Molecular mechanisms governing germ line development in zebrafish and the role of this lineage in sexual differentiation

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Tag der mündlichen Prüfung:

To Ina and Johann, the two
most important people for me

Abreviations

µ	micro
aa	amino acid
ab	antibody
Amp	Ampicillin
APS	ammoniumperoxodisulfate
as	anti sense
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CDS	coding sequence
Da	Dalton
DNA	deoxyribonucleic acid
Dnd	*dead end*
Dpf	days post fertilization
ds	double stranded
DT	*diphtheria* toxin
E. coli	Escherichia coli
ECL	enhanced chemiluminescence
EDTA	ethylendiamine tetraacetate
EST	expressed sequenced tag
GFP	green fluorescent protein
Glo	*globin*
Hpf	hours post fertilization
HRP	horse radish peroxidase
IgG	immunoglobulin G
IPTG	isopropyl-b-D-thiogalactopyranosid
kDa	kilo Dalton
m	mili
min	minute
miRNA	micro RNA

MO	morpholino oligonucleotide
mRNA	messenger RNA
Nos1	*nanos-1*
nt	nucleotide
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGC	primordial germ cell
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
s	seconds
SDF	stromal cell-derived factor
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
ss	single stranded
TBS	Tris(HCl) buffered saline
WT	wild type

Contents

I. Introduction

I.1. Zebrafish as a model organism

During the last 20 years a small tropical fish, *Danio rerio* (zebrafish), was gaining increasing popularity as a vertebrate model for various biological studies. Its small size, relatively short life cycle and ease of breeding was found useful by the researchers. The rapid *ex-utero* development and optical clarity of the embryos allow straightforward observation and manipulation during early developmental stages. These features attracted many scientists to conduct research in zebrafish resulting in rapid expansion of genetic and genomic information such as mapping panels, EST databases, BAC/PAC/YAC libraries and genome DNA sequence. Furthermore, large-scale mutagenesis screens have yielded thousands of mutant lines exhibiting a range of phenotypes reflecting specific defects in different developmental processes. Last, the value of the zebrafish model is enhanced by the fact that it shows high degree of conservation with mammals making it an attractive medically relevant system. This feature is a result of conservation of signaling pathways and organ function within vertebrates (e.g. chemokine signaling and adaptive immunity respectively).

The combination of these factors established zebrafish as a powerful model system for modern biological research. Taking advantage of these features, our group focuses on the development of the primordial germ cells (PGCs). These cells, which are specified in a position that is distinct from the position of the gonad where they differentiate into gametes, serve as an excellent *in-vivo* model for studying cell fate specification, differentiation and migration.

I. 2. Specification of the PGCs

Germ cells are the cells that give rise to the gametes, sperm and eggs, and ensure the transmission of genetic information between the generations in sexually reproducing organisms. Typically, PGCs are specified in distinct positions during early embryogenesis and actively migrate to reach the site where the gonad develops. However, germline specification differs among groups of animals with two main mechanisms described thus far.

In mammalians and urodele amphibians, germ cell specification is a result of induction by somatic cells shortly before and during gastrulation (Nieuwkoop, 1969; Tam

and Zhou, 1996). Consistently, no asymmetrically-localized maternally-provided determinants (termed germ plasm in other organisms, see below) that direct cells to the germline were identified. In the mouse embryo, germ cell induction is mediated by secreted factors of the bone morphogenetic protein (BMP) family (Lawson et al., 1999; Ying et al., 2001; Ying and Zhao, 2001).

The second mechanism for germ cell line specification relies upon the inheritance of a specific set of molecules collectively termed germ plasm. This mode of PGC specification is found in invertebrates (e.g. *Drosophila* and *C. elegans*) and evolutionary lower vertebrates (e.g. *Xenopus* and zebrafish). In *Caenorhabditis elegans,* germ plasm components are distributed uniformly throughout the cytoplasm of the one-cell-stage embryo. During cleavage stages, these components become sequentially restricted to a single cell that gives rise exclusively to germ cells (DeRenzo et al., 2003; Strome and Wood, 1982; Strome and Wood, 1983). In *Drosophila,* the germ plasm is localized to the posterior pole of the egg and at cellularization is incorporated into primordial germ cells, called pole cells (reviewed in (Rongo et al., 1997; Williamson and Lehmann, 1996)). Germ plasm is also found in cytological distinct islets at the vegetal pole of the unfertilized *Xenopus* eggs (Kloc et al., 2002; Robb et al., 1996; Savage and Danilchik, 1993).

In zebrafish, germ plasm components are maternally provided in the cytoplasm of the oocyte. During the first two cleavages it becomes enriched in four positions at the distal parts of the cleavage furrows (Fig. 1A). As the cleavages continue, the germ plasm remains associated with the furrows until 32 cells, when it is incorporated in the cytoplasm of four blastomers. In the following stages it is asymmetrically segregated during the cell divisions, such that only one of the daughter cells receives the determinants. As a result, compact germ plasm material is detected in four cells, found in four equidistant locations in the embryo. At sphere stage, the compact germ plasm undergoes a transition and becomes more diffuse and spreads allover the cytoplasm of the blastomers (Fig. 1A). This event marks the onset of symmetric germ plasm inheritance following cell divisions, leading to an increase of the PGCs number to about 25 – 40 by the time they complete their migration at the end of the first day of development. PGC migration towards the region where the gonad develops is a complex process guided by the chemokine *SDF1a* whose receptor *CXCR4b* is expressed in the migrating cells (Fig.1B, C) (Doitsidou et al., 2002).

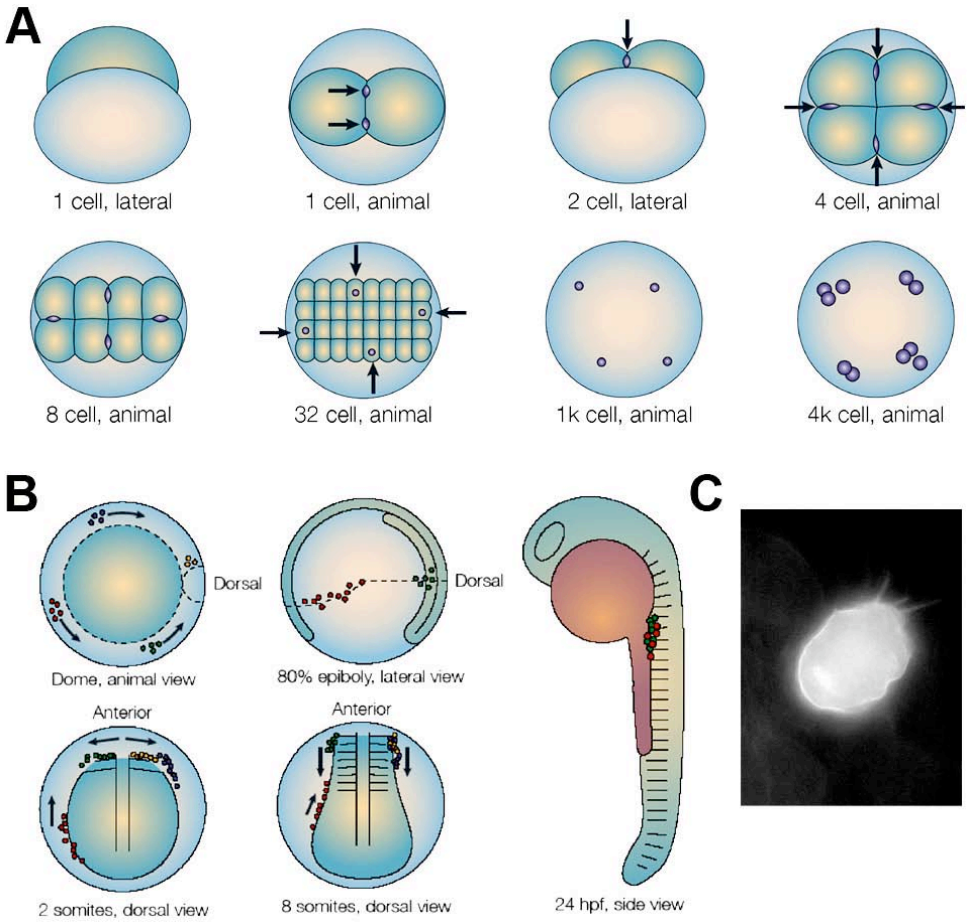

Fig.1. Germ cells specification and migration in zebrafish. A) Localization of the germ plasm in the developing embryos: at the cleavage planes in 2, 4 and 8 cells; inside four different blastomers from 32 – 1000 cells, which are giving rise to four clusters of primordial germ cells at 4000 cells. B) Migration of the four clusters of germ cells through the developing tissues towards the border of the yolk and the yolk extension at 24hpf. C) High magnification picture of fluorescently labeled actively migrating germ cell (bud stage), forming typical extensions and protrusions. Reproduced from (Raz, 2003).

I. 3. Germ plasm composition, dynamics and function

Molecular studies of the composition of the germ plasm in *C. Elegans*, *Drosophila, Xenopus laevis,* chick and zebrafish revealed striking similarities despite the evolutionary diversity of these species. In these organisms the germ plasm is made of conserved proteins, coding and non-coding RNA, and germ plasm specific granules. These components, often associated with clusters of mitochondria, appear electron-dense in EM studies and therefore have been termed *nuage*.

The function of the non-coding RNAs, found in the *nuage* remains largely

unknown. Nevertheless, some authors suggest that this fraction plays a role in the regulation of gene expression at post-transcriptional level (Deshpande et al., 2004; Iida and Kobayashi, 1998; Martinho et al., 2004). Similarly, the mitochondria found in association with the *nuage* are assumed to provide energy, but the biological relevance of production of large amounts of energy in proximity to the germ cell determinants is not clear (Mahowald, 1968; Pitt et al., 2000).

Germ plasm granules appear in two forms in different developmental stages, namely, cytoplasmic and associated with the nucleus. In the early *C. elegans, Drosophila*, and amphibian embryos, the granules are found in the cytoplasm. In *Drosophila,* polar granules become associated with the nuclei once the pole cells are formed (Hay et al., 1988a; Mahowald, 1968). In *C. elegans,* P granules remain associated with germ cell nuclei as the gonad forms during larval and adult development and then detach from the nuclei and become cytoplasmic in oocytes (Strome and Wood, 1982; Strome and Wood, 1983). Similarly, the dense bodies of amphibians are associated with nuclei prior to becoming cytoplasmic (Eddy and Ito, 1971; Mahowald and Hennen, 1971). This alteration in the appearance of the germ plasm granules might be correlated with a change in their function, suggesting multiple roles at different times in development.

Consistent with the idea that the granules play a multi-functional role in germ cell development, several proteins are part of the germ plasm of the early embryo, but are either not present or are present at reduced levels at later developmental stages. Example for such gene products are the *C. elegans* proteins Pie-1, Mex-1, and Pos-1 (Guedes and Priess, 1997; Mello et al., 1996; Tabara et al., 1999) and the *Drosophila* proteins Tudor and the long isoform of Oskar (Bardsley et al., 1993; Markussen et al., 1995). In *Drosophila,* mtlr-RNA is associated with the polar granules only until the pole cells are formed (Kobayashi et al., 1993). Similarly, amphibian mtlrRNA is associated with dense bodies only until the blastula stage (Kobayashi et al., 1998).

In contrast, other proteins such as *C. elegans* Glh-1, Glh-2, and Pgl-1 and Vasa, the *Drosophila* homologue of Glh-1 and Glh-2 are present continuously in perinuclear granules (Gruidl et al., 1996; Hay et al., 1988b; Kawasaki et al., 1998).

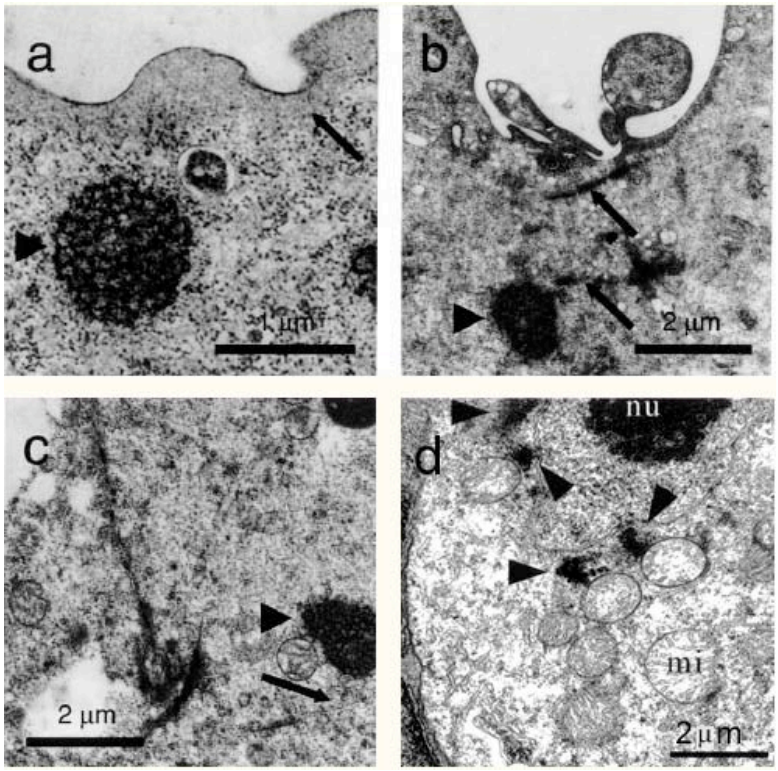

Fig. 2. Electron microscope images of germ cells at different developmental stages. (a), (b) and (c), sections along the animal-vegetal axis. In the 1-cell stage embryos, the germ plasm (arrowhead) is scattered in little patches that are in direct physical contact or close proximity to the indicated cortical actin network (a). After furrow initiation, these patches of germ plasm aggregate underneath the forming furrow and are seen in close proximity to microtubule bundles (b). After a second furrow indentation, the germ plasm aggregates are seen vegetal to the furrow, again in close proximity to bundles of microtubules (c). *Nuage* associated with mitochondria localizes close to the nucleus, which contains a clear nucleolus, at 4 dpf (d). Arrows indicate actin cortex (a) and microtubule (b and c). Reproduced from (Braat et al., 1999a; Knaut et al., 2000)

The dynamics and the function of germ plasm granules in zebrafish are poorly understood. Ultrastuctural studies reveal small patches of electron dense material, presumably *nuage*, in association with the actin cortex of the cytoplasm of the early oocyte. During the first and the second cleavages the granules increase in size and form rodlike structures underneath the incomplete furrows. These aggregated particles are no longer seen in proximity to the actin cortex, but rather are seen in close association with microtubule and mitochondria (Fig. 2) (Braat et al., 2000; Knaut et al., 2000). Moreover, embryos treated with latrunculin B, which sequesters actin monomers and depolymerizes actin filaments, failed to localize the nuage at the furrows, suggesting an actin-dependent step in early germ plasm localization (Knaut et al., 2000). At later stages (4 dpf) *nuage* is

detected in patches within the cytoplasm of the germ cells, always close to the nucleus and in association with clusters of mitochondria (Fig. 2d) (Braat et al., 1999b).

Although informative, following the germ plasm using ultrastructural criteria is difficult to perform. Studying germ cell development in zebrafish was thus made easier following the cloning of the zebrafish *vasa (vas)* homolog (Yoon et al., 1997). In *Drosophila* the Vasa protein, an RNA helicase, is a component of the pole plasm and is required for PGCs formation and oocyte differentiation (Schupbach and Wieschaus, 1986; Styhler et al., 1998; Tomancak et al., 1998). Homologues of *vas* have been identified in several other species with both, germ plasm mediated and inductive mode of germ cell specification (Fujiwara et al., 1994; Komiya et al., 1994; Olsen et al., 1997; Tanaka et al., 2000a; Yoon et al., 1997). In zebrafish, *vas* mRNA has been detected in the germline during all stages of development (Braat et al., 1999b; Knaut et al., 2000; Yoon et al., 1997) making the germline easy to trace. Nevertheless, the identification of more genes was crucial to obtain insights into the molecular mechanisms governing the development of this lineage. One method for identifying such genes is to isolate zebrafish genes based on their homology to germ cell markers identified in other organisms. Alternatively, large scale *in situ* hybridization screens can be used to identify genes expressed in the desired cell population with the advantage of identifying novel genes. Using the latter approach, several genes expressed in the germ cells of zebrafish have been identified. A list of genes expressed in zebrafish germ cells is provided in Table 1.

Table 1. Genes found in the germ cells of zebrafish

Gene	Expression pattern
vasa A DEAD-box RNA helicase	*vasa* RNA expressed in germ plasm, PGCs and germ cells during gametogenesis. Protein is initially uniformly distributed but becomes restricted to the PGCs during germgastrulation. Protein enriched in perinuclear locations. Function of early expression in PGCs unknown (Braat et al., 1999a; Braat et al., 2000; Braat et al., 2001; Wolke et al., 2002; Yoon et al., 1997)
nanos1 An RNA- binding zinc-finger protein	RNA is expressed in the region where the germ plasm protein resides and in the germ cells until 5 dpf. PGCs with lower levels of Nanos1 have migration defects and eventually die (Köprunner et al., 2001)
dazl A DAZ family RNA-binding protein. Contains an RNP-type RRM and DAZ motifs	RNA is expressed in the vegetal pole of the egg, transported to the blastomeres during cleavage stages and stops being expressed before gastrulation. Expressed in the ovary and testis. Function unknown (Maegawa et al., 1999); (Xu et al., 2001)
dead end A new protein that contains a single stranded RNA-binding domain	*dead end* RNA is enriched in the region where the germ plasm resides, and in the germ cells until 5 dpf. PGCs in which the protein level is low have defective migration and eventually die (Weidinger et al., 2003)
cxcr4b and *sdf-1a* Cxcr4b is a seven trans- membrane G-protein- coupled receptor for the chemokine Sdf1a	*cxcr4b* RNA is maternally provided and during gastrulation becomes expressed in specific tissues including the PGCs, which migrate towards domains of *sdf-1a* RNA expression. Sdf1a guides the PGCs towards their intermediate and final targets (Doitsidou et al., 2002)
H1M Linker histone	*H1M* transcripts are expressed in the germ line from the early gastrulation up to 18 h post-fertilisation Function: unknown (Müller et al., 2002; Wibrand and Olsen, 2002)
ziwi The zebrafish homologue of the *Drosophila piwi*	*Ziwi* is expressed in several tissues, including genital ridges after 24hpf. In adult fish it is found exclusively in the gonads. Function: unknown (Tan et al., 2002)

Despite the progress achieved in resolving the germ plasm composition, manifested in the number of the genes that are expressed in the this organelle, the mechanism of action of the germ line determinants remains enigmatic. Noteworthy, many of the proteins from the germ plasm contain various RNA-binding motifs (Raz, 2003; Santos and Lehmann, 2004; Wylie, 1999). One could speculate that these proteins bind to the mRNAs, regulating the gene expression in a germ cell specific manner. Consistently, the association of the germ cell granules with the nucleus, and in particular with the nuclear pore complex as shown in *C Elegans*, suggest that the granules may play a role in regulating transport or translation of newly synthesized mRNAs (Pitt et al., 2000; Schisa et al., 2001).

In zebrafish germ cells *vasa* is expressed throughout zebrafish development.

The Vasa protein is found in all of the cells of the early embryo, becomes enriched in the PGCs after 1000 cells and is exclusively localized to the perinuclear granules of the germ cells after somitogenesis (Braat et al., 2000). Interestingly, Knaut et al (Knaut et al., 2000) suggested that in fact only *vasa* mRNA and not its protein is part of the germ plasm. Analyzing 30 hpf old embryos these authors show that Vasa protein is localized strictly perinuclear in granules that do not colocalize with the nuclear pore as has been reported for *C. elegans* (Pitt et al., 2000). At this stage, *vasa* RNA is partially perinuclear partially cytosolic and does not strictly colocalize with Vasa protein. From these observations, the authors draw the conclusion that Vasa protein is not a component of the zebrafish germ plasm. Despite the well-characterized expression pattern of *vasa* in zebrafish, its function remains unclear. Specifically, knock down experiments using anti-sense morpholino oligonucleotides (MO) did not affect the development of the PGC (Braat et al., 2001). In contrast, *vasa* has been shown to be essential for proper germ cell development in other model organisms. Mutant flies fail to form pole plasm and lack germ cells (Styhler et al., 1998), in *C. eleganse* and *Xenpus, vasa* homologues are required for germ cell proliferation and gametogenesis (Ikenishi and Tanaka, 1997; Kuznicki et al., 2000) and vas-deficient mice exhibit spermatogenesis defects (Tanaka et al., 2000b).

