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Genome-wide screen identifies novel machineries required for both ciliogenesis and cell cycle arrest upon serum starvation

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ABSTRACT

Biogenesis of the primary cilium, a cellular organelle mediating various signaling pathways, is generally coordinated with cell cycle exit/re-entry. Although the dynamic cell cycle-associated profile of the primary cilium has been largely accepted, the mechanism governing the link between ciliogenesis and cell cycle progression has been poorly understood. Using a human genome-wide RNAi screen, we identify genes encoding subunits of the spliceosome and proteasome as novel regulators of ciliogenesis. We demonstrate that 1) the mRNA processing-related hits are essential for RNA expression of molecules acting in cilia disassembly, such as AURKA and PLK1, and 2) the ubiquitin–proteasome systems (UPS)-involved hits are necessary for proteolysis of molecules acting in cilia assembly, such as IFT88 and CPAP. In particular, we show that these screen hit-associated mechanisms are crucial for both cilia assembly and cell cycle arrest in response to serum withdrawal. Finally, our data suggest that the mRNA processing mechanism may modulate the UPS-dependent decay of cilia assembly regulators to control ciliary resorption-coupled cell cycle re-entry.

1. Introduction

For many years, the primary cilium had been considered an obscure structure of no importance. However, recent studies suggest that this cellular organelle acts as a major hub for multiple signal transduction pathways that are necessary for diverse cellular phenomena, including differentiation, polarity, and homeostasis [1–3]. The primary cilium is an immotile and microtubule-based sensory organelle that extends from a mother centriole in most types of cells. The mother centriole generally contributes to formation of the mitotic spindle in a dividing cell, but in a quiescent cell it becomes associated with a Golgi-derived vesicle, migrates to the cell surface, and attaches to the plasma membrane [4]. Once it attaches, the mother centriole is referred to as the basal body, which serves as a nucleation region for the growth of ciliary axonemal microtubules [5]. Because of the ambilateral roles of the mother centriole in ciliogenesis and cell division, the events of cilia biogenesis and cell cycle progression are mutually exclusive.

In general, assembly of the primary cilium is coupled with cell cycle exit and entry into quiescence, whereas its disassembly is coupled with resumption of proliferation [6–8]; these facts present long-standing evidence for a link between ciliogenesis and cell cycle progression. Although the idea that cilia are resorbed as the cells enter mitosis was once prevalent, recent studies have indicated that cilia disassembly proceeds in two waves as quiescent cells re-enter the cell cycle: the first wave occurs at the G1 → S transition, and the second wave occurs at the G2 → M transition [9]. It has also been suggested that the inhibition of ciliary resorption halts cell cycle progression. For example, depletion of Nde1, Tctex-1, and Aurora kinase A (AURKA) results in the delay of cell cycle re-entry with abnormal retention of cilia [10,11]. Thus, it appears that not only is a suppressive mechanism of ciliogenesis active during cell proliferation, but a stimulatory mechanism of ciliary resorption may also be active. Together, these observations imply that the disassembly of the primary cilium is a prerequisite for cells to cease being quiescent and re-enter the cell cycle.

Recent studies have shown that autophagy- and ubiquitin–proteasome system (UPS)-mediated proteolysis of cilia-related

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molecules are involved in cell cycle exit and subsequent cilia assembly [12,13]. For instance, UPS-mediated degradation of Trichoplein, which is composed of Cul3-RING ubiquitin ligases (CRL3s) and KCTD17 as a substrate-adaptor, inactivates AURKA and initiates ciliogenesis [13]. Therefore, it is conceivable that the formation of the primary cilium is regulated by degradation of proteins involved in cilia disassembly prior to exit from the cell cycle. Although there are several clues as to the basis of coordinated regulation of ciliogenesis and cell cycle progression, some questions remain about how resorption of the primary cilium modulates progression of the cell cycle. A mechanistic explanation does not exist for how the presence or absence of the primary cilium can serve as a checkpoint at the G1 → S transition.

In the present study, we perform a genome-wide high-content screen and uncover roles of mRNA processing and UPS mechanisms in cell cycle arrest and ciliogenesis. We find that mRNA processing-related hits are required to control RNA expression of cilia disassembly regulators (such as AURKA, PLK1, and NEK2), and UPS-related hits are necessary for the proteolysis of cilia assembly regulators (such as CPAP, KATANIN, and IFT88). Furthermore, our data suggest that mRNA processing is a stimulatory mechanism of cilia disassembly, whereas UPS is a suppressive mechanism of cilia assembly. Finally, we demonstrate that the mRNA processing and UPS-mediated ciliary resorption provide a link to the G1 → S transition during cell cycle re-entry.

2. Results

2.1. Identification of novel modulators of ciliogenesis and cell cycle progression

To obtain a list of candidates and to gain a comprehensive understanding of the molecular mechanism connecting ciliogenesis to cell cycle progression, we utilized a functional genomics approach. We performed a high-content screening (HCS) procedure using small interfering RNA (siRNA) [14] to analyze the genetic intersection of ciliogenesis and cell cycle state (Fig. 1A). This screen utilized a subclone of the hTERT-immortalized ciliary retinal pigment epithelial (RPE) cell line expressing both EGFP-tagged Smoothened, a transmembrane protein that accumulates in the cilium [15], and the N-terminal 110 amino acid residues of Geminin, a nuclear protein stabilized during S, G2, and M phases, fused to mCherry [16] (Fig. 1B). We predicted that this cell line, abbreviated SEMG (Smo-EGFP and mCherry-Geminin), would display a green fluorescent primary cilium during the G0/G1 phases and a red fluorescent nucleus during S, G2, and M phases (Fig. 1C).

