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A *Tlx2-Cre* mouse line uncovers essential roles for Hand1 in extraembryonic and lateral mesoderm

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SUMMARY

Hand1 regulates development of numerous tissues within the embryo, extraembryonic mesoderm and trophectoderm. Systemic loss of Hand1 results in early embryonic lethality but the cause has remained unknown. To determine if *Hand1* expression in extraembryonic mesoderm is essential for embryonic survival, *Hand1* was conditionally deleted using the *HoxB6-Cre* mouse line that expresses Cre in extraembryonic and lateral mesoderm. Deletion of *Hand1* using *HoxB6-Cre* resulted in embryonic lethality identical to systemic knockout. To determine if lethality is due to Hand1 function in extraembryonic mesoderm or lateral mesoderm, we generated a *Tlx2-Cre* mouse line expressing Cre in lateral mesoderm but not extraembryonic tissues. Deletion of Hand1 using the *Tlx2-Cre* line results in embryonic survival with embryos exhibiting herniated gut and thin enteric smooth muscle. Our results show that Hand1 regulates development of lateral mesoderm derivatives and its loss in extraembryonic mesoderm is the primary cause of lethality in Hand1-null embryos.

Keywords

mouse; embryo; Cre recombinase; Hand1; neural crest; nervous system

The Hand1 bHLH transcription factor is expressed in a number of lineages within the developing embryo including the heart, neural crest (NC) derivatives and lateral mesoderm while extraembryonically, it is expressed in mesoderm and trophectoderm (Cserjesi *et al.*, 1995; Morikawa and Cserjesi, 2004; Riley *et al.*, 1998). Systemic knockout of *Hand1* leads to early embryonic lethality but the cause of the lethality has not been determined (Firulli *et al.*, 1998; Morikawa and Cserjesi, 2004; Riley *et al.*, 1998). The role of Hand1 in extraembryonic mesoderm has been studied and shown to be essential during vasculogenesis (Morikawa and Cserjesi, 2004) while in the trophectoderm lineage, it is required during placental development (Riley *et al.*, 1998). These studies suggest that the early lethality in *Hand1* null embryos is due to its functions in one of these extraembryonic lineages. In support, conditional deletion of Hand1 in the NC lineage (Barbosa *et al.*, 2007) or heart (McFadden *et al.*, 2002) does not result in early lethality.

To determine if *Hand1* expression in lateral and extraembryonic mesoderm is essential for embryonic survival, we deleted *Hand1* in these tissues but not trophectoderm using the *HoxB6-Cre* deleter line (Lowe *et al.*, 2000). Deletion of *Hand1* using *HoxB6-Cre* (Fig. 1)

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recapitulates the phenotype of the *Hand1* systemic knockout (Firulli *et al.*, 1998; Morikawa and Cserjesi, 2004; Riley *et al.*, 1998). Mutant embryos survive in the expected Mendelian ratio until 8.5 dpc. Most survive to 9.5 dpc but fail to turn with develop halting at the 7-somite stage (8.5 dpc) (Fig. 1a, b), a phenotype identical to the *Hand1* systemic knockout. Previous analysis suggested that the lethality of the systemic *Hand1* knockout may be due to its function in trophectoderm (Riley *et al.*, 1998). To confirm that Cre is not expressed in trophectoderm, the *HoxB6-Cre* line was crossed with the *R26R* reporter line. No Cre activity was observed in trophectoderm derivatives (date not shown). These results show that lethality caused by systemic loss of Hand1 is due to loss of *Hand1* in lateral or extraembryonic mesoderm.

