Zebrafish Trap230/Med12 is required for Sox9 activity and limb induction

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To all the people who continuously encouraged me to keep going
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1 Introduction
1.1 Zebrafish as a model system

Over the last decade, the zebrafish (*Danio rerio*) has been established as a successful and popular model organism for studying vertebrate development (Anderson and Ingham, 2003; Detrich et al., 1999; Eisen, 1996). Zebrafish are easy to keep and breed, have a relatively short generation time (3-4 months), produce large numbers of embryos (100-200 per mating) and provide easy access to all developmental stages due to external fertilisation. The embryos are optically transparent and develop rapidly. Within three days, all important structures of an adult fish are established. Since defined strains are available and the sequencing of the zebrafish genome was started in 2001 by the Sanger Institute, zebrafish are also useful to study genetics. They present a unique opportunity to study not only embryology but also genetically inherited diseases and the genetics underlying developmental biology (Shin and Fishman, 2002), making zebrafish an attractive model system (Neumann, 2002).

In the context of limb development zebrafish offer the new possibility of using a forward genetics approach in a vertebrate, which allows the discovery of new genes involved in this process.

As part of this thesis, I analysed pectoral fin formation in a novel zebrafish mutant, *trapped*.

1.2 Screening

Already more than two decades ago, the aptness of zebrafish for random mutagenesis and mutant screening was pointed out (Streisinger et al., 1981) and consequently, a large scale screening protocol was developed at the Max-Planck-Institute for Developmental Biology in Tübingen (Mullins et al., 1994). This protocol was then employed in a large-scale screen in 1996 (Haffter et al., 1996). In the years 2000/01, a second large-scale screen, with the aim of reaching saturation and hitting every gene essential for embryonic development was performed (Aldhous, 2000).

Germ line cells of male zebrafish of the Tübingen (TU (Haffter et al., 1996) strain (P0 generation) were mutagenised by placing the fish in water containing ENU (1-
ethyl-1-nitrosourea (Werner, 1919)), a synthetic compound of pH-dependent stability which causes point mutations in the genome. This most likely leads to GC to AT transitions, although all types of transitions and transversions are possible (Knapik, 2000). By crossing three week old males to non-mutagenised females, premeiotic mutagenised germ cells were recovered to prevent mosaic offspring. A classical three-generation screen (Haldane, 1956) strategy was employed. P0 males were outcrossed to wild type Tübingen females and an F1 generation of non-mosaic founder fish, carrying one or more mutations in their germ line were raised. To drive the mutation to homozygosity, random pairwise matings between founder fish were performed, resulting in F2 families. Since half of the fish in each of the F2 families are heterozygotes for any segregating recessive mutation, crosses within the family will reveal homozygote embryos that display the mutant phenotype. F2 fish were subsequently visually screened for various phenotypes, including defects in pectoral fin development.

Heterozygous mutant lines of the Tübingen strain carrying an interesting phenotype, presumably due to a recessive point mutation, were then kept, and pectoral fin mutants brought to Heidelberg.

1.3 Mapping and positional cloning

The process of mapping and positional cloning involves unique issues for each organism. Success is usually based on experience. The mapping and cloning methods and facilities used for this dissertation were newly established in the lab in collaboration with William Norton.

Before embarking on the meticulous and time-consuming process of mapping (Fishman, 1999), it may be useful to set up complementation crosses of a heterozygous carrier of an unknown mutation with a heterozygous carrier of (known or unknown) mutants with similar phenotypes to identify multiple alleles of the same gene. If the mutations do not complement, i.e. one quarter of the offspring show the expected mutant phenotype, the mutants should be considered as alleles of the same gene. However, this test is not fool-proof and occasionally different genes acting in the same pathway may also fail to complement.
To identify the gene affected in a zebrafish ENU mutant, the most common mapping strategy uses SSLPs (single sequence length polymorphisms), CA-repeats whose lengths are polymorphic with respect to different strains of zebrafish (Beier, 1998; Postlethwait and Talbot, 1997). A mapping cross is up, outcrossing heterozygous mutant carriers - in this case of the Tübingen (TU) strain - to wild type fish of another – in this case the WIK (Wild India Kalkutta (Rauch, 1997)) strain (P0 generation, cf. Figure 1).

![Figure 1. Map cross and bulked segregant analysis of a recessive zebrafish mutation.](image)

(Geisler, 2002) WIK/WIK, reference fish; mut/Tü, fish carrying the mutation in Tü background. Band sizes and intensities of a representative SSLP marker are indicated schematically. In case of no linkage between the mutation and the marker, the intensities in the mutant and sibling pool are the same. In case of a linkage, the Tü band is stronger in the mutant pool and the WIK band in the sibling pool. Only a quarter of F1 crosses consist of two mutant carriers and yield mutant F2 as shown, the others are discarded.

However, these and all other widely available zebrafish strains are not entirely inbred. Genetic polymorphisms may be present within a given family of fish and the strains are not as isogenic as inbred strains of mice. Heterozygous F1 carriers of the mutation are then identified by multiple random incrossing. As many of the mutations are homozygous lethal, they are kept as heterozygote carriers. Individual
identified F1 carriers are set aside pairwise for ongoing incross embryo collection. Of these mapping pairs, embryos are collected and then visually sorted into phenotypically mutant and wild type siblings. According to Mendelian genetics, one quarter of the embryos should be mutants. Genomic DNA from individual embryos is then extracted.

The positional cloning approach is based on assessing meiotic recombination frequencies between the mutation to be mapped and defined SSLP markers of a known position within the genome. To this aim, linkage analysis with SSLP markers is performed.

For low resolution or rough mapping, bulked segregant analysis is employed (Michelmore et al., 1991). Its goal is to locate the linkage group (which equals one of the 25 chromosomes of zebrafish) - or better rough region of a linkage group - on which the mutation lies, by scanning microsatellite markers (SSLPs) throughout the genome. For this study, a set of 192 agarose scorable markers optimised for polymorphism between the TU and WIK strains were used. About two thirds of the markers in this set were usually polymorphic. This SSLP set covers the entire genome (2500cM) (Postlethwait et al., 1994) with an average distance of about 13 cM (centiMorgan, 1cM = 1 recombination event within 100 meioses) between markers. It has been developed in the lab of Robert Geisler (Geisler, 2002) and markers have previously been mapped to the meiotic MGH panel (Knapik et al., 1996), so their location within the genome is roughly known. Pools of DNA extracted from 48 mutant and wild type embryos each from the same F1 mapping pair are used to PCR-amplify the SSLPs set using a defined flanking primer pair for each marker. The products are electrophoretically separated on agarose gels. If an SSLP is polymorphic for the two strains used, the band size will be different for the two alleles, since the length of the CA repeats will differ between the strains. Bands are typically 100-400bp long and the size difference between alleles will be roughly between 20 and 150bp, which makes it necessary to perform the analysis under high resolution conditions.

For markers which are not physically close to the location of the mutation and therefore not linked to it, there will be no difference in the band pattern between mutants and wild types – both will show the mutant (TU) and wild type (WIK)
alleles (Figure 1). However, markers which are linked to the mutation, will co-segregate with it. Without meiotic recombination, the expected result for a marker on the same linkage group as the mutation would be that mutant pool DNA will show only the mutant (TU) band. This occurs since the embryos can only be phenotypically mutant if they are homozygous for the mutation, which was originally made on a chromosome of the mutant strain (TU). Pools of phenotypically wild type siblings will show both the mutant (TU) and wild type (WIK) bands, since they are a mixture of genotypically wild type and heterozygous embryos.

However, as meiotic recombination takes place, part of the F2 chromosome containing the mutation may actually be of wild type (WIK) origin. And if this part of the chromosome contains the marker being checked, the wild type (WIK) band will also show up in the PCR. The closer the marker being tested physically is to the mutation, the less often a recombination event will occur between the two. So the weaker the wild type (WIK) band amplified from a pool of mutant DNA for an individual marker is, the tighter its linkage to the mutation. Since it is not usually known which size the band should have for any given strain (due to polymorphism also within strains), the bands amplified by a pair of PCRs on DNA pools from mutant and wild type siblings (F2) with the same SSLP-flanking primer pair must be checked for a typical two-band-in-wild type-pool one-band-considerably-weaker-in-mutant-pool pattern (Figure 1). These will then be recorded as potential linkages.

False-positive linkages are not uncommon, therefore they must be confirmed by PCR on individual embryos. This also enables the calculation of the distance between marker and mutation by analysing the recombination rate between the marker and the mutation. DNA from the same individual embryos which were pooled for bulked segregant analysis is used. Mutant embryos should show either one (not recombinant) or two bands (recombinant), while wild type siblings will have two (heterozygotes) or either one (homozygous wild types or recombinants) of the two bands, if they are truly linked (Figure 2).

The distance between marker and mutation is calculated as follows:

\[
\text{Distance (cM)} = \frac{\text{number of recombinant mutant embryos} \times 100}{2 \times \text{number of all mutant embryos analysed}}
\]
The number of mutant embryos analysed has to be doubled since each embryo will represent two meioses and therefore possible recombination events – that of the father and the mother.

**Figure 2. Linkage analysis of individual embryos.**

(Geisler, 2002) In the heterozygous F1 the mutant Tü (red, bearing an asterisk indicating linkage with the mutation) and wild type WIK (blue) alleles from the P0 generation are meiotically recombined (R!) to the F2 generation. Individual wild type siblings (S) will have the wild type (blue) allele or be heterozygous (red and blue), plus some recombinants with the Tü allele (red). Mutants (M) will have the Tü allele (red). Two recombinant mutant individuals (red and blue) are represented. Here, there are two recombinants out of six (17% recombination, 17cM).

However, there are several caveats. First of all, the recombination rates of males is about 10-15 times lower than that of females, especially close to centromeres and telomeres, where recombination is generally repressed. The calculations above will give an average distance which will be the more accurate the higher the number of embryos that were actually counted and the closer the marker is to the mutation (up to a limit of about 0.1-0.3cM, where inaccuracy in a too-close-area begins again). It is also necessary to make sure that F1 parents are truly heterozygous for the marker in question. Otherwise, only the meioses of mother or father will be counted, and the number of embryos analysed in the formula above should not be doubled any more. The distance calculated will be much too low if only the father was heterozygous. To deal with this problem, F1 fish are regularly fin-clipped and DNA is extracted from a cut-off piece of the tail fin. Carriers are then genotyped for their allele composition of the marker being used to calculate the distance.

Another problem is caused by markers which give multiple bands. Band systems of one to five bands are common. Often it may be difficult to assess which bands are actually the ones that should be scored and markers will have to be abandoned.
Also, if the distance between marker and mutation is too high (more than about 5-10cM), double recombination events will obscure the correct recombination frequency, making it impossible to calculate distance.

At the same time, missorted wild type embryos can distort the calculations and appear as false-positive recombinants.

Once the distance between a marker and the mutation has been established, it is necessary to find out in which direction along the chromosome the mutation lies. To this aim, SSLP markers on the same linkage group are chosen accordingly (eg. from the meiotic MGH panel) to define a set of two markers between which the mutation lies. Since accurate distance calculation can be impaired for the above reasons, it is recommendable to define a pair of markers which can reliably be said to lie on either side of the mutation. To this end, the same individual embryos are tested with different markers. If mutually exclusive recombinants can be found, that is to say, if embryos can be found which are recombinant for one marker but not the other and vice versa, it can be assumed that the markers lie on different sides of the mutation. If they share a set of recombinant embryos, they will lie on the same side, the marker with less recombinants being closer to the mutation.

However, there are different scenarios in which the decision as to whether two markers lie on the same or opposite sides of the mutation is not that straightforward. One problem can again be missorted wild type embryos, which will then occur in markers on both sides of the mutation as false-positive recombinants. To address this problem, individual embryos then have to be tested with markers which were previously proven to definitely lie on different sides of the mutation. If they still appear recombinant for both, they must be either missorted wild types – or double recombinants, if the markers are too far apart. Thus, possible recombinants have to be confirmed, or markers can wrongly be assumed to lie on the same side of the mutation. Another problem occurs if for one of the markers tested only the F1 female is heterozygous, for the other marker only the F1 male. Such couples of markers will never show overlapping recombinants, no matter which side of the mutation they are on. If it is impossible to find markers for which both parents of a mapping pair are heterozygous, it is advisable to use markers for which at least the female meioses can be counted. Since the male recombination frequency is much lower, relying on such
markers means that 10-15 times more embryos need to be scored until a recombinant can be found, making a reasonable estimate of the distance virtually impossible.

Once a clear interval containing the mutation is identified, closer markers are sought to narrow it down as much as possible. This process is called fine mapping. To this end, markers from the meiotic MGH panel are used to exhaustion and tested for polymorphism and for which side of the mutation they are on. Several thousand individual mutant embryos from parents which are heterozygous for the respective markers have to be collected to refine the distance calculations. The aim of fine mapping is to get considerably closer than 1cM to the mutation from either side. On average, 1cM corresponds to 625kb of the zebrafish genome (Postlethwait et al., 1994). The SSLPs from the MGH panel can then be used to identify the actual genomic region encompassed by finding them in the zebrafish genome database (http://ensembl.org/Danio_rerio/), either by annotation or BLAST search of primer/marker sequences. This will sometimes prove difficult, since the genome is still patchy in parts and contains a large number of gaps. The genomic sequence between the closest markers can then be searched for simple repeats using the RepeatMasker program (http://www.repeatmasker.org), and flanking primers designed for these. These self-designed SSLP markers will then be tested for polymorphism and linkage, and typically about 20% of them yield useful results. By this method, the mutation can be fine-mapped until no further SSLP markers are available.

Should this region happen to contain sequence gaps, they can be filled by searching an assembly of sequenced zebrafish BAC (bacterial artificial chromosome) and PAC (P1-derived artificial chromosome) clones (http://vega.sanger.ac.uk/Danio_rerio/) or, failing that, by BAC-walking.

The available sequence can then be assessed for possible candidate genes using the distances calculated between markers and mutation and fitting predicted gene functions to the mutant phenotype. Candidate gene cDNA will then be cloned and sequenced from both mutant and wild type embryos to compare the sequence and look for possible point mutations. Failing that, introns, promoters and enhancers may also be cloned and analysed. RNA expression patterns and previously known data of individual genes can be taken into account when looking for possible candidates.
1.4 The Mediator complex

Much of biological regulation occurs at the level of transcription initiation. Protein-coding genes contain promoter sequences that are bound by transcriptional activators and repressors, which then recruit the transcription initiation machinery, consisting of RNA polymeras II (pol II) and at least 50 additional components. This complex contains DNA-binding factors, cyclin-dependent kinases (Cdks) and chromatin modifying enzymes (Lemon and Tjian, 2000).

Pol II itself is a 12-subunit complex in which the largest subunit contains a C-terminal domain (CTD) composed of a conserved heptapeptide sequence (YSPTSPS), rich in amino acids that can be phosphorylated. This phosphorylation is highly regulated and modulates the association of proteins with pol II. Elongating, transcriptionally active pol II contains a highly phosphorylated CTD (Cho et al., 1998).

The Mediator complex is a multifunctional key coactivator acting as a bridge between DNA-binding transcription factors (TF) and pol II, thus conveying regulatory information from enhancer elements to the basal transcription machinery (reviewed in (Bjorklund and Gustafsson, 2005; Kim and Lis, 2005; Malik and Roeder, 2005). It is involved in integrating both positive and negative transcriptional regulation (Carlson, 1997; Hampsey, 1998; Ito et al., 2000; Kuchin et al., 1995; Song et al., 1996) and stimulates basal, activator-independent transcription (Baek et al., 2002; Cho et al., 1998). Moreover, it is important to regulate and control the phosphorylation state of pol II CTD at Serines 2 and 5 in concert with TFIIH, a member of the transcription initiation complex (Bjorklund et al., 2001; Park et al., 2001a). Coactivators are defined as required for function of DNA-binding activators and by themselves show no site-specific binding (Malik and Roeder, 2000). Distinct activators can bind simultaneously to Mediator, providing a mechanism for synergistic functions of activators (Ptashne and Gann, 1997).

Recruitment of Mediator to promoters occurs by binding of TFs to different activation domains in a chromatin-dependent fashion (Naar et al., 1999). This is mostly preceded by chromatin-remodeling cofactors with histone acetyltransferase (HAT) activity like CBP/p300 and p160 making the DNA accessible (Fondell et al.,
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1999; Malik and Roeder, 2000). Yet, Mediator itself can also bind to nucleosomes, and one of its subunits, Med5 has HAT activity (Bjorklund et al., 2001; Boube et al., 2002; Zhang and Emmons, 2001).

Pol II is then recruited to the complex, rather than being a stoichiometric component of it (Park et al., 2001b; Rachez and Freedman, 2001). Mediator can override transcription factors of the basal machinery in some cases, in others they function synergistically (Malik and Roeder, 2000). It does not act instead of other coactivators, but mostly in concert with them (Malik and Roeder, 2000).

When transcription has been initiated, Mediator and some general transcription factors remain at the promoter, forming a scaffold onto which pol II and other components of the transcription initiation complex can reassemble to reinitiate transcription (Rachez and Freedman, 2001).

Apart from components of the transcription initiation machinery and chromatin remodelling enzymes, mammalian Mediator has also been copurified with RNA processing, DNA repair and elongation factors, suggesting a complex involvement in many DNA-related processes (Cho et al., 1998).

The first Mediator components were identified as suppressors of a yeast pol II CTD mutant phenotype (Thompson et al., 1993). Mediator complexes of differing composition were subsequently identified using different purification approaches and in different species (Malik and Roeder, 2000), but ultimately found to be essentially conserved as one (Figures 54, 55 appendix) (Boube et al., 2002; Sato et al., 2004). Consequently, a multitude of names for each subunit and subcomplex exist, which has recently been attempted to simplify by a unified nomenclature (Bourbon et al., 2004). There appears to be an intrinsic modular organisation, and the variations found may not only reflect different purification procedures, but also different physiological states of the cell (Malik and Roeder, 2000). Thus, the selectivity of transcriptional control may lie in subtle differences in the particular composition of Mediator. It may besides be influenced by combinations of activators and the context of enhancer sequences to which they are bound at the promoters of specific target genes (Rachez et al., 1999).
Electron-microscopic 3D-structures of yeast and murine Mediator are remarkably similar, implicating constraints imposed by the highly conserved shape of pol II, with which Mediator interacts (Asturias et al., 1999; Malik and Roeder, 2000). Essentially, the up to 30 components of Mediator are divided into three core parts - a head domain, closest to the CTD and most conserved among species, a less conserved middle domain, which interacts with other parts of pol II (Jiang et al., 1998; Malik and Roeder, 2000), and the divergent tail, which is thought to bind the TFs (Figure 3) (Bjorklund et al., 2001).

![Figure 3. Topological organisation of yeast Mediator.](image)

(Guglielmi et al., 2004) This model was made taking into account direct links between subunits found in (Guglielmi et al., 2004; Ito et al., 2001; Kang et al., 2001; Koh et al., 1998; Lee et al., 1998; Uetz et al., 2000).

Upon pol II binding, a conformational change from a compact structure to a form in which the three modules are wrapped around the globular pol II takes place (Asturias et al., 1999; Naar et al., 2002; Woychik and Hampsey, 2002). Moreover, human Mediator has been shown to change shape dramatically depending on which activator is bound to it (Taatjes et al., 2002). Apart from the core modules, a
detachable kinase subcomplex, composed of Trap230/Med12, Trap240/Med13, cyclinC (cycC) and its cyclin dependent kinase Cdk8, has been identified.

Still, there are some subunits which have not yet found their corresponding match across the phyla, namely Med2/3/5 in yeast, and Med23-30 as well as the recently identified Med12L, Med13L and Cdk11 in metazoans. Considering the discrepancy in numbers, it seems less likely that these subsets perform equivalent functions than that they represent species- or phylum-specific components added to an ancient protocomplex (Boube et al., 2002; Sato et al., 2004).

Interestingly, some of these derived subunits (Med28-30) are part of the Mediator head, which had been proposed as most conserved Mediator module. The others are part of the middle and tail modules (Med2/3/5), the kinase submodule (Med12L, Med13L, Cdk11) or still unassigned.

While some Mediator subunits appear to be universally required for the transcription of all genes, a number of subunits are dedicated to the regulation of specific genes (Bjorklund and Kim, 1996; Kwon et al., 1999). The specialised (activator-dedicated) subunits may fulfil a recruitment function, while a more kinetic, post-recruitment role could be carried out by a core complex (Malik and Roeder, 2000). Yeast Mediator subunits have been described to regulate distinct families of target genes of varying size and compositions (Holstege et al., 1998), while certain submodules are thought to constitute specialised targets for a subset of activators (Malik and Roeder, 2000).

For example, in yeast, C. elegans and Drosophila, mutations in Med6 are cell-lethal (Boube et al., 2002; Gim et al., 2001; Kwon and Lee, 2001) as well as Med17 in yeast and Drosophila (Boube et al., 2002) and Med21 in mouse (Tudor et al., 1999), all components of the Mediator head module (Figure 3), suggesting these subunits are essential for general functions of the Mediator complex. Nevertheless, Med6 is not required for integrity of the overall Mediator complex, nor for transcription of all genes in all tissues (Gim et al., 2001).

Mutations in yeast and metazoan Trap230/Med12 (hereafter called Trap230) and Trap240/Med13, (hereafter called Trap240) on the other hand, are cell-viable yet organism-lethal, due to their roles in environment-directed cell fate decisions and
development (Boube et al., 2002; Janody et al., 2003; Yoda et al., 2005). These subunits are named Trap (thyroid hormone receptor associated protein) after the specific Mediator complex in which they were identified, characterised by its binding ability to thyroid hormone receptors and other nuclear receptors (Yuan et al., 1998).

Notably, the *C. elegans* tail component Med14 is broadly required for transcription and phosphorylation of Serins 2 and 5 in the CTD, possibly indicating requirement at an early recruitment/initiation step (Shim et al., 2002), suggesting that the idea of the head module being most important in this respect may not exclusively hold true. Moreover, this subunit enhances ligand-dependent androgen receptor activity (Wang et al., 2002).

To date, little genetic evidence is available on the vertebrate Mediator complex. Mouse *Med1* mutants are viable and show specific defects in embryonic development, cell cycle regulation, cell survival, function of the pituitary-thyroid axis and a hypoplastic heart. The protein interacts with several nuclear hormone receptors. Additionally, fibroblasts derived from *Med1* mutants fail to differentiate into adipocytes in response to PPARγ2 (peroxisome proliferator-activated receptor gamma 2, a nuclear receptor essential for adipogenesis) (Ge et al., 2002; Ito et al., 2000).

