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RESEARCH ARTICLE

Wnt signaling and *tbx16* form a bistable switch to commit bipotential progenitors to mesoderm

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ABSTRACT

Anterior to posterior growth of the vertebrate body is fueled by a posteriorly located population of bipotential neuro-mesodermal progenitor cells. These progenitors have a limited rate of proliferation and their maintenance is crucial for completion of the anterior-posterior axis. How they leave the progenitor state and commit to differentiation is largely unknown, in part because widespread modulation of factors essential for this process causes organism-wide effects. Using a novel assay, we show that zebrafish Tbx16 (Spadetail) is capable of advancing mesodermal differentiation cell-autonomously. Tbx16 locks cells into the mesodermal state by not only activating downstream mesodermal genes, but also by repressing bipotential progenitor genes, in part through a direct repression of *sox2*. We demonstrate that *tbx16* is activated as cells move from an intermediate Wnt environment to a high Wnt environment, and show that Wnt signaling activates the *tbx16* promoter. Importantly, high-level Wnt signaling is able to accelerate mesodermal differentiation cell-autonomously, just as we observe with Tbx16. Finally, because our assay for mesodermal commitment is quantitative we are able to show that the acceleration of mesodermal differentiation is surprisingly incomplete, implicating a potential separation of cell movement and differentiation during this process. Together, our data suggest a model in which high levels of Wnt signaling induce a transition to mesoderm by directly activating *tbx16*, which in turn acts to irreversibly flip a bistable switch, leading to maintenance of the mesodermal fate and repression of the bipotential progenitor state, even as cells leave the initial high-Wnt environment.

KEY WORDS: Bipotential, Neuromesodermal, Wnt, Spadetail, Tbx16, Somitogenesis

INTRODUCTION

Vertebrates form an anterior-posterior axis through a process called posterior growth, in which a population of undifferentiated cells resides at the most posterior end of the embryo and gradually contributes cells to the trunk and tail during the somite-forming stages (Kimelman and Martin, 2012; Wilson et al., 2009). Germ layer specification had long been thought to be complete by the end of gastrulation, but recent work has demonstrated that the most posterior end of the growing embryo maintains a population of bipotential stem cell-like progenitors, which are capable of adopting either a neural or mesodermal fate depending on their exposure to canonical Wnt signaling (Garriock et al., 2015; Gouti et al., 2014; Jurberg et al., 2014; Martin and Kimelman, 2012; Takemoto et al., 2011; Tsakiridis et al., 2014). These bipotential cells provide the raw cellular material necessary to extend and complete the anterior-posterior axis. Owing to a limited rate of progenitor cell proliferation during posterior growth (Bouldin et al., 2014), the rate at which cells exit the bipotential progenitor zone and contribute to the body must be carefully controlled in order to prevent premature depletion of progenitor cells before the anterior-posterior axis is complete.

An essential component involved in maintaining the bipotential progenitors during posterior growth is the T-box transcription factor Brachyury (Papaioannou, 2014). In zebrafish, two *brachyury* genes, *no tail* (*ntl*) and *brachyury* (*bra*) [also known as *brachyury homolog a* (*ta*) and *b* (*tb*)], are expressed in the bipotential cells from the onset of gastrulation through the end of somitogenesis (Martin and Kimelman, 2008; Schulte-Merker et al., 1994). Loss of *ntl* results in embryos without a tail, whereas loss of both *ntl* and *bra* causes a more severe truncation of the axis (Halpern et al., 1993; Martin and Kimelman, 2008). Ntl and Bra act within the bipotential progenitor cells to maintain expression of the canonical Wnts *wnt3a* and *wnt8*, which together are essential for forming the mesoderm of the posterior body (Martin and Kimelman, 2008; Shimizu et al., 2005; Thorpe et al., 2005). The feedback between the canonical Wnts and the *brachyury* orthologs is broadly conserved among vertebrates as a crucial component of posterior growth (Martin and Kimelman, 2009).

As cells leave the bipotential progenitor state, they immediately activate another T-box gene crucial for mesodermal differentiation, *tbx16* (formerly known as *spadetail* in zebrafish), which is a member of the *tbx6/16* family of T-box genes (Ahn et al., 2012). *tbx16* mutant embryos have severe muscle defects due to loss of the cell-autonomous functions of Tbx16 in mesodermal cell differentiation and morphogenesis (Griffin et al., 1998; Ho and Kane, 1990; Kimmel et al., 1989). Interestingly, *tbx16* functions semi-redundantly with another transcription factor, *mesogenin 1* (*msgn1*), such that *tbx16;msgn1* mutants show a complete loss of muscle (Fior et al., 2012; Yabe and Takada, 2012). However, zebrafish *msgn1* mutants are essentially normal (Fior et al., 2012; Yabe and Takada, 2012), demonstrating that Tbx16, not Msgn1, is the major regulator of mesoderm formation during posterior growth in zebrafish.

A major unanswered question is how these mesoderm-inducing factors work together to let cells know when to leave the bipotential progenitor state and trigger the commitment to mesoderm. Since a key initial event in mesodermal differentiation during posterior growth is the activation of *tbx16* expression (Griffin and Kimelman, 2002), we tested the hypothesis that expression of Tbx16 is sufficient to initiate mesodermal cell differentiation. Because ubiquitous overexpression of factors such as Wnt, Msgn1 and Tbx16 causes severe alterations in posterior growth that preclude analysis of the effects on individual cells (Fior et al., 2012; Martin and Kimelman, 2012; Yabe and Takada, 2012; and below), we have

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Development (2015) 142, 2499-2507 doi:10.1242/dev.124024

developed a new quantitative assay that has allowed us to establish a cell-autonomous role for Tbx16 and Wnt in promoting progenitor cell exit from the tailbud. Intriguingly, this analysis demonstrated that, although Tbx16 and Wnt can hasten the exit of cells from the tailbud, they cannot force all bipotential cells from the progenitor zone immediately, suggesting that some other factor is required. Finally, we show that Tbx16 acts as a bifunctional transcriptional activator and repressor, and that its repressive function blocks the progenitor and neural state and creates an irreversible change that drives bipotential cells to mesodermal fates and establishes a clear distinction between the mesoderm, neural and progenitor cells of the tailbud.

RESULTS

tbx16 advances cells toward muscle differentiation

A key process in posterior growth is the epithelialization of blocks of mesoderm into somites (Holley, 2007; Pourquié, 2011). Somites function to compartmentalize and anchor developing myofibers, which are fused, elongated and morphologically distinguishable from their rounded myoblast precursors (Buckingham and Vincent, 2009; Stellabotte and Devoto, 2007). Because the somites form in a very stereotypical way, they provide a valuable landmark for position along the anterior-posterior axis and thus can be used as a clear measure of the time when cells begin the mesodermal differentiation process.

Because *tbx16* is immediately expressed when cells commit to mesoderm and a loss of *tbx16* results in loss of somitic mesoderm, *tbx16* is necessary for the initiation of muscle cell fate (Griffin and Kimelman, 2002; Griffin et al., 1998; Kimmel et al., 1989). At the outset of this study, we hypothesized that Tbx16 expression would be sufficient to trigger a bipotential cell to begin the process of mesodermal differentiation. We constructed a transgenic line to drive the expression of *tbx16* under the control of a heat shock promoter together with a co-expressed nuclear fluorescent protein *Tg(hsp70l:tbx16-2A-NLS-Kikume)*, hereafter called *HS:tbx16*, allowing us to bypass the severe gastrulation defects caused by expression of ectopic *tbx16* that is not temporally controlled (our unpublished results). A similar transgene rescues *tbx16* mutant cells during gastrulation and somitogenesis (Row et al., 2011). Whole embryos heat shocked during mid-somitogenesis were severely truncated, and somite formation was also disrupted (supplementary material Fig. S1). Similar results are observed when Wnt signaling or Msgn1 are ectopically activated or expressed (Fior et al., 2012; Martin and Kimelman, 2012; Yabe and Takada, 2012), showing the importance of these factors in body axis formation but precluding finer analysis of their specific roles as differentiation cues.

To circumvent defects in morphogenesis and somite formation, we developed an assay that allowed us to precisely measure the cell-autonomous effects of Tbx16. At the start of gastrulation we transplanted control or *HS:tbx16* cells to the ventral margin of wild-type embryos where the progenitors of the posterior mesoderm are located (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). The embryos were heat shocked at the 10-somite stage (ss) and allowed to complete somitogenesis (Fig. 1A). We then counted in each embryo the most posterior somite with an elongated muscle fiber containing a transplanted cell, similar to an assay that we used previously (Szeto and Kimelman, 2006). Whereas cells from non-transgenic embryos contributed to elongated fibers throughout the entire tail (Fig. 1B), cells from *HS:tbx16* embryos did not contribute cells to fibers in the most posterior; instead, the transplanted cells occupied more anterior positions (Fig. 1C). The interquartile ranges for the two conditions were well separated and the difference was statistically significant using non-parametric analysis (Fig. 1D).

