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LETTER

Nestin-Cre Mediated Deletion of *Pitx2* in the Mouse

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Summary: *Nestin-Cre* mice are widely used to generate gene deletions in the developing brain. Surprisingly, few *Nestin-Cre* lines have been characterized for their temporal and brain region-specific recombination. In addition, some *Nestin-Cre* lines express Cre outside the central nervous system, making it difficult to choose appropriate lines for targeting genes with brain region-restricted expression. Here we describe the properties of a *Nestin-Cre* transgenic line and its use for conditional deletions of *Pitx2*, a paired-like homeodomain transcription factor. We report that *Nestin-Cre* conditional *Pitx2* mutant mice have ocular and craniofacial defects consistent with the role of human *PITX2* in Rieger syndrome. Conditional mutants exhibit defects in midbrain neuronal development similar to those in *Pitx2* homozygous null embryos, but lack the abnormalities in subthalamic nucleus neurons that occur with complete loss of *Pitx2* function. These data indicate that normal differentiation of midbrain neurons depends upon adequate *Pitx2* function during the period of active neurogenesis. genesis 44:336–344, 2006. Published 2006 Wiley-Liss, Inc.†

Key words: neuronal development; transcription; conditional knock-out

Transcriptional regulation of neuronal development is critical for generating diversity and regional specificity in the brain. Gene targeting with Cre/loxP approaches makes it possible to study precise roles of transcription factors in discrete populations of developing neurons. Nestin is expressed in brain neural progenitors as early as e8.5, is down-regulated at the end of neurogenesis, and is transiently expressed in presomitic mesoderm and somites (Dahlstrand *et al*., 1995; Lendahl *et al*., 1990). Enhancer elements in the rat *nestin* second intron drive reporter gene expression in the neuroepithelium by e10.5 (Zimmerman *et al*., 1994), and have been used to generate several different lines of *Nestin-Cre* mice (Isaka *et al*., 1999; Petersen *et al*., 2002; Tronche *et al*., 1999; Trumpp *et al*., 1999).

The *Nestin-Cre* mice for our study have been used successfully to achieve gene deletions in the central nervous system during development (Bates *et al*., 1999; Fan *et al*., 2001; Groszer *et al*., 2001). This *Nestin-Cre* line is not brain specific; it also expresses Cre in branchial arch-derived tissues and the germline, and exhibits preferential expression from the male allele (Trumpp *et al*., 1999). Despite the availability and use of these mice for over six years, Cre expression sites in the brain and other tissues are not well characterized. Instead, prior studies relied on PCR or Southern based analysis of whole brain tissues to test for *Nestin-Cre* mediated recombination (Bates *et al*., 1999; Fan *et al*., 2001; Groszer *et al*., 2001).

To characterize *Nestin-Cre* activity during brain development, we mated male *Nestin-Cre* transgenic (*Tg*) mice with homozygous *Rosa26* reporter (*R26R*) females and processed embryos (e10.5–e14.5) for X-gal staining ($N =$ at least 3 for each time point). At e10.5, Cre recombination is high in the dorsal and ventral midbrain, dorsal and ventral hindbrain, ventral spinal cord, and branchial arches, and absent in future thalamus, hypothalamus, telencephalon, or mammillary recess (the area containing progenitors of subthalamic nucleus neurons) (see Fig. 1). By e12.5, β-galactosidase activity is extensive throughout the midbrain and hindbrain, and also present in the developing hypothalamus, thalamus, subthalamic nucleus region, and telencephalon (see Fig. 2). At e14.5, β-galactosidase activity is present throughout the brain

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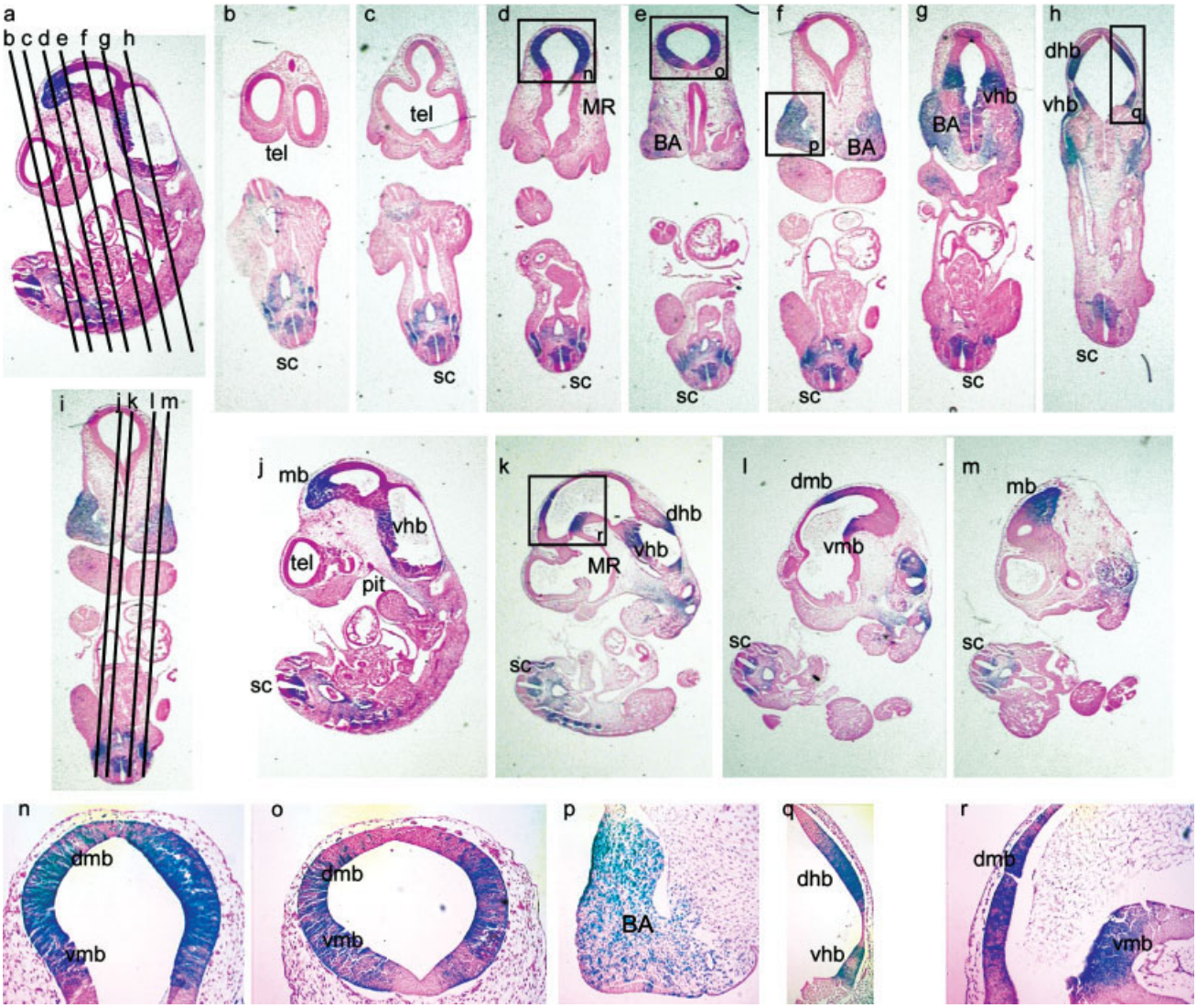

