Published in final edited form as:
Genesis. 2014 May ; 52(5): 417–423. doi:10.1002/dvg.22768.

A conditional mutant allele for analysis of *Mixl1* function in the mouse

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SUMMARY

Mixl1 is the only member of the *Mix/Bix* homeobox gene family identified in mammals. During mouse embryogenesis, *Mixl1* is first expressed at embryonic day (E)5.5 in cells of the visceral endoderm (VE). At the time of gastrulation, *Mixl1* expression is detected in the vicinity of the primitive streak. *Mixl1* is expressed in cells located within the primitive streak, in nascent mesoderm cells exiting the primitive streak, and in posterior VE overlying the primitive streak. Genetic ablation of *Mixl1* in mice has revealed its crucial role in mesoderm and endoderm cell specification and tissue morphogenesis during early embryonic development. However, the early lethality of the constitutive $Mixl1^{-/-}$ mutant precludes the study of its role at later stages of embryogenesis and in adult mice. To circumvent this limitation, we have generated a conditional *Mixl1* allele ($Mixl1^{cKO}$) that permits temporal as well as spatial control of gene ablation. Animals homozygous for the $Mixl1^{cKO}$ conditional allele were viable and fertile. $Mixl1^{KO/KO}$ embryos generated by crossing of $Mixl1^{cKO/cKO}$ mice with *Sox2-Cre* or *EIIa-Cre* transgenic mice were embryonic lethal at early somite stages. By contrast to wild-type embryos, $Mixl1^{KO/KO}$ embryos contained no detectable Mixl1, validating the $Mixl1^{cKO}$ as a protein null after Cre-mediated excision. $Mixl1^{KO/KO}$ embryos resembled the previously reported $Mixl1^{-/-}$ mutant phenotype. Therefore, the *Mixl1* cKO allele provides a tool for investigating the temporal and tissue-specific requirements for *Mixl1* in the mouse.

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Keywords

transcription factor; homeodomain; homeobox; Mixl1; primitive streak; gastrulation; mesoderm; endoderm

The Mix/Bix family of evolutionarily conserved transcription factors play critical roles during early embryogenesis. Members of the family are characterized by the presence of a 60 amino acid homeodomain motif which is highly conserved across species. *Mix.1* was first identified in *Xenopus* as a TGFβ signaling early response gene (Rosa, 1989). Eight additional family members have since been identified in *Xenopus*. By contrast, only a single *Mix/Bi*x gene, *Mixl1 (*also referred to as *Mml* or *Mix1)*, is present in the mouse and human (Pearce and Evans, 1999; Robb et al., 2000; Sahr et al., 2002), mapping to the distal region of MMU chromosome 1 (Robb et al., 2000), and chromosome 1 respectively (Sahr et al., 2002). The encoded homeodomain of the mouse protein shares 67% amino acid identity with *Xenopus* Mix.1 and Mix.2 (Robb et al., 2000; Sahr et al., 2002).

During mouse embryonic development, *Mixl1* mRNA is first detected at embryonic day (E)5.5 in cells of the posterior visceral endoderm (PVE) residing at the embryonic-extraembryonic junction (Rivera-Perez and Hadjantonakis, 2014), as well as in VE cells overlying the embryonic region (the emVE) (Robb et al., 2000). At the onset of gastrulation, *Mixl1* mRNA is localized to the posterior epiblast and in the posterior VE cells overlying the primitive streak (Mohn et al., 2003; Pearce and Evans, 1999; Robb et al., 2000). As gastrulation proceeds, *Mixl1* mRNA becomes broadly expressed in the primitive streak and nascent mesoderm. By the headfold stage (corresponding to E7.75), its expression is restricted to the posterior primitive streak, and, by E9.5, to the tail bud.

