Development 138, 5223-5234 (2011) doi:10.1242/dev.063776

Reelin sets the pace of neocortical neurogenesis

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SUMMARY

Migration of neurons during cortical development is often assumed to rely on purely post-proliferative reelin signaling. However, Notch signaling, long known to regulate neural precursor formation and maintenance, is required for the effects of reelin on neuronal migration. Here, we show that reelin gain-of-function causes a higher expression of Notch target genes in radial glia and accelerates the production of both neurons and intermediate progenitor cells. Converse alterations correlate with reelin loss-of-function, consistent with reelin controlling Notch signaling during neurogenesis. Ectopic expression of reelin in isolated clones of progenitors causes a severe reduction in neuronal differentiation. In mosaic cell cultures, reelin-primed progenitor cells respond to wild-type cells by further decreasing neuronal differentiation, consistent with an increased sensitivity to lateral inhibition. These results indicate that reelin and Notch signaling cooperate to set the pace of neocortical neurogenesis, a prerequisite for proper neuronal migration and cortical layering.

KEY WORDS: Reelin, Notch, Radial glia, Cortex development, Mouse

INTRODUCTION

During neocortical development, the biological programs underlying the generation, fate and migration of nervous cells are tightly connected, both temporally and spatially. Indeed, mechanisms underlying precursor cell proliferation adjust the timing of neuronal production for specific neocortical layers (for a review, see Caviness et al., 2009). Cortical development progresses through an early phase of lateral progenitor expansion, a middle neurogenic phase of radial expansion and a final phase of gliogenesis. During the neurogenic phase, the neocortical primordium, including the ventricular zone (VZ) and the subventricular zone (SVZ), balances the maintenance of neural precursor cells against the production of excitatory projection neurons (Miyata et al., 2010), which do not function in their birth places but undergo extensive radial migrations (Rakic, 2007). Molecular links between neurogenesis and migration have begun to be unraveled (Ge et al., 2006; Nguyen et al., 2006). However, migration of newborn neurons is often assumed to rely on purely post-proliferative events, such as the reelin signaling cascade, to establish the distinctive 'inside out' (later-born neurons past their predecessors) neocortical lamination pattern.

The gene mutated in *reeler* mice (*reelin*) encodes a secretable glycoprotein that controls the laminar position of cortical neurons by an unknown mechanism. Reelin is synthesized and secreted by Cajal-Retzius cells in the marginal zone. The plasma membrane apolipoprotein receptor 2 (ApoER2; Lrp8 – Mouse Genome Informatics) and the very low density lipoprotein receptor (Vldlr) along with the cytoplasmic adaptor protein disabled 1 (Dab1) are known to constitute the initial components of the reelin signaling pathway. Hence, mutant null mice for reelin, Dab1 or both ApoER2 and Vldlr, show similar layering defects in the neocortex. It is well established that reelin binding to ApoER2 and Vldlr induces Dab1 phosphorylation, a tyrosine kinase signal transduction cascade and Dab1-regulated turnover (Rice and Curran, 2001; Tissir and Goffinet, 2003; Cooper, 2008). Although reelin signaling decoding is often assumed to occur exclusively in neurons, neural progenitors located next to newborn neurons might receive a functional reelin signal (Luque, 2007). To date, the bulk of published analysis on reelin function has been limited to a loss-of-function approach. The *reeler* mice display no obvious problems with proliferative mechanisms, and their lineage sequences and the appropriate classes of neurons seem to arise in the appropriate order. Cortical neurons also seem to retain their principal class characteristic features of size and pattern of afferent and efferent connections despite a poor, rather 'outside in', pattern of lamination (Caviness et al., 2008).

Notch signaling, long known to keep neural progenitor character by inhibiting neuronal differentiation, has recently been shown to play a key role in mediating the effects of reelin on neuronal migration (Hashimoto-Torii et al., 2008). The fact that Notch can directly bind Dab1 (Keilani and Sugaya, 2008; Hashimoto-Torii et al., 2008), as does the *Drosophila* homolog (Giniger, 1998), lends support to the notion that reelin and Notch pathways might interact functionally (Gaiano, 2008). Although most previous studies have focused on the fact that reelin signaling is active in neurons, there is evidence that cells in the VZ, where neural progenitors reside, can respond to exogenous reelin by phosphorylating Dab1 (Magdaleno et al., 2002). An enrichment of functional reelin receptors (i.e. those present in the plasma membrane as mature forms) in the VZ-SVZ interface with a concomitant downregulation of reelin receptors in migrating projection neurons, also implies that primary reelin action occurs at early/pre-migratory stages (Uchida et al., 2009). Reelin receptors and Notch are expressed in radial glia (Luque et al., 2003; Luque, 2007; Gaiano et al., 2000) and activation of both pathways promotes a radial glial character, including expression of the radial glial marker brain lipid binding protein (Blbp; Fabp7 – Mouse Genome Informatics) (Hartfuss et al., 2003; Gaiano et al., 2000). Moreover, a reelin-dependent increase in Notch intracellular domain (NICD) concentration has been described in a human cortical progenitor cell line (Keilani and Sugaya, 2008). As neocortical radial glial cells give rise to most, if not all, projection neurons (Noctor et al.,

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Accepted 29 September 2011

2001; Miyata et al., 2001; Tamamaki et al., 2001) and are believed to serve as their primary migratory scaffold (Rakic, 1972), perturbations in radial glia could result in aberrant neurogenesis and/or neuronal migration. Understanding the early function of reelin is fundamental to interpret any later requirement based on a mutant phenotype, which might reflect altered neural determination and/or differentiation.

In the present study, we use both loss- and gain-of-function approaches to investigate the function of reelin in neocortical neurogenesis. We seek to reveal whether, earlier than in migrating neurons, the reelin and Notch pathways cooperate in the neural progenitor cells regulating their development. Our results show that reelin is necessary and sufficient to modulate the rate of neurogenesis during neocortical development. They suggest that reelin acting upstream of Notch signaling regulates the temporal specification of neural progenitors and neuronal differentiation.

MATERIALS AND METHODS

Mice

Heterozygous *reeler* mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The *nestin-reelin* (*ne-reelin*) transgenic mice were a generous gift from S. Magdaleno and T. Curran (Magdaleno et al., 2002). The GFP mice were a generous gift from O. Marín (Hadjantonakis et al., 2002). The day of vaginal plug appearance was considered to be embryonic day (E) 0. Animals were handled according to protocols approved by the European Union, NIH guidelines and the Animal Care and Use Committee of the Instituto de Neurociencias.

Genotyping

Primers used for *ne-reelin* were: Nerl-fwd 5′-GAGCAGGGCAG-GTGCTCATTTCC-3′, Nerl-rev 5′-GTTCAGGTCCTCCTCGGAATATC-3′, MBC (mouse beta casein)-fwd 5′-GATGTGCTCCAGGCTAAAGTT-3′, MBC-rev 5′-AGAAACGGAATGTTGTGGAGT-3′. The transgene amplified a 1000 bp band, and the MBC-control amplified a 500 bp band. The PCR conditions were: 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 2 minutes, final elongation at 72°C for 10 minutes.

Primers used for *reelin* were: GM75 5′-TAATCTGTCCTCACTC-TGCC-3′, 3R1 5′-TGCATTAATGTGCAGTGTTG-3′, 3W1 5′-ACAGTT-GACATACCTTAATC-3′. The wild type (WT) amplified a 242 bp band, and the mutated allele amplified a 275 bp band. The PCR conditions were: 94°C for 4 minutes, 30 cycles of 94°C for 1 minute, 55°C for 2 minutes and 72°C for 2 minutes, final elongation at 72°C for 10 minutes.

Reverse transcriptase polymerase chain reaction (RT-PCR) and western blotting

Total RNA was isolated from 10×10^6 cells/ml of undifferentiated neurospheres using TRIZOL (Sigma). cDNA was generated using a First Strand cDNA Synthesis Kit (Roche). RT-PCR sequences were: GAPDH-fwd 5′-TGATGACATCAAGAAGGTGGTGAAG-3′, GAPD-rev 5′-TCCTTGGAGGCCATGTAGGCCAT-3′, reelin-fwd 5′-GAGGTGTATG-CAGTG-3′, reelin-rev 5′-TCTCACAGTGGATCC-3′. Gapdh amplified a control band of 249 bp and reelin a 591 bp band.

The *Gapdh* PCR conditions were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, final elongation at 72°C for 5 minutes. The reelin PCR conditions were: 95°C for 3 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, final elongation 72°C for 5 minutes. Recombinant reelin was produced and western blot analysis was performed as previously described (Sáez-Valero et al., 2003).

