Molecular Mechanisms in Primordial Germ Cell Development in Zebrafish

Dissertation

In partial fulfilment of the requirements
for the degree “Doctor rerum naturalium (Dr. rer. nat.)”
in the Molecular Biology Program
at the Georg August University Göttingen,
Faculty of Biology

submitted by

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born in

Winterthur, Switzerland

2007

To my beloved wife Paola, my sweet daughter Sophie and in thankful honour to my parents.

Your work is to discover your world

and then with all your heart give yourself to it.

Buddha

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Affidavit

I affirm that the thesis “Molecular Mechanisms in Primordial Germ Cell Development in Zebrafish” has been written independently and with no other sources and aids if not otherwise stated.

Göttingen, 13.09.2007

Signature: Markus Strasser

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Abbreviations

AB	antibody
Amp	ampicilin
aRNA	antisense RNA
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Da	Dalton
Dig	Digoxigenin
DNA	deoxyribonucleic acid
dnd	*dead end*
dpf	days post fertilization
E. coli	Escherichia coli
EDTA	ethylendiamine tetraacetate
EST	expressed sequence tag
FACS	Fluorescent Activated Cell Sorter
FCS	Fetal calf serum
g	gravity
GFP	green fluorescent protein
gra	*granulito*
hpf	hours post fertilization
IgG	immunoglobulin G
kbp	kilo base pair
kDa	kilo Dalton
min	minutes
ml	mililiter
MO	morpholino antisense oligonucleotide
mRNA	messenger RNA
nos1	*nanos-1*
nt	nucleotide
o/n	overnight
ORF	open reading frame
PBS	phosphate buffered saline

PBT	phosphate buffered saline containing Tween 20
PBTX	phosphate buffered saline containing Tween 20 and TritonX-100
PCR	polymerase chain reaction
PGC	primordial germ cell
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDF-1	stromal cell-derived factor-1
sec	seconds
Tdrd7	Tudor domain containing protein 7
TGCTs	Testicular germ cell tumors
U	unit
URRs	uridine-rich regions
UTR	untranslated region
wt	wild-type

1 Abstract

In many organisms, primordial germ cells (PGCs) are specified early on in development at distant places from where the gonad will develop. therefore, the cells have to migrate to reach the place where they will differentiate into the gametes (sperm and egg). A common feature of germ cells is the presence of unique granular structures generally termed as germinal granules, which are thought to be involved in posttranscriptional regulation (including regulation through microRNAs). Interestingly, Dead end, a germinal granule component, is essential for several aspects of PGC development such as motility and fate maintenance in zebrafish. To understand the specific role germinal granules play in germ cell development it is important to identify their structural features as well as to characterize the function of Dead end and other germinal granule components.

Here I present the results of a microarray-based screen, which led to the identification and characterization of two novel germ-cell markers whose proteins are components of germinal granules, Granulito and Tdrd7. Based on the specific localization of Granulito to germinal granules, I generated a transgenic fish line which expresses a fluorescent fusion construct of Granulito early on in PGC development. This transgenic line allowed the description of the structural features of germinal granules during early development and how they change until the PGCs reach their targets. I found that initially, granules exhibit a wide size variation, but by the end of the first day of development they become a homogeneous population of medium size granules. Tdrd7 is required for this process as PGCs in which Tdrd7 was knocked down exhibit abnormal granule morphology and reduced granule numbers.

Last, I present evidences that the germinal granule component Dead end counteracts the function of microRNAs on specific targets. This derepression of microRNA activity through Dead end depends on uridine-rich regions within the 3'UTR of the target mRNA. As Dead end and components of the microRNA pathway are known to localize to germinal granules, their interaction is probably carried out within these structures.

2 Introduction

Many organisms undergo sexual reproduction to generate genetic variability, allowing a better adaptation to environmental factors and therefore ensuring the preservation of the species. A new generation is formed when two gametes from two individuals fuse to initiate the developmental process. Gametes are differentiated germ cells; and their progenitors are known as primordial germ cells (PGCs). In most species PGCs segregate from the somatic cell lineage early in development and typically have to migrate from the site of specification to the place where the gonad will develop and undergo further differentiation steps. (Molyneaux and Wylie, 2004; Raz, 2004; Saffman and Lasko, 1999; Santos and Lehmann, 2004; Starz-Gaiano and Lehmann, 2001; Wylie, 1999).

In addition to constituting an important general biological process, PGC migration serves as a model for long-range cell migration, an important theme in embryonic development, organogenesis and organ function as well as for situations in which abnormal cell movement leads to pathological conditions (i.e. cancer metastasis).

The zebrafish, *Danio rerio*, has become an important model system for studying PGC development as it offers several advantages over other vertebrate and invertebrate model organisms. The combination of extra-uterine development, a large number of progeny, the availability of genetic mutations and other genetic tools coupled with the optical translucency of the embryo is unique for this model organism. The features of this relatively new system facilitated the research on PGC development where novel contributions, especially in the field of PGC migration have been made (Raz, 2002; Raz, 2003; Raz, 2004; Raz and Reichman-Fried, 2006).

2.1 Mechanism of Germ Cell specification

Two modes of PGC specification have been described; specification by inheritance and specification by induction (Wylie, 2000). In some invertebrates (e.g *C.elegans* and *Drosophila*), as well as in some vertebrates such as chicken, fish and frog, germ cells are specified by inheritance of maternal factors (Hashimoto et al., 2004; Houston and King, 2000; Tsunekawa et al., 2000; Whitington and Dixon, 1975; Yoon et al., 1997), while in others, such as hydra, urodeles and mammals (Extavour and Akam, 2003), germ cells specify by induction through signaling molecules belonging to the BMP family (Hayashi et al., 2007; Lawson et al., 1999; Wakahara, 1996).

Interestingly, there are phylogenetic evidences that germ cell specification through inheritance arose several times in evolution (Johnson et al., 2003).

Specification of PGCs by inheritance requires the localization of cytoplasmic determinants within the forming embryo. These determinants are comprised of electron dense material containing maternal RNAs and proteins, collectively termed germ plasm (Mahowald, 1968; Saffman and Lasko, 1999). Transplantation experiments in *Drosophila* demonstrated that the germ plasm is sufficient for the induction of the germ cell fate (Illmensee and Mahowald, 1974). Similarly, in zebrafish it has been shown that removal of germ plasm leads to loss of germ cell specification (Hashimoto et al., 2004). The germ plasm localizes to specific sites in the fertilized oocyte that determine which cells will inherit these PGC determinants. In *Drosophila* oocytes, the localization of the germ plasm to the posterior pole depends on the posterior group genes *oskar*, *vasa*, *tudor* and *staufen* (Boswell and Mahowald, 1985; Lasko and Ashburner, 1988; Lehmann and Nusslein-Volhard, 1986; St Johnston et al., 1991). All these factors localize to germinal granules (Breitwieser et al., 1996), a discrete granulofibrous structure within the germ plasm, and flies mutant for these genes have a disturbed germ cell formation. Analysis of the 3'UTR of *vasa* (a known germ plasm component in PGCs) revealed an evolutionary conserved region, which is sufficient for localization of the mRNA to the germ plasm (Knaut et al., 2002). This localization mechanism is dependent on cytoskeletal components as well as on *trans*-acting factors that bind to sequences within the 3'untranslated region (UTR) of the RNAs to be localized (Micklem, 1995; Robb et al., 1996; Rongo and Lehmann, 1996; Seydoux, 1996; Zhou and King, 1996).

2.2 PGC specification in Zebrafish and the role of germinal granules

In zebrafish, the germ plasm is localized to the first and second cleavage furrows of the early embryo and this localization depends on the function of actin and microtubules (Pelegri et al., 1999; Theusch et al., 2006). At the 32-cell stage the germ plasm is incorporated into four cells that become the progenitors of the primordial germ cell lineage (Pelegri, 2003). During division of these progenitor cells, the germ plasm is inherited by only one daughter cell (Braat et al., 1999; Knaut et al., 2000), thereby maintaining the number of presumptive germ cells constant. However, after 4 hours of zebrafish development the germ plasm is segregated symmetrically, a change that coincides with an increase in PGC numbers (Whitington and Dixon, 1975). The

segragation of the germ plasm in zebrafish were described using *vasa* mRNA as a marker, which based on ultrastructural analysis reflects the distribution of maternally derived germ plasm material (Pelegri, 2003) (see Fig. 2-1).

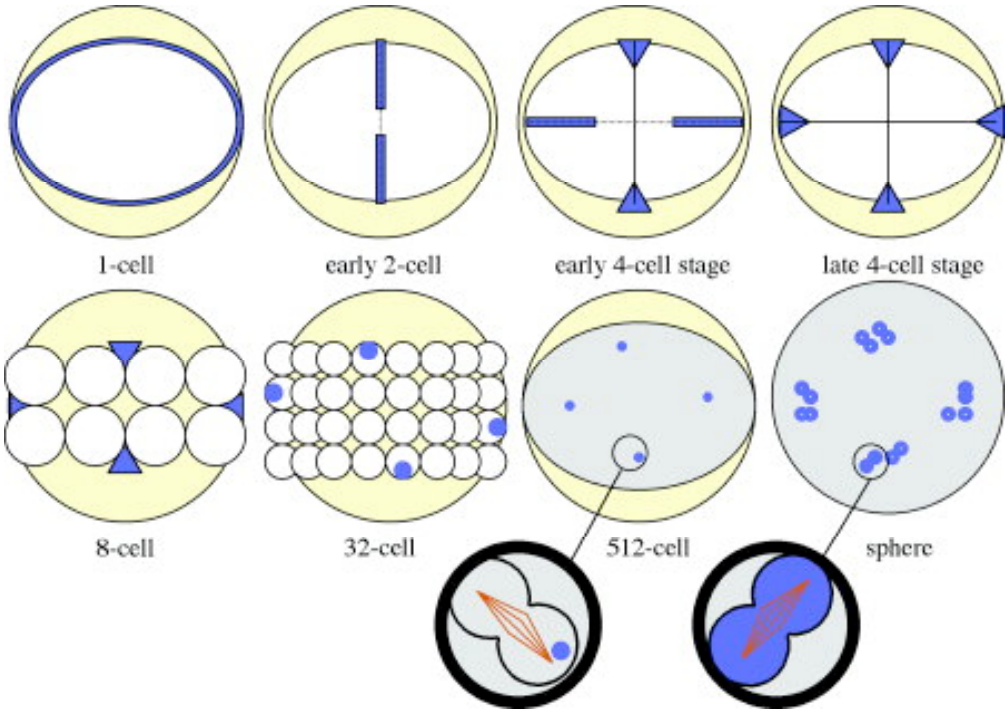


Fig. 2-1. Cartoon depicting the segregation of the germ plasm component *vasa* in zebrafish. Animal views of developing zebrafish embryos. *vasa* mRNA (blue), which becomes localized at the cytokinetic ring during egg activation, is recruited to the forming furrows of the first and second cleavage planes. Upon furrow maturation, the recruited mRNA forms a tight aggregate at the distal end of the furrow, a process that is dependent on microtubules of the furrow microtubule array. These aggregates ingress into four cells at the 32-cell stage, where they remain subcellularly localized and segregate asymmetrically during cell division (see insert at the 512-cell stage). At the sphere stage, *vasa* mRNA becomes evenly distributed within cells and segregates symmetrically during cell division (see insert) (adopted from Pelegri, 2003).

Upon initiation of symmetric cell division, the germ plasm then becomes localized around the nucleus, forming spherical structures known as perinuclear granules in zebrafish. These structures are a common feature of germ cells in all metazoans, including species where germ cells are specified by induction (McLaren, 1999), but have different names such as nuage (*Drosophila*), chromatoid bodies (mouse) or germinal granules (*Xenopus*). In this thesis these stuctures will be generally termed as germinal granules, as this term is most widely used across species. Although the precise function of the germinal granules in germ cell formation and development remains largely unknown, several RNAs and proteins localized to these structures were found to be essential for germ cell specification, survival and later development.

A common marker for germinal granules is Vasa, an RNA helicase, which shows significant sequence homology to the translation initiation factor eIF-4A (Hay et al., 1988; Lasko and Ashburner, 1988; Raz, 2000). In contrast to Vasa for which no function has been attributed yet during zebrafish PGC development, other components of the germinal granules, such as the RNA-binding proteins Dead end (Dnd) and Nanos1 (Nos1) are essential for germ-cell survival and maintenance of oocyte production in zebrafish (Draper et al., 2007; Köprunner et al., 2001; Weidinger et al., 2003). Interestingly, it was previously described that mitochondrial rRNA (mtlrRNA) is highly abundant in the germ plasm and tightly associated to germinal granules (Kobayashi et al., 1993). When mtlrRNA is decreased in the germ plasm, the number of germ cells is also reduced (Iida and Kobayashi, 1998). The presence of RNA-binding proteins, RNA helicases and the high abundance of mitochondrial rRNA in these structures suggests a role for germinal granules in posttranscriptional regulation and translation, which appear to be essential for PGC development (Houston and King, 2000; Seydoux and Strome, 1999).

Analysis of factors that are necessary for specification and formation of primordial germ cells in *Drosophila* demonstrated that the protein encoded by the *tudor* gene (Boswell and Mahowald, 1985) functions in the formation of a scaffold important for proper germ plasm assembly and granule structure (Arkov et al., 2006; Thomson and Lasko, 2005).

Although the distribution of germ plasm during early stages of zebrafish development has been described in detail (Knaut et al., 2000), little is known about its distribution and segregation during cell division after germinal granules adopt perinuclear localization. Understanding these processes would contribute to the understanding of the mechanisms that promote germ cell fate maintenance and those allowing for an increase in germ cell number during later stages of development (e.g. during migration and after arrival at the gonad).

2.3 PGC migration

In many organisms the germ cells are formed in regions distinct from the site where the gonad develops. Hence, PGCs have to migrate towards the region where the gonad develops, a process that has been studied in *Xenopus,* and particularly in *Drosophila* and mouse where modern genetic approaches have been applied (Starz-Gaiano and Lehmann, 2001; Wylie, 2000). The general conclusion from these studies

is that directed PGC migration relies on cues provided by the somatic environment. Therefore, during their migration the PGCs obtain cues from somatic cells, yet they have to ignore signals that would lead to their differentiation and assume cell identities similar to those of somatic cells in their environment.

The identity of the actual signal guiding PGCs in zebrafish was discovered in screens in which CXCR4b, the receptor for the chemokine SDF-1a (CXCL12a) was demonstrated to be required for the guidance of PGCs (Doitsidou et al., 2002; Knaut et al., 2003). *sdf-1a* was found to be expressed in positions where the PGCs were found or towards which they were migrating and conversely, translational inhibtion of the receptor or its ligand led to elimination of directed PGC migration towards the region of the gonad. This guidance cue for PGC migration was found to be conserved in mouse and chick {Molyneaux, 2003 #1336; (Stebler et al., 2004). Although PGCs lacking the SDF-1a signal exhibit a clear migration phenotype (Fig. 2-2, (Doitsidou et al., 2002)), they maintain several basic PGC characteristics.

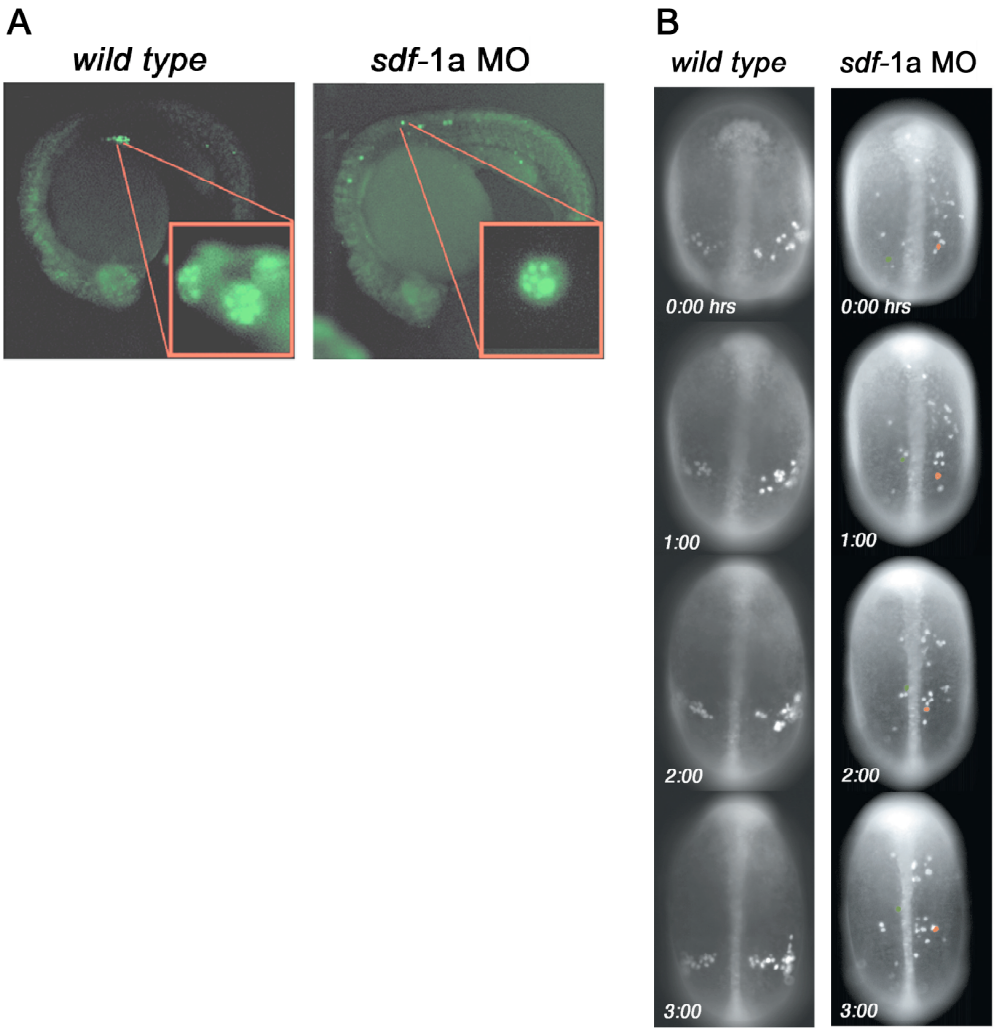


Fig. 2-2. PGCs that do not receive SDF-1a signals are motile and maintain their fate. A) Low magnification view of 24 hpf embryos expressing Vasa-GFP in PGCs which were treated with control (left panel) or SDF-1a morpholino (right panel). Insets show high magnification views of the indicated germ cells. PGCs possess characteristic Vasa-positive perinuclear granules independent of their successful migration towards the gonadal region. B) Snapshots from a low magnification timelapse movie of embryos expressing GFP within PGCs, treated with either control or SDF-1a morpholino, showing that in the embryo injected with *sdf*-1a antisense oligonucleotides the PGCs migrate relative to somatic tissues (see red labeled cell) (adopted from (Doitsidou et al., 2002)).

First, independent of SDF-1a signaling and location within the embryo, PGCs maintain the expression of PGC specific markers (Doitsidou et al., 2002) and possess characteristic cellular structures, such as germinal granules (Fig 2-2A). Second, although they do not show directed migration, the treated cells remain highly motile in the absence of SDF-1 signaling (Fig 2-2B). Consistently, subsequent studies revealed that G proteins of the Gi family, described to act downstream of chemokine signaling (Thelen, 2001), are essential for directional migration but not for PGC motility (Dumstrei et al., 2004). Thus, the molecular mechanisms that allow the PGCs to maintain their fate and exhibit their general morphological and behavioral characteristics are not governed by SDF-1a/CXCR4b signaling.

2.4 PGC fate maintenance

During the migration phase, PGCs need to maintain their cell fate while ignoring differentiation signals from nearby tissues. This requires a strong commitment to the PGC fate. Analyzing the loss-of-function of the RNA binding protein Dead end in zebrafish, whose mRNA is expressed in the PGCs during the first few days of development, revealed a critical role for the gene in PGC fate maintenance and motility (Weidinger et al., 2003). Specifically, in embryos injected with antisense oligoncleotides against *dead end*, the PGCs were immotile and lost the expression of several PGC specific RNAs (e.g. *vasa* and *nanos1*) (Fig. 2-3 and data not shown). At a later stage of development (between 10 hpf and 24 hpf) the PGCs died demonstating that the gene function is essential for PGC survival. In wild-type embryos, Dead end localizes to germinal granules, which are thought to regulate gene expression posttranscriptionally by modifying mRNA transport, stability and translation (Houston and King, 2000; Seydoux and Strome, 1999), suggesting a role of Dead end in one of these processes (Fig. 2-3D). The role of *dead end* in germ cell survival was

shown to be conserved in *xenopus* and mouse (Horvay et al., 2006; Youngren et al., 2005). Additionally in the mouse 129 inbred strain, loss of Dead end causes the formation of testicular germ cell tumors (TGCTs) (Youngren et al., 2005).

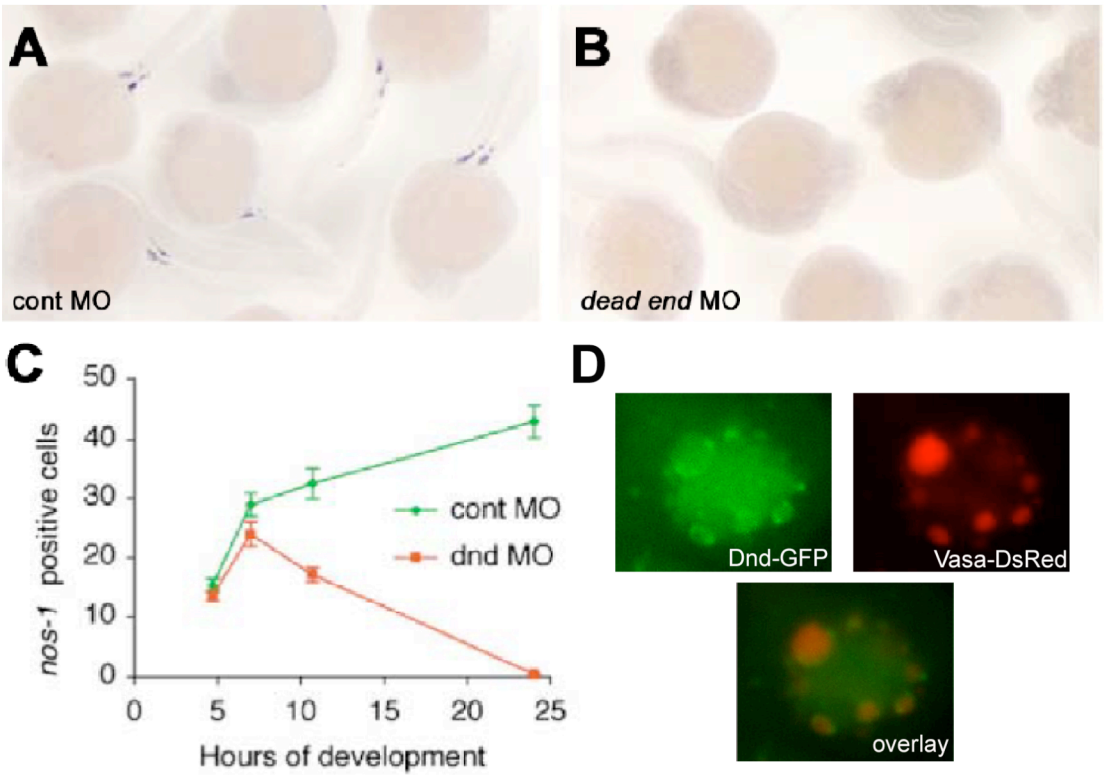


Fig. 2-3. *dead end* is required for maintenance of PGC fate and viability. A-B) Low magnification brightfield image of 24 hpf embryos treated with control or *dead end* morpholino and stained for *nanos1* mRNA. In 24 hours old control morpholino injected embryos the PGC marker *nanos1* mRNA is expressed (A), whereas expression of *nanos1* is not detected in embryos injected with the *dead end* morpholino. C) The number of *nanos1* expressing cells is initially normal indicating proper specification of the PGCs. Beginning at late gastrulation stages (10 hpf) PGC numbers decrease until at 24 hpf all PGCs have died. D) High magnification view of PGCs expressing Dead end-GFP and Vasa-DsRed at 14 hpf. Dead end localizes in speckles to germinal granules in partial colocalization with the granule marker Vasa (adapted from (Weidinger et al., 2003)).

2.5 miRNAs and their role in germ cell development

MicroRNAs (miRNA) were first described in C.elegans and plants (Cogoni and Macino, 1999; Lee et al., 1993). They constitute a family of 21 to 23-nucleotides (nt) RNAs widely expressed in metazoans (Pillai et al., 2007). These regulators of gene expression are capable of defining and altering cell fate. Recent estimations suggest the existence of 500-1000 miRNAs per genome, and that a large proportion of human protein coding genes are under the regulation of one or more miRNAs (Aravin and Tuschl, 2005; Lewis et al., 2005). In animals, miRNAs utilize a seed sequence at their

5’ end to associate with 3’UTR regions of mRNAs to suppress gene expression by inhibiting translation (Pillai et al., 2005). In some cases this suppression by miRNA is associated with mRNA decay (Bagga et al., 2005; Lim et al., 2005).

Several studies implicate that miRNAs participate in the regulation of many cellular processes and that a vast majority of miRNAs show tissue or developmental stage specific expression (Lagos-Quintana et al., 2002; Wienholds and Plasterk, 2005). Additionally, microarray data shows that some miRNAs downregulate a large number of target mRNAs (Lim et al., 2005). Importantly, aberrant expression or activity of miRNAs can lead to disease (Kloosterman and Plasterk, 2006; Pillai et al., 2007; Voorhoeve and Agami, 2007).

Recent reports indicate that miRNA mediated repression is reversible. Treatment of cultured rat neurons with BDNF leads to partial relief of miRNA-134-mediated Limk1 mRNA repression (Schratt et al., 2006). Limk1 is a serine-only kinase, which can phosphorylate cofilin upon extracellular stimuli. Phosporylated cofilin is unable to depolymerize actin filaments (Stanyon and Bernard, 1999). In Drosophila, external stimulation of olfactory neurons induces degradation of the Armitage protein, which is required for miRNP assembly (Ashraf and Kunes, 2006). As a result, the miRNA-controlled translation of the protein kinase CaMKII mRNA is enhanced. Last, the repression of CAT-1 mRNA by miR-122 is relieved in stress conditions by binding of HuR, an AU-rich-element binding protein, to a region at the end of the 3'UTR of CAT-1 (Bhattacharyya et al., 2006). Together, these observations indicate that the miRNA pathway can be controlled at different levels, from stability, processing, sequence identity and binding to target mRNAs.

Most miRNAs are transcribed by the RNA polymerase II as long RNAs that are then converted to ~70 nt long pre-miRNAs by the enzyme Drosha (Lee et al., 2003). The pre-miRNAs are then exported to the cytoplasm by Exportin 5, cleaved to about 21 to 23 nt mature miRNAs by the enzyme Dicer and one strand of the duplex is incorporated into the RNA induced silencing complex (RISC) (Gregory et al., 2005; Maniataki and Mourelatos, 2005). Repressed mRNAs, miRNAs, and most proteins constituting the miRNA-RISC complex (miRNP) are enriched in cytoplasmic processing bodies called P-bodies. P-bodies are known to be sites of translational repression and mRNA decay (Liu et al., 2005; Parker and Sheth, 2007).

P bodies have common features with germinal granules (Parker and Sheth, 2007), but the latter contain an array of cell-specific RNA-binding proteins giving PGCs a unique control over translation and the stability of mRNA and proteins (Kotaja et al., 2006a; Kotaja and Sassone-Corsi, 2007). Recent study shows that the chromatoid bodies, a form of germinal granules in mouse male germ cells, contains Dicer and components of the miRNP complex such as Argonautes and miRNAs (Kotaja et al., 2006a). Additionally, PIWI, a germ cell specific member of the Argonaute family that is conserved from *Drosophila* to humans, localizes specifically to germinal granules and interacts with Dicer (Houwing et al., 2007; Kotaja et al., 2006b; Megosh et al., 2006; Qiao et al., 2002). Interestingly, latest reports showed that germ cells possess a unique form of miRNA called piRNA, which are approximately 29 nt long and play a role in silencing transposons (Brennecke et al., 2007; Gunawardane et al., 2007; Houwing et al., 2007).

Interestingly, a recent report showed that, although miR-430 is able to repress certain targets equally well in somatic and germ cells, a subset of miR-430 targets appears to be protected from repression in germ cells (Mishima et al., 2006). In particular, miR-430 mediated repression of *nanos1* (Fig. 2-4) and *Tdrd7* is not effective in germ cells. Although Mishima *et al.* could not provide a mechanism for the specific inhibition of miRNA action in germ cells, they reasoned that regions in the 3'UTR of these genes counteract their repression in germ cells.

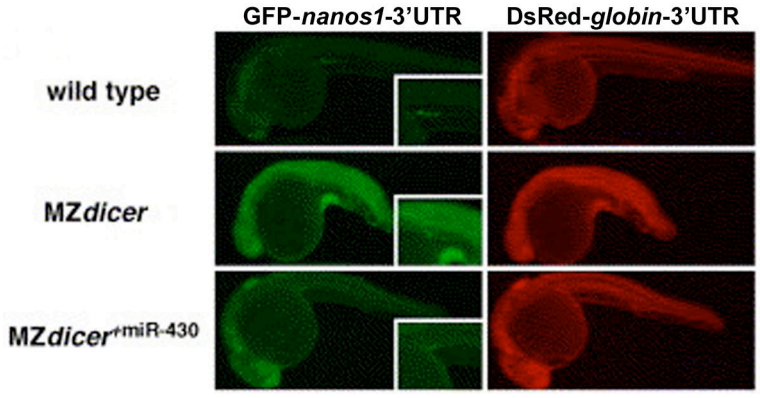


Fig. 2-4. miR-430 represses protein expression of Nanos1 in the soma but not in germ cells. Low magnification view of wild-type embryos and embryos mutant for the miRNA processing enzyme Dicer. In the presence of miRNAs (top row) GFP expression by a construct containing the *nanos1*-3'UTR is suppressed in the soma but not in germ cells (insert), while a control construct is uniformly expressed (red). In embryos devoid of Dicer (maternal zygotic mutants, middle row) the somatic repression of the *nanos1* 3'UTR is lost, but can be rescued through injection of mature miR-430 (bottom row) (adopted from Mishima et al., 2006).

2.6 Aim of the thesis

Dead end is a key player in the development of germ cells, as PGCs lacking Dead end are missing key features of this cell type, such as motility or expression of specific germ cell markers, and eventually die. The focus of this thesis is the identification of novel molecules important for germ cell development and further characterisation of the specific role of Dead end.

Here I present the identification and characterization of two novel germ cell markers *granulito* and *Tdrd7*, whose proteins are localized to germinal granules. I further describe the dynamics and structural properties of the germinal granules within the first 24 hours of embryonic development. Last I provide evidences that Dead end acts through alleviating the action of miRNA on specific targets.

3 Results

3.1 Identification of candidate genes, important for primordial germ cell development, using Affymetrix zebrafish microarray chips

In order to identify new genes important for PGC development I performed a microarray based screen where the trancriptional expression profile of wildtype PGCs was compared to somatic cells and to PGCs lacking Dead end, a protein essential for PGC motility and maintenance (Weidinger et al., 2003).

First, I established a protocol to manually isolate GFP-labeled PGCs that allowed direct cell isolation using a fluorescence stereo microscope followed by immediate freezing of the cells. This method proved to be superior to the use of Fluorescent Activated Cell Sorter (FACS) as it allowed more rapid freezing and overcame the adverse effect of FACS on morphology and behaviour of isolated PGCs, as well. RNA from wild type germ cells, Dead end knockdown germ cells and somatic cells were isolated separately, amplified, and hybridized to Affymetrix microarray chips as described in the Material and Methods section (chapters 6.11.1 – 6.11.5).

In a first round three criteria were chosen to define which candidate genes would be preselected for further investigation. The restriction parameters were defined such that known PGC markers fulfill the criteria.

1. The $\log_2$ signal in wildtype PGCs must be ≥ 8.
2. The difference of the $\log_2$ signal of wildtype PGCs to dead end knockdown PGCs is ≥ 2.
3. The difference of the $\log_2$ signal of wildtype PGCs to somatic cells is $\geq 2{,}6$.

192 genes fulfilled the selection criteria including five known PGC markers (*h1m* (Müller et al., 2002), *dazl* (Maegawa et al., 1999), *vasa* (Olsen et al., 1997; Yoon et al., 1997), *askopos* (Blaser et al., 2005) and *cxcr4b* (Doitsidou et al., 2002; Knaut et al., 2003)).

Additionally, in a second round, the data set of the dead end knock down embryos was excluded from the selection and candidate genes needed to fulfill only following two criteria to be selected, in order to increase the number of candidate genes.

1. The $\log_2$ signal in wildtype PGCs must be $\geq 6{,}5$.

2. The difference of the $\log_2$ signal of wildtype PGCs to somatic cells is $\geq$ 2,5.

Excluding the genes from the first round, 56 genes met the criteria, including one known PGC marker (*carbonic anhydrase IV*, unpublished data).

The list of all candidate genes, including gene information and the values for each selection criteria are supplied as Supplemental Material at the end of the thesis (Table 7-1).

3.2 Verification of increased expression in PGCs by *In Situ* hybridization

Based on the accession number provided by the Affymetrix annotation data, primers were designed to amplify the corresponding cDNA fragment from a cDNA mix (ovary + mid somite stage). As the reverse primers additionally contained the reverse strand sequence of the T3-Promoter, DIG-labeled antisense riboprobes were directly synthesized from the purified PCR products (see Material and Methods chapters 6.11.6 - 6.11.8, 6.11.10, 6.11.11, 6.11.23 and 6.12.4 for detail).

The DIG-labeled antisense RNA from each candidate gene was used in a subsequent *in situ* hybridization screen on embryos from 4 cell stage to 24 hpf. Their expression pattern were analyzed and validated for an increased expression in germ cells.

From 242 candidate genes (6 known markers already excluded) of the microarray screen, many genes showed a variety of different expression patterns (examples are shown in the Supplemental Material Fig. 7-1) and a distinct expression in the germ cells could be verified for 20 of them (Table 7-1; Fig. 7-2, 7-3 and 7-4 in the Supplemental Material section).

Table 3-1. Genes with newly identified distinct expression in PGCs during at least one of the tested stages in the first 24 h of zebrafish development.

Number	Gene	Homology to	Possible function
4A	Programmed Cell death 4 (PDCD4)	-	Involved in translation
7A	Granulito	other hypothetical proteins	unknown
10A	Unknown	Contains part of the retinoblastoma domain	unknown
19A	Superoxide dismutase2	-	Inorganic ion transport and metabolism
52A	G kinase anchoring protein1	-	Cyclic GMP signaling
58A	GTP binding protein 1	-	GTPase

86A	Longer transcript of granulito (3'UTR)	-	unknown
89A	Oogenesis-related protein	other hypothetical proteins	unknown
114A	DEAD box polypeptide 52 (ddx52)	ATP-dependent RNA helicase ROK1	RNA helicase
115A	Chromosome 6 open reading frame 194-like	NF-kappa B activating protein	
116A	HDCMA18P	HDCMA18 (human) Poly-sex comb (D.m.)	RNA processing
135A	Microfibrillar-associated protein 1	-	Component of extracellular matrix
151A	Hypothetical protein	Tumor suppressing transferable fragment 4	unknown
172A	Hypothetical protein	Daxx protein	Role in apoptosis
177A	microtubule-associated protein	-	Involved in cell cycle control
179A	unknown	unknown	unknown
183A	TATA-box binding protein.associated factor 5 (TAF5)	-	Transcription initiaton
186A	Hypothetical protein	TFIIH	Transcription factor
187A	RAD51	RecA	DNA repair/recombination
197A	Tdrd7	Tudor domains	Role in germ cell specification (drosophila)

3.3 Knock down experiments and subcellular localization of candidate genes

Based on the *in situ* expression and potential functional relevance we focused on 9 of these genes for characterisation of the phenotypic consequences in knock down studies and identification of the subcellular localization of the respective protein.

The phenotypic consequences of reducing the level of the identified proteins were studied following injection of morpholino antisense oligonucleotides (Table 3-2). Morpholinos were coinjected with *gfp-nos1*-3'UTR to follow PGC development. Embryos of 24 hpf and 48 hpf age were examined for proper migration and proliferation of PGCs. Subcellular localization was studied by PGC-specific expression of fusions of the respective protein with Yellow Florescent Protein (YFP) in order to determine localization and possible sites of cellular function (Table 3-2 and Fig. 3-1).

Table 3-2. Morpholino and YFP-fusion experiments on the 9 selected candidate genes

Nr.	Gene/possible	Morphant phenotype	Protein localization
4A	PDCD4	No phenotype observed	nucleus/cytoplasm
7A	Granulito (new)	Strong somatic defects, PGCs seem normal	germinal granules
10A	Unkown	No phenotype observed	nucleus
52A	GKAP1	No phenotype observed	cytoplasmic
115A	Transcription regulation	At higher conc. Mild somatic, PGC seem normal	Dots within nucleus
116A	RNA processing	No phenotype observed	nucleus/germinal granules
151A	Tumor surpression	Severe somatic defect	nucleus
187A	RAD51	No phenotype observed	cytoplasmic
197A	TDRD7	Somatic defects at higher conc. PGCs are normal in respect to migration and number	germinal granules/cytoplasmic

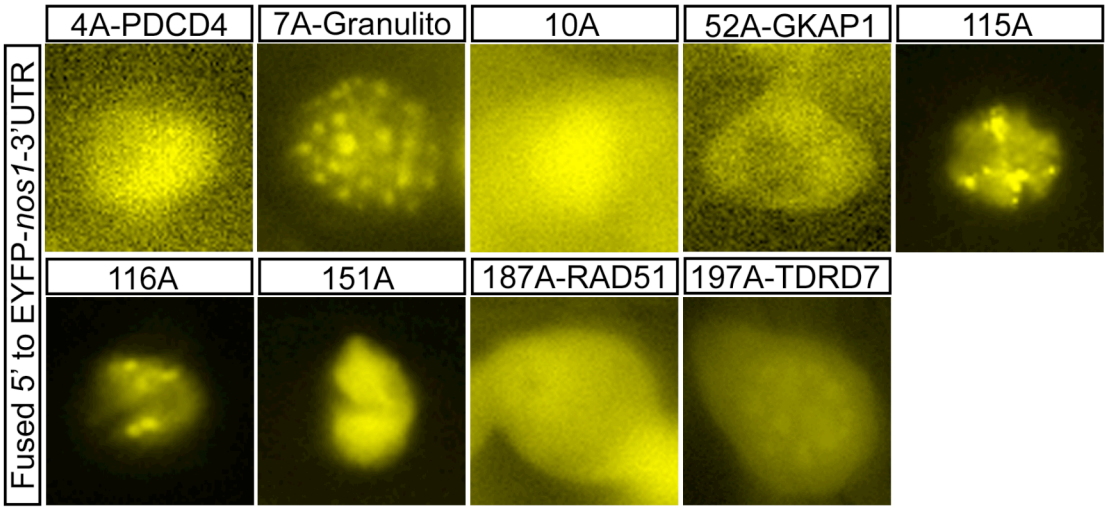


Fig. 3-1. Subcellular protein localization of the 9 selected candidate genes. High magnification views of PGCs expressing candidate gene fusions. The coding sequence of each candidate gene was fused upstream of EYFP and the 3'UTR of *nanos1*. Images were captured between 14 hpf and 20 hpf.