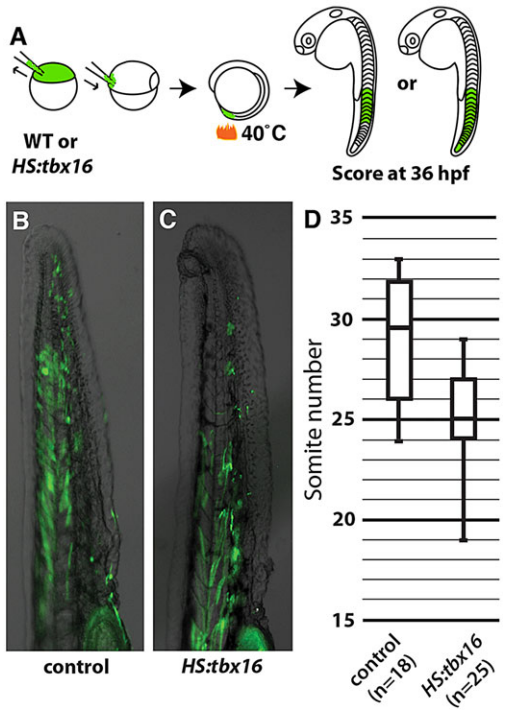

Fig. 1. *tbx16* expression causes cells to exit the tailbud. (A) Outline of the assay used to test and quantify the ability of ectopic *tbx16* to accelerate exit from the progenitor zone. (B,C) Overlays of transplanted cells labeled with fluorescent dextran (green) on a bright-field image from wild-type (B) or *HS:tbx16* (C) donor zebrafish embryos at 36 hpf. (D) Quantification of the most posterior somite occupied by a fluorescently labeled cell with fiber-like morphology in embryos heat shocked at the 10 ss. Data are significantly different (Mann-Whitney U-test, $P<0.05$).

tbx16 represses the progenitor and neural state

The developing tailbud can be subdivided into multiple domains of cells using markers for various stages of differentiation (Griffin and Kimelman, 2002). We examined whether *tbx16* was promoting cells toward mesoderm by acting on the earliest phase or on a later stage of differentiation. To distinguish these possibilities, we used the *HS:tbx16* line to drive expression of *tbx16* and then analyzed gene expression using whole-mount *in situ* hybridization. We chose three markers of the progenitor zone (*sox2*, *ntl* and *bra*), two markers that indicate the early stages of differentiation (*msgn1* and *tbx6l*) and four markers that are expressed a short time before cells enter somites (high levels of *pcdh8*, *her1*, *tbx6* and *mespba*) (Fior et al., 2012; Hug et al., 1997; Martin and Kimelman, 2012; Nikaido et al., 2002; Sawada et al., 2000; Yamamoto et al., 1998). To analyze the effect of *HS:tbx16* on endogenous *tbx16* transcription, we also created an *in situ* probe complementary to the 3′ UTR of *tbx16*, which is not included in the *tbx16* transgene.

The peak of ectopic Tbx16 protein accumulation, which occurred 4 h post-heat shock (supplementary material Fig. S2), coincided with an increase in endogenous *tbx16*, enhanced expression of markers of later stages of differentiation and subtle enhancement in the expression of the early differentiation markers (Fig. 2A; supplementary material Fig. S3 and Table S1). Several changes persisted to 6 h post-heat shock. For instance, *pcdh8* and *her1* were expanded anteriorly, which was expected because they are Tbx16 target genes (Fig. 2B,C) (Garnett et al., 2009; Yamamoto et al., 1998). Interestingly, *sox2* and *ntl* were reduced in the progenitor zone (Fig. 2D-G), suggesting that Tbx16 actively inhibits progenitor cell gene expression. *sox2* is also expressed in the neural

Development (2015) 142, 2499-2507 doi:10.1242/dev.124024

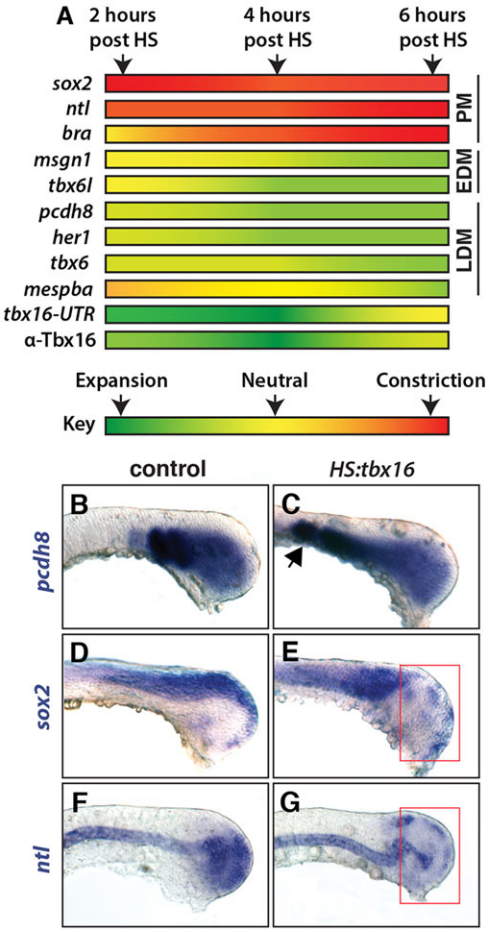

Fig. 2. *tbx16* expression represses genes involved in maintaining the progenitor zone. (A) Summary of changes in gene expression after the induction of ectopic *tbx16* (based on the data in supplementary material Fig. S3 and Table S1). Arrows indicate the number of hours post-heat shock (HS) and the colors represent expansion (green), constriction (red) or no change (yellow) to expression, with intermediate shades marking the degree of difference. PM, progenitor markers; EDM, early differentiation markers; LDM, late differentiation markers. α-Tbx16 indicates Tbx16 assessed by immunostaining. (B-G) *In situ* hybridization shows changes to gene expression in the tailbud of *HS:tbx16* (C,E,G) and control (B,D,F) embryos at 6 h after heat shock. The arrow (C) points to expansion of *pcdh8* and the red boxes (E,G) highlight the reduction of *sox2* and *ntl* in the tailbud.

tube, where it plays an important role in neural cell differentiation (Okuda et al., 2010). However, neural tube expression of *sox2* was unaffected by ectopic *tbx16*, as was the notochord expression of *ntl*. Taken together, these data show that ectopic *tbx16* expression strongly represses markers of the bipotential progenitor cells and enhances markers of later somite mesoderm differentiation, but does not affect notochord or neural gene expression.

Tbx16 is thought to act directly as a transcriptional activator and has been shown to be involved in activating the transcription of a large number of target genes (Garnett et al., 2009). We investigated whether the repression of *ntl* and *sox2* that we observed was a result of a repressive function of Tbx16 or of Tbx16-driven activation of a repressor. To distinguish these possibilities, we fused an Engrailed repressor domain (EnR), which converts the function of transcription factors to obligate repression (Smith and Jaynes, 1996), to *tbx16*. We created a new transgenic line *Tg(hsp70l:tbx16-EnR-2A-NLS-Kikume)*, hereafter called *HS:tbx16-EnR*, and examined its effect on the expression of *ntl* and *sox2*. Four hours after heat shock, expression of Tbx16-EnR repressed the Tbx16 targets *pcdh8* and *her1* and inhibited the maintenance of *tbx6l* (Fig. 3A-F), demonstrating that our fusion construct performs as expected. Interestingly, *sox2* was also repressed by Tbx16-EnR (Fig. 3G,H), as well as by wild-type Tbx16 (Fig. 2D,E), indicating that Tbx16 inhibits *sox2* through a novel repressive function. By contrast, Tbx16-EnR activated *ntl* expression (Fig. 3I,J), showing that the Tbx16-mediated repression of *ntl* is indirect. Our results reveal that Tbx16 is a dual-function activator/repressor that inactivates bipotential progenitor gene expression by repressing *sox2* and activating a repressor of *ntl*.

The summary of all of our results shows that *tbx16* is capable of cell-autonomously advancing the differentiation of bipotential cells to muscle by repressing the progenitor state. However, based on the time of heat shock and the time to make Tbx16 protein (supplementary material Fig. S2), it is clear that expression of Tbx16 does not force all cells to leave the progenitor zone immediately but instead hastens their exit (Fig. 1). Because we heat shocked at the 10 ss and the activation of induced mesodermal gene expression was at peak levels at the 16 ss, we would have expected the cells to be found in more anterior somites than we observed. Interestingly, we repeated the same experiment heat shocking 3 h earlier at the 2 ss, and saw a somitic distribution similar to that which we saw in Fig. 1 (supplementary material Fig. S4). Together, these data suggest that although the conversion to a mesodermal state is accelerated by overexpressing Tbx16, something in addition to *tbx16* expression is necessary for bipotential progenitor cells to exit from the tailbud.

