p38- and MK2-dependent signalling promotes stress-induced centriolar satellite remodelling via 14-3-3-dependent sequestration of CEP131/AZI1

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Centriolar satellites (CS) are small granular structures that cluster in the vicinity of centrosomes. CS are highly susceptible to stress stimuli, triggering abrupt displacement of key CS factors. Here we discover a linear p38-MK2-14-3-3 signalling pathway that specifically targets CEP131 to trigger CS remodelling after cell stress. We identify CEP131 as a substrate of the p38 effector kinase MK2 and pinpoint S47 and S78 as critical MK2 phosphorylation sites in CEP131. Ultraviolet-induced phosphorylation of these residues generates direct binding sites for 14-3-3 proteins, which sequester CEP131 in the cytoplasm to block formation of new CS, thereby leading to rapid depletion of these structures. Mutating S47 and S78 in CEP131 is sufficient to abolish stress-induced CS reorganization, demonstrating that CEP131 is the key regulatory target of MK2 and 14-3-3 in these structures. Our findings reveal the molecular mechanism underlying dynamic CS remodelling to modulate centrosome functions on cell stress.
Centriolar satellites (CS) are small granular structures with a diameter of 70–100 nm that cluster in the vicinity of the centrosome. CS are highly dynamic structures that travel along the microtubule network, and which can be readily observed by both electron and conventional light microscopy in the cytoplasm of mammalian interphase cells. By now, ~30 proteins have been classified as bona fide CS components, many of which also localize to and function at the centrosome. A growing list of CS factors is directly implicated in human pathologies, highlighting the clinical relevance of these structures. In one prevalent model, CS function to maintain centrosomal proteostasis through the replenishment of factors via active microtubule-mediated transport to the centrosome or by acting as proximal and temporary storage containers. Recent studies, however, have pointed to more elaborate functions of CS in promoting non-canonical roles of the centrosome such as primary cilia formation in quiescent cells and neurite outgrowth in neurons. Despite such recent progress, our understanding of the functions, molecular composition and regulation of CS remains rudimentary.

We recently discovered a novel p38 Mitogen-Activated Protein Kinase (MAPK)-dependent signalling pathway that mediates abrupt collapse of CS on stress stimuli such as ultraviolet irradiation. Accordingly, a range of CS components, including CEP131 (also known as AZI1), PCM1 and CEP290, undergo displacement from CS and disperse throughout the cytoplasm following exposure of cells to a number of different cellular stresses. Intriguingly, some CS components such as OFD1 do not change their localization under these conditions, suggesting that this novel stress response functions to rewire the composition and function of CS. Apart from the clear requirement of p38-dependent signalling, however, the molecular mechanisms underlying stress-induced CS reorganization are not known.

p38, similar to other MAPKs, acts to coordinate responses to environmental changes by regulating gene expression, cell growth, stress responses and apoptosis. The MAPK p38 (of which the α isoform is the prevalent one in proliferating cells) is a kinase downstream of p38, and previous work established an involvement of the p38-MK2 signalling axis in the regulation of processes such as cell cycle progression and RNA metabolism. We have recently shown that ultraviolet-induced CS reorganization is fully dependent on p38 activity, and we therefore reasoned that this process could also involve the MK2 kinase. To test this, we examined the localization of CS factors in response to ultraviolet irradiation in the presence or absence of small molecule inhibitors against p38 or MK2. In agreement with our previous findings, ultraviolet irradiation of human U2OS osteosarcoma cells led to a quantitative loss of CEP131, PCM1 and SSX2IP from CS in a p38-dependent manner (Fig. 1a–d and Supplementary Fig. 1a,b). The CS response was suppressed to a similar extent when MK2 function was abrogated by a specific chemical inhibitor, highlighting this kinase as an essential mediator of stress-induced CS remodelling downstream of p38. Importantly, this was not due to destabilization of any of a number of CS factors tested (Supplementary Fig. 1c). We made similar observations in immortalized human retinal pigment epithelial (RPE1) diploid cells (Supplementary Fig. 2), suggesting that stress-induced CS remodelling via p38 and MK2 is a general feature of human cells.

