The E3 ubiquitin ligase UBR5 regulates centriolar satellite stability and primary cilia

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ABSTRACT Primary cilia are crucial for signal transduction in a variety of pathways, including hedgehog and Wnt. Disruption of primary cilia formation (ciliogenesis) is linked to numerous developmental disorders (known as ciliopathies) and diseases, including cancer. The ubiquitin–proteasome system (UPS) component UBR5 was previously identified as a putative positive regulator of ciliogenesis in a functional genomics screen. UBR5 is an E3 ubiquitin ligase that is frequently deregulated in tumors, but its biological role in cancer is largely uncharacterized, partly due to a lack of understanding of interacting proteins and pathways. We validated the effect of UBR5 depletions on primary cilia formation using a robust model of ciliogenesis, and identified CSPP1, a centrosomal and ciliary protein required for cilia formation, as a UBR5-interacting protein. We show that UBR5 ubiquitylates CSPP1, and that UBR5 is required for cytoplasmic organization of CSPP1-comprising centriolar satellites in centrosomal periphery, suggesting that UBR5-mediated ubiquitylation of CSPP1 or associated centriolar satellite constituents is one underlying requirement for cilia expression. Hence, we have established a key role for UBR5 in ciliogenesis that may have important implications in understanding cancer pathobiology.

INTRODUCTION Primary cilia form at the surface of most mammalian cell types, and have been implicated in sensory perception, cell signaling, and development. Primary cilia comprise a microtubule (MT) axoneme and ciliary membrane extending from the basal body (Satir and Christensen, 2007), which is surrounded by granular structures known as centriolar satellites (Figure 1A; Lopes et al., 2011) largely composed of pericentriolar material 1 (PCM1) and other key proteins (Kubo et al., 1999). In contrast to motile cilia, primary cilia lack the core MT/dynein structure that provides motility to the former (Roth et al., 1988). During mammalian development, primary cilia...
Cilia were tightly linked (Figure 1B). For example, centriolar satellites regulate coregulated with cell cycle progression (Kim and Rhee, 2011), to (Tollenaere et al., 2015). Although the biology is poorly character related functions, including centriole formation and ciliogenesis (Michaud et al., 2016) demonstrated the highly organized and dynamic structure of primary cilia.

Ubiquitylation is one of the most common protein posttranslational modifications, resulting in the attachment of one or more ubiquitin (Ub) molecules to substrate proteins via an ATP-dependent enzymatic cascade known as the ubiquitin–proteasome system (UPS; Ciehanover et al., 1978; Hershko et al., 1981). The various signaling outcomes of protein ubiquitylation are determined by the topology of the attached poly-Ub chains (Passmore and Barford, 2004). For example, K48 linked poly-Ub target substrates for proteasomal degradation, but alternate linkages (K63, K29, etc.) are emerging as key regulators of cell signaling via modulation of protein function, localization, and protein–protein interactions. Key proteins associated with tumor initiation (e.g., p53, NF-κB) are regulated by the proteasome, and proteasome inhibitors such as PS-341 and bortezomib (Velcade) are under trial or approved for clinical use in hematological malignancies (Orlowski et al., 2002; Johnson, 2014).

The integrity of centriolar satellites is crucial for centrosome-related functions, including centriole formation and ciliogenesis (Tollenaere et al., 2015). Although the biology is poorly characterized, the distribution and appearance of centriolar satellites is coregulated with cell cycle progression (Kim and Rhee, 2011), to which cilia assembly/resorption and centrosome duplication are tightly linked (Figure 1B). For example, centriolar satellites regulate shuttling of cilia-related proteins to the basal body (Lopes et al., 2011), and aid in the assembly of the many centrosomal components required to duplicate the centrosome (Hori and Toda, 2017).

The UPS is emerging as a key regulator of ciliogenesis and centriolar satellite stability (Shearer and Saunders, 2016). E3 Ub ligases, which largely determine substrate specificity and are an important rate-limiting component of the UPS, can regulate expression of various proteins crucial to ciliary axoneme extension. For example, the Cullin Ub ligase Mindbomb 1 (MIB1) regulates multiple centriolar proteins, including PCM1 (Villumsen et al., 2013; Cajane et al., 2015), and extension of the axoneme is initiated by UPS-mediated degradation of the ciliogenesis inhibitor trichopein (Inoko et al., 2012; Kasahara et al., 2014). A genome-wide RNA interference (RNAi) screen for regulators of ciliogenesis identified a number of components of the UPS (Kim et al., 2010), including the E3 Ub ligase UBR5 (ubiquitin protein ligase E3 component N-recognin 5). UBR5 is a highly conserved gene (Mansfield et al., 1994; Callaghan et al., 1998) required for normal embryonic development, multiple aspects of the DNA damage response, and other cellular functions (Shearer et al., 2015). UBR5 is frequently deregulated in many cancer types by amplification and/or mutation (Clancy et al., 2003; O’Brien et al., 2008; Meissner et al., 2013), but the full complement of UBR5 substrates, and hence the mechanistic role of UBR5 in cancer, remain to be defined.

We sought to further investigate the role of UBR5 in ciliogenesis in a cancer context. We show that UBR5 regulates primary ciliogenesis and identify a novel interaction between UBR5 and the centriolar satellite protein CSPP1. CSPP1 is a direct substrate of UBR5 ligase activity, and depletion of UBR5 affects the subcellular localization of CSPP1 and CSPP1-associated centriolar satellites. Identification of UBR5 as a novel ciliary regulator has interesting implications for understanding cell signaling in development and cancer.

RESULTS
Depletion of UBR5 confers concurrent disruption of centriolar satellites and inhibition of primary cilia formation

UBR5 was identified as a putative regulator of primary cilia formation in a functional genomics screen (Kim et al., 2010) along with a number of other UPS components (Shearer and Saunders, 2016). We validated the effect of UBR5 depletion on cilia formation using a robust ciliogenesis assay (Figure 2A) and examined the functional significance of UBR5 depletion in the context of cilia/centriolar satellite stability/organization. Small interfering RNA (siRNA)-mediated depletion of UBR5 almost completely attenuated primary cilia formation in hTERT-RPE1 cells (i.e., observed in fewer than 20% of cells), as determined by labeling with an antibody against glutamylated tubulin (Figure 2, B and C). Furthermore, centriolar satellites were dispersed with UBR5 depletion (Figure 2, B and C). Centriolar satellite disruption with UBR5 depletion was similar to that observed with depletion of PCM1 (Figure 2, B and C). PCM1 has previously been shown to recruit another E3 Ub ligase, MIB1, to the centrosome (Wang et al., 2016). Cilia were absent in 89% of hTERT-RPE1 cells with UBR5 depletion under serum starvation, and 94% of these cells displayed dispersed centriolar satellites (Figure 2C). Approximately 77% of control hTERT-RPE1 cells expressed a primary cilia, of which 85% had distinct centriolar satellite formation around the basal body (Figure 2C). Cilia attenuation by UBR5 depletion was not cell cycle dependent, as hTERT-RPE1 cells did not accumulate in cell cycle phases considered nonpermissive for cilia formation (Supplemental Figure S1A).

