# How a differentiated cell can change its identity : study of the role of the LIN-12/Notch pathway in the establishment of the competence to transdifferentiate in vivo in C. elegans

Thomas Daniele

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# UNIVERSITE DE STRASBOURG

*ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ*

*ED414*

**Institut de génétique et de biologie moléculaire et cellulaire**

**CNRS UMR 7104**

# THÈSE présentée par :


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# How a differentiated cell can change its identity:

# Study of the role of the LIN-12/Notch pathway in the establishment of the competence to transdifferentiate *in vivo* in *C. elegans*

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# Résumé en français

L'acquisition d'une identité cellulaire différenciée est souvent considérée comme définitive et figée dans le temps; or un nombre croissant d'études démontre que les cellules différenciées peuvent faire preuve de plasticité sous certaines conditions. Il est par exemple possible de forcer une cellule différenciée à devenir soit une cellule pluripotente, ou directement un autre type cellulaire différencié, via la surexpression de facteurs de transcription clés, on parle alors de reprogrammation cellulaire. Ces phénomènes de plasticité cellulaire peuvent également prendre place naturellement chez certains organismes[1]. Le fait que l'identité cellulaire ne soit pas figée peut devenir préjudiciable dans le cas de pathologies tel que le cancer où certaines cellules normales changent d'identité pour devenir des cellules tumorales[2,3]. A l'opposé rendre possible la manipulation aisée de l'identité cellulaire permettrait à long terme de mettre en place de nouveaux protocoles de thérapie cellulaire. Il serait alors possible de remplacer des cellules malades ou endommagées par des cellules saines reprogrammées. Malheureusement, l'application thérapeutique de telles méthodes n'est pas encore à l'ordre du jour, d'une part les protocoles de reprogrammation actuels ne sont efficaces qu'à 0,1 à 1%, d'autre part la stabilité des cellules reprogrammées n'a pas encore été clairement évaluée. Il a été montré que certaines cellules reprogrammées pouvaient perdre leur équilibre et devenir tumorales[4]. Il est donc indispensable de comprendre les mécanismes sous-tendant la reprogrammation cellulaire afin de pouvoir mieux maitriser la manipulation de l'identité cellulaire.

Ainsi, afin de mieux comprendre les phénomènes de reprogrammation cellulaire prenant place naturellement ou lors de cancer, notre laboratoire a établi un modèle unique chez *Caenorhabditis elegans (C. elegans)* permettant l'étude d'un événement de reprogrammation dans un contexte physiologique à l'échelle de cellules uniques. Nous étudions particulièrement un événement qui prend place au sein du rectum, formé par six cellules. Une de ces cellules, nommée Y, présente un comportement extraordinaire. Y est une cellule rectale épithéliale qui, au cours du développement du ver, va migrer antérieurement puis changer d'identité pour devenir un motoneurone nommé PDA dont les signatures moléculaires et

morphologiques sont totalement distinctes de la cellule Y[5]. Ce changement direct d'identité cellulaire est défini par le terme de transdifférenciation. Ainsi, cet reprogrammation cellulaire prend place naturellement et de manière stéréotypée: contrairement à la reprogrammation in vitro, cet événement de transdifférenciation à lieu chez 100% des vers sauvages. De façon importante, la possibilité de prédire par avance quelle cellule va changer d'identité nous donne accès aux étapes précoces de ce processus.

Les travaux préliminaires du laboratoire ont montré que la voie de signalisation LIN-12/Notch est le signal le plus précoce nécessaire pour le bon déroulement de la reprogrammation de Y en PDA. De plus, la cellule Y acquière une compétence à changer d'identité et la voie Notch est nécessaire à l'établissement de cette compétence: chez certain mutants pour le gène *egl-38* ou *mab-9*, une cellule Y surnuméraire est formée mais n'est pas capable de se transdifférencier[6,7], alors que chez des mutants gain de fonction pour Notch, la cellule Y surnuméraire formée est compétente à se transdifférencier, résultant en la formation de 2 neurones PDA[5,8]. Mes travaux de thèse ont donc portés sur la compréhension de l'implication de la voie LIN-12/Notch dans cet évènement de reprogrammation cellulaire.

Au cours de ma thèse, nous avons pu mettre en évidence: **i)** que lors de l'embryogénèse, 2 ligands canoniques (*apx-1* et *lag-2*) semblent agir de façon redondante afin d'activer la voie Notch, et ce dans la cellule Y. **ii)** l'activation ectopique et contrôlée de la voie Notch est suffisante pour induire la formation d'un second neurone PDA (et ce dans une fenêtre de temps courte autour de la naissance de Y). De façon intéressante, ces résultats nous ont permis d'observer que seul un nombre limité de cellules est alors reprogrammé laissant suggérer l'importance d'un contexte cellulaire permissif. **iii)** Les facteurs nucléaires que le laboratoire a identifiés comme cruciaux pour l'initiation de cet évènement de transdifférenciation[6] sont également importants pour la reprogrammation induite de cette deuxième cellule en neurone PDA formée chez des vers portant une mutation gain de fonction pour Notch. Cela suggère que ces facteurs sont en aval du signal Notch. **iv)** La suractivation prolongée de la voie Notch dans la cellule Y maintien

l'identité épithéliale de cette dernière, ayant pour conséquence le blocage de la transdifférenciation de Y en PDA.

Ensemble, nos résultats montrent que la voie Notch est nécessaire et suffisante afin d'établir la compétence à transdifférencier et que cela ne peut être réalisé que si la voie Notch est régulée de façon très précise dans la cellule Y.

Plus généralement nos travaux apportent la preuve que la régulation fine et précise des voies de signalisation est indispensable au bon déroulement de la reprogrammation cellulaire.

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# List of abbreviations

Every genotypes, phenotypes, alleles, and transgenes have been named according to the nomenclature set by the *C. elegans* community (Horvitz, Brenner, Hodgkin, & Herman, 1979).

| | |
|---|---|
| **5-azaC** | 5-azacytidine |
| **AC** | Anchor Cell |
| **BEC** | Biliary epithelial cell |
| **bFGF** | Basic fibroblast growth factor |
| **BRN2** | POU domain 2 |
| **Btc** | Betacellulin |
| **CSL** | CBF1/Su(H)/LAG-1 |
| **DFAT** | Dedifferentiated fat |
| **DIC** | Differential interference contrast |
| **DNA** | Deoxyribonucleic acid |
| **dsRNA** | Double-stranded RNA |
| **DTC** | Distal tip cell |
| **EGF** | Epidermal Growth Factor |
| **FOXA3** | Forkhead box A3 |
| **gf** | Gain-of-function |
| **GFP** | Green fluorescent protein |
| **GSC** | Germline Stem Cell |
| **HAND2** | The heart and neural crest derivatives expressed 2 |
| **hiPSC** | Human induced pluripotent stem cell |
| **HNF1A** | HNF1 homeobox A |
| **HS** | Heat-shock |
| **iPSC** | Induced pluripotent stem cell |
| **JNK** | c-Jun N-Terminal Kinases |
| **kb** | Kilobase |
| **lf** | Loss-of-function |
| **MAFA** | V-maf musculoaponeurotic fibrosarcoma oncogene homologue A |
| **MCS** | Multiple cloning site |
| **MEF2C** | Myocyte-specific enahncer factor 2C |
| **miRNA** | MicroRNAs |
| **MYT1L** | Myelin transcription factor 1-like |
| **Ngn3** | Neurogenin 3 |
| **NICD** | Notch intracellular domain |
| **nt-ESC** | Nuclear-transfer-derived embryonic stem cells |
| **ORF** | Open reading frame |

| | |
|---|---|
| **PcG** | Polycomb-group |
| **PDX1** | pancreatic and duodenal homeobox 1 |
| **PEC** | Pigmented epithelial cell |
| **PTB** | RNA-binding polypyrimidine tract-binding |
| **RNA** | Ribonucleic acid |
| **RNAi** | RNA interference |
| **RPE** | Retinal pigmented epithelium |
| **SCNT** | Somatic-cell nuclear transfer |
| **TBX5** | T-box5 |
| **VPC** | Vulval precursor cells |
| **VU** | Ventral uterine precursor cell |
| **WT** | Wild-Type |

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# INTRODUCTION

# I) Cell plasticity

The term cell plasticity can be generally defined by the ability of a cell to change its identity. In this part of the introduction, general principles encompassed by the term "cell plasticity" will be discussed. However, before entering in the depths of cell plasticity, the historical perspectives of developmental biology will be presented to show that old concepts postulated with rudimentary tools are not that far from the actual known biological reality. Early scientists were able to postulate important concepts, which are still relevant today and others that turns to be less fixed than expected

## I.1) Historical view of development and cell differentiation

### I.1.1) Epigenesis versus preformationism – The opening debate

Epigenesis and preformation were two embryonic and developmental theories in competition during the past centuries. The epigenesis theory considers that the final form of an organism is built gradually. During development, the embryo gets more and more specified and acquires its final form over time. In contrary, the preformationist theory considers that an organism is already formed at its origin and just grows during development, in other words any adult being comes from the growth and consolidation of its miniature form.

The epigenesis theory arises with *Generation of Animals* (Aristotle *translated By Peck*, 1979) of Aristotle (384-322 B.C.) in which he describes the development of several organisms. By looking carefully at chicken development, he could notice that the early egg was not prepatterned, the egg does not contain a preformed little chick. Instead, he noted that the egg gradually acquires its form and specific features as a beating heart or developing eyes. He postulated that the starting material is certainly available at the beginning but the final form is obtained through embryonic development. At this period Aristotle thought that the epigenetic

elements affecting development are contained in the soul of the developing organism.

This concept of epigenesis was well accepted, even by the church, but new observations and interpretations of development made during the 17th and 18th century went for the preformationist theory. Jan Swammerdam (1637-1680) studied in detail the development of insects and amphibians and could observe that the same organism was preserved during development and was just growing packed inside different structure (as egg, larva or pupa). These findings were reinforced by the development of microscopy and the observations of spermatozoids by Nicolaas Hartsoeker (1656-1725). In is *Essai de Dioptrique* (Hartsoeker, 1694), Hartsoeker claimed that he could observe a microscopic men inside the sperm cell. He called this little human trapped in a spermatozoid: *homunculus* (Figure 1).

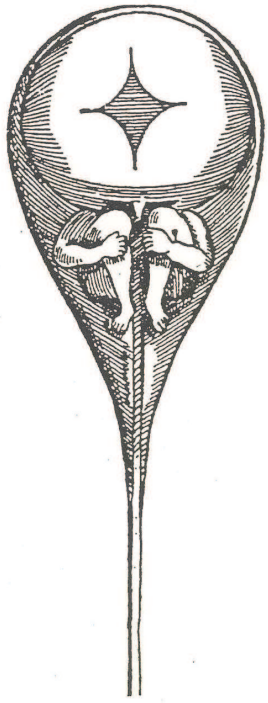

**Figure 1** – The Hartsoeker's homunculus (Hartsoeker, 1694)

Due to their scientific contribution and reputation Swammerdam and Hartsoeker (with many others) imposed the preformation theory, in which development is just a matter of growth and any living being is pre-determined. This pre-deterministic view of development stand for a long time and fueled the debate between epigenesist and preformationist, but in 1759, because of the evolution of

optics and microscopes, work performed by Kaspar Friedrich Wolff (1733-1794) allowed the complete reinterpretation of the observations made by previous researchers. Wolff studied meticulously the development of plants and animals and proposed that group of cells initially not specialized differentiate during development to give rise to tissues, organs and complete systems and qualified this phenomenon "morphogenesis" (Maienschein, 2012).

### I.1.2) The cell theory

The debate between preformationism and epigenesis ended with the emergence of the cell theory. During the 19$^{th}$ century, work from Matthias Schleiden (1804-1881), Theodore Schwann (1810-1882) and later Rudolf Virchow (1821-1902) showed that cells are the bases of all the elements constituting any living being. They went further by postulating that living cells can only come from other pre-existing cells. Many questions arise from these findings and especially how a single cell (the egg or the sperm) can give the information of all the traits found in an adult being? Or how the features found in an organism can be inherited through the egg or the sperm cell?

Observations of fertilization partially answered this last question, and showed the nuclei of the egg and the sperm cells have to fuse in order to let development to take place. From these observations scientists proposed that the nucleus was the vehicle of inheritance. In 1882, Walter Flemming (1843-1905) described mitosis and showed that in the nucleus, structures called chromosomes have to split equally between two sister cells during cell division. For the first time the physical nature of inheritance was described. It is only in 1910 that experimental embryology and genetics meet to prove that chromosomes and specific regions of chromosome support the inheritance information. Thomas Hunt Morgan (1866-1945) found some male mutant *Drosophila* having white-eyed among population of red-eyed wild-types flies. Morgan could show that this eye color trait is linked to the sexual chromosome giving for the first time the evidence that chromosomes carry the hereditary information (Heard, 2013).

Based on the available evidence and knowledge, scientists had different point of view on the behavior of the inheritable material during cell differentiation. Because cells become more and more specialized, some scientists thought that specialization was linked to a progressive loss of genetic material and only relevant material was kept in the cells (except for the germline where it had to be preserved). On the contrary, new generations of geneticists thought that genes and chromosomes were the same in every cell but their activity were different.

The ultimate demonstration that the genetic material was conserved all along cell differentiation was brought by experiments performed by Briggs (1911-1983) and King (1921-2000) (Briggs & King, 1952) in 1952 and ten years later by John Gurdon (Gurdon, 1962). By transferring purified nucleus from a somatic cell into an enucleated egg they could reconstitute fully a viable tadpole, showing that indeed the entire genetic material was kept through cellular differentiation and by consequence development is the result of gene expression changes.

### I.1.3) The Waddington's landscape

The biggest remaining question was to understand how the genetic information could be differentially used during cell differentiation. One brilliant paleontologist, zoologist, geneticist, embryologist and philosopher, Conrad H. Waddington (1905-1975) postulated several theories to try to bridge genetics and embryology. From his observations and personal experience, Waddington supposed that a single gene can have multiple effects on different organ and multiple genes can affect a single organ (Waddington, 2012). He later defined epigenetics as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" and formulated his famous epigenetic landscape theory (Waddington, 1957) (Figure 2, upper part). A schematic representation of his hypothesis shows a ball (representing a cell) rolling down a landscape made of valleys. Development is represented by the progression of the ball down the landscape. During its descent, the ball encounters several roads representing the different cell fates that a cell could acquire; once the ball has committed to a road, its destiny will be chosen and fixed for the rest of its life. Waddington imagines that differential gene expression is the foundation of the

topology of the landscape (Figure 2, lower part). At that time, Waddington was already arguing that mutation could change the topology of the landscape and by consequence change the road taken by the cell.

This unidirectional view of development (from a undifferentiated to differentiated cell) was really innovative for the era and has become the prevalent view since then.

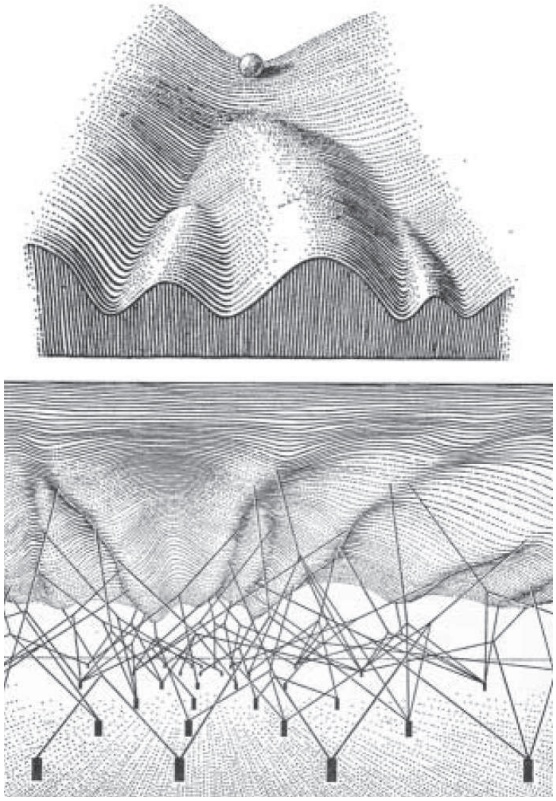

**Figure 2** – The Waddington's epigenetic landscape. In the upper part the ball represent a cell that will roll down the landscape. Each valley represents a possible cell fate. The lower part represents how the topology of the landscape is made because of genes represented by the black boxes.

This view of cell differentiation considers that commitment of a cell into a certain lineage and the acquisition of the final identity is a blocked phenomenon. Once the cell has reached its final destination, nothing can perturb this state, no cell fate switch or reversion could take place.

In the following parts we are going to discuss how this view of development and cell differentiation has changed. It is now well accepted that the acquisition of a

cell fate is not a fixed situation and that a cell can change its identity under certain circumstances.

## I.2) When a cell can exhibit plasticity

### I.2.1) Definition of cell potency

Cell potency is defined by the ability of a cell to differentiate into an other cell type, if we look back at the Waddington landscape (Figure 2), we can remark that according to this unidirectional view of development, cells possess a large potential of differentiation at the beginning its life. Then throughout the development this potency becomes more and more restricted.

According to their differentiation potential, cells can be group in different categories:

**Totipotent cells:** These cells are the most undifferentiated and potent cells found in early development. They can give rise to all the embryonic and extra embryonic tissues (Smith, 2006). (Example: mammalian embryonic cells up to the 8-cell stage embryo, plant meristem cells).

**Pluripotent cells:** can give rise to cells belonging to the three embryonic germ layers (endoderm, mesoderm, ectoderm) (De Miguel, Fuentes-Julian, & Alcaina, 2010; Ratajczak et al., 2012) (Example: embryonic stem cells)

**Multipotent cells:** can give rise to several types of cells constituting one or more tissues (Example: Mesenchymal stem cells (Augello, Kurth, & De Bari, 2010))

**Oligopotent cells:** can give rise to two or more cell types within a specific tissue. (Example haematopoietic stem cells (Metcalf, 2007) or oligopotent stem cells in mammalian eyes (Majo, Rochat, Nicolas, Jaoude, & Barrandon, 2008)

**Unipotent cells:** Can form a specific lineage and differentiate into one cell type only (example muscle stem cell (Bentzinger, Wang, von Maltzahn, & Rudnicki, 2013))

**Differentiated cells:** specialized cell performing a particular task in a organ or tissue.

During development, the first cells from the early embryo are totipotent. Throughout the cell divisions, this totipotency is gradually lost and the differentiation potential of the cell is reduced until the cells are differentiated and fulfill a specific function inside an organ or a tissue. Cell differentiation was seen as the last possible state for a cell. Once the differentiation program of cell is launched, we cannot go back to a primitive state or acquire another fate.

### I.2.2) Terminology of cell plasticity events

Even though evidence have been obtained by the scientific community that the fate of a cell can be modified, it is only since 2006 with the work of the Yamanaka's lab (Takahashi & Yamanaka, 2006) (detailed given in the following parts) that the fact that a cell can change its identity has been widely accepted. Since this date, the cell plasticity field is booming and many examples of cell fate manipulations have been reported.

Denomination and terminology problems are often encountered when a field is in rapid expansion. In order to be clear among them, scientists have to use the same terms and denomination to describe the same phenomena. For example, the epigenetic field had to set a nomenclature for histone denomination in order to simplify their description (Talbert et al., 2012). The same problem is encountered in the cell plasticity field. Unique cell plasticity events are sometimes defined by different terms. So far, no one really tried to unify the different terminologies used to describe cell plasticity phenomena. In order to be clear for the rest of the manuscript here are the definitions that I will use for the notions detailed in the following parts. The proposed definitions are the one currently used by the scientists of the cell plasticity field . Nevertheless it is possible that in a near future, theses definitions will be modified.

**Dedifferentiation:** is defined by the reversion of a terminally differentiated cell into a cell that does not exhibit any specialised or differentiated characters anymore. This encompasses what has been called by some retro-differentiation, the reversion of a terminally differentiated cell into a less differentiated (progenitor) stage within its

own lineage. Note that dedifferentiation is not automatically coupled to reversion to a more potent state.

**Nuclear reprogramming:** is defined by the reversal of the differentiation state of a mature cell to a pluripotent state (Hochedlinger & Jaenisch, 2006).

**Transdetermination:** is defined by the switch from a committed state to another (Manohar & Lagasse, 2009).

**Transdifferentiation:** is defined as the stable switch from a differentiated cell type to another (Eguchi, 1995).

### I.2.3) Dedifferentiation

#### *I.2.3.1) Naturally induced dedifferentiation*

Dedifferentiation has been reported to take place *in vivo* under specific circumstances. Some organisms have the capacity to regenerate some organs after amputation or injury and dedifferentiation processes take place to reform the missing organ.

Zebrafish can regenerate some organs such as the heart, spinal cord, retina or the fins (Poss, 2002). After amputation of up to 20% of the heart ventricle, regeneration can take place through dedifferentiation of cardiomyocytes (Jopling et al., 2010; Kikuchi et al., 2010). Cardiomyocytes present an important contractile sarcomeric apparatus. During regeneration, dedifferentiation of cardiomyocytes lead to the disassembly of the sarcomeres and decrease in the expression of sarcomeric genes. During this dedifferentiation process, cardiomyocytes separate and prior to proliferation start to express positive cell cycle regulators. After proliferation, this new pool of cells differentiates again into cardiomyocytes to reform the missing part of the heart.

Similar results have been reported during Zebrafish fin regeneration. After amputation of the fin, a zone of undifferentiated proliferating cells (called blastema) forms and gives rise again to the amputated part. Using careful cell tracking, it has been shown that after amputation, osteoblast cells dedifferentiate and become

proliferative in the blastema. The newly produced cells will then differentiate again into osteoblast to form the bones present in the fin (Knopf et al., 2011) (Figure 3).

In mammals very few examples of clear natural cell dedifferentiation have been reported. In the brain, Schwann cells are glial cells wrapping around neuronal axon and form a myelin sheath. During development, Schwann cell precursors are made from the neural crest cells. These precursors differentiate into proliferative immature Schwann cells and finally differentiate into mature Schwann cells able to wrap around axons. During injury, when Schwann cells lose the contact with the axons, they dedifferentiate by expressing immature Schwann cell genes, proliferate and differentiate again to form new mature Schwann cell (Mirsky et al., 2008; Woodhoo et al., 2009).

Surprisingly, only few well characterized cases of natural dedifferentiation have been reported. This absence of knowledge is due in particular to a lack of tools allowing proper cell tracking during development. Unfortunately, in the cell plasticity field this lack of tool was the source of several controversies that will be detailed later.

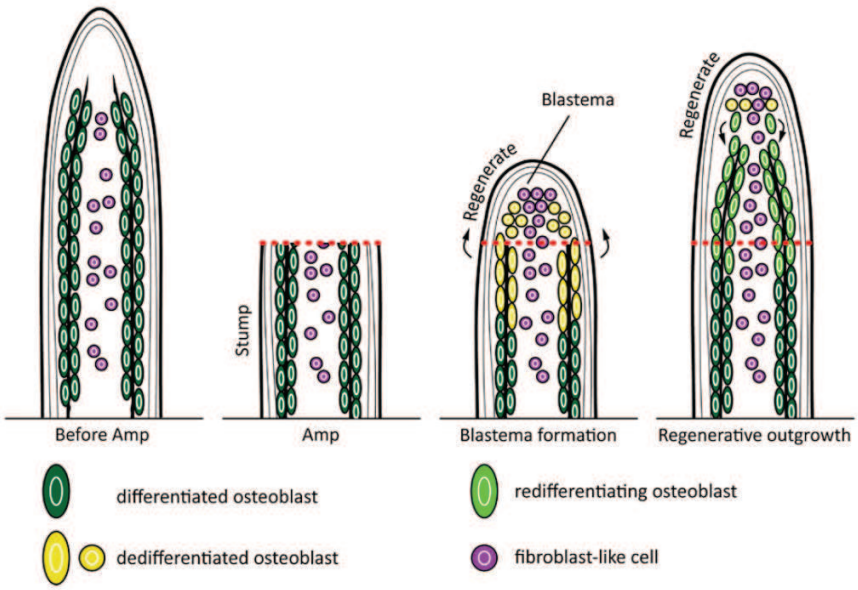


**Figure 3** – Regeneration of the Zebrafish fin through dedifferentiation of osteoblasts cells. After amputation, osteoblasts dedifferentiate and proliferate in a blastema. After proliferation, the new cells differentiate again into osteoblasts to reconstruct the fin (Knopf et al., 2011).

#### *I.2.3.2) Experimentally induced dedifferentiation*

To date, only few examples of experimentally induced dedifferentiation have been reported. Some of them were found *in vitro*, while only one clear example has been discovered *in vivo*.

In some organisms such as the newt, myotubes can dedifferentiate and proliferate whereas mammalian myotubes do not have this feature. This lack of plasticity could be due to several possibilities: mammalian myotubes lack specific genes allowing dedifferentiation or irreversibly express genes that lock the myotube fate. It can be possible as well that extrinsic factors present during newt myotube dedifferentiation are not available in mammals. To test these hypothesis scientists have applied extracts from newt regenerating limbs on C2C12 mammalian myotubes. They could observe that expression of myotube specific genes as MyoD, myogenin or troponin T was reduced and that cell can reenter the cell cycle suggesting a dedifferentiation followed by proliferation (McGann, Odelberg, & Keating, 2001). These data suggested that newt-specific extrinsic factors help to allow myotube dedifferentiation and could explain why this cell plasticity difference exists between newt and mammalian myotubes.

Purified human thyroid cells have been put in culture with a serum-free medium. A lot of the cultured cells died but a small proportion survived and proliferate. These proliferating cells show reduced expression in thyroid specific genes such as thyroglobulin or cytokeratine-18. If these cells are kept in these culture conditions, they do not express any mature thyroid specific markers. Surprisingly, these newly obtained undifferentiated cells were able to differentiate into thyroid cells and into neuronal or adipogenic lineages indicating that the starting cells dedifferentiate into multipotent cell (Suzuki et al., 2011) (Figure 4.A).

Human epidermal keratinocytes grown in culture with basic fibroblast growth factor (bFGF) and no extra intervention can dedifferentiate by re-expressing native keratinocyte stem cell markers such as ß-integrin, CK19 or CK14 (Sun et al., 2011) (Figure 4.B).

It has been reported that upon simple specific culture strategy, which allow adipocyte observation and prevent cell to fuse, that mature adipocyte (thought to be

non-proliferative) can dedifferentiate into cells called dedifferentiated fat (DFAT). These DFAT cells become multipotent and present adipogenic, osteogenic, chondrogenic and myogenic potential (Shen, Sugawara, Yamashita, Ogura, & Sato, 2011) (Figure 4.C).

Several cases of pancreatic cells dedifferentiation have been reported. In a first study, made *in vitro*, human islet cells embedded in collagen and in contact with epidermal growth factor have been shown to dedifferentiate into proliferative duct-like epithelial cells (Hanley, Assouline-Thomas, Makhlin, & Rosenberg, 2011) (Figure 4.D). In a second study, made *in vivo*, it has been reported that adult murine ß cells can modify their differentiation state under physiological stress conditions. Maintenance of the ß cell identity relies on the specific transcription factor FoxO1. Ablation of this gene in adult murine ß cell has no effect in normal condition. However under stress condition, the pancreas present a dramatic loss of ß cells. It was though that this loss is the result of increase cell death in insulin producing cells but instead ß cells seem to dedifferentiate into multipotent endocrine progenitors. This dedifferentiation could be an advantage allowing the ß cells to survive in case of strong metabolic stress (Talchai, Xuan, Lin, Sussel, & Accili, 2012) (Figure 4.E).

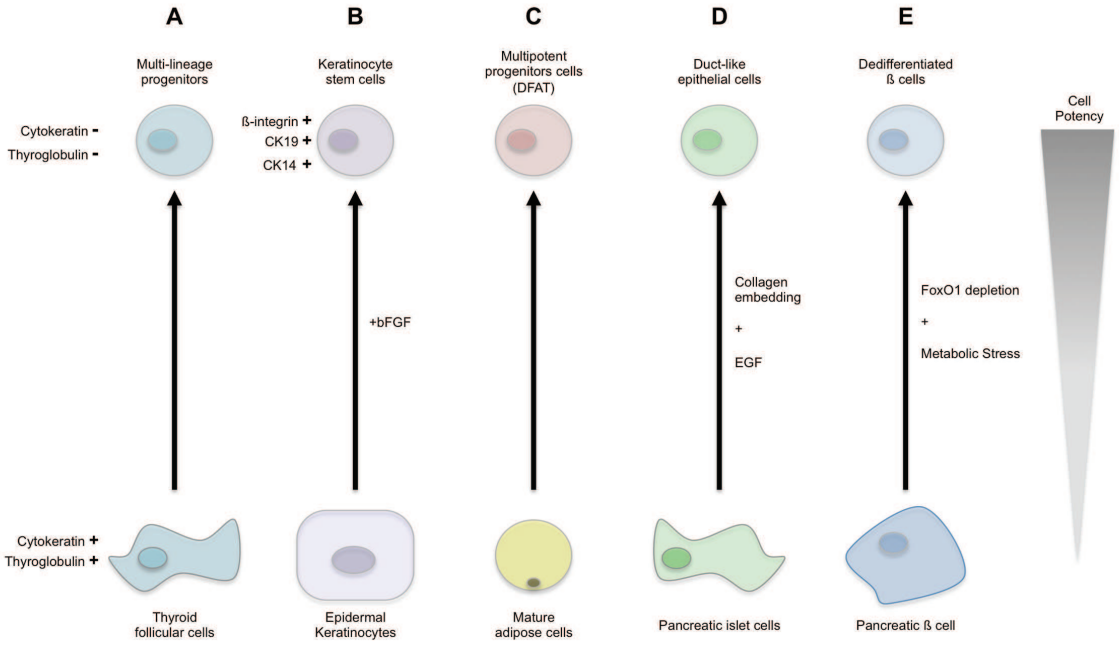


**Figure 4** – Examples of cell dedifferentiation adapted from (Eguizabal, Montserrat, Veiga, & Izpisua Belmonte, 2013) and (Puri & Hebrok, 2012).

All these examples point the fact that differentiated cells can revert into a less specialized state under specific conditions. Moreover, dedifferentiation is not limited to a specific embryonic origin (i.e. Pancreatic cells originate from the endoderm whereas keratinocytes originate from the ectoderm), suggesting that any cell type could increase its potency under specific circumstances.

### I.2.4) Nuclear reprogramming

As mentioned previously, nuclear reprogramming was used to describe the switch from a differentiated cell state to a pluripotent state. In other word, by nuclear reprogramming, a fully specialized cell fulfilling its role in a tissue can be reprogrammed into a non-specialized cell able to give rise to cells from the three germ layers. Different methods can be used to completely revert the differentiation state of cell. Nuclear reprogramming can be done by somatic-cell nuclear transfer (SCNT), by cell fusion, by using cell extracts or by over-expression of specific factors.

#### *I.2.4.1) Somatic-cell nuclear transfer (SCNT)*

During the 1950's the key question in developmental biology was whether the genome of cells was irreversibly modified during cell differentiation. To answer this question Briggs & King transferred the nucleus from a blastula into an enucleated frog egg and could obtain living tadpoles (Briggs & King, 1952). These experiments showed for the first time that the genetic information is not modified during cell differentiation. However, this view was challenged by Briggs and King themselves, when they tried to repeat this experiment with a nucleus coming from a late developmental stage. Indeed, using these new nuclei, they could not be able to obtain living tadpoles. They conclude that irreversible nuclear changes could take place when cells differentiate. Later, John Gurdon decided to do the same experiment in another species of frog. Somatic nuclei from post-blastula embryo and intestinal nuclei were transferred into enucleated eggs. In both cases, living and fertile frogs have been obtained bringing the proof that genetic information is not lost during cell differentiation. These results highlighted an other important concept: based on its experiments John Gurdon proposed that the fate of a differentiated nucleus can be reverted to a pluripotent one, which can drive the development and

formation of a complete new living organism. Indeed in the Gurdon's experiments, intestinal cells were extracted from a functional organ. The nucleus of these cells is "programmed" to execute the function of the cell. When this nucleus is transferred into an enucleated egg, its program and function are erased by intrinsic determinants of the egg. This newly reprogrammed nucleus is now totipotent, and can be committed into any cell differentiation program. At the time, the scientific community remained skeptical and thought that during the nuclear transfer procedure, Gurdon took the nucleus from a contaminating undifferentiated cell, which may exist in the post-blastula embryo or in the intestine (Figure 5).

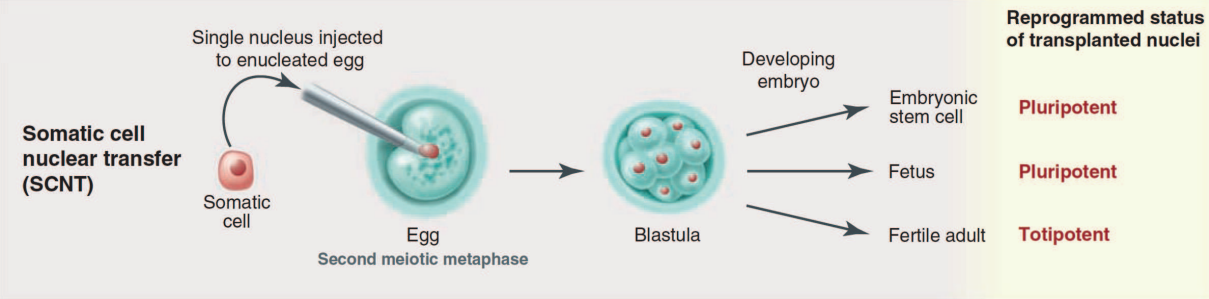


**Figure 5** – Somatic-cell nuclear transfer: A single nucleus from a somatic cell is injected into an enucleated egg. The developing embryo can give rise to a fertile adult, a fetus or can be used to derive embryonic stem cell line (Gurdon & Melton, 2008).

Although, John Gurdon showed that nuclei from skeletal muscle, skin, lung or kidney transferred into an egg could be reprogrammed (Gurdon, Brennan, Fairman, & Mohun, 1984; Laskey & Gurdon, 1970), it is only when the first mammal, Dolly the sheep, has been obtained by SCNT that the hypothesis that differentiated nucleus can be reprogrammed into a pluripotent state begun to be widely accepted (Wilmut, Schnieke, McWhir, Kind, & Campbell, 1997)]. Because all the nuclear reprogramming experiments performed so far have a success rate close to 1%, some people were still arguing that reprogramming is only due to the use of contaminating undifferentiated nuclei. The laboratory of Rudolf Jaenisch brought the ultimate evidence that this argument was wrong. They could show that reprogramming of highly specialized cells such as mouse olfactory neurons or mouse T and B lymphocyte can be performed showing that any differentiated nucleus can be reprogrammed into a pluripotent state (Eggan et al., 2004; Hochedlinger & Jaenisch, 2002). Indeed the viable mouse obtained by SCNT of T or B-lymphocytes carry the same genomic rearrangement than the donor cells.

The success rate of nuclear reprogramming has been shown to be dependent on the differentiation state of the donor cell. The more differentiated the cell is, the less reprogramming is efficient. The nucleus of differentiated cells acquires through development an epigenetic restriction. For example these restrictions can be brought by DNA methylation, histone modification, histone variants and non-coding RNAs (Pasque, Jullien, Miyamoto, Halley-Stott, & Gurdon, 2011). All these restrictions have to be suppressed at the same time to allow reprogramming explaining why this phenomenon hardly takes place.

Because nuclei from differentiated cells are more resistant to reprogramming, scientist passes through the formation of nuclear-transfer-derived embryonic stem cells (nt-ESCs). Rarely, transferring a nucleus (from any species) from a differentiated cell leads to the formation of a living blastula. From those embryos, nt-ESCs can be derived and used as a donor cell to perform new nuclear transfer into new enucleated egg or oocyte. When nt-ESCs are used more than 20% of the newly transferred nuclei are reprogrammed. Because of this technique nt-ESCs lines have been derived from cows, dogs, and non-human primates.

A very recent study showed that SCNT could work as well with human tissue. The recipient cell used in human SNCT is an oocyte blocked in metaphase II of the meiosis. During the SCNT experiment, the penetration of the needle used to transfer the somatic nucleus acts as a sign of fertilization for the oocyte, this manipulation triggers the completion of the meiosis in the oocyte and inhibits the reprogramming of the transferred nucleus. Caffeine has been applied to metaphase II oocytes to prevent premature completion of meiosis. Because of this molecule, the transferred nucleus can be reprogrammed; the embryo can develop until the blastocyst stage (around 100 cells) and can be used to derive embryonic stem cell lines (Tachibana et al., 2013).

*Note: This paper is under investigation because of potential mislabeling and figure duplication. The Cell press group is studying if these errors can completely change the conclusion of the paper and in the worst-case lead to a retraction.*

### *I.2.4.2) Nuclear reprogramming by cell fusion and cell extract*

Cells can be fused leading to the formation of a non-proliferative tetraploid cell called heterokaryon. During cell fusion the less differentiated cell is the dominant cell and impose its fate to the partner cell (Yamanaka & Blau, 2010). Reprogramming of differentiated cells such as mouse T lymphocytes can be done by fusion with an embryonic stem cell (Tada, Takahama, Abe, Nakatsuji, & Tada, 2001). The resulting heterokaryon expresses pluripotency genes after a few hours and when injected into mouse blastocyst can give rise to chimeric embryos. Similar results have been obtained with human cells (Cowan, 2005). Unfortunately the use of these heterokaryons is really limited. On one hand their non-proliferative status is not suitable to derive embryonic stem cell line, on the other hand their tetraploid status could lead to aberrant gene reactivation from one nucleus leading for example to loss of pluripotency (Figure 6).

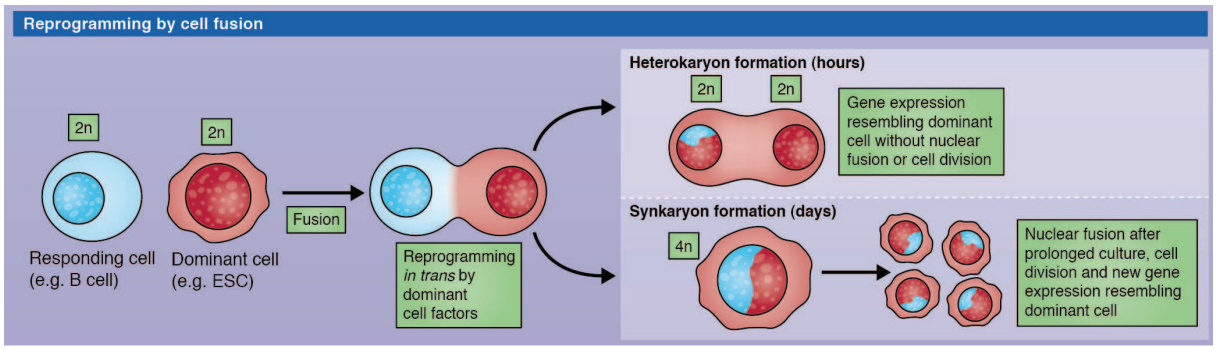


**Figure 6** – Nuclear reprogramming by cell fusion. A somatic cell can be reprogrammed by fusion with a dominant cell (e.g. an embryonic stem cell). The resulting cell can be a tetraploid heterokaryon or a synkaryon if the cell is kept under culture (Halley-Stott, 2013).

Nuclear reprogramming has been shown to be also possible after exposure to protein extracts from pluripotent cells (Taranger et al., 2005). Cultured epidermal cells (293T cells) have been permeabilized, exposed to embryonic stem cell or carcinoma cell extracts and re-sealed. After culture, the cells in contact with the extract represses 293T specific gene and begin to express genes normally up-regulated in the cells used for the extract. The reprogrammed cells can be differentiated into neurogenic, adipogenic, osteogenic, and endothelial lineages. The efficiency of reprogramming with this method is really low. Moreover we don't know if the resulting cells are fully pluripotent; their potency have not been tested with stringent test (i.e. teratoma or chimera formation) (Figure 7).

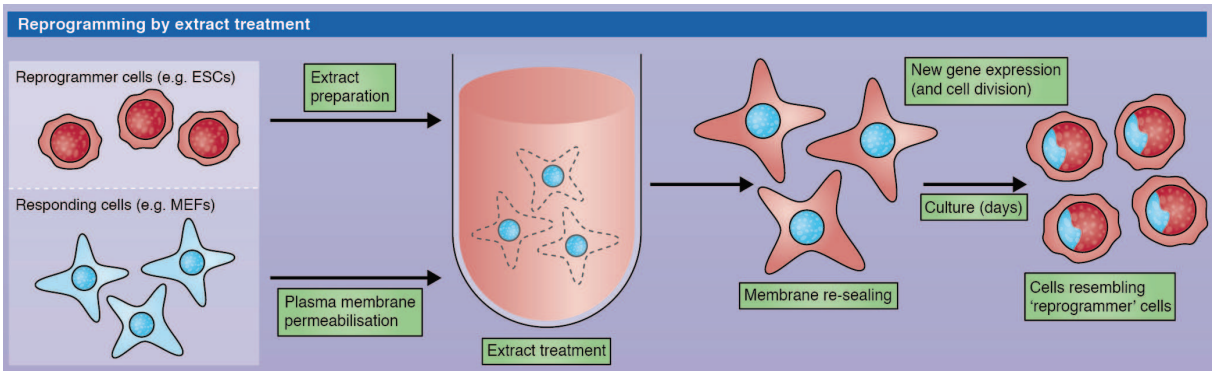


**Figure 7** – Nuclear reprogramming by cell extract treatment. Somatic cell can be reprogrammed after permeabilization and cultured with embryonic stem cell extract. The reprogrammed cell takes the identity of the cell used to make the extract (Halley-Stott, 2013).

### *I.2.4.3) Induction of pluripotency by defined factors*

In 2006, the lab of Shinya Yamanaka sat off of a revolution in the stem cell and cell plasticity field. They could show that mouse, then human embryonic fibroblasts can be reprogrammed into pluripotent stem cell by over-expression of specific factors (Takahashi & Yamanaka, 2006; Takahashi et al., 2007). A screen was designed to find the factors able to induce pluripotency in somatic cell. They developed a modified mouse fibroblast cell line in which a drug resistance gene can be express under an embryonic cell specific promoter (Fbxo1). They over-expressed 24 pluripotency associated factors in this cell line and could obtain drug resistant colonies with the morphology of embryonic stem cells. With consecutive series of elimination of individual factors they could define the minimal cocktail able to induce pluripotency in fibroblasts, which is composed by *Klf4*, *Sox2*, *c-Myc* and *Oct-4* (Figure 8). This newly pluripotent cells are called induced pluripotent stem cells (iPSC), express pluripotent stem cell markers such as SSEA-1 and Nanog and can generate teratomas when injected in immune-deficient mice.

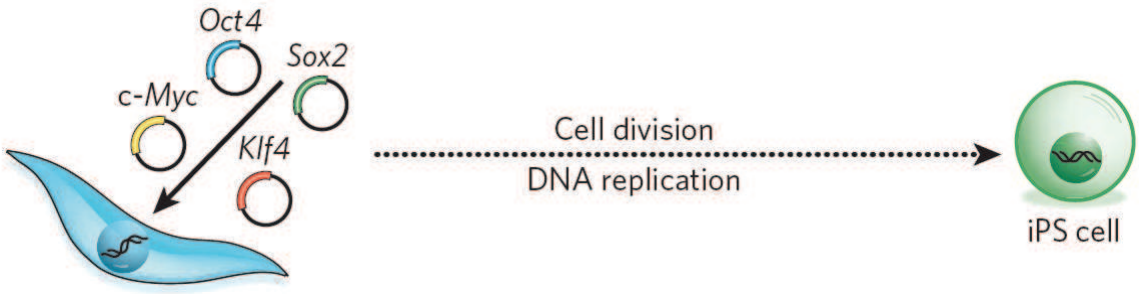


**Figure 8** – Nuclear reprogramming by over-expression of defined factors. Over-expression of *Klf4*, *c-Myc*, *Oct4* and *Sox2* allows reprogramming of fibroblast into induced pluripotent stem cell (Yamanaka & Blau, 2010).

However these first generations of iPSCs exhibit incomplete DNA demethylation of the Oct4 promoter compare to embryonic stem cells and were not able to generate chimeras when injected into blastocysts showing their plausible incomplete reprogramming. Later, improvements of this reprogramming technique, based on a more stringent method to select the reprogrammed cells, have been done resulting in iPSCs molecularly and functionally close to embryonic stem cells. These "new generation" of iPSCs were able to give rise to viable chimera attesting that these cells are fully pluripotent (Maherali et al., 2007; Okita, Ichisaka, & Yamanaka, 2007; Wernig et al., 2007). iPSCs have been derived from many species such as mouse, human, rats (Li et al., 2009), pig (Esteban et al., 2009), and rhesus monkey (Liu et al., 2008) showing that induction of pluripotency requires elements which have been conserved through evolution.

Since 2006, the number of publication related to iPSCs completely exploded (Scott, McCormick, Derouen, & Owen-Smith, 2011). An intensive race between labs was launched after the publication of the first iPSCs paper. Scientists tried to reprogram other cell type than fibroblast and could show that, for example, pancreatic ß cells (Stadtfeld, Brennand, & Hochedlinger, 2008), keratinocytes (Aasen et al., 2008) or hepatic and stomach cells can be reprogrammed (Aoi et al., 2008), indicating that reprogramming is not dependent on the starting cell fate. The first iPSCs were obtained using over-expression, after genomic integration, of the four reprogramming factors; people have tried to find alternative methods to reprogram cells and not modify the genome of the reprogrammed cells. Some non-integrative methods using adenoviruses (Stadtfeld, 2008) or plasmids (Okita, 2008) have been used. Some DNA free reprogramming methods have been developed using either recombinant protein (Zhou et al., 2009) or synthetic modified RNAs (Warren et al., 2010).

In parallel of this reprogramming improvement race, stem cell scientists tried to understand the mechanisms underlying induced pluripotency. The first stricking element is that efficiency rate of reprogramming with defined factors oscillate in average around 0,02% for mouse and human fibroblasts (Brambrink et al., 2008; Stadtfeld, 2008; Wernig et al., 2008). In addition to this, reprogramming is slow; it takes around 2 weeks to see the first reprogrammed clone appearing. From these

data, two different reprogramming theories have been proposed. An elitist or called as well deterministic model suggests that reprogramming efficiency is low because in a population of cultured fibroblast, a rare portion of them are close to a progenitor cell fate and by consequence are more susceptible to be reprogrammed. In the other hand a stochastic model has been proposed in which all the cells have the same probability to be reprogrammed but have to pass through stochastic events to acquire pluripotency. Because only few cells are able to pass through these events, the efficiency of reprogramming is low by consequence. Reprogramming of highly differentiated cells such as T or B lymphocyte, in which the initial level of differentiation can be assessed by looking at the genome (Hanna et al., 2008) and pancreatic ß cell (Stadtfeld et al., 2008) goes against the deterministic model where progenitor cells are supposed to exist in the starting cell population. Constant cell proliferation is needed to allow rare cells to acquire stochastic changes responsible of their reprogramming into pluripotent stem cells (Hanna et al., 2009).

Some precisions on the mechanisms of reprogramming have been reported recently because of the development of quantitative single-cell analysis. 48 genes (such as genes involved in proliferation, epigenetic modifiers, pluripotency markers and fibroblast markers) have been analyzed in parallel at different stages of the reprogramming process. This revealed that after expression of the reprogramming factors in fibroblasts, stochastic gene expression takes place and triggers cell fate modifications leading to the formation of senescent, apoptotic, transformed or reprogrammed cells. The reprogrammable cells will proliferate and modify their metabolism and epigenetic status and will enter in a poorly understood rate-limiting step, which delays the reprogramming. During this phase, stochastic expression of pluripotency markers takes place along with developmental regulators and activation of glycolysis. The last phase of reprogramming begins with the activation of *Sox2*. This activation leads to a series of deterministic and hierarchical events including activation of the core pluripotency circuit, silencing of the exogenous reprogramming factors, epigenetic resetting and others pluripotent stem cell specific features (Buganim et al., 2012) (Figure 9).

These data provide a basis to explain why the efficiency rate of nuclear reprogramming by over-expression of defined factors is so low. The fact that the early phase of reprogramming requires stochastic gene activation suggests that the probability to have the right sequence of reprogramming event is really low. This was confirmed by previous experiments showing that reprogramming efficiency of less differentiated cells such as haematopoietic stem cell of progenitor cells can raise to 40% (Eminli et al., 2009). It suggests that the differentiation state can affect the susceptibility of a cell to be reprogrammed. This can be explained by the fact that less differentiated cells are closer to the pluripotent state, and requires less stochastic event to be reprogrammed. By consequence, reprogramming can take place more easily.

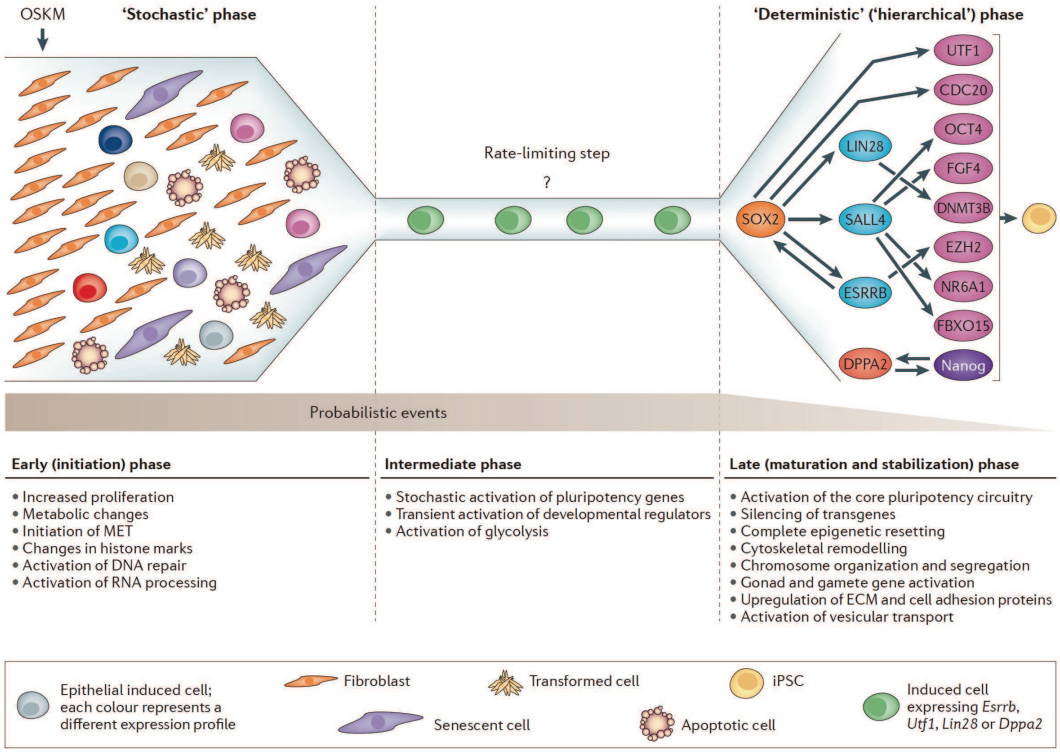


**Figure 9** – The phases of reprogramming: During the initiation phase, just after expression of the reprogramming factors (OSKM here for *Oct4*, *Sox2*, *Klf4* and *c-Myc*) stochastic gene expression takes place leading to the formation of different cell types including reprogrammable cells. These cells will proliferate and enter in a rate-limiting step where stochastic activation of pluripotency markers, developmental regulators and activation of glycolysis take place. This stochastic phase leads to the activation of *Sox2,* which will initiate the late deterministic phase of reprogramming. During this last phase, hierarchical gene expression will take place inducing for example activation of the core pluripotency circuitry, epigenetic resetting or cytoskeletal remodeling leading to the formation of an iPSC cell (Buganim, Faddah, & Jaenisch, 2013).

Because of the discovery of iPSC, the scientific community is less skeptical on the fact that a cell can become plastic under certain circumstances. The current challenge in the fundamental iPSC field is to clearly understand all the mechanism involved during reprogramming and why cellular differentiation can be reverted.

### I.2.5) Transdetermination

The switch from a committed state to another is defined as transdetermination. It has been discovered and extensively studied in *Drosophila* where imaginal discs can change their determination under specific conditions. Imaginal discs are groups of cells found in *Drosophila* larva at the origin of adult appendages such as legs, wings or antenna. The imaginal discs are specified during early embryogenesis at different places of the embryo (Figure 10). During development, cells proliferate inside the disc and restrict their developmental potential. At the late larval stages the disc reaches its final form and starts to differentiate to give a specific adult structure. The future fate of each imaginal disc is known (Figure 10) and because of this fate map transdetermination could be discovered.

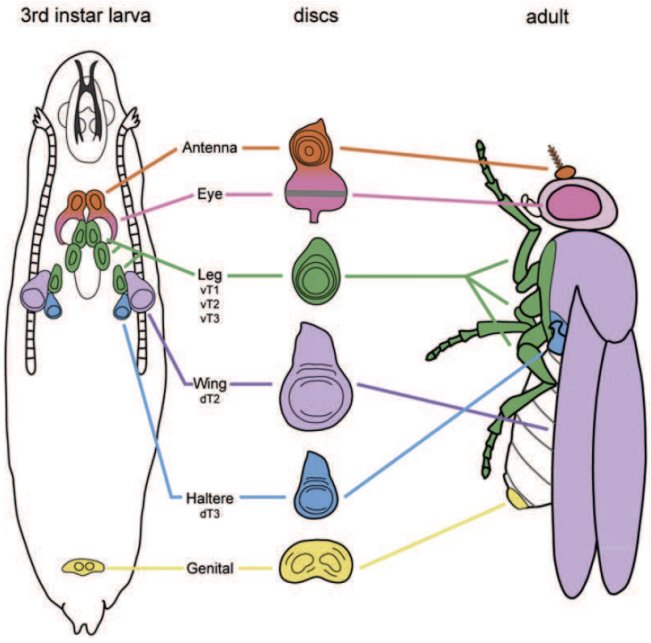


**Figure 10** – *Drosophila* imaginal disc map. Imaginal discs are formed during embryogenesis proliferate and differentiate into specific adult structures after metamorphosis. Antenna, eye, legs, wings, halters and genital organs are formed from imaginal discs (Jory et al., 2012).

There is a clear dichotomy between the determination and the differentiation of the imaginal disc. Determination takes place during embryonic development whereas differentiation occurs during metamorphosis. Cells from different discs and with different determinations were dissociated into individual cells, combined, reaggregated, and implanted into larvae. After metamorphosis, these cells gave rise only to structures that were appropriate to their disc of origin, showing that the determination state of imaginal disc is really stable (Gehring, 1966; Gehring, 1967).

The stability of the imaginal discs has been challenged by culturing disc fragments in the abdomens of adult flies. For example genital discs have been cut in two parts. One part, the test piece, was implanted into a larva and the structure formed by this implant was checked after metamorphosis suggesting that imaginal discs retain their differentiation potential. The other part, the stem piece, was cultured in an adult fly where it can proliferate. After proliferation the stem piece is extracted, divided and reimplanted into a larva to check its identity and in adult fly to expand the cell population. Surprisingly, using this proliferation method, disc culture can be maintained over 300 generations. It suggests that imaginal disc cells can be grown indefinitely in adult fly.

The test piece was keeping its determination after few transfers. However, after additional transfers some cultures acquired the ability to generate adult structure belonging to other type of imaginal discs (for example antenna discs cells form suddenly wing structure). This stable switch from one commited state to another was defined as transdetermination (Hadorn, 1968). These transfer experiments have been performed with all the existing imaginal discs and reveal that transdetermination has some restriction. Some transdetermination events take place more frequently than others and some of them can become irreversible. Culture from genital discs can give rise to leg or antenna, however opposite transdetermination is not possible. On the contrary, reciprocal transdetermination can take place, for example, between leg and antenna (Figure 11).

What is the behavior of cells during transdetermination? Are they reverting to an undifferentiated state before they acquire their new fate? To answer to this question position-effect variegation has been used to track the fate of the

transdeterminating cells. Position-effect variegation can be detected when the gene *white* or *yellow* is rearranged to a place adjacent to heterochromatin (Wallrath, 1998). Then cells will stochastically silence or express the gene during embryogenesis. This rearrangement will be transmitted to all the daughter cells and will generate areas of marked and unmarked tissue. Hence, if cells involved in transdetermination return to an undifferentiated state this will reset the variegation state of the cell of interest resulting in the formation of a mosaic of variegation states in the transdeterminating disc. However, culture of discs leads to the formation of one type of variegation (marked or unmarked) and this variegation state remains stable during transdetermination suggesting that the switch from one committed state to another do not imply reversion to a more primitive or undifferentiated state, transdetermination is a direct process (Hadorn, Gsell, & Schultz, 1970).

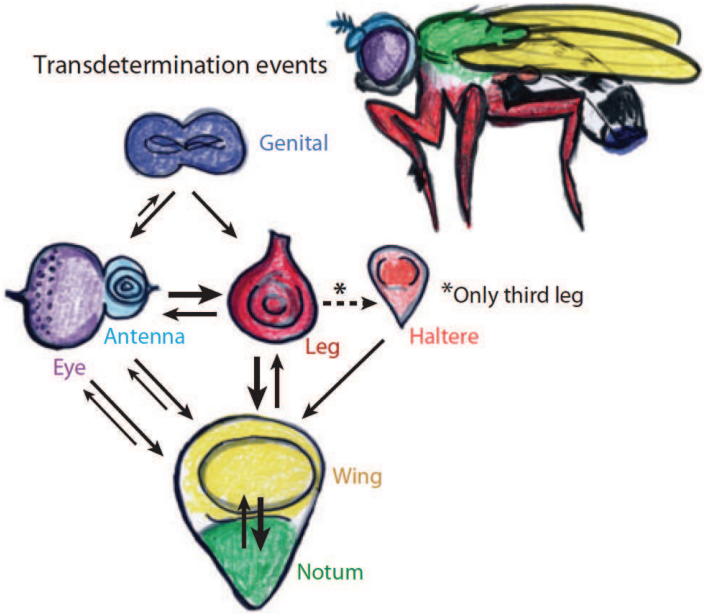


**Figure 11** – Transdetermination of imaginal discs: Transdetermination between imaginal disc can occur in a certain order. E.g. transdetermination of imaginal disc of leg into wing structure can take place and vice-versa. On the contrary, genital discs can transdeterminate into leg disc but the opposite never takes place. On this scheme the thickness of the arrows represent the probability for a transdetermination event to take place (Worley, Setiawan, & Hariharan, 2012).

Some mechanisms underlying transdetermination have been unraveled. For example, the c-Jun N-Terminal Kinases (JNK) pathway has an important role during leg to wing transdetermination. It has been shown that reduction of the JNK activity leads to a reduced frequency of transdetermination. In addition, transdeterminating

cells exhibit an increased JNK activity, which induces repression of Polycomb-group (PcG) genes. PcG activity stabilizes cell fate by repressing tissue-inappropriate genes, hence, during transdetermination, down-regulation of PcG allows the expression of these genes and facilitates the reprogramming process to take place (Lee, Maurange, Ringrose, & Paro, 2005).

### I.2.6) Transdifferentiation

Transdifferentiation is defined by the e switch from a differentiated cell type to another. This phenomenon remained controversial for decades. The work of Yamanaka and coworkers has eased the skepticism around cellular plasticity. For example, in a glossary published by the Nature journal, the term plasticity has been described as "Unproven notion that tissue stem cells may broaden potency in response to physiological demands or insults" (Smith, 2006). Although the iPSC cells technology has shown that cell fate can be plastic, some controversy still existed around phenomenon such as transdifferentiation. However, because of a tremendous number of recent reports, instance of transdifferentiation have been clearly and unambiguously described.

Transdifferentiation can take place naturally in some organisms such as *Drosophila*, *Caenorhabditis elegans*, or in mice. But transdifferentiation can occur as well during regeneration or being induced by over-expression of specific factors. Some examples, but not all, will be discussed in the following parts.

#### *I.2.6.1) Natural transdifferentiation events*

Two recent studies have shown that transdifferentiation takes place naturally in *Drosophila*. The eye of adult *Drosophila* contains a pair extra-retinal photosensory organ called eyelet. This adult structure is formed by four photoreceptors, which arise from twelve larval photoreceptors. Among these twelve larval photoreceptors, eight express a green light-sensitive Rhodopsin (Rh6); the remaining four photoreceptors express a blue light-sensitive Rhodopsin (Rh5). During development all the Rh6 photoreceptors die while the Rh5 photoreceptors survive. However, the adult eye is composed by only green-sensitive Rh6 photoreceptors. This suggests that a transdifferentiation of Rh5 to Rh6 photoreceptors took place during

metamorphosis. Indeed, it has been shown that the Rh6 photoreceptors found in the adult eyes are coming from the direct conversion of Rh5 photoreceptors in part because of a switch of rhodopsin expression (Sprecher & Desplan, 2008). This expression switch requires the *Ecdysone* hormone, which trigger Rh6 larval photoreceptors apoptosis and Rh5 to Rh6 switch. The *sens* gene seems to be involved in the survival of the four Rh5 photoreceptors through inhibition of apoptosis (Figure 12.A).

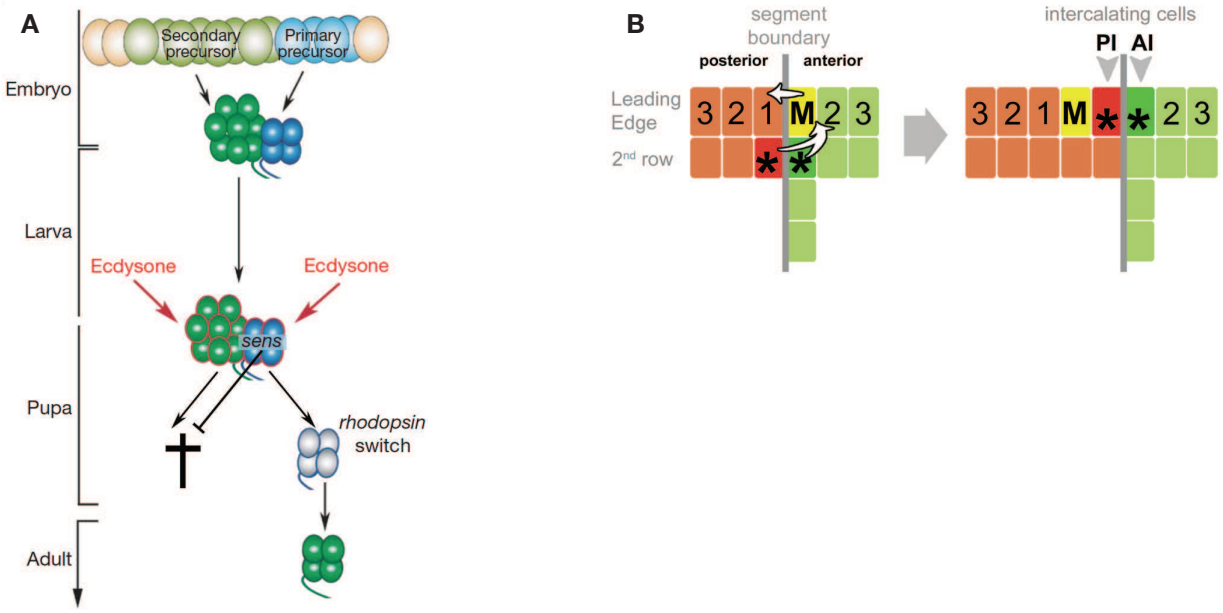


**Figure 12** – Examples of transdifferentiation in *Drosophila*. **A)** *Drosophila* eye arise from twelve eyelets. Eight of them express the green light-sensitive Rhodopsin (Rh6), the remaining four express the blue light-sensitive Rhodopsin (Rh5). During larval development, the *Ecdysone* hormone leads to apoptosis of the eight Rh6 positive eyelets and survival of the four Rh5 positive eyelets thanks to the *sens* gene. Between the pupa and adult stages a Rh5 to Rh6 switch occurs leading to the formation of an adult eye containing only four Rh6 positive eyelets. (Sprecher & Desplan, 2008). **B)** Epithelial mixer cells (yellow) move from an anterior to a posterior compartment. Crossing the compartment requires cell fate switch of the mixer cells in order to adopt the identity of the posterior compartment. Mixer cell movement allows cells from the second row (marked by a star) to intercalate in the leading edge and release tissue tension taking place during dorsal closure (Gettings et al., 2010).

*Drosophila* embryos are compartmentalized during embryogenesis. Each compartment is committed to a fate, which will give rise to specific adult structures. Epithelial cells of each compartment are known to never mix with cells from another compartment. However a recent study show that cells called "mixer cells" can move

from one compartment to the other during dorsal closure of the embryo. These mixer cells are located at each antero-dorsal part region of the segment (Gettings et al., 2010). When they cross the boundaries of two segments, these mixer cells change their fate in order to adopt the identity of their new location. This switch of identity relies on *de novo* expression of the selector gene *engrailed* (Figure 12.B). This switch from a compartment to another seems really important during dorsal closure. Indeed, the transition of mixer cell from a compartment to another allows underlying cells to intercalate in the leading edge. Increased tissue tension takes place during dorsal closure, which can lead to injury. Because of the movement of the mixer and the underlying cells, relaxation of the tissues can take place allowing proper embryogenesis.

To date, only few well-characterized case of natural transdifferentiation has been reported in mammals. One of them takes place in mouse. The developing heart is composed by the sinus venosus, which returns blood to the heart, the atrium and the ventricle. The vessel origin is still poorly understood and was source of debate in the heart development field. Using meticulous lineage tracing, it has been shown that coronary arteries arise from transdifferentiation of venous cells (Red-Horse, Ueno, Weissman, & Krasnow, 2010). Endothelial cells from the sinus venosus dedifferentiate and sprout to invade ventricle muscles. Invading cells in the upper part of the ventricle are reprogrammed into arterial cell fate. Cells from the bottom part stay dedifferentiate and form other sprouts, which will acquire arterial fate. The dedifferentiate cells not used to form arterial vessel will redifferentiate into vein.

Natural transdifferentiation seems to take place in all the phyla, but these events are certainly rare and are complicated to identify at the single cell level, explaining why they are so difficult to identify.

Note: The natural transdifferentiation event encountered in *Caenorhabditis elegans* will be discussed in the second part of the introduction.

### *I.2.6.2) Transdifferentiation during regeneration.*

Experiments performed during the 19th century by Colucci and Wolff have shown that adult newt can completely regenerate their lens after ablation. This regeneration process begins with proliferation and dedifferentiation of pigmented epithelial cell (PEC) coming from the dorsal iris. This dedifferentiation can be identified by the loss of pigmentation (Eguchi, 1963) and requires cell cycle re-entry, a pre-requisite for proliferation and regeneration. Several days after the lens ablation, a vesicle formed by depigmented PECs can be observed, in which synthesis of crystallins proteins begins. This lens regeneration has been identified as a clear transdifferentiation from a pigmented epithelial cell fate to a lens cell fate. This transdifferentiation event can also take place *in vitro* with less constraints (Eguchi, Abe, & Watanabe, 1974). Indeed during *in vivo* regeneration, only dorsal PEC can transdifferentiate into lens cells, however *in vitro* both dorsal and ventral iris cell can give rise to lens cells (Eguchi et al., 1974; Kodama & Eguchi, 1995).

Some amphibians can also regenerate their retina via transdifferentiation of the retinal pigmented epithelium (RPE). RPE dedifferentiate and re-enter the cell cycle to form a layer of neuroepithelium, which will be able to differentiate into different cell type of the retina leading to its regeneration (Filoni, 2009).

Heart regeneration in zebrafish has been described in many studies and requires dedifferentiation of cardiomyocyte (see part I.2.3.1). A recent study has identified a new mechanism underlying the Zebrafish heart regeneration. An atrial chamber connected to a ventricular chamber composes the Zebrafish heart. Distinct cell populations form these two regions. Injury can be induced in the ventricular chamber. Consistently with previous work (Jopling et al., 2010; Poss, 2002) the authors could see that heart regeneration occurs, in parts, through proliferation of ventricular cardiomyocytes, which were not affected by the injury but by using meticulous lineage tracing and time-lapse microscopy, the authors could observe that an other part of the heart regeneration was due to migration of atrial cardiomyocyte, which transdifferentiate afterwards into ventricular cardiomyocytes when they are in the site of injury (Zhang et al., 2013). These atrial cardiomyocytes

seem to pass through an atrial-progenitor-like state before turning into ventricular cells.

Together, these examples illustrate the fact that under certain circumstances such as injury, some calls can increase their plasticity and transdifferentiate to reform the missing organ or tissue and could take place in any organisms.

#### *I.2.6.3) Induced transdifferentiation*

Before the work of Yamanaka and coworkers several experiments provided evidence that under certain circumstances cells can change their identity. Transdifferentiation can be induced by several ways. The first one is to use ectopic expression of specific factors in a starting population of cells. These factors are often key master genes involved in specific developmental processes. The second approach uses cell fate destabilization. The cell population that is to be converted is turned first into a more primitive differentiation state. Then ectopic factors expression supplemented with specific culture conditions are used to induce the transdifferentiation. The following chapters review the induced transdifferentiation from the origin to the increased number of recent papers, which show that with the good combination of factors, any cell type could be converted into any other cell types. The selected examples have been accepted by the scientific community as true transdifferentiation event. However, our point of view on the limitations of these induced transdifferentiation will be discussed in another distinct chapter.

**The origin:**

In 1987, two studies demonstrated that over-expression of specific factors can lead to cell fate conversion. These studies have shown that immortalized fibroblasts treated with 5-azacytidine (5-azaC) differentiate into chondrocyte, adipocyte or muscle cells (Taylor & Jones, 1979).. Later, genomic DNA of fibroblasts turned into muscle was purified and transfected into untreated fibroblasts, and was proven to be sufficient to induce muscle formation (Lassar, Paterson, & Weintraub, 1986). In the following years, this led to the cloning of the helix-loop-helix transcription factor MyoD, which was shown to act as a strong inducer of myogenic genes. Indeed over-expression of MyoD in fibroblasts lead to muscle cells formation (Davis, Weintraub, & Lassar, 1987) and over-expression in other cell types such as

pigmented epithelium, nerve, fat or liver cell leads to activation of some muscle gene (but do not induce complete reprogramming) (Weintraub et al., 1989; Choi et al., 1990).

During the same year, it was shown that ectopic expression in larva of the leg fate-inducing homeobox gene *Antp* is able to convert antenna structure into ectopic legs suggesting that *Antp* can overcome the developmental program set in the antenna disc by installing a leg program (Schneuwly, Klemenz, & Gehring, 1987). In these experiments, *Antp* over-expression is done in the entire animal. Interestingly, few cells can be reprogrammed suggesting that the reprogramming effect of *Antp* is context dependent and cannot occur in any tissue.

These two findings showed for the first time that expression of specific factors could lead to cell fate conversion. These studies were a source of inspiration for Shinya Yamanaka to design a screen to find factors that can induce pluripotency in somatic cells.

Many examples of transdifferentiation events will be discussed in the following paragraphs. Something really striking is the fact that many examples of induced transdifferentiation were reported before the work of Yamanaka and colleagues but these results did not change the view of the scientific community on how cell can be plastic under certain conditions.

**Induced transdifferentiation in the haematopoietic system:**

Many example of induced transdifferentiation have been shown to take place in the haematopoietic system. The transcription factors GATA1 is known to direct haematopoietic progenitors in the erythrocytic and megakaryocytic lineage or the granulocytic and monocytic lineage (Arinobu et al., 2007; Iwasaki & Akashi, 2007). Forced expression of the transcription factor GATA1 in monocytic cell lines is sufficient to repress the monocytic markers and activate erythroid, eosinophil and megakaryocytic markers (Kulessa, Frampton, & Graf, 1995; Visvader, Elefanty, Strasser, & Adams, 1992). Similar over-expression in granulocyte and macrophage progenitors leads to their conversion into erythroid, eosinophil, basophil and megakaryocyte lineage. Ectopic expression of the transcription factor C/EBPα in committed B and T cell progenitors leads to their conversion into granulocyte and

macrophage precursors (Xie, Ye, Feng, & Graf, 2004; Zhang et al., 2004). This conversion has been shown to be only due to C/EBPα expression. Later it has been shown that co-expression of C/EBPα and PU.1 can induce conversion of 3T3 fibroblasts into macrophage-like cells (Feng et al., 2008; Xie et al., 2004). Genome-wide studies have shown that C/EBPα and PU.1 bind to specific macrophage genes and enhancers in fibroblasts. This results in the activation of the macrophage expression program, which induces the phenotypic conversion of fibroblast into macrophage-like cells (Natoli, 2010). All together these studies demonstrate that cell conversion in the haematopoietic system can occur through ectopic factor expression.