**Results**

**MK2 is required for CS remodelling.** MK2 is a major effector kinase downstream of p38, and previous work established MK2 phosphorylates CEP131 in response to ultraviolet. Prompted by these findings, we asked whether MK2 phosphorylates any of the known CS factors. To this end, we used Stable Isotope Labelling with Amino acids in Cell culture and mass spectrometry (SILAC/MS) to map p38- and/or MK2-dependent phosphorylation events in response to ultraviolet (Fig. 2a). Using this approach, where a SILAC ratio > 1 indicates an enrichment of the phosphorylated state, we identified three serine residues (S47, S78 and S731) in CEP131, whose phosphorylation increased on ultraviolet irradiation in a p38- and/or MK2-dependent manner (Fig. 2b). Notably, the amino-acid sequences surrounding each of the MK2-regulated serine residues corresponded to the known minimal consensus for MK2 phosphorylation (RXpS/T)11, suggesting that S47, S78 and S731 are direct targets of MK2-dependent phosphorylation (Fig. 2c). Consistently, endogenous CEP131 was immunoreactive with a generic phospho-specific antibody recognizing phosphorylated RXXpS/T peptides, and this phospho-signal increased after exposure to ultraviolet as well as the p38-activating compound anisomycin in a manner dependent on MK2 activity (Fig. 2d). In the case of both S47 and S78, but not S731, these motifs displayed a high degree of evolutionary conservation (Fig. 2c), indicating that S47 and S78 are functionally important substrates of MK2 phosphorylation. Importantly, we did not find phosphorylation sites in any other known CS components that robustly matched all of these criteria (Supplementary Data 1), suggesting that CEP131 might be a key target of p38/MK2-dependent phosphorylation in the stress-induced response leading to CS reorganization.

To test whether MK2 phosphorylates CEP131 directly, we generated and purified four overlapping CEP131 fragments (Fig. 2e), which were then subjected to MK2 phosphorylation in vitro. Strikingly, only the N-terminal fragment (CEP131-F1) containing S47 and S78, but not the region containing S731 (CEP131-F3), underwent phosphorylation by MK2 under these conditions (Fig. 2f). We then made alanine substitutions of S47 and/or S78 in F1 and examined the impact of these mutations on MK2 phosphorylation in the presence or absence of MK2 (Fig. 2g), demonstrating that both of these residues are targeted by MK2, in agreement with our MS data. Similarly, combined
mutation of S47 and S78 was sufficient to abolish phosphorylation of full-length CEP131 by MK2 (Fig. 2h), suggesting that S47 and S78 represent the major sites of MK2 phosphorylation in CEP131. These findings establish a direct role for MK2 in the stress-induced response leading to CS reorganization and suggest that CEP131 is a major CS-associated substrate of this kinase.

**MK2 phosphorylation of CEP131 promotes 14-3-3 binding.** In our previous work, we used SILAC/MS to pinpoint cellular-binding partners of CEP131 (ref. 7). Interestingly, on reanalysis of these data, we noticed that all members of the 14-3-3 family of proteins displayed enhanced interaction with CEP131 in response to ultraviolet irradiation, as indicated by a SILAC ratio > 1 when comparing heavy (H) versus light (L) peptides (Fig. 3a,b). 14-3-3 proteins form homo- or heterodimeric complexes that engage in phospho-dependent interactions with a range of client proteins, in many cases inhibiting specific protein–protein or protein–RNA interactions, modulating enzymatic activity or sequestering binding partners away from their normal sites of action13,16–18. Given the striking overlap between the MK2 phosphorylation site consensus (RXXpS/T) and reported 14-3-3-binding motifs (RSXpSXP and RXY/FXpSXP)19–21, we surmised that phosphorylation of CEP131 by MK2 in response to ultraviolet could promote binding to 14-3-3 proteins. To test this, we analysed binding of recombinant glutathione S-transferase (GST)-tagged 14-3-3 to CEP131 in cell extracts. Consistent with our MS data (Fig. 3b), we found that endogenous CEP131 bound to GST-14-3-3 in a manner that was strongly enhanced by ultraviolet (Fig. 3c). Importantly, the CEP131/14-3-3 interaction was abolished when MK2 was inhibited or depleted (Fig. 3c,d), suggesting that MK2 kinase activity plays a pivotal role in promoting interaction between CEP131 and 14-3-3 proteins. We verified the interaction between 14-3-3 and CEP131 in cells, where GFP-tagged 14-3-3 interacted with endogenous CEP131 in a ultraviolet-induced manner that was strictly dependent on both

![Figure 1](https://example.com/image1.png)

