

GemC1 controls multiciliogenesis in the airway epithelium

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Abstract

Multiciliated cells are terminally differentiated, post-mitotic cells that form hundreds of motile cilia on their apical surface. Defects in multiciliated cells lead to disease, including mucociliary clearance disorders that result from ciliated cell dysfunction in airways. The pathway controlling multiciliogenesis, however, remains poorly characterized. We showed that GemC1, previously implicated in cell cycle control, is a central regulator of ciliogenesis. GemC1 is specifically expressed in ciliated epithelia. Ectopic expression of GemC1 is sufficient to induce early steps of multiciliogenesis in airway epithelial cells *ex vivo*, upregulating *MclDas* and *Foxj1*, key transcriptional regulators of multiciliogenesis. GemC1 directly transactivates the *MclDas* and *Foxj1* upstream regulatory sequences, and its activity is enhanced by E2F5 and inhibited by Geminin. GemC1-knockout mice are born with airway epithelia devoid of multiciliated cells. Our results identify GemC1 as an essential regulator of ciliogenesis in the airway epithelium and a candidate gene for mucociliary disorders.

Keywords cell cycle; ciliary epithelia; ciliopathies; *MclDas*; respiratory disorders

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Development & Differentiation; Molecular Biology of Disease

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Introduction

Cilia are microtubule-based organelles that protrude from the surface of almost all cells of the human body [1]. Generating properly differentiated cilia during development and in the adult is pivotal, as evidenced by ciliopathies, human diseases that arise from cilia defects [2]. Multiple motile cilia are generated in

specific cell types and their concerted movement can generate flow to clear mucus in the airway epithelium, move cerebrospinal fluid in brain ventricles, and transport eggs in the female reproductive tract [3]. In the airway epithelium, a dynamic balance between ciliated and secretory cells and their progenitors is crucial for development, homeostasis and regeneration [4–6]. Pathological changes in the composition of the airway epithelium are observed in many respiratory conditions, including chronic obstructive pulmonary disease and hyperplasia–metaplasia of the epithelium, as well as hereditary mucociliary clearance disorders [7]. However, the pathways that control the generation of ciliated cells remain poorly characterized [8].

GemC1 was identified as an important cell cycle regulator [9]. It was shown to be required for the initiation of DNA replication by promoting the formation of replicative complexes at origins of replication during the G1-to-S-phase transition. GemC1 shares limited sequence similarity to Geminin, a central inhibitor of DNA replication [10–13] implicated in proliferation–differentiation decisions [14–20], and *MclDas* [21]. *MclDas* (also termed *Idas*) was originally identified as a Geminin binding partner required for cell cycle progression [21,22]. Moreover, it was shown to be specifically expressed in the developing choroid plexus, suggesting additional roles in differentiation [21]. Indeed, *MclDas* (also termed *multicilin*) was soon shown to constitute an important regulator of multiciliate cell differentiation, activating several genes required for ciliogenesis [23–25], including *Foxj1*, a transcription factor central for multiciliogenesis [26–29]. Mutations in *MCIDAS* were recently identified in patients with a severe mucociliary clearance disorder [30].

Here, we showed that GemC1 is a novel determinant of the early steps of multiciliate cell differentiation. GemC1 is essential for the formation of multiciliated cells in the mouse airway epithelium, promoting the transcription of *MclDas* and *Foxj1* in co-operation with the E2F5 transcription factor. Mice deficient for GemC1 are born with airway epithelia lacking multiciliated cells, underscoring the importance of GemC1 as a central regulator of the multiciliated cell fate.

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Results

GemC1 is specifically expressed in developing ciliated epithelia

To gain insight into the function of GemC1 at the organismal level, its expression pattern was assessed during mouse embryogenesis. *In situ* hybridization was performed on sections from mouse embryos at different developmental stages (Figs 1, EV1 and EV2). In the developing mouse brain, GemC1 exhibits highly specific expression in the epithelial cells of the choroid plexuses of all ventricles, throughout development (E10.5 days post-coitum (dpc), E12.5 dpc and E14.5 dpc). We previously showed that McIdas is also expressed in the developing choroid plexus [21]. We therefore directly compared GemC1 and McIdas expression in consecutive sections. During early embryogenesis (E10.5 dpc), GemC1 exhibits an overlapping expression pattern to McIdas in the choroid plexus epithelium of all ventricles (Fig 1A). At later developmental stages (E12.5 and E14.5 dpc, Figs EV1 and EV2), GemC1 expression remained to high levels throughout the choroid plexus epithelium, while McIdas expression was markedly reduced and was gradually confined to the base of the epithelium.

Specific expression of GemC1 was also detected in the developing mouse respiratory epithelium. GemC1 mRNA was detected throughout the airway epithelium, from the respiratory epithelium of the nasal cavity (Fig 1B, E15.5 dpc, upper and middle images) through the trachea, to the upper bronchial tree of the mouse lungs (Fig 1B, lower images), and exhibited an overlapping expression pattern to McIdas. Both the choroid plexus epithelium and the airway epithelium are ciliated epithelia. Double staining on sagittal sections of E18.5 dpc mouse lungs, with a probe for GemC1 mRNA and an antibody against Foxj1, a transcription factor specifically expressed in cells with motile cilia [31–33] and implicated in multiciliogenesis [26–29], showed that the great majority of GemC1-expressing cells also expressed Foxj1 (Fig EV3A), and this was similar to McIdas (Fig EV3B). This indicates that GemC1 is expressed in the developing multiciliated cells of the mouse airways.

To further characterize the expression of GemC1 during development and in the adult, quantitative RT-PCR was performed on various dissected embryonic and adult tissues. As shown in Fig EV4A, GemC1 exhibits high expression levels in the developing choroid plexus and shows specific expression in the adult choroid plexus, in embryonic and adult bronchi, and in ovaries/oviducts from adult mice, which contain multiciliated cells [3], but was not detectable in liver, a non-ciliated tissue. We directly compared the GemC1 expression pattern to the expression of McIdas and Geminin, with which GemC1 shares limited similarity [9,21], and to FoxJ1. GemC1 most closely resembles FoxJ1 expression, being exclusively confined to multiciliated epithelia. McIdas showed an overlapping expression pattern, but was also detectable in non-multiciliated tissues, such as the liver, while Geminin exhibited a more ubiquitous expression, consistent with its expression in all proliferating tissues [34,35]. We concluded that GemC1 shows specific expression in multiciliated tissues.

An *ex vivo* ciliogenesis model using isolated mouse tracheal epithelial cells (MTEC) cultured in an air–liquid interface (ALI) [36] was used to assess the timing of GemC1 expression during multiciliogenesis. GemC1 was not detected in the cycling

undifferentiated cell population (Fig 1C, ALI DAY -2 and 0). One day after differentiation induction (ALI DAY 1), GemC1 becomes detectable and its expression increases during multiciliate cell differentiation, dropping at ALI DAY 21, when ciliogenesis is completed [37]. A similar timing of expression was observed for McIdas and Foxj1. On the contrary, Geminin showed an inverted expression pattern: its expression diminishes as cells cease proliferation and progress toward differentiation.

Our data show that GemC1 is specifically expressed in ciliated epithelia and its expression closely mirrors multiciliate cell differentiation. This highly specific expression pattern suggests a role for GemC1 in multiciliogenesis.

GemC1 promotes early steps of multiciliogenesis in a mouse tracheal epithelial cell culture *ex vivo* system

Given the specific expression pattern of GemC1 during multiciliate cell differentiation, we wished to determine whether GemC1 can promote multiciliogenesis. To that end, we performed overexpression experiments *ex vivo*, using a lentiviral-based expression system in mouse tracheal epithelial cell cultures. MTECs were infected with lentiviruses expressing either a GFP-GemC1 fusion protein or GFP alone as a control. Two days after infection, ALI was established. Infected cells were analyzed at different time points post-ALI for the expression of McIdas and FoxJ1, two known regulators of multiciliate cell differentiation [8], by immunofluorescence. As shown in Fig 2 (representative images on the left and quantification on the right), at ALI DAY 1, 2 and 3, when ciliogenesis has not yet started in control cells, GemC1-GFP-overexpressing cells show a marked increase in the percentage of both McIdas- and FoxJ1-expressing cells, as compared to control GFP-overexpressing cells. Our analysis shows that ectopic GemC1 expression can upregulate McIdas and FoxJ1 expression, the earliest known markers of ciliogenesis, suggesting that GemC1 is a central and early determinant of ciliogenesis.

