Published in final edited form as:
Genesis. 2012 June ; 50(6): 490–495. doi:10.1002/dvg.20827.

A transgenic *Tbx6;CreER^{T2}* line for inducible gene manipulation in the presomitic mesoderm

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Abstract

The rhythmic segmentation process of the presomitic mesoderm (PSM) orchestrates the formation of somites, the fundamental units for the vertebrate axial body plan. To aid the investigation of molecular components governing the conversion from PSM into somites, we generated a transgenic mouse line that expresses a tamoxifen (tmx) inducible *CreER^{T2}* under the control of a 2.5kb enhancer element of *Tbx6,* a gene essential for PSM formation and somite patterning. Combined with *Cre* reporters, this *Tbx6;CreER^{T2}* line displays robust tmx-inducible *Cre* activity in the PSM at various embryonic stages. This tool should be useful for studying gene function during somitogenesis by either conditional inactivation or mis-expression, and potentially coupled with cell marking.

Keywords

Tbx6; presomitic mesoderm; somite; segmentation; Cre; tamoxifen

The vertebrate segmented body plan is established during embryogenesis by a process known as somitogenesis. During this process, somites form periodically as bilateral epithelial spheres flanking the spinal cord, from the presomitic mesoderm (PSM) (Christ and Ordahl, 1995; Dequeant and Pourquie, 2008; Tam, 1981). Periodicity of somite formation is correlated with the “segmentation clock”, a molecular oscillator that pulses waves of gene expression throughout the PSM (Cooke and Zeeman, 1976; Palmeirim *et al.*, 1997). Once the clock crosses a travelling determination front known as the “wavefront”, its activity ceases and synchronizes competent PSM cells to undergo somite formation (Cooke and Zeeman, 1976; Dubrulle *et al.*, 2001). As somites are forerunners to the axial skeleton, their segmented organization prefigures the reiterated nature of vertebrae and ribs.

Certain genetic components controlling somite formation have been identified. For example, mutations in the Notch signaling pathway lead to defects in somitogenesis in a manner related to a disrupted segmentation clock (Bessho *et al.*, 2001; Conlon *et al.*, 1995; Evrard *et al.*, 1998; Hrabe de Angelis *et al.*, 1997; Wong *et al.*, 1997; Zhang and Gridley, 1998). While most of these studies are based on germline mutations, recently conditional mutations and over-expression analysis have been conducted to tease out the mechanistic actions of selected players in the PSM (Aulehla *et al.*, 2008; Feller *et al.*, 2008; Wahl *et al.*, 2007). To date there are only a handful of PSM-specific constitutive Cre expressing mouse lines and

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Author contribution: Both authors contributed to experimental designs and manuscript writing.

Competing interest: The authors declare no potential conflict of interests, financially or otherwise.

none have the advantage of temporal control for gene manipulation at different anterior and posterior (A/P) levels (Ji *et al.*, 2006; Niwa *et al.*, 2007; Perantoni *et al.*, 2005; Wehn *et al.*, 2009). Towards this goal, we sought to generate a mouse line expressing the *CreER*^{T2} gene specifically in the PSM for tmx-inducible Cre activity for recombining flanked loxP sites (Feil *et al.*, 1996).

The T-box transcription factor *Tbx6* interacts with the clock and wavefront and has a dual role in somitogenesis. First it is an essential mediator of the PSM fate for generating posterior somites (Chapman *et al.*, 2003; Chapman and Papaioannou, 1998). Second, it has a critical role in A/P patterning of somites by synergizing with Notch signaling, a segmentation clock effector pathway (White and Chapman, 2005; White *et al.*, 2003). Expression of *Tbx6* commences on embryonic day 7.0 (E7.0) in the primitive streak and continues in the tailbud until the end of somitogenesis around E13.5 (Chapman *et al.*, 1996). Extensive studies on the *cis*-regulatory sequences of *Tbx6* identified a −2.3kb enhancer/promoter region that directs transgene expression in the PSM and newly formed somites (White *et al.*, 2005). This regulatory region should theoretically be useful to drive *CreER*^{T2} expression in the PSM.