Neurosphere culture and mosaic assays

Neural stem cells (NSCs) were isolated from the dorsal forebrain of E14 mouse embryos. Cells were cultured in serum-free Neurobasal medium (Gibco) with B27 supplement (Gibco), EGF (Sigma; 20 ng/ml), bFGF (Sigma; 10 ng/ml), mitogens and heparin (Sigma; 0.7 U/ml) in non-coated plastic. Cells were passaged every 4 days by complete cluster disintegration into a single cell culture. For differentiation assays, we used whole neurospheres after the first passage on pre-coated culture plastic dishes treated with laminin (Sigma; 0.5 mg/ml) for 5 hours. Cells were cultured for periods of 3 and 6 days. Differentiation medium consisted of Neurobasal medium (Gibco) with 10% FBS (Sigma), without EGF, bFGF and heparin. For mosaic assays, we used NSCs from WT GFP mice and *reeler ne-reelin* transgenic mice after the first passage. Different fractional combinations of neurospheres, varying from 15 to 90%, were seeded on pre-coated culture dishes and differentiated for 4 days before immunostaining.

Cell-cycle exit and ventricular length analysis

For cell-cycle exit analysis, the pregnant dam was injected with BrdU (100 mg/kg) at E13-14. *Reeler* and *ne-reelin* embryos were compared with WT in two different set of experiments. Embryos were harvested 18 hours after the injection and the brains processed for BrdU-Ki67 double immunolabeling. The fraction of cells that had exited the cell cycle was estimated by counting the number of BrdU+ cells and the number of BrdU+/Ki67– in a similar area. The cell-cycle exit fraction reported is the number of BrdU+/Ki67– cells divided by the total number of BrdU cells. Both the experimental procedure and the range of the control values in WT were similar to those previously published (Siegenthaler et al., 2009). Analysis of E14 dorsal forebrain length consisted of measuring the length of the ventricular surface from the pallial-subpallial boundary to the most dorsal point of the forebrain ventricular lumen in coronal sections from a similar rostral-caudal level.

Immunohistochemistry

Undifferentiated and differentiated NSCs were fixed with 4% paraformaldehyde (Sigma) in PBS for 20 minutes. After washing, cells were blocked for 1.5 hours in PBS plus 4% BSA (Sigma) and 0.5% Triton X-100 (Sigma). The cells were then incubated at 4°C overnight with primary antibodies in blocking solution, washed and then incubated with secondary antibodies for 2 hours at room temperature. After washing, DAPI was applied for 10 minutes and cells were mounted in Fluoromount media (Sigma). Undifferentiated neurospheres were mounted onto glass slides with spacers and Fluoromount-G mounting media (Southern Biotech). For immunohistochemistry, embryos or neonates were either fixed by immersion or perfused transcardially with 4% paraformaldehyde (Sigma) in phosphate buffer (PB, 0.01 M, pH 7.4). Brains were post-fixed for 48 hours in 4% paraformaldehyde followed by 24 hours in PB containing 20% sucrose (Sigma) for cryoprotection. Brains of different genotypes were arranged side by side and embedded together in the same block of gelatin matrix prior to cryosectioning at a thickness of 50 μm. This type of embedding enables us to perform the immunostaining using the same conditions for different brains. Floating sections were blocked for one hour in potassium phosphate buffered saline (KPBS) with 10% FBS and 0.025% Triton X-100. They were then incubated with primary antibodies in KPBS with 1% serum and 0.025% Triton X-100 overnight at room temperature. After washing, they were incubated with secondary antibodies in KPBS for 2 hours at room temperature. Conventional antigen retrieval and signal amplification procedures (Ochiai et al., 2009) were applied prior to NICD immunostaining. Finally, the sections were incubated with DAPI and mounted.

Primary antibodies were: mouse anti-reelin (G10, 1:500, from A. Goffinet, University of Louvain, Brussels, Belgium), rabbit anti-Tuj1 (1:3000, Covance), rabbit anti-GFAP (1:750, Dako), rabbit anti-BLBP (1:1000, Chemicon), rabbit anti-Tbr2 (1:250, Abcam and from R. Hevner, Seattle Children's Hospital Research Institute, Seattle, USA), mouse anti-H3P (1:200, Cell Signaling Technology), rabbit anti-Hes1 (1:250, Millipore), rabbit anti-Ctip2 (1:500, Abcam), rat anti-BrdU (1:250, Abcam), rabbit anti-Ki67 (1:50, Abcam), rabbit anti-cleaved caspase 3 (1:50, Cell Signaling Technology), rabbit anti-cleaved NICD (1:50, Abcam), rabbit anti-Par3 (1:500, Millipore), rabbit anti-S100 (1:300, DAKO), mouse anti-NeuN (1:50, Millipore). Secondary antibodies were: Cy2-donkey anti-rabbit, Cy2-donkey anti-mouse, Cy2-donkey anti-rat, Cy3-donkey anti-rabbit, Cy3-donkey anti-mouse, Cy5-donkey anti-mouse (1:200, all from Jackson ImmunoResearch). DAPI (4′,6-diaminobenzidine-2-phenylindole, dilactate; 300 nM, Invitrogen) was used for counterstaining.

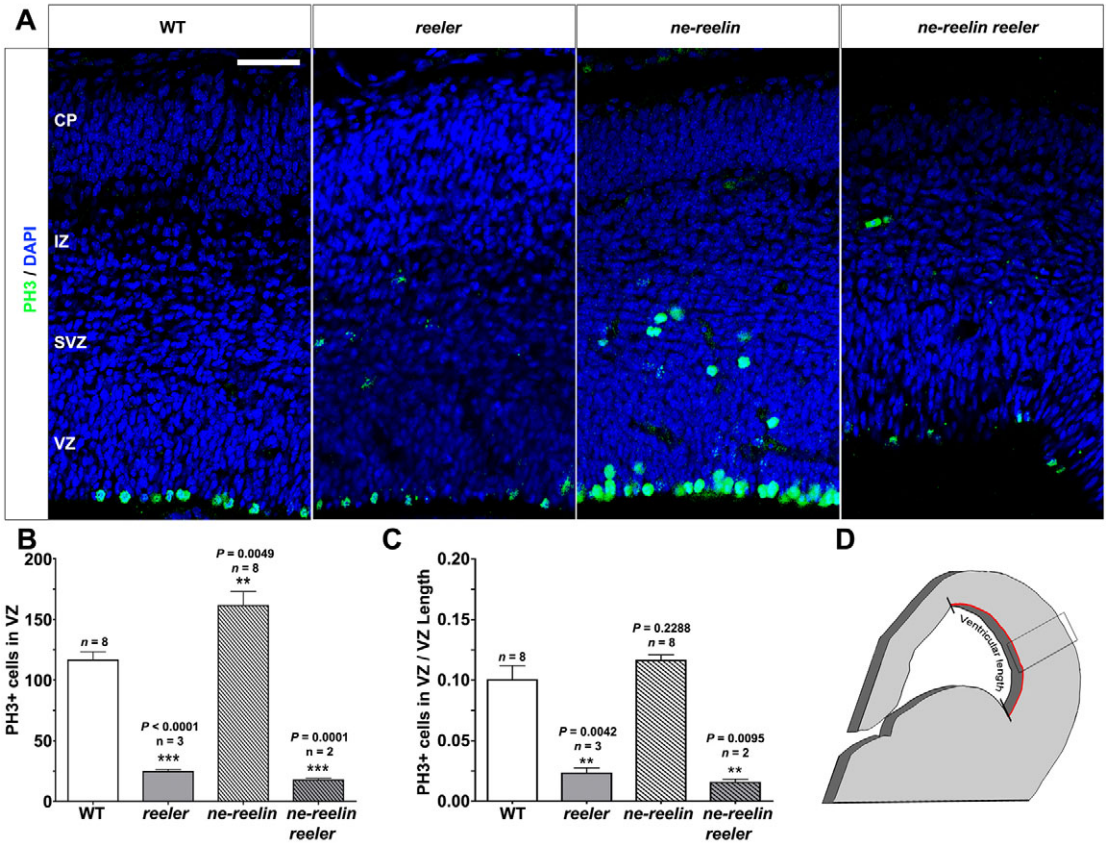

Fig. 1. Reelin regulates the number of dividing cortical NPCs. (**A**) E14 *reeler* cortex (LOF condition) shows a decreased number of VZ and SVZ mitosis (PH3 labeling). The E14 *ne-reelin* cortex (GOF condition) shows an increased density of mitosis in the VZ and the SVZ. In the *reeler* background, the *ne-reelin* transgene does not rescue the mitotic index to WT levels. Nuclei are stained with DAPI (blue). (**B**) Quantification of the number of PH3+ cells in the cortical VZ. (**C**) Ratio of the number of VZ PH3+ cells and the VZ length. (**D**) Schematic showing the dorsoventral and rostrocaudal level of the regions in which the immunofluorescence (box) and the length of ventricular surface (red line) were analyzed. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate. Scale bar: 50 μm. Error bars represent s.e.m.

