Polo-like kinase 4 maintains centriolar satellite integrity by phosphorylation of centrosomal protein 131 (CEP131)

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ABSTRACT

The centrosome, consisting of two centrioles surrounded by a dense network of proteins, is the microtubule organizing center of animal cells. Polo-like kinase 4 (PLK4) is a Ser/Thr protein kinase and the master regulator of centriole duplication, but may play additional roles in centrosome function. To identify additional proteins regulated by PLK4, we generated an RPE-1 human cell line with a genetically engineered ‘analog-sensitive’ PLK4* which genetically encodes chemical sensitivity to competitive inhibition via a bulky ATP analog. We used this transgenic line in an unbiased multiplex phosphoproteomic screen. Several hits were identified and validated as direct PLK4 substrates by in vitro kinase assays. Among them, we confirmed Ser-78 in centrosomal protein 131 (CEP131 also known as AZI1) as a direct substrate of PLK4. Using immunofluorescence microscopy, we observed that although PLK4-mediated phosphorylation of Ser-78 is dispensable for CEP131 localization, ciliogenesis, and centriole duplication, it is essential for maintaining the integrity of centriolar satellites. We also found that PLK4 inhibition or use of a non-phosphorylatable CEP131 variant results in dispersed centriolar satellites. Moreover, replacement of endogenous wildtype CEP131 with an S78D phosphomimetic variant promoted aggregation of centriolar satellites. We conclude that PLK4 phosphorylates CEP131 at Ser-78 to maintains centriolar satellite integrity.

The centrosome is the major microtubule organizing center of animal cells and consists of two orthogonal centrioles, which are cylindrical, ninefold symmetric arrays of microtubules that help to nucleate microtubule arrays. Centrioles are crucial for accurate chromosome segregation (1), primary cilia formation and neural development (2-4). Centriolar satellites are small, dynamic structures surrounding the centrosome. These satellites are important for the recruitment of proteins involved in microtubule organization (5), ciliogenesis (6), and centriole duplication (7).

Polo-like kinase 4 (PLK4) is a key regulator of centriolar duplication (8,9). Depletion of PLK4 impairs duplication resulting in loss of centrioles, whereas overexpression causes overduplication and increased centriole number (9-11). On a molecular level, the formation of a new...
(daughter) centriole in proximity to the preexisting (maternal) centriole is initiated by CEP152- and CEP192-mediated recruitment of PLK4 at the G1/S transition (12,13) of and involves the interactions of several additional centrosomal proteins including SASS6, STIL, CPAP, CEP135, and CP110 (14). After initiation of procentriole formation by PLK4 recruitment, cartwheel formation is initiated. The ninefold symmetric structure of the cartwheel is derived from the intrinsic ninefold symmetry of SAS6 oligomers that form the cartwheel (15). The next step is elongation. Lastly, the plus end of the centriole is capped by CP110 and associated proteins (16). In this manner, PLK4 regulates key steps of centriolar duplication.

In addition to its well-known role in duplicating centrioles, PLK4 is implicated in other critical roles in centrosome biology independent of its role in centriole duplication, such as ciliogenesis (17,18) and centriolar satellite maintenance (19). Regarding the latter, PLK4 phosphorylates PCM1 which partly regulates the integrity of centriolar satellite integrity (19). These data support a role for PLK4 in other centrosome functions, but a comprehensive list of substrates or functions is not available.

A number of centrosomal PLK4 substrates relevant to highly regulated centriole duplication are known (Table 1). Notably, PLK4 autophosphorylation results in ubiquitination and subsequent proteasomal degradation (20-23), which is critical for limiting PLK4-mediated centriole duplication to once per cell cycle. Substrates mediating centriole duplication include FBXW5 (24), GCP6 (25), and CP110 (26). Moreover, the substrates SAS6 (27) and STIL/Ana2 (28-31) mediate recruitment and stabilization of SAS6 at the centrosome during centriole duplication. Additionally, phosphorylation of CPAP allows for procentriole assembly and centriole elongation; and phosphorylation of CEP135 allows for recruitment of asterless/Cep152 to centrioles (32). Other known substrates of PLK4 mediate centriolar satellite integrity, PCM1 (19); trophoblast differentiation, HAND1 (33); and cell motility, APR2 (34). Additionally, CEP152 (28,35), CDC25C (36), CHK2 (37), and ECT2 (38) have been identified as PLK4 substrates, though the roles of these phosphorylation events remains unclear. Three previous studies have reported mass spectrometry (MS)-based screens to identify PLK4 interactors. One study purified Plx4 from Xenopus egg extracts by affinity chromatography and analyzed its associated binding partners by MS (35), the second study performed PLK4 BioID to identify proximity interactions (39), and the third study immunoprecipitated tagged-PLK4 from HeLa cells and examined binding partners by MS (28). To our knowledge, analysis of PLK4 regulated phosphorylation sites in human cells has not been reported. Here, we employed a chemical genetic system to perform an unbiased MS-based analysis of PLK4 phosphorylation targets in non-transformed human cells to discover novel substrates of PLK4. We identify CEP131 as a novel substrate of PLK4, and find that PLK4 phosphorylation of CEP131 is an important contributor to maintaining centriolar satellite integrity.

**Results**

**Generation of PLK4 analog sensitive cell line**

PLK4 is the master regulator of centriole duplication (8,9), and plays additional roles, but few substrates have been identified. In order to identify new substrates, we engineered a PLK4 conditional knockout cell line and a PLK4 analog sensitive cell line. First, recombinant adeno-associated virus (rAAV) was used to insert loxP sites around (flox) exons 3 and 4 of one endogenous PLK4 locus. The other genomic locus of PLK4 was deleted, so the genotype of this cell line is PLK4WT/Δ. Two clones were identified and validated by PCR (Figure S1A-B) and by examining the disappearance of centrosome components by immunofluorescence after treatment with adenoviral Cre (AdCre; Figure S1C-D). The efficiency of centrosome loss is similar to a previous report of 39% of cells losing
PLK4 Phosphorylates CEP131

As expected in p53 wildtype cells (40, 41), loss of PLK4 significantly reduced colony formation (Figure S1E).

Next, we used rAAV to engineer an analog sensitive (AS) cell line in immortalized, non-transformed RPE-1 cells. We targeted exons 3-4 to generate a point mutation at the conserved gatekeeper residue (L89G), enlarging the ATP-binding pocket for selective inhibition by the bulky ATP analog 3-MB-PP1 (42). To ensure that only Plk4ASH was expressed, exons 3-4 were deleted from the PLK4 locus edited colonies were screened by PCR (Figure S1F-G). The genotype of this cell line is PLK4AS/Δ, which is henceforth be labeled “AS” in the text and figures.

PLK4 inhibition is expected to prevent new centriole assembly without disassembling preexisting centrioles. Consistent with this, inhibition of AS with 3-MB-PP1 for 5 days yields about 65% of cells with fewer than 2 centrioles (Figure 1A-B), consistent with previous reports (40, 41).

Coincident with the centriole loss, there was a proliferation defect in the PLK4 AS cells after 5 days incubation in 10 µM 3-MB-PP1 (Figure 1C-E). Importantly, the effect was specific to AS cells, as PLK4 WT cells had normal cloning efficiency and proliferated well in the presence of 3-MB-PP1 (Figure 1C-E). Further characterization of the proliferation defect revealed cell cycle arrest, but not cell death, as evidenced by negative trypan blue and annexin V staining (Figure 1F) and positive β-galactosidase staining (Figure 1G). These findings are consistent with previous reports indicating cells lacking centrioles undergo cell cycle arrest, but not apoptosis (40, 41). Thus, this PLK4 AS cell line that allows for specific inhibition by 3-MB-PP1, resulting in loss of centrioles and cell cycle arrest.

**CEP131 is a substrate of PLK4**

To identify novel substrates of PLK4 using this system, we employed quantitative high resolution mass spectrometry for phosphoproteome analysis of PLK4 AS cells arrested at the G1/S boundary (Figure 2A) with or without 3-MB-PP1 challenge. Isobaric labeling with six-plex TMT tags allowed us to run three replicates each of AS cells with and without 3-MB-PP1 in a single MS experiment (43-46). The G1/S boundary was selected for this analysis (Figure 2A) because centriole duplication occurs at this phase of the cell cycle (14, 47).

