RESEARCH COMMUNICATION

Brachyury establishes the embryonic mesodermal progenitor niche

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Formation of the early vertebrate embryo depends on a Brachyury/Wnt autoregulatory loop within the posterior mesodermal progenitors. We show that exogenous retinoic acid (RA), which dramatically truncates the embryo, represses expression of the zebrafish *brachyury* ortholog *no tail* (*ntl*), causing a failure to sustain the loop. We found that Ntl functions normally to protect the autoregulatory loop from endogenous RA by directly activating *cyp26a1* expression. Thus, the embryonic mesodermal progenitors uniquely establish their own niche—with Brachyury being essential for creating a domain of high Wnt and low RA signaling—rather than having a niche created by separate support cells.

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Stem and progenitor cells play an essential role during the formation of the vertebrate body and the subsequent homeostasis of the adult form. A growing body of work has demonstrated that stem/progenitor cells exist throughout the embryo and adult in niches—unique physical and molecular environments that support the long-term maintenance of these populations (Morrison and Spradling 2008; Voog and Jones 2010). During vertebrate development, a population of mesodermal progenitor cells residing in the tailbud continuously releases differentiating cells throughout somitogenesis (Dubrulle and Pourquie 2004). We showed previously that the maintenance of this population is dependent on an autoregulatory loop between the T-box transcription factor Brachyury and canonical Wnt signaling, with a failure of this loop resulting in a truncated body axis (Martin and Kimelman 2008), explaining why Brachyury plays such a major role in vertebrate development (Martin and Kimelman 2009).

Retinoic acid (RA) signaling is another factor that can affect the formation of the body. Addition of RA to vertebrate embryos causes severe posterior truncations, superficially similar to a loss of Brachyury or Wnt signaling (Sive et al. 1990). Here we show that RA blocks the function of the autoregulatory loop by inhibiting *no tail* (*ntl*) expression, resulting in a failure to sustain Wnt signaling. We found that Ntl protects the mesodermal progenitors from RA produced in the adjacent somites by directly activating the expression of the RA-metabolizing enzyme Cyp26a1. Finally, we show that individual mesodermal progenitor cells do not need to express *ntl*, *wnts*, or *cyp26a1* as long as their neighbors express these genes, demonstrating that the essential role of Brachyury and its downstream targets is to create the niche in which the mesodermal progenitors are maintained in a progenitor state throughout somitogenesis.

Previously identified stem/progenitor cell niches are established by a separate population of niche support cells that exist adjacent to the stem/progenitor cells. The embryonic mesodermal progenitor population that we describe is unique in its ability to serve as both the niche support cells and the progenitor cells, without subfunctionalization into two separate cell types. These results have significant implications in understanding the pathogenesis of Brachyury-expressing human cancers.

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Results and Discussion

RA disrupts posterior growth by inhibiting ntl *expression*

RA has been known for many years to cause severe posterior truncations in vertebrate embryos exposed during gastrulation (Murakami and Kameyama 1965; Sive et al. 1990; Kessel and Gruss 1991; Shum et al. 1999). We found that this sensitivity persists throughout most of somitogenesis when the posterior half of the embryo is forming (Fig. 1B; data not shown), demonstrating that the posterior truncation caused by RA treatment is not the result of early patterning defects. We therefore investigated the Brachyury–Wnt autoregulatory loop, which is necessary for maintaining the mesodermal progenitors of the tailbud (Martin and Kimelman 2008, 2009). To determine if RA directly interferes with the maintenance of this signaling loop, we examined the expression of *ntl* and *wnt3a*. After just 1 h of RA treatment, *ntl* expression is completely lost in the mesodermal progenitors of the tailbud (100% of RA-treated embryos exhibited reduction in *ntl* expression, $N = 15$) (Fig. 1C,D), whereas *wnt3a* expression is normal (100% of RA-treated embryos exhibited normal *wnt3a* expression, $N = 7$) (Fig. 1E,F). After 2 h of RA exposure, *wnt3a* expression is down-regulated in the mesodermal progenitors (100% of RA-treated embryos exhibited reduced *wnt3a* expression, $N = 7$) (Fig. 1G,H). These results indicate that RA signaling impinges on the maintenance of the Brachyury–Wnt autoregulatory loop by first repressing *ntl* expression, which leads to a loss of *wnt* expression and a disruption in posterior growth.