We created a transgene construct by cloning a −2.5kb to +25kb *Tbx6* PSM-specific enhancer/promoter element which encompasses the −2.3kb region identified by White *et al.* (2005) upstream of a *CreER*^{T2} cassette (Fig.1a). Sixteen G0 lines were positive for the *Tbx6;CreER*^{T2} (referred to as *Tbx6;CreER* from here on) transgene as determined by polymerase chain reaction (PCR). To test for tmx-regulated Cre activity in the PSM, we crossed each transgenic line to *R26R*^{LacZ} Cre reporter mice (Fig.1b). Cre activity was induced by a single intraperitoneal (IP) injection of tmx into pregnant *R26R*^{LacZ} mice during mid-somitogenesis at E9.5. Embryos that were hemizygous for *Tbx6;CreER* and heterozygous for the reporter *R26R*^{LacZ} (*Tbx6;CreER;R26R*$^{LacZ/+}$) along with control littermates of various genotypic combinations were harvested 1 day later for β-galactosidase staining (using the X-gal substrate). We found that 6 transgenic lines were either sterile, negative for Cre activity, or ubiquitous for Cre activity in all mesodermal tissues (Fig.2a). In the remaining lines we observed low to high Cre activity in the PSM by this assay (compare Figs.2b and c). Line *Tbx6;CreER*^{Tg5578}*;R26R*$^{LacZ/+}$ is representative of 4 lines that had highly mosaic Cre activity in the PSM (Fig.2b). Six lines similar to *Tbx6;CreER*^{Tg5769}*;R26R*$^{LacZ/+}$ showed more uniform Cre activity in the PSM and its somite derivatives (Fig.2c). The *Tbx6;CreER*^{Tg5769} line displayed tightly regulated tmx-inducible Cre activity as control injection of corn oil, without tmx did not result in positive β-galactosidase activity for *Tbx6;CreER*^{Tg5769}*;R26R*$^{LacZ/+}$ embryos (not shown). We note however that tmx-induced *Tg5578* and *Tg5769* lines have small number of cells in the apical ectodermal ridge (AER) of the limb bud and 3rd brachial arch (Figs.2b,c) which may reflect either ectopic Cre activity due to insertion sites or differential sensitivities of using a permanent *R26R*^{LacZ} reporter (in this study) versus a status-quo *LacZ* reporter driven by the enhancer element (White *et al.*, 2005). Since *Tbx6;CreER*^{Tg5769} was responsive to tmx-induction with PSM-specificity, we further characterized this transgenic founder line.

After backcrosses to the *C57BL/6J* background, the tmx-inducible PSM-specific Cre activity remained in the *Tbx6;CreER*^{Tg5769} line when assayed at E9.5. As somitogenesis is a reiterated process that occurs over several days, we next wanted to ascertain the time window of *Tbx6;CreER*^{Tg5769} directed Cre activity. We found that the *Tbx6;CreER*^{Tg5769} transgene was active during most stages of somitogenesis by injecting tmx from E7.5 to E11.5 at daily intervals and examining reporter activity one day later (Figs.3 and 4). From E7.5 to E9.5, tmx-induced Cre activity was strongly active in the entire PSM and newly formed somites (Figs.3a and 4c). While tailbud stages (E10.5–12.5) had robust Cre activity in the anterior 3/4 of PSM and nascent somites, the tail bud was positive for only a few cells

(Figs.3b–e). We have titrated the tmx dosage, and found the optimal dosage for PSM specific Cre activity to be ~2.25 mg tmx/35 gram body weight. Above this amount we noticed embryonic toxicity, while below this dosage, PSM and somites displayed mosaic reporter expression (data not shown). In mice, one pair of somites forms every two hours (Tam, 1981). Given this rate, fewer than the expected 12 somites were labeled for Cre activity in the 24 hours following tmx injection. We had expected a delay in Cre activity, as the precise time frames of tmx action and sufficient accumulation of reporter product for assay (from recombination, transcription to translation) are unknown. Importantly, *Tbx6;CreER*^{Tg5769} confers tmx-inducible Cre activity in the PSM.

We further characterized tissue specificity of *Tbx6;CreER*^{Tg5769} by using the *R26R*^{YFP} Cre reporter and examined live tmx-induced E7.5 *Tbx6;CreER*^{Tg5769}*;R26R*$^{YFP/+}$ embryos at E8.5 (Figs.4a–c). We found a gradual wave of YFP signal intensity along the A/P axis indicative of Cre activity in the somites and PSM as with the LacZ reporter. To pinpoint the labeled cells, we performed immunofluorescence analysis on transverse sections (Figs.4d–i). In rostral somites (section I), we found few YFP positive cells (Figs.4d,g). In more caudal somites (section II), YFP labeling occurred in approximately ~42% of cells (Figs.4e,h). At the most anterior PSM level, ~68% are YFP positive (Figs.4f,i). Few scattered YFP positive cells were present in the lateral plate mesoderm, possibly through expression of the transgene in the primitive streak (Figs.4e,h and f,i). On the other hand, YFP positive cells were absent from the notochord, intermediate mesoderm, head and cardiac mesoderm.