*Med1* yeast and *C. elegans* mutants are also viable, and in *C. elegans* have a similar phenotype to those of *Trap230*, both being required for Wnt-dependently suppressing a Hox (homeobox transcription factor) gene (egl-5) in the postembryonic neuroectodermal lineage (Zhang and Emmons, 2001).

Knockdown of *Med15* is also not cell-lethal in yeast (Boube et al., 2002) and *Xenopus*, there leading to defects in Activin and Nodal, but not BMP, signaling through Smad transcription factors (Kato et al., 2002). Its specific function in signalling by these human tumour suppressors suggests it may itself be a tumour suppressor (Kato et al., 2002).

Concerning the metazoan-only-components, *Med24* mutant mice are embryonic lethal. They also lack the metazoan Med23 and conserved Med16 as well as having reduced amounts of Cdk8. The other three components of the kinase submodule are still present. This suggests a stabilising role for Med24 in the interaction of Cdk8
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with CycC, as well as the existence of a Med16/Med23/Med24 submodule (Ito et al., 2002), which, notably, contains both conserved and metazoan subunits. The residual complex shows unaltered binding to pol II and various activators. Med24, as Med1, interacts with nuclear receptors (Ito et al., 2002; Wang et al., 2002). Med24, Med1 and Trap240 have the same spatiotemporal expression pattern, being ubiquitous, but almost absent in small intestine and weak in pancreas and skeletal muscle (Ito et al., 2002).

1.4.1 Trap230 and the kinase subcomplex

The Mediator kinase subcomplex was identified as a part of the Mediator which is not present in all preparations. It consists of Trap230, Trap240, cyclin C and CDK8 (Myer and Young, 1998). Cyclins are a conserved family of proteins required for activation of a class of protein kinases termed CDKs (cyclin dependent kinases). Originally, cyclins were described as proteins whose abundance oscillated during the cell cycle (Evans et al., 1983). CycC was originally isolated in yeast assuming it to be a G1 cyclin, since its expression increases during G1 phase in the mammalian cell cycle (Lahue et al., 1991). It may be important for regulating cell proliferation, since it is expressed in response to growth factor stimulation and oscillates throughout the cell cycle (Liu et al., 1998b). However, it is not entirely clear whether this function is achieved independently of the Mediator complex or through it, by regulating the level of transcriptional activation. Trap230 and Trap240, the largest Mediator subunits, named after their molecular weight, seem to be required for the integrity of the subcomplex – loss of either one will abrogate function of the entire subcomplex, which is thought to be defined mainly via the cdk8 kinase function (Spahr et al., 2003).

In contrast to the Mediator core, this subunit has been implicated in transcriptional repression rather than activation. In both yeast and metazoans, a subset of genes has been found to be repressed by this subcomplex (Holstege et al., 1998; Samuelsen et al., 2003). This is a promoter-specific function, and thought to be mediated by phosphorylation of the CTD through cdk8 and cdk8-stimulated TFIIH before the binding of pol II to the transcription initiation machinery, inhibiting this (Bregman et al., 2000; Hengartner et al., 1998; Nair et al., 2005; Sun et al., 1998). This idea is
supported by the reduction of basal transcription levels in presence of the kinase submodule (Spahr et al., 2003). Nevertheless, CTD-independent repression through Mediator also occurs (Rachez and Freedman, 2001). Additionally, instances where the kinase subcomplex, or one of its members, may play an activating transcriptional role, have been described (Chang et al., 2001; Green and Johnson, 2004).

In addition to the CTD, CycC/Cdk8 in yeast phosphorylates other substrates within the initiation complex that could also influence transcriptional events. It can phosphorylate cycH, the cyclin partner of Cdk7, leading to inhibition of Cdk7 activity (Akoulitchev et al., 2000), subunits of the TFIID complex, Fal4 or Sip4, to increase the efficiency of inducing galactose respective glucose-responsive genes, transcription factors such as Gcn4 and Ste12, targeting them for ubiquitin-mediated degradation (Ansari et al., 2005; Chi et al., 2001), and other activators (Ansari et al., 2002; Baxter et al., 2004; Borggrefe et al., 2002; Boube et al., 2002; Hengartner et al., 1998; Lee et al., 2000; Song et al., 1998).

Consistent with the different interaction partners of Mediator copurified aside from the transcription machinery, cdk/cyclin complexes have been reported to connect transcription and RNA splicing, suggesting cdk8/cycC may be one of them (Loyer et al., 2005). Moreover, chromatin remodelling has also been attributed as a function to the human subcomplex (Cho et al., 1998).

Absence of the kinase subunit coincides with presence of the Med26 subunit in human cells, therefore distinguishing small and large Mediator (Naar et al., 2002; Taatjes et al., 2002; Wang et al., 2001). Absence of this subunit has been proposed as an alternative reason for the repressive function of large Mediator (Akoulitchev et al., 2000). However, since this subunit is metazoan-specific, this does not provide a sufficiently good explanation for the observations in yeast. The fact that small Mediator is still highly active in transcription confirms the idea that the kinase subcomplex is not required for Mediator function per se (Naar et al., 1999). Also, free Mediator was found to lack the kinase subcomplex (Myers et al., 1998) and large Mediator fails to bind the CTD (Naar et al., 2002). Moreover, different Cdk8-containing complexes have been separated in human cells, suggesting an even further modular composition of Mediator (Cho et al., 1998; Wilson et al., 1996).
Recruitment of Mediator to the yeast HO and Drosophila HSF promoters in absence of pol II supports the view that large Mediator may bind to activators, then, when pol II is recruited, the kinase submodule leaves (Figure 4). Lack of the submodule may have more profound effects on kinetics and timing of transcriptional activation at specific promoters, rather than on the overall levels of gene expression (Samuelsen et al., 2003).

Figure 4. Model of positive and negative regulation by yeast Mediator.

(Bjorklund and Gustafsson, 2005) (A) Mediator (blue) functions as a bridge between gene-specific activators (ACT; red) and the general pol II transcription machinery (purple) at the promoter. Activator interactions mainly take place within the tail region of Mediator, whereas contacts with pol II are localised to the head and middle region. A kinase subgroup of Mediator components – Srb8 (Med12), Srb9 (Med13), Srb10 (Cdk8) and Srb11 (CycC) – forms a module (pink) that is involved in negative regulation of transcription. (B) Only Mediator lacking the Srb8–11 module can associate with pol II. Gene-specific repressors (REPR; green) interact with specific Mediator subunits (blue) and recruit the complex to upstream regulatory DNA sequences. Mediator that is recruited by
repressors contains the Srb8–11 (Med12–Med13–Cdk8–CycC) module (pink), which prevents interactions with pol II and the basal transcription machinery (violet).

All members of the kinase subcomplex have been knocked down in yeast, and some also in metazoans. The general consensus is that they may perform very similar functions or all just aid Cdk8 kinase activity, since their phenotypes are said to be indistinguishable (Carlson, 1997; Hengartner et al., 1995). This is supposedly the case because loss of one subunit leads to disintegration of the whole subcomplex (Spahr et al., 2003).

Nevertheless, cyclin C and cdk8 have special functions in their roles as kinase and cyclin partners, also outside of Mediator, suggesting the situation may actually be more complex. Also Trap230 and Trap240, who have repeatedly been reported to share all functions and phenotypes, have been assigned differential functions, though this has mostly gone unnoticed. Some of these differences may be due to the analysis of only one of the two genes in a study, others, however, clearly mark them for distinction.

In yeast (*S. cerevisiae*), the components of the kinase subcomplex regulate genes involved in nutrient stress response, repressing genes required for gluconeogenic growth (Balciunas et al., 1999; Balciunas and Ronne, 1995; Holstege et al., 1998). The subcomplex itself is regulated by a stress response pathway under the influence of Ras, which by elevating intracellular cAMP levels activates a PKA homologue (Chang et al., 2004; Cooper et al., 1997; Woods et al., 1994). This PKA, which is inactive in presence of glucose, will mark the subcomplex for degradation, when a lack of nutrients occurs (Hengartner et al., 1998), by phosphorylation of Trap240 (Chang et al., 2004). Whether Trap230 is also phosphorylated, was not studied, but phosphorylation of Trap240 seems sufficient. Loss of kinase subcomplex function derepresses genes important for entry into stationary phase/G0 (Spahr et al., 2003). Therefore, mutants in these genes are also not cell-lethal (Boube et al., 2002). Another feature of these cells is then the formation of clumps (flocculation) in liquid culture, suggesting a change in cell affinities (Chang et al., 2004; Nair et al., 2005).

Some genes have been found to be regulated by both Cdk8 and and the head component Med18 (Holstege et al., 1998). Both Cdk8 and a HDAC1 (histone deacetylase 1) homologue in yeast mediate transcriptional repression by the Groucho
homologue Tup-1 (human TLE) through different mechanisms, either on their own or synergistically (Green and Johnson, 2004). Cdk8 plays a role in both Groucho-dependent and independent repression, and was also found to act as an activator in cases (Green and Johnson, 2004). Trap240 was also described to show an HDAC1-like phenotype, as well as an HMG box protein. Other than above, neither Cdk8 nor the other two members of the kinase subcomplex showed a similar function in this study (Larschan and Winston, 2005).

Trap240 plays a role in blocking transcript cleavage when pol II is arrested. For this, it interacts with the N-terminus of the elongation factor TFIIS on the outer surface of pol II (Kettenberger et al., 2003). This conserved N-terminus has significant homology to that of Med26 (Bourbon et al., 2004; Ryu et al., 1999). However, it is not clear, when an interaction between Med26 and Trap240 should take place, since they are mutually exclusive in Mediator. Trap240 was found to probably change the phosphorylation pattern of TFIIS in this context and interact with the SAGA coactivator (Wery et al., 2004). It is unclear whether this function is specific to Trap240 or whether Trap230 was just not mentioned.

Similar to *S. cerevisiae*, Trap230 and Trap240 mutants in *S. pombe* were described to show a nutrient limitation and flocculation phenotype, suggesting this may be a conserved specific process. Expression of only 10 genes was affected, especially those known to be involved in flocculation (Samuelsen et al., 2003).

In *Dictyostelium discoideum*, Trap230 and Trap240 were also found to affect a growth/differentiation transition in a cAMP-dependent signalling pathway upon nutrient-deprivation (Kon et al., 2000). A functional conservation from yeast to fly, involving specific effects on cell differentiation, notably in response to cell signalling pathways, has been suggested for Trap230 and Trap240 (Boube et al., 2002). *Dictyostelium* Cdk8 was found to be required specifically for spore differentiation (Lin et al., 2004).

*Trap230* and *Trap240* in *C. elegans* were found to have similar phenotypes and operate on common pathways. They are expressed ubiquitously and have maternal contribution, the protein localising to the nucleus. Yet, their loss does not increase all gene expression (Wang et al., 2004a; Zhang and Emmons, 2000). The functional interaction between the two is conserved from yeast to mammals, but the
subcomplex modulates metazoan-specific genetic pathways in addition to the ones in yeast. In vulval development, they relay signals downstream of Wnt, via β-catenin, to block activation of a homologue of the homeobox gene caudal through one of two known pathways (Zhang and Emmons, 2000; Zhang and Emmons, 2001). This results in a transcription factor activation cascade, from expression of a homologue of the Antennapedia homeobox gene (mab-5), to the Abdominal B homeobox gene (egl-5) and a homologue of the basic helix-loop-helix (bHLH) factor atonal (lin32) (Zhang and Emmons, 2000).

Trap230 and Trap240 are specifically required for the regulation of genes controlling asymmetric cell division in a complex with Med23 and Med6 (Yoda et al., 2005). In this function, they again repress a Hox gene (lin-39) through Wnt signalling via β-catenin. The canonical Wnt pathway shares some components with the Wnt pathway controlling polarity of cell division, suggesting that this pathway may be affected here. A different function of the Wnt/β-catenin pathway, the posterior migration of the QL neuroblast, is however not defective in Trap230/Trap240 mutants, indicating the tissue specificity of Trap230/Trap240 function (Yoda et al., 2005).

For Trap230, a Wnt-independent function in negative regulation of the RTK/Ras pathway by EGFR was identified. While the glutamine-rich C-terminus of Trap230 is important for β-catenin-dependent gene expression, it is dispensable for inhibition of Ras-dependent cell differentiation, therefore contributing to the specificity of protein (Moghal and Sternberg, 2003). The above lin-39 is a common target for Ras and Wnt signalling in C. elegans vulval development (Eisenmann et al., 1998; Maloof and Kenyon, 1998). Trap230 in one study is proposed to act largely on the Ras pathway rather than the Wnt pathway in this context (Moghal and Sternberg, 2003), consistent with its role in yeast an Dictyostelium. The metazoan-specific Med23 is also downstream of Ras, through the MAPK pathway, in C. elegans and human (Boyer et al., 1999; Singh and Han, 1995). Yet, its function is different from Trap230, suggesting that different Mediator components have selective effects in promoting different transcriptional regulatory signals, although Trap230 and Med23 interact to control asymmetric cell division (Zhang and Emmons, 2000). Also, it suggests that, unlike Med23, Trap230 may be activated through PKA
phosphorylation, as is the case for Trap240 in yeast. This agrees with a proposed ligand-independent activation of Trap230 (Zhang and Emmons, 2000).

EGFR function is inhibited by two pathways – by the Antennapedia homeobox gene mab-5 through Trap230/Trap240, and by HDAC complex components (Chen and Han, 2001; Lu and Horvitz, 1998; Solari and Ahringer, 2000). This is reminiscent of the situation in yeast, where HDAC1 and the kinase subcomplex have some common functions (Green and Johnson, 2004).

The intrinsic repressive function of C. elegans Trap240 has been located in one of four domains conserved to human, which affects specific genes (Wang et al., 2004a). Neither Trap230 nor Trap240 influence the phosphorylation at Serine 2 of the CTD (Wang et al., 2004a), indicating that a similarity to a Cdk8 phenotype may rather be due to a secondary loss of Cdk8 function.

The conserved C-terminus of Trap230 in yeast, Dictyostelium, C. elegans and Drosophila suggests its importance for their function, and was found to be essential for relaying Wnt downstream signals. Interestingly, strong and weak Trap230 loss-of-function mutants have opposite effects on mab-5 expression, which may suggest a tricky dose-dependent effect (Yoda et al., 2005).

As in C. elegans, Trap230 and Trap240 in Drosophila have similar effects, and, moreover, were shown to physically interact with one another, forming a submodule. They are required to regulate specific target genes, including those controlling cell affinity, possibly through Ci (Cubitus interruptus), although a direct interaction could not be shown (Janody, 2001). Mutants show misregulation of specific genes during imaginal disc development (Janody et al., 2003). Trap230/Trap240 do not seem to be required for cell proliferation but for differentiation, eg. of photoreceptors (Janody et al., 2004). Reminiscent of the strong and weak C. elegans Trap230 alleles, conflicting reports exist about the phenotypes elicited by different Trap240 alleles – some affect wing, eye and antenna, while others seem to be wing and leg-specific (Boube et al., 2000; Janody et al., 2003).

These discrepancies are further underlined, by Drosophila Trap230 and Trap240 alleles being divergingly described as members of the Trithorax or Polycomb groups of proteins, sometimes with opposing functions to each other (Bajusz et al., 2001;
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Gindhart and Kaufman, 1995; Gutierrez et al., 2003; Kennison and Tamkun, 1988; Papoulas et al., 1998). Mostly, however, they are grouped as trithorax type genes, which are defined as activators of homeotic genes (Janody et al., 2004; Kennison and Tamkun, 1988). Notably, this suggests they can have activating function, in agreement with reports from C. elegans, where they were found to both activate and repress diverse homeobox transcription factors (Yoda et al., 2005; Zhang and Emmons, 2000; Zhang and Emmons, 2001).

Trap230/Trap240 in Drosophila function as specific adaptors for signalling pathways including Notch and Wingless (Wnt). Supposedly, this effect is mediated by physical interaction with the transcriptional complexes regulated by these pathways, modulating the association of activating or repressing cofactors at downstream target genes (Carrera, 2003). Expression of Notch and Wingless themselves is normal (Janody, 2001). Interestingly, Cdk8/CycC in human cells have been shown to promote the degradation and turnover of the Notch ICD (intracellular domain) at target enhancers through phosphorylation (Fryer et al., 2004). The mutant eye phenotype observed is reminiscent of BMP overexpression, and both eye and wing discs show misregulation of some, but not all Hedgehog targets in both ways, indicating gene-specific functions (Janody et al., 2004). Interestingly, one of these targets is atonal, a bHLH TF which is also ectopically activated in C. elegans Trap230/Trap240 mutants as a downstream function of Wnt signalling (Zhang and Emmons, 2000).

Also in the eyes, Trap230 was found to be downstream of unpaired, a secreted ligand in the JAK/STAT pathway (Bach et al., 2003). Moreover, Trap230 has been reported to act downstream of the sevenless receptor tyrosine kinase in Drosophila, suppressing its Ras/RTK mutant phenotype (Maixner et al., 1998). Thus, Trap230/Trap240 have been implicated in a substantial number of signalling pathways, and it remains to be seen, which functions may be direct and specific, and which may result indirectly from crosstalk among the pathways.

Moreover, a cell-cycle regulated function for Trap230 downstream of cyclin E has been suggested (Brumby et al., 2002), in line with the proposed requirement of Trap230 for cell differentiation (Janody et al., 2004) and the implication of kinase subcomplex components in the mammalian cell cycle (Liu et al., 1998a; Liu et al.,
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CycC has been implicated in the vertebrate cell cycle, specifically an alternatively spliced smaller chick variant, which shows a pattern of cell cycle variation similar to cycB2 (Lew et al., 1991; Li et al., 1996; Liu et al., 1998b). CycC expression can be induced in response to serum or cytokine stimulation. The role in cell cycle regulation and mediating changes in cell adhesion in vertebrates has been demonstrated to be Cdk8-independent (Liu et al., 1998a) although the two are specific interaction partners for each other (Tassan et al., 1995).

Interestingly, this coincides with the cell adhesion phenotype observed in Drosophila Trap230/Trap240 mutants (Janody, 2001; Treisman, 2001) as well as the flocculation phenotypes in S. cerevisiae (Hengartner et al., 1998) and S. pombe (Samuelsen et al., 2003). This leaves the question open of whether it is a direct function of CycC for which an intact kinase subcomplex is needed, or whether it is an intrinsic function of another subcomplex component, eg. Trap240, indirectly attributed to CycC. The intriguing discrepancy is the flocculation phenotype observed also in yeast Cdk8 mutants, which, however, has been attributed to Trap240 phosphorylation (Chang et al., 2004) and the finding that the cell adhesion function of CycC is Cdk8-independent (Liu et al., 1998a).

Synergistic genetic interactions between Trap240 and Med17 have been observed in relaying the activity of a Hoxb5 homologue, indicating a shared function in specification of adult cell and segment identity within the Mediator complex (Boube et al., 2000). Med6 mutants show a significant reduction of Trap240 (Gim et al., 2001). However, Trap230 and Trap240, unlike Med6 and Med17 are not required for cell proliferation or survival (Boube et al., 2000; Gim et al., 2001; Treisman, 2001).

A differential function for Trap240 as opposed to Trap230 and Med17 has been reported concerning a strong interaction with tonally, a protein involved in posttranslational modification of transcriptional complexes (sumoylation), indicating that Trap240 may thus be modified, while the other two Mediator components are not ( Gutierrez et al., 2003). Moreover, a link between BEAF32A, with known chromatin insulator function, and Trap230, but not Trap240, has been described (Yamaguchi et al., 2001).

The repressive effect of Trap230/Trap240 could be mediated by preventing the Mediator complex from acting on certain enhancers while promoting its activity on
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others (Janody, 2001). These very large and highly conserved proteins are likely to present a large number of interaction surfaces or perhaps even exhibit enzymatic activities (Janody, 2001).

Interestingly, on the vertebrate side, while Trap240 is otherwise known to be ubiquitously expressed, its expression in newborn mice appears to be almost gone (Ito et al., 2002). The human protein contains two LXXLL sequences, potential nuclear receptor (NR) binding sites, suggesting a function in relaying nuclear hormone signals, as Med1 has (Ge et al., 2002; Ito et al., 1999; Ito et al., 2000). The phenotype of Med1 mutants has been described to resemble that of CycC in yeast (Balciunas et al., 1999) and Trap230 in C. elegans (Zhang and Emmons, 2001). It is negatively controlled by CycC-dependent phosphorylation (Balciunas et al., 1999; Bjorklund and Gustafsson, 2004). Med1 constitutes one of the core subunits to which the kinase module attaches, namely through Cdk8, which interacts also with Med4 and Med9. An additional interaction is that of Med21 with Cdk8 and CycC (Cho et al., 1998; Kang et al., 2001).

Recently, three new members of the kinase subcomplex have been identified in human cells (Sato et al., 2004), Med12L, Med13L and Cdk11, suggesting they may take the place of the canonical subunits under certain circumstances. Med13L was originally identified in human patients suffering from a congenital heart condition including looping defects. Subsequently, it was cloned from human and mouse and found to be rather ubiquitously expressed, but most strongly in heart and skeleton. This protein has conserved LXXLL putative NR binding sites (Musante et al., 2004).

Other interactions identified for the kinase subcomplex include a binding of Trap230 to the RTA transcription activator of Kaposi's sarcoma-associated herpesvirus (KSHV). This protein acts as a molecular switch for lytic reactivation of the virus and is essential in its life cycle. It also interacts with Med6, Med16, Med17, Med21 and Med30, but most strongly with the C-terminal domain of Trap230. The resulting complex includes the whole large Mediator and the RTA, as well as a chromatin remodelling complex (Gwack et al., 2003).

Most importantly for this work, the C-terminal domain of human Trap230 has also been found to interact with the transcription factor Sox9 (Zhou et al., 2002).
1.5  Sox9

The precise control of transcription is of central importance during the development of multicellular animals, as it leads to cell type-specific gene expression required for differentiation. Sox (Sry-type high-mobility group box) proteins are a large family of transcription factors implicated in the control of a variety of developmental processes not only in vertebrates, but also in *C. elegans* and *Drosophila* (Pevny and Lovell-Badge, 1997; Wegner, 1999). They often play roles in determining early cell fates and are suggested to act in a dose-dependent manner (Pevny and Lovell-Badge, 1997). They are characterised by the presence of a single HMG (high mobility group) box, a high affinity DNA-binding domain of 70-80 amino acids, which also contains a nuclear localisation signal (Wegner, 1999), and by their homology to SRY, the mammalian testis-determining factor (reviewed in (Pevny and Lovell-Badge, 1997; Wegner, 1999). Among HMG box proteins, DNA binding sequence specificity is unique to Sox and TCF/LEF (Laudet et al., 1993), to which they are closely related (Pevny and Lovell-Badge, 1997). Sox proteins show highly restricted tissue distribution (Ner, 1992).

