Temporal and spatial action of Tolloid (Mini fin) and Chordin to pattern tail tissues

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Abstract

In vertebrates, a bone morphogenetic protein (BMP) signaling pathway patterns all ventral cell fates along the embryonic axis. BMP activity is positively regulated by Tolloid, a metalloprotease, that can eliminate the activity of the BMP antagonist Chordin. A toloid mutant in zebrafish, mini fin (mfn), exhibits a specific loss of ventral tail tissues. Here, we investigate the spatial and temporal requirements for Tolloid (Mfn) in dorsoventral patterning of the tail. Through chimeric analyses, we found that Tolloid (Mfn) functions cell non-autonomously in the ventral-most vegetal cells of the gastrula or their derivatives. We generated a toloid transgene under the control of the inducible hsp70 promoter and demonstrate that toloid (mfn) is first required at the completion of gastrulation. Although toloid is expressed during gastrulation and dorsally and ventrally within the tail bud, our results indicate that Tolloid (Mfn) acts specifically in the ventral tail bud during a ∼4 h period extending from the completion of gastrulation to early somitogenesis stages to regulate BMP signaling.

Examination of the temporal requirements of Chordin activity by overexpression of the hsp70-tolloid transgene indicates that Chordin is required both during and after gastrulation for proper patterning of the tail, contrasting Tld’s requirement only during post-gastrula stages. We hypothesize that the gastrula role of Chordin in tail patterning is to generate the proper size domains of cells to enter the ventral and dorsal tail bud, whereas post-gastrula Chordin activity patterns the derivatives of the tail bud. Thus, fine modulation of BMP signaling levels through the negative and positive actions of Chordin and Tolloid, respectively, patterns tail tissues.

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Keywords: Tolloid; Metalloprotease; BMP; Chordin; Pattern formation; Tail; Tail bud; Dorsal–ventral; Heat-shock

Introduction

Dorsoventral pattern formation of the primary body axis depends on a bone morphogenetic protein (BMP) signaling pathway. Repression of BMP activity is necessary for neural development in dorsal regions and is mediated by BMP-binding antagonists, such as Chordin (Chd), Noggin, and Follistatin, which are secreted from dorsal tissue (reviewed in De Robertis et al., 2000; Munoz-Sanjuan and H.-Brivanlou, 2001). Models of dorsoventral pattern formation suggest that BMPs act as morphogens, with high levels of activity specifying ventral cell fates and lower levels specifying more lateral cell fates. Antagonists of BMP signaling are thought to establish a gradient of BMP activity as bmps are expressed relatively uniformly in ventral regions. A gradient of BMP antagonists, high dorsally and low laterally, sets up a reciprocal, post-translational BMP activity gradient that is high ventrally and low dorsally.

Analysis of dorsalized and ventralized zebrafish mutants demonstrates genetically the requirement for BMP signaling in patterning the vertebrate dorsoventral axis (reviewed in Hammerschmidt and Mullins, 2002). The zebrafish mutants swirl (bmp2b) (Kishimoto et al., 1997; Nguyen et al., 1998b), snailhouse (bmp7) (Dick et al., 2000; Schmid et al., 2000), somitabun (smad5) (Hild et al., 1999), and lost-a-fin (alk8) (Bauer et al., 2001; Mintzer et al., 2001) are defective in BMP pathway signaling components. These mutants display a loss of ventral gastrula-derived structures such as the tail, epidermis, blood, and pronephros and exhibit a concomitant expansion of dorsal cell fates such as neurectoderm and anterior somitic mesoderm. The ventralized zebrafish mutant chordino (chordin) confirms the necessity for BMP signal inhibition during early development (Fisher et al., 1997; Hammerschmidt et al., 1996a; Schulte-Merker et al., 1997). chordino mutants are characterized...
by multiplied tail fins, increased blood, and a variable reduction of the head, eyes, and anterior somitic mesoderm.

Chordin (Chd) is itself inhibited by the astacin metalloprotease Tolloid (Tld) (Blader et al., 1997; Marqués et al., 1997; Piccolo et al., 1997). The tld gene was originally identified as a *Drosophila* mutant defective in dorsoventral patterning and thought to enhance activity of Dpp, the *Drosophila* *bmp*2/4 homolog (Shimell et al., 1991). The discovery that Tld cleaves Chd in vitro, but not Noggin or Follistatin, and can inhibit Chd function in vivo (Blader et al., 1997; Marqués et al., 1997; Piccolo et al., 1997) provided a mechanism by which Tld increases BMP activity.

Several tolloid-related genes have been identified in vertebrates. Gene targeting experiments in mouse have not revealed a role for either of the mouse tolloid-related genes, *Bmp1* or *tl1*-1, in dorsoventral patterning (Suzuki et al., 1996; Clark et al., 1999). However, Tld, Xld, and XBMP1 when overexpressed ventralizes fish and frog embryos and were suggested to act during gastrulation to pattern the dorsoventral axis of the embryo (Blader et al., 1997; Goodman et al., 1998; Piccolo et al., 1997). We found that the dorsalized zebrafish mutant mini fin (mfn) encodes tld (Connors et al., 1999), demonstrating that at least one vertebrate *tld* acts in dorsoventral patterning. Homozygous mfn mutants show a variable loss of the ventral tail fin, posterior somites, and vasculature. The mfn mutant phenotype is less severe than *swirl* (*bmp2b*) or *snailhouse* (*bmp7*) homozygous mutants, a relationship similar to *Drosophila* tld and dpp mutants.

