Using microarray technology to find novel genes involved in somitogenesis

I n a u g u r a l - D i s s e r t a t i o n

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Table of contents:

Summary:

During the development of vertebrates the body is subdivided into repeated units, the somites. They are generated from the presomitic mesoderm (PSM) and are flanking the notochord on both sides. For this coordinated process components of the Delta-Notch signalling pathway are important.

Novel genes involved in somitogenesis were searched using microarray-technology. This technique had to be established in the lab and this was done using a cDNA library, which was prepared from embryos of mixed embryogenesis stages. The basic protocols were established like printing, labelling and hybridisation. The usage of spiked in controls was established as well. The controls were genes from the bacteriophage lambda.

Later the cDNA library was used to compare the expression of *wt* and *fss* embryos. The *fss* mutant is linked to the gene *Tbx24*, which is no component of the Delta-Notch pathway, but belongs to the fused somite type mutants.

To compare the other fused somite type embryos against *wt* the zebrafish oligo library from Sigma-Genosys was used. The other mutants are *des*, *bea* and *aei*. These mutants are defective in components of the Delta-Notch pathway, *des* in *notch1a*, *bea* in *deltaC* and *aei* in *deltaD*.

As these components are involved in the starting of the cascade and because of this some rescue mechanisms might occur the mediator of the pathway Su(H) was used as well. For this gene morpholino knock down embryos were produced.

The PSM consists of several compartments, namely a posterior, an intermediate and an anterior compartment. The PSM of the zebrafish was not dissected in these parts, as it is very small. The PSM of the mouse is bigger and therefore it was cut in an anterior and a posterior part, where the anterior part contains the intermediate and the anterior compartment. For this approach the mouse oligo library from Sigma-Genosys was used.

The candidate genes found in these screens had to be validated by another method. The *in situ* hybridisation was chosen, as this allows the localisation of the expression and validation. For the mouse PSM screen this method was not used. Here the literature was scanned to find the expression pattern of the genes found. Doing this validated two candidates from this approach.

The genes found in the zebrafish screens were validated using the *in situ* hybridisation. Genes with an interesting expression pattern will be further characterised, i.e. their functional role in somitogenesis will be elucidated.

Zusammenfassung:

Während der Entwicklung der Vertebraten wird der Körper in sich wiederholende Einheiten unterteilt, die sogenannten Somiten. Diese werden von dem präsomitischen paraxialen Mesoderm (PSM) gebildet und flankieren das Notochord zu beiden Seiten. Für den koordinierten Ablauf dieses Prozesses ist die Expression von Komponenten des Delta-Notch- Signaltransduktionsweges wichtig.

Um neue Gene, die eine Rolle in der Somitogenese zu finden, wurde die Mikroarraytechnologie verwendet, die allerdings zunaechst noch etalbiert werden musste. Dafür wurde eine cDNA Bank verwendet, die aus Embryonen in verschiedenen Entwicklungsstadien hergestellt wurde. Die grundlegenden Protokolle, wie Printen, Markieren und Hybridisierung, wurden mit dieser Bank etabliert. Die Verwendung von zugegebenen Kontrollen wurde ebenfalls eingeführt. Diese Kontrollen waren Gene des Bakteriophagen Lambda.

Danach wurde diese Bank verwendet um die Expression zwischen *wt* und *fss* Embryonen zu vergleichen. Die *fss*-Mutante ist in dem Gen *Tbx24* defekt. Dieses Gen gehört nicht zum Delta-Notch Signalweg, aber zu den fused somite type Mutanten.

Die Zebrafisch oligo Bank von Sigma-Genosys wurde für den Vergleich der fused somite type Mutanten mit den Wildtypembryonen herangezogen. Die Mutanten die noch zu dieser Klasse gehören sind *des*, *bea* und *aei*. Diese Mutanten haben Defekte in Komponenten des Delta-Notch Signalweges, *des* in *notch1a*, *bea* in *deltaC* und *aei* in *deltaD*.

Da diese Komponenten am Beginn der Kaskade stehen und da es deshalb sehr wahrscheinlich ist das andere Teile der Kaskade einspringen könnten, wurde der Mediator des Signalweges, Su(H) ebenfalls verwendet. Für dieses Gen wurden Morpholino knock down Embryos erzeugt.

Das PSM besteht aus mehreren Teilen, nämlich dem posterioren, dem intermediären und dem anterioren Kompartment. Das PSM des Zebrafisches wurde nicht in diese Teile zerschnitten, da es sehr klein ist. Das PSM der Maus ist grösser und deshalb wurde es hier in einen anterioren und einen posterioren Anteil geteilt, wobei der

anteriore Teil das anteriore und das intermediäre Kompartment umfasste. Für diesen Ansatz wurde die Maus Oligo Bank von Sigma-Genosys verwendet.

Die Kandidatengene die in diesen Versuchen gefunden wurden, mußten noch durch eine andere Methode überprüft werden. Die *in situ* Hybridisierung wurde ausgewählt, da sie zusätzlich zur Validierung auch eine Lokalisierung der Expression erlaubt. Für den Maus-PSM Screen wurde diese Methode allerdings nicht verwendet. In diesem Fall wurde in der Literatur nach Expressionsmustern der gefundenen Gene gesucht. Zwei Kandidaten wurden auf diese Art und Weise überprüft.

Die Gene, die in den Zebrafischversuchen entdeckt wurden, wurden durch die *in situ* Hybridisierung validiert. Gene, die ein interessantes Expressionsmuster zeigen werden weiter charakterisiert, das heißt ihre funktionelle Rolle in der Somitogenese wird untersucht werden.

1. Introduction:

1.1. Somitogenesis:

Somites are a feature shared by all vertebrates. They are formed during embryogenesis in an ordered manner from anterior to the posterior, are flanking the notochord on both sides and are generated from the presomitic mesoderm (PSM) (Fig. 1). Three phases can be distinguished in somitogenesis: first, prepatterning of the PSM and establishment of the rostro-caudal polarity of the future somites, second, the formation of the somite border and third, the differentiation of the somites to generate the muscle, vertebrae, intravertebral disks and ribs of the trunk and tail (Maroto and Pourquiè, 2001).
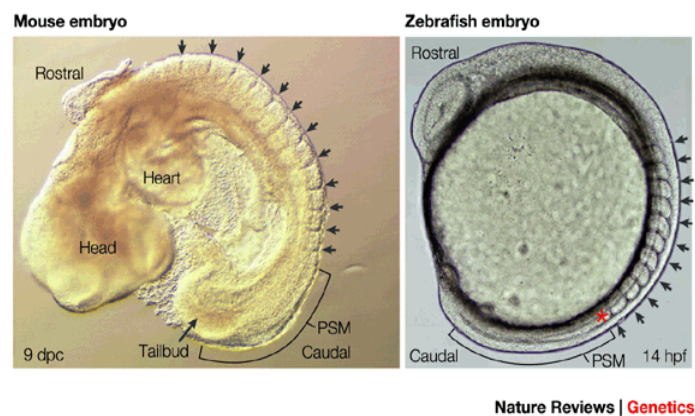
Figure 1: The left picture shows a mouse embryo and the right a zebrafishembryo during omitogenesis. The black arrows point to somite borders and the brackets demarcate the PSM (presomitic mesoderm), from which new somites are generated. (This picture was taken from Saga and Takeda, 2001).

Three classical models try to explain the periodicity of somite formation, namely Meinhardt's model, cell cycle model and the clock- and wavefront model. Meinhardt's model postulates that prior to the formation of each somite the cells in the presomitic mesoderm undergo several rounds of oscillation between two alternate stages corresponding to the prospective anterior and posterior compartment of the somite (Meinhard, 1986).

The cell cycle model tries to link the cell cycle to somitogenesis. Evidence for this comes from chicken embryos, which were heat-shocked and showed several segmentation abnormalities that are repeated along the AP (anterior-posterior) axis with a regular interval of six to seven somites (Primett et al., 1988). It takes

approximately 10 hours in the chicken to form this number of somites and this time-interval is needed to complete one cell cycle in the PSM (Primett et al., 1989). Therefore it was concluded that the cell cycle is the internal clock, which drives somitogenesis, but this theory has not been verified by further experiments. Nevertheless there is some linkage between somitogenesis and cell cycle, as Kawahara et al., 2004 suggest. They knocked down *GADD45beta*, which is involved in cell cycle control. The embryos displayed a disorganized cyclic expression of *her1* and of segmented expression of *MyoD*.

Cooke and Zeeman formulated the clock and wavefront model in 1976. Here two phenomena are needed for periodic somite formation. First, there must be an intrinsic clock driving oscillations of presomitic cells between a permissive and a non-permissive state. This is similar to the Meinhard's model, but they (Cooke and Zeeman, 1976) also postulated the existence of a wavefront travelling along the body-axis, which establishes a gradient of differentiation. Somite boundaries are formed, when oscillating cells of the PSM are reached by the wavefront. This model is most concurrent with the experimental data.

First molecular evidence for the existence of an oscillator was found in chicken (Palmeirim et al., 1997). It was detected that *hairy 1*, a basic Helix-Loop-Helix (bHLH) transcriptional repressor showed different patterns of expression in the PSM, although the chicken embryos used, were at the same developmental stage. Dynamic expression patterns were found to be repeated every 90 minutes, which is the time to form one pair of somites in the chick.

This oscillation was also detected to be an independent property of the PSM cells and not due to cell migration or a diffusible signal. Cells of the PSM undergo several rounds of oscillations before they become a somite.

Most of these cycling genes belong to the Hairy/Enhancer-of-Split (Hes) family which are targets of the Delta-Notch pathway as for example *hairy1* and *hairy2* in the chicken (Palmeirim et al., 1997), *hes1* and *hes7* in the mouse (Jouve et al., 2000; Bessho et al., 2001) and *her1* and *her7* in the zebrafish (Holley et al., 2000; Oates and Ho, 2002).

As Delta-Notch signalling plays an important role in somitogenesis it is interesting to see, which phenotypes are displayed by the mutants. Three mutants of the so-called fused somite type are defect in the Notch pathway, namely *des* (*notch1a*), *aei* (*deltaD*) (van Eeden et al., 1996) and *bea*(*DeltaC*) (personal communication Holley). These

8

mutants form several anterior somites and then cease somitogenesis. Therefore embryos of these mutants were chosen for the microrarray experiments described in this thesis. As the genes for which these mutants are defective are at the beginning of the signalling cascade and therefore "rescue and redundancy effects" are very likely to occur, the mediator of the Delta-Notch pathway, *Suppressor of Hairless (Su(H))* (Sieger et al., 2003) was used as well (Fig. 2). As no mutant for this gene has been generated so far, the morpholino knockdown embryos were used (kindly provided by Dirk Sieger).
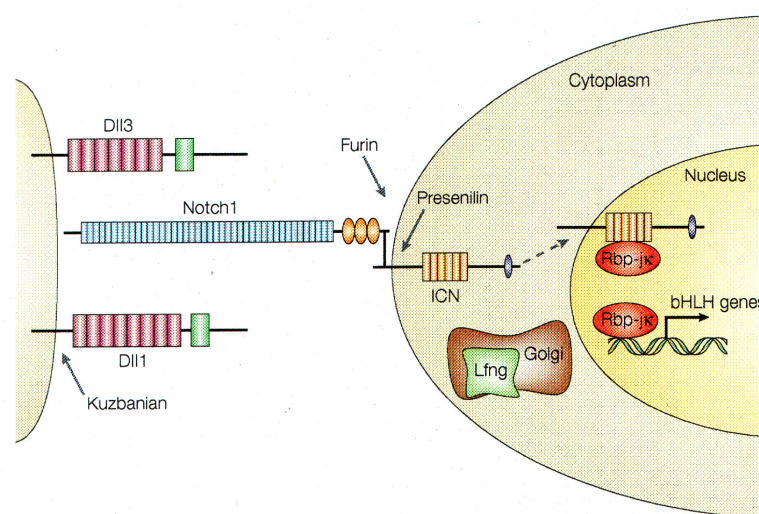
Figure 2: Scheme of the Delta-Notch pathway: The receptor Notch (Notch1) binds it ligand Delta (Dll1 or 3). The binding leads to cleavage (Furin and Presenilin) of the Intracellular Notch domain (ICN), which translocalises to the nucleus. In the nucleus the ICN binds Su(H) (Rbp-jκ) and activates the target genes. If Su(H) is not bound to the ICN it acts as repressor.
This picture was taken from Sada andTakeda, 2001.

There is another mutant belonging to the fused somite type, namely *fused somites (fss)* (van Eeden et al., 1996). The mutant embryos form no somites at all. The *fss* mutant was linked to the gene *Tbx24* (Nikaido et al., 2002), which is expressed in the intermediate and anterior PSM, but excluded in the posterior. This expression pattern might suggest that this gene plays a role in the wavefront, but the key-player of this part of somitogenesis seems to be *Fgf8*. It forms a gradient in the PSM were the concentration of *Fgf8* is highest in the posterior part (Dubrulle et al., 2001). As *Fgf8* is a growth factor and part of the FGF/MAPK (mitogen-activated protein kinase) signalling cascade it is perhaps needed to keep the cells in the posterior and intermediate compartment of the PSM in an undetermined or undifferentiated state. After the concentration falls under a certain threshold in the anterior PSM the cells are able to form the epithelia of the somites (Sawada et al., 2001).

1.2. Aim of this thesis:

Microarray technology was used to identify genes involved in the different somitogenesis events in zebrafish by comparing the expression in wildtype (wt) embryos with expression in different mutants belonging to the fused somite type class (van Eden et al., 1996) and Su(H)- morpholino knock down embryos (Sieger et al., 2003) that show defects in somite formation.

As experiments showed that there are several different compartments in the PSM it seemed worth to try to dissect it in at least a posterior and an anterior part. The zebrafish embryo is very small and the PSM even smaller, therefore it seemed worthwhile to do an experiment in the mouse, which has a bigger PSM. Here the PSM was cut in the two parts and those were hybridised on a chip. The border between anterior and posterior PSM was the umbilical chord, which was a good morphological feature and coincides with the expression of some genes, f.e. notch1 (Calceran et al., 2004) at E9.5.

The candidates found in the screens were validated using *in situ* hybridisations. Genes, which show a promising expression pattern are suitable for a further characterisation, i.e. their functional role in somite formation will be elucidated.

Before the microarray technology could be used for finding new somitogenesis genes it was necessary to be establish it.

1.3. Microarray technology:

This technology allows the comparison of gene expression between two or more states. This can be diseased and healthy, treated or untreated with chemicals or as used in this thesis between wildtype and mutant animals (Fig. 3).

A typical microarray experiment consists of several steps (Fig. 4). The beginning is the biological question (Fig. 4A). In this case it is to find genes, which are involved in somitogenesis (see 1.1). Parallel to this question and the designing of adequate experiments (Churchill, 2002; Yang at Speed, 2002) (Fig. 4B) the microarray technology had to be established in the laboratory, i.e. how to print the probes and in which printing buffer (Fig.4C). Another important feature was the production of the labelled targets. This means, which RNA extraction should be used, total RNA or poly A$^+$ enriched and before this, the tissue used for RNA extraction had to be chosen

10

(Fig. 4D). Intuitively one might suggest to use the presomitic region of the embryo, but as the zebrafish embryo is very small and a high amount of RNA is needed, we choose to use the entire embryo for RNA extraction. But in the case of the mouse microarray experiment we used parts of the PSM for RNA preparations. After this the right protocol for labelling the targets had to be established (Fig. 4E) and of course the hybridisation of the labelled targets to the printed probes as well (Fig. 4F). As mentioned above it is also very critical to design the experiments in a way that the most usage can be expected from the generated data. A keystone is the balance between technical and biological replicates (Yang and Speed, 2002). Technical replicates use the same labelled targets. Variances in these replicates stem for example from differences of the printed slides, differences during RNA isolation, labelling and so on. Biological replicates on the other hand use different samples. Here biological variance plays a role. This stems from individual differences of the fish (embryos and also parent fish), health state, water quality and so on. Another consideration should be, if the experimental design truly answers the biological question (Churchill, 2002). Following analogon should show what is meant with this. If for example, the difference between male and female fish should be investigated, one should be certain that one does not have old and young fish in the sample because then the differences of age will also fall in the pool of differences. This elucidates why thinking of sampling is very important.
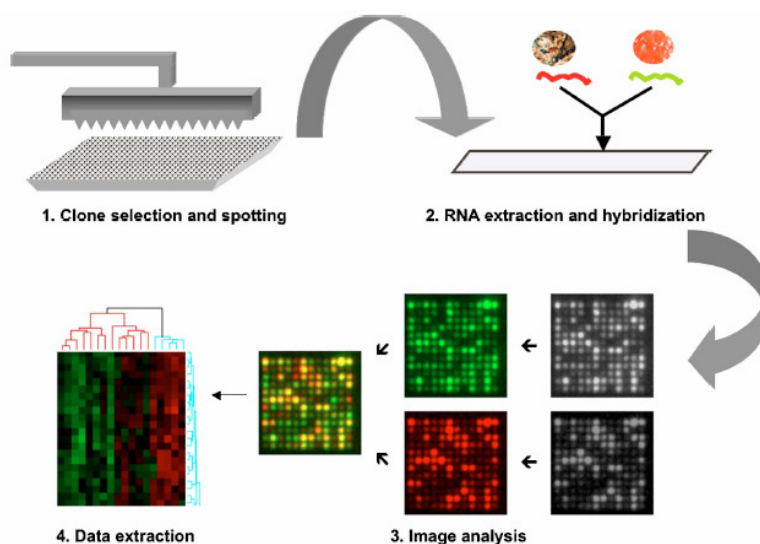
Figure 3: Flowchart of a microarray experiment: The clones, oligos or cDNAs (referred to as probes) are spotted on a coated glass surface (slide). RNAs are extracted from two different tissues. The RNA is transcribed into cDNAs and labelled with two different fluorphores (dyes). These labelled targets are hybridised to the slide. During scanning two different images, for each dye one are created and then analysed.

