In Vitro Oocyte Culture-Based Manipulation of Zebrafish Maternal Genes

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Abstract

In animals, females deposit gene products into developing oocytes, which drive early cellular events in embryos immediately after fertilization. As maternal gene products are present before fertilization, the functional manipulation of maternal genes is often challenging to implement, requiring gene expression or targeting during oogenesis. Maternal expression can be achieved through transgenesis, but transgenic approaches are time consuming and subject to undesired epigenetic effects. Here, we have implemented in vitro culturing of experimentally manipulated immature oocytes to study maternal gene contribution to early embryonic development in the zebrafish. We demonstrate phenotypic rescue of a maternal-effect mutation by expressing wild-type product in cultured oocytes. We also generate loss-of-function phenotypes in embryos through either the expression of a dominant-negative transcript or injection of translation-blocking morpholino oligonucleotides. Finally, we demonstrate subcellular localization during the early cell divisions immediately after fertilization of an exogenously provided maternal product fused to a fluorescent protein. These manipulations extend the potential to carry out genetic and imaging studies of zebrafish maternal genes during the egg-to-embryo transition.

Keywords

maternal gene; maternal-effect; reverse genetics; zebrafish; oocyte culture; oogenesis; *cellular island*; *futile cycle*; *mission impossible*; Sas-6; centrioles

Introduction

In animal embryos, development immediately after fertilization is driven solely by maternal mRNAs and proteins present in the egg and activated during fertilization at the egg-to-embryo transition (Lindeman and Pelegri, 2010). Perduring maternal factors also function together with zygotic genes to pattern the embryo (Lyman-Gingerich et al., 2005; Kotani and Kawakami, 2008; White and Heasman, 2008; Holloway et al., 2009; Putiri and Pelegri, 2011). Reverse genetics using conditional knock-out strategies targeting the germ line and expression of RNAi constructs during oogenesis have been successful in the study of maternal genes in mouse and *X. laevis* (Stein et al., 2003; Lykke-Andersen, 2006; Roy and Matzuk, 2006; Mir and Heasman, 2008; Matzuk and Burns, 2012). However, successful targeting of zebrafish maternal genes by reverse genetics remains to be demonstrated.

To observe a maternal effect (phenotype due to defects in the maternal genotype) by reverse genetic targeting of maternal genes, one must interfere with gene products deposited into

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oocytes as they develop into eggs, before fertilization. During oogenesis, with the exception of yolk precursor proteins such as vitellogenin, which is produced by the liver and acquired by the oocyte through endocytosis (Wallace, 1985), most RNAs and proteins are produced cell-autonomously by the oocyte. Such preloaded maternal products in the mature egg pose a problem for their functional manipulation, as standard reverse genetics methods to introduce exogenous products or reagents by injection into one-cell stage embryos or mature oocytes are ineffective for early developmental stages. For example, injection of mRNAs at these stages may not allow sufficient time to express products to have an effect on early embryonic processes, while morpholino oligonucleotides injected at these stages are unable to reduce already present maternal protein.

Currently, maternal products can be exogenously expressed by transgenic approaches (Knaut et al., 2002; Kotani and Kawakami, 2008; Yabe et al., 2009). However, the generation and evaluation of transgenic animals is labor intensive and requires fish husbandry for the months corresponding to a generation time. In addition, transgenes may be subject to position effects and silencing (Choo et al., 2006; Goll et al., 2009; Akitake et al., 2011), complicating both maintenance of stocks and analysis of the consequences of transgenic expression. Methods for the systematic down-regulation of maternal genes could also be achievable in principle through transgenic expression, however, RNA interference (RNAi) during oogenesis, which has been shown to be effective in the mouse (Roy and Matzuk, 2006), has not yet been reported in the zebrafish. Given the challenges of transgenic-based approaches for the functional manipulation of maternal factors in the zebrafish, an effective in vitro culture-based method that would allow both the up- and down-regulation of maternal gene product function using currently available tools would be a welcome alternative.

During oogenesis in zebrafish, stage I oocytes are arrested at meiosis I at the diplotene phase of prophase (Selman et al., 1993). Meiosis I resumes and completes only during stage IV, at the end of which the oocyte matures into stage V oocytes and re-arrests at metaphase of meiosis II (Selman et al., 1993). After defolliculation, exposure of eggs to water during natural matings or in vitro fertilization results in egg activation, which includes the expansion of the chorion membrane (Becker and Hart, 1996, 1999) and completion of meiosis II (Streisinger al., 1981; Selman et al., 1993; Dekens et al., 2003). Protocols for in vitro maturation of zebrafish oocytes have been previously reported (Selman et al., 1993, 1994; Seki et al., 2008). In this approach, ovaries that contain oocytes at various stages of oogenesis are removed from gravid females and cultured in maturation medium containing the hormone 17α-20β-Dihydroxy-4 Pregnen-3-one (DHP). DHP treatment allows oocytes to mature from stage III (undergoing vitellogenesis, arrested at diplotene of meiosis I with the oocyte nucleus in the center of the oocyte) through stage IV (meiosis I completed, oocyte nucleus closer to the surface of the egg) until the final stage of oogenesis at stage V (translucent, germinal vesicle breaks down, oocytes arrested in metaphase of meiosis II).

Although in vitro culture conditions for zebrafish oocyte maturation have been known for almost two decades (Selman et al., 1993, 1994), oocytes matured through such in vitro protocols could not be successfully in vitro fertilized. Recent modifications to the original culture conditions, such as a maturation medium pH closer to that of the ovarian fluid, has demonstrated that such in vitro matured oocytes can also be successfully fertilized in vitro to yield viable embryos (Seki et al., 2008). However, an in vitro approach for functional analysis of zebrafish maternal genes has not been documented.