The expression pattern of the mRNA of another germ plasm component, *nanos1 (nos)*, strongly resembles that of *vasa* (Köprunner et al., 2001). *nanos1* gene encodes a zinc finger containing protein that shows sequence similarity to the *Drosophila* Nanos protein. *nanos1* mRNA is provided maternally and during the first two mitotic divisions is enriched in the distal end of the cleavage furrows of the early embryo. During blastula stages, *nos* mRNA is more specifically expressed in the PGCs as mRNA that is expressed in somatic cells is degraded. Unlike *vasa*, which continues to be strongly expressed throughout germline development, *nos1* RNA is undetectable by *in-situ* hybridization after the fifth day of development. Remarkably, embryos injected with *nos*1 morpholino exhibit severe defects in PGC development leading to migration failure and reduction in the germ cell number (Köprunner et al., 2001). However, as maternally provided protein whose level is not affected by the antisense oligonucleotides could potentially mask an earlier role of Nanos1 in zebrafish PGC development such as in PGC specification. This matter could be investigated by using specific for Nanos1 antibodies to determine whether such maternal protein exists.

The most recently identified component of the germ plasm in zebrafish is *dead end (dnd)* RNA that encodes a novel protein containing several putative RNA binding domains (Weidinger et al., 2003). Similarly to *vasa* and *nanos1*, shortly after fertilization, maternal *dnd* RNA is present in numerous granules distributed throughout the cortex of the one-cell stage embryo. Subsequently, *dnd* RNA is enriched at the distal parts of the first two cleavage furrows and later (at 4.3 hpf) is expressed exclusively in the PGCs. *dnd* continues to be expressed in PGCs during their migration and as they reach the position of the presumptive gonad. At 5 days post fertilization, *dnd* RNA is still detectable in the PGCs, albeit at a lower level. Dead end-GFP fusion protein was found in the perinuclear germ granules in mid-somitogenesis stage embryos. Thus, Dead end protein is localized to the same cellular structures as Vasa and Nanos1. Knockdown of zebrafish *dead end* results in failure of PGCs to assume motile behavior followed by loss of specific marker gene expression and cell death (Weidinger et al., 2003). Nevertheless, as for *nanos1*, it is formally possible that maternally provided protein plays a role at earlier steps of PGC development, in particular in the specification stage.

I. 4. Sex determination among the animal model systems

In most organisms the sex is determined during early stages of development. In *Drosophila melanogaster* and *Caenorhabditis elegans* the primary signal for sex-determination is the ratio of *X* chromosomes to the autosomes. In these organisms *XX* animals become hermaphrodites (in worms) or female (in flies) while *XY* and *XO* animals develop as males (Reviewed in (Cline and Meyer, 1996)). Similarly, sex determination in mammals and birds depends on the chromosomal constitution of the organism. In mammals it is controlled by the Y-linked *SRY* gene, which initiates a cascade of genetic and cellular events leading to testicular differentiation (reviewed by (Capel, 2000; Swain and Lovell-Badge, 1999)). In birds the females are ZW, the heterogametic sex, and the males are ZZ, the homogametic sex, but master male or female promoting genes have not yet been identified and the precise mechanism of sex determination remains unclear (reviewed in (Smith and Sinclair, 2004)). In contrast to the dominant role of the genetic composition of the individual, in crocodilians, many turtles and some lizards, environmental conditions play a major role in sex determination (reviewed in (Western and Sinclair, 2001)). In these species

heteromorphic sex chromosomes have not been identified and sex is controlled by egg-incubation temperature.

In fish, both environmental as well as genetic (chromosome-based) mechanisms have been implicated in sex determination. In medaka and some poeciliid fishes for example, sex chromosomes can be distinguished from the autosomes (Matsuda et al., 2002; Volff and Schartl, 2001). Interestingly however, even fish with established sex chromosomes show strong dependence on environmental cues, the most prominent of which is temperature but other factors such as pH, pollutants and social effects have been shown to influence sex determination as well (reviewed in (Baroiller and D'Cotta, 2001; Lee et al., 2001)).

In other fish such as zebrafish and European eel, morphological differences in the chromosomes of the two sexes have not been identified by classical karyotyping. Furthermore, in zebrafish, a chromosomal locus controlling sex determination has not been found, implicating polygenetic or environmental signals in sex determination (Traut and Winking, 2001).

Sexual development culminates in formation of functional gametes that are derived from PGCs. The decision of PGCs to develop into sperm or eggs varies among the species with examples of mechanisms involving both cell autonomous control and inductive cues. In mammals, the decision to differentiate into male gametes depends on signals from somatic cells (e.g., (McLaren, 1995)) whereas in *Drosophila*, this process is mediated by cell-autonomous as well as inductive signals (reviewed in (Schutt and Nothiger, 2000))

In fish, the mechanisms governing the sexual fate of the PGCs are not clear. In some species, such as the channel catfish and medaka, an undifferentiated gonad develops, which then gives rise to ovaries in females and to testes in males (Patino et al., 1996; Tanaka et al., 2001). In other fish species, including zebrafish, an ovary-like structure is initially formed in all embryos. This structure subsequently develops into ovaries in females or following the death of the oocytes, into testis in males (Nakamura, 1984; Uchida et al., 2002).

Despite the extensive signaling between the germ cells and the somatic cells in the gonads of mouse or *Drosophila*, the presence of PGCs does not seem to be important for somatic sexual differentiation in these species since both sexual types are generated by animals bearing mutations causing germ cell depletion (Jongens et

al., 1992); (Beck et al., 1998). This data implies a unidirectional sex-determining signaling from the soma to the germ cells.

I. 5. RNA interference in zebrafish

The final topic studied in this thesis regarding primordial germ cell development relates to the property of these cells allowing them to "shield" themselves from undesired differentiation and retain their germline fate. The ability of the cells to maintain their fate appears to rely on their capacity to silence gene expression at the transcription and post-transcriptinal levels. In *C. elegans* and *Drosophila* it has been shown that the early germ cells are transcriptionally silenced (Reviewed in (Blackwell, 2004)). A key factor in this process in *C. elegans*, is PIE-1, a putative RNA-binding protein that can interfere with RNA elongation or associated RNA processing steps (Batchelder et al., 1999; Mello et al., 1996; Zhang et al., 2003). Upon the disappearance of PIE1 from the germline the silencing is maintained by low levels of transcriptionally active chromatin. This apparently inactive chromatin environment requires *C. elegans* Nanos1 action. (Schaner et al., 2003). In *Drosophila*, pole cells transcriptional quiescence also depends on the pole plasm component Nanos1 (Asaoka et al., 1998; Deshpande et al., 1999; Schaner et al., 2003), but the silencing mechanism there is complemented by the nuclear protein Germ cell-less and the non-coding RNA encoded by the polar granule component (pgc) (Deshpande et al., 2004; Martinho et al., 2004). In zebrafish, the activation of the zygotic genome at midblastula transition (MBT) occurs at 3.0 hpf (Kane and Kimmel, 1993) while zygotic *vasa* transcription starts approximately 1 h later, at 4.0 hpf. However, in contrast with *C. Elegans* and *Drosophila*, transcriptional quiescent of the germ cells was not detected, judged by the presence of the phosphorilated form of COOH terminal domain of the RNA polymerase II, important for RNA elongation (Knaut et al., 2000). Nevertheless, regulation by silencing on different level of the gene expression e.g. nuclear export, mRNA degradation or translational silencing could not be excluded.

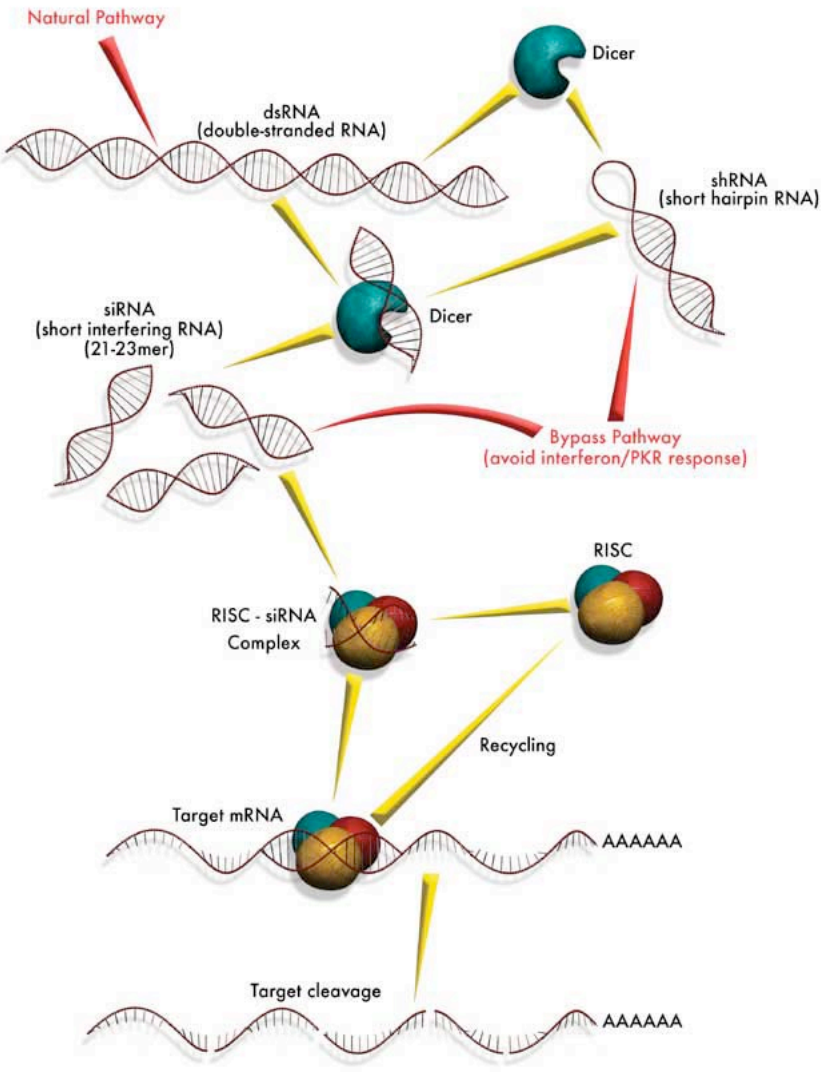

Fig. 3 The pathway of RNAi. The endonuclease dicer initiates RNAi by processing long dsRNAs or short hairpin RNAs to 21 nt long dsRNAs with 2 nt 3´-overhangs. Insertion of chemically synthetized siRNAs allows bypassing unspecific effects of the interferon response and PKR activation. The siRNA is then incorporated into RISC and unwound. The antisense strand guides the active RISC to the target position on the target mRNA, which in turn is sequence specifically cleaved and degraded.
(Modified from www.upstate.com/img/pathways/rnai.jpg)

An alternative, conserved mechanism of posttranscriptional gene silencing referred to as RNA interference (RNAi) is mediated by long or short double stranded RNA molecules (Fig. 3) (Elbashir et al., 2001; Fire et al., 1998; Hamilton and Baulcombe, 1999; Sijen et al., 2001; Zamore et al., 2000). This process was first demonstrated by injection of double-stranded (ds) RNA molecules into *Caenhorabditis elegans* initiating subsequent sequence-specific silencing of a homologous target gene (Fire et al., 1998). Ever since, a number of approaches to

establish RNAi mediated gene knockdown in model organisms have been carried. Interestingly, in *Drosophila*, Maelstrom, a protein localized to *nuage* in a Vasa-dependent manner, depends on two genes involved in RNAi phenomena, *aubergine* and *spindle-E* (*spn-E*), for its *nuage* localization. Furthermore, *maelstrom* mutant ovaries show mislocalization of two proteins involved in the microRNA and/or RNAi pathways, Dicer and Argonaute2, suggesting a potential connection between *nuage* and the microRNA-pathway (Findley et al., 2003).

However, the reports for induction of RNA interference in zebrafish have been controversial. Lim et al. (Lim et al., 2003) reported the presence of *dicer* mRNA and the active enzyme in the fertilized egg and the early embryo of the zebrafish, whereas Wienholds et al. (Wienholds et al., 2002) have shown that dicer is essential for embryonic development. Dicer knockout fish develop normally but die around day 14 post fertilization. If the translation of the maternal dicer mRNA pool is inhibited, fish survive only until day 8. These results suggest the presence of functional micro RNA/RNAi machinery, which is needed only at later developmental stages. Alternatively, a maternal pool of Dicer protein is fulfilling the requirements of the embryos until day 8 of their development. Detection of *mir-26a* and *let-7* (Wienholds et al., 2002), two highly conserved among the species micro RNAs, already at day 1 post fertilization argues in favor of the need of RNAi also during the early development of zebrafish. Interestingly, in other organisms, such as the fruit fly *Drosophila melanogaster* or the mouse *Mus musculus,* micro RNAs are expressed at very early stages of embryonic development (Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2002) and regulate gene expression at this time.

A number of groups have attempted to establish RNAi mediated gene silencing in zebrafish. Conflicting data is reported in the literature. Several groups report specific effects after delivery of dsRNA molecules into fish embryos; a specific reduction of exogenous GFP fusion proteins was observed after the introduction of the corresponding siRNAs in embryos of the rainbow trout (Boonanuntanasarn et al., 2003), whereas Hsieh and Liao reported specific silencing of the zebrafish M2 muscarinic acetylcholine receptor in the developing embryo after injection of dsRNA targeting the M2 mRNA (Hsieh and Liao, 2002). In addition, silencing of endogenous genes via injection of specific siRNAs into the yolk of two-cell zebrafish embryos was reported (Dodd et al., 2004). In contrast, other laboratories found only non-specific effects after injection of long dsRNAs and siRNAs into the embryo. A variety

of defects were observed after injection of either type of dsRNA (Oates et al., 2000; Zhao et al., 2001) independent of the targeted genes. A possible explanation for the nonspecific embryonic malformations might be the interferon response in the fish, which is activated in the presence of dsRNA (Collet and Secombes, 2002; Jensen and Robertsen, 2002). Since several groups attempted to target different genes, exogenous and endogenous, and reported conflicting results the question for the function of RNA interference in zebrafish, and more specifically in zebrafish germ cells, remains open.

The aim of this work is to investigate some aspects of the germ cell development in zebrafish. The work is divided into three chapters. The first chapter is describing an attempt to gain better insight of the germ plasm dynamics and function in zebrafish. We use three components of the germ plasm, *vasa, nanos1* and *dead end*, and follow their sub-cellular localization during the PGCs development. We show that the protein products of these three genes co-localize with the nuclear pore complex after the specification of the germ cells. Further, using mutated variants of Dead end, we demonstrate that an RNA-binding domain plays a crucial role for the function of this protein, and in particular for its restriction to the germ plasm granules.

Chapter number two is investigating the interactions between the germ cells and the somatic cells, manifested in development of male-only adult fish derived from *dnd* morpholino injected embryos. Via an independent method for germ cell ablation, we were able to demonstrate that the germ cell line is indispensable for the survival of the gonad and thus for the establishing of the female sex in zebrafish.

Finally, in the last chapter we describe an effort to determine if RNA interference mechanisms could function in regulating gene expression in the germ line. Here, we could not induce specific silencing of genes expressed in the germ cells, nor in the somatic cells of zebrafish embryos. However, successful experiments in zebrafish cell lines, as well as silencing of a GFP construct fused to the UTR of *lin41* UTR, confirmed the presence of the machinery required for the RNA interference response.

II. Materials and methods

II. 1. Materials

II. 1. 1. Equipment

Cameras	RT slider Spot, Diagnostic Instruments (USA) Leica DC 300, Leica RT SE Spot, Diagnostic Instruments (USA)
Injector	PV830 Pneumatic PicoPump, World Precisoin Instruments (USA)
Needle puller	PN-30 Microelectrode Puller, Science Products (Hofheim)
PCR machines	Cyclone 96, Peqlab, Erlangen Mastercycler Personal, Eppendorf (Hamburg)
Microscopes	Leica MZ FLIII, Leica Zeiss Axioplan 2, Zeiss Leica confocal mycroscope DMRXE, Leica
Centrifuges	Eppendorf 5415D, Eppendorf
Spectrophotometer	Eppendorf 6131, Eppendorf

II. 1. 2. Software

Image processing	Adobe Photoshop 7.0, Adobe
Microscopy	Metamorph, Universal Imaging Corp. (USA) Leica confocal software, Leica
Sequence analysis	Sequencher, Gene Codes Corp. (USA) Multiple sequence alignment
Virtual cloning	Vector NTI, Invitrogen (USA)
Text processing	Microsoft Word, Microsoft Corp. (USA)
Graphs and tables	Excel, Microsoft Corp. (USA)

II. 1. 3. Web pages

BLAST (basic Local Alignment search tool)	www.ncbi.nln.nih.gov./BLAST
PROSITE	www.expasy.ch/tools/scan/prosite
PROTINFO	www.chait-sgi.rockefeller.edu/cgi-bin/ protinfo
BLOCKS	www.blocks.fhcrc.org/
PubMed	www.ncbi.nln.nih.gov

II. 1. 4. Kits

Advantage HF II PCR Kit	Clontech (USA)
mMessage mMachine Kit	Ambion (USA)
QIAfilter Plasmid Midi Kit	Qiagen (Hilden)
QIAprep Spin Miniprep Kit	Qiagen (Hilden)
UltraCleanTM 15 DNA Purification Kit	MO BIO (USA)
Topo TA PCR Cloning Kit	Invitrogen (USA)

II. 1. 5. Bakteria and Media

E. Coli Top 10F'	Invitrogen (USA)
E.coli Rosetta	Invitrogen (USA)

II. 1. 6. DNA constructs

PCR 2.1-TOPO PCR II TOPO	TA cloning vector for cloning of PCR products (*Invitrogen*).
GFP-*nos-13*'UTR (#355)	The construct includes the mmGFP5 ORF fused to the 3'UTR of zebrafish *nos-1*. (Köprunner et al., 2001).
dnd-nos-13'UTR (#495)	ORF of *dead end* cloned in-between a bacterial transcription promoter Sp6 and a *nos1* 3'UTR (Weidinger et al., 2003).
Diphtheria Toxin (#675)	Diphtheria toxin (DT) A-chain; amplified by PCR from the plasmid pCGmIL3 (Liger et al., 1997) using specific primers and cloned into an RNA expression vector upstream of the *nanos-1* 3'UTR (Köprunner et al., 2001).
kid-nos-1 3'UTR (#754)	*kid* coding sequences was amplified from plasmid pcIneoKid (de la Cueva-Méndez et al., 2003) and cloned 5' from *nos-13*'UTR.
kis-globin UTR (#755)	*kis* coding sequences was amplified from plasmid p424Met25K (de la Cueva-Méndez et al., 2003) and cloned in-between *globin 5'* and 3'UTRs.
dnd-His tag (#697)	C-terminus of Dead end w/o the RNA-binding domain cloned into PeT 19b overexpression vector, N-terminal from the His tag
dnd-HAHA tag- *nos-1* 3'UTR (#776)	*dead end* ORF cloned N-terminal from HAHA-tag, followed by *nanos-1* 3'UTR
dnd-mutated-HAHA tag-*nos-1* 3'UTR	Dead end ORF carrying a single point mutation, cloned N-terminal from HAHA-tag, followed by *nanos1* 3'UTR
GFP-*lin41*UTR (#A008)	A 379 bp fragment containing two putative *let-7* target sites of the zebrafish *lin-41* 3'-UTR was cloned 3' from eGFP coding sequence (Kloosterman et al., 2004)
GFP-*lin41*UTR.*sdf* (#A008)	A 379 bp fragment containing 1 putative siSDF binding site and 1 *let-7* target site within the zebrafish *lin-41* 3'-UTR, cloned 3' from eGFP coding sequence
dnd-globin3'UTR (#487)	

II. 1. 7. Morpholino modified antisense oligonucleotide

Morpholino oligos were first devised by James Summerton in 1985 and were developed at Antivirals Inc. (now AVI BioPharma). They represent relatively cheap ribonucleoside analogs for antisense therapeutics, with high degradation stability and good RNA-binding affinity. A morpholino 25-oligomer complementary to the ATG region of *dnd* mRNA was designed and ordered (Gene Tools, LLC (Weidinger et al., 2003)). It was dissolved in Danieu's buffer and stored as 5µg/µl stock solution. Fresh working dilutions were generated with 10mM HEPES buffer immediately before the experiments.

