

Fig. S1 *Nkx1-2* expression in neurons. Mouse embryos analysed by whole-mount *in situ* hybridization for *Nkx1-2* at (A) E10.5 and (B) E12.5. (Aa) and (Bb) show transverse sections through the regions indicated in the corresponding whole-mount embryo. Scale bar in whole-mounts, 200 µm; in transverse sections, 50 µm.

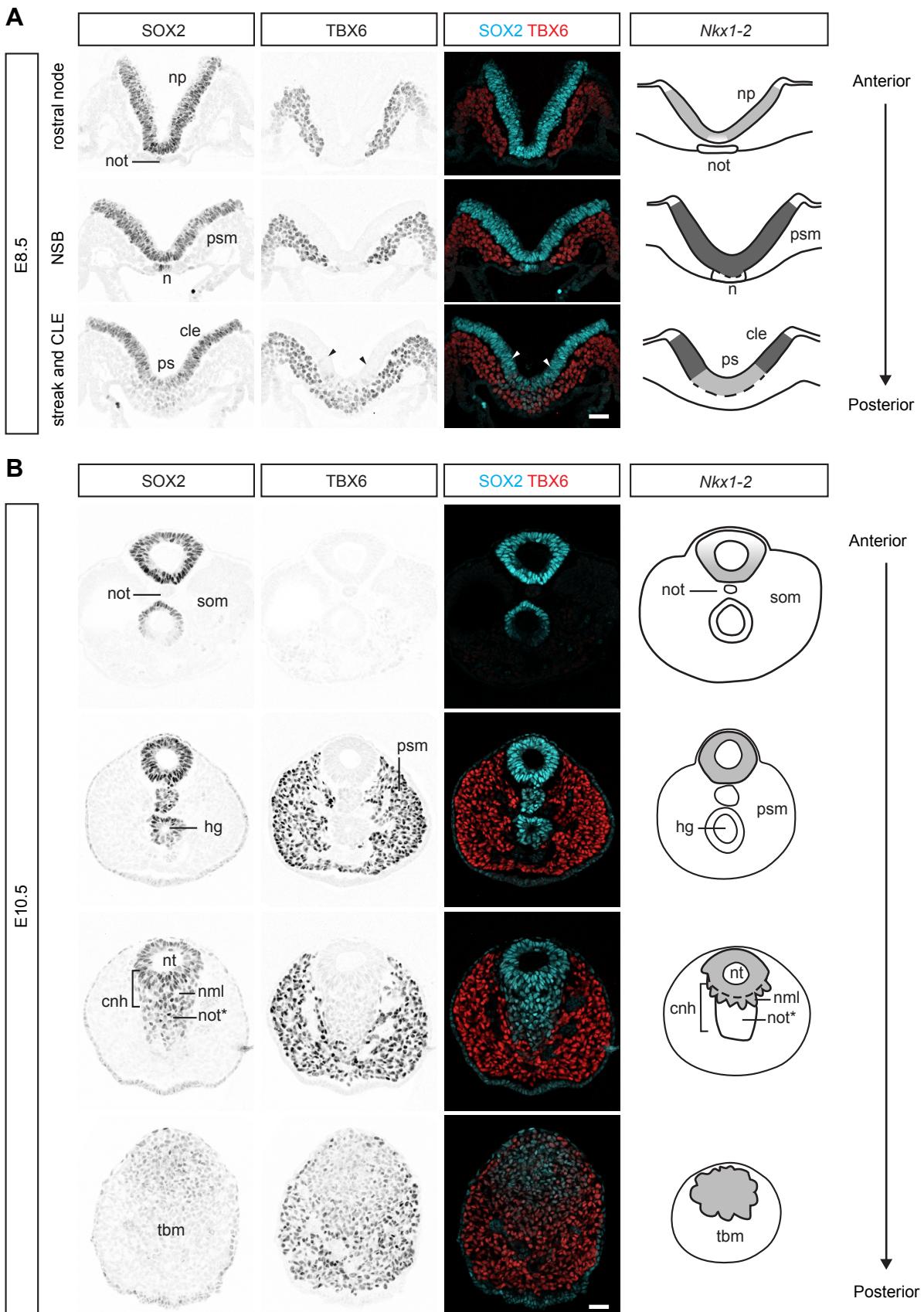


Fig. S2 SOX2 and TBX6 expression within *Nkx1-2* regions. (A) Transverse sections across the rostral node, NSB, and CLE of an E8.5 embryo immunolabelled for SOX2 and TBX6 ($n=4$). (B) Transverse sections across the tail end of an E10.5 embryo immunolabelled for SOX2 and TBX6 ($n=4$). The cartoons in (A) and (B) depict the expression pattern of *Nkx1-2* (as shown in Fig. 1). The arrowheads in (A) indicate the region that contains TBX6⁺ cells. The different levels of *Nkx1-2* expression (based on *in situ* hybridisation signal) are represented by different grey intensities (dark grey, high; light grey, low; white, no expression). The dashed lines delineate regions not limited by basement membrane. Abbreviations are the same as in Fig. 1. som, somite; nml, neuromesodermal lip. Scale bars, 50 μ m.