They display properties of both classical transcription factors and architectural components of chromatin (Pevny and Lovell-Badge, 1997). Upon binding to the minor groove of DNA (unusually for a transcription factor) at enhancers distal to the basal promoter, they cause DNA to bend at an acute angle (Ferrari and Kosher, 2002; Giese et al., 1992).

Five groups of Sox proteins are distinguished, termed A-G (Wegner, 1999). Group E features a conserved region of 36 amino acids N-terminal of the HMG box and a transactivation domain at the far C terminus (Figure 5) (Wegner, 1999).

**Group E**

![Figure 5. Conserved regions within SoxE proteins.](Wegner, 1999) black HMG box, TA transactivation domain, percent conservation between vertebrate members of this group.
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It consists of three members, Sox8, Sox9 and Sox10. Sox8 is expressed in the neural crest and its derivatives, in the pituitary and gonads (Cheung and Briscoe, 2003; Wegner, 1999).

Sox10 is expressed in the premigratory and migrating neural crest, restricted to its derivatives of the glial lineage – early in the embryonic PNS (peripheral nervous system), and late in the embryonic CNS (central nervous system), pigment cells, enteric nervous system and Schwann cells (Barembaum and Bronner-Fraser, 2005; Wegner, 1999). Sox10 is found to undergo nucleocytoplasmic shuttling, for which Sox9 contains identical regulatory sequences. Thus, regulating the balance of nuclear import and export might provide a mechanism to regulate transcriptional activity of these proteins (Rehberg et al., 2002).

Sox8 and Sox10 expression patterns overlap and functional redundancy among Sox E genes in the neural crest has been suggested (Cheung and Briscoe, 2003).

Expression of Sox9 is already found maternally (Spokony et al., 2002). During embryogenesis, Sox9 is expressed in premigratory neural crest, prior to other markers (Cheung and Briscoe, 2003), in mesenchymal condensations and later on in chondrocytes, in the kidney, sex-dysmorphically first in the genital ridge of both male and female, later only in the adult testis, notochord and epithelial placodes (otic and nasal), tubular heart structures and the CNS (Kent et al., 1996; Morais da Silva et al., 1996; Ng et al., 1997; Wagner et al., 1994; Wegner, 1999; Zhao et al., 1997).

A Sox9 homologue in Drosophila has been identified, named Sox100B. It is also expressed male-specifically at the time of initial gonadal formation, as in vertebrates. This is a strikingly conserved pathway controlling gonad sexual dimorphism (DeFalco et al., 2003).

Due to the additional genome duplication at the base of the teleost radiation, (Amores et al., 1998; Meyer and Schartl, 1999; Postlethwait et al., 1998; Taylor et al., 2001; Van de Peer et al., 2002) followed by nonfunctionalisation, zebrafish retain duplicate orthologs of about 30% of tetrapod genes (Postlethwait et al., 2000). Subfunctionalisation may preserve duplicate genes (Force et al., 1999; Stoltzfus, 1999) and ancestral functions may assort to different duplicate copies. Therefore, zebrafish have two Sox9 co-orthologs: Sox9a and Sox9b (Chiang et al., 2001).
Functional analysis indicates that these two Sox9 orthologs have partitioned the various functions of the ancestral Sox9 gene, leaving each with a subset of the original functions (Yan et al., 2002; Yan et al., 2005). This subfunctionalisation appears to be achieved at the level of transcription, as Sox9a and Sox9b are expressed in largely complementary domains that together approximate the expression of Sox9 in tetrapods (Cresko et al., 2003; Spokony et al., 2002; Wright et al., 1995; Yan et al., 2002). A phylogenetic tree for Sox9-related genes (Sox E group) can be found in the appendix.

There are substantial differences among expression and function of Sox genes throughout vertebrates. In chick, for example, Sox4, L-Sox5 and Sox11 also occur in neural crest progenitors and derivatives, while this is not the case for zebrafish Sox11 and has not been studied for Sox4 and L-Sox5 in the fish. Concerning members of the SoxE group, Sox8 is not expressed in the zebrafish neural crest, in contrast to mouse and chick, and Sox9a and b do not appear to be expressed in the heart, other than in mouse and chick. Zebrafish Sox10 is also different in that it is not expressed in pharyngeal arches, yet in the pectoral fin bud and otic placode. Nevertheless, common domains of Sox9 expression are the neural crest progenitors and pharyngeal arch crest derivatives, and for Sox10 the neural crest progenitors, PNS, pigment cells and enteric ganglia, which zebrafish share with other vertebrates (Hong and Saint-Jeannet, 2005).

Sox9 plays an important role in sex determination. Interestingly, in zebrafish gonads, Sox9b is expressed in the ovary and Sox9a in the testes (Chiang et al., 2001). In mammals, only the testes express Sox9 (Morais da Silva et al., 1996).

Besides sex determination, for which Sox9 is thought to be the ancestral protein, rather than SRY (Wegner, 1999), Sox9 plays important roles in development of neural crest and cartilage/bone, as well as epidermal placode formation. Neural crest and epidermal placodes (ears, olfactory organs, lens, lateral line and some cranial ganglia) are vertebrate innovations.

Human patients with mutations in Sox9 suffer from campomelic dysplasia, a skeletal malformation condition concurrent with renal malformations, absence of olfactory bulbs, heart and lung defects, deafness, mental retardation and male XY sex reversal. This disease reflects the Sox9 expression pattern in mouse (Wegner, 1999).
Neural crest cells are induced at the interface between non-neural ectoderm and neural plate, with both tissues contributing to them (Liem et al., 1995; Selleck and Bronner-Fraser, 1995). From there, they delaminate and migrate along specific routes to many destinations in the vertebrate embryo. They differentiate into a wide variety of cell types, including neurons and glial cells of the PNS, pigment cells (melanocytes), smooth muscle of the heart, head cartilage and skeleton (Christiansen et al., 2000). Migratory routes and cell types formed vary with their rostrocaudal position of origin along the embryonic axis. The frontmost, cranial neural crest, contributes to facial cartilage and bone as well as neurons and all the glia of the cranial ganglia. The vagal crest, arising from the caudal hindbrain, contributes to the heart and, together with the sacral neural crest at the most caudal part of the embryo, forms the enteric nervous system of the gut. Trunk neural crest gives rise to sensory and sympathetic ganglia and adrenal medulla. Pigment cells throughout the body arise from virtually all levels of neural crest (Barembaum and Bronner-Fraser, 2005).

All members of the Sox E group are expressed in the neural crest (Cheung and Briscoe, 2003), though not equally in all organisms. SoxE proteins can initiate neural crest formation, but are not sufficient for delamination from the neural tube (Cheung and Briscoe, 2003).

Growth factor signalling is essential for neural crest induction. An interplay of BMPs in the non-neural ectoderm, BMP antagonists like noggin and chordin in the ectoderm (Spokony et al., 2002), fine-tuning of Delta/Notch signalling to inhibit neurogenesis (Gammill and Bronner-Fraser, 2003), and Wnt signalling inducing the expression of Fgfs is necessary (Barembaum and Bronner-Fraser, 2005).

Sox9 provides competence for neural crest cells to undergo an EMT (epithelial-mesenchymal transition), essential for the cells to migrate, and is required for trunk neural crest survival – in its absence, cells undergo apoptosis prior to or shortly after delamination (Cheung et al., 2005).

Moreover, Sox9 is essential for cartilage and bone formation. In this context, it directly activates the cartilage extracellular matrix proteins col2a1, col11a2 and aggrecan (Bi et al 1999, Lefebvre & de Crombrugghe 1998).
1.6 Pectoral fin development

Classically, limb development has been most studied in *Drosophila* for invertebrates, chick and mouse for vertebrates and amphibians for regeneration (Capdevila and Izpisua Belmonte, 2001). Lately, zebrafish (*Danio rerio*) pectoral fins (Sordino et al., 1995), being homologous to tetrapod forelimbs, have also been investigated as a complementary model system for vertebrate limb development. One of their main advantages is the possibility to use forward genetics in a vertebrate to identify new genes involved in the process (Neumann, 2002).

However, it must be taken into account that in contrast to chondrichthyans and sarcopterygians (to which the tetrapods belong), zebrafish lack the structural equivalent of the distalmost limb, the autopod (Sordino et al., 1995) and first develop larval pectoral fins first which later undergo morphogenesis into adult structures (Grandel and Schulte-Merker, 1998).

Fins and limbs develop from buds formed by lateral plate mesoderm (LPM). The anteroposterior location of these along the embryonic flank is designated by combined expression of certain *Hox* genes, induced by retinoic acid, Fgfs and TGFβs (Capdevila and Izpisua Belmonte, 2001) (Figure 6). Bud induction is initiated by
interactions between LPM and more medial axial tissues, namely kidney-forming intermediate mesoderm (IM) and possibly also somites (Capdevila and Izpisua Belmonte, 2001). This interaction is supposedly mediated by expression of Fgf8 and Wnts, with Wnt2b/β-catenin mediating an Fgf8/Fgf10 loop between mesoderm and overlying ectoderm (Figure 6). In hindlimb buds, Wnt8c plays the role of Wnt2b (Kawakami et al., 2001). Budding is achieved through differential cell proliferation, bud cells proliferating faster than those around them (Searls and Janners, 1971).

Depending on the expression of specific transcription factors (Pitx, Tbx, Hox genes), pectoral or pelvic fins will develop (Capdevila and Izpisua Belmonte, 2001).

![Figure 7. Molecular interactions that coordinate limb growth and patterning along the three limb axes.](image)

(Niswander, 2003) Proximal–distal (Pr–D) axis is under the control of fibroblast growth factors (Fgfs; blue) from the apical ectodermal ridge (AER), the anterior–posterior (A–P) axis is under the control of Sonic hedgehog (Shh; red) from the zone of polarising activity in the posterior mesenchyme, and the dorsal–ventral (D–V) axis is under the control of bone morphogenetic proteins (Bmps) and Engrailed1 (En1; both in pink) from the ventral ectoderm and Wnt7a (green) from the dorsal ectoderm.
In the bud, there are organising centres for each of the three-dimensional axes. These three axes cross-talk to each other: Proximodistal patterning and bud outgrowth are directed by the apical ectodermal ridge (AER, Fgfs), an epidermal thickening on the dorsoventral border of the distal bud tip (Figure 7). Anteroposterior patterning is controlled by the zone of polarising activity (ZPA, Shh), a region in the posterior bud mesenchyme (Figure 7). The dorsoventral axis is patterned by signals from the non-ridge ectoderm (Wnt7a, Bmp/En1) (Figure 7) (Capdevila and Izpisua Belmonte, 2001; Niswander, 2003). Especially important is the epithelial-mesenchymal interaction between AER and ZPA, which maintain each other’s activity (Capdevila and Izpisua Belmonte, 2001; Neumann et al., 1999; Ng et al., 1999; Niswander, 2003; Panman and Zeller, 2003).

Members of all major growth factor families are involved in this process: Fgfs are secreted by the LPM, IM and AER, Bmps and Wnts by the LPM and ectoderm, Hedgehogs by the ZPA (Capdevila and Izpisua Belmonte, 2001; Niswander, 2003).

Patterning and growth mainly proceed in a proximal-to-distal fashion, whereby there are two controversial models. Positional information along this axis could either be specified very early on in individual cells or cell groups (early specification model) or be acquired in a time-dependent manner by a clock-like mechanism (progress zone model) (Duboule, 2002; Dudley et al., 2002; Saunders, 2002; Sun et al., 2002; Tickle, 2003; Wolpert, 2002).

1.7 Aim of this thesis

A novel zebrafish mutant named trapped (tpd), carrying a recessive point mutation had been identified in a chemical mutagenesis screen performed in Tübingen in 2000/01 by its pectoral fin phenotype. Two alleles, \( tpd^{25870} \) and \( tpd^{24970} \), were identified, of which \( tpd^{25870} \) is the stronger one. Tpd mutants show a complex pleiotropic phenotype including reduced embryonic pectoral fins, defects in neural crest derived structures, cartilage and placode formation as well as defects in other embryonic structures.

The aim of this thesis was the identification of the mutated gene responsible for the \( tpd \) phenotype and an initial characterisation of the mutant, since it is too complex
for a fully detailed analysis during the course of this work. One focus was on the developmental defect of the pectoral fins, since this is a strong phenotype by which the mutation was originally identified. A second focus became a comparative analysis of the tpd phenotype with that of Sox9a/Sox9b double mutants and the role of the mutated Trap230/Med12 gene in mediating Sox9 activity.

Both tpd and Sox9a/Sox9b mutants strongly resemble each other and Sox9 activity is impaired in tpd embryos, indicating that Trap230, a component of the Mediator transcriptional cofactor complex, is directly required for the activity of Sox9. Finally, I show that Trap230 functions downstream of Tbx5 and upstream of Fgf24 during pectoral fin induction, indicating that it also participates in Sox9-independent signaling events. This is the first genetic analysis of Trap230 in a vertebrate species, and reveals that vertebrate Trap230 is remarkably specific in mediating Sox9 activity.
2 RESULTS
2.1 Mapping and cloning the zebrafish \textit{trapped} mutant

2.1.1 Rough mapping

In a large-scale genetic screen to identify genes required for zebrafish embryonic development (Habeck et al., 2002), a number of mutants with defects in pectoral fin development were isolated. Before mapping, complementation crosses were set up for mutant lines with similar phenotypes. This showed that the mutant lines \textit{tpd}\textsuperscript{24970} and \textit{tpd}\textsuperscript{25870} are allelic, \textit{tpd}\textsuperscript{24970} being the weaker allele. However, they complement the known pectoral fin mutants \textit{heartstings} (\textit{tbx5}, (Garrity et al., 2002), \textit{dackel} (\textit{ext2}, (Grandel et al., 2000; Lee et al., 2004), \textit{boxer} (\textit{extl3})(Lee et al., 2004; Trowe et al., 1996), \textit{neckless} (\textit{raldh2}, (Begemann et al., 2001; Grandel et al., 2002), and also \textit{hand} (\textit{hand2}, (Yelon et al., 2000), which are all phenotypically related.

For \textit{tpd}\textsuperscript{24970} and \textit{tpd}\textsuperscript{25870}, sufficient embryos from four pairs each could be collected to start rough-mapping them. This was done using SSLP (single sequence length polymorphism) markers according to (Geisler, 2002) using bulked segregant analysis. Potential linkages were identified.

According to both the complementation analysis and the fact that similar linkages were found for both, \textit{tpd}\textsuperscript{24970}/\textit{tpd}\textsuperscript{25870} would presumably be two alleles of the same gene. This novel mutant was named \textit{trapped} (\textit{tpd}). Besides the defects in pectoral fin outgrowth, \textit{tpd} also has problems in craniofacial development, pigmentation, ear and eye development as well as slightly U-shaped somites and defective axon guidance and brain formation. In addition, \textit{tpd} mutants have a heart edema and a curly-down body axis.

The phenotypic analysis was mostly pursued with \textit{tpd}\textsuperscript{25870} embryos, this being the more penetrant allele, while for mapping more embryos from \textit{tpd}\textsuperscript{24970} were used, since more pairs showed polymorphisms in the available markers.

In order to identify the molecular nature of \textit{tpd}, bulked segregant analysis (Michelmore et al., 1991) was used to map it to linkage group 14 (Figures 8 and 9), close to marker Z20214 at 60.6cM from the top.
2.1.2 Fine mapping

Fine mapping using CA-repeat based markers from the meiotic MGH panel (Knapik et al., 1996), localised the mutation in a region at 49.8 cM on linkage group 14 between markers Z53264 (0.35 cM away, 5 recombinations in 1288 meioses) and Z11725 (0.58 cM away, 7 recombinations in 1207 meioses) (Figure 9).

While Z53264 was identified at 24.7 Mb on chromosome 14 in the Zv4 version of the zebrafish genome (http://ensembl.org/Danio_rerio/), Z11725 was found at 25.8 Mb.
RESULTS

The low recombination frequency corresponded to an apparently incompatibly large genomic region - 1100kb correspond to 0.93cM between the two markers, while the average distance corresponding to 1cM is 625kb (Postlethwait et al., 1994), so the expected distance for 0.93cM would be about 580kb. This genomic region still contained sequencing gaps which suggested the region may actually be even larger. It was supposed that the recombination in this region may be suppressed due to proximity of the centromere (Johnson et al., 1996).

However, detailed analysis of the various gene predictions annotated within this region finally revealed that part of the region in question had been triplicated in the genome assembly (Figure 10). This was reported to the Sanger Centre and the mistake confirmed.

Figure 10. Annotated genomic region of linkage group 14 in Zv4 containing the putative mutation.

Assembled contigs in blue in the centre. Homology annotation for predicted protein sequences in yellow, for mRNAs in red. Red and green circles indicate predicted open reading frames homologous to the same set of genes/proteins, which occur in triplicate with different orientations and lengths.

Z7495 is another marker identified to lie on the same side of the mutation as Z11725, also at 49.8cM on the MGH mapping panel. However, it is not to be found in the genomic zebrafish sequence by either annotation or sequence alignment (BLAST search). Its exact distance from the mutation remained elusive due to its failure to be
polymorphic in most of the mapping fish pairs used. Referring back to an assembly of sequenced zebrafish BAC and PAC clones (http://vega.sanger.ac.uk/Danio_rerio/), it was possible to identify an assembly of BAC sequences between Z53264 and Z7495, which also contained the two triplicated genes annotated in the genome sequence between Z53264 and Z11725 (Figure 11). Z11725 was annotated to be further away from the mutation, the distance between Z11725 and Z53264 now being about 710kb (including two gaps), a much more plausible number.

Figure 11. Annotated region containing assembled BAC sequences between Z53264 and Z7495 in VEGA.

Assembled BACs in blue in the centre. The black circle indicates a gap in the assembly. Red and green circles indicate the same pair of predicted genes found in Figure 10. Note the pink circle indicating a homology annotation for a short C-terminus of a protein. The red cross indicates position of marker P1.

By making use of the zebrafish genomic sequence (http://ensembl.org/Danio_rerio/ and http://vega.sanger.ac.uk/Danio_rerio/) available for this interval, new SSLP markers based on CA and other simple sequence repeats were designed. For one of these markers, termed P1, no recombinants out of 2894 meioses with $tpd$ ($tpd^{24970}$) were obtained, indicating that it must lie very close to the $tpd$ mutation (Figure 11, red cross), but failing to indicate to which side of P1 it had to be.
Importantly, the region which must include the mutation still contained a sequence gap, right next to P1. In the worst case the gene containing the mutation could be present within this gap, which should therefore be closed.

A first hint to tackle this problem was found in form of a tiny bit of a protein homology annotated right next to the gap (Figure 11, pink circle). Looking back at the scrambled triplicated genome sequence, a gene prediction with the same homology annotation could be found, this time containing an almost full-length gene (Figure 12).

![Figure 12. Annotated genomic region of linkage group 14 in Zv4 containing the putative mutation.](image)

Red and green circles indicate a triplicated set of similarly annotated gene predictions. Note the pink circle indicating an almost complete open reading frame of the 3’ end found in Figure 11.

The zebrafish BAC fingerprinting contig (BAC-FPC) database (http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish/small.shtml) revealed a BAC (zK87D3) whose ends aligned nicely with the BACs at either side of the gap. Therefore a BAC to bridge it was found. However, the shotgunning and sequencing of this BAC had only just begun, and none of the BAC sequence appeared to correspond to any part of the predicted sequence of the gene in the gap. The estimated length of the BAC from the fingerprinting data was about 150kb (Mario Caccamo, Sanger Centre, personal communication). Considering that its overlap with
the flanking BACs (as estimated from the alignment of the zK87D3 BAC end sequences with them) is about 41kb, the gap should then be about 109kb long. The sequence of the predicted gene covered 61kb plus an estimated 2kb for the missing 5' end predicted from homology to human, so this gene appeared to make up about two thirds of the gap, which was a reasonably good coverage.

2.1.3 Cloning

Since none of the genes annotated in the region was an obvious candidate for causing the \textit{tpd} phenotype according to previously published data, we considered several candidate genes in the region close to P1 and cloned their cDNAs from both wild type and \textit{tpd} embryos. I also tested their expression pattern by in situ hybridisation on wild type and \textit{tpd} embryos.

While one of the candidates, a gene for a homologue of the multi-drug-resistance protein MRP5, showed a spatiotemporal expression pattern corresponding to the mutant phenotypes, no mutation in the cDNA sequence could be identified.

Another of these candidates, Trap230/Med12, was ubiquitously expressed. Although it was therefore not considered a prime candidate, a difference in cDNA sequence between wild type and mutants was found (Figure 13). This happens to be the predicted gene in the gap.

The open reading frame of the predicted cDNA is about 6kb long and was therefore amplified in several parts for ease of cloning and sequencing (primers used cf. Materials and Methods). Firstly, polymorphisms and possible PCR artefacts had to be excluded as misinterpreted point mutations. Therefore, cDNA from several individual preparations was used, and several PCR reactions and individually picked clones were compared. Wild type sequences were aligned with sequences of both alleles. This excluded a large number of possible point mutations.

From the mapping data, the mutation in \textit{tpd}^{25870} was expected to lie further 5' in the affected gene than the mutation in \textit{tpd}^{24970} (Z53264 was unfortunately not polymorphic in any \textit{tpd}^{25870} fish, but Z11725 – which is downstream of Trap230 - had one recombinant in 154 meioses for \textit{tpd}^{25870}, corresponding to 0.65cM and 6 recombinants in 1053 meioses for \textit{tpd}^{24970}, corresponding to 0.57cM).
For \(tpd^{24970}\), several cDNA variants were found with various deletions within a range of three exons in the 3' end of the gene. None of them corresponded to the use of cryptic splice sites or to otherwise obvious specific point mutations. This allele also being the weaker one, it was decided to abandon its detailed analysis and concentrate on \(tpd^{25870}\).

As two different forms of cDNA were found in \(tpd^{25870}\) that could both be explained by aberrant splicing at the exon 12/intron 12 junction, the genomic locus of \(tpd^{25870}\) was also sequenced. The splice donor site of intron 12 was found to be altered from CAgtgag to CAgcgag (capitalized nucleotides belong to exon 12; Figure 13).

**Figure 13. Molecular characterisation of the tpd^{25870} locus.**

(A) \(tpd^{25870}\) maps to linkage group (LG) 14, between markers Z53264 and Z11725, close to marker P1. Two defectively spliced transcripts are generated as a result of this mutation. cDNA1 is spliced at a cryptic splice site (shaded in yellow) within exon 12. This leads to a frame shift and a stop codon after 93 amino acids. cDNA2 contains unspliced intron 12, leading to a stop codon after 6 amino acids. L: leucine-rich domain; LS leucine-and-serine-rich domain; PQL: proline-, glutamine-, and leucine-rich domain; OPA: glutamine-rich domain.

