A Motif from Lys$^{216}$ to Lys$^{222}$ in Human BUB3 Protein Is a Nuclear Localization Signal and Critical for BUB3 Function in Mitotic Checkpoint*

Received for publication, November 19, 2014, and in revised form, March 25, 2015. Published, JBC Papers in Press, March 26, 2015, DOI 10.1074/jbc.M114.598029

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Background: The amino acid residues in BUB3 that determine its localization remain unknown.

Results: BUB3 has a nuclear localization signal (NLS) involved in its nuclear and kinetochore localization.

Conclusion: BUB3 localization is regulated by its NLS and independent of CENP-A.

Significance: This study helps to dissect the role of BUB3 in mitotic checkpoint signaling.

Human BUB3 is a key mitotic checkpoint factor that recognizes centromeric components and recruits other mitotic checkpoint molecules to the unattached kinetochore. The key amino acid residues responsible for its localization are not yet defined. In this study, we identified a motif from Lys$^{216}$ to Lys$^{222}$ in BUB3 as its nuclear localization signal. A BUB3 mutant with deletion of this motif (Del216–222) was found to localize to both the cytoplasm and the nucleus, distinct from the exclusively nuclear distribution of wild-type BUB3. Further analysis revealed that residues Glu$^{213}$, Lys$^{216}$, Lys$^{217}$, Lys$^{218}$, Tyr$^{219}$, and Phe$^{221}$, but not Lys$^{222}$, contribute to nuclear localization. Interestingly, the nuclear localization signal was also critical for the kinetochore localization of BUB3. The deletion mutant Del216–222 and a subtle mutant with four residue changes in this region (E213Q/K216E/K217E/K218E (QE)) did not localize to the kinetochore efficiently or mediate mitotic checkpoint arrest. Protein interaction data suggested that the QE mutant was able to interact with BUB1, MAD2, and BubR1 but that its association with the centromeric components CENP-A and KNL1 was impaired. A motif from Leu$^{61}$ to Leu$^{65}$ in CENP-A was found to be involved in the association of BUB3 and CENP-A in cells; however, further assays suggested that CENP-A does not physically interact with BUB3 and does not affect BUB3 localization. Our findings help to dissect the mechanisms of BUB3 in mitotic checkpoint signaling.

*This work was supported by grants from the National Natural Science Foundation of China and the Ministry of Science and Technology (Grants 31171432, 2011CBA01100, 91219305, 31210103905, 31371510, 31101061, 81170499, 31201107, 31301208, 81201599, 2011CB956100, 2012CB966603, and 2013CB967600) and the Science and Technology Commission of Shanghai Municipality (Grants 11ZR143800 and 12ZR1450900).

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Cells use surveillance mechanisms, such as a mitotic checkpoint or spindle assembly checkpoint, to maintain the integrity of the genome (1). The mitotic checkpoint fulfills this function by monitoring the interaction between the chromosomal kinetochore and spindle microtubules (2). When the cell enters the mitotic phase of the cell cycle, the mitotic checkpoint is activated by kinetochores unattached to spindle microtubules (even a single unattached kinetochore can activate the checkpoint). When every kinetochore is attached to spindle microtubules and bi-orientation of sister chromatids is established, the mitotic checkpoint is satisfied, its signaling is silenced, and chromosome segregation proceeds (3). Prolonged mitotic checkpoint signaling activates cell death, which thus helps to eliminate the generation of aneuploid cells. The mitotic checkpoint also ensures the reductional division of chromosomes during meiosis (4–7). Defects in mitotic checkpoint signaling resulting from a dysregulation of the expression, modification, and localization of its components have been suggested to cause cancer, miscarriages, and birth defects (8–11).

Mitotic checkpoint components include the budding uninhibited by benzimidazole (BUB) genes BUB1 and BUB3; the mitotic arrest defective (MAD) genes MAD1, MAD2, and MAD3; HEC1 (highly expressed in cancer protein); and MPS1 (monopolar spindle 1 kinase, also known as TTK). Mitotic checkpoint proteins localize to kinetochores unattached to the spindle microtubule, and they assemble at the kinetochore as an active complex. The complex converts MAD2 to its inhibitory form, which binds to and inhibits CDC20, the activator subunit of the anaphase-promoting complex (APC). APC is an E3 ubiquitin ligase that targets cyclin B1, securin, and other mitotic proteins. Once APC is inhibited by MAD2, degradation of these proteins is blocked, the cell cycle progression to anaphase halts, and the initiation of premature chromosome segregation is prevented.