Data acquisition and statistical analysis

Images were captured on a Leica TCS SP2 AOBS inverted laser scanning confocal microscope or a Nikon i80 fluorescence microscope equipped with a confocal structured light system (Optigrid). Volocity 5.2, Image J (NIH, http://rsb.info.nih.gov/ij/) and Adobe Photoshop software were used for image capture and analysis. Gfap- and beta-tubulin-III positive cells were counted in at least ten randomly chosen fields of the culture plates. GraphPad Prism software was used to perform an unpaired Student's *t*-test for statistical significance. Values shown represent mean ± s.e.m.

RESULTS

To investigate the function of reelin in neocortical neurogenesis we took advantage of the classic *reeler* mice, which are deficient in reelin (loss-of-function, LOF), and the *ne-reelin* transgenic mice (Magdaleno et al., 2002), which express reelin under the control of a *nestin* promoter, which controls gene expression in neural precursor cells (NPCs) located in the VZ during corticogenesis (Lothian and Lendahl, 1997). Thus, *ne-reelin* embryos bear ectopic expression of the reelin protein in NPCs in the VZ and constitute gain-of-function (GOF) conditions. In the presence of the endogenous reelin protein, ectopic reelin did not seem able to alter cell migration in the neocortex. However, in the *reeler* background, ectopic reelin induced tyrosine phosphorylation of Dab1 in the VZ and partially rescued neuronal positioning, so giving rise to early pre-plate splitting (Magdaleno et al., 2002).

Reelin regulates the production of neuronal progenitors and the rate of neurogenesis

To assess the density of cells in the M-phase of the cell cycle during the cortical neurogenic phase we examined immunofluorescent labeling of phosphohistone-3 (PH3). PH3 labels both the primary proliferative population in the VZ, at the edge of the ventricular lumen (neuroepithelial/radial glial cells), and the secondary proliferative population in the SVZ at abventricular locations (intermediate progenitor cells, IPCs). IPCs are produced by the primary progenitors, radial glial cells (Kriegstein et al., 2006). When compared with WT by E14, reelin GOF cortex showed an increased density of PH3 positive (PH3+) cells in the VZ and SVZ. Reelin LOF cortex showed decreased VZ and SVZ proliferation when compared with WT cortex. This phenotype was similar in the *reeler ne-reelin* animal (Fig. 1A,B), suggesting that the GOF phenotype results from the summative effect of both ectopic and endogenous reelin. Though comparable to older WT cortices, the density of PH3+ cells decayed abruptly by E15 in reelin GOF cortices (supplementary material Fig. S1A,B). At E14, the reelin GOF dorsal forebrain was somewhat larger than those of WT and reelin LOF. The comparable WT and reelin LOF dorsal forebrain lengths (not shown) contrast with their mitotic index and this suggests a predominance of symmetric divisions in reelin LOF conditions at some earlier developmental time. We looked at this indirectly by analyzing the ratio between

the number of PH3+ cells and the length of the dorsal forebrain. The lower ratio in reelin LOF when compared with WT and reelin GOF conditions might be consistent with increased lateral expansion of the neuroepithelium. By contrast, the hint of a putative higher ratio in reelin GOF when compared with WT cortices would suggest a shortening of the neuroepithelium (Fig. 1C). We sought to strengthen the validity of this inference by examining the expression of the mammalian partition defective protein 3 (Par3) by E14. Par3 is a key cell polarity determinant that is enriched at the apical membrane of neuroepithelial cells undergoing symmetric divisions (Costa et al., 2008; Siegenthaler et al., 2009). The higher expression of Par3 in reelin LOF conditions compared with WT and GOF conditions (supplementary material Fig. S2) might support a role of reelin in the transition from the lateral expansion to the neurogenic phase. We then measured the output of asymmetric divisions, neurons and IPCs. A bromodeoxyuridine (BrdU)/Ki67 18-hour cell-cycle exit assay was used to examine neuron generation. BrdU-positive (BrdU+) but Ki67-negative (Ki67–) cells were counted as cells that had exited the cell cycle during the 18-hour interval. In the WT cortex, a band of BrdU+/Ki67– cells was apparent above the SVZ. When compared with WT, the number of this type of cells was somewhat lower in *reeler* (Fig. 2A,B) but higher in *ne-reelin* (Fig. 2C,D) cortices. This observation suggests a larger and smaller proportion of proliferative divisions, respectively (a higher percentage of NPCs remain proliferative in the former after the 18-hour period, whereas a lower percentage remain proliferative in the latter), before quitting the cell cycle. Therefore, although a more exhaustive analysis of the proliferation dynamics will be required to demonstrate that the duration of the NPC cell cycle is modulated by reelin, it seems clear that the neurogenic output in the 18-hour period of the BrdU 'pulse' comes under dynamic control of reelin expression. Tbr2 (Eomes – Mouse Genome Informatics) immunostaining was used to examine the IPC population. Dramatic increases in Tbr2 expression were observed in the E14 reelin GOF telencephalon, including the SVZ, the intermediate zone (IZ) and, interestingly, the lower part of the cortical plate (CP). Conversely, a significant decrease was observed in reelin LOF telencephalon at the same gestational age (Fig. 3A) and this was modestly ameliorated by ectopic expression of reelin (not shown). This observation is consistent with accelerated depletion of IPCs. However, abrupt decreases of Tbr2 expression were observed by E15 in reelin GOF cortices (supplementary material Fig. S3). To determine the consequences of the increased production of neurons and IPCs in reelin GOF conditions, we examined the expression of Tbr1, a marker of preplate derivatives and layer VI postmitotic neurons (Bulfone et al., 1995). Significant increases in Tbr1 expression were observed in the E14 reelin GOF cortex when compared with the WT cortex. Consistently, significant decreases in expression levels were observed in the reelin LOF cortex (Fig. 3A,B), which were barely rescued by ectopic expression of reelin (not shown). An abrupt decrease in Tbr1 expression was also observed by E15 in reelin GOF cortices, comparable to Tbr1 expression levels in older (E16-17) WT cortices (supplementary material Fig. S3). We also examined the expression of the transcriptional modulator Ctip2 (Bcl11b – Mouse Genome Informatics) (Arlotta et al., 2005) and found that at E14 layer V neurons were more abundant in reelin GOF cortices than in WT cortices (Fig. 3C). We estimated that this number is doubled in the reelin GOF condition compared with WT based on the number of cells displaying a signal above the 50% threshold of fluorescence. Thus, reelin GOF in the VZ correlates with an early increase in both neuron and IPC production, which would be consistent with an earlier shortening of the cortical neuroepithelium. Opposite phenotypes appear with reelin LOF, some of which tend to normalize with the ectopic expression of reelin. The lack of a stronger effect of the *ne-reelin* transgene on rescuing these *reeler* defects in cortex is not surprising, as the low amount of transgene-derived protein (~20% or less than endogenous reelin expression) (Magdaleno et al., 2002) corresponds to much less than a simple duplication of the gene. We conclude that reelin regulates the early production of neuronal progenitors and neurons.

Reelin enhances the expression of Notch target genes in radial glia

The decrease in neuron output and IPC production in the reelin LOF cortices might be caused by defects in forebrain patterning of the radial glia cell population. Radial glia cells arise early in development from the neuroepithelial cells lining the ventricles, around the time that neurons also start to appear. All neuronal populations in the mouse brain are derived from radial glial cells expressing Blbp (Anthony et al., 2004), a direct target of Notch signaling in radial glia (Anthony et al., 2005). We have previously shown a reduced expression of Blbp in the radial glia of reelin LOF cortices (Hartfuss et al., 2003). We further evaluated Blbp expression during reelin GOF and LOF cortical development together with that of Hes1, a classic Notch signaling sentinel target gene (Jarriault et al., 1995). Dramatic increases in both Blbp and Hes1 expression were observed in E14 reelin GOF when compared with WT cortices. Conversely, significant decreases were observed in reelin LOF cortices of the same gestational age, which were rescued by ectopic expression of reelin (Fig. 4A). To confirm that the LOF and GOF conditions of reelin affect Notch signaling itself, we evaluated the amount of cleaved NICD. A significant increase in the NICD signal was found in *ne-reelin* VZ when compared with the WT VZ at E14 (Fig. 4B). Nevertheless, possibly owing to limitations in the sensibility of the technique, we were unable to show a significant difference between *reeler* and WT (not shown). Consistent with premature maturation of the radial glial cell progenitor population and subsequent early increases in both neuron and IPC production, by E15 abrupt decreases of both Blbp and Hes1 were observed in reelin GOF cortices (supplementary material Fig. S4). We infer that radial glia responds to reelin signaling with enhanced Notch signaling activation, including direct targeting of Hes1 and Blbp.