Given this effect on UBR5 depletion on centriolar satellite stability, we hypothesized that UBR5 may act to stabilize centriolar
material and investigated potential protein–protein interactions with known structural components of the centrosome and regulators of centriolar satellite stability. We performed pull downs of GFP-tagged UBR5 in HEK293T cells, and observed coimmunoprecipitation of UBR5 and γ-tubulin, indicating that UBR5 can interact with structural components of the centrosome (Figure 2E). The UPS is known to regulate centriolar satellite stability via MIB1-mediated ubiquitylation of PCM1 (Villumsen et al., 2013; Wang et al., 2016). We could not detect an interaction between UBR5 and PCM1, and we observed only a very weak putative interaction with MIB1 (Figure 2E). Furthermore, UBR5 depletion in HEK293T cells did not significantly alter levels of PCM1, MIB1, or γ-tubulin proteins (Supplemental Figure S1B). Together, these data suggest that the role of UBR5 in ciliogenesis is not mediated via directly affecting PCM1/MIB1.

Coexpression of CSPP1 and UBR5 in human cancer cell lines and primary cancer biopsies

To identify potential mechanisms by which UBR5 regulates cilia/centrosome biology, we analyzed publicly available gene expression datasets to identify candidate genes coregulated with UBR5. Coordinate expression of genes functioning in common pathways (i.e., synexpression groups) is a widespread phenomenon in eukaryotes and coexpression analysis has proven to be a powerful
has established functional roles in regulation of spheroid formation, cell division, and ciliogenesis (Asiedu et al., 2009; Patzke et al., 2010; Sternemalm et al., 2015; Zhu et al., 2015a). Mutations in CSPP1 have been implicated in ciliopathies (Akizu et al., 2014; Shaheen et al., 2014; Tuz et al., 2014), and expression of CSPP1 isoforms display distinct restriction in breast cancer subtypes (Sternemalm et al., 2014). The large and predominantly expressed isoform of CSPP1, CSPP-L, localizes to the centrosome and centriolar satellites, where it is required for primary cilium formation in noncycling cells (Patzke et al., 2010; Gupta et al., 2015; Mick et al., 2015), and dynamically relocates to the spindle apparatus of dividing cells, where it aids chromosome movements and cytokinesis during cell division (Patzke et al., 2006; Asiedu et al., 2009; Zhu et al., 2015a).

Coexpression of CSPP1 with UBR5 in the NCI-60 cohort was confirmed by linear-regression analysis of NCI-60 cell line expression data ($R^2 = 0.3709$, $p < 0.0001$; Supplemental Figure S2A). Independent, complementary analysis of CSPP1 expression using the SEEK database (Zhu et al., 2015b) identified UBR5 as one of the top genes coexpressed with CSPP1 in cancer, ranked second (coexpression score 1.32, $p = 0.0001$) after OTUD6B (coexpression score 1.35, $p < 0.0001$), a deubiquitylase recently implicated in intellectual disability syndrome (Santiago-Sim et al., 2017). Coexpression of UBR5 and CSPP1 was validated in independent breast cancer gene expression datasets from The Cancer Genome Atlas (TCGA; Supplemental Figure S2B and Supplemental Table S1), Cancer Cell Line Encyclopedia (CCLE), and METABRIC cohorts (Supplemental Table S2). Coexpression of CSPP1 with UBR5 was evident in all cohorts, irrespective of subtype (Supplemental Tables S1 and S2). Overexpression of UBR5 and CSPP1 mRNA was the predominant form of altered expression, and coamplification did not correlate with increased fraction of altered genome (Supplemental Figure S2B).

There are three known protein products of the CSPP1 gene (Figure 3B). CSPP is a 101.5 kDa protein that contains both MT-organizing domain, and an additional 294 amino acids at the N-terminus, and an additional 51 amino acids in the middomain, which affects how CSPP1 interacts with MTs (Patzke et al., 2006). Of relevance to the ubiquitin context, the extended N-terminal region of CSPP-L harbors an N-degron motif (not present on CSPP) that likely targets CSPP-L for coexpression in NCI-60 cancer cell line panel, obtained via CellMiner (Reinhold et al., 1992). Analysis of mRNA coexpression in the NCI-60 panel using CellMiner (Stinson et al., 1999; Wu et al., 2002; van Noort et al., 2003). As UBR5 is frequently altered in multiple cancer types (Shearer et al., 2003), including breast cancer cell lines showed coexpression of UBR5 and CSPP-L at the protein level ($R^2 = 0.2532$, $p = 0.017$; Supplemental Figure S2, C and D), irrespective of subtype (Supplemental Figure S2, E and F). Note that the epitope recognized by the CSPP1 antibody used in Supplemental Figure S2C is specific to the CSPP-L isoform.

FIGURE 3: Coexpression of UBR5 gene. (A) Waterfall plot showing global correlation of gene expression against UBR5 expression in NCI-60 cancer cell line panel, obtained via CellMiner (Reinhold et al., 2012). Cutoff set at Pearson correlation of 0.254. Putative ciliary proteins are indicated, with putative top tier cilia localizing proteins labeled and indicated with larger red dots; second tier cilia localizing proteins are also labeled and shown with larger gray dots. Putative cilia localizing proteins based on cilia proximity labeling data (Mick et al., 2015). (B) Structure of CSPP1 protein isoforms. Note that the extra 294 amino acid N-terminal region of CSPP-L is the binding region of the selected CSPP1 antibody. N-Degron motif shown in red. Coiled-coil domains are shown with a black box. Proline-rich domains are shown with a white box. Glutamate-rich domains are shown with a white striped box.
UBR5 regulates ubiquitylation of CSPP-L

In the context of UBR5 and CSPP-L coexpression, common functional roles in ciliogenesis, and a recently established interaction between PCM1 and CSPP-L, and localization to centriolar satellites (Patzke et al., 2012), we hypothesized that UBR5 may regulate ciliogenesis via maintenance of centriolar satellite stability/organization and possibly involving CSPP-L. We therefore investigated a possible direct interaction between UBR5 and CSPP-L in cells using a panel of GFP-tagged UBR5 variants (wild-type and functional domain mutants UBR*, MLLE*, and HECT*, as detailed in Figure 4A). Coimmunoprecipitation using GFP-UBR5 in HEK293T cells detected an interaction between UBR5 and endogenous CSPP-L, while no interaction was observed using GFP-only control (Figure 4B and Supplemental Figure S3B). Mutation of predicted functional residues in the UBR-box of UBR5 (UBR*, Matta-Camacho et al., 2010), MLLE domain (MLLE*, Kozlov et al., 2010; Munoz-Escobar et al., 2015), or the known conserved cysteine (C2768A; Scheffner et al., 1995) within the HECT active site (HECT*) had no detectable effect on the immunoprecipitation of CSPP-L (Figure 4B), suggesting that the interaction between these proteins is independent of these domains in UBR5 (transfection efficiency for these experiments is detailed in Supplemental Figure S3A). The interaction between UBR5 and CSPP-L was confirmed using GFP-tagged CSPP-L to coprecipitate endogenous UBR5 from HEK293T cells, but not with GFP-only control (Figure 4C). A significant amount of CSPP-L was still detectable in the nonbound fraction of GFP-UBR5 pull downs (Figure 4B), suggesting that UBR5 is not sequestering the complete pool of cellular CSPP-L in this assay.