**Figure 1 | MK2 is required for stress-induced centriolar satellite reorganization.** (a) U2OS cells were incubated with inhibitors against p38 (p38i) or MK2 (MK2i) for 1 h and exposed to ultraviolet irradiation as indicated. Cells were fixed 1 h later and co-immunostained with CEP131 and γ-tubulin antibodies. Inserts show magnified regions around the centrosomes. (b) Quantification of CEP131 localization to CS in cells treated as in a. At least 100 cells were scored per condition. Results (mean ± s.d.) from three independent experiments are shown. P values were calculated from a one-tailed Student’s t-test. (c) As in a, except that cells were co-immunostained with PCM1 and γ-tubulin antibodies. (d) Quantification of cells with CS localization of PCM1 analysed as in b. Scale bars, 10 μm.
Figure 2 | MK2 phosphorylates CEP131 on S47 and S78. (a) Outline of SILAC-based phosphoproteomics experiment. U2OS cells were isotope-labelled in culture with light, medium and heavy amino acids and exposed to kinase inhibitors and ultraviolet irradiation for 1 h as indicated. Cell lysates were pooled, processed and analysed by MS. (b) Ultraviolet-regulated phosphorylation sites in CEP131 responsive to inhibition of p38 (p38i; left) or MK2 (MK2i; right), identified and quantified by SILAC and phosphoproteomic analysis. (c) Alignment of CEP131 amino-acid sequences surrounding three putative MK2 phosphorylation sites (S47, S78 and S731, highlighted in green) from different organisms. (d) U2OS cells were incubated for 1 h with MK2 inhibitor (MK2i) and exposed to ultraviolet irradiation or Anisomycin for 1 h as indicated. Input lysates (WCE) and immunoprecipitated (IP) CEP131 were analysed by immunoblotting with the indicated antibodies. (e) Schematic representation of human CEP131 with annotated domains, identified p38- and MK2-responsive phosphorylation sites and overlapping Strep-HA-CEP131 fragments used for in vitro kinase assays. (f) Overlapping Strep-HA-tagged CEP131 fragments (F1–F4) were expressed in U2OS cells, purified on streptavidin beads and subjected to phosphorylation by MK2 in vitro in the presence of radioactive ATP ($^{32}$P). Incorporation of radioactive phosphate was assessed using autoradiography. To further test the hypothesis that MK2-dependent phosphorylation of CEP131 promotes its association with 14-3-3, we sought to reconstitute MK2-mediated binding between these factors in vitro. To this end, we immunopurified wild-type (WT) or mutant forms of GFP-CEP131 expressed in cells, subjected
these proteins to phosphorylation by MK2 and assessed their interactions with recombinant GST-14-3-3. Strikingly, under these conditions, interaction between 14-3-3 and CEP131 only occurred when CEP131 had been phosphorylated by MK2 (Fig. 3f), further confirming that MK2-dependent phosphorylation of CEP131 is an important trigger of its binding to 14-3-3. Using this experimental set-up, we analysed whether the integrity of the MK2 phosphorylation sites we mapped in CEP131 (S47 and S78) was required for binding to 14-3-3. Indeed, single S47A or S78A mutations led to a robustly decreased interaction between CEP131 and 14-3-3, while the association was completely abolished on simultaneous mutation of both MK2 phosphorylation sites (Fig. 3f). In contrast, mutation of S731 had no effect on the interaction between CEP131 and 14-3-3 (Fig. 3f).
We conclude from these data that MK2-dependent phosphorylation of CEP131 on S47 and S78 generates binding sites for 14-3-3 proteins in response to ultraviolet.

14-3-3 promotes cytoplasmic localization of CS factors. The findings above suggested that MK2-dependent binding of 14-3-3 proteins to CEP131 might play an active role in promoting ultraviolet-induced CS restructuring. To test this, we took advantage of the peptide-based 14-3-3 inhibitor Difopein (Dimeric Fourteen-Three-Three-Peptide Inhibitor), which binds with high affinity to all 14-3-3 isoforms in a phospho-independent manner, suppressing their functionality (Fig. 4a)22. Expression of FLAG-Difopein quantitatively suppressed the ability of GFP-14-3-3 to interact with endogenous CEP131 in ultraviolet-irradiated U2OS cells (Fig. 4b), confirming that this construct efficiently blocks 14-3-3/protein interactions in cells. To determine whether 14-3-3 proteins are required for stress-induced CS remodelling, we assessed the subcellular localization of CEP131 and PCM1 in cells overexpressing FLAG-Difopein or a modified version (Difopein (Lys)) incapable of binding to 14-3-3 because of mutation of critical acidic residues in the two interaction domains (Fig. 4a)22,23. We found that moderate expression of Difopein, but not the Lys mutant, completely blocked the ultraviolet-induced displacement of CEP131 and PCM1 from CS (Fig. 4c,d and Supplementary Fig. 3a–c), while the overall appearance of CS in unstressed cells was not overtly affected. We further confirmed these observations in stable cell lines expressing FLAG-Difopein at low and uniform levels in an inducible manner (Fig. 4e and Supplementary Fig. 4a). Under these conditions, FLAG-Difopein bound strongly to endogenous 14-3-3 (Fig. 4f) and completely blocked ultraviolet-induced dispersal of the CS components CEP131, PCM1 and SXR2IP (Fig. 4g,h and Supplementary Fig. 4b). Importantly, induction of FLAG-Difopein did not interfere with ultraviolet-induced activation of p38 or MK2 (Supplementary Fig. 4c), thus indicating that 14-3-3 proteins function downstream of these kinases in promoting CS reorganization. We conclude that a linear p38-MK2-14-3-3 signalling cascade is responsible for disassembling CS in stressed cells by sequestering CEP131 and associated components in the cytoplasm.

