Mahogunin-mediated α-tubulin ubiquitination via noncanonical K6 linkage regulates microtubule stability and mitotic spindle orientation

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Mahogunin ring finger-1 (MGRN1) is a cytosolic ubiquitin ligase whose disruption or interaction with other isoforms of cytosolically exposed prion protein leads to spongiform neurodegeneration and also lack of which results in reduced embryonic viability due to mispatterning of the left–right (LR) axis during development. Here we demonstrate an interaction between the cytoskeletal protein α-tubulin and MGRN1. In cultured cell systems, loss of the ubiquitin E3 ligase activity of MGRN1 results in spindle misorientation and decreased α-tubulin polymerization, an effect also seen in primary cells. α-Tubulin was post-translationally modified by MGRN1 via noncanonical K6-linked polyubiquitination. This was significant because expression of catalytically inactive MGRN1 and/or ubiquitin mutant capable of only monoubiquitination resulted in similar mitotic spindle misorientation. The modulatory effect of MGRN1 was specific for α-tubulin and similar changes could not be detected in β- or γ-tubulin. However, catalytic inactivation of MGRN1 did not abrogate monoubiquitination of α-tubulin, thus unraveling a unique dual mode of ubiquitination by an unknown E3 ligase and MGRN1. MGRN1-mediated α-tubulin modification, and hence its stability, may highlight a key event in the LR patterning during embryogenesis.

Keywords: spindle misorientation; noncanonical polyubiquitination; tubulin polymerization

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Microtubules (MTs) are the fundamental structural elements of all cells that govern a myriad of cellular functions, including motility, maintenance of cell morphology, intracellular transport, mitosis and meiosis. A dynamic equilibrium exists between the assembly and disassembly of the MT network during the different phases of cell cycle.1 Proper assembly, positioning and orientation of the mitotic spindle are finely orchestrated by molecular players, like the dynein/dynactin complex that ensure correct spindle formation and generate the pulling forces required for proper cell division.2–4 These proteins are enriched at the spindle poles, and the disruption of a functional dynamin/dynactin motor protein complex impairs mitotic spindle morphology.5,6

Recent reports indicate that Huntingtin (HTT) protein, mutations of which cause Huntington’s disease (HD), localizes to spindle poles throughout mitosis. Downregulation of HTT in primary cells affects motor proteins and leads to spindle misorientation that translates to defects in murine neuronal progenitors and neuroblast precursors in Drosophila.7

Furthermore, Parkin, a ubiquitin E3 ligase,8 is also a tubulin-binding protein. Parkin–tubulin interaction in human cell lines leads to increased ubiquitination and accelerated degradation of α- and β-tubulins. Point mutants of Parkin (K161N, T240R and C431H) identified in Parkinson’s disease (PD) patients are incompetent in the E3 ligase activity toward tubulins.9

The role of post-translational modifications of tubulin, such as acetylation, methylation, detyrosination, glutamylation and glycation in MT formation and regulation, has been studied.10 However, our understanding of ubiquitination of tubulin is still premature and is primarily focused on regulating its degradation and turnover.

Recent reports indicate that BRCA1 (breast cancer-associated gene 1) protein binds to and ubiquitinates γ-tubulin that is crucial for maintaining appropriate centrosome number in cells.11–14 In addition, mutations in ubiquitin C-terminal hydrolase L1 (UCH L1), a cysteine hydrolase,15 expressed abundantly and exclusively in brain and reproductive tissues,16 are associated with PD and Alzheimer’s diseases (AD).17 Studies show that besides being a deubiquitinating

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Abbreviations: Ab, antibody; AD, Alzheimer’s diseases; BRCA1, breast cancer-associated gene 1; CysPrP, C-terminal transmembrane form of prion protein; cyPrP, cytosolic Prion; DMEM, Dulbecco’s modified Eagle’s medium; EGTA, ethylene glycol tetra acetic acid; ESCRT, endosomal sorting complex required for transport; FBS, fetal bovine serum; FCS, fetal calf serum; G2-M, Gap2–mitosis; GFP, green fluorescent protein; GTP, guanosine-5’triphosphate; HA, hemagglutinin; HD, Huntington’s disease; HEK293T, human embryonic kidney 293 transformed with T-antigen; He-Ne, helium–neon; HTT, Huntingtin; LR, left–right; MEF, mouse embryonic fibroblast; MGRN1, mahogunin ring finger-1; M phase, mitosis phase; MT, microtubule; Noc, nocodazole; NA, numerical aperture; p53, protein 53; P, pellet; PBS, phosphate buffer saline; PD, Parkinson’s disease; PIPES, 1,4-piperazinediethanesulphonic acid; PMSF, phenylmethylsulfonyl fluoride; PrP, prion protein; PrPSc, scrapie form of prion protein; Rad56, radiation gene 6; S, supemantant; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sRNA, small interfering RNA; TPA, 12-O-tetradecanoylphorbol 13-acetate; Tsg101, tumor suppressor gene 101; Tubgcp2, α-tubulin-associated protein; Ub, ubiquitin; UbcH2, ubiquitin conjugating enzyme 2 (human); UCH L1, ubiquitin C-terminal hydrolase L1

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enzyme, UCH L1 dimers have ubiquitin ligase activity in vitro and stabilize monoubiquitin in neurons.\textsuperscript{18–20} Prion diseases such as HD, PD and AD are late-onset neurodegenerative diseases. Prion protein (PrP), a highly conserved cell surface glycoprotein, is implicated in several of the prion diseases such as scrapie, bovine spongiform encephalopathy, Creutzfeldt–Jakob disease and Gerstmann–Strausser–Scheinker disease. Their pathogenesis is associated with the presence in brain of one or more abnormal isoforms of PrP (misfolded form of PrP (PrP\textsuperscript{Sc}), transmembrane isoform (PrP\textsuperscript{Sc}) and cytoplasmic (cyPrP)).\textsuperscript{21} Although the mechanisms leading to disease are not fully understood, recent reports suggest that some of the disease-causing isoforms of PrP can engage in atypical interactions with the cytosolic E3 ligase, mahogunin ring finger-1 (MGRN1). This results in inappropriate sequestration of MGRN1 in cell culture systems, and might contribute to late-onset neuronal dysfunction and disease.\textsuperscript{22} Similar to homozygous loss of MGRN1 function leading to late-onset spongiform neurodegeneration in mice.\textsuperscript{23} However, the role played by MGRN1 in prion diseases caused by PrP\textsuperscript{Sc} may not be as evident.\textsuperscript{24}

Separate studies indicate that although \textasciitilde 50\% of MGRN1-null mutants in mice develop late-onset spongiform neurodegeneration, rest of the animals exhibit severe developmental defects (like congenital heart defects, abnormal craniofacial patterning and mispatterning of the left–right (LR) body axis) and increased mortality by weaning age. In addition, \textasciitilde 1\% of animals also suffered from situs inversus (complete reversal of the left and right body axes).\textsuperscript{25,26} Hence, the effect of loss of function of MGRN1 on cell viability and ultimately on the development of animals seemed likely.

Here, we describe the serendipitous discovery of an interaction between MGRN1 and the cytoskeletal protein, \(\alpha\)-tubulin; and its effect on the axis of cell division during mitosis. Depletion of MGRN1 in cells affects ubiquitination of \(\alpha\)-tubulin, altering its polymerization state and not the protein turnover. Our analysis identifies a unique mode of ubiquitination of \(\alpha\)-tubulin by MGRN1 (utilizing K6 ubiquitin linkages) as a novel post-translation modification of this cytoskeletal protein, responsible for the formation and maintenance of the mitotic spindle fibers.

**Results**

**Interaction of endogenous MGRN1 with \(\alpha\)-tubulin in mitotic cells.** We observed in HeLa cells that the typical punctate/vesicular pattern of expression of endogenous MGRN1 with an obvious nuclear exclusion as seen in interphase cells gets altered and it now decorates mitotic spindles in dividing cells (Figure 1a). This pattern of close association of MGRN1 with mitotic apparatus could be detected through the different stages of mitosis (M phase) (Supplementary Figure S1A). Co-immunoprecipitation of synchronized cells indicates an interaction between MGRN1 and \(\alpha\)-tubulin (Figures 1b and c and Supplementary Figure S1B) and this requires the C-terminus of MGRN1 (Figure 1d and Supplementary Figure S1B). Co-immunocytochemistry also showed their pronounced colocalization on mitotic spindles (Figure 1e). Similar results were noted in mitotic HEK293T (human embryonic kidney 293 transformed with T-antigen) cells (Figure 1f), suggesting a cell line-independent phenomenon. Furthermore, co-immunoprecipitation of melanocytes\textsuperscript{27} (Supplementary Figure S1D) demonstrates an interaction between MGRN1 and \(\alpha\)-tubulin in melan a-6 (control) cells when compared with melan md1-nc (Mgrn1\textsuperscript{nd}) or Mgrn1 null cells (Figures 1g and h). These observations led to the following plausible hypotheses – either association with MGRN1 regulates the turnover of \(\alpha\)-tubulin or MGRN1 ubiquitinates \(\alpha\)-tubulin to affect its function. However, an indirect interaction with the spindle assembly via the dynein/dynactin complex seemed unlikely.

