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STEM CELLS AND REGENERATION

RESEARCH REPORT

Co-expression of Tbx6 and Sox2 identifies a novel transient neuromesoderm progenitor cell state

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ABSTRACT

Elongation of the body axis is a key aspect of body plan development. Bipotential neuromesoderm progenitors (NMPs) ensure axial growth of embryos by contributing both to the spinal cord and mesoderm. The current model for the mechanism controlling NMP deployment invokes Tbx6, a T-box factor, to drive mesoderm differentiation of NMPs. Here, we identify a new population of Tbx6$^+$ cells in a subdomain of the NMP niche in mouse embryos. Based on co-expression of a progenitor marker, Sox2, we identify this population as representing a transient cell state in the mesoderm-fated NMP lineage. Genetic lineage tracing confirms the presence of the *Tbx6*$^+$ NMP cell state. Furthermore, we report a novel aspect of the documented *Tbx6* mutant phenotype, namely an increase from two to four ectopic neural tubes, corresponding to the switch in NMP niche, thus highlighting the importance of *Tbx6* function in NMP fate decision. This study emphasizes the function of Tbx6 as a bistable switch that turns mesoderm fate 'on' and progenitor state 'off', and thus has implications for the molecular mechanism driving NMP fate choice.

KEY WORDS: Neuromesoderm progenitors, Tbx6, Axial elongation, Trunk-tail transition, Mouse

INTRODUCTION

The discovery of neuromesoderm progenitors (NMPs) has fundamentally impacted our understanding of the development of vertebrate body plan (Henrique et al., 2015; Stern et al., 2006; Steventon and Martinez Arias, 2017). NMPs constitute a distinct population of stem cells that allow axial growth of vertebrate embryos by contributing to the posterior spinal cord (neural tube) and the somite-forming paraxial mesoderm. In particular, clonal lineage analysis has revealed that NMPs include bipotent stem cells capable of generating either neural or mesodermal progenitors (Martin and Kimelman, 2012; Tzouanacou et al., 2009). In the progenitor state, NMPs are marked by co-expression of the mesoderm T-box transcription factor brachyury (T) and the progenitor/neural marker Sox2 (Cambray and Wilson, 2007; Delfino-Machin et al., 2005; Garriock et al., 2015; Martin and Kimelman, 2012; Wilson et al., 2009). The feedback loop involving Wnt/β-catenin signaling and its target T is speculated to be key for maintenance of the NMP pool (Martin and Kimelman, 2012). By contrast, another T-box factor, Tbx6, a downstream target of T, is implicated in favoring the mesodermal aspect of the binary fate choice. *Tbx6* is required for repression of *Sox2* (Takemoto et al., 2011; Nowotschin et al., 2012; Bouldin et al., 2015; Ruvinsky et al., 1998). Moreover, a negative feedback of *Tbx6* on *Wnt3a* may favor exit of NMPs from the undifferentiated progenitor state (Martin and Kimelman, 2012). Based on this evidence, Tbx6 has been proposed as a fate switch, tipping the balance between NMP and mesoderm towards the latter. The fate switch function would predict induction of Tbx6 in the progenitor state; however, Tbx6 has been detected only in the differentiated paraxial mesoderm and not in progenitors. This missing evidence leaves a gap in our understanding of the timing of NMP fate restriction.

Local signaling cues are central to fate determination of stem cell descendants and hence the anatomy of the niche is crucial. In mouse, fate mapping studies have located NMPs in the node-primitive streak border (NSB) and caudal lateral epiblast (CLE) in late gastrula embryos and in the chordo-neural hinge (CNH) in tail bud stage embryos (Cambray and Wilson, 2002, 2007; Delfino-Machin et al., 2005; Martin and Kimelman, 2012). The NMPs in the NSB and CLE contribute to axial growth of the interlimb (trunk) region. A recent study has refined the CLE fate map by showing that bipotent trunk NMPs are restricted to anterior CLE, i.e. near the node, and that other subdomains of the CLE predominantly harbor cells with neuromesoderm potency, but with their fate restricted to either the neural or mesodermal lineage (Wymeersch et al., 2016). This implies discrete microenvironments within the CLE that favor maintenance or lineage progression of NMPs. Nevertheless, owing to an absence of markers for distinct subpopulations, molecular evidence for discrete subdomains of the niche is lacking.

As trunk development ends, NMPs from the NSB/CLE are relocated to the CNH of the tail bud (Cambray and Wilson, 2002, 2007; Wymeersch et al., 2016). The CNH is the region abutting the posterior end of the notochord ventral to the developing neural tube. NMPs in the CNH continue to contribute to neural tube and mesoderm in the tail. Tail bud-mediated tail development represents a mode of axial growth distinct from that of gastrulation-driven development of the trunk (reviewed by Handrigan, 2003). The axial level of the hindlimb buds marks the trunk-to-tail developmental transition. Strikingly, the mechanism governing the trunk-to-tail transition also appears to regulate the relocation of NMPs, thereby underscoring a mechanistic coupling of the switch in the NMP niche and this important developmental transition (Jurberg et al., 2013). However, the role of the key regulators of NMP fate in this key aspect of their biology remains unexplored.