Here, we demonstrate functional manipulations of zebrafish maternal genes in immature stage IV oocytes through a modified version of the in vitro culture and fertilization method by Seki et al. We show that injection of mRNA encoding a wild-type maternal protein

rescues the corresponding maternal-effect phenotype. Conversely, a loss-of-function maternal-effect phenotype is mimicked by the injection of mRNA encoding a dominant-negative protein or translation-blocking morpholinos. We find that a morpholino targeting splice junctions is ineffective, possibly due to the presence of mature mRNAs in the developing oocyte. Finally, we show that mRNAs encoding fluorescent fusions of maternal proteins allow the visualization of subcellularly localized maternal protein immediately after fertilization. These results demonstrate an in vitro approach for functional manipulation and visualization of zebrafish maternal genes, extending the range of reverse genetics to encompass the egg-to-embryo transition.

Materials and Methods

Animal Husbandry

AB and mutant fish were maintained under standard conditions at 28.8°C. Before oocyte culture, AB and homozygous mutant females were paired for natural matings to purge the females of all mature eggs. Pairs that laid fertilized eggs were placed in smaller tanks in groups of up to 10 fish and fed twice a day. Day of egg-laying was day 0; ovaries were harvested on days 1–11 postpurging for oogenesis staging series and days 8–10 postpurging to isolate stage IV oocytes for all other experiments.

In Vitro Oocyte Culture

The general method is adapted from Seki et al. (2008) from which specific reagents described were used as follows: Leibovitz's L-15 medium with L-glutamine, pH 7.0 (Gibco), gentamycin 10 mg/ml (Sigma-Aldrich), 10% bovine serum albumin in distilled water (BSA, Sigma-Aldrich) and 17α-20β-dihydroxy-4 pregnen-3-one (DHP, Sigma-Aldrich and Santa Cruz Biotechnology). Sorting medium was 90% Leibovitz's pH 9.0 (adjusted with 10N NaOH), 0.5% BSA and 100 μg/ml gentamycin. Maturation medium was 90% Leibovitz's pH 9.0, 0.5% BSA, and 1 μg/ml DHP. For each experiment, ovaries from 1–2 females were harvested into sorting medium in a 35×10 mm plastic culture dish. Oocytes were dissociated by gentle pipetting with a plastic Pasteur pipette and a pair of forceps. Sorted/injected oocytes were transferred into maturation medium and cultured at 26°C for 4 hr. The duration between harvesting oocytes into sorting medium and transfer into maturation medium with or without injections can be up to 2 hours and was typically approximately 1.5 hr for all experiments described. Unmanipulated oocytes left in the sorting medium were found to spontaneously undergo chorion expansion 3–4 hr postharvesting.

In Vitro Fertilization

For in vitro matured translucent stage IV and mature stage V oocytes, the follicle membrane was removed manually using a pair of forceps by holding a part of the follicle membrane and gently rolling the oocyte out of it. Defolliculated oocytes were transferred into a clean dish in groups of 2–5 in 10–30 μl of maturation medium. 5–10 μl of sperm solution was added directly on top of the oocytes, followed by a drop of embryo medium ∼10 s later. The plate was flooded gently with embryo medium after 1 min. Sperm solution was made by shearing the testes from two AB males per experiment in 300 μl of Hank's Final solution kept on ice and used within 1 hr of preparation (Pelegri and Schulte-Merker, 1999). Fertilized embryos were observed to confirm normal chorion expansion, (indicative of successful defolliculation), which occurred maximally at 5–10 min postfertilization. In vitro fertilizations using sperm solution prepared as described resulted in ∼25% polyspermy in embryos in a given experiment. These embryos could be identified live at 40 minutes postfertilization (mpf) as described in the supplementary materials and were excluded from further analysis.

Oocyte Microinjections

The working stocks of all injected reagents were diluted in 0.2 M KCl as dilution in distilled water resulted in toxicity leading to inviable oocytes. Injections were performed by manually holding the oocytes directly on the culture dish with a pair of forceps. Oocytes were injected while in sorting medium and transferred to maturation medium immediately afterward.

Stage I–IV oocytes were injected with 100 pg of *GFP* mRNA. Stage IV oocytes were injected with 100 pg of wild-type *aurB* mRNA or 200 pg of *aurB-KD* mRNA (Yabe et al., 2009), 200 pg of *sass6*-Long-*mCherry* mRNA (Yabe et al., 2007), ~2 ng of Lrmp-MO (GeneTools, tggaccgggacaagagagaaagcct), ~2.5 ng of Dhx16-MO (Putiri and Pelegri, 2011) or 2 ng of sass6-spliceMO (GeneTools, gcctacacatgattacctcagagc). Also see the Reverse Transcriptase-Polymerase Chain Reaction section.

Immunolabeling and Imaging

Embryos were allowed to develop until the two- or eight-cell stage after which they were immunolabeled as described previously (Pelegri et al., 1999; Yabe et al., 2007). Confocal images of immunostainings were obtained on a Zeiss LSM510 and processed using ImageJ. To observe effects of the Dhx16-MO, live embryos were imaged on a Zeiss Axioscope using OpenLab at ~9 hours postfertilization (hpf).