Although *Hand1* is abundantly expressed in lateral mesoderm, and may be the cause of the early lethality, its role has not been investigated due to the lack of an appropriate Cre deleter line. To delete *Hand1* in lateral but not in extraembryonic mesoderm, we generated a transgenic mouse line expressing Cre under the control of Tlx2 regulatory sequences. The *Tlx2* gene (previously called Hox11L1, Enx, and Ncx) is first expressed in the primitive streak ectoderm (Tang *et al.*, 1998) and subsequently expressed in the peripheral nervous system (PNS) (Hatano *et al.*, 1997), neural tube (NT) (Uchiyama *et al.*, 1999) and posterior lateral mesoderm, extraembryonic ectoderm, and ectoplacental cone by 7.0 dpc (Tang *et al.*, 1998).

In order to find a regulatory region of the *Tlx2* gene that activates expression in the embryo but not extraembryonic lineages, we analyzed the *Tlx2* promoter region *in vivo*. We generated transgenic mouse lines with a 3 kb 5' sequence known to preferentially drives expression in NC-derived cell lines (Iitsuka *et al.*, 1999), linked to *LacZ*. The expression pattern was determined by examining embryos and adult tissues for β-galactosidase activity (Fig. 2). Unlike the expression of the endogenous *Tlx2* gene, which begins to express in the primitive streak, extraembryonic ectoderm, and ectoplacental cone by 7.0 dpc (Kapur *et al.*, 2005; Tang *et al.*, 1998), expression of the transgene begins at 8.5 dpc and is not detected in extraembryonic tissues. At 8.5 dpc, the transgene recapitulates expression of the endogenous *Tlx2* gene with expression first appearing in the neuroectoderm at the caudal half of the embryos at 8.5 dpc (Fig. 2a). By 9.5 dpc, expression is robust in the lateral mesoderm, caudal neural tube, and developing cranial nerves (CN) (Fig. 2b). In addition, ectopic expression is seen in the otic vesicle (Fig. 2b). At 10.5 dpc, expression is seen in much of the PNS (Fig. 2c). In the head, expression is seen in the glossopharyngeal ganglion (CN IX), inferior vagal ganglia (CN X), facioacoustic complex (CN VII–VIII) and trigeminal nerves (CN V). In the trunk, expression is high in the dorsal root ganglia (DRG), sympathetic ganglia (SG), enteric nervous system (ENS), with expression continuing in the lateral mesoderm (Fig. 2c). At 12.5 dpc, intense staining continues to be seen in the PNS but expression in the CNs and lateral mesoderm is decreased (Fig. 2d) resulting in weaker β-galactosidase staining by 14.5 dpc (data not shown). In the trunk, the transgene continues to be expressed in the PNS until birth (data not shown). In adults, transgene expression becomes highly restricted to the regions of endogenous gene expression (Parisi *et al.*, 2003). In the gut, expression remains high throughout the myenteric plexus and in a subset of smooth muscle cells (Fig 2e). Expression in the adrenal gland remains high (Fig. 2f) but in the trunk SG, expression is restricted in a subset of cells (Fig. 2g). The 3 Kb regulatory region of *Tlx2* closely resembles expression of endogenous gene in the embryos but not in extraembryonic tissues.

Since this regulatory region of the *Tlx2* does not promote expression in extraembryonic tissues, we generated a *Tlx2-Cre* deleter line. The *Tlx2-Cre* construct was generated by replacing the *LacZ* region of *Tlx2-LacZ* with the *Cre* recombinase gene. We generated a stable line of *Tlx2-Cre* and examined the expression of Cre by crossing with the *R26R*

reporter line. Analysis of β-galactosidase expression from 7.5 dpc to 10.5 dpc demonstrates that the *Tlx2-Cre* transgene recapitulates expression of the *Tlx2-LacZ* line (Fig. 3). Recombination of *LacZ* by *Tlx2-Cre* was not detected at 7.5 dpc (Fig 3a) but was observed at 8.5 dpc in the NT (data not shown) and lateral plate mesoderm in the caudal half of the embryo (Fig. 3b). By 9.5 dpc, β-galactosidase activity is seen in the otic vesicle and continues in the NT and lateral plate mesoderm (Fig. 3c). Unlike *Tlx2-LacZ*, a few cell in the extraembryonic mesoderm express β-galactosidase (Fig. 3d). At 10.5 dpc, Cre activity is found in the PNS (Fig. 3e, g, h), including CNs, DRG, SG and ENS. To visualize the distribution of Cre expressing cells in greater detail, embryos were sectioned. At 10.5 dpc, Cre activity is localized to the dorsal region of NT and lateral mesoderm and is not expressed in paraxial mesoderm (Fig. 3f).