**Catalytically inactive MGRN1 affects the axis of cell division.** To understand the functional significance of the interaction between MGRN1 and \(\alpha\)-tubulin, cells were transiently transfected with full-length (referred to as MGRN1) or a catalytically inactive MGRN1, lacking the RING domain (MGRN1\textsuperscript{AR}), or with point mutations (C316DMGRN1, C299EMGRN1) in the same domain (Supplementary Figures S2A and B). Although HeLa cells transiently transfected with MGRN1\textsuperscript{AR} completed M phase of cell cycle with a mitotic index comparable to those expressing either cytosolic green fluorescent protein (GFP) or MGRN1 (Supplementary Figure S1E), they displayed spindle misorientation.\textsuperscript{28} The spindle angle relative to the cell substrate adhesion plane (Supplementary Figure S2C) for MGRN1 RING mutant-expressing cells (\textasciitilde 55–70\%, depending on the construct) was \textasciitilde 10\°, whereas control spindles were usually parallel to the substratum (Figures 2a and b and Supplementary Figures S2 D and E and S3A). Whereas control cells show an angle mostly around 10\°, the average angle upon functional inactivation of MGRN1 was significantly higher (between 15\° and 30\°), indicating aberrant spindle orientation relative to the substratum (Figures 2b and d and Supplementary Figure S2E; right graphs). This phenotype was even more pronounced when MGRN1 was depleted from cells using small interfering RNA (siRNA) constructs (with knockdown efficiency of \textasciitilde 75\%), where spindle tilt of \textasciitilde 10\° was seen in \textasciitilde 80\% of cells (Figures 2c and d). Such experiments with catalytically inactive MGRN1 were possible only with transient transfections as three attempts to establish stable cell lines failed (data not shown); on the contrary, we could easily establish stable cells expressing full-length MGRN1 (Supplementary Figure S1C). Although no drastic effect of functionally inactive MGRN1 is obvious on the mitotic index or the completion of mitosis in the short term (Supplementary Figure S1E), the effect of spindle misorientation had a cumulative detrimental effect over multiple cycles of cell division.

We next examined the structural underpinnings of spindle misorientation induced by MGRN1 inactivation/depletion. The most notable defect was a significant loss and shortening of astral MTs that did not contact the cell cortex, a requirement for force generation during spindle orientation (Figures 2e–h). We also evaluated the effect of MGRN1 inactivation on the motor protein, dyenin1, as it is important for spindle pole assembly and orientation.\textsuperscript{7,28} Immunocytochemistry showed a decrease in dynein staining at the poles when MGRN1 was depleted compared with the control cells (Figures 3a and b).
Figure 1 Mahogunin interacts with α-tubulin. (a) Unsynchronized HeLa cells immunostained for endogenous MGRN1 show distinct nuclear exclusion during interphase but decorate the mitotic spindles in dividing cells. (b) Synchronized population of HeLa cells, enriched for mitotic cells, were lysed and immunoprecipitated with anti-MGRN1 antibody. Western blots analysis of this with anti-α-tubulin antibody shows co-immunoprecipitation of α-tubulin with MGRN1. Marks α-tubulin; — marks MGRN1; Ab, antibody; * indicates nonspecific band; and # indicates immunoglobulin G (IgG) heavy chain. (c) Synchronized HeLa cells were lysed and immunoprecipitated with anti-α-tubulin antibody. Immunoblotting with anti-MGRN1 antibody shows co-immunoprecipitation between the two proteins. ## Indicates IgG light chain. (d) HeLa cells transiently transfected with the indicated GFP-tagged MGRN1 constructs were synchronized, lysed and immunoprecipitated with anti-GFP antibody. Western blot analysis of this with anti-α-tubulin antibody shows co-immunoprecipitation of α-tubulin with MGRN1, MGRN1ΔR and MGRN1ΔN but not MGRN1ΔC (top panel). Control blot immunoprecipitated with anti-GFP antibody to show expression of different MGRN1 constructs (bottom panel). (e) Synchronized population of HeLa cells immunostained for MGRN1 and α-tubulin show colocalization between the two proteins on the mitotic apparatus. The channels for acquiring the images are indicated. Two representative cells (i) and (ii) are shown. (f) Colocalization between MGRN1 and α-tubulin is detected in mitotic HEK-293T cells. The channels for acquiring the images are indicated. Three representative cells (i), (ii) and (iii) are shown. (g) Asynchronous a-6 and md1-nc melanocyte cells were lysed and immunoprecipitated with anti-MGRN1 antibody. Western blots analysis of this with anti-α-tubulin antibody shows co-immunoprecipitation of α-tubulin with MGRN1 in case of melan a-6 but in the melan md1-nc cells. It should be noted that as IgG heavy chain also migrates at ~ 55 KDa, a band (of lower intensity) is detected even in melan md1-nc cells. Marks MGRN1. (h) Asynchronous a-6 and md1-nc melanocyte cells were lysed and immunoprecipitated with anti-α-tubulin antibody. Immunoblotting with anti-MGRN1 antibody shows co-immunoprecipitation between the two proteins in case of control melanocyte cell line (melan a-6) only but not in MGRN1-null cell line (melan md1-nc).
However, the spread and pattern of distribution of dynein at and around the spindle poles remained very similar in both the cell populations (Figure 3c). These results imply that the decrease in detectable dyenin at the spindle pole might be due to its reduced association with polymerized α-tubulin when MGRN1 is functionally inactive, rather than a direct effect on the inhibition in dynein/dynactin-dependent transport.7,29
MGRN1 affects α-tubulin polymerization. As mitotic spindles and asters are dynamic microtubule-based structures, we depolymerized microtubules with nocodazole (Noc) and followed their regrowth after washout of the drug in HeLa cells transiently expressing MGRN1, MGRN1ΔR or MGRN1 C316D at various time points (Figures 3d and e and Supplementary Figure S3B). Peripheral MT clusters, representing ectopic poles, were marginally more in number (1.5–1.7 times, across time points) upon functional inactivation of MGRN1 when compared with the control cells (Figure 3e). This difference remained very similar over the entire time period of the experiment, indirectly pointing to the fact that motor proteins do not directly govern this phenotype.

Furthermore, to biochemically establish the interaction between MGRN1 and polymerized MTs, co-sedimentation assay was performed using synchronized mitosis-enriched HeLa cell lysates. Results indicated that MGRN1 co-pelleted with taxol-stabilized MTs and not with Noc (negative control) (Figure 4a).

Finally, to check the status of endogenous microtubule polymerization in unsynchronized population of cells, HeLa cells and wild-type mouse embryonic fibroblasts (MEFs; primary cells) were transiently transfected with the indicated MGRN1 constructs. At 24h after transfection, the cells were lysed, 1/15th of this was retained for analysis as total lysate control and insoluble (pellet (P)) fractions and biochemically analyzed to detect any change in α-tubulin polymerization among the samples (Figures 4b–g). Melanocytes (melan a-6 and melan md1-nc cells) were grown for 24h, similarly fractionated and analyzed by western blots for the levels of α-tubulin. Our results showed that although the S/P ratio is ~1 in control HeLa cells expressing cytosolic GFP or MGRN1; the S/P ratio is ~2.5 in cells overexpressing catalytically inactive MGRN1 (MGRN1ΔR or MGRN1316D), hence indicating a decrease in the polymerized form of α-tubulin (Figures 4b and c). Similarly, the S/P ratio was >3 in the presence of MGRN1 siRNA as compared with control (S/P = 2; Figures 4d and e). The most pronounced change was observed with wild-type MEFs in a similar experiment – with S/P ratio of ~3.6 upon overexpression of MGRN1ΔR compared with the MGRN1 control having S/P ratio of ~1.4 (Figures 4f and g). These samples when analyzed for changes in β-tubulin polymerization failed to elicit similar differences, irrespective of the functional status of MGRN1 (Figures 4m–n). In melanocytes, whereas the S/P ratio was ~1.9 for melan a-6, it was ~3 for melan md1-nc cells (Figures 4h and i).

Furthermore, in Noc washout experiments with HeLa cells expressing MGRN1, MGRN1ΔR or C316DMGRN1 to study the MT network, the interphase cells showed that catalytic inactivation of MGRN1 distinctly compromised formation of a well-polymerized mesh – the MGRN1ΔR- or C316DMGRN1-expressing cells lag behind the MGRN1 cells by ~30 min (Figure 5a and Supplementary Figure S4). siRNA-mediated depletion of MGRN1 also severely affected microtubule re-assembly (Figure 5b). Wild-type MEFs subjected to similar microtubule regrowth assay after Noc treatment over a period of 10 and 15 min show a very punctate α-tubulin expression pattern in MGRN1ΔR cells as compared with a well-polymerized α-tubulin network and MT cluster nucleation centers in MGRN1 cells (Supplementary Figure S5B).

Furthermore, these results indicate a decrease in MT polymerization when the catalytic activity of MGRN1 is compromised in a cancer-derived cell line as well as primary cells.

MGRN1 does not affect γ-tubulin polymerization. Immunocytochemistry of HeLa cells for the endogenous levels of γ-tubulin and MGRN1 on mitotic cells showed that although γ-tubulin was present only on the spindle poles, MGRN1 decorates the entire spindle apparatus (Figure 6a). We next checked whether γ-tubulin was affected by MGRN1 depletion (Figures 6b and c). The expression of γ-tubulin, its intensity and spread at the poles remain independent of the presence of functional MGRN1, implying that in our system the formation and orientation of spindle poles represent two discrete events.

Furthermore, to check the status of endogenous γ-tubulin polymerization, HeLa cells expressing MGRN1, MGRN1ΔR,
C316DMGRN1 or depleted for the MGRN1 expression were lysed, separated into S and P fractions and biochemically analyzed (Figures 6d–g). Again, we could not detect any significant difference in the S/P ratios between the various experimental (with inactive MGRN1) and control samples.

These results again imply that MGRN1 dysfunction does not affect $\alpha$-tubulin polymerization, its localization or distribution and hence, in turn, does not compromise spindle pole formation while still severely altering the pole orientation.

MGRN1 affects $\alpha$-tubulin polymerization by its polyubiquitination. Tsg101 (tumor suppressor gene 101), a key component of the ESCRT-I (endosomal sorting complex required for transport-I), is the only known physiological substrate that is monoubiquitinated by MGRN1 at multiple sites.30 Like other ubiquitin E3 ligases, MGRN1 is capable of generating a polyubiquitin ladder and also its autoubiquitination in the presence of a functional RING domain.23 We therefore tested whether in vivo $\alpha$-tubulin was ubiquitinated by MGRN1. Lysates derived from HeLa cells co-transfected with either MGRN1 or MGRN1ΔR and separated into S and P fractions were immunoprecipitated with $\alpha$-tubulin antibody (Ab) and immunoblotted for endogenous ubiquitin (Figure 7b). Detection of stronger signal in the P fractions indicates the prevalence of ubiquitinated species in them and...
also strengthens the argument for the involvement of this modification in the polymerization of α-tubulin. Detectable level of ubiquitinated α-tubulin is less in the P fraction of MGRN1ΔR as compared with MGRN1.

