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Involvement of the eukaryotic initiation factor 6 and kermit2/gipc2 in *Xenopus laevis* pronephros formation

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ABSTRACT The translation initiation factor Eif6 has been implicated as a regulator of ribosome assembly, selective mRNA translation and apoptosis. Many of these activities depend upon the phosphorylation of eif6 Serine 235 by protein kinase C (PKC). Eif6-60S is probably part of the RNA-induced silencing complex (RISC). *eif6* over-expression in *Xenopus* embryos causes aberrant eye development. *kermit2/gipc2* morphants have an eye phenotype similar to that of the eif6 overexpressors. Eye formation is regulated by insulin growth factor (IGF) signalling. eif6 interacts with the IGF receptor (IGFR) and kermit2/gipc2, which also binds to igfr. *eif6* over-expression in *Xenopus* causes also the formation of antero-ventral oedema, suggesting a malfunction of the excretory system. Here we evaluated the pronephros phenotype. The oedema grows into the nephrocoel, expanding its boundary and is accompanied by a strong reduction of the pronephros. The three main components of the pronephros are severely impaired in *eif6* over-expressors, while are not affected in *eif6* morphants. Conversely, *gipc2* depletion induces the oedema phenotype and reduction of the pronephros, while *gipc2* overexpression does not. p110*, a constitutively active p110 subunit of the PI3 kinase partially recovers the oedema phenotype. We also determined that PKC-dependent phosphorylation of Ser235 in eif6 is not required to produce defective pronephroi. These results indicate that the levels of *eif6* are highly regulated during development and instrumental for proper morphogenesis of the pronephros. Moreover, it appears that for proper pronephros development the *gipc2* level should be kept within or over the physiological range and that the oedema phenotype is partly due to the inhibition of IGF signalling.

KEY WORDS: *pronephros, Xenopus laevis, eukaryotic initiation factor 6, kermit2/gipc2*

Eif6 [1](eukaryotic initiation factor 6) is a protein essential for cell survival that plays important roles in ribosome biogenesis and translation (see Ceci *et al.,* 2003). In particular, it is associated with the plasma membrane where it interacts with β4 integrin as well as the cytoskeleton (Biffo *et al.,* 1997). Eif6 can be regulated by extracellular signals, such as IGF (Gandin *et al.,* 2008), and is itself a regulator of translation at the 60S ribosomal subunit. When unphosphorylated, it impedes the joining of the 60S with the 40S ribosomal subunit. When phosphorylated by PKC, Eif6 separates from the 60S subunit. As a consequence, ribosomal subunits 60S and 40S may join, allowing mRNA translation to occur (Ceci *et al.,* 2003). Eif6-60S is probably part of the RISC (RNA-induced silencing complex) and as such may regulate the availability of mRNAs for translation (Chendrimada *et al.,* 2007). Because EIF6 appears to act selectively on specific mRNAs (Ji *et al.,* 2008; De Marco *et al.,* 2010), the study of this protein in embryogenesis is quite appealing because it may help to explain steps of anlagen determination or differentiation.

We previously showed that, in *Xenopus laevis*, eif6 is a factor regulating apoptosis upstream of bcl-2/bax (De Marco *et al.,* 2010). Moreover, *eif6* over-expression causes a delay in eye develop-

Abbreviations used in this paper: Akt, serine/threonine-protein kinase; βgal, β-galactosidase; *Clcnk* , chloride channel Kb; *cdh16* , human cadherin-16; Eif6 [1], eukaryotic initiation factor 6; IGF, insulin growth factor; Igfr, insulin growth factor receptor; gipc2, GAIP interacting protein, C terminus; *lhx1*, LIM homeobox 1; miR, microRNA; *myod1*, myogenic differentiation 1; *nphs1*, nephrin; p110*, protein 110 (subunit of the PI3 kinase); pi3/kinase, phosphatidylinositol 3-kinase; RISC, RNA-induced silencing complex; S235A, eif6 mutated in serine 235; *slc5a9*, solute carrier family 5 (sodium/glucose cotransporter), member 9.

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Note 1: We use official names (www.ncbi.nlm.nih.gov/sites/gquery) for each gene or protein according to the species to which they refer.