GemC1 transactivates the MCIDAS and FOXJ1 promoters and co-operates with E2F5

We showed that the expression of McIdas and FoxJ1 increased in GemC1-overexpressing mouse tracheal epithelial cells. In order to study whether GemC1 can directly activate the MCIDAS and FOXJ1 promoters, we performed a luciferase reporter gene assay using the upstream regulatory elements of the MCIDAS and FOXJ1 genes. GemC1 is able to induce expression from both the MCIDAS promoter (4.2-fold, Fig 3A) and the FOXJ1 promoter (5.5-fold, Fig 3B). A similar upregulation of both the MCIDAS and FOXJ1 promoters was shown by the McIdas protein, but not by Geminin. Interestingly, co-expression of Geminin strongly inhibited transactivation by both GemC1 and McIdas on the MCIDAS and FOXJ1 upstream regulatory sequences. In order to exclude that GemC1 indirectly induces FoxJ1 transcription through McIdas, siRNA was used to silence McIdas in cells. As shown in Fig 3C and D, GemC1 can induce expression from the FOXJ1 promoter irrespective of McIdas expression. McIdas, on the contrary, is unable to induce expression from the GEMC1 regulatory elements (Fig EV5E). Our data therefore identify GemC1 as an upstream transcriptional regulator of the earliest known transcriptional regulators that establish the multiciliated

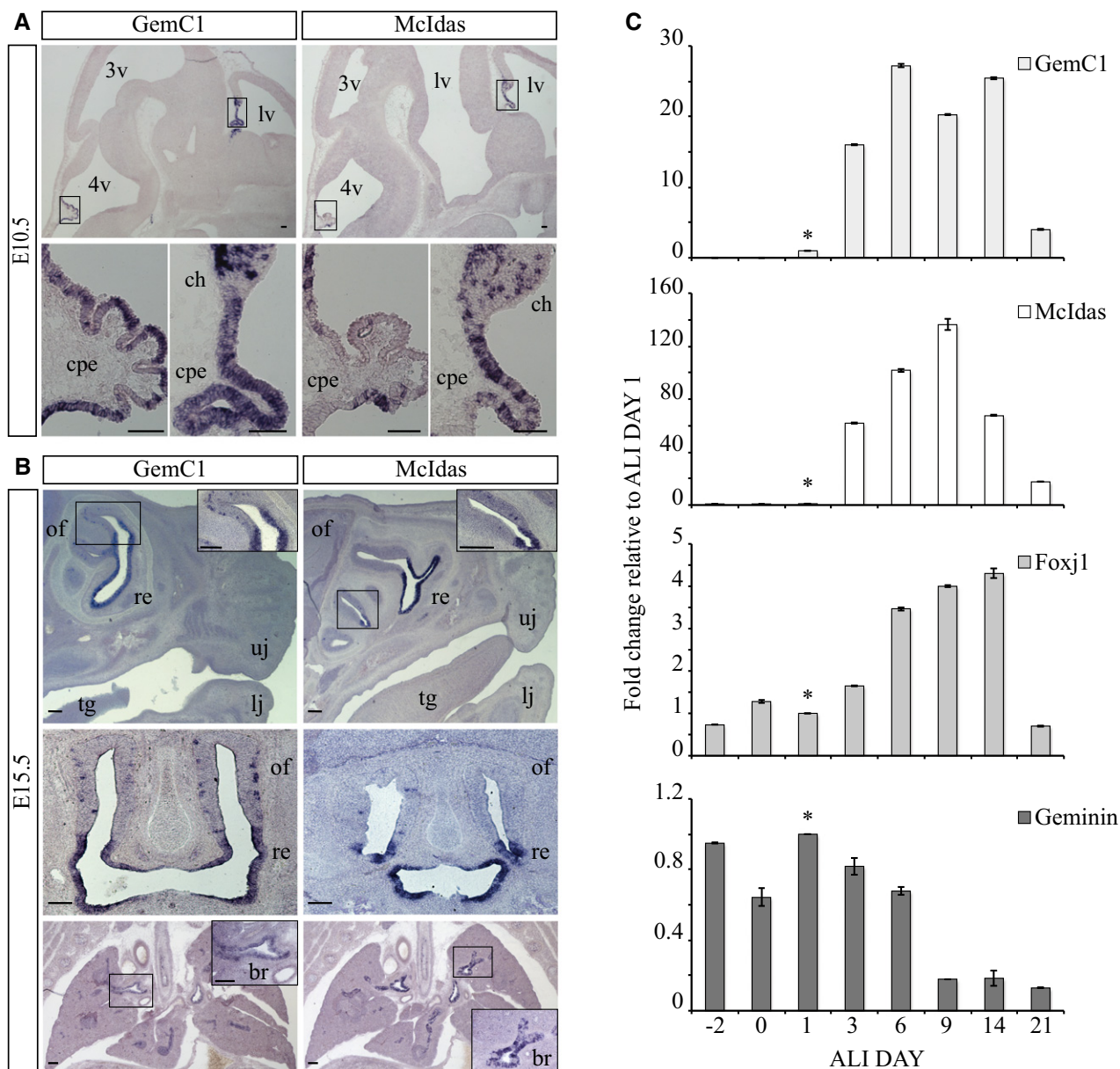


Figure 1. GemC1 is specifically expressed in developing multiciliated cells.

A *In situ* hybridization for GemC1 and McIDAS mRNA was performed on sagittal sections of E10.5 dpc mouse brain. GemC1 and McIDAS mRNA is specifically detected in the developing choroid plexuses of the ventricles. Lower panels show higher magnifications. Scale bars, 100 μ m.

B *In situ* hybridization on sagittal (upper) and coronal (middle and lower) sections of the airways of E15.5 dpc mouse embryos, depicting specific GemC1 and McIDAS mRNA expression in the respiratory epithelium of the nasal and oral cavity and in the upper bronchial tree in the mouse lungs. Inserts show higher-magnification images. Scale bars, 200 μ m.

C MTECs were cultured *ex vivo* and transitioned to ALI culture condition. Quantitative real-time PCR was used to measure the expression levels of GemC1, McIDAS, FoxJ1 and Geminin mRNA levels at different time points during multiciliogenesis. Values indicate expression levels (\pm standard error, s.e.) relative to ALI DAY 1 (marked by *), following normalization against the housekeeping HPRT mRNA. qPCR data were analyzed by the REST-MCS beta software from at least three independent experiments.

Data information: Abbreviations: lv: lateral ventricles, 3v: third ventricle, 4v: fourth ventricle, cpe: choroid plexus epithelium, ch: cortical hem, re: respiratory epithelium, of: olfactory epithelium, uj: upper jaw, lj: lower jaw, tg: tongue, br: bronchiole.

cell fate (McIDAS and FoxJ1) and suggest intricate positive and negative regulatory interactions between GemC1, McIDAS and Geminin.

