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Novel genetic tools facilitate the study of cortical neuron migration

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Abstract

Key facets of mammalian forebrain cortical development include the radial migration of projection neurons and subsequent cellular differentiation into layer-specific subtypes. Inappropriate regulation of these processes can lead to a number of congenital brain defects in both mouse and human, including lissencephaly and intellectual disability. The genes regulating these processes are still not all identified, suggesting genetic analyses will continue to be a powerful tool in mechanistically studying the development of the cerebral cortex. *Reelin* is a molecule which we have understood to be critical for proper cortical development for many years. The precise mechanism of *Reelin*, however, is not fully understood. To address both of these unresolved issues, we report here the creation of a novel conditional allele of the *Reelin* gene and showcase the use of an *Etv1-GFP* transgenic line highlighting a subpopulation of the cortex: layer V pyramidal neurons. Together, these represent genetic tools which may facilitate the study of cortical development in a number of different ways.

Introduction

Mammalian cerebral cortical development requires precise control of neuronal production and migration. Projection neurons of the cortex are generated at the ventricular surface and migrate radially towards the pial surface (Hatten 1999; Noctor et al. 2001; Rakic 1972). Proper migration carries these neurons beyond earlier generated neurons such that the final pattern is “inside-out,” where the latest-born neurons are at the outer edge of the cortical plate closest to the skull and the first-born neurons are closest to the ventricular surface. Failure to properly execute this blueprint can lead to a number of structural brain malformations, such as lissencephaly, or functional deficits including the intellectual disability disorders (Bielas et al. 2004; Hu et al. 2014; Jamuar and Walsh 2015; Manzini and Walsh 2011; Stouffer et al. 2015).

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The *Reelin* gene is known to partially control this “inside-out” pattern of radial migration. Reelin protein is secreted from Cajal–Retzius cells which migrate from the cortical hem (among other sources) and spread across the surface of the developing cortical plate [for a review, see (Kirischuk et al. 2014)]. The spontaneous mouse mutant, *reeler*, generated some of the first insight into this mechanism when the cortex of these animals was found to have lost the precise layering of the wild-type cortex (Falconer 1951; Mariani et al. 1977). Subsequent cloning efforts identified the *reeler* mutation to be an intergenic deletion in the *Reelin* locus (D’Arcangelo et al. 1995; Hirotsune et al. 1995). *RELN* mutations in humans cause lissencephaly and cerebellar hypoplasia (Hong et al. 2000). Multiple roles for reelin have been proposed with the classic view holding that it serves as a signal to stop radial migration, largely based on gene expression in overlying Cajal–Retzius cells and the *reeler* mouse phenotype. A full review of the *reeler* literature is not possible here but a recent study shows that genetic ablation of the reelin effector *Dab1* (*disabled1*) leads to cell-autonomous defects phenocopying loss of *reelin* function in cortical lamination, suggesting reelin is important for glial-independent somal translocation in latter stages of neuronal radial migration (Franco et al. 2011).

The layering of the cortical projection neurons was identified even in the earliest anatomical studies (e.g., y Cajal 1995) and further with techniques such as label retention, pulse-chase experiments (Bayer et al. 1991; Hatten 1999). The advent of the molecular age facilitated the identification of a large number of genes expressed in laminar-specific patterns (e.g., Arlotta et al. 2005; Lein et al. 2007; Molyneaux et al. 2007). However, the genetic mechanisms governing the patterning and migration of these diverse populations of neurons are not fully understood. Some reagents that might be useful to further probe these questions would include the ability to genetically disrupt the cortical lamination machinery and to rapidly visualize the lamination patterns, perhaps with a series of immunohistochemical experiments. The ability to genetically perturb the *reelin* locus in a targeted way should facilitate interesting studies. As a complementary tool, we highlight a mouse transgenic line with GFP expressed in a specific subset of cortical projection neurons. Together, these represent novel tools to facilitate further genetic analysis of basic mechanisms of cortical neuron migration and specification.