We next asked whether this was a mono- or polyubiquitination event. To address this, various hemagglutinin (HA)-tagged constructs of different ubiquitin species – wild-type ubiquitin (Ub), K0, K6, K11, K29, K48 or K63 (ubiquitin mutants with arginine substitutions at all lysine residues except the ones indicated) – were used (Figure 7a).31–33 Lysates derived from HeLa cells co-transfected with either MGRN1 or MGRN1ΔR along with the various ubiquitin constructs (Figure 7c) showed that α-tubulin underwent polyubiquitination in the presence of MGRN1 and Ub (lane 1, as evidenced by the detection of a polyubiquitin ladder); however, this was abrogated in the presence of MGRN1ΔR and Ub (lane 2), implying that the inhibition of the MGRN1 catalytic activity affects α-tubulin ubiquitination. As MGRN1 is known to multi-monoubiquitinate Tsg101,30 it was obvious to check the effect of K0 in a similar assay. However, unlike with Ub, we could not detect any multi-monoubiquitination in the presence of MGRN1 and K0 (lane 3) even on a darker exposure. Multi-monoubiquitination was, however, clearly evident in lane 4, having MGRN1ΔR and K0; this could probably mean than in the absence of a catalytically active MGRN1, there was enforced multi-monoubiquitination. Curiously though, lanes 2 and 3 did have a single band (*) corresponding to a monoubiquitinated α-tubulin species (Figure 7c); a similar band was also evident when analyzed for endogenous ubiquitin (Figure 7b). These observations led us to believe that monoubiquitination of α-tubulin occurred even in the absence of catalytic activity of MGRN1 (as in lanes 2 and 4), with enforced multi-monoubiquitination in the presence of MGRN1ΔR and K0 (lane 4). Results similar to that obtained with Ub and MGRN1/MGRN1ΔR were observed when instead of Ub, its mutant K6 was used (lanes 5 and 6). These data suggest that although MGRN1 promotes polyubiquitination, it also does not encourage multi-monoubiquitination of α-tubulin.

No discernible change was detected in the ubiquitination pattern of β- and γ-tubulins in the presence of the different HA–Ub constructs coexpressed with MGRN1 or MGRN1ΔR (Figures 7d and e). The polyubiquitination of α-tubulin in the presence of MGRN1, however, does not regulate its protein turnover. This was evident because of two reasons. First, K48-mediated polyubiquitination of α-tubulin occurred irrespective of the expression of MGRN1 or MGRN1ΔR. (Supplementary Figure S6A). Similar argument also negates the role of K11, K29 and K63 ubiquitin residues in the polyubiquitin chain extensions (Supplementary Figure S6A). Second, treatment with the proteasomal inhibitor, MG132, similarly affects the S/P ratio of lysates from cells overexpressing MGRN1, MGRN1ΔR or C316DMGRN1 (Supplementary Figure S6B), again stating that MGRN1 does not modulate α-tubulin protein levels.

Furthermore, to establish the role of MGRN1-mediated α-tubulin ubiquitination in endogenous MT polymerization, at 24 h after transfection, the cell lysates separated into cytosolic (soluble (S)) and insoluble (pellet (P)) fractions were biochemically analyzed to detect any change in α-tubulin polymerization among cells expressing Ub, K0 or K6 along with MGRN1 or MGRN1ΔR (Figures 8a and c). Our results showed that although the S/P ratio is ~1 in the presence of Ub/K6 and MGRN1, this ratio is ~1.5 in cells expressing K0 and/or MGRN1ΔR (Figures 8b and d).

However, similar changes in the ubiquitination and polymerization pattern of β- and γ-tubulins in the presence of the different HA-Ub constructs coexpressed with MGRN1 or MGRN1ΔR could not be detected (Figures 8e–h).

These results so far taken together hint towards a unique mode of ubiquitin-mediated post-translational modification of α-tubulin, where two ubiquitin E3 ligases act. An unknown ligase monoubiquitimates α-tubulin and this happens even in the presence of MGRN1ΔR. Either following this or independently, MGRN1 polyubiquitinates α-tubulin via noncanonical K6 linkages as a post-translational modification to ensure proper polymerization but not for its degradation. In the absence of a catalytically active MGRN1, multi-monoubiquitination occurs that, however, is insufficient for complete α-tubulin polymerization.

Such multiple E3 ligases working together and modifying a protein has been observed previously in case of some of the most important cell cycle regulators that in turn need to be under very tight regulation. The most studied protein to fall in this class is the tumor suppressor p53 (protein 53), known to be ubiquitylated by at least 11 different E3 ligases.34 Even though these E3 ligases have other targets, they all contribute individually in regulating p53 levels, which is vital for proper cell cycle progression.

**Polyubiquitination of α-tubulin affects spindle pole orientation.** To assess the physiological consequence of MGRN1-mediated polyubiquitination of α-tubulin, tilt in spindle poles was used as a read-out. HeLa cells were transfected with Ub or K0 along with MGRN1 or MGRN1ΔR, and the mitotic cells scored for the tilt in the axis of cell division. The spindle angle for cells expressing MGRN1 and Ub was ~10° for ~90% of cells (23 out of 27 cells; Figures 8i and j). However, a tilt of >10° was observed in ≥55% of cells expressing MGRN1 and K0 or MGRN1ΔR and Ub, and >10° tilt was observed in 60% of cells expressing MGRN1ΔR along with K0. Thus, just skewing the ubiquitination balance from polyubiquitinated α-tubulin to a more predominantly monoubiquitinated species was sufficient to cause spindle misorientation. Results similar to those with MGRN1 and Ub were also seen in the presence of MGRN1 and K6, where ~70% of transfected cells (21 out of 30 cells) showed angle of tilt ≤10°, whereas ~67% of cells (20 out of 30 cells) expressing K6 and MGRN1ΔR had >10° angle of tilt (Figures 8i and j). Polyubiquitination of α-tubulin by catalytically active MGRN1 was a crucial governing factor in the proper orientation of spindle poles.

**Discussion**

This study elucidates a novel interaction between the cytoskeletal protein α-tubulin and MGRN1, whose absence leads to spongiform neurodegeneration or causes significant developmental defects during embryogenesis. The present study for the first time highlights how loss of the ubiquitin
E3 ligase activity of MGRN1 affects spindle orientation. In cultured cell systems, MGRN1 physically associated with α-tubulin, with an increased preference for polymerized MTs. In cell lines and primary cells, functional inactivation of MGRN1 affected α-tubulin polymerization. This also simultaneously coincided with an increase in the angle of tilt in [Cell Death and Disease]
Figure 4  MGRN1 affects polymerization of α-tubulin. (a) Microtubule pulldown assay shows that MGRN1 co-pelleted with taxol-stabilized microtubules in mitotic HeLa cell lysates, indicated by (c). Nocodazole (Noc) inhibition of microtubule polymerization was used as negative control. The blot is a representative of at least three experiments. (b) Hela cells lysates, transiently expressing the indicated constructs, were fractionated to separate polymerized and unpolymerized fractions by high-speed ultracentrifugation; the fractionated samples along with total lysates were immunoblotted for α-tubulin. The levels of α-tubulin in the total lysates serve as loading control; the expression of the various constructs was biochemically analyzed with anti-GFP antibody. The blots are representative of at least five experiments. (c) The immunoblots generated in (b) were analyzed for the ratio of unpolymerized to polymerized α-tubulin. Note an increase in this ratio upon functional depletion of MGRN1, an indication of a compromise in tubulin polymerization. *P < 0.05, **P < 0.01, using Student’s t-test. Error bars, S.E.M. (d) HeLa cells treated with MGRN1 or GFP siRNAs were subjected to similar treatments as in (a), with recovery times of 30 and 60 min. Insets of higher magnification show punctate α-tubulin staining in the presence of MGRN1 siRNA, similar to MGRN1ΔR. 

Figure 5  MGRN1 affects microtubule regrowth. (a) HeLa cells transiently transfected with the indicated constructs were treated with nocodazole, allowed to recover for 30 and 60 min and microtubule regrowth was monitored by immunostaining with α-tubulin antibody. Insets of higher magnification show punctate α-tubulin staining in the presence of MGRN1ΔR contrary to a well-formed MT network in MGRN1-expressing cells. (b) HeLa cells treated with MGRN1 or GFP siRNAs were subjected to similar treatments as in (a), with recovery times of 30 and 60 min. Insets of higher magnification show punctate α-tubulin staining in the presence of MGRN1 siRNA, similar to MGRN1ΔR.
MGRN1 ubiquitinates α-tubulin to orient spindles
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Figure 6 MGRN1 does not affect the spindle poles. (a) Asynchronous HeLa cells were co-immunostained for MGRN1 and γ-tubulin; mitotic cells were imaged. γ-Tubulin is detected at the spindle poles, whereas MGRN1 is seen to decorate the entire mitotic apparatus. (b) HeLa cells treated with MGRN1 or GFP siRNAs were co-immunostained for MGRN1 and γ-tubulin; mitotic cells were imaged. Similar γ-tubulin staining is detected at the spindle poles, irrespective of the treatments. (c) Cells imaged in (b) were analyzed for the spread of γ-tubulin staining at the spindle poles. Histogram plotting the percentage of cells at different areas of spread as indicated on the X axis. Over 80 cells from 4 independent experiments were analyzed as represented for each of the siRNA treatments. (d) The same samples analyzed in Figure 4b were immunoblotted for γ-tubulin. The levels of γ-tubulin in the total lysates serve as loading control. The blots are representative of at least three experiments. (e) The immunoblots generated in (d) were analyzed for the ratio of unpolymerized to polymerized γ-tubulin. Note no change in this ratio across samples. (f) The same samples analyzed in Figure 4d were immunoblotted for γ-tubulin. The levels of γ-tubulin in the total lysates serve as loading control. The blots are representative of at least three experiments. (g) The immunoblots generated in (f) were analyzed for the ratio of unpolymerized to polymerized β-tubulin. Note no change in this ratio across samples.