Here, we show co-expression of Tbx6 and Sox2 in the primitive streak/CLE region. Later in development, in the tail bud, we find

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Tbx6$^+$ Sox2$^+$ NMPs in the CNH. The distribution in the progenitor compartment as well as the co-expression with a progenitor marker indicate that these Tbx6$^+$ cells represent a transient progenitor population intermediate in the NMP lineage between the stem cells and the descendant mesoderm. Furthermore, our analysis of *Tbx6* mutants reveals a novel aspect of the NMP developmental phenotype coincident with the relocation of the NMP niche during the trunk-to-tail developmental transition. In summary, the study reveals a new Tbx6$^+$ Sox2$^+$ transition state in the NMP lineage and underscores the pivotal function of *Tbx6* in these developmentally important stem cells. These findings also provide key missing evidence supporting the current model of NMP fate choice and emphasize the role of Tbx6 as a switch that drives NMPs to adopt mesoderm fate.

RESULTS AND DISCUSSION

Tbx6/Sox2 co-expression identifies a new population of mesoderm-primed trunk NMPs

Tbx6 is proposed to be the decisive factor favoring exit of NMPs from the progenitor state toward mesoderm differentiation. The model predicts that Tbx6 expression should precede NMP fate commitment to paraxial mesoderm. However, evidence for this is scant from documented *Tbx6* expression (Garriock et al., 2015; Nowotschin et al., 2012). To test the prediction of the model we assayed for Tbx6 expression in the trunk NMP compartment using a custom-made Tbx6 antibody. Following antibody specificity confirmation (Fig. S1), we performed immunostaining of cryosections from wild-type E8.5 mouse embryos. Transverse series were stained for Tbx6 and Sox2. In addition to paraxial mesoderm, Tbx6$^+$ cells were observed in the region encompassing the primitive streak and CLE (Fig. 1A). Remarkably, these cells co-expressed Sox2 (Fig. 1A). To assess the distribution of Tbx6$^+$ Sox2$^+$ cells in primitive streak versus CLE, adjacent sections of the series were stained for T and fibronectin 1 (Fn1), a marker of the extracellular matrix (ECM). Whereas CLE is bounded ventrally by unbroken ECM, primitive streak has discontinuous ECM, characteristic of epithelial-to-mesenchymal transition. The results show that the majority of double-positive cells are present in the primitive streak, with some present in the medial aspect of the CLE as well (Fig. 1A). However, Tbx6 could not be detected unambiguously in the NSB and in epithelium corresponding to the anterior one-fifth of the CLE (Fig. 1A), where the uncommitted bipotent progenitors reside (Wymeersch et al., 2016). This appears consistent with previous single-cell analysis indicating the absence of *Tbx6* RNA in NMPs at E8.5 (Gouti et al., 2017; Koch et al., 2017).

For quantitative analysis, we focused on the posterior part of the CLE expressing Tbx6 in whole-mount immunostained E8.5 mouse embryos. All confocal optical sections passing through the posterior part of the CLE and primitive streak were analyzed. As spatial references, we used the posterior tip of the epiblast and a midline running along the primitive streak. Consistent with the above data, Tbx6$^+$ Sox2$^+$ cells were distributed near the midline of the primitive streak/CLE zone (Fig. 1B,C, Fig. S2). Noticeably, Tbx6$^+$ cells dwindled caudorostrally within this zone (Fig. 1C, Table S1). These results locate the majority of Tbx6$^+$ Sox2$^+$ cells in the midline, which has no neuromesoderm potency (Cambray and Wilson, 2007). A proportion of double-positive cells were distributed in the posterior part of the CLE, a region documented to possess some neuromesoderm potency, but predominantly mesoderm-fated progenitors (Cambray and Wilson, 2007; Wymeersch et al., 2016). Nearly one-third of the progenitors in the analyzed region were Tbx6$^+$ Sox2$^+$ (Fig. 1C, Table S1), with the level of Tbx6 lower than that in presomitic mesoderm (Fig. 1C). Based on the anatomical distribution and co-expression with Sox2, we conclude that the Tbx6$^+$ cells represent a mesoderm-primed transient progenitor state of trunk NMPs.

These experiments are the first to formally test the prediction of a Tbx6$^+$ Sox2$^+$ transient state and provide strong support for the model ascribing a decisive role for Tbx6 in the mesodermal fate of NMPs. Further, we suggest that the low levels of Tbx6 in the transient state might function to inhibit neurogenesis via Sox2 suppression but may not be sufficient to activate paraxial mesoderm genes. Interestingly, suppressing *Sox2* in *Tbx6* mutants rescues ectopic neural tube formation yet fails to rescue paraxial mesoderm development (Takemoto et al., 2011). This points to a mechanistic uncoupling of *Tbx6* function in neural inhibition and transactivation of paraxial mesoderm genes such as *Msgn1* (Wittler et al., 2007; Chalamalasetty et al., 2014). Investigation of *Msgn1* induction in these cell compartments could shed light on the possible biphasic function of Tbx6 in the NMP lineage.

