Transcriptional regulation of multiciliated cell differentiation

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Abstract:

Multiciliated cells (MCC) project dozens to hundreds of motile cilia from the cell surface to generate fluid flow across epithelial surfaces or turbulence to promote the transport of gametes. The MCC differentiation program is initiated by GEMC1 and MCIDAS, members of the geminin family, that activate key transcription factors, including p73 and FOXJ1, to control the multiciliogenesis program. To support the generation of multiple motile cilia, MCCs must undergo massive centriole amplification to generate a sufficient number of basal bodies (modified centrioles). This transcriptional program involves the generation of deuterosomes, unique structures that act as platforms to regulate centriole amplification, the reactivation of cell cycle programs to control centriole amplification and release, and extensive remodeling of the cytoskeleton. This review will focus on providing an overview of the transcriptional regulation of MCCs and its connection to key processes, in addition to highlighting exciting recent developments and open questions in the field.

Keywords:

Multiciliated cells; E2F4; E2F5; GEMC1; MCIDAS; FOXJ1; MYB; p73; NOTCH; AHR; TRRAP; CCNO; CDC20B; centriole; basal body; airway; ependyma; germline; ciliopathy; infertility; hydrocephalus

Abbreviations

MCC:	Multiciliated cell
GMNC:	GEMC1, Geminin coiled-coil containing protein 1
MCIDAS:	Multicilin, Idas
GMNN:	Geminin
Air liquid interface:	ALI
Basal body:	BB
Radial Glial Cells:	RGC
Pericentriolar material:	PCM

Highlights

- GEMC1 and MCIDAS control multiciliated cell differentiation in a stepwise manner.
- p73 plays a major role in multiciliogenesis.
- Multiciliated cells activate a cell cycle program to regulate centriole amplification.
- PLK4, mother centrioles and deuterosomes are not strictly required for multiciliogenesis.
- Centriole numbers scale to cell surface area.

Multiciliated cells in vertebrates

Multiciliated cells (MCCs) are specialized epithelial cells that project multiple motile cilia required for respiratory, reproductive, renal and brain functions in many vertebrates[1]. In humans, MCCs are present in the ependyma and choroid plexus of the brain to direct the flow of cerebrospinal fluid, the airways to clear mucus and pathogens, and in the efferent ducts and oviducts for spermatozoa and egg transport, respectively. Depending on the tissue, dozens to hundreds of motile cilia are generated per MCC that can beat in a coordinated, directional manner or generate turbulence through whip-like movements[1,2].

The process of MCC differentiation, or multiciliogenesis, requires the activation of a unique transcriptional program that specifies cell fate and allows the massive amplification of centrioles; barrel-shaped, microtubule based organelles that dock at the cell surface with other factors to provide a basal body (BB) required to support the generation of the ciliary axoneme[3]. As several recent reviews have covered different aspects of multiciliogenesis in great detail[1,3–10], here we will focus on providing an overview of the transcriptional regulation of multiciliogenesis and how it connects to key cellular processes. In addition, we will highlight recent advances in our understanding of other cell biological aspects of MCC development and the consequences of their dysfunction.

MCC specification: Notch and the Geminin family proteins

The inhibition of Notch signaling has emerged as a consistent early event in MCC differentiation from the study of frog skin, zebrafish pronephros and murine ependymal, fallopian tube (oviduct) and airway epithelia (Figure 1) [11–18]. The precise details of Notch regulation remain unclear in all cases but the *Mir-34/449* family of miRNAs has been implicated in Notch inhibition in frogs, zebrafish and mice and this miRNA family plays redundant roles in MCC formation in several tissues, including the brain, airway and male germline[2,11,13,19–23]. In the murine airway, progenitor cells give rise to secretory (Clara) or MCC lineages in a Notch dependent manner[24]. Genetic or pharmacological inhibition of Notch causes the trans-differentiation of secretory cells into MCCs, demonstrating a central role for Notch inhibition in initiating the MCC differentiation program in the airway[15].

The full effects of Notch inhibition on transcription have not been clearly elucidated at early steps of MCC differentiation, but fate decisions following Notch inhibition are controlled by the interplay between the Geminin family proteins; Geminin (encoded by *GMNN*), GEMC1 (Geminin coiled-coil-domain containing protein 1, encoded by *GMNC*) and MCIDAS (Multicilin (MCI) and IDAS, encoded by *MCIDAS*). Geminin is a well-studied regulator of DNA replication that prevents re-replication through its cell cycle specific binding to CDT1, a factor required for origin licensing[25]. As its name reflects, Geminin acts as a dimer, homodimerizing through its central coiled-coil (CC) domain. Both

GEMC1 and MCIDAS were identified due to the presence of a similar CC domain and all 3 proteins can homo or heterodimerize[26–29]. This feature appears to be functionally relevant, as the CC domains are required for some of their key functions in all cases, although the physiological significance of heterodimerization remains unclear[30,31].

Studies in zebrafish and mice identified GEMC1 as a critical upstream mediator of the MCC differentiation program that is activated upon Notch inhibition[30,32–34]. GEMC1 deficient mice lack MCCs in every tissue where they are normally present, leading to penetrant hydrocephalus and infertility. While MCCs are absent from the murine airway in GEMC1 deficient mice, no respiratory defects have been reported, potentially due to sterile housing conditions, although significant discrepancies remain regarding the lifespan of GEMC1 deficient mice derived in different colonies where they range from 9 days to over 2 years[30,32].