Canonical Wnt signaling upregulates *tbx16* expression

Canonical Wnt signaling plays a crucial role in enabling bipotential cells to differentiate into mesoderm (Martin and Kimelman, 2012). There are at least two Wnts that activate canonical Wnt signaling in

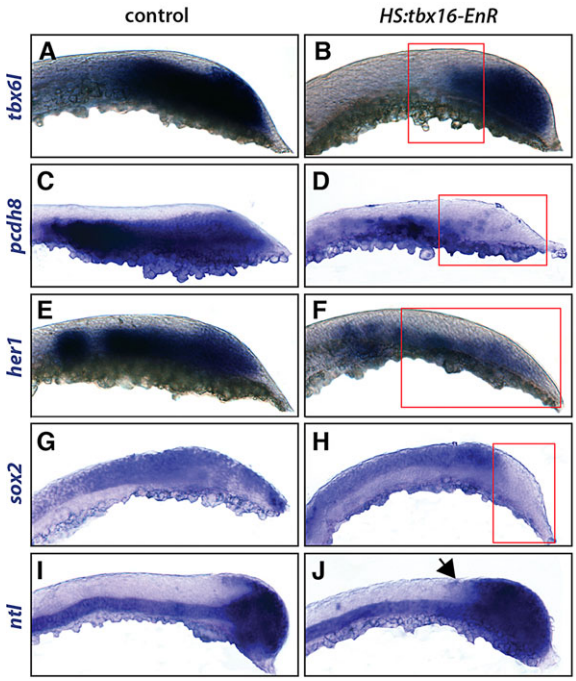

Fig. 3. *tbx16-EnR* expression represses *sox2* and activates *ntl*. *In situ* hybridization shows changes to gene expression with *HS:tbx16-EnR* (B,D,F,H,J) or in control (A,C,E,G,I) 4 h after heat shock. The red boxes (B,D,F,H) highlight the reduction of *tbx6l*, *pcdh8*, *her1* and *sox2* in the tailbud and the arrow (J) points to an expansion of *ntl*.

Development (2015) 142, 2499-2507 doi:10.1242/dev.124024

the tailbud, *wnt3a* and *wnt8*, and several antagonists of Wnt signaling, which combine to tune Wnt signaling (Row and Kimelman, 2009; Stulberg et al., 2012). The mix of Wnts and antagonists, each expressed in a unique pattern, makes it difficult to determine where Wnt signaling is active simply by looking at expression patterns.

To determine where Wnt signaling is active in the tailbud, we focused on levels of nuclear β-catenin, a hallmark of active canonical Wnt signaling, by using DAPI staining as a mask for β-catenin antibody staining, as we and others have done previously (Bajard et al., 2014; Row and Kimelman, 2009). Whereas earlier studies examined Wnt signaling in the anterior presomitic mesoderm, we focused on expression in the tailbud. Nuclear β-catenin was highest in the posterior of the tailbud in a region slightly anterior to the tip (Fig. 4A), which is known to be an area of high levels of *tbx16* expression, differentiation and cell movement (Fig. 4D) (Amacher et al., 2002; Kanki and Ho, 1997; Lawton et al., 2013; Martin and Kimelman, 2012; Uriu et al., 2014). Using a Sox2 antibody, we marked the bipotential progenitor cells at the periphery of the tailbud (Fig. 4B). When combined, we saw that the progenitor cells had only moderate levels of nuclear β-catenin and Sox2 relative to the more ventral and anterior cells (Fig. 4C). These results show that canonical Wnt signaling is active at high levels in early differentiating cells and not in progenitor cells.

Because the zone of high-level Wnt signaling corresponds to the domain where *tbx16* expression initiates and inhibition of Wnt signaling leads to a loss of new mesoderm and a progressive loss of *tbx16* expression (Martin and Kimelman, 2008, 2012), we reasoned that Wnt might be an important activator of *tbx16* expression. Therefore, we activated the Wnt pathway and analyzed events shortly after activation. For these experiments, we used a transgenic line that placed a heat shock promoter in front of a constitutively active form of β-catenin (caβ-catenin) and a fluorescent protein (Veldman et al., 2013), referred to here as *HS:caβ-catenin*. When we looked at the midline at 4 h post-heat shock when the fluorescent protein begins to be detectable, the domain of *tbx16* expression extended more posteriorly in the presence of caβ-catenin, with *tbx16* expression extending into the progenitor zone that normally does not express *tbx16* (Fig. 4D,F) (Martin and Kimelman, 2012). Interestingly, we also saw *tbx16* expanded anteriorly (Fig. 4E,G), showing that constitutively active Wnt signaling activates *tbx16* in both the mesoderm and bipotential cells of the tailbud.

To understand whether Wnt signaling directly activates *tbx16* expression, we created the transgenic line *Tg(tbx16-1.2:TagRFP)*, hereafter referred to as *tbx16-1.2*, that has a 1.2 kb fragment from upstream of the *tbx16* start codon driving the expression of a fluorescent protein (Fig. 5A). Within this 1.2 kb *tbx16* promoter we identified a highly conserved domain, found in all sequenced fish species, just in front of the start codon (Fig. 5A; supplementary material Fig. S5). Reporter expression recapitulates the normal activation of *tbx16* expression within the tailbud, although it lacks the continuing expression within the posterior presomitic mesoderm, which must be regulated by a distal genomic sequence (Fig. 5B).

Transcriptional activation of canonical Wnt signaling requires β-catenin binding to TCF sites, and the conserved region of *tbx16* contains two TCF sites that are present in all fish species (supplementary material Fig. S5). When these two sites were mutated in a new transgenic line [*Tg(tbx16-1.2ΔTCF:TagRFP)*,

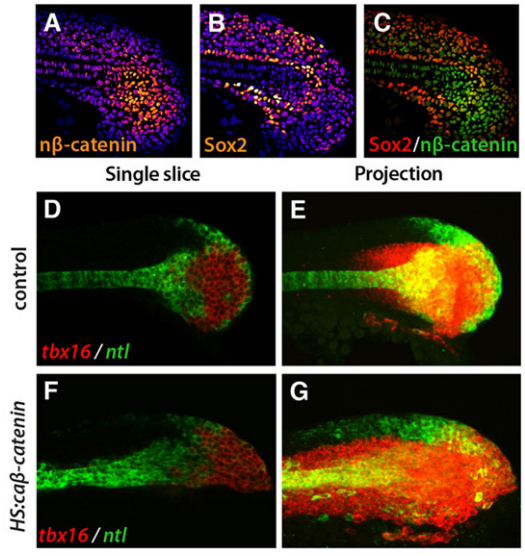

Fig. 4. *tbx16* is Wnt responsive. (A-C) Immunofluorescence shows cells in the tailbud with nuclear β-catenin (nβ-catenin; A, orange; cytoplasmic and membrane-bound β-catenin have been eliminated from the image; see Materials and Methods) or Sox2 protein (B, orange). DAPI labels nuclei (blue). The highest levels of nβ-catenin and Sox2 are white and the lowest levels are purple. (C) A composite image shows that there is little overlap between the Sox2 domain (red) and the nuclear β-catenin domain (green). (D-G) Fluorescent whole-mount *in situ* hybridization shows *tbx16* (red) and *ntl* (green) with *HS:caβ-catenin* (F,G) and in control (D,E) at 4 h post-heat shock. Single slices (D,F) and maximum intensity projections (E,G) are shown. In all images, anterior is to the left and posterior is to the right.

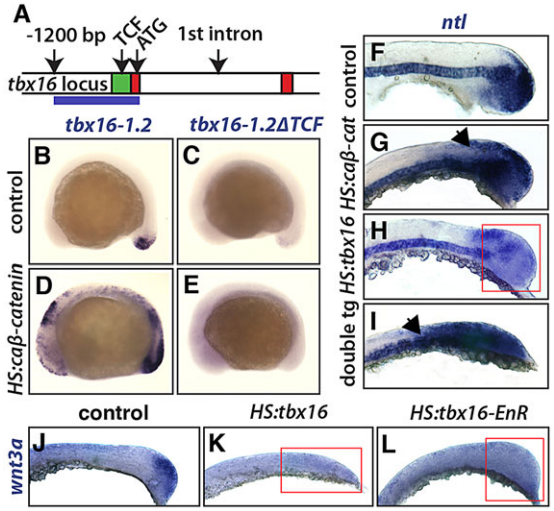

Fig. 5. Wnt drives *tbx16* reporter expression and *tbx16* represses Wnt. (A) The zebrafish *tbx16* locus, showing exons 1 and 2 (red) and a conserved region of the *tbx16* promoter (green), which contains two predicted TCF binding sites (see supplementary material Fig. S5). The blue line marks the region used for the *tbx16-1.2* construct. (B-E) Whole-mount *in situ* hybridization shows reporter expression from the *tbx16-1.2* transgene with *HS:caβ-catenin* (D) and in control (B). Another reporter line with mutations in both of the predicted TCF sites (*tbx16-1.2ΔTCF*) showed no expression with (E) or without (C) *HS:caβ-catenin*. (F-I) *In situ* hybridization shows wild-type *ntl* expression (F; *n*=22 embryos) and the changes in *ntl* expression after heat shock induction of *HS:caβ-catenin* (G; *n*=16 embryos), *HS:tbx16* (H; *n*=11 embryos), and both transgenes (I; *n*=9 embryos). The arrows (G,I) highlight the expansion of *ntl* and the red box (H) highlights the reduction of *ntl*. (J-L) *In situ* hybridization shows wild-type *wnt3a* expression (J) and the loss of *wnt3a* expression after heat shock induction of *HS:tbx16* (K) or *HS:tbx16-EnR* (L). Red boxes (K,L) highlight the reduction of *wnt3a* in the tailbud.