FIG. 1. Nestin-Cre mediated recombination in the e10.5 mouse embryo. *Nestin-Cre*, *R26R*/+ embryos stained at e10.5 with X-gal to detect β-galactosidase activity and counterstained with eosin. Lines over the sagittal section in (**a**) show the plane of frontal sections in (**b**–**h**). Lines over the frontal section in (**i**) show planes of sagittal sections in (**j**–**m**). Boxes in (**d**), (**e**), (**f**), (**h**), and (**k**) are enlarged and shown in (**n**), (**o**), (**p**), (**q**), and (**r**), respectively. β-Galactosidase activity (X-gal stain) is prominent in the dorsal and ventral midbrain (dmb and vmb), ventral spinal cord (sc), branchial arches (BA), and dorsal and ventral hindbrain (dhb and vhb). There is no β-galactosidase activity in the e10.5 mammillary recess (MR) (which contains subthalamic nucleus neuronal progenitors) or in the telencephalon (tel).

and the spinal cord, with mosaic expression in the pituitary, palate, tongue, facial mesenchyme, maxillary and mandibular processes, kidney, and adrenal gland (Fig. 2 and Supplementary Fig. S1). Many tissues exhibit mosaicism of β-galactosidase, suggesting not all cells have undergone Cre-mediated recombination.

There is a delay between Cre expression and β-galactosidase activity, and differences in mouse reporter strains can affect interpretation of Cre activity patterns (Branda and Dymecki, 2004). Thus, some early Cre-expressing cells may not have been identified in our studies. However, this seems unlikely since two other Cre reporter strains (*Z/AP* and *Z/EG* mice) yielded similar e9.5–e10.5 Cre activity patterns in the midbrain when mated with the same line of *Nestin-Cre* mice (unpublished observations, A. Trumpp). In addition, the initial report characterizing *R26R* mice demonstrated ubiquitous expression of the *lacZ* reporter in mouse embryos from as early as implantation (Soriano, 1999).

E10.5–15.5 is a period of intense neurogenesis throughout the developing mouse brain (Easter *et al.*, 1993); thus, we hypothesized that *Nestin-Cre* mediated recombination during this period could be valuable for studying late effects of *Pitx2* deficiency in neuronal development. *Pitx2* is a member of the paired-like family of homeodomain transcription factors essential for normal development in humans and mice (Gage *et al.*, 1999b; Kitamura *et al.*, 1999; Lin *et al.*, 1999; Liu *et al.*, 2001;