Tbx6/Sox2 co-expression marks NMPs in the tail bud

As gastrulation ends, the continued axial growth is assured by NMPs that translocate from the NSB/CLE to the CNH in the tail bud. To assess whether the progenitor compartment in the tail bud also expresses Tbx6, we performed immunostaining on E9.5 tail bud sagittal cryosections for Tbx6 and Sox2. We found Tbx6$^+$ Sox2$^+$ cells in the CNH, the region documented to harbor NMPs (Fig. 2). Co-expression of T and Tbx6 in this compartment, in adjacent sections, attests to Tbx6 expression in NMPs (Fig. 2). These results confirm Tbx6 induction prior to mesoderm differentiation in this NMP lineage. The difference in Tbx6 expression in the trunk versus tail NMP compartments might reflect differences in the signaling environment in the epiblast versus the tail bud niche. Thus, our findings suggest that the regulatory state of tail NMPs is different from that of trunk NMPs. This conclusion is consistent with the findings of a single-cell transcriptome study (Gouti et al., 2017), which reported distinct gene expression patterns in E8.5 and E9.5 NMPs.

Tbx6 lineage tracing marks the posterior neural tube

Given the co-expression with Sox2 in tail bud NMP progenitor compartment, we asked whether Tbx6$^+$ cells have neural differentiation potential. We generated a novel transgenic *Tbx6-Cre* mouse line for Cre-lox genetic tracing. First, we verified that the reporter matches the mesoderm expression of endogenous *Tbx6* (supplementary Materials and Methods, Figs S3, S4). To test whether the *Tbx6* lineage marks the posterior neural tube in addition to mesoderm, we analyzed transverse sections of *Tbx6-Cre:ROSA^{tdTom}* E10.5 embryos for tdTomato reporter expression at different levels on the anteroposterior axis. At the hindlimb level and in the tail, virtually the entire neural tube appeared to be *Tbx6-Tomato*$^+$ (Fig. 3A, Fig. S4), strongly supporting Tbx6 expression in tail bud NMPs. However, unexpectedly, the contribution begins at the level of cervical somites and the extent of contribution gradually increases in the interlimb neural tube (Fig. 3A), indicating *Tbx6-Cre* induction in trunk NMPs. Our analysis also revealed a contribution of *Tbx6* reporter-positive cells to a number of tissues including posterior neural crest (Fig. S4) (Concepcion et al., 2017).

Tbx6 lineage reporter expression in the neural tube at cervical and interlimb level appears discordant with undetectable Tbx6 in the NSB and anterior CLE (Fig. 1A) (Gouti et al., 2017). Therefore, we assessed *Tbx6* reporter expression in the CLE in E8.5 *Tbx6-Cre:ROSA^{tdTom}* embryos. The entire transverse section of the CLE,