Development (2015) 142, 2499-2507 doi:10.1242/dev.124024

hereafter referred to as *tbx16-1.2ΔTCF*], reporter expression was abolished (Fig. 5C; similar results were observed in multiple lines). In addition, expression of caβ-catenin strongly increased reporter expression from *tbx16-1.2* (Fig. 5D), but had no effect on reporter expression from *tbx16-1.2ΔTCF* (Fig. 5E), demonstrating that mutating the predicted TCF binding sites in the *tbx16* promoter eliminates Wnt responsiveness. Similar results were observed when multiple transgenic lines were produced using a 3.3 kb fragment of *tbx16* that also includes the first intron (data not shown). We conclude that *tbx16* is a direct target of Wnt signaling and that Wnt is essential for *tbx16* expression.

Tbx16-mediated repression of *ntl* occurs indirectly through *wnt3a*

Wnt and *ntl* create a feedback loop, which acts to maintain the expression of both in the progenitor cells of the tailbud (Martin and Kimelman, 2008, 2010). We hypothesized that the indirect repression of *ntl* by Tbx16 that we observed (Fig. 3F) was mediated by changes in Wnt signaling. In this scenario, *tbx16* could activate expression of a Wnt antagonist or repress Wnt gene transcription, either of which would cause loss of *ntl* expression. If we were correct, activation of the intracellular Wnt signaling pathway should rescue the Tbx16-mediated repression of *ntl*. To test this hypothesis, we crossed the *HS:tbx16* and *HS:ca*β*-catenin* lines, heat shocked embryos at the 2 ss, and fixed them 6 h later. Following *in situ* hybridization, embryos were genotyped. As expected, in embryos with only ectopic *tbx16* we saw reduced levels of *ntl* expression (Fig. 5H). In embryos with only *ca*β*-catenin*, we saw expansion of *ntl* expression (Fig. 5G). When both transgenes were present, *ntl* was also expanded, demonstrating that active Wnt signaling is capable of alleviating Tbx16-mediated repression of *ntl* (Fig. 5I). Additionally, when we injected *tbx16* morpholino oligonucleotides with and without *HS:ca*β*-catenin*, in each case we saw an increase in *ntl* expression, indicating that Wnt signaling can induce *ntl* even in the absence of *tbx16* (supplementary material Fig. S6).

We next focused on the transcription of specific Wnt genes, *wnt3a* and *wnt8*, which are expressed in the tailbud during posterior growth (Krauss et al., 1992). Transcription of *wnt3a* was reduced in both the *HS:tbx16* and *HS:tbx16-EnR* lines (Fig. 5J-L), indicating that the reduction of *wnt3a* is due to repression by Tbx16. *wnt8* was similarly reduced after expression of *HS:tbx16*, although the effect was more moderate (data not shown). Our results show that Tbx16 inhibits progenitor gene expression by acting as a direct repressor of *sox2* and an indirect repressor of *ntl* mediated by effects on Wnt signaling.

Wnt signaling advances cells toward muscle differentiation

After establishing that Wnt activates *tbx16*, we asked if activation of canonical Wnt signaling is capable of advancing muscle differentiation, as Tbx16 does (Fig. 1). To focus specifically on cell-autonomous effects, we used the cell transplantation assay described above (Fig. 1A). Non-transgenic cells were capable of contributing muscle fibers to the entire length of the host tail (Fig. 6A), whereas cells positive for the *HS:ca*β*-catenin* transgene did not contribute to the most posterior somites, indicating that they are incapable of long-term residency in the tailbud as bipotential progenitor cells (Fig. 6B), similar to results observed with ectopic Tbx16 (Fig. 1B). Results of several replicates were compiled in a box plot and the differences in distribution were found to be statistically significant (Fig. 6C).

We also examined the ability of transplanted cells expressing the *HS:ca*β*-catenin* transgene to cell-autonomously activate *tbx16*

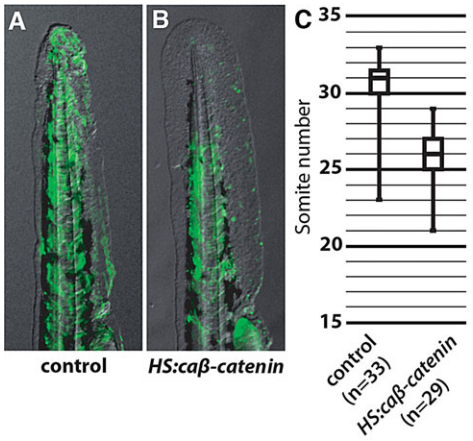

Fig. 6. *caβ-catenin* expression causes early exit of cells from the progenitor zone. (A,B) Overlays of transplanted cells labeled with fluorescent dextran (green) onto a bright-field image from control (A) or *HS:caβ-catenin* (B) donor embryos into wild-type hosts. (C) Quantification of the most posterior somite occupied by a fluorescently labeled cell in host embryos. Data are significantly different (Mann-Whitney U-test, $P<0.05$).

expression within the progenitor pool, which normally only expresses *ntl*. Whereas control transplanted cells in the progenitor zone express *ntl* and not *tbx16*, transplanted cells expressing *ca*β*-catenin* showed induced *tbx16* expression in the absence of *ntl* (supplementary material Fig. S7A-D). When we examined all transplanted cells in the tailbud, *ca*β*-catenin*-expressing cells showed a reduced number of cells expressing only *ntl* and an increase in cells expressing *tbx16*, when compared with controls, which increased as time post-heat shock continued (supplementary material Fig. S7E).

Taken together, these data identify a role for canonical Wnt signaling, acting through Tbx16, in advancing cells toward muscle differentiation and creating a regulatory network that represses the progenitor state.

DISCUSSION

Tbx16 creates a bistable switch for mesoderm in zebrafish bipotential cells

Although the presence of Wnt-regulated bipotential progenitors that form the posterior body in vertebrates is well established (Garriock et al., 2015; Gouti et al., 2014; Jurberg et al., 2014; Martin and Kimelman, 2012; Tsakiridis et al., 2014), how cells actually make the commitment to begin differentiation has remained unclear. Using a novel assay that examines the ability of single cells to contribute to specific somites during posterior growth, we established that Tbx16 is capable of advancing cells from the tailbud to become muscle. In pursuing this observation, we uncovered the regulatory logic that irreversibly commits cells to a mesodermal fate.

During posterior growth, because bipotential progenitors can remain in a progenitor state or adopt a mesodermal fate equally well, they can be described as bistable. In the progenitor steady state, cells express both the pro-neural gene *sox2* and the mesodermal gene *ntl*, which is maintained by intermediate levels of Wnt signaling (Fig. 7, progenitor cell). Entrance of cells into a high-Wnt zone induces a change to levels of nuclear β-catenin, which has been shown to be more important than the absolute level of β-catenin (Goentoro and Kirschner, 2009), and induces expression of Tbx16. Activation of Tbx16 is then sufficient to commit cells to a mesodermal fate. Importantly, Tbx16 not only