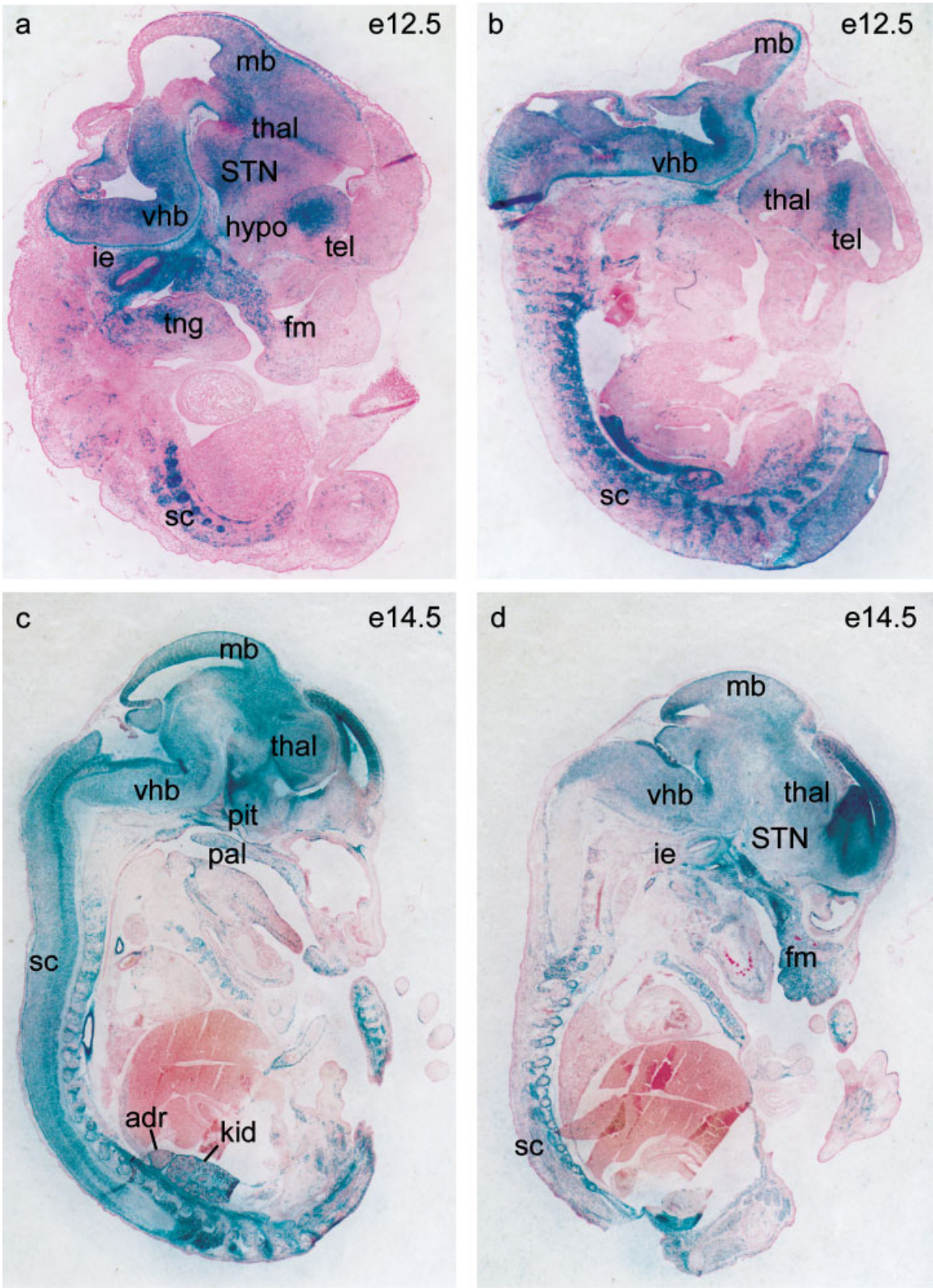

FIG. 2. *Nestin-Cre* mediated recombination in the e12.5–14.5 mouse embryo. Sagittal sections from X-gal stained e12.5 (**a**, **b**) or e14.5 (**c**, **d**) *Nestin-Cre*, *R26R*/+ embryos. In addition to the areas of recombination at e10.5, X-gal is present at e12.5 in the telencephalon (tel), thalamus (thal), and hypothalamus (hypo). At e14.5, X-gal stain is present throughout the region containing the subthalamic nucleus (STN) and in scattered cells of the facial mesenchyme (fm), pituitary (pit), palate (pal), inner ear (ie), kidney (kid), and adrenal gland (adr). Other abbreviations – tng, tongue; sc, spinal cord; vhb, ventral hindbrain; mb, midbrain.

Semina *et al*., 1997). In humans, autosomal dominant haploinsufficient *PITX2* mutations cause Rieger syndrome, characterized by eye abnormalities, umbilical defects, hypodontia, craniofacial abnormalities, and occasional central nervous system defects (Idrees *et al*., 2006; Semina *et al*., 1997).

In mice, *Pitx2* is expressed in the developing heart, eye, pituitary, teeth, tongue, maxillary and mandibular epithelia, and in discrete regions of the developing central nervous system, including the mammillary region, subthalamic nucleus, superior colliculus, hindbrain, and zona limitans intrathalamica (Gage *et al*., 1999a; Kitamura *et al*., 1997; Martin *et al*., 2002; Mucchielli *et al*., 1996). Mice with complete loss of *Pitx2* function ($Pitx2^{-/-}$) die by e15 with severe defects in heart, craniofacial structures, teeth, eyes, and pituitary gland (Gage *et al*., 1999b; Kitamura *et al*., 1999; Lin *et al*., 1999; Liu *et al*., 2001). In the pituitary, *Pitx2* is required at several different stages of development, with roles in early progenitor cell formation and later lineage specification (Charles *et al*., 2005; Suh *et al*., 2002). Our prior work showed that gene expression in *Pitx2* homozygous null embryos is disrupted in the subthalamic nucleus and mislocalized in the midbrain, suggesting delayed or arrested neuronal migration or differentiation (Martin *et al*., 2004).

To generate *Pitx2NCre* mutants (see Fig. 3), we mated male *Nestin-Cre Tg*, $Pitx2^{+/-}$ mice with $Pitx2^{flox/flox}$ females, since the *Nestin-Cre* transgene is preferentially expressed from the paternal allele (Trumpp *et al*., 1999). Offspring from this mating are homozygous *Pitx2* null in *Nestin-Cre* lineage cells, and heterozygous *Pitx2* null elsewhere. Conditional $Pitx2^{flox/flox}$ mice contain *loxP* sites flanking the *Pitx2* homeodomain-containing exon 5 and exhibit no phenotypic abnormalities (Evans and Gage, 2005; Gage *et al*., 1999b). To examine postnatal survival of *Pitx2NCre* mutants, pups were checked daily, tail DNA isolated at postnatal days 1–7, and DNA genotyped for *Nestin-Cre* and *Pitx2* wild-type, null, and floxed alleles, as previously described (Gage *et al*., 1999b; Trumpp *et al*., 1999). Pups of genotype *Nestin-Cre Tg*, $Pitx2^{-/flox}$ (*Pitx2NCre* mutants; $N = 5$) survived to postnatal day 1, whereas all pups of the three other genotypes (*Nestin-Cre Tg*; $Pitx2^{+/flox}$ $N = 8$; *Non-Tg*; $Pitx2^{+/flox}$ $N = 14$; *Non-Tg* $Pitx2^{-/flox}$ $N = 6$) survived to postnatal day 7, the latest timepoint analyzed. Extended survival of *Pitx2* conditional mutants beyond e15 enabled us to expand our analysis of the *Pitx2* deficient brain to late embryonic periods.

