The Cul3–KLHL21 E3 ubiquitin ligase targets Aurora B to midzone microtubules in anaphase and is required for cytokinesis

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Cul3 (Cullin3)-based E3 ubiquitin ligases recently emerged as critical regulators of mitosis. In this study, we identify two mammalian BTB (Bric-a-brac–Tramtrack–Broad complex)-Kelch proteins, KLHL21 and KLHL22, that interact with Cul3 and are required for efficient chromosome alignment. Interestingly, KLHL21 but not KLHL22 is necessary for cytokinesis and regulates translocation of the chromosomal passenger complex (CPC) from chromosomes to the spindle midzone in anaphase, similar to the previously described BTB-Kelch proteins KLHL9 and KLHL13. KLHL21 directly binds to Aurora B and mediates ubiquitination of Aurora B in vitro. In contrast to KLHL9 and KLHL13, KLHL21 localizes to midzone microtubules in anaphase and recruits Aurora B and Cul3 to this region. Together, our results suggest that different Cul3 adaptors nonredundantly regulate Aurora B during mitosis, possibly by ubiquitinating different pools of Aurora B at distinct subcellular localizations.

Introduction

Cell division is a highly coordinated process that ensures faithful segregation of genetic material to daughter cells. Accurate progression through the cell cycle requires ubiquitination of key regulators (Pines, 2006; Sumara et al., 2008), a process involving a cascade of three enzymes (ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3). Ubiquitinated substrates are often degraded by 26-S proteasomes (Hershko and Ciechanover, 1998; Welchman et al., 2005), whereas in other cases, ubiquitination serves as a targeting signal or regulates complex assembly (Hicke, 2001). CRLs (cullin-ring–E3 ligases) are composed of a cullin scaffold that interacts through conserved regions with substrate adaptors and the ring-finger protein Rbx1 (Petroski and Deshaies, 2005). Cul3 (Cullin3) assembles with BTB (Bric-a-brac–Tramtrack–Broad complex) domain adaptors, which bind to specific substrates via distinct protein interaction domains (Pintard et al., 2004).

Recently, we discovered that the BTB proteins KLHL9 and KLHL13 form a complex with Cul3, which is required for ubiquitination of the mitotic kinase Aurora B (Sumara et al., 2007). Aurora B is a member of the chromosomal passenger complex (CPC) together with the inner centromere protein (INCENP), Borealin/Dasra B, and survivin (Andrews et al., 2003; Ruchaud et al., 2007). Aurora B kinase activity depends on association with its coactivator INCENP and on autophosphorylation of Thr232 (Yasui et al., 2004). Importantly, the CPC is also regulated at the level of subcellular localization. In early mitosis, it localizes to centromeres, where it regulates kinetochore assembly and function and thereby plays essential roles in chromosome alignment, segregation, and the spindle assembly checkpoint (Gorbsky, 2004; Meraldi et al., 2004; Vigneron et al., 2004). After anaphase onset, the CPC accumulates at the spindle midzone and the midbody, where it ensures completion of cytokinesis (Tatsuka et al., 1998; Terada et al., 1998; Gassmann et al., 2004). Although the mechanisms of this dynamic localization are poorly understood, recent evidence
suggests a critical role for the ubiquitination of CPC components. Ubiquitination of survivin may trigger CPC binding to centromeres (Vong et al., 2005), whereas Cul3–KLHL9–KLHL13 E3 ligase–dependent ubiquitination of Aurora B may regulate recruitment of the CPC to the spindle midzone (Sumara et al., 2007). Indeed, KLHL9 and KLHL13 bind Aurora B in vivo and in vitro, and Aurora B is ubiquitinated in a KLHL9- and KLHL13-dependent manner (Sumara et al., 2007). In Xenopus laevis egg extracts, the AAA-ATPase p97 in complex with the cofactors Ufd1-Npl4 binds ubiquitinated Aurora B and may extract it from mitotic chromosomes (Ramadan et al., 2007). Therefore, it is possible that Cul3–KLHL9–KLHL13 ubiquitinates Aurora B, thereby promoting translocation of the CPC to the spindle midzone. However, the mechanism of how ubiquitination of Aurora B results in CPC translocation remains to be elucidated.