The zebrafish *tld* (mfn) gene is expressed throughout gastrulation in a ventral-animal domain, as well as dorsally and ventrally around the margin. In post-gastrula stages, it is expressed dorsally and ventrally in the tail bud with its dorsal limit of expression directly abutting chd expression (Connors et al., 1999). However, it is unknown if some or all of these expression domains are crucial to Tolloid’s role in tail patterning. Moreover, the period when Tld and Chd modulate BMP signaling levels to mediate the correct patterning of the tail is not known. Here, we investigate the spatial and temporal mechanism by which Tld and Chd activity regulate BMP signaling in tail patterning.

We show by inducing *tld* expression via a heat-shock promoter transgene that *tld* is first required just after gastrulation for proper tail development. These results together with chimeric studies indicate that *tld* is required in the ventral (posterior) tail bud, although the dorsal (anterior) tail bud expression domain of *tld* lies in closer proximity to chd-expressing cells in the tail bud. Furthermore, using the heat-shock-inducible *tld* transgene, we found that Chd functions in tail patterning both during and after gastrulation. We postulate that graded BMP signaling, achieved at least in part by Chd and Tld in the tail bud, patterns dorsoventral derivatives of the tail.

Materials and methods

*Generation of transgenic zebrafish and phenotypic analysis*

The coding region of an myc-tagged tolloid (tolloid-MT; Blader et al., 1997) was cloned downstream of the *hsp70/4* promoter (Halloran et al., 2000), resulting in the *hsp70-tld* construct. The *hsp70* promoter drives gene expression ubiquitously in the embryo (Halloran et al., 2000). Transgenic lines were generated by injecting one cell *mfntm124a/mfntm124a* embryos with approximately 1 nl of 50 µg/ml supercoiled *hsp70-tld* DNA and 100 pg of tld mRNA. Synthetic *tld* mRNA was made from pCS2:Ztld-3MT (Blader et al., 1997) using the mMessageMachine (Ambion) system. To identify founders with germline transmission, injected embryos (F0) were raised to adulthood and were crossed to each other. DNA from pools of the resultant progeny (50–75 embryos) was made by overnight incubation in lysis buffer (10 mM Tris (pH 8.0), 100 mM EDTA, 0.5% SDS, 100 µg/ml Proteinase K, and 20 µg/ml RNase A) followed by phenol/chloroform extraction and ethanol precipitation. PCR was performed to identify the presence of the transgene in the F1 progeny (forward primer (*hsp* promoter): TCCCCGACGAGGTATTCTC; reverse primer (tld): AGTC-TAACACAGCTGTCACAG). Alternatively, the F1 embryos were heat-shocked during gastrulation for 1 h at 37°C and scored at 1 dpf for rescue of the mutant phenotype. F0 fish positive for germline transmission were crossed to nontransgenic *mfntm124a/mfntm124a* to establish an F1 generation. *mfntm124a* transgenic carriers are heterozygous for the *Tg(hsp70-tld)* and were crossed to nontransgenic *mfntm124a* mutants to generate F2 embryos. Thus, 50% of the F2 embryos are expected to carry the transgene. All experiments were performed in F2 embryos, and the 50% non-transgenic embryos served as controls for each experiment. Multiple transgenic lines were used to confirm all results unless otherwise indicated.

Of 60 injected fish, 8 were positive for the transgene as determined by PCR. The transgene was found to be inducible in 7 of the 8 positive fish. Of 90 fish tested for germline transmission via heat induction, 11 were found to carry the transgene. Thus, of 150 F0 fish tested, 18 inducible founders (12%) were identified and kept to analyze. Transmission through the germline of the F0 founders ranged from 1 to 19%. Several founders were discarded because of low transmission rate, phenotypes associated with the heat-shock, or leakiness of the transgene as seen by rescue or partial rescue of the *mfn* phenotype in the absence of heat-shock treatment. Nine lines were used for experimentation.

*Heat induction of the hsp-tolloid transgene and genotyping transgenic embryos*

For most experiments, heat-shock induction of the *Tg(hsp70/tld)* was performed in a water bath at 37°C for 10 min. Embryos were transferred from embryo medium at 29°C to medium at 37°C for heat-shock treatment and then transferred back to 29°C medium for the remainder of development. All embryos were scored at 1 dpf for rescue of the *mfntm124a* mutant phenotype. Duplex PCR with the genotyping primers listed above and a positive control (*Bmp7*; forward: TTTGATGACAGTCCAAACG, reverse: TCAGAAATCAAGTG-CAATG) was used to verify that phenotypically rescued fish carried the *Tg(hsp70/tld)*, alternatively, a PCR machine was used for heat-shock treatments (Chordin inhibition experiments). Embryos in E3 medium were heat-shocked in 96-well plates. One round (1+) of inhibition included 1 h of heat-shock treatment at 37°C followed by 2 h at 29°C.

