

# **Germ Cell Development and Migration**

**Dissertation**

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**by  
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**In thankful honor to my parents  
Heidi and Alois Stebler**

# Table of Contents

<b>TABLE OF CONTENTS</b>	<b>III</b>
<b>ABBREVIATIONS</b>	<b>VI</b>
<b>1. INTRODUCTION</b>	<b>1</b>
1.1 BIOLOGY OF THE DEVELOPMENT	1
1.2 GERM CELLS	2
1.3 GERM CELL SPECIFICATION AND MIGRATION IN MOUSE	3
1.4 GERM CELL SPECIFICATION AND MIGRATION IN CHICK	6
1.5 GERM CELL SPECIFICATION AND MIGRATION IN ZEBRAFISH	7
1.6 IDENTIFICATION OF GENES EXPRESSED IN PGCs OR FUNCTION IN GERM CELL MIGRATION	10
1.7 AIM OF THIS THESIS	12
<b>2. RESULTS</b>	<b>13</b>
<b>2.1 THE ROLE OF THE CHEMOKINE SDF-1 IN AVIAN AND MURINE GERM CELL MIGRATION</b>	<b>13</b>
2.1.1 IDENTIFICATION OF CHICKEN SDF-1	13
2.1.2 CHICK SDF-1 IS EXPRESSED IN POSITIONS WHERE PGCs ARE FOUND DURING THE LAST STAGES OF THEIR MIGRATION	15
2.1.3 PGCs CAN BE ATTRACTED TOWARDS A SOURCE OF SDF-1 $\alpha$	18
2.1.4 MOUSE PGCs TRANSMIGRATE THROUGH THE HINDGUT EPITHELIA EXCLUSIVELY IN THE REGION OF SDF-1 EXPRESSION	20
<b>2.2 DEAD END IS REQUIRED FOR PRIMORDIAL GERM CELL MIGRATION AND SURVIVAL</b>	<b>24</b>
2.2.1 DEAD END IS A NOVEL PUTATIVE RNA-BINDING PROTEIN, WHICH IS LOCALIZED TO PERINUCLEAR GERM GRANULES	24
2.2.2 DEAD END OVEREXPRESSION IN PGCs AND SOMATIC TISSUE DOES NOT ALTER GERM CELL SPECIFICATION AND NUMBER	26
2.2.3 DEAD END IS REQUIRED FOR PGC MIGRATION	27
2.2.4 ZEBRAFISH <i>DEAD END</i> IS REQUIRED FOR PGC SURVIVAL	29
2.2.5 <i>DEAD END</i> EXPRESSION IS CONSERVED IN OTHER VERTEBRATE SPECIES	31
2.2.6 IDENTIFICATION OF DEAD END INTERACTION PARTNERS	32

## Table of Contents

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<b>3. DISCUSSION</b>	<b>36</b>
<b>3.1 GERM CELL MIGRATION</b>	<b>36</b>
<b>3.2 THE ROLE OF THE CHEMOKINE SDF-1 IN AVIAN GERM CELL MIGRATION</b>	<b>36</b>
<b>3.3 THE ROLE OF THE CHEMOKINE SDF-1 IN MURINE GERM CELL MIGRATION</b>	<b>37</b>
<b>3.4 CONSERVATION OF THE MECHANISM OF PGC GUIDANCE</b>	<b>38</b>
<b>3.5 BASIS AND RELEVANCE TO SCREEN FOR GERM PLASM COMPONENTS: THE IDENTIFICATION OF <i>DEAD END</i></b>	<b>39</b>
<b>3.6 <i>DEAD END</i>, A GERM PLASM COMPONENT IS ASSOCIATED WITH PERINUCLEAR GRANULES</b>	<b>40</b>
<b>3.7 DEAD END AND GERM CELL SPECIFICATION</b>	<b>40</b>
<b>3.8 DEAD END IS IMPORTANT FOR PGC MOTILITY</b>	<b>41</b>
<b>3.9 DEAD END IS ESSENTIAL FOR PGC SURVIVAL</b>	<b>42</b>
<b>3.10 DEAD END, PGCs AND ZEBRAFISH SEX DETERMINATION</b>	<b>43</b>
<b>4. SUMMARY &amp; CONCLUSIONS</b>	<b>44</b>
<b>5. MATERIAL AND METHODS</b>	<b>46</b>
<b>5.1 BACTERIA</b>	<b>46</b>
<b>5.2 CHEMICALS</b>	<b>46</b>
<b>5.3 KITS</b>	<b>46</b>
<b>5.4 PRIMARY AND SECONDARY ANTIBODIES</b>	<b>47</b>
<b>5.5 DNA CONSTRUCTS USED IN THIS WORK</b>	<b>47</b>
<b>5.6 EQUIPMENT</b>	<b>49</b>
<b>5.7 PROGRAMS, DATABASE,</b>	<b>49</b>
<b>5.8 MOLECULAR BIOLOGY</b>	<b>50</b>
5.8.1 PLASMID DNA ISOLATION FROM <i>E. COLI</i>	50
5.8.2 TOTAL RNA ISOLATION FROM EUKARYOTIC CELLS OR EMBRYOS	51
5.8.3 DNA AND RNA ELECTROPHORESIS AND PURIFICATION FROM AGAROSE GEL	51
5.8.4 DNA DIGESTION WITH RESTRICTION ENZYMES	51
5.8.5 DEPHOSPHORYLATING AND BLUNTING OF DNA FRAGMENT	51
5.8.6 LIGATION	52
5.8.7 STANDARD PCRS	52
5.8.8 HIGH FIDELITY PCRS	52
5.8.9 AMPLIFICATION OF 5' AND 3' END OF cDNAs	52
5.8.10 STANDARD REVERSE TRANSCRIPTION	53
5.8.11 PGC SPECIFIC FULL LENGTH cDNA LIBRARY SYNTHESIS	53
5.8.12 YEAST-TWO-HYBRID SCREEN	55
5.8.13 PREPARATION OF ELECTROCOMPETENT <i>E. COLI</i> CELLS AND TRANSFORMATION BY ELECTROPORATION	55
<b>5.9 BIOCHEMISTRY</b>	<b>56</b>
5.9.1 IMMUNOPRECIPITATION (IP)	56
5.9.2 PROTEIN GEL ELECTROPHORESIS	58
5.9.3 COOMASSIE BLUE STAINING	59

5.9.4	SILVER STAINING FOR MASS SPECTROMETRY	59
5.9.5	WESTERN BLOTTING	60
<b>5.10</b>	<b>CELL BIOLOGY</b>	<b>62</b>
5.10.1	CELL CULTURE MEDIUM	62
5.10.2	CELL REVIVAL	62
5.10.3	CELL PASSAGE AND FREEZE	62
5.10.4	CELL TRANSFECTIONS WITH PLASMIDS	63
<b>5.11</b>	<b>ZEBRAFISH</b>	<b>64</b>
5.11.1	FISH BREEDING AND INCUBATION	64
5.11.2	MORPHOLINOS	65
5.11.3	LINEARIZATION OF PLASMID FOR <i>IN VITRO</i> TRANSCRIPTION	65
5.11.4	MRNA SYNTHESIS FOR INJECTION	65
5.11.5	INJECTION OF ZEBRAFISH EMBRYOS	66
5.11.6	IMMUNOSTAINING OF ZEBRAFISH EMBRYOS	66
5.11.7	DIG- AND FLUO- LABELED RNA PROBE SYNTHESIS	67
5.11.8	ZEBRAFISH ONE- AND TWO-COLOUR WHOLE MOUNT <i>IN SITU</i> HYBRIDIZATION	67
5.11.9	MICROSCOPY AND TIME-LAPSE ANALYSIS	73
<b>5.12</b>	<b>CHICKEN</b>	<b>73</b>
5.12.1	CULTIVATION OF CHICKEN EMBRYOS	73
5.12.2	EARLY CHICK (EC)-CULTURE	73
5.12.3	IMPLANTATION OF COS 7 CELLS INTO CHICK EMBRYOS	76
5.12.4	ELECTROPORATION	76
5.12.5	CHICK WHOLE-MOUNT <i>IN SITU</i> HYBRIDIZATION	77
5.12.6	HISTOLOGY – PREPARATION OF PARAFFIN SECTIONS	80
<b>5.13</b>	<b>MOUSE</b>	<b>81</b>
5.13.1	MOUSE WHOLE-MOUNT <i>IN SITU</i> HYBRIDIZATION	81
5.13.2	MOUSE ORGAN CULTURE AND TIME LAPSE	85
<b>6.</b>	<b>REFERENCES</b>	<b>86</b>
<b>7.</b>	<b>ACKNOWLEDGMENTS</b>	<b>97</b>
<b>8.</b>	<b>APPENDIX</b>	<b>98</b>
<b>8.1</b>	<b>AFFIDAVIT</b>	<b>98</b>
<b>8.2</b>	<b>LIST OF PUBLICATIONS</b>	<b>99</b>
<b>8.3</b>	<b>CURRICULUM VITAE</b>	<b>100</b>

# Abbreviations

Amp	Ampicillin
Dig	Digoxigenin
<i>dnd</i>	<i>dead end</i>
dpf	day post fertilization
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetra actetic acid
EST	Expressed sequence tag
FCS	Fetal calf serum
Fluo	Fluorecein
g	gravity
GFP	Green fluorescence protein
<i>glo</i>	<i>globin</i>
HH	staging according to Hamburger and Hamilton (Hamburger and Hamilton, 1951)
	hours post fertilization
Hpf	horse radish peroxidase
HRP	Immunoblot
IB	Immunoglobulin G
IgG	Immunoprecipitation
IP	kilo Dalton
kDA	minutes
min	milliliter
ml	Morpholino antisense oligonucleotide
MO	<i>nanos-1</i>
<i>nos1</i>	overexpression
<i>o/e</i>	open reading frame
ORF	Poly acrylamide gel electrophoresis
PAGE	Phosphate buffered saline
PBS	Polymerase chain reaction
PCR	primordial germ cell
PGC	Phenylmethylsulfonyl fluoride
PMSF	rotations per minute
rpm	Room temperature
RT	Stromal cell-derived factor 1
SDF-1	Sodium dodecyl sulfate
SDS	seconds
sec	units
U	Untranslated region
UTR	Volt
V	volume
v	Western blot
WB	

# 1. Introduction

## 1.1 Biology of the Development

During the course of development, a single cell, the zygote, will after numerous divisions ultimately give rise to a complex organism consisting of various different cell types. These cells, first regionally specified by asymmetric distribution of cytoplasmic determinants, by inductive signals and/or cell autonomous processes organize into functionally diverse tissues and organs of the body. A central aim in developmental biology is to understand the molecular processes that enable a single cell (the zygote) to generate multicellular and highly organized tissues, organs and organisms. The numerous aspects of this discipline include the formation of an organism as well as the investigation of aberrant cell behavior that can cause diseases. A multitude of questions have to be solved to understand these complex processes, such as: How are the different cells specified to give rise to a certain cell type? How cells communicate with neighboring cells to organize complex structures e.g. an embryo or a partially formed organ? What are the reasons that some cells divide more than others? What are the molecular mechanisms that allows only a part of the genome to be used and how is it differentially regulated in the thousands of different cell types of higher organisms?

The multidisciplinary field of developmental biology, combining among other fields genetics, anatomy, embryology, molecular- and cell biology, contributed to the growing understanding of the mechanism responsible for the formation of a multicellular organism. However, the present understanding is in most processes still basic and many questions remain unanswered.

## 1.2 Germ Cells

Continuation of the germ cell lineage guarantees transmission of genetic information from one generation to the next by sexual reproduction, the fusion of gametes to create a zygote. All sexually reproducing organisms arise from gametes (sperm and egg), which develop from primordial germ cells (PGCs), a small population of cells that set aside from other cell lineages early in embryonic life in most animal species. Differentiation of the germ cells into gametes occurs in the gonad, an organ comprised of the germ cells and somatic tissue, which supports and directs proper gamete differentiation. During the process of differentiation, which includes meiosis and DNA recombination, germ cells generate unique gametes and consequently unique individuals (Niewkoop and Sutasurya, 1979; Starz-Gaiano and Lehmann, 2001; Wylie, 1999; Zhao and Garbers, 2002).

Primordial germ cell specification marks the initiation of the life cycle of the germ cell lineage. The basic mechanism of PGCs being set aside as a distinct cell population early during embryogenesis is common throughout the phylogeny from invertebrates to mammals (Houston and King, 2000a; Ikenishi, 1998; McLaren, 1999; Seydoux and Schedl, 2001; Starz-Gaiano and Lehmann, 2001; Wylie, 1999). However, the molecular mechanisms responsible for PGC specification differ in different organisms. In many species, including *Xenopus*, zebrafish, *Drosophila* and *C.elegans*, germ cells are specified by the inheritance of maternal factors deposited in the egg during oogenesis that contain germ cell determinants, the germ plasm (Houston and King, 2000a; Houston and King, 2000b; Lehmann and Nusslein-Volhard, 1991; Seydoux and Schedl, 2001; Yoon et al., 1997). In contrast to these organisms, PGCs in mammals and urodeles segregate from the somatic cell lineage by inductive signals from tissues at an early gastrulation stage (Lawson et al., 1999; Ying et al., 2001).

In many species the germ cells are specified in regions distinct from the site where the gonad will form. Therefore, PGCs have to migrate from their site of specification towards the region where the gonad develops. The mechanisms allowing directed germ cell migration in *Drosophila*, *Xenopus*, zebrafish and mouse have been studied intensively over the last decades (reviewed in (Molyneaux and Wylie, 2004; Raz, 2004; Santos and Lehmann, 2004; Starz-Gaiano and Lehmann, 2001; Wylie, 2000). The general conclusion from these studies is that the movements of the PGCs towards the gonadal region rely on directional cues provided by the somatic environment. The precise molecular mechanisms responsible for this feature of the germ cells were and still are, however, largely not known.



### 1.3 Germ Cell Specification and Migration in Mouse

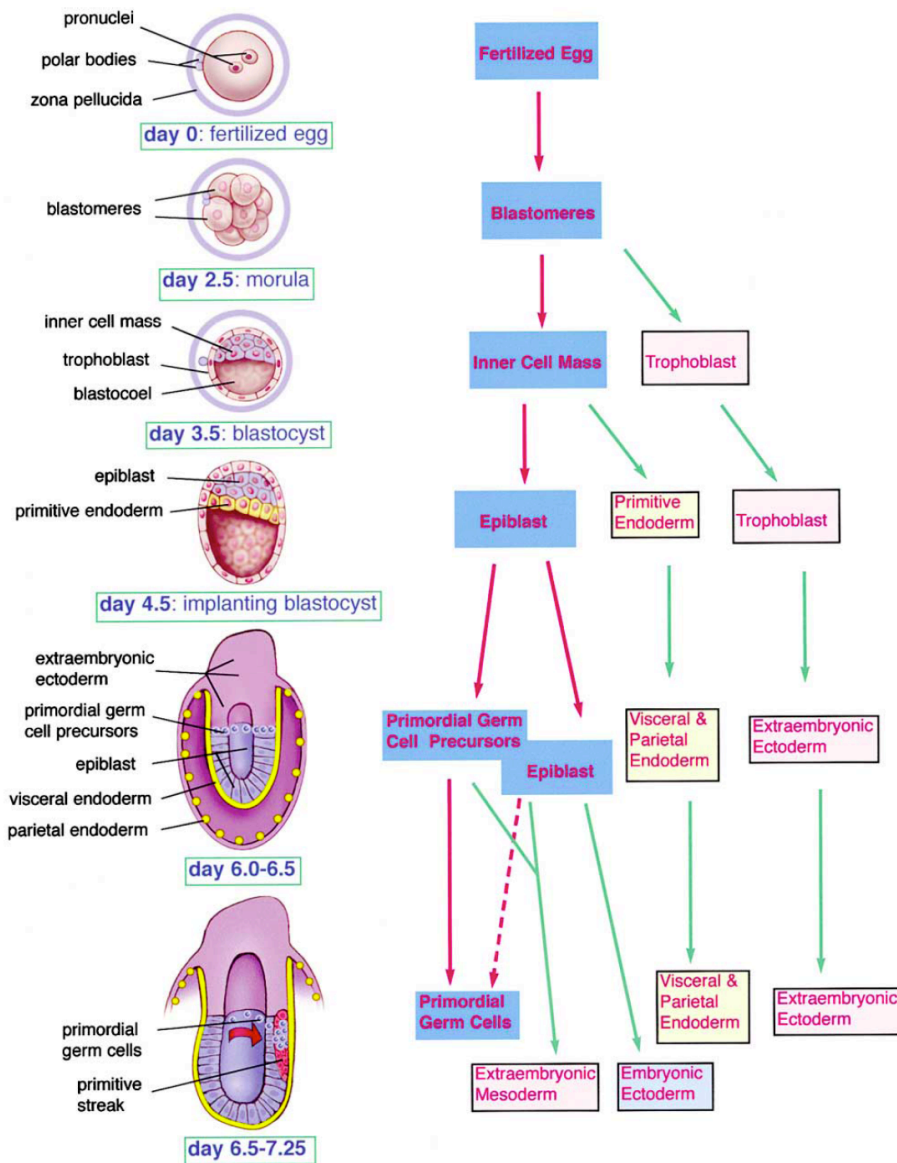
In early mammalian embryogenesis, the newly formed zygote divides three times to give rise to eight cells with each equal totipotency. At the 16-cell stage, cellular differentiation occurs, whereas the cells at the center remain pluripotent and give rise to the inner cell mass (ICM), while cells at the periphery become the first differentiated embryonic cell types, called the trophoectoderm or trophoblast (Beddington and Robertson, 1999). At the 32-cell stage, the trophoectoderm forms the wall of a sphere and the ICM is enclosed in one hemisphere, while a second inner cavity, the blastocoel, is formed. Subsequently, cells on the surface of the ICM, facing the blastocoel, differentiate into primitive endoderm that forms visceral and parietal endoderm, while the rest of the ICM remains pluripotent and becomes primitive ectoderm or epiblast. Eventually, epiblast cells of the early gastrula give rise to primordial germ cell precursor at a stage of E5.5-E6.5 (de Sousa Lopes et al., 2004; Lawson et al., 1999; Tam and Zhou, 1996; Ying et al., 2001) (Figure 1-1).

Transplantation experiments of different tissues of the mouse embryo led to the conclusion that signals produced by the extraembryonic ectoderm induce epiblast cells to become PGC precursors, which then eventually migrate toward the primitive streak and segregate into PGC and allantois lineages (Ginsburg et al., 1990; Starz-Gaiano and Lehmann, 2001; Tam and Zhou, 1996),

The signal transduction cascade activated by bone morphogenetic protein 4 (BMP4), the type I BMP receptor ALK2 and the downstream Smad1 and Smad5 were demonstrated to be necessary for the generation of PGCs (Chang and Matzuk, 2001; de Sousa Lopes et al., 2004; Hayashi et al., 2002; Lawson et al., 1999; Tremblay et al., 2001). In addition, the BMP family members BMP8b and BMP2 have been shown to play a role in the formation of PGCs as well (Ying et al., 2000; Ying et al., 2001; Ying and Zhao, 2001). Recent findings showed that the zinc-finger containing DNA-binding transcriptional repressor Blimp1 is essential for specification of PGCs (Vincent et al., 2005), whereas the gene targets important for the germ cell specification process are currently unknown.

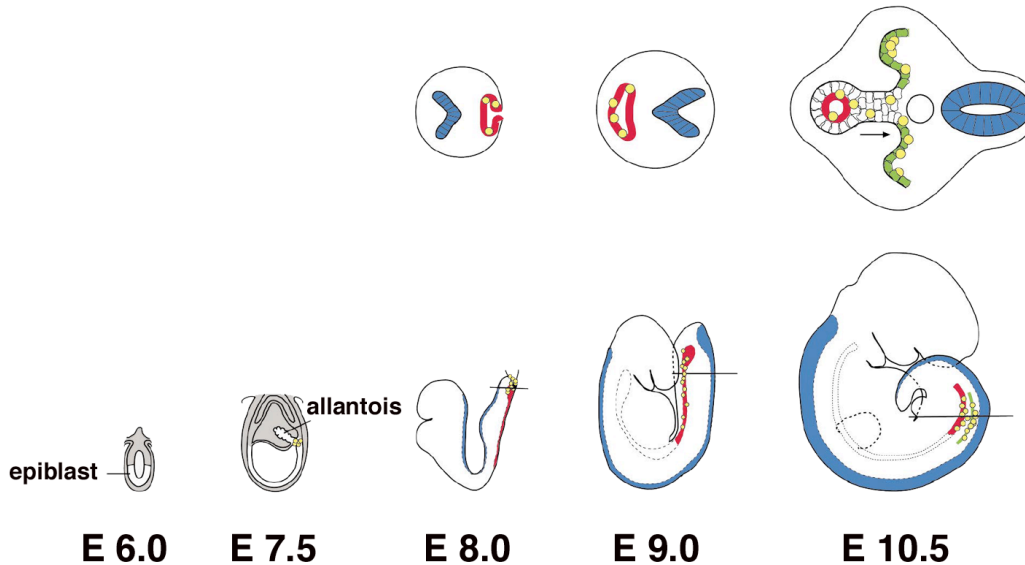
Following primordial germ cell precursor (pPGCs) specification at E5.5-E6.5, the cells migrate toward the primitive streak where they can be identified by alkaline phosphates and *stella* expression at the root of the allantois around E7.5 (Ginsburg et al., 1990; Saitou et al., 2002; Tanaka and Matsui, 2002). Germ cells incorporate into the hindgut pocket of the developing hindgut epithelium and move in random directions along the anterior-posterior axis in the closed hindgut (Anderson et al., 2000). At E9.0,

all PGCs



**Figure 1-1:** From fertilized egg to primordial germ cell specification. The development of a mouse embryo from fertilization (embryonic day 0) to the time of PGC specification at day 7.25 is shown on the left. PGC precursors in the proximal epiblast and PGCs in the primitive streak area are depicted as blue circles with darker smaller circles as nuclei. The appearance of different cell types during early development is shown on the right as boxes. Arrows are used to show differentiation. Cells considered totipotent or pluripotent are boxed in blue. These include the fertilized egg, blastomeres, cells in the inner cell mass (ICM), epiblast, PGC precursors, and PGCs. Red arrows are used to indicate the path from totipotency and pluripotency to PGCs. In addition to the PGC precursors, some epiblast cells have the potential to form PGCs in vitro (shown by a dashed red arrow). Green arrows are used to indicate differentiation of cells that do not have pluripotent character. (Adapted from (Zhao and Garbers, 2002))

are found in the hindgut and are motile. About 12 hours later, PGCs start to actively transmigrate the hindgut epithelium and migrate via the dorsal mesentery towards the genital ridges between E10.0 and E11.5 (Molyneaux et al., 2001) (Figure 1-2).



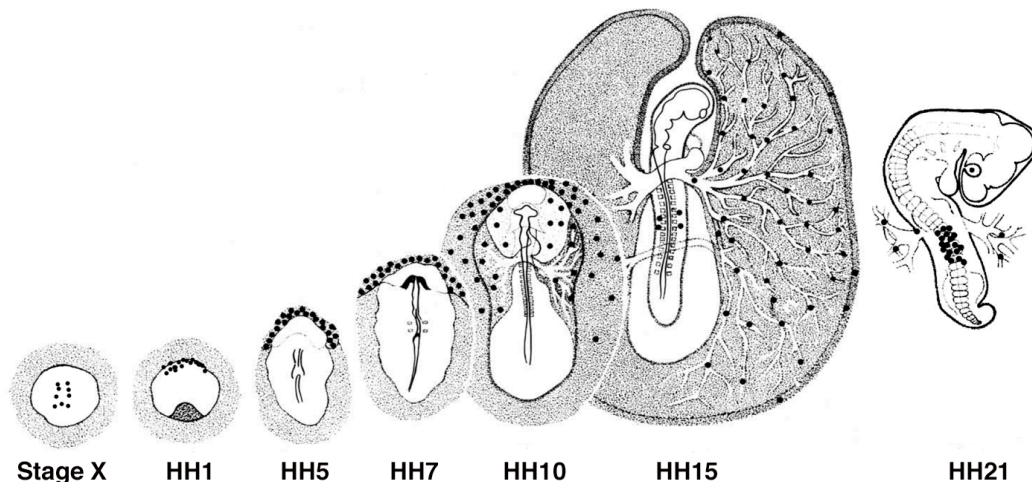
**Figure 1-2:** Germ cell migration in the mouse embryo. E6: The primordial germ cell precursors are formed. E7.5: The germ cells (yellow) are specified. E8: Germ cells occupy the developing hindgut epithelium (hindgut pocket, red) and are highly motile. Neural plate in blue. E9: Embryo has turned, PGCs are still in the hindgut. Following 24 hours (E9.0-10.0), PGCs start to leave the hindgut epithelium and migrate via the dorsal mesentery towards the genital ridges (green). E10.5: Germ cells migrate toward the gonad and until E11.5. (Adapted from (Molyneaux et al., 2001; Starz-Gaiano and Lehmann, 2001))

Numerous factors have been implicated in controlling mouse PGC migration and these include extracellular matrix components such as integrins and signaling molecules such as the receptor tyrosine kinase *steel* and its ligand ((Anderson et al., 1999; Bernex et al., 1996; Garcia-Castro et al., 1997; Gomperts et al., 1994; Pellas et al., 1991) and reviewed by (Molyneaux and Wylie, 2004; Santos and Lehmann, 2004; Starz-Gaiano and Lehmann, 2001; Wylie, 2000)). Nevertheless, the actual cues, guiding mouse primordial germ cells into the hindgut pocket or from the hindgut via the dorsal mesentery towards the genital ridges were not known at the time the work described in this thesis was initiated.

## 1.4 Germ Cell Specification and Migration in Chick

In contrast to other model organisms such as mouse, *Xenopus*, zebrafish, *Drosophila* and *C.elegans*, germ cell specification and migration in the chick embryo were less intensively studied and therefore very little were known regarding the molecular mechanisms governing these processes.

