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Retention of Exogenous mRNAs Selectively in the Germ Cells of the Sea Urchin Requires Only a 5'-Cap and a 3'-UTR

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SUMMARY

The abundance of an mRNA in a cell depends on its overall rates of synthesis and decay. RNA stability is an important element in the regulation of gene expression, and is achieved by a variety of processes including specific recruitment of nucleases and RNAi-associated mechanisms. These mechanisms are particularly important in stem cells, which, in many cases, have attenuated transcription. Here we report that exogenous mRNA injected into fertilized eggs of the sea urchin is selectively retained in the small micromeres, which contribute to the germ line in this organism, beginning in blastulae, when compared to adjacent somatic cells. We show that modification of this exogenous RNA using cap analogs and poly-adenosine tail deletions do not affect its selective retention in the small micromeres, but removal of the cap or of the 3'-untranslated region eliminates any selective mRNA retention in the presumptive germ line. Our results illuminate a likely ancient mechanism used by stem cells to prolong the lifespan of RNAs—either through RNA protection or by the absence of basic RNA degradation mechanisms, which are employed by most other cells of an organism.

INTRODUCTION

Germ line determination often occurs early in animal development, and the cells dedicated to the germ-line lineage become insulated from influence by the somatic cells. In many organisms used to study germline determination, such as the fruit fly *Drosophila*, the primordial germ cells (PGCs) acquire their fate by inheriting molecular determinants stored asymmetrically within the oocyte (Forrest et al., 2004; Forrest and Gavis, 2003; Gao and Arkov, 2013). Once these cells are fated to the germ line, they usually adopt a different phenotype, including low transcriptional activity, likely as a result of overall repressive chromatin organization, and a slow cell cycle. Nanos, a translational repressor of the germ line, often targets *cyclin* mRNAs for translational repression and thereby slows the cell cycle (Asaoka-Taguchi et al., 1999). The absence of nanos in the several animals that have been studied leads to cell cycling rates in PGCs more compatible with adjacent somatic cells, followed by induction of apoptosis in the putative PGCs (Sato et al., 2007). It is thought that

many of the germ-line characters, such as low transcriptional, metabolic, and cell cycle activities, protect the selected cells from incurring metabolic damage or from exhausting their nutritional reserves, especially since PGCs often have a large nuclear–cytoplasm ratio. Yet, PGCs are capable of high metabolic activity, become active in migration to the somatic gonad, and display rapid cell divisions that expand the population of germ-line stem cells once settled in their somatic niche (Seydoux and Braun, 2006). Thus, early metabolic efficiency may be a common characteristic of PGCs.

In sea urchins, development of the germ line depends on the small micromeres (sMics), which are set aside during early embryogenesis after two unequal cleavage divisions (Horstadius, 1973; Pehrson and Cohen, 1986; Tanaka and Dan, 1990; Yajima and Wessel, 2011). In blastula, the sMics reside at the vegetal plate where they divide once before being transported to the tip of the archenteron during gastrulation. The eight sMic descendants then partition into the left and right coelomic pouches, the site of adult rudiment formation in the pluteus larva. Specific mRNAs are expressed in the sMics, such as *nanos* (Juliano et al., 2010) and *vasa* (Voronina et al., 2008), whereas other RNAs, such as *gustavus*, are excluded (Gustafson et al., 2011). The sea urchin does not appear to have localized determinants for the germ line in the egg, as found in *Drosophila*, *Caenorhabditis elegans*, and *Xenopus*; in fact, the mechanism of germline determination in this animal remains unknown (Wessel GM, et al. The Biology of the Germline Lineage in Echinoderms. submitted 2013). Instead, some of the mRNAs encoding germ-line determinants, such as *vasa* and *piwi*, are present uniformly in eggs and early embryos, and during development these mRNAs are cleared by somatic cells, but are retained in the PGCs. Remarkably, a synthetic RNA containing *Xenopus* β -globin untranslated regions (UTRs) and injected into sea urchin eggs was found to be selectively retained in the sMics following gastrulation (Gustafson and Wessel, 2010). The β -globin UTRs stabilize the transcripts in oocytes, and lead to high levels of translation (Krieg and Melton, 1984). These UTRs represent a good control for in vitro translation assays and microinjection experiments to study translation regulation in organisms such as human (Kruys et al., 1987), *Xenopus* (Robbie et al., 1995), zebrafish (Koprunner et al., 2001), and sea urchin (Lepage and Gache, 2004). Here we test the mRNA requirements for this selective retention in the germ line.