*Western blot analysis*

Decelerated embryos were lysed by pipetting in 2× Laemmli sample buffer (2.5 µl per embryo). An equal amount of distilled water was then added. Samples were boiled for 5 min, centrifuged for 5 min, then subjected to SDS-PAGE analysis in a 7.5% gel (2 embryos per lane). After transfer to PVDF, membranes were probed with a 1:1000 dilution of anti-myc 9E10 antibody followed by a 1:5000 dilution of HRP-conjugated sheep anti-mouse antibody. Western blots were developed with ECL-Plus Kit (Amersham Biosciences) according to manufacturer’s instructions.

*Generation of chimeric animals*

Wild type embryos were cojected with rhodamine-dextran (10,000 MW, Molecular Probes) and *tolloid* mRNA (150–300 pg) at the one-cell stage. This level of mRNA produced weak to moderate ventralization in wild type embryos. At the sphere stage, cells were removed from a wild type donor and transplanted into the margin of a *mfntm124a/mfntm124a* at *mfntm15a*/*mfntm15a* host. Homozygous *mfn* mutant adults were intercrossed to generate the hosts. Hosts were sorted...
between the shield and bud stages of development for the position of the transplanted cells. Embryos were photographed and allowed to develop until 1 dpf at which time they were scored for rescue of the mfn phenotype. Fluorescent and DIC images of the mosaics were captured with a Leica DC200 camera.

**In situ hybridizations**

In situ hybridizations were performed with standard methods, using the following probes: tld (Blader et al., 1997), chd (Miller-Bertoglio et al., 1997), bmp4 (Chin et al., 1997), dx3 (Akimenko et al., 1994), eve1 (Joly et al., 1993), fkl1 (Thompson et al., 1998), mnxB (Akimenko et al., 1995), mnxD (Akimenko et al., 1995), myoD (Weinberg et al., 1996), Krox 20 (Oxtoby and Jowett, 1993), pax2.1 (Krauss et al., 1991), and gata1 (Detrich et al., 1995). In situ hybridizations were performed on at least 10 mutant embryos, 1/2 of which carried the transgene. Embryos were cleared in benzylbenzoate:benzylalcohol (2:1) and were scanned with a Progres 3012 digital camera (Kontron Elekoniks). All images were processed with Adobe software.

**Results**

**Tolloid activity is sufficient ventral-vegetally**

Tolloid is expressed at low levels in both dorsal and ventral regions of the embryo before gastrulation. During gastrulation, tololoid expression becomes restricted to a ventral domain, except around the margin of the gastrulating embryo, where it is strongly expressed both dorsally and ventrally (Blader et al., 1997; Connors et al., 1999). In a first step to determine which of these tololoid gene expression domains is sufficient to antagonize Chordin in dorsoventral patterning, we generated chimeric embryos to examine where tld-expressing cells must be present to rescue the mfn mutant phenotype. We transplanted wild type cells cojected with tld mRNA (see below) and a lineage tracer into mfn homozygous mutant embryos at sphere stage, a time point before cell fate commitment (Ho and Kimmel, 1993). Embryos were sorted during or at the end of gastrulation (bud stage) into one of four groups based on the location of the transplanted wild type cells in either a dorsal, lateral, animal-ventral, or vegetal-ventral region (Fig. 1A). Embryos were allowed to develop to 24–30 h post-fertilization (hpf) when they were scored morphologically for rescue of the mfn mutant phenotype.

We found that Tld-expressing cells present in dorsal, lateral, or animal-ventral domains of the embryo rarely, if ever, rescued the mfn phenotype (Figs. 1A–C). When rescue was observed, it appeared relatively weak: either a small reduction of the posterior ventral fin was still apparent or the ventral tail fin was thin, i.e. not as wide as a wild type fin (data not shown). Because of the variability of the mfn phenotype, it is difficult to assess whether these weak phenotypes were the result of partial rescue of a strong mfn phenotype or represented an unrescued, weak mfn phenotype that is occasionally seen in mfn mutant clutches (Connors et al., 1999).

In contrast, Tld-expressing cells that contributed to the ventral-vegetal region rescued the mfn mutant phenotype in approximately 50% of the chimeras (Figs. 1A, D, E). Transplantation of wild type cells that were not injected with tld-mRNA did not rescue the mfn mutant. This is likely due to the inability to target sufficiently large numbers of cells to the ventral tail bud, due to the extensive dorsal convergence during gastrulation, combined with the negative autoregulation of bmp component gene transcription (Schmid et al., 2000; Nguyen et al., 1998b; Schulte-Merker et al., 1997), which turns off tld gene expression when Bmp signaling is reduced (Connors et al., 1999). However, the ability of ventral-vegetally located tld-injected cells to rescue the mfn mutant indicates that Tld activity is most critical in the ventral marginal domain of the gastrula and/or ventral (posterior) regions of the tail bud (or its derivatives) to modulate Chordin antagonism of BMP signaling and pattern ventral tail cell fates. Thus, although the dorsal–marginal tld expression domain lies closest to the chd expression domain (Connors et al., 1999), this domain does not appear crucial to Tld function.

At 24 to 30 hpf, we examined the chimeric embryos for the location of the transplanted wild type cells in rescued mfn embryos. Interestingly, transplanted cells rarely gave rise to clones of cells in the ventral tail fin (Fig. 1E). Descendants of the Tld-expressing transplanted cells were almost always in the trunk or anterior tail somites and occasionally also gave rise to epidermal cell fates in the tail. These results demonstrate that Tld functions cell non-autonomously to promote the development of ventral tail tissues.

