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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Mcidas and GemC1 are key regulators for the generation of multiciliated ependymal cells in the adult neurogenic niche

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ABSTRACT

Multiciliated cells are abundant in the epithelial surface of different tissues, including cells lining the walls of the lateral ventricles in the brain and the airway epithelium. Their main role is to control fluid flow and defects in their differentiation are implicated in many human disorders, such as hydrocephalus, accompanied by defects in adult neurogenesis and mucociliary disorder in the airway system. Here we show that Mcidas, which is mutated in human mucociliary clearance disorder, and GemC1 (Gmnc or Lynkeas), previously implicated in cell cycle progression, are key regulators of multiciliated ependymal cell generation in the mouse brain. Overexpression and knockdown experiments show that Mcidas and GemC1 are sufficient and necessary for cell fate commitment and differentiation of radial glial cells to multiciliated ependymal cells. Furthermore, we show that GemC1 and Mcidas operate in hierarchical order, upstream of Foxj1 and c-Myb transcription factors, which are known regulators of ependymal cell generation, and that Notch signaling inhibits GemC1 and Mcidas function. Our results suggest that Mcidas and GemC1 are key players in the generation of multiciliated ependymal cells of the adult neurogenic niche.

KEY WORDS: Mcidas, Geminin coiled-coil domain containing, GemC1, Lynkeas, Gmnc, Multicilin, Radial glia, Multiciliated cell, Ependymal cell, Cell fate commitment

INTRODUCTION

The adult mouse brain largely consists of terminally differentiated neural and glial cells, which are generated from neural progenitor cell populations during development. Neuroepithelial cells are the initial neural stem cells (NSCs) located in the ventricular zone (VZ), from which radial glial cells (RGCs) arise, shortly after the initiation of neurogenesis. RGCs constitute the main neural progenitor population in the developing cerebral cortex and generate the intermediate progenitors, which lose their apical-basal polarity and reside in the subventricular zone (SVZ) (Borrell and Reillo, 2012; Gotz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). Recently, additional neuronal progenitor cell populations have been identified, namely short neuronal precursors located in the VZ (Gal et al., 2006; Hartfuss et al., 2001; Stancik et al., 2010), as well as basal RGCs, which share radial glia characteristics although they reside in the SVZ (Borrell and Gotz, 2014; Pilz et al., 2013; Shitamukai et al., 2011; Wang et al., 2011).

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RGCs comprise a heterogeneous population (Pinto and Gotz, 2007) during cortical development. Initially, they carry out mostly self-renewing divisions in order to expand their number, whereas at later stages they undergo differentiating divisions to generate neurons or glial cells (Haubensak et al., 2004; Malatesta et al., 2003, 2000; Noctor et al., 2004, 2007). A subpopulation of RGCs, which has not yet differentiated at the end of embryogenesis, generates the adult neural stem cells (aNSCs) and the multiciliated ependymal cells of the adult subependymal zone (SEZ) niche (Merkle et al., 2004; Spassky et al., 2005; Young et al., 2007).

RGCs become committed to the ependymal cell lineage around embryonic day (E) 16; however, ependymal cell differentiation is initiated around birth and is completed within the first two postnatal weeks (Spassky et al., 2005). It has been shown that Foxj1 and c-Myb regulate the differentiation of RGCs towards the ependymal cell lineage and maintain their identity (Jacquet et al., 2009; Malaterre et al., 2008), and that Six3 expression is crucial for the loss of radial glial characteristics of the differentiating ependymal cells (Lavado and Oliver, 2011). Ependymal cells contain in their apical surface multiple motile cilia and reside in a single cell layer facing the lateral ventricles (Doetsch et al., 1997), forming rosette-like structures surrounding a type B1 NSC (Mirzadeh et al., 2008). In the adult SEZ niche, ependymal cells control the proliferation and differentiation of aNSCs by receiving secreted molecules from the cerebrospinal fluid through their motile cilia. Additionally, they facilitate cerebrospinal fluid flow, which is required for the migration of neuroblasts towards the olfactory bulbs (Del Bigio, 1995, 2010; Lim et al., 2000; Sawamoto et al., 2006). Beyond their role in regulating aNSC fate decisions, ependymal cells can enter the cell cycle and give rise to neuroblasts and astrocytes after stroke, exhibiting a functional role in adult neurogenesis and regeneration upon injury (Carlen et al., 2009).

We have previously shown that geminin regulates self-renewal and differentiation decisions of cortical progenitor cells (Spella et al., 2007, 2011). Two novel proteins, Mcidas (also known as Idas) and GemC1 (also known as Gmnc and GemC1/Lynkeas), that share homology with geminin have been recently characterized (Balestrini et al., 2010; Pefani et al., 2011). Mcidas was initially characterized by its ability to regulate DNA replication and cell cycle progression through its interaction with geminin (Caillat et al., 2013; Pefani et al., 2011), and its mRNA and protein expression are restricted to the choroid plexus and cortical hem epithelium during early brain development (Pefani et al., 2011). Later studies proposed that Mcidas controls multiciliated cell differentiation in *Xenopus* skin and kidney as well as in the human and mouse airway epithelium (Boon et al., 2014; Stubbs et al., 2012). The third member of the geminin superfamily, GemC1, was shown in *Xenopus* to mediate preinitiation complex formation through interactions with TopBP1 and Cdc45 recruitment (Balestrini et al., 2010), although no role in cellular differentiation has been described.

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Here we provide evidence in mouse that Mcidas and GemC1 regulate fate decisions of RGCs. More specifically, we show that Mcidas and GemC1 are expressed in RGCs at the end of embryogenesis and that their expression persists during early stages of RGC differentiation towards the multiciliated ependymal cell lineage. Ectopic expression of Mcidas or GemC1 in RGCs promotes the loss of their apical characteristics and differentiates them into multiciliated ependymal cells, whereas downregulation of Mcidas or GemC1 expression reduces the ability to differentiate towards this lineage. Furthermore, we propose that GemC1 and Mcidas act upstream of c-Myb and Foxj1 in order to induce the generation of multiciliated ependymal cells, and that Mcidas and GemC1 activity is inhibited by the Notch pathway.

RESULTS

Mcidas and GemC1 are expressed in periventricular zone progenitor cells during the initial stages of ependymal cell generation

We have initially characterized Mcidas and GemC1 spatial and temporal expression patterns in neural progenitor populations of the developing mouse brain, as RGCs become committed and differentiate towards different neuronal lineages. *In situ* hybridization (ISH) was performed on sections of E16.5 and E18.5 mouse embryos using probes specific for *Mcidas* and *GemC1* mRNA (Fig. 1A-D′). *Mcidas* and *GemC1* mRNAs were detected in the developing brain in a population of cells lying next to the lateral ventricles, in the septal area of ventral telencephalon at E16.5 (Fig. 1A,A′,C,C′). *GemC1* mRNA expression in this region is decreased at E18.5 (Fig. 1B,B′) and is not detected at postnatal day (P) 0 and P7 (Fig. S1A-B″). *Mcidas* mRNA is detected in a larger population at E18.5 compared with E16.5, and its expression is maintained until postnatal stages (Fig. 1D,D′, Fig. S1C-D″). A small number of Mcidas$^+$ cells is also present in caudal regions of the dorsal telencephalon at E18.5 and it is expanded in the caudal-rostral axis and maintained until P7 (Fig. S1D′; data not shown).

To characterize the cells in which Mcidas is expressed, we compared its expression with that of *Foxj1*, a gene expressed in RGCs committed to the ependymal cell lineage (Jacquet et al., 2009). Mcidas protein expression is detected in the periventricular cell layer of E16.5 embryos, where about half of the Mcidas$^+$ cells colocalize with Foxj1$^+$ cells (Fig. 1E-E″). Two days later (E18.5), the vast majority of Mcidas$^+$ cells are double labeled with Foxj1, while expression of Foxj1 is detected in a larger population than that of Mcidas$^+$ cells (Fig. 1F-F″). Approximately 70% of Mcidas$^+$ cells coexpress S100β, a marker of differentiated ependymal cells (Fig. 1G-G″), but not Tuj1 (Tubb3), a neuronal marker (Fig. 1H-H″). We also performed wholemount immunofluorescence of the wall of the lateral ventricles at P0, P6, P15 and in adult mice using anti-Mcidas and anti-Foxj1 or anti-S100β antibodies. Foxj1$^+$ cells were identified from the first postnatal days until adulthood in the wall of the lateral ventricles, both in the anterior-dorsal and in the posterior-ventral part. Mcidas$^+$ cells were detected only until the first postnatal week in the anterior-dorsal part of the lateral wall (Fig. S2A-A″; data not shown). In this area, Mcidas$^+$ cells at P0 and P6 comprise 65.6% and 52.7%, respectively, of the Foxj1$^+$ cells (Fig. S2C), while all Mcidas$^+$ cells coexpress Foxj1 (data not shown). During the first postnatal week a small number of S100β$^+$ cells present also express Mcidas (Fig. S2D-D″; data not shown). The percentage of Mcidas$^+$ S100β$^+$ cells corresponds to 40.6% at P0 and 52.7% at P6 of the total Mcidas$^+$ cell population (Fig. S2F; data not shown). In the posterior-ventral part of the lateral ventricle wall of P15 and adult mice a small number of Mcidas$^+$ cells is detected. This population represents 45.5% and 53.4% of Foxj1$^+$ cells and 25% and 5.6% of S100β$^+$ cells of P15 and adult mice, respectively (Fig. S2G-J; data not shown). In all other areas, from P15 until adulthood, Mcidas expression is undetected, whereas Foxj1 and S100β are expressed in the whole periventricular cell layer, where fully differentiated ependymal cells are located (Fig. S2B-B″,E-E″; data not shown).