The expression pattern of the *Tlx2-Cre* line suggests it will be useful for conditional inactivation of genes in a number of lineages. Of particular interest will be its use in development studies of the NC-derived PNS. Early deletion of genes regulating sympathetic, sensory and enteric nervous system development have relied to a great extent on the *Wnt1-Cre* mouse line which expresses Cre in the premigratory NC (Danielian *et al.*, 1998). However, *Wnt1-Cre* deletes genes in all NC often resulting in cardiovascular and facial defects leading to death at birth (Morikawa and Cserjesi, 2008; Morikawa *et al.*, 2009; Stottmann *et al.*, 2004; Xiong *et al.*, 2009). The *Tlx2-Cre* line deletes genes in the NC-derived PNS (Fig. 3h, g) and in placode derived cranial ganglia (Fig. 3e) but does not express Cre in the cranial NC (Fig. 3e) or cardiac NC (Fig. 3i, j), allowing deletion of the genes regulation of PNS development without affecting the cranial or cardiac NC lineages.

Tlx2-Cre and *Hand1* are co-expressed in the SG and lateral mesoderm (Cserjesi *et al.*, 1995; Hatano *et al.*, 1997; Kapur *et al.*, 2005; Morikawa and Cserjesi, 2004). To investigate the role of Hand1 in lateral mesoderm, we deleted *Hand1* conditionally by crossing with the *Tlx2-Cre* line. The level of *Hand1* recombination was determined by PCR analysis. In enteric smooth muscle (ESM), which is derived from Cre expressing lateral mesoderm, recombination occurs at a high level (Fig. 4a). Survival analysis of mutant embryos shows that all survive until 14.5 dpc with 6 out of 8 mutants dying between 14.5 dpc and birth with the remaining surviving to adulthood. Gross analysis of 18.5 dpc mutant embryos shows that the defects in conditional knockout embryos range from no apparent defects (Fig. 4b, c) to those with ventral closure defects resulting in gut hernias of variable severity (Fig. 4d). The results show that Hand1 plays an important function in lateral plate derived mesoderm.

Hand1 is expressed in the ESM throughout development and in adults (Cserjesi *et al.*, 1995; D'Autreaux *et al.*, 2007; Morikawa and Cserjesi, 2004) and we have shown a role in vascular smooth muscle cell recruitment in extraembryonic mesoderm (Morikawa and Cserjesi, 2004). We examined if *Hand1* deletion using *Tlx2-Cre* affects ESM development (Fig. 4e–h). Compared to control embryos (Fig. 4e, g), the ESM layers in mutant embryos were thinner and disorganized (Fig. 4f, h). To determine if the ESM cells are differentiated, the guts were analyzed for expression of α-smooth muscle actin (α-SMA) by immunofluorescent analysis (Fig. 4i, j). The cells in longitudinal and circular muscle layers of the gut express α-SMA in both control (Fig. 4i) and conditional mutant (Fig. 4j) embryos, suggesting that as in extraembryonic mesoderm, *Hand1* is not required for differentiation of ESM cells but is required for its organization.

The combined deletional analysis of *Hand1* using the *HoxB6-Cre* and *Tlx2-Cre* lines shows for the first time that the early lethality of systemic loss of *Hand1* is due to its function in extraembryonic mesoderm. In addition, our analysis of Hand1 function in lateral mesoderm has uncovered roles in ventral wall closure and ESM development.