McIDAS was previously shown to form a ternary complex with E2F4/5 and DP1 transcription factors to upregulate genes important

for ciliogenesis [24]. In order to address whether GemC1 co-operates with E2F family members, GemC1 was co-expressed with E2F5 (Fig 4A and B) and E2F1 (Fig EV5C and D) in luciferase reporter assays. While E2F5 shows no transactivation activity on its own

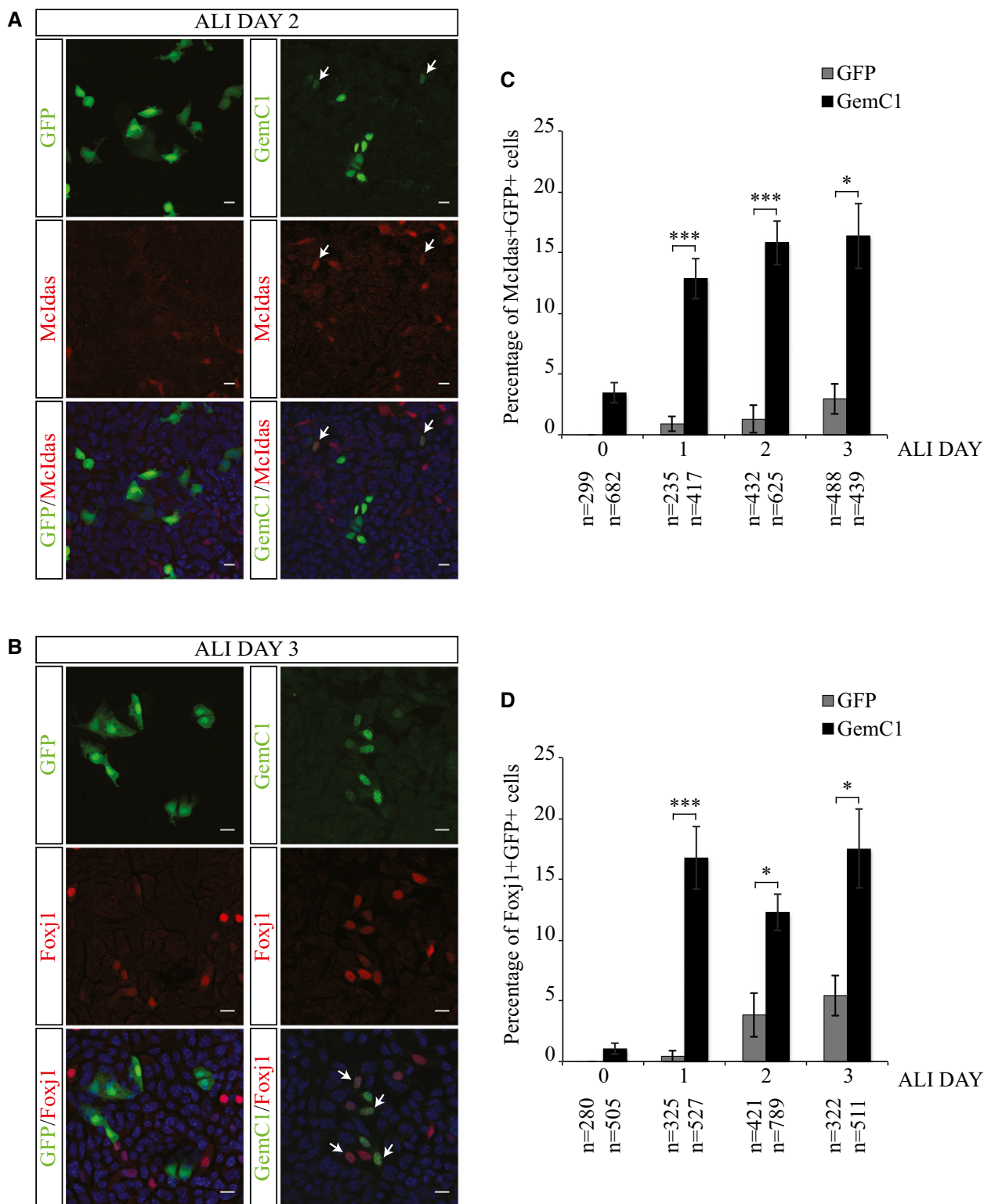


Figure 2. GemC1 induces multiciliate cell differentiation of airway epithelial cells *ex vivo*.

A, B MTEC cultures were infected with a lentivirus expressing either GFP-GemC1 (GemC1) or GFP as a control and two days later transitioned to ALI culture conditions. Cells harvested at different time points during differentiation were co-stained with antibodies against GFP (green) to mark infected cells and endogenous McIdas (A) or Foxj1 (B) (red). Arrows indicate infected cells that are McIdas or Foxj1 positive. Scale bars, 10 μ m.

C, D The percentage of infected cells expressing McIdas (C) or Foxj1 (D) at each time point was quantified. Data are presented as the mean values of at least 10 independent fields obtained from at least two independent experiments for each condition. Error bars indicate \pm SEM. * $P < 0.05$, *** $P < 0.001$. *P*-values were calculated by the nonparametric two-tailed Mann–Whitney test.

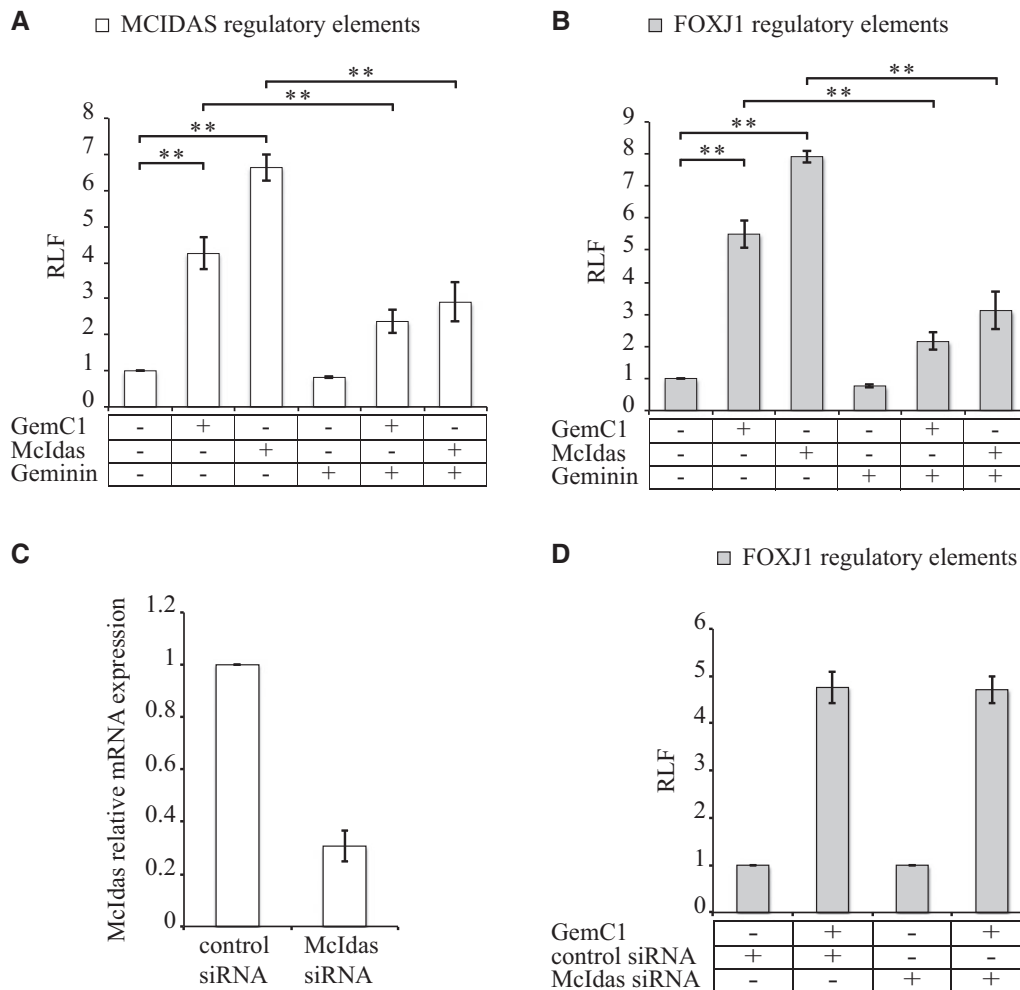


Figure 3. GemC1 directly activates the upstream regulatory sequences of MCIDAS and FOXJ1.

A–D MCIDAS (A) and FOXJ1 (B, D) regulatory elements (see Materials and Methods) cloned upstream of the luciferase gene were co-transfected into HEK293T cells with vectors expressing GemC1, McIdas and Geminin, as indicated or an empty vector (–) as a control. In (D), McIdas siRNAs or a control siRNA were also co-transfected. McIdas mRNA levels after McIdas RNAi were assessed by qPCR (C). All luciferase experiments (A, B, D) were normalized for transfection efficiency with an expression vector for Renilla luciferase. Fold induction is the ratio between the normalized luciferase activity induced by the expression constructs and that induced by the empty expression vector. Data are the mean values of at least three independent experiments, and error bars indicate \pm SEM. $**P < 0.01$. *P*-values were calculated by the nonparametric two-tailed Mann–Whitney test. Abbreviations: RLF: relative luciferase fold induction.