Materials and methods

Mouse generation

Chimeric mice were generated from two independent, *Reelin*-targeted clones of ES cells (*Reln^{tm1a}*) created by the Knockout Mouse Project using standard methods. 5′ long-range PCR was performed to show correct insertion of the vector with primers pCSD-Reln-LF1 and pCSD-Reln-LF2 in conjunction with the reverse primer LR-5En2frt-R (all primers for this study are indicated in Table 1). Targeting of the *Reelin* locus was confirmed via Southern blotting as genomic DNA from the two *Reln* clones and control ES cells were digested with BsrG1. Blots were detected with a probe external to the 5′ targeting arm (5′ Southern) or a probe internal of the 3′ targeting arm (3′ Southern, Fig. 1a, b). Progeny were genotyped for *lacZ* with internal primers (*lacZF*; *lacZR*) and/or with primers specifically designed to this gene trap (*pRelnlacZF*; *pRelnlacZR*). Chimeras and offspring were also

judged by coat color as the ES cells were injected in albino B6 blastocysts and chimeras bred to albino mice. *Etv1-GFP* mice were generated with standard in vitro fertilization protocols with sperm from the GEN-SAT project (Gong et al. 2003).

Mouse husbandry

All animals were maintained in accordance with Cincinnati Children's Hospital Medical Center IACUC guidelines. Matings were monitored and noon of the day of copulation plug was determined to be embryonic day (E) 0.5. Embryos were collected via Cesarean section after the pregnant dams were sedated and euthanized. Postnatal animals were sedated and euthanized prior to harvest of brain tissues.

RNA analysis

RNA was harvested from the forebrain of *Reln^{tm1a/+}* animals using TRIZOL reagent (Life Technologies) and transcribed into cDNA using the Superscript III First Strand Synthesis System (Life Technologies) per manufacturer's protocols. Control mouse DNA was harvested from inner medullary collecting duct (IMCD) cells. Primers used for cDNA analysis were as follows: exon1F, exon2F, exon2R, and exon3R (Table 1).

Creation of a floxed allele

Reln^{tm1a} mice were crossed with FLPe mice to remove the genetrap and neomycin resistance cassettes (Fig. 2b). The *Reln* locus was analyzed as indicated in Fig. 2a (p1–p6, Table 1). The FLPe allele was genotyped with FLP-TgF, FLP-TgR, FLP-WtF, and FLP-WtR (Table 1).

Histology and immunohistochemistry

Samples for histological analysis were fixed in Bouin's fixative, 4 % paraformaldehyde or 10 % formalin, prepared using a Leica TP1020 automated tissue processor, sectioned at 10–14 μ m, and Nissl stained using established protocols. Immunohistochemistry was performed on cryosections (formalin fixed and cryo-preserved) with an Alexa-Fluor488 conjugated Rabbit IgG anti-GFP (Molecular Probes) antibody using manufacturer protocols. Fluorescence microscopy was performed on Zeiss Discovery V.8 or Zeiss ApoTome microscopes.

Results and discussion

We obtained ES cells (two independent clones) from the Knockout Mouse Project (KOMP) repository (Lloyd 2011) targeted with a conditional gene trap construct (*Reln^{tm1a(KOMP)Mbp}; Reln^{tm1a}*) in JM8A3.N1 ES cells (Fig. 1a). Data presented here are for Clone A03, which resulted in germline transmission. Multiple analyses were performed as part of the KOMP project. ES cells were 71–80 % euploid. Multiple PCR analyses were performed to verify insertion: long-range PCR at the 5' end of the vector and short-range PCR for the confirmation of the 3' loxP site (Fig. S1; Table 1). Vector integrity and genomic locus integrity were also tested via PCR (Fig. S1). The targeting vector copy number was determined by real-time qPCR data with a deltaCT of –1.014 (1–1.4 indicates a single copy of the vector is present; data not shown). Loss of allele analysis indicated homologous

recombination as performed using standard methods (Frendewey et al. 2010; Valenzuela et al. 2003). Southern blotting performed at CCHMC confirmed targeting of the gene trap cassette to the *Reelin* locus (Fig. 1b). Chimeras were produced by blastocyst injection of the correctly targeted ES cells. Multiple strong chimeras were born but only one male proved to be a germline chimera transmitting the gene trap cassette to the next generation. This was determined by coat color as well as PCR genotyping for the lacZ gene in all progeny.