Using this approach, we identified 11,501 phosphopeptides (Table S1), corresponding to 3711 proteins, of which 15 were significantly downregulated (P value < 0.05 and fold change >1.5) by inhibition of PLK4. The most strongly regulated phosphopeptides were found in RUNX1, PTPN12, IL6ST, TRIM3, and SCRIB. Given that none of these proteins are known to localize to the centrosome or play any role in centrosome or microtubule biology, these phosphorylation events are likely to be indirectly regulated by PLK4. To increase the likelihood of identifying direct substrates, we therefore focused on proteins known to localize to the centrosome or play any role in centrosome or microtubule biology, and reduced our fold-change and statistical significance thresholds, as PLK4 is a low abundance kinase with rare phosphorylation events that may not reach robust fold-change or statistical significance thresholds. We considered proteins to be centrosomal if their Uniprot entry mentioned localization to the centrosome, centriole, or minus ends of microtubules or if the protein was identified in a phosphoproteomic screen of purified centrosomes (48) (Figure 2C). Many such phosphopeptides had only modest changes in abundance, possibly due to the moderating effect of feedback.
regulation of PLK4 catalytic activity (42). Two centrosome proteins identified in our screen, CEP152 and PCM1, were previously identified as PLK4 substrates (Table 1), providing credence that this screening approach identified PLK4 substrates. From the remaining list, we selected the proteins known to be associated with centriole duplication: CEP131 (7,49), CEP215/CDK5RAP2 (50), CEP350 (51). None of these proteins have been previously identified as PLK4 substrates. Among the potential phosphorylation sites, we identified residues that are evolutionarily conserved: S78 and S89 on CEP131, S1238 on CEP215, and S1648 on CEP350 (Figure S2). To evaluate these as potential phosphorylation sites, direct phosphorylation was tested with GST-tagged 13-mer peptide fragments with the serine of interest located centrally (sequences listed in Table S2). STIL (S1108 ± 6 amino acids) was used as a positive control (29). Of the potential fragments identified, only CEP131 S78 produced a strong signal when incubated with WT PLK4 (Figure 2D). We also incubated PLK4 with a larger N-terminal fragment of CEP131 (amino acids 1-250) and observe that PLK4 is capable of phosphorylating this fragment (Figure S3). Importantly, phosphorylation is abolished when the PLK4 inhibitor, centrinone B (40), is added to the reaction (Figure 2D). Moreover, a CEP131 fragment containing the S78A mutation is not phosphorylated (Figure 2E). We conclude that CEP131 S78 is directly phosphorylated by PLK4 in vitro. To assess which domain of CEP131 interacts with PLK4, we performed co-immunoprecipitation experiments. Full length PLK4 was co-expressed with different domains of CEP131. We find that PLK4 interacts with the N-terminus of CEP131 (Figure S5).

**CEP131 S78 phosphorylation is required for centriolar satellite integrity but is dispensable for centriole duplication and ciliogenesis**

CEP131 is a component of the centrosome and centriolar satellites and was previously identified as a PLK4 interactor in BioID experiments (39). Furthermore, CEP131 and PLK4 co-localize (Figure S4). CEP131 is known to have three major functions in the cell: centriole duplication, ciliogenesis, and centriolar satellite formation. Moreover, PLK4 activity is implicated in each of these activities (8,9,19). Therefore, we tested whether PLK4 phosphorylation of CEP131 is required for these functions. To do this, we inhibited PLK4 in RPE-1 AS cells with 3-MB-PP1 and in HeLa cells using centrinone B. As expected, inhibition of PLK4 blocked centriole duplication (Figure S6A-B), ciliogenesis (Figure S6C-D), and centriolar satellite organization (Figure S3E-F).

To test if CEP131 S78 phosphorylation is important for any of these three functions, we expressed siRNA-resistant, EGFP-tagged, non-phosphorylatable (S78A) or phospho-mimetic (S78D) CEP131 mutants in HeLa cells. These mutants properly localize to centrioles and centriolar satellites (Figure 3A), suggesting that this phosphorylation event is not required for CEP131 localization. Next, we depleted endogenous CEP131 with siRNA (Figure 3B). Knockdown-addback efficiency was also assessed by qRT-PCR (Figure 3C). We next analyzed the ability of these cells to duplicate centrioles and nucleate primary cilia. No defects in centriole numbers were observed among conditions (Figure 3D-E). With regard to primary cilia formation, CEP131-depleted cells exhibited a reduction in primary cilia formation, consistent with previous reports (52,53). However, defects in primary cilia formation did not occur in either the non-phosphorylatable (S78A) or
PLK4 Phosphorylates CEP131

phosphomimetic (S78D) mutant cell lines (Figure 3F-G). Combined, these data suggest that PLK4 phosphorylation of CEP131 S78 is dispensable for centriole duplication and primary cilia formation.

PCM1 comprises a structural platform for centriolar satellites (54) and PLK4 phosphorylation of PCM1 is required for proper organization of centriolar satellites (19). To test if CEP131 S78 phosphorylation is also important for centriolar satellite integrity, we quantified PCM1 as either normal, dispersed, or aggregated, as has been previously reported (19,55). Nocodazole served as positive control for dispersion.(56) We find that depletion of CEP131 increases the dispersion of centriolar satellites (Figure 3H-I). Additionally, addback of WT and S78D CEP131 rescues this phenotype, but addback of the S78A non-phosphorylatable mutant does not rescue this phenotype.

Furthermore, we devised a more objective method of quantifying centriolar satellite organization by computing the percentage of PCM1 intensity found within 2.5µm of the center of the centrosome (Figure 3J). Similarly, we find that addback of the S78A non-phosphorylatable mutant does not rescue centriolar satellite dispersion (Figure 3K). Additionally, addback of the S78D phosphomimetic mutant causes increased aggregation of centriolar satellites. These data support a model where phosphorylation of S78 by PLK4 helps restrain dispersion of centriolar satellites.

CEP131 S78 was previously reported to be phosphorylated in response to ultraviolet (UV) irradiation by mitogen-activated protein kinase-activated protein kinase 2 (known as MK2 or MAPKAP2) (57). In the presence of UV and other cellular stresses, centriolar satellites disperse in a manner dependent on p38-mediated MK2 phosphorylation of CEP131 at both S47 and S78, thereby allowing 14-3-3 proteins to bind and sequester CEP131 to the cytoplasm. Given our findings, we tested whether PLK4 is important for this stress-induced remodeling. Indeed, p38 is required for this remodeling in response to UV, but PLK4 activity is dispensable (Figure 4).

This suggests that PLK4 may also regulate centriolar satellite integrity via CEP131 phosphorylation in a similar manner to MK2, albeit under different conditions.

CEP131 is known to enhance CEP152 recruitment to the centrosome. CEP152 then recruits WDR62 and CEP63 and promotes the centrosomal localization of CDK2 (7). We therefore tested whether CEP131 S78 phosphorylation alters the recruitment of CEP152 to the centrosome by quantitative immunofluorescence in our knockdown-depletion experiment. However, we did not observe any differences in CEP152 localization to the centrosome (Figure 5).

CEP131 knockout cell lines exhibit reduced centriolar satellite organization and increased sensitivity to nutrient stresses

To assess the effects of chronic loss of endogenous CEP131, we engineered CEP131 knockout cell lines using CRISPR/Cas9 editing; this is possible as it is not essential (58). As expected, we recovered monoclonal CEP131 knockout cell lines, and confirmed these by CEP131 immunoblotting (Figure 6A) and immunofluorescence (Figure 6B). We utilized two different knockout clones for our experiments, denoted KO4E and KO9. As expected, knockout cells had dispersed centriolar satellites, as visualized by PCM1 (Figure 6C-D), a decrease in CEP152 expression at the centrosome (Figure 5B), and an increase in mitotic errors and multinuclei (Figure S7), consistent with previous reports (7,49). However, no differences were seen in centriole number (Figure 6E) or formation of primary cilia (Figure 6F-G).

Next, we assessed proliferation in CEP131 knockout cell lines and found slightly reduced proliferation (Figure 6H), as shown previously by CEP131 knockdown (49). Centriolar satellites are important for response to cellular stresses (54,57,59).

Therefore, we tested whether our knockout cell lines were more sensitive to nutrient...
stresses. Indeed, both knockout cell lines are more sensitive to both serum-free media (Figure 6I) and 1% serum media (Figure 6J), as the CEP131 knockout cell lines demonstrated a greater decrease in cell proliferation in response to these treatments.