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ment, evident at stage 35, independent of the eif6-regulated apoptosis. Analyses of *eif6* over-expressors and morphants led to the conclusion that eye morphogenesis is achieved only at a tightly regulated level of eif6 (De Marco *et al.,* 2011). In *Xenopus*, eye formation is regulated by igf signalling (Pera *et al.,* 2001; Wu *et al.,* 2006), and eif6 is able to modulate the translation of specific mRNA upon stimulation of growth factors such as Igf (Gandin *et al.,* 2008). Recently, it was found that eif6 interacts with igf receptor (igfr) and kermit2/gipc2 (GAIP interacting protein, C terminus) (Wu *et al.,* 2006; De Marco *et al.,* in preparation), which also binds to igfr (Booth at al. 2002). *gipc2* morphants have an eye phenotype similar to that of the eif6 gain-of-function embryos (Wu *et al.,* 2006; De Marco *et al.,* in preparation). It should be mentioned that the gipc2 protein is a powerful modulator of signals as it interacts not only with igfr but also with a variety of molecules implicated in cell signalling, such as semaphorins-F and 4C (Wang *et al.,* 1999), and integrins (Spicer *et al.,* 2010) (see Wu *et al.,* 2006, for detailed references).

In addition to the above-mentioned effects, eif6 over-expression in *Xenopus* causes the formation of antero-ventral oedema, suggesting a malfunction of the excretory system. Importantly, Wu *et al.,* (2006) showed that a *gipc2* riboprobe labels *X. laevis* pronephroi, where igfr is expressed as well (Groigno *et al.,* 1999). The connections existing between eif6 and gipc2 prompted us to investigate whether these two proteins have a role in the pronephros formation, highlighting the interesting possibility that they act along the igf pathway.

The pronephros is the embryonic kidney, present and fully functional in amphibian tadpoles. It originates mostly from the intermediate mesoderm. At metamorphosis, the pronephros regresses, and its function is replaced by the adult mesonephros. Importantly, as many events regulating pronephros induction, patterning and differentiation have been revealed, it is now evident that the genetic programs underlying pronephros formation are conserved across evolution, being similarly present in the kidneys of higher vertebrates (Vize *et al.,* 1997; Jones, 2005). *Xenopus* pronephros is composed of the pronephric corpuscle, with the glomus as the filtration unit, the pronephric tubules and the pronephric duct. The filtration chamber of the pronephros is the nephrocoel, initially contiguous to the coelom. Later, the nephrocoel and the coelom will separate into two distinct cavities. Splanchnic mesoderm surrounds the glomus (Vize *et al.,* 1997). Convoluted pronephric tubules are composed of ciliated nephrostomes and connecting tubules linked to a common tubule which joins the pronephric duct. The first histological indication of pronephric development occurs around stage 21 when cells begin to condense away from the intermediate mesoderm below somites 3 to 5. These cells will form the main body of the pronephros. At about the same time, below somites 5 to 7, a similar cellular condensation occurs that will give rise to the pronephric duct. The primordia of these two structures fuse, and the body of the pronephros forms a lumen.