Because serum starvation induces cell cycle arrest in the G0/G1 phases and ciliary assembly in RPE cells, we hypothesized that serum withdrawal would yield a majority of cells with EGFP-positive cilia and mCherry-negative nuclei, whereas addition of serum would cause the majority of the cells to display mCherry-positive nuclei with EGFP-negative cilia. The cells were subjected to automated fluorescent imaging and scoring, and we found that ~70% of SEMG cells that were cultured in the presence of fetal bovine serum (FBS) showed mCherry-positive nuclei, with few if any EGFP-positive cilia. In contrast, 24 h after serum deprivation, ~60% of the cells had EGFP-positive cilia with few mCherry-positive nuclei (Fig. 1D). Additionally, we found that the depletion of positive control genes including *KIF3A*, *ACTR3*, and *CRNKL1*, which are involved in ciliogenesis or cell cycle regulation, led to abnormal numbers of EGFP-cilia and mCherry-nuclei in the serum-starved SEMG cells [14]. The rationale for performing our genome-wide RNAi screen with SEMG cells was to identify genes that, when downregulated, interfered with the mutually exclusive expression of mCherry and EGFP.

A total of 18,055 target genes representing the known human ENCODE transcriptome were individually knocked down in duplicate with the siRNA library for 72 h, including 24 h of serum starvation (Fig. 1A). The siRNA library was pooled such that four individual siRNAs targeted each gene (Supplementary Table S1). The cells were then subjected to automated fluorescent imaging and scoring followed by visual inspection (Fig. 1E) and secondary screening in triplicate. Through manual visual inspection, we confirmed that the non-specific scrambled siRNA-transfected cells under serum starvation had mostly EGFP-labeled cilia and few cells with mCherry-labeled nuclei. Finally, we obtained the screen hits, which displayed abnormal numbers of EGFP-cilia and mCherry-nuclei with Z-scores >2; the hits were clustered into three outcome groups (Fig. 1F). Among the classified hits that surpassed the threshold, we identified 201 in Group A, 460 in Group B, and 164 in Group C (Supplementary Table S2). In addition, we found that several genes such as *NEK2*, *PLK1*, *APCs*, and cyclins, which are known to be associated with ciliogenesis-linked cell cycle control, were included in our screen hits.

Because the hits from Groups A, B, and C were likely to be largely non-overlapping, we profiled the results separately. Group A had a normal number of EGFP-cilia but a higher than normal number of mCherry-nuclei. This result indicated that silencing of Group A genes might cause inhibition of ciliary resorption and failed entry into mitosis. Group B had a lower than normal number of EGFP-cilia but a normal number of mCherry-nuclei, suggesting that the Group B genes might have no direct effect on the cell cycle but might block ciliogenesis. Furthermore, we found that the Group B list included many known ciliopathy genes and, accordingly, identified a novel Joubert Syndrome causative gene, *KIAA0586*, from the list [14]. Group C had a lower than normal number of EGFP-cilia and a higher than normal number of mCherry-nuclei. This finding suggested that depletion of Group C genes triggered a blockade of entry into or maintenance of a quiescent state and a concurrent (or resulting) failure of ciliation. Therefore, we focused on Group C because the results suggested dual regulation of ciliogenesis and cell cycle progression. To extract information regarding the mechanisms by which ciliogenesis is coupled to cell cycle regulation, we analyzed the list of hits using public databases such as DAVID [17] and GeneMANIA [18]. The results of combined analyses showed enrichment within two functional clusters: mRNA processing and the ubiquitin–proteasome system (UPS) (Fig. 1G). The mRNA processing module (GO: 0006397) is involved in the conversion of a primary mRNA transcript into one or more mature mRNAs prior to translation into a polypeptide. The UPS module (proteasome-mediated ubiquitin-dependent protein catabolic process; GO: 0043161) is involved in the breakdown of a protein (this process is initiated by attachment of ubiquitin, which is mediated by the proteasome).

2.2. The machineries of mRNA processing and UPS are necessary for ciliogenesis and cell cycle control

To validate the role of hit genes in the coupling of ciliogenesis with cell cycle progression, we performed fluorescent imaging and fluorescence-activated cell sorting (FACS) analyses on knockdown cells transfected with individual siRNAs against representative hits. For the validation tests, we prioritized groups of genes that were predicted to have roles in similar pathways within each cluster shown in Fig. 1G. The individual siRNAs used to target each hit were selected based on a qRT-PCR knockdown efficiency test (Supplementary Fig. S1). We counted Smo-EGFP-positive ciliated cells and mCherry-Geminin-positive cycling cells from the imaging data and compared the results to the FACS data. In the mRNA processing pathway cluster, crooked neck pre-mRNA splicing factor 1 (*CRNKL1*); eukaryotic translation initiation factor 3, subunit E (*EIF3E*); pre-mRNA-processing factor 6 (*PRPF6*); and pre-mRNA-processing factor 8 (*PRPF8*) were selected. As expected, the depletion of each hit resulted in a decrease of Smo-EGFP-positive cells and an increase of mCherry-Geminin-positive cells (Fig. 2A). Further analysis using FACS implied that the increased number of cycling cells might be due to an increase of S phase cells (Fig. 2B). In the UPS pathway cluster,