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from whole ovaries at day 1 postpurging and from stage IV oocytes using Trizol (Invitrogen) and DNAase treated. cDNA was synthesized using oligodT and random hexamer primers (Invitrogen) and standard polymerase chain reactions (PCRs) were performed to amplify ~300 bps of α-*tubulin*, β-*actin*, or *sass6* products. Exon primers were used on oligodT primed and intron primers on random hexamer primed cDNAs. *sass6*-SpliceMO efficacy was confirmed by reverse transcriptase-PCR (RT-PCR) on oligodT primed 24 hpf cDNA from embryos injected with 2ng of MO at the one-cell stage, using exon primers flanking the targeted intron. Gene-specific primers used to amplify cDNA sequences were as follows: *sass6*-Exon-F: cgtcatcttcatcagcgttc; *sass6*-Exon-R: tctgctggacagagaggttg; *sass6*-Intron-F: tt tccagaacaactgaccacag; *sass6*-Intron-R: ggcaatttcaaatctgtcacc; *β-actin*-Exon-F: gcccatctatgagggttacg; *β-actin*-Exon-R: ag gaaggaaggctggaagag; *β-actin*-Intron-F: ccacattaaactctcatttgtgc; *β-actin*-Intron-R: accgagaaatgaggaactgc. Primers to corroborate the effectiveness of the *sass6* splice MO were SpliceMO-*sass6*-F: gaggaaaag cagcagttgcag; SpliceMO-*sass6*-R: gctcaga gactttggtctggagctg.

Results and Discussion

Oogenesis Occurs Synchronously in Zebrafish During Which Immature Oocytes Are Translation Competent

For the in vitro culture procedure described by Seki et al., random selection of gravid females required 4–5 females for a clutch of 40–60 in vitro matured oocytes, as we could not predict whether a female would carry immature stage IV oocytes or mature stage V oocytes. We sought to overcome this unpredictability and reduce the number of euthanized females, especially homozygous maternal-effect mutant females, whose numbers are generally limiting.

To maximize the probability of isolating immature stage IV oocytes on a given day, females were paired in natural matings to "purge" mature eggs and used to establish an oogenesis time-line. Ovaries were harvested 1–11 days postpurging (dpp) and oocytes were staged as

described previously (Fig. 1; Selman et al., 1993). Oogenesis resumed predictably: stage I and II oocytes (IA: 7–20 μm; IB: 20–140 μm, both translucent; II: 140–340 μm, translucent with beginning accumulation of cortical alveoli) were first observed at 1–2 dpp (Fig. 1A), early stage III oocytes (lower range of 340– 690 μm, opaque, oocytes becoming opaque due to initiation of vitellogenesis, germinal vesicle in central position) at 3–4 dpp (Fig. 1E), late stage III oocytes (nearing 690 μm, opaque, germinal vesicle in central position) at 4–7 dpp (Fig. 1I), stage IV oocytes (690–730 μm, opaque, germinal vesicle asymmetrically located, near the cortex) at 8–10 dpp (Fig. 1M) and mature stage V oocytes (730–750 μm, translucent, germinal vesicle fully disassembled) at 11 dpp and beyond. Thus, under standard husbandry conditions, purged female zebrafish undergo oogenesis to reliably yield immature stage IV oocytes between 8 and 10 dpp. Oogenesis occurred fairly synchronously, as on a given day postpurging, in addition to oogonia, most of the immature oocytes were predominantly of one stage (this study; Selman et al., 1993).

We next injected *GFP* mRNA to assay the translation competency of oocytes during in vitro culturing (Fig. 1). Consistent with previous reports for stage I and II oocytes (Csenki et al., 2010; Bontems et al., 2009), immature zebrafish oocytes of all stages (I–IV) translated the exogenously injected *GFP* mRNA and accumulated detectable levels of green fluorescent protein (GFP) protein within 1.5–2 hr postinjection (Fig. 1B–D,F–H,J–L,N–P).

GFP-expressing oocytes were further assayed for their competence to mature into stage V oocytes. Stage I and II oocytes failed to mature and degenerated (Fig. 1A–H), while stage III oocytes matured into stage IV oocytes with germinal vesicles (Fig. 1I,J), which disassembled indicating their further maturation into stage V oocytes. However, unlike freshly isolated stage IV oocytes, which characteristically contain a germinal vesicle and are opaque, becoming translucent as they mature into stage V oocytes, stage III oocytes that matured in vitro into stage IV oocytes with germinal vesicles were translucent before germinal vesicle disassembly (Fig. 1J, compare with M). As germinal vesicle disassembly did occur in these stage IV oocytes and a previous study reported successful fertilization of such eggs (Li et al., 1993), we attempted to in vitro fertilize such eggs. However, we were unsuccessful in fertilizing eggs obtained from in vitro culture of stage III oocytes. Thus, although stage III oocytes mature in response to exogenous hormone, they may be limited in their potential to develop in vitro into fully mature eggs. For such stage I–III oocytes that are amenable to experimental manipulations but fail to mature in vitro, transplantation into foster female fish for in vivo maturation into eggs may be a viable strategy (Csenki et al., 2010).

Unlike stage I–III oocytes, stage IV oocytes matured into translucent stage V oocytes within 3.5–4 hr of in vitro culturing, a duration that is ideal for successful in vitro fertilization (Fig. 1N; Selman et al., 1993, 1994; Seki et al., 2008). We, therefore, restricted our in vitro oocyte culture duration to 4 hr postinjection, which based on our GFP expression results allows a maximum period of 2–2.5 hr for exogenous maternal protein expression before fertilization.

