Biology Of Reproduction
August 2015, Volume 93 Issue 2 Pages 42, 1-11
http://dx.doi.org/10.1095/biolreprod.115.130575
http://archimer.ifremer.fr/doc/00280/39099/

X-Linked Retinitis Pigmentosa 2 Is a Novel Maternal-Effect Gene Required for Left-Right Asymmetry in Zebrafish

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Abstract :

Retinitis pigmentosa 2 (RP2) gene is responsible for up to 20% of X-linked retinitis pigmentosa, a severe heterogeneous genetic disorder resulting in progressive retinal degeneration in humans. In vertebrates, several bodies of evidence have clearly established the role of Rp2 protein in cilia genesis and/or function. Unexpectedly, some observations in zebrafish have suggested the oocyte-predominant expression of the rp2 gene, a typical feature of maternal-effect genes. In the present study, we investigate the maternal inheritance of rp2 gene products in zebrafish eggs in order to address whether rp2 could be a novel maternal-effect gene required for normal development. Although both rp2 mRNA and corresponding protein are expressed during oogenesis, rp2 mRNA is maternally inherited, in contrast to Rp2 protein. A knockdown of the protein transcribed from both rp2 maternal and zygotic mRNA results in delayed epiboly and severe developmental defects, including eye malformations, that were not observed when only the protein from zygotic origin was knocked down. Moreover, the knockdown of maternal and zygotic Rp2 revealed a high incidence of left-right asymmetry establishment defects compared to only zygotic knockdown. Here we show that rp2 is a novel maternal-effect gene exclusively expressed in oocytes within the zebrafish ovary and demonstrate that maternal rp2 mRNA is essential for successful embryonic development and thus contributes to egg developmental competence. Our observations also reveal that Rp2 protein translated from maternal mRNA is important to allow normal heart loop formation, thus providing evidence of a direct maternal contribution to left-right asymmetry establishment.

Keywords : developmental biology, egg developmental competence, egg quality, fish, fish reproduction, maternal-effect gene, *ndpk*, *nme10*, oocyte, oocyte-specific, ovum, teleost, zygote

Supported by the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement no. 222719 LIFECYCLE and French National Research Agency grant (ANR-13-BSV7-0015-Maternal Legacy) to J.B.

INTRODUCTION

Human retinitis Pigmentosa 2 (RP2) gene is responsible for up to 20% of X-linked retinitis pigmentosa, a severe heterogeneous genetic disorder resulting in progressive retinal degeneration [1–6]. Rp2 protein is composed of two domains: a Tubulin Binding Cofactor C (TBCC) domain highly similar to the functional domain of metazoan Tbcc proteins, and a partial NDPk domain similar to the domain found in Nme ("Non-Metastatic cells, Expressed in") proteins [7]. Interestingly, like human TBCC protein, RP2 acts as GAP (GTPase-Activating Protein) proteins towards tubulin in a TBCC-dependent manner but, unlike TBCC, RP2 does not promote tubulin heterodimerization *in vitro* [8]. However, the GAP activity of RP2 and TBCC relies on the TBCC domain and many of the RP2 mutations involved in the retinitis pigmentosa pathology were found in this domain [3,9]. It has been shown that RP2 interacts with, and is a GAP to, Arl3, a small GTP-binding protein of still unclear function but shown to have a microtubule cytoskeleton-related role and important role in cilium protein-trafficking [8,10]. In mouse photoreceptor cells, Arl3 localizes mainly to the connecting cilium [11] and has been shown to be required for the assembly of cilia in mouse and flagella in the parasite *Leishmania donovani* [12]. Interestingly, deregulation of Arl3 by Rp2 silencing in mammalian cells leads to the fragmentation of Golgi apparatus and alters vesicle trafficking from Golgi to the cilium [13]. Transport of Rp2 protein to the basis of the cilium has been shown to be dependent on the interaction with Importin-β2, a member of the Importin-β family that regulates nuclear–cytoplasmic shuttling [14]. In addition, several mutations were shown to occur in the carboxy-terminal part of the Rp2 protein which is post-translationally modified by myristoylation and palmitoylation to regulate its localization either in the cytoplasm or at the cellular and cilia membrane [15–17]. More specifically, RP2 protein has been shown to be addressed in a myristoylated-dependent manner to the basal body and the associated centriole of the photoreceptor cilium [18]. Additionally, Rp2 localizes at the basal body of *Trypanosoma brucei* flagellum, where it was proposed to participate in a tubulin quality control mechanism prior to incorporation of tubulin heterodimers in the axoneme [19]. In mammalian cells, RP2 depletion does not seem to affect cilia biogenesis even though RP2-silenced cells present swelled cilia with accumulation of the calcium release channel polycystin-2, suggesting that RP2 could play a role in cilia biogenesis and function [18]. Additionally, morpholino-mediated Rp2 knock-down in zebrafish (*Danio rerio*) has been shown to alter embryonic development through defects such as delayed epiboly, hydrocephaly, kidney cysts, and left-right asymmetry establishment, phenotypes already well-established as related to ciliopathies, as well as retinal degeneration [18,20,21]. In these studies, authors used Rp2 knock-down and its rescue in zebrafish as an *in vivo* assay system to functionally experiment effects of known human *RP2* mutations on retinal degeneration [18,20]. Finally, it was reported that RP2 also interacts with the Nethylmaleimide-sensitive factor (NSF), a protein involved in vesicle-membrane fusion suggesting a role in membrane protein trafficking [22]. Altogether, these data have clearly established an important role of RP2 in cilia genesis and/or function [23].

Surprisingly, the ovarian predominant expression of *rp2* in zebrafish was reported [7] and confirmed [20]. This pattern was unexpected given the well-documented implication of Rp2 in retinal and cilia functions. This pattern of expression is characteristic of oocyte predominant genes, not only in mammals (see [24] for a review on early mouse development) but also in fish [25–27]. This typical expression pattern is shared by several maternal-effect genes that are key players of egg developmental competence (i.e. the egg ability to be fertilized and subsequently develop into a normal embryo) and subsequent successful embryonic development [28–31]. In all animals, early embryonic development starts with a period during which the genome of the embryo is transcriptionally silent. Throughout this

period, embryonic development entirely relies on maternal gene products (mRNAs and proteins) that have been stored in the oocyte during oogenesis. The transition between maternal gene products-based development and zygotic gene expression is called the Maternal-to-Zygotic Transition (MZT) and has been well characterized in several model species, including zebrafish (See [32,33] for review). MZT is characterized by the clearance of maternal mRNAs and by the progressive onset of transcription in the zygote genome, namely the Zygote Genome Activation (ZGA). Interestingly, recent studies have suggested a maternal-inheritance of the *rp2* transcript in the zebrafish egg [18,20]. Altogether, these observations suggest an implication of *rp2* gene in oogenesis and/or early development that remains to be demonstrated. The aim of the present study was thus to investigate the maternal inheritance of *rp2* gene products in zebrafish in order to reveal a possible maternal-effect required for successful embryonic development. Here we show that *rp2* is a maternal-effect gene and that maternal *rp2* mRNA is essential for successful embryonic development. Rp2 protein translated from maternal message is important to allow normal gastrulation, organogenesis, providing an example of maternally-inherited gene product acting after the onset of embryonic genome activation. The present study also provides evidence of direct maternal contribution to cilia-mediated development and more specifically to left-right asymmetry establishment.

MATERIALS AND METHODS

Animals and sampling

Investigations were conducted according to the guiding principles for the use and care of laboratory animals in compliance with French and European regulations on animal welfare. Protocols were approved by the Rennes ethical committee for animal research (CREEA) under approval # R2012-JB-01.

