CHAPTER 3
MGRN1: NOVEL INTERACTOR OF $\alpha$-TUBULIN AND IMPLICATIONS IN CELL DIVISION
3.01 INTRODUCTION

Although vertebrates exhibit bilateral symmetric externally, most internal organs, such as the heart, viscera, and brain display asymmetric structure and/or unilateral positioning with respect to the left–right (LR) axis. A common defect in LR patterning is the loss of conformity among the sidedness of individual organs known as heterotaxia. In human beings, abnormalities in the proper development of laterality occur in more than 1 in 8,000 live births and often have significant medical consequences (Desai and Mitchison, 1997).

Until recently, it was believed that the presence of cilia-driven extracellular fluid flow (Spe´der et al, 2007; Gros, 2009) or recruitment of ‘ciliary’ proteins, such as left–right dynein (Levin and Palmer, 2007; Klar, 2008) acts as the factor governing this asymmetry. However, a very recent report has shown in the plant model system (Arabidopsis thaliana) that mutations in α-tubulin and in a γ-tubulin-associated protein (Tubgcp2) play an important role in the symmetry properties of the plant’s axial organs (Starita et al, 2012). Wild-type A. thaliana axial organs do not twist during normal elongation, and its flowers are radially symmetrical. This symmetry can be broken by mutations in tubulin and tubulin-associated protein complexes. The tubulin mutations (spiral1, spiral2, and spiral 3) produce right-handed helical growth mutants. Lefty (lefty1 and lefty2) mutants have been shown to be suppressor mutants of spiral1, and when outcrossed, displayed a prominent left-handed helical growth (Thitamadee et al, 2002; Abe et al, 2004; Lobikin et al, 2012). Both α-tubulin and γ-tubulin complexes are ubiquitous in eukaryotes and involved in the formation and nucleation of microtubules. Recent report shows a cilia-independent role of tubulin in the generation of LR asymmetry. Similar reports, indicating the importance of tubulin in LR axis of symmetry has also been found in nematodes and frog embryos. This raised the possibility that alterations in α-tubulin might as well have a drastic effect on the LR axis of symmetry (Lobikin et al, 2012).
The craniofacial patterning defects, congenital heart defects, mis-patterning of the left–right (LR) body axis and *situs inversus* in some of the *Mgn1* null mice (Cota et al, 2006; Jiao et al, 2009) suggests that MGRN1 could possibly play a role in determination of the LR body axis. My study shows that the modulation of α-tubulin by MGRN1 hints at a plausible explanation for these observations.

My work involves a serendipitous discovery of an interaction between MGRN1 and the cytoskeletal protein, α-tubulin; and its effect on the axis of cell division during mitosis. Depletion of MGRN1 in cells affects ubiquitination of α-tubulin, altering its polymerisation state and not the protein turnover. My analysis identifies a unique mode of ubiquitination of α-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 fibres.

3.02 OBSERVATIONS

3.02.01 Interaction of endogenous MGRN1 with α-tubulin in mitotic cells. MGRN1 shows a typical punctate pattern of expression of endogenous MGRN1 with an obvious nuclear exclusion in interphase cells. I observed that this localization of MGRN1 gets altered and it decorates mitotic spindles in dividing cells (Figure 3.1 A). This pattern of close association of MGRN1 with mitotic apparatus could be detected through the different stages of mitosis (M phase) (Figure 3.1 B).
Figure 3.1: Mahogunin localizes at the spindles during mitosis

(A) Unsynchronized HeLa cells immunostained for endogenous MGRN1 show distinct nuclear exclusion during interphase but decorate the mitotic spindles in dividing cells.

(B) Endogenous MGRN1 localizes on the spindle apparatus through different stages of mitosis. (Thin arrow, DNA stained with propidium iodide; thick arrow, MGRN1 staining on the mitotic spindles).

I proceeded to study this interaction by biochemical and imaging techniques in various cell lines. Co-immunoprecipitation studies to validate the interaction of MGRN1 and α-tubulin in G2/M synchronised cells indicated an interaction between MGRN1 and α-tubulin in HeLa cells (Figures 3.2 A and B) via the C-terminus of MGRN1 (Figure 3.2 C).
Figure 3.2: Mahogunin interacts with α-tubulin (Biochemical evidence)

(A) Synchronized HeLa cells were lysed and immunoprecipitated with anti-α-MGRN1 antibody. Immunoblotting with anti-α tubulin antibody shows co-immunoprecipitation between the two proteins. Ab denotes antibody; ## IgG light chain.
(A) Synchronized HeLa cells were lysed and immunoprecipitated with anti-α-tubulin antibody. Immunoblotting with anti-MGRN1 antibody shows co-immunoprecipitation between the two proteins. Ab denotes antibody; ## IgG light chain.

(B) HeLa cells transiently transfected with the indicated GFP-tagged MGRN1 constructs were synchronized, lysed and immunoprecipitated with anti-GFP antibody. Western blots analysis of this with anti-α-tubulin antibody shows co-immunoprecipitation of α-tubulin with MGRN1, MGRN1ΔR and MGRN1ΔN but not MGRN1ΔC (top panel). Reverse immunoblot control (bottom panel). ▲ marks α-tubulin; * non-specific band; # IgG heavy chain.