While the addition of RA efficiently represses *ntl* expression in mesodermal progenitors, it has little effect on *ntl* expression in axial mesoderm during both the gastrula and tailbud stages (Fig. 1D; Supplemental Fig. S1). The lack of *ntl* repression in axial mesoderm was determined to be due to the lack of RA receptor (RAR) expression in this region. While four RARs are expressed in the tailbud, there is a clear absence of RAR expression in zebrafish axial mesoderm (Thisse and Thisse 2008), suggesting that exogenous RA cannot repress *ntl* in axial mesoderm due to a lack of receptor expression. Indeed, misexpression of RAR in the axial mesoderm led to RA-induced repression of *ntl* in this tissue (Supplemental Fig. S1). We further

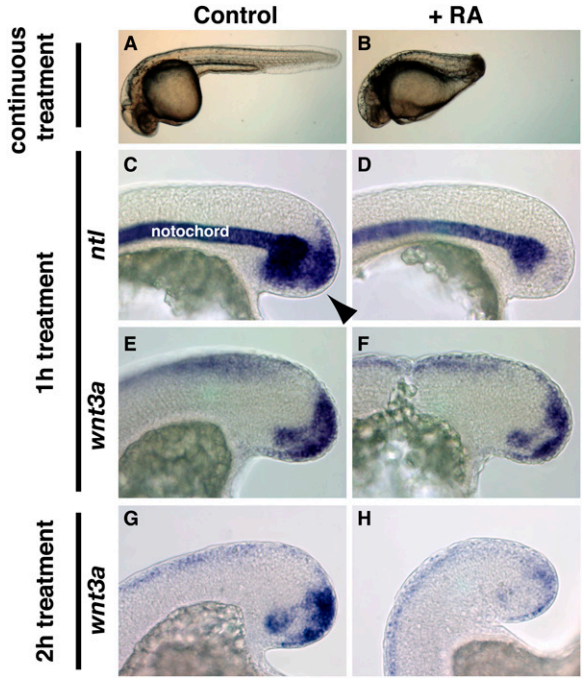

Figure 1. RA impinges on the Ntl—Wnt autoregulatory loop by repressing *ntl* expression. (*A*,*B*) Embryos (36 h post-fertilization [hpf]) treated with vehicle alone (*A*) or 1 μM RA (*B*), beginning at bud stage. (*B*) RA treatment causes severe posterior truncation. *ntl* (*C*,*D*) and *wnt3a* (*E*,*F*) expression after 1 h treatment with vehicle (*C*,*E*) or RA (*D*,*F*), beginning at the 12-somite stage. Within 1 h, mesodermal progenitor (arrowhead in *C*) *ntl* expression is lost (*D*), whereas *wnt3a* expression is normal (*F*). After 2 h of RA treatment, *wnt3a* expression is lost from the mesodermal progenitors (*H*) as compared with vehicle treatment (*G*). (*D*) Note that the expression of *ntl* in the notochord is not affected by RA treatment. (*C*–*H*) Lateral view of the posterior end.

investigated the mechanism of RA-induced repression of *ntl* and discovered that RA/RAR likely acts indirectly through the activation of an unknown *ntl* repressor (Supplemental Fig. S2).

Ntl protects mesodermal progenitors from RA by regulating cyp26a1 *expression*

During normal development, RA is produced in the posterior somites, where it plays an important role in ensuring that the somites form symmetrically (Kawakami et al. 2005; Vermot and Pourquie 2005; Sirbu and Duester 2006). The production of RA is controlled by the localized expression of an aldehyde dehydrogenase (*aldh1a2*) (Niederreither et al. 1997; Dobbs-McAuliffe et al. 2004), whereas the posterior end of the embryo expresses the RA-metabolizing enzyme *cyp26a1* (Abu-Abed et al. 2001; Sakai et al. 2001; Emoto et al. 2005). The expression of *cyp26a1* is directly activated by RA signaling, but the posterior tailbud expression domain is independent of this regulation, indicating that another factor regulates posterior activation (Hu et al. 2008). Since *ntl* and *cyp26a1* are both expressed in the mesodermal progenitors, and since exogenous RA is capable of repressing *ntl*, we tested the hypothesis that Ntl protects its own expression by regulating *cyp26a1*. In *ntl* mutant embryos at a stage before defects in somite formation are observed, *cyp26a1* expression is completely lost from the mesodermal progenitors (100% of *ntl* mutants affected, $N = 6$) (Fig. 2A,B). In contrast, embryos mutant for the tailbud-specific T-box transcription factor *spt* showed no reduction in *cyp26a1* (100% with strong *cyp26a1* expression, $N = 20$) (Fig. 2C,D). The expanded *cyp26a1* domain is due to the failure of *spt* mutant cells to complete differentiation (Griffin and Kimelman 2002). These results indicate that Ntl is specifically necessary for *cyp26a1* expression in the mesodermal progenitors.

