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Running title: XRLIP GAP activity needs the N-terminal sequence of RLIP to control F-actin and is required for gastrulation

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Abstract

The transduction pathways that branch out of fibroblast growth factor signaling are essential for the induction of the mesoderm and the specification of the vertebrate body plan. One of these pathways is thought to control remodeling of the actin cytoskeleton through the Ral binding protein (RLIP also known as RalBP1), an effector of the small G protein Ral. RLIP contains a region of homology with the GTPase-activating protein (GAP) domain involved in the regulation of GTPases of the Rho family. We demonstrate here that the GAP domain of RLIP is responsible for the stability of the actin cytoskeleton in *Xenopus laevis* embryos. We also demonstrate that the complete N-terminal domain of RLIP containing the μ2 binding domain (μ2BD) and the GAP domain induces disruption of the actin cytoskeleton when targeted to the plasma membrane. Neither domain, however, has any effect on the actin cytoskeleton when individually targeted to the plasma membrane. We also determined that Cdc42-GDP, but neither Rac-GDP nor Rho-GDP, rescues the effect of expression of the membrane-localized *Xenopus* RLIP on the actin cytoskeleton. We show that the GAP domain of RLIP interacts *in vivo* with Cdc42-GTP and Cdc42-GDP. Finally, a single mutation (K244A) in the GAP sequence prevented embryos from gastrulating. These results demonstrate that to participate in the control of the actin cytoskeleton, RLIP needs its complete N-terminal region coding for the μ2BD and the GAP domain. We suggest that RLIP, by coordinating two complementary mechanisms, the endocytosis of clathrin-coated pits and the remodeling of cortical actin, participates in the gastrulation process.

Key words: RLIP, GAP, Cdc42, actin cytoskeleton, *Xenopus*, gastrulation, Ral

Introduction

Gastrulation, a primary event of morphogenesis, results from the coordination of cell differentiation and cell movement. Various complex processes such as changes in cell morphology and cell-cell and cell-extracellular matrix adhesion lead to cell movement. Each process is the result of multiple intra-cellular events such as endocytosis, remodeling of the actin cytoskeleton, gene transcription etc, which are all controlled by a network of protein interactions activated by growth factor signaling. Thus, fibroblast growth factors (FGFs), acting via branched pathways, participate in induction of the mesoderm and in cell movement (Amaya et al., 1991; Ciruna and Rossant, 2001). Whereas the involvement of the FGF pathway in mesodermal gene expression is relatively well documented, its involvement in cell movement during gastrulation remains poorly understood. FGF is however, known to regulate different cellular events essential for gastrulation such as cell shaping and migration (Burdine et al., 1997; Ribeiro et al., 2002; Schumacher et al., 2004; Yang et al., 2002). Studies in *Xenopus* have shown that FGF activates a signaling cascade that controls mesodermal gene expression through activation of the small GTP-binding protein Ras (Whitman and Melton, 1992; Umbhauer et al., 1995), and induces cell motility and lamellipodia formation at the gastrula stage (Wacker et al., 1998). It has also been shown that the FGF signal directly affects morphogenetic cell movements during gastrulation (Ciruna and Rossant, 2001; Yang et al., 2002) and that through the Sprouty protein, FGF signaling prevents convergent extension movements, independently of the Ras/MAPK pathway (Nutt et al., 2001). Thus, FGF appears to be required for the coordination of cellular behavior during gastrulation. The coordination of different targets and cell functions by FGF signaling has also been described in the canonical pathway involving Ras. Furthermore, it is now clearly accepted that Ras coordinates many cell functions through its interaction with various effectors such as the serine-threonine kinase Raf, the phosphatidylinositol 3-kinase (PI3K), and the Ral GDP dissociation stimulator RalGDS (for a review see (Vojtek and Der, 1998)). When Ras mutants containing different point mutations in the Ras effector domain were used, they could interact selectively with each of the three downstream effectors characterized; the Raf, the p110 catalytic subunit of PI3K and the Ral guanine nucleotide exchange factors (RalGEFs) (White et al., 1995). In contrast to the Raf and the PI3K effectors, the RalGDS effector does not have its own kinase activity, but induces the formation of the GTP-bound state of the GTPase members of the Ral-family. Ral belongs to the Ras superfamily (Wolthuis and Bos, 1999).

The Ral-GTPases and Ras are important actors in various aspects of cell behavior such as cell migration (Gildea et al., 2002), transformation (Urano et al., 1996; Yu and Feig, 2002), growth and differentiation (Gille and Downward, 1999; Verheijen et al., 1999), and have been shown to be involved in metastasis (Ward et al., 2001; Tchevkina et al., 2005). The overexpression of the mRNA that codes for the activated form of Ral (Ral-GTP) induces a local depigmentation in the animal hemisphere of the *Xenopus* embryo and is correlated with the disruption of the cortical actin cytoskeleton. These observations allowed us to demonstrate that the actin network is one of the targets of the Ras/Ral pathway during early development of *Xenopus* embryos (Moreau et al., 1999). While it is now well established that Ras activates canonical pathways by Raf and the PI3Ks, the exploration of the RalGEF pathway is just beginning (Feig, 2003).,During early amphibian development, the FGF-inducing signal is mediated by Ras and is induced through activation of the MAPK cascade (MacNicol et al., 1993) and PI3K (Carballada et al., 2001), two pathways that control the expression of many mesodermal genes such as Xbra expressed in the marginal zone. FGF/Ras has thus been shown to play a role in the patterning of the marginal zone (Kumano and Smith, 2000), and through activation of Xbra expression maintains cell migration and convergent extension as mutually exclusive behaviors (Conlon et al., 1996; Kwan and Kirschner, 2003). Nevertheless, although the expression of activated MAPK has been observed during gastrulation in mesodermal cells prior to their involution around the blastopore lip (Curran and Grainger, 2000), it has never been detected when mesendodermal cells migrate on the blastocoel roof. Consequently, even if the FGF signal plays a major role in cell migration, this could be independent of the MAPK pathway.

We have focused our attention on the Ras/RalGDS pathway during early development of *Xenopus* embryos. We showed that the final target of this transduction pathway is the dynamic cortical actin network, independently from the activation of new genes (Lebreton et al., 2004), and that FGF activates the Ral pathway in the marginal zone during gastrulation (Lebreton et al., 2003). The expression in the blastula marginal zone of a dominant negative form of Ral (RalS28N) or the expression of the Ral binding domain of RLIP, its effector, inhibits this branched pathway, inducing gastrulation defects (i.e. spina bifida, shortening of the anterior-posterior axis, reduction or lack of anterior embryonic structures). Ral, as do many small G proteins, behaves like a molecular switch, alternating between its active and inactive forms to interact with its partners: when Ral is in its activated form (Ral-GTP), it interacts with an effector. In *Xenopus* embryos we have identified RLIP (Lebreton et al., 2004) as a partner of Ral.

RLIP, the effector of Ral (Jullien-Flores et al., 1995; Cantor et al., 1995; Ikeda et al., 1998; Lebreton et al., 2004), interacts with at least three other proteins, the μ2 subunit of the clathrin adaptor complex AP-2 (Jullien-Flores et al., 2000), POB1/Reps (Morikana et al., 1999) and the Activin type II receptors (ActRIIs) (Matsuzaki et al., 2002), and possesses a sequence that is similar to the primary sequences of protein families that share a 170-200 amino acid region of homology designated RhoGAP. The GAP domain of RLIP activates the GTPase activity of Cdc42, and, to a lesser extent, that of Rac1 (Park and Weinberg, 1995; Jullien-Flores et al., 1995). Using a mutagenesis approach, we demonstrate that the central GAP domain of RLIP is involved in controlling the stability of the actin cytoskeleton network via Cdc42. In addition, we demonstrate that, *in vivo,* the μ2 interacting domain present in the N-terminal region of RLIP is necessary for the action of the GAP domain on the actin cytoskeleton. We also show that interaction between Cdc42 and the GAP domain of RLIP is required for gastrulation movements. Thus, during gastrulation, FGF signal transduction controls involution of dorsal mesodermal cells via the Ral/RLIP pathway.

Materials and methods

Microinjections. They were performed as described (Lebreton et al., 2004). For overexpression studies, either 4.6 or 9.2 nl of mRNA at the appropriate concentration was injected near the surface of the animal hemisphere of two-cell stage embryos or in the marginal zone of four-cell stage embryos with a Nanoject microinjector (Drummond Sci.).