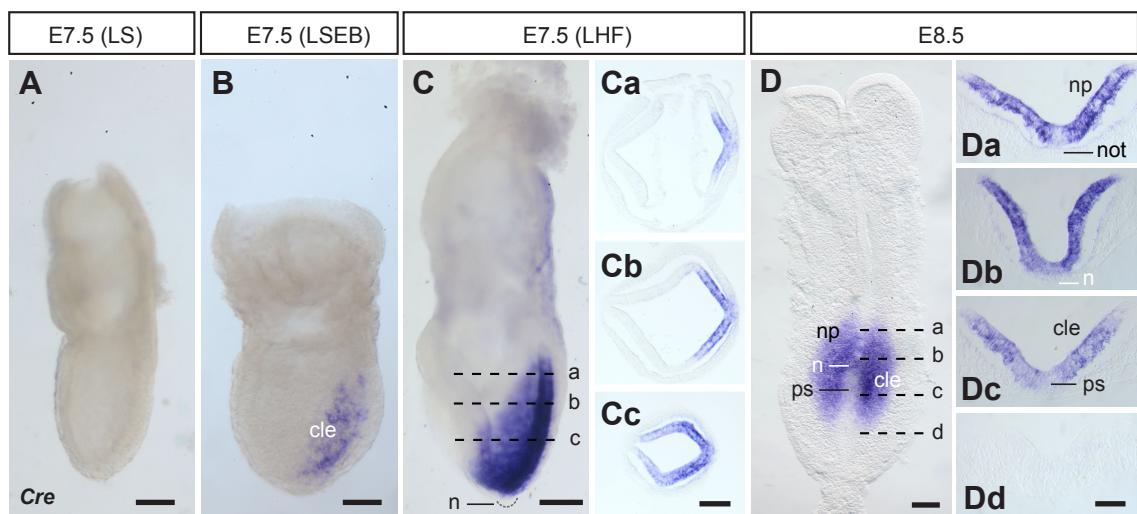


Fig. S3 CreERT^{T2} expression in Nkx1-2CreERT^{T2} embryos recapitulates endogenous *Nkx1-2* expression. Cre *in situ* hybridisation in whole-mount and transverse sections of Nkx1-2CreERT^{T2} mouse embryos. (A) Lateral view of a late streak (LS) stage embryo. CreERT^{T2} expression is undetectable at this stage. (B) Lateral view of a late streak, early allantoic bud (LSEB) stage embryo. (C) Lateral view of an early to late headfold (LHF) stage embryo. (Ca-Cc) Transverse sections through the regions indicated in C. Note that at this stage Cre might be expressed in a few cells in the posterior endoderm (Ca). Posterior is to the right (n=15 E7.5 embryos at different developmental stages). (D) Dorsal view of an E8.5 embryo (n=8). (Da-Dd) Transverse sections through the regions indicated in D. cle, caudal lateral epiblast; ps, primitive streak; np, neural plate; n, node; not, notochord. Scale bars, 100 µm.