Development (2017) 144, 4522-4529 doi:10.1242/dev.153262

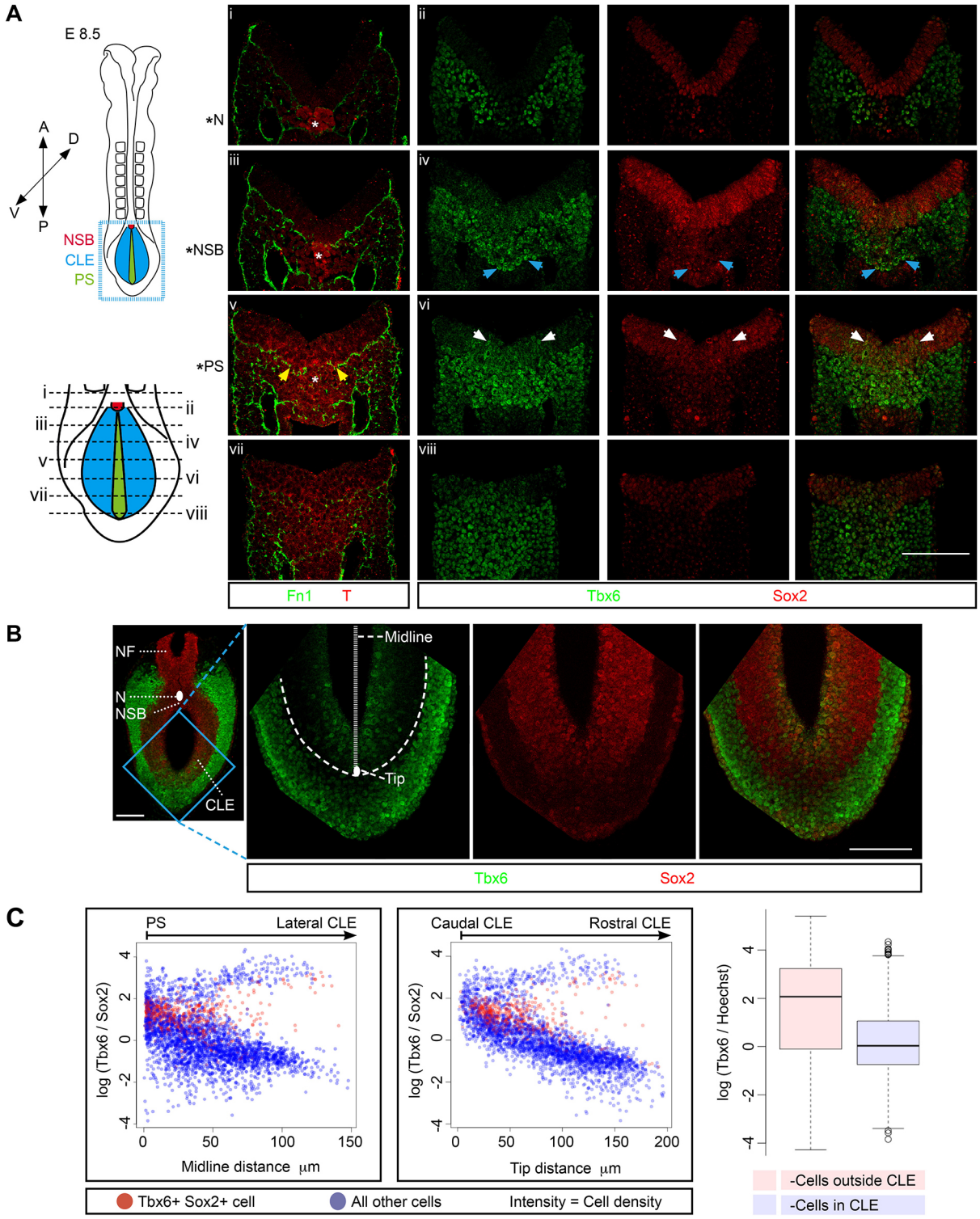


Fig. 1. See next page for legend.

including the T^+ $Sox2^+$ pool, was reporter positive (Fig. 3B). To address this discordance in *Tbx6* and reporter expression, we compared the expression of *Cre* RNA with that of endogenous *Tbx6*. We observed *Cre* expression in the NSB, while *Tbx6* RNA was unambiguously detected only in primitive streak posterior to the NSB (Fig. S5). In addition, we tested whether Tbx6 is induced in the NMP compartment earlier than E8.5, which could also explain the wider expression of reporter in NMP derivatives observed. As documented, we detected rare $Tbx6^+$ $Sox2^+$ cells in the NSB at E7.5 (Fig. S6) (Hadjantonakis et al., 2008). Taken together, transient induction of *Tbx6* in the NSB at E7.5 combined with expression of *Cre* transgene earlier in the lineage might account for reporter expression in the entirety of NMP derivatives. However, Tbx6 expression in the tail bud NMPs is independently corroborated by our analysis of the knock-in $Tbx6^{H2B\text{-}EYFP}$ [$Tbx6^{YFP}$ (Hadjantonakis et al., 2008)] reporter mouse line, which acts as a short-term tracer.

Development (2017) 144, 4522-4529 doi:10.1242/dev.153262

Fig. 1. Tbx6 and Sox2 co-expression marks a new transient progenitor state of the neuromesoderm lineage. (A) (Left) Illustration of NMP anatomy in an E8.5 mouse embryo. A, anterior; P, posterior; D, dorsal; V, ventral; NSB, node-primitive streak border; CLE, caudal lateral epiblast; PS, primitive streak. Box indicates the area shown in B. (Right) Transverse series (at the levels indicated bottom left) of cryosections co-immunostained for Fn1 and brachyury (T) or for Tbx6 and Sox2. Blue arrows mark ingressed cells; yellow arrows delimit discontinuous ECM and thus primitive streak; white arrows mark Tbx6$^+$ Sox2$^+$ cells in the CLE. Asterisks mark the node (N), NSB or PS. (B) Single confocal optical section at a dorsal level of immunostained whole-mount embryos. NF, neural folds. The midline of the primitive streak and the caudal tip of the primitive streak/CLE zone are indicated; these were used as geographical references for measurements performed in C. The gaps in the sections reflect invaginations in the neural plate (top; seen in the low-magnification image) and in the primitive steak (bottom). (C) Quantitative image analysis (from Embryo 1, see Table S1) of Tbx6 and Sox2 expression. The dots in the scatter plot represent cells in the geometrically defined CLE region and primitive streak. Fluorescence is represented by the logarithm of intensity normalized to Hoechst intensity. The dots represent the ratio of Tbx6 to Sox2 fluorescence, i.e. (Tbx6/Hoechst)/(Sox2/Hoechst). The dots are semi-transparent so that overlap of multiple dots is indicated by higher intensity. The box plot shows low levels of Tbx6 in the CLE compared with paraxial mesoderm (outside the CLE). The difference between the means in the two groups is fourfold; P=2.2×10^{-16} for the pairwise comparison. n=3 embryos. Scale bars: 100 µm.

We found strong and abundant YFP expression in the Sox2$^+$ posterior neural tube (Fig. 3C). Taking this result together with *Tbx6-Cre* tracing, we conclude that the *Tbx6* locus is 'open' in the NMP lineage much earlier than anticipated.

Understanding the regulatory cell state of stem cells is essential to deciphering the mechanisms controlling their maintenance and function. NMPs could either be a lineage non-committed 'naïve' population or a committed pool equipoised to both the neural and mesodermal lineages. NMPs have been proposed to represent the latter (Steventon and Martinez Arias, 2017) based on the co-expression of promesodermal T and proneural Sox2 factors. Transient induction of the *Tbx6* locus or its 'open' state does not equate to the presence of functional protein in trunk NMPs, but reflects a poised regulatory state. Furthermore, we suggest that mere induction of *Tbx6* alone might not be sufficient to effect a fate switch and that a threshold level might be required, at least in the tail. This idea is in accordance with the apparent difference in Tbx6 levels between the CNH and tail bud mesenchyme (Fig. 2).