Co-immunocytochemistry also showed their pronounced co-localization on mitotic spindles in HeLa (Figure 3.3 A) and mitotic HEK293T (human embryonic kidney 293 transformed with T antigen) cells (Figure 3.3 B), suggesting a cell line-independent phenomenon. For further experiments, I tried to establish stable cells expressing full-length MGRN1 or catalytically inactive MGRN1, lacking the RING domain (MGRN1ΔR). However, three attempts to establish stable cell lines expressing catalytically inactive MGRN1 failed (data not shown); on the contrary, I could easily establish stable cells expressing full-length MGRN1 (Figure 3.3 C). The co-localization between MGRN1 and α-tubulin was observed in stable cells, expressing MGRN1 as well.

Furthermore, co-immunoprecipitation of melanocytes (Hida et al, 2009) (Figure 3.4 A) demonstrated an interaction between MGRN1 and α-tubulin in melan a-6 (control) cells when compared with melan md1-nc (Mgrn1md-nc or Mgrn1 null) cells (Figure 3.4 B, C). These observations led to the following plausible hypotheses – either association with MGRN1 regulates the turnover of α-tubulin or MGRN1 ubiquitinates α-tubulin to affect its function.
Figure 3.3: Mahogunin interacts with α-tubulin (Immunocytochemistry evidence)

(A) Synchronized population of HeLa cells immunostained for MGRN1 and α-tubulin show co-localization between the two proteins on the mitotic apparatus. The channels for acquiring the images are indicated. Two representative cells (i) and (ii) are shown.

(B) Co-localization 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.
(C) HeLa cells stably transfected with MGRN1 show a pronounced co-localization with α-tubulin in mitotic cells, irrespective of the stage of cell division. Inset (i) represents metaphase cell while inset (ii) is an anaphase cell.

**Figure 3.4:** Mahogunin interacts with α-tubulin as seen in melanocytes

(A) Bright field images of melan a-6 control cells and melan md1-nc cells.

(B) 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 since IgG heavy chain also migrates at ~55KDa, a band (of lower intensity) is detected even in melan md1-nc cells. ▲ marks MGRN1; Ab denotes antibody ; # IgG heavy chain, ## IgG light chain.
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). Ab denotes antibody; ## IgG light chain

3.02.02 Catalytically inactive MGRN1 affects the axis of cell division. To study the functional significance of the interaction between MGRN1 and α-tubulin, cells were transiently transfected with full-length MGRN1 (referred to as MGRN1) or a catalytically inactive MGRN1, lacking the RING domain (MGRN1ΔR), or with functionally inactivating point mutations of the RING domain (C316DMGRN1, C299EMGRN1). Surprisingly, HeLa cells transiently transfected with MGRN1ΔR were able to complete the M phase of cell cycle with a mitotic index comparable to those expressing either cytosolic green fluorescent protein (GFP) or MGRN1 (Figure 3.5).

However, they displayed spindle misorientation along the z-axis, (Delaval et al, 2011) one of the aberrations, leading to defects in spindle positioning. The spindle angle relative to the cell substrate adhesion plane for MGRN1ΔR mutant-expressing cells (~55–70%,
depending on the construct) was >10°, whereas control spindles were usually parallel to the substratum (Figures 3.6 A–D; Figure 3.7). While an angle mostly around 10° was observed for control cells, the average angle upon functional inactivation of MGRN1 was significantly higher (between 15° and 30°), indicating aberrant spindle orientation relative to the substratum (Figures 3.6 B and D).
Figure 3.6: Functional depletion of MGRN1 results in tilt in the axis of cell division in cells over expressing MGRN1 or its inactive mutants.

(A) HeLa cells transiently transfected with the indicated constructs were synchronized and immunostained for α-tubulin. Mitotic cells positive for GFP expression were imaged by taking z-sections. The images are representative mid-sections (of the z-stacks) from at least 30 cells for each of the constructs. A larger proportion of cells expressing catalytically inactive MGRN1 have angle of tilt ≥10°, compared to the control. LUT images are represented to efficiently locate the spindle poles.

(B) Histogram plotting the distribution and average of the angles of tilt for cells transfected with cytosolic GFP (control), MGRN1ΔR-GFP or C316DMGRN1-GFP. Over 30 cells were analysed as in panel A are represented for each of the constructs. Graph shows results from 3 independent experiments. ** p≤0.001, using Student’s t-test. Error bars, SEM.

(C) HeLa cells transiently transfected with MGRN1 GFP (control) or C299E MGRN1 GFP (catalytically inactive MGRN1) were synchronized and immunostained for α-tubulin. Mitotic cells positive for GFP expression were imaged by taking z-sections. Two cells for each construct ((i) and (ii) are shown. The images are representative mid-sections (of the z-stacks) for each of the constructs. A larger proportion of cells expressing C299E MGRN1 GFP have angle of tilt >10°, compared to the MGRN1 GFP control.