In this study, we identify two BTB proteins, KLHL21 and KLHL22, as novel regulators of mitosis. Unlike KLHL22, KLHL21 regulates CPC translocation at the onset of anaphase and is required for completion of cytokinesis. KLHL21 directly interacts with Aurora B and mediates ubiquitination of Aurora B in vitro. In contrast to KLHL9 and KLHL13, KLHL21 localizes to midzone microtubules during anaphase and targets Cul3 and Aurora B to this region. We propose that KLHL21 is a specificity factor for Cul3-dependent ubiquitination of Aurora B at the central spindle, which ensures midzone recruitment of the CPC and completion of cytokinesis.

Results and discussion

The BTB proteins KLHL21 and KLHL22 interact with Cul3 and are required for faithful cell division

To identify novel regulators and components of the Cul3–KLHL9–KLHL13 E3 ligase, we immunoprecipitated KLHL9 and KLHL13 and analyzed associated proteins by mass spectrometry (Fig. 1 A and Fig. S1 A). Both adaptors copurified the BTB proteins KLHL22 and KLHL21, a protein found to associate with KLHL12 (Angers et al., 2006). Moreover, like KLHL9, KLHL13, KLHL12, BTBD1, and BTBD2 (Sumara et al., 2007), KLHL21 and KLHL22 were present in Cul3 immunoprecipitates (Fig. 1 A and not depicted). To confirm these interactions, we generated antibodies against KLHL22 and KLHL21 (Fig. S1 B). Indeed, endogenous KLHL21 interacted with Cul3, KLHL9, and KLHL13 but not with BTBD1, BTBD2, or KLHL22 (Fig. 1 B). Furthermore, ectopically expressed KLHL21 interacted with Cul3 in a BTB domain–dependent manner (Fig. S1 C). Similar results were obtained for KLHL22 (unpublished data), indicating that KLHL21 and KLHL22 interact with Cul3 and may form distinct complexes with KLHL9 and KLHL13. These results identify KLHL21 and KLHL22 as novel adaptors for Cul3 E3 ligases in human cells.

To address possible cell cycle functions of KLHL21 and KLHL22, we performed time-lapse microscopy of HeLa cells stably expressing H2B–monomeric RFP (mRFP) and mEGFP–α-tubulin (chromatin and microtubule markers) upon down-regulation of KLHL21 or KLHL22 by siRNA. Interestingly, siRNA against KLHL21 or KLHL22 led to severe defects during mitosis. 43.0% (±5.2%) of KLHL21- and 65.3% (±9.2%) of KLHL22-depleted cells displayed a prometaphase delay (promphase–anaphase >50 min) and failed to form proper metaphase plates in comparison with control cells, in which only 14.47% (±4.7%) showed this phenotype (Fig. 1, C and D; and Videos 1–4). Similarly, Cul3 down-regulation led to an increase in cells with a prometaphase delay (31.0 ± 7%). The defect was less severe compared with KLHL21 or KLHL22 siRNA, most likely as the result of pleiotropic phenotypes caused by Cul3 depletion, including defects in the spindle assembly checkpoint (Sumara and Peter, 2007).

Although about half (47.3%) of the delayed KLHL22–down-regulated cells died in prometaphase, the remaining cells resumed mitosis and completed cytokinesis. In contrast, most of the delayed KLHL21–down-regulated cells continued cell division, but out of these, 48.8% subsequently failed to complete cytokinesis (Video 5). To confirm these observations, we used immunofluorescence (IF) microscopy to analyze cells siRNA depleted for KLHL21 or KLHL22 for 72 h. Strikingly, down-regulation of KLHL21 but not KLHL22 led to 23.8% (±1.2%) of multinucleated cells (Fig. 1 E and Fig. S1 D), which is a phenotype reminiscent of cells lacking KLHL9, KLHL13, or Cul3 (Sumara et al., 2007). These results suggest that like KLHL9 and KLHL13, KLHL21 forms a complex with Cul3 to ensure faithful mitosis and completion of cytokinesis.