The presence of higher molecular weight smears detected using anti-CSPP-L following pull down with GFP-UBR5 (Figure 4B) suggested the presence of polyubiquitylated forms of CSPP-L in HEK293T cells. Probing with an anti-Ub antibody following pull down of GFP-CSPP-L confirmed the presence of ubiquitylated CSPP-L (CSPP-L-Ub) in HEK-293T cells (Figure 4C). To ensure the high molecular weight Ub smears were indeed covalently bound Ub, and not a ubiquitylated CSPP-L interactor, the GFP-CSPP-L co-IP was also performed using denaturing conditions (Figure 4C, far right). These presumptive polyubiquitylated forms of CSPP-L were attenuated in pull downs using the catalytically inactive form of UBR5 (HECT*), suggesting direct ubiquitylation of CSPP-L by UBR5 (Figure 4B and Supplemental Figure S3B). However, it should be noted that variation in *HECT interacting modified CSPP-L was observed between experiments, likely due to varied GFP-UBR5 transfection efficiency coupled with the presence of endogenous wild-type UBR5 (Supplemental Figure S3D). Despite this observation, a clear reduction in modified CSPP-L was observed interacting with *HECT when compared with
wild-type UBR5. Lower molecular weight CSPP-L species in WCE are likely degradation products of endogenous CSPP-L. Importantly, this experiment also confirmed the presence of ubiquitylated forms of endogenous CSPP-L (i.e., not GFP labeled). Overexpression of UBR5 did not alter total cellular CSPP-L levels (Supplemental Figure S3B), suggesting that UBR5-mediated ubiquitylation of CSPP-L is not targeting the protein for degradation by the proteasome.

**UBR5 interacts with CSPP-L at the centriolar satellites**

We confirmed that GFP-tagged UBR5 localizes to the nucleus and cytoplasm (Supplemental Figure S3A) and CSPP-L displays centrosomal and centriolar satellite localization (Supplemental Figure S3E, white arrows), consistent with previous reports (Patzke et al., 2010). As UBR5 antibodies show cross-reactivity with cytoplasmic proteins by immunohistochemistry (unpublished data), we sought to further characterize the UBR5 and CSPP-L interaction in a cellular context using protein complementation assays to define subcellular localization. Bimolecular fluorescence complementation (BiFC) analysis (Figure 5A) indicated the presence of protein–protein interactions between CSPP-L and UBR5 in discrete perinuclear foci in HEK293T cells, consistent with the primary subcellular localization of CSPP-L to and around centrosomes (white arrows, Figure 5A). A similar subcellular distribution was observed for the BiFC signal generated by an interaction between CSPP-L and Ub (Figure 5A), suggesting that CSPP-L is ubiquitylated around the centrosome and consistent with our results above (Figure 4). A positive control assay between UBR5 and Ub showed strong nuclear and cytoplasmic foci (Figure 5A) consistent with the previously described localization of UBR5 to the nucleus and cytoplasm (Supplemental Figure S3A; Fuja et al., 2004). Correct expression of V1- or V2-tagged BiFC fusion proteins was confirmed by immunoblotting for both the expression protein and the respective Venus fluorescent protein fragment (Figure 5B).

We next used BiFC to examine ubiquitylation of CSPP-L interaction pairs in hTERT-RPE1 cells and costained for PCM1 (which marks the centriolar satellites) to confirm detailed localization. A BiFC signal indicating Ub-CSPP-L was detected at the centrosome proximal centriolar satellites (Figure 5C), indicating that the perinuclear BiFC signal observed in HEK293T cells (Figure 5A) was indeed localized at centriolar satellites surrounding the centrosome. These data indicate that CSPP-L is ubiquitylated at the centriolar satellites.

**UBR5 is necessary for efficient recruitment of CSPP-L to the centrosome and centriolar satellites**

Consistent with the effects of UBR5 overexpression (Figure 4B), we observed no change in total cellular CSPP-L following UBR5 depletion by short-hairpin RNA (shRNA; Figure 6A). Further, we did not observe an accumulation of ubiquitylated CSPP-L following treatment with the proteasome inhibitor MG-132 (Supplemental Figure S3B). Together, these data strongly indicate that UBR5-mediated ubiquitylation of CSPP-L is not targeting the protein for degradation. Analysis of GFP-CSPP-L pull downs using chain-specific Ub antibodies indicated the presence of both K48- and K63-linked polyubiquitin chains on CSPP-L (Figure 6B). Nondegrading K63-linked polyubiquitin chains have been implicated in endocytosis trafficking and signal transduction (Ikeda and Dikic, 2008).

Interestingly, transiently coexpressed V1-CSPP-L and V2-Ub fusion proteins resulted in a BiFC signal that colocalized with PCM1 in hTERT-RPE1 cells (Figure 5C), indicating the presence of ubiquitylated CSPP-L at centriolar satellites. Moreover, the V1-CSPP-L–V2-Ub BiFC signal was confined to centriolar satellites (and likely the pericentriolar material) alone, because excessive ectopic (and endogenous) CSPP-L was detected at the cytoskeleton (microtubules [MTs] as inferred by Patzke et al., 2006) by a CSPP-L–specific antibody (Figure 5D).

Depletion of UBR5 diminished localization of CSPP-L to the centrosome (Figure 6, C and E) and adjacent satellites as determined by a pericentriolar quantitation mask (Figure 6D). This effect was independent of disruption of centriolar satellites, as depletion of PCM1 did not prevent aggregation of CSPP-L at the centrosome (Figure 6C and Sternemalm et al., unpublished data). Loss of PCM1 is known to disrupt centriolar satellite stability and ciliogenesis (Wang et al., 2016), and accordingly PCM1 disruption depletes centriolar satellite accumulation of CSPP-L (Figure 6C). It is apparent that CSPP-L and UBR5 are integral to satellite stability, as depletion of either almost completely attenuates detectable PCM1 directly adjacent to the centrosome (Figure 6C).

**DISCUSSION**

We demonstrate a novel function for the E3 Ub ligase UBR5 in regulation of ciliogenesis via maintenance of centriolar satellite stability. We also demonstrate a novel protein–protein interaction between UBR5 and the CSPP-L isoform of CSP1, predominantly at the centrosome and surrounding centriolar satellites. CSPP-L is an established positive regulator of ciliogenesis (Patzke et al., 2010) and a prominent cilia-localizing protein (Mick et al., 2015). Cilium assembly is tightly linked to exit from mitosis into G1 phase (Rieder et al., 1979). CSPP-L is increasingly recruited to the centrosome during G2/prophase in cell cycle progression, and detected on spindle MTs during mitosis, concomitant with centriolar satellite dispersal (Patzke et al., 2006; Kim et al., 2016). This cell cycle–dependent localization of CSPP-L to the primary cilium and spindle apparatus is likely regulated via posttranslational regulatory mechanisms. Indeed, the UPS is a known regulator of centriolar satellite stability (Shearer and Saunders, 2016) and we observed that UBR5 is necessary to stabilize not only centriolar satellite organization, but also CSPP-Ls centrosomal localization.