**Generation of tololoid transgenic zebrafish**

**tld (mfn)** expression begins before gastrulation and extends throughout and beyond gastrulation. The chimeric studies above indicate a ventral-vegetal spatial requirement for tld expression but do not reveal when Tld is modulating BMP signaling. To determine the temporal action of Tld (Mfn) in antagonizing Chordin, we generated transgenic homozygous mfn mutant fish carrying the tld cDNA under the control of the heat-shock70-inducible promoter (Halloran et al., 2000). Founder fish carrying the heat-shock promoter tld transgene (Tg(hsp70::tld)) were generated and used to produce independent F1 lines. Heat induction of the transgene in F2 embryos rescued the mfn mutant phenotype to a wild type (Figs. 2A, B) or ventralized phenotype (Fig. 2C) in the expected 50% of the progeny (see Materials and methods). PCR was used to verify that the observed rescue correlated with the presence of the transgene (Fig. 2M). The heat-shock treatment had no significant effect on wild type or non-transgenic mfn mutant embryos, although development was slightly retarded by repeated heat-shock treatments (data not shown).

Molecular marker analysis demonstrates that the Tg(hsp70::tld) rescued the expression of genes that are normally reduced in mfn mutant embryos (Connors et al., 1999). Wild type levels of bmp4 (Figs. 2D–F) and eve1 (Figs. 2G–I) expression were restored in the ventral tail bud after transgene induction. Furthermore, mnxB (Figs. 2J–L) expression in the median fin fold returned to normal, correlating with the morphological rescue of the fin seen at 1 day post-fertilization (dpf). These results show that the Tg(hsp70::tld) can rescue all aspects of the mfn mutant phenotype from bud stage to 1 dpf.
We next verified that in gastrulation stage embryos the transgene is induced shortly after the start of the heat-shock treatment. We examined expression of the Tg(hsp70:tld) via RNA in situ hybridization at different time points following the start of heat induction. Expression of the Tg(hsp70:tld) was visible within as little as 10 min of initiating heat induction and was not spatially restricted (Figs. 3A–C and data not shown). Expression of the transgene was much stronger than endogenous tld expression. We also examined the expression of the myc-Tld protein at various time points following the start of heat-shock (Fig. 3G). Anti-myc Western blot analysis of heat-shocked embryos shows that Tld protein is evident within 30 min after the start of a 10-min heat-shock. Peak protein levels are reached by 1 h, and Tld protein is present through 2.5 h after the start of heat induction. We examined the response of eve1 expression, a gene downstream of tld and the BMP pathway (Connors et al., 1999; Mullins et al., 1996), as an indication that Tld protein was active soon after transgene induction and could upregulate BMP activity. eve1 expression was increased in the transgenic embryos at 1 h after the start of the heat-shock (Figs 3D–F), indicating that BMP activity had increased in the embryo by that time. Thus, the Tg(hsp70:tld), Tld protein and Tld activity are induced shortly after heat-shock treatment, indicating that the Tg(hsp70:tld) is an effective tool to examine the temporal requirement for Tld function in embryonic development.

Fig. 1. Tolloid activity is required in ventral-vegetal cells. (A) Schematic of cell transplantations (location of wild type cells in red) in gastrula or bud stage embryos, N values and percent of rescue observed. The dorsal group also includes dorsolateral clones (0/32 rescued). Not included are 7/30 (23%) cases of rescue seen in transplants that were scored as ventrolateral at the margin. (B) Fluorescence image of an embryo at bud stage with cells located dorsally and (C) the same embryo at 24 hpf displaying the mfn phenotype, indicating no rescue. (D) Fluorescence image of an embryo at bud stage with transplanted cells located ventrally, and (E) the same embryo at 24 hpf shows rescue of the mfn phenotype. (C, E) Fluorescence images are overlaid on the bright field image to show the location of transplanted cells at 24 hpf. All are lateral views except (D), which is slightly ventral-lateral. (A, B, D) Dorsal is to the right and anterior is up. (C, E) Anterior to the left.
Tolloid acts at the end of gastrulation

To establish when Tld is first required for patterning the embryo, the Tg(hsp70:tld) was induced in mfn mutants at different stages of development. Rescue of the mutant phenotype to wild type was assayed morphologically at 1 dpf. As little as 6- to 10-min heat-shock during or at the end of gastrulation rescued fully all mfn mutants carrying any one of several of the transgenes (Fig. 3H, blue line, Table 1). Induction of tld expression at stages following the end of gastrulation revealed a progressive decline in the ability to rescue the mfn mutant embryos to a wild type phenotype (Table 1). This progressive decline in rescuing ability, as opposed to a dramatic decrease, likely reflects the variability in the strength of the mfn mutant phenotype. tld expression at the end of gastrulation may be required to rescue strongly affected mfn mutants, whereas tld expression during early somitogenesis may be sufficient to rescue more weakly affected mfn mutants. The results show that heat-shock-induced tld expression during early, mid, or late gastrulation and early somitogenesis can rescue the mfn mutant phenotype. However, the latest time point at which tld expression can rescue all mfn mutants is the end of gastrulation. These results indicate that tld is not required until this stage for normal embryonic development.