II. 1. 8. DNA oligonucleotides

List of the DNA oligonucleotides used as PCR primers:

Primer	Used for	Sequence
470	*dnd* amplification fwd	TTTTTTTTTTTTTTTAAGTCTAGAGAAAATGT
471	*dnd* amplification rev	CAATTAATACATAACCTTATGTATC
737	*dnd* amplification for PET vector fwd	CCGCATCCATGGCTAAGAAAGTGCTCG
738	*dnd* amplification for PET vector rev	CATGCCATGGAGAAAGGCCGTAAATTTG
807	DToxin Achan fwd	GGCATGGGCGCTGATGATGTTGTTG
808	DToxin Achan rev	TTATCGCCTGACACGATTTCCTGCA
818	T7-PET sequencing	TCCCGCGAAATTAATACG
A023	*kid* amplification fwd	CGGGATCCACCATGGAAAGAGGGGAAATCT
A024	*kid* amplification rev	CCCTCGAGTCAAGTCAGAATAGTGGACAGG
A025	*kis* amplification fwd	CCCTCGAGCCATGCATACCACCCGACTGAA
A026	*kis* amplification rev	TAAAGCGGCCGCTCAGATTTCCTCCTGACC
A144	eYFP-SDF amplification fwd	CCGGACTCAGATCTTAATTCCTGCAGCCCG
A145	eYFP-SDF amplification rev	CGGGCTGCAGGAATTAAGATCTGAGTCCGG
A455	*sdf-lin41* mutation fwd	ATTGGAAATCGGTGCATGAAGAAGATTATGAATTC
A456	*sdf-lin41* mutation rev	TTCATGCACCGATTTCCAATCTAGCTTATGTATGA
K474-K547	*dnd* mutagenesis	Single nucleotide difference from the original *dnd* sequence

II. 1. 9. siRNA oligonucleotides

RNA oligonucleotides were chemically synthesised in the Group for combinatorial biochemistry (Dr. T. Tuschl, AG 105, Max-Planck Institute for biophysical Chemistry, Göttingen) or commercially supplied by Dharmacon (Lafayette, Colorado).

To design siRNA duplexes against a specific target mRNA it is necessary to know the correct sequence of at least 20 nucleotides of the targeted mRNA. Since recent studies show that the silencing (RNA interference) occurs in the cytoplasm, any intron sequences should be neglected. Therefore the target regions were selected from the open reading frame of the corresponding cDNA, preferably within the first few hundred nts downstream of the start codon. Search criterion was the sequence 5´-AA(N19)UU where N is any nucleotide. Candidate sequences had a G/C-content of 40 to 60%. siRNA

oligonucleotides were synthesised as 5´-(N19)TT for sense and 5´-(N´19)TT (N and N´ indicate any ribonucleotide, T is 2´-deoxythymidine) for antisense orientation. To ensure that only one gene will be targeted the siRNA sequences were subjected to a Blast-search against zebrafish EST libraries and known mRNA sequences.

These criteria led to a variety of siRNA sequences (listed below) targeting the ORF of the selected genes. As a control siRNA a sequence against firefly (*Photinus pyralis*) luciferase (accession number X65324) was used (GL2 siRNA). Single stranded siRNA oligonucleotides were duplexed as described (Elbashir et al., 2001) and used for injections into ZF embryos or transient transfection of cell lines.

List of used RNA oligonucleotides

CXCR4b

Name	Target region	Duplex
CXCR4b-1	333-351 5′ CATGATCTACACTCTGAAT 3′	5′ CAUGAUCUACACUCUGAAUTT 3′ 3′ TTGUACUAGAUGUGAGACUUA 5′
CXCR4b-2	455-473 5′ GGGTGATCTACATTGGAGT 3′	5′ GGGUGAUCUACAUUGGAGUTT 3′ 3′ TTCCCACUAGAUGUAACCUCA 5′
CXCR4b-3	721-739 5′ CTGAAGACCACCGTCATCC 3′	5′ CTGAAGACCACCGTCATCCTT 3′ 3′ TTGACTTCTGGTGGCAGTAGG
CXCR4b-4	721-739 5′ CTGAAGACCACCGTCATCC 3′	5′ pCTGAAGACCACCGTCATCCTT 3′ 3′ TTGACTTCTGGTGGCAGTAGGp
CXCR4b-5	721-739 5′ CTGAAGACCACCGTCATCC 3′	5′ CTGAAGACCACCGTCATCCTT 3′ 3′ TpsTpsGpsACTTCTGGTGGCAGTAGG

CXCR4b

Name	Target region	Hairpin
CXCR4b-6	721-739 5′ CTGAAGACCACCGTCATCC 3′	5′ CTGAAGACCACCGTCATCCTATTTTCG 3′ TTGACTTCTGGTGGCAGTAGGATAA-
CXCR4b-7	721-739 5′ CTGAAGACCACCGTCATCC 3′	5′ ATAACTGAAGACCACCGTCATCCTT 3′ -GCUUUAUUGACTTCTGGTGGCAGTAGG

Dead end

Name	Target region	Duplex
DED-1	655-677 5′ GAAAGTGCTCGTGGAAGCT 3′	5′ GAAAGUGCUCGUGGAAGCUTT 3′ 3′ TTCUUUCACGAGCACCUUCGA 5′
DED-2	1263-1285 5′ CCTTGTGCCGAGTCTCAAA 3′	5′ CCUUGUGCCGAGUCUCAAATT 3′ 3′ TTGGAACACGGCUCAGAGUUU 5′

Floating head

Name	Target region	Duplex
FLH-1	314-332 5′ CAACAUGUCGUGGAGCUGU 3′	5′ pCAACAUGUCGUGGAGCUGUTT 3′ 3′ TTGUUGUACAGCACCUCGACAp 5′

Name	Target region	Hairpin
FLH-2	2-20 5′ UGCAGAUUCCCGGAAGAGC 3′	5′ UGCAGAUUCCCGGAAGAGCUUACGAUUCG 3′ TTACGUCUAAGGGCCUUCUCGAAUGCU-
FLH-3	314-332 5′ CAACAUGUCGUGGAGCUGU 3′	5′ CAACAUGUCGUGGAGCUGUGUACGCUUCG 3′ TTGUUGUACAGCACCUCGACACAUGCG-
FLH-4	837-855 5′ CUUACACACGCCAUUGCUC 3′	5′ CUUACACACGCCAUUGCUCUUAUAAUUCG 3′ TTGAAUGUGUGCGGUAACGAGAAUAUU-

mGFP

Name	Target region	Duplex
mGFP-1	222-240 5′ TCCAGATCATATGAAGCGG 3′	5′ pUCCAGAUCAUAUGAAGCGGTT 3′ 3′ TTAGGUCUAGUAUACUUCGCCp 5′
mGFP-2	136-154 5′ TTTATTTGCACTACTGGAA 3′	5′ UUUAUUUGCACUACUGGAATT 3′ 3′ TTAAAUAAACGUGAUGACCUU 5′
mGFP-3	178-196 5′ CTTGTCACTACTCTCACTT 3′	5′ CUUGUCACUACUCUCACUUTT 3′ 3′ TTGAACAGUGAUGAGAGUGAA 5′
mGFP-4	315-333 5′ CTACAAGACACGTGCTGAA 3′	5′ CUACAAGACACGUGCUGAATT 3′ 3′ TTGAUGUUCUGUGCACGACUU 5′
mGFP-5	404-422 5′ ACATCCTCGGCCACAAGTT 3′	5′ ACAUCCUCGGCCACAAGUUTT 3′ 3′ TTUGUAGGAGCCGGUGUUCAA 5′

Name	Target region	Hairpin
mGFP-6	222-240 5′ TCCAGATCATATGAAGCGG 3′	5′ UCCAGAUCAUAUGAAGCGGCACGACUUCG 3′ UUAGGUCUAGUAUACUUCGCCGUGCUG-

GL2

Name	Zielregion /Sequenz	Duplex
GL2-1	155-173 5′ CGUACGCGGAAUACUUCGA 3′	5′ pCGUACGCGGAAUACUUCGATT 3′ 3′ TTGCAUGCGCCUUAUGAAGCUp 5′

Name	Target region	Hairpin
GL2-2	155-173 5′ CGUACGCGGAAUACUUCGA 3′	5′ CGUACGCGGAAUACUUCGAAAUGUCUUCG 3′ TTGCAUGCGCCUUAUGAAGCUUUACAG-

SDF1a

Name	Target region	Duplex
SDF1a-1	47-65 5′ TTCATGCACCGATTTCCAA 3′	5′ UUCAUGCACCGAUUUCCAATT 3′ 3′ TTAAGUACGUGGCUAAAGGUU 5′
SDF1a-2	77-95 5′ TCAGCCTGGTAGAGAGATG 3′	5′ UCAGCCUGGUAGAGAGAUGTT 3′ 3′ TTAGUCGGACCAUCUCUCUAC 5′
SDF1a-3	133-151 5′ AGCATTCGCGAGCTCAAGT 3′	5′ AGCAUUCGCGAGCUCAAGUTT 3′ 3′ TTUCGUAAGCGCUCGAGUUCA 5′
SDF1a-4	200-218 5′ ACAACAAGGAGGTGTGCAT 3′	5′ ACAACAAGGAGGUGUGCAUTT 3′ 3′ TTUGUUGUUCCUCCACACGUA 5′
SDF1a-5	222-240 5′ TCCAGATCATATGAAGCGG 3′	5′ pUCCAGAUCAUAUGAAGCGGTT 3′ 3′ TTAGGUCUAGUAUACUUCGCCp 5′

SDF1a

Name	Target region	Hairpin
SDF1a-6	222-240 5′ TCCAGATCATATGAAGCGG 3′	5′ UCCAGAUCAUAUGAAGCGGCACGACUUCG 3′ TTAGGUCUAGUAUACUUCGCCGUGCUG-
SDF1a-7	77-95 5′ TCAGCCTGGTAGAGAGATG 3′	5′ UCAGCCUGGUAGAGAGAUGCUGGUGUUCG 3′ TTAGUCGGACCAUCUCUCUACGACCAC-
SDF1a-8	243-261 5′ GCAGTACCTGAAGAACGCC 3′	5′ GCAGUACCUGAAGAACGCCAUCAACUUCG 3′ TTCGUCAUGGACUUCUUGCGGUAGUUG-

Spade tail

Name	Target region	Hairpin
SPT-1	2-20 5′ TCCAGATCATATGAAGCGG 3′	5′ UGCAGGCUAUCAGAGACCUCAAGCAUUCG 3′ TTACGUCCGAUAGUCUCUGGAGUUCGU-
SPT-2	736-754 5′ TCCAGATCATATGAAGCGG 3′	5′ CAAGAUGUCCAGCCGUCAUCGUGUGUUCG 3′ TTGUUCUACAGGUCGGCAGUAGCACAC-
SPT-3	1552-1570 5′ TCCAGATCATATGAAGCGG 3′	5′ CUGUGAGAGGACGCACUAACUACACUUCG 3′ TTGACACUCUCCUGCGUGAUUGAUGUG-

let-7

Target region	Duplex
let-7 **mi**RNa 5′ CTGCATTACACCTACCTCA 3′	5′ CTATACAACCTACTACCTCATT 3′ 3′ TTGATATGTTGGATGATGGAGT 5′
let-7 **si**RNA 5′ CTGCATTACACCTACCTCA 3′	5′ CTGCATTACACCTACCTCATT 3′ 3′ TTGACGTAATGTGGATGGAGT 5′

II. 1. 10. Buffers

Buffer	Composition
Blocking buffer	-5% BSA in TTBS
Coupling buffer	-0.1M NaHCO3 -0.5M NaCl; pH 8.3
Danieu's solution, 10x, for 1l	-1.74M NaCl 101.7 g -21mM KCl 1.57 g -12mM MgSO4[7H2O] 2.96 g -18mM Ca(NO3)2[4H2O)] 4.25 g -150mM HEPES 35.75 g pH 7.6
Elution buffer I (pH 4.8)	-0.1M Acetic acid -0.5M NaCl pH to 4.8 with AcAc and NaOH
Elution buffer II (pH 2.6)	-0.2M Acetic acid -0.5M NaCl pH to 2.6 with AcAc and NaOH
Eosin solution	-1g Eosin powder in 100 ml MP water. -Mix 1% Eosin solution 1:1 with MP water. -Filter -add 1-2 drops conc acetic acid per 100ml (the solution becomes clear and lighter)
Glycin HCl, 0,1M	-3,75 g Glycin -add H_2O to 400 ml -adjust pH to 2,2 -fill to 500 ml with H_2O. + 5 ml Tween, 10%
Hematoxylin solution	Use commercially provided solution (Sigma)
Hybridisation buffer for 1l	-250 ml SSC, 20x =5x -500 mg Torula yeast RNA (Sigma) =500µg/ml -50 mg Heparin (Sigma) = 50µg/ml -10 ml Tween 20, (Sigma) 10% = 0.1% -1,89 g Citric Acid (Monohydrate) = 9mM - H_2O to 500ml -last: 500 ml Formamide, deionized. = 50% The final pH of hyb buffer should be 6,0 – 6,5
LB media for 1l	-10g trypton, -5g yeast extract, -10g NaCl. -Add MP water to 1l. Autoclave, cool down to 55°C and add 100mg/l desired antibiotics. For plates, 1.5% agarose is added to the media

Buffer	**Composition**
Na-carbonate dyalise buffer	-0.1M NaHCO3 -0.5M NaCl, pH 8.3 -250 mM EDTA pH 8.0
NBT	Stock: 75 mg/ml in 0.7 ml of DMF+ 0.3 ml H_2O. Store at –20°C
NTMT for 20ml	-100 mM Tris HCl pH 9.5, 2ml Tris HCl pH 9.5, 1M -50 mM $MgCl_2$, 1ml $MgCl_2$, 1M -100 mM NaCl, 2ml NaCl, 1M -0.1% Tween 20 200 µl Tween 20, 10% -14.8 ml H_20
PBS 10x, for1l	-80 g NaCl -2 g KCl -18 g Na_2HPO_4 (2 H_2O) -2,4 g KH_2PO_4 adjust pH to pH 7,2
PBT, for1l	-100 ml 10x PBS -900 ml H_2O -adjust the pH to 7,4 (by adding a few drops of 15% HCl) + 10 ml Tween 20, 10%
PBTB	PBS + 0,2% Triton X-100 + 1% BSA
STOP solution	-0.05M phosphate buffer pH 5.8 -1mM EDTA -0.1% Tween
Transfer buffer	-3.03 g. TRIS -14,42 g. Glycin -200ml MeOH
TBS	-100mM TRIS-Cl, pH 7.5 -0.9% Na Cl
TTBS	-0.1% Tween-20 in TBS
X-Phosphate (BCIP)	Stock: 50mg/ml in 100%DMF. Store at –20°C

II. 2. Methods

II. 2. 1. Isolation of plasmid DNA

Bacterial Top 10F' electro-competent cells were transformed according to the protocol for One Shot Electroporation of competent cells (Invitrogen). Transformed bacterial cells were plated overnight on Amp selective plates. Single positive colonies were propagated overnight (10h) in 2ml Amp selective LB media. DNA was isolated according to the Qiagen spin miniprep protocol and dissolved in 50μl milipore water.

II. 2. 2. Mutagenesis

The method used to generate single nucleotide mutations was Site-specific Mutagenesis by Overlap Extension (Sambrook and Russell, 2001). Four primers and three PCR reactions were used to create a site-specific mutation by overlap extension. One pair of primers was used to amplify DNA that contained the mutation site together with upstream sequences. The second pair of primers was used in a separate PCR to amplify DNA that contained the mutation site together with downstream sequences. The mutation of interest was located in the region of overlap and therefore in both amplified fragments. The overlapping fragments were mixed, denatured, and annealed to generate heteroduplexes that can be extended and, in a third PCR, amplified into a full-length DNA using two primers that bind to the extremes of the two initial fragments.

II. 2. 3. Cloning procedures

TOPO cloning of PCR fragments was carried according to the standard protocol of TOPO kit (Invitrogen). Two variants TOPO PCRII and TOPO 2.1 were used.

Blunt ends generation: Blunt ends of DNA fragments were generated by incubating the DNA in a mix of 1xPCR buffer, 1xdNTPs and 1x T4 polimerase for 15min at 37°C.

Dephosphorilation of the 5' end of the vector DNA to prevent re-ligation when needed, was performed by incubation with shrimp alkaline dephosphorilase (Roche Diagnosticts) for 15min at 37°C.

Cloning of DNA fragments obtained upon digestion: The fragment and the vector were mixed in molar ratio 3:1, 2µl 5x ligation buffer, 1µl T4 ligaze (Biozym) and MP water to 10µl final volume were added. The reaction was incubated for 1h at RT or overnight at 16°C.

In all cloning procedures electro-competent Top 10F' bacterial cells (Invitrogen) were used for transformations and DNA multiplication.

II. 2. 4. Production of capped mRNA

The mRNA for injections was produced according to the standard protocol of mMESSAGE mMACHINE™ Kit by Ambion. The kit utilizes Ambion's patented high yield transcription technology to allow the routine synthesis of 7.5-17 μg of 7-methyl guanosine capped RNA from 0.5 μg of template DNA with Sp6, T7 or T3 bacterial promoters, in a 10 μl volume, during a 2 hour reaction. The amount and the quality of the obtained RNAs were estimated by gel electrophoresis and measurements of the UV absorption at λ 260/280nm.

II. 2. 5. Production of antisense DIG-labeled RNA probe

Antisense RNA probes labeled with digoxygenin (DIG) were used for the *in situ* hybridizations. A plasmid containing the relevant cDNA template was linerized with a restriction enzyme at the 5'end of the insert. An appropriate bacteriophage RNA polymerase according to the promoter (T3, T7 or SP6; provided by Roche Diagnosticts) was used for transcription of the antisense strand of the cDNA. For subsequent antibody detection of this probe, DIG- labeled UTP was included in the NTP mix of the transcription reaction.

The reaction mix was incubated at 37°C for 2hrs. DNA template was removed by adding 1µl DNAse-I and incubating the reaction for additional 30min. RNA was precipitate by adding 1 volume of 7.8M NH_4Acetate and 3 volumes of ethanol, and centrifugation for 40min at room temperature. The RNA pellet was washed with 80% ethanol and dissolved in 20µl DPC-treated H_2O and 80µl hybridization buffer (see below). RNA quantity and quality was checked on a gel, where the RNA should form a defined band.

II. 2. 6. Antibody staining

For the antibody staining embryos were fixed for 24-48h in 4% PFA at 4°C, dechorionated and incubated in 100% MeOH for min. 1h at -20°C. After rehydration and dechorionation, embryos were blocked in PBS with 0.2% Triton X-100 and 1% BSA (PBTB) for 1 h at room temperature, incubated with the primary antibody at appropriate dilution (1:2000 for anti Vasa and MAb414, 1:1500 for anti Nos and 1:100 for purified

anti Dnd) in PBTB at 4°C overnight, washed with PBTB four times for 20 min, incubated with the secondary antibody (Alexa 546 goat anti–rabbit IgG, Alexa 488 goat anti guinea pig or mouse (Molecular probes), goat anti–rabbit Cy3 or Cy2, (Dianova)) for 2 h at room temperature, and washed with PBT four times for 20 min.