The distribution and localization of mitotic checkpoint components during the cell cycle are dynamic, and the regulatory mechanisms have been of great interest (12, 13). The kinase...
MPS1 predominately localizes to the cytosol during interphase of the cell cycle and relocates from the cytosol to the nucleus at the G2/M-phase boundary prior to nuclear envelope breakdown (14, 15). In both yeast and mammalian cells, Mad1 and Mad2 localize to the nuclear pore complex during interphase. Upon mitotic checkpoint activation, Mad2 (but not Mad1) is released from the nuclear pore complex and accumulates on kinetochores (16). Most of the mitotic checkpoint components are kinetochore dynamic proteins, and they localize to the kinetochore hierarchically (17–20). In yeast, Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F, and Mad2 (20). MPS1 is required for the recruitment of Mad1 and Mad2 to kinetochores, and the kinetochore localization of MPS1 itself depends on the calponin homology domain of HEC1 (21). Constituents of the kinetochore are also critical for mitotic checkpoint proteins to localize to the unattached kinetochore. It is thought that certain exposed kinetochore components are subjected to phosphorylation by a mitotic kinase, such as MPS1 or the Aurora kinases, and that these events further recruit mitotic checkpoint signaling components to assemble into a large complex at kinetochores (22–24). The centrrome-specific variant of histone H3, CENP-A, is required for sustained kinetochore association of BubR1 under conditions of checkpoint activation (25).

Human BUB3 is an essential component for the mitotic checkpoint. It has been demonstrated that BUB3 enrichment at the unattached kinetochore is essential for BUB1 and BubR1 to localize to the kinetochore (26). Biochemical studies suggested that BUB3 interacts with BUB1, BubR1, MAD2, and CDC20 through its WD40 repeat (27–29). In Saccharomyces cerevisiae, it was reported that Bub3 recognizes multiple MPS1-phosphorylated Met-Glu-Leu-Thr (MELT) motifs of Spc105/Knl1 (also known as CASC5, Blinkin, and AF15Q14 and referred to as Spc105 in yeast) (30). Human BUB3 shares 34% identity and 69% similarity with the S. cerevisiae protein Bub3 throughout the entire length of the protein (26). There are significant differences between human BUB3 and yeast Bub3. The domain(s) or amino acid residues in human BUB3 that are responsible for recognizing the kinetochore component have not been defined to date (31).

We previously reported the regulation of BUB3 expression by bone morphogenetic protein (BMP)/TGF-β signaling and MPS1 translocation (14, 32). We are interested in the mechanism by which the localization of human BUB3 is regulated. In this study, we report the identification of a motif in BUB3 that is required for its nuclear and kinetochore localization and also a region in the kinetochore component CENP-A that mediates BUB3/CENP-A interaction.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**—HeLa and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Life Technologies) at 37 °C in a humidified incubator under 5% CO₂. The medium was supplemented with 10% fetal bovine serum, 100 units/ml streptomycin, and 100 units/ml penicillin. For nocodazole or Taxol treatment, cells were treated with 100 ng/ml nocodazole or 1 μM Taxol for the indicated times.

**Plasmids, Transfections, and Virus Generation**—Two siRNA sequences against human BUB3 were #1 UGACGCAUUUGAACCAGUGU ATT (4) and #2 AGCGACTGTGCCCAATTCATT (33), and siRNA sequence against human CENP-A was CACAGTCGGCAGACAGTT (34). DNA fragments coding for these siRNA were cloned into pLKO.1 for lentivirus generation. The lentivirus was produced according to the protocol from Addgene. BUB3 and its mutant constructs were cloned into pEGFP-C2, pEGFP-N1, and the lentiviral vector FUGW for overexpression. Mutations were made with the QuikChange kit from Agilent. All constructs and mutations were verified by DNA sequencing. DNA transfections were performed using a transfection reagent from Exlgene. Virus infection procedure was as described previously (35). For immunofluorescence staining, cells were seeded on polylysine-coated glass coverslips and stained as described previously (14). For immunofluorescence staining of mitotic cells, HeLa cells were treated with 1 μM Taxol for 4 hours and subjected to immunofluorescence staining. Stained cells or GFP-BUB3-transfected cells were observed on a Leica microscope (Leica, Tokyo, Japan). The following antibodies were used for Western blot analysis: BUB3, Abcam Ab4180 and Santa Cruz Biotechnology sc-376506; BUB1, mouse monoclonal antibody (14H5) to BUB1, Abcam Ab4636; BubR1, mouse monoclonal antibody (BG1) to BubR1, Abcam Ab4637; HEC1 mouse monoclonal clone 9G3, Abcam Ab3613; MAD2 (C-19), Santa Cruz Biotechnology SC-6329; CREST sera, Antibodies Inc.; CENP-A, Cell Signaling Technology, CST2186; KNL1, (CASIC5, K-14), Santa Cruz Biotechnology sc-242311; GAPDH Abclonal Inc.; and HRP-conjugated secondary antibodies, Cell Signaling Technology. Alexa Fluor-conjugated secondary antibodies were obtained from Invitrogen.