Pitx2NCre mutant embryos exhibit kinked tails, a pointed snout, and a fused mouth (see Fig. 3), and similar ocular anomalies (medially displaced eyes) as in homozygous *Pitx2* null embryos (Gage *et al*., 1999b). *Pitx2NCre* embryos also have hypoplastic teeth and other craniofacial tissues (see Fig. 3), consistent with β-galactosidase activity in the developing branchial arches (see Fig. 1) and with prior reports of *Nestin-Cre* mediated *Fgf8* deletion in branchial arch-derived tissues (Trumpp *et al*., 1999). The *Pitx2NCre* mutant craniofacial phenotype resembles that of human Rieger syndrome, providing evidence for conversion of the $Pitx2^{flox}$ to $Pitx2^{null}$ allele in craniofacial tissues; thus, *Pitx2NCre* embryos are also useful for understanding the morphogenesis of *Pitx2*-dependent craniofacial structures.

Since homozygous *Pitx2* null embryos have defects in neuronal differentiation, we hypothesized that similar effects might also occur in *Pitx2NCre* mutants. To test this, we used a *Pitx2* in situ probe that recognizes wildtype, $Pitx2^{flox}$, and recombined ($Pitx2^{null}$ or $Pitx2^{-}$) alleles (Martin *et al*., 2002, 2004) to track wildtype, heterozygous, and homozygous mutant *Pitx2*-expressing neurons. We examined *Pitx2NCre* embryos at e14.5 (the latest surviving stage of homozygous *Pitx2* null embryos) to compare subthalamic nucleus and midbrain phenotypes between complete and conditional null mutants. As in homozygous *Pitx2* null embryos (Martin *et al*., 2004), the midbrain of e14.5 *Pitx2NCre* mutants exhibits a shift in *Pitx2* mRNA expression toward the midline (see Fig. 4). This effect persists in e18.5 embryos, in which *Pitx2* mRNA is dispersed throughout the *Pitx2NCre* midbrain, in contrast to the tight layer of *Pitx2* mRNA seen in control embryos (see Fig. 5). As in homozygous *Pitx2* nulls, PITX2 immunofluorescence is undetectable in the e14.5 (and e18.5) midbrain of *Pitx2NCre* mutants (Figs. 4 and 5), consistent with absence of PITX2 protein from a null (recombined) allele (Hjalt *et al*., 2000; Martin *et al*., 2004). These data argue against a simple developmental delay or change in gene expression as the explanation for shifted locations of *Pitx2* mutant midbrain neurons, and suggest an ongoing requirement (between e14.5 and e18.5) for *Pitx2* in midbrain neuronal differentiation or survival.

The homozygous *Pitx2* null e14.5 subthalamic nucleus region fails to express multiple neuronal markers, including *Pitx2* mRNA, PITX2 immunofluorescence, and calretinin (Martin *et al*., 2004). In contrast, both *Pitx2* mRNA and PITX2 immunofluorescence are present in the subthalamic nucleus of e14.5 (see Fig. 4) and e18.5 (see Fig. 6) *Pitx2NCre* mutants, albeit at slightly reduced levels and in a disorganized pattern compared with control littermates. Unlike in homozygous *Pitx2* nulls, in *Pitx2NCre* mutants there is no increase in *Pitx2* mRNA around the third ventricle to suggest an accumulation of mutant cells (Martin *et al*., 2004). The majority of subthalamic nucleus neurons are generated in the mouse between e10.5 and e14.5 (Martin *et al*., 2002, 2004), raising the possibility that *Pitx2NCre* mutant neurons born between e10.5 and e12.5 (prior to the onset of *Nestin-Cre Tg* expression in the mammillary region (Figs. 1 and 2)) escape recombination at the *Pitx2* locus. These genotypic $Pitx2^{flox/-}$ neurons would still express PITX2 protein and could migrate through the neuroepithelium toward the subthalamic nucleus.

The slight reduction in size and disorganization of the subthalamic nucleus in *Pitx2NCre* mutants suggest that some neurons (i.e. those born at e12.5 or later) may undergo Cre-mediated recombination, lose *Pitx2* function, and either fail to migrate to the nucleus, die, or