### Induced transdifferentiation into cardiomyocyte:

Cardiac muscle cells can be obtained by induced transdifferentiation. Microarray analyses have been performed to identify genes specifically expressed in the developing mouse heart. Data analysis has lead to the identification of 14 factors potentially essential for the heart development. These factors were screened for their ability to convert fibroblast into cardiomyocyte-like cells. After refinement, GATA4, myocyte-specific enhancer factor 2C (MEF2C) and T-box5 (TBX5) have been identified as the minimal cocktail necessary to reprogram fibroblasts into cardiomyocyte-like cells (Ieda et al., 2010). The resulting cells present an expression profile close to primary cardiomyocytes suggesting that complete reprogramming of fibroblasts may occur. Later, it has been shown that *in vivo* conversion of cardiac fibroblasts into cardiomyocytes can be done using the same cocktail of factors (Qian et al., 2012). In this study, the resulting cells are exhibiting cardiomyocyte-like properties. They generate action potential and can rescue partially the cardiac failure encountered in a cardiac dysfunction mouse model. A parallel study, using the same factors supplemented with the heart and neural crest derivatives expressed 2 (HAND2) gene, showed that the obtained cardiomyocytes improve cardiac function and regeneration of cardiac ischemic mouse (Song et al., 2012).. Similar induced transdifferentiation has been obtained by using cocktail of microRNAs (miRNAs). Several miRNAs have been identified to act during heart development. For example by itself miR-1 can convert fibroblasts into immature cardiomyocytes. Combined ectopic expression of miR-1, miR-133, miR-208 and

miR-499 leads to *in vitro* and *in vivo* conversion of fibroblasts into mature cardiomyocytes (Jayawardena et al., 2012).

### Induced transdifferentiation into cartilage cells:

Cartilage cells are difficult to collect and maintain. In order to overcome this problem, induced transdifferentiation of fibroblasts into cartilage cells can be performed. Over-expression of KLF4, MYC and the chondrogenic gene SOX9 converts skin fibroblasts into polygonal chondrocytes (Hiramatsu et al., 2011). These conversion leads to the formation of colony expressing chondrocyte specific genes. The newly obtained cells can be maintained during 6 weeks in culture and have to potential to make cartilage tissue. Induced transdifferentiation of fibroblast into cartilage does not go through a pluripotent state even if the reprogramming cocktail comprises two genes of the Yamanaka's cocktail (KLF4 and MYC) (Outani, Okada, Hiramatsu, Yoshikawa, & Tsumaki, 2011)

### Induced transdifferentiation into hepatocytes:

Transdifferentiation of mouse fibroblasts into hepatocyte-like cells have been recently reported by two independent groups (Huang et al., 2011; Sekiya & Suzuki, 2011). In both studies a screen for reprogramming factors has been performed. Two distinct cocktails have been found to have similar reprogramming properties. The first one uses a combination of GATA4, HNF1 homeobox A (HNF1A) and forkhead box A3 (FOXA3), the second uses only two factors, HNF4 with either FOXA1, FOXA2 or FOXA3. Ectopic expression of one of these cocktail induces expression of specific hepatocyte markers such as albumin, tryptophan 2,3-dioxygenase and transthyretin. Moreover, the resulting cells, when engrafted into mouse liver, were able to increase the survival time and restore the liver function of mouse model for liver failure suggesting that reprogramming of the fibroblasts was complete.

### Induced transdifferentiation of exocrine into endocrine pancreatic cells:

Expression patterns of 1100 transcription factors have been screened to find factors, which are predominantly expressed in the developing pancreas. From these observations, pools of several transcription factors have been tested for their capacity to convert exocrine cells into endocrine ß cells. This screen led to the identification of 3 genes, neurogenin 3 (NGN3), pancreatic and duodenal homeobox

1 (PDX1) and v-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MAFA), sufficient to reprogram *in vivo* acinar exocrine cell into endocrine cells. The morphology of the reprogrammed cells was indistinguishable from the endogenous ß cells. This *in vivo* induced transdifferentiation can restore the level of blood glucose in a mouse model for diabetes (Zhou, Brown, Kanarek, Rajagopal, & Melton, 2008). This suggests that the converted cells are fully functional and can fulfill their physiological role.

**Induced transdifferentiation into differentiated neurons or neuronal precursors:**

Many of the recent papers describing induced transdifferentiation by defined factors described conversion into neurons or neuronal precursors. Here again a screen for factors involved in neuronal development and epigenetic reprogramming has been conducted. Nineteen candidate genes have been selected and tested for the ability to reprogram mouse fibroblasts into neurons. Among them *Ascl1* has been shown to convert by itself fibroblasts into immature cells with neuronal morphology and expressing neuronal markers. However, in order to get electrophysiologically functional neurons capable of making synaptic circuits, the POU domain 2 (BRN2) protein and myelin transcription factor 1-like (MYT1L) had to be supplemented to ASCL1 (Vierbuchen et al., 2010). Later, similar induced transdifferentiation have been performed with human fibroblast (Pang et al., 2011) and hepatocytes (Marro et al., 2011). Surprisingly, the previous cocktail was able to partially reprogrammed human cells. All the obtained neurons were functionally immature. To have a complete transdifferentiation the gene *NeuroD1* had to be added to the original reprogramming cocktail (Pang et al., 2011). Transdifferentiation of fibroblasts into neurons has been possible by using miRNAs or by modulating their activity. Combined expression of miR-9/9* with miR-124 converts fibroblasts into neuron-like cells (Yoo et al., 2011). Another recent study demonstrates that repression of the RNA-binding polypyrimidine tract-binding (PTB) protein induces conversion of fibroblasts into functional neurons (Xue et al., 2013). The PTB protein seems to be involved in the blockage of miRNA action on a complex called REST (for RE1-silencing transcription factor), which leads to the derepression of neuronal specific genes.

The therapeutical applications of the use of such technology are tremendous and will be discuss in a following part. But we can easily envision that making neurons from fibroblasts will help the design of new regenerative medicine approaches. Neurons are by nature post-mitotic, then neurons obtained by induced transdifferentiation can be kept in culture but their population cannot be expanded. To overcome this problem conversion of fibroblasts into neuronal stem cells or neuronal precursors can be used. The proteins ASCL1, NGN2, HES1 (hairy and enhancer of split 1), ID1 (inhibitor of DNA binding 1), PAX6, BR2, SOX2, MYC and KLF4 are highly expressed in neural stem cells and have been used to reprogram sertoli cells into neural precursors (Sheng et al., 2012). These precursors can be differentiated into different neural cell types such as functional neurons, oligodendrocytes or astrocytes. A recent study showed that over-expression of Sox10, Olig2 and Zfp536 converts fibroblasts into oligodendroglial cells. These cells can differentiate into oligodendrocytes, which are able to form myelin sheath *in vitro*, when co-cultured with dorsal root ganglion cells, or *in vivo*, when transplanted into brain of shiverer mice (Yang et al., 2013). Unlike neurons, these precursors can be cultured and expanded *in vitro* to obtain a larger pool of cell. Then directed differentiation can be applied to obtain the desired cells.

**Induced transdifferentiation by cell fate destabilization:**

To improve reprogramming efficiency transient expression of the Yamanaka's cocktail has been used recently as an alternative to transdifferentiate somatic cells. This strategy is based on the fact that the over-expressed factor(s) will push the cell to a dedifferentiated or a destabilized state. When the cells are in this transition phase, their redifferentiation can be induced by expression of other factors or because of specific culture conditions. This method has been used to produce multilineage blood progenitors from fibroblasts. Over-expression of OCT4 with culture condition supplemented with cytokines leads to the activation of haematopoietic transcription factors and expression of the pan-leukocyte marker CD45 in fibroblasts. The obtained blood progenitors can be differentiated into the granulocyte, megakaryocyte, monocyte and erythroid lineages (Szabo et al., 2010). This induced transdifferentiation seems to be direct but no clear investigations have been made to know if fibroblasts pass through an undifferentiated state before their

conversion. Using the three factors OCT4, SOX2 and KLF4 supplemented with inhibitors of the JAK-STAT pathway and BMP4, fibroblasts have been induced to transdifferentiate into cardiomyocytes (Efe et al., 2011). Other examples in which neural progenitors have been obtained have been reported as well (Koch, Opitz, Steinbeck, Ladewig, & Brustle, 2009; Matsui et al., 2012; Thier et al., 2012). But in all the cases, it is not clear whether cells go or have to go through a dedifferentiated or a destabilized state. This key question has to be assessed to evaluate the possible therapeutic use of this approach.

**Induced transdifferentiation by transcriptome transfer:**

Fibroblasts have been converted into cardiomyocytes using a transcriptome transfer method. Total poly A+ mRNAs have been purified from cultured cardiomyocytes. These coding mRNAs have been transfected twice into fibroblasts using cationic lipids. In two weeks, the transfected fibroblasts acquire cardiomyocyte morphology. Global gene expression analysis of these obtained cells shows a decreased expression of fibroblast genes combined with an increase expression of cardiomyocyte genes. These cardiomyocytes are electrically excitable indicating that at least an important part of the cardiomyocyte program has been activated in fibroblasts. The same strategy has been used to convert astrocytes leading to the same results; cardiomyocytes could be obtained (Kim et al., 2011c).

According to the authors this method can be used to convert any cell type into any other cell type (James Eberwine, personal communication). To confirm this hypothesis, further investigations have to be performed and other cell types have to be converted. For example, it could be interesting in a fundamental point of view to try to convert post-mitotic cells such as neurons into completely unrelated differentiated cells such as intestinal cells or keratinocytes.

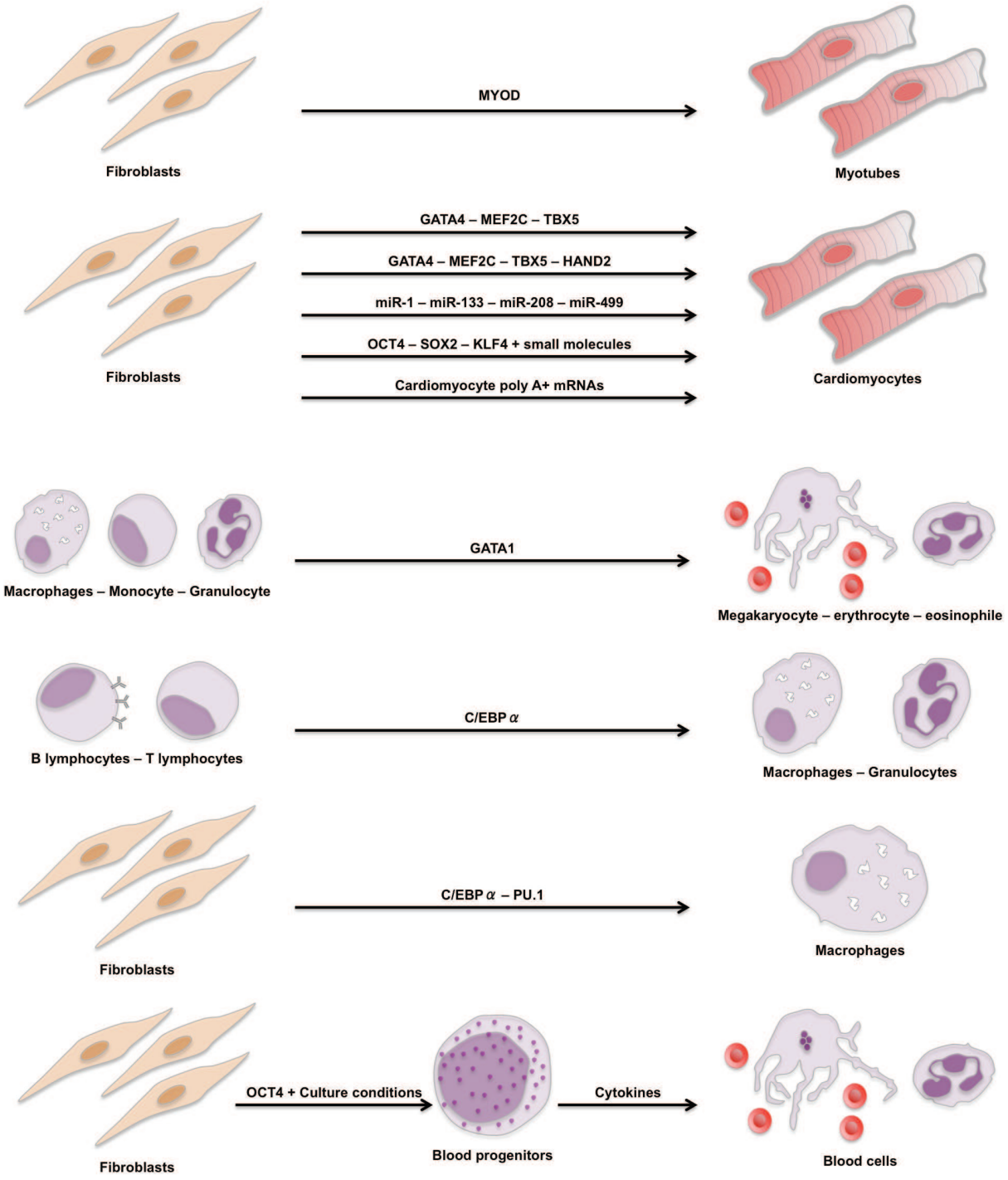


**Figure 13 Part 1** – Summary of the described induced transdifferentiation events

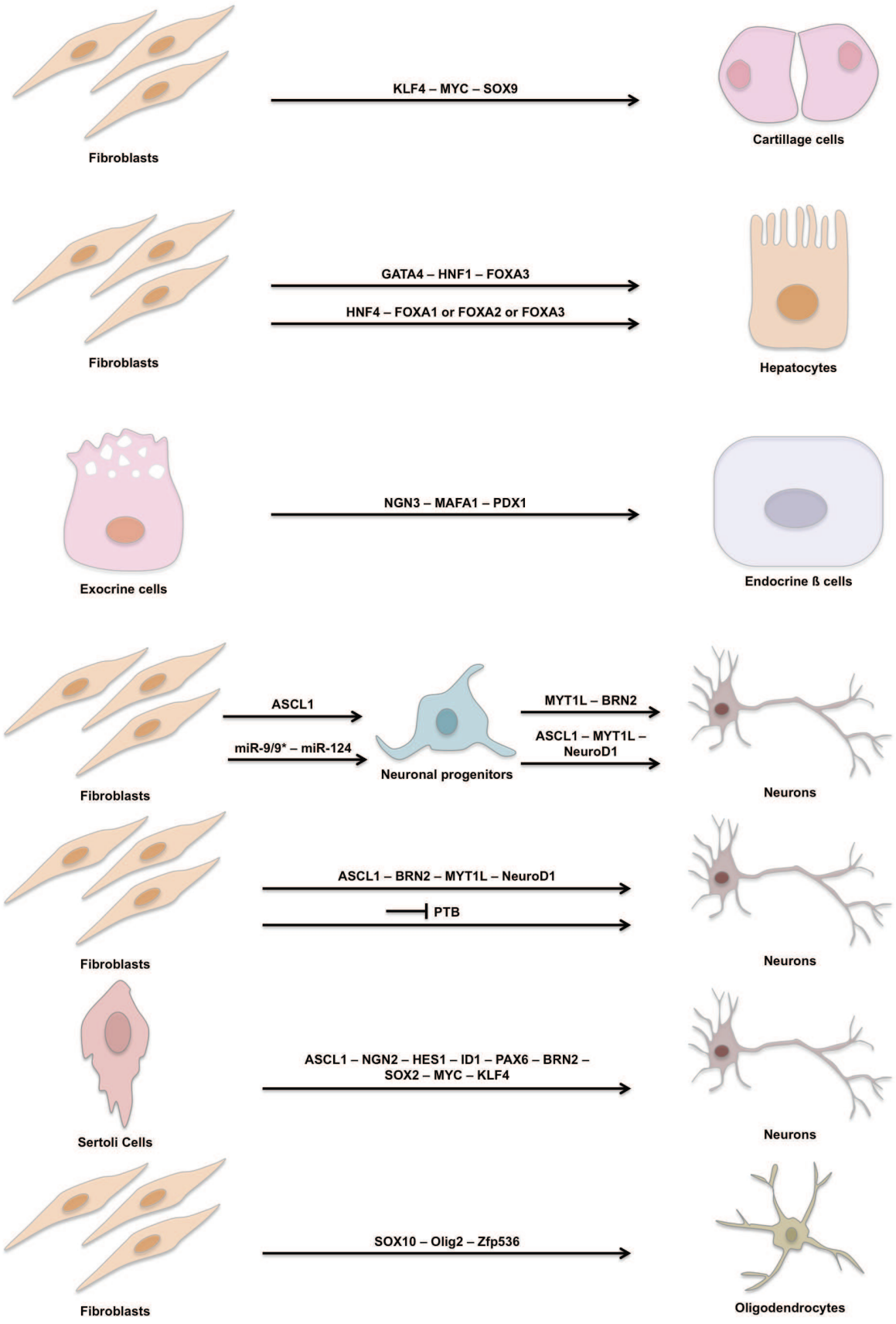


**Figure 13 Part 2** – Summary of the described induced transdifferentiation events

### I.2.7) Limitations and future challenges in the cell plasticity field

The previous chapter presents the induced transdifferentiation with an idealistic view. Every described reprogramming event seems to work perfectly and the conversion of one cell type to another appears complete. Nevertheless, most of the studies describing the induced conversion of one cell type to another lack key experiments that provide the evidence that reprogramming is indeed complete in every sense of the term.

To validate the completion of reprogramming several important features of the reprogrammed cells must be assessed. However not all of them are always studied in the studies described above.

Molecular markers have to be studied in the reprogrammed cells. These cells should express markers of the cell fate of interest and must not express markers of the cell fate of origin. It has to be carefully checked that the reprogrammed cells do not have a mixed identity after reprogramming.

In some cases, transcriptome analyses are performed on the reprogrammed cells and are compared to the transcriptome of cell with the fate of interest. In most cases, transcriptomes of both populations appear to be really close. However, these analyses are only made on cell population, thus scientists cannot assess if partially reprogrammed cells exist in the population. Single-cell transcriptome on several cells could be an alternative to these global analyses. By analyzing a large number of reprogramming cells at the single-cell level we can clearly assess if reprogramming is complete at the transcriptomic level. In these analyses, scientists have to be sure if the transcriptomic program of the cell of origin is switched off and if the transcriptomic program of the fate of interest is completely turned on. However, these single cell analyses require to be able to predict which cell will be reprogrammed before it starts to do so, which is, to date, almost impossible because of the low efficiency of the current reprogramming metods. Thus, many efforts are still remaining to be able to convert cells at high efficiency to perform these transcriptome analyses.

None of the current induced studies have shown if in the reprogrammed cells the epigenetic landscape is different to the one of the cell of origin. Indeed it has been shown that IPS cells exhibit an epigenetic signature associated with their cell type of origin (Kim et al., 2010; Polo et al., 2010; Kim et al., 2011b). Thus the same scenario could be encountered during induced transdifferentiation. The reprogrammed cells could keep the epigenetic signature of the cell of origin. This feature has to be assessed carefully to confirm that complete reprogramming took place at all level. If not, additional studies are required to determine the consequences of this partial epigenetic reprogramming on the final cells, and this on the long term, and also after re-implantation *in vivo* in a tissue.

Functionality of the reprogrammed cells has to be assessed, are the reprogrammed cells able to fulfill their physiological function? This feature has been assessed for most of the recent induced transdifferentiation studies. For example, exocrine cells converted to endocrine ß cell are able to produce insulin and restore glycaemia of diabetic mice (Zhou et al., 2008). Converted cardiomyocytes are beating as normal cardiomyocytes and are able to rescue partially the cardiac failure encountered in a cardiac dysfunction mouse model (Ieda et al., 2010). Converted neurons have the same electrophysiological features as endogenous neurons (Vierbuchen et al., 2010; Pfisterer et al., 2011). However, in this case, an added layer of complexity is brought up by the fact that in none of these studies where induced neurons are obtained, scientists do not have a clear idea of which type of neurons they got (besides being able to say, which sort of neurotransmitter these neurons can produce). This lack of precision is mostly due to the extreme complexity of the neuronal system, which is still not completely understood. In summary, functionality has to be assessed in each reprogramming study, and is one important argument showing that reprogramming is complete.

For most of the studies it is not clear if during reprogramming, the cell of origin is going through an undifferentiated and if this is accompanied by reversion to a more pluripotent or multipotent state. This aspect has to be studied in detail. Indeed, going through a more potent state could be dangerous if the reprogrammed cells are used for therapy. In the reprogrammed cell population, some of the cells

could keep their undifferentiated state and could be source of aberrant proliferation in patients, which could lead to diseases such as cancer.

Finally, it is not clear if reprogrammed cells are stable. Indeed, even though the reprogrammed cells are able to fulfill their function after transplantation in mice, to date, stability over long term of the reprogrammed cells has never been assessed. Moreover, it is not clear if the stability of the reprogrammed cells is dependent on the maintained expression of the reprogramming factors. Only one study has shown that cardiomyocytes are stable (although a limited period of time has been assessed) when reprogramming factor expression is abolished (Ieda et al., 2010). However this feature has never been studied in detail in most of the work describing induced transdifferentiation.

All these points have to be studied in details to assess that reprogramming of differentiated cell type to another is complete. Moreover, if induced transdifferentiation is the chosen strategy in the case of regenerative medicine, the reprogrammed cells must be stable and must not have any feature, which can lead to potential issue in the treated patients.

### I.2.8) Controversy around cell plasticity

All the previous examples of dedifferentiation, nuclear reprogramming, transdetermination and transdifferentiation are the evidence that cell identity can be plastic. Terminally differentiated cell can be turned back to a more primitive state or to another cell type. The Yamanaka's work brought another piece of evidence that pluripotency can be induced in somatic cells. But despite all these data, transdifferentiation has elicited much skepticism. It is maybe, from 2010, when a wave of papers demonstrating induced transdifferentiation have been published that most of the skepticism has vanished, at least when transdifferentiation is induced (mostly in *in* vitro studies).

This lack of consideration for the transdifferentiation is in part due to several controversial studies. One famous example of misinterpretation of transdifferentiation event took place between 1998 and 2002. Several studies demonstrated that neural and haematopoietic stem cells are not limited to give rise

to just cells of the nervous or blood system. They could be able to give rise to a plethora of different cell types such as liver, intestinal cells, heart or skeletal muscle (Brazelton, Rossi, Keshet, & Blau, 2000; Ferrari, 1998; Krause, 2001; Lagasse, 2000; Mezey, Chandross, Harta, Maki, & McKercher, 2000; Petersen, 1999; Pittenger, 1998; Theise, 2000). At the time, scientists suggested that this switch from one lineage to another is due to a transdifferentiation event. These findings were a revolution, in which tissue-specific stem cells displayed the developmental potential of embryonic stem (ES) cell, opening new doors for regenerative medicine. Indeed the need to destroy embryos to engineer ES cells would be bypassed. This was before the discovery made by two independent groups. They set culture conditions, in which neural stem cells and haematopoietic stem cells are co-cultured with ES cells. They thought that such culture condition would lead to transdifferentiation of the neural and haematopoietic stem cells into embryonic–like stem cells. Surprisingly, they found that the tissue-specific stem cells can fuse spontaneously with the ES cells and take their characteristics (Terada et al., 2002; Ying, Nichols, Evans, & Smith, 2002). This discovery was possible because of a clever lineage-tracing method. In both cases the starting tissue-specific stem cells are modified and express the green fluorescent protein (GFP) and a puromycin resistance cassette under the influence of the *Oct4* promoter (a gene only expressed in ES cells). In another hand, the ES used during the co-culture are resistant the hygromycin antibiotic. Both cell types were co-cultured in a medium containing puromycin, to select against the original ES cells. After several weeks, GFP positive cells resistant to puromycin could be obtained in the petri dish. This suggested that tissue-specific stem cells express ES cell-like characteristics. A quick glance at these results could have led the authors to conclude that transdifferentiation took place. However the obtained cells were, as well, resistant to hygromycin indicating that fusion of both cell types certainly occured. This fusion was confirmed by the fact that the obtained ES-cell-like cells were tetraploid (Terada et al., 2002; Ying et al., 2002).

A second example of controversy has been brought by the study of limb regeneration in axolotl. Urodele can regenerate some of their appendages such as the tail or the limb. Limb regeneration has been extensively studied and models of

the mechanisms underlying regeneration have been reinterpreted several times. Limb regeneration takes place in different phases. After amputation, wound healing takes place, then a mass of proliferating cells called blastema is formed at the tip of the regenerative part. This blastema proliferates and gradually starts to form differentiated structures such as bones, nerves or muscles. After several weeks a complete new limb is formed. Surprisingly the regenerated limb has no scar of the amputation (Figure 14) (Brockes & Kumar, 2002). Because in the blastema, all the cells present the same morphology and appear undifferentiated, it was thought that the blastema was a mass of homogenous pluripotent or multipotent cells coming from the dedifferentiation of adjacent cells of the amputation zone (Brockes & Kumar, 2002). It has been as well suggested that some cell transdifferentiates from their original lineage to give rise to other cell types during the regeneration process. After amputation, spinal cord cells were shown to migrate to the regeneration site and transdifferentiate to muscle or cartilage cells (Echeverri & Tanaka, 2002). Here again, precise tracing of the cell behavior has challenged this view of regeneration. Using GFP based tracing methods, in which GFP-positive cells from a donor salamander are engrafted into the regenerating part of a recipient salamander, the lab of Elly Tanaka (which was the first lab to postulate that salamander tail regeneration takes place via transdifferentiation) could demonstrate that cells of the blastema are not pluripotent but their differentiation potential is dependent on their cell-of-origin identity. For example, muscle cells will give muscle and cartilage cells will give muscle. This study completely redefined the composition of the blastema. It is not composed by homogenous population of pluripotent cells, instead it is an heterogeneous mass of tissue-specific progenitors (Kragl et al., 2009).

All these controversial studies highlight one key problem when studying cell plasticity in multicellular organisms: the lack of traceability between the starting cells in a population and the reprogrammed cells. Sub-optimal experimental designs, due to the lack of tools allowing complete traceability of cells *in vitro* or in a multicellular organism, coupled with over-reaching interpretations of the data obtained are at the origin of the misinterpretations described above. Nowadays, advanced CRE/LOX based methods (Alcolea & Jones, 2013) can be used to trace cells in multicellular organisms. However, CRE/LOX tracing methods are dependent on the use of

specific promoters, which tissue and dynamics of expression have to be perfectly characterized to allow unambiguous tracing. Indeed, if the used promoter labeled few unseen stem cells, the obtained results may be erroneous. Added to this linage tracing technology, the evolution of single cell analysis (such as single-cell transcriptomic (Hashimshony, Wagner, Sher, & Yanai, 2012)) will allow easier unambiguous identification of the different steps taking place during reprogramming.

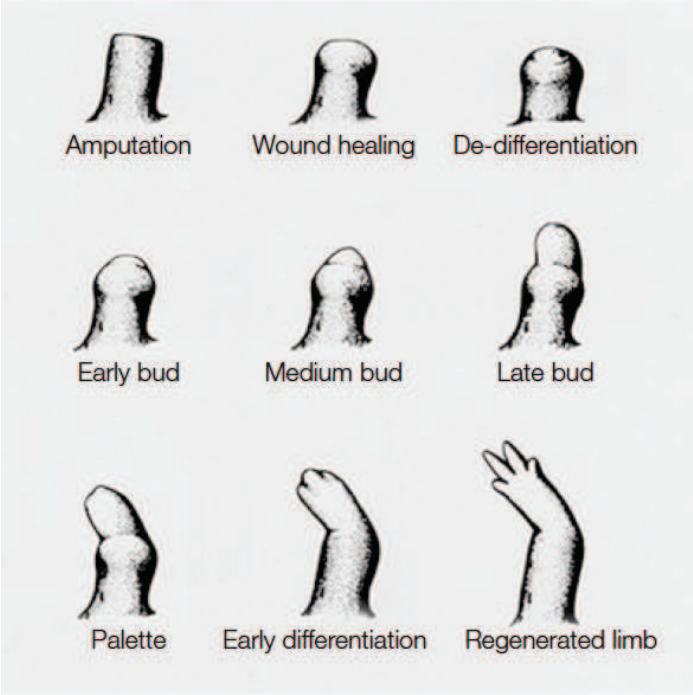


**Figure 14** – Regeneration of the urodele limb. After amputation, wound healing takes place and is followed by the formation of a blastema at the tip of the amputated zone. This blastema will proliferate and differentiate to give rise to a completely reformed limb (Brockes & Kumar, 2002).

### I.2.9) Maintenance of cell identity

Production of iPSCs, natural and induced transdifferentiation or dedifferentiation, all these phenomena clearly demonstrate that the differentiated state is not irreversible. However recent studies suggest that the more differentiated a cell is, the more difficult it is to reprogram (Pasque et al., 2011).. To understand how a cell can be reprogrammed, we have to understand how cell identity is maintained. Is it an active process or a passive process? Is it easy to modify? We have some answer since the 1980's but recent knowledge have brought a more precise view of cell fate maintenance.

Cellular reprogramming experiments demonstrate the need of continuous instructive regulation to maintain cell fate. Cell fusion of differentiated cells leads to the formation of multinucleated non-dividing cells called heterokaryon. Experiments implying fusion of cell of different species makes possible the tracking of species-specific markers in the obtained heterokaryon. Such experiments demonstrate that specific regulators of one cell can force the expression of cell-type specific genes in the second cell (Blau, Chiu, & Webster, 1983). For example, fusion between mouse myotubes with different human cells such as fibroblasts, neural cells, keratinocytes B-lymphocytes or hepatocytes induces suppression of their differentiated traits and human muscle cell markers are induced (Blau et al., 1983; Wright, 1984; Miller, Pavlath, Blakely, & Blau, 1988; Terranova, Pereira, Du Roure, Merkenschlager, & Fisher, 2006; Palermo et al., 2009). These experiments demonstrate that the differentiated state is certainly continuously preserved because of actively maintained instructive regulators even when the cell is fully differentiated. The same conclusion can be made because of the induced transdifferentiation experiments. In the case of conversion of fibroblasts into cardiomyocytes, neurons or macrophages with defined factors, cells may not to go trough an (undifferentiated) pluripotent or multipotent state before conversion (Feng et al., 2008; Ieda et al., 2010; Vierbuchen et al., 2010; Kim et al., 2011a; Pfisterer et al., 2011; Sekiya & Suzuki, 2011). See also the contemporary data presented in the *C. elegans* section, where this issue has been rigorously addressed. Indeed, even if in most studies striking evidence are missing, conversion from one cell type to another with defined factors may be direct. The passage from a cell fate to another does not seem to recapitulate the developmental phases needed to acquire normally the final cellular fate. The factors used to convert cells have a strong instructive capacity. These observations suggest that instructive regulation is one source of the maintenance of cell fate.

In physiological conditions instructive transcription factors exist and are expressed to actively maintained cell identity. For example, in *Caenorhabditis elegans* (*C. elegans*) neuron identity is maintained by continuous expression of terminal selector genes (Hobert, 2008). These factors are often regulating downstream genes giving to a cell its particular phenotype. It has been shown that terminal selector genes can auto-regulates their expression to ensure stable cell fate.

In *C. elegans*, sensory neurons (such as ASE neurons) are maintained because of the *che-1* gene, which regulates ASE-specific genes. In *che-1* null mutant, ASE neurons do not acquire their identity but still express pan-neuronal markers indicating that *che-1* is certainly a terminal selector gene continuously required to maintain ASE fate (Uchida, 2003; Chang, Johnston, & Hobert, 2003; Etchberger et al., 2007). The same mechanism is found in AIY fate maintenance. Maintained activity of 2 heterodimers formed by MEC-3/UNC-86 and TTX-3/CEH-10, which control the continuous expression of *unc-3*, are required to maintain the fate of AIY neurons (Xue, Tu, & Chalfie, 1993; Duggan, Ma, & Chalfie, 1998; Altun-Gultekin et al., 2001; Zhang et al., 2002; Wenick & Hobert, 2004; Kratsios, Stolfi, Levine, & Hobert, 2012). These simple models from invertebrate are true as well in mammals, but they are often more elaborated. They imply more factors and more hierarchical interactions while the general principle remains the same. For example, a first wave of instructive factors can lead to the expression of a second wave of downstream instructive factors, which will give to the cell its identity. Then, the genes of the first wave can be downregulated, while the genes of the second wave manage cell fate maintenance.

It is clear that cell fate maintenance is due in a large part to continued and active maintenance of transcriptional networks. Epigenetic factors and chromatin modifications are important as well, but all the details concerning their role in cell fate maintenance will not be detailed in this manuscript. Nevertheless, we can add that the stability of a cell fate can be maintained through chromatin compaction or decompaction of specific loci. For example, genes required to maintain cell identity are made accessible that way to the transcription machinery while genes not required for this process may not be accessible. During cell plasticity events, when cells change their identity, they have to overcome the entire transcriptional program set to maintain cell fate and to remodel partially their epigenetic status. Thus, understanding the key event allowing this switch from one transcriptional program to another will certainly be the key to manipulate cell fate at will. Maybe one day, we will be able to take any cell of the organism and efficiently turn it into the cell fate we desire.

### I.2.10) Cell-plasticity based therapeutical approaches

The recent explosion of the cell plasticity field has been driven by the thirst of knowledge from scientists, who would like to understand the mechanisms underlying these phenomena, but the therapeutical potential of cellular plasticity is tremendous and has also sparked several studies. As attested by the myhology the mythology, people always dreamed to be able to regenerate missing organs or appendages. In human, the only tissue able to clearly regenerate is the liver. Replacing missing tissue or damaged organs is the challenge of regenerative medicine.

To date, grafts are used to replace missing or damages organs and tissues. However, lack of donor and potential immunological incompatibilities are important issues. Moreover some organs such as the brain cannot by engrafted. To face this problem, scientists have thought to use ES cells. Because of their pluripotency, they can give rise to any tissue, however their use is source of troubles. The first and biggest problem is that to obtain patient-specific ES cells embryos are needed. The only way to obtain patient-specific ES indeed is the use of therapeutical cloning that will lead to the death of a living embryo, both of which are ethically unacceptable. The second problem is that *in vitr*o differentiation protocols are not controlled, remained poorly efficient, and the growing of new tissue or organs is to date, out of reach.

The discovery of iPS cells brought a new hope for regenerative medicine. Using this technique, it is now possible to take any cell of a patient, turn them into pluripotent stem cells and differentiate them into the tissue of interest. This was the first prospect for the future use of iPS cells in regenerative medicine. In order to be used in regenerative medicine the cells used to replace the damaged tissue have to be safe. Unfortunately it is not the case for the iPS cells. The first known issue was the potential reactivation of the factors used during reprogramming. They are known to be highly oncogenic, so their reactivation could be really deleterious and could lead to tumors formation in the healing patient. Many effort have been done to overcome this problem, iPS cells can now be created by using many new techniques in which the inducing factors cannot be reactivated (Gonzalez, Boue, &

Izpisua Belmonte, 2011). Second, IPS cells are prone to acquire genetic aberrations such as copy number variation, aneuploidy, or aberrant epigenetic profiles (Mayshar et al., 2010; Laurent et al., 2011; Gore et al., 2011; Hussein et al., 2011). These aberrations are acquired during the reprogramming and the subsequent culture of the iPS cells, and again this can lead to really problematic issues in the patient. One last point, which is still open to debate, concerns the putative immunogenicity of iPS cells. One key argument for the use of iPS cells in regenerative medicine was the fact that these cells are directly coming from the patient himself to treat. Thus, the obtained pluripotent cells would have exactly the same immunological profile than the patient. However a recent study suggests that injection of iPS cells coming from the same mice can lead to immune reaction (Zhao, Zhang, Rong, & Xu, 2011), which is going against one fundamental precept of the use of the iPS cells. Recently two publications have suggested that this immunogenicity is certainly negligible (Araki et al., 2013; Guha, Morgan, Mostoslavsky, Rodrigues, & Boyd, 2013). Because of all these potential risks, iPS cell are far of their clinical use in regenerative medicine. To date, iPS cells are mostly used to create disease-specific cell line to try to understand the physiopathology of specific disorders and to design new potential drugs (Cherry & Daley, 2012).

To avoid all these issues the use of transdifferentiation can be a good alternative. Many of induced transdifferentiation studies have suggested that passing through a pluripotent or multipotent state is not required to switch from one cell type to another. Thus, the risk to use cells partially reprogrammed with ES cell features is avoided. The fact that induced transdifferentiation can be done *in situ* and *in vivo* open new doors to regenerative medicine. In fact, ß cells (Zhou et al., 2008), cardiomyocyte (Song et al., 2012) and recently neurons (Torper et al., 2013) demonstrate that healthy cells of a patient could be reprogrammed into the needed cells directly on site. This would avoid all the *ex vivo* cell manipulation of cells, which can leads in some case to appearance of aberration (as it is for iPS cells).

The understanding of cell plasticity has exponentially increased recently. The tremendous potential of cell fate manipulations for regenerative medicine is one big driver for research. For example, it has been very recently shown by a Japanese

team that vascularized and functional human liver can be recreated from iPS cells (Takebe et al., 2013). This work demonstrates how far we can go by using the potential of cell plasticity and what could be the possibilities in regenerative medicine when the issues (i.e. tumorigenicity or immunogenicity of IPS cells) linked to cell fate manipulation will be fixed.

## I.3) A refreshed view of the Waddington's landscape

Conrad Waddington proposed his theory in 1957. Today in 2013, the knowledge we have acquired during all these years allows us to reinterpret the Waddington's view of development. It has to be noted that the concept proposed during the 1950's were really innovative, when we consider the data available about development at this time.

If we consider the definition given in I.2.2. We know now that the rolling ball of the Waddington's landscape can follow different downward and upward directions.

In the case of dedifferentiation (or retro-differentiation), the cell (the rolling ball) acquired its final identity but can go back slightly to reach a less differentiated state in its own lineage (Figure 15.A). In the case of nuclear reprogramming, the ball has reached its final position and goes back to its original starting point (Figure 15.B). The ball can go transversally to another valley before it reaches its end point when transdetermination occurs (Figure 15.C). In the same idea, during transdifferentiation the rolling ball reaches its final position but can cross the valley to reach another final position (Figure 15.D). In all these cases, genes are still at the origin of the valley, but they are as well at the origin of the directional changes of the rolling ball. We can then conclude that development and more specially cell differentiation are not fixed. The next challenges are to understand all these phenomena in details.

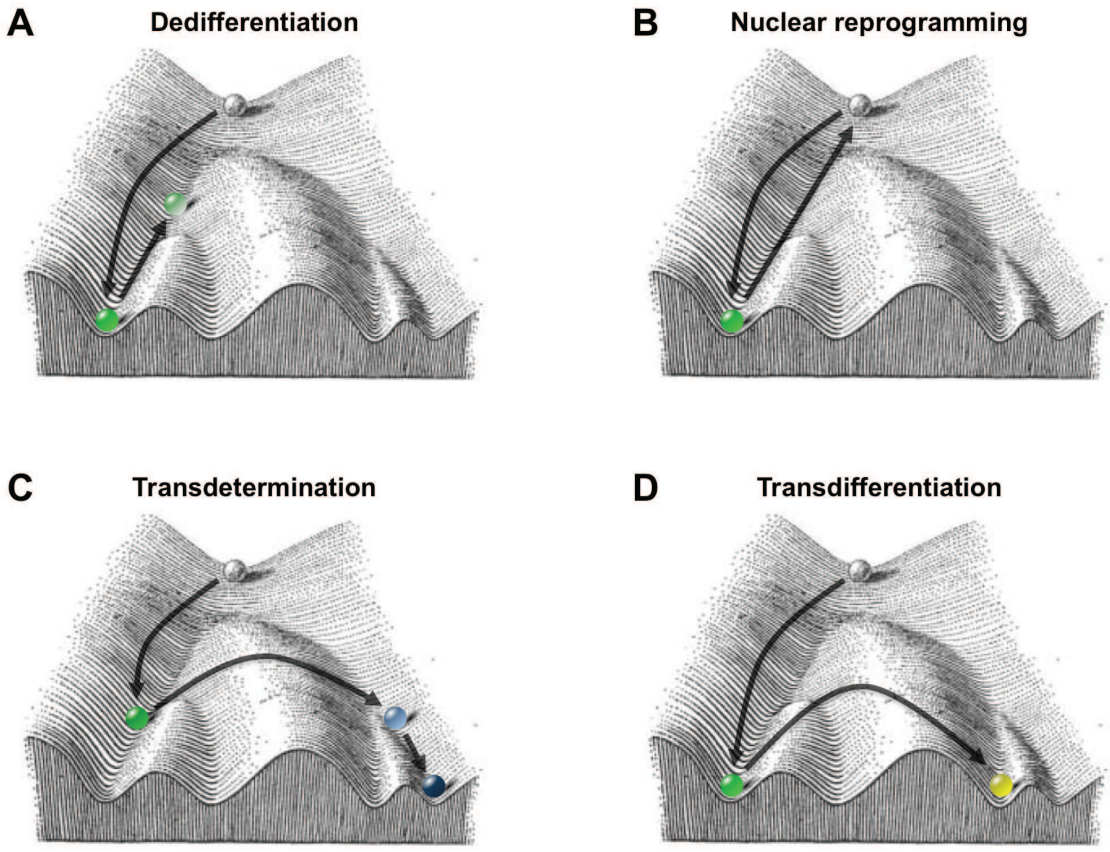


**Figure 15** – A refreshed view of Waddington's landscape. **A)** During dedifferentiation, cells can reach their final identity and revert to a less differentiated or to unspecialized cell, which is not pluripotent **B)** During nuclear reprogramming; fully differentiated cells can be reprogrammed to return to a pluripotent state. **C)** During transdetermination committed cells can change their commitment and differentiate afterwards into another cell type. **D)** During transdifferentiation a cell can switch its final differentiation state to another.

# II) *Caenorhabditis elegans* as a model to study cell plasticity

## II.1) The worm *Caenorhabditis elegans* (*C. elegans*)

In 1900, Emile Maupas described for the first time this small bacteriovorous nematode (also called round-worm) (Maupas, 1900). *C. elegans* has been studied essentially by nematologists during many years. During the 1960's-1970's, Sydney Brenner was looking for the "perfect" model that was easy to observe, easy to keep and easily manageable for genetics to study "how genes might specify the complex structures found in higher organisms" (Brenner, 1974). He selected *C. elegans* to start a new program in genetic research at Cambridge University. This was the start of the *C. elegans* story as a model organism (Brenner, 1974).

In the lab, *C. elegans* does not need fancy equipment to grow. *C. elegans* feeds on bacteria and can be maintained at 15°C to 25°C on agar plates. The worm is small, an adult is approximately 1mm in length, thus millions of worms can be kept in a small place. It has the advantage to be see-through throughout its life cycle, and then observations can be made at the cellular level under a microscope without the need of specific tissue treatment. The *C. elegans* life cycle is short (Figure 16). It takes around 3 days from an egg to an adult, making genetic experiments and worm population expansion quick. In a worm population, self-fertilizing hermaphrodites are in majority. Males naturally exist at low percentage (0,1%) and can be used for genetic crossings. *C. elegans* can be cryopreserved at -80°C, stock of worms can be kept indefinitely at this temperature.

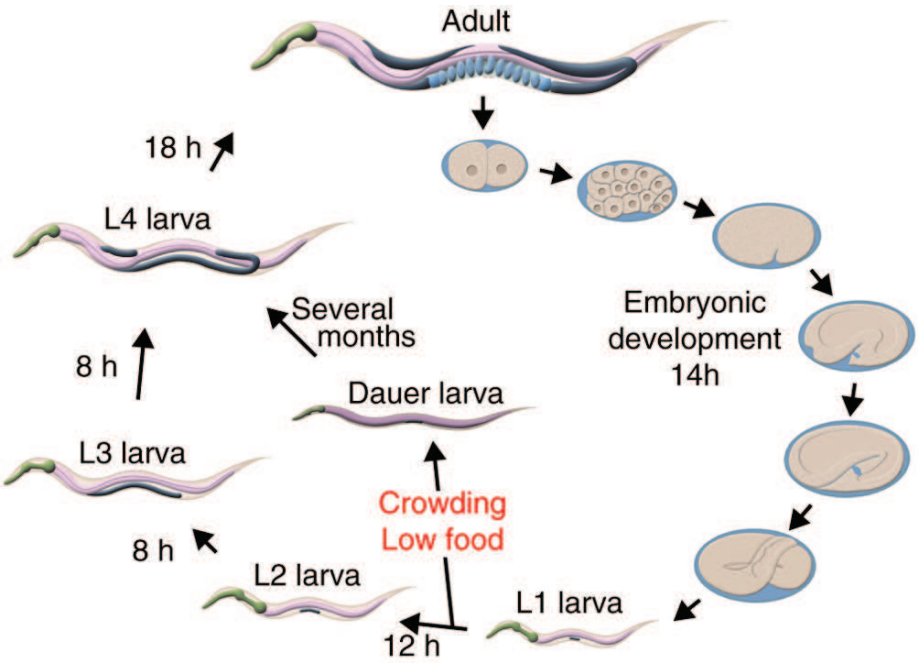


**Figure 16** – The *C. elegans* life cycle at 22°C takes around 3 days from embryo to adult (Murgatroyd & Spengler, 2010.

*C. elegans* is a simple model but display all the features of any animal; the worm possesses muscles, digestive system, skin, an elaborate nervous system and many other tissues. Interestingly all these tissues and organs are built with few cells. The complete cellular lineage of the embryo and larvae has been described ans is essentially invariant (Sulston & Horvitz, 1977; Sulston, Schierenberg, White, & Thomson, 1983). It shows that the hatching larva is composed of 558 nuclei, and the adult hermaphrodite is made of 959 somatic nuclei. The lineage analysis brought to the *C. elegans* community a complete map of all the cell division taking place during development from the eggs to the adult worm (Figure 17).

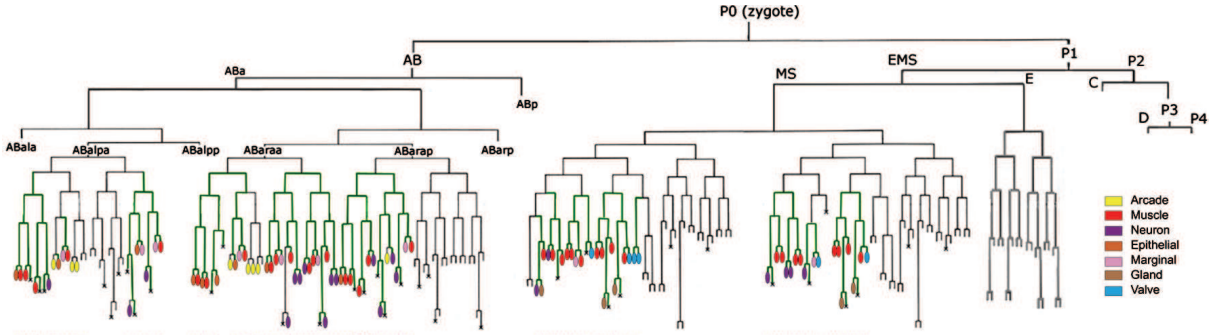


**Figure 17** – Example of the power of the cell lineage map. The green lines represent all the cells, which will participate to the pharynx formation. Each color represents a pharyngeal cell type. This scheme represents by itself the power of the cell lineage identification. For each cell we can determinates its past and its future without bias (Mango, 2007).

Sydney Brenner performed the first mutagenesis screen in *C. elegans* and published 619 mutant strains in his first paper (Brenner, 1974).. These mutation have been used to define six linkage groups indicating that hermaphrodite worms have 6 pairs of chromosomes, sex being determined by the number of sexual X chromosomes (hermaphrodites have two, noted XX; males have one, noted XO).

The genome of *C. elegans* is around 100 megabases (Sulston & Brenner, 1974) and because a physical map of overlapping cosmids already existed, these elements were favorable to launch the *C. elegans* genome project which lead to the first entire genome sequenced (The *C. elegans* Sequencing Consortium, 1998). The availability of this sequence completely changes the way of working of the *C. elegans* community. Large-scale projects could be launched such as the study of gene expression pattern, systematic generation of knockout mutant or for more focused project, the analysis of a single gene.

Over the years, *C. elegans* has been accepted as a powerful animal model. Many tools have been developed to allow scientists to answer their biological questions. For example, the worm is a perfect model to conduct forward and reverse genetic screens. These two unbiased approaches can be used to discover genes involved in a specific biological process. The mutation in a gene important for a process will lead to a mutant phenotype, which can be used as readout for the performed screen. In forward genetic screen, mutagen agents are applied on *C. elegans* population leading to the appearance of all possible phenotypes. Researchers can select the worms having the phenotype of interest and try to identify the related mutations. This mutation can be mapped by different methods (for example by deep-sequencing (Zuryn, Le Gras, Jamet, & Jarriault, 2010)) to identify the mutated gene and maybe identify a new actor for a biological process. Forward genetic screen was the first method used on *C. elegans* by Sydney Brenner to isolate mutant worms (Brenner, 1974). In the worm, double stranded RNA can be used to knockdown specific genes. This process is called RNA interference (RNAi) and has been characterized for the first time in the worm (Fire et al., 1998). Bacteria expressing double stranded RNA against a gene of interest can be used to feed the worms. We can then observe if a given gene, when knocked-down, can cause a

particular phenotype we are interested. To conduct genome-wide reverse screens by RNAi, the laboratory of Julie Ahringer has developed a library of bacteria expressing double stranded RNA, which cover almost all the genome (Kamath et al., 2003). Thus, it is possible to screen all the gene of this library for the process we are studying. GFP has been used for the first time in a living animal in *C. elegans* (Chalfie, Tu, Euskirchen, Ward, & Prasher, 1994). Today, this protein is used by every lab all over the world and has been used to develop a plethora of cellular markers available for the community. The expression pattern of any gene of interest can be deducted simply by looking at transcriptional reporters (promoter fused to GFP) or translational reporters (promoter and open reading frame (ORF) fused to GFP).

Today, *C. elegans* is a model for many research fields. One third of the somatic worm body is made of neurons (302 neurons in the adult worm), which makes it a really good model to study neurobiology and behavior. All the neurons have been identified, their positions and their synapses have been described because of meticulous electron microscopy studies (White, Southgate, Thomson, & Brenner, 1986). Many micro-RNA processes have been unraveled by using the worm. Cell death has been identified in *C. elegans*. Aging is now extensively studied as well as centrosome biology, cellular polarity or the emerging field of trans-generational epigenetic inheritance. Many conserved and very important pathways and mechanisms have been described in the nematodes (EGF, Notch, Wnt, insulin or BMP pathway). Of course it could be a hard and long task to talk about all the biology of *C. elegans*, but today many new opening labs choose *C. elegans* to conduct their research, emphasizing how this model is suitable to study any biological process.

## II.2) *C. elegans* as a model to study stem cell biology

Stem cell biology is a constantly growing field. It encompasses many research aspects such as the control of totipotency, interactions between tissue stem cell and their niche or how stem cell renewal occurs. To answer to these questions, many animal models can be used but accessible tools such as genetics

or cellular and biochemical analysis can be achieved more easily in invertebrate models. Moreover, unbiased systematic analyses (such as genetic screens) can be performed easily. In C. elegans several systems are used for stem cell biology; i) the germline, for its totipotency and the implication of germ cell-niche interaction for its homeostasis ii) the seam cells for their division pattern mimicking asymmetric cell division used by stem cell to self renew. These two models will not be discussed in profound details; we will give an overview of why they are good models to answer questions relevant to the stem cell field.

### II.2.1) The C. elegans germline, a model to study stem cell-niche interactions

In hermaphrodites, two polarized U-shaped tubes form the gonad. Somatic cells are covering the external part of the gonads while the internal part is formed by a syncytium of individual germ cell nuclei. These nuclei are positioned circumferentially in open membranes and share a common cytoplasm called the rachis (Figure 18). Although the germline is a syncytium, each nucleus behaves as if there were in individual cells. The germline is regionalized; the distal part of the gonad contains proliferating mitotic germ line stem cells. These cells move proximally and enter a meiotic zone. Then cellularization takes place, which will gives rise to arrested oocytes.

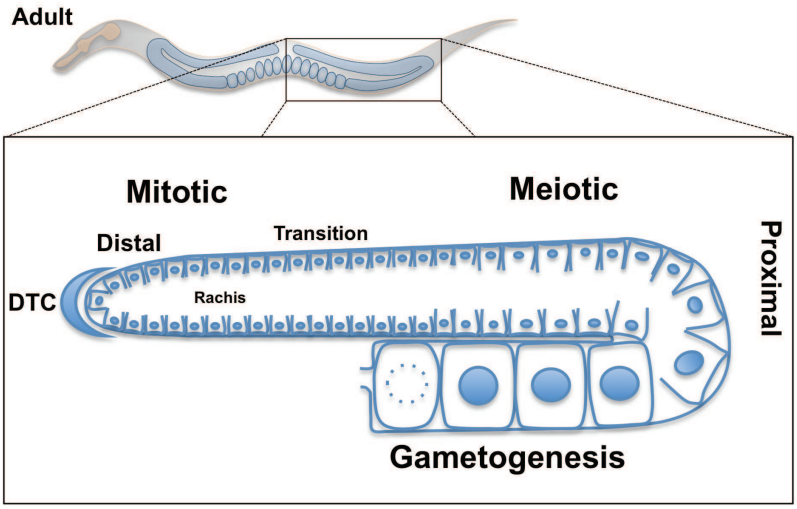


**Figure 18** – The *C. elegans* germline. Schematic representation of one gonadal arm. The germline is polarized syncytium with a mitotic distal part and a meiotic proximal part. The DTC represent the niche, which control germ cells proliferation. Adapted from (Zuryn, Daniele, & Jarriault, 2012).

Pioneer work on the *C. elegans* germline identified for the first time at the cellular level a stem cell niche (Hirsh, Oppenheim, & Klass, 1976). The germline stem cells (GSCs) are kept under a proliferative state in a niche defined by a somatic cell called the distal tip cell (DTC). The DTC is at the distal end of the gonad and cover it until the tenth row of germ cell nuclei. Manipulation of the DTC leads to proliferation aberration or gonadal problems. Ablation of the DTC leads to inhibition of the GSC proliferation and induces their differentiation into gametes. Altering the position of the DTC results on ectopic proliferation of the germ cells in contact with this misplaced DTC. Thus, the DTC is a key component of the germ cell niche. (Byrd & Kimble, 2009). The proliferative state of the germ cell in contact with the DTC is maintained because of the Notch signaling pathway (discussed later). In the proliferative zone, germ cells divide extensively and move away from the DTC. The nuclei are not under the influence of Notch signaling anymore and switch from mitosis to meiosis. The stem cell niche of *C. elegans* has structural similarities to niches found in other species such as the *Drosophila* female germline stem cell niche; the hub or the *Drosophila* germinal tip (Lehmann, 2012). It has been shown that adhesion through cadherins and integrins retains the *Drosophila* GSCs in the niche (Lehmann, 2012). These adhesions orient the axis of division of GSCs resulting in asymmetric cell division. Asymmetric division gives rise to two daughters with different fates. The first one stays in contact with the niche and keeps its germ cell identity. The second daughter cell progresses in the germline and differentiates into gametes. By contrast with the situation in drosophila, asymmetric cell division does not take place in the *C. elegans* germline. There is no bias in the orientation of the cell close to the niche (Crittenden, Leonhard, Byrd, & Kimble, 2006).

Using genetics, many genes have been found to be involved in germ cells proliferation, differentiation and control of their totipotency. Some of these aspects will be discussed in the following parts.

### II.2.2) The seam cells, a model to study stem cell-like lineages

To ensure proper tissue homeostasis the balance between proliferation and differentiation has to be tightly regulated. This regulation takes place at the stem cell level where divisions are controlled to avoid over-proliferation. Indeed tissue stem cell can divide following two models; Stem cells can divide asymmetrically to give rise to one cell, which will proliferate and keep the stem cell identity (self-renewal), while the second one will differentiate. On the other hand, stem cells can divide symmetrically to increase the pool of stem cells. The molecular mechanism underlying the regulation of these modes of division are still not well understood. This is an important issue to understand how stem cells control the choice between differentiation and self-renewal expansion.

The lateral epidermal cells of *C. elegans*, the seam cells, can be used to study this aspect of stem cell biology. Seam cells proliferate to self-renew and divide asymmetrically to give rise to differentiated neural and epidermal cells. Seam-cell lineage is an interesting model to study stem cell division patterns. The seam cells are born during embryogenesis and form ten bilateral pairs of cells (H0 to H2, V1 to V6 and T). During larval development these cells will display a stem cell-like division pattern. During the first larval stage, V1 and V6 cells divide. The posterior cell keeps a seam-cell identity whereas the anterior cell will fuse to the hyp7 syncytium, exit the cell cycle and differentiate. At the L2 stage, V1-V4, V6 and the T cell symmetrically divide to self-renew. Surprisingly, the V5 division will be different to the other seam cells. Indeed, its anterior daughter cell will form a neuroblast, which will give rise, in hermaphrodites, to a neural sensory structure afterwards. During L3 and L4 stages, V1-V6 descendant cell will divide again through a self-renewal division pattern. At each division, the anterior cell fuses to hyp7 while the posterior cell keeps its proliferative state. At the end of L4, the anterior cell will fuse again with hyp7; the posterior cell will fuse with the other posterior cells to form a syncytium responsible for alae formation (Sulston & Horvitz, 1977) (Figure 19). Some mechanisms controlling the regulation of this expression pattern have been identify and can be used to understand how stem cell are controlling their division pattern.

For the example, miRNA have been shown to tightly control the timing of seam cell proliferation and differentiation. Two miRNAs, *lin-4* and *let-7* control the timing of seam cell division through stage specific inhibition of heterochronic genes such as *lin-14*, *lin-28*, *lin-41*, *hbl-1*, *daf-12* and *lin-29* (Slack & Ruvkun, 1997). In the worm, loss of function mutants for *let-7* or *lin-4* exhibit seam cell overproliferation. This phenotype correlates with human data where *let-7* downregulation has been associated with cancer. Furthermore *let-7* expression seems to suppress tumor formation is some cancers (Nimmo & Slack, 2009). In the seam cells, *let-7* and *lin-4* induce cell differentiation and block self-renewal and this mechanism is conserved in human. Indeed, human homologues of *let-7* and *lin-4* are not expressed in embryonic stem cell but in late developmental stages, confirming their pro-differentiation action. These data show that the study of a simple model such as seam cell biology leads to the discovery of conserved mechanisms found during developmental processes in vertebrates.

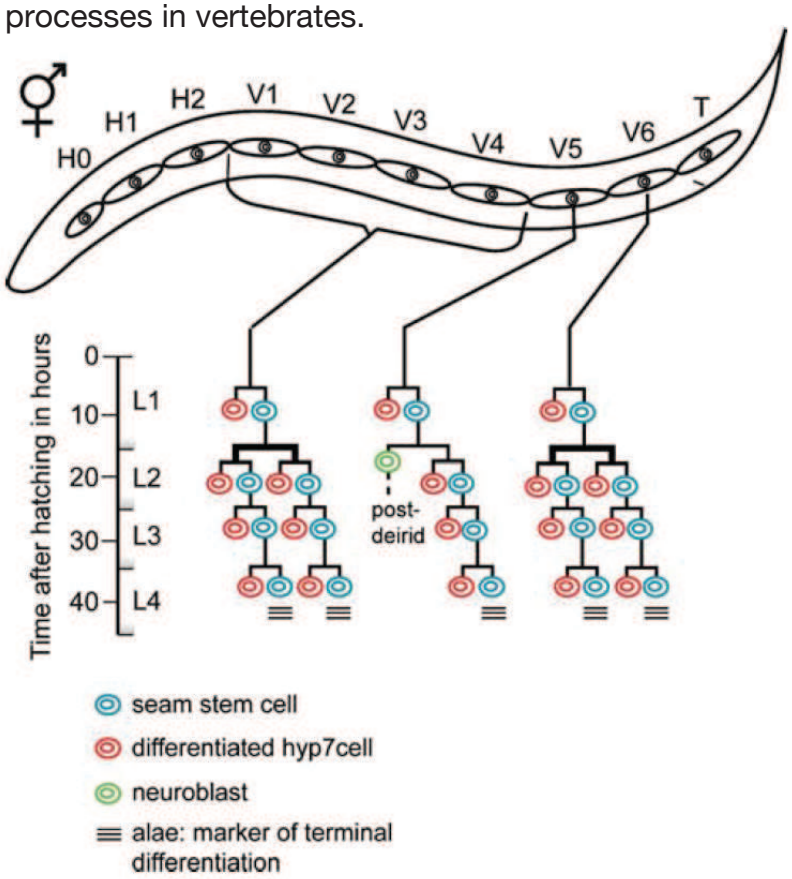


**Figure 19** – Seam cell division pattern. During development seam cells divide in a stem cell manner. At each developmental stage, seam cells divide to give two different daughter cells. One fuse with hyp7 and differentiates, the second keeps its seam cell identity and will divide the same way at the next developmental stage (Nimmo & Slack, 2009).

## II.3) *C. elegans* as a model to study cellular reprogramming

As it has been discussed above, study of cellular plasticity and cellular reprogramming led to some controversy. This was due to the lack of traceability between the initial and the reprogrammed cells. In *C.elegans*, this issue does not exist. Indeed, the essentially invariant lineage and the plethora of available markers make *C. elegans* a perfect model to study cellular reprogramming. We can follow without any ambiguity the cellular switch from a cell type A to a cell type B. Moreover, by using this lineage, reprogramming events can be predicted. We know in advance which cell will be reprogrammed and we have access to all the reprogramming steps including the early ones. All these observations can be done *in vivo*, in really time at the single-cell level. Furthermore, using genetic tools and rapid transgenesis, genes involved in cellular reprogramming and their mechanism of action can be identified very quickly.

### II.3.1) Induced reprogramming in *C. elegans*

#### *II.3.1.1) Induced reprogramming of C. elegans blastomere by ectopic factors expression*

During embryogenesis some blastomeres will be engaged in the exclusive formation of specific organs. For example the E (endoderm) blastomere will be the progenitor of the entire gut (20 cells in the adult worm). The number of cells arising from the E blastomere can be used as a reference to time developmental stages during embryogenesis (Figure 20.A). Until the beginning of the gastrulation (corresponding to the 2E stage, 24 cells in total), embryo patterning and cell fate specification are the result of the expression of maternally inherited products. During this period, the blastomeres remain pluripotent and restrict their developmental potential during early embryogenesis. After gastrulation, organ specification and tissue identity are gradually acquired by the embryo because of zygotic genes. The entire *C. elegans* development is stereotyped but during early embryogenesis cell fate of embryonic blastomeres can be manipulated by several methods. During embryogenesis, the position of the blastomeres is important to define their future fate because of intercellular signals. Physical manipulations of blastomeres lead to

modification of these signals, leading to acquisition of alternative cell fates. The ABp blastomere can give rise to pharyngeal muscle when physically positioned next to the Aba blastomere, while this situation never takes place in a non-manipulated embryo (Priess & Thomson, 1987).

Fate conversion of early embryo blastomeres can be achieved by ectopic expression of key developmental factors. Ectopic expression of myogenic transcription factor *hlh-1*/MRF is sufficient to convert almost all blastomeres into body wall muscles (Fukushige & Krause, 2005) (figure 20.B and C1). The reprogrammed cells express muscle specific markers such as MHCa, *myo-3* and filamentous actin. Markers of other tissues such as intestine, pharynx or hypodermis are lost indicating that blastomeres reprogramming is certainly complete. It has been observed that conversion into muscle cells is an active consequence of *hlh-1* activity. Indeed, ectopic activation of *hlh-1* does not set muscle fate by default by repressing other cell fate. Indeed, the reprogramming activity of *hlh-1* lasts until the 8E stage, when lineages are established and some cells start to differentiate. This suggests that *hlh-1* overexpression, activates muscle fate program along with repression of endogenous cell fate program.

Similar experiments have been done with other factors. GATA transcription factors *elt-2*/GATA4-6 and *end-1* overexpression lead to conversion of most if not all blastomeres into intestinal cells (Fukushige et al., 1998; Zhu, Fukushige, McGhee, & Rothman, 1998) (Figure 20.B and C2). Both of these genes are involved in endoderm specification and like *hlh-1*, ectopic expression of *end-1* during embryogenesis trigger repression of lineage markers of other cell types (such as muscle, ectoderm, pharyngeal muscle) and activation of intestinal specific features. Indeed, the reprogrammed cells have birefringent granules, typical of gut cells, and express intestine specific markers such as *elt-2*, *ges-1* and specific intestine antigen.

Epithelial identity has been induced in the embryo by ectopic expression of the zinc finger transcription factor *lin-26*, during early gastrulation (between 2E and 8E stage) (Quintin et al., 2001) (Figure 20.B and C3). The converted blastomeres express adherens junction protein such as AJM-1 and DLG-1. The apical trafficking gene *che-14*, has been found to be ectopically expressed upon *lin-26*

overexpression. At the opposite, markers for other tissue types were not expressed in the reprogrammed blastomeres. However, some epidermal-specific genes were missing in the reprogrammed cells leading to the conclusion that blastomeres were not converted into fully differentiated epithelial cells. Overexpression of the GATA transcription factor *elt-1* or *elt-3*, involved in epidermis specification, has lead as well to conversion blastomeres into epidermis (Gilleard & McGhee, 2001; Zhu et al., 1998) (Figure 20.B)

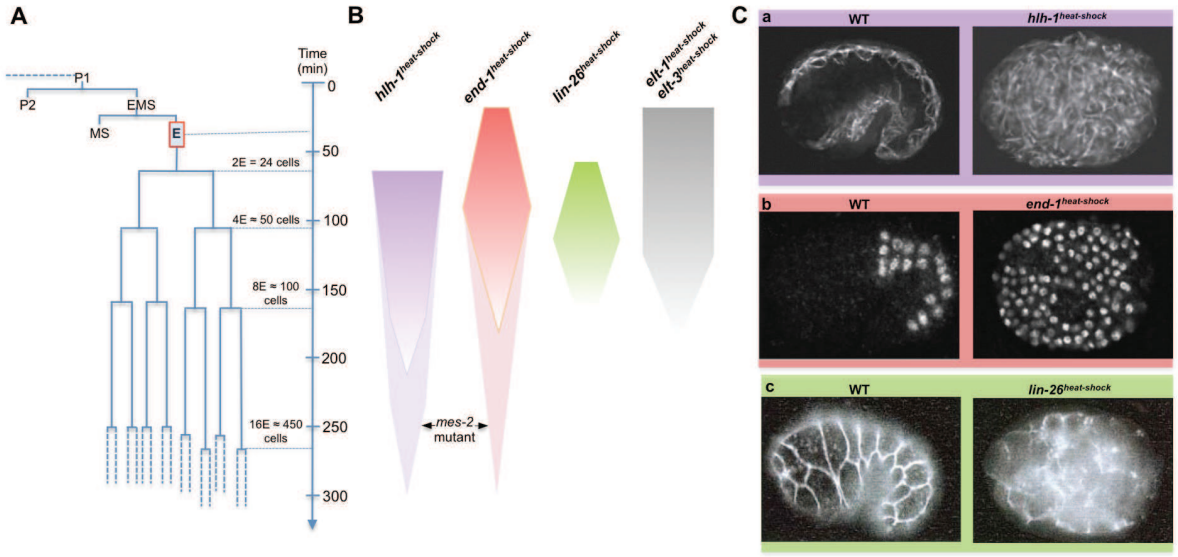


**Figure 20** – Blastomeres conversion by ectopic factors expression. **A)** Lineage of the E blastomere. The total number of embryonic cells is precised with the corresponding E stage. The time scale starts at the first embryonic cleavage. **B)** Competence window for blastomere conversion. The width of the diagram for each factor represents the efficiency of reprogramming. This competence window is extended in *mes-2* mutants. **C)** Pictures of wild-type embryos compared to embryos converted with the indicated factor. (1) Blastomere conversion into muscle is observed by staining of MHCa. (2) Blastomere conversion into intestinal cells observed by the expression of *elt-2*::GFP. (3) Blastomere conversion into epithelial cells identified by *dlg-1*::GFP (Zuryn et al., 2012).

On the same line, ectopic expression of the transcription factor *pha-4*/FoxA induces the conversion of blastomeres into pharyngeal cells (Horner et al., 1998). Here again markers for other tissue types were missing while the reprogrammed cells expressed pharyngeal muscle and pharyngeal marginal cells markers. In this case, *pha-4* with the TRIM protein TAM-1 act together to activate pharyngeal genes whereas *pha-4* with NuRD component are repressing other developmental genes.

Interestingly, all these studies on blastomere conversion have led to the same conclusions. i) Single transcription factor overexpression is sufficient to force specific fate changes in the blastomeres. ii) Blastomeres are sensitive to reprogramming until the 8E stage, after this point they become resistant to these factors overexpression.

This resistance to reprogramming has been associated to the *C. elegans* homologue of the PRC2 epigenetic complex. In the worm the PRC2 complex is composed, among others, by *mes-2*, *mes-3* and *mes-6*. *mes-2* mutants embryos challenged by *hlh-1* or *end-1* overexpression are twice as responsive to ectopic reprogramming than normal at the 4E and 8E stage (Yuzyuk, Fakhouri, Kiefer, & Mango, 2009) (Figure 20 B). This suggests that *mes-2* helps terminate plasticity in the developing cells, loss of which extends the window of developmental plasticity. This loss of plasticity is probably the consequence of global *mes-2*-dependent chromatin organization such as compaction.

#### *II.3.1.2) Induced reprogramming in the germline by factors elimination*

During metazoan development, germ cell primordia are set aside and segregated from somatic cells. Differentiation is inhibited in these cells because of multiple genetic insulators such as establishment and maintenance repressive histone modifications or the inhibition of the RNA polymerase II (Seydoux & Braun, 2006). This differentiation inhibition can be removed by loss of the PIE-1 CCCH-zinc finger protein (Unhavaithaya et al., 2002), which mediates global transcriptional repression through PcG factors. Indeed *pie-1* mutants display a conversion of their primordial germ cells into somatic cells (Figure 21.A) (Mello, Draper, Krause, Weintraub, & Priess, 1992; Mello et al., 1996; Seydoux et al., 1996).

As we have described above, the germline is a reservoir of proliferative totipotent germ cells. The totipotency of the germ cells is locked in the gonad and will gradually be restricted during embryogenesis. Germ cells totipotency is essentially maintained by two proteins; GLD-1 and MEX-3 (Ciosk, DePalma, & Priess, 2006). Abnormal looking germ cells have been found in the meiotic zone of *gld-1; mex-3* double mutants gonad. Observation of specific markers and electronic microscopy analyses revealed that abnormal germ cells acquired differentiated

features of somatic cells from various types (Figure 21.D). Body and pharyngeal muscles, neurons and intestinal cells are ectopically localized in the gonad of *gld-1; mex-3* double mutants. The presence of extensive cellular processes, like those of regular neurons, suggested that the germline neurons were fully differentiated. Moreover, the presence of filaments and contractile activity also suggested that the muscle cells, at least, were functional, suggesting that cellular reprogramming found in this case is complete. Germ cells traits such as presence of P-granules and expression of P-granules proteins were not present in the final somatic cells. Both GLD-1 and MEX-3, two RNA binding proteins, have been shown to repress translation of key development mRNAs (Draper, Mello, Bowerman, Hardin, & Priess, 1996; Hunter & Kenyon, 1996; Mootz, Ho, & Hunter, 2004). Thus, the wide variety of cell types found in the gonad of *gld-1; mex-3* double mutants may be the results of stochastic translation of different specific fate factors. It could be possible that several factors are expressed simultaneously but the dominant developmental factor determines the fate of an individual germ cell.

A second example of germline conversion has been reported recently. Knockdown of *lin-53* by RNAi, a component of several histone remodeling and modifying complexes, allows ectopic overexpression of genes involved in the specification of different neuronal subtypes to induce direct conversion of germ cell into neurons (Tursun, Patel, Kratsios, & Hobert, 2011) (Figure 21.B). As in *gld-1; mex-3* double mutants P-granules are lost in the reprogrammed cells. However none of these cells co-expressed several pan-neuronal of fate specific markers suggesting an incomplete germ cell conversion.