Zebrafish (*Danio rerio*), of the AB strain, were obtained from the fish rearing facility at INRA-LPGP (Rennes, France) and cmlc2:eGFP zebrafish strain from the University of Oregon fish facility (Eugene, USA). For all tissue collections, zebrafish were anesthetized and subsequently euthanized. For the oogenesis study, stages I to IV ovarian follicles were dissected from ovaries collected from four mature zebrafish females according to the developmental stages defined by Selman *et al* [34]. After removal from the body cavity, ovaries were subsequently incubated for 5 min in a Petri dish containing Hank's Full Strength solution [31] supplemented with Collagenase (0.35 mg/ml), then washed three times in large volume of Hank's Full Strength solution containing BSA (0.5 mg/ml). Ovarian follicles were then separated manually using forceps. Stage V oocytes (i.e. unfertilized eggs) were obtained from ovulating females by gentle manual stripping. For the early embryonic development study, fertilized eggs were collected within 5-10 min following fertilization. For each batch, 15-20% of the eggs were kept to assess developmental success and used to monitor survival at 24 hpf (hours post fertilization) and hatching rate. In order to analyze the developmental sequence of embryos originating from developmentally competent eggs, only egg batches exhibiting hatching rates above 80% were kept for further analysis. Remaining eggs of each batch were serially sampled at fertilization (0.25 hpf), 1 cell (0.75 hpf), sphere stage (4 hpf), shield stage (6 hpf), and 24 hpf, according to previously described developmental stages [35]. For *in situ* hybridization, whole mount *in situ* hybridization and immunohistochemistry, ovaries or embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C and then transferred in 100% methanol after three successive short incubations in methanol solution of increasing concentrations, and subsequently stored at -20°C until further processing. For western blot experiments, yolk was manually removed from the embryo in a deyolking buffer

(0.1M NaCl; 0.03M KCl; 0.02M $MgSO_4$, $7H_2O$; 0.04M $CaCl_2$, $2H_2O$; 0.1M Saccharose; 0.02M Hepes; 0.02M Bicine; pH 7.5; pOsm 315mOs), and samples stored at -80°C until protein extraction.

QPCR analysis

Samples for quantitative RT-PCR (QPCR) analysis were prepared as previously described [36]. QPCR reactions were performed with 400nM of each primer in order to obtain PCR efficiency between 95 and 100%. In order to avoid unspecific signal due to genomic DNA contamination, primers were designed on exon boundaries [4]. The relative abundance of target cDNA within a sample set was calculated from serially diluted cDNA pool (standard curve) using Applied Biosystem StepOne™ V.2.0 software. After amplification, a fusion curve was obtained to validate the amplification of a single PCR product. The fusion curves obtained showed that each primer pair used was specific of a single transcript. Negative control reactions were used to estimate background level. As 9 pg of exogenous *luciferase* RNA (Promega) per oocyte/embryo were added to the sample prior to homogenization in Tri-reagent, data were thus normalized using exogenous *luciferase* transcript abundance in samples diluted to 1/25. Genes were considered significantly expressed when measured level was significantly above background at $p<0.05$ and within the range of the standard curve.

In situ and whole-mount in situ hybridization

rp2 digoxigenin-labeled anti-sense RNA probes were produced as previously described [36] using the Promega SP6/T7 RNA polymerase Riboprobe Combination System following manufacturer's instructions, using a full-length PCR product as DNA template. Bacterial clones containing zebrafish *rp2/nme10* (GenBank Acc# EH538403.1 - FDR202-P00007-BR_G22) cDNA were obtained from the Genome Institute of Singapore and sequenced. For *in situ* hybridization (ISH), fixed samples were paraffin-embedded and serial cross-sections of 5 μm were treated as previously described using the In situ Pro, Intavis AG robotic station [36]. Digoxigenin-labeled RNA probes were diluted in hybridization buffer at a final concentration of 8 ng/μl. The digoxigenin signal was then revealed with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics) and a NBT/BCIP revelation system (Roche Diagnostics) as recommended by the manufacturer. Slides were mounted with mowiol 4–88 (Calbiochem). Whole-mount *in situ* hybridization (WISH) was also performed using the In situ Pro, Intavis AG robotic station. Ovaries digestion was carried out for 20 min at 37°C with 25 μg/ml of proteinase K. Embryo samples of early developmental stages (1cell, 4hpf, 6hpf, 12- and 18-somite stages) were not digested with proteinase K. Embryo samples at 24hpf, 48hpf and 72hpf were digested with 5μg/ml proteinase K solution for 5 min at 20°C. Digoxigenin-labeled RNA probes were diluted in hybridization buffer at a final concentration of 2 ng/μl. The digoxigenin signal was then revealed as described above for ISH. The time of revelation was kept identical for 1cell, 4hpf and 6hpf developmental stages. For 24hpf, 48hpf, and 72hpf the revelation time was 50% longer due to lower signal. Samples were then rinsed overnight in PBS and subsequently observed under binocular (Zeiss, Stemi 2000-C).

RP2 antibody

A polyclonal antibody raised against a synthetic peptide (AGRSIVPLTKGSRR, Fig.1A) and purified by affinity was ordered from Genscript (Piscataway, NJ, USA). In order to validate the antibody specificity, a 24hpf protein extract was loaded on four different tracks and hybridized under different conditions (Fig.1). The first strip was hybridized with the purified antibody (Ab), the second with the pre-immune serum (PPI), the third with the antibody

which had been pre-adsorbed on the synthetic peptide for one hour (Ab+Pept), and the fourth with no primary antibody. On the strip hybridized with the purified antibody, two separated bands are observed at the expected size. When the antibody is pre-adsorbed on the synthetic peptide, the upper band is totally absent whereas the lower band remains present. Furthermore, on the pre-immune serum strip, a band at the exact same size of the lower band can be observed. Together this indicates that this lower band does not correspond to Rp2 protein but to another protein that can be revealed using the pre-immune serum. This lower band was thus considered as non-specific in all subsequent analyses. The presence of a single Rp2 band is also consistent with recent data obtained using a full-length recombinant human RP2-GFP protein to generate an antibody used for zebrafish Rp2 protein detection [18].

Western Blot

Protein extracts from whole oocytes or embryos were prepared in protein extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 10 mM Benzamidine) supplemented with an anti-protease cocktail (1 mM AEBSF, 10 µM Leupeptin, 1 mM EDTA, 0.5 mM DTT). Homogenates were centrifuged at 14,000g for 15min at 4°C. Proteins (10 µg in each lane) in supernatants were resolved on denaturing 15% polyacrylamide gels and transferred to nitrocellulose membranes. Saturation was performed during 1h30 in incubation buffer (TBS, 0.1% Tween 20, 1% of non-fat dry milk). Membrane was then incubated overnight at 4°C with 1:250 Rp2 primary antibody diluted in incubation buffer. β-Actin antibody, as loading control, was co-incubated at 1:5000. Excess of primary antibodies was washed with incubation buffer (5x5min) and membranes were then incubated for 1h at room temperature in incubation buffer containing anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies. After 5 successive 5-min washes with washing buffer (TBS, 0.1% Tween 20), proteins were detected using Uptima UptiLight chemiluminescent revelation kit. Images were taken under Fusion FX7 Imager with Fusion software v15.11.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on Discovery XT automated station (Roche, Illkirch, France). Sections (4 µm) were dewaxed during 8min at 75°C using EZ Prep (Roche) and unmasked during 48min at 95°C with CC1 buffer and endogenous biotins blocked (Roche). Rp2 primary antibody was incubated at 1:50 during 60min at 37°C and anti-rabbit biotinylated secondary antibody incubated (Vector Laboratories) at 1:700 during 32min at same temperature. Signal detection was performed using DAB/MAP system (Roche) and slides were counter-stained using Mayer's hematoxylin before mounting.

Microinjections

To perform and validate zebrafish Rp2 protein knock-down, antisense morpholino oligonucleotides (MO) were designed (Gene-Tools, LLC). The translation-blocking morpholino oligonucleotide (ATG-MO 5′- TTTGGAGAAGAAGCACCCCATTTAT), 5 bases mismatch morpholino oligonucleotide (mMO 5'-TTTcGAcAAcAAGcACCCcATTTAT, lower case letters indicate mispaired residues) and splicing morpholino oligonucleotide (Splicing-MO 5'-GCGTCACAAATAAGTTCTAACCTCA) were used. The MOs were resuspended in nuclease-free water and 1-2 nl of 0.25, 0.5, 0.75, or 1 mM MO were injected into zebrafish embryos at 1cell stage. The rescue of the phenotype was performed using the pcDNA-6.2-EmGFP vector created and published by Hurd and coworkers [18] and containing synonymous mutations into the ATG-MO target region. The capped RNA was synthesized with mMessenger mMachine T7 kit (Ambion) and about 100 ng were co-injected with the

ATG-MO. For each injection experiment, approximately 30% of each egg batch was kept as uninjected control to assess developmental success through the monitoring of survival rates at 24 hpf and hatching period as well as the occurrence of developmental defects. Remaining eggs were injected to characterize the knock-down of Rp2. Only egg batches exhibiting hatching rates above 80% were considered for further analysis. Embryos development was followed at 4hpf (sphere stage), 6hpf (shield stage), 10hpf (bud stage), 24hpf, 48hpf, and 72hpf (hatching). Pictures were taken using a Nikon AZ100 macroscope. Survival and developmental defects were checked at 24 hpf, 48hpf, and 72 hpf.

