Leukemia inhibitory factor signaling in *Xenopus* embryo: Insights from gain of function analysis and dominant negative mutant of the receptor

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Abstract

Leukemia inhibitory factor (LIF) is a cytokine member of the interleukin 6 family (IL6) of cytokines. It signals through a heterodimer receptor complex that consists of the LIF receptor (or LIFR formerly known as gp190) and the Interleukin 6 signal transducer (or IL6ST formerly known as gp130). LIF signaling is mediated mainly by signal transducer and activator of transcription 3 (STAT3) and has a wide variety of biological activities with pleiotropic effects on many cell types and organs among which are stem cell renewal and implantation process in mammalian embryo. Despite the wealth of data on LIF in mammalian cells, there is a paucity of information on its functions in lower vertebrates. Here, we provide information on the status and the function of LIF signaling in *Xenopus* amphibian. The IL6 cytokine family is highly conserved in *Xenopus* genome both at ligands and receptors levels. All cytokines and receptors of the family, except oncostatin M (OSM) and IL27, can be identified in the genome including the orthologs of LIF, cardiotrophin 1 (CTF1), ciliary neurotrophic factor (CNTF), cardiotrophin like cytokine factor 1 (CLCF1), LIFR, IL6ST, IL6R, IL11RA and CNTFR. *Lif* mRNA is zygotically expressed after midblastula transition while *lifr* and *il6st* are maternally expressed. We have investigated the functions of LIF in *Xenopus* early development with a gain-of-function analysis combined to the use of a dominant negative form of the receptor. The overexpression of *Xenopus* lif in embryo activates STAT3 phosphorylation and induces a dramatic phenotype where embryos are ventralised and show a reduction of anterior structures with microcephaly. This results mainly from BMP signal stimulation and antagonism towards IGF signals. In addition, most embryos develop tumor-like cell masses according to both autonomous and non-autonomous processes. Through the use of a dominant negative form of the receptor, we demonstrate for the first time that a functional LIF signaling is required for normal vertebrate kidney development. Owing to its experimental advantages, the *Xenopus* embryo constitutes a useful model to identify the molecular actors that may account for the pleiotropic functions of LIF and their role in vertebrate development.

1. Introduction

Leukemia inhibitory factor (LIF) is a glycoprotein cytokine that belongs to the interleukin 6 (IL6) family that comprises interleukins IL11, IL27, oncostatin M (OSM), cardiotrophin 1 (CTF1), ciliary neurotrophic factor (CNTF) and cardiotrophin like cytokine factor 1 (CLCF1) (Auernhammer and Melmed, 2000; Trouillas et al., 2009). All those structurally related cytokines bind to plasma membrane receptor complexes containing the common signal receptor subunit glycoprotein gp130 (interleukin 6 signal transducer or IL6ST) (Heinrich et al., 2003). In the case of LIF, the receptor complex consists of the IL6ST subunit combined to the low-affinity LIF specific receptor (gp190 or leukemia inhibitory factor receptor beta or LIFR) (Nicola and Babon, 2015). Although termed LIFR, the receptor is also shared by OSM, CTF1, CNTF and CLC. While OSM and LIF can signal through a simple IL6ST:LIFR dimer, CTF1, CNTF and CLCF1 require an alpha-chain receptor (CNTFR) to form a complex in order to initiate signaling (Nicola and Babon, 2015). LIF signaling is mainly mediated by the activation of JAK/STAT family members and more specifically by Stat3 (Auernhammer and Melmed, 2000). Since its purification and cloning 30 years ago, a large amount of data has been accumulated on the pleiotropic function of LIF in mammals on various cell types and organ sometimes having opposite effects on proliferation, differentiation and survival. Among the diverse array of physiological effects regulated by LIF are inflammatory response, bone formation, hormone production and formation and survival of neurons (Mathieu et al., 2012). Knock out studies in mouse have revealed that the cytokine has a crucial function in blastocyst implantation and that *Lif* expression is under the control of p53 pathway for the implantation process (Stewart et al., 1992; Hu et al., 2007). Surprisingly, LIF has been shown to be a negative regulator of p53 and this could explain its role in cancer displaying a promoting effect on tumorigenesis in many solid tumors (Yu et al., 2014a). Besides this, LIF possesses the outstanding ability to held lineage commitment of murine embryonic stem cells therefore maintaining their pluripotency (Smith et al., 1988; Williams et al., 1988). The culture of mES cells grown with or without LIF has allowed the identification numerous stemness genes (Mathieu et al., 2012). Among these, we have shown that *mRAS* is involved not only in mES cells pluripotency but also neurogenesis in *Xenopus* embryo (Mathieu et al., 2013). A large amount of data has been accumulated on the pleiotropic functions of LIF in mammals, but so far we have little insight on what could be its functions in non-mammalian vertebrate with respect to early development and differentiation.

LIF belongs to class I-helical cytokine family which is considered to constitute a monophyletic group having evolved from a single ancestral gene through successive

duplication events mainly in the vertebrate lineage (Huising et al., 2006). LIF cDNA was first cloned from mouse and human (Gearing et al., 1987; Gough et al., 1988; Moreau et al., 1988). Since then, orthologs have been identified through cDNA cloning and *in silico* analysis in several non-mammalian vertebrates. Chicken cDNA LIF has been cloned and shown to be able to maintain blastodermal cells into an undifferentiated state (Horiuchi et al., 2004). cDNA encoding LIF ortholog has also been cloned in teleost species as zebrafish, carp and goldfish (Fujiki et al., 2003; Abe et al., 2007; Hanington and Belosevic, 2007). The finding of LIF-like cytokine in fishes indicates that the cytokine already existed before the fish-tetrapod divergence that occurred approximatively 450 million years ago. Although the amino acid conservation of LIF sequences between mammalian and non-mammalian vertebrate species is rather low (20% - 40%), those sequences share a conserved tridimensional fold. LIF receptor has been identified in chick and zebrafish and functional analysis performed in both species. In chicken, LIFR is involved in the control of vasoactive intestinal peptide expression in sympathetic neurons (Duong et al., 2002). Functional experiments in zebrafish using morpholino based mediated knock-down have shown that, unlike LIF whose knock-down has no obvious effects on development, LIFR knock-down impairs proper neural development (Hanington et al., 2008).

Since there is currently nothing known about LIF in *Xenopus* and owing the advantages of this amphibian as a model system in development, we have examined the expression and function of LIF during *Xenopus* development. LIF and its receptor as well as other members of the IL6 cytokine family are conserved between amphibian and mammals in term of gene structure and synteny. We show here that *lif* expression is barely detectable before gastrula stage and increase thereafter being ubiquitously expressed in the embryo. *Lifr* and its partner *il6st* are expressed at all developmental stages with a predominant expression in brain and embryonic kidney. Overexpression of *lif* in the embryo induces a specific stat3 phosphorylation that can be abolish with the co-expression of a truncated form of its receptor whom intracellular domain has been deleted. Upon *lif* mRNA injection, there is a specific overexpression of a subset of genes that are known to be Stat3 target genes in mouse embryonic stem cells. Embryos in which *lif* has been overexpressed are strongly ventralized and are deficient in anterior structures with complete absence of head. Those embryos display tumor-like structures that appear in both autonomous and non-autonomous manner. The use of a dominant negative mutant of the *lif* receptor allowed us to show that *lif* signaling is essential for embryonic kidney formation at a late stage of its development.

2. Materials and methods

2.1. Ethics Statement

This study was carried out in accordance with the European Community Guide for Care and Use of Laboratory Animals and approved by the “Comité d’éthique en expérimentation de Bordeaux”, N° 33011005-A.

2.2. Sequences analysis and plasmid constructions

Sequences for LIF were as follows: human, AAA51699; bovine, AAC27535.1; mouse, AAA37211; rat, NP_071532; chicken, BD187371; *Xenopus laevis*, BC128936; *Xenopus tropicalis*, XP_012825419. Sequences for LIFR were: Human, NP_001121143; bovine, NP_001179192; mouse, NP_038612; chicken, NP_989906.1; rat, NP_112310.1; *Xenopus laevis*, XP_018121814; *Xenopus tropicalis*, XP_002938237.2. Synteny analysis was performed using ensembl and xenbase support. The clone IMAGE BC128936 encoding *Xenopus laevis* LIF cDNA was obtained from Geneservices and the open reading frame was subcloned into pCS2-FLAG by PCR using the primers listed in Table S1. LIF was then subcloned into HindIII/XbaI sites of pXEX vector (Johnson and Krieg, 1994). Cardiotrophin 1, ciliary neurotrophin factor and lif receptor mutant (Δ lifr) cDNA sequences were subcloned into pCS2-HA vector using primers listed in Table S1. For *in situ* hybridization probes, sequences for *lif*, *il6st* and *lifr* were subcloned into bluescript vector.