14-3-3–CEP131 binding underlies stress-induced CS remodelling. While both CEP131 and PCM1 interact with 14-3-3 in an MK2-dependent manner (Fig. 3d,e), the apparent absence of MK2 phosphorylation sites in CS components other than CEP131 (Supplementary Data 1) suggested that this protein could provide a direct handle for 14-3-3-mediated reconfiguration of these structures after cell stress. To address this, we asked whether CEP131 was required for binding of PCM1 to 14-3-3 and vice versa. We found that, while CEP131/14-3-3 binding occurred independently of PCM1, the interaction between PCM1 and 14-3-3 was strongly impaired in CEP131-depleted cells (Fig. 5a). This supports the notion that CEP131 is a major CS-associated factor capable of interacting with 14-3-3, and that interactions between 14-3-3 to other CS components such as PCM1 are likely indirect and bridged by CEP131. To further test this model, we generated cell lines expressing low levels of WT or MK2 phosphorylation-deficient (S47A/S78A) forms of GFP-CEP131. Consistent with our previous observations, only GFP-CEP131 WT, but not the S47A/S78A mutant, reacted with an antibody specific to phosphorylated RXSps/T peptides and co-precipitated with GST-14-3-3 in a ultraviolet-inducible manner in these cell lines (Fig. 5b,c).

To test whether mutation of the MK2 phosphorylation sites in GFP-CEP131 interferes with stress-induced CS remodelling, we next examined the subcellular localization of CS components in these cell lines (Fig. 5d,e). Whereas cells ectopically expressing GFP-CEP131 WT showed a normal stress-induced CS response characterized by ultraviolet-induced displacement of GFP-CEP131 and endogenous PCM1, expression of the MK2 phosphorylation-deficient S47A/S78A allele completely abrogated this response (Fig. 5d,e). The ultraviolet-induced displacement of other CS factors such as SXX2IP and CEP290 was also fully suppressed in cells expressing S47A/S78A, but not WT, GFP-CEP131 (Fig. 5e and Supplementary Fig. 5a,b). Importantly, these effects could not be attributed to a defect in p38 pathway activation as measured by ultraviolet-induced phosphorylation of p38, MK2 and HSP27 (Fig. 5b and Supplementary Fig. 5c). The persistent CS localization of all of these factors in ultraviolet-irradiated cells expressing CEP131 S47A/S78A suggests that CEP131 phosphorylation by MK2 and ensuing 14-3-3 binding is essential for the displacement of CS-associated factors on cell stress. We also established a cell line expressing an allele of GFP-CEP131 with acidic residues at positions 47 and 78 (S47E/S78E); however, this mutant behaved essentially like the alanine mutant, suggesting that phosphate groups are required to mediate the interaction with 14-3-3 (Supplementary Fig. 6).

CEP131 (S47A/S78A) supports CS dynamics in unstressed cells. Ectopic expression of CS components such as CEP131 is challenging because of their inherent propensity to assemble into large and insoluble protein aggregates (our unpublished observations). To address these concerns, and to further characterize the impact of p38-MK2 signalling on CS biology, we carefully compared the behaviour of WT and mutant GFP-CEP131 with that of the endogenous protein. Importantly, in our cell lines both WT and mutant GFP-CEP131 required PCM1 for targeting to CS (but still displayed PCM1-independent affinity for the centrosome24,25; Supplementary Fig. 7a), and this localization was lost on mitosis26,27 (Supplementary Fig. 7b). In addition, time-lapse studies showed that, consistent with the reported dynamic behaviour of CS in live cells28, all of the previously reported behaviours of CS, including fusion and dissociation events and step-wise transport towards the centrosome along microtubules could be observed in cells expressing WT and mutant GFP-CEP131 (Supplementary Movies 1 and 2). This demonstrates that the GFP-CEP131-positive granules in our cell lines recapitulate all the hallmarks of physiological CS.

Prompted by these observations, we utilized the cell lines stably expressing GFP-CEP131 constructs to replace endogenous CEP131 with near-physiological levels of the GFP-CEP131 transgenes, by combining short interfering RNA (siRNA)-mediated knockdown and carefully optimized dosing of low concentrations of doxycycline (Supplementary Fig. 8a). Consistent with our previous observations, only GFP-CEP131 WT, but not the S47A/S78A mutant, efficiently co-purified with GST-14-3-3 in a ultraviolet-dependent manner (Supplementary Fig. 8b). Furthermore, we established cell lines recapitulating all the hallmarks of physiological CS.