Analysis of mutant mice has revealed that *Mixl1* is essential for axial patterning, mesoderm and definitive endoderm morphogenesis (Hart et al., 2002). Though precursors of the mesoderm, endoderm and axial tissues are specified in *Mixl1*$^{-/-}$ embryos, they display severe defects in the morphogenesis of mesoderm and definitive endoderm derivatives. *Mixl1*$^{-/-}$ embryos fail to form a proper node and notochord, do not elongate their anterior-posterior (AP) axis, fail to form a heart, and display defects in gut tube morphogenesis (Hart et al., 2002). Experiments with mouse embryonic stem (ES) cells have shown that overexpression of *Mixl1* is sufficient to drive their differentiation into mesoderm (Willey et al., 2006), mesendoderm (Zhang et al., 2009) or definitive endoderm (Lim et al., 2009). Similarly, the analysis of embryo chimeras revealed that *Mixl1*$^{-/-}$ cells exhibit a very poor contribution to the embryonic gut, suggesting it to be crucial for the potency of embryonic cells to differentiate into definitive endoderm derivatives (Hart et al., 2002).

To circumvent the early embryonic lethality of *Mixl1* deficiency, and to achieve tissue and temporal-specific *Mixl1* inactivation, we created a conditional *Mixl1* allele in mice (Fig. 1).

First, we sought to demonstrate that the complete deletion product of our conditional allele would produce a protein null, and that this would resemble the previously reported *Mix1*$^{-/-}$ allele. To do this, three independent lines of ES cells containing the correctly targeted *mMixl1 cKO-Neo* allele (*lox*P-neo-*lox*P-exon2-*lox*P, Figs. 1C) were used to generate

germline transmitting chimeras. Complete deletion (ΔneoΔexon2, which we designate as the *Mixl1* KO allele) was achieved in one of the lines, 3D7 (Fig. 1F), by mating F1 animals with constitutive Cre deletor mice carrying an *EIIa-Cre* transgene (Lakso et al., 1996) (data not shown).

We also sought to generate mice carrying the *Mixl1* conditional knockout (cKO) allele (l*ox*P-exon2-*lox*P, Fig. 1D), to verify that this configuration did not interfere with endogenous *Mixl1* function, and produced animals that were viable and fertile. We analyzed two independently targeted ES cell lines in which the NEO cassette had been excised (Δneo-*lox*P-exon2-*lox*P) by transfection of a *pCAG-Cre* to create the cKO allele. In the *Mixl1* cKO allele, the region of exon 2 that encodes helix 3 of the homeodomain is flanked by *lox*P sites 1 and 3 (Fig. 1D). Male chimeras carrying the *Mixl1* cKO allele were bred with CD1 females to test for the germline transmission of the targeted *Mixl1* allele. F1 mice were genotyped using polymerase chain reaction (PCR) to detect the cKO allele (Fig. 1G). F_1 *Mixl1*cKO/+ mice were intercrossed. $Mixl1^{cKO/cKO}$ mice, identified using PCR, were grossly indistinguishable from $Mixl1^{cKO/+}$ and $Mixl1^{+/+}$ littermates based on their weight, size, morphology of external features (craniofacial, limbs, tail) and fertility (data not shown). $Mixl1^{cKO/cKO}$ embryos were recovered at Mendelian ratios. Evaluation of embryos based on size, including crown-rump length at midgestation and fetal stages, or morphological features using criteria defined by Downs & Davies (Development 1994), for gastrula to early somite stages, suggested $Mixl1^{cKO/cKO}$ embryos were indistinguishable from $Mixl1^{cKO/+}$ and $Mixl1^{+/+}$ littermates.

To confirm that we could convert the $Mixl1^{cKO}$ into the $Mixl1^{KO}$ allele in mice (Fig. 1E, G), we performed Cre-mediated gene inactivation, by crossing $Mixl^{cKO/cKO}$ animals with a *Sox2-Cre* strain (Hayashi et al., 2002). The *Sox2-Cre* allele was bred out of the colony. The resulting $Mixl1^{KO/+}$ animals displayed no overt phenotype and were intercrossed to generate $Mixl1^{KO/KO}$ progeny. Embryos from these intercrosses were recovered at Mendelian rations at E7.5-E8.5 and were analyzed. At E7.5, by gross morphology, $Mixl^{KO/KO}$ embryos exhibited an accumulation of cells in the vicinity of the primitive streak (Fig. 2A). This defect resembled that described for a $Mixl1^{-/-}$ allele in a previous study (Hart et al., 2002). At later stages, in contrast with wild-type embryos, E7.5–8.0, $Mixl1^{KO/KO}$ embryos lacked morphologically recognizable midline structures such as the node and notochord (Fig. 2B).