II. 2. 7. Paraffin sections and Eosin-hematoxylin staining

The embryos were fixed for 24-48h in 4% PFA at 4°C washed 3x with PBS and Dehydrate in increasing ethanol solutions:

3x 50% EtOH	for 1h
3x 70% EtOH	for 1h
3x 90% EtOH	for 1h
3x 100% EtOH	for 1h
100% Tuluol	for 1h

The dehydrated embryos were incubated 3-4x in hot paraffin minimum for several hours. Oriented and embed in paraffin blocks and sectioned on a microtome at 8μm thick. The sections were transferred on superfrost objective slides. Drayed 1h on the heater next to the microtome water bath (40°C) and incubated at 37° O/N.

The paraffin from the slides was removed by dipping 2x 100% Xylol (Hystoclear) for 2' and the tissue was rehydrated in decreasing, 2x100%, 2x90%, 2x75%, 2x50% and 2x water, EtOH solutions for 2' per step.

Nuclear staining (Blue):

The sections were dipped in Hematoxylin solution for 4-6 min. and washed with tab water for 10'

Cytoplasmic staining (Red):

The sections were incubated 2x in water for 2 min and stained in Eosin solution for 2-6 min. Then washed briefly 2x in water and dehydrated by dipping in reverse order in the above EtOH solutions for about 20 sec. Final step 2x in 100% Xylol (Hystoclear) for 2 min, drayed shortly and mounted with EU KIT.

II. 2. 8. Discontinuous polyacrylamide gel electrophoresis

Molecular weight dependent separation of proteins was performed by discontinuous SDS-PAGE. SDS is a non-ionic detergent, which denatures the proteins and neutralises

the charges. In the stacking gel (upper) the proteins are concentrated, in the separation gel (lower) separated.

Gels with a SDS content of 0.1% (w/v) and an acrylamide content of 12% (w/v) in the separating gel were used. Polymerisation of the liquid gel solution was started by addition of the radical starter APS and TEMED, a catalysator. The separating gel was covered with 2-butanol and rinsed with water after polymerisation. Subsequently the stacking gel was added and the combs placed so as to form troughs in the stacking gel. Protein samples were mixed with 5x sample buffer (so the concentration in the sample was 1x), sonicated for 30 sec in ice and boiled for 2-5 min (water bath). After re-cooling to room temperature they were loaded on the gel or stored at –20°C. Electrophoresis was carried out with 60mV. After electrophoresis the resolving gel was either stained with Coomassie Brilliant Blue and subsequently photographed or was used for Western blotting experiments.

Separating gel (lower):

20ml H_20, 16ml acrylamide/bis solution, 12ml resolving gel buffer (1.5M Tris-HCl, pH 8.8, 0.4% (w/v) SDS), 150µl APS, 20µl TEMED

Stacking gel (upper):

9ml H_2O, 2.25ml acrylamide/bis solution, 3.75ml stacking gel buffer (0.5M Tris-HCl, pH 6.8, 0.4% (w/v) SDS), 150µl APS, 20µl TEMED

APS 10% (w/v):

Ammoniumperoxodisulfate in water (stored at -20°C in the dark)

Acrylamide/bis solution:

30% (w/v) acrylamide, 0.8% (w/v) N,N´-methylenbisacrylamide (ratio 37.5:1)

II. 2. 9. Western Blotting

Protein blotting was done using Mini-PROTEAN 3 tank transfer system (Biorad)

The acrilamide gel was released from the glas plates and the stacking gel was removed. The gel was incubated in Transfer buffer for 5’ and the transfer system was assembled as follow: Sponge; Whatman paper, soaked in transfer buffer; gel (keep wet by adding transfer buffer); Nitrocellulose membrane soaked the membrane in H2O for 1 min.; Whatman paper, soaked in transfer buffer; sponge. All air bubbles were removed by rolling with a glass pipette. The plastic holder with the assembled transfer system was

closed (white side on top) and insert into blotting chamber filled with transfer buffer. The transfer was performed for1h at 100V or overnight at 20 volts, at 4°C.
The membrane was processed immediately or dried and stored at 4 degrees in Saran Wrap.
Immuno-staining of the blotted proteins was performed as follow:
The membrane was rinsed 1x in PBT, blocked in PBT + 5% dry milk powder for 1h at RT or 4°C ON and rinsed again 2x in PBT.
The first antibody was diluted to appropriate concentrations (1:2000 for anti Nos and 1:100 for the purified anti Dnd) in PBT+ 5% dry milk powder in BSA. The membrane was incubated in the first Ab solution for 1h at RT, rinsed 2x in PBT and washed 3x 10 min in PBT on a shaker at RT.
The membrane was then incubated with the 2nd antibody (HRP-coupled Goat anti-rabbit, 1:10 000 in PBT + 5% dry milk) for 1h at RT and washed again 5-6 times with PBT on a shaker at RT.
Visualization of the secondary antibody was performed using Chemoluminescent Detection (ECL Western blotting detection reagents: see also manual):
Equal volumes of detection solutions 1 and 2 were mixed (total amount of 1ml/blot) The washed membranes were drained from the excess buffer and placed on Saran Wrap – protein side up. The detection reagent was added to the protein side of the membrane, incubated for 1 minute at room temperature, drained from the detection reagent and placed on fresh Saran Wrap. The blots were placed in a film cassette, protein side up and exposed for the required time (30 sec to 30 min).

II. 2. 10. Protein Expression

Expression of a protein in a bacterial host requires the transcription of DNA to the corresponding mRNA and the subsequent translation into the primary amino acid structure at the ribosomes. The pET19b plasmid contains the strong T7 promoter, which acts as a binding site for the polymerase holoenzyme in the presence of IPTG (2-Isopropyl-ß-D-galactopyranoside). IPTG causes gratuitous induction of the promoter and the cDNA of the protein is therefore permanently transcribed. For protein expression transformed bacteria were incubated over-night in 20ml LB medium at 37°C as a preculture. The precultures were transferred to 800ml LB medium, which was subsequently incubated on a shaker (37°C) until an optical density of ~0.6 (measured at a wavelength of 595nm) was observed and then induced with 10mM IPTG. After 3hrs

incubation cells were harvested for protein purification. The cells washed by re-suspending the pallet in 1xPBS. Spin again. The pallet was re-suspend in 10 ml 1xPBS and cells were sonicated on ice for 5min and spun down for 10 min at full speed. If the protein is soluble in PBS it is present in the supernatant, otherwise it is still in the pallet. In this case add 10 ml of denaturing buffer (6M Urea binding buffer). Dissolve the pallet and spin down for 10 min at max speed.
Now the protein is in one of the two supernatants. Confirm it by loading on SDS gel (10µl sample + 10µl load dye. Load 5µl).

II. 2. 11 Coomassie staining

Separated proteins in the gels were visualised by staining with Coomassie Brilliant Blue (Roth, Munich). The gel was placed in the staining bath for 20 min, rinsed with water and destained in destaining solution on a shaker overnight at room temperature.
Staining solution:
6.25 g Coomassie Brilliant Blue, 180 ml acetic acid (96%), 900 ml methanol to 2000 ml with water (final volume).
Destaining solution:
1500 ml methanol, 1000 ml acetic acid (96%) to 4000 ml with water (final volume).

II. 2. 12. Protein purification

The recombinant protein was purified using metal affinity resins, according to the protocol for His-tagged protein purification of TALON purification kit (Clontech). Normally 2ml of the bead solution (1ml after settle down) were enough to purify up to 2mg of protein. When the purification required denaturing conditions, all buffers were supplemented with 6M. Upon purification, the amount and the purity of the obtained protein was estimated from Comasie brilliant blue stained gels, by comparing the size and the intensity of the band with a standard.

II. 2. 13. Production and purification of polyclonal antibodies

Animals were immunised with the recombinant Dead end protein (commercial immunisation, BioScience, Göttingen).
The antibody purification was performed using affinity chromatography with BrCN activated Sepharose 4B (Amersham, MA).

The ligand to be coupled was dissolved in coupling buffer. About 5-10mg protein per ml is recommended. As the protein was dissolved in denaturing buffer, it was dialysed overnight against coupling buffer. Some times aggregates form upon dialysis, just pipet everything in the coupling mixture.

1 g BrCN activated Sepharose was dissolved in some ml 1mM HCl solution (1 g sepharose gives rise to about 3 ml gel matrix), purred onto a glass filter column (Glassfritte), activated with 250 ml 1 mM HCl with constant swirling with a pasteur pipette and transferred in a 14 ml Sarstedt-tube to sediment the matrix (5min 600 rpm). The supernatant was discarded. The protein ligand (dissolved in sodium-carbonate-dialysis buffer) was added and coupled over night at 4 ºC with end-over-end rotation. On the next day the gel matrix was sediment (5 min, 600 rpm), resuspend again in 10 ml of 1 M Tris (pH 8) and incubated for 1 hour end-over-end, (to block the free sites of the matrix). Then the gel matrix was washed 5x 10 ml PBS (5 min, 600 rpm) by consecutive sedimentation and resuspention.

10 ml from the serum to be purified was added to the gel matrix in a 50 ml tube and incubated over night at 4 ºC end-over-end.

On the next day the matrix was separated from the serum (5 min, 600 rpm) and washed 3x 10 ml PBS. The gel matrix was then resuspend in 10 ml PBS and transferred into the Sephadex column. Let the PBS flow through.

The bound Abs were eluted with 0 ml elution buffer I (pH 4,8) and stored at 4 ºC. The column was washed again with 10ml PBS and again eluted with 4 ml elution buffer II (pH 2,6). The two eluates were immediately dialysed at 4 ºC overnight in 2 l PBS. The purified antibodies were aliquot and stored at -20 ºC

II. 2. 14. Whole mount *in-situ* hybridization

In situ Day 1

Embryos were fixed for 24-48h in 4% PFA at 4°C, dechorionated and incubated in 100% MeOH for min.1h at -20°C. After rehydration and dechorionation, embryos were treated with Proteinase K (10 µg/ml in PBT) according to the stage:

gastrula stages:	0-3 min
1 somite:	10 min
5 somites:	15-20 min
24 h embryos:	30 min

36 h-72 h embryos: 30 min at 4x the concentration (=40µg/ml)

The embryos were washed 2x in PBT to stop the digest. (First wash: 1min, second wash: 5 min.), refixed in 4% PFA in PBS for20 min at room temperature, washed in PBT, 5 x 5 min and subjected to prehybridization in Hyb buffer for 2 to 5 hrs at hybridization temperature (here: 67°C) in a water bath.

Hybridization was performed in hybridization mix (=probes in Hyb buffer) wit different concentration of the probe:

Examples: vasa-Dig for 24h embryos: 0,5µl probe in 200µl of Hyb buffer.
vasa-Dig for sphere stage: 0,2µl probe in 200µl Hyb buffer.

At 67°C (water bath) overnight.

In situ Day 2:

The embryos were washed with preheated to hybridization temperature washing solutions:

1: Hyb buffer, at 67°C, 20 min

2: 50% SSCT 2 x / 50% Formamide, at 67°C, 3x 20 min

3: 75% SSCT 2 x / 25% Formamide, at 67°C, 20 min

4: SSCT 2x at 67°C, 2x 20 min

5: SSCT 0.2 x + 4 x 30 min, at 67°C

6: PBT, 67°C, 5 min

The samples were blocked at room temp for several hrs (minimum 1 h) in 5% sheep serum + 10mg/ml BSA in PBT and then incubated with anti-Dig-AP antiserum, preabsorbed against embryos, 1:2000- 1:4000 in PBT+2 mg/ml BSA (optional: +2% sheep serum) overnight at 4°C.

In situ Day 3:

The samples were washed at room temperature on shaker/ wheel etc (slow agitation) 10-12 times distributed over a total time of ca 3h.

Fresh staining buffer NTMT was prepared and the embryos were washed 3 x 5 min. During last wash, the samples were transfer to a 24 well plate.

Staining was performed in staining solution at room temp. or in a 28.5°C incubator, in the dark, without shaking, and the staining reaction was monitored using a dissecting microscope. The reaction was stopped washing and storing in STOP solution.

Staining solution BCIP/ NBT (blue staining):
4.5 µl NBT (75 mg/ml in 70% DMF / 30% H2O)
3,5 µl X-phosphate (=BCIP) (50 mg/ml in 100% DMF)
1 ml NTMT buffer

II. 2. 15. Fish stocks and maintenance

Zebrafish embryos were raised, maintained and staged as described by Kimmel (Kimmel et al., 1995). Embryos were obtained from natural spawning and incubated at 28°C until the desired stage in Daniou's solution.

II. 2. 16. mRNA and morpholino microinjection

For the injection the RNA as well as the morpholino oligonucleotides reagents were diluted in Hepes (10 mM, pH 7,4) and stored on ice until use. After harvesting, the eggs were placed into agarose ramps and oriented with the animal pole up. Injections were performed with a microinjector (see Material), using constant backpressure. Every egg was injected in the yolk below the first developing cell with about 0.5nl solution.

II. 2. 17. Time-Lapse analysis of PGC migration

For the time lapse analysis control and experimental embryos whose PGCs were labeled with GFP by injection of GFP-nanos-1 RNA (Köprunner et al., 2001) were oriented in agarose ramps and overlaid with 0.3x Danieau's solution (Westerfield, 1995). Time-lapse movies were generated using Metamorph software (Universal Imaging) controlling a Zeiss Axioplan2 microscope.

III. Results

III. 1. Dynamics and function of germ plasm components

III. 1. 1. Generation of antibodies directed against Nanos1 and Dead end

To investigate the distribution of Nanos1 and Dead end in the zebrafish embryos, we decided to raise specific polyclonal antibodies, recognizing these proteins. An N-terminal His-tagged version of the zebrafish full size *nanos1* gene was cloned in viral pBACPAK9 (Clontech) expression vector. The protein was over-expressed in insect cells, and purified by Mark Neumann (a diplom student in the lab). A recombinant His-tagged fragment of *dead end* containing 200 amino acids from its C-terminal side was cloned in PET 19b vector (Novagen), over-expressed in *E. coli* and purified using TALON purification kit (Clontech) under denaturing conditions. Purified Nanos1 protein was then send to Neurogentec (Belgium) and Dead end to Biolabs (Germany) for animal immunizations.

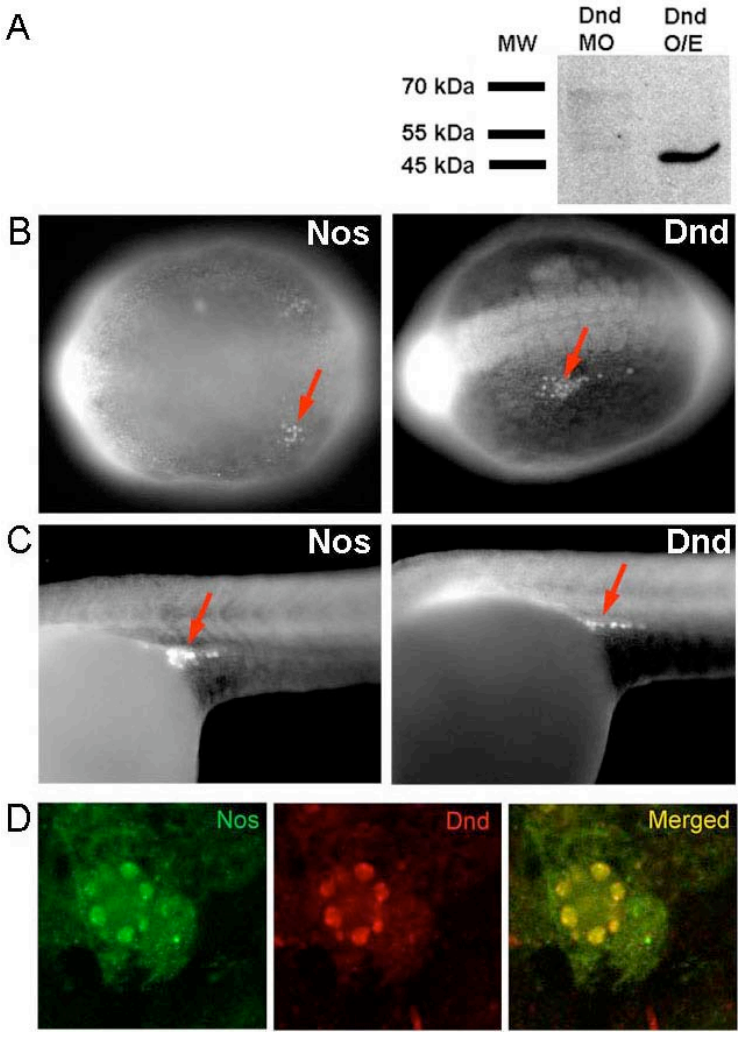

Fig. 4. Tests of Nos and Dnd antibodies.
(A) Western blot assay of immuno-affinity purified Dnd antibody. A 45 kDA band was detected in lyzates of embryos injected with *dnd-globin3*'UTR mRNA. The same band was absent in lysates from *dnd* MO injected embryos. (B, C) Whole mount fluorescent antibody staining with anti-Nos (left panels) and anti-Dnd (right panels) antibodies of embryos in the segmentation (B) and pharyngula (C) stages. The arrows point to the germ cells. (D) High magnification confocal images of a PGC stained for Nos (green) and Dnd (red) antibody. The two proteins co-localize in the perinuclear granules (merged).

The final blood serum from the animals injected with both proteins was then examined in whole mount antibody staining. 6- somite stage and 24hpf old embryos incubated with 1:1000 serum dilutions from one rabbit and one guinea pig, immunized with Nanos1 protein, exhibited higher signal in a group of cells, which judged by positional and morphological criteria appeared to be the germ cells (Fig. 4 B, C). In contrast, no specific staining was observed in embryos stained with the serum from animals immunized with Dnd protein (data not shown). Therefore, an additional immuno-affinity purification step was performed using recombinant Dnd protein coupled to CNBr-activated agarose beads, followed by pH-based elution of the bound antibodies (See materials and methods for details). The purified antibody recognized a 45kDa band in Western blot assays of zebrafish embryo lisates, in which Dnd was over expressed by mRNA injection (Fig. 4 A). This band was absent in dead end MO injected embryos. In addition, when used in whole mount staining, purified Dnd antibody labelled the germ cells of zebrafish embryos (Fig. 4 B, C).

III. 1. 2. Germ plasm granules co-localize with the nuclear pores

To examine the dynamics of Dead end and Nanos1 distribution we performed high magnification microscopic analysis of immunostained embryos at different stages. Prior to shield stage (6 hpf), we failed to detect any enrichment of these proteins in specific cells or cell structures (data not shown). After this stage, in some cells aggregates of these proteins started to accumulate in the cytoplasm, forming specific granules, similar to the ones described in Vasa antibody studies (Braat et al., 2000). Since Vasa, Dnd and Nos co-localize in these structures (Fig.4 D and data not shown) and fusion Dnd and Nos proteins are found in the germ cell granules at later stages (Köprunner et al., 2001; Weidinger et al., 2003), we suggest that the endogenous Dead end and Nanos1 proteins become localized to the granules of the germ cells after Shield stage (data not shown).

Previous studies of the germ plasm granules in zebrafish demonstrated that at later stages of the development (30 hpf) they are found in close proximity to the nucleus, but unlike the prinuclear granules in *C. elegans,* do not co-localize with the nuclear pores of the germ cells (Knaut et al., 2000). To examine this point during the earlier stages of zebrafish development we stained the granules with Vasa polyclonal antibody (Knaut et al., 2000) and compared their localization with that of the nuclear pores detected by

MAb414 antibody (Hiss Diagnostics). Similarly to the Dead end and Nanos1 proteins, we were able to detect Vasa-positive granules as early as shield stage. Laser confocal images of germ cells at this stage revealed that the granules are found in close proximity with the nucleus but a significant portion of Vasa protein, and presumably the rest of the components of the granules, was not in direct contact with the nuclear pores. At the same time, a considerable number of the evenly distributed pores appeared free from the granules components. Therefore, we conclude that at his stage the germ plasm granules are not associated with all nuclear pores of the germ cells (Fig. 5 upper panel).