In the chick embryo, germ cells are specified in the epiblast in the central zone of the *area pellucida* (Karagenc et al., 1996; Petite et al., 1997; Tsunekawa et al., 2000), whereas the factors important for this process are unknown. From this position the cells, expressing the chicken homologue of *vasa* (Tsunekawa et al., 2000), are translocated by morphogenetic movements to the anterior extraembryonic region called the germinal crescent where they incorporate into the forming extraembryonic vascular network (stage 8-10 according to Hamburger and Hamilton (HH 8-10), (Hamburger and Hamilton, 1951)) and start to circulate within the blood stream (stage HH 11) (Ginsburg and Eyal-Giladi, 1987; Tsunekawa et al., 2000). Later in development, germ cells leave the vascular system and migrate into the region where the gonad develops (HH 15-29) (reviewed by (Niewkoop and Sutasurya, 1979)).



**Figure 1-3:** Germ cell specification and migration in the chick embryo. At stage X, PGCs are specified in the central zone of the *area pellucida* and translocated by morphogenetic movement to the anterior extraembryonic region, the germinal crescent. From HH1 to HH10, Germ cells proliferate and incorporate into the forming vascular system. When the heart starts to pulsate at HH10, PGCs are passively circulating through in the blood stream until HH15. From HH15 on, PGCs leave the vascular system and migrate actively towards the genital ridge. (Adapted from (Niewkoop and Sutasurya, 1979)).

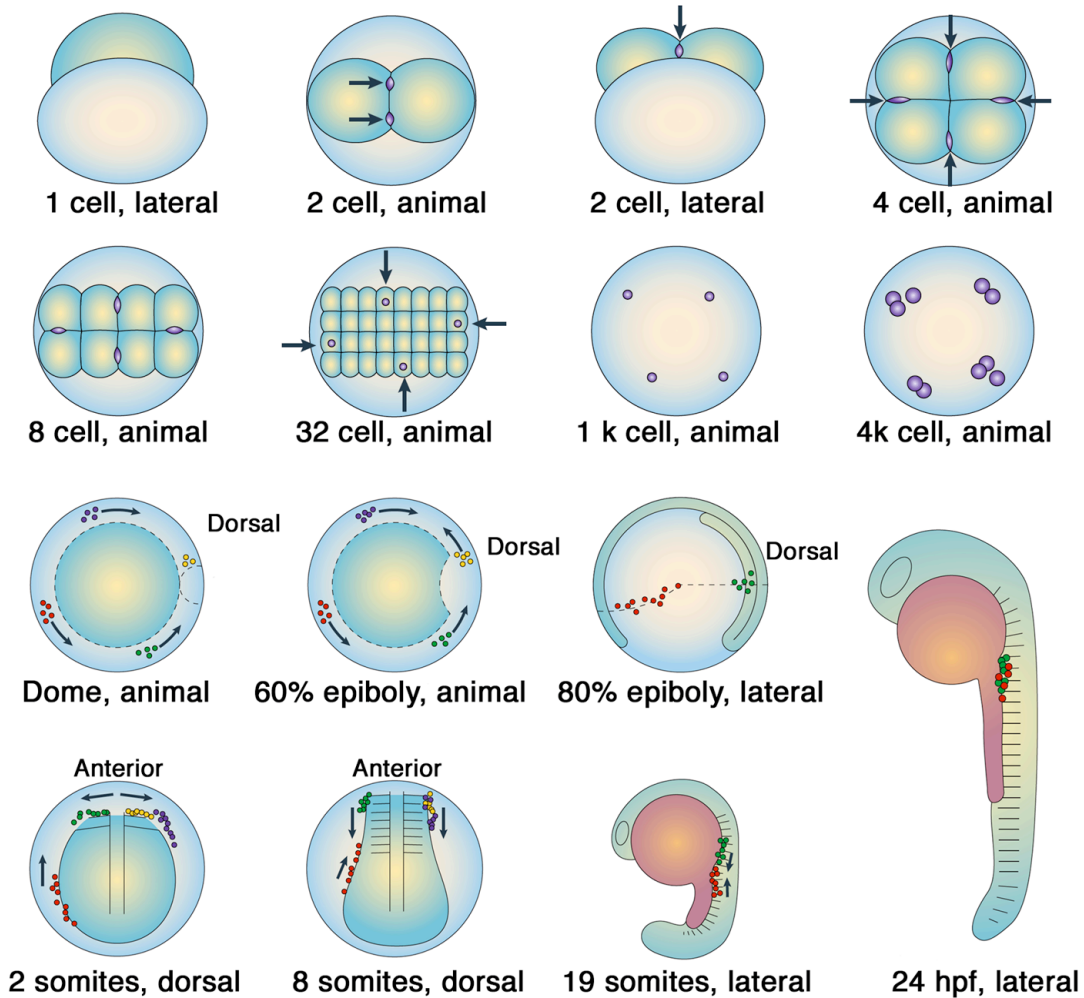
The migration path of PGCs in the chick is strikingly similar to the route taken by leukocytes during normal development and in response to an immune challenge as well as to the route metastatic cells follow on their way to form secondary tumors. In all of those cases, the vascular system serves as a vehicle to transport the cells to distant locations and migration through the vessel wall takes place in the vicinity of the target tissue.

Although the molecular mechanism responsible for germ cell migration including extravasation from the endothelial system was not known, the existence of a guidance cue directing the migrating cells to the developing gonad was supported by results of transplantations- and *in vitro* experiments (Rogulska, 1969; Rogulska et al., 1971).

## 1.5 Germ Cell Specification and Migration in Zebrafish

The zebrafish, *Danio rerio*, has become an important model system for studying PGC development and migration as it offers several advantages over other vertebrate and invertebrate model organisms (Kimmel, 1989). The combination of extra-uterine development, a large number of progeny, the availability of genetic mutations coupled with the optical clarity of the embryo is unique for this model organism. In addition to mutagenesis-based screens, the analysis of protein function *in vivo* became extremely rapid thanks to a new technology that allows knock-down of specific genes in zebrafish by injection of modified antisense oligonucleotides (Nasevicius and Ekker, 2000).

As mentioned above zebrafish germ cells are specified by the maternal inheritance of asymmetric localized cytoplasmic determinants, referred to as germ plasm. During the 1-cell stage, germ plasm components such as *vasa* RNA (Braat et al., 1999; Knaut et al., 2000; Yoon et al., 1997) and *nanos-1* RNA (*nos-1*) (Kopranner et al., 2001) are uniformly distributed in the embryo, but accumulate at the distal parts of the cleavage furrows after the first and second cell division (Figure 1-4, 2-/4-cell arrow). Following additional cell divisions, the zebrafish germ plasm is incorporated into four blastomeres. The number of blastomeres harbouring germ plasm remain constant until blastula stages as a result of asymmetric cell divisions where germ plasm material is distributed only to one daughter cell (Knaut et al., 2000). At the late blastula stage (4 hpf, 4 k cell, late sphere stage) PGC specification occurs, and as a result in subsequent divisions the germ plasm is symmetrically distributed to both daughter cells resulting in an increase in the PGC population (Knaut et al., 2000).



**Figure 1-4:** Germ cell specification and the six steps of early PGC migration in zebrafish. Schematic drawings of embryos from 1 cell stage to 24 hpf showing the process of PGC specification and positions/movements of the four PGC clusters.

**(1 cell)** Maternal inherited and asymmetric localized cytoplasmic determinants, referred to as germ plasm, is uniform distributed in the cell.

**(2/4 cell)** At the end of the first two cell divisions, germ plasm components accumulate at the distal parts of the cleavage furrows.

**(32 cell – 1 k cell)** During early cleavage stages, the zebrafish germ plasm is incorporated into four blastomeres, which asymmetrically divide and inherit the germ plasm to only one daughter cell .

**(4 k cell)** Germ cells are specified and divide symmetrically.

**(Dome)** At dome stage, four clusters of PGCs are found close to the blastoderm margin in a symmetrical 'square' shape. All possible orientations of the square relative to the dorsal side of the embryo can be observed. Here, an intermediate arrangement is shown with one cluster close to, but not directly at the dorsal side. Before gastrulation, lateral and ventral clusters move towards the dorsal, with ventral clusters migrating more slowly (step I, convergence

towards the dorsal). This movement is shared with somatic cells and can be attributed to early compaction before gastrulation and dorsal convergence of hypoblast cells during gastrulation.

**(60% epiboly)** Clusters located very close to the dorsal migrate away from the dorsal midline and are therefore rarely found on the notochord from the 60% epiboly stage on (step II, exclusion from the dorsal midline).

**(80% epiboly)** Dorsally located PGCs align along the border between the head and trunk paraxial mesoderm depicted by a dashed line (step IIIa, alignment along the anterior border of the trunk mesoderm). Ventrally located clusters align at the lateral border of the mesoderm (step IIIb, alignment along the lateral border of the mesoderm).

**(2 somites)** At the 2-somite stage, most PGCs have arrived in two lines at the level of the first somite. These anterior located PGCs migrate towards the lateral (step IV, formation of two lateral PGC clusters). Cells that were initially located ventrally migrate towards the anterior (step V, anterior migration of trailing PGCs). In this illustration, the positions of the PGCs are drawn relative to the adaxial cells, the somites and the lateral border of the pronephric anlage.

**(8 somites)** At the 8-somite stage, all anterior PGCs are found lateral to the paraxial mesoderm in a cluster extending from the 1<sup>st</sup> to the 3<sup>rd</sup> somite. These clusters start to move towards the posterior (step VI, posterior positioning of the PGC clusters), while the trailing cells migrate anteriorly. Here, the PGCs are drawn relative to the expression domains of *myoD* in the adaxial cells and somites and *pax2.1* in the pronephros.

**(19 somites)** At the 19-somite stage, the main clusters have shifted to more posterior positions and in 60% of embryos some trailing cells are still seen.

**(24 hpf)** At 24 hpf, the PGC clusters are located at the anterior end of the yolk extension, which corresponds to the 8<sup>th</sup> to 10<sup>th</sup> somite level. In most embryos, all PGCs have reached this region, only a few trailing cells are found close to the main clusters. (Adapted from (Raz, 2003; Weidinger et al., 1999a)).

For different germ plasm components, a substantial fraction is not incorporated into the PGCs and remains detectable in somatic cells as late as up to early epiboly stages ( 5 hpf - Dome, (Braat et al., 1999; Knaut et al., 2000; Kopranner et al., 2001; Olsen et al., 1997; Wolke et al., 2002; Yoon et al., 1997)). Germ plasm components, such as *vasa* and *nanos-1* RNA have been shown to be actively degraded in the somatic tissue in a process mediated by *cis*-acting elements in the RNA (Kopranner et al., 2001; Wolke et al., 2002). Similarly, RNA elements were shown to inhibit the translation of those RNAs in somatic cells (Kopranner et al., 2001).

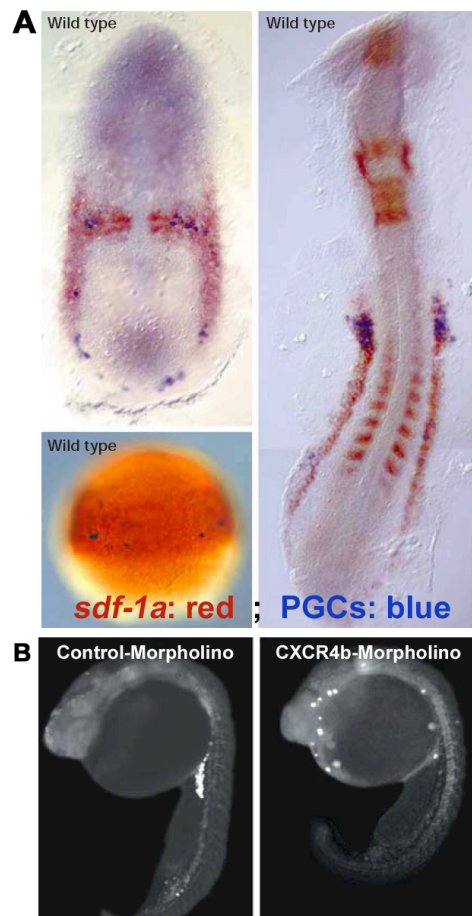
As a result of the position where the germ cells are specified, zebrafish PGCs start their migration from positions that are random with respect to the dorso-ventral body axis. Hence, in contrast to many other organisms, where germ cells are specified on one site and therefore start to migrate from one place, PGCs in zebrafish start migrating from four different positions (Figure 1-4, Dome stage). The route, germ cell follow in the course of their migration during of the first day of development, could be divided into six distinct steps and culminates in the formation of two clusters in the region where the gonad will form (Figure 1-4, (Weidinger et al., 1999a)).

## 1.6 Identification of Genes Expressed in PGCs or Function in Germ Cell Migration

In a large-scale screen, our lab and others have identified the chemokine receptor CXCR4b (Doitsidou et al., 2002; Knaut et al., 2003) and its ligand SDF-1a (Doitsidou et al., 2002) as key proteins essential for guided migration of primordial germ cells from their site of specification to the region of the zebrafish gonad.

SDF-1 – CXCR4 signaling has previously been shown to regulate numerous processes in development (Aiuti et al., 1997; Bleul et al., 1996a; Bleul et al., 1996b; Ma et al., 1998; Nagasawa et al., 1996; Tachibana et al., 1998; Zhu et al., 2002) and in diseases (e.g. tumors/metastasis and inflammatory processes) (Abi-Younes et al., 2000; Buckley et al., 2000; Epstein, 2004; Hernandez et al., 2003; Muller et al., 2001; Staller et al., 2003; Zeelenberg et al., 2003). In zebrafish embryos, *sdf-1a* was found to be expressed in positions where the PGCs were found or towards which they were migrating (A).

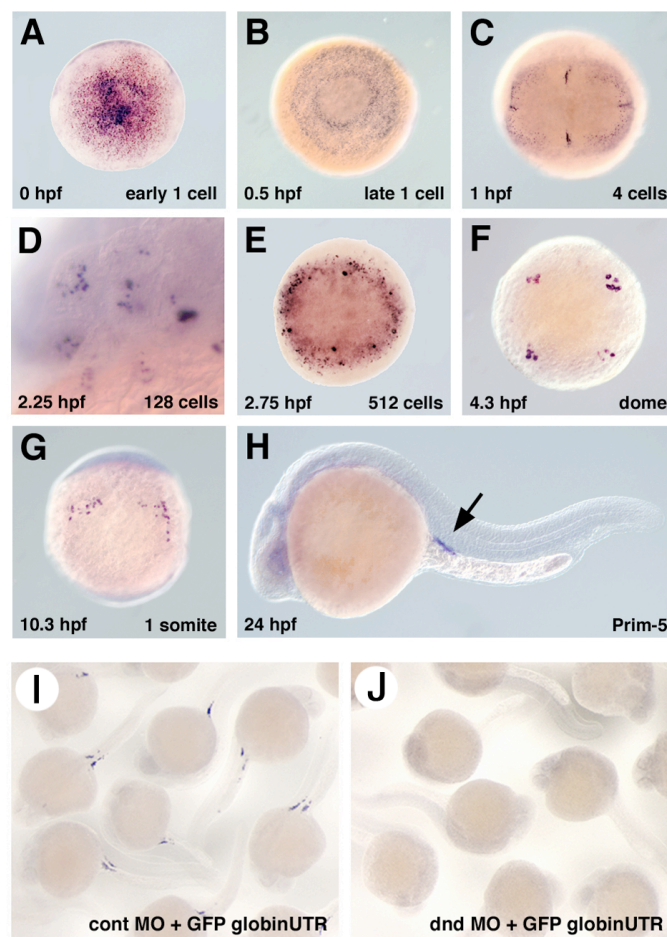
**Figure 1-5:** The role of Cxcr4b and Sdf-1a in germ-cell migration. (A) In zebrafish embryos, the germ cells (blue) are found in positions where the *sdf-1a* RNA (red) is highly expressed. (B) Alterations in the level of Sdf-1a signaling in the primordial germ cells result in defects of germ-cell migration. In contrast to wild-type embryos, in which most of the germ cells are found in the region of the gonad by the end of the first day of development, inhibiting the translation of *cxcr4b* or Sdf1a (not shown) in the germ cells results in migration defects.





In embryos, in which the receptor or ligand was knocked down, PGCs lost their ability to migrate directionally towards the region of the gonad and were therefore found in ectopic positions within the embryo (B (Doitsidou et al., 2002). Furthermore, in embryos in which the ligand or the receptor was knocked down and an ectopic source of SDF-1a was introduced, germ cells migrated towards the new location where SDF-1a was highly expressed (Doitsidou et al., 2002).

In another screen, based on whole mount in situ hybridization to identify genes expressed in zebrafish PGCs, we identified a novel gene, *dead end* (*dnd*), which is specifically expressed in germ plasm and primordial germ cells (Weidinger et al., 2003).



**Figure 1-6:** *dead end* expression during the first day of development and loss of PGCs in *Dead end* depleted zebrafish embryos. (A-H) Whole mount in situ hybridizations of embryos with *dnd* antisense RNA probe at the indicated stages. (D) is a high magnification view of ectopic *dnd*-RNA containing granules in somatic cells. Note that the RNA is detected exclusively in PGCs from dome stage onwards (F). (I and J) *nos-1* expression at 24 hpf in embryos injected with the indicated MOs (200 pg) plus RNAs ( $1.7 \times 10^{-16}$  moles). Expression of *nos-1* is lost in embryos coinjected with *dnd* MO and control GFP globinUTR RNA (Weidinger et al., 2003).

Shortly after fertilization, maternally provided *dead end* RNA is present in numerous granules distributed throughout the cortex of the one cell stage embryo (Figure 1-6 A). During the later phase of the first cell cycle, these granules disappear from the animal pole of the embryo and concentrate at the vegetal part of the blastomere (Figure 1-6 B). Subsequently, *dead end* RNA accumulates at the distal parts of the first two cleavage furrows (Figure 1-6 C). A substantial fraction of maternal *dead end* RNA is not incorporated into the PGCs and remains in somatic cells up to early gastrulation stages (Figure 1-6 D and E). This and the following expression pattern during blastula, gastrulation and segmentation stages is similar to that of *vasa* RNA, which is known to reside within the zebrafish germ plasm (Figure 1-4, Figure 1-6 E-H, (Knaut et al., 2000; Yoon et al., 1997)).

Strikingly, in contrast to control, germ cells depleted of Dead end protein by injection of a specific morpholino antisense oligonucleotide were immotile and lost *nos-1* expression at 24 hpf indicating an important role of *dead end* in early germ cell development (Weidinger et al., 2003).

### 1.7 Aim of this thesis

The identification of the mechanisms governing PGC migration is a fundamental question in Developmental Biology. The identification of SDF-1/CXCR4 signaling as a key pathway guiding zebrafish PGCs towards their targets provided an answer to this question in one organism. Thereafter, it was important to check whether this pathway represent a conserved mechanism for directional PGC migration. In the first part of this thesis we addressed this question by determining the role of CXCR4 and SDF-1 in germ cell migration in different organisms.

In the second part of this thesis I was focusing on the other aspect of germ cell development, namely the molecular mechanisms underlying germ cell motility and fate maintenance. Here, the function a novel germ plasm component, which is essential for these processes, was analyzed.

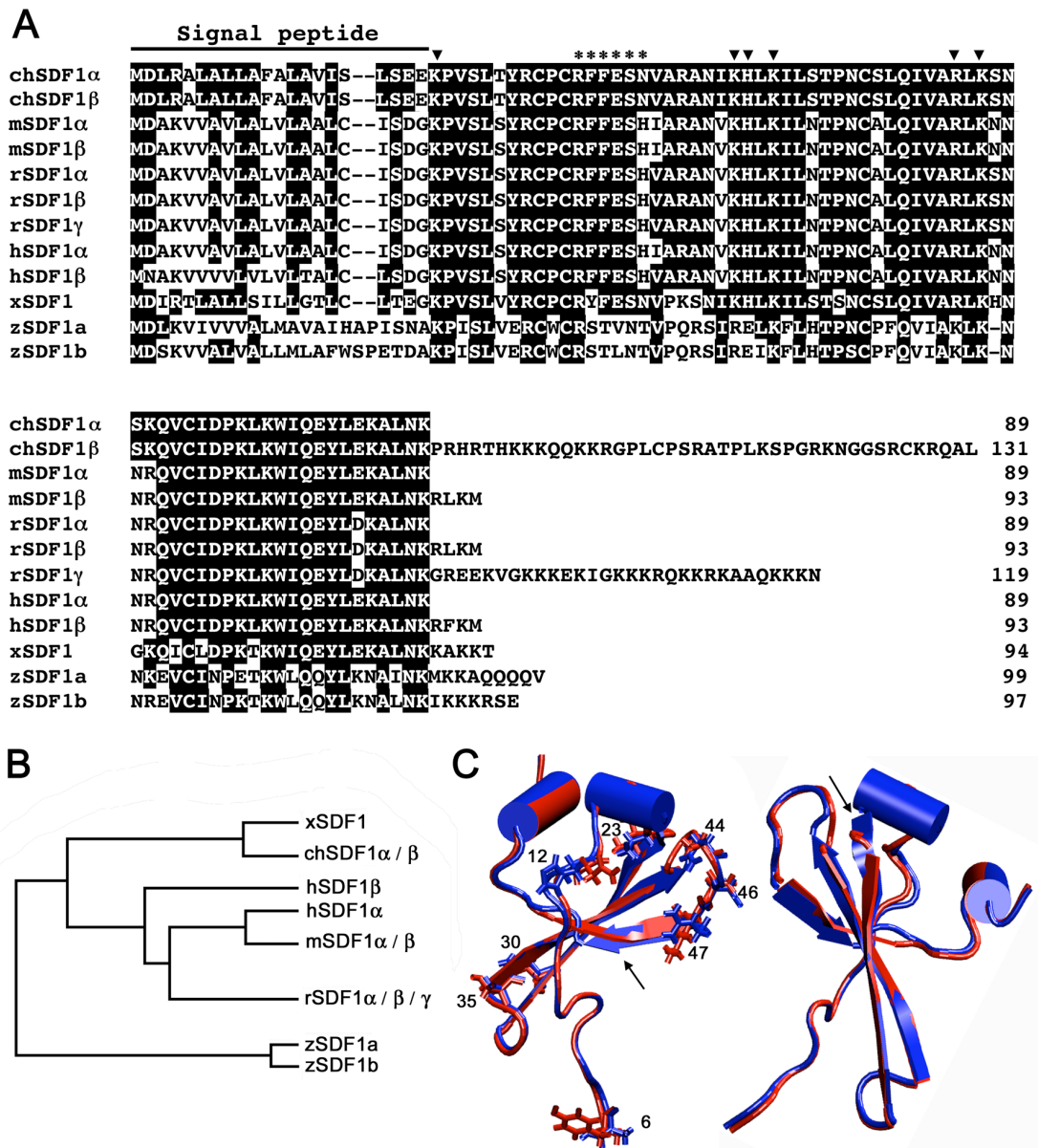
## 2. Results

### 2.1 The Role of the Chemokine SDF-1 in Avian and Murine Germ Cell Migration

#### 2.1.1 Identification of Chicken SDF-1

Avian and reptile primordial germ cells (PGCs) are transported by the vascular system to the region of the gonad where they exit the blood vessels and migrate into the genital ridge. The mechanisms that direct the PGCs along this route are largely unknown. Considering recent evidence implicating SDF-1 signaling in guiding germ cell migration in zebrafish (Doitsidou et al., 2002), SDF-1 is a good candidate molecule for providing PGCs with directional cues in these organisms. To investigate this point, the chick SDF-1 cDNA was cloned and two transcripts were identified, which were termed SDF-1 $\alpha$  and SDF-1 $\beta$ . These two transcripts are identical except for the C-terminal domain in which the  $\beta$  form is 42 amino acid longer. SDF-1 amino acid sequence alignment of chicken, mouse, rat, human, *Xenopus laevis* and zebrafish shows extensive conservation of the chick SDF-1 with its mammalian counterparts (Figure 2-1 A and B). For example, the chicken SDF-1 $\alpha$  protein is 90% identical to the mature mouse SDF-1 $\alpha$  (identical residues in respect to chicken SDF-1 $\alpha$  are indicated in black). Six residues that are essential for binding to glycosaminoglycans (i.e. Lys<sup>1</sup>, Lys<sup>24</sup>, His<sup>25</sup>, Lys<sup>27</sup>, Arg<sup>41</sup>, Lys<sup>43</sup>) (Figure 2-1 A, arrowheads) (Amara et al., 1999; Sadir et al., 2001) as well as the R<sup>12</sup>FFESH (Arg<sup>12</sup>PhePheGluSerHis) motif, (Figure 2-1 A, stars) proposed to be an important initial docking site of mouse SDF-1 to its receptor (Crump et al., 1997), are identical except His<sup>17</sup> which is replaced by a similar amino acid Asn. The chicken SDF-1 $\beta$  protein is more similar in length to the recently cloned rat SDF-1 $\gamma$  for which a function has not been assigned yet (Gleichmann et al., 2000). The similarity between the mouse and the chick proteins at the primary sequence level can be extended by way of calculation, to imply conservation at the level of the tertiary structures (Figure 2-1 C). To visualize these structural differences, the backbones of mouse SDF-1 $\alpha$  crystal structure (blue) and modeled chicken SDF-1 $\alpha$  tertiary structure (red) were superimposed and side chains of non-identical residues were drawn. The

overlay shows extensive similarities ( $\text{RMSD}_{\text{backbone}} = 0.56$ ) between these two structures. The residues that differ between the chicken SDF-1 $\alpha$  and the mouse SDF-1 $\alpha$  do not critically change the  $\alpha$ - $\beta$ - $\beta$ - $\beta$ - $\alpha$  topology of the protein. The main conformational differences are in the length of the first and third  $\beta$ -sheet.