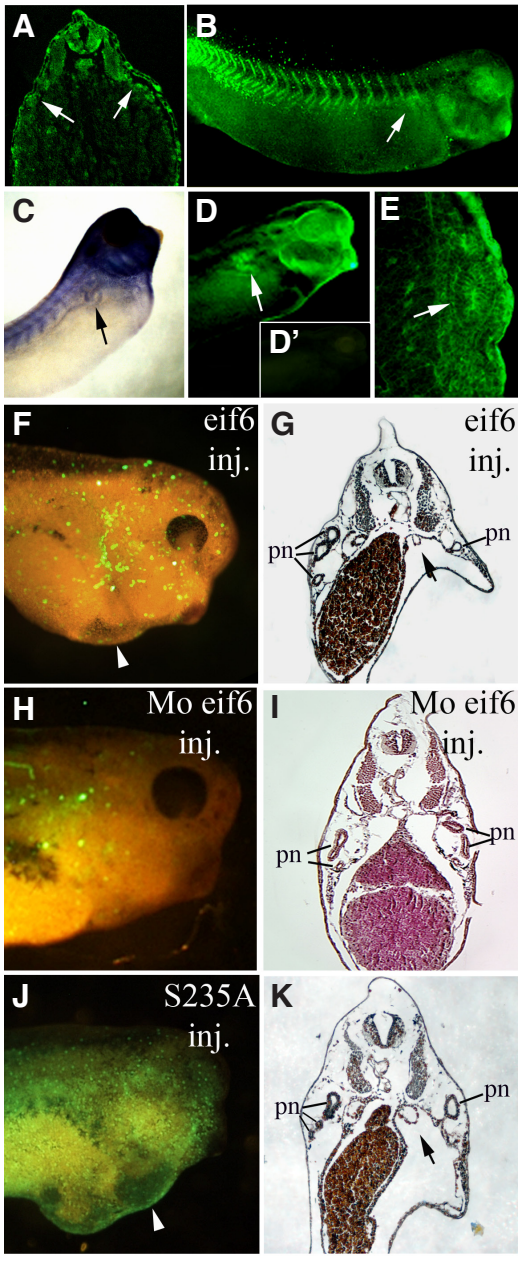

Fig. 1. The pronephros phenotype in *eif6* overexpressors. (A-E) *eif6 mRNA and protein are expressed in the pronephros (arrows) of stages 24, 32 and 38* Xenopus laevis *embryos.* **(A,B)** *Immunofluorescence using anti-Eif6 antibody of section* **(A)** *or whole mount* **(B)** *of respectively stage 24 and stage 32 embryos indicating that eif6 is present in the pronephros as well as in most tissues; the arrows indicate the pronephros anlagen.* **(C)** *Whole mount* in situ *hybridisation using* eif6 *antisense probe (stage 38).* **(D,E)** *Immunofluorescence with anti-Eif6 antibody of whole mounts* **(D)** *or sections* **(E)** *of stage 38 embryo.* **(D')** *Whole mount immunofluorescence using only secondary antibody BODIPY-conjugates as negative control. Staining is absent.* **(F)** *Oedema formation (arrowhead) is present in embryos injected with 400 pg of* eif6 *and 300 pg of* GFP *into one blastomere at the two-cell stage and harvested at stage 38.* **(G)** *Histology with haematoxylin and eosin on the embryonic sections showed in (F). A reduction of pronephric tubules is evident. The arrow indicates that the glomus is not surrounded by the splanchnic mesoderm, as the oedema has grown in the nephrocoel, expanding the splanchnic mesoderm boundary (see also K).* **(H)** *A stage 38 embryo injected with* eif6 *morpholino: there is no oedema.* **(I)** *A section of the same embryo stained with haematoxylin and eosin.* eif6 *depletion did not produce oedema.* **(J,K)** *Embryos injected with 400 pg of S235A and 300 pg of* GFP *show the oedema (arrowhead).* **(J)** *The pronephric reduction is evident in the histological section of embryo shown in (K). The arrow indicates that the glomus is not surrounded by the splanchnic mesoderm. pn, pronephros.*

In this study, we found that the oedema phenotype is present in *eif6* over-expressors and *gipc2* morphants and is due to a defect of the pronephros. Indeed, *in situ* hybridisation showed that the three main components of the pronephros are severely impaired in *eif6* over-expressors, while in *eif6* morphants the oedema is absent. Conversely, *gipc2* depletion induces the oedema phenotype and *gipc2* gain-of-function has no effect on the pronephros. A constitutively active p110 subunit (p110*) of the PI3 kinase partially recovers the oedema phenotype, suggesting that the oedema is at least partly due to the inhibition of the igf signalling. Therefore, the interplay between igfr, eif6 and gipc2 appears to be relevant during pronephric development.

Results

The pronephros phenotype in eif6 gain-of-function and gipc2 loss-of-function embryos

Anti-eif6 immunostaining shows the pronephric anlagen in stage 24 and 32 embryos (Figs. 1A-B). The pronephros of stage 38 embryos is marked by *eif6* riboprobe in *eif6* whole mount *in situ* hybridisation, (Fig. 1C, and see Vaccaro *et al.,* 2006) as well as by anti-eif6 immunostaining (Fig 1D,D',E). It should be observed that eif6 is present in most tissues at various level in agreement with previous data (Biffo *et al.,* 1997; Vaccaro *et al.,* 2006) (Figs. 1). Overall, 65% of the embryos injected with *eif6* (400 pg; n=91) displayed oedema in the antero-ventral region, a condition often caused by impaired osmoregulatory function of the pronephros. Indeed, upon sectioning, we found a strong reduction of the pronephric tubules in the injected side. Moreover, in contrast to the uninjected side, the splanchnic mesoderm delineating the nephrocoel does not surround the glomus. The oedema grew into the nephrocoel, expanding its boundary (Figs. 1F,G). The eif6 morpholino does not cause oedema. Accordingly, in *eif6* loss-of-function embryos, the pronephros of the injected side was not affected (Figs. 1H,I).