In wild-type embryos, Mixl1 protein is localized to the vicinity of the primitive streak (Fig. 2C). By contrast, no Mixl1 protein was detected in $Mixl^{KO/KO}$ embryos (Fig. 2C). These data suggest that the $Mixl1^{KO}$ allele is a protein null.

Notably, by contrast to anti-Mixl antibody staining, wholemount *in situ* hybridization with a *Mixl1* antisense riboprobe revealed that we could detect *Mixl1* transcripts in $Mixl1^{KO/KO}$ mutants and that the domain of *Mixl1* expression was expanded compared to wild-type (Fig. 3A, B). This reflected an accumulation of cells and resulting enlargement of tissue in the vicinity of the primitive streak, evident by gross morphology (Fig. 2A). This accumulation of primitive streak and/or nascent mesoderm cells is a hallmark of gastrulation mutants. *Brachyury*, a marker of the primitive streak and nascent mesoderm at E7.5 (Fig. 3C, C′), was expressed in an expanded domain encompassing both the thickened primitive streak and

adjacent mesoderm of *Mixl1*$^{KO/KO}$ embryos (Fig. 3D, D′). Despite their inability to form a proper node (Fig. 2B) and notochord, cells with a midline identity were specified in *Mixl1*$^{KO/KO}$ embryos, as revealed by *FoxA2* (Fig. 3E, F) and *Shh* expression (Fig. 3G, H). Both *FoxA2* and *Shh* were expressed in an expanded domain at the distal tip of *Mixl1*$^{KO/KO}$ mutant embryos (Fig. 3F, H). A probe for *Nkx2.5* revealed that, at E8.5, cardiac progenitors were specified in *Mixl1*$^{KO/KO}$ embryos, but they failed to form a proper heart tube (Fig. 3J). In wild-type embryos, *Twist* mRNA was detected in paraxial locations corresponding to the presumptive lateral plate mesoderm and underneath the presumptive neural plate (Fig. 3K). By contrast, *Twist* was absent from mesoderm underneath presumptive cranial neural plate of *Mixl1*$^{KO/KO}$ embryos (Fig. 3L), as previously reported (Hart et al., 2002).

In summary, the data presented here validate the *Mixl1*^{cKO} allele for Cre-mediated conditional gene ablation, and confirm that the derivative *Mixl1*^{KO} allele recapitulates the previously reported mutant phenotype. Therefore, the *Mixl1*^{cKO} line will provide a valuable tool for the tissue and stage-specific dissection of *Mixl1* function in mice.

MATERIALS AND METHODS

Generation of Mixl1^{cKO} allele

Three independent murine genomic *Mixl1* subclones were isolated from a 129/SvJ bacterial artificial chromosome (BAC) library, as described (Sahr et al., 2002). A ~7.5 kb *Eco*RI fragment was isolated from one of the clones and was analyzed using Southern blot hybridization, PCR, and DNA sequencing. The fragment was found to contain the entire *Mixl1* gene, with two exons, ~2.4 kb upstream sequence, and ~1.2 kb downstream sequence. To generate a conditional targeting construct from the genomic fragment, a *lox*P sequence was inserted into the unique *Nco*I site of exon 2, just downstream from the translation stop signal. A floxed *neomycin* gene (*lox*P-neo-*lox*P) was then subcloned into the unique *Kpn*I site of intron 1 in reverse orientation with respect to the *Mixl1* gene. The orientation of the *lox*P sequences and all subcloning boundaries were verified by DNA sequence analysis. The resulting targeting vector (Fig. 1B), containing 4.4 kb of upstream and 2.6 kb of downstream *Mixl1* homology arms, was electroporated into R1 embryonic stem (ES) cells (Nagy et al., 1993). After selection for G418-resistance, clones were picked and replica plated onto 96 well dishes. ES cell clones containing the correctly targeted *mMixl* gene having the arrangement of *lox*P-neo-*lox*P-exon2-*lox*P, which we refer to as the *Mixl*$^{Neo\text{-}cKO}$ allele (Fig. 1C), were identified using Southern blotting (Fig. 2A). Excision of the Neo cassette, and creation of a conditional knock-out (cKO) *Mixl*^{cKO} allele containing *lox*P sites 1 and 3 flanking the 5′ end of exon 2, was achieved in ES cells by electroporation of a *pCAG-Cre* plasmid using standard protocols (Nagy, 2003). Chimeric animals generated by the MSKCC mouse genetics core facilities were bred with CD1 mice; germline transmission was confirmed using Southern blot and PCR analyses. The *Mixl1*^{cKO} mouse line will be made available to interested investigators.