MATERIALS AND METHODS

Generation of Transgenic Constructs and Mouse Lines

A *Tlx2* sequence containing −3003 bp to +706 was amplified from C57BL6 genomic DNA (Promega) using LA Taq polymerase (TaKaRa) and the primers 5'-AGA CAC CCT CAC CCC TAC CCC AAC CCT CAA -3' and 5'-TCC TCC CCC AAC CAC AGA AGC CTC ATC AAG -3'. The amplified region was cloned into pCR-XL-Topo vector (Invitrogen) to generate Tlx2-pCR-XL. To generate the *Tlx2* promoter/enhancer β-galactosidase fusion construct pTlx2-LacZ, the Tlx2-pCR-XL construct was digested with *Hin*dIII and *Nco*I to produce a fragment containing *Tlx2* sequence from −3003 bp upstream to the start of translation. The fragment was cloned into the *Hin*dIII and *Nco*I sites of pBS-Hsp68-LacZ (Kothary *et al.*, 1989) resulting in the simultaneous loss of the Hsp68 promoter sequence and fusion of the Tlx2 translational start site of *LacZ*. The pTlx2-LacZ insert was excised for proneural injection by digestion with *Sal*I.

The pTlx2-Cre vector was generated by replacing the *LacZ* of pTlx2-LacZ with the gene encoding Cre recombinase. The *Cre* gene was excised as an *Nco*I-*Not*I fragment from Hsp68-Cre (provided by Brian Black, University of California San Francisco) and cloned into the *Nco*I-*Not*I site of pTlx2-LacZ. The *Tlx2-Cre* insert was released by digestion with *Sal*I.

Generation of the transgenic lines was by pronuclear injection by the Tulane Transgenic Mouse Facility. *Tlx2-LacZ* transgenic mouse lines were identified by PCR analysis of tail clippings using the primers GCA ATT TAA CCG CCA GTC AGG and AGG CGG TCG GGA TAG TTT TCT T to amplify a fragment of 462 bp located in the *LacZ* gene. Mice carrying *Tlx2-Cre* were identified by PCR genotyping using primer sequences CTG GAA AAT GCT TCT GTC CGT TTG and ACG AAC CTG GTC GAA ATC AGT GCG located within the *Cre* gene producing a 316 bp fragment (Lowe *et al.*, 2000).

Mouse Husbandry

Tlx2-LacZ males were mated with Black Swiss females (Taconic) and the resulting *Tlx2-Cre* males were mated with *R26R* (*Gt(ROSA)26Sor*^{tm1Sho}) reporter females (Mao *et al.*, 1999). *HoxB6-Cre* (Lowe *et al.*, 2000) and *Tlx2-Cre* lines were mated with the *Hand1*$^{\beta\text{-}gal/+}$ line (Morikawa and Cserjesi, 2004) to obtain *Hand1*$^{\beta\text{-}gal/+}$; *HoxB6-Cre* or *Hand1*$^{\beta\text{-}gal/+}$; *Tlx2-Cre* lines. These mice were crossed with *Hand1*$^{fx/fx}$female mice (provided by Dr E. Olson, UT Southwestern Medical Center)(McFadden *et al.*, 2005) to obtain conditional knockout embryos. To obtain embryos, mice were sacrificed using CO_2 in accordance with Tulane's Institutional Animal Care and Use Committee.

LoxP site and recombined allele were genotyped by PCR analysis. Primer set 1 (GGG AGG GAC ATA GGC GGG GCG GGT TTT and GG GTG CGG CGG GTG TGA GTG GTG) amplifies across the first loxP site producing a 600 bp product. The recombined allele was genotyped using primer set 2 (CCT TCA GGC TCC CAC GAT and AGC AAA ATT CTA TGT TCA CTC AGC) that amplifies an 800 bp region.

β-galactosidase staining and histological analysis

Embryos and tissues were fixed in 4% paraformaldehyde in PBS for 15 minutes, rinsed in PBS, and stained overnight with 1 mg/ml X-gal, in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS. For analysis at 14.5 dpc, embryos were cleared after staining by dehydration in ethanol then clearing in BABB (2:1 benzylaclohol:benzyl benzoate). For histological analysis, tissues were embedded in paraffin and sectioned at 10 μm. Sections were counter-stained for eosin.