These conversions are not spontaneous; knockdown of *lin-53* alone does not trigger germ cell conversion. Thus the mechanisms underlying this reprogramming event are different of the RNA inhibition caused by GLD-1 and MEX-3. Instead, *lin-53* removal by RNAi seems to set a reprogramming capacity in the germ cells. Other differences have been pointed with the *gld-1; mex-3* double mutants. In fact, during *lin-53* RNAi, germ cells were converted into very specific neurons corresponding the particular factor ectopically expressed. The germ cell-to-neurons conversion takes place in the mitotic zone as opposed to *gld-1; mex-3* double mutants in which

conversions take place in the meiotic zone. These differences point the fact that germ cell conversions by loss of GLD-1/MEX-3 or by LIN-53 use two completely distinct mechanisms.

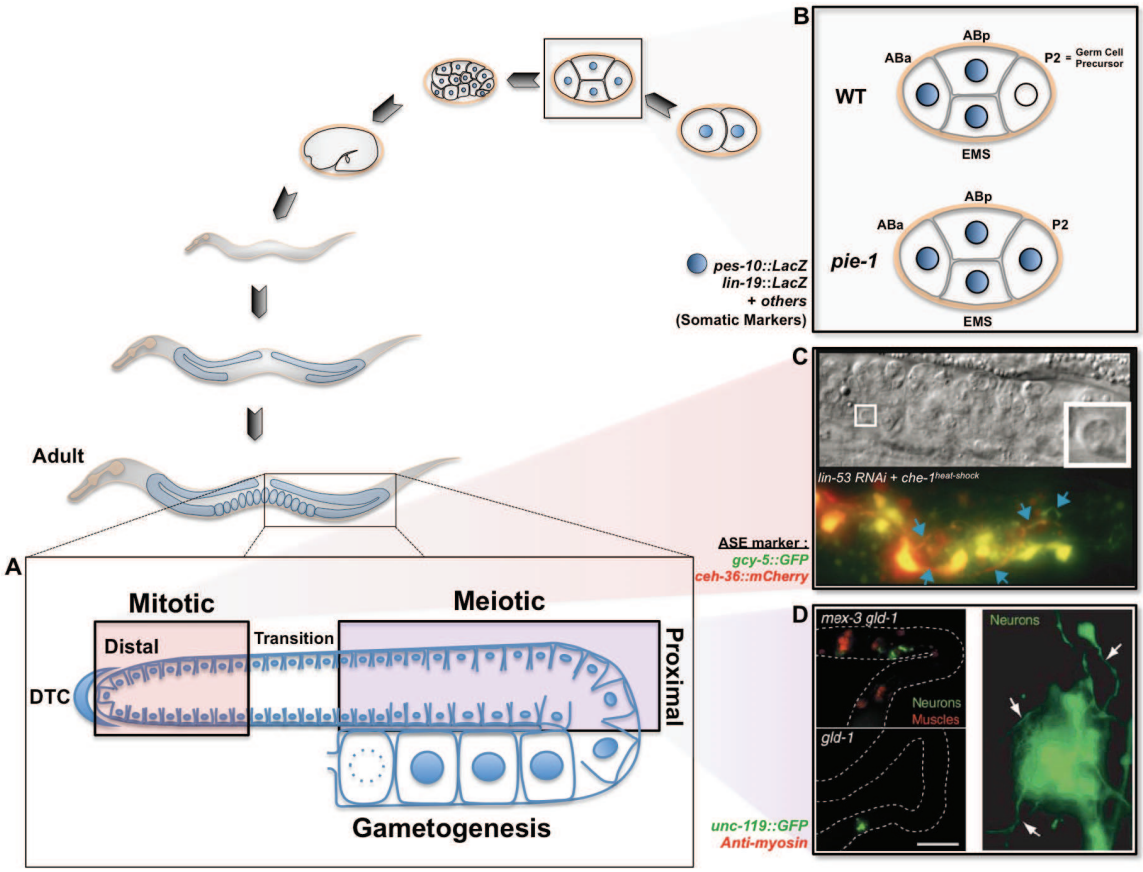


**Figure 21** – Germ cell conversion. **A)** Schematic representation of the *C. elegans* adult gonad. **B)** Ectopic expression of somatic markers (in blue) in the germ cell primordium P2 in *pie-1* mutant 4-cell embryo. **C)** Conversion of mitotic germ cells into specific neurons (ASE neuron) after *lin-53* RNAi and ectopic expression of *che-1*. The Nomarsky picture shown abnormal looking nucleus in the germline. The fluorescent photograph shows co-localization of two different ASE markers in the same reprogrammed cells. **D)** Conversion of germ cells into neuron and muscle in the meiotic region of *gld-1; mex-3* double mutants (Zuryn et al., 2012).

This germ cell-to-neuron conversion has been phenocopied by removal by RNAi of the methyltransferase PRC2. Here again, neurons could be obtained by expression of terminal selector genes in the mitotic region of worms knocked down for PRC2 subunit such as *mes-2*, *mes-3*, *mes-6* (Patel, Tursun, Rahe, & Hobert, 2012). Contradicting the previous study, germ cell-to-muscle conversion could be obtained by overexpression of *hlh-1*, which has never been observed upon *lin-53*

removal. In mammals, LIN-53 has been shown to recruit histone modifying enzyme such as PRC2 specifically to loci to silence transcription (Loyola & Almouzni, 2004; Eitoku, Sato, Senda, & Horikoshi, 2008). In this model of germ cell-to-neuron conversion, LIN-53 is maybe recruiting PRC2 specifically at neuronal loci. Thus upon *lin-53* RNAi, these loci, which should stay silenced, are accessible to the ectopically expressed terminal selector genes. These factors are in favorable epigenetic complex to induce germ cell-to-neuron conversion. However it could be possible that in normal situation PRC2 is bound at pan-neuronal loci and to other cell fate specific loci such as muscle loci. Then upon PRC2 removal, muscle loci could be in a favorable epigenetic context to be ectopically activated in germ cells by *hlh-1*.

In both studies, germ cell-to-neuron conversions are not spontaneous. These conversions work only when specific transcription factors are overexpressed in worms exposed to RNAi against *lin-53* or PRC2 components. These elements raise several interrogations about the mechanisms involved in these conversions. Indeed, in wild-type worms, multi-copy transgene are generally silenced in the germline. It could be possible that *lin-53* or PRC2 components RNAi triggers the desilencencing of these transgenes. Therefore germ cell conversions would not be the result of epigenetics changes. Moreover, germ cell conversion occurs only when worms are exposed to RNAi. The only germ cell conversion trials made in genetic mutants, such as *mes-2* or *mes-3* mutants, did not succeed. Thus, it could be imaginable that germ cell conversion is RNAi dependent and has nothing related with the knocked down genes.

#### *II.3.1.3) Induced reprogramming of the adult soma by factor elimination*

In the previous paragraph, we have seen that early embryos and the germline can be reprogrammed under certain conditions. Blastomere reprogramming can occur during a short time window only, then the cells become refractory to be reprogrammed. Several studies have shown that larval somatic cells are refractory to transcription factor-induced reprogramming (Richard et al., 2011). However in several mutants, somatic cell identity have been shown to be destabilized. In early larvae subject to RNAi against a conserved Kruppel type zinc-finger protein, MEP-1, hypodermal and intestinal cell are converted into cells exhibiting germ cell

morphology and expressing P granules (Unhavaithaya et al., 2002). In wild-type situation, MEP-1 acts through the NuRD complex to repress germline specific genes. In this RNAi condition, cell conversion does not seem to be complete. Indeed, upon reprogramming hypodermal and intestinal specific genes are still expressed in the reprogrammed cells. Similar results have been found when the retinoblastoma (Rb) pathway is downregulated (Wang et al., 2005). These findings, could be interesting to explore. Although conversion of hypodermal and intestinal cell in germ cell is incomplete, these cells express P granule indicating that they gain in plasticity and partially revert into an undifferentiated state.

#### *II.3.1.4) Chemically induced reprogramming*

Germline cell identity has been recently reprogrammed after exposition to several MAPK pathway inhibitors (Morgan, Lee, & Kimble, 2010). In the worm, hermaphrodite germline produce both oocytes and sperm. Masculinized hermaphrodites germline exists and produces only sperm whereas feminized hermaphrodite germline produce only oocytes (Ellis, 2006; Kimble & Crittenden, 2007). Double mutant for *lip-1* (MAPK phosphatase) and *puf-8* (Pumilio/FBF-RNA-binding protein) have MAPK hyperactivity and masculinized germline. Application of specific inhibitor of MEK1/2 kinases (involved in the MAPK pathway) on *lip-1; puf-8* double mutant leads to the formation of functional oocytes in masculinized gonads (Figure 22). The induced oocytes were fully functional; they could produce embryos that developed into adults. It is not clear if this reprogramming event is due to germ cell induced differentiation into oocyte or to dedifferentiation of spermatocytes. The molecular mechanisms of this reprogramming event are not well understood, but the work provides the first example of chemically induced reprogramming *in vivo*.

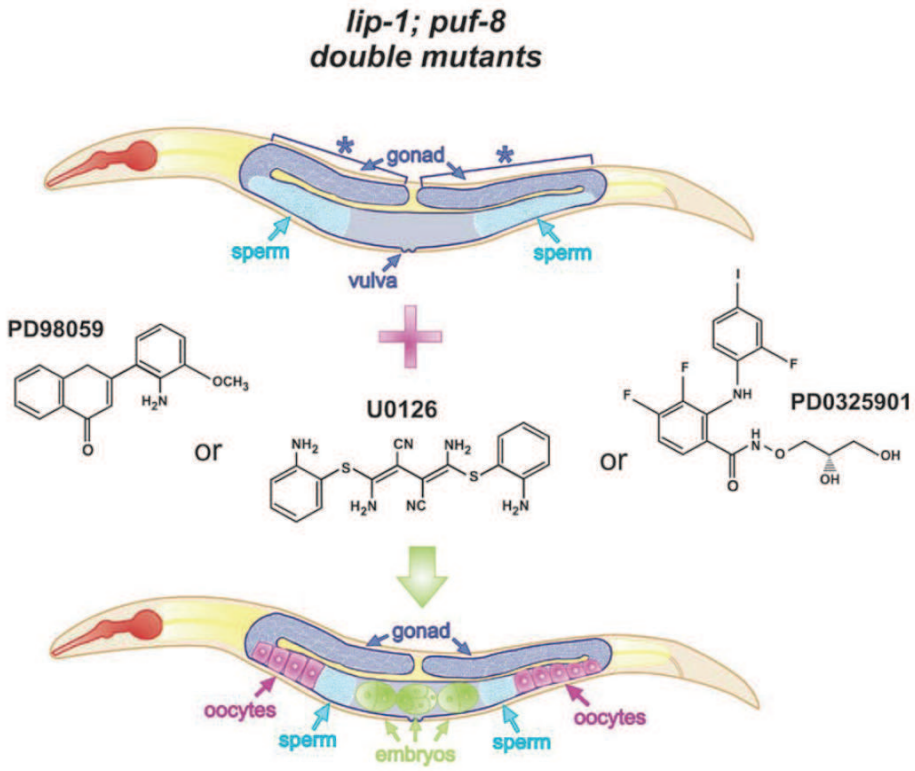


**Figure 22** – Chemical reprogramming. Gonads of *lip-1; puf-8* double mutants are masculinized; they only produce sperm cells. Application of any of the three MAPK pathway inhibitors to adult worms can restore the production of functional oocytes via cell reprogramming (Hajduskova, Ahier, Daniele, & Jarriault, 2011).

## II.3.2) Natural reprogramming in *C. elegans*

### *II.3.2.1) Cell fate reprogramming during vulva formation*

In *C. elegans*, the vulva takes its origin in the differentiation of three of the six multipotent vulval precursors cells (VPCs). The VPCs are committed and divide during the third larval stage. These divisions give rise to daughter cells, which will adopt distinct vulval cell fates. In *lin-28* heterochronic mutants, the VPCs divide at the second larval stage (Euling & Ambros, 1996). *Lin-28* mutants exposed to unfavorable living conditions enter in the dauer stage, the commitment of their VPCs is erased and the VPC daughter cells are reprogrammed back into the multipotent VPC state. When the worms enter back into normal living condition, the reprogrammed VPCs can generate again a functional vulva. This example is the only one where *lin-28* is involved in a cell reprogramming event in *C. elegans*, which is surprising when the human homologues of *lin-28* has been identified as a stem cell marker and can be used to reprogram fibroblasts into iPS cells (Yu et al., 2007).

#### *II.3.2.2) Transdifferentiation of a rectal epithelial cell into a neuron*

An unambiguous transdifferentiation event has been recently characterized in the worm (Jarriault, Schwab, & Greenwald, 2008). Classical works on the cell lineage of *C. elegans* suggest that some cells change their morphology during larval development (Sulston & Horvitz, 1977).. Our laboratory hypothesized that these could be due to cell identity switched. No clear characterization of these putative cells fate switch had been performed until now. Our laboratory chose to focus on one of them, which take place in the rectal region of the worm.

*C. elegans* rectum is a tube formed by three pairs of epithelial cells named K, K', U and F, and B and Y (Figure 23.A). At the beginning of the second larval stage, the Y cell starts to migrate away from the rectum and transdifferentiates into a neuron called PDA (Figure 23.B). The Y cell is replaced in the rectum by another epithelial cell, P12.pa, to reform a functional rectum.

This transdifferentiation event has been characterized at the cellular and molecular level. Before transdifferentiation, the Y cell expresses epithelial markers such as *che-14*, *ajm-1*, *dlg-1*, *elg-26* and *lin-26* (Jarriault et al., 2008). Moreover, electron microscopy observations of the Y cell reveal that Y has the ultrastructure of an epithelial cell and makes apical junctions with its neighboring cell, B (Jarriault et al., 2008). After transdifferentiation the resulting PDA cell expresses neuronal markers such as *unc-119*, *F25B3.2*, *ace3/4* and *cog-1* (Jarriault et al., 2008). None of the neuronal markers are expressed in the Y cell, and none of the epithelial markers are found in PDA indicating that Y is purely epithelial when in the rectum and purely neuronal when it has become a PDA.

Over the years, several steps have been identified during the transdifferentiation of Y into PDA. i) Y acquires its rectal epithelial identity. ii) Y retracts from the rectum, erases its initial identity. iii) Y migrates antero-dorsally to its final position. iv) The migrating cell acquires its final PDA identity.

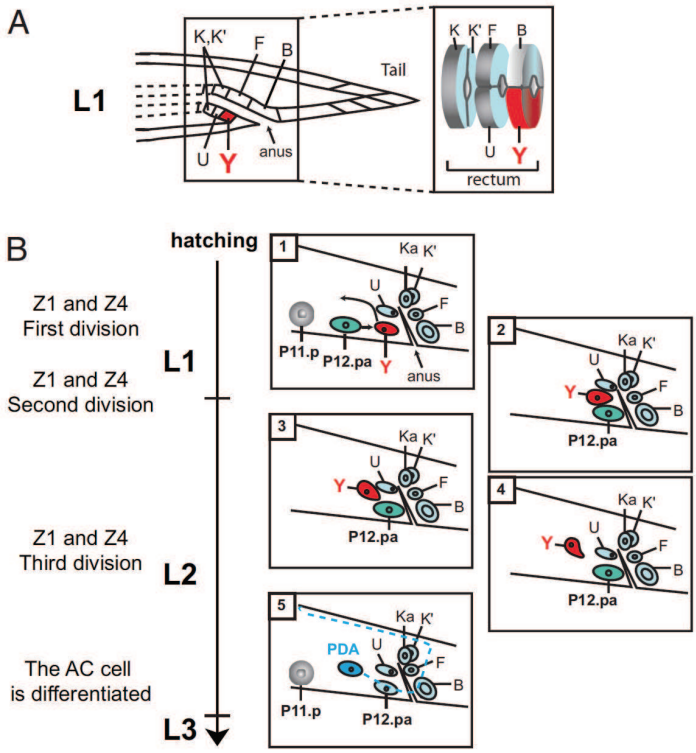


**Figure 23** – The Y-to-PDA transdifferentiation. **A)** In the first larval stage, the rectum is a vital organ formed by six pairs of cells, K, K', U, F, B and Y. Each pair of rectal cells forms a ring because of adherens junctions **B)** At the beginning of the second larval stage, Y retracts from the rectum and migrate antordorsally to reach its final position. During its migration, Y looses its epithelial identity and will transdifferentiate into a motor neuron called PDA. Y is replaced in the rectum by P12.pa to reform an intact rectum (Jarriault et al., 2008).

From a molecular and cellular point of view, rectal cells are very similar. Despite this fact, only Y is able to transdifferentiate into PDA. This suggests that only Y integrates specific signals that prime transdifferentiation. Because of these signals Y acquires the capacity, also called the competence, to transdifferentiate. The molecular origin of the acquisition of the competence to transdifferentiate in Y has been partially identified. Indeed, in some mutant backgrounds a supernumerary Y unable to transdifferentiate can be found. In *egl-38/Pax5* mutants, the rectal cell U takes a Y identity (Chamberlin et al., 1997; Jarriault et al., 2008). It has been confirmed by loss of U specific marker, acquisition of Y specific marker and analysis of cell division pattern in males. Similarly, in *mab-9/T-box transcription factors*, the B cell acquires a Y identity (Jarriault et al., 2008; Woollard & Hodgkin, 2000). In both mutant backgrounds, the supernumerary Y is not able to give rise to a PDA; only the original Y is able to do so and that is still true if the original Y is ablated (Jarriault et

al., 2008). These observations indicate that adoption of an ectopic Y cell identity is not sufficient to become a PDA; these supernumerary Y cells are not competent to transdifferentiate. At the opposite, gain-of-function mutations in *lin-12/Notch* lead to the acquisition of a second Y cell coming from the conversion of the DA9 cell, the contralateral homologue of Y (Greenwald, Sternberg, & Horvitz, 1983; Jarriault et al., 2008). In this situation, both Y cells are able to transdifferentiate and give rise to two PDA neurons (Jarriault et al., 2008). Together, these observations suggest that *lin-12* is not only necessary and sufficient to form the Y rectal cell, but appears to be the signal that primes transdifferentiation. Hence, Y competency to transdifferentiate seems to be set by the LIN-12/Notch pathway.

Y-to-PDA transdifferentiation is a stepwise process. After *lin-12* action, Y is competent and will subsequently initiate transdifferentiation. In some mutants identified in our laboratory, the Y-to-PDA transdifferentiation is not initiated. For these mutants, the Y cell keeps its epithelial identity and stays in the rectum. A conserved nuclear complex involved in this initiation step has been recently identified. Indeed, members of the NODE complex such as *sem-4/Sall*, *egl-27/Mta* and *ceh-6/Oct* in association with *sox-2* and upstream of *egl-5/Hox* have been shown to promote the initiation of the Y-to-PDA transdifferentiation (Kagias, Ahier, Fischer, & Jarriault, 2012). Several of these components have human homologues essential for pluripotency of human ES cells (Zhang et al., 2006; Wong, Gaspar-Maia, Ramalho-Santos, & Reijo Pera, 2008; Liang et al., 2008) and because some of these factors trigger or enhance iPS cells formation, it is possible that they confer cellular plasticity via a conserved mechanism.

After the initiation, Y migrates to reach its final position. This migration is not necessary for Y to acquire its final identity. In fact, when the precursor cell of P12.pa is ablated using a laser, even if Y migration does not occur, transdifferentiation still takes place. In the same line, ablation of the other rectal cells during the first larval stage does not impact the Y-to-PDA transdifferentiation, suggesting that the microenvironment surrounding Y in the larva is not important for the reprogramming to take place. Nevertheless, redundant role of the rectal cells has not been excluded from these ablation experiments.

The switch from an epithelial cell to a neuron can take place according to two different models. In a first case, Y is losing its epithelial features while it acquires the neuronal one. In this scenario a cell with a mixed identity should exist during the reprogramming event. In a second case, the epithelial identity has to be completely erased before the acquisition of the neuronal fate. In *unc-3* mutant background, the Y cell is blocked at an intermediate phase of transdifferentiation (Richard et al., 2011). Analysis of this mutant suggested a step-wise Y-to-PDA conversion where Y first erases its identity and go through into an intermediary cell (called Y.0), which has neither epithelial nor neuronal identity. Y.0 becomes an early neuronal cell (called Y.1) that expresses the pan-neuronal marker *unc-33* and that finally differentiates into PDA (Figure 24). During all these steps, cells with mixed identity have never been found supporting the scenario in which the Y cell loses its epithelial features before acquiring neuronal ones.

During these steps, Y dedifferentiation does not go through a multipotent state. Indeed, ectopic expression of endodermal (*end-1*), muscular (*hlh-1*) or neuronal (*unc-30*) cell fate determinants failed to reprogram the fate of the Y.0 cell. The Y.0 cell not only lacks the potential to be converted into distinct cell fates, but it also cannot be forced to re-differentiate back into an epithelial cell. Thus, conversion of Y into PDA is tightly regulated and cannot be modified easily.

To date, this model of *in vivo* transdifferentiation is one of best characterized. Although taking place *in vivo* in a multicellular organism, the Y-to-PDA conversion is an unambiguous transdifferentiation event. Many factors have been found to be involved in this process, and others are under characterization. The identification of all the required steps allowing an epithelial cell to change its identity into a neuron can lead one day to the development of new protocols of cell conversion.

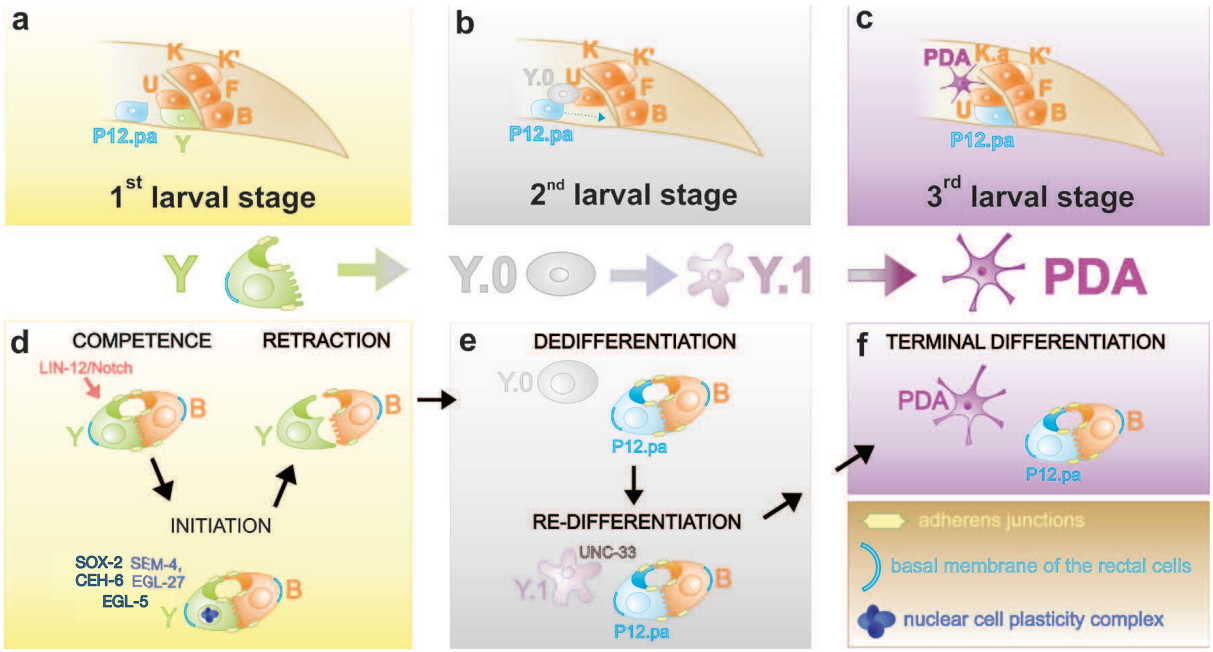


**Figure 24** – **(a-c)** Summary of the Y-to-PDA step at the rectum level. Y migrates to reach its final position and transdifferentiate into PDA while P12.pa takes place in the rectum to reform it. **(d-e)** Y-to-PDA steps at the Y level. **d)** Y and B interact together through adherens junction to form one of the three rectal ring. LIN-12/Notch signaling give to Y its identity and the competence to transdifferentiate. A complex formed by the NODE-like complex (EGL27, CEH-6 and SEM-4) in association with SOX-2, and acting upstream of EGL-5, is essential for the initiation of the reprogramming event. **e)** P12.pa takes place in the rectum to preserve its function. Y erases iits identity and go through into an intermediate cell called Y.0, which is neither epithelial nor neuronal. Y.0 begins to re-differentiates and become a neuronal precursor called Y.1, which expresses the pan-neuronal marker *unc-33*. **f)** The Y.1 cell finally differentiates into the PDA motor neuron. Adapted from (Hajduskova et al., 2011).

The power of this model is not only that it takes place naturally. The reprogramming of Y into PDA is predictable and occurs with 100% efficiency. It leads to a defined, functional and stable final identity, all features that contrast with to induced and *in vitro* cell conversions that reach, in the best case, 10 to 20% efficiency, are not tracable and where sometimes reprogramming is not complete. Thus understanding robust and efficient *in vivo* reprogramming could lead to the discovery of factors and signals, which can be important to reprogram efficiently any cell type. Our data could be used as a support to develop new reprogramming strategy. Moreover, the study of Y-to-PDA transdifferentiation will unravel the mechanisms underlying *in vivo* cell plasticity.

# III) The *C. elegans* Notch signaling

Signaling pathways are one of the main actors governing developmental processes. As Raphael Kopan suggested, signaling pathways could be seen as musical notes. To make an infinite numbers of melodies only seven notes are needed. Thus, during development around 20 signaling "cassettes", which can be seen as notes, are responsible for the entire diversity of metazoan cell types, organs and life forms, which can be the melodies. Thus, Wnt pathway could be the 'Do", the TGFß pathway the "Re", the Notch pathway the "Mi".

The Notch pathway has been extensively studied over the past decades. Many components and mechanisms of action have been unraveled because of studies performed in invertebrates such as *C. elegans* and *Drosophila*. Many differentiation, homeostasis and developmental processes involve the Notch pathway. By consequence making a list of all the processes involving the Notch pathway would need a thesis in several volumes. In this part, general principles and components of the *C. elegans* Notch pathway will be described. Then the different type of signaling induced by Notch will be discussed. To finish, involvement of Notch signaling in cell plasticity will be reviewed.

## III.1) Mechanistic aspects and components of the *C. elegans* Notch pathway

### III.1.1) General mechanisms

The Notch pathway components and signal transduction are extremely conserved between species. Notch signaling involves cell-cell interactions. One signaling cell expresses a ligand, while the receiving cell expresses the Notch receptor (Figure 25.A). When the ligand and the receptor are not in contact, the pathway is not activated and the Notch target genes are not expressed. Indeed the nuclear effector CSL (for CBF1/Su(H)/LAG-1) is associated to transcriptional repressors to inhibit target genes expression. Interaction between the receptor and

the ligand will trigger several events: i) Ligand-Receptor interaction trigger endocytosis of the ligand in the signaling cell. ii) Conformational changes of the receptor take place because of pulling-force driven by the ligand-endocytosis (Meloty-Kapella et al., 2012; Shergill et al., 2012). iii) After these conformational changes, the receptor is now accessible to proteases (Figure 25.B). A protease belonging to the ADAM family will cleave the extracellular part of the receptor in a site called S2 (Figure 25.C). A second constitutive cleavage occurs in a transmembrane S3 site by the ɣ-secretase Figure 25.D), which will release the intracellular domain of the Notch receptor (NICD). This domain will translocate into the nucleus (Figure 25.E) (Jarriault et al., 1995) and will interact with the sequence binding specific protein CSL and a co-activator of the Mastermind family to promote target gene expression (Figure 25.F).

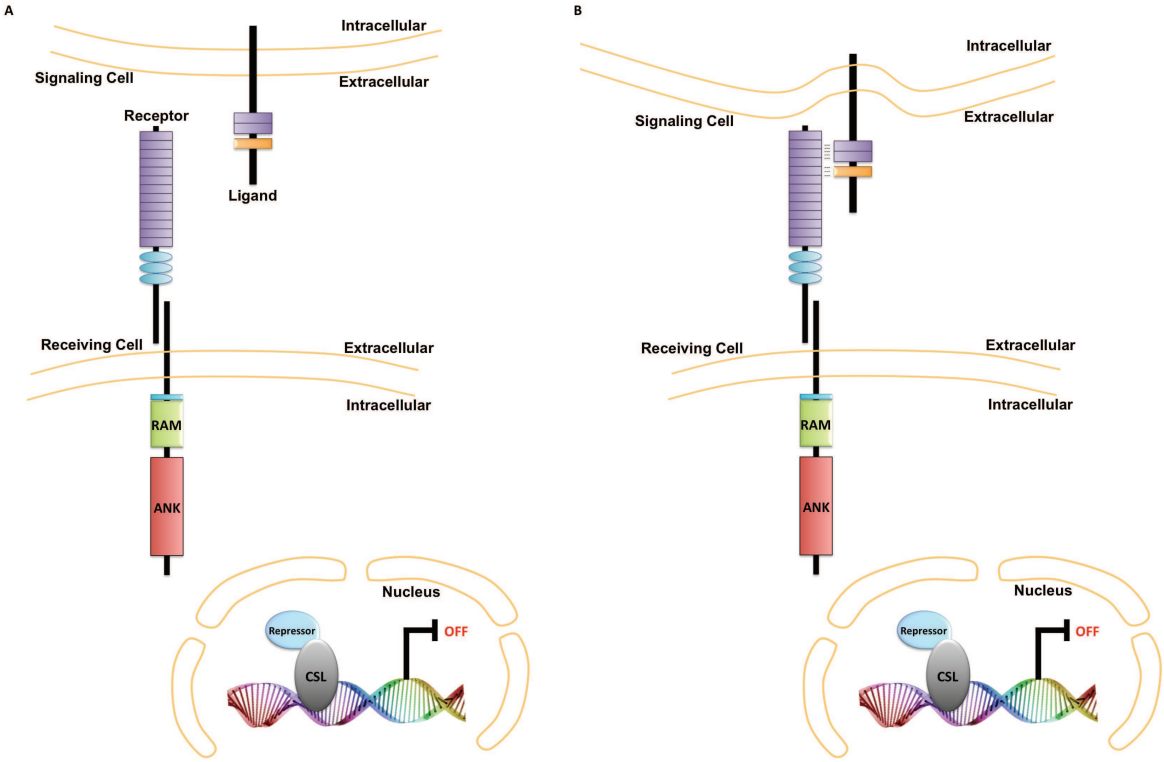


**Figure 25** – Key events of the Notch pathway. **A)** A signaling cell expresses a ligand, while a receiving cell expresses the Notch receptor. Without ligand-receptor interaction, the Notch pathway is off. A sequence specific DNA binding protein, called CSL, associated to a repressor complex inhibits the Notch target genes. **B)** Ligand-receptor interaction triggers ligand endocytosis and leads to receptor conformational changes. These conformational changes make the receptor accessible to proteases.

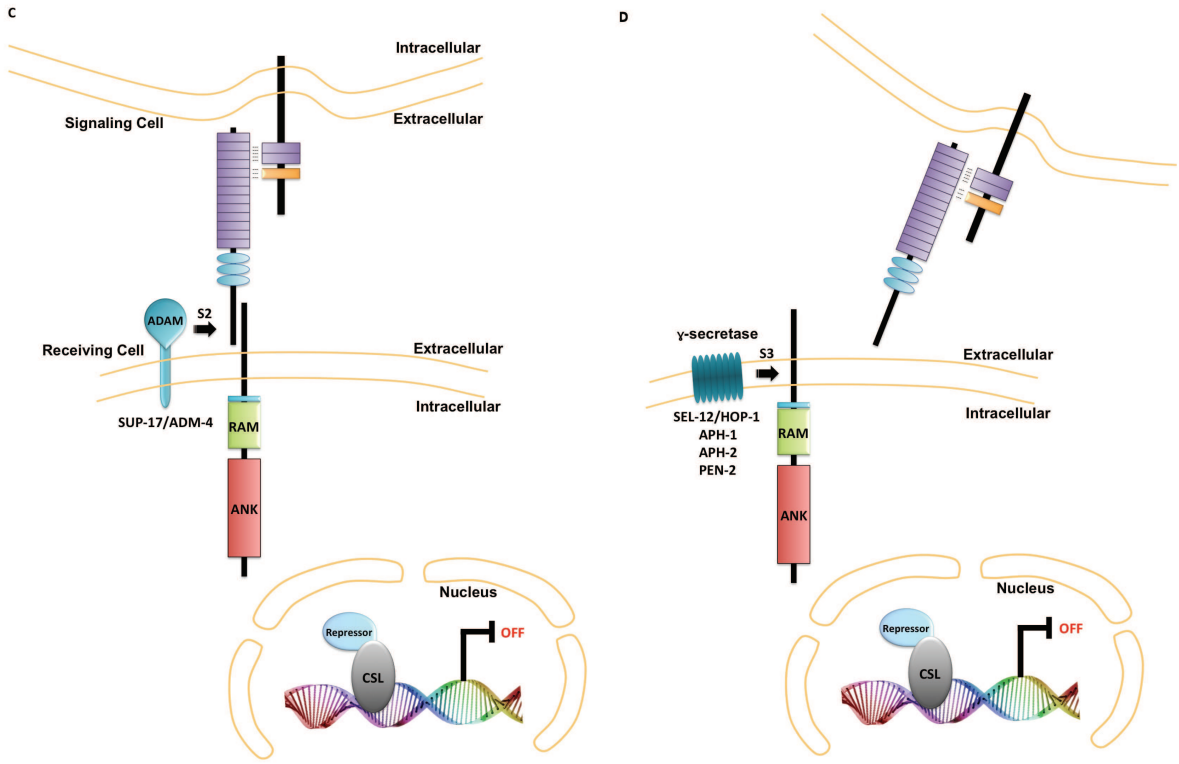


**Figure 25** – Key events of the Notch pathway. **C)** Conformational changes of the Notch receptor allow cleavage of the extracellular domain of the receptor by an ADAM protease at site 2. **D)** The ADAM-mediated cleavage triggers constitutive cut of the remaining receptor by the γ-secretase. This cleavage takes place at the transmembrane site 3.

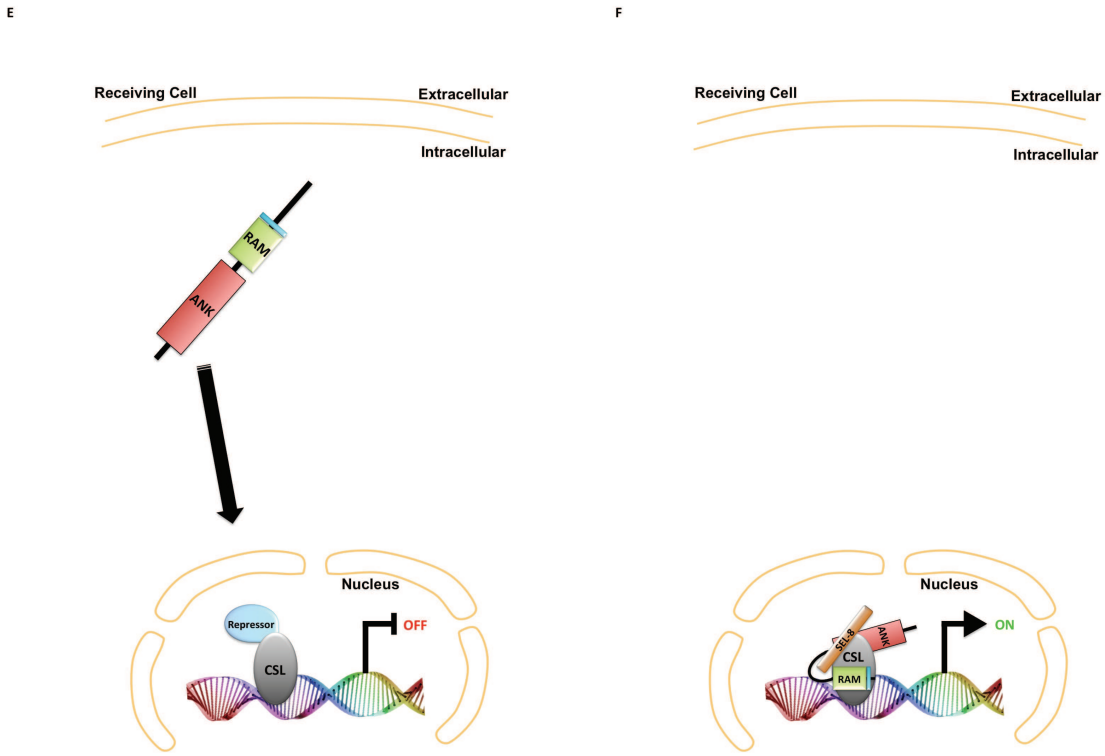


**Figure 25** – Key events of the Notch pathway. **E)** The released NICD translocates to the nucleus. **F)** Interactions between, CSL, a Mastermind protein and NICD induce target genes expression.

### III.1.2) Core components and mechanistic of the *C. elegans* Notch signaling

#### *III.1.2.1) The C. elegans Notch receptors: LIN-12 and GLP-1*

LIN-12 and GLP-1 are the two proteins encoding a Notch receptor in *C. elegans*. Their sequence motifs are described in the Figure 26

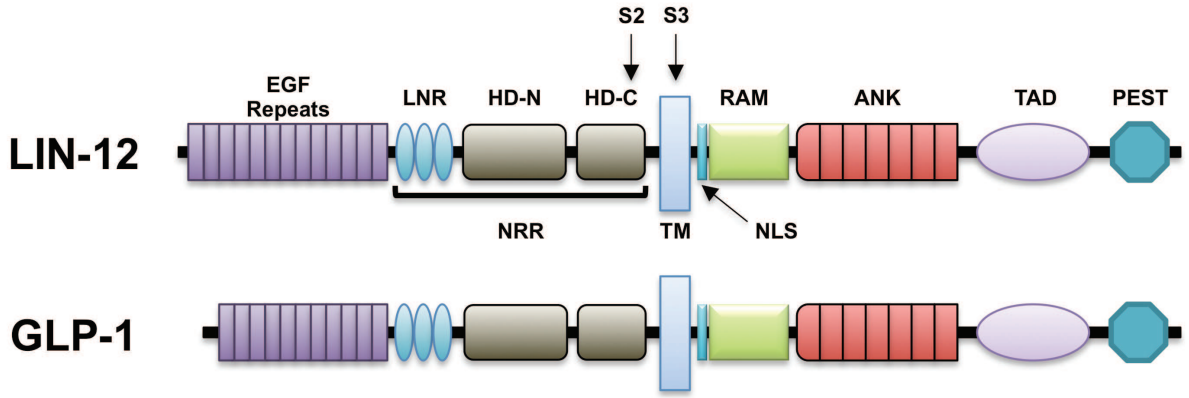


**Figure 26** – Organization of the LIN-12 and GLP-1 Notch receptors. Both receptors have an extracellular and an intracellular part. The extracellular part is followed by a single transmembrane region (TM) and the intracellular domain (NICD). S2 indicates the cleavage site of the ADAM proteases. S3 indicates the cleavage site of the -secretase. The extracellular domains of the receptors are composed of EGF repeats, LIN-12/Notch repeats (LNR), HD (Heterodimerization domain) composed of its amino terminal segment HD-N and its carboxy terminal segment HD-C. LNR, HD-N and HD-C together are part of the Negative Regulator Region (NRR). The TM domain allows anchorage in the cell membrane. The intracellular part of the receptor encompasses a nuclear localization sequence (NLS), a RAM domain, which allows interactions with the Notch nuclear effector, an ankyrin repeat motif (ANK), a transcriptional activation domain (TAD), and a PEST domain (involved in protein degradation).

*lin-12* has been identified for the first time in *C. elegans* in a genetic screen for mutations affecting vulval development (Greenwald et al., 1983; Ferguson & Horvitz, 1985). *glp-1* has been identified later by two independent screens. A first screen for sterile mutants revealed that loss of zygotic *glp-1* causes sterility, which was the consequence of premature entry in meiosis of the germ cells (Austin & Kimble, 1987). The second screen was focused on maternal-effect embryonic lethal mutations and showed that loss of maternal *glp-1* inhibits cell fate induction during early embryogenesis. (Priess, Schnabel, & Schnabel, 1987). The existence of two Notch receptors in *C. elegans* suggests potential redundancy between them. Double

mutants lacking zygotic *lin-12* and *glp-1* die as L1 larvae with various defects. This phenotype is different from both single mutants phenotype and is defined as the "Lag" phenotype (**L**in-12 **a**nd **G**lp-1) and suggested that despite the sequence divergence between LIN-12 and GLP-1 they are functionally redundant (Lambie & Kimble, 1991). An other study showed that GLP-1 could substitute for LIN-12 in cell fate decisions, which confirms their functional redundancy (Fitzgerald, Wilkinson, & Greenwald, 1993).

#### *III.1.2.2) The C. elegans Notch ligands*

The Notch ligands are named DSL proteins, which is an acronym derived from three canonical ligands, the *Drosophila* Delta and Serrate, and the *C. elegans* ligand LAG-2. In *C. elegans*, many DSL proteins have been identified based on their primary sequence and domain organization but their involvement in Notch signaling is still poorly understood. The first canonical ligands identified were *lag-2* and *apx-1*. *lag-2* has been identified by its null allele which confers the "Lag" phenotype (Lambie & Kimble, 1991) and by its suppressor effect of *lin-12* gain-of-function mutations (Tax, Yeargers, & Thomas, 1994; Tax, Thomas, Ferguson, & Horvitz, 1997). *apx-1* has been isolated during studies on the early embryo (Mello, Draper, & Priess, 1994; Mango, Thorpe, Martin, Chamberlain, & Bowerman, 1994). *arg-1* has been identified as a putative Notch ligand (Mello et al., 1994) but its role in Notch signaling has never been determined. This ligand appears to be functional; expression of the ARG-1 protein under the control of *lag-2* regulatory sequence can rescue *lag-2* mutants (Fitzgerald & Greenwald, 1995) (Figure 27.A). These canonical ligands are composed of several DSL domains followed by some EGF repeats and a transmembrane domain, which allows anchorage of the ligand in the membrane of the signaling cell. (Figure 26.A)

Bioinformatics analyses identified several DSL proteins, which could be potential ligands for Notch. Among them, DSL-1 has been found to have a discrete role during vulval cell fate decision (Chen & Greenwald, 2004; Hoyos et al., 2011). DSL-3 has been recently shown to restrict embryonic developmental plasticity (this study will be discussed later) (Djabrayan, Dudley, Sommermann, & Rothman, 2012). Surprisingly, some of the computationally identified DSL proteins lack a

transmembrane (TM) domain. This suggests that some ligands could be secreted. To date, evidence of long distance Notch signaling has been reported only for the non-canonical ligand, *dsl-1*, but single mutants for these non-canonical Notch ligands have no particular phenotypes, which make the identification of their role difficult. As *apx-1* and *lag-2*, these DSL proteins have EGF repeats and DSL domains (Figure 27.B)

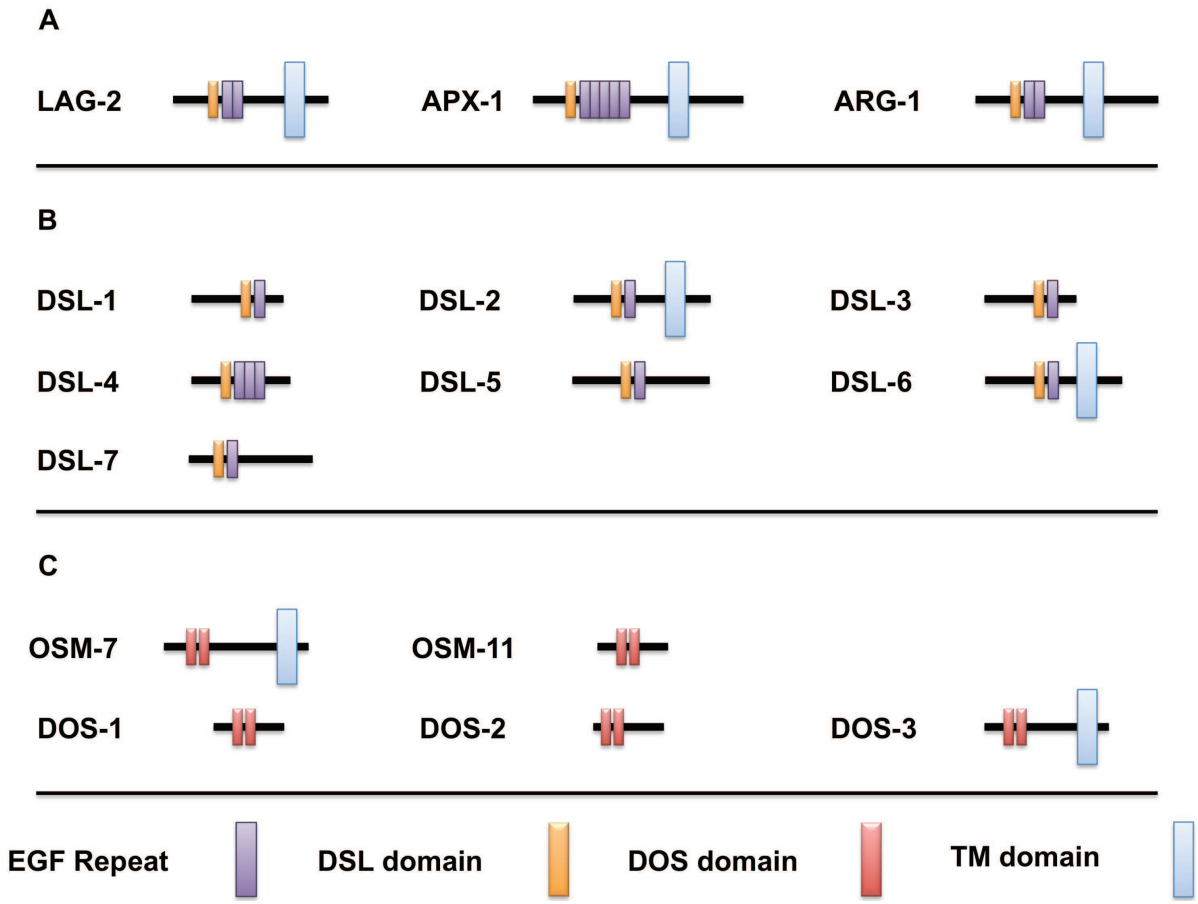


**Figure 27** – The *C. elegans* Notch ligands and coligands. **A)** LAG-2 and APX-1 were the first *C. elegans* Notch ligands identified. ARG-1 has been qualified as a putative ligand. There are composed of EGF repeats, a DSL domain and a TM domain. **B)** DSL proteins identified by bioinformatics analyses. DSL-1 has been identified as a bona fide ligand. Some of them do not have a TM domain, and could be secreted. **C)** OSM-11 has been identified as functional co-ligands. OSM-7, DOS-1, DOS-2 and DOS-3 have been included in this category because of their sequence similarities with OSM-11.

### *III.1.2.3) The C. elegans Notch coligands*

*Drosophila* and mouse Notch ligands are composed of DSL domains, EGF repeats and another class of domain called DOS domain (for Delta and OSM-11). DOS domains are not found in the *C. elegans* DSL proteins. However some proteins

are only composed of DOS domain associated or not with a TM domain. These proteins could function as co-ligands, which facilitate the activity of other ligands. For example OSM-11 has been described to facilitate the activation of Notch receptor during vulval precursor cells specification (Komatsu et al., 2008). The DOS proteins are represented by OSM-11, OSM-7, DOS-1, DOS-2 and DOS-3 (Figure 27.C). To date, only OSM-11 and OSM-7 was shown to be involved in the *C. elegans* Notch pathway (Komatsu et al., 2008; Singh et al., 2011).

#### *III.1.2.4) The C. elegans Notch proteases*

As described above, the LIN-12 and GLP-1 protein have two identified cleavage sites. Site 2 is located in the extracellular part of the receptor. In wild-type conditions, cleavage at site 2 exclusively requires ligand-receptor interactions. The site 2 protease is a member of the ADAM (A Disintegrin And Metalloprotease) family. Genetic analysis suggests that site 2 cleavage is mainly operated by the gene *sup-17* (Tax et al., 1997; Wen, Metzstein, & Greenwald, 1997). Loss-of-function allele of this gene is able to suppress the phenotypes triggered by gain-of-function mutations of *lin-12* (Tax et al., 1997). Further studies on the site 2 demonstrate that a second gene, *adm-4*, also mediates site 2 cleavage and can act redundantly with *sup-17* (Jarriault & Greenwald, 2005). Thus, two different proteins are involved in the cleavage of the Notch extracellular part.

After the action of *sup-17* or *adm-4*, the site 3 becomes accessible in the transmembrane domain. The cleavage by the ɣ-secretase is constitutive. This protease cuts membrane proteins with a short ectodomain (Struhl & Adachi, 2000), which is exactly the structure of the Notch receptor after site 2 cleavage. The ɣ-secretase is multi-subunit complex composed of the catalytic subunit, the presenilin, in association with APH-1, APH-2 and PEN-2, which are involved in the substrate recognition and stability. Two redundant genes, *sel-12* and *hop-1*, can encode the presenilin. Alone, each single mutant is viable and presents an incomplete Notch loss-of-function phenotype. In contrary, double mutants for these two presenilins cause hallmarks of the Lag phenotype indicating their redundant function in Notch signaling (Li & Greenwald, 1997).

### *III.1.2.5) The C. elegans Notch nuclear complex*

NICD is released after the ɣ-secretase cleavage, goes into the nucleus, and interacts with a CSL protein and Mastermind to form a transcriptional activation complex that promotes expression of target genes. Many other proteins involved in general transcriptional activation are also recruited by this ternary complex.

CSL is the acronym for "CBF1/Su(H)/LAG-1 and can recognize DNA in a sequence specific manner. In *C. elegans*, the CSL protein is coded by the *lag-1* gene. Null mutation of *lag-1* causes "Lag" phenotype, indicating its crucial role in Notch signaling (Lambie & Kimble, 1991). Molecular characterization revealed that LAG-1 is a CSL protein that has the same sequence specificity as its *Drosophila* and mammalian orthologs (Christensen et al., 1996). The LAG-1 protein is composed of a three highly conserved domains: an N-Terminal domain (NTD), a ß-trefoil domain (BTD) and a C-Terminal domain (CTD). The NTD and CTD belong to the Rel homology domains, which are similar to other DNA binding transcription factors, such as NF-kappaB. The NTD and CTD domains form a continuous electropositive surface that makes specific contacts in the major and minor grooves to recognize DNA (Kovall & Hendrickson, 2004) (Figure 28).

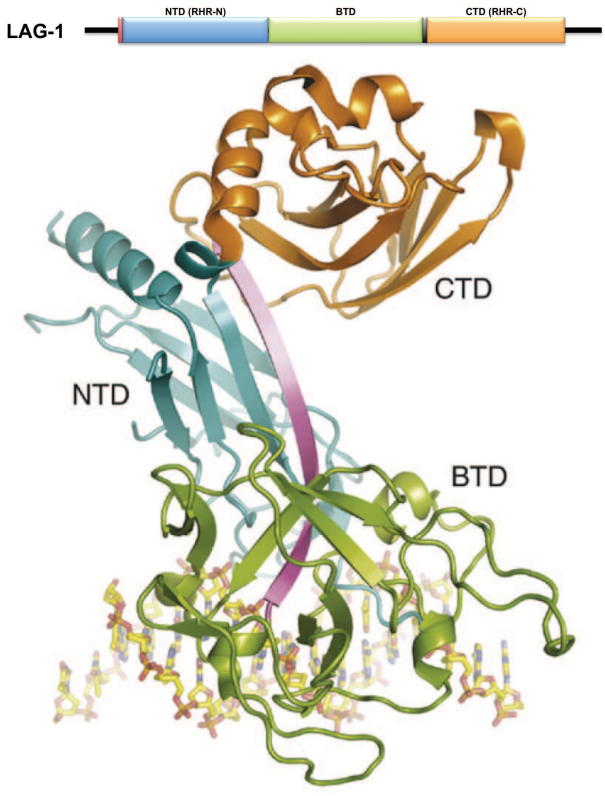


**Figure 28** – Structure of LAG-1 bound to DNA. NTD domain is in blue, BTD in green and the CTD in orange. Together the NTD and CTD domain make specific contacts with DNA.

Mastermind is encoded by *sel-8* in *C. elegans* and as been identified because of the suppressor effect of *sel-8* loss-of-function allele on gain-of-function mutants for *lin-12* (Tax et al., 1997; Doyle, Wen, & Greenwald, 2000). SEL-8 can form a ternary complex with LAG-1 and the intracellular part of the GLP-1 receptor (Petcherski & Kimble, 2000). These data suggest that SEL-8 has the same function as the *Drosophila* and mammalian Mastermind. SEL-8 is composed of a CSL-ANK N-terminal domain that interacts with LAG-1 and the ANK repeats of the Notch intradomain, a CBP/p300 domain and CycC/CDK8 domain, together involved in the recruitment of transcriptional coactivators (Figure 29).

**Figure 29** – Domain organization of the SEL-8 nuclear effector. SEL-8 is made of three distinct domains. A CSL-ANK interaction domain, a CBP/p300 domain and a CycC/CDK8 domain

The ternary complex made by LAG-1/SEL-8/NICD constitutes a favorable context to recruit transcriptional coactivators. All together they will trigger expression of the Notch target genes.

#### *III.1.2.6) The C. elegans Notch target genes*

LAG-1 binds to the sequence consensus RTGGGAA (Christensen et al., 1996). Consensus sequences have been used to computationally identify Notch target genes by using knowledge acquire from studies in mammals and *Drosophila.* One generic Notch target in *Drosophila* is the *E(spl)* gene. The regulatory sequence of *E(spl)* contains a SPS site (SuH paired site), which consists of two CSL binding sites in a head-to-head arrangement separated by approximately 16 base pairs (Cave, Loh, Surpris, Xia, & Caudy, 2005). In mammals this configuration is also found in the general Notch target *hes-1* (Jarriault et al., 1995). The SPS sequence binds two CSL/NICD/Mastermind complexes in order to lower the amount of NICD necessary to activate a target gene (Nam, Sliz, Pear, Aster, & Blacklow, 2007). To date, only few validated *C. elegans* Notch target genes have been identified (Figure 30). Surprisingly, none of them possess a SPS in their regulatory sequence, which raises the question as to whether the SPS motif is used in *C. elegans* to activate Notch

target genes. This indicates as well that knowledge on Notch target genes acquired from other modesl are maybe not suitable to find *C. elegans* Notch target genes. Extensive studies in *C. elegans* have to be performed to better understand the transcriptional network underlying Notch receptor activation.

| Target Gene | Product | References |
|---|---|---|
| ***lin-12*** | Notch receptor | (Wilkinson, Fitzgerald, & Greenwald, 1994) |
| ***ref-1*** | bHLH transcription factor | (Neves, English, & Priess, 2007) |
| ***mir-61/250*** | microRNA | (Yoo & Greenwald, 2005) |
| ***lip-1*** | ERK phosphatase | (Berset, et al., 2001; Lamont et al., 2004; Lee & Schedl, 2006) |
| ***lst-1*** | Novel nematode specific | (Yoo, Bais, & Greenwald, 2004; Singh et al., 2011) |
| ***lst-2*** | FYVE finger protein | (Yoo & Greenwald, 2005) |
| ***lst-3*** | SAP domain protein | (Yoo & Greenwald, 2005) |
| ***lst-4*** | SNX9 sorting nexin | (Yoo & Greenwald, 2005) |
| ***fbf-2*** | RNA binding protein | (Lamont et al., 2004; Lee & Schedl, 2006) |
| ***egl-4*** | cGMP-dependant protein kinase | (Singh et al., 2011) |
| ***let-502*** | Rho-kinase | (Farooqui et al., 2012) |
| ***hlh-16*** | bHLH transcription factor | (Bertrand, Bisso, Poole, & Hobert, 2011) |

**Figure 30** – Validated *C.elegans* Notch target genes.

## III.2) Diversity of Notch signaling in *C. elegans*

Notch signaling is involved in several cell fate decisions in *C. elegans*. Although the core component can be the same between these cell fate decisions, the interaction between the signaling cell and the receiving cell can differ. Indeed, three main mechanisms will be discussed in the following parts. The Notch lateral interaction and specification during the AC/VU decision. The biased Notch mediated interaction during VPCs specification. To finish, the inductive Notch signaling during germline development, π cell specification and early embryogenesis will be described.

### III.2.1) Lateral specification by the Notch signaling

Lateral interactions and specifications occur between two or more equivalent cells and results in the generation of cells of different types. The cells communicate together before their commitment in one fate or another. In normal conditions lateral specification is stochastic, thus any of the cells involved in this interaction can adopt a default fate. Studies in *C. elegans* and drosophila have highlighted a mechanism in which, during some cell fate decisions, differences in Notch activity are amplified by a feedback mechanism that affect ligand and receptor expression. This mechanism has been first identified by the study of two equivalent cells during gonadogenesis in *C. elegans*.

Two gonadal cells, Z1.ppp and Z4.aaa are initially equivalent in their developmental potential. Each cell has the same probability to become the Anchor Cell (AC), a terminally differentiated cell type, or a ventral uterine precursor cell (VU) that contributes to the ventral uterus (Kimble & Hirsh, 1979). However, in every hermaphrodite, only one of these cells will become the AC, while the other one takes the VU identity (Kimble, 1981; Seydoux & Greenwald, 1989).

The lateral interaction between Z1.ppp and Z4.aaa is mediated by *lin-12* and *lag-2*. Strong gain-of-function mutants for *lin-12* (which becomes constitutively activated in this context) have no AC. At the opposite, mutant worms with no *lin-12* activity have two ACs (Greenwald et al., 1983). Together these results suggest that

activation of the *lin-12* receptor induces the VU fate, while failure in its activation leads to acquisition of the AC fate. Analysis of genetic mosaics in which *lin-12(+)* (=*lin-12* wild-type allele) and *lin-12(0)* (=*lin-12* null allele) cells are juxtaposed suggested the existence of a feedback mechanism (Seydoux & Greenwald, 1989). When Z1.ppp or Z4.aaa lack *lin-12* activity while the other cell carry a wild-type allele of *lin-12*, the *lin-12* negative cell always become an AC. In these genetic mosaics, the *lin-12(+)* cells always becomes a VU, while in wild-type this happens in 50% of the cases (Figure 31).

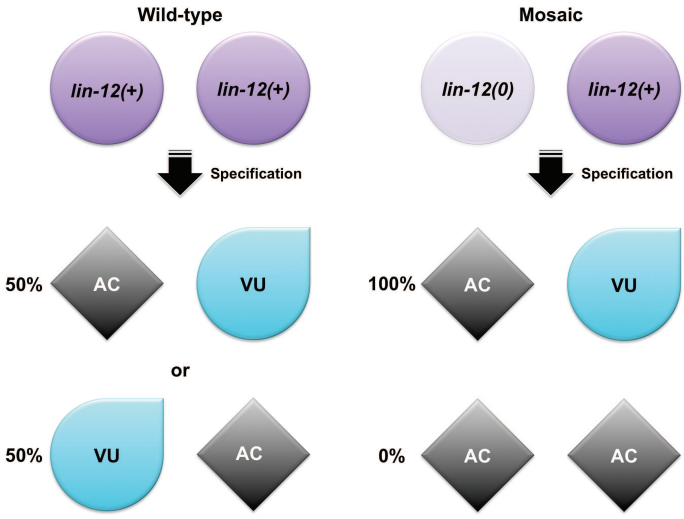


**Figure 31** – AC-VU decision in wild-type conditions and genetic mosaics. In wild-type condition, the AC/VU decision is stochastic. Both precursor cells have equal chance to become an AC or a VU cell. In genetic mosaics, the *lin-12* positive cell always become a VU, while the cell lacking *lin-12* activity takes the AC fate by default.

Precise expression pattern analysis of *lin-12* and *lag-2* during the AC/VU decision suggest that a feedback mechanism takes place at the transcriptional level of these genes (Wilkinson et al., 1994). Before their specification Z1.ppp and Z4.aaa express *lin-12* and *lag-2*. However, this expression pattern changes in a reciprocal manner. During the AC/VU specification, *lin-12* becomes restricted to the presumptive VU, while *lag-2* become restricted to the presumptive AC. These expression pattern changes take place before commitment of both Z1.ppp and Z4.aaa and appear to be dependent on *lin-12* activity. Indeed, *lin-12* activity

positively regulates its own expression and represses at the same time *lag-2* expression (Figure 32). This model of lateral specification (also called lateral inhibition) appears to be conserved. A similar mechanism takes place during the specification of sensory organ precursors in *Drosophila*.

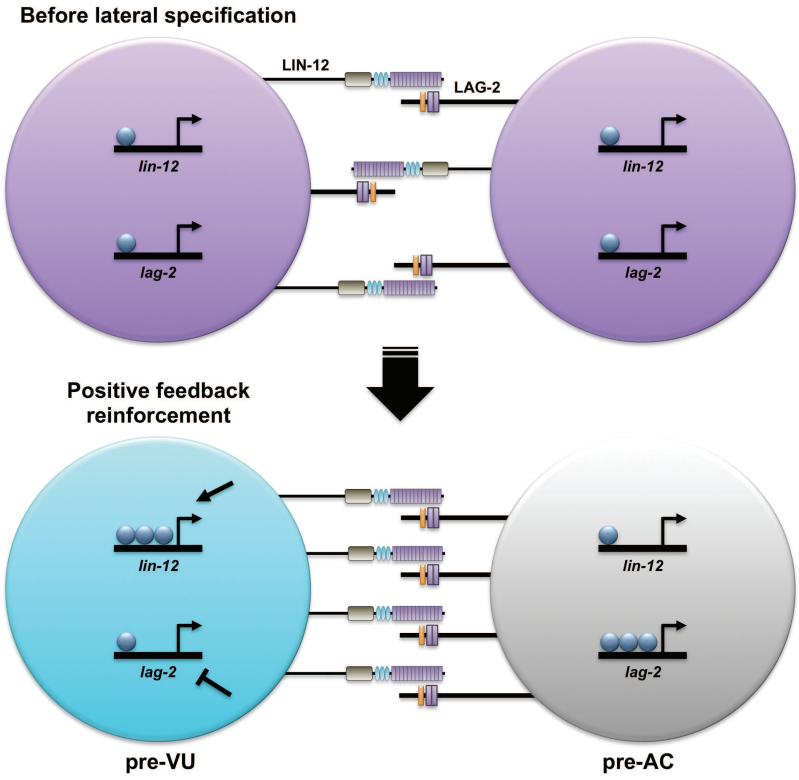


**Figure 32** – Positive feedback mechanism during the AC/VU decision. Before specification both cells express *lin-12* and *lag-2* in equal amount. Stochastic small differences in ligand or receptor activity is amplified by a feedback mechanism, which leads to transcriptional activation of *lin-12* in the pre-VU cell and transcriptional activation of *lag-2* in the pre-AC cell.

### III.2.2) Biased Notch interaction and specification

Biased Notch signaling takes place during VPCs specification and vulva formation. In wild-type hermaphrodites, three hypodermal cells, P5.p, P6.p and P7.p will be the cells responsible for the formation of the vulva. They are positioned in the ventral part of the worm lying under the somatic gonads. P5.p and P7.p are positioned at the right and left side of P6.p respectively. P6.p is positioned just under the Anchor Cell. It has been shown that these three hypodermal cells (+ 3

others) have the same developmental potential and adopt a pattern of vulval fates that can be represented as 2° fate-1°fate-2°fate (Sulston & White, 1980; Sternberg & Horvitz, 1986). This cell fate pattern is the result of the coupling of two different signaling pathways. The first engaged pathway is the EGF signaling pathway. P5-7.p cells express the LET-23/EGF receptor. At the same time the AC expresses the LIN-3/EGF ligand, which is diffused and spatially graded (Katz, Hill, Clandinin, & Sternberg, 1995; Katz et al., 1996; Yoo et al., 2004). Thus, P6.p receives the highest dose of LIN-3 while P5.p and P7.p are in contact with less ligand. The second actor of this specification is the LIN-12 pathway. *lin-12* appears to be expressed uniformly in P5-7.p during their specification suggesting that lateral specification is not taking place as for the AC/VU decision (Wilkinson & Greenwald, 1995). A cross-talk between the EGF and the Notch pathway takes place during VPC specification. Indeed, activation of LET-23 by LIN-3 in the P6.p cell appears to up-regulate ligands expression and down-regulate *lin-12* expression which leads to acquisition of the 1° fate (Levitan & Greenwald, 1998). This inductive signal governed by LIN-3 will lead to biased *lin-12* and ligand expression. The up-regulation of the ligands in P6.P leads to activation of LIN-12 in P5.p and P7.p. This activation of the Notch pathway induces the acquisition of the 2° fate and inhibition of the 1° fate in these two cells (Figure 33).

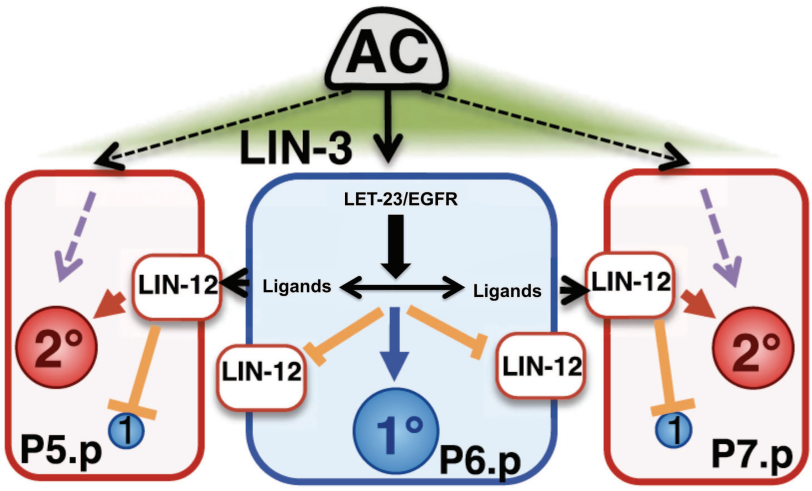


**Figure 33** – Model of biased Notch signaling during VPC specification. The EGF pathway activation in P6.p induces 1° fate, ligand expression and represses *lin-12* expression, resulting in the activation of *lin-12* in P5.p and P7.p leading to the acquisition of the 2° fate (Adapted from (Barkoulas et al., 2013)

At the opposite of the AC/VU decision, the asymmetric repartition of *lin-12* in VPC is not stochastic. The EGF pathway triggers the expression bias of *lin-12* allowing proper specification of the VPCs and formation of the vulva.

### III.2.3) Inductive Notch interactions

Inductive Notch interactions involve signaling between nonequivalent cells. One receiving cell will always express a Notch receptor, while a signaling cell expresses ligand(s). Inductive Notch interactions take place during blastomere specification in the early embryo, during gonadal morphogenesis and germ cell proliferation.

#### *III.2.3.1) Inductive Notch interactions in the early embryo*

Many inductive Notch interactions have been identified in the early embryos. In this part only two of them will be used to illustrate this model of Notch signaling.

After fertilization, the embryo undergoes a series of cleavage. The first division produces two cells called AB and $P_1$. Each of these blastomeres has a different, but reproducible, pattern of division and differentiation. $P_1$ divides asymmetrically along the antero-posterior axis to give rise to two daughters blastomeres EMS and $P_2$ that express different proteins. $P_2$ expresses the Notch ligand *apx-1* (Mickey, Mello, Montgomery, Fire, & Priess, 1996). Meanwhile, the AB blastomere divides to give the posterior daughter cell ABp and the anterior one ABa. Both of these cells have the same developmental potential and express the GLP-1 Notch receptor (Priess & Thomson, 1987; Evans, Crittenden, Kodoyianni, & Kimble, 1994). Due to the 4-cell embryo organization, only ABp can be in contact with the ligand-expressing $P_2$. The Notch pathway is then activated in ABp but not in ABa.

The second inductive Notch interaction in the early embryo takes place at the 12-cell stage. Maternally-expressed GLP-1 remains on the surfaces of the ABa descendants while two new descendants of $P_1$, E and MS, become signaling cells. MS will activate the GLP-1 receptor in ABalp and ABara blastomeres (Hutter & Schnabel, 1994; Mango et al., 1994) (Figure 34).

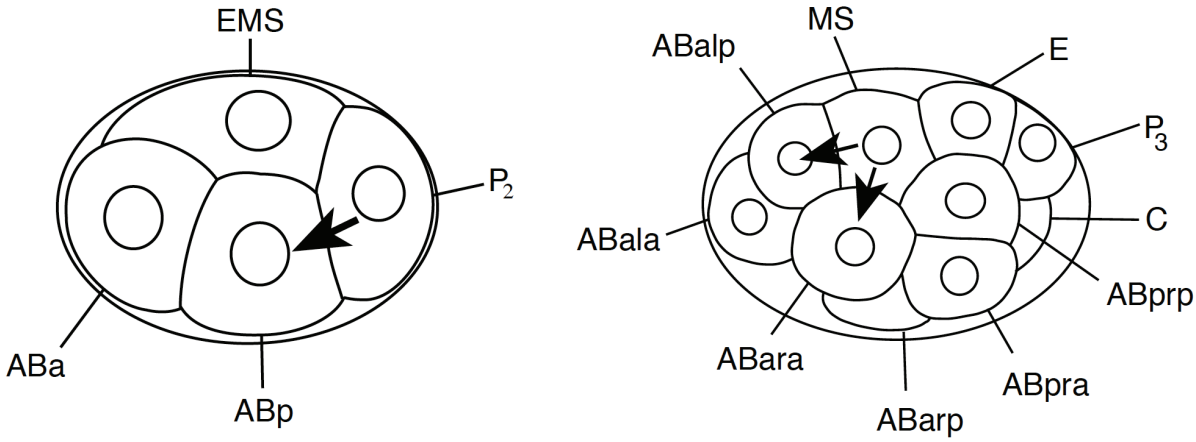


**Figure 34** – Notch inductive interactions during early embryogenesis. Black arrow represents the inductive signaling by the APX-1 ligand. In the 4-cell stage, $P_2$ induces Notch signaling activation in ABp. In the 12-cell stage, MS activates the Notch pathway in the ABalp and Abara blastomeres (Hutter & Schnabel, 1994)

These two Notch inductive interactions are essential to produce pharyngeal cells. The *C. elegans* pharynx is a vital and complex organ formed by muscle cells, gland cells, support cells and neurons. The master gene of pharynx development is the forkhead transcription factor PHA-4 (Mango, Lambie, & Kimble, 1994; Azzaria, Goszczynski, Chung, Kalb, & McGhee, 1996; Horner et al., 1998; Kalb et al., 1998). During early embryogenesis, ABa descendants express PHA-4 and give rise to pharyngeal cells, while ABp descendant do not express this gene and will be at the origin of other types of tissue. During the first inductive interaction between ABp and $P_2$, GLP-1 activation induces expression of the *ref-1* Notch target gene that will repress the expression of two functionally redundant T-box transcription factor *tbx-37 and tbx-38* (Neves et al., 2007). These genes have been shown to be essential for the production of pharyngeal tissue (Good et al., 2004). Thus, preventing the expression of *tbx-37* and *tbx-38* in ABp descendants inhibits their commitment in the pharyngeal lineage. At the opposite, because the ABa blastomere is not induced by Notch signaling, *tbx-37* and *tbx-38* can be expressed and induce commitment to the pharyngeal lineage. In summary, the first inductive Notch signaling prevents, and the second interaction induces pharynx development.

#### *III.2.3.2) Inductive Notch interaction during gonadal morphogenesis*

Inductive Notch interaction takes place during gonadal morphogenesis. As discussed above, the AC induces and patterns the vulva but later the AC also patterns the ventral uterus. The AC cell will induce the nearest six of twelve granddaughters of the Ventral Uterine cell to adopt a π fate. This induction takes place because of the LAG-2 ligand expressed by the AC and the LIN-12 receptor expressed by the future π cells. A well-detailed gene network has been identified allowing this inductive interaction. Indeed, *lag-2* expression in the AC cell has been shown to be triggered by the Zn-finger gene *lin-29,* while *lin-12* expression is induced by the NHR family member*, nhr-67* in the π cell (Newman, Inoue, Wang, & Sternberg, 2000; Verghese et al., 2011). Once *activated, lin-12* activates the expression of *egl-43, egl-13* and *lin-11* to allow proper π cell specification and vulva morphogenesis (Newman, et al., 1999; Newman et al., 2000; Cinar, Richards, Oommen, & Newman, 2003; Marri & Gupta, 2009; Verghese et al., 2011).