The effect of MGRN1 on spindle poles was specific for α-tubulin as depletion of this protein did not affect either the expression pattern or polymerization status of β- and γ-tubulins. Evidence for the importance of the E3 ligase activity of MGRN1 further came from the results that α-tubulin polyubiquitination was achieved only in the presence of MGRN1 and Ub and not when MGRN1.DR and/or K0 were expressed, suggesting that although MGRN1 promotes polyubiquitination, it also does not encourage multi-mono-ubiquitination of α-tubulin. Mono ubiquitination of α-tubulin by an unknown E3 ligase and MGRN1-mediated polyubiquitination (utilizing the noncanonical K6 linkages) were most likely unrelated, independent events. However, modification of α-tubulin by MGRN1 was crucial for proper spindle orientation. Accordingly, catalytic inactivation of MGRN1
Figure 7  MGRN1 mediates $\alpha$-tubulin polyubiquitination. (a) A cartoon representation of the sequence of ubiquitin, the lysine residues involved in ubiquitination and their putative role in the cell. (b) HeLa cells transiently transfected with MGRN1-GFP or MGRN1ΔR-GFP were fractionated to separate polymerized and unpolymerized fractions by high-speed ultracentrifugation, and the fractionated samples were immunoprecipitated with anti-$\alpha$-tubulin antibody. Enhanced in vivo ubiquitination was detected in pellet fractions compared with the supernatants by immunoblotting with anti-ubiquitin antibody. Faint and dark exposures of the anti-ubiquitin blot are shown. Expression of MGRN1ΔR compromises the polyubiquitination in the pellet fraction. Expressions of $\alpha$-tubulin in the various fractions and total lysates along with that of MGRN1 in the total lysates serve as loading control. * Indicates monoubiquitinated $\alpha$-tubulin; # indicates immunoglobulin G IgG heavy chain; ## indicates IgG light chain. (c) HeLa cells transiently co-transfected with HA-Ub, HA-K0 or HA-K6 constructs along with MGRN1-GFP or MGRN1ΔR-GFP were lysed and immunoprecipitated with anti-$\alpha$-tubulin antibody. In vivo ubiquitination was detected by immunoblotting with anti-HA antibody. Polyubiquitination is detected in the presence of MGRN1 along with either Ub or K6. The input levels of $\alpha$-tubulin in the total lysates serve as loading control. (d) HeLa cells transiently co-transfected with HA-Ub, HA-K0 or HA-K6 constructs along with MGRN1-GFP or MGRN1ΔR-GFP were lysed, immunoprecipitated with anti-$\beta$-tubulin antibody and immunoblotted with anti-HA antibody. The input levels of $\beta$-tubulin in the total lysates serve as loading control. (e) HeLa cells transiently co-transfected with HA-Ub, HA-K0 or HA-K6 constructs along with MGRN1-GFP or MGRN1ΔR-GFP were lysed, immunoprecipitated with anti-$\gamma$-tubulin antibody and immunoblotted with anti-HA antibody. The input levels of $\gamma$-tubulin in the total lysates serve as loading control.
and consequential monoubiquitination of α-tubulin resulted in misoriented mitotic spindle apparatus. We therefore conclude that ubiquitin-mediated post-translational modification of α-tubulin that eventually affects its polymerization occurs via multiple E3 ligases, with MGRN1 being one of them (Figure 9).
Vertebrates in general exhibit external bilateral symmetry; however, most internal organs such as the heart, lungs and kidney display asymmetry in structure and/or unilateral positioning with respect to the LR axis; interestingly, defects in laterality affects more than 1 in 8000 live births. Various models predict that the LR axis depends on either the presence of cilia-driven extracellular fluid flow or recruit ‘ciliary’ proteins, such as left–right dynein, as factors governing this asymmetry. However, a very recent report has shown in the plant model system (Arabidopsis thaliana) that mutations in α-tubulin and in its γ-tubulin-associated protein (Tubgcp2) play an important role in the symmetry properties of the plant’s axial organs. Similar mutations in vertebrate (Xenopus laevis), nematode (Caenorhabditis elegans) and mammalian cells affect the generation of LR asymmetry in a cilia-independent manner. Furthermore, expressions of mutant tubulins alter laterality by affecting proper localization of laterality related cargo molecules across the LR axis. This raised the possibility that alterations in α-tubulin might as well have a drastic effect on the LR axis of symmetry.

A later study has further shown that mutations in β-tubulin disrupt spindle orientation while affecting MT dynamics, thus again emphasizing the importance of tubulins and their polymerization status in spindle orientation. However, this does not rule out the possibility that because of mutations, the association between the MT and the motor proteins may be compromised.

Although the association of tubulins with the motor proteins and hence their role cannot be undermined, the nature of the tubulin subunits themselves are also equally important for MT polymerization, stability and dynamics. Evidence already exist in varied experimental systems where changes in tubulins alter their polymerization status and MT stability, in turn playing a significant role in spindle orientation at the cellular level and the LR axis of symmetry at the organismal level. Hence, we hypothesize that instead of mutations in tubulin, a post-translational modification that would affect its polymerization status as is affected by the E3 ligase, MGRN1, would similarly alter α-tubulin monomers and affect α-tubulin functions at the cellular as well as the organismal levels.

When studying tubulin polymerization, MT stability and spindle orientation, the emphasis has been on various post-translational modifications other than ubiquitination—the roles of PARKIN and BRC1 as ubiquitin E3 ligases in their expression patterns in the same samples, thus uncoupling the expression of Nodal from its responsive genes. However, the Nodal-responsive genes (Lefty1, Lefty2 and Paired-like homeodomain transcription factor2) showed aberration in their expression patterns in the same samples, thus uncoupling the expression of Nodal from its responsive genes in the presence of MGRN1 mutants. In plants, tubulin

Figure 8 MGRN1 ubiquitinates α-tubulin to orient spindles. (a) HeLa cells transfected with MGRN1-GFP or MGRN1AR-GFP along with HA–Ub or HA–K0 were fractionated and immunoblotted with α-tubulin to check for the status of α-tubulin polymerization. The levels of α-tubulin in the total lysates serve as loading control. The blot is representative of at least three experiments. (b) The immunobots from (a) were quantitated and analyzed for the ratio of polyubiquitinated to non-ubiquitinated tubulin. Note that the functional MGRN1 and Ub are required for efficient α-tubulin polymerization. Error bars, S.E.M. (c) HeLa cells transfected with MGRN1-GFP or MGRN1AR-GFP along with HA–K6 were similarly treated as in (a). The levels of α-tubulin in the total lysates serve as loading control. The blot is representative of at least three experiments. (d) Quantitation and analyses of immunobots from (c) show that significantly more α-tubulin exists in the polymerized state in the presence of MGRN1 and K6. Error bars, S.E.M. (e) The same samples generated in (a) and (d) were immunoblotted with β-tubulin. The levels of β-tubulin in the total lysates serve as loading control. The blot is representative of at least three experiments. (f) Quantitation and analyses of immunobots from (e) show similar pattern of β-tubulin polymerization across different samples. Error bars, S.E.M. (g) The cells transfected with MGRN1-GFP or MGRN1AR-GFP along with HA–Ub, HA–K0 or HA–K6 were imaged and mitotic cells analyzed for the tilt in axis of division as in Figure 2. Note that conditions that support polyubiquitination (as in the presence of MGRN1 along with Ub or K6) only result in normal axis of cell division (with spindle tilt ≤ 10°). The cells imaged in (h) were analyzed the amount of tilt in the axis of cell division was calculated similar to Figure 2. The graph shows the percentage of cells with abnormal tilt (> 10°, in dark gray) and those with normal axis of division (tilt ≤ 10°, in light gray). Similar effect on the spindle angles was observed in the Ub and K6

Although the ubiquitin molecule contains seven lysine residues in positions 6, 11, 27, 39, 48 and 63, the most studied have been homogenous polyubiquitin linkages involving residues 48 and 63. The involvement of the other lysine residues in regulating different cellular functions is progressively becoming evident – K6 polyubiquitin chains being less known. The K6 chains have been observed in E3-independent reactions catalyzed by radiation gene 6 (Rad6), the yeast ortholog of UbcH2 (ubiquitin conjugating enzyme 2 (human)). More recently, evidences suggest that autoubiquitination of BRCA1 in vivo is mediated by K6 or K29 residues. Our present study identifying the polyubiquitination of α-tubulin by MGRN1 preferentially utilizing K6, an unconventional site for ubiquitin polymerization, was unexpected. Although our results indicated that MGRN1-mediated α-tubulin ubiquitination did not involve K11, K29, K48 or K63 residues, it does not rule out the possibility of utilizing other lysine (K27, K33) residues or by linear chain extension.
Figure 9 Schematic diagram summarizing the results. Tb denotes α-tubulin. Although MGRN1-mediated polyubiquitination of α-tubulin is crucial for its polymerization and proper orientation of the axis of cell division, its monoubiquitination by an unknown E3 ligase may be an independent event. Monoubiquitination of α-tubulin preceeding MGRN1-mediated polyubiquitination also remains a possibility.

Mutations (spiral1, spiral2 and spiral3) produce right-handed helical growth; Lefty1 and Lefty2 mutants can act as suppressors of spiral1.40,41 However, in Xenopus, α-tubulin mutations affecting the LR axis also alter Nodal expression pattern,42 hence elucidating that there is a close cross-talk between the MT and the motor proteins during such developmental events.