The induction of *Gemc1* and *Mcidas* in the developing murine brain is temporally distinct and MCIDAS deficient mice develop morphologically identifiable MCCs that express early transcription factors, including p73 and FOXJ1, but fail to amplify centrioles or generate cilia[33,35]. Air liquid interface (ALI) cultures from MCIDAS deficient mice showed no increase in the expression of genes implicated in centriole amplification, including *CCNO*, *CDC20B*, *CCDC78* or *DEUP1*, indicating that GEMC1 is not sufficient to activate these genes when under its normal physiological regulation[35]. Thus, a two-step process has been proposed; GEMC1 activates *MCIDAS* and other key transcription factors to promote MCC specification and MCIDAS subsequently activates the expression of genes required for multiciliogenesis[35]. The overexpression of *MCIDAS* can trigger MCC differentiation in frog skin and mouse cells (the latter in conjunction with an E2F4-activation domain fusion), indicating that *MCIDAS* is one of the most crucial targets of GEMC1[36,37]. Consistent with this, overexpression of GEMC1 in frog embryos or murine ALI cultures also generates supernumerary MCCs in a manner that requires MCIDAS in ALI cultures[34,35]. Curiously, the overexpression of human or zebrafish GEMC1 is not sufficient to generate supernumerary MCCs in zebrafish, potentially indicating that essential cofactors or important post-translational modifications of GEMC1 are limiting[34].

Transcriptional activation by GEMC1 and MCIDAS

As GEMC1 and MCIDAS lack clear DNA binding domains, it remained an open question as to how they could activate transcription. Work in frogs first showed that a C-terminal domain of MCIDAS, absent in Geminin, dubbed the TIRT domain due to a repetitive amino acid motif sequence, interacted with the E2F4 and E2F5 transcription factors heterodimerized with DP-1[38]. This connected the activity of MCIDAS to previous and subsequent work that established a key role for E2F4 and E2F5 in murine brain, airway and germline MCC development[38–41]. While it lacks the specific TIRT amino-acid repetition, the C-terminus of GEMC1 contains a homologous “TIRT” domain that is also required for E2F4/5-DP1 binding and transcriptional activity[30,32]. Recent evidence suggests that the TIRT

domains of GEMC1 and MCIDAS are not functionally identical. In immunoprecipitation experiments, mouse and human GEMC1 showed a higher affinity for E2F5 than E2F4 and this specificity could be eliminated by replacing the TIRT domain of GEMC1 with that of MCIDAS[35]. Moreover, co-expressing E2F5, but not E2F4, with GEMC1 enhanced the activation of FOXJ1. In contrast, MCIDAS was shown to have similar affinity to both E2F4 and E2F5, potentially enabling more efficient activation of the genes necessary for centriole amplification[35]. One possibility that is consistent with current data is that GEMC1 displaces pocket protein inhibitors of E2F5, such as RB, p107 or p130, but does not strongly activate the MCC transcriptional program until it is reinforced by *MCIDAS* expression and its interactions with both E2F4 and E2F5. While more detailed biochemical and structural studies remain to be performed, the emerging picture is that GEMC1 and MCIDAS play sequential roles that utilize specific E2F4/5-DP1 interactions influenced by differences in their C-terminal domains.

While both E2F4 and E2F5 have been clearly linked to MCC generation, discerning their specific roles has been challenging in mice due to their essentiality and influence on the development of many tissues. E2F4 and E2F5 have been typically characterized as repressive E2Fs, in contrast to the activating E2Fs, E2F1-3, and bind to the regulatory regions of hundreds of genes to repress cell cycle genes during G0 and G1 in conjunction with the RB family proteins and interactions with the MuvB/DREAM complex[42]. Recent work in zebrafish showed that some tissue specificity exists with regards to their role in MCC generation, but that this reflects the relative expression levels of *E2f4* or *E2f5* in those tissues, rather than specific functions of either factor, consistent with work in frogs that suggested redundancy between E2F4 and E2F5[38,43]. Surprisingly, *Mcidas* was not required for MCC generation in the nasal placode of zebrafish, indicating that in some circumstances, GEMC1 is sufficient to promote centriole amplification through E2F4/5[43]. Whether this reflects inherent differences in target gene activation, increased expression of GEMC1 or the relative numbers of centrioles needed in MCCs in this tissue remains to be determined.

In overexpression experiments in frogs or human cells, Geminin acted as a potent inhibitor of transcriptional activation by GEMC1 and MCIDAS through CC-mediated interactions and the formation of a ternary complex with the E2F4 or E2F5-DP1 transcription factors[30,32,38]. It was proposed that Geminin may prevent the activation of the multiciliogenesis program until dividing cells exit the cell cycle, as the consequent centriole amplification would cause mitotic spindle defects[38]. Geminin was previously implicated in transcriptional regulation through interactions with the BRG1 component of the SWI/SNF complex, that is known to interact with E2F4/5, and controls the expression of a number of transcription factors[44–47]. Whether Geminin acts only by impairing GEMC1 or MCIDAS homodimerization and transcriptional activation through E2F4/5, or has a more complex role involving SWI/SNF or transcription factor interactions, has not been clearly established.

Recent work using lineage tracing in the mouse ependyma demonstrated that in adult mice, the direct generation of MCCs from B1 astrocyte progenitors can occur[48]. This is reminiscent of previous studies showing that following damage to the airway, MCCs can be generated directly from p63+ basal cells[49]. In the ependyma, the rate of symmetric or asymmetric divisions can be influenced by the transient overexpression of either Geminin, that favors symmetric divisions and the B1 cell fate, or GEMC1 that promotes MCC differentiation[48]. While this suggests that the expression and stoichiometry of Geminin and GEMC1 is highly relevant to cell fate, it remains unclear if direct interactions between Geminin and GEMC1 play a role in E2F4/5-mediated MCC specification under physiological conditions.