(Weeraratna et. al, 2004)

Scanning follows the hybridisation (Fig.4G) and gridding converts this scanned images into columns of numbers referring to the measured intensities and background values (Fig. 4H). Normalisation eliminates differences stemming from differences in dye efficiency (coupling to the cDNA, produced from the RNA) (Fig. 4I) (Yang et al., 2002; Quackenbush, 2002;Park et al., 2003). After this, statistic methods had to be chosen (Fig. 4J), which allow to differentiate between true expression differences and variable differences coming from random events. They should be easy to use and suit the special requirements of the biological question (Leung and Cavaliere, 2003). As we are interested in differently expressed genes it might seem valid to set a certain threshold of up- or downregulation for a given gene and this is it what we did in the beginning of the experiments, later, experience showed that it would be better to compare expression changes to housekeeping genes and/or genes, which were known to be differently expressed (Chaudhuri, 2005).

A	Biological question
B	Experimental design
C	Printing of probes
D	mRNA isolation
E	Labeling of targets
F	Hybridization
G	Scanning
H	Gridding
I	Normalization
J	Statistical analysis
K	Selection of candidates
L	Validation
M	further analysis
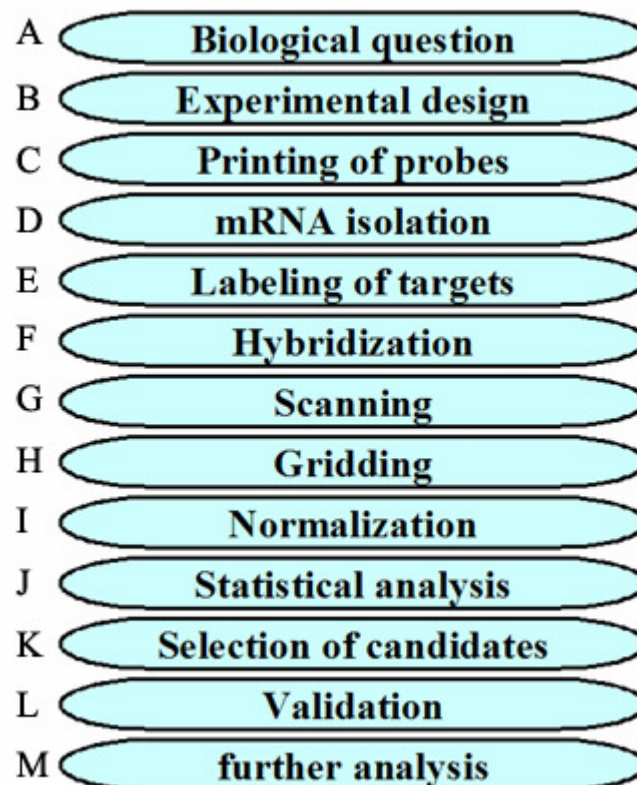
Figure 4: Steps in a microarray experiment.

2. Materials and Methods:

2.1. Materials:

2.1.1 Chemicals, kits and enzymes:

μMACs Kit (Miltenyi)

Fairplay labelling Kit (Stratagene)

Cyscribe labelling Kit (Amersham)

SYBR Green II (Molecular probes)

Ethanol (Merck)

Diethyl Pyrocarbonate (DEPC) (Sigma)

NaOH (Fluka)

HCl

LiCl (Sigma)

Oligo dT primer

Reverse transcription buffer (Promega)

Reverse transcriptase (Promega)

10x transcription buffer (Roche)

dNTPs (Sigma)

NTPs (Roche)

DIG RNA labelling mix (Roche)

SP6 RNA polymerase (Roche)

T7 RNA polymerase (Roche)

DNaseI (Roche)

Glycogen (Ambion)

BM Purple (Roche)

RNase Inhibitor (Roche)

Anti DIG AP Fab fragments (Roche)

$Na_2HPO_4*2H_2O$ (Merck)

$Na_2SO_4*10H_2O$ (Merck)

KH_2PO_4 (Sigma)

NaCl (Merck)

$MgCl_2$ (Sigma)

Sodiumcitrate (Sigma)

KCl (Sigma)

Tween-20 (Merck)

Sodium Acetate (Fluka)

Cy3 reactive dye (Amersham Pharmacia)

Cy5 reactive dye (Amersham Pharmacia)

Alexa 555 (Molecular Probes)

Alexa 647 (Molecular Probes)

Sodium bicarbonate (Sigma)

Formamide

Boehringer Block (Roche)

Yeast RNA (Sigma)

Heparin

3-(Cyclohexylamino)-propan-1-sulfonacid (Chaps) (Sigma)

Paraformaldehyde

Triethanolamine (Sigma)

BSA (Bovine serum albumin) (Sigma)

Levamisol

DMSO (Sigma)

Ethidium bromide

Iso-propanol (Merck)

Methanol (Merck)

Taq DNA polymerase (selfmade)

10x PCR Buffer (selfmade)

Taq DNA polymerase (Ampliqon)

10x PCR buffer (Ampliqon)

Bruce Apple cDNA library

Lambda Phage DNA (Sigma)

Sigma Genosys mouse oligo library

Sigma Genosys zebrafish oligo library

Control oligos: lambda genes Q, N and G (Sigma)

LB Broth Base (Gibco BRL)

LB Agar (Gibco BRL)

SOLR cells (Stratagene)

Virkon

Ampicillin (Sigma)

Kanamycin (Sigma)

EDTA (Sigma)

PCR purification kit (Quiagen)

Rapid PCR purification and Gel extraction system (Biocat)

Phenol/Chloroform (Roth)

Chloroform (Roth)

Tris-base (Merck)

Ultra Pure H_2O

Glycerol (Roth)

2.1.2 Equipment:

Tubes 1.5 and 2ml (Eppendorf)

PCR tubes (Mβb)

Incubator for fish

Incubator for bacteria

Bacterial shaker

Incubators

12 well dishes (Nunc)

pH meter

Gel chambers

Power supply (Biorad)

Microscopic slides (Roth)

Stereomicroscope (Zeiss)

In situ machine (Intavis)

Heating blocks (Eppendorf)

Mastercycler personal (Eppendorf)

DNA Engine Tetrad (MJ Research)

Centrifuge (Eppendorf)

Centrifuge 5810 (Eppendorf)

Pipettes (Gilson): P2, P20, P200, P1000

Pasteur-pipettes glass (KMF)

Pasteur-pipettes plastic (KMF)

Forceps (Inox no. 11)

Aspiratory tube assembly for microcapillary pipettes (Sigma)

glasscapillaries

Reusable columns

GAPSI slides (Corning)

GAPSII slides (Corning)

Elipsa slides (Eppendorf)

Schott H slides (Schott)

Gene Tac G3 (Genomic solution): Spotter and picker

Gene Tac Hybridization (Genomic solution)

Gene Tac LS IV (Genomic solution): Laserscanner

Multiprobe II Robotic liquid handling system (Packard)

Hybridisation chambers (Corning)

Coverslips (Corning)

Lifterslips (Eerie)

Slide-rack (Roth)

Glass dishes (Roth)

96 well culture plate (Nunc)

96 well plate (Brand, ABgene)

96 well filter plate (Nunc)

384 microarray plates (ABgene)

airpore tape (Deelux)

PCR plate tapes (Brand)

Robotic tips (Mβb, Neptun): 20, 200, 1000μl conductive and nonconductive

Spectrometer (Eppendorf)

2.1.3. Animals:

Fish (*Danio rerio*):

Wildtype fish were obtained from animal store "Schlepps"

Mice wildtype strain:

FVB/N (Charles River Laboratories)

2.1.4. Hormones:

Pregnant mare serum (PMS)

Human gonadotropic hormone (HGC)

2.1.5. Primers:

Table 1: Primer used

	Name	**Accession-no**	**Sequence**
Lambda control genes			
	Q-forward	M38285	TAT TTA GGT GAC ACT ATA GGG CGC ATG AGA CTC GAA AGC GTA GC
	Q-reverse		(T)18 CAT GCT GCT AAC GTG TGA CCG CAT TC
	N-forward	V00637	TAT TTA GGT GAC ACT ATA GTG GAC TGA ATT AGT TGC CAG CTA TG
	N-reverse		(T)18 TGG CGG TGT TGA CAG AAA TAC CAC TG
	G-forward	X00166	TAT TTA GGT GAC ACT ATA GGA TCA GCC AAA CGT CTC TTC AGG CC
	G-reverse		(T)18 GGT GTT AGA TAT TTA TCC CT TGC GGT
Primer used for amplification of fragments of cDNA library			
	T3-forward		AAT TAA CCC TCA CTA AAG GG
	T7-reverse		TAA TAC GAC TCA CTA TAG GG
Primers for candidates of « Des » chip			
(forward primers include T3 sequence, reverse primer include T7 sequence)			
	Cx43.4 forward	L46801	AAT TAA CCC TCA CTA AAG GGT CGA GGA CTG AGA CG
	Cx43.4 reverse		TAA TAC GAC TCA CTA TAG GGC GGT CAA TGT CTC GC
	D249 forward	AB055680	AAT TAA CCC TCA CTA AAG GGA ACA AGC ACG GAG TG
	D249 reverse		TAA TAC GAC TCA CTA TAG GGT GTC TCA GGT GCA GC
	Her6 forward	X97333	AAT TAA CCC TCA CTA AAG GGA ACA GAC TGT GAC AT
	Name	**Accession-no**	**Sequence**
	Her6 reverse		TAA TAC GAC TCA CTA TAG GGC CTC GGA

Name	Accession-no	Sequence
		GTC TAC CA
Hsp90 forward	AF068772	AAT TAA CCC TCA CTA AAG GGA CCA AGA TGC CTG AA
Hsp90 reverse		TAA TAC GAC TCA CTA TAG GGG AAG AGC GCG GAA CT
Mesogenin forward	AJ309314	AAT TAA CCC TCA CTA AAG GGA GGA CAG GTC ATT TG
Mesogenin reverse		TAA TAC GAC TCA CTA TAG GGT GCC GGA TAA CTC TT
Mta forward	AF097875	AAT TAA CCC TCA CTA AAG GGG GAC AGT GTC TAC TA
Mta reverse		TAA TAC GAC TCA CTA TAG GGC TCC AGC AGG AAG AA
P38a forward	AB030897	AAT TAA CCC TCA CTA AAG GGA GAC TAG GAG CTG CG
P38a reverse		TAA TAC GAC TCA CTA TAG GGA TTC CTC TTG GGC AT
Pbx4 forward	AF162696	AAT TAA CCC TCA CTA AAG GGG CTT GGG AAC AAA CC
Pbx4 reverse		TAA TAC GAC TCA CTA TAG GGC GGT TGA CCG AGC GA
MMP1A forward	BI867183	AAT TAA CCC TCA CTA AAG GGG CTT GAT TCA AAA TT
MMP1A reverse		TAA TAC GAC TCA CTA TAG GGG TGC GAT TCT GGG AT
ZfdIII forward	BG304258	AAT TAA CCC TCA CTA AAG GGG TTA GTT CCT GTC CG
ZfdIII reverse		TAA TAC GAC TCA CTA TAG GGC GCA CAT TTC CCA GC
Ziro5 forward	AY017309	AAT TAA CCC TCA CTA AAG GGA CTC AAG TGA GAA GC
Ziro5 reverse		TAA TAC GAC TCA CTA TAG GGG AGA ATG AAT AAC AG
Ziro7 forward	AF398433	AAT TAA CCC TCA CTA AAG GGA AAC TTC TTC ATG GA
Ziro7 reverse		TAA TAC GAC TCA CTA TAG GGT CTG TTA TGC AAA CA
Name	**Accession-no**	**Sequence**
Ztsg1 forward	AF332096	AAT TAA CCC TCA CTA AAG GGG ATG GGG TCT TCA TC
Ztsg reverse		TAA TAC GAC TCA CTA TAG GGG TCC AGC

		AGA AAC AC

2.1.6. Solutions:

DEPC-H$_2$O:

0.5ml DEPC was added to 1l H$_2$O. The solution was left overnight at room temperature. Later the DEPC was inactivated by autoclaving.

20 x SSC :

175.3g NaCl and 88.2g Sodiumcitrat were dissolved in 1l H$_2$O. The pH was adjusted to 4.7. Finally the solution was autoclaved.

20 x PBS:

160g NaCl, 6g KCl, 23g Na$_2$HPO$_4$ and 4.8g KH$_2$PO$_4$ were dissolved in 1l DEPC H$_2$O. The pH was adjusted to 7.5 and the solution was autoclaved.

1xPBS:

20x PBS was diluted to 1x.

4% PFA:

40g paraformaldehyde powder was dissolved in 1l 1xPBS. The solution was heated until it became clear. Then it was aliquoted.

Tris-HCl:

121.1g Tris-base was dissolved in H$_2$O. The pH was adjusted by adding HCl.

5M NaCl:

292g NaCl was dissolved in 1l DEPC H$_2$O. The solution was autoclaved.

0.5M MgCl$_2$:

101.7g MgCl$_2$ were dissolved in 1l DEPC H$_2$O. The solution was autoclaved.

50x TAE-buffer:

19

For 1l 242.2g Tris and 57.1g Acetanhydrid were dissolved in water. 100ml 0.5M EDTA pH 8 were added and the solution was filled up with water.

For electophoresis the buffer was diluted to 1x TAE.

Agarose-gel loading buffer:

15% Ficoll was dissolved in 45ml H_2O. One drop of Bromphenol (0.25%) and Xylene Blue (0.25%) were added. The bluejuice was aliquoted in 1.5ml Eppendorf tubes.

Sephadex G50:

10g of the sephadex were suspended in 150 ml water and autoclaved. The sephadex was stored at 4°C

Printing buffer (Schott H and Elipsa slides):

To approximately 800 ml of water 1.78g of $Na_2HPO_4*2H_2O$ (100mM) was added. To this 10g of $Na_2SO_4*10H_2O$ (10%) was also added. Mixing was done until the solution became clear. Then the solution was adjusted to pH 9. Finally water was added to get 1 l of 1x printing buffer.

Hybridisation and washing solutions (GAPSI and II slides):

The hybridisation solution contains 3xSSC, 0.3%SDS and 200ng/µl yeast tRNA.

Medium stringency buffer: 2xSSC and 0.1%SDS

High stringency buffer: 0.1xSSC and 0.05% SDS

Hybridisation and washing solutions (Elipsa):

The Pre-hybridisation-solution was 3xSSC, 0.3%SDS, 1%BSA

The Hybridisation-solution was the same as the Pre-hybridisation-solution except for the lack of BSA (3x SSC, 0.3%SDS).

Washing solution 1: 2xSSC, 0.1 SDS

Washing solution 2: 0.1xSSC, 0.1%SDS

Washing solution 3: 0.1xSSC

Hybridisation and washing solutions (Schott H):

Blocking solution: 50mM Ethanolamine was dissolved in 50mM borate buffer. The solution was adjusted to pH 9.0.

Hybridisation solution: 2xSSC, 0.1% SDS

Washing solution 1: 2xSSC, 0.1% SDS

Washing solution 2: 0,1xSSC, 0.1% SDS

Washing solution 3: 0.1xSSC

Binding solution (PCR-product purification):

5.4 M Guanidinium-Hydrochlorid was dissolved in 20mM Tris-HCl, pH 7.5; 5mM EDTA; 50% Ethanol

1 x PBST:

20x PBS was diluted to 1x and 0.1% Tween-20 added.

2 x SSCT:

20 x SSC was diluted to 2x SSC and Tween-20 was added to a final concentration of 0.1%.

0,2 x SSCT:

20 x SSC was diluted to 0.2x SSC and Tween-20 was added to a final concentration of 0.1%.

Hybridisation-solution:

25ml Formamide, 12.5ml 20x SSC, 0.5g Boehringer Block, 500μl Heparin (10mg/ml), 500μl Denhard's, 500μl 10% Tween-20, 500μl Chaps and 500μl 0.5M EDTA were mixed and brought to the final volume of 50ml.

0.1M Triethanolamine (TEA):

0.93g TEA was dissolved in 50ml DEPC H_2O and the pH adjusted to pH 7.8.

Blocking solution I (*in situ* Hybridisation):

0.1 g BSA was dissolved in 50 ml 1xPBST.

Blocking solution II (*in situ* Hybridisation):

20ml Blocking solution I was mixed with heat-inactivated sheep serum (5%)

Alkaline Phosphates (AP)-buffer:

5ml 1M Tris, 5ml 0.5 M $MgCl_2$, 1ml 5M NaCl, 500µl 10% Tween and 250µl 1M Levamisol were mixed and brought to final volume of 50ml.

TE-buffer:

10mM Tris-Cl and 1mM EDTA (pH 8)

2.2. Methods:

2.2.1: Preparation of embryos:

2.2.1.1: Fish:

Fish were mated in separate small plastic boxes filled with water. On the bottom of the box were small glass balls to prevent eating of the eggs by the adult fish. One female fish was mated to one or two male fish. Mating started in the next morning after the light-cycle began.

Two hours later the eggs were collected. The fish were grown overnight in a 22°C incubator. The fish grew to 10 somite stage. Then the fish were either used for RNA extraction (described below) or dechorionised and fixed in 4% PFA overnight at 4°C. After fixing the fish were dehydrated in MeOH (5min in 33% MeOH in PBST, 5min in 66% MeOH in PBST and 5min in 100% MeOH). The embryos were stored in 100% MeOH at -20°C until use for *in situ* hybridisation.

2.2.1.2: Mice:

To increase the number of embryos, female mice were superovulated. For this purpose FVB/N mice between four to six weeks were injected with PSM at midday. 48h later HCG was injected as well. Then the mice were mated. At E9.5 the females were killed. Noon after the day the vaginal plug was observed is considered as E0.5. The embryos were dissected out of the embryonic membranes in ice-cold PBS. Then in a first step the PSM below the umbilical chord was cut off and put into RNA later. After this the PSM above the umbilical and below the first somitic border was removed and put into another tube containing RNA later. The RNA of these two samples was extracted as described below.