Immunohistochemistry

Immunohistochemical analysis on cryosections was performed as previously described (Morikawa and Cserjesi, 2004; Morikawa *et al.*, 2007). The antibody used in this study was FITC conjugated anti-α-SM actin, (Sigma) at a 1:200 dilution.

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LITERATURE CITED

Barbosa AC, Funato N, Chapman S, McKee MD, Richardson JA, Olson EN, Yanagisawa H. Hand transcription factors cooperatively regulate development of the distal midline mesenchyme. Dev Biol. 2007; 310:154–168. [PubMed: 17764670]

Cserjesi P, Brown D, Lyons GE, Olson EN. Expression of the novel basic helix-loop-helix gene eHAND in neural crest derivatives and extraembryonic membranes during mouse development. Dev Biol. 1995; 170:664–678. [PubMed: 7649392]

D'Autreaux F, Morikawa Y, Cserjesi P, Gershon MD. Hand2 is necessary for terminal differentiation of enteric neurons from crest-derived precursors but not for their migration into the gut or for formation of glia. Development. 2007; 134:2237–2249. [PubMed: 17507395]

Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr Biol. 1998; 8:1323–1326. [PubMed: 9843687]

Firulli AB, McFadden DG, Lin Q, Srivastava D, Olson EN. Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. Nat Genet. 1998; 18:266–270. [PubMed: 9500550]

Hatano M, Iitsuka Y, Yamamoto H, Dezawa M, Yusa S, Kohno Y, Tokuhisa T. Ncx, a Hox11 related gene, is expressed in a variety of tissues derived from neural crest cells. Anat Embryol (Berl). 1997; 195:419–425. [PubMed: 9176664]

Iitsuka Y, Shimizu H, Kang MM, Sasagawa K, Sekiya S, Tokuhisa T, Hatano M. An enhancer element for expression of the Ncx (Enx, Hox11L1) gene in neural crest-derived cells. J Biol Chem. 1999; 274:24401–24407. [PubMed: 10446220]

Kapur RP, Clarke CM, Doggett B, Taylor BE, Baldessari A, Parisi MA, Howe DG. Hox11L1 expression by precursors of enteric smooth muscle: an alternative explanation for megacecum in HOX11L1-/- mice. Pediatr Dev Pathol. 2005; 8:148–161. [PubMed: 15803212]

Kothary R, Clapoff S, Darling S, Perry MD, Moran LA, Rossant J. Inducible expression of an hsp68-lacZ hybrid gene in transgenic mice. Development. 1989; 105:707–714. [PubMed: 2557196]

Lowe LA, Yamada S, Kuehn MR. HoxB6-Cre transgenic mice express Cre recombinase in extra-embryonic mesoderm, in lateral plate and limb mesoderm and at the midbrain/hindbrain junction. Genesis. 2000; 26:118–120. [PubMed: 10686603]

Mao X, Fujiwara Y, Orkin SH. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. Proc Natl Acad Sci U S A. 1999; 96:5037–5042. [PubMed: 10220414]

McFadden DG, Barbosa AC, Richardson JA, Schneider MD, Srivastava D, Olson EN. The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development. 2005; 132:189–201. [PubMed: 15576406]

McFadden DG, McAnally J, Richardson JA, Charite J, Olson EN. Misexpression of dHAND induces ectopic digits in the developing limb bud in the absence of direct DNA binding. Development. 2002; 129:3077–3088. [PubMed: 12070084]

Morikawa Y, Cserjesi P. Extra-embryonic vasculature development is regulated by the transcription factor HAND1. Development. 2004; 131:2195–2204. [PubMed: 15073150]