RESULTS

Exogenous RNAs Are Retained in the sMics

Injection of a synthetic RNA containing the open reading frame of *mCherry* surrounded by the *Xenopus* β -globin 5'- and a poly-adenosine (polyA)-tailed 3'-UTR into eggs was shown previously to result in selective retention of the RNA in the sMics at the gastrula and prism stage (Gustafson et al., 2011). To determine the dynamics of this selective retention, the same synthetic RNA was injected in *Strongylocentrotus purpuratus* fertilized eggs, and embryos were fixed throughout development for in situ hybridization (Fig. 1). The *mCherry* RNA was detectable at a high level at the 2-cell stage, 2 hr after fertilization, and is present uniformly in all blastomeres until mesenchyme blastulae (Fig. 1). In mesenchyme blastulae, the RNA signal is higher in the sMics when compared to all other cells of the embryo (Fig. 1M). A few hours later, with the beginning of gastrulation, the difference between retention

in the sMics compared to the somatic cells was marked, and soon after RNA was detectable only in the sMics and their descendants (Fig. 1N–P). Background levels of staining were standardized using uninjected embryos that were otherwise treated equally in the hybridization and labeling process. These results show that exogenous RNAs are degraded in the somatic cells before gastrulation, but are selectively retained in the sMics and are detectable in larvae as late as 7 days after fertilization (data not shown).

Follow-up experiments measured RNA retention by quantitative reverse-transcription PCR (q-RT-PCR) to evaluate the rate of turnover for this exogenous RNA during development. Again, a synthetic, capped RNA containing the β -globin 5'-UTR followed by the *mCherry* open reading frame and then by the polyadenylated β -globin 3'-UTR was injected into fertilized *S. purpuratus* eggs, and total RNAs were extracted from embryos just after fertilization, at the 2-cell stage, at hatching and mesenchyme blastula, and at gastrula. The percentage of exogenous RNA retained was then measured by q-RT-PCR (Fig. 2). Independent of the experimental variations due to injection, the results suggest that the exogenous RNA is constantly degraded during the first 40 hr of development: 55% of the injected RNA is retained in mesenchyme blastula (20 hr after fertilization) while 1.93% is retained at gastrula (40 hr after fertilization). Interestingly, the RNA remains detectable in gastrula. A gastrula-staged embryo contains approximately 1,000 cells, 8 of which are sMics, meaning that the sMics represent approximately 0.8% of the cells at gastrula. Considering this *mCherry* RNA is mostly detected in the sMics at gastrula by in situ hybridization (Fig. 1), this result suggests that the sMics retain the exogenous RNA at concentrations that were originally present when the sMics were formed. Thus, sMics appear to retain the vast majority of RNA they acquire during cell division.

The m⁷GTP Cap and the PolyA Tail Are Not Required for Selective RNA Retention in the Germ Line

To better understand the mechanisms protecting RNA from degradation in the sMics, we injected different forms of *mCherry*-encoding RNAs. As observed before, RNA with an m⁷GTP and a polyA tail is selectively retained in the sMics during gastrulation (Fig. 3A). To test the importance of the cap in this process, a cap analog was used during RNA synthesis. The A cap does not interact with the cap binding protein eIF4E, but protects the RNA from 5' to 3' rapid degradation by exonucleases. Interestingly, this A cap RNA is also retained in the sMics (Fig. 3B). Thus, translation of an mRNA is not a necessary part of the retention mechanism. To test if the presence of any cap is required for the retention, an uncapped RNA also was injected into fertilized *S. purpuratus* eggs at an equimolar ratio. This RNA was not retained in any cells, including the sMics (Fig. 3F). Thus, the sMics do have RNA-degradation machinery that target uncapped transcripts. The role of the polyA tail was then tested by injection of an RNA containing a regular m⁷GTP followed by the β -globin 5'-UTR, the *mCherry* open reading frame, and the β -globin 3'-UTR without a polyA tail. To prevent endogenous polyadenylation after injection, the polyadenylation signal present in the β -globin 3'-UTR was mutated to make it non-functional; no evidence of polyadenylation was found in this exogenous mRNA by sequencing of this injected RNA at the blastula stage (data not shown). This untailable mRNA is still retained in the sMics,

however (Fig. 3C). Altogether, these data suggest that the sMics retained exogenous RNAs because they use different cap and polyA-tail removal mechanisms than somatic cells.