Here, we describe a new genetic tool that provides conditional manipulation of gene function in the PSM and somites at any time during somitogenesis. The *Tbx6;CreER*^{Tg5769} line is unique among Cre lines that are currently used in the somite field (Ji *et al.*, 2006; Niwa *et al.*, 2007; Perantoni *et al.*, 2005; Wehn *et al.*, 2009). For example, the transgenic *T-Cre* exhibits high Cre activity in all mesodermal tissues, in addition to notochord, head and cardiac mesoderm (Perantoni *et al.*, 2005). Similar to *T-Cre*, *Dll1-msd Cre* is widely expressed in the intermediate and lateral plate mesoderm, although Cre activity does overlap with *Tbx6;CreER*^{Tg5769} in the anterior PSM and somites (Wehn *et al.*, 2009). Aside from greater tissue specificity, *Tbx6;CreER*^{Tg5769} provides temporal control that does not exist in constitutively active Cre lines in use to study somitogenesis. This genetic tool has the potential to test the function of segmentation clock or somite patterning genes at the desired A/P level of an embryo by either inactivation or mis-expression. Furthermore, since we observed mosaic Cre activity at lower tmx dosages, we envision that titrating tmx concentrations will enable the study of cell autonomous gene function at the single cell level. This would require the concomitant labeling of genetically modified cells by a cis-linked Cre reporter provided by the investigator.

Materials and Methods

Vertebrate animals

All experimental procedures involving live mice complied with institutional and national animal welfare laws, guidelines and policies, and are approved by Institutional Animal Care and Use Committee of Carnegie Institution of Washington.

Generation of *Tbx6;CreER*^{T2} transgenic mice

To obtain the *Tbx6* enhancer and promoter fragment, we amplified a region −2.5 kb upstream and +25bp downstream from the transcriptional start site of *Tbx6* using polymerase chain reaction (PCR) on a BAC template (CHORI BAC ID: RP24-239G12): primers 5′-ATAGCGGCCGCTGGAT GCCCCATTGCAAAGACAGTC-3′ and 5′-GCGTCTAGAGTTGTAGTTTCTTCTGGCCTTGTGTCC-3′. This product was digested

with Not1 and Xba1, cloned 5′ to the *CreER^{T2}* expression cassette with a 3′ SalI site (Feil *et al.*, 1997) in the pBluescript SK+ vector (Stratagene), and sequence verified. This plasmid was Not1-SalI digested and the transgene fragment was purified for pronuclear injection into C57BL/6 × DBA/2 F1-hybrid embryos. Of 150 injected embryos, 54 grew to adulthood, and 16 were positive for Cre by PCR, (Feil *et al.*, 1996). The *Tbx6;CreER^{Tg5769}* founder line was backcrossed to the *C57BL/6J* (JAX) background for eight generations.

Preparation of tamoxifen

A 20 mg/ml tmx citrate (Sigma T5648) solution dissolved in corn oil (Sigma C8267) was used in this study. After solubilization, 10 ml aliquots were flash frozen and were stored up to 6 months at −80°C.