Wild-type mRNAs Injected Into Stage IV Oocytes Rescue Maternal-effect Phenotypes in Embryos

Translation of exogenously injected transcripts occurs between 2 and 3 hr postinjection, a temporal delay that precludes rescue of early maternal-effect phenotypes when the wild-type transcript is injected at the one-cell stage. Hence, phenotypic rescue of maternal-effect mutations by mRNA injections into one-cell embryos have been observed only at or after midcleavage stages (Kelly et al., 2000; Yabe et al., 2007; Holloway et al., 2009; Fukazawa et al., 2010; Putiri and Pelegri, 2011; Abrams et al., 2012). To circumvent this temporal translation lag, maternal gene expression during oogenesis has been achieved using transgenic fish (Knaut et al., 2002; Yabe et al., 2009). However, despite increases in

transgenic generation efficiency (Kawakami, 2005; Kwan et al., 2007), this approach requires at least one generation-time, possibly more if transgene expression is required in a specific mutant background. Moreover, transgenes can be subject to epigenetic modifications, complicating analysis. Direct assessment of maternal gene function by injection into developing oocytes would avoid such drawbacks and simultaneously expedite the time-frame of the experiment.

Embryos from females homozygous for the maternal-effect mutation *cellular island* (*cei*) fail to undergo early cell divisions and the mutation was positionally cloned as a missense mutation in the zebrafish Aurora Kinase B (Stka/AurB; Dosch et al., 2004; Yabe et al., 2009). Phenotypic rescue of the *cei* mutation was previously achieved through transgenesis as injection of wild-type *aurB* mRNA at the one-cell stage failed to rescue the early maternal-effect phenotype in *cei/aurB* mutant embryos (Yabe et al., 2009). We attempted to directly rescue the *cei/aurB* maternal-effect phenotype by injecting wild-type *aurB* mRNA into stage IV oocytes from homozygous *cei/aurB* females. In *cei/aurB* mutant embryos from such injections, cleavage furrows ingressed and were robustly maintained, reflecting rescue of the mutant phenotype, while sibling *cei/aurB* mutant embryos from an uninjected subset of in vitro mature eggs showed characteristic rudimentary furrows (Supp. Table S1, which is available online). We confirmed rescue of the *cei/aurB* maternal-effect phenotype by assaying for β-catenin, which accumulates at mature cleavage furrows in wild-type (Fig. 2A–D), but not *cei/aurB* mutant embryos (Fig. 2E–H; Yabe et al., 2009). Confirming our observations in live embryos, β-catenin protein was found at the furrows in *cei/aurB* mutant embryos that were injected with wild-type *aurB* mRNA as stage IV oocytes (Fig. 2I–L; Supp. Table S1). These results recapitulate the rescue of the *cei/aurB* maternal-effect phenotype obtained through transgenic wild-type AurB copies (Yabe et al., 2009).

In addition to *cei/aurB*, we have shown that early maternal-effect phenotypes in fertilized embryos of *futile cycle* (Lindeman and Pelegri., 2012) and *motley* (Nair et al., in revision) can also be rescued by injecting mRNAs of the corresponding wild-type genes into stage IV mutant oocytes. In *motley*, phenotypes associated with oogenesis in mature eggs are additionally rescued (Nair et al., in revision). Taken together, our results demonstrate that early maternal-effect phenotypes can be effectively rescued by injecting wild-type mRNAs into stage IV oocytes cultured to maturity in vitro.

Mutant Transcripts Predicted to Interfere With Endogenous Protein Function Cause a Maternal Dominant-Negative Effect When Injected Into Stage IV Oocytes

We next tested whether we could interfere with endogenous maternal function by injecting transcripts encoding mutant versions of a protein. A mutation in the kinase domain of AurB functions as a dominant-negative in mammalian cell lines (Terada et al., 1998). Injections of an mRNA with the same mutation engineered in the zebrafish AurB kinase domain (*aurB-KD*) at the one-cell stage failed to mimic the maternal-effect *cei/aurB* mutation (i.e., it did not affect early cell divisions), resulting instead in brain necrosis later (Yabe et al., 2009). We injected wild-type stage IV oocytes with the *aurB-KD* mRNA to test whether we could obtain a maternal dominant-negative effect resulting from reduced wild-type AurB function during early cleavage stages. In embryos derived from stage IV oocytes injected with the *aurB-KD* mRNA, we observed rudimentary cleavage furrows that failed to fully ingress and mature (Supp. Table S1). Compared with wild-type embryos (Fig. 3A–D), β-catenin also failed to accumulate at the cleavage furrows in the *aurB-KD* mRNA injected embryos, confirming that cytokinesis had indeed failed (Fig. 3I–L; Supp. Table S1). This phenotype resembles the maternal-effect cell division phenotype seen in *cei/aurB* mutant embryos from natural matings of *cei/aurB* homozygous females (Yabe et al., 2009) and in vitro matured *cei/aurB* stage IV oocytes (Figs. 2E–H, 3E–H). Thus, these results suggest that the *aurB-KD*

mRNA injected into stage IV oocytes successfully interfered with the function of the endogenous maternal wild-type AurB protein, resulting in a maternal dominant-negative effect in embryos observed immediately after fertilization.