Our findings suggest that the restricted expression of Mcidas and GemC1 correlates with the region where RGCs are located, while the timing of expression coincides with specification events leading to the generation of multiciliated ependymal cells and aNSCs of the SEZ (Merkle et al., 2004; Spassky et al., 2005). Interestingly, Mcidas and GemC1 expression precedes the expression of Foxj1, a known marker of RGCs committed to the ependymal cell lineage (Jacquet et al., 2009), suggesting that their expression might determine early stages of RGC commitment towards the ependymal cell fate.

Ectopic overexpression of Mcidas and GemC1 in embryonic RGCs results in premature ependymal cell differentiation

To elucidate the *in vivo* role of Mcidas and GemC1 in RGC fate decisions we performed overexpression experiments using *in utero* electroporation (IUE) in the developing cortex of E14.5 mouse embryos. Plasmids expressing internal ribosome entry site (IRES)-GFP (referred to as GFP) alone or in conjunction with Mcidas or GemC1 were injected into the telencephalic ventricles of E14.5 embryos. Ectopic overexpression of Mcidas and GemC1 was confirmed by ISH and immunofluorescence, and electroporated cells were monitored by GFP expression. Mice were analyzed 5 days after electroporation (Fig. 2A). When the control plasmid was overexpressed, GFP-expressing cells had migrated away from the VZ and colonized the cortex, where they differentiated into neurons (Fig. 2B; data not shown). However, upon Mcidas and GemC1 overexpression, GFP$^+$ cells resided next to the lateral ventricles (Fig. 2C,D). To quantify the localization of GFP$^+$ cells, the developing cortex was subdivided into five equal bins, with bin A corresponding to the VZ of the cortex, and bin E to the pial surface. GFP$^+$ cells were scored separately in each bin (Fig. S3). The percentage of GFP$^+$ cells in the VZ (bin A) was increased from a mean of 7.2% in the control to 75.2% and 80.4% following Mcidas and GemC1 overexpression, respectively (Fig. 2E). These data indicate that overexpression of either Mcidas or GemC1 entraps cells in the VZ of the cortex.

In order to characterize these cells further, we performed immunofluorescence using anti-Ki67 (Fig. 2F-H″) and anti-BLBP (Fabp7) or anti-Pax6 antibodies (Fig. 2I-K″, Fig. S4). Despite remaining at the ventricle, Mcidas and GemC1 overexpression cells did not proliferate and lost their radial glial character, as cells double positive for GFP and Ki67 or BLBP could not be identified. Only a small fraction of GFP$^+$ cells that overexpressed Mcidas or GemC1 colocalized with the neural progenitor marker Pax6 (24.7% and 13%, respectively; Fig. S4C-E). By contrast, when only GFP was expressed few of the GFP$^+$ cells were detected in bin A and, of these, 50% coexpressed Pax6 (Fig. S4B,B′,E). Moreover, the vast majority of the GFP$^+$ cells following Mcidas and GemC1 overexpression colocalized with S100β, which labels ependymal cells (Fig. 3C-D″). By contrast, upon GFP overexpression S100β$^+$ cells were not detected (Fig. 3B,B′), as under physiological conditions ependymal cell differentiation has not yet been initiated at this developmental stage (Fig. 3A).

Fully functional ependymal cells carry in their apical surface clusters of basal bodies and motile cilia, which distinguish them from other cells located close to the lateral ventricles. Thus, ependymal cells exhibit high levels of γ-tubulin and acetylated

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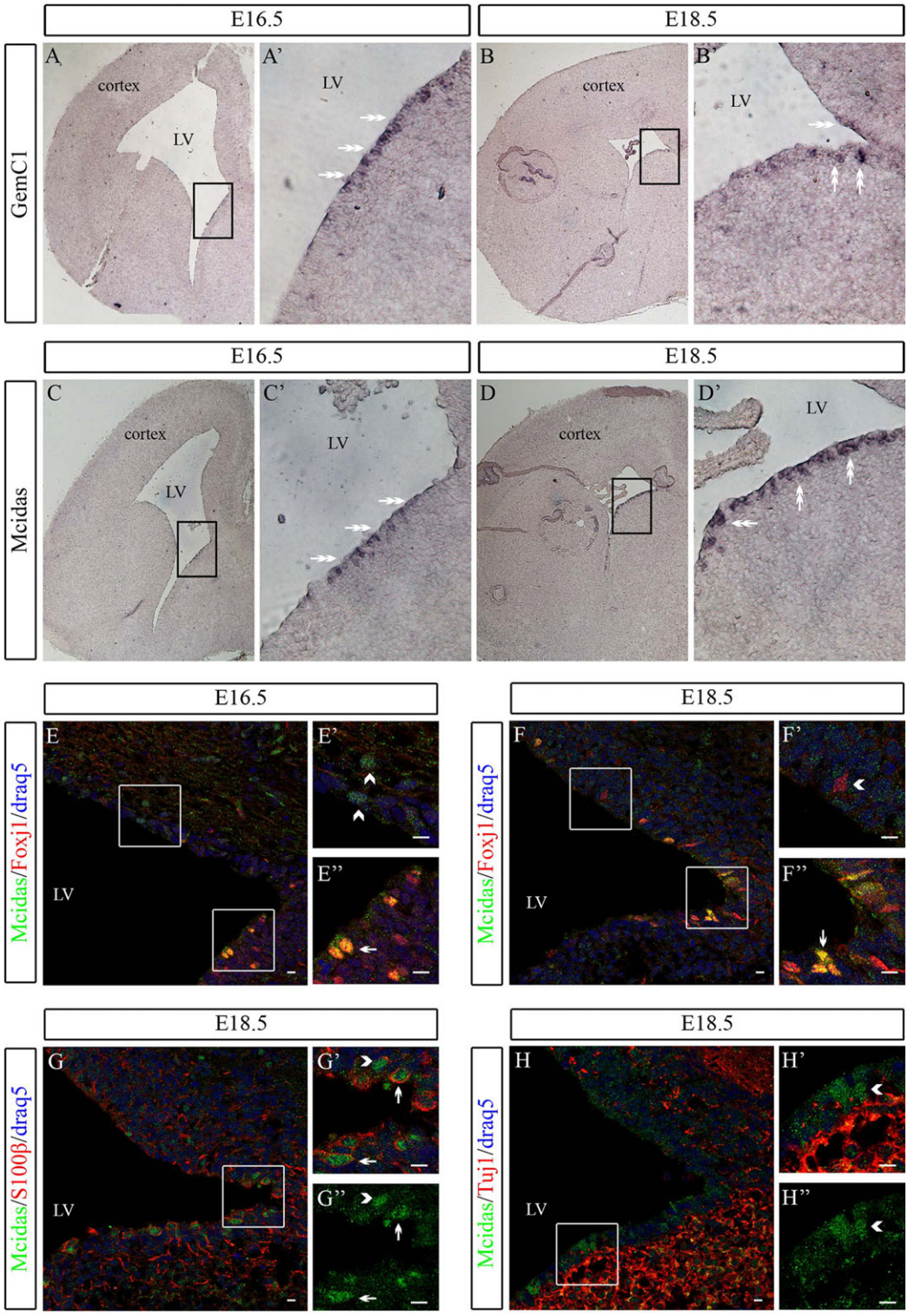

Fig. 1. Mcidas and GemC1 are expressed during late embryogenesis in RGCs that will give rise to ependymal cells. (A-D′) *In situ* hybridization (ISH) for *GemC1* (A-B′) or *Mcidas* (C-D′) mRNA expression was performed on coronal sections of E16.5 and E18.5 mouse embryos. *GemC1* and *Mcidas* are expressed in cells lying next to the lateral ventricles of the brain. Double-headed arrows indicate cells expressing *GemC1* or *Mcidas*. (E-F″) Immunofluorescence using specific antibodies against Mcidas and Foxj1 was performed on E16.5 (E-E″) and E18.5 (F-F″) embryos. (G-H″) Coronal sections of brains from E18.5 embryos were stained with specific antibodies against Mcidas and S100β, a marker of differentiated ependymal cells (G-G″), or Tuj1, a neuronal marker (H-H″). Arrows indicate double-positive cells and arrowheads indicates cells that are Mcidas^{+} or Foxj1^{+}. DNA was stained with draq5 (blue). LV, lateral ventricles. Scale bars: 20 µm.

tubulin, which localize in basal bodies and motile cilia, respectively (Mirzadeh et al., 2010, 2008). To investigate whether cells overexpressing Mcidas and GemC1 have fully differentiated into multiciliated cells, immunofluorescence was performed using antibodies against these proteins (Fig. 3E-J″). Under control conditions, the periventricular zone progenitor cells have only