Because heat-shock induction of Tg(hsp70:tld) rescued equally well during early gastrulation, late gastrulation, and bud stages of development, we further investigated the critical time period of Tld activity by heat-shocking embryos for a period of time that was “sub-optimal.” We reasoned that strong induction of tld would create high levels of protein that might rescue the mfn phenotype over several stages of development. This possibility was strengthened by the observation that the Tg(hsp70:tld) is more highly expressed than endogenous tld (Figs. 3A–C, and data not shown). Therefore, we tested whether shorter heat-shock induction times, and consequently lower levels of Tld, would reveal that Tld was critical during a smaller window of development.

Fig. 2. An hsp-tolloid transgene rescues the mini fin phenotype. All embryos were heat-shocked for 10 min at 37°C during late gastrulation. (A–C) Heat-shocked embryos at 1 dpf. Non-transgenic mfn embryos (A) still display the mfn phenotype (arrowheads indicate missing fin), but transgenic mfn embryos are rescued to a phenotype indistinguishable from wild type (B) or are ventralized (C), as demonstrated by excess blood (arrow) and duplicated tail fins (arrowhead). Heat-shock-treated mfn transgenic (E, H, K) and non-transgenic sibling (D, G, J) and wild type (F, I, L) embryos. (D–F) bmp4 expression at bud stage. (G–I) eve1 and goosecoid (gsc, as a control) expression at the 1-somite stage (N = 18). (J–L) msxB expression at the 20-somite stage (N = 18). Arrowheads indicate absent msxB expression domain in mfn (I), which is restored in the transgenic mutant (K). (D–L) Lateral views, dorsal to the right, anterior to the top. (M) Duplex PCR for Tg(hsp70:tld) and bmp7. All rescued wild type or ventralized embryos carry a copy of the Tg(hsp70:tld) and endogenous bmp7 (control), whereas non-rescued mutants carry only the bmp7 gene. PCR from two different transgenic lines are shown (top, line 30; bottom, line 1).
Fig. 3. Temporal rescue of mini fin mutants by hsp-tld. (A–C) tld and (D–F) eve1 expression in transgenic mfn mutant embryos heat-shocked for 10 min during late gastrulation (85–90% epiboly). Expression of tld (N = 30) and eve1 (N = 15) in embryos fixed before the heat-shock treatment (A, D). Expression of tld 10 (N = 33) (B) or 30 (N = 43) (C) min after the start of the heat-shock. Expression of eve1 1 h after the start of the heat-shock in non-transgenic (E) and transgenic embryos (N = 15) (F). (G) Expression of myc-tagged Tld protein after a 10-min heat-shock, or in the last lane a 60-min heat-shock, during late gastrulation. Embryos were collected at stated time points following the start of the heat-shock period and assayed by anti-myc Western blot analysis. A lower non-specific cross-reacting band serves as a loading control. (H) Graphic representation of rescue of mfn transgenic embryos after a 10-min heat-shock treatment at different time points during development. The blue graph indicates an average of the percent rescue seen in multiple transgenic lines heat-shocked. The red graph shows a “sub-optimal” 7-min heat-shock performed in transgenic line 75, so that less than 100% of the transgenic embryos at any time point were rescued.
In these “sub-optimal” experiments, we determined the minimal heat-shock time required to rescue 100% of the transgenic embryos. We found that a 10-min heat-shock rescued all transgenic mutants of the Tg(hsp70:tld) line 75. We then decreased the heat-shock time to 7 min to determine if a heat-shock early in gastrulation was as effective as a heat-shock later in gastrulation or at the bud stage (end of gastrulation). A 7-min heat-shock of mfn; Tg(hsp70:tld) line 75 embryos at early gastrulation did not rescue the mutant phenotype as robustly as a heat-shock at very late gastrulation or bud stage (Fig. 3H, red line, Table 1). After the bud stage, the ability of the tld transgene to rescue the mfn mutant declined similarly to that seen for a 10-min heat-shock. These results suggest that at the end of gastrulation or stages just following the completion of

![Image](https://via.placeholder.com/150)

**Fig. 4.** Overexpression of tollloid phenocopies the chordino mutant. A Tg(hsp70:tld) embryo heat-shocked during and after gastrulation to remove Chd activity (A) can phenocopy din/chd mutants (B). Eye and head size is reduced, the blood increased (arrow), and the tail fins duplicated (arrowhead). Dorsal-anterior views of a representative 7-somite stage non-transgenic mfn embryo (N = 37) (C), transgenic mfn embryo (N = 42) (D), or din homozygote (E) stained for myoD, krox20, gata1, and pax2.1. R3 and R5 refer to rhombomeres 3 and 5, respectively. (F–H) More posterior dorsal views of embryos shown in panels (C–E). Arrows indicate smaller somites, and arrowheads indicate more laterally located pronephric and blood precursor domains in transgenic and din mutants.

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**Table 1** Temporal rescue of the mini fin mutant phenotype by Tg(hsp70:tld)

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a Up to 50% rescue is expected since heterozygous transgenic fish were crossed to non-transgenic fish yielding half of the progeny carrying the transgene.
gastrulation are the critical time points when Tld (Mfn) activity is first required for proper patterning of the tail.