(D) Histogram plotting the percentage of cells for each of the angles of tilt as indicated on the X-axis. The histogram on the right shows that the average angle of tilt is more than 20° for cells expressing C299E MGRN1 GFP.
3.7 Tilt in axis of cell division. This figure shows z-stack images of representative cells used to calculate tilt in the axis of cell division. The constructs used are indicated in the figure. In each image, the poles have been marked with a white arrow head (►) when in focus. Note that control cells expressing either cytosolic GFP or MGRN1-GFP have maximum number of images with both poles in focus, for rest of the constructs only one pole is in focus.
This phenotype was even more pronounced when MGRN1 was depleted from cells using small interfering RNA (siRNA) constructs (with knockdown efficiency of ~75%), where spindle tilt of >10° was seen in ≥80% of cells (Figures 3.8 A and B). The fact that I was unable to establish cells line expressing catalytically inactive MGRN1ΔR was indicative of the fact that though no drastic effect of functionally inactive MGRN1 is obvious on the mitotic index or the completion of mitosis in the short term, the effect of spindle misorientation had a cumulative detrimental effect over multiple cycles of cell division.

Figure 3.8: Functional depletion of MGRN1 results in tilt in the axis of cell division in cells depleted for MGRN1.

(A) HeLa cells treated with MGRN1 siRNAs or irrelevant siRNAs (GFP siRNAs) were imaged and analysed as in panel A. Note that MGRN1 knockdown closely phenocopies MGRN1ΔR expression in affecting the orientation of the axis of cell division. The immunoblot and graph shows a knockdown efficiency of ~75%. LUT images are represented to efficiently locate the spindle poles.

(B) Histogram plotting the distribution and average of the angles of tilt for cells treated with MGRN1 siRNAs or irrelevant siRNAs (GFP siRNAs). Over 40 cells were analysed as in panel C are represented for each of the siRNA
The functional significance of spindle misorientation induced by MGRN1 inactivation/depletion was studied next. 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 3.9 A–D). I also evaluated the effect of MGRN1 inactivation on the motor protein, dyenin1, as it is important for spindle pole assembly and orientation (Carminati and Stearns, 1997; Busson et al, 1998; O’Connell and Wang, 2000; Merdes et al, 2000; Godin et al, 2010; Delaval et al, 2011). Immunocytochemistry showed a decrease in dynein staining at the poles when MGRN1 was depleted compared with the control cells (Figures 3.10 A and B).
Figure 3.9: Functional depletion of MGRN1 results in tilt in the axis of cell division

(A) HeLa cells either untransfected (control) or transiently transfected with the indicated constructs were synchronized and immunostained for α-tubulin. Mitotic cells positive for GFP expression were imaged by taking z-sections. The images are representative z-projections of at least 30 cells for each of the constructs. The images were acquired with high detector gains and saturating pixels to enable efficient visualization of the thin aster rays and the limiting boundary of the cells. Note shortening of aster rays in MGRN1ΔR-GFP in comparison with either MGRN1-GFP or control cells. LUT images are represented to efficiently locate the spindle poles and thin aster rays.

(B) HeLa cells treated with MGRN1 siRNAs or irrelevant siRNAs (GFP siRNAs) were imaged and analysed as in panel A. The images were acquired with high detector gains and saturating pixels to enable efficient visualization of the thin aster rays and the limiting boundary of the cells. Note that MGRN1 knockdown closely phenocopies MGRN1ΔR expression in length of the aster rays. LUT images are represented to efficiently locate the spindle poles and thin aster rays.

(C) Histogram plotting the percentage of cells with long asters (ratio of the aster length to the cell diameter > 0.15) upon expression of the constructs as indicated on the X-axis. Over 30 cells were analysed for this. Graph shows results from 3 independent experiments.

(D) Histogram similar to panel C was plotted to check the effect of MGRN1 knock-down on aster length. Note a marked decrease in aster length in the absence of MGRN1. About 30 cells were analysed. Graph shows results from 3 independent experiments.

3.02.03 No change in expression of motor protein dynein. I next checked the effect of MGRN1 inactivation on the motor protein, dyenin1, as it is essential for spindle pole assembly and orientation (Carminati and Stearns, 1997; Busson et al, 1998; O’Connell and Wang, 2000; Merdes et al, 2000; Godin et al, 2010; Delaval et al, 2011). Immunocytochemistry showed a reduction in dynein staining at the poles in cells depleted for MGRN1 compared with the control cells (Figures 3.10 A and B). However, the spread and pattern of distribution of dynein at and around the spindle poles remained very similar in both the cell populations (Figure
These results indicated that the decrease in detectable dynein at the spindle pole might be an indirect phenotype observed due to a reduction in its association with polymerised α-tubulin when MGRN1 is functionally inactive, rather than a direct effect on the inhibition in dynein/dynactin-dependent transport.

Figure 3.10: Interaction of MGRN1 with α-tubulin is independent of motor protein dynein.

(A) HeLa cells treated with MGRN1 siRNAs or irrelevant siRNAs (GFP siRNAs) were co-immunostained for dynein intermediate chain (dynein) and MGRN1; mitotic cells were imaged. Note reduced intensity of detectable dynein with MGRN1 knockdown when compared with the control cells. Both the cell populations were imaged with identical microscope settings. The channels for acquiring the images are indicated.
(B) Histogram plotting the average intensity of dynein on the spindles (in arbitrary units) in cells imaged in panel A. Note a significant decrease in spindle dynein intensity with MGRN1 siRNA treatment; ** ps<0.01, using Student’s t-test. Error bars, SEM. Graph shows results from 3 independent experiments.