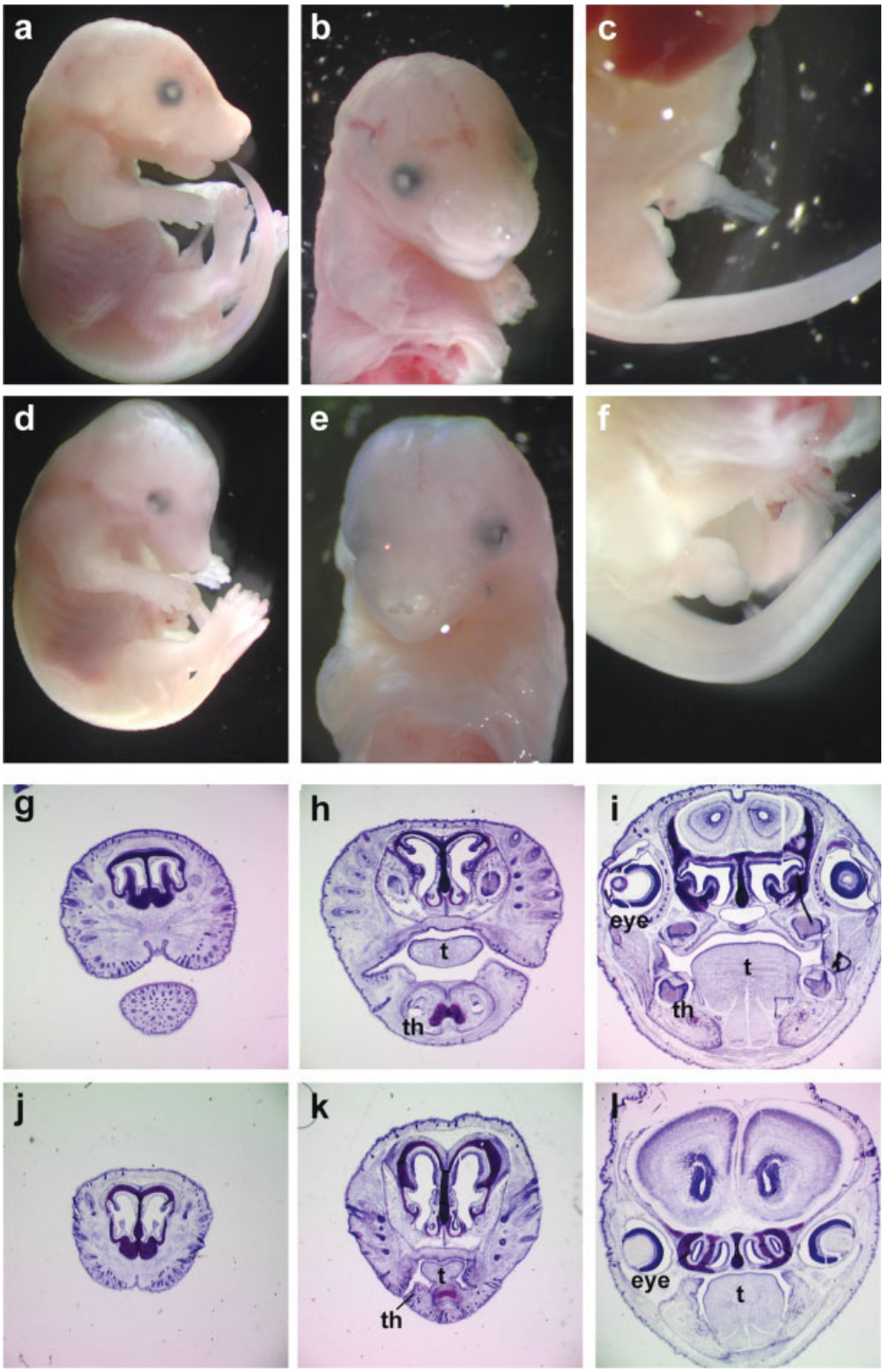

FIG. 3. *Pitx2NCre* mutants have craniofacial and distal tail abnormalities. *Nestin-Cre Tg*, $Pitx2^{+/flox}$ e18.5 mice (controls, (**a**–**c**)) compared with *Nestin-Cre Tg*, $Pitx2^{-/flox}$ mice (*Pitx2NCre* mutants, (**d**–**f**)). *Pitx2NCre* mutants exhibit a small pointed snout, fused mouth, kinked tail, and slightly reduced size compared to control embryos. *Pitx2NCre* mutants (**j**–**l**) also have a hypoplastic tongue (t), teeth (th), and medially displaced eyes in comparison with controls (**g**–**i**).

adopt alternative fates. Additional studies using reporter tagged alleles are necessary to distinguish among these possibilities. There is detectable β-galactosidase activity in the subthalamic nucleus of e18.5 *Nestin-Cre*, *R26R* embryos (see Fig. 6); however, low level mosaicism for Cre-negative cells in the developing subthalamic nucleus might have been missed in our analysis. An intriguing possibility is that deletion of *Pitx2* in dividing progenitors may have more severe effects on neuronal differentiation or migration than deletion of *Pitx2* in postmitotic migratory subthalamic nucleus neurons, but this remains to be determined. Further experiments using neural spe-