Another E3 ubiquitin ligase, MIB1, is also known to regulate ciliogenesis via ubiquitin-mediated regulation of PCM1 function (Vilumsen et al., 2013; Wang et al., 2016). We observed a weak interaction between UBR5 and MIB1, but not between UBR5 and PCM1. This may indicate large multiprotein complexes arising at centriolar satellites but as these putative complexes would also presumably accumulate PCM1—a major component of centriolar satellites—this explanation is improbable (Kubo et al., 1999). More likely, as UBR5 also interacts with γ-tubulin, the apparent weak interaction between UBR5 and MIB1 may reflect a general accumulation of UBR5 around the centrosome.

Even though we detected ubiquitylation of CSPP-L in association with UBR5, siRNA-mediated depletion of UBR5 did not alter cellular levels of CSPP-L, suggesting that UBR5 is not targeting CSPP-L for proteasomal degradation. BiFC experiments indicate that ubiquitylated CSPP-L is primarily confined to centriolar satellites, while excessive and nonubiquitylated CSPP-L (i.e., not detected by BiFC for Ub-CSPP-L) localizes to MTs (Figure 5D). Notably, both K48- and K63-linked poly-Ub conjugated forms of CSPP-L were detected interacting with UBR5, indicating the presence of nondegrading Ub signaling events. UBR5 is known to assemble nondegrading ubiquitin chains on β-catenin (Hay-Koren et al., 2011) and ATMIN (Zhang et al., 2014). Nondegrading Ub signaling is also involved in other aspects of centriolar satellite maintenance. For example, MIB1 monoubiquitylates PCM1, A2I, and CEP290 in the absence of UV cellular stress and maintains these proteins in an inactive form until monoubiquitylation is reversed (Vilumsen et al., 2013). However, another study found PCM1 to be degraded by MIB1-mediated polyubiquitylation (Wang et al., 2016) with discrepancies in results.
FIGURE 5: UBR5 interacts with CSPP-L at the centrosome. (A) Left to right, CSPP-L interacts with UBR5 in large foci adjacent to the nucleus (arrows). CSPP-L interacts with Ub in large foci adjacent to the nucleus (arrows). Interaction between UBR5 and Ub in HEK293T cells shows nuclear and cytoplasmic localization, with large foci of UBR5 and Ub in the nucleus. Untransfected cells show no visible signal. Nuclear marker is H2B-mCherry (magenta). High-powered inset fields are indicated by a dashed box (bar = 30 µm) and schematic of BiFC analysis for protein–protein interactions included. Data are representative of two experiments. (B) Immunoblot data showing correct production of fusion proteins in BiFC assay. Expected fusion protein sizes are V2-CSPP-L 152 kDa, V1-UBR5 329 kDa, V1-Ub 28 kDa, and V2-Ub 19 kDa. (C) CSPP-L/Ub interacting pairs colocalize with PCM1 (centriolar satellite marker) in hTERT-RPE1 cells. Bar = 20 µm. High magnification of ROI bar = 2 µm. (D) High-level expression of CSPP-L/Ub BiFC vectors shows CSPP-L and Ub interaction is confined to the centriolar satellites, despite strong detection of CSPP-L at the MTs in HEK293T cells. Region of interest (ROI) 1 shows relatively high BiFC pair expression, ROI 2 shows relatively low BiFC pair expression, and ROI 3 shows no BiFC pair expression. Bar = 10 µm. High magnification of ROI bar = 2 µm.
FIGURE 6: UBR5 maintains CSPP-L at the centrosome/centriolar satellites, and is required for centriolar satellite stability. (A) Depletion of UBR5 by shRNA in HEK293T cells does not decrease CSPP-L levels. shRNA induced with 1 μg/ml doxycycline 48 h before harvest. Data summary represents results from four independent experiments; n = 6 replicates per experiment. (B) Immunoprecipitation of GFP-CSPP-L coimmunoprecipitates CSPP-L–bound lysine-48 (K48) linked Ub chains and lysine-63 (K63) linked Ub chains. Transfections performed 24 h before harvest. (C) Depletion of UBR5 (but not PCM1) causes dispersion of centrosomal CSPP-L in RPE1 cells. (D) Centrosome/centriolar satellite mask. (E) Quantitation of loss of CSPP-L/PCM1 at the centrosome according to the mask described in D. At least 30 cells were scored per sample and statistical analysis performed using two-tailed t test.
likely due to different methodologies used in each study (Shearer and Saunders, 2016). Depletion of either CSPP-L or UBR5 disrupted centriolar satellite organization (as defined by PCM1), indicating that UBR5-mediated ubiquitylation of CSPP-L promotes localization of CSPP-L to centriolar satellites. The UBR5/CSPP-L interplay is a potential regulatory mechanism controlling the timely release and activity of ciliogenesis promoting factors, including CSPP-L itself; however, further study is required to confirm this model. Further work should also examine CSPP-L interactors for other significant E3 Ub ligase enzymes, as K48-linked Ub chains detected on CSPP-L may not be generated by UBR5 and there is significant redundancy and multiplicity in the UPS (Iconomidou and Saunders, 2016).

Primary cilia are an important component of the Hh signal transduction pathway during development (Rohatgi et al., 2007; Goetz and Anderson, 2010) and autocrine Hh signaling is reactivated in some cancer types (Kubo et al., 2004; Liu et al., 2014; Ertao et al., 2016). Several studies have implicated UBR5 as a modulator of Hh signaling and a variety of model organisms with UBR5 mutations display developmental defect phenotypes. Hence, it is worth considering a potential underlying role for UBR5 in Hh signaling via the novel role in regulating ciliogenesis.

Mutations in the Drosophila orthologue of UBR5 (Hyd) display a range of severe developmental phenotypes (reviewed in Shearer and Saunders, 2016). Specifically, Hyd directly regulates Hh and Dpp signaling (Lee et al., 2002) and can also indirectly affect Hh signal transduction by regulating Ci promoter binding (Wang et al., 2014). Ubr5-null mouse embryos die at midgestation due to failure of yolk sac vascular development (Saunders et al., 2004). Coincidently, this timing corresponds to the developmental stage at which primary cilia first appear on epiblast-derived mesothelial and endothelial cells (Bangs et al., 2015). Conditional deletion of Ubr5 in the early embryonic limb-bud mesenchyme of mice resulted in decreased Hh ligand production and decreased Hh pathway activity (Kinsella et al., 2016). It was not determined whether these effects on Hh signaling were direct (cell autonomous) or indirect (noncell autonomous) and so the underlying mechanism for this effect remains elusive. UBR5 has not been shown to directly bind Hh pathway components in human cells apart from GLI2 (Moncrieff et al., 2015); however, multiple Hh pathway components including PTCH1, GLI1, and HHIP are direct transcriptional targets of active Hh signaling (Gupta et al., 2010). It cannot be excluded that a reduction in general Hh activity with reduced UBR5 may indicate a more general disruption in signal transduction caused by failed ciliogenesis.