Translation Blocking Morpholino Oligonucleotides Phenocopy Known Maternal-Effect Mutations

For maternal genes, the standard reverse-genetics approach of injecting morpholino oligonucleotides (MOs) into one-cell embryos, which successfully target zygotic transcripts (Eisen and Smith, 2008), is ineffective. This is because the wild-type maternal protein (for translation MOs), and mature mRNAs (for splice MOs), present in the egg before fertilization override any effect of the injected MO. Thus, injecting MOs into one-cell embryos for genes expressed both maternally and zygotically and for which there are maternal-effect mutations, cause zygotic phenotypes but fail to phenocopy the known maternal-effect phenotype at earlier stages (Yabe et al, 2009; Putiri and Pelegri, 2011).

To test the potential of MO injections into in vitro cultured oocytes to study maternal gene function, we targeted two maternal genes, for both of which maternal-effect mutants have been isolated by traditional forward genetics. The maternal-effect mutant *futile cycle*(*fue*) encodes a vertebrate-specific Lymphoid restricted membrane protein (Lrmp), which is required for nuclear-centrosome attachments (Lindeman and Pelegri, 2012). Translation blocking morpholinos targeting Lrmp (Lrmp-MO) when injected into one-cell stage embryos failed to phenocopy the maternal-effect phenotypes seen in *fue* mutant embryos from homozygous *fue* females: morphant embryos obtained in this manner gastrulated normally and did not manifest any nuclear-centrosome detachments typical of the maternal-effect *fue* mutants (our unpublished observations). We injected the same translation blocking Lrmp-MO into wild-type stage IV oocytes, which were in vitro matured into stage V oocytes, defolliculated and fertilized. In embryos derived from uninjected wild-type stage IV oocytes, at the eight-cell stage, nuclei always associated with γ-tubulin labeled centrioles and centrosomes (Fig. 4A–C). In *fue* mutants obtained by in vitro maturation of stage IV oocytes from homozygous *fue* females, karyokinesis does not occur and the nuclear patches do not associate with centrosomes (Fig. 4D–F; Lindeman and Pelegri, 2012). In Lrmp morphants obtained by injecting the translation blocking Lrmp-MO into wild-type stage IV oocytes, we observed nuclear-centrosome detachments characteristic of the maternal-effect *fue* mutation: failed karyokinesis, with nuclear patches that failed to associate with centrosomes (Fig. 4G–I; Supp. Table S1). Thus, injecting the Lrmp-MO into wild-type stage IV oocytes recapitulated the maternal-effect *fue* mutation in the resulting Lrmp morphants.

We further validated this approach by targeting a second maternal-effect mutation *mission impossible*(*mis*), which encodes a maternal RNA heli-case, Dhx16 (Putiri and Pelegri, 2011). *mis* mutant embryos from homozygous *mis* females fail to undergo epiboly and thus fail to complete gastrulation (Putiri and Pelegri, 2011). A translation blocking morpholino targeting Dhx16 (Dhx16-MO), when injected into one-cell stage embryos, results in brain necrosis (Putiri and Pelegri, 2011), a phenotype common in zygotic knockdowns of essential genes with both maternal and zygotic contributions (Putiri and Pelegri, 2011; Yabe et al., 2011). Even though the gastrulation phenotype characteristic of the maternal-effect *mis* mutation occurs 4 to 9 hours after fertilization and MO injection, Dhx16 morphants obtained by injecting one-cell embryos do not manifest any gastrulation defects (Putiri and Pelegri, 2011), presumably due to the presence of functional Dhx16 protein preloaded in the egg. To test whether MO injection during oogenesis could phenocopy the maternal-effect *mis* mutant phenotype, we injected wild-type stage IV oocytes with translation blocking Dhx16-MO, which were in vitro matured and fertilized. In wild-type embryos derived from uninjected wild-type stage IV oocytes, epiboly and gastrulation occurred normally (Fig. 4J), while in

control *mis* mutants derived from stage IV oocytes from homozygous *mis* females, epiboly fails to occur normally resulting in defective gastrulation and embryonic lysis (Fig. 4K; Putiri and Pelegri, 2011). In Dhx16 morphants derived from wild-type stage IV oocytes injected with Dhx16-MO, epiboly fails and such morphants fail to complete gastrulation and lyse (Fig. 4L; Supp. Table S1), demonstrating phenocopy of the maternal-effect *mis* phenotype.

Together with the *aurB-KD* mRNA injections, the Lrmp and Dhx16 translation blocking morpholino experiments in stage IV oocytes reaffirm the feasibility of the in vitro oocyte manipulation strategy as a reverse genetics methodology for functional studies of maternal genes. In addition to facilitating phenocopying of maternal-effect mutations obtained by forward genetics, this methodology can also aid in the dissecting of maternal vs. zygotic functions of genes, and testing the functional contribution of maternal genes with as yet unknown functions.

We further tested if splice-blocking morpholinos targeting another maternal-effect gene *cellular atoll*(*cea*), encoding the centriolar component Sass6, could recapitulate its maternal-effect cell division phenotype (Yabe et al., 2007). The *sass6*-spliceMO effectively caused mis-splicing of endogenous *sass6* transcripts when injected at the one-cell stage, as we detected the expected alternatively spliced transcript containing the retained intron (data not shown). However, embryos derived from wild-type stage IV oocytes injected with the *sass6*-spliceMO appeared wild-type with no early cell divisions defects, and gastrulated normally (data not shown).