Increased ectopic neural tubes in *Tbx6* null embryos at the trunk-tail transition correlates with NMP niche relocation

Since Tbx6 is expressed in NMPs of the CNH and virtually all cells in the tail are *Tbx6* lineage reporter positive, we wondered whether *Tbx6* loss of function would impact tail development more severely. In fact, a bulged tail bud with multiple ectopic neural rosettes in *Tbx6* mutants (Chapman and Papaioannou, 1998) hints at increasing phenotypic severity in the tail. We revisited the analysis of *Tbx6* mutant tail phenotype. No overt posterior neural tube patterning defect was observed (Fig. S7); however, strikingly, we found an increased number of ectopic neural tubes along the anteroposterior axis. By analyzing a series of transverse sections in E10.5 *Tbx6*$^{YFP/YFP}$ mutant embryos, we found a switch from two ectopic tubes at the base of the forelimb bud to four ectopic tubes at the axial level in register with the top of the hindlimb bud (Fig. 4A, Fig. S7). This phenotypic switch correlates with the transition from trunk to tail development, which entails reallocation of NMPs from the NSB/CLE to the CNH in the tail bud.

A priori, the supernumerary neural tubes could be due to expansion of the neurogenic progenitor domain. In fact, a broader domain of Sox2$^+$ neural progenitors in *Tbx6* mutant tail bud has been documented (Nowotschin et al., 2012). First, we ruled out the

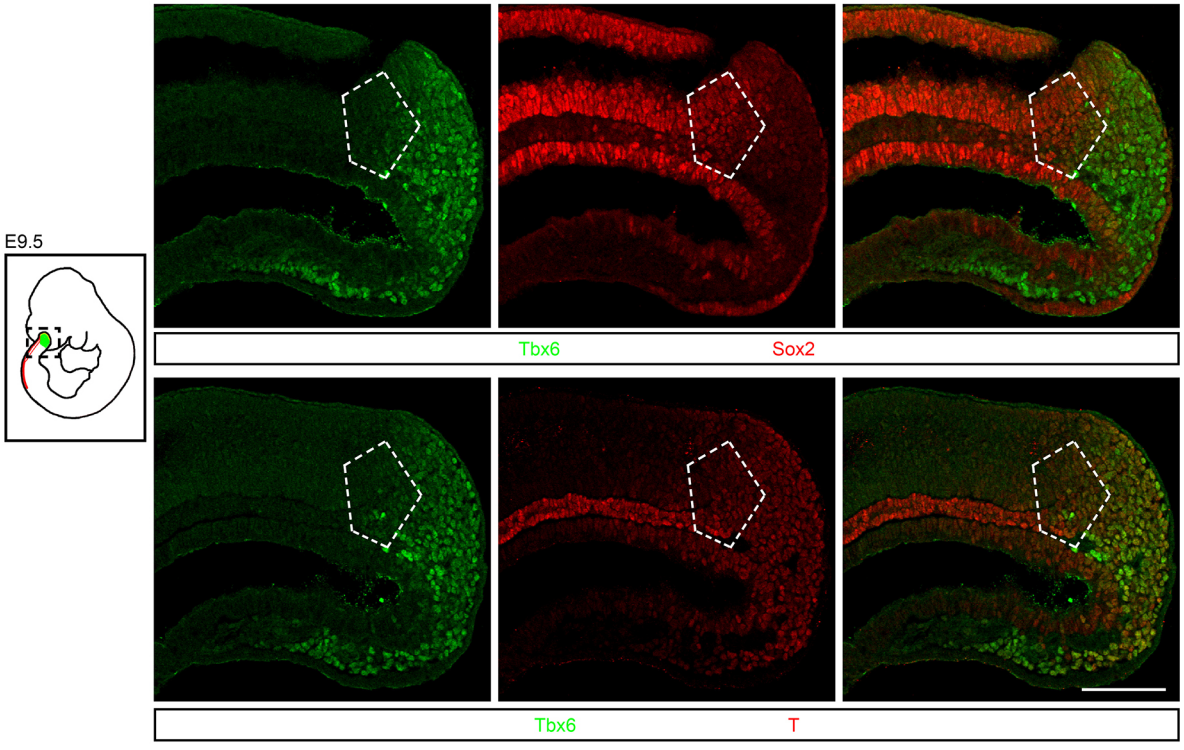
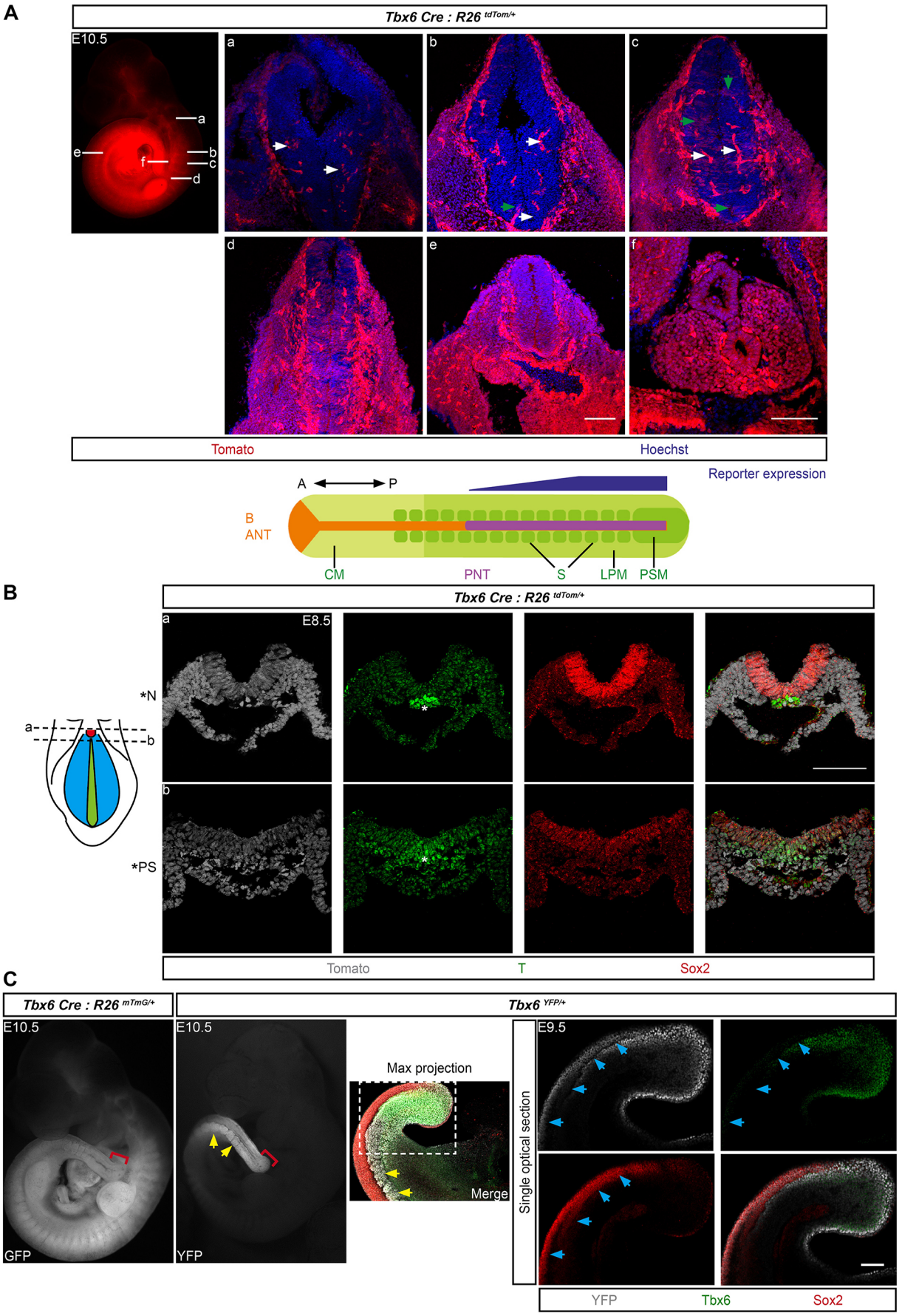