This mutation changes the highly conserved GU found at the intronic 5' splice site to GC, and results in two types of aberrant splicing in \(tpd^{25870}\) mutants: In type 1 cDNAs, a cryptic splice site (CTgtgct) located 7 bases 5' of the correct splice site is used, resulting in a frame shift and a stop codon 93 amino acids downstream of this point (Figure 13). In type 2 cDNAs, intron 12 fails to be spliced at all, and its translation generates a stop codon 6 amino acids downstream of this point (Figure...
13). In both cases, the $tpd^{25870}$ mutation results in a severely truncated Trap230 protein, in which more than half the C-terminus is missing (Figure 13). Nevertheless, this $tpd$ allele may be hypomorphic, as it shows some variability in its phenotype (Figure 13).

Several attempts were made at cloning the missing 5' end of the gene, using 5' RACE (rapid amplification of cDNA ends) and degenerate PCR methods making use of a putative conservation of the ATG region as defined by comparison of the sequences of *Homo sapiens* and *Fugu rubripes*. Nevertheless, the sequence of an estimated three exons and 142 amino acids at the 5' end remains to be ultimately identified. The full-length wild type and predicted sequences can be found aligned in the appendix.

Taking advantage of the fact that the point mutation of $tpd^{25870}$ lies within the recognition site of the restriction enzyme Est1, this enzyme was used to confirm the mutation and identify further carriers with the point mutation as SNP (single nucleotide polymorphism). A band of 282bp comprising the exon12/intron12 boundary was PCR-amplified from genomic DNA of individual $tpd^{25870}$ mutant and sibling embryos and then cut with Est1. While two bands are visible for the $tpd^{25870}$ embryos, corresponding to 25% of the batch, one third of the wild type siblings has one band (being homozygous wild type embryos) and two thirds have three bands, showing one cut (mutant) and one uncut (wild type) allele and therefore corresponding to heterozygous embryos (Figure 14).

![Figure 14. The mutation in $tpd^{25870}$ as a SNP.](image)

Restriction enzyme Est1 cuts genomic $tpd^{25870}$ but not wild type DNA due to the point mutation in its recognition site. *het*: DNA from a heterozygous embryo, *WT*: wild type, *tpd*: $tpd^{25870}$.

### 2.1.4 Expression of Trap230

The $trap^{230}$ transcript is expressed ubiquitously throughout the embryo, and is also deposited maternally (Figure 15). This is in agreement with the findings in other
organisms (Janody, 2001; Treisman, 2001; Wang et al., 2004a; Zhang and Emmons, 2000). The sequence used for the in situ probe comprised the 3’ end of the gene. No change in the expression was found in $tpd^{25870}$ embryos compared to wild type. Hereafter, $tpd$ will be used to indicate the $tpd^{25870}$ mutant allele, since phenotypic characterisation was carried out with this allele.

**Figure 15. Trap230 RNA expression.**
(A) 4-cell-stage, animal pole to the top. (B) 3-somite stage, lateral view, dorsal to the top, anterior to the left.

### 2.1.5 Phenotype of the embryo

The $tpd$ mutants were originally identified for their defect in pectoral fin development (Figure 16).

**Figure 16. The $tpd$ pectoral fin phenotype.**
Methylene blue-stained (Humphrey and Pittman, 1974) transverse sections at the level of the pectoral fin buds (arrows and asterisks) through 3dpf wild type (WT) and $tpd$ embryos showing absence of buds in the mutant.

**Figure 17. The overall $tpd$ phenotype.**
Wild type and $tpd$ embryos at 3dpf.
Moreover, defects are apparent in craniofacial development, pigmentation, ear and eye development, and it shows slightly U-shaped somites, defective axon guidance and brain formation. In addition, *tpd* mutants have a heart edema and a curly-down body axis (Figure 17).

### 2.1.6 Phenocopy by morpholino injections

To further investigate the possibility that *tpd* disrupts Trap230, a morpholino oligonucleotide was designed to block splicing of the *trap230* transcript at the exon 26/intron 26 splice junction (Figure 18). It is not targeted against the exon-intron boundary containing the actual mutation because this experiment preceded the cloning of the point mutation.

**Figure 18.** The Trap230-morpholino targets the exon 26/intron 26 splice junction.

Partial genomic sequence of zebrafish Trap230 as predicted in the Zv4 genome annotation. Sequences of exon 26 and start of exon 27 in red, intron 26 in black. The sequence targeted by the Trap230-morpholino is shaded in yellow.

It was found that injection of this morpholino into fertilised 1-cell stage zebrafish embryos generates a phenocopy of *tpd* mutants, including absence of pectoral fins, identical ear, pigmentation and craniofacial defects, heart, brain and somite phenotype and a curly-down body axis (Figure 19).

**Figure 19.** Trap230 morphant at 3dpf.

To confirm the functionality of the Trap230 morpholino, an RT-PCR product was amplified from exons 26 and 27 of both wild type and morpholino-injected embryos.
RESULTS

Morpholino-injected embryos lack this product, since intron 26 fails to be spliced out (Figure 20).

![Figure 20. Trap230 MO effectively targets splicing of the exon 26/intron 26 splice junction. Trap230 morphants lack the RT-PCR product amplified from exons 26 and 27, since intron 26 fails to be spliced out.]

Taken together, these data show that the *tpd* phenotype is due to disruption of zebrafish Trap230 function.

2.2 Trap230 and Sox9

2.2.1 The Trap230 loss-of-function phenotype resembles loss of Sox9 activity in many different tissues

Since several of the phenotypes observed in *tpd* mutants affect cell types known to depend on Sox9 function, the characterised phenotypes of zebrafish *Sox9a/Sox9b* single and double mutants (Yan et al., 2005) were systematically compared to the phenotypes present in *tpd* embryos. In *Sox9a/Sox9b* double mutants, the ear fails to form, while it is only partially reduced in *Sox9a* or *Sox9b* single mutants. One of the prominent functions of Sox9 is its requirement for the development of epidermal placodes, like the otic placode (Saint-Germain et al., 2004; Yan et al., 2005). We find the ear is variably reduced in *tpd* mutants (Figure 21), similar to *Sox9b* mutants, showing an overall size reduction and smaller otoliths of equal size instead of one large and one small otolith (Figure 21 A). Additionally, some embryos show three otoliths instead of two, a phenotype so far only described for zebrafish *runx1* mutants (Kalev-Zylinska et al., 2002), a gene otherwise implicated in hematopoiesis.
RESULTS

Figure 21. Ear phenotype of tpd mutants.
Ears of WT (A) and tpd (B and C) embryos at 4dpf. Note partial reduction of the overall ear, smaller otoliths and irregular number in tpd.

In Trap230 morphants, however, the ear is either partially reduced or completely absent (Figure 22), similar to Sox9a/Sox9b double mutants, suggesting the morphant gives a stronger reduction of Trap230 function than the tpd mutation, and further indicating that \( tpd^{25870} \) is a hypomorphic mutation.

Figure 22. Ear phenotype of Trap230 morphants at 4dpf.
(A) reduced size and absence of otoliths. (B) absent ear.

Sox9 activity is also important for specification of neural crest cells. Pigment cells derive from the neural crest – in zebrafish three types are known: melanophores, xanthophores and iridophores. In Sox9b or Sox9a/Sox9b double mutants, there is an absence of iridophores, which can be detected as bright, shiny cells in darkfield microscopy. As in Sox9b single mutants or Sox9a/Sox9b double mutants, no iridophores are detected in tpd mutants (Figure 23).

Figure 23. tpd iridophore phenotype.
Dark field microscopy, revealing iridophores (bright shiny cells), present in WT, but not in tpd mutants at 4dpf in dorsal view.
The decision to form either pigment cells or neural derivatives is supposedly made early during migration. In zebrafish, medially located cranial neural crest cells are induced by local Wnt1 and Wnt3a signals to form pigment cells, whereas lateral cells that are further from the Wnt signals form neurons (Dorsky et al., 1998).

This is mediated through Tcf/Lef1 directly activating *nacre/mitfa*, which is essential for melanophore differentiation (Dorsky et al., 2000; Lister et al., 1999) and was reported to be normally expressed in *sox9a/sox9b* double mutants (Yan et al., 2005).

*Sox9* mutants have largely unperturbed pigment cells, since *Sox9* is primarily involved in cranial neural crest development and pigment cells derive from neural crest all along the embryonic crest (Spokony et al., 2002).

Therefore, melanocytes are present in *Sox9a/Sox9b* mutants, but are larger than in wild types, due to dispersed melanosomes even in bright light (Yan et al., 2005). Melanocytes are similarly enlarged in *tpd* mutants and in Trap230 morphants (Figure 24). This is a physiological rather than developmental consequence because both *Sox9a/Sox9b* and *tpd* mutants have eye defects and are presumably blind, so that light cannot be detected to elicit a light-dependent response of the melanophores.

Formation of xanthophores, the third pigment phenotype, is not impaired in either *Sox9* or *tpd* mutants (Yan et al., 2005) (not shown).

![Figure 24. Trap230 loss-of-function melanophore phenotype.](image)

Trunk melanocytes (dark cells, arrows) in wild type embryos contract under influence of light, but not in *tpd* mutants and Trap230 morphants.

*Sox9b* mutants fail to straighten the body axis, resulting in a curly-down tail, probably due to *Sox9b* function in the notochord (Yan et al., 2005). A similar curly-down body axis is observed in *tpd* mutants (90% of embryos in *tpd<sup>25870</sup>*, n=272) and Trap230 morphants (Figures 17 and 19).

Taken together, these results show that Trap230 loss-of-function strongly resembles zebrafish *Sox9a/Sox9b* mutants with respect to ear, pigmentation, and body axis development.
2.2.2 Trap230 is required for Sox9-dependent neural crest fates and craniofacial cartilage formation

To further investigate the role of Trap230 in Sox9 signaling, an analysis of neural crest and cartilage-related phenotypes was performed. Craniofacial cartilage derives from head neural crest. Therefore, the expression of col2a1 was examined, which is expressed during the differentiation of craniofacial cartilage elements, and known as a direct target of Sox9 binding and activation (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhou et al., 1998). In Sox9b and Sox9a/Sox9b double mutants, col2a1 expression is strongly reduced, although not completely absent, in the pharyngeal arches. It is also present in reduced amounts in the eye capsule and otic vesicle (Yan et al., 2005). A similar reduction of col2a1 in these expression domains is found in tpd embryos (Figure 25).

While either Sox9a or Sox9b single mutants show partial absence of craniofacial cartilages, the Sox9a/Sox9b double mutant animals lack all traces of pharyngeal cartilages and the neurocranium (Yan et al., 2005). Likewise, tpd mutants have a complete absence of both the pharyngeal cartilages and the neurocranium, as well as total absence of the pectoral fin skeleton (Figure 26). In contrast to Sox9a/Sox9b double mutants, tpd mutants also lack the entire shoulder girdle including a cleithrum, hinting to a further function of Trap230.
The earliest known response to neural crest induction through Sox9 is expression of slug/snail zinc finger transcription factors (Nieto et al., 1994; Sefton et al., 1998). Snail-mediated repression is an important mechanism for the induction of an EMT and hence for initiating neural crest migration. This is thought to occur through targeting of cadherins (Christiansen et al., 2000). Loss of Sox9 impairs expression of slug/snail as well as foxd3, sox10 and other transcription factors in premigratory neural crest dose-dependently (Bronner-Fraser, 1986; Cheng et al., 2000; Nakagawa and Takeichi, 1998; Spokony et al., 2002).

In zebrafish premigratory cephalic and trunk neural crest cells of Sox9b single mutants or in Sox9a/Sox9b double mutants, expression of snail1b is reduced, as are foxd3 and sox10. This can also be seen in tpd mutants (Figure 27).

Figure 27. Expression of neural crest markers at 3 somites, dorsal view.

Neural crest cells originating from the hindbrain express dlx2a during their migration into the visceral arch primordia (Akimenko et al., 1994; Ellies et al., 1997). In Sox9b and Sox9a/Sox9b double mutants, expression of the migratory neural crest marker dlx2a is only slightly reduced, with the strongest reduction in the posterior branchial arches, indicating that the cells are specified normally and also start to migrate (Yan et al., 2005).

Figure 28. Expression of dlx2a at 24hpf in the pharyngeal arches, dorsal view.

In tpd mutants, there is a similar reduction of dlx2a, which is also more pronounced in the posterior arches (Figure 28). Trap230, as Sox9 function, is not essential for
cranial crest migration, but Sox9b and Trap230 may help determine the size of the dlx2a-expressing cell population (Yan et al., 2005).

Both in mouse Sox9 mutants and in zebrafish Sox9b and Sox9a/Sox9b double mutants, neural crest cells are induced, but then undergo apoptosis, indicating that Sox9 acts as a survival factor (Cheung et al., 2005; Yan et al., 2005). A similar increase of apoptotic cells was detected by TUNEL labeling in tpd mutants (Figure 29).

![Figure 29. Apoptosis at 4dpf.](image)

TUNEL staining reveals apoptotic neural crest cells in tpd but not wild type embryos.

An important derivative of trunk neural crest are the neurons and glia found in dorsal root ganglia (DRG), and in mouse Sox9 mutants, these cell types are strongly reduced (Cheung et al., 2005). Similarly, no Hu-positive DRG neurons could be detected in Trap230 morphants, Hu being a neuron-specific RNA-binding protein in vertebrates (Marusich et al., 1994) (Figure 30).

![Figure 30. Dorsal root ganglion neurons at 3dpf (arrows).](image)

Dorsal root ganglion neurons detected by Hu and Islet1 (Isl1) antibodies at 3dpf are absent in tpd embryos. Note the circular structure of the ganglia stained with Islet1.
Staining with Islet-1 (Isl1) as another neuronal marker (Cheung et al., 2005) was similarly reduced in DRGs (Figure 30).

Altogether, these results show that Trap230 activity is crucial for Sox9-dependent neural crest development and cartilage differentiation, and that many of the defects found in *tpd* embryos are identical to those found in Sox9 mutants. While some of the phenotypes can be explained by loss of Sox9b activity only, others like the ear and cartilage phenotypes clearly indicate that functions depending on both Sox9a and Sox9b synergistically are impaired in *tpd*.

### 2.2.3 The role of Trap230 in Sox9-dependent ear development

The vertebrate inner ear develops from a thickening of the embryonic ectoderm, adjacent to the hindbrain, known as the otic placode. Otic Sox9 expression, initiated in the prospective otic placode, a bilateral cell patch adjacent to the cranial neural crest shortly after gastrulation by Fgf and Wnt signalling (Pfeffer et al., 1998; Saint-Germain et al., 2004), is required for specification of the otic placode. Patients suffering of campomelic dysplasia due to mutations in Sox9 are often deaf (Saint-Germain et al., 2004).

Sox9 is one of the earliest genes expressed in the otic placode, and knockdown of Sox9 activity in *Xenopus* leads to complete absence of the ear (Saint-Germain et al., 2004). *pax8* is also expressed very early in the otic placode, and *pax8* expression fails to be activated following Sox9 knockdown (Saint-Germain et al., 2004). Similarly, no *pax8* expression is detected in the otic placode of Trap230 morphants (Figure 31 E and F), even though *Sox9a* and *Sox9b* expression are present in the presumptive otic placode of these morphants, although at partially reduced levels (Figure 31 A-D).
**RESULTS**

Figure 31. Expression of *sox9a, sox9b* and *pax8* in the otic placode of Trap230 morphants.

Expression of *sox9a* (A, B) and *sox9b* (C, D) in the otic preplacode region of wild type (A, C) and Trap230 morphant (B, D) embryos at 3 somites. Expression of *pax8* (E, F) in wildtype and Trap230 morphant embryos at tailbud stage. Arrows indicate expression domain.

Figure 32. Expression of *pax2.1* in the otic placode at 24hpf in wild type and Trap230 morphant embryos.

In the majority of Trap230 morphants, there is a partial recovery of ear development later on, leading to a reduced number of cells expressing *pax2.1* compared to wild type siblings at 24hpf (Figure 32).

This partial recovery of morphants is likely due to incomplete blockage of *trap230* splicing, since a small number of morphants do not have any signs of ear development (cf. Figure 22 B).

These results show that Trap230 is required for Sox9-dependent activation of *pax8* in the ear and for Sox9-dependent ear development, and further indicate that Trap230 is required for Sox9 activity.
2.3 Trap230 is required for Sox9 activity

As *tpd* mutants are very similar to *Sox9a/Sox9b* mutants, one can hypothesise that this could potentially reflect two different modes of regulatory interaction between Trap230 and Sox9. Since Trap230 is a transcriptional regulator, it might be required for expression of *Sox9a* and *Sox9b*. Alternatively, it might participate together with Sox9 proteins in regulating Sox9 targets.

2.3.1 Sox9 expression in *tpd* is mostly normal

To distinguish between these possibilities, first the expression of *Sox9a* and *Sox9b* mRNA in the absence of Trap230 activity were examined. *Sox9a* expression was found to be largely normal in *tpd* mutants compared to wild type siblings, both at 10hpf and at 24hpf (Figure 33 A-D).

![Figure 33. Expression of *sox9a* and *sox9b* is mostly normal in Trap230 loss-of-function embryos.](image)

Expression of *sox9a* (A-D, I, J) in wild type and *tpd* embryos at tailbud stage (animal pole to the top) and 24hpf (lateral view). Expression of *sox9b* (E-H, K, L) in wild type an Trap230 morphant embryos at 3 somites (dorsal view) and 24hpf (lateral view). Expression of *sox9a* and *sox9b* in the pectoral fin buds is lost in *tpd* or Trap230 morphant embryos at 24hpf (I-L, dorsal view). Arrows indicate fin bud expression, asterisks its absence.
The exceptions to this are the pectoral fin buds, in which Sox9a expression fails to be activated (Figure 33 C, D, I, J), and the presumptive otic placodes, in which Sox9a expression is activated, but at reduced levels compared to wild type siblings (Figure 31 A and B). Sox9b expression is also largely normal in Trap230 morphants (Figure 33 E-H), although there is a weak reduction in the neural crest region at 3 somites (Figures 31 C, D and 33 E, F) and a loss of pectoral fin bud expression at 24hpf (Figure 33 G, H, K, L). The weak reduction of Sox9a in the otic placode region and Sox9b in neural crest might be due to the fact that Sox9a and Sox9b activate each other's expression (Yan et al., 2005), and thus reflects a stimulatory effect of Sox9 activity on its own transcription in these cells.

2.3.2 Sox9 overexpression cannot rescue tpd mutants

Since failure to activate Sox9a or Sox9b expression does not appear to be the cause of the tpd phenotype, the ability of Sox9b mRNA to trigger downstream target gene activation in the absence of Trap230 activity was examined next. Since snail1b expression is reduced in Sox9b mutants, but is upregulated and/or its expression expanded rostrally in wild type embryos injected with Sox9b mRNA injection (Yan et al., 2005), Sox9b mRNA was injected into 1-cell stage wild type and tpd embryos, and its ability to up-regulate snail1b transcription in the presence or absence of Trap230 activity compared. While Sox9b mRNA injection leads to strong upregulation of snail1b expression in wild type embryos, no effect was detected on tpd embryos, which still show reduced snail1b expression (Figure 34).

Similar results were obtained with foxd3 and Sox10, which also fail to respond to Sox9b overexpression in the absence of Trap230, although the effect observed is not as strong (Figure 34). Interestingly, the overexpression of Sox9b mRNA seems to lead to an expansion of neural plate tissue (between the two neural crest expression domains). However, such a function has so far not been described for Sox9 and the presumptively expanded tissue of Sox9b-mRNA-injected embryos in this study has not yet been analysed for expression of neural plate markers to confirm this observation.
RESULTS

Figure 34. Overexpression of sox9b-mRNA fails to rescue neural crest marker expression in tpd embryos.

Expression of snai1b, foxD3 and sox10 is strongly activated in wild type embryos injected with sox9b-mRNA, but not in sox9b-mRNA-injected tpd embryos. Expression of these markers is reduced in tpd embryos. Embryos at 3 somites, dorsal view. Note concomitant expansion of neural plate in sox9b-mRNA injected embryos.

Taken together, these results show that Trap230 activity is not necessary for transcription of Sox9 genes in most tissues, including many tissues showing a Sox9 loss-of-function phenotype in tpd embryos. Instead, Trap230 is required for Sox9 activity and for activation of Sox9 downstream targets. Importantly, this study not only confirms the data from mammalian chondrocytes, which indicated that a direct interaction between Trap230 and Sox9 may be required for Sox9 function (Zhou et al., 2002), but extends this requirement to activity of Sox9 in a large variety of tissues and functional contexts, moreover in a vertebrate in vivo system, showing genetic epistasis.

2.4 A Sox9-independent role of Trap230 in forelimb development

Since tpd mutants show a complete absence of pectoral fins in the most strongly affected mutants (Figure 16), it was interesting to determine how Trap230 fits into
the cascade of genes involved in limb induction. It is well established that both *tbx5* mutants (Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002) and *fgf24* mutants (Fischer et al., 2003) show a similar absence of pectoral fins. Therefore the expression of these two genes, as well as other genes known to be regulated by them, in *tpd* mutants were examined.

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**Figure 35. The pectoral fin phenotype of tpd mutants.**


*tbx5* expression (Tamura et al., 1999) is activated in *tpd* mutants, but *tbx5*-expressing cells fail to congregate towards the fin bud and stay dispersed in the lateral plate mesoderm (Figure 35 B, F), as in both *tbx5* and *fgf24* mutants (Ahn et al., 2002; Fischer et al., 2003; Garrity et al., 2002). *fgf24* expression, in contrast, fails to be activated at all in *tpd* mutants (Figure 35 C, G). Consistent with this result, Fgf24- and Tbx5-dependent expression of *dlx2a* in the fin bud ectoderm (Akimenko et al.,
1994; Fischer et al., 2003), and of shh in the fin bud mesenchyme (Ahn et al., 2002; Fischer et al., 2003; Krauss et al., 1993; Roelink et al., 1994), are not detectable in tpd mutants (Figure 35 I, J, M, N). Similarly, pectoral fin activation of erm1, pea3 (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001), and msx-c (Akimenko et al., 1995) fails to occur, or is strongly reduced, in tpd mutants (Figure 35 D, H, K, L, O, P). Since these three genes are normally activated in the absence of fgf24 activity (Fischer et al., 2003), these results indicate that Trap230 functions upstream of Fgf24 activation, but downstream of Tbx5 during limb development. This is further confirmed by the absence of a cleithrum, a feature of tbx5 but not fgf24 loss-of-function (Fischer et al., 2003; Garrity et al., 2002). Consistent with this proposal, wnt2b, which in zebrafish functions upstream of Tbx5 (Ng et al., 2002), is expressed normally in tpd mutants (Figure 35 A, E).

