Ccdd13; a novel human centriolar satellite protein required for ciliogenesis and genome stability.

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Summary
Here we identify coiled-coil domain-containing protein 13 (Ccdc13) in a genome-wide RNA interference screen for regulators of genome stability. We establish that Ccdc13 is a novel centriolar satellite protein that interacts with PCM1, Cep290 and pericentrin, which prevents the accumulation of DNA damage during mitotic transit. Depletion of Ccdc13 results in loss of microtubule organisation in a manner similar to PCM1 and Cep290 depletion, although Ccdc13 is not required for satellite integrity. We show that microtubule regrowth is enhanced in Ccdc13-depleted cells, but slowed in cells overexpressing Ccdc13. Furthermore, in serum-starved cells, Ccdc13 localises to the basal body, is required for primary cilia formation, and promotes the localisation of the ciliopathy protein BBS4 to both centriolar satellites and cilia. These data highlight the emerging link between DDR factors, centriolar/peri-centriolar satellites and cilia-associated proteins and implicate Ccdc13 as a centriolar satellite protein that functions to promote both genome stability and cilia formation.

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Introduction

The mammalian centrosome is a major site of microtubule nucleation and organisation during both interphase and mitosis, and is also the origin of primary cilia formation. It is also important for establishing a mitotic spindle to ensure efficient chromosome segregation and genome stability. The mammalian centrosome consists of two orthogonally orientated microtubule barrels called centrioles, which are surrounded by a mesh of electron-dense pericentriolar material (PCM). This is in part comprised of particles termed centriolar satellites. The formation of an organised microtubule array is dependent on satellite proteins such as pericentriolar material 1 (PCM1), which is thought to act as a scaffold to recruit other proteins including pericentrin, ninein, Cep90 and Cep290 to the PCM (Dammermann and Merdes, 2002; Kim and Rhee, 2011; Lopes et al., 2011; Mogensen et al., 2000). This involves complex and incompletely characterised interactions between numerous satellite proteins and the dynein-dynactin microtubule minus end motor system (Dammermann and Merdes, 2002; Kim et al., 2008; Kim et al., 2004; Kodani et al., 2010; Lee and Rhee, 2010).

A number of centriolar satellite proteins such as PCM1 and the ciliopathy proteins Cep290 and Bardet-Biedl Syndrome protein 4 (BBS4) co-operate in the formation of the primary cilia (Kim et al., 2008; Lopes et al., 2011; Stowe et al., 2012), an organelle only recently appreciated as a crucial signalling hub for a number of pathways including Wnt and Sonic hedgehog (Logan et al., 2011). Recent work has demonstrated that a number of proteins involved in the DNA damage response (DDR) also function during ciliogenesis. Mutations in Mre11, ZNF423 and Cep164 are causative for a sub-set of renal ciliopathies (Chaki et al., 2012), while FAN1 mutations cause karyomegalic interstitial nephritis (Zhou et al., 2012). Interestingly, the DDR kinase ATR has been recently shown to localize to the photoreceptor connecting cilium, and ATR-deficient mice exhibit ciliary defects and photoreceptor degeneration (Valdes-Sanchez et al., 2013). Furthermore, it has been recently demonstrated that the renal ciliopathy-associated kinase Nek8 also plays a role in ATR signalling during the cellular responses to replication stress (Choi et al., 2013). Finally, mutations in several DNA replication factors impair ciliogenesis and are causal for Meier-Gorlin syndrome (Stiff et al., 2013).
In a recent human genome-wide siRNA-based screen for regulators of genome stability, we identified Cep131, a protein required for both cilia formation and genome integrity (Staples et al., 2012). Through our on-going analyses of candidates identified in this screen, the uncharacterised protein coiled-coil domain-containing protein 13 (Cccd13) was identified as a potential regulator of genome stability. We report here that Cccd13 is a novel centriolar satellite protein required for cilia formation and genome stability. Depletion of Cccd13 results in increased levels of post-mitotic DNA damage. At the molecular level, Cccd13 binds to a number of satellite proteins including PCM1, Cep290, pericentrin and Cep131, and its depletion causes defects in ciliogenesis and reduced BBS4 recruitment to centriolar satellites. Modulation of Cccd13 levels also alters microtubule organisation in interphase cells, and microtubule regrowth following depolymerisation. Consistent with recent findings that mutations in several DDR proteins give rise to human ciliopathies, our data reveal that an emerging subset of centriolar satellite proteins function both to promote ciliogenesis and maintain genome stability.

Results

Identification and validation of Cccd13 as a novel regulator of genome stability.

We recently carried out a genome-wide siRNA screen to identify novel regulators of genome stability (Staples et al., 2012), using phosphorylation of the histone variant H2AX on Ser139 (termed γH2AX) as a marker of increased DNA damage (Bonner et al., 2008). As part of this screen we identified a coiled-coil domain-containing protein 13 (Cccd13), which yielded a high z-score of 2.2. Cccd13 is an SMC domain-containing protein, which is encoded on the short arm of chromosome 3 in humans (3p22.1), and is conserved in primate, dog, cow, rat, chicken and zebrafish.