Fig. 2. Tail bud NMPs co-express Tbx6 and Sox2. (Top) Immunostaining of sagittal sections shows co-expression of Tbx6 and Sox2 in the CNH (pentagon). (Bottom) Adjacent section stained for the mesoderm marker T and Tbx6. n=3 embryos. Scale bar: 100 µm.

Development (2017) 144, 4522-4529 doi:10.1242/dev.153262



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Development (2017) 144, 4522-4529 doi:10.1242/dev.153262

Fig. 3. *Tbx6* lineage reporter marks virtually all NMP derivatives. (A) Live tdTomato reporter expression in a whole-mount embryo; the axial level of the transverse sections (a-f) is indicated. White arrows, endothelial cells (see Fig. S4); green arrows, neuroepithelial progenitors. The schematic beneath illustrates the findings. Blue gradient highlights the increase in reporter expression along the anteroposterior axis. A, anterior; P, posterior; B, brain; ANT, anterior neural tube; PNT, posterior neural tube; CM, cranial mesoderm; S, somites; LPM, lateral plate mesoderm; PSM, presomitic mesoderm. (B) Immunostained transverse cryosections (at levels a, b) show *Tbx6* reporter expression throughout the entire epithelium of the CLE fully overlapping the T^+ $Sox2^+$ zone. Asterisks indicate the node (N) or primitive streak (PS). (C) Comparison of the *Tbx6-Cre* reporter and the $Tbx6^{YFP}$ knock-in reporter shows the short-term tracing potential of the latter. The red bracket flags the domain of endogenous Tbx6 protein expression (see Fig. S1). Yellow arrows show the chase of *Tbx6* expression by YFP reporter in the somites. Sagittal confocal optical sections of the tail bud show $Tbx6^{YFP}$ reporter expression in $Sox2^+$ neural tube (blue arrows). *n*=3 embryos. Scale bars: 100 µm.

unlikely possibility that lateral plate mesoderm fate transformation contributes to the additional ectopic tubes (Fig. S7). Then, to directly assess whether the majority of the tail bud mesenchyme acquires neural fate, we analyzed the tail bud of $Tbx6^{YFP}$ heterozygotes and null mutants by immunostaining. Exploiting the *YFP* reporter, we found that 74.7±1.4% (*n*=4 embryos; total 4024 nuclei) overlap in YFP and Sox2 expression in mutants, as opposed to a more restricted co-expression of 28.3±2.9% (*n*=4 embryos; total 3213 nuclei) in the CNH region of heterozygotes (Fig. 4B). Based on these data we suggest that *Tbx6* function is required to restrict the neurogenic NMP pool to the CNH region.