Chordin function during and after gastrulation

We used the Tg(hsp70:tld) fish to examine the role of Chd in dorsoventral pattern formation during embryonic development. Because Tld cleaves and inactivates Chd (Marqués et al., 1997; Piccolo et al., 1997), the Tg(hsp70:tld) lines allowed us to induce tld expression and thus remove Chd activity from the embryo at different time points during development. We first investigated the ability of Tg(hsp70:tld) to phenocopy the chordino/chordin mutant phenotype, which would indicate that most or all Chd activity could be removed from transgenic embryos. Repeated heat-shock treatments between the high stage, a time point before chd is expressed in the embryo, until the 16–19 somite stage was used to eliminate Chd activity. In these experiments, 6 rounds of heat-shock treatment were performed, where one round corresponds to a 1 h heat-shock treatment at 37°C, which induces robust amounts of Tld protein (Fig. 3G), and a 2 h recovery at 29°C. Transgenic embryos at 30 hpf looked remarkably similar to homozygous chordino mutant embryos (Figs. 4A, B), displaying excess amounts of blood, multiplicated ventral tail fins, and a reduced head and eye size. These observations indicate that the Tg (hsp70:tld) was effective in strongly reducing or eliminating Chd activity.

We examined the expression of molecular markers to evaluate further if the observed ventralized phenotype in multi-heat-shock-treated Tg(hsp70:tld) embryos phenocopied the chordino mutant phenotype. Embryos at the 6-somite stage showed defects consistent with the loss of Chd activity, such as a reduction of the neural plate and anterior somitic tissue. pax2.1 expression in the prospective midbrain–hindbrain boundary and krox20 expression in rhombomeres 3 and 5 were clearly reduced in domain size, similar to that observed in homozygous chordino mutants (Figs. 4C–E, and Fisher et al., 1997; Hammerschmidt et al., 1996a). Similar reductions in the

Fig. 5. Chordin activity is required during gastrulation and segmentation stages. (A) Non-transgenic mfn and (B) Tg(hsp70:tld); mfn embryo heat-shocked twice for 1 h, at high stage (~3.5 hpf) and then early gastrula stage (~6.5 hpf) (2× early). (C) Representative Tg(hsp70:tld); mfn embryo heat-shocked for 6 rounds from pregastrulation to the 18-somite stage (6× early, N = 10). Note the stronger ventralization in the embryo lacking Chd activity during both gastrulation and somitogenesis (C). Non-transgenic (D) and Tg(hsp70:tld) (E–F) mfn embryos heat-shocked 4 times between the bud and 18-somite stages. A weak ventralized phenotype is observed as excess blood (arrow), blistering of the ventral tail fin (arrowhead in panel E), and small duplications of the ventral tail fin (arrowhead in panel F). krox20 expression in non-transgenic (G) and transgenic (H) mfn embryos heat-shocked 3 times prior to and during gastrulation (N = 22). krox20 expression in non-transgenic (I) and transgenic (J) mfn embryos (N = 20) at the 9-somite stage that were heat-shocked twice after gastrulation (2× late). A 2× rather than 4× heat-shock was performed so that krox20 expression could be examined at the 9-somite stage. Note a reduction in krox20 expression only when Chd is inhibited during gastrulation.
neural plate were seen in embryos analyzed at bud stage (data not shown). myoD expression in the anterior somitic mesoderm was also reduced, whereas blood and pronephric precursors expressing pax2.1 and gata1, respectively, were laterally displaced (Figs. 4E, G). These defects are also observed in chordino mutants (Fig. 4H and Fisher et al., 1997; Hammerschmidt et al., 1996a). These results show that the Tg(hsp70:tld) can effectively inhibit Chd function.

To determine the contribution played by Chd in patterning tissues during versus after gastrulation, we performed modified heat-shock experiments in the Tg(hsp70:tld) lines. Embryos were heat-shocked beginning prior to gastrulation and extending to either the end of gastrulation (2 to 3 rounds of heat-shock) or to the approximately 18-somite stage (9 h after gastrulation; 6 rounds of heat-shock). Embryos that were heat-shocked during and after gastrulation to remove Chd activity were more strongly ventralized than embryos where Chd activity was removed only during gastrulation (Figs. 5A–C). The transgenic embryos heat-shocked for both periods showed a more severe ventralization of tail tissues, specifically a larger increase in blood cells and stronger duplications of the ventral tail fin. This stronger phenotype provides evidence that Chd modulates BMP activity not only during gastrulation, but also after gastrulation to pattern the dorsoventral axis.

To further test the role of Chd following gastrulation, we removed Chd activity by repeated heat-shock treatments beginning at the end of gastrulation (2 to 4 rounds). Embryos lacking Chd activity during post-gastrula stages showed indications of ventralization, including ventral tail fin blisters and/or small duplications of the ventral tail fin, as well as excess amounts of blood (Figs. 5D–F). Embryos heat-shocked only during post-gastrula stages did not display a reduced head and eye size seen in gastrula heat-shocked embryos (compare Figs. 4A, 5B to Figs. 5E, F and Figs. 5G, H to I, J). Altogether, these data show that Chd activity is necessary both during and after gastrulation for proper formation of ventral-posterior tissues.

Discussion

Utility of heat-shock promoter transgenics

We generated in zebrafish a tld transgene under the control of an inducible heat-shock promoter (Halloran et al., 2000) and show that tld induction can rescue mfn mutants to a wild type phenotype. This method may be generally useful to test for the identification of a gene defective in a mutant. It does not necessitate cloning endogenous gene promoters, a task that can be arduous. Furthermore, it can turn on gene expression to rescue late manifesting mutant phenotypes that are not feasible to rescue by microinjection of mRNA at early cleavage stages due to lack of perdurance of the mRNA.