Characterization of *Tbx6;CreER* transgenic mice for PSM-specific Cre activity

Tbx6;CreER^{T2}(Tbx6;CreER) transgenic lines were crossed to *R26R$^{LacZ/LacZ}$* or *R26R$^{YFP/YFP}$* Cre reporter mice (Soriano, 1999; Srinivas *et al.*, 2001). Pregnant mice were intraperitoneal (IP) injected with a single dose of ~2.25 mg tmx/35 gram of body weight at stages specified in the text (E7.5–E11.5; E=embryonic day; the vaginal plug date was designated as E0.5). Embryos were harvested 24 hours later in L-15 media (Gibco #11415064) and the yolk sac was used for genotyping by PCR. β-galactosidase staining followed standard procedures using the X-gal substrate (Hogan, 1994). Live fluorescence of *Tbx6;CreER;R26R^{YFP}* embryos (in PBS) was visualized under a Leica fluorescent dissecting scope and images were captured with a SPOT camera. For immunofluorescence, embryos were fixed in 4% paraformaldehyde (PFA)/PBS at room temperature for 2 hours, equilibrated in 20% sucrose/PBS, followed by quick freeze-embedding in OCT. 10 μm transverse sections were taken, post-fixed for 5 minutes in 4% PFA/PBS, blocked with 10% bovine serum albumin (Sigma A9418) in 0.1% TritonX-100/PBS for 1 hour, then blocked in 10% goat serum in 0.1% TritonX-100/PBS for another hour. Chick anti-GFP (1:200; Aves labs #GFP-1020) was used for overnight incubation at 4°C. After washing, Goat anti-chick Alexa fluor 488 (1:1000; Molecular Probes #A-11039) was incubated for 2 hours. After wash, slides were counterstained with DAPI, rinsed, and mounted in Fluoromount-G (Southern Biotech #0100-01). Fluorescent images were taken with an AxioCam. Digital images were processed by Metamorph and Photoshop software for merging and brightness/contrast adjustments respectively.

Acknowledgments

The authors thank the Fan lab members for reading the manuscript, Evan Siple and Samantha Satchell for genotyping assistance. The Harvard transgenic facility performed the pronuclei injection as a paid service. This work is funded by the Carnegie Institution Endowment and NIH grants to CMF and TPL (HD035596 and HD035596-S).

References

Aulehla A, Wiegraebe W, Baubet V, Wahl MB, Deng C, Taketo M, Lewandoski M, Pourquie O. A beta-catenin gradient links the clock and wavefront systems in mouse embryo segmentation. Nat Cell Biol. 2008; 10:186–193. [PubMed: 18157121]

Bessho Y, Sakata R, Komatsu S, Shiota K, Yamada S, Kageyama R. Dynamic expression and essential functions of Hes7 in somite segmentation. Genes Dev. 2001; 15:2642–2647. [PubMed: 11641270]

Chapman DL, Agulnik I, Hancock S, Silver LM, Papaioannou VE. Tbx6, a mouse T-Box gene implicated in paraxial mesoderm formation at gastrulation. Dev Biol. 1996; 180:534–542. [PubMed: 8954725]

Chapman DL, Cooper-Morgan A, Harrelson Z, Papaioannou VE. Critical role for Tbx6 in mesoderm specification in the mouse embryo. Mech Dev. 2003; 120:837–847. [PubMed: 12915233]

Chapman DL, Papaioannou VE. Three neural tubes in mouse embryos with mutations in the T-box gene Tbx6. Nature. 1998; 391:695–697. [PubMed: 9490412]

Christ B, Ordahl CP. Early stages of chick somite development. Anat Embryol (Berl). 1995; 191:381–396. [PubMed: 7625610]

Conlon RA, Reaume AG, Rossant J. Notch1 is required for the coordinate segmentation of somites. Development. 1995; 121:1533–1545. [PubMed: 7789282]

Cooke J, Zeeman EC. A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. J Theor Biol. 1976; 58:455–476. [PubMed: 940335]

Dequeant ML, Pourquie O. Segmental patterning of the vertebrate embryonic axis. Nat Rev Genet. 2008; 9:370–382. [PubMed: 18414404]

Dubrulle J, McGrew MJ, Pourquie O. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. Cell. 2001; 106:219–232. [PubMed: 11511349]

Evrard YA, Lun Y, Aulehla A, Gan L, Johnson RL. lunatic fringe is an essential mediator of somite segmentation and patterning. Nature. 1998; 394:377–381. [PubMed: 9690473]

Feil R, Brocard J, Mascrez B, LeMeur M, Metzger D, Chambon P. Ligand-activated site-specific recombination in mice. Proc Natl Acad Sci U S A. 1996; 93:10887–10890. [PubMed: 8855277]

Feil R, Wagner J, Metzger D, Chambon P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem Biophys Res Commun. 1997; 237:752–757. [PubMed: 9299439]

Feller J, Schneider A, Schuster-Gossler K, Gossler A. Noncyclic Notch activity in the presomitic mesoderm demonstrates uncoupling of somite compartmentalization and boundary formation. Genes Dev. 2008; 22:2166–2171. [PubMed: 18708576]

Hogan, BBR.; Constantini, F.; Lacy, E. Manipulating the Mouse Embryo: A Laboratory Manual. 2. Cold Spring Harbor Laboratory Press; 1994.