#### *III.2.3.3) Inductive Notch interaction during germ cells proliferation*

In the *C. elegans* gonad, inductive Notch signaling regulates the switch from mitotic germ cells to meiotic germ cells. Germ cells proliferation defect is found in loss-of-function mutants for the ligands *apx-1* and *lag-2* (Henderson, et al., 1994; Tax et al., 1994; Nadarajan, Govindan, McGovern, Hubbard, & Greenstein, 2009) the receptor *glp-1* (Austin & Kimble, 1989; Yochem & Greenwald, 1989), or the nuclear effector *lag-1* (Christensen et al., 1996). LAG-2 and APX-1 are expressed in the DTC, which activates GLP-1 receptor expressed on the surface of the germ cells (Crittenden, Troemel, Evans, & Kimble, 1994). Notch pathway activation leads to expression of genes necessary for the proliferative fate. Then, germ cells migrate proximally, away from the DTC. Germ cells are not making contact with the DTC anymore, by consequence Notch signaling levels decrease, allowing the cells to enter into meiosis.

The switch from mitotic to meiotic state is controlled by two pathways downstream of GLP-1/Notch signaling. In one hand, in the germ cells, Notch signaling maintains cells in the proliferative fate through inhibition of RNA regulatory pathways involved in the promotion of the meiotic entry and the inhibition of

proliferative fate. These RNA regulatory pathways are the GLD-1 and GLD-2 pathways. In another hand, the GLD pathway appears to promote meiotic entry and is regulating by the FBF pathway, which is composed of a direct GLP-1 target gene.

GLD-1 and GLD-2 act redundantly, single mutant for each gene has no phenotype while *gld-1;gld-2* double mutants have germ cell over-proliferation (Kadyk & Kimble, 1998; Eckmann, Crittenden, Suh, & Kimble, 2004; Hansen, Hubbard, & Schedl, 2004a; Hansen, Wilson-Berry, Dang, & Schedl, 2004b). GLD-1 is a translational inhibitor homologous to mouse Quacking (Jones & Schedl, 1995; Lee & Schedl, 2010) and is able to bind 3'UTRs of mRNAs to inhibit their translation. The GLD-1 pathway also includes *nos-3* (Hansen et al., 2004a; Hansen et al., 2004b), the homologue of the transcriptional regulator *Nanos* in *Drosophila* (Kraemer et al., 1999). The GLD-2 pathway includes *gld-2,* a catalytic portion of the poly(A) polymerase, and *gld-3* BicC homologue (Kadyk & Kimble, 1998; Eckmann et al., 2004). Activities of the GLD-1 and GLD-2 pathways are low or absent in the distal end of the gonad where GLP-1 levels are high. At the opposite, when germ cells move proximally, and are not in contact with the DTC anymore, GLP-1 activity decrease causing GLD-1 and GLD-2 pathway activity to increase. Thus, cells enter in meiosis. The relation between GLP-1 and GLD-1 has been well described. GLD-1 protein is undetectable in the distal end of the gonad and rises gradually in the proliferative zone until reaching a maximum level where cells enter in meiosis (Jones, Francis, & Schedl, 1996). When GLP-1 is removed, GLD-1 protein is located in the distal end of the gonad, suggesting that GLP-1 suppresses GLD-1 accumulation in the distal part of the gonad (Hansen et al., 2004a; Hansen et al., 2004b). GLD-2 and GLD-3 also display a nonuniform expression pattern (Eckmann, Kraemer, Wickens, & Kimble, 2002; Wang, Eckmann, Kadyk, Wickens, & Kimble, 2002; Eckmann et al., 2004) but, to date, the role of this spatial distribution in the proliferative fate versus meiotic entry has not been assessed. Molecular and functional characterization of these GLD-1/GLD-2 pathways revealed that GLD-1 inhibits translation while GLD-2 and GLD-3 have a role in mRNA stabilization. Thus GLD-1 and GLD-2/3 have RNA targets with opposite functions. GLD-1 represses translation of mRNAs that promote proliferative fate, while GLD-2 promotes translation of mRNAs that promote the meiotic fate (Hansen & Schedl, 2013).

Notch signaling is mainly known for its transcription activation function. However, the GLD-1 and GLD-2 pathways appear to be inhibited by the GLP-1 Notch signaling. Thus, inhibition of GLD pathways is certainly indirect. Indeed this inhibition is due to the action of *fbf-1* and *fbf-2*. FBF proteins are translational regulators homologous to the *Drosophila Pumilio* (Zhang et al., 1997) and have redundant function in the decision between proliferative fate and meiotic entry. *fbf-2* has been shown to be a direct target of the GLP-1 receptor. The regulatory sequence of *fbf-2* contains LAG-1 binding site, and can be bound by LAG-1 in vitro (Crittenden et al., 2002). Moreover, FBF-2 protein is localized in the distal region of the gonad where GLP-1 activity is the highest. Surprisingly, even though *fbf-1* and *fbf-2* are redundant, only *fbf-2* appears to be directly regulated by LAG-1. When *fbf-1* and *fbf-2* are eliminated, germ cells enter into meiosis prematurely (Crittenden et al., 2002). Thus these genes promote the proliferative fate. Some evidence suggest that FBF-1 and FBF-2 may have a reciprocal inhibitory feedback, as they bind each other's 3'UTR (Lamont et al., 2004), by consequence FBF-2 could have a dual role, where it promotes and inhibits proliferative fate and could be regulated by its interaction with FBF-1.

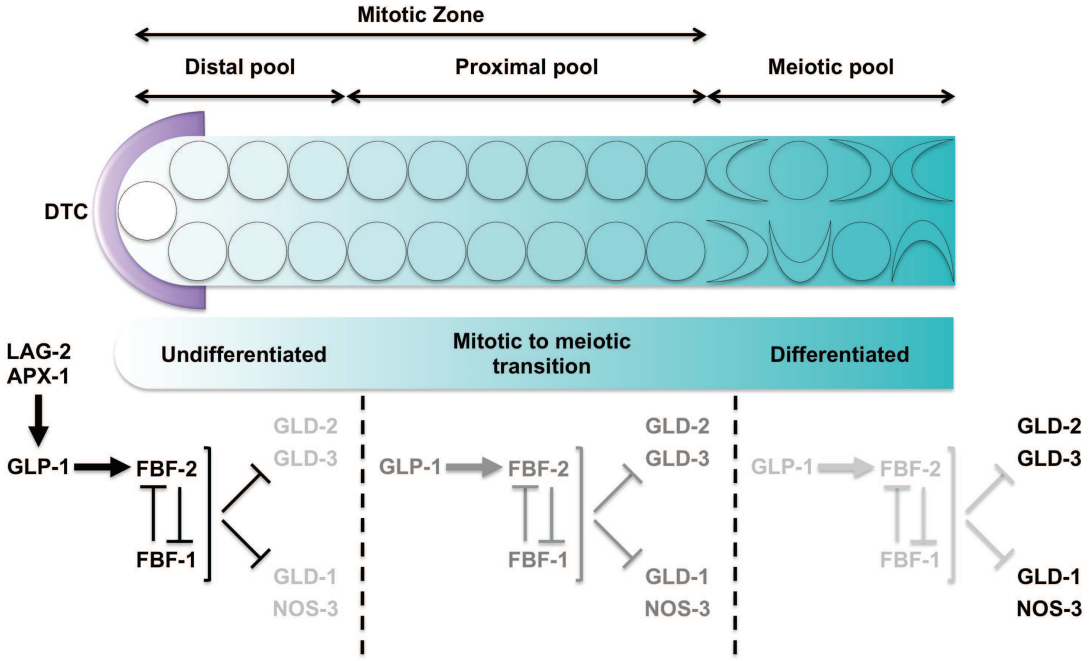


**Figure 35** – Notch inductive interactions during germ cell proliferations. The DTC expresses the APX-1 and LAG-2 ligands leading to activation of the GLP-1/Notch pathway in distal germ cells. Notch signaling activates expression of *fbf-1* and *fbf-2*, which are inhibitors of the GLD-1 and GLD-2 pathway. As germ cells migrate away from the DTC GLP-1 activity is decreased, inducing activation of GLD-1 and GLD-2 pathways, which promote meiosis entry.

FBF proteins appear to promote proliferative fate by inhibiting the activity of the GLD-1 and GDL-2 pathways. Indeed, FBF binds the 3'UTR of GLD-1 mRNA (Suh et al., 2009). In summary, in the distal part of the gonad, LAG-2 expressed by the DTC activates through inductive interactions the GLP-1 receptor expressed in germ cells. This activation leads to expression of *fbf-1* and *fbf-2*, which will promote the proliferative fate by inhibiting GLD-1 and GLD-2 pathways. Germ cells will migrate away from the DTC and will have less active GLP-1 receptor, leading to decrease of FBF expression, increase activity of GLD-1 and GLD-2 pathway, which will induce meiotic entry (Figure 35).

## III.3) Notch signaling and cell plasticity

Notch signaling is involved in many biological processes and pathologies. Over the years, an increasing number of studies involving the Notch signaling have been published. However, only few of them concern the role of Notch signaling during cell plasticity events. This can be explained by the fact that cell plasticity events are hard to identify and follow, by consequence only few precisely observed cases have been described. An other explanation could come from the lack of precise lineage tracing tools making analysis of cell plasticity difficult. Recently, several studies have made a link between Notch signaling and cell plasticity; they will be discussed in the following parts.

### III.3.1) Notch signaling and cell plasticity during organ regeneration

Recent studies have made a link between regeneration and Notch signaling in zebrafish and mammals. As discussed in the part I.2.3.1 and I.2.6.2, Zebrafish can regenerate organs, after injury, such as the heart or fin.

Heart regeneration takes place because of dedifferentiation of cardiomyocytes and transdifferentiation of atrial cardiomyocytes into ventricular cardiomyocytes. During cardiac ventricular regeneration, transdifferentiation appears to be mediated by Notch signaling. After injury, Notch expression is activated in atrial cardiomyocytes prior transdifferentiation. Activation of Notch

signaling may lead to sarcomeric reorganization, cardiomyocyte migration and re-expression of key early cardiac transcriptional regulators such as Gata4, Hand2, Mef2, Nkx2.5, Tbx5 and Tbx20. These results have been confirmed by inhibition of Notch signaling which leads to transdifferentiation blockade (Zhang et al., 2013).

Two independent groups have recently reported that Notch signaling is crucial for Zebrafish fin regeneration. This regeneration event occurs through a mechanism called epimorphic regeneration whereby a population of mesenchymal cells, the blastema, appears at the wound site. Progenitor cells are located in the blastema and will divide, differentiate and organize to restore the lost tissues. Notch pathway genes are expressed in the blastema cells after injury and are maintained in the proliferative cells of the blastema. Inhibition of Notch activity reduces proliferation in the blastema leading to regeneration alterations. Conversely, Notch over-activation induces increase proliferation and maintenance of the undifferentiated state of the blastema cells. These Notch dependent events are due to activation of the *msxe*, *msxb* gene and the retinoic acid pathway, which are respectively blastema cells markers and proliferation regulator. Together, these study brought the evidence that Notch signaling maintains blastema cells in a plastic, undifferentiated and proliferative state (Grotek, Wehner, & Weidinger, 2013; Munch, Gonzalez-Rajal, & de la Pompa, 2013).

A last example has been also reported recently, and takes place during mouse liver regeneration. The liver is the mammalian organ with the best regenerative capacity. After amputation of up to 75%, the liver can completely regenerate. This regeneration takes place, in part, because of transdifferentiation of hepatocytes into biliary epithelial cells (BEC). Notch signaling appears to be sufficient to induce this switch from one fate to the other. Hepatocyte-to-BEC transdifferentiation requires an intermediate state where cells co-express hepatocyte and BEC markers and adopt morphological features of BECs (Yanger et al., 2013).

### III.3.2) Notch signaling and cell plasticity in *C. elegans*

Pluripotency of the *C. elegans* blastomeres and their reprogramming by expression of specific factors has been discussed in the part II.3.1.1. The wild-type embryo becomes refractory to reprogramming around the 8E stage, marking a transition from plasticity to restriction in developmental potential. *C. elegans* Notch signaling has been described to restrict embryonic developmental plasticity.

Elimination of the GLP-1 Notch receptor before the 2E stage extends the period during which nonendodermal cells can be reprogrammed into endoderm (by over-expression of the endoderm-promoting END-3 factor). In these conditions the reprogramming window is expanded until the early 20E stage (In wild-type reprogramming can be performed only until the 8E stage). Surprisingly, reactivation of GLP-1 after the 2E stage does not reverse this effect. Conversely, when GLP-1 activity is abolished after the 2E stage, embryos become developmentally committed and cannot be reprogrammed. Thus, GLP-1 sets a memory state in the early embryo, which delays plasticity to commitment transition. GLP-1 appears to act with LIN-12 to restrict developmental plasticity. Indeed, before the 2E stage only GLP-1 is required, whereas after this stage both LIN-12 and GLP-1 are required. Moreover, Notch signaling restricts developmental plasticity specifically in the AB blastomeres.

One of the most surprising results of this study is that none of the canonical Notch ligands (LAG-2 or APX-1) appears to be involved in the establishment of this developmental plasticity restriction. Indeed, developmental plasticity restriction is suppressed only when the secreted DSL-3 or DSL-1 ligand are eliminated.

In summary, two fundamentally different modes of action for Notch signaling take place during embryogenesis. The first one involved one set of ligand that control specification of cell identity, while another regulates developmental plasticity (Djabrayan et al., 2012).

To finish, LIN-12/Notch signaling appears to be involved in the establishment of the competence to transdifferentiate during Y-to-PDA transdifferentiation, the subject of my thesis, as I will try to demonstrate in the next sections.

# MATERIEL AND METHODS

This part of the manuscript will describe all the strains, material and methods used to produce the discussed results. Note: our lab name is IS and our lab allele is fp.

## I) *C. elegans* maintenance, strains and alleles

Standard methods as described in (Brenner, 1974) were used for worm handling, maintenance and genetic analysis. Experiments were mostly performed at 25°C unless otherwise indicated. The strains are ordered according to their use in the discussed results:

| | |
|---|---|
| **Wild-type background** | |
| **IS98** | *syIs63[cog-1p::gfp;unc-119(+)] IV (outcrossed 8x)* |
| ***lin-12* mutant characterization** | |
| **IS791** | *lin-12(n950) III; syIs63[cog-1p::gfp;unc-119(+)] IV* |
| **IS2120** | *syIs63[cog-1::gfp;unc-119(+)] IV; fpEx683[mig-13p::mCherry; myo-2::mCherry]* |
| **IS2121** | *syIs63[cog-1::gfp;unc-119(+)] IV; fpEx684[mig-13p::mCherry; myo-2::mCherry]* |
| **IS2123** | *syIs63[cog-1::gfp;unc-119(+)] IV; fpEx686[mig-13p::mCherry; myo-2::mCherry]* |
| **IS2118** | *lin-12(n950) III; syIs63[cog-1p::gfp;unc-119(+)] IV; fpEX682[mig-13p::mCherry; myo-2::mCherry]* |
| **IS2115** | *syIs63[cog-1p::gfp;unc-119(+)] IV; wyEx1902[itr-1p::mCherry; odr-1p::GFP]* |
| **IS2116** | *lin-12(n950) III; syIs63[cog-1p::gfp;unc-119(+)] IV; wyEx1902[itr-1p::mCherry; odr-1p::GFP]* |
| **IS2199** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpIs67[itr-1p::mCherry; odr-1p::GFP]* |
| **IS2235** | *unc-32(e189) lin-12(n676n930)III; syIs63[cog-1p::gfp;unc-119(+)] IV; fpIs67[itr-1p::mCherry; odr-1p::GFP]* |
| ***glp-1* mutant characterization** | |
| **IS790** | *glp-1(e2141ts) III; syIs63[cog-1p::gfp;unc-119(+)] IV* |
| **IS2153** | *glp-1(ar202ts) IIII; syIs63[cog-1p::gfp;unc-119(+)] IV* |
| ***hsp::ICLGFP* transgenic strains** | |
| **IS2093** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx664[hsp-16.2::ICLGFP; myo-2::mCherry]* |
| **IS2094** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx665[hsp-16.2::ICLGFP; myo-2::mCherry]* |
| **IS2095** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx666[hsp-16.2::ICLGFP; myo-2::mCherry]* |
| **IS2231** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpIs67[itr-1p::mCherry; odr-1p::GFP]; fpEx665[hsp-16.2::ICLGFP; myo-2::mCherry]* |
| **IS2232** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpIs67[itr-1p::mCherry; odr-1p::GFP]; fpEx666[hsp-16.2::ICLGFP; myo-2::mCherry]* |
| **RNAi sensitized background** | |
| **IS85** | *rrf-3(pk1426) II; syIs63[cog-1p::gfp;unc-119(+)] IV* |
| **IS1675** | *rrf-3(pk1426) II; lin-12(n950) III; syIs63[cog-1p::gfp;unc-119(+)] IV* |

| | |
|---|---|
| ***lin-12* reporter strains** | |
| **GS4335** | *arIs41[LIN-12::gfp; pRF4]* |
| **HA2182** | *pha-1(e2123)III; rtEx727[lin-12p::gfp, myo-2p::gfp, pha-1(+)]* |
| **GS956** | *smg-1(r861) unc-54(r293)I; arIs11[lin-12p::lacZ; rol-6(su1006)]* |
| ***promoter-specific::ICLGFP* transgenic strains** | |
| **IS834** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx84[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (20ng), myo-2p::GFP]* |
| **IS835** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx85[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (20ng), myo-2p::GFP]* |
| **IS836** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx86[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (20ng), myo-2p::GFP]* |
| **IS837** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx87[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (20ng), myo-2p::GFP]* |
| **IS838** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx88[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (20ng), myo-2p::GFP]* |
| **IS1052** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx217[col-34p::ICLGFP; myo-2p::GFP]* |
| **IS1053** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx218[col-34p::ICLGFP; myo-2p::GFP]* |
| **IS1054** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx219[col-34p::ICLGFP; myo-2p::GFP]* |
| **IS1055** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx220[col-34p::ICLGFP; myo-2p::GFP]* |
| **IS1056** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx221[col-34p::ICLGFP; myo-2p::GFP]* |
| **IS1388** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx334[lin-48p::ICLGFP::SL2::mCherry; myo-2p::GFP]* |
| **IS1389** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx335[lin-48p::ICLGFP::SL2::mCherry; myo-2p::GFP]* |
| **IS1390** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx336[lin-48p::ICLGFP::SL2::mCherry; myo-2p::GFP]* |
| **IS1391** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx337[lin-48p::ICLGFP::SL2::mCherry; myo-2p::GFP]* |
| **IS1392** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx338[lin-48p::ICLGFP::SL2::mCherry; myo-2p::GFP]* |
| **IS1623** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx459[egl-20p::ICLGFP::SL2::mCherry; myo-2p::mCherry]* |
| **IS1624** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx460[egl-20p::ICLGFP::SL2::mCherry; myo-2p::mCherry]* |
| **IS1625** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx461[egl-20p::ICLGFP::SL2::mCherry; myo-2p::mCherry]* |
| **IS1626** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx462[egl-20p::ICLGFP::SL2::mCherry; myo-2p::mCherry]* |
| **IS1627** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx463[egl-20p::ICLGFP::SL2::mCherry; myo-2p::mCherry]* |
| **IS1504** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpIs22[egl-5(6,2kb)Δpes10p::ICLGFP(20ng)::SL2::mCherry (20ng); myo-2::GFP]* |
| **IS1880** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]* |
| **Reporter strains used to assess Y identity** | |
| **IS1908** | *fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]; mcIs17[lin-26p::NLS-GFP;pRF4]* |
| **IS1900** | *fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]; kuIs36[egl-26p::gfp; unc-119(+)]* |
| **IS1904** | *jcIs1[AJM-1::GFP; pRF4] IV; fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]* |
| **IS1902** | *fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]; mcIs47[DLG-1::gfp; pRF4]* |
| **IS1912** | *otIs117[unc-4(+); unc-33p::GFP] IV; fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]* |

| | |
|---|---|
| **IS1911** | *ncIs3[tag-168p::GFP] III; fpIs54[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]* |
| **IS1910** | *edIs6[unc-119p::gfp; rol-6] IV; fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]* |
| **IS1915** | *wyIs75[unc-47p::DsRed; exp-1p::GFP; odr-1p::RFP] III; fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]* |
| **IS1916** | *wyIs75[unc-47p::DsRed; exp-1p::GFP; odr-1p::RFP] III; fpIs54[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]* |
| **Reporter strains used to assess rectal cells identity** | |
| **IS2198** | *fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]; saIs14[lin-48p::GFP; unc-119(+)]* |
| **IS2149** | *eIs34[mab-9p::gfp; pCes1943 (rol-6D)] III; fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]* |
| **Transgenic strains with reduced activity of *ICLGFP*** | |
| **IS1172** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx263[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (2ng), myo-2p::mCherry]* |
| **IS1173** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx264[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (2ng), myo-2p::mCherry]* |
| **IS1174** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx265[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (2ng), myo-2p::mCherry]* |
| **IS1175** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx266[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (2ng), myo-2p::mCherry]* |
| **IS1176** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx267[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (0.2ng), myo-2p::mCherry]* |
| **IS1177** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx268[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (0.2ng), myo-2p::mCherry]* |
| **IS1178** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx269[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (0.2ng), myo-2p::mCherry]* |
| **IS1179** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx270[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (0.2ng), myo-2p::mCherry]* |
| **IS1180** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx271[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (0.2ng), myo-2p::mCherry]* |
| **Canonical Notch ligand mutant strains** | |
| **IS794** | *syIs63[cog-1p::gfp;unc-119(+)] IV; sel-12(ar171) unc-1(e538) X* |
| **IS792** | *lin-12(n950 III; syIs63[cog-1p::gfp;unc-119(+)] IV; sel-12(ar171) unc-1(e538) X* |
| **IS1361** | *syIs63[cog-1p::gfp;unc-119(+)] IV; apx-1(zu347ts)V* |
| **IS1362** | *syIs63[cog-1p::gfp;unc-119(+)] IV; apx-1(zu347ts)V; sel-12(ar171) unc-1(e538) X* |
| **IS1364** | *syIs63[cog-1p::gfp;unc-119(+)] IV; lag-2(q420ts)V* |
| **Non-canonical ligand mutant strains** | |
| **IS1925** | *syIs63[cog-1p::gfp;unc-119(+)] IV; arg-1(ok3127)X* |
| **IS2009** | *rrf-3(pk1426) II; syIs63[cog-1p::gfp;unc-119(+)] IV; arg-1(ok3127)X* |
| **IS770** | *dsl-1(ok810) V; fpIs11[exp-1:p:mCherry; myo-2p::GFP]* |
| **IS973** | *dsl-1(ok810) IV; sel-12(ar171) unc-1(e538) X; fpIs10[exp-1p::mCherry; myo-2p::GFP]* |
| **IS1925** | *syIs63[cog-1p::gfp;unc-119(+)] IV; arg-1(ok3127) X* |
| **IS1895** | *wyIs75[unc-47p::DsRed; exp-1p::GFP; odr-1p::RFP] III; dsl-3(ok3411) IV* |
| **IS1894** | *wyIs75[unc-47p::DsRed; exp-1p::GFP; odr-1p::RFP] III; dsl-3(ok3411) IV; sel-12(ar171) unc-1(e538) X* |
| **IS1890** | *syIs63[cog-1p::gfp;unc-119(+)] IV; dsl-4(ok1020)X* |
| **IS1958** | *rrf-3(pk1426) II; syIs63[cog-1p::gfp;unc-119(+)] IV; dsl-4(ok1020)X* |
| **IS1872** | *wyIs75[unc-47p::DsRed; exp-1p::GFP; odr-1p::RFP] III; dsl-5(ok588) IV* |

| | |
|---|---|
| **IS1873** | *wyIs75[unc-47p::DsRed; exp-1p::GFP; odr-1p::RFP] III; dsl-5(ok588) IV; sel-12(ar171) unc-1(e538) X* |
| **IS1874** | *wyIs75[unc-47p::DsRed; exp-1p::GFP; odr-1p::RFP] III; dsl-6(ok2265) IV* |
| **IS1875** | *wyIs75[unc-47p::DsRed; exp-1p::GFP; odr-1p::RFP] III; dsl-6(ok2265) IV; sel-12(ar171) unc-1(e538) X* |
| **IS1887** | *osm-7(n1515) III; syIs63[cog-1p::gfp;unc-119(+)] IV* |
| **IS1888** | *osm-7(n1515) III; syIs63[cog-1p::gfp;unc-119(+)] IV; sel-12(ar171) unc-1(e538) X* |
| **IS1924** | *syIs63[cog-1p::gfp;unc-119(+)] IV; osm-11(rt192) X* |
| **IS1960** | *rrf-3(pk1426) II; syIs63[cog-1p::gfp;unc-119(+)] IV; osm-11(rt192)X* |
| **IS1876** | *dos-1(ok2398) III; syIs63[cog-1p::gfp;unc-119(+)] IV* |
| **IS1877** | *dos-1(ok2398) III; syIs63[cog-1p::gfp;unc-119(+)] IV; sel-12(ar171) unc-1(e538) X* |
| **IS1889** | *dos-2(tm4515) II; syIs63[cog-1p::gfp;unc-119(+)] IV* |
| **IS1878** | *dos-2(tm4515) II; syIs63[cog-1p::gfp;unc-119(+)] IV; sel-12(ar171) unc-1(e538) X* |
| **IS1879** | *dos-3(tm4899) III; syIs63[cog-1p::gfp;unc-119(+)] IV* |
| **IS1881** | *dos-3(tm4899) III; syIs63[cog-1p::gfp;unc-119(+)] IV; sel-12(ar171) unc-1(e538) X* |
| **Ligand reporter strains** | |
| **GS3795** | *dpy-20(e1282) IV; arIs98[apx-1p::2NLS::YFP; ceh-22::gfp; pMH86]* |
| **JK2049** | *qIs19[lag-2p::GFP; pRF4] V* |
| **Transgenic strains used to assess ligand availability over time** | |
| **IS2164** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx713[col-34p::lin-12cDNA(n941); myo-2p::mCherry]* |
| **IS2166** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx714[col-34p::lin-12cDNA(n941); myo-2p::mCherry]* |
| **IS2169** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx717[col-34p::lin-12cDNA(n941); myo-2p::mCherry]* |
| **IS2193** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx729[col-34p::lin-12cDNA(ΔANK); myo-2p::mCherry]* |
| **IS2195** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx731[col-34p::lin-12cDNA(ΔANK); myo-2p::mCherry]* |
| **IS2196** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx732[col-34p::lin-12cDNA(ΔANK); myo-2p::mCherry]* |
| **IS2161** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx710[col-34p::lin-12cDNA(n137); myo-2p::mCherry]* |
| **IS2162** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx711[col-34p::lin-12cDNA(n137); myo-2p::mCherry]* |
| **IS2163** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx712[col-34p::lin-12cDNA(n137); myo-2p::mCherry]* |
| **IS2130** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx689[col-34p::lin-12cDNA; myo-2p::mCherry]* |
| **IS2132** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx691[col-34p::lin-12cDNA; myo-2p::mCherry]* |
| **IS2133** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx692[col-34p::lin-12cDNA; myo-2p::mCherry]* |
| **IS2135** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx694[col-34p::lin-12cDNA::SL2::mCherry; myo-2p::mCherry]* |
| **IS2138** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx697[col-34p::lin-12cDNA::SL2::mCherry; myo-2p::mCherry]* |
| **IS2139** | *syIs63[cog-1p::gfp;unc-119(+)] IV; fpEx698[col-34p::lin-12cDNA::SL2::mCherry; myo-2p::mCherry]* |

# II) Plasmid constructions

Note: In our study, *ICLGFP* sequence includes most of the domains of the intracellular part of the LIN-12 receptor and starts just after the predicted TM domain. GFP as been inserted in frame in this sequence (Sophie JARRIAULT personal communication)

**pSJ3177 – *MCS::ICLGFP::unc-54 3'UTR***

*ICLGFP* was amplified by PCR from the plasmid pSJ201 – *ICLGFP in pBSKII*$^{+}$ with the following primers:

F-KpnI – 5' AACGGTACCAGAAAAAATGGTTGTTCTGATGTTAGGAGCATTACC 3'

R-KpnI – 5' TTGGTACCTCAAAAATAATGAGCTGGTTCGGAGTATCG 3'

The obtained PCR product was digested with KpnI and inserted in the multiple cloning site (MCS) 2 of pSJ901 – *MCS1::MCS2::unc-54 3'UTR*

**pSJ3171 - *hsp-16.2::ICLGFP::unc-54 3´UTR***

*hsp-16.2* was excised from pPD49.78 with BamHI and HindIII, and was inserted in MCS1 of pSJ3177 – *MCS::ICLGFP::unc-54 3'UTR.*

**pSJ6003 - *egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry::unc-54 3´UTR***

ICLGFP was amplified by PCR from the plasmid pSJ201 with the following primers:

F-KpnI – 5' AACGGTACCAGAAAAAATGGTTGTTCTGATGTTAGGAGCATTACC 3'

R-KpnI – 5' TTGGTACCTCAAAAATAATGAGCTGGTTCGGAGTATCG 3'

The obtained PCR product was inserted in the MCS of pSJ671 - *egl-5(6,7kb)Δpes10p::MCS::SL2::mCherry* at a unique KpnI site.

**pSJ3173 – *col-34p::ICLGFP::unc-54 3´UTR***

*col-34p* was amplified by PCR from genomic DNA with the following primers:

F-SphI – 5' ACAGCATGCGACATGTAAAGTACATCCGTTACATC 3'

R-XmaI – 5' CCCCCCGGGTGTATGCAGTGGTGGTTTGG 3'

The obtained PCR product was digested by SphI and XmaI, and inserted in the MCS of pSJ3177 – *MCS::ICLGFP::unc-54 3'UTR*

**pSJ3169 – *lin-48p::ICLGFP::SL2::mCherry::unc-54 3´UTR***

*lin-48p* was amplified by PCR from genomic DNA with the following primers:

F-SphI – 5' ACATGCATGCGGATCCAAAAAACCTGCATTTTTTTCAG 3'

R-XmaI – 5' CCCCCCGGGCTGAAATTGAGCAGAGCTGAAAATTTTTG 3'

The obtained PCR product was digested with SphI and XmaI, and inserted in the MCS of pSJ3177 – *MCS::ICLGFP::unc-54 3'UTR resulting in pSJ3169 – lin-48p::ICLGFP::unc-54 3'UTR*

The SL2::mCherry sequence was amplified by PCR from pJG7-psm-SL2-Mcherry (Gift from Cory Bargmann laboratory) with the following primers:

F-NotI – 5' GCGGCCGCGCTGTCTCATCCTACTTTCACC 3'

R-NotI – 5' GCGGCCGCCTACTTATACAATTCATCCATGCC 3'

The obtained PCR product was digested by NotI and inserted after ICLGFP in *pSJ3169 – lin-48p::ICLGFP::unc-54 3'UTR*

**pSJ3162 – *egl-20p::ICLGFP::SL2::mCherry::unc-54 3´UTR***

*egl-20p* was amplified by PCR from genomic DNA with the following primers :

F-SphI – 5' AAAGCATGCGAAGTCATCCTACTAACTAACAATATGACGC 3'

R-XmaI – 5’ AAACCCGGGTATTTCTGAAATTGAGATGTTTTAGAATTTC 3’

The obtained PCR product was digested with SphI and XmaI, and inserted in the MCS of pSJ3177 – *MCS::ICLGFP::unc-54 3’UTR resulting in pSJ3164 – egl-20p::ICLGFP::unc-54 3´UTR*

SL2::mCherry sequenced as been inserted as previous i*n pSJ3164 – egl-20p::ICLGFP::unc-54 3´UTR*

**pSJ3103 - *egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry::unc-54 3´UTR***

The GFP of ICLGFP sequence was deleted by inverse PCR on pSJ6003 - *egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry::unc-54 3´UTR* with the following primers:

F – 5’ GACTCAACTCATCTGACACCTCC 3’

R – 5’ GGGTCGAGTTACTTTTCTTGAAGG 3’

**pSJ3215** - ***col-34p::lin-12cDNA::unc-54 3´UTR and* pSJ3217 - *col-34p::lin-12cDNA::SL2::mCherry::unc-54 3´UTR***

*lin-12* cDNA was amplified from pLM2.4 – *lin-12cDNA in pBSKII*$^{+}$ (a gift from the Greenwald laboratory) with the following primers:

F-KpnI – 5’ AAAAGGTACCATGCGGATCCCTACGATTTG 3’

R-KpnI – 5’ TTTTGGTACCTCAAAAATAATGAGCTGGTTCGG 3’

ICLGFP was excised by KpnI from pSJ3173 – *col-34p::ICLGFP::unc-54 3´UTR and* pSJ3148 - *col-34p::ICLGFP::SL2::mCherry::*unc-54 3´UTR and replaced by the obtained PCR product of *lin-12* cDNA digested by KpnI

**pSJ3218 - *col-34p::lin-12cDNA(n137)::unc-54 3´UTR***

TCT to TTT point mutation was generated by site directed mutagenesis on pSJ3215 - *col-34p::lin-12cDNA::unc-54 3´UTR* with the following primers:

F-n137 – 5' GTGTTGTTGACTCAATATTTGCAAGGCTTGC 3'

R-n137 – 5' GCAAGCCTTGCAAATATTGAGTCAACAACAC 3'

**pSJ3222 - *col-34p::lin-12cDNA(n941)::unc-54 3´UTR***

TGG to TAG Premature stop codon was generated by site directed mutagenesis on pSJ3215 - *col-34p::lin-12cDNA::unc-54 3´UTR* with the following primers:

F-n941 – 5' GGATTCGGTGGGAAATAGTGTGACGAGCCATTG 3'

R-n941 – 5' CAATGGCTCGTCACACTATTTCCCACCGAATCC 3'

**pSJ3223 - *col-34p::lin-12cDNA(ΔANK)::unc-54 3´UTR***

Deletion of the seven ankyrin repeats contained in the intracellular part of the lin-12 receptor was performed by inverse PCR according to the recommendations of Rhett Kovall with the following primers:

F – 5' CCAGAACGAGAATATTCAATGGATC 3'

R – 5' AGGTTCAGGTTCAGTTGGAATTTG 3'

## III) Germline transformations and integrations

*fpEx683*, *fpEx684* and *fpEx685[mig-13p::mCherry; myo-2::mCherry]* were generated by injecting pCM327 – *mig-13p::mCherry::unc-10 3' UTR* (a gift from the Shen laboratory) at 8 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^{+}$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx682* was generated by injecting pCM327 – *mig-13p::mCherry::unc-10 3' UTR* (a gift from the Shen laboratory) at 8 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS791 *lin-12(n950) III; syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx664*, *fpex665* and *fpEX666[hsp-16.2::ICLGFP; myo-2::mCherry]* were generated by injecting pSJ3171 - *hsp-16.2::ICLGFP::unc-54 3´UTR* at 10 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^+$ (up to 200 ng/μL final concentration) into *hsf-1(sy441)I* background

*fpEx84*, *fpEx85*, *fpEx86*, *fpEx87* and *fpEx88*[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (20ng), myo-2p::GFP] were generated by injecting pSJ6003 - *egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry::unc-54 3´UTR* at 20 ng/μL with 5 ng/μL of pPD118.33 – *myo-2p::GFP* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx217*, *fpEx218*, *fpEx219*, *fpEx220* and *fpEX221[col-34p::ICLGFP; myo-2p::GFP]* were generated by injecting pSJ3173 – *col-34p::ICLGFP::unc-54 3´UTR* at 20 ng/μL with 2 ng/μL of pPD118.33 – *myo-2p::GFP* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx334*, *fpEx335*, *fpEx336*, *fpEx337* and *fpEx338[lin-48p::ICLGFP::SL2::mCherry; myo-2p::GFP]* were generated by injecting pSJ3169 – *lin-48p::ICLGFP::SL2::mCherry::unc-54 3´UTR* at 20 ng/μL with 2 ng/μL of pPD118.33 – *myo-2p::GFP* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx459*, *fpEx460*, *fpEx461*, *fpEx462* and *fpEx463[egl-20p::ICLGFP::SL2::mCherry; myo-2p::mCherry]* were generated by injecting pSJ3162 – *egl-20p::ICLGFP::SL2::mCherry::unc-54 3´UTR* at 20 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx263, fpEx264, fpEx265 and fpEx266[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (2ng), myo-2p::mCherry]* were generated

by injecting pSJ6003 - *egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry::unc-54 3´UTR* at 2 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx267, fpEx268, fpEx269, fpEx270 and fpEx271[egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry (0,2ng), myo-2p::mCherry]* were generated by injecting pSJ6003 - *egl-5(6,2kb)Δpes10p::ICLGFP::SL2::mCherry::unc-54 3´UTR* at 0,2 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx713*, *fpEx714* and *fpEx717[col-34p::lin-2cDNA(n941); myo-2p::mCherry]* were generated by injecting pSJ3222 - *col-34p::lin-12cDNA(n941)*::*unc-54 3´UTR* at 10 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx729*, *fpEx731* and *fpEx732[col-34p::lin-12cDNA(ΔANK); myo-2p::mCherry]* were generated by injecting pSJ3223 - *col-34p::lin-12cDNA(ΔANK)::unc-54 3´UTR* at 10 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx710*, *fpEx711* and *fpEx712[col-34p::lin-12cDNA(n137); myo-2p::mCherry]* were generated by injecting pSJ3218 - *col-34p::lin-12cDNA(n137)::unc-54 3´UTR* at 10 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx689*, *fpEx691* and *fpEx692[col-34p::lin-12cDNA; myo-2p::mCherry]* were generated by injecting pSJ3215 - *col-34p::lin-12cDNA::unc-54 3´UTR* at 10 ng/μL with 2 ng/μL of pCFJ90 – *myo-2p::mCherry* and pBSKII$^+$ (up to 200 ng/μL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpEx694*, *fpEx697* and *fpEx698[col-34p::lin-12cDNA::SL2::mCherry; myo-2p::mCherry]* were generated by injecting pSJ3217 - *col-34p::lin-12cDNA::SL2::mCherry::unc-54 3´UTR* at 10 ng/μL with 2 ng/μL of pCFJ90 – *myo-*

*2p::mCherry* and pBSKII$^+$ (up to 200 ng/µL final concentration) into IS98 *syIs63[cog-1p::gfp;unc-119(+)] IV*.

*fpIs22[egl-5(6,2kb)Δpes10p::ICLGFP(20ng)::SL2::mCherry (20ng); myo-2::GFP]*, *fpIs51[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]*, *fpIs54[egl-5(6,2kb)Δpes10p::ICL::SL2::mCherry]* and *fpIs67[itr-1p::mCherry; odr-1p::GFP]* were generated by X-ray integrations.

## IV) RNAi

RNAi experiments were performed by injection of double stranded RNA (dsRNA) synthetized with a T7 polymerase from PCR fragment amplified from a given clone or from genomic DNA.

*egl-27*, *sem-4* and *egl-5* clones were taken from the Ahringer-MRC feeding RNA interference library (Kamath et al., 2003).

Two clones called *ceh-6 target 1* and *sox-2 target 2* (Kagias et al., 2012) were used.

A *sel-7* clone (Chen, Li, & Greenwald, 2004) was used.

dsRNA against *sel-12* were synthetized from a T7-containing PCR fragment amplified from genomic DNA with the following primers:

F-T7promoter – 5’ taatacgactcactatagggATGCCTTCCACAAGGAGACAAC 3’

R-T7promoter – 5’ taatacgactcactatagggGAGATCGCTCAAGATATAATCGAAAAG 3’

Synthetized dsRNA were purified on RNeasy columns (Qiagen) and injected into gonads and pseudocoelome of young adult worms.

## V) Crossing of *lin-12(n950)* allele

In *lin-12(n950)* mutant background, the vulva is not correctly specified, therefore these mutant worms are Egl and cannot mate in normal culture conditions. To allow these worms to cross RNAi against *sel-7,* a positive regulator of the LIN-12/Notch signaling (Chen et al., 2004), has been performed.

We injected dsRNA synthetized from the *sel-7* clone described above into *lin-12(n950)* young adults. In the next generation, mothers able to lay eggs were used to perform our genetic crossing.

## VI) Heat-shock experiments

Heat-shock experiments were performed similarly for IS2093, IS2094, IS2095, IS2231 and IS2232.

Induction of ICLGFP expression around Y birth:

Egg-pulses were performed with around 100 gravid transgenic hermaphrodites at 20°C for one hour on plate with food. Mothers were removed and embryos were left on plate for 15 minutes. Plates with embryos were sealed and immerged in a water bath at 34°C for 30 minutes. The embryos were immediately cooled down in a 20 °C water bath and left to grow at 20 °C until the L4 stage, when they were scored. Both transgenic and non-transgenic siblings were handled and scored in parallel.

Induction of ICLGFP expression at mid-embryogenesis:

Egg-pulses were performed with around 100 gravid transgenic hermaphrodites at 20°C for one hour on plate with food. Mothers were removed and embryos were left on plate for 3 hours. Plates with embryos were sealed and immerged in a water bath at 34°C for 30 minutes. The embryos were immediately cooled down in a 20 °C water bath and left to grow at 20 °C until the L4 stage, when

they were scored. Both transgenic and non-transgenic siblings were handled and scored in parallel.

## VII) Temperature-shift experiments

Temperature-shift experiments of *glp-1(ar202ts) and glp-1(e2141ts)*:

Egg-pulses were performed on pre-warmed plates at 25°C for one hour with gravid hermaphrodites grown at 15°C. Mothers were removed and embryos were left at 25°C until the adult stage, to score for PDA phenotype and to monitor gonads sterility and morphology.

Temperature-shift experiments of *apx-1(zu347ts) and lag-2 (q420ts)*:

Non-sensitized and sensitized backgrounds were treated the same way during temperature-shift experiments. Worm populations grown at 15°C were bleached. The resulting embryos were put on pre-warmed plates at 26,5°C without food. After 7 hours, all the synchronized hatched L1 larva were washed away with M9 and discarded. We monitored that only embryos were remaining on the plate. OP50 was added subsequently on plates to allow embryo to grow until the L4 stage, when they were scored.

## VIII) LacZ staining

LacZ staining on GS956 *smg-1(r861) unc-54(r293)I; arls11[lin-12p::lacZ; rol-6(su1006)]* has been performed according to (Fire, 1992). LacZ expression is induced only at 25°C in the GS956 strain. However, *arls11* seems toxic at this temperature. To stain L1 larva, mixed worm population was cultured at 15°C and subsequently bleached. The obtained eggs were cultured on plate without food at 25°C to induce LacZ expression and obtain synchronized L1 worms. Staining was performed on this synchronized worm population.

## IX) Microscopic observations

DIC and epifluorescence observations were performed using a Zeiss Z1 imager microscope. Worms were mounted on a 2% agarose pad and anesthetized with 50mM of sodium azide.

# RESULTS

# I) Scientific context

Previous studies made by our laboratory have brought new knowledge on the mechanism underlying the *in vivo* transdifferentiation of a rectal epithelial cell into a neuron (see part II.3.2.2). We have shown that transdifferentiation occurs in a step-wise manner. Y acquires the competence to transdifferentiate, initiates reprogramming and become a neuron after going through a dedifferentiated and probably unipotent state (Jarriault et al., 2008; Kagias et al., 2012; Richard et al., 2011). Molecular factors involved in the initiation of the transdifferentiation have been recently identified (Kagias et al., 2012). However many other aspects of this reprogramming event have not been elucidated for the moment. For example, how the Y cell acquires its competence to transdifferentiate remains an open question.

Some answers have been brought by previous work in the laboratory, and have shed light on the potential role of the LIN-12/Notch pathway.

A link between Y fate and the LIN-12/Notch pathway had been discovered when the *lin-12* locus was identified (Greenwald et al., 1983). Lineage analyses of *lin-12* gain-of-function (gf) and loss-of-function (lf) mutant revealed that LIN-12 activity plays a role in Y fate specification.

During wild-type embryogenesis ABp**r**pppaaaa is the future Y and its contralateral homologue ABp**l**pppaaaa is the future DA9 neuron (Sulston et al., 1983). The fate of these cells changes according to LIN-12 activity. Indeed, in *lin-12* null mutant Y is not specified and takes on DA9 fate, while DA9 keeps its identity. At the opposite, in *lin-12(gf)* mutants, DA9 acquires the Y fate while Y keeps its identity (Greenwald et al., 1983). Thus *lin-12* is both necessary and sufficient for the acquisition of the Y fate. This fate acquisition is not the result of a binary lateral signaling process (Y and DA9 fate is independent of each other and is only dependent on the dose *lin-12* activity). This led us to ask whether the supernumerary Y cell found in *lin-12(gf)* mutants is competent to transdifferentiate and whether being a Y cell is sufficient to be able to transdifferentiate.

In fact, a supernumerary Y has been described to exist in several mutant backgrounds. In *egl-38*/PAX mutant background, the rectal cell U is transformed into Y (Chamberlin et al., 1997). Similarly in *mab-9*/T-box mutant background, the rectal cell B takes the Y identity (Chisholm & Hodgkin, 1989). These cell fate conversions have been identified firstly by lineage analysis in males, and confirmed by molecular markers expression (Chamberlin et al., 1997; Woollard & Hodgkin, 2000; Jarriault et al., 2008). Indeed the transformed U and B cells express Y epithelial markers, which is not the case in wild-type background and behave as rectal cells. Furthermore, in males both converted cells exhibit the exact same division pattern than Y. However despite exhibiting characteristics of a bona fide Y cell, the supernumerary Y cell found in these mutant backgrounds never becomes a PDA neuron, nor do they change their identity. These observations suggest that being a Y cell is not sufficient to become a PDA. In contrast to these results, analysis of *lin-12(gf)* mutants shows that the supernumerary Y coming from DA9 conversion is competent to transdifferentiate. In this mutant background, two PDAs are formed. All together these data suggest that *lin-12* may be necessary to induce Y fate and its competence to transdifferentiate.

Even though, we know that *lin-12* is crucial for the transdifferentiation to take place, precise characterization of its function in Y-to-PDA reprogramming has never been performed. By consequence, my work was focused on the elucidation of the role of the LIN-12/Notch pathway in the acquisition of the competence to transdifferentiate *in vivo*.

# II) Results

## II.1) Characterization of the lin-12 mutants

### II.1.1) Phenotype of the *lin-12(gf)* mutants

*C. elegans* Notch gain-of-function mutants have the particularity to have two Y cells able to transdifferentiate into two PDAs. This has been identified because of lineage analysis and observations of PDA specific markers (Jarriault et al., 2008).

Observations of PDA specific markers such as *cog-1p*::GFP (Palmer, 2002) in *lin-12(gf)* mutants reveal that several phenotypes can be obtained. We introduced an integrated array, *syIs63*, carrying the PDA marker *cog-1p::GFP*, in a *lin-12(n950)* background, which is one of the strongest *lin-12* gain-of-function allele (Greenwald et al., 1983; Tax et al., 1997). We selected this allele for its strong gain of function phenotype and its relative easy genetic use. Indeed, the *n950* allele can be maintained and crossed easily in homozygous state (See Materiel and Methods), compare to the strongest gain-of-function allele, *n137*, which is difficult to maintain and cross.

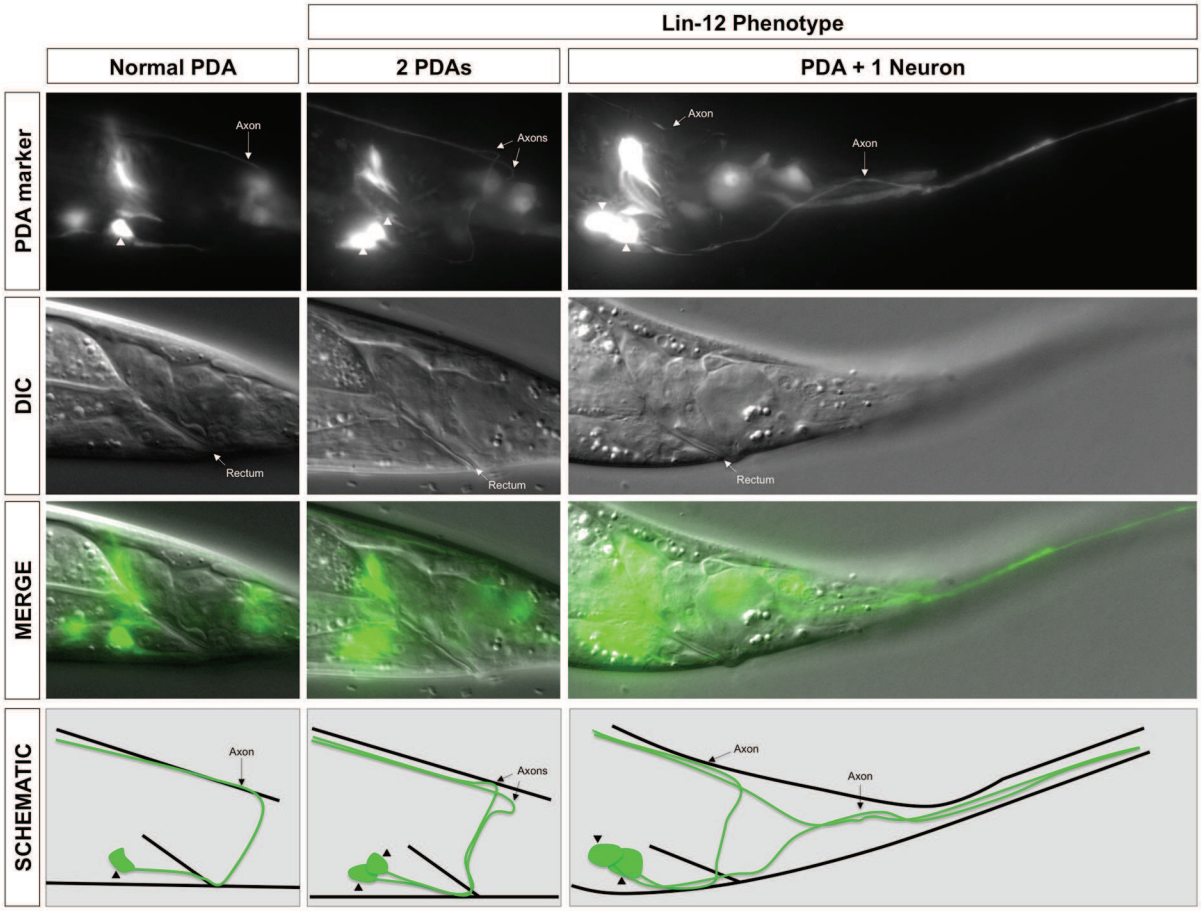


**Figure 36** – Description of the *lin-12(gf)* phenotypes. Upper panel represents an epifluorescent picture of the *cog-1p*::*GFP* PDA marker. Second panel represents differential interference contrast (DIC) pictures. Third panel represents a merge picture of the GFP signal and the DIC. Bottom panel is a schematic representation of the different phenotypes observed in *lin-12(gf)* mutants. Gain-of-function mutants for *lin-12* exhibit different phenotypes for PDA: a portion of the worm population has one PDA as in wild-type (left panel), or two neurons expressing the PDA maker, *cog-1p*::*GFP*. These neurons can be two PDAs (middle panel) or one PDA associated to a neuron resembling to an other neuron called PDB, based on its axonal trajectory (right). These phenotypes where two *cog-1* positive neurons are made, are included in a category called "Lin-12 phenotype". White and black arrowheads show the cell body of the concerned neurons.

Several classes of PDA phenotypes can be observed in *lin-12(n950)* mutant background. A proportion of worms have only one PDA motor neuron (Figure 36, left panel), while other worms have two neurons expressing the PDA marker *cog-1p*::GFP. In one hand, two clear PDAs can be observed (Figure 36, middle panel). In another hand, two *cog-1* positive neurons are formed in *lin-12(n950)* background. The first neuron is a clear PDA, whereas the second neuron exhibits a long axon going through the entire tail and coming back by the dorsal side of the worm. This shape resembles the PDB neuron (Figure 36, right panel). We considered this "looking-like PDB" neuron as a PDA for the following reasons: i) *cog-1p::GFP* is never expressed in PDB (Palmer, 2002), while in wild-type, PDA always expresses this marker. ii) Notch has been reported to have a role in axon guidance during the development of the *Drosophila* nervous system (Giniger, 2012). Thus, it is possible that ectopic activation of *lin-12* could lead to axon guidance failure resulting in the formation of an atypical axonal trajectory. In summary, the "two PDAs" phenotype and the "PDA + one neuron cog-1-positive" phenotype are included together in a class called "Lin-12 phenotype" (figure 36, middle and right panel) and will be referred as such for the following parts.

### II.1.2) Conversion of the supernumerary Y into PDA in *lin-12(gf)* mutant is complete

As described in the scientific context, the first work in which *lin-12* mutants have been identified suggested that the fate of the Y cell and the DA9 neuron are dependent on Lin-12/Notch signaling (Greenwald et al., 1983). ABp**l**pppaaaa will be the future Y, and ABp**r**pppaaaa will be the future DA9 cell. By lineage analysis and DIC morphology they could observe that in *lin-12(lf)* mutant, both ABp**l**pppaaaa and ABp**r**pppaaaa acquire a DA9 fate. Conversely in *lin-12(gf)* mutants, these cells take together the Y fate and we showed that both appear to be competent to transdifferentiate into PDA. These cell fate decisions are not dependent on a LIN-12/Notch lateral signaling between Y and DA9, instead it is the dose of *lin*-12 encountered independently by each cell that determines which fate is adopted.

To verify at the molecular level these observations we looked simultaneously at DA9 and PDA markers in *lin-12(lf)* and *lin-12(gf)* mutant backgrounds. *mig-*

*13p::mCherry* and *itr-1p::mCherry* have been reported to be expressed in DA9 but not in PDA (Dal Santo, Logan, Chisholm, & Jorgensen, 1999; Teichmann & Shen, 2011) while *cog-1p*::GFP is expressed in PDA but not in DA9. We have introduced together these markers either in *lin-12(n950)* gain-of-function mutants or in *lin-12(n676n930)* loss-of-function mutants.

In wild-type, 100% of the worms have one DA9 expressing *mig-13p::mCherry* or *itr-1p::mCherry* and one PDA expressing *cog-1p::GFP* (Figure 37.A and D). In *lin-12(n950)* mutants carrying *itr-1p::mCherry* and *cog-1p::GFP* transgenes, 23,7% (± 11,2%) of the worms exhibit a wild type phenotype with one PDA and one DA9 neuron while 67,3% (± 11,2%) have a "Lin-12 phenotype". This proportion of "Lin-12 phenotype" worms is equivalent to the proportion of worms having No DA9 neuron (Figure 37.B and D). This correlation between the "Lin-12 phenotype" and the absence of DA9 has been confirmed by the use of the second DA9 marker *mig-13p*::*mCherry*: in *lin-12(gf)* mutants 29% of worms are wild type and 71% have a "Lin-12 phenotype", which is the same percentage of worms having no DA9 (Figure 37.D). In fact, in worms lacking a DA9 marker, a second *cog-1*-positive neuron is always found, showing that DA9 cell is at the origin of the supernumerary *cog-1*-positive cell. Thus, these data suggest that in *lin-12(gf)* mutants, DA9 is not formed and the supernumerary Y cell formed from DA9 does not keep any DA9 features. These data confirm that complete conversion occurs in *lin-12(gf)* mutants when DA9 acquires the Y fate and subsequently gives rise to a PDA.

The same experiments has been performed with *lin-12(n676n930)* loss-of-function mutants. In this background, 100% of the worms have two DA9 and no PDA are formed (Figure 37.C and E). This confirms the previous results suggesting that in *lin-12* loss-of-function mutants, ABp**r**pppaaaa do not acquire its Y fate but instead the DA9 fate and although the microenvironment is similar, this extra DA9 cell never changes its fate (to PDA) subsequently.

Together these data confirm the observations made in previous studies (Greenwald et al., 1983; Jarriault et al., 2008). In wild-type DA9 and PDA are correctly formed. In *lin-12(lf)* mutants, two DA9 and no PDA are present because Y acquires a DA9 fate and by consequence is not able to transdifferentiate into PDA.

Conversely, in *lin-12(gf)*, DA9 acquires a Y fate and appears to be able to transdifferentiate into PDA. This conversion of DA9 into Y appears to be complete, no DA9 features are found in the subsequent supernumerary PDA.

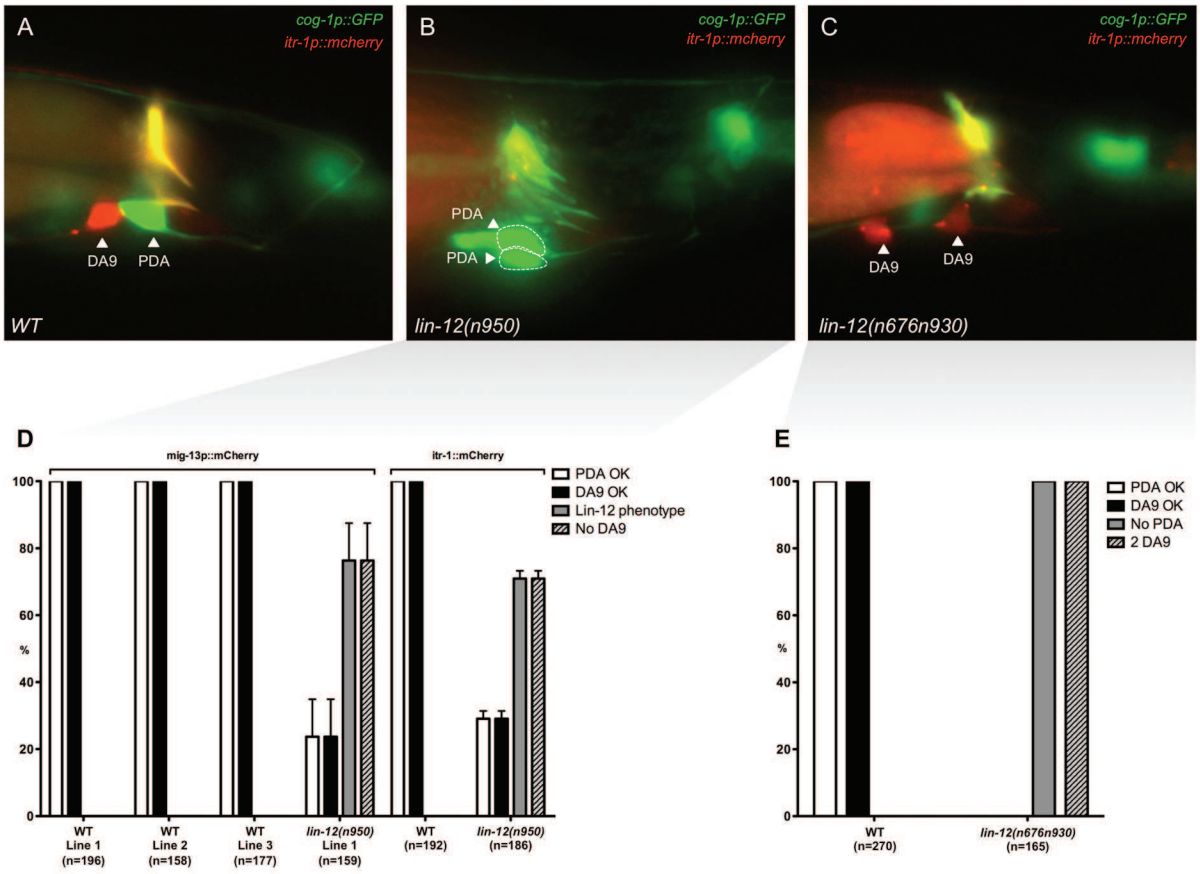


**Figure 37** – DA9 and PDA fates in *lin-12* gain *(n950)* and loss *(n676n930)* of function. **A)** Wild-type worms with DA9 labeled with *itr-1p::mcherry* (red) and PDA labeled with *cog-1p*::*GFP* (green). **B)** Phenotype observed in *lin-12(n950)* gain-of-function mutants. No DA9 are formed while 2 PDAs have been produced. **C)** Phenotype of *lin-12(n676n930)* loss-of-function mutants. No PDA is detected; instead two DA9 cells are formed. **D)** Simultaneous scoring of DA9 and PDA markers in *lin-12(n950)* mutants. Three independent lines carrying *mig-13p*::*mCherry* in *cog-1p::GFP* background have been obtained and scored. Only one line could be obtained when *mig-13p::mCherry* has been injected in *lin-12(n950); cog-1p*::*GFP* background. To confirm these results; a second DA9 marker, *wyEx1902[itr-1p::mCherry; odr-1p::GFP*], has been scored alone and crossed into *lin-12(n950); cog-1p::GFP* background. **E)** Simultaneous scoring of DA9 and PDA markers in in *lin-12(n676n930)* mutants. The array *wyEx1902[itr-1p::mCherry; odr-1p::GFP]* has been introduced in *lin-12(n676n930); cog-1p::GFP* background. Data represent the mean of three biological replicates. Error bars represent the standard deviation. Anterior is on the left and dorsal is up.

## II.2) Only one Notch receptor is involved in Y-to-PDA transdifferentiation

In *C. elegans*, two different genes encode a Notch receptor, *lin-12* and *glp-1*. These genes have redundant functions during *C. elegans* embryogenesis (Lambie & Kimble, 1991). Moreover, GLP-1 can substitute for LIN-12 in cell fate decisions (Fitzgerald et al., 1993). In order to test this potential functional redundancy, we looked at the Y-to-PDA phenotype of *glp-1* gain and loss-of-function mutants. If a functional redundancy exists between the two receptors, *glp-1(gf)* mutants should have the same phenotype as *lin-12(gf)* mutants, i.e. appearance of a "Lin-12 phenotype" (two *cog-1*-positives neurons). At the opposite, *glp-1(lf)* should have the same phenotype as *lin-12(lf)* mutants, which is characterized by the appearance of a "No PDA" phenotype.

*glp-1* loss-of-function mutant are embryonic lethal and sterile. GLP-1 is required during the first embryonic divisions to induce specific fate in blastomeres, by consequence its elimination inhibits proper embryonic development. GLP-1 is as well involved in germ cells proliferation and differentiation. In loss-of-function mutants, germ cells are only meiotic leading to sterility of the worms. To overcome these phenotypic issues, we decided to use the *glp-1(e2141ts)* thermo-sensitive loss-of-function allele (Priess et al., 1987). *glp-1(e2141ts)* worms are wild-type at 15°C, the permissive temperature and exhibit a strong loss of function phenotype at 25°C, the restrictive temperature. To performed our experiments, we put gravid mothers grown at 15°C onto plates warmed at 25°C. We let the mothers laying their eggs and we scored the subsequent larvae for the presence or not of PDA. At the same time we verified if the scored worms were sterile, which attests of the proper elimination of GLP-1 activity. The eggs are laid by the gravid worms at 25°C at around 150 minutes and the Y cell is born at around 300 minutes after fertilization (Sulston et al., 1983), by consequence elimination of GLP-1 activity was induced before the birth of the Y cell. The GLP-1/Notch pathway should not be able to be activated in ABp**r**pppaaaa to induce the Y fate and its competence to transdifferentiate. Temperature-shifted *glp-1(lf*) mutants exhibit complete sterility

and 100% of the worms have a PDA. No worms having "No PDA" were observed (Figure 38).

The exact same experiment has been performed with the *glp-1(ar202ts)* thermo-sensitive gain-of-function allele (Pepper, Killian, & Hubbard, 2003). This allele behave as a wild type at 15°C, while strong gain-of-function phenotypes are obtained at 25°C. As described above we shifted embryos at 25°C before Y birth and scored the obtained larvae for their PDA phenotype and their sterility. Temperature-shifted *glp-1(gf)* mutants are completely sterile but do not have any "Lin-12 phenotype". All the scored worms have a normal PDA (Figure 38).

Together these results suggest that GLP-1 is not involved in any steps of the Y-to-PDA transdifferentiation. Only LIN-12 appears to be necessary for this reprogramming event.

| Genotype | PDA OK | No PDA | Lin-12 phenotype | Sterility | n = |
|---|---|---|---|---|---|
| ***glp-1(e2141ts)*** **loss-of-function** | **100%** | 0% | NA | 100% | **100** |
| ***glp-1(ar202ts)*** **gain-of-function** | **100%** | NA | 0% | 100% | **100** |

**Figure 38 -** Scoring of loss and gain-of-function mutants for *glp-1*. *glp-1(e2141ts)* and *glp-1(ar202ts)* do not exhibit any particular PDA phenotype at the restrictive temperature. In both cases 100% of the worms have a PDA formed. Data represent pooled biological duplicates; NA, non applicable.

## II.3) Canonical but not non-canonical ligands are involved in the activation of the LIN-12/Notch pathway in Y.

### II.3.1) Identification of a sensitized background to study the role of LIN-12/Notch ligands in Y-to-PDA transdifferentiation

We tried to identify members of the LIN-12/Notch pathway involved in the establishment of the competence to transdifferentiate in Y. We built combinations of mutants carrying a gain-of-function mutation for *lin-12* and a loss-of-function

mutation for the factors we would like to study. We hypothesized that if a specific LIN-12/Notch factor is involved in this process, single loss-of-function mutants would give rise to "No PDA" phenotype and association of this mutation with *lin-12(gf)* mutants would lead to suppression or reduction of "Lin-12 phenotype". We built a strain carrying a strong loss-of-function allele for the presenilin gene, *sel-12(ar171)* (Levitan & Greenwald, 1995), associated with the strong gain-of-function allele, *lin-12(n950)*.

*sel-12(ar171)* single mutant worms are wild-type for Y-to-PDA transdifferentiation (Figure 39), indicating that loss of *sel-12* alone is not problematic for the establishment of the competence to transdifferentiate and suggests a possible redundancy with its ortholog *hop-1*. At the opposite, *lin-12(n950); sel-12(ar171)* double mutants exhibit a significant decrease of "Lin-12 phenotype" compared to *lin-12(n950)* single mutants (figure 39). This indicates that although loss of its activity does not cause a PDA defect by itself, *sel-12(ar171)* does play a role during Y-to-PDA transdifferentiation. This role can be revealed in certain genetic contexts, such as mutants in the LIN-12/Notch pathway. Depending on the genetic background, *sel-12(ar171)* can be silent or enhance certain phenotypes: thus *sel-12(ar171)* mutation can be used as a sensitized background, in which discrete phenotypes of some single mutants can be increased, a property we took advantage of, as described below.

### II.3.2) APX-1 and LAG-2 act redundantly to activates LIN-12 in the Y cell

We took advantage of this sensitized background to study the potential role of the canonical and non-canonical ligands in Y-to-PDA transdifferentiation (see figure 27). We first investigated if *apx-1* could activate the LIN-12/Notch pathway in Y to set the competence to transdifferentiate. This ligand is important during the first cleavage of the embryo, by consequence strong loss-of-function for *apx-1* have a highly penetrant embryonic lethality phenotype, preventing us from analyzing PDA phenotype in larva. To overcome this issue, we decided to use the thermosensitive allele *apx-1(zu347ts)*, which has been described as a strong loss-of-function at 26°C (Mickey et al., 1996). We can now let *apx-1* fulfilling its role during early

embryogenesis and eliminate it before Y birth by shifting embryos to the restrictive temperature, 26°C. We did these temperature shifting experiments with *apx-1* single mutants and *apx-1; sel-12* double mutants. Single mutants have a low penetrant (but significant) "No PDA" phenotype when embryos are grown at 26°C. This penetrance slightly increases in double mutants, in which the "No PDA" phenotype oscillates around 5% (Figure 40.A). The appearance of the "No PDA" phenotype when APX-1 activity is eliminated indicates that *apx-1* is involved in the activation of the LIN-12/Notch pathway in Y to set the competence to transdifferentiate.

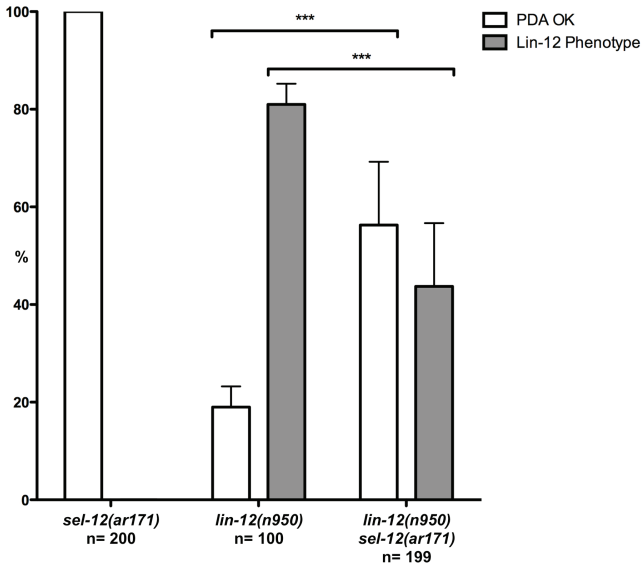


**Figure 39 –** Identification of a sensitized background for the analysis of LIN-12/Notch pathway. Scoring of *sel-12(ar171)* single mutants compare to *lin-12(n950)* gain-of-function mutant and double *lin-12(n950);sel-12(ar172)* mutants. *sel-12* single mutants are wild type while association between *lin-12(n950)* and *sel-12(ar171)* leads to reduction of the "Lin-12 phenotype" induced by *lin-12(n950).* Data represent the mean of two biological replicates; error bars represent the standard deviation. Two-tailed P value is calculated using Fisher's exact test; ***P < 0.001; **P < 0.01; *P < 0.05.

If *apx-1* had been the only ligand involved, we would have expected a higher penetrance of the "No PDA" phenotype in *apx-1* mutants. Our results suggest that *apx-1* is maybe not working alone in the activation of LIN-12 in Y and could act redundantly with another ligand. We next examined the involvement of the second canonical ligand, *lag-2.* LAG-2 is required during embryogenesis to specify several cell fates. Strong loss-of-function alleles of *lag-2* display a "Lag" phenotype, and

lead to larval lethality at the L1 stage preventing us again from analyzing the PDA phenotype of *lag-2* mutants. To overcome this problem, we used the *lag-2(q420ts)* allele, which has been described as a strong thermosensitive loss-of-function allele (Henderson et al., 1994). At 25°C, the restrictive temperature, LAG-2 activity is abolished, by consequence we can suppress this activity before Y birth by shifting developing embryos at 25°C.

We could only work with *lag-2* single mutants. Indeed, *lag-2; sel-12* double mutants appear to not be viable. Single mutants shifted at 25°C display a low penetrant (but significant) "No PDA" phenotype (Figure 40.B), which suggests that LAG-2, as APX-1, is involved in the activation of LIN-12 in Y to set the competence to transdifferentiate.

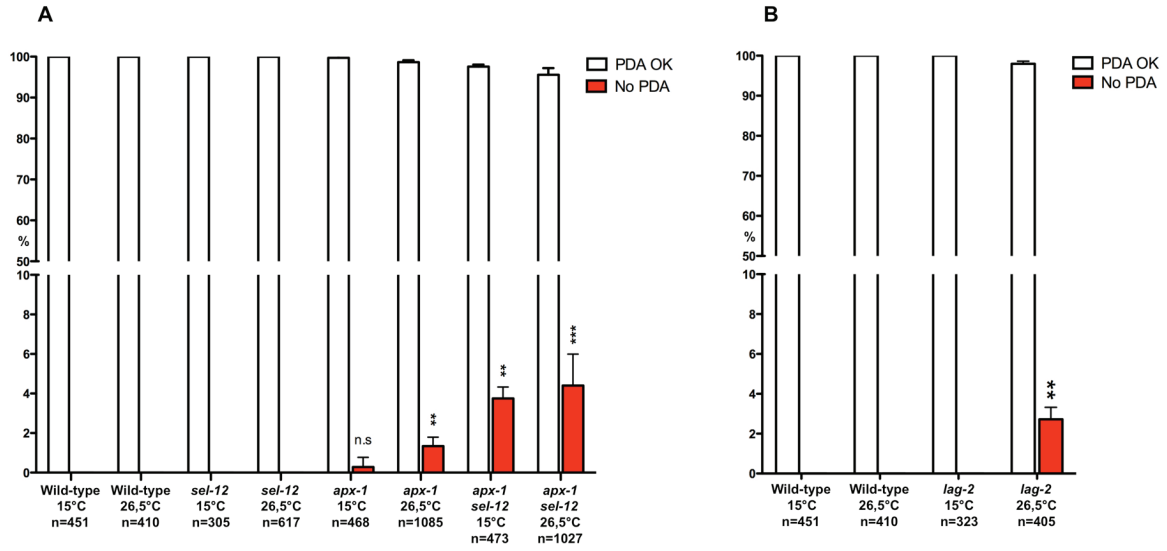


**Figure 40 –** APX-1 and LAG-2 act redundantly in Y-to-PDA transdifferentiation. **A)** Scoring of *apx-1(zu347ts)* mutants, alone or associated with *sel-12(ar171)* mutation. 15°C is the permissive temperature, 26,5°C is used as restrictive temperature. **B)** Scoring of l*ag-2(q420ts)* mutants. 15°C is the permissive temperature, 26,5°C is used as restrictive temperature. Data represent the mean of three biological replicates; error bars represent the standard deviation. Two-tailed P value is calculated using Fisher's exact test; ***P < 0.001; **P < 0.01; *P < 0.05.