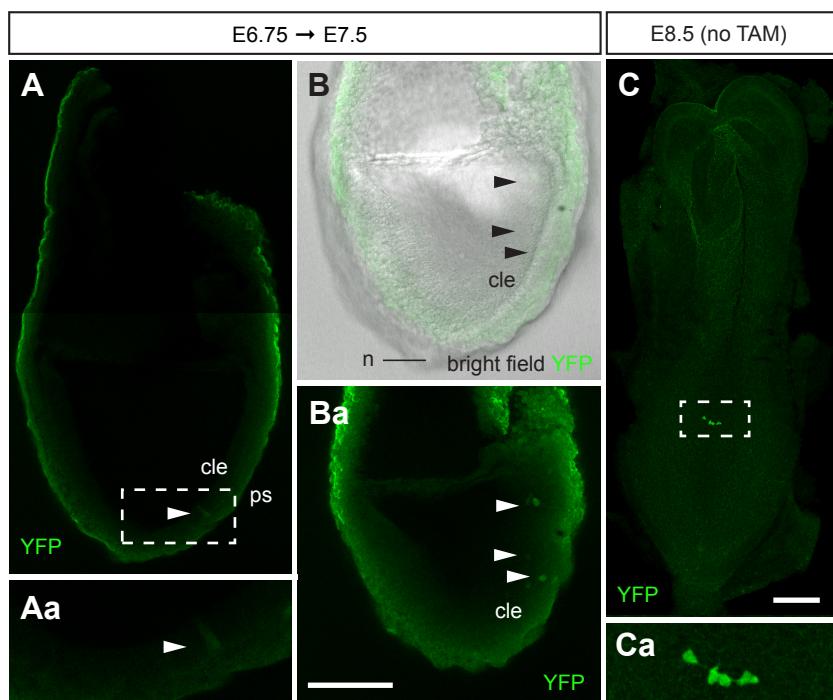


Fig. S4 Tamoxifen administration to *Nkx1-2*Cre $\text{ER}^{\text{T}2}$ /YFP mice drives Cre $\text{ER}^{\text{T}2}$ -mediated recombination specifically in cells within *Nkx1-2* regions. (A) and (B) Timed-pregnant *Nkx1-2*Cre $\text{ER}^{\text{T}2}$ /YFP mice received tamoxifen at E6.75 and embryos assessed for YFP expression 18 hours later (A) Parasagittal optical section through a late bud stage *Nkx1-2*Cre $\text{ER}^{\text{T}2}$ /YFP embryo (around E7.5) exposed to tamoxifen at E6.75 and immunolabelled for YFP on whole-mount. YFP $^{+}$ cells were found in the NSB region and CLE which indicates that the expression of the Cre $\text{ER}^{\text{T}2}$ transgene is consistent with the pattern of endogenous *Nkx1-2* expression (Figure 1) (Schubert et al., 1995) (n=6). (Aa) Higher magnification of the region indicated in A. (B) Maximum intensity projection (MIP) of four optical sections (i.e. 16 μm) of the embryo in A. YFP $^{+}$ cells were also found more posteriorly throughout the CLE (arrowheads). (Ba) YFP channel of B. Note that even if early post-implantation embryos are highly autofluorescent (diffuse green signal in A to Ba), cells with clear cytoplasmic YFP stood out above the background signal. (C) To assess the extent of potential spontaneous recombination in *Nkx1-2*Cre $\text{ER}^{\text{T}2}$ /YFP mice, embryos not exposed to tamoxifen (no TAM) were analysed for YFP $^{+}$ cells on whole-mounts at E8.5. (D) MIP of an *Nkx1-2*Cre $\text{ER}^{\text{T}2}$ /YFP embryo at E8.5 in the absence of tamoxifen induction. All embryos analysed (n=5) showed low levels of spontaneous recombination, from 4 to 9 YFP $^{+}$ cells each (6 ± 1 cells/embryo). The close proximity and number of YFP $^{+}$ cells suggest that they originate from a single recombination event at around E7.5 because cells have a cell cycle of ~6-7h (Snow, 1977; Tzouanacou et al., 2009) and it takes ~4h to detect YFP (data not shown). (Ca) Higher magnification of the region indicated in C. Abbreviations are the same as in Fig. 1. Scale bars, 100 μm .