(C) Same samples as in panel A were analysed and plotted for the average spread of dynein staining at the mitotic apparatus. This difference in the spread between the cells was not significant (p≥0.1) using Student’s t-test. Error bars, SEM. Graph shows results from 3 independent experiments.
Figure 3.1: MGRN1 affects α-tubulin polymerization

(A) HeLa cells transiently transfected with the indicated constructs were treated with nocodazole and allowed to recover for various time points (30mins, 60mins, 90mins). The cells were immunostained for α-tubulin. Note an increase in ectopic poles with the functional depletion of MGRN1. The channel for acquiring the images is indicated.

(B) Cells imaged in panel A were analysed for the total number of ectopic poles. The ratio of the average number of ectopic poles between MGRN1ΔR/C316DMGRN1 and MGRN1 was plotted for each of the time points. Note that this ratio remains similar over time. Graph shows results from 3 independent experiments.

(C) Additional representative images of cells treated as panel A show the formation of ectopic poles upon nocodazole removal. α-Tubulin immunostaining in red; GFP in green shows expression of the different constructs in the cells.

3.02.04 MGRN1 affects α-tubulin polymerisation. Microtubules are under dynamic equilibrium, undergoing polymerisation and depolymerisation continuously. This principle was used to study the polymerisation of α-tubulin by depolymerising 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 3.11 A, C). 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 3.11 B). 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.

To biochemically establish the interaction between MGRN1 and polymerised MTs, co-sedimentation assay was performed using mitotically enriched HeLa cell lysates. Results indicated that MGRN1 co-pelleted with taxol-stabilized MTs and not with Noc (negative control) (Figure 3.12). Taxol stabilizes and promotes MT polymerisation. When lysates are subjected to treatment with taxol under appropriate conditions in vitro, it co-pellets the proteins that are bound to the microtubules polymerising under such conditions. Lysates treated with nocodazole under similar conditions in vitro act as control, since nocodazole is known to be a microtubule depolymerising agent.

**Figure 3.12: Co-sedimentation assay** Microtubule pulldown assay shows that MGRN1 co-pelleted with taxol-stabilized microtubules in mitotic HeLa cell lysates, indicated by (►). Nocodazole (Noc), inhibition of microtubule polymerization was used as negative control. The blot is a representative of at least 3 experiments.
I next checked the status of endogenous microtubule polymerisation in unsynchronised population in HeLa cells and wild-type mouse embryonic fibroblasts (MEFs; primary cells) which were transiently transfected with the indicated MGRN1 constructs. At 24 h after transfection, the cells were lysed, 1/15th of this was retained for analysis as total lysate control and the rest was separated into soluble [supernatant (S)] and insoluble [pellet (P)] fractions and biochemically analysed to detect any change in α-tubulin polymerisation among the samples (Figures 3.13 A-F). The results showed that although the S/P ratio was ~1 in control HeLa cells expressing cytosolic GFP or MGRN1; the ratio was ~2.5 in cells overexpressing catalytically inactive MGRN1 (MGRN1ΔR or MGRN1316D), hence indicating a decrease in the polymerised form of α-tubulin (Figures 3.13 A and B). Similarly, the S/P ratio was >3 when MGRN1 was depleted from cells via siRNA mediated knockdown as compared with control (S/P=2; Figures 3.13 C and D). The most significant 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 3.13 E and F).

Figure 3.13: MGRN1 affects α-tubulin polymerization in HeLa cells:

(A) Hela cells lysates, transiently expressing the indicated constructs, were fractionated to separate polymerised and unpolymerised 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 analysed with anti-GFP antibody. The blots are representative of at least 5 experiments.

(B) The immunoblots generated in panel A were analysed for the ratio of unpolymerised to polymerised α-tubulin. Note an increase in this ratio upon functional depletion of MGRN1, an indication of a compromise in tubulin polymerisation. *p<0.05, **p<0.01, using Student’s t-test. Error bars, SEM.
(C) HeLa cells treated with MGRN1 or GFP siRNAs were fractionated and immunoblotted as in panel A. The blots are representative of at least 3 experiments. The inset immunoblot shows an efficient knockdown of MGRN1.

(D) The immunoblots generated in panel C were analysed for the ratio of unpolymerised to polymerised α-tubulin. Note that MGRN1 siRNA treatment phenocopies expression of catalytically inactive MGRN1 expression. Error bars, SEM.

(E) MEF cell lysates, transiently expressing the indicated constructs, were fractionated and immunoblotted as in panel A. Note that faint (F) and dark (D) exposures of the blot show negligible amounts of detectable α-tubulin in the pellet fraction with MGRN1ΔR.