Statistical analysis

Significant differences observed in QPCR analyses and in knock-down experiments were analyzed using Wilcoxon Rank Sum Test with superiority or inferiority alternatives hypothesis. All statistical analyses were performed using R v.2.6.2.

RESULTS

Rp2 mRNA and protein profiling during oogenesis

rp2 mRNA expression was monitored by quantitative PCR (QPCR) (Fig 2A) in the ovary throughout oogenesis. *rp2* was significantly expressed at all stages of ovarian follicular development (Fig 2A). As shown by *in situ* hybridization, *rp2* expression was restricted to the oocyte cytoplasm and no signal could be observed in follicular layers or in the oocyte nucleus (Fig 2C, Supplemental Figure S1; Supplemental Data are available online at www.biolreprod.org) thus demonstrating the strict germinal expression of the gene in the ovary. During oogenesis, mRNA expression levels per oocyte were high in stage I and remained elevated in stage II and vitellogenic stage III oocytes (Fig 2). Expression subsequently decreased during the final steps of vitellogenesis and oocyte maturation (Fig 2A). It is noteworthy that in stage V oocytes, which correspond to ovulated metaphase II oocytes (i.e. unfertilized eggs), *rp2* mRNA was still present at significant levels (Fig 2A) and can therefore be considered as maternally inherited.

Rp2 protein expression was also monitored throughout oogenesis using western blot and immunohistochemistry (Fig 3). The western blot analysis demonstrated the presence of the Rp2 protein in stage I-IV oocytes, whereas no Rp2 band could be detected in stage V oocytes (Fig 3A). The Rp2 protein could not be detected by immunohistochemistry in stage I oocytes (Fig 3B-C). A moderate and homogenous labeling was however detected in the ooplasm of stage II oocytes (Fig 3B-C). In stage III oocytes, the labeling remained moderate but was restricted to the outer part of the ooplasm (Fig 3B). Finally, in stage IV oocytes, no signal could be observed in the oocyte (Fig 3C). No labeling in the nucleus could be observed at any stage of oocyte development (Fig 3B-C).

Rp2 mRNA and protein profiling during early development

During early development, *rp2* mRNA was significantly detected in the zebrafish embryo at all studied stages using QPCR (Fig 4A). The highest expression levels per embryo were detected before the mid-blastula transition (MBT) in fertilized eggs, 1-cell embryos, and 4hpf embryos (Fig 4A). After the MBT, *rp2* mRNA levels dramatically decreased between 4hpf and 6hpf and subsequently remained low until hatching (Fig 4A).

Using whole-mount *in situ* hybridization (WISH), we observed a strong *rp2* expression in 1-cell stage (Fig 4B) and 4hpf embryos (Fig 4D). At 6hpf, a weaker *rp2* labeling was detected in the embryo (Fig 4E). At 10-somite stage, a weak *rp2* expression was observed in the cephalic region and in the developing otic vesicles (Fig 4F-G). No expression was

detected in the Kuppfer's vesicle at that stage (Fig 4H). At 18-somite stage, weak *rp2* labeling was observed all over the embryo with higher expression in the developing otic vesicles (Fig 4I-J). A strong *rp2* expression was also observed in the Kuppfer's vesicle at that stage (Fig 4I). At 24hpf, *rp2* transcript was detected at high levels in the eyes, the developing brain, the otic vesicles, all along the pronephric duct, and at the uro-genital opening (Fig 4K). At 48hpf, *rp2* transcript was detected in several parts of the developing brain, the olfactory pit, the otic vesicles, and in the eyes (Fig 4L-M). In the eyes, a very strong signal was observed at the basis of the ventral fissure of the retina, possibly at the basis of the optic nerve (Fig 4M). At 48hpf, a strong labeling was also observed in the pronephric glomeruli and along the pronephric duct (Fig 4L). Finally, a weak signal was observed in the somites (Fig 4L). At 72hpf, the labeling was similar to the labeling observed at 48hpf with weak labeling in somites and olfactory pit and strong expression in eyes, different parts of the brain, pronephric glomeruli and pronephric ducts (Supplemental Figure S2). It is noteworthy that the strong signal observed at 48hpf in the lower part of the ventral fissure of the retina could not be observed any longer at 72hpf (Supplemental Figure S2).

Western Blot analysis of whole embryo protein extracts showed that Rp2 protein was undetectable in 8- to 32-cell embryos but could be detected from 4hpf to 72hpf (Fig 5A). Using immunohistochemistry, no Rp2 protein could be detected in cytoplasm or nucleus of 2-cell embryos (Fig 5B). At 4hpf and 6hpf, Rp2 protein was expressed in all embryonic cells and strong staining was observed on cellular membranes and in the cytoplasm (Fig 5C-G).

Rp2 knock-down

Maternal and zygotic Knock-down. Using increasing concentration of antisense morpholino-oligonucleotide (MO) directed against the translation initiation site of the *rp2* transcript (ATG-MO) (Fig 1A) we were able to obtain an increase in the severity of the phenotype. Microinjection of this ATG-MO at the concentration of 0.75 mM led to a total knock-down of the Rp2 protein from 4hpf to 72hpf as shown by both western blot analysis (Fig 6A-B) and immunohistochemistry (Fig 6C). In contrast, the ATG-MO at a concentration of 0.5 mM only resulted in a partial knock-down of the protein as shown by western blot analysis (Fig 6A). At all tested concentrations (0.25, 0.5, 0.75 and 1mM), all injected embryos displayed developmental defects (Fig 6D) increasing in severity with increased ATG-MO concentrations. Using concentrations of 0.25mM and 0.5mM, no mortality increase was observed at 24hpf and 72hpf (Fig 6D), whereas with 0.75mM and 1mM ATG-MO concentration, survival at 24hpf was significantly reduced compared to non-injected embryos (Fig 6D). At 72hpf, survival rate was significantly decreased when using ATG-MO concentrations of 0.75mM and 1mM with survival rates of 42% and 21%, respectively (Fig 6D). At the same concentrations, a 5-base mismatch ATG-MO did not induce any significant increased mortality at 24hpf and 72hpf (Supplemental Figure S3A). The working ATG-MO concentration was thus set up at 0.75mM.

From fertilization to the onset of somitogenesis at 10hpf, no obvious macroscopic phenotype could be observed in ATG-MO-injected embryos at 0.75mM (Fig 6E-F, Supplemental Figure S3, C-D, F-G). Delayed development could however be detected as early as 6hpf with 25-min and 60-min delay to reach shield stage (6hpf) (Supplemental Figure S3, F-G) and bud stage (10hpf) (Fig 6E-F), respectively. ATG-MO-injected embryos never reached a similar development stage to control at 24hpf because some features, such as the vitellin extension, never developed (Fig 6 H-I). At 48hpf, important developmental defects could be easily identified with non-round and clearer eyes, hydrocephaly, pericardial edema, reduced or missing vitellin extension and bended tails (Fig 6K-L). At 72hpf, developmental defects were similar but more important to that observed at 48hpf with hydrocephaly, pericardial edema,

thin or no vitellin extension and bended tail (Fig 6N-O, Supplemental Figure S3I). More specifically, at 72hpf, the eyes of injected embryos were degenerating as shown by their shape and the lack of dark pigmented retina (Fig 6N-O, Supplemental Figure S3, J-K). Furthermore, in cmlc2:eGFP transgenic zebrafish, specifically expressing GFP in the heart, a significantly higher proportion of ATG-MO-injected fish (37%) exhibited abnormal heart loop compared to their non-injected siblings (3%) at 48hpf (Fig 6Q-S). The proportion of embryos exhibiting abnormal heart loop remained significantly different from the proportion of injected embryos exhibiting normal heart loop (63%) (Fig 6Q-S). A partial, yet significant, rescue of this phenotype was observed when the ATG-MO was co-injected with *in-vitro* transcribed mutated *rp2* mRNAs (Fig 6Q).

Zygotic Knock-down. We then used a MO directed against the exon 2 / intron 2 boundary of the transcript (splicing-MO) (Fig 1A) in order to specifically target *rp2* mRNAs transcribed from the zygote genome but not maternal *rp2* mRNAs that are already spliced. After injection of the splicing-MO in one-cell embryos, Rp2 protein was detected at 4hpf, 6hpf, and 24hpf (Fig 6B). At 48hpf, a very weak Rp2 band was detected in embryos injected with the splicing-MO and became almost undetectable at 72hpf (Fig 6B). After injection of the splicing-MO at a concentration of 0.75mM, Rp2 labeling observed by immunohistochemistry at 4hpf was similar to the signal observed in non-injected embryos (Fig 6C). Altogether, these results validate the specificity of the zygotic transcripts targeting.