**Figure 2-1:** (A) SDF-1 amino acid sequence alignment of chicken (chSDF1 $\alpha$  and chSDF1 $\beta$ ), mouse (mSDF1 $\alpha$  and mSDF1 $\beta$ ), rat (rSDF1 $\alpha$ , rSDF1 $\beta$  and rSDF1 $\gamma$ ), human (hSDF1 $\alpha$  and hSDF1 $\beta$ ), *Xenopus laevis* (xSDF1) and zebrafish (zSDF1a and zSDF1b) shows extensive conservation of the chick SDF-1 $\alpha$  with its mammalian counterparts including essential residues for binding to CXCR4 and glycosaminoglycans (identical residues in respect to chicken SDF-1 $\alpha$

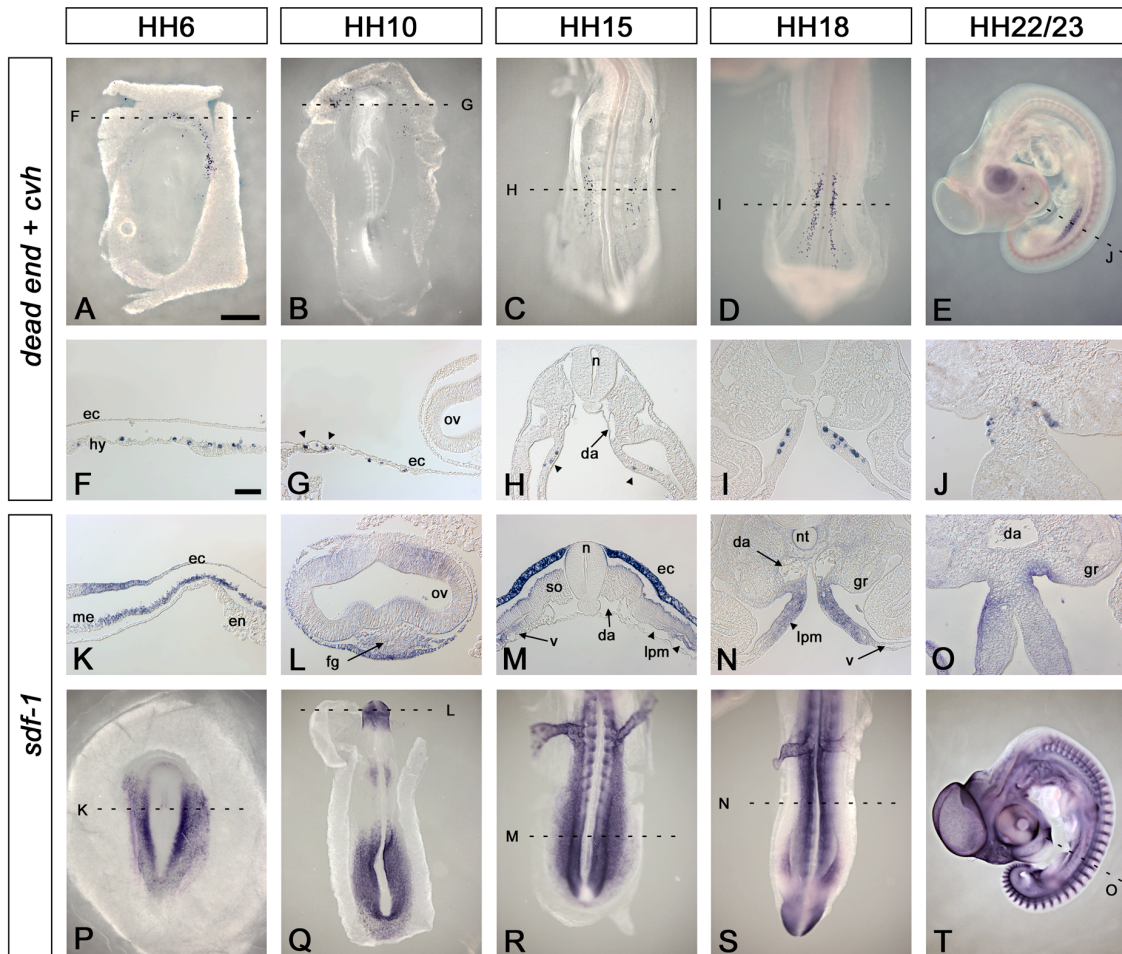
are indicated in black). (B) The unrooted phylogenetic tree of SDF-1 homologues shows diversion of zebrafish SDF-1 from the other species. (C) Modelling of the chicken SDF-1 $\alpha$  was performed using the mouse SDF-1 $\alpha$  crystal structure (Ohnishi et al., 2000) as an initial template for the backbone structure. Visualization of structural differences of the backbones of mouse SDF-1 $\alpha$  crystal structure (blue) and modeled chicken SDF-1 $\alpha$  tertiary structure (red).

### 2.1.2 Chick SDF-1 is Expressed in Positions Where PGCs are Found During the Last Stages of Their Migration

To determine whether SDF-1 and CXCR4 play a role in chicken PGC migration, the expression pattern of the chicken *sdf-1* and *cxcr4* RNAs relative to the position of the PGCs and their migration path was examined at different stages. The PGCs were detected by employing a cocktail of the chick *vasa* (*cvh*) and *dead end* (*dnd*) probes, while *sdf-1* was detected using a probe recognizing both the  $\alpha$  and the  $\beta$  forms (see Material and Methods). At stage HH 6, *sdf-1* is expressed in the posterior region of the ectoderm and mesoderm (Figure 2-2 K and P) but not in the hypoblast of the germinal crescent, the site where most PGCs are located (Fig. 2A and 2F). Later in development, during the first phases of somitogenesis, the PGCs migrate out of the hypoblast and enter the developing vascular network (Figure 2-2 F and G, arrowheads). At this stage, a few PGCs are frequently found in the developing head (data not shown). As the heart starts to beat (HH 11), PGCs are transported passively by the blood stream and are distributed throughout the entire vascular network (Niewkoop and Sutasurya, 1979). Finally, between embryonic stage HH 15 and HH 23, PGCs start to transmigrate the vascular endothelium and migrate within the lateral plate mesoderm (lpm) towards the genital ridge (Figure 2-2 C-E and H-J).

Analysis of the spatio-temporal expression of *sdf-1* revealed a strong correlation between *sdf-1* expression domains and the position of the PGCs during different developmental stages. During HH 8-10, SDF-1 expression becomes restricted to the posterior part of the embryo and to the head region (Figure 2-2 Q and L), thus raising the possibility that SDF-1 is responsible for the localization of some PGCs to the developing head. As expression in the head region is strongly reduced at stage HH 15 (data not shown), *sdf-1* is expressed in a broad area of the posterior part of the embryo in the lateral plate mesoderm where the germ cells are found after leaving the blood vessels (Figure 2-2 C, H, M and R). In the following stages, *sdf-1* expression becomes stronger and more spatially restricted in the lateral plate mesoderm. Thus, during later development, the expression pattern of *sdf-1* becomes gradually overlapping with the sites at which PGCs are found (Figure 2-2 D, E, I, J, N, O, S and

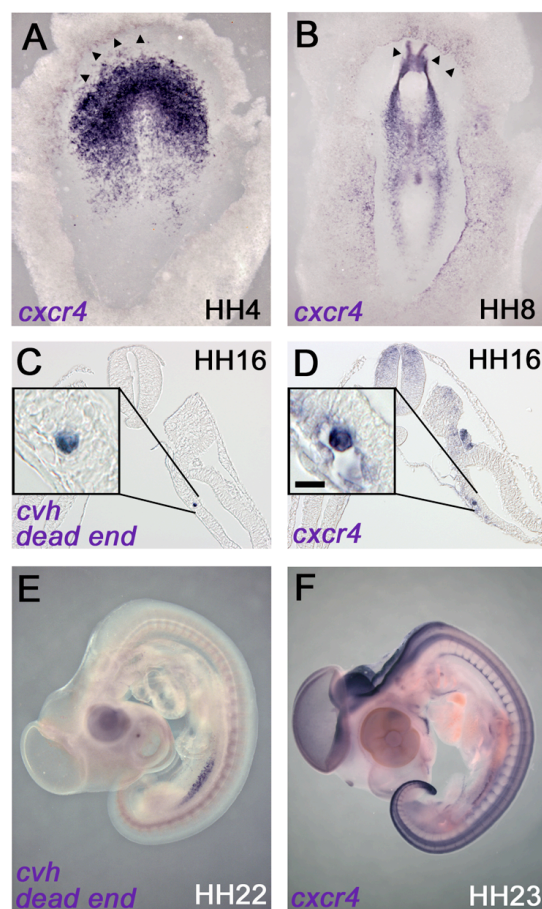
T).



**Figure 2-2:** Correlation between *sdf-1* expression and the position of the germ cells. (A-E) Whole mount in situ hybridization pictures of chicken embryos stained with PGC marker *dead end* and *cvh* or (P-T) *sdf-1*. (F-O) Sections from the corresponding embryo performed at the level indicated by the black dashed line. (A, B, F, G) PGCs marked by *dead end* and *cvh* are located in the germinal crescent within the hypoblast at stage HH 6 and within forming blood vessels (arrowhead and insert) at stage HH 10. (K, L, P, Q) At these stages *sdf-1* is expressed more posterior in the ectoderm and mesoderm and later additionally in the head region. (C and H) At HH 15, circulating PGCs (arrowhead) leave the vesicular endothelium and migrate within the lateral plate mesoderm. (M and R) *sdf-1* is expressed in the lateral plate mesoderm and in the ectoderm while expression in the head region is almost completely diminished (data not shown). (D, E, I, J) Germ cells within the lateral plate mesoderm migrate towards the genital ridge between HH 18 and HH 23. (N, O, S, T) Expression of *sdf-1* within the lateral plate mesoderm (arrowhead) becomes more restricted and overlaps with the position of the germ cells. Indicated are dorsal aorta (da), ectoderm (ec), endoderm (en), foregut (fg), genital ridge (gr), hypoblast (hy), lateral plate mesoderm (lpm), neural tube (n), notochord (nt), mesoderm (me), optic vesicle (ov), somite (so), vein (v). The scale bar in A represents 1mm in A and P,

0.8 mm in B and Q, 0.5 mm in C-D and R-S and 1.5 mm in E and T. The scale bar in F-O represents 200  $\mu\text{m}$  and in insert 600  $\mu\text{m}$ .

The correlation between the position of the PGCs during later stages of migration and the expression pattern of SDF-1 raised the possibility that SDF-1 provides the cells with directional cues by activating its receptor, CXCR4, expressed by the migrating cells. To examine this supposition, the distribution of *cxcr4* mRNA was examined by *in situ* hybridization. At stage HH 4 and HH 8, *cxcr4* is weakly expressed in the germinal crescent, where PGCs reside (Figure 2-3 A and B, arrowheads). Before stage HH 14, *cxcr4* is widely expressed in the lateral plate mesoderm (data not shown).



**Figure 2-3:** *cxcr4* is expressed in the germinal crescent and in cells with typical morphological characteristics of PGCs in the lateral plate mesoderm. (A and B) At stage HH 4-8, *cxcr4* is expressed in low amount in the germinal crescent, the site where germ cells are (arrowheads). (C) At stage HH 16 PGCs expressing *dead end* and *cvh* are located in the lateral plate mesoderm. (D) At this stage cells located in the lateral plate mesoderm showing the typical morphology and size of germ cells express *cxcr4*. (E and F) At stage HH 23 PGCs expressing *dead end* and *cvh* are located in the region of the gonad (E) and *cxcr4* is expressed in

numerous tissues including the region of the gonad (F). The scale bar in B represents 280  $\mu\text{m}$  in A and B, 200  $\mu\text{m}$  in C and D, 17  $\mu\text{m}$  in inserts and 0.9 mm in E and F.

Significantly, at the time of PGC migration, *cxcr4* positive cells can be clearly detected showing the typical morphology (large cell size, 14-17  $\mu\text{m}$ ) and location characteristic of PGCs at this stage (Figure 2-3 C and D). At the time PGCs are colonizing the gonad (Figure 2-3 E), *cxcr4* is expressed in numerous tissues whereas mRNA expression in the region where the germ cells reside is significantly reduced (Figure 2-3 F).

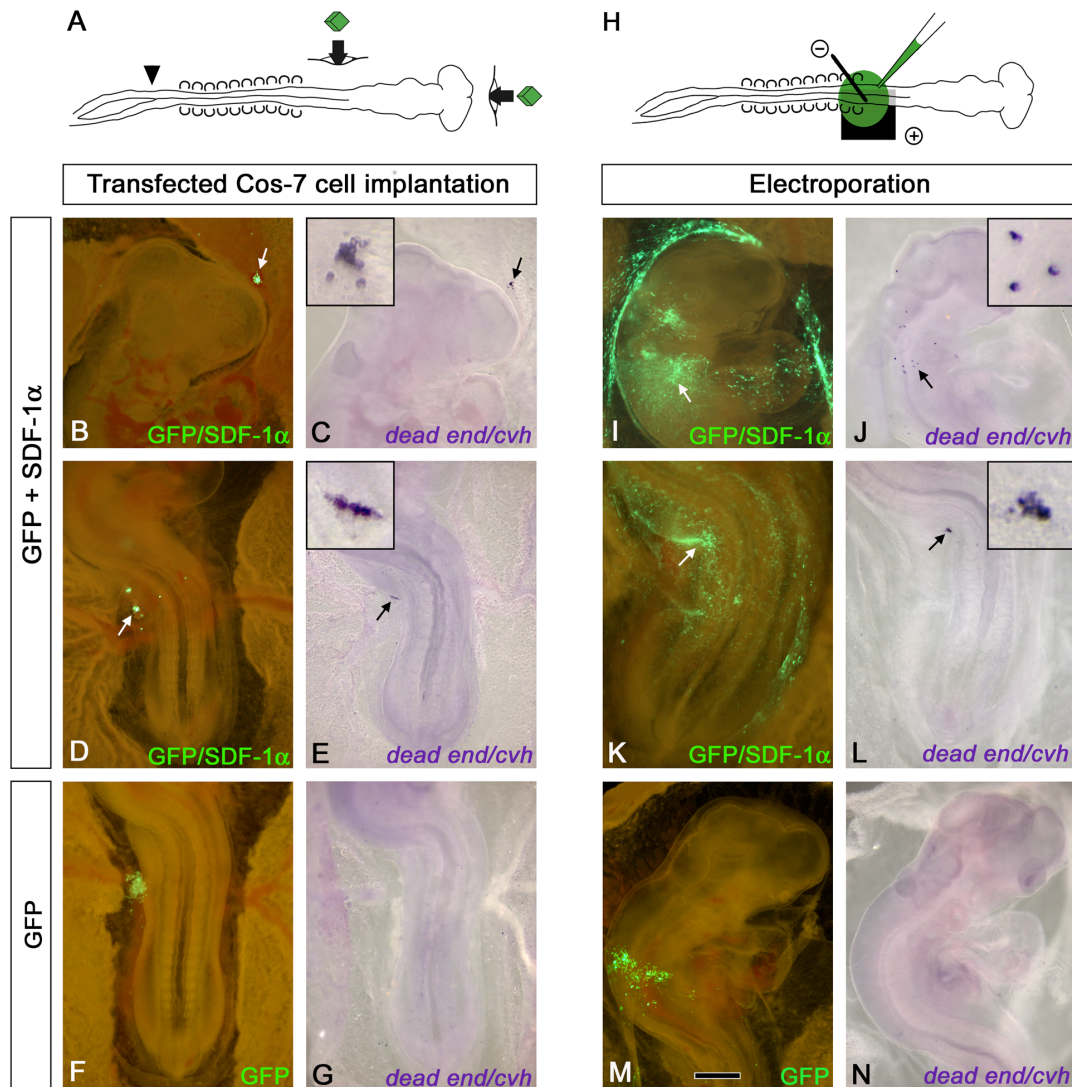
### 2.1.3 PGCs Can Be Attracted Towards a Source of SDF-1 $\alpha$

To directly test the notion that SDF-1 can attract chick PGCs, SDF-1 $\alpha$  and SDF-1 $\beta$  were ectopically expressed and the ensuing PGC response was examined. Aggregates of Cos 7 cells, co-transfected with either the  $\alpha$  or  $\beta$  forms of SDF-1 and GFP, were implanted into chicken embryos at stage HH 4 and HH 10 at different regions (Figure 2-4 A). Embryos manipulated at stage HH 4 were cultured until stage HH 10, at which time the PGC localization relative to the site of the implant was examined. Embryos harbouring implants expressing either SDF-1 $\alpha$  and GFP (n = 16), SDF-1 $\beta$  and GFP (n = 13) or GFP alone as control (n = 10) in the *area opaca* or head region did not show any effect of SDF-1 on germ cell localization as no PGCs were found adjacent to the implants (data not shown). Considering that SDF-1 expressing cells were transplanted adjacent to the region where PGCs reside and close to positions where blood vessel develop, we conclude that at least under the conditions of this experiment, PGCs do not respond to SDF-1 between stages HH 4 and HH 10.

To explore the possible function of SDF-1 in germ cell migration during later stages, embryos were similarly manipulated at stage HH 10 and analysed at stage HH 17 (Figure 2-4 B-G, at these stages all embryos showed high numbers of PGCs in the gonad which responded to the endogenous signals, but these cells can be visualized only from the ventral side of the embryo). 4 of the 13 embryos containing implanted Cos 7 cells that express SDF-1 $\alpha$  and GFP (Figure 2-4 B and D) showed ectopic *dead end* and *cvh* labelled PGCs exactly and exclusively at the position of the GFP-labelled graft (Figure 2-4 C and E). This result was further validated by sectioning the embryos and confirming the overlapping localization of the SDF-1 $\alpha$  source and the attracted PGCs (data not shown). In contrast, none of the embryos in which SDF-1 $\beta$ /GFP- or GFP- expressing cells were implanted showed PGCs in close proximity to the graft (n = 17, data not shown and n = 17, Figure 2-4 F and G, respectively). Taking into



consideration that the exogenous SDF-1 $\alpha$  source has to be precisely positioned near a blood vessel such



**Figure 2-4:** Germ cells are attracted towards ectopically expressed SDF-1 $\alpha$ . (A) Aggregates of Cos 7 cells expressing either SDF-1 $\alpha$  and GFP, SDF-1 $\beta$  and GFP or GFP alone were implanted at different anterior-posterior position into the chicken embryo at stage HH 10. Embryos were cultured until stage HH 17 and after *in situ* hybridization using the PGC markers *dead end* and *cvh* analysed for the position of germ cells relative to the graft. Arrowhead indicates region of the future gonad. (B, C, D, E) GFP and SDF-1 $\alpha$  expressing Cos 7 cells (white arrow) attracted numerous germ cells (insert), which are marked with *dead end* and *cvh* (dorsal view). (F and G) GFP expressing Cos 7 cells do not attract germ cells. (H) CMV-SDF-1 $\alpha$  and CMV-GFP or CMV-GFP alone were co-electroporated at different positions into the chicken embryo, cultured until stage HH 17 and analysed for the position of the PGCs relative to cells expressing high levels of SDF-1 $\alpha$ . (I, J, K, L) Germ cells (insert) are attracted to areas of CMV-GFP and CMV-SDF-1 $\alpha$  electroporated cells (white arrow). (M and N) Germ cells are not attracted to areas of CMV-GFP electroporated cells. Insert shows PGCs from the area

indicated by the arrow. All embryos showed high numbers of PGCs in the gonad, which were attracted by the endogenous signals (visible from ventral side view only, data not shown). The scale bar in M represents 515  $\mu\text{m}$  in B and C, 500  $\mu\text{m}$  in D-G and I-N.

that it can subsequently be presented on the luminal side to circulating PGCs and that it has to compete with the endogenous SDF-1 $\alpha$ , the number of embryos showing these results supports the idea that SDF-1 $\alpha$  acts as a chemoattractant for chicken PGCs.

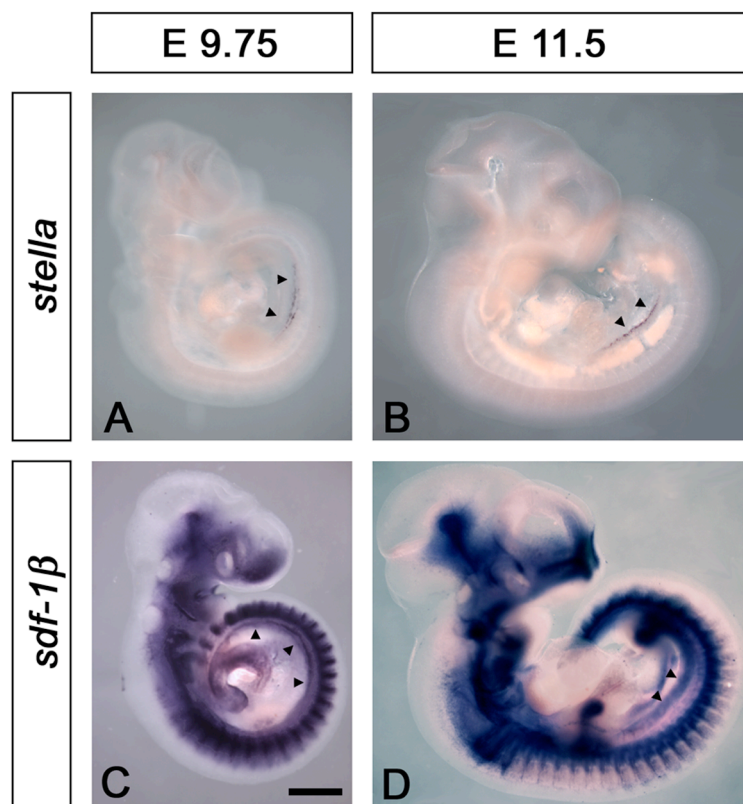
To confirm this finding, we employed an independent method, in which we electroporated chicken embryos at stages HH 4 and HH 10 with SDF-1 $\alpha$  and GFP or GFP alone to generate an ectopic SDF-1 $\alpha$  source at different positions (Figure 2-4 H). These embryos were cultured in EC-whole embryo culture until stage HH 17. Indeed, 19.1%  $\pm$  5.5% (s.e.m. standard error of the mean) of PGCs in embryos electroporated with SDF-1 $\alpha$  and GFP (n = 10 embryos, n = 519 cells) reached the region of high SDF-1 $\alpha$  expression (marked by high GFP expression) away from the region of the gonad (Figure 2-4 I-L). In contrast, only 1.7%  $\pm$  0.6% of the PGCs in GFP electroporated control embryos (n = 20 embryos, n = 1472 cells P < 0.0001) were found within high GFP expressing regions (Figure 2-4 M and N).

These data, along with *sdf-1* expression pattern, clearly implicate SDF-1 $\alpha$ , but does not support a role for SDF-1 $\beta$ , in guidance of migrating PGC in the chick. Interestingly, in zebrafish too, only one of the two SDF-1 proteins (SDF-1a which differs from SDF-1b in its C-terminus) is considered to be relevant for activating CXCR4b in the PGCs (Doitsidou et al., 2002). According to our findings, the function of SDF-1 as a PGC attractant in the chick appears to be temporally restricted to the second phase of PGC migration, the stage at which germ cells leave the vascular network and migrate towards the genital ridge (HH 15-29).

### **2.1.4 Mouse PGCs Transmigrate Through the Hindgut Epithelia Exclusively in the Region of SDF-1 Expression**

Analogous to our findings in chick, mouse SDF-1 has been recently shown to be essential for PGC migration from the hindgut via the mesenchyme to the gonad (Ara et al., 2003; Molyneaux et al., 2003), but is not required for the earlier migration phases. Based on these results, it was proposed that during early steps of PGC migration SDF-1 might be complemented by other chemokines or cytokines that play a redundant role (Ara et al., 2003). To address this notion, we examined the expression pattern of mouse SDF-1 $\alpha$  and SDF-1 $\beta$  with respect to the position of the germ cells by *in situ*

hybridization from developmental stages E8.5 to E11.5. During this time window, the PGCs exit the hindgut and migrate towards the gonad. *sdf-1 $\beta$*  (and *sdf-1 $\alpha$* , data not

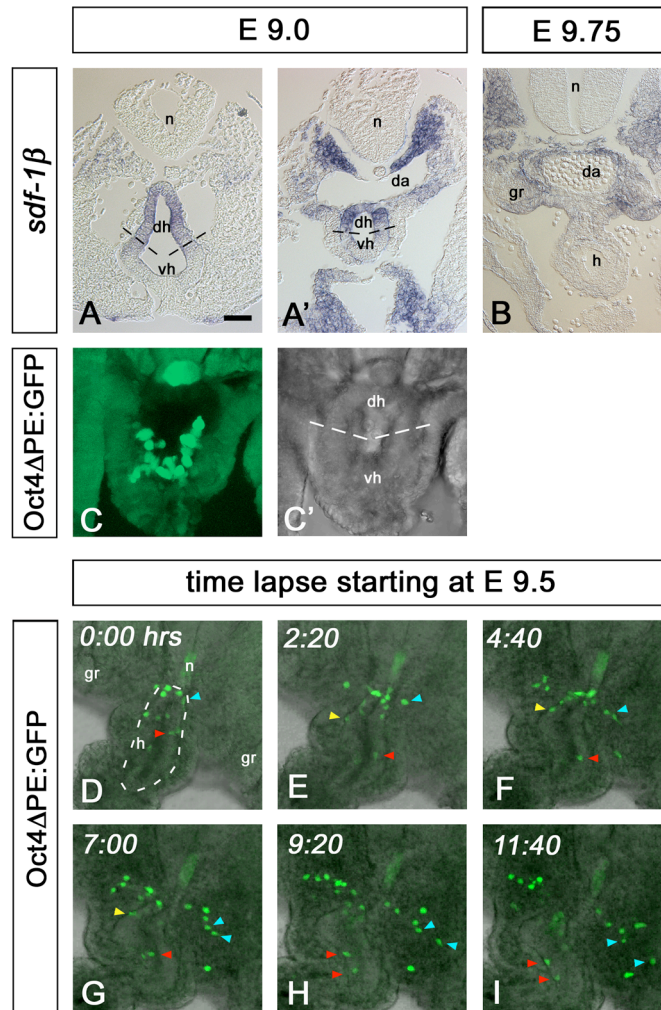


**Figure 2-5:** Mouse SDF-1 expression correlates with the position of the germ cells (A) An E9.75 embryo with germ cells (arrowhead) stained by in situ hybridization with *stella* at the onset of directional PGCs migration from the dorsal body wall towards the genital ridge. (B) At E11.5, germ cells (arrowhead) are found within the genital ridge. (C and D) In embryos of the same stages as in (A) and (B) *sdf-1 $\beta$*  is expressed over the whole anterior-posterior axis along the germ cell migration route and in the gonad (arrowhead). The scale bar in C represents 1 mm in A and C and 2.5 mm in B and D.

shown) is expressed along the migratory route in the dorsal mesentery and in the genital ridge (Figure 2-5 and Figure 2-6 D-I).