Morikawa Y, Cserjesi P. Cardiac neural crest expression of Hand2 regulates outflow and second heart field development. Circ Res. 2008; 103:1422–1429. [PubMed: 19008477]

Morikawa Y, D'Autreaux F, Gershon MD, Cserjesi P. Hand2 determines the noradrenergic phenotype in the mouse sympathetic nervous system. Dev Biol. 2007; 307:114–126. [PubMed: 17531968]

Morikawa Y, Zehir A, Maska E, Deng C, Schneider MD, Mishina Y, Cserjesi P. BMP signaling regulates sympathetic nervous system development through Smad4-dependent and -independent pathways. Development. 2009; 136:3575–3584. [PubMed: 19793887]

Parisi MA, Baldessari AE, Iida MH, Clarke CM, Doggett B, Shirasawa S, Kapur RP. Genetic background modifies intestinal pseudo-obstruction and the expression of a reporter gene in Hox11L1-/- mice. Gastroenterology. 2003; 125:1428–1440. [PubMed: 14598259]

Riley P, Anson-Cartwright L, Cross JC. The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. Nat Genet. 1998; 18:271–275. [PubMed: 9500551]

Stottmann RW, Choi M, Mishina Y, Meyers EN, Klingensmith J. BMP receptor IA is required in mammalian neural crest cells for development of the cardiac outflow tract and ventricular myocardium. Development. 2004; 131:2205–2218. [PubMed: 15073157]

Tang SJ, Hoodless PA, Lu Z, Breitman ML, McInnes RR, Wrana JL, Buchwald M. The Tlx-2 homeobox gene is a downstream target of BMP signalling and is required for mouse mesoderm development. Development. 1998; 125:1877–1887. [PubMed: 9550720]

Uchiyama K, Otsuka R, Hanaoka K. CHox11L2, a Hox11 related gene, is expressed in the peripheral nervous system and subpopulation of the spinal cord during chick development. Neurosci Lett. 1999; 273:97–100. [PubMed: 10505625]

Xiong W, He F, Morikawa Y, Yu X, Zhang Z, Lan Y, Jiang R, Cserjesi P, Chen Y. Hand2 is required in the epithelium for palatogenesis in mice. Dev Biol. 2009; 330:131–141. [PubMed: 19341725]

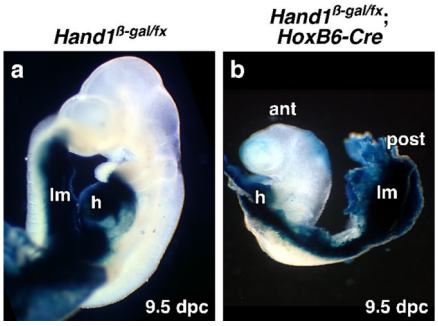

Fig. 1. Deletion of Hand1 in extraembryonic and lateral mesoderm results in embryonic lethality

Hand1 was conditionally deleted in lateral and extraembryonic mesoderm using the *HoxB6-Cre* deleter line and Hand1 expressing tissues were marked using a β-galactosidase gene inserted into the *Hand1* gene. (a) Control embryos show the distribution of Hand1 expression at 9.5 dpc. (b) Conditional mutant embryos at 9.5 dpc are developmentally retarded failing to turn and do not develop past the 7-somite stage. ant: anterior, h; heart, lm; lateral mesoderm, post: posterior.

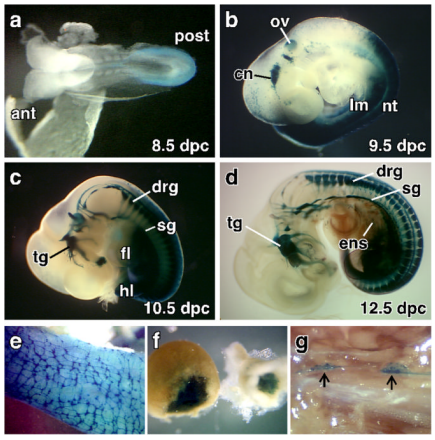

Fig. 2. A 3 kb region of the *Tlx2* gene promotes endogenous gene expression within the embryos