Selective mRNA Retention Is Independent of the Coding Region, and Depends on the 3'-UTR

To test if the *mCherry* open reading frame is required to prevent RNA degradation in the sMics, a similar RNA containing the *GFP* open reading frame surrounded by β -globin UTRs was synthesized in vitro and injected into fertilized *S. purpuratus* eggs. mCherry and GFP share 57.81% and 29.15% identity at the nucleotide and amino acid level, respectively. Both RNAs were retained equally in the sMics at gastrula stage, arguing that the mRNA retention is independent of any sequence in the open reading frame (Fig. 4A,C). To test if the β -globin UTRs are both required for the selective RNA retention, a construct containing only the *GFP* open reading frame without any β -globin UTRs was injected. This RNA is not protected from degradation in the sMics (Fig. 5B). Moreover, an RNA containing the β -globin 5'-UTR followed by the *mCherry* open reading frame without the β -globin 3'-UTR is not detectable in the sMics during gastrulation (Fig. 5E). These results indicate that a non-coding 3'-UTR is required for the selective RNA retention in the sMics. Along with the 5'-exonuclease activity seen above, these findings suggest that the sMics do have substantial RNA degradation machinery, but points to specific steps of normal RNA-turnover mechanisms that appear to be downregulated in the presumptive PGCs.

DISCUSSION

The abundance of an mRNA in a cell is the outcome of many functions, including its synthesis, processing, rate of nuclear export, and rate of degradation in the cytoplasm. An mRNA can be degraded by mechanisms of quality control that eliminate the production of potentially toxic proteins, and by mechanisms that shorten its half-life for the purpose of changing protein abundance (Schoenberg and Maquat, 2012). mRNA decay depends on cis-acting elements within each RNA and on the mRNA degradation machinery present at a specific time in specific cells. The degradation rates often change during development, differentiation, and/or in response to a stimulus to meet the cell's needs (Wu and Brewer, 2012).

By injecting mRNA into the cytoplasm of a fertilized egg, it is possible to bypass the complications and checkpoints associated with transcription, processing, and mRNA export. The data from this manipulation in sea urchins show that an exogenous mRNA is highly and selectively retained in the germ-cell precursors in early development. This retention could result from selective RNA decay in the non-small micromere cells, or selective RNA protection in the sMics. In most organisms, specification of the germ line depends on mechanisms that also inhibit the expression of somatic genes (Seydoux and Braun, 2006). For example, in *Drosophila* and *C. elegans* embryos, somatic cell nuclei initiate mRNA transcription 1 hr after fertilization, but the PGCs and their precursors do not accumulate embryonic mRNA until after the onset of gastrulation (Blackwell, 2004). Results using phospho-specific antibodies against RNA polymerase II suggest that the transcription is blocked at a step between initiation and elongation in early germ cells before gastrulation

(Blackwell, 2004), and the cells must therefore make new protein from pre-existing (retained) mRNAs. If the sMics are largely transcriptionally repressed, they might also be inhibiting RNA decay machinery in order to protect the RNA present for protein synthesis, which may function to retain sMic viability for the prolonged period of transcriptional silence during early development. Here, we show that neither the m⁷GTP cap or the polyA tail are required for selective RNA retention in the sMics, suggesting that the mRNA retention is independent of protein translation, that is, prolonged ribosome engagement is not an explanation for long-term retention of mRNAs. Complete removal of the cap or the 3'-UTR, however, leads to a uniform degradation of the mRNAs in the embryos, indicating that some components of the RNA degradation machinery are present in the germ-cell precursors. The selective RNA retention observed in Figure 1 could result from the absence of one or several components of the degradation machinery or by their specific inactivation in the sMics.