However, at E9, before the PGCs migrate dorsally towards the point where they exit the gut, *sdf-1 $\beta$*  (and *sdf-1 $\alpha$* , data not shown) is expressed in the dorsal half of the hindgut, but is clearly absent from the ventral half (Figure 2-6 A and A'), where PGCs are predominantly found at this stage (Figure 2-6 C and C'). These data show that most PGCs are distant from SDF-1 expression domain prior to the time point at which they migrate dorsally (E9-E9.5). We conclude that SDF-1 does not play a role in guiding the cells anteriorly along the ventral side of the hindgut. The migration of the

cells to the position adjacent to the genital ridge is therefore guided by other factors.



**Figure 2-6:** Mouse SDF-1 expression correlates with germ cell position from the time PGCs start to emerge from the hindgut. (A and A') Two sections of a stage E9 embryo show *sdf-1β* staining in the dorsal half of the hindgut but not in the ventral half. Both sections are from the same embryo whereas A is more posterior than A'. (B) Section through the gonad of the E9.75 embryo displayed in Figure 2-5 C shows that *sdf-1β* is expressed along the PGC migration route in the dorsal mesentery and in the gonad but not anymore in the hindgut. (C) GFP expressing germ cells in cultured hindgut transverse slices are located in the ventral half of the hindgut. (C') shows (C) in bright field. (D-I) Six confocal snapshots from a 11 hours 40 minutes long time lapse movie showing GFP expressing PGCs in Oct4ΔPE:GFP transgenic mouse which emerge from the dorsal-most part of the hindgut and migrate towards the genital ridge (blue arrowhead). Germ cells located in the ventral half of the hindgut (red and yellow arrowheads) do not transmigrate the gut epithelia and eventually die (supplementary Movie 1). Indicated are dorsal aorta (da), dorsal hindgut (dh), genital ridge (gr), hindgut (h), neural tube

(n), and ventral hindgut (vh). The scale bar in C represents 1 mm in A and C and 2.5 mm in B and D. The scale bar in A represents 200  $\mu\text{m}$  in A-B, 300  $\mu\text{m}$  in D-I and 360  $\mu\text{m}$  in C and C'.

To examine the events occurring at this stage more directly, we monitored the behaviour of GFP-expressing mouse germ cells in cultured transverse slices (Anderson et al., 1999; Molyneaux et al., 2001) from E9.5 hindgut by time-lapse microscopy (Figure 2-6 D-I). We found that the germ cells emerge exclusively from the dorsal-most part of the hindgut (blue arrowhead), where *sdf-1* is expressed, whereas ventrally positioned PGCs move while colliding with the epithelium but do not transmigrate through it (red and yellow arrowhead) and eventually die (Molyneaux et al., 2001). Together, these results are consistent with the idea that SDF-1 is required specifically for directing the dorsal migration of PGCs to the position in the hindgut from which they migrate into the mesentery. Our data suggest a common function of SDF-1 in promoting PGC transmigration through epithelial like structures such as the hindgut epithelium in mouse and the endothelium in chick.

## 2.2 *dead end* is Required for Primordial Germ Cell Migration and Survival

### 2.2.1 *Dead end* is a novel putative RNA-binding protein, which is localized to perinuclear germ granules

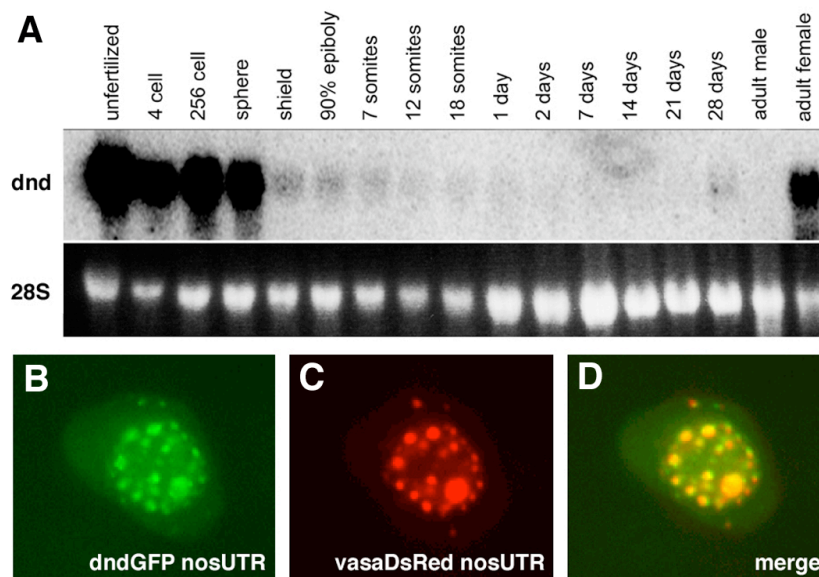
In a large scale whole mount in situ screen for genes expressed in zebrafish PGCs we identified a novel gene, *dead end* (*dnd*). The full-length zebrafish *dead end* cDNA encodes a protein of 411 amino acids containing a putative single strand RNA binding domain (Prosite database profile PS50102) in its N-terminal half (Figure 2-7).



**Figure 2-7:** Multiple alignment of the zebrafish *dead end* and orthologs. In the red frame, highly conserved putative RNA binding domain is highlighted.

This RNA binding motif is found in a large variety of RNA binding proteins such as heterogeneous nuclear ribonucleoprotein particles (hnRNPs), small nuclear ribonucleoprotein particles (snRNPs) and other pre-RNA and mRNA associated proteins. Multiple alignment of zebrafish *dead end* and ESTs or genomic sequences encoding closely related *dead end* genes in *Xenopus laevis*, mouse and human shows that the single strand RNA binding motif is conserved 58-59% between zebrafish and orthologs from other species.

*dead end* is specifically expressed in germ plasm and primordial germ cells (Weidinger et al., 2003). The expression pattern is similar to that of *vasa* RNA, which is known to reside within the zebrafish germ plasm (Knaut et al., 2000; Yoon et al., 1997). Northern blot analysis revealed rapid degradation of the maternally provided *dnd* RNA just after PGC specification at 4 hpf (sphere stage) (Figure 2-8 A). Low levels of RNA persist during embryogenesis, which presumably corresponds to exclusive expression of the gene in the PGCs.

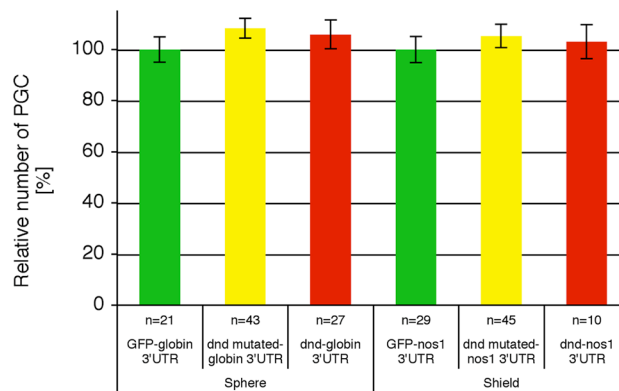


**Figure 2-8:** Expression and subcellular localization of Dead end. (A) Northern blot analysis using *dead end* as probe shows that maternally provided *dead end* is degraded after sphere stage but low level of expression persists during embryogenesis. (B-D) Fluorescent pictures of a PGC in a 10-somite stage embryo coinjected with 100 pg *dndGFP nos1-3'UTR* (green channel in B) and *vasaDsRed nos1-3'UTR* (red channel in B). The merge picture in D shows that Vasa and Dead end proteins colocalize to perinuclear germ granules.

To determine the subcellular localization of the Dead end protein, a Dead end-GFP fusion protein was expressed in the PGCs. As shown in Figure 2-8 B-D, Dead end protein is localized to perinuclear germ granules, which also contain a Vasa-DsRed fusion protein in mid-somitogenesis stage embryos. Thus, Dead end is localized to the same cellular structure where other zebrafish germline-specific proteins (e.g. Vasa and Nanos (Knaut et al., 2000; Kopranner et al., 2001; Yoon et al., 1997)) are found.

### 2.2.2 Dead end Overexpression in PGCs and Somatic Tissue Does not Alter Germ Cell Specification and Number

As described above, germ cells in many organisms are specified by the inheritance of cytoplasmic determinants, the so-called germ plasm. To date, little is known about the mechanism of this process but it is assumed that cells harboring high concentration of germ plasm become PGCs, whereas the fate of cells with low concentration acquire a somatic cell fates. To investigate, if germ cell specification and/or proliferation is influenced by *dead end*-concentration, we over-expressed *dead end* in the zebrafish embryo by injection of *dead end-globin UTR* RNA (over-expression in the whole embryo, 300 ng/ $\mu$ l) or *dead end-nos1 3'UTR* RNA (over-expression in the germ cells, 300 ng/ml) and compared PGC number at sphere stage (PGCs have just specified) and shield stage (just after PGCs specification, 4 hours post fertilization) and shield stage (6 hours post fertilization) relative to controls (embryos injected with GFP and a non-functional mutated form of *dead end* (Figure 2-9).



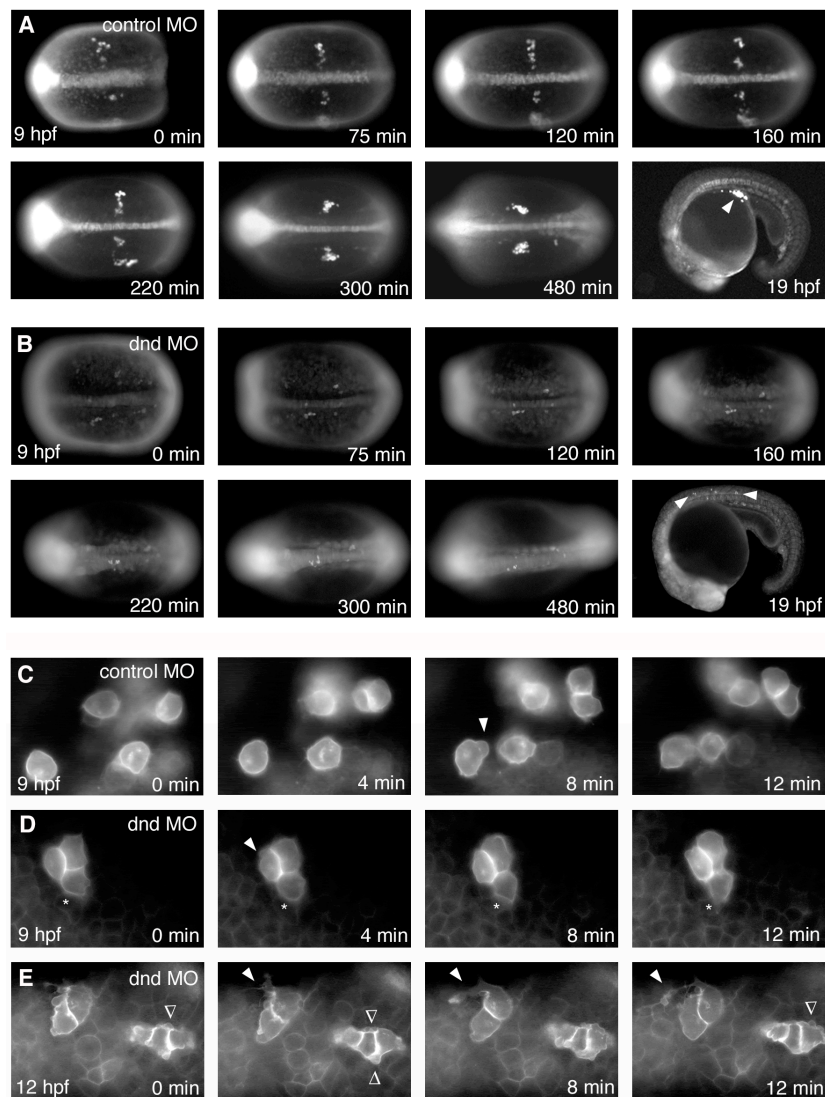
**Figure 2-9:** Over expression of *dead end* in the zebrafish embryo does not affect PGC specification or proliferation.



Over-expression of *dead end* did not alter the specification or PGC number at sphere and shield stage (Figure 2-9). We therefore conclude that an increased expression of Dead end protein is not sufficient to affect any of these processes.

### 2.2.3 Dead end is Required for PGC Migration

To investigate the function of *dead end* on PGC development, we knocked down the expression of Dead end protein by injection of *dead end*-specific morpholino antisense oligonucleotide (MO) into one-cell stage zebrafish embryos. Coinjection of *Farnesyl-GFP-nos1-3'UTR* (membran bound GFP) enabled to follow PGC behavior by time-lapse microscopy.



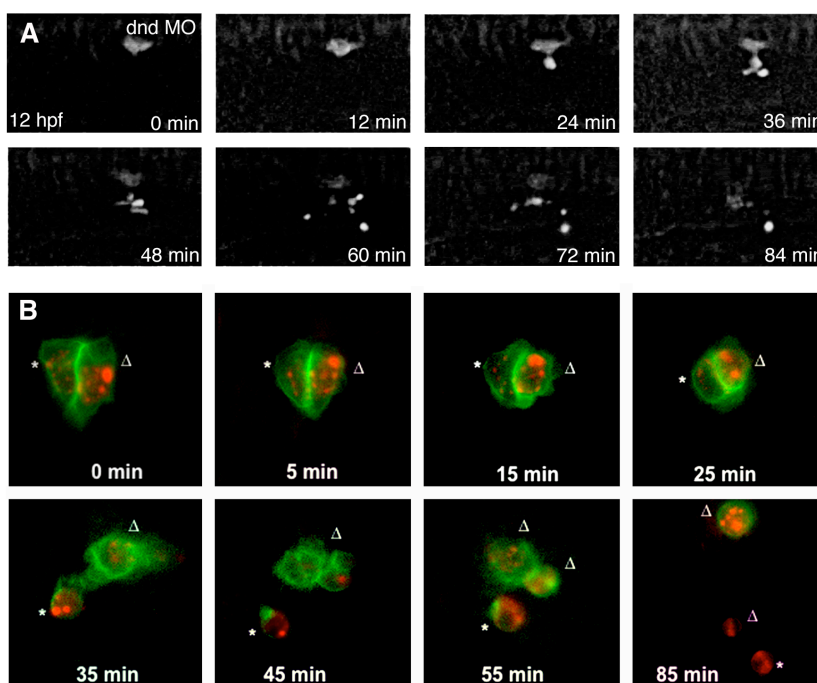
**Figure 2-10:** Dead end is required for zebrafish PGC migration. Frames taken at the indicated times from time lapse movies showing membrane-localized GFP labeled PGCs in an zebrafish embryo injected with 400 pg control MO or 400 pg *dnd* MO. (A) PGCs in control MO injected embryos migrate actively, align at the border of the trunk mesoderm, later form to lateral cluster and subsequently migrate dorsally to the region of the 8th to 10th somite. (B) PGCs in *dnd* MO injected embryos are not motile, move with the gastrulation movements towards the embryonic midline and disappear. (C) Control PGCs migrate actively as individual cells relative to their somatic neighbors. (D) In contrast, at the same developmental stage, *dnd* knockdown-PGCs do not actively migrate, form stable close cell-cell contacts and do not form long stable protrusions. (E) However, during mid-segmentation stages, Dead end depleted PGCs form atypical long protrusion.

In control MO injected zebrafish embryos (Figure 2-10 A) as in untreated embryos (Weidinger et al., 1999b; Weidinger et al., 2002; Yoon et al., 1997), PGCs start to migrate before gastrulation from four different positions where they are specified and align along the anterior border of the trunk mesoderm, with the exception of the dorsal midline (9 hpf - 11 hpf). The PGCs then migrate laterally to form two PGC cluster (11 hpf – 13 hpf). Subsequently, clustered PGCs migrate posteriorly towards the position of the 8<sup>th</sup> to 10<sup>th</sup> somite, where they are found at 24 hpf (arrowhead). In *dead end* MO injected embryos, PGCs do not exhibit active migration. Rather, the germ cells move by the convergence and extension movements together with the somatic tissue and therefore arrive at ectopic positions including the dorsal midline (Figure 2-10 B, arrowhead). In contrast to control embryos, GFP expressing PGCs in *dead end* MO injected embryos gradually disappear during somitogenesis stages.

To determine the basis for the migration phenotype described above, we examined Dead end depleted PGCs in live embryos on the cellular level and compared their behavior to that in control embryos. In contrast to GFP-labeled PGCs in control-MO injected embryos, which exhibit active migration relative to each other and their somatic neighbors and form stable big pseudopodia (Figure 2-10 C, arrowhead), there is no evidence for active PGC migration in *dead end* knockdown embryos as PGCs do not move relative to somatic neighbors (Figure 2-10 D and E). Furthermore, unlike control PGCs, which migrate as individual cells or show loose transient contacts to other germ cells, *dead end* knockdown PGCs often remain in groups of cells, which maintain in close cell-cell contact (Figure 2-10 D and E). During gastrulation and early somitogenesis, PGCs in *dead end* knock down embryos form only small transient pseudopodia (Figure 2-10 D), whereas during later segmentation stages more stable atypical long lamellipodia and filopodia can be formed (Figure 2-10 E). However, the later morphological phenotype may reflect indirect effects associated with the *dead end* knock down (see below).

### 2.2.4 Zebrafish *dead end* Is Required for PGC Survival

As mentioned above, while PGCs in which *Dead end* was knocked down expressed GFP (Figure 2-10 B), all of the GFP-positive PGCs disappeared by the end of the first day of development (Figure 2-10 B and data not shown). To determine the fate of these PGCs, we tracked individual GFP-labeled PGCs in live embryos. We found that in contrast to control-MO injected embryos, all PGCs in *dnd*-knockdown embryos eventually exhibited the morphological hallmarks of apoptotic cells (Rich et al., 1999), including membrane blebbing and fractionation into small apoptotic bodies (Figure 2-11 A). Although we cannot exclude the possibility that a small number of PGCs assumes a different fate, we conclude that PGCs lacking functional *Dead end* lose expression of characteristic genes (*vasa*, *nanos-1*, data not shown) and eventually die.



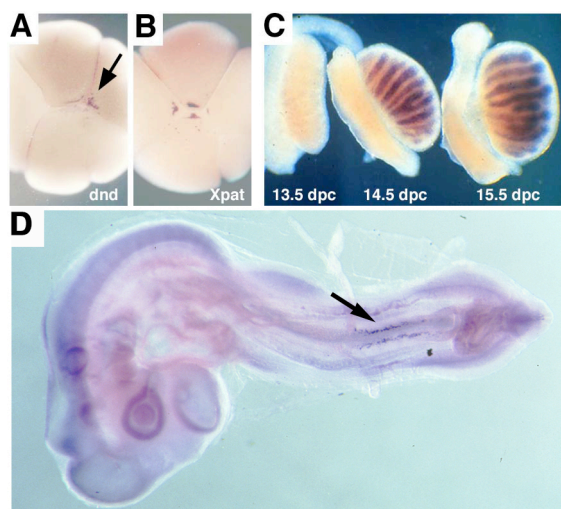
**Figure 2-11:** Zebrafish *dead end* is essential for PGC survival. (A) Frames taken at the indicated times from a time-lapse movie that starts at 12 hpf showing GFP labeled PGC in a zebrafish embryo injected with 400 pg *dnd* MO. PGC is fractionating as it dies and forms numerous apoptotic bodies (B) Two closely attached PGCs labeled by membrane-localized GFP and VasaDsRed fusion protein to visualize perinuclear granules in an embryo injected with 400 pg *dnd* MO. Note that the perinuclear granules are present in the germ cells until fractionation into apoptotic bodies.

One of germ cell characteristics are perinuclear granules, a cellular structure where zebrafish germline-specific proteins (e.g. Vasa and Nanos) are found. Perinuclear granules contain multiple putative RNA binding proteins are found in germline cells of many organisms and are associated with nuclear pores in *C. elegans* (Pitt et al., 2000). It has been proposed that germ granules are play an important role in the PGC specific development by regulating gene expression in PGCs post-transcriptionally, by controlling mRNA transport, stability and translation (Houston and King, 2000a; Seydoux and Strome, 1999). As PGCs in Dead end depleted zebrafish embryos do not maintain their fate, it might be possible that these cells transdifferentiate prior to their death. To address this question, we monitored the presence of perinuclear granules in PGCs of Dead end knock down embryos using the *vasaDsRed* fusion protein to label the granules. The fact that PGCs maintain the perinuclear granule staining until they die indicates that these cells maintain thiere fate at least partially until they die

The current understanding of the germline origin in zebrafish has been based on the expression of specific molecular markers such as vasa by cells that arrive at the region of the gonad and expression of the same markers later during gametogenesis. In *cxcr4* morphants or mutants, the PGCs exhibit severe migration defects, yet a large proportion of adult *cxcr4* mutants is fertile (Knaut et al., 2003). This finding might indicate that a few PGCs, which arrive at the region of the gonad by chance, can found a functional germ cell population. However, there is no evidence that no other cells can potentially contribute to the germline during normal development, or when the number of vasa-expressing cells is reduced. To investigate this aspect, we raised control and *dnd* MO injected embryos to adulthood and crossed them with wild-type fish. Remarkably, while on average 84% of the eggs were fertilized in crosses with control-MO injected males (1333 of 1592 eggs), only 1% was fertilized by *dnd* MO males (20 of 1486, all derived from one male). 14 of 15 *dnd* MO injected males did not fertilize a single egg in several crosses, while all 7 of the control-MO injected males fertilized eggs at least in one cross. This strong correlation between loss of cells expressing PGC markers like vasa and *nos-1* and sterility further corroborates the notion that the only cells capable of populating the germline in the zebrafish gonad are the cells expressing these markers at earlier stages. Indeed, this finding allows for the complete replacement of the germ-line in *dnd* MO injected embryos with that of genetically marked donor embryos by transplantation, an achievement that significantly simplifies the production of maternal effect mutants (Ciruna et al., 2002).

### 2.2.5 *dead end* Expression is Conserved in Other Vertebrate Species

While *dead end* function is clearly crucial for proper development of zebrafish PGCs, no homologous proteins have been described when the work on this thesis was initiated. Nevertheless, ESTs and genomic sequences encoding closely related genes in *Xenopus laevis*, chick, mouse and human were identified (Figure 2-7). These *dead end* orthologs are expressed in the germ plasm of *Xenopus* and in germ cells of mouse and chick (Figure 2-12), implying that they might play a role in germline development in these organisms, too. In *Xenopus* maternal *dead end* RNA is present along the cleavage planes at the vegetal pole of early cleavage stage embryos (Figure 2-12 A, arrow) in a pattern very similar to that of *Xpat*, a germ plasm component in that organism (Figure 2-12 A and B, done by Gilbert Weidinger) (Hudson and Woodland, 1998). Expression of *dead end* in the mouse also



**Figure 2-12:** *dead end* orthologs are expressed in germ plasm and germ cells in *Xenopus*, mouse and chick. (A, B) Vegetal views of *Xenopus laevis* embryos at the 16-cell stage stained for expression of *dead end* (arrow in A) and *Xpat* (B) RNAs. Both are present in aggregates at the cleavage planes where the *Xenopus* germ plasm resides. (C) Mouse gonads plus attached mesonephroi explanted from embryos at the indicated days post coitum (dpc) stained for expression of *dead end* RNA. (D) Expression of chick *dead end* in PGCs (arrow) after their arrival at the region of the gonad (stage 18 according to (Hamburger and Hamilton, 1951))

appeared to be in the germ cells, as seen at 14.5 dpc, where expression is very strong within the testis cords (Figure 2-12 C, done by Clare Wise and Robin Lovell-Badge). This expression pattern is reminiscent of the expression pattern of the mouse *vasa* homolog (*mvh*), which is expressed in the PGCs as they arrive at the region of the

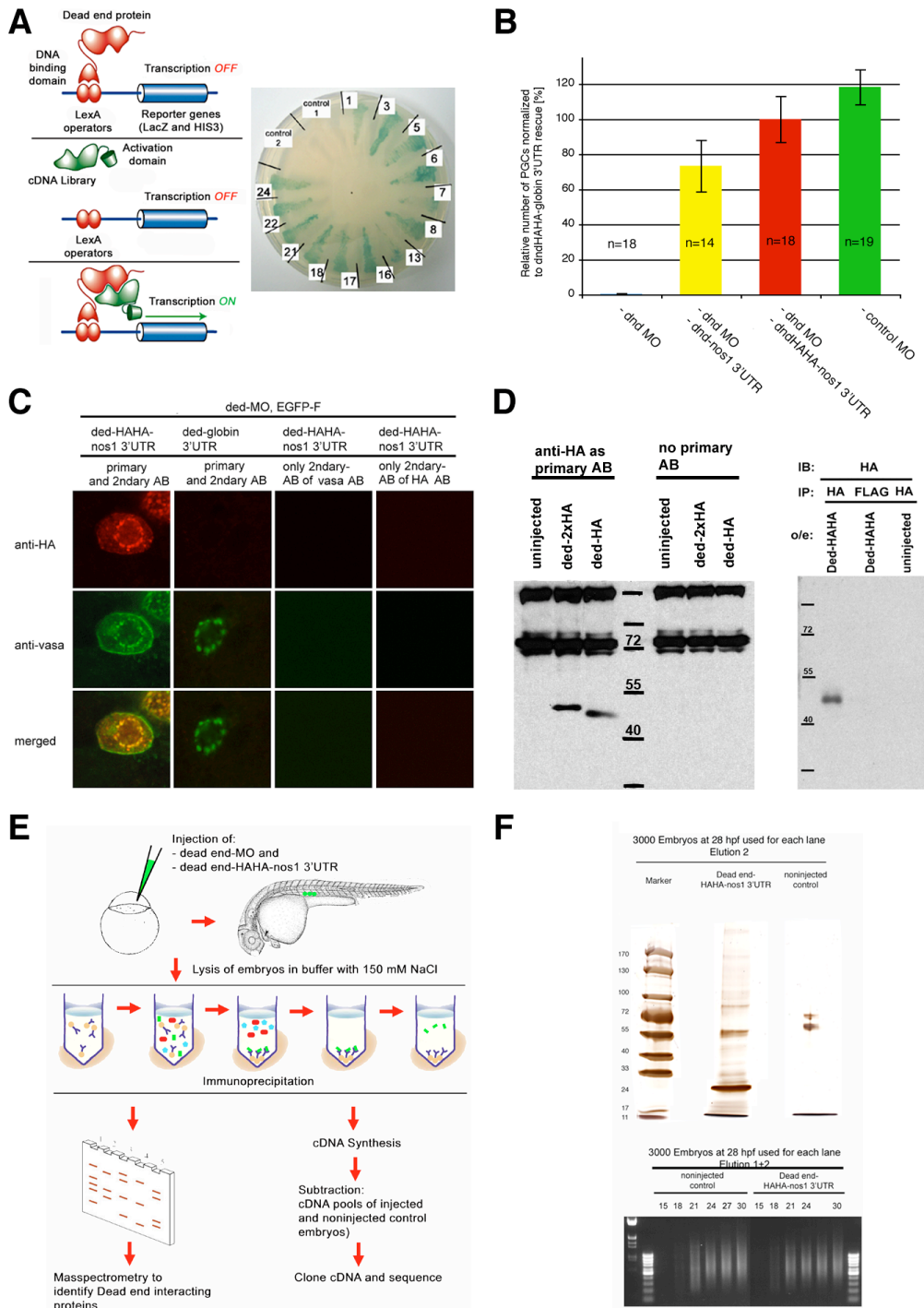
gonad (Noce et al., 2001). The chick *dead end* was expressed in the PGCs, in a similar pattern to that of chick *vasa* (Tsunekawa et al., 2000) and could be detected in those cells before, during and after their arrival at the gonad (Figure 2-12 D and data not shown). Therefore, based on the similarity in sequence and expression pattern it is likely that *dead end* plays a general role in germline development in other vertebrates, as well. Interestingly, we failed to identify *dead end* orthologues in the fully sequenced genomes of *Drosophila melanogaster* and *Caenorhabditis elegans*. Specifically, proteins from these invertebrates showing similarity to *dead end* orthologs show significantly higher degree of similarity to other proteins in vertebrates. This raises the possibility that *dead end* is the first vertebrate-specific germ plasm component known to be essential for germ cell development.