2.3. *Xenopus* embryo injections, dissection and explant cultures

Xenopus laevis embryos were obtained and staged as previously described (Newport and Kirschner, 1982; Nieuwkoop and Faber, 1967). All mRNAs were synthesized *in vitro* by using the Message Machine kit (Ambion) and injected at the following doses: *lif* (2-200 pg), *cntf* and *ctf1* (1ng), *lifr* (20-2000 pg). For targeted injections, mRNAs were injected with 250 pg of β -gal (lacZ staining) or *gfp* mRNAs as a lineage tracer. The phenotype of embryos was scored and correspond the sum of at least three independent experiments with different batches of embryos. Explants from stage 22, 28, 35-36 and 45 were dissected under the stereomicroscope with fine watchmaker’s forceps and then proceed for RNA extraction. For animal cap assay, both blastomeres of two-cell stage embryos were injected into the animal pole region. Animal caps were then dissected from stage 9 embryos and cultured until appropriate stages before RNA extraction.

2.4. RNA extraction, RT-PCR and real time PCR analysis

RNA extraction was performed as previously described (Tréguer et al., 2013). cDNA was synthesized from 1µg of RNA in 20µL final volume with Promega reverse transcriptase. Semi quantitative PCR was performed with the PCR primers listed in Table S1 and analysed on agarose gel as previously described (Tocco et al., 2015). Real time PCR was performed in a final volume of 20µL with the Mx3000P QPCR system (Stratagene-Agilent). The reaction mixture consisted of 10µL of 2x Brilliant III SYBR green QPCR master mix (Agilent), 5µM of each primer (Table S1) and 2µL of 1/100 dilution of cDNA. The cycling conditions were as follows: denaturation at 95°C (15 s), annealing at 60°C (30 s) and extension at 64°C (22 sec). A single specific product was amplified and confirmed by melting curve analysis. Each reaction includes a control without template and a standard curve of serial dilution points (10-fold step). *Odc* was used as an internal reference and the relative expression of each gene was calculated using the $\Delta\Delta C_t$ method. Expression was calculated relative to uninjected controls. Each data point represents the mean +SEM of at least three independent experiments.

2.4. Whole mount *in situ* hybridization and immunodetection

Whole-mount *in situ* hybridization was carried out with digoxigenin-labelled (Roche) RNA probes prepared from linearized plasmids using T3 or T7 RNA polymerase (Promega) and visualized with BM purple staining (Roche). For histological analysis, 7µm serial sections were performed on embryos post fixed in MEMFA for 1hr at RT and embedded in paraffin. Immunofluorescence was performed on paraffin sections with rabbit anti-P-Stat3 as primary antibody (1:50, Cell Signaling Technology, D3A7). As secondary antibody, Alexa Fluor 555 (1:500, Thermofisher Scientific, A-31572) was used. Nuclei were stained with DAPI (1:10.000).

2.5. Western blot

Once stage of development reached, embryos were lysed directly in cold buffer (100mM NaCl, 50mM Tris-HCl pH 8.0, 1% NP40, 0.05% SDS, 1 µg/ml protease inhibitor cocktail (Roche)), sonicated and centrifuged 10 min at 10000 rpm. Proteins extracted from the equivalent of one embryo were loaded on 10% SDS-PAGE and transferred on nitrocellulose membranes. Proteins were reacted with the primary antibodies Stat3 (C-20 Santa Cruz at 1/1000) and pTyr705-Stat3 (Cell Signaling at 1/1000). The blot were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody and the revelation was done using the enhanced chemiluminescence detection kit (GE Healthcare). ImageJ program was used for quantification.

3. Results

3.1. Identification and characterization of *Xenopus* *lif* and *lif* receptor

The *Xenopus laevis* *lif* mRNA sequence deposited in databank (GenBank BC128936) has been cloned from adult spleen, and encodes a 216 amino acid protein. When blasting the last release of *Xenopus laevis* genome (Xenbase 9.1), two sequences are found and they correspond to homeolog genes *lif.L* and *lif.S* as expected from the allotetraploid nature of the genome (Uno et al., 2013). A predicted *Xenopus tropicalis* sequence (GenBank XP_012825419) is also found and encodes a 218 amino acid protein that shows 80% identity with the *Xenopus laevis* *lif* protein (Fig. 1A). Although the amphibian protein shows only 30 to 35% identity with the mammalian proteins respectively, it contains six cysteine residues that can make three disulfides bridges allowing a conserved tridimensional structure similarly to mammalian LIF proteins (Nicola et al., 1993; Robinson et al., 1994) (Fig. 1A). Moreover, the synteny analysis shows a noteworthy conservation of the gene between human, mouse and *Xenopus* (Fig. 1B). The structural organization of the amphibian gene is partially conserved with human and mouse orthologous genes with three common exons (Fig. S1). However, the mammalian LIF gene displays two alternative promoters associated with a different first exon leading to either a soluble form (LIF-D) or an extracellular matrix form (LIF-M) (Rathjen et al., 1990). There is no evidence for two promoters in the amphibian genes and, from protein sequence and gene structure, we may hypothesize that the *Xenopus* *lif* protein is homolog to the diffusible mammalian form.

With respect to the *lif* receptor gene (*lifr* or *gp190*), a predicted 43.3 kb gene with 17 exons is found in Xenbase corresponding to a transcript (XeXenL6RMv10000165m) encoding a 964 amino acid protein. The identity of the gene is confirmed by synteny analysis which is conserved between mammals and amphibian (Fig. S2A). In order to construct an expression plasmid vector, we cloned by RT-PCR from *Xenopus laevis* embryo RNA, a full length cDNA clone encoding *lifr* (see Materials and Methods). This cDNA encodes a 1091 amino acid protein that shows 45% identity with the human, bovine, murine or chicken proteins (Fig S3). The amphibian protein displays conserved features of vertebrate LIFR such as cysteine residues, WSxWS motifs, three Fibronectin-type III domain and a 26 amino acid transmembrane domain (Fig S3). Moreover, the intracellular domain of the receptor contains six tyrosine residues whose location is conserved with the mammalian and chicken proteins and the last three are located in the motif YXXQ which is known as a STAT3 consensus domain (Auernhammer and Melmed, 2000) (Fig S3). Surprisingly, the comparison between the *Xenopus* cDNA we have isolated and the genomic sequence in Xenbase reveals that two

exons were not annotated in the genomic sequence. Therefore the *Xenopus lifr* gene is composed of 19 coding exons, like the human and mouse genes (Fig. S4A). It should be noted that all three genes display the same intron/exon splicing pattern where the only difference being between the length of the exons (Fig S4B). While this work was in preparation, a recent completion of the *Xenopus laevis* genome was published and confirmed that the *X. laevis lifr* gene contains 19 exons (Session et al., 2016). The *Xenopus* gene encoding the co-receptor *il6st* is highly conserved in sequence and synteny with its mammalian ortholog (Fig. S2 and data not shown).

3.2. Identification and characterization of *Xenopus* interleukin 6 family

LIF signals through a heterodimeric receptor complex, composed the interleukin 6 signal transducer (IL6ST) and LIFR. In mammals, this heterodimer is bound not only by LIF but also by oncostatin M (OSM) which also bind to a heterodimer made of its receptor (OSMR) and IL6ST (Metcalf, 2003). *IL6ST* gene synteny is conserved between mammals and *Xenopus* (Fig. S2B). Surprisingly, although the *Xenopus* genome contains an OSM receptor gene (*osmr*) that displays a conserved synteny with its mammalian orthologs, there is no *osm* gene found (Fig.1B and Fig. S2A). Indeed, while *OSM* and *LIF* genes are tightly linked in mammalian genomes, there is no *osm* encoding sequences in the vicinity of the *Xenopus lif* gene (Fig. 1B). This is consistent with the hypothesis that OSM and LIF have resulted from a duplication event of a common ancestral gene and this event has occurred after radiation of mammals from amphibians 340 million years ago (Rose et al., 1993; Blair and Hedges, 2005). The LIF receptor is also shared by three additional cytokines, namely ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CTF1) and cardiotrophin like cytokine factor 1 (CLCF1) (Fig. S5) (Nicola and Babon, 2015). Those three cytokines bind to the LIFR:IL6ST heterodimer with the help of a specific receptor alpha chain (CNTFR). Mammalian *CNTF*, *CTF1*, *CLCF1* and *CNTFR* genes have orthologs in *Xenopus* genome and they display a conserved synteny (Fig. S5). While *ctf1* is expressed at all stages of development and in adult tissues, *cntf* expression is barely detectable in embryo and adult tissues (data not shown). In mammals, IL6 and IL11 signaling relies on a complex that has an hexameric structure consisting of two molecules each of IL6 (or IL11) that bind a receptor consisting of two specific IL6 receptor (IL6R) or IL11 receptor (IL11RA) (Nicola and Babon, 2015). IL6, IL11 as well as their receptor subunits IL6R and IL11RA are present in *Xenopus* genomes (Fig. S5).