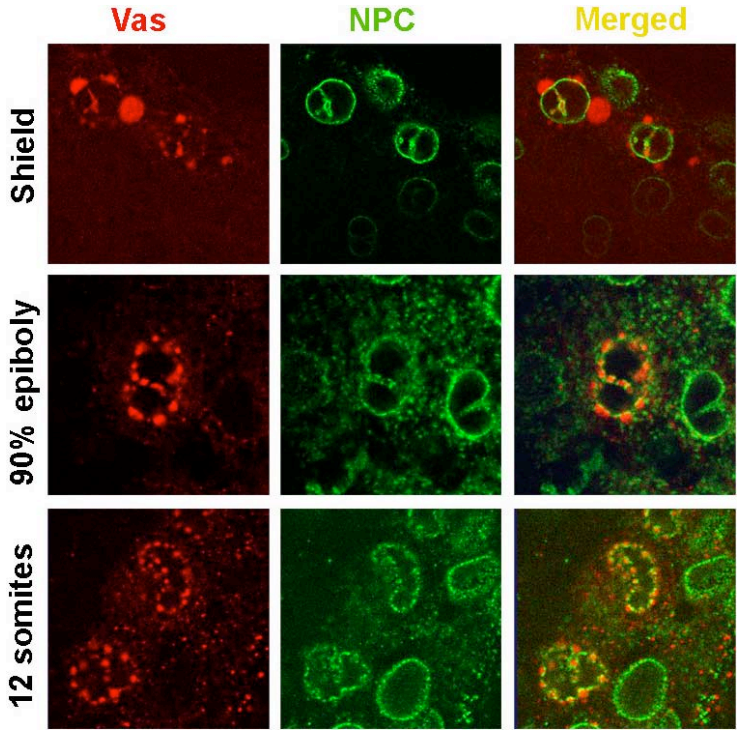

Fig. 5. Germ cell granules co-localize with the nuclear pores.
Vasa positive granules (red) at Shield stage (upper panel) are associated with the nucleus but do not co-localize with all nuclear pores (green). At later stages, 90% epiboly (middle) and 12 somites (lower panel), the majority of the nuclear pores are found in close proximity to the Vasa granules.

However, as the development proceeds, Vasa granules in the germ cells assumed more even distribution around the nucleus and at 90% epiboly we found this organelle surrounded by the granules. Furthermore, every granule was found in close proximity with one or more nuclear pores and almost no free pores were observed. Interestingly, the overall appearance of the nuclear pores changed from evenly distributed in shield stage to more clustered around the granules, whereas the distribution of the nuclear pores in the somatic cells remained unchanged (Fig. 5). Similar results for the relative positions of these two cellular structures were obtained for the germ cells of 12 somites (Fig. 5 lower panel) and 24 hpf (data not shown) old embryos. Thus, we hypothesize that during the period 5 hpf to 24 hpf of the zebrafish development the germ cell granules are associated with the majority of the nuclear pores.

III. 1. 3. Functional studies of Dead end and the loss of function mutated variants

As shown in previous studies, *dead end* encodes an RNA-binding protein, containing several putative RNA-binding domains, the most prominent of which is localized close to the N-terminus RNP domain. Dead end protein is indispensable for the proper germ cell development. In particular, embryos injected with anti-sense morpholino oligonucleotides targeting the ATG region of *dnd* are lacking germ cells at 24 hpf. Co-injection of "morpholino resistant" *dead end* mRNA can rescue the PGCs (Weidinger et al., 2003).

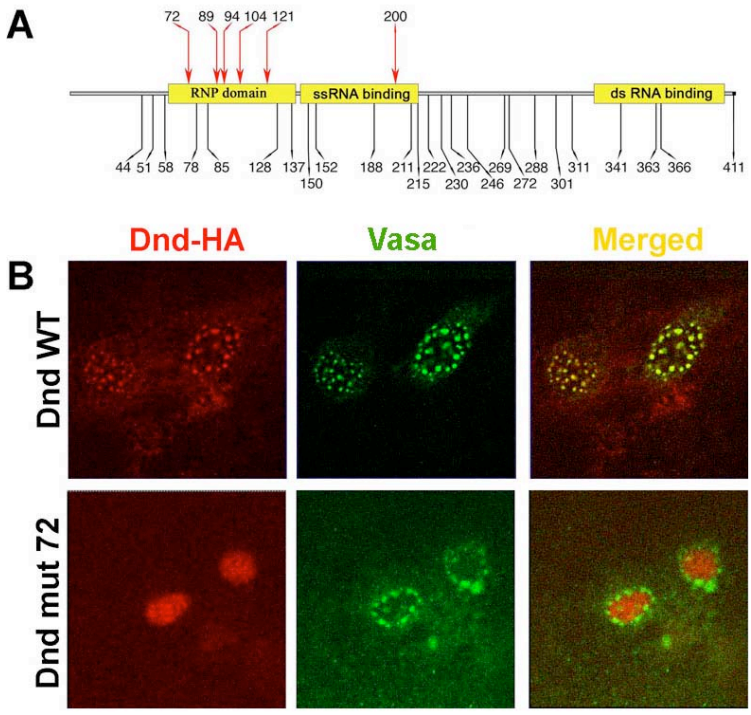

Fig. 6. Functional studies of the domains important for Dnd function.
(A) Schematic representation of Dnd protein with its putative RNA-binding domains and the distribution of the introduced point mutations along the sequence. Mutation of 6 amino acids (red) caused loss of function of the protein. (B) Fluorescent staining of injected HA-tagged wild type Dnd (upper panel) and a loss-of-function mutated Dnd variant (lower panel) (red). Upon the amino acid exchange, the non-functional protein is found in the nucleus, whereas the wild type form is in the granules. Vasa localization remained unaltered (green).

During my Master thesis research performed in the same lab, we investigated the importance of the putative domains within the Dnd for the protein function *in vivo* by mutating single key amino acids alternating the primary sequence. To this end, we introduced 31 point mutations along the DNA encoding full size Dnd protein, exchanging with each mutation one amino acid for a different one in the translated protein. At the level of RNA these mutated variants of the protein were designed to be "morpholino resistant" due to nucleotide exchanges in the morpholino binding region, which did not

alter the protein sequence. These modified *dnd* constructs were sub-cloned in RNA-expression vector upstream from *nanos1* 3'UTR, transcribed in-vitro and injected in 1-cell stage embryos, together with Dead end MO. We assumed that the mutated variants of Dnd, in which a crucial amino acid was altered, would not be able to fulfill the function of the protein and thus will not rescue the PGCs from the action of the MO targeting the endogenous *dnd* mRNA. All injected embryos were fixed at 24 hpf, subjected to *in situ* hybridization staining with a germ cell specific probe, and their PGCs were counted. We found out that out of 31 mutated variants of Dnd 6 failed to rescue the germ cells and therefore we consider them as loss of function mutations. Importantly, in 5 of them the mutations were localized within the putative RNP domain (Fig. 6 A).

Our further efforts performed in the present work were directed towards determining the potential changes in the intra-cellular role of Dnd leading to the loss-of-function effect caused by the mutations. As the wild type Dead end protein is localized to a very specific cellular structure within the germ cells, the perinuclear granules (see above), tests of the localization of the mutated variants appeared as a straightforward assay for proper function. Wild type Dnd as well as its mutated variants were tagged with HA-tag and injected in 1-cell stage embryos as *in vitro* transcribed mRNA. Consequently, the embryos were fixed and the localization of the fusion protein was examined using anti HA antibody. Our preliminary results form this experiment show dramatic alteration in the localization of the mutated variants of Dnd in comparison to the wild type. As shown in (Fig. 6 B), in some cases (here mut 72) the mutated proteins were no longer found in perinuclear positions, but appeared in the nucleus, whereas the positions of the perinuclear granules remained unaltered as judged by the Vasa staining.

III. 2. Sex differentiation in zebrafish

III. 2. 1. Embryos lacking germ cells develop into sterile adult males

To determine the role of the germline in somatic development of zebrafish we ablated the PGCs using *dead end* antisense morpholino (MO) oligonucleotides. As previously described, *dnd* is essential for normal migration and survival of PGCs and therefore embryos devoid of this protein develop to become sterile adults (Ciruna et al., 2002; Weidinger et al., 2003). Interestingly, in contrast to embryos injected with control mopholino that developed into fish of both sexes, all embryos injected with *dead end* mopholino developed into fish that appeared phenotypically males as determined by their body shape and color (Fig. 7 A-C). Moreover, when these males were mated with wild type females, the fish displayed normal male sexual behaviour as judged by their ability to induce females to lay eggs.

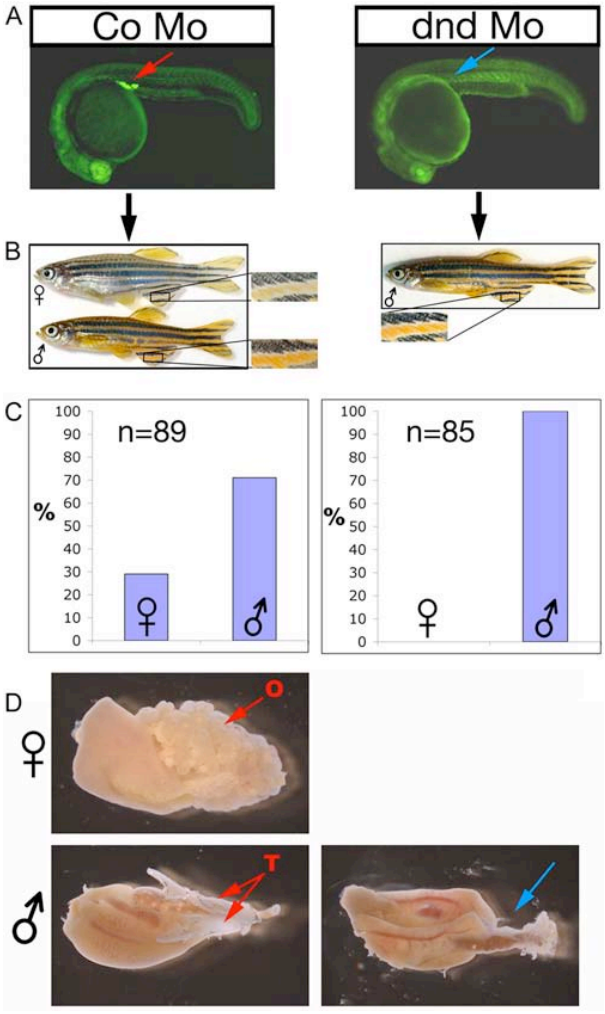

Fig. 7. Dead end Morpholino (MO) injected embryos develop into sterile adult male fish.
(A) Fluorescent images of 24hpf old embryos injected with 200pg control morpholino antisense oligonucleotide or *dnd* morpholino and GFP-*nos1*-3'UTR to visualize the PGCs. The red arrow points to the germ cells in control embryos and the blue arrow shows the region where the PGC are normally found. (B) Adult fish derived from control (left) or *dnd* (right) morpholino-injected embryos and (C) a quantitative analysis of the female/male ratio. (D) Dissected gonads from control and *dnd* morpholino-injected embryos. Red arrows point to ovary (O) or testis (T) in the control fish. Blue arrow shows the region of the gonad in a phenotypically-appearing male fish derived from *dnd*-MO injected embryos.

Nevertheless, as expected from the early loss of PGCs, the males were sterile and did not fertilize the eggs. To characterize the *dnd* MO phenotype further we examined the adult fish. This analysis revealed the lack of any gonadal structures in these fish (Fig. 7 D). These results clearly show that Dead end function is essential for female development as well as for proper development of the gonad. It should be noted however, that this requirement could represent either a specific and direct function of *dead end* in sex determination or an indirect consequence of the loss of PGCs or the gonad. To distinguish between these two possibilities, we set out to ablate the PGCs using an independent method and determine the effect of the treatment on sex determination in zebrafish.

III. 2. 2. Germ cell ablation using *Diphtheria* toxin

To ablate zebrafish PGCs by an independent method we decided to express bacterial toxins in these cells. One cell stage embryos were injected with mRNA encoding for the *Diphtheria* toxin (DT) catalytic A-chain (Lord et al., 1999) fused to the 3' untranslated region (UTR) of the zebrafish *nanos-1 (nos1)* gene, which directs the expression of the DT protein preferentially to the PGCs (Köprunner et al., 2001). Embryos injected with 0.05pg or more of the *Diphtheria* fusion mRNA died within the first hours of their development with severe malformations (Data not shown). Reduction of the injected amounts of RNA led to a decrease in the observed malformations, which presumably resulted from residual toxin expression in the somatic cells (Fig. 8 A, C).

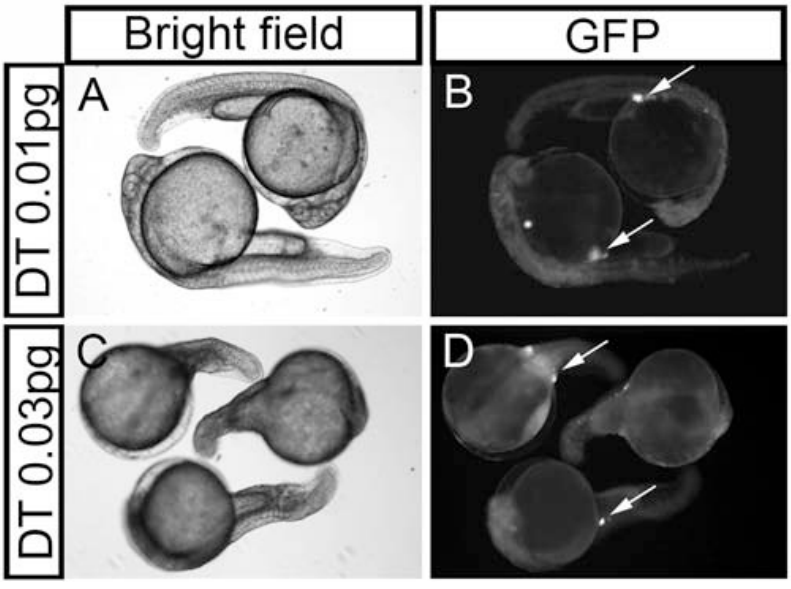

Fig. 8. ***Diphtheria*** **toxin A-chain-*nos1*-3'UTR mRNA injected embryos exhibit somatic malformations.**
(A, C) Bright field images of embryos injected with 0.01 and 0.03pg of *DT-nos1*-3'UTR mRNA show concentration-dependent increase of somatic defects. (B, D) Fluorescent pictures of the DT-injected embryos demonstrating that in the

defective treated embryos some PGCs survive. Arrows in B, D point at the PGCs.

Our attempts to define injection conditions in which the injected embryos would lose all of their PGCs yet develop to reach adulthood failed (Fig. 8 B, D). This result probably reflects a high sensitivity to DT of some somatic cell lineages that are essential for viability. Thus, under conditions in which all the PGCs were ablated, low level of DT in somatic cells led to embryonic lethality.

III. 2. 3. Prokaryotic toxin Kid and antitoxin Kis are functional in zebrafish

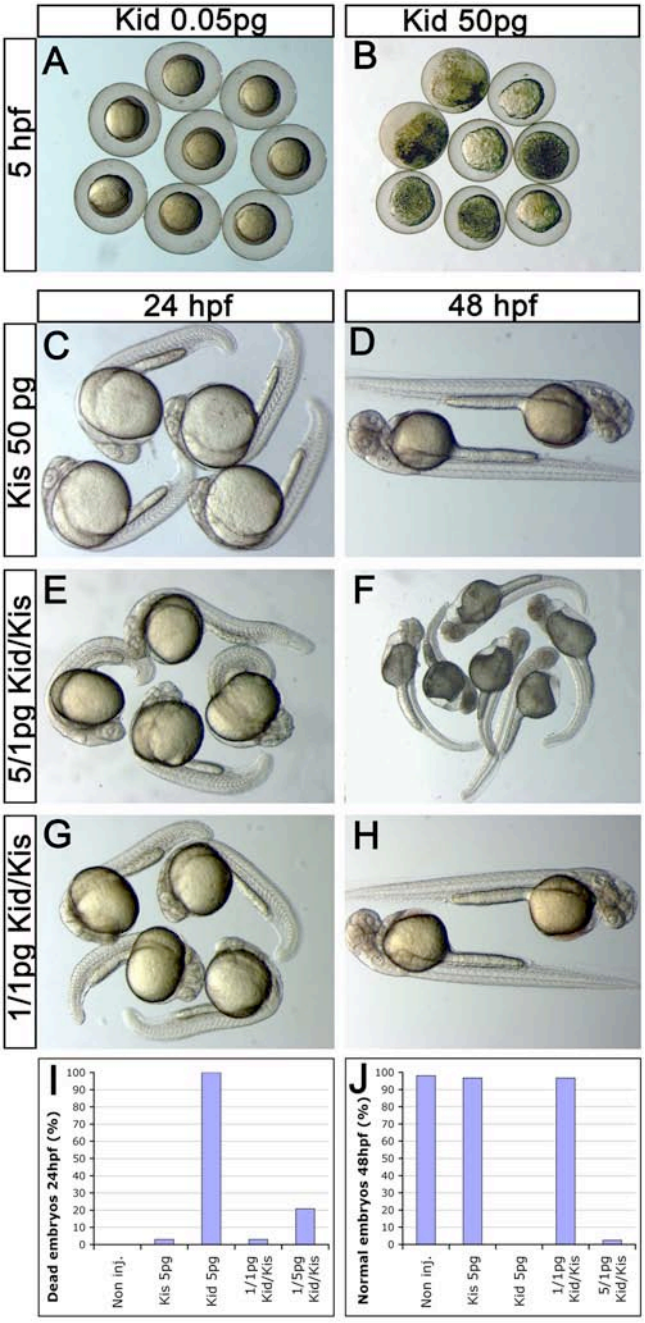

Fig. 9. The Prokaryotic toxin Kid and anti-toxin Kis are functional in zebrafish.

(A, B) High concentrations of Kid result in rapid embryonic lethality in embryos injected with *kid-nos1-3'UTR* RNA. (C, D) Embryonic development is not affected by high levels of somatically expressed antidote *kis-globin-UTR* RNA. (E) 1pg of *kis-globin-UTR* efficiently counteracts the deleterious effect of 5pg of Kid on somatic cell development up to 24 hours, but not at 48hpf (F). (G, H) A ratio of 1pg/1pg of *kid-nos1-3*'UTR/*kis-globin-UTR* mRNAs allows proper somatic development during the first two days of development. (I, J) Quantitative representation of Kid-induced somatic phenotypes during the first two days of development.

To overcome the problem of toxicity to somatic cells we chose to utilize a system that allows ablating the PGCs while protecting somatic cells from the toxin. The prokaryotic *par*D system consists of a toxin, *kid*, and an antidote, *kis*; In cells expressing the Kid protein cell growth is inhibited whereas co-expression of Kis inactivates the toxin and neutralizes this effect (Ruiz-Echevarria et al., 1991). This system has been shown to inhibit cell proliferation in yeast, frog embryos and in mammalian cell lines, where it also induces cell death (de la Cueva-Méndez et al., 2003), but ablation of a specific cell type in a developing organism using this system has not been demonstrated thus far.

To determine whether the toxin anti- toxin components of the *parD* system could function in zebrafish we expressed the toxin preferentially in the PGCs as described above (injection of mRNA, which included the Kid open reading frame (ORF) fused to 3'UTR of the *nos1* gene). Indeed, this treatment effectively eliminated the PGCs demonstrating that Kid is functional in zebrafish cells (see below). Nevertheless, similar to our findings using *Diphtheria* toxin, injections of *kid-nos1*-3'UTR resulted in somatic defects and embryonic death. The extent of the Kid-induced embryonic death was concentration dependent such that embryos injected with 50pg of the toxin fusion mRNA died at the very early developmental stages while amounts lower than 0.05pg had no effect on the embryos (Fig. 9 A, B).