(Fig EV5A and B), GemC1, when co-expressed with E2F5, markedly increases expression from both the MCIDAS promoter (17.73-fold, Fig 4A) and the FOXJ1 promoter (10.84-fold, Fig 4B). A similar co-operation is seen between E2F5 and McIdas, but not Geminin, which cannot induce expression either alone or in combination with E2F5. In contrast, GemC1, McIdas, or Geminin did not affect E2F1-mediated transactivation of the MCIDAS and FOXJ1 promoters (Fig EV5C and D). E2F4 exhibited a pronounced transactivation activity on the MCIDAS and FOXJ1 promoters on its own (Fig EV5A and B) and could therefore not be assessed for co-operative interactions with GemC1.

In order to assess whether GemC1-containing protein complexes directly bind to the MCIDAS and FOXJ1 promoter elements in these reporter assays, chromatin immunoprecipitations were carried out. Cells were transfected with GemC1-GFP or GFP as a control,

together with E2F5, DP1 and the MCIDAS or FOXJ1 regulatory elements, and co-precipitation of specific DNA fragments was assessed by real-time PCR in GFP immunoprecipitates. As shown in Fig 4C and D, a subset of fragments from both the MCIDAS and FOXJ1 regulatory elements are enriched in the GemC1-GFP immunoprecipitates. This co-precipitation is specific, as different promoter fragments or a non-related genomic fragment is not enriched in GemC1-GFP immunoprecipitates, while no fragments are enriched in GFP immunoprecipitates.

We concluded that GemC1 specifically induces transcription from the upstream regulatory elements of both MCIDAS and FOXJ1 and co-operates with the E2F5 transcription factor for this induction. Our data identify GemC1 as an upstream transcriptional regulator, which controls the expression of other transcription factors (McIdas and FoxJ1) important for inducing ciliogenesis.

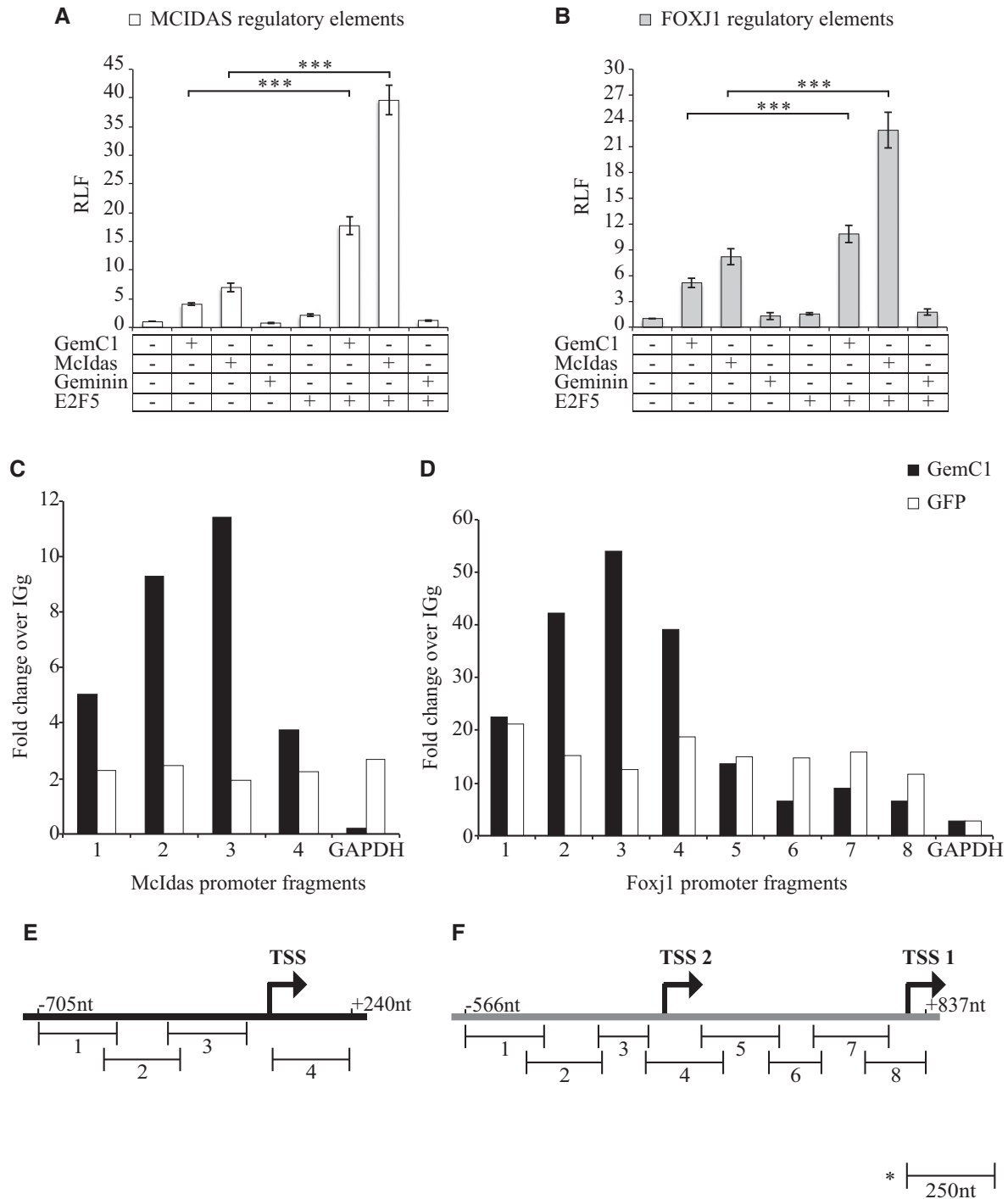


Figure 4. GemC1 co-operates with E2F5 and associates with MCIDAS and FOXJ1 promoter regions.

A, B MCIDAS (A) and FOXJ1 (B) regulatory elements cloned upstream of the luciferase gene were co-transfected into HEK293T cells with vectors expressing GemC1, MclDas, Geminin and E2F5, as indicated, or an empty vector (–) as a control. Luciferase values were normalized against co-transfected Renilla Luciferase and depicted as fold induction over the control empty vector. Data shown are mean values of at least three independent experiments, and error bars indicate \pm SEM. $***P < 0.001$. *P*-values were calculated by the nonparametric two-tailed Mann–Whitney test. Abbreviations: RLF: relative luciferase fold induction.

C, D Chromatin immunoprecipitation of MCIDAS and FOXJ1 promoter fragments by GemC1. HEK293T cells were co-transfected with vectors expressing GemC1–GFP (GemC1) or GFP as a control, E2F5 and DP1, together with the regulatory elements of either MCIDAS (C) or FOXJ1 (D). Following chromatin immunoprecipitation, DNA fragments of the MCIDAS and FOXJ1 promoters were detected in immunoprecipitates by qPCR. Data from a representative experiment are shown as fold enrichment of MCIDAS and FOXJ1 promoter fragments in anti-GFP immunoprecipitates over control IgG immunoprecipitates. At least two independent experiments were performed.

E, F Schematic representation of the promoter fragments from the MCIDAS (E) and FOXJ1 (F) regulatory elements assayed by chromatin immunoprecipitation. For FOXJ1, two putative transcription start sites (TSS) have been proposed and are indicated (see Materials and Methods).

GemC1 is essential for the formation of multiciliated cells in the respiratory epithelium

In order to determine whether GemC1 is necessary for multiciliate cell differentiation *in vivo*, we generated GemC1-knockout (KO) mice. A KO allele, generated by the insertion of a lacZ-neo cassette [38] between exons 2 and 3 (Fig 5A, see Materials and Methods), was bred to homozygosity.

GemC1-KO mice are born at normal ratios and without gross abnormalities. They, however, show poor postnatal weight gain, even though they exhibit normal feeding behavior and exhibit severe growth retardation in comparison with wild-type or heterozygous littermates (Fig 5B). GemC1-KO animals die within the first postnatal week (Fig 5C).