Expression of Reelin in individual clones of NPCs causes a severe imbalance in the number of differentiating neurons

Reelin binding to the surface of neuroepithelial and radial glial cells (Luque et al., 2003; Luque, 2007) might induce the enhanced expression of Notch target genes and drive radial glia maturation, including morphological changes (Hartfuss et al., 2003; Keilani and Sugaya, 2008) (present results). This would cause a shift from the proliferative symmetric division phase (lateral expansion) to the neurogenic asymmetric division phase (radial expansion) that will result in the generation of neurons. In addition, reelin might be acting via Notch upon the lateral inhibition mechanism in neurogenic radial glial cells. Either or both of these alternatives in turn strongly predict a differential behavior between isochronically isolated neural progenitors expressing reelin versus those devoid of the protein upon differentiation. We tested this prediction using the neurosphere assay (Rietze and Reynolds, 2006) to derive individual clones of neural stem cells isolated from E14 cortices. First, we

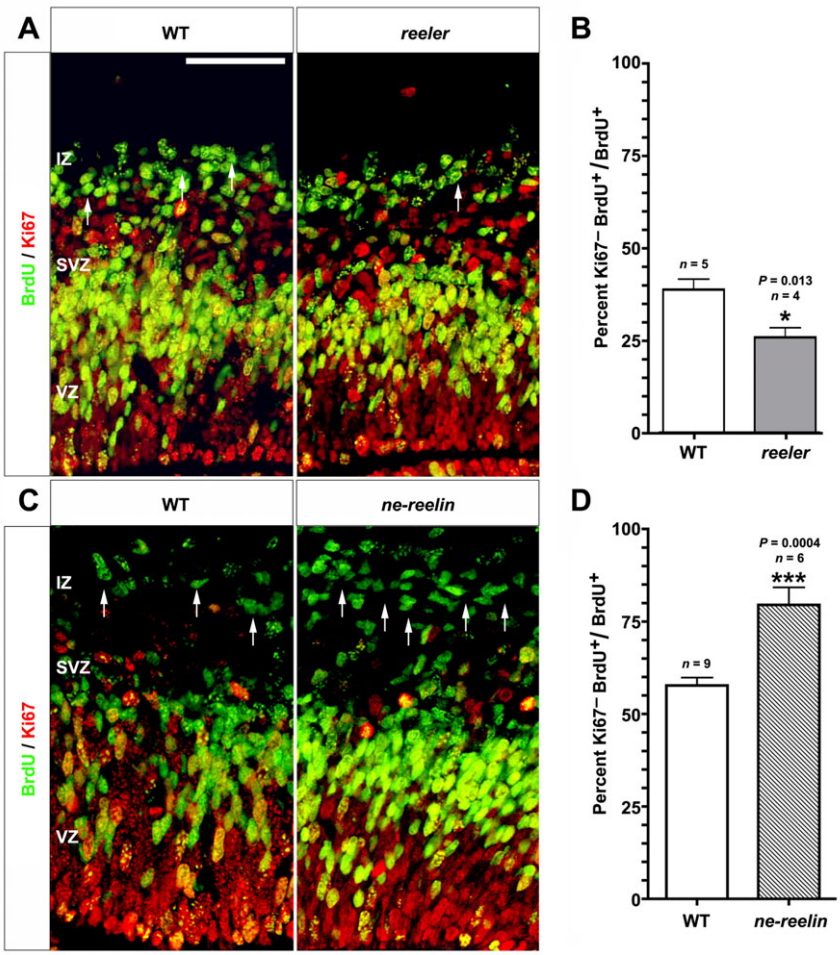

Fig. 2. The output of cortical neurogenic divisions relies on reelin expression. In this cell-cycle exit assay at E13-14, a bromodeoxyuridine (BrdU) pulse was followed 18 hours later by Brdu/Ki67 immunostaining. BrdU-positive but Ki67-negative cells were considered to be cells that had exited the cell cycle. (**A**) Representative staining from BrdU/Ki67 cell-cycle exit assay in WT versus *reeler* cortex. When compared with WT, the number of cells that had exited the cell cycle was somewhat lower in *reeler* mutants. (**B**) Percent of BrdU+/Ki67– cells in WT versus *reeler* cortices. (**C**) Representative staining from BrDU/Ki67 cell-cycle assay in WT versus *ne-reelin* cortex. When compared with WT, the number of cells that had exited the cell cycle was significantly higher in *ne-reelin* cortices. (**D**) Percentage of BrdU+/Ki67– cells in WT versus *ne-reelin* cortices. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone. Arrows indicate the putative migratory front of postmitotic neurons. Scale bar: 50 μm. Error bars represent s.e.m.

confirmed the presence of reelin signaling partners, including ApoER2 and Dab1, in undifferentiated WT neurospheres (supplementary material Fig. S5A), consistent with the reported expression of both proteins in WT cortical radial glia (Luque et al., 2003; Luque, 2007). We were unable to detect significant expression of reelin in WT undifferentiated neurospheres (not shown). We then decided to use more sensitive techniques such as RT-PCR (mRNA) and immunoblotting (protein). For comparative purposes, recombinant reelin was produced as previously described (D'Arcangelo et al., 1997). Extremely low levels of both *reelin* mRNA (Fig. 5A) and reelin protein (supplementary material Fig. S5B) were detected in undifferentiated WT neurosphere lysates, consistent with the reported absence of reelin in cortical radial glial cells (Schiffmann et al., 1997). By contrast, because neural stem cells are highly enriched with nestin, the undifferentiated neurospheres derived from E14 reelin GOF transgenic cortices strongly express reelin, irrespective of the endogenous *reelin* genetic background (Fig. 5A; supplementary material Fig. S5B,C). We found that the expression of reelin in undifferentiated neurospheres did not result in any significant morphological change in neurosphere size (Fig. 5B), proliferation rate or cell death (as assayed with PH3 and cleaved caspase 3, respectively) when compared with WT-derived neurospheres (Fig. 5C). This finding suggests that under non-differentiating conditions, expression of reelin has no significant effect on in vitro progenitor growth. Upon neural stem cell differentiation, we observed a modest but not significant reduction in neuronal production [as assayed by Tuj1 (Tubb3 – Mouse Genome Informatics) expression] from differentiating neurospheres derived from E14 *reeler* cortices. Remarkably, a dramatic and highly significant reduction in neuron numbers was observed from transgenic *ne-reelin* differentiating neurospheres, irrespective of their endogenous *reelin* genetic background. Astroglial numbers in turn (as assayed by Gfap expression) showed a reciprocal trend in all assayed genotypes (Fig. 5D,E).

Reelin expression sensitizes NPCs to lateral inhibition

Notch signaling is pivotal for lateral inhibition, contributing to binary cell fate specification from an initially equipotent population (Heitzler and Simpson, 1991). During lateral inhibition, Notch signals cell autonomously after binding to its ligand (e.g. delta) to maintain a non-neuronal fate (progenitor, epithelial or glial) in cells neighboring a neural-committed cell (which expresses delta). Lateral inhibition mediated by delta-Notch signaling has been recently shown to govern neocortical neurogenesis, segregating equipotent NPCs into two alternative fates: NPCs and neurons (Kawaguchi et al., 2008). To analyze further the function of reelin in neurogenesis and whether reelin acts on NPCs to favor lateral inhibition, we designed a mosaic neurosphere assay. We mixed WT neurospheres derived from green fluorescent protein (GFP) mice and *reeler ne-reelin* (non-GFP) neurospheres in compositions ranging from 15% to 90% of *ne-reelin* NPCs, and induced simultaneous differentiation. Remarkably, the number of