All together, these results indicate that both ligands APX-1 and LAG-2 are involved in the activation of LIN-12 in Y to set the competence to transdifferentiate. Both ligand single mutants have really low "No PDA" penetrance, which support the idea that they certainly act together. These two genes are really close genetically (less than 0,2 cM separates *apx-1* and *lag-2*), by consequence we could not build a

double mutant strain. Nevertheless, we are currently trying to use other approaches to verify if indeed *apx-1* and *lag-2* are redundant in Y-to-PDA transdifferentiation.

### II.3.3) Individual non-canonical ligands are not required for the establishment of the competence to transdifferentiate

The canonical ligands APX-1 or LAG-2 have been shown to be involved in all the identified LIN-12/GLP-1 cell fate decision. The role of the non-canonical *C. elegans* Notch ligands is still poorly understood. Few mechanisms involving non-canonical ligands have been identified. *dsl-1* is involved in lateral signaling during vulval cell specification and in restriction of embryonic plasticity (Chen & Greenwald, 2004; Djabrayan et al., 2012). *dsl-3*, as *dsl-1*, is involved in restriction of embryonic plasticity (Djabrayan et al., 2012). *osm-11* facilitate LIN-12/Notch activation during vulval development and is involved with *osm-7* in the regulation of chemosensory response and larval molting quiescence (Komatsu et al., 2008; Singh et al., 2011). No role has been found so far for the other non-canonical ligands.

To investigate the possible role of the non-canonical ligands on Y-to-PDA transdifferentiation, we analyzed the phenotype of all the available non-canonical ligand mutants. As for *apx-1* and *lag-2*, we analyzed these mutants alone and associated with *sel-12(ar171)* sensitized background. For some mutations, it was not possible to build a double mutant with *sel-12* (due to a too small genetic distance): to overcome this issue, we crossed the mutation of interest to the RNAi hypersensitive allele, *rrf-3(pk1426)* in which we knocked down *sel-12* by dsRNA injection.

None of the generated strain displayed a "No PDA" phenotype (figure 41). Single and double mutants are all wild-type for Y-to-PDA transdifferentiation.

These results show that individual non-canonical ligands are not required for the establishment of the competence to transdifferentiate. However we cannot completely exclude the possibility that some of these ligands act redundantly with *apx-1* and *lag-2*.

| | Genotype | RNAi | T°C | PDA OK | No PDA | n = |
|---|---|---|---|---|---|---|
| DSL | *arg-1(ok3127)X* | - | 25°C | 100% | 0% | 582 |
| | *rrf-3(pk1426)II; arg-1(ok3127)X* | *sel-12* | 20°C | 100% | 0% | 582 |
| | *dsl-1(ok810)III* | - | 25°C | 100% | 0% | 367 |
| | *dsl-1(ok810)III; sel-12(ar171)unc-1(e538)X* | - | 25°C | 100% | 0% | 441 |
| | *dsl-3(ok3411)IV* | - | 25°C | 100% | 0% | 389 |
| | *dsl-3(ok3411)IV; sel-12(ar171)unc-1(e538)X* | - | 25°C | 100% | 0% | 287 |
| | *dsl-4(ok1020)X* | - | 25°C | 100% | 0% | 285 |
| | *rrf-3(pk1426); dsl-4(ok1020)X* | *sel-12* | 20°C | 100% | 0% | 697 |
| | *dsl-5(ok588)IV* | - | 25°C | 100% | 0% | 349 |
| | *dsl-5(ok588)IV; sel-12(ar171)unc-1(e538)X* | - | 25°C | 100% | 0% | 344 |
| | *dsl-6(ok2265)IV* | - | 25°C | 100% | 0% | 310 |
| | *dsl-6(ok2265)IV; sel-12(ar171)unc-1(e538)X* | - | 25°C | 100% | 0% | 252 |
| DOS | *osm-7(n1515)III* | - | 25°C | 100% | 0% | 253 |
| | *osm-7(n1515)III; sel-12(ar171)unc-1(e538)X* | - | 25°C | 100% | 0% | 308 |
| | *osm-11(rt192)X* | - | 25°C | 100% | 0% | 396 |
| | *rrf-3(pk1426)II; osm-11(rt192)X* | *sel-12* | 20°C | 100% | 0% | 412 |
| | *dos-1(ok2398)III* | - | 25°C | 100% | 0% | 353 |
| | *dos-1(ok2398)III; sel-12(ar171)unc-1(e538)X* | - | 25°C | 100% | 0% | 299 |
| | *dos-2(tm4515)II* | - | 25°C | 100% | 0% | 480 |
| | *dos-2(tm4515)II; sel-12(ar171)unc-1(e538)X* | - | 25°C | 100% | 0% | 306 |
| | *dos-3(tm4899)III* | - | 25°C | 100% | 0% | 425 |
| | *dos-3(tm4899)III; sel-12(ar171)unc-1(e538)X* | - | 25°C | 100% | 0% | 393 |

**Figure 41 –** Individual non-canonical ligands are not required for the establishment of the competence to transdifferentiate. Here are shown the results of scoring of all the available mutants for non-canonical ligands, alone or associated with *sel-12(ar171)*. Data represent pooled biological triplicates.

## II.4) LIN-12 is necessary and sufficient around the Y birth to induce the competence to transdifferentiate

### II.4.1) LIN-12 is necessary and sufficient only around Y birth to induce the competence to transdifferentiate

Previous studies have demonstrated that *lin-12* is necessary for the Y cell to acquire its fate and its competence to transdifferentiate (Jarriault et al., 2008). Indeed *lin-12* is necessary to trigger the competence to transdifferentiate and its action appeared to be required during a wide time window going from the Y birth to the 3-fold stage (Jarriault et al., 2008). However, it is not know if *lin-12* is sufficient to induce the competence, and when precisely its activity is required. To answer this question we built a construct allowing the expression of a constitutively active form of the LIN-12 receptor under the control of a heat-shock promoter. This construct consists of the intracellular part of the LIN-12 receptor (called here ICL for **I**ntra**C**ellular **L**IN-12) fused to GFP. A similar construct has been shown to constitutively activate the LIN-12/Notch pathway in worms and *Drosophila* (Struhl, Fitzgerald, & Greenwald, 1993). Heat-shock (HS) promoters are heat inducible promoters, at the temperature used to grow worms, these promoters are partially or completely silent and can be induced by a pulse of heat (Stringham, Dixon, Jones, & Candido, 1992). These promoters are used to temporally control the expression of a transgene of interest. In our experiments, a combination of heat-shock promoter with ICLGFP allows us to activate the LIN-12/Notch pathway at precise time points during any stage of the development.

We encountered several issues during the generation of transgenic strains carrying a construct made of a HS promoter fuse to ICLGFP. Indeed, heat-shock promoters appear to have some leakiness. Their activity is not completely silent even at the restrictive temperature. Moreover expression of *lin*-12(IC) under certain promoters promotes toxicity (Iva Greenwald, personal communication), by consequence association of a fairly ubiquitous leaky promoter with ICLGFP leads to high toxicity. This toxicity is at the origin of a counter-selection in which low-

expressing transgenic lines are promoted and high-expressing transgenic lines are not selected. We could not obtain lines with a good expression in our first injection trials. To overcome this issue, we decided to inject the construct of interest in the *hsf-1(sy441)* heat-shock resistant background. It has been shown that this mutant background has no heat-shock response (Stringham et al., 1992), and we thought that injection in such background would avoid the counter-selection taking place during injection in wild-type worms. Three independent lines have been obtained in *hsf-1(sy441)*. The obtained transgenes were subsequently reintroduced into wild-type background to carry out our experiments.

*lin-12* seems to endow Y with the competence to transdifferentiate. We wanted to test if *lin-12* was sufficient to endow other cells with the competence to transdifferentiate. To answer to this question, we ectopically induced the activation of the LIN-12/Notch pathway at precise time point during embryogenesis and we use the appearance of the "Lin-12 phenotype" as a readout. Indeed, if LIN-12 activity is sufficient to promote transdifferentiation, its ectopic activation in wild-type should lead to the formation of supernumerary PDA.

We chose to induce the activation of the LIN-12/Notch pathway at two different time points during embryogenesis. The first one takes place around the Y birth (around 300 minutes after fertilization), the second one takes place around the 2-fold stage (between 450-500 minutes after fertilization) (Figure 42.A).

Activation of the LIN-12/Notch pathway around the Y birth leads to the appearance of a "Lin-12 phenotype" in transgenic worms of the three analyzed lines, while their non-transgenic siblings do not exhibit any particular phenotype. The penetrance of this Lin-12 phenotype oscillates between 17% for the lowest expressing line and 49% for the highest expressing line (Figure 42.B). These results show that single ectopic activation of the LIN-12/Notch pathway around the Y birth is sufficient to trigger the formation of an extra PDA. Thus, *lin-12* appears to be sufficient to promote transdifferentiation.

Induction of the LIN-12/Notch pathway during mid-embryogenesis leads to different results. Indeed, in one line, the transgenic and non-transgenic worms are wild-type. At the opposite, in the two other lines, the fraction of "Lin-12 phenotype"

worms has drastically reduced and surprisingly, some transgenic worms have a "No PDA" phenotype (Figure 42.C). These results lead to the following conclusions: **i)** *lin-12* is sufficient to promote transdifferentiation in a short time window mostly just after Y birth. **ii)** Outside of this time window, ectopic activation of the LIN-12/Notch pathway is not able to promote transdifferentiation. The few worms having a "Lin-12 phenotype" are certainly coming from embryos, which were at the limit of this time window. **iii)** Ectopic activation of LIN-12/Notch pathway when it is not required may block Y-to-PDA transdifferentiation as evidenced by the appearance of a low penetrant "No PDA" phenotype.

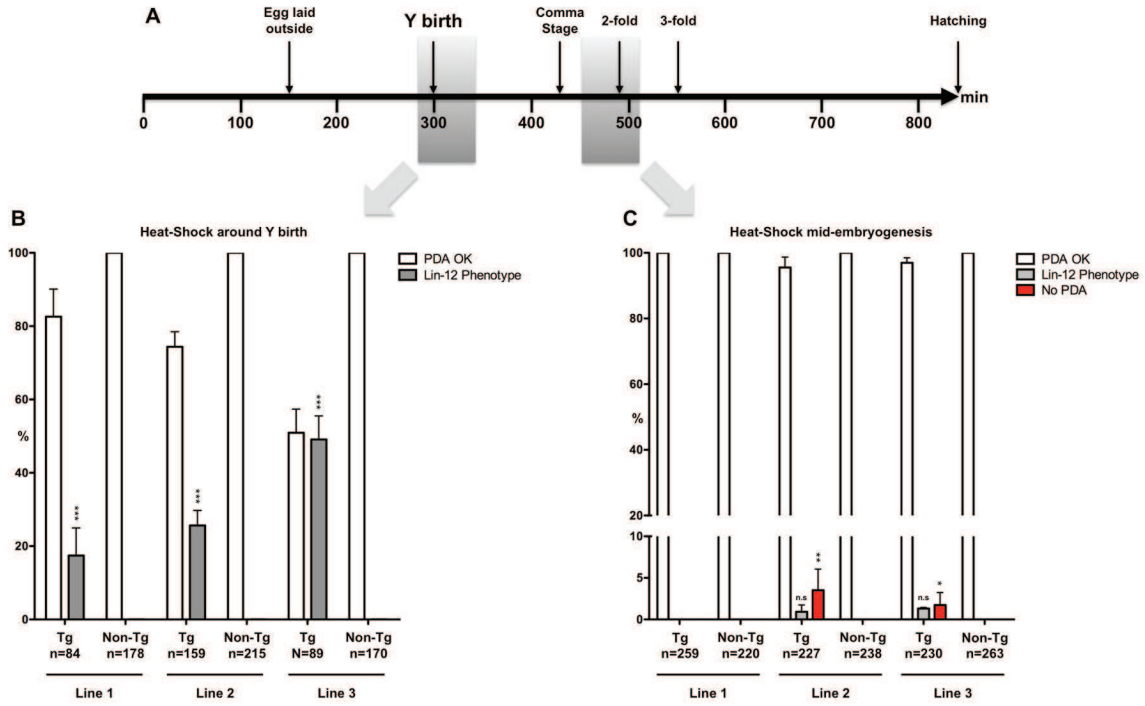


**Figure 42 -** *lin-12* is sufficient to promote transdifferentiation. **A)** Timeline of embryogenesis. Y birth takes place around 300 minutes. The grey boxes represent the period during which the ectopic LIN-12/Notch pathway activation is induced. **B)** Scoring of the worms heat-shocked around Y birth. In the three lines, "Lin-12 phenotype" appears in transgenic worms but not in non-transgenic siblings. **C)** Scoring of the worms heat-shocked during mid-embryogenesis. In one line, no particular phenotype is detected. In two other lines, the percentage "Lin-12 phenotype" worms has dropped and a "No PDA" phenotype is found is some worms. Data represent the mean of three biological replicates; error bars represent the standard deviation. Two-tailed P value is calculated using Fisher's exact test; ***P < 0.001; **P < 0.01; *P < 0.05; n.s., not statistically significant. Tg, transgenic worms; Non-tg, non-transgenic siblings

Together these results suggest that the competence to transdifferentiate has to be triggered during Y life, after its birth. They also suggest that *lin-12* is necessary and sufficient during a short time window around Y birth to promote transdifferentiation, and confirm and extend the previous published data using a thermosensitive loss-of-function *lin-12* allele suggesting that *lin-12* is not required after the 3-fold stage (Jarriault et al., 2008)..

### II.4.2) The induced extra PDA is coming from DA9 conversion and suggests a context-dependent action of *lin-12*

When the LIN-12/Notch pathway activation is induced around the Y birth, two PDAs are formed. We tried to identify the origin of this extra PDA. The most evident scenario is that as in *lin-12(gf)* mutants, DA9 takes the Y fate and is then competent to transdifferentiate into PDA. To verify this hypothesis, we introduced an integrated DA9 marker in the two most penetrant lines carrying the *Heat-shock promoter::ICL* constructs. Expression of this heat-sensitive transgene was triggered around Y birth as in the previous experiments.

In the heat-shocked worms and in both lines, non-transgenic worms are wild-type, they have one PDA and one DA9. Conversely in heat-shocked transgenic worms, "Lin-12 phenotype" is induced and the penetrance of this phenotype completely correlates with the penetrance of the "No DA9" phenotype (Figure 43). These results show that the formation of an extra PDA elicit the disappearance of the DA9. Thus, DA9 turns into Y and is competent to transdifferentiate into PDA.

Interestingly, ectopic activation of the LIN-12/Notch pathway around Y birth induces the competence to transdifferentiate only in the DA9 cell. Indeed, no other extra PDA (or *cog-1*-positive neuron) is found during these experiments. This suggests that the action of *lin-12* to induce a competence is dependent on a specific cellular context, which can respond to this Notch signal.

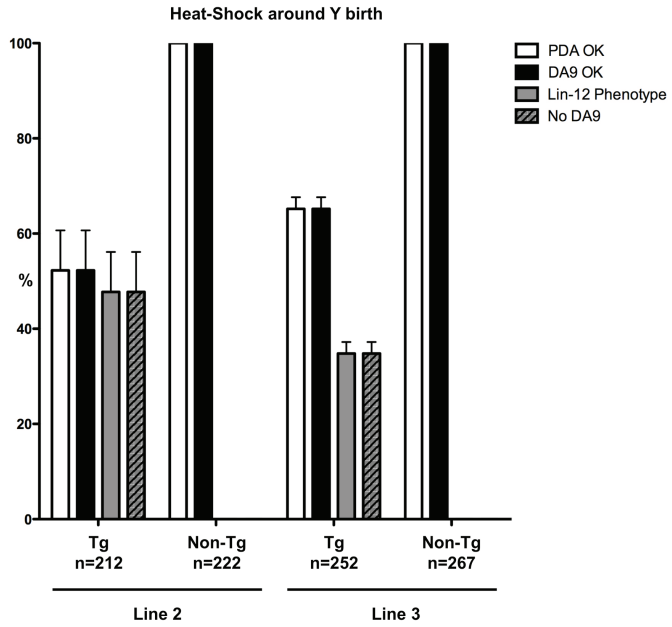


**Figure 43 –** Extra PDA formed after ectopic LIN-12/Notch activation around Y birth is coming from DA9. Scoring of two independent lines, carrying a *Heat-shock promoter::ICL* construct with integrated markers for PDA and DA9. Complete correlation between the "Lin-12 phenotype" and the "No DA9" is found in transgenic worms. Thus, the extra PDA is coming from the conversion of the DA9 cell into a competent Y. Data represent the mean of three biological replicates; error bars represent the standard deviation.

## II.5) The supernumerary PDA formed in *lin-12(gf)* mutants requires the same principles as the endogenous PDA.

Previous work carried out in our laboratory highlighted the different steps and factors involved in Y-to-PDA transdifferentiation (Jarriault et al., 2008; Richard et al., 2011; Kagias et al., 2012). After the induction of the competence in Y by *lin-12*, several other factors are then involved in the initiation of transdifferentiation. These factors are composed of a NODE-like complex, made of *egl-27/Mta, ceh-6/Oct, sem-4/Sall,* which interact with *sox-2*, and acts upstream of the *egl-5/Hox* gene. This nuclear complex is required to properly initiate transdifferentiation of Y into PDA. If one of these genes is eliminated (in mutant background or by RNAi) the Y cell is formed but stay in the rectum as an epithelial cell not able to initiate its conversion

into a neuron. We investigated whether these genes are also involved in the formation of the extra PDA present in *lin-12(gf)* mutants.

In *C. elegans*, RNAi response is not the same from one cell type to the other. Indeed, some cell types such as neurons appear to be refractory to RNAi (Timmons, Court, & Fire, 2001) and we found that this phenomenon occurs as well in rectal cells. In order to increase the sensitivity of our RNAi experiments, we used the RNAi hypersensitive mutant *rrf-3(pk1426)*. We used two different strains to perform our experiments, the first one carries the *rrf-3(pk1426)* loss-of-function mutation associated with a PDA marker and will be used as control strain. The second strain carries two mutations, the *rrf-3(pk1426)* mutation and the gain-of-function allele *lin-12(n950)* together with a PDA marker. We first evaluated in *rrf-3(pk1426)* mutants the efficiency and the phenotype triggered after injection of double-stranded RNA (dsRNA) of genes of interest. We use *dpy-8* RNAi as a control, which has no effect on Y-to-PDA transdifferentiation (Figure 44.A). Conversely, RNAi of *sem-4*, *egl-27*, *sox-2*, *ceh-6* and *egl-5* all led to a "No PDA" phenotype with diverse penetrance (Figure 44.A).

We did the exact same experiments in *rrf-3(pk1426); lin-12(n950)* background. RNAi against *dpy-8* has no effect on the "LIN-12 phenotype" of the double mutant worms: a fraction of the worms have a normal PDA while the majority exhibits a "Lin-12 phenotype". At the opposite, RNAi of *sem-4*, *egl-27*, *sox-2*, *ceh-6* and *egl-5* leads to a significant appearance of the "No PDA" phenotype and a significant decrease of the "Lin-12 phenotype" (Figure 44.B). Together, these results show that the five tested genes are important for the initiation of the original and the supernumerary PDA formed in *lin-12(gf).*

These results point to several hypothesis: **i)** transdifferentiation-promoting factors (NODE-Like complex and EGL-5) could be induced by Lin-12/Notch pathway activation in DA9, which will allowing then its proper transdifferentiation. **ii)** *mab-9* and *egl-38* mutants in which a non-competent Y cell is formed may lack LIN-12/Notch pathway activation and expression of transdifferentiation-promoting factors that could explain why the extra Y cell is not able to give rise to a PDA.

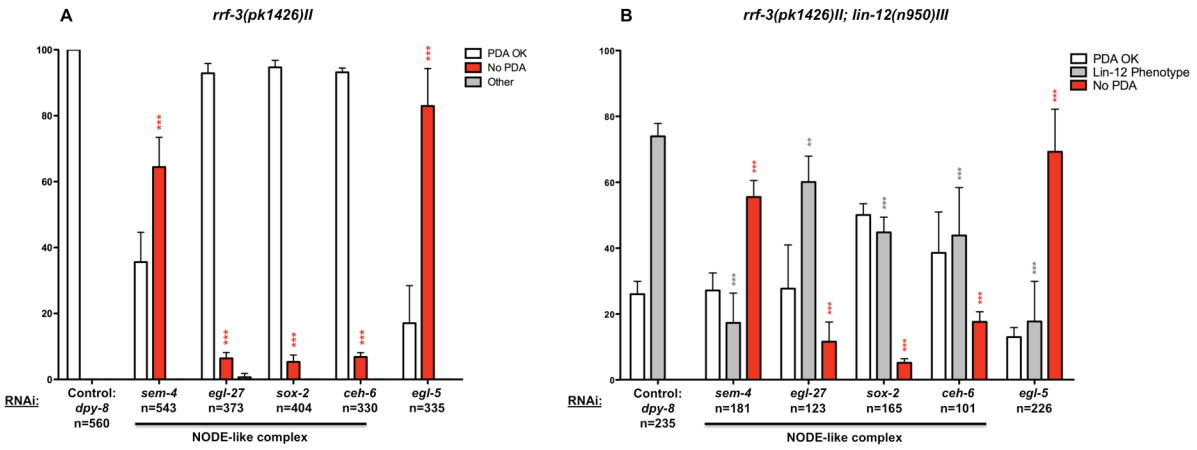


**Figure 44 –** Supernumerary PDA formed in *lin-12(gf)* mutant requires the same factors as the endogenous PDA. **A)** RNAi by injection of double-stranded RNA against *dpy-8* (negative control), members of the NODE-Like complex (*sem-4, egl-27, sox-2 and ceh-6)*, and *egl-5* in the RNAi hypersensitive mutant *rrf-3(pk1426)II*. **B)** RNAi by injection of double-stranded RNA against *dpy-8* (negative control), members of the NODE-Like complex (*sem-4, egl-27, sox-2 and ceh-6)*, and *egl-5* in the double mutant *rrf-3(pk1426)II; lin-12(n950)III.* Data represent the mean of three biological replicates; error bars represent the standard deviation. Two-tailed P value is calculated using Fisher's exact test; ***P < 0.001; **P < 0.01.

## II.6) Expression pattern and dynamics of *lin-12* and its activating-ligands

We have shown that *lin-12* is necessary and sufficient, during a precise time window during embryogenesis, to induce competence to transdifferentiate. These results suggest that *lin-12* should be active in Y during embryogenesis at the same precise time window. We hypothesized that *lin-12* activity could be regulated by either its own expression or the availability of its ligands (or both), and we tested these hypotheses. In this chapter we examined if the activity of the receptor can be regulated at the level of its presence. For this, we analyzed the expression pattern of translational *LIN-12::GFP* and transcriptional *lin-12p*::GFP reporters during embryogenesis and larval development.

### II.6.1) LIN-12 protein is expressed in Y during a short embryonic time window

To identify the expression pattern of the LIN-12 protein we used a rescuing translational reporter developed by the Greenwald laboratory (Levitan & Greenwald, 1998). This construct is functional and can be observe in Y using conventional microscopic devices. We could observe that LIN-12 is expressed in Y around its birth (Figure 45, top panel). This expression is found and maintained in Y until the 2-fold stage, where the LIN-12 expression begins to shut down. After the 2-fold stage, LIN-12 is not detected anymore in Y (Figure 45). This expression pattern confirms that the LIN-12 receptor is present in the Y cell during a short time period of embryogenesis, when it is required.

### II.6.2) *lin-12* mRNA is expressed in Y and DA9 during a short embryonic time window

To observe the transcriptional expression pattern of *lin-12* we used a transgenic line developed by the Hart laboratory. This reporter consists of the regulatory sequence of *lin-12* fused to GFP (Singh et al., 2011). This reporter is expressed in Y around its birth and expression is maintained until the 2-fold stage. *lin-12* expression is not detectable anymore at the 3-fold stage indicating that *lin-12* expression is down-regulated before this stage (Figure 46). Thus, the presence of the LIN-12 receptor in the Y cell follows the same dynamics as the *lin-12* mRNA: this strongly suggests that *lin-12* expression is regulated at the RNA level in the Y cell.

We could detect the exact same expression pattern in the DA9 cell suggesting that DA9 expresses *lin-12* with the same dynamic as Y (Figure 46). We can speculate that the LIN-12/Notch pathway is activated during embryogenesis only in the Y cell, or only at high levels in the Y cell, explaining why DA9 keeps its fate and do not take Y identity.

Together, these results indicate that *lin-12* is expressed in Y and DA9 during a short time window of embryogenesis and bring out some information on why an extra PDA can be obtained when the LIN-12/Notch pathway is ectopically activated around Y birth and not later on. Indeed, LIN-12 is probably never activated in DA9 in

wild-type animals, but the cell is in a latent state where any activation of the receptor during this time window can lead to the acquisition of the Y fate and the competence to transdifferentiate.

These results suggest as well that the *lin-12* permanent downregulation that we observed in the Y cell (after the 2-fold embryonic stage) may be important for the Y fate. Indeed ectopic activation of Lin-12/Notch pathway during mid-embryogenesis triggered a low penetrance Y-to-PDA inhibition. Thus, Lin-12/Notch pathway activation outside of its period of action may be deleterious for Y-to-PDA transdifferentiation, a hypothesis that we will test in the next chapter.

The downregulation of *lin-12* during embryogenesis has been confirmed by the expression pattern obtained in another transgenic strain, which expresses ß-galactosidase inserted in the whole *lin-12* genomic locus (and thus where most if not all of the *lin-12* regulatory regions are present (Wilkinson et al., 1994)). We analyzed stained L1 larvae and used as staining positive control a cell of the excretory system, which appears to express *lin-12* (Figure 44). This cell may be the G2 and/or the W cell of the excretory canal, which both have been shown to express *lin-12* (Abdus-Saboor et al., 2011). Whereas in all the observed worms this excretory cell was stained, the Y cell was never found to express the ß-galactosidase at this larval stage. Thus, downregulation of *lin-12* takes place during embryogenesis and the *lin-12* gene is not re-expressed later in the Y cell when its transdifferentiation takes place.

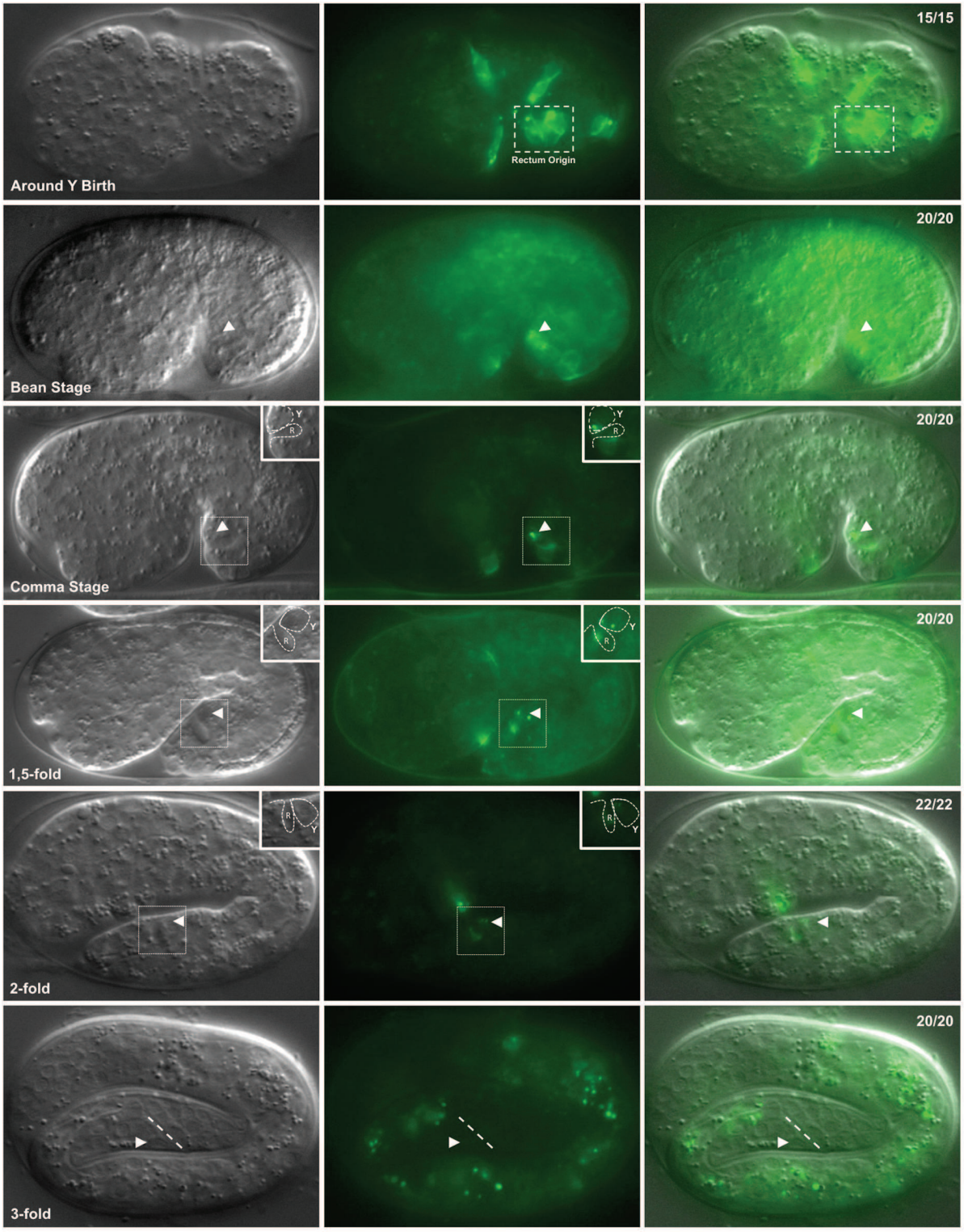


**Figure 45 –** Expression pattern and dynamics of the LIN-12 protein. LIN-12 is present in Y during embryogenesis from its birth to the 2-fold stage. Arrowheads indicate Y; R, rectum; dotted line, rectal slit; numbers indicates the fraction of worms displaying this representative expression pattern

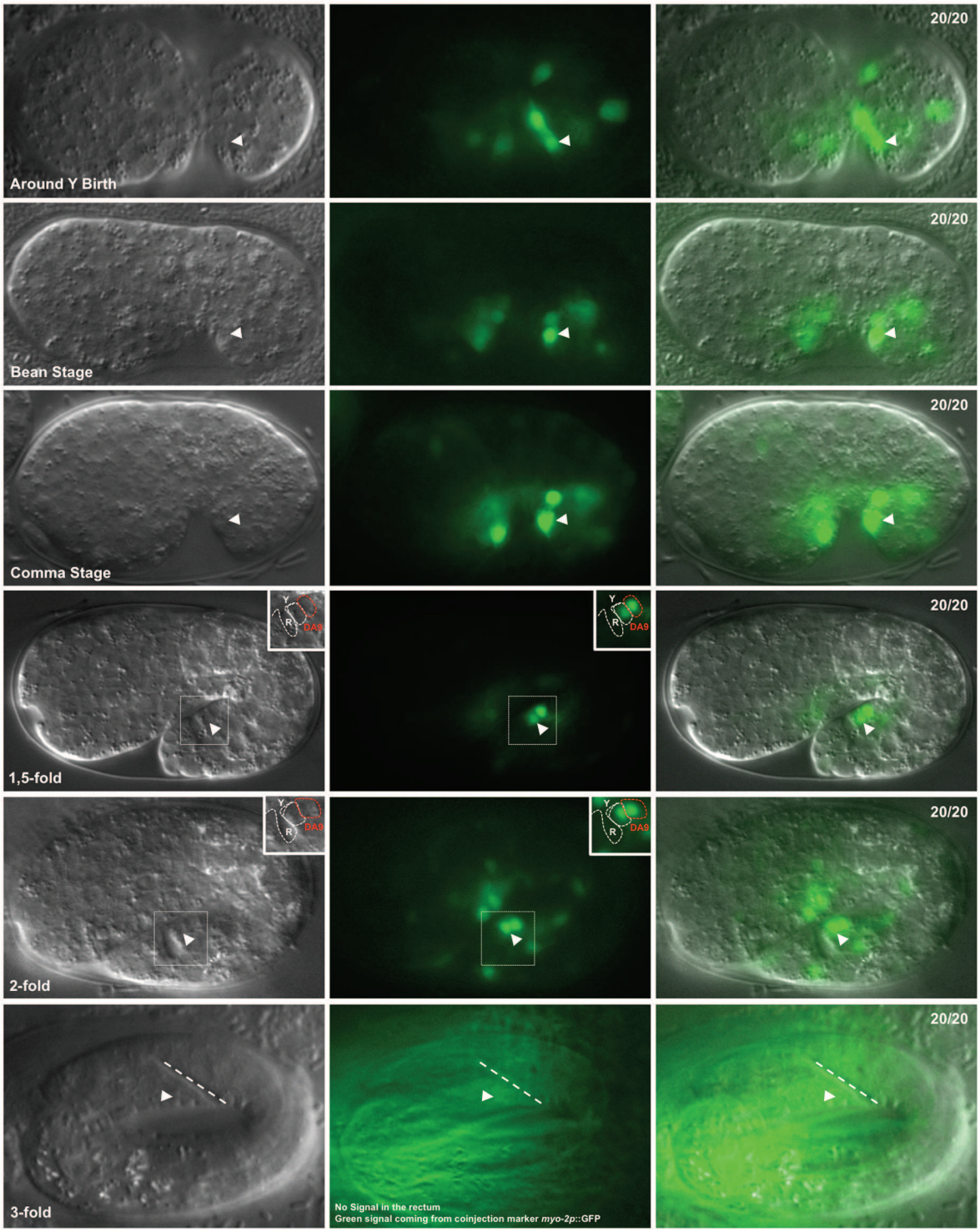


**Figure 46 –** Expression pattern and dynamics of *lin-12* mRNA. *lin-12* is expressed in Y during embryogenesis from its birth to the 2-fold stage. Arrowheads indicate Y; R, rectum; dotted line, rectal slit; numbers indicates the fraction of worms displaying this representative expression pattern. DA9 is indicated in red. In the 3-fold stage, GFP signal is coming from the coinjection marker (*myo-2p*::GFP*)* used to generate this transgenic line and which starts to be expressed at this stage; no *lin-12* signal is detected in Y nor in DA9 at this stage. Arrowheads indicate Y; R, rectum; dotted line, rectal slit; numbers indicates the fraction of worms displaying this representative expression pattern

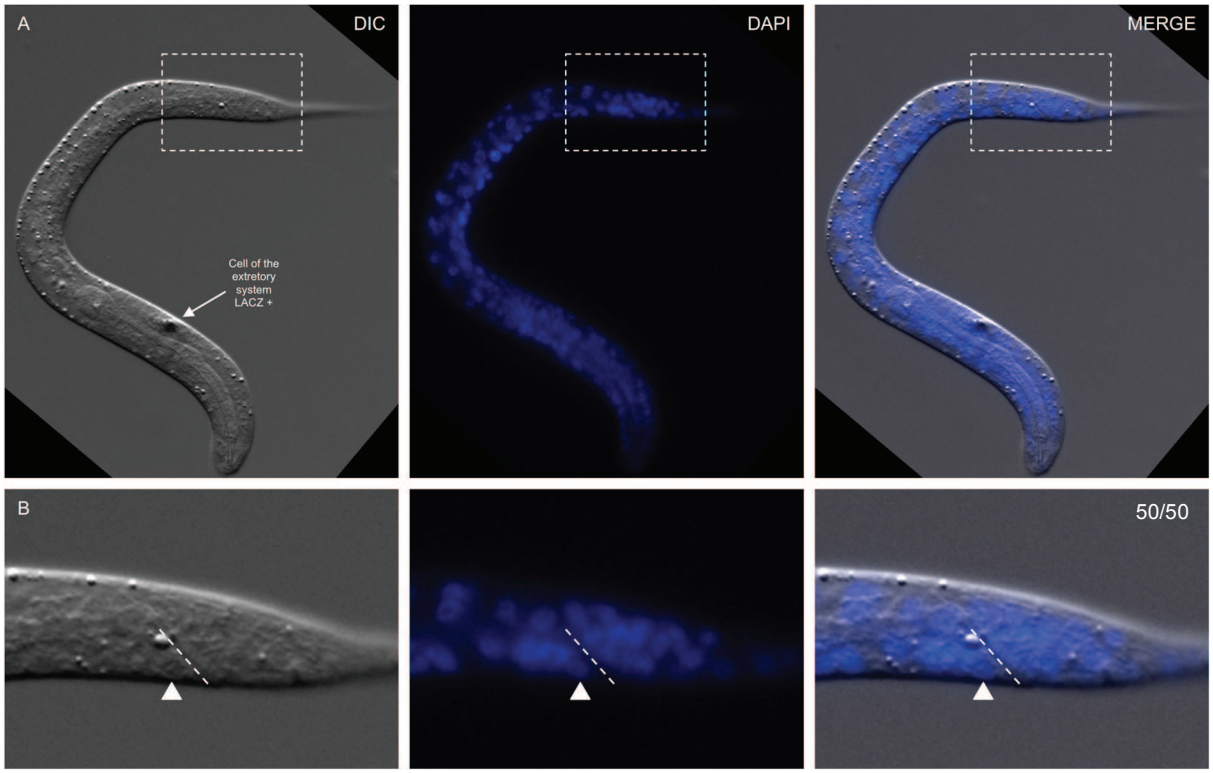


**Figure 47 –** Expression pattern of *lin-12* in L1 larva. **A)** ß-galactosidase staining of L1 larva, the excretory cell indicated by the arrow, is used a staining positive signal. **B)** Magnification of the rectal region, Y (indicated by the arrowhead) does not express *lin-12.* Dotted line represents the rectal slit. The blue channel represents DAPI staining. Numbers indicate the fraction of worms displaying this representative expression pattern.

## II.6.3) Expression pattern and dynamics of *apx-1* and *lag-2*

Our genetic analyses suggested that *apx-1* and *lag-2* act redundantly in the activation of the LIN-12/Notch pathway in Y to set the competence to transdifferentiate. We next investigated the expression pattern and dynamics of *apx-1* and *lag-2* around the rectum to better map the source of the LIN-12 activation in Y. We used two published and established transcriptional reporter for *apx-1* (Li & Greenwald, 2010; Pengpeng, Kevin, Michael, & Kang, 2013) and for *lag-2* (Blelloch et al., 1999; Siegfried, Kidd, Chesney, & Kimble, 2004; Chesney, Lam, Morgan, Phillips, & Kimble, 2009).

Around Y birth, *apx-1* is expressed in four cells close to the rectum origin. Two of these cells maintain this expression and will be positioned close to the rectum from the bean stage, where they can certainly make contacts with Y. *apx-1*

expression is maintained at the 2-fold and L1 stage (Figure 48). Note that we could not ascertain the identity of these two *apx-1* positive cells in early embryos, although it is likely that the *apx-1* positive cell found in L1 and L2 are same cells found close to the rectum during embryogenesis. We can conclude that *apx-1* is expressed in close proximity to the rectum when LIN-12 is expressed in Y supporting the fact that this ligand is involved in Y-to-PDA transdifferentiation.

For *lag-2*, the scenario is even simpler. Indeed, *lag-2* is expressed in the rectal B cell, which directly contacts and makes adherens junctions with Y, from Y birth until the late L1 stage (Figure 49) and is not expressed in the rectum afterwards. These observations support the fact that *lag-2* as well can activate the LIN-12/Notch pathway in Y during embryogenesis. Together these data show that both ligands are expressed close to the rectum and could be together the source of LIN-12 activation in Y, which is in accordance with the genetic data.

Interestingly, both ligand expressions appear to be maintained during embryogenesis and beginning of larval development in cells close to the rectum. These expression dynamics are different of the LIN-12 receptor, which indicates that LIN-12 downregulation during embryogenesis is certainly not due to ligand downregulation. This hypothesis will be tested in a future chapter.

Before our observations, we could imagine a model with ressembling aspects of the AC/VU decision, in which Y expresses LIN-12 and the surrounding cells express the ligands. Ligand availability would trigger a positive feedback loop, which will induce *lin-12* activation and reinforce *lin-12* expression in Y. Then, ligand expression would stop at some point leading to *lin-12* downregulation in Y. But the obtained data suggest another model in which, ligand expression is maintained around Y, while LIN-12 is expressed transiently. This model suggests an active mechanism, which will downregulate *lin-12* expression.

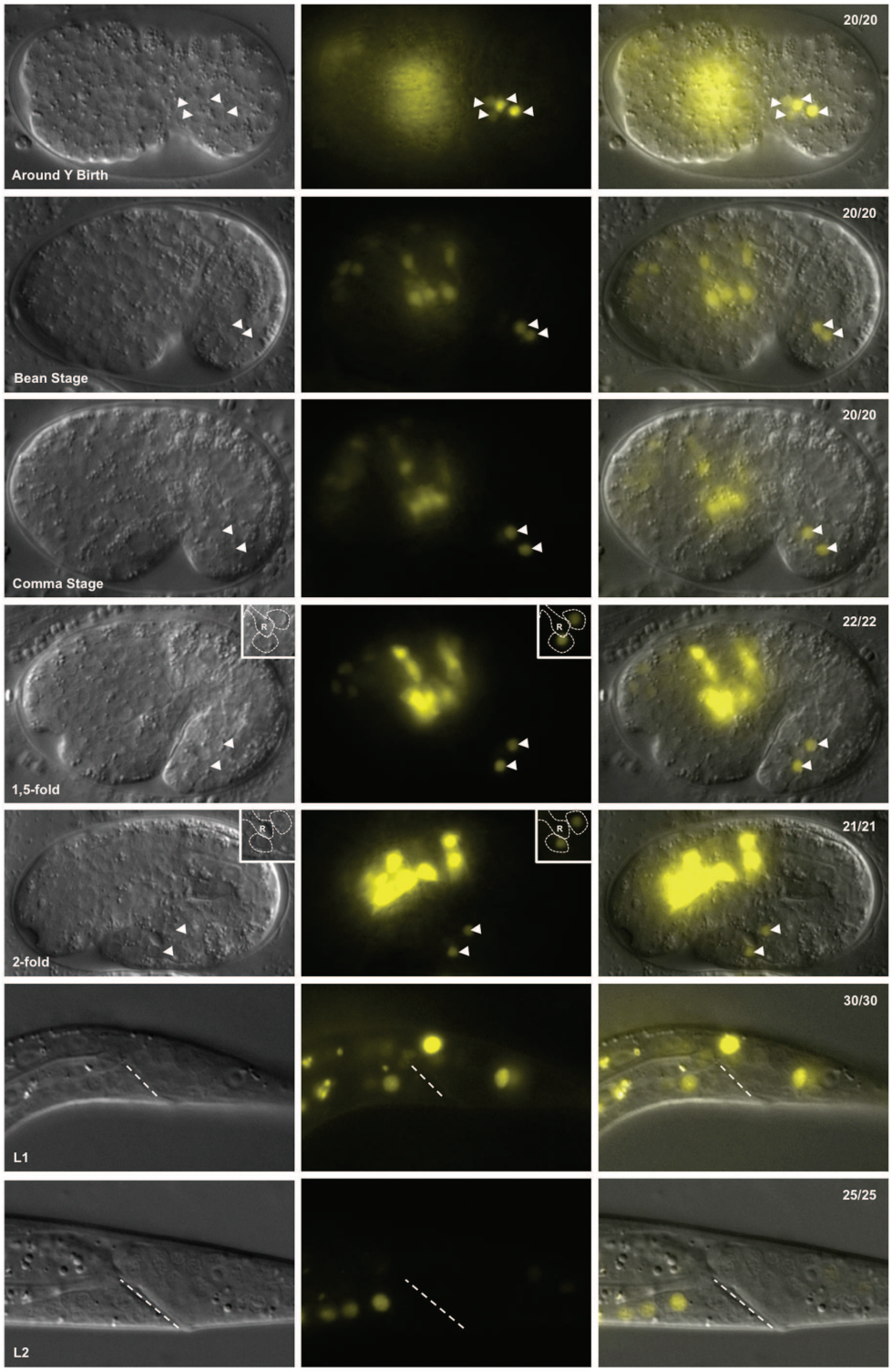


**Figure 48 –** Expression pattern and dynamics of *apx-1*. *apx-1* is expressed in four cells close to the rectum during embryogenesis. Part of this expression is maintained during larval stages. Arrowheads show *apx-1* expressing cells; R, rectum; doted line represent the rectal commissure. Numbers represent the fraction of worms displaying this representative expression pattern.

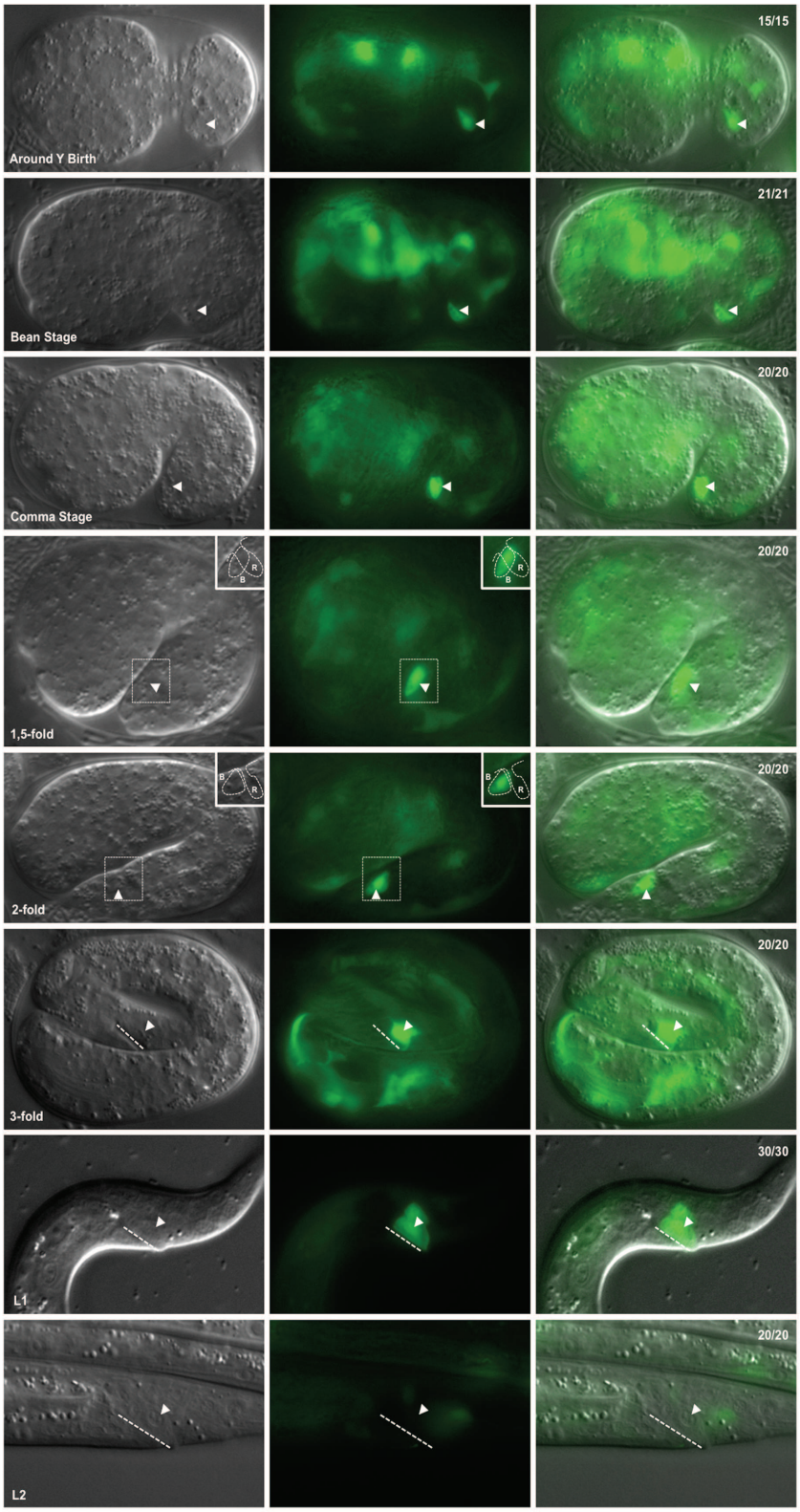


**Figure 49 –** Expression pattern and dynamics of *lag-2*. *lag-2* is expressed in B from Y birth until the L1 stage. Arrowheads show the *lag-2* expressing B cell; R, rectum; doted line represent the rectal commissure. Numbers represent the fraction of worms displaying this representative expression pattern.

## II.7) Maintained LIN-12/Notch activity in Y is deleterious for Y-to-PDA transdifferentiation

Several results we have obtained suggest that LIN-12 activity may need to be eliminated during embryogenesis. Expression pattern analysis reveals that *lin-12* is expressed only from Y birth to the 2-fold stage; it is thus not present anymore when Y transdifferentiation is initiated. Activation of LIN-12/Notch pathway outside of this window via our heat-shock experiments did not allow the formation of 2 PDAs and even led to low penetrance Y-to-PDA inhibition. Thus, we investigated whether *lin-12* must be actively downregulated during embryogenesis to allow proper Y-to-PDA transdifferentiation, by misregulating the LIN-12/Notch pathway at different time point during Y-to-PDA transdifferentiation.

### II.7.1) Maintained LIN-12 activity inhibits Y-to-PDA transdifferentiation

To investigate if tight regulation of the LIN-12/Notch pathway is important for Y-to-PDA transdifferentiation, we triggered misregulation of this pathway by overexpressing our constitutively active ICLGFP under a 6,2kb portion of the *egl-5* promoter (Kagias et al., 2012) in *cog-1p::GFP* background (to monitor PDA status).

*egl-5*/*Hox* expression is regionalized and can be found mainly posteriorly in rectal cells and surrounding tissues. The regulatory sequence of *egl-5* encompasses many enhancers, which allow cell specific expression (Ferreira, Zhang, Zhao, & Emmons, 1999; Teng, Girard, Ferreira, Sternberg, & Emmons, 2004). Several rectal specific enhancers have been identified and are used by our laboratory to express transgenes in rectal cells from their birth to the end of the *C. elegans* life cycle (Kagias et al., 2012).

As described in II.4.1), ICLGFP is made of the intracellular domains of the LIN-12 protein fused in frame with GFP. ICL has a PEST domain, which is involved in protein degradation and confers to protein a short lifetime (Rogers, Wells, & Rechsteiner, 1986). Because we did not know whether ICLGFP would be detectable

upon microscopic observations, we add to our construct a *SL2::mCherry* sequence to track transgene expression. From our transgene, a polycistronic mRNA containing ICLGFP and mCherry will be produced. This mRNA will be trans-spliced because of the SL2 sequence and will give rise to two individual mRNAs, encoding ICLGFP in one hand and mCherry in the other hand (Blumenthal, 2005; Macosko et al., 2009). This system allows us to monitor the expression level and localization of our transgene without affecting its function.

Overactivation of the LIN-12/Notch pathway by *egl-5p::ICLGFP* led to inhibition of Y-to-PDA transdifferentiation in the five independent lines we scored. Nuclear GFP and mCherry signal indicated that ICLGFP is expressed in rectal cells since around their birth and its expression is maintained during development (Figure 50.A). This expression leads to inhibition of Y-to-PDA in around 50% of all the transgenic worms, while non-transgenic siblings are wild-type (Figure 50.B). Thus, activation of the LIN-12/Notch pathway initiated early and maintained throughout is deleterious for Y-to-PDA transdifferentiation. Moreover, three epithelial cells are found in the rectum (U, Y and P12.pa), which is the phenotype found in mutant worms having transdifferentiation initiation problems, as it is found in NODE-Like complex or *egl-5* mutants (Jarriault et al., 2008; Kagias et al., 2012). Thus, in these transgenic animals, Y seems to be blocked in the rectum at the initiation step (Figure 50.A).

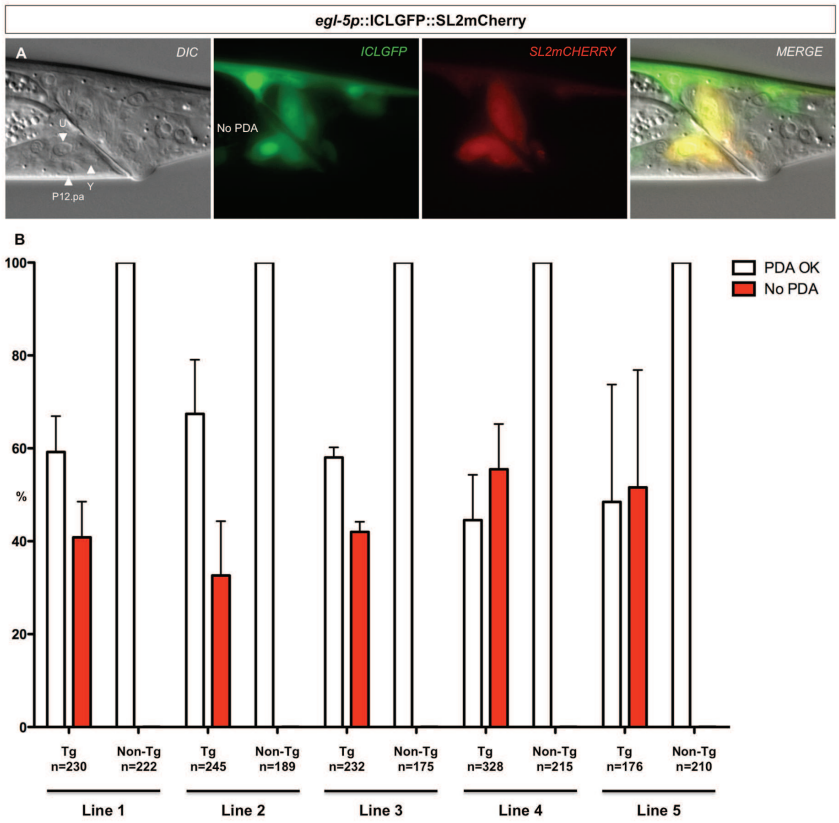


**Figure 50 –** Maintained LIN-12/Notch pathway activation is deleterious for Y-to-PDA transdifferentiation. **A)** ICLGFP is expressed in rectal cells (GFP and mCherry signal) and its expression leads to a "No PDA" phenotype. **B)** Scoring of five independent lines expressing *egl-5p*::*ICLGFP::SL2mCherry* injected at 20 ng. In all the lines high penetrance of a "No PDA" phenotype is found in transgenic worms, while non-transgenic worms are wild-type. Data represent the mean of three biological replicates; error bars represent the standard deviation. Tg, transgenic worms; Non-tg, non-transgenic siblings

### II.7.2) Y-to PDA transdifferentiation inhibition by maintained LIN-12/Notch signal is not dependent of an early activation time point

A pulse of activation of the LIN-12/Notch pathway, outside of its window of activity leads to very low penetrance of "No PDA" phenotype in the challenged worms (see II.4.1), Conversely, maintained LIN-12/Notch overactivation from Y birth leads to high penetrance Y-to-PDA transdifferentiation inhibition. We asked if this difference of penetrance is due to the timing when LIN-12/Notch pathway is overactivated. Indeed, high level of transgene expression during Y specification and acquisition of the competence to transdifferentiate could be the source of this Y-to-PDA inhibition. To answer this question, we expressed ICLGFP under the *col-34*

promoter, which is rectal specific and starts to be expressed after the embryonic 3-fold stage (i.e. after the LIN-12/Notch expression window in Y) (Liu et al., 2009; Kagias et al., 2012).

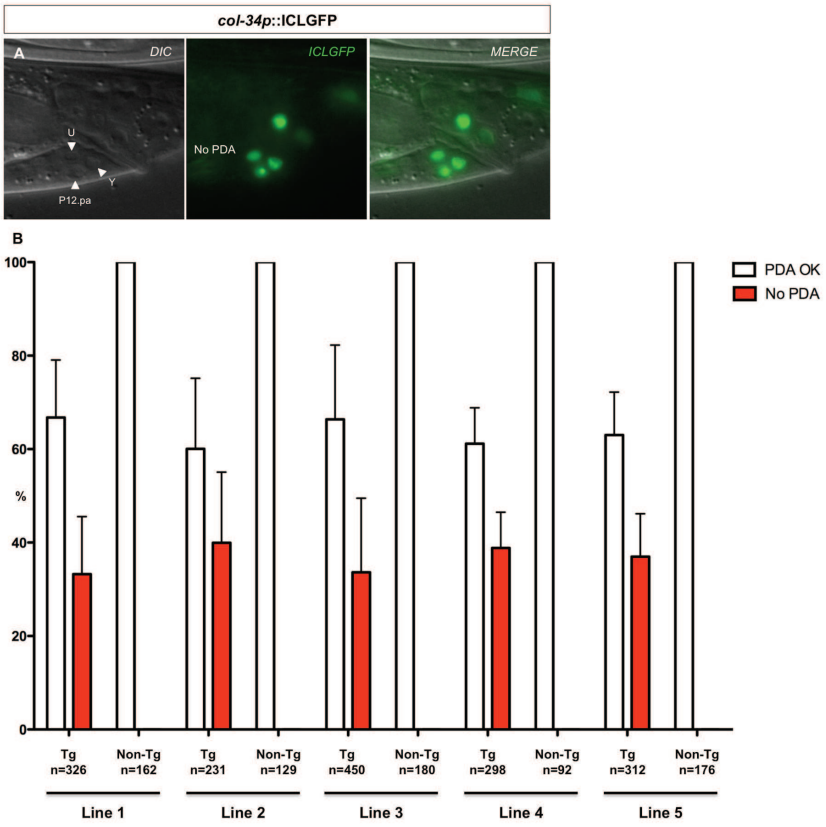


**Figure 51 –** Y-to-PDA transdifferentiation inhibition by maintained LIN-12/Notch pathway is independent of an early activation. **A)** ICLGFP is expressed in rectal cells and its expression leads to a "No PDA" phenotype. **B)** Scoring of five independent lines expressing *col-34p::ICLGFP* injected at 20 ng. In all the lines high penetrance of the "No PDA" phenotype is found in transgenic worms, while non-transgenic worms are wild-type. Data represent the mean of three biological replicates; error bars represent the standard deviation. Tg, transgenic worms; Non-tg, non-transgenic siblings.

As previously, overactivation of the LIN-12/Notch pathway in rectal cell leads to a "No PDA" phenotype appearance in transgenic worms. In each line, the penetrance of the "No PDA" phenotype is comparable to *egl-5p::ICLGFP* expressing lines (Figure 51.B). Moreover, in these conditions, Y appears to be blocked again during the transdifferentiation initiation step (Figure 51.A). These results suggest that ectopic and maintained activation of the LIN-12/Notch pathway inhibits Y-to-PDA

transdifferentiation. It reinforces the fact that LIN-12/Notch pathway has to be downregulated and not activated anymore after its action during embryogenesis.

### II.7.3) Ligands are still available and functional when *lin-12* is downregulated

Expression pattern analyses of *apx-1* and *lag-2* suggest that their expression is maintained around the rectum from Y birth until at least the L1 stage. At the opposite *lin-12* mRNA and protein are downregulated in Y after the embryonic 2-fold stage. This maintenance of ligand expression is maybe not biologically relevant. Indeed, our expression pattern analyses rely on the observations of transcriptional reporters made of *apx-1* promoter fused to NLS-YFP or *lag-2* promoter fused to GFP. Both of these fluorescent proteins are stable in cells, by consequence the signal detected in later stages could be coming from fluorescent protein stability and not reflect the actual activity of the promoter used to drive expression. We checked if ligands are still functional and available for the Y cell after the embryonic 2-fold stage, when LIN-12 is not expressed anymore.

To perform these experiments, we used an approach based on the fact that ectopic overactivation of the LIN-12/Notch pathway inhibits Y-to-PDA. We expressed the full-length *lin-12*cDNA in the rectal cells under the control of *col-34p*. The activity of this promoter begins after the 3-fold stage, thus LIN-12 will be expressed and available again to ligands when it is not supposed to be there anymore. If the ligands are still present around the rectum and functional, they should activate the newly expressed LIN-12 receptor, which will lead to Y-to-PDA inhibition. Indeed, the LIN-12/Notch pathway would be ectopically activated and thus mimic *col-34p::ICLGFP*. No fluorescent protein was inserted in the first construct we made; by consequence we were not able to track its expression. We subsequently add the SL2::mCherry sequence to monitor where *col-34p::lin-12cDNA* was expressed. We could see that the transgene was correctly expressed in Y (Figure 53.A).

To validate our approach we designed several constructs, which will be used as negative and positive controls. Two different negative controls have been

designed. In one hand, *lin-12(n941)* has been characterized as a null mutation for *lin-12*, which is induced by a premature stop codon in the extracellular EGF repeats of the receptor (Wen & Greenwald, 1999). We introduced the exact same mutation in the *lin-12*cDNA of our construct, thus the expressed protein will be truncated or produced mRNA will be degraded by the nonsense-mediated decay. No ectopic activation of the LIN-12/Notch pathway and no inhibition of Y-to-PDA transdifferentiation should take place. The second negative control is made by a deletion of the seven ankyrin repeats of the intracellular part of LIN-12 receptor. This deletion should not affect the extracellular structure of the receptor (Rhett Kovall, personal communications), thus the receptor can be activated, but the intracellular part should not be able to interact with the LIN-12/Notch nuclear effector *lag-1* to transcriptionally activate the LIN-12/Notch target genes. Finally, the positive control contains the same mutation as the *lin-12(n137)* allele, which has been shown to induce ligand-independent receptor activation (Greenwald & Seydoux, 1990; Seydoux, Schedl, & Greenwald, 1990; Komatsu et al., 2008). Expression of this form of the *lin-12*c DNA will trigger LIN-12/Notch pathway activation and will inhibit Y-to-PDA transdifferentiation. For both negative controls, each of the three independent lines scored displayed a wild-type phenotype (Figure 52). At the opposite, the three lines of the positive control show a variably penetrant "No PDA" (Figure 52), validating our approach. Indeed, negative controls appear to be silent and are not able to ectopically activate the LIN-12/Notch pathway in Y, whereas in the positive control activation takes place and inhibit Y-to-PDA transdifferentiation.

Examination of three independent lines expressing the full length *lin-12* cDNA with or without SL2::mCherry revealed that a "No PDA" phenotype was induced in transgenic worms while non-transgenic siblings were wild-type (Figure 53.B). Moreover, Y appears to be blocked at the initiation step as in previous experiments (Figure 53.A). These results show that LIN-12 receptor has been ectopically activated by the surrounding ligands and confirm the fact that ligand expression reflects the presence of these functional proteins at the surface of the cells when *lin-12* is not expressed anymore in Y. Together these observations support the idea that *lin-12* downregulation actively takes place in Y at the level of its mRNA and is ligand-independent.

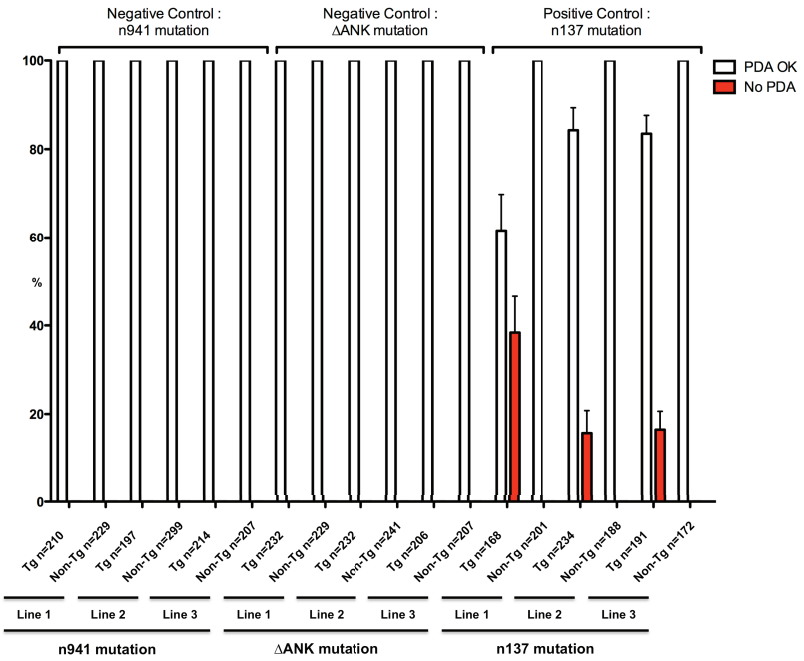


**Figure 52 –** Ligand are still available when *lin-12* is downregulated: Validation of the approach. Scoring of independent lines carrying the negative control transgenes, *col-34p::lin-12cDNA(n941)* or *col-34p*::*lin-12cDNA(ΔANK)* and the positive control *col-34p::lin-12cDNA(n137)*. All the worms of the negative controls are wild-type. Conversely, in positive control, a "No PDA" phenotype is found in transgenic worms. Data represent the mean of three biological replicates; error bars represent the standard deviation. Tg, transgenic worms; Non-tg, non-transgenic siblings.

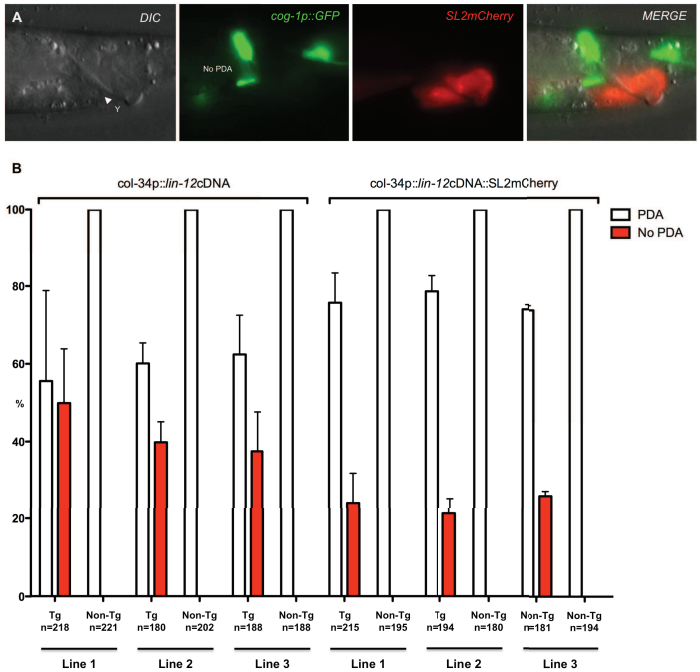


**Figure 53 –** Ligand are still available and functional when *lin-12* is downregulated **A)** *col-34p::lin-12cDNA::SL2mCherry* is expressed in Y and inhibits Y-to-PDA transdifferentiation **B)** Scoring of three independent lines for each construct tested *col-34p*::lin-12cDNA and *col-34p::lin-12cDNA::SL2mCherry*. Data represent the mean of three biological replicates; error bars represent the standard deviation. Tg, transgenic worms; Non-tg, non-transgenic siblings.

## II.8) Misregulated LIN-12/Notch signaling inhibits Y-to-PDA transdifferentiation by maintenaining Y epithelial fate and is cell autonomous

### II.8.1) Y-to PDA transdifferentiation inhibition by maintained LIN-12/Notch pathway is cell-autonomous

To date, no Y-only specific promoter has been identified; only rectal specific promoters are available to express our transgenes. Our hypothesis is that inhibition of Y-to-PDA transdifferentiation by ICLGFP overexpression is due to transgene expression in the Y cell, but it could be possible that overexpression in the other rectal cells disorganized a “niche”, which would be necessary for Y-to-PDA transdifferentiation to take place. Thus, the "No PDA" phenotype triggered by LIN-12/Notch pathway overactivation could be a non-cell autonomous consequence. To test this possibility, we checked whether overexpression of ICLGFP in rectal cells but not in Y induces inhibition of Y-to-PDA transdifferentiation. Several promoters have been tried to express ICLGFP in the rectal cells except in Y, but due to their non-rectal specific action, no high transgene expressing lines could be obtained. Only two promoters allowed us to express ICLGFP at satisfying levels, *lin-48* and *egl-20* promoters.

The *lin-48* gene is expressed in U, F, K and K’ (Johnson, Fitzsimmons, Hagman, & Chamberlin, 2001; Sewell, Zhang, Uttam, & Chamberlin, 2003). We used the regulatory sequence described to drive expression of ICLGFP with this pattern. Such construct is indeed expressed in the indicated rectal cells (Figure 54.A) but does not induce inhibition of Y-to-PDA transdifferentiation. Transgenic worms and their non-transgenic siblings are wild-type in the five independent lines we scored (Figure 54.B). This suggests that overactivation of the LIN-12/Notch pathway in U, F, K and K’ has no influence on transdifferentiation. By consequence these cells are probably not making a “niche”, which help Y-to-PDA to take place. It suggests that inhibition of Y-to-PDA transdifferentiation by prolonged LIN-12/Notch signal activation is cell-autonomous.

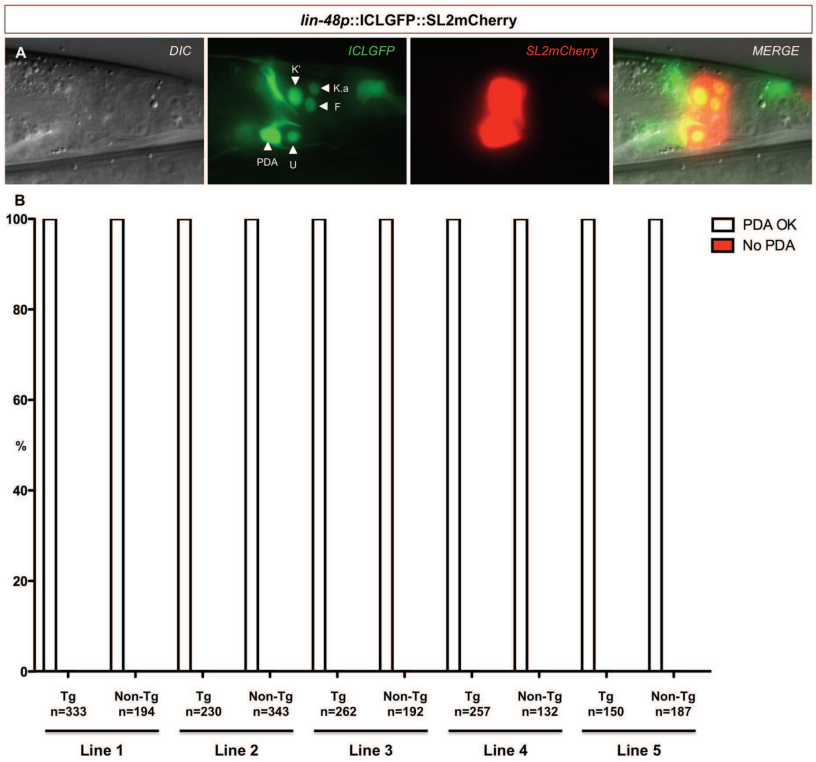


**Figure 54 –** Activation of the LIN-12/Notch pathway in U, F, K and K' has no effect on Y-to-PDA transdifferentiation. **A)** ICLGFP is expressed in U, F, K and K' and does not block PDA formation. In the five observed lines, ICLGFP and mCherry has never been detected in Y nor PDA **B)** Scoring of five independent lines expressing *lin-48p::ICLGFP::SL2mCherry* injected at 20 ng. All the scored transgenic and non-transgenic worms are wild-type. Data represent the mean of three biological replicates; error bars represent the standard deviation. Tg, transgenic worms; Non-tg, non-transgenic siblings.