Heat-shock promoter-driven transgenes are effective tools to study gene function in the zebrafish. They can serve as a tool to analyze the temporal requirement for gene function during development, as we have done here for Tld and Chd. Additionally, for genes that function at multiple stages during development, an inducible transgene can specifically rescue an earlier phenotype, so that later phenotypes may be studied. Moreover, ectopic overexpression can be induced to study gain-of-function phenotypes, as previously shown by Halloran et al., 2000, and we show here.

Tolloid/Mini fin patterns tail tissues after gastrulation

Our heat-shock results indicate that Tld (Mfn) activity is first required at the end of gastrulation for proper embryonic development. BMP pathway mutant analyses indicate that BMPs and Chd act before and during gastrulation in the zebrafish (Fisher et al., 1997; Hammerschmidt et al., 1996b; Mullins et al., 1996; Nguyen et al., 1998b). Since Tld is expressed during gastrulation (Blader et al., 1997; Connors et al., 1999) and it functions to cleave Chd to modulate BMP activity (Blader et al., 1997; Marqués et al., 1997; Piccolo et al., 1997), it might be expected to function at this time when BMPs and Chordin pattern the dorsoventral axis. Here, we show that Tld (Mfn) activity is dispensable during gastrulation and instead is first required at the end of gastrulation to pattern tissue that will form the tail.

Our studies show that ventral tail tissues can be restored in all transgenic mfn mutants, if tld is first expressed at the end of gastrulation. However, a small fraction of presumptive weak mfn mutants were also rescued when Tld was first expressed at the 9- to 10-somite stage (Table 1). Together, these results indicate that Tld patterns tail tissues between the end of gastrulation (10 hpf) and at least the 10-somite stage (14 hpf). These results are supported by recent studies of Pyati et al. (2005), which show through BMP receptor inhibition that BMP signaling is required during similar stages for tail patterning.

Tld could conceivably block all Chd function within the tail bud. However, our heat-shock experiments demonstrate a requirement for Chd activity after gastrulation, indicating that Tld does not block all Chd activity. Rather, proper modulation of BMP activity within the tail bud is achieved through both Tld and Chd activity. If too much or insufficient Chd extends ventrally within the tail bud, BMP activity is lowered below a critical threshold or is increased abnormally, respectively, causing a failure in proper patterning of the ventral tail fin, somite, and vasculature.

We have shown that tld (mfn) does not play an essential role during gastrulation in the zebrafish, although it is expressed at these stages. Multiple tld genes have been found in the frog, mouse, and human. It is possible that another tld-related gene product in zebrafish plays an overlapping role with Tld (Mfn) in regulating Chd activity during gastrulation. There is some evidence to support this notion (Little and Mullins, 2004, S. Fisher, personal communication). In the frog, Xld is expressed uniformly at low levels until neurulation at which time it becomes localized near chd-expressing cells in the chordoneural hinge of the tail bud (Goodman et al., 1998; Piccolo et al., 1997). Xld may play a role similar to zebrafish Tld (Mfn) in regulating Chd activity levels in the frog tail bud to pattern dorsoventral tail tissues.
Tolloid acts cell non-autonomously in the ventral tail bud

During gastrulation, *tld* is expressed strongly dorsally and ventrally around the margin and in a ventral-vegetal region, while *chd* is expressed in a near complementary fashion, but restricted to dorsal regions (Miller-Bertoglio et al., 1997). At the end of gastrulation, the marginal *tld* expression domain becomes part of the tail bud, with expression predominantly ventrally within the tail bud, but also extending into the dorsal tail bud, similar to its dorsal-marginal expression domain during gastrulation (Connors et al., 1999). *chd*-expressing cells continue to be juxtaposed to *tld*-expressing cells in the dorsal tail bud, consistent with these gene products acting to pattern the tail. Within the tail bud region, *tld* is strongly expressed in a ventral domain that partially overlaps with ventral *bmp4* tail bud expression, but it extends closer to the dorsally located *chd* expression domain than does *bmp4* expression (Connors et al., 1999). Thus, between the *bmp4* and *chd* expression domains, there is an area where *tld* alone is expressed within the dorsal tail bud.

Through chimeric studies, we found that Tld activity is sufficient in the ventral-vegetal cells that eventually reside in the ventral tail bud (Fig. 1). The spatial and temporal requirements for Tld activity together indicate that Tld acts within the ventral tail bud to modulate BMP activity to pattern ventral tail tissues. Thus, the crucial location for Tld appears to be its ventral, rather than its dorsal domain, which directly apposes *chd*-expressing cells. The ventral Tld domain overlaps with the ventral tail bud expression domain of *bmp4*. One possible reason for the requirement of Tld in this domain is that cleavage of Chd by Tld may depend on Chd being bound to BMP, as found in *Drosophila* (Marqués et al., 1997; Nguyen et al., 1998a), so only in a region where BMPs are present can Tld cleave Chd. Thus, dorsal expression of Tld would be ineffective at blocking Chd. Alternatively, critical post-translational modification(s) or co-factors required for Tolloid’s activity, such as Twisted gastrulation (Little and Mullins, 2004; Oelgeschlager et al., 2000, 2003; Xie and Fisher, 2005; Yu et al., 2000), may be ventrally restricted. Since Tld can block all Chd activity in our heat-shock experiments, we know that it can function in all regions where Chd antagonizes BMP signaling. However, these regions may be predominantly ventral, at least for Tld’s repression of Chd that normally occurs within the tail bud.