Previous data showed that, in *X. laevis*, PKC-dependent phosphorylation of Ser235 is necessary for regulating both translation and the anti-apoptotic activities of eif6, but it is not required for proper eye development (De Marco *et al.,* 2010). Here, by injecting the mutated form of eif6, S235A, the oedema and pronephric reduction are present as well (Figs. 1J,K), indicating that phosphorylation of Ser235 in eif6 is not required to produce defective pronephros development. Therefore, the aberrant kidney phenotype produced by *eif6* over-expression is not directly linked to the PKC-regulated effects on translation and ribosomal subunit interaction. This finding is similar to what we have found for the eye phenotype in *eif6* over-expressors (De Marco *et al.,* 2011). As gipc2 depletion leads to an eye defect similar to *eif6* over-expression (Wu *et al.,* 2006; Marco *et al.,* 2011), we investigated the involvement of gipc2 in the pronephros phenotype. We injected *gipc2*-morpholino (40 pg, n=82) and found that in 65% of the morphants, oedema develops similar to the oedema found in eif6 over-expressors but not in the embryos injected with *gipc2* mismatch-morpholino (Fig. 2). The phenotype appears at stage 32, when the pronephros starts functioning, and is particularly evident at stage 38 in both *gipc2* morphants and in *eif6* over-expressors (Fig. 2). It should be mentioned that *gipc2* over-expressors do not display oedemas (data not shown and see Wu *et al.,* 2006).

eif6 over-expression and gipc2 depletion affect pronephros markers

The primordia of the pronephros starts forming at stage 21 (see Vize *et al.,* 1997). Whole mount *in situ* hybridisation of stage 22 *eif6* over-expressors using *lhx1*, an early marker of the pronephros anlagen (Agrawal *et al.,* 2009) was performed. Staining shows that the pronephric anlagen of the injected side is markedly less extended with respect to the contra-lateral uninjected side (70% n=60) (Fig. 3A,B). It should be noted that, according to Wu *et al.,* (2006), although the pronephros is not marked by gipc2 *in situ* hybridisation before stage 33, gipc2 is maternally detected and expressed throughout early development. At stage 22 of *gipc2* morphants, the injected side shows little evidence of the pronephric anlagen (66% n=60) (Fig. 3C). Previous data showed that the *gipc2* morpholino phenotype can be rescued by coinjection of gipc2 RNA lacking the morpholino

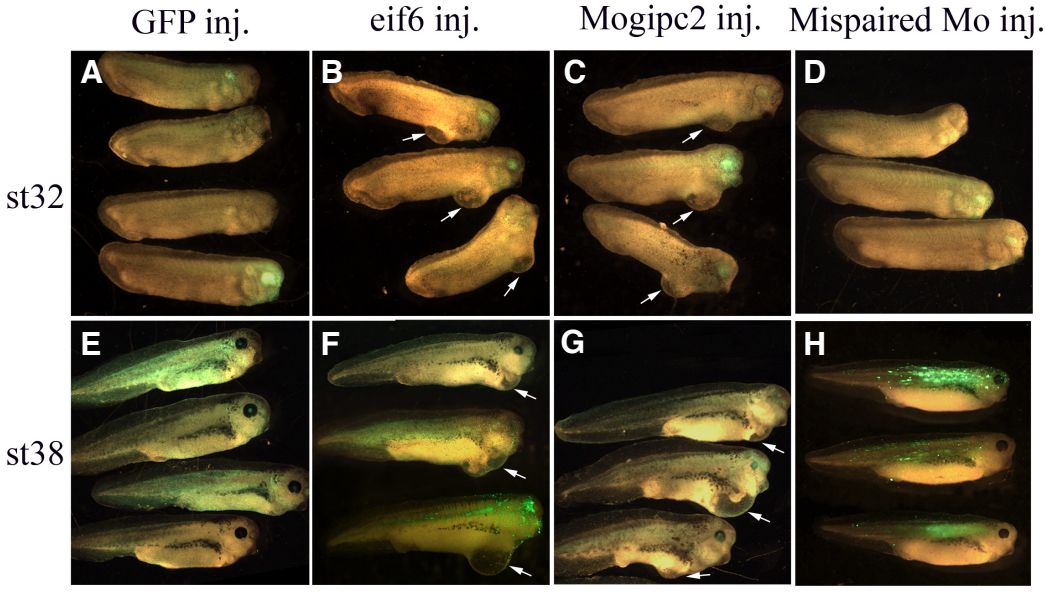

Fig. 2. Oedema phenotype in *eif6* overexpressors and in *gipc2* loss-of –function embryos. **(A-D)** *Stage 32 and stage 38 embryos* **(E-H)** *injected into one blastomere at the two-cell stage with 400 pg of* GFP *alone* **(A,E)**, *400 pg of* eif6 *and 300 pg of* GFP **(B,F)** *40 ng of* gipc2 *morpholino and 300 pg of* GFP **(C,G)** *or 40 ng of* gipc2 *mispaired morpholino and 300 pg of* GFP **(D,H)**. *In the* eif6 *over-expressors* **(B,F)** *and* gipc2 *morphants* **(C,G)**, *the oedema phenotype (arrow) appears at stage 32 and increases at stage 38.*