Construction of deletion mutants and site-directed mutants. To generate the deletion mutants of XRLIP, XRLIP cDNAs in the pRN3 vector, the sequences were amplified by PCR with a 5' primer containing the EcoRI site in phase with either the tag Myc sequence or the XRLIP sequence corresponding to the 5' limit of the domain to be cloned. The 3' primer contained a hybridization sequence corresponding to the XRLIP domain to be cloned with the CAAX sequence, a termination codon and a NotI site. Its general design was: 3'-(hybridization sequence): TTCCCGAAGTTTGTTGCGACAAATGAGACTCGCCGGCGATTGAGC 5' (termination codon : double underline and Not site: single underline). The XRLIP mutants in the GAP sequence were generated by oligonucleotide-directed mutagenesis of XRLIP in the pRN3 vector by the PCR based second extension amplification method with the Quick Change site-directed mutagenesis kit as recommended (Stratagene) and primers containing the desired mutations. The products were

amplified from the cDNA coding for XRLIP with primers sandwiching the junction site and the 5' or 3' end of the mutated coding sequences containing a 5' EcoRI site and an internal SacI site. The resulting chimeric inserts were sequence-proofed by Genome Express.

***In vitro* transcription of RNA for injection.** cDNAs were cleaved after the poly dT sequence with Asp718, and RNA transcripts were produced using T3 polymerase. Capped mRNAs were obtained with the mMessage Machine System (Ambion) according to the manufacturer's instructions.

GAP assay. GAP activity was assessed by prebinding Cdc42 with [γ-^{32}P]GTP. The GTPase activity was quantified by measuring the decrease in the radiolabeled protein-GTP complex following incubation with proteins synthesized *in vitro*. The XRLIP construct was cloned in the GEX4TI vector fused to GST at its N-terminus. GST and GST-Cdc42 from transformed *Escherichia coli* cells were prepared according to classical methods as described in the Pharmacia manual. Purified GST fusion proteins were cleaved with thrombin. [γ-^{32}P] GTP-bound Cdc42, was prepared by incubating 200 nM protein with 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.2 mM $MgCl_2$, 0.1 mg/ml of BSA, 1 mM DTT, 10 mM [γ-^{32}P] GTP (30 mCi/mmol (Amersham)) in 50 µl for 15 min at room temperature. GTP hydrolysis was initiated in the presence of 400 to 800 nM of protein in 20 mM $MgCl_2$ and 200 µM GTP. After 15 min, hydrolysis was stopped with buffer containing 50 mM Tris-HCl pH 8.0, 35 mM $MgCl_2$, 1 mM DTT and 150 mM NaCl, filtered on BA 85 nitrocellulose filters and the radioactivity retained on the filter was measured.

Immunostaining of embryos and confocal microscopy analyses. *Xenopus* embryos at the appropriate stage of development were either incubated in permeabilization buffer (80 mM Hepes pH 6.8, 1 mM $MgCl_2$, 100 mM EDTA, 30% glycerol and 0.1% Triton X-100) for 15 min at room temperature before being fixed overnight in 1 X PBS and 1.4% formaldehyde, or their vitellin membrane was manually removed. For either procedure, the samples where incubated with 9E10 anti-myc antibody was diluted 1:100 in 1 X PBS, 1% BSA and 0.1% Triton X-100. Confocal microscopy observations were performed as described (Moreau et al., 1999) with a Leica SB2 AOBS confocal imaging system (Leica Instruments, Heidelberg, Germany).

Western blotting. Embryos were harvested at stage 6-7 and disrupted in 40 µl of lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100 and 1 mM PMSF) per

embryo by pipetting several times with a yellow tip (Gilson) and treated twice with the same volume of Freon (Merck) to remove the yolk (Gusse et al., 1989). The crude protein extracts were mixed with Laemmli buffer, subjected to SDS-PAGE and transferred to PVDF membranes (Hybond-P, Amersham) that were probed overnight with the appropriate antibodies (Santa Cruz Lab.) and peroxidase-conjugated secondary antibodies (Sigma). Signals were detected by chemiluminescence (ECL, Amersham).

Synthesis and purification of the GST-fusion proteins. cDNAs coding for XRLIP, Rac-GTP and Cdc42-GTP were fused to GST in the pGEX-4T-1 vector (Pharmacia) and cloned. Protein synthesis in *E coli* was induced by 1 mM IPTG (Euromedex). Clones containing GST-RalAG23V were subsequently incubated at 37°C, whereas clones containing GST-XRLIP, XRLIP, GST-Rac-GTP and GST-Cdc42-GTP were incubated at 30°C to limit protein degradation. The cultures were then centrifuged and the bacterial pellets resuspended in buffer D (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ and 1% Triton X-100). Cells were lysed by adding 1 mg/ml lysozyme (Euromedex), and after 1 h at 4°C, the samples were sonicated, centrifuged at 4°C for 45 min at 14,000 rpm to remove cell debris and insoluble material and the supernatants were immediately used.

Recombinant GST-proteins were purified on glutathione Sepharose 4B (Amersham) at room temperature in buffer D according to standard procedures (Amersham). The purified products were analyzed by SDS-PAGE, and the concentration of the purified recombinant GST-proteins was estimated by Coomassie Brilliant blue staining using a BSA standard.

GST pull-down. GST pull-down experiments were carried out with 5 µg of GST-RalA, Rac or Cdc42. The small G proteins were preloaded with GTP γ-S in Mg^{++}-free loading buffer (20 mM Tris-HCl pH 7.5, 25 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.05% BSA and 1 mM GTP γ-S) for 1 h at 37° prior to use. The reaction was stopped by adding 20 mM $MgCl_2$. Using this method, the yield of GTP loading was about 30%. Reticulocyte extracts (20 µl) were pre-incubated with 5 µg of GST in lysis buffer for 1 h at room temperature and centrifuged. The supernatants were then incubated with the corresponding GST-fusion protein for 2-3 h at room temperature and centrifuged. All pellets were washed 3 times with ice-cold buffer D and re-suspended in Laemmli buffer prior to Western blot analysis.

Co-immunoprecipitation. Embryos were injected with mRNA coding for the appropriate Myc-tagged cDNA. At the 2,000 cell stage, proteins were extracted by lysis of 15 to 20 embryos in 800 ml of buffer A^- (50 mM Tris-HCl pH 7.5, 100 mM NaCl and 2 mM PMSF). After 15 min on ice the extracts were centrifuged at 13,000 rpm for 30 min. The supernatants were incubated with slow shaking at 4° in the presence of 3 µl of anti-myc-agarose monoclonal antibody (c-Myc (9E10), Santa Cruz) and centrifuged at 13,000 rpm for 6 min. The supernatant were discarded and 32 µl of Laemmli buffer added to the pellet. The precipitated proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. Western blotting was performed as above.

2. Results

The N-terminal region of XRLIP is involved in disruption of cortical F-actin

Previous experiments have shown that Ras recruits and activates the RalGDS protein family at the plasma membrane (Wolthuis and Bos, 1999). Then the guanine-nucleotide exchange factor activity of RalGDS activates Ral, which in turn recruits the RLIP protein to the membrane. So, to mimic the active location of RLIP independently from activation of Ras/Ral pathway, we artificially targeted RLIP to the plasma membrane by adding a CAAX amino acid sequence (CAAX assay). Previously, we observed that in this condition RLIP induces the disappearance of pigment (also called bleaching) (Moreau et al., 1999; Lebreton et al., 2004) on the actin cytoskeleton. To determine whether a specific sequence of RLIP is responsible for disruption of the cortical actin cytoskeleton, RLIP was divided into two parts, both of which were fused to CAAX to test their action on pigmentation by microinjection into the animal cap (Fig. 1A). These two segments of RLIP share the Ral binding domain (RalBD, amino acids 379–477). The N terminus (XRLIP (1-495)) contains the µ2 binding domain (µ2BD) (Jullien-Flores et al., 2000) and the domain coding for a RhoGAP domain (amino acids 187-330). The C-terminus (XRLIP (330-636)) contains the POB1/Reps interacting domain. mRNA (0.5 ng) of each construct was injected into the animal hemisphere of two-cell stage embryos (the animal pole is the pigmented part of the embryo). All the RLIP constructs contained a myc tag at their N-terminus, thus, for each mRNA the location of the protein expressed in the plasma membrane was checked by confocal microscopy using the anti-myc antibody (Fig. 1A and B middle panel). All embryos injected with the N-terminal mRNA of RLIP (XRLIP (1-495)—CAAX) showed clear bleaching of their ectodermal cells (100%, n=135) (Fig. 1A left panel), as observed when expression of the full length XRLIP-CAAX (XRLIP (1-636)-CAAX) was studied. Conversely, embryos injected with mRNA coding for the C-terminal region

(XRLIP (330-636)-CAAX) did not display any depigmentation and developed normally up to the mid blastula transition (MBT) stage. The ectodermal cells derived from a blastomere injected with XRLIP (330-636)-CAAX showed a hyperpigmentation phenotype from the end of the cleavage stage, a phenotype that persisted throughout development and in which no other developmental abnormalities appeared (in preparation). To confirm that depigmentation is correlated with disruption of the actin cytoskeleton (as seen after overexpression of either the Ral-GTP or XRLIP-CAAX), we analyzed the integrity of the actin network using confocal microscopy. Whereas embryos injected with XRLIP (330-636)-CAAX, like the control embryos, possess a cortical cytoskeleton (Fig. 1A right panel), we observed a dramatic alteration of the cortical actin cytoskeleton in the blastomeres of animal caps injected with XRLIP (1-495)-CAAX as in the case of XRLIP-CAAX expression (Fig. 1A right panel). The expression of mRNA coding for XRLIP (1-495) without the CAAX sequence (2 ng per blastomere) did not alter pigmentation and development of the embryos (not shown). Surprisingly, when higher quantities of this mRNA were injected, bleaching of the blastomere was observed, an effect never seen when the full-length of XRLIP was overexpressed.