In essence, defining the axial level at which the phenotypic transition occurs reveals an important link between *Tbx6* function and NMP behavior. The increase from two to four neural tubes appears to correspond to the change in NMP niche. Although conclusive demonstration is awaited, relocation from the CLE to CNH might involve a transition of NMPs from epithelium to mesenchyme. We suggest that the expansion of the neurogenic progenitor zone in mutants, together with the differences in the morphogenetic processes consequent to the epithelial-to-mesenchymal switch in the NMP niche, might explain the difference in the phenotype at the trunk and tail axial levels.

In summary, our findings establish the presence of a $Tbx6^+$ $Sox2^+$ transient progenitor population intermediate in the differentiation

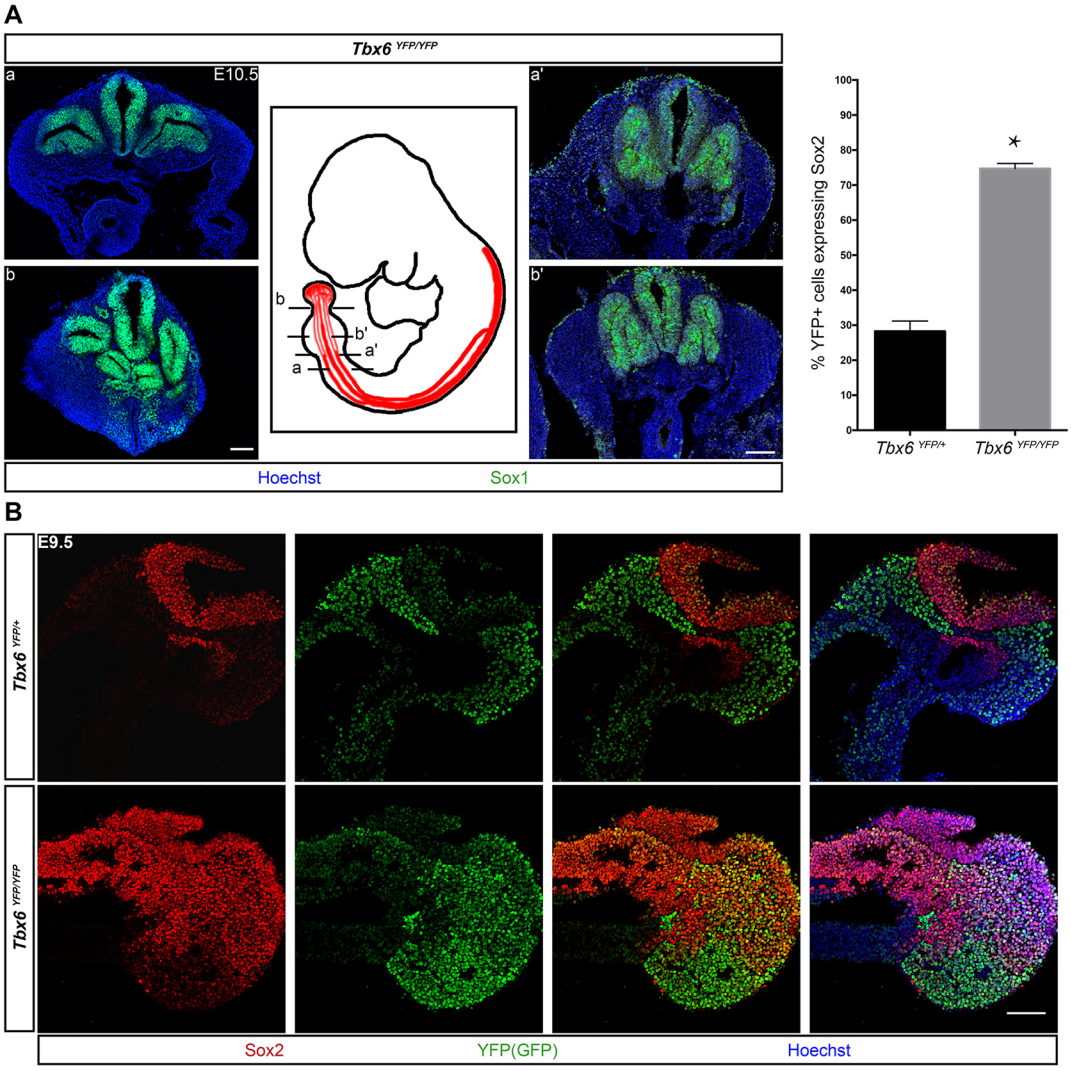

Fig. 4. Increase in ectopic neural tubes in the *Tbx6* mutant correlates with NMP niche relocation. (A) Immunostained cryosections (at levels a-b′) show four ectopic neural tubes in the tail bud (b). The switch from two to four ectopic neural tubes begins at the anterior end of hindlimb bud. *n*=6 embryos. (B) Sagittal sections of tail bud stained for YFP (antibody against GFP) and Sox2. Bar chart shows quantification of the increased proportion of $Sox2^+$ cells expressing YFP. *n*=3 embryos. Mann–Whitney test, two-tailed; error bars are s.e.m.; **P*=0.0286. Scale bars: 100 µm.