To confirm the screen data (which was carried out in the human colorectal cell line HCT116 using pools of four siRNA), we transfected cervical carcinoma-derived HeLa cells with four individual siRNAs targeting Cccd13. All four siRNA resulted in increased numbers of cells exhibiting γH2AX foci (Figure 1A), thus
validating the results from the initial screen and strongly diminishing the likelihood of any off-target effects of the siRNA. In order to confirm efficient knockdown of Ccdc13, we tested 2 separate commercially available Ccdc13 antibodies in combination with siRNA treatments. However, neither antibody was able to recognise endogenous Ccdc13, and only weakly recognised overexpressed Ccdc13 (data not shown). Therefore, we confirmed that all four Ccdc13-directed siRNA conferred an efficient knockdown of exogenous forms of Ccdc13 (supplementary Figure 1A). Additionally, we confirmed that two Ccdc13 siRNA that we use for all subsequent studies result in significant reduction of endogenous Ccdc13 mRNA levels as assessed by quantitative RT-PCR (supplementary Figure 1B).

Increased endogenous DNA damage can arise as a consequence of defective damage response (DDR) signalling (Chapman et al., 2012). Therefore we assessed the phosphorylation of the DDR effector kinases Chk1 and Chk2 following exposure to hydroxyurea and ionising radiation, and also assessed the effect of Ccdc13 depletion on cell survival following these treatments. Neither effector kinase phosphorylation, nor cell survival was altered by depletion of Ccdc13 (supplementary Figure 1C & data not shown), suggesting that Ccdc13 does not play a significant role in the activation and/or maintenance of the DDR. To ascertain the origin of the increased DNA damage observed in Ccdc13-depleted cells, we performed cell cycle block-release experiments using the iron chelator mimosine, which induces cell cycle arrest in G1 phase without leading to centrosome over-duplication (Prosser et al., 2009). Treatment with mimosine suppressed the increased γH2AX observed following Ccdc13 depletion (Figure 1B). Furthermore, in a similar manner to that previously reported for Cep131 (Staples et al., 2012), increased γH2AX was only observed in Ccdc13-depleted cells in G1 following mitotic transit (Figure 1B). However, in contrast to our findings with Cep131, depletion of Ccdc13 did not cause centrosome amplification or alterations in the size of the pericentriolar material (PCM; data not shown).

To investigate how Ccdc13-depleted cells respond to the increased endogenous DNA damage, we assessed nuclear focus formation for phosphorylated ATM, 53BP1, Rad51, phosphorylated RPA, and phosphorylated DNA-PK (Bekker-
Jensen et al., 2006). Consistent with increased amounts of DNA damage in Ccdc13-depleted cells (Figure 1A), depletion of Ccdc13 led to a significant increase in the number of cells exhibiting activated ATM (phospho-Ser1981), activated DNA-PK (phospho-Thr2609) and 53BP1 nuclear foci compared to control siRNA treated cells (Figure 1C). However, Rad51 and activated RPA (phospho-Thr21) foci were unaltered in Ccdc13-depleted cells compared to control siRNA (Figure 1C). These data suggest that cells deficient in Ccdc13 generate DNA breaks during mitosis, and given the non-toxic effects of Ccdc13 depletion, we predict that the majority of these lesions are repaired by NHEJ in the subsequent G1 phase of the cell cycle (Chapman et al., 2012; Giunta et al., 2010). Indeed, Ccdc13-depleted cells exhibit increased amounts of micronuclei (supplementary Figure 1D), which are a marker of post-mitotic DNA damage (Janssen et al., 2011). Post-mitotic repair of such lesions would also be consistent with an intact DDR observed in Ccdc13-depleted cells (supplementary Figure 1C). It is worth noting that these phenotypes are a result of significantly reduced levels of Ccdc13, and may be more severe following complete abolishment (knock-out) of the endogenous protein.