To determine precisely whether the μ2 BD or the GAP domain of the N-terminal region of RLIP is responsible for the destabilization of the actin cytoskeleton, each domain was fused to a CAAX sequence (XRLIP (1-230)-CAAX and XRLIP (177-387)-CAAX)) and their mRNA (0.5 ng/blastomere) was injected into the blastomeres of the animal hemisphere of embryos at the two-cell stage. Overexpression of these domains individually had no effect on either pigmentation of the blastomere (Fig. 1B left panel) or late embryonic development. To investigate whether these two domains act on the actin cytoskeleton, by a possible cooperation when they are associated in a same protein, XRLIP (1-230)-CAAX and XRLIP (177-387)-CAAX were coinjected into embryos. As is the case for individual domains, no abnormal phenotype or disturbance of the actin cytoskeleton was observed (data not shown).

It was previously demonstrated that the N-terminal domain of 165 amino acids of the human RLIP is sufficient to interact with μ2 (Jullien-Flores et al., 2000). Therefore, we tested whether the targeting to the membrane of the N-terminal domain of XRLIP deleted of μ2BD and coding only for the GAP domain (XRLIP (148-387)-CAAX)), affects pigmentation of the embryos. As observed with the individual domains, no effect on development was induced by this deletion (Fig. 1B left panel). Confocal microscopy analyses (Fig. 1B right panel) confirmed the absence of effect on the actin cytoskeleton. These observations suggest that, in the N-terminal RLIP domain, the association

of the μ2 BD and the RhoGAP domain of RLIP acts cooperatively to disassemble the actin cytoskeleton.

Effect of mutations of the GAP domain on the actin cytoskeleton

The N terminus of XRLIP (1-387) containing the RhoGAP sequence is the minimum region required to mediate of the effect of RLIP on the actin network. The GAP domain of the RhoGAP family contains at least three highly conserved residues (Arg^{85}, Lys^{122} and Asn^{194}), which are located in the putative G-protein binding helix pocket and are involved in binding or catalysis of Rho GTPases (Musacchio et al., 1996; Barrett et al., 1997). To examine the requirement of RhoGAP activity, we constructed three XRLIP site-specific mutants: R208L (208L), K244A (244A) and N317V (317V) corresponding to Arg^{85}, Lys^{122}, and Asn^{194}, respectively, of the consensus GAP sequence. Since Arg^{85} is required for the catalytic activity of GAP (Li et al., 1997), prior to phenotypic analysis we investigated whether the GAP mutant 208L of XRLIP is as efficient as the mutated Arg^{85} described in p190GB (Li et al., 1997). An activity test (Settleman et al., 1992) was performed *in vitro*, in which the level of [γ^{32}P]GTP retained on Cdc42 bound to GTP was determined when Cdc42 loaded with GTP was incubated with either wild type RLIP, XRLIP (1-387), or XRLIP (1-495). For each construct the RhoGAP domain had a similar residual level of bound GTP (35.0±12.0%, 33.7±10.8% and 26.3±4.6%, respectively) to which is indicative of a similar activity (Fig. 2A). As reported elsewhere, the level of activity of the GAP domain in RLIP can be considered low compared to that of other proteins containing a GAP domain (Jullien-Flores et al., 1995). Moreover, when tested alone the GAP domain (177-387) also exhibited a very low level of Cdc42 GTPase activity, since 76.3±11% of [γ^{32}P]GTP remained bound to Cdc42 after incubation with the GAP domain alone. As expected, XRLIP (1-387) 208L and XRLIP (1-495) 208L showed a decrease in GAP activity towards Cdc42-GTP (Fig. 2A) compared with the native form, but the GAP activity towards Cdc42-GTP was never totally lost. GAP activity, however, seemed more efficient when it was associated with the μ2 binding domain alone than when it was associated with the μ2 and Ral binding domains together.

The biological activity of XRLIP GAP mutants on the actin cytoskeleton of blastomeres was investigated by examining the effect of the mutations on embryonic pigmentation. The effects on the actin cytoskeleton of overexpression of XRLIP (1-387), containing the RhoGAP domain mutated or not, were compared when the overexpressed protein was translocated to the plasma

membrane. Each mRNA (1 ng per /blastomere), corresponding to either mutated or non-mutated XRLIP (1-387)-CAAX, was injected into the animal hemisphere at the two-cell stage and the embryos were examined for any alteration of their pigmentation from the 32-cell stage onwards. Whereas XRLIP (1-387)-CAAX induced depigmentation (Fig. 2B) as did XRLIP (1-495)-CAAX) (Fig. 1A), the same XRLIP construct with the 208L, 244A or 317V mutations did not (Fig. 2B). Using confocal microscopy, we verified that this absence of depigmentation was associated with the absence of damage to the actin cytoskeleton (data not shown). To confirm that this inhibition of the effect of the GAP domain on actin cytoskeleton was due to the presence of the mutation, protein expression was evaluated by Western blotting and compared to that of unmutated XRLIP (1-387)-CAAX, which induces blastomere depigmentation (Fig. 2C). In all cases the amount of mutated protein synthesized was equivalent or superior to that of XRLIP (1-387)-CAAX (Fig. 2C). With the mutants 208L and 317V the embryos were normal when the amount of mRNA injected was less than 1.5 ng/ blastomere, and at higher levels they were depigmented. With the mutant 244A they were always depigmented whatever the level of mRNA (data not shown). Therefore, the 208L, 244A and 317V mutations all alter the capacity of RLIP to affect both the pigmentation and organization of the actin cytoskeleton of the embryos. The two mutants 208L and 317V, which do not totally lose their GAP activity, are less efficient in inhibiting the phenotypic effect induced by RLIP than the 244A mutant, which appears not to bind to GTPase proteins (Li et al., 1997). Consequently, in our CAAX assay, mutations that have been shown to affect GAP activity, lead a milder bleaching phenotype when the RLIP GAP domain is targeted to the plasma membrane. These observations provide clear evidence that the GAP domain of RLIP is involved in the control of the integrity of the actin network.

Cdc42 is a putative target of XRLIP GAP activity *in vivo*

The target of the GAP domain of RLIP is Cdc42, and, to a lesser extent, Rac (Jullien-Flores et al., 1995; Cantor et al., 1995). To determine which small G protein is the target of RLIP during early development, the GAP activity of XRLIP was examined for its possible effect on the activated forms of various members of the Rho GTPase family. mRNAs coding for Cdc42 12V, Rac 12V or Rho 12V were injected into embryos alone or with XRLIP (1-387)-CAAX. Individually, each activated small G protein mutant induced bleaching of the ectoderm. The bleaching effect was also observed in the presence of XRLIP (1-387)-CAAX. We therefore examined whether the action of RLIP GAP on the integrity of the actin cytoskeleton could be abolished by co-expression of the dominant negative forms of Rho GTPases. mRNAs (1 ng/blastomere) coding for WT Cdc42 or a

dominant negative form Cdc42 17N, Rho 19N or Rac 17N were injected alone or with 0.75 ng /blastomere of mRNA coding for XRLIP (1-387)-CAAX (0.75 ng mRNA/blastomere). When the blastomeres were injected with XRLIP (1-387)-CAAX alone (Fig. 3B and C) or co-injected with Rho 19N, Rac 17N or Cdc42 WT (Fig. 3H, I, K, L, N, and O) depigmentation was observed in more than 90% of the embryos (n= 40 to 60 for each mRNA) from the cleavage stages (32 or 64-cell) onwards and evolved towards ectoderm lesions during their development. Because of the lesions these embryos did not survive beyond the neurula stage. In contrast, co-injection of XRLIP (1-387)-CAAX mRNA with Cdc42 17N (1 or 0.5 ng mRNA/blastomere) did not affect pigmentation during the cleavage stage (Fig. 3E end F) (92%, n=50). Nevertheless, during gastrulation, these embryos failed to close their blastopores and did not survive beyond stage 32 as determined with sibling embryos. Hence, only the dominant-negative form of Cdc42 rescues the deleterious effects caused by targeting of the N-terminal region of RLIP to the membrane. This indicates that the sequestration of Cdc42 GEFs was sufficient to block the deleterious effects of the GAP domain of RLIP on the integrity of the actin network. These results are consistent with observations made *in vitro* (Jullien-Flores et al., 1995; Park and Weinberg 1995, Cantor et al., 1995) and indicate that the GAP domain of RLIP requires Cdc42-GTP to induce bleaching of the embryos. Hence, during early development, RLIP controls the dynamics of the actin cytoskeleton through the small G protein Cdc42.