Other transcription factors required for MCC generation

A crucial function of GEMC1 and MCIDAS is to activate numerous downstream transcription factors (TFs). This expanding list includes FOXJ1, FOXN4, RFX2, RFX3, MYB and P73, among others (Table S1). Each of these TFs has been demonstrated to play critical, and in some cases cooperative, roles in enabling the gene expression of numerous proteins involved in MCC differentiation.

The TF p73 (encoded by *TP73* and *Trp73* in humans or mice) is a member of the p53 family that also includes p63, a marker of basal progenitor cells that give rise to MCCs in the airway[15]. Given its relationship to p53, one of the most well studied tumor suppressors, p73 was extensively studied in mice and was shown to cause a number of developmental phenotypes, many of which have been recently linked to defects in MCC differentiation[50]. The TP73 gene generates 2 major isoform groups via 2 promoters; the activating (TAp73) or an N-terminally truncated (DNp73) form lacking the transactivating domain. Reduced numbers of MCCs were observed in the airway, oviducts and efferent ducts of mice lacking both isoforms, or only TAp73, and ChIP experiments linked p73 directly to genes involved in multiciliogenesis, such as *FoxJ1*, *Rfx2*, or *Rfx3*[51,52]. P73 forms a ternary complex with GEMC1 and E2F5 that is stabilized by both the CC and TIRT domains of GEMC1, and this complex can activate the *TP73* promoter[31,51,53]. P73 is induced by both GEMC1 and MCIDAS through their respective E2F4/5-DP1-containing complexes, although it remains unclear if MCIDAS also interacts with p73. P73 expression has been reported in p63+ basal cells and Radial Glial Cells (RGCs), that act as MCC progenitors in the airway and brain, respectively, suggesting an early role for p73 in MCC fate specification independent of GEMC1 or MCIDAS, although this remains controversial[50–52,54]. The requirement for p73 appears to also vary depending on the isoform, tissue and cell type. In contrast to the airway, where TAp73 deletion impaired MCC formation, these mice did not exhibit hydrocephalus or show impaired MCC formation in the brain due to compensation from the *miR-449a-c* cluster[20]. Combined deletion of *TAp73* and *miR-449a-c* impaired MCC generation in the choroid plexus (CP) and deletion of both TP73 isoforms impaired MCC generation in ependymal cells[20,55]. Therefore, MCC

fate may be subjected to tissue-specific feedback modulation, warranting further comparison of the transcriptional regulation and signaling pathways involved in different tissues.

FOXJ1 was the first transcription factor shown to be required for MCC differentiation and is frequently used as a marker of MCCs in all tissues[56–59]. FOXJ1 is now a well-established target of p73 and GEMC1, that likely work together to activate its expression at early stages of multiciliogenesis[30,32,35,51,52]. FOXJ1 is also regulated by MCIDAS, as the absence of MCIDAS activity strongly reduced FOXJ1 expression in frog skin and human airway cells, but airway MCCs in MCIDAS deficient mice were FOXJ1 positive[35,37,60]. FOXJ1 promotes MCC differentiation by regulating a cohort of genes involved in the production, assembly, transport, and docking of the inner and outer dynein arms, radial spokes and the central pair as well as genes that encode intraflagellar transport (IFT) proteins[56,61–65]. Studies in the human airway demonstrated that the RFX3 TF functions as a transcriptional coactivator of FOXJ1, helping to induce the expression of cilia genes involved in differentiation towards the MCC lineage[61]. Similarly, recent work demonstrated that FOXJ1 preferentially binds enhancers and is stabilized at promoters of cilia genes through cooperative interactions with the TF RFX2[66]. The stability of the MCC lineage is reportedly dependent on a constant FOXJ1 protein expression level in order to prevent cellular de-differentiation back into a glial-like morphology in mouse ependymal MCCs[67]. Recently, human patients with FOXJ1 mutations were shown to have defects in motile cilia, left right patterning defects and impaired basal body docking in MCCs[68].

Another Forkhead family transcription factor, FOXN4, was implicated in MCC generation in frog skin and identified as an early target of MCIDAS[69]. Similar to FOXJ1, the promoter binding of FOXN4 was enhanced by RFX2, and its depletion using morpholinos or CRISPR/CAS9 editing impaired multiciliogenesis to a similar extent as FOXJ1, although through effects on distinct target genes. Its role in MCCs in other organisms has yet to be explored.

An shRNA based screening strategy in cultured primary human basal cell ALI cultures identified TRRAP, a component of several histone acetyltransferase complexes, to be required for MCC generation[70]. Analysis of ALI cultures using immunofluorescence showed that TRRAP acted at an early time following NOTCH inhibition and accumulated in MCCs prior to FOXJ1. Moreover, it was required for MCIDAS expression, suggesting that it was necessary for GEMC1 activity, a possibility that remains to be formally tested. In addition to TRRAP, ATAD2B, a AAA domain containing ATPase, was also identified in the screen and validated in subsequent experiments[70]. While it was not pursued further, it contains a bromodomain, binds acetylated histones, is regulated by E2Fs and has been implicated in transcription in cancer, suggesting it may also play a transcriptional role in MCCs [71,72].