To confirm these results, we used the *egl-20* promoter to drive ICLGFP expression. We used an *egl-20* regulatory sequence described to be expressed in B, U, F, K and K' (Whangbo & Kenyon, 1999). Surprisingly, scoring of transgenic worms carrying the *egl-20p::ICLGFP::SL2mCherry* transgene reveal that they exhibits a highly penetrant "No PDA" phenotype. Careful observations of transgene expression revealed that ICLGFP was expressed in Y (Figure 55.A) explaining why the five independent lines scored exhibited a "No PDA" phenotype with Y blocked at the initiation step (Figure 55.A and B). These results confirm the previous observations obtained using *egl-5* and *col-34* promoters, which showed that ectopic and maintained LIN-12/Notch pathway activation in Y is certainly deleterious for Y-to-PDA transdifferentiation. However, here we cannot use these results to definitely conclude on the possible cellular autonomy of Y-to-PDA inhibition. Moreover, these

experiments leave open the possibility that expression of ICLGFP in B is the origin of Y-to-PDA inhibition.

After we did this experiment, the Korswagen laboratory published a study in which they use single molecule FISH (smFISH) to look at *egl-20* expression (Harterink et al., 2011). SmFISH is a very sensitive method, which allows the *in situ* detection of single mRNA for a given gene (Raj, et al., 2008). Using this technique, *egl-20* mRNAs have been detected in Y, a conclusion that had never been reported for any translational and transcriptional reporters for *egl-20* and confirmed the results we obtained.

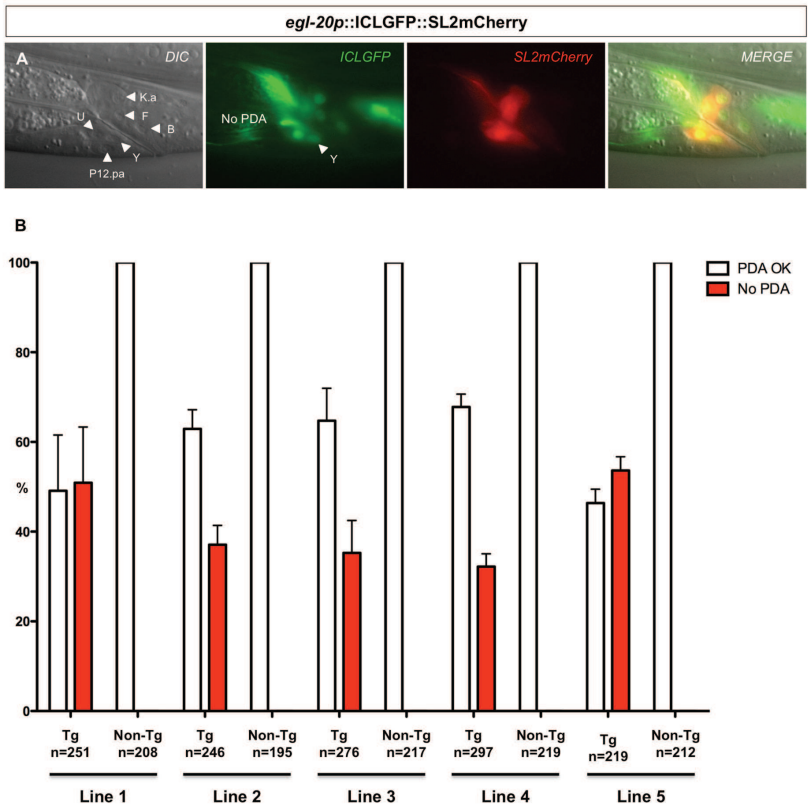


**Figure 55 –** Surprisingly *egl-20*::*ICLGFP* is expressed in all the rectal cells including Y. **A)** ICLGFP is expressed in all the rectal cells, a result that had never been reported before with similar constructs. This expression leads to a "No PDA" phenotype. **B)** Scoring of five independent lines expressing *egl-20p::ICLGFP::SL2mCherry* injected at 20 ng. In all the lines a high penetrance of "No PDA" phenotype is found in transgenic worms, while non-transgenic worms are wild-type. Data represent the mean of three biological replicates; error bars represent the standard deviation. Tg, transgenic worms; Non-tg, non-transgenic siblings.

To assess if ICLGFP expression in the B cell induces Y-to-PDA inhibition we took advantage of specific features we found in *egl-5p::ICLGFP* and *egl-*

*20p::ICLGFP* expressing lines. In our hands, *egl-5p* appears to have a low degree of moisaicism compare to *egl-20p*, which is very mosaic. This difference of moisaicism allowed us to test if expression of ICLGFP in the B cell blocks Y-to-PDA transdifferentiation. We scored the fraction of transgenic worms, which exhibit a "PDA OK" or a "No PDA" phenotype. Among these phenotypes we scored the fraction of worms, which expressed the transgene in B and checked if a correlation exists between ICLGFP expression in B and "No PDA" phenotype.

In transgenic worms expressing *egl-5p*::*ICLGFP*, the majority of the worms having a "No PDA" phenotype (60,7%) expressed ICLGFP in the B cell. Similarly, the majority of the worms with a PDA (39,3%) expressed as well ICLGFP in B (Figure 56.A). These observations suggest that no link exists between inhibition of Y-to-PDA transdifferentiation and overactivation of the LIN-12/Notch pathway in B. These results were confirmed by analysis of *egl-20p::ICLGFP* expressing lines. In this case, the majority of the worms did not express ICLGFP in B. In the "No PDA" phenotype population, almost all the worms did not express ICLGFP in B, indicating that the "No PDA" phenotype is not triggered by ICLGFP expression in B (Figure 56.B).

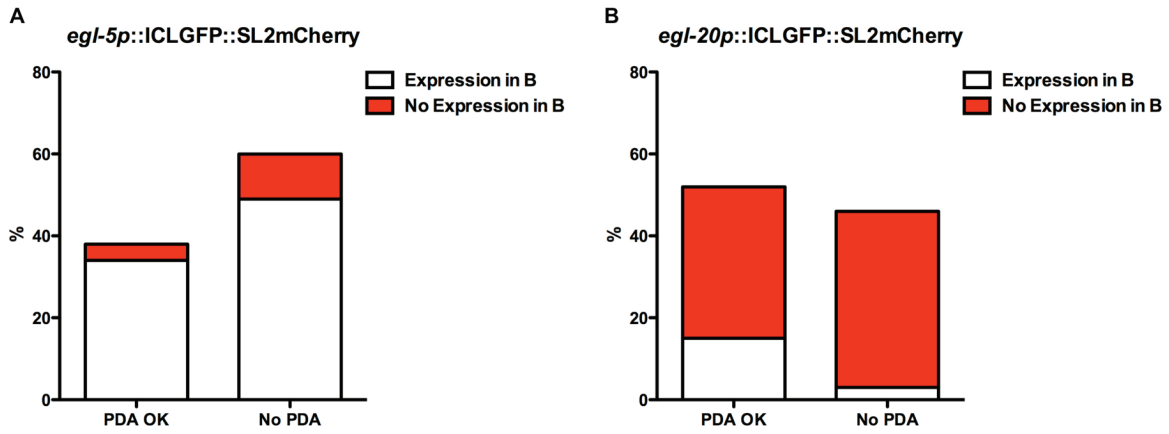


**Figure 56 –** Mosaic analysis of ICLGFP expression. **A)** Scoring of "PDA OK" versus "No PDA" phenotypes in a line expressing *egl-5p::ICLGFP*. In both phenotype classes, majority of the worms express the transgene in B. **B)** Scoring of "PDA OK" versus "No PDA" phenotype in a line expressing *egl-20p::ICLGFP*. In both phenotype classes, the majority of the worms do not express the transgene in B. Together, these analyses indicate that no correlation exists between ICLGFP expression in B and inhibition of Y-to-PDA transdifferentiation.

All together these results show that Y-to-PDA inhibition by maintained LIN-12/Notch pathway activation is cell-autonomous and is the result of pathway

activation in Y. Overexpression of ICLGFP in the other rectal cells has no influence on Y-to-PDA transdifferentiation.

### II.8.2) Prolonged LIN-12/Notch signaling inhibits Y-to-PDA transdifferentiation by maintaining Y fate

The previous experiments demonstrate that LIN-12/Notch expression around Y birth is necessary and sufficient to promote transdifferentiation. Misregulation of this pathway during development leads to inhibition of the reprogramming event. As described before, Y appears to be blocked at the initiation step. However, we do not know in which differentiation state the cell is. Indeed, before transdifferentiation Y is epithelial and turns into a neuron through discrete non-pluripotent steps (Richard et al., 2011). In the case of a misregulation of the LIN-12/Notch signal, it could be possible that Y identity is mixed up or that the cell is blocked in an epithelial, transition or neuronal state. To test these hypotheses, we looked at expression of cell fate markers in the blocked Y when the LIN-12/Notch pathway is misregulated.

To do this experiment we had to develop new strains that express prolonged ectopic LIN-12/Notch signal. Most of the cell fate markers available in the laboratory are labeled with GFP, by consequence they are not compatible with the ICLGFP construct we have. It is difficult to distinguish whether the GFP signal we see is coming from the cell fate marker or by ICLGFP. We modified our current *egl-5p::ICLGFP::SL2mCherry* construct by removing GFP. This newly obtained construct, *egl-5p::ICL::SL2mCherry*, can be tracked because of mCherry and is functional. Expression of ICL in rectal cells inhibits Y-to-PDA transdifferentiation as ICLGFP does (Figure 57).

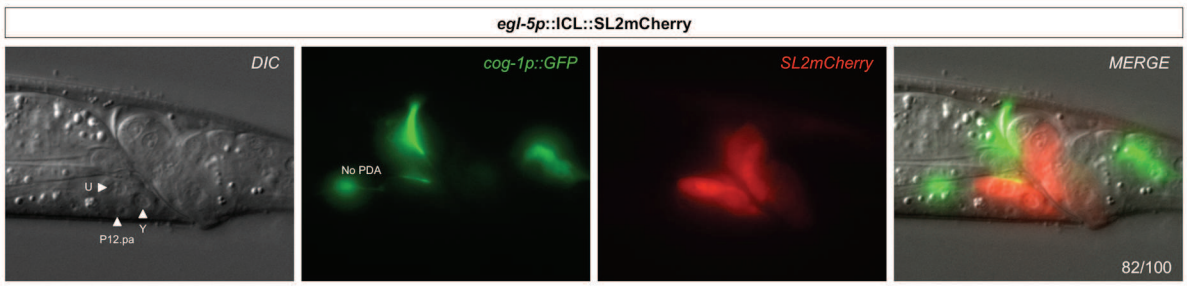


**Figure 57 –** Verification of ICL functionality. GFP as been removed from the ICLGFP construct previously used We injected it into *cog-1p*::GFP background and integrated it. The integrated array induces 82% of "No PDA" phenotype.

### *II.8.2.1) Y expresses epithelial markers when LIN-12/Notch pathway activity is maintained*

To investigate whether Y keeps its epithelial fate when LIN-12/Notch activity is maintained, we crossed an integrated *egl-5::ICL* array we built with epithelial or rectal specific markers. We used two epithelial junction markers, *AJM-1::GFP* (Figure 58.A), *DLG-1::GFP* (Figure 58.B), a rectal specific marker, *egl-26p::*GFP (Figure 58.C) and an epithelial differentiation marker, *lin-26p*::NLS-GFP (Figure 58.D). Each of these markers is expressed in the blocked Y cell indicating that misregulation of the LIN-12/Notch pathway in Y leads to maintenance of its epithelial fate.

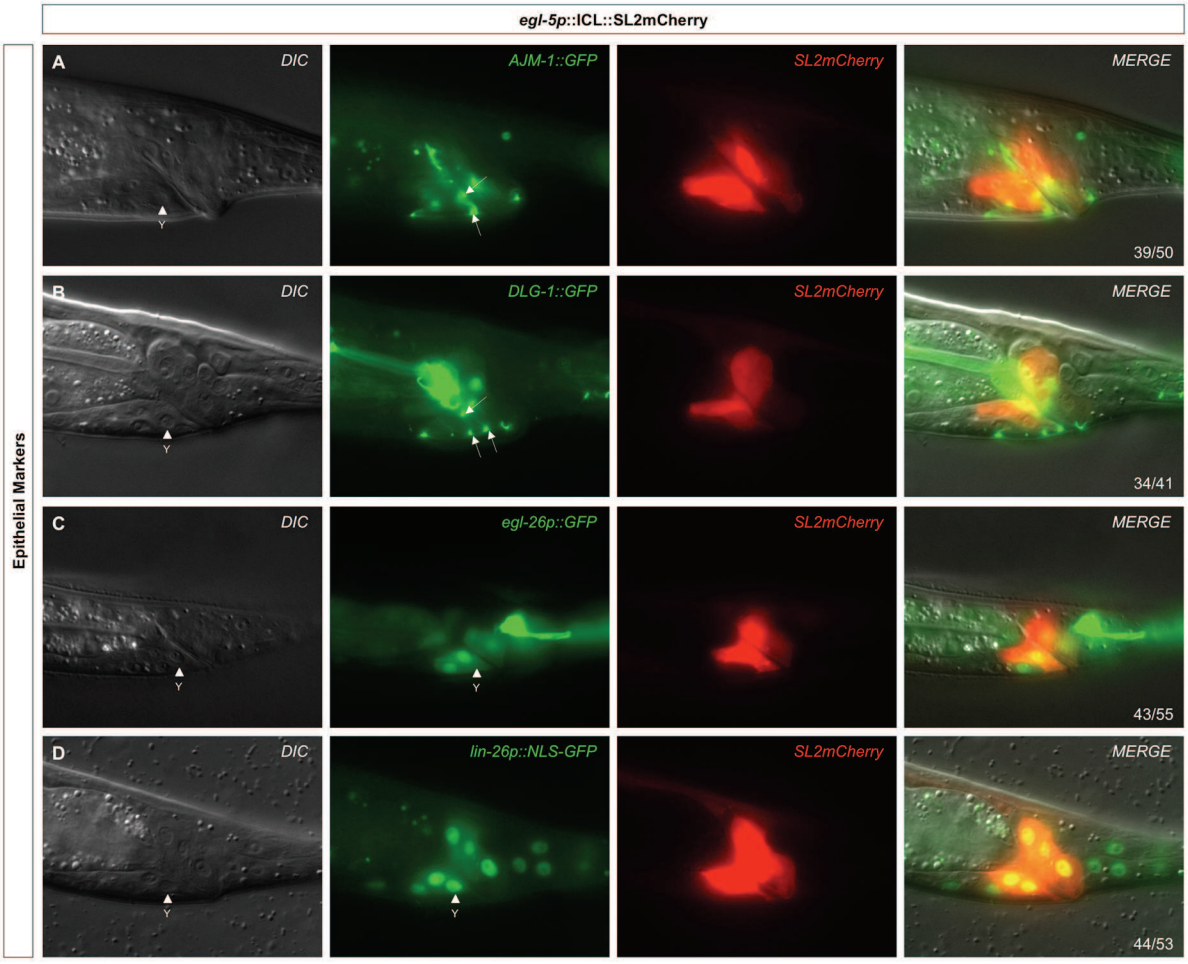


**Figure 58 –** Blocked Y cell expresses epithelial markers. **A)** The epithelial junction marker, *AJM-1::GFP*, is expressed (arrow) in the blocked Y (Arrowhead). **B)** The epithelial junction marker, *DLG-1::GFP*, is *expressed* (arrow) in the blocked Y (Arrowhead). **C)** The rectal cell marker, *egl-26p*::GFP, is expressed in the blocked Y (Arrowhead). **D)** The epithelial differentiation marker, *lin-26p*::NLS-GFP, is expressed in the blocked Y (Arrowhead). Numbers represent the fraction of worms showing this representative phenotype. Anterior is on the left and dorsal is up.

### *II.8.2.2) Y does not express neuronal markers when Lin-12/Notch pathway activity is maintained*

Y appears to be blocked in its original epithelial fate. However we could imagine that Y is blocked in a mixed identity state in which both epithelial and neuronal markers are expressed something that never happens during the wild-type process. To clarify this point we looked at the expression in the blocked Y of another PDA marker, *exp-1p::GFP* (Figure 59.A) and three pan-neuronal markers, *tag168p::GFP* (Figure 59.B), *unc-33p::GFP* (Figure 59.C) and *unc-119p::GFP* (Figure 59.D) (Richard et al., 2011). By contrast with the previous results, the blocked Y cell does not express any of these neuronal markers. Thus, when the LIN-12/Notch pathway is misregulated, Y keeps its epithelial fate and Y-to-PDA transdifferentiation is blocked.

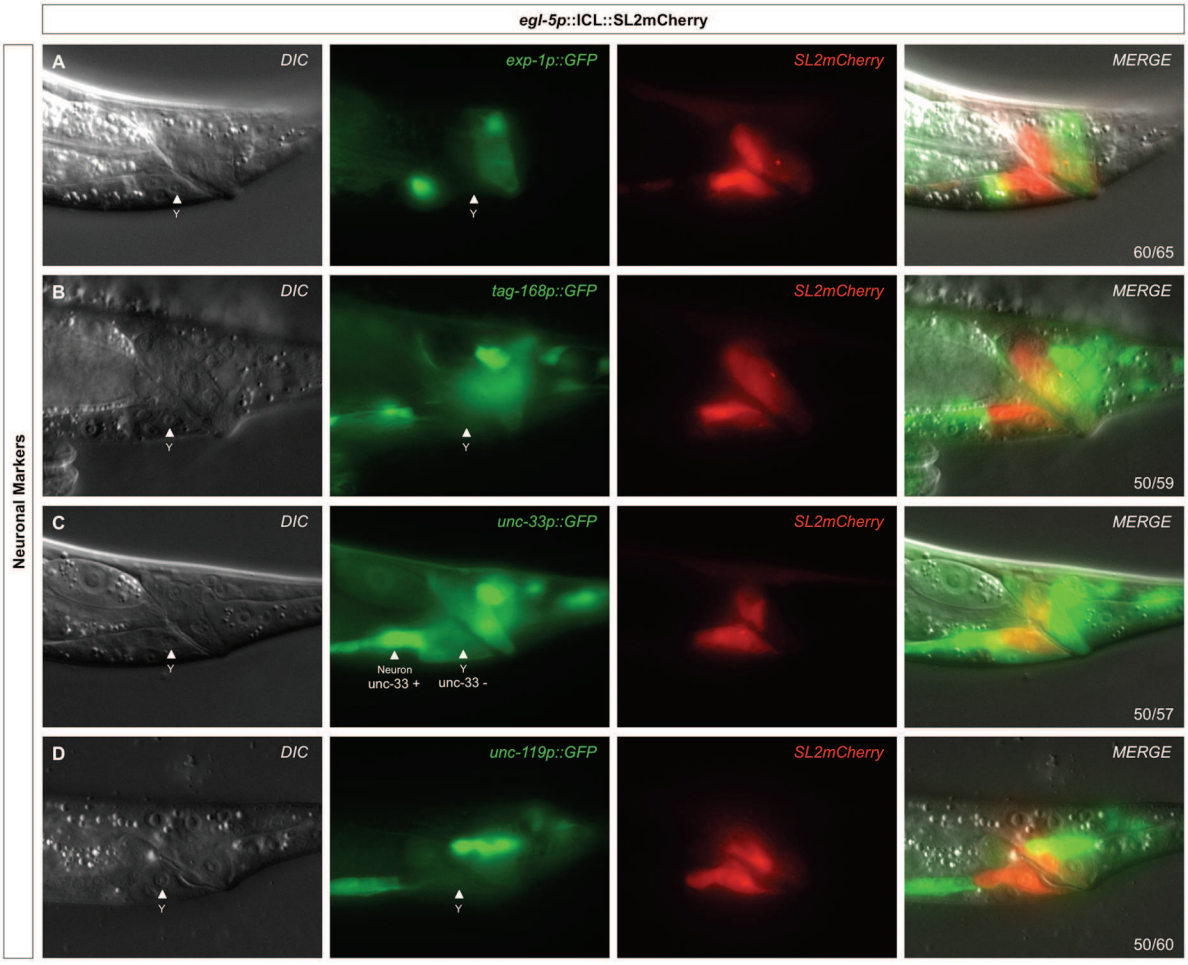


**Figure 59 –** Blocked Y cell does not express neuronal markers. **A)** The PDA marker, *exp-1p*::GFP, is not expressed in the blocked Y (Arrowhead). None of the following pan-neuronal markers are expressed in the blocked Y cell (Arrowhead)**:** *tag-168p*::GFP **(B)**; *unc-33p*::GFP **(C)**; *unc-119p*::GFP **(D)**. Numbers represent the fraction of worms showing this representative phenotype. Anterior is on the left and dorsal is up.

### II.8.3) The identity of U, B, F, K and K' cells is not affected by LIN-12/Notch misregulation

During the previous experiments overactivation of the LIN-12/Notch pathway is not exclusively done in the Y cell, but in all the other rectal cells as well. We checked if the identity of B, U, F, K and K' was not affected by LIN-12/Notch overactivation. To monitor the identity of these cells, we observed several rectal cell markers in strain carrying an integrated array of *egl-5p::ICL*.

We found that expression of *lin-26p::NLS-GFP* (expressed in all rectal cells) is not affected by LIN-12/Notch overactivation (Figure 60.A). Similar results are obtained by observations of *lin-48p::GFP* (expressed in F, U, K and K') (Figure 60.B) and *mab-9p::NLS-GFP* (expressed in B and F) (Figure 60.C). Together these observations indicates that the identity of U, B, F, K and K' does not appear to be affected when LIN-12/Notch pathway is overactivated.

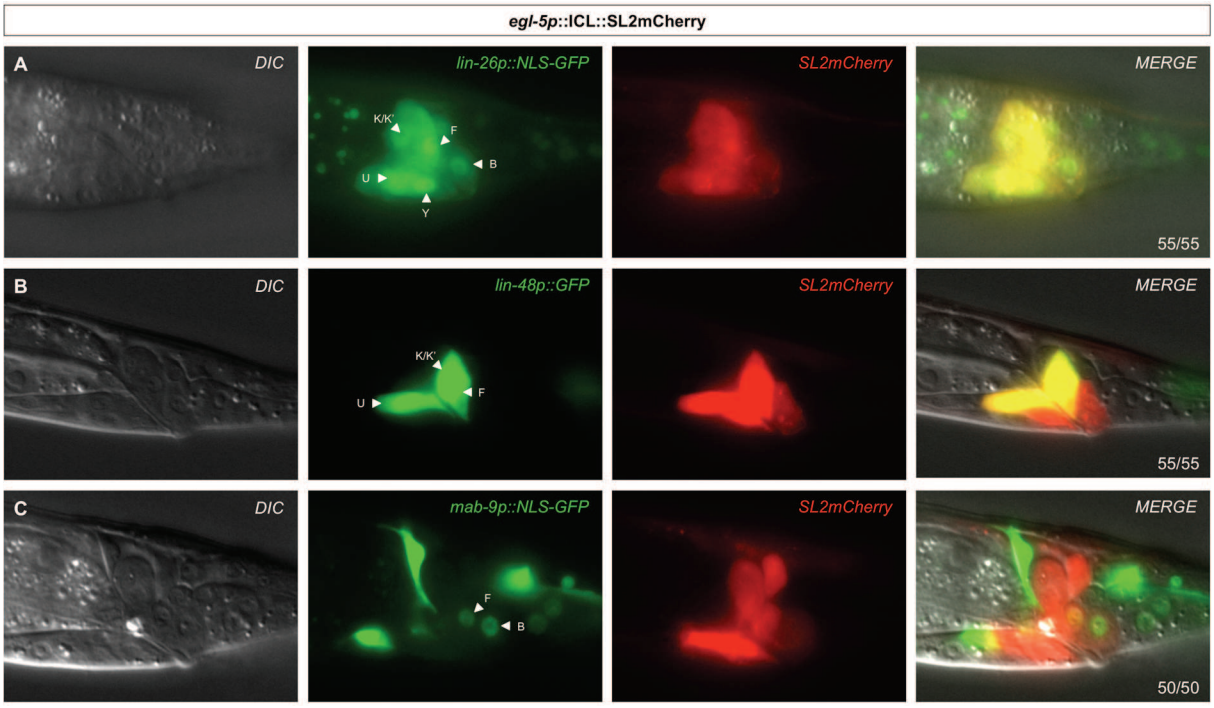


**Figure 60 –** The Identity of B, F, U, K and K' is not affected by LIN-12/Notch overactivation. **A)** *lin-26p*::*NLS-GFP* is properly expressed in rectal cells. **B)** *lin-48p::GFP* is properly expressed in F, U, K and K'. **C** *mab-9p::NLS-GFP* is properly expressed in B and F. Numbers represent the fraction of worms having this representative phenotype. Arrowheads show the position of the designated cells.

### II.8.4) A threshold of LIN-12/Notch signaling is necessary to be deleterious for Y-to-PDA transdifferentiation

Misregulation of LIN-12/Notch signaling is deleterious for Y-to-PDA transdifferentiation. We could observe that overactivation of this pathway maintains Y in its original epithelial fate. We wanted to characterize if a precise dose of ectopic LIN-12/Notch activation becomes deleterious. We injected serial dilutions of *egl-5p::ICLGFP::SL2mCherry* and looked at the obtained phenotype. The previous experiments were done with the construct injected at 20 ng, thus we decided to try the effect of the *egl-5p::ICLGFP::SL2mcherry* construct injected at 2 ng and 0,2 ng.

In *C. elegans*, injection of transgenes in the gonad leads to the formation of multi-copy extrachromosomal arrays, which are transmitted to progeny (Mello et al., 1991). The generation of these arrays is not controlled and forms randomly, although there is a correlation between the concentration injected and the expression levels of the construct (Barkoulas et al., 2013); we thus expected to obtain different ranges of penetrance of the "No PDA" phenotype, due to the delivery of different doses of activated LIN-12/Notch.

However, we did not obtain a range of phenotypes. Four independent lines carrying *egl-5p::ICLGFP::SL2mcherry* injected at 2 ng were analyzed. Penetrance of the "No PDA" phenotype dropped drastically (Figure 61.B and C), for three lines this penetrance is close to 1%, while for one line no "No PDA" transgenic worms are found (Figure 61.A and C). ICLGFP is not detectable under the microscope. However we could see low detectable mCherry signal indicating that the transgene is expressed. When the construct is injected at 0,2 ng, all the five independent lines observed are wild-type (Figure 61.D and E). In this case, both GFP and mCherry are not detectable, by consequence we are not sure if the transgene is correctly expressed or not, even at really low level. We tried to quantify the amount of expressed transgene in several lines by quantitative PCR or by smFISH. Unfortunately, we could not obtain satisfying results due to some technical issues and lack of sensitivity for the quantitative PCR.

However, we can conclude that only high doses of ectopic LIN-12/Notch activation are deleterious for Y-to-PDA transdifferentiation. Our data suggest that in the lines expressing the transgene injected at 2 ng, it is possible that LIN-12/Notch pathway activation is low and does not reach a certain threshold after which "No PDA" phenotype appears. Importantly these high doses of ICLGFP remain close to physiological ones. Indeed, in experiments using the prolonged expression of the full-length *lin-12*cDNA, ectopic activation of the receptor is done by endogenous ligands and lead to a "No PDA" phenotype. In this case the level of this ectopic activation may be the same as the one present in Y during the establishment of the competence to transdifferentiate.

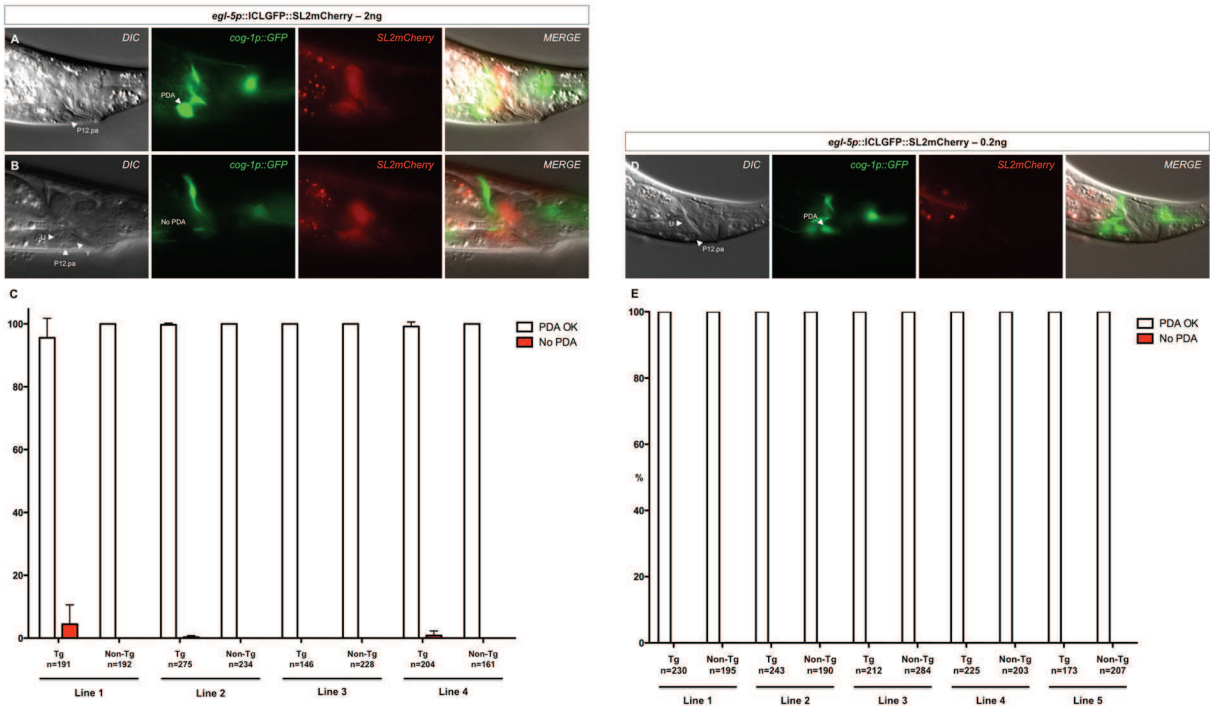


**Figure 61 –** High dose of LIN-12/Notch signaling is deleterious of Y-to-PDA transdifferentiation. **A)** The majority of the worms carrying *egl-5p::ICLGFP::SL2mCherry* expresses the transgene and are wild type. **B)** Example of "No PDA" worms found with low expressed transgene. **C)** Scoring of four independent lines expressing *egl-5p::ICLGFP::SL2mCherry* injected at 2 ng. **D)** *egl-5p::ICLGFP::SL2mCherry* injected at 0,2 ng is not detectable. **E)** Scoring of five independent lines expressing *egl-5p::ICLGFP::SL2mCherry* injected at 0,2 ng. Data represent the mean of three biological replicates; error bars represent the standard deviation. Tg, transgenic worms; Non-tg, non-transgenic siblings.

In summary for this part, we could demonstrate that LIN-12/Notch has to be tightly regulated in Y to set the competence to transdifferentiate and allow proper Y-to-PDA transdifferentiation. If this pathway is misregulated, transdifferentiation is blocked and Y fate is maintained throughout the entire worm lifetime.

# DISCUSSION

# I) Results summary

Our work provides new insights on the acquisition of the competence to transdifferentiate and the role of the LIN-12/Notch pathway in *C. elegans* during *in vivo* cellular reprogramming. We showed that LIN-12/Notch signaling is necessary and sufficient to induce the competence to transdifferentiate in the Y rectal cell. The activity of this pathway appears to be tightly regulated during embryogenesis at the level of *lin-12* mRNA. Indeed, LIN-12 protein, which is very short lived and mRNA are expressed in Y during a short embryonic time window around Y birth. We could determine that LIN-12/Notch activation outside of this window of activity inhibits Y-to-PDA transdifferentiation and blocks the Y cell in its original epithelial fate. This reprogramming inhibition is cell-autonomous and takes place only at a threshold dose of LIN-12/Notch activity.

Our work highlighted that the LIN-12/Notch pathway is activated in Y through the redundant action of the canonical ligands *apx-1* and *lag-2*. Several types of signalization are known to be mediated by the LIN-12/Notch pathway such as lateral signaling or inductive signaling. In the case of Y-to-PDA, inductive LIN-12/Notch interactions take place between the signaling cells and Y. Surprisingly, LIN-12 is transiently expressed in Y while ligands expression is maintained in the surrounding cells. LIN-12/Notch overactivation is detrimental and thus *lin-12* expression has to be actively downregulated to not be activated anymore by the available ligands. No positive feedback loop between ligands and *lin-12* expression seems involved.

Together, our data suggest that tight regulation of LIN-12/Notch signaling is required for proper cellular reprogramming.

# II) Discussion

Many questions and poorly understood facts remain during Y-to-PDA transdifferentiation. This part will be dedicated to interpret some of our results and

speculate on the possible LIN-12/Notch-dependent mechanisms, which take place during this cellular reprogramming event.

## II.1) ABprpppaaaa and ABplpppaaaa have the same developmental potential

As we described previously, ABpr**r**pppaaaa is the future Y cell and ABpl**l**pppaaaa becomes the DA9 neuron. These two blastomeres have the same developmental potential depending on LIN-12/Notch signaling. Indeed, in *lin-12(lf)* mutants, both blastomeres take the DA9 fate. On the other hand, we showed that in *lin-12(gf)* mutants both blastomeres acquire the Y identity and can transdifferentiate into PDA. We show for the first time that *lin-12* is expressed in Y and DA9 during embryogenesis and that the LIN-12/Notch pathway is necessary and sufficient to set the competence to transdifferentiate. DA9 can become a competent Y only if the LIN-12/Notch pathway is activated in it. We propose that, in wild-type, DA9 keeps its fate because the LIN-12/Notch pathway is never activated even though *lin-12* is expressed in this cell. At the opposite in ligand-independent *lin-12(gf)* mutants, the pathway is activated, which leads to conversion of DA9 into a competent Y.

Why does DA9 never become a competent Y in wild-type worms? After observations of the *lin-12* mRNA and ligand expression patterns, we think that DA9 is never in contact with ligand-expressing cells. In the rectum, the rectal B cell expresses *lag-2* and is in direct contact with Y (Figure 49). Two *apx-1* positive cells are close to the rectum as well, and can make close contact with Y (Figure 48). These contacts are favorable for LIN-12/Notch pathway activation in Y. However the position of these signaling cells suggest that they cannot be in contact with DA9, by consequence neither *lag-2* nor *apx-1* can activate the Lin-12/Notch pathway in DA9. This situation is similar to the GLP-1/Notch decision, which is taking place at the 4-cell embryonic stage. The ABa and ABp blastomeres are equivalent and both express the GLP-1 receptor. However, the organization of the embryo at this stage only allows contact between ABp and the signaling cell $P_2$. Thus, GLP-1/Notch pathway is activated in ABp but not in ABa even though this blastomere expresses the receptor.

A natural question arising from these observations is why does DA9 expresses LIN-12 receptor? LIN-12/Notch signaling does not appear to be important for DA9 at any step of the development: in *lin-12(lf)* mutants, DA9 is normally formed. Some elements of answer have been postulated in the first paper identifying the *lin-12* locus (Greenwald et al., 1983). From their observations, it was suggested that cells involved in LIN-12/Notch interactions are multipotent. Cell-cell interactions specify the level of *lin-12* activity in each of these multipotential cells, which will trigger specific gene expression that direct one cell into one fate and the other one into another fate. In other words, two equivalent cells can interact and acquire two different cell fates depending on their intrinsic *lin-12* activity level. However they could identify that the fate of Y and DA9 was not following this model of cell-cell interactions and that these cells are not multipotent anymore but can only acquire one fate depending on *lin-12* activity. They suggested that Y and DA9 were certainly multipotent in an ancestral nematode species, in which they were involved in cell-cell interactions (by consequence they were both expressing *lin-12* in this ancestral species) but this feature was lost through evolution. Thus it could be interesting to see whether a similar transdifferentiation event exists in less evoluted nematode species, and if this reprogramming event relies on *lin-12*-dependent interactions of two equivalent cells.

In summary, both Y and DA9 may have been certainly equivalent in an more primitive species, and had the same developmental potential. In *C. elegans*, both cells still have the same developmental potential (Y can become DA9 and vice-versa), but LIN-12 activation has been biased and is only working in Y explaining why only this cell acquires a rectal fate and subsequently become a PDA.

## II.2) Acquisition of the Y fate and the competence to transdifferentiate are indistinguishable

Being a Y cell is not sufficient to become a PDA. Indeed, in *mab-9* and *egl-38* loss-of-function mutants, a supernumerary Y cell is formed but is not competent to transdifferentiate. These supernumerary Y cells express some Y specific markers (Jarriault et al., 2008), have the same division pattern in males than Y (Chamberlin et

al., 1997; Woollard & Hodgkin, 2000) and appear to be functional in the rectum as epithelial cells but are not competent. It is possible that these supernumerary Y cells express the initiation factors (*egl-5*, *sem-4*, *egl-27*, *ceh-6* and *sox-2*) but they do not express the LIN-12/Notch receptor. Because these cells have never seen a LIN-12/Notch signal, they are not competent to transdifferentiate. By contrast in *lin-12(gf)* mutants, two competent Y cells are formed, which will give rise to two PDAs. Our results showing that this second PDA comes from a complete conversion of DA9 into a competent Y indicates that LIN-12/Notch signaling is essential for the acquisition of the Y fate, but at the same time primes a "program" that provides Y with the competence to change its identity. We think that both phenomena take place at the same time. By consequence, after LIN-12/Notch pathway activation Y fate and competence acquisition cannot be easily separated. It may be possible that these two distinct phenomena could be distinguished by the use of specific LIN-12/Notch target genes involved in either Y fate establishment or the acquisition of the competence to transdifferentiate. The strategies used to identify these target genes will be detailed in a future chapter.

## II.3) Transient *lin-12* activity is crucial for Y-to-PDA transdifferentiation

Expression pattern analyses revealed that *lin-12* protein and mRNA expression are transient during embryogenesis. Their expressions take place from Y birth until the 2-fold stage. During this time window, *lin-12* is necessary and sufficient to prime the competence to transdifferentiate. Conversely, outside of this window, LIN-12/Notch activation inhibits Y-to-PDA transdifferentiation and maintains Y in its original fate. Thus, *lin-12* must be downregulated during embryogenesis to allow proper Y-to-PDA transdifferentiation.

Meanwhile, ligands are expressed, available and functional around Y during an expanded time window, which goes from Y birth until at least the first larval stage. If *lin-12* is not downregulated, ligands surrounding Y and can still activate the LIN-12/Notch pathway, which will have a deleterious effect on Y-to-PDA transdifferentiation. By consequence regulated transient *lin-12* expression is crucial.

This model in which ligands are constantly available while transient Notch activation takes place has also been identified during chicken myogenesis (Rios, Serralbo, Salgado, & Marcelle, 2011). Expression of a Notch ligand is maintained in chicken neural crest cells. Neural crest cells migrate next to muscle progenitors, which express transiently Notch receptors (and delaminate within the myotome following Notch activation). This transient activation of NOTCH is essential for proper differentiation of muscle progenitors into mature muscle cells. If Notch signaling is maintained activated in these progenitors, myogenesis is blocked and cells keeps their original fate. These observations indicate that transient NOTCH activation is the key event, which allow proper myogenesis. However, the mechanisms of downregulation of Notch activity may differ, as in this case, and by contrast with the Y cell, the ligand expressing cells and receptor expressing cells are also only transiently in contact.

In our case, it is likely that prolonged *lin-12* activity stabilizes and reinforces the rectal epithelial identity of the Y cell and makes it so stable that it becomes a barrier to reprogramming and could explain why transient LIN-12/Notch activity is required for proper transdifferentiation.

The fact that Notch expression can be transient to allow cell differentiation (in the case of chicken myogenesis) or cellular reprogramming (in Y-to-PDA transdifferentiation) has to be considered in any experimental design. Indeed, to show that Notch is important for cell differentiation, scientists mostly use loss-of-function strategies (i.e. knock-out of the receptor, knock-down of Notch pathway component, etc.) and ectopic gain-of-function strategies in which they over-express the intracellular part of the Notch receptor under specific promoters. As our results underscore, the use of such gain-of-function strategy can lead to misinterpretation of the obtained results. If the studied process requires a transient Notch expression the use of gain-of-function strategy could lead to inhibition of the process and give the opposite results. By consequence, providing a pulse of signal (as we have done with our *heat shock promoter::ICLGFP* construct) to activate transiently the Notch pathway should be considered in gain-of-function experiments.

## II.4) Mechanisms of *lin-12* downregulation

We have shown that *lin-12* is transiently expressed in Y during embryogenesis. This transient expression is certainly the results of an active downregulation of *lin-12* expression. We did not determine the factors responsible for this downregulation. However we think that *lin-12* downregulation takes place at its promoter level. Indeed both translational and transcriptional reporters have the same dynamics. The translational reporter we used is composed of the complete *lin-12* locus (promoter, coding sequence and 3'UTR) in which the GFP is inserted in frame (Levitan & Greenwald, 1998). This translational construct has LIN-12 endogenous PEST domain, known to trigger protein degradation, by consequence the fusion protein has a short half-life that should reflect the endogenous receptor's one. Thus, the fact that this reporter does not last longer (or shorter) than the *lin-12* mRNA strongly suggests that no additional regulation takes place at the protein level. However, we cannot exclude that this degradation could be reinforced by an additional targeted protein degradation mechanism. The first transcriptional reporter we analyzed is only composed of the *lin-12* promoter upstream of the GFP and the 3'UTR of the *unc-54* gene (Singh et al., 2011). The only common feature between these two constructs is the use of the same *lin-12* promoter, which strongly suggests that *lin-12* dynamics of expression in Y is the result of *lin-12* promoter activity. In addition, the same expression dynamics is observed with a transcriptional reporter that encompasses the whole *lin-12* locus (Wilkinson et al., 1994), including its own 3'UTR: this strongly suggests that the additional *lin-12* possible regulatory sequences and its own 3'UTR do not impact significantly on *lin-12* dynamics of expression in Y. To verify this hypothesis, we are currently trying to obtain strains carrying a transgene made of *lin-12p::ICL::lin-12 3'UTR*. This transgene should transiently activate the LIN-12/Notch pathway in Y and DA9 (according to our transcriptional expression pattern observations), and induce the appearance of a "2 PDAs" phenotype in transgenic worms. At the opposite, if our hypothesis is wrong and if *lin-12* dynamics is not driven by the *lin-12* promoter activity, our transgene should lead to the appearance of a "No PDA" phenotype.

We are currently working on the injection conditions of *lin-12p::ICL::lin-12 3'UTR,* which is known to be highly toxic (Iva Greenwald, personal communication and (Struhl et al., 1993)). Next, lines carrying this transgene will be integrated and may be used to perform a genetic screen to find the factors responsible of *lin-12* active downregulation in Y.

## II.5) Establishment of the competence to transdifferentiate is context dependent

During our heat-shock experiments, we could obtain a supernumerary PDA after a pulse of LIN-12/Notch signaling around Y birth. We could determine that this supernumerary PDA originates from the conversion of DA9. Surprisingly, in these experiments only DA9 is able to give rise to a supernumerary PDA. We never found any extra neurons formed after a pulse of LIN-12/Notch activity. These observations indicate that in the embryo and at this precise stage, only DA9 is responsive to LIN-12/Notch pathway activation. No other embryonic cell was able to integrate this signal and give rise to a competent cell that will give rise to a supernumerary PDA. Thus, establishment of the competence to transdifferentiate after LIN-12/Notch pathway activation can take place only in a specific cellular context, which is restricted to Y and DA9.

We looked at the formation of supernumerary PDAs, but we cannot exclude the probable fact that ectopic LIN-12/Notch pathway activation can lead to the formation of other supernumerary Y cells, which are not able to transdifferentiate, or which are not able to transdifferentiate into a PDA, ie that lack the information necessary for the correct re-differentiation step. In addition, an interesting experiment would be to test. We could verify, using Y markers, if after a pulse of LIN-12/Notch activation ectopic Y cells are formed in the embryos.

## II.6) Interconnection between LIN-12/Notch signaling and the initiation-promoting factors

Our laboratory has recently shown that a NODE-like complex (composed by *sox-2*, *ceh-6*, *egl-27* and *sem-4*) in association with *egl-5* are together involved in the promotion of the initiation to transdifferentiate. Mutation or elimination of any of these initiation-promoting factors leads to the same phenotype; Y is blocked in the rectum as an epithelial cell and cannot turn into a PDA (Kagias et al., 2012). The initiation step takes place after the acquisition of the Y fate and the competence to transdifferentiate. Indeed, Y birth takes place during the first third of embryogenesis, while the initiation of Y-to-PDA transdifferentiation morphologically takes place at the end of the L1 larval stage. Then, is there a link between the LIN-12/Notch pathway and these initiation-promoting factors? We showed that RNAi against initiation-promoting factors on *rrf-3(pk1426);lin-12(n950)* double mutants leads to the appearance of a "No PDA" phenotype: thus both PDA formed in *lin-12(n950)* are affected. These results suggest that these factors act certainly downstream of *lin-12* and that their expression, which is normally not observed in DA9, is induced, directly or not by the LIN-12/Notch signal. By consequence, some of these genes could be direct targets of the LIN-12/Notch pathway. For example, *sox-2* has been reported to be a direct Notch target in mouse neural stem cells (Ehm et al., 2010; Li, Hibbs, Gard, Shylo, & Yun, 2012). Thus, it could be possible that in the context of Y-to-PDA transdifferentiation *sox-2* is a direct target of *lin-12*. Other arguments are comforting this hypothesis: we looked rapidly at putative *lag-1* binding sites in the upstream 15 kilobases (kb) region of *sox-2*. As described in the chapter III.1.2.5 of the introduction, *lag-1* is a nuclear effector of the LIN-12/GLP-1 pathway. *lag-1* is bound to DNA and is a core member of the nuclear complex that activates LIN-12/GLP-1 target genes after receptor activation. The *lag-1* binding site consensus has been defined as RTGGGAA (Christensen et al., 1996). The orientation of the site is not important, thus we searched for sens and antisens *lag-1* binding sites in *sox-2* regulatory sequence. In the 15 kb upstream of *sox-2* we could identify 8 different *lag-1* binding sites and a ninth located in the second intron of *sox-2* coding region. The evidence of a direct link between *sox-2* and Notch in neural stem cells

associated with the presence of several *lag-1* binding sites in *C. elegans sox-2* regulatory sequence indicates that this NODE-like component could be a direct target gene of *lin-12* during Y-to-PDA transdifferentiation.

We did not find, in the literature, any evidence of a direct link between Notch and the homologs of *egl-27* (MTA1), *ceh-6* (OCT4) *or egl-5* (HOX). *sem-4* (SALL4) has been suggested to be a *lin-12* target during vulval cell specification (Grant, Hanna-Rose, & Han, 2000), but, to date no direct links have been described in worms or in other models between these two genes. Nevertheless we found for *ceh-6, egl-5* and *sem-4*, one or several *lag-1* binding sites in their regulatory sequence. They could be as well direct target genes of the LIN-12/Notch pathway. These hypotheses can be verified by the building of rescuing constructs in which we can delete some or all of the *lag-1* sites to see if indeed they are necessary and whether these initiation-promoting factors are LIN-12/Notch direct target genes or not.

The expression of these initiation-promoting factors may be initiated by *lin-12*. However, we know that these genes remain expressed even when *lin-12* signal disappears during embryogenesis. It suggests that *lin-12* is not required for their maintenance. It is possible that *lin-12* triggers the expression of these initiation-promoting factors, which will set up an autoregulatory loop, as described for (*sox-2* and *ceh-6*/Oct-4 in ES cells (Jaenisch & Young, 2008)) and/or a positive feedback loop to maintain their expression.

To date, we can only speculate on the putative links between the LIN-12/Notch pathway and the other factors we have identified in the laboratory. Assessing these links will require further studies.

## II.7) LIN-12/Notch signaling and cell plasticity in *C.elegans*: two opposite roles?

A recent study shows that *glp-1* and *lin-12* are essential to restrict embryonic developmental plasticity (see III.3.2 of the introduction) (Djabrayan et al., 2012). When both of *glp-1* and *lin-12* are eliminated during early embryogenesis, embryonic blastomeres are more prone to be reprogrammed. However they did not show if excess of activity of *glp-1* and *lin-12* leads to stronger restriction of the developmental plasticity. In the case of Y-to-PDA transdifferentiation, *lin-12* primes cellular plasticity. Lin-12/Notch signaling sets up the conditions, which will allow a rectal epithelial cell to become a motor neuron.

During Y-to-PDA transdifferentiation, initiation-promoting factors are downstream of *lin-12* (at least they are involved in a step taking place after the priming of the competence). The homologs of these factors have been shown to be crucial for the maintenance of pluripotency in ES cells (Kagias et al., 2012). Cell identity switch from an epithelial cell into a neuron is elicited by these initiation factors. Due to the sequential action of *lin-12* and these initiation factors, cellular plasticity features are installed in Y. At the opposite, during embryogenesis and because it is a different context, we can speculate that the factors downstream of the GLP-1/Notch pathway are inhibiting cellular plasticity. Together these studies show that in different contexts, Notch signaling can have opposite roles on cellular plasticity.

## II.8) General discussion around the PDA motor neuron

The origin of the PDA motor neuron is unique in *C. elegans* hermaphrodites. It is surprising to see that to generate PDA, Y migrates and is replaced in the rectum by another epithelial cell, P12.pa. It is also surprising to see that formation of PDA utilizes a slightly different process in *C. elegans* males (Sulston & Horvitz, 1977; Sulston et al., 1983). In fact, during male post-embryonic development Y divides and gives rise to two different daughter cells, the first one becomes PDA, while the

second one will give rise to a cell at the origin of the ten cells constituting the post-cloacal sensilia. Division does not occur in hermaphrodite probably because these sensilia are not required in this sex. However we could imagine a scenario in which Y divides in hermaphrodites and gives rise to PDA and a second cell that is epithelial and replace Y in the rectum.

Why does such a complicated mechanism takes place for PDA formation? Is it originating from an ancestral species? It could be interesting to perform lineage analysis in ancestral *Caenorhabditis* species, to see if: i) PDA exists ii) PDA is originating from the transdifferentiation or the division of a differentiated cell. iii) Similar factors are involved in the formation of PDA.

Another missing information about PDA is its function. PDA has been described to innervate the posterior dorsal body muscles. Mutants lacking PDA do not display any particular locomotion defect or uncoordinated phenotype. We have never found any obvious phenotype linked to PDA defect, although detailed investigations remain to be performed. PDA is apparently making contacts with neurons involved in the innervation of the intestinal and anal depressor muscles (White et al., 1986). Thus, PDA could allow proper defecation in collaboration with other neurons and as the worm grows, acquiring one more neuron that facilitates defecation, a vital function, may be an advantage for the worm. To date, this aspect of the PDA function has never been investigated.

## III) Future Directions

This last part will be focused on the immediate and important future questions we would like to assess concerning the role of the LIN-12/Notch pathway during Y-to-PDA transdifferentiation.

### III.1) Re-evaluation of the *apx-1* and *lag-2* redundancy

Our work showed that *apx-1* and *lag-2* are certainly the source of the activation of the LIN-12/Notch pathway in Y. In both cases, mutant worms for these

genes display a really low penetrance of worms, which fail to form a PDA. From our observations we conclude that *lag-2* and *apx-1* have a redundant role.

Technically it is really challenging to create *apx-1; lag-2* double mutants. Indeed these genes are separated by only 0,2 cM. By consequence during genetic crossing the probability to get a double recombinant mutant worm is close to 2 for 1000. This low probability associated with the defect (early lethality) brought by these mutations makes the realization of such double mutant almost impossible.

To overcome these issues, we will try to perform RNAi against *lag-2*, in the sensitized mutant background *apx-1(zu347ts); sel-12(ar171)*. We will have to define the best culture and RNAi conditions to obtain escapers that we can score. We hope that such experiment will lead to an increase of the "No PDA" phenotype compared to single mutants. If this increase is significant, we will be able to conclude that elimination of *apx-1* and *lag-2* has an additive effect, which proves that both of these genes acts redundantly in the first step of Y-to-PDA transdifferentiation. These data will allow us to define the minimal cellular microenvironment necessary for Y-to-PDA transdifferentiation.

### III.2) Induction of competence in non-competent supernumerary Y cell

A supernumerary Y exists in *egl-38* and *mab-9* mutant backgrounds. This cell is not competent to become a PDA. We think that supernumerary Y cells are not competent to transdifferentiate because they never activate the LIN-12/Notch pathway. To induce the activation of this pathway in these supernumerary cells, we are going to use our *heat-shock promoter::ICL* construct in *egl-38* and *mab-9* mutants and assess if another extra PDA can be formed. Functionnaly, cellularly and molecularly these supernumerary Y cells appear to be close to the original Y (Jarriault et al., 2008), however we do not know if the factors allowing Y to become a PDA are as well available in these supernumerary Y cells. It remains possible that in *egl-38* and *mab-9* mutants, the conversion of U and B, respectively, lead to incomplete conversion into Y. Indeed, *lin-12* is necessary and sufficient to induce

the rectal Y fate and the competence to transdifferentiate. These mutants cells become Y in mutant background without the action of *lin-12*, thus these cells are maybe Y-like for some rectal specific features but are lacking key components that will allow them to transdifferentiate. For example, supernumerary Y cells may be lacking some initiation-promoting factors or LIN-12/Notch target genes, which are only available in the original Y cell.

Our data on the extra PDA induced by a pulse of LIN-12/Notch signal suggest that initiation-promoting factors are downstream of *lin-12.* Thus, It is possible that ectopic activation of the LIN-12/Notch pathway in these converted cells could lead to the expression of factors suffiencient for the priming and the initiation of the transdifferentiation and will allow proper transdifferentiation into PDA.

### III.3) What is a permissive context?

What are the elements that give to Y this exclusive cell plasticity? From our work *lin-12* seems to be the earliest key factor that allows Y to become plastic and transdifferentiate into a neuron. All the rectal cells are cellularly and molecularly close (so close that finding a marker that is expressed in Y and not the other rectal cells has proved a challenge); however only Y seems to be able to uniquely receive and integrate a *lin-12* signal to be able to subsequently transdifferentiate. Indeed, in our heat-shock experiments, ICLGFP is expressed in all the cell of the embryo thus, all the rectal cells have received a dose of *lin-12* signal but they did not integrate it and are not permissive to change their identity. To understand the specificity of the Y cell to be able to transdifferentiate we have decided to develop strategies to identify the transcriptome and the LIN-12/Notch target genes in the Y cell

We are trying to identify the genes downstream of the LIN-12/Notch pathway. We have envisioned several complementary strategies. The first one we have started does not allow the identification of direct LIN-12/Notch target genes instead we can find the genes modulated by this pathway.

We decided to use microarray analyses to identify the transcriptome of wild-type, loss-of-function and gain-of-function mutants for *lin-12*. These transcriptomes

are made at two different stages, during embryogenesis (as close as possible of the Y birth) and during the L1 larva. These experiments will be useful to make transcriptome comparison between the different stages and the different genetic backgrounds. By using this approach, genes modulated by the LIN-12/Notch pathway can be detected. This pathway is known to activate the transcription of target genes, thus by comparison of the different genetic background we could identify upregulated and downregulated genes. We think that LIN-12/Notch target genes (direct or indirect target genes) can be highlighted by this approach. The purification of mRNA from the different genetic backgrounds and the hybridization on the microarray chip in quadruplicate have been performed, however the data are still under investigation by our bio-informaticians. The modulation of identified genes will be confirmed by quantitative PCR, reporter gene expression pattern analysis and smFISH in Y and the role of these genes in Y-to-PDA transdifferentiation will be challenged by RNAi. These results will be compared to the other approaches described in the following parts to try to identify common target genes.

To determine the transcriptome of the Y cell and to understand the molecular mechanisms underlying the inhibition of Y-to-PDA transdifferentiation when the LIN-12/Notch pathway is misregulated, we decided to undertake a challenging approach. Recent work has shown that isolation of cells from *C. elegans* larvae is possible after specific treatments (Zhang, Banerjee, & Kuhn, 2011). We would like to use this protocol to purify wild-type and blocked Y cells when the Lin-12/Notch pathway is misregulated.

We did not discuss this point during this manuscript, but a part of my thesis was focused on the development of a Y specific fluorescent marker. To date, we have never found a marker specific of the Y cell. Each promoter available in our laboratory, which marks the rectum, is never specific to one particular cell. Either the promoter is expressed in one rectal cell with many others in the worm body or more commonly the promoter is expressed only in the rectum in all or a subsets of the rectal cells, a testimony to their high similarity. By consequence we could not use any of these promoters to mark specifically Y. To overcome this issue we considered the use of the CRE/LOX system (Macosko et al., 2009), the FLP/FRT

system (Davis, Morton, Carroll, & Jorgensen, 2008; Voutev & Hubbard, 2008), or the use of reconstituted fluorescent protein (Zhang, Ma, & Chalfie, 2004). However in our hands none of these approaches lead to satisfactory results. Although reconstituted fluorescent protein works in the PDA neuron, it does not in Y, we could not get the CRE/LOX system to work and the efficiency of the FLP/FRT system was very low on multicopy arrays, which is necessary to see any expression.

We are now trying to use overlapping promoters to mark specific rectal cells. The use of two or more overlapping promoters allows us to have all the rectal cells marked differently. For example, all the rectal cell can be marked in green by a *egl-5p::GFP* construct, U, F, K and K' can be marked in red because of *lin-48p::mCherry,* finally F and B can be marked in blue by a construct carrying *mab-9p::CFP*. Therefore, Y is green only, U, K and K' are red, B is blue and F is blue and red. Thus, we could try to purify each of these cells by cell sorting and study their transcriptome. We have designed a number of possible combinations taking advantage of all the markers we have now identified and we are developing the best combination of overlapping promoters, but we already tried some larval cell purifications.

We developed two strains; a control strain carrying a *col-34p::GFP* and an *egl-5p::mCherry* integrated arrays, which allow specific labeling of all the rectal cells in red and green, and a second strain carrying a *col-34p::GFP* and an *egl-5p::ICL::SL2mCherry* integrated arrays, which allows rectum specific labeling and overactivation of the LIN-12/Notch pathway in all the rectal cells (Figure 62). Our strategy consists on the extraction of cells from L1 larva and specific isolation of rectal cells by cell sorting. RNAs from these purified cells can be extracted (amplified if necessary) and sequenced to obtain the complete transcriptome of the rectal cells (Figure 62). This purification and sequencing will be performed in parallel in the control strain and in the strain with overactivated LIN-12/Notch pathway. The comparison of these transcriptomes will allow us to understand the molecular mechanisms involved in the inhibition of Y-to-PDA transdifferentiation during misregulation of LIN-12/Notch pathway.

Several larval cell isolations have been tried and we realized that the published protocol is really tricky to implement. In order to avoid mRNA amplification prior sequencing, we would need approximately thousands of cells. However this protocols is not made to treat a large amount of worms. Moreover, many steps of this protocol are critical and have to be carefully monitored. We can also note that the yield of isolated cells from larva is really low.

Many optimizations have to be done, and we already tried to adapt this protocol for large culture of worms. From our trials we could obtain some isolated cells and rectums (Figure 63). Moreover, some purification of rectal cells by FACS has been performed in the lab (Nadine Fisher and Sophie Jarriault). We are really optimistic on the feasibility of this approach to determine the transcriptome of our cell of interest.

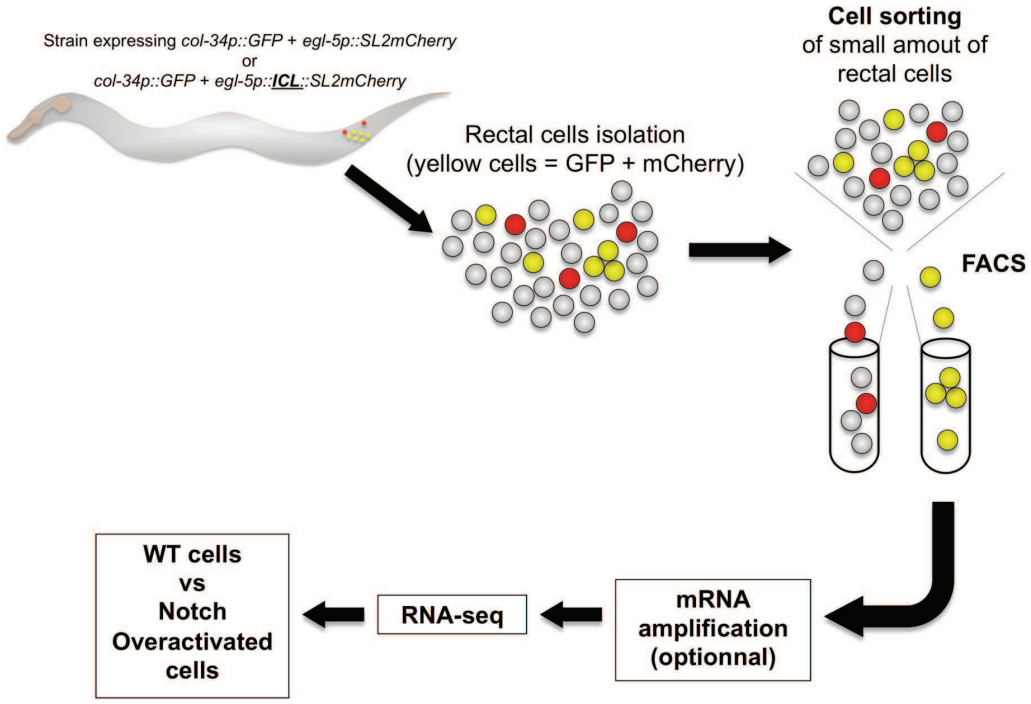


**Figure 62 –** Experimental design to identify the transcriptome of the wild-type Y cell and the blocked Y cell when LIN-12/Notch is misregulated. The used strains have the rectal cells labeled in green and red (giving yellow rectal cells). Larval cells can be isolated; among them rectal cells can be purified by cell sorting. RNAs of these purified cells are extracted and sequenced. Wild-type transcriptome will be compared the transcriptome of cells having the LIN-12/Notch pathway overactivated. This comparison will allow the identification of the molecular mechanisms involved in the inhibition of Y-to-PDA transdifferentiation during misregulation of LIN-12/Notch pathway.

If these experiments do not lead to satisfactory results, an alternative strategy is available. Indeed, the Yanai laboratory has recently developed a technique that gives access to the transcriptome of single *C. elegans* blastomere (Hashimshony et al., 2012). These single cell strategies could be used to identify the transcriptome of single Y cell extracted from embryos or larva (trials are currently on-going in the lab). We could extend this approach to the other rectal cells and compare their transcriptome to the Y transcriptome to identify the genes, which give to Y this capacity to be reprogrammed.

We also consider to make a transcriptomic time course of the Y cell during development, to see how genes are modulated during the different phases of the Y-to-PDA transdifferentiation. These experiments could allow us to identify the molecular networks involved in each phases of the Y-to-PDA transdifferentiation, such as the acquisition of the rectal epithelial fate, the competence to transdifferentiate and during the initiation of the reprogramming.

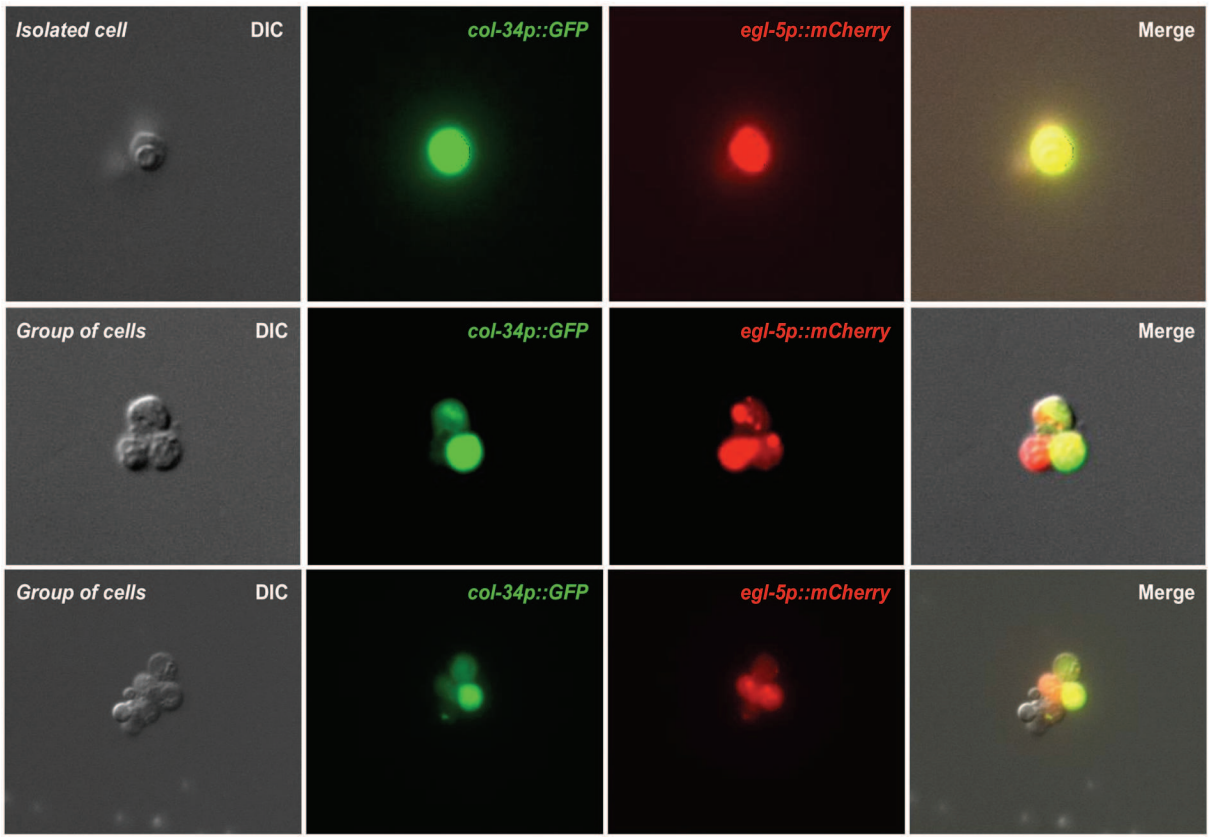


**Figure 63 –** Isolation of rectal cells from larva. We used an established protocol to isolate cells from *C. elegans* larva (Zhang et al., 2011). The strain we used expresses two different integrated transgenes; *col-34p::GFP* and *egl-5p::mCherry*. This picture show isolated rectal cells and part of the rectum. Thess cells can be isolated afterwards by cell sorting to extract and determine their transcriptome.

All together these data will allow us to understand, at the transcriptomic level, what are the differences between Y and its neighboring rectal cells, and what are the elements that give to Y this so specific features.

# ANNEXES

# I) Essay on the induced transdifferentiation

## La transdifférenciation induite : nouvel Eldorado de la médecine régénérative ?

Auteurs : Thomas DANIELE et Sophie JARRIAULT

**Résumé en français** : Il était classiquement considéré que le destin différencié d'une cellule était figé, cependant de nombreuses études récentes ont pu démontrer qu'il est possible de changer l'identité d'une cellule somatique en l'exposant à des conditions adéquates. Ainsi, il semble possible de convertir des cellules somatiques différenciées en cellules pluripotentes induites (cellules iPS) ou en un autre type cellulaire différencié (par transdifférenciation induite). Ces données suggèrent que toutes cellules différenciées possèderaient un potentiel de reprogrammation latent pouvant être activé sous certaines conditions. L'exploitation de cette plasticité cellulaire serait alors une nouvelle solution pour la médecine régénérative permettant de s'affranchir des greffes ou de l'utilisation de cellules souches embryonnaires posant tout deux, divers problèmes techniques et éthiques. Dans cette revue nous discutons en quoi les cellules iPS ne peuvent pas encore avoir d'utilisation clinique et pourquoi la transdifférenciation induite semble être une solution plus sûre pour la médecine de demain.

### La notion de plasticité cellulaire enfin appréciée à sa juste valeur

De manière générale, chez les organismes multicellulaires, la rencontre entre un gamète mâle et femelle donne lieu à la formation d'un zygote pluripotent qui a la capacité de se différencier en tout type cellulaire constituant un organisme entier. Au cours du développement, le zygote se divise et donne naissance à des cellules dont le potentiel de différenciation se réduit de plus en plus jusqu'à donner une cellule totalement spécialisée et dédiée à une fonction très spécifique dans l'organisme qu'elle compose. Historiquement, il était considéré que la différenciation

cellulaire était issue d'une perte irréversible d'information génétique permettant à la cellule de s'engager dans un processus développemental précis. Cette théorie a tenu bon jusqu'aux années 50 où Robert Briggs, Thomas King suivis ensuite par John Gurdon, montrèrent que la transplantation d'un noyau de cellule somatique spécialisée de grenouille transféré dans un ovocyte énuclé donne naissance à une nouvelle grenouille totalement saine (Briggs & King, 1952), (Gurdon, 1962). Ces expériences ont montré pour la première fois que tous les gènes requis pour la création d'un organisme entier étaient contenus dans le noyau d'une cellule spécialisée et qu'ils pouvaient être réactivés à tout moment après exposition à certains facteurs présents dans l'ovocyte. Ces expériences de reprogrammation nucléaire démontrent que la spécialisation cellulaire est issue d'un changement dans l'expression des gènes et non dans le contenu du matériel génétique ce qui laisse suggérer que la différenciation cellulaire est un phénomène biologique réversible. Malgré l'importance de ces découvertes, ce n'est que plusieurs décennies plus tard que certaines équipes de recherche se sont réellement penchées sur la plasticité cellulaire. En 1987, il a pu être démontré que l'expression forcée du gène *myoD* (un régulateur clé de la myogenèse), dans des fibroblastes, suffisait pour convertir ces derniers en cellules musculaires (Davis, Weintraub, & Lassar, 1987). C'est à partir de ces travaux que Shinya Yamanaka a établi sa stratégie pour transformer des cellules somatiques en cellules pluripotentes (Takahashi & Yamanaka, 2006). Ces travaux démontrent que l'expression forcée de 4 facteurs de transcription (Sox-2, Oct-3/4, Klf4 et c-Myc) suffisent à convertir des fibroblastes en cellules pluripotentes induites (iPS). Cette découverte fut l'effet d'une bombe dans la communauté scientifique en démontrant qu'il serait possible de rendre pluripotente n'importe quelle cellule somatique de notre organisme et que par conséquent celles-ci possèderaient un potentiel de reprogrammation latent pouvant être activé sous l'effet de certains facteurs. En plus de révolutionner la vision classique que l'on avait de l'aspect plus ou moins figé de la différenciation cellulaire, ces travaux ont ouvert de nouvelles portes pour la médecine régénérative. En effet, un des grands défis de la médecine moderne est de pouvoir remplacer tout organe, tissu ou cellules lésés d'un organisme malade. Or, à l'heure actuelle la seule alternative à de telles lésions est la greffe d'organe, qui présentent bons nombres

d'inconvénients tels que la faible disponibilité des greffons ainsi que le rejet possible de ces derniers, ou l'utilisation de cellules souches embryonnaire (ES), qui possèdent la capacité de donner naissance à tout type cellulaire mais qui posent des problèmes éthiques quant à l'utilisation d'embryon humain pour leur obtention. Cette nouvelle technique de reprogrammation cellulaire permettrait ainsi de créer des lignées cellulaires pluripotentes patient-spécifiques qui ne subiraient aucun rejet et permettrait de s'affranchir de l'utilisation d'embryon. La différenciation de ces cellules pourrait alors être dirigée permettant ainsi d'avoir une population cellulaire spécialisée qui pourra remplacer celles du corps malade.

**La face cachée des cellules pluripotentes induites**

Cela fait maintenant 5 ans que les cellules IPS ont vu le jour, depuis bon nombre de travaux ont été réalisés pour comprendre les mécanismes sous-tendant à la reprogrammation cellulaire mais une grande partie des efforts s'est concentrée vers la création de nouvelle stratégie de reprogrammation. En effet les premières cellules iPS ont été obtenues par surexpression de quatre facteurs de transcription via l'utilisation de rétrovirus. Cette méthode présente plusieurs inconvénients rendant les cellules pluripotentes obtenues inapplicables pour une utilisation clinique : parmi les facteurs utilisés, l'un d'entre eux, c-Myc, a clairement été établi comme étant très fortement oncogénique, de plus les 3 autres facteurs, sox-2, oct-3/4 et klf-4, sont fortement exprimés dans différents type de cancers (Schoenhals et al., 2009), les vecteurs rétroviraux quant à eux permettent l'intégration aléatoire des facteurs utilisés au sein même du génome, pouvant conduire à l'invalidation de gène essentiel à la stabilité des cellules tels que des gènes suppresseurs de tumeurs. Il est également possible que les facteurs intégrés puissent se réactiver après différenciation des cellules iPS rendant les cellules obtenues potentiellement tumorales. Depuis, il est possible de reprogrammer des cellules somatiques en utilisant des vecteurs adénoviraux (qui ne s'intègrent pas au sein du génome), en transfectant des ARNs messager modifiés ou en faisant pénétrer directement les protéines SOX-2, OCT-3/4, KLF-4, et c-MYC dans la cellule à reprogrammer (Gonzalez, Boue, & Izpisua Belmonte, 2011). Grâce à ces nouvelles techniques, il est

alors possible d'avoir une expression transitoire des facteurs reprogrammant, permettant ainsi la création de cellules iPS plus sûres.