The lack of a maternal-effect phenotype with the *sass6*-spliceMO prompted us to investigate the splice status of *cea/sass6* together with α-*tubulin* (data not shown) and β-*actin*, as control maternally expressed genes. Wild-type whole ovaries harvested at day 1 (stage I oocytes) and day 9 (stage IV oocytes) postpurging both contained exonic and intronic RT-PCR products for all three genes (Fig. S1A,B). Thus, despite ongoing splicing in stage IV oocytes, the presence of mature transcripts in stage I oocytes indicates that protein function would remain unaffected by the injection of splice MOs at stage IV, explaining the lack of a maternal-effect using *sass6*-spliceMO. However, whole ovaries isolated at day 1 postpurging also contain cell types such as follicle cells and immature oogonia, precluding definitive conclusion that the mature transcripts are exclusively from stage I oocytes. Regardless, our data caution that splice MOs may not be effective for the down-regulation of maternal genes, as mature transcripts at early stages of oogenesis may obscure effects of splice MOs injected into stage IV oocytes.

Transcript Encoding a Fluorescent Fusion of Maternal Protein Injected Into Stage IV Oocytes Allows Visualization of Subcellular Protein Localization in Early Embryos

Many maternal proteins are required for fundamental processes during egg activation, such as meiosis, and embryonic development postfertilization, such as male and female pronuclei fusion, karyokinesis, and cytokinesis, all of which require correct subcellular localization for biological function (Yabe et al., 2007; Bontems et al., 2009; Yabe et al., 2009; Lindeman and Pelegri, 2012; Nair et al., in revision). Expression of transcripts encoding maternal proteins fused to fluorescent reporters during oogenesis would provide a read-out of subcellular localization during early cleavage stages, especially in the absence of species-specific antibodies. We tested this rationale for zebrafish Sass6, an essential component of centrioles. It had been shown previously that fusion protein from transcripts encoding *sass6:mCherry* injected at the one-cell stage retained biological function and localized to centrioles (Yabe et al., 2007). However, in such injections the fusion protein did not become visible until early gastrulation, precluding its use to visualize centrioles during the early cleavage stages. We injected mRNA coding for the same fusion construct into stage IV

oocytes and assayed for localization of Sass6: :mCherry to centrioles in fertilized embryos (Fig. 5). We found that within 40 mpf, Sass6: :mCherry protein localized to foci on either side of the nuclei during prophase, presumably the centrioles (Fig. 5A–J). These Sass6: :mCherry foci colocalized with intense staining of centrosomal γ-tubulin, a known component of the peri-centriolar material associated with centrioles (Fig. 5C–E,H–J). Thus, exogenous Sass6: :mCherry translated during oogenesis localizes to centrioles in embryos immediately upon fertilization. Such expression of maternal proteins as fluorescent fusions in stage IV oocytes provides an alternative to transgenesis (Knaut et al., 2002) for analyzing subcellular localization of maternal products in the early embryo. Combined with the optical clarity of zebrafish embryos, this method should also allow live imaging of dynamic changes in maternal product subcellular localization patterns during the early cleavage stages.

The methods and reagents described in this study are a necessary complement to traditional forward genetics studies of maternal gene function. Furthermore, the approaches described herein could potentially facilitate functional assessment of maternal genes for which mutants are yet to be isolated or that have overlapping maternal and zygotic functions. In conclusion, our results provide proof-of-principle for effective functional manipulation and visualization of maternal gene products in zebrafish.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key findings

- In vitro maturation method coupled to in vitro fertilization for the functional manipulation of maternal genes in zebrafish.

- Injection of wild-type mRNAs into oocytes rescues maternal-effect mutations.

- Functional knock down/reverse genetics can be achieved by expression of dominant-negative products or injection of translation-blocking morpholino oligonucleotides.