In order to determine whether Ntl is sufficient for *cyp26a1* expression during the posterior growth phase, we generated a transgenic line with the *ntl* coding region under the control of a synthetic heat-shock promoter. Heat-shock activation of *ntl* caused ubiquitous expression of *cyp26a1* throughout the embryo, demonstrating that Ntl is sufficient for *cyp26a1* expression (100% of transgenic embryos had ubiquitous *cyp26a1* expression [$N = 30$], whereas *cyp26a1* expression was normal in all nontransgenic siblings [$N = 17$]) (Fig. 2E,F). Together, these results indicate that Ntl protects its own expression and the mesodermal progenitor population from the effects of endogenous somite-produced RA by activating *cyp26a1* expression.

In mice, posterior *cyp26a1* expression is lost when *Fgfr1* is conditionally mutated in all *brachyury*-expressing mesodermal cells (Wahl et al. 2007). This and experiments in chicks showing that RA inhibits Fgf signaling (Diez del Corral et al. 2003) raised the possibility that Ntl regulates *cyp26a1* expression indirectly through activation of Fgf signaling. In contrast, our previous work demonstrated that Fgf signaling is maintained in mesodermal progenitors lacking Ntl function (Martin and Kimelman 2008), suggesting that Ntl regulation of *cyp26a1* is not through Fgf activation. We determined that Fgf signaling is not involved in the posterior regulation of *cyp26a1* using a heat-shock-inducible transgenic line expressing dominant-negative Fgfr1 from the *hsp70* promoter (*HS:dnfgfr1*) (Lee et al. 2005). We used this line to inhibit Fgf signaling after the end of gastrulation (bud stage) so that we could bypass the early gastrula requirement of Fgf for all posterior mesoderm formation in zebrafish (Griffin et al. 1995). Despite complete loss of expression of the direct Fgf targets *pea3* and *erm* after heat-shock activation of *dnfgfr1* (100% of transgenic embryos had complete *pea3* and *erm* loss, $N = 20$ and $N = 19$, respectively) (Fig. 2G–J), *cyp26a1* expression in the posterior tailbud was normal (100% of transgenic embryos had normal *cyp26a1* expression, $N = 19$) (Fig. 2K,L). Correspondingly, we also found that *ntl* expression was normal in the tailbud after Fgf loss of function (100% of transgenic embryos had normal *ntl* expression, $N = 20$) (Fig. 2M,N). These data confirmed that zebrafish *cyp26a1* is not regulated by Fgf signaling, and raised the possibility that Ntl directly activates *cyp26a1* expression in mesodermal progenitors.

Ntl directly activates cyp26a1

To determine if Ntl regulation of *cyp26a1* is direct, a Ntl-glucocorticoid receptor (Ntl-GR) fusion protein was overexpressed in embryos. This fusion protein enters the nucleus only when the inducer dexamethasone is added (Kolm and Sive 1995). To examine direct regulation, cycloheximide (Chx) was added 30 min prior to the addition of dexamethasone. In the absence of protein synthesis, the Ntl-GR protein expanded *cyp26a1* expression