### 2.2.6 Identification of Dead end Interaction Partners

Whereas the RNA binding domain in Dead end provides an important hint regarding its function, the precise biochemical activity of this protein is unknown. Identification of proteins and RNAs that interact with Dead end could provide clues regarding this question. To identify such molecules we employed yeast-two-hybrid technology and immunoprecipitation to identify putative Dead end interacting proteins and RNAs.

To identify Dead end – protein complexes by yeast-two-hybrid technology, we first produced a PGC specific full-length cDNA library using 635 PGCs from embryos whose germ cells express GFP. This technique has the advantage of immediate freezing of the PGCs following their isolation and therefore maintaining the transcription profile more representative of the *in vivo* situation as compared to other techniques such as FACS. The mRNA pool was then reverse transcribed, amplified and subsequently cloned into a plasmid vector. With this library, a yeast-two-hybrid screen was performed and 13 positive clones were identified (Figure 2-13 A and chapter Material and Methods).

For the identification of Dead end–RNA or Dead end–protein complexes by immunoprecipitation, we decided to follow the strategy of using a commercially available monoclonal antibody against the HA-tag, a tag that was fused to the Dead end protein. This fusion protein normally interacts with other molecules as judged by its ability to support the survival and migration of PGCs depleted for the endogenous Dead end protein using morpholino antisense oligonucleotides (Figure 2-13 B).



**Figure 2-13:** Identification of Dead end interacting proteins and RNAs by yeast—two-hybrid technology and immunoprecipitation.

A further proof for the fact that the tagged Dead end protein normally interacts with other molecules is the fact that it assumes the proper subcellular localization. This point was demonstrated by co-injection of *dead end-HAHA-nos1 3'UTR* (for specific expression of the tagged *dead end* in the PGCs) together with *EGFP-Farnesyl-nos1 3'UTR* (specific expression of membrane bound EGFP in PGCs) and *dnd* MO (400 pg). After whole mount immunostaining of zebrafish embryos with anti-HA and anti-Vasa antibody, we analyzed the localization of Dead end-HAHA and Vasa. We show that Dead end-HAHA protein colocalize with Vasa in the perinuclear granules (Figure 2-13 C). Taken together, we conclude that the Dead end-HAHA protein is functional as evident by the “rescue” experiments and subcellular localization analysis and therefore Dead end-HAHA protein should still interact with partners that are critical for its function.

A pre-requisite for isolating the protein and RNA that bind the tagged Dead end is, that the Dead end-HA fusion protein expressed in zebrafish embryos is recognized by the anti-HA antibody. To check this point, we injected *dnd-HAHA-nos1 3'UTR* RNA or *dnd-HA-nos1 3'UTR* RNA (each at the concentration of 150ng/μl) into the one cell stage zebrafish embryo and 6 hours later transferred the embryos into loading buffer for SDS-PAGE followed by immunoblotting (Figure 2-13 D, right side). Indeed, HA-tag fused to Dead end is recognized specifically (at about 50 kDa) by the anti-HA antibody. Furthermore, the double HA-tagged Dead end shows higher detection sensitivity than single HA-tagged version.

To determine whether Dead end-HAHA protein can be immunoprecipitated using anti-HA antibody, we injected *dnd* MO (1 ng) and *dead end-HAHA-SV40 3'UTR* RNA (at a concentration of 150ng/μl) to knock down the endogenous Dead end and for expression of the tagged version in the whole embryo. Using 160 embryos of the stage 50-90% epiboly (6-8 hours post fertilization), Dead end-HAHA could be specifically immunoprecipitated and detected after IP and IB (Figure 2-13 D left side).

In conclusion, these results demonstrate that the double-HA-tagged version of Dead end in combination with the anti-HA antibody could serve as a system for the immunoprecipitation of Dead end interacting proteins and RNAs. Since we overexpressed Dead end-HAHA in the whole embryo for the immunoprecipitation but are interested in the co-immunoprecipitation of PGC specific factors together with Dead end-HAHA from germ cells only, we estimated to use a minimum of 3000 embryos when Dead end-HAHA is expressed in the germ cells. For the isolation of Dead end interacting factors, we injected two pools of each 3000 zebrafish embryos with *dead end-HAHA-nos1 3'UTR* RNA (at a concentration of 150ng/μl) together with



*dnd* MO (1 ng) to knock down endogenous Dead end and rescuing PGCs with the tagged version expressed specifically in the PGCs. As control, we used the same number of noninjected embryos, which express non-tagged Dead end. Embryos were lysed, subjected to co-immunoprecipitation and eluted with HA-peptide (Figure 2-13 E and F). One pool was used for SDS-PAGE for mass spectrometry (MS) to identify Dead end interacting proteins and several strong bands, observed after silver staining, are currently analyzed by MS (Figure 2-13 F). The second pool was used for the isolation of RNA and synthesis of amplified cDNA for the isolation of Dead end interacting RNAs (Figure 2-13 E and F). The PCR-amplified RNAs from control- and experiment-IP, will be molecularly subtracted and candidate genes will be analyzed by sequencing, in situ hybridization and knock-down experiments (Figure 2-13 E).

## 3. Discussion

### 3.1 Germ Cell Migration

In the first part of this work, we have addressed the question of how germ cells are guided from their site of specification towards the region where the somatic part of the gonad will develop. In a large-scale antisense oligonucleotide based screen and in a forward genetic screen, we and others have shown that the chemokine SDF-1a and its receptor CXCR4b are essential and sufficient for guiding the migration of primordial germ cells from their site of specification to the region of the zebrafish gonad (Doitsidou et al., 2002; Knaut et al., 2003). To investigate the basis for germ cell guidance in other species, we have chosen to examine the function of SDF-1 in this process in the chick and mouse model systems as avian and murine/mammalian representatives. We did not make similar attempts in the fruit fly *Drosophila melanogaster*, which is a popular system for studying PGC migration and guidance (Coffman et al., 2002; Kunwar et al., 2003; Moore et al., 1998; Starz-Gaiano, 2002; Starz-Gaiano et al., 2001; Starz-Gaiano and Lehmann, 2001; Stein et al., 2002; Zhang et al., 1997), since chemokines and their receptors are not found in the genome of this model organism.

### 3.2 The Role of the Chemokine SDF-1 in Avian Germ Cell Migration

The migration path of PGCs in chick is remarkably similar to that taken by leukocytes during normal development and disease as well as by metastatic cells. In this study, we provide evidence that implicates SDF-1 $\alpha$  in guiding chicken germ cells as they leave the blood vessels on their way to the region of the gonad.

Ectopic expression of SDF-1 $\alpha$ , but not SDF-1 $\beta$ , could attract PGCs at the time at which they migrate through the endothelium but not in earlier stages. Our interpretation of these results is that SDF-1 does not play a role in PGC migration before stage HH 11, nor is it responsible for the commonly observed early ectopic localization of PGCs in the head region (Nakamura et al., 1988), as could have been inferred from SDF-1

expression domains at early developmental stages. We therefore favor the idea that early migration of chick PGCs, as well as the early ectopic positioning of PGCs, reflects the function of another signaling molecule or results from non-guided cell movements. As we were not able to label the PGCs simultaneously with germ cell markers and CXCR4, we could not exclude the formal possibility that the cells do not respond to SDF-1 at these stages due to lack of receptor on their cell membrane.

The proposed role for SDF-1 in migration of chick PGCs is highly reminiscent of the mechanism underlying the migration of hematopoietic precursor cells and lymphocytes as they exit the blood vessels. Here, SDF-1 stimulates integrin-mediated arrest of CD34 expressing cells (Peled et al., 1999) and mediates transendothelial migration of T lymphocytes (Phillips and Ager, 2002) possibly through rapid increase of integrin avidity (Campbell et al., 1998; Grabovsky et al., 2000).

### **3.3 The Role of the Chemokine SDF-1 in Murine Germ Cell Migration**

In collaboration with other groups, we could show that in mice colonization of the gonad by germ cells and PGC survival were impaired in animals lacking functional SDF-1 or CXCR4 (Molyneaux et al., 2003) (similar findings were reported by another group (Ara et al., 2003)). Since PGCs are still migrating in the direction of the gonad of animals lacking functional SDF-1 or CXCR4 and were properly positioned in the hindgut before the stage of directed migration towards the gonads, it was proposed that SDF-1 might be complemented by other chemokines or cytokines that play a redundant role during early steps of PGC migration (Ara et al., 2003). To address this notion, we examined the expression pattern of mouse SDF-1 $\alpha$  and SDF-1 $\beta$  with respect to the position of the germ cells by *in situ* hybridization from developmental stages E8.5 to E11.5. At E9, before the PGCs migrate dorsally towards the point where they exit the gut, *sdf-1* expression and the position of germ cells in the gonad are clearly distinct. Since these data show that most PGCs are distant from SDF-1 expression domain prior to the time point at which they migrate dorsally (E9-E9.5), we conclude that SDF-1 does not play a role in guiding the cells anteriorly along the ventral side of the hindgut. The migration of the cells to the position adjacent to the genital ridge is therefore guided by other chemokines or cytokines.

The observation that transepithelial migration of mouse PGCs occurs specifically in the

dorsal-most part of the hindgut, where *sdf-1* is expressed, is consistent with the suggestion that the cells are attracted towards this region. Interestingly, cells located at ventral positions never exit the hindgut despite the fact that they clearly bounce against its walls. It would be interesting to determine whether in analogy to the situation in *Drosophila* (Jaglarz and Howard, 1994; Jaglarz and Howard, 1995; Starz-Gaiano and Lehmann, 2001), only parts of the mouse hindgut epithelium become competent for transmigration due to specific alterations in epithelial morphology.

### **3.4 Conservation of the mechanism of PGC guidance**

The demonstration that SDF-1/CXCR4 signaling is important for germ cell migration in birds (aves), together with similar findings in ray-finned fish (actinopterygii) and mammals (mammalia) suggest that this mechanism had evolved before the phylum Chordata diverged. Moreover, the requirement for seven transmembrane domain receptors for PGC migration could represent a more ancient mechanism since *Drosophila* PGCs, which transmigrate through the gut epithelium on their way to the gonad also appear to depend on the function of such proteins (Starz-Gaiano, 2002). Despite the apparent evolutionary conservation with respect to the use of the CXCR4-SDF-1 signalling in PGC migration, an important difference should be pointed out. In contrast to the role of SDF-1a in fish, which is essential for PGC guidance throughout their migration (Doitsidou et al., 2002), chicken and mouse germ cells appear to perform the first phases of migration independently of SDF-1 function (Molyneaux et al., 2003) and this work). Furthermore, the function of SDF-1 in PGC migration is required for different processes in PGC development in different organisms. Whereas in zebrafish, SDF-1a is the key guidance cue that directs PGCs towards the developing gonad, germ cells in mice are initially directed by another signaling cue and depend on SDF-1 function for survival.

### **3.5 Basis and Relevance to screen for Germ Plasm Components: The Identification of *dead end***

The second part of this work is focused on a better understanding of the molecular mechanism involved in germ cell specification, motility and fate maintenance by the identification of new germ plasm/germ cell components.

Despite the fact, that germ plasm was found in a wide range of species, its precise composition and biochemical function are poorly understood. Interestingly, the molecular composition of the germ plasm appears to be conserved across species as exemplified by the *vasa* gene whose RNA or protein serves as a universal molecular marker for the germline (Raz, 2000). Moreover, in some cases it has been demonstrated that certain germ plasm components play an analogous role in the development of PGCs in evolutionary distant organisms (e.g. the Nanos protein in *Drosophila*, *Caenorhabditis elegans* and zebrafish (Kobayashi et al., 1996; Kopranner et al., 2001; Subramaniam and Seydoux, 1999; Wang and Lehmann, 1991)). While many germ plasm components have originally been identified based on their role in PGC specification and development or their distribution in the early embryo, the same molecules appear to function also at later steps of germ cell development. For example, *Vasa* which is important for early PGC formation in *Drosophila* is also expressed during oogenesis stages where it is important for cyst development and oocyte differentiation (Hay et al., 1988; Kobayashi, 1998; Lasko and Ashburner, 1988; Styhler et al., 1998; Tomancak et al., 1998). Several molecules expressed in the germ plasm of lower organisms are known to be expressed in mammalian germline cells upon arrival at the region of the gonad and thereafter. Furthermore, these molecules are found within structures resembling germ plasm (Eddy, 1975; Noce et al., 2001). The requirement for the function of such molecules at later stages is revealed by spermatogenesis defects in mice deficient for *Vasa* (Tanaka et al., 2000) as well as by male fertility defects in humans deficient for *DAZ*, which belongs to a gene family whose members are expressed in the germ plasm of *Xenopus* (Houston and King, 2000b; Houston et al., 1998; Xu et al., 2001).

Thus, identification and functional analysis of additional germ plasm/germ cell components should elucidate the molecular mechanisms of early and late germline development. Such molecules could also be important for gametogenesis in mammals with relevance for infertility syndromes in humans.

To identify such molecules and analyze the molecular basis of PGC specification, motility and fate maintenance, we have benefited from a large scale *in situ*

hybridization screen to identify new RNAs that are expressed specifically in PGCs. One of the genes isolated in this screen is the putative RNA binding protein Dead end. This gene is expressed in the PGCs of zebrafish during the first days of their development (Weidinger et al., 2003).

### **3.6 *dead end*, a Germ Plasm Component is Associated with Perinuclear Granules**

*In situ* hybridization experiments to visualize *dead end* RNA show specific localization/expression of *dead end* RNA in the maternally provided germ plasm and in primordial germ cells (Weidinger et al., 2003). We examined the subcellular localization of Dead end protein and show that Dead end-GFP fusion protein is localized to the perinuclear granules. Hence, Dead end is localized to the same cellular structure where other zebrafish germline-specific proteins (e.g. Vasa and Nanos) are found (Knaut et al., 2000; Kopranner et al., 2001). Similar germ granules that contain multiple putative RNA binding proteins are found in germline cells of many organisms and intriguingly, in *C. elegans* these structures are found in association with nuclear pores (Pitt et al., 2000). It has been proposed that germ granules regulate gene expression in PGCs posttranscriptionally, by modifying mRNA transport, stability and translation (Houston and King, 2000a; Seydoux and Strome, 1999). The existence of a putative RNA-binding domain in the Dead end protein and its localization to germ granules make it likely that *dnd* functions in one of these processes (Weidinger et al., 2003; Youngren et al., 2005).

### **3.7 Dead end and Germ Cell Specification**

To investigate if germ cell specification and/or proliferation depend on Dead end protein levels, we over-expressed *dead end* in zebrafish embryos. As the number of germ cells was not altered by this treatment, we conclude that an increased expression of Dead end protein is not sufficient to regulate PGC specification or proliferation. Consistently, inhibition of *dead end* mRNA translation (see below) did not affect PGC specification. It should be noted however that injection of morpholinos

antisense oligonucleotides into early embryos does not interfere with the function of proteins deposited into the egg during oogenesis. Thus, it is formally possible that maternally provided Dead end protein does play a role in PGC specification.

### 3.8 Dead end is Important for PGC Motility

In most species, PGCs migrate from the site at which they are specified towards the developing gonad (Starz-Gaiano and Lehmann, 2001). Zebrafish PGCs start to migrate shortly before gastrulation and follow six distinct migration steps before arriving at the region where the gonad develops (Doitsidou et al., 2002; Weidinger et al., 1999a; Weidinger et al., 2002; Yoon et al., 1997).

To determine the role of *dead end* in PGC development, knock-down experiments were conducted in which the translation of the mRNA was inhibited using morpholino antisense oligonucleotides. In these experiments, it was found that in early developmental stages, *dead end* is essential for the active migration of PGCs, one of the earliest manifestations of PGC identity. In knock-down embryos, PGCs are not motile and are carried along with somatic cells that perform gastrulation movements.

Nevertheless, in most cases, Dead end-depleted PGCs did show some morphological features of motile cells. Specifically, while formation of lamellipodia was strongly reduced, the number of PGCs extending filopodia was less affected compared to controls (Weidinger et al., 2003) and competence to stabilize long atypical protrusion is elevated during somitogenesis. Taken together, functional Dead end is required for one of the fundamental properties of PGCs, which is their ability to migrate. To the best of our knowledge this is the first description of a gene product required for the transition of PGCs from stationary to motile cells. It will therefore be interesting to identify the proteins and RNAs with which Dead end interacts as these may provide a better understanding of the molecular basis of the control of epithelial-mesenchymal transition, motility and migratory cell behavior of germ cells *per se* (Dumstrei et al., 2004; Reichman-Fried et al., 2004; Thorpe et al., 2004). Importantly, the dead end knock-down phenotype is strikingly different from that described for PGCs lacking CXCR4b signaling in zebrafish (Doitsidou et al., 2002; Knaut et al., 2003). While showing severe defects in directional migration, zebrafish PGCs lacking CXCR4b are motile and exhibit active migration relative to their somatic neighbors (Doitsidou et al., 2002; Knaut et al., 2003).

### 3.9 Dead end is Essential for PGC Survival

At low magnification, we observed that GFP expressing PGCs in which Dead end was knocked down disappeared by the end of the first day of development. To trace the fate of these PGCs, we tracked individual germ cells in live embryos and found that PGCs in Dead end knockdown embryos die after exhibiting the morphological hallmarks of apoptotic cells (Rich et al., 1999), including membrane blebbing and fractionation into small apoptotic bodies. Remarkably, a similar effect could be demonstrated when the zebrafish *nanos-1* expression was knocked down (Koprunner et al., 2001) but physical or genetic interaction would have to be done to prove a functional interaction between these two genes. As PGCs in Dead end-depleted zebrafish embryos do not maintain their fate, it could be possible that these cells transdifferentiate prior to their death. We show that germ cells in *dead end* knock down embryos maintain the perinuclear granules, a cellular structure specific for germ cells (Knaut et al., 2000; Pitt et al., 2000), until to the time of cell fractionation. Even though germ cells start to lose the expression and stabilization of germ cell specific genes several hours before they die (Weidinger et al., 2003), this cells maintain at least partially their germ cell specific fate. Recent finding have shown that the *Ter* allele, which has been mapped to the mouse chromosome 18 (Asada et al., 1994) encodes for the mouse homologue of the zebrafish *dead end* (Youngren et al., 2005). The teratoma (*Ter*) mutation in laboratory mice shows germ cell deficiency in all inbred strain backgrounds that have been examined (Asada et al., 1994; Stevens and Hummel, 1957; Stevens and Little, 1954) and causes dramatically increased susceptibility to spontaneous testicular teratocarcinomas in strain 129 inbred mice (Asada et al., 1994; Noguchi and Noguchi, 1985; Stevens, 1970; Stevens, 1973; Stevens and Hummel, 1957; Stevens and Little, 1954).

Taken together, Dead end deficient germ cells lose their fate and die whereas under specific genetic background conditions, some germ cells in mice survive, and form teratocarcinomas. The strong similarities between zebrafish and mouse *dead end* loss of function phenotypes suggest that Dead end plays a conserved role in both species. We conclude that Dead end plays a key regulatory function essential for germ cell fate maintenance and that molecular analysis of Dead end function will contribute to the understanding of the mechanisms responsible for the unique features of these cells (Kanatsu-Shinohara et al., 2004; Matsui et al., 1992).



### 3.10 Dead end, PGCs and Zebrafish Sex Determination

Whereas germ cells in *Dead end* depleted embryos undergo apoptosis at the end of the first day of development, no effect on the somatic part of the embryo could be observed. Strikingly, when these embryos were raised to adulthood, all the *dead end* knockdown fish turned out to be males. Moreover, morphological analysis of these adult fishes revealed that no gonadal structures are present in the animals.

This finding implicating PGCs in sexual differentiation differs from findings in other organisms such as mouse and *Drosophila*, where both sexual types are generated in animals lacking germ cells (Beck et al., 1998; Jongens et al., 1992). To determine whether development of females is a result of loss of *Dead end* function or reflects the loss of the PGCs, we set out to eliminate these cells by different means. For that purpose we have developed a novel method for targeted cell ablation in zebrafish that was applied to the PGCs. This method is based on the bicistronic protein killer bacterial system *parD*. Preferential expression of the toxin *kid* in the PGCs, while protecting somatic cells by expression of the natural antidote *kis*, allowed specific ablation of the PGCs. We show that embryos depleted of the germ cells with this method develop into sterile male fish similar to the findings in *dead end* depleted animals. Thus it is not a direct function of *Dead end* but rather the PGCs that are important for the development of the gonad which in turn is essential for development of female fish (this work was done in collaboration, see thesis of Krasimir Slanchev and (Slanchev et al., 2005)).

## 4. Summary & Conclusions

In this thesis, I studied the function of SDF-1 in germ cell migration in the chick and the mouse embryo. As in many other animals, the primordial germ cells in the avian embryo are specified in positions distinct from the positions where they differentiate into sperm and egg. Unlike in other organism however, in these embryos the PGCs use the vascular system as a vehicle to transport them to the region of the gonad where they exit the blood vessels and reach their target. We show that *sdf-1* mRNA is expressed in locations where PGCs are found and towards which they migrate at the time they leave the blood vessels. Ectopically expressed chicken SDF-1 $\alpha$  led to accumulation of PGCs at those positions.

In the mouse embryo, we show that SDF-1 is expressed in locations where PGCs are found when these cells transmigrate the hindgut epithelium and migrate towards the genital ridge, but not during earlier stages. This expression based analysis as well as analysis of PGC behaviour in the mouse embryo suggests that similar to our findings in chick, SDF-1 functions during the second phase of PGC migration, but not at earlier phases.

Whereas in zebrafish, SDF-1a is essential and sufficient for the guidance of PGCs towards the developing gonad, germ cell migration in mice lacking SDF-1 function is impaired but still some cells colonize the gonad. We therefore assume, that germ cells in mice are directed by another signaling cue.

While SDF-1 signaling is important for directional migration of PGCs in zebrafish, cell motility requires the function of *dead end*. We show that *dead end* RNA resides where the germ plasm is located, becomes restricted to the germline during the first hours of development. Dead end protein is localized to the perinuclear granules. Overexpression of *dead end* does not alter germ cell specification and number. We demonstrate that germ cells in Dead end depleted zebrafish embryos are not motile and die by the end of the first day of development resembling cells undergoing apoptosis. Finally, we show that the expression of *dead end* in the germ plasm or in germ cells is conserved among different vertebrate species. Recent finding have shown that the *Ter* locus encodes the mouse homologue of the zebrafish *dead end*. Mutation in this gene causes a dramatic increase in the susceptibility to spontaneous testicular teratocarcinomas in strain 129 inbred mice and leads to germ cell death in other strains. The strong resemblance between the zebrafish and the mouse phenotypes implies that Dead end plays a conserved role in both species.

Currently, the precise molecular function of Dead end is not known. The analysis molecules with which Dead end interacts will contribute to a deeper understanding of the function of Dead end.