The initial knock down analysis using morpholino antisense oligonucleotides showed no phenotype in PGC migration or proliferation for any of the selected genes. The morpholinos were checked for their ability to sequester the fluorescent signal by coinjection with mRNA coding for the corresponding YFP fusion protein in order to rule out lack of efficiency in blocking translation by the designed morpholino. All morpholinos significantly reduced the expression of the target construct while translation of the control mRNA (*CFP-nos1*-3'UTR) was unaffected (data not shown).

Regardless of the apparent lack of PGC phenotype I decided to further characterize the two genes which have the most specific expression pattern to PGCs and localize to the germinal granules: 197A (*Tdrd7*) and the novel gene 7A (named *granulito*, a word construct from granules, the protein's localization, and –ito, a Spanish form for defining an object as small, referring to its size).

3.4 *granulito* mRNA is a germ plasm component and is localized in PGCs until 24 hpf

granulito (*gra*) was one of the first genes (7A) to be identified in the Affymetrix Microarray screen, as it showed a high expression level in wildtype PGCs (log_2=10,45) and a strong reduction in dead end knock down PGCs (log_2=3,52). The subsequent whole mount *in situ* hybridization screen revealed that *granulito* mRNA is maternally provided and a component of the germ plasm, as it localizes to the cleavage furrows at the 4-cell stage embryo (Fig. 3-2). As development proceeds and PGCs become specified, *granulito* mRNA is expressed in PGCs until the end of the first day of development (Fig. 3-2 and data not shown). This expression pattern is similar to that of previously described germ cell markers such as *dead end* and *nanos1* (Köprunner et al., 2001; Weidinger et al., 2003).

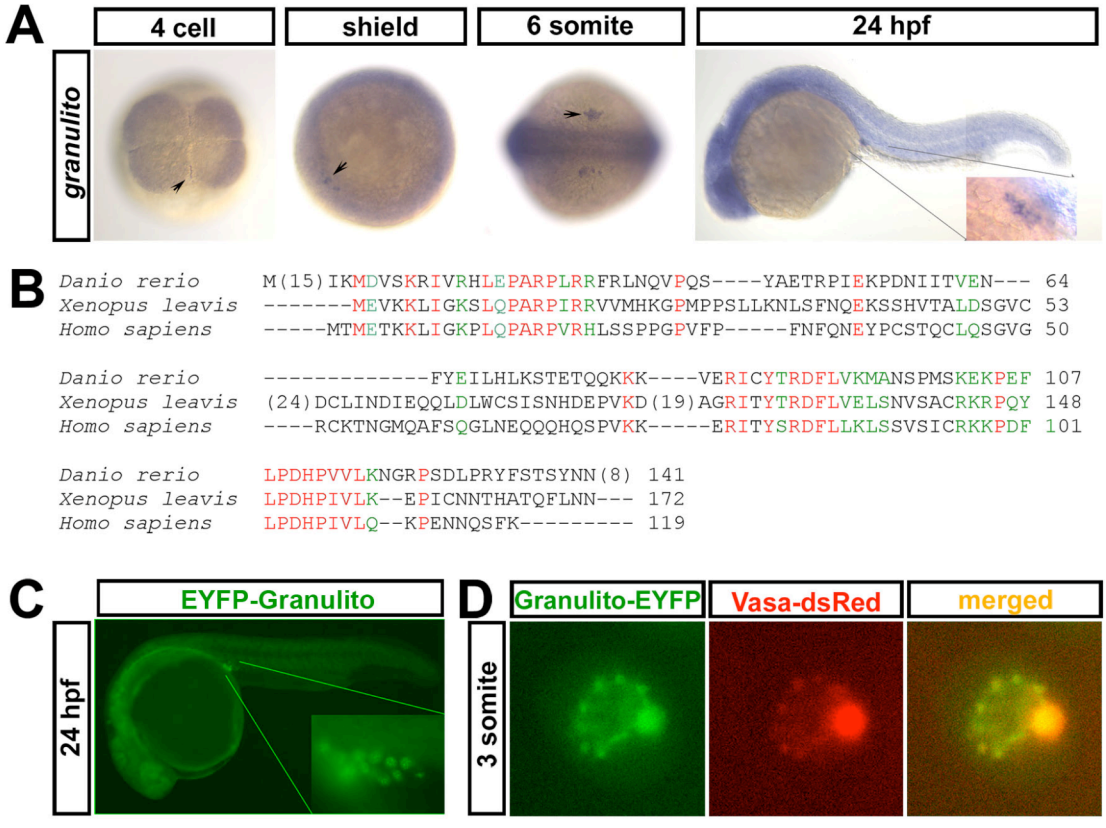
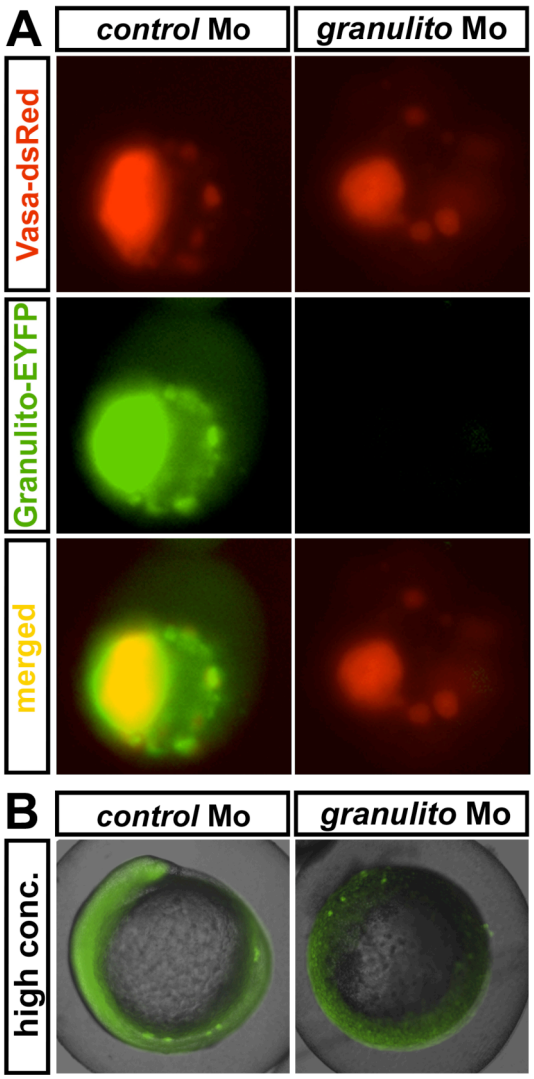


Fig. 3-2. Expression pattern of *granulito* RNA and subcellular localization of the Ganulito protein. A) Low magnification views of whole-mount *in situ* hybridizations using *granulito* antisense RNA probe at the indicated stages. *granulito* is enriched at the region where the germ plasm resides (cleavage furrows, arrowheads) and is expressed in the primordial germ cells at later stages (arrowheads). B) Alignment of the zebrafish Granulito protein with those from *Xenopus leavis* and *Homo sapiens*. Red signifies conservation in all 3 species, green labels conserved substitutions. C) Low magnification view of en embryo injected with *EYFP-granulito-gra*-3'UTR. This labels the germinal granules in PGCs (see insert). D) High magnification view of PGCs expressing Granulito-EYFP and Vasa-DsRed. Granulito localizes specifically to germinal granules as it colocalizes with Vasa protein.

3.5 Granulito is a novel protein conserved in vertebrates and localizes specifically to germinal granules

granulito mRNA encodes a 141 amino acid protein with significant homologs only in the vertebrata (Fig. 3-2B). Granulito-EYFP fusion protein is localized to germinal granules in PGCs, the structure in which the Vasa protein is found (Fig. 3-2D). This localization was identical for both types of EYFP fusions (n-terminal or c-terminal) and whether the construct contained the *nanos1*-3'UTR or the *granulito* 3'UTR (Fig. 3-2C,D and data not shown).



To determine whether Granulito plays a role in zebrafish PGC development its function was knocked down using antisense morpholino oligonucleotides. While injection of antisense oligonucleotides directed against the 5' sequence of *granulito* efficiently blocked translation of *granulito-eyfp-nos1*-3'UTR (Fig. 3-3A), germ cell development proceeded normally for at least 24 hours post fertilization (hpf). When high amount of antisense oligonucleotides was injected, embryonic development was arrested during gastrulation, while germ cell development appeared unaffected (Fig. 3-3B).

Fig. 3-3. *granulito* morpholino inhibits translation of *granulito-yfp* mRNA and leads at high concentration to developmental arrest. A) High magnification views of PGCs specifically expressing Vasa-dsRed and Granulito-YFP treated with control morpholino (left panel) or granulito morpholino (right panel). While in control both Vasa-dsRed and Granulito-YFP are visible, only Vasa-dsRed can be detected in *granulito* morphants. This demonstrates that the translation of *granulito* mRNA is efficiently and specifically blocked by the morpholino. B) Low magnification view of embryos coinjected with *mGFP-nanos1*-3'UTR to label the germ cells and a high concentration (300 μM) of control or granulito morpholino. Injection of granulito morpholino leads to developmental arrest of the embryo during gastrulation, while embryos injected with the same concentration of control morpholino develop normally. Despite the severe somatic defects in the Ganulito morphants, PGCs still specified (green glowing cells) and are migratory (data not shown). Embryos were coinjected with *mGFP-nanos1*-3'UTR to label the germ cells. Pictures desplay an overlay of bright field and fluorescent image.

Therefore, if this protein plays a role in early PGC development, its function is either redundant with that of another gene product, or maternally provided Granulito protein (which is not affected by the antisense oligonucleotides treatment) is sufficient for performing the early role of the protein. Despite the apparent lack of function for the protein in our experimental setting, the localization of Granulito to the perinuclear granules could serve as a tool to investigate these structures during early stages of PGC development.

3.6 Dynamic variation of germinal-granule size during early embryonic development

Germinal granules in live zebrafish embryos were previously visualized by injection of the GFP fusion constructs *nanos1-gfp-nos3'UTR*, *deadend-gfp-nos3'UTR* and *vasa-gfp-nos3'UTR* (Köprunner et al., 2001; Weidinger et al., 2003; Wolke et al., 2002). However, mRNA injection does not allow adequate visualization of granules before 6 hpf due to high fluorescent background in somatic cells and poor specific signal in primordial germ cells. Additionally, Nanos1-GFP and Dead end-GFP localization appears to be diffused and not unique to granules. In order to visualize the granules at an earlier developmental stage, I generated a transgenic fish expressing *granulito-dsRedEx-nos1*-3'UTR under the control of the *askopos* promoter (Blaser et al., 2005) (Fig. 3-4A). In progeny of transgenic females expressing *egfp-nos1*-3'UTR fusion under the control of the maternal promotor of *askopos*, germ cells could be visualized as early as 3 hpf. The localization and specific translation of *granulito-*

dsRedEx-nos1-3'UTR in PGCs allowed me to observe germinal granules beginning at 4 hpf in living embryos (Fig. 3-4B). At this stage and until 6 hpf, in most germ cells a single to few large granules were observed along with several smaller ones (Fig. 3-4B,C). As development proceeds, average size and size variation of the granules decreased (Fig. 3-4B,C) until at 24 hpf, at the end of PGC migration, PGCs show a uniform distribution of granules around the nucleus with similar small sizes. This strong reduction in granule size variation within the first 24 hours of development is presented in Fig. 3-4C,D.

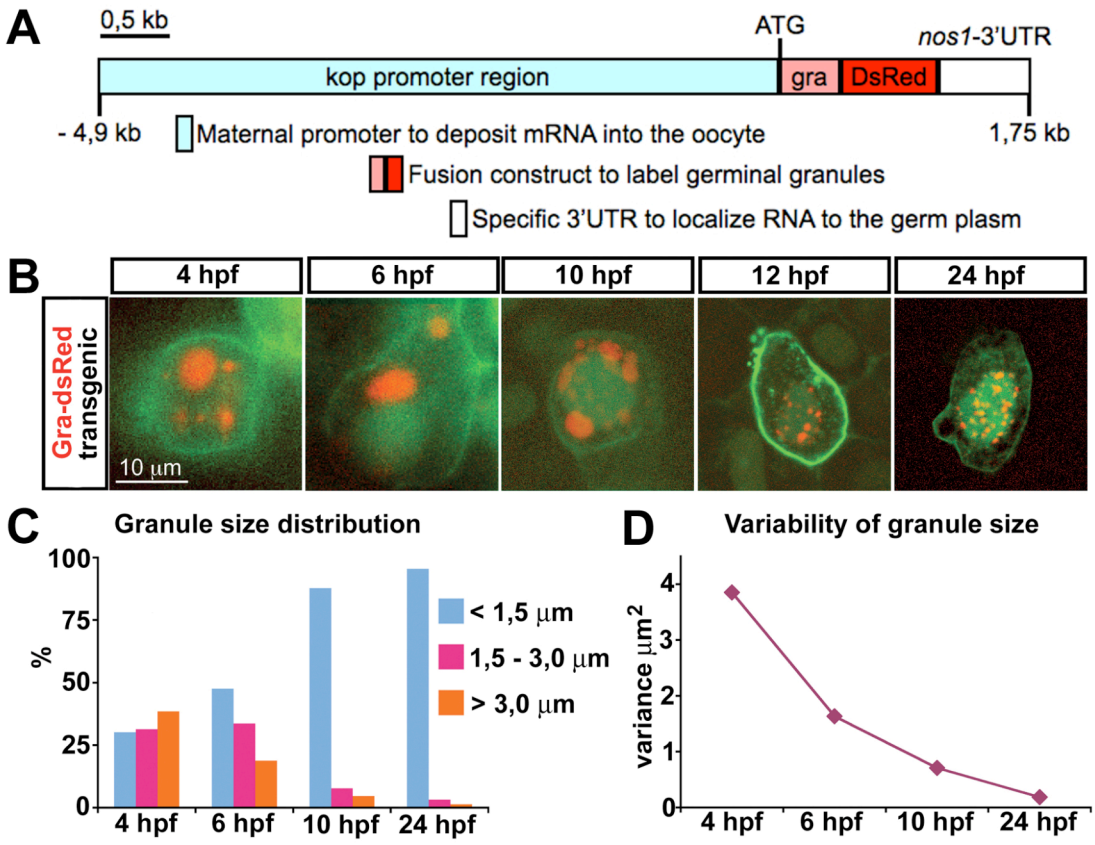


Fig. 3-4. Transgenic line expressing *granulito-DsRed-nos1*-3'UTR maternally uncovers a dynamic process for the establishment of perinuclear granules. A) Schematic representation of the transgenic construct. B) Epifluorescence images of PGCs of *kop-granulito-dsRedex-nos1*-3'UTR transgenic fish whose membrane and nucleus are labeled in green (except for the 4 hours stage in which the nucleus is not labeled). Initially, germinal granules with a large variety of sizes are observed. As development proceeds, the variation in germinal granule size decreases. C) Granule size distribution at different stages. D) Variance of granule size throughout the first 24 hours of development. n >100 granules for each time point.

3.7 *Tdrd7* mRNA is a germ plasm component and is expressed in PGCs until at least 7dpf

Another gene identified in the Affymetrix microarray screen with germinal granule localization is the Tudor-repeat-containing gene 7 (*Tdrd7*). *Tdrd7* was identified in the screen as number 197A due to its high relative expression level in wildtype PGCs (log_2=11,69) as compared to somatic cells (log_2=3,78). In contrast to *granulito*, *Tdrd7* is not reduced in dead end knock down PGCs (log_2=11,22). *Tdrd7* belongs to a family of Tudor domain containing genes, of which several are described to be expressed in vertebrate germ cells (Chuma et al., 2006; Hirose et al., 2000; Hosokawa et al., 2007; Pan et al., 2005; Smith et al., 2004). In *Drosophila,* Tudor function is important for germ cell specification and for the structural integrity of polar granules (Boswell and Mahowald, 1985,Arkov, 2006 #3035).

Zebrafish Tdrd7 is 1079 amino acids long and contains 3 tudor domains located in the C-terminus half of the protein (Fig. 3-5A). Interestingly, the Drosophila Oskar protein exhibits sequence homology with an 80 amino acid sequence stretch at the N-terminus of Tdrd7. Oskar is required for the pole plasm assembly and localization by recruiting other germline determinants in Drosophila oocytes (Ephrussi and Lehmann, 1992). In connection with the importance of Drosophila Tudor for germ cell specification and structural integrity of polar granules this raises the possibility that Tdrd7 might serve a similar function. The transcript of *Tdrd7* is localized to the germ plasm and is expressed in PGCs for at least the first 7 days of development (Fig. 3-5B). This expression pattern is similar to that of the previously described germ cell marker *vasa* (Yoon et al., 1997).

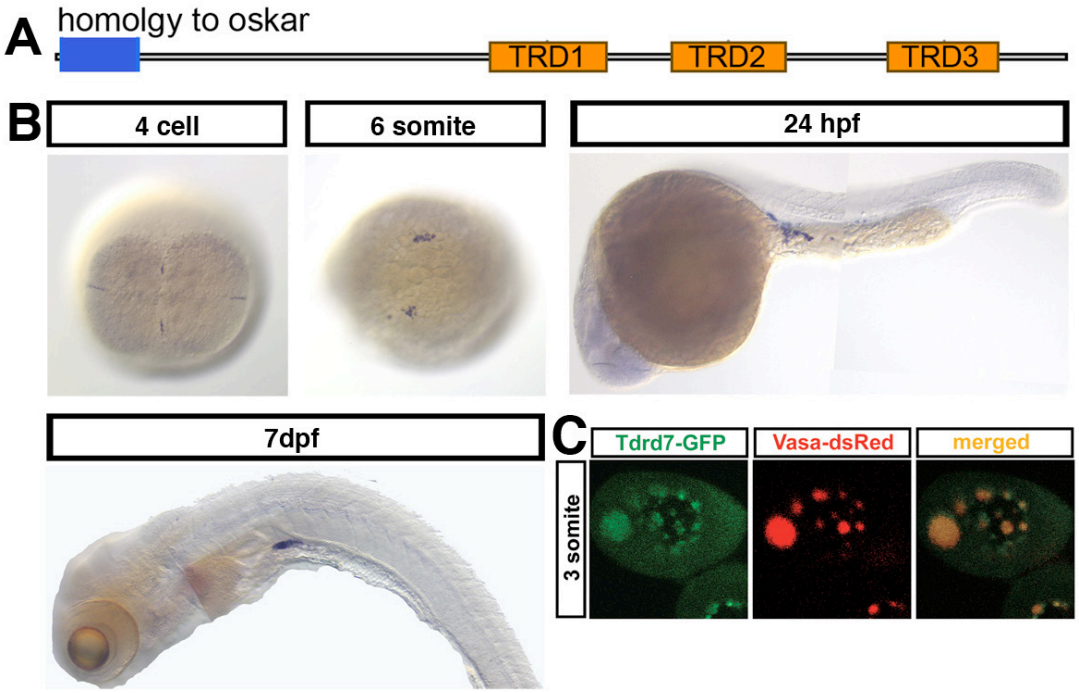


Fig. 3-5. Expression pattern of *Tdrd7* mRNA and subcellular localization of the Tdrd7 protein. A) Protein domain structure of Tdrd7. The blue box shows homogy to *Drosophila* Oskar, the 3 orange boxes depict the Tudor domains. B) Whole-mount *in situ* hybridizations using *Tdrd7* antisense RNA probe at the indicated stages. *Tdrd7* is specifically localized at the region where the germ plasm resides (cleavage furrows) and is expressed in the primordial germ cells at later stages. C) High magnification images of PGCs expressing Tdrd7-GFP and Vasa-dsRed. Tdrd7 localizes specifically to germinal granules as it colocalizes with Vasa protein.

3.8 Tdrd7 protein is enriched in the germinal granules

To examine Tdrd7 function, I initially determined the subcellular localization of the protein by expressing a Tdrd7-GFP fusion protein in germ cells and found it to be localized to germinal granules. Interestingly, whereas Vasa-dsRed was equally detected in granules of all sizes, Tdrd7-GFP was found preferentially in granules of smaller size (Fig. 3-5C and data not shown).

3.9 The potency of the 3'UTR of Tdrd7 and granulito to direct protein expression preferentially into PGCs.

Based on the specific expression localization of *Tdrd7* and *granulito* mRNA to the germ plasm as wells as PGCs, I asked whether the 3'UTRs of these genes would be sufficient to achieve germ cell specific expression, similar to the 3'UTR of *nanos1* (Köprunner et al., 2001). Indeed, both 3'UTRs of *Tdrd7* and *granulito* are principally able to direct GFP expression to PGCs when the RNA is injected into the one-cell

stage embryo, albeit with strong somatic background when using the *granulito* 3'UTR (Fig. 3-6).

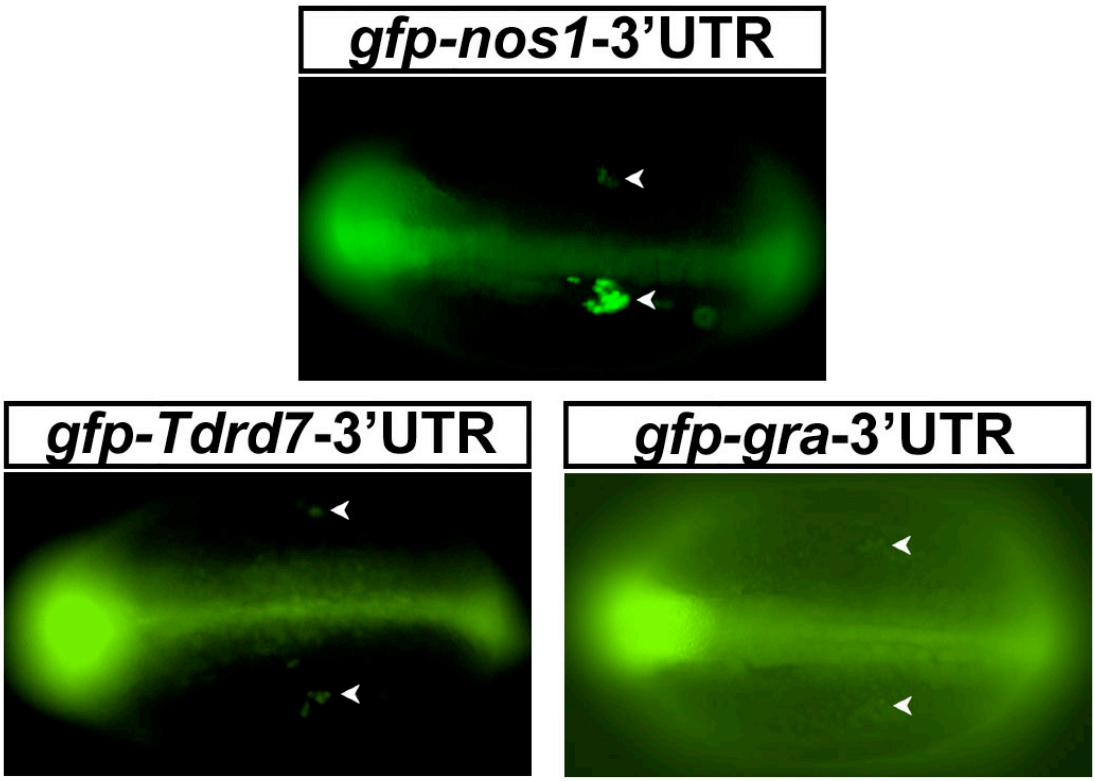


Fig. 3-6. Potency of the 3'UTR of *Tdrd7* and *granulito* to facilitate protein expression into PGCs. Low magnification views of 12 hpf embryos injected with mRNA coding for GFP expression under the control of the 3'UTRs of *nanos1*, *Tdrd7* and *granulito.* Arrowheads depict PGC clusters.

3.10 PGC formation and migration is not affected in Tdrd7 morphants

To determine whether Tdrd7 participates in PGC development in zebrafish, embryos were treated with morpholino antisense oligonucleotides to inhibit *Tdrd7* translation. No adverse effect on PGC specification, division or migration upon inhibition of *Tdrd7* translation was observed, as judged by the number, position, and expression of PGCs markers (*vasa*, *nanos*, *dead end*, *h1m*) during the first day of development (Fig. 3-7). Additionally, the expression of the differentiation marker *ziwi* (Houwing et al., 2007; Tan et al., 2002) at 60 hpf indicates normal PGC fate maintenance in Tdrd7 knocked-down embryos (Fig. 3-7).

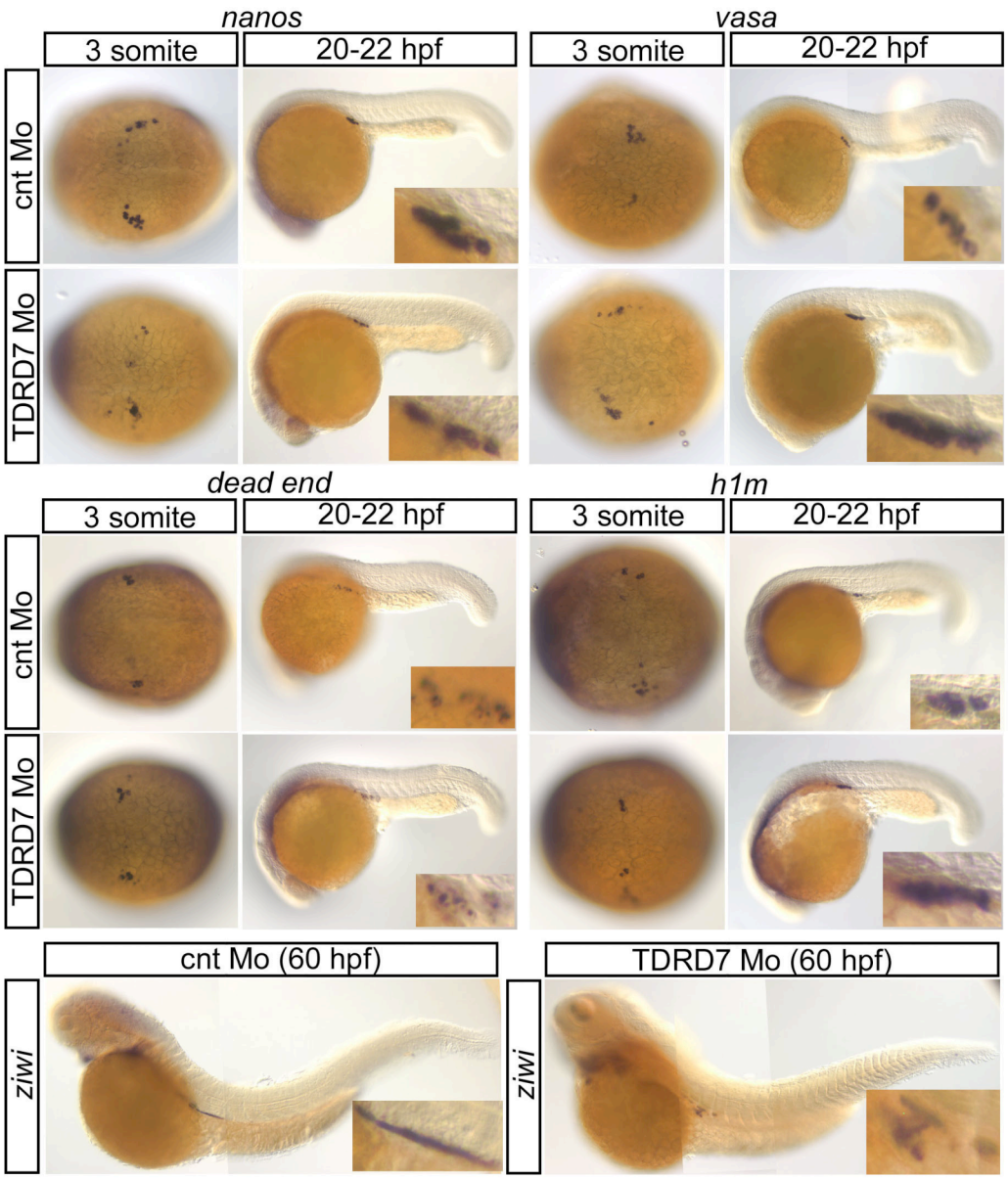


Fig. 3-7. Expression of germ cell markers in PGCs and their migration proceeds normally in Tdrd7 morphants. Low magnification images of whole-mount *in situ* hybridizations on control morpholino and Tdrd7 morpholino-treated embryos. The early PGC markers *nanos1*, *vasa*, *dead end* and *h1m* and the PGC differentiation marker *ziwi* are expressed normally in PGCs of Tdrd7 morphants during the first 3 days of development.

3.11 Tdrd7 plays a crucial role for structural integrity of granules in PGCs

Despite the normal migration and differentiation of PGCs in Tdrd7 knocked-down embryos a prominent phenotype of abnormal germinal granule morphology was observed in treated embryos at 24 hpf (Fig. 3-8A middle panel). In these embryos, large granules were found that resembled those observed in early developmental stages in wild-type embryos (Fig. 3-4B). In PGCs lacking Tdrd7, the average size of the largest granule in a cell is significantly increased (from 1,9 µm to 3,5 µm, Fig. 3-

8B,D). In addition, we observed an increase in the number of very small granules (Fig. 3-8D), which is reflected by an alteration of the granule size distribution in favor to extreme sizes (Fig. 3-8D). This phenotype was readily reverted by co-injection of an antisense resistant mRNA encoding Tdrd7, demonstrating the specificity of the antisense oligonucleotide-induced phenotype (Fig. 3-8A right panel). To verify whether the obtained phenotype in Tdrd7 knocked-down embryos is specific to Vasa protein localization or indeed represents an alteration of the germinal granules, I confirmed the granule phenotype using Granulito as a marker for germinal granules (Fig.3-9A,B). Thus, Tdrd7 facilitates the development of homogenous medium size granules over time and is thus important for normal number of granules in the germ cells (Fig. 3-8C).

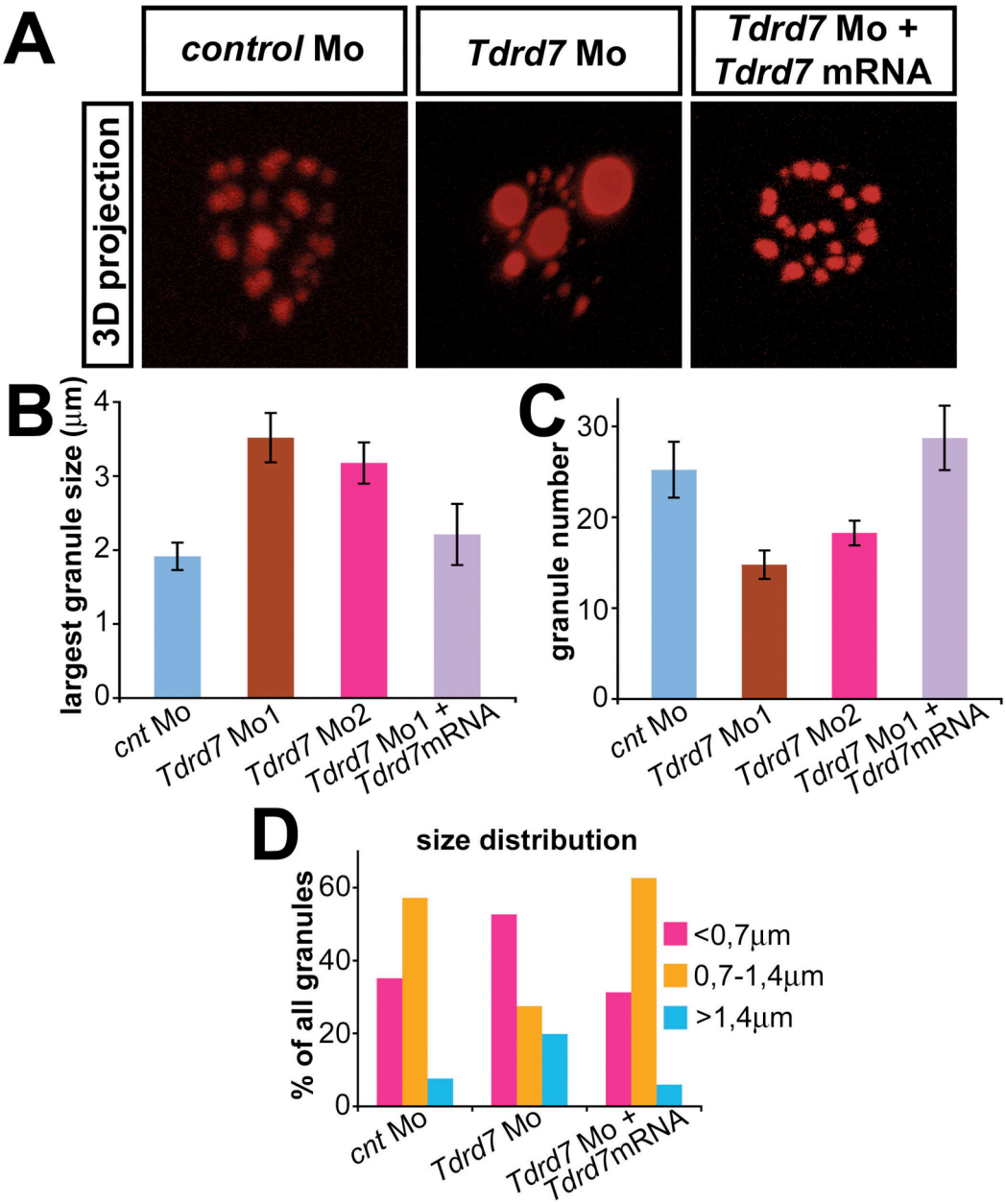


Fig. 3-8. Tdrd7 function is required for proper germinal granule architecture. A) High magnification 3D projections of PGCs stained for Vasa protein in 24 hpf embryos treated with control morpholino (left panel), Tdrd7 morpholino (middle panel), and in embryos co-injected with Tdrd7 morpholino and morpholino-resistant *Tdrd7-nanos1*-3'UTR mRNA (right panel). B) Size of the largest granule in PGCs of 24 hpf embryos treated with control morpholino, Tdrd7 morpholinos and after rescue of Tdrd7 knockdown by co-injection of morpholino-resistant *Tdrd7-nanos1*-3'UTR mRNA. n=18 cells for control (cnt Mo), 16 cells for morpholino 1 (Tdrd7 Mo1, t-test compared with the control, $p<0.001$), 19 cells for morpholino 2 (Tdrd7Mo2), and 16 cells in rescued embryos. C) Number of granules in PGCs of 24 hpf embryos treated with control morpholino, Tdrd7 morpholinos and after rescue of Tdrd7 knockdown by co-injection of morpholino-resistant *Tdrd7-nanos1*-3'UTR mRNA. n=13 cells for control (cnt Mo), 19 cells for morpholino 1 (Tdrd7 Mo1, t-test compared with the control, $p<0.01$), 26 cells for morpholino 2 (Tdrd7 Mo2), and 13 cells in rescued embryos. D) Distribution of germinal granule sizes in embryos injected with control morpholino, Tdrd7 morpholino and after rescue of Tdrd7 knockdown by co-injection of morpholino-resistant *Tdrd7-nanos1*-3'UTR mRNA. n=223 granules for control morpholino (cnt Mo), 150 granules for morpholino 1 (Tdrd7 Mo1) and 132 granules in rescued embryos.

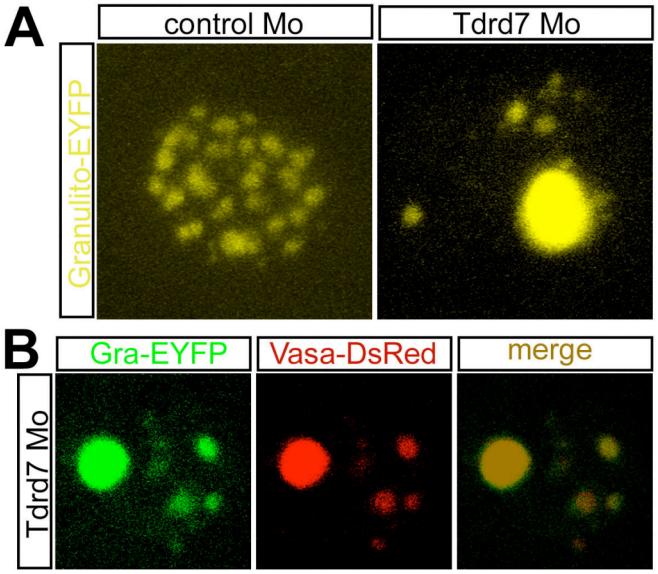


Fig. 3-9. Verification of the Tdrd7 knock down phenotype. A) High magnification 3D projection of granules labeled with Granulito-EYFP in 24 hours old control cells (left panel), in Tdrd7 depleted cells (right panel) verifies that Tdrd7 function is indeed required for the formation of uniform normal sized granules. B) Granulito and Vasa colocalize as well in the Tdrd7 morphant situation with each other.

3.12 Tdrd7 function is presumably independent on microtubule function

In our laboratory a colleague could show that the microtubule network and the associated machinery, in particular the motor protein dynein, participates in regulating granules distribution, size and number in migratory germ cells. To test whether this machinery is affected by Tdrd7 morpholino treatment I studied the localization of dynein and the microtubular network in PGCs. In both wildtype and Tdrd7-depleted PGCs, Dynein is localized to the nucleus and displays a dynamic localization in germinal granules with preference for large granules. (Fig.3-10A). Similarly, in both wild-type and Tdrd7 knocked-down embryos, granules seem to be associated to the

microtubule network which forms a cage around the nucleus of interphase PGCs and the spindle apparatus in mitotic cells (Fig. 3-10B). Tdrd7 knockdown apparently does not interfere with the functional interaction of granules with the microtubule network suggesting that the impact of Tdrd7 on germinal granule architecture is probably not dependent on the microtubular network.

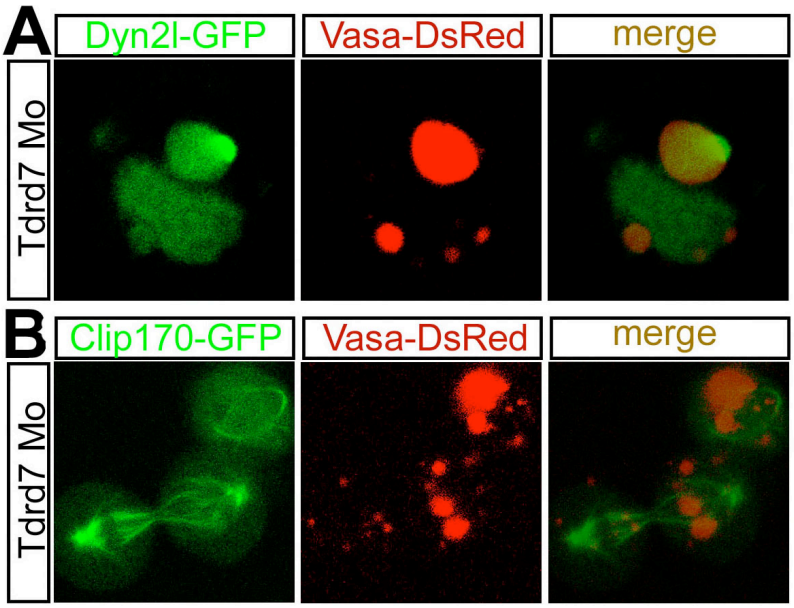


Fig. 3-10. Tdrd7 knockdown does not interfere with the association of granules to the microtubule network. A) High magnification images of PGCs coexpressing GFP-tagged dynein light chain2 and Vasa-DsRed in Tdrd7 morphants. The dynamic localization of Dynein in germinal granules is comparable to how it is known for wildtype PGCs. D) High magnification images of PGCs expressing Clip170-GFP as a marker for microtubular networks and Vasa-DsRed. In the upper right corner a interphase cell is shown, where the microtubles form a nuclear cage. In the lower half a dividing cell is depicted where the microtubules form mitotic spindles.