PCR genotyping

PCR primers used were TTGAGCAGAGAGTCAAGGCT (KS51, sense) and TGTGCAGTCTTGGAGAGTCA (KS52, antisense, Fig. 1E). Amplicons were 0.8 kb for the wild-type, 0.836 kb for the cKO, and 0.24 kb for the KO allele (Fig. 1G).

Mouse lines and embryo collection

In addition to the *Mixl1*^{cKO} allele, mouse lines used in the study were wild-type CD1, purchased from Charles River, and *EIIa-Cre* (Lakso et al., 1996) and *Sox2-Cre* (Hayashi et al., 2002). Animals were maintained on a mixed 129/CD1 background. To generate the *Mixl1*^{KO} allele, *Mixl1*^{cKO} animals were bred to *Sox2-Cre* animals. In all data presented, wild-type controls are wild-type littermates of mutant embryos recovered from *Mixl1*$^{KO/+}$ inter-crosses. None of the embryos analyzed or depicted in the figures contained the *Sox2-Cre* transgene. Embryos were collected in Dulbecco's Modified Eagle's Medium (DMEM)-F12 (Gibco) supplemented with 5% fetal bovine serum (FBS, Lonza) at embryonic day (E)7.0–8.5. Gestational age of the embryos was considered E0.5 on the morning of detection of a vaginal plug. Embryos were staged according to Downs and Davies (Downs and Davies, 1993). Adult mice were evaluated based on their weight (for example, ~30g for 6-week old females), size, morphology of external features (craniofacial, limbs, tail) and fertility. Embryos were evaluated for their overall size (for example, crown-rump length from midgestation onwards), or based on morphological features defined by Downs & Davies from before gastrulation to the beginning of organogenesis (Downs and Davies, 1993), for early post-implantation stages. Representative litter sizes and transmission ratios for the *Mixl*^{cKO} and *Mixl1*^{KO} alleles are provided in Supplementary Table 1.

Whole mount in situ hybridization and immunofluorescence

Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 24 hr at 4°C. Embryos were then washed twice in PBS, dehydrated in a graded series of methanol and stored in 100% methanol at −20°C. Whole mount *in situ* hybridizations were performed as described previously (Henrique et al., 1995). The following plasmids were used for *in vitro* transcription of anti-sense riboprobes: *FoxA2* (Sasaki and Hogan, 1993), *Mixl* (Mohn et al., 2003), *Nkx2.5* (Lyons et al., 1995), *Shh* (Echelard et al., 1993), *T*/*Brachyury* (Wilkinson et al., 1990), and *Twist* (Wolf et al., 1991). Stained embryos were photographed using a Zeiss Axiocam MRc CCD camera on a Leica MZ165FC microscope. Embryos were post-fixed in 4% PFA overnight, washed in PBS, then embedded in a solution containing BSA and sucrose, cross-linked using glutaraldehyde. Sections were cut using a VT1000 vibrating microtome (Leica). Immunohistochemistry was carried out as previously described (Nowotschin et al., 2013). An anti-Mixl1 antibody was used at 1:500 dilution (Zhang et al., 2009). Embryos were counterstained with Hoechst nuclear stain (Invitrogen).