The stability of mRNAs can vary from minutes to days, with a median half-life of 21 min in yeast and 10 hr in mammals (Yang et al., 2003). Deadenylation is often the first step towards RNA decay. It can be triggered by the PolyA-binding protein-dependent deadenylase PAN2-PAN3, the cap dependent deadenylase PARN, and the CCR4-NOT complex, which is the main deadenylase found in *Saccharomyces cerevisiae* (Garneau et al., 2007). After deadenylation, exonucleases may be recruited to degrade the mRNAs in a 3'-to-5' direction. The 3'-to-5' degradation pathway is carried out by a tightly controlled exonuclease complex, the RNA exosome (Schmid and Jensen, 2008). The eukaryotic exosome consists of 10–12 subunits that adopt a ring-like structure. This core can associate with a variety of enzymatically active subunits and co-factors (Garneau et al., 2007; Lykke-Andersen et al., 2011). The scavenger mRNA decapping enzyme DcpS hydrolyzes the residual cap structure resulting from this 3'-to-5' decay (Wang and Kiledjian, 2001; Liu et al., 2002). In eukaryotic cells, the 5'-to-3' degradation pathway depends first on removal of the cap by the decapping proteins Dcp1 and Dcp2 (Liu and Kiledjian, 2006). A third component, called Hedls (human enhancer of the decapping large subunit), is required for the Dcp1/Dcp2 interaction in human cells (Simon et al., 2006). A set of accessory proteins, such as Pat1 protein and the DEAD box RNA helicase Dhh1, are also part of the decapping complex (Coller and Parker, 2005). In the cytoplasm, the resulting uncapped 5' end is then targeted to initiate the degradation by the 5' exoribonuclease Xrn1 (Larimer et al., 1992; Hsu and Stevens, 1993). Currently we do not know the status of any of these proteins in the early sea urchin embryo.

mRNA can also be subject to endonuclease-mediated decay, followed by degradation of the upstream and downstream decay products by exonucleases. Endonuclease cleavage can be activated by cellular stress, extracellular signals, or by ribosome stalling, as might occur at a premature termination codon (Otsuka and Schoenberg, 2008). Several endonucleases have been identified, such as PMR1 (Yang and Schoenberg, 2004), MRP (Xiao et al., 2002), G3BP-1 (Gallouzi et al., 1998), IRE-1 (Hollien and Weissman, 2006), and Dom34 (Lee et al., 2007).

Messenger RNAs can also be degraded by the RNAi pathway. Both miRNAs and piRNAs have recently been implicated in mRNA turnover. We found the seed sequence of *S. purpuratus* miR2010 in the β -globin 3'-UTR (Jia Song, personal communication), but

mutation of this sequence did not affect the RNA retention in the sMics (Supplemental Fig. 1). We do not believe that miRNAs or piRNAs are functioning in rapid soma RNA turnover since (1) the UTRs and coding regions tested are exogenous sequences; (2) removal of the UTRs enhance degradation, not reduce it as might otherwise be expected; and (3) the different coding regions tested were treated equally by the cell.

The selective retention could also be explained by a differential RNA storage mechanism. A typical eukaryotic cell contains several types of spatially formed granules, such as P bodies (processing bodies). These granules accumulate in the cytoplasm and are described as specific sites for mRNA storage, reversible mRNA repression, and mRNA decay (Olszewska et al., 2012). In contrast, germ cells contain granules related to P bodies, called germ granules. They are localized near the cell nucleus and have similar functions to P bodies, such as regulating RNA accumulation and decay. The germ cell granules regulate maternal RNA required for germ cell specification (Anderson and Kedersha, 2006). In this study, exogenous RNA was injected in the embryo at the one-cell stage, so the embryos likely utilized it similarly to maternal RNA, protecting it from degradation once the germ cell lineage formed at the 5th cell division. Germ granules could be more efficient than P bodies at protecting maternal RNA to promote germ cell development, yet normally these perinuclear structures (nuage) are thought to see newly exported mRNAs. If nuage is also protecting the microinjected mRNAs, they must be providing equal access to the structures for both newly synthesized and pre-existing transcripts. Moreover, results from *C. elegans* identified three distinct structures during oogenesis and embryogenesis: somatic P bodies, germ granules, and storage bodies (Boag et al., 2008). The authors revealed that many maternal mRNAs are regulated not only by interactions with individual RNA binding protein that control their translation, but also by a CGH1 (Dhh1/RCK)-dependent protective mechanism. In somatic tissues, CGH1 forms Pat1-dependent P bodies that are associated with RNA decay, but in developing oocytes, Pat1 is not required to form CGH1 RNP particles (which are referred as storage bodies). Depending on the proteins present in the sMics, specific granules could be enriched to indiscriminately bind to and protect RNAs from degradation. How the 3'-UTR guides the mRNA into a protective state, though, is unclear.