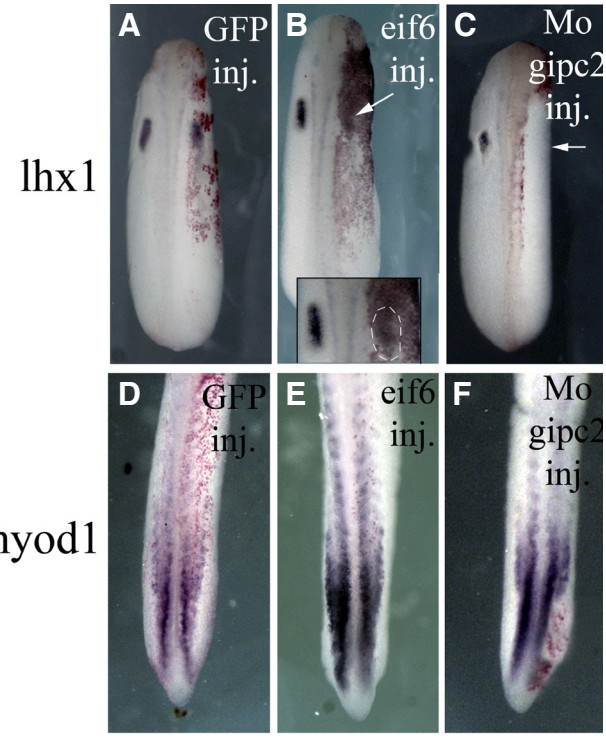

Fig. 3. *eif6* over-expression and *gipc* depletion affect *lhx1* expression. *Embryos injected into one blastomere at the two-cell stage with* GFP **(A,D)**, eif6 **(B,E)** *and* gipc2 *morpholino* **(C,F)**. βgal *mRNA was co-injected for tracing lineage (stained red).* **(A-C)** *Embryos were cultured until stage 22 and whole mount* in situ *hybridised for expression of* lhx1*, an early marker of the pronephric anlagen. Injection of the GFP alone had no effect on* lhx1 *expression* **(A)**. eif6 *over-expression* **(B)** *and* gipc2 *depletion* **(C)** *inhibited most pronephric anlagen formation (arrow). The white lines in the inset of (B) surround the reduced pronephric anlagen.* **(D-F)** *Embryos were cultured until stage 25-26 and whole mount* in situ *hybridised for expression of* myod1*, a marker of differentiating muscle. No difference in the* myod1 *expression pattern was found in the* eif6 *over-expressors* **(E)** *and* gipc2 *morphants* **(F)**.

target sequence (Wu *et al.,* 2006). This phenotype is not related to the closely positioned paraxial mesoderm. Indeed, embryos fixed at stage 25/26 and hybridised with *myod1*, have identical hybridisation patterns in the injected and uninjected side, both in the case of *eif6* over-expressors (n=50) and *gipc2* morphants (n=50) (Figs. 3D-F).

The pronephros is organised along its proximal-distal axis in a manner that is highly similar to the metanephric nephron, thus allowing the use of molecular markers of metanephric terminal differentiation for the identification of various segments (Zhou and Vize, 2004). By using nephrin (*nphs1*) as marker of the glomus (see Jones, 2005), it can be seen that at stage 38 the glomus of the injected side appears reduced when compared to the uninjected side in *eif6* over-expressors (78% n=70) (Figs. 4B,B') and *gipc2* morphants (78% n=60) (Figs. 4C,C'). The proximal tubule marker *slc5a9* (Zhou and Vize, 2004) shows that this region is partially affected by eif6 over-expression (Figs. 4E,E') (60% n=70) as well as by *gipc2* depletion (62% n=70) (Figs. 4F,F'). Whole mount *in situ* hybridisation was performed using a *clcnkb* or *cdh16* riboprobe, both markers of intermediate and distal tubules and the duct. In *eif6* over-expressors, the pronephric anlagen of the injected side is markedly less developed with respect to the counter-lateral uninjected side (81% both *clcnkb* n=65 and *cdh16*n=70) (Figs. 4H,H',K,K'). Very similar hybridisation patterns are obtained in the injected side of *gipc2* morphants (Fig. 4I,I',L,L').