While Sox9 activity is important for development of the fin cartilage elements, the initial induction, patterning, and outgrowth of pectoral fin buds is normal in Sox9a/Sox9b double mutants (Yan et al., 2005), indicating that the tpd fin phenotype cannot be attributed to loss of Sox9 activity, and that Trap230 regulates a Sox9-independent mechanism in forelimb development.

### 2.5 Other Phenotypes

The tpd mutant has a number of interesting phenotypes, as would be expected from a component of the Mediator complex. However, a mutation in Trap230 is not immediately lethal in zebrafish, similar to what has been found in yeast, C. elegans and Drosophila. Homozygotes survive up to one week and many structures develop rather normally considering and show surprisingly specific defects.

For completeness and with regard to possible involvement of the Sox9 pathway or genes/functions affected in development of the pectoral fin, the other phenotypes displayed by tpd mutants shall be reported here. They have not been analysed in detail yet, but may raise some interesting aspects.

Beside the described neural crest, cartilage, otic placode, curled body and pectoral fin phenotypes, tpd mutants have defects in brain, eye formation and axon guidance defects as well as heart problems, slightly U-shaped somites and edemae at the
colon/cloaca. Whole mount in situ hybridisation studies also revealed an overall reduction in the expression of several fibroblast growth factors (Fgfs).

2.5.1 Brain phenotype

The earliest phenotype by which tpd mutants can easily be distinguished while alive (from around 28hpf), is when midbrain-hindbrain boundary (MHB) formation is retarded (Figure 36 A, B).

![Figure 36. Defective formation of the MHB in tpd embryos.](image)

(A, B) Lateral view of embryos at 28hpf. Arrow indicates MHB, note impaired formation in tpd embryos. (C-F) Expression of fgf3 in the MHB is reduced in tpd embryos at 24hpf (C, D lateral views, E, F dorsoposterior views).

Concomittantly, expression of fgf3 in the MHB (Raible and Brand, 2001) is strongly reduced in tpd embryos at 24hpf (Figure 36 C-F).

The forebrain also appears to be smaller (cf. Figures 17, 19, 36, 37) and the midbrain ventricle fails to inflate (Figure 37).

![Figure 37. Midbrain ventricle inflation.](image)

Brackets indicate size of the midbrain ventricle at 24hpf. Note failure to inflate and reduced size in tpd embryos (right panel, dorsal views).
Together with the lack of cartilage, this leads to an overall smaller head. An almost complete lack of touch response and hence hatching problems suggest either further neural disorders or muscular problems (cf. 2.5.4).

2.5.2 **Eye and axon guidance phenotypes**

The second most prominent phenotype visible in *tpd* embryos beside the pectoral fin defect is a defect in eye formation. As well as having slightly fused eyes (cyclopia), they are reduced in size and misshapen (Figure 38). Moreover, methylene blue staining of transverse cryosections reveals that lamination of the *tpd* retina is not entirely intact, i.e. the different layers cannot be distinguished properly and a reduction of the lens is visible (Figure 38 B, C).

![Figure 38. Eye phenotype of *tpd* embryos.](image)

(A) The eyes of *tpd* embryos at 4dpf (bottom) are smaller than in wild type embryos (top) and misshapen. Embryos are also slightly cyclopic. (B, C) Methylene blue stained transverse sections across eyes of embryos at 3dpf. Note the shape of the *tpd* eye, small lens and problems with proper lamination. GCL: ganglion cell layer, INL: inner nuclear layer, IPL: inner plexiform layer, L: lens, ONL: outer nuclear layer, OPL: outer plexiform layer, RPE: retinal pigmented epithelium.

Seven major cell types compose the zebrafish retina. They are arranged in three major layers of neural retina, interconnected by two plexiform layers (Figure 38 B). From outer to inner layer, these are: the outer nuclear layer (ONL) containing cone and rod photoreceptors (Malicki, 2000); the outer plexiform layer (OPL), made up from synaptic connections between the photoreceptors and bipolar and horizontal cells (Bilotta and Saszik, 2001); the inner nuclear layer (INL) with amacrine, bipolar and horizontal interneurons and Mueller glia cells (Hogan, 1963); the inner plexiform layer (IPL) formed by dendrites of retinal ganglion cells and processes of bipolar and amacrine cells (Bilotta and Saszik, 2001); and finally the retinal ganglion
RESULTS

Cell layer (GCL) containing retinal ganglion cells (RGCs), which transmit the visual signals to the brain and are the first neurons to be born in the retina (Malicki, 2000).

Figure 39. Eye morphology of \(tpd\) embryos at 2 and 3 dpf visualised by antibody staining.

Sections through the eye, dorsal view. All embryos counterstained with F-actin (red) to visualise membranes. (A-C) zn5 antibody staining (green) at 2 dpf labels ganglion cells (asterisk). Note failure of \(tpd\) embryos to form a proper ganglion cell layer (B, C), though some retinal ganglion cells can be identified in the posterior part of the retina (asterisk in B). Note also the defasciculated optic nerve (yellow arrow in C) exiting the retina (compare with D, G). (D-F) zpr1 antibody staining (green) at 3 dpf labels red/green double cone photoreceptors. \(tpd\) embryos form a partial outer nuclear layer (E, F in green) at the back of the eye, but misplaced groups of cone photoreceptors are found, especially in the posterior half of the eye (light blue arrows in E and F), which form rosettes (arrows an close-ups in F and I) and disrupt the inner plexiform layer (bright red). Note also the small lens in \(tpd\) embryos (compare D, E). (G-I) zpr3 antibody staining (green) at 3 dpf labels rod photoreceptors. A similar phenotype to cone photoreceptors is seen. Note misplaced and rosette-forming groups of photoreceptors in H and I (light blue arrows), also disrupting the inner plexiform layer (bright red, compare G and I).

To further analyse the lamination defect seen in \(tpd\) embryos, antibody stainings on transverse sections of the eye were performed at 2 and 3 dpf. Inner surfaces of plasma membranes were visualised by F-actin staining with phalloidin. This confirmed the
reduced size of the lens and defective shape of the \textit{tpd} eye (Figure 39, in red). Moreover, the IPL can thus be detected, since phalloidin strongly stains it at 3dpf due to its high plasma membrane density (Figure 39 D, G, in red). In the \textit{tpd} retina, the IPL forms, but it does not appear as smooth and gets disrupted, especially in the ventroposterior half of the eye (Figure 39 E, F, H, I, in red, arrows).

To detect whether ganglion cells are present in the \textit{tpd} retina at 2dpf, zn5 antibody staining was performed. This shows that the GCL is strongly affected in \textit{tpd} mutants and not formed correctly (Figure 39 A-C, in green, asterisks). RGCs are either not visible at all or occur as a small disorganised group in the ventroposterior retina (Figure 39 B-C, in green), where they normally start to form (Hu and Easter, 1999).

The ONL was visualised by labelling of either red/green cone photoreceptors with zpr1 antibody or rod photoreceptors with zpr3 antibody at 3dpf (Figure 39 D-I, in green). Similar to the IPL, the ONL in \textit{tpd} embryos does form, however, is not as smooth in shape as in wild type embryos and again the defects occur mainly in the ventroposterior part of the retina (compare Figure 39 D and G with E, F, H and I, in green). Notably, photoreceptor cells are found ectopically throughout the ventroposterior half of the \textit{tpd} eye, disrupting the IPL, among other layers (Figure 39 E, F, H and I, in green, arrows). Most interestingly, these photoreceptors seem to aggregate in small groups and form rosettes (Figure 39 F, I, arrows and close-ups), seemingly around a strongly F-actin expressing centre, reminiscent of the \textit{n-cadherin} mutant retina phenotype in zebrafish (Malicki et al., 2003; Pujic and Malicki, 2001).

Another phenotype visible in retinal sections is the defasciculation of the optic nerve exiting the \textit{tpd} retina in comparison to wild type embryos (compare Figure 39 D, G with C, yellow arrow). This is further confirmed by anti-acetylated tubulin antibody stainings of the axons (Devine and Key, 2003) in 2dpf old embryos (Figure 40, asterisks), which moreover reveal a defect in guidance of the retinotectal axons, which misproject anteriorly towards the anterior commissure in \textit{tpd} embryos (Figure 40, arrow). Whether this is accompanied by a misprojection within the optic tectum, remains to be seen.
RESULTS

Figure 40. Axons stained with anti-acetylated tubulin antibody at 2dpf.
In tpd embryos, retinotectal axons misproject anteriorly (arrow) and optic nerves do not form orderly fascicles (asterisks).

As retinotectal axons are guided to the diencephalon along the optic stalk, a transitory structure, which is lost in wild type embryos by 3dpf (Figure 41 D), it was interesting to look at this in tpd embryos. Indeed, the optic stalk in tpd embryos fails to degenerate (Figure 41 E, arrows) and persists at 3dpf instead, disrupting the RPE, so that the choroid fissure in the ventral eye does not close, leading to a coloboma phenotype in tpd embryos, which is even stronger in Trap230 morphants (Figure 40 A-C).

Figure 41. Loss of Trap230 leads to formation of a coloboma in the eye.
(A-C) Eyes at 4dpf (MO: Trap230 morphants). Note failure of the choroid fissure (ventrally) to close. (D, E) Methylene blue stained sections of embryos at 3dpf at the level of the eye. The optic stalk (arrows) fails to degenerate in tpd embryos.
Concomittantly, expression of pax2a, a marker for the optic stalk, which is also required for its development (Macdonald et al., 1997), is still present in tpd embryos at 52hpf, while it is absent from the optic stalk expression domain of wild type embryos at that age (Figure 42).

Expression of fgf3 is downregulated in the tpd optic stalk at 28hpf (Figure 43), a feature that has been reported to concur with expansion of the optic stalk tissue (Walshe and Mason, 2003).

Since the ventral eye in tpd embryos appears to be most strongly affected, judging from the severe coloboma and the disorganised lamination in the ventroposterior retina, fgfs which are expressed in the ventral eye were analysed by wholemount in situ hybridisation. This revealed that expression fgf24 and fgf10 in the tpd ventral retina is lost (Figure 43). Of note, expression of fgf10 in the tpd nasal placode at 24hpf is also lost.

Figure 42. Expression of pax2a in the optic stalk.

At 52hpf, pax2a expression in the optic stalk persists in tpd embryos, while it is gone in wild types (ventral view).

Figure 43. Expression of fgfs in the eye of tpd embryos.

At 24hpf, expression of fgf24 (lateral view) and fgf10 (dorsal view) in the retina (ret, arrow) is reduced. Expression of fgf3 at 28hpf is lost in the optic stalk (os) of tpd embryos (lateral view). Reduced expression of fgf10 in the posterior retina of tpd embryos (arrow) at 30hpf (lateral view). Note also loss of fgf10 expression in the tpd nasal placodes (topmost expression domain).
Since the reduced expression of markers for the ventral retina suggested a possible dorsoventral patterning defect, expression of tbx5, which is a known dorsal marker in the retina (Lupo et al., 2005), was studied in tpd embryos. There, it was found to be strongly reduced at 24hpf (Figure 44), indicating that there is no additional expansion of dorsal fates beside the optic stalk persistence at the expense of ventral tissues.

2.5.3 Heart and circulation phenotype

The tpd embryos show a heart phenotype reminiscent of tbx5 mutants (Garrity et al., 2002), with variably small/dysmorphic ventricle, as (Deborah Yelon, personal communication, Figure 45 B). The heart forms severe edemae, the tube fails to loop and forms a thin string instead (Figure 45 A). As seen in Figure 44, expression of tbx5 in the tpd heart domain seems to be reduced at 24hpf.

The heart rate of tpd embryos at 3dpf is reduced compared by 37% (n=32) in tpd<sup>24970</sup> and by 55% (n=25) in tpd<sup>25870</sup> compared to wild type embryos (150-160 beats per minute, n=20). Bradycardia is another feature of Tbx5 loss-of-function (Garrity et al., 2002). Moreover, blood islands form in the embryo (not shown).
2.5.4 Somite phenotype

A lack of touch response and motility was observed in tpd embryos, indicating a possible neuromuscular problem. Somites in tpd mutants appear slightly U-shaped instead of having the wild type V-shape (not shown). This is a classical feature of defects in the Hedgehog pathway (Ingham and Kim, 2005; van Eeden et al., 1996) and has been related to function of the repressive transcription factor Prdm1 (Baxendale et al., 2004; Roy et al., 2001). Alternatively, since sox9a is expressed in the somites, its function there may relate to the tpd muscle phenotype.

2.5.5 Overall downregulation of Fgf signalling and gut phenotype

The tpd mutant shows reduced expression of fgfs and downstream genes (erm1, pea3) (Roehl and Nusslein-Volhard, 2001) not only in the pectoral fin buds (Figures 35 C, G, K, L, O, P and 46), but also in other structures including the eye (Figure 43), midbrain-hindbrain-boundary (Figure 36 C-F), branchial arches (Figure 47), nasal placodes (Figure 43) and midgut (Figure 48 A, B). Especially fgf24 is absent in all expression domains (Figures 35 C, G, 43, 47, 48 A, B). This suggests that further investigation of the role of Trap230 in Fgf regulation may be interesting.

Figure 46. Expression of fgf10 is reduced in the fin bud of 30hpf tpd embryos (dorsal view).
Apart from the lack of fgf24 expression (Figure 48 A, B) in the midgut, tpd embryos show another gut phenotype, namely the development of a strong edema at the colon/cloaca.
3 Discussion
3.1 \textit{tpd} encodes a mutation in the Mediator complex component Trap230

The \textit{tpd} mutant was found to encode a splice-site mutation in a zebrafish homologue of the conserved Mediator complex component Trap230. The Mediator complex is a general coactivator for most RNA pol II driven transcription in eukaryotes (Bjorklund and Gustafsson, 2004). While some of its up to 30 components are conserved from yeast to human, others have so far been identified in only some of the organisms (Sato et al., 2004). The Trap230 subunit is one of the conserved components, indicating an important function. The zebrafish Trap230 protein is presumably 2155 amino acids long. This is an estimation, since the sequence of the first three exons of the gene is so far only predicted and cannot be identified in any of the zebrafish sequence databases nor has been possible to clone so far. Considering the high degree of conservation between the gene structures and protein sequences of \textit{Fugu rubripes} and \textit{Homo sapiens}, however (cf. appendix Figures 51, 52, 56), a high similarity of the zebrafish sequence and gene structure can be postulated for the N terminus encoded by the first three exons.

Similar to \textit{C. elegans} and \textit{Drosophila} Trap230, the expression in zebrafish is found to be maternal and ubiquitous (Treisman, 2001; Wang et al., 2004a; Zhang and Emmons, 2000). In zebrafish, the only Mediator components cloned so far are \textit{cdk8} and part of \textit{cycC} (Brabazon et al., 2002), and they appear to be expressed similarly, although, of note, the published pictures seem to show no expression of \textit{cdk8} in the pectoral fin buds.

The Trap230 protein consists of four domains: a leucine-rich domain (L) comprising the first 450 amino acids of the N terminus, followed by a leucine-and-serine-rich domain (LS) up to amino acid 1590, a proline-, glutamine-, and leucine-rich domain (PQL) of 424 amino acids length and finally a C-terminal glutamine-rich domain (OPA) starting at amino acid 2015.

The OPA (opposite paired) domain was originally identified in \textit{Drosophila} proteins. It consists of (CAX)$_n$ repeats in the cDNA encoding poly-glutamine (Q) stretches, where X stands for either G or A and n is $\leq 30$. They occur in developmentally
regulated genes, e.g. in Notch, also in mice, where the first description was actually that of the Trap230 OPA domain (Duboule et al., 1987). Yet there, in contrast to the fly, they are not apparent in homeobox transcription factors. They have been speculated to function as hinges/spacers or protein interaction domains. (Duboule et al., 1987; Wharton et al., 1985)

The conserved Trap230 OPA domain (Ito et al., 1999) has been described as essential for Wnt-dependent but not Ras-dependent activity in *C. elegans* (Moghal and Sternberg, 2003). In human cells, the domain has shown activation functions in Gal4-protein fusion assays. However, it was not reported which genes were tested (Ito et al., 1999).

Such CAG repeats as in Trap230 have been associated with many hereditary diseases, most notably neuropsychiatric disorders (Ito et al., 1999). Susceptibility to several neuropsychiatric disorders including autism, depression, schizophrenia, X-linked dementia and hypothyroidism in humans has controversially been attributed to a dodecamer insertion in the Trap230 OPA domain (Beyer et al., 2002; DeLisi et al., 2000; Friez et al., 2000; Hung et al., 2003; Kirov et al., 2003; Michaelis et al., 2000; Philibert et al., 2002; Philibert et al., 2001; Sandhu et al., 2003; Spinks et al., 2004). Of note, a possible use for zebrafish in studying the causes of autism has been proposed, and Trap230 may be suitable as a possible object for such studies (Tropepe and Sive, 2003).

The link to hypothyroidism is especially interesting, since other Mediator components including Med1 - which in *C. elegans* has a similar Wnt-dependent repressor phenotype to Trap230 (Zhang and Emmons, 2000; Zhang and Emmons, 2001) - are known to mediate ligand-dependent nuclear hormone receptor signalling including that through thyroid hormones (Ito et al., 2000). Moreover, maternal thyroid deficiency during pregnancy can have an adverse effect on the subsequent neuropsychological functioning of the offspring (Haddow et al., 1999).

The Trap230 protein contains two overlapping LXXLL motifs (Ito et al., 1999) near the N-terminus, known as nuclear receptor recognition motifs (Voegel et al., 1998) in various coactivators including Med1. This motif interacts with nuclear receptors in vitro, but has only a modest effect on nuclear receptor function in cells (Ito et al.,
Fittingly, Trap230 has been described to be associated with androgen receptor in the presence of testosterone (Wang et al., 2002).

Unlike for Sox9, no subfunction partitioning appears to have occurred for the members of the Mediator kinase subcomplex in zebrafish. In the Zv5 genome assembly, only one homologue each for Trap230, Trap240, Cdk8 and CycC could be identified. However, this also means that Med12L and Med13L still remain to be found, since they should have come up in such a homology search. Nevertheless, the fact that it took several years to identify homologues for Mediator subunits in several species shows that this will not always be easy, as structure may be conserved rather than sequence.

Still, the severe phenotype of tpd mutants makes it unlikely that there should be redundancy, although the even stronger morphant phenotype might hint to another Trap230-like gene being targeted and knocked down. Yet, morpholino oligonucleotides are known to be highly sequence specific, with a 2 or 3bp mismatch already abolishing their function – so another target sequence would perchance have to be essentially identical to the here targeted exon26-intron26 boundary of Trap230. Considering the high level of polymorphism in the zebrafish genome, this is highly unlikely.

However, the strong Trap230 phenotype may be argued not to be sufficient proof of no other Trap230-like gene in zebrafish, since loss of one may be enough to abrogate the function of another, as is seen in the case of Trap230 and Trap240 in Drosophila and C. elegans. In these two organisms, one might also suspect at least homologues of Med12L and Med13L, but still the strong phenotypes visible for Trap230 or Trap240 mutants are there, suggesting the two novel subunits will have non-redundant functions. This may also be the case for any Trap230-like gene still out there in the zebrafish genome – which, as has to be remembered, is not in a good enough state yet anyway to entirely exclude sequence homologues to be found eventually.

Possibly, a comparative microarray analysis of invertebrate and vertebrate Trap230 mutants may elucidate whether the functions are entirely similar or whether essential invertebrate Trap230 functions are still intact in tpd mutants. Yet, already the fact
that vertebrate-specific structures as neural crest and epithelial placodes are affected in *tpd* embryos, makes the comparison more difficult.

Interestingly, the N-terminus of Trap230 has been found to interact with the BEACH domain of the lysosomal trafficking regulator protein (LYST), whose mutation causes Chediak-Higashi syndrome, an inherited immunodeficiency disease, characterised by giant lysosomes and impaired leukocyte degranulation (Tchernev et al., 2002). Unexpectedly, this may suggest a so far undescribed prevalence of Trap230 in the cytoplasm, since LYST is a cytosomal protein.

### 3.2 Interaction of Sox9 + Trap230

#### 3.2.1 The trapped mutation shares many phenotypes with Sox9 mutants

Although the Mediator complex functions as a general coactivator for most RNA Pol II driven transcription in eukaryotes, it seems that different parts of Mediator regulate distinct sets of genes by interacting with specific DNA-binding transcriptional activators. In this study, zebrafish Trap230 was shown to be required for Sox9 activity. *trapped*, a novel Trap230 mutant, was isolated, and the characterisation of this mutant shows that it strongly resembles the *Sox9a/Sox9b* double mutant in many different tissues.

The phenotypes shared between *tpd* and *Sox9a/Sox9b* mutants include the complete absence of craniofacial cartilages, the absence or strong reduction of otic placodes, the absence of iridophores, the presence of expanded melanophores, and the presence of a curly-down body axis and heart edema. The absence of craniofacial cartilages correlates with strongly reduced *col2a1* expression in this region. *col2a1* is a direct target of transcriptional activation by Sox9 (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhou et al., 1998) and is strongly reduced in *Sox9a/Sox9b* mutants (Yan et al., 2005), therefore raising the possibility that Trap230 functions as a transcriptional coactivator participating in the regulation of Sox9 targets such as *col2a1*.

In trunk neural crest, *tpd* mutants show reduced expression of *snai1b, foxd3* and *sox10*, which is also observed in zebrafish *Sox9b* mutants (Yan et al., 2005).
Expression of the postmigratory neural crest marker *dlx2a* in *tpd* and *Sox9a/Sox9b* mutants is only slightly reduced, especially in the posterior branchial arches. This indicates that cranial neural crest cells are specified correctly, although the population of *dlx2a* expressing cells is smaller than usual. Migration of cranial neural crest cells also starts normally, as is the case in *Sox9a/Sox9b* double mutants and *Sox9* mutants in chicken, frog or mouse.

However, Sox9 in mouse and Sox9b in zebrafish are known to act as survival factors for neural crest cells. Accordingly, both zebrafish *Sox9b* and *Sox9a/Sox9b* double mutants and mouse *Sox9* mutants show elevated apoptosis in pre-and post-migratory neural crest cells, which is also observed in *tpd* mutants.

This eventually leads to a loss of neural crest derived structures in *Sox9* mutants. One instance of this is the absence of DRG neurons, which has been previously described in mouse *Sox9* mutants (Cheung et al., 2005) and was again confirmed in *tpd* mutants.