Microinjection of the splicing-MO at a concentration of 0.75mM did not affect survival rates at 24hpf and 72hpf (Supplemental Figure S3B) and none of the injected embryos displayed developmental defects at any studied stages from fertilization to 24hpf (Fig 6G, J, Supplemental Figure S3, E, H). At 48hpf, weak developmental defects could be noticed with limited hydrocephaly and small pericardial edema (Fig 6M). In contrast to ATG-MO, the shape and pigmentation of the eyes were similar to non-injected embryos even though their size seemed lightly smaller. In addition, a limited number of embryos displayed a bended tail or reduced vitellin extension (Fig 6M). At 72hpf, developmental defects were similar to those observed at 48hpf with little hydrocephaly and small pericardial edema (Fig 6P). At 48hpf, no significant difference in heart loop side was observed in splicing-MO-injected cmcl2:eGFP transgenic zebrafish (6%) compared to their non-injected siblings (3%) (Fig 6Q-S).

DISCUSSION

Oocyte-specific rp2 *mRNA is maternally-inherited in the zebrafish egg*

Using *in situ* hybridization, we were able to demonstrate that *rp2* was exclusively expressed in the oocyte during oogenesis and could not be detected in somatic follicular layers or in extra-follicular tissue. This germinal expression is consistent with the strong ovarian predominant expression previously reported in zebrafish [4] that is often observed for the so called oocyte-specific or oocyte-predominant genes in vertebrates [24,28,31,37–39]. In the present study, we also report the expression of Rp2 protein in the oocyte during oogenesis. The drop in protein expression in fully-grown zebrafish oocytes suggests a role in developing oocytes during oogenesis that remains undocumented and deserves further attention. Expression data in stage V oocytes (i.e. unfertilized eggs) and fertilized eggs also clearly indicate that *rp2* mRNA is maternally-inherited and confirms previous observations obtained using non-quantitative PCR [18,20]. The quantitative expression profile reported here during early development further indicates that *rp2* transcript exhibits a typical maternal mRNA profile with a dramatic decrease in abundance at the time of zygotic genome activation and maternal transcript clearance [33,40]. During gastrulation and organogenesis, *rp2* gene remains expressed but at a much lower level per embryo compared to pre-MBT stages. This suggests that, in addition to its maternal-inheritance, *rp2* gene also exhibits a mild zygotic

expression. In contrast to the corresponding transcript, Rp2 protein is not maternally-inherited in the zebrafish egg as demonstrated by the lack of expression in ovulated stage V metaphase II oocytes and in 8- to 32-cell embryos. In consistency with this observation is the lack of signal observed by immunohistochemistry at 2-cell stage. In contrast, the protein is detected in embryos beyond 4 hours post-fertilization and in the oocyte during oogenesis. This lack of expression before the onset of zygotic genome activation is consistent with the abundance profile of the *rp2* mRNA. The mRNA and protein expression profiles suggest a role of Rp2 protein translated from maternal mRNA in zebrafish development from the mid-blastula transition and the beginning of epiboly.
Together, our expression data suggest that Rp2 protein may first play a role in intra-ovarian oocyte development during oogenesis. We also show that *rp2* mRNA is maternally-inherited in the zebrafish egg and that Rp2 protein is translated from maternal mRNAs before or around the full activation of the zygotic genome and the onset of epiboly, suggesting a participation in embryonic development.

Maternally-inherited rp2 *mRNA contributes to egg developmental competence*

rp2 mRNA is required for successful early embryonic development as shown by the reduced embryonic survival and the major developmental defects observed after ATG-MO injection. Using an ATG-MO concentration sufficient to induce a complete knock-down of the protein, embryonic development was delayed, embryonic survival was low, and all embryos displayed severe developmental defects. Embryonic malformations were characterized by a delayed progression of epiboly, a reduced or missing vitellin extension and, from 24hpf, by hydrocephaly, pericardial edema, retinal and eye degeneration, and bended tail. At lower ATG-MO concentrations (*i.e.* 0.25mM and 0.5mM), that did not induce a complete knock-down of the protein, milder effects were obtained but all developing embryos exhibited similar abnormal phenotypes. This dose-effect response in the intensity of the observed phenotype would be in favor of an important role of maternal *rp2* mRNA in egg developmental competence (*i.e.* the egg ability to be fertilized and subsequently develop into a normal embryo). Furthermore, when another morpholino-oligonucleotide was used to specifically knock-down translation of zygotic but not maternal *rp2* transcripts by targeting a splicing site (splicing-MO), embryos developed normally until 24hpf while weak developmental defects could be detected after 48hpf. Together, these results clearly indicate that maternally-inherited *rp2* mRNA is required for successful early embryonic development and thus contributes to egg developmental competence. Our study also reveals that *rp2* is a maternal-effect gene (i.e. a gene that encodes mRNA and/or protein that are produced in the oocyte during oogenesis and play an essential role during early embryogenesis [25,40,41]). By using two types of morpholino-oligonucleotides, ATG-MO or splicing-MO, we show that the Splicing-MO, specifically targeting transcripts of zygotic origin, did not induce any developmental delay at 24hpf while a noticeable delay could be observed with the ATG-MO from the onset of epiboly. These observations indicate that the knock-down of the Rp2 protein translated from maternal *rp2* mRNA delays the gastrulation process and alters embryonic phenotype at 24hpf. Interestingly, the QPCR analysis showed a dramatic decrease in *rp2* mRNA abundance between 4 and 6 hours of development, a moment when Rp2 protein can be detected in the embryo. Our data therefore indicate that *rp2* mRNA from maternal origin allows the expression of the Rp2 protein before the onset of epiboly and clearly suggest a role of Rp2 in embryonic development from the beginning of the gastrulation process. As Rp2 partially overlaps with Tubulin Binding Cofactor C (TBCC) protein function [8], it has been suggested to contribute to cell cycle progression through microtubule

polymerization/nucleation in cell centrioles [42]. The role of Rp2 in the gastrulation process has however not been studied so far and deserves further attention.
Together, our observations indicate that maternally-inherited *rp2* mRNA contributes to egg developmental competence. Our observations also reveal that Rp2 protein translated from maternal message is crucial to allow normal gastrulation, a process occurring after the onset of embryonic genome expression. While several maternal-effect mutations have been characterized in zebrafish (See [25–27,29] for review), the present work demonstrates a maternal contribution essential for normal development exclusively provided through a maternally-inherited mRNA [31,43].

Unsuspected role of maternal rp2 *mRNA in retina*

As indicated above, several mutations in the *Rp2* gene have been shown to account for a significant proportion of a human disease called Retinitis Pigmentosa [3] and to cause Opsin mislocalization and photoreceptor degeneration when suppressed in mice [44]. When using zebrafish as a model to study retinal development, recent knock-down studies also reported that Rp2 was necessary for retinal development in zebrafish [18,20,21]. These results are consistent with the strong expression observed in the developing eye and abnormalities in eye development reported here, even at the lower doses of ATG-MO used. However, the strong expression at the basis of the ventral fissure of the eye, possibly at the basis of the optic nerve, has never been reported before and may be of importance in regard of the implication of *rp2* in eye development and maintenance. In addition, it should be noted that recent studies, that have used zebrafish as an *in vivo* model to study human *RP2* mutations, were carried out using the same ATG-MO as the one used in the present study but at a lower dose, most likely to overcome the strong maternal effect in the gastrulation process reported here. Indeed, investigators specifically indicated that higher doses induced mortalities [21]. In these studies, the investigators were able to rescue the phenotype induced by this specific ATG-MO and used different mutated rescue RNAs to investigate the effect of specific mutations on eye development [18,20,21]. In our hands, the rescue of the phenotype induced by the ATG-MO, using the same synthetic RNA used by Hurd and coworkers [18], was partial but significant. Together, the lack of effect reported here when injecting a mismatch ATG-MO as well as all western blot and immunohistochemistry analyses carried out, and previous reports of successful rescued phenotype demonstrate the specificity of the MO used herein to target *rp2* transcripts. Altogether, these results also clearly indicate a crucial role for *rp2* maternally-inherited mRNA in eye/retina formation and maintenance during development. The zebrafish has recently emerged as a developmental model for eye pathologies and the present study sheds new light on investigations in this field in which maternal contribution has possibly been overlooked.