The Aryl hydrocarbon receptor (AHR), a ligand activated transcription factor that responds to a variety of xenobiotic chemicals or oxygen levels, has been linked to MCC generation through work in both mice and frogs[73,74]. In ALI cultures from *Ahr*$^{-/-}$ mice, the expression of *Mcidas*, *Ccno* and *Cdc20b* were all strongly impaired, suggesting that AHR may function in conjunction with GEMC1 to activate *Mcidas*, and potentially facilitate the ability of MCIDAS to activate *Ccno*, that was identified as a direct AHR target[74]. In both murine ALI cultures and depletion experiments in frog skin, MCC numbers were reduced and many of the FOXJ1 positive cells showed disorganized ciliary patterns. At late embryonic stages, AHR deficient mice showed reduced numbers of airway MCCs *in vivo* but adult mice did not exhibit the same defects, indicating that redundant pathways may provide compensation for AHR loss at later developmental stages. Whether AHR plays a role in MCC generation in other tissues, such as the brain or germline, remains unclear, but hydrocephalus, that usually accompanies loss of ependymal MCCs, was not reported, and although AHR loss has been linked to reduced fertility in aging male mice, available evidence does not suggest that this is due to defects in the MCCs of the efferent ducts[75].

Additional regulators of MCC transcription and differentiation, including IL-6, STAT3, FANK1 and JAZF1 have been identified using in vivo and in vitro models[76,77] and there is little doubt that many additional transcriptional regulators will be involved in this complex differentiation program. Future work will be needed to further deconvolve their functions, regulation and target genes, as well as their tissue specific roles.

Centrioles, cell cycle regulators and the deuterosome

In proliferating cells, canonical centriole duplication is highly restricted to a single round per cell cycle to avoid mitotic defects that can result from multipolar spindle formation. This canonical mother-daughter (MD) pathway is tightly regulated and has been well described to involve the interplay of PLK4, CEP63, CEP152, and SAS6, among other factors[78]. The mother and daughter centrioles inherited by the cell following mitosis serve as templates for pro-centriole generation that gives rise to 2 new daughter centrioles in a process coordinated with cell cycle progression. Aside from contributing to the formation of the microtubule-organizing center (MTOC) of the mitotic spindle, the mother centriole contains distal appendages that facilitate docking to become a BB that can act as a base for the extension of a primary cilium that functions to integrate a diverse range of environmental signals[79]. A defining characteristic of post-mitotic MCCs is the massive amplification of centrioles to form the BBs needed for generating multiple motile cilia. The MD pathway of centriole duplication is estimated to account for only around 10% of BB production in MCCs[80]. The bulk of centriole production in MCCs is normally accomplished by the deuterosome-dependent (DD) pathway, that uses a ring-shaped, electron dense structure called the deuterosome to facilitate centriole expansion[1,81,82]. Many deuterosomes can form during multcilliogenesis, each nucleating multiple procentrioles, thereby

evading the restrictions that regulate the MD centriole duplication pathway to allow rapid BB production.

While the deuterosome was first described many decades ago only recently has its major structural component, DEUP1, been identified[81,83]. *DEUP1* evolved through a duplication of *CEP63,* that plays a key role in centriole duplication in the MD pathway. In addition to DEUP1, CCDC78 that localizes to the acentriolar sites of centriole biogenesis and CDC20B that localizes to the perideuterosomal region are the only other deuterosome specific proteins identified in both mouse and frog MCCs[82,84]. Other components of the deuterosome are common to the MD pathway, including Pericentrin (PCNT), γ-tubulin, and CEP152, that are all located on the peripheral ring[6]. However, there are likely additional proteins that comprise the center core or outer wall of the deuterosome that remain to be identified.

Until recently, the prevailing view was that deuterosome synthesis occurred *de novo* and did not require centrosomal centrioles. Detailed live imaging of newly synthesized centrioles during cultured brain MCC differentiation showed that deuterosomes were seeded by the daughter centrosomal centriole[80]. However, in the last year, a handful of studies showed that deuterosomes with multiple centrioles could be produced when both parent centrioles were depleted[85–87]. Deuterosomes were able to spontaneously synthesize from the pericenteriolar material (PCM) in a manner that did not require PLK4, in contrast to earlier reports[81]. This opens up a possibility that deuterosomes are created in the PCM, and then only briefly associate with the daughter centrioles to facilitate the loading of procentrioles that occur there[87]. Recent work has implicated cytoplasmic E2F4 in the initiation of centriole amplification[88]. A cytoplasmic pool of E2F4 was shown to co-localize with various components of centriole amplification, including PCM1, and it was proposed that this forms the core of the fibrous granules that have been observed overlapping with or adjacent to deuterosomes. It will be important in future work to fully elucidate the composition of the PCM in MCCs and explore potential species-specific differences.

The ordered stages of the deuterosome cycle have come in to clearer view through the use of mouse models and advancements in cell culture systems and super-resolution microscopy techniques over the last few years. At the onset of centriole amplification, a Centrin-2 cloud or ‘halo’ accumulates around the pre-existing centrosome (Stage-I or Amplification (A)) and this signal intensifies to adopt a ‘flower-like’ shape (Stage-II or Growth(G)), corresponding to maturing centrioles that appear in a synchronized manner. And finally, the coordinated disengagement (Stage III or D) and release of centrioles and their migration to the apical surface as BBs takes place[80].