We first determined if the gene trap resulted in appropriate expression of the lacZ reporter allele. Multiple samples were collected at embryonic and postnatal stages from multiple males but no lacZ activity was detected in any of the offspring. PCR genotyping confirmed inheritance of the *Reln^{tm1a}* lacZ gene trap allele (Fig. 1c). To determine if animals were producing transcript from a gene-trapped allele, we purified RNA from a transgenic adult brain and made *Reelin* cDNA. We were able to detect *Reelin* cDNA sequence corresponding to exons 1–2, 1–3, and 2–3 (Fig. 1c). The gene trap cassette is between exons two and three. Consistent with our inability to detect lacZ activity in situ, however, we were never able to amplify product from multiple primer pairs targeting the lacZ cassette or the lacZ cassette and adjacent exons (data not shown). Furthermore, we generated animals which were genotyped to be homozygous for the lacZ gene trap but never exhibited the stereotypic behavior of the *reeler* mice. We conclude from these experiments that the targeting cassette correctly inserted into the genome but the gene trap construct did not effectively disrupt the normal splicing of the *Reelin* gene and create a lacZ reporter allele. In order to test the hypothesis the neomycin cassette interfered with the lacZ gene trap, we mated *Reln^{tm1a}* mice with a germline Cre and selected mice where the loxP recombination removed the neomycin cassette (i.e., recombination between loxP₁ and loxP₂, Fig. 1a). Progeny of these mice also did not express lacZ (data not shown).

We next sought to use the conditional gene trap cassette to generate a traditional loxP allele by deleting the entire lacZ/neomycin cassette with a FLP deleter mouse [B6.129S4-*Gt(ROSA)26Sor-tm1(FLP1)^{Dym/RainJ}*; (Farley et al. 2000)]. FLP-mediated recombination of the FRT sites with excision of the intervening sequence should produce a traditional loxP allele: referred to as *Reln^{fllox}* (Fig. 2a, b). PCR genotyping confirmed progeny carrying the FLP allele no longer carried the intervening sequence (Fig. 2c). Animals that were FLP positive were also loxP positive (Fig. 2d).

Some of the classic *reeler* phenotypes are disrupted lamination of cerebral cortical, hippocampal, and cerebellar structures (Caviness 1976; Mariani et al. 1977; Stanfield and Cowan 1979a, b). We tested if our conditional allele could indeed inactivate the *Reelin* locus and phenocopy the *reeler* mutation in a tissue-specific manner by genetically ablating *Reelin* with the *Emx1-Cre* allele (*B6.129S2-Emx1^{tm1(cre)Krl/J}*) which is expressed throughout the telencephalon (Gorski et al. 2002). We crossed mice doubly heterozygous for *Emx1-Cre* and the *reeler* allele *Reln^{rl}* (*Emx1-Cre;Reln^{rl/+}*) with mice homozygous for the *Reln^{fllox}* allele. We then histologically examined *Emx1-Cre;Reln^{rl/fllox}* mice at early postnatal stages in comparison to wild-type and *Reln^{rl/rl}* homozygous mice. We confirmed that *Reln^{rl/rl}* homozygotes have abnormal neuronal migration in the cerebral cortex (Fig. 3d), hippocampus (Fig. 3e), and cerebellum (Fig. 3f). *Emx1-Cre* mediates recombination in the telencephalon but not the cerebellum (Gorski et al. 2002). *Emx1-Cre;Reln^{rl/fllox}* mice show

cortical and hippocampal defects consistent with loss of Reelin function (Fig. 3g, h) but have phenotypically normal cerebella (Fig. 3i). These data are consistent with a properly floxed allele of *Reelin* that was indeed recombined in the *Emx1-Cre* domain leading to loss of *Reelin* function.