To identify the role of phosphorylation at S78, we expressed GFP-tagged WT, S78A, and S78D CEP131 constructs in the CEP131 knockout cell lines and evaluated integrity of centriolar satellites. Only cells with visible GFP signal were analyzed. Consistent with previous experiments, addback of WT and S78D rescues the dispersed satellites, while addback of the S78A mutant does not (Figure 6K-M). Additionally, there is an increase in centriolar satellite aggregation in the S78D addback. To confirm that this effect observed using PCM1 as a centriolar satellite marker is indeed a disruption in centriolar satellite integrity versus other causes (e.g. disruption in CEP131 interaction with PCM1), we assessed centriolar satellites in these same conditions using a different marker of centriolar satellites: CEP72. Similarly, we find that CEP72 is dispersed in CEP131 knockout cells and is rescued by addback of WT or S78D transgenes but not S78A (Figure 7).

Next, we assessed whether this aggregation defect observed in S78D mutants is dependent on dynein. Previous reports have demonstrated that PCM1 (5,56,60) and CEP131(49) localization to centriolar satellites is dynein-dependent. We expressed WT CEP131 and CEP131 S78D phospho-mimetic mutant in CEP131 knockout cells and treated with the dynein inhibitor ciliobrevin D. Ciliobrevin D caused dispersion of PCM1 and/or accumulation of PCM1 aggregates outside of the centrosome (Figure 8A-B). Further, treatment with ciliobrevin D in CEP131 knockout cells expressing the S78D mutant significantly reduced the aggregation of PCM1 at the centrosome (Figure 8C-D). These findings suggest that aggregation of satellites by PLK4 phosphorylation of CEP131 is dynein-dependent.

To verify that these observed effects of CEP131 S78 mutants depend on PLK4 activity, we overexpressed WT, S78A, or S78D constructs and GFP control in WT HeLa cells (Figure 9A), treated with the PLK4 inhibitor centrinone B, and observed the effects on centriolar satellite organization by PCM1 immunofluorescence (Figure 9B). Consistent with previous findings, PLK4 inhibition increased the dispersion of centriolar satellites, and the S78D phospho-mimetic mutant restored clustered centriolar satellites in the presence of PLK4 inhibition (Figure 9C-D).

Additive effects of PLK4 phosphorylation of PCM1 and CEP131 on centriolar satellite organization

The effect of PLK4 phosphorylation of CEP131 on centriolar satellite organization is reminiscent of the previously reported effect of PLK4 phosphorylation of PCM1 (19). Therefore, we tested whether these two phosphorylation events cooperate to control centriolar satellite integrity. We overexpressed both phosphomimetic versions of FLAG-CEP131 (S78D) and GFP-PCM1 (S372D) in WT HeLa cells (Figure 10A) and treated with centrinone B. We observe an increase in centriolar satellite aggregation in cells co-expressing both CEP131 S78D and PCM1 S372D phospho-mimetic constructs compared to cells expressing just one of these phospho-mimetic constructs (Figure 10B-C), suggesting that phosphorylations of both CEP131 and PCM1 by PLK4 are important for centriolar satellite integrity. In summary, PLK4 phosphorylates CEP131 and PCM1 to maintain the integrity of centriolar satellites in interphase (Figure 10D).

Discussion

Herein, we have coupled a chemical genetic system of selective PLK4 inhibition and an unbiased, multiplex phosphoproteomic screen to identify novel substrates of PLK4. We identify CEP131 S78 as a substrate of PLK4 and find that PLK4 phosphorylation of this site is important for maintaining the integrity of
Plk4 Phosphorylates Cep131

PLK4 Phosphorylates CEP131

Centriolar satellites in interphase. Centriolar satellites are typically dispersed during mitosis (61), so we speculate that a cellular phosphatase regulates this process by reversing phosphorylation events prior to mitotic onset. However, other regulatory mechanisms are also possible. Additionally, our phosphoproteomic screen has uncovered many other potential substrates that could be explored.

This is the first study to our knowledge to characterize cellular knockout of human PLK4 protein using a Cre/lox conditional system versus prior work that focused solely on chemical inhibition or knockdown. However, the phenotype is highly similar to knockdown PLK4 with RNAi (8), auxin-inducible degradation (41), or inhibited PLK4 catalytic activity (29,40). This is most consistent with PLK4 playing a primarily catalytic role without additional structural roles which would impart additional differences in phenotype between chemical inhibition and depletion or knockout.

PLK4 regulates other centrosomal activities independent of centriole duplication, including phosphorylation of PCM1 to maintain centriolar satellite integrity and primary cilia formation (19), and phosphorylation of CEP135 to regulate its interaction with CEP152 and influence the radial positioning of CEP152 on centrioles (32). Herein we have contributed to our understanding of one such non-canonical role of PLK4: the organization of centriolar satellites. Centriolar satellites are structures located around the centrosome and are important for proper microtubule nucleation, centrosome assembly, and primary cilia formation (5,54,56). Our data suggest that PLK4 phosphorylation of both PCM1 and CEP131 is required to maintain centriolar satellite organization and/or integrity. However, PLK4 phosphorylation of CEP131 is dispensable for centriole duplication and primary cilia formation.

Given the previously reported findings that intact, functional centriolar satellites and/or their components are important for centriole duplication (7,49) and ciliogenesis (19,62), it is likely that different methods of centriolar satellite perturbations affect these pathways differently. It will be important to determine whether it is centriolar satellites or the individual components themselves that are important for these processes.

A number of centriolar satellite components are important for accurate mitosis. For example, we and others (49) have demonstrated that depletion of CEP131 increases multipolar spindles and chromosome missegregation. Given this increase in chromosomal instability with loss of CEP131, it might be suspected that CEP131 is a tumor suppressor. However, data from The Cancer Genome Atlas (TCGA) indicates that genomic deletion of CEP131 or decreased CEP131 gene expression are extremely rare events in cancer and are likely not drivers of chromosomal instability in cancer. Furthermore, increased CEP131 expression has been reported in hepatocellular carcinoma and correlates with worse outcomes (63). The specific mechanism by which centriolar satellites regulate proper chromosome segregation remains to be elucidated.

As the cell enters mitosis, centriolar satellites disperse, and upon completion of mitosis and entry into G1, the satellites reorganize around the centrosome (61). PLK4 phosphorylation of PCM1 and CEP131 may help regulate the reorganization of centriolar satellites in G1 in preparation for centriole duplication. Previous evidence has suggested that centriolar satellites are required for recruiting many key regulators of centriole duplication to the centrosome. Other possible mechanisms explaining centriolar satellite dispersion at the onset of mitosis include NEK2A kinase-dependent loss of C-NAP1 from the centrosome leading to reduced centriolar satellite density (64). There may be additional mechanisms of centriolar satellite dispersion in mitosis and reorganization in G1 that remain to be elucidated, and this is an important area of future research. One additional question is why and how do some centriolar satellite
proteins remain at the centrosome when centriolar satellites are dispersed in mitosis and with microtubule depolymerization (e.g. OFD1 and CEP290), while others (e.g. BBS4 and PCM1) do not remain at the centrosome under these conditions (6). Further work is required to elucidate this mechanism of centriolar satellite dispersion and aggregation during the cell cycle and its functional significance.

Centriolar satellites are also important for response to stresses (54), such as UV, heat shock, proteotoxic reagents, and nutrient deprivation. In response to serum starvation, MIB1 E3 ubiquitin ligase activity is reduced, which decreases PCM1 and CEP131 ubiquitination.(59) Furthermore, loss of centriolar satellites by depletion of PCM1 sensitizes glioblastoma cells to temozolomide (65), a DNA alkylating agent commonly used to treat glioblastoma. Further work will be needed to further elucidate these molecular mechanisms regarding centriolar satellites and response to cellular stresses. For example, since PLK4 inhibition causes centriolar satellite dispersion, does PLK4 inhibition also sensitize glioblastoma and other cancer types to temozolomide and other stress-inducing agents? As PLK4 inhibition is a potential strategy to treat cancer (40,66-68), this is an important area of future research.