14-3-3–CEP131 interaction blocks formation of CS. CS are dynamic structures, which continuously form and dissociate as they travel towards the centrosome along the microtubule
Thus, CEP131 and other CS components continuously shuttle between mobile cytoplasmic and immobile CS-bound pools. To better understand why 14-3-3 binding to CEP131 results in CS depletion, we considered two possible scenarios underlying this phenomenon: (1) MK2 phosphorylation and 14-3-3 binding target the CS-resident CEP131 pool and extract the protein from this locale or (2) the same events target CEP131 in the cytoplasm to block de novo formation of CS after stress. To test the first hypothesis, we carefully assayed for localization of MK2 or 14-3-3 to CS during the ultraviolet-induced stress response. However, we failed to observe detectable accumulation of these proteins at CS (Supplementary Fig. 9a,b). This was also the case for MK2 when activated by p38 after ultraviolet but chemically inhibited, so that it was unable to promote CEP131 phosphorylation and CS remodelling (Supplementary Fig. 9b). In addition, we found that MK2-dependent phosphorylation of CEP131 and ensuing 14-3-3 binding were independent of PCM1 and thus did not require PCM1γ.

**Figure 4 | 14-3-3 proteins are required for stress-induced displacement of centriolar satellite factors.** (a) Schematic representation of unmodified and 14-3-3-binding-deficient (Lys) FLAG-Difopein constructs. (b) U2OS cells co-transfected with GFP-14-3-3 and FLAG-Difopein and exposed to ultraviolet irradiation, as indicated, were lysed and subjected to GFP pull-downs. Binding between GFP-14-3-3 and endogenous CEP131 was analysed by immunoblotting with the indicated antibodies. (c) Quantification of cells with CS localization of CEP131 after ultraviolet irradiation in cells transfected with FLAG-Difopein or FLAG-Difopein (Lys) constructs. At least 100 cells with low to moderate FLAG staining were scored per condition. Results (mean ± s.d.) from three independent experiments are shown. *P* values were calculated from a one-tailed Student's *t*-test. Representative images are shown in Supplementary Fig. 3a. (d) As in (c), except that CS were counterstained for PCM1. Representative images are shown in Supplementary Fig. 3b. (e) U2OS/FLAG-Difopein cells incubated or not with Doxycycline (Dox) for 24 h were lysed and subjected to FLAG immunoprecipitation followed by immunoblotting with the indicated antibodies. (f) As in (e), except that an antibody broadly recognizing 14-3-3 isoforms (14-3-3 pan) was used for immunoblotting. (g) U2OS/FLAG-Difopein cells grown in the presence or absence of Doxycycline and exposed to ultraviolet irradiation as indicated, were fixed and co-immunostained with antibodies to CEP131 and γ-tubulin. Inserts show magnified regions around the centrosomes. (h) As in (g), except that cells were co-immunostained with PCM1 and γ-tubulin antibodies. Scale bars, 10 μm. Unprocessed original scans of western blots are shown in Supplementary Fig. 11.

network\(^{28}\) (Supplementary Movies 1 and 2). Thus, CEP131 and other CS components continuously shuttle between mobile cytoplasmic and immobile CS-bound pools. To better understand why 14-3-3 binding to CEP131 results in CS depletion, we considered two possible scenarios underlying this phenomenon: (1) MK2 phosphorylation and 14-3-3 binding target the CS-resident CEP131 pool and extract the protein from this locale or (2) the same events target CEP131 in the cytoplasm to block de novo formation of CS after stress. To test the first hypothesis, we carefully assayed for localization of MK2 or 14-3-3 to CS during the ultraviolet-induced stress response. However, we failed to observe detectable accumulation of these proteins at CS (Supplementary Fig. 9a,b). This was also the case for MK2 when activated by p38 after ultraviolet but chemically inhibited, so that it was unable to promote CEP131 phosphorylation and CS remodelling (Supplementary Fig. 9b). In addition, we found that MK2-dependent phosphorylation of CEP131 and ensuing 14-3-3 binding were independent of PCM1 and thus did not require
localization of CEP131 to CS (Figs 5a and 6a). These observations argued against a model, in which CEP131 and other factors are directly extracted from CS on cell stress and prompted us to examine the kinetics of CS remodelling. Remarkably, we found that ultraviolet-induced CS remodelling, as measured by the presence of CEP131-positive granules in cells, progressively evolved to reach a peak around 1 h after ultraviolet treatment, suggesting that this response is caused by a gradual loss of intact CS rather than by abrupt stress-induced collapse of these structures (Fig. 6b and Supplementary Fig. 10). Importantly, the kinetics of CEP131 loss from CS was strikingly different from that of p38 and MK2 activation and 14-3-3/CEP131 complex formation, which was maximal already 15 min after ultraviolet (Fig. 6c). On the basis of these observations, we propose that phosphorylation and cytoplasmic sequestration of CEP131 occur rapidly after ultraviolet to block the formation of new CS, thereby leading to a net progressive depletion of these structures after stress.