Development (2015) 142, 2499-2507 doi:10.1242/dev.124024

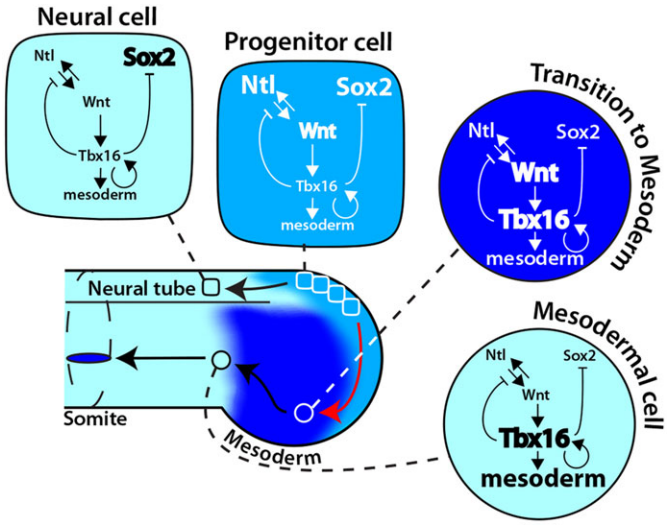

Fig. 7. High levels of Wnt and activation of Tbx16 irreversibly commit progenitors to a mesodermal fate. The status of the regulatory network in different cell types in the tailbud. Levels of Wnt exposure are indicated by shades of blue. Progenitor cells are exposed to a moderate level of Wnt (medium blue), which sustains *ntl* expression but is insufficient to activate *tbx16*. If cells leave the progenitor zone and are exposed to a low-Wnt environment (light blue), they become neural. Cells exposed to high levels of Wnt (dark blue) as they leave the progenitor zone activate *tbx16* and transition to a mesodermal fate. Cells expressing *tbx16* are irreversibly committed to the mesodermal fate even as levels of Wnt eventually drop to a level comparable to that of neural cells, since Tbx16 activates mesodermal genes and turns off the progenitor genes *ntl* and *sox2* through its repressive function. The red arrow highlights the movement of cells from the tailbud toward the domain of early mesodermal differentiation, which we propose requires an additional factor.

activates downstream mesodermal genes such as *pcdh8* but, as shown here, also acts to repress pro-neural and progenitor genes (Fig. 7, transition to mesoderm cell). The multiple functions of Tbx16 place it centrally in the regulatory logic of a bistable switch, which requires a non-linear feedback circuit that creates two balanced states (Ferrell, 2002). Moderate levels of Wnt signaling, in this case, create one balanced state, and high levels of Wnt signaling are necessary to activate Tbx16, which then provides feedback and establishes the second self-perpetuating mesodermal state. Even after levels of Wnt signaling are reduced by Wnt antagonists in the presomitic mesoderm (Row and Kimelman, 2009), cells retain memory of their commitment to mesoderm because Tbx16 has irreversibly activated the mesodermal fate and inactivated the progenitor state (Fig. 7, mesodermal cell). Alternatively, when progenitors leave the tailbud and enter a zone of low Wnt, which is likely to be due to the presence of Wnt antagonists in the neural tube (Kagermeier-Schenk et al., 2011; Miyake et al., 2012), they adopt a neural fate (Fig. 7, neural cell). Prospective neural cells continue *sox2* expression but lose *ntl* and fail to activate *tbx16* and thus become committed to a neural fate. Through this regulatory network, mesodermal cells become clearly separate from the adjacent neural and progenitor cells.

Our results also explain the *tbx16* (*spadetail*) and *tbx16;msgn1* mutant phenotypes, in which undifferentiated cells accumulate in the tail (Fior et al., 2012; Griffin et al., 1998; Kimmel et al., 1989; Yabe and Takada, 2012). Without *tbx16*, not only are cells unable to activate mesodermal differentiation genes but they are also unable to repress progenitor genes. Thus, unlike cells that no longer see Wnt and can proceed to a neural fate (Martin and Kimelman, 2012), cells without *tbx16* and its enhancing factor *msgn1* maintain both *ntl* and *sox2* and remain stuck in the progenitor state (supplementary material Fig. S8) (Griffin and Kimelman, 2002).

Tbx16 is a dual-function activator/repressor

Because it induces the expression of many genes, several of which have been shown to be direct targets, Tbx16 in zebrafish has been considered a transcriptional activator (Garnett et al., 2009). Similarly, the *Xenopus* Tbx16, VegT, also activates many genes, including those of the nodal-related family (Agius et al., 2000; Hyde and Old, 2000; Takahashi et al., 2000). Here, we show that zebrafish Tbx16 also functions as a repressor, since a repressive form of Tbx16 functions like wild-type Tbx16 to inhibit *sox2* expression. Additionally, although Tbx16 also shuts off *ntl* expression, our data suggest that this occurs by repressing the expression of *wnt3a*, which breaks the autoregulatory loop between *ntl* and Wnt genes (Fig. 7) (Martin and Kimelman, 2008). We therefore place Tbx16 together with Tbx20 as T-box factors that are known to be dual-function activators/repressors (Stennard and Harvey, 2005).

Regulation of mesodermal fate by Wnt

Understanding the role of Wnt in the newly forming mesoderm of the posterior body is complicated, since loss of function results in both loss of *ntl* (and *bra*) within the progenitors and depletion of presomitic and somitic mesoderm (Martin and Kimelman, 2008, 2012). Here we show that *tbx16* is a direct target of Wnt signaling and that this pathway is essential for *tbx16* expression. Intriguingly, using nuclear β-catenin as a precise readout we find that Wnt signaling is present in a gradient, with moderate levels in the progenitor zone and high levels anterior and ventral to the progenitor zone in differentiating mesoderm that also expresses *tbx16*. Moreover, we show that ectopic activation of the Wnt pathway causes *tbx16* to be expressed in the progenitor zone, suggesting that the activity of Wnt signaling is precisely regulated in the tailbud to ensure the correct *tbx16* expression pattern. Finally, using our single-cell assay we demonstrate that activation of the Wnt pathway causes the same degree of acceleration of cells from the tailbud as does ectopic expression of *tbx16*. From all of these results, we suggest that feedback between *wnt3a*/*wnt8* and *ntl*/*bra* acts to maintain the progenitor steady state with intermediate levels of Wnt, and then increased Wnt is used to activate *tbx16* in some of the cells exiting the progenitor zone, committing them to a mesodermal fate (Fig. 7).

Recent work has highlighted the importance of Wnt signaling dynamics. For instance, a link has been established between repression of Wnt signaling, slowing of axis elongation and termination of the anterior-posterior axis (Denans et al., 2015). Additionally, Wnt signaling is involved in controlling somite length by providing positional information in differentiating mesoderm (Bajard et al., 2014). Our work adds to that of others by clearly establishing the importance of a dynamic Wnt response to commit bipotential cells to the mesodermal fate.

Additional factors to Tbx16 and canonical Wnt signaling may regulate cell exit from the progenitor zone

Heat shock activation of Tbx16 expression caused a rapid inhibition of progenitor genes and activation of mesodermal genes with peak effects occurring 4 h post-heat shock, consistent with peak levels of ectopic Tbx16 protein. Surprisingly, although ectopic expression of Tbx16 or Wnt causes cells to exit from the tailbud, it only sped up exit by approximately five somites, and we therefore suggest that an additional factor needs to work with Tbx16 to allow cells to physically leave the tailbud. Although, from our data, we cannot conclude which step of cell movement from the tailbud is blocked

Development (2015) 142, 2499-2507 doi:10.1242/dev.124024

after cells have initiated the differentiation program, we suspect that, even with Wnt or Tbx16 overexpression, cells are held until an epithelial-to-mesenchymal transition (EMT) is initiated (red arrow in Fig. 7). This hypothesis is consistent with our previous observation that Tbx16 is essential for completion of EMT, but not for its initiation (Row et al., 2011). How EMT is triggered in fish is still unclear and merits further study.

Comparison with mouse

While recent evidence has established that the formation of the posterior body from bipotential cells is regulated by Wnt and is well conserved in fish and mouse (Gouti et al., 2014; Jurberg et al., 2014; Martin and Kimelman, 2012; Takemoto et al., 2011; Tsakiridis et al., 2014), interesting differences occur within this general framework. For example, whereas *Msgn1* is essential for the formation of presomitic mesoderm in the mouse, in fish loss of *msgn1* has virtually no effect unless *tbx16* is also removed (Fior et al., 2012; Yabe and Takada, 2012). In mouse, which lacks an ortholog of *tbx16* (Lardelli, 2003), a recent study has shown by overexpression that Msgn1 acts as a major regulator of presomitic mesoderm formation, capable of forcing bipotential cells to adopt a mesodermal fate (Chalamalasetty et al., 2014). Interestingly, Msgn1 directly activates expression of Tbx6 (Chalamalasetty et al., 2014), which is a member of the Tbx6/16 family (Ahn et al., 2012), and so the effects of Msgn1 overexpression in mouse are most likely to be a combination of an increase in both Msgn1 and Tbx6.