It is known that eif6 interacts with igfr and gipc2 and that gipc2 binds to igfr, leading to the activation of the tyrosine kinase pi3/akt kinase pathway (Wu *et al.,* 2006; De Marco *et al.,* in prepa-

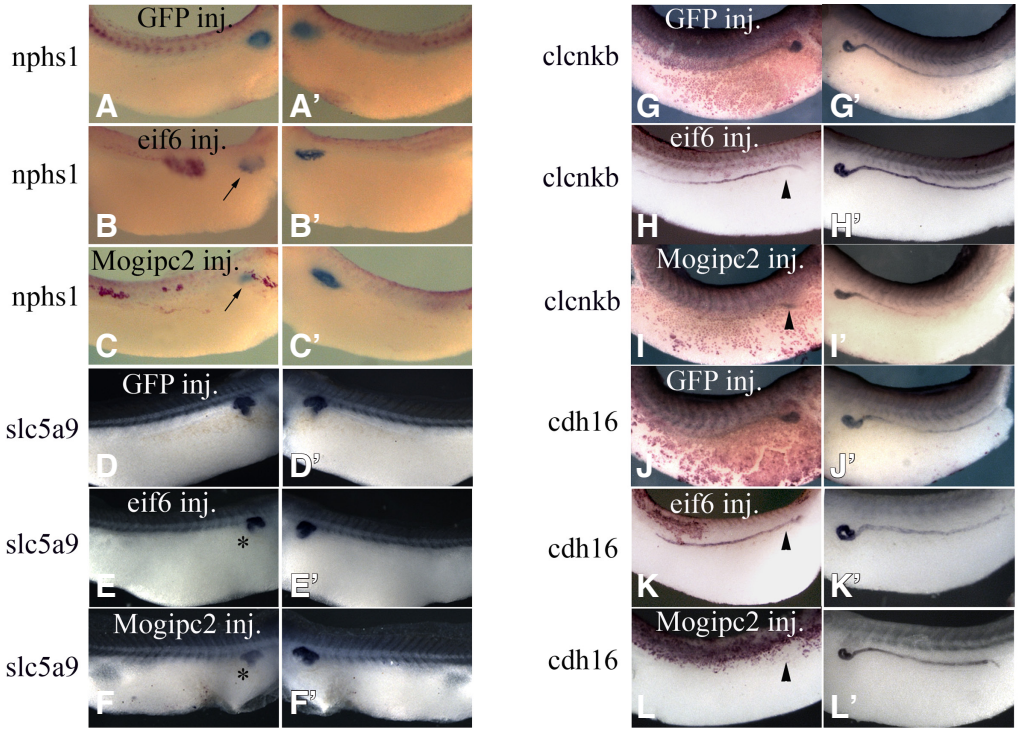

Fig. 4. Whole mount *in situ* hybridisation for markers of terminal pronephros differentiation. *Stage 38 embryos injected with* GFP *alone* **(A,D,G,J)**, eif6 **(B,E,H,K)** *or* gipc2 *morpholino* **(C,F,I,L)**. *Micrographs of the two sides of the same embryos are shown* (**A,A'**, **B,B',** *etc.). Co-injection of* **(D-F)** GFP *mRNA as tracer, and* **(G-I)** *βgal mRNA (stained red). It should be observed that the red stained tissue distributed in the most superficial tissues is often partially removed during manipulation. The glomus, (arrow) marked by* nephrin*; proximal tubule (asterisk), marked by* slc5a9*; intermediate and distal tubule domain (arrowhead), marked by* clcnkb *and* cdh16*, respectively, were reduced in* eif6 *over-expressors and in* gipc2 *morphants.*