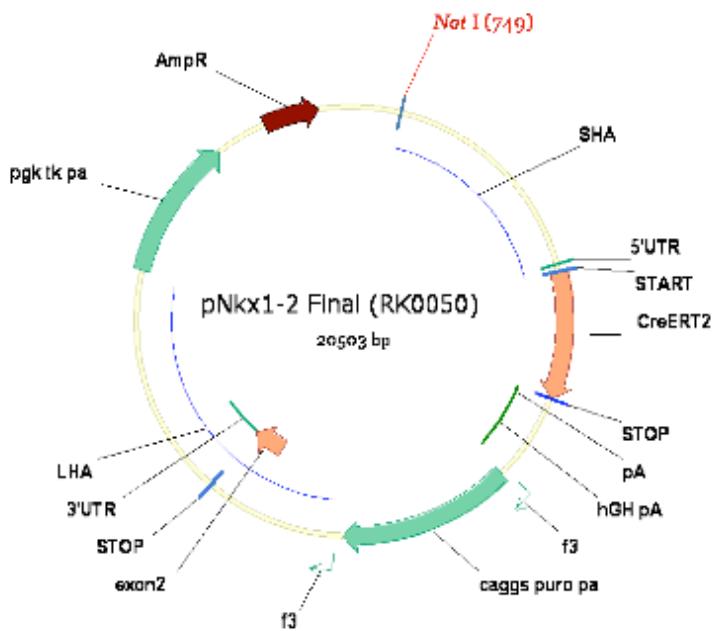
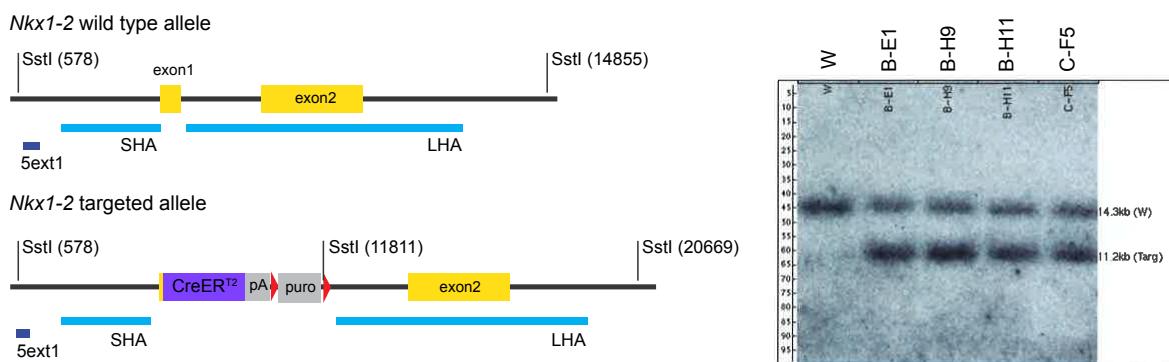
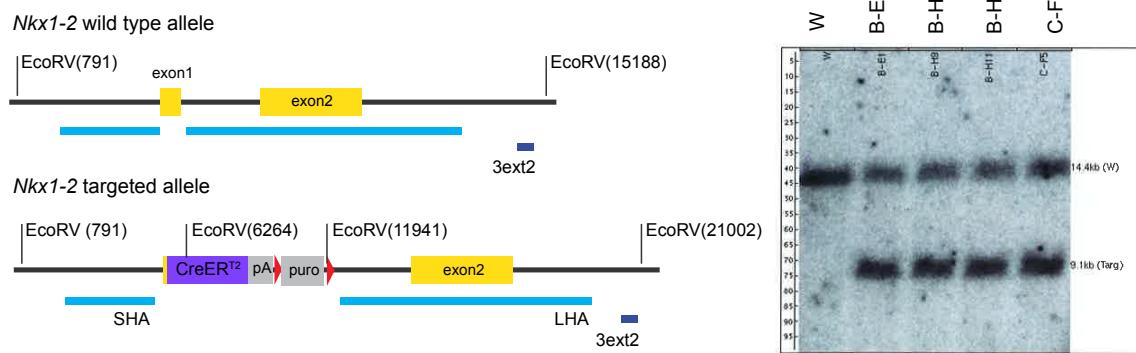
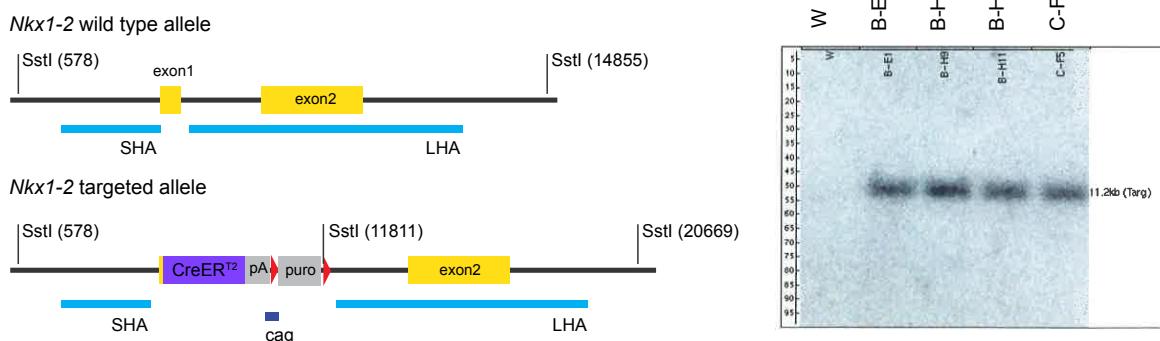