3.13 Identification of familiy member of Tudor domain containing genes in PGCs of zebrafish

As mentioned before, several other Tudor domain containing proteins are known to be expressed in vertebrata germ cells (*RNF17*, *Tdrd1*, *Tdrd5* and *Tdrd6* (Chuma et al., 2006; Hirose et al., 2000; Hosokawa et al., 2007; Pan et al., 2005; Smith et al., 2004)). Therefore, *Tdrd7* could be redundant to another yet-to-be-identified gene of the same family that would prevent proper analysis of Tdrd7 function in the context of germ cell specification and maintenance. To test this hypothesis I cloned the zebrafish homolgs of the genes mentioned above and studied their expression pattern within the first 7 days of development (Fig. 3-11) Indeed, following the second day of development, *Tdrd1* and *RNF17* are found to be specifically expressed in germ cells

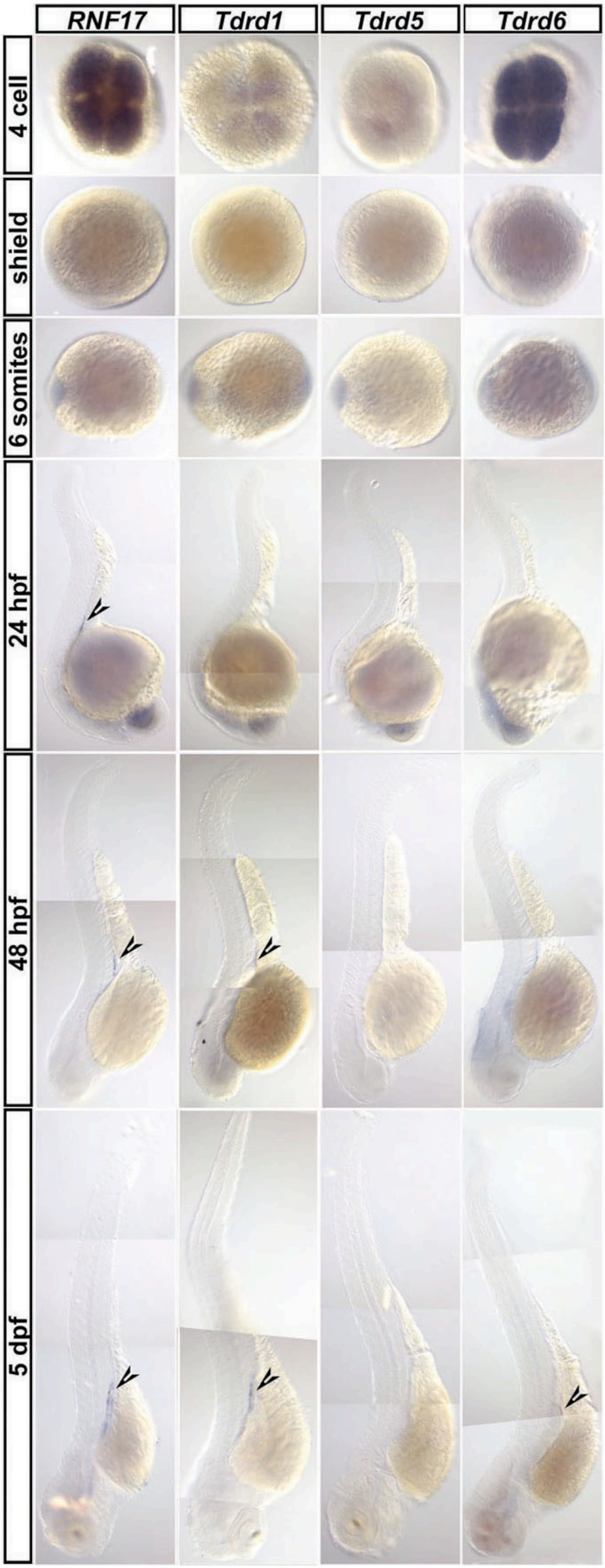


Fig. 3-11. Expression pattern of Tudor domain containing proteins in zebrafish embryos and larvas. Low magnification pictures of embryos from *in situ* hybridization for zebrafish homologs of previously described tudor domain containing genes *RNF17*, *Tdrd1*, *Tdrd5*, *Tdrd6* and *Tdrd7* at different stages starting from 4 cell stage until 5 dpf. For *RNF17* germ cell specific expression was observed starting from 24 hpf. For *Tdrd1* germ cell specific expression was observed starting from 48 hpf. For *Tdrd5* no expression could be observed in the analyzed stages. For *Tdrd6* faint staining at the region where germ cells reside is visible at 3 dpf and 5 dpf.

along with *Tdrd7,* suggesting a possible role for these genes in zebrafish germ cell development at later stages. Yet, *Tdrd7* has been the only identified tudor domain-containing gene to be expressed during early zebrafish germ cell development. Examination of the roles of the different tudor domain-containing genes at later stage of zebrafish development would require the availability of mutants as morpholino injection are only efficient within the first 2 dpf.

3.14 Dead end function is required for efficient translation of fusion proteins containining the 3'UTR of *nanos1* or *Tdrd7*

The 3'UTR of *nanos1* serves as a powerful tool to specifically express proteins in PGCs of zebrafish embryos. mRNA constructs carrying the *nanos1*-3'UTR are stabilized in PGCs but degraded in somatic tissues (Köprunner et al., 2001). How this cell specific regulation is achieved remained unknown. Interestingly, a recent publication demonstrated that the somatic repression of the PGC marker genes *nanos1* and *Tdrd7* was due to binding of the microRNA miR-430 to target sequences within the 3'UTR of these genes (Mishima et al., 2006). Importantly, the authors suggested a yet-to-be-identified mechanism that protects these genes specifically in germ cells from translational inhibition by the miRNA pathway.

One phenomenon I observed when manually isolating germ cells from dead end knocked-down embryos was that, besides the failure to maintain marker gene expression, the cells also showed a pronounced reduction of the GFP signal intensity of the injected *gfp-nanos1*-3'UTR mRNA as compared to control cells. Thus it seemed that Dead end knockdown had an effect on the translation and/or stability of mRNAs containing the *nanos1*-3'UTR. Based on these observations and the fact that Dead end contains two single stranded RNA recognition motifs (RRM), I asked in collaboration with the group of Dr. Reuven Agami (Netherlands Cancer Institute, Netherlands), whether Dead end might be a key player in the protection of mRNAs from translational inhibition by microRNAs. First, I verified the reduction of expression levels of germ cell-specific mRNAs in Dead end knocked-down embryos by quantifying the signal intensity obtained from a *DsRedex* fusion construct to either *nanos1*-3'UTR or *Tdrd7*-3'UTR in control or dead end knock down PGCs. A *gfp-vasa*-3'UTR construct was co-injected, which does not contain a miR-430 binding site and should thus not be affected by Dead end knockdown. Indeed, the signal intensity originating from the *gfp-vasa*-3'UTR construct was not reduced in *dnd* knocked–down embryos (Fig. 3-12), while the signal originating from *Dsred-nanos1*-3'UTR (fig. 3-12A) and from *Dsred-Tdrd7*-3'UTR (fig. 3-12B) was significantly reduced. Thus, Dead end facilitates the germ-cell specific expression of genes under the control of the *nanos1*- and *Tdrd7*-3'UTR.

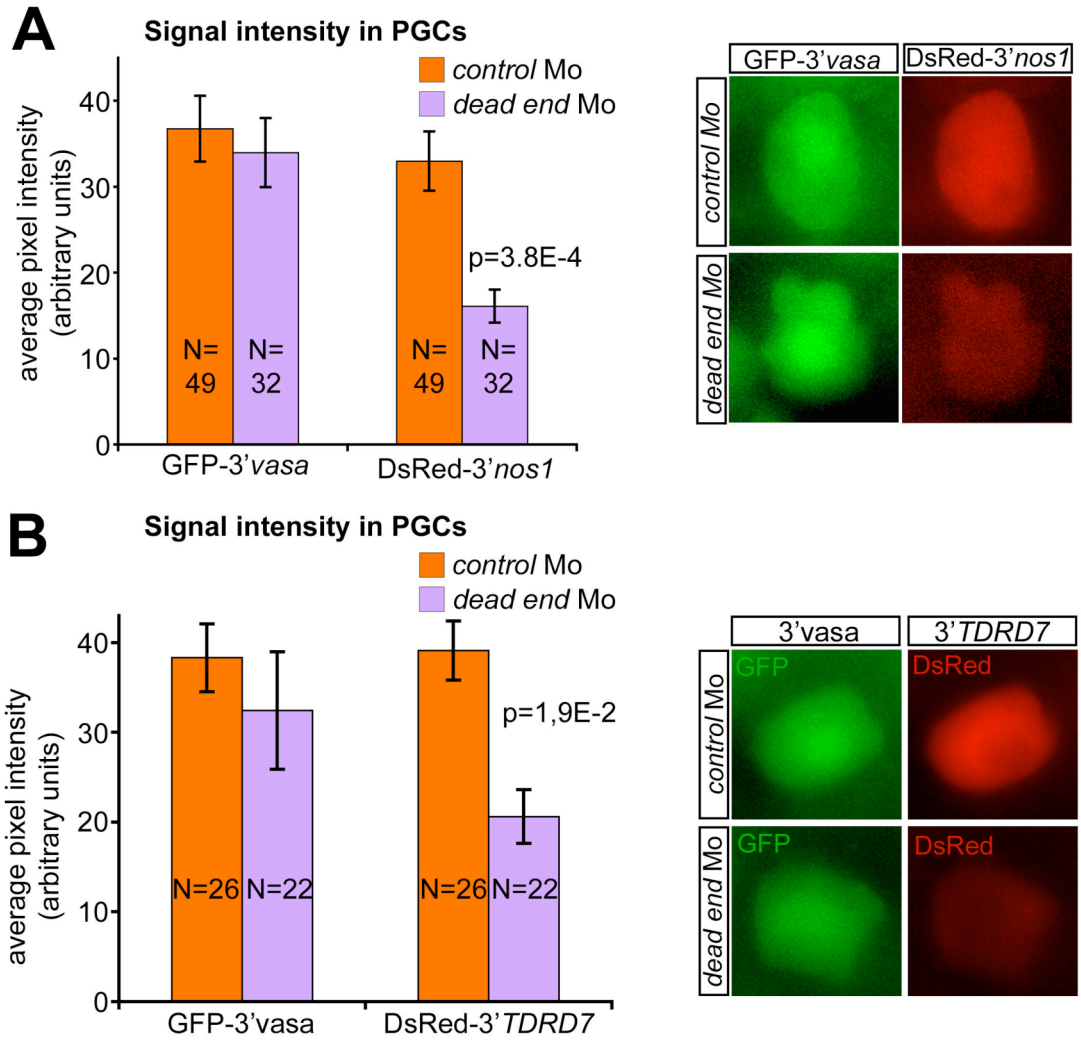


Fig. 3-12. Dead end is required for efficient translation of Nanos1 and Tdrd7. A) One-cell-stage zebrafish embryos were co-injected with *DsRed-nanos1*-3'UTR and *gfp-vasa*-3'UTR together with dead end morpholino or control morpholino. The signal intensity was determined and representative single cells are shown in the right panels. B) One-cell-stage zebrafish embryos were co-injected with *DsRed-Tdrd7*-3'UTR (3'*TDRD7*) and *gfp-vasa*-3'UTR (3'*vasa*) together with dead end morpholino or control morpholino. The signal intensity was determined and representative single cells are shown in the right panels. Error bars depict the standard error of the mean (SEM), p-value was calculated using t-test.

3.15 Mutation in the miR-430 binding site of *nanos1* and *Tdrd7* releases translational inhibition even in absence of Dead end

To test the hypothesis that Dead end acts through alleviation of translational repression by miR-430, I mutated the miR-430 binding site in the 3'UTR of *nanos1* and *Tdrd7*. If this hypothesis holds true, protein expression should be obtained from these mutated RNAs even in the absence of Dead end. Although the signal is reduced from constructs containing the mutated 3'UTRs in Dead end knock down germ cells

at 10 hpf as compared to wt, this reduction is significantly less as compared to wt 3'UTR (Fig. 3-13). These results indicate that the loss of signal intensity when using constructs containing the *nanos1*-3'UTR or *Tdrd7*-3'UTR in absence of Dead end depends on miR-430 function.

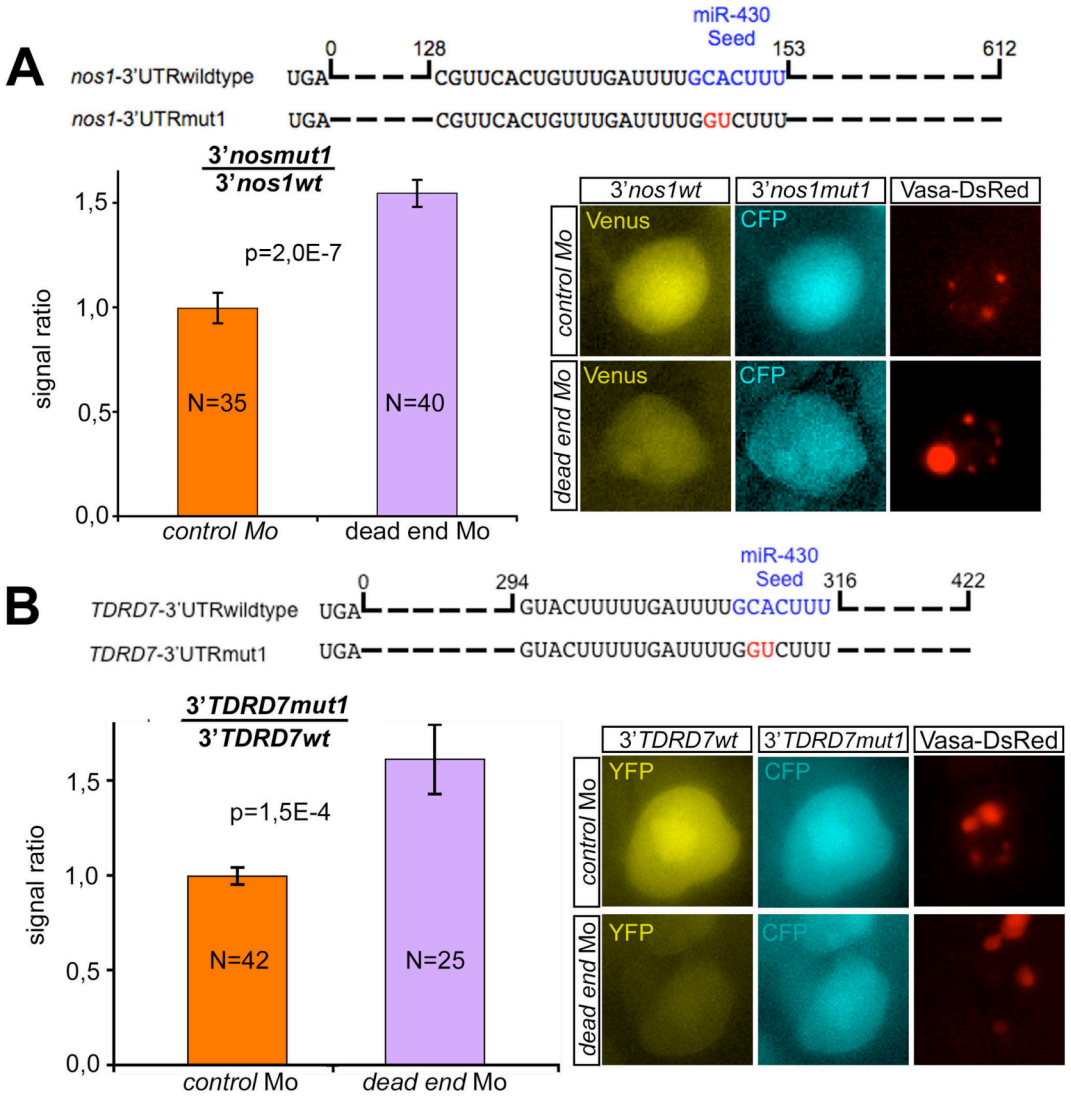


Fig. 3-13. Dead end counteracts inhibition of *nanos1* and *Tdrd7* by miR-430. A) One-cell-stage zebrafish embryos were co-injected with RNA containing the *venus* open reading frame fused to the wild-type *nanos1*-3'UTR (*3'nos1wt*), RNA containing the *cfp* open reading frame fused to the miR-430 binding site mutated *nanos1*-3'UTR (*cfp-3'nos1mut1*) and vasa-dsRed (for labeling the germinal granule for easier identification of germ cells) together with dead end morpholino or control morpholino. The ratio between the signal intensity provided by the fluorescent protein whose open reading frame was fused to the mutated *nanos1*-3'UTR was divided by that originating from a wild-type *nanos1*-3'UTR. Representative single cells are shown in the right panels. B) One-cell-stage zebrafish embryos were coinjected with RNA containing the *yfp* open reading frame fused to the wild-type *Tdrd7*-3'UTR (*3'TDRD7wt*), RNA containing the *cfp* open reading frame fused to the miR-430 binding site mutated *Tdrd7*-3'UTR (*cfp-3'TDRD7mut1*) and vasa-DsRed (for labelling the germinal

granule for easier identification of germ cells) together with dead end morpholino or control morpholino. The ratio between the signal intensity provided by the fluorescent protein whose open reading frame was fused to the mutated *Tdrd7*-3'UTR was divided by that originating from a wild-type *Tdrd7*-3'UTR. Representative single cells are shown in the right panels. Error bars depict the standard error of the mean (SEM), pvalue was calculated using t-test.

3.16 U-rich regions located closely to miRNA-target sequences mediate Dead end binding and function

To further investigate how Dead end interferes with the action of microRNA on specific targets, our collaborators blasted the Dead end RRMs for homologs. A BLASTP search of the mouse homolog of Dead end, DND1 revealed several significant homologues (E-values of 10^{-5} and 10^{-3}) and allowed homology-based modelling using the SWISS-MODEL server (Schwede et al., 2003). Superimposing the two homology-modeled domains in the structure of the similar *Drosophila* sex-lethal protein bound to uridine-rich single-stranded RNA, indicated that DND1 has all the hallmarks necessary to bind U-rich single stranded RNA (Handa et al., 1999). Therefore it was proposed that Dead end might also bind U-rich regions (URRs).

Interestingly, two URRs are found in close proximity to the miR-430 binding sites in the *nanos1*-3'UTR and one URR in close proximity to the miR-430 binding site in the *Tdrd7*-3'UTR. I therefore mutated each of these URRs and tested the effect on the derepression by Dead end. Mutating the URR downstream of miR-430 site or both URRs significantly hampered the Dnd effect on *nos1*-3'UTR (Fig. 3-14A) and similar results were obtained with the *Tdrd7*-3'UTR (Fig. 3-14B). Altogether, these results point to a model by which Dnd positively regulates gene expression by antagonizing miRNA-mediated translational inhibition through association with URRs adjacent to the miRNA recognition site.

To exclude the possibility that Dead end acts rather as a translational activator than as an inhibitor of miRNA function, I generated mutation constructs containing the *nanos1*-3'UTR bearing both mutations at the miR-430 binding site as well as at the URRs (Dead end interacting sequences). If Dead end were to act as a translational activator independent of miR, constructs carrying mutations in the Dnd interacting sequences should have a smaller increase in fluorescent signal as a mutation only in the miR-430 binding site. Figure 3-15 shows that the signal of constructs carrying mutations both at the miR-430 binding site as well as at the URRs is increased to the same level as a mutation only in the miR-430 binding site. This supports the

hypothesis that Dead end acts as an inhibitor of miRNAs rather than as an activator of translation.

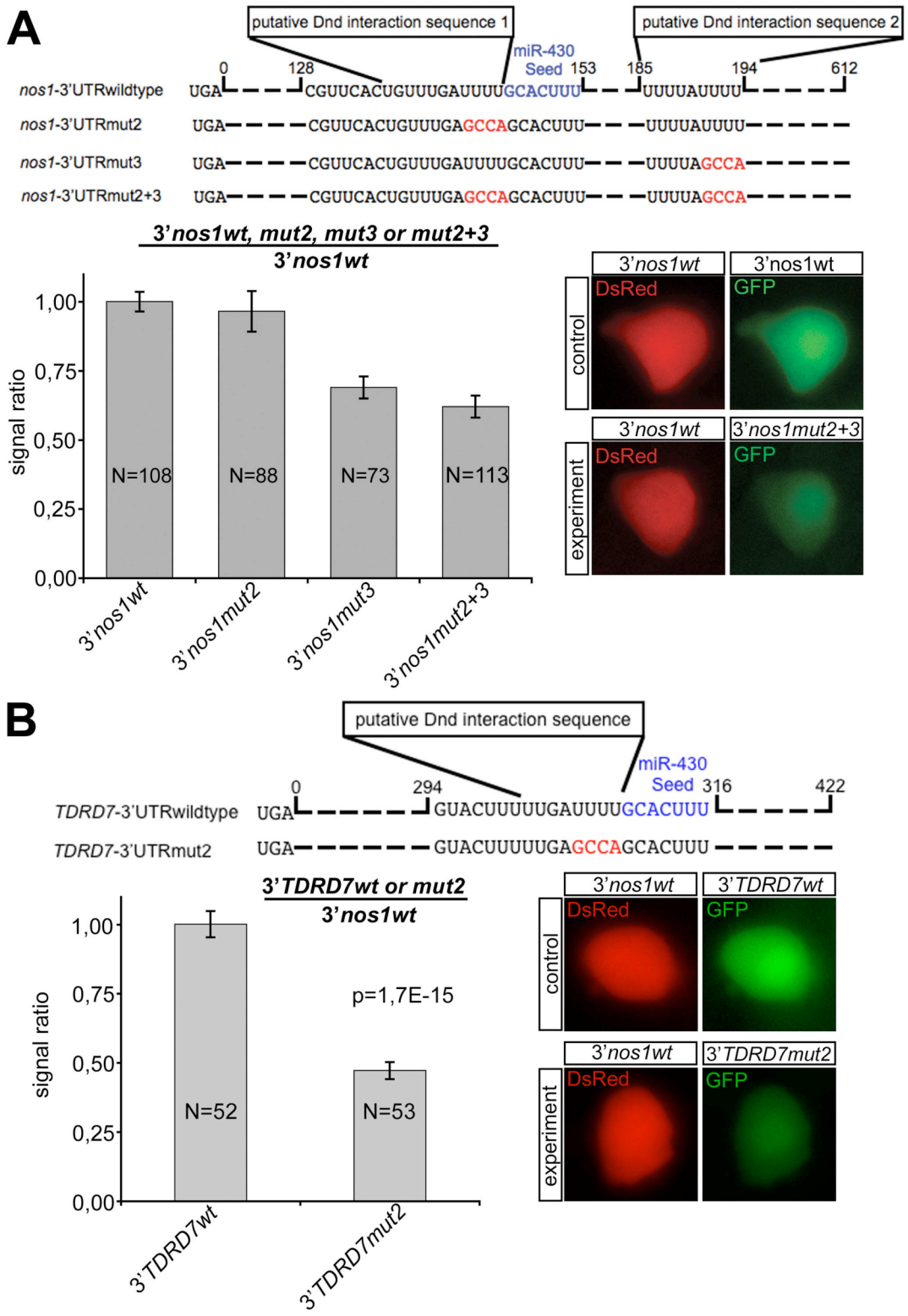


Fig. 3-14. URRs are required for Dnd to efficiently repress miR-430 mediated Nanos1 and Tdrd7 inhibition. A) RNA containing the *DsRed* open reading frame fused to the wild-type *nanos1*-3'UTR (*3'nos1wt*) was co-injected into one-cell-stage zebrafish embryos together with RNA containing the *gfp* open reading frame fused to different versions of the *nanos1*-3'UTR. The different *nanos1*-3'UTRs

used were: wild-type *nanos1*-3'UTR (*3'nos1wt*), *nanos1*-3'UTR where the putative Dnd interaction sequence 1 was mutated (*3'nos1mut2*), *nanos1*-3'UTR where the putative Dnd interaction sequence 2 was mutated (*3'nos1mut3*) or *nanos1*-3'UTR where the putative Dnd interaction sequences 1 and 2 were mutated (*3'nos1mut2+3*). The ratio between the signal intensity provided by the fluorescent protein whose open reading frame was fused to wild-type or either one of the mutated *nanos1*-3'UTRs was divided by that originating from a wild-type *nanos1*-3'UTR. B) RNA containing the *DsRed* open reading frame fused to the wild-type *nanos1*-3'UTR (*3'nos1wt*) was co-injected into one-cell-stage zebrafish embryos together with RNA containing the *gfp* open reading frame fused to different versions of the *Tdrd7*-1 3' UTR. The different *Tdrd7*-3'UTR used were: wild-type *Tdrd7*-3'UTR (*3'TDRD7wt*) or *Tdrd7*-3'UTR where the putative Dnd interaction sequence was mutated (*3'TDRD7mut2*). The ratio between the signal intensity provided by the fluorescent protein whose open reading frame was fused to wild-type or the mutated *Tdrd7*-3'UTR was divided by that originating from a wild-type *nanos1*-3'UTR. Error bars depict the standard error of the mean (SEM), p-value was calculated using t-test.

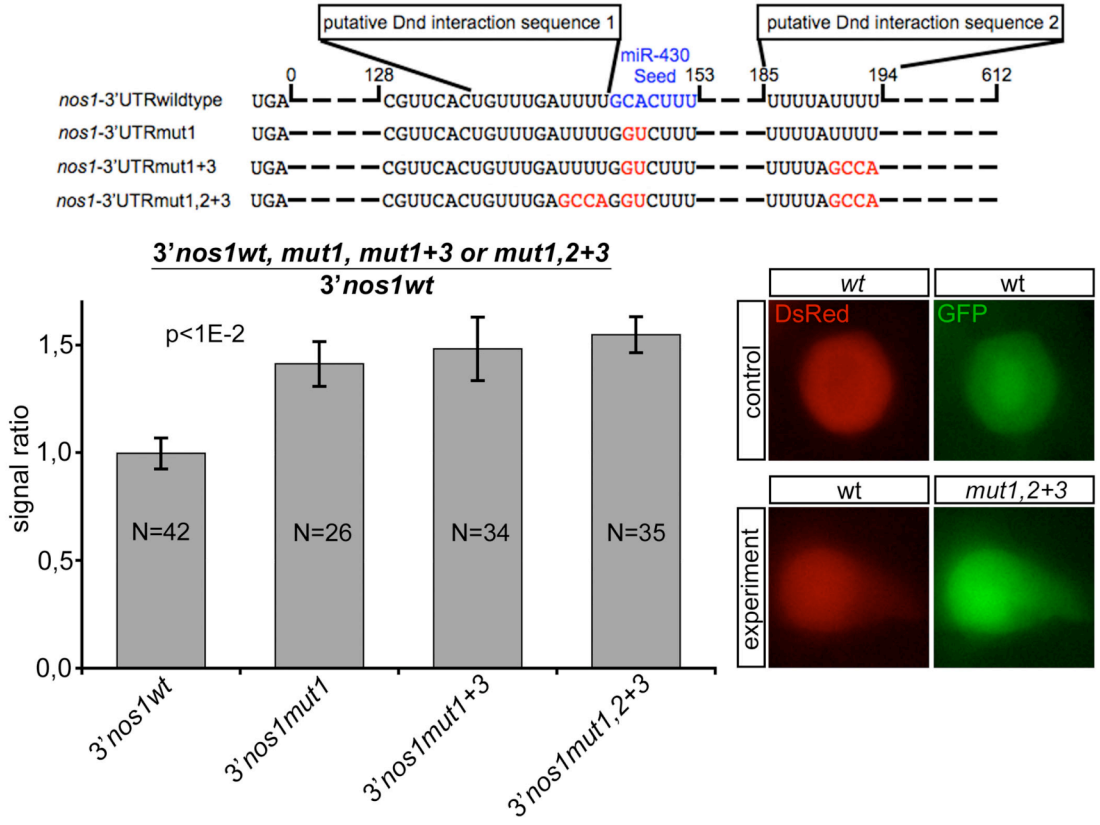


Fig. 3-15. Increase of signal intensity through Dnd depends of miRNA action inhibition rather than an independent role in translational activation. RNA containing the *DsRed* open reading frame fused to the wild-type *nanos1*-3'UTR (*3'nos1wt*) was co-injected into one-cell-stage zebrafish embryos together with RNA containing the *gfp* open reading frame fused to different versions of the *nanos1*-3'UTR. The different *nanos1*-3'UTRs used were: wild-type *nanos1*-3'UTR (*3'nos1wt*), *nanos1*-3'UTR where the miR-430 binding site was mutated (*3'nos1mut1*), *nanos1*-3'UTR where the miR-430 binding site and the putative Dnd interaction sequence 2 was mutated (*3'nos1mut1+3*) or *nanos1*-3'UTR where the miR-430 binding site and the putative Dnd interaction sequences 1 and 2

were mutated (*3'nos1mut1,2+3*). The ratio between the signal intensity provided by the fluorescent protein whose open reading frame was fused to either one of the mutated *nanos1*-3'UTRs was divided by that originating from a wild-type *nanos1*-3'UTR. Representative single cells are shown in the right panels. Error bars depict the standard error of the mean (SEM), p-value was calculated using t-test.

3.17 Inhibition of the five most abundant miR-430s rescues partially loss of signal in Dead end morphants but not the loss of germ cells

As presented above, the action of miR-430 repressing the translation of *nanos1* and *Tdrd7* can be blocked by Dead end, I therefore asked whether reducing the levels of available miR-430 would revert the phenotype in dead end morphants. For this purpose, five morpholino antisense oligonucleotides were designed to base pair with the most abundant miR-430s in zebrafish and were coinjected with control or dead end morpholino, respectively. At higher concentrations of the miR-430 morpholinos the embryos displayed a severe somatic defect, manifested in developmental delay, distorted gastrulation and somitogenesis (Fig. 3-16A). At these miR-430 morpholinos concentration, PGCs were still lost at 24 hpf, when dead end morpholino was coinjected (Fig. 3-16A, lower panel). Nevertheless we performed measuremens on the PGCs at 10 hpf and found that reduction of the five miR-430 increased the fluorescent signal originating from *nanos1*-3'UTR in otherwise wildtype PGCs (p-value<0,01) and counteracted the decrease of signal by additional Dead end morpholino injection, compared to dead end morpholino alone (p-value<0,02) (Fig. 3-16B).

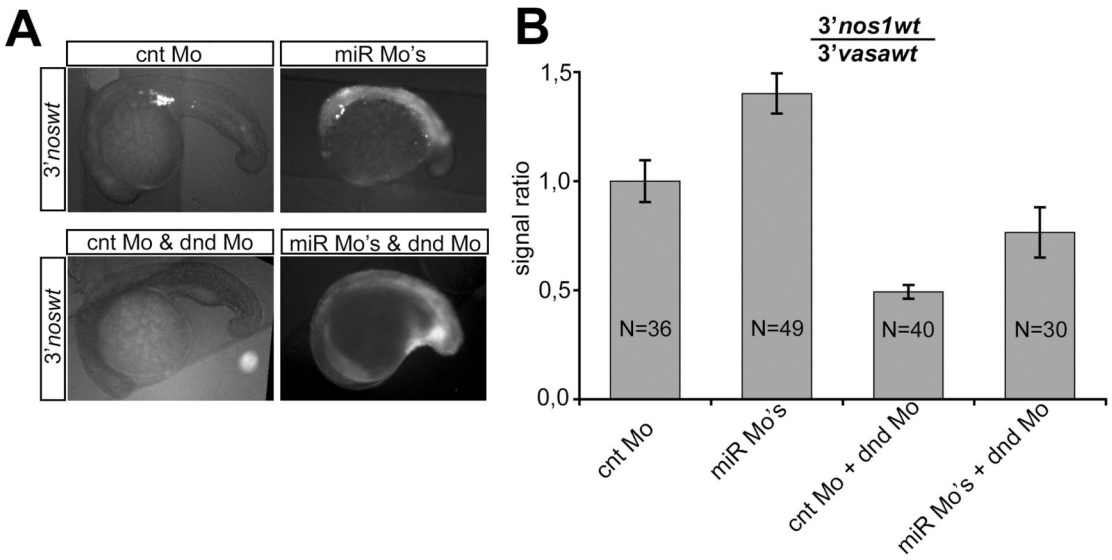


Fig. 3-16. Ablation of miR-430 rescues partially loss of signal intensity in the dead end morphants but not the loss of germ cells. A) one-cell-stage zebrafish embryos were co-injected with RNA containing the *DsRed* open reading frame fused to the wild-type *nanos*-1 3' UTR (*3'nos1wt*)

together with RNA containing the *gfp* open reading frame fused to *vasa*-3'UTR and either control morpholino, the five miR-430 morphilnos, control morpholino plus dnd morpholino or the five miR-430 morpholinos plus dnd morpholino. The pictures were captured by using the rhodamine filter. **Note**: Most PGCs in the miR Mo's embryo, which are in an ectopic location, reside at a position in the head with a strong SDF source. This is caused by the defects in the soma rather than in the migration properties of those cells. B) PGCs from A were captured at bud stage (10 hpf). The ratio between the signal intensity provided by the fluorescent protein whose open reading frame was fused to the wild-type *nanos1*-3'UTRs was divided by that originating from a wild-type *vasa*-3'UTR.

4 Discussion

4.1 Identification of candidate genes using microarray chips and whole mount in situ hybridization

As presented in the result section, the Affymetrix microarray chip serves as a powerful tool to assess differential expression in cells. Upon establishment of an isolation procedure for PGCs and defining the parameters for the selection of candidate genes, 248 candidate genes were selected. The parameters were defined so that 6 known PGC markers were included in the selection. The subsequent *in situ* hybridization screen confirmed 20 candidate genes with enriched expression in PGCs, two of them being localized to the germ plasm (the novel gene *granulito* and *Tdrd7*). The rather low verification percentage (8,2 %) of increased expression in PGCs between in situ and microarray has several possible causes. First, Microarray data are prone to generate false positive candidates caused by the large data set (Allison et al., 2006). Generation of biological replicas would reduce the number of false positives, however the advantage of such a reduction would not outweigh the time consuming procedure of PGC isolation, as the secondary *in situ* hybridization screen was designed to exclude those false positives from further analysis. Second, the low amount of initially isolated RNA made it necessary to amplify the RNA in two rounds, which can be a cause for artifacts. To minimize alterations in the amplified transcriptome I used the Eberwine method for amplification, which was shown to have only a minimal alteration in the amplified transcriptome (Van Gelder et al., 1990). Third, it cannot be completely excluded that the manually isolated PGCs were contaminated with somatic cells during their isolation.

Of the 20 new genes, which were found to be enriched in PGCs by the secondary in situ hybridization screen, 9 were analyzed with loss of function experiments using morpholino antisense oligonucleotides. These knockdown studies could only reveal a function for Tdrd7 in PGCs in regulating the structural properties of germinal granules. As morpholino-mediated gene knock down is only efficient during the first days of development and the effect decreases rapidly later on, it cannot be excluded that these genes play a possible function only later in development or that maternal protein is sufficiently provided to rescue the first days of development. Further analysis would require a dominant-negative approach or isolation of mutants for the

corresponding gene, which in turn requires the identification of corresponding mutants from mutagenesis screens.

Based on the critical role Dead end plays in PGC development (Weidinger et al., 2003), 192 of 248 candidate genes were selected for their higher expression in wildtype PGCs as compared to PGCs in dead end knockdown PGCs. The notion was that loss of Dead end leads to reduced transcription or reduced level of mRNA of genes important for PGC development. Interestingly, *Tdrd7* mRNA level was not significantly decreased in PGCs of dead end knocked-down embryos, but as presented here, Dead end knock down causes a dramatic decrease of its translation. These findings imply that many developmental processes such as PGC specification may significantly depend on changes in protein expression that are not regulated on the level of transcription.

Since important genes for germ cell development, such as *dead end, dazl*, *nanos, pumilio* and *vasa* have RNA binding domains or RNA helicase activity (Asaoka-Taguchi et al., 1999; Hay et al., 1988; Houston et al., 1998; Weidinger et al., 2003) and therefore could alter the translation of target mRNAs, future investigations for novel candidate genes important for PGC development and migration should aim at the identification of alterations in the proteome. For example, in Drosophila it was shown that Nanos together with Pumilio, both important for germline maintenance (Forbes and Lehmann, 1998), repress translation of Hunchback in the posterior pole by binding to the 3'UTR of *hunchback* mRNA (Wreden et al., 1997; Zamore et al., 1999) and Vasa is required for accumulation of Gurken in the Drosophila oocyte (Styhler et al., 1998).

4.2 The novel gene *granulito* encodes a marker protein for germinal granules

granulito was identified in this study as a novel germ cell component. The mRNA of this gene is maternally provided and becomes localized to the germ plasm, while the protein localizes to germinal granules. Protein blasts did not reveal known domains and significant homologs are found only in the vertebrata (all hypothetical proteins). Knockdown experiments using *granulito* morpholino antisense oligonucleotides failed to induce a discernible phenotype suggesting either that the function of the protein might be masked by a redundant protein(s), or that *granulito* function is not essential during early development, or that a maternally-provided protein is sufficient

for carrying out the role at the stages tested as it has been described for other processes in zebrafish development (Gritsman et al., 1999). To test the maternal contribution scenario, I attempted to generate an antibody directed against the endogenous Granulito protein, which could reveal the presence of maternal protein. Unfortunately, this obtained serum failed to recognize the protein in immunohistochemistry.

4.3 Characterization of germinal granules in zebrafish PGCs

Based on the specific localization of Granulito to germinal granules, I have taken advantage of the newly identified gene and generated *granulito-dsRedEx* transgenic fish. This fish line serves as a valuable tool for investigating germ plasm distribution during very early stages of PGC development that had previously been inaccessible for *in vivo* analysis in live embryos. I found that the morphology of the germ plasm is transformed from large aggregates seen at early developmental stages into small granules that adopt perinuclear localization in the cell, later in development. This transition is believed to allow the symmetrical distribution of the material to both daughter cells during cell division, thereby enabling an increase in germ cell number as the cells proliferate (Whitington and Dixon, 1975).

Although all cells that contain germinal granules are committed to the germ cell fate, the data shows that number and size of granules are highly variable among PGCs during early stages of development. Following the distribution of germinal granules during the first 24 hours of embryonic development, I observed that as PGCs proliferate, the variation in granule sizes decreases.

A colleague in the lab could provide evidence that segregation of granules among daughter cells is not a random but rather an ordered process, dependent on the microtubular network and its motor protein Dynein. Altering the levels of microtubules or Dynein caused a change in number and size of germinal granules. This is in line with previous studies in *C.elegans* and zebrafish earlier in development, which showed that the microtubular network is required for proper localization of germ plasm for the purpose of specifying the germ cells (Hird et al., 1996; Pelegri et al., 1999; Theusch et al., 2006).

4.4 TDRD7 is required for germinal granule distribution within the cell

Another germinal granule resident from the screen was *Tdrd7*. Similar to the *Drosophila tudor* (Boswell and Mahowald, 1985), *Tdrd7* is localized to the germ plasm, yet formation of germ cells in Tdrd7-depleted zebrafish embryos appears unaffected. As the presented experimental approach with morpholino only donwnregulates the translation of zygotic mRNA and does not inhibit the function of maternally provided protein, I cannot exclude a role for Tdrd7 protein in germ cell specification during early stages of zebrafish development.