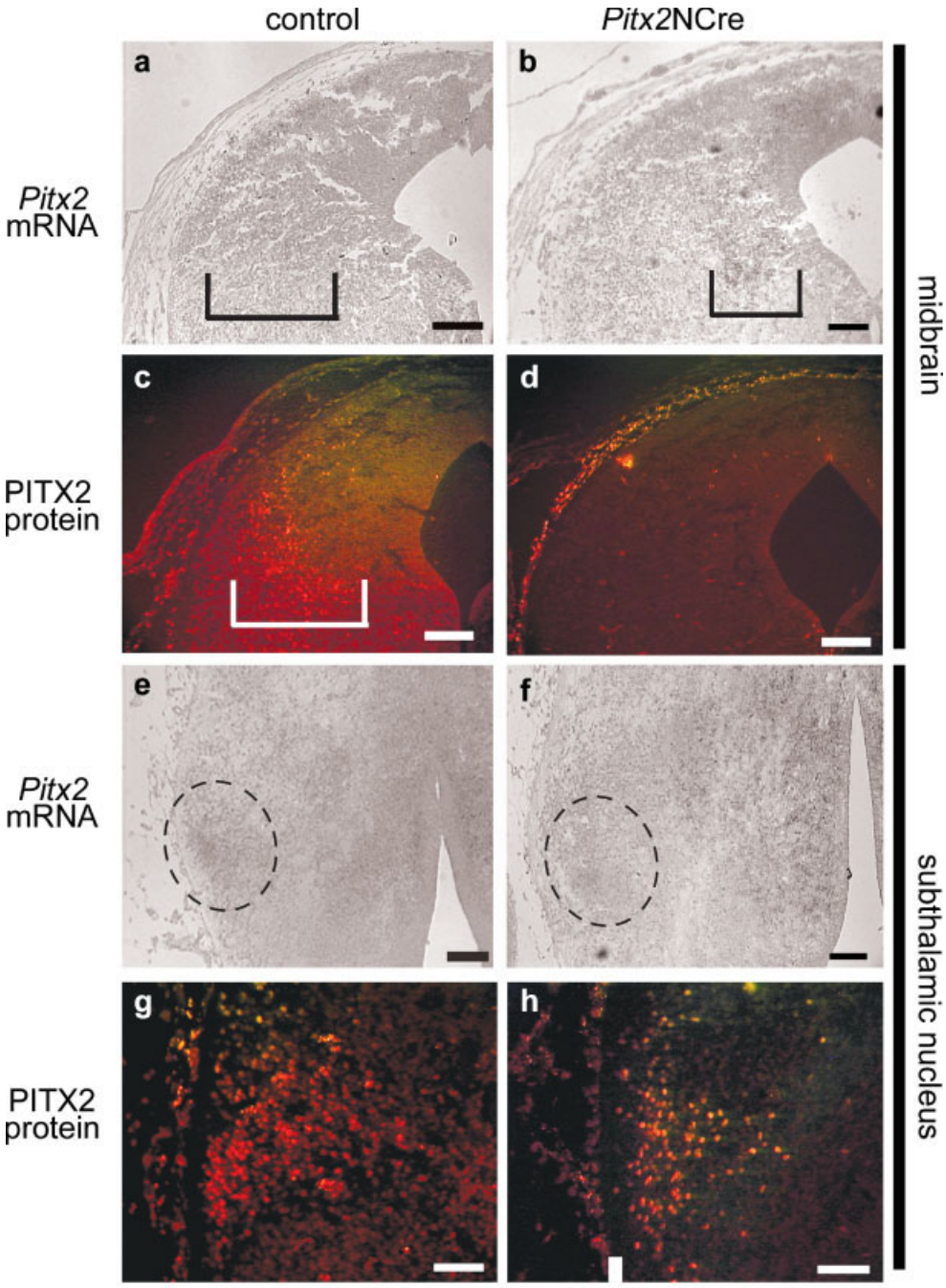

FIG. 4. *Nestin-Cre* conditional mutants exhibit brain region specific *Pitx2* deficiency. Frontal (coronal) sections of control (*Pitx2*$^{+/-}$, *Nestin-Cre Tg*) (**a**, **c**, **e**, **g**) and *Pitx2NCre* mutant (**b**, **d**, **f**, **h**) embryos at e14.5. *Pitx2* mRNA (a, b, e, f), which detects wildtype, flox, and recombined (null) alleles, is shifted medially (b) and PITX2 immunofluorescence is absent in the midbrain of *Pitx2NCre* mutants (d) compared to controls (a, c). These results are similar to the midbrain phenotype of e14.5 homozygous *Pitx2* null embryos (Martin *et al*., 2004). Subthalamic nucleus *Pitx2* mRNA (e, f; hatched ovals) and immunofluorescence (g, h) are slightly reduced in *Pitx2NCre* mutants, unlike homozygous e14.5 *Pitx2* null embryos, which exhibit complete loss of *Pitx2* mRNA and protein in the subthalamic nucleus (Martin *et al*., 2004). Scale bars are 100 μm in (a–f) and 50 μm in (g) and (h).

cific Cre deleter strains with temporal control and earlier onset of expression will help determine whether *Pitx2* has distinct roles in neurogenesis versus neuronal migration and differentiation, and whether these effects are unique to specific brain regions.

METHODS

Mice

All procedures involving the use of mice were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA). *Nestin-Cre* transgenic mice were obtained from Gail Martin (Trumpp *et al*., 1999) and maintained on a C57BL/6J background. *Rosa26* reporter mice (*R26R*), line B6.129S4-*Gt*(Rosa)26Sor^{tm1Sor}/J (Soriano, 1999), were obtained from The Jackson Laboratory (www.jax.org) and maintained in our mouse colony as homozygotes. *Pitx2*$^{-}$ and *Pitx2*^{flox} alleles were as previously described (Gage *et al*., 1999b; Martin *et al*., 2004), and were maintained on a C57BL/6J background to N7 generation. *Pitx2* alleles were genotyped by PCR using previously described primers (Gage *et al*., 1999b).