To assess whether multiciliate cell differentiation is affected in the absence of GemC1, airways of neonatal mice (P0) homozygous for the GemC1 deletion (GemC1^{KO/KO} mice) were compared with control wild-type littermates. Immunofluorescence for acetylated α -tubulin, which labels ciliary axonemes, and for pericentrin, which marks nascent centrioles/basal bodies, was used to compare the sections of the trachea of GemC1-knockout animals with their wild-type littermates. As shown in Fig 6A, cilia are absent in

GemC1^{KO/KO} mouse tracheal epithelial cells, while pericentrin reveals only a couple of centrioles per cell. On the contrary, multiple cilia are detected on the tracheal cells from GemC1^{WT/WT} mice, protruding from multiple basal bodies docked in the cell membrane. Lack of centriole amplification in the tracheas of GemC1-knockout animals was also verified using γ -tubulin (Fig 6B). We concluded that ciliogenesis is blocked at an early stage in tracheal epithelial cells homozygous for the GemC1 deletion.

To assess the reason for failed ciliogenesis in GemC1-knockout mice, the effect of the absence of GemC1 on McIdas and FoxJ1 expression in the mouse tracheal epithelium was examined in newborn mice by immunofluorescence using specific antibodies against McIdas and FoxJ1. Both McIdas and FoxJ1 were undetectable in GemC1-deficient trachea, but not in the tracheal cells from control littermates, where McIdas- and FoxJ1-positive cells were clearly detected (Fig 6C). To assess whether McIdas and FoxJ1 reduction is apparent early during airway development in mutant mice, McIdas and Foxj1 mRNA expression levels were assessed by real-time PCR in the tracheas isolated at embryonic stage E16.5 from GemC1-knockout and control littermates. As shown in Fig EV6, McIdas and Foxj1 mRNA expression was markedly reduced already from E16.5 in the tracheas of animals

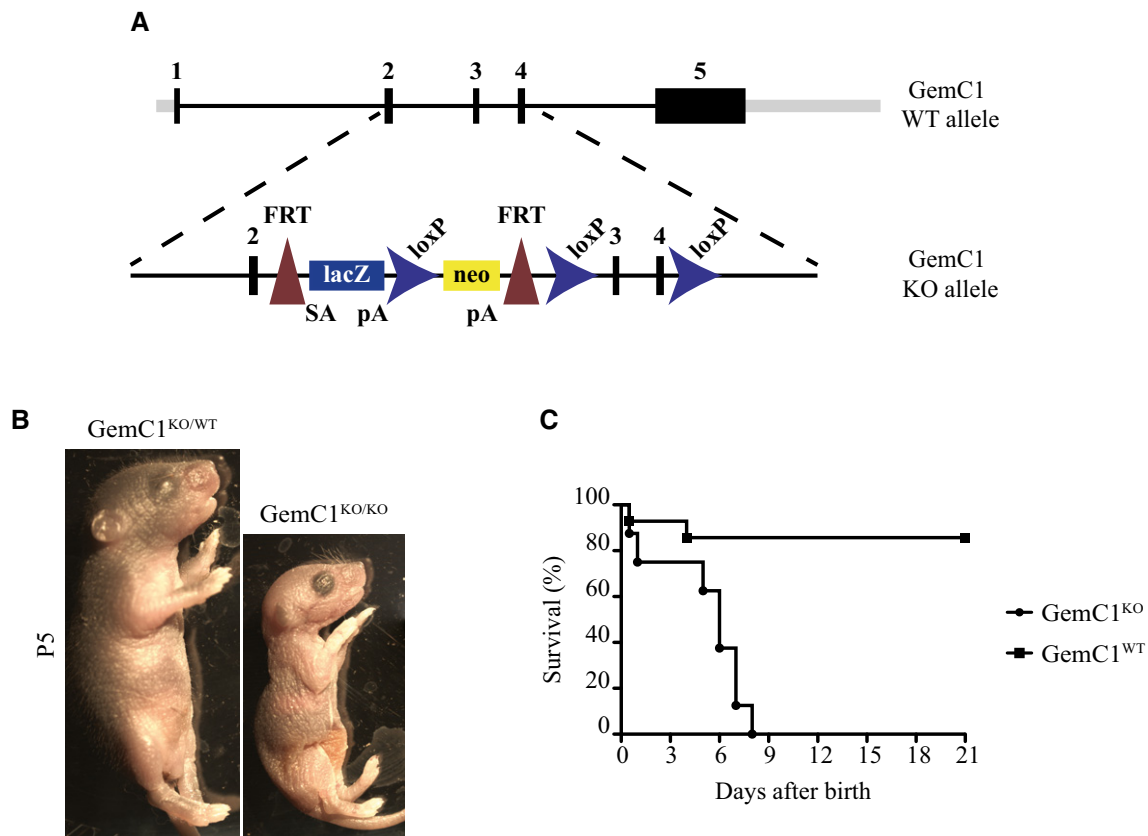


Figure 5. GemC1-deficient mice die within the first postnatal week.

A Schematic representation of the wild-type (WT) and knockout (KO) GemC1 alleles. A lacZ-neo cassette has been inserted between exons 2 and 3 of the GemC1 gene. A splicing acceptor site (SA) upstream of lacZ ablates gene function (knockout-first allele, [38])

B GemC1^{KO/KO} mice compared to their wild-type littermates (GemC1^{KO/WT}) at P5.

C Survival plot of mice homozygous for the GemC1 deletion (GemC1^{KO/KO}, $n = 8$) and control wild-type or heterozygous littermates GemC1 (GemC1^{WT}, $n = 14$). GemC1-knockout mice exhibit markedly reduced growth after birth and die within the first postnatal week.