Despite normal PGC specification and migration observed in *Tdrd7* morphants, germinal granule structure integrity is abnormal. This finding is in agreement with the proposed role for the *Drosophila* Tudor, which is thought to provide docking sites for other proteins thereby allowing proper germinal granule assembly (Arkov et al., 2006). The severe disruption of granule architecture due to Tdrd7 protein loss-of-function in early stages may point towards a critical role of the protein during later stages of germ cell development. For example, it is known that germ cells of mice deficient in Tdrd1 initially migrate and develop normally, however, male mice are sterile due to lack of mature sperm (Chuma et al., 2006). In these mutants, the inter-mitochondrial cement, an essential subset of germinal granules, is strongly reduced. Remarkably, mice carrying mutant forms of RNF17, another Tudor domain containing protein, show a similar phenotype to that of Tdrd1 knockouts (Pan et al., 2005). RNF17 localizes to a new form of germinal granules, RNF17-granules; the loss of these granules in RNF17 mutants is the cause of male infertility. Additionally, analyzing germ cells in mice mutant for the mouse vasa homolog, revealed that the colocalization of Tdrd1, Tdrd6 and Tdrd7 to germinal granules depends on Vasa (Hosokawa et al., 2007).

Since several genes contain Tudor domains, *Tdrd7* could be redundant with another yet-to-be-identified genes of the same family. As presented in Figure 3-11, I sought to identify other Tudor domain containing proteins that are expressed in zebrafish germ cells. Indeed, following the second day of development, *Tdrd1* and *RNF17* were found to be specifically expressed in germ cells along with *Tdrd7,* suggesting a possible redundant role for these genes in zebrafish germ cell development at later stages. Yet, Tdrd7 has been the only identified Tudor domain-containing gene to be expressed during early zebrafish germ cell development. Interestingly, *Tdrd7* mRNA in zebrafish is protected from degradation in PGCs, and is efficiently degraded in the

soma by the same miRNA mechanism as for *nanos1* mRNA (Mishima et al., 2006), which further supports a role in germ cell development.

To elucidate the role of germinal granules architecture in germ cell development, alteration of the function of genes described here during later stages of development will be required. For example, the identification of *Tdrd7* zebrafish mutants or mouse knockouts would allow the analysis of this effect during more mature stages of germ cell development.

4.5 Dead end is required for efficient translation of Nanos1 and Tdrd7

Recent studies have implied that germinal granules and their component are linked to posttranscriptional regulation by microRNAs (Kotaja et al., 2006a; Megosh et al., 2006). However germ-cell specific mechanism of microRNA action was so far not described. Until today it was unknown why some miR-430 RNA targets, such as *nanos1* and *Tdrd7*, are efficiently translated in primordial germ cells in the presence of miR-430 (Mishima et al., 2006). The presented results suggest that Dead end protects these mRNAs from miR-430-mediated inhibition depending on URRs within the target mRNAs.

The precise mechanism by which Dnd exerts its function is still unknown, but recent publications have implicated mechanisms that counteract the activity of miRNAs on specific mRNAs (Ashraf and Kunes, 2006; Bhattacharyya et al., 2006; Schratt et al., 2006). The most relevant work showed that HuR, an AU-rich element binding protein, relieves *CAT-1* mRNA from miR-122-mediated repression in a process that involves binding of HuR to the 3′-UTR of *CAT-1* mRNA (Bhattacharyya et al., 2006). Here, I could demonstrate that Dnd has a comparable activity to HuR. Dnd presumably also binds to mRNAs and thereby relieves miRNA-mediated translation repression, however Dnd seems to inhibit miRNAs in a different fashion than HuR. Dnd depends on URRs that are positioned in close proximity to the miRNA-binding sequences to relieve the miRNA repression, unlike HuR, which depends on AU-rich elements at the end of the 3'UTR of its target (Bhattacharyya et al., 2006). Additionally, while the expression of HuR is induced following stress in liver cells, Dnd is constitutively expressed in PGCs and certain neuronal tissues in zebrafish and mouse, playing a role in normal development (Weidinger et al., 2003; Youngren et al., 2005).

Interestingly, Dnd does not only alleviate the repression of *nanos1* and *Tdrd7* in

PGCs, but, as suggested by cell culture experiments of our collaboration partner, additionally antagonizes the repression of p27 by miR-221 (le Sage et al., 2007), LATS2 by miR-372, Connexin 43 (Cx43) by miR-1 and miR-206 (Anderson et al., 2006). In line with these findings, the inhibition of miR-430 by morpholinos did not rescue germ cell death in dead end knock down embryos. Although I cannot exclude that the inhibition of miR-430 was incomplete due to experimental limitations I suggest that Dead end might antagonize the action of other miRNAs in PGCs as well. Genome-wide proteome and RNome analysis comparing normal cells to cells lacking Dnd should address these issues in the future.

An important step towards resolving the precice mechanism of Dnd is the identification of Dnd interaction partners. Hints towards the existence of proteins interacting with Dnd stem from the implication of Dnd in cancer research. Both in zebrafish and in mouse, Dead end is usually essential for germ-cell survival, whereas in the mouse strain 129 Dead end knock down induces testicular germ cell tumors (TGCTs) arising from the few germ cells that develop in the absence of Dnd (Weidinger et al., 2003; Youngren et al., 2005). These tumors resemble human testicular germ cell tumors, which are the most common cancers affecting young men (Oosterhuis and Looijenga, 2005; Youngren et al., 2005). Interestingly two target mRNAs of Dnd, p27 and LATS2 are both tumor supressors (Li et al., 2003; Nakayama et al., 1996), supporting an additional role of Dnd in tumor suppression next to the requirement in germ cell development (Fig. 4-1). It remains to be established which mutation(s) from the 129 strain synergize with the Dnd mutation to cause the development of TGCTs, but it is tempting to speculate that some of the yet-to-be-identified genes might be Dnd interaction partners. The finding that disruption of the *C. elegans* germ plasm by loss of the two translational regulators MEX-3 and GLD-1 may also lead to the development of similar tumors (Ciosk et al., 2006) supports the idea that Dead end is a member of a complex protein machinery involved in specific posttranscriptional regulation in germ cells.

The herein suggested concept of repression of miRNA bears several important consequences. As my results suggest that the 3'UTRs of at least some mRNAs are binding platforms for both miRNAs and their regulators. RNA binding proteins might restore gene expression in the presence of inhibitory miRNAs. First, as such a regulation affects protein synthesis rather than expression of miRNA in the cells

(which may take several cell cycles, unless the miRNAs are subjected to rapid destruction) it is a potentially fast mechanism. Second, relief of repression can be exerted simultaneously only on a subset of the miRNA-targeted mRNAs, thereby giving modularity to miRNA function. Third, it adds robustness to expression patterns as different RNA binding proteins can in principle relieve the repression of different sets of mRNAs, even if they are regulated by the same miRNA. Finally, this mechanism allows differential gene regulation in different tissues while keeping the expression of both miRNAs and mRNAs constant in the cell. Thus, co-expression of both miRNA and target mRNA does not necessarily result in repression, as exemplified by the PGC-specific derepression of *nanos1* and *Tdrd7* by Dead end.

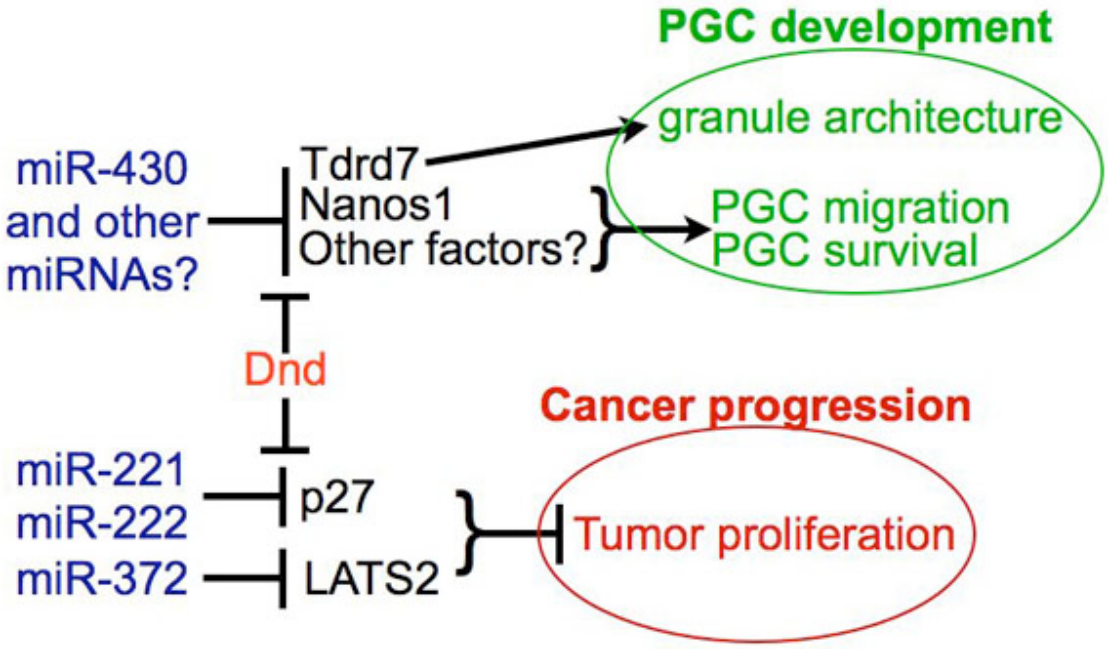


Fig. 4-1. Role of Dead end in alleviating the action of miRNAs. The presented results and the results of our collaborators identified a role for Dead end in alleviating the action of miRNA and therefore allowing the proper development of PGCs as well as the suppression of cancer cell proliferation.

5 Summary and Conclusion

Here I present the results of a microarray-based screen, which led to the identification, and characterization of two novel germ cell markers: *granulito* and *Tdrd7*. *granulito* mRNA localizes to the germ plasm and is expressed in PGCs until the end of the first day of development. It encodes a short protein of 143 amino acids without homology to any known domains, which localizes specifically to germinal granules, a structure unique to germ cells. Although a specific function for *granulito* could not be revealed, its property as a marker for germinal granules in a transgene allowed the description of the dynamics and architectural properties of germinal granules during the first day of PGC development in zebrafish. After specification of germ cells in zebrafish, germinal granules are inhomogeneous in size, but as development proceeds they become uniform in size and are organized around the nucleus.

Tdrd7 mRNA localizes to the germ plasm and is expressed in germ cells until at least 7 dpf. Tdrd7 protein contains three Tudor domains. Family members of Tudor domain containing proteins were already described to be important for germ cell development. Here I showed that Tdrd7 protein localizes to germinal granules and is essential for the proper architecture of these structures during PGC development. However, these structural abnormalities did not interfere with other aspects of PGC development such as migration and fate maintenance in the first days of embryonic development.

Last, I provided evidences that Dead end, a germinal granule component essential for PGC survival and motility that has been linked to the formation of testicular germ cell tumors, antagonizes the action of microRNA action on specific targets in germ cells. Particularly, I showed that two targets of miR-430, *nanos1* and *Tdrd7*, loose their protection from miRNA action in germ cells upon Dead end knock down. In collaboration with the group of Dr. Reuven Agami (Netherlands Cancer Institute, Netherlands) it was found that Dead end requires uridine rich regions (URRs) within the 3'UTR of these targets to exert its protective function.

In conclusion, my data gives new insights into the structural identity and to the function of some of the components of germinal granules, a unique structure only found in germ cells. Germinal granules undergo a structural transformation after the

specification of germ cells, and this transformation requires specific factors such as Tdrd7. Additionally, I could show that the germinal granule component Dead end, which is essential for PGC development, is involved in microRNA-mediated gene regulation. Altogether these findings support the idea that germinal granules are essential for germ cells and argue for a unique regulation of mRNA stability and translation in this cell type executed within the germinal granules.

6 Material and Methods

6.1 Bacteria strains

Table 6-1. List of bacteria strains

Bacteria	Company	Description
Top 10F'	Invitrogen	*E. coli* competent cells, for plasmid DNA electroporation
DH5a	Invitrogen	*E. coli* competent cells, for plasmid DNA electroporation

6.2 Chemicals

All chemicals, if not noted otherwise, were purchased from the companies Applichem, Merck, Roth and SIGMA.

6.3 Primer list of candidate genes from the Microarray Screen

Table 6-2. List of Candidate genes and primers

Number	Accesion No	Forward Primer	Reverse Primer
1A	BQ093700	AATGTGCTAGTGGAAAGTGC	TGACACTTGGGTTGACTTTG
2A	BI474808	GATGCTGAGGTTGAGGGAGA 2. Fw primer CTGTGCAGATGGCTCAGTTC	CAATAAATTGTTTTGCATTGG
3A	BI983354	TTCAAACACAATGGGTGCAT	TTCATTTGCATTTGGCGTTA
4A	BC045513.1	TCGAGAAGACGGTCACTCCT	TCAACAACTGCAGGAGCATC
5A	BC044551.1	GAGGCTGAACATTGGGACAT	TGAGGGCACAGAAACAACTG
6A	AL922740	GCAGTACATGAAGCTGAGCA	GGGAAATGCACCAGATGACA
7A	BQ450026	AAAAATGGTGACCAAAGTGG	TGACAGTTCTATTTTGCTTGC
8A	BC046012.1	CATGTCCAGAACGGTTTGTG	AACCAGTTTGGAGGCATCAG
9A	AW567529	CGCTGCAAAATGAAAGATT	CCTGAAGACTTTTAGTCCCAAT
10A	BM103315	CAACGCCCAGACTGATGA	TCAGCGACGTGGTCTCTTC
11A	BM101604	GGGGAAAACAGACCATCTGA	TCATTCCAAACAAGTGCAAA
12A	AI721440	GGTGGTCACCAGTGAAAGGT	CGGAAGCCCAAGTTCGAG
13A	AL915830	ATGGCGGCAAAGACTCAG	TTCATCATGCCCTTGAAGC
14A	BM316210	ACGAGGAGCTGGGGAAATTA	GCATGAAGCTCATCGGTTGT
15A	BG728541	TCGCAGAGGAGGAGGAGA	ACCTCACTGCCCGAGTCA
16A	BM095926	GCAGGTTTGATCCCAGCTC	CGATTACAACCCTACATCAGC
17A	AI641729	TGTGCCAAAAGAGAGTCACG	AAGGTCAGGCAACCTTTACA
18A	BM571673	CGCACTTTGCATGTTTGTG	CACGTGGTTCCAGCCTTC
19A	AW076961	TGTCACACTCATTGTCCAGA 2. Fw primer AGCGTGACTTTGGCTCATTT	CCAAGCCTCAGTTTTGTTTG
20A	BI843295	AGGATCATCTGCTGACTCATT 2. Fw primer AACGAATCTATAAATGCGACCA	ACAGCATCAGAACTCGAAACT 2. Rv primer CAATACAGCATCAGAACTCGAA
21A	BI672117	AACCAAAAGAACTCCAGCAA	ACTCAAGCAAAGAGCCGATG
22A	BU710607	AAAGCGGTGGCCGAGTTTA	TATTCGAGATCCCACTGAGG
23A	BM104070	GCGGATTAGTGGGCATCTT	AAGGGGCATTGATTCACAAG
24A	BM154132	GTGATCAGCCGGATCTGC	CAGGAGTCCTCGGGTTCA
25A	AW170913	TGGGTGGGAGGTGAAGAATA	TTGCTGGAATGCTGATTGTC
26A	NM_130948	ACCCATGTGTGGTGTCTGAA	CGCTCAGAGGAAAAGGACAC
27A	AI558276	TGTGTTTCACGGAAGAAGGA	CGTCAGTGAATTTTTGGTTCC

28A	BQ616930	CCTACTGGACCGTTGTGGAC	CGTTCTGACGGGCATTTATT
29A	AW421114	TGATTGTGTGTGTGGGTGTG	ATTGGGCTTTGAAATGAAGC 2. Rv primer ACATTCTGGACAGGCGAGAG
30A	BQ262910	AGCAGAAAACGCTCACATCC	CCAAAATGGTCAACAGACAG
31A	BC046081.1	AAAACCCTGATCGTCCCTCT	TCCAAACAGTCCTCCGAATC
32A	BM095897	GGTTTACACCCGTTCCTGTG	AGTTTGCTCAAAAGCATGTC
33A	BM154752	CTCTTGCAGTCGCAGCAG 2. Fw primer TCTGTGGTCCCCTTTACCAC	CAAGACAGCAATGCCACAG
34A	BQ285167	AGATTCACGCATGGTCCTG	GCTGATCCGTGAACTTTGCT
35A	BQ078419	TGGATGTGAGCCAACGATTA	CTTGGATCAACCATGGCATT
36A	CD605341	GATGCATAAAACGCCACAAA 2. Fw primer CCACAAAGCAGCTCAAAACA	TAAAACCCTAAATGCAGCAA 2. Rv primer AACCAAAAGAACTCTGAAATGC
37A	BC044165.1	GAACTTTGCAACGCAGTGAA	GAAGAGCAGAAGTGGGTTGG
38A	BQ261266	AAGTGGTGCAGGCAAAGC	GGCAACAATGGAACAAGAGA
39A	BQ262324	TGGAGCAACTGCAGACGA	GCCTCACATTGGCATAGCA
40A	BC044447.1	CAGTGGTCCTGGCTTCTCTC	AGCTTCTTCTCGCTGTGGAG
41A	AW232570	GCCGGGATTACACTCAGATG	GCTGTTCAGCCTGGACTTTT
42A	BM571456	AACCGGCTGGTGTCAGAG	ACTGCAACACACTAATGACGA
43A	BQ479662	CAGGCTGATATGAGCAGGAT	TCGTGTTGAGGTCTTGTGAA
44A	BM956864	TCTCCAGAAAGCCCAGAAGA	GCACATTGCAAAAGGCAGTA
45A	BC045926.1	TTCAGAGTGGCCTTCGAGTT	TGAAGGGCATTGTGGTGATA
46A	BQ093634	CAGAGGAGCCTTCGTGGA	CAGTTGTGCTTATGCAGGAT
47A	BM530127	GCGAGGATTCATGCTGCT	AGGCATGCAATGTAACAAAA
48A	BQ092687	CCCATCAAGGATCAGAATGG	CCCAGCATAGAGGGGTTC
49A	BM316143	GCGCATACAGCCAACACA	TGTGCCAAGAAATGCACTG
50A	BM024151	CGAGCAGTTGCTGCTGTTT	TGTGCTTTGCAAGGATGC
51A	BQ783871	GCAGCTGTTCTGGGGTGT	GCAGCGAGCACATTGTTG
52A	BI889921	TCCGAACCCTGAAGCTTTTA	TTTCCAGATCCGCCTCATAC
53A	BC049516.1	GGCTCTGCTGAAGACCAAAC	GGATAGTCCACTGCCATGCT
54A	BE017931	TGAAATGTGAGACGGAGCTG	TAGTCTCGGCTCTGCTGGAT
55A	BM571646	GGAAAGCAGAGATGAAGCAG	CGACTTTGAAAGGTGCACAA
56A	BC050170.1	CCGCATAGAGCTGGAGAAAG 2. Fw primer ACCGTAACAGGGACTTGCAG	GGGATGCTCTTCTGATGGAA 2. Rv primer CTGAGGAACACTGGGATGCT
57A	NM_131649	GAGCATCGCAAACACTGAAA	TTGTTGGAAGCTCAGTGCAG
58A	BQ285243	GTCAGCCGATGGATTCCTAA	GCCCGTACAGGTCAATTTGT
59A	BI879940	CCCTAGCAAGCATGAAGCA	GACTGCAATGGCAGAAGGA
60A	BQ479881	ACCGCAGTCCAGAGCAAC	AAAGGCGAGCAGCCATTA
61A	BG308403	GCCCTCCAGGTCAATAGGA	GGTCCAGCGGCATTGTAA
62A	AI544978	GACTGGCTGGAGGTGCAT	TGAGGCCATTGTGCTCTG
63A	BC049451.1	TGCAGATCACCGACAGGA	GCTCAAACAGCCCCAAAA
64A	AW281891	GAAAGCGTCGCCAAACAT	CAACGCAATGCAGACACTG
65A	BM857906	CAGAGAAGCCTTTTGCGTGT 2. Fw primer CTGCAGGATTGCTGAAGAAG	GGCACAATAATTCCAATCCA 2. Rv primer TGAATGAGGAGAAGGGTTTTT
66A	BM534431	CATCGGCTGCTGAGAGAGA	AGTCTGAGCTCCAGCACCA
67A	BM082457	GGCCCAAACACAAATGGT	AATGCCTCCTGGGGTTTC
68A	BI709398	CCAGTCCTGCATCCTCCA	GCCCTGGTGCAGTCCTCT
69A	BM529903	ACGCCACGAGGATCCATA	TTGTCATGTTGGATGGCATA
70A	BI878749	CCTGAAGCACCAGCTGAAA	GAACGAACACAAGAGCCACA
71A	AW077628	GCTTGTTCACGCACAACACT	AAAGCAGTGGATGCACGA
72A	BI879024	GCCTTCCATCAGTGTGGTG 2. Fw primer GCTTTAGTCTCTGCAGGGTCAG	TGAGGTAAAGGCACGGTTG 2. Rv primer GAGGTAAAGGCACGGTTGAG

73A	BC053222.1	CATGCGTCAAGCCACAAG	TAGCGCTGGTCTCGAAGC
74A	BQ074694	GACCACCCGTACGCTGAC	CTCCTGCAGGGGTAACCA
75A	AA494741	CAGCACCACCAGAGACTCC	GCAAAAACAGCACATGTTGA
76A	BI983550	TGCGTGTGAGCACTCTGTC	ACGTTCTGAAGGGCGTTT
77A	BI430208	GGAGGAGTCATCTGGAGGAT	TCAGTGGTTTGAGTCTTTCG 2. Rv primer GAACATGTACGACAGCTTCATC
78A	AF409097.1	ACCCGCGTCGACACAATA	TTGACAGTTCTGGGAACAGG
79A	BI896378	AGCTGGGCACGCTTATTC	TCCCGCTCTTGTCTCCAG
80A	BQ616045	CCCCGTCTTTCCACAGTC	CGGTCAGGCTTAAAATGCAG
81A	BM571619	AGCAGCGGGATCATCAGA	CAGCCAATGTGAGCACCA
82A	BI326702	TGCATGAATACGTCATGTTGAG	TGACAGTAAAACTGCAAACACA
83A	BI983434	CCGGTTTGGGAGCTGATA	GGACGAAATACAGGGTGTTGA
84A	AL907236	ATGGGTGGATTTCGCTCA	TCGCGACATCAGAGGACA
85A	AW171527	GGGTGTCGCTCTCAGGAA	GGAAGACACAAGCTGCCATT
86A	BM776698	CCGATATGGCTAGGAACTTGT	GGAGGAGGATCCTTGCACA
87A	BI867164	TTGCGTTTCCGGAATGAT	GGCACAGCTCCAACCCTA
88A	BM182245	TTACATGGATCAGGAGAATGG	CAGGAGGCAAGTCAACTTCA
89A	BM777971	TGCAACTACGCCGTTGAG	TCGTTGTGGGACCGGTAT
90A	BC045489.1	TTGCAGAGGACCGCTTTC	GGAGTGCCGCAGTTCTGT
91A	AW826425	CGGGGCTTCATTTGCTAT	TCACACAAAGCACTGACAGTAT
92A	CD014176	TCCATCGGCTCTGGAAAA	TTCTCGTGCTGTGCGAGT
93A	BC053246.1	GACATCCGTCAGGGCATC	AATGCAGGGGACCAAACA
94A	BG728739	GCGGCCAGTCTGACAGTT	AAGCAGACGGTGGTGAGC
95A	AW171210	GGTCTGCTGTGCATTTTGC	AAGCAGCACATGAAGGTCA
96A	BM181739	CAGGAGTCCAGCGAGGAG	AAAAGCATAAAAGTTCAATTTAC
97A	BM104000	GGAGGCTGGTGACAATGG	TAACCAGAGGCCCAAGCA
98A	BM026548	CGAGCGATATTGGGGAGA	CCCTGGAAGAGCTGCACTA
99A	BM036980	ACTCGAGGCCCAGTCAGA	TGTCCCGCATTTTAAAGCA
100A	BC049044.1	ACGACCGTCTGCAACCAT	AAACGCACCTCCCCTTTC
101A	BM082897	AATGAAGGAGCGCACGAG	CGGGGACAGTGAAACACC
102A	BM530945	CCCCTTCGAGAACACACG	GTACCGCCCCTCTGGTTT
103A	CD604918	TACCCTACGACTAACATTTTG	TTAAACGTCTTACCTTTGGTT
104A	BM812123	GTCATATAAATTCGGTATAGAGC	GCCTTTTTAGTGGTTTATTTAG
105A	BI878221	TGCTCGGTGGTGAACAGA	ACCTCAGCGAGCCACATC
106A	BI896246	ACTGGCAGACGGCCAAAT	GACGCTGAGCACTCGTGA
107A	AY070267.1	AGGAGCTGCACAGCGAGT	GCGCCAGAAAGACACGAT
108A	BM140763	TCCAGTCTGGCCTTCAGC	TCCAGGAACTGACATCAGCA
109A	BI979582	TGCATGGATTCTGACCTTCA	CCAGAACACGGATCAGAGAA
110A	BI430015	ACCGGAGTCTGACGCTTG	CCCTCCAGCGAAGAACAA
111A	BQ285080	GGTATCCGTGCCTGATCG	ATTCCGAAGAAGTGAATGC
112A	NM_13117	CACGTTCTTCGCCTGCTT	ATGGGCAGGCATCTACCA
113A	AI588346	ATTTGCGTCCGCTTCAAA	TCATGTGTGCAGTTGAGGA
114A	BM342451	CTGCTTGCCCATCTTCGT	TTCCTGAGCGGAAACTGC
115A	BM342614	AGGGAAAGGGACCGACAG	CCTCCATACGCCGATGTC
116A	BC049455.1	CGCAAGAGCAAGCACAAA	TCTTGCTCGGCAGAGGTT
117A	BI880553	CATCTGCTGCGAGTGTGG	GCAAAGCCTTCCACATGAA
118A	BI980890	TTAGCCCCCTTCAATCCA	AAGATGCAGCAAAACACCA
119A	AW116978	TTTTGGGACGGGATCTCA	GTGCAGTGAGGCATCGTG
120A	BI884157	TGCTTGACACTTGGGTTAGA	CAGATGGGAGTGCAGCAA
121A	BQ284849	ACATACAGGAACATACATCAGG	AAGGCAGAATTAAGAGCACAA
122A	AL911045	CCAGGTGGCGATGGAATA	TCAGTACAGCGTAGAAAAGGA
123A	AI584334	GTGGGGAAGGACATGCAG	GCCAGGCTCTTCCACAGA
124A	BC046088.1	GGCTGGATCCATCACCTG	TTTGCTGGGGGTCTCTGA
125A	AW175497	TGCGGCTCATGTTCTCAA	CCGGAGACAACCCACAAC
126A	BQ262294	TTCAGCTGGAGCTCAGAACA	AAAAAGCCATTTACATTTGCTA

127A	NM_178298	GCTTTGCTGGGAGCTTCA	GGGTGGCTCCTTTGGTTT
128A	BI891683	AGGCCAGCGAACACAATG	TTGGTTGGTTTTCACAGG
129A	NM_130914	GCCTGGAGGGAGAAGGAG	CTGCGCATGAACTGCAAC
130A	BI673624	CGCTAGCTGTGGAACCAA	GTCCCGTCCGTTCTGATG
131A	BC049410.1	GATCAGGTGGCGATGACC	AAGGCTGTGCCGTCTGTC
132A	BI866379	GGAGAGTGCAGCCTGGAG	TTTGCAAATCGGGATGCT
133A	AL925292	CTGCGAAAAAGGCAGCAT	CGGAAATTGGGTGGTGAG
134A	BG985458	GAAGGGACGAAACGGACA	CAAAAAGAAGCGCCAAACA
135A	BC052118.1	GGTGGAGGAGGAGGGAAA	GAGCCCAGGCTGAGTCAA
136A	AW171424	GCCTGCTGCACTTTCGAT	ACCGTCGTTCCTGGTTCA
137A	BM571837	CACACCGGGTTTGTCTCA	TGCGTTTCTGCTCCTGGT
138A	BC048052.1	CTGCCACCACCAATCCTC	CCGCATTCTGAAGCCACT
139A	BI428480	CGATGCCGGTTCATAAGC	ATGCACTGCAGGTGCTCA
140A	BM736017	CTGGAAGCCCAGCTGAAA	TAGCTGCCTGTGCTGTGC
141A	BM957968	ACTTTCTGCGGCCAGTTG	TGTGCCAAAGCCATTTCA
142A	BM071848	GCCGCTTTTGGCACTTTA	CACAAAGGAAATCTCGCAAT
143A	AL911455	TCGGAGGATGAGGCTCTG	GTCACCACCCGCAGTTTC
144A	BQ480688	CTGGATGCAGGGCTGACT	GCAGGTGATGAGAACAGCA
145A	AW058783	TGTCGCAGCCATAGACCA	CGGCTGGAAGAACCTCAA
146A	BI897382	GGCGCCCACTGCTATAAG	ATCAGGCGTCTCCACAGC
147A	BI984262	GGCCAGCTTTCACTCAGC	CCACCTCCACCTGACTGG
148A	BI709943	AAAACCTGCGGGGAAAAC	GGCCAAAACCACGACAAG
149A	BC051623.1	GTGGCTCAGAACGGCACT	ACCCCATCCCATTGGTTT
150A	BI673296	CGGGATGAAAGGAGAGCA	AAGCACGTGGCTCCTGAC
151A	BM530072	CGGCCAAGAGCTTACCAA	CATGGCCAAAGCCAAGAT
152A	AI397123	ATGGCGTCTCCGAAAACA	TGTGGACTCGCTGGATGA
153A	AL920484	GGACAAGAGAAGAACCAGCA	GGGCCAGCTGACTCTGAA
154A	AW421046	AATCTGCCGCTGTGAACC	CCTGCCGTTTCAGCATTT
155A	BI670934	TGATGCCAGCACCTTTCA	TCATTGCAACAACAGTGTCA
156A	NM_131331	GCCCTGTTGTTGGTCAGG	TTGGCAAAGGAGCAGGAC
157A	AI942949	AACCCCGTCCTGGAGAAG	CTCCAGCACACGCATCAC
158A	AI497193	TCATTGTTAGAATGCTTCTTTT	TCAAAGTCTAATGAACCTATCA
159A	BC044485.1	GACTCACGGGGTCACGTC	GTGCCACGCTGTGTCAAG
160A	AI964212	ACCCACCATAGGCAGCAA	GAGGCCAGTCAAGGCAGA
161A	AI331186	CACAGGCCAAGGAGGAAA	ATCTGGCGAAGCGTCTTG
162A	AL715712	GTCATGCGGCTCTGTGTG	AAATTGAGCAGCGTGAAGG
163A	BM530311	TACGGTGCTGGAGGGTTT	TCATGTCGGAGGTGACGA
164A	BQ618152	AGACCCCCAAGCGAAGTC	TTGCATACCAGACCATTGGA
165A	AW420720	GGGGGCTGTTAATAATGGA	TCCCCACCTACACGGAGA
166A	AI437377	CTGTTTCTCCGCGTGTCC	TCCGGCTTGTTTTGTGGT
167A	BC052141.1	GATCTCAGCGCCTTTCCA	GAAATGATGGCCGAGGTG
168A	AL925202	CAAGCCAGGGGAAGGAAT	GCATGCAAGTGTGGCTTG
169A	BI475189	GGCCATGTGGACTGGAAG	AAATACGCACGCACAGCA
170A	BM156878	TGCGATAAGAGATTTAGGCAGT	GGCTTCTTCTATGTATGAACTC
171A	AW077423	ACTCCCCCTCCAAACAGC	TGTGCTGCGTTTCCTCAA
172A	AI667252	CCGAACAGGTTTCCTCCA	TGTGTCCAAACCCTCAAAAA
173A	BI984278	CACATTGAGGATTCTGCACTC	GGCCGTCTGTATTTTGTGC
174A	AI964108	TATACGGGCAGCCGAAGA	GGCAAAATCCACCAGTGC
175A	AI723162	GCTCGGTGGAGATGGAGT	ACATGCTGGTGTGCAGGA
176A	BI983410	CCTTTCCGAAGGCAACCT	AAACCCTGAGCCCACCTC
177A	BC044167.1	TGTGCACAGGTGCTGCTT	CCCCATGGATTGGTCAAA
178A	AI942930	CTCTCCTCAACCGGAGCA	ACTCGGGGCATAAGCAGA
179A	BM156735	CAGCAAACAGAAGCTTCATCA	TCATCAAATGTTTTCCCATTTT
180A	AA497149	TGGCGAGACCTTCTCCAG	GATCCATGGTCCGCTCAC
181A	BM095864	CAATCACAACGCGAGACG	CCGCTCGGTCACCATTAC

182A	BC047169.1	ACCTGGCGCTGAAGACAC	CCTTCAGGGTGGAGCAAA
183A	BI980350	AGCTGAAGGGTCACACTGG	GCAGAACACGTGTCATTTTC
184A	BM082811	CATGAGCCCAAGGTTTCG	CGCAGGCCTGAAGTCGTA
185A	BI984521	GGACTCGACCCCAGAGATG	CGGAAAGCAGAAGATGAGC
186A	BI980257	GGAAGACCCCGTCCTGTT	ATCCGTGCTGCTCGTCTC
187A	AI437173	TTTTGGGCCACAACCAGT	CCCCTCGTCCCGAGTAAT
188A	AW281850	AGATGCTGCAGGGTCAGG	CTGGACACAGGCCGAGAT
Below are without dnd as selection criteria			
189A	BM957986	CCGGTATTTGGGCTCCTT	TCACCCCACACACACACC
190A	BM533995	GGCCTGTTTTGCATCCTG	GCAACATGGGAGAAAAGC
191A	AI882824	GGGCGTCACTGATGTCAA	GAGGCATCGCCATTGAAG
192A	BM957900	GGACGCCAATGAAATCTCC	TCACGTGACCCATCCATTT
193A	CD014975	ATCATCGGCATGGATGTG	GTGATCTGCTGCGACACG
194A	AW019691	CTGCCCTGGTTTTGTTGG	CGCGAGCCACAAGTTTTT
195A	BC053235	AAGCGAGCGTCACAGGAC	GTCCAAAAGGTGGCGATG
196A	BM402113	GCGTTGTTCATGCAGCAG	GCCACAAAAATGCCACCT
197A	BC052137	CAGGAGGCATTTGGCTGT	TCCCACTAGCGCTCAGAA
198A	AL728293	CCCAGGCATCTCCTCTCA	ACAGCCCCTGCCTCTGTC
199A	AL718042	Not necessary, known not to be specific to PGCs	
200A	BG306502	CGCCTTGCTGTATCGTCA	GGTTGACGGTTGCATGGT
201A	BM080957	AAGGAACGTGCTGGAGGA	TGGCACCATGAATTGTCA
202A	AW115837	GGCTTCAAGAGGCCAACA	CAGGCTTCTGAGGCGATG
203A	AL926145	TGAGATCGCTGGTAAATGA	TTTATTCCTAAACAAATCTCCA
204A	NM_173222	GCAGCAGCAGCTGATTGA	GGAGGAAGGGAGGACTGG
205A	NM_131778	TTGCCTTTCTTGCCTTGC	ACGGTTTGACGCACCAGT
206A	AW019440	GCTGAAGGCACTGGAGGA	TGACCCACCAAGCAGTTT
207A	AW058961	TTTTTCAGACACAGAACTGGA	GGTTTAGTCCTCAATGACAGC
208A	NM_152953	CACATTCCAGCAGGGACT	TGCCCAACTCACACACAA
209A	BM182911	ATTCAGCTCTCATCATGTCG	TCTTGCGTGTGTTTGCTCA
210A	BM534432	TCGTGGAGGCTCCCATTA 2. Fw primer AGCAACTGCGTGTGAGCA	CGCCTGCAGCAGTATTTG 2.Rv primer CACAACATATGGACGACAGGA
211A	BG308620	GGACGAAACCGGGTGTAA	GTGCGATGGTGCTGGTCT
212A	BI705782	ACCCCCTGACCTGTGTGA	GGCAGGCACATGGAGAAC
213A	BQ262207	TTGTTGGATTGCAGTCAGGA	TCACCTTGTGTTTCTTTAGAGC
214A	NM_131128	CCAGGCCAATTTGTTCCA	CCTTGGCGATGGTCTCAT
215A	BQ077741	AAGCAATGCCTCCTGCAC	TCCTTCCAGGCACGTGAT
216A	BM184021	GCCCTGTTGTTGGTCAGG	TTGGCAAAGGAGCAGGAC
217A	AI477980	GCTGGAGCCCTACACTGC	TGAGTGAAATTTCTGCATCTGG
218A	BQ284855	ACGCACCTCACAAGCACA	AGAACGTCGCCTCGACAG
219A	AY283178	ACCGACGCGTCTATCCAG	GGGTCTTTTGGGGTCCAC
220A	BM533791	AGCCTGCTCAATCAGGTCA 2. Fw primer GAAGACTCCCGCGCAGAT	TGTACGTCCAGCGTCAGG
221A	BM103345	GGGAGAGAGCCATGACCA	TCAACTCAAAACTGGCCTGA
223A	BQ616904	GACAGACCTGCTGAGCTCCT	CCTTCATGCGCTTGGACT
224A	BC045858	AACGCCGTGGAAACAAAA	ATTGGCGCAAAAAGCAAC
225A	BM103343	AGCCTGACCAACGACTGG	TCTGCGTGTCCTTCATGC
226A	AW116113	GCTCTCAATGCGGGAAGA	CTGCTTCCTCAGTTCAGTGG
227A	BM777122	CTGGCAGGCTCAGTGGTT	CAGCCCAGAGGAGATCCA
228A	AL919330	GCCGAATGCAAAGGTGAG	GCTGGATGGTTTTCCACAA
229A	BM082834	CAAGGCCCTCCAAGGTAAA	TTGGACCACATGACCGTTT
230A	CD606373	CCTGAAGTGCGTGTGTGC	TCAATTATTCAAATAGGAGTCAG
231A	CD606429	GAGCTGACGCTGCAACAA	TCAACACCAAAAACAAACACG
232A	BM777683	GACCGTTTCCGGAAGAGAA	TTTCAAGCCATTTCAAACCA

233A	BQ618177	TGATTCATTGTTTTGCTTTATGT 2. Fw primer TGTGAGCACTGCACAGATTG	AAAACAGCTTCAACACACTCCA
234A	AI793494	AACGGACAGCCCAGTGTC	CAGCAGGTTGTCCGTGTG
235A	NM_131676	TCAATGGCGGAGTCATCA	CCAGGCAAAAATCTGCAC
236A	BI867507	GAAGACTTGCGCCTCTGG	CGAGCACAACAATGGGAGT
237A	BI887400	CCTGACCCCTCACACCTG	GTAGGTGGCGTGCATACG
238A	AL916489	TGAAGATGGTTCCCAAGATG	AAATTTGAAGCATGATTGTTAGA
239A	BI672084	TTTGGAGGTCTGGGAGGA	GCTGTGGTTTGCTGAAGGA
240A	BQ260855	CGATGTCAGATCGAGCATTAG 2. Fw primer GGCTTTTCGGAAACGTGA	AGCCACATTTGCCTGAGC
241A	AW233610	CTCCACCTGCCACCATTC	GATGGGCAGGGTTTGACA
242A	BG728987	GCGGCAAAAAGAAACAGC	GAGGACTGCTGCCAGGTC
243A	AW420842	TCCGCTGTCTTATGATTTCTCC	CCAACAAAACTTCATTTTGCAT
244A	BM184278	TCATATCTCCCAATGTTGCAC	TTTCTGCCATTTGGTTTCA

Note: 5' to all reverse primer was added following sequence to generate the T3-Promoter sequence directly at the PCR product:

5'-AATTAACCCTCACTAAAGGG-3'