Rp2 from maternal origin contributes to left-right asymmetry establishment

In vertebrates, many studies have demonstrated the role of cilia protein in embryonic development and the high incidence of developmental disorders and diseases resulting from alteration of cilia-related mechanisms [45,46]. Recent studies have highlighted the role of Rp2 in the cilia basal body through the study of its function as a regulator in cilia protein trafficking [4–6,13,14,18,22,23,47]. Among those studies, several demonstrated that known *RP2* mutations causing Retinitis Pigmentosa altered Rp2 function in the ciliated cells [14,20–23]. Interestingly, most knock-down experiments of cilia-related genes in zebrafish led to developmental defects such as gastrulation delay, left-right asymmetry disruption, hydrocephaly, body curvature, pericardial edema, kidney cyst, and retinal degeneration [48–53]. Consistently with the role of Rp2 in cilia function, we report herein the expression of *rp2*

in a variety of ciliated tissue and most of the above phenotypes could be observed after Rp2 knock-down in the present study and in previous ones [18,20,21]. A 20% incidence of kidney cyst was also reported in Rp2 knocked-down zebrafish embryos at 60hpf [18]. Furthermore, during zebrafish development, dorsal forerunner cells form a ciliated epithelium located inside a fluid-filled organ called the Kupffer's vesicle (KV) which is transiently visible from early to late somite stages [54,55]. It was demonstrated that inside the KV a directional fluid flow was created by motile cilia and was responsible for asymmetric expression of specific genes that further induces left-right asymmetric organ development [50,56–58]. Here we report a 36% incidence of right heart loop phenotype in ATG-MO injected fish reflecting a defect in left-right asymmetry establishment following Rp2 knock-down. This result is in total agreement with the partial randomization of *southpaw* mRNA expression pattern and increased incidence of heart looping defects previously reported, even though authors also showed that Rp2 knock-down does not affect Kupffer's vesicle cilia biogenesis [18]. It is noteworthy that the observed incidence of left-right asymmetry establishment defects in Rp2 knocked-down embryos remained significantly different from a 50% incidence expected in the case of a total randomization of the left-right asymmetry establishment, suggesting that KV's cilia still partially function. Together with the localization of *rp2* expression in developing ciliated organs reported here (e.g. otic vesicle, KV, and pronephric duct) our observations support the role of Rp2 as an important actor of cilia formation or function and thus of cilia-mediated development.
Furthermore, when only targeting zygotic *rp2* mRNA, morphological phenotypes related to ciliopathies are much weaker, and no significant increase in heart loop defect could be observed revealing that no left-right asymmetry establishment defects are triggered when translation from maternally inherited *rp2* transcripts is not inhibited. This indicates that Rp2 protein translated from maternally inherited RNAs has an important role in early cilia-mediated development. In addition, the lack of detection of *rp2* expression in the Kupffer's vesicle (KV) of embryos before 18-somites stage also suggests that Rp2 protein function in the KV, revealed by heart asymmetry, is initially handled by Rp2 proteins transcribed from maternally-inherited mRNAs. *rp2* transcripts would then be produced at later stages by the zygote in the KV to compensate for protein turn-over and sustain Rp2 function in cilia. Altogether, we provide a clear and rare example of a maternally-inherited gene product regulating post-ZGA embryonic development and demonstrate here the direct implication of a maternally-inherited gene product in zebrafish cilia-mediated development and particularly in left-right asymmetry establishment. Our results are consistent with previous reports of a maternal contribution to left-right asymmetry in zebrafish but for other genes [59–61].

In the present work, we demonstrate that *rp2* is a maternal-effect gene exclusively expressed in oocytes within the zebrafish ovary. While both Rp2 mRNA and corresponding protein are expressed during oogenesis, Rp2 protein is not maternally-inherited, in contrast to *rp2* mRNA. Furthermore, we show that maternal *rp2* mRNA is essential for successful embryonic development and thus contributes to the egg developmental competence. Our observations also reveal that Rp2 protein translated from maternal message is important to allow normal gastrulation and organogenesis, providing an example of maternally-inherited gene product acting after the onset of embryonic genome activation. Finally, the present study provides evidence of direct maternal contribution to cilia-mediated development and more specifically to left-right asymmetry establishment. Together, our observations stress the importance of maternal contribution for successful embryonic development and illustrate the wide variety of biological processes that remain under maternal control in the developing embryo.

ACKNOWLEDGMENTS

Authors thank A Fostier and JC Gabillard for helpful discussions, the Singapore Genome Institute for providing zebrafish *rp2* cDNA, T Hurd for gracefully providing his *rp2* rescue plasmid, C Cauty and H2P2 histopathology platform for their help in histological analyses, and J Nichols for providing cmlc2:eGFP transgenic zebrafish. Also, authors would like to particularly thank F Borel and A Patinote for zebrafish rearing and egg production.

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Figure Legends

Figure 1. Rp2 QPCR primers, oligo morpholinos and antibody. **A**) QPCR primers, translation blocking (ATG-MO) and splicing-blocking morpholino (Splicing-MO) oligonucleotides used in this study are aligned on zebrafish *rp2* transcript exon structure. Location (Ab) of synthetic peptide used for antibody production is shown on protein domain representation. **B**) Antibody validation on same sample using antibody (Ab), pre-immune serum (PPI), antibody pre-adsorbed on synthetic peptide (Ab+Pept) and no antibody (No Ab). The molecular weight (M.W.) of proteins in the ladder is indicated. Rp2 and non-specific (ns) bands are indicated.

Figure 2. Zebrafish *rp2* gene expression during oogenesis. Oocyte development stages according to Selman *et al* [43]. **A**) QPCR analysis. Mean and SEM are shown (n = 4 for all

stages I, II, III, and V, n=5 for stage IV). Different letters indicate significantly different levels of expression at $p<0.05$. For all stages, gene expression level is normalized to exogenous *luciferase* RNA abundance using the ΔΔCt. WISH of *rp2* gene in ovary with antisense and sense (insert) probes (**B**) and ISH picture of ovary section with antisense and sense (insert) probes (**C**). Ia, Stage Ia oocytes; Ib, Stage Ib oocytes; II, Stage II oocytes; III, Stage III oocytes; IV, Stage IV oocytes.

Figure 3. Zebrafish Rp2 protein expression during oogenesis. Oocyte development stages according to Selman *et al* [43]. **A**) WB analysis with β-Actin as loading control. Rp2 and non-specific (ns) bands are indicated. **B**-**C**) IHC of Rp2 protein in ovaries with oocytes at successive developmental stages. Ia, Stage Ia oocytes; Ib, Stage Ib oocytes; II, Stage II oocytes; III, Stage III oocytes; IV, Stage IV oocytes.

Figure 4. Zebrafish *rp2* mRNA abundance and localization during zebrafish embryonic development. Embryonic stages according to Kimmel et al [45]. **A**) QPCR analysis. Mean and SEM are shown (n=3 for 1-cell stage, 48hpf, and 72hpf; n = 4 for zyg, 4hpf, and 6hpf; n=7 for 24hpf). Different letters indicate significantly different levels of expression at $p < 0.05$. For all stages, gene expression level is normalized to exogenous luciferase RNA abundance using the ΔΔCt method. Zyg corresponds to the fertilized egg, before the formation of the first cell. **B**-**M**) WISH of embryonic developmental stages at 1-cell (**B**), 4hpf (**D**), 6hpf (**E**), 10-somites (**F**-**H**), 18-somites (**I**-**J**), 24hpf (**K**), and 48hpf (**L**-**M**). Sense probe hybridization on 1-cell stage is shown (**C**). b, brain; e, eyes; kv, Kupffer's vesicle; on, optic nerve; op, olfactory pit; ov, otic vesicle; pd, pronephric duct; pg, pronephric glomeruli; s, somites; ugo, uro-genital opening; Ant., anterior part of the embryo.

Figure 5. Zebrafish Rp2 protein expression in zebrafish embryonic development. Embryonic developmental stages according to Kimmel et al [45]. **A**) WB analysis with β-actin as loading control. Rp2 and non-specific (ns) bands are indicated. **B**-**F**) IHC of Rp2 protein in embryonic developmental stages at 2 cells (**B**), 4hpf (**C**-**D**), 6hpf (**E**-**G**). es, embryonic shield; n, nucleus; vit, vitellus. Bars = 100 µm.