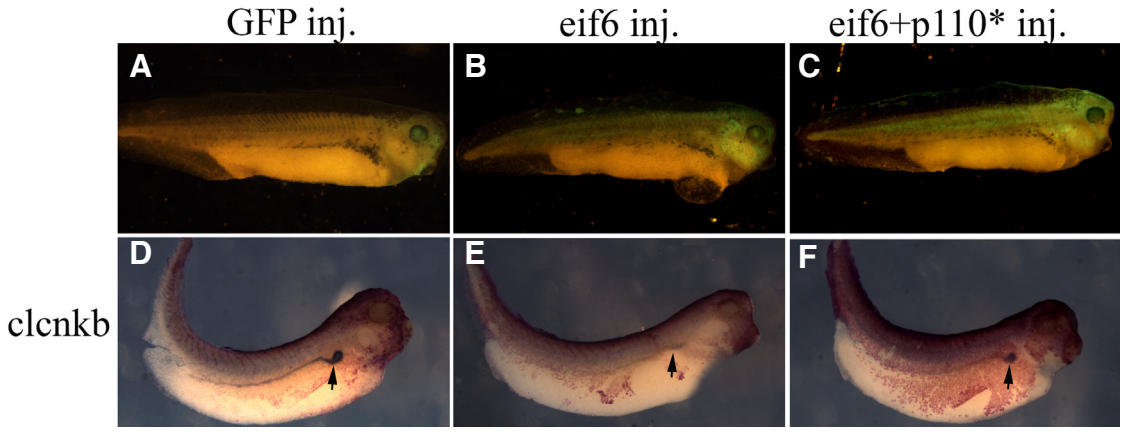

Fig. 5. p110* partially recovers the phenotype in eif6 overexpressors. (A-F) *Stage 38 embryos injected into one blastomere at the two-cell stage with 400 pg of* GFP *only* **(A,D)**, *400 pg of* eif6 *and 300 pg of* GFP **(B,E)**, *or 400 pg of* eif6, *300 pg of* GFP *and 1 ng of p110** **(C,F)**. **(D-F)** *Whole mount* in situ *hybridisation for* clcnkb, *an intermediate and distal tubule marker (arrow). p110* partially recovers the oedema phenotype* **(C)** *and* clcnkb *expression* **(F)**.

ration). To investigate whether the pronephric defect from eif6 over-expression is related to a decrease in akt phosphorylation/activation, we used a constitutively active p110 subunit (p110*) of the pi3 kinase, which is upstream of akt and downstream of igf1r. Indeed, when we coinjected embryos with *eif6* and p110* and compared to eif6 over-expressors, a partial rescue of the oedema phenotype and *clcnkb* marker expression was obtained (72% n=60) (Fig 5). Thus, p110* partially recovers the oedema phenotype, suggesting that this phenotype is at least partly due to the inhibition of the pi3 kinase/akt pathway.

Discussion

In conclusion, our results indicate that in *Xenopus* eif6 over-expression produces an aberrant kidney phenotype that is not directly linked to the pkc-regulated effects of eif6 on translation and ribosomal subunit interaction. This finding is in contrast to eif6 anti-apoptotic function and to the translation of *b-catenin* that require the conserved S235 in *Xenopus* embryogenesis (De Marco *et al.,* 2010, 2011). Our data suggest that eif6 regulates gipc2 levels in *Xenopus* embryogenesis through a different mechanism. In fact, for the kidney phenotype, eif6 may act through RISC (Chendrimada *et al.,* 2007; De Marco *et al.,* 2011) down-regulating *gipc2* translation. Indeed, analyses of the 3'UTR of *gipc2* through the miRBase (www.mirbase.org) and miRanda (www.microrna.org) programs depict binding sites for miRNAs implicated in kidney and eye development. Examples of miRNA regulation include the following: the miR-30 family (Agrawal *et al.,* 2009), which appears to regulate *Xenopus* pronephros development; miR467e*, which is implicated in eye development of the mouse (Karali *et al.,* 2010); and miR-532-5p, which is involved in kidney cancer and human retinoblastoma (Hoon and Kitago, 2010).

In embryos injected with eif6 morpholino, the pronephros does not differ from that of w.t. embryos, leading us to speculate that the levels of eif6 are highly regulated during development and instrumental for proper morphogenesis of the pronephros. This finding is in agreement with previous data on eif6 activity in eye development (De Marco *et al.,* 2011). On the other hand, this result also suggests that *gipc2* expression beyond threshold levels is necessary for pronephric formation.

Is igf signalling involved in the pronephric formation? gipc2 binds to igfr and is involved in the maintenance of igf/pi3k/akt stimuli. Embryos coinjected with *gipc2* morpholino and p110* partially recover the defective eye phenotype of gipc2 morphants (Wu *et al.,* 2006). This finding is in agreement with our findings showing a partial rescue of the pronephric defect upon coinjection of *eif6* and p110*. In *Xenopus*, igfr (Groigno *et al.,* 1999) as well as the adaptor proteins of the igfr substrate family (IRS-1 family) are expressed in the pronephros. However, it has not been determined whether IRS-1 acts through the pi3k/akt pathway. Because two organs, the eye and pronephros, are regulated by eif6 and gipc2 (this paper and De Marco *et al.,* in preparation), the question can be raised as to whether any other anlagen development is also dependent upon the interaction of these two molecules. Comparing the major expression patterns of *gipc2* and *eif6* suggests that this could be the case (Vaccaro *et al.,* 2006; Wu *et al.,* 2006). Further studies should establish the functionality of these colocalisations and whether eif6 and gipc2 interactions are required for igf signalling. As the gipc2 protein is a potential interactor not only of igfr but also of a variety of molecules implicated in cell signalling, we hypothesise that during *Xenopus* embryogenesis the eif6/gipc2 partnership is a crucial step for anlagen development. In fact, eif6 may regulate the gipc2 levels, consequently leading to organ-specific downstream-signalling events.