Cdc42 exerts a synergistic effect on XRLIP-CAAX

Since RLIP requires Cdc42 during early development we examined whether RLIP targeted to the plasma membrane could recruit a cytosolic form of Cdc42 such as Cdc42-GTP. To inhibit normal targeting of Cdc42 12V to the plasma membrane, the cysteine at position 188, which is part of the CAAX sequence, was mutated to serine (Cdc42 12V C188S). No phenotype occurred when at most 0.4 ng/blastomere of mutated mRNA were injected (n=54) (Fig. 5D). One blastomere of the animal hemisphere at the two-cell stage was injected with the amount of XRLIP-CAAX mRNA (0.5 ng/blastomere) that does not induce depigmentation (Fig. 4B) alone or together with 0.2 ng/blastomere of Cdc42 12V C188S mRNA (Fig. 4C). As expected no effect on the phenotype was observed (n=45) when XRLIP-CAAX was injected alone. In contrast, when the same amounts of XRLIP-CAAX mRNA and Cdc42 12V C188S mRNA were co-injected, as above, there resulted a clear depigmentation of the animal pole at the 32-cell stage (93%, n=56) (Fig. 4C). To ensure that the depigmentation was not due to an unexpected ratio of translation of the two proteins, the levels of XRLIP and Cdc42 expressed were determined by Western blot. All the XRLIP constructs were

fused with the Myc epitope, and their protein products were detected by an anti-myc antibody and the Cdc42 protein by anti-Cdc42 antibodies. The results show that the levels of expression of XRLIP and Cdc42 were equivalent (Fig. 4E). These results provide evidence that, in embryonic blastomeres, activated Cdc42 and XRLIP-CAAX have a synergistic effect on the integrity of the actin cytoskeleton and strongly suggest that this could result from their direct interaction with each other.

Cdc42 interacts *in vivo* with XRLIP

To examine whether a possible direct molecular interaction between the proteins Cdc42 and XRLIP occurs *in vitro*, the lysates of embryos expressing Myc–XRLIP were incubated with GST-RalA 23V, GST-Rac 12V or GST-Cdc42 12V, each one charged with [γ-^{32}P]GTP. GST-RalA 23V and GST-Cdc42 12V precipitated RLIP but not GST or GST-Rac 12V (Fig. 5A), showing RLIP and GST-Cdc42 12V interact *in vitro* with apparently high affinity. To determine whether these interactions occur *in vivo*, the embryos were injected with mRNAs corresponding to the full-length Myc-XRLIP (Fig. 5B) or mRNAs composed of different XRLIP constructs containing or not the RhoGAP domain as did the XRLIP (330-636) (Fig. 5C). Anti-myc agarose beads served to analyze the amount of endogenous Cdc42 retained with Myc-XRLIP. When Myc-XRLIP or Myc-XRLIP-CAAX were expressed in the embryo, endogenous Cdc42 protein was retained on the beads (Fig. 5B) as well as when the mutants were expressed (Fig. 5C). Except for XRLIP (330-636), the deletion mutants that contained the RhoGAP domain all bound to endogenous Cdc42 (Fig. 5C). The 208L 317V double mutant exhibited a weaker reproducible co-immunoprecipitation signal. This confirms the study carried out on the GAP domain of p190 showing that mutations of Arg^{85} or Asn^{194} are not essential for the binding of Rho (Li et al., 1997). Even if the amounts of Cdc42 retained on the beads were not equivalent in the different experiments, the presence of Cdc42 in the protein fraction retained by the beads is sufficient on its own to show that Cdc42 can interact with the XRLIPs *in vivo* and establishes a direct interaction between Cdc42 and the GAP domain of XRLIP in embryonic cells.

The rescue and synergistic experiments described here suggest a link between XRLIP and Cdc42-GTP. The pull down and GST experiments described above, in Figure 4 and two-hybrid experiments (data not shown), confirm that this interaction is due to the specific affinity of the GAP domain of XRLIP for Cdc42-GTP and not for Cdc42-GDP or Rac.

We therefore examined whether the interaction with Cdc42 *in vivo* is specific to the GTP form or whether it can be extended to the GDP form. Myc-Cdc42 harboring either the 12V or 17N mutation was expressed, and the embryos analyzed for their ability to interact with RLIP using co-immunoprecipitation. Figure 5D shows that comparable amounts of XRLIP were recovered in each Myc-Cdc42 co-immunoprecipitation complex. Hence not only Cdc42-GTP but also Cdc42-GDP interacts with the GAP sequence of XRLIP *in vivo*.

The N-terminal region of XRLIP is involved in the gastrulation

In previous studies, we showed that RalB is necessary for gastrulation (Moreau et al., 1999; Lebreton et al 2003.), and here we show that RLIP, its effector, has to target the dynamic cytoskeleton actin. We therefore sought to determine whether the N-terminal domain of RLIP implied in the dynamic cytoskeleton actin is also involved in gastrulation. To this end, we were able to express in the marginal zone of embryos the full length XRLIP (1-636), the C-terminal (379-636) part containing the RalBD and Reps BD or the C-terminal (468-636) part containing only the RepsBD, each one in the absence of the CAAX sequence. Thus targeting of XRLIP (1-636) and XRLIP (379-636) to the plasma membrane is dependent on the activation of Ral, whereas XRLIP (468-636) cannot interact with Ral.

When 800 pg of XRLIP (379-636) mRNA were injected into the equatorial zone of each blastomere, gastrulation of the embryos was arrested and no blastopore closure (100%, n=46) (Fig. 6Be) occurred. Cross-sections of these embryos (Fig. 6Ab) clearly show the lack of archenteron and internalization of mesodermal cells. In contrast, overexpression with 2 ng/blastomere of wild type RLIP mRNA (corresponding to XRLIP (1-636)) had no effect on development (Fig. 6Aa and d). As well as defects in blastopore closure, the blastocoel roofs of some embryos injected with XRLIP (379-636) mRNA showed a thick layer of cells. To confirm that this gastrulation defect was due to competition for Ral between endogenous RLIP and XRLIP (379-636), the part of RLIP coding for the POB1/Reps interaction domain without the Ral binding domain (XRLIP (468-636)) was injected. No effect on blastopore closure was observed (Fig. 6Ac and f). When wild type XRLIP was co-injected with XRLIP (379-636), less than 50% (n=54) of the embryos were completely blocked at the gastrulation stage. The rescue experiment was restricted by the size of the XRLIP mRNA (1.9 Kb), which limits any increase in the ratio between wild type and mutant mRNA that must be co-injected to achieve a complete rescue. Partial rescue, however, indicates that XRLIP can compete for the XRLIP (379-636) mutant, whereas co-injection of wild type XRLIP mRNA, with an equimolar amount of ß-Gal mRNA does not interfere with the phenotypic effect (Lebreton et al.,

2004). Together, these data show that XRLIP (379-636) is able to compete for the interaction of the endogenous RLIP with Ral and interferes with the gastrulation process.

These results clearly demonstrate that the local bleaching phenotype and actin disruption are caused exclusively by the μ2 BD and the GAP sequence of the N-terminal part of RLIP, when they are targeted together to the plasma membrane. Moreover, they suggest that the N-terminal domain of RLIP is also necessary for a complete gastrulation; since the expression of RLIP without this domain, when it competes with the endogenous RLIP for interaction with Ral, leads to the arrest of gastrulation.

Expression of GAP mutants results in altered gastrulation and cell movements

Once targeted to the plasma membrane, the effect of the N-terminal region of RLIP on the actin network depends on the integrity of the GAP domain. The same region of RLIP is essential for gastrulation. To determine whether the effect of the GAP domain is also required for gastrulation, the effect on gastrulation of two GAP activity mutations (208L and 244A) was tested. These mutations confer different molecular properties; 208L acts on the GAP activity directly, while 244A has been shown to be critical for binding to Rho proteins (Li 1997). mRNAs coding for these XRLIP mutants were injected (1 ng/blastomere) with GFP mRNA, either radially into the marginal zone or into the dorsal marginal zone of four-cell *Xenopus* embryos. In all embryos injected with either mutant the onset of blastopore closure was delayed (Fig. 6C), leading to failure of blastopore closure at the neurula stage. At the tadpole stage, anterior-posterior structures were greatly reduced and, for some embryos, the anterior structures were absent. In embryos that exhibited the most severe gastrulation defects, the antero-posterior axis was not established. Mutant 244A produced more pronounced effects than mutant 208L (Fig. 6D). The XRLIP 244A mRNA, coinjected with GFP mRNA into the marginal zone of two dorsal blastomeres at the four-cell stage, induced a defect in the blastopore lip in the zone where GFP expression (Fig. 6F) was observed. Later, embryos showed severe gastrulation defects, such as a shortened trunk or an absence of axis (Fig. 6E and F). Moreover, the GFP fluorescence signal shows that the cells expressing XRLIP 244A failed to migrate in the embryos (Fig. 6F) compared to embryos coinjected with wild type XRLIP (Fig. 6F). Together, these data provide additional evidence that the GAP sequence of RLIP is needed for gastrulation.