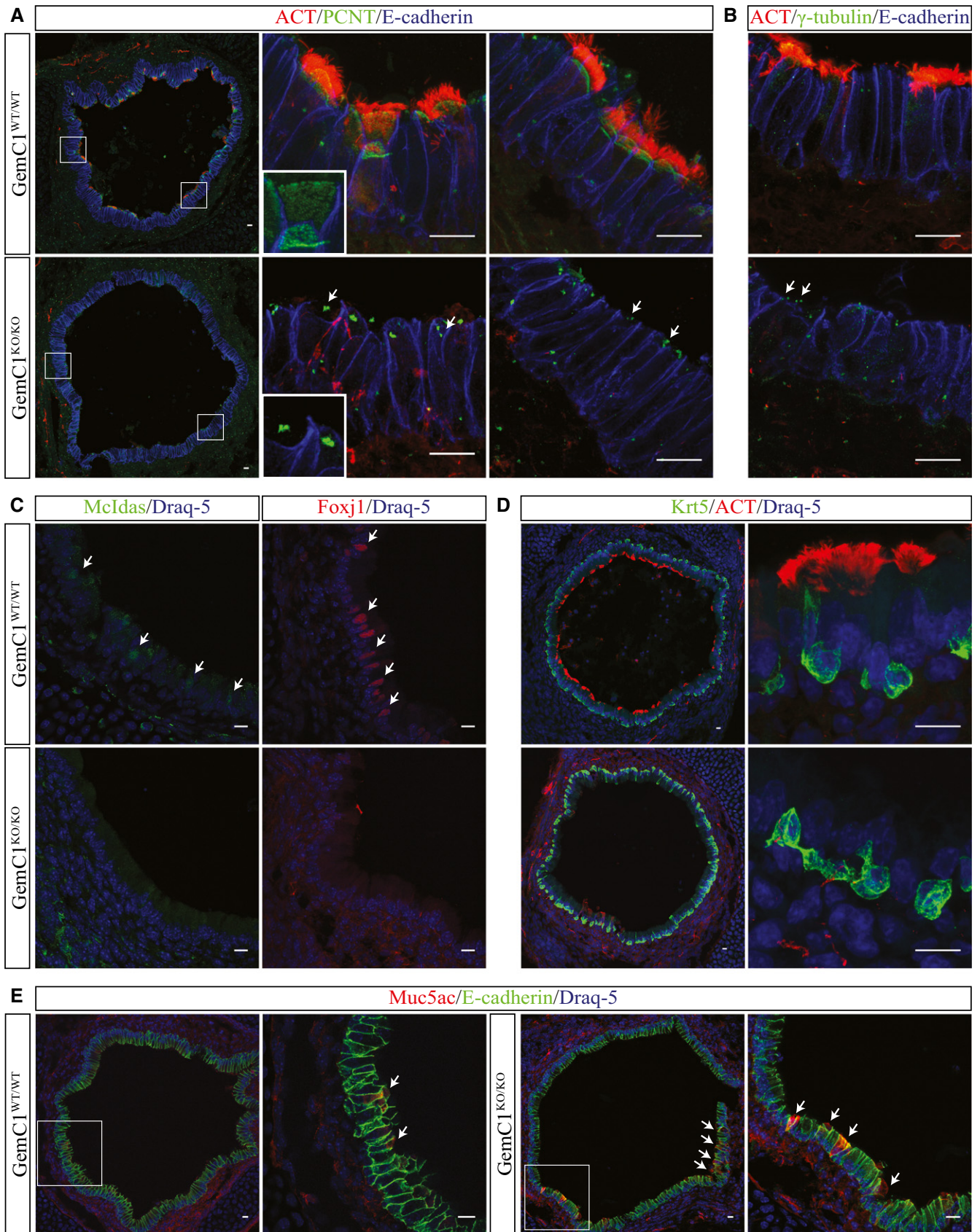


Figure 6.

Figure 6. Multiciliate cell differentiation is blocked in the absence of GemC1.

- A, B Transverse sections from trachea isolated from GemC1^{WT/WT} (upper) or GemC1^{KO/KO} (lower) P0 mice, immunostained with antibodies against acetylated α -tubulin (ACT, cilia marker, red), pericentrin (PCNT, marker for nascent centrioles, green), γ -tubulin (centriole marker, green) and E-cadherin (marker for cell boundaries, blue). Serial images have been merged to show the complete trachea section (left). Inserts show higher-magnification images for acetylated α -tubulin and pericentrin staining in GemC1^{WT/WT} and GemC1^{KO/KO} trachea, respectively. Scale bars, 10 μ m.
- C McIdas- and FoxJ1-specific antibodies were used for immunofluorescence on trachea from GemC1^{WT/WT} (upper) or GemC1^{KO/KO} (lower) P0 mice. Arrows show representative positive cells for McIdas (green) and FoxJ1 (red) in the GemC1^{WT/WT} mice. DNA was stained with Draq-5 (blue). Scale bars, 10 μ m.
- D Transverse sections from P0 GemC1^{WT/WT} (upper) or GemC1^{KO/KO} (lower) trachea, immunostained with antibodies against keratin 5 (Krt5, basal cell marker, green) and acetylated α -tubulin (ACT, cilia marker, red). DNA was stained with Draq-5 (blue). Serial images have been merged to show the complete trachea section on the left. Higher-magnification images are shown on the right. Scale bars, 10 μ m.
- E Transverse sections from P0 GemC1^{WT/WT} (left two images) or GemC1^{KO/KO} (right two images) trachea, immunostained with antibodies against Muc5ac (marker for mucus-expressing cells, red) and E-cadherin (marker for cell boundaries, green). DNA was stained with Draq-5 (blue). Serial images have been merged to show the complete trachea section (left). Higher magnifications from the same section are shown on the right. Scale bars, 10 μ m.

homozygous for the GemC1 deletion. We therefore concluded that in the absence of GemC1, McIdas and FoxJ1 fail to be expressed early during airway development and multiciliated cell differentiation is blocked.

We next wished to investigate whether other cell types in the airway epithelium are affected in GemC1-knockout animals. We therefore stained tracheas from newborn GemC1-knockout and control littermates with markers for basal cells (keratin 5) and mucus-producing cells (Muc5ac). As shown in Fig 6D, basal cells appear unaffected in GemC1-knockout animals. Mucus cells are clearly detected and their numbers appear increased in newborn mice homozygous for the GemC1 deletion in comparison with control littermates.

We therefore concluded that in the absence of GemC1, the generation of multiciliated cells is specifically blocked early in the ciliogenesis process, consistent with a central role for GemC1 as an early regulator of multiciliogenesis.

Discussion

Our findings showed that GemC1 is a central regulator of multiciliate cell differentiation in the airway epithelium. GemC1 was originally identified as a factor implicated in DNA replication [9]. It was shown to interact directly with replication factors, to be required for the formation of preinitiation complexes onto origins of replication and to stimulate chromosomal DNA replication. We showed that GemC1 exhibits a highly specific expression pattern in ciliated epithelia during the development and in the adult. This suggests a role of GemC1 in the differentiation of ciliated cells. The expression pattern of GemC1 is highly overlapping with McIdas and FoxJ1 expression, suggesting that they co-operate in establishing the multiciliated cell fate. Indeed, in a mouse tracheal epithelial cell culture system, GemC1 ectopic expression is sufficient to induce the expression of both McIdas and FoxJ1. GemC1 directly activates transcriptionally the upstream regulatory elements of these genes, in co-operation with the E2F5 transcription factor. Chromatin immunoprecipitation assays showed that GemC1-containing complexes are associated with MCIDAS and FOXJ1 regulatory elements under these conditions. Consensus E2F4/5 sites are present in these regulatory elements, suggesting that GemC1 association with promoter DNA may be mediated through E2F family members. It would be interesting to investigate whether GemC1 binds to E2F family members and whether this binding may regulate its activity. MCIDAS is flanked in the genome [21] by two more

genes implicated in multiciliogenesis: CCNO, required for multiciliogenesis downstream of McIdas [39,40], and CDC20B, which contains intronic miRNAs of the miR-34/449 family, also implicated in multiciliogenesis [41,42]. It is intriguing to speculate that GemC1 may activate this entire genomic locus in cells initiating a multiciliogenesis differentiation program.

Mice lacking GemC1 are born at normal ratios and without apparent developmental defects. This suggests that GemC1 is not essential for DNA replication in the early embryo and during development. This differs from Geminin deletion mice, which die at the preimplantation stage [18]. An involvement of GemC1 in DNA replication, however, is not incompatible with this observation, as several cell cycle regulators are known to act redundantly and their deletion does not lead to embryonic lethality. GemC1, however, does have an essential function in multiciliogenesis. GemC1-knockout mice are born with airway epithelia devoid of multiciliated cells and die soon after birth. This phenotype is reminiscent of FoxJ1-knockout mice [26], with death likely caused by defects in airway mucus clearance. Situs inversus has not been observed in these mice, though a low penetrance phenotype cannot be excluded. Similarly, MCIDAS-deficient patients do not have situs inversus. Consistent with GemC1 acting as an upstream transcriptional regulator during multiciliogenesis, McIdas and FoxJ1 expression is severely decreased in GemC1-knockout animals already from early development of the airway epithelium (E16.5) and is undetectable in newborn mice. Multiciliation is inhibited at an early stage: Airway epithelial cells in mice homozygous for GemC1 deletion exhibit only a couple of centrioles and no detectable cilium, in sharp contrast to the hundreds of centrioles and cilia present in wild-type airways. This complete block of ciliogenesis observed in GemC1-knockout mice is reminiscent of and appears more severe than the phenotype seen in patients with mutations in McIdas [30], where most airway epithelial cells lack cilia or occasionally exhibit one or two cilia, and the phenotype of CCNO deletion mice, where small numbers of basal bodies and cilia are formed [39]. This severe phenotype is consistent with an early role for GemC1 during ciliogenesis. Consistent with a role for GemC1 in fate decisions in the airway epithelium, keratin 5-expressing basal progenitor cells appear unaffected in tracheas from newborn mice homozygous for GemC1 deletion, while mucus-producing cells appear increased. In the airway epithelium, a dynamic balance between ciliated and secretory cells is essential for homeostasis and regeneration [4–6]. Our data suggest that GemC1 is a central regulator of the multiciliate cell fate likely to play a key role in airway physiology and pathology.