As part of our ongoing studies in genetic mechanisms of cortical lamination, we also took advantage of the Gene Expression Nervous System Atlas (GENSAT) project (Gong et al. 2003) and imported a transgenic line expressing EGFP under the control of the *Etv1* promoter: *Tg (Etv1-EGFP)^{BZ192Gsat}*, hereafter referred to as *Etv1-GFP*. *Etv1* (formerly *Er81*) is highly expressed in a subset of layer V neurons of the cerebral cortex of multiple mammalian species (Hevner et al. 2003; Hirokawa et al. 2008; Watakabe et al. 2007; Yoneshima et al. 2006). We reasoned this reagent could be a useful reporter transgene in a number of experimental contexts, including forward genetic approaches to identify novel genes controlling cortical laminar fate and migration (Ha et al. 2015; Stottmann et al. 2011). We first confirmed that the expression of the *Etv1-GFP* transgene was layer specific in early postnatal stages, at postnatal (P) 4 and P8 (Fig. 4a, b). The GFP transgene was expressed at sufficiently high levels to be visualized with whole mount fluorescent microscopy without any immunohistochemical labeling. In contrast to the documented expression of *Etv1* itself, the BAC transgenic did not result in any detectable GFP expression at any embryonic stage we examined (data not shown).

To highlight the utility of this transgenic as a reporter of laminar fate in the cortex, we created *Reln^{rl/rl}; Etv1-GFP/+* complex heterozygotes and compared them to control *Reln^{rl/+}; Etv1-GFP/+* doubly heterozygous littermates. As expected, immunohistochemistry on control animals highlighted a discrete layer of cortical projection neurons with robust axonal and dendritic extensions (Fig. 4c, d). Consistent with known radial migration phenotypes in *Reln^{rl/rl}* homozygotes (Boyle et al. 2011), the *Etv1-GFP* transgene was expressed in neurons scattered across the width of the cortical plate (Fig. 4e). Neurite extensions in the mutant were also shorter in the *Reln^{rl/rl}; Etv1-GFP* tissue (Fig. 4f).

Here we present a pair of tools that may be employed to facilitate genetic analyses of the mechanisms of both Reelin function and cortical neuron migration and laminar fate. We have created a novel conditional allele of *reelin* from the gene trap allele from KOMP. Although the reporter function of this allele seems to be inoperable, the derived conditional allele is a novel reagent. This conditional allele of *reelin* may be especially useful to genetically dissect the role of the Reelin protein in cortical migration and cellular behaviors. A number of models for Reelin activity have been proposed and the complete mechanism is not yet understood (Zhao and Frotscher 2010; Sekine et al. 2014). One approach would be to define which cell populations in the developing CNS require *reelin* function for normal development by comparing the results of different Cre recombinase-mediated ablations. Ongoing work in our laboratory is focused on precisely these questions.

We also highlight the utility of a *Etv1-GFP* transgenic reporter allele in analysis of cortical lamination and migration. The *Etv1-GFP* allele allows rapid analysis of cortical neuron radial migration as demonstrated by the *Reln^{rl/rl}; Etv1-GFP* experiment and is well suited for use in genetic analyses. The *Etv1-GFP* allele does have some similarity to a *Thy1-YFP-H*