3. Discussion

RLIP is a crucial element in the Ras cascade leading to cell spreading and migration (Oshiro et al., 2002 210; Goldfinger et al., 2006). Studies on *Xenopus* embryos have shown that the Ras/Ral/RLIP pathway plays an important role in mesendoderm migration during gastrulation (Lebreton et al., 2004). Here we extend these observations to examine how RLIP participates in the control of the dynamics of the actin cytoskeleton and in gastrulation. To this end, we defined the N-terminal domain of RLIP involved in cortical actin disruption.

In early embryos, when different combinations of the XRLIP domains were targeted to the plasma membrane, the N-terminal sequence (amino acids 1 to 387) containing both the μ2 BD and the RhoGAP sequence was necessary and sufficient to act on the cortical actin. In the absence of the first 148 amino acids, the GAP sequence was unable either to induce the depigmentation phenotype or to act on the cytoskeleton of the cortical actin (Fig. 1A and B). However, the XRLIP GAP domain alone can interact with Cdc42-GTP (Fig. 6C). This suggests that the GAP sequence of RLIP is not sufficient to ensure the biological properties of RLIP *in vivo*, but requires that other parts of RLIP itself interact with an additional protein(s) to regulate the action of RLIP on the actin cytoskeleton. To date, μ2 is the only protein known to bind the N-terminal RLIP sequence (Jullien-Flores et al., 2000) and thus might be a good candidate to participate in the action of RLIP *in vivo*. The μ2 BD is a subunit of the AP-2 complex, an important structural component of the plasma membrane clathrin-coated vesicles. Reorganization of the actin cytoskeleton is essential for cell migration, and it has been shown to be involved in the formation and internalization of clathrin pits (Yarar et al., 2005). Cdc42 controls the activity of N-WASP and the recruitment of proteins involved in remodeling of the actin skeleton (Benesch et al., 2005).

Using an *in vitro* assay to test GAP activity and by titration of a specific GEF protein, we and others (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995) have been able to show that the GAP sequence of RLIP displays a greater specificity towards Cdc42 than towards Rac and the other small G proteins tested. Pull-down GST and co-immunoprecipitation experiments confirm that Cdc42 is the direct target of RLIP GAP. Co-immunoprecipitation of endogenous RLIP with Cdc42 GTP or Cdc42 GDP reveals that Cdc42, whether activated or not, interacts with the GAP domain. This observation and the low level of GTPase activity suggest that, as for IQGAP (Hart et al., 1996), RLIP could be an effector for Cdc42 and might provide a direct link between the GTPase and the actin cytoskeleton. Our experiments demonstrating that XRLIP-CAAX requires Cdc42-GTP to induce bleaching of embryos and the synergistic effect of Cdc42-GTP on XRLIP-CAAX support the hypothesis of a direct interaction between these two proteins to mediate actin rearrangement. The emerging evidence favors a model (Fig. 7) where Ral recruits RLIP at the

plasma membrane and this can in turn recruit Cdc42-GTP by its GAP domain. This complex, which is focused on RLIP, mightlocally control the cortical actin cytoskeleton and influence vesicle endocytosis and cytoskeleton formation. This mechanism might be controlled by the low GAP activity of RLIP and thereby depend on the half-life of the interaction of Cdc42-GTP with Ral/RLIP.

Using mutations of the GAP domain, we can correlate the activity of this domain with both the actin cytoskeleton and gastrulation. The 208L mutation that acts on the GAP activity (Li et al., 1997) affects the closure of the blastopore less severely than the 244A mutation, which showed a clear delay in the onset of blastopore closure and at later stages this delay resulted in anterior-posterior axis failure (Fig. 6C and D). Therefore the RLIP GAP sequence may not really be functional on its own; instead it is possible that Cdc42 is recruited to the site where it is necessary for its action on the actin cytoskeleton. Yet as suggested above and by the group of Camonis (Jullien-Flores et al., 1995), the weak GAP activity of RLIP might control this action by prolonging the interaction between GAP and Cdc42. Due to the great number of reports concerning the control of cortical actin networks in cell migration (for reviews see Suetsugu and Takenawa, 2003 and Ridley, 2001), our data strengthen the hypothesis that RLIP is involved in regulating the migratory behaviour of ingressing mesendodermal cells during gastrulation.

In addition, our results demonstrate the involvement of RLIP on gastrulation. The maternal expression of RLIP and its lengthy half-life are a limitation when morpholino experiments are performed. However, the fact that a single specific mutation (244A or 208L), which interferes either with the interaction between RLIP and Cdc42 or with GAP activity, prevents blastopore closure, reinforces the possibility that RLIP is involved in this developmental process. Moreover, expression of these mutants induced defects in dorsal mesendodermal cell migration (Fig. 6F) inside the embryo and suggests that, during early stages of gastrulation, RLIP is required to maintain the mesodermal migration property. The shortened axis induced by RLIP mutants (Fig. 6E) also suggests that convergent extension movements are perhaps disrupted. Thus, during gastrulation, the FGF signal could participate in morphogenetic movement through two distinct transduction pathways that act on two types of cell behavior. In addition, these combined results are consistent with the proposal that Ras, an element of the FGF signaling pathway, acts as a spatial-temporal switch for the activation of mesodermal genes through its interaction with Raf and later for cell migration through interaction with the effector RalGDS.

To summarize, although the possibility remains that RLIP binds GDP- or GTP-loaded GTPases with different affinities under non-saturating conditions, the results we present here suggest that RLIP might be important both in regulating Cdc42 via its GAP sequence and in properly localizing different components within the plasma membrane microdomain of the cell where it acts on the dynamics of F-actin. The Ral pathway appears to be involved in EGF receptor endocytosis (Nakashima et al., 1999; Jullien-Flores et al., 2000). By recruiting different partners, RLIP might participate in the internalization of a FGF receptor, which activates the Ral protein in the marginal zone of migrating cells (Lebreton et al., 2003), but also in the internalization of integrins for cell migration of mesendodermal cells. In the cell motility process, the integrins mediate interaction with the extracellular matrix and are recycled by endocytosis from the rear of the cell to its leading edge (Lawson and Maxfield, 1995). This would mean that RLIP is directly involved in cell migration when recruited by Ral. The Ral mutant lacking affinity for RalBP1 (RalBP1 is also designated RLIP) exhibits a much lower cell motility (Suzuki et al., 2000). Internalization might be achieved by μ2 BD and clathrin pit formation, and might be facilitated by local remodeling of the cortical actin cytoskeleton by the GAP domain (Fig. 8). Further studies are necessary to confirm the function of RLIP on endocytosis during gastrulation, and interaction of RLIP with μ2. These data show that the interaction of RLIP with Cdc42 participates in the remodeling of the actin cytoskeleton and in mesodermal cell movement during gastrulation. Yet it remains to be established how RLIP regulates Cdc42 to control actin cytoskeleton remodeling. It will also be necessary to specify which of the following mechanisms - cell adhesion, cell shaping or cell migration - are controlled by Ral//RLIP and which type of behavior - mesendoderm migration or convergent extension - depends on RLIP during gastulation

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Figure legends

Figure 1. **Identification of the RLIP domain that acts on the actin cytoskeleton**. Myc-tagged XRLIP deletion mutants were addressed to the plasma membrane by the CAAX sequence in their C terminus. To compare their relative activities on the actin cytoskeleton, embryos at the two-cell stage were injected into the animal pole of one blastomere with 300 to 500 pg mRNA. (A) Myc-XRLIP (1-636)-CAAX (full-length), and XRLIP divided in two parts: Myc-XRLIP (1-495)-CAAX (coding for μ2BD, RhoGAP and RalBD) or the Myc-XRLIP (330-636)-CAAX (coding for RalBD and POB1/Reps) deletion mutants were tested. For XRLIP (1-636)-CAAX and the mutant containing RhoGAP, and the μ2 and Ral binding domains, depigmentation was observed. **(**B) Each individual domain of the XRLIP N-terminus: RhoGAP domain (XRLIP (177-387)-CAAX), the μ2 BD (XRLIP (1-230)-CAAX), or the RhoGAP domain did not induce depigmentation. Left panel: Pictures of the animal hemisphere of embryos at the large-cell blastula stage. Middle Panel: Localization of XRLIP-CAAX mutants was analyzed with the Myc antibody 9E10 and secondary antibodies conjugated to FITC. Right panel: Confocal microscopy analysis of the effect of the same mutants on the cortical actin cytoskeleton from the animal pole. The immunofluorescence images of actin cortical cytoskeleton were obtained by rhodamine-phalloidin. Disruption of cortical actin is observed in embryos injected with the same mutants that induced bleaching. On the right, schematic presentation of mutants used. The numbers in brackets represent the amino acids corresponding to the full-length XRLIP that delimits each mutant.