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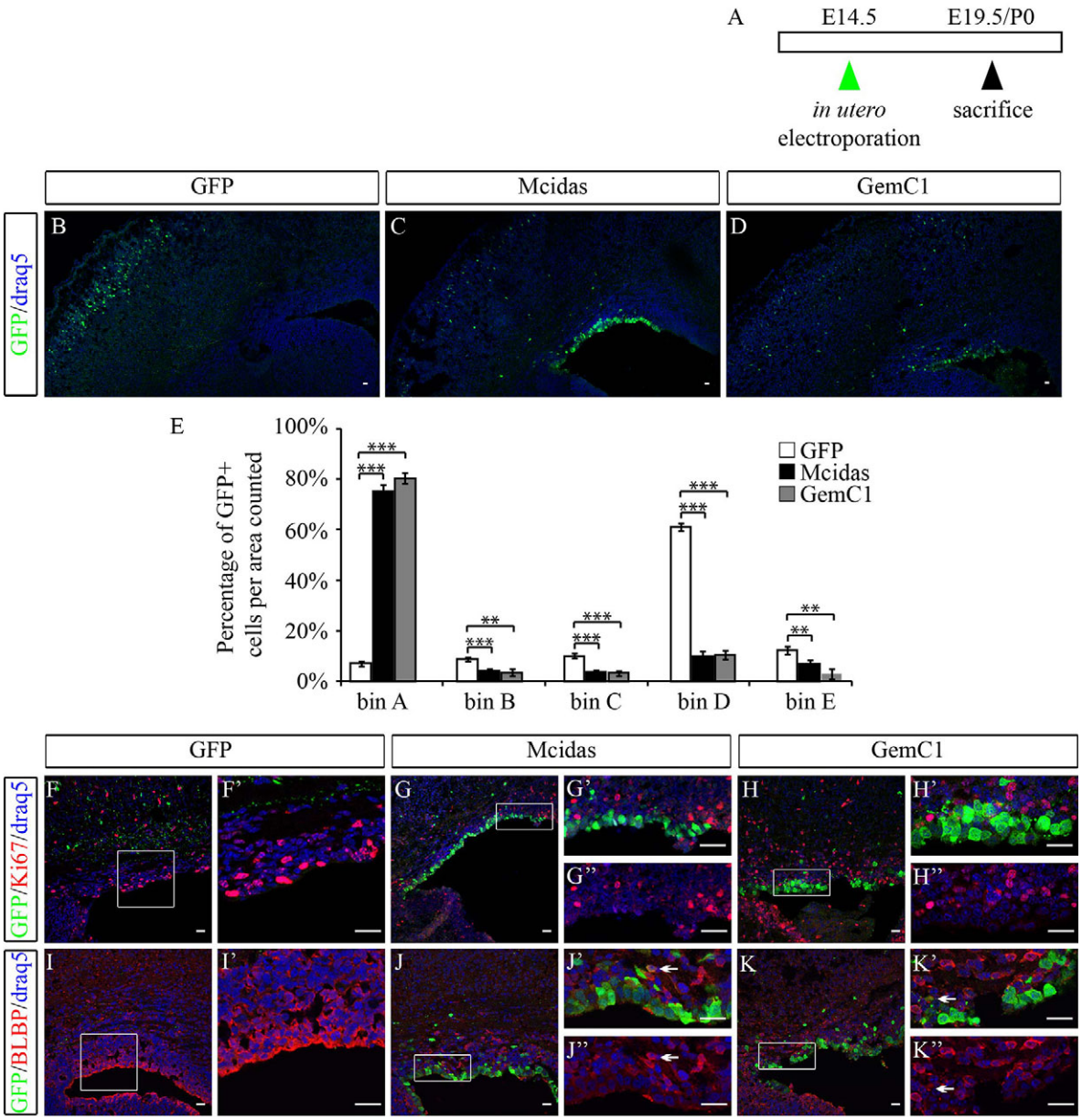

Fig. 2. Mcidas and GemC1 overexpression entraps cells in the VZ of the cortex. (A) IUE was performed in E14.5 embryos, using vectors expressing IRES-GFP (GFP), Mcidas-IRES-GFP (Mcidas) or GemC1-IRES-GFP (GemC1). (B-D) Low-magnification confocal images of the mouse cortex showing the distribution of cells overexpressing GFP (B), Mcidas (C) and GemC1 (D) 5 days following IUE. (E) The developing cortex was subdivided in five equal bins and the percentage of GFP$^+$ cells located in each bin is shown. (F-K″) E14.5 embryos were electroporated with plasmids expressing GFP, Mcidas or GemC1 and analyzed 5 days later using anti-GFP and antibodies against Ki67, a marker of cycling cells (F-H″) or BLBP, a marker of RGCs (I-K″). Arrows indicate GFP$^+$ BLBP$^+$ cells. Data are the mean±s.e.m. from quantification of at least three independent experiments in which at least four sections were analyzed. $^{**}P$<0.01, $^{***}P$<0.001. Scale bars: 20 µm.

two γ-tubulin dots and one primary cilium, as is characteristic of apical progenitor cells (Fig. 3E,E′,H,H′), whereas upon Mcidas or GemC1 electroporation the vast majority of GFP$^+$ cells exhibited multiple basal bodies (Fig. 3F-G″) and multiple cilia (Fig. 3I-J″), indicating that they have differentiated into multiciliated ependymal cells.

In order to examine whether Mcidas and GemC1 expression directly promotes the acquisition of ependymal cell fate and differentiation, analysis was performed 2 days following IUE (Fig. 4A). Following Mcidas overexpression, GFP$^+$ cells were mainly localized in bin A (67.4% of GFP$^+$ cells, Fig. 4C,E), similar to what was observed 5 days following IUE, whereas upon GFP overexpression only 36% of the overexpression cells remained in bin A (Fig. 4B,E). We found that 7.6% and 51.4% of GFP$^+$ cells coexpress Ki67 upon Mcidas and GFP overexpression, respectively (Fig. 4F-G″,O). Additionally, actively proliferating progenitors were determined by BrdU incorporation (Fig. S5A). Immunofluorescence with BrdU antibody was performed 30 min following a single BrdU injection. Whereas 36.4% of GFP-expressing cells incorporated BrdU, only 1.8% of the Mcidas-overexpressing cells incorporated BrdU (Fig. S5B-C″,E). Following GemC1 overexpression, 24.1% of the GFP$^+$ cells coexpressed Ki67 and 10.3% incorporated BrdU (Fig. 4H-H″,O, Fig. S5D-E). These results suggest that periventricular zone progenitor cells exit the cell cycle immediately after Mcidas

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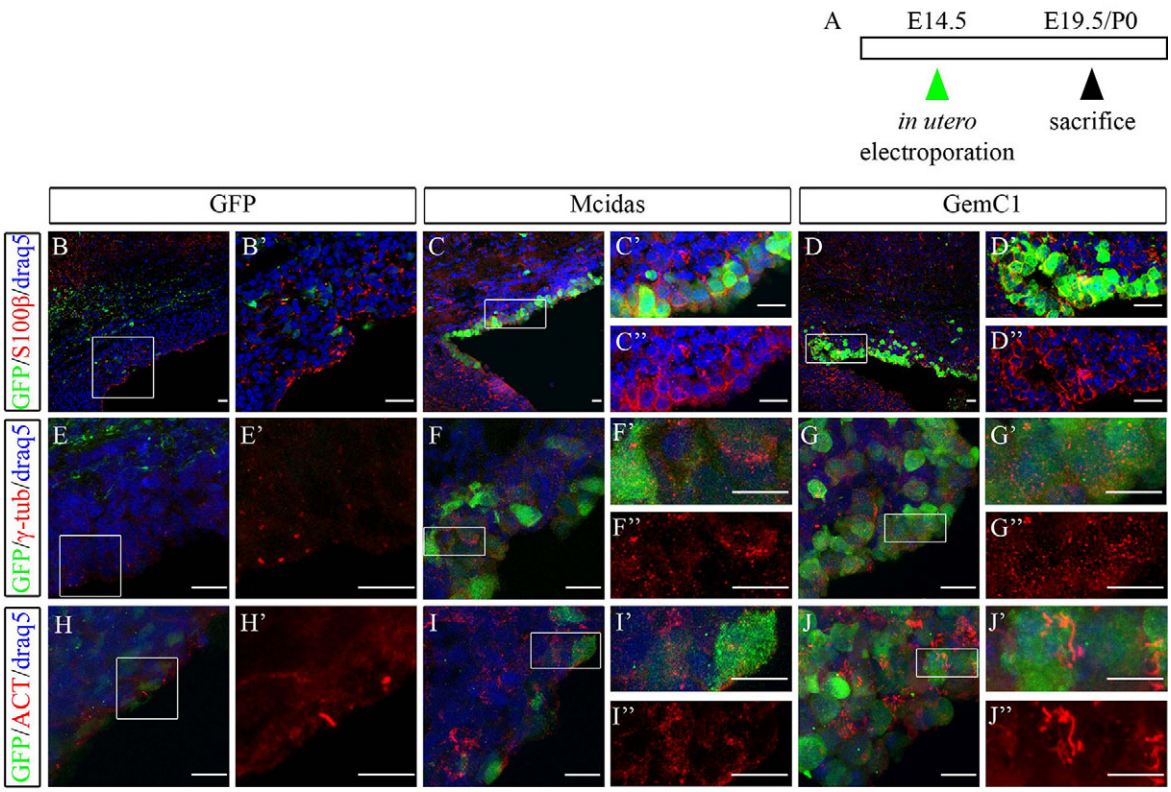

Fig. 3. Apical progenitor cells prematurely differentiate into multiciliated ependymal cells upon Mcidas or GemC1 overexpression. (A) E14.5 embryos were electroporated with plasmids expressing GFP, Mcidas or GemC1 and analyzed 5 days later. (B-J″) GFP, Mcidas and GemC1 overexpressing brains were immunostained for GFP and S100β (B-D″), γ-tubulin, a marker of basal bodies (E-G″), or acetylated tubulin (ACT), which labels cilia (H-J″) in 50 µm coronal cryosections. Approximately 20 stacks of the sections were taken using a confocal microscope and maximal projections are shown. Scale bars: 20 µm.

overexpression, while a proportion of GemC1-overexpressing cells remain in the cell cycle and undergo additional divisions.