**RESULTS**

**Localization of Endogenous and GFP-tagged BUB3 during Both Interphase and the Mitotic Phase of the Cell Cycle**—We detected the localization of endogenous BUB3 in HeLa cells by immunostaining. The specificity of the antibody for BUB3 was confirmed by Western blotting. Two shRNAs against BUB3 (4) (shBUB3) were transfected into HeLa cells (Fig. 1A). The BUB3 antibody recognized a single band with a predicted molecular mass of 37 kDa. The intensity of the BUB3 decreased upon transfection of HeLa cells with shBUB3 (Fig. 1A). The shRNA specifically targeted BUB3 without effect on other checkpoint-related proteins, BUB1, MAD2, and CENP-A, and had very minor effects on BubR1 and KNL1, suggesting that the antibody is relatively specific for BUB3. In contrast to the cytosolic localization of MPS1 during interphase, as we reported previously (14), BUB3 localizes to the nucleus during interphase (Fig. 1B) in HeLa cells. During the mitotic phase of the cell cycle, BUB3 was found as discrete dots and was localized to...
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the kinetochores, which were visualized by CREST antiserum staining (Fig. 1C).

We next generated an N-terminal GFP-tagged BUB3 expression vector (GFPBUB3) and expressed this construct in HeLa cells (Fig. 1F). Confocal microscopy showed that in prometaphase (Fig. 1D) and prophase (Fig. 1E), GFPBUB3 was presented as discrete dots, as observed with endogenous BUB3. The kinetochore localization of GFPBUB3 was further confirmed by its co-localization with endogenous centromeric marker CENP-A protein (Fig. 1G). This observation is consistent with other studies (26, 33, 36) and suggests that GFPBUB3 recapitulated the localization behavior of endogenous BUB3 in cells.

A Motif of BUB3 Is Critical for Its Nuclear Localization—To understand the mechanism controlling the nuclear localization of BUB3, we examined its amino acid sequence and noted the presence of two interesting motifs: one containing Lys216, Lys217, Lys218, Tyr219, Ala220, Phe221, and Lys222, and a second one containing Lys266, Lys267, and Arg268. Both motifs contained tandem polar basic amino acid residues typical of a protein nuclear localization signal. We created two mutants: one with a deletion of the region from Lys216 to Lys222 (Del216–222) (Fig. 2E), and the other with the polar charged amino acid residues Lys266, Lys267, and Arg268 mutated to the polar uncharged amino acid Gln (K266Q/K267Q/R288Q) (Fig. 2A). Western blots confirmed that both mutants were intact and recognized by the BUB3-specific antibody when expressed in HeLa cells (Fig. 2E). We found that wild-type BUB3 (GFPBUB3WT) localization was exclusively nuclear (Fig. 2B), which was marked by mCherryH2B and DAPI staining. The deletion mutant Del216–222 (GFPDel216–222) was distributed in both the nucleus and the cytoplasm (Fig. 2C). The localization of the K266Q/K267Q/R288Q mutant remained the same as that of the wild-type protein (Fig. 2D).