The *Tlx2-LacZ* transgenic line was analyzed for expression during development and in adults. Expression of the transgene is first observed at 8.5 dpc (a) with expression restricted to the dorsal region of the closing neural tube (NT) rostrally and neural folds caudally. At 9.5 dpc (b), *Tlx2-LacZ* expression is seen in the condensing cranial ganglia, otic vesicle, lateral mesoderm and NT. At 10.5 dpc (c), expression begins to be detected in the DRG and SG while expression is maintained in the cranial nerves, neural tube and lateral mesoderm. In the lateral mesoderm derivatives, expression is restricted caudal to the posterior half of forelimb. At 12.5 dpc (d), expression of the transgene remains robust in cranial ganglia, DRG, SG and express can be seen in the ENS with decreased expression in the NT and lateral mesoderm. Prior to imaging, 12.5 dpc embryos were cleared in benzyl alcohol and benzyl benzoate. Expression of *Tlx2-LacZ* continues in adult ENS (e), adrenal gland (f), and a subset of the cells in the SG (g). ant: anterior, cn: cranial nerves, drg: dorsal root ganglia, ens; enteric nervous system, fl: forlimb, hl: hindlimb, lm: lateral mesoderm, nt: neural tube, ov: otic vesicle, post: posterior, sg: sympathetic ganglia, tg: trigeminal ganglia,

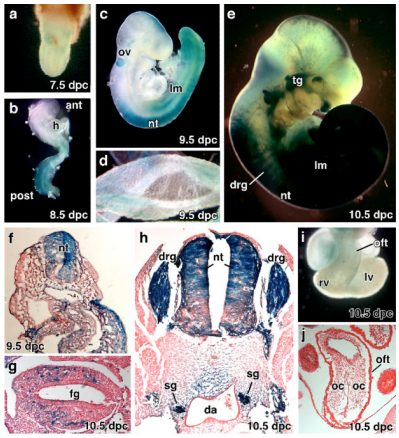

Fig. 3. The *Tlx2-Cre* line expresses Cre in the PNS, otic vessel and lateral mesoderm

The *Tlx2-Cre* mouse line was analyzed for β-galactosidase expression by crossing with the *R26R* reporter line. (a) Little or no expression of β-galactosidase was observed at 7.5 dpc. (b) Cre recombinase activity was observed at 8.5 dpc in caudal half of the embryo. (c) At 9.5, β-galactosidase expression is seen in the neural tube, lateral mesoderm, and otic vesicle. (d) Limited punctate expression of β-galactosidase was observed in yolk sac. (e) β-galactosidase expression is activated in the PNS by 10.5 dpc and remains expressed in the neural tube and lateral plate mesoderm but not in NC-derived cranial tissues. (f, g, h) Histological analysis of 9.5 dpc (f) and 10.5 dpc (g, h) embryos shows Cre activity in the dorsal neural tube, lateral mesoderm and the NC-derived sensory, sympathetic and enteric nervous systems. (i, j) β-galactosidase is not expressed in the outflow tract (oft) of the heart. ant: anterior, drg: dorsal root ganglia, ens: enteric nervous system, fg: forgut, h: heart, lm: lateral mesoderm, lv: left ventricle, nt: neural tube, oc: outflow tract cardiac cushion, oft: outflow tract, ov: otic vesicle, post: posterior, rv: right ventricle, sg: sympathetic ganglia, tg: trigeminal ganglia.