Fig. S5 Map of the targeting vector used to knock-in the CreERT^{T2} cassette into the *Nkx1-2* locus. NotI was used to linearise the vector before homologous recombination. Puromycin (puro) and thymidine kinase (tk) were used as selection markers as described in Materials and Methods. SHA, short homology arm; LHA, long homology arm; pA, polyadenylation site.

A Homologous recombination at the 5' side**B Homologous recombination at the 3' side****C Homologous recombination at the 5' side and single integration****Fig. S6 Southern blot and PCR analysis confirms that targeted clones carry the CreERT^{T2} cassette.**

Southern blot analyses of genomic DNA from wild type (W), and targeted clones B-E1, B-H9, B-H11 and C-F5. The sizes of the wild type allele (W) and the targeted allele (Targ) are shown in the Southern blot. (A) The genomic DNA was digested with the restriction enzyme SstI and a 5' probe (5ext1) was used to confirm correct homologous recombination at the 5' side. (B) The genomic DNA was digested with the restriction enzyme EcoRV and a 3' probe (3ext2) was used to confirm correct homologous recombination at the 3' side. (C) The genomic DNA was digested with SstI and a probe (cag) that detects a region located within the puro selection cassette used to confirm both correct homologous recombination at the 5' side and single integration of the cassette in all clones. The primer sequences for the PCR amplification of the external probes can be found in Table S2. The black line in the schematics represents the genomic DNA. SHA, short homology arms; LHA, long homology arms.

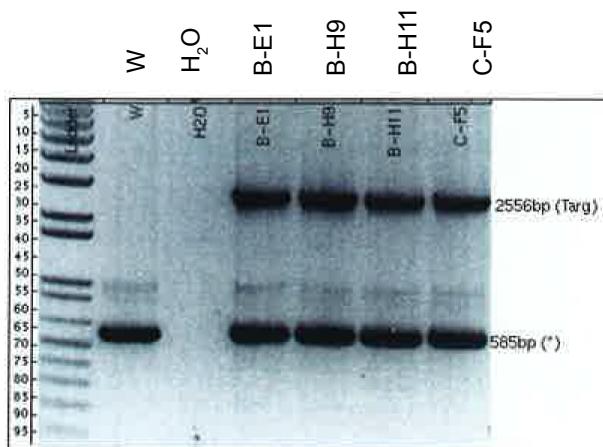


Fig. S7 PCR analysis confirms that targeted clones carry the CreERT^{T2} cassette. The insertion of the CreERT^{T2} cassette as detected in all targeted clones (B-E1, B-H9, B-H11, C-F5) by sequencing the 5' junction between the *Nkx1-2* and CreERT^{T2} regions but not in wild type clones (W). Targ, targeted allele; *, internal control for PCR amplification. Primer sequences can be found in Table S3.

Data S1 *Nkx1-1* expression is undetectable in *Nkx1-2*-expressing cells.

The *Nkx1-1* gene is a paralog of *Nkx1-2* and thus both genes could carry out similar functions during embryonic development. To test this, we set out to investigate whether *Nkx1-1* is also expressed in *Nkx1-2* regions by *in situ* hybridisation. Simon and Lufkin (Simon and Lufkin, 2003) reported that *Nkx1-1* expression "can be detected as early as embryonic stage E10.5". We have tried to revisit *Nkx1-1* expression using the following strategies:

1. We used the primers described in the paper (covering the end of intron2/start of exon2) to try and clone the gene, but were unable to amplify the correct product. We think that this could be due to the high GC content of the primers (GC 80%, Tm 70+).

Primer pair 1: mNkx1-1 forward 5'-TCCTGGGGCGGAGCGGGCAGGGCGG-3', mNkx1-1 reverse 5'-
CGGCGCGCTGGTGTCAAGCGCCT-3'
2. We redesigned primers with lower GC content to target a similar region (end of intron2/start of exon2), but these did not amplify the correct product either.