# 5. Material and Methods

## 5.1 Bacteria

<i>E. Coli</i> Top 10F'	Invitrogen
<i>E. Coli</i> ElectroTen-Blue	Stratagene
<i>E. Coli</i> ElectroMAX DH10B	Invitrogen
<i>E. Coli</i> DH5 $\alpha$	Invitrogen

## 5.2 Chemicals

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

anti-DIG antibody	Roche 1093274
anti-fluorescein antibody	Roche 1426338
DIG RNA labeling Mix	Roche 1277073
DAB Diaminobenzidine	SIGMA
ECL	ECL Western Blotting Detection Reagent, Amersham RPN2109
RNA isolation	Trizol, Invitrogen
Pistil	Dstroy-S-16, Biozyme

## 5.3 Kits

ABC-Kit Vectastain	Vector Laboratories
anti-HA Affinity Matrix	Roche
mMessage mMachine Kit	Ambion
Topo-TA Cloning Kit	Invitrogen
UltraClean <sup>TM</sup> 15 DNA Purification Kit	MO BIO
QIAquick Gel extraction Kit	Quiagen
OmniScript Kit	Quiagen
SuperSMART PCR cDNA Synthesis and Creator SMART cDNA Library Construction	Clontech
FLAG Tagged Protein Immunoprecipitation Kit	SIGMA
Advantage HF 2 PCR Kit	Clontech

QIAquick PCR purification Kit	Qiagen
QIAfilter Plasmid Mini/Midi/Maxi Kit	Qiagen
5'/3'Race system for Rapid Amplification of cDNA Ends Vers. 2.0,	Invitrogen

## 5.4 Primary and Secondary Antibodies

No	Primary antibody	Specificity	Description	Company	Assay and used dilution
P2	Anti-vasa K12/4	Zebrafish Vasa	Rabbit polyclonal	From Holger Knaut	Immunostaining 1:1500
P4	Anti-HA	HA-tag	Mouse Monoclonal (12CA5)	Roche	Immunostaining: 1:200 WB: 1:1000
P11	Anti-HA	HA-tag	Rat Monoclonal	Roche	Immunostaining: 1:200 WB: 1:1000
P12	Anti-FLAG	FLAG-tag	Mouse Monoclonal	Sigma	WB: 1:1000

No	Secondary antibody	Specificity	Description	Company	Assay and used dilution
S7	HRP	Goat-anti-rabbit IgG	HRP conjugated Polyclonal	Vector Laboratories	WB: 1:5000
S9	Alexa Fluor 546 (orange)	Goat-anti-mouse IgG	Alexa 546 conjugated Polyclonal	Molecular probes	Immunostaining 1:200
S10	Biotinylated	Horse-anti-mouse IgG	Biotin- conjugated Polyclonal	Vector Laboratories	WB: 1:10'000
S11	Cyanine (green)	Goat-anti-rabbit IgG	Cy2- conjugated Polyclonal	Jackson Immunoresearch	Immunostaining: 1:200
S14	Biotinylated	Donkey-anti-rat IgG	Biotin- conjugated Polyclonal	Jackson Immunoresearch	WB: 1:10'000

## 5.5 DNA Constructs used in this work

DNA constructs produced in this work		
Plasmid No.	Name	Description
651	pSP6-nanos1-pT7	Nanos1 without 3'UTR; for antisense probe
667	pSP6-Chv-pT7	Chicken homologue of vasa; for antisense probe
668	pSP6-mStella-pT7	Mouse Stella; for antisense probe
669	pSP6-mTNAP-pT7	Mouse Tissue nonspecific alkaline phosphatase; for antisense probe

## Material and Methods

670	pT7-mSDF-1alpha-pSP6	Part of mouse SDF-1alpha 3'UTR; for antisense probe
671	pSP6-mSDF1beta-pT7	Part of mouse SDF-1beta 3'UTR; for antisense probe
672	pSP6-mCXCR4-pT7	Part of mouse CXCR4; for antisense probe
678	Xdead end	Xenopus dead end; for sequencing the ORF
693	pCRIITopo-chCXCR4	Chick CXCR4; for antisense probe
707	CMV-chSDF1a	Chick SDF1a in pcDNA3.1(-); for expression in cells and chick embryos
742	CMV-chSDF1b	Chick SDF1b in pcDNA3.1(-); for expression in cells and chick embryos
775	deadend-HA-SV40 3'UTR in pCS2+	Dead end tagged with HA; for expression in cells and embryos
776	deadend-HAHA-SV40 3'UTR in pCS2+	Dead end tagged with HAHA; for expression in cells and embryos
777	deadend-FLAG-SV40 3'UTR in pCS2+	Dead end tagged with FLAG; for expression in cells and embryos
778	deadend-FLAGFLAG-SV40 3'UTR in pCS2+	Dead end tagged with 2xFLAG; for expression in cells and embryos
857	deadend-HAHA-nos1 3'UTR in pCS2+	Dead end tagged with HAHA; for expression in PGCs

Other plasmids used in this work		
Plasmid No.	Name	Description
40	pCS2+	Expression vector for DNA driven by the simian CMV IE94 enhancer/promoter and for RNA transcription from SP6 (sense) or T7, T3 (antisense)
355	pSP6-GFP-nos1 3'UTR	GFP fused to nos1 3'UTR for expression of GFP in the PGCs specifically
363	Supergerm Red	N-terminal Vasa protein fragment sufficient for localization to germ granules fused to DsRed, RNA stabilized in PGCs by nanos 3'UTR; efficiently labeling of PGCs in red
393	EGFP-F-globin 3'UTR	Farnesylated GFP protein is localized to the plasma membrane in mammalian- and zebrafish-cells; for GFP labelling of somatic tissue
487	RN3 rescORF deadend-globin 3'UTR	dead end ORF with Morpholino 2 binding site mutated that Morpholino2 can not bind - amino acids are not changed; for rescue and o/e experiments
493	pSP64 EGFP-F nos1 3'UTR	Expression of farnesylated EGFP to label membrane-localized germ cells by the nos1 3'UTR
495	SP6 ded nos1 3'UTR	For expression of full-length zebrafish dead end protein insensitive to the dead end morpholino 2, specifically expressed in PGCs driven by the nos1 3'UTR
516	RN3 dedGFP nos1 3'UTR	Dead end-GFP fusion protein expressed specifically in PGCs by the nos1 3'UTR and localized in the perinuclear granules.
640	pSP6-SDF1a-nos1 3'UTR	For o/e of zebrafish SDF-1a in PGCs
652	pT7-chickded-pSP6	Chick homologue of dead end; for antisense probe
653	pT7-chickSDF1-pSP6	Chick SDF-1; for antisense probe, unspecific for SDF-1a or SDF-1b

## 5.6 Equipment

Cameras	- RT slider Spot, Diagnostic Instruments - Leica DC 300, Leica - RT SE Spot, Diagnostic Instruments
Injector	- PV830 Pneumatic PicoPump, World - Precision Instruments (USA)
Needle puller	PN-30 Microelectrode Puller, Science Products
PCR machines	- Cyclone 96, Peqlab, Erlangen - Mastercycler Personal, Eppendorf
Microscopes	- Leica MZ FLIII, Leica - Zeiss Axioplan 2, Zeiss - Leica confocal microscope DMRXE - Olympus SZX12
Centrifuges	Eppendorf 5415D, Eppendorf
Spectrophotometer	Eppendorf 6131, Eppendorf
Western blotting	- Mini-PROTEAN 3, BioRad - Mini-Trans-Blot Cell, BioRad

## 5.7 Programs, Database,

Image processing	Adobe Photoshop 7.0, Adobe
Microscopy	- Metamorph, Universal Imaging Corp. - Leica confocal software, Leica
Multiple sequence alignment	Sequencher, Gene Codes Corp.
Cloning	Vector NTI, Invitrogen (USA)
Office application	Microsoft
Literature	PubMed <a href="http://www.ncbi.nlm.nih.gov">www.ncbi.nlm.nih.gov</a>
Databases	- Endnote 8.0, Thomson - FileMaker Pro 7, FileMaker Inc.

BLAST-programs	- bl2seq	<a href="http://www.ncbi.nlm.nih.gov">www.ncbi.nlm.nih.gov</a>
	- blastn	
	- blastp	
	- tblastn	
1/2/3D protein analysis	ExPASy Proteomics Server	<a href="http://www.expasy.org">www.expasy.org</a>
	EMBL-EBI	<a href="http://www.ebi.ac.uk/services/">www.ebi.ac.uk/services/</a>
Multiple sequence alignment	clustalW	<a href="http://www2.ebi.ac.uk/clustalw">www2.ebi.ac.uk/clustalw</a>
Molecular Modeling	- AMBER7	
	- VMD 1.8	

## 5.8 Molecular Biology

### 5.8.1 Plasmid DNA Isolation from *E. coli*

*E. coli* containing a certain plasmid were inoculated into 5 ml (Miniprep), 50 ml (Midiprep) or 150 ml (Maxiprep) LB Standard Medium (1% Bacto-Tryptone (Gibco BRL), 0.5% Bacto-Yeast Extract (Gibco BRL), 1% NaCl in Millipore water) with 50 µg/ml ampicillin or kanamycin, and incubated at 37 °C shaking for 16 hours. The plasmid was extracted using Plasmid Mini, Midi or Maxi Kit (Qiagen) according to the manufacturer instruction, eluted in millipore water and the concentration was measured using a BioPhotometer (Eppendorf).

For transcription, cell transfection and chick embryo electroporation the Mini/Midi/Maxiprep DNA was further purified by extraction with 1 volume of phenol/chloroform/isoamylalcohol (25:24:1) once or twice, followed by 13,500 rpm centrifugation for 10 minutes. The supernatant was carefully collected and subjected twice to 1 volume of chloroform, again followed by centrifugation and supernatant collection. Then, the DNA was precipitated with 0.1 volume of 3 M NaAc, pH 5.2 and 2.5 volume of ethanol 100 % on ice for 30 min or for low amounts of DNA at -20 °C for 15 minutes. Afterwards, the DNA was pelleted by centrifugation at 13,500 rpm and 4 °C for 15 minutes, washed with 1 ml 70% ethanol, centrifuged at 13,500 rpm for another 5 minutes, air dried, and dissolved in a proper volume of Millipore H<sub>2</sub>O.



### **5.8.2 Total RNA Isolation from Eukaryotic Cells or Embryos**

Total RNA from cells or zebrafish embryos was isolated using Trizol as described by the manufacturer.

### **5.8.3 DNA and RNA Electrophoresis and Purification from Agarose Gel**

0.5-2% agarose gel was prepared by melting agarose (Biozym) in 1×TAE Buffer (400 mM Tris, 0.2M Acetic acid, 10 mM EDTA, pH 8.0) and subsequently adding ethidium bromide to a final concentration of 0.3 µg/ml. DNA and RNA sample were mixed with 5×DNA Loading Buffer (25% Ficoll, 100 mM EDTA, 0.05% Bromophenol Blue), and electrophoresis was performed under 1-7 V/cm in 1×TAE buffer.

For DNA isolation, the DNA band was cut from the gel and DNA was isolated using the UltraClean™ 15 DNA Purification Kit (MO BIO) or the QIAquick Gel extraction Kit as described by the manufacturers.

### **5.8.4 DNA Digestion with Restriction Enzymes**

For DNA analysis, about 50-200ng DNA was digested with 3-10 U restriction enzymes at the appropriate temperature for 45-60 minutes. For DNA preparation, 1-10 µg DNA was incubated with 20-40 U restriction enzymes at the appropriate temperature for at least 4 hours.

### **5.8.5 Dephosphorylating and Blunting of DNA Fragment**

5'-ends of DNA fragment dephosphorylation was performed by directly adding 1 µl Alkaline Phosphatase (1U/µl, Roche) into the restriction enzyme digestion mixture and incubating at 37 °C for 1 hour.

DNA polymerase I large fragment (Klenow fragment) (5U/µl, NEB) was used to fill-in the ends of 5'-overhang DNA fragment. DNA in restriction enzyme NEBuffer supplemented with 33 µM dNTPs was incubated with Klenow at a concentration of 1 U per µg DNA at 25 °C for 30 minutes.

3'-overhang DNA fragment was blunted using T4 DNA Polymerase (5U/µl, Roche) in the restriction enzyme NEBuffer supplemented with 50 µM dNTPs.

### 5.8.6 Ligation

25-100 ng purified vector fragment was mixed with 3-10 folds (molecular ratio) of purified insert fragment, 1  $\mu$ l 10 $\times$  T4 DNA ligase buffer (MBI Fermentas), and 1  $\mu$ l T4 DNA ligase (3 U/ $\mu$ l, MBI Fermentas) in a total volume of 10  $\mu$ l. This ligation mixture was incubated at RT for at least 2 hours or overnight at 16  $^{\circ}$ C.

Alternatively, insert of e.g. PCR fragments were cloned by TOPO cloning according to the standard protocol of the Topo-TA Cloning Kit (Invitrogen) and PCR II, PCR 2.1 and PCR 4 vectors were used.

### 5.8.7 Standard PCRs

For standard PCR, primers were designed with 15-24 nucleotides and a melting temperature ( $T_M$ ) between 50  $^{\circ}$ C and 68  $^{\circ}$ C. The reaction mixture contains 1  $\mu$ l template DNA (10pg-100ng DNA), 2.5  $\mu$ l of forward primer (5mM), 2.5  $\mu$ l of reverse primer (5mM), 2.5  $\mu$ l 10 $\times$  Amersham PCR Buffer, 2.5  $\mu$ l 2.5 mM dNTPs, and 1  $\mu$ l Tag Polymerase (5U/ $\mu$ l) in a total volume of 25  $\mu$ l.

The thermocycling program (Table 5-1) was carried out as described below.

Step	Temperature	Duration
1. Initial Denaturation	92 $^{\circ}$ C	1 minute
2. Denaturation	92 $^{\circ}$ C	15 seconds
3. Annealing	$T_M - 4$ $^{\circ}$ C	20 seconds
4. Annealing and Elongation	72 $^{\circ}$ C	1 minute/kb
5. Go to Step 2, 15-30 cycles		
6. Final Elongation	72 $^{\circ}$ C	3-15 minutes

**Table 5-1:** The thermocycling program for standard PCR.

### 5.8.8 High Fidelity PCRs

The Advantage HF 2 PCR Kit (Clontech) was used for high fidelity PCR and PCRs starting from low amount of template DNA according to manufactures instruction.

### 5.8.9 Amplification of 5' and 3' End of cDNAs

To amplify 5' and 3' ends of cDNAs the 5'/3'Race system for Rapid Amplification of cDNA Ends Vers. 2.0 (Invitrogen) was used according to manufactures instructions.

### 5.8.10 Standard Reverse Transcription

The OmniScript Kit (Qiagen) was used to reverse transcribe RNA to cDNA. The reaction mixture contains 5 ng to 2 µg template RNA, 2 µl Oligo-dT-primer (10µM), 2 µl 10× Buffer RT, 2 µl dNTPs mix (5 mM each dNTP), 1 µl RNase inhibitor (10 U/µl) and 1 µl Omniscript reverse transcriptase in a total volume of 20 µl. Reaction mixture was incubated for 60 minutes at 37 °C.

### 5.8.11 PGC specific full length cDNA library synthesis

To synthesis a zebrafish PGC specific full-length cDNA library, 635 single germ cells from the stage 10 somite – 24 hpf were isolated according to the procedure in Table 5-2.

---

1. Injection of fish in the evening with 80 – 120 ng/ml 355 (GFP-nos1 3'UTR) to label PGCs with GFP

---

Incubation of embryos until stage 10 somite – 24 hpf

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2. Embed the tube of a syringe into dry ice within a polystyrol box and place a 1.5 ml tube into the tube to collect the isolated cell in a frozen drops of TM1 buffer (100mM NaCl, 5mM KCL, 5mM Hepes, 1%PEG 20000, pH 7.0, 0.2 micron filtered) or RNAlater (Qiagen).

---

3. Pump with the installed syringe mineral oil (SIGMA M-5904) through the “Transplantation-Manual injector” tube system to remove air bubbles, cut a glass needle and install it into the “TM injector”

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4. Choose under the binocular 5 – 7 embryos with good-labelled PGCs in the right position in the embryo and dechorionate them.

---

5. Transfer embryos with a 200 µl pipette-tip into a 10 cm diameter glass petridish with about 450 µl TM1 buffer in the middle. Pipet up and down “the embryos” (keep pipet rectangular) and check from time to time under the binocular if cells are dissociated well. Let cells settle down for about 5 min.

---

6. Search via GFP under the binocular the germ cells, bring needle with the micromanipulator to the germ cell and suck it with the Transplantation-Manual injector.

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7. Transfer cell into a 1-2 µl drop (TM1 buffer or RNAlater), which is placed onto a coated glass slide (SIGMAcoate).

## Material and Methods

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8. Place glass slide into dry ice filled polystyrol box and scratch with a scalpel-tip the ice drop containing the cell into the e-cup.

9. Store cells at – 80 °C until RNA isolation with Trizol will be done.

---

**Table 5-2:** Single PGC isolation.

RNA of isolated PGCs was than purified and isolated according to the manufactures instruction with Trizol (Invitrogen) with modifications as described below (Table 5-3).

---

1. Add 10 times Trizol (Invitrogen) to the original volume of the sample. Vortex thoroughly.

---

2. Incubate the homogenized samples for 5 minutes at RT.

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3. Add 0.2 ml of chloroform per 1 ml of Trizol Reagent.

---

4. Shake tubes vigorously by hand for 15 seconds and incubate them at RT for 2 to 3 minutes.

---

5. Centrifuge the samples at maximum speed for 15 minutes at 4°C.

---

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

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7. Transfer the aqueous phase to a fresh 1.5 ml tube. Add 7.5 ug (= 0.5 ml) glycogen and add 0.8 volume of isopropanol to each tube.

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8. Incubate samples over night at -20 °C.

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9. Centrifuge at maximum for 15 minutes at 4 °C. (The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.)

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

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11. Remove with a thin pipette all remaining liquid and air-dry the RNA pellet for 5 min. Dissolve RNA in RNase-free water.

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**Table 5-3:** RNA isolation from less than 1000 germ cells with Trizol.

For reverse transcription, PCR amplification and subsequent directional cloning of full length cDNA into pDNR-LIB vector (Clontech), a combination of SuperSMART PCR

cDNA Synthesis Kit and Creator SMART cDNA Library Construction Kit (Clontech) was used as described by the manufacturers. For the reverse transcription and cDNA amplification, primer from Creator SMART cDNA Library Construction Kit only were used.

### **5.8.12 Yeast-two-hybrid screen**

In collaboration with the Dualsystems Biotech AG (Zurich) Zebrafish PGC specific full-length cDNA library from the stage of 10 somite – 24 hpf was subcloned into bait vector pGAD-DS and yeast-two-hybrid screen was performed according to their standard procedure.

### **5.8.13 Preparation of Electrocompetent *E. coli* Cells and Transformation by Electroporation**

Preparation of electrocompetent *E. coli* cells was performed as described below (Table 5-4)

1. Inoculate two single colonies of *E. coli* into 2×10 ml LB medium and cultured at 37 °C shaking overnight (250 rpm).
  2. Inoculate the 2×10 ml overnight cultures into 2×1 liter prewarmed LB medium and culture them at 37 °C shaking until the O.D.<sub>600</sub> reached 0.6-0.8 (about 3 hours).
  3. Chill the cells on ice for 10-30 minutes.
  4. Centrifuge at 5,000 rpm at 4 °C for 20 minutes to harvest the cells.
  5. Discard the supernatant, wash each pellet from 1 liter culture with 1 liter prechilled water (1:1 wash), then centrifuge at 5,000 rpm at 4 °C for 20 minutes.
  6. Discard the supernatant, wash each pellet with 100 ml prechilled 10% glycerol (1:10 wash), then centrifuge at 6,000 rpm (Sorvall HS-4 rotor) at 4 °C for 10 minutes.
  7. Discard the supernatant, wash each pellet with 20 ml prechilled 10% glycerol (1:50 wash), then centrifuge at 6,000 rpm at 4 °C for 10 minutes.
  8. Discard the supernatant, wash each pellet with 2 ml prechilled 10% glycerol (1:500 wash), then centrifuge at 6,000 rpm at 4 °C for 5 minutes.
  9. Aspirate the supernatant, resuspend each pellet in 2-3 ml 10% glycerol. 40 µl or
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80  $\mu$ l resuspension was aliquoted into each tube on ice, frozen in liquid nitrogen, and stored at  $-80$  °C.

---

**Table 5-4:** Preparation of electrocompetent *E. coli* cells.

50  $\mu$ l competent cells were thawed on ice and transferred into a prechilled 0.1 cm electrode Gene Pulser Cuvette (Bio-Rad). 1-5  $\mu$ l DNA solution (1-100 ng/ $\mu$ l) or 1-5  $\mu$ l ligation product was added directly into the competent cells and mixed well by gently flicking. Then, the surface of the cuvette was completely dried and the electroporation was performed using Gene Pulser (Bio-Rad) under the condition of 1.8 kV, 200  $\Omega$  resistance, 25  $\mu$ F capacitance. Afterwards, 960  $\mu$ l prewarmed LB medium was immediately supplied to the electroporated *E. coli* for recovery. The cells were recovered at 37 °C rotating for 1 hour, followed by plating 100  $\mu$ l and 900  $\mu$ l on separate selective plates.

## 5.9 Biochemistry

### 5.9.1 Immunoprecipitation (IP)

Lysis buffer (50 mM Tris HCl pH 7.5, 150-200 mM NaCl , 1 mM EDTA, 1 mM EGTA (Sigma), 10 % Glycerol , 1 % Triton X-100) in RNase free water was autoclaved and before use supplemented with 1 mM MgCl<sub>2</sub> , 50 U/ml Rnasin, Roche “ complete mini” Protease inhibitor tablet with EDTA (1 tablet per 10 ml lysis buffer), 1.6 mM vanadyl ribonucleosid complex (Sigma) and 100  $\mu$ g/ml PMSF (Sigma).

It is recommended to use  $10^6 - 10^7$  cells/ml of lysis buffer, which equals to 100 – 1000 80 % epiboly embryos/ml lysis buffer. For IP of 50 – 90 % epiboly embryos, 160 embryos/ml lysis buffer were used and for IP of 28 hpf embryos, 500-1500 embryos/ml lysis buffer were used. Embryo lysate was prepared according to Table 5-5

Embryo Preparation for 28 hpf Embryo Lysis
1. Injection zebrafish embryo at the one cell stage
2. Grow embryos up to 28hpf
3. Dechorionate with forceps
4. Transfer embryos into 50 ml Falcon tube and count them (all done in Danieaus – see chapter “Fish breeding and incubation”)

5. Wash 5x with PBS pH 7. Embryos were sediment by gravity.
6. Remove almost all PBS – pipet with 200 $\mu$ l tip embryos up and down to disrupt yolk
7. Wash embryos about 10 times with PBS. Embryos were sediment by gravity.
8. Transfer embryos into a 1.5 ml eppendorf cup and spin embryos for 10 s at 1200g
9. Remove all supernatant and add e.g 0.5 ml ice cold lysis buffer supplemented with all inhibitors /1000 embryo.
10. On ice: disrupt embryos with a pistil (Dstroy-S-16, Biozyme) and pipette 10 times gently up and down to disrupt all cells (clear lysate should result within 5-10 min)
11. Centrifuge cell lysate for 10 min at 12000g at 4 °C
12. Transfer supernatant into a new pre-cooled eppendorf cup
13. Freeze and store at – 70 °C
<b>Embryo Preparation for 50 % Epiboly Embryo Lysis</b>
1. Injection zebrafish embryo at the one cell stage
2. Grow embryos up to 50 % epiboly
3. Transfer embryos in PBS 25 °C or better cooler
4. Dechorionate by forceps and dissect animal proper from yolk
5. Transfer embryo proper into 1.5 ml eppendorf cup in PBS 4 °C
6. May wash 1x with 4 °C PBS (led embryo proper sediment by gravity – or spin embryos for 5 s at 1000g
7. Remove all supernatant and add e.g 0.8-1 ml ice cold lysis buffer supplemented with all inhibitors/160 embryo.
8. Pipette 3-10 times gently up and down to disrupt all cells and incubate on ice for 5–10 minutes (clear lysate should result)
9. Centrifuge cell lysate for 10 min at 12000g at 4 °C
10. Transfer supernatant into a new pre-cooled eppendorf cup
11. Freeze and store at – 70 °C

**Table 5-5:** Preparation of embryo lysate for immunoprecipitation

For immunoprecipitation, 50 to 100  $\mu$ l beads with precoupled FLAG- or HA-antibody (FLAG Tagged Protein Immunoprecipitation Kit (SIGMA) and anti-HA affinity matrix (Roche) were used as following (Table 5-6).

All the work was done at 4 °C

1. Resin were washed 6 times with 1.5 ml lysis buffer (spined at 10'600 g for 5 seconds).
2. 0.8 – 1 ml of lysate/1.5 ml tube were added to the beads and tagged protein were immunoprecipitated for 4-6 hours in a rotator.
3. Wash beads with lysis buffer with 200mM NaCl: 2x 5 minutes, 2x 10 minutes and 1x 15 minutes.
4. Elution 3x 20 min with 40-80 µl FLAG-peptide (150 ng/µl) or HA-peptide (300 ng/µl) dissolved in lysis buffer with 200mM NaCl.
5. 37.5 µl of elute were mixed with 12.5 µl 4x loading buffer, boiled at 95 °C for 5 minutes, cooled down and loaded on an SDS-PAGE gel.

**Table 5-6:** Procedure of immunoprecipitation.

### 5.9.2 Protein Gel Electrophoresis

SDS-polyacrylamide gels (SDS-PAGE) were prepared with protein gel preparation system (Bio-Rad). 12% separating gel and 6% stacking gel (Tab. X-x) were applied to detect proteins smaller than 50 kDa, while 10% separating gel and 5% stacking gel (Tab. X-x) were used to analyze proteins larger than 50 kDa. A protein sample (e.g. 0.6 zebrafish embryos/µl loading buffer) was mixed with the same volume of 2×SDS loading buffer (125 mM Tris, pH 6.8, 20% glycerol, 0.02% bromophenol blue, 2% β-mercaptoethanol, 4% SDS), and heated at 95 °C for 5 minutes or in boiling water for 3 minutes. A maximum of 25-50 µl sample were loaded into the slot, empty slots were filled with the same amount of loading buffer only and electrophoresis was performed in 1×SDS buffer (25 mM Tris-base, 0.1% SDS, 192 mM glycine, pH 8.75) under 20 mA/gel, following 10-20 minutes preelectrophoresis under the same condition.

Separating Gel	10%	12%
1.5 M Tris, pH 8.8	7.5 ml	6 ml
30% Acrylamide-Bisacrylamide Solution (Roth)	10 ml	10 ml
H <sub>2</sub> O	12ml	7.6 ml
10% SDS	300 µl	240 µl
10% APS	150 µl	120 µl



TEMED	15 $\mu$ l	12 $\mu$ l
<b>Total Volume</b>	<b>30 ml</b>	<b>24 ml</b>

Stacking Gel	5%	6%
1 M Tris, pH 6.8	3 ml	2.5 ml
30% Acrylamide-Bisacrylamide Solution (Roth)	2 ml	2 ml
H <sub>2</sub> O	6.82 ml	5.35 ml
10% SDS	120 $\mu$ l	100 $\mu$ l
10% APS	60 $\mu$ l	50 $\mu$ l
TEMED	12 $\mu$ l	10 $\mu$ l
<b>Total Volume</b>	<b>12 ml</b>	<b>10 ml</b>

**Table 5-7:** The components of SDS-polyacrylamide gels.

### 5.9.3 Coomassie Blue Staining

After gel electrophoresis, for coomassie blue staining, the stacking gel was removed from the resolving gel, which was stained with Coomassie blue staining solution (0.25% Coomassie brilliant blue R 250, 45% methanol, 10% acetic acid) at RT for 0.5-1 hour. The Coomassie stained gel was rinsed twice with water and subsequently destained in destain buffer (5% methanol, 10% acetic acid) for several hours to overnight.

### 5.9.4 Silver Staining for Mass Spectrometry

Silver staining for mass spectrometry was performed as described below (Table 5-8). All reagents have *pro analysis* purity.