**Ccdc13 is a novel human centriolar satellite protein.**

To gain insight into the molecular function of Ccdc13, we generated both HeLa and HEK293 cell lines stably expressing tetracycline-inducible N-terminally FLAG- or YFP-tagged Ccdc13 (supplementary Figure 2A). In interphase cells expressing low levels of YFP-Ccdc13, it localised to the centriolar core region as evidenced by strong co-localisation with gamma tubulin (Figure 2A). However, in cells expressing high levels of YFP-Ccdc13, it also localised diffusely around the centrosome in an array reminiscent of centriolar satellites. Indeed, the YFP-Ccdc13 structures co-localised strongly with the archetypal centriolar satellite PCM1 (Figure 2A). Furthermore, immunofluorescent analysis of cells ectopically expressing N-terminally Myc-tagged Ccdc13 (supplementary Figure 2A) also demonstrated significant co-localisation of tagged Ccdc13 and PCM1 (Figure 2A). In keeping with the range of known centrosomal proteins that, like Ccdc13, contain at least one SMC domain, further examination of YFP-Ccdc13 expressing cells by immunofluorescence confirmed that Ccdc13 is a centrosomal protein with dynamic localisation. As cells traversed interphase, YFP-Ccdc13 expression
became more restricted in localisation to both separating centrosomes through late G2 (Figure 2B). In early mitosis, YFP-Ccdc13 localised to both spindle poles, and remained there during metaphase and anaphase (Figure 2B), although there is a qualitative suggestion that this localisation may be somewhat diminished following metaphase. However, due to a lack of good Ccdc13 antibodies, it’s not clear at this time if this is representative of endogenous Ccdc13, or if persistence of YFP-Ccdc13 at anaphase spindle poles is an artefact of over-expression. As cells progress through telophase and cytokinesis, YFP-Ccdc13 once again assumes a centriolar satellite distribution in both daughter cells (Figure 2B).

Centrosomal localisation of satellites is often dependent on the presence of intact microtubules and a functional dynein-dynactin transport system (Dammermann and Merdes, 2002; Staples et al., 2012). To determine if a similar mechanism might underlie Ccdc13 recruitment to centrosomes, we disrupted microtubules using the microtubule depolymerising agent nocodazole. Treatment of cells with nocodazole caused a marked disruption to the centrosomal localisation of Ccdc13 (Figure 2C). Consistent with this finding, disruption of microtubules by ice treatments also led to dispersal of Ccdc13 from the centrosome (data not shown). PCM1 is transported to the centrosome via the dynein-dynactin transport system, via which it also carries protein cargos to the centrosome (Dammermann and Merdes, 2002). We therefore wanted to assess the effect of disruption of the dynactin transport system on centrosomal localisation of Ccdc13. Over-expression of p50-dynamitin, which disrupts the dynactin transport system, caused mislocalisation of YFP-Ccdc13 from the centrosome (Figure 2C), an observation suggestive that Ccdc13 is itself a protein cargo for PCM1-mediated transport.

The dynamic cell cycle localisation of Ccdc13 coupled with mislocalisation following microtubule disruption provides further evidence that Ccdc13 is a centriolar satellite protein. Indeed, tet-inducible YFP-Ccdc13 almost exclusively co-localised with the centriolar satellite protein Cep215 and the centriolar sub-distal appendage protein Cep170 in both interphase (G1) cells, and in late G2/early mitotic cells (supplementary Figure 2B). Furthermore, we found that depletion of PCM1 caused complete disruption to the centriolar satellite
localisation of Ccdc13 (Figure 2D); an observation in keeping with the known role of PCM1 in satellite formation via the loading of dynein protein cargoes (Dammermann and Merdes, 2002; Lopes et al., 2011). Interestingly, we found that PCM1 depletion did not affect the localisation of a subset of Ccdc13, which remained at the centriolar core; a finding also observed in our investigations into the centriolar satellite protein Cep131 (Staples et al., 2012). In a manner similar to Cep131, depletion of pericentrin B resulted in complete disruption of Ccdc13 localisation from both the centriolar core and the pericentriolar satellites (Figure 2D).

Centrosomal satellites often form multimeric protein complexes (Barenz et al., 2011). Therefore, to further define Ccdc13 as a novel centrosomal satellite protein, we carried out proteomic analyses of FLAG-Ccdc13 complexes purified from tetracycline-inducible HEK293 stable cell lines (supplementary Figure 2A). Putative Ccdc13 interactors with the greatest peptide coverage were PCM1, pericentrin, Cep290, Cep215, Cep131, Cep90 and Cep72 (Figure 3A), which have all been previously shown to localise to centriolar satellites (Balczon et al., 1994; Dammermann and Merdes, 2002; Kim et al., 2008; Kim et al., 2012; Lee and Rhee, 2010; Lopes et al., 2011; Ma and Jarman, 2011; Stowe et al., 2012). Additionally, we confirmed that YFP-Ccdc13 co-localises with PCM1 and PCNT (Figure 3B), and that FLAG-Ccdc13 interacts with endogenous pericentrin, PCM1 and Cep290 (Figure 3C). Furthermore, reciprocal experiments in which the satellite proteins PCM1, PCNT, Cep290 and Cep131 were immunoprecipitated from HeLa cells stably expressing tetracycline-inducible FLAG-tagged Ccdc13 (supplementary Figure 2A) demonstrated robust interactions between these proteins and FLAG-Ccdc13 (Figure 3D). Identical experiments with using a HeLa line expressing tetracycline-inducible YFP-Ccdc13 (supplementary Figure 2A) yielded the same results (supplementary Figure 2C). Collectively, these data are consistent with Ccdc13 being a bone fide centrosomal satellite protein.