From a disease perspective, UBR5 has not been specifically examined in the context of ciliopathies. Rare UBR5 missense mutations have been linked to familial epilepsy (Kato et al., 2007) and cilia have been implicated in some forms of epilepsy (Delgado-Escueta, 2007), but this link is speculative. Exome sequencing data from the Exome Aggregation Consortium (ExAC) demonstrates a very low loss of function (LoF) mutation rate for UBR5 in healthy somatic tissue. Only four LoF UBR5 variants were observed, with a very low frequency (Lek et al., 2016). These indicate strong selective pressure against deactivating UBR5 mutations and support a critical role for UBR5 in human development, consistent with observations in model organisms.

Correlated expression of CSPP1 and UBR5 mRNA in human cancer cell lines and primary breast cancer gives additional relevance of CSPP-L and UBR5 in normal and transformed mammary epithelium. However, further work is required to confirm whether UBR5/CSPP-L signaling is mechanistically responsible for cilia maintenance, and to understand the putative impact of epithelial lineage-specific expression of CSPP1 isoforms (which cannot be distinguished by mRNA expression data) and their coexpression and interaction with UBR5 (Sternemalm et al., 2014). It is interesting to note that ciliary Hh signaling controls branching morphology of mammary gland xenografts (McDermott et al., 2010) and the Hh signaling pathway is a growth-promoting factor for breast cancer subgroups (Kubo et al., 2004; O’Toole et al., 2011). Future analysis may focus on determining putative correlations between UBR5 controlled cilia formation and mammary epithelial cell differentiation and transformation.

In summary, we have demonstrated a highly novel role for the E3 Ub ligase UBR5 in primary cilia maintenance/formation, with potential implications for understanding the molecular basis of key signaling pathways in development and disease.

MATERIALS AND METHODS

Gene expression correlation

Global gene expression correlation in the NCI-60 panel of cancer cell lines (n = 60; Stinson et al., 1992) was analyzed using the Pattern comparison tool from CellMiner (Reinhold et al., 2012). Expression intensity Z-scores for 26,065 genes were correlated against UBR5 expression based on Affymetrix microarray transcript intensity level and significance determined using Pearson’s correlation coefficient with p < 0.05 without multiple comparisons. Linear-regression analysis was performed comparing intensity Z-scores for UBR5 and CSP1 expression using data obtained from the Cross-correlations tool from CellMiner. Coexpression data was validated using TCGA (Ciriello et al., 2015), CCCLE (Barretina et al., 2012), and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC; Curtis et al., 2012) cohorts. Analyses were performed using cBioPortal (Cerami et al., 2012; Gao et al., 2013). Coexpression measured on mRNA expression determined using RNA-seq data (Z-score, threshold +/-2.0). Analysis of genes coexpressed with CSPP1 in 1671 datasets representing all major cancer types was performed using the SEEK database (Zhu et al., 2015b).

Plasmids

Full-length UBR5 ORF was obtained from pEGFP-C1 EDD (K37190; Addgene, Cambridge, MA; Henderson et al., 2002) and used to create pENTR221-UBR5 (Addgene; #81062). UBR5 cDNA was digested from pEGFP-C1 EDD and was cloned into pENTR221 containing synthetic fragments (GeneArt, Life Technologies, Carlsbad, CA) of the 5’ and 3’ region (base pairs 1–1129 and 6933–8404, respectively) of UBR5. Mutagenesis of UBR5 was achieved by subcloning synthetic fragments (GeneArt, Life Technologies) of the HECT domain (base pairs 6933–8404) with a mutation (base pairs 8302g and 8303c) corresponding to C2768A (UBR5C2768A, herein HECT*; Addgene; #81065), the UBR5 MLLE domain (Kozlov et al., 2007) (base pairs 6933–8404) with mutations (base pairs t7204g, a7205c, t7206c, a7243g, a7244c, and a7245c) corresponding to Y2402A and K2415A (UBR5Y2402A/K2415A, herein MLLE*; Addgene; #81064), and the UBR5 domain (base pairs 3163–4217) with a mutation (base pair g3704t) corresponding to W1235L (UBR5W1235L, herein UBR*; Addgene; #81063). A Gateway entry vector encoding full-length CSPP-L was generated by PCR using modified flanking primers and the previously described pCSPP-L-EGFP vector (Patzek et al., 2006).

Gateway entry vectors were used to generate expression clones using the following destination vectors: Vivid Colors pcDNA 6.2/N-EmGFP-DEST (V356-20; Invitrogen, Carlsbad, CA), BifC vectors pDEST-V1-ORF (Addgene; #73635), and pDEST-V2-ORF (Addgene; #73636; Croucher et al., 2016). GFP-UBR5 (Addgene; #52050) and GFP-UBR5 HECT* (Addgene; #52051) expression vectors are described (Gudjonsson et al., 2012). V1-Ub and V2-Ub expression vectors are described (Lee et al., 2015). Recombination was
catalyzed by Gateway LR clonase II enzyme mix (11791-020; Invitrogen) according to manufacturer’s instructions.

shRNA sequences to UBR5 were obtained from the RNAi codex project (Olson et al., 2006). Hairpins were cloned into pEN_TmiR3 (Addgene; #25748), before shutting into pSLIK Gateway compatible expression vectors encoding Venus (Addgene; #25734) or G418 (Addgene; #25735) selectable markers as described (Shin et al., 2006). The hairpin sequence 5’-CGAGTAGTAATGATTCAGAAA-3’ (HP_6400, herein shUBR5; Addgene; #81066) was found to efficiently silence UBR5 and was used for experiments. A scrambled sequence 5’-TCGATGTCTAAGGTCTATC-3’ (herein shNT; Addgene; #81067) was used as a nontargeting control. pLV-CCN-H2B-mCherry was a kind gift from Marc Giry-Laterriere (Kinghorn Cancer Centre).

PAGE and immunoblot
All lysates were made using RIPA buffer supplemented with protease inhibitors (1183617001; Roche Diagnostics, Basel, Switzerland), and 10 mM N-ethylmaleimide (E3876-5G; Sigma-Aldrich, St. Louis, MO). Where indicated, denaturing lysis was performed using freshly prepared 8 M Urea lysis buffer supplemented with 10% glycerol (vol/vol), 20% SDS (wt/vol), 5 mM dithiothreitol, and 10 mM Tris (pH 6.8). Cultured cells were washed with phosphate-buffered saline (PBS) and scraped from culture vessels in the presence of lysis buffer on ice. Lysates were cleared by centrifugation at 4°C and the total protein concentration determined using protein assay dye reagent (500-0006; Bio-Rad, Hercules, CA) according to manufacturer’s instructions. Denaturing lysates were sonicated before clearing. Samples were separated using SDS-PAGE, transferred to Immobilon-P PVDF 0.45 µm membrane (IPVH00010; Merck Millipore, Darmstadt, Germany) and subsequently immunoblotted using standard procedure. Densitometry was performed using ImageJ. Lane density was plotted and relative band intensity determined by area under curve analysis. Densitometry was restricted to a comparison of lanes from the same exposure and run on the same gel. Intensity was normalized to loading control and was standardized to the first lane of each gel. The theoretical size of fusion proteins was calculated using the Compute pI/Mw tool available on the ExPASy server (Bjellqvist et al., 1993) via the average resolution setting.