3.3. Developmental expression of *lif* and its receptors

We examined by RT-PCR the mRNA levels of *lif*, *lifr* and *il6st* genes in *Xenopus* embryo from different stages. *Lif* mRNA is not detected from 2-cell stage to blastula stage (stage 9) and starts to accumulate in stage 11 indicating a zygotic expression (Fig. 2A). On the contrary, *lifr* and *il6st* mRNAs are expressed maternally, and their expression remains constant until tadpole stage (Fig. 2A). There is no major difference in the expression levels of *lif*, *lifr* and *il6st* in various regions of stage 11 embryo (Fig. 2B). In the adult, *lifr* and *il6st* are expressed in all tissues analysed while *lif* mRNA level show variations with a peak of expression in spleen, heart, testis and skeletal muscle (Fig. 2C).

To address the spatial distribution of *lif*, *lifr* and *il6st* mRNAs in the embryo, we performed *in situ* hybridization. *Lif* mRNA expression is hardly detectable above background in stage 35 embryo suggesting a very low level of expression (Fig. 2D). *Lifr* and *il6st* mRNAs are detected in stage 35 embryo in brain, otic vesicle, kidney and somites (Fig. 2D). When stage 22, 28 and 35-36 embryos are dissected into pieces, *lif* mRNA together with *lifr* and *il6st* mRNAs are found to be expressed in all portions of the embryo indicating a widespread co-expression of the ligand and its receptors (Fig. S6).

3.4. *Lif* signaling in embryo

A major step in LIF signaling is the activation of Stat3, after ligand binding, by phosphorylation on Tyr705. We therefore asked whether *lif* overexpression in the embryo could activate Stat3 phosphorylation. *Lif* mRNA was injected into 2-cell stage embryos and the phosphorylation status of Stat3 was assayed in developing embryo using a pTyr705-Stat3 antibody. Stat3 phosphorylation on Tyr705 (α -Tyr 705) is detected in late gastrula (stage 12) and stage 27 embryos injected with *lif* mRNA but not in uninjected embryos (Fig. 3A). Stat3 protein (α -Stat3) is detected in all embryos, injected or not (Fig. 3A). These data suggest that *lif* overexpression activates the downstream effector Stat3 (Fig. 3A). The activation of Stat3 has been confirmed by immunofluorescence. A strong nuclear P-Stat3 signal is observed in stage 22 injected embryo (Fig. 3B).

Next, we have tested whether in our experiments, *lif* signaling was indeed transduced through its receptor. For this, we have engineered a truncated version of *lifr* (or Δ *lifr*) where the intra-cytoplasmic domain has been deleted. A similar dominant negative form of the LIFR has been shown to attenuate LIF signaling in cell culture (Ng et al., 2003). Embryos were injected with 20 pg of *lif* mRNA together with 2 ng Δ *lifr* mRNA at the two cell stage and embryos were fixed at gastrula stage and analysed by western blot for Stat3 phosphorylation

status (Fig. 3C). Embryos injected with *lif* mRNA show a significant decrease in the phosphorylation status of Stat3 in the presence of Δ *Lifr* mRNA. Together these data indicate that a dominant negative receptor of *lif* (Δ *Lifr*) inhibits *lif*-Stat3 dependent phosphorylation.

Several comprehensive studies have identified primary targets of LIF/Stat3 pathway in cancer cells and mouse ES cells. Among the genes whose expression is stimulated by Stat3 are *Socs3*, *Fos*, *JunB*, *Myc*, *Zfp36*, *Klf4* and *Klf5* (Bourillot et al., 2009; Dauer et al., 2005). We have tested whether those genes were upregulated in embryo upon *lif* overexpression. Embryos were injected with *lif* mRNA and then analysed at stage 12.5 by RT-PCR for the content of the relevant mRNA. While uninjected control embryos express very low level of *socs3*, *fos* and *junb* mRNAs, a strong increase of those mRNAs level, between 10 to 30 times, is observed in embryo injected with *lif* mRNA whatever the stage analysed (Fig. 4). The mRNA levels for *myc* is also increased upon *lif* overexpression likewise *zfp36*, *klf4* and *klf5* mRNA levels albeit to a lesser extent (Fig. 4). We have also observed the stimulation of *socs3* and *junb* expression upon *lif* overexpression in animal cap assay (data not shown). Moreover, this expression is abolished when Δ *Lifr* mRNA is co-injected with *lif* mRNA (data not shown). This strengthens the effectiveness of Δ *Lifr* in blocking *lif* signaling and together confirms that LIF/Stat3 pathway activates in both *Xenopus* embryo and murine ES cells identical subset of target genes. All the genes we have tested in our study have been shown to contribute to the maintenance of the undifferentiated state of murine ES cells (Bourillot et al., 2009). Surprisingly, in our experiments, *lif* overexpression stimulates, also moderately, the pluripotency gene *oct4* (Fig. 4).

3.5. *Lif* overexpression induces ventralisation and microcephaly

In order to analyze a possible role of *lif* during *Xenopus* embryonic development, we performed a gain-of-function analysis by microinjection increasing amounts of *lif* mRNA into the animal pole of 2- to 4-cell stage embryos. Developing embryos were then analysed for their phenotype (Fig. 5A). At neurula stage (stage 18), injected embryos show a narrowing of the neural plate in their anterior region (Fig. 5A). At early tailbud stage (stage 22), the embryos display a reduction of anterior structure and a belly shape. At later stage (stage 33-34), embryos displayed a complete loss of head structures with ventral enlargement (Fig. 5A). Surprisingly, no clear dose dependence could be observed and the phenotype alteration is induced with as low as 2 pg of *lif* mRNA and this phenotype is very penetrant as more than 95% of injected embryos (n>300) displayed this phenotype. No phenotype was observed when *LacZ* mRNA was injected at the same concentrations (data not shown).

In those experiments, the injection of *lif* mRNA in two cell-stage embryos mimics a maternal effect of the ligand. To determine what is the effect of *lif* zygotic overexpression, i.e. after midblastula transition, a plasmid DNA expressing *lif* under the control of the *EF1 α* promoter (pXEX) was injected into embryos. The embryos obtained have the same phenotype than when injected with *lif* mRNA, indicating similar effect for maternal and zygotic *lif* overexpression (data not shown).

The effect of *lif* overexpression on anterior development was confirmed by the expression analysis of the forebrain/midbrain marker *otx2*, the hindbrain markers *vgl3* (Faucheux et al. 2010) and *krox20* and the eye marker *pax6* (Fig. 5B). *Otx2* expression was strongly reduced in embryos injected with *lif* mRNA while *vgl3/krox20* gene expression was drastically reduced (Fig. 5B). *Pax6* expression is reduced or even absent. Anterior views of embryos clearly show the dramatic decrease of all markers analysed (Fig. 5B, insets). RT-qPCR analysis of *lif* injected embryos confirmed the decreased expression of the gene markers (Fig. 5C).

In order to confirm that the dramatic phenotype observed in *lif* mRNA-injected embryos is dependent on an intact signaling receptor, the mRNA encoding the dominant negative form of the *lifr* (Δ *lifr*) was co-injected with *lif* mRNA and embryos were scored for phenotype at stage 22, 29-30 and 35-36. A clear rescue of the phenotype is observed upon increasing amounts of Δ *lifr* mRNA injected (Fig. 6A). At the highest dose of Δ *lifr* mRNA injected, more than 95% of stage 35-36 injected embryos have a normal phenotype (Fig. 6A, B). Injection of (Δ *lifr*) alone does not lead to any phenotypic disorders (Fig. 6C). This confirms that *lif* signaling through its receptor is responsible for the altered phenotype.

LIF receptor is also shared by CTF1 and CNTF that can signal via a LIFR:IL6ST heterodimer with the requirement of an α -chain receptor (CNTFR) to form a quaternary complex (Nicola and Babon, 2015) (Fig. S5A). We found that the overexpression of *ctf1* and *cntf* mRNAs, even at the dose of 2ng, has no effect on embryonic development (Fig. S7). This eliminates the possible redundancy of cytokine effect signaling through *lifr:il6st* receptor complex in the embryo. Together, we may conclude that the ventralisation and microcephaly phenotypes observed in *lif* mRNA-injected embryos are caused by *lif* signaling activation. Human or mouse *Lif* mRNAs, even injected at high doses, have no effect at all on embryonic development (data not shown).

3.6. *Lif* overexpression upregulates *bmp* signaling and antagonizes *igf* signal

Head induction is a complex process that is mediated by extracellular inhibition of wnt by DKK1, and by inhibition of BMP by Cerberus (Glinka et al., 1997, 1998). In our experiments, *lif* mRNA-injected embryos show indeed a decrease in *dkk* and *cerberus* expression although modest for the latter (Fig. 7A). The phenotype of *lif*-injected embryos with reduced head and ventralization is reminiscent of what has been observed when BMP signaling is overexpressed in embryo (Cao et al., 2006; Jones et al., 1992). Indeed overexpression of *msx1* or *ventx1/2* that are downstream effectors of BMP signaling, produce embryos resembling *lif*-injected embryos (Suzuki et al., 1997; Onichtchouk et al., 1998; Yamamoto et al., 2000, 2001). In our experiments, the expression of *msx1*, and *ventx1/2* is increased in *lif* mRNA-injected embryos (Fig. 7A). Together, our data suggest that *lif* signaling has a positive effect on BMP signaling, at least at the level of *msx1* and *ventx1/2*.