transgenic line, which is reported to be largely restricted to layer V in the cortex (Feng et al. 2000). However, multiple reports employing the *Thyl-YFP-H* line indicate it is more widely expressed in comparison to the *Etv1-GFP* (Angata et al. 2007; Demyanenko et al. 2004; Porrero et al. 2010). Furthermore, the *Thyl-YFP-H* line seems to express GFP earlier in cortical development than *Etv1-GFP* (Feng and Walsh 2004). *Thyl-YFP-H* also seems to highlight more neuronal processes than *Etv1-GFP* (e.g., (Buskila et al. 2013). Together, these applications of the *Thyl-YFP-H* line show it to have much broader expression than *Etv1-GFP*, which may render it more useful for some applications. As a biomarker of cortical lamination, however, the reduced expression of the *Etv1-GFP* may allow this transgene to highlight more subtle changes in layer formation than the more broadly expressed *Thyl-GFP-H* line.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A conditional gene trap allele of *Reelin*. **a** KOMP “knockout first” allele design: *FRT* flippase recognition target site, *En2SA engrailed2* splice acceptor, *IRES* internal ribosomal entry site, *lacZ*, β -galactosidase sequence, *pA* polyadenylation sequence, *hBactP* human Beta actin promoter, *neo* neomycin, *B₁₋₄*, *BsrGI* enzyme recognition sites. *Orange bars* represent sequences used for Southern probes in the 5' and 3' portion of the gene. Relative locations of select genotyping primers are represented by *arrows*. **b** Southern blotting confirms targeting of the *reelin* locus. A 14.4 kb band represents the entire sequence from *B₁* to *B₄*. 5.4 kb is the sequence between *B₁* and *B₂* resulting from integration of the gene trap cassette and 8.8 kb is the sequence between *B₃* and *B₄*. **c** PCR genotyping results for the *lacZ* allele and the non-targeted wild-type locus. **d** cDNA analysis of RNA from *Reln*^{tm1a} forebrain, with IMCD cell lysate as a control. Primers spanning exons 1–2, exons 1–3 and exon 2–3 all amplified *Reelin* from the forebrain lysate. No PCR product was amplified with a variety of primer combinations designed against *lacZ* sequence

Fig. 2.

A conditional allele of Reelin. *Reln*^{tm1a} locus before (a) and after (b) FLP-mediated recombination. Relative locations of genotyping primers show the location of primers designed to test for FLP/FRT recombination and for genotyping are indicated. c Primers ppA and pex3R amplify sequence found only in the native gene trap state which is absent after FLP recombination. d PCR genotyping of the *Reln*^{flox} mice with p5/p6 to distinguish between *Reln*^{wt/wt} (approx. 320 bp); *Reln*^{flox/wt}; *Reln*^{flox/flox} (approx. 400 bp) mice

Fig. 3.

Histological analysis of Reelin mutants. Nissl-stained sections of cerebral cortex (**a, d, g**), hippocampus (**b, e, h**), and cerebellum (**c, f, i**) from wild-type at P17 (**a–c**), *Reln*^{rl/rl} mutants at P17 (**d–f**), and *Emx1-Cre;Reln*^{flox/rl} mutants at P21 (**g–i**). The wild-type postnatal cerebral cortex (**a**) has a distinct marginal zone (MZ) and layered cortical plate (CP) dorsal to the striatum (str). This organization, especially of the CP, is lost in the *Reln*^{rl/rl} (**d**) and *Emx1-Cre;Reln*^{flox/rl} (**g**) mutants. The wild-type hippocampus (**b**) also has discrete structures such as the CA1, CA3 areas, and the dentate gyrus (DG). The formation of the hippocampus, most notably the DG, is significantly disrupted in both mutant genotypes (**e, h**). The cerebellum in wild-type animals is highly foliated (**c**). This patterning is lost in the hypoplastic *Reln*^{rl/rl} cerebellum (**f**) but not the *Emx1-Cre;Reln*^{flox/rl} mutants (**i**) indicating the fidelity of the *Emx1-Cre*-mediated deletion. All paired images are shown at the same magnification

Fig. 4.

The *Etv1-GFP* transgene highlights layer V cortical neurons. Whole mount fluorescent imaging of coronal brain slices at P4 (a) and P8 (b) showing the discrete expression pattern of the *Etv1-GFP*. c-f Sections of *Reelin*^{*r1/+*};*Etv1-GFP* $+/+$ brains at P11 showing the *Etv1-GFP* expressing neurons aligned in one layer (c) as compared to the *Reelin*^{*r1/r1*};*Etv1-GFP* $+/+$ brains (e) where *Etv1-GFP* expressing neurons are scattered across the width of the cortex. d, f are higher magnification images of sections similar to c, e, respectively, to highlight the robust cellular process in control neurons (d), which are stunted in the mutant neurons (f). All paired images are shown at the same magnification