Development (2017) 144, 4522-4529 doi:10.1242/dev.153262

of NMP stem cells into paraxial mesoderm. This proof of Tbx6 expression marking the transition cell state strongly supports the current model of NMP fate decisions and emphasizes the role of Tbx6 as a fate switch or a 'fate dial' controlling mesoderm differentiation. These findings, along with the graded *Tbx6* mutant phenotype during the trunk to tail transition coincident with NMP niche relocation, underscore the central role of Tbx6 in NMP biology. Broadly, NMPs are crucial to the construction of the vertebrate body plan and our findings have a bearing on understanding the biology of this important pool of developmental stem cells.

MATERIALS AND METHODS

Mouse strains

All experiments on mice were carried out in compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) national guidelines. All experimental procedures were approved by the inStem Institutional Animal Ethics Committee. Strains used: $Tbx6^{YFP/+}$ (Hadjantonakis et al., 2008), *Tbx6-Cre* (see supplementary Materials and Methods), $R26^{mTmG/+}$ (Muzumdar et al., 2007), $R26^{tdTom/+}$ (Madisen et al., 2010) and $R26^{nlacZ/+}$ (Tzouanacou et al., 2009).

Immunostaining

For whole-mount immunostaining, embryos were fixed in 4% paraformaldehyde (PFA) in PBS for 90 min at 4°C. Embryos were blocked from non-specific antibody binding in blocking solution containing 0.5% Triton X-100 and 10% normal donkey serum. The embryos were treated overnight at 4°C with primary antibodies diluted in the blocking solution, followed by 1 h incubation in PBS containing 0.1% Tween 20, 10 mg/ml Hoechst and 4 mg/ml secondary antibodies.

For cryosections, embryos were embedded in PBS containing 7% gelatin and 15% sucrose following fixation in 4% PFA in PBS for 90-120 min and cryoprotection by equilibration in 15% sucrose in PBS overnight. The embryos were sectioned at 16 µm thickness using a Leica CM 1850 UV cryostat. Select sections were stained as described for whole-mount immunostaining.

For immunostaining cultured cells, the cells were fixed with 4% PFA for 15 min at ambient temperature and permeabilized using 0.3% Triton X-100 in PBS for 30 min and further blocked in PBS with 0.3% Triton X-100 and 5% normal donkey serum for 1 h. Incubation with primary antibody in blocking solution was carried out overnight at 4°C. The secondary antibody treatment was performed as described for whole-mount immunostaining.

Polyclonal anti-Tbx6 antibody used in this study was raised in rabbit against mouse Tbx6 using a peptide immunogen of the following sequence CFHGAPSHLPARTPSFAEAPDPGRPAPYS and affinity purified (Imgenex India). The specificity of the antibody has been tested by immunostaining as well as in immunoblot assays (Fig. S1). Images were acquired using a confocal or inverted fluorescence microscope. See the supplementary Materials and Methods for details and Table S2 for information about the antibodies used.

Quantitative image analysis

Images were analyzed using a probabilistic approach, which relies on nuclear segmentation using a Hough transform-based voting algorithm to detect cell positions in space, followed by pixel classification using the nuclear marker. The image analysis platform 'SilentMark' (Leonavicius et al., 2017 preprint) is available as an online data analysis application provided by Droplet Genomics (http://data.dropletgenomics.com/new/experiment/SilentMark). See the supplementary Materials and Methods for details.

In situ RNA hybridization

Whole-mount RNA *in situ* hybridization was performed following an established protocol (Henrique et al., 1995). The incubation period for proteinase K treatment was empirically determined for different stages of the mouse embryos. To detect *Dermo1*, a probe against its 3′ UTR was used, while *Tbx6* and *Cre* probes were prepared against the coding sequences. The antisense riboprobes were generated by *in vitro* transcription incorporating Digoxigenin-11_UTP (Roche). Washes following the incubation with alkaline phosphatase (AP)-conjugated anti-Digoxigenin-11 antibodies were extended for 48 h and BM Purple (Roche) was used as the colorigenic substrate for AP.

X-gal staining

Embryos were fixed for 40 min to 1 h in 4% PFA, washed in PBS and stained overnight at room temperature in a standard X-gal staining solution (Sanes et al., 1986).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.J., A.M., R.S.; Methodology: A.J., A.M., K.L., D.A., B.V.; Software: K.L.; Validation: A.J., A.M., B.V.; Formal analysis: A.J., A.M., K.L., R.S.; Investigation: A.J., A.M., D.A., B.V.; Writing - original draft: R.S.; Writing - review & editing: A.J., A.M., K.L., R.S.; Visualization: A.J., A.M., K.L., D.A., B.V.; Supervision: R.S.; Project administration: R.S.; Funding acquisition: R.S.

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Supplementary information

Supplementary information available online at
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