Figure 2. **Mutations that affect the activity of the GAP domain reduce the effect of XRLIP-CAAX on the actin cytoskeleton.** (A) Effect of the mutation R208L on the GAP activity of XRLIP. Constructs of wild type XRLIP (1-636) or XRLIP containing the RhoGAP domain alone (XRLIP (177-387)), associated with the μ2 BD and Ral binding domains (XRLIP (1-495)), associated with the μ2BD (XRLIP (1-387)) or without the 208L mutation were incubated with (γ-^{32}P) GTP-Cdc42. The products were separated by filtration and the residual filter-bound radioactivity was determined after incubation. (B) Embryos were injected with mRNAs coding for XRLIP containing the μ2BD linked to the GAP domain carrying the CAAX sequence (XRLIP (1-387)-CAAX. Three mutations were tested, 208L, 244A and 317V. None of the embryos injected

with a mutated GAP domain presented flaws in their pigmentation. (C) Analysis by Western blot of protein expression. Each lane was loaded with protein extracts corresponding to one half embryo and loading was controlled by anti-tubulin and anti-Myc antibodies.

Figure 3. **Only the dominant negative form of Cdc42 rescues the depigmentation phenotype induced by XRLIP-CAAX.** The animal hemisphere of embryos was injected with mRNAs coding for Cdc42, Rho or Rac in an inactive form, with Cdc42 WT alone, or with RLIP-CAAX containing the μ2BD and the GAP domain (amino acids 1-387). Individually, the small G proteins of the Rho family do not induce pigmentation (D, G, J and M), whereas XRLIP-CAAX co-injected with either Rho, Rac inactivated forms or Cdc42 wt induces bleaching of the animal hemisphere (H, I, K and L). Only Cdc42 17N rescues the bleaching of embryos (E and F). Right panel corresponds to an enlargement of the area delimited by the yellow rectangle.

Figure 4. **Synergistic effect of XRLIP-CAAX and Cdc42 12V C188S**. (A) The animal hemisphere of embryos were injected with 200 pg XRLIP-CAAX mRNA or 50 pg Cdc42 V12 C188S mRNA alone, or coinjected with the same amounts of mRNAs. Only coinjection induced bleaching of the embryo. At least 40 embryos of two independent experiments were scored for each mRNA microinjected. (E) Each lane was loaded with protein extracts corresponding to one half embryo and tested with the appropriate antibodies (anti Myc for the XRLIP-CAAX constructs and anti-Cdc42).

Figure 5. **Cdc42 interacts with the GAP domain of XRLIP**. (A) *In vitro,* GST-RalA, Rac and Cdc42 loaded with γ-^{32}P GTP were mixed with or without protein extracts from 10 embryos, precipitated with glutathione-Sepharose beads, washed, eluted in SDS and separated by SDS-PAGE followed by autoradiography. Cdc42 and RalA interacted with XRLIP, whereas GST or Rac did not present a signal with anti-Myc antibody. (B) Co-immunoprecipitation (Co-IP) of endogenous Cdc42 with Myc-XRLIP or XRLIP-CAAX. (C) Co-IP of endogenous Cdc42 with different deletion mutants of XRLIP (see Figure 1 for details of domains tested). (D) Co-IP of endogenous RLIP with Myc-Cdc42 17N or 12V. Data are representatives of at least three independent experiments.

Figure 6. **Effect of RLIP deletion mutants, and GAP domain mutant induces severe on gastrulation.** (A) Embryos at four-cell stage, were injected into the marginal zone. (d, e and f) Vegetal view of embryos at 12.5 stage. (a, b and c) cross-section of embryos corresponding to the same stage of development. White arrows indicate the archenteron and the arrowheads the site of blastopore formation. (B) Schematic representation of the XRLIP deletion mutants without the CAAX sequence, used in embryos shown in the upper pictures. Letters in bracket corresponds to the mutants used in the pictures. (C) Embryos injected with mRNAs coding for WT XRLIP or mutants into the four blastomeres in the marginal zone. (D) Quantification of embryos with an abnormal phenotype at the 35-36 stage. Embryos were injected with 0,8 ng mRNAs in the marginal zone at the four-cell stage in the two dorsal blastomeres (grey) and in all four blastomeres (white). (E) Phenotypes of *Xenopus* embryos injected in the dorsal marginal zone with RNA encoding XRLIP 244A. (F) Embryos coinjected with XRLIP or XRLIP 244A and GFP mRNAs into the two dorsal blastomeres at the four-cell stage. The cells that express XRLIP are identified by GFP fluorescence during the different stages of early development. The embryos injected with RLIP 244A show fluorescence in a more posterior location than embryos injected with XRLIP.

Figure 7. **Model for recruiting of protein complex by RLIP during gastrulation.** Activation of FGF sequentially recruits proteins that activate Ras. Activated Ras interacts with RalGDS that in turn activates Ral. RLIP then interacts with Ral and is used as a platform allowing interaction with many proteins such as Cdc42, through its GAP domain, and μ2 a subunit of the adaptor protein complex AP-2. Together Cdc42 and μ2 are involved in endocytosis and locally control the dynamics of cortical cytoskeleton actin. (?) Corresponds to the interaction with unidentified proteins.

Figure

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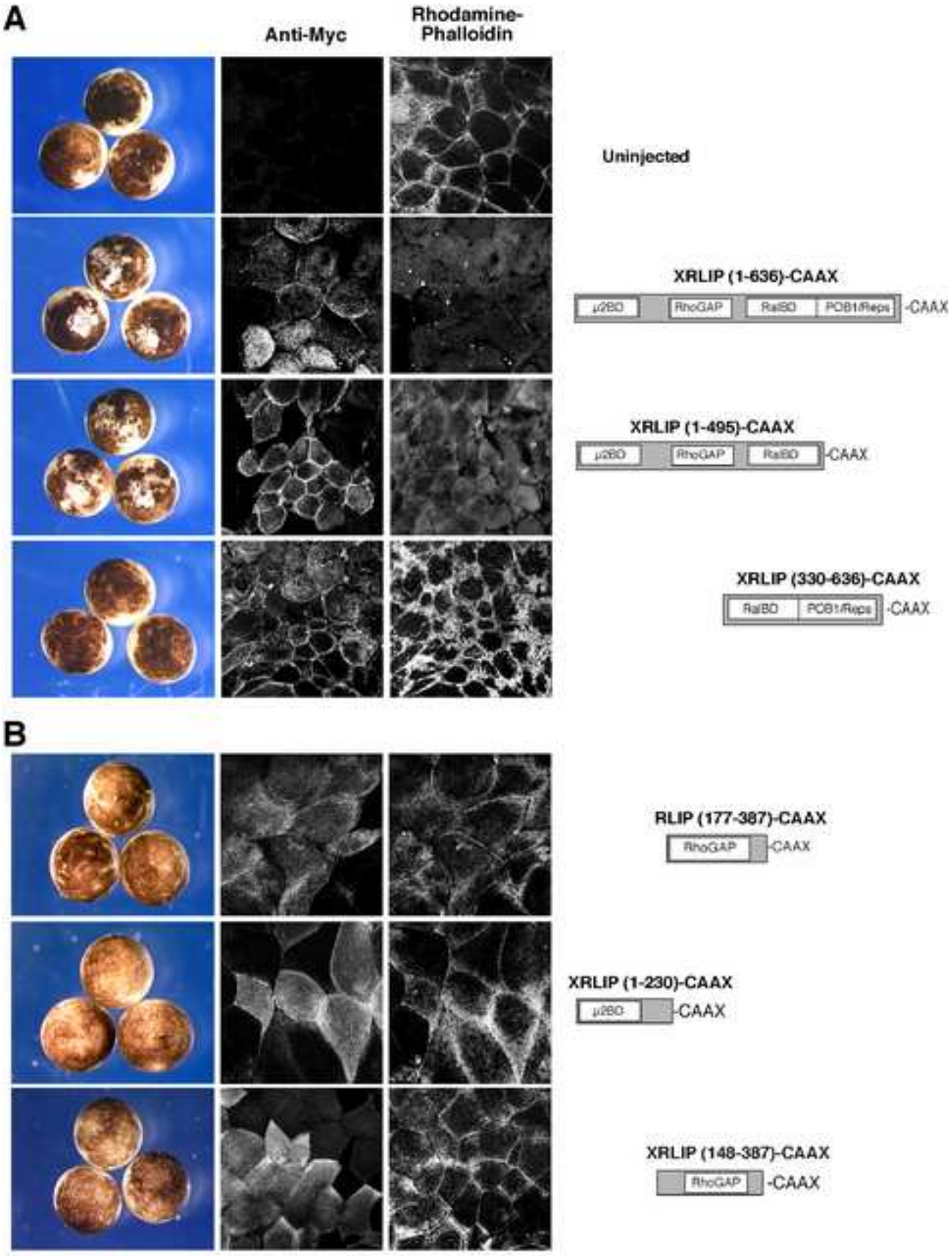