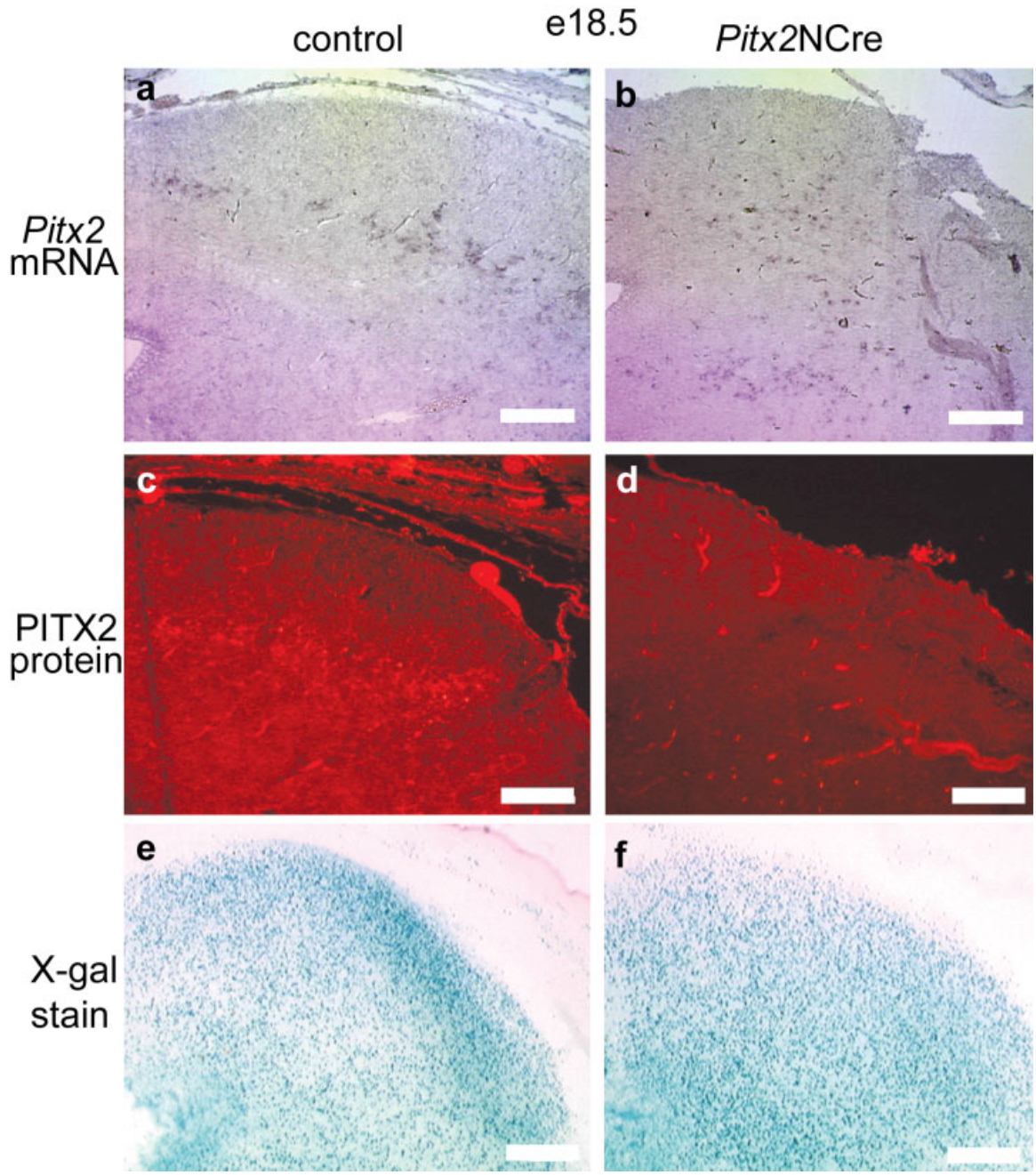

FIG. 5. Deletion of *Pitx2* in *Pitx2NCre* midbrain neurons disrupts neuronal location. Frontal sections of e18.5 embryos at the level of the midbrain show *Pitx2* mRNA (**a**, **b**) and immunofluorescence (**c**, **d**) in controls (*Pitx2*$^{+/-}$, *Nestin-Cre Tg*) (a, c) and *Pitx2NCre* mutants (b, d). *Pitx2* mRNA is dispersed in *Pitx2NCre* mutants, and PITX2 immunofluorescence is absent. Autofluorescence from tissue folds is present in c and d. β-Galactosidase activity (X-gal staining) is distributed throughout the e18.5 midbrain, in both control (**e**) and *Pitx2NCre* mutant (**f**) embryos that also contain the *R26R* reporter. Scale bars are 100 μm.

The *Nestin-Cre* transgene was detected using forward primer 5′-CCGGGGTGTCTGGCTGTATCTCAA-3′ and reverse primer 5′-CGGTGCTAACCAGCGTTTTC-3′ (Invitrogen, Carlsbad, CA). For *R26R* mice, genotyping was done using primers that detect wild-type and floxed alleles as indicated on The Jackson Laboratory website (www.jax.org).

Immunofluorescence and In Situ Hybridization

Timed pregnancies were established between *Nestin-Cre Tg*, *Pitx2*$^{+/-}$ males and *Pitx2*$^{flox/flox}$ females or between *Nestin-Cre Tg* males and homozygous *R26R* females. The morning of vaginal plug identification was designated as embryonic day 0.5. Embryos (e10.5–e18.5) were harvested from the uterus after cervical