Head formation in *Xenopus* embryo has been shown to rely on extracellular pathways that elicit neural anterior development through the inductive capacity of Insulin Growth Factor (IGF) (Richard-Parpaillon et al., 2002; Pera et al., 2001). We therefore tested whether *lif* could interfere with IGF pathway in animal caps explants that are made of naive ectodermal cells. As expected, *igf1* mRNA injection upregulates the expression of the two anterior neural markers *otx2* and *pax6* (Fig. 7B) (Richard-Parpaillon et al., 2002; Pera et al., 2001). However, in the presence of *lif*, a clear decrease in the expression of both markers is observed suggesting that *lif* can antagonize *igf1*-induced expression (Fig. 7B). The co-injection of *igf1* mRNA with *lif* mRNA gives an embryo with an almost normal head confirming that *lif* and IGF signaling have opposite effects in the early embryo (data not shown). The anterior development of the *Xenopus* embryo has also been showed to be promoted by IGF binding protein 5 (IGFBP-5) (Pera et al., 2001). *Lif* mRNA overexpression in embryo induces a decrease in *igfbp5* expression confirming an antagonism between *lif* and *igf* signaling (Fig. 7C).

3.7. *Lif* overexpression induces localized hyperplasia with aspect of “tumor-like” masses

During our experiments, we have repeatedly observed that, in addition to head defects, embryos injected with *lif* mRNA developed abnormal hyperplasia (Fig. 8A, a-c for instance). Those large cell masses are reminiscent of tumor-like structures that have been previously described to be induced by overexpression of well-characterized oncogenes (Gli1, Xrel3 and Kras^{G12D}) or by p53 mutant tumor suppressor (Dahmane et al., 1997; Wallingford et al., 1997; Yang et al., 1998).

To determine whether the localisation and the size of tumor-like masses were dependent on the site of injection, 20 pg of *lif* mRNA were injected into single blastomere of 8-cell stage embryos (Fig. 7B). To follow the fate of injected blastomeres, *lif* mRNA was co-injected with mRNA encoding nuclear β -galactosidase or GFP as lineage tracers (Fig. 8B). Injected embryos were then raised to stage 30-35 fixed and stained for β -galactosidase activity or observed under fluorescent light. Injection of *lacZ* or *GFP* mRNA alone had no effect on development (data not shown). Embryos injected with *lif* mRNA into ventral blastomere V1 show a tumor-like mass of cells in their anterior ventral part (80%, n=45) while injection into blastomere V2 give embryos with a tumor-like mass found more posteriorly (70%, n=42) (Fig. 8B, V1, V2). In both cases, head development was barely affected. Injection of *lif* mRNA into blastomere D1 leads to embryos with tumor-like in dorsal anterior part but with normal posterior end (85%, n=55) (Fig. 8B, D1). Embryos injected with *lif* mRNA in blastomere D2 show tumor-like mass in their dorsal posterior end with head defect less pronounced (54%, n=52) (Fig. 8B, D2). These cell masses persist through to tadpole stages afterwards embryos die through slow disintegration. Histological analyses revealed tumor-like masses in the epidermis with a strong disorganized structure adopting a scalloped aspect partially covered with pigmented melanocytes (Fig. 8C, a-d).

In most embryos, β -galactosidase-positive cells were found clustered into the tumor-like masses but some of them do not show staining (see D2 in Fig. 8B). To investigate this further, embryos were microinjected with *GFP* mRNA together with *lif* mRNA in order to follow the progression of the tumor-like mass. Most tumors contain GFP expressing cells, indicating that they are derived cell-autonomously from *lif* mRNA-injected cells (Fig. 8D, a-d). However, we have reproducibly observed GFP negative tumors, indicating they have arisen non-autonomously (Fig. 8D, e-h). This could be related to a paracrine action of *lif* that signals at the extracellular level on adjacent cells originating from uninjected cells.

Some of the *lif* mRNA injected embryos that develop tumors strongly resemble embryos that have been injected with mRNA encoding member of the *rel* oncoprotein family (Yang et al., 1998). Surprisingly, we have found that the overexpression of *lif* mRNA in animal cap cells can stimulate *rel* expression (data not shown) suggesting that *rel* could, at least partially, relay the effect of *lif* on tumor-like formation.

To assess whether the formation of tumor-like masses was dependent on *lif* signaling, *Alif* mRNA was co-injected with *lif* mRNA. More than 90% of embryos injected with 20pg of *lif* mRNA show developmental defects and the presence of tumor-like structure (n=45). However only 5% of injected embryos (n=21) show altered phenotype when *Alif* mRNA is

coinjected with *lif* mRNA (data not shown). This indicates that the development defects and the formation of tumor-like structures is dependent on *lif* signaling through its receptor.

3.8. Overexpression of a dominant negative *lif* receptor impairs normal kidney development

Lifr and *il6st* that transduce *lif* signaling are both expressed in embryonic kidney suggesting a potential function of this pathway during organ formation (see Fig. 2C and Fig. S6). Although *Lif* knockout has no obvious kidney phenotype in mouse, it has been showed that *Lif* can induce nephrogenesis in culture in synergy with TGF β 2 and FGF2 (Barasch et al., 1999; Plisov et al., 2001). To test whether *lif* signaling could be involved in *Xenopus* kidney development, 8-cell stage embryos were injected unilaterally with Δ *lif*r mRNA in the ventral vegetal cells that are fated to contribute to the pronephros, the larval kidney of the frog (Jones, 2005). Embryos were analysed for pronephros formation by immunohistochemistry with 3G8 antibody that allows the detection of the pronephric proximal tubules (Vize et al., 1995). Embryos show on their injected size a clear reduction of the pronephric tubules (75%, n=61) (Fig. 9A, B). They display a glomus with a reduced size as attested by the expression of *wt1* (75%, n=46) (Fig. 9C). In those embryos, the intermediate and distal tubule as well as the pronephric duct seem unaffected according to *clcnkb* expression (Fig 9D). In our experiments, *lif* signaling blockade does not affect *pax8* expression which is a major actor of tubule fate specification during *Xenopus* kidney development (Buisson et al., 2015). This precludes any role in the early steps of nephrogenesis (data not shown). Three other cytokines, *clcf1*, *ctf1* and *cntf*, can also signal through the *lif* receptor. As we mentioned it before, *cntf* expression is barely detectable in the embryo while *ctf1* is expressed at all stages of development. We tested by RT-PCR analysis whether *clcf1*, *ctf1* and *cntfr* were expressed in embryonic kidney of stage 45 embryo. *Clcf1* expression is not detected in embryonic kidney while *ctf1* and *cntfr* are expressed in all tissues tested (Fig. S7B). Despite the fact that *ctf1*, *cntf* and *cntfr* is expressed in embryonic kidney, overexpression of *ctf1* and *cntf* in the embryo, even at high dose, has no effect at all on development (Fig. S7A). This suggests that this is *lif* signaling through its receptor that is responsible of the kidney phenotype observed in embryos overexpressing Δ *lif*r. However we cannot totally exclude the implication of *ctf1* in the kidney phenotype.

4. Discussion

In this paper, we have characterized for the first time the *Xenopus* leukemia inhibitory factor (lif) and its receptors. We describe the effects of its overexpression in the embryo and the impact of inhibiting its pathway through the use of a dominant negative receptor.

4.1. Conservation of interleukin 6 family in *Xenopus*

LIF is member of the IL6 family of cytokines which is a group of cytokines consisting in mammals of IL6, IL11, IL27, LIF, OSM, CTF1, CNTF and CLCF1. They are grouped into one family because the receptor complex of each cytokine contains the signaling receptor subunit IL6ST, previously known as glycoprotein 130 or gp130. The IL6 family is highly conserved in *Xenopus* genomes with very few differences with that of the mammalian genomes. Among all the genes that make up the IL6 family, only *OSM* and *IL27* genes have no ortholog in the amphibian genome while all other genes are present and display a conserved synteny with their human and mouse orthologs. *LIF* and *OSM* genes are tightly linked in the human and mouse genomes and it is admitted that they have evolved from a common ancestral gene very lately in evolution and specifically in the mammalian lineage (Rose et al., 1993). In the case of *IL27* or its receptor *IL27r*, none orthologs of the mammalian genes are found in fishes, amphibian and birds, suggesting that these genes appeared during evolution after the radiation of birds and mammals 310 million years ago (Hedges, 2002).