In order to determine whether cells overexpressing Mcidas and GemC1 maintain neural progenitor markers or if they have differentiated towards the ependymal cell lineage, anti-Pax6 and anti-S100β antibodies were used. We found that 43.8% and 54.9% of cells expressing Mcidas and GemC1 were Pax6$^+$, compared with 79.3% of the GFP-expressing cells (Fig. 4I-K″,P). The percentage of S100β$^+$ cells among GFP$^+$ cells was 4.5%, 29% and 16.9% when GFP, Mcidas or GemC1 was expressed, respectively (Fig. 4L-N″,Q). Our experiments suggest that Mcidas or GemC1, soon after their expression, can modify the fate of RGCs and promote the differentiation of multiciliated ependymal cells at the expense of neuronal differentiation.

Depletion of Mcidas or GemC1 reduces their ability to promote ependymal cell differentiation

To determine whether Mcidas and GemC1 are necessary for ependymal cell generation we knocked down their expression in postnatal stages. RGCs isolated from P0 mice (pRGCs) can differentiate into SEZ niche cells, forming rosette-like structures with one or two astrocyte-like stem cells surrounded by multiciliated ependymal cells (El Zein et al., 2009; Paez-Gonzalez et al., 2011). *Mcidas* and *GemC1* mRNAs are expressed in pRGCs cultured under proliferating conditions and their expression decreases when differentiation is promoted (data not shown). pRGCs were infected with lentiviruses expressing control-IRES-GFP (control), Mcidas-IRES-GFP (Mcidas) or GemC1-IRES-GFP (GemC1) shRNAs and differentiating medium was applied 4 days post infection. pRGCs were cultured for an additional 5 or 15 days (Fig. 5A). The efficiency of the Mcidas and GemC1 shRNAs was examined at the mRNA and protein level (Fig. S6). Immunofluorescence experiments were performed using antibodies against GFP, pericentrin and acetylated tubulin. The percentage of infected cells with clusters of nascent basal bodies based on pericentrin (Pcnt) accumulation (Fig. 5B-G′) and fully differentiated cells demonstrating multiply acetylated tubulin (ACT) were scored (Fig. 5H-M′). pRGCs infected with viruses expressing control, Mcidas or GemC1 shRNAs and cultured for 5 days under differentiating conditions reveal multiple basal bodies in 31.3%, 12% and 11.7% of cells, respectively (Fig. 5B-D′,N). Additionally, the percentage of GFP$^+$ cells showing Pcnt accumulation at 15 days post initiation of differentiation under control conditions was 45.1%, whereas when Mcidas and GemC1 shRNAs were used GFP$^+$ cells were decreased to 23.1% and 22.1%, respectively (Fig. 5E-G′,N). The percentage of pRGCs developing multiple cilia was reduced by half upon Mcidas or GemC1 depletion (Fig. 5H-M′,O). Furthermore, a higher percentage of cells infected with Mcidas and GemC1 shRNAs express the marker Gfap compared with the control condition (Fig. S7), suggesting that Mcidas- or GemC1-deficient pRGCs exhibit a preference towards the astrocytic lineage at the expense of the multiciliated ependymal cell lineage.

Inactivation of Mcidas and GemC1 was also performed *in vivo* in E16.5 embryos (Fig. S8A). Wholemount immunofluorescence was performed on the wall of the lateral ventricles 18 days post infection using anti-GFP and anti-Pcnt or anti-ACT antibodies (Fig. S8B-G′). Mice infected with Mcidas or GemC1 shRNAs showed reduced Pcnt staining and reduced accumulation of

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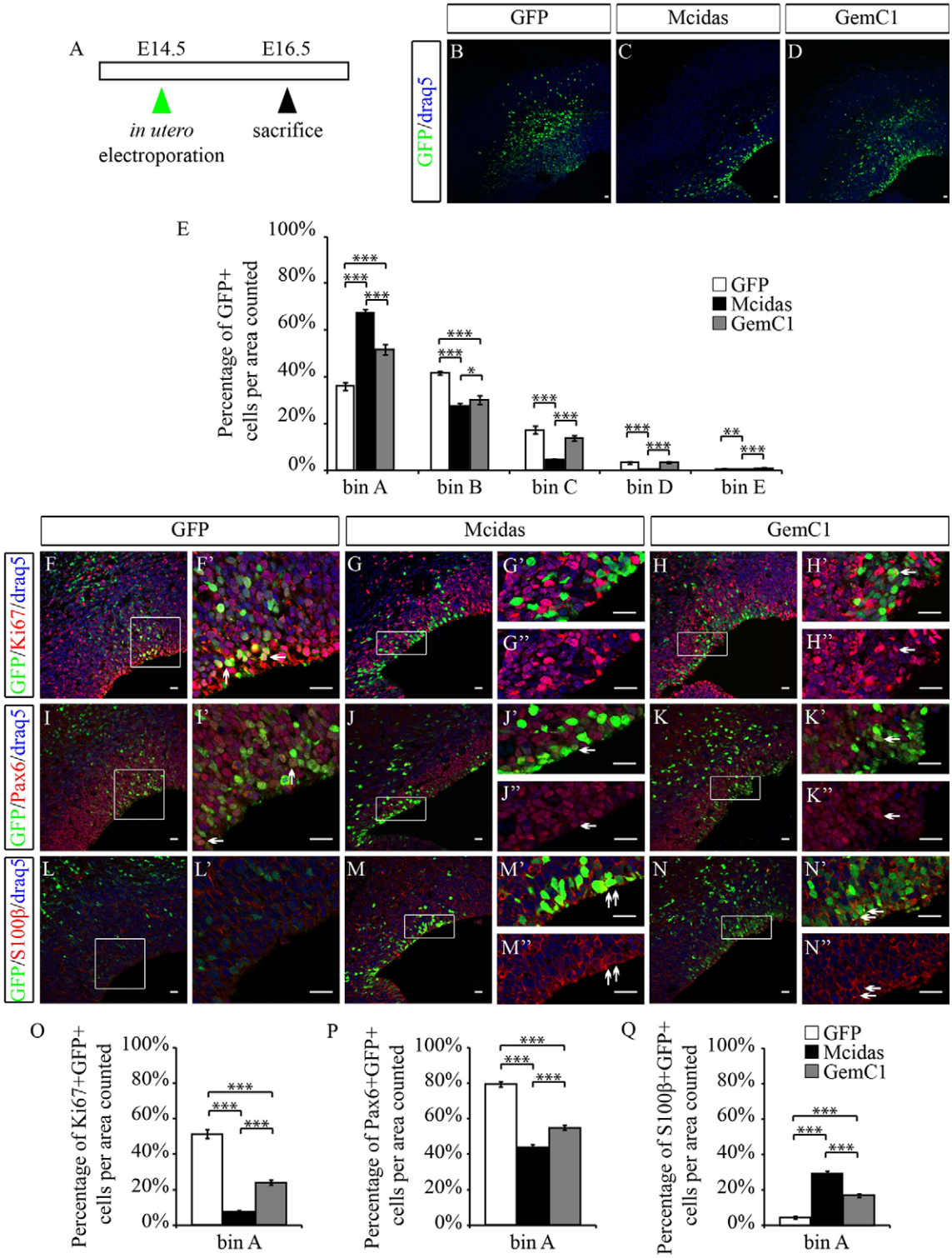

Fig. 4. RGCs prematurely differentiate into ependymal cells 2 days following Mcidas or GemC1 overexpression. (A) Embryos at E14.5 were electroporated in the developing cortex with vectors expressing GFP, Mcidas or GemC1 and analyzed 2 days later. (B-D) Coronal sections were immunolabeled with GFP antibody. (E) The developing cortex was divided into five equal bins and the percentage of GFP$^+$ cells in each bin was measured. (F-N″) Immunofluorescence using GFP and Ki67 (F-H″), Pax6 (I-K″) or S100β (L-N″) antibodies. Arrows point to double-positive cells. (O-Q) The percentage of GFP$^+$ cells that coexpress Ki67 (O), Pax6 (P) or S100β (Q) in bin A. Data are represented as mean±s.e.m. of at least three independent experiments analyzing at least four different sections per brain. **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars: 20 µm.

multiple cilia (Fig. S8H,I), suggesting that they fail to differentiate into multiciliated ependymal cells. These results indicate that Mcidas or GemC1 depletion results in defective differentiation of multiciliated ependymal cells, suggesting that their expression is necessary for RGC commitment and differentiation towards the multiciliated ependymal cell lineage.