Fig. 1

Figure

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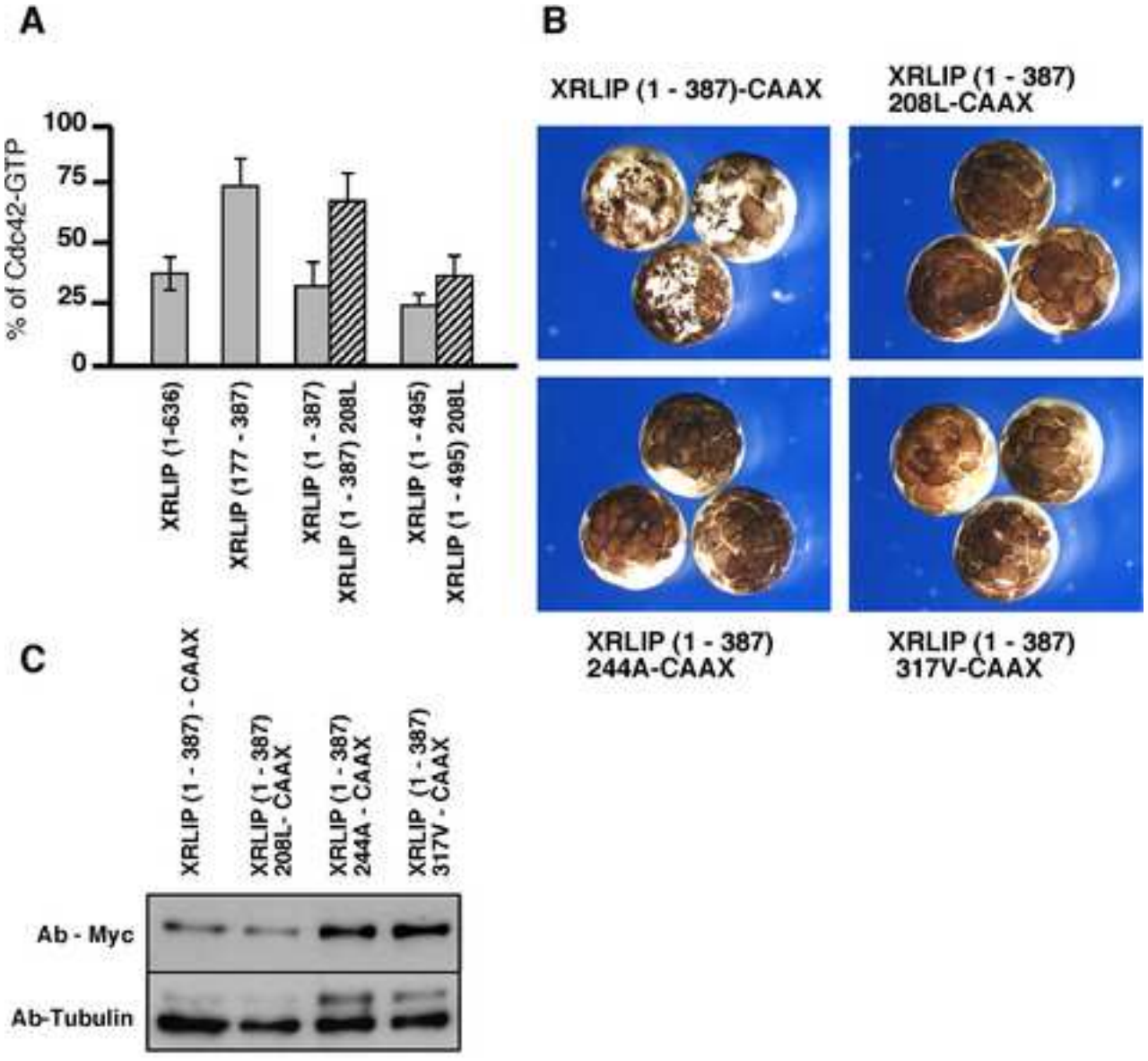

Fig 2

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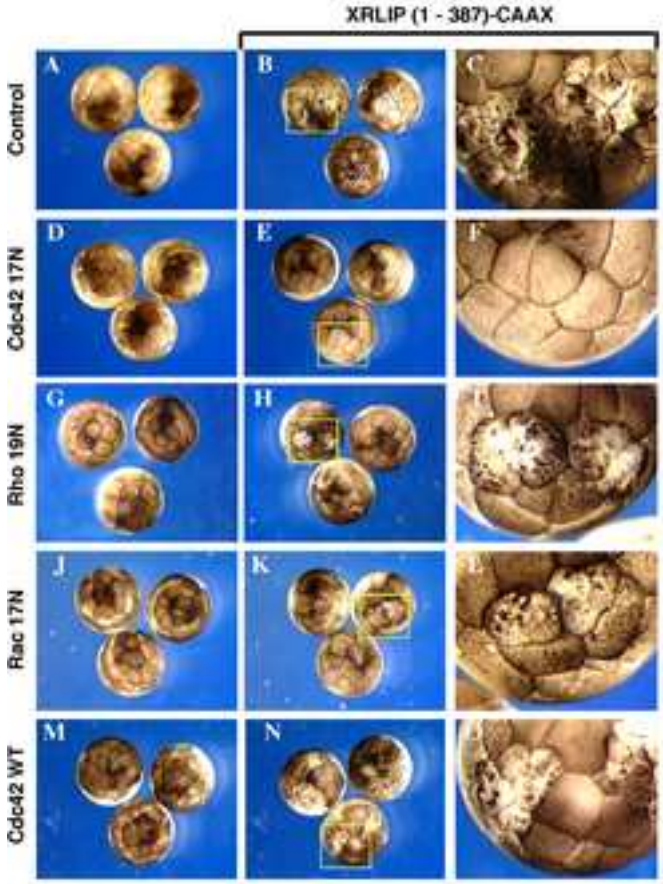

Fig 3

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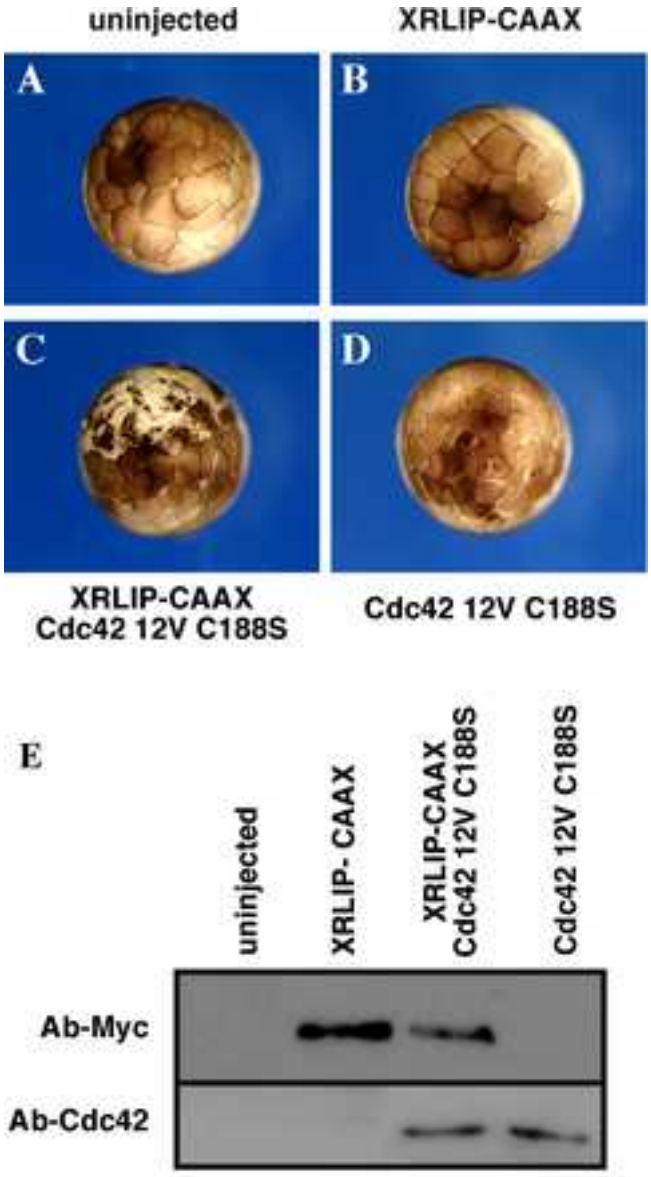