KLHL21 regulates localization of the CPC in anaphase

To test whether KLHL21 down-regulation alters the localization of the CPC in anaphase, we stained cells for Aurora B. In contrast to KLHL22 down-regulation, Aurora B remained on segregating chromosomes in KLHL21-depleted cells, with only weak staining at the spindle midzone (Fig. 2, A and C). The same defects could be observed for survivin, INCENP, and Borealin (Fig. 2 B and Fig. S2, A and B), suggesting that the entire CPC fails to relocalize in anaphase. Aurora B retained on anaphase chromosomes in KLHL21-depleted cells was at least partially active, as revealed by IF using an antibody specifically recognizing Aurora B–phosphorylated histone H3 (Fig. 2 D).

To address whether a general defect in spindle midzone assembly might cause mislocalization of the CPC in KLHL21–down-regulated cells, we analyzed cells by IF using antibodies against PRC1 and anillin, two cytokinesis regulators which accumulate at the midzone or the cleavage furrow by CPC-independent mechanisms. PRC1 efficiently localized to the midzone in the absence of KLHL21 (Fig. 3, A and C), whereas KLHL22-dependent manner (Sumara and Peter, 2007). In contrast, KLHL21 was necessary for efficient midzone recruitment of the kinesin-6 family member Mklp2 (Fig. 3, C and F), supporting the notion that Mklp2 and the CPC are mutually dependent for midzone localization (Hümmer and Mayer, 2009).
Figure 1. **The BTB proteins KLHL21 and KLHL22 interact with Cul3 and regulate mitosis.** (A) Immunoprecipitation of human KLHL9 and KLHL13 was analyzed by mass spectrometry, and associated Cul3 (circle) and BTB proteins (boxes) are shown (dark gray). Immunoprecipitation of Cul3 identified KLHL12, KLHL22, and the highly homologous KLHL9 and KLHL13 and BTBD1 and BTBD2 (light gray). (B) HeLa extract was immunoprecipitated with control (anti-IgG) or anti-KLHL21 antibodies. Input, supernatants (Sup), and eluates (IP) were analyzed by immunoblotting. (C) HeLa cells expressing H2B-mRFP and mEGFP–α-tubulin were treated with the indicated siRNAs for 48 h and analyzed by live video microscopy. (D) Hela cells expressing H2B-mRFP and mEGFP–α-tubulin were treated with the indicated siRNAs for 48 h and analyzed by live video microscopy. (D) Quantification of the phenotype in C. The percentage of cells with a delay from prophase to anaphase (>50 min) was quantified (n = 50). (D) HeLa cells were treated with the indicated siRNAs for 72 h, and the percentage of multinucleated cells was quantified (n = 500). Example images are shown in Fig. S1 D. (D and E) Bars represent the mean with standard deviation of three independent experiments. Bars, 10 µm.
Collectively, these results suggest that unlike the CPC and CPC-dependent factors, other midzone components localize normally in KLHL21-depleted cells.

KLHL21, KLHL9, and KLHL13 form multiple Cul3 complexes that may independently regulate CPC localization