Primer pair 2: mNkx1-1 forward 5'-GCAAGCAATCAAGGAGGAAC-3', mNkx1-1 reverse 5'-
ACAGGTAGCGAGTGGCCTTA-3'
3. We used a new set of primers covering ~800 nucleotides of the *Nkx1-1* coding sequence (out of 1.3kb full length) on oligo dT-primed cDNA from E13.5 and E9.5 embryos. Again, the products generated were not the correct product/sequence.

Primer pair 3: mNkx1-1 forward 5'-GGCTACAGCTCGGGACACTA-3', mNkx1-1 reverse 5'-
GAGCTGCTCGTAGGTGAAGG-3'
4. We re-checked the genomic sequence of *Nkx1-1* and found that the Ensembl version misses out a bit of 5' coding sequence in the first exon. The Kozak sequence is there so it should be translated. Based on Simon and Lufkin 2003, *Nkx1-1* mRNA might be more abundant at later stages. We tried again cloning the gene from E13.5 oligodT-primed cDNA, but were still unable to amplify the predicted product.

Given these difficulties we took a different approach and checked whether *Nkx1-1* is normally expressed in NMPs by interrogating two published single-cell RNA-sequencing data sets: the Koch (Koch et al., 2017) and the Gouti (Gouti et al., 2017) data sets. We found that *Nkx1-1* is undetectable in any of the 53 NMP cells of the Koch data set. Similarly, *Nkx1-1* is also undetectable in the E8.5 and E9.5 NMPs of the Gouti data set, and in their *in vitro*-derived NMPs. *Nkx1-1* expression is also effectively undetectable in bulk RNA-sequencing data of *in vitro*-derived NMPs from Gouti et al. (Gouti et al., 2014) whereas *Nkx1-2* expression is indeed detected (Fig. S8, Robert Blassberg, James Briscoe lab, The Francis Crick Institute, personal communication).

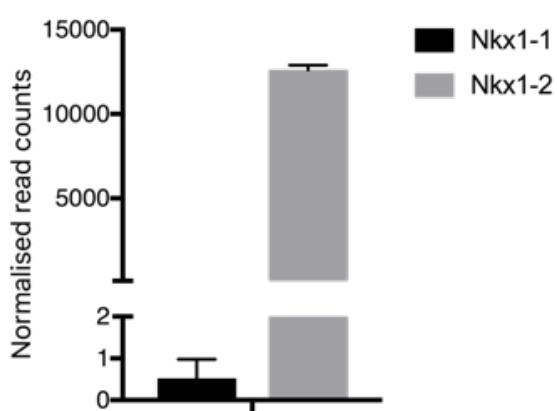


Fig. S8 Bar plot showing the relative expression of *Nkx1-1* and *Nkx1-2* generated from the bulk mRNA-sequencing data of *in vitro*-derived NMPs (data kindly provided by Robert Blassberg from (Gouti et al., 2014)). The normalised read counts for *Nkx1-1* were so low that did not pass filtering steps before downstream analysis.

Taken all together, the lack of *Nkx1-1* expression does not seem to reflect a limitation of single-cell RNA-sequencing technologies in sampling low-expressed genes; but rather, that *Nkx1-1* is not expressed in NMPs before E10.5. All in all, we have not found any evidence suggesting that *Nkx1-1* is co-expressed with *Nkx1-2* in axial progenitors. However, we still do not know whether *Nkx1-1* is upregulated to compensate for the loss of *Nkx1-2* function.

Table S1 Sequence of the targeting vector used to knock-in CreER^{T2} into the *Nkx1-2* locus.

NotI Homology arms

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TGCCTTCTAGTGTAGCGTAGTTAGGCCACCACTCAAGAACTCTGTAGCACC
GGATAAGGCCAGCGGTGCGGCTGAACG

Table S2 Primer sequences for the amplification of the probes for Southern blot analysis.

Probe	Forward primer	Reverse primer
5ext1	GGTGGTCAGCTTACTTGCTAGAGC	TCAAATCAAGCTTCAAAATCCC
3ext2	TAAACTGAGTCTTGACAGGACATG	TCCCATGCACGCTAGGCAGGCTTC

Table S3 Primer sequences for the detection of the inserted CreER^{T2} sequence by PCR.

PCR product	Forward primer	Reverse primer
CreER ^{T2} cassette	GGTGGTCAGCTTACTTGCTAGAGC	TCAAATCAAGCTTCAAAATCCC
internal control	TAAACTGAGTCTTGACAGGACATG	TCCCATGCACGCTAGGCAGGCTTC

Supplementary references

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