Discussion
In this study, we have elucidated the molecular mechanism governing p38-dependent CS reorganization during cellular stress responses (Fig. 6d). We have shown that this process is fully dependent on the activity of MK2, a key effector kinase...
downstream of p38, and that MK2-mediated phosphorylation of the CS factor CEP131 generates stress-induced binding sites for 14-3-3 proteins, leading to sequestration of CEP131 in the cytoplasm. Several 14-3-3 ligands have been shown to contain dual 14-3-3 interaction motifs, required to initiate and stabilize the interaction, respectively. This is also likely to be the case for CEP131, which is phosphorylated by MK2 on two residues (S47 and S78), both of which are required for efficient association with 14-3-3. While 14-3-3 directly interacts with CEP131, the interaction with PCM1 appears to be bridged by CEP131 (Fig. 5a). In addition, the dispersal of both CEP131 as well as other CS components including PCM1 can be completely abrogated by mutating S47 and S78 in CEP131 (Fig. 5d,e and Supplementary Fig. 5a,b). Our data therefore suggest that phosphorylated CEP131 is likely to be the main, if not the only, CS-associated factor capable of interacting with 14-3-3 proteins, and that this interaction underlies the overall reorganization of CS following cell stress, characterized by the concomitant loss of other CS factors including PCM1, CEP290 and SSX2IP from these structures. Our experiments further suggest that binding of 14-3-3 to CEP131 does not disrupt existing CS, but rather prevents the de novo formation of new CS, thereby causing a net progressive loss of these structures after stress (Fig. 6a–c and Supplementary Figs 9 and 10). These conclusions are in line with other studies, where 14-3-3 binding was found to prevent the integration of client proteins into larger protein complexes.

Figure 6 | MK2- and 14-3-3-dependent CEP131 sequestration blocks CS formation after cell stress. (a) U2OS cells were transfected with indicated siRNAs and exposed to MK2 inhibitor for 1 h and ultraviolet irradiation (1 h) as indicated. Input lysates and CEP131 IP were analysed by immunoblotting with the indicated antibodies. (b) U2OS cells were exposed to ultraviolet irradiation, fixed at the indicated time points and co-immunostained with CEP131 and γ-tubulin antibodies. At least 100 cells were scored per condition. Results (mean ± s.d.) from three independent experiments are shown. P values were calculated from a one-tailed Student’s t-test. Representative images are shown in Supplementary Fig. 10. (c) Lysates from cells in b were incubated with GST-14-3-3. Input lysates and GST-co-purified material were analysed by immunoblotting with the indicated antibodies. (d) Model of stress-induced CS remodelling. In unstressed cells (upper panel), CS are maintained through an equilibrium between formation and dissolution events. On ultraviolet irradiation and other stress stimuli (lower panel), p38 kinase is activated, resulting in phosphorylation and activation of its downstream kinase MK2. MK2 directly phosphorylates two residues on CEP131, S47 and S78, to create dual binding sites for 14-3-3 proteins. The resulting interaction mediates cytoplasmic sequestration of CEP131 that blocks de novo CS formation, leading to a net progressive loss of CS in cells. Unprocessed original scans of western blots are shown in Supplementary Fig. 11.
p38-mediated stress responses. Intriguingly, similar dynamic remodelling of CS was shown to underlie a variety of centrosome-associated biological responses. For example, primary cilium formation is associated with the exclusion of BBS4 from CS and degradation of CS-associated OFD1 (ref. 43). In addition, proliferation of CS, as visualized by PCM1- and centrin-positive granules, was shown to precede and be functionally linked to amplification of centrosomes at prolonged times after exposure of cells to DNA-damaging agents29,39,40. Together, these studies highlight CS as inherently dynamic structures, whose context-specific remodelling can have diverse impacts on a variety of cellular processes. Thus, CS are emerging as central regulatory hubs for a host of cellular responses that can alter the composition and/or stability of these structures, with poorly understood functional consequences.

In conclusion, our study adds stress-induced reorganization of CS to the growing cellular repertoire of p38-MK2-14-3-3 effector pathways. Despite these insights, we are still far from understanding the exact biological ramifications of this novel aspect of cellular stress responses, hampered by a lack of published insights into the cellular functions of CS and their underlying mechanisms. Studies over the last decade have suggested roles for CS in ciliogenesis4,33,41, mitotic spindle pole maintenance32, centrosome maintenance/duplication25,31 and even neurogenesis6,24. In our previous work, we reported that ultraviolet irradiation induces cilium formation in RPE1 cells in a partially p38-dependent manner7. It is thus formally possible that the p38-MK2-mediated pathway described in the present study is involved in this response. Future work will be required to further elucidate the supporting roles of CS for centrosome-associated functions and shed light on how such processes are modulated in response to cellular stress to protect cellular homeostasis.

