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E2F4/5-mediated transcriptional control of multiciliated cell differentiation: redundancy or fine-tuning?

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Centrosomes consist of a pair of cylindrical, microtubule based structures called centrioles, surrounded by proteinaceous pericentriolar material (Nigg and Holland, 2018). Centrosomes are key regulators of mitotic spindle formation and the docking of one of the centrioles on the cell surface allows it to function as a basal body for primary cilium formation. In interphase cells, similar to DNA replication, the mother centriole-dependent (MCD) pathway of duplication is regulated to occur once per cell cycle in order to ensure monociliation and prevent abnormal mitoses resulting from spindle defects caused by numerical aberrations in centrosomes (Nigg and Holland, 2018).

While centrioles are normally duplicated only once per cell cycle, multiciliated cells (MCCs), characterized by the projection of dozens to hundreds of motile cilia, have the unique requirement of massive centriolar expansion in order to generate the basal bodies required for multiciliation (Spassky and Meunier, 2017). MCCs promote luminal flow in the brain ventricle, airways, oviducts and epididymis, and defects in this cell type are associated with a rare ciliopathy, termed Reduced Generation of Multiple motile Cilia (RGMC) (Boon et al., 2014; Wallmeier et al., 2014). RGMC patients present with hydrocephalus, severe respiratory distress and infertility, likely all resulting from the loss of MCC function.

In order to more efficiently amplify the multiple centrioles needed to form the basal bodies, MCCs utilize the MCD pathway, but appear to rely primarily on an additional deuterosome-dependent (DD) pathway (Al Jord et al., 2014; Zhao et al., 2013). Deuterosomes are poorly characterized electron dense structures that mediate centriole amplification. DD mediated amplification is regulated in a stepwise manner and controlled by numerous cell cycle components, including CDK1, CDK2, PLK1 and the APC/C cyclosome, many of which normally regulate centrosome duplication, mitotic progression and chromosome segregation in cycling cells (Al Jord et al., 2017; Vladar et al., 2018). The coordinated, stepwise assembly of deuterosomes and the involvement of key cell cycle regulators suggests the existence of deuterosome checkpoints, analogous to those that monitor DNA integrity or centrosome duplication in mitotic cells.

Recent work in frogs, zebrafish and mice identified the Geminin family proteins GEMC1 (encoded by *GMNC* and called Gmnc in fish) and MCIDAS (also called IDAS in humans, Multicilin in frogs and Mcidas in fish) as key activators of the MCC transcriptional cascade, that includes the specific genes involved in the DD pathway of centrosome amplification (Arbi et al., 2016; Ma et al., 2014; Terre et al., 2016; Zhou et al., 2015). The Geminin family members are characterized by a central coiled coil (CC) domain that facilitates their homo and heterodimerization. In Geminin, this domain is required to regulate CDT1, ensuring that DNA replication occurs once per cell cycle (Arbi et al., 2017). While implicated in cell cycle control through Geminin interactions, *Gemc1* and *Mcidas* appear to be expressed exclusively in MCCs in mice (Kyrousi et al., 2015). The encoded proteins contain the central Geminin-like coiled coil domain, as well as a C-terminal domain absent from Geminin, dubbed the TIRT domain, due to a repeated amino acid motif, that interacts with heterodimers of either E2F4 or E2F5 and DP1 (Arbi et al., 2016; Ma et al., 2014; Terre et al., 2016; Zhou et al., 2015). Both GEMC1 and MCIDAS are necessary for MCC differentiation and their ectopic expression is sufficient to activate the transcriptional program and drive multiciliogenesis. As neither protein has a recognizable DNA binding domain, the E2F4/5-DP1 factors are presumably the primary contact point for transcriptional activation. Consistent with this, reduced dosage both of E2F4 and E2F5 led to defects in MCC formation in some tissues of the mouse (Danielian et al., 2007; Danielian et al., 2016).