To investigate why multiple Cul3 adaptors are needed to regulate CPC recruitment to the spindle midzone, we compared the levels of KLHL9, KLHL13, KLHL21, and Cul3 in cells depleted for each of these components. Although siRNA against Cul3 led to an accumulation of all three adaptors, most likely by blocking an autocatalytic degradation mechanism (Wirbelauer et al., 2000), down-regulation of the individual adaptors did not change the abundance of Cul3 or the other BTB proteins (Fig. 3 G). Moreover, KLHL9 and KLHL13 interaction with Cul3 did not depend on KLHL21, and KLHL21 binding to Cul3 increased in the absence of KLHL9 and KLHL13 (Fig. 3 H). These results imply that Cul3 can assemble different complexes with KLHL9/KLHL13 and KLHL21 in vivo and that KLHL21 may compete with KLHL9 and KLHL13 for binding to Cul3. Sucrose gradient and gel filtration experiments of complexes from Sf9 insect cells and HeLa cell extracts, respectively, confirmed that KLHL21 and KLHL9 fractionate into overlapping but also distinct
Figure 3. **KLHL21 regulates midzone recruitment of Mklp2 but not PRC1 or anillin.** [A–C] HeLa cells treated with control or KLHL21 siRNA were analyzed by IF for PRC1 (A), anillin (B), or Mklp2 (C) and Aurora B. DNA was stained with DAPI. [D and F] Quantification of the relative intensities of PRC1 (D) and Mklp2 (F). The intensity on the midzone was divided by the intensity in the cytoplasm (n = 20). [E] Percentage of anaphase cells with anillin recruitment to the cleavage furrow (n = 50). In KLHL21 siRNA, only cells with mislocalized Aurora B were included. [D–F] Bars represent the mean with standard deviation of three independent experiments. (G) HeLa cells were treated with the indicated siRNAs for 72 h, and extracts were analyzed by immunoblotting. α-Tubulin was used as a loading control. (H) Extracts prepared from cells treated with control siRNA, siRNA depleting KLHL21, or siRNA depleting both KLHL9 and KLHL13 were immunoprecipitated with anti-Cul3 antibodies. Inputs and eluates (Cul3 IP) were analyzed by immunoblotting. Bars, 5 µm.
complexes (Fig. S2 C), whereas KLHL13 cofractionates with KLHL9 (Sumara et al., 2007). Finally, we could not detect differences in the timing of complex formation during mitosis (Fig. S2 D), indicating that all Cul3 complexes can exist simultaneously. However, as KLHL21 interacted with KLHL9 and KLHL13 even in the absence of Cul3 binding (Fig. S1 C), we conclude that KLHL21, KLHL9, and KLHL13 form both homo- and heterodimers in vivo (Perez-Torrado et al., 2006; Wimuttisuk and Singer, 2007). Collectively, these results suggest that KLHL21, KLHL9, and KLHL13 assemble distinct Cul3 complexes that regulate CPC localization during mitosis by non-redundant mechanisms.

KLHL21 directly binds and mediates the ubiquitination of Aurora B

We next investigated whether, like KLHL9 and KLHL13, KLHL21 binds directly to Aurora B. Indeed, endogenous KLHL21 was present in CPC complexes purified by immunoprecipitation of GFP-tagged Aurora B (Fig. 4 A). Moreover, overexpressed HA–KLHL21 but not HA–BTBD1 communoprecipitated with GFP–Aurora B (Fig. 4 B). To test whether complex formation of KLHL21 with Cul3 is required for binding to Aurora B, we generated a mutated version of KLHL21 containing amino acid substitutions in the BTB domain (D114A/L115A/Q117A; referred to as DLQ), which is in analogy to the Caenorhabditis elegans BTB protein MEL26 (Xu et al., 2003), specifically disrupts Cul3 binding. As expected, HA–KLHL21-DLQ was unable to bind Cul3 (Fig. S1 C) but communoprecipitated with GFP-tagged Aurora B (Fig. 4 B), suggesting that Aurora B interacts with the Kelch domains of KLHL21 in vivo. Indeed, recombinant Aurora B but not survivin specifically bound Escherichia coli–expressed GST-tagged Kelch domains of KLHL21 and KLHL9 (Fig. 4, C and D), supporting the hypothesis that the interaction between KLHL21 and Aurora B is specific and direct.