**Ccdc13 is required for microtubule organisation and ciliogenesis.**

PCM1 and Cep290 are required for the maintenance of a radial microtubule array in U2OS cells (Dammermann and Merdes, 2002; Kim et al., 2008). To assess whether Ccdc13 might have a similar role, we depleted U2OS cells of Ccdc13 and
examined microtubule organisation by staining for alpha-tubulin. PCM1 siRNA was used as a positive control, and as expected, depletion of PCM1 resulted in a marked loss of microtubule organisation (Figure 4A). Interestingly, Ccdc13 depletion caused a similar but less marked loss of interphase microtubule organisation (Figure 4A), indicating that Ccdc13 plays a role in maintaining a stable microtubule array. Ccdc13-depleted cells also exhibited disrupted mitotic microtubules (supplementary Figure 3A), however, any such disruption to the mitotic spindle in Ccdc13-depleted cells did not appear to activate the spindle assembly checkpoint, as the duration of mitotic transit was unaffected in Ccdc13-depleted cells when assessed over multiple experiments (supplementary Figure 3B). Additionally, any role Ccdc13 may have in interphase microtubule organisation appears independent of PCM1 satellite localisation, as PCM1 integrity was unaffected by Ccdc13 depletion (supplementary Figure 3C).

It has been previously shown that some centrosomal satellites such as pericentrin are required for efficient microtubule regrowth, while others such as PCM1 are not (Dammermann and Merdes, 2002; Dictenberg et al., 1998). To determine if Ccdc13 is required for this process, we assessed microtubule regrowth following rapid depolymerisation by treatment with ice (Dammermann and Merdes, 2002). Cells depleted of Ccdc13 exhibited increased microtubule regrowth, whereas cells ectopically over-expressing Ccdc13 conversely exhibited reduced microtubule regrowth post-depolymerisation (supplementary Figure 3D). Additionally, we noted that disrupted YFP-Ccdc13 following ice treatments rapidly re-engaged with the centrosome during early phases of microtubule re-polymerisation (supplementary Figure 3D). These data suggest that local concentrations of Ccdc13 around the MTOC may influence microtubule growth and possibly delay microtubule nucleation.

Many centriolar satellite proteins play crucial roles in ciliogenesis (Lopes et al., 2011; Stowe et al., 2012). We therefore depleted RPE-1 cells of Ccdc13 to examine a possible role in ciliogenesis. Ciliogenesis was induced in RPE-1 cells by serum withdrawal for 48 hours and confirmed by a pronounced increase in the proportion of ciliated cells, as assessed by positive acetylated tubulin staining (Figure 4B). Depletion of Ccdc13 using two individual siRNA caused a significant
decrease in the proportion of ciliated cells (Figure 4B). To confirm that this represents a specific effect, flow cytometric analyses and immunofluorescence studies showed that decreased ciliogenesis in Ccdc13-depleted cells was not respectively due to altered cell cycle dynamics (Figure 4C), or due to loss of PCM1 from the basal body (data not shown). In keeping with a functional role for Ccdc13 in ciliogenesis, we also observed that YFP-Ccdc13 localised to the basal body centriole pair following serum withdrawal, although YFP-Ccdc13 did not appear to localise to the cilium itself (Figure 4D).

PCM1 and Cep290 are thought to cooperate with BBS4 to promote ciliogenesis (Kim et al., 2008; Lopes et al., 2011; Stowe et al., 2012). Given the severe effects of Ccdc13 depletion on ciliogenesis and the fact that it interacts with both PCM1 and Cep290 (Figures 3A, 3C, 3D and supplementary Figure 2C), we proceeded to assess the effect of Ccdc13 depletion on the localisation of BBS4 using a previously established RPE-1-derived cell line stably expressing LAP-tagged BBS4 (Nachury, 2008). PCM1 siRNA was used as a positive control, and as expected, PCM1 depletion resulted in an almost complete loss of BBS4 localisation to both centriolar satellites and the primary cilium (Figure 5A). Ccdc13 depletion also resulted in a decrease in BBS4 localisation to both centriolar satellites and the primary cilium (Figure 5A), although this was less severe than in PCM1-depleted cells (Figure 5B). Since Ccdc13 depletion does not affect PCM1 localisation (supplementary Figure 3C), it may facilitate the ability of PCM1 to recruit BBS4 to centriolar satellites and thus aid in cilia formation. However, since we do not have any evidence to suggest that Ccdc13 binds directly to BBS4; it was not identified in either our proteomic analyses of purified Ccdc13 complexes or detectable in co-IP studies, we suggest that this effect may be indirect. Collectively, these data indicate that although it does not appear to be essential for this process, Ccdc13 acts at the basal body to promote efficient cilia formation.
Discussion