The sMics do not retain all the various mRNAs that are present at the birth of this cell lineage. For example, *Gus* is present ubiquitously early in development, but then is cleared from the sMics shortly after their formation (Gustafson et al., 2011). This suggests that the sMics do have mRNA degradation capability. *Gus* mRNA degradation could be targeted by specific decapping enzymes, or nucleases that would be differentially expressed between the sMics and the non-small micromere cells. This transcript could be specifically excluded from some storage granules only present in the sMics, leading to its degradation, or it could also be targeted, perhaps by miRNAs that target distinct transcripts in the sMics. The sea urchin embryo has ~56 miRNAs, and an active miRNA-mediated pathway for regulating mRNA translational repression and turnover (Campo-Paysaa et al., 2011; Kadri et al., 2011; Wei et al., 2011; Song et al., 2012), including each of the major components of the miRNA biosynthesis and mRNA degradation mechanisms (Song and Wessel, 2007). Differential

miRNA expression between the sMics and the non-small micromere cells could explain selectivity in this example of RNA retention.

MATERIALS AND METHODS

Animals

S. purpuratus were housed in aquaria with artificial seawater (ASW) at 16°C (Coral Life Scientific Grade Marine Salt, Carson, CA). Gametes were acquired by either 0.5 M KCl injection or by shaking. Eggs were collected in ASW or filtered seawater, and sperm was collected dry. To obtain embryos, fertilized eggs were cultured in filtered seawater at 16°C.

Plasmid Constructions

The *mCherry* constructs were generated from the vector pSP6T4 (Gustafson and Wessel, 2010). This vector contains either the *mCherry* or the *GFP* open reading frame flanked by *Xenopus* β -globin 5' – and 3'-UTRs. The β -globin 3'-UTR is followed by a polyA tail composed of 30 adenosines. A construct without polyA tail was made using the following primers: F(5'-AGGTACTAGTGACTGACTAGGATCTGGTTAC) and R (5'-CCGGGATCCAGAATGTGAAGAACTTTCTTTTATTAG). The polyadenylation signal AATAAA present in the β -globin 3'-UTR was mutated to AAGAAG using the following primers: F (5'-GCCATTCGTATCTGCTCCTAAGAAGAAGAAAGTTTCTTCAC) and R (5'-GTGAAGAACTTTCTTCTTCTTAGGAGCAGATACGAATGGC). The miR2010 seed sequence ACCAGCC was mutated to ACGAGTC using the primers F: (5'-TCTGGTTACCACTAAACGAGTCTCAAGAACACCCGAATGG) and R (5'-CCATTCGGGTGTTCTTGAGACTCGTTTAGTGTAACCAGA). Mutations were made using the Quick Change II Site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA).

In Vitro RNA Synthesis

mCherry or *GFP* RNA were synthesized using the Maxiscript SP6 or T7 Kit (Ambion, Austin, TX), yielding RNA concentrations between 0.15 and 0.2 µg/µl. m⁷GTP or A-capped RNAs were obtained using respectively the 3'-O-Me-m⁷G(5')ppp(5')G (New England Biolabs, Ipswich, MA; S1411S) or the G(5')ppp(5')A (New England Biolabs-S1406S) cap analog during the RNA synthesis. Injection solutions contain: 20% glycerol, RNA at 100 ng/µl, and either FITC or rhodamine dye to identify and follow the injected embryos. Approximately 2 pl of each RNA mixture was injected into each fertilized egg.

Microinjections

Microinjection of zygotes was performed as previously described (Cheers and Ettensohn, 2004). In brief, eggs were de-jellied with acidic sea water (pH 5.0), washed three times with filtered sea water, rowed with a mouth pipette onto protamine sulfate-coated 60 mm × 15 mm Petri dishes, fertilized in the presence of 1 mM 3-AT, and injected using the Femto Jet injection system (Eppendorf, Hamburg, Germany). To make the injection needles, 1 × 90 mm glass capillaries with filaments (Narishige, Tokyo, Japan) were pulled on a vertical needle puller (Narishige). Injected embryos were cultured in seawater at 16°C.