6.4 Plasmids/Constructs

Table 6-3. List of constructs generated during this work

Name	Description/Cloning	Injection amount
TOPOII-*granulito*	Full length granulito (EF643555) for sequencing and antisense RNA synthesis	For antisense
	Granulito was amplified using primer TTTAAAAATGACAGAGGCAGAGG and GTTACCGATCAGATTTAATAATTGTC from ovary cDNA and cloned into TOPOII	
pSP64T-*granulito-eyfp-nos1*-3'UTR	Was used to identify subcellular localization of Granulito: Labels specifically the germinal granules	150 – 300 pg
	Granulito was amplified using primer TTTAGATCTGTTTAAAAATGACAGAGGCAG and TTTGGATCCAAGTCAGACAAGTGTTTGTAGT from ovary cDNA and cloned into a plasmid containing *EYFP-nos1*-3'UTR	
pSP64T-*granulito-morphRes-nos1*-3'UTR	Rescue mRNA for granulito, contains 5 point mutation at the morpholino binding site	90 – 600 pg
	Granulito was amplified with forward primer AAAGGATCCACCATGACGGAAGCTGAAGATCGGTGAATGAAGCTTG and reverse primer AAACTCGAGTTAGTCAGACAAGTGTTTGTAG and cloned into a vector containing the *nos1*-3'UTR	

T3-*granulito-morphRes-globin*-3'UTR	Rescue mRNA for granulito in the soma, contains 5 point mutation at the morpholino binding site	90 – 600 pg
	Granulito was amplified with forward primer AAAGGATCCACCATGACGGAAGCTGAAGATCGGTGAATGAAGCTTG and reverse primer AAACTCGAGTTAGTCAGACAAGTGTTTGTAG and cloned into a vector containing the *globin*-3'UTR	
pSP64T-*eyfp-granulito-gra*-3'UTR	Granulito coding sequence plus its own 3'UTR fused downstream of EYFP to check localization	300 pg
	Granulito cds + 3'UTR was amplified from ovary cDNA using primer AAACTCGAGATGACAGAGGCAGAGGACG and AAATCTAGAGTTACCGATCAGATTTAATAATTG and cloned into a vector containing EYFP w/o STOP	
pSP64T-*mgfp-gra*-3'UTR	Was used to check the potency of the 3'UTR of Granulito to label PGCs	150 pg
	The 3'UTR of granulito was amplified from ovary cDNA using primer AAACTCGAGATTGGAAAATTTTTATTGTTTTGTTT and AAATCTAGAGTTACCGATCAGATTTAATAATTG and cloned into a vector containing *mgfp*	
kop-gra-DsRedex1-nos1-3'UTR	Transgenic construct, maternal expression, labels germinal granules, early on. DNA was coinjected with 150 pg Tol2 transposase RNA	15 pg (DNA)
	Granulito was amplified from pSP64T-*granulito-eyfp-nos1*-3'UTR using primer AAACCATGGATGACAGAGGCAGAGGACG and GGGTCAGCTTGCCGTAGGTG and cloned into a vector containing the *kop* promoter and *DsRedex1-nos1*-3'UTR	
TOPOII-*PDCD4*	Full length *PDCD4* (BC045513.1) for antisense RNA and cloning purposes.	
	PDCD4 was amplified using primer attttaggtgctaaggcacatttc and cacatctcacatccaccagtcta from ovary + mid-somite cDNA and cloned into TOPOII	
pSP64T-*PDCD4-eyfp-nos1*-3'UTR	Was used to identify subcellular localization of *PDCD4*: It is enriched in the nucleus	300 pg
	PDCD4 was amplified using primer CAGGAAACAGCTATGACC and TTTAGATCTAAGTAGTTCTGCAGTTTGAGGT from plasmid TOPOII-*PDCD4* cloned into a plasmid containing *EYFP-nos1*-3'UTR	
TOPOII-*10A*	Full length *10A* (BM103315) for antisense RNA and cloning purposes	
	10A was amplified using primer gttgtttgaatctcgctgtgtc and tctcttggatgaaggtctctcag from ovary + mid-somite cDNA and cloned into TOPOII	

pSP64T-*10A-eyfp-nos1*-3'UTR	Was used to identify subcellular localization of *10A*: Is enriched in nucleus	300 pg
	10A was amplified using primer CAGGAAACAGCTATGACC and TTTAGATCTAATGACTCCCTTTCGGTCTGGGTC from plasmid TOPOII-*10A* cloned into a plasmid containing *EYFP-nos1*-3'UTR	
TOPOII-*GKAP1*	Full length *GKAP1* (BI889921) for antisense RNA and cloning purposes	
	GKAP1 was amplified using primer gtgtagcagtttccacttgcac and gaacttaagattgttggccgttc from ovary + mid-somite cDNA and cloned into TOPOII	
pSP64T-*GKAP1-eyfp-nos1*-3'UTR	Was used to identify subcellular localization of *GKAP1*: cytoplasmic	300 pg
	GKAP1 was amplified using primer TTTAAGCTTTGGATATCTGCAGAATTCGC and TTTAGATCTAATACATCCCCTTCTGAGGCTT from plasmid TOPOII-*GKAP1* cloned into a plasmid containing *EYFP-nos1*-3'UTR	
TOPOII-*115A*	Full length *115A* (BM342614) for antisense RNA and cloning purposes	
	115A was amplified using primer tgtctttaggtttcacggtgtg and cacgtgacccatccatttattt from ovary + mid-somite cDNA and cloned into TOPOII	
pSP64T-*115A-eyfp-nos1*-3'UTR	Was used to identify subcellular localization of *115A*: dots within nucleus	300 pg
	115A was amplified using primer CAGGAAACAGCTATGACC and TTTGGATCCGGCTTCTCGTCTTTGCCTTTGG from plasmid TOPOII-*115A* cloned into a plasmid containing *EYFP-nos1*-3'UTR	
TOPOII-*116A*	Full length *116A* (BC049455.1) for antisense RNA and cloning purposes	
	116A was amplified using primer tcgtaaacgaacgatttactttga and tcaaaaccaatcaaataggaataatg from ovary + mid-somite cDNA and cloned into TOPOII	
pSP64T-*116A-eyfp-nos1*-3'UTR	Was used to identify subcellular localization of *116A*: nucleus/geminal granules	300 pg
	116A was amplified using primer CAGGAAACAGCTATGACC and TTTGGATCCGGATCATCAAAGCGGATGTGAT from plasmid TOPOII-*116A* cloned into a plasmid containing *EYFP-nos1*-3'UTR	
TOPOII-*151A*	Full length *151A* (BM530072) for antisense RNA and cloning purposes	
	151A was amplified using primer AGGAAGAAGAGGACAGAATCGAG and TGAAAAGCATCCATAAAAACTTGA from ovary + mid-somite cDNA and cloned into TOPOII	

pSP64T-*151A-eyfp-nos1*-3'UTR	Was used to identify subcellular localization of *151A*: Is enriched in nucleus	300 pg
	151A was amplified using primer CAGGAAACAGCTATGACC and TTTGGATCCGGCTCTTTGTCACCATCATTATC from plasmid TOPOII-*151A* cloned into a plasmid containing *EYFP-nos1*-3'UTR	
TOPOII-*RAD51*	Full length *RAD51* (AI437173) for antisense RNA and cloning purposes	
	RAD51 was amplified using primer GCAGCAGCAGTAGCTTAGCG and AGGAAAAAGTCCACACAGACAAA from ovary + mid-somite cDNA and cloned into TOPOII	
pSP64T-*RAD51-eyfp-nos1*-3'UTR	Was used to identify subcellular localization of *RAD51*: Cytoplasmic	450 pg
	RAD51 was amplified using primer CAGGAAACAGCTATGACC and TTTGATATCAAGTCTTTAGCATCTCCCACTC from plasmid TOPOII-*RAD51* cloned into a plasmid containing *EYFP-nos1*-3'UTR	
TopoII-*TDRD7*	Full length TDRD7 (EF643554) for sequencing and antisense RNA synthesis	
	TDRD7 was amplified using primer CGCATTAACGGCGAAAAA and GCAAACAAACCAAAGTGCAA from ovary cDNA and cloned into TOPOII	
pSP64T-*Tdrd7-eyfp-nos1*-3'UTR	Was used to identify subcellular localization of TDRD7: Labels germinal granules but less specific as Granulito	540 pg
	TDRD7 was amplified using primer TTTAAGCTTTGGATATCTGCAGAATTCGC and TTTAGATCTAAAGCATCTCTGCTGATCTCATC from ovary cDNA and cloned into a plasmid containing *EYFP-nos1*-3'UTR	
pSP64-*mgfp-Tdrd7*-3'UTR	Was used to check the potency of the 3'UTR of TDRD7 to label PGCs	90 – 240 pg
	The 3'UTR of TDRD7 was amplified with primer AAACTCGAGTACTCTCAGAACTGCACTTTC and AAATCTAGATAATACAACAAAACCTGAACACC and cloned into a plasmid containing *mgfp*	
pSP64-*morphRes* -*Tdrd7*-3'UTR	Rescue mRNA for Tdrd7, contains 5 point mutations at the morpholino binding site	540 pg
	TDRD7 was amplified with primer TTTAGATCTACCATGAGCGATGTCGAATTAGTGAAGAAGATGCTGCG AGC and AAAGTCGACTCAAGCATCTCTGCTGATCTC and cloned into a plasmid containing the 3'UTR of TDRD7	

TopoII-*RNF17*	Partial sequence of *RNF17* (XM_692362.1) for antisense RNA synthesis	
	Partial sequence of *RNF17* was amplified using primer ACCAGCCCAAGTCAAACAAC and AATTAACCCTCACTAAAGGGAACACTGGTCTGGTGGAAGG from cDNA mix (ovary + mid somites + 3dpf) and cloned into TOPOII	
TopoII-*TDRD1*	Partial sequence of *TDRD1* (XM_679932) for antisense RNA synthesis	
	Partial sequence of *TDRD1* was amplified using primer TGTCTTGCAGTGGCACTTTC and AATTAACCCTCACTAAAGGGCAAGCAGGAGAACCAACTCC from cDNA mix (ovary + mid somites + 3dpf) and cloned into TOPOII	
TopoII-*TDRD5*	Partial sequence of *TDRD5* (XM_681163) for antisense RNA synthesis	
	Partial sequence of *TDRD5* was amplified using primer CTGGTGTCAAAGCAACGAGA and AATTAACCCTCACTAAAGGGCCTGTTGGACTGGAAGTGGT from cDNA mix (ovary + mid somites + 3dpf) and cloned into TOPOII	
TopoII-*TDRD6p1*	Partial sequence of *TDRD6p1* (XM_687668) for antisense RNA synthesis	
	Partial sequence of *TDRD6p1* was amplified using primer CCCATTCAGGCTGTTCAGTT and AATTAACCCTCACTAAAGGGTTTTCACCTGCTGCCTCTTT from cDNA mix (ovary + mid somites + 3dpf) and cloned into TOPOII	
TopoII-*TDRD6p2*	Partial sequence of *TDRD6p2* (XM_688932) for antisense RNA synthesis	
	Partial sequence of *TDRD6p2* was amplified using primer GGTGCACAGCACGAGTTTTA and AATTAACCCTCACTAAAGGGTTTTTCACTCTCGGGCTCAT from cDNA mix (ovary + mid somites + 3dpf) and cloned into TOPOII	
TopoII-*TDRD6p3*	Partial sequence of *TDRD6p3* (BX000362) for antisense RNA synthesis	
	Partial sequence of *TDRD6p3* was amplified using primer GACCAATTTGGATCCACCAC and AATTAACCCTCACTAAAGGGAATGCAATGCAGAGCGTAA from cDNA mix (ovary + mid somites + 3dpf) and cloned into TOPOII	
pSP64-*DsRedEx*-*Tdrd7*-3'UTR	To label germ cells red using the TDRD7 3'UTR (used as internal control for mutation experiments)	240 pg
	The 3'UTR of TDRD7 was amplified with forward primer AAACTCGAGTACTCTCAGAACTGCACTTTC and reverse primer AAATCTAGATAATACAACAAAACCTGAACACC and cloned into plasmid containing *DsRedEx*	

pSP64-*YPet-Tdrd7*-3'UTR	To label germ cells yellow using the TDRD7 3'UTR (used as internal control for mutation experiments)	240 pg
	The 3'UTR of TDRD7 was amplified with forward primer AAACTCGAGTACTCTCAGAACTGCACTTTC and reverse primer AAATCTAGATAATACAACAAAACCTGAACACC and cloned into plasmid containing *Ypet*	
pSP64T-*ecfp-nos1*-3'UTRmut1	Mutation at the MiR-430 binding site of the nos1-3'UTR	180 pg
	Plasmid pSP64T-*ecfp-nos1*-3'UTR was amplified with primer GTCTTTTTGTGTGTGTGTAT and CAAAATCAAACAGTGAACGC	
pSP64T-*mgfp-nos1*-3'UTRmut2	Mutation at the DND interacting sequence 1 of the nos1-3'UTR	180 pg
	Plasmid pSP64T-*mgfp-nos1*-3'UTR was amplified with primer CAGCACTTTTTGTGTGTGTGTATA and GCTCAAACAGTGAACGCACACAT	
pSP64T-*mgfp-nos1*-3'UTRmut3	Mutation at the DND interacting sequence 2 of the nos1-3'UTR	180 pg
	Plasmid pSP64T-*mgfp-nos1*-3'UTR was amplified with primer CAGTGTGCACTGGTGTTGTGTT and GCTAAAACACAGCAAACACACACA	
pSP64T-*mgfp-nos1*-3'UTRmut2+3	Mutation at both DND interacting sequence 1+2 of the nos1-3'UTR	180 pg
	Plasmid pSP64T-*mgfp-nos1*-3'UTRmut2 was amplified with primer CAGTGTGCACTGGTGTTGTGTT and GCTAAAACACAGCAAACACACACA	
pSP64T-*mgfp-nos1*-3'UTRmut1+3	Mutation at DND interacting sequence 2 and the MiR-430 binding site of the nos1-3'UTR	180 pg
	Plasmid pSP64T-*mgfp-nos1*-3'UTRmut1 was amplified with primer CAGTGTGCACTGGTGTTGTGTT and GCTAAAACACAGCAAACACACACA	
pSP64T-*mgfp-nos1*-3'UTRmut1+2+3	Mutation at both DND interacting sequence and the MiR-430 binding site of the nos1-3'UTR	180 pg
	Plasmid pSP64T-*mgfp-nos1*-3'UTRmut2+3 was amplified with primer GTCTTTTTGTGTGTGTGTAT and ACCTGGCTCAAACAGTGAACGC	

pSP64T-*mgfp-TDRD7*-3'UTRmut1	Mutation at the MiR-430 binding site of the TDRD7-3'UTR	180 pg
	Plasmid pSP64T-*mgfp-TDRD7*-3'UTR was amplified with primer TCTTTGGTTTGTTTTGCTGTGTTT and CCAAAATCAAAAAGTACAAACAATG	
pSP64T-*ecfp-TDRD7*-3'UTRmut1	To follow the TDRD7 MiR-430 binding site mutation in the cyan channel	180 pg
	The MiR-430 binding site mutated 3'UTR of TDRD7 was subcloned into a plasmid containing *ecfp*	
pSP64T-*mgfp-TDRD7*-3'UTRmut2	Mutation at the DND interacting sequence of the TDRD7-3'UTR	180 pg
	Plasmid pSP64T-*mgfp-TDRD7*-3'UTR was amplified with primer CAGCACTTTGGTTTGTTTGCT and GCTCAAAAAGTACAAACAATGC	

Table 6-4. List of other plasmids used in this work kindly provided by collegues and former lab members

Name	**Description**	**RNA/DNA conc.**
pSP64T-*mgfp-nos1*-3'UTR	Labels PGC green (Köprunner et al., 2001)	150 – 300 pg
pSP64T-*ecfp-nos1*-3'UTR	Labels PGC cyan blue	180 pg
T3-*venus-nos1*-3'UTR	Labels PGC yellow	180 pg
pSP64T-*mgfp-vasa*-3'UTR	Labels PGC with GFP, (not as specific as the *nos1*-3'UTR) (Wolke et al., 2002)	90 pg
pSP64T-*DsRedex1-nos1*-3'UTR	Labels PGC with DsRedex1	300 pg
H1M-gfp-globin-3'UTR	Labels all nuclei in the embryo with GFP	60 pg
T3-*vasa-DsRedex1-nos1*-3'UTR	Labels the germinal granules in PGCs with DsRedex1	300 pg
pSP64T-*Clip170-gfp-nos1*-3'UTR	Labels the microtubular network in PGCs	300 pg
pSP64T-*egfp-Dyn2L-nos1*-3'UTR	Labels Dynein localization in PGCs	300 pg
pCS-Tol2-transposase	Transposase used for coinjection with DNA containing Tol2 sites for genomic integration (transgenic). (Kawakami et al., 2004)	150 pg

6.5 Antisense probes for in situ hybridization

From all PCR products from the candidate genes of the Affymetrix screen DIG-labeled antisense probes were direcly synthesised. The list below contains only additionally used antisense probes.

Table 6-5. List of antisense Probes

Name	Description of Expression pattern
Nanos1-Dig	Nanos1 RNA is localized first to the germ plasm and later restricted to germ cells
Vasa-Dig	Vasa RNA is localized first to the germ plasm and is later restricted to germ cells with some additional tissues in the embryo
GFP-Dig	GFP RNA is only detected upon injection of RNA, which includes GFP sequence into the zebrafish embryo
H1m-Dig	H1m RNA is zygotically expressed in PGCs but not part of the germ plasm
Granulito-Dig	Granulito RNA is localized to the germ plasm and is then weakly expressed in Germ cells until 24 hpf
TDRD7-DIG	TDRD7 RNA is specifically expressed in the germ plasm and is later restricted to the germ cells until at least 7 dpf
RNF17-DIG	RNF17 RNA was first detected in germ cells of 24 hpf old embryos. Germ cells specific expression was confirmed till at least 7 dpf
TDRD1-DIG	TDRD1 RNA was first detected in germ cells of 48 hpf old embryos. Germ cells specific expression was confirmed till at least 7 dpf
TDRD5-DIG	No specific expression was detected for the first 7 days of development
TDRD6-DIG	Mix of the 3 paralogs (see constructs above); Weak expression in germ cells was detected at 3 dpf till 7 dpf

6.6 Morpholino antisense oligonucleotides

Table 6-6. List of morpholinos

Name	Conc.	Sequence
cnt-Mo	600 pg – 2 mM	5’-CCTCTTACCTCAGTTACAATTTATA-3’
dnd-Mo	600 pg	5’-GCTGGGCATCCATGTCTCCGACCAT-3’
4A -PDCD4	200 µM – 1,6 µM	5’-CCATGCCTCGCAATCAGTTGCCATG-3’
7A	50 µM – 1 mM	5’-CGTCCTCTGCCTCTGTCATTTTTAA-3’

7A-2	50 µM – 1 mM	5'-TCCTCTGCCTCTGTCATTTTTAAAC-3'
7A-3	50 µM – 1,6 mM	5'-TCCATTTTTATGTGCTCAAAGCTCC-3'
10A-Mo	200 µM – 1 mM	5'-AACCTGTAGTGAACACAAGACATAC-3'
52A-GKAP1	200 µM – 1 mM	5'-GAGATCACTGCTGATGCCATCCTAG-3'
115A	200 µM – 1 mM	5'-ACGTCTAGCTCAGGCATTACTACAC-3'
116A	200 µM – 1,2 mM	5'-GTCTTGCGTTGACACACTTTCATCC-3'
151A	200 µM	5'-CCTTTGCGCTTTCGGTCACTCATTG-3'
187A-RAD51	200 µM – 1,2 mM	5'-CATATTTACTCCCGCTAAGCTACTG-3'
197A-TDRD7	100 µM – 600 µM	5'-AACCAACTCCACGTCACTCATCCTG-3'
197A-2	100 µM – 600 µM	5'-TCCTGCCGTTTTCTCTTCACACTTG-3'
RNF17	200 µM – 1,2 mM	5'-TACACGTTACCGCTGTCTCCGACAT-3'
dre-miR430a-2	80 µM – 300 µM	ACTACCCCAACAAATAGCACTTACCAATTAC
dre-miR430b-1	80 µM – 300 µM	TCTACCCCAACTTGATAGCACTTTCTACTTT
dre-miR430c-1	80 µM – 300 µM	ACTACCCCAAAGAGAAGCACTTATGAACAAA
dre-miR430i	80 µM – 300 µM	ACTACGCCAACAAATAGCACTTACCAAATAC
dre-miR430j	80 µM – 300 µM	CTACCCCAATTTGATAGCACTTTCTACTTTG

6.7 Antibodies

Table 6-7. List of primary antibodies

Name	Epitope	Host	Conc.	Company/Publication
anti-GFP	GFP, monoclonal	mouse	1:200	Santa Cruz Biotechnology
anti-vasa	Zebrafish vasa (K12/4)	rabbit	1:2000	(Knaut et al., 2000)

Table 6-8. List of secondary antibodies

Name	Host	Fluorophore	Conc.	Company
Alexa Fluor 488-anti-mouse IgG	goat	Alexa Fluor 488- conjugated (green)	1:200	Molecular Probes
Alexa Fluor 546-anti-rabbit IgG	goat	Alexa Fluor 546-conjugated (red)	1:200	Molecular Probes

6.8 Kits

Table 6-9. List of Kits

Product-Name	Company	Description
5'RACE system	Invitrogen	Rapid amplification of cDNA 5' ends
MessageAmp™	Ambion	Representational amplification of small amounts of RNA for array analysis
OmniScript RT Kit	QIAGEN	Standard reverse transcription
QIAquick Gel Extraction Kit	QIAGEN	DNA fragment (70 bp – 10 kb) extraction from TAE/TBE agarose gels
QIAquick PCR Purification Kit	QIAGEN	Direct purification of double- or single-stranded PCR products (100 bp – 10 kb)
PCR clean-up / Gel extraction	Macherey-Nagel	Direct purification of PCR fragments (65 bp – 10 kb) and DNA extraction from TAE/TBE agarose gels in one kit
QIAprep Spin Miniprep Kit	QIAGEN	Plasmid DNA isolation from bacteria cultures (Minicultures up to 20 µg DNA)
Plasmid DNA Purification	Macherey-Nagel	Plasmid DNA isolation from bacteria cultures (Minicultures ~2 ml)
QIAfilter Plasmid Midi Kit	QIAGEN	For fast purification of up to 100 µg transfection grade plasmid DNA
EndoFree Plasmid Kit	QIAGEN	Endotoxin-free ultrapure Plasmid DNA isolation
Phusion Kit	Finnzymes	High fidelity DNA amplification/PCR
TOPO TA Cloning Kit	Invitrogen	Enables fast, efficient cloning of Taq-amplified PCR products
Message Machine Kit	Ambion	Capped sense RNA

6.9 Tools/Hardware

Table 6-10. List of Tools and Hardware

Product Description	Company
PN-30 Needle puller	Science Product
Microinjector PV830 Pneumatic PicoPump	World precision Instruments
Cell tram (transplantation apparatus)	Eppendorf
MZ FLIII stereomicroscope	Leica
Confocal microscope TCS SL	Leica

Axioplan 2 microscope	Zeiss
RT slider spot camera	Diagnostic Instruments
RT SE spot camera	Diagnostic Instruments
GeneChip® Hybridization Oven 640	Affymetrix
GeneChip® Fluidics Station 450	Affymetrix
GeneChip® Scanner 3000	Affymetrix
PCR machine Cyclone 96	PEQLAB
PCR machine Mastercycler personal	Eppendorf
PCR machine MyCycler	Bio Rad
Insitu machine BioLane HTI	Hölle & Hüttner AG
water bath WB-7	Memert GmbH
pH-meter MP220	Mettler Toledo
Electroporation MicroPulser	Bio Rad
BioPhotometer Eppendorf for DNA, RNA and protein concentrations	Eppendorf
Gel chambers ComPhor L Mini, Midi	Bioplastics BV
Power supply EC105	E-C Apparatus Corporation
Bacteria incubator Type 3031	GFL
Incubator Oven FD (for Agarplates)	Binder
Heating block Type BBA2	Grant-Boekel
Cooling block Thermomixer comfort	Eppendorf
Centrifuge Biofuge primo R, rotor #7590, max. 8500 rpm	Heraeus
Centrifuge Eppendorf 5415D, rotor F45-24-11, max. 13200 rpm	Eppendorf
Fish incubator BK600	Heraeus, Kendro

6.10 Software

Table 6-11. List of Tools and Hardware

Software-Name	Product Description
Vector NTI (Invitrogen)	Application for DNA and protein sequence analysis.
dChip (Harvard, Cheng Li Lab) http://biosun1.harvard.edu/complab/dchip/	Analyses gene expression data from microarray data (e.g. Affymetrix)
ZFIN (The Zebrafish Information Network) http://zfin.org	ZFIN serves as the zebrafish model organism database.
The *Danio rerio* Sequencing Project (Sanger Institute) http://www.sanger.ac.uk/modelorgs/zebrafish.shtml	the genome sequencing project of the zebrafish (*Danio rerio*) following two strategies: clone mapping and sequencing from BAC and PAC libraries and whole genome shotgun sequencing with subsequent assembly
PubMed http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed	A service of the National Library of Medicine and the National Institutes of Health. Tool for literature search
NCBI-BLAST (Basic Local Alignment Search Tool) http://130.14.29.110/BLAST/	Finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches
The ExPASy (**Ex**pert **P**rotein **A**nalysis **Sy**stem) proteomics server http://expasy.org/	Used for the analysis of protein sequences and structures
ClustalW (EMBL-EBI) http://www.ebi.ac.uk/clustalw/	Tool to generate multiple alignments
Primer3 (Whitehead Institute for Biomedical Research) http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi	Tool to identify optimal primer pairs for amplification of a given sequence
Sequencher 4.5 (Gene Codes Corporation)	Aligns sequences in respect of their similarity to create contigs
Metamorph (Universal Imaging)	Image acquisition/archival system, processing capabilities (motion analysis, co-localization, multi-dimensional acquisition and analysis, etc.)
Leica confocal software	Performs system control, 2D-3D imaging and processing
MS Office (Microsoft Inc.)	Software package for writing documents (word), analysing and graphic representation of data (excel), etc
Adobe Photoshop	Image processing
ImageJ http://rsb.info.nih.gov/ij/	Image processing and analysis

6.11 Molecular Biology – general protocols

6.11.1 Germ cell isolation

Fertilized zebrafish eggs were co-injected with 240 pg *mgfp-nos1*-3'UTR and 600 pg dead end morpholino or control morpholino, respectively. Embryos were incubated at 28°C until 13 hpf. They were then selected for good germ cell labelling and little background signal under UV Stereomicrocope. Selected embryos were decorionated and 3 embryos were transferred at a time onto a glass-petridish, having 200 µL TM1-buffer in the center. Embryos were dissociated by pipetting up and down with a 20 µl pipettor. Cells needed 2-3 minutes to settle down on the glass. Germ cells were identified by the GFP signal under the UV-Stereomicroscop and by morphology under light. Cells were collected using cell tram. (Neddle were opened by using forceps, width approx. 1,5 cell diameter.) After collecting 5 to 10 germ cells, avoiding somatic cells, they were transferred onto a 5 µl drop of TM1-buffer on a glass slide and immediately frozen on dry ice. The frozen droplet was transferred into a tube and stored on dry ice. A total of about 300 control morphant germ cells, 300 dead end morphant germ cells and 300 somatic cells (selected for no GFP signal and smaller cell size) were isolated.

TM1-Buffer

NaCl	100mM
KCL	5mM
Hepes	5mM
PEG 20000	1%

PH 7.0- Filter though 0.2 micron filter

6.11.2 RNA isolation from 300 cells

Protocol:

1. Add 10 times Trizol (Gibco BRL-Life Technologies) to the original volume of the sample. Vortex thoroughly.

2. Incubate the homogenized samples for 5 minutes at RT. Add 0.2 ml of chloroform per 1 ml of Trizol Reagent. Shake tubes vigorously by hand for 15 seconds and incubate them at RT for 2 to 3 minutes. Centrifuge the samples at maximum speed for 15 minutes at 4°C.

Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of Trizol Reagent used for homogenization.

3. Transfer the aqueous phase to a fresh tube. Add 10 μg/ml glycogen and add 0.8 volume of isopropanol to each tube. Incubate samples over night at-20°C and centrifuge at maximum speed for 15 minutes at 4°C.
   The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4. Remove the supernatant. Wash the RNA pellet with 80% ethanol, adding 1 ml of 80% ethanol to the tube. Mix the sample by vortexing and centrifuge at maximum speed for 10 minutes at 4°C.

At the end of the procedure, remove with a thin pipette all remaining liquid and air-dry the RNA pellet for 5 min. Dissolve RNA in RNase-free water (6 µl per tube) by passing the solution a few times through a pipette tip, and incubating for 5 minutes at room temperature.

6.11.3 Target synthesis for Affymetrix Microarray

The Message Amp kit and protocol (Ambion) was adapted to be compatible with the Affymetrix arrays.

Protocol:

A. First Strand cDNA Synthesis

Table 6-12. Components for first strand cDNA Synthesis

Component	**Volume**
Isolated RNA	5,5 µl
T7 Oligo(dT) Primer	0.5 µl

Mix and incubate for 10 min at 70 °C, centrifuge and place on ice.

10X First Strand Buffer	1 µl
Ribonuclease Inhibitor	0.5 µl
dNTP Mix	2 µl
Reverse Transcriptase	0.5 µl

Mix well by gently pipetting up and down or flicking the tube a few times. Centrifuge briefly (~5 sec) to collect the master mix at the bottom of tube and place on ice and incubate for 2 h at 42 °C in a thermocycler.
After incubation centrifuge tubes briefly, place on ice and proceed immediately.

B. Second Strand cDNA Synthesis

Table 6-13. Components for second strand cDNA Synthesis

Component	Volume
cDNA sample (from above)	10 µl
Nuclease-free Water	31.5 µl
10X Second Strand Buffer	5 µl
dNTP Mix	2 µl
DNA Polymerase	1 µl
RNase H	0.5 µl

Flick tube a few times to mix, centrifuge briefly and incubate for 2 h at 16°C in thermocycler. After incubation proceed directly or freeze at –20°C.

C. cDNA Purification

Table 6-14. Protocol for cDNA Purification

Step	Description
1	Before beginning the cDNA purification, preheat the 5 ml bottle of Nuclease-free Water to 50°C for at least 10 min.
2	Equilibrate one cDNA Filter Cartridge per cDNA sample, immediately before starting the cDNA purification: a. Check that the cDNA Filter Cartridge is firmly placed in a 2 ml Wash Tube (supplied), and pipet 50 µl cDNA Binding Buffer onto the filter in the cDNA Filter Cartridge. b. Incubate at room temperature for 5 min. Do NOT spin the cDNA Binding Buffer through the cDNAFilter Cartridge.
3	Add 250 µl of cDNA Binding Buffer to each cDNA sample and mix thoroughly by repeated pipetting or gentle vortexing.
4	Apply DNA sample on equilibrated cDNA Filter Cartridge a. Pipet the cDNA sample/cDNA Binding Buffer (from step 3) onto the center of an equilibrated cDNA Filter Cartridge (from step 2).

	b. Centrifuge for ~1 min at 10,000 x g, Add flow-through over the column again and repeat centrifugation. c. Discard the flow-through and replace the cDNA Filter Cartridge in the 2 ml Wash Tube.
5	Wash the cDNA Filter Cartridge with 500 µl cDNA Wash Buffer a. Apply 500 µl cDNA Wash Buffer to each cDNA Filter Cartridge. b. Centrifuge for ~1 min at 10,000 x g. c. Discard the flow-through and spin the cDNA Filter Cartridge for an additional minute to remove trace amounts of ethanol. d. Transfer cDNA Filter Cartridge to a cDNA Elution Tube.
6	Elute cDNA with 2 X 10 µl Nuclease-free Water a. To the center of the filter in the cDNA Filter Cartridge, apply 10 µl of Nuclease-free Water that is preheated to 50°C. b. Leave at room temperature for 2 min and then centrifuge for ~1.5 min at 10,000 x g, or until all the Nuclease-free Water is through the filter. c. Elute with a second 10 µl of pre-heated Nuclease-free Water. The double-stranded cDNA will now be in the eluate (~16 µl). d. Discard the cDNA Filter Cartridge.
7	Bring the volume of the cDNA samples to 8 µl via speed vac Speed vac for approx. 4 min and check the volume, if the volume is 8 µl, place samples on ice or at –20°C. Transfer the 8 µl of cDNA to a 0.5 ml siliconized tube.

D. In Vitro Transcription to Synthesize antisense RNA (aRNA)

Assemble the 20 µl transcription reaction components at RT in the order shown.

Table 6-15. Components for aRNA transcription

Component	Volume
double-stranded cDNA	8 µl
T7 ATP Soln (75 mM)	2 µl
T7 CTP Soln (75 mM)	2 µl
T7 GTP Soln (75 mM)	2 µl
T7 UTP Soln (75 mM)	2 µl
T7 10X Reaction Buffer	2 µl
T7 Enzyme Mix	2 µl

Flick tube a few times to mix, centrifuge tubes briefly and incubate the reaction for 14 h at 37°C.

Add 1 µl DNase I to each reaction, mix and incubate 30 min at 37°C. After incubation add 80 µl Elution Solution to each aRNA sample, mix thoroughly by repeated pipetting. Transfer to a 1.5 ml tube.

E. aRNA Purification

Table 6-16. Protocol for aRNA Purification

Step	Description
1	Preheat Nuclease-free Water or Elution Solution to 50–60°C (≥10 min)
2	Add 350 µl of antisense RNA (aRNA) Binding Buffer to aRNA sample, and mix by repeated pipetting.
3	Add 250 µl of ACS grade 100% ethanol to aRNA sample, and mix by repeated. Proceed immediately to the next step.
4	Apply mixture to an aRNA Filter Cartridge(s) a. Place an aRNA Filter Cartridge in an aRNA Collection Tube, and pipet each sample mixture from step 3 onto the center of the filter in the aRNA Filter Cartridge. b. Centrifuge for ~1 min at 10,000 X g, Add flow-through over the column again and repeat centrifugation. c. Discard the flow-through and replace the aRNA Filter Cartridge in the aRNA Collection Tube.
5	Wash the aRNA Filter Cartridge(s) with 650 µl aRNA Wash Buffer a. Apply 650 µl aRNA Wash Buffer to each aRNA Filter Cartridge. b. Centrifuge for ~1 min at 10,000 x g. c. Discard the flow-through and spin the aRNA Filter Cartridge for an additional ~1 min to remove trace amounts of ethanol. d. Transfer Filter Cartridge(s) to a fresh aRNA Collection Tube.
6	Elute aRNA with 2 X 50 µl preheated Nuclease-free Water a. To the center of the filter, add 50 µl Nuclease-free Water that is preheated to 50°C. Replace Nuclease-free Water back in the 50°C incubator. b. Leave at room temperature for 2 min and then centrifuge for ~1.5 min at 10,000 x g. c. Repeat the elution with a second 50 µl Nuclease-free Water. The aRNA will now be in the aRNA Collection Tube in 100 µl of the solution used for elution. d. Discard the aRNA Filter Cartridge.

Measure aRNA concentration: Dilute 2 µl of the eluted aRNA in 98 µl nuclease free water (ambion). And measure at the eppendorf spectrometer.

F. Spike in of controls

Table 6-17. Dilutions for Spike in of controls

Total RNA	First	Second	Third	Spike in volume
1 µg	1:20	1:50	1:50	2 ul
5 µg	1:20	1:50	1:10	2 ul
10 µg	1:20	1:50	1:5	2 ul

The dilutions are made in the poly-A control dilution-buffer. The first dilution can be stored for 6 weeks at -20 C and thawed up to 8 times.

G. Second Round, first strand synthesis

Have the desired amount of RNA concentrated in 10 µl dH_2O.

Table 6-18. Components for second round of first stand cDNA synthesis

Component	**Volume**
aRNA	9 µl
T7 Oligo(dT) Primer	1 µl

Mix and incubate for 10 min at 70 °C, centrifuge and place on ice.

10X First Strand Buffer	2 µl
Ribonuclease Inhibitor	1 µl
dNTP Mix	4 µl
Reverse Transcriptase	1 µl

Mix well by flicking the tube a few times. Centrifuge briefly (~5 sec) and place on ice and incubate for 2 h at 42 °C in a thermocycler. After incubation centrifuge tubes briefly, place on ice and proceed immediately.

H. Second Strand synthesis

Table 6-19. Components for second round of second stand cDNA synthesis

Component	**Volume**
cDNA sample (from above)	20 µl
Nuclease-free Water	63 µl
10X Second Strand Buffer	10 µl
dNTP Mix	4 µl
DNA Polymerase	2 µl
RNase H	1 µl

Flick tube a few times to mix, centrifuge briefly and incubate for 2 h at 16°C in thermocycler. After incubation proceed directly or freeze reactions immediately at–20°C.

I. cDNA purification

Proceed exactly as described in C (above) until step 6.

Add 7.5 ul 10 mM biotin-11-CTP and 7.5 ul 10 mM biotin-16-UTP to the eluted cDNA sample and bring the volume of the cDNA samples to 18 µl via speed vac. Transfer the 18 µl of cDNA to a 0.5 ml siliconized tube and place on ice or store at –20°C.

J. In vitro Transcription

Assemble the 20 µl transcription reaction components at RT in the order shown.

Table 6-20. Components for biotinylated aRNA transcription

Component	**Volume**
double-stranded cDNA, biotin CTP, UTP mixture	18 µl
T7 ATP Soln (75 mM)	4 µl
T7 CTP Soln (75 mM)	3 µl
T7 GTP Soln (75 mM)	4 µl
T7 UTP Soln (75 mM)	3 µl
T7 10X Reaction Buffer	4 µl
T7 Enzyme Mix	4 µl

Flick tube a few times to mix, centrifuge tubes briefly and incubate the reaction for 14 h at 37°C

Add 2 µl DNase I to each reaction, mix and incubate 30 min at 37°C. After incubation add 60 µl Elution Solution to each aRNA sample, mix thoroughly by repeated pipetting. Transfer to a 1.5 ml tube.

K. aRNA Purification

As described in E (above).

L. RNA fragmentation

Note: It is usually best to perform the fragmentation on the same day as hybridization Whenever it is possible, to minimize freeze-thawing effects on aRNA.

Table 6-21. Protocol for RNA fragmentation

Step	Description
1	Quantify aRNA concentration and A260/A280 ratio. aRNA must be at a minimum concentration of 0.6 µg/µl. If it is not, concentrate with speed vac. A260/A280 ratio should be between 1.9 and 2.1.
2	Use 2 µl of 5X Fragmentation Buffer for every 8 µl of aRNA plus water together (ratio is 2:8). Optimal: 20 µg aRNA in a 40 µl fragmentation reaction.
3	Incubate at 94°C for 35 minutes. Cool to 4°C and place on ice.
4	Check quality of aRNA by Agilent Bioanalyzer using mRNA Smear Nano Assay. Run 100 ng of unfragmented aRNA side-by-side with 1 µl fragmented undiluted aRNA directly from the fragmentation reaction. The assay should reveal long unfragmented aRNAs, majority length greater than 400 bp, and fragmented cRNAs ideally between 35 and 200 bp.
5	Use all remaining fragmented aRNA in the target hybridization cocktail immediately.

5X Fragmentation Buffer

Tris-acetate 200 mM
KOAc 500 mM
MgOAc 150 mM
PH 8.1- Filter though 0.2 vacuum filter

Aliquot and store at room temperature.

6.11.4 Target hybridization on Affymetrix zebrafish microarray chip

Protocol:

Equilibrate probe array at RT before use and heat 20x eukaryotic hybridization controls at 65 °C for 5 min.