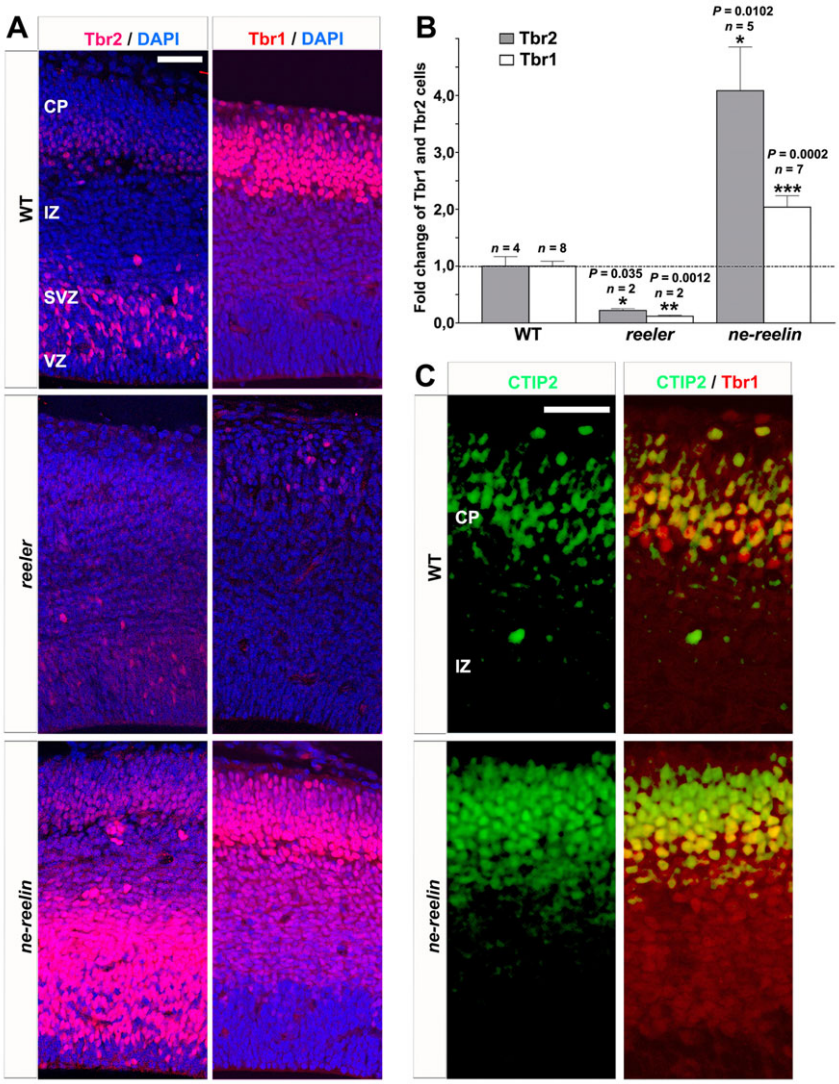

Fig. 3. Reelin regulates the timed production of IPCs and projection neurons. (**A**) Expression of the transcription factor Tbr2 reveals IPCs in the most basal part of the VZ, the SVZ and the most apical part of the IZ. When compared with E14 WT, Tbr2 expression was reduced in *reeler* but dramatically increased in *ne-reelin* cortices. Note that even the lower part of the cortical plate expresses small amounts of Tbr2 in the *ne-reelin* cortex. Expression of the transcription factor Tbr1 reveals layer VI cortical neurons and preplate derivatives. Tbr1 expression was reduced in *reeler* but strongly increased in *ne-reelin* cortices. Note that even cells in the IZ express low levels of Tbr1 in the *ne-reelin* cortex. (**B**) Quantitative analysis of the number of Tbr2+ and Tbr1+ cells. (**C**) Expression of the transcriptional modulator Ctip2 reveals layer V cortical neurons. Based on their immunofluorescence, layer V neurons were approximately twice as abundant in *ne-reelin* cortices than in WT cortices. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate. Nuclei are stained with DAPI (blue). Scale bar: 50 μm. Error bars represent s.e.m.

differentiated WT-GFP versus *ne-reelin* neurons did not correspond to that expected for mosaic composition. Although no significant changes were observed in the number of neurons differentiated from WT NPCs, *reeler ne-reelin* NPCs differentiated far fewer neurons than expected based on their behavior in homogeneous culture conditions (Fig. 6A-C). Therefore, *ne-reelin reeler* NPCs are especially sensitive to the presence of WT cells with a higher proneural capacity. This reflects either, or both, a sensitization to lateral inhibition by neural-committed WT NPCs, or a sensitization to reelin produced by some WT differentiating neurons. To distinguish between these alternatives, we stained the differentiating mosaic compositions for reelin expression. We found a similar very low proportion of WT reelin-expressing neurons in all mosaic compositions (not shown), suggesting that the enhanced sensitivity of *ne-reelin reeler* NPCs in avoiding neuronal differentiation relates to a higher susceptibility for lateral inhibition signals. These results strongly suggest that reelin-primed NPCs have an enhanced Notch function that prevents them from entering the neuronal differentiation pathway. Likewise, no changes were observed in the number of astrocytes differentiated from WT NPCs. However, at high concentrations (75-90%) of WT NPCs (those with a higher number of neurons and more faithfully mimicking the shift from neurogenesis to gliogenesis at later developmental stages) *reeler ne-reelin* NPCs significantly differentiated more astrocytes than expected based on their behavior in homogeneous culture conditions (Fig. 6B,C). Together, these results strongly support the notion that reelin modulates lateral inhibitory signals between NPCs, possibly by enhancing Notch function in NPCs, thereby contributing to the determination of cell fate in the developing neocortex.

DISCUSSION

The present study demonstrates that reelin functions prior to its widely known physiological role in neurons, i.e. the regulation of neuronal migration and positioning in the developing neocortex. Neocortical NPCs respond to reelin expression with enhanced Notch activation, as revealed by the expression of NICD, Hes1 and Blbp in radial glia. This, in turn, regulates both neuron and IPC production. Consistent with reelin acting upstream of Notch signaling in NPCs, the expression of reelin in isolated progenitors causes a severe reduction in the number of neurons that differentiate. Moreover, in mosaic cell cultures, the reelin-expressing progenitor cells respond to the higher pro-neuronal capacity of WT cells by further decreasing neuronal differentiation,