Strikingly, the deuterosome cycle appears to rely on much of the same cell cycle machinery that plays a role during stepwise MD centriole duplication in proliferating cells, although the precise regulatory

details remain unclear. Following cell cycle exit, radial glial progenitors re-express cell cycle markers, including KI67, CDK1, CDK2 and phosphorylated histone H3-Serine10, without performing DNA synthesis or undergoing mitotic division[89]. Through the use of a number of small molecule inhibitors or agonists, key roles in the regulation of the deuterosome cycle were demonstrated for CDK1, CDK2, PLK1, APC/C and CDC20[7,89,90]. This repurposing of the mitotic oscillator also appears to require the poorly understood CDC20B, as well as CCNO, that has its highest expression levels during the earliest phase of deuterosome formation[84,91,92]. CCNO mutant mice exhibit large, malformed deuterosomes and produce fewer BBs, suggesting that CCNO was required for deuterosome stability[91]. MCCs generated in ALI cultures derived from CCNO knockout mice also display significant transcriptional dysregulation, suggesting that CCNO functions to restrain the activation of key MCC genes, including MCIDAS[91]. This role of CCNO could prevent the overproduction of deuterosome components and contribute to the observed structural abnormalities. Repurposing the existing cell cycle regulation machinery appears to provide an elegant solution to regulating centriole amplification in the context of MCCs. Future work will be needed to understand its regulation and the specific roles of factors such as CDC20B and CCNO that are expressed primarily in MCCs.

Recently, surprising work has questioned the necessity of the deuterosome for centriole amplification in MCCs. In mouse tracheal MCCs in vitro, depletion of DEUP1 caused a reduction in centriole amplification and CEP63 depletion did not influence centriole numbers in MCCs, due to apparent compensation from DEUP1[81]. Depletion of CEP63 in vivo, that reduces MD pathway centriole duplication in neural progenitor cells, also did not have an impact on centriole numbers in MCCs[93,94]. However, the deletion of DEUP1 in vivo revealed that mice were able to generate MCCs normally in the absence of deuterosomes, indicating they are dispensable for procentriole amplification during multiciliogenesis in mice and frogs[94]. Concomitant depletion of CEP63, to impair the MD pathway, led to a slight reduction in centriole amplification in vitro, indicating that there is likely minimal compensation from the MD pathway in the absence of DEUP1[94]. This finding remains to be extended to MCCs in all tissues but it opens up the field to a number of exciting possibilities. They speculate that centriole synthesis occurs within a cloud of fibrogranular material and PCM, and that deuterosomes function to relieve the parental centrioles of the high numbers of procentrioles that form along their length. Supporting this possibility, flatworms and some species of ray-finned fish (such as zebrafish) are capable of producing MCCs and do not encode DEUP1 or generate deuterosomes[95–97]. This establishes that another route for de novo procentriole generation in MCCs can function independently of both the MD and DD pathways and provide compensation for their loss. It was proposed that this pathway may be equivalent to pericentriolar satellites that occur in non-MCCs given that they are scaffolded by PCM1 that is enriched in fibrogranular material[94,98].

Late transcriptional roles, polarization and cilia assembly

Like many other types of epithelial cells, MCCs are polarized and aligned by the planar cell polarity (PCP) pathway[99]. Additionally, MCCs in both the frog skin and mammalian airway originate in a basal epithelial layer and migrate apically, through a process known as radial intercalation[100,101]. PCP and radial intercalation are both coordinated with ciliogenesis in MCCs through the actions of many proteins, including, dystroglycan, Rab11, the Par complex, RFX2, Slit2, Septins and RhoA[102–106]. Among these, the transcription factor RFX2 was identified as a central regulator of PCP, radial intercalation and ciliogenesis in frogs and other vertebrates[105,107,108]. Recent analysis of mice lacking RFX2 identified a key role in the regulation of cilia related genes during spermiogenesis, but suggested that it is dispensable for MCC generation in higher mammals, as hydrocephalus and respiratory defects were not reported[109–111]. So while RFX2 is expressed in cells with motile cilia in mice, further analysis of tissues that contain MCCs will be needed to establish its roles in higher mammals and determine if RFX3 or other factors may compensate for its loss[108–111].

How MCCs control the number of centrioles/BBs and cilia generated remains unclear. Studies using frog larvae showed that during radial intercalation, a network of apical and subapical actin forms in a lattice connecting the centrioles, and generates a 2D force that expands the apical surface of MCCs to accommodate the required number of BBs and ensure the even distribution of cilia[100,112]. Recent work demonstrated that centriole numbers remain normal in mouse cells lacking DEUP1 and a striking correlation between cell surface area and centriole number in MCCs was reported[85,94]. Manipulation of cell surface area in mouse tracheal epithelial cells, using differences in collagen density, showed that centriole numbers were impacted accordingly[85]. This suggests that either cell surface area, or potentially cortical stiffness, dictates the number of centrioles generated via early stage transcriptional mechanisms, and suggests potential links to the cytoskeletal rearrangements that occur during PCP and radial intercalation.

The YAP transcription factor, a central component of the Hippo signaling pathway, responds to cell surface tension and mechanical forces, regulates epithelial size during airway development and has been linked to both actin cytoskeletal rearrangements and ciliogenesis[113,114]. YAP is expressed in mouse airway MCCs, several YAP interactors have been localized to BBs, and in frogs, YAP nuclear translocation and increased tissue stiffness occur during regeneration of the multiciliated epithelium[115,116]. However, YAP is not required for MCC generation per se and it remains unclear if YAP plays an important role in dictating MCC size or centriole numbers[117].