Image acquisition

Widefield images were acquired using a Zeiss Axiocam MRc camera on a Leica M165FC stereo dissecting microscope. Laser scanning confocal data wer acquired using a Zeiss LSM510 META. Fluorophores were excited using a 405-nm diode laser (Hoechst), and 543-

nm HeNe laser (Alexa543). Objectives used were Plan-Apo 20x/NA0.75 and Fluar 5x/NA0.25. Embryos were imaged in wholemount in MatTek dishes (Ashland). Confocal images were acquired as z-stacks of (2D) optical sections taken at 2 μm intervals. 3D projections of z-stacks were generated using ZEN software (Zeiss).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Memorial Sloan Kettering Cancer Center (MSKCC) Mouse Genetics Core and Mount Sinai School of Medicine (MSSM) Mouse Genetics Shared Resources Facilities for blastocyst injections, and Dr. Kevin Kelley (MSSM) for valuable technical advice. Work in our laboratories is supported by National Institutes of Health (NIH) RO1-HL62248 (MHB), and RO1-HD052115 and RO1-DK084391 (AKH). SN is supported by a Muscular Dystrophy Association Development Grant (186552).

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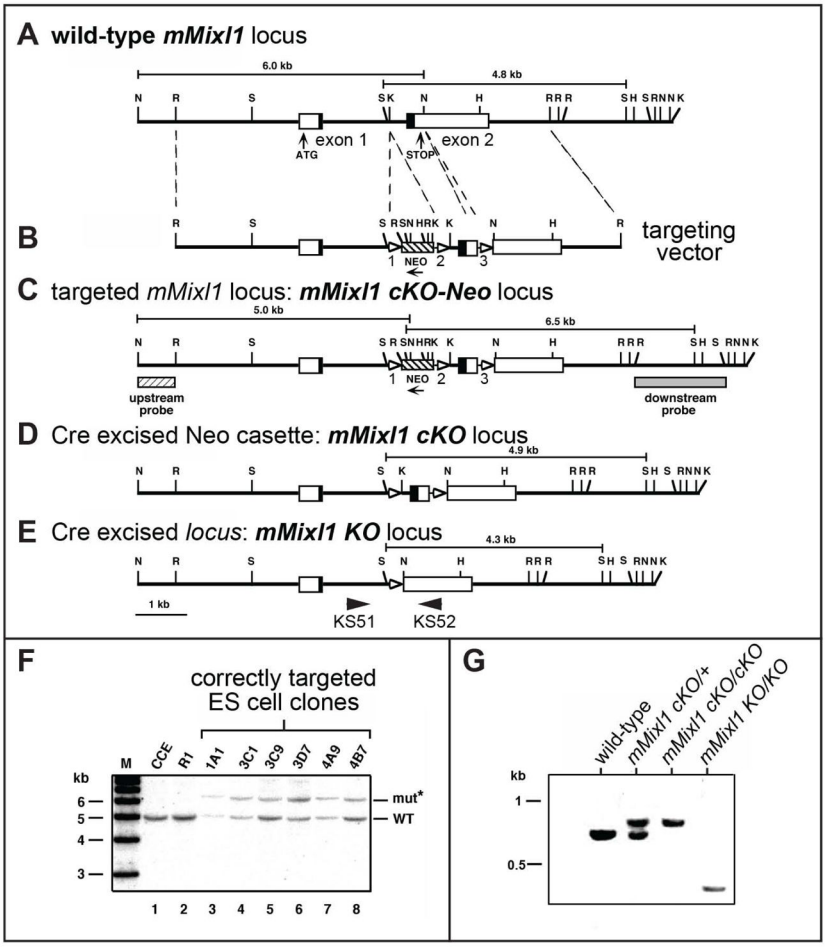

Figure 1. Strategy for generation and validation of a conditional *Mixl1*^{cKO} allele