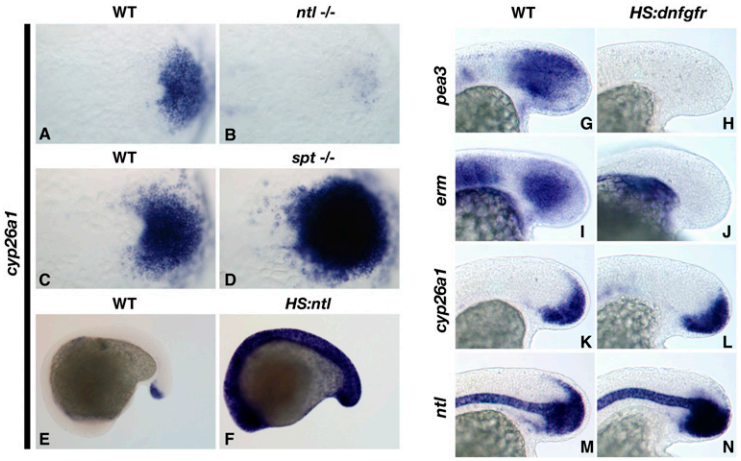

Figure 2. Ntl protects mesodermal progenitors from RA by regulating *cyp26a1* expression. (*A—D*) Ntl but not Spt is necessary for *cyp26a1* expression. *cyp26a1* expression is absent in *ntl* mutants at the eight-somite stage (*B*) and increased in *spt* mutants (*D*) as compared with wild-type siblings (*A*,*C*). (*E*,*F*) Ntl is sufficient for *cyp26a1* expression. A *HS:ntl* transgenic embryo (*F*) expresses *cyp26a1* in all cells 3 h after heat shock, as opposed to the posteriorly restricted expression in a nontransgenic embryo (*E*). (*G–N*) Fgf signaling is not required for *cyp26a1* or *ntl* expression during tailbud stages. *HS:dnfgfr* embryos completely lack expression of the direct Fgf targets *pea3* (*H*) and *erm* (*J*) 3 h after heat shock at bud stage compared with normal expression in nontransgenic embryos (*G*,*I*), but maintain normal expression of *cyp26a1* (*L*) and *ntl* (*N*), as compared with nontransgenic embryos (*K*,*M*). (*A–D*) Dorsal (flat-mounted) view of the posterior end at the eight-somite stage. (*G–N*) Lateral view of the posterior end at the 16- to 18-somite stage.

only when dexamethasone was added (81% of Chx/Dex-treated embryos hexpanded *cyp26a1* expression [*N* = 27], whereas 9% of embryos treated with Chx alone had expanded *cyp26a1* expression [*N* = 23]) (Fig. 3A,B). In order to further substantiate that Ntl directly activates *cyp26a1* expression, we identified potential Ntl-binding sites in the 2.5-kb region upstream of the *cyp26a1* transcriptional start site, which has been shown previously to fully recapitulate endogenous *cyp26a1* expression (Hu et al. 2008). Using a Ntl position-weighted matrix (Garnett et al. 2009), we identified seven putative Ntl-binding sites with a *P*-value of <0.001 (Fig. 3C,E). A luciferase activity assay in 293T cells demonstrated that Ntl is capable of strongly activating the wild-type *cyp26a1* promoter (Fig. 3D). A combination of deletions and mutational analysis of individual Ntl-binding sites indicated that the two sites proximal to the *cyp26a1* transcriptional start site are essential for the majority of Ntl activation of the *cyp26a1* promoter (Fig. 3D). Combined with the Ntl-GR result, these data indicate that Ntl acts directly through Ntl-binding sites to activate *cyp26a1* expression. The presence of at least five putative Ntl-binding sites with a *P*-value of <0.001 within 2.5 kb of the *cyp26a1* transcriptional start site in five different fish species indicates that Ntl regulation of *cyp26a1* is likely evolutionarily conserved (Fig. 3E).

Ntl establishes the embryonic mesodermal progenitor niche

In a whole-embryo context, loss of Ntl results in a loss of *wnt3a*, *wnt8*, and *cyp26a1* expression (Fig. 2; Martin and Kimelman 2008), causing a failure to sustain the mesodermal progenitors and a subsequent posterior truncation. Surprisingly, we found that the loss of Ntl function within single mesodermal progenitor cells transplanted into a wild-type environment has no consequence on their development, and they can contribute to somites at all axial levels (Martin and Kimelman 2008). One possibility is that single cells lacking Ntl are rescued by surrounding wild-type progenitor cells, which provide Wnt ligands and reduce RA levels in the progenitor environment such that any single cell does not need to produce the cytosolic enzyme Cyp26a1 to degrade RA itself. Alternatively, the surrounding wild-type cells could produce a signal that would induce the transcription of the *wnts* and *cyp26a1* in the *ntl* mutant cells. To distinguish between these two possibilities, *ntl* morphant cells containing fluorescein dextran were transplanted into wild-type hosts, which were later fixed and examined for the expression of *wnt3a*, *wnt8*, and *cyp26a1*. While control wild-type transplants express these genes normally, *ntl* morphant cells fail to express all three genes (80%, *N* = 10; 90%, *N* = 10; and 70%, *N* = 17, respectively) (Fig. 4, cf. D–F and A–C). Therefore, Ntl functions cell-autonomously to activate the *wnt* genes and *cyp26a1* in the progenitor domain.