Table 6-22. Hybridization mix

Component	Volume/amount
fragmented aRNA	15 µg
control oligonucleotide B2 (3 nM)	5 µl
20 x eukaryotic hybridization controls	15 µl
Herring sperm DNA (10 mg/ml)	3 µl
BSA (50 mg/ml)	3 µl
2x Hybridization buffer	150 µl
DMSO	30 µl
DEPC-water	up to 300 µl

Table 6-23. Protocol for target hybridization on Affymetrix zebrafish microarray

Step	Description
1	Heat hybridization mix (hyb mix) for 5 min at 95 °C
2	Transfer the hyb mix from the 95 °C block to 45 °C for 5 min
3	Spin hyb mix at max speed for 5 min
4	Pre-wet the array with 250 µl 1 x hyb buffer. Use 2 pipet tips, one for filling and one for venting
5	Remove buffer solution from the array and add 250 µl hyb mix. Avoid any insoluble matter at the bottom of the tube
6	Place array in to the hyb oven. Balance it and rotate at 60 rpm, set to 45 °C and hybridize for 16 h

2x Hybridization buffer

8.3 ml	12x MES stock buffer
17.7 ml	5 M NaCl
4 ml	0.5 M EDTA
0.1 ml	10 % Tween 20
19.9 ml	water

Store at 4 to 8 °C and shield from light.

12x MES stock buffer

64.61 g	MES hydrate
193.3 g	MES sodium salt
800 ml	water

Mix and adjust to 1l. pH=6.6,
Steril filter and store at 2 to 8°C
Shield from light.

Washing

After 16 h hybridization, remove hyb mix and fill with 250 µl wash buffer A.

Washing and antibody staining is done at the Affymetrix workstation as well as scanning the chips and transforming them into intensity values for each spot.

Buffers:

20x SSPE

3 M	NaCl
0,2 M	NaH_2PO_4
0,02 M	EDTA

Wash buffer A

300 ml	20x SSPE
1 ml	10 % Tween 20
699 ml	water

Sterile filter

Wash buffer B

83,3 ml	12x MES
5,2 ml	5 M NaCl
1 ml	10% Tween 20
910,5 ml	water

Sterile Filter and shield.

Prepare Staining reagents:

SAPE-stain solution

600 µl	2x stain buffer
48 µl	50 mg/ml BSA
12 µl	1mg/ml SAPE
540 µl	DI water

Mix and divide into 2 aliquot of 600 µl

2x Stain buffer

41.7 ml	12 x MES stock buffer
92.5 ml	5 M NaCl
2.5 ml	10 % Tween 20
113.3 ml	Water

Filter and store at 2 - 8 °C, shield

Antibody solution:

300 µl	2 x stain buffer
24 µl	50 mg/ml BSA
6 µl	10 mg/ml goat IgG stock
3.6 µl	0.5 mg/ml biotinylated antibody
266.4 µl	DI water

10 mg/ml goat IgG stock: resuspend 50 mg in 5 ml of 150 mM NaCl and store at 4 °C. (Can be aliquoted and stored at – 20 °C, do not refreeze).

6.11.5 Analyzing the Affymetrix data using dChip

The data of the Affymetrix zebrafish genome array were normalized and analysed using the dChip software (Li and Hung Wong, 2001).

In a first round, a group of candidate genes were selected using following parameters:

Log2 control germ cell signal: ≥8

Log2 difference of control germ cells to dead end: ≥2

Log difference of control germ cells to somatic cells: ≥2,6

Due to the low number of candidates, we redefine the parameters for selection to be applied in a second round.

The new candidate genes were selected using following parameters:

Log2 control germ cell signal: ≥6,5

Log difference of control germ cells to somatic cells: ≥2,5

Genes from the first round were excluded.

6.11.6 RNA extraction from tissue and embryos and subsequent cDNA synthesis

RNA extraction from zebrafish embryos:

One ovary from an adult female or about 50 embryos were grown to a certain stage of development, transferred into 200 µl Trizol reagent (Gibco BRL-Life Technologies) and homogenized with a glass-teflon pistil. Following homogenization, 800 µl Trizol reagent were added then the cell suspension was centrifuged (12 min, 12000 rpm, 4°C) to further process the liquid phase and to discard the pellet. Next, the liquid phase was incubated at RT for about 5 min to dissociate associated proteins on the RNA. 200 µl of chloroform was added, shaked and incubated for about 2 min at RT followed by spinning (15 min, 12000 rpm, 4°C). The upper (aqueous phase including the RNA) was transferred into a new Eppendorf-tube and 500 µl iso-propyl alcohol

was added before shaking and spinning (10 min, 12000 rpm, 4°C). The supernatant was discarded and the residual RNA pellet was washed with 1 ml of 75% ethanol/water before spinning again (5 min, 7000 rpm, 4°C). The ethanol was removed, the pellet got air-dried and finally the RNA was dissolved in 20 µl HEPES solution (10mM, pH 7.4).

First-strand cDNA synthesis (reverse-transcription):

1. Thaw template RNA on ice.
2. Thaw the primer solutions, 10x Buffer RT, dNTP Mix, and RNase-free water at room temperature
3. Prepare a fresh master mix on ice according to bellow's Table 1.
   Note: The protocol is optimized for use with 50 ng to 2 µg RNA.
4. Add template RNA to the individual tubes containing the master mix.
5. Incubate for 60 min at 37°C.
6. Store the cDNA at -20°C (can be used directly for amplifications by PCR)

Master mix (standard example):

Table 6-24. Reverse-Transcription Reaction Components

Component	Vol/Reaction	Final Concentration
10x Buffer RT	2 µl	1x
dNTP Mix (5 mM each dNTP)	2 µl	0.5 mM each dNTP
Oligo-dT primer (10 mM)	2 µl	1 mM
RNase inhibitor (10 units/ML)	1 µl	10 units (per 20 µl reaction)
Omniscript Reverse Transcriptase	1 µl	4 units (per 20 µl reaction)
RNase-free water	Variable	
Template RNA,	Variable	Up to 2 µg (per 20 µl reaction)
Total volume	**20 µl**	

6.11.7 Polymerase chain reaction (PCR)

For initial amplification of candidate genes form the Affymetrix data:

To subsequently analyze the expression pattern of the candidate genes selected from the Affymetrix Microarray data, transcripts were amplified from a cDNA mix (ovary + mid somite stage) with the according primers mentioned in section 6.3. All reverse

primers contained additionally the T3-Promoter sequence (sequence attached to the 5' of the reverse primers: AATTAACCCTCACTAAAGGG) for direct synthesis of the antisense probe from the PCR product.

All primers used for the initial amplification of the candidate genes were ordered by VBC-Genomics (Vienna, Austria). The primers were designed to obtain a PCR product with a length between 500 and 1000 bp (if enough sequence information was available).

For all amplifications the following standard protocol was used:

Table 6-25. Taq-PCR reaction mix

Component	**Concentration**	**Volume**
cDNA	50 ng/Reaction	1 µl
Primer fw	10 µM	1 µl
Primer rev	10 µM	1 µl
dNTPs	2.5 mM	1.5 µl
PCR buffer	10x	2 µl
dH_2O		13 µl
Taq-polymerase	5 U/µl	0,5 µl
Total		**20 µl**

Table 6-26. Taq-PCR program

Temperature	**Time**	**Cycles**	**Steps**
94 °C	2 min	1	Initial denaturation
94 °C	30 sec	30	Denaturation
55 °C	20 sec		Primer annealing
72 °C	1 min		Extension
72 °C	4 min	1	Final Extension
4 °C	hold	1	Cool down

If no PCR product was observed, PCR was repeated at an annealing temperature of 50°C. If still no specific band was observed a new primer was ordered.

Amplification for cloning purposes:

All other primers, which were used for PCR amplification and sequencing were produced by the following manufacturers; IBA (Göttingen, Germany), MWG (Ebersberg, Germany) or VBC-Genomics (Vienna, Austria).

Table 6-27. Phusion-PCR reaction mix

Component	Concentration	Volume
Plasmid or cDNA	1-50 ng/Reaction	1 µl
Primer fw	10 µM	1 µl
Primer rev	10 µM	1 µl
dH_2O		7 µl
Phusion-mix	2 U/µl	10 µl
Total		**20 µl**

Note: Phusion mix contains, buffer, nucleotides and enzyme.

Table 6-28. Phusion PCR program

Temperature	Time	Cycles	Steps
98 °C	30	1	Initial denaturation
94 °C	10 sec	30	Denaturation
50-60 °C	20 sec		Primer annealing
72 °C	2 kb/min		Extension
72 °C	4 min	1	Final Extension
4 °C	hold	1	Cool down

If subsequent cloning into TOPO vector was desired, 1 µl of Taq-polymerase was added prior to the final extension to provide the PCR product with adenine overhangs, which are required for the TOPO cloning.

6.11.8 DNA separation on agarose gels

TAE Agarose Gel (standard example):

0,6 g SeaKem LE-Agarose (Biozyme) was dissolved in 50 ml TAE agarose running buffer by heating in a microwave. The agarose solution was cooled down to about 40 - 50°C before 5 µl of a 1% Ethidium-bromide solution (Merck) was added before the

viscous mixture was poured into the sledge of a ComPhor Gel chamber. Combs of different sizes (vary in loading volume) are provided by the supplier. After the agarose gel polymerized, the chamber was filled with TAE running buffer until the gel was covered by a 3-5 mm. DNA got supplemented with 6x blue loading dye (Fermentas) and loaded into the slots of the gel. To compare the sizes of the loaded DNA fragments, a 'DNA ladder' (Fermentas; λDNA-EcoRI/HindIII, 100 bp, 1 kb) was loaded into an empty slot next to the DNA samples. The power-supply was used between 50-120 milliamps to run the DNA through the agarose gel.

6.11.9 Enzymatic DNA digestions and modifications for cloning purposes

Restriction enzyme digest:

Enzymes were provided by NEB (New England Biolabs, Frankfurt, Germany) and Fermentas (St. Leon-Rot, Germany).

Standard digestion:

Table 6-29. Restriction Enzyme reaction mix

Component	**Concentration**	**Volume**
Plasmid-DNA, PCR product	1-3 µg/Reaction	1 - 5 µl
Reaction buffer	10x	3 µl
BSA (optional)	100x	0,3 µl
Enzyme	5-20 U/µl	0,5 - 1 µl per enzyme
dH2O		add to 30 µl
Total		**30 µl**

Duration: 2-4 hours depending on the amount of DNA and additionally on the enzyme activity. The reactions were incubated at 25°C, 37°C or 50°C corresponding to the optimal activity of the used enzymes. Most of the restriction enzymes can be heat inactivated at 65 - 80°C for 10 – 20 min. Digested DNA was purified by using the PCR-Purification Kit from Qiagen.

Klenow fragment (Fermentas):

The Klenow fragment is a large fragment of the DNA Polymerase I of *E.coli*. It exhibits the 5'-3' polymerase activity, the 3'-5' exonuclease activity but lacks the 5'-3' exonuclease activity. The Klenow fragment was used (instead of T4-Polymerase) to fill-up (blunt) sticky double strand DNA ends.

Klenow reaction (standard example):

Table 6-30. Klenow reaction mix

Component	**Concentration**	**Volume**
linearized Plasmid-DNA, PCR product	2-3 µg/Reaction	25,5 µl
dNTPs	2.5 mM	1 µl
Klenow buffer	10x	3 µl
Klenow fragment	10 U/µl	0.5 µl
Total		**30 µl**

Duration: 15 min at RT, and 10 min heat inactivated at 70°C. Blunted DNA was purified by using the PCR-Purification Kit from Qiagen.

CIAP (Calf Intestine Alkaline Phosphatase, Fermentas):

CIAP catalyzes the release of 5'- and 3'-phosphates from DNA, RNA, NTPs, dNTPs, and proteins. It is most often used for removing the phosphate groups of a linearized plasmid to avoid self-religation

Reaction (standard example):

Table 6-31. CIAP reaction mix

Component	**Concentration**	**Volume**
linearized Plasmid-DNA, PCR product	2-3 µg/Reaction	26 µl
CIP reaction buffer	10x	3 µl
CIP enzyme	10 U/µl	1 µl
Total		**30 µl**

30 min incubation at 37 °C and heat inactivated at 85 °C. Modified DNA was purified by using the PCR-Purification Kit from Qiagen.

Phosphorylation for ligation of PCR amplified plasmids:

Unmodified primers do not contain a phosphate group at their 5' termini. For direct ligation of PCR products, e.g of amplified plasmid for the purpose of mutation, phosphate groups will be needed. The T4 polynucleotide kinase is an enzyme that in presence of ATP can phosphorylate the 5' termini of DNA fragments.

Table 6-32. PNK reaction mix

Component	Concentration	Volume
PCR product	2-3 µg/Reaction	23 µl
T4 Ligase buffer	5x	6 µl
PNK enzyme	10 U/µl	1 µl
Total		**30 µl**

30 min incubation at 37 °C and heat inactivated at 65 °C for 20 min. Phosphorylated product was then gel isolated prior to the ligation reaction.

6.11.10 DNA isolation from gel slices

To obtain single DNA fragments from PCRs and digestions. DNA fragments were separated according to their size on a TAE agarose gel as described above.

Table 6-33. Protocol using QIAquick Gel Extraction Kit from Qiagen

Step	Description
1	Excise the DNA band from the agarose gel with a clean, sharp scalpel.
2	Weigh the gel slice in an Eppendorf tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
3	Dissolve the gel by incubating it for 10 min at 50 ºC; mix by vortexing the tube every 2–3 min during the incubation.
4	After the gel slice has dissolved completely, add 1 gel volume of isopropanol to the sample and mix. (100 mg gel = 100 µl isopropanol)
5	To bind the DNA, apply the sample to the QIAquick column, which was inserted into a provided 2 ml collection tube and centrifuge (16000 x g) for 1 min.
6	Discard the flow-through, place the QIAquick column back in the same collection tube and wash the QIAquick column by adding 750 µl of Buffer PE. Centrifuge (16000 x g) for 1 min.
7	Discard the flow-through and place the QIAquick column back in the same collection tube and centrifuge again to remove residual PE traces (16000 x g) for 1,5 min.

8	Place the QIAquick column into a clean 1,5 ml Eppendorf tube. To elute DNA, add 30 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or dH_2O to the center of the QIAquick membrane, wait for 2 min and centrifuge the column (16000 x g) for 1,5 min.

Table 6-34. Protocol using Gel extraction kit (Macherey-Nagel)

Step	Description
1	Excise the DNA band from the agarose gel with a clean, sharp scalpel.
2	Mix 1 volume of gel slice (100 mg ~100 µl) with 2 volumes of buffer NT. Incubate sample at 50 ºC until gel slice is completely dissolved (ca. 5 min). Mix every 2 min by vortexing.
3	Place a NucleoSpin® Extract II column into a 2 ml collecting tube and load sample of step 2. Bind DNA on column by centrifugation (11000 x g for 1 min).
3	Discard flow-through. Wash column by adding 600 µl buffer NT3. Centrifuge (11000 x g for 1 min)
4	Discard the flow-through and place the column back in the same collection tube and centrifuge again to remove residual NT3 buffer traces (11000 x g for 1,5 min).
5	Place the column into a clean 1.5 ml Eppendorf tube. To elute DNA, add 30 µl of water or Buffer NE to the center of the column membrane, wait for 1 min and centrifuge the column (11000 x g for 1,5 min).

6.11.11 Direct purification of linearized DNA or PCR products

Table 6-35. Protocol using Qiaquick PCR purification kit (Qiagen)

Step	Description
1	Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.
2	Place a QIAquick spin column in a provided 2 ml collection tube and apply the sample of step 1 on the column. Bind the DNA by centrifugation (16000 x g for 1 min).
3	Discard flow-through and wash by adding 750 µl of Buffer PE to the QIAquick column and centrifuge (16000 x g for 1 min).
4	Discard the flow-through and place the QIAquick column back in the same collection tube and centrifuge again to remove residual PE traces (16000 x g for 2 min).
5	Place the QIAquick column into a clean 1.5 ml Eppendorf tube. To elute DNA, add 30 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the QIAquick membrane, wait for 2 min and centrifuge the column (16000 x g for 2 min).

Table 6.36. Protocol using PCR clean-up kit (Macherey-Nagel):

Step	Description
1	Mix 1 Volume of sample with 2 volumes of buffer NT
2	Place a NucleoSpin® Extract II column into a 2 ml collecting tube and load sample of step 1. Bind DNA on column by centrifugation (11000 x g for 1 min).
3	Discard flow-through. Wash column by adding 600 µl buffer NT3. Centrifuge (11000 x g for 1 min)
4	Discard the flow-through and place the column back in the same collection tube and centrifuge again to remove residual NT3 buffer traces (11000 x g for 1,5 min).
5	Place the column into a clean 1.5 ml Eppendorf tube. To elute DNA, add 30 µl of water or Buffer NE to the center of the column membrane, wait for 1 min and centrifuge the column (11000 x g for 1,5 min).

6.11.12 DNA ligation reaction

T4-DNA ligation:

Table 6-37. T4-Ligation mix

Component	Concentration	Volume
Vector DNA	100 ng/µl	1 µl
Insert DNA	100 ng/µl	3 µl
T4-ligation buffer	5x	2 µl
dH2O		3.5 µl
T4-DNA Ligase	1U/µl	0.5 µl
Total		**10 µl**

Incubation: 1-4 h at RT, o/n at 16 °C. T4-DNA ligase was purchased by invitrogen.

TOPO ligation:

Table 6-38. TOPO-Ligation mix

Component	Concentration	Volume
PCR product	100 ng/µl	2 µl
Salt solution	1:4 dilution	0.5 µl
TOPO-system mix (Invitrogen)		0.5 µl
Total		**3 µl**

Incubation: 20 min at RT.

6.11.13 Generation of Electro-Competent Bacteria Cells

A single colony of *E.coli* cells was inoculated in 10-15 ml LB-medium (+Tetracycline for TOP 10 F' cells) and grown over night (o/n), shaking (200 rpm) at 37 °C. 10 ml o/n grown bacteria were inoculated in 1 l of pre-warmed (37 °C) LB-medium and grown at 37 °C, shaking 250 - 300 rpm, to an OD_{600} of = 0.5 - 0.6. The OD was measured by using a spectrophotometer at 600 nm (simply LB-medium was used for the blank-value). The cells got chilled on ice for 10 to 15 min and transferred to pre-chilled 0.5 l centrifuge bottles (cells were kept at 2 °C for all subsequent steps).
Afterwards, the cells were centrifuged for 15 min at 5000 x g. The supernatant was poured off and the cells got re-suspended in 5 ml of pre-cooled fresh water, then the centrifuge bottles got filled up to 1 l before the centrifuge step above was repeated. The supernatant got immediately discharged following re-suspension of the pellet in 5 ml cold fresh water, then the centrifuge bottles got filled up to 500 ml before the centrifuge step above was repeated once more. The supernatant got decanted following the addition of 15 ml 10% cold glycerol and transferred into two 50 ml falcon-tubes to centrifuge again as mentioned before. The supernatant got discharged and the pellet was re-suspended in 2.5 – 3 ml 10% glycerol. 50 µl aliquots of the competent cells were immediately thrown into a bucket with liquid nitrogen and frozen at -80 °C.

6.11.14 DNA transformation into bacteria strains (electro-competent cells)

Table 6-39. Protocol for DNA transformation into electro-competent cells

Step	Description	Temp.	Time
1	Thaw competent cells on ice. (one tube contains 50 µl)	4 °C	10 min
2	Add 2 µl of pCR-TOPO-ligation or T4-ligation to the competent cells and mix thoroughly with a pipette tip.	4 °C	
3	Transfer the cell suspension including the DNA-ligation to a electroporation cuvette and transform the cells by using the MicroPulser of BioRad (Program EC2)		
4	Add 400 µl of LB-bacteria medium to the cells and transfer everything into a pre-cooled Eppendorf tube.	4 °C	
5	keep the tube including the transformed cells on ice.	4 °C	2 min

6	Incubate the cells before plating.	37 °C	30-60 min
7	50 – 100 µl of the total 450 µl got plated on a LB-bacteria plate supplemented with Ampicillin (Ampicillin stock 100 mg/ml => final conc. 1000x diluted)	37 °C	o/n

6.11.15 Bacteria liquid-cultures

Single colonies of *E.coli* cells were picked with sterile tooth-pick were inoculated in LB-medium (Luria-Bertani medium) supplemented with Ampicillin 100 µg/ml (2-3mL for mini-cultures, 30-50 ml for midi-cultures) and grown overnight (o/n), shaking (200 rpm) at 37°C.

6.11.16 Plasmid DNA isolation from Mini-cultures

Table 6-40. Protocol QIAprep Spin Miniprep Kit (Qiagen)

Step	Description
1	Transfer the bacteria culture from the mini-culture to an Eppendorf tube and centrifuge at 3000 x g for 5 min.
2	Discard supernatant and resuspend bacteria pellet in 250 µl buffer P1.
3	Add 250 µl of buffer P2 (lysis buffer) and invert tube 6 times. Incubate for 3 to maximal 5 min.
4	Add 350 µl of buffer N3 (neutralization buffer) and invert tube immediately 6 times. Centrifuge at 16000 x g for 10 min.
5	Apply supernatant to the QIAprep Spin Column and centrifuge at 16000 x g for 1 min.
6	Discard flow-through and wash column by adding 750 µl buffer PE. Centrifuge at 16000 x g for 1 min.
7	Discard flow-through and centrifuge again for 1 min to remove residual wash buffer.
8	Place column in a new Eppendorf tube. To elute the DNA add 50 µl dH_2O, let stand for 1 min and centrifuge for 1 min at 16000 x g.

Table 6-41. Protocol Plasmid DNA Purification Kit (Macherey-Nagel)

Step	Description
1	Transfer the bacteria culture from the mini-culture to an Eppendorf tube and centrifuge at 11000 x g for 30 sec.

2	Discard supernatant and resuspend bacteria pellet in 250 µl buffer A1.
3	Add 250 µl of buffer A2 (lysis buffer) and invert tube 6 times. Incubate for 3 to maximal 5 min.
4	Add 300 µl of buffer A3 (neutralization buffer) and invert tube immediately 6 times. Centrifuge at 11000 x g for 10 min.
5	Apply supernatant to NucleoSpin® Plasmid Column plced in a collecting tube and centrifuge at 11000 x g for 1 min.
6	Discard flow-through and wash column by adding 600 µl buffer A4. Centrifuge at 11000 x g for 1 min.
7	Discard flow-through and centrifuge again for 2 min to remove residual wash buffer.
8	Place column in a new Eppendorf tube. To elute the DNA add 50 µl dH_2O, let stand for 1 min and centrifuge for 1 min at 11000 x g.

6.11.17 Plasmid DNA isolation from Midi-cultures

Table 6-42. Protocol QIAfilter Midi Kit (Qiagen)

Step	Description
1	Transfer the bacteria culture to a 50 ml Falcon-Tubes and centrifuge the bacteria culture at 6000 x g for 15 min at 4 ºC and discard the supernatant.
2	Resuspend the pelleted bacterial cells in 4 ml Buffer P1 (includes RNase A) by vortexing.
3	Add 4 ml Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Let stand 3 to max. 5 min.
4	Add 4 ml chilled Buffer P3 and mix immediately and thoroughly by inverting the tube 4–6 times. Pour the lysate into the barrel of the QIAfilter Cartridge and incubate for 10 min at RT.
5	Equilibrate a Qiagen-tip 100 by applying 4 ml QBT Buffer and allow the column to empty by gravity flow.
6	Remove the cap of the QIAfilter Cartridge outled nozzle. Insert the plunger into the QIAfilter Cartridge and filter the cell lysate into the previously equilibrated Qiagen-tip 100. Allow the cleared lysate to enter the resin by gravity flow.
7	Wash the Qiagen-tip 100 with 2 x 10 m Buffer QC. Allow Buffer QC to move through the Qiagen-tip 100 by gravity flow.
8	Elute DNA with 5 ml Buffer QF. Collect the eluate in a 15 ml Falcon tube.
9	Precipitate DNA by adding 3.5 mL (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at 15000 x g for 30 min at 4 ºC.

10	Decant supernatant wash DNA pellet with 2 ml ethanol and centriguge at 15000 x g for 10 min.
11	Carefully remove supernatant without disturbing the pellet, Air dry pellet for 10 min.
12	Disolve the DNA by adding 100 µl dH_2O.

6.11.18 Ultrapure DNA for generation of a transgenic fishline

Table 6-43. Protocol (Plasmid DNA Purification Using the EndoFree Plasmid Maxi Kit, according to Qiagen)

Step	Description
1	Transfer the bacteria culture to a 50 ml Falcon-Tubes and centrifuge the bacteria culture at 6000 x g for 15 min at 4 ºC and discard the supernatant.
2	Resuspend the pelleted bacterial cells in 10 ml buffer P1 (includes RNase A) by vortexing.
3	Add 10 ml buffer P2 (lysis buffer) and mix thoroughly by inverting the tube 4–6 times. Incubate 3 – max. 5 min.
4	Add 10 ml chilled buffer P3 and mix immediately and thoroughly by inverting the tube 4–6 times.
5	Pour the lysate into the barrel of the QIAfilter Cartridge and incubate. Do not insert the plunger! Let stand for 10 min.
6	Remove the cap of the QIAfilter Cartridge outled nozzle. Gently insert the plunger into the QIAfilter Maxi Cartridge and filter the cell lysate into a 50 ml tube.
7	Add 2.5 ml buffer ER to the filtered lysate, mix by inverting the tube approximately 10 times, and incubate on ice for 30 min.
8	Equilibrate a QIAGEN-tip 500 by applying 10 ml buffer QBT, and allow the column to empty by gravity flow.
9	Apply the filtered lysate from step 7 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
10	Wash the QIAGEN-tip with 2 x 30 ml buffer QC. Allow buffer QC to move through the QIAGEN-tip by gravity flow.
11	Elute DNA with 15 ml buffer QN. Collect the eluate in a 30 ml endotoxin-free or pyrogen-free tube.
12	Precipitate DNA by adding 10.5 ml (0.7 volumes) isopropanol to the eluted DNA. Mix and centrifuge immediately at 15000 x g at 4 ºC for 30 min..
13	Wash DNA pellet with 5 ml of endotoxin-free 70% ethanol (add40 ml of 96–100% ethanol to the endotoxin-free water supplied with the kit) and centrifuge at 15000 x g at RT for 10 min..
14	Air-dry the pellet for 5–10 min, and redissolve the DNA in 200 µl endotoxin-free Buffer TE.

Filter the DNA sample in a Spin-X tube by centrifugation at maximum speed for 1 min. (contains a 0.45 µm cellulose acetate filter) before using it for injections.

6.11.19 Diagnostic Restriction digest to verify plasmid DNA

Reaction (standard example):

Table 6-44. Diagnostic restriction enzyme mix

Component	Concentration	Volume
Plasmid-DNA	200 – 400 ng	1 - 3 µl
Reaction buffer	10x	1,5 µl
BSA (optional)	100x	0.15 µl
Enzyme	5-20 U/µl	0.3 µl per enzyme
dH2O		variable
Total		**15 µl**

6.11.20 Sequencing of plasmid DNA

Plasmids used for this work were verified by sequencing performed by Seqlab (Göttingen, Germany) and MWG (Ebersberg, Germany).

6.11.21 Linearization of plasmid DNA for RNA synthesis

To produce a DNA template for sense RNA production, a restriction enzyme was chosen which cuts 3' after coding and 3'UTR sequence. To produce a template DNA for antisense production a restriction enzyme was chosen which cuts 5' of the sense sequence of the gene fragment.

Table 6-45. Restriction digestion mix for linearization

Component	Concentration	Volume
Plasmid-DNA	5 -10 µg	4 -20 µl
Reaction buffer	10x	10 µl
BSA (optional)	100x	1 µl
Enzyme	5-20 U/µl	2 µl enzyme
dH2O		variable
Total		**100 µl**

Linearized DNA was purified with the QIAquick PCR purification kit as described above (section 6.11.11).

Sense RNA:

The Message machine kit (Ambion) was used to produce capped sense RNA for injection.

6.11.22 Sense RNA synthesis

Table 6-46. Sense RNA reaction mix

Component	Concentration	Volume
Linearized DNA	50 - 500 ng	1 – 3,2 µl
NTP mix, Cap analog	2x	5 µl
GTP (optional for long transcripts only)		1 µl
T3, T7 or SP6 buffer	10x	1 µl
dH2O		variable
T3, T7 or SP6 RNA-Polymerase	1U/µl	0.8 µl
Total		**10 µl**
Incubate: 2 – 3.5 hours at 37°C		

After incubation, add 0,5 µl DNAseI (to remove template DNA), mix well and incubate for 20 min at 37°C.

RNA purification by phenol-chloroform extraction:

1) add 30 µl Ammonium Acetate Stop Solution (Message Machine Kit)
2) add 260 µl DEPC water (AppliChem) to the sense reaction
3) add 300 µl PCI (Phenol-Chloroform-Isoamylalcohol, 25:24:1, pH=6,6, Ambion)
4) vortex for about 10 s, spin for 15 min at 16000 x g at RT
5) carefully transfer the upper phase to a new Eppendorf tube and discard the lower phase.
6) add 300 µl CI (Chloroform-Isoamylalcohol, 49:1), shake vigorously and spin for 7 min at 16000 x g at RT

7) transfer the upper phase to new Eppendorf tube
8) repeat step 5 – 7 again
9) add equal volume of 100% Isopropanol
10) centrifuge immediately for 40 min at 16000 x g., cool down to 4°C while spinning
11) carefully remove the supernatant
12) wash 2x with 80% EtOH
13) after the second wash, remove large drops and air dry the RNA pellet for a minimum time.
14) dissolve the RNA in 20 µl HEPES (10mM, pH 7,4, DEPC water)

Alternative method for steps 3-8 using PhaseLock Heavy Gel tube (Eppendorf):

a) Do steps 1 to 3 as described
b) Spin a new PhaseLock Heavy Gel tube for 1 min at 16000 x g to bring down the gel
c) Transfer the RNA-containing solution to the top of the gel
d) add 300µl Phenol-Chloroform-IAA (25:24:1, pH=6,6, Ambion)
e) Mix well by shaking for 10s
f) Spin for 3 min at max speed, the aqueous and organic phase will now be separated by a stable gel barrier
g) Repeat steps 6 through 8 twice using chloroform-IAA (49:1)
h) Transfer the aqueous phase to a new tube by pipetting or decanting. To not touch the gel when pipetting. Proceed with step 9

Analysis of the RNA:

- Spectrophotometer:

  dissolve 1 µL in 99 µL 10 mM Tris buffer use the RNA mode of the spectrometer, Good RNA should have a ratio of 260 nm/280 nm between 1,9 to 2,1!

- Agarose gel:

  1 µL RNA + 1µL Ambion RNA loading buffer

  heat the mixture for 3 min at 85°C, put on ice for 2 min and load the gel.

Intact RNA should have a concise band. Smear indicates degradation.

6.11.23 Antisense RNA synthesis

Note: For the In Situ screen using the candidate genes from the Affymetrix microarray screen. The purified PCR products were directly used as templates for the antisense RNA synthesis as they carried the T3-Promoter sequence added with the reverse primers. Additionally the antisense reaction mix shown below was scaled down to ¼ (red text in table)

Table 6-47. Antisense RNA reaction mix

Component	**Concentration**	**Volume**
Linearized DNA, PCR product	0,5 – 1 µg	2 µl, 2 µl
DIG labelled NTPs (Roche)	10x	2 µl, 0,5 µl
T3, T7 or SP6 buffer (Fermentas)	5x	4 µl, 1 µl
RNAse Inhibitors (Roche)		1 µl, 0,25 µl
dH2O		Variable, 0,75 µl
T3, T7 or SP6 RNA-Polymerase (Fermentas)	1U/µl	2 µl, 0,5 µl
Total		**20 µl, 5 µl**
Incubate: 2 – 3.5 hours at 37°C		

After incubation, add 0,5 µl (0,2 µl) DNAseI (Roche)(to remove template DNA), mix well and incubate for 30 min at 37°C.

Add 10 µg glycogen (to later visualize RNA pellet) and fill reaction mix to 20 ul with DEPC treated H_2O.

NH_4 Ac-Precipitation for RNA purification:

1) add 11µL of NH_4Ac (7,8 M)
2) add 63 µL of 100% EtOH
3) mix well by using the vortex and spin at 13000 rpm at RT
4) let the RNA precipitate for 30 - 50 min at RT (keep dark)
5) discard supernatant
6) wash the RNA pellet 1x with 1 mL 80% EtOH at RT
7) spin for 5 – 10 min at 13000 rpm
8) discard supernatant
9) remove big residual drops and air dry the RNA pellet for a minimum time
10) dissolve the RNA in 20 µL H_2O + 80 µL Hyb-buffer

Analysis of the RNA:

- Agarose gel:

  3 µL RNA + 3 µL Ambion RNA loading buffer

  heat the mixture for 3 min at 85°C, put on ice for 2 min and load the gel

6.12 Zebrafish – experimental animal model

6.12.1 Zebrafish strain and fish maintenance

Zebrafish (Danio rerio) of the AB genetic background were maintained under standard laboratory conditions at a constant light-dark cycle (14 h light/10 h dark (Kimmel et al., 1995; Westerfield, 1995). For breeding, 2 fish were placed in a mating tank in the evening. The following morning, females start normally to lay eggs. To avoid parental cannibalism the cage separated parents from eggs. The eggs were collected and transferred to methylene blue egg water or Danieau's solution to prevent the growth of fungi or bacteria. The eggs were maintained at 28 °C until the desired stages. Alternatively, to accelerate or slow down the development, embryos were incubated at 30 °C or 24 °C, respectively.
Morphological features were used to determine the stage of the embryo (according to Kimmel et al., 1995). For analyzing fishes at stages older then 24 hpf, embryos were kept in Danieaus solution containing 0.005 % phenylthiourea (PTU), to prevent pigmentation.

Egg water: Dissolve 300 mg of Red sea salt per 1 l.

Methylene blue solution in egg water (blue water)

stock solution of methylene blue: 1 g methylene blue to 1 liter dH_2O

working solution: Dilute 0,5 ml of stock solution in 1 l egg water

30x Danieau's stock solution

NaCl	1,74 M
KCl	21 mM
$MgSO_4$	12 mM
$Ca(NO_3)_2$	18 mM
HEPES	150 mM

pH=7,6

For working solution (0,3x Danieaus's) dilute stock solution 1:100 and readjust pH to 7,6 by adding few drops of 5M NaOH. To prevent bacterial growth Penicilin and Streptomycin antibiotic solution (P/S) was added to final concentration of 50 U/ml.

6.12.2 Injection of morpholinos and sense RNA into zebrafish 1 cell stage embryo

Injection protocol:

The day before injection:

1) Prepare 1 l of 0.3x Danieau's solution as described above (for pouring and overlaying the injection ramps where the embryos are placed during the injection)
2) Add 2 g of agarose and 100 ml 0.3x Danieau's solution into a Erlenmeyer flask. Dissolve agarose by boiling in a microwave. Pour the hot liquid agarose directly into petridishes and immediatly place a desired ramp template (different types for injection, microscopy, with and without chorion and for different stages) carefully in the center of the petridish. Remove the ramp template carefully after the agarose solidified. Overlay the agarose with 0.3x Danieau's solution to prevent it from drying out. Store at 4˚ C.
3) Pull needles and cut the tip with a scalpel. The opening width should be such that air bubbles start to be generated by a pressure between 10 and 20 psi.
4) Set-up of mating couples: fill tanks with fish-facility water, place one female fish into a net inside the tank, add a net above it and place one male fish into it. One male and female will be in one tank but separated by a net.

The day of injection:

1) Add Penicillin/Streptomycin (P/S) to 0.3x Danieau's solution and use it to exchange the 0.3x Danieau's solution on the injection ramps. Transfer the ramps to 28 ºC.
2) Dilute RNA, DNA and/or morpholinos in HEPES (10 mM) to the appropriate concentration and keep on ice.

Note: Prior to use, morpholinos need to be heated up to 65 ºC for 10 min each time to avoid aggregates. Centrifuge afterwards for 5 min at max. speed.

3) Bring male and female together, by removing the net, which separates them. One net remains in the tank, which will keep the parents away from already laid eggs.
4) Collect the eggs in a tea strainer and transfer the eggs in a clean petridish containing 1x methylene blue/egg water solution.
5) Transfer eggs into the injection ramps with a Pasteur pipette and orient the embryos in the ramp by using a metal needle.
6) Add injection solution into an injection needle and connect needle to the injector.
7) Pierce the chorion membrane and inject RNA and morpholinos into the yolk. DNA samples have to be injected directly into the cell.
8) After injection, take the embryos out of the ramps and transfer them into a new clean petridish containing 0.3x Danieau's solution with P/S (do not add more than 100 embryos in one dish).
9) Transfer the eggs into the appropriate incubator (24°C, 28°C or 30°C), after 2-3 hours take a look to them and place the fertilized ones into a new petridish with 0.3x Danieau's solution P/S. Incubate them further at 24°C - 30°C.

Progress to perform live imaging of embryos:

Chorions, which surround the embryos are removed by hand (forceps) and embryos are transferred and oriented into agarose ramps for microscopy.

Progress to grow embryos until adulthood:

Transfer morphologically "good looking" embryos on the next day into methylene blue water. At the age of 5 dpf, put fish larva into the so-called baby wall of the fish facility to grow them under appropriate conditions for their developmental stage.

6.12.3 Fixation of embryos

For in situ hybridization:

1) Embryos 24 hpf and older: chorion was removed by using forceps before fixation. Fixation o/n at 4°C in 4% PFA in PBS.
2) Embryos younger than 1-2 somite stage were fixed for 2 days.
   After fixation the embryos were rinsed 2-3 times in PBT and the chorion was removed by using forceps.
3) Embryos got transferred to 100% MeOH by passing them through a gradient of MeOH/PBT; 25%, 50%, 75% and 100%.
4) Embryos were stored in 100% MeOH at –20°C for several weeks or months.

To dechorionate embryos enzymatically, 0.5–1 mg Pronase E was added per 1 ml of Danieau's and following dechorionation washed 7 times with Danieau's.

For immunohistochemistry:

Chorion was removed with forceps before fixation. Embryos were transferred in pre-warmed (RT) PFA (4%) in PBS and fixed for 2-3 h at RT and washed 3 times in PBT.

6.12.4 In situ hybridization of whole mount zebrafish embryos

One-colour whole-mount in situ hybridization was performed as described previously (Jowett and Lettice, 1994) with modifications described elsewhere (Hauptmann and Gerster, 1994; Weidinger et al., 2002). In short: A specific DIG-labeled antisense probe is hybridized to embryos of different stages. A DIG specific antibody, which is coupled to alkaline phosphatase, binds to the probe. The enzyme is used for the staining reaction with following dye combination X-phosphate/NBT, which gives a blue color.