Figure 6. Zebrafish Rp2 protein knockdown. A) ATG-MO dose effect on Rp2 knockdown at 24 and 72 hpf visualized by Western Blot. Rp2 and non-specific (ns) bands are indicated. **B**) Evolution of Rp2 protein abundance in control, ATG-MO injected and Splicing-MO injected embryos at 4hpf, 6hpf, 24hpf, 48hpf and 72hpf. Rp2 and non-specific (ns) bands are indicated. **C**) IHC labeling of Rp2 proteins in control, ATG-MO injected and splicing-MO injected embryos at 4hpf. **D**) Dose effect of ATG-MO injection on embryonic survival at 24hpf and 72hpf and successful embryonic development at 72hpf between un-injected controls (Control) and Injected fish. At 72hpf, the global survival of the injected embryos is given (Injected all) and compared to the proportion of injected embryos displaying a wild-type phenotype (Injected wild-type phenotype). For each studied morpholino-oligonucleotide concentration, experimental replicates are given (N=Number of cluches/n=total number of non-injected embryos/n=total number of injected embryos). * indicates significant difference to control embryos at $p<0.05$. **E**-**P**) Phenotype of control embryos, ATG-MO injected embryos and splicing-MO injected embryos at 10hpf (**E**-**G**), 24hpf (**H**-**J**), 48hpf (**K**-**M**) and 72hpf (**N**-**P**). Time given in F is the mean delay observed in ATG-MO injected embryos to reach corresponding stage. No delay was observed in splicing-MO injected embryos. bt, bended tail; hc, hydrocephaly; pce, pericardial edema; ve, vitellin extension; e, eyes. **Q**-**S**) Effect of ATG-MO (N=5/n=112), Rescue (ATG-MO and rp2-mRNA) (N=5/n=269), and Splicing MO

(N=6/n=154) injections on heart looping. Different letters indicate significant differences at $p<0.01$, except between Rescue and Splicing ($p=0.0427$). ** indicates significant difference at $p<0.01$. SEM are given. Normal left (**R**) and abnormal right (**S**) heart looping in ATG-MO injected embryo at 48hpf. The working concentration for oligonucleotide was 0.75mM (**C**, **E-S**). Bars = 250 µm.

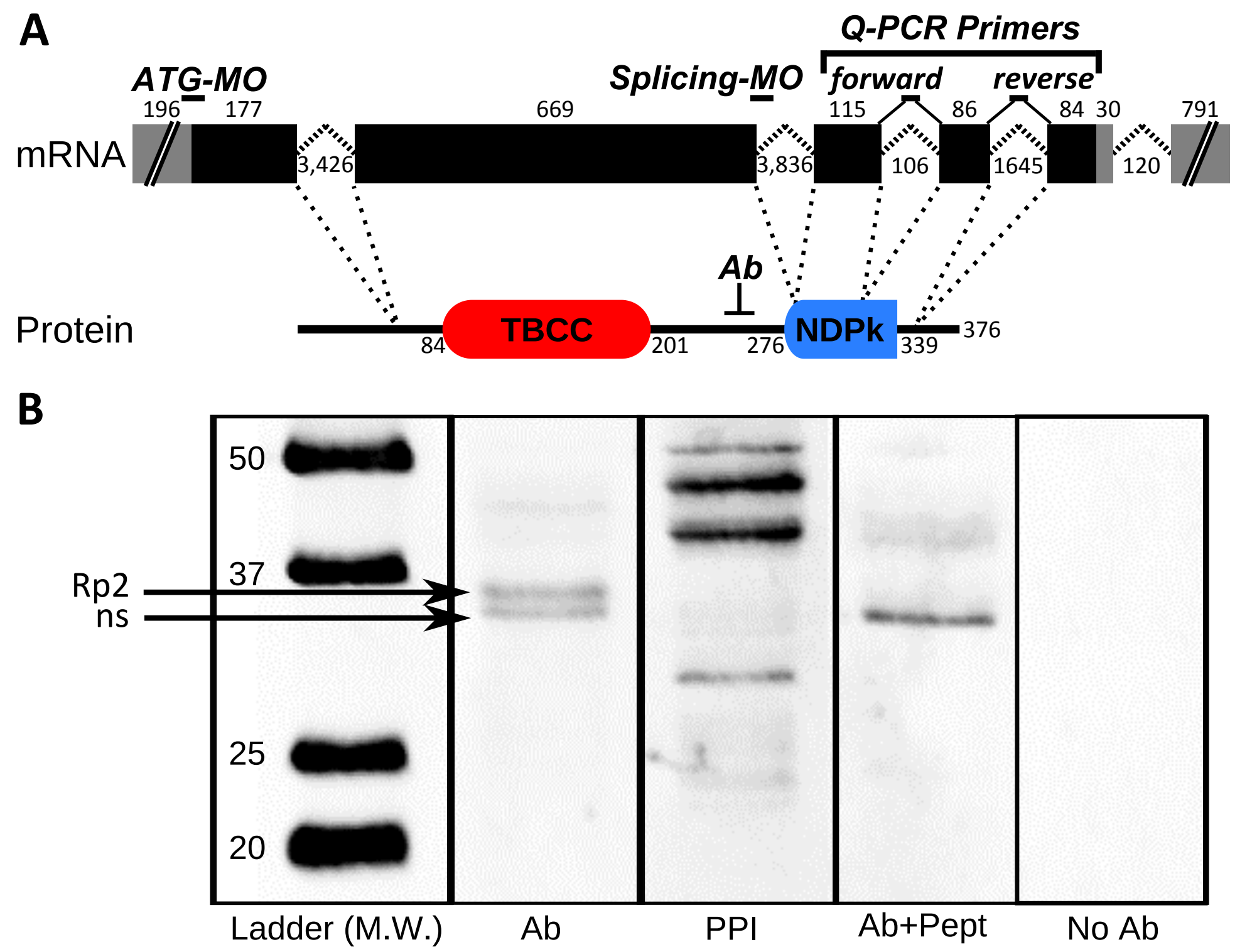
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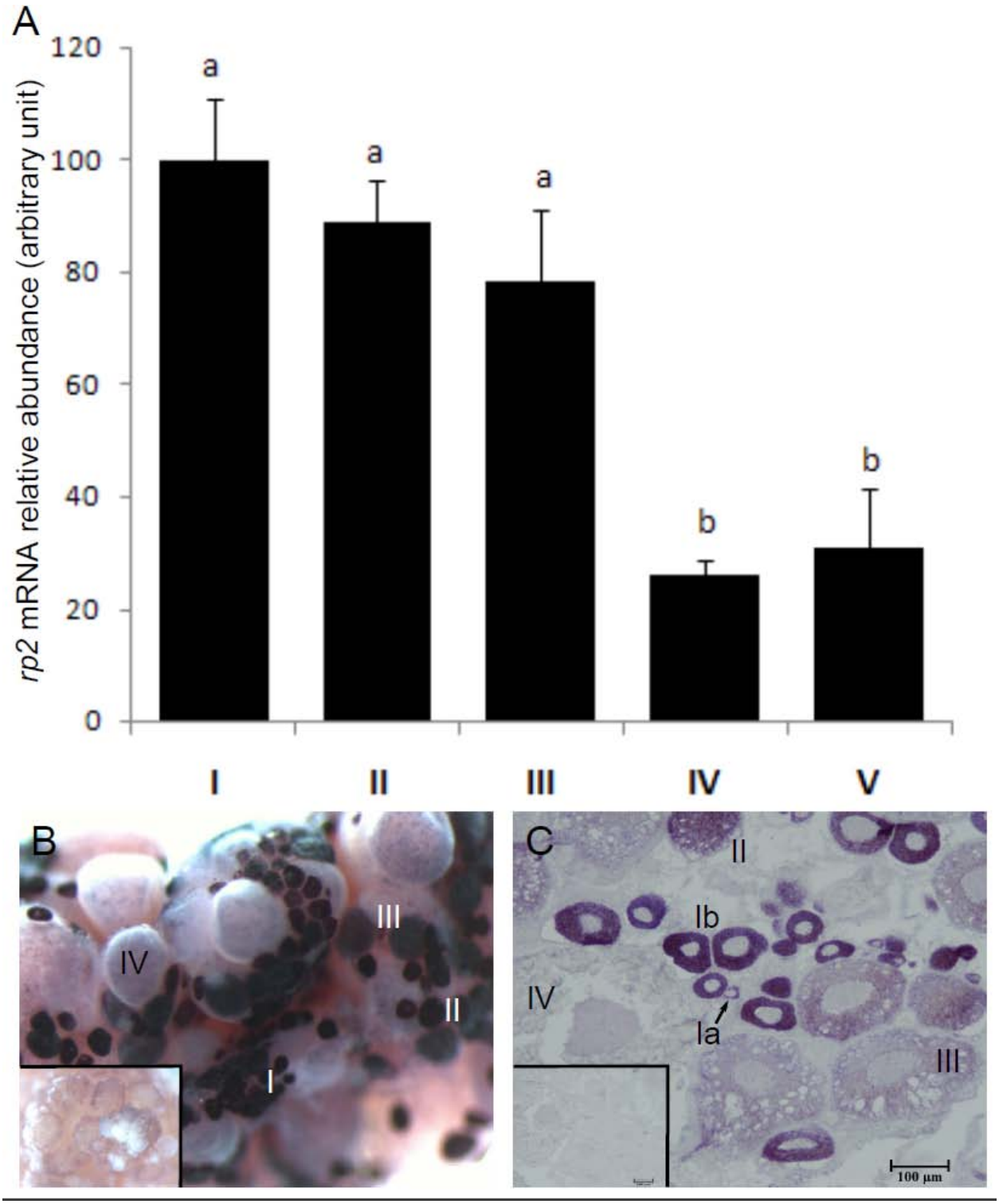
Figure 2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rp2 mRNA relative abundance (arbitrary unit)
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B
IV
III
II
I
C
II
Ib
IV
Ia
III
100 µm