(F) The immunoblot generated in panel E was analysed for the ratio of unpolymerised to polymerised α-tubulin. Note that the functional depletion of MGRN1 has very similar effects on primary and HeLa cells.
Melanocytes (melan a-6 and melan md1-nc) cells were grown for 24 h, similarly fractionated and analysed by western blots for the levels of α-tubulin. When checked for similar S/P ratio of α-tubulin polymerisation, they showed a ratio of ~1.9 for melan a-6, while it was ~3 for melan md1-nc cells (Figures 3.14 A and B). When HeLa lysates either overexpressing exogenously expressed MGRN1 or its mutants, or depleted for MGRN1, were studied for changes in β-tubulin polymerisation, these samples failed to elicit similar differences, irrespective of the functional status of MGRN1 (Figures 3.14 C–F).
**Figure 3.14: MGRN1 affects α-tubulin polymerisation in melanocytes:**

(A) Lysates from melanocytes, melan a-6 and md1-nc were fractionated into polymerised and unpolymerised fractions; these samples along with the total cell lysates were immunoblotted for α-tubulin. The blot shown is representative from 3 experiments.

(B) The immunoblots generated in panel A were analysed for the ratio of unpolymerised to polymerised α-tubulin. Note that this ratio in melan md1-nc (MGRN1 null cells) is very similar to that of MGRN1 knockdown in HeLa cells as shown in Figure 3.13 A and B. Error bars, SEM.

(C) The same samples analysed in panel 3.13 A were immunoblotted for β-tubulin. The levels of β-tubulin in the total lysates serve as loading control. The blots are representative of at least 3 experiments.

(D) The immunoblots generated in panel C were analysed for the ratio of unpolymerised to polymerised β-tubulin. Note no change in this ratio across samples.

(E) The same samples analysed in panel 3.13 C were immunoblotted for β-tubulin. The levels of β-tubulin in the total lysates serve as loading control. The blots are representative of at least 3 experiments.

(F) The immunoblots generated in panel D were analysed for the ratio of unpolymerised to polymerised β-tubulin. Note no change in this ratio across samples.

_Nocodazole washout experiments were carried out to study the kinetics of the microtubule mesh in vivo in interphase. In such washout experiments with HeLa cells expressing MGRN1, MGRN1ΔR or C316DMGRN1 showed that the catalytic inactivation of MGRN1 distinctly compromised formation of a polymerised mesh – the MGRN1ΔR or C316DMGRN1 expressing cells lag behind the MGRN1 cells by ~30 min (Figure 3.15 A and 3.16). siRNA - mediated depletion of MGRN1 also severely affected microtubule reassembly (Figure 3.15 B)._

**Figure 3.15: MGRN1 affects α-tubulin polymerization as seen by nocodazole washout assay:**

(A) HeLa cells transiently transfected with the indicated constructs were treated with nocodazole, allowed to recover for 30 and 60 minutes 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 panel A, with recovery times of 30 and 60 minutes. Insets of higher magnification show punctate α-tubulin staining in the presence of MGRN1 siRNA, similar to MGRN1ΔR.
3.16 Nocodazole washout experiment: The panel displays more representative images of Figure 3.15A. α-Tubulin immunostaining in red; GFP in green shows expression of the different constructs in the cells.
Wild-type MEFs subjected to similar microtubule regrowth assay after Noc treatment over a period of 10 and 15 minutes showed a very punctate α-tubulin expression pattern in MGRN1ΔR cells as compared with a well-polymerised α-tubulin network and MT cluster nucleation centres in MGRN1 cells (Figure 3.17 A). Similar to HeLa cells, MEFs also endogenously express MGRN1, as indicated by immunocytochemistry studies (Figure 3.17 B). Taken together, these results indicate a decrease in MT polymerisation when the catalytic activity of MGRN1 is compromised in a cancer-derived cell line as well as primary cells. Further, biochemical data clearly states that the effect is specific for only α-tubulin and not β-tubulin.
3.17 MGRN1 in mouse embryonic fibroblast cells (MEFs)

(A) MEF cells were transiently transfected and subjected to similar treatment as in Figure 3.14 with recovery times of 10 and 15 mins. Insets of higher magnification show punctate α-Tubulin staining in the presence on MGRN1ΔR while a well formed MT network in MGRN1 expressing cells.

(B) MEFs endogenously express MGRN1. MEFs immunostained to check the expression of endogenous MGRN1 showed punctate localization of the protein throughout the cytosol, with clear nuclear exclusion. MGRN1 expression in 4 different cells (numbered i-iv) is depicted.

3.02.05 MGRN1 does not affect γ-tubulin polymerisation. The next step in the study was to check if MGRN1 had any effect on of γ-tubulin. This was essential since similar defects were observed when the spindle pole formation by of γ-tubulin was compromised (Parvin and Sankaran, 2006; Sankaran et al, 2007). Immunocytochemistry study of HeLa cells for the endogenous levels of γ-tubulin and MGRN1 in mitotic cells showed that although γ-tubulin was present only on the spindle poles, MGRN1 was present all along the entire spindle apparatus (Figure 3.18 A).

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Figure 3.18: 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, while 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 panel B were analysed 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 analysed as represented for each of the siRNA treatments.