The actual pathway by which MGRN1 alters nodal-responsive genes or helps orient the LR axis is yet under speculation; however, it would be prudent to extrapolate that MGRN1 through its post-translational modification of tubulins, Nodal and nodal-responsive genes; this need not follow a completely linear pathway.

Finally, our study linking MGRN1 to the MT network via post-translational modification of α-tubulin utilizing a more noncanonical K6 polyubiquitin linkage may highlight a key event in the LR patterning during early development in systems where stability of α-tubulin subunits supersedes the association between the MT and the motor proteins during such developmental events.

Materials and Methods
Constructs and antibodies. MGRN1, MGRN1ΔR, MGRN1ΔN and MGRN1ΔC constructs have been described before.22 The C316DMGRN1 and C299EMGRN1 constructs were generated by standard site-directed mutagenesis techniques. HA-tagged wild-type Ub was a gift of Rafael Mattera (Bethesda, MD, USA); K6, K8 and K63 ubiquitin mutants were gifts of Kah-Leong Lim (Singapore); HA-tagged K6, K11 and K29 ubiquitin mutants were gifts of Tomohiko Ohta (Kawasaki, Japan). Antibodies were from the following sources:

- α-tubulin (Santa Cruz Biotechnology, Dallas, TX, USA; J-tubulin (Abcam, Cambridge, UK), γ-tubulin (Sigma-Aldrich, St. Louis, MO, USA), dynemin (Santa Cruz Biotechnology) and ubiquitin (Sigma-Aldrich). The MGRN1, GFP, RFP and HA antibodies were gifts of Ramanujan S Hegde (Cambridge, UK).

Cell culture, synchronization and immunocytochemistry. Cell lines used for the experiment were HeLa (human cervical cancer cell line), HEK 293T (human embryonic kidney cell line), MEF (mouse embryonic fibroblast cells), immortal melanocytes (control melan-a6, MGRN1-null mutations, melan md1-nc).22 Culture of HeLa, transient transfections, preparation of stable cell lines, immunofluorescent staining and fluorescence microscopy of fixed cells was as before.22,24 Briefly, cells were grown in 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA)/Dulbecco’s modified Eagle’s medium (DMEM; Himedia, Mumbai, India) media at 37 °C and 5% CO2. At ~90% confluence, cells were transfected with DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s instructions. At ~24h after transfection, cells were lysed using suitable lysis buffer. Immortal melanocytes were grown in 10% fetal calf serum (FCS; Gibco)/RPMI-1640 (Gibco)/200 μM 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma-Aldrich) media at 37 °C and 10% CO2, as per the guidelines of the Wellcome Trust Functional Genomics Cell Bank. HEK293T (gift of Subrata Banerjee, Kolkata, India) and MEF (ICP-1 strain) graft of Mitras M Panicker, Bengaluru, India) cells were also grown under standard cell culture conditions. Immortal melanocytes (gift of Ramanujan S Hegde) were obtained from the Wellcome Trust Functional Genomics Cell Bank.

All tissue culture plasticware and Lab-Tek 8-well chambered slides used for microscopy were from Nunc, Roskilde, Denmark, and bottom coverglass dishes used for microscopy were from SPL Lifesciences, Gyeonggi-do, Korea. For synchronization of HeLa cells, thymidine–synchronization method was followed. Briefly, cells were subjected to 5 mM thymidine (Sigma-Aldrich)/DMEM at 37°C and 10% CO2. After ~7 days, cells were again reseeded for 1 day and then treated with serum (10% FBS/0.1% saponin) for 16 h. This was followed by a nocodazole (Sigma-Aldrich) block at 100 ng/ml for 16 h. The cells, now blocked at G2-M, were then allowed to enter mitosis for 60 min and then fixed (as in case of immunocytochemistry) or lysed (for biochemical studies).

Immunohistochemistry was done with minor modifications of earlier methods.22,50 For immunocytochemistry, cells were fixed with either 10% formaldehyde or methanol as per the requirement of the Ab. Cells were permeabilized using 1% FBS/phosphate buffered saline (PBS)/0.1% saponin (Sigma-Aldrich) for 60 min. Following overnight staining in primary Ab at 4 °C and 60 min of incubation in secondary Ab at room temperature. The samples were then imaged using confocal microscope.

Western blotting and immunoprecipitation. The protocol for western blotting was as before.22 Briefly, 10 or 12% tris-tricine gels were run as per the

Stable α-tubulin polymer

Properly formed spindles with no tilt. (both poles in focus on the same plane)

Improper spindles with pronounced tilt in axis of division (poles on different planes along the z-axis)

Alternate fates? Protoplast degradation??

?

Unknown E3 ligase

MGRN1

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molecular weight being probed and then the proteins were transferred by wet electrophoretic transfer method for 55 min. at 100 V. For immunoprecipitation, the protocol was as described previously with antibodies as indicated in the figures. Briefly, at 24 h after transfection, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton-X-1% IGEPAL, 1 mM PMSF, protease inhibitor (Sigma-Aldrich)) and then immunoprecipitated using the standard protocol.

Separation of tubulin into polymerized and unpolymerized fractions. Whole-cell lysates were taken from 52 mm culture dishes with 1 ml of lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) at 4°C for 20 min. Soluble and insoluble fractions of cell lysates were separated by ultracentrifugation at 75 000 r.p.m. for 1 h (rotor TLA 120.1, Beckman Coulter, CA, USA). A portion of the lysate (1/15th of the total) was saved before centrifugation. The soluble fraction (S) and insoluble fraction (P) were collected, and pellet was resuspended in Laemmli buffer (1/3 volume of initial lysis buffer buffer used). Equal volumes of supernatant and pellet fractions were then loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed with analysis by western blotting.

FACS analysis. Cell cycle analysis was done on cells synchronized as before and labeled with 0.01 mM propidium iodide (Thermo Scientific) at 37°C for 20 min. This was done on BD FACS Calibur flow cytometer (Becton Dickson, San Jose, CA, USA).

Co-sedimentation assay. This was performed as described previously with minor modifications. Briefly, cells, synchronized and enriched in mitosis were lysed at 4°C in 100 mM 4,1-piperazineethanesulfonic acid (PIPES), at pH 6.8, 1 mM MgCl2, 2 mM ethylene glycol tetra acetic acid (EGTA) and 1% Triton X-100, and spun at 13 000 × g for 30 min. To the supernatant thus obtained, purified tubulin (Cytoskeleton, Denver, CO, USA; 4 μg), dithiothreitol (1 mM), guanosine-5’-triphosphate (GTP; 1 mM) and taxol (Sigma-Aldrich) (10 μM) were added to cleared lysates, and incubated for 1 h at 37°C. Nocodazole (100 μM) was added as a negative control. Lysates were layered over a 25% sucrose cushion in the above buffer and spun at 48 000 r.p.m. for 1 h at room temperature (rotor TLA 120.1, Beckman Coulter). Microtubule pellets were collected after removing lysate and cushion, bound proteins were separated by SDS-PAGE and analyzed by western blotting.

Microtubule regrowth assay. At 20 h after transfection, microtubules were depolymerized in 10-20 μM nocodazole in culture medium for 1 h at 37°C. Cells were then washed and incubated in culture medium without nocodazole at 37°C to allow regrowth. Cells were fixed at different time intervals in 15% formalin and processed for immunofluorescence microscopy to examine microtubule regrowth (∼tubulin) from spindle poles in metaphase cells and the microtubule meshwork in interphase cells. To study the same in MEFs, the procedure was as described by Godin et al. Briefly, microtubules were depolymerized by treating cells with 5 μM nocodazole for 1 h at 37°C and 30 min on ice. After treatment, cells were washed twice with CO2 equilibrated medium. Microtubules were allowed to re-grow for different times (10 and 15 min). This was followed by fixing and permeabilization of cells as described above.

In vivo ubiquitination assay. In vivo ubiquitination assays were performed as described previously. Briefly, lysates of HeLa cells expressing the indicated GFP-tagged MGRN1, wild-type HA-tagged wild-type Ub or HA-tagged ubiquitin mutants (Ub-K0, Ub-K6, Ub-K11, Ub-K29, Ub-K48 and Ub-K63) were immunoprecipitated with various indicated anti-tubulin antibodies. Ubiquitinated tubulin was detected by immunoblotting with anti-HA antibodies. For analysis of ubiquitination of fractionated samples, equal volumes of S and P (resuspended in same as initial volume of lysis buffer) were immunoprecipitated with α-tubulin Ab and immunoblotted with ubiquitin Ab.

Knockdown with siRNA. ON-TARGETplus SMARTpool siRNAs against MGRN1 and GFP (catalog LG-022620-00-005 and D-013000-01-20; Thermo Scientific) Dharmacon Products, Lafayette, CO, USA) were transfected using Lipofectamine 2000 following the manufacturer’s instructions. Cells to be imaged were divided into two parts, trypsinized and replated – one set fixed 45 h after siRNA treatment, permeabilized and stained for immunocytochemistry, whereas another set lysed to check for knockdown efficiency biochemically.

Fluorescence microscopy and imaging. Fluorescence microscopy was performed utilizing LSM710-Meta and LSM710/ConfoCor 3 microscopy systems (Zeiss, Jena, Germany) equipped with an Ar-ion laser (for GFP excitation or Alexa-Flour 488 with the 488 nm line), a helium-neon (He-Ne) laser (for RFP, Alexa-Flour 546 and 594 excitation with the 543 nm line) and a He-Ne laser (for Alexa-Flour 633 with the 633 line). For all imaging, 63 × 1.4 numerical aperture (NA) oil immersion objective was used. For quantitative analyses and comparisons between multiple samples, images were collected using identical excitation and detection settings. The detector gain settings were chosen to allow imaging of the desired cells within the linear range of the photomultiplier tube without saturating pixels, unless otherwise specified.