We report here the first characterisation of the human protein coiled-coil domain-containing 13; Ccdc13, which we identified in a human genome-wide RNAi screen for novel regulators of genome stability. Indeed, depletion of Ccdc13 leads to increased spontaneous levels of post-mitotic DNA damage. However, Ccdc13-depleted cells appear to have intact DDR mechanisms following exogenous insults. We therefore suggest that subtle microtubule defects in Ccdc13 cells enhance mitotic chromosome segregation errors leading to increased levels of DNA breaks and activation of the DDR through previously described mechanisms (Janssen et al., 2011). Indeed, our proteomic analyses of purified Ccdc13 complexes suggest that interacts with a number of centrosome and spindle proteins with known functions in maintaining both interphase and mitotic microtubule organisation. These include PCM1 (Dammermann and Merdes, 2002), Cep90 (Kim et al., 2012; Kim and Rhee, 2011), CAP350 (Yan et al., 2006) and Cep290 (Kim et al., 2008; Lopes et al., 2011; Stowe et al., 2012). Given the non-toxic effects of Ccdc13 depletion, we predict that the majority of these lesions are repaired by NHEJ in the subsequent G1 phase of the cell cycle (Giunta et al., 2010), which would be consistent with a lack of overt DDR defects in Ccdc13-depleted cells.

We also show here that Ccdc13 localises with PCM1 at pericentriolar satellites, and is also found at the centrosome and the basal body of primary cilia, revealing that Ccdc13 is a novel centrosomal satellite protein. Centriolar satellites have been extensively studied in flies and mammals (Barenz et al., 2011). They are electron-dense granules composed of numerous proteins involved in microtubule organization and nucleation. These granules become more tightly localised to the centrosome throughout interphase before redistributing to the cytoplasm during metaphase. Like PCM1, Ccdc13 localises more tightly to the centrosome in late G2. In contrast however, Ccdc13 remains centrosome-bound during metaphase and anaphase (Figure 2B). Recent work has demonstrated that although a subset of satellite proteins such as Cep131, PCM1 and BBS4 are lost from the centrosome during mitosis (Balczon et al., 1994; Kim et al., 2004; Staples et al., 2012), others including OFD1 and Cep290 remain centrosomal during metaphase...
(Kim et al., 2008; Lopes et al., 2011; Stowe et al., 2012). The fact that Ccdc13 localisation to inter-phasic satellites but not the centrosome core is dependent on PCM1, suggests that specific and distinct protein complexes retain different Ccdc13 pools and/or sub-complexes within isolated centrosome compartments. Interestingly, we identified several uncharacterised coiled-coil domain-containing proteins in proteomic analyses of purified Ccdc13 complexes, suggesting that some of these may also be novel centrosomal satellites that may facilitate localisation of Ccdc13. Certainly, further characterisation of these coiled-coil domain-containing in terms of centrosome biology and ciliogenesis is worthy of future study.

It is well known that centriolar satellites participate in cilogenesis (Lopes et al., 2011). We find that Ccdc13 localises to the basal body following serum withdrawal and remains associated with the basal body throughout ciliogenesis. Furthermore, Ccdc13 is required for cilia formation although the observed defect is markedly less severe than that induced by PCM1 depletion (Figure 5B). PCM1 is an absolute requirement for Cep290 and BBS4 localisation to centriolar satellites (Kim et al., 2008; Kim et al., 2004; Lopes et al., 2011). In turn, BBS4 is part of a protein complex termed the BBSome that promotes ciliogenesis by facilitating the Rabin8-dependent activation and ciliary localization of the Rab8 GTPase (Kim et al., 2008). We observed decreased BBS4 localisation to centriolar satellites in serum-starved RPE-1 cells depleted of Ccdc13, without a concomitant effect on PCM1 or Cep290 localisation. This suggests a specific role for Ccdc13 in the recruitment of BBS4 to centriolar satellites during ciliogenesis. However, from co-immunoprecipitation and proteomic studies, we do not have any compelling evidence for an interaction between Ccdc13 and BBS4 (data not shown). We therefore suggest that Ccdc13 depletion might alter the composition of centriolar satellites and thus decrease the ability of PCM1 to recruit BBS4. We are currently studying the role of Ccdc13 in ciliogenesis further using a zebrafish mutant model, which will hopefully also yield insight into potential functions for Ccdc13 in organelle development where ciliogenesis is important (Malicki et al., 2011).
In conclusion, our data establishes Ccdc13 as a novel centrosomal satellite protein which joins an increasing number of human centrosomal proteins including Cep63, Cep131, Cep152, Cep164 and pericentrin that are important for maintaining genome stability (Griffith et al., 2008; Kalay et al., 2011; Sivasubramaniam et al., 2008; Smith et al., 2009; Staples et al., 2012). Interestingly, several of these are either known or putative substrates for the key DDR regulating kinases ATM and ATR (Matsuoka et al., 2007; Smith et al., 2009), which may point to DDR-related regulation of these proteins. Collectively, these findings, together with recent genetic studies (Chaki et al., 2012; Choi et al., 2013; Kalay et al., 2011; Sivasubramaniam et al., 2008; Stiff et al., 2013; Valdes-Sanchez et al., 2013; Zhou et al., 2012), highlight emerging functional links between centrosomal satellites, the DNA damage response, genome stability and human ciliopathies (Chavali and Gergely, 2013). These findings open up new exciting avenues of research, and suggest that mutations in other DDR-related proteins may be causal for a sub-set of human ciliopathies, and potentially, mutations in some centrosomal satellite proteins may be causal for a sub-set of cancer predisposition disorders.