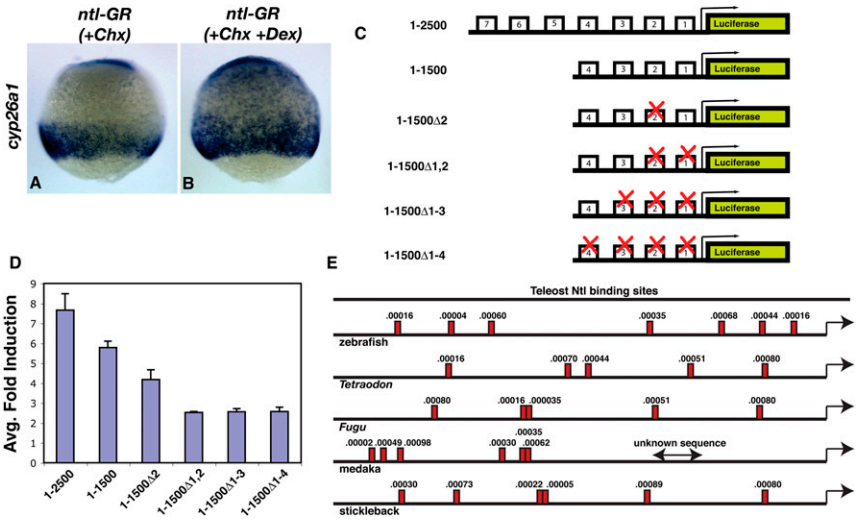

Figure 3. Ntl directly activates *cyp26a1* transcription. (*A*,*B*) Ntl activates *cyp26a1* expression in the absence of protein synthesis. Embryos injected with *ntl-GR* mRNA and treated with Chx (*A*) show no increase in *cyp26a1* expression, whereas treatment with Chx and dexamethasone (*B*) causes a strong expansion of *cyp26a1*. (*C*) *Pcyp26a1:luciferase* constructs used for testing Ntl activation of the *cyp26a1* promoter. Boxes 1–7 represent potential Ntl-binding sites (*E* shows the true locations of these sites within the promoter). (*D*) The two most proximal Ntl-binding sites are essential for *cyp26a1* induction by Ntl. The graph shows the fold induction of *Pcyp26a1:luciferase* by Ntl for each of the constructs shown in *C*. (*E*) A minimum of five Ntl-binding sites with a *P*-value of <0.001 are found in a 2.5-kb region upstream of the *cyp26a1* transcriptional start site in five different fish species. The numbers *above* the boxes show the calculated *P*-value. (*A*,*B*) Lateral view at 75% epiboly.