Next, the effect of MGRN1 depletion on γ-tubulin was analysed (Figures 3.18 B and C). The expression of γ-tubulin, its intensity and spread at the poles remained independent of the presence of functional MGRN1, implying that in mammalian system, the formation and orientation of spindle poles represent two discrete events.
Furthermore, to check the status of polymerisation of endogenous γ-tubulin, HeLa cells expressing MGRN1, MGRN1ΔR, and C316DMGRN1 or depleted for the MGRN1 expression were lysed, separated into S and P fractions and biochemically analysed (Figures 3.19 A–D). No significant difference was detected in the S/P ratios between the various experimental (with inactive MGRN1) and control samples. These results again point towards the fact that MGRN1 dysfunction does not affect γ-tubulin polymerisation, its localization or distribution and hence, in turn, does not compromise spindle pole formation while still severely altering the pole orientation.
**Figure 3.19: MGRN1 does not affect γ-tubulin polymerization**

(A) The same samples analysed in panel of Figure 3.14 C 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 3 experiments.

(B) The immunoblots generated in panel A were analysed for the ratio of unpolymerised to polymerised γ-tubulin. Note no change in this ratio across samples.

(C) The same samples analysed in panel Figure 3.14 E 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 3 experiments.

(D) The immunoblots generated in panel C were analysed for the ratio of unpolymerised to polymerised β-tubulin. Note no change in this ratio across samples.

**3.02.06 MGRN1 affects α-tubulin polymerisation by its polyubiquitination.** 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. Among substrates, TSG101 (Tumor Suppressor Gene 101), a key member 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 (He et al, 2003; Kim et al, 2007). Hence it was obvious to study if α-tubulin was ubiquitinated by MGRN1 in vivo. Lysates from HeLa cells co-transfected with either MGRN1 or MGRN1ΔR and separated into S and P fractions were immunoprecipitated with α-tubulin antibody (Ab) and immunoblotted for endogenous ubiquitin (Figure 3.20 B). Presence of stronger signal in the P fractions indicated the prevalence of ubiquitinated species in them and also supported the argument for the involvement of this modification in the polymerisation of α-tubulin. Detectable level of ubiquitinated α-tubulin was less in the P fraction of MGRN1ΔR as compared with MGRN1. I next sought to find whether this was a mono- or polyubiquitination event. To determine 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 3.20 A) (Mattera et al, 2004; Tan et al, 2008; Nishikawa et al, 2004).
**Figure 3.20: MGRN1 mediates α-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 polymerised and unpolymerised fractions by high speed ultracentrifugation, the fractionated samples were immunoprecipitated with anti-α-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 α-tubulin in the various fractions and total lysates along with that of MGRN1 in the total lysates serve as loading control. (*) indicates monoubiquitinatated α-tubulin; # IgG heavy chain; ## 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 α-tubulin antibody. In vivo ubiquitination was detected by immunoblotting with anti-HA antibody. Faint and dark exposures of the anti-HA blot are shown. Polyubiquitination is detected in the presence of MGRN1 along with either Ub or K6. The input levels of α-tubulin in the total lysates serve as loading control. (*) indicates monoubiquitinatated α-tubulin.

Lysates derived from HeLa cells co-transfected with combinations of either MGRN1 or MGRN1ΔR along with the various ubiquitin constructs (Figure 3.20 C) 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), suggesting that the ligase function of MGRN1 was necessary for ubiquitination of α-tubulin. I next checked the effect of a K0 ubiquitin mutant in a similar assay since MGRN1 is known to multi-monoubiquitinate TSG101 (Kim et al, 2007). However, unlike with Ub, no multi-monoubiquitination was detected 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 that 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 3.20 C); a similar band was also evident when analysed for endogenous ubiquitin (Figure 3.20 B). These observations led to the plausibility that monoubiquitination of α-tubulin occurred independent of the catalytic activity of MGRN1 (as in lanes 2 and 4), with enforced multi-monoubiquitination in the presence of MGRN1ΔR and K0 (lane 4). Results comparable to that obtained with Ub and MGRN1/MGRN1ΔR were observed when instead of Ub, a K6 mutant of Ub was used (lanes 5 and 6). These data suggested that although MGRN1 promotes polyubiquitination, it also does not encourage multi-monoubiquitination of α-tubulin.

No perceptible change was seen in the ubiquitination pattern of β- and γ-tubulins in the presence of the different HA–Ub constructs co-expressed with MGRN1 or MGRN1ΔR in similar assays (Figures 3.21 A and B).

3.21 MGRN1 does not ubiquitinate β-tubulin and γ- tubulin

(A) 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 β-tubulin antibody and immunoblotted with anti-HA antibody. The input levels of β-tubulin in the total lysates serve as loading control.
The polyubiquitination of α-tubulin in the presence of MGRN1 does not regulate its protein turnover. This was established in two ways. First, the popular lysine residue of ubiquitin used to mark proteins for degradation, namely K48 showed polyubiquitination of α-tubulin occurred irrespective of the expression of MGRN1 or MGRN1ΔR (Figure 3.2 A). Similar argument also disproved the role of K11, K29 and K63 ubiquitin residues in the polyubiquitin chain extensions (Figure 3.2 A). Second, treatment with the proteasomal inhibitor, MG132, had similar effects on the S/P ratio of lysates from cells overexpressing MGRN1, MGRN1ΔR or C316DMGRN1 (Figure 3.2 B), again reinstating that MGRN1 does not modulate α-tubulin protein levels.
Figure 3.22: MGRN1 mediated α-tubulin polyubiquitination does not utilize commonly used ubiquitin-lysine residues or regulates its turn-over