Another stressor, UV light, induces restructuring of centriolar satellites, including the displacement of PCM1, CEP131, CEP290 from satellites (57,59). Phosphorylation of CEP131 at the S47 and S78 residues by the p38 effector kinase MK2 promotes centriolar satellite remodeling and dissociation of CEP131 from PCM1 in response to UV light (57). Surprisingly, this previous report found that expression of the both non-phosphorylatable and phospho-mimetic CEP131 prevented dispersion in response to UV light, whereas we have found that the non-phosphorylatable mutant causes centriolar satellite dispersion while the phospho-mimetic mutant promotes centriolar satellite aggregation. Although the reason for the discrepancy for the function of the phosphoimetic is unclear, we speculate that the regulatory mechanisms during DNA-damage stresses, such as UV, may differ from non-DNA damage cellular stresses.

CEP131 is thought to be involved in centriole duplication or, at least, controlling centriole numbers. However, one report demonstrated that CEP131 depletion increases centriole numbers (49), while another report demonstrated that CEP131 depletion decreases centriole numbers (7). Additional evidence from Cep131-mutant MEFs found no differences in centrin foci or centrosome amplification (percent of cells with >2 gamma tubulin foci) (52). Furthermore, USP9X is a deubiquitinase that was shown to stabilize CEP131. This prior report also found that USP9X is required for centriole duplication, providing some indirect evidence that CEP131 may be required for centriole duplication (69). However, herein we find that depletion of CEP131 does not significantly alter centriole number, regardless of the degree or duration of knockdown.

A limitation of our experiments is that PLK4 has low abundance with rare phosphorylation events, making it particularly challenging to detect direct substrates. As a result, we reduced our fold-change and statistical significance thresholds and focused on centrosomal proteins. Because of this, any future use of our phosphoproteomic dataset to determine potential substrates of PLK4 will also require robust in vitro and in vivo validation. Furthermore, our analysis did not identify some of the previously identified PLK4 substrates, such as STIL. Despite this limitation, we detected other known substrates including CEP152 and PCM1, as well as CEP131, which is validated as a direct substrate in biochemical assays. Another limitation of our work is that we narrowly focused our potential substrates (e.g., evolutionary conservation, role in centriole duplication), and these criteria may have been too strict to uncover novel PLK4 functions and substrates. Lastly, the identified phosphosite on CEP131, S78, does not conform to the canonical PLK4
PLK4 Phosphorylates CEP131

9

...consensus motif (S/T followed by two hydrophobic residues) (70). However, this consensus motif does not hold in all cases. Despite this, we did validate that direct phosphorylation occurs with purified proteins.

In summary, we developed a chemical genetic method to interrogate PLK4 signaling and utilized multiplex phosphoproteomics to discover novel substrates of PLK4. We identified CEP131 as a novel substrate of PLK4 and find that PLK4 phosphorylation of CEP131 S78 is important for proper organization and integrity of centriolar satellites and may help explain the changes seen in centriolar satellite organization during the cell cycle.

Experimental procedures

Generation of PLK4 conditional knockout and analog sensitive cell lines

Gene editing was performed using adeno-associated virus (AAV) in RPE-1 (ATCC, Manassas, VA) an immortalized, non-transformed human epithelial cell line. Procedures for preparation of infectious AAV particles, transduction of RPE-1 cells, and isolation of properly targeted clones was performed as described previously (71,72). Briefly, a targeting vector was made by inserting a neo cassette surrounded by FRT sites in the intron upstream of exons 3-4 of PLK4, all flanked by loxP sites (Figure S1). To generate adenovirus producing this targeting vector, HEK293 cells were transfected with pRC and pHelper. RPE-1 cells were infected with adenovirus collected from the supernatant and subcloned in the presence of G418 (0.4 μg/mL) and screened/verified. This cell line was targeted in two ways: (1) to make a conditional knockout cell line; and (2) to make an analog sensitive cell line. To make the conditional knockout cell line, one copy of PLK4 was deleted by treating with Cre, then cells were re-targeted with the same initial vector (neo cassette, exons 3-4, all flanked by loxP sites). To make the analog sensitive cell line, cells were transfected with pFLIPE to remove the neo cassette, subcloned and re-targeted with the initial targeting vector in which the gatekeeper residue was mutated to a glycine (L89G) to enlarge the ATP binding pocket and genetically encode chemical sensitivity to the bulky ATP analog 3-MB-PP1 (42) (Figure S1). The resulting ASflox/WTflox cell lines were treated with Cre and subcloned in the presence G418. Clones were screened by PCR with primers both inside and outside the neo cassette (Figure S1). In addition, RNA was isolated from the AS cells, converted to cDNA, exon 3 was PCR-amplified and sequenced to confirm the presence of only the mutant (L89G).

Cell Culture and Assays

All cell lines were propagated at 37 °C in 5% CO₂. RPE-1-derived cell lines were grown in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 2.5 mm l-glutamine, with 10% fetal bovine serum and 100 units/mL penicillin-streptomycin. HeLa cells (ATCC) were grown in high glucose DMEM supplemented with 2.5 mm l-glutamine, with 10% fetal bovine serum and 100 units/mL penicillin-streptomycin. For stable retroviral transduction, constructs were co-transfected with a VSV-G envelope plasmid into Phoenix cells. Fresh medium was applied at 24 hours post-transfection, harvested 24 hours later, clarified by centrifugation and filtration through a 0.45 μm membrane to remove cell debris, and diluted 1:1 with complete medium containing 10 μg/mL polybrene. Target cells were infected at 40-60% confluence for 24 hours. Polyclonal transductants were further purified by limiting dilution to obtain individual clones.

To test for a proliferative defect, sub-cloning was performed in presence of 10 μM 3-MB-PP1. Poisson correction was used to determine cloning efficiency. Cellular senescence was assayed using a pH-dependent β-galactosidase staining kit (Cell Signaling Technology) according to the manufacturer’s instructions.

Chemicals used in this study include aphidicolin (Sigma), 3-MB-PP1 (Toronto Research Chemicals), doxycycline (Fisher),...
puromycin (Thermo), SB203580 (p38 inhibitor, MedChem Express), etoposide (obtained from UW Oncology Pharmacy), doxorubicin (Fisher), paclitaxel (Fisher), vincristine (Fisher), thapsigargin (Fisher), tunicamycin (Fisher), chloroquine (Fisher), and bafilomycin A1 (Fisher).

siRNA directed against CEP131 (5′-AGGCCCTCAGGCCCAACAA-3′) was purchased from GE Healthcare, and control siRNA (Universal Negative Control #1) was purchased from Thermo Scientific.

For CEP131 knockdown-addback experiments, HeLa cells were grown to 80-90% confluence in 6-well plates. 2 µg CEP131 cDNA plasmid and 4 µL of 20 µM siRNA were co-transfected simultaneously using lipofectamine 2000 according to the manufacturer’s instructions. Cells were given fresh media 4 hours after the transfection. 24 hours after the transfection, cells were either transferred to coverslips for fixation and immunofluorescence or harvested for Western blotting and qRT-PCR.

For ciliogenesis, 5x10^4 cells were plated on coverslips, allowed to adhere overnight, washed 3 times with HBSS, given serum-free media for 48 hours, then fixed with methanol and stained for acetylated tubulin to mark primary cilia.

CRISPR/Cas9

The CRISPR/Cas9 system was used to create CEP131 knockout cell lines in HeLa cells. Four gRNAs targeting CEP131 were cloned into lentiCRISPRv2(73,74) from Feng Zhang (Addgene plasmid #52961). The four guides used were: GCGCTCCGGGACGCTGCCGA, CCGGGACGCTGCGATGGCC, TTGACTCACCAGGGGCCC, GGCACGCTGCACTCCGGGCAT. Cutting efficiency of each guide was assessed by surveyor assay (IDT, 706025) following PCR of 600bp fragments from genomic DNA encompassing the guide RNA locus. lentiCRISPRv2 vectors were transfected into HeLa cells using Fugene (Promega). Prior to transfection, media was replaced with serum- and antibiotic-free media. 24 hours after transfection, cells were selected with puromycin (1µg/mL) for 72 hours, then allowed to recover for 24 hours in fresh media. The guide RNA yielding the greatest cutting by surveyor assay (TTGACTCACCAGGGGCCC) was transfected again into HeLa cells, which were then subcloned by limiting dilution. Colonies were selected and screened by PCR of the region flanking the cut site and sequencing. Verification of knockout lines was performed with CEP131 Western blotting and immunofluorescence.