In mouse, loss of *Tbx6* causes a phenotype that closely resembles that of zebrafish *tbx16* mutants, and a large tailbud of undifferentiated tissue forms (Chapman and Papaioannou, 1998; Chapman et al., 2003; Nowotschin et al., 2012). As with zebrafish *tbx16* mutants, loss of *Tbx6* in mouse causes a reduction of *Msgn1* and an increase in brachyury and *Sox2* expression (Chapman and Papaioannou, 1998; Griffin and Kimelman, 2002; Nowotschin et al., 2012; Takemoto et al., 2011; our results), all supporting the close functional parallels between mouse *Tbx6* and zebrafish *tbx16* despite the sequence divergence (Ahn et al., 2012). It would be interesting to examine whether Msgn1 (and/or Tbx6) in the mouse is able to cell-autonomously drive cell exit from the progenitor zone as we have shown with zebrafish Tbx16. It would also be valuable to determine if Tbx6, and perhaps Msgn1, has the ability to act as a dual-function transcriptional activator/repressor.

Conclusions

Over the course of this work, we found a previously undescribed role for Tbx16 in advancing bipotential cell exit from the tailbud toward a mesodermal fate. We have also identified Wnt signaling as an activator of *tbx16* that is necessary for its expression and showed that Tbx16 functions dually as an activator and a repressor. Finally, we propose that Wnt activation of *tbx16* is the transitional node in a bistable switch, which takes cells from a steady progenitor state to a committed mesodermal fate. Our results emphasize the importance of temporally manipulating gene expression in single cells within a wild-type background using cell-autonomous regulators as a means to study the complex dynamics of cell behavior *in vivo*, since ubiquitous alterations in gene expression affect many different populations of cells and confound this type of analysis.

MATERIALS AND METHODS

Transgenic lines

All transgenic lines were created using Tol2 transposase (Kawakami et al., 2000). For the *Tg(hsp70l:tbx16-2A-NLS-Kikume)* line, full-length *tbx16* was cloned without a STOP codon upstream of a 2A viral peptide (Provost et al., 2007) and Kikume Green-Red with a nuclear localization signal (NLS); genotyping was performed using the following primers (5′-3′): forward, AATTTCAGCCTCCCTCCTCC; reverse, ACTCATGCTTCGTTCCCAAG. The *Tg(hsp70l:tx16-EnR-2A-NLS-Kikume)* line was made similarly, with an Engrailed repressor domain inserted between *tbx16* and the viral 2A sequence. The *Tg(hsp70l:ca*β*-catenin-2A-TFP)* line was described previously (Veldman et al., 2013); genotyping was performed using the following primers: forward, TGCTGTTGTGTTCCACCAAT; reverse, GCCAACGTTTGGTTCAGAAT. For the *Tg(tbx16-1.2:TagRFP)* line, a 1.2 kb fragment of the *tbx16* locus upstream of, and including, the ATG was placed in front of TagRFP. To make the *Tg(tbx16-1.2ΔTCF:TagRFP)* line, two predicted TCF binding sites were altered (see supplementary material Fig. S5 for details). The distal site was changed from TTTGAT to TTacAT and the proximal site from TTCAAA to TTgAAt. This line also has a *crystallin* promoter driving the expression of GFP in the eyes to identify transgenic lines. Seven independently isolated reporter lines showed the same result as in Fig. 5C.

Morpholino treatment

Morpholino oligonucleotides (MOs) were used as follows: 1.1 ng of *tbx16* MO1 and 0.58 ng of *tbx16* MO2 from Lewis and Eisen (2004) and 2 ng of *msgn1* MO from Fior et al. (2012) were injected into single-cell embryos, which were then incubated until they reached the 12 ss.

Heat shock, transplantation assay and statistical analysis

All heat shocks were performed with a mix of transgenic embryos and control siblings. Embryos were placed at 40°C in a circulating water bath for 30 min. Post-heat shock, embryos were sorted and allowed to grow at 30°C until the described time. For the transplantation assay, donor embryos were labeled with Rhodamine-dextran and cells were transplanted into a gastrula stage host embryo on the ventral side, approximately ten cell diameters from the margin. Based on fate-mapping studies, that position will cause the transplanted cells to become long-term residents of the bipotential progenitor cell population (Bouldin et al., 2014). Post-transplantation, embryos were grown to the appropriate stage for heat shock. *HS:tbx16* embryos were heat shocked at both the 2 ss and the 10 ss and *HS:ca*β*-catenin* embryos were heat shocked at different time points (either bud stage, 6 ss or 8 ss), which also had no apparent effect on outcome. After heat shock, embryos were grown to 48 hpf and both DIC and fluorescent images were captured. The most posterior somite with a fluorescent cell that morphologically resembled a myofiber was recorded. Data were plotted using a box and whisker plot (with maximum and minimum values) and statistical significance was tested using a Mann-Whitney U-test with an *a priori* α of 0.05.

Whole-mount *in situ* hybridization and immunofluorescence

Alkaline phosphatase *in situ* hybridizations were performed as previously described (Griffin et al., 1995), with more than 15 embryos for each condition. Fluorescent *in situ* hybridization was performed as described by Lauter et al. (2011). Immunofluorescence was performed with anti-Tbx16 (anti-VegT from the ZIRC, 1:1000), anti-Sox2 (Epitomics, clone EPR3131, 1:200) and anti-β-catenin (BD Biosciences, clone 14, 1:200) and detected with the appropriate Alexa Fluor-conjugated secondary antibody (Molecular Probes). To separate nuclear staining from membrane staining, embryos processed for β-catenin immunofluorescence were counterstained with DAPI and imaged using an Olympus Fluoview 1200 laser scanning confocal microscope. Image processing was performed in ImageJ (NIH) and presented using the Fire lookup table as described previously (Row and Kimelman, 2009).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.M.B. and D.K. developed the approach. C.M.B., Y.-H.P., D.K., G.H.F., K.L.H., A.J.M. and A.D. performed experiments. C.M.B., Y.-H.P. and D.K. analyzed data. C.M.B. and D.K. prepared the manuscript. A.J.M. edited the manuscript prior to submission.

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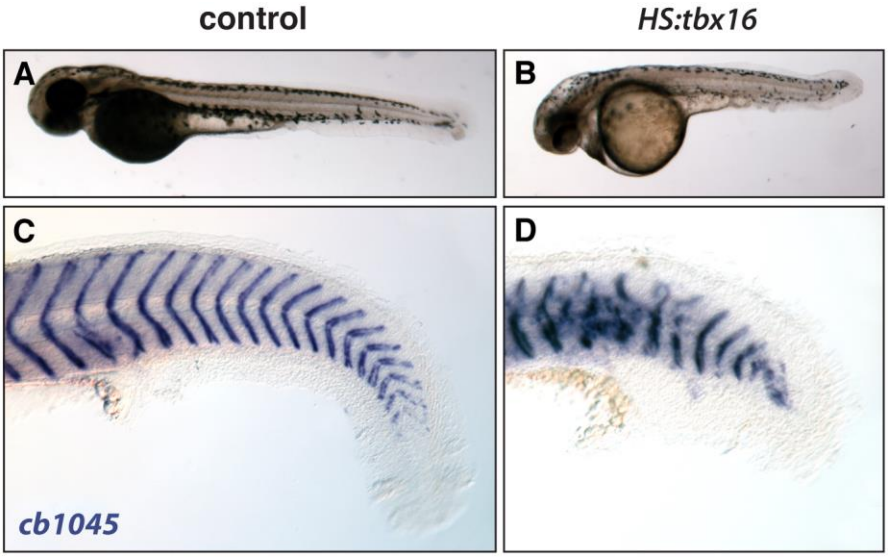

Figure S1. Ubiquitous *tbx16* expression causes organism-wide defects.

At 36 hpf, bright field images show that control siblings (A) are longer than embryos with the *HS:tbx16* transgene (B) after heat shock. In situ hybridization with *cb1045*, a probe that normally marks the somite boundaries in control siblings (C), shows severe disruptions in somite formation in *HS:tbx16* embryo (D). The left-most somite shown in C is the 14th somite and the left-most somite shown in D is the 13th somite. All embryos were heat shocked at the 12 ss.

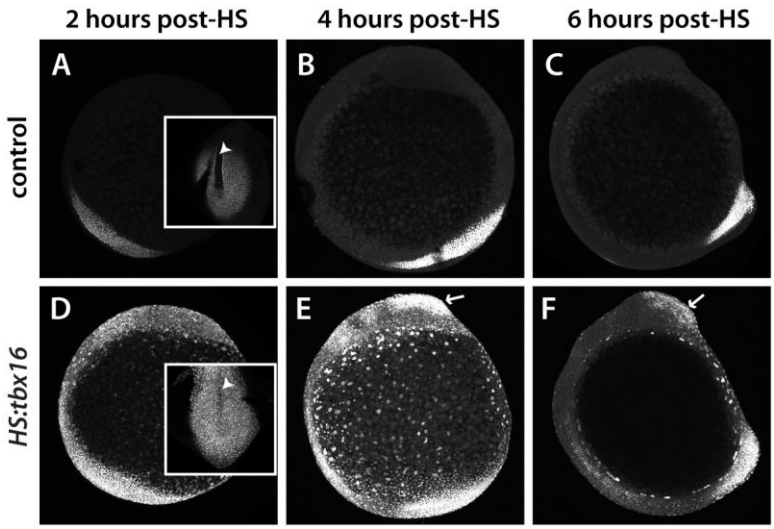

Figure S2. Ectopic Tbx16 is ubiquitous, labile and peaks at 4 hours post-heat shock.