(A) Wild-type mouse *Mixl1* locus. **(B)** Targeting construct. **(C)** Homologously targeted *Mixl1* locus *(lox*P1-neo-*lox*P2-exon2-*lox*P3) referred to as *mMixl1 cKO-Neo* allele. **(D)** Cre recombinase mediated excision of the Neo cassette is expected to produce the (Δneo-*lox*P2-exon2-*lox*P3) *mMixl1 cKO* allele and leaves uninterrupted exons at the *Mixl1* locus. **(E)** Complete excision produces the (ΔneoΔexon2) predicted null allele. Expected structure of allele upon Cre excision between *lox*P sites 1 and 2 results in removal of the neomycin cassette (Δneo). Expected structure of allele upon Cre excision between *lox*P sites 1 and 3 or 2 and 3 results in removal of the beginning of exon 2 (ΔneoΔexon2), which includes the portion of the gene encoding the critical third helix of the DNA-binding homeodomain region. LoxP sites are marked as 1, 2, 3 in targeting vector (**B**) and homologously targeted allele (**C**). Letters refer to restriction endonuclease sites. The position of the genotyping primers (KS51 and KS52), and Southern hybridization probes is provided (**C, E**). **(F)** Southern blot analysis illustrating two wild-type ES cell lines (CCE and R1) and 6 correctly targeted *Mixl1*^{cKO} ES cell lines. Genomic DNA was digested with *Sph*I, blotted and hybridized against a 3′ (downstream) probe (**C**) that detected fragments of ~5 kb in the wild-type locus, and 6.5 kb in the correctly targeted *mMixl1 cKO-Neo* allele. Targeted ES cells were generated in a parental R1 ES cell line (Nagy et al., 1993). **(G)** PCR genotyping

of *Mixl1*$^{cKO/cKO}$ mouse ear biopsy DNA to amplify an 0.8 kb fragment in the wild-type, a 0.84 kb fragment from the cKO allele, and a 0.24 kb fragment from the KO allele. Gel image is inverted to increase contrast.