Materials and Methods

Animals

Adult *Xenopus laevis* females were obtained from Nasco (Fort Atkinsons, Wisconsin, USA). They were kept and utilised at the Department of Structural and Functional Biology of the University of Naples, Federico II, according to the guidelines and policies dictated by the University Animal Welfare Office and in agreement with international rules. To obtain eggs, *X. laevis* females were injected in the dorsal lymphatic sac with 500 units of Gonase (AMSA) in amphibian Ringer's solution (111 mM NaCl, 1.3 mM CaCl2, 2 mM KCl, 0.8 mM MgSO4, and 25 mM Hepes, pH 7.8). Fertilised eggs and embryos were obtained by standard insemination methods (see De Marco *et al*., 2010) and staged according to Nieuwkoop and Faber.

Microinjections

The pCS2eif6 and pCS2S235A plasmids were used as described in De Marco *et al.,* (2010). The pCS2p110˚ plasmid was a generous gift of Laurent Kodjabachian (Université de la Méditerranée, France). The pCS2βgal was kindly supplied by M. Ori (University of Pisa, Italy). Capped synthetic RNAs were generated by *in vitro* transcription using the Sp6 Message Machine kit (Ambion, Austin, TX, USA). *gipc2* antisense morpholino (5'-AGAGGCATCTTTCTTTCAGCGAAGG-3'), eif6 antisense morpholino (5'-GCGGACGGCCATGTTGGCTTCTTAG-3') and the mispaired morpholino control (5'-GCcGACcGCCATcTTGcCTTCTaAG-3') were purchased from Gene Tools LLC, Philomath, OR, USA. pCS2MTGFP or

pCS2βgal mRNA was always co-injected to label the injected side. RNAs were injected into the animal hemisphere of a single cell of one- or two-cell embryos using a Drummond 'Nanoject' apparatus. During injection, embryos were cultured in 3% Ficoll in 0.1% Ringer. The phenotype of the injected embryos was scored when the uninjected embryos reached stage 32 or 38. The samples were photographed with a Leica MZ16F UV stereomicroscope, equipped with a Leica DFC 300Fx camera and IM50 image manager software.

Immunofluorescence and histology

X. laevis embryos were fixed in 4% formaldehyde at 4°C and stored in 100% MetOH at –20°C. For *in toto* immunofluorescence, the samples were incubated in hydrogen peroxide/methanol 1:2 (v/v) for about 2 days, then incubated with rabbit anti-Eif6 antibody (a gift of PC Marchisio and S. Biffo, DIBIT, Milan, Italy) diluted 1:500 in PBS/0.5%BSA/0.1% Triton X-100. The secondary antibody was goat anti-rabbit IgG BODIPY FL conjugated (Molecular Probes). Next, the embryos were dehydrated in graded methanol and cleared in benzyl alcohol/benzyl benzoate 1:2 (v/v). Whole mounts were photographed with a Leica MZ 16F. Frozen 10 μm-thick sections were obtained after embedding and freezing in Killik (Bio Optica). For histology, sections were stained with haematoxylin and eosin. For immunofluorescence, non-specific background was blocked by incubating the sections for 30 min in normal goat serum, 3% in PBS/0.5% BSA/0.1% Tween, prior to exposure O/N at 4ºC to rabbit anti-Eif6 (generous gift of P.C. Marchisio and S. Biffo, DIBIT, Milan, Italy) diluted 1:500 in PBS/0.5% BSA/0.1% Tween. Staining was completed by incubating the samples with anti-rabbit goat IgG BODIPY FL-conjugated (Molecular Probes) and mounting in PBS/glycerol (9:1, v/v). Sections were observed and photographed with a Leica CTR 6500 UV microscope equipped with the Leica application suite.

In situ *hybridisation*

In situ hybridisation was performed as described previously (Vaccaro *et al.*, 2006). Antisense digoxigenin-labelled RNA probes of *lhx1*, *myod1* (a gift from M. Ori, University of Pisa, Italy) *slc5a9*, *clcnkb*, *cdh16* (generously supplied by O. Wessely, LSU, New Orleans, USA) and *nphs1* (courtesy of European *Xenopus* Resource Centre, University of Portsmouth) were synthesised with RNA T7 or SP6 polymerases (Roche, Mannheim, Germany). β-galactosidase activity was visualised in embryos with Red-Gal (Biosynth, Staad, Switzerland). Sections were observed and photographed using a Leica 6500 microscope. Whole mounts were photographed with a Leica MZ16F.

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