We further examined the role of the individual amino acid residue of the deleted region in BUB3 nuclear localization. We made three mutants with the individual point mutations (Y219A, F221A, and K222A) and a QE mutant (Fig. 3A) in which the polar acidic residue glutamic acid was mutated to the polar uncharged residue glutamine and three of the polar basic residue lysines were changed to glutamic acid residues. All mutants were GFP-tagged. After expression in HeLa cells (Fig. 3F), we found that, with the exception of K222A, all of the mutants were cytoplasmically distributed in both the nucleus and the cytoplasm (Fig. 3, B–E). This suggested that the nuclear localization capacity of these mutants was impaired and that residues Glu213, Lys216, Lys217, Lys218, Tyr219, and Phe221 all contribute to BUB3 nuclear localization.

The QE and Del216–222 Mutants Displayed Impaired Kinetochore Localization—BUB3 function in the mitotic checkpoint depends on its kinetochore localization. We asked whether these BUB3 mutants were still able to localize to the kinetochore. HeLa cells were transfected with GFPBUB3 or its mutants, treated with Taxol for 4 h, and then subjected to immunostaining with an antibody specific for CENP-A, which is a kinetochore marker. We found that BUB3WT colocalized very well with CENP-A (Fig. 4A). However, the kinetochore localization of both the QE and the Del216–222 mutants was impaired but not abolished (Fig. 4, B and C). This suggested that the nuclear localization signal of BUB3 is also involved in its kinetochore localization.

The QE and Del216–222 Mutants Are Unable to Mediate Mitotic Checkpoint Signaling—To further examine the role of the BUB3 nuclear localization signal in mitotic checkpoint signaling, we measured the ability of the QE and Del216–222 mutants to arrest cells at mitotic phase. We first knocked down endogenous BUB3 using the shBUB3 lentivirus and then restored the BUB3WT, QE or Del216–222 proteins in the BUB3-depleted cells. All of the overexpression plasmids were made to be resistant to shBUB3. We then activated the mitotic checkpoint with nocodazole and measured mitotic checkpoint signaling using multiple assays (Fig. 5A). The shBUB3 virus depleted BUB3 efficiently, and the expression levels of WT, QE, and Del216–222 were comparable with each other (Fig. 5C).

We observed mitotic arrest by microscopy when these cells were treated with nocodazole for 18 h (Fig. 5B, panels a–e). A high percentage of the control group cells was able to arrest during mitosis upon nocodazole treatment, as indicated by the presence of many rounded cells. As expected, cells in the shBUB3 group were not able to arrest during mitosis. The restored expression of BUB3WT partially rescued the ability of cells to arrest during mitosis. However, neither QE nor Del216–222 could rescue the mitotic arrest to the extent of the wild-type protein. To further confirm this observation, these cells were harvested and subjected to biochemical analysis of mitotic markers including MPM2, cyclin B1, and phospho-Ser10 of histone H3 (Fig. 5D). The levels of multiple bands of MPM2 decreased significantly upon shBUB3 infection, and the major signal at ~55–75 kDa (but not the other bands) largely rescued in the WT group. In the QE and Del216–222 groups (Fig. 5D, upper panel), none of the MPM2 signals were rescued. Both cyclin B1 and phospho-Ser10 of histone H3 were largely rescued in the WT group but not in the QE and Del216–222 groups.

We further performed FACS analysis to quantify the mitotic 4N population in the cell pool of each group, and the result was consistent with the microscopic observation and biochemical analysis (Fig. 5E), further confirming the impaired function of the QE and Del216–222 mutants. We noticed that the levels of mitotic markers cyclin B1 and histone H3 phospho-Ser10 were largely rescued to levels similar to control upon the wild-type BUB3 restoration in shBUB3 cells; however, the mitotic index

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The following mutant designation was used: QE, E213Q/K216E/K217E/K218E.
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(Fig. 5E) was not fully rescued. These data underscored the importance of unperturbed expression BUB3 in mitotic checkpoint signaling. Combined with its kinetochore localization data, we concluded that the motif from Lys216 to Lys222 is critical for BUB3 function in mitotic checkpoint signaling.