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Materials and Methods

Antibodies
Abcam; Cep131 (ab84864 for immunofluorescence and ab99379 for western blotting), phospho-RPA Thr21 (ab61065), phospho-DNA-PKcs Thr2609 (ab18356), rabbit anti-53BP1, pericentrin (ab4448), Cep290 (ab85728), phospho-histone H3 Ser10 (ab14955) and β-actin (ab8224). Cell Signaling Technologies; γH2AX Ser139 (no. 2577), and rabbit anti-PCM1 (Q-15, no. 5259). Abnova: mouse anti-PCM1 (4152-B01). Millipore: γH2AX Ser139 (JBW301, no. 05-636). Santa Cruz: rabbit anti-Rad51 (H-92). For western blotting, primary antibodies were visualised using HRP-conjugated secondary antibodies from DAKO. For immunofluorescence, Invitrogen anti-mouse or rabbit Alexa Fluor 488 or Alexa 594 were used.

Cell culture
HCT116, HeLa, U2OS and HEK293 cells were maintained as an adherent monolayer in DMEM media containing 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide. HeLa Flp-in T-Rex and HEK293 Flp-In T-Rex cells (Invitrogen) were maintained in DMEM media containing 10% FBS and 1% penicillin/streptomycin, supplemented with 4 µg/ml Blasticidin S (Melford) and 100 µg/ml Zeocin (Invitrogen). HeLa cells stably expressing GFP-tagged Histone H2B were maintained in identical media supplemented with 2 µg/ml Blasticidin S.

Stable cell line generation
Stable tetracycline-inducible HEK293 Flp-In cell lines expressing FLAG-tagged Ccdc13 cDNA (NM_144719.2) and HeLa Flp-In cell lines expressing YFP or FLAG-tagged Ccdc13 were created by co-transfection of these cell lines with pPGKFLPobpA-Flp recombinase and either empty pDEST-Flag/FRT/TO or pDEST-Flag/FRT/TO-Ccdc13 according to the Flp-In manufacturer’s protocol. Recombinants were selected in media containing 4 µg/ml Blasticidin S and 150 µg/ml Hygromycin B (Invitrogen). All transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.
Transfections and drug treatments
HEK293, U2OS, RPE-1 and HeLa cells were transfected with between 30-50 nM siRNA using Lipofectamine 2000 (Invitrogen), RNAiMAX (Invitrogen) or Dharmafect 1 (Dharmacon) according to the manufacturer’s instructions. Cells were collected, lysed or fixed for analysis after 48 hrs unless otherwise indicated. Cells were treated with 1 µM nocodazole (Sigma) or 10 µM Taxol (Sigma) for 3 hrs or 300 µM mimosine (Sigma) before fixation or release into drug-free medium for the times indicated.

Cell lysis and western blotting
For whole-cell extracts, the cells were solubilised on ice in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM DTT and 1 mM EDTA) supplemented with 50 U/µl benzonase (Novagen), protease and phosphatase inhibitors (Sigma). Cleared lysates were produced by centrifugation of the resulting samples at 16,000 g for 15 min at 4°C. Gel electrophoresis was performed using the NuPAGE system (Invitrogen). Briefly, samples were resolved on 4–12% Bis-Tris gels in MOPS buffer, transferred to a PVDF membrane which was then probed for the protein of interest using antibodies diluted in PBS containing 5% Marvel and 0.1% Tween-20 (Sigma).

Immunoprecipitation
For purification of FLAG-tagged proteins, 1 mg of the whole-cell extract was incubated with 20 µl of M2-anti FLAG beads (Sigma) for 16 hrs at 4°C. For immunoprecipitations using endogenous antibodies, 1–2 µg of antibody was incubated with the sample for 1–2 hrs before addition to 20 µl of washed Protein A/G beads (Santa Cruz) and incubation for 16 hrs at 4°C. Beads were then pelleted and washed three times in 20×bed volume of the lysis buffer. The bound protein was eluted either by heating the beads at 95°C for 5 min with 2×LDS buffer (Invitrogen) or by incubation with FLAG peptide (Sigma) according to manufacturer’s instructions. Inputs represent 1/40th of the extract used for the immunoprecipitation.

Immunofluorescence and live-cell imaging
Cells were grown on glass coverslips and treated as indicated, then fixed with
either methanol or 3% buffered paraformaldehyde for 10 min at RT, and permeabilised in PBS containing 0.2% Triton X-100 for 5 min at RT. Cells were then incubated with primary antibody for 2 hrs at RT, and detected with a secondary Alexa 488 or Alexa 594 conjugated goat anti-rabbit or anti-mouse IgG. Antibody dilutions and washes after incubations were performed in PBS. DNA was stained with DAPI (1 µg/ml) and coverslips were mounted in Shandon Immumount medium (Thermo). Fluorescence microscopy was performed on a Nikon Eclipse T200 inverted microscope (Melville), equipped with a Hamamatsu Orca ER camera and a 200 W metal arc lamp (Prior Scientific, United Kingdom), with a 100×objective lens. Images were captured and analysed using Volocity 3.6.1 software (Improvision).