Fig. 4

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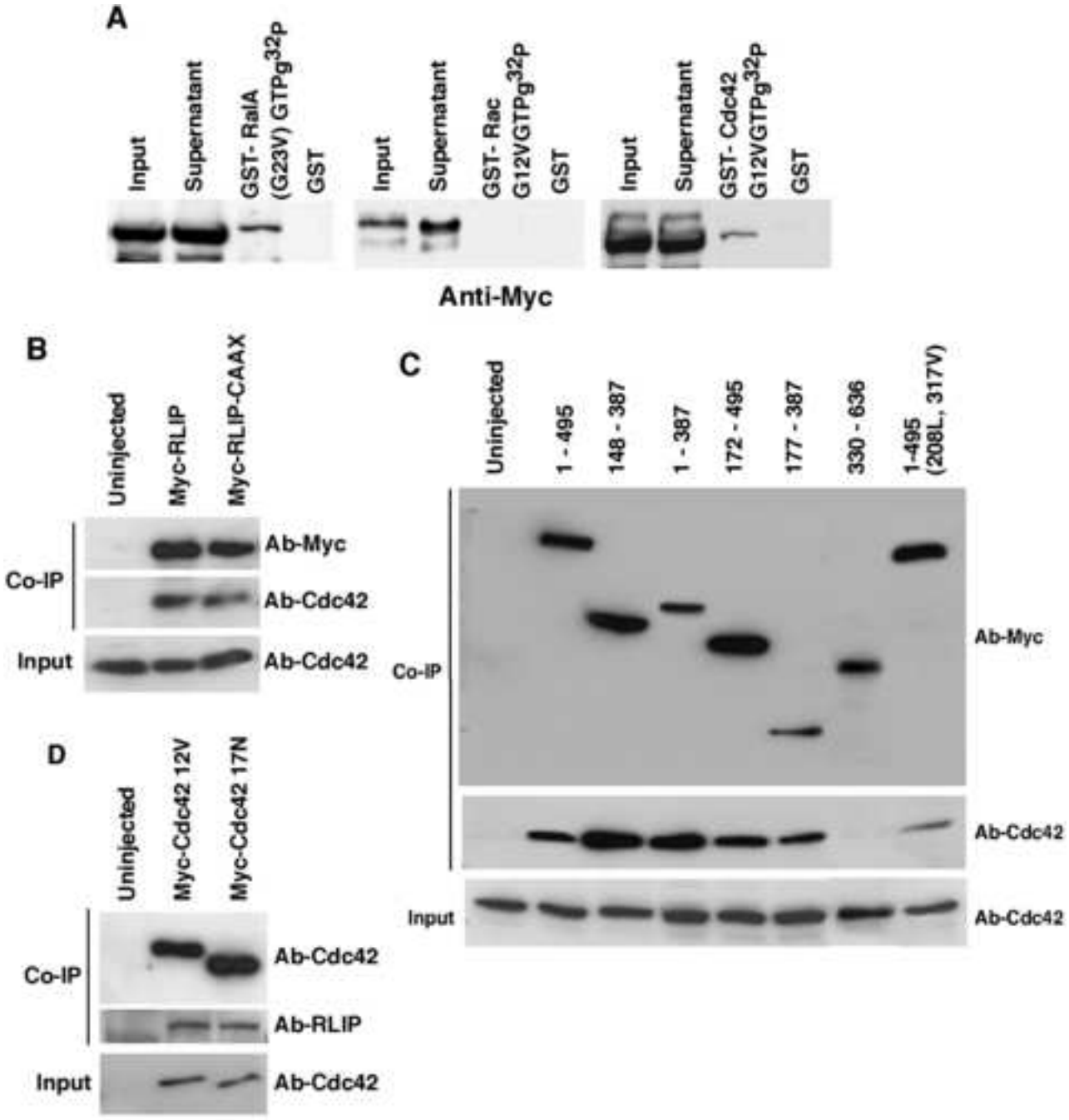

Fig. 5

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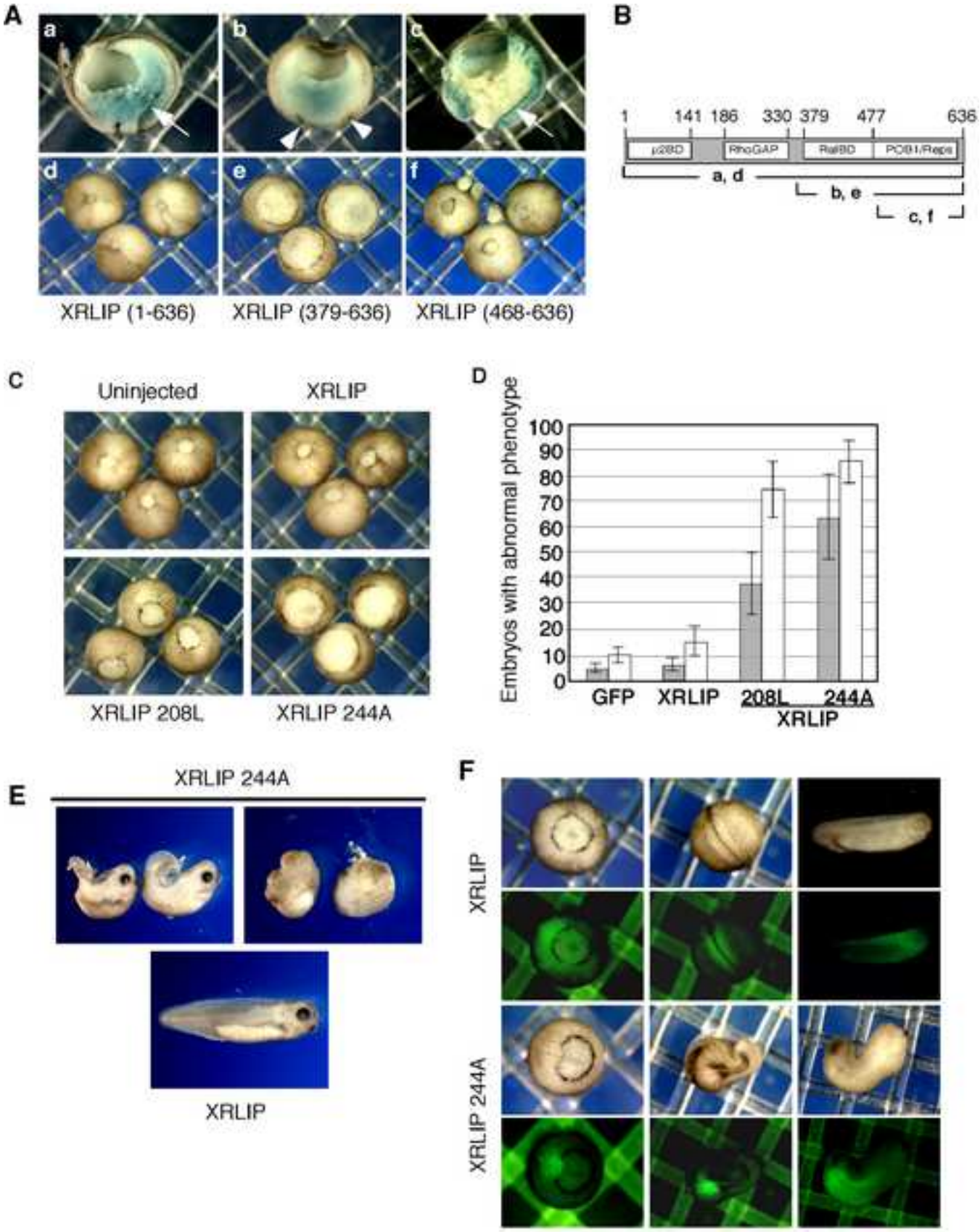

Fig. 6

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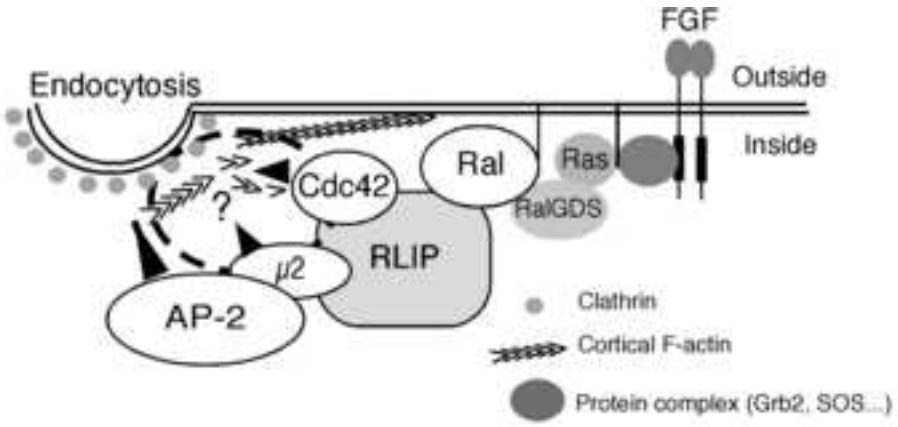

Fig. 7