Association of the BUB3 Mutants with the Mitotic Checkpoint and Centromeric Proteins—Previous studies demonstrated that BUB3 interacts with MAD2, BubR1, and the kinetochore protein KNL1 (29, 30, 37, 38). We were interested in whether the tandem basic lysine residues in the identified motif mediate the interaction with these proteins. We measured the interaction of the QE mutant with BUB3WT and Del216–222, and GFPBUB3 K266Q/K267Q/R288Q were cotransfected with PGK-H2B-mCherry into HeLa cells seeded on glass coverslips. Cells were stained with DAPI, fixed, and mounted. The localization of both wild-type and mutant BUB3 was observed using confocal microscopy. E, Western blotting confirmed the expression of the mutants, and the mutated hBUB3 was still recognized by the hBUB3-specific antibody. The green arrow indicates GFPBUB3, and the black arrow indicates endogenous BUB3. Scale bar, 25 μm. endo., endogenous; D216–222, Del216–222.
However, the point mutation mutants, even when being expressed at low levels (Fig. 6E), can still be efficiently immunoprecipitated, indicating that their interaction with CENP-A was not affected.

The association of BUB3 and CENP-A has not been reported previously. We thus further investigated the association of CENP-A and BUB3. Given that the C terminus of CENP-A is mainly involved in its folding and association with canonic histones for nucleosome assembly, we hypothesized that the N terminus of CENP-A might mediate its interaction with BUB3. A series of mutations and truncations at the N terminus of CENP-A (Fig. 6F) was generated, and their interaction with BUB3 was evaluated. We found that deletion of the first 29 or 53 amino acid residues did not affect CENP-A interaction with BUB3. However, deletion of five amino acid residues from Leu<sup>61</sup>–Leu<sup>65</sup> (Del61–65) greatly impaired CENP-A interaction with BUB3 (Fig. 6, F and G), suggesting that this motif of CENP-A is critical for BUB3 binding. As two controls, CENP-A mutants with mutations R42A/R43A/R44A (3A) or K49A/R52A/K53A/K56A (4A), which are located very close to the region Leu<sup>61</sup>–Leu<sup>65</sup>, interacted with BUB3 as efficiently as wild-type CENP-A (Fig. 6, F and G).

To further investigate the relationship of BUB3 and CENP-A, we performed GST pulldown assay to find whether...
the association between CENP-A and BUB3 observed in cells is a direct interaction. The result (Fig. 7A) revealed that there is no physical interaction between recombinant CENP-A and recombinant BUB3.

We went on to evaluate the role of CENP-A in BUB3 localization. CENP-A was efficiently depleted with shRNA against CENP-A (Fig. 7B). BUB3 nuclear localization in interphase of the cell cycle was not affected upon CENP-A knockdown (Fig. 7C versus 7D). However, examination of BUB3 kinetochore localization in mitotic phase in CENP-A knockdown cells is difficult because the mitotic cell in which CENP-A was knocked down cannot be found. We found that depletion of CENP-A inhibits cell cycle progression to mitotic phase G2/M of the cell cycle, which was reported previously (39–41), and is evidenced by the reduced cyclin B1 level in CENP-A shRNA-treated cells in our experiment (Fig. 7B).

Combined with above data, we concluded that the nuclear localization signal motif of BUB3 is involved in its kinetochore localization. Region Leu61–Leu65 of the centromeric constituent CENP-A is potentially involved in BUB3 kinetochore recruitment through a non-direct interaction mechanism.

DISCUSSION

Nuclear enrichment of mitotic checkpoint proteins is critical for checkpoint signaling (15, 16). We found that BUB3 localizes to the nucleus during interphase, which is distinct from the timely translocation of MPS1 and MAD2 (15). A prevailing mechanism for importing proteins to the nucleus is through the importin pathway. BUB3 may contain nuclear localization signal sequences that are thus recognized by members of the importin family of proteins. Classical nuclear localization signal sequences are either monopartite or bipartite. Monopartite nuclear localization signals appear as a consensus sequence, (K/R)4–6, and bipartite nuclear localization signals appear as two smaller clusters separated by 11 amino acid residues, (K/R)2X10–12(K/R)3 (where X denotes any other amino acid residue). Both forms of nuclear localization signal sequences are marked by clusters of basic amino acids, essentially lysines or arginines (42, 43). The motif we found in human BUB3 consists of a stretch of basic lysines and two aromatic amino acid residues, Tyr219 and Phe221. Deletion of this motif impaired but did not abolish BUB3 nuclear distribution. There was no motif