Treatment		Solution	Duration
Fixation	Slow agitation	50 % (v/v) Ethanol 12% acetic acid 0.05% (v/v) of 37% formaldehyd	90 minutes to overnight
3x Wash		in 50% ethanol in H <sub>2</sub> O	20 minutes each
Impregnation		0,01 % (w/v) Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> x 5 H <sub>2</sub> O in H <sub>2</sub> O	1 minute exact!!

3x Wash		H <sub>2</sub> O	20 seconds exact!!
Colorization		0.1 % (w/v) AgNO <sub>3</sub> 0.075 % (v/v) of 37% formaldehyd	20 min
3x Wash		H <sub>2</sub> O	20 seconds exact!!
Development		3% (w/v) Na <sub>2</sub> CO <sub>3</sub> , 0,05 % (v/v) of 37% formaldehyd, 2 x 10 <sup>-4</sup> % (w/v) Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> x 5 H <sub>2</sub> O in H <sub>2</sub> O	2 – 10 minutes
Stop		10 mM Na <sub>2</sub> EDTA	10 minutes
Storage		H <sub>2</sub> O for HPLC	days to weeks

**Table 5-8:** Silver staining for mass spectrometry

### 5.9.5 Western Blotting

Western blotting and subsequent immunostaining were performed as described below (Table 5-9) using antibodies against HA-tag, FLAG-tag, Dead end, Nanos and Vasa.

1. Cut a Protran Nitrocellulose Transfer Membrane (Schleicher & Schell BioScience) into a similar size than the gel and 4 pieces of Whatman paper the slightly larger than the gel and presock them in blot buffer (25 mM Tris-base – 3 g/l, 192 mM glycine – 14.4 g/l, 20% methanol).
2. Between two double-layered presocked Whatman paper, the SDS-polyacrylamide gel was placed tightly onto a presocked nitrocellulose membrane avoiding any air bubble in between, with the membrane close to the anode and the gel close to the cathode.
3. Press this sandwich including Whatman paper, gel, and membrane tightly together into a clamp. Electroblot was performed in blot buffer using western blotting system (Bio-Rad) at 15 V overnight or at 100 V for 1 hours with a cooling chamber.
4. Stop the membrane transfer, and stain for 1–3 min the membrane with Ponceau S staining solution( 0.2 % Ponceau S, 3 % trichlor acetic acid, 3 % sulfosalicylic acid) to check the membrane transfer efficiency.
5. Destain the Ponceau S with water and then TBST buffer (20 mM Tris, pH 7.6,

0.8 % NaCl, 0.05% Tween 20).

6. Block the membrane in blocking solution (1-5% low fat milk powder in TBST) at RT for 1 hour, rocking on a rocky machine (Biometra).

7. Dilute the primary antibody 1:100-1:1000 in blocking solution, and incubate at 4 °C shaking overnight (preferred) or at RT rocking for 1-2 hours.

8. Wash with TBST at RT rocking for 15 minutes.

9. Wash with TBST at RT rocking for 10 minutes.

10. Wash with TBST at RT rocking for 10 minutes..

11A. Incubate the membrane with 1:10'000 diluted biotinylated anti-rabbit, -anti-rat or -anti-mouse IgG secondary antibody in blocking solution at RT rocking for 1 hour.

12. Wash with TBST at RT rocking for 1x15 minutes, 1x30 minutes, 3x10 minutes

13. Prepare Vectastain Elite ABC Kit (Vector Laboratories) solutions to enhance the signal by forming a macromolecular complex of biotin-avidin-biotinylated horse radish peroxidase: Mix 10 µl A and 10 µl B in 1 ml TBST and incubate this mixture 30 min on a shaker. Dilute this 1 ml with 50 ml TBST and incubate the membrane in this final solution for 30 min

14. Wash with TBST at RT rocking for 1x15 minutes, 4x5 minutes

15. Detection of horse radish peroxidase with ECL Western blotting detection reagents (Amersham): Mix 500 µl Solution 1 and 500 µl Solution 2 containing the chemiluminescent substrate for the HRP. The TBST-washed membranes were drained from the excess buffer and placed on Saran Wrap – protein side up. The detection reagent was equally distributed 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, and the protein side was exposed for the required time (30 sec to 30 min) to a Kodak photo film which was subsequently developed according to standard procedure.

11B. When Horseradish Peroxidase (HRP) conjugated secondary antibody was used (1:10000) and the membrane incubated at RT rocking for 1 hour, washing steps 8.–10. were done followed by the detection (step 15.)

**Table 5-9:** Western blotting onto nitrocellulose membranes and subsequent immunostaining

## 5.10 Cell Biology

### 5.10.1 Cell Culture Medium

Cos-7 cells were cultured at 37 °C in DMEM high glucose medium containing 4.5 mg/ml glucose and supplemented with 10% FCS, 100 U/ml penicillin G, 100 µg/ml Streptomycin.

### 5.10.2 Cell Revival

Cells from liquid nitrogen are revived in a 37 °C water bath as quickly as possible, then transferred into a 15 ml Falcon tube containing 5 ml proper culture medium, and centrifuged at 1,000 rpm for 5 minutes. The supernatant medium was aspirated, 5 ml fresh medium was added to the cell pellet and pipetted up and down for at least 15 times to break cell aggregates. Then, the cell resuspension was distributed in a 10 cm petridish containing 5 ml culture medium (10 ml in total). The dish was gently shaken left-right and backward-forward to achieve equally distribution of cells. Then, the cells were cultured in a BBD 6220 incubator (Heraeus) at 37 °C under 5% CO<sub>2</sub> concentration.

### 5.10.3 Cell Passage and Freeze

The medium of cultured 70-95% confluent cells in a 10 cm dish was aspirated. Cells were washed with 10 ml PBS, which was then aspirated from the dish. 3 ml 1×Trypsin-EDTA solution (Gibco BRL) was equally distributed onto the washed cells, and incubated at 37 °C for about 2 minutes. The dish was shaken until all the cells became floating. 11 ml culture medium with 10 % FCS was added to stop the trypsin digestion, pipetted up and down for several times to blow the cells, and then transferred into a 15 ml Falcon tube. Centrifugation was carried out at 1,000 rpm for 5 minutes to pellet the cells, followed by aspirating the supernatant.

For passage, the cell pellet was resuspended in 12 ml culture medium by pipetting 15 to 20 times to break cell aggregates. 3 ml resuspension was finally equally distributed into a 10 cm dish containing 9 ml medium (1:4 dilution), gently shaken and incubated.

For freeze, the cell pellet was resuspended in 2 ml culture medium, and every 500 µl cell resuspension was transferred into a cryotube (Nunc) containing 500 µl culture medium and 10% DMSO, mixed well by inverting, and sequentially frozen at -20 °C overnight, at -80 °C for a week to a month, and finally in liquid nitrogen.

#### 5.10.4 Cell Transfections with Plasmids

The cultured cells were transfected with plasmids using Lipofectamine 2000 transfection reagent (Invitrogen) (Table 5-10).

1. The day before transfection, trypsinize and count the cells, plating them so that they are 90-95% confluent on the day of transfection. Cells are plated in 12 ml of their normal growth medium containing serum and without antibiotics. Best results are obtained when cells are transfected at a high cell density.
2. For each well (10 cm diameter petridish) of cells to be transfected, dilute 19.2  $\mu\text{g}$  of DNA into 1.2 ml of DMEM without serum.
3. For each well of cells, dilute 72  $\mu\text{l}$  of LIPOFECTAMINE 2000 (LF2000™) Reagent into 1.2 ml of DMEM without serum and incubate for 5 min at room temperature. Once the LF2000 Reagent is diluted, combine it with the DNA within 5 min. Longer incubation times may result in decreased activity. This dilution can be prepared in bulk for multiple wells.
4. Combine the diluted DNA (from step 2) with the diluted LF2000 Reagent (from step 3); Volume total 2.4 ml. Incubate at room temperature for 20 min to allow DNA-LF2000 Reagent complexes to form.
5. Remove growth medium from cells, wash them once with medium and add 12 ml of medium without serum to each well. Add the DNA-LF2000 Reagent complexes (2.4 ml) directly to each well and mix gently by rocking the plate back and forth.
6. Incubate the cells at 37°C in a CO<sub>2</sub> incubator for 4-5 h. Add 12 ml of growth medium containing 20% FBS for a final concentration of 10% FBS. Incubate cells until 24 h post-transfection or until they are ready to assay for transgene expression. It is not necessary to remove the complexes or change the medium. Alternatively, growth medium may be replaced after 4-5 h without loss in transfection activity.

**Table 5-10:** Transfection of plasmids or siRNAs into cultured cells using lipofectacmine.

## 5.11 Zebrafish

### 5.11.1 Fish breeding and incubation

Zebrafish were raised and kept under standard laboratory conditions at a constant light-dark cycle (14 h light/10 h dark (Kimmel et al., 1995; Westerfield, 1995). For breeding, 2 fish were placed in a mating tank, separated by a net over night. In the following morning, the separating net was removed that zebrafish could lay. To avoid parental cannibalism the cage separated parents from eggs. The eggs were collected and transferred to methylene blue egg water or Danieau's solution to prevent the growth of fungi or bacteria. The eggs were maintained at 28.5 °C until the desired stages. Alternatively, to accelerate or slow down the development, embryos were incubated at 30 °C or 25 °C, respectively.

Morphological features were used to determine the stage of the embryo according to Kimmel et al., 1995. To dechorionate embryos enzymatically, 0.5–1 mg Pronase E was added/1 ml of Danieau's and following dechoriation washed 7 times with Danieau's. When fish older than 24 hpf were used, 0.005 % phenylthiourea (PTU) was added to the Danieaus to prevent melanisation.

<b>30x Danieau's stock solution</b>		<b>for 1 liter</b>
1.74M NaCl (M=58.44 g/mol)		101.7 g
21mM KCl (M=74.56 g/mol)		1.57 g
12mM MgSO <sub>4</sub> [7H <sub>2</sub> O] (M=246.48 g/mol)		2.96 g
18mM Ca(NO <sub>3</sub> ) <sub>2</sub> [4H <sub>2</sub> O]] (M=236.15 g/mol)		4.25 g
150mM HEPES (M=238.31 g/mol)		35.75 g
	pH 7.6	
<b>0.3x Danieau's solution = working solution</b>		
1:100 dilution of stock and pH adjustment with a view drop of 5M NaOH to pH 7.6		
5 ml Penicilin and Streptomycin (100x stock solution – 10'000U/ml, Gibco) / 1 liter of Danieau's may added to suppress bacterial growth.		

### 5.11.2 Morpholinos

Morpholinos are ribonucleoside analogs with high degradation stability, good RNA-binding affinity and morpholino antisense oligonucleotides are used for efficient expression-inhibition of a specific protein. The dead end morpholino (*dnd* MO, 5'-GCTGGGCATCCATGTCTCCGACCAT-3') and standard control MO were obtained from Genetools (Philomath, OR, USA). Morpholino stock solutions were dissolved in water, 1mM HEPES pH7.4 or Danieau's buffer. Fresh working dilutions were generated with 10mM HEPES pH7.4 buffer immediately before the experiments.

### 5.11.3 Linearization of Plasmid for *in vitro* transcription

5-10  $\mu$ g plasmid DNA was linearized by incubating with 2-4  $\mu$ l restriction enzyme at 37 °C for 4 hours, purified with PCR Purification Kit (Qiagen), and eluted in 30  $\mu$ l H<sub>2</sub>O. 1  $\mu$ l elution was loaded on an agarose gel to check the linearization efficiency.

### 5.11.4 mRNA Synthesis for Injection

Synthesis of 7-methyl-guanosine capped sense mRNA for injections was produced according to the standard protocol of mMACHINE mMACHINE Kit (Ambion) (see in table below)

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1. 1  $\mu$ l 10x Reaction buffer, 5  $\mu$ l 2x Nucleotide Mix / Cap analog, 50-500ng linearized DNA template, 0.8  $\mu$ l T3, T7 or SP6 Enzyme Mix, H<sub>2</sub>O to a final volume of 10  $\mu$ l were mixed and incubated for 2h at 37° C.

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2. To degrade DNA, 0,5  $\mu$ l DNaseI were added and incubate for 20 min at 37 °C.

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#### Phenol-Chloroform extraction

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3. Add 260  $\mu$ l DEPC water

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4. Add 30  $\mu$ l Ammonium Acetate Stop Solution (Message Machine Kit) and mix

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5. Add 300  $\mu$ l PCI (= Phenol-Chloroform-Isoamylalcohol, 25: 24 : 1, „for RNA“, pH=6,6, from Ambion)

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6. Shake vigorously (or vortex) 10 seconds; spin 15 minutes at RT; carefully transfer upper phase to a new tube. Do not take interphase – discard lower phase.

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7. Add CI (= Chloroform-Isoamylalcohol, 24:1), shake vigorously, spin 7 minutes, transfer supernatant to new tube.

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8. repeat CI step once.

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### Precipitation and Analysis

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9. Add equal volume of 100% Isopropanol (room temp.). Do not incubate at  $-20^{\circ}\text{C}$  as recommended by the Ambion kit.

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10. Centrifuge immediately, 40 minutes. Don't precool the centrifuge, cool down to  $4^{\circ}\text{C}$  while spinning.

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11. Carefully remove the supernatant using a pipette.

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12. Wash 2x with 80% EtOH (RT)

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13. After second wash, remove large drops using a pipette; air dry for a minimum time

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14. Dissolve in 20  $\mu\text{l}$  HEPES (10mM, pH 7,4, DEPC water). Pipette up and down to dissolve.

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The amount and the quality of the obtained RNAs were estimated by gel electrophoresis and measurements of the UV absorption at  $\lambda$  260/280nm.

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**Table 5-11:** Synthesis of sense mRNA for injection of zebrafish embryos.

#### 5.11.5 Injection of Zebrafish Embryos

mRNA was diluted with HEPES (10mM, pH 7,4, DEPC water) at different concentration. After fertilized zebrafish eggs were collected, aligned in a 1.5 % agarose ramp, about 5 nl RNA solution was injected into each yolk of the embryo. Post-microinjection embryos were kept at  $28.5^{\circ}\text{C}$  in Danieau's medium and were checked several hours later to remove the unfertilized and dead eggs.

#### 5.11.6 Immunostaining of Zebrafish Embryos

Immunostaining of zebrafish embryos was performed as described below in Table 5-12.

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1. Embryos were fixed in PFA 4% for 2h at  $4^{\circ}\text{C}$  and 2h at RT

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2. Wash PBTB (PBS pH 7.4, 0.1% Tween-20, 0.2% Triton X-100, 1% BSA) for 3h at RT on shaker.

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3. Primary antibody: rabbit-anti vasa (1:1500; P2), anti-HA (1:200; P4) dissolved in PBTB o/n at  $4^{\circ}\text{C}$ .

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4. Wash PBTX (PBS pH 7.4, 0.1% Tween-20, 0.2% Triton X-100) for 7h at RT on shaker, in total 12 changes



- 
5. Secondary antibody: goat-anti-mouse 546 (1:200; orange; S9) and goat-anti-rabbit Cy2 conjugated (1:200; green; S11) in PBTB overnight at 4 °C

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  6. Wash PBTX for 7h at RT on shaker, in total 12 changes

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  7. Store at 4 °C for microscopy.
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**Table 5-12:** Immunostaining of zebrafish embryos.

### 5.11.7 DIG- and Fluo- Labeled RNA Probe Synthesis

DIG- and Fluo- labeled antisense RNA probes were synthesized by incubating 1 µg – 100ng linearized DNA, 4 µl 5×Transcription Buffer (Fermentas), 2 µl DIG-/Fluo-Labeling Mixture (Roche), 1 µl RNase inhibitor, 2 µl SP6, T3 or T7 RNA Polymerase (Fermentas, Roche) in a total volume of 20 µl at 37 °C for 2-2.5 hours and kept dark when Fluorescein (Fluo-) was used. To degrade DNA, 0,5 µl DNaseI (Fermentas) were added and incubate for 20 min at 37 °C.

For RNA probe precipitation, 11µl NH<sub>4</sub>Ac 7,8M (=1/2 Vol) and 63 µl ethanol 100 % RT (= 3 Vol) were added, mixed and let precipitate for 30-50 minutes at RT. Following, precipitate were collected by spinning 30-40 minutes at maximum speed at 20 °C and washed once with 1 ml ethanol 80 % RT. Air dried pellet was dissolved in 20 µl H<sub>2</sub>O and 80 µl Hyb-buffer (see below).

Alternative to RNA probe precipitation, the transcription product was supplemented with 30 µl H<sub>2</sub>O, purified with a G-50 Sephadex Micro Columns according to manufactures instruction and 80 µl Hyb-buffer added to the elut. 3 µl purified probe was checked on an agarose gel.

### 5.11.8 Zebrafish One- and Two-Colour Whole Mount *In Situ* Hybridization

Zebrafish embryos were fixed in 4% PFA/PBS at 4 °C overnight, embryos younger than 2 somite stage were fixed for 2 days. Fish were twice washed with PBS 8 g/l NaCl, 0.2 g/l KCl, 1.8 g Na<sub>2</sub>HPO<sub>4</sub> \*2 H<sub>2</sub>O, 0.24 g KH<sub>2</sub>PO<sub>4</sub>, pH 7,2 ) or PBT (PBS, 0.1 % Tween 20), dechorionated after fixation until the 16-somite stage. Later stages were dechorionated prior to fixation, using forceps and up to 50 embryos per 1.5 ml tube were subjected to whole-mount *in situ* hybridization (Table 5-13).

## Material and Methods

	Treatment and Solution	T	Duration
Dehydration	1. 5x methanol 100%	RT	5 minutes
The embryos in 100% methanol can be stored in $-20^{\circ}\text{C}$ for several months.			
1. Day	1. 75% methanol/PBT, shaking.	RT	5 minutes
	2. 50% methanol/PBS, shaking.	RT	5 minutes
	3. 25% methanol/PBT, shaking.	RT	5 minutes
	4. Wash 4x PBT	RT	5 minutes each
	5. Digest with 5 $\mu\text{g/ml}$ Proteinase K (1:1000 dilution from stock in PBT).	RT	< bud : no 1 somite : 30 sec 5 somite : 1-3 min 24 hpf : 3 – 5 min
	6. Wash 3x PBT	RT	1 minutes each
	7. Refixation with 4% PFA in PBS	RT	20 minutes
	8. Wash 5x PBT	RT	5 minutes each
	9. Prehybridize in prewarmed Hyb buffer (50% formamide, 5xSSC, 9mM Citric acid monohydrate to pH 6.0 – 6.5, 0.1% Tween 20, 500 $\mu\text{g/ml}$ PCI extracted Torula yeast tRNA (SIGMA), 50 $\mu\text{g/ml}$ heparin), incubated in a waterbath. [ 20x SSC; 3M NaCl, 0.3 M $\text{Na}_3\text{Citrat}$ ]	67 $^{\circ}\text{C}$	2 -5 hours
	10. Dilute 1:200 to 5:200 Dig or Fluo labeled RNA probe into Hyb-buffer. Hybridize with 200 $\mu\text{l}$ Hyb-buffer per 1.5 ml tube of this solution	67 $^{\circ}\text{C}$	Overnight
2. Day	1. Wash with Hyb buffer	67 $^{\circ}\text{C}$	20 min
	2. Wash 3x with 50% SSCT 2x/50% Formamide	67 $^{\circ}\text{C}$	20 min
	3. Wash 1x with 75% SSCT 2x/25% Formamide	67 $^{\circ}\text{C}$	20 min
	4. Wash 2x with SSCT 2x	67 $^{\circ}\text{C}$	20 min
	5. Wash 4x with SSCT 0.2x	67 $^{\circ}\text{C}$	30 min

	6. Wash 1x with PBT	67 °C	5 min
	7. Blocking with Blocking solution (5% sheep serum and 10 mg/ml BSA in PBT)	RT	1 to several hours
	8. Antibody Incubation 1 with 200 µl preabsorbed Antibody solution: 1:2000 Anti-Dig or anti-Fluo (Roche), 2% sheep serum and 2 mg/ml BSA in PBT	4 °C	Overnight
3. Day	1. Wash 3x with PBT; keep dark, if anti-Fluo antibody is used	RT	5 minutes
	2. Wash 8x with PBT	RT	Within 3 hours
	3. Wash 3x with NTMT (100 mM Tris HCl pH 9.5, 50 mM MgCl <sub>2</sub> , 100 mM NaCl, 0.3% Tween 20) for 20 ml use: 2ml Tris HCl pH 9.5, 1M / 1ml MgCl <sub>2</sub> , 1M / 2ml NaCl, 1M / 600 µl Tween 20, 10% / 14.8 ml H <sub>2</sub> O	RT	5 minutes each
	4. Transfer embryo into a 24 well plate, using a cut 1 ml pipet tip		
	5. Staining (blue): remove most NTMT and add 0.5 ml staining solution (1 ml NTMT, 4.5 µl NBT - Nitro Blue Tetrazolium, Sigma N6876, 75 mg/ml in 70% DMF / 30% H <sub>2</sub> O), 3.5 µl X-phosphate (=BCIP, 50 mg/ml in 100% DMF) to each well.	37 °C	Various

## Material and Methods

		6. Stop reaction by removing staining solution and wash 2x with PBT and (only for one colour <i>in situ</i> hybridization experiment) 3x with Stop solution (1mM EDTA, 0.1% Tween, 0.05M phosphate buffer pH 5.8 [for 200ml: 92ml 0.1M NaH <sub>2</sub> PO <sub>4</sub> , 8ml 0.1M Na <sub>2</sub> HPO <sub>4</sub> - this mixture produce a pH of 5.8])	RT	5 min each
For 2 colour <i>in situ</i> hybridization experiment	Remove first antibody	If a two colour <i>in situ</i> hybridization experiment with both, Dig- and Fluor- labeled antisense probe will be performed, the first antibody has to be inactivated previous incubation of the embryos with the second antibody.		
		1. transfer embryos to 1.5 ml tubes		
		2. Wash 2x embryos in 0,1 M Glycin /HCl pH 2.2 and 0.1% Tween. Shake well!	RT	5 minutes each
		3. Wash 4x with PBT	RT	5 minutes each
Clearing	If the yolk turned brownish during the staining procedure with NBT/ X-phosphate, washing with ethanol gives clear these staining and intensify the staining if the embryo. After a few days of storage this brown precipitate would turn into a black, stable precipitate that cannot be dissolved away. Therefore, this ethanol treatment has to be done soon after the completion of the staining reaction. This step cannot be performed after the red staining reaction. (The red stain dissolves in ethanol very fast).			
	1. Wash 3x with ethanol 100 %	RT	5 minutes, 2x 10 minutes	
	2. Wash 75% ethanol/PBT	RT	5 minutes	
	2. Wash 50% ethanol/PBT	RT	5 minutes	
	2. Wash 25% ethanol/PBT	RT	5 minutes	
	4. Wash 4x PBT	RT	5 minutes	
	5A. For storage: wash 3x with Stop solution	RT	5 minutes	
For 2 colour <i>in situ</i> hybridization experiment				

	5B. For second color staining: Antibody Incubation 2 with 200 $\mu$ l preabsorbed Antibody solution: 1:2000 Anti-Dig or anti-Fluo (Roche), 2% sheep serum and 2 mg/ml BSA in PBT	4 °C	Overnight
4. Day	1. Wash 3x with PBT; keep dark	RT	5 minutes
	2. Wash 8x with PBT	RT	Within 3 hours
	3. Wash 3x with NTMT (100 mM Tris HCl pH 9.5, 50 mM MgCl <sub>2</sub> , 100 mM NaCl, 0.3% Tween 20) for 20 ml use: 2ml Tris HCl pH 9.5, 1M / 1ml MgCl <sub>2</sub> , 1M / 2ml NaCl, 1M / 600 $\mu$ l Tween 20, 10% / 14.8 ml H <sub>2</sub> O	RT	5 minutes each
	4. Transfer embryo into a 24 well plate, using a cut 1 ml pipet tip		
	5. Staining (blue): remove most NTMT and add 0.5 ml staining solution (1 ml NTMT, 3.5 $\mu$ l INT; 4-Iodonitrotetrazolium violet, Sigma, 50 mg/ml in DMSO), 3.5 $\mu$ l X-phosphate (=BCIP, 50 mg/ml in 100% DMF) to each well.	37 °C	Various
	6. Stop reaction by removing staining solution and wash 2x with PBT and 3x with Stop solution (1mM EDTA, 0.1% Tween, 0.05M phosphate buffer pH 5.8 [for 200ml: 92ml 0.1M NaH <sub>2</sub> PO <sub>4</sub> , 8ml 0.1M Na <sub>2</sub> HPO <sub>4</sub> - this mixture produce a pH of 5.8])	RT	5 min each
Transfer embryos in 80% Glycerol / 20% Stop solution.			

**Table 5-13:** Whole-mount *in situ* hybridization of zebrafish embryos.

## Material and Methods

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The preparation of the yeast torula RNA for the Hyb buffer was done as described in Table 5-14.

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1. 500 mg torula yeast total RNA (Sigma) were dissolved in 25 ml (in 50 ml falcon tube) water by vortexing for 30 min
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### Phenol-Chloroform extraction

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2. Add 25 ml PCI (= Phenol-Chloroform-Isoamylalcohol, 25: 24 : 1, „for RNA“, pH=6,6, from Ambion)
  3. Vortex 1 min; spin 30 minutes (6500 rpm) at 4°C; carefully transfer upper phase to a new tube. Do not take interphase – discard lower phase.
  7. Add 20 ml CI (= Chloroform-Isoamylalcohol, 24:1), vortex, spin 20 minutes (6500 rpm) at 4°C, transfer supernatant to new tube.
  8. repeat CI step once.
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### Precipitation and Analysis

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9. Add equal volume of 100% Isopropanol (room temp.). Do not incubate at –20°C as recommended.
  10. Centrifuge immediately, 1.4 hours at 4°C.
  11. Carefully remove the supernatant using a pipette.
  12. Wash 2x with 80% EtOH (RT)
  13. After second wash, remove large drops using a pipette; air dry (can take 2 hours)
  14. Dissolve in 5 ml 20x SSC. Pipette up and down to dissolve and store at -20°C
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**Table 5-14:** Synthesis of sense mRNA for injection of zebrafish embryos.

The pre-absorption of anti-Dig- and anti-Fluo-antibody (Roche) was done as described below.