Image analyses and calculation of spindle tilt. ImageJ (NIH, Bethesda, MD, USA) was used for all the image analyses reported in the text. For calculation of tilt in cells, z-stack images were taken with a z-spacing of 0.5–1.0 μm and normalized as required. The distance between the poles was estimated by using ImageJ and then the spindle tilt was calculated as described by Delaval et al. The intensity or spread (i.e., area) was calculated using ImageJ. For analysis of long aster MTs, the length of aster rays was measured and the ratio of the length of aster ray to the cell diameter was determined. The central value of the ratio for control cells of 0.15 was taken as a cutoff mark. The number of cells with aster length to cell diameter ratio of >0.15 was calculated and plotted.

Conflict of Interest
The authors declare no conflict of interest.

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MINIREVIEW

Ubiquitin in regulation of spindle apparatus and its positioning: implications in development and disease

Devika Srivastava and Oishee Chakrabarti

Abstract: Emerging data implicates ubiquitination, a post-translational modification, in regulating essential cellular events, one of them being mitosis. In this review we discuss how various E3 ligases modulate the cortical proteins such as dynein, LGN, NuMa, Gor, along with polymerization, stability, and integrity of spindles. These are responsible for regulating symmetric cell division. Some of the ubiquitin ligases regulating these proteins include PARK2, BRCA1/BARD1, MGRN1, SMURF2, and SIAH1; these play a pivotal role in the correct positioning of the spindle apparatus. A direct connection between developmental or various pathological disorders and the ubiquitination mediated cortical regulation is rather speculative, though deletions or mutations in them lead to developmental disorders and disease conditions.

Key words: mitosis, ubiquitination, spindle positioning, cortical regulation, spindle integrity.


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Mots-clés : French, French, French, French, French.

Introduction

One of the earliest events that occur in a cell, post fertilization, is rapid cell division. It serves two purposes: (i) a proliferative function to increase in cell number and (ii) gives rise to cellular diversity in the growing organism. While the former is taken care of by symmetric cell division, asymmetric cell division serves to generate cells of different lineages. Errors that are incorporated during this stage get permanently incorporated in the cell. Aberrations during cell division can lead to multiple problems that range from developmental anomalies, defective tissue organization, and even uncontrolled cell proliferation leading to cancer. Cell division is under complex regulation from multiple players such as DNA compaction, regulation of cyclin/CDKs, and check points. Another important determinant of cell division that ensures normal growth and development of an organism, and simultaneously also determines the future of daughter cells, is the axis of cell division (Gönczi 2008; Morin and Bellaïche 2011; Gillies and Cabernard 2011).

Orientation of cell division establishes the content, position, and fate of daughter cells within tissues. First, this helps generate elongated cell sheets and define tissue shapes. Secondly, it determines cellular diversity. To achieve these two mutually non-exclusive functions, the orientation of the mitotic spindle needs to be controlled. Oscar Hertwig first recognized that spindle orientation and cell division orientation were instrumental in determining cell shape; he further discovered that cells divide along their long cell axis, an observation known as the ‘long axis rule’ (Hertwig 1884). Symmetric cell division refers to the equal partitioning of cellular constituents, giving rise to two identical daughter cells. On the other hand, asymmetric cell division gives rise to two non-identical daughter cells with unequal partitioning of cellular constituents.

Depending on their spatial orientation, shape, structure, and function, cells may be broadly classified into two categories – polarized and non-polarized. During the early stages, post fertilization in mammals, rapid cell division is observed. These cells are pluripotent stem cells that undergo symmetric cell division to give rise to clonal population of cells. At some point during this clonal expansion, polarity is established using external and internal cues. After this stage, the stem cells undergo both symmetric division (for self-renewal) and asymmetric cell division (to give rise to differentiated cells of various lineages) (Fig. 1a). The F1 differentiated cells may be polarized or non-polarized in nature (Gonczy 2008; Knoblich 2008; Morin and Bellaïche 2011; Gillies and Cabernard 2011; Williams and Puch 2013; Lu and Johnston 2013). Non-polarized cells typically undergo symmetric cell division that serves to maintain cell number and homeostasis in an organism (Siller and Doe 2009). However, under the influence of external cues or improper spindle positioning, these might be able to divide with unequal partitioning of cellular constituents (i.e., asymmetric cell division) (Lancaster and Baum 2011) (Fig. 1b). In the case of polarized cells, the axis of division becomes a defining feature for the shape and characteristics of the daughter cells generated. Cells divide along the apico–basal axis to give rise to cells that belong to different niches. At an organismal level, this acts as one of the driving forces that guide the formation of the neural tube or alimentary canal from the polarized neuronal or...
Polarized cells can also undergo division along the anterior–posterior axis, and this division is governed by planar cell polarity (PCP pathway) (Simons and Mlodzik 2008; Devenport 2014; Morin and Bellaïche 2011) (Fig. 1c). A battery of proteins and their gradients define the plane of the axis of division. These proteins determine the position and plane of divisional axis, which in turn gives rise to symmetric or asymmetric cell division. Both these divisions occur in the case of polarized cells, based on external and internal cues. While the discussion on the effect of external cues is beyond the scope of the present discussion, the internal cues regulating the same are discussed briefly in this section.

Central to the machinery that regulates cell division axis and spindle positioning are partitioning family cell polarity regulator proteins. These determine the axis of division and gradient of proteins governing the same. Besides these, there are a host of cortically enriched proteins that assist in the correct division by translating the external and internal cues into cortical forces. These molecular interactions at the cell cortex in turn translate into the force necessary to hold the spindle apparatus in place and along the thus determined axis of division. The force required to stabilize the spindle apparatus in correct position and orientation, is generated by the interaction of astral microtubules with the cortical proteins and minus end-directed motor protein, dynein. Dynein is recruited to the membrane by the help of an evolutionarily conserved ternary complex of proteins consisting of NuMa, LGN, and G/H9251 subunit of the trimeric G-protein complex (Lin-5/GPR-1/2/G/H9251, respectively, in worm and Mud/Pins/G/H9251 in flies) (Du and Macara 2004; Siller and Doe 2009; Stevermann and Liakopoulos 2012; McNally 2013). Dynein serves as an anchor to maintain a connection with the depolymerizing microtubule, which then exerts the force necessary to position the spindle apparatus (Kotak et al. 2013) (Fig. 1d). The details and intricacies of this process are discussed in the course of this review in the context of different mitotic defects leading to disease conditions. Recent reports suggest that proteins participating in this regulation are mitotic motor protein KIF15 (kinesin superfamily protein), Eg5 (also known as KIF11) (van Heesbeen et al. 2014), certain RanGTP and CLASP (Cytoplasmic linker associated protein 1) proteins (Bird et al. 2013). While CLASP1 is required for correct

![Fig. 1. Types of cell division in the various cell types in the body. (a) Schematic diagram showing the relevance of symmetric/asymmetric division in stem cells. (b) This shows one of the possible ways by which external cues could cause erroneous spindle positioning in the cell leading to asymmetric division in non-polarized cells. Internal cues, like altered distribution of the cortical regulators, could also give rise to a similar situation. (c) Symmetric and asymmetric division in case of polarized cells is shown. While the former is seen along the anterior–posterior axis, division along the apico–basal axis leads to asymmetric cell division. (d) An overview of different cortical players important for spindle orientation. LGN acts as an adaptor protein that holds NuMa and G/H9251. This ternary complex recruits dynein at the cell cortex with the help of motor proteins. NuMa helps establish a cortical zone enriched in dynein by binding to it. Dynein then binds the astral microtubules and regulates spindle positioning in cells. Polo Kinase 1, present on spindles, serves to negatively regulate the cortical dynein localization, while the RanGTP gradient present on the chromosomes negatively regulates NuMa/LGN distribution in the lateral cell cortex. It is the cooperative balance of these two gradients that positions the cortical proteins in the right place, thus ensuring correct spindle positioning. Further, players like CLASP1 (not shown in the figure), belonging to a family of microtubule associated proteins (MAPs), are involved in the correct capture of the astral microtubules and their attachment at the cell cortex.

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capture of astral microtubules, RanGTP and PLK1 ensure that the ternary complex is restricted to only the cell cortex and not the membrane area in vicinity of the metaphase plate. RanGTP has also been shown to promote astral microtubule elongation along the long axis of cells in Xenopus egg extracts (Zhang et al. 1999). Another aspect that is essential for the proper functioning of these cortical regulators is the integrity of the spindles formed during mitosis. If these fail to form stable structures during mitosis, no amount of cortical regulation can ensure accurate cell division.

Our understanding of the importance of post-translational modifications that regulate cortical proteins, spindle, and astral microtubule polymerization and positioning is still premature. In this review, we discuss the emerging importance of ubiquitination in modulation of the molecular players affecting the orientation of cell division, leading to pathological abnormalities and disease conditions during their functional inactivation.

**Microtubule assembly regulators**

**Ubiquitin E3 ligases**

The covalent attachment of ubiquitin to specific protein substrates is achieved by the activity of E3 ubiquitin ligases. These ligases are divided into multiple classes defined by the structural elusiveness of their E3 functional activity. They are broadly classified into HECT (homologous to E6-AP C-terminus)-family E3 ligase and RING (really interesting new gene) domain and cysteine residue usually restricted to HECT family. Hence these ligases are also known as ‘RING/HECT hybrid’ enzymes (Wenzel et al. 2011; Stiegltz et al. 2012).