Hrabe de Angelis M, McIntyre J 2nd, Gossler A. Maintenance of somite borders in mice requires the Delta homologue DII1. Nature. 1997; 386:717–721. [PubMed: 9109488]

Ji SJ, Zhuang B, Falco C, Schneider A, Schuster-Gossler K, Gossler A, Sockanathan S. Mesodermal and neuronal retinoids regulate the induction and maintenance of limb innervating spinal motor neurons. Dev Biol. 2006; 297:249–261. [PubMed: 16781703]

Niwa Y, Masamizu Y, Liu T, Nakayama R, Deng CX, Kageyama R. The initiation and propagation of Hes7 oscillation are cooperatively regulated by Fgf and notch signaling in the somite segmentation clock. Dev Cell. 2007; 13:298–304. [PubMed: 17681139]

Palmeirim I, Henrique D, Ish-Horowicz D, Pourquie O. Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. Cell. 1997; 91:639–648. [PubMed: 9393857]

Perantoni AO, Timofeeva O, Naillat F, Richman C, Pajni-Underwood S, Wilson C, Vainio S, Dove LF, Lewandoski M. Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. Development. 2005; 132:3859–3871. [PubMed: 16049111]

Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999; 21:70–71. [PubMed: 9916792]

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol. 2001; 1:4. [PubMed: 11299042]

Tam PP. The control of somitogenesis in mouse embryos. J Embryol Exp Morphol. 1981; 65(Suppl): 103–128. [PubMed: 6801176]

Wahl MB, Deng C, Lewandoski M, Pourquie O. FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. Development. 2007; 134:4033–4041. [PubMed: 17965051]

Wehn AK, Gallo PH, Chapman DL. Generation of transgenic mice expressing Cre recombinase under the control of the Dll1 mesoderm enhancer element. Genesis. 2009; 47:309–313. [PubMed: 19298012]

White PH, Chapman DL. Dll1 is a downstream target of Tbx6 in the paraxial mesoderm. Genesis. 2005; 42:193–202. [PubMed: 15986483]

White PH, Farkas DR, Chapman DL. Regulation of Tbx6 expression by Notch signaling. Genesis. 2005; 42:61–70. [PubMed: 15864811]

White PH, Farkas DR, McFadden EE, Chapman DL. Defective somite patterning in mouse embryos with reduced levels of Tbx6. Development. 2003; 130:1681–1690. [PubMed: 12620991]

Wong PC, Zheng H, Chen H, Becher MW, Sirinathsinghji DJ, Trumbauer ME, Chen HY, Price DL, Van der Ploeg LH, Sisodia SS. Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. Nature. 1997; 387:288–292. [PubMed: 9153393]

Zhang N, Gridley T. Defects in somite formation in lunatic fringe-deficient mice. Nature. 1998; 394:374–377. [PubMed: 9690472]