The following antibodies were used for immunoblotting: goat anti-EDD N-19 (sc-9561; Santa Cruz, Dallas, TX) diluted 1:5000; rabbit anti-EDD1 (A300-573A; Bethyl Laboratories, Montgomery, TX) diluted 1:5000; rabbit anti-c-SSP1 (binds CSPP-L only; 11931-1-AP; ACR110PT; Acris-Antibodies) diluted 1:10,000; rabbit anti-ubiquitin (MMS-118P; Covance, Princeton, NJ) diluted 1:5000; mouse anti-GFP (11814460001; Roche Diagnostics) diluted 1:5000; rabbit anti-CSPP1 (binds CSPP-L only; 11931-1-AP; MMS-118P; Covance, Princeton, NJ) diluted 1:5000; rabbit anti-EDD N-19 (sc-9561; Santa Cruz, Dallas, TX) diluted 1:5000; rabbit anti-ubiquitin (linkage-specific K48; ab140601; Abcam) diluted 1:5000; rabbit anti-ubiquitin (linkage-specific K63; ab179434; Abcam) diluted 1:5000; rabbit anti-α-tubulin (T3320; Sigma-Aldrich) 1:5000; mouse anti-γ-tubulin (T6557; Sigma-Aldrich) diluted 1:5000; and rabbit anti-CEP290 (ab85728; Abcam) diluted 1:2000. All antibodies were diluted in 5% (wt/vol) bovine serum albumin Tris-buffered saline solution.

Immunoprecipitation
GFP-tagged fusion proteins were isolated from whole cell extract (WCE) using GFP-Trap (GTA-100; Chromotek, Planegg-Martinsried, Germany) affinity purification reagent according to manufac-
turer’s instructions. In brief, 20 µl of bead slurry was washed twice in 10 mM Tris-HCl (pH 7.5) with 150 mM NaCl and 0.5 mM EDTA before addition of 250 µg WCE. Samples were incubated for 1 h at room temperature with gentle end-over-end mixing. Beads were washed twice, and bound proteins eluted by heating at 95°C for 10 min in 1x SDS gel loading dye.

Cell culture
The following cell lines were cultured at 37°C with 5% CO2 and passage according to American Type Culture Collection (ATCC, Manassas, VA) recommendations. Cell line identity was verified using standard in-house authentication. hTERT-RPE1 (CRL-4000; ATCC) human hTERT immortalized retina pigmented epithelial cells were maintained in DMEM-F12 culture medium (31331-028; Life Technologies) supplemented with 10% fetal bovine serum (FBS; vol/vol; 10270-106; Life Technologies) and 1% penicillin-streptomycin solution (vol/vol; P4333; Sigma-Aldrich). Human embryonic kidney (HEK293T; CRL-3216, ATCC) cells were grown using DMEM culture medium (11995-065; Life Technologies) supplemented with 10% FBS (vol/vol), MEM nonessential amino acids (11140-050; Life Technologies), and sodium pyruvate (11360-070; Life Technologies). MDA-MB-231 (HTB-26; ATCC) human mammary carcinoma cells were grown with RPMI 1640 culture medium (11875-085; Life Technologies) supplemented with 10% (vol/vol) FBS, 10 mM HEPES buffer (15630-080; Life Technologies), and 0.2 IU/ml human insulin (Actrapid Penfill; Novo Nordisk). Inhibition of proteasomal degradation was achieved where indicated using 10 µM MG-132 (474790-1MG; Calbiochem) in complete growth media before harvest.

HEK293T cells expressing H2B-mCherry were generated by stable transduction of pLV-CCN-H2B-mCherry viral supernatant. Cells expressing H2B-mCherry were grown under 500 µg/ml G418 selection and sorted for moderate expression as described (McCloy et al., 2014). HEK293T cells expressing a short hairpin to UBR5 (shUBR5) or a nontargeting scrambled control (shNT) were generated by stable transduction of pSLIK (see above) viral supernatant. Viral transduction to achieve minimal multiplicity of infection (MOI) performed visually in serial dilution as described (Shearer and Saunders, 2015). Sorting was performed by Garvan Flow Cytometry Facility staff.

Plasmid transfections were performed using Xtreme Gene 9 HP transfection reagent (06366236001; Roche). Cells were plated out 24 h before achieving roughly 50% confluency at the time of transfection. Plasmid DNA was mixed with transfection reagent in a 1:3 (µg DNA:µl reagent) ratio, diluted in a total volume of 100 µl Opti-Mem I reduced serum media (31985-070; Life Technologies). Complexes were incubated for 15 min at room temperature before being added to cells with complete growth media. Transient gene silencing in HEK293T cells using siRNA was performed using lipofectamine 2000 (11668019; Life Technologies) according to manufacturer’s instructions. 5 × 105 cells were plated in a six-well plate 24 h before transfection. siRNA (25 pmol) was combined with 7 µl transfection reagent diluted in 100 µl total volume. After plating, cells were subject to imaging for proliferation assay (see below) before harvest and lysis at 24–48 h as indicated. Epifluorescence imaging was performed using a Leica DMS50 microscope.

Ciliogenesis assay
Cells (5 × 105) were seeded on high precision glass coverslips (0.17 ± 0.01 mm; 1014/10; Hecht Assistent, Sondheim/Rhön, Germany) in 30-mm wells, allowed to adhere overnight, and transfected with siRNA using RNAiMAX (13778-150; Life Technologies) for transient gene silencing. Posttransfection (48 h) cells were washed twice in serum-free DMEM-F12 and cilia formation induced by continued
incubation in serum-free medium for 48 h. For cilia detection, cells were fixed in methanol (−20°C) and stained subsequently with a glutamylated tubulin–specific mouse monoclonal antibody (clone GT335; AG-20B-0020-C100; Adipogen San Diego, CA) and a mouse-specific Cy3 labeled secondary antibody (715-165-151; Jackson Immuno Research, West Grove, PA) for labeling of ciliary axonemes. At least 150 cells were scored by manual inspection on an Axioimager.Z1 epifluorescence microscope equipped with a 40×/NA 0.95 and a 63×/NA 1.4 Plan-Apochromat lens and a HXP120 Metal-Halide Illuminator (Carl Zeiss, Jena, Germany).

**Immunofluorescence microscopy**

Cells were grown on heat-sterilized cover glasses (0.17 ± 0.01 mm; 1014/10; Hecht Assistant), fixed for 15 min in 1% neutral buffered formalin solution at room temperature before postfixation in methanol (−20°C). Cells were rehydrated for IFM staining by three consecutive washes in PBS and blocked and permeabilized for 15 min in PBS-AT (PBS containing 5% wt/vol bovine serum albumin [A4503; Sigma-Aldrich] and 0.1% vol/vol Triton X-100 [T9284; Sigma-Aldrich]). Cells were stained with primary antibodies for 2 h at room temperature, washed three times in PBS, and stained with secondary antibodies for 1 h. All antibody incubations were performed in PBS-AT. Cells were washed three times in PBS, counterstained for DNA (Hoechst 33258 in PBS; 14530; Sigma-Aldrich), washed briefly in distilled water, dried, and mounted on object glasses using Prolong Gold (P36930; Life Technologies). Fluorescence images were acquired using appropriate optical filters on a multifluorescent bead calibrated Axioimager Z1 ApoTome microscope system (Carl Zeiss) equipped with a 100× or a 63× lens (both PlanApo, NA 1.4) and an AxioCam MRm camera. To display the entire cell volume, images are presented as maximal projections of z-stacks using Axiosvision 4.8.2 (Carl Zeiss).