**PARK2**

Parkin (PARK2), an RBR ligase is widely implicated in the onset and progression of Parkinson’s disease. Numerous reports suggest the dysregulation of the ubiquitin proteasome system (UPS) in the pathogenesis of Parkinson’s disease (Hristova et al. 2009). Recent studies show a more detailed and direct influence of PARK2 on cell division (Sun et al. 2013). Studies with pancreatic tumor samples from patients exhibit reduced expression levels of PARK2. Various mitotic defects were detected in these cases, viz., a-vis multiple spindle formation, chromosomal instability, spindle tilt along the axis of division, and increase in the vertical distance between the spindle poles. These observations could be replicated in pancreatic cancer cell line, EPP85, with the depletion of PARK2. These observations indicate an obvious involvement of PARK2, direct or indirect, in cell division (Sun et al. 2013). It has been hypothesized that PARK2 modulates proteins critical for mitotic spindles via its regulation of the microtubule motor protein Eg5 expression (Sun et al. 2013). Eg5 is a bipolar, plus-end-directed microtubule motor protein (Tanebaum and Mitchison, 1995; Whitehead et al. 1996), while in Drosophila embryos, its homolog does not have a similar role in prophase (Sharp et al. 1999). Moreover, even in mammalian systems, prophase centrosomes have been suggested to move independently of each other without the involvement of Eg5 (Waters et al. 1993). Hence, the importance/function of Eg5 in bipolar spindle assembly is far from clear.

Thus, though there is evidence for the regulation of spindle tilt by PARK2, the exact mechanism still needs to be clearly understood. It is plausible that PARK2 regulates spindle tilt and pole formation in an Eg5 independent manner.

**BRCA1/BARD1**

Germline mutations in breast cancer-associated gene 1 (BRCA1) have been associated with familial breast and ovarian cancers. Further, downregulation of BRCA1 has been reported in sporadic breast and ovarian cancers. BRCA1 undergoes heterodimerisation with BRCA2-associated RING domain protein 1 (BARD1) (Wu et al. 1996). The RING domains of BRCA1 and BARD1 form a dimerisation interface, and the BRCA1/BARD1 heterodimer functions as an ubiquitin ligase (Parvin 2009). This has been shown to participate in cell proliferation and chromosome stability.

Homozygous Braf knockout mice die in utero between 10 and 13 days of gestation (E10–E13). These mice have abnormalities in the neural tube, with 40% of the embryos showing varying degrees of spina bifida and anencephaly. Further, the neuroepithelium in Braf-deficient embryos also seem to be disorganized (Parvin 2009). At the cellular level, BRCA1 and BARD1 are required for the centrosome during the cell cycle (Sankaran et al. 2005). Embryonic fibroblasts from Braf knockout mice show centrosome amplification and genome instability (Kais et al. 2012). BRCA1 is known to bind to γ-tubulin, a major component of centrosomes. Studies in Xenopus egg extracts and HeLa cells show that this BRCA1/BARD1 complex then mono-ubiquinates γ-tubulin on Lysine 48 (K48) and K44 (Joukov et al. 2006). The BRCA1/BARD1 ubiquitin ligase activity regulates centrosome number and function. Further, BRCA1/BARD1-dependent ubiquitination activity modulates the microtubule nucleation and centrosomal hyperplasty (Sankaran et al. 2005). Cells with depleted BRCA1/BARD1 show suppressed aser formation in interphase (Tarapore et al. 2012). BRCA1/BARD1 is also required for mitotic spindle pole assembly and for the accumulation of Tpx2 (targeting protein for Xklp2), a major spindle organizer and a downstream target of Ran-GTP, on spindle poles. This function is centrosome independent, operates downstream of Ran GTPase, and depends upon BRCA1/BARD1 E3 ubiquitin ligase activity (Daniels et al. 2009) (Fig. 3).

As indicated earlier, the microtubule aster polymerization and spindle pole assembly is an important player that regulates the positioning of the spindle apparatus in the center of the cell, along the planar axis of division during in case of polarized cells. These observations about BRCA1/BARD1 can thus be extrapolated to explain the increased propensity for breast cancer in individuals who harbor mutations in these genes. BRCA1/BARD1 is known to localize to centrosomes during metaphase, where this ubiquititates and degrades Aurora B (Ryser
et al. 2009). The same study has elucidated that various cancer
cells express RING-deficient isoforms of BARD1, called BARD1/H9252.
This isoform can have BRCA1-independent functions (Ryser et al.
2009). It has been shown that BARD1/H9252 localizes to the mid body at
telophase and cytokinesis, where it associates with and stabilizes
Aurora B, a protein crucial for mid body formation and abscission
(Delaval et al. 2004; Ryser et al. 2009). Thus, the elevated levels of
BARD1/H9252 and the imbalance between the BARD1/BARD1/H9252
levels in cancer cells could partially contribute to their increased rate of
cell division.

VHL
von Hippel-Lindau disease, an inherited cancer syndrome is as-
associated with an E3-ligase, VHL (von Hippel-Lindau tumor sup-
pressor) in which patients are predisposed to develop various
vascular tumors and clear cell renal cell carcinomas (cRCC), high-
lighting distinct roles for VHL in tissue morphogenesis and tumor-
genesis (Kaelin 2007). It is the substrate recognition component of
the cullin-RING ubiquitin ligase complex (CRL) that includes
cullin-2, Rbx 1, and elongins B and C (Deshaies and Joazeiro 2009;
Kaelin 2008). VHL is best characterized for its role as a key com-
ponent of an E3 ubiquitin ligase that targets hypoxia-inducible
factor (HIF) for degradation (Kaelin 2008; Jaakkola et al. 2001). The
homozygous knockout of Vhl leads to embryonic lethality at E14.5
to E18.5. Heterozygous knockout mice, however, appear normal
in their development, growth, and reproductive (Zhang et al.
2012).

VHL is known to play a role in spindle orientation, as its
knockdown in MEFs and HeLa causes spindle misorientation by
eliminating astral microtubules. The defects observed by VHL
downregulation bear striking resemblance to that of APC (adeno-
matus polyposis coli) inactivation, which includes compromised
spindles and misaligned chromosomes (Thoma et al. 2009). One of
the possible reasons might be that VHL positively regulates MAD2
(mitotic arrest deficient 2) levels in cells (Thoma et al. 2009; Liu
et al. 2011). MAD2 is a member of mitosis checkpoint complex at
the kinetochore, where it interacts with other proteins like Bub1
(budding uninhibited by benzimidazoles 1 homolog). MAD2 re-
duction is seen to cause aneuploidy in cells, an observation widely
prevalent in VHL-defective cells as well. This is consistent with the
known contribution of VHL to microtubule stability and cell cycle
regulation. Further, VHL may be required for the degradation of
incorrectly folded tubulin, suggesting that VHL could also contribu-
to MT dynamics (Deshgehy et al. 2006).

MGRN1
Mahogunin ring finger-1 (MGRN1) was identified by positional
cloning of mahoganoid (md) (Miller et al. 1997), a mutation char-

Fig. 2. PARK2 and spindle defects. Schematic of the possible mode of action of PARK2 by which it affects spindles and causes spindle
multipolarity.

Fig. 3. BRCA1: centrosomal and spindle defects. Summary of the mode of action of BRCA1 by which it affects spindles and causes genomic
instability.
SIAH1

Besides the above discussed E3-ligases, there are several other ubiquitinating enzymes that regulate spindle positioning and thus might be potential players in cancer. The human homologue of the Drosophila seven in absentia (Sina) (SIAH1 and SIAH2), have been implicated in ubiquitin-mediated proteolysis of different target proteins and are known to regulate HIFs protein stability. SIAH1 and SIAH2 bind propyl hydroxylases (PHD1 and PHD3) and target them for proteasomal degradation (Nakayama et al. 2004). Double knockout mice for Siah1 and Siah2 are not viable, but single knockouts are able to live. Growth in Siah1 knock-out mice is severely retarded, along with poor bone formation (House et al. 2009). Also, Siah1 null mice have poor viability and display a meiotic block at metaphase I that impairs spermatogenesis and causes sterility in male mice (Dickins et al. 2002). Murine Siah-1 is a P53-induced gene; its human homolog, SIAH-1, is activated during tumour suppression and p53-induced apoptosis. When overexpressed, it has been shown to inhibit cell growth by causing defects in mitosis and increasing apoptosis in breast cancer cell line, MCF-7. Mitotic defects include anomalies of the mitotic spindle and abnormal chromosomal segregation, multinucleate giant cells along with failure of cytokinesis. Established targets of SIAH1 include α-tubulin and Kid (kinesin like DNA binding protein, also known as KIF22), identified by yeast two hybrid assays (Germani et al. 2000). While the significance of interaction with α-tubulin is mostly speculative, it has been shown to ubiquitinate and degrade Kid. Kid has been proposed as a linkage regulator of chromosome movement along microtubules during mitosis. Reports showed that the Xenopus homologue of Kid (Xkid) is the motor protein that provides the necessary force to push chromosome arms toward the equator of spindle. Its degradation is necessary for anaphase chromosome movement. These observations provide a plausible explanation for the mitotic anomalies observed in the absence of SIAH1 (Antonio et al. 2000; Funabiki and Murray, 2000; Germani et al. 2000).

Interestingly, SIAH1 is also shown to polyubiquitinate microtubule plus-end tracking proteins (+TIP) binding protein EB3 (end binding protein 3), and mediate its degradation through the ubiquitin-proteasome system. EB3 has been shown to facilitate cell cycle progression at prometaphase and is downregulated during the transition to G1 phase in cell culture-based studies (Ban et al. 2009). Thus, the interaction between SIAH1 and EB3 could be used as an alternative explanation for the observed mitotic defects and spindle anomalies.

SMURF2

Another interesting ligase is SMURF2 (Smad specific ubiquitin regulatory factor 2), which is a HECD E3 ligase, originally characterized as a negative regulator of the TGF-β signaling pathway by targeting receptors, signaling intermediates, and other pathway-specific transcription factors for degradation (Tang et al. 2011). Smurf2−/− mice (>99%) do not exhibit any pronounced developmental defects during embryogenesis, and they are phenotypically indistinguishable from their wildtype or heterozygous counterparts.