The complete procedure for in situ hybridization is described in the following table:

Table 6-48. Protocol *In situ* Day 1

Step	Description	Temp.	Time
1	Rehydration 75 % MetOH – 25 % PBT	RT	5 min
2	Rehydration 50 % MetOH – 50 % PBT	RT	5 min
3	Rehydration 25 % MetOH – 75 % PBT	RT	5 min
4	Rehydration 100 % PBT	RT	4x 5 min
5	Proteinase K (5 µg/ml in PBT): - before shield: no treatment - shield - bud: 0 - 30 sec - 1 somite: 30 - 60 sec - 5 somite: 3 - 4 min - 24 hpf embryo: 4 - 5 min - 36 - 72 hpf embryo: 10 - 20 min with 10 µg/ml - 4 dpf -7 dpf larva: 20 min -30 min with 60 µg/ml	RT	
6	PBT	RT	10 s
7	PBT	RT	10 s
8	PBT	RT	1 min
9	PBT	RT	5 min
10	Refixation in 4 % PFA in PBS	RT	20 min
11	Wash in PBT	RT	5x 5min
12	Prehybridization: incubate in 200 µl of Hyb-buffer	67 °C	2 – 5 h
13	Hybridization: 0,5 µl – 3 µl of antisense probe in 200 µl of Hyb-buffer to replace the prehybridization. *Heat the hybridization mix to 67°C (water bath) for about 10 min.*	67 °C	12 h

Table 6-49. Protocol *In situ* Day 2

Step	Description	Temp.	Time
Recover the probes! They can be reused three times. Always preheat the washing solutions to hybridization temperature!			
1	Wash with Hyb-buffer	67 °C	20 min
2	Wash with 50 % SSCT 2x / 50 % Formamide	67 °C	3x 20 min
3	Wash with 75 % SSCT 2 x / 25 % Formamide	67 °C	20 min

4	Wash with SSCT 2x	67 °C	2x 20 min
5	Wash with SSCT 0.2x	67 °C	4x 30 min
6	PBT	67 °C	5 min
7	Blocking: 5% sheep serum + 10 mg/ml BSA in PBT *Slow agitation and keep dark*	RT	> 1 h
8	Antibody-incubation I: Incubate in 100 - 200 µl antibody solution. *Antiserum pre-absorbed against embryos, 1:2000- 1:4000 in PBT + 2 mg/ml BSA (optional: +2 % sheep serum). For storage at 4°C: add Sodium Azide to 0,02 %*	4 °C	14 h

Table 6-50. Protocol *In situ* Day 3

Step	**Description**	**Temp.**	**Time**
Recover the antibody! It can be reused two times.			
1	Wash with PBT with slow agitation, dark	RT	2x 5 min
2	Wash with PBT with slow agitation, dark	RT	8x 20 min
3	Wash with NTMT buffer	RT	3x 5 min
5	Transfer embryos to a 24 well plate		
6	Staining reaction: add 500 µl of BCIP/NBT mix *Staining at 4 °C overnight is also possible.*	37 °C	
7	Stop the reaction: removing of the staining solution and wash in Stop-Solution	RT	3x 1 min
8	Removal of the first antibody: add 0,1 M Glycin/HCl, pH 2.2 + 0.1 % Tween20, *shake well*	RT	2x 5 min
9	Wash with PBT with slow agitation, dark	RT	4x 5 min
10	Clearing (optional): incubate embryos in 100 % EtOH with slow agitation *This step cannot be performed after the red staining reaction. (The red stain dissolves in ETOH very fast.)*	RT	2x 10 min
11	75 % EtOH / 25 % PBT	RT	5 min
12	50 % EtOH /50 % PBT	RT	5 min
13	25 % EtOH / 75 % PBT	RT	5 min
14	PBT	RT	4x 5 min

Solutions:

PBS 10x for 1 l
80 g NaCl
2 g KCl
18 g Na_2HPO_4 (2 H_2O)
2,4 g KH_2PO_4
adjust to pH 7,2

PBT 1x for 1 l
100 ml 10x PBS
900 ml H_2O
adjust to pH 7,4 by adding HCl
add 10 ml 10% Tween 20

SSC 20x for 8 l
1400 g NaCl
704 g Trisodium Citrate[$2H_2O$]
adjust to pH 7,0 (by adding 15% HCl)

Hyb-buffer for 1 l
250 ml 20x SSC
500 mg tRNA* (Sigma)
50 mg Heparin (Sigma)
10 ml 10% Tween 20 (Sigma)
1,89 g Citric Acid (Monohydrate)
adjust pH 6,0 – 6,5
H_2O to 500 ml
last: add 500 mL deionized formamide
Store at –20°C

* Rnase free tRNA: torula yeast RNA (Sigma R6625): 500 mg tRNA in 20 mL 10x SSC extracted with Phenol/Chloroform – Chloroform/Isoamylalcohol (see protocol for purifying sense or antisense RNA), after 2x wash with 80% EtOH, air dry and resuspend in 5 mL 10x SSC

NTMT staining buffer for 20 ml
2 ml Tris HCl pH 9.5, 1M
1 ml $MgCl_2$, 1M
2 ml NaCl, 1M
200 µl 10% Tween20
add 14.8 ml H_20

Staining solution BCIP/NBT (blue staining):

4.5 µl NBT (Nitro Blue Tetrazolium, Sigma; 75 mg/ml in 70% DMF / 30% H_2O)

3,5 µl X-phosphate (=BCIP) (50 mg/ml in 100% DMF)

1 ml NTMT buffer

Stop-Solution

- 0.05M phosphate buffer pH 5.8
- 1mM EDTA
- 0.1% Tween20

For 200 ml: 92 ml 0.1M NaH2PO4
8 ml 0.1M Na2HPO4
(this mixture should produce a pH of 5.8)

Preabsorption of the antibody:

1) Harvest hundreds of embryos, at different stages.
2) Fix overnight in 4 % PFA in PBS.
3) Transfer to MeOH, 4-5 changes. Store embryos at –20 °C.
4) For preabsorbtion, take about 200 embryos (for ca. 20 ml of final 1:2000 antibody solution).
5) Wash embryos 2x in PBT and dechorionate by hand.
6) Wash another 5x in PBT.
7) Spin the concentrated antibody 5 min. at 13000 rpm.
8) Incubate antibody (dilution 1:400) on the embryos in BSA, 2 mg/ml in PBT Shake at RT for at least 1 h.
9) Spin down after incubation and carefully transfer the supernatant to a separate tube before diluting to the final concentration.
10) Dilute antibody to a final dilution of 1:2000 in PBT (+2% sheep serum, +2 mg/ml BSA).
11) Filtrate antibody using a sterile filter (0,2 – 0,45 µm Cellulose Acetate filter).
12) Keep the antibody at this concentration at 4 °C. For storage, add Sodium Azide to 0,02 %.

6.12.5 Immunohistochemistry of whole mount zebrafish embryos

Table 6-51. Protocol *whole mount* immunochemistry

Step	Description	Temp.	Time
1	Fixation of embryos: Remove chorion with forceps before fixation. Embryos were transferred in pre-warmed (RT) PFA (4 %) in PBS.	RT	2-3 h
2	Wash with PBTX (pre-cooled at 4 °C)	4 °C	6x 10 min
3	Wash with PBTB	4 °C	2x 30 min
4	Blocking: PBTB	4 °C	12 h
5	Primary antibody: dilute antibody in 1 ml PBTB, Combination of several primary antibodies during the incubation step possible. Dilution depends on antibody. *slow agitation and keep dark*	4 °C	2-3 days

6	Recover the antibody.		
7	Wash with PBTX, *slow agitation and keep dark*	4 °C	8x 1 h
8	Wash with PBTX, *slow agitation and keep dark*	4 °C	o/n
9	Secondary antibody: dilute antibody in 1 mL PBTB, Combination of several secondary antibodies during the incubation step possible. Dilution depends on antibody. *slow agitation and keep dark*	4 °C	o/n
10	Recover the antibody.		
11	Wash with PBTX, *slow agitation and keep dark*	4 °C	8x 1 h
12	Wash with PBTX, *slow agitation and keep dark*	4 °C	o/n
13	Store embryos in PBTX	4 °C	

Solutions:

PBTX

PBS (as described above in the *in situ* protocol)
add 0,2 % Triton X-100

PBTB

PBTX
add 1 % BSA

6.12.6 Microscopy and data processing

Stereomicroscopy:

Images were obtained using a Zeiss Axioplan2 microscope controlled by the Metamorph software (Universal Imaging). *In situ* pictures were taken using 10x magnification. Pictures of individual cells were taken at 63x magnification. Signal intensity measurements of PGCs were performed using ImageJ. 63x magnification time-lapse movies were generated capturing frames at 10-second intervals.

Confocal-Microscopy:

Images were obtained using a Leica TCS Confocal microscope controlled by the internal Leica software. Pictures were recorded in the xyzt modus (allows z-stacks) with 512 x 512 pixel resolution. The laser intensity, potentiometer, gain and offset were adjusted according to the individual conditions of the experiments (fluorescent

intensity based on the probe characteristics). The recorded frames of z stacks were processed to generate 3 D reconstruction using ImageJ (3D project). To measure granule size the Quantity-Mode (Stack profile/Statistics) of the TCS SL confocal software provided by Leica was used.

For processing and documentation of pictures ImageJ and Adobe Photoshop 8.0 was used. Statistical analysis and graphical representation of numerical data was carried out using Microsoft Excel.

7 Supplemental material

7.1 List of candidate genes from the Affymetrix Zebrafish microarray

Known germ cell specific genes were either excluded and are put at the end of the list, or marked with an x.

The Affymetrix raw data were analyzed by the dChip software, which averaged and normalized the pixel signal intensity from several spots corresponding to one EST. The log signal PGC values in the list are presented as the logarithm to the base 2 (log_2) of the signal intensities.

Table 7-1. Table of candidate genes from the Affymetrix microarray screen.

Nr.	Gene name	Accesion Nr.	log signal PGC	Logdiff dnd	Logdiff som
1	weak similarity to protein pir:JC4913 (H.sapiens) JC4913 anti-sigma cross-reacting protein homolog I alpha precursor - human	BQ093700	11,19	3,87	2,88
2	weak similarity to protein sp:P20742 (H.sapiens) PZP_HUMAN Pregnancy zone protein precursor	BI474808	11,07	3,71	2,97
3	weak similarity to protein sp:O75593 (H.sapiens) FXH1_HUMAN Forkhead box protein H1 (Forkhead activin signal transducer 1) (Fast-1) (hFAST-1) (Forkhead activin signal transducer 2) (Fast-2)	BI983354	10,94	10,94	3,74
4	programmed cell death 4	BC045513.1	10,69	3,35	2,94
5	zgc:55944	BC044551.1	10,60	3,40	3,27
6	moderate similarity to protein sp:P54098 (H.sapiens) DPG1_HUMAN DNA polymerase gamma subunit 1 (Mitochondrial DNA polymerase catalytic subunit) (PolG-alpha)	AL922740	10,51	3,53	2,83
7	Danio rerio transcribed sequences	BQ450026	10,45	6,93	3,17
8	zgc:56233	BC046012.1	10,31	3,50	3,24
9	Danio rerio transcribed sequences	AW567529	10,21	3,83	2,71
10	zgc:77614	BM103315	10,09	5,80	3,79
11	zgc:103482	BM101604	9,93	5,27	2,89
12	Danio rerio transcribed sequences	AI721440	9,74	3,38	3,53
13	sequence not in UniGene	AL915830	9,64	3,32	2,77
14	zgc:76877	BM316210	9,58	4,37	2,98
15	hypothetical protein MGC10471-like protein	BG728541	9,56	3,56	3,25
16	disabled homolog 2 (Drosophila)	BM095926	9,43	4,74	3,22
17	weak similarity to protein pir:T00383 (H.sapiens) T00383 KIAA0632 protein - human (fragment)	AI641729	9,40	3,30	2,90
18	weak similarity to protein ref:NP_066937.1 (H.sapiens) serine protease inhibitor, Kazal type, 2 (acrosin-trypsin inhibitor) (Homo sapiens)	BM571673	9,38	5,26	2,89
19	superoxide dismutase 2, mitochondrial	AW076961	9,33	3,56	3,46

20	weak similarity to protein pir:I37570 (H.sapiens) I37570 zinc finger protein - human (fragment)	BI843295	9,30	3,69	3,09	
21	Danio rerio transcribed sequences	BI672117	9,18	3,66	3,71	
22	moderate similarity to protein ref:NP_068753.1 (H.sapiens) hypothetical protein FLJ11773 (Homo sapiens)	BU710607	9,17	3,32	3,69	
23	zgc:63792	BM104070	9,16	5,15	3,25	
24	Danio rerio transcribed sequences	BM154132	9,15	3,07	3,71	
25	zgc:66221	AW170913	9,12	3,17	2,72	
26	decapentaplegic and Vg-related 1	NM_130948.1	9,09	4,56	2,94	
27	Danio rerio transcribed sequences	AI558276	9,09	3,75	2,73	
28	Danio rerio, clone MGC:56493 IMAGE:5777308, mRNA, complete cds	BQ616930	9,08	5,68	2,93	
29	weak similarity to protein ref:NP_001298.1 (H.sapiens) claudin 7; Clostridium perfringens enterotoxin receptor-like 2; claudin 9 (Homo sapiens)"	AW421114	9,01	3,11	2,94	
30	moderate similarity to protein pdb:1D9T (H.sapiens) A Chain A, Human Glucosamine-6-Phosphate Deaminase Isomerase At 1.75 A	BQ262910	8,99	3,80	2,80	
31	ankyrin repeat, SAM and basic leucine zipper domain containing 1	BC046081.1	8,98	5,32	4,08	
32	Transcribed locus, weakly similar to NP_808790.1 slinky [Rattus norvegicus]	BM095897	8,95	6,50	2,98	
33	Danio rerio transcribed sequences	BM154752	8,94	3,21	3,62	
34	zgc:73273	BQ285167	8,9	5,20	4,30	
35	weak similarity to protein pir:A36000 (H.sapiens) A36000 sperm-binding glycoprotein ZP3 precursor - human	BQ078419	8,86	6,73	3,91	
36	Danio rerio transcribed sequences	CD605341	8,83	4,17	4,87	
37	zgc:55413	BC044165.1	8,82	4,96	3,33	
38	Danio rerio transcribed sequences	BQ261266	8,81	3,52	3,51	
39	weak similarity to protein ref:NP_004634.1 (H.sapiens) poly(A)-binding protein, nuclear 1; poly(A) binding protein II; oculopharyngeal muscular dystrophy; poly(A) binding protein 2 (Homo sapiens)"	BQ262324	8,75	4,98	2,81	
40	zgc:55656	BC044447.1	8,75	3,23	3,53	
41	zgc:101700	AW232570	8,72	3,76	6,37	
42	weak similarity to protein ref:NP_079177.1 (H.sapiens) hypothetical protein FLJ22457 (Homo sapiens)	BM571456	8,71	4,37	2,90	
43	zgc:103655	BQ479662	8,71	3,32	3,66	
44	Danio rerio transcribed sequences	BM956864	8,70	4,82	4,32	
45	zgc:56121	BC045926.1	8,70	3,96	3,14	
46	zgc:73241	BQ093634	8,68	3,53	3,04	
47	askopos	BM530127	8,66	8,52	3,19	x
48	weak similarity to protein ref:NP_006461.2 (H.sapiens) tripartite motif-containing 16; estrogen-responsive B box protein (Homo sapiens)"	BQ092687	8,66	4,38	3,73	
49	Danio rerio transcribed sequences	BM316143	8,63	3,74	3,19	
50	weak similarity to protein ref:NP_115733.1 (H.sapiens) hypothetical protein MGC12981 (Homo sapiens)	BM024151	8,63	3,21	3,10	
51	zgc:100786	BQ783871	8,61	4,25	3,05	

52	zgc:85804	BI889921	8,57	4,31	4,31
53	zgc:56653	BC049516.1	8,56	3,92	2,78
54	weak similarity to protein pir:KRHUE (H.sapiens) KRHUE keratin 14, type I, cytoskeletal - human	BE017931	8,55	3,52	2,96
55	zgc:103708	BM571646	8,48	3,49	2,71
56	zgc:56571	BC050170.1	8,44	3,67	4,20
57	novel immune-type receptor 2.2 /// novel immune-type receptor 2.5 /// novel immune-type receptor 2b /// novel immune-type receptor 2.8 /// novel immune-type receptor 2.3	NM_131649.1	8,43	5,20	2,88
58	GTP binding protein 1, like	BQ285243	8,40	3,69	3,20
59	moderate similarity to protein pir:T46490 (H.sapiens) T46490 hypothetical protein DKFZp434K046.1 - human	BI879940	8,36	4,67	3,97
60	Danio rerio transcribed sequences	BQ479881	8,32	7,09	7,98
61	zgc:73223	BG308403	8,32	3,57	3,19
62	weak similarity to protein sp:Q13164 (H.sapiens) MK07_HUMAN Mitogen-activated protein kinase 7 (Extracellular signal-regulated kinase 5) (ERK-5) (ERK4) (BMK1 kinase)	AI544978	8,30	3,32	3,54
63	zgc:56462	BC049451.1	8,27	3,56	3,12
64	weak similarity to protein ref:NP_064515.1 (H.sapiens) chromosome 8 open reading frame 4; C8ORF4 protein (Homo sapiens)"	AW281891	8,26	5,82	3,60
65	weak similarity to protein ref:NP_060730.1 (H.sapiens) hypothetical protein FLJ10891 (Homo sapiens)	BM857906	8,26	3,61	2,81
66	zgc:92661	BM534431	8,26	3,46	2,87
67	peptidylprolyl isomerase F (cyclophilin F)	BM082457	8,24	3,02	3,42
68	ring finger protein 146	BI709398	8,23	4,38	2,72
69	Danio rerio transcribed sequences	BM529903	8,19	3,08	4,32
70	moderate similarity to protein ref:NP_003277.1 (H.sapiens) DNA topoisomerase I; type I DNA topoisomerase (Homo sapiens)"	BI878749	8,14	6,06	3,01
71	zgc:91894	AW077628	8,14	3,22	2,74
72	FabG (beta-ketoacyl-[acyl-carrier-protein] reductase, E. coli) like	BI879024	8,12	3,32	2,80
73	zgc:64047	BC053222.1	8,12	3,26	2,88
74	l-isoaspartyl protein carboxyl methyltransferase, like	BQ074694	8,12	3,25	3,70
75	weak similarity to protein sp:Q99676 (H.sapiens) Z184_HUMAN Zinc finger protein 184	AA494741	8,11	3,24	2,80
76	weak similarity to protein ref:NP_036434.1 (H.sapiens) latrophilin; KIAA0786 protein; lectomedin-1 (Homo sapiens)"	BI983550	8,09	8,09	5,80
77	weak similarity to protein sp:O15342 (H.sapiens) VA0H_HUMAN Vacuolar ATP synthase subunit H (V-ATPase H subunit) (Vacuolar proton pump H subunit) (V-ATPase M9.2 subunit) (V-ATPase 9.2 kDa membrane accessory protein)	BI430208	8,08	4,12	2,94

78	seven in absentia homolog 2 (Drosophila)-like	AF409097.1	8,05	5,32	3,98
79	zgc:66313	BI896378	8,05	4,05	3,34
80	weak similarity to protein ref:NP_036596.1 (H.sapiens) tolloid-like 1 (Homo sapiens)	BQ616045	8,04	4,54	4,88
81	Danio rerio transcribed sequences	BM571619	8,03	4,55	3,72
82	Danio rerio transcribed sequences	BI326702	7,2	3,82	4,2
83	weak similarity to protein ref:NP_001432.1 (H.sapiens) fatty acid amide hydrolase (Homo sapiens)	BI983434	7,68	2,19	5
84	zgc:56685	AL907236	10,07	2,16	10,07
85	weak similarity to protein sp:Q93075 (H.sapiens) Y218_HUMAN Putative deoxyribonuclease KIAA0218	AW171527	8,55	2,58	8,55
86	Danio rerio transcribed sequences	BM776698	10,05	2,59	6,73
87	zgc:55413	BI867164	12,27	2,48	6,52
88	weak similarity to protein ref:NP_004210.1 (H.sapiens) pituitary tumor-transforming protein 1; ESP1-associated protein 1; tumor-transforming protein 1; securin (Homo sapiens)"	BM182245	9,56	2,18	6,33
89	Danio rerio transcribed sequences	BM777971	10,73	2,57	6,32
90	solute carrier family 16 (monocarboxylic acid transporters), member 3	BC045489.1	11,09	2,05	5,77
91	Danio rerio transcribed sequences	AW826425	8,41	2,26	5,61
92	weak similarity to protein sp:P05090 (H.sapiens) APOD_HUMAN Apolipoprotein D precursor (ApoD)	CD014176	8,00	2,58	5,56
93	zgc:64091	BC053246.1	9,06	2,34	5,56
94	TAF5-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor	BG728739	8,19	2,39	5,47
95	kinesin-like 5	AW171210	8,42	2,18	5,40
96	weak similarity to protein pir:T29919 (C.elegans) T29919 hypothetical protein ZC449.5 - Caenorhabditis elegans	BM181739	9,78	2,18	5,27
97	weak similarity to protein pir:A36000 (H.sapiens) A36000 sperm-binding glycoprotein ZP3 precursor - human	BM104000	9,82	2,66	4,98
98	moderate similarity to protein sp:O14490 (H.sapiens) DLP1_HUMAN Disks large-associated protein 1 (DAP-1) (Guanylate kinase-associated protein) (hGKAP) (SAP90PSD-95-associated protein 1) (SAPAP1) (PSD-95SAP90 binding protein 1)	BM026548	8,50	2,54	4,92
99	weak similarity to protein ref:NP_056535.1 (H.sapiens) endoglycan; podocalyxin-like 2 (Homo sapiens)"	BM036980	8,60	2,26	4,92
100	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	BC049044.1	8,51	2,27	4,83
101	general transcription factor IIIA	BM082897	8,16	2,22	4,69
102	zgc:92791	BM530945	8,46	2,50	4,48
103	Danio rerio transcribed sequences	CD604918	9,42	2,44	4,42
104	Danio rerio transcribed sequences	BM812123	8,2	2,18	4,34
105	fanconi anemia complementation group G protein	BI878221	8,43	2,16	4,27
106	Glutathione peroxidase 4a	BI896246	10,29	2,04	4,20

107	oncomodulin B	AY070267.1	9,42	2,43	4,17
108	Similar to chaperonin containing TCP1, subunit 6A (zeta 1), clone MGC:55415 IMAGE:2639244, mRNA, complete cds	BM140763	8,62	2,45	4,13
109	weak similarity to protein pir:T46611 (R.norvegicus) T46611 CL2BB protein - rat	BI979582	8,44	2,17	4,13
110	zgc:92102	BI430015	9,10	2,55	4,08
111	Danio rerio transcribed sequences	BQ285080	9,34	2,29	4,00
112	opsin 1 (cone pigments), long-wave-sensitive, 1	NM_131175.1	10,28	2,05	3,91
113	weak similarity to protein pir:S47068 (H.sapiens) S47068 finger protein HZF6, Krueppel-related - human (fragment)	AI588346	8,99	2,55	3,90
114	hypothetical protein LOC407696	BM342451	10,83	2,22	3,87
115	chromosome 6 open reading frame 194-like (H. sapiens)	BM342614	10,50	2,54	3,85
116	zgc:56476	BC049455.1	9,52	2,52	3,85
117	weak similarity to protein sp:P52742 (H.sapiens) Z135_HUMAN Zinc finger protein 135	BI880553	8,12	2,55	3,84
118	moderate similarity to protein pir:I53013 (H.sapiens) I53013 kinesin light chain - human	BI980890	8,03	2,15	3,83
119	annexin A6	AW116978	8,07	2,31	3,81
120	weak similarity to protein sp:P41208 (H.sapiens) CAT1_HUMAN Caltractin, isoform 1 (Centrin 2)	BI884157	8,42	2,58	3,77
121	Danio rerio transcribed sequences	BQ284849	8,83	2,19	3,77
122	Danio rerio transcribed sequences	AL911045	10,05	2,25	3,76
123	zgc:92476	AI584334	8,68	2,06	3,72
124	cryptochrome 3	BC046088.1	8,58	2,08	3,70
125	Transcribed locus, weakly similar to NP_034368.1 bromodomain containing 2 [Mus musculus]	AW175497	9,17	2,10	3,67
126	Danio rerio transcribed sequences	BQ262294	8,05	2,27	3,61
127	selenoprotein P, plasma, 1b	NM_178298.2	8,11	2,50	3,60
128	Danio rerio transcribed sequences	BI891683	8,07	2,24	3,58
129	T-box 4	NM_130914.1	9,84	2,09	3,52
130	moderate similarity to protein pir:T12476 (H.sapiens) T12476 hypothetical protein DKFZp564L0562.1 - human	BI673624	10,04	2,16	3,51
131	zgc:56365	BC049410.1	8,93	2,52	3,48
132	selenoprotein P, plasma, 1a	BI866379	8,36	2,27	3,46
133	zgc:103692	AL925292	10,19	2,53	3,42
134	Transcribed locus, weakly similar to XP_226471.2 similar to homolog of yeast mRNA transport regulator 3 [Rattus norvegicus]	BG985458	10,44	2,63	3,40
135	microfibrillar-associated protein 1	BC052118.1	9,59	2,26	3,40
136	Transcribed locus, weakly similar to NP_057020.1 CGI-04 protein [Homo sapiens]	AW171424	8,68	2,12	3,40
137	zgc:101084	BM571837	10,92	2,33	3,34
138	zgc:55580	BC048052.1	8,59	2,20	3,34
139	Danio rerio transcribed sequences	BI428480	8,84	2,56	3,33
140	cyclin G2	BM736017	9,22	2,08	3,32
141	zgc:77929	BM957968	9,48	2,55	3,29

142	weak similarity to protein ref:NP_055182.1 (H.sapiens) hypothetical protein, estradiol-induced 5 (Homo sapiens)	BM071848	10,02	2,51	3,28
143	zgc:66477	AL911455	8,87	2,24	3,28
144	similar to crystallin, zeta (quinone reductase) (cDNA clone IMAGE:5915682)	BQ480688	8,03	2,17	3,26
145	weak similarity to protein pir:S60466 (D.melanogaster) S60466 transposase - fruit fly (Drosophila melanogaster) transposon element S	AW058783	9,34	2,28	3,24
146	weak similarity to protein ref:NP_061147.1 (H.sapiens) acetyl-CoA synthetase, isoform a; cytoplasmic acetyl-coenzyme A synthetase; acetate-CoA ligase; acyl-activating enzyme; acetate thiokinase (Homo sapiens)"	BI897382	8,43	2,23	3,21
147	zgc:91962	BI984262	8,23	2,59	3,20
148	Danio rerio transcribed sequences	BI709943	10,77	2,33	3,20
149	zgc:56620	BC051623.1	9,77	2,49	3,18
150	zgc:73340	BI673296	8,09	2,66	3,17
151	weak similarity to protein ref:NP_005697.1 (H.sapiens) tumor suppressing subtransferable candidate 4; tumor-suppressing STF cDNA 4; TSSC4 tumor-suppressing STF cDNA 4 (Homo sapiens)"	BM530072	10,58	2,53	3,09
152	Transcribed locus, moderately similar to NP_081415.1 TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor [Mus musculus]	AI397123	10,94	2,54	3,08
153	weak similarity to protein sp:P06727 (H.sapiens) APA4_HUMAN Apolipoprotein A-IV precursor (Apo-AIV)	AL920484	8,13	2,05	3,08
154	zinc finger protein 183	AW421046	9,97	2,22	3,07
155	Danio rerio transcribed sequences	BI670934	8,12	2,45	3,06
156	zona pellucida glycoprotein 3	NM_131331.1	11,07	2,00	3,05
157	zgc:77760	AI942949	11,22	2,51	3,00
158	Danio rerio transcribed sequence	AI497193	10,12	2,37	3,00
159	zgc:55777	BC044485.1	8,49	2,71	2,99
160	zgc:92193	AI964212	9,93	2,30	2,99
161	zgc:85640	AI331186	8,07	2,39	2,97
162	weak similarity to protein ref:NP_079653.1 (M.musculus) RIKEN cDNA 1110001A07 (Mus musculus)	AL715712	9,47	3,14	2,96
163	Danio rerio transcribed sequences	BM530311	8,73	2,50	2,96
164	zgc:92670	BQ618152	9,38	2,46	2,95
165	Danio rerio transcribed sequences	AW420720	8,31	2,26	2,95
166	zgc:85702	AI437377	9,16	2,29	2,94
167	swelling dependent chloride channel	BC052141.1	9,25	2,69	2,93
168	calpain, small subunit 1	AL925202	9,00	2,52	2,93
169	zgc:100907	BI475189	8,03	2,78	2,92
170	Danio rerio transcribed sequence with weak similarity to protein pir:A54661 (H.sapiens) A54661 zinc finger protein ZNF41 - human (fragment)	BM156878	8,30	3,06	2,91
171	zgc:92894	AW077423	8,30	3,00	2,91
172	Danio rerio transcribed sequences	AI667252	9,29	2,40	2,91

173	Danio rerio transcribed sequence	BI984278	9,74	3,13	2,90	
174	mitochondrial ribosomal protein L38	AI964108	9,85	2,10	2,90	
175	moderate similarity to protein ref:NP_073585.6 (H.sapiens) tumor endothelial marker 6; thyroid specific PTB domain protein (Homo sapiens)"	AI723162	8,01	2,32	2,88	
176	zgc:85752	BI983410	11,27	2,54	2,87	
177	microtubule-associated protein, RP/EB family, member 1, like	BC044167.1	10,96	2,22	2,87	
178	Danio rerio transcribed sequence with moderate similarity to protein sp:Q99708 (H.sapiens) RBB8_HUMAN Retinoblastoma-binding protein 8 (RBBP-8) (CtBP interacting protein) (CtIP) (Retinoblastoma-interacting protein and myosin-like) (RIM)	AI942930	8,74	3,35	2,86	
179	weak similarity to protein sp:Q9UHN1 (H.sapiens) DPG2_HUMAN DNA polymerase gamma subunit 2, mitochondrial precursor (Mitochondrial DNA polymerase accessory subunit) (PolG-beta) (MtPolB) (DNA polymerase gamma accessory 55 kDa subunit) (p55)	BM156735	8,24	2,66	2,86	
180	zgc:73187	AA497149	9,14	2,88	2,85	
181	zgc:63573	BM095864	9,35	2,19	2,84	
182	zgc:55376	BC047169.1	10,00	2,69	2,83	
183	Transcribed locus, moderately similar to NP_008882.2 TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa [Homo sapiens]	BI980350	10,53	2,00	2,83	
184	Danio rerio transcribed sequence with moderate similarity to protein sp:O14525 (H.sapiens) ACN1_HUMAN Astrotactin 1	BM082811	8,84	2,80	2,82	
185	Transcribed locus, moderately similar to NP_004854.1 serine palmitoyltransferase, long chain base subunit 2 [Homo sapiens]	BI984521	9,79	2,28	2,82	
186	zgc:92126	BI980257	9,36	2,17	2,82	
187	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	AI437173	8,16	4,30	2,81	
188	zgc:77462	AW281850	8,09	2,61	2,81	
Below are without dnd as selection criteria						
189	Carbonic anhydrase 4	BM957986	10,36	1,74	5,91	x
190	Danio rerio, clone MGC:55385 IMAGE:2602021, mRNA, complete cds /FL=gb:BC044387.1	BM533995	10,24	1,41	4,30	
191	weak similarity to protein ref:NP_001602.1 (H.sapiens) tartrate resistant acid phosphatase 5 precursor (Homo sapiens)	AI882824	9,37	1,36	2,73	
192	moderate similarity to protein ref:NP_078804.1 (H.sapiens) hypothetical protein FLJ22626 (Homo sapiens)	BM957900	9,34	1,39	2,65	
193	ribonucleotide reductase protein r1 class I	CD014975	9,13	1,46	3,40	
194	weak similarity to protein sp:Q08426 (H.sapiens) ECHP_HUMAN Peroxisomal bifunctional enzyme (PBE) (PBFE) (Includes: Enoyl-CoA hydratase ; 3,2-trans-enoyl-CoA isomerase ; 3-hydroxyacyl-CoA dehydrogenase)	AW019691	7,63	2,43	2,61	

195	membrin	BC053235	7,55	2,15	2,81
196	Danio rerio transcribed sequences	BM402113	7,20	1,53	2,99
197	Tdrd7, TRAP	BC052137	11,69	0,47	7,91
198	ribonucleotide reductase protein r2 class I	AL728293	10,56	-0,39	5,10
199	cathepsin L, b	AL718042	11,02	0,13	4,51
200	strong similarity to protein pdb:1YER (H.sapiens) Human Hsp90 Geldanamycin-Binding Domain, closed Conformation	BG306502	9,20	0,77	4,41
201	weak similarity to protein pir:S35342 (H.sapiens) S35342 Golgi-associated particle 102K chain - human	BM080957	7,44	0,36	4,29
202	cellular nucleic acid-binding protein mRNA, complete cds	AW115837	11,86	0,53	4,18
203	weak similarity to protein prf:2208436B (H.sapiens) 2208436B hepatocyte nuclear factor 4 (Homo sapiens)	AL926145	7,29	0,76	3,85
204	creatine kinase, brain (ckb)	NM_173222	8,88	0,60	3,84
205	myeloid ecotropic viral integration site 3 (meis3)	NM_131778	7,94	-0,74	3,81
206	Danio rerio transcribed sequences	AW019440	9,04	0,04	3,76
207	Danio rerio transcribed sequences	AW058961	7,91	0,39	3,75
208	cytochrome P450, subfamily XIA, polypeptide 1 (cyp11a1)	NM_152953	7,67	0,86	3,70
209	weak similarity to protein sp:P07148 (H.sapiens) FABL_HUMAN FATTY ACID-BINDING PROTEIN, LIVER (L-FABP)	BM182911	10,88	0,51	3,67
210	Danio rerio, clone MGC:56493 IMAGE:5777308, mRNA, complete cds	BM534432	9,17	0,92	3,50
211	Danio rerio transcribed sequences	BG308620	8,32	0,25	3,50
212	moderate similarity to protein sp:Q93088 (H.sapiens) BHMT_HUMAN Betaine--homocysteine S-methyltransferase	BI705782	11,22	0,88	3,44
213	Danio rerio transcribed sequences	BQ262207	7,79	0,65	3,43
214	apolipoprotein A-I (apoa)	NM_131128	10,72	0,60	3,37
215	moderate similarity to protein sp:P04406 (H.sapiens) G3P2_HUMAN Glyceraldehyde 3-phosphate dehydrogenase, liver	BQ077741	10,34	0,97	2,55
216	zona pellucida glycoprotein 3 (zp3)	BM184021	10,76	1,09	2,56
217	weak similarity to protein sp:P06727 (H.sapiens) APA4_HUMAN Apolipoprotein A-IV precursor (Apo-AIV)	AI477980	9,12	1,00	3,12
218	weak similarity to protein pir:S42626 (H.sapiens) S42626 ER-golgi intermediate compartment protein - human	BQ284855	7,97	1,03	3,35
219	zygote arrest 1 (zar1)	AY283178	7,25	1,28	2,58
220	casein kinase 2 beta (ck2b)	BM533791	6,88	1,31	3,46
221	moderate similarity to protein sp:P11142 (H.sapiens) HS7C_HUMAN Heat shock cognate 71 kDa protein	BM103345	6,81	1,77	3,63
223	weak similarity to protein ref:NP_079081.1 (H.sapiens) hypothetical protein FLJ21172 (Homo sapiens)	BQ616904	6,68	2,40	2,58
224	coilin p80	BC045858	6,68	1,41	2,69
225	heat shock protein 90-alpha	BM103343	6,65	1,52	2,79
226	weak similarity to protein prf:2208306A (H.sapiens) 2208306A metal-binding protein (Homo sapiens)	AW116113	6,60	1,74	3,03

227	Danio rerio transcribed sequences	BM777122	6,56	1,95	2,60	
228	Danio rerio transcribed sequences	AL919330	8,04	1,94	4,53	
229	Danio rerio transcribed sequence	BM082834	7,72	2,02	2,69	
230	Danio rerio transcribed sequence	CD606373	7,98	3,57	3,31	
231	Danio rerio transcribed sequence	CD606429	7,96	3,10	3,91	
232	weak similarity to protein ref:NP_116222.1 (H.sapiens) hypothetical protein FLJ14744 (Homo sapiens)	BM777683	7,90	3,29	4,34	
233	Danio rerio transcribed sequence	BQ618177	7,85	3,23	4,14	
234	zgc:63714	AI793494	7,83	3,01	3,00	
235	proteasome (prosome, macropain) subunit, beta type, 11 (psmb11)	NM_131676.1	7,64	3,64	4,94	
236	weak similarity to protein ref:NP_116012.1 (H.sapiens) ovary-specific acidic protein (Homo sapiens)	BI867507	7,62	3,35	3,31	
237	weak similarity to protein ref:NP_056513.2 (H.sapiens) T54 protein (Homo sapiens)	BI887400	7,61	3,07	4,00	
238	Danio rerio transcribed sequence	AL916489	7,61	4,30	3,24	
239	zgc:101596, RAD51 homolog	BI672084	7,60	2,90	3,2	
240	weak similarity to protein sp:Q92664 (H.sapiens) TF3A_HUMAN Transcription factor IIIA (Factor A) (TFIIIA)	BQ260855	7,53	4,75	7,53	
241	weak similarity to protein ref:NP_502332.1 (C.elegans) C42C1.4.p (Caenorhabditis elegans)	AW233610	7,53	2,98	3,75	
242	zgc:85910	BG728987	7,52	3,62	3,12	
243	Danio rerio transcribed sequences	AW420842	7,52	3,06	3,68	
244	moderate similarity to protein ref:NP_536711.1 (M.musculus) protein O-fucosyltransferase 1 (Mus musculus)	BM184278	7,52	2,78	3,10	

Below known PGC specific genes excluded from further analysis.

	vasa	Y12007.1	9,24	2,05	9,24	x
	h1m	AF499607.1	12,34	4,34	9,33	x
	dazl	NM_131524.1	11	2,23	7,42	x
	cxcr4b	NM_131834.1	10,98	2,06	2,88	x

7.2 Examples of Expression Pattern varieties of the candidate genes

For many genes selected in the Affymetrix microarray screen a PGC specific or enriched expression in PGCs could not be confirmed. Instead they showed a wide variety of expression pattern (examples shown in Fig. 7-1).

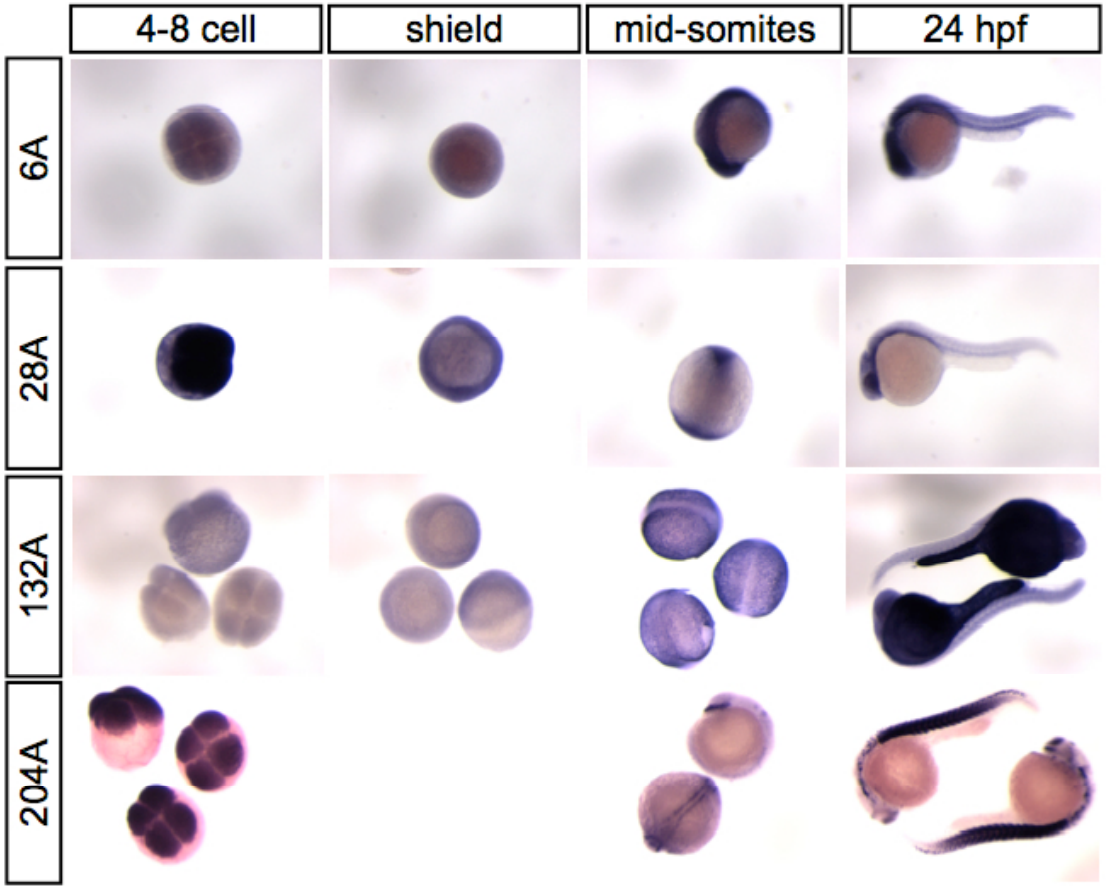


Fig. 7-1. Examples of Expression pattern variety in the In Situ hybridization screen. Some genes are maternally provided, see 28A and 204A as examples, some are not like 6A and 132A. Expressions were detected in a variety of tissues. 6A shows an increased expression in the head at 24 hpf. 132A (selenoprotein 1a plasma) is expressed in the YSL cells. 204A (creatine kinase brain) shows a strong expression in the diencephalon at mid somite stage and at 24 hpf it is predominantly expressed in the central nervous system and spinal chord.