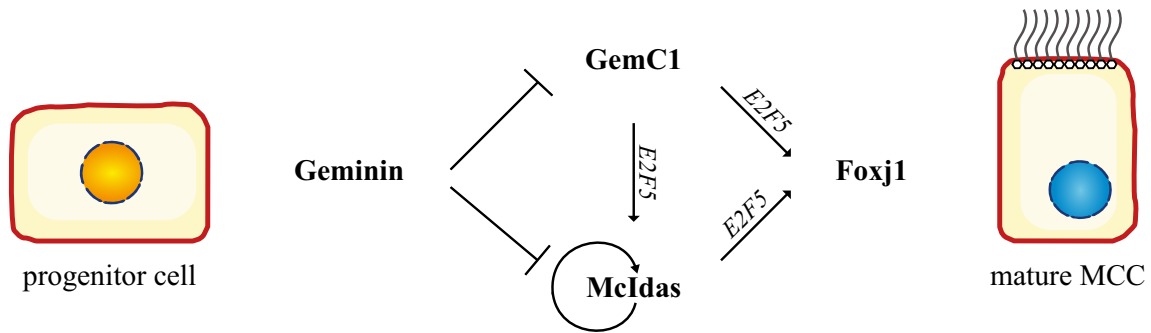


Figure 7. GemC1 is a central regulator of multiciliate cell differentiation.

Model illustrating GemC1 as an early regulator of multiciliogenesis through the control of transcription factors McIdas and Foxj1. Abbreviations: MCC: multiciliated cells.

It is likely that the function of GemC1 may not be restricted to the airway epithelium, but may be extended to other ciliated tissues. McIdas was shown to affect centrosome numbers in cycling cells [21] and to induce multiciliogenesis in the *Xenopus* embryonic skin [23]. Very recently, McIdas and GemC1 were shown to affect the differentiation of ependymal cells in the mouse brain [43], while a GemC1 orthologue was shown to control multiciliate cell differentiation in the zebrafish embryo [44]. GemC1 and McIdas may impose a state permissive for centrosome amplification and ciliogenesis in different cell types, across evolution. Their involvement in DNA replication may serve to link events during a normal cell cycle to the transition to ciliogenesis. It would be interesting to investigate other defects in GemC1-knockout animals which could link GemC1 to centrosome and cilia formation and pathology in multiple systems.

GemC1 shares limited sequence similarity with McIdas [21], evident in a small, phylogenetically conserved region located at the C-termini of both proteins. This region in McIdas has been implicated in interactions with E2F family members [24] and is mutated in patients with a rare hereditary mucociliary disorder [30]. The conservation of this region in GemC1, together with the co-operative interactions we observed between GemC1 and E2F5 for transcriptional regulation of the FoxJ1 and McIdas upstream regulatory sequences, makes it likely that GemC1 may directly bind to E2F family members. It also highlights the C-terminus of GemC1 as a candidate region for mutations in patients. GemC1 and McIdas also contain a coiled-coil region with similarity to the coiled-coil of the cell cycle regulator Geminin. The McIdas, GemC1 and Geminin coiled-coils form tight heterodimers [22,45]. We showed that Geminin antagonizes both GemC1 and McIdas, inhibiting their transactivation potential. Geminin has been shown to be expressed in proliferating cells [34,35] and to regulate proliferation–differentiation decisions [14–19,46,47]. Consistently, we observed the expression of Geminin in proliferating MTECs, which is decreased as cells progress toward differentiation, in parallel with the induction of GemC1, McIdas and FoxJ1. Our data point to the following model for the regulatory events that control multiciliogenesis (Fig 7): In proliferating progenitor cells, Geminin is expressed and GemC1 and McIdas are at low levels and inhibited by Geminin. Notch signaling may be important for maintaining this state [48,49]. As cells commit to differentiation, Geminin levels decrease and GemC1 levels increase, leading to the expression of McIdas and FoxJ1. McIdas is

able to transactivate its own promoter, creating a positive feedback loop that locks commitment toward multiciliation. In this model, GemC1 is central for the establishment of the multiciliate cell fate. It would be highly interesting to investigate how GemC1 expression and interactions with Geminin and McIdas are regulated to control multiciliogenesis in health and disease.

Materials and Methods

RNA purification and real-time PCR

Total RNA from MTECs and mouse embryonic tissues was isolated using the RNeasy micro kit (Qiagen), while RNA from adult mice tissues was extracted with TRIzol (Ambion). RNA from HEK293T cells was extracted using the Nucleospin RNA II kit (Macherey-Nagel); 1 µg RNA was converted to cDNA using M-MLV reverse transcriptase (Invitrogen). GemC1, McIdas, Foxj1 and Geminin mRNA expression levels were assessed by quantitative real-time PCR (Applied Biosystems StepOne), using the Kapa SYBR Fast qPCR kit (KapaBiosystems, KK4605). HPRT or YWHAZ mRNA expression levels were used for normalization. Primer sequences are shown in Table EV1. At least three independent experiments were analyzed in duplicates for all data shown. For qPCR data analysis, the REST-MCS beta software was used.

Lentiviral production

Lentiviral expression vectors were kindly provided by Dr. M. Götz, Helmholtz Center, Munich. GFP or GemC1 with an N-terminal GFP tag (GFP-GemC1) was initially cloned into the BamHI/XhoI and KpnI/XbaI restriction sites of the pENTR1AminusCmR vector, respectively. An LR recombination reaction, using the Gateway LR Clonase II enzyme mix (Invitrogen, 11791), was performed between the attL-containing entry clone and the attR-containing destination pLVDest-CAG vector.

A second-generation packaging system [50] was used. HEK293T cells, grown in DME medium (Gibco) with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco), were used as producer cells. In short, lentiviral particles were generated by transient co-transfection of HEK293T cells with the expression vector carrying either the GFP or the GFP-GEMC1 gene and the two helper plasmids psPAX2

(packaging vector, Addgene 12260) and pMD2.G (envelope vector, Addgene 12259), using Turbofect (Fermentas) as transfection reagent. The lentiviral supernatant was harvested 48 h later and filtered with 0.45- μ m filter.

Mouse tracheal epithelial cell culture (MTEC) and lentiviral infection

MTECs were isolated from wild-type C57/Bl6 mice of 8 weeks of age or older, as described previously [36]. Briefly, epithelial cells were isolated from mouse trachea by protease digestion (Pronase, Roche), seeded onto collagen-coated supported, semipermeable membranes (Transwell filters, Corning), and cultured at 37°C and 5% CO₂ in MTEC complete medium [51] to favor proliferation. Two days after confluency, an ALI condition was established by adding a serum-free differentiation medium [51] only in the basal chamber. Ciliogenesis initiates 2–3 days after ALI creation.

To infect MTECs, 5 days after the initiation of the culture, epithelial tight junctions were disrupted by treating cells with 12 mM EGTA in 10 mM Hepes, pH 7.4, at 37°C for 20 min. Cells were spin-infected at 1,300 g for 80 min with an unconcentrated lentiviral preparation (approximately 10⁶ infectious units per milliliter) containing 5 μ g/ml hexadimethrine bromide (Sigma-Aldrich). The infection mix was removed 24 h later, and an ALI condition was set up 2 days after the infection.

Mouse-knockout strain, *in situ* hybridization and immunohistochemistry

C57/Bl6 and Parkes mice were housed in the animal house of the University of Patras. C57/Bl6 mice carrying a knockout (KO) allele of the GemC1 gene were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP repository. The KO allele was generated by the insertion of a lacZ-neo cassette between exons 2 and 3. Specifically, a “knockout-first” strategy that ablates gene function by the insertion of RNA processing signals without the deletion of any of the target gene was followed, as described previously [38]. All experiments involving animals were approved by the Veterinary Administrations of the Prefecture of Achaia, Greece, and were conducted in strict accordance with EU Directives.

Mouse embryos at E10.5, E12.5, E14.5, E15.5 and E18.5 dpc were fixed overnight with 4% PFA, cryopreserved using 30% sucrose, frozen in 7.5% gelatin plus 15% sucrose, and sectioned at 10 μ m. The day of the vaginal plug was defined as embryonic day E0.5. Non-radioactive *in situ* hybridization was performed using DIG-labeled RNA probes, as described previously [19]. For the generation of mouse GemC1 RNA probe, the cDNA was cloned in a pBlue-script vector between SalI/EcoRV restriction sites. The mouse McIDAS cDNA was used as described in Pefani *et al* [21].