- Expression of mRNAs coding for fluorescent fusion products allows visualizing the subcellular localization of maternal factors immediately after fertilization.
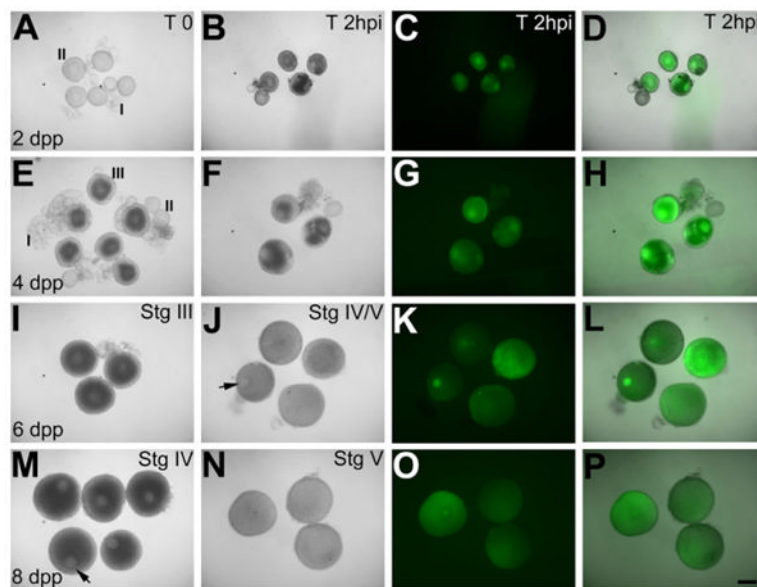
Fig. 1.
Oogenesis occurs predictably after purging and oocytes of all stages are translation competent. Live images of oocytes. **A–P:** Oocytes immediately after isolation from purged females on the indicated dpp at time 0 (A,E,I,M) and 2 hr after *GFP* mRNA injection (B,F,J,N), expressing green fluorescent protein (GFP) protein (C,D,G,H,K,L,O,P). B,F,I,J: Injected Stage I and II (B) and early III (F) oocytes degenerate, while late stage III oocytes (I) mature into stage IV transluscent oocytes (J) with germinal vesicle (arrow). Injected Stage IV oocytes (M) containing germinal vesicle (arrow) mature into stage V oocytes (N, note germinal vesicle breakdown). Scale bar = 300 μm in A–P.
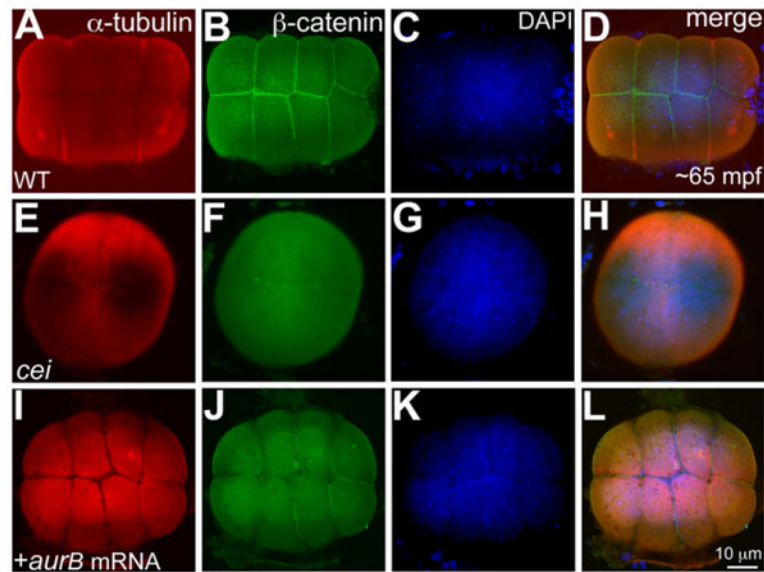
Fig. 2.
Wild-type *aurB* mRNA injected into stage IV *cei/aurB* oocytes rescues the maternal-effect phenotype in cei/aurB embryos. **A–L**: Animal views of 65 mpf blastodiscs immunolabeled to detect α-tubulin (A,E,I) and β-catenin (B,F,J), with DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride) stainings (C,G,K) and panel merges (D,H,L). A,B,E,F: Wild-type embryos show robust furrows between cells and β-catenin accumulation at the furrows (A,B), while *cei/aurB* embryo from uninjected *cei/aurB* stage IV oocyte shows partial, rudimentary furrows (E), which do not accumulate β-catenin (F). I,J: Rescued *cei/aurB* embryo from *cei/aurB* oocyte injected with wild-type *aurB* mRNA labeled with α-tubulin (I) show robust furrows which accumulate β-catenin (J), indicating furrow maturation. C,G,K: DAPI images show that nuclear division proceeds normally in all cases.
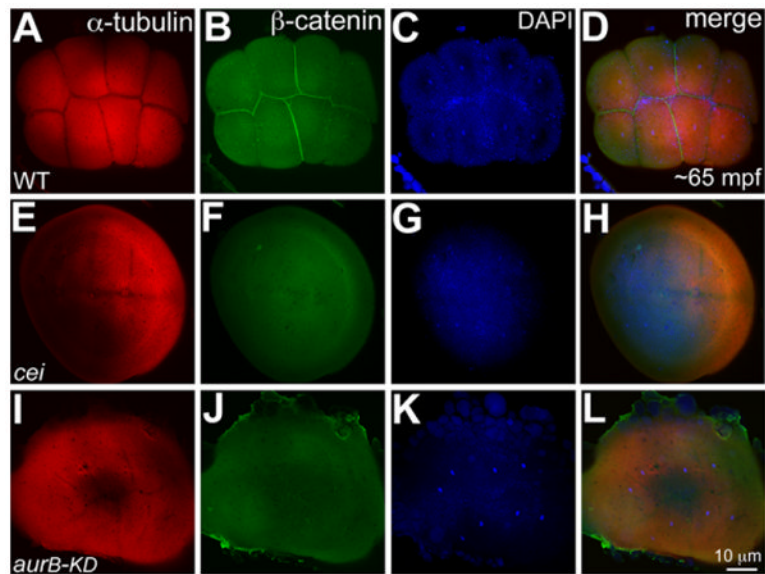
Fig. 3.
Stage IV oocyte injection of *aurB-KD* mRNA phenocopies the *cei/aurB* maternal-effect phenotype. **A–L:** Animal views of 65 mpf blastodiscs immunolabeled to detect α-tubulin (A,E,I) and β-catenin (B,F,J), with DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride) stainings (C,G,K) and panel merges (D,H,L). A,B: Robust furrows in wild-type embryos labeled with α-tubulin (A), which accumulate β-catenin (B). E,F: α-Tubulin labeling of control *cei/aurB* embryos from mutant stage IV oocytes show partial rudimentary furrows (E) with no β-catenin labeling (F). I,J: α-tubulin labeled partial, immature furrows in embryos from wild-type stage IV oocytes injected with *aurB-KD* mRNA (I), which also fail to accumulate β-catenin (J), recapitulating *cei/aurB* maternal effect phenotype. C,G,K: As in Figure 2, DAPI images show that nuclear division proceeds normally in all cases.