Figure 3

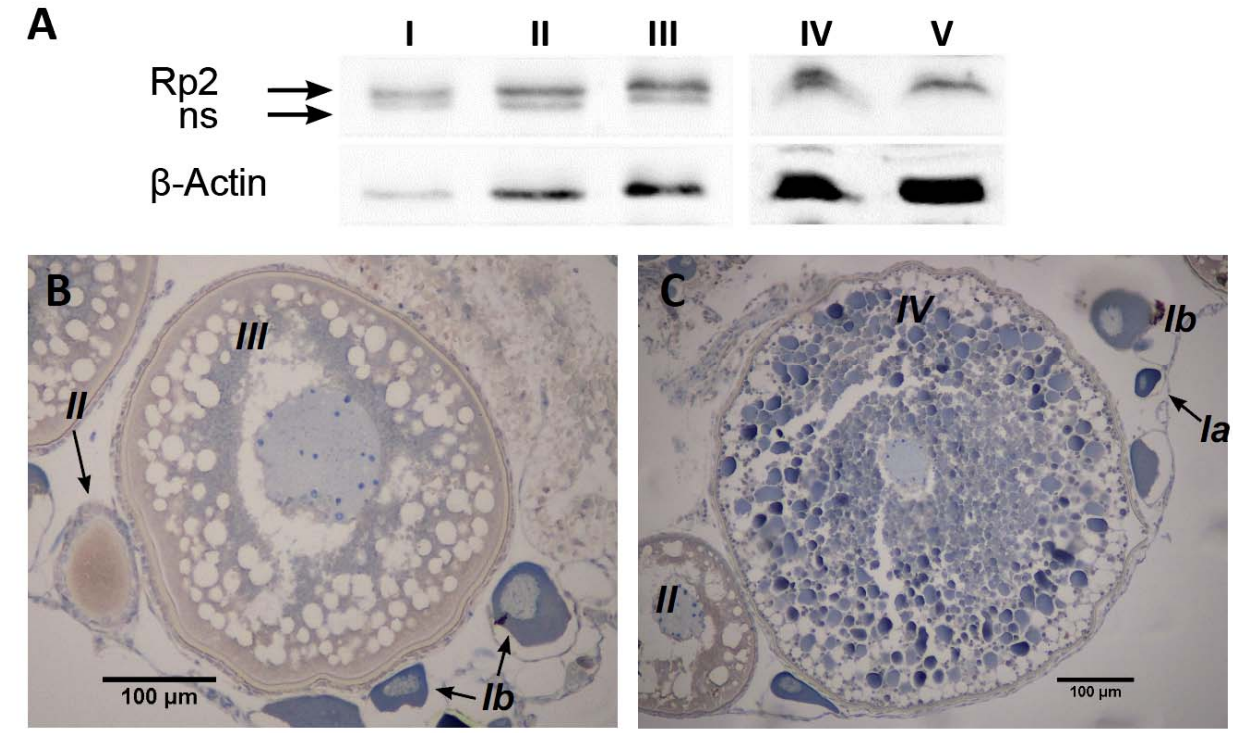


Figure 4

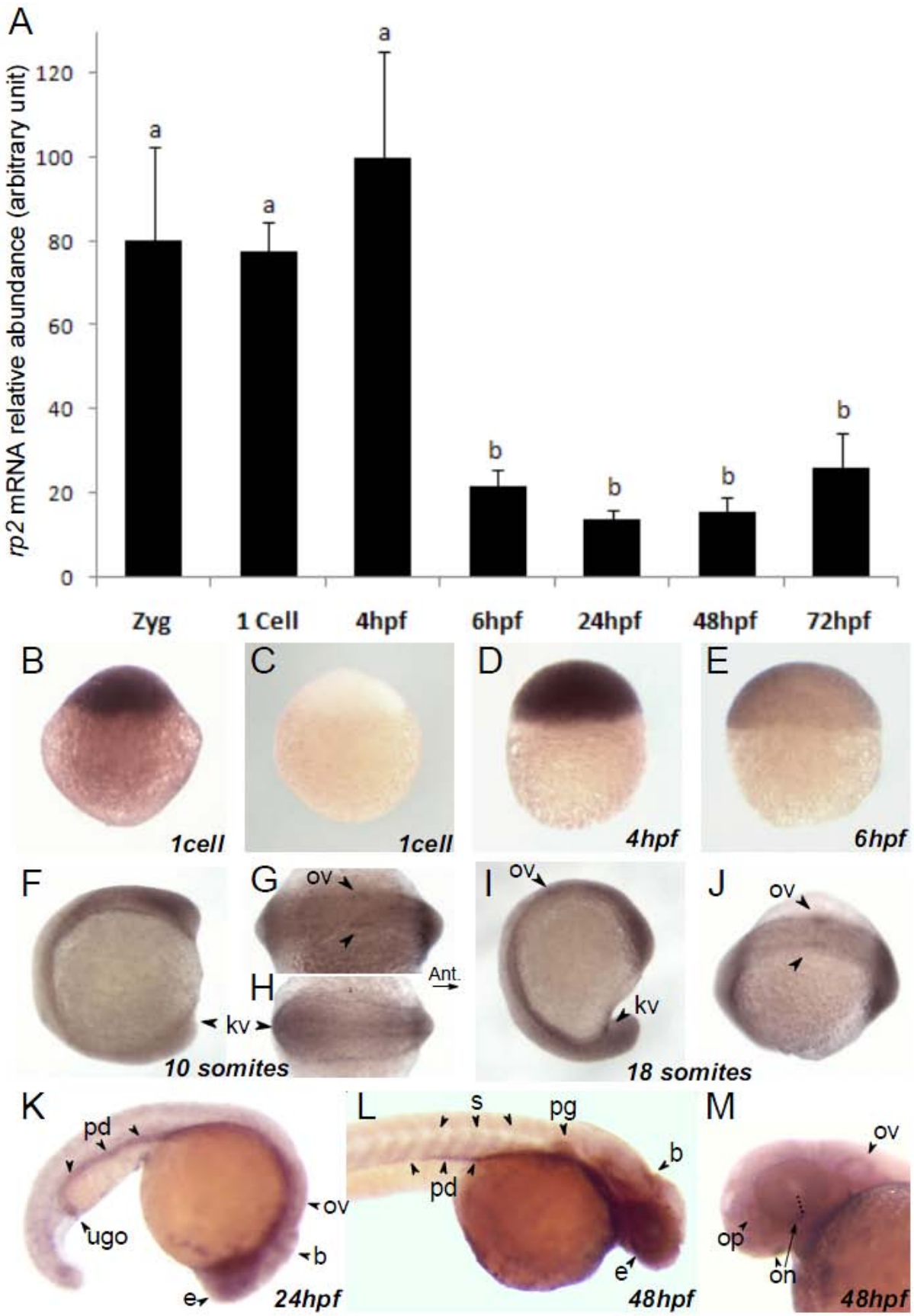

A
rp2 mRNA relative abundance (arbitrary unit)
120
100
80
60
40
20
0
a
a
a
b
b
b
b
Zyg
1 Cell
4hpf
6hpf
24hpf
48hpf
72hpf
B
1cell
C
1cell
D
4hpf
E
6hpf
F
10 somites
G
ov
H
Ant.
kv
I
ov
kv
18 somites
J
ov
K
pd
ugo
ov
b
e
24hpf
L
s
pg
b
pd
e
48hpf
M
ov
op
on
48hpf