Immunofluorescence (IF) and microscopy

IF and imaging were carried out as previously described (75,76). Cells were seeded on glass coverslips in 24-well plates and fixed with 100% ice-cold methanol for 30 minutes. Fixed cells were then blocked for 30 minutes in 3% bovine serum albumin (BSA) and 0.1% triton X-100 in PBS (PBSTx + BSA). Primary antibodies were incubated in PBSTx + BSA for 1 hour at room temperature and washed three times in PBSTx, followed by secondary antibody incubation in PBSTx + BSA for 30 minutes at room temperature and two washes with PBSTx. Cells were counterstained with DAPI and mounted on glass slides with Prolong Gold antifade medium (Invitrogen). Image acquisition was performed on a Nikon Eclipse Ti inverted microscope and Hamamatsu ORCA Flash 4.0 camera. Where indicated in the figure legends, optical sections were taken at 0.2-µm intervals and deconvolved using Nikon Elements. Where appropriate, the observer was blinded to treatment condition during image acquisition and analysis. Images were processed and analyzed using Nikon Elements.

Qualitative analysis of centriolar satellites was performed by categorizing cells as either normal, dispersed, or aggregated, as previously described (19). Further, quantitative analysis of centriolar satellites was performed by taking Z stack images, deconvolving, and quantifying the percentage of PCM1 or CEP72 intensity
within a 2.5 μm radius of the center of the centrosome (identified by gamma tubulin).

Primary antibodies used were: CEP131 (Thermo, PA5-38978, 1:500), CEP72 (Proteintech, 19928-1-AP, 1:500), CEP152 (Bethyl, A302-480A, 1:500), alpha tubulin (Abcam, ab4074, 1:5000), acetylated tubulin (Santa Cruz, sc-23950, 1:1000), pericentrin (Abcam, ab4448, 1:1000), polyglutamylated tubulin (Adipogen, AG-20B-0020, 1:500), PCM1 (Cell Signaling, 5259, 1:500), centrin (Millipore, 04-1624, 1:500), gamma tubulin (Abcam, ab27074, 1:1000), beta actin (Abcam, ab6276, 1:5000), FLAG (Sigma, F1804, 1:1000), GFP (Invitrogen, A-11120, 1:1000). Alexa Fluor-conjugated secondary antibodies were used at 1:350 (Invitrogen).

Western blotting and immunoprecipitation

Cells were lysed in buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 0.5% NP-40, 10% glycerol) containing phosphatase inhibitors (10 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 50 mM NaF, 0.3 mM Na3VO4), 1 mM PMSF, protease inhibitor cocktail (Thermo Scientific), and 1 mM dithiothreitol. Samples were sonicated and heated in SDS buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membrane (Millipore), and blocked for at least 30 minutes in 5% milk and 0.1% Tween 20 in Tris-buffered saline, pH 7.4 (TBST + milk). Membranes were incubated 1 hour at room temperature or overnight at 4°C with primary antibodies diluted in TBST + 5% milk, washed three times with TBST, and incubated for 1 hour at room temperature in secondary antibodies conjugated to horseradish peroxidase in TBST + 5% milk. Membranes were washed and developed with luminol/peroxide (Millipore) and visualized with film.

Antibodies utilized for immunoblotting include: actin (DSHB, JLA20, 1:1000), CEP131 (Thermo, PA5-38978, 1:1000), FLAG (Sigma, F1804, 1:5000), GFP (Invitrogen, A-11120, 1:1000), and alpha tubulin (DSHB, 12G10, 1:1000). HRP-conjugated secondary antibodies were used (Jackson, 1:5000). Relative intensities of bands were calculated using ImageJ from scanned images and normalized to their respective loading control intensity.

For immunoprecipitation experiments, T75 flasks of HeLa cells were transfected with FLAG-PLK4 and GFP-tagged CEP131 fragments. Two days after transfection, cells were lysed in the aforementioned lysis buffer used for western blotting. Extracts were incubated with anti-FLAG M2 agarose beads (Sigma). Beads were washed three times with lysis buffer and immunoprecipitated protein complexes were analyzed by SDS-PAGE and immunoblotting with anti-FLAG and anti-GFP antibodies.

Preparation of Samples for Mass Spectrometry

PLK4 AS cells were cultured in T175 plates. At 75% confluence, cells were synchronized at the G1-S phase with aphidicolin (5 μM) for 24 hours. To assess cell cycle, a fraction of cells from MS sample preps were taken for flow cytometry. Cell pellets were fixed in cold 70% EtOH for at least 24 hours, washed once in PBS, and resuspended in PBS with 0.5 mg/mL RNase A and 50 mg/mL propidium iodide. Samples were incubated at 4°C overnight and analyzed on a flow cytometer (FACSCalibur, BD). 3-MB-PP1 (10 μM) was added for the last 6 hours to half of the plates. 3 independent replicates were utilized for each of the 2 conditions. Cells were collected, pelleted, snap frozen in liquid nitrogen and stored at -80°C until ready for lysis. Cell pellets were then lysed in buffer (8 M urea, 50 mM Tris pH 8.0, 100 mM CaCl2) containing dissolved protease and phosphatase inhibitor tablets (Roche) and then sonicated for 20 minutes. Protein concentrations were determined using a BCA kit (Thermo Pierce). Cell lysates were then reduced by addition of dithiothreitol (final concentration of 5 mM), incubated at 37°C for 45 minutes, and alkylated by adding iodoacetamide (15 mM final concentration). Next, lysates were incubated for 45 minutes in the dark at room...
PLK4 Phosphorylates CEP131

temperature, and remaining iodoacetamide was quenched by bringing each lysate back to a final 5 mM dithiothreitol concentration. Lysates were diluted to a 1.5 M urea concentration using a 50 mM Tris and 100 mM CaCl$_2$ solution. Trypsin was added to each lysate in a 50:1 (protein: enzyme) ratio and digested overnight at ambient temperature. The six samples were desalted using 100 mg C18 Sep-Paks (Waters) and dried down using a vacuum centrifuge to obtain tryptically digested peptides. 1 mg of peptides for each of the six samples was incubated with 6-plex tandem mass tags (TMT) reagents (Thermo Scientific) for three hours at room temperature. An aliquot of each sample was mixed in a 1:1 ratio and run on an Orbitrap Elite mass spectrometer (Thermo Scientific) to ensure complete TMT peptide labeling. The 6 samples were mixed in a final 1:1 ratio across all 6 TMT channels and desalted using a 500 mg C18 Sep-Pak (Waters) to produce a single pooled sample containing chemically labeled peptides from all 6 samples. The pooled sample was fractionated using Strong Cation Exchange (SCX) chromatography to produce 12 total peptide fractions, which were subsequently lyophilized and desalted. The resultant 12 peptide fractions were each enriched using Immobilized Metal Affinity Chromatography (IMAC) Ni-NTA magnetic agarose beads (Qiagen), leading to 12 final SCX fractionated enriched phosphopeptide and unenriched peptide fractions. Each fraction was dried down using a vacuum centrifuge and resuspended in 0.2% formic acid for MS analyses.

**LC-MS/MS**

Each sample was introduced to an Orbitrap Fusion mass spectrometer (Thermo Scientific) during a 90 minute nano-liquid chromatography separation using a nanoAcquity UPLC (Waters). A “Top N” Fusion method was used to analyze eluting peptides, using a 15,000 resolving power survey scan followed by MS/MS scans collected at 15,000 resolving power. Peptides were fragmented using higher-energy collisional dissociation (HCD) at a normalized collision energy of 35%. Phosphopeptide fractions were analyzed with 200 msec maximum injections times for MS scans and 120 msec maximum injection times for MS/MS scans, while unenriched fractions were analyzed with 100 msec maximum injections times for MS scans and 75 msec maximum injection times for MS/MS scans. Only peptides with charge states from +2 to +8 were selected for MS/MS with an exclusion duration of 30 seconds. The 12 phosphopeptide samples were run in duplicate.