Uniform somatic expression of the antidote upon injecting 50pg *kis-globin*-3'UTR mRNA by itself, did not lead to any visible affect on embryos at 24 hours post fertilization (hpf), 48 hpf (Fig. 9 C, D) and adults. Importantly, co-injection of the antidote mRNA effectively neutralized the deleterious effects of Kid on somatic development. Specifically, embryos coinjected with 5pg *kid-nos1*-3'UTR and 1pg *kis-glo*-UTR mRNA appeared morphologically normal at 24 hpf and thus sharply contrasting embryos that did not receive the antidote (Fig. 9 E, I, J). Nevertheless, these embryos did exhibit somatic defects at 48 hpf (Fig. 9 F). Embryos injected with a ratio of 1pg/1pg from the toxin mRNA/antidote mRNA showed PGC loss (see below), but appeared normal at 24 hpf, 48 hpf (Fig. 9 G, H) and could be raised to adulthood.

III. 2. 4. Embryos injected with kid/kis constructs lack PGCs and develop as sterile male adult fish

To examine further the effect of PGCs on sex determination we compared control embryos injected with 2pg of *kis-globin*-UTR (Fig. 10 A-F) with such injected with 1pg of each *kid* and *kis* RNA-constructs. These embryos consequently exhibited dramatically reduced number of fluorescent PGCs or completely lacked them at 24 hpf (Fig. 10 H). By 48 hpf the majority of toxin-injected embryos had no fluorescent PGCs (Fig.10 K), an observation confirmed by *in situ* hybridization using the germ cell specific marker *vasa* (Fig. 10 F, L). Significantly, these embryos showed no somatic defects during their development (Fig. 10 G, J) and could be raised to adulthood.

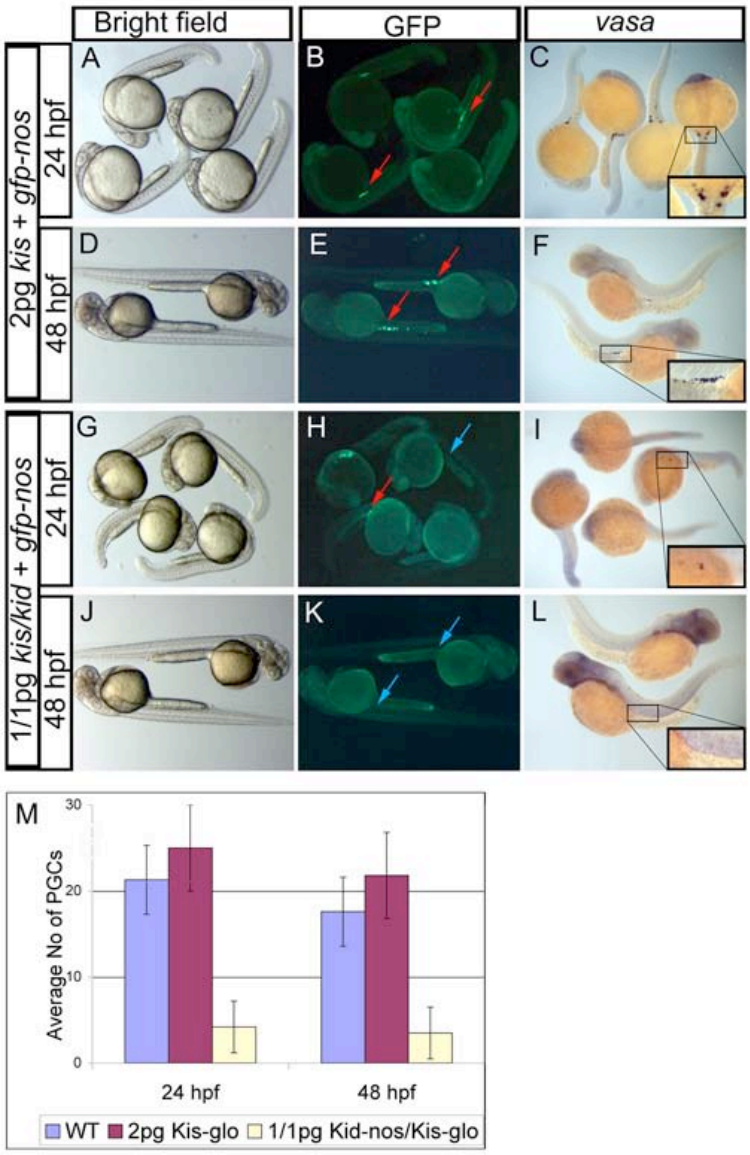

Fig. 10. *kid* and *kis* allow effective ablation of the germline without affecting embryonic morphology. (A, D) Control embryos injected with 2pg of the antidote RNA developed without apparent somatic defects and are morphologically similar to embryos injected with 1pg *kid-nos1*-3'UTR + 1pg *kis-glo* (G, J). Reduction in the number and complete lack of GFP (B, E, H, K) or *vasa* RNA (C, F, I, L) labelled germ cells at 24 and 48 hours post fertilization. Red arrows point to the germ cells, blue arrows show the regions where PGCs are normally found. (M) A quantitative representation of the number of germ cells following different treatment at 24 and 48 hour post fertilization as determined by *in-situ* hybridization using

vasa anti-sense RNA probe.

Analysis of *kid/kis* treated fish revealed high efficiency of the germ cell ablation manifested in frequent generation of sterile adult fish (54 out of 71). We attribute the presence of the 17 fertile fish (4 females and 13 males) to a small number of germ cells that survived the treatment and succeeded in populating the gonad. Importantly, all sterile fish appeared phenotypically males and were capable of inducing females to lay eggs. In contrast with the striking male bias in the experimental fish, only 44 out of 87 control fish developed as males (Fig. 10 M). Dissection of the sterile experimental fish revealed that similar to *dnd*-morpholino injected fish, these males too lacked gonadal structures (data not shown).

By Time-lapse movie we recorded fluorescently labelled germ cells in *kid/kis* -treated embryos. This analysis demonstrated that PGCs appear abnormally large relative to the wild type germ cells (Fig. 11 A, B), presumably signifying an effect on cell proliferation by Kid (de la Cueva-Méndez et al., 2003). These large germ cells subsequently die while exhibiting morphology typical of apoptotic cells (Fig. 11 C).

These results demonstrate that the germ cells are essential for female development and that the phenotype observed in *dnd* MO-treated fish results from germ cells loss rather than reflecting a specific function of the *dnd* gene in this process.

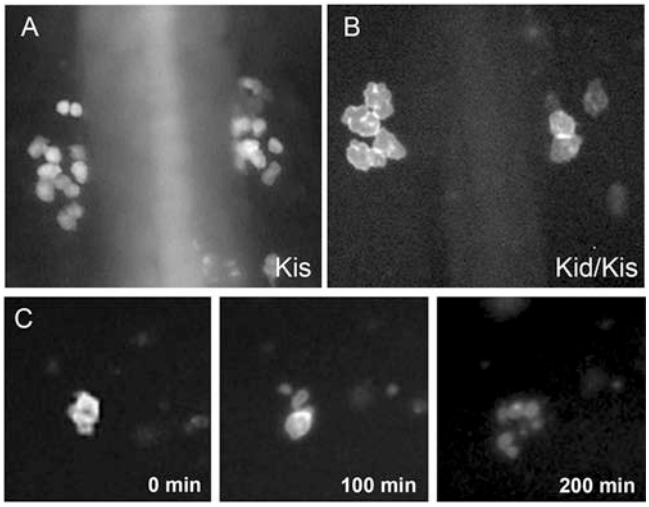

Fig. 11. Primordial germ cells in *kid-nos1-3*'UTR treated embryos exhibit abnormal cell morphology culminating in cell death.

(A) Fluorescently labelled germ cells in *kis-glo*-UTR and (B) *kid-nos1*-3'UTR/*kis-glo*-UTR injected embryos at 12hpf. More PGCs are found in the control embryos and these appear smaller in size relative to *kid*-treated PGCs. (C) Frames from a time-lapse movie of a *kid-nos1*-3'UTR treated embryo showing a primordial germ cell undergoing cell death.

III. 2. 5. Germ cells are important for gonad survival

To investigate the role of germ cells in gonad development, we sectioned fish derived from embryos in which the germline has been ablated using *dnd* morpholino. It was previously shown that initially (25-35 days post fertilization (dpf)) the zebrafish gonads do not undergo morphological sex specific differentiation and appear similar to an ovary (Uchida et al., 2002). Sex differentiation in the male gonad is first manifested by massive early-oocyte death. Prior to this transition we find that the gonadal tissue shows similar morphology in both control and *dnd* MO-treated fish (e.g., at 20 dpf, Fig. 12 A, D). Shortly upon gonad differentiation however, approximately half of the control embryos developed ovaries that were full with oocytes (35dpf, Fig. 12 B) while the rest developed testis (Fig. 12 C).

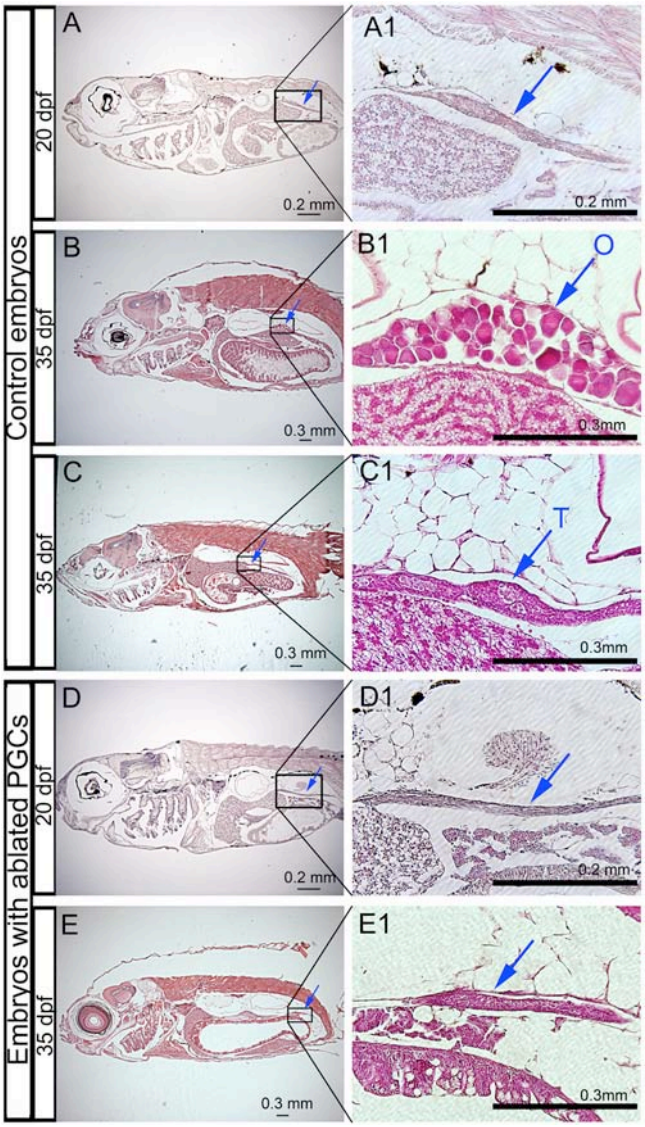

Fig. 12. The formation of the zebrafish gonads does not depend on colonization by PGCs.

(A-C) Eosin/Hematoxylin-stained paraffin sections of 20 and 35 days post fertilization (dpf) fish derived from embryos injected with control MO. (A1-C1) The region of the gonad is boxed in panels A-C and is magnified in the right panel. Blue arrows are pointing at the gonads labelled with (T) for testis, (O) for ovary and blank for undifferentiated gonads. (D, E) Sections of 20 and 35dpf fish injected with 200pg *dnd* MO. Arrows point at gonadal tissue, which is detected at the correct position(D1, E1).

In contrast, in the experimental fish the gonadal structure appeared smaller in size and histologically uniform. Moreover, at 90 dpf no gonad-like structures were observed in *dnd* MO-injected fish, suggesting that the gonads degenerated in the absence of PGCs. Hence, our results imply that in zebrafish germ cells are not required for the formation of the gonad but rather are essential for the differentiation and survival of this organ.

III. 2. 6. The role of the gonad in sex hormone regulation

The analysis of fish lacking the germline demonstrated a critical role for this lineage in female sexual differentiation. A compelling hypothesis would be that the function of the germline in this context is to support the survival of the gonad that in turn, is important for controlling the levels of the sex hormones testosterone and estrogen.

To examine this option, we have generated germline-depleted fish by knocking down the function of *dead end* and treated these fish with estrogen thus, supplementing the sex hormone that presumably is normally generated in the female gonad. Indeed, we could bypass the requirement for the gonad for female development by providing the germline-ablated fish with estrogen; 12 out of 18 fish treated in this manner developed into adult fish that appear phenotypically females despite the lack of gonadal structures (the sex of the other six fish could not be determined). In addition to the female morphological characteristics, the experimental fish were unable to induce wild-type females to lay eggs. Specifically, none of the female appearing estrogen-treated fish were able to induce egg lay as compared to 13 of 16 germline-ablated control (not treated with the hormone) fish.

III. 3. RNA interference

III. 3. 1. RNAi in mammalian and zebrafish cell culture

The finial goal of this part of our study was to determine if small interfering or micro RNA oligonucleotides could induce gene silencing in the somatic cells or germ cells in zebrafish embryos. Here we have tested endogenous zebrafish genes as well as exogenous ones (the latter were introduced as injected mRNA or as transgenes). Before proceeding with experiments on fish embryos, we set out to test if the RNA oligonucleotides were able to induce specific RNAi response in cell culture. HeLa cells were chosen for these tests as well-established, easy-to-use and reliable system for targeting of various genes by interfering double stranded RNAs. The first target gene was GFP that was expressed under the control of a strong constitutive CMV promoter. Four individual siRNAs (siGFP-1-4), each targeting the mGFP mRNA, were synthesized (See Material and Methods for sequence). The siRNAs were transfected into the cells either by co-transfection/co-injection with the GFP expressing plasmid, or transfected sequentially after the plasmid DNA. Fluorescence microscopy and Western blot analysis of transfected HeLa cells showed that three of the siRNAs (siGFP-1, -2 and -4) could silence the GFP protein production from the co-transfected plasmid (Fig. 13 A).

In addition, siRNAs directed against the endogenous gene *sdf-1a*, were tested in HeLa cells. To determine the efficiency of the interference in this case a fusion between Sdf-1a and GFP was constructed and anti-GFP antibodies were used in Western blot assays to assess the level of the translated protein. Four siRNA directed against *sdf-1a* mRNA were tested in HeLa cells together with a plasmid expressing the *sdf*-GFP fusion construct under the control of a CMV promoter. As judged by Western blot analysis, all four oligos were able to silence the expression of the fusion protein in HeLa cells (Fig. 13B).

Further experiments, demonstrating the efficacy of the used oligos as well as the presence of the RNAi machinery in zebrafish cell lines, were conducted in the lab of Prof. Weber at Max-Planck Institute for Biophysical Chemistry. In these experiments siGFP-1 and -2 were used to silence mGFP expression in two zebrafish cell lines (Fig. 13 C). In this experiments both siGFP1 and siGFP2 were able to silence GFP expression as judged by the fact that only 5% of the experimental cells expressed GFP compared with 35% in the control (Fig. 13 C). In addition, the endogenous β-actin, lamin A and lamin B2 genes were targeted and effective gene silencing was obtained (data not shown). Taken

together, these results demonstrate that the siRNAs used could induce target specific gene silencing in mammalian as well as in zebrafish cell lines.

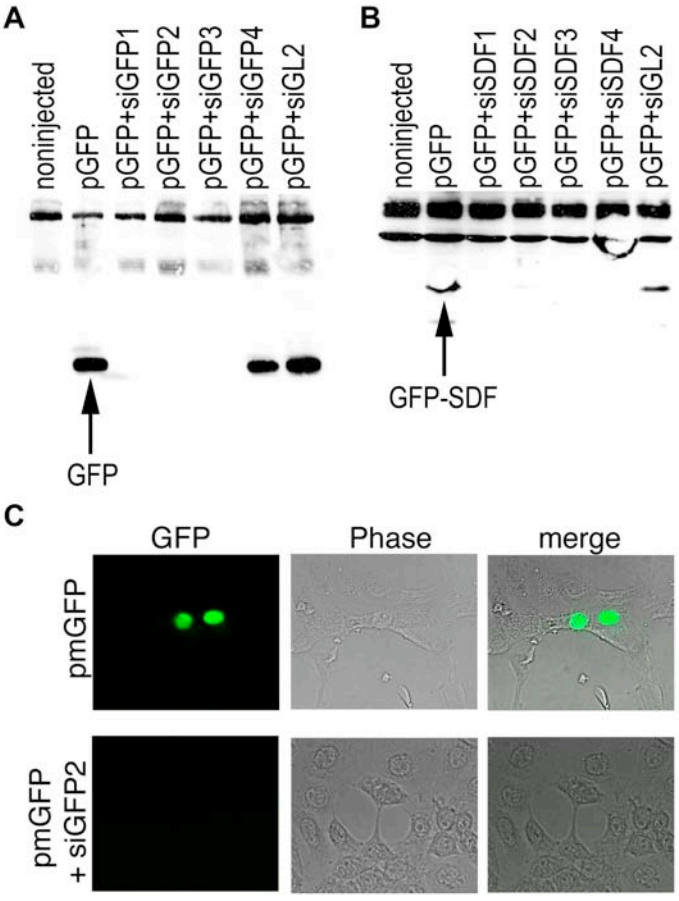

Fig. 13. siRNA oligonucleotides induce gene silencing in HeLa and in zebrafish cell lines.
(A) Western Blot analysis of HeLa cells co-transfeceted with plasmid carrying the CDS of the mGFP together with the siGFP-1 -4. Immunostaining of the blot with anti GFP antibody demonstrated successful GFP targeting in three of four examined siRNA. (B) Tests of the SDF-1 -4 siRNA. All investigated SDF oligos induced silencing of the expression of a GFP-SDF fusion protein from a co-transfected plasmid, detected with anti-GFP staining. (C) Fluorescent and phase-contrast images of zebrafish cell line co-transfected with GFP plasmid and siGFP-1 and -2. Less than 5% of the siGFP oligos transfected cells exhibited fluorescence, compared to 35% of the cells transfected with the plasmid alone.

III. 3. 2. Injections of high amounts of siRNAs into zebrafish embryos cause general malformations.

To determine the maximal amount of siRNA that can be delivered into zebrafish embryos we injected 1-cell stage embryos with different concentrations of double stranded 21bps RNA oligomers and examined the morphology of the embryos at 24 and 48 hpf. We found that all embryos injected with siRNA in concentrations higher than 200μM exhibited similar developmental defects regardless of the targeted sequence (Fig. 14). These severe malformations lead to the subsequent death of these embryos. Decreasing the concentrations of injected siRNA solutions resulted in a decrease in the number of severely affected embryos and concentrations below 10 μM resulted in less than 10% malformed embryos (Fig 14 A).