Although the *Xenopus* lif protein shows a rather low sequence conservation with the mammalian proteins, it contains six conserved residues at identical position that can make disulfide bridges allowing the adoption of a four alpha-helical bundle structure similarly to other IL6 family cytokines (Robinson et al., 1994). Moreover, there is a significant synteny conservation between human, mouse and *Xenopus* genomes around LIF gene and this extends also to chicken genome (data not shown), confirming a strong evolutionary constraint for more than 370 million years. In zebrafish, the complete sequence of the genome does not reveal clearly identifiable ortholog of LIF, although the receptors *lifr* and *osmr* are present in the genome, as in *Xenopus* (Howe et al., 2013). However a protein with similarity to IL6 cytokine was identified in carp (named M17), in goldfish and zebrafish and was considered to be equivalent to mammalian LIF (Fujiki et al., 2003; Hanington and Belosevic, 2007). The *Xenopus* lif receptor (or *lifr*, previously named gp190) and its co-receptor *il6st* show a strong conservation with their mammalian orthologs at both gene level, in terms of exon number and splicing pattern, and at protein level. Finally the mammalian *IL6R*, *IL11R* and *CNTFR* have their orthologs in *Xenopus* genome and show a conserved synteny. The IL6 family of

cytokines has thus not undergone major evolutionary changes since the divergence time of amphibian about 370 million years ago.

4.2. Expression of *lif* and its receptors in *Xenopus*

Lif gene expression in *Xenopus* embryo is not detected before blastula stage while *lifr* and *il6st* genes encoding receptor and co-receptor respectively are expressed maternally. From gastrula stage, *lif* expression is ubiquitous. *Lifr* and *il6st* have also a widespread expression in tadpole embryo although they show a substantial increase in brain, otic vesicle, branchial arches and prospective kidney. *Lif* expression is more precocious in *Xenopus* development than in zebrafish since in the latter, *lif* mRNA is not detected before 24 to 42 hrs of development, a time when organogenesis has already started (Abe et al., 2007; Hanington et al., 2008). In zebrafish, *lif* and *lifr* are expressed at roughly the same time during early development, indicating that there is no maternal contribution for both genes while in *Xenopus* *lifr* is maternally expressed. In mouse, *Lif* mRNA is first detected at morula stage one day before *Lifr* and *Il6st* mRNAs that are first expressed at blastocyst stage (Nichols et al., 1996). The human and mouse *LIF* genes can produce different isoforms of the protein through the use of two alternate promoters associated with their first exon. Thus, a soluble form (LIF-D) and an extracellular matrix associated form (LIF-M) have been described and are expressed in an independent way during embryogenesis and in the adult (Rathjen et al., 1990; Robertson et al., 1993). The genomic sequence between the first two exons of the *Xenopus* gene does not contain an initiation codon that might give an in phase sequence when spliced to exon 2 like this is the case in the mammalian genes. We therefore hypothesize that there is a single form of *lif* produced in *Xenopus* and it is similar to the mammalian diffusible LIF-D.

4.3. *Lif* signaling in the embryo

The JAK-STAT signaling cascade is a common signaling pathway shared by all IL6 family of cytokines and JAK1/STAT3 have been showed to be the most important signal transducers following stimulation by LIF and the one which mediates most of its cellular effects (Stahl et al., 1994; Niwa et al., 1998). Briefly, LIF forms a ternary complex with the complex receptor LIFR/IL6ST that is subsequently bound by JAK1 that initiates the signaling cascade. JAK1 phosphorylates tyrosine residues on each receptor chain (LIFR and IL6ST) and phosphorylated receptors act as scaffold to recruit STAT3 which is itself phosphorylated on tyrosine 705 (Tyr 705) by JAK1 (Auernhammer and Melmed, 2000; Nicola and Babon, 2015). Once activated, STAT3 form a signaling competent dimer that can translocate to the

nucleus to upregulate the transcription of LIF responsive genes. We have showed that overexpression of *lif* in the embryo, through mRNA microinjection, induces a specific Stat3 phosphorylation on Tyrosine 705 at dose as low as 2 pg of mRNA. This phosphorylation is rapid and can be detected in 32-cell stage embryo (data not shown) and persists until at least stage 27 embryo. It should be noted that there is no detectable level of Stat3 phosphorylation in uninjected and unperturbed embryo. Stat3 phosphorylation in embryonic explant can be interpret as a response to injury as it has been observed for immediate-early genes and genes involved in wound healing processes (Krain and Nordheim, 1999; Nichane et al., 2010; Ding et al., 2017). Surprisingly, it has been shown very recently that the expression of *lif* and *il11*, two members of the IL6 family, is stimulated during *Xenopus* tadpole tail regeneration after injury (Tapia et al., 2017; Tsujioka et al., 2017). In those cases phospho-stat3 is strongly expressed at the site of injury. In our experiments we demonstrate that *lif* mRNA injection induces the bona fide *lif* signaling pathway as attested by stat3 phosphorylation on Tyr 705. Moreover, this is confirmed by the use of a dominant negative *lif* receptor whose intracellular domain has been removed, thus impairing signal transduction. We may conclude that *lif* signaling pathway is functional in early embryo and can be activated by *lif* mRNA injection.

A number of genes have been shown to be primary target of *lif* signal, in mouse ES cells, through Stat3 (Bourillot et al., 2009). For instance, *Socs3* which encodes the suppressor of cytokine signaling 3 and functions as a feedback regulator of ES cells self-renewal has been described as the most heavily induced gene upon Lif/Stat3 stimulation (Bourillot et al., 2009). Here we show that is also the case in *Xenopus* embryo that barely expresses *socs3* but, whose expression level is dramatically induced upon *lif* overexpression. *Junb*, *fos* and *myc* are known direct targets of Lif/Stat3 signaling and see their expression level stimulated as well as *klf4*, *klf5* or *zfp36* albeit to a much lesser extent. We may conclude that *lif* signaling is conserved between *Xenopus* and mammals and can activate through stat3 phosphorylation a common subset of genes.

4.4. *Lif* overexpression induces ventralization of embryo

Overexpression of *lif* in the embryo, even in the range of picogram of injected mRNA, is highly effective and penetrant and induces major changes in development characterized by a dramatic ventralisation. Two hints allow us to conclude that this results from *lif* signaling impairment. First, the co-expression of a dominant negative receptor of *lif*, lacking the intracellular domain, completely restore a wild type phenotype. Secondly, the overexpression in the embryo of *ctf1* or *cntf*, two other cytokines that signal through *lifr*, has no effect at all

on development. However, we cannot totally exclude that those two latter cytokines do not signal in amphibian like their mammalian counterparts through the use of *lif* receptor.

Dorso-ventral patterning of the *Xenopus* embryo is an evolutionary conservative mechanism that relies on the formation of a morphogenetic gradient established by members of the BMP4 family that is antagonized by several secreted molecules at the level of the Spemann organizer (De Robertis and Kuroda, 2004; De Robertis and Moriyama, 2016). The overexpression of members of BMP signaling pathway such as BMP2/4 or *ventx1/2*, that are nuclear targets and mediator of BMP signaling, leads to ventralized embryos that are phenotypically resembling embryo injected with *lif* mRNA (Jones et al., 1992; Cao et al., 2006; Gawantka et al., 1995; Onichtchouk et al., 1998). In our hands, *lif* overexpression stimulates *ventx1/2* genes supporting the hypothesis of an effect of LIF signaling on BMP pathway at their downstream effectors level.

Stat3 has been previously found to be activated upon *il6st* stimulation (Nishinakamura et al., 1999). In those experiments, embryos were injected with mRNA encoding the human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) together with a chimeric cytokine receptor consisting of extracellular regions of the human GM-CSF receptor and transmembrane-intracellular regions of mouse *Il6st*. Stat3 activation was then revealed with a Stat3-dependent reporter gene (Nishinakamura et al., 1999). Although the phenotype of embryos in this study closely resemble to *lif*-phenotype embryos, the authors conclude that the ventralisation of embryos is independent of BMP4. Although those findings are in contrast with our data we may rely those differences to heterologous expression of human cytokine in *Xenopus* embryo.

4.5. *Lif* overexpression induces anterior defects through *Igf* antagonism

Embryos injected with *lif* mRNA display also anterior defaults affecting the head with a more or less severe phenotype that can ultimately lead to headless embryos. Anterior neural development and head formation requires the concomitant repression of *wnt*, *nodal* and BMP signaling in addition to an active pathway mediated by IGF (Glinka et al., 1997; Pera et al., 2001; Richard-Parpaillon et al., 2002). Our data indicate that *lif* overexpression in animal cap cells can antagonize *igf*-dependent induction of the two anterior neural markers *otx2* and *pax6* (Pera et al., 2001; Richard-Parpaillon et al., 2002). We also found that *lif* overexpression induces a decrease in IGF binding protein 5 (*igfbp5*) expression which is known to promote anterior development and head induction (Pera et al., 2001). We have also found that the overexpression of *igf1* can rescue headless phenotype induced by *lif* confirming that the two pathways have antagonistic effects in the embryo (data not shown). It will be interesting to

know whether the antagonistic effects between the two pathways operate at the extracellular level or downstream at the subcellular level. The overexpression of *lif* before or after midblastula transition gives the same phenotype indicating a similar effect of the pathway when activated either maternally or zygotically. Surprisingly, the injection of AP-1 complex mRNA (*c-jun* and *c-fos*) leads to embryo with a ventralized phenotype and a loss of anterior structures that is resembling *lif*-injected embryos (Knöchel et al., 2000). Since we found that *lif* overexpression induces *junb* and *fos* expression (Fig. 3C) we may hypothesize that this could partially contribute to the headless phenotype observed.