Following their amplification, centrioles are transported to the apical surface in an actin-myosin dependent manner and the organization and attachment of the BBs takes place, regulated in part by ERK7, DVL1/2, CapZIP, CELSR2/3, WDR5, GTPases and the miR-34/449 family to ensure correct cilia function and integrity of the epithelium [19,37,50,99,118–126]. While the details of transcriptional crosstalk with the cytoskeleton and PCP pathways remains to be fully elucidated, MYB, FOXJ1, p73

RFX2 and RFX3, all contribute to the regulation of genes that ensure correct BB migration and docking, axoneme outgrowth, and motility, as well as cilia beating in frogs and mice[61,65,66,105,123,127–129].

Consequences of MCC dysfunction

While present only in a few tissues, defective MCCs contribute to congenital disorders, such as primary ciliary dyskinesia (PCD) and Reduced generation of multiple motile cilia (RGMC), the latter due to defects specifically in MCCs[60,130,131]. Thus far, mutations in 2 genes have been implicated in RGMC: *MCIDAS* and *CCNO*. Both genes are located adjacent to each other on Chromosome 5 (5q11.2) that also contains *CDC20B* that encodes both a protein and the *Mir449a/b/c* genes. RGMC patients present clinically with hydrocephalus, recurrent airway infections and bronchiectasis. Due to the ages of the patients, little data regarding fertility has been reported, although some female patients have presented with infertility[60,130,131]. While RGMC represents an extreme case, it is possible that less severe mutations or tissue specific defects in the function or expression of key MCC factors may result in more subtle pathologies, such as subfertility, scoliosis or normal pressure hydrocephalus[132,133]. Consistent with this possibility, a mutation in *GEMC1* was recently identified in a patient with congenital hydrocephalus but respiratory defects or fertility issues were not reported[134]. *MCIDAS* mutations that have been identified in RGMC are located in the TIRT domain of MCIDAS and interfere with binding of the E2F4/5 transcription factors, highlighting again the central importance of MCIDAS in the MCC transcriptional pathway[60]. A new report identified the NEK10 kinase in familial bronchiectasis and demonstrated its expression in airway MCCs[135]. While these patients exhibited respiratory distress, similar to that observed in RGMC, they generated airway MCCs with subtle morphological defects and shorter cilia that were incapable of mucociliary clearance. Proteomic analysis of NEK10 deficient MCCs revealed widespread effects on the phosphorylation of many proteins required for motile cilia function, indicating that NEK10 may be a key regulator of many aspects of MCC function.

Conclusions and open questions

Rapid advances have been made in determining the molecular events that control MCC generation in multiple tissues. However, many open questions remain unresolved. The structural and functional similarity of GEMC1 and MCIDAS raises the question as to whether they play distinct or redundant roles in transcription. The sequential nature of their activation, their antagonism with Geminin and their distinct contributions to MCC differentiation have been well documented[25,26,28,30,32,33,35,38,48]. Whether this reflects differential regulation of their activities, post-translational regulation of their stoichiometry or distinct functional domains that have yet to be identified remains unknown. Geminin levels are well established to be regulated by the Ubiquitin proteasome system, exemplified by the use of its N-terminal D-box degron in the Fucci system to monitor cell cycle[136]. MCIDAS also appears

to have a functional D-box motif and is degraded during anaphase in cancer cells but whether this is relevant to its activity in post-mitotic MCCs has not been established[27]. The 3'UTR of *GEMC1* contains overlapping *Mir449* and CPE sites, required for binding of CPEB translational regulators, that may function to tightly control mRNA and protein levels in response to regulatory cues[30]. Tissues from mice lacking CCNO have been reported to exhibit high levels of *Mcidas* expression, suggesting that modulation of CDK activity, that is clearly important for the centriole amplification process, may also play a direct role in regulating transcriptional feedback via GEMC1 and MCIDAS but this remains to be formally demonstrated[89,91]. MCCs in many tissues, particularly in the airway, must be periodically replaced due to damage to the epithelium. The maintenance of mRNA levels of GEMC1 and other transcriptional activators may serve to prime the rapid replenishment of cilia in existing MCCs following damage, and whether GEMC1 is required for the direct generation of MCCs from progenitor cells in this context also remains to be tested[48,49].

A common feature reported for both GEMC1 and CCNO deficient mice that remains unexplained was growth defects[30,32,92]. We speculated that this could be secondary to fluid pressure on the developing brain that could influence pituitary gland function, although we could not detect alterations in its function. Alternatively, it could reflect functions unrelated to MCC generation, for example in the regulation of DNA replication at early developmental stages. Mutations in Geminin are associated with Meier-Gorlin syndrome that is characterized by primordial dwarfism[137]. While the expression of GEMC1 and MCIDAS appears to be restricted to MCCs, CCNO expression is detected in other tissues, including the pituitary and thyroid glands, and may have distinct functions. Future work using conditional mouse models can begin to address these possibilities.

Aberrant expression of key MCC specific genes can also be observed in many cancers and some evidence suggests they may play functional roles[138–140]. The aberrant expression or depletion of *GEMC1* and *MCIDAS* in cancer cell lines has been shown to impact DNA replication and could potentially activate transcription factors such as E2F4, p73 and FOXJ1 that have been implicated in cancer development, and could provoke centriole amplification and mitotic defects[26–28]. The potential of CCNO and CDC20B to impact cell cycle regulation or apoptosis is also potentially relevant to their expression in human cancers[138,141].