Otic placodes are also Sox9-dependently induced. Therefore, it is not surprising to find that loss of either *Sox9a/Sox9b* or *Trap230* function leads to smaller otic vesicles and otoliths, which in the strongest cases are entirely absent. Interestingly, *tpd* mutants show three otoliths instead of two in some cases. The only instance where to my knowledge occurrence of three otoliths has been described, is a zebrafish knockdown of the *runx1* gene (Kalev-Zylinska et al., 2002). Otherwise, Runx1 has been mainly described as a hematopoietic factor. Besides, a role in neurogenesis has been suggested.

Expression of *fgf3* and *fgf8* during late gastrula stages cooperatively induces otic placode formation and is required for expression of *pax8* and *pax2.1* in preplacode tissue (Phillips et al., 2001). Also, loss of Sox9 has been correlated with loss of *pax8* expression in the presumptive otic placodes (Saint-Germain et al., 2004). Indeed, *tpd* mutants show a similar loss of *pax8* and reduced *pax2.1* expression.

While some of the phenotypes can be explained by loss of Sox9b activity only, especially the cartilage and ear phenotypes observed in *tpd* are as strong as otherwise only found in *Sox9a/Sox9b* double mutants (Yan et al., 2005), suggesting activity of both genes is lost in *tpd* mutants.
These results indicate that Trap230 is crucial for Sox9 activity, and is required for both Sox9a and Sox9b in zebrafish, suggesting that the split of the ancestral Sox9 gene into two teleost copies did not affect their dependence on Trap230 activity. My results suggest that Trap230 specifically interacts with Sox9, since most tpd phenotypes can be explained by disrupted Sox9 activity. However, Trap230 clearly also functions in Sox9-independent events, such as limb induction. It therefore remains to be seen how many different vertebrate transcription factors depend on Trap230. The present study has focused on the role of zygotically expressed trap230, and it may be that maternally expressed trap230 is required for distinct transcription factors.

### 3.2.2 The role of Trap230 in Sox9 activity

Two possible scenarios could explain the apparent similarity between the tpd and Sox9a/Sox9 mutant phenotypes: Trap230 could either be upstream of Sox9a and Sox9b and necessary for the transcriptional activation of these two genes, or else it could be a competence factor necessary for Sox9-dependent transcription through interaction with the Mediator complex.

Since transcription of Sox9a and Sox9b is activated normally in most tissues in the absence of Trap230 activity, this suggests that the activity of Sox9 is disrupted in these mutants. The exception to this are the pectoral fin buds, where both Sox9a and Sox9b fail to be expressed. However, the fin development defect observed in tpd mutants is very early, the disruption taking place already at the bud initiation stage, while the role of Sox9a and Sox9b in the limb buds is a later one, namely during chondrogenesis. Since tpd fin buds never reach that stage, it is not surprising that in this case Sox9 expression should be lost and Trap230 function be upstream of Sox9.

Consistent with the proposal that Trap230 acts as a competence factor for Sox9 rather than as a transcriptional regulator of Sox9 expression, injection of Sox9b mRNA can trigger target gene activation in wild type embryos, but not in tpd mutants. Consequently, Sox9 activity appears to require the presence of at least part of the Mediator kinase subcomplex.

Similar to the situation in Sox9a/Sox9b double mutants and mouse Sox9 mutants, col2a1 expression persists in some expression domains in tpd embryos (Ng et al.,
DISCUSSION

Since col2a1 is known to be directly activated not only by Sox9 but in cooperation with Sox5 and Sox6 (Ng et al., 1997; Zhou et al., 1998), this may explain the residual expression of col2a1 in both Sox9a/Sox9b and tpd mutants. A similar cooperation has been observed for the aggrecan gene, suggesting that Sox genes coordinately affect the composition of the extracellular matrix (Wegner, 1999). Importantly, the residual col2a1 expression in tpd embryos indicates the specificity of the interaction between Sox9 and Trap230, since the tpd mutant does apparently not affect the activities of Sox5 and Sox6 in this context, but specifically that of Sox9.

These results are in agreement with the finding that the transactivating domain of human SOX9 protein binds directly to TRAP230 in vitro, both in a yeast two-hybrid assay, and in a GST pull-down assay (Zhou et al., 2002). Furthermore, Zhou et al. also showed that TRAP230 co-immunoprecipitates with SOX9 from a human cell line, thus demonstrating that this binding also occurs in vivo. The PQL domain of TRAP230 is both necessary and sufficient for this interaction with SOX9. The PQL domain, which is located near the C-terminus, is deleted both in tpd mutants and in Trap230 morphants, and therefore suggests these truncated forms are unable to bind Sox9. The in vivo results of this study thus confirm and extend the significance of the regulatory interaction between Trap230 and Sox9.

Other regions of Sox9 could also contact the TRAP complex. The PQA motif (Wagner et al., 1994) adjacent to the Sox9 TA domain is known to contribute to its function. Nevertheless, this region is poorly conserved in Sox9 between different species and because of its PQA-rich sequence may only work as a flexible hinge for the TA-domain (Zhou et al., 2002).

The study of (Zhou et al., 2002) also found the PQL domain of TRAP230 to interact with the transactivating domains of human SOX8 and SOX10, the two other members of the E group of SOX transcription factors. This may indicate a similar function of Trap230 for the activities of Sox8 and Sox10 and remains to be tested.

A general Trap230 function for Sox10 activity can however be excluded, since zebrafish Sox10 mutants lack all melanophores, as tpd embryos clearly do not (Dutton et al., 2001).
Interestingly, both Sox9 and Trap230 have been suggested to be regulated by Wnt and through PKA phosphorylation (Harley et al., 2003), suggesting they might be regulated in parallel on top of Trap230 being required for the activity of Sox9. Moreover, Sox9 is involved in the induction of migratory neural crest through modulating cadherin expression (Cheung and Briscoe, 2003), indicating a possible involvement in the cell affinity defects observed in tpd mutants, like failure of pectoral fin bud cells to migrate into a coherent patch, or the axon guidance and photoreceptor defects.

C. elegans Trap230 has been described as modulator of β-catenin stimulated Wnt pathway activity (Zhang and Emmons, 2000). This could reinforce the capacity of different Sox proteins to interfere with TCF/LEF binding to β-catenin and modulate Wnt signalling (Zorn, Williams 1999 XSox3; Takash, Poulat, Mattei 2001 Sox7).

The binding of Sox9 at enhancers and concurrent bending of the DNA may facilitate the interaction of Sox9 with the promoter-bound Mediator and allow context-dependent coregulation by bringing other enhancer-bound factors close to the Mediator machinery (Pevny and Lovell-Badge, 1997).

Importantly, my findings not only support the previous data suggesting an interaction of Trap230 and Sox9 but extend it, indicating that Trap230 and Sox9 in vivo not only interact in chondrogenesis but also in all other tissues, reflecting in vivo Sox9 activity. Besides, the fact that some of the phenotypes seen in tpd reflect interference with the Sox9a/Sox9b double activity rather than that of only one of them (eg the chondrogenesis defect), indicates that this interaction has survived the division and subfunctionalisation into two Sox9 proteins. Moreover, the fact that the C-terminal sequences containing the transactivating domain of Sox9a and Sox9b are reasonably different (Chiang et al., 2001), nevertheless not abolishing the functional interaction with Trap230, supports the idea that this interaction is structure rather than sequence specific, a possibility that has also been postulated by (Zhou et al., 2002) in view of the fact that the TA domains of human SOX8 and SOX10, the other two E box transcription factors, also show in vitro interaction with the Trap230 PQL domain, despite their sequence dissimilarities. Sox genes from other groups, which presumably will have different 3D structures, show no such interactions (Zhou et al.,
2002). Unfortunately, Sox5 and Sox6, the two prime candidates for interaction with Sox9 in chondrogenic regulation, have not been tested.

One possible scenario for the function of Trap230-Sox9 interaction in transcriptional activation (at least in the case of Col2a1, my study has demonstrated Trap230 to be a direct coactivator rather than a corepressor) could be similar to the scenario observed in activation of the retinoic acid pathway, where PARP-1 interacts with Cdk8 (Pavri et al., 2005) to mediate RA-induced activity – which actually comes about through uncoupling the kinase module from the core complex and hereby sterically allowing for RNA Pol II binding, resulting in constitutively active RA signalling activity if Cdk8 – and through this presumably the integrity of the kinase subcomplex – is lost. This would also be in accordance with the dominant-negative activity of an ectopically supplied Trap230 PQL/OPA domain for Sox9 (Zhou et al., 2002). If this truncated protein cannot interact properly with endogenous members of the kinase subcomplex, only endogenous Trap230 will be part of the complex and binding of Sox9 to the ectopic Trap230 will not disrupt endogenous Mediator structure, resulting in its dominant negative activity.

Another possibility would be that the interaction of Sox9 with Trap230 leads to a conformational change, but without detaching the kinase subcomplex from the rest of Mediator. This seems to be the case, as it was possible to purify an entire Mediator complex (including core components) through a Sox9 interaction. Nevertheless, there are two caveats in the approach used – one is the fact that thyroid hormone receptor (TR) is still bound to the purified Mediator complex, which could positively be viewed as steric non-inhibition of Sox9 and TR binding together to the Mediator complex, or negatively as TR holding together the complex artificially and overriding a possible disengagement of parts that could be the normal result of Sox9 interaction. The other possibility that cannot be ruled out is that Sox9 can also bind to other parts of the Mediator complex, possibly also of the core, and it is this interaction that has actually been monitored by this assay.
3.2.3 Trap230 as a transcriptional co-regulator during vertebrate development

The Trap230/Med12 and Trap240/Med13 subunits are part of a Mediator subcomplex that also contains Cdk8 and CycC (reviewed in Bjorklund and Gustafsson, 2005; Malik and Roeder, 2005). This module is variably present in the Mediator complex, and its presence correlates with transcriptional repression in yeast (Holstege et al., 1998; Samuelsen et al., 2003; Spahr et al., 2003) and in mammalian cells (Naar et al., 2002; Wang et al., 2001), leading to a model in which recruitment of Mediator containing this module represses transcription, whereas Mediator devoid of this module activates transcription. The role of this module may be more complex, however, since Cdk8 is linked to the positive regulatory effect of Mediator on pol II in Drosophila (Park et al., 2001a), and activation of the Notch signaling pathway leads to the recruitment of Cdk8 and CycC to a Notch target promoter for Notch degradation and turnover (Fryer et al., 2004). Mediator containing this module may thus be involved both in transcriptional activation and repression. In C. elegans, Trap230 is required for transcriptional repression (Yoda et al., 2005), while in Drosophila it is not yet clear if Trap230 participates in transcriptional repression or activation (Janody et al., 2003).

My results are consistent with a role for Trap230 in transcriptional activation by Sox9. Thus col2a1, a direct transcriptional target of Sox9 activation (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhou et al., 1998), shows strongly reduced expression in tpd mutants, and the same is true of genes known to be downstream of Sox9 signaling in neural crest and otic placodes. Furthermore, injection of Sox9b mRNA can upregulate snai1b, foxd3 and sox10 expression in wild type, but not in tpd mutant embryos. Consistent with this proposal, overexpression of a Trap230 subfragment, which only contains the Sox9-binding domain, acts as a dominant negative and blocks the ability of Sox9 to activate target gene expression in vitro, suggesting that binding of full-length Trap230 is crucial for transcriptional activation by Sox9 (Zhou et al., 2002). Alternatively, for targets that are not known to be direct, Sox9 may achieve some of its activation effects indirectly, by repressing the transcription of a repressor. In that case, Trap230 might mediate such an effect as a corepressor while in the case of col2a1 it must surely act as a coactivator.
3.3 The role of Trap230 in forelimb initiation

This study has shown that *tpd* mutants fail to form pectoral fin buds, and lack all pectoral fin structures. Since Sox9 signaling is not involved in the early steps of limb development (Yan et al., 2005), this is a clear example of Trap230 involvement in a Sox9-independent pathway.

The data indicate that Trap230 functions upstream of Fgf24 during limb development. *fgf24* expression fails to be activated in *tpd* mutants, as do the *fgf24*-dependent genes *dlx2* and *shh*. Also, several genes that are activated independently of Fgf24 in the limb bud mesenchyme, including *msx-c*, *erm1*, and *pea3* (Fischer et al., 2003), are strongly reduced or absent in *tpd* embryos, thus further indicating an event upstream of Fgf24 is blocked in the absence of Trap230 activity.

*fgf24*, *dlx2* and *shh* are all downstream of Tbx5 in the limb signalling cascade (Fischer et al., 2003). Since *tbx5* expression is activated in *tpd* mutants, the data of this study suggest that Trap230 is required for an event between Tbx5 and Fgf24 activation. Similarly, the heart and eye phenotypes observed in *tpd* embryos may also be related to loss of Tbx5 activity during their development. In the simplest scenario, Trap230 might bind directly to Tbx5, and function as a coregulator, similar to its interaction with Sox9. Alternatively, it could bind to a transcription factor acting downstream of Tbx5. Future experiments will help to distinguish between these possibilities.

Tbx5 has been proposed to act directly on the promoters of human and mouse *fgf10* (Ng et al., 2002). In zebrafish, however, *fgf24*, a member of the fibroblast growth factor family for which a mammalian ortholog still waits to be identified (Draper et al., 2003), has been placed in the early limb outgrowth signalling cascade between Tbx5 and Fgf10 (Fischer et al., 2003; Norton et al., 2005), suggesting that the *fgf24* promoter is a worthwhile candidate for Tbx5 binding site analysis. A direct role for zebrafish Trap230 mediating Tbx5 function on this promoter seems plausible. Depending on whether a mammalian *fgf24* ortholog can be identified, this may point to a specialised role of Trap230 in zebrafish and thus to the versatility of a highly conserved pan-eukaryotic complex such as Mediator.
While *tbx5*-expressing cells in zebrafish *tpd* and *tbx5* mutants fail to migrate towards the mesenchymal core of the developing limb bud, they nevertheless occasionally turn perpendicular to the basement membrane, but do not proliferate further (Garrity et al., 2002 and this study). This effect of missing Tbx5 activity has not been confirmed in the mouse, though (Agarwal et al., 2003; Ahn et al., 2002). It is likely mediated through Fgf24, as the same effect is observed in *fgf24* mutants (Fischer et al., 2003). A general role has been proposed for *T-box* family genes in cell-autonomously mediating cell migration, possibly through changes in adhesive properties (Griffin et al., 1998; Ho and Kane, 1990; Russ et al., 2000; Wilson et al., 1995). Trap230 has also been implicated in cell affinity changes in Drosophila, *C. elegans* and yeast, suggesting a possible cooperation.

*T-box* genes, like *Hox* genes, have been proposed to often work within gene networks comprised of related family members (Goering et al., 2003). Moreover, Tbx5 in the heart has been shown to interact with the Nkx2.5 homeodomain for direct transcriptional activation of targets like atrial natriuretic factor and Connexin 40 (Cx40, (Fan et al., 2003). Cx40 is a gap junction component that has recently also been reported as a direct target of Tbx5 in the mouse limb (Pizard et al., 2005). There, it is required at a later stage, during bone formation. Interestingly, this is mediated through modulating Sox9 expression (Pizard et al., 2005). Whether there is another function of Cx40 in early zebrafish pectoral fin development, possibly mediating Tbx5 dependent cell migration in parallel to or downstream of Fgf24, remains to be seen. A modulation of gap junctions could well be implicated in this process.

The connection of *tbx5* to bone formation indicates a possible pathway by which formation of the cleithrum in the pectoral girdle of both *tpd* and *tbx5* mutant embryos is lost (or significantly reduced) in contrast to mutations in *sox9a/sox9b* or *fgf24* (Fischer et al., 2003; Yan et al., 2005).

Cooperation with other transcription factors is a common theme for both Sox9 and Tbx5. Thus, should Trap230 prove to be directly required for Tbx5 function, Trap230-Mediator may be envisaged as an integrating platform for combined transcription factor activity. Whether transcription factors bound to Sox9 and Tbx5 would then also interact directly with the Mediator complex, and with which
component, remains speculative. The size of Trap230 as the second-largest component of the complex may already permit simultaneous binding of several transcription factors. Protein-protein interaction sites outside the Sox9-interacting PQL domain remain still to be identified.

In tpd embryos, expression of \textit{wnt2b} upstream of Tbx5 in pectoral fin induction is normal. While this requirement for Wnt signalling in zebrafish is well established (Ng et al., 2002), experiments in mouse failed to confirm it (Agarwal et al., 2003; Galceran et al., 2001; Yamaguchi et al., 1999). In chick, on the other hand, a dominant negative form of Tbx5 lead to reduced \textit{wnt2b} expression, while inhibition of canonical Wnt signalling did not affect Tbx5. This suggests a role for Tbx5 in maintenance of Wnt signalling (Takeuchi et al., 2003). Nevertheless, the lack of Tbx5 activity in tpd pectoral fins constitutes a modulation of a Wnt signalling downstream effect. This is consistent with a role for Trap230 as Wnt signalling modulator reported in \textit{Drosophila} and \textit{C. elegans}.

3.4 Other phenotypes

3.4.1 Brain and muscle phenotypes

The brain of tpd embryos shows several defects: the forebrain is reduced, the midbrain ventricle fails to inflate and the MHB is not specified properly.

These defects may, at least in part, again be mediated by loss of Sox9 activity, since \textit{sox9a} is expressed in the MHB and forebrain (both diencephalons and telencephalon), \textit{sox9b} in the ventral midbrain, and both \textit{sox9a} and \textit{sox9b} are expressed in the epiphysis and hindbrain at 24hpf (Chiang et al., 2001; Yan et al., 2005). Moreover, patients suffering from campomelic dysplasia due to a defect in Sox9, have also been described to display brain abnormalities (Houston et al., 1983).

Wnt signalling is necessary for proper MHB specification in zebrafish. This is a function conserved among vertebrates (Buckles et al., 2004). With regard to the proposed and known functions of Sox9 and Trap230 in Wnt signalling mentioned above, this may add to explanation of the phenotype.
3.4.2 Eye and axon guidance phenotypes

The eye phenotype of tpd embryos is the most prominent one beside that in the pectoral fins. They are overall reduced in size and misshapen, plus they are closer together than normal anteriorly (slight cyclopia), which may correlate with the observed reduction of forebrain tissue (cf. 3.4.1).

Derivatives of the eye premordium (optic stalk, neural retina, retinal pigmented epithelium) and patterning events responsible for their formation are determined along the dorsoventral axis of the eye (Chow and Lang, 2001). tpd mutant embryos show severe defects in dorsoventral patterning. Specifically, ventral, and to some extent also dorsal retina tissue is reduced, while the optic stalk fails to degenerate and is therefore expanded relative to the rest of the eye. This can not only be visually assessed by the coloboma which tpd embryos show, but also molecularly, by in situ hybridisation. While expression of tbx5 as a marker for the dorsal retina prior to retinal lamination (Begemann and Ingham, 2000; Lupo et al., 2005) and fgf24 and fgf10 as markers for the ventral retina are lost, expression of pax2a in the optic stalk (Lupo et al., 2005) persists overly long, while expression of fgf3 in the same domain (Walshe and Mason, 2003) is prematurely lost. All this is consistent with the observed tpd phenotype. Strong expansion of optic stalk tissue at the expense of mainly ventral retina as in tpd has been described as a function of strong retinoic acid, Hedgehog or Fgf receptor signalling or their combination (Lupo et al., 2005). However, expression of at least fgf3, fgf10 and fgf24 in the eye is entirely lost rather than enhanced. Fgf3 and Fgf10 both act mainly through Fgf receptor 2 (Fgfr2IIIB) (Herzog et al., 2004), therefore, enhanced signalling through this Fgf receptor is possibly not expected to be the trigger.

Moreover, lamination defects are seen in the tpd retina later on. While the ganglion cell layer fails to develop to more than a few disorganised cells at 2dpf, both the outer nuclear and inner plexiform layers do form at 3dpf, although with a less smooth appearance than usual and strong disruptions in the ventroposterior part of the retina, which is strongly affected in tpd embryos. This disruption is seen mostly in form of displaced photoreceptor cells, both rods and cones, mainly in the inner plexiform layer. There, they form rosettes around an expression centre of F-actin. Intriguingly, this is a specific phenotype observed also in zebrafish n-cadherin
mutants (Malicki et al., 2003; Pujic and Malicki, 2001), suggestive of a cell affinity specification defect in *tpd* embryos.

Here, zebrafish *tpd* mutants are clearly different from mutants in *Drosophila* Trap230, which have been reported to lack photoreceptor differentiation (Janody et al., 2004). Still, another Trap230 *Drosophila* allele has been described, which shows no overt eye defect (Boube et al., 2000).

Guidance of retinal ganglion cell axons along the optic stalk is also defective. They are not fasciculated into an orderly optic nerve as in wild type embryos (Culverwell and Karlstrom, 2002), and misproject anteriorly to the anterior commissure along their way. The lack of melanophore condensation displayed by *tpd* embryos furthermore suggests they are blind and therefore unable to detect light. With regard to the disorganised retina and optic nerve, this seems likely.

Different types of retinotectal axon pathfinding mutants are known in zebrafish (Karlstrom et al., 1997). The defects seen in *tpd* resemble the no-isthmus phenotype seen in *pax2a* mutants, which fail to close the choroid fissure and have defasciculated retinotectal axons extending rostrally inappropriately (Macdonald et al., 1997). Intriguingly, however, expression of *pax2a* in *tpd* embryos is upregulated rather than downregulated, suggesting that possibly *pax2a* may be another transcription factor for whose activity Trap230 is necessary. Its transcription may then be upregulated due to the failure to activate a negative feedback loop. Alternatively, *pax2a* misregulation may lead to a similar phenotype, no matter whether its transcription is up- or downregulated, and Trap230 may play a role in finetuning the transcriptional level of *pax2a*, normally repressing it either directly or indirectly.

As obvious candidates for transcription factors requiring Trap230 as a competence factor, expression of *tbx5*, *Sox9a* and *Sox9b* in the *tpd* eye were considered. Expression of *tbx5* in the eye of *tpd* embryos appears to be reduced by 24hpf (Figure 44). However, zebrafish *tbx5* mutants do not have an overt eye phenotype (Garrity et al., 2002), suggesting that *tbx5* is anyway not a prime candidate in this context.

*Sox9a* does not seem to be expressed in the zebrafish eye (Chiang et al., 2001; Yan et al., 2005) and this study, and *Sox9b* expression in the *tpd* eye at 24hpf did not seem
altered (Figure 33 G, H). This is in agreement with my other findings concerning the interaction of Sox9 and Trap230. As the role of Sox9 in the eye has not been reported yet, it remains to be seen whether the defects described in *tpd* embryos can be attributed to a lack of Sox9, in this case Sox9b activity. Although this has not actually been reported, Sox9b mutant embryos appear to have a coloboma of the eye, as well (Yan et al., 2005), suggesting that a closer analysis is certainly worthwhile.