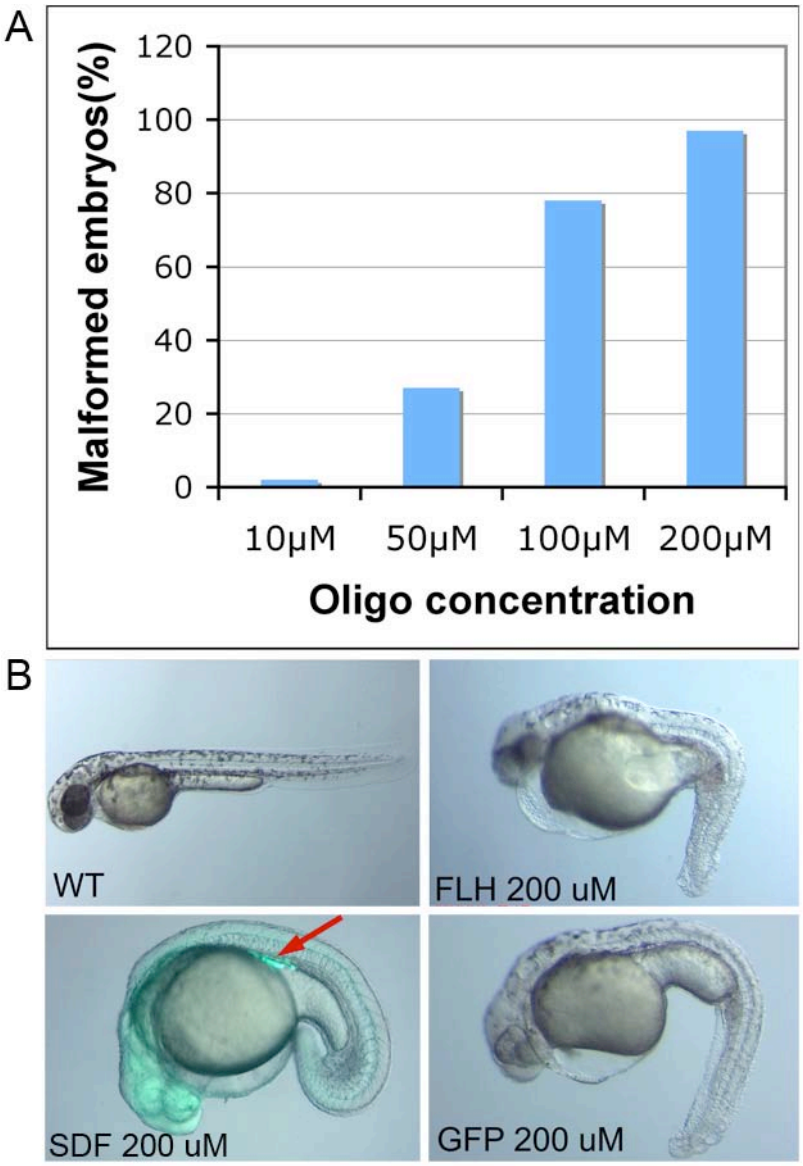

Fig. 14. siRNA oligos induce concentration dependent malformations in zebrafish embryos.
(A) Graphical representation of the concentration dependent malformations. 100% of the embryos injected with siRNA solutions with concentrations higher than 200µM, irrespective of the sequence, developed severe malformations.
(B) Images of embryos injected with 200µM solutions of different siRNAs. In the case of SDF siRNA, the bright field image was superimpose with a fluorescent picture of the same embryo, showing GFP labelled PGCs located at the correct position, pointed by an arrow.

III. 3. 3. Lack of specific RNAi in zebrafish embryos injected with siRNA

Following the demonstration of the efficacy of the oligos to induce RNAi in cell cultures, we set out to examine their ability to silence genes in developing zebrafish embryos. To this end we tested the efficiency of silencing GFP expression from an *in vitro* transcribed capped mRNA encoding for mGFP. 1-cell stage embryos injected with 100pg of GFP mRNA expressed the GFP protein ubiquitously at 24 hpf (Fig. 15). Embryos injected with GFP mRNA and a control oligonucleotide targeting the firefly (*Photinus pyralis*) luciferase gene (GL2), exhibited similar levels of GFP fluorescence (Fig. 15). In attempt to specifically silence GFP expression, a mixture of siGFP-1 and siGFP-2 was injected in three different concentrations, 3μM, 15μM and 75μM. Despite the increasing level of embryonic malformations seen when the siRNA concentration was

increased we could not detect a reduction of the fluorescence in embryos at 24 hpf (Fig 15).

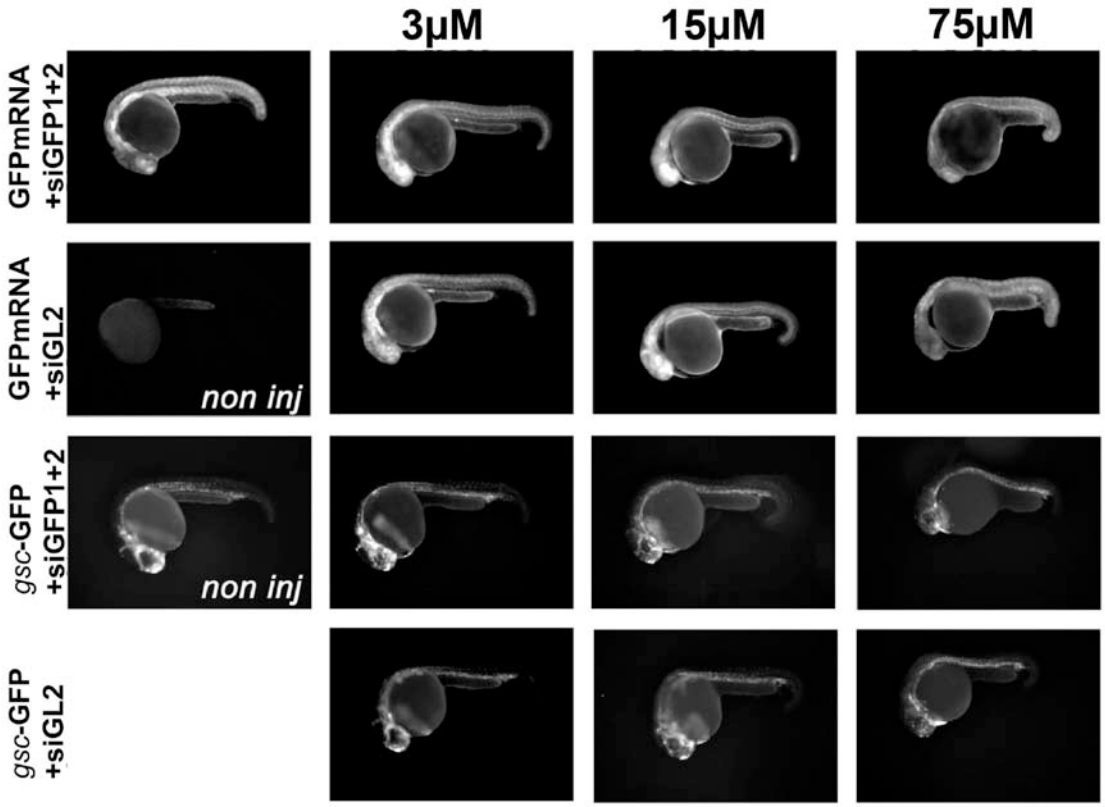

Fig. 15. The siGFP1 and 2 failed to silence GFP expression in zebrafish embryos.
Fluorescent images of embryos injected with a control siRNA or a mixture of siGFP-1 and -2 in increasing concentrations. The GFP oligos failed to silence the expression of GFP from co-injected GFP mRNA (first two rows) or *goosecoid*-GFP transgenic fish line (second two rows), despite the increasing levels of observed embryonic malformation.

To test whether genes transcribed in the embryos (as opposed to *in vitro* transcribed RNA) could be silenced, we injected the mix of siGFP-1 and siGFP-2 into transgenic zebrafish embryos expressing GFP under the control of *goosecoid* promoter (*gsc-GFP*). *gsc* gene exhibits two independent phases of expression: an early one in cells anterior to the presumptive notochord, and a later one in neural crest derivatives in the larval head (Schulte-Merker et al., 1994). GFP fluorescence is detected in the region of the notochord and the head of the 24hpf old embryos carrying the *gsc*-GFP transgene. Similarly to the previous experiment, a reduction of the fluorescence was not observed in embryos injected with different concentrations of mixed siGFP-1 and siGFP-2, compared to the control ones. These experiments demonstrated that using short interfering RNA we

could not specifically silence the expression of GFP neither from injected mRNA nor from such GFP expressed in the genome.

Analyzes of the siRNA pathway have shown that the siRNA might derive from a long double stranded RNA or from hairpin precursor, both of which are processed by the endonuclease Dicer and subsequently bind RISC complex (Fig. 3). One of the possibilities for the failure to induce GFP silencing using siRNA could be the inappropriate presentation of the oligos to the zebrafish RISC complex. To circumvent this potential problem one could synthesize the precursors of the siRNa, long double stranded RNA or hairpin RNA, and inject them in zebrafish embryos. However, previous experiments have demonstrated that injections of long double stranded RNA in zebrafish cause non-specific malformations in the injected embryos, probably due to induction of the interferon response (Oates et al., 2000; Zhao et al., 2001)). Thus, we have chosen to synthesize RNA hairpins, which would be processed by Dicer and produce siRNA targeting the desired sequence (See Materials and methods for sequence). Injections in 1-cell stage embryos of the hairpin RNA giving rise to siGFP1 (siGFP-6) in concentrations similar to those used in the siRNAs injections failed to silence the GFP expression (data not shown).

Another possible explanation for the negative results obtained using GFP was suggested by the observation in other organisms that some genes are more prone to silencing using siRNA, whereas others tend to be “difficult” to be targeted. In an effort to exclude such bios from our experiments, we have chosen to target other three genes, *floating head* (*flh*), *spade tail* (*spt*) and *sdf-1a*. Previous studies have shown that zebrafish floating head mutants lack a notochord, but develop prechordal plate and other mesodermal derivatives, indicating that *flh* functions specifically in notochord development. The *spadetail* gene is required for non-notochordal trunk mesoderm formation; *spadetail* mutant embryos have major trunk mesoderm deficiencies, but exhibit normal tail and notochord development (Griffin et al., 1998; Talbot et al., 1995). The chemokine SDF-1a is dynamically expressed in somatic cells providing guiding cues for migrating cells, such as germ cells and lateral line primordium (David et al., 2002; Doitsidou et al., 2002). In *sdf-1a* MO mediated knock down and mutant embryos the guided migrating cells fail to reach their final target and are found in ectopic positions. All three genes are zygotically expressed in a variety of tissues, lack maternal contribution and show a clear loss of funcction phenotype. Injection of 3 different hairpins targeting *spt*, 3 hairpins and one siRNA duplex targeting *flh*, 3 hairpins and 5 duplexes directed

against *sdf-1a* (See Materials and Methods for sequence), in low concentrations (5μM) did not cause any specific phenotype in the injected embryos, whereas higher concentrations produced non-specific malformations, similar to the ones observed in embryos injected with control oligonucleotides (Fig.14 and data not shown).

Whereas in mammalian cell lines a broad spectrum of genes have been targeted using siRNA (Reviewed in (Dykxhoorn et al., 2003)), it is possible that in the live zebrafish embryo only certain cells (or developmental stages) are prone to gene silencing by introduced oligonucleotides. As the germ cells exhibit extensive use of posttranscriptional regulation over gene expression (Reviewed in (Raz, 2003)), we set out to inhibit genes that are expressed within these cells. To this end we have made attempts to inhibit the expression of CXCR4b, a chemokine receptor whose activity is essential for directed PGC migration (Doitsidou et al., 2002; Knaut et al., 2003) and of Dead end, an RNA binding protein whose function is required to promote PGC motility and survival (Weidinger et al., 2003) . 7 siRNA duplexes and 2 hairpin precursors targeting these two genes were synthesized and injected in 1-cell stage embryos. Importantly, as in the experiments described before, no specific phenotype could be induced and general malformations appeared with the increase of the injected amounts of oligonucleotides (data not shown).

III. 3. 4. GFP silencing in zebrafish embryos can be achieved by siRNA and miRNA oligonucleotides when the target is within a specific UTR environment

As described above, using a variety of methods, we failed to induce specific gene silencing in zebrafish embryos. Nevertheless, observations by other labs of successful reduction of gene expression upon RNA oligonucleotides injection (Boonanuntanasarn et al., 2003; Dodd et al., 2004), the presence of Dicer and other components from the siRNA pathway in embryos (Wienholds et al., 2003) and detection of some miRNAs in zebrafish embryo lysates (Wienholds et al., 2003, Lim, 2003 #1666) suggest that the RNA interference machinery is presented and might play an unknown role in the development of zebrafish. Recent studies from Kloosterman et al. (Kloosterman et al., 2004) demonstrated successful translation repression of GFP expression from a construct containing 3'UTR from *lin41,* a gene that appears to be a natural target of *let-7* miRNA in *C. elegans* (Lee et al., 1993).

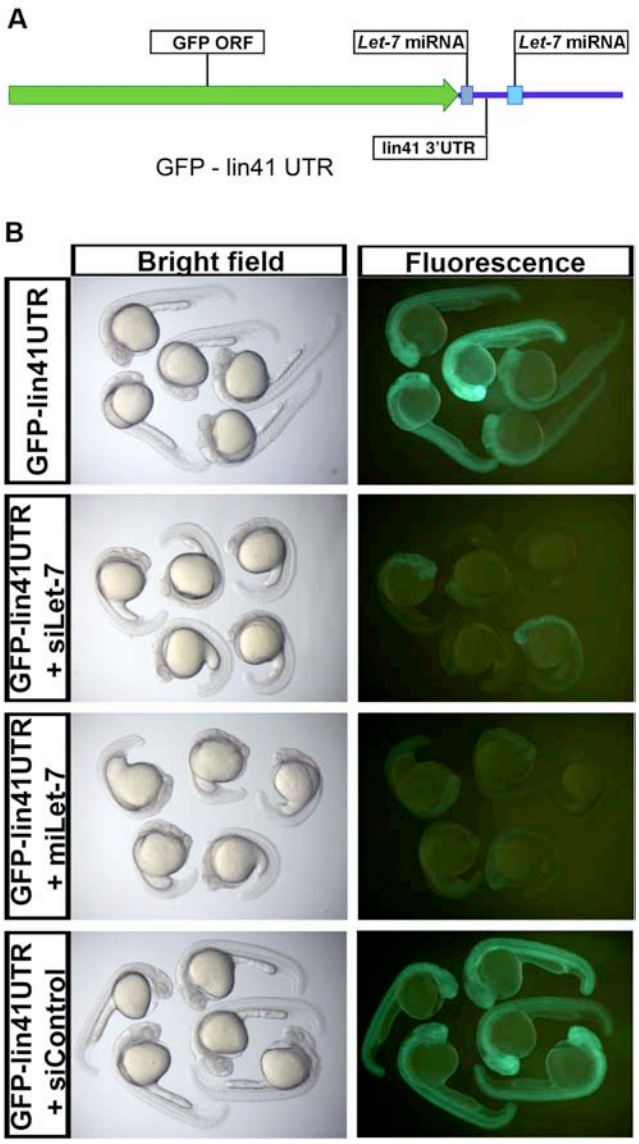

Fig. 16. Let-7 miRNA and siRNA are able to induce silencing of the GFP expression from a construct containing *lin41* UTR.

(A) Schematic representation of the mRNA construct containing GFP ORF upstream from the 3'UTR of *lin41* containing two binding sites for let-7 miRNA.

(B) Bright field and fluorescent pictures of 24hpf zebrafish embryos injected with 70ng/μl of mRNA from the construct depicted in A and 7.5μM of siLet-7, miLet-7 or control siRNA. Reduction of GFP fluorescence was detected in the cases where Let-7 oligos were provided. In addition, despite the same concentrations, these two oligonucleotides had stronger effect in inducing malformations in injected embryos, when compared with the control ones.

A construct provided by the lab of Prof Plasterk, was used to synthesize RNA encoding GFP that includes the 3'UTR from *lin41*with 2 putative binding sites for *let-7* miRNA (Fig. 16 A). This RNA was injected together with *let-7* miRNA and siRNA. Indeed, in this case GFP expression was silenced by the miRNA. In addition, we found that the *let-7* siRNA, fully complementary to the 5' *let-7* binding site within the UTR, was also causing significant reduction of the fluorescence measured at 24 hpf (Fig. 16 B).

This experiment demonstrated gene silencing induced by siRNA when its potential endogenous target mediates the regulation. To check if there is specific sequence requirement for *let-7* siRNA, the 5' *Let-7* miRNA binding site from the lin41 UTR was replaced by sequence homologous to our siSDF-1 (Fig. 17 A). Significantly, when the in-vitro transcribed mRNA from this construct was injected together with siSDF-1 we observed strong reduction of the GFP at 24 hpf (Fig. 17 B).

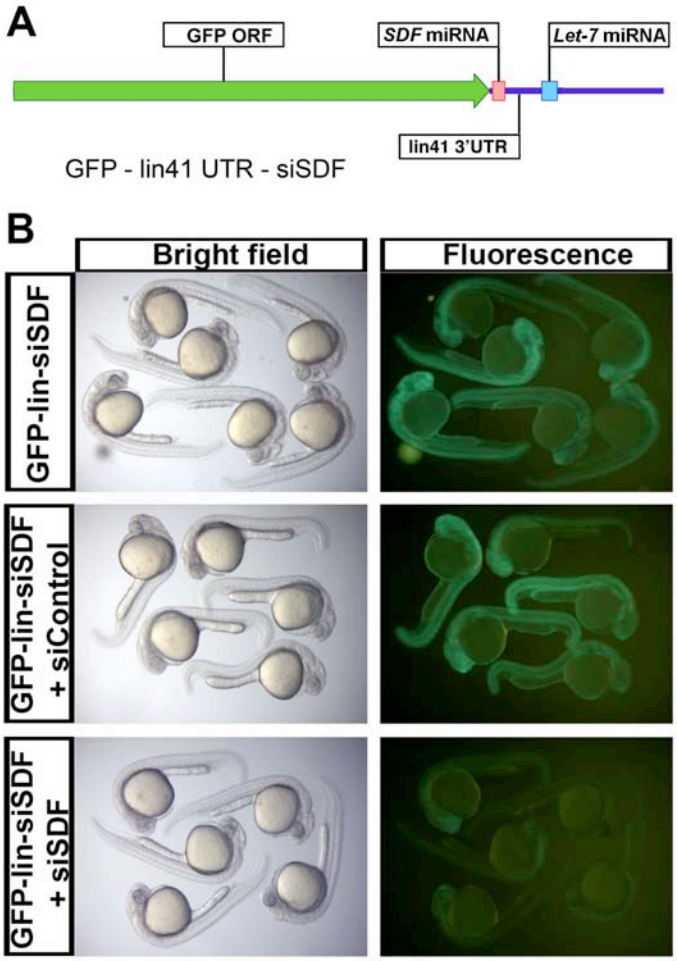

Fig. 17. SDF siRNA oligo induced silencing of the GFP expression from a construct containing modified *lin41* UTR with a binding site complementary to the siSDF sequence.

(A) Schematic representation of the mRNA construct containing GFP ORF upstream from the modified 3'UTR of *lin41* containing one SDF and one let-7 miRNA binding sites.

(B) Bright field and fluorescent pictures of 24hpf zebrafish embryos injected with 70ng/μl of the construct depicted in (A) and 7.5μM of siSDF or control siRNA. The SDF specific siRNA caused reduction of the level of GFP fluorescence in comparison to the one of the embryos injected with control oligos or mRNA only.

III. 3. 5. The GFP silencing is mediated by RNA degradation

As we have used siRNA, which is promoting degradation of the targeted mRNA, we performed *in-situ* hybridization with Dig-labelled anti-sense RNA probe to follow the fate of the injected GFP mRNA. The amount of RNA was judged by the intensity of the staining at 90% epiboly and 24 hpf (Fig. 18).