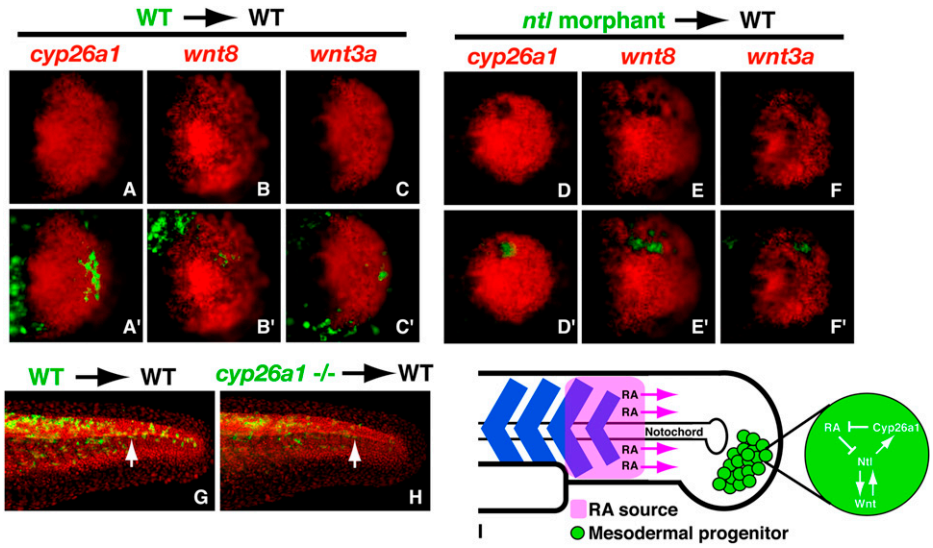

Figure 4. Ntl establishes the mesodermal progenitor niche. (*A–F′*) Ntl is cell-autonomously required for the expression of *cyp26a1*, *wnt8*, and *wnt3a*. (A–C′) Transplanted wild-type cells have no effect on *cyp26a1* (*A*,*A′*), *wnt8* (*B*,*B′*), or *wnt3a* (*C*,*C′*) expression, whereas transplanted *ntl* morphant cells do not express any of the three genes: *cyp26a1* (*D*,*D′*), *wnt8* (*E*,*E′*), or *wnt3a* (*F*,*F′*). The *top* panels show the in situ hybridization alone (red), whereas the *bottom* panels are a merge showing the transplanted cells (green) along with the in situ hybridization. (*D–F*) Note the absence of expression in the *top* panels in locations where the transplanted *ntl* morphant cells are found. (*G*,*H*) Cyp26a1 functions non-cell-autonomously in mesodermal progenitors. *cyp26a1* mutant cells (*H*) can contribute to the posterior-most somites of wild-type embryos equally as well as wild-type cells (*G*) in a wild-type embryo (arrows). Transplanted cells are green, whereas all nuclei are labeled in red to show the structure of the embryo. (*I*) Model of Ntl function in the mesodermal progenitors. Ntl creates the progenitor niche by regulating *wnt* ligand expression and *cyp26a1* expression, which prevents RA produced from neighboring somites from having an effect on the progenitors.

Although *cyp26a1* expression was clearly very reduced in *ntl* morphant cells (Fig. 4D), it remained possible that a low level of *cyp26a1* expression could still provide a protective function. We therefore asked if cells lacking all Cyp26a1 would be able to function normally. Cells from *cyp26a1* mutants (*giraffe*) (Emoto et al. 2005) were transplanted into wild-type hosts and, like *ntl* mutant transplants, were found to develop normally and populate somites at all axial levels (50% host embryos with *cyp26a1* mutant cells in somites posterior to the 24th somite, $N = 18$; 52% host embryos with control wild-type cells in somites posterior to the 24th somite, $N = 21$) (Fig. 4G,H). Since *cyp26a1* mutants have a truncated body (Emoto et al. 2005), we conclude that the mesodermal progenitor population functions as a community to degrade somite-derived RA at the posterior end of the embryo, whereas any individual cell does not have to degrade RA. Combined with our observation that *cyp26a1* mutants have reduced *ntl* expression during the post-gastrula stages (data not shown), we conclude that an essential role of Ntl is to activate *cyp26a1* expression within the community of progenitor cells in order to prevent endogenous RA from repressing *ntl*, which would then suppress the Ntl/Wnt autoregulatory loop and thereby cause truncation of the embryo.

Conclusion

Common examples of stem or progenitor cell niches in both embryonic and adult organisms consist of at least two general cell types: the stem/progenitor cells and the support cells, which provide the physical and molecular environment necessary for the maintenance of the stem/progenitor cells (Morrison and Spradling 2008; Voog and Jones 2010). Our data provide evidence of a unique type of progenitor cell niche consisting of only one cell type, in which mesodermal progenitor cells of the zebrafish tailbud act as their own support cells. Mesodermal progenitors express *ntl*, *wnt3a*, *wnt8*, and *cyp26a1*, all of which are required within the progenitor population as a whole, but none of which are required by individual progenitor cells in a wild-type environment. This demonstrates that the wild-type mesodermal progenitor cells act as support cells for the genetically deficient progenitors and can sustain them by creating an environment of high Wnt and low RA signaling. The primary function of Ntl, therefore, is to create the mesodermal progenitor niche through direct regulation of canonical *wnt* ligands and *cyp26a1* (Fig. 4I). While our analysis focused specifically on zebrafish, the common phenotypes of *brachyury* loss of function and RA treatment in different vertebrates (Dobrovolskaïa-Zavadskaïa 1927; Murakami and Kameyama 1965; Sive et al. 1990; Halpern et al. 1993; Schulte-Merker et al. 1994; Conlon et al. 1996; Martin and Kimelman 2008), as well as the conservation of expression patterns of *brachyury*, *wnts*, and *cyp26a1*, indicates that the same mechanism is common to all vertebrates. Thus, we suggest that expression of *brachyury* in the progenitor domain was a vertebrate adaptation that allowed the progenitor cells to be sustained during the long process of somitogenesis, which in some species can last for many days (Gomez et al. 2008). This unique function of Brachyury is particularly relevant, given that recent molecular analysis of various human cancers has demonstrated that *brachyury* is commonly up-regulated in tumors (for example, Vujovic et al. 2006; Palena et al. 2007; Yang et al. 2009; Yoshida et al. 2010). The up-regulation of *brachyury* may, in effect, be creating a cancer cell niche that maintains high Wnt signaling and low RA signaling, both of which have been extensively