Once again, loss of Trap230 function seems to be implicated in altered cell adhesion properties and migration defects, in this case defined by retinal cells which do not form smooth layers, and, in the case of photoreceptors, even aggregate ectopically as rosettes of cells, a phenotype that has been related to defects in N-cadherin, a cell adhesion molecule. Moreover, retinotectal axons fail to form fascicles and are misguided, phenotypes that also suggest defective cell affinity properties.

Mutations in *Trap230* have been reported to alter cell affinity already in *Drosophila* eye, antennal and wing discs, where mutant clones disrupt expression boundaries and/or have smooth borders (Janody et al., 2003; Treisman, 2001). A possible link to E-cadherin has been drawn (Dahmann and Basler, 2000), interestingly correlating with the *n-cadherin*-like photoreceptor phenotype of *tpd* eyes. Moreover, Trap230 in *Drosophila* has been implicated in the RTK/Ras pathway (Maixner et al., 1998), which through phosphorylation of Trap240 in yeast stimulates a flocculation phenotype of cell aggregation, therefore also a change in cell affinity (Chang et al., 2004).

### 3.4.3 Heart and circulation phenotype

Tbx5 deficiency in humans leads to Holt-Oram syndrome, a defect in heart and limb development (Basson et al., 1994; Newbury-Ecob et al., 1996). Zebrafish *tbx5* mutants exhibit not only a similar limb but also heart phenotype as *tpd* embryos (Garrity et al., 2002) and Tbx5 function is involved in heart formation (Begemann and Ingham, 2000; Fan et al., 2003). With regard to this, a direct role for Trap230 in Tbx5 activity similar to that it has with Sox9, seems plausible not only in the context of pectoral fin induction, as discussed above, but also of heart development.

Sox9 expression in the heart during development has also been reported (Ng et al., 1997). Therefore, Sox9 loss of activity may explain the *tpd* heart phenotype. Since
regulation of Sox9 by Tbx5 through Connexin 40 in limb bone development has recently been reported (Pizard et al., 2005), a similar cascade may function in the heart, involving Trap230 at multiple steps.

3.4.4 Somite phenotype

In tpd mutants, a motility problem and slightly U-shaped somites were observed. As sox9a is expressed in the somites, a requirement for Trap230 in this context may explain the tpd muscle phenotype. However, the phenotype described for sox9a mutants consists of shorter and thicker muscles (Yan et al., 2002) and it is not clear whether this is identical with the phenotype displayed by tpd embryos. Alternatively, Prdm1, a zinc finger and SET domain transcription factor with histone methyl transferase activity, which has been implicated in this process (Baxendale et al., 2004; Roy et al., 2001) and is known to function mainly as a repressor (Wilm and Solnica-Krezel, 2005), may recruit Trap230 for its function – especially since Trap230 is thought to act mainly as a corepressor rather than a coactivator. Strikingly, loss of Prdm1 function also leads to a failure of fin bud induction and the gene is expressed in the photoreceptor cell layer (Wilm and Solnica-Krezel, 2005) (cf. tpd eye phenotype in 2.5.2).

3.4.5 Overall downregulation of Fgf signalling and gut phenotype

Zebrafish tpd mutants show a downregulation of fgf and Fgf-downstream-target expression in several tissues apart from pectoral fin buds, namely in branchial arches, midbrain hindbrain boundary, ventral eyes, nasal placodes and the midgut.

fgf-expression in the nasal placodes may be reduced due to lack of Sox9 activity, caused by the function of Sox9 in epidermal placode formation, not only for the otic but also the nasal placodes (Yan et al., 2005).

Genetic interactions between Fgf signalling pathways and T-box transcription factors, where the latter are required for their induction and maintenance, have started to become a common theme in vertebrate development (Draper et al., 2003; Ng et al., 2002). Should the possibility of a rule for Trap230 in mediating T-box
transcription factor activity, firstly in the case of Tbx5, hold true, this may provide an explanation for reduced expression of Fgfs in tpd embryos to be proven.

Another hint may come from cloning the gene affected in the zebrafish aussicht mutant, which shows a general fgf overexpression (Heisenberg et al., 1999) and may point to a universal Fgf-regulatory mechanism which could possibly also be conversely affected and lead to general downregulation of fgfs.

A combined effect of deficient Sox9 and Tbx5 activity with disruption of a general Fgf-regulatory mechanism and indirect components attributable to misregulated targets of other major signalling pathways as the Wnt pathway, which is known to intersect with the Fgf pathway on several levels and in many instances, appears plausible though complex. Possibly, a simpler and more specific explanation may turn out to hold true.

In addition to fgf24 downregulation in the midgut, tpd embryos also show an edema at the colon/cloaca. This may be a secondary effect due to osmotic problems caused by renal failure, as Sox9 is also known to have a function in the kidney (Morais da Silva et al., 1996). However, it also seems feasible that this should hint to a possible interaction of Trap230 with Sox10, since a mouse Sox10 mutant, DOM (dominant megacolon) shows exactly this phenotype (Herbarth et al., 1998). On the other hand, zebrafish Sox10 mutants have an intrinsic lack of melanophores (Dutton et al., 2001), which tpd embryos certainly do not have (Figure 48 C). Therefore, Trap230 could only be required for some functions of Sox10, but not all. Interestingly, an expression of Sox9 in the gut has also recently been reported (Blache et al., 2004), suggesting that there may be a function of Sox9 whose loss could lead to the tpd gut phenotype. Yet, Sox9a/Sox9b double mutants have not been reported to show such edemae, making this explanation less likely. The Sox9 function in the gut, interestingly, appears to involve maintenance of an undifferentiated cell state through Wnt signalling regulation (Blache et al., 2004), relating back to loss of Trap230/Mediator kinase subcomplex being involved in entry of cells into a G0/stationary phase/differentiated state (Spahr et al., 2003).

A regulator of Fgf24 in the gut has unfortunately also not been reported yet. So the cause of this tpd phenotype remains elusive.
3.4.6 Outlook

Certainly, one recurrent theme for Trap230 and the Mediator kinase subcomplex is its involvement in the control of differentiation. Intriguingly, as seems to be the case for HDAC1 (Stadler et al., 2005), which is generally thought to be a repressor and has been proposed as an opponent of the Mediator complex (Kwon and Lee, 2001), although they share some phenotypes, the Mediator kinase subcomplex appears to be involved in activation as well as in its canonical repression of genes, and has been implicated in both maintaining an undifferentiated cell state and promoting cell differentiation, depending on the context. Its cdk and cyclin components link it to the cell cycle, making this role more feasible. Apparently, phosphorylation of Trap240 and possibly also Trap230 through a Ras/cAMP pathway seems to be recurrently involved in this function. Moreover, the proposed chromatin remodelling activity in the subunit may come to aid there, and complex differential regulation of the Mediator kinase submodule through other Mediator components, co- and transcription factors as well as directly through secondary modifications like phosphorylation or possibly sumoylation explain the fact that Trap230 and Trap240 have been identified in Drosophila as members of both the Trithorax and Polycomb group, maintaining either an active or repressed transcriptional state. Indeed, Trap230/Trap240 are involved in regulating the activity or expression of a number of homeobox transcription factors in various organisms.

Differentiation is inhibited - respective an undifferentiated state maintained - in presence of the kinase complex in case of yeast cells, where it represses entry into G0/stationary phase. This is also the case for much of the Sox9 functions, which through Wnt signalling have been implicated in maintaining undifferentiated cell states and require Trap230 to be relayed. On the other hand, in C. elegans vulval development, Drosophila eye development and Dictyostelium spore formation, Trap230 appears to promote differentiation.

Moreover, another (or possibly the same) connection exists between Trap230/the Mediator kinase subcomplex and regulation of cell affinity in yeast, mammalian cells, Drosophila and zebrafish. Whether this is an indirect effect, eg. through modulating Wnt/Sox9 signalling and thereby the composition of the ECM, the actin
cytoskeleton bound to β-catenin or the planar cell polarity pathway, or a more direct function, remains to be seen.

Clearly, the emerging picture of Mediator is that of an all-integrative platform for transcriptional regulation as well as other DNA-related activities, at which signalling pathways and transcription factor activities converge and cross-talk. It is a complex and versatile machinery with manifold regulatory features. Different proteins may bind to different subunits, modify them, eg. by phosphorylation, sterically inhibit each other or be brought in contact with one another. The chromatin structure is altered to bring enhancer- and promoter-bound proteins in contact with one another, nucleosomes are removed and acetylated, individual Mediator components may modify each other and regulate each other, submodules may be recruited depending on the context – and ultimately, structure and state of the entire complex are thus modified to intricately regulate the activity of pol II for transcribing the gene close to which Mediator is bound.

Such a complex system is probably more than the sum of its components, yet, understanding the function of individual subunits will promote the understanding of the whole. Therefore, it is essential to analyse a component like Trap230 and study its function. Again, analysing individual instances of Trap230 activity will help to get an idea, like its interaction with Sox9 or a possible interaction with Tbx5 and resulting effects. Nevertheless, it will be most important to understand the general idea behind it and find the rules that this piece in the whole framework obeys to. One may think of Trap230 as a protein which randomly interacts with some transcription factor or other as a competence factor for its activity, yet I believe the emerging picture to be more specific. A number of possibilities present themselves for now, which may not encompass the whole scope yet and can also include the truth in a combination of them. Trap230 may be specific for relaying transcription factor activity downstream of certain signalling pathways and not others – so far, the Wnt, Ras, Notch, Hedgehog and JNK pathways have been proposed. It may be specific for certain tissues, although its ubiquitous expression suggests otherwise. Its specificity may lie in a role for regulating cell cycle and proliferation, as has been implicated at several points also for other components of the Mediator kinase subunit. Alternatively, it may serve to regulate cell-cell or cell-matrix affinity as a general
rule, as some data suggest. Or, it may be specific for a set of transcription factors with common structural or other features, like those which need to synergise with others for their function, as Sox and T-box proteins do. Moreover, unknown functions of Trap230 may remain to be found, like enzymatic activities or a role in the cytoplasm. These possible functions may also differ from one species or phylum to the other – or be conserved. It remains to be seen.

Another important point are the possible implications of Trap230 and Mediator defects for human diseases. They have been linked to neurodegenerative and neuropsychiatric diseases, as well as to congenital defects of many organs and cancer.

In summary, both a detailed analysis of specific Trap230-interactions and a broader analysis, eg. by microarray studies, should help to provide us with a clearer picture of its function within the Mediator complex. The same holds true for its other components, and comparative analysis of the clockwork components will be important to understand how the Mediator clock ticks.
4 MATERIALS AND METHODS
4.1 Fish stocks

The *trapped* alleles used were $t^{25870}$ and $t^{24970}$. Embryos were cultured in E3 medium, with or without the addition of 0.003% 1-Phenyl-2-thiourea (PTU, Sigma) to inhibit pigmentation. Embryos were staged according to hours post fertilisation (hpf) or days post fertilisation (dpf); (Westerfield, 1995).

4.2 Chemicals and solutions

The following overview comprises general buffers and solutions. Special solutions and reagents are mentioned together with the methods. All chemicals, if not noted otherwise, were purchased from the companies Applichem, Merck, Roth and Sigma. Agarose was purchased from Pharmacia, Bromophenol blue and Xylene cyanol from Serva.

60x stock solution E3 saline

- 34.8g NaCl
- 1.6g KCl
- 5.8g CaCl$_2$ x 2H$_2$O
- 9.78g MgSO$_4$ x 6H$_2$O
- H$_2$O ad 2l
- pH 7.2 with NaOH autoclave
- for 1x use 16ml/l + 100µl methylene blue solution

10x loading buffer:

- 50% glycerol
- 1x TE
- 0.25% bromophenol blue
- 0.25% xylene cyanol

TE:

- 10 mM Tris/HCl pH 7.4
- 1mM EDTA pH 8.0

50xTAE

- 242 gm Tris base
- 57.1 ml glacial acetic acid
100ml 0.5M EDTA
Add ddH₂O to 1 liter and adjust pH to 8.5.

10xPBS 70g NaCl; 62.4g Na₂HPO₄.2H₂O; 3.4g KH₂PO₄
pH 7.4

4.3  Linkage analysis and genetic mapping

4.3.1  Quick lysis of embryos
- Fix embryos in 100% MeOH
- Place at -20°C for at least 30min
- Place embryos individually into a 96-well-plate
- Mix 250µl proteinase K (20mg/ml) and 2250µl TE
- Pipet 25µl into each well (multipipet)
- Close plate firmly with lids
- Digest 4h-O/N at 50-70°C
- Boil 5min at 96°C to destroy proteinase K
- Dilute with 75µl H₂O per well
- Store at -20°C
- Use 1-5µl per PCR

4.3.2  Finclipping
Anesthetise the fish in 100ml H₂O + 4ml 0.4% Triacin (MS222, Sigma). After 30-60sec, the fish will be anesthetised. Scoop the fish out with a tea-strainer, cut off a tiny bit of tail fin using a sharp sterile blade and let the fish recover in fresh water.

Finclipping solution:

400mM Tris
5mM EDTA
150mM NaCl
0.1% SDS
pH 8.0
- Digest finclip in 100µl finclip solution + 5µl proteinase K (20mg/ml) for several hours at 60°C or O/N at 50°C
- Boil 5min to destroy proteinase K
- Purify and concentrate DNA using a Qiagen PCR Purification Kit, elute with 10-50µl

4.3.3  SSLP markers used
Primer sequences for all markers used from the meiotic MGH panel can be found in the ZFIN database (http://www.zfin.org).
For fine mapping of *tpd*, SSLPs were generated using the Sanger genome database. The closest SSLP marker to the *tpd* mutation, P1, uses the primer pair GCATCCACCCAAACATGAGG (forward) and GCAGTGCGATTGATGTTGGG (reverse), at a distance of <0.08cM south of the mutation.

### 4.3.4 Bulked segregant analysis

For bulked segregant analysis, 5µl each of quick lysis extracted DNA from 48 mutant or wild type embryos (cf. 4.3.2) were pooled. PCR using SSLP primers was performed as follows:

**PCR MIX**
- 5µl DNA
- 2µl 10x PCR Buffer
- 1.6µl DNTPs (2.5µmol each)
- 0.08µl each primer at 100pmol/µl
- 0.4µl Taq polymerase
- ad 20µl H₂O

**PROGRAM**
- 2min 94°C
- 35 – 40 cycles:
  - 30sec 94°C
  - 30sec 58°C
  - 1min 72°C
- 5min 72°C

For initial linkage analysis of the 192 marker set, PCRs were performed in 96-well-plates, for analysis of single markers, 8-strip or single PCR tubes.

### 4.3.5 Fine mapping

Individual embryo PCR was performed essentially as described for bulked segregant analysis, but using 2-5µl of DNA from individually lysed embryos (cf. 4.3.2) and doing the PCRs in 96-well-plates.

### 4.3.6 Websites

- [http://www.zfin.org](http://www.zfin.org) Zebrafish database
- [http://www.repeatmasker.org](http://www.repeatmasker.org) Program to find CA repeats
- [http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish/small.shtml](http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish/small.shtml) Zebrafish BAC-FPC map
- [http://vega.sanger.ac.uk/Danio_rerio/](http://vega.sanger.ac.uk/Danio_rerio/) Zebrafish BAC/PAC sequence assembly
- [http://zebrafish.mgh.harvard.edu/](http://zebrafish.mgh.harvard.edu/) Zebrafish meiotic MGH mapping panel
- [http://helix.wustl.edu/dcaps/dcaps.html](http://helix.wustl.edu/dcaps/dcaps.html) Program to find SNPs
- [http://www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/) Sequence alignment program
4.4 Histochemical methods

4.4.1 Whole mount in situ hybridization (WMISH)

4.4.1.1 WMISH probes

col2a1 (Yan et al., 1995)
dlx2 (Akimenko et al., 1994)
erm1 (Roehl and Nusslein-Volhard, 2001)
fgfβ3 (courtesy of Michael Tsang) (Kiefer et al., 1996)
fgf10 (Ng et al., 2002)
fgf24 (Fischer et al., 2003)
foxd3 (courtesy of Darren Gilmour) (Odenthal and Nusslein-Volhard, 1998)
msx-c (Akimenko et al., 1995)
pax2a (Krauss et al., 1991)
pax8 (Pfeffer et al., 1998)
pea3 (Roehl and Nusslein-Volhard, 2001)
shh (Krauss et al., 1993)
sna11b (courtesy of Stephen Wilson) (Thisse et al., 1995)
sox9a (courtesy of Stephen Wilson) (Chiang et al., 2001)
sox9b (Chiang et al., 2001)
sox10 (courtesy of Darren Gilmour) (Dutton et al., 2001)
tbx5 (Begemann and Ingham, 2000)
wnt2b (Ng et al., 2002)

The trap230 in situ probe was synthesized using the following primers:

forward primer GGTGGGTGGGATGTTTGAC
reverse primer TTCACAGAACAACGCCAGTATG.

4.4.1.2 IN SITU PROBE SYNTHESIS

Preparation of DNA template:

- Digest 4-5µg plasmid DNA in 100µl volume 2h at 37°C
- Purify DNA with Qiagen PCR purification kit
  - Add 5 vol. buffer PB to 1 vol. PCR sample, mix
  - Apply sample on Qiaquick spin column on 2ml tube, spin 30-60s, discard flow-through
  - Wash with 750µl Buffer PE, spin 30-60s, discard flow-through, spin 1min max. speed
  - Place column on clean 1.5ml tube
  - Add with 30µl Buffer EB, let stand for 1min, spin 1min max. speed
- Measure concentration

Transcription reaction (20µl final):

- 1µg linearised DNA in 9µl H2O
- 4µl 5x transcription Buffer (Promega DIG kit no. 8)
- 2µl 100mM DTT (shelf 5 in box)
MATERIALS AND METHODS

• 2µl 10x DIG-labelled nucleotide mix (DIG kit no. 7)
• 1µl RNAse inhibitor (DIG kit no. 10)
• 2µl RNA polymerase (DIG kit no. 12, shelf 7)
• incubate 2h 37°C

Nucleotide removal:

• use Qiagen RNeasy kit
  o use maximum of 100µg RNA for Mini kit
  o Buffer RLT should be redissolved by warming if precipitated, then at RT
  o Adjust sample to 100µl with RNase-free water
  o Add 350µl Buffer RLT, mix thoroughly (β-ME should be in Buffer!)
  o Add 250µl EtOH, mix thoroughly by pipetting, don’t spin
  o Apply to RNeasy column in 2ml tube, spin 15s at max. speed (≥8000g), discard flow-through
  o Place column on new tube
  o Add 500µl Buffer RPE, spin 15s at max. speed, discard flow-through
  o Add 500µl Buffer RPE, spin 2min at max. speed, discard flow-through
  o Optional: place column in new tube, spin 1min at max. speed
  o Place column on new 1.5ml tube
  o Add 30µl H2O, spin 1min at max. speed
• Check RNA concentration (should be ~100ng/µl)
• Add 30µl formamide
• Store at −20°C

4.4.1.3 WMISH protocol

BUFFERS:

20xSSC:
175.3 g NaCl
88.2 g NatriumCitrat
in 1l H2O pH 7.0/NaOH

Hyb-:
50% formamide
5x SSC
0.1% Tween-20
(store at -20°C)

Hyb+:

Hyb-
50µg/ml Heparin
50mg/ml torula (yeast) RNA
(store at -20°C)

Staining buffer:
100mM Tris pH 9.5 (50ml 1M stock for 500ml)
50mM MgCl2 (25ml 1M stock for 500ml)
100mM NaCl (16.7ml 3M stock for 500ml)
0.1% Tween-20 (add shortly before use!)
**MATERIALS AND METHODS**

2x malate buffer:
- 200mM Malate pH 7.5 (50ml 2M stock for 500ml – adjust pH in stock!!)
- 300mM NaCl (50ml 3M stock for 500ml)
- 0.1% Tween-20 (add shortly before use!)