**MS data analysis**

Data was searched using Proteome Discoverer 1.4.1.14 (Thermo Fisher) with the Sequest search algorithm. Thermo RAW files were searched against a Homo sapiens target-decoy database (UniProt, downloaded 11/06/2014). Peptide and phosphopeptide datasets were searched using a 50 ppm precursor mass tolerance and 0.02 Da fragment tolerance for b- and y-type ions produced by HCD fragmentation. All fractions were searched with static carbamidomethyl of cysteine residues, static TMT 6-plex modifications of peptide N-termini and lysines, dynamic methionine oxidation, and dynamic TMT 6-plex modification of tyrosine residues. Phosphopeptide fractions were searched with additional dynamic phosphorylation modifications of serine, threonine, and tyrosine residues. Resulting peptide identifications were filtered to 1% false discovery rate (FDR) and exported to tab-delimited text files compatible with the COMPASS software suite (77). COMPASS calculated the six-plex TMT protein and phosphopeptide quantitation for all the 12 peptide fractions and 12 phosphopeptide fractions. Peptides were mapped back to their parent proteins using COMPASS, and Phospho RS (78) was used to localize phosphorylation to amino acid residues with a fragment tolerance of 0.02 Da automatically considering neutral loss peaks for HCD and considering a maximum of 200 maximum position isoforms per phosphopeptide. The raw 6-plex reporter ion
PLK4 Phosphorylates CEP131

intensities of localized phosphopeptide isoforms were log₂ transformed and normalized against inhibited PLK4 (+3-MB-PP1) to obtain the relative phosphopeptide and protein quantitation for each cell line and condition.

**Kinase assays**

The PLK4 kinase domain (amino acids 1–390) of human PLK4 was cloned into pGEX-6P-1 vector (GE Healthcare). The catalytically inactive mutant, K41M, was generated using Phusion site-directed Quikchange mutagenesis. 15-amino acid fragments of the identified potential PLK4 substrates (putative phosphosite ±7 amino acids) were also cloned into pGEX-6P-1. Sequences of these peptides can be found in Table S2. Protein was expressed in Rosetta DE3 bacteria, extracted with Glutathione Sepharose 4B (GE Healthcare, 17-0756-01), and eluted by addition of glutathione for the production of the intact GST fusion proteins.

All kinase assay reactions were incubated at 30°C for 30 minutes in buffer (50mM Tris-HCl, pH 7.5, 10mM MgCl₂, 0.1mM NaF, 10μM Na₃VO₄) with 1mM DTT, 1μM cold ATP, 2μCi [γ-³²P] ATP, 5μg substrate, and 100ng PLK4. Reactions were resolved by SDS-PAGE. Dried gels were exposed to a storage phosphor screen (GE Healthcare), and γ-³²P incorporation was visualized by Typhoon TRIO imager (GE Healthcare).

To confirm that CEP131 S78 is a substrate of PLK4, S78A mutant (15 amino acid fragment, as above) was generated using Phusion site-directed mutagenesis, purified, and utilized in kinase assays, as described above.

**Molecular biology**

CEP131 cDNA was obtained as a kind gift from Drs. Spencer Collis and Katie Myers (corresponds to Uniprot isoform 2, Q9UPN4-2). Phusion site-directed mutagenesis was used to generate S78A and S78D mutants and to make CEP131 siRNA-resistant (mutated AGGCCCTCAAGGCAACAA to AAGCATTGAAGCAAAATA).

For construction of AS cells stably expressing mNeonGreen-centrin, CETN2 and mNeonGreen were cloned into pQCXIX retroviral vector. Retrovirus was generated by transfecting Phoenix cells (ATCC) in a T25 flask with 2 μg pQCXIX-mNeonGreen-CETN2 and 1 μg pVSV-G packaging vector. Virus was collected 48 hours later and used to infect AS cells, which were then subcloned to isolate a monoclonal cell line stably expressing mNeonGreen-centrin.

**Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)**

RNA was isolated from cells using TRI Reagent (Molecular Research Center) per manufacturer’s protocol. DNase treatment was used to remove DNA contamination (Promega). RNA was then converted to cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit and manufacturer’s protocol. Primer sequences are provided in Table S3. Primers were used at a final concentration of 0.67 μM each, and 25ng cDNA was used per reaction. A StepOne Plus (Applied Biosystems) real-time PCR thermal cycler was used for amplification with iQ SYBR Green Supermix (Bioard) per manufacturer’s protocol. Quantification of CEP131 mRNA was normalized to three housekeeping genes (RRN18S, GAPDH and ACTB). The ΔΔCₜ method was employed to calculate the fold change in expression (79).

**Drug screening**

To assess the sensitivity of CEP131 knockout cell lines to chemicals, 1000 cells/well were plated in 96-well plates and allowed to adhere for 24 hours. Chemicals were added the next day. Four days later, media was removed and cell viability was determined using Vita-Orange Cell Viability Reagent (Biotool) according to the manufacturer’s instructions. The Vita-Orange reagent detects metabolically active cells, which linearly correlates with the number of viable cells. Relative proliferation was determined by subtracting absorbance values of wells containing only Vita-Orange reagent from the experimental wells and...
then normalizing them to untreated control wells for each cell line.

**Statistics**
Statistical evaluations were performed using Prism software (GraphPad). Two-tailed t-tests and one-way ANOVA were used for comparisons. P-values <0.05 were considered significant for all tests, and designations are made in the figures for statistical significance.

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**FOOTNOTES**
The abbreviations used are: AS, analog sensitive; MS, mass spectrometry; KO, knockout; PLK4, polo-like kinase 4; TMT, tandem mass tags; UV, ultraviolet; WT, wild type.
Table 1: Previously identified substrates of PLK4.

| Gene   | Uniprot ID | Other Names and Orthologs | Residue(s) | Protein function | Effect of phosphorylation | Ref            |
|--------|------------|----------------------------|------------|------------------|--------------------------|----------------|
| PLK4   | O00444     | Sak, STK18, ZYG-1, Plx4    | T170, S305; S293 and T297 in Drosophila | Coordinates centriole duplication | Results in ubiquitination and degradation, limiting centriole duplication to once per cell cycle | (20-23,42,80) |
| FBXW5  | Q969U6     | F-box/WD repeat-containing protein 5, FBW5,PP397 1 | S151 | Substrate recognition component of both SCF (SKP1-CUL1-F-box protein) and DCX (DDB1-CUL4-X-box) E3 ubiquitin-protein ligase complexes | Prevents its ability to phosphorylate SAS6, keeping SAS6 at the centrosome and enabling centriole duplication | (24) |
| TUBGCP 6 | Q96RT7 | GCP6, KIAA1669 | Undetermined | Microtubule nucleation | Required for centriole duplication | (25) |
| SAS6   | Q6UV10     | SASS6, HsSAS-6, MCPH14 | S123 (in C. elegans) | Centriole cartwheel formation | Allows maintenance of SAS6 and emerging centriole | (27) |
| HAND1  | O96004     | BHLHA27, EHAND | T107, S109 | Transcription factor that plays an essential role in both trophoblast-giant cells differentiation and in cardiac morphogenesis | Frees HAND1 from the nucleolus, allowing for cell differentiation | (33) |
| ECT2   | Q9H8V3     | Epithelial Cell Transforming 2, ARHGEF31 | Undetermined | Guanine nucleotide exchange factor (GEF), facilitates myosin | Unclear, only in vitro kinase assay performed | (38) |
| Protein | UniProt ID | Description | Function | Notes |
|---------|------------|-------------|----------|-------|
| STIL    | Q15468     | SCL/TAL1-interrupting locus, SIL, MCPH7, Ana2, SAS5 | S1108 and S1116 in human STIL; S318, S365, S370, S373 in Drosophila Ana2 | Recruits SAS6, allowing for proper centriole duplication | Allows for SAS6 recruitment (28,30,31) |
| CEP110  | O43303     | CCP110, CP110, KIAA0419 | S98 | Centriole duplication, caps mother centriole | Promotes centriole duplication, may stabilize SAS6 (26) |
| CEP152  | O94986     | KIAA0912, MCPH9, SCKL5, MCPH4, asterless, Asl | Undetermined | Scaffold for recruitment of PLK4 and CENPJ | Unclear (35) |
| CDC25C  | P30307     | M-phase inducer phosphatase 3 | Undetermined | Tyrosine protein phosphatase required for progression of the cell cycle | Unclear, only in vitro kinase (36) |
| CHK2    | O96017     | Checkpoint kinase 2, CHEK2, CDS1, RAD53 | Undetermined | DNA damage response and cell-cycle arrest | Unclear, only in vitro kinase assay performed (37) |
| PCM1    | Q15154     | Pericentriolar material 1, PTC4 | S372 | Centrosome assembly, ciliogenesis, forms centriolar satellites | Required for centriolar satellite formation and ciliogenesis (19) |
| CPAP    | Q9HC77     | Centromere protein J, CENPJ, LAP, LIP1 | S595 | Recruits centrosome components, inhibits microtubule nucleation from centrosome | Allows for procentriole assembly and centriole elongation (81) |
| Protein | Modification | Co-PIs | Status | Function  |
|---------|--------------|--------|--------|-----------|
| CEP135  | Q66GS9       | CEP4, KIAA0635 | Undetermined | Centriole duplication, recruitment of centriolar satellite proteins |
|         |              |        |        | Allows for recruitment of asterless to centrioles    |
| ARP2   | P61160       | ACTR2  | T237, T238 | Actin organization |
|         |              |        |        | Enhances cell motility |