(A) HeLa cells transiently co-transfected with HA-K63, HA-K48, HA-K29 or HA-K11 constructs along with MGRN1-GFP or MRN1ΔR-GFP were lysed and immunoprecipitated with anti α-tubulin antibody. In vivo ubiquitination was detected by immunoblotting with anti HA-antibody. Faint and dark exposures of the anti-HA blot are shown. Note that polyubiquitination is detected in all the lanes, irrespective of the presence of MGRN1 or MGRN1ΔR, hence negating the role of any of these lysine residues in participating in MGRN1-mediated polyubiquitination of α-tubulin.

(B) HeLa cells transiently transfected with the indicated constructs and treated with 5μM MG132 for 6 hours to block the proteasome-mediated degradation of α-tubulin. Cells were lysed, fractionated and checked for the levels of α-tubulin amongst the different cellular samples. The pellet fractions of all the samples are affected similarly by blocking this mode of protein degradation. Hence again showing that polyubiquitination mediated by MGRN1 does not regulate turn-over of α-tubulin.

To ascertain the role of MGRN1-mediated α-tubulin ubiquitination in endogenous MT polymerisation, at 24 h post transfection, the cell lysates separated into cytosolic (soluble (S)) and insoluble (pellet (P)) fractions were biochemically analysed to detect any change in α-tubulin polymerisation among cells expressing Ub, K0 or K6 along with MGRN1 or MGRN1ΔR (Figures 3.23 A and C). Experimental data suggests 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 3.23 B and D).
Figure 3.23: MGRN1 promotes α-tubulin polymerization by polyubiquitination

(A) HeLa cells transfected with MGRN1-GFP or MGRN1ΔR-GFP along with HA-Ub or HA-K0 were fractionated and immunoblotted with α-tubulin to check for the status of α-tubulin polymerisation. The levels of α-tubulin in the total lysates serve as loading control. The blot is representative of at least 3 experiments.

(B) The immunoblots from panel A were quantitated and analysed for the ratio of unpolymerised to polymerised tubulin. Note that the functional MGRN1 and Ub are required for efficient α-tubulin polymerisation. Error bars, SEM.

(C) HeLa cells transfected with MGRN1-GFP or MGRN1ΔR-GFP along with HA-K6 were similarly treated as in panel A. The levels of α-tubulin in the total lysates serve as loading control. The blot is representative of at least 3 experiments.

(D) Quantitation and analyses of immunoblots from panel C show that significantly more α-tubulin exists in the polymerised state in the presence of MGRN1 and K6. Error bars, SEM.

However, no changes were observed in the ubiquitination and polymerisation pattern of β- and γ-tubulins in the presence of the different HA-Ub constructs co-expressed with MGRN1 or MGRN1ΔR (Figures 3.24 and Figure 3.25).
Figure 3.2: MGRN1 does not promote β-tubulin polymerization

(A) The same samples generated in panels 3.23 A and C 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 3 experiments.

(B) Quantitation and analyses of immunoblots from panel A show similar pattern of β-tubulin polymerisation across samples. Error bars, SEM.
Figure 3.25: MGRN1 does not promote γ-tubulin polymerization

(A) The same samples generated in panels 3.23 A and C were immunoblotted with γ-tubulin. The levels of γ-tubulin in the total lysates serve as loading control. The blot is representative of at least 3 experiments.

(B) Quantitation and analyses of immunoblots from panel A show similar pattern of γ-tubulin polymerization across different trasfections. Error bars, SEM.
The data hints at the existence of a unique mode of ubiquitin-mediated post-translational modification of α-tubulin, where two ubiquitin E3 ligases act. An unknown ligase monoubiquitinates α-tubulin and this happens even in the presence of MGRN1ΔR. Either in tandem or independently, MGRN1 polyubiquitinates α-tubulin via non-canonical K6 linkages as a post-translational modification to ensure proper polymerisation but not for its degradation. In the absence of a catalytically active MGRN1, multi-monoubiquitination occurs; that, however, is insufficient for complete α-tubulin polymerisation and its stabilisation.

Phenomenon of multiple E3 ligases working together and modifying a protein has been observed previously in case of some of the critical cell cycle regulators which need to be under very tight regulation. The most popular example of such a protein is tumor suppressor p53 (protein 53), known to be ubiquitinated by at least 11 different E3 ligases (Brooks and Gu, 2011). Even though these E3 ligases have other targets, they all contribute individually in regulating p53 levels, which is vital for proper cell cycle progression.

3.02.07 Polyubiquitination of α-tubulin affects spindle pole orientation. To evaluate the physiological consequence of MGRN1-mediated polyubiquitination of α-tubulin, spindle tilt in axis of division 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 3.26 A and B). 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 tilting the ubiquitination balance between polyubiquitinated to a mono-ubiquitinated α-tubulin 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. Hence, this work establishes polyubiquitination of α-tubulin by catalytically active MGRN1 as a crucial governing factor in the proper orientation of spindle poles.