The intriguing observations that MCCs coordinate cell area with centriole numbers and can perform this function in the absence of deuterosomes by repurposing cell cycle machinery opens up a number of new mechanistic questions to be addressed[85,89,94]. And finally, microarrays, RNAseq and more recently, single cell sequencing, of multiciliated cells has identified new genes in their transcriptional programs that have yet to be functionally characterized[30,38,66,69,142–144]. The careful comparisons between in vitro systems and MCCs in distinct tissues and organisms will no doubt also reveal new

aspects of their diversity and specialized functions that are important for human development and health.

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Author contributions

M.L. and T.H.S. co-wrote and edited the manuscript.

Declaration of Interests

The authors declare no competing interests.

Figure

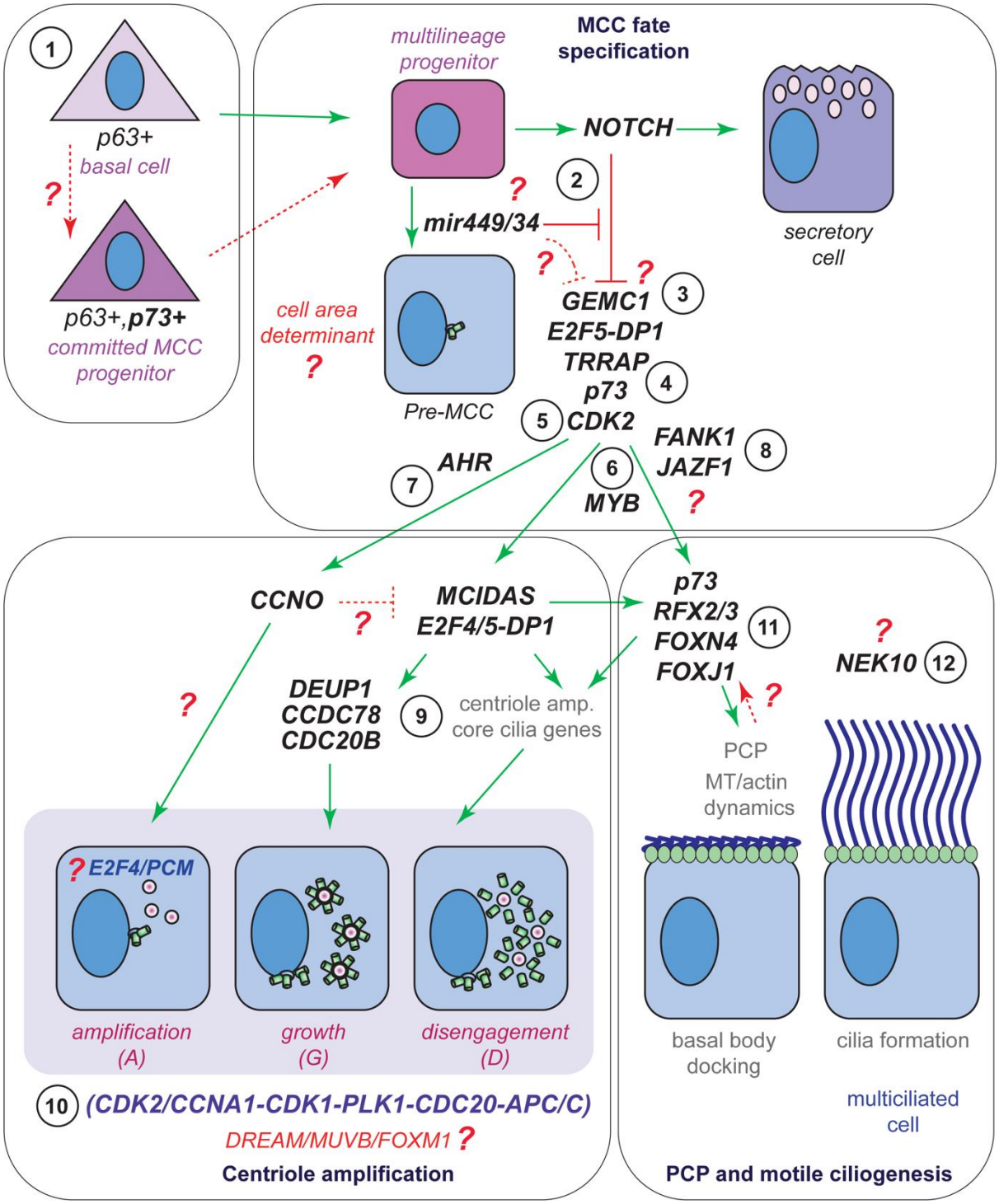

Figure 1: Knowns and unknowns of MCC transcription. The mammalian airway (trachea) is a well studied epithelial layer containing MCCs. Basal cells, that generate all other cell types of the trachea are p63 positive (+). 1) Induction of p73 at an early stage was proposed to mark p63+p73+ basal cells for an MCC fate, (?) although the regulation of this step remains unknown. 2) Inhibition of NOTCH instructs MCC fate and the activation of the mir-34/449 family was implicated in this step, (?) but how their induction is regulated remains an open question. (?) GEMC1 contains a mir-34/449 consensus site in its 3'UTR that may limit its expression levels. 3) (?) NOTCH inhibition activates GEMC1 through