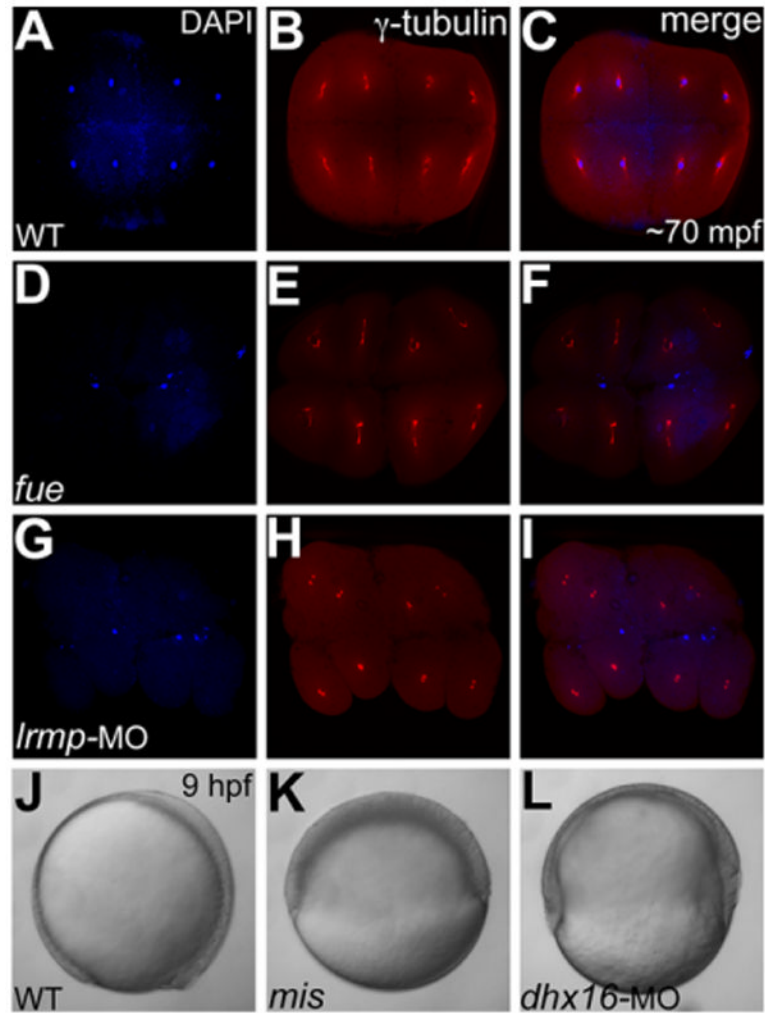
Fig. 4.
Stage IV oocyte injections of Lrmp and Dhx16 morpholinos phenocopy *fue* and *mis* maternal-effect phenotypes, respectively. **A–I**: Animal views of immunolabeled 70 minutes postfertilization (mpf) blastodiscs stained for DAPI (A,D,G) immunolabeled to detect γ-tubulin (B,E,H) and panel merges (C,F,I). A–C: In wild-type embryos, each nucleus associates with centrosomal γ-tubulin staining. D–F: In maternal-effect *fue* mutants, nuclei fail to divide, resulting in two to three patches of nuclei stainings corresponding to unfused parental pronuclei and the polar body for meiosis II (D,F), which fail to associate with γ-tubulin (E,F). G–I: In Lrmp morphants where maternal Lrmp function was inhibited, the nuclei similarly fail to divide (G) and also fail to associate with γ-tubulin (H,I). J–L: Lateral views of live embryos at 9 hours postfertilization (hpf). J,K: Wild-type embryos at 9 hpf where axis formation is evident (anterior to the top and dorsal to the right) (J), while in maternal-effect *mis* mutants, epiboly fails to occur (K). L: In Dhx16 morphants where maternal function was inhibited, epiboly similarly arrests, resulting in gastrulation failure.
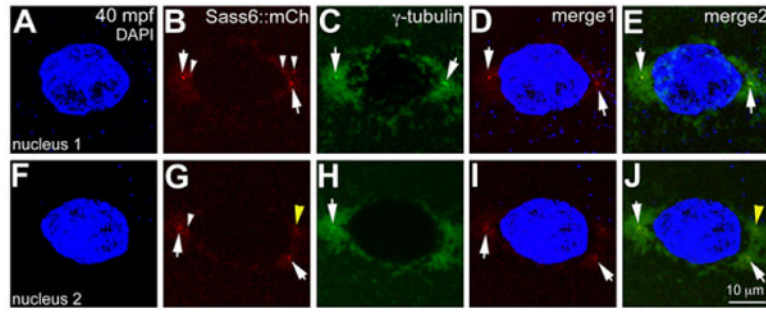
Fig. 5.
Sass6-mCherry protein from exogenous mRNA injected into stage IV oocytes localizes to centrioles during early cleavage stages. Immunolabeling for γ-tubulin and DAPI staining for nuclei in a Sass6-mCherry expressing blastodisc during prophase (40 mpf), which contains two nuclei, both of which are shown. C,E,H,J: γ-tubulin labels pericentriolar material around the nuclei, with intense foci in presumptive centrioles flanking each nuclei (arrows). B,D,E,G,I,J: Ectopically expressed Sass6-mCherry protein also localizes to foci flanking the nuclei (arrows in B,D,G,I) and colocalizes with γ-tubulin foci (arrows in E,J). B,G: Ectopic Sass6-mCherry is also observed as additional foci near the nucleus periphery (white arrowheads in B,G) and in the cytoplasm (not shown), consistent with the production of supernumerary centrioles after overexpression of maternally-derived Dsas-6 in *Drosophila* embryos (Peel et al., 2007). G,J: The observed putative supernumerary foci can also be associated with extra γ-tubulin foci (yellow arrowhead). Merge1: DAPI and Sass6: :mCherry; merge2: DAPI, Sass6: :mCherry and γ-tubulin.