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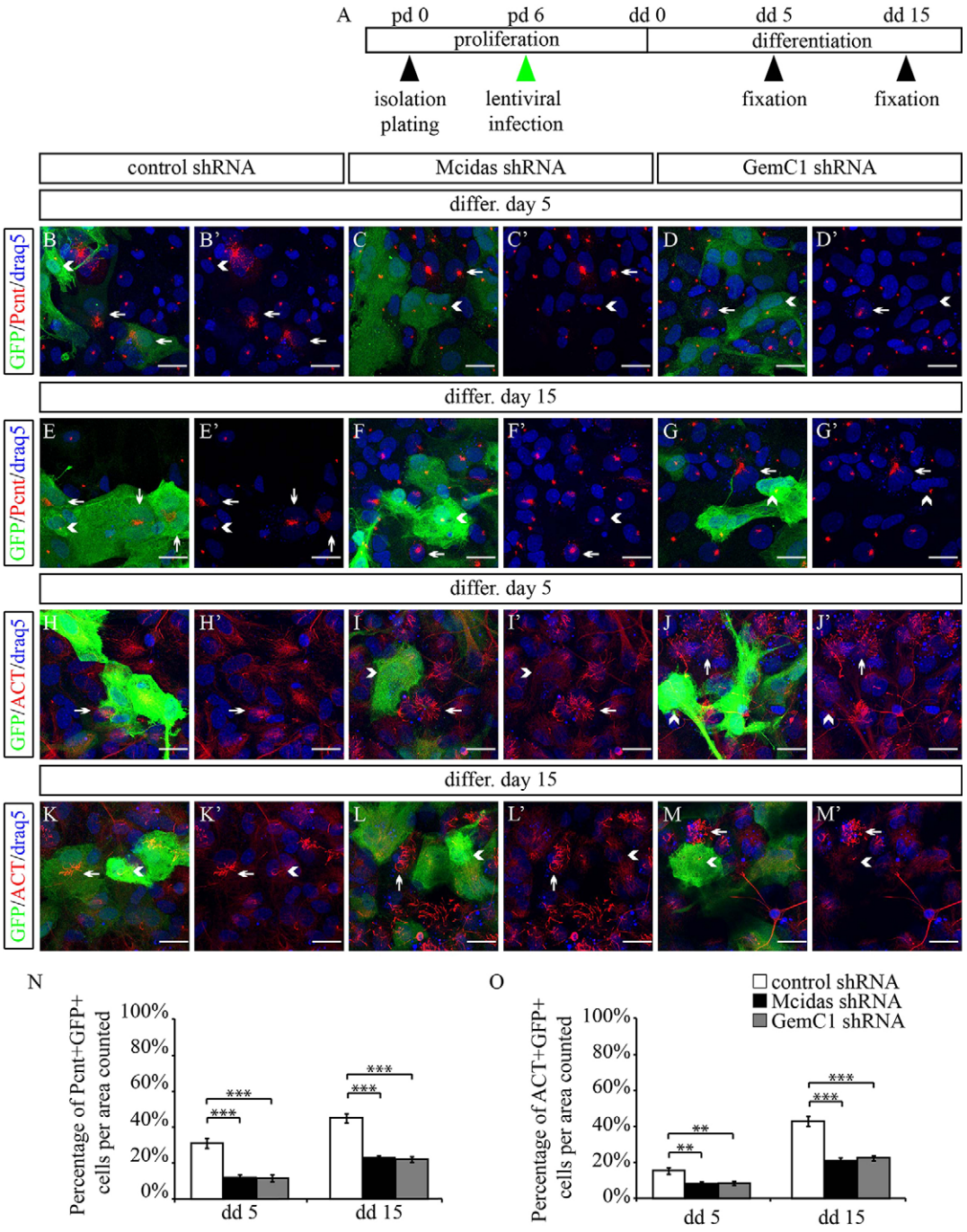

Fig. 5. Inactivation of Mcidas or GemC1 blocks generation of multiciliated ependymal cells. (A) pRGCs isolated form P0 mice were cultured *in vitro* under proliferating conditions and infected with lentiviruses expressing control-GFP (control), Mcidas-GFP (Mcidas) or GemC1-GFP (GemC1) shRNAs. Subsequently, differentiation medium was added 4 days following infection, and analysis was performed 5 or 15 days later. (B-M′) Immunofluorescence was performed 5 (B-D′,H-J′) or 15 (E-G′,K-M′) days following differentiation using antibodies against GFP and Pcnt, marker of nascent basal bodies (B-G′), or ACT, a marker of cilia (H-M′). (N,O) The percentage of GFP^+ cells that are ciliated based on Pcnt accumulation (N) and the number of cilia based on ACT staining (O). Arrowheads indicate cells with no Pcnt accumulation or with one cilium, and arrows indicate cells with Pcnt accumulation or those with clusters of cilia. Data are represented as mean±s.e.m. of at least three independent experiments. ***P*<0.01, ****P*<0.001. pd, proliferating conditions; dd, differentiating conditions. Scale bars: 20 µm.

Mcidas and GemC1 promote the generation of ependymal cells through Foxj1 and c-Myb activation

Foxj1 and c-Myb are known to be key transcription factors controlling RGC differentiation towards the ependymal cell lineage (Jacquet et al., 2009; Malaterre et al., 2008). To gain insight into the molecular pathway by which Mcidas and GemC1 regulate ependymal cell generation we examined the expression of Foxj1 and c-Myb following deletion or overexpression of Mcidas and GemC1.

Foxj1 expression was determined in pRGCs cultured under differentiating conditions for 5 and 15 days following Mcidas or GemC1 depletion. At 5 days, 43.5% and 44.4% of pRGCs express Foxj1 upon Mcidas and GemC1 knockdown, respectively, compared with 68.6% of cells in control conditions, a reduction that is maintained at 15 days (Fig. 6A-D). In order to test whether Mcidas and GemC1 overexpression can promote the activation of Foxj1 and c-Myb expression ectopically, we performed

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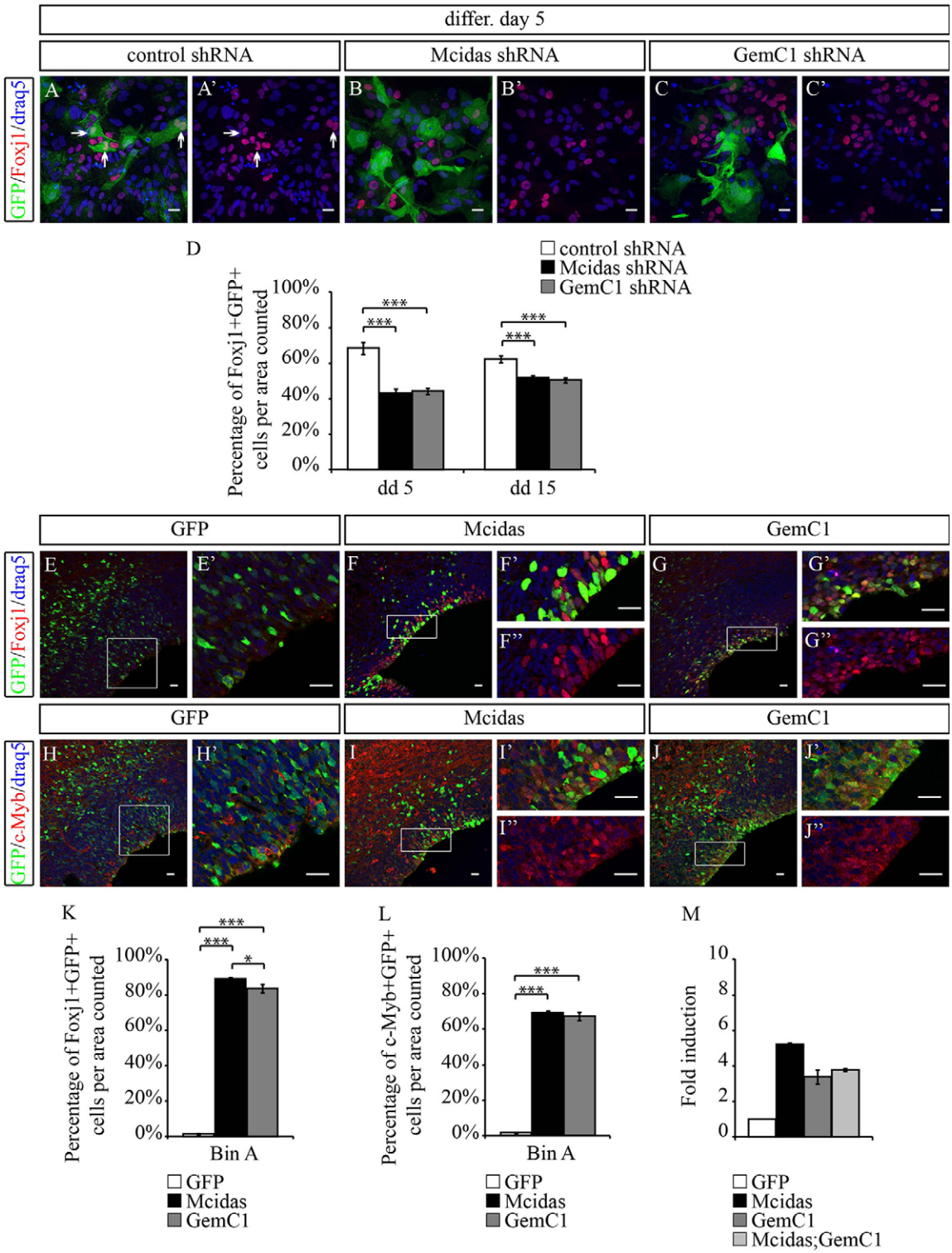

Fig. 6. Mcidas and GemC1 control the activation of Foxj1 and c-Myb expression. (A-C′) Anti-GFP and anti-Foxj1 antibodies were used for immunofluorescence on pRGC cultures infected with control, Mcidas or GemC1 shRNAs. Arrows point to GFP$^+$ Foxj1$^+$ cells. (D) The percentage of GFP$^+$ pRGCs that coexpress Foxj1. (E-J″) IUE was performed on E14.5 developing cortex using plasmids encoding GFP, Mcidas or GemC1 and embryos were analyzed 2 days later with anti-GFP and anti-Foxj1 (E-G″) or anti-c-Myb (H-J″) antibodies. (K,L) The percentage of GFP$^+$ cells that coexpress Foxj1 (K) and c-Myb (L) in bin A. (M) Fold increase of luciferase reporter gene expression driven by *c-Myb* regulatory elements upon Mcidas and GemC1 overexpression in 293T cells. Data are represented as a mean±s.e.m. of three independent experiments. **P*<0.05, ****P*<0.001. Scale bars: 20 µm.

immunofluorescence using antibodies against Foxj1 and c-Myb 2 days following IUE of GFP, Mcidas or GemC1 (Fig. 6E-L). The percentage of GFP$^+$ cells coexpressing Foxj1 and c-Myb was 1.4% and 1.5%, respectively, when plasmids expressing GFP were electroporated, as ependymal cell differentiation has not yet initiated (Fig. 6E,E′,H,H′,K,L). By contrast, 88.9% and 83.7% of

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GFP$^+$ cells were colabeled with anti-Foxj1 and 69.3% and 67.4% were colabeled with anti-c-Myb (Fig. 6F-G″,I-L), respectively, when plasmids expressing Mcidas or GemC1 were used.