Table 1

Primers and probes used

Name	Purpose	Sequence
pCSD-ReIn-LF1	Long-range PCR for ES cells	CCCTGTGAGTCTCATTATCTCTGTAACC
pCSD-ReIn-LF2	Long-range PCR for ES cells	CAGGTTGGAGAACTGCTGTCCTAGAC
pLR-5En2frt-R	Long-range PCR for ES cells	GGTGGTGTGGGAAAGGGTTCGAAG
pCSD-5FRT-F2	Vector integrity (2 kb)	GAAAGTATAGGAACCTTCGAACCCCTTTC
pCSD-LacZ-R1	Vector integrity	GATCGCACTCCAGCCAGCTTTC
pLR-wtF3	Genomic integrity (7 kb)	GAACATACAGCACATTTTCATTTGGACTC
pLR-wtR1	Genomic integrity (7 kb)	GTTAGGTAGAACAGATTCAGGGCTGTC
pCSD-ReIn-R	3'loxP junction	ACCTGTTTCTGCAACCACGATG
pCSD-LoxPcom-F1	3'loxP junction	GAGATGGCGCAACGCAATTAAT
5' Southern Probe	Southern blotting of ES cells	TGACCTAGACATCGACTCAGTCCCTAAGAGAAATTTTGAGGAAAGAG AAGCTACTTGACATGAATCACCTTACAAAAGTTTGAAACCTTAGTCT GTTCCATTAATTTCCCTTCCCACATGATTATAGCTGGAGTGTTTCATT TTCCTTGTGGCTAGACTGACTTCCAGATGTGTCATTTCACACATT GGGTCCCTGCGTCGTGCTTGACCAGGAGAGGAGTGGCAGTTCTCTGAG TCATGGCTCTTGAGTCTGCT
3' Southern Probe	Southern blotting of ES cells	TGTGACTCAGCTGACAGGACCGCACTCATCCACAGAGCCGGCCAAC ATTTAATCTCCTTGCTTCTTCTCTGGGGAAGCTTTTGTGTCTGAGTC TTTCTCTGCAGGACTACTACGGGCACTGCAAAAGTTCCACAACCTCC ACTTCTCCTTGCTCTGTCTCTATCCCTGGTGCCGTGCTAACCTTCTTA GGCTCAATGATCATTTCACTCAGAATTTGGAGAAGCCTGAAATGCTCT CCTGAAGCCAGGCATTTGGCAAGGATCTGTAGT
placZF	Genotyping LacZ-positive mice	TTTAACGCCGTGCGCTGTTTCG
placZR	Genotyping LacZ-positive mice	GATCCAGCGATACAGCGCGTC
pReInlacZF	Genotyping	TGGCTCTCCTCAAGCGTATT
pReInlacZR	Genotyping	AAATTCAGACGGCAAACGAC
exon1F	ReIn cDNA analysis	TCGCCCTTTCTTTTCTCTGTG
exon2F	ReIn cDNA analysis	GTACCGGGACAGGAATACCA
exon2R	ReIn cDNA analysis	GCCTCCAATGCTCTGAGAAG
exon3R	ReIn cDNA analysis	AACTGGTGGTCGGACATGAT
p1	Genotyping floxed allele	CCCCCTGAACCTGAAACATA
p2	Genotyping floxed allele	GTGGACATCTCTTGGGCACT
p3	Genotyping floxed allele	CCCAGAGCATCTCTGTGCTA
p4	Genotyping floxed allele	AACTGGTGGTCGGACATGAT
p5	Genotyping floxed allele	CACCTGCCTACAACCAACCT
p6	Genotyping floxed allele	CCTCTCCTCCTCACATCAGC
ppA	Vector insertion site verification	CCCCCTGAACCTGAAACATA
pex3R	Vector insertion site verification	AACTGGTGGTCGGACATGAT
pFLP-TgF	Genotyping FLP allele	CACTGATATTGTAAGTAGTTTGC
pFLP-TgR	Genotyping FLP allele	CTAGTGCGAAGTAGTGATCAGG
pFLP-WtF	Genotyping FLP allele	TGTTTTGGAGGCAGGAAGCACTTG
pFLP-WtR	Genotyping FLP allele	AAATACTCCGAGGCGGATCACAAG