an unknown mechanism. GEMC1 interacts with E2F5-DP1 through its C-terminal domain, to activate the MCC differentiation program. 4) The transcriptional co-activator TRRAP and p73 act at an early step, prior to the induction of MCIDAS and p73 has been shown to interact with GEMC1. 5) CDK2 activity was demonstrated to play an early but thus far uncharacterized role in transcriptional activation. 6) MCIDAS induction required MYB, that also acts upstream of FOXJ1. 7) During embryogenesis, but not in adults, AHR is required for GEMC1-mediated induction of CCNO. 8) Both GEMC1 and MCIDAS activate additional TFs, including FOXJ1, FOXN4, RFX2, RFX3 and p73 in a manner that requires FANK1 and JAZF1, (?) that remain to be functionally characterized in more detail. (?) Cell area correlates with centriole/cilia number and appears to be specified prior to centriole amplification through unknown mechanisms. 9) MCIDAS and multiple TFs activate genes required for deuterosome-mediated centriole amplification and ciliogenesis. (?) CCNO mutants showed elevated levels of MCIDAS expression, suggesting a negative feedback role, but the mechanism of this has yet to be determined. (?) E2F4 promotes initiation of centriole duplication through a cytoplasmic role that remains to be fully elucidated. 10) The deuterosome cycle is regulated at distinct steps, in part by the canonical cell cycle machinery, as well as specialized factors including CCNA1, CCNO and CDC20B. (?) Whether the DREAM/MUVB-FOXM1 proteins regulate expression of the canonical or MCC-specific factors in a manner similar to a normal cell cycle remains unknown. 10) CCNO interacts with CDK1 and CDK2 and is required for normal deuterosome formation, (?) but its precise role remains unclear. (?) Similarly, the function of CDC20B in the deuterosome cycle remains unclear but appears to be independent of APC/C regulation. 11) MYB, FOXJ1, FOXN4, RFX2/3 and p73 all influence the expression of genes involved in PCP, cytoskeletal dynamics, basal body docking and cilia formation. (?) The molecular crosstalk between these processes and transcription remains unclear. 12) NEK10 was implicated in cilia length control and function through the phosphorylation of numerous proteins, (?) the molecular impact of which remains unknown. See main text for more detailed description and references. (?) Denotes open questions indicated in the figure.

Supplementary Table S1:

Gene	Organism	Function	Domains	References
AHR	X, M	Upstream ligand-activated transcription factor that promotes airway MCC differentiation	A basic helix-loop-helix (bHLH), DNA binding, Per-Arnt-Sim (PAS) 1&2	[74]
CCNO	M, H	Suppresses MCIDAS expression levels and influences deuterosome	Cyclin domain	[91,130,131]

		stability. Mutated in RGMC patients.		
DP1	X, M	Heterodimerizes with E2F4 or E2F5 to regulate transcription. Interacts with GEMC1 and MCIDAS.	Coiled-coil and marked box (CM); sequence specific DNA-binding	[36,38,39,41,43,88]
E2F4 **E2F5**	X, D, M	Heterodimerizes with DP1 to regulate transcription. Interacts with GEMC1 and MCIDAS.	Coiled-coil and marked box (CM); sequence specific DNA-binding; transactivation	[36,38,39,41,43,88]
FANK1	M	Promotes MCC differentiation upstream of FOXJ1 in vitro.	Fibronectin type III; 6 Ankyrin repeat domains	[76]
FOXJ1	X, D, M, H	Activates genes involved in ciliogenesis, basal body trafficking, BB docking at the apical membrane and axoneme growth.	Fork-head domain	[56–59,64,65,68,120]
FOXN4	X	Binds to many of the same promoters as FOXJ1 and complements its activity.	Fork-head domain	[69]
GEMC1	X, D, M	Upstream transcriptional activator, critical for MCC specification.	Geminin coiled coil (CC); TIRT domain (E2F/DP1 interaction)	[30–33,53]
JAZF1	M	Promotes MCC differentiation upstream of FOXJ1 in vitro.	2 x C2H2-type zinc-finger (ZF); 1 x C2H2- degenerate (ZF)	[76]
MCIDAS	X, D, M, H	Downstream of GEMC1 a crucial activator of key transcription factors and genes required for centriole biogenesis	Geminin coiled coil (CC); TIRT domain (E2F/DP1 interaction)	[33,35,37,38,53,60]
MIR34/ 449a-c	X, D, M	Negatively regulate genes in the MCC program. Inhibit NOTCH signaling by targeting ligands.		[2,13,19–23]
MYB	M, H	Early transcription factor that can induce FOXJ1	H-T-H motif DNA binding 1,2 & 3; transactivation domain	[127,129]
P73	M, H	Induced by GEMC1 and MCIDAS, interacts with GEMC1, directly regulates a large number of genes required for MCCs	DNA binding; transactivation domain	[20,51,52,123]

RFX2	X, D	Stabilizes FOXJ1 at promoters to activate cilia genes. May recruit distal enhancers to promoters.	RFX-type winged-helix DNA-binding	[18,66,105,107]
RFX3	M, H	Downstream of GEMC1, modulates FOXJ1 to activate genes required for ciliogenesis and motility.	RFX-type winged-helix DNA-binding	[61,128]
TRRAP	H	Component of several histone acetyltransferase complexes. Acts downstream of NOTCH and upstream of MCIDAS.	FRAP, ATM and TRRAP (FAT) and FAT C-terminal (FATC) domain; PI3K/PI4K domain	[70]

Table S1: Transcriptional regulators implicated in MCC differentiation are hyperlinked to uniport.org for domain information. Species are abbreviated: *Xenopus laevis* (X, frogs), *Danio Rerio* (D, zebrafish), *Mus musculus* (M, mice), *Homo sapiens* (H, humans).

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