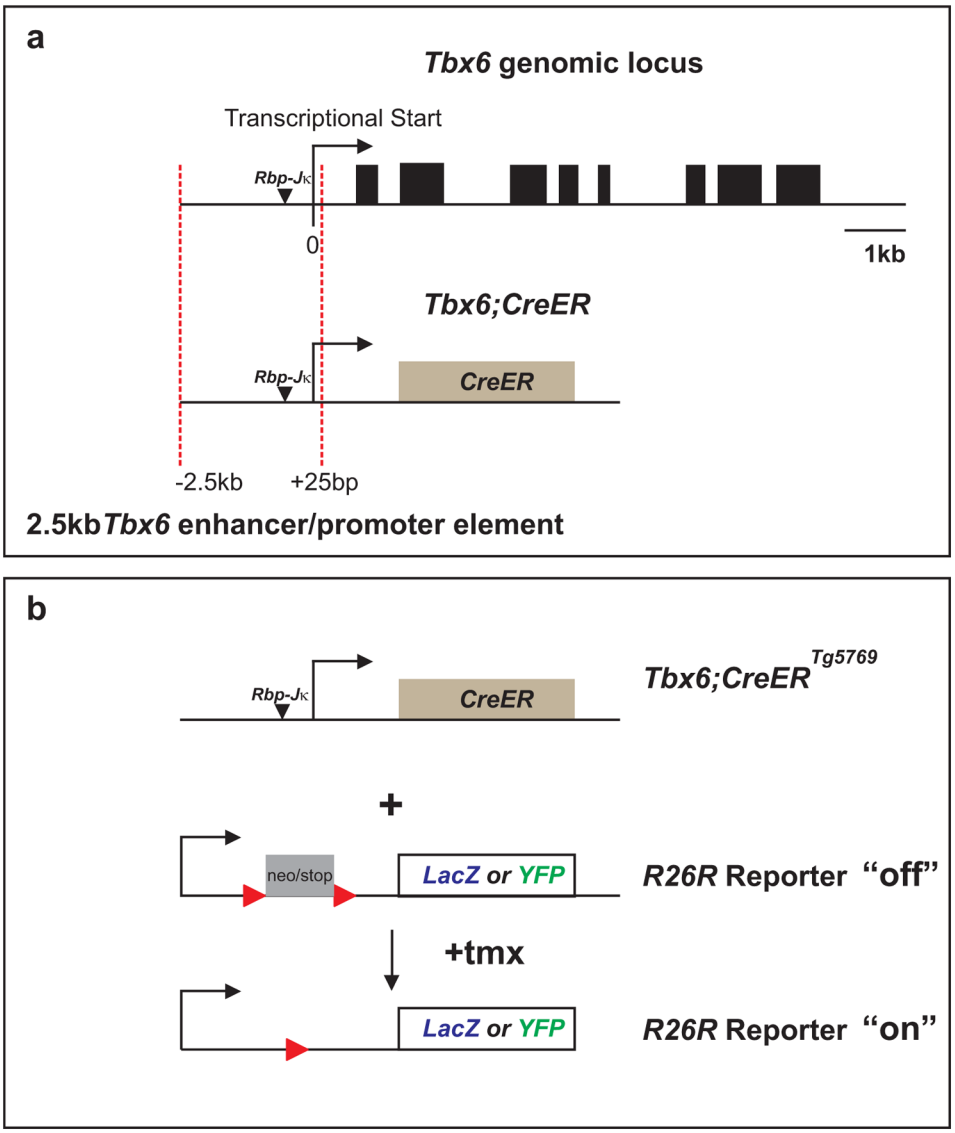

Fig. 1.

Diagram depicting *Tbx6;CreER* transgene construction and strategy used to screen for Cre activity in embryonic mice, *Tbx6* genomic diagram was adapted from (White *et al.*, 2005). **(a)** A $CreER^{T2}$ *(CreER)* cassette was cloned downstream to a 2.5kb enhancer and promoter element from the *Tbx6* genomic locus (red dotted lines). **(b)** Transgenic candidates such as $Tbx6;CreER^{Tg5769}$ were screened by crossing to LacZ or YFP report lines ($R26R^{LacZ/LacZ}$ or $R26R^{YFP/YFP}$), followed by a single dose of tmx to activate CreER to remove the floxed neo/stop cassette (Soriano, 1999; Srinivas *et al.*, 2001). Arrows mark the *Tbx6* promoter. Black arrowheads mark Rbp-Jκ binding sites. Red arrowheads represent the location of lox-P sites in reporter mice.

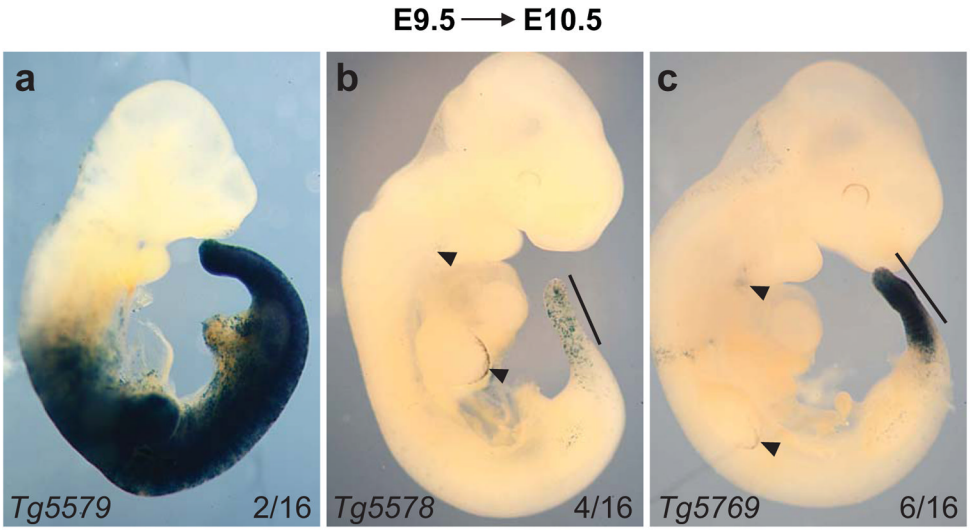

Fig. 2.