Mouse tracheas isolated from neonatal mice were fixed overnight with 4% PFA at 4°C, cryopreserved using 30% sucrose, frozen in Tissue-Tek, and sectioned at 10 μ m. For immunohistochemistry, transverse sections were fixed with 4% PFA for 10 min, treated with 0.3% Triton X-100 for 5 min, and incubated in blocking solution containing 10% FBS, 3% BSA, 0.1% Tween 20 in 1 \times PBS, for 1 h. Samples were incubated with primary antibodies in blocking solution at 4°C, overnight. The primary antibodies used were as follows:

rabbit anti-hMcIDAS [21] (1:250), mouse anti-Foxj1 (1:500, eBioscience), rabbit anti-Foxj1 (1:500, Sigma), rabbit anti-pericentrin (1:1,000, Covance), rabbit anti- γ -tubulin (1:1,000, Sigma), mouse anti-acetylated α -tubulin (1:2,000, Sigma), rat anti-E-cadherin (1:500, Invitrogen), rabbit anti-Krt5 (1:2,000, Covance), and mouse anti-Muc5ac (1:500, ThermoFisher Scientific). The following Alexa Fluor-labeled secondary antibodies (Invitrogen) were used at 1:500 in blocking solution, for 1 h: Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 568 goat anti-mouse, Alexa Fluor 568 goat anti-mouse IgG1. DNA was stained either with Hoechst 33258 (1:1,500, Sigma) or with Draq-5 (1:1,000, Biostatus). Sections were mounted in Mowiol 4-88 (Calbiochem).

For combined *in situ* hybridization–immunofluorescence experiments (Fig EV3), sections were first labeled using the DIG-labeled GemC1 or McIDAS RNA probes, as described previously [19]. Following hybridization, the slides were incubated simultaneously with an alkaline phosphatase-conjugated sheep anti-DIG-AP, Fab fragments (1:5,000, Roche) and an antibody against Foxj1 (1:200, Sigma) in blocking solution at 4°C, overnight. Staining for the DIG-labeled RNA probes was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (Roche). After the development of the signal, the slides were blocked for 1 h and incubated with a secondary antibody (Alexa Fluor-labeled, Invitrogen) at 1:500 in blocking solution at 4°C, overnight. Sections were mounted with Glycergel (DakoCytomation, Carpinteria, CA, USA).

Immunofluorescence in MTECs was performed as described previously [37]. Cells were analyzed at different time points using the following antibodies: rabbit anti-hMcIDAS [21] (1:500), rabbit anti-Foxj1 (1:500, Sigma), and mouse anti-GFP (1:500, Molecular Probes) and Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse as secondary antibodies. Quantifications were carried out on filters from at least two independent infections. Statistical analysis was done with the nonparametric two-tailed Mann–Whitney test.

Images from *in situ* hybridization were captured on a Nikon Eclipse TE 200-U inverted microscope using 4 \times and 20 \times lenses. Images from MTECs and mouse tracheas were recorded on a Confocal Leica SP5 microscope, using 40 \times and 63 \times lenses. Digital images were processed with Adobe Photoshop, Adobe Illustrator, and Fiji software.

Luciferase reporter assay

1-kb, 1.5-kb and 2-kb genomic regions upstream of the human MCIDAS, FOXJ1 and GEMC1 (GMNC) translation start sites, respectively, were cloned upstream of the coding sequence for firefly luciferase in the pGL3 basic vector (Promega) to create pGL3-MCIDAS, pGL3-FOXJ1 and pGL3-GEMC1 constructs, respectively. Specifically, the upstream regulatory sequences used were nucleotides –767 to +233 for MCIDAS (hg19, chr5: 54522910–54523910) and –535 to +1,465 for GEMC1 (GMNC, hg19, chr3: 190579000–190581000), with respect to the predicted transcription start site. For the FOXJ1 gene, two putative transcription start sites have been predicted, TSS1 (hg19, chr17: 74136645) and TSS2 (hg19, chr17: 74137380) (see Fig 4). Foxj1 regulatory sequence used were hg19, chr17: 74136502–74138069, corresponding to nucleotides –1,424 to +143 with respect to the TSS1 and nucleotides –566 to +837 with respect to the TSS2. Expression vectors used for co-transfections were as

follows: cDNAs for mouse GemC1 and McIdas and human Geminin cloned in the pCAGGS expression vector and human E2F1, E2F4 and E2F5 in the pCMV vector (Addgene 24225, 37966 and 24213, respectively).

HEK293T cells (3.5×10^4 cells) were co-transfected in a 96-well plate, using Turbofect (Fermentas), with a total of 130 ng DNA, containing 25 ng of pGL3-MCIDAS or pGL3-FOXJ1 with 50 ng of the expression constructs (empty vector, pCAGGS-GemC1, pCAGGS-McIdas, pCAGGS-Geminin, pCMV-E2F5) and 5 ng of the Renilla luciferase phRL-SV40 vector (Promega), as a transfection efficiency control.

For McIdas RNAi experiments, the following STEALTH siRNAs (Invitrogen), 5'-CCACCAAACGGAAGCAGACTTCAAT-3' and 5'-CCAAACAGGAACGGACATTCCCAAT-3', were used as described previously [21]. An irrelevant control siRNA was also used: 5'-CCAAACAGGAACGGACATTCCCAAT-3'. Briefly, HEK293T cells (3.5×10^4 cells) were co-transfected in a 96-well plate, using Turbofect (Fermentas), with a total of 130 ng DNA, containing 25 ng pGL3-FOXJ1 with 50 ng of the expression constructs (empty vector, pCAGGS-GemC1), 5 ng of the Renilla luciferase phRL-SV40 vector (Promega), as a transfection efficiency control, and 9 nM of each McIdas siRNAs or 18 nM of control siRNA, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. A second treatment with the siRNAs followed 24 h later and cells analyzed either with qPCR for the quantification of McIdas mRNA, or for the Firefly and Renilla luciferase activity, 24 h after the second transfection.

Firefly and Renilla luciferase activity was measured in cell lysates 48 h after transfection with the Dual-Luciferase Reporter Assay Kit (Promega, E1980), in a Victor Light Luminescence plate reader. The transcriptional activity of the regulatory elements was measured as the ratio of Firefly to Renilla luciferase signal, normalized to the values obtained when the empty vector was co-transfected. Results from at least three independent transfections are presented as mean fold induction relative to the control expressing vector, with standard error of the mean. Statistical analysis was done with the nonparametric two-tailed Mann–Whitney test.

Chromatin immunoprecipitation assay

HEK293T cells were co-transfected in a 6-well plate, using Turbofect (Fermentas), with a total of 1 μ g DNA, containing 250 ng each of the following plasmid DNAs: pGL3-MCIDAS or pGL3-FOXJ1, pCDNA3.1-GemC1-GFP or pCDNA3.1-GFP, E2F5 and DP1. Forty-eight hours after transfection, the cells were fixed sequentially with 2 mM di(N-succinimidyl) glutarate (Sigma) and 1% formaldehyde (Sigma) and quenched with 0.125 M glycine, followed by lysis with 1% SDS, 10 mM EDTA, and 50 mM Tris pH 8. Sonication was performed in a Bioruptor (Diagenode) for 50 cycles (30 s on/off, power settings high), using 3×10^6 cells; 20 μ g of chromatin was precipitated with 5 μ g of either a rabbit GFP antibody (Molecular Probes) or a rabbit control IgG (Sigma). Immunoprecipitates were retrieved with 50 μ l of magnetic Dynabeads conjugated to protein G (Invitrogen) and analyzed by quantitative real-time PCR (Applied Biosystems StepOne), using the Kapa SYBR Fast qPCR kit (KapaBiosystems, KK4605). The primers used to amplify different fragments of the promoter regions of MCIDAS and FOXJ1 are listed in Table EV2. Primers for GAPDH were used as a negative control.

Two independent experiments were analyzed in duplicate for all data shown. Fold change was calculated relative to the control IgG signals.

Expanded View for this article is available online.

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Author contributions

MA designed the study, performed experiments, performed data analysis and interpretation, and wrote the paper. DEP, CK, MEL, AK, and ADP performed experiments. ST designed the study. ZL designed the study, performed data analysis and interpretation, and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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