To examine the ability of Mcidas and GemC1 to promote the differentiation of NSCs into ependymal cells *in vitro*, NS5 cells, which are derived from mouse embryonic stem cells, were used (Conti et al., 2005). NS5 cells were infected with lentiviruses expressing IRES-RFP (RFP), Mcidas-IRES-RFP (Mcidas) or GemC1-IRES-RFP (GemC1). Cells were cultured in differentiating conditions without EGF and FGF for 2 days and immunofluorescence was performed using anti-RFP and anti-Foxj1, anti-c-Myb or anti-Pcnt. Foxj1 expression was detected in 89.5% of Mcidas-overexpressing and 83.7% of GemC1-overexpressing cells, compared with 1.5% in control infected cells (Fig. S9B-D′,H). Additionally, Mcidas-infected and GemC1-infected NS5 cells expressed c-Myb at 85.7% and 65.7%, respectively, compared with 3.3% in control infected cells (Fig. S9E-G′,I). Furthermore, Mcidas and GemC1 overexpression was sufficient to initiate early steps of multiciliation *in vitro* as judged by the accumulation of pericentriolar material, as an accumulation of pericentrin signal was detected by immunofluorescence (Fig. S9J-L‴). Our data show that Mcidas and GemC1 promote the differentiation of RGCs towards the ependymal cell lineage through Foxj1 and c-Myb, two transcription factors crucial for ependymal cell fate acquisition and differentiation.

It has previously been suggested that c-Myb acts upstream of Foxj1 in multiciliogenesis (Tan et al., 2013). In order to examine whether Mcidas and GemC1 can activate c-Myb expression, 293T cells were transfected with a plasmid containing a luciferase reporter gene under the control of regulatory elements of *c-Myb* together with Mcidas or GemC1. Luciferase activity increased more than 5.2-fold and 3.2-fold following Mcidas and GemC1 overexpression, respectively. No additive effect on reporter gene activity was detected when Mcidas and GemC1 were coexpressed (Fig. 6M). These results indicate that Mcidas and GemC1 can activate c-Myb expression, an essential step for RGC commitment towards the multiciliated ependymal cell fate.

These results were reinforced by experiments in which Mcidas or GemC1 overexpression was combined with c-Myb knockdown. Upon Mcidas or GemC1 overexpression approximately 85% and 75% of cells were localized near the lateral ventricle, respectively, whereas when c-Myb was knocked down only 22% and 9% of the cells expressing Mcidas and GemC1 remained in the area near the lateral ventricles. Upon c-Myb inhibition the majority of cells migrated into the upper cortical layers, similar to the distribution of cells observed when control vector was overexpressed (Fig. S10). Our data suggest that Mcidas and GemC1 control fate decisions of RGCs and their differentiation into multiciliated ependymal cells by acting upstream of c-Myb and Foxj1.

GemC1 promotes Mcidas expression

To examine whether Mcidas and GemC1 act in the same pathway, we determined whether Mcidas or GemC1 could activate each other's expression. Mcidas or GemC1 was overexpressed using IUE at E14.5 and ISH was performed 2 days later using GemC1 and Mcidas cRNA probes, respectively. *GemC1* mRNA expression was not detected following Mcidas overexpression (Fig. S11A-C′); however, upon GemC1 overexpression, *Mcidas* mRNA was ectopically upregulated (Fig. 7A-B′). Similarly, 43.3% of GemC1-overexpressing cells were positively immunolabeled for Mcidas protein (Fig. 7C-E).

To determine whether the GemC1-overexpressing cells that upregulate Mcidas expression maintain RGC characteristics, we performed immunolabeling on GemC1-overexpressing cortices using Mcidas and Pax6 antibodies (Fig. 7F-G″). GFP$^+$ Mcidas$^+$ cells that were negative for Pax6 represented 33.7% of the GemC1-overexpressing cells, whereas GFP$^+$ Mcidas$^+$ Pax6$^+$ cells represented only 7.2% (Fig. 7H), indicating that GemC1-overexpressing cells that have upregulated Mcidas expression downregulate Pax6 expression. Additionally, *in vitro* depletion of GemC1 in pRGCs resulted in reduced numbers of Mcidas$^+$ cells: 31.3% of cells expressed Mcidas compared with 43.7% in control conditions (Fig. 7I-K). Our findings suggest that GemC1 can activate Mcidas expression as part of a functional hierarchy.

Notch signaling negatively regulates GemC1 function

We investigated whether Notch can regulate Mcidas and GemC1 function, as it was previously shown that Mcidas expression and function in the generation of multiciliated cells in *Xenopus* is negatively regulated by Notch signaling (Stubbs et al., 2012). We examined the distribution of GFP$^+$ cells in the developing cortex, the expression of Foxj1 (which is upregulated upon Mcidas and GemC1 overexpression and leads to multiciliated ependymal cell generation) and the expression of Mcidas (which is upregulated upon GemC1 overexpression) upon Mcidas or GemC1 overexpression in the presence of activated Notch signaling. Notch intracellular domain (NICD), the catalytic subunit of Notch, was used to promote ectopic activation of the Notch pathway.

We performed IUE using plasmids expressing GFP, Notch, GemC1, Mcidas, or Notch together with GemC1, or Notch together with Mcidas, at E14.5 and analysis was performed 2 days later (Fig. 8A). Ectopic overexpression of Notch in RGCs alters the distribution of GFP$^+$ cells, comparing with control (GFP), as the vast majority of Notch-overexpressing cells are located in bin A and fail to differentiate into cortical neurons (Fig. 8B,C,H,I). This phenotype simulates the distribution of GFP$^+$ cells observed upon GemC1 or Mcidas overexpression (Fig. 8E,G-I). However, Notch-overexpressing cells maintain their undifferentiated state, as they express the radial glial marker Pax6 (data not shown) and do not express Foxj1 (Fig. 8J,K) or Mcidas (Fig. 8L). Cells in which Notch is overexpressed together with GemC1 or Mcidas are also localized in bin A, and no difference was observed in the bin distribution of cells overexpressing GemC1 with or without Notch, nor Mcidas with or without Notch (Fig. 8D-I). However, a decrease in the number of Foxj1$^+$ cells from 72.7% to 56.7% was observed when GemC1 or Notch plus GemC1 was overexpressed (Fig. 8J) and from 81.2% to 63.7% when Mcidas or Notch plus Mcidas was overexpressed, respectively (Fig. 8K). Additionally, the number of Mcidas$^+$ cells was reduced from 40.9% to 30.1% when GemC1 or Notch plus GemC1 was overexpressed, respectively (Fig. 8L). Finally, the percentage of Pax6$^+$ cells in Notch plus GemC1 or Notch plus Mcidas overexpression was increased when compared with GemC1 overexpression (data not shown). These results indicate that loss of radial glial characteristics of periventricular cells and the ectopic increase of Foxj1- and Mcidas-expressing cells that was observed upon GemC1 or Mcidas overexpression are inhibited when Notch is co-overexpressed, suggesting that the Notch pathway reduces the ability of GemC1 and Mcidas to promote RGC commitment to the ependymal cell lineage.

DISCUSSION

In the adult brain the SEZ, which is located in the wall of the lateral ventricles, is one of the two main neurogenic niches that generates new neurons throughout adulthood that migrate away from the niche