Identification of a *Tbx6;CreER* line that mediates efficient Cre activity in the presomitic mesoderm (PSM) after 24 hour tmx-induction (E9.5→E10.5). Three types of transgenic lines with distinct patterns of Cre activity are shown here as *Tbx6;CreER;R26R*$^{LacZ/+}$ X-gal stained embryos **(a–c)** representative of each category found. **(a)** 2/16 lines were positive for Cre activity in most mesodermal structures. **(b)** 4/16 lines had mosaic Cre activity in the PSM and newly formed somites (solid black line). **(c)** 6/16 embryos such as *Tg5769* had robust Cre activity in the PSM and nascent somites (solid black line). Arrowheads mark X-gal stained cells in the apical ectodermal ridge (AER) and 3rd brachial arch. The name of each transgenic line is located in the bottom left corner of each image.

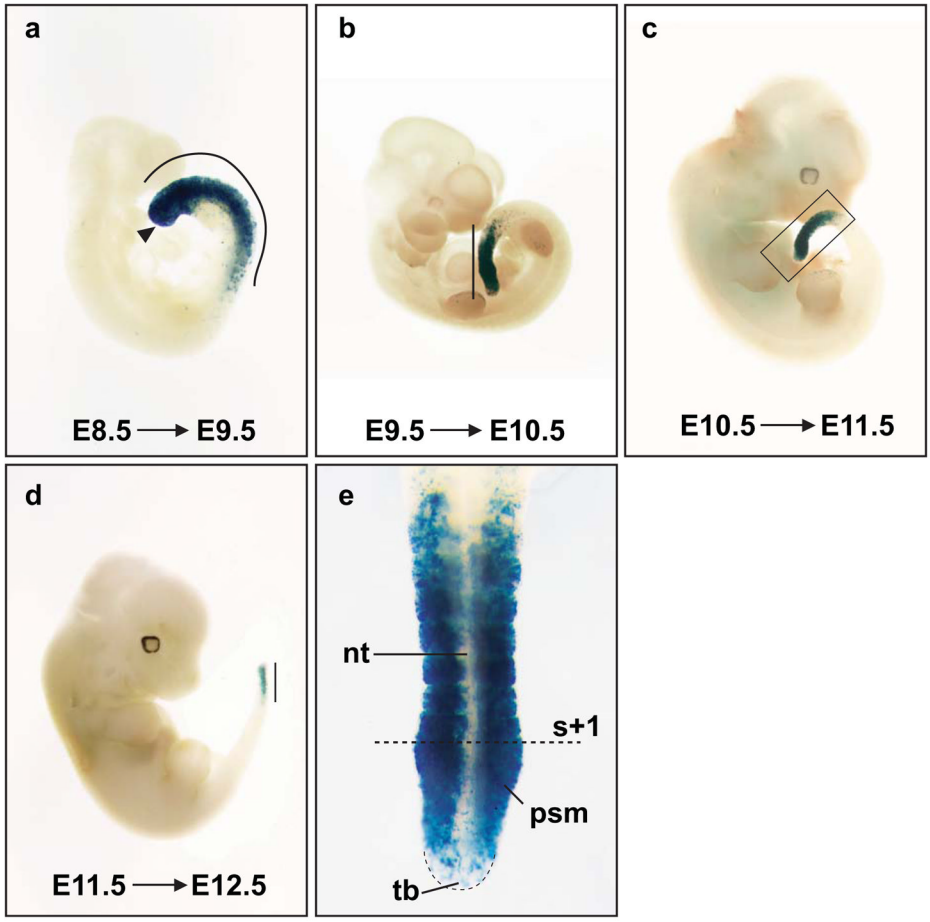

Fig. 3.