4.6. *Lif* overexpression stimulates pluripotent genes

In mouse, the overexpression in the embryo of the Diffusible form (D-LIF) has no gross effect on the development while overexpression of the Matrix form (M-form) is lethal with gastrulation defect resulting from inner-cell mass differentiation into primitive ectoderm (Conquet et al., 1992; Shen and Leder, 1992). *Lif* maintains the pluripotency of mouse embryonic stem cells in cooperation with Oct4 by stimulating *Klf4* and *Klf5* expression (Hall et al., 2009). In our study, *klf4* and *klf5* stimulation is rather low but we found that *oct4* and *ventx1/2* expression is increased upon *lif* overexpression. Surprisingly, it is known that in *Xenopus*, *ventx1/2* is the ortholog of *nanog* (Scerbo et al., 2012). Although we do not know whether this stimulation is direct or not, it seems that *lif* could stimulate the expression of pluripotency genes in a way distinct from mouse embryonic stem cells. Furthermore, Stat3, the downstream effector of *Lif* has been found to be able to maintain *Xenopus* pluripotent neural crest cells in an undifferentiated state downstream of FGF signals (Nichane et al., 2010). Together we may conclude that *Lif* can maintain pluripotency on *Xenopus* embryonic cells.

4.7. *Lif* overexpression induces tumor-like structures in the embryo

Xenopus embryo has long proposed to provide an effective model system for *in vivo* studies of tumorigenesis (Wallingford, 1999). Our work completes this since we have consistently observed tumor-like structures in embryos injected with *lif* mRNA. Those structures form both autonomously and non-autonomously. The non-autonomous process can be interpreted as a paracrine signal, produced by cells descendant from injected cells, and acting on adjacent cells that have not received *lif* mRNA. Most of the tumors generally develop in the epidermis and interestingly, those tumor-like structures are very resembling to those found in embryos injected with *xrel3* mRNA, an oncoprotein of the Rel family (Yang et al., 1998). Similar tumors have been also induced by overexpression of *gli*, an oncogene that

is up-regulated in basal cell carcinoma (Dahmane et al., 1997). However, the effectiveness of tumor-like masses induction by *lif* is much greater than those described for *xrel3* or *gli*, since tumors are formed in embryos injected with as low as 2pg of mRNA ie 100 times lower than in aforementioned studies. One interesting finding is that *lif* overexpression does induce *xrel* expression in embryo (data not shown). Since *lif* stimulates also the proto-oncogenes *fos* and *jun*, we may hypothesize that altogether, the induction of oncogenes might contribute, at least partially, to the formation of tumors.

Nevertheless, the formation of tumor-like masses in *lif*-injected embryo is not unexpected given the large number of studies that link LIF and cancer. Indeed LIF has been shown to promote the development and progression of many types of solid tumors and to be involved in the metastasis of several cancers like breast cancer, rhabdomyosarcoma, melanoma and nasopharyngeal (Li et al., 2014; Yu et al., 2014a). Moreover, Stat3 the downstream effector of LIF is known for a longtime as a potent oncogene and has a crucial role in tumor cell formation through the activation of a wide range of genes that contribute to cell proliferation and tumor progression (Bromberg et al., 1999; Yu et al., 2014b). One striking observation is that Stat3 injected embryos do not develop the same phenotype (data not shown) suggesting that the *lif*-induced phenotype does not result solely on Stat3 signaling but probably involves additional downstream effectors. Indeed, besides Stat3 signaling, the activation of JAK1 kinase by LIF also activate MAP-kinase and PI(3) kinase pathways (Nicola and Babon, 2015). The use of inhibitors specific of each pathway might give clues about the respective contribution of the different downstream effectors. The process of tumor-like formation is strictly dependent on *lif* signaling pathway since it can be rescued by the dominant negative form of the *lif* receptor. Therefore, the *Xenopus* embryo might constitute a useful model for phenotype-based chemical screening and identification of bioactive compounds that could block tumor-like formation.

4.8 Lif signaling blocking impairs proper embryonic kidney development

An efficient way to block *lif* signaling in the embryo is to express a dominant negative mutant of its receptor, in which the intracellular domain has been deleted (Δ *lifr*). This strategy has been successfully used in the case of the FGF receptor (Amaya et al., 1991). We engineered such a mutant and it can efficiently block the first step of *lif* signaling, i.e. Stat3 phosphorylation, and can also rescue embryos injected with *lif* mRNA. Embryos injected with Δ *lifr* mRNA develop normally until tadpole stage without any gross phenotypic alterations. However, the embryos display an impairment in pronephros development. This is conspicuous when embryos are analysed by immunohistochemistry for 3G8 expression and *in*

situ hybridization analysis for *wtl* mRNA. In those embryos, only the glomus and proximal tubule are affected but not the distal tubule. Altering kidney development when blocking lif signaling is consistent with the expression of *lifr* and *il6st* receptors in pronephros region. In these experiments, early steps of pronephros development, such as specification, seems not affected as attested by normal *pax8* expression. Lif signaling is therefore required for pronephros development but only at a late stage of development. However since we found that *ctf1* is also expressed in kidney at late stage we cannot exclude a role in kidney formation via *lifr*. Interestingly, it has been reported that LIF plays an important role in nephrogenesis in mammals. Indeed, LIF is synthesized by renal epithelial cells and found to be secreted by ureteric bud and induces the mesenchyme to epithelial conversion (Barasch et al., 1999; Morel et al., 2000). Moreover, LIF cooperates with TGF β 2 and FGF2 to enhance and accelerate renal tubule formation from mesenchymal explants (Plisov et al., 2001). Finally LIF has been shown to be involved in tubular regeneration after acute renal failure (Yoshino et al., 2003). However, although Lif knockout mice do not show kidney abnormalities, our data strongly suggest an underestimated function of Lif on kidney development at an early stage of development. *Zfp36*, a well-described Lif target gene, could help to reconcile the present data with our earlier observations where we showed that *zfp36* has a critical role during kidney development (Tréguer et al., 2013). Therefore LIF function in kidney development has a long evolutionary history and an intact LIF signaling is required for its normal development.

In conclusion, the mammalian IL6 cytokine family is highly conserved in *Xenopus* both at ligands and receptors levels. Among the members of the family, Lif signaling is present in early embryo and upon overexpression, it activates primary targets of the Lif/STAT3 pathway identical to those identified in mouse ES cells. Lif signaling pathway activation causes ventralization of the embryo and anterior defects. This is the result of a complex integration at the cellular level of BMP signaling activation, blockade of head inducers and antagonism towards IGF signal. Finally, we propose for the first time that a functional LIF signaling is required for normal vertebrate kidney development (Figure 10). Owing to its experimental advantages, the *Xenopus* embryo may constitute a useful model to identify the molecular actors that may account for the properties of LIF in vertebrate development and its pleiotropic functions.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

NT and PT conceived and designed the experiments; SJ, PV, SF and PT performed the experiments. HRR contributed to reagents, provided funding and intellectual inputs. NT and PT analysed the data and wrote the paper.

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Figure Legends

Figure 1. Conservative evolution of vertebrate Leukaemia Inhibitory Factor. (A)

Deduced amino acid sequence comparison between vertebrate LIF proteins. Numbers indicate the position of the relevant sequences relatively to the complete sequences. Identical residues are shaded in black, conservative differences are shaded in gray, and white represents nonhomology. The position of conserved cysteine residues are marked by a black triangle.

Dashes have been introduced for best alignment. GenBank accession numbers for sequences are as follows: *X. laevis* (BC128936); *X. tropicalis* (XP_012825419); Human (AAA51699); Bovine (AAC27535.1); Mouse (AAA37211.1), Rat (NP_071532); Chicken (XP_425293.2).

(B) Conserved syntenic regions between human (HSA), mouse (MMU), *Xenopus laevis* (XLA) and *Xenopus tropicalis* (XTR) chromosome regions containing *LIF* locus. Gene symbols are according to HUGO. The chromosome number for each species is indicated and the two subgenomes of *Xenopus laevis* (XLAL and XLAS). Genes are represented as colored boxes with the arrow indicating the orientation of the transcription unit. Boxes with the same color correspond to ortholog genes and white boxes correspond to genes without orthologues in the species shown here. The following versions of the genomes used to establish are as follows: Human, GRCH38; p7; mouse, GRCm38; *Xenopus laevis* Version 9.1; *Xenopus tropicalis*, Version 7.1. The drawing is not on scale to avoid complexity.

Figure 2. Temporal and spatial expression of *Xenopus laevis* *lif* and *lif* receptors encoded genes. (A) RT-PCR analysis of embryo from stage 2 to stage 33. (B) RT-PCR analysis of

dissected parts of stage 11 embryo. AC, animal cap; Do, dorsal mesoderm; Em, total embryo; En, endoderm; Ve, ventral mesoderm. *Chordin* and *wnt8* expression is used as control. (C)

RT-PCR analysis of adult tissues. Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Mu, skeletal muscle; Ov, ovary; Sk, skin; Sp, spleen; St, stomach; Te, testis. Ornithine decarboxylase (*odc*) and ribosomal protein L8 (*rpl8*) gene expression was used as control. -

Control without reverse transcription. (D) *In situ* hybridization on stage 37/38 embryo with *lif*, *lifr* and *il6st* antisense probes. Ba, branchial arches; ki, embryonic kidney; nt, neural tube; ot, otic vesicle; so, somites.