To test whether Cul3–KLHL21 complexes are sufficient to ubiquitinate Aurora B, we coexpressed and purified Cul3 complexes from insect cells (Fig. S3 A) and analyzed their ubiquitination activity toward Aurora B in vitro. In contrast to Cul3–KLHL22, Cul3–KLHL21 complexes ubiquitinated recombinant Aurora B (Fig. S3 B), and this activity was not increased by coexpression of KLHL9 and KLHL13. To corroborate these results and to investigate which E2 enzyme or enzymes most efficiently promote Aurora B ubiquitination, we reconstituted Cul3–Rbx1 complexes with maltose-binding protein (MBP)–tagged adaptors expressed in E. coli. Although most E2s were unable to function in this context, UbcH5a and, to a lesser extent, UbcH5b catalyzed monoubiquitinated species of Aurora B (Fig. 4 E and Fig. S3 C). Monoubiquitination on multiple sites of Aurora B was best revealed in the presence of methyl-ubiquitin (Fig. 4 F), which is defective in its ability to form chains. We conclude that KLHL21 directly interacts with Aurora B and that KLHL21 in complex with Cul3–Rbx1 and the E2 UbcH5a mediates Aurora B ubiquitination in vitro. Although it remains to be elucidated whether Aurora B is mono-ubiquitinated by Cul3 complexes in vivo, these results suggest that Cul3–KLHL21 may not target Aurora B for degradation but rather promote its relocalization during anaphase.

KLHL21 localizes to the midzone and targets Cul3 to this region

To examine why multiple Cul3 complexes are required to regulate the CPC, we compared the localization of KLHL9, KLHL13, KLHL21, and Cul3 by IF of pre-extracted mitotic cells. Interestingly, KLHL21 accumulated at the spindle midzone in early anaphase concomitant with Aurora B recruitment and remained at the midbody until abscission (Fig. 5 A). The midzone and midbody staining was specific, as it disappeared in KLHL21-depleted cells (Fig. 5 C and Fig. S3 D). Like KLHL21, Cul3 was detected at the midzone during anaphase B. Interestingly, Cul3 no longer accumulated at the midzone in cells depleted for KLHL21 (Fig. 5 B). Likewise, KLHL21 localization to the midzone was dependent on the presence of Cul3 (Fig. 5 C), suggesting that complex formation of Cul3 and KLHL21 is needed for their recruitment to the midzone. KLHL13 remained diffuse during all mitotic stages, and KLHL9 only concentrated on the midbody during telophase (Fig. 5 A). Although KLHL21 was also found on midbodies at this stage, its localization pattern was distinct from KLHL9 and Cul3, which were focused to a single site and flanked by KLHL21, which colocalized with Aurora B (Fig. S3 E). The few midbodies observed in KLHL21-depleted cells displayed significantly decreased levels of Cul3 (Fig. S3 D), which is consistent with cytokinesis failure occurring in these cells.

Together, these results suggest that differential localization of distinct Cul3 complexes with KLHL9, KLHL13, and KLHL21 may target different pools of Aurora B during mitosis. KLHL21 may function as a specificity factor to ubiquitinate Aurora B on the midzone, whereas KLHL9 and KLHL13 may mainly target the cytoplasmic pool of Aurora B at this stage. This hypothesis may explain the drastic phenotypes of Aurora B mislocalization observed upon Cul3 or KLHL21 down-regulation.

KLHL21 is a specificity factor for Cul3-mediated regulation of Aurora B during anaphase