Figure 5

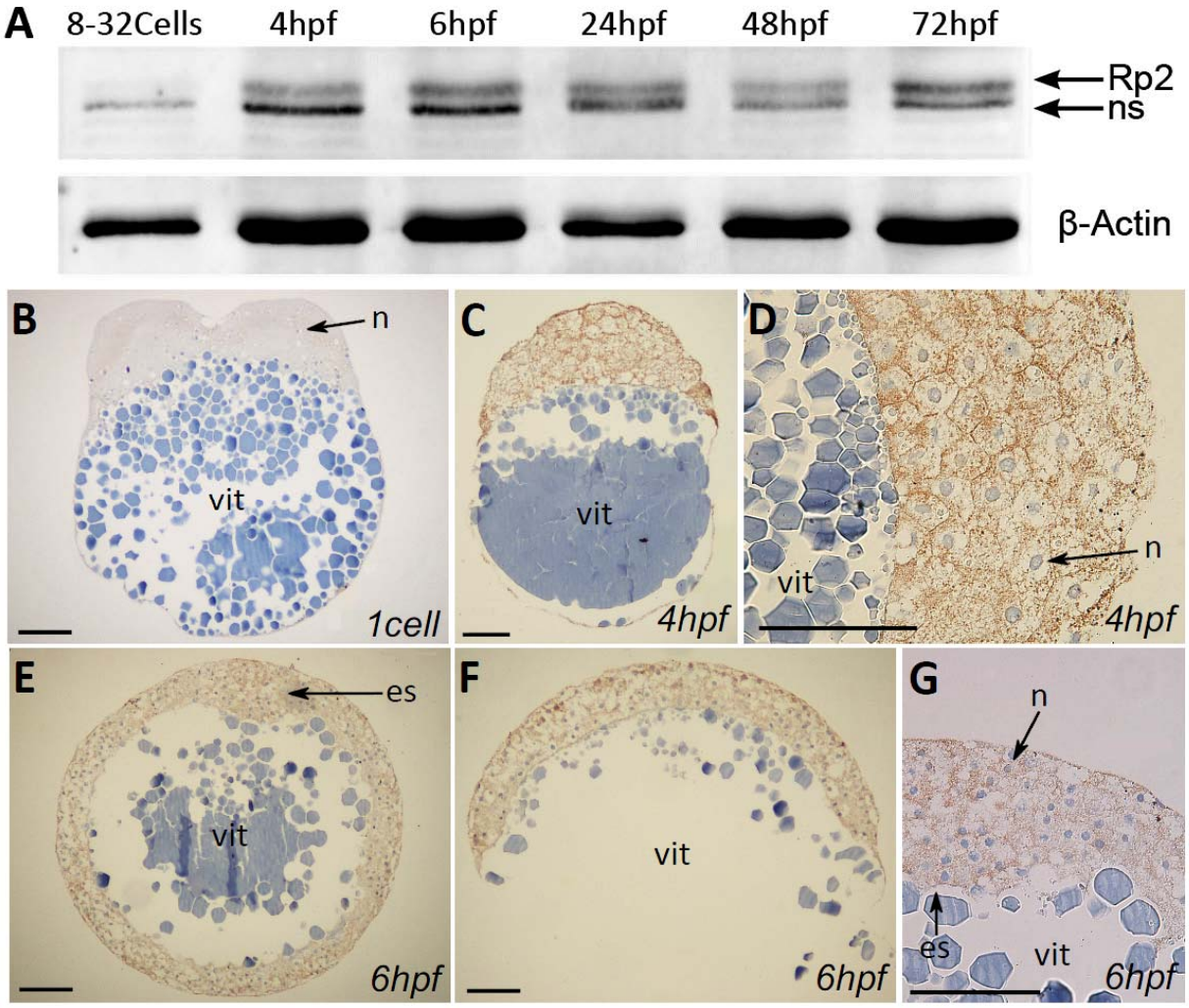
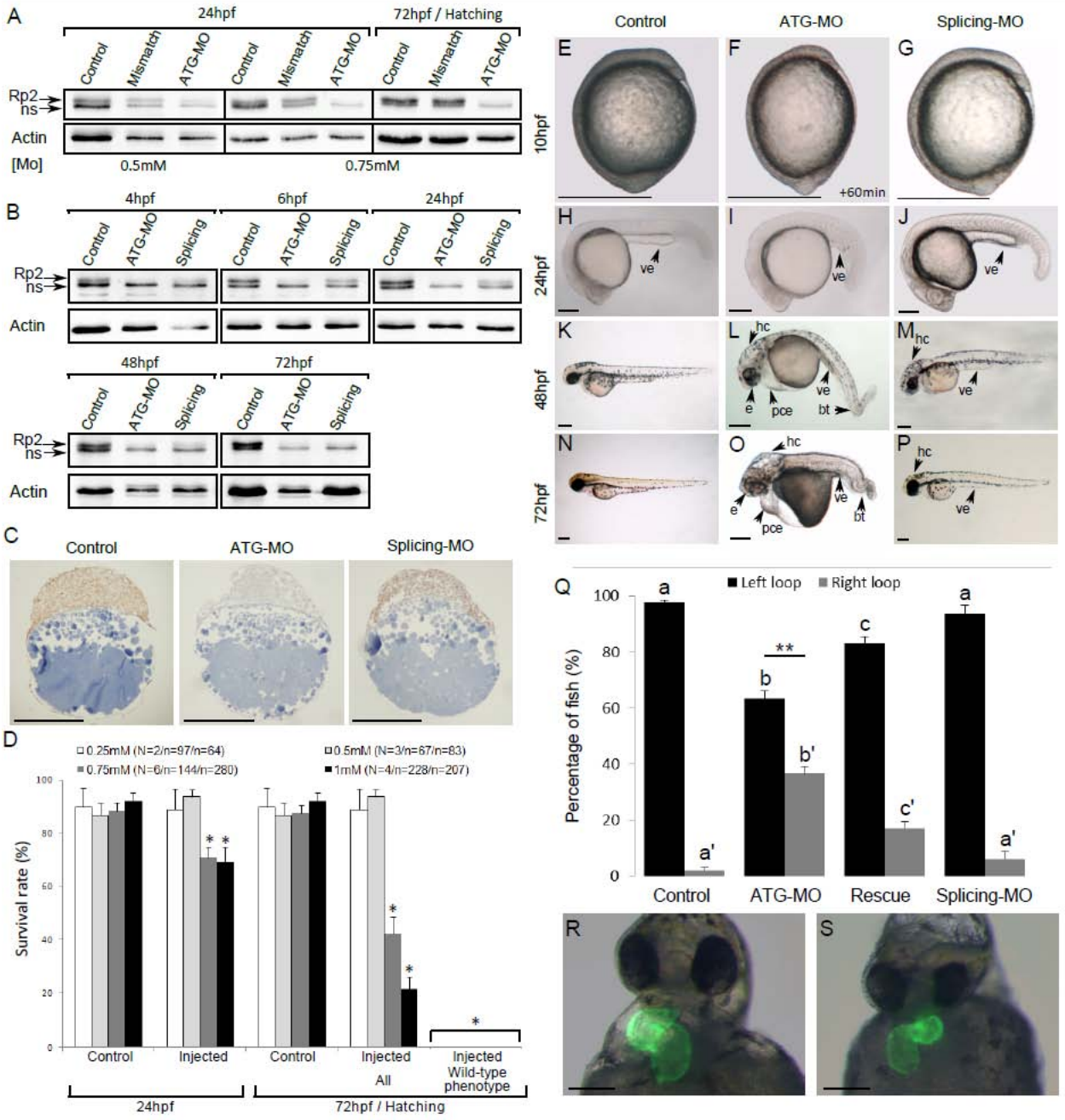

A
8-32Cells
4hpf
6hpf
24hpf
48hpf
72hpf
Rp2
ns
β-Actin
B
n
vit
1cell
C
vit
4hpf
D
n
vit
4hpf
E
es
vit
6hpf
F
vit
6hpf
G
n
es
vit
6hpf

Figure 6


A
24hpf
72hpf / Hatching
Control
Mismatch
ATG-MO
Rp2
ns
Actin
[Mo]
0.5mM
0.75mM
B
4hpf
6hpf
24hpf
48hpf
72hpf
Control
ATG-MO
Splicing
C
Control
ATG-MO
Splicing-MO
D
0.25mM (N=2/n=97/n=64)
0.5mM (N=3/n=67/n=83)
0.75mM (N=6/n=144/n=280)
1mM (N=4/n=228/n=207)
Survival rate (%)
Control
Injected
All
Injected
Wild-type phenotype
24hpf
72hpf / Hatching
E
F
G
H
I
J
K
L
M
N
O
P
10hpf
24hpf
48hpf
72hpf
+60min
ve
hc
e
pce
bt
Q
Left loop
Right loop
Percentage of fish (%)
Control
ATG-MO
Rescue
Splicing-MO
R
S