2.2.2 Mixed embryogenesis stage cDNA -Library:

This library was kindly provided by Bruce Appel. It was created using the Stratagene ZAP-cDNA synthesis kit using 12-20 somite stage embryos. PCRs, Purification and Rearraying were done together with Dr. Martin Gajewski, Eva Schetter and Vladimir Simovic.

Mass excision was done following the instruction of the kit. The phagmids were transformed into SOLR cells and the bacteria were streaked out on LB Agar plates (described in Stratagene instruction manual: cDNA Synthesis Kit, ZAP-cDNA Synthesis Kit, and ZAP-cDNA Gigapack III Gold Cloning Kit).

2.2.2.1: Picking of colonies:

Colonies were picked using the Gene Tac Picker tool. 96 well culture plates were filled with LB medium containing Ampicillin. The plates were loaded into the picker together with the colonies on the LB-amp agar plates. The picking device was also equipped with washing baths allowing the washing and sterilisation of the 48 pin picking head before, during and after the picking process. The picking tool was first washed in 50% EtOH and Virkon. Then the tool moved to the ultrasonic bath containing water and was washed for 5 seconds. Afterwards the tool was washed in 70% EtOH for 5 seconds. Finally the tool was sterilised with heat for five seconds. To allow cooling down of the pins the head rested for 5 seconds before the next picking step.

The automated vision system (CCD camera and frame grabber card) could detect colonies after setting the parameters: size, circularity, grey range and kernel size. It was necessary to set this parameter carefully because otherwise merged colonies or air bubbles in the agar would have been picked. The picked colonies were transferred to the 96 well culture plates. After picking the plates were sealed with air pore tape and incubated o.n. on the shacker (300rpm) at 37°C.

2.2.2.2: Automated colony PCR in 96 well format:

From one 96 culture well plate two identical 96 colony PCRs in 96 well plates were made.

The mastermix (1U Taq-polymerase, 1.5 mM Magnesium buffer, 1x Taq buffer, 200μM dNTPs, 200nM T3 forward primer, 200nM T7 reverse primer) was filled into

a container and put into the machine on a cooling socket. The empty 96 well plates were also put on cooled holds. The 96 culture well plate was loaded in the machine, too. 200μl conductive tips were used by the machine to pipette the mastermix into the 96 well plates (95μl per well). Afterwards 5μl bacterial suspension was pipetted to the mastermix. As two PCR plates were produced the total volume of bacterial suspension, which was pipetted was 10μl. To the remaining bacteria 100μl of 60% Glycerol was added and stored at -20°C.

The PCR in the 96 well plates was done in a tetrad-thermocycler. Reaction profile: 95°C for 2minutes, 35 cycles: 95°C for 30 seconds, 55°C for 45 seconds, 72°C for 2 minutes and a final extension of 72°C for 5minutes. The PCRs were checked on a 1% Agarose Gel.

2.2.2.3: PCR-product purification in 96 well format:

The protocol used for PCR product purification was established by Dr. Martin Gajewski and was also done with the pipetting-robot. A 96-well filter plate was mounted on a vaccum manifold. The PCR products of both produced plates were transferred together with the Binding solution into the 96-well filter plate. Then vacuum was applied. 700μl wash solution washed the bound DNA. After this again vaccum was applied, this time longer to allow the entire removal of washing solution. With 50μl water (warmed to 60°C) in each well the DNA was eluted and collected in a fresh 96-well plate. The elution was checked on a 1% agarose gel.

2.2.2.4: Rearraying of purified PCR products:

This step was done using the pipetting-robot. Four 96 well plates containing purified PCR products were rearrayed into a 384 well plate. To do this 10 μl were pipetted out of each well and transferred to the 384 plate containing 10μl DMSO (printing buffer). The scheme of rearraying can be seen in Fig. 5.

Positions 1-96 refer to plate 1, 97-192 to plate 2, 193-288 to plate 3 and 289-384 to plate 4. Tables were created to allow an easy reference to the original 96 well plate.

2.2.3 Sigma Genosys mouse oligo library and zebrafish library:

Both libraries were delivered in 384 well format. The oligos were lyophilised and each well contained 0.5nmole. The oligos are 65-mers with a 5'-C6 amino modification, helping in covalent binding of the oligo to the slide coating substrate. One oligo has been designed for each gene. Control oligos were present in the library and were housekeeping genes, i.e. beta-actin. The oligos were dissolved in the

Printing buffer to a final concentration of 20μM and a daughter-plate-set was created, which was used for printing.

Mouse oligo library: This library consisted of 22 228 oligos, including 231 control oligos. Dissolvation and creating of daughter plates was done together with Chriz Voolstra.

Zebrafish oligo library: This library was made up of 16 399 oligos, including 171 controls.
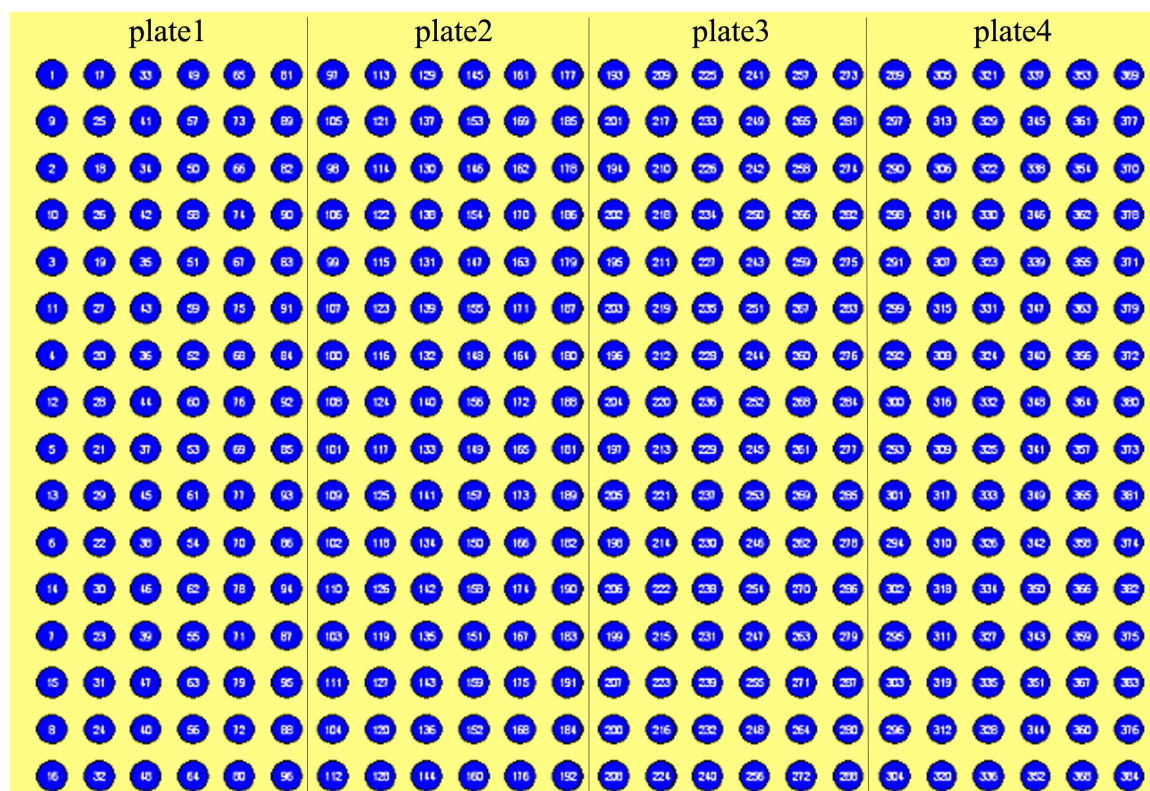
Figure 5: Scheme of rearraying. Four 96 well plates were rearrayed into one 384 well plate. The PCRs of plate 1 are in postions 1-96; of plate2 in 97-192; of plate 3 in 193-288 and of plate 4 in 289-384.

2.2.4. PCR and *in vitro* transcription of the Lambda-Control genes for the cDNA-chip

Out of Lambda DNA three control genes were amplified, namely Q, N and Gama cI (G).

The mastermix contained 1x PCR buffer, 2.5mM $MgCl_2$, 5% DMSO, 200nM forward primer, 200nM reverse primer, 1U Taq polymerase, 1μl lambda phage DNA. The primers

had an overhang carrying the SP6 polymerase sequence.

Reaction profile: 94°C for 15 minutes, 40 circles: 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds and a final extension of 72°C for 2min.

The PCR products were purified using the Quiagen PCR purification kit. In the case of the Gene G more than one band was obtained. The product band was cut out and gel extracted using the Gel extraction kit.

For all genes one reamplification was done. This product was purified, too.

The purified product was Phenol- Chloroform extracted: One volume of Phenol/Chloroform was added and the tube was vortexted and afterwards centrifuged at maximum speed for 7 minutes. The upper phase (containing the DNA) was transferred to a new tube and mixed with one volume of Chloroform and again centrifuged. Again the upper phase was pipetted into a new tube. The DNA was precipitated by adding 1/20 volume 5M NaCl and 2.5 Volumes Ethanol at -20°C for 1 hour. After this time the tube was centrifuged at maximum speed for 20 minutes and the supernatant was discarded. The pellet was washed once with 70% Ethanol in DEPC-H_2O and afterwards dried completely. The pellet was dissolved in 20μl TE-buffer and the concentration was measured. 200-400ng were used for transcription together with 2μl 10x transcription buffer, 2μl 10mM NTPs, 1μl RNase-Inhibitorand 20U SP6 RNA Polymerase. The final volume of the reaction was 20μl. The reaction was incubated at 37°C for 3hours. To destroy the template DNA 1μl DNase was added after the transcription and incubated for 15 minutes at 37°C. After this the reaction was stopped by adding 1μl 0.2M EDTA. To get rid of the DNase the sample was again Phenol-Chloroform extracted and precipitated as described above. This time the pellet was dissolved in 20μl DEPC-H_2O. The transcription was checked on a 1.5% Agarose-gel.

2.2.5. Printing on Microarray slides:

General:

Slides, which were stored at 4°C (Elipsa) or –20°C (Schott H) were put to room temperature 15 minutes prior to printing. The 384 plates were also allowed to warm to room temperature and afterwards spun down at 240g for 3 minutes. Slides and plates were put into the spotter. An antistatic brush was used to remove dust from the slides. The sonic bath was filled with water as well as one of the washing-station. The second washing-station was filled with 50% Ethanol. The washing protocol was 3 seconds

washing in sonic bath, 3 seconds in water with brushing, 3 seconds washing in 50% EtOH, 3 seconds heater and 5 seconds air-dry.

After spotting the slides were left for 10 minutes in the spotter to allow drying.

GAPSI and II: On this slide the PCR-products from the Bruce Appel cDNA were printed. As controls, Q and GFP DNA was printed on each sub-grid. As printing buffer 50% DMSO was used. To covalently attach the DNA to the chip surface the slides were incubated for 2 hours at 80°C.

Elipsa: On this type of slide the mouse oligo library was printed. Four slides were necessary to print the entire library. Three different controls were spotted on the chips. The Q oligo was printed on each subgrid and served additionally as a landmark. The oligos N and G were just printed into each subgrid on slide2 and 4. 100mM Sodium Phosphate Buffer was used for printing. Spotting on this type of slides was done together with Chriz Voolstra. Post-processing was done as described for the GAPS-slides.

Schott H: On this slide the zebrafish oligo library was printed. Two slides were needed to print the entire library. The control Q was again in each subgrid. The controls N and G could only be seen on slide2. The same printing buffer as for Elipsa slides were used. Post-printing incubation was done in a humidity chamber (wet towels in a box) for 2 hours at room temperature. Finally the slides were dried, by leaving them overnight in a closed box.

2.2.6. SYBR green II staining:

After printing the success of the spotting process, i.e. spot morphology and spot size, could be checked. One slide of each printing run was incubated in a 10 000 fold dilution of SYBR green II stain in 0.5x TBE buffer for 3minutes. Afterwards the slides were washed four times in 0.5x TBE buffer. Finally the slides were dried by centrifugation and scanned in the Microarray Scanner at 560nm.

2.2.7. Labelling using the Fairplay-kit (Stratagene):

The RNAs (approximately 1μg per sample) in precipitation was spun down (maximum speed for 20minutes). Then the pellet was washed with 70% Ethanol and dried completely at room temperature.

cDNA synthesis:

The dried RNA was dissolved in 8.5µl DEPC-H$_2$O. Control RNAs were added in following amounts: To the vial containing the RNA, which was later labelled with the green fluorphor 1µl Q, 0.5µl N and 2µl G were added. To the other vial 1 µl Q, 2µl N and 0.5µl G were added.

To obtain a complete redissolvation, the RNAs were heated for 15 minutes at 37°C. Afterwards the RNAs were cooled down on ice and spun down. 2µl 10x Stratascript reaction buffer, 1µl 20x dNTP mix, 1.5µl 0.1DTT, 0.5ul Rnase Block (40U/µl) and 1µl Stratascript reverse transcriptase (50U/µl) were added to each tube. Incubation was done at 48°. After 25minutes 1µl of Stratascript reverse transcriptase was added again and the tubes were incubated for additional 35 minutes.

10µl 1M NaOH were added to the reactions and incubated at 70°C for 10 minutes to hydrolyse the RNA. After this the reactions were slowly cooled down to room temperature. The cDNA-solutions were neutralized by adding 10µl 1M HCl. The cDNA was purified by precipitation (4µl 3M Sodium Acetate, 1µl Glycogen and 100 µl Ethanol). Precipitation was done overnight at -20°C. The reactions were spun down at maximum speed for 15 minutes. After washing the pellet with 70% Ethanol, it was dried completely.

Fluorescent Dye Coupling :

Using Amersham Pharmacia dyes :

The pellets were dissolved in 10µl 2x Coupling Buffer. 45µl DMSO was added to one Cy3 and one Cy5 reactive dye package. After vortexing the dyes were spun briefly. 10 µl of the dissolved dye were added to the cDNA sample. In darkness the tubes were incubated for 30 minutes at room temperature.

Using Alexa Dyes :

The pellets were dissolved in 5µl nuclease free H$_2$O and incubated at 42°C for 5 minutes. Then 3µl of labelling buffer (Sodium bicarbonate buffer) were added.

2µl of DMSO were added to each vial of Alexa Dyes (555 and 647). The 8µl of amine-modified DNA were added to the dye. In darkness the tubes were incubated for 1 hour at room temperature. Finally 10µl H$_2$O were added to the reaction.

Purification:

Again a precipitation step was performed (2µl 3M sodium acetate and 50µl 100% ethanol) at -20°C for at least 30 minutes. The fluorescent DNA was spun down at

maximum speed for 15 minutes and washed with 70% Ethanol. The dried pellet was dissolved in 20µl deonized H_2O.

850µl sephadex were added to reusable columns. The column was placed into a 2ml tube and spun at 300xg for 1 minute. The flow-through was discarded and the spin column placed in a new 1.5ml tube. On the center of the column the labelled probe was added and spun for 2 minutes at 300xg.

To check for the quality of the labelled material, 2µl probe were loaded together with 2µl loading buffer on a 1% agarose gel without ethidium bromide. The gel was run for 1hour at 70 Volt. In the Microarray-scanner the labelled cDNA could be checked.

2.2.8. Labelling using the Cyscribe-labelling kit:

The RNA was handled as described in the fairplay labelling section. Control RNAs were added in the same amount as described above. 1µl of anchored oligo(dT) was added and incubated for 5 minutes at 70°C. After cooling down 4µl of 5x CyScript buffer, 2µl 0.1M DTT, 1µl dUTP nucleotide mix, 1µl dUTP CyDye-labelled nucleotide (red or green) and 1µl of reverse transcriptase were added and incubated at 42°C for 1.5 hours. After the cDNA production the RNA was degraded by adding 2µl of 2.5M NaOH to the reaction and incubating it at 37°C for 15 minutes. 2µl of 2.5M HCl neutralised the cDNA solution.

The labelled cDNAs were purified in the G-50 Sephadex Spin Columns as described above.

2.2.9. Hybridisation on microarray slides:

GAPSI and GAPSII slides:

To 100µl of hybridisation solution 10µl of labelled cDNA was added (5µl green and 5µl red labelled cDNA). The cDNA-mixture was denatured at 85°C for 3minutes and then at 65°C for 20 minutes.

The hybridisation was done in the GeneTAC Hybridisation-station and the slides were mounted according to the instructions.

The hybridisation program consisted of following steps:

The probe was introduced at 75°C. Then a step-down hybridisation was performed; 3hours at 65°C, 3 hours at 55°C and 12 hours at 50°. Afterwards the slides were washed in the machine: medium stringency buffer 6 times at 50°C for 20 seconds. High stringency buffer 3 times at 25°C for 20 seconds and post wash buffer 3times at 25°C for 20 seconds.

The slides were dried by centrifugation at 235xg for 5minutes.

Elipsa slides:

The prehybridisation solution was heated to 42°C and the arrays incubated in it for 45 minutes, keeping the temperature at 42°C. Afterwards the slides were rinsed in water at room temperature. The slides were shortly dipped in iso-propanol and finally dried by centrifugation at 235g for 2 min.

The hybridisation was done in hybridisation chambers. Into the tiny holes 3xSSC was pipetted to allow a humid environment in the chambers during hybridisation. To the labelled and combined cDNAs hybridisation-solution was pipetted (For four slides 265µl). The cDNAs were denatured by heating the solution for 10 minutes at 80°C. The cDNA-hybridsation-solution was pipetted on the slides and a coverslip was placed onto the slide. The slide was put into the chamber and the chamber was submerged into a waterbath at 42°C. Hybridisation was done overnight (at least 16 hours).