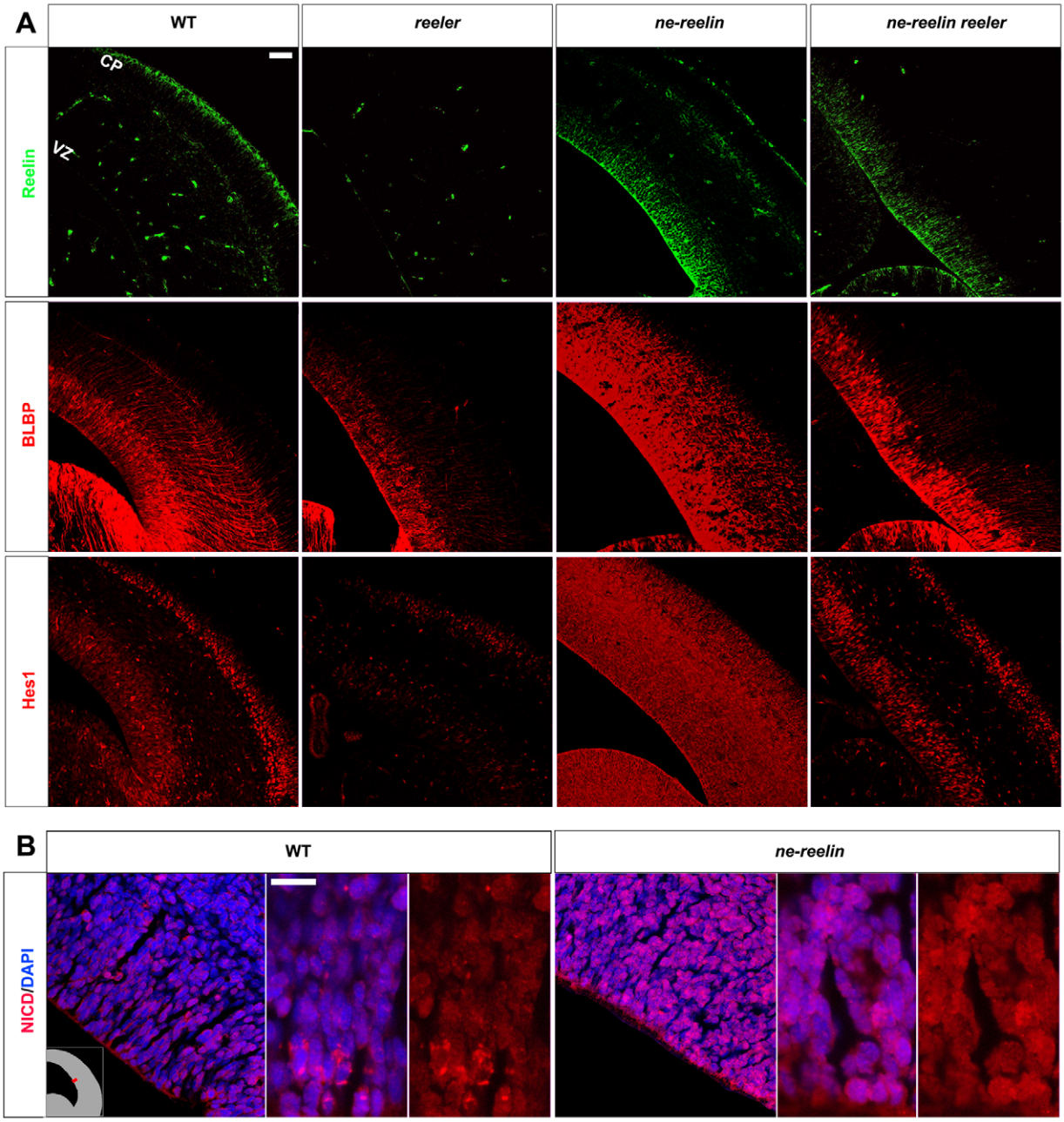

Fig. 4. Reelin enhances the concentration of NICD and the expression of the Notch target genes Blbp and Hes1 in NPCs. (**A**) Reduced expression of both Blbp and Hes1 is noticeable in *reeler* cortices by E14 when compared with WT. By contrast, a conspicuous increase of both Blbp and Hes1 appears in *ne-reelin* cortices. Ectopic expression of reelin rescues the expression of these Notch-target genes in compound *reeler ne-reelin* cortices. (**B**) The *ne-reelin* transgene enhances the expression of cleaved NICD in the ventricular zone (schematic indicates region shown). VZ, ventricular zone; CP, cortical plate. Scale bars: 50 μm in A; 25 μm in B.

presumably because they are more sensitive to lateral inhibition than the WT progenitors. These results strongly suggest that reelin enhances Notch signaling within progenitor cells.

The classical *reeler* mutant mouse 'has contributed both insight and consternation' to the topic of proliferative versus post-proliferative determinants of cortical architectonic patterns (Caviness et al., 2008). Despite consistent anomalies in cell position, the attributes of cell class were found to be preserved in terms of the many aspects of cell appearance and the specificity of connections (Caviness and Rakic, 1978). The present results indicate that cortical NPCs respond to reelin expression before postmitotic newborn neurons. Reelin GOF under a *nestin* driver expressed by NPCs correlates with an early increase in both neuron and IPC production. Reciprocal phenotypes correlate with reelin LOF, consistent with previous observations (Polleux et al., 1998), indicating that changes in the rate of neuron production are attributable to changes in the proportion of neurogenic divisions. The fact that reelin GOF does not produce an obvious migratory phenotype (Magdaleno et al., 2002) precludes the possibility that this effect is secondarily or fundamentally controlled by the

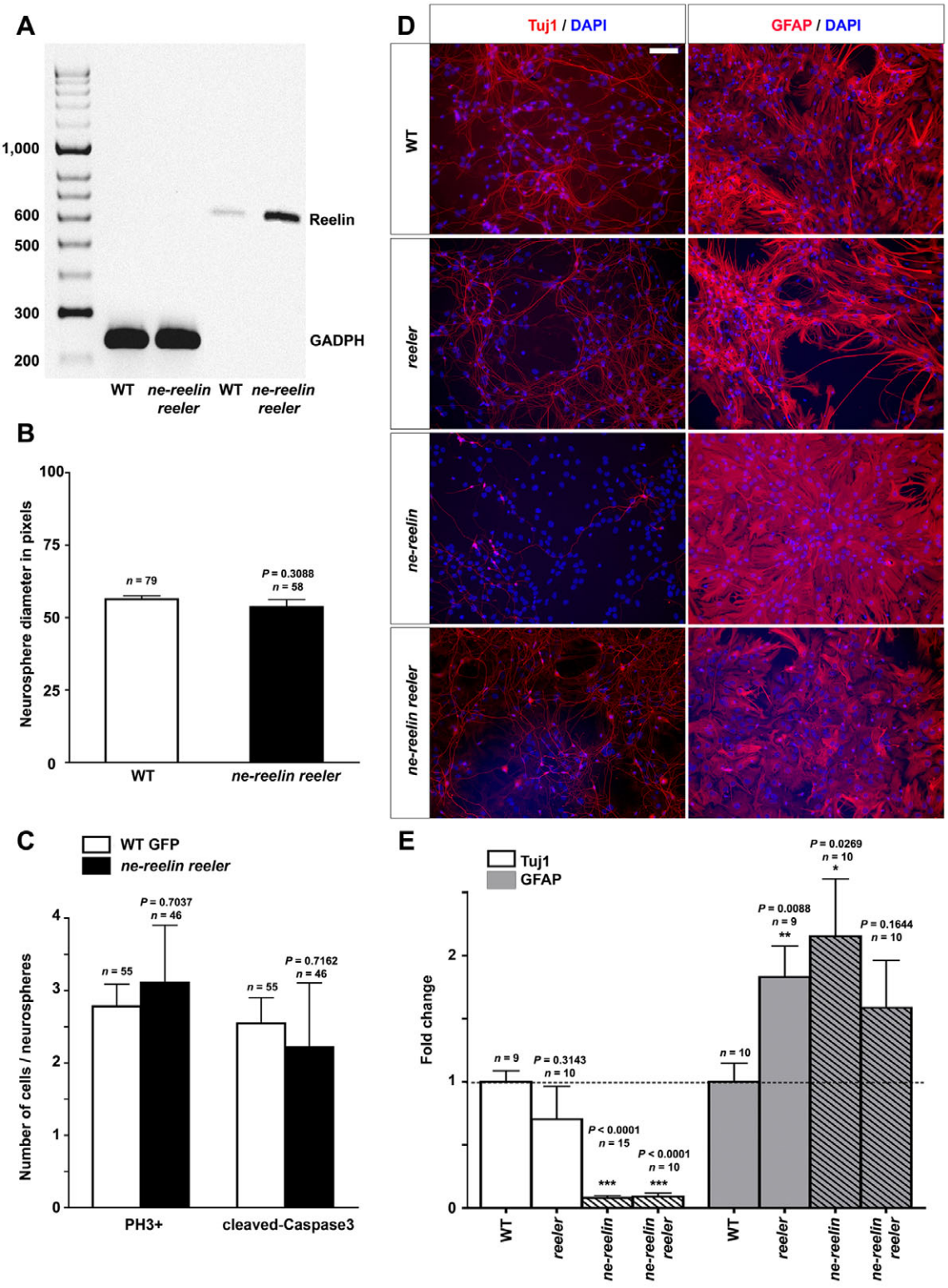

Fig. 5. The expression of reelin in individual clones of NPCs strongly reduces neuronal differentiation. (**A**) Extremely low levels of *reelin* mRNA are detectable in lysates of undifferentiated neurospheres generated from E14 dorsal forebrain, consistent with the reported absence of reelin in NPCs. By contrast, *ne-reelin* neurospheres, even those with an endogenous *reeler* background, strongly express *reelin*. (**B**) Expression of the *ne-reelin* transgene does not modify the size of undifferentiated neurospheres. (**C**) Under non-differentiating conditions, expression of the *ne-reelin* transgene does not result in any significant change in proliferation rate or cell death, as assayed with PH3 and cleaved caspase 3, respectively. (**D**) Representative fields showing young neurons (Tuj1+) and astrocytes (Gfap+) in differentiating neurospheres. Scale bar: 50 μm. (**E**) Quantification of neurons and astrocytes in differentiating neurospheres. A dramatic reduction in neuron number is observed in transgenic *ne-reelin* neurospheres, irrespective of their genetic background for endogenous reelin. Astroglial numbers showed a reciprocal trend in all assayed genotypes. Error bars represent s.e.m.