Recent work revealed that in Xenopus extracts, the AAA-ATPase p97 in association with the ubiquitin-binding heterodimer Ufd1-Npl4 may remove ubiquitinated Aurora B from mitotic chromosomes (Ramadan et al., 2007). Although in the absence of KLHL21, KLHL9, or KLHL13, Aurora B remains on chromosomes and fails to accumulate at the spindle midzone, none of these adaptors detectably localizes to mitotic chromosomes. However, FRAP experiments suggest that Aurora B rapidly exchanges between centromeres and the cytoplasm (Murata-Hori and Wang, 2002), and Aurora B dissociation from mitotic chromosomes may require prior ubiquitination by specific Cul3 E3 ligases in the cytoplasm. Alternatively, ubiquitination of Aurora B may promote its accumulation on midzone microtubules. In the latter scenario, ubiquitin may serve as a retention signal to stabilize the microtubule-bound pool of Aurora B. Indeed, the dynamic behavior of Aurora B is faster on centromeres compared with midzone microtubules (Murata-Hori and Wang, 2002). These two models are not mutually exclusive: Aurora B ubiquitination may stabilize its association with the spindle midzone while it may at the same time serve as a removal signal on
Figure 4. **KLHL21 binds and mediates ubiquitination of Aurora B.** [A] HeLa cells expressing photoactivatable GFP (PA-GFP) or GFP–Aurora B were treated with 100 ng/ml nocodazole for 16 h followed by mitotic shake-off. Extracts were prepared and immunoprecipitated with anti-GFP antibodies. Inputs and eluates (GFP IP) were analyzed by immunoblotting. [B] HeLa cells expressing GFP–Aurora B were transfected with an empty vector or vector containing HA-KLHL21, -KLHL21-DLQ, or -KLHL21-DLQ. Extracts were immunoprecipitated with anti-GFP, and inputs and eluates (GFP IP) were analyzed by immunoblotting. [C] Recombinant GST and GST-6-Kelch-KLHL9 and –KLHL21 were incubated with recombinant Aurora B (C) or survivin (D). Mixtures were bound to glutathione Sepharose beads, and inputs and eluates (GST pd) were analyzed by immunoblotting. [E and F] Recombinant Aurora B was added to in vitro ubiquitination reactions containing GST-Cul3–Rbx1 complexes and UbcH5a. Reactions were incubated for 1 h with MBP or MBP-KLHL21, -KLHL9, or -KLHL13 and analyzed by immunoblotting. In the presence of ATP, Aurora B migrated slower because of autophosphorylation (asterisks). In F, methyl-ubiquitin was added instead of ubiquitin. Note that Aurora B was mainly monoubiquitinated (arrows) under these conditions, but multiple sites were used with lower efficiency.
Figure 5. **KLHL21 localizes to the midzone and targets Cul3 to this region.** (A) HeLa cells pre-extracted before fixation were analyzed by IF for KLHL21, Cul3, KLHL9, and KLHL13 and α-tubulin. Boxes in the telophase rows outline areas magnified in the panels below. (B and C) Cells treated with the indicated siRNAs were analyzed by IF for Cul3 (B) or KLHL21 (C) and α-tubulin. DNA was stained with DAPI. Bars, 5 µm.

Materials and methods

Cell culture

HeLa cells were grown in DME with 10% FBS, 0.2 mM Glu, 100 U/ml penicillin, and 100 µg/ml streptomycin. Double thymidine block and release experiments (Sumara et al., 2004) and HeLa cells expressing GFP–Aurora B or H2B-mRFP, mEGFP–α-tubulin or mCherry–α-tubulin, and photo-activatable mGFP were described previously (Steigemann et al., 2009). For live video microscopy, images were taken at 37°C with a 20× NA 0.8 differential interference contrast objective using a laser-scanning confocal microscope (LSM 510; Carl Zeiss, Inc.) and LSM software (Carl Zeiss, Inc.). Data were processed with the Image Browser (Carl Zeiss, Inc.). siRNA oligonucleotides used were control nonsilencing siRNA (5'-TTCTCCGAACGTGTCACGT-3'), KLHL21 siRNA (pool of 6 siRNAs: 5'-GGGCGTAAGGGCTGGGTTT-3', and 5'-TGTCATTGCTGTCGGGTTA-3'), and KLHL22 (pool of 3 siRNAs: 5'-GCAACAACGATGCCGGATA-3', 5'-CCTATATCCTCAAAAACTT-3', and 5'-GGACTGGCTCTGTGATAAA-3'), and KLHL21 [Thermo Fisher Scientific], KLHL22 (RZPD), and BTBD1 (provided by P. D’Arpa, National Institutes of Health, Bethesda, MD). The GST-6-Kelch fragment of KLHL21 encompasses aa 273–598 (full length, 1–598); the GST-6-Kelch fragment of KLHL9 was described previously (Sumara et al., 2007).

Collectively, our data suggest that KLHL21 functions as a novel specificity factor to regulate CPC localization in early anaphase. Although we cannot exclude that Cul3–KLHL21 may have additional mitotic substrates, our results are consistent with a model in which the Cul3–KLHL21 E3 ligase ubiquitinates Aurora B at the spindle midzone, thereby preventing CPC accumulation on mitotic chromosomes not only by promoting active removal but also by increased retention on midzone microtubules. Future studies are needed to identify additional components, in particular ubiquitin-interacting proteins, that play a role in this essential mitotic pathway.