Whole-Mount RNA In Situ Hybridization

Antisense digoxigenin (DIG)-labeled RNA probes against *GFP* and *mCherry* were constructed using a DIG RNA labeling kit (Roche, Indianapolis, IN). Whole-mount RNA in situ hybridization experiments were performed as previously described (Minokawa et al., 2004), and the alkaline phosphatase reaction was carried out for 1 hr. All steps were carried out in 96-well round-bottom PVC plates (ThermoFisher Scientific, Rockford, IL). Samples were imaged on a Zeiss Axiovert 200 M microscope equipped with a Zeiss color AxioCam MRc5 camera (Carl Zeiss, Inc., Thornwood, NY).

Real-Time Quantitative Reverse-Transcriptase PCR

At each time point, total RNA was extracted from 200 embryos using the RNeasy Micro Kit (Qiagen, Valencia, CA). cDNA was prepared using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems) with the FastStart Universal Probe Master (Roche Applied Science, Penzberg, Germany). *mCherry* was amplified using the following primers: F (5'-CCCCGTAATGCAGAAGAAGA) and R (5'-TCTTGGCCTTGTAGGTGGTC). Experiments were run in triplicate, and the data were normalized to *Ubiquitin* RNA levels amplified using F (5'-CACAGGCAAGACCATCACAC) and R (5'-GAGAGAGTGCGACCATCCTC).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PGCs	primordial germ cells
polyA	poly-adenosine
sMics	small micromeres
UTR	untranslated region

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Figure 1.

RNA containing β -globin UTRs is selectively retained in the sMics. Asynthetic mRNA containing the *mCherry* open reading frame flanked by β -globin 5'- and 3'-UTRs was injected into *S. purpuratus* fertilized eggs (**I** to **P**). Non-injected eggs were used as a control (**A** to **H**). In situ hybridizations were performed using a probe against *mCherry* after fixation of the embryos at the following stages: 2 cells (**A**, **I**), morula (**B**, **J**), early blastula (**C**, **K**), hatching blastula (**D**, **L**), mesenchyme blastula (**E**, **M**), and during gastrulation (**F**, **G**, **H**, **N**, **O**, **P**).

Figure 2.

sMics primarily retain exogenous RNAs. A synthetic mRNA was made using the *mCherry* open reading frame flanked by β -globin 5'- and 3'-UTRs, including a tail of 30 adenosines. The RNA was injected into *S. purpuratus* fertilized eggs, and total RNA was extracted at different time point just after fertilization (30 min), at the 2-cell stage (3 hr), in hatching blastula (12 hr), in mesenchyme blastula (20 hr), or in gastrula (40 hr), for quantitative RT-PCR analysis.

Figure 3.

RNA retention involves a mechanism independent of the m⁷GTP cap and polyA tail. Synthetic mRNAs were made using the *mCherry* open reading frame flanked by the β -globin 5'- and 3'-UTRs (A–C, E, F). The 5'-UTR was capped by m⁷GTP (A, C, E), an A-cap analog (B), or uncapped (F). The 3'-UTR contained a polyA tail (A, B, E, F) or not (C). RNAs were injected into *S. purpuratus* fertilized eggs. Non-injected eggs were used as a background labeling control (D, G). In situ hybridizations were performed using a probe against *mCherry* after fixation of the embryos at the gastrula stage.

Figure 4.

Selective RNA retention in the sMics is independent of the open reading frame. Synthetic mRNAs were made using β -globin 5'- and 3'-UTRs, surrounding the *mCherry* (A) or *GFP* (C) open reading frame (ORF). RNAs were injected into *S. purpuratus* fertilized eggs. Non-injected eggs were used as a background labeling control (B,D). In situ hybridizations were performed using a probe against *mCherry* (A,B) or *GFP* (C,D) after fixation of the embryos at the gastrula stage.

Figure 5.

Exogenous RNAs without 3'-UTRs are not selectively retained in the sMics. Capped and polyA tailed mRNAs containing the *GFP* (A,B) or *mCherry* open reading frame (D,E) and flanked by either both β -globin 5'- and 3'-UTRs (A,D), no UTRs (B), or only the β -globin 5'-UTR (E), were synthesized and injected into *S. purpuratus* fertilized eggs. Non-injected embryos (C,F) were used as a control. In situ hybridizations were performed using a probe against *GFP* (A–C) or *mCherry* (D–F) after fixation of the embryos at the gastrula stage.