FIGURE 4. Evaluation of the kinetochore localization of BUB3 mutants. HeLa cells seeded onto polylysine-coated coverslips were transfected with GFPBUB3WT, GFPQE, and GFPDel216–222. Cells were treated with 1 μM Taxol for 4 h and subjected to immunostaining with CENP-A-specific antibody and Alexa Fluor-conjugated secondary antibodies. The kinetochore localization of BUB3 was observed using confocal microscopy. A–C, kinetochore localization of BUB3WT (A), QE (B), and Del216–222 (C). Scale bar, 5 μm.
found in BUB3 that matches the bipartite nuclear localization signal sequences pattern. There is a cluster of basic lysine residues, Lys^{266}, Lys^{267}, and Arg^{268}, located ~40 residues downstream of the Lys^{216}–Lys^{222} motif. However, the mutation of Lys^{266}, Lys^{267}, and Arg^{268} did not affect BUB3 nuclear localization. On the other hand, most of the amino acid residues in this motif, including the two aromatic residues Tyr^{219} and Phe^{221}, contributed significantly to BUB3 nuclear localization. Thus, the Lys^{216}–Lys^{222} motif in BUB3 is neither a monopartite nor a component of a bipartite form of typical nuclear localization signal sequences. This motif is a novel and atypical nuclear localization signal.

The function of BUB3 in the mitotic checkpoint is conserved from yeast to humans. However, human BUB3 shares only 34% identity with ScBub3 (S. cerevisiae Bub3) throughout the entire length of the protein (26). It was demonstrated that the protein ScBub3 binds to multiple MELT motifs phosphorylated by MPS1 of the kinetochore subunit Spc105/Knl1 (30, 38). The crystal structure of the ternary complex containing a fragment of ScBub1, ScBub3, and a synthetically phosphorylated MELT peptide suggested that multiple residues of ScBub3 are responsible for the interaction with Knl1 and the recruitment of Bub3 to the kinetochores (38). These residues include Arg^{217} (corresponding to Arg^{202} in human BUB3), Phe^{236} (not conserved and...
corresponding to Tyr219 in human BUB3), Phe238 (corresponding to Phe221 in human BUB3), Arg239 (not conserved and corresponding to Lys222 in human BUB3), Arg242 (corresponding to Arg225 in human BUB3), Trp278 (corresponding to Trp262 in human BUB3), and Arg283 (corresponding to Lys266 in human BUB3). Given the significant divergence between ScBub3 and human BUB3, we concluded that in human BUB3, the tandem lysine residues Lys216, Lys217, and Lys218 play critical roles in its association with the centromeric component CENP-A but have only a minor role in its interaction with MAD2, BUB1, BubR1, and HEC1. These observations help greatly in dissecting the mechanism of the role of human BUB3 in mitotic checkpoint signaling.

Another novel and interesting finding in our study is that the N terminus of CENP-A plays a potential role in mitotic checkpoint signaling. CENP-A has been considered to be the epigenetic marker and the built-in block of the centromere on which the kinetochore can be assembled. In yeast, it has been shown that Bub3 is recruited to the kinetochore through its interaction with Knl1. However, human KNL1 shares very low similarity with the S. cerevisiae Knl1 throughout the entire protein, and this mechanism in yeast may not occur in humans. We found that the motif Leu61–Leu65 of CENP-A is involved in its association with BUB3. These data suggest that, beyond serving as the platform for kinetochore assembly, CENP-A is potentially involved in recruitment of mitotic checkpoint signaling component(s), such as BUB3, through its N-terminal.

In summary, in this study, we identified a motif in human BUB3 involved in its nuclear and kinetochore localization. We also found that the Leu61–Leu65 motif of CENP-A is critical for its association with BUB3. Given the importance of BUB3 and CENP-A in the mitotic checkpoint, meiosis, and tumor sup-

FIGURE 6. Analysis of the interaction of BUB3 and its mutant with the mitotic checkpoint proteins BUB1, MAD2, and BubR1 and centromeric components. A–D, HEK293T cells (A and B) or HeLa cells (C and D) were transfected with BUB3WT or BUB3QE as indicated. Cells were treated with nocodazole for 18 h and harvested. Protein interactions were measured by co-immunoprecipitation (IP) and Western blotting (IB) as indicated. E, interaction of BUB3 mutants and CENP-A in 293T cells. F, schematics of CENP-A mutants and their binding capacity to BUB3. G, interaction of CENP-A mutants and BUB3 in 293T cells. NC, negative control.
pression (4, 9, 44), further investigation of this interaction in the context of these biological processes will greatly enhance our current understanding of the functions and mechanisms of BUB3 and CENP-A.

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