Chromosomes. The resulting equilibrium shift toward microtubule binding may ultimately lead to rapid accumulation of Aurora B at spindle microtubules in early anaphase.

IF Cells were fixed in −20°C MeOH for 10 min and stained as described previously (Sumara et al., 2007). For H2B, KLHL13, KLHL21, or Cul3 staining, cells were extracted before fixation for 1 min (300 mM sucrose, 3 mM MgCl2, 1 mM EDTA, 50 mM NaCl, 25 mM Hepes, pH 7.5, and 0.5% Triton X-100). The fluorochromes used were Alexa Fluor 488 and 568. Images were captured using a 63x NA 1.4 oil differential
interference contrast objective with a microscope (Axioskop 2; Carl Zeiss, Inc.), AxioCAM Mr5c (Carl Zeiss, Inc.), and the AxioVision 4.2 software (Carl Zeiss, Inc.). Images were processed with ImageJ (National Institutes of Health).

Protein purification and in vitro assays

Baculoviruses expressing 6x His–KLHL21 or –KLHL22 were generated by L. Zhiqin and F. Sicheri (Mount Sinai Hospital, Toronto, Ontario, Canada) using the bac to bac method (Invitrogen). SF9 cells were coinected with different viruses, and assembled complexes were purified as described previously (Sumara et al., 2007). GST and GST-6-Kelch–KLH9 and –KLHL21 were purified from E. coli and used in in vitro binding assays together with reconstituted Aurora B (Millipore) or survivin (Prospeck) as described previously (Sumara et al., 2007). MBP and MBP-KLHL21, –KLH9, and –KLHL13 were purified from E. coli analogous to GST purification using amnylose resins (New England Biolabs, Inc.).

For in vitro ubiquitination assays, either 1 µg of in vitro neddylated, SF9-purified Cul3–Rbx1–adapter complex or SF9-purified Cul3–Rbx1 mixed with 2 µg MBP and MBP-KLHL21, –KLH9, and/or –KLHL13 purified from E. coli was used as E3 ligase. The reaction was incubated for 1 h at 30°C with 0.06 µg Aurora B (US Biological), 15 µg ubiquitin or methyl-ubiquitin, 550 ng rabbit E1 [UBE1], 850 ng E2, 12.5 mM Mg-ATP, and 1x of ubiquitination reaction buffer. Reagents were purchased from Boston Biochem.

Immuno precipitation, liquid chromatography–tandem mass spectrometry, and fractionation experiments

Antibodies were crosslinked to Affipur prep protein A beads (BioRad Laboratories) at 1 µg of antibodies/1 µl of beads. HA and GFP immunoprecipitations were performed with anti-HA agose (Sigma-Aldrich) or GFP-Trap_A (ChromoTek). For mass spectrometry analysis, KLH9- and KLHL13-containing complexes were eluted in SDS buffer, fractionated by SDS-PAGE, tryspin digested, and analyzed by liquid chromatography–tandem mass spectrometry as described previously (Grill et al., 2007). For protein identification, the UniProt database release 7.4 was used, and Scaffold (Proteome Software, Inc.) was used for probability-based validation with a threshold at 95% (Keller et al., 2002; Nesvizhskii et al., 2003). Proteins detected in the IgG-negative control were subtracted.

Cell fractionation, chromatography of Hela 100,000 g fractions was performed using a Superdex 200 column (GE Healthcare). 500-µl fractions were collected and tested by immunoblotting. Marker proteins (albunin 67 kD, catalase 232 kD, ferritin 440 kD and thyroglobulin 669 kD) were observed using the bac to bac method (Invitrogen). Sf9 cells were coinfected with E. coli Sf9-purified Cul3–Rbx1–adaptor complex or Sf9-purified Cul3–Rbx1 mixed with 550 ng rabbit E1 (UBE1), 850 ng E2, 12.5 mM Mg-ATP, and 1x of ubiquitination reaction buffer. Reagents were purchased from Boston Biochem.

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