After hybridisation the slides were washed using the glass-rack and the glass-chambers. The first washing step was done at 42°C with washing solution 1 and was done to remove the coverslips. Afterwards the slide were put into another washing bath also containing washing solution 1 at 42°C and incubated for 5minutes. Then the slides were washed five times in washing solution 2 for 5 minutes at room temperature. Finally the slides were rinsed five times in washing solution 3 and dried by centrifugation at 235xg for 5minutes.

Schott H slides:

Prior to hybridisation the slides were incubated in the blocking solution for 1 hour at room temperature. Afterwards the slides were washed two times with water and then dried by centrifugation at 240xg for 6minutes. The cDNAs were combined with the hybridisation solution (65µl per slide were needed. Therefore 5µl of each labelled

cDNA was mixed with 55μl of hybridsation solution). The cDNA in hybridisation solution was denatured at 80°C for 10 minutes and then kept at 42°C until pipetting on the slide. A coverslip and later a lifter slip were placed onto the slide. Then the slide was put into a Corning chamber (Preparation of Corning chambers see Elipsa slides) and the chamber was submerged into a waterbath at 42°C. Hybridisation was done overnight (at least 16 hours). The slides were put shortly into washing solution 1 to remove the cover- or lifterslips. Then the slides were incubated for 5 minutes in washing solution 1 at room temperature. Afterwards the slides were incubated for 5 minutes in washing solution 2. Finally the slides were rinsed five times in washing solution 3 and the slides dried by centrifugation at 240xg for 6minutes.

2.2.10. Scanning of microarray slides:

The micorarray slides were scanned using the Genetac Scanner following the instruction manual. The green fluorphor was scanned at 560 nm and the red one at 647nm.

2.2.11. Preparation of DIG labelled Antisense probes:

The template DNA was created by PCR using genespecific primers with an T7 sequence overhang. The PCR was purified. 100ng of the purified DNA was used as template. 2μl DIG RNA labelling mix, 2μl 10 transcription buffer, 2μl T7 RNA polymerase and 1μl of RNase Inhibitor was added to the template. The transcription reaction volume was 20μl. The reaction was incubated for 2hours at 37° C. After this the template DNA was destroyed by adding 2μl DNaseI and incubation at 37°C. Purification was done by precipitation using 1/10 LiCl, 2.5Volumes 100% Ethanol

and 1μl Glycogen. The precipitation was done at -20°C over night. The RNA was spun down at maximum speed for 20 minutes and washed once with 75% Ethanol in DEPC-H$_2$O. The dried pellet was dissolved in 20μl DEPC-H$_2$O. To prevent degradation of the DIG labelled RNA 20μl of Formamide was added and the RNA was stored at -20°C.

2.2.12. *in situ* Hybridisation:
2.2.12.1. Rehydration of embryos:

The dehydrated embryos were rehydrated by incubating them for 5minutes in 66% Methanol in PBST, 5minutes in 33% Methanol in PBST and washing them 3 times in PBST.

2.2.12.2. Heat-treatment of zebrafish embryos

The embryos in 1ml PBST were put in nearly boiling water for 10 minutes. The embryos were whirled up every two minutes to avoid sticking together of the embryos. After this the embryos were put for 5minutes on ice. Again the embryos were whirled up every minute. Finally the embryos were washed once with PBST.

2.2.12.3. in situ Hybridisation using the hybridization machine:

After heat-treatment the embryos were put together with the solutions in the machine.

Following steps were done by the machine:

2 times incubation in PBST

10 minutes incubation in 0.1M Triethanolamine and Acetanhydrid

Four times washing with PBST for 5minutes

Incubation in 50% hybridisation solution in PBST at 65°C

Prehybridisation in hybridisation solution at 65°C for 1 hour

The hybridisation buffer was replaced by hybridisation solution containing DIG labelled probe (2-4μl labelled probe in 1ml hybridisation solution). Incubation was for 18hours at 65°C.

After hybridisation the embryos were washed in hybridisation solution for 30 minutes at 65°C and twice in 50% hybridisation solution in 2x SSCT at 65°C. Two washing steps in 0.2xSSCT at 65°C followed.

Before antibody incubation the embryos were washed four times with PBST at RT. Blocking was done for 20 minutes in blocking solution I and for 60 minutes in blocking solution II at room temperature. The antibody (Anti-Dig alkaline phosphates linked) was diluted 1:2000 in blocking solution II and pipetted on the the embryos and incubated for 4 hours.

Finally the embryos were washed 4 times in PBST and then incubated two times in AP-buffer.

2.2.12.4. Staining reaction with BM-Purple:

The embryos were removed from the machine at put into a 12 well plate. 400µl of BM-purple (staining substate) was added and the staining reaction was done in the dark.

To stop the staining reaction the embryos were washed 2 times in PBST.

2.2.12.5. Photographing of stained embryos:

Either the embryos were photographed as whole mounts under a stereomicroscope or the embryos were flat-mounted on a microscopic slide and the picture was taken under a microscope. For flat-mounting the yolk of the embryo was removed using acupuncture needles. The flattened embryo was covered with a cover-slip.

2.2.13. DNA- Sequencing with the ABI- Sequencer:

1 to 50 ng of PCR product was mixed with 2µl terminator ready reaction mix (Big Dye) and 2µl of sequencing primer. Water was added to a final concentration of 10µl. Sequencing programm: 1 minutes at 96°C, 30 times: 10 seconds 96°C, 15 seconds at the annealing temperature of the primer, 4 minutes at 60°C. Afterwards to the reaction 10µl water was added. Purification and loading of the sequencer was done by the sequencing facility personal.

The sequenced fragments were checked with Vector NTI and blasted against the NCBI database.

2.2.14. mRNA Isolation using the µMACs-Kit:

Danio rerio embryos at 10 somite stage were collected as described above. Approximately 100 animals were used per isolation. The embryos were transferred

into an eppendorf tube and the water was removed using a glass Pasteur pipette. Lysisbuffer (1ml) was pipetted into the tube. With a small plastic pistil the dechorionated embryos were homogenized and vortexed for 3 minutes to lyse them completely. A centrifugation step at 4000g for 3 minutes reduced the foam produced during vortexing. The lysate was transferred into a column attached to a tube. The lysate was run through the column by centrifugation at 13 000g for 3 minutes to reduce viscosity. 50µl microbeads were added to the lysate. Then this mixture was transferred onto a column fixed in the magnetic field. Before this the column was equilibrated with 100µl Lysis buffer.

The lysate run through the column by gravity. Afterwards the column was washed twice with 200μl lysis-buffer and four times with 100μl washing buffer. These washing steps removed proteins, RNA and DNA.

120μl Elution buffer (nuclease free H_2O) eluted the enriched mRNA from the column. The first drop of the eluate was discarded and the rest was collected.

The concentration of the RNA was obtained by measuring the absorbance. Finally the RNA was put into precipitation by adding 1μl Glycogen, 10μl 4M LiCL and 250μl 100% EtOH.

2.2.15. 1st synthesis:

1μg precipitated mRNA (obtained with the μMACs Kit) was centrifuged at 14 000 rpm for 20 minutes. The pellet was washed with 70% EtOH in DEPC- H_2O and centrifuged again for 7minutes. After the pellet was dried completely the RNA was redissolved in 11μl DEPC-H_2O. To help the redissolvation the tube was incubated at 37° C for 10 minutes. After this 1μg of oligo dT-primer was added and incubated at 70°C for 5 minutes. Then the tube was put on ice and after cooling down, the contents of the tube were spun down. 5μl Transcription buffer, 2.5μl 25mM dNTPs, 2.5μl DEPC-H_2O, 1μl RNase Inhibitor and 1μl Reverse Transcriptase were added and incubated for one hour at 48°C. The enzymes were inactivated by incubating the reaction for 10 minutes at 70°.

3. Results:

3.1.: General remarks on the different approaches:

Several approaches were used to find novel genes involved in somitogenesis. The first one, using a cDNA library was also used to establish the technology in the lab. Several protocols were tried out until the ones described here where chosen and established (3.2).

The PSM consists of several different compartments and it seemed promising to investigate in the differences between them. The PSM of the zebrafish is very small and therefore the dissection in an anterior and posterior part was easier in the mouse system. This decision was also facilitated by the fact that the mouse oligo library (Sigma-Genosys) was at this time-point available in the lab (3.3).

Finally genes, which are dependent on the Delta-Notch pathway, were detected using the zebrafish oligo libray (Sigma-Genosys) (3.4).

3.2.: Establishment of microarray-technology:

3.2.1: Printing:

Three 96 well PCR plates were used for establishing the printing protocol. The DNA was mixed with 50% DMSO. This buffer was chosen because it denatures the double stranded DNA and therefore no heating step was needed prior to spotting. Additionally it reduces evaporation. One drawback of DMSO is that the spots tend to spread, but as not too many plates were used it was possible to print in the necessary distance to avoid merging of spots (Diehl et al, 2001).

Corning GAPSII slides were used for the first printing runs. As the spotting morphology was good, this slide type was used for further experiments (Fig. 6), although other slide-types were tested as well, for example Arraylink (Genescan).
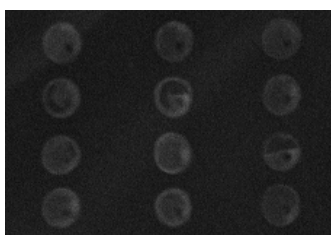
Fig 6: SYBR green staining of one of the printing runs. Most of the spots show a round, even surface.

3.2.2.: Labelling and Hybridisation:

Two different labelling methods were used in the beginning, a direct (Cyscribe labelling kit) and an indirect (Fairplay labelling kit) method. In the direct mode of incorporation of the nucleotides, the fluorescent molecules are coupled to one of the nucleotides (a dCTP or dUTP) prior to the first strand synthesis. The Fairplay labelling kit, on the other hand, uses aminoallyl modified nucleotides for incorporation during 1st strand synthesis. In a second step the fluorescent dye is coupled to the modification on the nucleotide. The indirect method was chosen

because in this case no sterical hindrance due to the dye during 1st synthesis can occur and no additional bias because of this is introduced (Hoen et al., 2003).

Parallel to this, the lambda control genes (Q, G and N) were produced and spotted on a chip. The control RNAs to the corresponding "lambda spots" were spiked into the zebrafish RNA prior to labelling. By doing this, landmarks were produced in each sub grid, because at least the lambda DNA should hybridise and by adding these RNAs in different ratios to the fish DNA, these control spots could later be used for normalization and calibration of the chip. Thus in the case of a bias in the two dyes, the ratio could be normalized using the control spots because their mRNA ratio is known.

For our experiments we also used the control genes to check the hybridisation success and also the amount of DNA needed for spotting. Therefore we spotted the three lambda control genes in three different concentrations on the chip (500ng, 250ng and 125ng). Then the lambda RNAs for Q, N and G were labelled. The success of the labelling was checked on a gel (data not shown) and afterwards the labelled cDNA was hybridised to the chip. Figure 7 shows the result of this hybridisation. All three concentrations (represented by each subgrid) show good hybridisation and the ratios of the used RNAs are as expected. The yellow-orange spot corresponds to the Q-gene. The RNA in both labelling reaction was the same. The N-spot was red and here four times more N-RNA was spiked in the red-labelled reaction than in the green one. G had the opposite ratio to N and therefore the spot was green.

3.2.3: First results using the mixed embryogenesis stages cDNA library:

1344 purified cDNA-inserts (four plates) were spotted on chips together with the controls. The amount of DNA per well was estimated by loading them on an agarose gel. To be included into the experiment, they had to have a concentration of 250-500ng. This spotted part of the library was used for testing the fss mutant against wild type fish. Several rounds of labelling and hybridisation were done to fine-tune the labelling protocol also a switch from Cy-dyes to Alexa-dyes were performed. For the "true" experiment two independent samples of fss and wt fish were generated and labelled. Fss was labelled red and wt RNA was labelled green. The independent samples were hybridised to four chips each, resulting in two biological and two technical replicates, but the technical replicates were over represented.

Gridding and normalization was done using the Analyzer (Genomic Solution) software. Afterwards the data of all slides were imported into the Array Scout software (Lion Bioscience) and a gene-list was produced containing genes, which show more than 2x down-regulation in the fss mutant condition on at least four slides. This gene list contained 43 genes. The corresponding cDNAs were sequenced and afterwards ten candidates were chosen for a second round of analysis by *in situ* hybridisation (Table 2).

Of these only three candidates show expression in the somites, namely *Pax-like*, *rack1* and *unnamed protein II*. All three are expressed in the somites in the wt situation. In the fss embryos there are no somites and no expression can be detected in this region. (Fig.8)
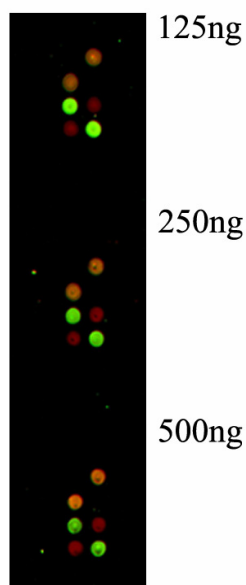
Figure7: Hybridisation of Lambda control genes. Lambda controls were spotted in three different concentrations on the chip. The hybridisation result was in all three cases the same. The yellow/orange spot corresponds to Q, which RNA ratio in both reaction was 1:1, G is the green spot with a ratio red:green=1:4 and N is red with a ratio of 4:1.

Table 2: Genes found in the fss vs wt microarray experiment and chosen for validation with *in situ* hybridisation.

Gene	Accessionnumber	Down-regulation	Down-regulation (log2 ratio)
Celsr	AF025330	2.3x	-1.20
Hsp90	AF068772	2x	-1
PAI I	XP536673	2.3x	-1.20
Pax-like	AJ299411	4.3x	-2.10
Rack1	AF025330	2.9x	-1.54
Sox2	NM213118	3.4x	-1.77
Unnamed Protein I	XM688017	2.8x	-1.49
Unnamed Protein II	AJ299411	2.3x	-1.20

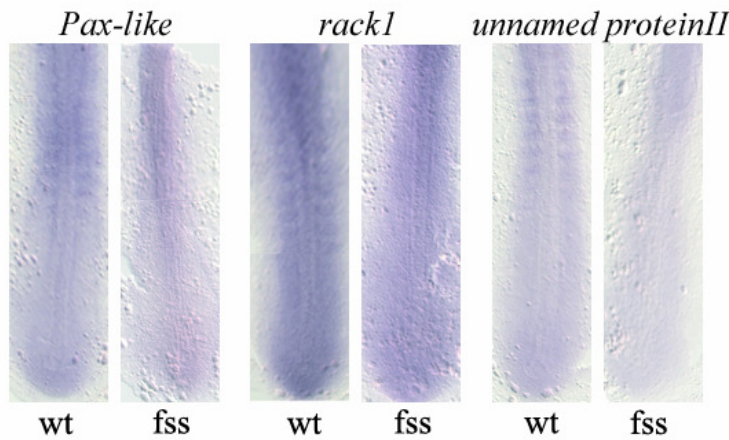| Unnamed Protein III | U94592 | 2x | -1 |
| Unnamed Protein IV | XM694942 | 2.1x | -1.07 |

Figure 8: Three of the candidates showed expression in the somites. In the fss embryos this expression is not present.

3.3.: Mouse oligo library:

As hybridisation on GAPSII slides on which the mouse oligo library was spotted resulted in a bad ratio between background and signal a new slide type was used. The manufactors (Sigma-Genosys) recommended Elipsa slides, and therefore this slide type was used. The hybridisation protocol for this slide type was mostly established by Chris Voolstra. As the mouse oligo library consisting of 22 228 oligos could not be spotted on one slide due to technical limitations of the spotting robot, four slides were used.

The mouse oligo-array was used to test gene expression in the different compartments of the PSM, namely the anterior versus the posterior part. The PSM of the E9.5 embryos were extracted and then cut into a posterior and anterior part. The border between these two compartments was the umbilical chord. This border coincides with

some expression domains, f.e notch1 (Calceran et al., 2004). The anterior part was labelled with the green fluorphor and the posterior one with the red dye. Six slide sets were used for hybridisation. Three different samples were taken, meaning three different litters from different female mice mated to different males were used. After hybridisation, gridding was done using the Spotfinder 224 (Tigr) and normalization with the MIDAS (Tigr) software. Further steps of analysis were done in Excel

(Microsoft). First the standard deviation of all six sets was calculated and all values above 30% Standard Deviation were excluded. 394 genes were detected to be differently expressed in the two compartments. 184 of them were higher expressed in the posterior part and 210 in the anterior part. 41 genes were chosen, which suggested an involvement in development (Table 3).

Table 3 (mouse): Genelist of the mouse microarray experiment. The expression differences between the anterior and posterior compartment were tested. For two genes an expression pattern in the PSM was found (highlighted in blue). One false positive was also detected (red colour).