In control (A-C) and *HS:tbx16* (D-F) embryos, immunofluorescence shows that Tbx16 protein levels have increased by 2 hours post-heat shock (A, D) with a peak at 4 hours post-heat shock (B, E), and that levels of Tbx16 have largely returned to normal by 6 hours post-heat shock (C, F). Inset in D shows ectopic Tbx16 in the notochord and neural tube (marked by arrowheads) when compared to the control inset in A (both dorsal views of the tailbud); arrows in E, F highlight the telencephalon, which retains ectopic Tbx16.

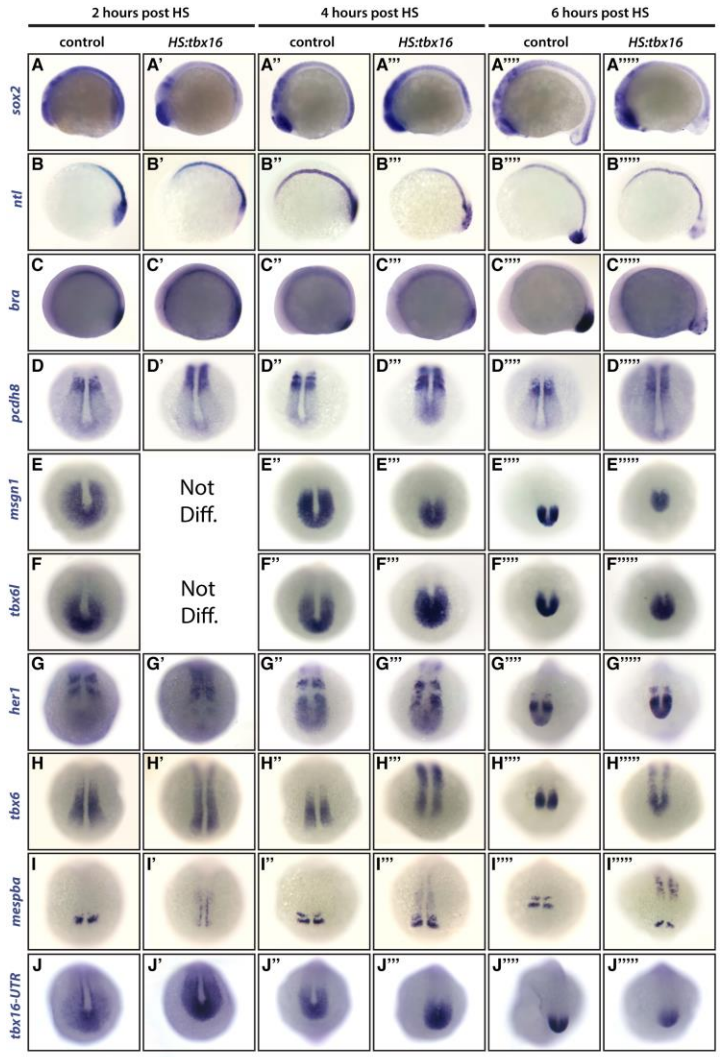

Figure S3. Gene expression after heat shock activation of *HS:tbx16*.

Whole mount in situ hybridization data used for the summary of changes to genes after the expression of ectopic *tbx16* (shown in Figure 2A). Because of the large number of embryos screened for this analysis, transgenic embryos were not separated from their nontransgenic siblings prior to in situ hybridization. A minimum of 30 embryos was used for each condition. In all cases except where noted as Not Diff. (not different), approximately 50% of the embryos matched previously published wild-type expression patterns and 50% showed the altered pattern. See Table S1 for quantification of the results.

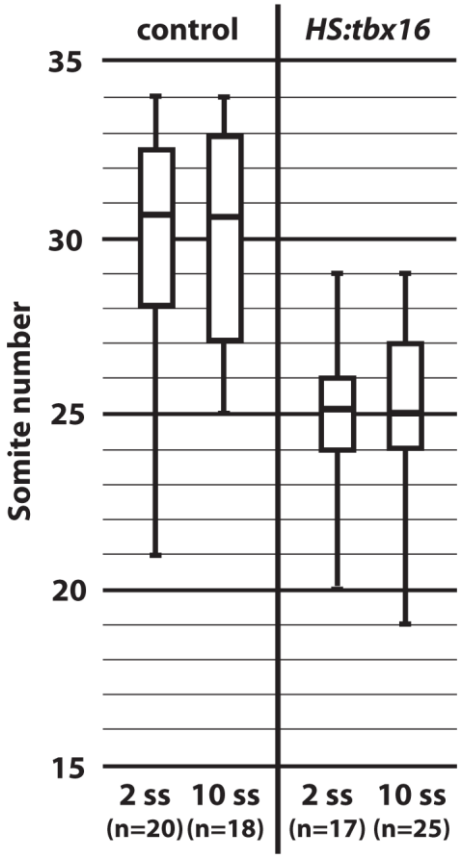

Figure S4. The timing of heat shock does not determine the timing of the cell exit driven by *HS:tbx16*.

Quantification of the most posterior somite in a 36 hpf embryo occupied by a fluorescently labeled cell with fiber-like morphology when embryos were heat shocked at the 2 ss and the 10 ss. Somitic distribution of *HS:tbx16* expressing cells were significantly different from controls at each time point (Mann Whitney U, $P < 0.05$).

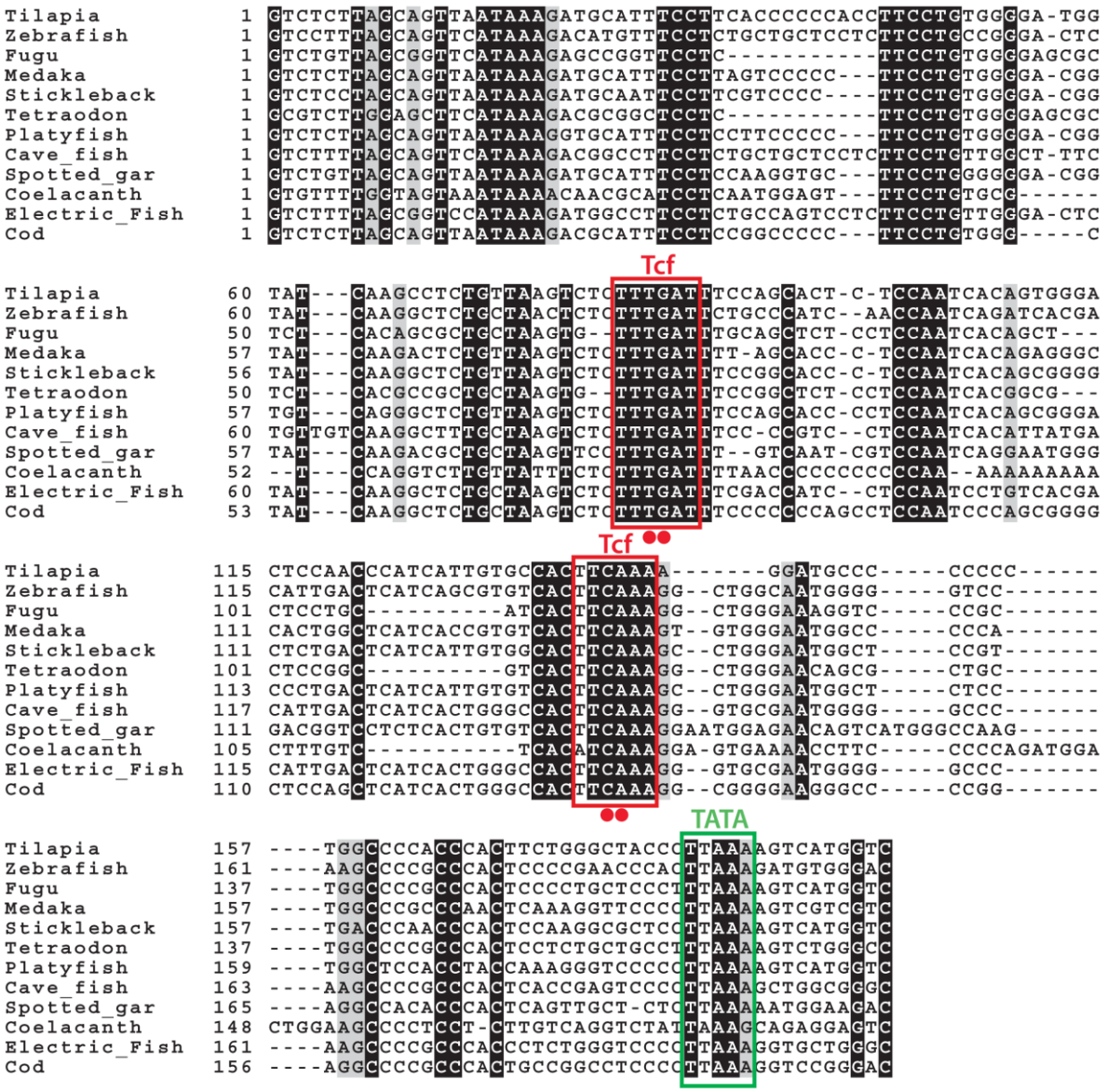

Figure S5. A conserved region in the *tbx16* promoter contains Tcf sites.