(32)  
(34)
Figure 1: Validation of PLK4 chemical genetic system.  
(A) Representative images of RPE PLK4 AS cells treated with 10 μM 3-MB-PP1 for 5 days. Individual cells are outlined with dotted lines in the centrin image, and the arrow points to the one cell in the image with centrioles. Scale bar = 5 μm.  
(B) Centrosomes were quantified after treatment with 3-MB-PP1 for the indicated time points. Bars represent the average of 3 biological replicates of 100 cells each ± SEM.  
(C) PLK4<sup>AS/Δ</sup> and PLK4<sup>WT/Δ</sup> were subcloned by limiting dilution, and cloning efficiency was assessed using a Poisson correction.  
(D) Proliferation was assessed by culturing cells in the indicated concentration of 3-MB-PP1 for 7 days, then staining with crystal violet.  
(E) 1000 cells were plated per well in a 6-well plate (day -2), and 3-MB-PP1 was added on day 0. Cells were counted every 2 days. Each point represents the average of 3 technical replicates ± SEM.  
(F) Cells were cultured for 7 days with DMSO or 3-MB-PP1, and apoptosis was assessed by both trypan blue exclusion assay and staining with propidium iodide and anti-annexin V followed by flow cytometric detection. The percentage of cells staining negative for both propidium iodide and annexin V are plotted.  
(G) Senescence was assessed by β-galactosidase staining after 14 days of culture with 3-MB-PP1. The micrographs are representative images of the staining, and the bar graphs quantifies the percentage of senescent cells. All experiments in this figure utilized RPE cells.
Figure 2: PLK4 phosphorylates CEP131 S78.

(A) Cell cycle profile of cells arrested with aphidicolin to enrich RPE PLK4 AS cells in the G1/S transition, which is when centriole duplication occurs and therefore when PLK4 is active. These cells were utilized for mass spectrometry. (B) Volcano plot of –log10(p value) versus log2(fold change) for the centrosome components (red dots) that were identified in the MS screen. (C) Diagram of the centrosome showing where each of these proteins are thought to localize. Adapted from (82). (D) GST-tagged peptides and PLK4 kinase domain were purified from E. coli and utilized for in vitro kinase assays. GST serves as a negative control and STIL serves as a positive control. Centrinone B is a PLK4 inhibitor. (E) An S78A mutation was created in the CEP131 peptide and purified for in vitro kinase assay to confirm that PLK4 phosphorylates CEP131 S78.
Figure 3: PLK4 phosphorylation of CEP131 maintains centriolar satellite integrity.
(A) Localization of WT CEP131, S78A and S78D mutants in WT HeLa cells. The dotted white box is enlarged for the adjacent gray scale images showing GFP, PCM1, and centrin at the centrosome. Scale bar sizes are indicated on the images. (B) Knockdown addback experiment timeline. (C) Quantitative RT-PCR experiment demonstrating the efficiency of knockdown and addback of CEP131. Asterisks demonstrate significant enrichment of GFP-tagged CEP131 versus endogenous GFP (black bars) or significant depletion of endogenous CEP131 versus untreated cells (gray bars). (D-E) Images and quantification of centriole duplication. Each dot represents one cell, and bars represent means ±SD of at least 100 cells per condition. Centrioles were identified by counting centrin foci. For example, the image in D shows 2 centrosomes with 4 total centrioles. (F-G) Images and quantification of ciliogenesis. Cilia were marked by staining for acetylated tubulin. At least 300 total cells were analyzed per condition. (H-I) Images and quantification of centriolar satellites. At least 300 total cells were analyzed per condition. (J) Schema of a more quantitative approach to assess centriolar satellites. The yellow circle measures the PCM1 intensity within a 2.5 radius of the center of the centrosome, while the blue circle measures the PCM1 intensity in the entire cell. (K) Quantification of centriolar satellites using the schema described in panel J. Nocodazole is used as a positive control for centriolar satellite dispersion. All experiments in this figure utilize HeLa cells. Bars represent means ±SEM from 3 independent experiments. Scale bars = 10µm. *P value < 0.05. NS = not significant.
Figure 4: PLK4 activity is not required for centriolar satellite remodeling in response to UV.
(A) Representative images of centriolar satellites (PCM1 staining) in HeLa cells under the indicated conditions. The p38 inhibitor (p38i) utilized was SB203580. Scale bar = 10 μm. (B) Quantification of centriolar satellites as aggregated or dispersed. Bars represent averages ±SD for 1 independent experiment. *P value < 0.05; ns = not significant. Scale bar = 5 μm.
Figure 5: Effects of S78A and S78D mutations on CEP131 interaction with CEP152.
(A) HeLa cells were simultaneously transfected with CEP131 siRNA and the indicated GFP-tagged CEP131 construct. Cells were then fixed and stained for CEP152. The amount of CEP152 at the centrosome was quantified and is shown in the adjacent dotplot. Insets on CEP152 images show magnified CEP152 at the centrosome. (B) WT HeLa cells and CEP131 knockout cell lines (KO4E and KO9) were stained for CEP152, and the amount of CEP152 at the centrosome was quantified. Insets show magnifications of the centrosome. Each dot represents a single cell. Bars represent means ±SD. Scale bars = 10μm. *** P value < 0.0001; ns = not significant.
Figure 6: Chronic CEP131 depletion causes dispersion of centriolar satellites and is rescued by CEP131 phosphorylation.
(A) CEP131 Western blot in WT HeLa cells compared to two CEP131 knockout cell lines, KO4E and KO9. (B) Immunofluorescent images showing loss of CEP131 in the knockout cell lines. (C) Immunofluorescent imaging of centrioles and centriolar satellites in the knockout cell lines. (D) Quantification of centriolar satellites in WT HeLa cells and the two CEP131 knockout lines. (E) Quantification of centrioles (centrin foci) in WT and CEP131 knockout HeLa lines. (F) Representative images of primary cilia in WT and CEP131 knockout HeLa lines. (G) Quantification of primary cilia in WT HeLa cells and the two CEP131 knockout lines. Scale bars = 10µm. (H) Cell counts over 12 days in WT HeLa cells compared to two CEP131 knockout cell lines. (I-J) Proliferation in cells treated with serum-free media (I) or with 1% serum (J). Proliferation was normalized to untreated cells within each cell line. (K) Representative images of centriolar satellites (marked by PCM1) in CEP131 knockout cells transfected with either WT, S78A, or S78D CEP131 transgenes. (L) Quantification of the percent of cells with dispersed and aggregated PCM1. (M) Quantification of the percent of PCM1 intensity within a 2.5 µm radius of the center of the centrosome. Nocodazole is used as a positive control for centriolar satellite dispersion. All experiments in this figure utilize HeLa cells. Bars represent means ±SEM. Scale bars = 10µm. *P value < 0.05, ns = not significant.
Figure 7: CEP72 staining recapitulates regulation of centriolar satellite integrity by CEP131 S78 phosphorylation status.

(A) To ensure that our results regarding PLK4 phosphorylation of CEP131 regulating centriolar satellite integrity were not specific to PCM1, we also analyzed another marker of centriolar satellites, CEP72. Wild type (WT) or CEP131 knockout (KO) HeLa cell lines were transfected with GFP-tagged CEP131 constructs (WT, S78A, S78D) versus GFP-only control. Nocodazole treatment served as a positive control for satellite dispersion. Representative images are shown. 

(B) CEP72 was assessed as either dispersed or aggregated, just as done for PCM1 staining of centriolar satellites in other figures. All experiments in this figure utilize HeLa cells. Bars represent means ±SD from three replicates. Scale bars = 10µm. At least 300 cells were analyzed for each condition. *P value < 0.05.
Figure 8: Dynein is required for satellite aggregation with CEP131 S78D phosphomimetic. (A) Representative images of HeLa cells treated with nocodazole and the dynein inhibitor ciliobrevin D. (B) Quantification of centriolar satellites (PCM1 staining) in the conditions shown in panel A. (C) Representative images of WT HeLa cells transfected with CEP131 S78D and treated with ciliobrevin D. (D) Quantification of centriolar satellites (PCM1 staining) in the conditions shown in panel C. Bars represent means ±SD from three replicates. Scale bars = 10µm. At least 300 cells were analyzed for each condition. *P value < 0.05.
Figure 9: PLK4 phosphorylation of CEP131 at S78 is critical for proper centriolar satellite integrity.