The current model of MCC differentiation posits that the inhibition of NOTCH results in the activation of GMNC, which initiates the MCC transcriptional program through its interactions with E2F4/5-DP1 (Spassky and Meunier, 2017). This is followed by the activation of MCIDAS that also uses E2F4/5-DP1 to sustain a transcriptional cascade involving the FOXJ1, FOXN4, Myb, p73 and RFX2/3 transcription factors, and likely many additional regulators that remain to be identified. This model therefore predicts that MCCs in every tissue in which they are present will require some combination of E2F4-DP1 and E2F5-DP1. However, due to the complications of deleting both factors in all MCC containing tissues of the mouse, it has remained an open question whether that is indeed the case.

Now, work from Sudipto Roy's lab sheds some light on this question using CRISPR/CAS9 engineered zebrafish with mutations in E2f4, E2f5 or both factors (Chong et al., 2018). While E2F4 has been considered the more important factor in mice, where it is essential for MCC generation in the airway, E2f4 null zebrafish are fully competent to generate MCCs in the pronephric ducts and the nasal placode. In contrast, E2f5 was absolutely required for pronephric MCCs and E2f5 mutants also showed a

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reduction in MCC numbers in the nose. Of note, the defects of E2f5 mutants in the pronephric duct could be rescued by ectopic E2f4 expression, indicating that relative expression levels, rather than specific functions of either protein may explain the apparent specificity. The deletion of both genes led to a complete absence of MCCs in all tissues, demonstrating that while there are some tissue specific differences, some level of either E2f4 or E2f5 is required for MCCs in all tissues of the fish where they are known to differentiate.

Notably, the investigators also used the mutant fish to address the influence of E2f4 and E2f5 on the activation of *gmnc* and *mci* during multiciliogenesis. They found that the activation of *gmnc* was independent of both proteins, but that Gmnc required E2fs to activate *mci* and other downstream genes. This further supports the idea that Gmnc is the primary first responder to cell fate signals, such as Notch inhibition, and that this step is E2F4/5 independent. Gmnc then initiates the E2F4/5-dependent activation of *Mci*, and other transcriptional activators, that subsequently induce key components of the MCC differentiation program.

Similar to E2f4 and E2f5, the level of redundancy between Gmnc and Mci remains unclear. The analysis of expression in zebrafish revealed that *Mc*i is not detectably expressed in the nasal placode MCCs, indicating that Gmnc is sufficient and Mci may be highly, or completely, redundant with Gmnc and subject to differential regulation. This could correspond to the relative cilia numbers needed in particular MCC types or the relative contribution of other transcription factors expressed in a given tissue.

The latter conclusion is potentially consistent with another new report from the Roy lab describing the phenotypes of *Mcidas* knockout mice (Lu et al., in press). The deletion of *Mcidas* in the mouse led to defects in MCC formation as expected, however, in contrast to *Gemc1* knockouts, MCCs were specified and key transcription factors, such as FOXJ1, were expressed at apparently normal levels. This and other data led to a model of stepwise activation of the MCC program with Gemc1 using primarily E2F5 to specify MCC precursors and activate MCIDAS that through the use of both E2F4/5 activates the expression of genes needed for the DD pathway for MCC differentiation.

While the zebrafish work clarifies a requirement for either E2F4 or E2F5 for the generation of all MCCs and identifies Mci-independent MCC differentiation in the nasal placode, many questions still remain. Do these observations extend to mice, where in contrast to fish, GEMC1 shows preference for E2F5 binding? Given that GEMC1/Gmnc and MCIDAS/Mci appear to regulate distinct targets in their respective organism, it seems unlikely that differential E2F binding is the *sole* determinant of target gene specificity, so what other factors are involved? How do Notch inhibition, or other signals, activate *Gmnc* expression? Do heterodimers of Gmnc and Mci have distinct functions in the transition from specification to multiciliation? The comparative analysis of zebrafish, mice and other systems will no doubt continue to be important for addressing these questions and unraveling the complexity and diversity of the MCC differentiation program.

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