Images for quantitative IFM imaging were acquired on a multifluorescence submicron beads calibrated CellObserver microscope system (Carl Zeiss) equipped with a 40×/1.3 PlanApo Phase 3 lens and an AxioCam MRm camera. Images were acquired with constant exposure times at 10 random positions per coverslip and in seven optical sections at 0.5 μm distance, centered around focal planes for cilia. Central focal planes were identified by γ-tubulin labeling as centrosome reference using a contrast-based autofocus routine (AxioVision 4.8.2). Image analysis was performed in Fiji/ImageJ (Schindelin et al., 2012). Sum projections of individual channels were background corrected using a 5 pixel rolling circle algorithm and segmented by signal intensity and morphological thresholds. Thresholded γ-tubulin signals defined the centrosomal compartment mask. The radius of the centrosomal area was iteratively dilated (20×) to cover the pericentrosomal area, and subtracted for the core centrosomal area to create the centriolar satellite mask. Fluorescence signal intensities in thresholded areas under each mask were measured in all channels to obtain integrated signal intensities.

The following antibodies were used for immunofluorescence: rabbit anti-PCM1 (ab72443; Abcam) diluted 1:1000; mouse anti-γ-tubulin (T6557; Sigma-Aldrich) diluted 1:500; rabbit anti-CSPP1 (binds CSPP-L only; 11931-1-AP; Proteintech) diluted 1:500; and mouse anti-glutamylated tubulin (GT335; Enzo Life Sciences) diluted 1:500. All statistical analyses were performed using Prism (Graphpad Software).

**BiFC**

BiFC allows fluorescence visualization of binary protein–protein interactions (Kerppola, 2008). Proteins of interest were expressed fused to either Met4-Gln157 (V1) or Lys158-Lys232 (V2) separated by a 2x GGGGS linker sequence (Croucher et al., 2016). Recombination of the Venus fluorescent protein indicates a positive interaction. A total of 5 × 10⁴ HEK293T cells (containing stable H2B-mCherry) were plated in a six-well plate containing a round coverslip 24 h before transfection. Cells were transfected with 500 ng of each plasmid construct (see above) and incubated for 18 h before coverslips were mounted with Vectashield mounting medium (H-1400; Vector Laboratories, Burlingame, CA). Remaining cells were harvested for immunoblot analysis to ensure correct fusion protein translation. Confocal imaging was performed using a Leica TCS SP8 confocal microscope optimized for at least 75 nm resolution. Gain and resolution were maintained across all samples within experiments. Fluorescent images were pseudocolored with an appropriate lookup table (LUT) and merged using ImageJ.

**Flow cytometry**

HTERT RPE1 cells (2 × 10⁴) were seeded in 60-mm dishes and reverse transfected with siRNA using lipofectamine RNAiMAX (13778-150; Invitrogen). Asynchronous samples were harvested 48 h post-transfection, while serum-starved samples were washed three times with serum-free medium and starved 48 h before harvest 96 h post-transfection. Cells were stained with Pacific Orange (P30253; Invitrogen) for live/dead labeling before ice-cold methanol fixation. Mitotic cells were labeled using antibody against phospho-histone H3 (06-570; Merck) and a rabbit-specific R-phycocerythrin (PE) antibody (P2771MP; Life Technology) secondary. DNA were stained using Fx-Cycle FarRed (F10348; Invitrogen) and RNaseA (12091-021; Invitrogen). Samples were run on a LSRII flow cytometer with BD FACSDiva software and further analyzed using FlowJo v10 (BD Biosciences, San Jose, CA).

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**REFERENCES**

Akizu N, Silhavy JL, Rosti RO, Scott E, Fenstermaker AG, Schroth J, Zaki MS, Sanchez H, Gupta N, Kabra M, et al. (2014). Mutations in CSPP1 lead to classical Joubert syndrome. Am J Hum Genet 94, 80–86.

Aisiedu M, Wu D, Matsumura F, Wei Q (2009). Centrosome/spindle pole-associated protein regulates cytokinesis via promoting the recruitment of MyoGEF to the central spindle. Mol Biol Cell 20, 1428–1440.

Bangs FK, Schrode N, Hadjantonakis AK, Anderson KV (2015). Lineage specificity of primary cilia in the mouse embryo. Nat Cell Biol 17, 113–122.

Barretta J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehr J, Kryukov GV, Sonkin D, et al. (2012). The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483, 603–607.

Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez JC, Frutiger S, Hochstrasser D (1993). The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis 14, 1023–1031.

Boldt K, van Reeuwijk J, Lu Q, Koutroupas K, Nguyen TM, Tesier Y, van Beersum SE, Horn N, Willer JR, Mans DA, et al. (2016). An organelle-specific protein landscape identifies novel diseases and molecular mechanisms. Nat Commun 7, 11491.

Cajanek L, Glatter T, Nigg EA (2015). The E3 ubiquitin ligase Mib1 regulates Plik4 and centrosome biogenesis. J Cell Sci 128, 1674–1682.
Callaghan MJ, Russell AJ, Woollat E, Sutherland GR, Sutherland RW, Watts CK (1998). Identification of a human HECT family protein with homology to the Drosophila tumor suppressor gene hyperplastic discs. Oncogene 17, 3479–3491.

Cerami E, Gao J, Dogrusoz U, Gross BE, Umer S, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larson E, et al. (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomi- cks data. Cancer Discov 2, 401–404.

Ciechanover A, Hod Y, Hershko A (1978). A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. Biochem Biophys Res Commun 81, 1100–1105.

Ciriello G, Gatza ML, Beck AH, Wilkerson MD, Rhie SK, Pastore A, Zhang H, McLellan M, Yau C, Kadooth C, et al. (2015). Comprehensive molecular portraits of invasive breast cancer. Cell 163, 506–519.

Clancy JL, Henderson MJ, Russell AJ, Anderson DW, Bova RJ, Campbell IG, Choong DY, Macdonald GA, Mann GN, Nolan T, et al. (2003). EDD, the human orthologue of the hyperplastic discs tumour suppressor gene, is amplified and overexpressed in cancer. Oncogene 22, 5070–5081.

Croucher DR, Iconomou M, Hastings JF, Kennedy SP, Han JZ, Shearer RF, Clancy JL, Henderson MJ, Russell AJ, Anderson DW, Bova RJ, Campbell IG, Choong DY, Macdonald GA, Mann GN, Nolan T, et al. (2003). EDD, the human orthologue of the hyperplastic discs tumour suppressor gene, is amplified and overexpressed in cancer. Oncogene 22, 5070–5081.