Sommes-nous alors proches de l'application clinique de ces cellules ? La réponse est malheureusement non… L'intégrité génomique des cellules iPS était communément vérifiée via l'analyse du caryotype de ces dernières qui ne montrait en général aucun défaut. Cependant de nouvelles études utilisant des méthodes beaucoup plus précises tel que l'hybridation génomique comparative, l'utilisation de puces à polymorphisme nucléotidique et plus récemment par séquençage à haut débit (Mayshar et al., 2010) (Laurent et al., 2011) (Hussein et al., 2011; Gore et al., 2011) ont pu démontrer que les cellules iPS possèdent un nombre assez conséquent d'aberrations génétiques (incluant aneuploïdies, variation du nombre de copie de gène et mutation ponctuelles) **(Figure 1)**. Ces études ont été réalisés sur un grand nombre de souches différentes de cellules iPS, obtenues par différentes méthodes (intégratives et non intégratives) et comparées directement aux cellules somatiques dont elles sont issues. Les chercheurs s'accordent sur le fait que ces aberrations apparaissent à différentes phases de la reprogrammation. Pendant les phases précoces, des aberrations apparaitraient suite au stress réplicatif que subiraient les cellules en reprogrammation. En effet il a été démontré que lorsque l'activité du gardien du génome, la protéine p53, est invalidée, l'efficacité de la reprogrammation augmenterait, démontrant ainsi que l'acquisition d'aberrations chromosomiques faciliterait l'obtention de cellules iPS (Ben-David & Benvenisty, 2011). Cependant ces aberrations précoces ne semblent pas être maintenues lorsque la culture des cellules iPS obtenues est prolongée. Les défauts les plus communs semblent être issus d'une adaptation aux conditions de cultures. Ces aberrations sont représentées pour leur majorité par une duplication de certaines régions des chromosomes 12 et 20 (comportant des gènes impliqués dans la pluripotence tel que NANOG et démontrés comme surexprimés dans certain type de cancer). Les mutations ponctuelles semblent quant à elles assez différentes d'une souche à l'autre de cellules iPS mais généralement concentrées dans des gènes impliqués dans la régulation du cycle cellulaire. (Gore et al., 2011).

Un des grands avantages de l'utilisation de cellules iPS était qu'elles ne seraient pas immunogènes car issues directement du patient à traiter. Or une étude

récente vient contredire cet argument de poids et éloigne un peu plus les cellule iPS du monde clinique (Zhao, Zhang, Rong, & Xu, 2011). En générale lorsque des cellules ES autologues sont injectés dans des souris, celles-ci forment des tératomes démontrant la tolérance du système immunitaire vis-à-vis de ces cellules. À l'opposé lorsque des cellules allogéniques sont injectées, ces dernières sont rejetées par l'organisme hôte. Dans ces travaux récents, les auteurs ont pu démontrer que les tératomes issus de cellules iPS syngéniques induisent une réponse immunitaire conduisant à la destruction partielle des cellules injectées. L'origine de ce rejet serait la surexpression de certains gènes tel que *hormad1* (connu pour être un antigène tumoral) ou *spt1* (un antigène tissu spécifique) responsable de l'activation d'une réaction immunitaire spécifique des cellules T. Cette réaction immunitaire apparaît majoritairement lorsque la reprogrammation utilise des vecteurs intégratifs. A l'opposé, les transgènes ne s'intégrant pas ou pouvant être excisés semblent induire une réponse immunitaire beaucoup moins importante. Néanmoins ces travaux démontrent le danger potentiel de l'utilisation des cellules iPS mais démontrent également que les efforts portés vers les nouvelles technologies de reprogrammation semblent porter leurs fruits, les cellules obtenues avec des transgènes non-intégratifs semblent plus sûres.

Même si, pour le moment, les cellules iPS ne peuvent pas être utilisées en thérapie cellulaire, elles représentent néanmoins un formidable outil pour comprendre les mécanismes cellulaires sous-tendant la reprogrammation, mais également pour la création de modèle cellulaire mimant certaines pathologies (Wu & Hochedlinger, 2011). Ces modèles cellulaires sont utilisés après différenciation pour comprendre les mécanismes physiopathologiques de maladies rares mais également pour le crible de nouvelles molécules chimiques qui pourraient traiter ces pathologies. Il est donc évident que les recherches sur les cellules iPS doivent s'intensifier, à la fois pour trouver de nouvelles méthodes de reprogrammation qui permettrons d'obtenir des cellules plus « sures » applicables en thérapie cellulaire, mais également pour comprendre comment une cellule spécialisée, peut, par la simple surexpression de facteurs spécifiques, devenir une cellule pluripotente.

**La transdifférenciation induite : une solution plus sûre ?**

Avant les travaux de l'équipe Shinya Yamanaka, il avait déjà été montré que la reprogrammation cellulaire pouvait avoir lieu après l'expression de facteurs spécifiques. À la fin des années 80, des travaux ont pu montrer qu'il était possible de reprogrammer des fibroblastes en cellules musculaires par la simple surexpression du facteur myogénique, MYOD (Davis et al., 1987). Plus tard, l'équipe de Thomas Graf a pu montrer qu'il était possible de convertir des lymphocytes B en macrophages par la simple surexpression du facteur C/EBPα impliqué dans la mise en place de ces derniers (Xie, Ye, Feng, & Graf, 2004) (Une reprogrammation similaire a pu être réalisée en surexprimant C/EBPα/β et le facteur PU.1 dans des fibroblastes (Feng et al., 2008)). Ces travaux de reprogrammation (ou transdifférenciation) induite démontrèrent, bien avant les travaux de Yamanaka, que les cellules somatiques possèdent une certaine plasticité leur permettant d'être reprogrammer sous certaines conditions. Partant de l'hypothèse que des cellules somatiques pouvaient être reprogrammer par surexpression d'un cocktail précis de facteurs, plusieurs équipes de recherche ont pu démontrer que des fibroblastes peuvent être transdifférenciés en neurones, cardiomyocytes, progéniteurs sanguins ou encore en hépatocytes. Les stratégies de transdifférenciation induite sont presque toutes élaborées sur le même modèle. Tout d'abord il est indispensable de mettre en évidence le cocktail de facteur minimum requis à la reprogrammation cellulaire, ensuite les cellules obtenues doivent être caractérisées en détail afin de s'assurer de la totale reprogrammation des cellules nouvellement obtenues. Dans certains cas, le bon fonctionnement de ces cellules est testé en système *in vitro*, mais également en système in vivo afin de s'assurer de la totale fonctionnalité des cellules nouvellement obtenues.

Récemment, des fibroblastes ont pu directement être transdifférenciés en une population hétérogène de neurones (neurones GABAergiques, Glutmatergiques…) (Vierbuchen et al., 2010) **(Figure 2A)**. Les auteurs sont partis d'une liste de 19 gènes impliqués dans le développement neuronal, parmi eux Ascl1, Brn2 et Mytl1 semble indispensable pour la reprogrammation des fibroblastes. Les neurones ainsi obtenus expriment bon nombre de protéines neurone-spécifiques et semblent former des synapses fonctionnelles. Peu de temps après, deux équipes

ont démontré que l'expression forcée de Ascl1, Nurr1 et Lmx1a induit la transdifférenciation de fibroblastes en neurones dopaminergiques (Caiazzo et al., 2011) (Pfisterer et al., 2011) **(Figure 2B)**. Les neurones nouvellement obtenus présentent une activité électrique spontanée, synthétisent de la dopamine et semblent n'exprimer que le programme génétique de la machinerie dopaminergique (aucune expression de programme adrénergique ou sérotoninergique détectée), démontrant une claire transdifférenciation des fibroblastes. De la même manière, des fibroblastes ont pu être reprogrammés en cellules cardiaques (Ieda et al., 2010) **(Figure 2C)**, 14 facteurs impliqués dans le développement cardiaque ont été criblés pour identifier le meilleur cocktail reprogrammant. L'expression de 3 facteurs, Gata4, Mef2c et Tbx5 sont suffisants pour transformer des fibroblastes en cardiomyocytes ayant une organisation sarcomérique, exprimant des protéines spécifiques des cellules cardiaques ainsi qu'une activité contractile et électrique spontanée. Grâce à un suivi rigoureux du devenir des cellules reprogrammées, les auteurs ont pu mettre en évidence que cette transdifférenciation induite n'impliquait pas d'intermédiaire pluripotent, mais que le passage de fibroblaste à cardiomyocyte se faisait de manière directe. Il est assez surprenant de voir que l'efficacité de reprogrammation avec les 3 facteurs utilisés est nettement supérieure comparée à l'efficacité d'obtention de cellule iPS (20% et <0,1% respectivement) démontrant que de tel stratégie pourrait présenter un intérêt important en médecine régénérative. Ce sont ensuite des progéniteurs sanguins qui ont pu être obtenus suite à la reprogrammation de fibroblastes (Szabo et al., 2010) **(Figure 2D)**, cependant dans ces travaux la stratégie utilisée diffère totalement des travaux décrits précédemment. La surexpression du simple facteur OCT-4 et l'exposition aux cytokine SCF (stem-cell factor) et FLTL3G (FMS-like tyrosine kinase 3 ligang) suffisent à reprogrammer des fibroblastes en progéniteurs sanguin caractérisés par l'expression des marqueurs membranaires CD45 et CD34. Ces progéniteurs une fois exposés à un cocktail de cytokines spécifiques sont capables de se différencier en cellules de la lignée érythroïde et myéloïde (à l'opposé aucune cellule de la lignée lymphoïde n'a pu être obtenue). De manière tout à fait surprenante la surexpression de OCT-4 semble être une des clés de cette transdifférenciation induite alors que ce gène n'a aucun rôle dans le développement sanguin (aucun éléments de l'hématopoïèse ne

requiert OCT-4), néanmoins OCT-4 serait capable de se fixer et d'activer des gènes spécifiquement impliqué dans l'hématopoïèse rendant ainsi possible la reprogrammation des fibroblastes. Les exemples présentés ci-dessus, laisse suggérer qu'il est possible de reprogrammer des fibroblastes en tout type de cellules, si les bons facteurs ou les bonnes conditions sont utilisés. Deux équipes de recherches confirment ces suggestions en rendant possible la transdifférenciation de fibroblastes en hépatocytes (Huang et al., 2011) (Sekiya & Suzuki, 2011) **(Figure 2E)**. Un ensemble de 14 facteurs ont été testés et plusieurs d'entre eux semble être la clé d'une possible transdifférenciation. Hnf1α associé à Foxa1, Foxa2 ou Foxa3 et Gata4 semble être suffisant pour convertir des fibroblastes en hépatocytes. Ces cellules nouvellement obtenues expriment des facteurs de transcription ainsi que des protéines hépatiques sécrétées, de plus ces cellules semblent posséder une réelle activité métabolique *in vitro* (capacité à métaboliser la testostérone par exemple). Afin de vérifier que ces hépatocytes induits sont totalement fonctionnels, les auteurs ont testé le potentiel de ces cellules en milieu physiologique. Des souris mutantes pour la fumarylacetoacetate hydrolase présentent une défaillance hépatique qui conduit à la mort de l'animal. Lorsque des hépatocytes induits non mutant sont injectés dans des foies malades, les souris survivent dans 50% des cas. Les cellules injectées semblent coloniser le foie malade et restore la fonction de l'organe après arrêt de leur prolifération. En plus de restaurer les fonctions hépatiques de l'animal, ces hépatocytes induits semblent également présenter les mêmes capacités de régénération que possède les hépatocytes normaux indiquant ainsi que la reprogrammation induite ici est totale.

La transdifférenciation induite peut également être réalisée *in situ*, en effet de célèbre travaux ont démontrés que des cellules pancréatiques exocrines peuvent être reprogrammées en cellules endocrines β par la surexpression de 3 facteurs, Ngn3, Pdx1 et Mafa directement au sein de pancréas de souris (Zhou, Brown, Kanarek, Rajagopal, & Melton, 2008) **(Figure 2F)**. Les cellules endocrines induites expriment les bons marqueurs et sont capables de restaurer la glycémie de modèles murins de diabète de type 1.

En résumé tous ces travaux laissent penser que les cellules somatiques peuvent être reprogrammées sous certaines conditions. La grande difficulté de ce

type d 'expérience est de trouver la bonne combinaison de facteurs permettant d'induire cette transdifférenciation. Pour cela la biologie du développement à un rôle capital dans la mise en évidence de ces facteurs clé impliqués dans la mise en place des tissus et organes mais également dans la compréhension des mécanismes moléculaires et cellulaires sous-tendant la transdifférenciation.

D'un point de vue clinique, la transdifférenciation induite apporte bon nombre d'avantage par rapport aux cellules iPS. En effet tous les événements de transdifférenciation décrits ici, ne semblent pas passer par un intermédiaire pluripotent permettant ainsi de s'affranchir du risque de formation de tumeurs et de tératomes pouvant exister avec les cellules iPS. De plus l 'efficacité de reprogrammation semble être très nettement supérieure dans les cas de transdifférenciation induite, suggérant que le facteur limitant ici semble être la disponibilité des fibroblastes à reprogrammer (facilement cultivable, mais il est indispensable de vérifier que la culture prolongée de ces cellules n'induise pas d'aberrations génétiques). Cependant, malgré tous ces résultats prometteurs, les mécanismes en jeu dans la transdifférenciation induite reste encore très mystérieux rendant la route vers l'application clinique de cette méthode encore longue et sûrement très sinueuse.

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**Légendes Figures :**

**Figure 1 :**

Sources, origines et type d'aberrations génétiques observées dans les cellules iPS. Ces aberrations peuvent provenir des cellules reprogrammées originellement mutantes, apparaître durant la reprogrammation ou lors de l'adaptation aux conditions de cultures des cellules iPS obtenues. Ces aberrations peuvent être des aneuploïdies, des mutations ponctuelles ou des variations dans le nombre de copie de certains gènes. Le nombre de ces dernières semble croître pendant la reprogrammation, mais étant délétères, leur nombre réduit par la suite.

**Figure 2 :**

Exemples de transdifférenciation induite, des fibroblastes en culture ont pu être convertis en neurones grâce aux facteurs Ascl1, Brn2, Mytl1, en neurones dopaminergiques avec les facteurs Ascl1, Nurr1, Lmx1a, en cardiomyocytes avec les facteurs Gata4, Mef2c, Tbx5, en progéniteurs sanguins par la surexpression de OCT-4 et l'exposition aux cytokines FTL3G, SCF, Ces progéniteurs sanguins ont pu être différenciés en cellules de la lignée erythroïdes et myéloïde. Des fibroblastes ont pu être transdifférenciés en hépatocyte par surexpression de Hif1α, Foxa1, 2 ou 3 (et Gata4). Des cellules pancréatiques exocrines ont pu être directement converties en cellules endocrines B en système *in vivo.*

**Figure 1**

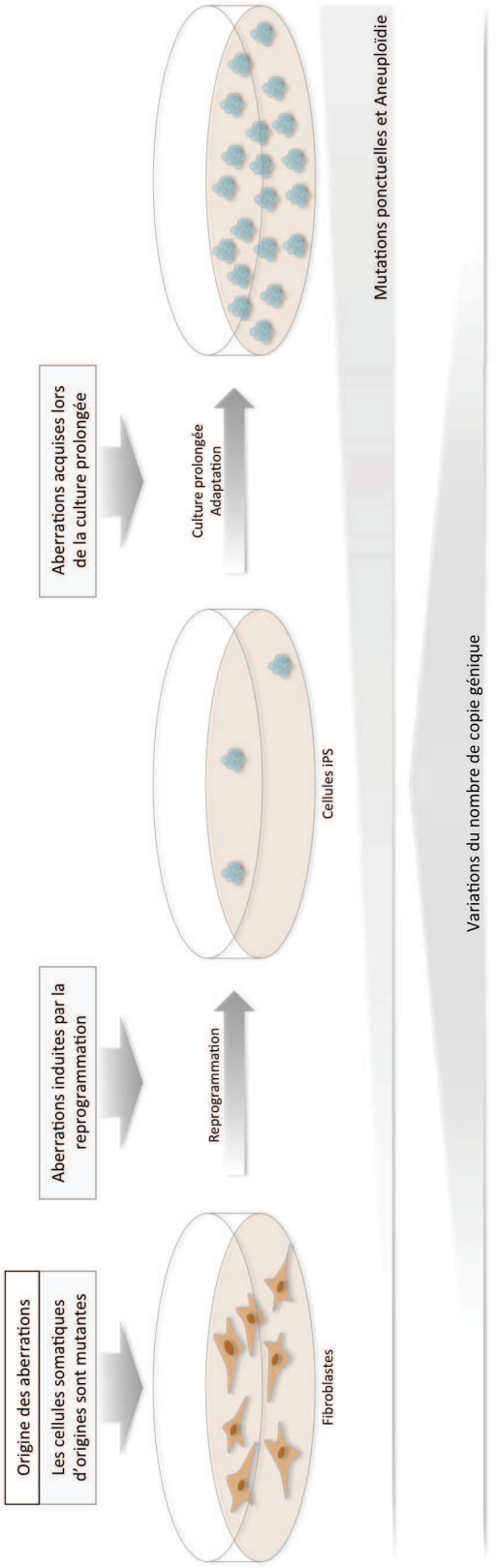
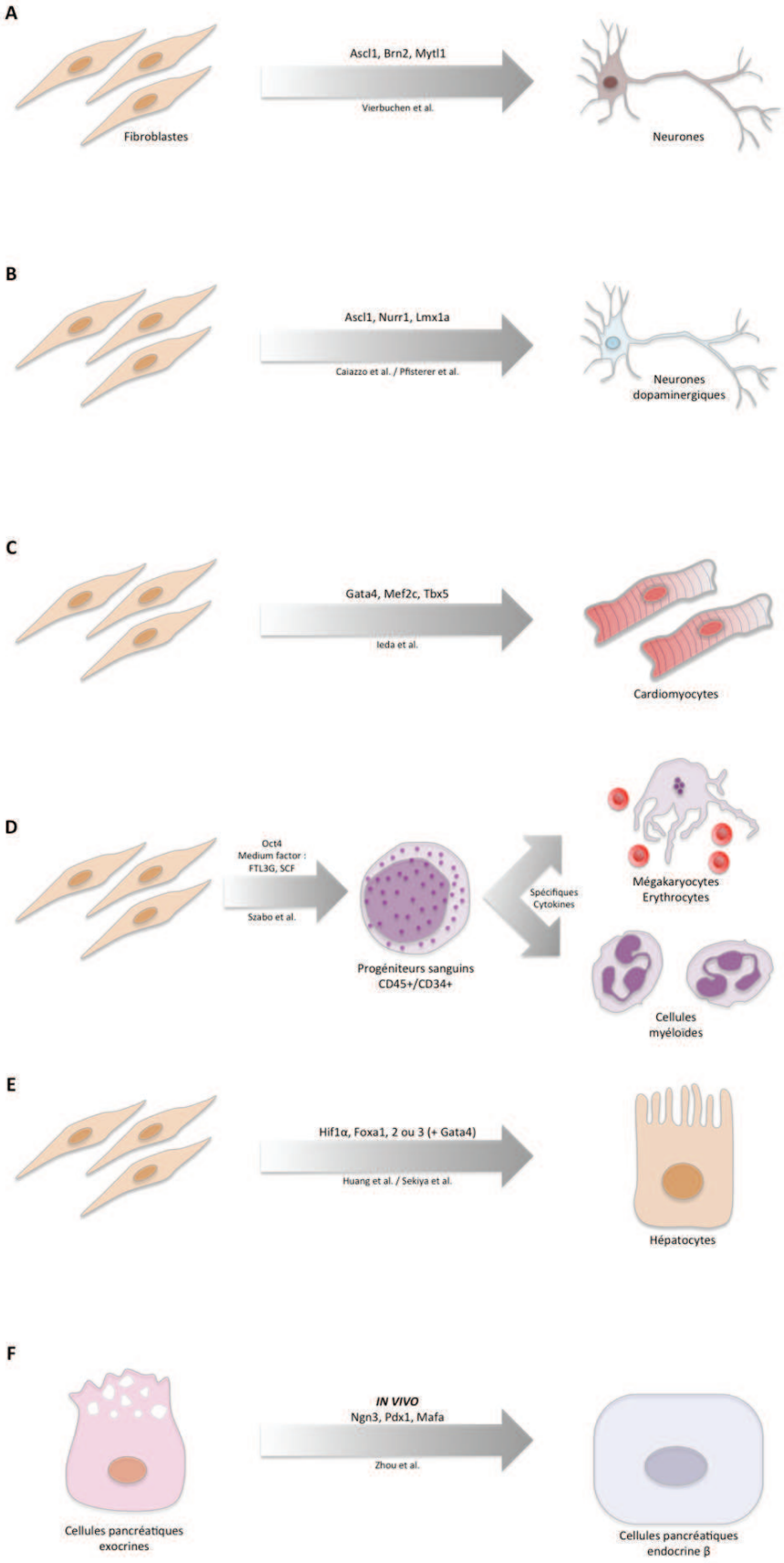

Origine des aberrations
Les cellules somatiques d'origines sont mutantes
Fibroblastes
Aberrations induites par la reprogrammation
Reprogrammation
Cellules iPS
Aberrations acquises lors de la culture prolongée
Culture prolongée
Adaptation
Mutations ponctuelles et Aneuploïdie
Variations du nombre de copie génique

**Figure 2**


A
Fibroblastes
Ascl1, Brn2, Mytl1
Vierbuchen et al.
Neurones
B
Ascl1, Nurr1, Lmx1a
Caiazzo et al. / Pfisterer et al.
Neurones dopaminergiques
C
Gata4, Mef2c, Tbx5
Ieda et al.
Cardiomyocytes
D
Oct4
Medium factor : FTL3G, SCF
Szabo et al.
Progéniteurs sanguins CD45+/CD34+
Spécifiques Cytokines
Mégakaryocytes Erythrocytes
Cellules myéloïdes
E
Hif1α, Foxa1, 2 ou 3 (+ Gata4)
Huang et al. / Sekiya et al.
Hépatocytes
F
IN VIVO
Ngn3, Pdx1, Mafa
Zhou et al.
Cellules pancréatiques exocrines
Cellules pancréatiques endocrine β

# II) Participation in the writing of two reviews

**1: Hajduskova M, Ahier A, Daniele T, Jarriault S.**

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**2: Zuryn S, Daniele T, Jarriault S.**

Direct cellular reprogramming in Caenorhabditis elegans: facts, models, and promises for regenerative medicine. Wiley Interdiscip Rev Dev Biol. 2012 Jan-Feb;1(1):138-52. doi: 10.1002/wdev.7. Epub 2011 Nov 17. PubMed PMID: 23801672.

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REVIEW

# Cell Plasticity in *Caenorhabditis elegans*: From Induced to Natural Cell Reprogramming

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**Summary: Achieving controlled reprogramming of differentiated cells into a desired cell type would open new opportunities in stem-cell biology and regenerative medicine. Experimentation on cell reprogramming requires a model in which cell conversion can be induced and tracked individually. The tiny nematode, *Caenorhabditis elegans*, owing to its known cellular lineage, allows the study of direct cell type conversion with a single-cell resolution. Indeed, recent advances have shown that despite its invariant cell lineage, cellular identities can be reprogrammed, leading to cell conversion in vivo. In addition, natural transdifferentiation events occur in the worm, providing a powerful model for the study of cellular plasticity in a physiological cellular microenvironment. Here, we review pioneer studies on induced and naturally occurring reprogramming events in *C. elegans* and the new notions that have emerged. genesis 00:1–17, 2011. © 2011 Wiley-Liss, Inc.**

**Key words:** transdifferentiation; direct reprogramming; epigenetics; germ line; direct cell type conversion; *C. elegans*

## HISTORY OF CELL REPROGRAMMING

During metazoan development, oocyte fertilization gives rise to the totipotent zygote that has the capacity to form an entire organism. The first series of zygotic divisions leads to a gradual loss of totipotency and to the formation of pluripotent blastomeres. In the course of development, the cellular capacity to assume diverse cell fates is restricted, and cells become specified for a particular cell type. Initially, the differentiated identity was considered to represent a complete loss of cellular potential and believed to be irreversible. However, during the last three decades, work from many laboratories has shown that cells committed to specific cell fates, and even fully differentiated cells, can still change their identity naturally or upon artificial induction. These findings have revolutionized the traditional view and spurred an increasing number of studies on the mechanisms underlying cell identity changes. The cellular reprogramming of differentiated cells can lead to cell dedifferentiation or to direct cell type conversion (a.k.a. transdifferentiation or direct reprogramming).

The first evidence indicating that nuclear reprogramming of somatic cells could be achieved was established via nuclear transfer in frogs (Gurdon, 1962a,b; Gurdon *et al*., 1958). The nucleus from a fully differentiated cell was able to initiate the entire developmental program and produce a viable and fertile frog when transplanted into an enucleated oocyte. The transplanted nucleus reverted to an embryonic state and became totipotent. These experiments proved that the genetic instruction essential for development persists in nuclei of fully differentiated cells. It further suggested that domains in the genome are silenced in specialized cells and that these can be re-activated by factors present in the oocyte cytoplasm. Similar nuclear transfer experiments were conducted to derive a live lamb from differentiated fetal and adult cells (Wilmut *et al*., 1997), demon-

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strating that dedifferentiation is not a peculiarity of amphibians.

Alternatively, partial or complete dedifferentiation can be achieved by forced fusion of two cells in distinct differentiation states (Gurdon and Melton, 2008; Tada *et al*., 1997). Such fusions either give rise to a nonproliferative heterokaryon or to a proliferative hybrid cell, in which the more specialized nucleus often adopts the fate of the less differentiated one. For instance, fusion with embryonic stem (ES) cells has been successfully used to reprogram somatic cells toward pluripotency, indicating that factors present in the pluripotent ES cells are also sufficient to revert somatic cells to a more potent state (Cowan *et al*., 2005). These cell fusion experiments further showed that cell identity is not irreversibly locked but rather that, under certain circumstances, it can be reprogrammed.

What exactly are the factors that confer toti- or pluripotency to cells? Pioneer work on fibroblast to myocyte-like direct cell type conversion pointed to transcriptional modulators (Davis *et al*., 1987, 1990). Indeed, the master regulatory gene MyoD, a transcription factor required for myogenesis during development, was sufficient to induce the myogenic program in differentiated cells when ectopically expressed. Yamanaka and colleagues reasoned that if cytoplasmic factors present in oocytes or ES cells can trigger dedifferentiation of somatic cells and genetically re-set the nucleus, and if single transcription factors, such as MyoD, suffice for fibroblasts to adopt muscle features, one should be able to reprogram somatic cells back to a pluripotent state using specific proteins. They achieved this with a small number of ES cell-specific transcription factors. By systematically combining subsets of 24 transcription factors implicated in ES cell pluripotency, Yamanaka and colleagues defined a cocktail of four, Sox-2, Oct-3/4, Klf-4, and c-Myc, capable of bestowing pluripotency in human or murine somatic cells, which were henceforth dubbed the induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Takahashi *et al*., 2007; Wernig *et al*., 2007). A similar cocktail with Nanog and Lin-28 replacing Klf-4 and c-Myc has been shown to induce human iPS cells (Yu *et al*., 2007). iPS cells exhibit morphology and molecular characteristics similar to ES cells and can give rise to derivatives of all three germ layers, demonstrating restoration of pluripotency. This remarkable discovery has sparked a boom in the research of cellular reprogramming and stem-cell biology, making conversion of most if not all cell types into iPS cells feasible.

Remarkably, in addition to transcription factor-induced reprogramming, a recent study demonstrates that a mixture of four small molecules can substitute for three crucial transcription factors, *Sox2*, *Klf4*, and *c-Myc*, in reprogramming mouse fibroblasts into iPS cells (Li *et al*., 2011). Valproic acid, a GSK-3β inhibitor CHIR99021, a histone H3 K4 demethylation inhibitor tranylcypromine, and a TGF-β inhibitor 616452 all combined with a single transcription factor *Oct4* were sufficient to trigger fibroblast reprogramming (Li *et al*., 2011). Similarly, an inhibitor of the MAPK pathway PD0325901 has been shown to promote dedifferentiation of the neural stem cells to iPS cells in mice, thus making the transcription factor *Sox2* dispensable for this process (Silva *et al*., 2008). Such findings pave the way for chemical approaches to cellular therapies enabling the identification of chemical compounds that can induce specific target cells to differentiate or undergo cell fate reprogramming in vivo. Chemical strategies offer several advantages including instant regulation of protein activity, fine-tuning of specific effects by adjusting the concentration of the chemical, and often regulation of more than one target within a protein family (Xu *et al*., 2008).

Besides inducing cell dedifferentiation, an increasing number of studies have focused on the challenging task of directly reprogramming one somatic cell type into another. The work of Weintraub and colleagues suggested that ectopic expression of tissue-specific master regulatory genes involved in cell fate differentiation or specification during development could be used to initiate ectopic tissue-specific transcription programs (Davis *et al*., 1990). Lately, these studies, combined with the recent findings from the Yamanaka team, have inspired others to use combinations of developmental determinants for direct cell type conversion. For example, mature B cells were reprogrammed into macrophage-like cells via overexpression of only two transcription factors, C/EBPα and PU.1 (Xie *et al*., 2004). Functional neuron-like cells (Vierbuchen *et al*., 2010) and even cardiac myocytic cells were also obtained in vitro via reprogramming of fibroblasts by a defined cocktail of developmental factors (Ieda *et al*., 2010). The first example of induced direct cell type conversion achieved in vivo was the reprogramming of murine pancreatic exocrine cells into endocrine β-cells in mice in situ by overexpression of Ngn3, Pdx1, and MafA1 (Zhou *et al*., 2008). These studies provided further evidence that master regulatory genes can impose a new developmental program on fully differentiated cells, resulting in a switch of their identity even in a complex tissue context in vivo. It should be noted that modulation of signaling pathways can also result in cellular reprogramming: the conversion of exocrine acinar cells into the ductal cells was observed in response to ectopic EGFR signaling, for instance (Means *et al*., 2005).

Interestingly, some cells possess the inherent capability to naturally transdifferentiate under certain circumstances such as regeneration processes in amphibians. Lens and retina regeneration after removal or damage in newts and frogs occurs solely by transdifferentiation of pigment cells, and this process requires a dedifferentia-

tion step (Del Rio-Tsonis and Tsonis, 2003). The regeneration capacity of lens and/or retina has been shown in other vertebrates including fish and birds and probably also occurs in mammals. However, it is not clear in all cases whether transdifferentiation is an exclusive mechanism to achieve organ regeneration (Del Rio-Tsonis and Tsonis, 2003). Another example of natural cell capacity to transdifferentiate is the occurrence of hepatocytes in rat pancreas upon feeding on a copper-deficient diet (Rao *et al*., 1986). These ectopic hepatocytes were later shown to result from the direct conversion of pancreatic cells (Thowfeequ *et al*., 2007).

Remarkably, some direct cell reprogramming events take place spontaneously under physiological conditions, suggesting that cells have an inherent flexibility. In invertebrates, described examples include photoreceptor conversion in *Drosophila* (Sprecher and Desplan, 2008) and the cellular switch of a rectal cell into a motor neuron in *Caenorhabditis elegans* (Jarriault *et al*., 2008). In mammals, natural transdifferentiation is exemplified by the formation of coronary arteries from venous cells (Red-Horse *et al*., 2010) and by neurons generated from astrocytes during adult neurogenesis (Doetsch, 2003).

Although it has become clear that somatic cells have a latent potential to change their identity, how and when this potential can be revealed is still poorly understood. The understanding of the mechanisms at work will answer fundamental questions about cell identity and plasticity. In vivo studies at the single cell level will be particularly important to understand how a defined cellular identity is maintained or can be compromised and changed.

In this review, we focus on the use of *Caenorhabditis elegans* as an emerging powerful model for the study of cell plasticity and reprogramming both via candidate gene and unbiased approaches.

## *Caenorhabditis elegans*, A MODEL FOR CELL PLASTICITY AND REPROGRAMMING

A promising strategy for cellular therapy is to trigger replacement of the diseased/damaged cells with alternative cells from the same patient, directly in situ (Xu *et al*., 2008). However, the complexity of multicellular organisms limits most studies to experimenting in vitro, in which case cell populations are used. Reprogrammed cells can be selected for, but a major limitation is that it remains difficult to predict which individual cells will undergo reprogramming and therefore to follow the early steps of the process. Since Sydney Brenner established the nematode *Caenorhabditis elegans* as a genetic model organism, the worm has proven a powerful model for cellular and developmental biology (Brenner, 1974). The small, transparent body of the worm and the invariant number and fate of its somatic cells allowed the determination of all cell lineages from the zygote to the adult stage (Sulston and Horvitz, 1977; Sulston *et al*., 1983). Because of its essentially invariant lineage, *C. elegans* is an outstanding model for such in vivo studies as it allows unambiguous traceability of unique cell reprogramming events. Owing to the availability of a broad range of transgenic cell markers in *C. elegans* (http://www.wormbase.org/db/gene/transgene_tbls), the identity of a cell can be established and subsequently tracked during its cell fate reprogramming in living worms. Thus, reprogramming events can be studied at a single cell resolution, yet in a complex tissue context. These observations open an innovative branch of *C. elegans* research highly relevant to stem-cell biology and regenerative medicine. In the following chapters, we review the pioneering studies on cellular reprogramming events in *C. elegans* (Table 1) and highlight how these have contributed to our understanding of the process.

## INDUCED REPROGRAMMING

The aim of regenerative medicine is to reconstruct damaged or diseased tissues. A future direction is to produce replacement cells directly from patient tissues. One way to achieve this is to stimulate the differentiation or the transdifferentiation of resident cells in their natural niches to a desired cell type (Xu *et al*., 2008). *C. elegans* enables one to study the effects of induced reprogramming within the context of a living organism. Both chemical compounds and forced expression of defined factors have been used successfully to direct cell type conversions in vivo in *C. elegans*.

### Chemical Reprogramming in *C. elegans*

In *C. elegans*, MAPK pathway inhibitors have been used to reprogram cell fate of the germline (Morgan *et al*., 2010). The hermaphrodite worm gonad produces both oocytes and sperm. Genetic screens for regulators of the oocyte versus sperm germ-cell fate decision yielded mutants with either masculinized (producing only sperm) or feminized (only oocytes) germlines (Ellis and Schedl, 2006; Kimble and Crittenden, 2007). Newly characterized double mutants of *lip-1* (MAPK phosphatase) and *puf-8* (Pumilio/FBF-RNA-binding protein) have masculinized germlines and exhibit MAPK pathway hyperactivity (Morgan *et al*., 2010). Functional oocytes have been chemically induced in the masculinized gonad of *lip-1; puf-8* double mutants (Morgan *et al*., 2010; Fig. 1). Morgan and colleagues achieved this chemical reprogramming with compounds that had been identified as specific inhibitors of the MEK1/2 kinases (Alessi *et al*., 1995; Barrett *et al*., 2008; Fig. 1). Each of the three small-molecules, U0126, PD0325901, and PD098059, was alone sufficient to induce oocyte formation by downregulating Ras/ERK signaling

**Table 1**
*Induced and Natural Reprogramming Events in C. elegans*

| Type of reprogramming | Inducing factor(s) | Cell type/tissue reprogrammed | Induced cell type | Other players implicated in the process | Citation |
|---|---|---|---|---|---|
| Induced, chemical | U0126<br>PD98059<br>PD0325901 | Masculinized gonad in *lip-1;puf-8* double mutants | Oocytes | MAPK signaling | Morgan *et al*., 2010 |
| Induced, ectopic expression of CFDG | *end-1* | Blastomeres | intestinal cells | ? | Zhu *et al*., 1998 |
| Induced, ectopic expression of CFDG | *med-1, med-2* | Blastomeres | mesendoderm | *end-1*, *end-3* | Maduro *et al*., 2001 |
| Induced, ectopic expression of CFDG | *hlh-1* | Blastomeres | body wall muscle-like cells | ? | Fukushige and Krause, 2005 |
| Induced, ectopic expression of CFDG | *lin-26* | Blastomeres | epithelial-like cells | ? | Quintin *et al*., 2001 |
| Induced, ectopic expression of CFDG | *elt-1,elt-3* | Blastomeres | Epithelial/epidermal-like cells | *lin-26* | Gilleard and McGee, 2001 |
| Induced, elimination of the translational regulators | Simultaneously mutated *mex-3* and *gld-1* | Germ line in *mex-3 gld-1*double mutants | Teratoma | *hlh-1*, *pal-1* | Ciosk *et al*., 2006 |
| Induced, ectopic expression | *che-1, unc-3 or unc-30* misexpression and simultaneous *lin-53* RNAi | Germ line in *lin-53(RNAi)* background | Glutamatergic, cholinergic, GABAergic neurons | *glp-1* | Tursun *et al*., 2011 |
| Natural[a], dauer-larva developmental loop | Mutated *lin-28* and dauer-larva developmental loop | Descendants of VPCs: Pn.px and Pn.pxx in *lin-28* mutants | VPCs | ? | Euling and Ambros, 1996 |
| Natural | *lin-12/Notch* | Rectal cell Y | PDA neuron | *egl-5/Hox*, *sem-4/Sall4*, *unc-3/COE* | Jarriault *et al*., 2008<br>Richard *et al*., 2011 |

CFDG cell fate determining genes. VPCs vulval precursor cells.
[a]Natural, based on the authors interpretation of this process, see text for explanation.

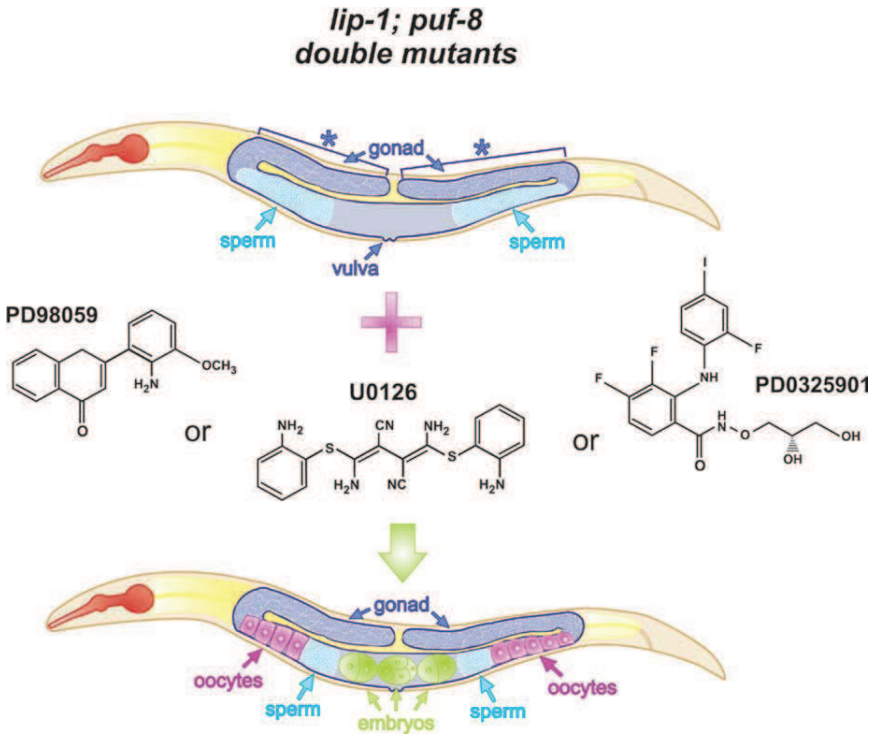


**FIG. 1.** Chemical reprogramming. Oocytes are chemically induced in the masculinized germline of mutant hermaphrodites lacking both LIP-1 and PUF-8 that produce only sperm. The gonad of *lip-1; puf-8* double mutants contains extended sperm area (bright blue) while the female germ cells are blocked at the state of the mitotic cycle and meiotic prophase (indicated by stars). Application of any of the three MAPK pathway inhibitors, PD98059, U0126, or PD0325901 to adult worms can restore production of functional oocytes via cell reprogramming.

(Morgan *et al.*, 2010). Nevertheless, only PD0325901, which was also used in the iPS cells studies (Lin *et al.*, 2009; Silva *et al.*, 2008), and U0126 could induce sperm-to-oocyte reprogramming with nearly full efficiency. The induced oocytes were functional, because the U0126-treated worms produced embryos that developed to adults. However, it remains unclear whether the oocytes were induced from the germ stem cells or via dedifferentiation of spermatocytes. Moreover, a direct link between MAPK pathway activity and the function of the *lip-1* and *puf-8* genes is yet to be demonstrated. Although the exact molecular mechanism of this event remains to be elucidated, this work represents a prime example of chemical reprogramming in vivo. It shows that chemical approaches can be an alternative to genetic manipulations aimed at obtaining iPS cells or to reprogram cell identity not only in cultured cells but also in whole animals.

## Reprogramming Induced by Ectopic Expression of Cell Fate Determinants During *C. elegans* Embryogenesis

Because the cell lineages of *C. elegans* embryogenesis are entirely mapped and each particular cell can be identified (Sulston *et al.*, 1983), worm embryos offer an attractive model for studying cell plasticity and cell fate decision. The blastomeres of the *C. elegans* embryo become committed to distinct lineages during the first few embryonic cleavages (Gönczy and Rose, 2005). Five asymmetric divisions generate six founder cells: AB, MS, E, C, D, and $P_4$, each of which undergoes a unique developmental program to ensure formation of all germ layers (Fig. 2a). Blastomeres were believed to have restricted cell plasticity from the instant the founder cells are born. However, four studies introduced in this chapter demonstrate that the embryonic cells of *C. elegans* have the potential to change their developmental program even after the founder cells of every lineage have been established (Fukushige and Krause, 2005; Maduro *et al.*, 2001; Quintin *et al.*, 2001; Zhu *et al.*, 1998).

## *C. elegans* Blastomere Reprogramming Into Endodermal Lineage In Vivo

The somatic blastomeres of *C. elegans* embryos showed a capacity to be reprogrammed to the endodermal lineage (Zhu *et al.*, 1998), mesendodermal lineage (Maduro *et al.*, 2001), to epithelial-like cells (Gilleard

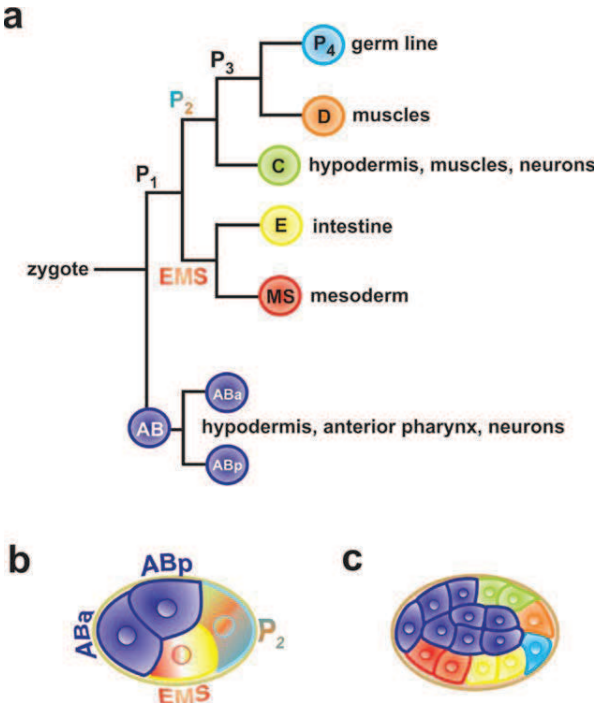


**FIG. 2.** Establishment of cell lineages during early *C. elegans* embryogenesis. (**a**) A lineage diagram of first zygotic divisions that yield six founder cells, AB, MS, E, C, D, and $P_4$, each of which gives rise to distinct cell types. The AB founder cell generates an anterior daughter ABa and a posterior daughter cell ABp. (**b, c**) Schematic drawings of the 4-cell-stage and 16-cell-stage embryo, respectively. Individual blastomeres are shown in colors corresponding to their origin and to tissue types that derive from them. Anterior is to the left, dorsal up.

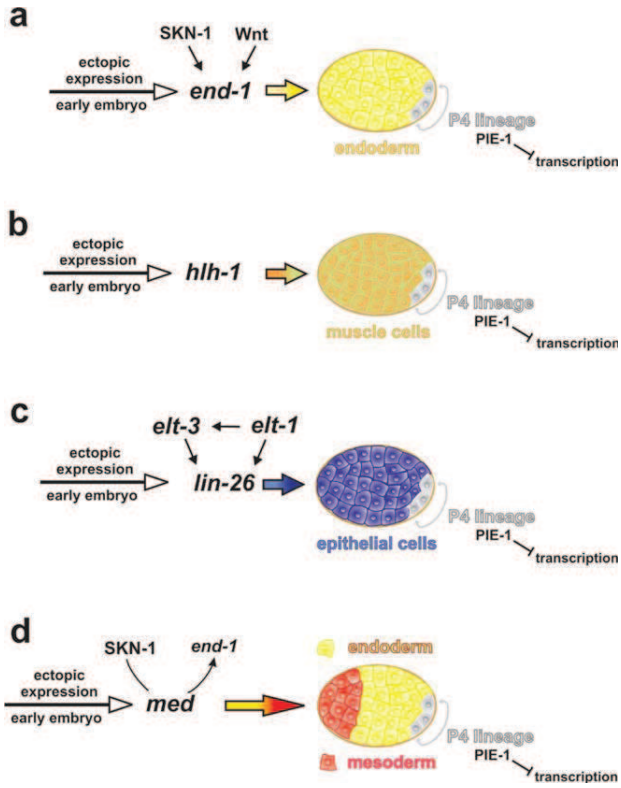


**FIG. 3.** Examples of cell reprogramming achieved by ectopic gene expression. Depicted are four scenarios resulting from misexpression of regulatory genes, *end-1*, *hlh-1*, *lin-26*, and *med*, respectively. The manipulated ectopic induction of these genes overrides the normal cell-specific regulation by the indicated transcription factors and causes reprogramming of the original embryonic cell lineages toward a particular cell type. The potential of the P4 lineage (germline) to be reprogrammed is limited, possibly due to transcriptional repression by PIE-1. See text for further details.

and McGhee, 2001; Quintin *et al.*, 2001) and to body wall muscle-like cells (Fukushige and Krause, 2005), respectively, upon forced expression of specific cell-fate determinants (Fig. 3). One of these is a zinc finger transcription factor of the GATA family, END-1, which specifies endoderm (Zhu *et al.*, 1998). Based on current knowledge, END-1 is the earliest zygotic protein expressed in the E (endodermal) lineage. However, when expressed ubiquitously during embryonic development, the nonendodermal lineages also assume endoderm-like cell fate instead of producing ectodermal or mesodermal cells (Fig. 3a). Their endodermal identity was determined based on cell morphology, expression of the endoderm-specific GATA factor gene *elt-2*, presence of antigens specific for the intestinal brush border, and of the gut-specific GES-1 esterase. Embryos with ectopic *end-1* activity not only exhibited widespread endoderm formation, but also broad suppression of the ectodermal fate as assessed by staining for the epithelial-specific marker LIN-26. This result demonstrated that ectopic *end-1* expression induced endoderm respecification and simultaneously inhibited other cell fates in other lineages. The strongest effect was observed when *end-1* expression was triggered in the 20- to 50-cell stage embryos; inducing *end-1* at the 200-cell stage was no longer effective. These experiments provided the first evidence that the blastomeres of *C. elegans* embryos can be redirected to a different cell fate until quite late in development and defined a window of developmental plasticity. Surprisingly, this plasticity extends beyond the time point when the lineage founder cells have been specified.

Interestingly, the ectopic expression of *end-1* bypasses two pathways that are normally required for the endoderm formation (Zhu *et al.*, 1998; Fig. 3a): Wnt signaling (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997) and a cascade that includes the transcription factor SKN-1 (Blackwell *et al.*, 1994; Bowerman *et al.*, 1992). Mutants for *skn-1* or Wnt pathway components fail to generate endodermal cells, yet *end-1* overexpression is sufficient to overcome their absence and induces the endodermal lineage in the background of *skn-1* or Wnt

pathway mutants (Zhu *et al*., 1998). Therefore, maternally provided SKN-1 and Wnt pathway components are dispensable for endodermal cell fate if *end-1* expression is forced. This finding, supported by the evidence that SKN-1 binds to *end-1* regulatory sequences, suggests that maternal SKN-1 and the Wnt pathway might regulate *end-1* expression, which in turn is sufficient to initiate endoderm formation (Fig. 3a).

Excitingly, similar blastomere reprogramming was also observed following misexpression of other lineages determinants (Fig. 3).

### *C. elegans* Blastomere Reprogramming Into Muscle-Like Cells In Vivo

Mutation of *hlh-1*, a *C. elegans* MRF-related gene, does not cause any myogenic defect, having all body wall muscles formed in embryos. This demonstrates that *hlh-1* is dispensable for embryonic myogenesis and that other myogenic factor(s) are sufficient to trigger myogenic fate in blastomeres of wild-type embryos. However, widespread conversion of embryonic blastomeres into body wall muscle cells is induced by ectopic expression of *hlh-1* (Fukushige and Krause, 2005; Fig. 3b). Based on the presence of myosin heavy chain A (MHC-A), filamentous actin and factors required for proper muscle cell structure and attachment, most if not all cells undergo muscle-like fate upon *hlh-1* misexpression. At the same time, antibody staining for markers specific to the hypodermis (LIN-26), the intestine (ELT-2), and the germline (P-granules) shows vast depletion of other (nonmuscle) cell types in embryos ectopically expressing *hlh-1*.

To prove that ectopic *hlh-1* itself re-specifies other blastomeres toward the muscle-like cell fate, the authors misexpressed *hlh-1* in embryos whose entire anterior part is transformed to the intestinal fate (ELT-2 positive) as a consequence of depleted *mex-1* and *pop-1*. In *mex-1, pop-1* RNAi embryos misexpressing *hlh-1*, most if not all cells expressed the MHC-A muscle marker, while almost no cells expressed the ELT-2 intestinal marker, indicating that the blastomeres initially committed to intestinal fate were re-directed towards muscle-like cell type (Fukushige and Krause, 2005; Fig. 3b).

Similarly to ectopic *end-1*, ectopic *hlh-1* can reprogram blastomeres during a broad time window of embryogenesis, up to the 8-E-cell-stage, when eight intestinal precursors and over 100 cells in total have been generated (Fukushige and Krause, 2005). At this stage, cells that will give rise to the intestine express the intestine-specific markers *end-1* and *elt-2*, indicating that they are already committed to the intestinal fate. Thus, the capacity of *hlh-1* to convert blastomeres to a muscle-like fate even after they passed the stage in which lineage founders have been specified reflects the surprisingly high level of cell plasticity in the *C. elegans* embryo.

### *C. elegans* Blastomere Reprogramming Into Epithelial-Like Cells In Vivo

LIN-26, a zinc finger transcription factor, has also been shown to be capable of reprogramming *C. elegans* embryonic cells (Quintin *et al*., 2001). LIN-26 is essential for the differentiation of the ectodermal and mesodermal epithelial lineages and its loss causes embryonic lethality. Ubiquitous ectopic expression of *lin-26* transforms most of the blastomeres into epithelial-like cells, while eliminating other cell types in the embryo (Fig. 3c). The ectopic epithelial-like cells express *ajm-1*, *dlg-1*, and *che-14*, that is, two components of the adherens junctions and an apical membrane marker, respectively. However, misexpressed *lin-26* alone is not competent to induce epidermal differentiation, because epidermal-specific genes, including cuticle collagen or muscle attachment genes, are not activated in the epithelial-like cells. Therefore, although *lin-26* is sufficient to induce a basic epithelial character of the cells, it does not behave as a master epithelial regulatory gene.

By contrast, the GATA factors ELT-1 and ELT-3 act in both epithelial and epidermal specification in *C. elegans* (Gilleard and McGhee, 2001; Page *et al*., 1997). Forced expression of either *elt-1* or *elt-3* individually not only induces activation of the epithelial-specific marker *ajm-1*, but also of the epidermal collagen gene *dpy*-7 (Gilleard and McGhee, 2001). In addition, misexpression of *elt-1* or *elt-3* activates expression of *lin-26* (Fig. 3c). The ectopic expression of *elt-1* or *elt-3* is sufficient to activate hypodermal cell differentiation while suppressing other developmental programs in blastomeres: cell type-specific markers including *myo-3* (body wall muscles), MH33 (gut), and *rgef-1* (neurons) are repressed as a consequence of *elt-1* or *elt-3* misexpression (Gilleard and McGee, 2001).

The competence of nonepithelial founder cells to be redirected to the epithelial program has been further tested by laser cell killing experiments. The authors eliminated three blastomeres at the four-cell-stage, leaving one nonepithelial blastomere, and subsequently ectopically expressed *lin-26*, *elt-1*, or *elt-3* in such embryos. All nonepithelial blastomeres were capable of generating epithelial-like cells upon ectopic expression of any of the three genes. A restricted potential to be reprogrammed was observed for the founder cell that gives rise to the germline (Quintin *et al*., 2001), most probably as a consequence of the transcriptional inhibition by the germline-specific repressor PIE-1 (Fig. 3). By analogy to *end-1* or *hlh-1* misexpression, the most robust effect on blastomeres was observed when *lin-26* was ectopically activated in 20- to 50-cell stage embryos, defining the time window during worm

embryogenesis when blastomere reprogramming is most effective.

### *C. elegans* Blastomere Reprogramming Into Mesodermal Lineage In Vivo

The GATA transcription factors *med-1* and *med-2* are direct targets of SKN-1, and they establish mesodermal cell fate within the MS lineage (Maduro *et al*., 2001; Fig. 2b). Misexpression of the *med-1* and *med-2* factors is sufficient to redirect non-EMS blastomeres to an EMS-like lineage, and this *med*-dependent blastomere transformation does not require maternal SKN-1 (Bowerman *et al*., 1992; Maduro *et al*., 2001; Fig 3d). Therefore, ectopic expression of multiple transcription factors can lead to blastomere reprogramming in *C. elegans*, suggesting that somatic blastomeres offer a permissive environment for most lineage determinant-mediated reprogramming and thus are multipotent.

The study of cell reprogramming in *C. elegans* embryos has revealed a remarkable plasticity of lineage founder cells, determined during early embryogenesis but still able to switch to another developmental program upon ectopic expression of specific cell-fate determinants. Four important points can be drawn from these experiments: (i) the individual developmental regulator genes tested are sufficient to convert *C. elegans* embryonic blastomeres into specific cell types. This is a testimony to the plasticity retained by the early embryonic lineage founder blastomeres; (ii) although the extent of blastomere reprogramming may vary depending on the factor that is used to re-specify cell fate, most if not all blastomeres of the embryo are reprogrammed. Thus, *C. elegans* embryonic cells exhibit a high efficiency of reprogramming, when compared with other experimental systems; (iii) this induced re-specification involves simultaneous repression of the endogenous differentiation program, rather than resulting in a state of mixed fates; (iv) the ability of a *C. elegans* cell to be reprogrammed is stage-specific and goes from extremely high during the embryonic developmental plasticity window, to almost null during larval development. When exactly, and why, blastomeres become refractory to reprogramming by single transcription factors remains an open question. In addition, because most of the developmental genes are conserved, the worm model provides an opportunity to investigate specific roles of vertebrate factors and the regulatory pathways within a multicellular organism.

### Reprogramming Induced by Elimination of Translational Repressors or Histone Modifiers in the *C. elegans* Germ Line

The hallmark of the germ line is the maintenance of totipotency to retain the reproductive capacity of the germ cells. To date, two mechanisms to preserve totipotency have been described. The first is by means of transcriptional inhibition [for a review, see Seydoux and Schedl (2001)] and the second is at the level of translation (Ciosk *et al*., 2006). An outstanding finding has revealed that the coincident loss of two translational regulators, MEX-3 and GLD-1, causes ectopic transdifferentiation of germ cells (Ciosk *et al*., 2006). The authors have detected differentiated somatic cells in the gonad of *mex-3 gld-1* double mutants (Fig. 4b). The identity of these ectopic somatic cells has been assessed by cell morphology, by immunostaining, or by using transgenic reporters for cell-specific genes. The direct conversion

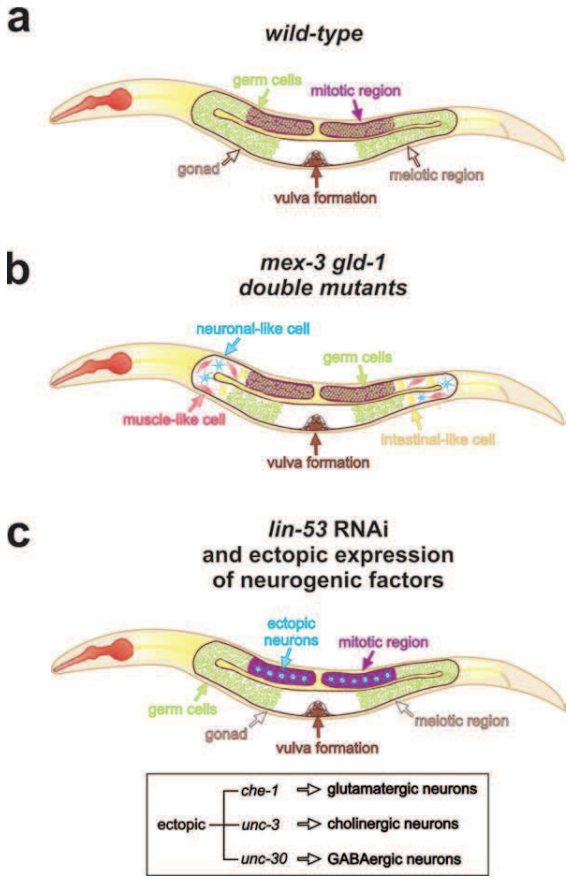


**FIG. 4.** Cell reprogramming in the *C. elegans* germline. (**a**) A wild-type hermaphrodite at the fourth larval stage. The distal zone of the gonad, represented here in purple, contains the mitotic germ cells. Moving proximally, the germ cells (green nuclei) enter and progress through meiosis; the meiotic region is represented by a white background. (**b**) Distinct somatic cell types appear as a result of spontaneous germ cell reprogramming in the gonad of the *mex-3 gld-1* double mutant at the fourth larval stage. Note that this is observed in the meiotic zone where *mex-3* and *gld-1* are expressed. (**c**) Direct germ cell conversion into glutamatergic, cholinergic, or GABAergic neurons occurs upon ectopic expression of neurogenic transcription factors, *che-1*, *unc-3*, and *unc-30*, respectively, in a *lin-53* RNAi background. In contrast to the *mex-3 gld-1* mutants, neuronal cells have been suggested to arise from the reprogramming of mitotic germ cells.

of germ cells into somatic cells in young *mex-3 gld-1* mutants is further supported by the smaller size of these cells and their reduced content of P granules, distinctive particles that are specifically inherited by the germ line lineage. Ectopic somatic cells in the gonad display either neuronal, muscle, or intestinal character (Fig. 4b) and resemble human germ-cell tumors called teratomas. To examine whether these somatic cells derive from germ cells, cell ablation experiments have been performed (Ciosk *et al.*, 2006). When the germ-cell precursors Z2 and Z3 are ablated, no somatic cells are found in the gonad of *mex-3 gld-1* mutants, suggesting that the germ cells themselves are converted into somatic cells. Importantly, the competence of the germ cells for reprogramming strictly depends on their transition to meiosis, as *mex-3 gld-1* double RNAi does not produce somatic cells in *glp-1* gain-of-function mutants, whose germ cells remain mitotically active and do not enter meiosis.

Further analysis focused on the nature of ectopic muscle cells in the gonad of *mex-3 gld-1* mutants implicated HLH-1 and PAL-1 in this process (Ciosk *et al.*, 2006). The myogenic factor HLH-1 is absent from the wild-type germ line, whereas it is detected in the nuclei of reprogrammed gonads of *mex-3 gld-1* mutants. Moreover, depletion of the *hlh-1* regulator PAL-1 diminishes the number of HLH-1-positive nuclei and the number of the ectopic muscle cells. This result demonstrates that ectopic muscle cell formation in *mex-3 gld-1* mutants is controlled by a pathway reminiscent of myogenesis during wild-type embryogenesis. Both MEX-3 and GLD-1 are involved in the suppression of *pal-1* translation in the wild-type gonad (Draper *et al.*, 1996; Hunter and Kenyon, 1996; Mootz *et al.*, 2004), yet the germ cells in meiotic phase occasionally express *pal-1* without inducing reprogramming (Mootz *et al.*, 2004). Ciosk and colleagues thus proposed that two changes are essential for the germ-cell reprogramming. One is the activation of factor(s) inducing somatic differentiation, and the second is a capacity of germ cells to respond to this factor(s) (Ciosk *et al.*, 2006). The molecular mechanisms allowing *mex-1 gld-1* mutant germ cells to adopt a somatic identity have not yet been elucidated. They may include a potential role for the P-granules and/or chromatin modification. P granules are particles that occur specifically in the *C. elegans* germ line and contain maternal mRNAs and some regulators of RNA metabolism (Seydoux and Schedl, 2001). The diminished pool of the P-granules observed in *mex-3 gld-1* mutants (Ciosk *et al.*, 2006) might indicate that some P-granules have broken down and released their mRNA content. The subsequent ectopic occurrence of mRNAs may contribute to the germ-line transdifferentiation. In addition, besides developmental regulators, targets of GLD-1 include components of histone modifier complexes (Fong *et al.*, 2002; Xu *et al.*, 2001); loss of *gld-1* could therefore result in a chromatin state more favorable to somatic gene expression. Whether the translational regulators MEX-3 and GLD-1 crosstalk with chromatin modifying complexes remains to be tested.

PUF-8, a pumilio-like protein is another RNA binding factor that acts in the *C. elegans* germ line to control the balance between cell proliferation and differentiation (Subramaniam and Seydoux, 2003). PUF proteins inhibit the expression of target mRNAs either by repressing translation or by promoting mRNA degradation. PUF-8 together with MEX-3 functions redundantly to maintain self-renewal of germ stem cells (Ariz *et al.*, 2009). Conversely, PUF-8 has a different function in germ cells that have entered meiosis. In primary spermatocytes, PUF-8 sustains meiosis and prevents reversion to the mitotic state (Subramaniam and Seydoux, 2003). Primary spermatocytes that lack PUF-8 are therefore unable to complete meiosis although they have successfully entered it. Instead, they dedifferentiate into mitotically active germ cells and subsequently form tumors. Apparently, the control of gene expression at the translational level is crucial for preventing transition from the germ-cell proliferation to differentiation and vice versa. Removal of translational inhibition leads to cell dedifferentiation or reprogramming in *C. elegans* germ line.

Recently, an example of a specific neuronal induction has been demonstrated in the *C. elegans* germ line, revealing a third layer of control of germ cell identity (Tursun *et al.*, 2011). Depletion of the histone-associated protein LIN-53 by RNAi does not affect germ cells, whereas ectopic expression of neurogenic transcription factors in the absence of LIN-53 activity results in germ-cell reprogramming into specific neurons (Fig. 4c). Mitotic germ cells are converted into glutamatergic, cholinergic, or GABAergic neurons upon ectopic activation of the Zn finger transcription factor *che-1*, the EBF-like transcription factor *unc-3*, and the Pitx-type homeobox gene *unc-30*, respectively (Fig. 4c). The identity of the resulting ectopic cells has been determined based on cell morphology, expression of neuronal markers, axon/dendrite projection, and localization of presynaptic components.

Unlike the teratoma formation encountered in *mex-3 gld-1* mutants, which requires meiosis, here, it is the mitotic germ-line population that appears to yield somatic cells after neuronal induction in a *lin-53* RNAi background (Tursun *et al.*, 2011). It remains to be shown whether *lin-53* acts in germ cells autonomously. Nevertheless, as LIN-53 is predicted to bind histone H4, and as a histone deacetylase inhibitor similarly renders germ cells permissive to neural induction, it is possible that loss of *lin-53* impacts on chromatin architecture in germ cells. It is likely that future work will assess the involvement of additional chromatin modulators in this reprogramming event.

Interestingly, the *lin-53*-depleted germ cells seem to be incompetent to assume a muscle developmental program in response to ectopic *hlh-1*. This suggests that removal of *lin-53* by RNAi renders germ cells permissive to neural reprogramming, as seen upon overexpression of all neural determinants assessed, but not to reprogramming to other lineages. The basis for this pan-neural permissivity is presently unclear.

In summary, the three studies introduced in this chapter demonstrate that the *C. elegans* germ line possesses a high capacity to be reprogrammed to a variety of somatic cells depending on inducing factors and genetic background. At least three layers of insulating mechanisms were found to protect germ cells identity: inhibition of transcription, inhibition of translation, and modulation of the chromatin environment. Additionally, an interesting concept is emerging from the studies presented earlier: specific mechanisms may render a given cell type primed for reprogramming into one lineage specifically. Whether this is true for somatic cell types and what is the basis for permissivity to specific lineages will be the aim of future studies.

## NATURAL CELL TRANSDIFFERENTIATION IN *C. elegans*

Spontaneous reprogramming of differentiated cells without cell division represents a fascinating phenomenon that will allow to focus on defined cells and to follow their transdifferentiation step by step, an impossible task when working with cell populations. In the literature, this phenomenon has been referred to as "natural direct reprogramming," "natural direct cell type conversion," or "transdifferentiation". In *C. elegans*, few natural reprogramming examples have been described. We first introduce a reprogramming event, which is triggered by transition through an alternative larval stage, and is at the margin of the induced and natural cell conversion. Next, we focus on a new intensively studied *C. elegans* model of a rectal-to-neuronal cell transdifferentiation.

### Cell Fate Reversion During Vulva Formation

In *C. elegans*, three of the six multipotent vulval precursor cells (VPCs) are specified to adopt a vulval fate, and their descendants form an egg-laying organ, called the vulva. These VPCs become committed and divide at the third larval stage, producing daughter cells that will adopt distinct vulval cell fates. In *lin-28* heterochronic mutants, VPCs divide at the second larval stage, starting vulval development precociously (Euling and Ambros, 1996). Interestingly, when the *lin-28* mutants enter the dauer larval stage, a mode of developmental arrest induced by unfavorable environmental conditions, the commitment of the VPC descendants is reset, and the VPC progeny is reprogrammed back to the multipotent VPC state. The progeny of the reprogrammed VPCs are capable to generate a functional vulva due to the restored sensitivity to the inducing signal. The authors speculate that this reprogramming allows coordination and synchronization of developmental processes that might facilitate the transition between the larval arrest and the ensuing postdauer development. This finding, so far the only example implicating *lin-28* in cell reprogramming in *C. elegans*, is quite surprising, because a homolog of LIN-28 marks undifferentiated human stem cells and has been used to increase the efficiency of the iPS cells production.

### Transdifferentiation of a Rectal Cell Into a Neuron In Vivo

A unique transdifferentiation event, which occurs naturally during *C. elegans* development in absence of cellular division, has been recently characterized (Jarriault *et al*., 2008). This phenomenon provides an excellent opportunity to study the molecular mechanisms of spontaneous reprogramming in the context of a complex multicellular organism. The rectal tube of *C. elegans* larvae consists of three pairs of cells, named K and K′, U and F, and Y and B (Fig. 5a). At the beginning of the second larval stage, the Y cell starts to migrate out from the rectum and transdifferentiates into a neuron called PDA (Fig. 5b). The vacant position in the rectum is taken by another epithelial cell P12.pa to preserve a functional rectal tube structure (Fig. 5b,c).

Based on the expression of cell-specific markers, ultrastructure, and synaptic junctions, Y is a fully differentiated and purely epithelial cell when in the rectum. Although the Y cell expresses epithelial markers, it neither expresses pan-neuronal markers nor the PDA-specific markers (Jarriault *et al*., 2008, Richard *et al*., 2011). In contrast, upon transdifferentiation, PDA displays a fully neuronal character: the PDA neuron never shows any residual signal of the epithelial/rectal-specific markers, whereas it displays the pan-neuronal and PDA-specific markers (Hall and Russell, 1991; Jarriault *et al*., 2008; Thomas, 1994). At least four different morphological states have been discerned within the Y-to-PDA transdifferentiation event (Jarriault *et al*., 2008). (1) The rectal epithelial identity of the Y cell is established; (2) Y retracts from the rectal tube, mimicking an epithelial-to-mesenchymal transition; (3) Y migrates to its final position; (4) Y, which has completely lost its former rectal identity, becomes the PDA motor neuron (Fig. 5). The characterization of these individual steps of the Y-to-PDA transdifferentiation and the elucidation of the mechanisms governing each particular state will be a key to our understanding of how cells control endogenous cellular potential.

### Competence of Y to Change its Identity

Why does the Y cell, and not the neighboring rectal cells, change its identity? To understand how the com-

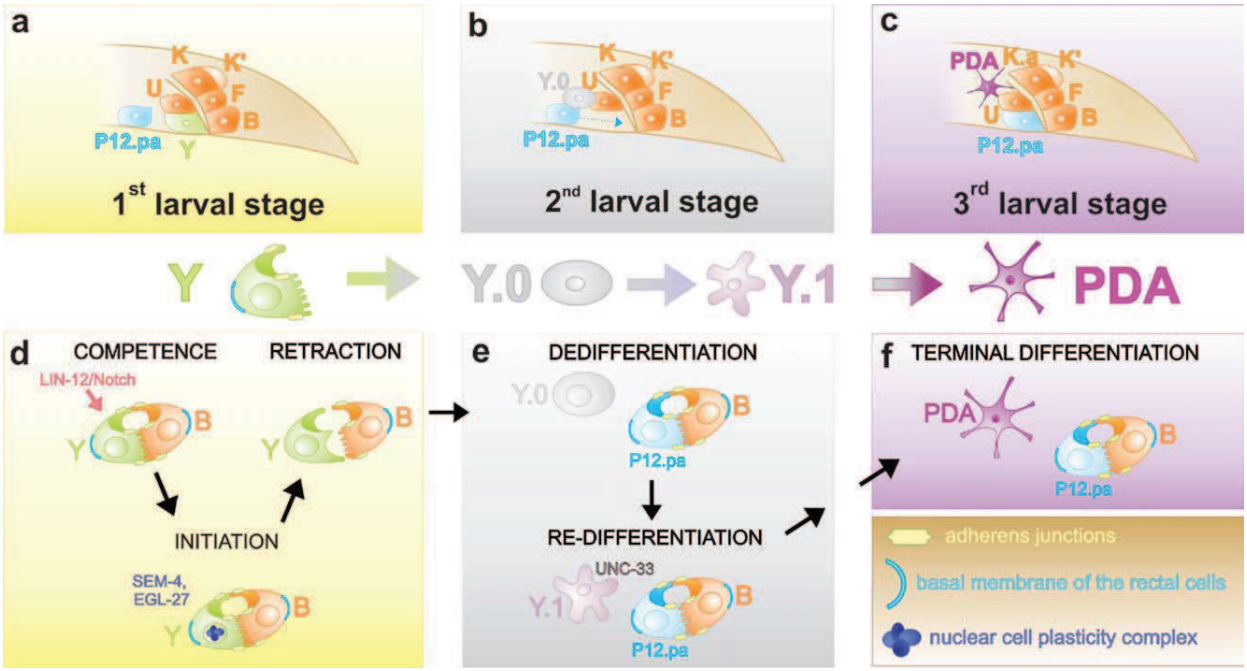


**FIG. 5.** Transdifferentiation of the rectal cell Y to the PDA neuron in *C. elegans* larvae. (**a–c**) Drawings of a tail area show organization of the rectal cells during Y-to-PDA cell type conversion. Six rectal cells, K, K′, U, F, Y, and B form a rectal tube in the *C. elegans* first larval stage (a). During the second larval stage, the Y cell migrates out of the rectum and dedifferentiates into an intermediate cell type called Y.0 (b). An epithelial cell P12.pa that resides in the close proximity of the rectum replaces Y in the rectal tube to maintain the organ function (c). By the third larval stage, the Y.0 cell has re-differentiated into the PDA neuron. (**d–f**) Individual steps of the Y-to-PDA transdifferentiation in their chronological order. The adherens junctions at the apical side of the rectal cells are represented by yellow blocks, and their basal side is indicated in blue. (d) The Y and B cells interact to form one of the three rectal rings. LIN-12/Notch endows the Y cell with a competence to undergo cell conversion. A nuclear plasticity complex, comprising SEM-4/SALL and EGL-27/MTA1, is essential for initiation of the reprogramming. The Y cell has to extinguish the adherens junctions to be able to migrate out of the rectal tube and initiate its transdifferentiation. (e) The epithelial cell P12.pa preserves the functional rectum by taking the vacant position in the rectal ring. Y dedifferentiates into an intermediary cell termed Y.0, which has neither epithelial nor neuronal identity. Y.0 begins to re-differentiate and becomes an early neuronal cell Y.1 that expresses the pan-neuronal marker *unc-33*. (f) The Y.1 cell finally differentiates into the PDA motor neuron. Anterior is to the left, dorsal up.

petence to switch identity is established, the fate of a supernumerary Y cell was examined in mutants where two Y cells are formed. For instance, a supernumerary Y cell is made in the *egl-38/Pax5* and *mab-9/T-box transcription factor* mutants. The extra Y cell is generated at the expense of either the rectal U or B cell in the *egl-38* and *mab-9* mutants, respectively. The supernumerary Y-like cells express Y-specific markers, while they have lost expression of specific markers of their wild-type identity (Jarriault *et al.*, 2008) and divide in mutant males following the division pattern of the wild-type Y blast cell (Chamberlin *et al.*, 1999), indicating that the extra Y cell acquired a proper Y-cell-identity in the *egl-38* and *mab-9* mutants. However, these ectopic supernumerary Y-like cells are not competent to transdifferentiate into the PDA neuron. In addition, the ablation of the endogenous Y cell does not force the supernumerary Y-like cell to reprogram, excluding the existence of a counting mechanism to preclude transdifferentiation of more than one rectal cell. Thus, adoption of an ectopic Y cell identity is not sufficient per se to become PDA. Conversely, a gain-of-function mutation in *lin-12/Notch* leads to a supernumerary Y cell that derives from the DA9 cell, the contralateral lineal homolog of Y. In this case, both Y cells are capable to transdifferentiate and generate two PDA neurons. Therefore, *lin-12/Notch* is not only essential and sufficient to form the Y rectal cell, but is also necessary to endow it with the competence to become PDA, suggesting that the cellular environment experienced by the Y cell at its birth is important to allow it to become PDA (Jarriault *et al.*, 2008).