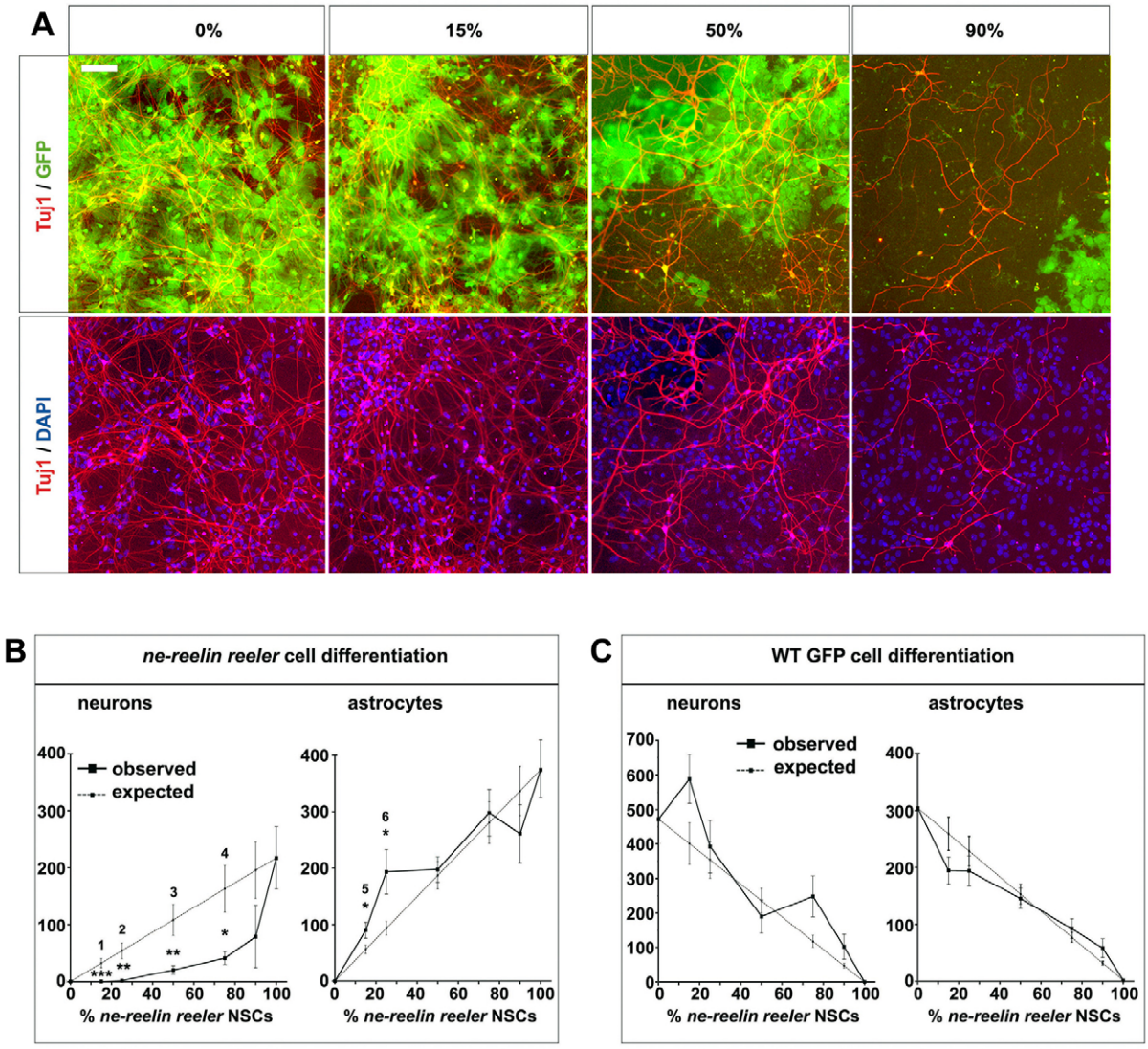

Fig. 6. Reelin acts on NPCs during lateral inhibition. (**A**) Mosaic neurosphere differentiation assay. Representative fields depicting young neurons (Tuj1+) in a mix of WT neurospheres derived from green fluorescent protein (GFP) mice and *reeler ne-reelin* (non-GFP) mice in compositions ranging from 15% to 90% of *ne-reelin* NPCs. Top and bottom images show the same field in the red-green and red-blue channels, respectively. (**B**) Quantification of *reeler ne-reelin* neurons and astrocytes differentiated in mosaic conditions. Note that *reeler ne-reelin* NPCs differentiate far fewer neurons than expected based on their behavior in homogeneous (non-mosaic) culture conditions. The strength of this inhibitory effect clearly correlates with the proportion of WT NPCs in the mosaic, suggesting that reelin expression sensitizes NPCs to lateral inhibition. At high concentrations of WT NPCs (interestingly, those that more faithfully mimic the developmental shift from neurogenesis to gliogenesis), *reeler ne-reelin* NPCs differentiate more astrocytes than expected based on their behavior in homogeneous cultures. 1, P=0.0010, n=10; 2, P=0.0038, n=10; 3, P=0.0061, n=10; 4, P=0.0109, n=10; 5, P=0.0484, n=10; 6, P=0.0170, n=10. (**C**) Quantification of WT neurons and astrocytes differentiated in mosaic conditions. No significant changes are observed as WT NPCs differentiate the expected numbers of neurons and astrocytes based on their behavior in homogeneous culture conditions. Error bars represent s.e.m.

postmigratory compartment. As cells isolated from the cortical VZ are competent to respond to exogenous reelin by Dab1 phosphorylation (Magdaleno et al., 2002), and the reelin receptor machinery is expressed in cortical NPCs (Luque et al., 2003), all modifications of events in the VZ/SVZ are most likely the consequence of reelin signaling in NPCs. Thus, the reciprocally modified proliferative behavior of the pool of NPCs in reelin GOF and LOF conditions provides evidence for a direct reelin influence on the neocortical primordium. A single study has demonstrated lower rates of differentiative divisions in early stages of corticogenesis in *reeler* mice, followed by increased rates in the final stages of corticogenesis (Polleux et al., 1998). We found a comparable WT and reelin LOF dorsal forebrain length at E14. This contrasts with the lower mitotic index found in reelin LOF cortices, suggesting a predominance of symmetric divisions or a delayed entry into the neurogenic phase (enlargement of the neuroepithelium) in reelin LOF conditions early during cortical development. Reciprocal levels of Par3 expression under reelin LOF (higher) and GOF conditions (lower) also support this notion. Accordingly, the low cell cycle quitting fraction (neurogenic output) observed in reelin LOF conditions suggests that precursors might remain longer on proliferative divisions or have a longer cell cycle. Reciprocally, the higher cell cycle quitting fraction observed in E14 reelin GOF conditions suggests that precursors generating

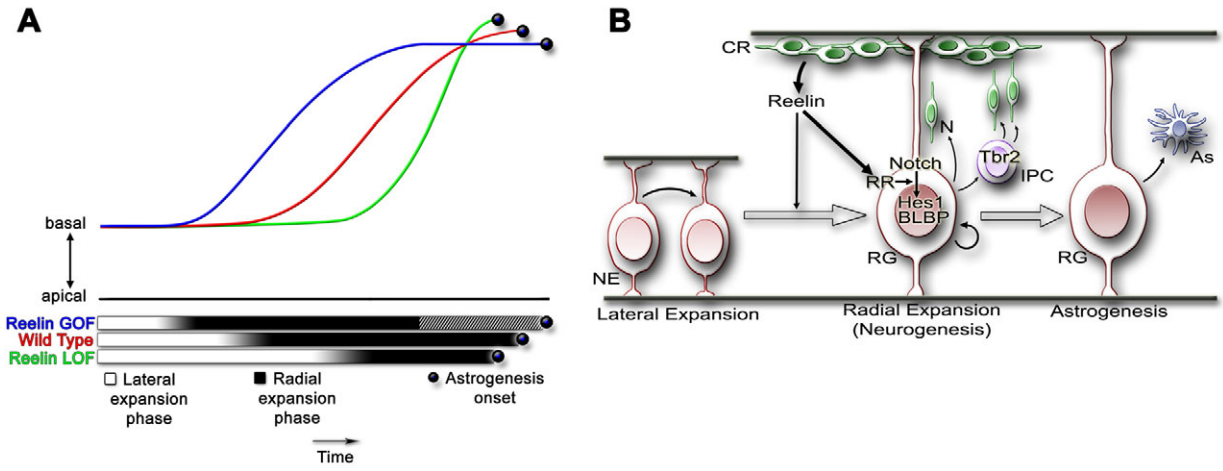

Fig. 7. Proposed model in which reelin upstream of Notch regulates the temporal specification of NPCs and neuronal differentiation. During neocortical development, NPCs sequentially pass through phases of lateral expansion, radial expansion (neurogenesis) and gliogenesis (astrogenesis). During the expansion phase, NPCs, referred to as neuroepithelial cells (NE), expand their population by symmetric cell divisions. However, around the onset of the neurogenic phase, NPCs mature into radial glia (RG), which simultaneously self-renew and generate neurons (N) either directly or indirectly via intermediate progenitor cells (IPCs) through asymmetric cell divisions. (**A**) Inferred dynamics of cortical neurogenesis in reelin loss-of-function (LOF) and gain-of-function (GOF) conditions compared with WT. Reelin might cooperate first with Notch in the maturation of NE to RG, thus regulating the timed onset of (and maintenance of) the neurogenic phase. Reelin LOF might delay and reelin GOF might advance the onset of neurogenesis. Reelin regulates the temporal specification of cortical NPCs (regardless of later compensatory or secondary refinements, such as trophic effects or apoptosis, which might also affect the total number of neurons). By contrast, reelin LOF might advance and reelin GOF might delay the onset of astrogenesis. Regardless of the completion of their neurogenic program, reelin GOF might retain NPCs in 'neurogenic standby' (striped bar) precluding the timed onset of astrogenesis. (**B**) Reelin enhances Notch function during neurogenesis. The emergence of Cajal-Retzius cells (CRs) expressing and secreting reelin roughly coincides with the onset of neurogenesis. Reelin via the reelin receptor machinery (RR) expressed by RG cooperates with Notch, also expressed by RG, to regulate the expression of target genes, such as Blbp and Hes1. This, in turn, contributes to the maintenance of the progenitor pool, thereby setting directly and indirectly (via IPCs expressing Tbr2) the pace of neocortical neurogenesis. Likewise, the disappearance of CRs roughly coincides with the generation of astrocytes (As), suggesting that reelin functions also in the shift of NPCs from the neurogenic phase to astrogliogenesis.