A sequence alignment of the *tbx16* promoter from multiple fish species shows a conserved region of approximately 150 bp. Black highlighted bases are completely conserved, and grey highlighted bases are mostly conserved. Two Tcf consensus elements are boxed in red, and a predicted TATA sequence is boxed in green. Below the Tcf sites are dots indicating the bases mutated in zebrafish *tbx16* to make *tbx16-1.2ΔTCF*.

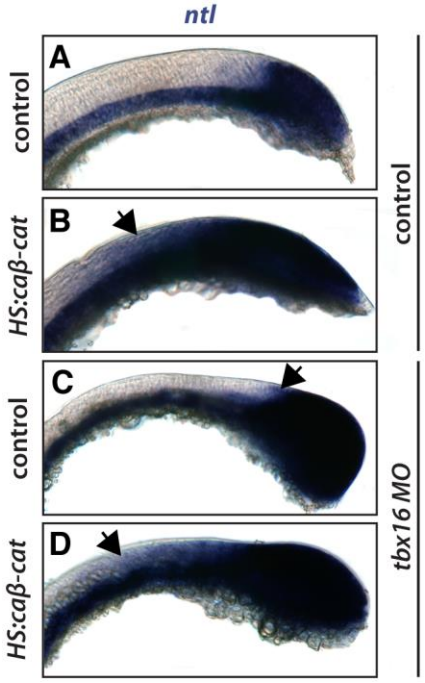

Figure S6. Wnt signaling regulates *ntl* independently of *tbx16*.

Expression of *ntl* is expanded in *HS:ca*β*-catenin* when compared to control embryos (A, B; 17 control and 15 transgenic); with *tbx16 MO, ntl* is similarly expanded in *HS:ca*β*-catenin* when compared to control embryos (C, D; 17 control and 26 transgenic).

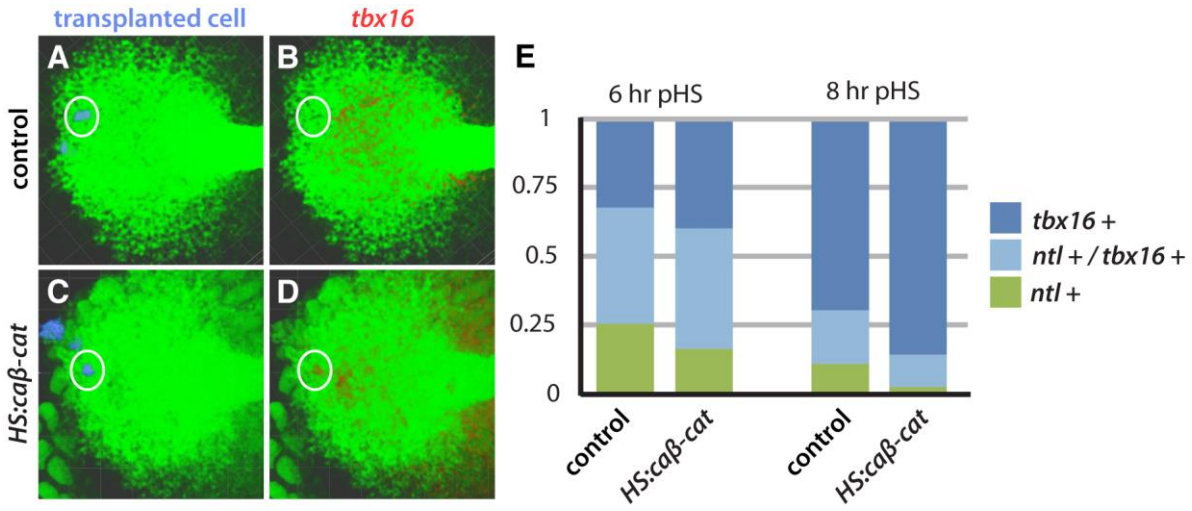

Figure S7. Transplanted *HS:caβ-catenin* cells activate *tbx16* cell-autonomously.

(A, C) Transplanted cells in the progenitor population are identified in blue. Cell-autonomous expression of *tbx16* is not apparent in control cells (B) but is apparent in cells with *HS:ca*β-*catenin* (D). (E) Quantification of all transplanted cells within the tailbud from the same experiment as shown in A-D. At both 6 and 8 hrs post-heat shock, cells expressing *caβ-catenin* were less likely to express *ntl* and more likely to express *tbx16* than control transplanted cells. At 6 hours post-heat shock, 151 cells were analyzed from 16 embryos (control) and 190 cells were scored from 19 embryos (*HS:ca*β-*catenin*); the distribution of control cells was different from *HS:ca*β-*catenin* cells (chi-square; P=0.052). At 8 hours post-heat shock, 131 cells were analyzed from 15 embryos (control) and 99 cells were scored from 14 embryos (*HS:ca*β-*catenin*); the distribution of control cells was significantly different from *HS:ca*β-*catenin* cells (chi-square; P<0.01).

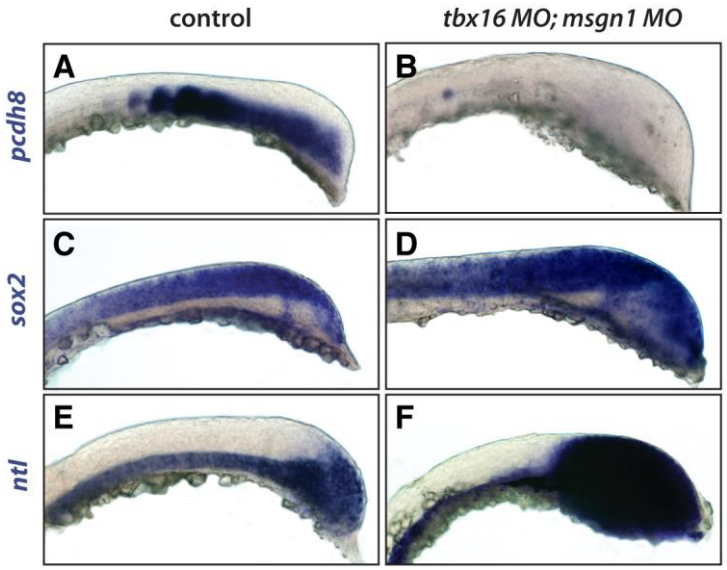

Figure S8. *sox2* and *ntl* increase when Tbx16 and Msgn1 are depleted.

Whole mount in situ hybridization shows a decrease in *pcdh8* expression in embryos (A, B; 13 controls and 12 morphants) injected with *tbx16 MO;msgn1 MO* (B). *sox2* expression (C, D; 11 controls and 13 morphants) and *ntl* expression (E, F; 16 controls and 10 morphants) increase in embryos injected with *tbx16 MO;msgn1 MO* (D, F).

Table S1: Summary of the number of embryos analyzed

		# of control embryos			# of transgenic embryos		
Transgene	**Transcript analyzed**	**2 hr**	**4 hr**	**6 hr**	**2 hr**	**4 hr**	**6 hr**
HS:tbx16	***sox2***	17	18	15	19	19	21
	ntl	22	18	20	18	22	21
	bra	17	19	25	13	14	23
	msgn1	ND (37)	16	22	ND (37)	16	19
	tbx6l	ND (36)	23	20	ND (36)	16	17
	pcdh8	18	18	17	22	22	23
	her1	19	24	20	17	16	19
	tbx6	21	20	22	19	19	18
	mespba	21	25	20	19	17	16
	tbx16-UTR	13	18	18	10	15	16
	wnt3a		20			22	
	Tbx16 protein (Immuno Fluor)	3	4	5	5	3	3
HS:tbx16-EnR	***tbx6l***		22			12	
	pcdh8		22			16	
	her1		23			17	
	sox2		28			13	
	ntl		30			16	
	wnt3a		23			13	
HS:caβ-catenin	***tbx16/ntl* (Fluor In Situ)**		4			6	
	tbx16-1.2			11			10
	tbx16-1.2ΔTCF			7			22
HS:tbx16	***cb1045* (33 hr pHS)**	15			6		