## Initiation of the Y-to-PDA Transdifferentiation Process

A number of mutants have been identified in which Y transdifferentiation is not initiated (M.C. Morin and S. J., unpublished; Richard *et al.*, 2011). For example, in *egl-5/Hox* and *sem-4/Sall* mutants, the Y cell does not undergo reprogramming to the PDA neuron but instead persists in the rectum and maintains an epithelial char-

acter (Jarriault *et al*., 2008). Therefore, *egl-5* and *sem-4* activities are crucial for early steps of the Y-to-PDA transdifferentiation, likely for the Y cell to initiate its reprogramming. Recently, we have identified a nuclear complex comprising SEM-4/SALL and conserved transcriptional modulators, which is necessary for the initial step of Y-to-PDA cell conversion (Fig. 5d; K. Kagias, A. A. and S. J., unpublished data). Because homologs of all components forming this complex are essential for pluripotency of ES cells in mammals (Liang *et al*., 2008), and because these factors trigger or enhance iPS cell reprogramming, it is possible that they confer cellular plasticity via a conserved mechanism.

Y cell retraction from the rectum represents an early step of the Y-to-PDA reprogramming, and its impact on the Y cell transdifferentiation has been addressed. Laser and genetic cell ablations identified the neighboring "P12" cell as being necessary for Y migration away from the rectum (Jarriault *et al*., 2008). However, even when this migration is precluded, a PDA neuron is made, showing that migration is dispensable. Thus, migration and reprogramming can be uncoupled, indicating that a new cellular microenvironment, which Y experiences while migrating, does not contribute to the conversion of Y to PDA. Interestingly, none of the five rectal cells that are in contact with Y (Fig. 5a) appear to be essential for the Y cell transdifferentiation, because laser cell ablation of an individual rectal cell during early larval development does not prevent the Y-to-PDA conversion (Jarriault *et al*., 2008). However, it is possible that the cellular microenvironment does contribute to the Y-to-PDA transdifferentiation during embryogenesis. Alternatively, this function might be shared redundantly by more than one neighboring rectal cell.

## Switch From the Epithelial to the Neuronal Identity

A fascinating aspect of the Y-to-PDA reprogramming is how Y loses its epithelial identity and how it acquires the neuronal properties of PDA. Two scenarios of Y cell identity change are possible. Y might concomitantly forfeit epithelial features while gaining neuronal character. In this case, a cell with mixed identity should occur during the reprogramming event. Alternatively, the epithelial identity needs to be erased before the acquisition of neuronal fate (Fig. 5). A recent genetic screen identified a COE transcription factor mutant whose Y cell is blocked at an intermediate phase of transdifferentiation (Richard *et al*., 2011). Analysis of this mutant suggested a step-wise Y-to-PDA conversion where Y first dedifferentiates into an intermediary cell (termed Y.0), which has neither epithelial nor neuronal identity (Fig. 5b,e). Subsequently, Y.0 becomes an early neuronal cell Y.1 that expresses the pan-neuronal marker *unc-33* and that finally differentiates into the PDA motor neuron (Fig. 5c,e,f; Richard *et al*., 2011). None of these steps involves cells with mixed identity, supporting the scenario in which the Y cell loses its epithelial features before acquiring neuronal ones.

Surprisingly, the apparent dedifferentiation of Y does not represent reversion to a multipotent state, because ectopic expression of endodermal (*end-1*), muscular (*hlh-1*), or neuronal (*unc-30*) master cell fate determinant genes fails to induce the anticipated cell fate in Y.0 (Richard *et al*., 2011). Strikingly, the Y.0 cell not only lacks the potential to be converted into distinct cell fates, but it also cannot be forced to re-differentiate back into an epithelial cell. These findings demonstrate that the successive cellular transitions during the Y-to-PDA transdifferentiation are tightly controlled. In addition, it suggests that dedifferentiation and multipotency are uncoupled in vivo. The absence of multipotency in dedifferentiated cells might be safer for an organism, making such cells less likely to divert to an unwanted cell type. This feature is therefore relevant for stem-cell therapies, where instability of the desired cell identity and potential malignant transformation present major risks of induced cellular reprogramming. Because the Y-to-PDA transdifferentiation is a natural event, yet occurring in a stereotyped manner, it represents an outstanding model to disclose the molecular mechanisms that govern direct cell conversion within an entire multicellular organism.

## Mechanistic Considerations

Differentiated cells can be forced to revert to a pluripotent state or change to another differentiated identity naturally. Do these induced and natural reprogramming events rely on a common molecular basis? Although the exact mechanisms underlying cell plasticity are still unknown, work from several laboratories has indicated promising directions.

## Epigenetic Phenomena as a Unifying Theme to Cell Plasticity Events

**Polycomb group complexes.** Because cell-type conversion involves the switching off a whole expression program while activating a new one, nuclear events are expected to play an important role in the modulation of cellular identity. The capacity of a cocktail of specific transcription factors to dedifferentiate somatic cells (Takahashi and Yamanaka, 2006) suggests that transcriptional reprogramming is central to cell conversions. Substantial changes in transcriptional profiles likely involve various levels of transcriptional regulation, including the modulation of chromatin architecture. Indeed, a balance between the activities of the Polycomb and Trithorax groups, that comprises histone binding and modifying activities, has been proposed to maintain cellular identity (Ringrose and Paro, 2007;

Schuettengruber and Cavalli, 2009). Polycomb proteins, typically associated with silenced chromatin, have been linked with the potential of mouse ES cells to generate diverse cell types (Landeira *et al*., 2010; Pasini *et al*., 2007; Shen *et al*., 2008, 2009). Moreover, genome-wide mapping studies in human and mouse ES cells have shown that their epigenetic landscape is dynamic and changes upon differentiation and that genes promoting differentiation are de-repressed in ES cells deficient for a functional Polycomb repressive complex (PRC; Boyer *et al*., 2006). Nonetheless, the role of Polycomb group proteins in mammalian ES cells remains controversial, as other studies have reported that certain PRC components may not be essential for the maintenance of ES cell pluripotency (Chamberlain *et al*., 2008; Niwa, 2007).

Could similar mechanisms be involved during in vivo reprogramming/cell plasticity events? A recent study by Yuzyuk *et al*. (2009) suggests that this might be the case. The authors examined the ability of the early embryonic *C. elegans* blastomeres to change their fate upon misexpression of cell-fate determining genes (e.g., *hlh-1* or *end-1*; see Fig. 3) in mutants with compromised Polycomb function. They found that mutants in a component of the PRC2 Polycomb complex, *mes-2*, displayed a prolonged developmental plasticity (Yuzyuk *et al*., 2009). Accordingly, microarray analyses showed that the gene expression profile of developmentally advanced *mes-2* mutants resembled that of wild-type embryos at an earlier pluripotent stage. Therefore, the *mes-2* mutant phenotype may result from a failure to repress genes that should only be expressed during a defined time window within early development. These results were further correlated with changes in chromatin morphology of an artificial chromosome (a transgenic DNA array) during early embryogenesis (Yuzyuk *et al*., 2009). This pseudo-chromosome became compacted while the blastomeres were losing their pluripotency in wild-type embryos, whereas it remained decondensed in *mes-2* mutants, a state it normally exhibits only during the pluripotent stage. Thus, *mes-2*/PRC2 may be associated with facultative (i.e., developmentally regulated) heterochromatin formation during *C. elegans* development, a process that could contribute to termination of the cell plasticity window during *C. elegans* embryogenesis.

Taken together, the Polycomb group proteins may participate in controlling cell plasticity both in vivo in a physiological context as well as in cultured ES cells. However, Polycomb complexes, described as a transcriptionally repressive complex, seem to operate by two different modes. Although in mammalian, ES cells PRC represses genes promoting differentiation and hence exit from the pluripotent state; in *C. elegans*, blastomeres PRC appears to inhibit expression of genes whose activity would otherwise prevent acquisition of the new cell fates.

**Other epigenetic actors.** In addition to the members of the Polycomb complexes, others factors belonging to chromatin bound complexes have been identified that control cellular plasticity in vivo. The induced reprogramming of *C. elegans* germ cells into neurons was only possible after RNAi knockdown of *lin-53* (Tursun *et al*., 2011; Fig. 4c). *lin-53* encodes the *C. elegans* ortholog of the retinoblastoma binding protein RbAp46/48, a component of the Nucleosome Remodelling and Deacetylation (NuRD) complex. This suggests that LIN-53, and maybe a NuRD-like complex, serves as a safeguard against exogenously or aberrantly expressed transcription factors in the germ cells. Although the actual mechanism is unknown, it is plausible that LIN-53 acts either through nucleosome remodeling or histone modifications, as both of these activities have been associated with the NuRD complex. The latter mode of action is supported by the fact that a chemical histone deacetylase inhibitor permits the germ cell-to-neuron reprogramming in a way similar to depletion of LIN-53 (Tursun *et al*., 2011). In fact, the involvement of complexes that impact on chromatin dynamics may be a general feature of in vivo reprogramming. For instance, we have recently implicated additional chromatin-associated factors in the transdifferentiation of the rectal Y cell to the PDA neuron in *C. elegans* (K. Kagias, A. A., and S. J., unpublished data). EGL-27/MTA1, a component of the NuRD and NODE (Nanog- and Oct4-associated deacetylase) complexes (Liang *et al*., 2008) and SEM-4/SALL, a DNA-binding factor that interacts with NuRD and NODE (Lauberth and Rauchman, 2006), are required for the initiation step of the Y-to-PDA conversion (Jarriault *et al*., 2008; K. Kagias, A. A. and S. J., unpublished data; Fig. 5d).

Thus, besides Polycomb group proteins, various chromatin bound factors known to be associated with histone modification or chromatin remodeling activities impact on the ability of a cell to change identity. Importantly, similar activities are necessary during a physiological cell-type conversion, as in experimentally induced ones. Studies on primary human fibroblasts have found that their reprogramming into iPS cells is enhanced by valproic acid, a histone deacetylase inhibitor (Huangfu *et al*., 2008), reinforcing the notion that similar mechanisms may be used to either erase the initial identity, or to potentiate the adoption of a new one, in various types of reprogramming events across phyla. Different types of cellular reprogramming (natural vs. induced, pluripotent vs. direct, etc.) may therefore be characterized by small variations on a common epigenetic mechanism theme. Future studies will determine if, besides histone deacetylation, other epigenetic activities are required to increase cellular potential in vivo.

## Cell Context-Dependent Cues for Cell Reprogramming

Although similar epigenetic strategies are being unravelled in various cell plasticity processes, the actual factors that mediate these effects will probably differ. In the recent years, it has become clear that chromatin-bound complexes are dynamic entities whose composition varies between cell types and probably during cellular life and that would be better described as families of complexes. The Polycomb PRC1 complex provides a good example of this (Schuettengruber and Cavalli, 2009). In addition, many components are shared between different complexes, so that the definition of a complex on the basis of its unique components can be reduced to a few factors. This has two implications: (i) removal of a given factor may affect more than one complex or a family of complexes; (ii) depletion of a given factor is likely to have different effects in distinct cell types, depending on the number and combinatorial of complexes for which this component's activity is critical. Analysis of the molecular requirements for induced and natural cell type conversions affecting two different tissues in vivo in the worm illustrates these notions. Although both *lin-53* and *egl-27* are expressed fairly ubiquitously in the worm, their function in transdifferentiation is cell specific. Indeed, LIN-53 acts to prevent neural induction of germ cells (Fig. 4c), while it has no role in the Y-to-PDA transdifferentiation (A. A., S. J., unpublished data). Conversely, *egl-27/MTA1* functions in Y rectal-to-neuronal cell conversion but does not potentiate neural determinant-induced germ-cell conversion (Fig. 5d; A. A., S. J., unpublished data, Tursun *et al*., 2011). In fact, loss of *lin-53* only potentiates the reprogramming of few cell types including germ cells and a handful of head neurons and has been suggested to specifically permit conversion to neurons only but to no other cell types. One interesting corollary of these results is that the mere misexpression of a cell fate determinant during postembryonic development does not suffice to cause the in vivo conversion of any cell. A permissive cellular context is needed, and it can be provided either by wild-type early embryonic blastomeres (for all cell fate determinants tested thus far; Fig. 3) or by germ cells deprived of *lin-53* activity (for proneural determinants; Fig. 4c). Knowing what makes a cell permissive to reprogramming in vivo, and what underlies the cell type-restricted ability of certain cell fate determinants, but not others, to induced direct reprogramming, will be of high interest.

## Processes Outside of the Nucleus Can Also Impact on a Cell's Identity

In addition to molecular mechanisms directly affecting the chromatin transcriptional state, translational inhibition appears to be an important mechanism to protect one's cell potential, at least in the germ cells. This may be more acutely needed in the germline as germ cells contain important levels of RNA stocks that have to be tightly regulated to preserve germline integrity. Accordingly, it has been reported that transcriptional inhibition of germ-cell RNA stocks could contribute to maintain totipotency of germ-cell precursors in early *C. elegans* and *Drosophila melanogaster* embryos (Nakamura and Seydoux, 2008; Seydoux and Schedl, 2001). Loss of two translational repressors, MEX-3 and GLD-1, causes spontaneous differentiation of multiple cell types from the germline, a phenotype called teratoma (Ciosk *et al*., 2006; Fig. 4b). Translational inhibition could ensure maintenance of the germ-cell totipotency by counteracting unwanted synthesis of cell fate determinants, thereby precluding germ-cell differentiation (Ciosk *et al*., 2006). It will be interesting to know the contribution of this mechanism to other reprogramming events in the worm or in vertebrates.

Just as new models and data are emerging in this fast moving field, many questions still lie ahead. One is whether induced reprogramming events borrow mechanisms that are used by naturally reprogramming cells. To which degree can similarities be found, and how can we eventually exploit these similarities to steer cell fate decisions in a desired direction? It may be that the general strategy executed during the reprogramming of different cell types in an organism or between the different types of reprogramming across phyla share common elements, but that the actual molecules and complexes at work come in different flavors. In addition, as natural direct reprogramming involves transition through discrete nonpluripotent identities, identifying the genetic networks controlling each step of reprogramming will be of high importance. Furthermore, the study of natural reprogramming events will also allow the mechanisms driving re-differentiation to be addressed. For example, does direct cell type conversion involve step-wise re-differentiation mechanisms such as the ones used during embryogenesis, or a different strategy?

We predict that invertebrate models of induced and natural cellular reprogramming have much to contribute to answer these questions. These models are amenable to single cell level approaches, providing unmatched cellular precision, and allow the implementation of unbiased creative genetic screens. More importantly, maybe, studies in the worm bring integrated answers by analyzing the behavior of cells in their natural environment, answers that may differ from those obtained with cells in a petri dish. These studies have important implications for human therapeutic strategies, for example, to design safe cellular therapies in regenerative medicine.

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Focus Article

# Direct cellular reprogramming in *Caenorhabditis elegans*: facts, models, and promises for regenerative medicine

Steven Zuryn, Thomas Daniele and Sophie Jarriault*

*In vitro* systems of cellular reprogramming [induced pluripotent stem (iPS) cells and direct reprogramming or transdifferentiation] are rapidly improving our repertoire of molecular techniques that can force cells in culture to change into a desired identity. However, the new frontier for regenerative medicine is *in vivo* cellular reprogramming, which in light of concerns about the safety of *in vitro* cell manipulations, is an increasingly attractive approach for regenerative medicine. Powerful *in vivo* approaches are currently being undertaken in the genetic model *Caenorhabditis elegans*. Several very distinct cell types have been induced to change or have been discovered to transform naturally, into altogether different cell types. These examples have improved our understanding of the fundamental molecular and cellular mechanisms that permit cell identity changes in live animals. In addition, the combination of a stereotyped lineage with single cell analyses allows dissection of the early and intermediate mechanisms of reprogramming, as well as their kinetics. As a result, several important concepts on *in vivo* cellular reprogramming have been recently developed. © 2011 Wiley Periodicals, Inc.

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## INTRODUCTION

Recent findings have shown that cells of many types are capable of retaining cellular plasticity, which can be exploited under the right circumstances. The classical view[1] of a totipotent (Box 1) embryonic cell sequentially shifting down a differentiation landscape that irreversibly specializes and restricts the cells' developmental options is contradicted by naturally observable exceptions as well as by recent breakthroughs in induced cellular reprogramming (Box 1). Differentiated cells cultured *in vitro* can be forced to directly adapt another identity or to revert to a pluripotent stem cell-like state by overexpression of just a handful of genes (for review, see Ref 2). To date, examples of induced reprogramming of a fully differentiated somatic cell *in vivo*[3] are very rare, which suggests that the challenges posed by cell intrinsic and extrinsic factors that maintain stable cellular identity *in situ* are difficult to overcome.

An understanding of cellular reprogramming *in vivo* will improve our knowledge of disease mechanisms whereby cells ectopically undergo transformation, such as in cancer. Moreover, besides the *in vitro* production of replacement cells, an important challenge of regenerative medicine (Box 1) is to implement and control cellular reprogramming *in vivo*, thereby replacing damaged or diseased tissue *in situ*. Thus, a better understanding of the requirements for direct *in vivo* reprogramming, which as we discuss below occurs in a more tightly controlled context, can facilitate better approaches for both *in vivo* and *in vitro* direct conversion. Indeed, a growing number of concerns are arising about the safety of cells induced to undergo pluripotent reprogramming *in vitro* and their proposed reintroduction into the body because of aberrant modifications.[4,5] However, to overcome the apparent difficulties of *in vivo*

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**BOX 1**

**DEFINITIONS OF SOME TERMS USED IN THIS REVIEW**

*Totipotency:* Describes the ability of a cell to give rise to all of the cell types of the body plus all of the cell types that make up the extraembryonic tissues such as the placenta.

*Dedifferentiation:* Process in which a differentiated cell loses its specialized characteristics and reverts to a less differentiated cell type.

*Redifferentiation:* Process in which a differentiated cell returns to a differentiated state (including alternative states to the original cell) after undergoing dedifferentiation.

*Reprogramming:* Describes the complete erasure of an initial differentiated identity and the adoption of a new identity, and thus represents an increase in cellular potential. It can lead to a dedifferentiated cell (e.g., during amphibian regeneration), a pluripotent cell (e.g., iPS cells) or, when 'direct', to a final identity that is also differentiated.

*Direct cell-type conversion/direct reprogramming/transdifferentiation:* Describes the switch from one differentiated state to another, different differentiated state. Can be induced by the experimenter or occur naturally.

*Regenerative medicine:* aims at reconstructing damaged/diseased tissue by stimulating resident cells or by providing replacement cells.

cellular reprogramming, an adequate understanding of the fundamental cellular and molecular mechanisms that regulate several aspects of cell identity is required. Particularly important are mechanisms that cells use *in vivo* to maintain a particular fate and how these can be compromised and redirected.

*Caenorhabditis elegans* offers an outstanding model in which to understand these basic principles and to then test them in an *in vivo* setting for several reasons. An essentially invariant cell lineage is defined[6] and thus allows unambiguous traceability of cell reprogramming events. Multiple tissue types and transparency at all developmental stages allow tracking of distinct cell-type reprogramming events in live animals. Amenability to genetic analysis allows unbiased rather than candidate-based approaches to uncover genes that can mediate cell-type conversion. In addition, rapid transgenic techniques that allow cell specific expression avoid the heavy investment in resources needed to test ideas in vertebrate models such as mouse or Zebrafish. Finally, the occurrence of stereotyped natural reprogramming events allows probing of the mechanisms involved at the much needed single cell level. Because of these amenities, all cellular transitions throughout the reprogramming process can be traced, a feat that is unreachable when cell populations are studied.

In this study, we discuss the growing number of examples of cell conversion in *C. elegans*, and how these have contributed to our understanding of cell reprogramming. The review is structured into three sections that cover three distinct categories of cells: germ cells, early embryonic cells (blastomeres), and postembryonic somatic cells.

## CELL CONVERSION IN THE GERMLINE

At the beginning of *C. elegans* development, like in many metazoans, only a few primordial germ cells are set aside and segregated from somatic cells. They are protected from differentiation-inducing signals by multiple layers of genetic insulation that include the establishment and maintenance of repressive histone modifications and the inhibition of RNA polymerase II (for review, see Ref 7). Removal of this repression, e.g., by loss of the PIE-1 CCCH-zinc finger protein that mediates global transcriptional repression through Polycomb (PcG) factors,[8] induces transformation of primordial germ cells into cells with a somatic fate[9–11] (Figure 1(b)). Germ cells later on proliferate in the gonad, giving rise to the many gametes necessary for successful reproduction. Within the gonad of adult worms, they exist in a sequentially positioned, developmentally linear relationship. A reservoir of proliferating germ stem cells is maintained close to a distal niche and is separated from a proximal region of gametogenesis by a centrally located meiotic zone (Figure 1(a)). Although germ cells are totipotent—able to give rise to every other cell in the organism—this potential is locked away until later.

Two proteins, GLD-1 and MEX-3, predicted to function as translational regulators, were found to maintain totipotency in the *C. elegans* germline.[12] Abnormal looking germ cell nuclei in *mex-3 gld-1* double mutants were revealed through a number of techniques to have acquired differentiated features of somatic cells from various lineages (Figure 1(d)). Two types of muscle (body and pharyngeal), neurons, and intestinal cells were all ectopically localized within *mex-3 gld-1* mutant gonads.[12] The presence of extensive cellular processes, like those of regular neurons, suggested that the germline neurons were fully differentiated. Moreover, the presence of filaments and contractile activity also suggested that the muscle

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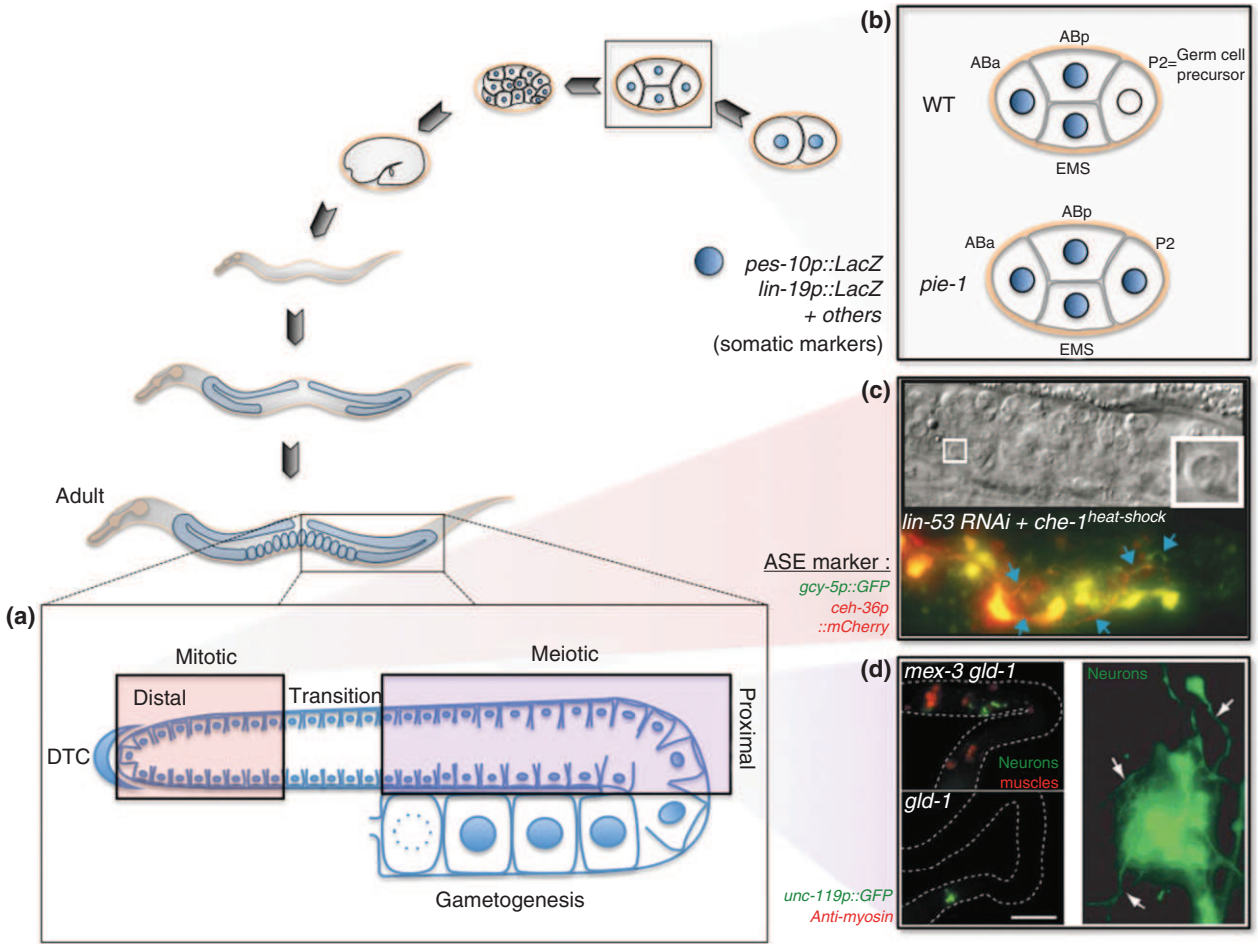


**FIGURE 1** | Germ cell conversion. (a) Schematic layout of the *Caenorhabditis elegans* adult gonad. The distal tip cell (DTC) creates a niche in which mitotic germ cells reside. The proximal gonad, where gametogenesis occurs, is separated from the mitotic region by germ cells undergoing meiosis. (b) Ectopic expression of somatic marker genes (represented in blue) in the germline precursor cell, P2, in *pie-1* mutant 4-cells embryos. (c) Conversion of germ cells into ASE neurons after *lin-53* RNA interference (RNAi) and ectopic expression of *che-1*. The Differential interference contrast (DIC) photograph shows an abnormal looking nucleus in the mitotic region of the gonad. The fluorescent photograph shows coexpression of two different ASE specific markers in these same cells. (Reprinted with permission from Ref 21. Copyright 2011 AAAS) (d) Conversion of germ cells into both neuron and muscle cells within the meiotic region of *mex-3 gld-1* double mutant gonads. (Reprinted with permission from Ref 12. Copyright 2006 AAAS)

cells, at least, were even functional. Total conversion of cellular identity appeared complete as germ cell characteristics, such as the presence of P-granules and the expression of P-granule proteins PGL-1, GLH-1, and GLH-4, were lost in the final somatic cells.[12]

The wide mixture of cell types present in the gonad suggests that *mex-3 gld-1* loss-of-function induces germline teratoma-like transformation. *gld-1* was previously described as a germline-specific tumor suppressor gene.[13–15] Strong loss-of-function or null mutations in *gld-1* lead to ectopic proliferation of germ cells following meiosis.[15] Germ cells normally destined to develop into oocytes enter meiosis but then inappropriately exit the pachytene stage of meiotic prophase and re–enter mitosis. GLD-1, a member of the signal transduction and activation of RNA (STAR) family of KH-domain RNA binding proteins,[16] was shown to repress translation of *cye-1* (cyclin E), a key cell cycle regulator, during meiotic prophase.[17] Derepression of *cye-1* was not only found to promote mitotic reentry in the germline (thus causing tumor formation) but also lead to transcriptional activation of early embryonic genes (*vet-1*, *vet-4* [*pes-2.1*], and *vet-6*), processes that could enhance acquisition of somatic cellular identity.

Both GLD-1 and MEX-3, which contains two RNA-binding KH domains,[18] have also been shown to repress the translation of key developmental mRNAs such as *pal-1* (Caudal in humans) in the germline.[18–20] In fact, a general role in suppression of inappropriate

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developmental mRNAs has been determined for MEX-3 in the embryo as well.[18] In *mex-3 gld-1* mutant animals, germ-to-muscle cell transformation apparently mimics that of normal somatic muscle development in the embryo, as both require a pathway involving PAL-1 and the downstream factor HLH-1 (similar to MYOD1 and MYF6 in humans).[12] It is therefore plausible that the coincident derepression of key cell cycle, early embryonic, and fate determination factors lead to the conversion of germ cells into various somatic cell types in *mex-3 gld-1* mutants.

The remarkably wide variety of cell types found within a single gonadal arm of *mex-3 gld-1* animals may result from stochastic translation of different fate-specifying factors. In this scenario, the dominant developmental factor would determine the fate of each individual germ cell. Indeed, lowering levels of *pal-1* mRNA by RNA interference (RNAi) repressed body muscle cell transformation in *mex-3 gld-1* mutants but had no effect on the conversion of germ cells into other cell types.[12] However, it remains to be seen if the converted individual germ cells simultaneously express markers of more than one cell type, which could originate from derepression of multiple tissue specific mRNAs.[12]

Whereas MEX-3 and GLD-1 appear to control germ cell plasticity through posttranscriptional regulation, a recent study pointed to another—latent—control layer, probably acting at the transcriptional level.[21] Removal of LIN-53 by RNAi (RBBP7 and RBBP4 in humans, formerly known as RbAp46 and RbAp48), a component of several histone remodeling and modifying complexes, allowed ectopic overexpression of genes driving various neuronal subtype identities (*che-1*$^{heat\text{-}shock}$, *unc-30*$^{heat\text{-}shock}$, and *unc-3*$^{heat\text{-}shock}$, see Table 1 for the transgenes used) to induce direct conversion of germ cells into neurons (Figure 1(c)). As in *mex-3 gld-1* mutants, P granules are lost in transformed cells, suggesting complete conversion in at least some cells. However, unlike *mex-3 gld-1* double mutants, where germ cells spontaneously underwent direct conversion, *lin-53* inactivation alone had no effect on germ cell fate. Thus, absence of *lin-53* activity does not appear to potentiate developmental factors that are endogenously expressed or stored as mRNAs in germ cells for postfertilization development. Instead, it appeared that removal of *lin-53* primed the potential of the germ cells to undergo inducible reprogramming into neurons. In further contrast to *mex-3 gld-1* mutant animals, where a mixture of somatic cell types were observed, germ cells were converted into very specific neuron types corresponding to the particular fate determinant factor ectopically expressed. Also, in *mex-3 gld-1* mutants, germ cell conversion required entry into meiosis.[12] This was not the case after *lin-53* inactivation, which allowed germ cell conversion within the mitotic pool (Figure 1(a)). These and other differences between germline reprogramming mediated by the loss of MEX-3/GLD-1 and that primed by LIN-53 removal suggest that they each represent a completely different mode by which germ cells can be converted into fully differentiated somatic cells.

Although the mechanisms are not yet understood, it is intriguing that distinct forms of genetic repression (posttranscriptional and transcriptional) are employed in different regions of the gonad to prevent inappropriate transformations in cell identity. For LIN-53-primed reprogramming, it is conceivable that its activity represses developmental gene expression in the mitotic germ cells like it does in vulval precursor cells.[22] Mammalian LIN-53/ RBBP7/RBBP4 is involved in recruiting various histone modifiers and remodelers (such as the Polycomb repressor complex, PRC2, and the nucleosome remodeling complex, NuRD) to loci to silence transcription (for review, see Refs 23 and 24). Its inactivation may allow artificially induced neuronal terminal selector factors to enact expression of downstream terminal differentiation genes, which would otherwise be kept silent in a LIN-53 dependent manner. Indeed, chemical inhibition of histone deacetylases (HDACs), enzymes which participate in condensing and thus silencing chromatin, also primes germ cells for reprogramming, like *lin-53* inactivation.[21] Whereas it remains possible that deacetylation of targets other than histones are involved, these results suggest that perturbing the establishment or maintenance of characteristically repressive histone modifications potentiates the induced acquisition of somatic cell identity in germ cells. One intriguing aspect of LIN-53 primed germ cell reprogramming is that it only allows acquisition of neural somatic identity, and not that of other tissue types, such as muscle.[21] It could be that LIN-53 mediates recruitment of histone modifying factors specifically to pan-neural loci in mitotic germ cells. In this respect, it would be telling to determine whether the broad inhibition of HDACs allows a much wider, unspecific, acquisition of somatic cell identity in these cells. Moreover, do other LIN-53-like factors specifically allow acquisition of different cell types in the germline? If so, as RBBP7/RBBP4 proteins have been shown to bind histones rather than DNA, what mechanism drives the specificity of the priming for a tissue, e.g., pan-neural for LIN-53? With recent breakthroughs in tools that allow automated and faster large-scale forward genetics (mutant sorting[25] and

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**TABLE 1** | Molecular Toolbox for Cell Reprogramming in *Caenorhabditis elegans*

| Conversion Type | Reprogramming Factor | Allele, Array, or Strain | Reprogramming Conditions | Cell Type | Marker Transgene | Allele, Array, or Strain | References |
|---|---|---|---|---|---|---|---|
| Germ cells to neurons | *hsp::che-1*<br>*hsp::unc-3*<br>*hsp::unc-30* | *otIs284*<br>*otEx4441*<br>*nEx648* | RNAi of *lin-53* + heat-shock for 30 min at 37°C<br>Stage: larval (L2 best efficiency) | ASE neuron | 8xASEmotif::*gfp* | *otIs300* | Tursun et al.[21] |
| | | | | | *gcy-5p::gfp* | *ntIs1* | |
| | | | | | *gcy-7p::rfp* | *otIs131* | |
| | | | | | *ceh-36p::Tagrfp* | *otIs264* | |
| | | | | | *eat-4p::mCherry* | *otIs291* | |
| | | | | Pan-sensory neurons | *osm-6::gfp* | *oyIs59* | |
| | | | | Pan-neuronal | *unc-33p::gfp* | *otIs118* | |
| | | | | | *F25B3.3::DsRed2* | *otIs301* | |
| | | | | | *rab-3p::NLS-Tagrfp* | *otIs314* | |
| | | | | | *snb-1::NLS-Tagrfp* | *otEx4445* | |
| | | | | | *unc-119p::gfp* | *otIs45* | |
| | | | | Dopaminergic and serotonergic neurons | *cat-1p::mCherry* | *otIs266* | |
| | | | | Command interneuron | *glr-1p::mCherry* | *hdIs30* | |
| | | | | Cholinergic AIY interneuron | *ttx-3p::mCherry* | *otIs133* | |
| | | | | D-type motor neurons | *ttr-39p::rfp* | *juIs224* | |
| | | | | Muscle | *myo-3p::mCherry* | *otEx4440* | |
| | | | | AWB neuron | *str-1p::rfp* | *oyIs54* | |
| | | | | Cholinergic A/B-type motor neurons | *acr-2p::gfp* | *juIs14* | |
| | | | | GABAergic motor neurons | *unc-47p::gfp* | *oxIs12* | |
| | | | | Germ cells | PGL-1 antibody | — | |
| Blastomere to muscle | *hsp::hlh-1* | *KM267 & KM289* | Heat-shock for 30 min at 34°C<br>Stage: 2E to 10.2E[1] | Muscle | MHC A antibody | — | Fukushige et al.[29] |
| | | | | | UNC-89 antibody | — | |
| | | | | | UNC-98 antibody | — | |
| | | | | | PAT-3 antibody | — | |
| | | | | | *hlh-1p::gfp* | PD7963 | |
| | | | | | *myo-3p::gfp* | PD4251 | |
| | | | | Intestine | *elt-2p::gfp* | JM63 | |
| | | | | Hypodermis | LIN-26 antibody | — | |
| | | | | Pharynx | 3NB12 antibody | — | |

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**TABLE 1** | Continued

| Conversion Type | Reprogramming Factor | Allele, Array, or Strain | Reprogramming Conditions | Cell Type | Marker Transgene | Allele, Array, or Strain | References |
|---|---|---|---|---|---|---|---|
| Blastomere to endoderm | *hsp::end-1* | *wIs47* | Heat-shock for 30 min at 33–34°C<br>Stage: 2E–8E | Germ cells | P-granules antibody | — | Zhu et al.[32] |
| | | | | | ELT-2 antibody | — | |
| | | | | Intestine | 1C84 antibody | — | |
| | | | | | MH33 antibody | — | |
| | | | | | GES-1 activity | — | |
| | | | | Hypodermis | Lin-26 antibody | — | |
| | | | | Body wall muscle | *myo-3p::gfp* | *ccIs4251* | |
| Blastomere to epithelium | *hsp::lin-26* | *mcIs22 & mcIs23* | Heat-shock for 25 min at 33°C<br>Stage: 2E–8E | Epithelial | *che-14p::gfp* | *mcEx242* | Quintin et al.[34] |
| | | | | | *dlg-1::gfp* | *mcIs47* | |
| | | | | | *ajm-1::gfp* | *jcIs1* | |
| | | | | | MH27 antibody | — | |
| | | | | Epidermis | *dpy-7p::gfp* | *ijIs12* | |
| | | | | | *let-502::gfp* | *sbEx136* | |
| | | | | Hypodermis | *elt-1p::gfp* | *vpIs2* | |
| | | | | | LIN-26 antibody | — | |
| | | | | Pharynx | PHA-4 antibody | — | |
| | | | | | 3NB12 antibody | — | |
| | | | | Body wall muscle | NE8-4C6 antibody | — | |
| | | | | Intestinal Cells | 1CB4 antibody | — | |
| Blastomere to intestine | *hsp::elt-2* | *caIs8* | Heat-shock for 30 min at 33–34°C<br>Stage: from 4E | Intestine | ELT-2 antibody | — | Fukushige et al.[31] |
| | | | | | *elt-2p::LacZ* | — | |
| | | | | | GES-1 activity | — | |
| Blastomere to hypoderm | *hsp::elt-1* | *caIs4/caIs5/ caIs6* | Heat-shock for 40 min at 33.5°C<br>Stage: from 4-cell stage to 8E | Epidermis | *dpy-7p::gfp* | *ijIs12* | Gilleard et al.[35] |
| | | | | Hypodermis | LIN-26 antibody | — | |
| | | | | | *elt-3p::gfp* | *VpIs1* | |
| | *hsp::elt-3* | *caIs9/caIs10/ caIs11* | | Epithelial | MH27 antibody | — | |
| | | | | Muscle | *myo-3p::gfp* | *ccIs4251* | |
| | | | | Pan-neuronal | *rgef-1p:gfp* | *evIs111* | |



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**TABLE 1** | Continued

| Conversion Type | Reprogramming Factor | Allele, Array, or Strain | Reprogramming Conditions | Cell Type | Marker Transgene | Allele, Array, or Strain | References |
|---|---|---|---|---|---|---|---|
| Intestinal or hypodermal to germ cells | *mep-1* | — | RNAi of *mep-1* | Germ cells | PGL-1 antibody | — | Unhavaithaya et al.[8] |
| | | | | | GLH-2 antibody | — | |
| | | | | | GLH-3 antibody | — | |
| Epithelial to neuronal | *sem-4*<br>*egl-5*<br>*lin-12*<br>*unc-3* | | Naturally occurring | PDA (motoneuron) | *exp-1p::gfp* | *wyIs75* | Jarriault et al.[54] and Richard et al. [55] |
| | | | | | *cog-1p::gfp* | *syIs63* | |
| | | | | | *ace-3/4p::gfp* | *fpIs1* | |
| | | | | Pan-neuronal | *unc-119p::gfp* | *edIs6* | |
| | | | | | *tag-168p::gfp* | *ncIs3* | |
| | | | | | *unc-33p::gfp* | *otIs117* | |
| | | | | Y (rectal epithelial) | *egl-26p::gfp* | *kuIs36* | |
| | | | | | *egl-5p::gfp* | *bxIs7* | |
| | | | | | *cki-1p::gfp* | *maIs113* | |
| | | | | Epithelial | *che-14::gfp* | *mcEx177* | |
| | | | | | *ajm-1::gfp* | *jcIs1* | |
| | | | | | *dlg-1::gfp* | *mcIs47* | |
| | | | | | LIN-26 antibody | – | |
| | | | | Body wall muscle | *myo-3p::gfp* | *ccIs4251* | |
| | | | | Hypodermal | *dpy-7p::gfp* | *arIs99* | |
| | | | | Endodermal | *elt-2p::gfp* | *wIs84* | |
| | | | | Mechanosensory neuron | *mec-7p::gfp* | *muIs35* | |
| | | | | GABAergic neuron | *unc-47p::gfp* | *krIs6* | |
| | | | | DA1-9 neuron | *unc-4p::gfp* | *wdIs4* | |
| | | | | DA3-7 neuron | *dlb-1p::gfp* | *ctIs43* | |
| | | | | DA9 neuron | *ser-2p::gfp* | *otIs107* | |
| | | | | DA9 neuron | *mig-13p::gfp* | *muIs62* | |

[1]The 10.2E stage corresponds to an incubation time of 210 min at 22°C after 1–2 cell stage embryo is isolated. Note that *unc-3*$^{heat\text{-}shock}$, for instance, represents actual induction of transcription by heat shock, whereas *hsp::unc-3* describes the transgene used.

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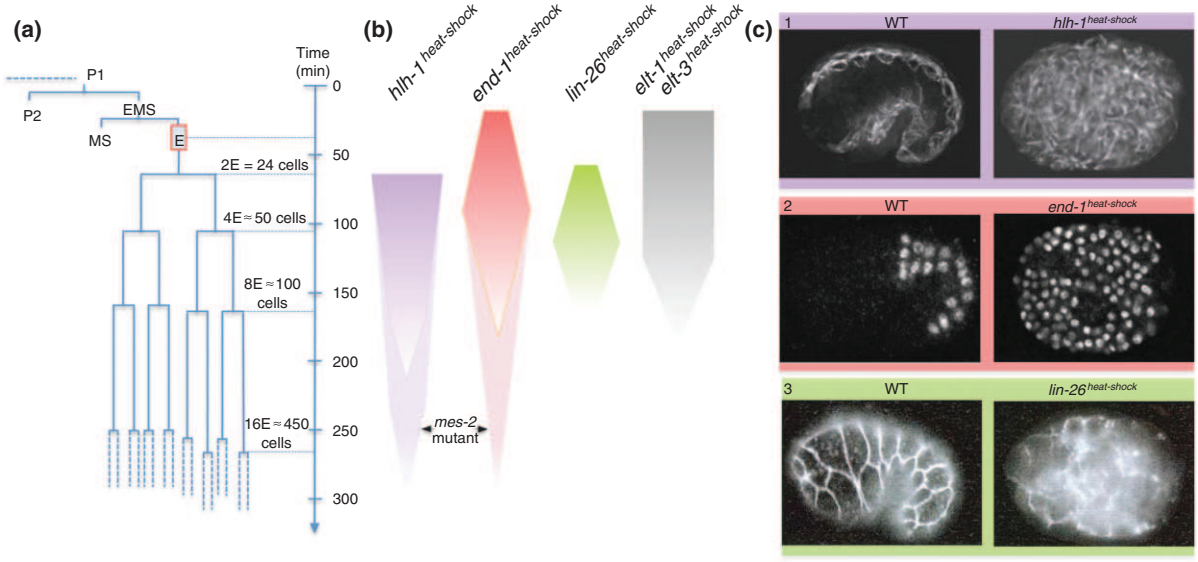


**FIGURE 2** | Blastomere conversion. (a) Endodermal (E) cell lineage in the embryo; (nE), number of E descendants, followed by the total number of embryonic cells; the time scale starts at the first embryonic cleavage. (b) Competence windows for widespread blastomere conversion following ectopic expression of the indicated transgenes. Widespread conversion of blastomeres into muscle cells, *hlh-1*$^{heat\text{-}shock}$; intestinal cells, *end-1*$^{heat\text{-}shock}$; epithelial cells, *lin-26*$^{heat\text{-}shock}$; and epidermal cells, *elt-1*$^{heat\text{-}shock}$ and *elt-3*$^{heat\text{-}shock}$ is shown. Both *hlh-1*$^{heat\text{-}shock}$ and *end-1*$^{heat\text{-}shock}$ induced reprogramming of blastomeres can be extended in a *mes-2* mutant background (different color shade). The widest section of each window represents the embryonic stage where the efficiency of reprogramming is the highest. Data are compiled from Refs 29, 32, 34, 35, and 45. (c) 1–3: Comparison of WT embryos versus embryos carrying either (1) *hsp::hlh-1*, or (2) *hsp::end-1*, or (3) *hsp::lin-26* transgenes and induced under heat shock conditions. (1) Muscle cell identity is observed with an antibody against MHCa. (Reprinted with permission from Ref 29. Copyright 2005 Company of Biologists) (2) Intestinal cell identity is observed through expression of *elt-2p::gfp*. (Reprinted with permission from Ref 32. Copyright 1998 Cold Spring Harbor Laboratory Press) (3) Epithelial identity is observed through expression of *dlg-1::gfp*. (Reprinted with permission from Ref 34. Copyright 2001 Elsevier)

rapid mutation identification[26,27]), it may only be a matter of time as to when new factors are discovered that prime the adoption of different tissue types.

## CELL CONVERSION IN THE EMBRYO

Upon fertilization, the zygote undergoes a set of cleavages forming six founder cells (AB, MS, E, C, D, and $P_4$).[6] The E (endoderm) blastomere is the clonal progenitor of the entire gut (20 cells in total in the adult) and its subsequent number of daughters will serve here as a reference to the developmental stage of the embryo (Figure 2(a)). Until the onset of gastrulation (the two endoderm or 2E stage, 24 cells in total), maternally inherited products help to control patterning and fate specification. As the experiments presented below demonstrate, blastomeres remain pluripotent during this time. After gastrulation, zygotic genes that specify organ and tissue identities gradually undergo activation, which proceed to control the rest of differentiation. Morphogenesis begins at the 550-cell stage and the embryo hatches as a larvae with 558 cells.[6]

Adoption of alternative cell fates in embryonic blastomeres can be achieved through various means in *C. elegans*. For instance, when cells are physically manipulated into new positions within the embryo, intercellular signals are mistakenly received by the inappropriate cell, leading to ectopic acquisition of an alternative fate.[28] For example, the ABp blastomere that does not normally give rise to pharyngeal muscle cells does so when moved into the position of the ABa blastomere.[28] Alternatively, blastomere reprogramming has been induced through forced ubiquitous expression of potent *C. elegans* developmental factors. In such cases, reviewed below, reprogramming is mediated by the sole expression of just one potent transcription factor, sufficient to erase the natural commitment of blastomeres and induce the partial or apparently complete expression program of a defined tissue.

Induced *hlh-1*$^{heat\text{-}shock}$ expression is sufficient to reprogram almost all blastomeres into body muscle cells, e.g., Figure 2(b) and (c)1.[29] The converted cells express several muscle markers including major histocompatibility complex a (MHCa) and a *myo-3p::gfp* transgene (Table 1) and are positive for filamentous actin, although filaments are disorganized and the cells do not contract. Furthermore, markers characteristic of other tissues (intestine,

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hypodermis, and pharyngeal muscle) are lost in these reprogrammed embryos, indicating the cells are not of a mixed identity.[29] It was determined also that the myogenic activity of HLH-1 was not solely a result of repression of other cell fates, leading to a default muscle cell fate, but rather an active consequence of HLH-1 activity. Similar results were found upon *pal-1*$^{heat\text{-}shock}$ induction, which drove ectopic body wall myogenesis through the activation of endogenous *hlh-1* and possibly other redundant factors.[29] However, in the presence of POP-1, the *C. elegans* TCF-like Wnt effector,[30] *pal-1*$^{heat\text{-}shock}$ induction promotes hypodermal fate.

The window during which *hlh-1*$^{heat\text{-}shock}$ activation is able to alter cell fate was found to be surprisingly broad, spanning 3 h into embryonic development[29] (Figure 2(b)). At 3 h into embryonic development, which is around the 8E cell stage (>100 total cells), lineages are established in wild type embryos and cells even begin to express nonmuscle lineage-restricted markers (e.g., *end-1* and *elt-2*). This suggests that forced expression of HLH-1 is not simply directing muscle development but is rather extinguishing endogenous cell fate lineage programs and respecifying an altogether different cell fate course.

The blastomere reprogramming activities of *hlh-1* and *pal-1* are not unique. Blastomere-to-intestine through *end-1*$^{heat\text{-}shock}$ or *elt-2*$^{heat\text{-}shock}$ induction,[31,32] blastomere-to-pharyngeal cells through *pha-4*$^{heat\text{-}shock}$ induction,[33] blastomere-to-epithelia through *lin-26*$^{heat\text{-}shock}$ induction,[34] and blastomere-to-epidermis through either *elt-3*$^{heat\text{-}shock}$ or *elt-1*$^{heat\text{-}shock}$[32,35] have all been described in *C. elegans* embryos (Figure 2 and Table 1). Thus, blastomeres can be reprogrammed into cell types of all three germ layers.

Like *hlh-1*$^{heat\text{-}shock}$, *end-1*$^{heat\text{-}shock}$ (GATA-like transcription factor) induction could reprogram blastomeres into intestinal cells after the onset of gastrulation and also attenuated lineage markers of other cell types (ectoderm, body muscle, and pharyngeal muscle).[32] The reprogrammed cells contained birefringent granules (a characteristic of gut cells) and several other morphological characteristics of intestinal cells. They also displayed molecular hallmarks of intestine such as ELT-2 expression, GES-1 esterase activity, and intestine specific antigen recognition.[32]

*lin-26*$^{heat\text{-}shock}$ activation was found to induce ectopic epithelial identity the most strongest during early gastrulation (28–100 cell stages).[34] Ultastructural analysis revealed that the reprogrammed blastomeres developed adherens-like junctions, a characteristic of epithelial cells. Accordingly, they expressed the adherens junction protein AJM-1 and DLG-1, an essential AJM-1 assembly factor. Furthermore, CHE-14 that is involved in apical trafficking was also ectopically expressed. Despite this, *lin-26*$^{heat\text{-}shock}$ activation did not appear to lead to fully differentiated epidermal cells, as polarity defects and the absence of induction of several other epidermal-specific genes suggested. This nonepidermal epithelial-like identity coincided with loss of other tissue types (body wall muscle, pharynx, rectum, and intestine). Laser microdissection of embryos also showed that each of the first four blastomeres (ABa, ABp, EMS, and $P_2$) was able to be reprogrammed upon induction of *lin-26*$^{heat\text{-}shock}$. However, P2 blastomeres, which generate the germline, were more refractory to ectopic reprogramming, a result also seen with *end-1*$^{heat\text{-}shock}$ induction.[32]

Ectopic PHA-4 expression (FoxA in mammals) showed its strongest effect on the embryo at the 4E stage.[33] Staining with pharyngeal muscle and pharyngeal marginal cell antibodies revealed widespread, albeit incomplete, pharyngeal identity acquisition. Activation of *pha-4*$^{heat\text{-}shock}$ also strongly reduced the presence of other tissue types (body wall muscle, epidermal, and neuronal support cells). In this case, it appears that PHA-4 acts to both activate pharyngeal genes while cooperating with the TRIM protein TAM-1 and NuRD components to repress other developmental genes such as *lin-26*.[36,37] Indeed, removal of *pha-4* causes pharyngeal precursors to transform into ectodermal cell types expressing *lin-26*.[33,38]

These potent transcription factors, which normally act to specify distinct cell and tissue types,[33,39–44] are all sufficient to force ectopic and specific changes in blastomere cell fate. However, in all cases, blastomeres are only responsive up until approximately the 8E cell stage (Figure 2(b)), after which they are remarkably resistant to these potent factors.

One of the mechanisms that restrict the competence of later stage blastomeres to be reprogrammed involves a complex similar to PRC2 in *Drosophila* and mammals.[45] In *C. elegans*, a complex of MES-2/EZH2 (Human)/EZ (*Drosophila*), MES-3/novel, and MES-6/EED (Human)/ESC (*Drosophila*) methylates H3K27 *in vitro* and *in vivo*.[46] When challenged with *hlh-1*$^{heat\text{-}shock}$ or *end-1*$^{heat\text{-}shock}$, *mes-2* mutant embryos were approximately twice as responsive to ectopic reprogramming than normal at the 4E and 8E stages.[45] This suggested that *mes-2* helps terminate plasticity in the developing cells, loss of which extends the window of developmental plasticity (Figure 2(b)). It was also found that PRC2 performs this role independently of

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cell fate specification, in that misregulation of developmental regulators such as *end-1* and *pha-4* is not responsible for extending the developmentally plastic window. Instead, *mes-2* might help terminate plasticity in developing embryos through global chromatin reorganization (i.e., compaction and localization in the nuclear periphery).[45]

## CHANGES IN CELLULAR IDENTITY IN THE LARVAL/ADULT SOMA

Relatively simple genetic, chemical, or physical manipulation can induce complete conversion of germ cells or blastomeres into alternative fates. Germ cells and early blastomeres are, however, totipotent and pluripotent, respectively, and are localized within an environment of developmentally naïve cells. Therefore, these cells can be considered quite amenable to reprogramming. During gastrulation, a more differentiated identity is established and somatic cells progressively adapt a refractory position toward transcription-factor-induced reprogramming.[29,31–35] A major challenge is to reprogram these cells that already have an established, differentiated identity. Cellular reprogramming conducted on cultured cells *in vitro* have shown that such a goal is achievable.[2] Moreover, pancreatic exocrine cells have been converted into $\beta$-cells *in vivo* in mice, although both cell types do originate from the same pancreatic endoderm.[3] To progress further, a deeper understanding of *in vivo* cell fate maintenance and plasticity is required.

Several mutants have been described in *C. elegans* that lead to misexpression or destabilization of cell identity. These examples provide fascinating insights into the mechanisms that establish as well as maintain cell identity. For instance, hypodermal and intestinal cells in early larvae can be misdirected by RNAi of a conserved Kruppel-type zinc-finger protein, MEP-1, to develop into cells exhibiting germ cell morphology and P granule expression[8] (Figure 3(a)). It appears that MEP-1 acts through the NuRD complex to transcriptionally suppress germline specific genes. Indeed, continued expression of somatic genes normally specific to the hypodermis and intestine suggest that the cells are of mixed identity. Mutations in the retinoblastoma (Rb) pathway have also been shown to change hypodermal and intestinal cells into P granule-expressing cells.[47] Recently, these cells were also shown to upregulate numerous other germ cell markers, such as meiosis proteins, a characteristic that was enhanced with higher environmental temperatures.[48] Despite incomplete conversion, it is intriguingly possible that these germ-like cells gain developmental plasticity (especially at higher temperatures), the potential of which might be exploited through techniques used previously in the germline.[12,21]

In another example, inactivation of *bet-1*, which encodes a double bromodomain-containing protein that associates with chromatin marked with acetylated histones, can induce several cell types to change into alternative lineage relatives in postembryonic worms.[49] This suggests BET-1 acts in accordance with epigenetic criteria to maintain cell fate after helping to establish it. In contrast to the germline and early embryo, where epigenetic mechanisms appear to solely repress inappropriate expression of genes, in differentiated somatic cells, epigenetic mechanisms appear to both repress inappropriate expression as well as maintain expression of genes that specify correct cell type. This twofold level of regulation of cellular identity probably explains the difficulty associated with complete reprogramming of fully differentiated somatic cells. Thus unlike in the germline, where only one molecular factor (an inhibitor of expression) needs to be overcome to reprogram cells, it is imaginable that multiple molecular factors need to be overcome in somatic cells. In addition, cell extrinsic factors, such as the differentiated environment within which the cell is situated, add to the complexity.

One strategy to overcome these multiple hurdles artificially is to firstly understand what exactly they are and how they have been overcome naturally through evolution. In nature, there are instances whereby differentiated cells naturally change identity. Prominent examples include photoreceptor conversion during metamorphosis[50] as well as segment boundary cell reprogramming in the fly,[51] astrocytes to neuron conversion during adult neurogenesis,[52] and the formation of coronary arteries by developmental reprogramming of venous cells.[53]

There is at least one instance of natural direct cellular reprogramming in *C. elegans* that has been characterized—transdifferentiation of the Y cell into PDA[54] (Figure 3(b)). This stereotyped process, where a rectal–epithelial cell (named Y), born at the 300 cell embryonic stage and lasting until the L2 larval stage,[6] transforms into a motoneuron (named PDA) after retraction from the rectal tube, occurs without cell division.[55] It is not yet understood why this process occurs, but it is possibly an adaptation to a rigid cell number in the postmitotic *C. elegans* soma. By contrast, the other neighboring cells of the rectal tube, a vital organ made of three pairs of cells arranged into three rings, are born and remain rectal the entire length of the animal life. Thus in this model, as

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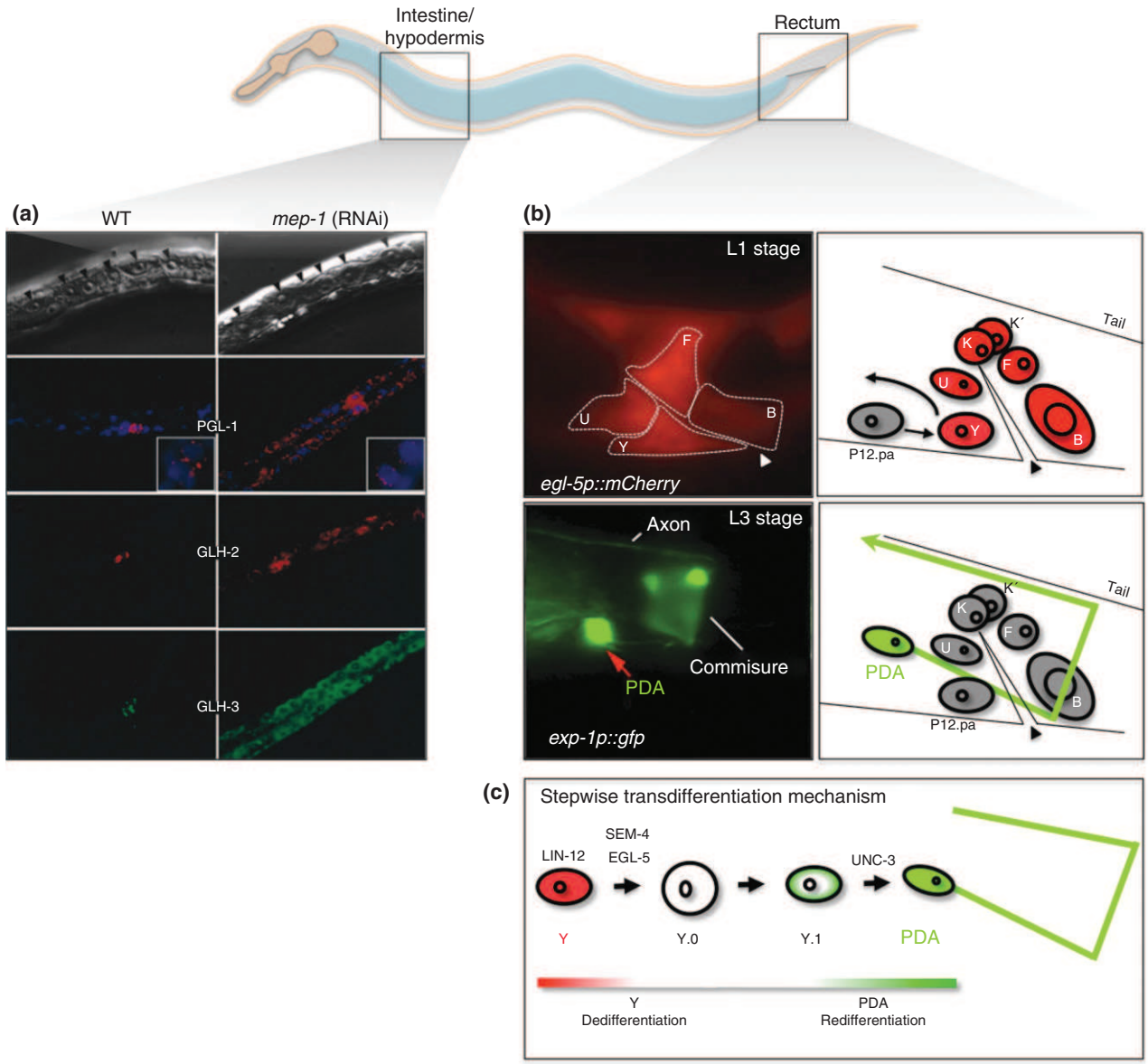


**FIGURE 3** | Postembryonic somatic cell conversion. (a) Conversion of intestinal cells into germ-like cells that express the P-granule proteins PGL-1, GLH-2, and GLH-3 after *mep-1* RNAi. (Reprinted with permission from Ref 8. Copyright 2002 Elsevier) (b) Natural conversion of an rectal epithelial cell named Y (marked by *egl-5p::mCherry*) into a motoneuron named PDA (marked by *exp-1p*::GFP) during the second larval stage. Fluorescent image of PDA is taken from Ref 55. (c) Y-to-PDA transdifferentiation occurs in a stepwise manner whereby Y first loses all of its differentiated epithelial characteristics (Y.0, dedifferentiation) and then begins to acquire PDA neural characteristics (Y.1, redifferentiation), before becoming a fully differentiated motoneuron. LIN-12, SEM-4, and EGL-5, as well as UNC-3, are all required at different steps of the process.

opposed to models involving cell populations, the initiation of direct reprogramming can be predicted and the process followed at the single cell level, a key asset to dissect the steps involved. The LIN-12/notch-signaling pathway is required to give competence to Y to change its identity and *sem-4* and *egl-5*, which encode a zinc-finger transcriptional regulator and a homeodomain transcription factor, respectively, are required for initiation of the process.[54] Initiation leads first to the complete loss of the rectal–epithelial identity of Y, suggesting a dedifferentiation, which is followed by a stepwise redifferentiation into an early neural cell, and then into a motoneuron with PDA identity[55] (Figure 3(c)). The COE transcription factor UNC-3 is required to redifferentiate the intermediate Y.1 early neural cell into PDA, and could thus represent a terminal differentiation factor.[55] Interestingly, the intermediate Y.0 dedifferentiated

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step of Y-to-PDA transdifferentiation is refractory to ectopic reprogramming suggesting an uncoupling of dedifferentiation and pluripotentcy in this context (Figure 3(c)). Moreover, none of the intermediates could be forced backward to adapt an epithelial identity, suggesting that tight controls are in place to restrict acquisition of an aberrant identity at all steps.[55] This could represent safeguard mechanisms to protect the organism against a reprogrammed event gone awry. Indeed, in mutants where this process is blocked midway, the intermediate cell does not die but remains remarkably stable at its position in the animal. An important next goal is to elucidate the nature of these tight controls, which in light of other examples discussed here, are likely to involve the control of the transcriptional activity of rectal and neural loci through chromatin modifications.

## CONCLUSION

There are several important concepts that can be drawn from studies performed thus far on cellular reprogramming in *C. elegans*. Firstly, cellular context greatly impacts on the permissibility of reprogramming. Ectopic expression of cell fate determinant factors is sufficient to drive cellular reprogramming of early embryonic cells into a wide variety of cell types derived from all three germ layers.[29,31–35] Reprogramming of meiotic germ cells requires removal of an inhibitory factor,[12] which leads to spontaneous cellular conversion. However, mitotic germ cell reprogramming requires both removal of an inhibitory factor as well as ectopic expression of cell fate determinant factors, and only thus far has been shown with neural cell conversion.[21]

Postembryonic somatic cells are perhaps on an entirely different level: switches in cell fate in mutant contexts have led to either cells with mixed identity[8] or cells switching between very similar or lineally related types.[49] In addition, these switches are obtained following removal of activities that appear important for cell identity maintenance. By contrast, forced expression of cell fate determinants in early larvae, where blast cells are present, or late larvae leads to very restricted cases of cell-type conversions, if any, in the worm[21,55] (S. Zuryn and S. Jarriault, unpublished). Thus, the cellular context impacts greatly on the latent plasticity of a cell, and variations are observed between both cell types and developmental stage. It seems that, in the worm, both the expressed potential, i.e., the range of cell types naturally derived from a cell, and the latent potential, i.e., the ability and the range of tissues a cell can be forced to be reprogrammed into, become more restricted during development. In this context, elucidating the molecular mechanisms of a complete and natural direct reprogramming event, where latent potential is either retained or reacquired (such as the Y-to-PDA model), may prove useful for devising improved strategies for induced somatic reprogramming.

A second important concept is that DNA replication and/or cell division is not absolutely required for cellular conversion. Although the impact of cell division has not been determined in blastomere cell-type conversion or that mediated/primed in the germline by MEX-3/GLD-1 or LIN-53 removal, Y-to-PDA conversion explicitly occurs in the absence of division (as well as fusion to another cell).[54] In terms of regenerative medicine, this is a beneficial feature, as requirements for cell divisions and thus DNA replication may increase the likelihood of introduction of aberrant chromosomal alterations, as has been observed in *in vitro* reprogramming,[4,5] In addition, it ensures faster production of the desired cells.

Thirdly, redifferentiation into the new identity apparently employs the same mechanisms as regular developmental differentiation. Meiotic germ cells undergo transformation into muscle cells through *pal-1*, which is required for normal muscle development in the embryo.[12] Transdifferentiation of Y-to-PDA, which has revealed an important cellular mechanism that allows direct conversion *in vivo*, namely a stepwise dedifferentiation and then redifferentiation process into the new cellular identity, also requires the neural terminal differentiation gene *unc-3*,[55] shown to be involved in development of other neurons.[56]

The molecules that mediate and drive *in vivo* cellular reprogramming in *C. elegans* are highly conserved. Mammalian orthologs of the potent transcription factors that can ectopically drive cell fates in *C. elegans* have developmentally conserved roles in tissue differentiation. Also, a conserved set of interacting pluripotency factors has been found to be strictly required to initiate Y dedifferentiation (A. Ahier, K. Kagias, and S. Jarriault, unpublished). Thus, much of what has been learned and will be learned using *C. elegans* as an *in vivo* model system is likely applicable to cells of other species, including human. Detailed processes that may be obscured in other systems because of their complexity (vertebrates) or lack of physiological cues (*in vitro* cell culture) are fully accessible in *C. elegans*. It is in such details that fundamental requirements for exploiting and controlling *in vivo* cellular reprogramming may be found.

Indeed, being able to manipulate cell identity so that abundant patient cells could be used to produce

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replacement cells opens new avenues for cellular therapy strategies. Various alternatives are being considered to produce replacement cells that would bypass the problems associated with immunorejection: resident stem cells from patient, redifferentiation of patient-derived induced pluripotent stem (iPS) cells, or direct reprogramming of patient somatic cells. Before implementation into the clinic, an accurate evaluation of the risks associated with each strategy is needed. For example, recent research has pointed to the chromosomal abnormalities and the tumorigenic risks associated with the use of stem cell-like cells. Because of its simplicity, comparatively lower cost and no cell division requirement, direct *in vitro* or *in vivo* reprogramming may thus represent a promising alternative. In addition, the existence of safeguard mechanisms to restrict the potential of intermediates during natural direct *in vivo* reprogramming in *C. elegans* further underlines the potential of such strategy. Finally, other challenges associated with cellular therapy include the maintenance of the newly reprogrammed identity in its complex *in vivo* environment and the functional integration of the replacement cells in the relevant tissue. In these respects, inducing resident somatic cells that are already integrated within an organ or tissue, to adapt the identity of the missing cells might represent an advantage. It is thus hoped that the research on cellular reprogramming will widen our cellular therapy horizon.

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UNIVERSITÉ DE STRASBOURG

# Thomas DANIELE

## How a differentiated cell can change its identity: Study of the role of the LIN-12/Notch pathway in the establishment of the competence to transdifferentiate *in vivo* in *C. elegans*

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## Résumé en français

L'acquisition d'une identité cellulaire différenciée est souvent considérée comme définitive et figée dans le temps; or un nombre croissant d'études démontre que les cellules différenciées peuvent faire preuve de plasticité sous certaines conditions.

Afin de mieux comprendre ces phénomènes, notre laboratoire a établi un modèle unique chez *Caenorhabditis elegans* (*C. elegans*) permettant l'étude d'un événement de transdifférenciation dans un contexte physiologique à l'échelle de cellules uniques. Au cours du développement, une cellule épithéliale du rectum de *C. elegans*, nommé Y, va migrer antérieurement puis changer d'identité pour devenir un motoneurone nommé PDA.

Les travaux préliminaires du laboratoire ont montré que la voie de signalisation LIN-12/Notch est le signal le plus précoce nécessaire pour le bon déroulement de la transdifférenciation de Y en PDA.

Nous avons pu mettre en évidence : **i)** que lors de l'embryogénèse, deux ligands canoniques (*apx-1* et *lag-2*) semblent agir de façon redondante afin d'activer la voie Notch. **ii)** l'activation ectopique et contrôlée de la voie Notch est suffisante pour induire la formation d'un second neurone PDA. **iii)** Les facteurs nucléaires que le laboratoire a identifiés comme cruciaux pour l'initiation de cet évènement de TD sont également importants pour la reprogrammation induite de cette deuxième cellule en neurone PDA par l'activation ectopique de Notch. **iv)** La suractivation prolongée de la voie Notch dans la cellule Y maintien l'identité épithéliale de cette dernière, ayant pour conséquence le blocage de la transdifférenciation de Y en PDA.

L'ensemble de nos résultats montrent que la voie Notch est nécessaire et suffisante afin d'établir la compétence à transdifférencier et que cela ne peut être réalisé que si la voie Notch est régulée de façon très précise dans la cellule Y.

## Résumé en anglais

The acquisition of a differentiated cell identity is often considered as final and frozen in time. However, a growing number of studies showed that differentiated cells can exhibit plasticity under certain conditions.

To better understand these cell plasticity phenomena, our laboratory has developed a unique model in *Caenorhabditis elegans* (*C. elegans*) to study a transdifferentiation event in a physiological context and at the single-cell level. During the worm development, an epithelial rectal cell, named Y, will migrate anteriorly and change its identity to become a neuron named PDA.

Preliminary work performed by our laboratory showed that the LIN-12/Notch signalling pathway is the earliest signal necessary for the proper conduct of the transdifferentiation of Y into PDA.

In our study, we showed that: **i)** during embryogenesis, two canonical ligands (*apx-1* and *lag-2*) appear to act redundantly to activate the Notch pathway in Y. **ii)** ectopic and controlled activation of the Notch pathway is sufficient to induce formation of a second PDA neuron. **iii)** Nuclear factors indentified in our laboratory as crucial for the initiation of this event are also important for transdifferentiation of the second PDA obtained by ectopic activation of Notch. **iv)** A prolonged activation of the Notch pathway in the Y cell maintains its epithelial identity, which results in the inhibition of the transdifferentiation of Y into PDA.

Together, our results showed that the Notch pathway is necessary and sufficient to establish the competence to transdifferentiate. This can only be achieved if the Notch pathway is regulated very precisely in the Y cell.