7.3 Expression Pattern with confirmed enriched expression in PGCs

For 20 genes, from the microarray screen, an enriched expression in PGCs was observed in at least one of the 4 stages examined. The stages analyzed cover typical steps of germ cell development within the first 24 hpf: 4 cell stage (germ plasm localized to cleavage furrows), shield (PGCs migrate individually from the place of specification), mid-somite (PGC cluster) and 24 hpf (PGCs reached the place where the gonad will develop).

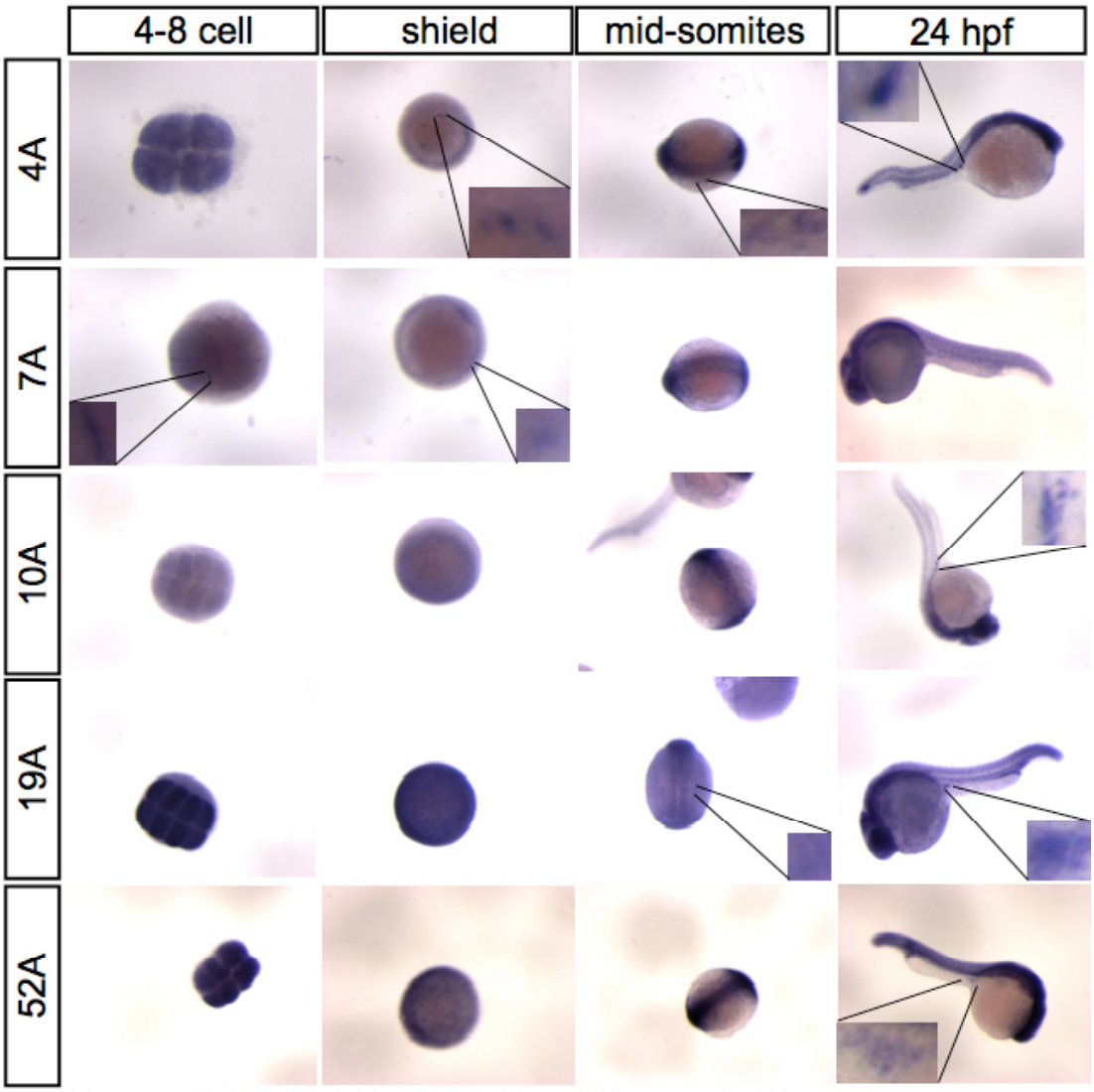


Fig. 7-2. Expression pattern of the first 5 genes with confirmed expression in PGCs. Expression in PGCs was observed in at least one of the depicted stages. 4A (PDCD4) was not found in the germ plasm but is expressed in PGCs of all 3 stages during PGC migration. 7A (novel protein, named later Granulito) is localized in the germ plasm (staining in the cleavage furrow) at the 4 cell stage and is later on found in PGCs. 10A (novel gene) is only faintly expressed in PGCs at 24 hpf. 19A (superoxide dismutase 2) is expressed at mid-somite and 24 hpf stage in the PGCs. 52A (G kinase anchoring protein 1) was found in PGCs at 24 hpf. Pictures were taken at 5x magnification.

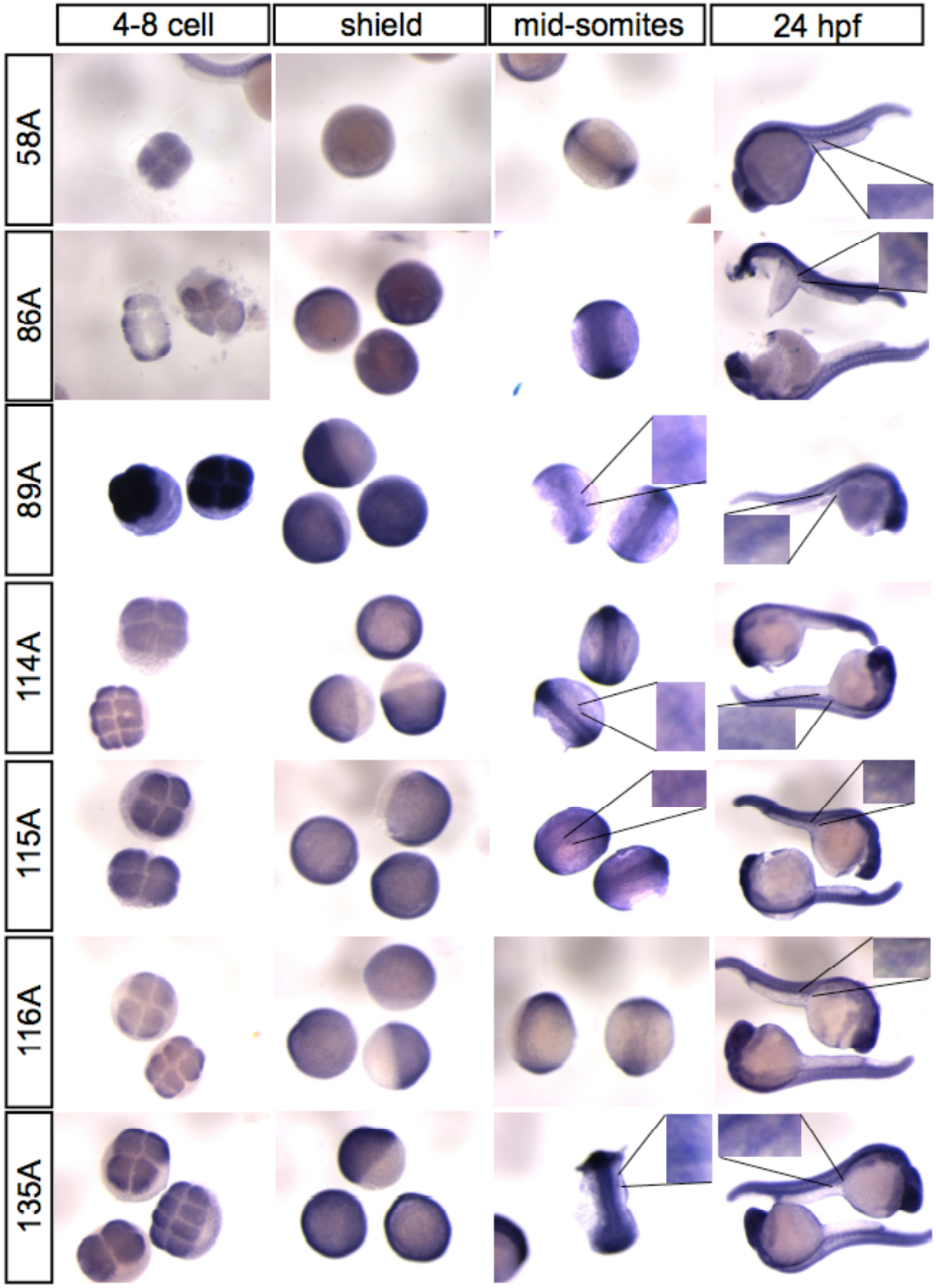


Fig. 7-3. Expression pattern of genes 6-12 with confirmed expression in PGCs. 58A (GTP binding protein 1) was found in PGCs at 24 hpf. 86A (alternative 3'UTR of Granulito) was found in germ plasm and in PGCs at 24 hpf. 89A (oogenesis-related protein) a strong maternally supplied RNA had a fainted expression in PGCs at 24 hpf. 114A (DEAD box polypeptide 52) is expressed at mid-somite and 24 hpf stage in PGCs. 115A (chromosome 6 open reading frame 194-like) is expressed at mid-somite and 24 hpf stage in PGCs. 116A (hypothetical protein) is expressed at 24 hpf in PGCs. 135A

(microfibrillar-associated protein 1) is expressed at mid-somite and 24 hpf stage in PGCs. Pictures were taken at 5x magnification.

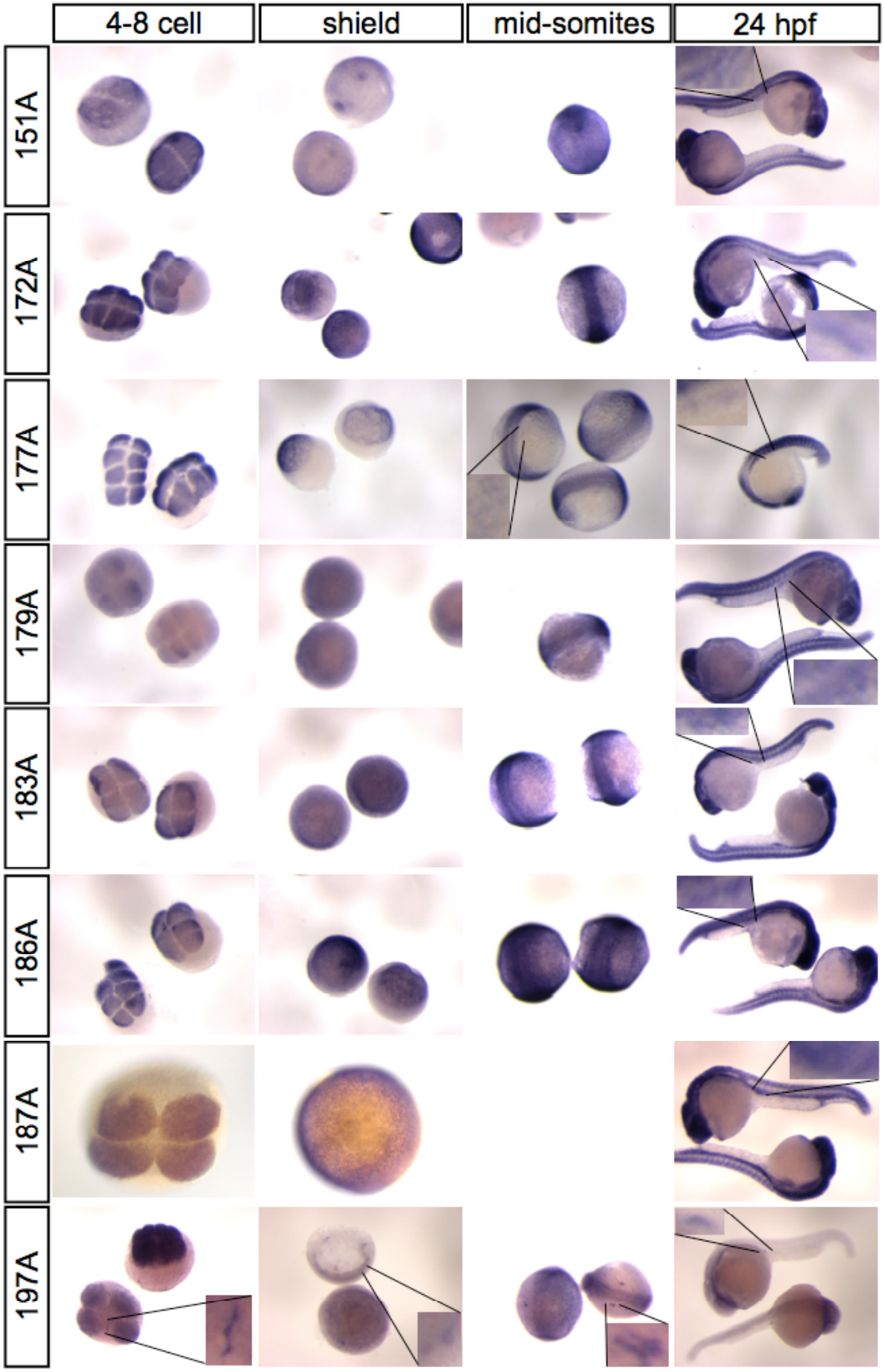


Fig. 7-4. Expression pattern of genes 13-20 with confirmed expression in PGCs. 151A (hypothetical protein) is expressed at 24 hpf stage. 172A (hypothetical protein) is expressed at 24 hpf stage. 177A (microtubule associated protein) (hypothetical protein) is expressed at mid-somite and 24 hpf stage. 179A(unknown) is expressed at 24 hpf stage. 183A (TAF5) is expressed at 24 hpf stage. 186A (hypothetical protein) is expressed at 24 hpf stage. 187A (RAD51) is expressed at 24 hpf stage. **Note:** 4 cell stage and shield stage picture were captured at 10x magnification. 197A (TDRD7) is specifically expressed in the germ plasm and all 3 stages of PGC development in the first 24 hpf. Pictures were taken at 5x magnification.

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10 References

Allison, D. B., Cui, X., Page, G. P. and Sabripour, M. (2006). Microarray data analysis: from disarray to consolidation and consensus. *Nature reviews* **7**, 55-65.

Anderson, C., Catoe, H. and Werner, R. (2006). MIR-206 regulates connexin43 expression during skeletal muscle development. *Nucleic Acids Research* **34**, 5863-71.

Aravin, A. and Tuschl, T. (2005). Identification and characterization of small RNAs involved in RNA silencing. *FEBS Letters* **579**, 5830-40.

Arkov, A. L., Wang, J. Y., Ramos, A. and Lehmann, R. (2006). The role of Tudor domains in germline development and polar granule architecture. *Development* **133**, 4053-62.

Asaoka-Taguchi, M., Yamada, M., Nakamura, A., Hanyu, K. and Kobayashi, S. (1999). Maternal Pumilio acts together with Nanos in germline development in Drosophila embryos. *Nat Cell Biol* **1**, 431-437.

Ashraf, S. I. and Kunes, S. (2006). A trace of silence: memory and microRNA at the synapse. *Current Opinion in Neurobiology* **16**, 535-9.

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R. and Pasquinelli, A. E. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* **122**, 553-63.

Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I. and Filipowicz, W. (2006). Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* **125**, 1111-24.

Blaser, H., Eisenbeiss, S., Neumann, M., Reichman-Fried, M., Thisse, B., Thisse, C. and Raz, E. (2005). Transition from non-motile behaviour to directed migration during early PGC development in zebrafish. *Journal of Cell Science* **118**, 4027-38.

Boswell, R. E. and Mahowald, A. P. (1985). tudor, a gene required for assembly of the germ plasm in Drosophila melanogaster. *Cell* **43**, 97-104.

Braat, A. K., Speksnijder, J. E. and Zivkovic, D. (1999). Germ line development in fishes. *International Journal of Developmental Biology* **43(7**, 745-60.

Breitwieser, W., Markussen, F. H., Horstmann, H. and Ephrussi, A. (1996). Oskar protein interaction with Vasa represents an essential step in polar granule assembly. *Genes and Development* **10**, 2179-88.

Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R. and Hannon, G. J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell* **128**, 1089-103.

Chuma, S., Hosokawa, M., Kitamura, K., Kasai, S., Fujioka, M., Hiyoshi, M., Takamune, K., Noce, T. and Nakatsuji, N. (2006). Tdrd1/Mtr-1, a tudor-related

gene, is essential for male germ-cell differentiation and nuage/germinal granule formation in mice. *Proc Natl Acad Sci U S A* **103**, 15894-9.

Ciosk, R., DePalma, M. and Priess, J. R. (2006). Translational regulators maintain totipotency in the Caenorhabditis elegans germline. *Science (New York, N.Y* **311**, 851-3.

Cogoni, C. and Macino, G. (1999). Homology-dependent gene silencing in plants and fungi: a number of variations on the same theme. *Current opinion in microbiology* **2**, 657-62.

Doitsidou, M., Reichman-Fried, M., Stebler, J., Koprunner, M., Dorries, J., Meyer, D., Esguerra, C. V., Leung, T. and Raz, E. (2002). Guidance of Primordial Germ Cell Migration by the Chemokine SDF-1. *Cell* **111**, 647-59.

Draper, B. W., McCallum, C. M. and Moens, C. B. (2007). nanos1 is required to maintain oocyte production in adult zebrafish. *Developmental Biology* **305**, 589-98.

Dumstrei, K., Mennecke, R. and Raz, E. (2004). Signaling Pathways Controlling Primordial Germ Cell Migration in Zebrafish. *Journal of Cell Science* **117**, 4787-4795.

Ephrussi, A. and Lehmann, R. (1992). Induction of germ cell formation by oskar. *Nature* **358**, 387-92.

Extavour, C. G. and Akam, M. (2003). Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development* **130**, 5869-84.

Forbes, A. and Lehmann, R. (1998). Nanos and Pumilio have critical roles in the development and function of Drosophila germline stem cells. *Development* **125**, 679-90.

Gregory, R. I., Chendrimada, T. P., Cooch, N. and Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* **123**, 631-40.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W. S. and Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* **97**, 121-32.

Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H. and Siomi, M. C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in Drosophila. *Science (New York, N.Y* **315**, 1587-90.

Handa, N., Nureki, O., Kurimoto, K., Kim, I., Sakamoto, H., Shimura, Y., Muto, Y. and Yokoyama, S. (1999). Structural basis for recognition of the tra mRNA precursor by the Sex-lethal protein. *Nature* **398**, 579-85.

Hashimoto, Y., Maegawa, S., Nagai, T., Yamaha, E., Suzuki, H., Yasuda, K. and Inoue, K. (2004). Localized maternal factors are required for zebrafish germ cell formation. *Developmental Biology* **268**, 152-61.

Hauptmann, G. and Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and Drosophila embryos. *Trends in Genetics* **10**, 266.

Hay, B., Jan, L. and Jan, Y. (1988). A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence
similarity to ATP-dependent helicases. *Cell* **55**, 577-587.

Hayashi, K., de Sousa Lopes, S. M. and Surani, M. A. (2007). Germ cell specification in mice. *Science (New York, N.Y* **316**, 394-6.

Hird, S. N., Paulsen, J. E. and Strome, S. (1996). Segregation of germ granules in living Caenorhabditis elegans embryos: cell-type-specific mechanisms for cytoplasmic localisation. *Development* **122**, 1303-12.

Hirose, T., Kawabuchi, M., Tamaru, T., Okumura, N., Nagai, K. and Okada, M. (2000). Identification of tudor repeat associator with PCTAIRE 2 (Trap). A novel protein that interacts with the N-terminal domain of PCTAIRE 2 in rat brain. *European journal of biochemistry / FEBS* **267**, 2113-21.

Horvay, K., Claussen, M., Katzer, M., Landgrebe, J. and Pieler, T. (2006). Xenopus Dead end mRNA is a localized maternal determinant that serves a conserved function in germ cell development. *Developmental Biology*.

Hosokawa, M., Shoji, M., Kitamura, K., Tanaka, T., Noce, T., Chuma, S. and Nakatsuji, N. (2007). Tudor-related proteins TDRD1/MTR-1, TDRD6 and TDRD7/TRAP: domain composition, intracellular localization, and function in male germ cells in mice. *Developmental Biology* **301**, 38-52.

Houston, D. and King, M. (2000). Germ plasm and molecular determinants of germ cell fate. *Current Topics in Developmental Biology* **50**, 155-81.

Houston, D. W., Zhang, J., Maines, J. Z., Wasserman, S. A. and King, M. L. (1998). A Xenopus DAZ-like gene encodes an RNA component of germ plasm and is a functional homologue of Drosophila boule. *Development* **125**, 171-80.

Houwing, S., Kamminga, L. M., Berezikov, E., Cronembold, D., Girard, A., van den Elst, H., Filippov, D. V., Blaser, H., Raz, E., Moens, C. B. et al. (2007). A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. *Cell* **129**, 69-82.

Iida, T. and Kobayashi, S. (1998). Essential role of mitochondrially encoded large rRNA for germ-line formation in Drosophila embryos. *Proc Natl Acad Sci U S A* **95**, 11274-8.

Illmensee, K. and Mahowald, A. P. (1974). Transplantation of posterior polar plasm in Drosophila. Induction of germ cells at the anterior pole of the egg. *Proc Natl Acad Sci U S A* **71**, 1016-20.

Johnson, A. D., Drum, M., Bachvarova, R. F., Masi, T., White, M. E. and Crother, B. I. (2003). Evolution of predetermined germ cells in vertebrate embryos: implications for macroevolution. *Evolution & development* **5**, 414-31.

Jowett, T. and Lettice, L. (1994). Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes. *Trends in Genetics* **10**, 73-4.

Kawakami, K., Takeda, H., Kawakami, N., Kobayashi, M., Matsuda, N. and Mishina, M. (2004). A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. *Dev Cell* **7**, 133-44.

Kimmel, C., Ballard, W., SR, K., B, U. and TF, S. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics* **203**, 253-310.

Kloosterman, W. P. and Plasterk, R. H. (2006). The diverse functions of microRNAs in animal development and disease. *Developmental Cell* **11**, 441-50.

Knaut, H., Pelegri, F., Bohmann, K., Schwarz, H. and Nusslein-Volhard, C. (2000). Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. *Journal of Cell Biology* **149**, 875-88.

Knaut, H., Steinbeisser, H., Schwarz, H. and Nusslein-Volhard, C. (2002). An Evolutionary Conserved Region in the vasa 3'UTR Targets RNA Translation to the Germ Cells in the Zebrafish. *Current Biology* **12**, 454-66.

Knaut, H., Werz, C., Geisler, R. and Nusslein-Volhard, C. (2003). A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. *Nature* **421**, 279-82.

Kobayashi, S., Amikura, R. and Okada, M. (1993). Presence of mitochondrial large ribosomal RNA outside mitochondria in germ plasm of Drosophila melanogaster. *Science* **260**, 1521-4.

Kotaja, N., Bhattacharyya, S. N., Jaskiewicz, L., Kimmins, S., Parvinen, M., Filipowicz, W. and Sassone-Corsi, P. (2006a). The chromatoid body of male germ cells: similarity with processing bodies and presence of Dicer and microRNA pathway components. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 2647-52.

Kotaja, N., Lin, H., Parvinen, M. and Sassone-Corsi, P. (2006b). Interplay of PIWI/Argonaute protein MIWI and kinesin KIF17b in chromatoid bodies of male germ cells. *Journal of Cell Science* **119**, 2819-25.

Kotaja, N. and Sassone-Corsi, P. (2007). The chromatoid body: a germ-cell-specific RNA-processing centre. *Nature reviews* **8**, 85-90.

Köprunner, M., Thisse, C., Thisse, B. and Raz, E. (2001). A zebrafish nanos-related gene is essential for the development of primordial germ cells. *Genes and Development* **15**, 2877-85.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W. and Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. *Current Biology* **12**, 735-9.

Lasko, P. and Ashburner, M. (1988). The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A. *Nature* **335**, 611-617.

Lawson, K. A., Dunn, N. R., Roelen, B. A., Zeinstra, L. M., Davis, A. M., Wright, C. V., Korving, J. P. and Hogan, B. L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes and Development* **13**, 424-436.

le Sage, C., Nagel, R., Egan, D. A., Schrier, M., Mesman, E., Mangiola, A., Anile, C., Maira, G., Mercatelli, N., Ciafre, S. A. et al. (2007). Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation.

Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843-54.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415-9.

Lehmann, R. and Nusslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in Drosophila. *Cell* **47**, 141-52.

Lewis, B. P., Burge, C. B. and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**, 15-20.

Li, C. and Hung Wong, W. (2001). Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biology* **2**, RESEARCH0032.

Li, Y., Pei, J., Xia, H., Ke, H., Wang, H. and Tao, W. (2003). Lats2, a putative tumor suppressor, inhibits G1/S transition. *Oncogene* **22**, 4398-405.

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P., Linsley, P. S. and Johnson, J. M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433**, 769-73.

Liu, J., Rivas, F. V., Wohlschlegel, J., Yates, J. R., 3rd, Parker, R. and Hannon, G. J. (2005). A role for the P-body component GW182 in microRNA function. *Nature cell biology* **7**, 1261-6.

Maegawa, S., Yasuda, K. and Inoue, K. (1999). Maternal mRNA localization of zebrafish DAZ-like gene. *Mechanisms of Development* **81**, 223-6.

Mahowald, A. P. (1968). Polar granules of Drosophila. II. Ultrastructural changes during early embryogenesis. *Journal of Experimental Zoology* **167**, 237-61.

Maniataki, E. and Mourelatos, Z. (2005). A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. *Genes & development* **19**, 2979-90.

McLaren, A. (1999). Signaling for germ cells. *Genes and Development* **13**, 373-6.

Megosh, H. B., Cox, D. N., Campbell, C. and Lin, H. (2006). The role of PIWI and the miRNA machinery in Drosophila germline determination. *Current Biology* **16**, 1884-94.

Micklem, D. R. (1995). mRNA localisation during development. *Developmental Biology* **172**, 377-95.

Mishima, Y., Giraldez, A. J., Takeda, Y., Fujiwara, T., Sakamoto, H., Schier, A. F. and Inoue, K. (2006a). Differential regulation of germline mRNAs in soma and germ cells by zebrafish miR-430. *Current Biology* **16**, 2135-42.

Molyneaux, K. and Wylie, C. (2004). Primordial germ cell migration. *International Journal of Developmental Biology* **48**, 537-43.

Müller, K., Thisse, C., Thisse, B. and Raz, E. (2002). Expression of a linker histone-like gene in the primordial germ cells in zebrafish. *Mechanisms of Development* **117**, 253-257.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D. Y. and Nakayama, K. (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**, 707-20.

Olsen, L. C., Aasland, R. and Fjose, A. (1997). A vasa-like gene in zebrafish identifies putative primordial germ cells. *Mechanisms of Development* **66**, 95-105.

Oosterhuis, J. W. and Looijenga, L. H. (2005). Testicular germ-cell tumours in a broader perspective. *Nature reviews* **5**, 210-22.

Pan, J., Goodheart, M., Chuma, S., Nakatsuji, N., Page, D. C. and Wang, P. J. (2005). RNF17, a component of the mammalian germ cell nuage, is essential for spermiogenesis. *Development (Cambridge, England)* **132**, 4029-39.

Parker, R. and Sheth, U. (2007). P bodies and the control of mRNA translation and degradation. *Molecular cell* **25**, 635-46.

Pelegri, F. (2003). Maternal factors in zebrafish development. *Developmental Dynamics* **228**, 535-54.

Pelegri, F., Knaut, H., Maischein, H., Schulte-Merker, S. and Nusslein-Volhard, C. (1999). A mutation in the zebrafish maternal-effect gene nebel affects furrow formation and vasa RNA localization. *Current Biology* **9**, 1431-40.

Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E. and Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science (New York, N.Y* **309**, 1573-6.

Pillai, R. S., Bhattacharyya, S. N. and Filipowicz, W. (2007). Repression of protein synthesis by miRNAs: how many mechanisms? *Trends in cell biology* **17**, 118-26.

Qiao, D., Zeeman, A. M., Deng, W., Looijenga, L. H. and Lin, H. (2002). Molecular characterization of hiwi, a human member of the piwi gene family whose overexpression is correlated to seminomas. *Oncogene* **21**, 3988-99.

Raz, E. (2000). The function and regulation of vasa-like genes in germ-cell development. *Genome Biol* **1**, 1017.1-1017.6.

Raz, E. (2002). Primordial germ cell development in zebrafish. *Semin Cell Dev Biol* **13**, 489-95.

Raz, E. (2003). Primordial germ-cell development: the zebrafish perspective. *Nature Reviews Genetics* **4**, 690-700.

Raz, E. (2004). Guidance of primordial germ cell migration. *Current Opinion in Cell Biology* **16**, 169-73.

Raz, E. and Reichman-Fried, M. (2006). Attraction rules: germ cell migration in zebrafish. *Current Opinion in Genetics and Development* **16**, 355-9.

Robb, D. L., Heasman, J., Raats, J. and Wylie, C. (1996). A kinesin-like protein is required for germ plasm aggregation in Xenopus. *Cell* **87**, 823-31.

Rongo, C. and Lehmann, R. (1996). Regulated synthesis, transport and assembly of the Drosophila germ plasm. *Trends in Genetics* **12**, 102-9.

Saffman, E. E. and Lasko, P. (1999). Germline development in vertebrates and invertebrates. *Cellular and Molecular Life Sciences* **55**, 1141-63.

Santos, A. C. and Lehmann, R. (2004). Germ cell specification and migration in Drosophila and beyond. *Current Biology* **14**, R578-89.

Schratt, G. M., Tuebing, F., Nigh, E. A., Kane, C. G., Sabatini, M. E., Kiebler, M. and Greenberg, M. E. (2006). A brain-specific microRNA regulates dendritic spine development. *Nature* **439**, 283-9.

Schwede, T., Kopp, J., Guex, N. and Peitsch, M. C. (2003). SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Research* **31**, 3381-5.

Seydoux, G. (1996). Mechanisms of translational control in early development. *Current Opinion in Genetics and Development* **6**, 555-61.

Seydoux, G. and Strome, S. (1999). Launching the germline in Caenorhabditis elegans: regulation of gene expression in early germ cells. *Development* **126**, 3275-83.

Smith, J. M., Bowles, J., Wilson, M., Teasdale, R. D. and Koopman, P. (2004). Expression of the tudor-related gene Tdrd5 during development of the male germline in mice. *Gene expression patterns* **4**, 701-5.

St Johnston, D., Beuchle, D. and Nusslein-Volhard, C. (1991). Staufen, a gene required to localize maternal RNAs in the Drosophila egg. *Cell* **66**, 51-63.

Stanyon, C. A. and Bernard, O. (1999). LIM-kinase1. *International Journal of Biochemistry and Cell Biology* **31**, 389-94.

Starz-Gaiano, M. and Lehmann, R. (2001). Moving towards the next generation. *Mechanisms of Development* **105**, 5-18.

Stebler, J., Spieler, D., Slanchev, K., Molyneaux, K. A., Richter, U., Cojocaru, V., Tarabykin, V., Wylie, C., Kessel, M. and Raz, E. (2004). Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. *Developmental Biology* **272**, 351-61.

Styhler, S., Nakamura, A., Swan, A., Suter, B. and Lasko, P. (1998). vasa is required for GURKEN accumulation in the oocyte, and is involved in oocyte differentiation and
germline cyst development. *Development* **125**, 1569-78.

Tan, C. H., Lee, T. C., Weeraratne, S. D., Korzh, V., Lim, T. M. and Gong, Z. (2002). Ziwi, the zebrafish homologue of the Drosophila piwi: co-localization with vasa at the embryonic genital ridge and gonad-specific expression in the adults. *Gene Expr Patterns* **2**, 257-60.

Thelen, M. (2001). Dancing to the tune of chemokines. *Nat Immunol* **2**, 129-34.

Theusch, E. V., Brown, K. J. and Pelegri, F. (2006). Separate pathways of RNA recruitment lead to the compartmentalization of the zebrafish germ plasm. *Developmental Biology* **292**, 129-41.

Thomson, T. and Lasko, P. (2005). Tudor and its domains: germ cell formation from a Tudor perspective. *Cell Res* **15**, 281-91.

Tsunekawa, N., Naito, M., Sakai, Y., Nishida, T. and Noce, T. (2000). Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. *Development* **127**, 2741-50.

Van Gelder, R. N., von Zastrow, M. E., Yool, A., Dement, W. C., Barchas, J. D. and Eberwine, J. H. (1990). Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 1663-7.

Voorhoeve, P. M. and Agami, R. (2007). Classifying microRNAs in cancer: the good, the bad and the ugly. *Biochimica et Biophysica Acta* **1775**, 274-82.

Wakahara, M. (1996). Primordial germ cell development: is the urodele pattern closer to mammals than to anurans? *The International journal of developmental biology* **40**, 653-9.

Weidinger, G., Stebler, J., Slanchev, K., Dumstrei, K., Wise, C., Lovell-Badge, R., Thisse, C., Thisse, B. and Raz, E. (2003). *dead end*, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. *Current Biology* **13**, 1429-1434.

Weidinger, G., Wolke, U., Koprunner, M., Thisse, C., Thisse, B. and Raz, E. (2002). Regulation of zebrafish primordial germ cell migration by attraction towards an intermediate target. *Development* **129**, 25-36.

Westerfield, M. (1995). The Zebrafish Book. Oregon: University of Oregon Press.

Whitington, P. M. and Dixon, K. E. (1975). Quantitative studies of germ plasm and germ cells during early embryogenesis of Xenopus laevis. *J Embryol Exp Morphol* **33**, 57-74.

Wienholds, E. and Plasterk, R. H. (2005). MicroRNA function in animal development. *FEBS Letters* **579**, 5911-22.

Wolke, U., Weidinger, G., Köprunner, M. and Raz, E. (2002). Multiple levels of post-transcriptional control lead to germ line specific gene expression in the zebrafish. *Current Biology* **12**, 289-294.

Wreden, C., Verrotti, A. C., Schisa, J. A., Lieberfarb, M. E. and Strickland, S. (1997). Nanos and pumilio establish embryonic polarity in Drosophila by promoting posterior deadenylation of hunchback mRNA. *Development* **124**, 3015-23.

Wylie, C. (1999). Germ cells. *Cell* **96**, 165-74.

Wylie, C. (2000). Germ cells. *Current Opinion in Genetics and Development* **10**, 410-3.

Yoon, C., Kawakami, K. and Hopkins, N. (1997). Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. *Development* **124**, 3157-65.

Youngren, K. K., Coveney, D., Peng, X., Bhattacharya, C., Schmidt, L. S., Nickerson, M. L., Lamb, B. T., Deng, J. M., Behringer, R. R., Capel, B. et al. (2005). The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. *Nature* **435**, 360-4.

Zamore, P. D., Bartel, D. P., Lehmann, R. and Williamson, J. R. (1999). The PUMILIO-RNA interaction: a single RNA-binding domain monomer recognizes a bipartite target sequence. *Biochemistry* **38**, 596-604.

Zhou, Y. and King, M. (1996). Localization of Xcat-2 RNA, a putative germ plasm component, to the mitochondrial cloud in Xenopus stage I oocytes. *Development* **122**, 2947-2953.

11 Acknowledgements

I would like to thank Prof. Erez Raz for giving me the opportunity to conduct my PhD thesis in his lab and for all his support during this time. His broad experience, which he shared with me, has helped me to find my way in daily work as well as in science generally.

A special thank to my former colleague Karin Dumstrei with whom I started the microarray project. I benefited a lot from here experience and I enjoyed the time working with her. Work was not the same anymore without her.

I am grateful to Prof. Michael Kessel and Prof. Tomas Pieler for the helpful guidance and discussions during the committee meetings. A special thank to Dr. Steffen Burkhardt, the coordinator of our PhD program for all his effort in to keep the program organized in the best possible way.

I also thank all former and current lab member of the Raz lab a lot for the time I could spend with them in the lab, working or chatting. Specially, I am thankful to our former and current technicians, Rebecca, Helene, Uschi and Julia, who assisted me a lot in the daily work routine. Additional I would like to express my gratitude to Bijan for his comments on the manuscript of this thesis.

Meinen Eltern Sigrid und Johann Strasser möchte ich ganz herzlich danken für die Liebe und Geborgenheit, sowie ihrer Unterstützung auf meinem Lebensweg; dieser Dank gilt auch meinen Geschwistern, Anita und Uwe, sowie deren Familien.

Finally, I would like to express my love and gratitude to my little family, who was build up in the time of my PhD. To my beloved wife Paola: I love you and I am happy to have found you in my life. Your presence and support meant a lot to me to pass the difficult times in my PhD. To my little daughter Sophie: Du bist der Sonnenschein in meinem Leben. Deine Existenz bringt die Arbeit wieder in Relation und zeigte mir, dass bei all dem was die Entwicklungsbiologie schon erklären kann, das Leben dennoch ein unglaublich herrliches Wunder ist.

12 List of publications

***dead end* regulates gene expression by prohibiting microRNA function**
Martijn Kedde[#], Markus J. Strasser[#], Joachim A.F, Oude Vrielink, Krasimir Slanchev, Carlos le Sage, Remco Nagel, P. Mathijs Voorhoeve, Josyanne van Duijse, Ulf Andersson, Anders H. Lund, Anastassis Perrakis, Erez Raz, and Reuven Agami
Resubmitted to Cell, September 2007

Control over the morphology and segregation of Germinal Granules during embryonic Zebrafish development
Markus J. Strasser[#], Natalia C. Mackenzie[#], Karin Dumstrei, Dietmar Riedel, La-Iad Nakkrasae, Jürg Stebler, Erez Raz
Under revision for MoD

[#]Equal contributions

13 Curriculum Vitae

PERSONAL INFORMATION

Citizen: Switzerland
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Marital status: married
Place of birth: Winterthur (Switzerland)

EDUCATION

2003 - 2007	Max Planck Institute for Biophysical Chemistry, Dept. Germ Cell Development, Göttingen (Germany) *Doctoral Thesis*
2002 - 2003	Int. Max Planck Research School, University of Göttingen (Germany) *Graduation in the Int. Master-PhD Program*
1997 - 2000	Zurich University of Applied Sciences Winterthur, (Switzerland) *Graduation as Chemist FH*
1994 - 1997	Ciba AG (Novartis) Basel, (Switzerland) *Graduation of the "Technical Professional Maturity" and Apprenticeship as laboratory Assistant*