Gene-name (Accession number)	Fold-change (log2 ratio)
Tdrd1 (NM_031387)	-0.54
zinc finger protein 281 (BC003243)	-0.51
MEF-2 (U13262)	-0.51
Zrf2 (NM_009584)	-0.44
Vpreb2 (NM_016983)	-0.40
Refbp2 (NM_019484)	-0.37
Myg1 (AF289484)	-0.36
Crtap (NM_019922)	-0.35
Hoxb13 (NM_008267)	-0.33
Balb/C (Z31362)	-0.32
Pint1 (AY029599)	-0.26
Sept6 (Sint1) (NM_017380)	-0.26
patched homolog (NM_008957)	-0.25
Rab20 (X80332)	-0.21
WAC (AF320996)	-0.21
GPR7 (U23807)	-0.20
Sox8 (Z18957)	-0.20
Bcl2-like 2 (NM_007537)	-0.20
integrin alpha 7 (NM_008398)	-0.19
zinc finger transcription factor GLI (AF189287)	0.19
***Ors25* (NM_020291)**	0.19
Tcea2 (NM_009326)	0.19
DR6 (AF322069)	0.19
Asb8 (AF398969)	0.20
Rec8 (NM_020002)	0.20
Mapk8ip3 (NM_013931)	0.20
Gene-name (Accession number)	**Fold-change (log2 ratio)**
Klk16 (NM_008454)	0.20
claudin 11 (NM_008770)	0.21
Spred-1 (AB063495)	0.22
semaphorin 3B (NM_009153)	0.22
Acdp1 (AF202994)	0.22
Doc2b (NM_007873)	0.22
mk2e (X74784)	0.22

Tubulin alpha 6 (NM_009448)	0.23
Alg-4 (AF055668)	0.23
BRAP2 (AF321921)	0.24
RANBP20 (AY029528)	0.24
Fiz1 (NM_011813)	0.24
zinc finger protein 103 (NM_009543)	0.25
Dp1 (NM_007874)	0.25
Daf1 (NM_010016)	0.26
TOB3 (AF343079)	0.27
Clc1 (AJ011106)	0.29
Fkbp6 (AF367710)	0.30
Tbx19 (NM_032005)	0.31
integrin beta 3 (Itgb3) (NM_016780)	0.31
Krt2-18 (NM_016879)	0.32
Krtap16.4 (AF345294)	0.33
Krox-20 (X06746)	0.35
Mox-2A (Z16406)	0.36
Lbh (AF317517)	0.36
Dvl2 (NM_007888)	0.40
retinoic acid receptor beta 2 (X56573)	0.43
presenilin 2 (NM_011183)	0.45
Pou2af1 (NM_011136)	0.46
Dscr6 (AB063284)	0.52

The literature was scanned for pictures showing the expression of these genes in the somites. Unfortunately, only two papers were found showing the expression of the gene in the PSM. These two genes are *Hoxb13* and *Sint1* (Fig. 9). Both genes are higher expressed in the posterior compartment as the microarray result predicts. A gradient of the respective expression reaches into the anterior compartment. *Hoxb-13* expression is shown at age E9.0 (Fig. 9A) and *Sint1* at age E11.5 (Fig. 9B). Another candidate where the expression is known is Krox20, but as this gene is only expressed in the brain it is a false positive. As only expression of these three genes was known it is difficult to calculate the rate of false positives.
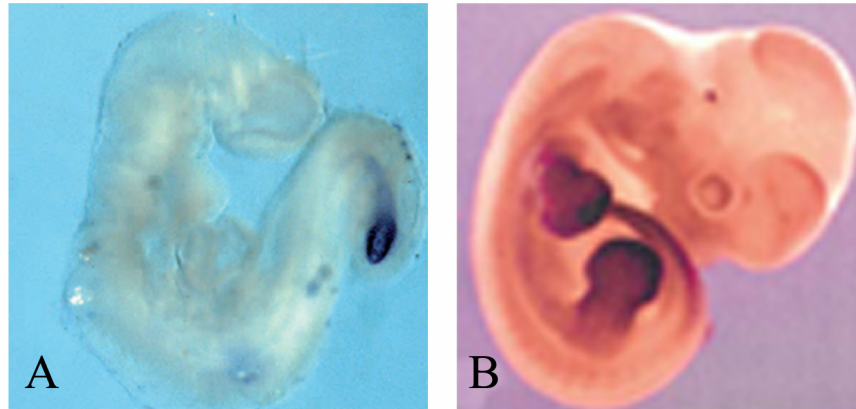
Figure 9: Two genes, which were found as candidates in the mouse microarray experiment. Both genes are higher expressed in the posterior part of the PSM. A gradient of expression reaches into the anterior part. A: *Hoxb-13* expression in an E9.0 embryo. Picture taken from Zeltser et al., 1996 B: *Sint1* expression in an E11.5 embryo. Picture taken from Sørensen et al., 2002).

3.4.: Zebrafish oligo library:

3.4.1. General comments:

A new slide type was used for the zebrafish oligo library, namely the Schott H slide. The oligos carry an amino modification, which is linked to the amine reactive NHS esters on the slide. First tests showed that the spots were smaller than on the chips previously used (170-190μm in contrast to 230-300μm). As the library consisted of 16 399 oligos it was not possible to spot all of them on one slide, but with the smaller spot morphology it was possible to print it on two slides.

This time the hybridisation success on the first slides was not only estimated by eye, meaning checking the colours of the control spots. A run was done were both samples were wildtype, coming from the same batch. The RNA was separated in two parts. They were labelled with the two different dyes. Two slides were hybridised (each containing the first half of the library). Another hybridisation was done with *des* embryos versus wild-type. Analysis was performed as described below and the result plotted on an MA plot (Fig 10). The *wt* against *wt* hybridisation should result in equal expression ratios on all spots, whereas the *des* vs. *wt* hybridisation should show differential expression on some spots. The datapoints in the *wt* against *wt* plot cluster narrower around the 0-axis as the *des* against *wt* plot. As just one sample, although on two slides was hybridised, one has to expect a certain numbers of outliers on the *wt* vs *wt* plot (Fig 10: datapoints between -0.75 and –1.75 wtvswt plot)), but the tendency can be seen.
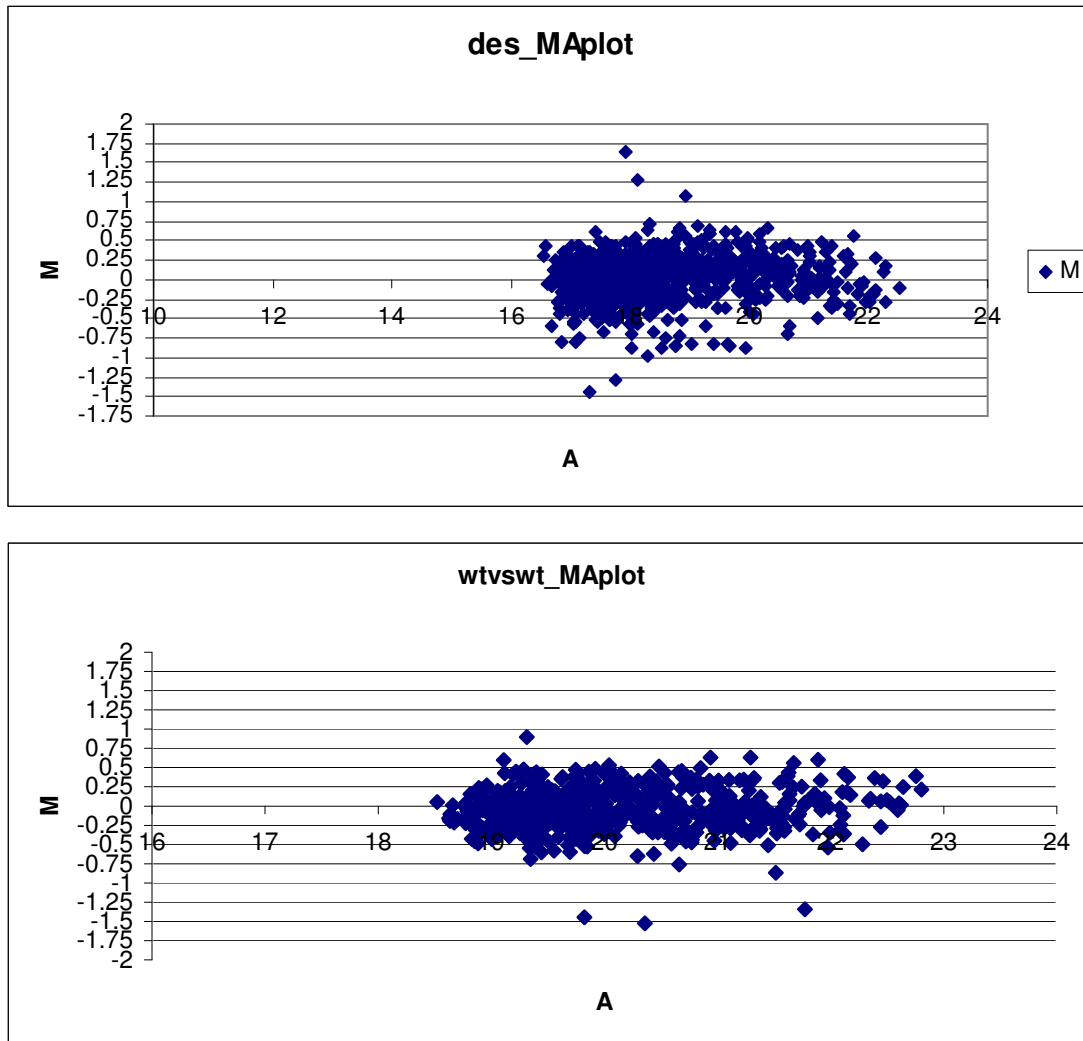
41

Figure 10: MA plots of the first hybridisations using Schott H slides. The upper plot shows a plot, where the des mutant was tested against wt. A broad cloud of datapoints can be seen. In the wt vs wt plot, where the datapoints should be near Zero, the cloud is much narrower, although some outliers can be detected.

M= log2 (green/red) A=log2 $\sqrt{\text{green} \times \text{red}}$; green= labelled with the green fluorphor, red= labelled with the red fluorphor.

The different delta-notch mutants were tested against wild-type fish. For each mutant three independent samples were prepared, the same was done for the wild-type fish. This means, that RNA was isolated from three different batches of eggs, laid on different days. The mutant RNA was always labelled with the red dye and the wild-type RNA with the green one.

The labelled cDNA was hybridised to two sets of slides and by doing this creating a technical replicate. This sums up to six slide sets hybridised per mutant. Gridding was done using the TIGR spotfinder 224 software. After normalization, which was done with the MIDAS (Tigr) software, the values above a Standard Deviation of 30% were excluded. This calculation was done with Excel (Microsoft). Instead of choosing an

arbitrary fold change the internal control genes of the zebrafish-oligo library were used. The house keeping gene beta-actin should not change in the different mutants. Therefore ratios above or below the "Fold change" of beta-actin were chosen and the corresponding genes added to the list. The lowest "fold-change" of beta-actin was –0.05 and the highest 0.10.

The list of genes was further shortened by including only known genes, meaning members of important pathways, for example the WNT pathway, transcription factors, or genes were motifs were discovered, suggesting transcription factors, i.e. zinc finger domains, leucin zipper etc.

In the following chapters the genes containing zinc finger domains were numbered by the experimentator. This means, that a gene called *zfdI*, is an unknown gene containing a zincfinger domain and was discovered first during analysis of the microrarrays.

As microarray results have a certain amount of false positives, a validation must be done. In this case the whole mount *in situ* hybridisation was chosen, as it not only allows the validation, i.e. see the expression difference between wild-type and mutant embryos, but also the determination of the expression site. This means that genes having an expression in the PSM or the somites are interesting candidates for further characterization. The validation of 13 candidates out of 34 found in the *des*-chip series, showed that the microarray results could be verified using *in situ* hybridisation.

3.4.2.:Des/notch1:

507 genes were found to be differently expressed in the *des* mutant, i.e. above or below the beta actin expression. More genes, namely 386, were down-regulated. Only 121 genes were up-regulated. Most of these genes were unknown. Only 33 candidates remained meeting the above-mentioned criteria. 9 genes were up-regulated and 25 genes were down-regulated in the *des*-mutant embryos. Gene names and fold-changes are in Table 4.

Table 4 (des): 9 genes were up-regulated and 25 were down-regulated. Genes which were used for validation are in bold.

Gene-name (Accession number)	**Fold-change (log2 ratio)**
Cx43.4 (L46801)	0.37
Pbx4 (AF162696)	0.36
Similar to *SP-1* (AI882784)	0.33
FXR (U93467)	0.3
p38a (AB030897)	0.29

Gene-name (Accession number)	Fold-change (log2 ratio)
zfdI (BI877718)	0.25
Similar to *MMP1-A* (BI867183)	0.22
Ziro 7 (AF398433)	0.22
zfd II (AW116027)	0.16
Similar to *Bmp1* (BI884917)	-0.11
Similar to *DP-1* (BI867054)	-0.13
Unc119b	-0.13
Hoxd12 (Y14547)	-0.15
ndr2 (AF056327)	-0.17
twisted gastrulation like protein (AF261692)	-0.18
mdm2 homolog (AF010255)	-0.18
zfdIII (BG304258)	-0.18
Similar to *SPT4* (AI883716)	-0.19
Her6 (X97333)	-0.21
D249 (AB055680)	-0.22
Mta (AF097875)	-0.22
Semaphorin Z1b (AF083382)	-0.25
ztsg1 (AF332096)	-0.25
Wnt11 (AF067429)	-0.25
Reggie 2a (AF315947)	-0.25
Mesogenin (AJ309314)	-0.28
Cecr1 (AF384217)	-0.30
Fzd7b (was *Zg13*) (U49417)	-0.40
Similar to *Matrix Metalloproteinase-14* (BM186907)	-0.44
***Ziro* 5** (AY017309)	-0.45
Hsp90beta (AF068772)	-0.48
Similar to *CTCF* (BI883421)	-0.53
Pbx1a (AJ245962)	-0.53
Similar to *nemo-like-kinase* (BG304985)	-0.60

3.4.3.: *aei/deltaD*:

131 genes were differently expressed in the *aei* mutant embryos. Of these 54 genes were up-regulated and 77 genes down-regulated. Again this list was shortened by excluding genes, which did not meet the chosen criteria. Eight genes remained, which were up-regulated and 12 genes, which were down-regulated (Table 5).

Three genes were detected, which came up on different experiments, too, namely *Semaphorin Zb1* (des and bea), *Hoxd12* (des) and *ndr* (des).

Table 5 (aei): Genes found in the microarray experiment aei vs wt. 8 genes were up-regulated and 12 genes were down-regulated.

Gene-name (Accession number)	Fold-change (log2 ratio)
Semaphorin Z1b (AF083382)	0.49

Gene-name (Accession number)	Fold-change (log2 ratio)
Similar to *XRC9* (BI878221)	0.29
Zg03 (U49407)	0.22
Similar to *UBI-D4* (AW420405)	0.20
Similar to *Thioredoxin* (BI864190)	0.18
Similar to *Calsenilin/Dream* (BI706537)	0.18
Similar to *P8* (BF717555)	0.14
Hoxd12 (Y14547)	0.14
MCM7 (AW777430)	-0.15
H3.3 (BI879444)	-0.15
Similar to *Kinesin-C* (BM025946)	-0.18
Similar to *Eset* (BE017589)	-0.19
Similar to *Dystroglycan precursor* (AW171079)	-0.25
Similar to *Thymosin Beta-4*	-0.28
Junctional adhesion molecule (BQ133340)	-0.30
D204 (*plexin A4*) (AB055678)	-0.43
zcry1a (AB042248)	-0.44
ndr2 (AF056327)	-0.45
Similar to *Cytochrome C Oxidase subunit* (BM186826)	-0.60
Similar to *Nuclear factor NF-Kappa-B p52* (AW059384)	-0.65

3.4.4.: *bea/deltaC:*

109 genes were detected to be differently expressed in the *bea* mutant condition. 38 genes were up- and 71 down regulated. In these experiments shortening of the list just left 10 candidates for further validation (Table 6). One of them was up-regulated and 9 down-regulated in the mutant embryos. Again it was checked if some of the genes found were also candidates on other chips. There were four genes, namely *Cecr1* (des), *Semaphorin Zb1* (aei and des), *Unc119b* (des, bea and Su(H)) and *Pbx1a* (des).

Table 6 (bea): 10 genes were found to be up- or downregulated. One of them was up-, the others down-regulated.

Gene-name (Accession number)	Fold-change (log2 ratio)
rtk8 (AJ005029)	0.25
Similar to *Cathepsin Z precursor* (BM103154)	-0.11
Six7 (AF030282)	-0.11
Similar to *Pas domain protein 1* (BG883983)	-0.14
Similar to *p53-binding protein 53BP1* (AI793650)	-0.18
Similar to *Melanocyte-specific protein 1*	-0.22

(AW342944)	
Gene-name (Accession number)	**Fold-change (log2 ratio)**
Unc119b (AF387341)	-0.22
Cecr1 (AF384217)	-0.24
semaphorin Z1b (AF083382)	-0.28
Pbx1a (AJ245962)	-0.31

3.4.5.: *Su(H):*

In this experiment morpholino injected embryos were used. The injections were done by Dirk Sieger. The Su(H) knock down embryos were tested against wildtype embryos. These embryos were from the same batch as the ones, which were injected. 132 genes were differently expressed. 70 of them were up-regulated and 62 were down regulated. Again the list of these genes was searched for genes meeting the criteria mentioned in the General Comments section. The final list shows 16 up-regulated genes and 13 genes, which were down-regulated in the morpholino injected embryos (Table 7).

Three genes were also found on other chips: *Pbx4* (des), *Wnt11* (des) and *Unc119b* (des and bea.)

Table 7 (Su(H)): Morpholino injected embryos were tested against wildtype embryos. 16 genes are up-regulated and 13 genes down-regulated in the injected embryos.

Gene-name (Accession number)	**Fold-change (log2 ratio)**
Pbx4 (AF162696)	0.66
CK2 alpha (X99964)	0.60
Similar to *Integrin alpha-6* (BM186155)	0.56
Similar to Actin-like protein 2 (AW154456)	0.54
fkd8 (AF052251)	0.50
Similar to *His 1 protein* (BI533460)	0.48
Hua (AF184244)	0.47
G1-Related zinc finger protein (BI673966)	0.46
Zfd IV (BM104037)	0.38
Zfd V (AI942517)	0.34
Similar to *zic3* (BM183276)	0.34
Similar to *Meprin A Beta- subunit precursor* (BM184851)	0.30
Similar to *Ece1* (BM182470)	0.30
Similar to *Ese-3A* (BI705456)	0.29
Similar to *Znf72=Kruppel-Type zinc finger* (BM071786)	0.27
Similar to *Ring finger protein AO7* (BM096183)	0.26

Gene-name (Accession number)	Fold-change (log2 ratio)
FBX25 (BG303968)	-0.54
Similar to *zinc finger 5 protein* (AW422582)	-0.55
Similar to *NP95* (AW594981)	-0.55
Mindin 2 (AB006085)	-0.59
Unc119b (AF387341)	-0.62
Similar to *Hypothetical protein CGI-126* (BM025947)	-0.63
Zfd VI (AI958897)	-0.63
Similar to *Nucleoporin p54* (BI878193)	-0.63
Wnt11 (AF067429)	-0.65
Tie-2 (AF053632)	-0.68
Similar to *Enhancer of filmentation 1* (BI883121)	-0.72
Paracaspase (AF316598)	-0.72
F-spondin 1 (AB006086)	-0.74

3.4.6.: Validation:

3.4.6.1. General remarks:

Thirteen candidates of the *des* micorarray experiment were used for validation. Primers for these genes were designed and amplified using PCR. As the primers carried a T7 sequence overhang, the PCR products could directly be used for antisense insitu probe production. The *in situ* hybridisation was repeated at least two times with embryos around the 10-somite stage. Staining reactions for the wild-type and mutant embryos were stopped at the same time point.