Fig. 4. MGRN1 and spindle tilt. An overview of the effect of MGRN1 on spindles and its plausible mode of action.
littermates at weaning (Lin et al. 2000). Surprisingly, mice mutant for both Smurfl and Smurfr display planar cell polarity (PCP) defects in the cochlea and abnormalities in convergence and extension movements (CE), which include a failure to close the neural tube. Further, it has also been illustrated that SMURF1s are involved in non-canonical Wnt signaling pathway via ubiquitin-mediated degradation of the core PCP protein Prickle1 (Narimatsu et al. 2009).

SMURF2 exhibits a dynamic localization pattern throughout mitosis, moving from centrosomes in late prophase and metaphase, to the mitotic midzone in anaphase, and ultimately to the mid body in telophase. Study shows that SMURF2 participates in post-translational control of MAD2 (mitotic arrest deficient 2), stabilizing the same. SMURF2 depletion results in enhanced polyubiquitination and degradation of MAD2. MAD2 mislocalizes in SMURF2-depleted cells, suggesting that SMURF2 regulates the localization and stability of MAD2, thus regulating spindle check point. In addition to its role as a regulator of the spindle checkpoint, the localization of SMURF2 to other critical mitotic structures implies its participation in other aspects of mitotic control. Further studies would be required to establish this hypothesis (Osmundson et al. 2009).

TRIM E3 ligases
A family of E3 ligases worth mentioning is TRIM family (the conserved arrangement of a RING-finger domain, B-box, and coiled-coil domains). Several members of this family of ligases have been implicated in disease conditions namely, MID1 (Midline-1), TRIM5α (triptapite motif protein S-5), and Xnf7 (Xenopus nuclear factor 7). While TRIM5 mediates resistance to the human virus in rhesus monkey cells (Stremlau et al. 2004), MID1 is implicated in developmental disorder, Opitz Syndrome, an inborn error of cholesterol synthesis (Cow et al. 2000). Xnf7 (Xenopus nuclear factor 7), discovered from Xenopus egg extracts as an inhibitor of the anaphase promotor complex (APC), is a maternally expressed nuclear protein that is retained in the cytoplasm from oocyte maturation until the midblastula transition (MBT) and is shown to be involved in dorsal/ventral patterning (El Hodiri et al. 1997). Xnf7 has not yet been implicated in disease conditions. Although both MID1 and Xnf7 have E3 ligase activities, their roles in governing spindle dynamics do not involve this enzymatic activity. These proteins bind to microtubules and drive their bundling, thus acting as key players in the regulation of spindle dynamics (Maresca et al. 2005).

Other UPS associated proteins regulating spindle dynamics
Huntingtin
Huntingtin (HTT), implicated as the foremost player in the onset and progression of the severely debilitating neurodegenerative disease, Huntington’s disease (HD), is also suggested to modulate spindle assembly and orientation. HD is a neurodegenerative disorder characterized by severe psychiatric, cognitive, and motor defects and selective neuronal death in the brain, caused by HTT gene. Although the mechanisms leading to disease are not completely known, increasing evidence indicates that in addition to the gain of new toxic properties, loss of wild-type HTT function also adds to pathogenesis (Cattaneo et al. 2005). Various studies in cell models and with patient samples have reported that the UPS is impaired in HD, which could be the underlying cause of the neurotoxicity.

HTT is widely expressed in the early embryonic stages in mice, where it plays an important role in several processes including cell differentiation and neuronal survival. Inactivation of the mouse gene results in developmental retardation and embryonic lethality at E7.5 (Nasir et al. 1995; Zeitlin et al. 1995). Studies have now conclusively shown that HTT is involved in neurogenesis and brain development (Goddin et al. 2010).

Reports suggest that HTT is ubiquitinated and that it also interacts with a ubiquitin conjugating enzyme E2 (Kalchman et al. 1996). The ubiquitinated species detected may be parts of either processed or partially degraded forms of HTT. Studies in SHSY5Y cells show that stable expression of long polyglutamine tracts lead to cell cycle arrest and inhibition of proteasomal degradation pathway. The impaired proteasomal activity does not elevate protein aggregation under basal conditions, however it severely compromises the ability of the proteasome to respond to stress leading to increased stress-induced protein aggregation. This is further accompanied by altered expression of the 20 s proteasomal subunits (Ding et al. 2009).

Recent studies have shed a lot of light on the function of HTT in the cell. Wild type HTT is shown to interact with microtubules, the dynein/dynactin complex, and kinesin to regulate the microtubule-dependent transport of organelles in neurons (Caviston et al. 2007, McGuire et al. 2006). HTT is found in high levels in dividing cells, where it associates with the centrosomal region and microtubules (Hoffner et al. 2002). Interestingly, HTT has been shown to be required in murine neuronal progenitors for appropriate spindle orientation and for cell fate determination (Goddin et al. 2010). Also, it has been demonstrated that mutant forms of HTT disturb the cortical localization of proteins like dynein, NuMa (Molina-Calavita et al. 2014). A study in mammmary cells throws light on the mechanistic cause of the mitotic abnormalities observed earlier. This study shows that HTT is not only important for bipolar spindle assembly but also for the cortical localization of the dynein/dynactin/NUMA/LGN complex. Thus HTT may be a connecting point between the microtubule cytoskeleton and the nucleus (Caviston et al. 2007). Recent studies indicate that KIF5 is a molecular plus-end motor, which in association with HTT regulates the trafficking of the dynactin/dynein/NUMA/LGN complex along the astral microtubules to the cell cortex during mitosis. Localized at the cell cortex, the dynactin/dynein/NUMA/LGN complex generates pulling forces on astral microtubules for mitotic spindle positioning in the center of the cell (Elias et al. 2014).

Yet unclear, it will be interesting to closely study the role of HTT and see if there is any direct or indirect connection between its influence on the regulation of UPS cells, the subsequently reported cell cycle inhibition, and the regulation of the spindle apparatus.

Deubiquitinases
Besides the ubiquitin ligases, the deubiquitinating enzyme, ubiquitin C-terminal hydrolase L1 (UCH L1) also plays a significant role in modulating microtubule dynamics. UCH L1 is a cysteine hydrolase that contains a typical active-site triad of cysteine, histidine, and aspartic acid and catalyzes hydrolysis of C-terminal esters and amides of ubiquitin. This enzyme is expressed in abundance in brain and reproductive tissues (Setsue and Wada 2007). Reports indicate that mutations in the UCH L1 gene have been involved with Parkinson’s and Alzheimer’s diseases (Betarbet et al. 2005). Recent studies indicate that UCH L1 is a multi-functional protein: besides being a deubiquitinating enzyme, UCH L1 dimer has an ubiquitin ligase activity in vitro. It has been implicated in mitosis by studies in cell culture systems, where it binds to tubulin and regulates microtubule dynamics by ubiquitination (Bheda et al. 2010).

The deubiquitinating enzyme cyclinomatosis (CYLD) specifically removes lysine 63 (K63)-linked polyubiquitin chains and is a regulator of microtubule dynamics (Gao et al. 2008; Haraj and
Fig. 5. A summary of the different target proteins/structures regulated by ubiquitination in the cortex of the cell during mitosis. Ligases like MGRN1, VHL, Xnf7, and SIAH regulate microtubule assembly or polymerization, while BRCA1/BAARD1 regulates the centrosomal apparatus. PARK2 and HTT affect microtubule motors, thus in turn regulating the correct localization of the cortical effectors of mitosis.

Dixit 2011). It stabilizes astral microtubules and stimulates the formation of the Dvl (disheveled)-NuMa-dynein/dynactin complex at the cell cortex in HeLa cells and mice models. The deubiquitination of Dvl enhances its interaction with nuclear mitotic apparatus, stimulating its cortical localization in association with the dynein/dynactin motor complex. Thus generates pulling forces on astral microtubules (Yang et al. 2014). These studies suggest a role for CLYD in tumor progression, as it is well known that the tumor suppressor protein is mutated in familial cylindromatosis and multiple familial trichoepithelioma, genetic conditions associated with the development of skin-appendage tumors (Massoumi 2011).

Conclusion

From the few E3 ligases discussed above, it is evident that these proteins play an important role in cell division. This review only highlights the importance of the ubiquitin E3 ligases that directly interact with or alter mitotic spindle generation, positioning, and orientation (Fig. 5). It is essential to point out that there is an elaborate list of other E3 ligases, like APC and Skp, Cullin, F-box containing complex (or SCF complex), which also affect cell cycle progression and mitosis, either directly by modulating the cell cycle checkpoints or indirectly by altering the functions of other MT interacting proteins. Surprisingly, while all the ligases discussed in this review are crucial for successful mitosis, all of them except BRCA1 and VHL are non-essential — in other words their loss of function does not lead to embryonic lethality. One probable explanation could be a phenomenon similar to what is observed in Drosophila during development, “telophase rescue”. It redistributes fate determinants in accordance with spindle orientation just before cytokinesis in the majority of mutant neuroblasts, irrespective of the AB polarity axis. Mutations in mud (homolog of NuMa present in Drosophila) are only able to cause defects in spindle orientations but not affect the polarity axis during division. The mechanism of telophase rescue are not entirely clear (Schober et al. 1999; Wodarz et al. 2000; Peng et al. 2000; Neumüller and Knoblich, 2009; Morin and Bellaiche 2011). It is plausible to hypothesize that similar salvage mechanisms exist in mammalian systems as well, thus ensuring correct division during development. Alternatively, it also raises the possibility of functional redundancy between the isoforms of the proteins and amongst different types of proteins as well. Though it may not have been studied in depth in the context of the ubiquitin E3 ligases, this concept is not new as there exists examples of functionally redundant proteins in the genome (Kafri et al. 2006). It has been shown that responsive backup circuits may function as devices for filtering non-genetic noise from transcriptional pathways, thus rendering an added layer of protection to the organism against stress and mutations (Massoumi 2011). More extensive genome-wide studies are required to prove that similar backup circuits exist with respect to E3 ligases, as they may seem functionally irreplaceable at the cellular levels, yet they are non-essential at the organismal level during development.

References


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