Blocking buffer:
- for 300ml:
  - 6g blocking reagent (Roche Cat. No. 1096176) to make 2% solution
  - 150ml H$_2$O
  - 150ml 2x malate buffer with 0.1% Tween

(Store at –20°C)

**EMBRYO FIXATION AND STORAGE**
- Fix embryos in 4% PFA (paraformaldehyde) in PBS at RT 1-4h or O/N at 4°C
- Wash 2-3x in PBS
- Dechorionate in small petridish with forceps in PBS
- Wash in PBS
- Replace PBS by 50% methanol (in PBS)
- Replace by 100% methanol
- Wash 1-2x in 100% methanol
- Store at -20°C 30min - storage

**WMISH**

**DAY 1**
- 5min 50% MetOH in PBS at RT
- 5min 30% MetOH in PBS at RT
- 2-4x 5min PBST (0.1% Tween 20 in PBS) at RT
- digest with $5\mu$g/ml Proteinase K (2ml PBST + 0.5$\mu$l 20mg/ml stock) at RT, time depends on embryo stage
  - 10h 3min
  - 3s 5min
  - 14s 7min
  - 16s 8min
  - 20s 10min
  - 24h 12min
  - 52h 15min
  - 68h 15min
- wash 2-3x in PBST
- transfer to 0.5ml tube (20-30 embryos per tube)
- prehybridise in 200$\mu$l Hyb- (RT liquid) 5min at 67°C (may be left out)
- may be stored in Hyb- up to 24h at -20°C
- prehybridise in 200$\mu$l Hyb+ (RT liquid) at least 1-2h at 67°C
- take off 100-120$\mu$l Hyb+ (don’t let embryos touch air)
- add 1-2$\mu$l RNA probe
- hybridise at 65-68°C O/N – 48h

**DAY 2**
- take off probe, store at -20°C and reuse
- wash 2x 30min in 50% formamide / 50% 2xSSCT (0.1% Tween) at 67°C
- wash 15min with 2xSSCT at 67°C
• wash 2x 30min with 0.2x SSCT at 67°C
• wash 2x with 1x malate buffer
• block at least 1h with blocking buffer at RT
• incubate in 500µl alkaline phosphatase coupled Fab antibody (0.5µl in 2ml blocking buffer) for 4h at RT or 4°C O/N

DAY3
• wash 4x 25min with 1x malate buffer at RT (make fresh!!)
• (leave in PBST at 4°C O/N)
• wash 3x 5min with staining buffer at RT (make fresh)
• stain in 500µl BM Purple AP substrate solution (Roche Cat. No. 1442074) (warm & shake before use) in 24-well-plates at 37°C in the dark, check every 10-15min
• wash 3x with PBST
• fix in 4% PFA, store at 4°C
• replace by 75% glycerol/PBS, store at 4°C

4.4.2 Cryosections
• Fix dechorionated embryos in 4% PFA/PBS for 1h at RT or O/N at 4°C
• Wash with PBS
• 2h at RT (O/N – a few days at 4°C) in 20% sucrose/PBS
• take embryos out from sucrose and place in plastic moulds (Polysciences)
• take off sucrose as well as possible
• cover embryos with Tissu Tek (Plano)
• align embryos in required position (ventral to the mold bottom) under the microscope
• place molds on dry ice so tissue-tek hardens (about 20min)
• detach blocks by pressing hard with the thumb against the mold bottom
• place blocks on holders
• place a holder in the cryostat and start to cut
• try to find the plane containing embryos by trimming 30-50µm sections
• cut embryos in 12µm sections
• transfer sections in a line to slide (super frost slides, Roth), 5-7 sections per line are optimal
• leave to dry at RT at least 1h

4.4.3 Antibody stainings

4.4.3.1 Antibodies
The following primary antibodies were used:
mouse anti-acetylated tubulin antibody (Santa Cruz; 1:1000)
mouse anti-Hu (Marusich et al., 1994), kindly provided by Darren Gilmour; 1:200)
mouse anti-Islet1 (Developmental Studies Hybridoma Bank; 1:50)
mouse anti-Zn5 (University of Oregon; 1:500)
mouse anti-Zpr1 (University of Oregon; 1:200)
mouse anti-Zpr3 (University of Oregon; 1:200)
Secondary antibodies:
anti-mouse antibody used was either coupled with AlexaFluor488 (green) or AlexaFluor 549 (red) (Molecular Probes, 1:500)
Alexa Fluor 568-conjugated phalloidin (Molecular Probes; 1:40)

4.4.3.2 Hu staining protocol

- Fix embryos in 4% PFA (PBS) for 3h at RT or O/N at 4°C
- Rinse once + wash 3x with PBST (0.1% Tween 20)
- Dehydrate in MetOH series (33%, 66%, 2x100%, 5min each, place at -20°C for at least 1h). Important for penetration of later stage samples.
- Permeabilise in 1ml Proteinase K solution (30µg/ml in H2O). Time depends on stage of embryos – 10min should be enough for most later stages.
- Rinse once + wash 3x with PBST (0.1% Tween 20)
- Block 1h in GS-PDT (10% goat serum in 0.1% Tween 20, 1% DMSO in PBS)
- Dilute primary antibody in GS-PBST (no DMSO), add 100-200µl to the samples and incubate O/N at 4°C or 4h at RT
- Wash 4-6x 30min in GS-PBST
- Incubate with secondary antibody (anti-mouse AlexaFluor 488; 1:500) in GS-PBST 4h at RT or O/N at 4°C
- Rinse once + wash 2x 30min in GS-PDT
- Rinse once + wash 2x 30min in PDT

4.4.3.3 Islet1 staining protocol

This is a tedious but worthwhile protocol, since embryos have to be digested in every possible way to let the antibody through to the dorsal root ganglia.

- Dechorionate embryos
- Fix in 4% PFA 0.1% Tween 1h at RT
- Wash embryos 2x 5min in PBSTw (0.1% Tween/PBS)
- Wash 5min in H2O, then 1h in H2O, then through a series of 25%, 50%, 75% into 100% MetOH in H2O to keep at -20°C O/N
- Rehydrate through a MetOH series into PBS 0.1% Tween20 0.5% Triton
- Pass embryos into a glass tube (acetone will dissolve plastic)
- Short wash with H2O
- Permeabilise with cold (-20°C) acetone for 20min at -20°C
- Short wash with H2O
- Wash 3x 5min with PBS 0.1% Tween20 0.5% Triton
- Short wash in 2% goat serum, 1% BSA, 1% DMSO, 0.5% Triton in PBS (PBDST)
- Transfer embryos back into Eppendorff tubes
- Block 1h in PBDST
- Primary antibody incubation: Islet-1 1:30 in PBDST O/N at 4°C or 4h at RT
- 4x 30min wash in PBDT (no goat serum)
- secondary antibody incubation: (anti-mouse AlexaFluor 488; 1:500) In PBDT O/N at 4°C or 4h at RT
• 4x 30min wash in PBDT

4.4.3.4 Acetylated tubulin staining protocol

SOLUTIONS

TCA: 10% trichloroacetic acid stock in distilled water, stored at 4°C, dilute to 2% in PBS
PBT: 0.5% Triton X-100 in PBS, 10% Triton, stored at RT
Trypsin: from Gibco-BRL (2.5% solution), stored at -20°C in aliquots (200µl), dilute to 0.25% in PBT before use
Primary and secondary antibodies, goat serum and DAB stocks are stored at -20°C in aliquots.

PROTOCOL

• Fix embryos in 2% TCA (trichloroacetid acid) in PBS for 3h exactly at RT
• Wash 3x 5min in PBS
• Store at 4°C in PBTx (0.5% Triton in PBS) until use

Careful: TCA fixed fish are quite fragile

DAY1

• Wash 2x 10min in PBS after several rinses
• Trypsin treatment (to permeabilise the embryos)
  o Prechill trypsin solution (dilute frozen stock 10x in PBTx 100µl stock) on ice
  o Prechill aliquot of PBTx (for stop solution)
  o Incubate the embryos in trypsin solution on ice
    4min for 44hpf, 8min for 58hpf, 12min for 72hpf embryos
• Rinse in prechilled PBTx
• Wash 5x 5min in PBTx at RT
• Blocking: incubate in 10% goat serum, 1% DMSO in PBTx for at least 1h at RT
• Replace with primary antibody diluted in 10% goat serum, 1% DMSO in PBTx
• Incubate O/N at 4°C

DAY2

• Remove primary antibody solution and keep in fresh tube at 4°C for reuse
• Rinse embryos 2x with PBTx
• Wash 6x 30min in PBTx on a shaker at RT
• Wash in PBTx for 30min
• Incubate in secondary antibody (anti-mouse AlexaFluor 488; 1:200) diluted in 1% goat serum in PBT O/N at 4°C

DAY3

• Remove secondary antibody (and discard)
• Rinse 3x with PBTx
• Wash 6x 30min in PBTx on shaker at RT
• Final wash in PBS after several rinses
• Transfer to 70% glycerol / 30% PBS and store at 4°C to view

4.4.3.5 Eye sections double labelling protocol

Cryosections of 2 and 3dpf old embryos were used.
• Wash 2x 5min in PBST (0.1% Tween)
• Block 1h at RT in 1% BSA/PBS in a wet chamber, cover sections with parafilm, 150µl per slide
• Primary antibody:
  o Zn5 1:500; or Zpr1 or Zpr3: 1:100
  o 4°C O/N incubation
• Wash 3x 5min in PBST
• Secondary antibody:
  o Anti-mouse AlexaFlour 488 (1:500) and Alexa-conjugated phalloidin (1:40)
  o 150µl per slide in blocking buffer
  o 1h at RT in wet chamber, slides covered with parafilm
• Wash 3x 10min with PBST
• Mount with Moviol

4.4.4 Methylene blue staining
Methylene blue solution:
• Add 25ml of 4% NaOH solution to 100ml of 1% silver nitrate solution
• Allow the precipitated silver oxide to sediment and decant supernatant
• Dissolve 1g methylene blue in 100ml distilled water, heat and add to the washed silver oxide
• Heat for a further 5min
• Allow to cool and filter
Staining procedure:
• Wash cryosections 2x in PBS
• Wash sections with distilled water
• Stain for 15sec – 2min in methylene blue
• Wash in running tap water around 10min (dependent on section thickness and age of staining solution)
• Wash 30sec in 70% EtOH
• Wash 2x 30sec in 100% EtOH
• Place 5min in xylene
• Check staining quality under microscope
• Mount with Entellan

4.4.5 Alcian blue staining
H₂OT: tap water with 0.1% Tween 20
• Fix embryos O/N at 4°C in 4% PFA (PBS)
• Place embryos in 70% EtOH for 10min (can be stored at 4°C for about a week)
• For storage (optional): take through a MetOH series and store in 100% MetOH at -20°C
• Stain O/N in 0.1g Alcian Blue in 0.1N HCl pH1 at 37°C
• Rinse 2x 5min in H₂OT
4.4.6 TUNEL staining

TUNEL staining was performed on whole mount embryos using the in situ cell death TMR red kit (Roche).

4.5 Total RNA isolation from embryos

- Collect 30-50 embryos at 3dpf and shock-freeze with liquid nitrogen
- place at -80°C for a few hours
- resuspend in 400μl TES solution:
  - 10mM TrisCl pH 7.5
  - 10mM EDTA
  - 0.5% SDS
- add 400μl acid phenol, vortex vigorously 10sec
- incubate 60min at 65°C with occasional, brief vortexing
- place on ice 5min, centrifuge 5min at top speed, 4°C
- transfer aqueous (top) phase (400μl) to a clean 1.5ml tube, add 400μl acid phenol, vortex vigorously, place on ice 5min, centrifuge at top speed, 4°C
- repeat
- transfer aqueous phase to a new tube, add 40μl of 3M sodium acetate, pH 5.3 and 1ml of ice-cold 100% EtOH
- precipitate 1h at -80°C
- centrifuge 5min at top speed, 4°C
- take off EtOH
- wash RNA pellet by vortexing briefly in ice-cold 70% EtOH
- centrifuge as before to pellet RNA
- take off EtOH
- resuspend pellet in 50μl H2O, determine OD (1:100), store at -20°C

- Bleach and defat 30min at 37°C in:
  10μl 30% H2O2
  100μl 10% KOH
  890μl H2O (freshly made, invert tube 2x to mix)
- Rinse 2x 5min in H2O
- Rinse 10min in:
  300μl saturated Borate (Borax)
  600μl H2O
- Digest: replace with the same solution including 0.25mg/ml Trypsin, digest 30min at 37°C (invert tube 2x to mix)
- Rinse 2x 5min in H2O
- Rinse in 25% glycerol/75% H2O for 30min
- Transfer to 50% glycerol/50% H2O
- Transfer to 75% glycerol/25% H2O for long term storage at RT/4°C
4.6 cDNA synthesis

cDNA first strand synthesis (for cloning) was performed with SuperScriptII Reverse transcriptase (Gibco) according to the manufacturer’s protocol.

4.7 General gene cloning procedure

Programs and PCR mixtures used depended on the aim of amplification and primers. If not noted otherwise, 1000bps/min were used as standard to calculate the extension time. Promega Tfl polymerase was used. PCR products were separated electrophoretically on agarose gels, cut out and extracted using a Qiagen MinElute Kit.

**Redo TA overhangs:**

<table>
<thead>
<tr>
<th>Volume</th>
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<td>DNA</td>
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<tr>
<td>2µl</td>
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<tr>
<td>1µl</td>
<td>dATP 0.2mM (1:50 dilution of stock from stores)</td>
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<td>1µl</td>
<td>Promega Tfl polymerase from RT kit (Promega)</td>
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<tr>
<td>0.4µl</td>
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**Ligation TopoTA Kit:**

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<td>1µl salt solution</td>
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<tr>
<td>0.5µl vector</td>
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<td>2.5µl water</td>
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**Ligation pGEMT Kit:**

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Ligated plasmids were chemically transformed into Top10 cells (Invitrogen) according to product sheet, grown O/N on agar plates. Individual clones were picked and a bacterial mini preparation performed. Restriction digests using EcoRI were done to check insert size on agarose gel. Clones containing inserts of correct size were then sequenced in house.

4.7.1 Trap230 primers

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<th>forward primer(s)</th>
<th>reverse primer(s)</th>
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<td>GTTCCCATCTTTCAGCAAGAAG</td>
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<td>ACACGAGTTCCTCACCCTGG</td>
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## Materials and Methods

### Primer name

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Figure 49. Sequences of Trap230 primers used in this study.

### 4.8 Sequencing

DNA sequencing was performed by the EMBL internal service unit of Vladimir Benes.

### 4.9 Sequence alignments

DNA sequences were analysed and aligned using the DNAMAN program. DNA and protein sequences of a selected number of species were obtained from the database and aligned using the online CLUSTALW algorithm at the EBI, Cambridge.

### 4.10 Microinjection of morpholino oligonucleotides and mRNA

TRAP230 splice morpholino oligonucleotide (MO) was purchased from GeneTools LLC. The MO, designed to target the exon26-intron26 splice junction, has the
sequence CAGATCCTCTAAAAATCATCACCTG. A MO stock solution was formed by dilution in water and was stored at –20°C prior to use. Embryos were injected at the single cell stage with 0.25 mM MO.

sox9b-mRNA was synthesized using the SP6 mMessage mMMachine kit (Ambion) from a full-length clone obtained at RZPD (IMAGp998A0514858Q) and injected at single cell stage with a concentration of 90ng/µl.

## 4.11 Further molecular standard techniques

DNA restriction digests and agarose gel electrophoresis (1-2% agarose, TAE buffer) were performed according to standard protocols (Sambrook, 1989). Ethidium bromide was added in a 1:10000 dilution directly to the gels prior to solidifying. Gels were used for gel extraction or photography on a gel documentation station.

Bacterial mini preparations were done using an Eppendorff FastPlasmid Mini kit.

DNA was eluted from agarose gels by cutting the respective piece out of gel under long-wave UV conditions (λ=366nm) and subsequently purifying it using a Qiagen MinElute kit and eluted in a final volume of 10µl.
5 References


REFERENCES


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6 Appendix
### 6.1 Sequence alignments

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<td><strong>ATTAAAGGCAAGGGCAGCTTTGACCA</strong></td>
<td><strong>ATTAAAGGCAAGGGCAGCTTTGACCA</strong></td>
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**氨基酸序列对齐和预测**
Figure 50. Alignment of zebrafish Trap230 sequences.

Trap230_WT: self-cloned wild type sequence from Tübingen strain; Trap230_tpd25870: mutant sequence; Trap230_prediction: sequence as predicted by zebrafish genome annotation; Note differences between prediction and self-cloned sequence and the actual mutation around 1360bp.

Alignment was done using Clustalw.
Figure 51. Alignment of Trap230 and related proteins across species (Clustalw).

z_Trap230: zebrafish Trap230, own cloned sequence; z_Trap230_pred: zebrafish Trap230, predicted sequence; d_kto: Drosophila kohtalo (Trap230 homologue); m_TNRC11: mouse TNRC11 (Trap230 homologue); h_MED12: human MED12 (Trap230 homologue); h_MED12L: human MED12L (Trap230-like); c_TNRC11: chick TNRC11 (Trap230 homologue); f_Trap230: Takifugu rubripes Trap230; sc_srb8: Saccharomyces cerevisiae srb8 (Trap230 homologue); ss_srb8: Schizosaccharomyces pombe srb8 (Trap230 homologue); d_skd: Drosophila skuld (Trap240 homologue); h_MED13: human MED13 (Trap240 homologue); h_MED13L: human MED13L (Trap240-like). Chick sequence part of 3086AA.
6.2 Phylogenetic trees

Figure 52. Cladogram of Trap230 and related proteins across species (Clustalw).

Abbreviations as in Figure 52.

Figure 53. A phylogenetic tree for Sox9-related genes.

(Cresko et al., 2003) Numbers are bootstrap values for 1,000 trials. Dre, *Danio rerio*, zebrafish; Gac, *Gasterosteus aculeatus*, threespine stickleback; Gga, *Gallus gallus*, chicken; Hsa, *Homo sapiens*, human; Mal, *Monopterus albus*, rice eel; Mmu, *Mus musculus*, mouse; Tru, *Takifugu rubripes*, pufferfish. Dre Sox8 AW153579; Dre Sox9a AYO00034; Dre Sox9b AAG09815; Dre Sox10 AF402677; Gga Sox9 U12533; Gga Sox10 AF152356; Gga Sox8 AF228664; Hsa Sox8 NP_055402; Hsa Sox9 NP_000337; Hsa Sox10 NP_008872; Mal Sox9a AF378150; Mal Sox9b AF378151; Mmu Sox8 XP_128601; Mmu Sox9 NP_035578; Mmu Sox10 XP_128139; Tru Sox9a AAL32172 (mayfold000587); Tru Sox9b mayfold 000421 (fugu assembly 3 at http://fugu.hgmp.mrc.ac.uk/). Mediator complex components.
### Mediator complex components

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<th>S. pombe</th>
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<th>C. elegans</th>
<th>H. sapiens*</th>
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<td>MDT-1.1</td>
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**Figure 54. Mediator subunits. New nomenclature and comparison of synonyms across species.**

(Bourbon et al., 2004)

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<tr>
<th>New name</th>
<th>S. cerevisiae*</th>
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</tr>
</tbody>
</table>

**Figure 54. Mediator subunits. New nomenclature and comparison of synonyms across species.**

(Bourbon et al., 2004)

*From SGD.
*From WormBase.
*From FlyBase.

*Acronyms given to MEDs identified from various mammalian MED-like complexes (Malik and Roeder, 2005). Many of the components listed under Others recently have been found in both the larger and smaller complexes; however, the MED12, MED13, COK5, and CycC components clearly are not present in the smaller complexes, consistent with their absence in a subpopulation of yeast Mediator complexes. Asterisks indicate that the corresponding proteins have not yet been identified in purified MED complexes.
Figure 55. Comparison of mammalian Mediator-like complexes.

(Conaway et al., 2005) Mediator subunits identified in different Mediator preparations are indicated with blue; Mediator subunits not identified are indicated with yellow. Subunits are grouped according to which module they are believed to be in. On the left, composition of S.cerevisiae Mediator is summarised in comparison. On the right, a summary of Mediator components identified by MudPIT (multidimensional protein identification technology) via immunoaffinity chromatography of nuclear extracts prepared from extracts of HeLa cells expressing the indicated subunits with an N-terminal epitope tag with the amino sequence Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (the FLAG epitope). f denotes the FLAG epitope; proteins identified following immunoaffinity chromatography of extracts from parental HeLa or 293 cells are indicated.
Figure 56. Location of conserved regions within human Mediator subunits.

(Blazek et al., 2005) Extended stretches of high homology can be found in the MED23 and MED31 proteins and the Cdk8/Cyclin C pair. Regions that share at least 20% homology with the corresponding sequences in the respective Drosophila melanogaster subunits are coloured according to the degree of identity. Systematic as well as common names of human and corresponding yeast subunits are listed. In addition, tentative locations of subunits within the Head, Body and Kinase (KIN) module of the complex have been assigned. Sequences were aligned using the SIM program (BLOSUM62 Matrix) and visualized with the LALNVIEW (Duret et al., 1996) program (both available at http://www.expasy.ch). The data were extracted for further manipulation. Alignments of up to 20 regions per subunit were generated. Within the LALNVIEW program the "similarity score threshold" was set to a value above 40 to ignore insignificant alignments.
## APPENDIX

<table>
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<tr>
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**Figure 57. The role of Mediator in cellular signalling.**

(Blazek et al., 2005) Transcription factors that exert their function via the Mediator complex are listed along with their target subunit within the Mediator complex and the corresponding reference(s)
6.4 Abbreviations

SI units (système international d’unités) and symbols of standard multiples (m, µ, etc.) are not listed below. Additional abbreviations are introduced in the text at the site of their first appearance.

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<td>amino acid</td>
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<td>AER</td>
<td>apical ectodermal ridge</td>
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<td>AT</td>
<td>adénin-thymine</td>
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<td>bovine serum albumin</td>
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<td>CA</td>
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<td>centiMorgan</td>
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<td>MO</td>
<td>morpholino oligonucleotide</td>
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Short Summary

This thesis deals with the mapping and cloning of trapped (tpd), a novel zebrafish mutant found to disrupt an ortholog of Trap230, a member of the Mediator complex. Mediator is a coactivator complex transducing the interaction of DNA-binding transcription factors with RNA polymerase II.

The vertebrate Sox9 transcription factor directs the development of neural crest, otic placodes, cartilage, and bone. In zebrafish, there are two Sox9 orthologs, Sox9a and Sox9b, which together perform the functions of the single-copy tetrapod Sox9. The mutant phenotypes of tpd and the Sox9a/Sox9b double mutant are remarkably similar. The results of this thesis show that Trap230 is required for Sox9 activity. In addition, I show that Trap230 is required for an early step in pectoral fin induction, indicating that it also participates in Sox9-independent signaling events. Moreover, additional phenotypes in brain, eye, heart, muscle and gut development as well as axon guidance and a general downregulation phenotype of fibroblast growth factor expression in tpd mutants are described. These phenotypes may or may not be Sox9-dependent. This is the first characterisation of a vertebrate Trap230 mutant, and reveals a surprisingly specific requirement for Trap230 in mediating Sox9 activity.
Zusammenfassung


Erklärung

Ich versichere, daß ich meine Dissertation

"Zebrafish Trap230/Med12 is required for Sox9 activity and limb induction"

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den 08. November 2005

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A. Education and research

University of Marburg, Marburg, Germany: 1998 to present

10/2002 to present  PhD student of Dr. Renate Renkawitz-Pohl at the University of Marburg in the lab of Dr. Carl Neumann at the EMBL, Heidelberg

08/2002  German Diploma degree (grade 1.3)

11/2001– 08/2002  Diploma student of Dr. Renate Renkawitz-Pohl in the lab of Dr. Christof Niehrs at the DKFZ Heidelberg, working on the role of Unc5H2 in *Xenopus laevis* axis development

10/2001  final exams in developmental biology, cell biology/applied botany and informatics

04/2001-07/2001 and 12/2000-02/2001  Research student in the lab of Dr. Renate Renkawitz-Pohl at the University of Marburg, characterising the function of *alien* in *Drosophila melanogaster* by RNAi, yeast-two-hybrid screening and expression in S2-cells

02/2001-03/2001  Internship in the lab of Dr. Christof Niehrs at the DKFZ Heidelberg, characterising *Xenopus laevis* Dkk1 by morpholino microinjection and in situ hybridisation

07/2001 and 08/2000-10/2000  Research student in the lab of Dr. Tina Trenczek at the University of Giessen, isolating and purifying an innate immunity protein from *Manduca sexta*

04/2000-02/2002  Economic studies for scientists and engineers (degree Diplomwirtschaftsbiologin) at the Fernuniversität Hagen

04/2000-10/2000  Research student in the lab of Dr. Uwe Homberg at the University of Marburg, immunological work on the brain development of *Manduca sexta*

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Main studies in biology (diploma degree), subjects: developmental biology, cell biology/applied botany, informatics

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Basic studies in biology and informatics (diploma degree) and in biology and English (highschool teaching degree)

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B. Scholarship:

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Rau MJ, Fischer S, Neumann CJ: Zebrafish Trap230/Med12 is required for Sox9 activity and limb induction; in revision


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