Figure 3. *Lif* signaling in *Xenopus* embryo. (A) 5 or 100 pg of *lif* mRNA were injected in

two cell-stage embryos and Stat3 phosphorylation status (α -Tyr705, top) was analysed by western blot analysis in stage 12 or 27 injected embryos. The total content of Stat3 protein was revealed by anti α -Stat3 antibody (bottom). Uninjected embryos are used as control. (B)

Immunofluorescence analysis of P-Stat3 in *lif* mRNA-injected embryo. Transverse section of stage 22 embryo with dorsal side on the top (a) was analysed for immunostaining of P-Stat3 (c, red) and DAPI (b, blue). b and c are magnification of the white square in a. Arrows indicate representative P-Stat3 cells. Scale bar represents 50 μ m. (C) Western blot analysis of stage 12 embryos injected with 20 pg of *lif* mRNA at the two cell stage with 2000 pg of *Alif*r mRNA using anti-phosphorylated Tyr705 Stat3 (α -Tyr705, top) and anti-Stat3 (α -Stat3, bottom) antibodies. Uninjected embryos are used as control. Relative quantification of phospho-Stat3 (P-Stat3) is displayed on the right. The diagram represents the mean of three independent measurements with standard deviations.

Figure 4. Lif activation of target genes. Real-time RT-PCR analysis of *lif* mRNA injected embryos. Embryos were either uninjected (-) or injected with 20 pg of *lif* mRNA (*lif*) cultured until stage 12.5 and processed for RT-qPCR of the indicated genes. Five embryos were used in each lot. All values were normalized and calibrated to the expression of the reference gene *odc*. Bar plots show the mean of three independent $\pm$ SEM of normalized fold induction compared to uninjected control embryos.

Figure 5. Lif overexpression in *Xenopus* embryo causes ventralization and anterior truncation. (A) Two-cell stage embryos were injected in each blastomere at the animal pole with 2, 20 or 200 pg of *lif* mRNA (+*lif*). Representative phenotypes observed at stage (St) 18, 22 or 33-34 are shown (anterior view for St18 embryos, lateral view and anterior to the left with dorsal to the top for St 22 and St 33-34 embryos). (-*lif*) uninjected embryos. (B). Embryos injected with 20 pg of *lif* mRNA were fixed at late neurula stage and analysed by *in situ* hybridization for the expression of the indicated genes. Embryos are observed on lateral view with anterior to the left and dorsal to the top. Insets are anterior view of the embryos. (C) RT-qPCR analysis of *lif* mRNA injected embryos (*lif*) versus uninjected control embryos (-). Embryos were injected with 20 pg of *lif* mRNA and cultured until stage 22 before processing for RT-qPCR. Five embryos were used in each lot. All values were normalized and calibrated to the expression of the reference gene *odc*. Bar plots show the mean $\pm$ SEM of at least three independent experiments.

Figure 6. A dominant negative of the *lif* receptor can rescue *lif*-induced phenotype. (A) *Xenopus* embryo were injected at the two cell stage in both blastomeres with 20 pg of *lif* mRNA together with increasing amounts (20, 200, 2000 pg) of the dominant negative mutant receptor (*Alif*r). Embryos were scored for their phenotype at stage (St) 22, 29-30 or 35-36. (B)

Three independent batches of embryos were scored for wild type (Wt) or *lif*-phenotype. The total number of embryos analysed is indicated above the bars. (C) Stage 35-36 embryos injected with increasing amounts of *Alifr* mRNA alone.

Figure 7. *Lif* overexpression upregulates BMP signaling and antagonizes IGF signal.

(A) Embryos were either not injected (-) or injected with 20 pg of *lif* mRNA (*lif*) at the two-cell stage. They were cultured until stage 12.5 and processed for RT-qPCR analysis. (B) Embryos were injected with 1 ng of *igf1* mRNA (*igf*) or with 1ng of *igf1* together with 20pg of *lif* mRNA (*igf+lif*). Animal caps were explanted at blastula stage and cultured until stage 18 before RT-qPCR analysis. (C) Embryos were either not injected (-) or injected with 20pg of *lif* mRNA (*lif*). They were cultured until stage 18 and processed for RT-qPCR analysis. *Soc3* was used as a control of *lif* signaling activation. Five embryos or 15-20 caps were used in each lot. All values were normalized and calibrated to the expression of the reference gene *odc*. Bar plots show the mean $\pm$ SEM of at least three independent experiments.

Figure 8. *Lif* overexpression in *Xenopus* embryo induces tumor-like masses formation.

(A) Control uninjected stage 28 embryo (a) or stage 28 (b) and stage 33-34 (c) embryos injected with 20pg of *lif* mRNA. Tumor-like mass are visible in ventral position (arrowhead). (B) 20 pg of *lif* mRNA and 250 pg of *lacZ* mRNA were co-injected in ventral (V1, V2) or dorsal (D1, D2) blastomeres of 8-cell stage embryos (schematic diagram). Developing embryos were fixed at stage 31 for β -galactosidase coloration. Tumor-like mass formed are indicated by arrow in embryos derived from targeted injections. (C) Histological analysis of embryos injected with 20pg of *lif* mRNA. Tumor-like structures in black boxes in a and c are magnified in b and d respectively. (D) 20 pg of *lif* mRNA and 250 pg of *GFP* mRNA were co-injected in ventral (V2) or dorsal (D1) blastomeres of 8-cell stage embryos. Developing embryos were observed under white light (a, c, e, g) or fluorescent light (b, d, f, h). Tumor-like masses in white boxes are in dotted circle when observed under fluorescent light. g and h are magnification of e and f respectively.

Figure 9. Blocking *lif* signaling in *Xenopus* embryo impairs normal kidney formation.

Embryos were injected co-injected unilaterally at the 8-cell stage in the prospective pronephros region with 2 ng of *Alifr* mRNA (*Alifr*) and 250pg of *lacZ* mRNA as tracer control. Injected side is revealed by β -galactoside activity (red). Uninjected side is used as control (-). (A) Dorsal anterior view of a stage 42 embryo. The embryo was fixed before immunohistochemistry analysis to reveal the expression of pronephros specific marker 3G8.

Arrow marks the proximal tubule. (B) Close up lateral views of representative phenotypes of embryos injected with *Alifr* mRNA. Histogram on the right shows the classification of 3G8 phenotypes into three categories: no effect, weak or strong effect. The total number of embryos analysed is indicated above the bars. (C) Developing embryos were fixed at stage 35-36 and analysed by *in situ* hybridization for *wt1* expression. a, b, and c are representative phenotypes of *wt1* reduced expression. Glomus region is indicated by an arrow. Histogram on the right shows the classification of phenotypes into no effect or reduced *wt1* expression. The total number of embryos analysed is indicated above the bars. (D) Developing embryos were fixed at stage 35-36 and analysed by *in situ* hybridization for *clcnkb* expression. Embryos are observed on lateral view with anterior to the left and dorsal to the top.

Figure 10. Proposed model showing the multiple roles of Lif signaling in *Xenopus* embryo from gain of function and the use of dominant negative of its receptor. Lif is involved in tumor-like formation, ventralization, head and kidney formation through the use of three distinct signaling pathways: STAT3 activation (P-STAT3), PI3K and MAPK. Data from this study are figured in blue. Data from literature are figured in red. Lif activates STAT3 target genes like the oncogenes *fos*, *jun* and *myc* that can contribute to tumor-like formation. Lif activates the expression of BMP effectors *msx1* and *ventx1/2* that are known to ventralize the embryo. Ventralisation can also depend on STAT3 activation independently of BMP (Nishinakamura et al., 1999). P-STAT3 may also activate BMP gene expression (Fukuda et al., 2007). Lif repress cerberus, DKK and IGF that are required for head development (Glinka et al., 1997, 1998; Pera et al., 2001). Lif signaling hindrance leads to *wt1* decrease and normal kidney formation.

Figure S1. Schematic representation of LIF genes from human, mouse and *Xenopus*.

Coding exons are in black boxes. Introns are figured as a solid line. Numbers above the coding exons and introns correspond to the size of the sequences in nucleotides. Protein size in amino acids (aa) is indicated. For the mammalian genes, the two promoters that give rise to the alternative forms D and M are figured together with the size of the corresponding proteins. XLA1L, Chromosome 1 of the *Xenopus laevis* subgenome L, XLA1S, Chromosome 1 of the *Xenopus laevis* subgenome S; XTR1, Chromosome 1 of the *Xenopus tropicalis* genome. HSA22, Chromosome 22 of the human genome; MMU11, Chromosome 11 of the mouse genome. An additional exon of 10 nucleotides is present in the human and mouse gene and absent from the *Xenopus* gene.