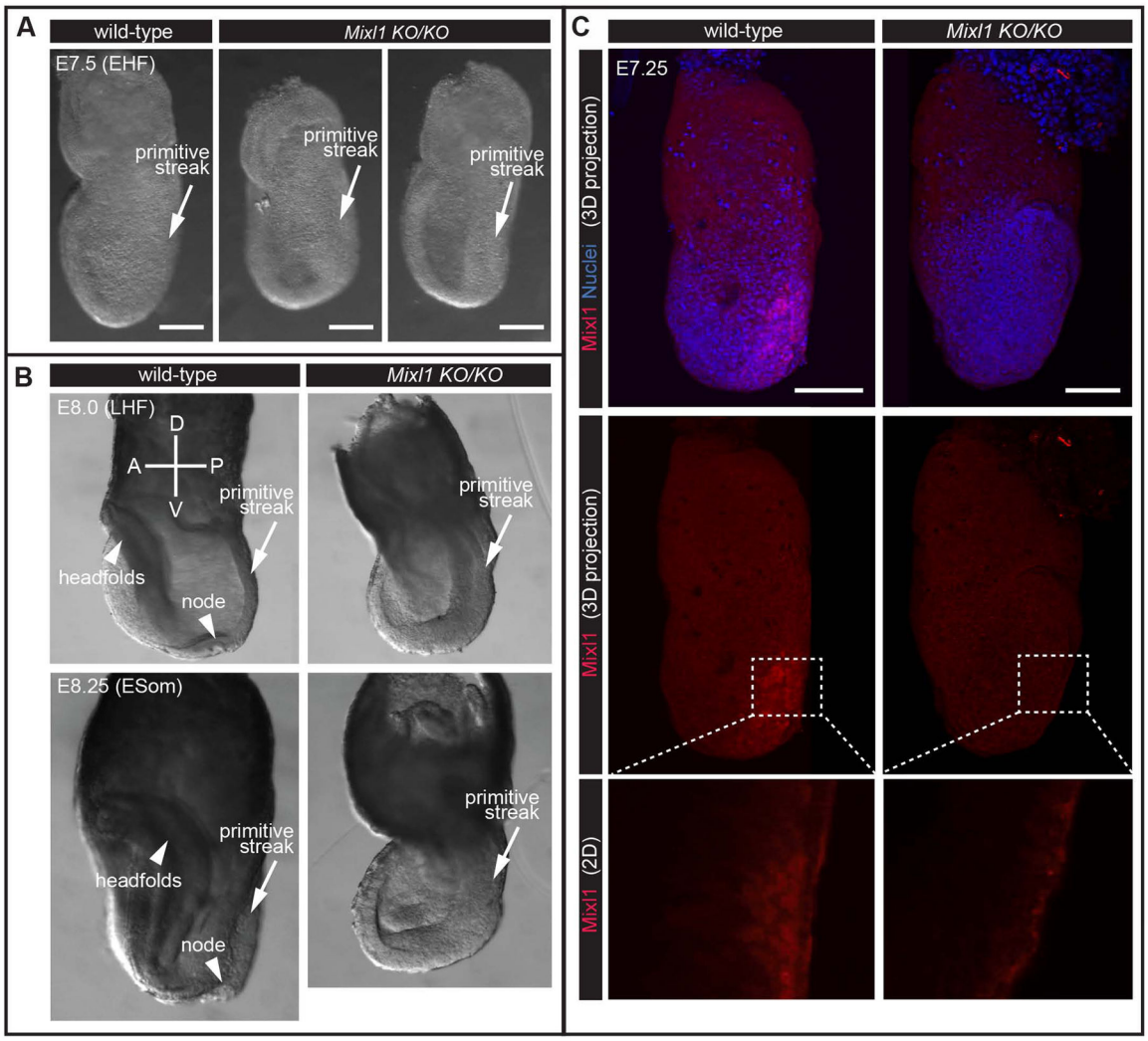

Figure 2. *Mixl1*$^{KO/KO}$ embryos exhibit gastrulation defects, resemble the previously reported *Mixl1*$^{-/-}$ allele, and are a protein null

(A) Lateral views of E7.5 wild-type and two *Mixl1*$^{KO/KO}$ embryos exhibiting an enlarged primitive streak region. **(B)** Lateral views of E8.0 (late headfold stage, LHF) and E8.5 (early somite stage, ESom) wild-type and *Mixl1*$^{KO/KO}$ embryos. The mutants exhibit defects within the primitive streak and its derivative tissues, which are characterized by an accumulation of cells within the vicinity of the primitive streak, and absence of a node and headfolds. This phenotype closely resembles that described previously for *Mixl1*$^{-/-}$ embryos (Hart et al., 2002). **(C)** Lateral views of E7.25 wild-type and *Mixl1*$^{KO/KO}$ embryos demonstrating detection of Mixl1 protein in the vicinity of the primitive streak in the wild-type embryo, and absence in *Mixl1*$^{KO/KO}$ mutant. Scale bars are 50μM in A and 100μM in C. Wild-type controls are wild-type littermates of mutant embryos that were generated from *Mixl1*$^{KO/+}$ inter-crosses.

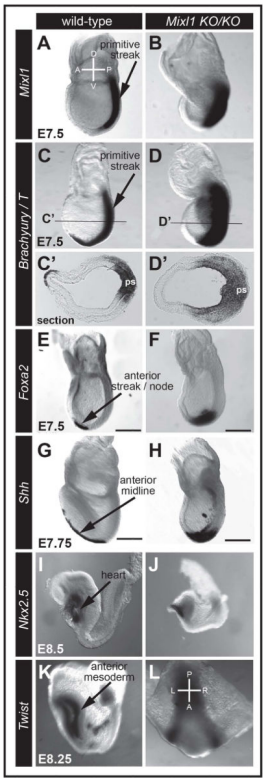

Figure 3. *Mixl1*$^{KO/KO}$ embryos exhibit defects in the primitive streak and its derivative tissues

(A–F) Lateral views of E7.5 wild-type and *Mixl1*$^{KO/KO}$ mutant embryos assayed for the expression of *Mixl1* (**A, B**), *Brachyury/T* (**C, D**) and *Foxa2* (**E, F**). Transverse sections at approximately the levels indicated by black lines (**C′, D′**), reveal thickening and accumulation of cells within the primitive streak (ps) of the *Mixl1*$^{KO/KO}$ mutant embryos **(D′). (G, H)** Lateral views of E7.75 wild-type and *Mixl1*$^{KO/KO}$ mutant embryos analyzed for the expression of *Shh.* Lateral views of E8.5 (somite stage) wild-type and *Mixl1*$^{KO/KO}$ mutant embryos analyzed for the expression of *Nkx2.5* (**I, J**). Lateral (**K**) and posterior (**L**) views of E8.25 (early somite stage) wild-type and *Mixl1*$^{KO/KO}$ mutant embryos analyzed for the expression *Twist.* Scale bars are 50 μM in all panels.