Figure 3.26: Polyubiquitination of α-tubulin affects spindle pole orientation.

(A) HeLa cells transfected with MGRN1-GFP or MGRN1ΔR-GFP along with HA-Ub, HA-K0 or HA-K6 were imaged and mitotic cells analysed for the tilt in axis of division. 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°).

(B) The cells imaged in panel A were analysed the amount of tilt in the axis of cell division was calculated. The graph shows the percentage of cells with abnormal tilt (>10°, in dark grey) and those with normal axis of division (tilt ≤10°, in light grey). Similar effect on the spindle angles was observed in the Ub and K6.
3.03 DISCUSSION

This study has established 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 polymerised MTs. Functional inactivation of MGRN1 affected α-tubulin polymerisation in immortalized cells and also primary cells. This also simultaneously coincided with an increase in the angle of tilt in the axis of cell division, resulting in spindle misorientation. The effect of MGRN1 on spindle poles was specific for α-tubulin as depletion of this protein did not alter the expression pattern or polymerisation 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 MGRN1ΔR and/or K0 were expressed, suggesting that although MGRN1 promotes polyubiquitination, it also does not encourage multi-monoubiquitination of α-tubulin. Monoubiquitination of α-tubulin by an unknown E3 ligase and MGRN1-mediated polyubiquitination (utilizing the noncanonical K6 linkages) were most likely unrelated, independent events. However, ubiquitination of α-tubulin by MGRN1 was a critical determinant of proper spindle orientation. Accordingly, the monoubiquitination of α-tubulin observed during the catalytic inactivation of MGRN1 resulted in misorientated mitotic spindle apparatus. Therefore it was deduced that ubiquitin-mediated post-translational modification of α-tubulin that eventually affects its polymerisation occurs via multiple E3 ligases, with MGRN1 being one of them (Figure 3.27). Although the significance of the association of tubulins with the motor proteins cannot be undermined in the context of spindle dynamics,
the nature of the tubulin subunits themselves are also equally important for MT polymerization, stability and dynamics. Existing data from varied experimental systems hint that changes in tubulins affect their polymerisation status and MT stability. This in turn, plays a significant role in spindle orientation at the cellular level and the LR axis of symmetry at the organismal level. Hence, I hypothesize that instead of mutations in tubulin, a post-translational modification that would affect its polymerisation 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.
While the significance of different posttranslational modification in tubulin polymerisation, MT stability and spindle orientation, has been widely and extensively studied, the importance of ubiquitination as a posttranslational modification of tubulin in similar context, has not been covered. The roles of PARKIN and BRCA1 as ubiquitin E3 ligases of α-/β and γ-tubulins, respectively, have become evident only recently (Ren et al, 2003; Parvin and Sankaran, 2006; Cuschieri et al, 2007; Sankaran et al, 2007). It was, hence, plausible to speculate that several other E3 ligases might exist as tubulins are one of the fundamental proteins whose functions regulate a plethora of cellular activities and in turn would require stringent regulation by multiple modifiers. This would be similar to some of the most important cell cycle regulators (like p53, core histone proteins and Rev-erba) that are under very tight regulation by employing multiple E3 ligases (Nishikawa et al, 2004; Singh et al, 2012; Yin et al, 2010). Several E3 ligases have been implicated to regulate MT dynamics and spindle positioning like SIAH1, SMURF2 etc, though there has been no conclusive report stating the requirement of polyubiquitin chains for MT stability and assembly. Thus, this study, for the first time, establishes ubiquitination as an important post-translational modification of α-tubulin.

Polyubiquitination involves the formation of a ubiquitin chain on a single lysine residue on the substrate protein. After addition of a single ubiquitin molecule to a protein substrate, further ubiquitin molecules get added to the first, yielding a polyubiquitin chain. These chains arise by linking the glycine residue of a ubiquitin molecule to a lysine of ubiquitin bound to a substrate. Although the ubiquitin molecule contains seven lysine residues in positions 6, 11, 27, 29, 33, 48 and 63, the most popular residues for homogenous polyubiquitin linkages have been residues 48 and 63 (Pickart, 2001; Kravtsova-Ivantsiv and Ciechanover, 2012). However, the involvement of the other lysine residues in regulating different cellular functions is
progressively becoming evident (Yin et al, 2010) – K6 polyubiquitin chains being one such lesser known lysine residue used for attachment of Ub chains. The K6 chains have been reported in E3-independent reactions catalysed by radiation gene 6 (Rad6), the yeast ortholog of UbcH2 (ubiquitin conjugating enzyme 2 (human)) (Baboshina and Haas, 1996). More recently, there have been reports suggesting that autoubiquitination of BRCA1 in vivo is mediated by K6 or K29 residues (Tan et al, 2008; Wu-Baer et al, 2003). This study identifying the polyubiquitination of α-tubulin by MGRN1 preferentially utilizing K6, as an unconventional site for ubiquitin polymerisation, was unexpected. Although the 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 utilising other lysine (K27, K33) residues or by linear chain extension.

My study establishes MGRN1 as a determinant for the stability of MT network via post-translational modification of α-tubulin via a non-canonical K6 residue of Ub. This highlights polyubiquitin linkage as 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.