The validated genes can be grouped in following categories:

I: down-regulated in the PSM: *mesogenin, zfdIII*

II: down-regulated in the PSM and other regions: *her6, ztsg, mta*

III: down-regulated in other regions of the embryo: *ziro5, D249*

IV: up-regulated in the PSM: *Cx43.3*

V: up-regulated in other regions of the embryos: *ziro7, p38, MMP1-A*

VI: Genes, were the regulation pattern could not be validated: *Hsp90beta, Pbx4*

3.4.6.2. Category I: Down-regulation in the PSM:

3.4.6.2.1. *Mesogenin*:

zfMesogenin plays a role in development and is expressed in the PSM (Fig. 11A and C) (Yoon and Wold, 2000). The expression in the wt situation seems to be somewhat

47

stronger around the notochord and in the lateral parts a little bit weaker (Fig. 11C). In the des embryos the expression is in the same domain, but much more weaker (Fig. 11B,D). This is what the microarray experiment has predicted.
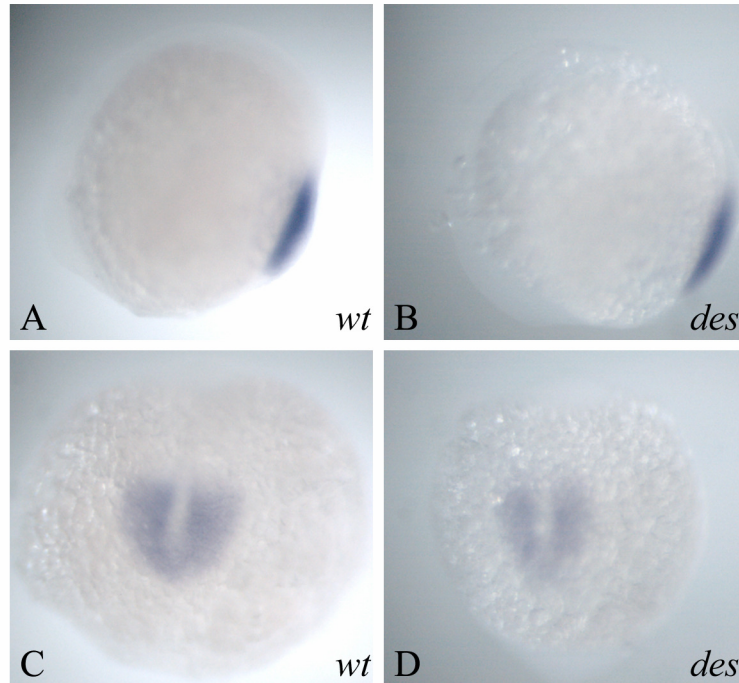

Figure 11: Expression of *Mesogenin*: *Mesogenin* is expressed in the PSM. In the wild-type embryos the expression near the notochord seems to be stronger than in the more lateral parts (C). The expression in the mutant is weaker (B, D).

3.4.6.2.2. *zfdIII* (zinfinger domain):

About this gene nothing is known, except that it contains a zincfinger domain, suggesting that *zfdIII* is a transcription factor. This gene was found to be down-regulated in the mutants.

In situ hybridisations showed that this gene was expressed in the PSM (Fig. 12A). In the *des* mutants this expression was down-regulated (Fig. 12B).
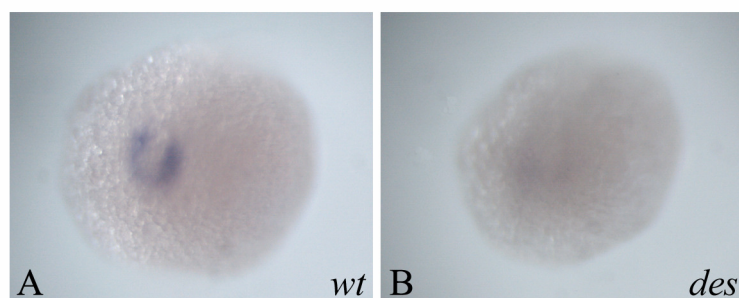

Figure 12: Expression of *zfdIII*: This gene is expressed in the PSM (A) and down-regulated in the mutant situation (B).

3.4.6.3. CategoryII: Down-regulation in the PSM and other regions:

3.4.6.3.1. *Her6*:

This gene belongs to the Hairy/Enhancer of split gene family (Pasini et al., 2003) and was found to be down-regulated in the mutant embryos at least in the somite and posterior body part of the embryos. Wild-type embryos display expression of *Her6* in the brain, the somites and the posterior embryo (Fig. 13A, C, E). In the head this gene is expressed in the telencephalon and the midbrain/hindbrain boundary (MHB) (Fig. 13A). In the mutant embryos an up-regulation could be seen at the midline and an expression at the posterior border of the midbrain appeared. The expression in the telencephalon is in a smaller domain (Fig. 13B). A weak expression could be detected in the wildtype somites. Anterior to the somite strong expression of *Her6* is present (Fig. 13C). The mutant expression is a smear spanning the entire region, where the somites normally form. The expression anterior of the somites has vanished (Fig. 13D). In the posterior part of the body of wt embryos the expression can be detected in the posterior lateral mesoderm (Fig. 13E). This expression domain is missing in the *des* embryos (Fig. 13F).

As most expression domains are smaller or not present in the des embryos Her6 could be validated as being down-regulated.
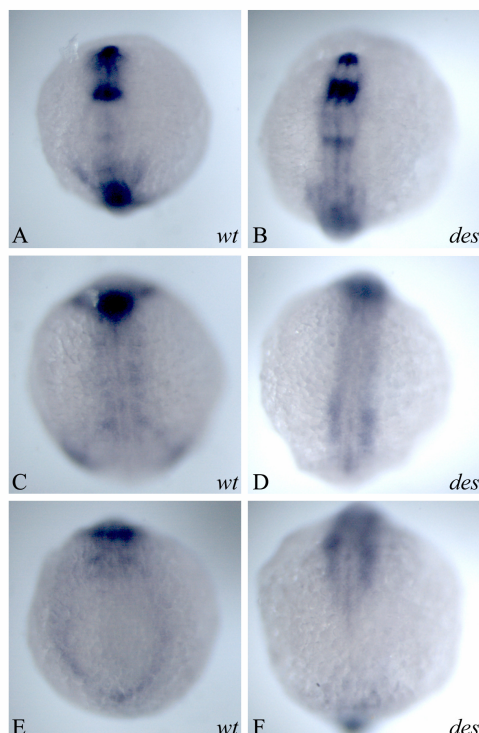
Figure 13 to picture on previous page: Expression of *her6*: In the wildtype embryos her6 is expressed in the brain, somites and PSM. In the head *her6* is expressed in the midline the teleencephalon and the MHB (A). Expression in the somites is weak at the 10 somite stage. Anterior to the somite a high expression of *Her 6* can be seen (C). In the posterior part of the embryo *her6* is expressed in the posterior lateral mesoderm (E). In the mutant the expression in the telencephalon is reduced, but the expression in the midline and MHB is stronger. The posterior border of the midbrain is also demarketed by expression of *her6* (B). The expression in the somite region is dysregulated (D) and the expression in the posterior lateral mesoderm has vanished (F).
MHB: midbrain/hindbrain boundary

3.4.6.3.2. *mta*:

Mta is a gene involved in DNA-methylation and plays a role in development (Martin et al., 1999). In the wild-type embryos this gene is expressed in the entire brain and the PSM (Fig. 14 A, C ,E). As predicted by the microarray, this gene is down-regulated in the mutants. In the brain the expression is weak (Fig. 14 D) and no expression in the PSM can be seen anymore (Fig. 14F).
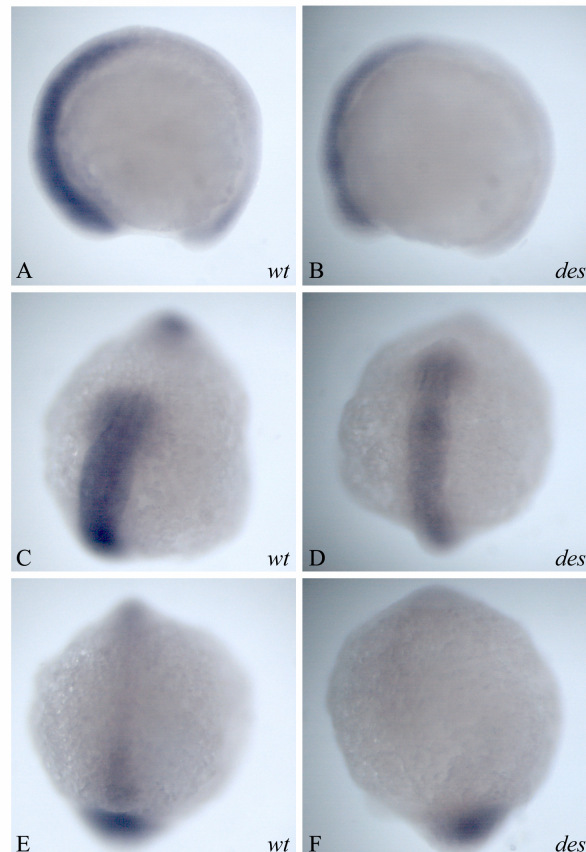
Figure 14: Expression of *mta*: In the wt embryos *mta* can be detected in the entire brain and the PSM (A, C, E). The expression in the *des* brain is much weaker as in the wildtype situation (D) and the expression in the PSM is not detectable (F).

3.4.6.3.3. *ztsg*:

This gene was found to be down-regulated and is called *twisted gastrulation protein* and plays a role in development (Scott et al., 2001; Ross et al., 2001). It is expressed

in the anterior half and the PSM of the embryo (Fig. 15A and C). As predicted by the microarray the expression is down regulated in the des embryos (Fig. 15B). In the PSM the expression was nearly not detectable (Fig. 15D).
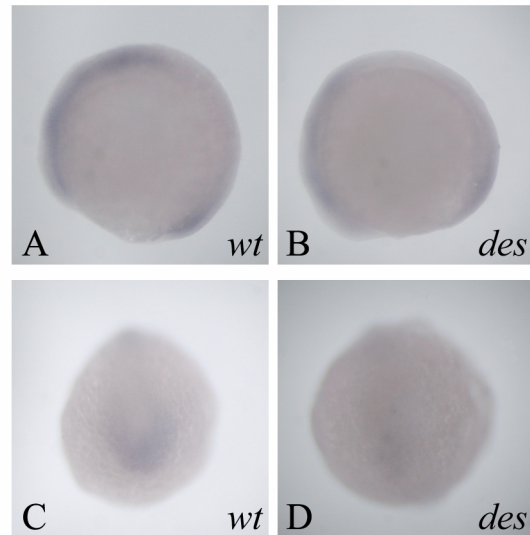

Figure 15: Expression of *ztsg*: This gene is expressed in the anterior part of the embryos and the PSM (A, C). In the mutants the expression was fainter in the anterior part (B) and nearly gone in the PSM (D).

3.4.6.4. Category III: Down-regulation in other regions of the embryo:

3.4.6.4.1. *D249*:

Nothing is known about the function of the gene. The gene ontology for this gene suggests an involvement in signal transduction. In the microarray experiment this gene was found to be down-regulated. The gene is expressed in the anterior head structure around the developing eye (Fig. 16A). The dorsal view (Fig. 16C) shows a strict expression border at the forebrain/midbrain boundary. In the *des* embryos the expression is much fainter, but remains at the same domains (Fig. 16B, D). In the lateral view the expression seems to be restricted to the eye primordia.

3.4.6.4.2.. *ziro5*:

This gene belongs to the Iroquois homeobox containing gene family (Wang et al., 2001) and was found to be down-regulated in the des mutant. *Ziro5* is expressed in the notochord and the brain. Expression in the notochord reaches down until it stops before the posterior PSM (Fig. 17A, C and E). In the mutants this expression seems to be a little bit weaker (Fig. 17D). In the brain *ziro5* is normally expressed at the MHB

and the posterior midbrain boundary. Further an expression domain lateral to the rhombomers could be seen (Fig. 17E). In the des embryos the expression domains have got broader and more diffuse. This expression was a little bit weaker, but as the domains are broader it might be argued, if this is really down-regulation (Fig. 17 B and F).
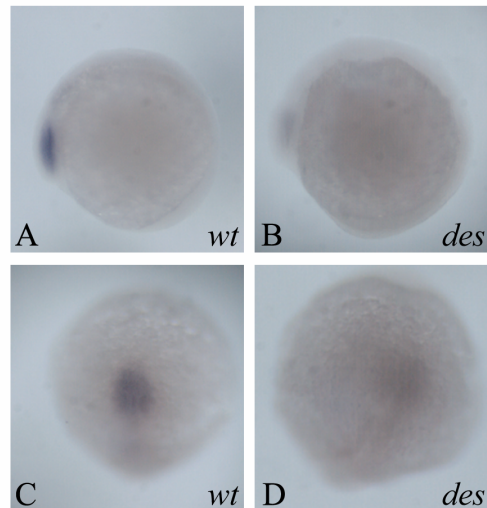
Figure 16: Expression of *D249*: The gene is expressed in the embryonic head around and in the eye primordia (A, C). The expression of *D249* in the mutant embryos is at the same place, but very reduced (B, D).
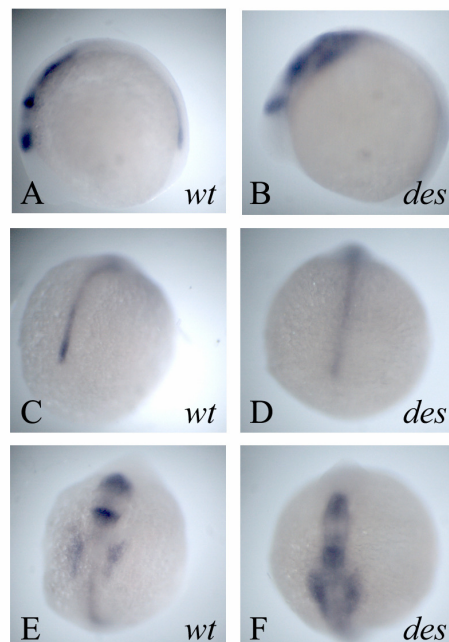
Figure 17: Expression of *ziro5*: This gene is expressed in the brain and the notochord (A, C, E). The expression in the notochord stops anterior to the posterior PSM (C). In the brain *ziro5* is expressed in the MHB and the posterior midbrain boundary. Expression of *ziro5* lateral to the rhombomeres was also detectable (E). In the *des* mutant the expression in the notochord seems a little bit weaker (D). The brain expression looks more diffuse and the sharp borders of expression observed in the wt embryos have vanished (B, F).
MHB: midbrain/hindbrain boundary

3.4.6.5. Category IV: Up-regulation in the PSM:

3.4.6.5.1. *Cx43.4* (*Connexin 43.4*):

Connexin 43.4 plays a role in the cell-cell communication by building gap junctions between cells (Wei et al., 2004). This gene should be up-regulated and the expression-pattern verified this (Fig. 18). In the wild-type embryos the strongest expression can be seen in the most posterior part of the PSM (Fig. 18A, C). A faint gradient reached towards the intermediate and anterior part of the PSM. In the *des* situation the expression is stronger and the domain broader reaching to the anterior PSM. The gradient cannot be detected. It seems as in the des embryos, instead of the gradient-like expression, *Cx43.3* is equally strong expressed in the entire PSM (Fig. 18B, D).
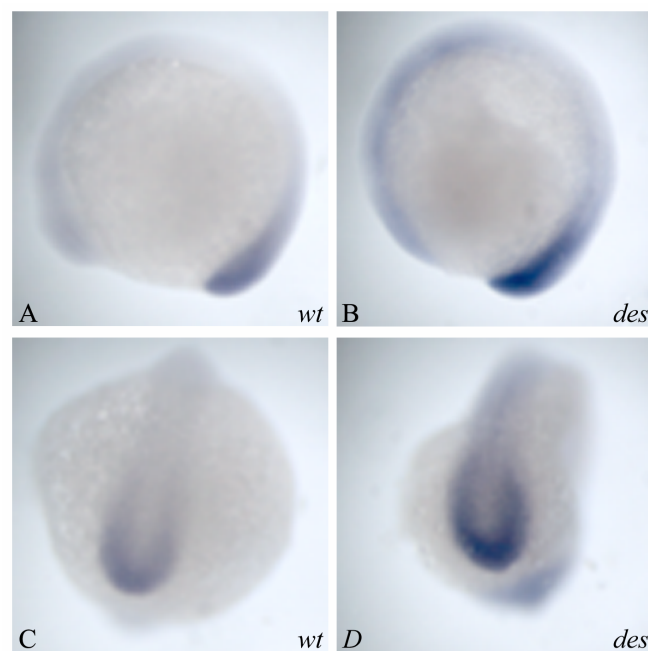
Figure 18: Expression of Cx43.3. At the 10 somite stage the expression is restricted to the PSM. In the wild type embryos the expression is restricted to the more posterior part of the PSM. A faint gradient in the intermediate PSM can be seen (A, C). In the mutant embryos the expression is stronger and reaches more to the anterior (B, D).