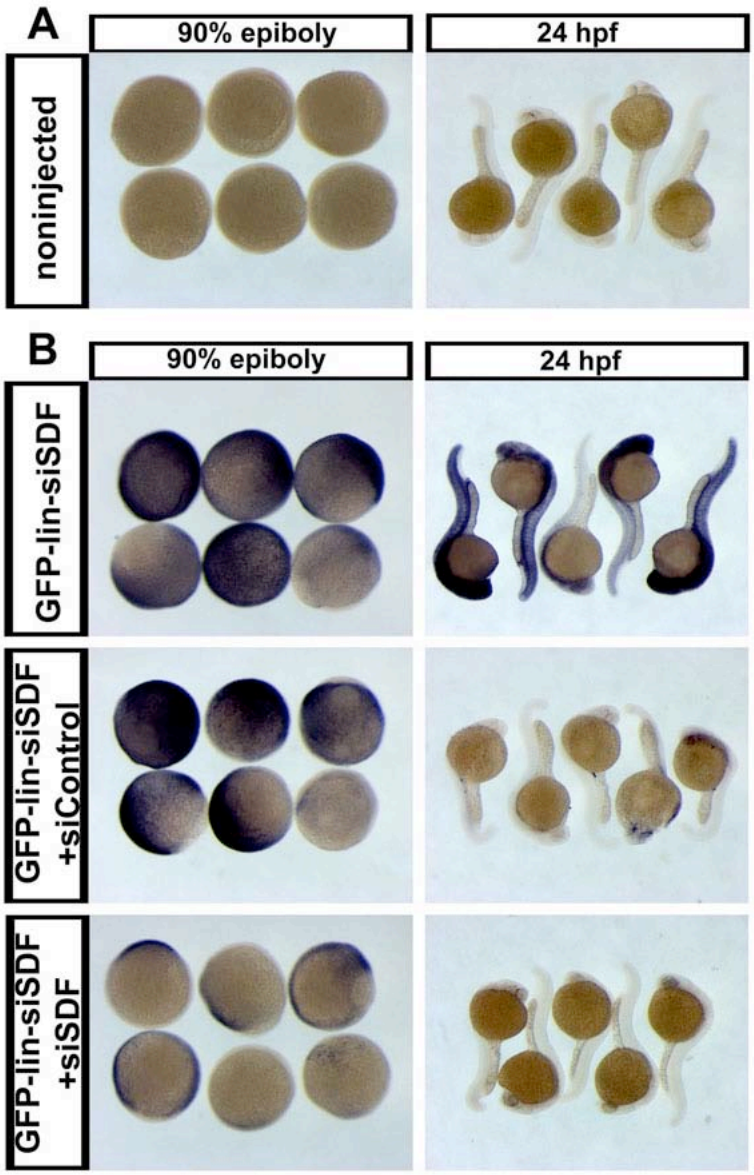

Fig. 18. Injections of miRNA and siRNA oligonicleotides targeting GFP-*lin41*'UTR constructs induce the degradation of the mRNA.

In situ staining of embryos injected with siSDF, siLet-7, miLet-7 and control siRNA oligonucleotides. The amount of the GFP-*lin41* constructs mRNA was detected using labelled anti-sense GFP RNA probe.

(A) Control embryos processed in the same manner as the experimental ones show no staining, confirming the specificity of the used probe. The levels of GFP-*lin41-SDF* mRNA were strongly reduced in the embryos injected with siSDF (B) compared with the one injected with the mRNA only.

As expected, embryos injected with siSDF exhibited lower staining intensity compared with controls already at 90% epiboly, suggesting that the injected mRNA was rapidly degraded in an siRNA dependent manner. Interestingly, injections of the control oligos also caused mRNA degradation, albeit only at latter stages.

IV. Discussion

IV. 1. Dynamics and function of germ plasm components

In the animal species in which PGC specification occurs by inheritance of a subset of molecules, the germ plasm is required to determine the germ cell fate. However, the mechanisms of action of the germ plasm remain unclear. Expression pattern studies and molecular function analysis of components of the germ plasm could help to address this issue. In chapter III.1 we describe generation of specific polyclonal antibodies for two proteins encoded by germ cell specific marker genes, *dead end* and *nanos1*. We show that the generated antibodies recognize specifically the endogenous proteins in whole mount antibody staining assays and Dnd and Nos co-localize in the germ cell granules after shield stage. There are three possible mechanisms leading to restriction of proteins specifically to the germ cells: degradation of maternally contributed protein in the somatic cells, mechanism suggested for some proteins in *C. elegans* and zebrafish (DeRenzo et al., 2003; Wolke et al., 2002); recruitment of maternal protein from the soma to germ cells, and de novo synthesis of protein from localized mRNA upon germ cell specification (Weidinger et al., 2003, Köprunner, 2001 #1092). Prior to shield stage we could not detect any enrichment of the studied proteins in particular cells or cell structures. Since the ubiquitous weak staining observed during the early stages might be due to low maternal protein presence or its absence, we could not draw any conclusions about how the localization was mediated. Further experiments, such as Western blot assays of Dnd and Nos proteins levels in lysates of different stages zebrafish embryos, might be used to detect if there is maternal contribution of these proteins and are they subjected to degradation during the embryonic development.

Previous studies of the germ cells in zebrafish revealed that at about 1000-cell stage the germ plasm undergoes a transition from a compact structure to more diffused one, which leads to a net increase in PGC number following cell division (reviewed in (Raz, 2003)). Moreover, EM studies of the germ plasm described the nuage forming discrete aggregates in the cells (Braat et al., 1999; Knaut et al., 2000). Therfore, as we could not detect any localization of the signal during the early stages of development, we hypothesize that the positions of Nanos1 and Dead end proteins (if present) differ from that of the nuage, as described for the zebrafish Vasa protein (Knaut et al., 2000).

As the development proceeds, the studied proteins accumulate in the germ plasm granules, which in *C. elegans* have been shown to associate with the nuclear pores of the

germ cells (Pitt et al., 2000). However, similar relations between the zebrafish germ cell nucleus and Vasa positive granules were not observed during studies of 30 hpf old embryos (Knaut et al., 2000). Our experiments, in contrast, show that at 90% epiboly a clear correlation between the positions of the germ plasm granules, stained for Vasa, and the majority of the nuclear pores does exist. Once established, this co-localization persists during the development and is still present at 24 hpf, the latest developmental stage examined. Our findings reinforce the hypothesis that zebrafish germ plasm granules are forming a complex with the nuclear pores and might be involved in mRNA packaging, processing and export from the nucleus in a germ cell specific manner. Interestingly, such level of regulation does not appear required during the early stages of the development, given that prior to 50% epiboly only a limited number of nuclear pores are associated with the granules. Alternatively, it is possible that the number of granules associated with the nuclear pores represents a requirement for the amount of material from the granules that functions at this developmental stage. Thus, an attractive hypothesis is that during the stages between 50% epiboly and 24 hpf, the maintenance of the most important germ cells properties, such as suppressing the differentiation and retaining active migration towards attractant, requires large amount of germ cell granule molecules in contact with the nuclear pore complexes. Therefore, the majority of the pores appear associated with the granules at those stages. Interestingly, this period of the development coincides with the most active migration of the germ cells, and knocking down of some perinuclear granules components, e.g. *nanos1* or *dead end*, leads to migration defects in the PGCs and their subsequent death, but does not disrupt the PGC formation (Weidinger et al., 2003, Köprunner, 2001 #1092). However, inhibition of the translation of other components, such as *vasa*, appears to be non-crucial for PGC development (Braat et al., 2001).

Finally, the preliminary results from our last experiment strongly support the idea that germ plasm components are involved in the export of RNA molecules from the nucleus. Here, we describe a screen for amino acids crucial for Dead end function. Summarizing the information obtained from the screen we were able to demonstrate that the RNP domain is indispensable for the protein function and mutations within this region often lead to a complete inactivation of the protein. Furthermore, we demonstrated that disruptions in this domain could alter the localization of Dnd from perinuclear to nuclear. This important finding gives a hint for the role of one of the components of the

germ cell granules, suggesting that upon its synthesis, Dnd is imported in the nucleus where it binds RNA and is again exported to the granules. In this process the RNA binding domain appears crucial for the export of the protein from the nucleus. Despite the absence of Dnd, the germ cell granules appear to form normally, judged by the localization of Vasa protein. The identity of the RNA target or targets of Dnd remains to be determined. Future experiments such as immuno-precipitation assays might help to address these questions and consequently shed light on the important issue for the role of the germ plasm in maintenance the germ cell fate.

IV. 2. Sex differentiation in zebrafish

In this study we have examined the role of the germline in the development of somatic tissues in zebrafish. We have utilized two independent methods to ablate the germ cells and both resulted in the generation of sterile adults. Remarkably, all these sterile adults developed as males as judged by morphological and behavioural criteria. We therefore conclude that the germline is essential for the development of female zebrafish, but is dispensable for the development of male somatic tissues with the exception of the gonad.

Targeted cell ablation is commonly used as a tool for studying the role of a particular cell line in the multi-cellular context of the entire organism. Genetic methods for expressing toxic molecules under the control of tissue specific promoters allow high specificity of cell targeting (Arase et al., 1999; Roman et al., 2001). Nevertheless, in many cases, as shown in our experiments with DT, even minute amounts of the toxin presented outside of the targeted cells can compromise the viability of the organism. Obstacles of a similar nature often prevent the use of targeted cell ablation for medical purposes such as in cancer treatment. Our results clearly show that toxin-antitoxin pairs such as the prokaryotic Kid and Kis proteins can be successfully used to refine the specificity of the toxic action to a well-defined cell population, the PGCs in this case. These findings demonstrate for the first time that these proteins can be applied for highly specific ablation of targeted eukaryotic cells, and lend support to the general idea that they could become invaluable tools in developmental studies and anti-cancer therapies (de la Cueva-Méndez et al., 2003).

Sex determination in zebrafish is affected by environmental factors and exogenous hormonal treatments (Baroiller and D'Cotta, 2001)). In addition, removal of

the germ cells acts as a sexual modulator promoting male development. It could be hypothesized that environmental cues and lack of germ cells operate through a common mechanism namely, the endocrine system. Thus, the cross talk between the germline, somatic gonadal tissue and additional somatic tissues that are relevant for sex differentiation could be influenced by environmental factors.

Molecules that are likely to participate in such signaling are sex-determining hormones such as testosterone or estrogen (Baroiller and D'Cotta, 2001)). A key enzyme controlling the relative levels of sex hormones, cytochrome P450, catalyzes the transition of testosterone into estrogen (Callard et al., 2001). Inhibition of the biochemical activity of cytochrome P450 using a non-steroidal aromatase inhibitor, fadrozole, promotes male development conceivably due to its effect on the hormonal balance of the organism (Chiang et al., 2001; Fenske and Segner, 2004; Uchida et al., 2004). Consistent with the critical role of the ovary in zebrafish feminization, this enzyme is expressed at higher levels in the ovary than in the testes (Chiang et al., 2001). Furthermore, the observed elevated expression in the follicles surrounding maturing oocytes (Chiang et al., 2001) hints to a potential crosstalk between the germ cells, particularly the oocytes, and this key regulator of the hormonal balance in zebrafish. In our experiments the absence of germline cells resulted in lack of gonadal tissue, presumably decreasing the conversion of testosterone into estrogen. Consistent with this suggestion, treating fish that lack the gonad with estrogen resulted in the production of adult fish exhibiting female characteristics. Thus, once female gonadal tissue is determined through primary signals (currently unknown), this tissue could be responsible for specifying and maintaining the feminized fate of somatic tissue by converting testosterone into estrogen. Whereas the germline plays an essential role in female somatic development, it is dispensable for the male development and behaviour. Therefore, with respect to the role of the germline, male development represents the “default state” which is then modified in female fish. Apart from its role in female development, irrespective of the gender, zebrafish germ cells are crucial for the survival of the gonads, but not for their formation. The integrity of the somatic tissue in the gonad of the fish is therefore dependent on the germline. This is different from mammals where in the absence of germ cells the gonads are present albeit in a smaller size (Beck et al., 1998).

Finally, our findings are of practical importance for fish ecology. First, chemicals in the environment affecting germ cell development and migration in fish (e.g., (Thorpe et al., 2004)) constitute a severe threat for the fish populations. The alteration of the sex

ratio amplified by the fact that sterile males are able to mate with wild type females thereby producing unfertilized eggs, would lead to a rapid decrease in population size. Second, when required, the *kid/kis* method for ablating the germline could be utilized for generating genetically modified fish whose fertility could be controlled by induction of Kid toxicity. Such a system could be valuable in preventing the contamination of wild-type fish populations with genetically modified fish, which represents a major concern hampering the use of genetically modified fish in the aquaculture (Stokstad, 2002).

IV. 3. RNA interference

From the time of their specification as a separate cell population in the developing embryo, the germ cells are subjected to tight regulation of the gene expression, maintaining their specific properties while suppressing expression of genes characteristically expressed in somatic cells. A tempting option for controlling gene expression in PGCs is the recently discovered RNA interference pathway. Indeed, a number of experiments in *C. elegans, Drosophila* and mammalian cell cultures have demonstrated that it is possible to target almost any gene by delivering long or short double stranded RNA oligonucleotides into the cells (Dykxhoorn et al., 2003; Huppi et al., 2005; Soutschek et al., 2004). However, despite the presence of some key players in the RNA interference pathway, such as Dicer and proteins from the RISC complex, the existence of such regulatory mechanism in zebrafish remained questionable, with a number of conflicting experimental evidences arguing for and against it. In this work we assayed for RNA interference in zebrafish somatic and germ cells by designing and injecting a number of 21 bps oligonucleotides as well as their hairpin precursors. We targeted the coding sequence of a reporter gene, GFP, three genes widely expressed in the soma, *spt*, *flh* and *sdf1a*, and two genes specific for the germ cells, *cxcR4b*, and *dead end*. In none of the cases were we able to detect the phenotype characteristic for the disruptions in the targeted gene, but rather observed non-specific embryonic malformations correlated with the increase of the injected amount of oligonucleotides. Finally, we were able to induce RNA silencing upon injection of one of the siSDF oligos by inserting its target site sequence in the context of the *lin-41* UTR in a GFP reporter construct.

Our data suggest that targeting of zebrafish genes using oligonucleotides complementary to the coding sequence of a desired gene without taking neighbouring

RNA sequences into consideration is not likely to yield effective inhibition. This differs from other model organisms and cells in culture, where such experiments are becoming routine (Reviewed in (Dykxhoorn et al., 2003; Soutschek et al., 2004). Moreover, oligos demonstrating good RNAi efficiency in cell cultures failed to induce similar effect when their activity was assayed in the embryo. Currently we do not have a straightforward explanation for the observed differences observed between the zebrafish cell lines and embryos. Nevertheless, several possible explanations can be suggested. First, the immortal cultured cells perform RNA interference, a feature that is lost in the cells of the embryo as their differentiation proceeds. Second, the oligos are efficiently delivered in the cytoplasm of the first embryonic cell upon injection in the yolk (data not shown), but they are efficiently degraded in embryonic cells as compared to their relative stability in cell culture, which allows efficient RNAi. Last, whereas the treated zebrafish culture cells were viable and appeared normal, we did not exclude the option that apart from the targeted gene other genes were inhibited as well. Such a phenomenon occurring in embryonic cells would lead to embryonic death and an apparent lack of specific inhibition.

Nevertheless, the fact that RNA interference mediated by siRNA or miRNA could be achieved in zebrafish embryos ((Kloosterman et al., 2004) and our own results) argues that this mechanism does operate *in vivo*. Thus, it could be proposed that the most important requirement for successful RNA interference in zebrafish embryos is for the targeted sequence to be presented in the appropriate context. Therefore, sites that are not natural targets could be used for gene silencing in cell cultures, whereas in the developing embryo, due to more complete regulation, these sites are not subjected to RNAi. To date very few naturally occurring sites for micro RNAs have been identified, predominantly located in the 3'UTR of the regulated genes (Brennecke et al., 2005). Klosterman et al demonstrated GFP silencing by targeting the coding sequence of this gene in which two *let-7* miRNA binding sites with a 5-bps spacer were introduced (Kloosterman et al., 2004). However, such construct could still be satisfying the requirements for appropriate environment, generated by the insertion of the exogenous sequence. Yet, a reliable study of the non-targeted genes expression was not presented, leaving the opportunity of general down-regulation of the gene expression, caused by the ubiquitous presence of the *let-7* miRNA and manifested in embryonic malformations.

In conclusion, increasing number of evidences suggest that RNA interference is an

important regulator of the gene expression conserved from plants to mammals. Practically, this feature have been utilized for knocking down specific genes in cell cultures by delivering siRNAs or miRNAs. However, the two sides of the processes, miRNA translational inhibition and siRNA mediated mRNA degradation, are demonstrating different potential for gene silencing in zebrafish. miRNA oligos could be used for targeting specific sequence at strictly defined positions with a not well determined sequence requirements. siRNAs produce occasional, gene dependent, reduction of the mRNA levels. Yet, the limited knowledge regarding successful prediction of miRNA-binding sites makes the use of this technology in zebrafish more challenging especially when compared to the well-established technology of using morpholino antisense oligonucleotides for inhibiting mRNA translation. As we failed to demonstrate RNAi of endogenous genes both in somatic and in germ cells, we could not draw any conclusion regarding the possibility that RNAi regulates gene expression in either one of these two cell populations.

V. Summary

Primordial germ cells (PGCs) in zebrafish are specified early in the development by inheritance of a specific type of cytoplasm termed germ plasm. The asymmetrically distributed germ plasm contains factors that direct cells to assume the fate of a germ cell. RNAs of several genes, such as *vasa, nanos1* and *dead end* are enriched in the germ plasm and together with the encoded proteins are expressed in the germline.

The first chapter of this thesis concerns the protein localization of Dead end and Nanos1 during different developmental stages. To address this question we generated polyclonal antibodies against the two proteins. Both antibodies co-localize to the perinuclear granules, a germ cell specific organelle with unknown function in which proteins encoded by the components of the germ plasm are concentrated. Further characterization of the perinuclear granules show that they are positioned adjacent to the nuclear pores. We hypothesize that some of the components of the perinuclear granules might be involved in posttranscriptional regulation of gene expression such as mRNA protection, transport and/or degradation. In accordance with this hypothesis loss of function point mutation in *dead end* altered its protein localization from the perinuclear granules to the nucleus.

Chapter two of the thesis addresses the role of Dead end in sex determination. Embryos injected with a Morpholino that blocks the translation of *dnd* loose the germ cells. Furthermore, these embryos develop exclusively into sterile male adults. This suggests that sex determination in zebrafish may either involve Dead end function or depend on the presence of germ cells. To discriminate between these two possibilities an independent method for germ cell ablation was applied. The method was based on bi-cistronic toxin-antidote system, which allowed expression of the toxin in the germ cells while protecting the somatic cells with an antidote. Germ-cell-less embryos generated by this “dead end independent” method also exhibited a male biased sex ratio, showing that germ cells are important for sex determination of zebrafish.

The last chapter deals with the role of RNA interference in germ cell development in zebrafish. RNA interference is a widely used method for gene silencing in many organisms. In zebrafish however, only partial and controversial data regarding the function of RNA interference exists. As gene silencing is a common theme in germline development of many organisms, we investigated if small interfering RNAs and micro

RNAs function in *Danio rerio* are important for germline specification and development. Our results demonstrate that the two sides of the processes, miRNA translational inhibition and siRNA mediated mRNA degradation, exhibit different potential for gene silencing in zebrafish. miRNA oligos could be used for targeting specific sequence at strictly defined positions with a not well determined sequence requirements. siRNAs produce occasional, gene dependent, reduction of the mRNA levels. Yet, the limited knowledge regarding successful prediction of miRNA-binding sites makes the use of this technology in zebrafish more challenging especially when compared to the well-established technology of using morpholino antisense oligonucleotides for inhibiting mRNA translation. As we failed to demonstrate RNAi of endogenous genes both in somatic and in germ cells, we could not draw any conclusion regarding the possibility that RNAi regulates gene expression in either one of these two cell populations.

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VIII. Appendix

Tables and Figures

Tables

Figures

Appendix

Erklärung

Hiermit erkläre ich, dass die Resultate meiner Dissertation mit dem Titel “Molecular mechanisms governing germ line development in zebrafish and the role of this lineage in sexual differentiation” von mir selbst angefertigt wurden, mit Ausnahmen von den Resultaten der erwähnten Zusammenarbeiten.

List of publications

Slanchev K, Stebler J, de la Cueva-Méndez G, Raz E.
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Lebenslauf

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Januar 2002	Beginn der experimentellen Arbeit zur vorliegenden Dissertation