**Methods**

**Plasmids and siRNA.** Full-length CEP131 cDNA was amplified using PCR and inserted into pEGFP-C1 (Clontech) to generate a mammalian expression plasmid for GFP-tagged CEP131. Plasmids pGEX2TK-P-GST-14-3-3 and pGEX2TK-P-GST-14-3-3Δ were a gift from Alistair Cook, Gurdon Institute, University of Cambridge, UK. Plasmids pEGFP-14-3-3Δ and pEGFP-14-3-3Δ, were a gift from Max Douglas, London Research Institute, UK. The S47A, S47E, S78A, S78E and S78K mutations in CEP131 were introduced by site-directed mutagenesis using KOD DNA polymerase (Millipore) according to the manufacturer’s instructions. For generation of inducible GFP-CEP131 expression plasmids, GFP-CEP131 (WT, S47A/S78A, S47E/S78E) was cloned into pcDNA5/FRT/TO (Life Technologies) using the BamHI and EcoRV restriction sites. For generation of FLAG-Difopein expression plasmids, Difopein (Lys) expression plasmids were subcloned into pcDNA4/TO (Life Technologies) using KpnI and BamHI restriction sites. All constructs were verified by sequencing. Plasmid transfections were performed using FuGene 6 (Promega) or Lipofectamine (Life Technologies), and siRNA transfections were carried out with Lipofectamine RNAiMAX (Life Technologies) following the manufacturer’s protocol. siRNA target sequences (Eurofins) used in this study were as follows: Control (siCTRL; 5'-CCGGAUUGACUUGAGCUCU-3'); MK2 (5'-CCGAACAUAGGACGCAUCU-3'); CEP131 (5'-UCACUAGUACAGCAGGUTT-3'); CEP131 3'UTR (5'-GUAGAAGGCAGCCAGCAGUTT-3'); and PCMI (5'-GGUUUUAACUAAUUAUGGATT-3').

**Cell culture and reagents.** Human U2OS osteosarcoma cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin. For SILAC labelling, cells were cultured in media containing either L-arginine and L-lysine (Light), L-arginine [15C6] and L-lysine [15N4] (Medium) or L-arginine [15C6-15N4] and L-lysine [15C6-15N2] (Heavy; Cambridge Isotope Laboratories). All cells were cultured at 37°C in a humidified incubator containing 5% CO₂. To generate cell lines stably expressing FLAG-Difopein, cells were co-transfected with pcDNA4/TO-FLAG-Difopein and pcDNA6/TR (Life Technologies) in a 1:1 ratio and selected with Zeocin and Blasticidin (5 μg ml⁻¹).
for 14 days. Individual clones were picked and analysed for FLAG-Difopein expression using immunofluorescence (IF) and western blot (WB) analyses. To generate cell lines expressing GFP-CEP131, U2OS Flp-In T-rex cells were co-transfected with pOG44 recombinase construct (Life Technologies) and pcDNAS/FRT/TO-GFP-CEP131 (WT, S47A/S78A, S47E/S78E) for 14 days. Individual clones were picked and analysed for green fluorescence using fluorescence microscopy and WB analyses. RPE1 (hTERT-immortalized human retinal pigment epithelial cells) was obtained from ATCC and maintained in DMEM/F12 medium containing 10% fetal bovine serum. For GFP expression using fluorescence microscopy and WB analyses. RPE1 (hTERT-immortalized human retinal pigment epithelial cells) was obtained from ATCC and maintained in DMEM/F12 medium containing 10% fetal bovine serum. Ultraviolet irradiation (50 mJ cm⁻²) was delivered in a BS-02 irradiation chamber equipped for 254 nm UV radiation (Grobelt Electron Tube Industry). Irradiation was performed on p83 inhibitor SR203580 (10 μM, Cell Signaling), MK2 inhibitor PF6464022 (10 μM, Sigma) and MK2 inhibitor III (10 μM, Calbiochem). Anti-cytokeratin (1 μg ml⁻¹), Sigma-Aldrich). GST-tagged 14-3-3-3 and 14-3-3-5 were purified from E. coli. GST-tagged 14-3-3-3 and 14-3-3-5 were mixed in a 1:1 ratio for use in GST-pull-down experiments. For SILAC experiments, we labelled U2OS cells with stable isotypes of lysine and arginine to obtain cultures with light (L), medium (M) and heavy (H) proteins, the differential mass of which can be conveniently discriminated by MS. These cell cultures were then exposed to ultraviolet and/or kinase inhibitors and the lysates were mixed and processed for phosphoproteomics.