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1. A total volume of 300-500 µl zebrafish embryos is harvested in various stages, fixed in 4 % PFA, transferred in methanol and 1. Day of *in situ* hybridization protocol without prehybridization and hybridization was done as described in above.
  2. Embryos are transferred in a 15 ml Falcon tube, 5 ml blocking solution (2 mg/ml BSA, 2 % sheep serum, PBT), 25 µl anti-Dig- and anti-Fluo-antibody (1:200) were added and with agitation incubated overnight at 4°C.
  3. Optional, to rescue antibody within the embryos, transfer embryos into a 1.5 ml tube
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and dissociate them with a pistil, add 0.5 ml clear antibody solution to the tube and filter this suspension with a 0.2  $\mu\text{m}$  syringe filter.

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4. 45 ml blocking solution is added to the 5 ml clear antibody solution (1:2000) and supplemented with 100  $\mu\text{l}$  of a 10 %  $\text{NaN}_3$  solution (end-concentration: 0.02 %).

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### **5.11.9 Microscopy and Time-Lapse Analysis**

Bright field and fluorescence pictures were made with a Zeiss Axioplan 2 microscope or a Leica confocal DMRXE microscope.

For the time lapse analysis embryos were oriented in agarose ramps overlaid with 0.3x Danieau's solution. Time-lapse movies were generated using Metamorph software (Universal Imaging) controlling a Zeiss Axioplan2 microscope.

## **5.12 Chicken**

### **5.12.1 Cultivation of Chicken Embryos**

Fertilized eggs were incubated at 38 °C in a humid with rolling during the whole incubation time.

### **5.12.2 Early Chick (EC)-culture**

Early chick (EC)-culture (Chapman et al., 2001) is a simple method of chick whole-embryo culture, which uses a filter paper carrier to hold the early blastoderm and vitelline membranes under tension while the embryo grows on a substratum of agar-albumen (Table X-x). This is a quick and efficient means of setting up cultures of chick embryos beginning at pre-primitive streak stages to stage 10.

Preparation of Agar-Albumen Culture Dishes:

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1. Heat a water bath to 49°C.
2. Add 120 ml saline (7.19g NaCl/ 1 l distilled water, autoclaved) to a sterile 500 ml flask and bring it to boiling, using a hot plate/stirrer. Add 0.72 g Bacto-Agar (Difco) and stir until it is dissolved.
3. While the agar is dissolving, collect 120 ml of thin albumen (collected from 2

## Material and Methods

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dozen unincubated eggs) in sterile Falcon tube (50 ml) or similar container. Place the tubes into the water bath at 49°C.

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4. Once the agar is dissolved, put the flask into the water bath. Allow the liquid to equilibrate at 49°C.

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5. On a flat surface, lay out 80 35-mm sterile Petri dishes with their lids removed.

---

6. Add the albumen to the flask containing the dissolved agar, and mix by swirling for 30-60 sec. Also add the penicillin/streptomycin (Sigma, P0906) to this mixture, 5 U/ml.

---

7. Using a sterile 10 ml pipette and pipette-aid (e.g., Drummond, Bibby Jet) or similar device, aliquot 2.5 ml of the mixture per Petri dish. Do this reasonably quickly, without introducing bubbles into the dishes. If more than 2.5 ml agar-albumen mixture is pipetted, the substrate will be too thick, subsequently impractical for the imaging of embryos with transillumination.

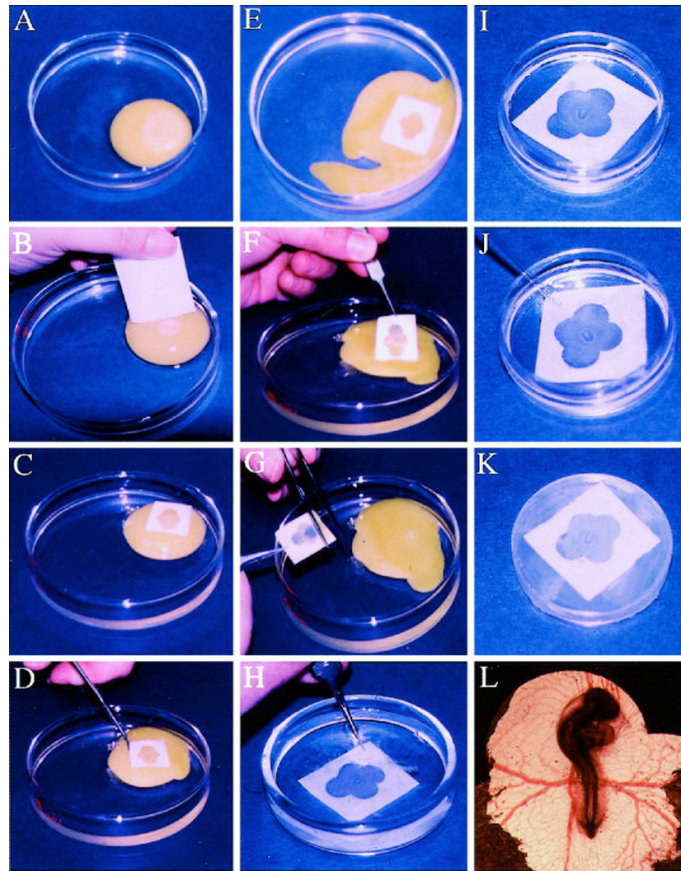
---

8. Once the aliquoting is complete, replace the lids of the Petri dishes and leave the dishes for several hours or overnight at room temperature to dry. Place dishes upright in an airtight container at 4°C and use them as required. The dishes can be stored at 4°C for 1-2 weeks, provided that sterile conditions are maintained.

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**Table 5-15:** Preparation of Agar-Albumen EC-culture dishes.





**Figure 5-1:** Preparation of EC-culture. (Adapted from (Chapman et al., 2001)).

The preparation of the embryo EC-culture was then performed as described below in Table 5-16.

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A: Eggs should be incubated on their side until the desired stage (HH4 to HH10) is reached. After cooling for 15-30 min, break each egg into a glass Petri dish.

---

B: Using a piece of tissue paper (e.g., Kimwipe) neatly folded, place the tissue paper on the thick albumen and pull the thick albumen away from the blastoderm in a centrifugal direction.

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C: Center the filter paper over the blastoderm. If the craniocaudal axis is apparent, align the filter paper with respect to this axis as desired.

---

D: Cut the vitelline membranes around the filter paper, ensuring a complete cut.

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E: The filter paper will remain attached to the vitelline membranes after cutting if the albumen has been properly removed.

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F: Pull the filter paper away from the yolk in an oblique direction; that is, usually in

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the direction of yolk flow and/or the craniocaudal axis.

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G: Using blunt forceps, wipe excess yolk off the filter paper in a centrifugal direction.

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H: For removing as much of the remaining excess yolk as possible, use a small glass Petri dish, filled with saline and 5 U/ml Penicillin/Streptomycin, and swirl the EC culture to wash away excess yolk.

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I: Place the filter and contained blastoderm onto the substrate and cover the dish with a lid. Incubate dishes in a larger Petri dish containing moistened tissue paper lining the bottom of the dish.

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J: As an alternative method of washing away yolk, place the filter paper directly onto an agar-albumen culture dish. Using a Pasteur pipette, wash the yolk off with a jet of saline. After washing, remove excess fluid while tilting the dish.

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K: For an alternative culture substrate, construct a Parafilm covered Petri-dish lid, containing a central hole. Fill the lid up to the level of the hole with thin albumen. The culture is placed on top of the Petri dish, over the central hole, ventral-side up.

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L: An embryo after 44 hr of EC culture

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**Table 5-16:** Preparation of embryos for EC culture.

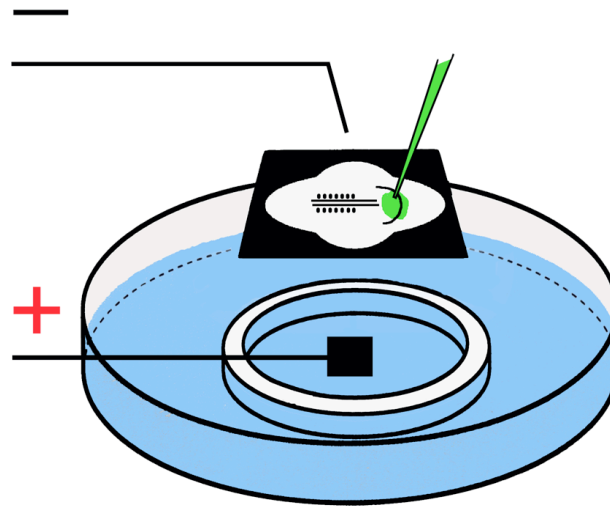
### 5.12.3 Implantation of Cos 7 cells into Chick Embryos

Cos 7 cells were co-transfected with either CMV- SDF-1a CMV-GFP or CMV-SDF-1 $\beta$  and CMV-GFP as described in chapter “Cell Transfections with Plasmids”. As a control, the same total DNA amount of CMV-GFP was used. 24 hours after transfection, an aggregate of transfected Cos 7 cells was grafted between ectoderm and endoderm into embryonic and extra embryonic tissues at different anterior-posterior position of stage HH 4 or HH 10 chicken embryos. Chicken embryos were cultured in Early-Chick (EC)-whole embryo culture either up to stage HH 10 or HH 17, imaged and analyzed after in situ hybridization.

### 5.12.4 Electroporation

Chick embryos were electroporated by a new method, developed by Dr. Derek Spieler and based on the methods from (Chapman et al., 2001; Endo et al., 2002). In respect to conventional *in-ovo* electroporation, the advantage of this method is the

possibility to electroporate high numbers of young chick embryos (HH4) (Spieler, 2004 #1572). For electroporation, the chamber is filled in both compartments with PBS up to the border of the inner ring. Next, the embryo together with the filter paper is taken out of the EC-culture dish and placed with the ventral side down just above the 2 mm<sup>2</sup> platin plate cathode. The area, which is electroporated, is placed directly above the cathode. With a glass injection needle, expression vector solution (2.5 µg/µl – 5 µg/µl) together with 0.025 % Fastgreen (to visualize injection volume)



**Figure 5-2:** Electroporation of embryos in EC culture.

were injected between vitelin membrane and ectoderm. The anode, a platin wire of 0.2 mm diameter, was than placed carefully onto the embryo and three pulses of 7 volts for 25 milliseconds within 200 milliseconds (using Electro Square Porator ECM 830, Btx Inc.) were applied. Finally, the embryo was brought back into a EC-culture dish with the dorsal side up and incubated for the appropriate time.

### 5.12.5 Chick Whole-Mount *In Situ* Hybridization

Chick embryos were fixed in 4% PFA/PBS at 4 °C for 4 hours to overnight, and subjected to whole-mount *in situ* hybridization (Table 5-17).

	Treatment and Solution	T [°C]	Duration
Dehydration	1. Wash the fixed embryos with PBT 3 times, shaking.	on ice	10 min each
	2. 25% methanol/PBT, shaking.	on ice	10 min
	3. 50% methanol/PBS, shaking.	on ice	10 min

## Material and Methods

	4. 75% methanol/PBT, shaking.	on ice	10 min
	5. 100% methanol, shaking, twice	on ice	10 min each
The embryos in 100% methanol can be stored in $-20^{\circ}\text{C}$ up to 2 weeks.			
Day 1	1. 75% methanol/PBT, shaking.	on ice	10 min
	2. 50% methanol/PBS, shaking.	on ice	10 min
	3. 25% methanol/PBT, shaking.	on ice	10 min
	4. Wash with PBT twice, shaking.	on ice	10 min each
	5. Bleach with 6% $\text{H}_2\text{O}_2$ /PBT, shaking.	on ice	< HH 13: 30 min HH 14-26: 1 hour
	6. Wash with PBT 3 times, shaking.	on ice	5 min each
	7. Digest with 10 $\mu\text{g}/\text{ml}$ Proteinase K (1:1000 dilution from stock in PBT).	on ice	< HH7: 0.5 min HH8-10: 1 min HH11-13: 1.5 min HH14-16: 5 min HH17-19: 9 min >HH21: 15 min
	8. Stop the Proteinase K digestion with about 2 mg/ml glycine in PBT.	on ice	5 min
	9. Wash with PBT 3 times. No agitation	on ice	5-10 min each
	10. Treat with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% Sodium deoxycholate)	on ice	< HH11: 1x10min 10-16: 3x10min >HH16: 3x20min
	11. Wash with PBT 3 times	on ice	5-10 min each
	12. Refix with 4% PFA, 0.2% glutaraldehyde, and 0.1% Tween20 in PBS	on ice	20 min
	13. Wash with PBT 3 times, shaking.	on ice	10 min each
	14. Prehybridize in prewarmed Prehyb solution (50% formamide, 5xSSC, pH 4.5, 1% SDS, 50 $\mu\text{g}/\text{ml}$ yeast tRNA, 50 $\mu\text{g}/\text{ml}$ heparin), shaking. [ 20x SSC; 3M NaCl, 0.3 M $\text{Na}_3\text{Citrat}$ ]	67-70 $^{\circ}\text{C}$	4 hours

	Or before prehybridization, the embryos can be stored in store mix (50% formamide, 5×SSC, pH 4.5) at –20 °C up to months.		
	15. Dilute 1:100 the DIG labeled RNA probe (endconcentration: 1 µg/ml) into the Prehyb solution for hybridization	70 °C	Overnight
Day 2	1. Incubate with prewarmed “Solution1” (50% formamide, 5×SSC, pH 4.5, 1% SDS) twice, shaking.	70 °C	30 min each
	2. Incubate with prewarmed “Solution3” (50% formamide, 2×SSC, pH 4.5, 0.1% Tween20) twice, shaking.	70 °C	30 min each
	3. Wash with MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween20, pH 7.5) 5 times, shaking. Flush embryos if necessary to remove probe from body cavities.	RT	5 min each
	4. Incubate with 2% Block Reagent (Roche) in MABT, shaking.	RT	5 min
	5. Block with blocking solution (2% Blocking Reagent Roche, 20% FCS in MABT), shaking.	RT	2-8 hours
	6. Incubate with 1:2000 diluted Anti-DIG-AP Fab fragment (Roche) in blocking solution, shaking.	4 °C	Overnight
Day 3-4	1. Wash with MABT 6 times. Flush after three wash steps the embryos if necessary to remove antibody from body cavities.	RT	5 min each
	2. Transfer the embryos into a sealed 5 ml bottle and wash with MABT for 48 hours rotating. Refresh the solution several times.	RT	48 hours

Day 5	1. Equilibrate embryos with freshly prepared NTMT/Lev (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM MgCl <sub>2</sub> , 1% Tween20, 2 mM Levamisol) three times, shaking. Levamisol may be not added to the buffer.	RT	5 min each
	2. Incubate with NTMT/Lev.	RT	4 hours
	3. Stain with 1:50 diluted NBT/BCIP (Roche) in NTMT/Lev in a dark environment shaking. Control the staining every 10 minutes.	RT	10 min-2 hours
	4. Stop staining reaction by washing with PBT twice, shaking.	RT	10 min each
	5. Incubate with 50% glycerol/PBT, shaking.	RT	15-60 min
	6. Incubate with 80% glycerol/PBT, shaking.	RT	15-60 min
	7. Take pictures		
Then, the embryos can be stored in 80% glycerol/PBT at 4 °C. For long time storage, embryos after staining is stopped fixed overnight in 4% PFA / PBT.			

**Table 5-17:** Whole-mount *in situ* hybridization of chick embryos.

### 5.12.6 Histology – Preparation of Paraffin Sections

Paraffin section after *in situ* hybridization of chick and mouse embryos was done as described below.

	Treatment and Solution	T [°C]	Duration
Fixation	1. Embryos were fixed or post-fixed after <i>in situ</i> hybridization in 4% PFA	4 °C	Overnight to 24 hours
Dehydration	2. 2x wash with PBS	RT	10 min each

	3. Wash with ethanol 50 %/PBS	RT	<HH14: 5 min >HH14: 1 hour
	4. Wash with ethanol 70 %/PBS	RT	<HH14: 5 min >HH14: 1 hour
	5. Wash with ethanol 80 %/PBS		<HH14: 5 min >HH14: 1 hour
	6. Wash with ethanol 90 %/PBS	RT	<HH14: 5 min >HH14: 1 hour
	7. Wash with ethanol 100 %	RT	<HH14: 5 min >HH14: 1 hour
	8. Wash with isopropanol 100 %	RT	<HH14: 10 min >HH14: 1 hour
	8. 1x wash with Toluol	RT	1 hour
Paraffin infiltration	1. Infiltrate with Paraffin (Paraplast-Plus)	60 °C	<HH14: 1x 30 min embryos will curl when longer washed >HH14: 1x 1 hour 1x 24 hours
	2. Embryos are oriented and embedded in paraffin.	60 °C	
Paraffin blocks, stored at RT, are sectioned 8_μm thick on a Leica microtome. Sections were placed onto the surface of a 42 °C waterbath for smoothening, transferred on Superfrost®Plus (Menzel-Gläser, Germany) objective slides and dried at 37 °C overnight.			
Deparaffinization and embedding	1x 100% xylol or hystoclear	RT	5 min each
Embed with Eukit and dry overnight			

**Table 5-18:** Preparation of histological paraffin section of chick and mouse embryos.

## 5.13 Mouse

### 5.13.1 Mouse Whole-Mount *In Situ* Hybridization

Embryos were dissected from the uterus in PBS (cold PBS was used for the stages E9.0-E9.5 and RT warm PBS was used for the stages E10.0-E11.5 that the heart

## Material and Methods

keeps contracting and the blood is washed out of the endothelial system) and fixed in 4% PFA/PBS at 4 °C for 12 hours to overnight, and subjected to whole-mount *in situ* hybridization (Table 5-19).

	Treatment and Solution	T [°C]	Duration
Dehydration	1. Wash the fixed embryos with PBT 3 times, shaking.	on ice	10 min each
	2. 25% methanol/PBT, shaking.	on ice	10 min
	3. 50% methanol/PBS, shaking.	on ice	10 min
	4. 75% methanol/PBT, shaking.	on ice	10 min
	5. 100% methanol, shaking, twice	on ice	10 min each
In 100% methanol on ice, one hole in the forebrain the hindbrain the heart and the region of the gonad, all on the same side of the embryo, were done with a tungsten needle.			
The embryos are incubated in 100% methanol overnight and may stored for a view weeks at -20 °C.			
1. Day	1. 75% methanol/PBT, shaking.	on ice	10 min
	2. 50% methanol/PBS, shaking.	on ice	10 min
	3. 25% methanol/PBT, shaking.	on ice	10 min
	4. Wash with PBT 3x, shaking.	on ice	10 min each
	5. Bleach with 6% H <sub>2</sub> O <sub>2</sub> /PBT, shaking.	on ice	1 hour
	6. Wash with PBT 3 times, shaking.	RT	5 min each
	7. Digest with 10 µg/ml Proteinase K (1:1000 dilution from stock in PBT).  No agitation!	RT	E6.0/6.5: 2 min E7.0/7.5: 4 min E8.0/8.5: 6 min E9.0/9.5: 8 min E10.0/10.5:12min E11.0/11.5:15min >E12.0: 30min
	8. Stop the Proteinase K digestion with about 2 mg/ml glycine in PBT.	RT	5 min
	9. Wash with PBT 3 times. No agitation	RT	5-10 min each



	10. Treat with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% Sodium deoxycholate)	RT	E6.0/7.5:3x 5min E8.0/9.5:3x10min E10.0/11.5:3x15min E12.0/13.5:3x20min >E14.0: 3x30min
	11. Wash with PBT 3 times	RT	5-10 min each
	12. Refix with 4% PFA, 0.2% glutaraldehyde, and 0.1% Tween20 in PBS	RT	20 min
	13. Wash with PBT 3 times, shaking.	RT	10 min each
	14. Aquilibrate in prewarmed 50% PBT/50% PBS Prehyb solution (50% formamide, 5xSSC, 9mM Citric acid mono-hydrate to pH 6.0–6.5, 0.1% Tween 20, 500 µg/ml PCI extracted Torula yeast tRNA (SIGMA), 50 µg/ml heparin), shaking. [20x SSC; 3M NaCl, 0.3 M Na <sub>3</sub> Citrat]	68-70 °	1 hours
	15. Prehybridize in prewarmed Prehyb solution (50% formamide, 5 x SSC, 9mM Citric acid monohydrate to pH 6.0 – 6.5, 0.1% Tween 20, 500 µg/ml PCI extracted Torula yeast tRNA (SIGMA), 50 µg/ml heparin), shaking. [20x SSC; 3M NaCl, 0.3 M Na <sub>3</sub> Citrat]	68-70 °C	1.5-4 hours
	16. Dilute 1:100 the DIG labeled RNA probe (endconcentration: 1 µg/ml) into the Prehyb solution for hybridization.	68-70 °C	Overnight
Day 2	1. Wash with Prehyb solution 2 times, shaking.	68-70 °C	30 min each

## Material and Methods

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	2. Wash with 50% Prehyb solution/50% RNase-buffer (500 mM NaCl, 10 mM Tris pH7.5, 0.1 % Tween-20), shaking.	RT	5 min
	3. RNaseA digestion (RNase-buffer supplemented with 100 µg/ml RNaseA)	37 °C	60 min
	4. Wash with 50% RNase-buffer /50% "Solution2" (50% Formamid, 2x SSC pH7.0, 0.1 % Tween-20), shaking.	68-70 °C	5 min
	5. Wash 10x with "Solution2"	68-70 °C	2x 5 min 3x 10 min 5x 30 min
	6. Wash with 50% "Solution2"/ 50% MABT ((100 mM maleic acid, 150 mM NaCl, 0.1% Tween20, pH 7.5).	RT	5 min
	7. Wash 3x with MABT	RT	10 min each
	8. Block with blocking solution (2% Blocking Reagent Roche, 20% FCS in MABT), shaking.	RT	6 hours
	9. Incubate with 1:2000 diluted Anti-DIG-AP Fab fragment (Roche) in blocking solution, shaking.	4 °C	Overnight
Day 3-6	1. Wash 3x with blocking solution with 5 % FCS only.	RT	In total 4-6 hours
	2. Wash with MABT. Change 2-3 times per day MABT.	RT	In total 4 days

Day 6	1. Equilibrate embryos with freshly prepared NTMT/Lev (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM MgCl <sub>2</sub> , 1% Tween20, 2 mM Levamisol) three times, shaking. Levamisol may is not added to the buffer.	RT	20 min each
	2. Stain with 1:50 diluted NBT/BCIP (Roche) in NTMT/Lev in a dark environment shaking. Control the staining every 10 minutes.	RT	10 min-2 hours
	3. Stop staining reaction by washing 3x with PBT, shaking.	RT	10 min each
	4. Postfixation in 4% PFA / PBT	4 °C	Overnight
Day 7-9	1. Incubate with 30% glycerol/PBT, shaking.	4 °C	Overnight
	2. Incubate with 50% glycerol/PBT, shaking.	4 °C	Overnight
	3. Incubate with 80% glycerol/PBT, shaking and store at 4 °C	4 °C	Overnight - months
Take pictures			

**Table 5-19:** Whole-mount *in situ* hybridization of mouse embryos.

### 5.13.2 Mouse Organ Culture and Time Lapse

Transverse slices from the hindgut regions of heterozygous Oct4 $\Delta$ PE:GFP transgenic mouse embryos expressing GFP in the germ cell lineage were cultured and filmed as previously described (Molyneaux et al., 2003). Images for time lapse were captured every 7 minutes starting at E 9.5. This work was done in collaboration with Kathleen Molyneaux at the Cincinnati Children Hospital.

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# 8. Appendix

## 8.1 Affidavit

Here I declare that my thesis entitled “Germ Cell Development and Migration” has been done independently and with no other sources and aids than quoted and listed below.

Figure 2-6 D-I: Time lapse analysis of migrating mouse PGCs in a section through the gonad was done in collaboration with Kathleen Molyneaux.

Figure 2-7: The human and mouse *dead end* homologues were cloned by Gilbert Weidinger.

Figure 2-12 A-C: The *Xenopus in situ* hybridization was done by Gilbert Weidinger, the mouse *in situ* hybridization was done by Clare Wise and Robin Lovell-Badge.

Jürg Stebler  
August 27<sup>th</sup>, 2005  
Göttingen



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## 8.2 List of Publications

Vasyutina E., **Stebler J.**, Brand-Saberi B., Raz E., and Birchmeier C., CXCR4 and Gab1 co-operate to control the development of migrating muscle progenitor cells. Submitted for publication.

Slanchev. K., **Stebler. J.**, de la Cueva-Méndez. G., and Raz. E.. Development without germ cells: the role of the germline in zebrafish sex differentiation. PNAS. 2005 Feb 23.

**Stebler J.**, Spieler D, Slanchev K., Molyneaux K.A., Richter U., Cojocar V., Tarabykin V., Wylie C., Kessel M., Raz E. (2004). Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. *Developmental Biology* 15;272(2):351-61

Spieler, D., Baumer, N., **Stebler, J.**, Kopranner, M., Reichman-Fried, M., Teichmann, U., Raz, E., Kessel, M. and Wittler, L. (2004) Involvement of Pax6 and Otx2 in the forebrain-specific regulation of the vertebrate homeobox gene ANF/Hesx1. *Developmental Biology* 269: 567-579.

Molyneaux, K.A., Zinszner, H., Kunwar, P.S., Schaible, K., **Stebler, J.**, Sunshine, M.J., O'Brien, W., Raz, E., Littman, D., Wylie, C. and Lehmann, R. (2003) The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development* 130: 4279-4286.

Weidinger, G., **Stebler, J.**, Slanchev, K., Dumstrei, K., Wise, C., Lovell-Badge, R., Thisse, C., Thisse, B. and Raz, E. (2003) dead end, a Novel Vertebrate Germ Plasm Component, Is Required for Zebrafish Primordial Germ Cell Migration and Survival. *Current Biology* 13:1429-1434.

Doitsidou, M., Reichman-Fried, M., **Stebler, J.**, Köprunner, M., Dörries, J., Meyer, D., Esguerra, V.C., Leung, T. and Raz, E. (2002) Guidance of primordial germ cell migration by the chemokine SDF-1. *Cell* 111: 647-659.

## 8.3 Curriculum Vitae

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### SCIENTIFIC EDUCATION

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2002- present      Dissertation, Germ Cell Development, Max Planck Institute for Biophysical Chemistry, Germany

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