demonstrated to be key components of cancer growth (Freemantle et al. 2003; Reya and Clevers 2005; Fodde and Brabletz 2007; Osanai et al. 2010).

Materials and methods

HS:ntl-myc *transgenic zebrafish*

myc-tagged *ntl* (also referred to as *ntla*) (Goering et al. 2003) was inserted into a previously described vector (Bajoghli et al. 2004) containing a bi-directional heat-shock promoter, *GFP*, and *tol2* transposable elements. Tol2-mediated transgenesis was used to produce a stable transgenic line as described previously (Kawakami 2004). Heat shocks were performed for 30 min at 41°C on embryos from an outcross of *HS:ntl-myc* hemizygous adults; transgenic embryos were separated from nontransgenic embryos based on GFP fluorescence.

HS:dnfgfr1-GFP *transgenic zebrafish*

Tg(hsp70I:dnfgfr1-EGFP) fish were kindly provided by Ken Poss (Lee et al. 2005). Hemizygous adults were outcrossed and progeny were heat-shocked at the 12-somite stage for 30 min at 37°C. Transgenic embryos were sorted based on GFP fluorescence and fixed 3 h after the end of the 30-min heat shock.

Cell transplantation

Embryos were injected with 2% fluorescein dextran either alone,or in combination with the *ntl* MO (Martin and Kimelman 2008), and were transplanted into the ventral margin of shield stage uninjected WIK/AB host embryos using a CellTram (Eppendorf) (Martin and Kimelman 2008). The same procedure was used for *cyp26a1* mutant (*giraffe*) transplants. Donor embryos were examined at 24 h post-fertilization to identify homozygous mutants.

RNA injection and in situ hybridization

The *ntl-GR* injections and in situ hybridizations were performed as described previously (Martin and Kimelman 2008; Griffin et al. 1995).

cyp26a1 *promoter analysis*

The *Pcyp26a1*-luciferase construct (Hu et al. 2008), which contains ~2500 base pairs (bp) upstream of the start codon, was kindly provided by Qingshun Zhao. The 1–1500 construct was made by cutting with AgeI (at −1500) and the SacI site in the vector, blunting the ends, and religating. Individual sites were mutated using the QuickChange method (Stratagene) to change positions 4 and 5 of the Ntl motif from CA to AT (Garnett et al. 2009). The mutagenic oligonucleotides used were (base changes shown in lowercase) site 1, CCACAATTAAAGATGAACTTTGatTGAACTAATTTATCTGAGGAAGTTAACAGGAGG; site 2, GTGTCGGGGGAATTAAatCCTTTTCAAAGTGAAATCTCAGGATTGTCTGC; site 3, GAGTAGGCTGTCTAACATGatTTATAGCCTTTTAAGGACCCCCAGAGC; and site 4, CATGTATAGAACTTTACATTTATAGAAATATCCAGAAATCAatCTATTATAATCAACTTTTGAAAATAAAATGCTTTCTATTAT. Luciferase assays used the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. 293T cells were transfected with Lipofectamine 2000 (Invitrogen) in a 24-well plate using 50 ng of each *Pcyp26a1*-luciferase construct, 20 ng of a TK-Renilla luciferase plasmid (Promega), and 150 ng of Ntl-myc expressed from a CMV promoter (Goering et al. 2003) and assayed 48 h after transfection.

For identification of Ntl-binding sites in *cyp26a1* promoters from different fish species, the 2500-bp upstream region was identified from the ENSEMBL database, and the PATSER program (http://rsat.ulb.ac.be/rsat/patser_form.cgi) was used with the Ntl position-weighted matrix (Garnett et al. 2009). Only sites with a *P*-value of ≤0.001 were counted as binding sites.

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