3.4.6.6. Category V: Up-regulation in other regions of the embryo:

3.4.6.6.1. Similar to *MMP1A* (*Matrix Metalloproteinase-1A*):

MMP1A is a Matrix Metalloproteinase and is important for the connection of cells (Mott and Werb, 2004). A weak, kind of background expression of this gene can be

detected in the normal embryos (Fig. 19A, C). The expression in the *des* embryos was up-regulated, according to the result of the microarray analysis (Fig. 19B, D).

Figure 19: Expression of *MMP1-A*: This gene is expressed weakly in the entire *wt* embryo (A, C). The expression is highly up-regulated in the *des* embryos as predicted by the microarray analysis.

3.4.6.6.2. *p38a*:

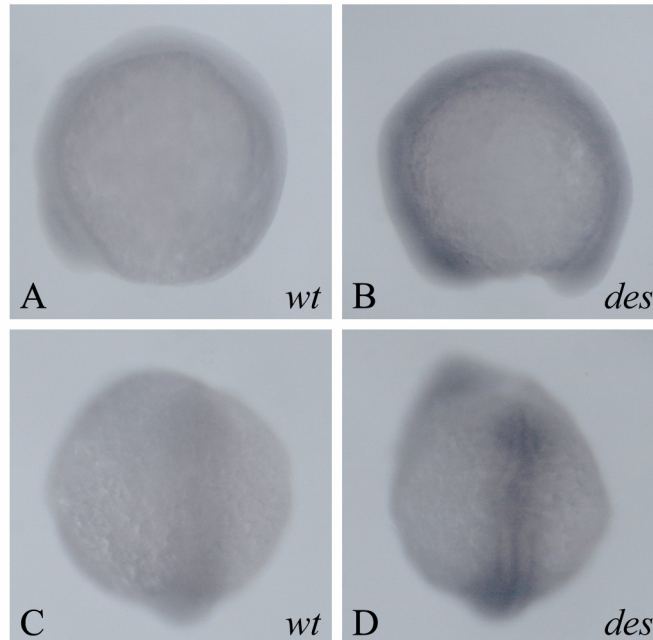This gene plays a role in cell motility and is part of the MAPK pathway (Mori et al., 1999). In *wt* embryos this gene is expressed in the brain and the PSM (Fig. 20A). In the brain several compartments with higher expression of *p38a* can be detected, namely the telencephalon and the midbrain. Between these parts the expression is downregulated (Fig. 20A and C). This expression is perturbed in the mutant embryos (Fig 20B and D) as the expression is higher and no compartments can be detected. The normal expression of *p38a* is also detectable in the PSM (Fig. 20E), but this expression seem to be unaffected in *des* embryos (Fig. 20F).

3.4.6.6.3. *ziro7*:

This gene also belongs to the Iroquois homeobox containing gene family (Lecaudy et al., 2001), but was up-regulated in contrast to *ziro5*, which was down-regulated. This gene is only expressed in the brain (Fig. 21). In the wt situation it is expressed in two boundaries flanking the midbrain. It is also expressed in the first rhombomere (Fig. 21A, D and G). Checking the expression in *des* embryos reveals an interesting feature,

namely that two types exist. In the Type I expression the normal wild type expression can be seen, but additionally expression in the lateral parts connection this patterns can be detected. The expression in the rhombomere has vanished, but the lateral region flanking the rhombomers shows now expression of *ziro7* (Fig. 21B, E and H). Type II shows the same expression as Type I, but there is a strong expression in the midbrain.
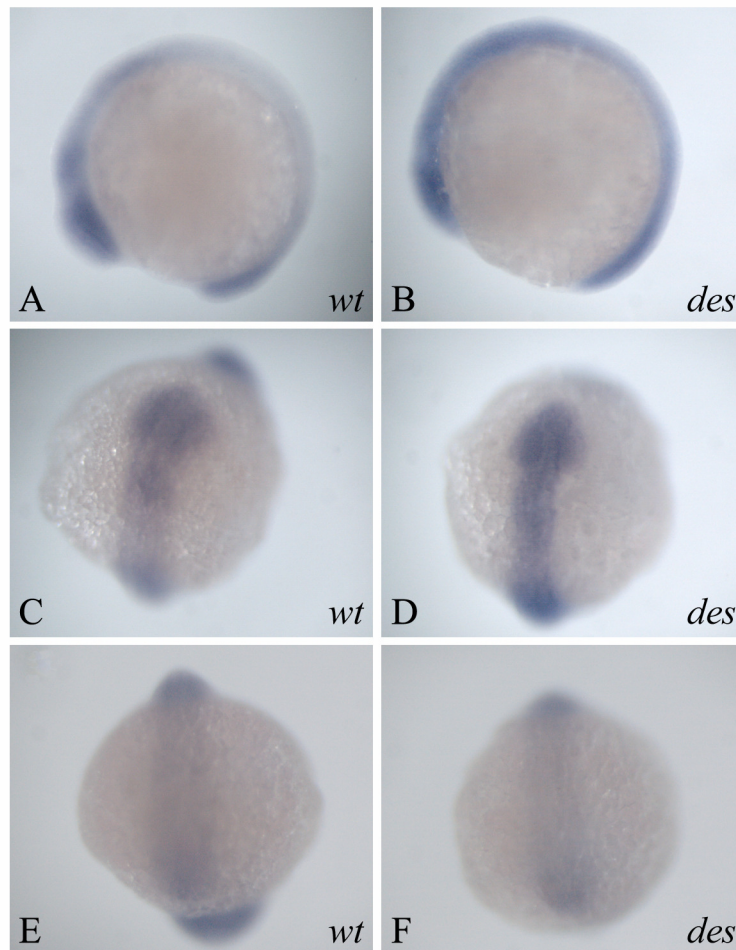
Figure 20: Expression of *p38a*: In *wt* embryos this gene is expressed in brain (A, C) and weakly in the PSM (E). In the brain a higher expression in the telencephalon and the midbrain can be seen (A, C). This pattern in the brain is perturbed in the *des* embryos as the expression is up-regulated in the entire brain region (B, D). The expression in the PSM seems to be unaffected (F).

3.4.6.7. Category VI: The regulation pattern could not be verified:

3.4.6.7.1. *Hsp90beta*:

Hsp90beta is heat shock-protein, which also plays a role in cell-cycle (Krone et al., 2003). This gene is ubiquitously expressed and no difference between wt and des

situation can be detected (Fig. 22). This can be due to the sensitivity of the validation method chosen, or this gene might be a false positive.
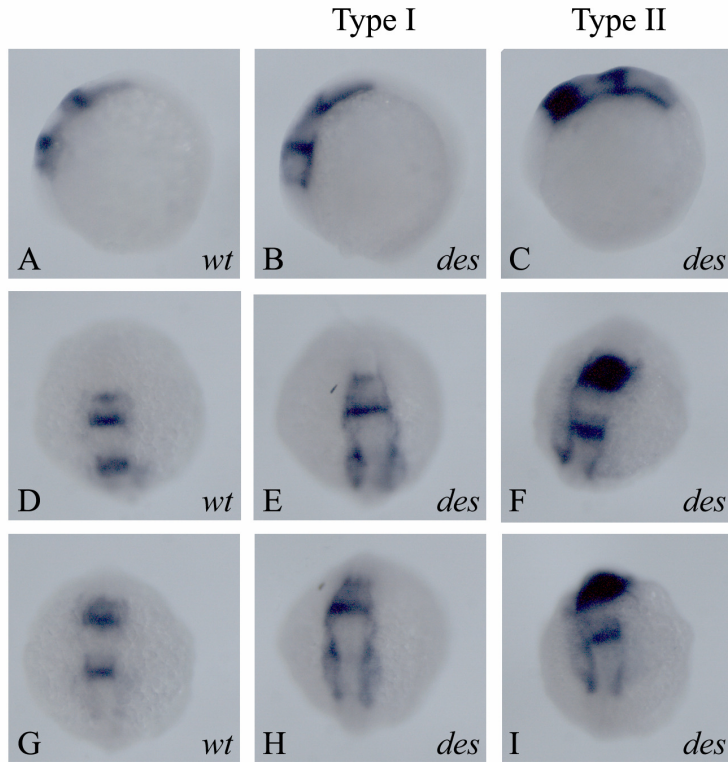
Figure 21: Expression of *ziro7*: This gene is expressed in the brain. Expression in the two boundaries flanking the midbrain and the first rhombomere can be detected in the normal situation (A,D,G). In the mutant embryos two types of expression were observed. In TypeI the wildtype domains are present and additionally the lateral structures show also expression, connecting the different expression domains in the brain. No expression in the rhombomers can be seen, just in the lateral parts (B, E, H). TypeII is the same as TypeI, but there is expression in the midbrain (C, F,I).
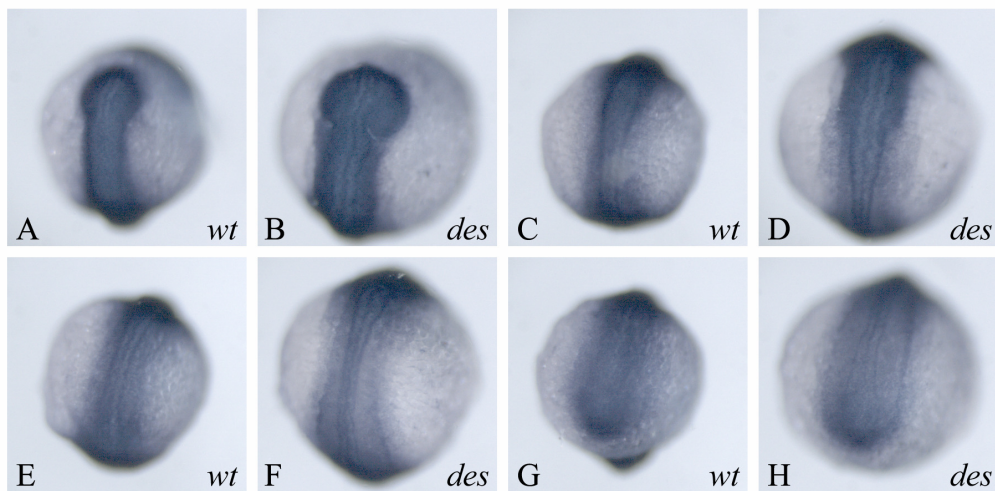
Figure 22: Expression of *Hsp90beta*: *Hsp90* is ubiquitously expressed. No difference in expression between *wt* and *des* embryos can be seen.

Pbx4 is a transcription factor containing a homeo-domain (Vlachakis et al., 2000). Expression is strong in the anterior half of the embryo in the *wt* and *des* embryos (Fig. 23). The up-regulation found in the microarrays cannot be verified by these *in situ* patterns, but there is a difference in the expression in the forebrain (Fig. 23C and D). This might be just the morphological difference between the two embryos and not a difference in expression.
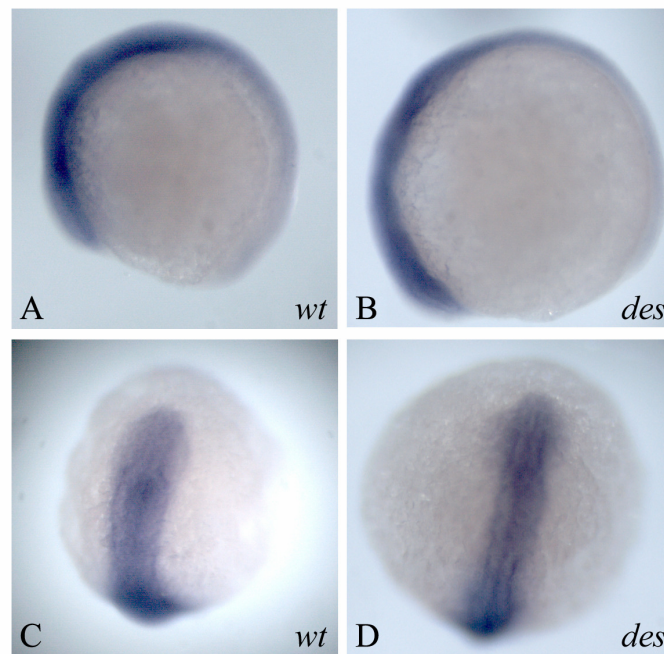
Figure 23: Expression of *Pbx4*: This gene is widely expressed in the anterior part of the embryo. Expression signal seems to be equally expressed in *wt* and *des*, but the patterns in the brains seems to be a little bit deregulated in the *des* embryos (C, D).

4. Discussion:

4.1. Microarray:

The microarray experiments performed in the practical work of this thesis were different from each other. Nevertheless all of them gave results, which could be validated either by *in situ* hybridisation or by comparing the data with information present in the literature. In the following paragraphs the different approaches in the different steps are compared with each other. As the different experiments were done one after the other, an improvement can be detected.

4.1.1: Experimental design and sampling:

4.1.1.1.Sampling:

Entire zebrafish embryos were used for RNA isolation. This decision allowed to detect differences in brain and PSM development in the different mutants. But on the other hand some of these differences cannot be separated by microarray analysis anymore and in the worst case, the differences might cancel each other out. This might be the case if there is up-regulation in the brain and down-regulation in the PSM. The observed difference in this case would be zero.

In the case of *fss*, this is not a problem because the gene defect in *fss* is *Tbx24* and it is expressed only in the intermediate and anterior PSM (Nikaido et al., 2002). In the other *fss* type mutants one might argue that their phenotype in the brain is rather mild and not easily observable morphologically (vanEden et al, 1996). But except for *DeltaC/bea* there are roles in neurogenesis described (Haddon et al., 1998, Gray et al., 2001 and Wright et al, 2004) and expression of DeltaC in brain regions is very weak (Haddon et al., 1998 and Gajewksi personal communication). This is reflected by the fact that the list of candidates for *bea* is the shortest. This might be because there are no or only a few neurogenesis genes transcribed. *Notch1/des* plays a role in neurogenesis as migration of some neural crest population is aberrant and crest-derived dorsal root ganglion neurons are misplaced in *des* embryos. Additionally an increased number of motor neurons are present. (Gray et al., 2001). Accordingly, from 13 genes validated 4 show an exclusive expression in brain domains.

DeltaD/aei plays a role in primary neurogenesis. Together with *DeltaA* and *B* it is expressed in cells, which will become primary neurons. Primary neurons emerge from proneural clusters of neuro-epithelial cells by lateral inhibition (Haddon et al., 1998 and Wright et al., 2004). Here genes involved just in brain-development can be found, for example plexin A4 (Miyashita, T. et al., 2004), which shows the efficiency of the procedure. To focus in further experiments on PSM genes it might be worthwhile to perform microarrays with RNA isolated of PSM, which has been amplified using one of the procedures, which are on the market (f.e. SenseAMP/IMPLEN).

4.1.1.2: Experimental design:

Careful design is important to get the maximum on information. Biological and technical variances must be taken into account to estimate the necessary number of repetitions (Churchill, 2002; Yang and Speed, 2002). In the case of the *fss* cDNA microarray experiments the experimental design was sub-optimal compared to the one used for the oligo chips. In the first experiments only two independent samples were taken to cancel out biological variance. Both samples were hybridised to four chips each. These are technical replicates to cancel out differences in spotting, labelling and hybridisation. Two technical replicates per biological sample would have been sufficient (Yang and Speed, 2002). One more biological sample would have resulted in a good experimental design as a simple way of assessment of the adequacy of the design shows, namely by determination of the degrees of freedom (df). Here the number of independent units are counted and then the number of treatments is subtracted. At least one df should be left (Churchill, 2002). In the case of fss, we had two independent samples minus two treatments (as two dyes were used). The result is zero, meaning that all statistical tests relied on technical variance alone. This results in candidates, which might be just due of individual differences in the fish batches taken. As pools of 100 embryos each were used, this effect was smoothed out a little bit. The zebrafish oligo approach on the other hand had three independent samples minus 2 treatments, giving one degree of freedom, which is satisfying, according to the above mentioned assumptions. The same is true for the mouse oligo arrays.

4.1.2. Normalization:

Two different modes of normalization were used. The fss cDNA microarrays were normalized using the lambda control spots. As 48 lambda Q-spots, distributed over the entire chip are present, this is indeed valid. As the amount of labelled material should be equal, aberration of this ratio can be easily detected. With this calculated correction factor the ratio for the other genes can be normalized as well. The Lowess method later chosen, uses all spots and is therefore more sensitive than the other method. In addition, this normalization method allows the division of the chip in some subunits and normalization is then done for each unit separately. This is a good option, if the hybridisation is not even on the entire chip (Yang et al., 2002; Park et al., 2003; Quackenbush, 2002).

4.1.3. Statistics and choosing of candidates:

For analysis of microarrays many different statistical approaches can be used (Leung and Cavaliere, 2003). Two possibilities seemed reasonable, the first, using a t-test with subsequent estimation of the false discovery rate (Grant et al., 2005) or an approach in which only these genes are kept, which show a similar ratio (in a given Standard Deviation) of expression in all experiments. The latter was used, although this method might lead to losing of some of the truly differently expressed genes, as it is very strict. On the other hand the amount of false positives is reduced, allowing the assumption that nearly all candidate genes found are differently expressed.

In the cDNA approach all genes, which were at least 2 times up or down regulated in at least four experiments were chosen as candidates (log 2 ratio =1), as this is the threshold for the fold-change used by many groups.

On the oligo chips this fold change approach was not chosen as here the observed fold changes were much lower compared to the cDNA chips perhaps because of the different slide surface, i.e. planarity to the coating or spot morphology, f.e. different sizes (Dudley et al., 2002).