Figure S2. Conserved syntenic regions between human, mouse and *Xenopus* chromosome regions containing *LIFR* and *IL6ST* locus. (A) Syntenic regions comparison of *LIFR* between mammals and *Xenopus*. (B) Syntenic regions comparison of *IL6ST* between mammals and *Xenopus*. Gene names and symbols are according to HUGO. The chromosome number for human (HAS), mouse (MMU) and the two subgenomes of *Xenopus laevis* (XLAL and XLAS) is indicated. Genes are represented as colored boxes with the arrow indicating the orientation of the transcription unit. Boxes with the same color correspond to ortholog genes. The following versions of the genomes used to establish are as follows: Human, GRCH38.p7; mouse, GRCm38; *Xenopus laevis* Version 9.1; *Xenopus tropicalis*, Version 8.0. The drawing is not on scale to avoid complexity.

Figure S3. Sequence comparison between vertebrate *LIFR* proteins. Numbers indicate the position of the relevant sequences relatively to the complete sequences. Identical residues are shaded in black, conservative differences are shaded in gray, and white represents non homology. Arrowheads indicate the position of conserved cysteine residues. The WSxWS conserved motifs are in blue. The Fibronectin-type III domains (amino acids 414-496, 516-604 and 709-808) are figured by a black bar. The position of the 26 amino-acids transmembrane domain and the YxPQ sites are highlighted in yellow and red respectively. Dashes have been introduced for best alignment. GenBank accession numbers for sequences are as follows: human, NP_001121143; Bovine, NP_001179192; mouse, NP_038612; rat, NP_112310.1; chicken, NP_989906.1; *Xenopus laevis*, XP_018121814.

Figure S4. The *LIFR* gene structure is conserved between mammals and amphibian. (A) Schematic representation of the human *LIFR* gene structure. Exons are figured in box and intron with a black solid line. White boxes correspond to non-coding sequences. The different domains of the protein are figured with a black line below the gene structure: CD, cytoplasmic domain; CRH, cytokine receptor homology domain; FNIII, type III fibronectin domain; IG, Ig-like domain; SP, signal peptide. (B) Exon size comparison between human, mouse and *Xenopus LIFR* genes. End phase (0,1,2) for each exon is indicated.

Figure S5. Conserved syntenic regions between mammalian and *Xenopus* chromosome regions containing Interleukin 6 family cytokines and receptors. (A) Table showing interleukin 6 family members in human genome together with their receptors. Crosses indicate the components of each cytokine receptor complex. * Genes unidentified in *Xenopus* genome. (B) Interleukin 6 (IL6) gene synteny. (C) Interleukin 11 (IL11) gene synteny. (D)

Cardiotrophin 1 gene synteny (CTF1). (E) Ciliary neurotrophic factor (CNTF) and cardiotrophin like cytokine 1 factor (CLCF1) genes synteny. (F) Ciliary neurotrophic factor receptor (CNTFR) and Interleukin 11 receptor alpha (IL11RA) gene synteny. (G) Interleukin 6 receptor (IL6R) gene synteny. Gene names symbol are according to HUGO. The chromosome number for human (HAS), mouse (MMU) and the two subgenomes of *Xenopus laevis* (XLAL and XLAS) is indicated. For *Xenopus ctfl* genes, XLAL and XLAS correspond to scaffold 22 and 87 respectively. Genes are represented as colored boxes with the arrow indicating the orientation of the transcription unit. Boxes with the same color correspond to ortholog genes. The following versions of the genomes used to establish are as follows: Human, GRCH38.p7; mouse, GRCm38; *Xenopus laevis*, Version 9.1; *Xenopus tropicalis*, Version 8.0. The drawing is not on scale to avoid complexity.

Figure S6. *Lif*, *lifr* and *il6st* genes are ubiquitously expressed in *Xenopus* embryo. (A) Schematic diagram of stage 22 embryo with the dissected parts (top) used for RT-PCR expression analysis (bottom). (B) Stage 28 embryo analysis. (C) Stage 35/36 embryo analysis. E, total non-dissected embryo; - control without reverse transcription. Ornithine decarboxylase (*odc*) expression was used as control.

Figure S7. Overexpression in *Xenopus* embryos of *ctfl* or *cntf* has no effect on embryonic development. (A) Embryos were injected with 20 pg of *lif* mRNA or 2 ng of either *ctfl* or *cntf* mRNAs. Embryos were observed at stage (St) 27 or 37-38 for phenotype alteration. (B) RNA extracted from stage 45 embryonic tissues was analysed by RT-PCR. Br, brain, He, heart; Ki, kidney; So, somites. E, total non-dissected embryo; - control without reverse transcription. Ornithine decarboxylase (*odc*) expression was used as control.

Table S1. Sequence of the primers used in the study. Appropriate restriction sites incorporated in the primers are in underlined italics lowercases.

Semi-quantitative RT-PCR and subcloning PRIMERS		
Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>lif</i>	GGAGTCAAGGGAATTCTATCC	GGGGCTCAAGCATCGATGAC
<i>lif</i> -FLAG	<i>ccgcggatcc</i> ATGAGGCACATGCCAGGAATTGTG	<i>ccgcctaga</i> AGAAGGCATTGAAGGATTTGCGTTTC
<i>lifr</i>	CTGGATACAGAACTGCACTC	TCATCCACAGGAGGAATCAG
Δ <i>lifr</i>	<i>gccggaattc</i> ATGAAGAACAAGCTGCATTGCTG	<i>cgcgctcga</i> AGGCATTGAAGGATTTGCGTTTC
<i>il6st</i>	TCCAGCACCGTCCAGTATTC	TGGCATGTAACTCCACTCT
<i>ctf1</i>	GGTCACCACTATCCATGAAC	ACCAGGATCTCCATATCTCG
<i>cntf</i>	ATGGATTACAGACACTCTTC	GATTGTACCCATGTAACCTCC
<i>ctf1</i> -HA	<i>cgcgctcga</i> ATGGAAGTGCTCAGAGCCATTG	<i>cgcgctcga</i> ACACTGATGTCTCGGCCACCAG
<i>cntf</i> -HA	<i>cgcgctcga</i> ATGGATTACAGACACTCTTCAG	<i>cgcgctcga</i> ATGATATGGTCTTCTTTCCGCAG
<i>clcf1</i>	CTCTTACAGAGTCTGTATG	TGAGACGGTTGAAGTCCTTG
<i>cntfr</i>	CTGGAAGTCACTTGGCAGATCC	GCTCACACACCTTGGTAGATGG
<i>chordin</i>	CCTCCAATCCAAGACTCCAGCCAG	GGAGGAGGAGGAGCTTTGGGACAA
<i>wnt8</i>	TGGCAAGAACTGTCCAGT	TTGTGGAATGCCGTCATCTC
<i>rpl8</i>	GACGACCAGTACGACGAG	CACCATGCCTGAAGGCAC
<i>odc</i>	GTCAATGATGGAGTGTATGGATC	TCCATTCCGCTCTCCTGACCAC

RT-qPCR PRIMERS		
Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>socs3</i>	CTGCAGGAATATGACTCCAC	AGGAAACCAGAACCAAGGTC
<i>junb</i>	CCAACCCTCTGCCACAATTAAC	TCACCCTCACCTTCAGTGCTC
<i>fos</i>	TCTGGGTCTGATTGCAGAGACC	ACTGCTCATTGCTGCTGCTTCC
<i>myc</i>	GCCTCAATACTGGGATTTGG	GACTGCTTCTGTGTTACTAGG
<i>oct4</i>	ACCTGTCTGTCCGCTTTAC	GCTCCAGACAGAGACATATC
<i>klk4</i>	CCTCCAACCCAGCTACAATA	CCTAGAGAAAGCTCGATCAC
<i>klf5</i>	ATAGGCAAGCAGAGCTTCTC	ATGGAGGGCTAAGTGATCAG
<i>zfp36</i>	GTTGCAACTTCATCCACCACC	CCACTGCTGTAGCATTCTGTC
<i>cerberus</i>	ACAGAATACAAGCCATGGCAG	TGCAGGGTAGTAGATGTATCC
<i>dkk</i>	GTAGCAACGGAATTTGTGTCC	AAATGTGTAGCCCGTGAGAC
<i>msx1</i>	ACTGGTGTGAAGCCGTCCT	TTCTCTCGGACTCTCAGGC-
<i>ventx1.2</i>	GATGGAAAGGACTCCATGAGC	TGCTTGTTGAATGCCTGTTCC
<i>ventx2.2</i>	TGATACCTCAGATGAAGAGGG	CTGCATGCTGGAATACAGGAG
<i>msx1</i>	333 A COMPLETER	334 A COMPLETER
<i>igfbp5</i>	AAGAAGACCCAACCACTCA	CATTGCAGGTCTCCATTGAC
<i>otx2</i>	TCTTCATGCATGCAGAGGTC	CCAAGCAGTCAGCATTGAAG
<i>pax6</i>	CAACCTGGCGAGCGATAAGC	CCTGCCGTCTCTGGTTCCGT

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6

Figure S7