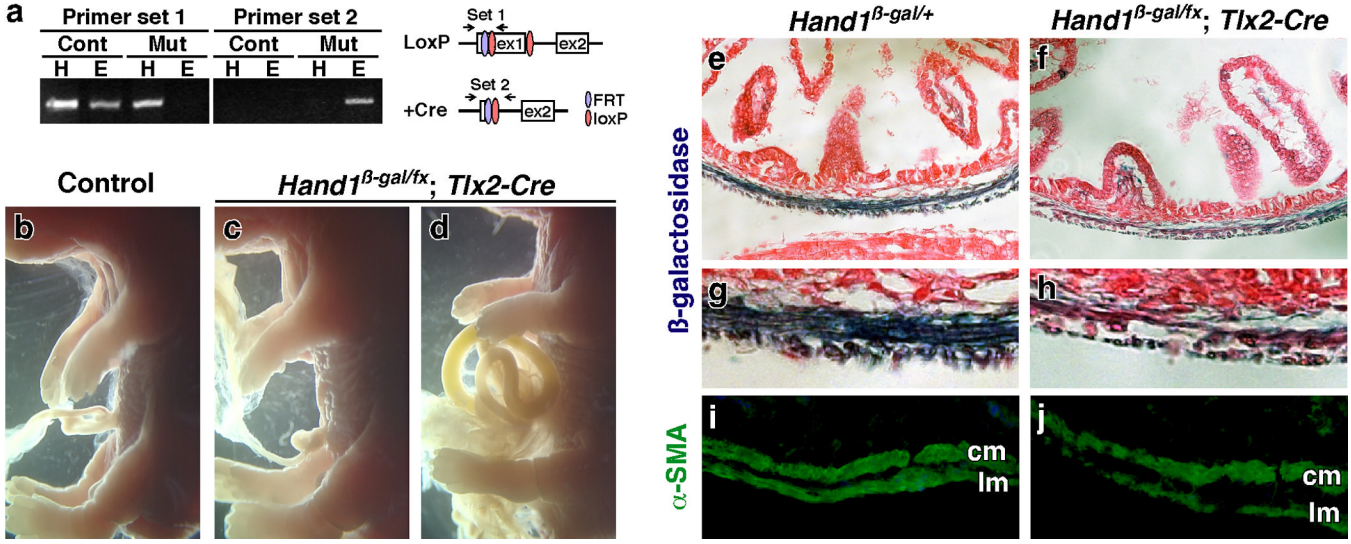

Fig. 4. Deletion of Hand1 in lateral mesoderm results in gut herniation and enteric smooth muscle defects

Hand1 was deleted in lateral mesoderm by crossing the *Hand1*$^{\beta\text{-}gal/fx}$ and *Tlx2-Cre* mouse lines. (a) PCR analysis of recombination efficiency of the conditional Hand1 allele. Recombination of *Hand1*$^{\beta\text{-}gal/fx}$ (cont) and *Hand1*$^{\beta\text{-}gal/fx}$; *Tlx2-Cre* (mut) tissues from heart (H) and enteric smooth muscles (E) was determined by analysis of the first loxP site (primer set 1) and the recombined loxP site (primer set 2). A high level of recombination is seen in enteric smooth muscle. (b–d) Morphological analysis of control (b) and conditional *Hand1* knockout (c, d) embryos at 18.5 dpc shows that loss of *Hand1* can result in gut herniation. Mutant embryos present variable penetrance ranging from no gut herniation (c) to severe herniation (d). (e–j) Histological analysis of gut musculature of *Hand1*$^{\beta\text{-}gal/+}$ heterozygous (e, g, i) and conditional *Hand1* mutant (f, h, j) 18.5 dpc embryos. Staining for β-galactosidase expression from the *Hand1*$^{\beta\text{-}gal}$ allele (d, f) shows that *Hand1* is expressed in a subset of cells in the smooth muscle layers. Loss of *Hand1* in lateral mesoderm results in thinner and disorganized smooth muscle layers. (i, j) Enteric smooth muscle cells in control (i) and conditional *Hand1* mutant (j) embryos were analyzed for α-smooth muscle actin (α-SMA) expression by immunohistochemistry. Enteric smooth muscle in conditional *Hand1* knockout embryos is thin and disorganized. cm: circular muscle, lm: longitudinal muscle.