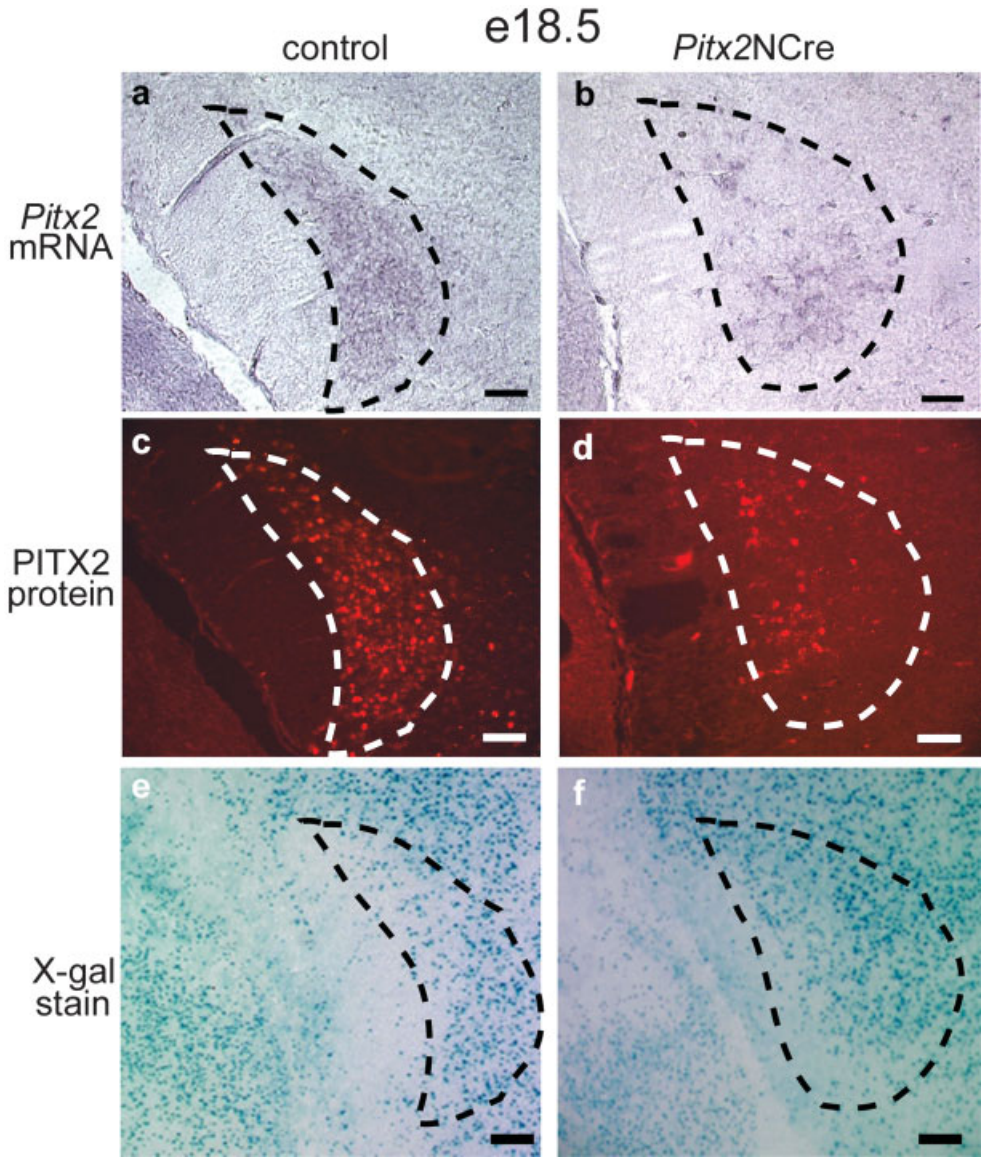

FIG. 6. PITX2 is maintained in the subthalamic nucleus of *Pitx2NCre* mutants. Frontal (coronal) sections at the level of the subthalamic nucleus (hatched areas) show *Pitx2* mRNA (**a**, **b**) and immunofluorescence (**c**, **d**) in *Pitx2NCre* mutants. The pattern of *Pitx2* expression at both mRNA and protein levels appears disorganized and reduced relative to control (*Pitx2*$^{+/-}$, *Nestin-Cre Tg*) littermates. β-Galactosidase activity (X-gal staining) is present in the e18.5 subthalamic nucleus region in both control (**e**) and *Pitx2NCre* mutant (**f**) embryos that also contain the *R26R* reporter. Scale bars are 50 μm.

dislocation and hysterectomy, and embryos were dissected into 0.1 M PBS, pH 7.2. Yolk and amniotic sacs or caudal aspects of each embryo were processed for genotyping as described (Martin *et al.*, 2004). Embryos (e14.5 and e18.5) were fixed for 3 h in 4% paraformaldehyde (Sigma, St. Louis, MO), washed in PBS, and then dehydrated in an increasing series of ethanol concentrations to 100%. Dehydrated embryos were rinsed in methylsalicylate (Fisher) and embedded in paraffin wax. Embryos were sectioned at 7 μm (e10.5–e14.5) or 8 μm (e18.5). All embryos were sectioned on a microtome (Leitz) and mounted on baked super frost plus slides (Fisher, Pittsburgh, PA). Immunofluorescence and in situ hybridization were performed as described (Martin *et al.*, 2002, 2004); the *Pitx2* in situ probe we used recognizes Exon 5 and the 3′ UTR, which is present in the *Pitx2* somatic recombined null, germline null, floxed, and wildtype alleles (Martin *et al.*, 2004).

β-Galactosidase Activity Assay

Embryos (e10.5–e14.5) were harvested as described before and fixed in 0.5% formaldehyde, 1.25 mM EGTA, 2 mM $MgCl_2$ in PBS overnight at 4°C, and then in 30% sucrose containing 2 mM $MgCl_2$ in PBS overnight at 4°C. Embryos were embedded in Optimal Cutting Temperature embedding medium (Sakura Finetek, Torrance, CA), cryosectioned (15 μm), and stored at −80°C. Sections were thawed and fixed in 0.5% glutaraldehyde (PBS, 1.25 mM EGTA, 2 mM $MgCl_2$) for 20 min, washed in buffer (sodium phosphate buffer pH 7.4 with 2 mM $MgCl_2$ and 0.02% NP-40 (Sigma)), and incubated for 2–24 h in X-gal wash buffer containing 1 mg X-gal (Invitrogen), 5 mM potassium ferro-

cyanide (Fisher), 5 mM potassium ferricyanide (Fisher), and 0.33% *N-N*-dimethylformamide (Sigma). Upon completion of staining, slides were transferred to wash buffer, post-fixed, and mounted.

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