(A) WT HeLa cells were transfected with GFP or GFP-tagged WT CEP131, S78A or S78D mutants. Western blot demonstrating transfection efficiency. (B) Images of centriolar satellites in these transfected cells after treatment with the PLK4 inhibitor centrinone B. Scale bar = 10µm. (C) Qualitative assessment of centriolar satellites as either dispersed or aggregated. (D) Quantitative assessment of centriolar satellite dispersion/aggregation. All experiments in this figure utilize WT HeLa cells. Bars represent means ±SEM. Scale bars = 10µm. *P value < 0.05; **P value < 0.01; *** P value < 0.0001; ns = not significant.
Figure 10: PLK4 phosphorylation of CEP131 cooperates with its phosphorylation of PCM1. (A) Western blotting demonstrating transfection efficiency of GFP-PCM1 and FLAG-CEP131 constructs. (B) Images of centriolar satellites in the transfected cell before and after PLK4 inhibition with centrinone B. Scale bar = 10µm. (C) Quantification of dispersed and aggregated centriolar satellites. *P value < 0.05. All experiments in this figure utilize HeLa cells. (D) Diagram summarizing the regulation of centriolar satellite integrity by PLK4 phosphorylation of CEP131 and PCM1. Red = cytoplasm, light blue = DNA, green = centrosome, yellow = centrioles, dark blue = centriolar satellites.
Figure 1

A

B

C

D

E

F

G

AS/Δ + 3-MB-PP1
AS/Δ + DMSO
WT/AS + 3-MB-PP1
WT/Δ + 3-MB-PP1

Days of treatment with 3-MB-PP1

Percent of cells with <2 centrin foci

AS/Δ
WT/Δ

Cloning Efficiency

DMSO
3-MB-PP1

β galactosidase-positive cells

Days of 3-MB-PP1 treatment

Percent alive (negative for trypan blue)

AS/Δ
WT/Δ

Days of 3-MB-PP1 treatment

Percent alive (negative for both annexin V and PI)

AS/Δ
WT/Δ

% β galactosidase-positive cells

AS/Δ + 3-MB-PP1
AS/Δ + DMSO
AS/WT + 3MBPP1
WT/Δ + 3-MB-PP1
Figure 2

A

Untreated

Aphidicolin

B

log2(fold change)

-log10(p value)

Potential Substrates

C

Exact Location Unknown:

Centriolar Satellites

CEP131
PCM1

CAMSAP2
CEP170B

Centriolar Subdomains

Microtubules

GST
STI
CEP131 S78
POC5

E

Centrinone 32p
CBB

Centrinone 32p
CBB

-35 kDa
-25 kDa
Figure 3

A

Merge with DAPI  GFP-CEP131  PCM1  Centrin

WT

S78A

S78D

B

Plate 10  cells/well

Transfect with cDNA and siRNA

Fresh media

Analyze cells and use for experiments

24 hours - 48 hours

C

qRT-PCR

Addback:  WT  GFP  S78A  S78D  WT  GFP  S78A  S78D

Scrambled siRNA:  -  -  -  -  +  +  +  +

CEP131 siRNA:  +  +  +  +  -  -  -  -

Addback:  WT  GFP  S78A  S78D  WT  GFP  S78A  S78D

Scrambled siRNA:  -  -  -  -  +  +  +  +

CEP131 siRNA:  +  +  +  +  -  -  -  -

Addback:  WT  GFP  S78A  S78D  WT  GFP  S78A  S78D

Scrambled siRNA:  -  -  -  -  +  +  +  +

CEP131 siRNA:  +  +  +  +  -  -  -  -

Addback:  WT  GFP  S78A  S78D  WT  GFP  S78A  S78D

Scrambled siRNA:  -  -  -  -  +  +  +  +

CEP131 siRNA:  +  +  +  +  -  -  -  -

D

Merge with DAPI

E

Centrioles per cell

G

Percent of Cells with Cilia

Addback:  WT  GFP  S78A  S78D  WT  GFP  S78A  S78D

Scrambled siRNA:  -  -  -  -  +  +  +  +

CEP131 siRNA:  +  +  +  +  -  -  -  -

Addback:  WT  GFP  S78A  S78D  WT  GFP  S78A  S78D

Scrambled siRNA:  -  -  -  -  +  +  +  +

CEP131 siRNA:  +  +  +  +  -  -  -  -

Addback:  WT  GFP  S78A  S78D  WT  GFP  S78A  S78D

Scrambled siRNA:  -  -  -  -  +  +  +  +

CEP131 siRNA:  +  +  +  +  -  -  -  -

H

Merge with DAPI  γ Tubulin  PCM1

Normal

Dispersed

Aggregated

I

Centriolar Satellites

Centriolar Satellite Dispersion Score =

Higher Score = More Aggregated Satellites Around Centrosome

Lower Score = More Dispersed Satellites

K

Percent of PCM1 Intensity within 2.5 µm Radius of Centrosome

Centriolar Satellite Dispersion Score =

Higher Score = More Aggregated Satellites Around Centrosome

Lower Score = More Dispersed Satellites
Figure 4

A

Table of images showing the effect of various treatments on centriolar satellites.

- Untreated
- p38i
- Centrinone
- UV
- Nocodazole

B

Bar graph showing the percent of cells with dispersed or aggregated centriolar satellites across different treatments.

- HeLa
- HeLa p38i
- HeLa PLK4i
- HeLa + UV
- HeLa p38i + UV
- HeLa PLK4i + UV
- HeLa + Nocodazole

Legend:
- Dispersed
- Aggregated

ns

*
Figure 5

A

Addback

siCEP131

WT

GFP

S78A

S78D

merge with DAPI

GFP-CEP131

CEP152

Centrin

CEP152 Expression at the Centrosome

B

WT

KO4E

KO9

merge with DAPI

Centrin

CEP152

CEP152 Expression at the Centrosome
### A

| Cell Line | Treatment | WT | None | Nocodazole | WT | None | Nocodazole | WT | None | Nocodazole | WT | None | Nocodazole | WT | None | Nocodazole | WT | None | Nocodazole |
|-----------|-----------|----|------|------------|----|------|------------|----|------|------------|----|------|------------|----|------|------------|----|------|------------|----|------|------------|
| WT        | None      |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |
| WT        | Nocodazole|    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |
| CEP131 KO | None      |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |
| CEP131 KO | WT        |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |
| CEP131 KO | GFP       |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |
| CEP131 KO | S78A      |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |
| CEP131 KO | S78D      |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |    |      |            |

### B

![Histogram](image)

**Percent of Cells**

- WT
- WT + nocodazole
- KO9
- KO9 + WT
- KO9 + GFP
- KO9 + S78A
- KO9 + S78D

- Dispersed
- Aggregated

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* Indicates significant difference.
Figure 9

A. WT HeLa Cells

- GFP
- Actin

B. Merge with DAPI

- GFP
- PCM1

C. Percent of Cells

- WT
- GFP
- S78A
- S78D

* Dispersed  Aggregated

D. Centriolar Satellite Dispersion Score

- WT
- GFP
- S78A
- S78D

***  ***  *  ns
Figure 10

A

FLAG-CEP131: WT       WT     S78D    S78D
GFP-PCM1:    WT    S372D     WT    S372D
GFP Actin     CEP131    WT     WT     WT     WT   S78D  S78D  S78D  S78D
PCM1       WT     WT   S372D S372D WT    WT   S372D S372D
Centrinone     -   +     -    +    -   +     -    +

B

CEP131
WT    WT
WT    S372D
S78D  WT
S78D  S372D

PCM1
WT    WT
WT    S372D
S78D  WT
S78D  S372D

C

Dispersed
Aggregated

Percent of Cells

D

PLK4 phosphorylation of CEP131 and PCM1
Dephosphorylation of CEP131 and PCM1
Polo-like kinase 4 maintains centriolar satellite integrity by phosphorylation of centrosomal protein 131 (CEP131)

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