neurons and IPCs might undergo either a relatively shorter period of proliferative divisions or a faster cell cycle before quitting the cell cycle. In any event, it seems clear that the regulation of neocortical neurogenesis comes under dynamic control of reelin expression.

reeler mutants show a significant reduction in VZ cells with long radial processes in the cortex, correlating with a decrease in the expression of Blbp. In vitro, reelin addition increases both Blbp expression and process extension of cortical radial glia via Dab1 (Hartfuss et al., 2003). Here, we show that the effect of reelin on radial glia occurs via Notch activation. Whereas reelin GOF increases the amount of cleaved NICD, reelin LOF decreases Notch signaling (as revealed by the expression of both Hes1 and Blbp). Moreover, the ectopic expression of reelin in the *reeler* background rescued Hes1 and Blbp expression in radial glia. Consistent with this, recent work in a human progenitor cell line showed that reelin treatment led to elevated NICD levels and enhanced radial glial characteristics (Keilani and Sugaya, 2008). Furthermore, in the postnatal hippocampus reelin enhances Notch signaling contributing to the formation of the radial glial scaffold (Sibbe et al., 2009), and decreased neurogenesis is found in *reeler* mutants (Zhao et al., 2007). Reelin signaling is thought to inhibit NICD degradation via Dab1 (Hashimoto-Torii et al., 2008), making extremely unlikely that a reelin-null condition would create (or produce a phenocopy of) a Notch-null condition (some type of mild hypomorphic Notch condition at most). Indeed, we expect the Notch-dependent processes of development to occur in the absence of reelin, although their progression would be delayed or altered. Notch signaling which inhibits proneural basic helix-loop-helix transcription factors and, hence, neuronal differentiation also appears to advance initiation of the neurogenic phase (Miyata et al., 2010). The emergence of delta-like 1, a Notch ligand responsible for the activation of Notch signaling in the developing neocortex (Kawaguchi et al., 2008) roughly coincides with the onset of neurogenesis (Hatakeyama et al., 2004), and the forced activation or inactivation of the Notch pathway respectively increased and decreased expression of radial glia markers such as RC2 (Ifaprc2 - Mouse Genome Informatics) and Blbp (Gaiano et al., 2000; Anthony et al., 2005), suggesting that Notch signaling contributes to the mechanics of the switch from lateral expansion (symmetric) to neurogenic (asymmetric) divisions. Moreover, molecules involved in or susceptible to cross-talk with Notch signaling, have been recently identified as being central in this transition (Schmid et al., 2003; Yoon et al., 2004; Sahara and O'Leary, 2009; Siegenthaler et al., 2009). The expression of reelin, secreted by Cajal-Retzius cells in the marginal zone, also roughly coincides with the onset of neurogenesis in the cortical plate. The close proximity to the radial glial endfeet enables a potent, short-range signal that does not need to influence directly IPC proliferation events. A further possibility is that reelin signaling on NPCs comes from the cerebrospinal fluid (Sáez-Valero et al., 2003; Ignatova et al., 2004). In any event, reelin might cooperate with Notch signaling to advance the transit from the lateral expansion phase to the neurogenic phase by regulating the biochemical maturation from neuropithelial progenitors to radial glia.

Our in vitro studies confirm that reelin acts upstream of Notch signaling in its most classical function, i.e. to inhibit neuronal differentiation and maintain progenitor/glial fate. Indeed, the transgenic expression of reelin in isolated progenitors causes a dramatic reduction in the number of neurons that differentiate. Because the situation in vitro is not exactly comparable with in vivo embryonic development, these results complement our in vivo study. In vitro, E14-isolated NSCs are artificially maintained as precursors (avoiding neurogenesis), then all of them are compelled to differentiate when FGF and EGF are removed from the culture medium. Thus, NSCs are forced to take a differentiating alternative between neuron (low Notch function) and non-neuron (high Notch function) during the lateral inhibition process. As the precursor radial glia fate is not a differentiation alternative for NSC after removal of growth factors, NSCs that differentiate as astrocytes in vitro might represent cells that would be maintained as radial glia in vivo. In fact, in vitro neurosphere culture constitutes a developmental snapshot that should reflect the diverse developmental history and degree of maturation by E14 of the isolated progenitor cells. Because neurosphere cultures select only NPCs to survive and the *nestin* promoter driving reelin expression functions in NPCs but not in differentiating neurons, these results suggest that reelin expression affects NPCs just before or very early during the neurogenesis process. Together with previously published results (Hartfuss et al., 2003; Anthony et al., 2005; Keilani and Sugaya, 2008; Gaiano, 2008), they are consistent with the notion of reelin acting upstream of Notch signaling in NPCs to regulate cell fate before differentiation. Our results also emphasize the need for combined GOF and LOF approaches, as conflicting results have been obtained in two reports upon differentiation of *reeler*-derived neurospheres (Kwon et al., 2009; Massalini et al., 2009). The recent proposal that Dab1 suppresses astroglial differentiation, albeit independently of reelin (Kwon et al., 2009), is seemingly incompatible with previously published results showing how the lack of reelin accelerates the transition of radial glial cells to astrocytes (Hunter-Schaedle, 1997) and our own observations showing reciprocal behavior in the reelin GOF condition (supplementary material Fig. S6). The Delta-Notch pathway contributes to the lateral signaling between NPCs that segregates equipotent mouse neocortical NPCs into two alternative fates: NPCs and neurons (Kawaguchi et al., 2008). In our mosaic cell cultures, the reelin-expressing progenitor cells respond through a lateral inhibition mechanism to the higher proneural capacity of WT cells by further decreasing their neuronal differentiation with a concomitant increase in astroglial differentiation. Taken together, these results strongly support a role for reelin in enhancing Notch function during neocortical neurogenesis. The question arises of why it would be important to amplify Notch signaling. The slow or delayed neurogenesis during early neocortical development in *reeler* mutants might reflect the fact that that the promotion of progenitor proliferation is not robust enough. An amplifier of the Notch-Delta signaling, such as reelin, that could add proliferative-promoting capacity to low Delta signals (improving the robustness of progenitor proliferation) would certainly be an advantage in a system that has to grow exponentially. Thus, reelin might enhance neurogenesis in the same way as an upward compressor of the Notch signaling dynamic range in NPCs. The schematic in Fig. 7 summarizes our results and proposal.

Here, we have provided experimental evidence supporting the hypothesis that reelin acts as an operational amplifier of Notch signaling in neocortical NPCs. At first, reelin appears to advance the transit from the lateral expansion phase to the neurogenic phase by regulating the biochemical maturation from neuroepithelial progenitors to radial glia progenitors. Reelin then enhances the precise timing of neurogenesis by promoting radial glial fate while inhibiting neuronal differentiation. Confirming and extending previous work, we propose now that reelin regulates the temporal specification of NPCs and thus couples neocortical neurogenesis to neuronal migration, and probably also to astrogliogenesis. Further elucidation of the underlying molecular mechanism of reelin-Notch signaling action should reveal novel concepts and patterns that provide a clear link between their seemingly distinct proliferative and post-proliferative functions. In the meantime, it seems increasingly evident that molecules regulating NPC proliferation, neurogenesis and neuronal fate also regulate neuronal migration.

Acknowledgements
We thank Drs S. Magdaleno and T. Curran for *ne-reelin* mice; Dr O. Marín for GFP-mice; Drs A. Goffinet and R. Hevner for reelin and Tbr2 antibodies; Dr M. Giménez y Ribotta, Dr A. Fairén, Dr E. Soriano and G. Expósito for reactives and expert technical advice.

Funding
This work was supported in part by grants from the Generalitat Valenciana-Prometeo [2008-134 to L.G.-A.]; the Spanish Ministry of Science and Innovation [SAF2004-07685 to J.M.L.]; and the Fundación Médica Mútua Madrileña [to J.M.L.]. J.L. holds a Junta para la Ampliación de Estudios (JAE) Programme/Spanish National Research Council (CSIC) pre-doctoral fellowship.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.063776/-/DC1

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