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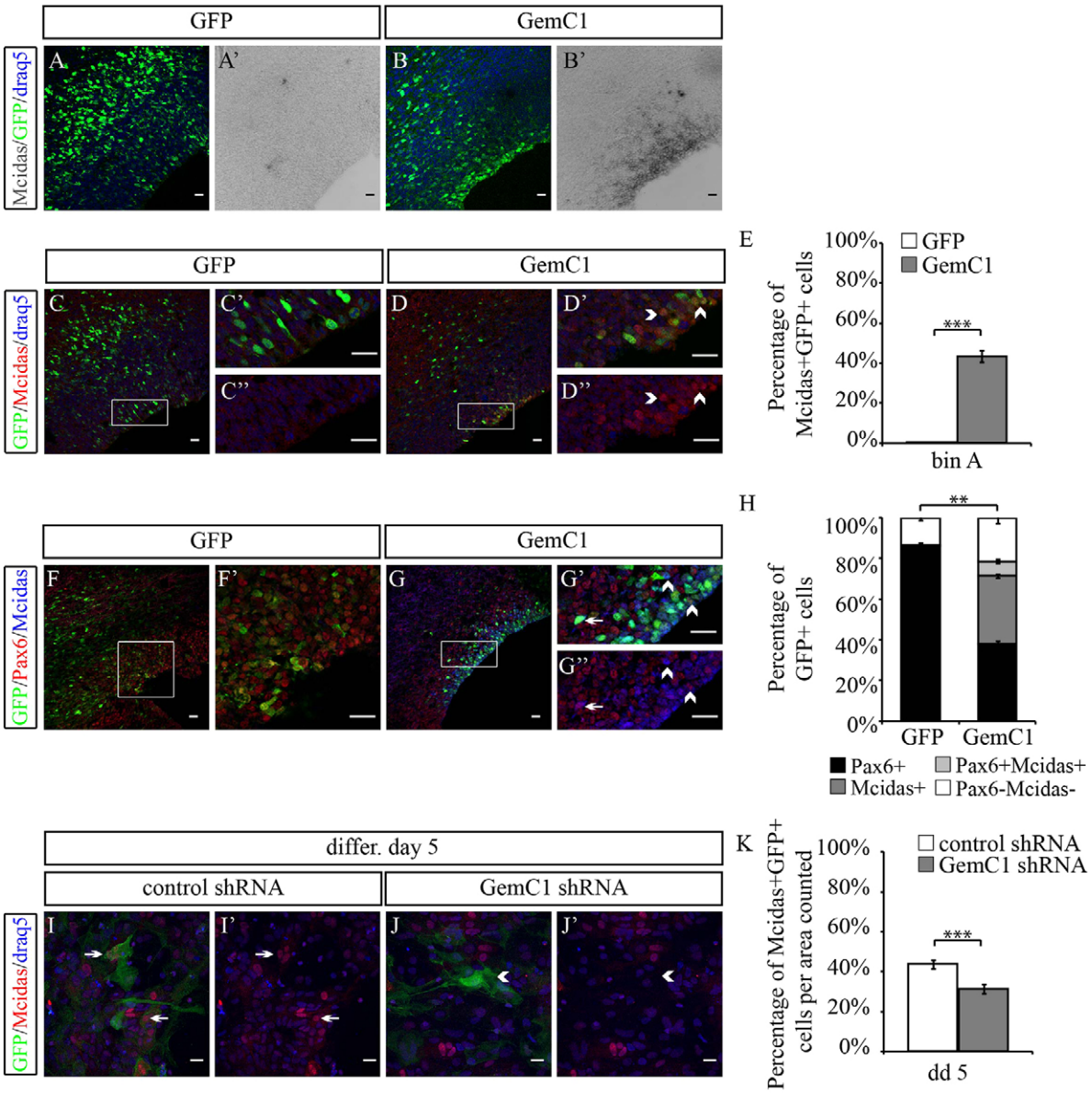

Fig. 7. Mcidas expression is regulated by GemC1. (A-B′) Embryos at E14.5 were electroporated with plasmids expressing GFP or GemC1 and analyzed 2 days later. ISH was performed on GFP (A,A′) and GemC1 (B,B′) overexpressing cortices using RNA probes complementary to *Mcidas* mRNA. (C-D″,F-G″) Immunofluorescence was performed on coronal sections using anti-GFP and anti-Mcidas (C-D″), or anti-GFP, anti-Mcidas and anti-Pax6 (F-G″) antibodies 2 days following IUE. Arrowheads indicate GFP^+ cells that coexpress either $Mcidas^+$ or $Pax6^+$, and arrows indicate GFP^+ $Mcidas^+$ $Pax6^+$ cells. (E) The percentage of GFP^+ cells that were colabeled with Mcidas antibody in bin A. (H) The percentage of GFP^+ cells that coexpress Pax6, Mcidas, Mcidas and Pax6 or none of these. (I-J′) pRGCs were infected with control or GemC1 shRNAs and cultured for 4 days in proliferating conditions and an additional 5 days in differentiating conditions. Immunolabeling experiments were performed using anti-GFP and anti-Mcidas antibodies. Arrows indicate GFP^+ $Mcidas^+$ cells and arrowheads indicate GFP^+ cells. (K) The percentage of GFP^+ $Mcidas^+$ cells at 5 days in differentiating conditions. Data are the mean±s.e.m. of three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$. Scale bars: 20 µm.

and colonize the olfactory bulbs (Alvarez-Buylla et al., 2008). The proper organization of the neurogenic niche is essential for the correct function of aNSCs; however, little is known about the mechanisms that regulate the assembly of the niche. It has been proposed that RGCs at the end of embryogenesis will give rise to cells forming the adult SEZ niche, consisting mainly of multiciliated ependymal cells and aNSCs (Merkle et al., 2004; Spassky et al., 2005; Young et al., 2007). RGCs must undergo differential cell fate decisions that are organized in a spatial and temporal manner during brain development. Several proteins have been implicated in the differentiation of RGCs towards different progeny (Guerout et al., 2014); however, the specific molecular pathway responsible for RGC commitment into adult SEZ cells and specifically into multiciliated ependymal cells is not well understood. It has been suggested that RGCs responsible for the generation of adult SEZ cells, including multiciliated ependymal cells, are $Gsh2^+$ cells in lateral and medial ganglionic eminence as well as $Emx1^+$ cells of the developing cortex (Merkle et al., 2004; Young et al., 2007). These cells are committed to the ependymal cell lineage at E14 to E16, when they perform their last division (Spassky et al., 2005). $Gsh2^+$ and $Emx1^+$ cells generate a wide range of differentiated cells and, therefore, identification of novel genes that will specifically mark the committed RGC that will generate the ependymal cell lineage is required.

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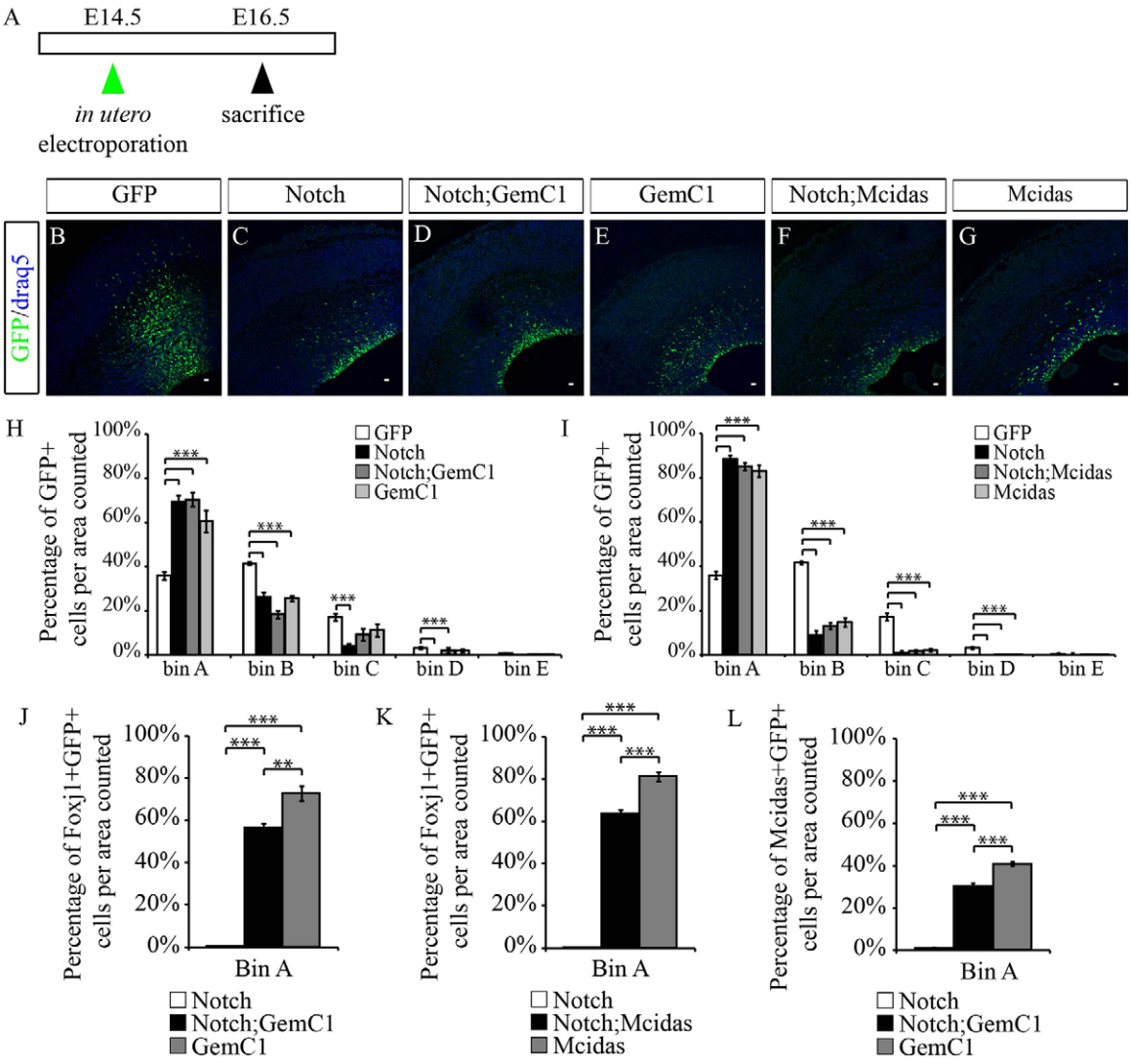

Fig. 8. Notch overexpression reduces the ability of Mcidas and GemC1 to activate Mcidas and Foxj1 expression. (A) IUE was performed in E14.5 embryos using vectors expressing IRES-GFP (GFP), Notch intracellular domain transcript (Notch), Notch together with GemC1 (Notch;GemC1), GemC1, Notch together with Mcidas (Notch;Mcidas) or Mcidas. (B-G) Immunofluorescence was performed in cortex using anti-GFP antibody. The distribution of cells overexpressing GFP (B), Notch (C), Notch;GemC1 (D), GemC1 (E), Notch;Mcidas (F) or Mcidas (G) 2 days following IUE is shown. (H,I) The developing cortex was subdivided in five equal bins and the percentage of the GFP^+ cells located in each bin is shown. (J,K) The percentage of GFP^+ cells coexpressing Foxj1 when Notch, Notch;GemC1 or GemC1 were expressed (J) or when Notch, Notch;Mcidas or Mcidas were expressed (K). (L) The percentage of GFP^+ cells coexpressing Mcidas when Notch, Notch;GemC1 or GemC1 were expressed. Data are mean±s.e.m. from the quantification of at least three independent experiments in which at least four sections were analyzed. $^{**}P<0.01$, $^{***}P<0.001$. Scale bars: 20 µm.