For live cell imaging, H2B–GFP HeLa cells were grown on 24-well glass bottomed plates (Scientific Laboratory Supplies) in selective media. Cells were transfected with siRNA, then cells were imaged on a Leica AF6000 LX inverted microscope fitted with an environmental chamber set at 37°C providing humidity and 5% CO2. Leica LAS AF Lite software was used for image acquisition and analysis.

**Cell cycle analyses by flow cytometry**

Cells were trypsinised from dishes and pelleted, then washed with PBS and fixed in 70% ice-cold ethanol. Cells were then washed with PBS and stained with a propidium iodide solution (50 µg/ml) containing RNase A (25 µg/ml) for 30 min before flow cytometry was performed on a Becton Dickinson FACScalibur instrument. Cells were gated for size and granularity to remove cell debris from the analyses, and 10,000 live cells were quantified for each treatment per experiment.
References


Kim, K., Lee, K. and Rhee, K. (2012). CEP90 is required for the assembly and centrosomal accumulation of centriolar satellites, which is essential for primary cilia formation. PloS one 7, e48196.


Ma, L. and Jarman, A. P. (2011). Dilatory is a Drosophila protein related to AZI1 (CEP131) that is located at the ciliary base and required for cilium formation. Journal of cell science 124, 2622-30.


Figure legends

Figure 1. Depletion of Ccdc13 results in increased post-mitotic DNA damage.
A: HeLa cells were transfected with non-targeting control siRNA, or four individual siRNAs targeting Ccdc13 as indicated. After 48 hrs cells were fixed, stained for γH2AX and counterstained with DAPI (upper panel) and cells containing >5 foci quantified over three independent experiments (lower panel). B: HCT116 cells were transfected with non-targeting control or Ccdc13 targeting siRNA. After 48 hrs cells were treated with 300 µM mimosine for 24 hrs then released into normal medium for the indicated times. Cells were then fixed, stained for γH2AX and counterstained with DAPI. Data shown represents quantification of cells containing >5 γH2AX foci in 3 independent experiments. C: HeLa cells were transfected with non-targeting control siRNA, or two individual siRNA targeting Ccdc13. After 48 hrs these were stained for either phospho-RPA Thr21), phospho-ATM (Ser1981), phospho-DNA-PK (Thr2609), Rad51 or 53BP1 and counterstained with DAPI. Cells with >5 foci indicating sites of damage or replication stress were counted and displayed as a percentage of the total number of cells. All experiments were performed at least three times with error bars representing the standard errors of the means, with representative images shown.

Figure 2. Fluorescently-tagged Ccdc13 localises to centriolar satellites in a PCM1 and PCNT-dependent manner.
A: Upper 2 panels; HeLa cells stably expressing tetracycline-inducible YFP-tagged Ccdc13 (supplementary Figure 2A) were mock-treated or treated with 1 µg/ml tetracycline for 24 hrs to induced Ccdc13 expression. Lower panel; Additionally, HeLa cells were transfected with either an empty plasmid or a plasmid expressing Myc-Ccdc13 (supplementary Figure 2A). Cells were fixed, and co-stained for Myc or GFP and either γ-tubulin or PCM1 as indicated, then counterstained with DAPI. Average Pearson’s coefficients for co-localisation with PCM1 was calculated as 0.760 and 0.815 for YFP-Ccdc13 and Myc-Ccdc13 respectively. Inset images highlight the centrosome/satellite region for a given image. B: Left panel; Representative images of HeLa cells stably expressing low amounts of tetracycline-inducible YFP-tagged Ccdc13 (fixed and stained for GFP) at various stages of the cell cycle. Right panel; Quantification of mean area occupied and mean fluorescence intensity for YFP-Ccdc13 (relative arbitrary units to interphase cells) at the various stages of the cell cycle. Error bars represent standard deviations of the means. C: Upper panels; YFP-Ccdc13-expressing HeLa cells were treated with 1 µg/ml nocodazole and fixed after 3 hrs before staining for GFP and counterstaining with DAPI. Lower panels; Cells were transfected with either empty vector and Myc-Ccdc13, or Myc-Ccdc13 and a vector encoding GFP-tagged p50-dynamitin. After 24 hrs, cells were fixed and stained with antibodies against GFP and Myc, then counterstained with DAPI. D: YFP-Ccdc13 cells were transfected with non-targeting control siRNA or siRNA targeting PCM1 or PCNT as indicated. After 48 hrs, 1 µg/ml tetracycline was added to induce YFP-Ccdc13 expression and after a further 24 hrs cells were fixed, stained for GFP and PCM1 or PCNT and counterstained with DAPI. All experiments were performed three times, and representative images are shown throughout. Inset images highlight the centrosome/satellite region for a given image.
**Figure 3. Ccdc13 interacts and co-localises with several centriolar satellite proteins.**