The log2 ratio fold changes of her7 shows -0.53 (log2) fold change and a difference can be observed (Fig. 24). The genes showing a higher or lower "fold-change" of beta-actin, which should not change, were taken as candidates.
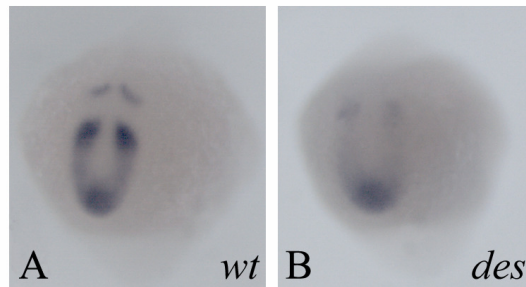
Figure 24: her7 showed a down-regulation of –0.53 in the des embryos and this can be seen after *in situ* hybridization. Therefore even this small log2 ratios can be used for detecting differently expressed genes.

4.2. Genes found using microarray technology, which might play a role in somitogenesis.

4.2.1. General notes on candidates:

The genes found on the microarrays using zebrafish RNA can be, as described above, involved in other processes than somitogenesis. Especially brain development, as the Notch-Delta pathway plays an important role there, can lead to true positives, which are not involved in somitogenesis.

The genes validated show this. Here the genes *D249*, *Pbx4*, *ziro5* and *ziro7* show expression in the brain, but not in the PSM and/or the somites.

I focus here, to validate the approach on the candidate genes, for which information, i.e. expression pattern, functional information etc. could be found in the literature or which have been validated by *in situ* hybridisation.

4.2.2. Zebrafish microarrays:

4.2.2.1: cDNA Microarray:

Ten genes were validated and three genes of them showed differently expression: *Pax-like*, *rack1* and an unnamed protein (*unnamed proteinII*). *Hsp90beta*, which was also found, showed ubiquitous expression and no difference between *wt* and *fss* could be seen. Interesting is that the genes validated were expressed in the somites, where *Tbx24/fss* is not expressed, but this situation is comparable with the expression of

61

Fgf8 in fss embryos. There the expression of *Fgf8* in the tailbud is unchanged, but the expression in the somites is disturbed (Nikaido et al., 2002).

The role of Pax-genes in somitogenesis is the patterning of sclerotome (*Pax1*) and the dermamyotome (*Pax3*) (Cossu et al., 1996b), this means it acts in late stages in somitogenesis. In chicken (Suetsugu et al., 2002) *Pax2* show a cycling expression at the future border of the forming somite, but as this gene is not expressed in mouse PSM, it might be that this expression is avian specific. As the gene-fragment found in the microarray is similar to *Pax* genes it is not possible to tell, which *Pax* it is and one just can speculate about the role it might play in somitogenesis. At it is expressed in the somites, but not in the PSM it might be involved in patterning of the somite into the sclerotome and dermomyotome, but not in pre-patterning of the PSM.

In *Xenopus, receptor for activated c-kinase 1* (*rack1*) is expressed in the somites of embryos comparable to the expression found in zebrafish (Kwon et al., 2001). In another study (Lilienthal et al., 1998) it was shown that *rack1* interacts with the integrin beta subunit. As *integrin alpha 5* plays a role in mouse (Goh et al., 1997) and zebrafish somitogenesis (Jülich et al, 2005 and Koshida et al., 2005) and other *integrins* (see below) were detected in the microarrays conducted in this thesis it seems as *rack1* might play an important role in somitogenesis and it might be worthwhile to do functional analysis with this gene. The role r*ack1* might play is that it forms together with an integrin beta a receptor for fibronectin and/or Laminin and by doing this helps to form and maintain the epithelia of the somites by accumulation of fibronectin at the somite boundaries.

It seems as if these two validated genes together with *unnamed protein II* play a role in epithelialization or later events in somitogenesis.

Another gene, which was tested using *in situ* hybridisation, but showed no difference between *wt* and *fss* embryos, was *Hsp90beta*. Because of this it was thought of a false positive, but this gene also showed-up in the *des*-chip. There again no difference in the expression in the mutant embryos could be detected, but it plays a role in somitogenesis (Krone et al., 2003). Blockage with Geldanamycin leads to somites, varying in size and shape, the trunk is shortened and the tailbud malformed (Lele et al., 1999). This chemical binds specific to *Hsp90α* and *β* and interacts with the ATP binding domain. Normally *Hsp90* interacts with and modulates the activity of several signalling molecules and transcription factors, for example *myoD* (Shaknovich et al., 1992 and Kotz et al, 1994). *Hsp90* can stimulate the DNA binding activity of *MyoD*.

Having this in mind it seems logical that this gene appeared in the microarray experiments, but it is interesting that it did not display a difference in the *in situ* hybridisation. A plausible explanation might be that *Hsp90beta* is really expressed differently, but that the sensitivity of the validation method is too low. It might be that in both cases, wildtype and mutant, the gene is expressed in an amount that the output signal (Staining) is over-saturated in both conditions and therefore no difference can be seen. Another explanation might lay in the difference of embryo handling in microarray experiments and embryo fixation before *in situ* hybridisation. The embryos, used for microarrays are killed quickly by smashing them in lysis buffer, the other embryos are put in 4%PFA and die perhaps slower and as this induces stress, stress chaperones as *Hsp90beta* are activated, but this seems less likely as the response to this must be in a time-interval of maximal 20minutes. This is the time to fixate the embryos in PFA.

4.2.2.2. zebrafish oligo microarrays:

Here the genes found in the *des/notch1* chip, which have been validated will be presented.

Thirteen genes, which showed different expression on the des chip genes, have been tested using *in situ* hybridisation. This was not only done to validate the genes, but also to check for the site of expression, as we are interested in genes involved in somitogenesis. The genes, which show an interesting pattern in the PSM and or the somites will then be further characterized.

Of the thirteen genes 11 genes could be validated. Two genes showed no expression differences namely *Hsp90beta* (see section 4.2.2.1) (Fig. 22) and *Pbx4* (Fig. 23). *Pbx4*, which was found to be up-regulated, just showed some deregulation in the forebrain region, which might be due to the role of *notch1a* in brain development.

Ziro5 (Fig. 17), *ziro7* (Fig. 21) and *D249* (Fig. 16) are just expressed in the brain and are not interesting for a further analysis, but they show the expression difference predicted by the microarray analysis. *P38a* shows expression in the PSM, but the up-regulation of the expression can only be detected in the brain (Fig. 20).

Connexin43.3: This gene belongs to a gene-family, which function is to form gap-junctions (Wei et al., 2004). Gap-junctions are 2-3nm in diameter and mediate the passage of ions, metabolites and small signalling molecules less than 1 kDa in size. Gap-junctions play a role in establishment and/or maintenance of differences in cell

adhesion, migration and the interpretation of positional information (Meyer et al., 1992, Warner et al., 1992, Paul et al., 1995). *Cx43.3* function can lead to cross talk with cell signalling pathways that regulate cell adhesion, cell motility and the actin cytoskeleton (Essner et al., 1996). This gene was up-regulated in the des-mutant. In the wild-type situation this gene was expressed as a gradient in the PSM, with the highest expression in the posterior part. This gradient was not observable in the des-mutant. In the mutant an even expression in the entire PSM can be seen (Fig. 18). The expression in the wildtype situation is similar to the gradient expression of *Fgf8* (Dubrulle et al., 2001, Sawada, 2001). It seems as if the undetermined cells cross talk via gap-junctions and later this communication is shut down. This restriction of cross-talk might be necessary for the cells to establish or maintain their more determined fate in the anterior part of the PSM. The communication in earlier stages can be necessary for prepatterning of the somites. After the prepatterring is done a shut-down of additional signals seems to be necessary.

Cx43.4 might interact with other genes found in the microarray experiments. On the *aei*-chip a *junctional adhesion protein* was found, which might interact with the Connexin to form a Connexon. As mentioned above Connexins can interact with the actin cytoskeleton and on the Su(H) chip the *actin-like protein2* was found.

<u>Her6:</u> Pasini et al., 2003 showed that *Her6* is Notch-dependent by blocking Su(H). In this case *her6* is down-regulated in the PSM and the somites. The AP polarity in the somites is disrupted as well. The embryos displayed a phenotype like embryos of the *des*, *bea*, *aei* and *mib* mutations. Interesting it is not only the case that *Her6* depends on *notch1a*, but that regular expression of *notch1a* also depends on *Her6*. Therefore it was no surprise that in the des mutant embryos *Her6* is down-regulated in the somites and the PSM (Fig. 13).

<u>ZfMesogenin:</u> For *zfmeso* no information was present, whether it is linked to the Delta-Notch pathway. Here *zfmeso* is downregulated in the des mutant embryos (Fig. 11). Mesogenin mouse mutants lack posterior somites. *Brachyury* expression is up regulated indicating that Mesogenin normally represses T-Box gene expression (Yoon and Wold, 2000). This might link Delta-notch signalling with FGF signalling as *Tbx24* is a T-box gene and *Fgf8* expression is disturbed in the somites of *fss* embryos (Nikaido et al, 2002).

<u>Similar to MMP1-A:</u> Matrix Metalloproteinases are endopeptidases playing a role in growth, development, wound healing, arthritis and cancer. Until recently, it was

thought that these roles were only fulfilled by degrading ECM (Extracellular matrix), but this activity also releases information of the ECM. The ECM consists of laminins, fibronectins, integrins, heparan sulfate proteoglycans etc. and also serves as a reservoir for embedded cytokines and growth factors. MMPs cleave insoluble ECM fragments and ECM associated molecules. This leads to free bioactive fragments and Growth-factors (members of the VEGF and TGF-beta family for example) and change ECM architecture (reviewed in Mott and Werb, 2004). In the *des* mutant *MMP1-A* was up regulated (Fig. 19). This might just be an effect of deregulation, meaning that *notch1a* regulates this gene allowing proper function of this gene, on the other hand it is also possible that the high activity is necessary to allow functioning and survival of the cells by releasing the factors stored in the ECM.

Mta: This gene plays a role in DNA methylation, which is an important factor in the control of genetic information. Embryos treated with chemicals inducing DNA hypo-methylation (5-aza cytidine and 5-axa-2 deoxycytidine) exhibited developmental perturbation, namely a loss of tail and abnormal patterning of the somites. The head was affected as well. This phenotype implies that *mta* plays a role in somite formation and posterior body formation. DNA methylation normally occurs to stabilize a certain state of cells in which genes not required are silenced (Martin, et al., 1999). In the *wt* embryos *mta* is expressed in the tailbud and the head (Fig. 14), as the phenotype of blocking methylation suggested. As methylation patterns change during development, it might be that in the tailbud, genes are inactivated, which would drive the cells to a more determined fate. The role of *mta* might be to allow many rounds of mitosis without differentiation in the posterior part of the body. Later and more anterior the genes, which were inactivated via methylation must be activated again to allow specification into cells, which can now be prepatterned into somites. As *mta* is not or only residually active in the mutant this might lead to an early prepatterning, disturbing the possibility of a normal somite formation at least in the somites formed after the first seven somites. During the building of the first somites the differentiation might not be that advanced as in the latter ones and therefore normal somites are formed. Interesting in this aspect is that normally cells expressing the Notch-receptor on the surface are hindered to adopt a certain cell fate, when binding with the ligand Delta occurs at least during neurogenesis (Schroeder et al., 1998).

ZfdIII: This gene is down-regulated in the des mutant embryos. The only fact about that gene known is that it contains a zinc-finger domain, which marks this gene as a

transcription factor, but without any functional data nothing more can be said about this gene.

Ztsg1: In *Xenopus* it has been shown that twisted grastrulation (Tsg) can increase the binding of Chordin to BMP4 and by doing so acts as cofactor in antagonizing BMPs. (Scott et al., 2001). If knocked down in zebrafish the expression of *MyoD* is reduced. (Ross et al., 2001). In zebrafish *ztsg1* is down regulated in the *des* mutants suggesting that *ztsg1* connects Delta-Notch and BMP signalling. Interestingly a gene similar to *Bmp1* and a gene, called *twisted gastrulation like* (*tg*l) have been found as well on the des-chips.

4.2.2.3. Genes found in microarrays using zebrafish embryos, which have not been validated and comparison of genes found:

In this section only some of the genes found are described. The choice, which genes should be included here was based on the information in the literature, if expression or functional data was present. Is should be noted that because these genes have not been validated by own *in situ* hybridisation, this section is even more speculative as the previous one. The genes chosen were found in more than on one chip (Table 8).

Table 8: Genes found on more than one chip series.

	des	*aei*	*bea*	*Su(H)*	*fss*
Cecr1	down		down		
Hoxd12	down	up			
Hsp90-beta	down				down
Ndr2	down	down			
Pbx1a	down		down		
Pbx4	up			up	
Semaphorin Zb1	down	up	down		
Unc119b	down		down	down	
Wnt11	down			down	

The regulation-pattern was on all chips the same except for *Hoxd12* and *Semaphorin Z1b*. These genes were down-regulated on the *des* chip and up-regulated on *aei*. There are two possible explanations for this. First, it can be that on one chip the regulation predicted is not true or second, that there is a difference if the receptor (*notch1a*) or ligand (*deltaD*) is defective.

Cecr1: Cecr1 belongs to the family of adenosine deaminase-related growth factor (ADGF) (Maier et al., 2001). The deamination of adenosine is required for mitogenic action as imaginal discs cell-cultures depleted of adenosine by using bovine adenosine deaminase show stimulation of proliferation. Addition of adenosin inhibits proliferation (Zurovec et al., 2002). In the *bea* and *des* experiments this gene is down-regulated, which might mean that proliferation is also somewhat down-regulated. BrdU (5-bromo-2'-deoxyuridine) labelling of *des/bea* and *wt* embryos, showing proliferation might verify this thesis, together with the *in situ* test.

Hoxd12: Until now, for this gene only a role in patterning of the anterior-posterior polarity of the nascent limb bud has been described (Deschamps, 2004), but it is not unlikely that it also is involved in the A/P-patterning of the embryonic axis, as *Hoxd12* was found to be up-regulated on the *aei* chip and down-regulated on the *des* chip. Therefore an important first step is to validate this via in situ hybridisation and later after determining the expression pattern a theory might be formulated.

Hsp90beta: see above

Ndr2(nodal related 2): This gene is an early mesendodermal inducer and overexpression experiments lead to an elongation of the anterior and posterior somites. In later stages (24h) the body axis was kinked (Erter et al., 1998). On the *des* and *aei* chip this gene was down regulated and it seems reasonable that this gene is necessary for proper somite formation.

Pbx1a: This gene is an important Co-factor for transcriptional regulation as it modifies the selectivity and affinity to the DNA (Vlachakis et al., 2000). The gene was down regulated in the des and bea microarray-experiments. The literature suggests that this gene is only expressed in the brain.

Pbx4: Pbx4 is as *Pbx1a* a Co-factor for transcriptional regulation (Vlachakis et al., 2000). Interestingly this gene was up regulated on the *des* and *Su(H)* chips, suggesting an involvement in different regulations as *Pbx1a*. This gene is also not expressed in the somitic region and/or the PSM.

Semaphorin Z1b: This gene plays a role in caudal primary (CaP) motor axon guidance. Not much is known about the signals that define the CaP pathway, but they are channelled into the anterior half of the somites and it is theorized that the posterior half possess an inhibitory environment. *Semaphorin Zb1* is a likely candidate to play a role in this process of inhibiting as it is expressed in the posterior half of the somites (Bernhardt et al., 1998). It was very surprising to find out that this gene was down-

regulated in the *bea* and *des* mutant, but up-regulated on *aei*-chips. Maybe *Zb1* cannot be expressed in its compartment because the patterning of the posterior somites has not worked in the mutants and for unknown other factors *Zb1* is expressed widely in the entire somites in *aei* embryos (leading to an up-regulation) and down-regulation in *des* and *bea*.

Unc119b: UNC-119 proteins are highly conserved at both sequence and functional levels in many metazoans (Drosophila, *C.elegans*, *Danio rerio*, Humans etc.). In zebrafish, as in the other animals this gene is expressed in various neural tissues (Manning et al., 2004). In *des*, *bea* and *Su(H)* embryos this gene is down-regulated according to the microarray. It is not unlikely that Delta-Notch components regulate *Unc119b* in brain development.

Wnt11: The mutant for *Wnt11* silberblick (*slb*) made clear that this gene is needed for convergent extension movements during gastrulation. *Slb* mutants also show abnormal extension of axial tissue. No role in somitogenesis had been described (Heisenberg et al., 2000), but *Wnt11* is expressed in the tailbud from the early segmentation stages on, later expression in the notochord can be detected. At 16-somite stage *Wnt11* is induced in the medial part of the somite and from 24h on this gene is expressed in the myotome (Makita et al, 1998). Makita et al. also showed that *Wnt11* is induced by FGF by implanting a bead soaked in this protein. On the *des* and *Su(H)* chips this gene was down-regulated. Perhaps *Wnt11* acts at the interface between the Notch and the WNT pathway in somitogenesis and/or neurogenesis.

4.2.3. Mouse oligo library:

Only two genes have been validated by literature, namely *Hoxb13* and *Sint1(Sept9)*. *Hoxb13* is expressed in the tailbud and a gradient of expression reaches into the anterior PSM. This gene has a role in the patterning of the A/P axis (Zeltser et al., 1996) Therefore this gene might not be involved directly in somitogenesis. *Sint1 (Sept9)* belongs to the Septin gene family, which encode nucleotide-binding proteins. They have been described as cell division cycle regulated proteins (Sørensen et al., 2002). This gene is also higher expressed in the posterior part of the PSM as the anterior one. Furthermore it is expressed in the somites. This gene might be involved in the linkage between cell cycle and the segmentation clock (Primett et al., 1988 and 1989).

There is another gene, which might be expressed in the predicted pattern, namely *Retinoic acid receptor beta 2 (RAR beta2)*. This gene is up regulated in the anterior PSM, according to the microarray experiment. Retinoic acid plays a role in fine-tuning of the wavefront (Diez del Corral et al., 2003, Moreno et al., 2004). It forms an opposite gradient to FGF8, showing exactly the regulation pattern predicted by the mouse-chips.

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