In 1 dpf rescued chimeric embryos, the transplanted wild type *tld*-expressing cells most often resided in somitic mesoderm of the trunk and anterior tail, not in the posterior somitic mesoderm or ventral tail fin, the tissues rescued by Tld activity. The fact that wild type cells do not contribute to the tissues absent in *mfn* mutants to rescue those tissues demonstrates that Tld acts cell non-autonomously. The cell non-autonomy of Tld is consistent with Tld acting extracellularly to cleave Chd and thus increase BMP activity. Since cells leave the tail bud at progressively later stages to form posterior trunk and then more posterior tail segments (Kanki and Ho, 1997), the fact that most *tld*-rescuing cells reside within the posterior trunk and anterior tail suggests that Tld modulates BMP signaling prior to the imergence of these cells from the tail bud. Since the trunk is comprised of ~15 somites and the cells that form the 15th somite (at 16.5 hpf) leave the tail bud before formation of the somite (~2 h earlier; Muller et al., 1996) at about 14.5 hpf, our chimeric studies also support our heat-shock studies, which indicate a requirement of Tld during a 4-h window from the end of gastrulation to the 10-somite stage (14 hpf).

**Gastrula versus post-gastrula function of Chordin**

The only described target of zebrafish Tld is Chd. Mouse Tll-1 can cleave procollagen in addition to Chd (Scott et al., 1999), suggesting that zebrafish Tld may have additional substrates. However, *chordino;mfn* double mutants are indistinguishable from *chordino* homozygotes (Wagner and Mullins, 2002), indicating that Tld (Mfn) does not have another target during early zebrafish development. Moreover, multiply heat-shocked transgenic embryo display defects identical to those of *chordin* mutants. Thus, the Tg(*hsp70:tdl*) likely acts specifically on Chd in the studies presented here.

Chd is required to regionalize the gastrula into proper dorsal and ventral domains (Fisher et al., 1997; Hammerschmidt et al., 1996a; Sasai et al., 1994). Chd plays an important role in establishing the neural plate, and loss of Chd activity before and during gastrulation causes reductions of the neural plate (Fig. 5). In contrast, removal of Chd activity after gastrulation leaves intact rostral neural tissue, indicating that its formation by Chd antagonism of BMP activity is complete by the end of gastrulation.

We found that Chd patterns tail tissues both during and after gastrulation, unlike Tld (Mfn), which only acts after gastrulation. Post-gastrula or gastrula removal of Chd causes weakly ventralized tail phenotypes, whereas loss of Chd in pre- and post-gastrula stages causes stronger ventralizations of the tail (Fig. 5). The weak tail ventralization seen in post-gastrula Chd inhibition is consistent with the weak tail dorsalization of *mfn* mutants and Tld (Mfn)’s post-gastrula role in tail patterning.

What is the gastrula role of Chd in patterning the tail? A BMP activity gradient is thought to be established during gastrulation by BMP antagonists, including Chd, which leads to a BMP-level-dependent specification of cell fates (Dosch et al., 1997; Jones and Smith, 1998; Knecht and Harland, 1997; Neave et al., 1997; Wilson et al., 1997). Loss of Chd significantly changes the gastrula fate map, causing a larger domain of ventral cells to come under the influence of BMP activity and adopt a ventral identity at the expense of dorsal cells (Fisher et al., 1997; Hammerschmidt et al., 1996a; Myers et al., 2002; Schulte-Merker et al., 1997). Most, if not all, of the distinct cell types along the dorsoventral axis form in the absence of Chd, indicating that a gradient of BMP activity is still present (Gonzalez et al., 2000; Wagner and Mullins, 2002). However, a larger number of cells with ventral identity are established. Since ventral-vegetal cells of the late gastrula form the ventral tail bud (Agathon et al., 2003; Kanki and Ho, 1997; Myers et al., 2002), a larger domain of ventral cells in gastrula-depleted Chd embryos causes an enlarged ventral tail bud, which then generates an enlargement of ventral tail bud derivatives in the
tail. Thus, we postulate that the gastrula role of Chd in tail development is to generate the proper size domain of precursor cells to enter the dorsal and ventral tail bud regions. Consistent with this model, these ventral marginal cells have been shown to be part of a tail organizer, which can induce tail formation when transplanted to an ectopic position. As shown by others, formation of this organizer depends on BMP and other signaling pathways (Agathon et al., 2003; Beck et al., 2001; Pyati et al., 2005).

In contrast to gastrula Chd function, we hypothesize that post-gastrula Chd patterns the cells of the ventral tail bud to form different tail derivatives. Loss of post-gastrula Chd or Tld likely shifts oppositely the fate map of the tail bud, similar to removal of Chd or BMP activity during gastrulation causing reciprocal shifts of the gastrula fate map (Hammerschmidt and Mullins, 2002). The requirement for both Tld and Chd after gastrulation indicates that BMP activity is finely modulated during post-gastrula stages, suggesting graded BMP activity patterns different dorsoventral aspects of the tail, as it does during gastrulation for the trunk.

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