**A:** HEK293 cells stably expressing tetracycline-inducible FLAG-tagged Ccdc13 (supplementary Figure 2A) were grown in large-scale culture. Cells were mock-treated or 1 μg/ml tetracycline was added to induce FLAG-Ccdc13 expression, and after 24 hrs cells were lysed and FLAG-Ccdc13 was immunoprecipitated using FLAG M2 antibody conjugated to agarose beads. FLAG-Ccdc13 was eluted using 150 ng/ml 3xFLAG peptide and resulting eluates boiled and resolved by SDS-PAGE. Following SYPRORuby staining, the gel was dissected and sent for mass spectrometric analysis. A shortlist of proteins significantly enriched in the tetracycline-induced sample (as evidenced by the enrichment of recovered unique peptides) is shown alongside the associated peptide coverage.

**B:** YFP-Ccdc13-expressing HeLa cells were mock-treated or treated with 1 μg/ml tetracycline to induce expression. After 24 hrs cells were fixed and stained for GFP and co-stained for either PCM1 or PCNT. Inset images highlight the centrosome/satellite region for a given image.

**C:** FLAG-Ccdc13-expressing HeLa cells were mock-treated or treated with 1 μg/ml tetracycline. After 24 hrs cells were lysed and FLAG-Ccdc13 immunoprecipitated using FLAG antibody-conjugated agarose beads and eluted with 3xFLAG peptide. Eluates were resolved by SDS-PAGE and blots probed using antibodies against PCNT, PCM1, Cep290 and FLAG.

**D:** HeLa cells stably expressing tetracycline-inducible FLAG-tagged Ccdc13 (supplementary Figure 2A) were mock-treated, or treated with 1 μg/ml tetracycline. After 24 hrs, cells were lysed and immunoprecipitations performed on Protein G-Sepharose beads using antibodies against the centrosomal satellite proteins PCM1, PCNT, Cep290 and Cep131 as indicated. Eluates were resolved by SDS-PAGE, and western blotting performed with the indicated antibodies.

**Figure 4: Ccdc13 is required for interphasic microtubule organisation and ciliogenesis**

**A:** Microtubule (MT) organisation in U2OS cells transfected with non-targeting control siRNA, or siRNA targeting Ccdc13. After 48 hrs, cells were fixed, stained for PCNT and α-tubulin and counterstained with DAPI to assess microtubule architecture. Left panel show representative images with white arrows highlighting the MTOC. Inset images highlight the centrosome/satellite region for a given image. Right panel shows quantification of MT defects in cells treated with either control or Ccdc13 siRNA.

**B:** RPE-1 cells were transfected with non-targeting control siRNA, or two individual siRNA targeting Ccdc13 and then serum-starved for 48 hrs before fixation. Cells were then stained for PCNT and acetylated tubulin, and counterstained with DAPI (left panel). Right panel; the proportion of ciliated Ccdc13-depleted cells was quantified and displayed relative to control siRNA-transfected cells. Inset images highlight basal bodies for a given image.

**C:** Cell cycle analyses of cells treated with either control or Ccdc13-targetted siRNA as indicated.

**D:** RPE-1 cells were transfected with a vector encoding YFP-tagged Ccdc13. After 24 hrs cells were serum-starved for the indicated times before fixation, staining with antibodies recognizing GFP and acetylated tubulin, and counterstained with DAPI. White arrows highlight site of basal body and inset images highlight basal bodies for a given image. All experiments shown were performed at least three times with error bars representing the standard errors of the mean. Representative images are shown for each experiment.
Figure 5: Ccdc13 promotes BBS4 centriolar satellite localisation and ciliary recruitment.
A: RPE-1 cells stably expressing LAP-tagged BBS4 were transfected with non-targeting control siRNA, or siRNA targeting PCM1, Cep131 or Ccdc13. After 48 hrs, cells were fixed and stained for GFP and acetylated tubulin, and counterstained with DAPI. Inset images highlight basal bodies for a given image. B: Proportion of cells with centriolar satellite BBS4 and ciliary BBS4 relative to cells transfected with control siRNA, as determined from 3 independent experiments with error bars representing the standard errors of the means.
A

Control siRNA  |  PCM1 siRNA  |  Ccdc13 siRNA

PCNT

α-Tubulin

Merge

B

Control siRNA  |  Ccdc13 siRNA  |  PCM1 siRNA

DAPI
Acetyl-Tubulin
PCNT

C

Cell cycle phase

Sub-G1  |  G1  |  S  |  G2/M

Cells (%)

Control  |  Ccdc13 si1  |  Ccdc13 si2

siRNA

D

0 hrs  |  6 hrs  |  24 hrs

YFP-Ccdc13

Acetyl-Tubulin

Merge

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