Croucher DR, Iconomou M, Hastings JF, Kennedy SP, Han JZ, Shearer RF, Clancy JL, Henderson MJ, Russell AJ, Anderson DW, Bova RJ, Campbell IG, Choong DY, Macdonald GA, Mann GN, Nolan T, et al. (2003). EDD, the human orthologue of the hyperplastic discs tumour suppressor gene, is amplified and overexpressed in cancer. Oncogene 22, 5070–5081.

Croucher DR, Iconomou M, Hastings JF, Kennedy SP, Han JZ, Shearer RF, Clancy JL, Henderson MJ, Russell AJ, Anderson DW, Bova RJ, Campbell IG, Choong DY, Macdonald GA, Mann GN, Nolan T, et al. (2003). EDD, the human orthologue of the hyperplastic discs tumour suppressor gene, is amplified and overexpressed in cancer. Oncogene 22, 5070–5081.

Croucher DR, Iconomou M, Hastings JF, Kennedy SP, Han JZ, Shearer RF, Clancy JL, Henderson MJ, Russell AJ, Anderson DW, Bova RJ, Campbell IG, Choong DY, Macdonald GA, Mann GN, Nolan T, et al. (2003). EDD, the human orthologue of the hyperplastic discs tumour suppressor gene, is amplified and overexpressed in cancer. Oncogene 22, 5070–5081.

Croucher DR, Iconomou M, Hastings JF, Kennedy SP, Han JZ, Shearer RF, Clancy JL, Henderson MJ, Russell AJ, Anderson DW, Bova RJ, Campbell IG, Choong DY, Macdonald GA, Mann GN, Nolan T, et al. (2003). EDD, the human orthologue of the hyperplastic discs tumour suppressor gene, is amplified and overexpressed in cancer. Oncogene 22, 5070–5081.
Meissner B, Kriel R, Lim RS, Rogn C, Tse K, Scott DW, Moore R, Mungall AJ, Marra MA, Conners JM, et al. (2013). The E3 ubiquitin ligase UBR5 is recurrently mutated in mantle cell lymphoma. Blood 121, 3161–3164.

Michaud EA, Yoder BK (2006). The primary cilium in cell signaling and cancer. Cancer Res 66, 6463–6467.

Mick DU, Rodrigues RB, Leib RD, Adams CM, Chien AS, Gygi SP, Nachury MUF (2015). Cytoskeleton and bioenergetics of primary cilium by proximity labeling. Dev Cell 35, 497–512.

Moncieff N, Moncan M, Scalpi F, Ditzel M (2015). Regulation of hedgehog ligand expression by the N-end rule ubiquitin-protein ligase hyperplastic discs and the Dro sophila GSK3β homologue, Shaggy. PLoS One 10, e0136760.

Munoz-Escobar J, Matta-Camacho E, Kozlov G, Gehring K (2015). The MURF domain of the ubiquitin ligase UBR5 binds to its catalytic domain to regulate substrate binding. J Biol Chem 290, 22841–22850.

Niehrs C, Pellet N (1999). Synexression groups in eukaryotes. Nature 402, 483–487.

O’Brien PM, Davies MJ, Scorry JP, Smith AN, Barton CA, Henderson MJ, Saunders DN, Gloss BS, Patterson KI, Clancy JL, et al. (2008). The E3 ubiquitin ligase EDD is an adaptive prognostic factor for serious epithelial ovarian cancer and modulates cisplatin resistance in vitro. Br J Cancer 98, 1085–1093.

Olson A, Sheth N, Lee JS, Hannon G, Sachidanandam R (2006). RNAi: a portal/database for short-hairpin RNA (shRNA) gene-silencing constructs. Nucleic Acids Res 34, D153–D157.

Orłowski RZ, Stinchcombe TE, Mitchell BS, Baldwin AS, Stahl S, et al. (2002). Phase I trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies. J Clin Oncol 20, 4420–4427.

Ottoa SA, Machalek DA, Shearer RF, Millar EK, Nair R, Schofield P, McLeod D, Cooper CL, McNeil E, McFarland AF, et al. (2011). Hedgehog overexpression is associated with stromal interactions and predicts for poor outcome in breast cancer. Cancer Res 71, 6002–6014.

Passmore LA, Barford D (2004). Getting into position: the catalytic mechanism of the proteasome. Biochem Soc Trans 32, 513–525.

Patzke S, Hauge H, Sioud M, Finne EF, Sivertsen EA, Delabie J, Stokke T, Aasheim HC (2006). CSPP and CSPP-L associate with the desmosome of polarized epithelial cells and are required for cilia formation. Mol Biol Cell 21, 2555–2567.

Patzke S, Heinrichs J, Schreiber J, Kridel R, Lim RS, Rogic S, Tse K, Scott DW, Murga-Zamalloa CA, Khanna H, Doxsey S, Stokke T, Sivertsen EA, Delabie J, Stokke T, et al. (2014). Nuclear CSP1 expression defined subtypes of basal-like breast cancer. Br J Cancer 111, 326–338.

Pommier Y, Liu J, Lee JS, Hannon G, Sachidanandam R, Altschuler SJ, Stoughton RB, Wu LF, Hughes TR, Davierwala AP, Robinson MD, Shen Y, et al. (2012). CellMiner: a web-based suite of genomic and pharmaceutical databases. Nat Methods 9, 676–682. 81–83.

Scheffner M, Nuber U, Huibregtse JM (1995). Protein ubiquitination involving an E1–E2–E3 enzyme ubiquitin thioester cascade. Nature 373, 81–83.

Scheidlin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods 9, 676–682.

Serrao VH, Alessandro F, Caldas VE, Marcal RL, Pereira HD, Thiemann OH, Carratt RC (2011). Promiscuous interactions of human septins: the GTP binding domain of SEPT7 forms filaments within the crystal. FEBS Lett 583, 3868–3873.

Shaheen R, Shamseldin HE, Loucks CM, Seidahmed MZ, Ansari S, Ibrahim K, Al-Yacoub N, Davis EE, Mola NA, Szymanska K, et al. (2014). Mutations in CSP1, encoding a core centrosomal protein, cause a range of ciliopathy phenotypes in humans. Am J Hum Genet 94, 73–79.

Shearer RF, Iconomidou M, Watts CK, Saunders DN (2015). Functional roles of the E3 ubiquitin ligase UBR5 in cancer. Mol Cancer Res 13, 1532–1533.

Shearer RF, Saunders DN (2015). Experimental design for stable genetic manipulation in mammalian cell lines: lentiviruses and alternatives. Genes Cells 20, 1–10.

Shearer RF, Saunders DN (2016). Regulation of primary cilia formation by the ubiquitin–proteasome system. Biochem Soc Trans 44, 1252–1257.

Shin KJ, Wall EA, Zavadzajdan JR, Santal LA, Liu J, Hwang JI, Rebres R, Roach T, Seidahmed MZ, Ansari S, Ibrahim K, Al-Yacoub N, Davis EE, Mola NA, Szymanska K, et al. (2014). Mutations in CSP1, encoding a core centrosomal protein, cause a range of ciliopathy phenotypes in humans. Am J Hum Genet 94, 73–79.