In vitro phosphorylation assays. GFP- or Strep-HA-tagged CEP131 was immunopurified from transfected U2OS cells lysed in high-salt EBC buffer (50 mM Tris, pH 7.5; 300 mM NaCl; 1 mM EDTA; 0.5% NP40; 1 mM dithiothreitol (DTT)). Beads were washed three times with lysis buffer once with kinase buffer (25 mM Hepes, pH 7.2; 25 mM MgCl2; 2 mM DTT). Reactions were initiated by adding 200–450 ng recombinant MK2 (Abcam) and 25 μM ATP to each sample. To assay MK2-mediated phosphorylation, ATP was spiked with [γ-32P]-ATP (10 μCi, Perkin-Elmer). Indicated samples were supplemented with 100 ng recombinant HS2TP (Promega) to serve as positive control for MK2 activity. Reactions were incubated for 30 min at 30 °C with gentle shaking and terminated by adding Laemmli buffer and boiling the samples at 95 °C for 10 min. Samples were then run on SDS–PAGE and vacuum-dried on a Whatman filter paper (Sigma). Relative phosphorylation was assayed by radioblotting. To assay MK2-dependent 14-3-3-3 binding in vitro, GFP CEP131 immobilized on beads was treated with 1 μl Lambda Phosphatase (New England Biolabs) and incubated for 30 min at 30 °C before in vitro phosphorylation. The subsequent MK2 kinase assay was performed as above but without radioactive ATP. Following the kinase reaction, beads were re-suspended in low-salt EBC buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.5% NP40; 1 mM DTT) and washed three times with GST pull-down buffer. Subsequently, purified GST-14-3-3-3 was added to each sample and incubated for 1 h at 4 °C while rotating, and then washed five times in low-salt EBC buffer and analysed by WB analysis.

Immunofluorescence methods. GFP immunoprecipitations were performed with GFP-Trap agarose beads (Chromotek) and CEP131 immunoprecipitations were performed with CEP131 antibody (ab84864, Abcam) coupled to Protein A Sepharose beads (GE Healthcare). FLAG pull-down was performed using FLAG-M2 agarose (Sigma-Aldrich) and GST pull-down was performed with Glutathione Sepharose beads (GE Healthcare). All immunoprecipitations were carried out in low-salt EBC lyss buffer (150 mM NaCl; 50 mM Tris, pH 7.5; 1 mM EDTA; 0.5% NP40). Antibodies used in this study included the following: Rabbit polyclonal to Phospho-GSK3β (Ser21) (9326S, Cell Signaling) and mouse monoclonal to GST (14-3-3) 40/1.35 WD 0.10 dry objective. Seven Z-Stacks with intervals of 1.5 μm were taken using differential interference contrast and a GFP filter set (32% intensity, 0.1-s exposure time). All data analyses were performed using the SoftWoRx software (GE Healthcare).

MS analysis. Phosphorylated peptides were enriched by conventional methods. Peptide fractions were analysed on a quadrupole Orbitrap mass spectrometer (Q Exactive or Q Exactive Plus, Thermo Scientific) equipped with a UHPLC system (EASY-nLC 1000, Thermo Scientific) for 2 days before imaging. Culture medium was changed to L-15 (Life Technologies) supplemented with 10% fetal bovine serum (HyClone) and Penicillin/Streptomycin (Life Technologies) immediately before imaging. Slides were mounted on a DeltaVision Elite microscope (GE Healthcare) and cells were imaged for 16h in 10-min intervals using a ×40/0.15 WD 0.60 dry objective. Seven Z-Stacks with intervals of 1.5 μm were taken using differential interference contrast and a GFP filter set (32% intensity, 0.1-s exposure time). All data analyses were performed using the SoftWoRx software (GE Healthcare).

Peptide identification. Raw data files were analysed using MaxQuant46. Parent ion and MS2 spectra were searched against a database containing 88,473 human protein sequences obtained from the UniProtKB released in December 2013 using the Andromeda search engine47. Spectra were searched with a mass tolerance of 6 p.p.m. in the MS mode, 20 p.p.m. in the higher-energy C-trap dissociation MS2 mode, strict tryptic specificity and allowing up to three miscleavages. Cysteine carbamidemethylation was searched as a fixed modification, whereas protein N-terminal acetylation, methionine oxidation and phosphorylation of serine, threonine and tyrosine were searched as variable modifications. Site localization probabilities were determined by MaxQuant using the PTM scoring algorithm46. The data set was filtered based on posterior error probability to arrive at a false discovery rate of below 1% estimated using a target-decoy approach48.

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M.A.X.T., B.H.Y., M.B. and J.C.N. performed the biochemical and cell biological experiments. S.A.W. and P.B. performed and analysed the proteomics experiments. J.B. supervised M.B. and analysed data. N.M. and S.B.-J. conceived the project. M.A.X.T., B.H.Y., N.M. and S.B.-J. designed the experiments, and M.A.X.T., N.M. and S.B.-J. wrote the manuscript. All authors discussed and interpreted the data together.

**Additional information**

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