Tbx6;CreER^{Tg5769} drives robust Cre activity during most stages of somitogenesis. *Tbx6;CreER*^{Tg5769}*;R26R*$^{LacZ/+}$ embryos were tmx-induced (E8.5–E11.5) and harvested one day later for β–galactosidase staining. **(a–d)** presomitic mesoderm (PSM) and somite derivatives were positive for X-gal in all stages examined. **(a)** Cre activity is positive in the entire PSM, nascent somites (curved line) and primitive streak (black arrowhead). **(b–e)** Cre activity is down-regulated in the tailbud (tb) by stage E10.5 and onward. **(e)** Higher magnification of the boxed area in **(c)**, the tb has few X-gal positive cells, while the anterior PSM and newly formed somites contains high levels of β-galactosidase activity. The dotted line marks the boundary in between the first budded somite (s+1) and the PSM. All solid lines mark the length of Cre activity in embryos. The blue tint in the neural tube (nt) **(e)** is a result from light scattering of the X-gal positive somites.

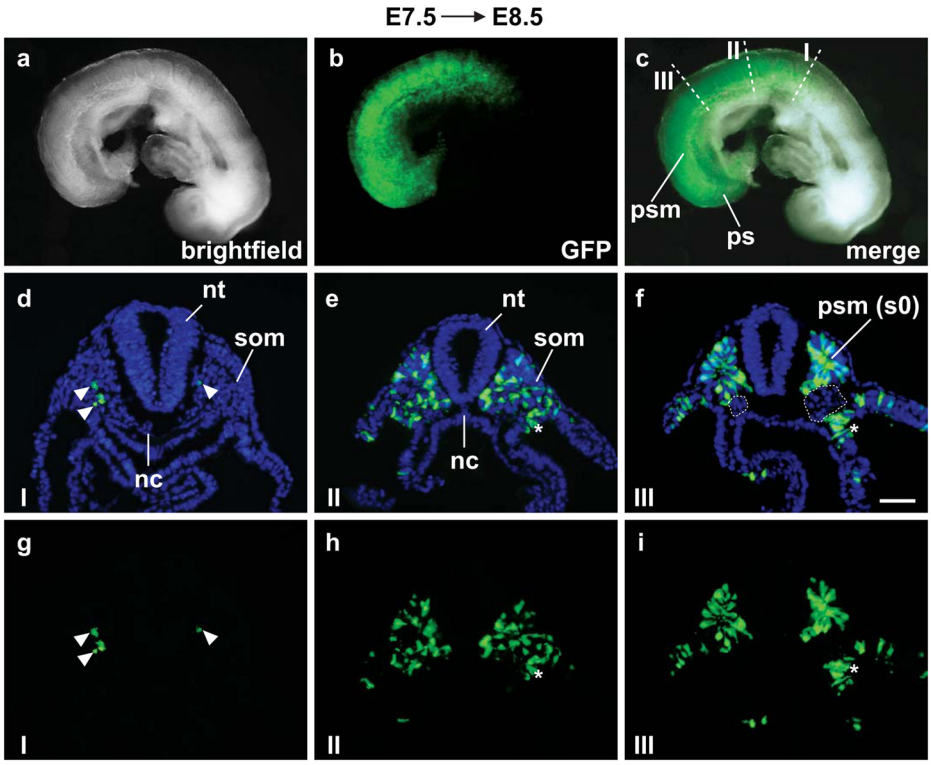

Fig. 4.

Tbx6;CreER^{Tg5769} is active on the first day of somitogenesis (E7.5→E8.5). **(a–c)** Live *Tbx6;CreER*^{Tg5769}*;R26R*$^{YFP/+}$ embryos exhibit fluorescent reporter activity along the A/P axis of the embryo including the presomitic mesoderm (psm), somites (som) and primitive streak (ps). **(c)** White dotted lines mark sections (I, II and III) merged for GFP and DAPI staining **(d–f). (g–i)** GFP only. **(d and g)** Section I has few +YFP cells (white arrowheads). Reporter activity increases posteriorly along the A/P axis of the embryo from moderate **(e and h)** to high in the presumptive somite (s0) of the psm **(f and i).** Cre activity was mosaic in the lateral plate mesoderm (white asterisks), labeling a few cells **(e,h and f,i). (d–f)** Cre activity was absent in the neural tube (nt), notochord (nc), intermediate mesoderm (white dotted circles), head and cardiac mesoderm **(b,c)**. Scale bar=50 μm.