Here, we propose that GemC1 and Mcidas are key regulators of late RGC commitment and differentiation into multiciliated ependymal cells. GemC1 and Mcidas were initially described for their role in cell cycle progression (Balestrini et al., 2010; Caillat et al., 2013; Pefani et al., 2011). In addition, Mcidas was shown to control differentiation of multiciliated cells in lung and epidermis (Boon et al., 2014; Stubbs et al., 2012; Tadokoro et al., 2014; Tan et al., 2013; Wallmeier et al., 2014); however, GemC1 has not been implicated in cell differentiation and fate decisions. Here we show that GemC1 and Mcidas expression is mainly restricted to a population of cells lying next to the lateral ventricles in the ventral telencephalon. Their expression is initiated around E16.5, and at E18.5 Mcidas partially colocalizes with Foxj1 and S100β. Thus, according to their localization and coexpression with Foxj1 and S100β, we propose that Mcidas and GemC1 are expressed in RGCs during late embryogenesis that are committed to the multiciliated ependymal cell lineage. Although ependymal cell fate commitment takes place in late embryogenesis, the ciliogenesis in these cells begins after birth and is completed after the second postnatal week (Spassky et al., 2005). As GemC1 and Mcidas expression is maintained only until E18.5 and P7, respectively, it is more likely that their main role concerns the initial events of RGC commitment and differentiation and not the maintenance of differentiated ependymal cells. Moreover, we show that upon Mcidas and GemC1 overexpression, RGCs lose their neural progenitor markers, exit the cell cycle and prematurely differentiate into multiciliated ependymal cells, whereas their deletion prevents ependymal cell generation. Our findings suggest that Mcidas and GemC1 constitute the earliest known markers of RGCs committed to the ependymal cell lineage and that they are sufficient, as well as necessary, for RGC commitment and differentiation towards this lineage.

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Once RGCs are committed to the ependymal cell lineage, they will differentiate into multiciliated cells. This process requires the generation of multiple basal bodies, which will be translocated to the apical surface of ependymal cells and nucleate the formation of clusters of motile cilia. Foxj1 was shown to be essential for centriole docking (Stubbs et al., 2008; Yu et al., 2008), although recently it was shown to control RGC differentiation towards multiciliated ependymal cells, acting upstream of Ank3, which is necessary for the correct assembly of ependymal cells in the adult SEZ (Jacquet et al., 2009; Paez-Gonzalez et al., 2011). Moreover, c-Myb was also proposed to be crucial for multiciliated cell differentiation, acting upstream of Foxj1 in airway epithelium (Tan et al., 2013). Using gain- and loss-of-function analyses, we propose that Mcidas and GemC1 regulate c-Myb and Foxj1 expression. Overexpression of either Mcidas or GemC1 upregulates c-Myb and Foxj1 expression, whereas knockdown of either decreases c-Myb and Foxj1 expression. These findings suggest that Mcidas and GemC1 act upstream of these transcription factors in the regulation of RGC fate commitment and differentiation of multiciliated ependymal cells. Additionally, we show that Mcidas and GemC1 promote multiciliated ependymal cell differentiation through c-Myb and therefore Foxj1, as depletion of c-Myb does not allow the differentiation process to be initiated even after Mcidas or GemC1 overexpression. Therefore, Mcidas and GemC1 determine the subpopulation of RGCs that will give rise to multiciliated ependymal cells of the SEZ by regulating the expression of genes essential for ependymal cell differentiation, and thus Mcidas and GemC1 function initiates the RGC commitment program.

Furthermore, our findings show that GemC1 expression results in ectopic upregulation of Mcidas, whereas upon GemC1 deletion the endogenous levels of Mcidas protein are reduced. This suggests that GemC1 regulates Mcidas expression, revealing a functional hierarchy between the two geminin homologs in RGC commitment and differentiation. This hypothesis is in agreement with the transient expression of GemC1 in RGCs as they become committed to the ependymal cell lineage, while Mcidas expression is maintained until the first postnatal week, overlapping with the timing of ependymal cell differentiation. Moreover, activation of Notch signaling reduces the ability of GemC1 to upregulate Mcidas and Foxj1 expression and the ability of Mcidas to upregulate Foxj1 expression, maintaining RGCs in an undifferentiated state, suggesting that GemC1 and Mcidas act downstream of Notch signaling. This hypothesis is also supported by recent findings showing that Mcidas expression and function are altered upon manipulation of Notch (Stubbs et al., 2012). GemC1 and Mcidas expression is thus key in promoting the commitment and differentiation of multiciliated ependymal cells, competing with Notch signaling, which maintains the RGC population.

Based on the spatiotemporal expression of GemC1 and Mcidas and their ability to activate c-Myb and Foxj1 expression, we propose that GemC1 and Mcidas, in a sequential hierarchy, control RGC commitment and differentiation towards the multiciliated ependymal cell lineage (Fig. S12). Our results suggest that Mcidas and GemC1 are key factors in initiating the mechanisms that determine the generation of the cellular components of the adult neurogenic niche.

In addition to the SEZ niche, multiciliated cells appear in the ependyma of many other tissues and organisms. All multiciliated cells share common features in morphology, as they carry multiple basal bodies and motile cilia, and in function, their main role being the regulation of fluid flow. Thus, a universal molecular pathway controlling their differentiation could exist. Previous findings showed that Mcidas has a key role in multiciliated cell differentiation in *Xenopus* skin and kidney and in mouse and human airway epithelium upstream of c-Myb and Foxj1, acting antagonistically to the Notch pathway (Boon et al., 2014; Stubbs et al., 2012; Tan et al., 2013). Our results show that Mcidas controls the generation of multiciliated ependymal cells in the mouse brain. Moreover, we provide evidence that a protein previously uncharacterized for its role in cellular differentiation, GemC1, plays a key role in the generation of ependymal cells and controls Mcidas expression. Our data make GemC1 an ideal candidate for involvement in ciliogenesis in other tissues and, in addition, this might aid the identification of mutations underlying humans conditions involving mucociliary clearance disorder or impaired adult neurogenesis.

MATERIALS AND METHODS

Plasmid constructs

Constructs used to prepare ISH probes, shRNAs and for IUE are described in the supplementary Materials and Methods.

Mice, *in situ* hybridization (ISH) and immunohistochemistry

C57BL/6 and Parkes mice were housed in the animal house of the University of Patras. Animal care and experiments were approved by the Veterinary Administrations of the Prefectures of Achaia, Greece, and were conducted in strict accordance with EU directives. Noon of the day of the vaginal plug was defined as E0.5. Brains dissected from mouse embryos and pups were fixed overnight with 4% paraformaldehyde (PFA), cryopreserved using 30% sucrose, frozen in 7.5% gelatin plus 15% sucrose and sectioned at 10 µm or 50 µm.

Non-radioactive ISH was performed using DIG-labeled RNA probes, and immunofluorescence in frozen sections and BrdU labeling and detection were performed as described elsewhere (Spella et al., 2011). Wholemount immunofluorescence in the wall of lateral ventricles was performed as described previously (Mirzadeh et al., 2010). The primary and secondary antibodies used and the processing and analysis of ISH and immunohistochemistry confocal images are described in the supplementary Materials and Methods.

In utero electroporation (IUE) and infection

IUE was performed in mouse embryos at E14.5 as described previously (Pilz et al., 2013). *In utero* infections were performed in mouse embryos at E16.5, in which 1 µl of lentiviral particles were injected into the lateral ventricles of each embryo. For details, see the supplementary Materials and Methods.

Lentiviral production

For lentiviral production a second-generation packaging system was used, as described in the supplementary Materials and Methods.

Cell culture

For *in vitro* differentiation of embryonic RGCs towards multiciliated ependymal cells two cell lines were used: pRGC primary cultures, which were isolated and cultured as described previously (Paez-Gonzalez et al., 2011); and NS5 NSCs, which were differentiated from mouse embryonic stem cells (Conti et al., 2005). Cells were incubated at 37°C under 5% CO_2 atmosphere. For details, see the supplementary Materials and Methods.

Luciferase assay

Luciferase assay was performed in cell lysates from 293T cells using the Dual-Luciferase Reporter Assay Kit (Promega) and analyzed in a Victor light luminescence plate reader (PerkinElmer). For further details, see the supplementary Materials and Methods.

Statistics

Statistical significance of differences was analyzed with the non-parametric two-tailed Mann–Whitney test. All results are presented as mean and s.e.m.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
C.K.: experimental concept and design, performed experiments, data analysis and interpretation, manuscript writing. M.A., G.-A.P., D.-E.P. and M.-E.L.: performed experiments. J.N., M.G. and Z.L.: experimental concept and design. S.T.: experimental conception and design, data analysis and interpretation, manuscript writing.

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Supplementary information
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