Chapter 4: Mahogunin RING finger 1 does not affect lysosomal competence
4.01 Introduction

In the previous chapter I established that functional aberration of MGRN1 leads to incompetence in lysosomal degradation by disrupting both autophagic and endocytic pathways. Blocked fusions of vesicles comprising both these pathways are the key events for such disruption. A number of neurodegenerative diseases are associated with dysregulation of autophagy and endocytic pathways, and lysosomal incompetence is one of key phenomena found to be associated with such neurodegenerations (Levine and Kroemer, 2008, Nixon et al., 2008).

Lysosome contains a large repertoire (more than 50) of soluble acid hydrolases which participate in degradation of macromolecules. These enzymes are synthesized and primarily glycosylated in rough endoplasmic reticulum (Kornfeld, 1990). The glycosylated proteins are then transported to golgi body where further modifications take place. In trans golgi network (TGN), mannose residues of lysosomal enzymes get phosphorylated, recognized and attached to mannose-6-phosphate receptor (MPR) (Ghosh et al., 2003, Braulke and Bonifacino, 2009). MPR-ligand complexes are packed in clathrin-coated intermediate vesicles and fuse with endosomal structures (Meel and Klumperman, 2008). Some lysosomal enzymes are transported via MPR-independent pathways (Dittmer et al., 1999). In I-cell disease (ICD), sortilin is responsible for the MPR-independent targeting of the sphingolipid activator proteins (SAPs) and acid sphingomyelinase (ASM) (Lefrancois et al., 2003; Ni and Morales, 2006). In late endosomes, the receptor-ligand complexes dissociate due to low pH. The MRP and sortilin recycle back to TGN owing to their interaction with the retromer complex (Kornfeld and Mellman, 1989; Seaman, 2004; Canuel et al., 2008). β-glucocerebrosidase (βGC) is also transported in a MPR independent manner. It is bound to lysosomal integral membrane protein (LIMP)-II, in a pH-dependent manner and transported to the lysosome, where the complex dissociates because of the acidic pH (Reczek et al., 2007). In certain cell types and disease conditions, Cathepsin D (which is normally transported via MPR) reaches lysosomes in absence of MPR (Rijnboutt et al., 1991; Glickman and Kornfeld, 1993). The transport of cargoes to lysosomes from endosomal structures is mediated either by maturation of late endosomes to lysosomes or by formation of hybrid organelles by fusion between late endosomes or lysosomes. In the acidic compartments, the lysosomal soluble enzymes undergo a number of proteolytic cleavages to obtain their matured forms. Enzymes active in acidic pH participate in such proteolysis (Kornfeld, 1990; Braulke and Bonifacino, 2009). Acidic pH of lysosome is
maintained by vacuolar-type H+-ATPase (V-ATPase) which uses ATP hydrolysis to pump protons into the lumen of the lysosome (Forgac, 2007).

For lysosome to be functional, its resident enzymes should reach their final destination. The acidic environment of late endo-lysosomal vesicles control maturation and activity of these enzymes which in turn regulates the lysosomal competence.

I have seen that depletion of MGRN1 causes incompetence in lysosomal degradation as well as aberration in lysosomal phenotype by affecting vesicular fusions in the autophagosomal and endocytic pathways. The results does not rule out the possibility that lysosomal biogenesis (lysosomal activity as well) may be hampered when MGRN1 is inactive. To understand this, the following possibilities were addressed:

First, it was plausible that the formation of functional late endosomes/ MVBs could be perturbed upon knockdown of MGRN1. Transport of lysosomal resident proteins which are required for lysosomal biogenesis need appropriate formation of late endosomes/ MVBs.

Second, whether MGRN1 depletion exerted any effect on the activity of lysosomal enzymes as well as the pH of acidic vesicles.

I observed that depletion of MGRN1 has no effect on the generation of functional multivesicular bodies. Similarly, activity of lysosomal enzyme Cathepsin D and the pH of lysosomes remain unchanged. Hence it can be concluded that MGRN1 does not affect biogenesis as well as competence of lysosomes. Rather it only affects at the level of fusion between vesicles participating in degradative pathways that terminate at lysosomes.

4.02 Observations

4.02.01 Functional MBVs are formed irrespective of MGRN1 activity

In the previous chapter, I established that depletion of MGRN1 led to blocked fusion of MVBs and amphisomes with lysosomes. MBVs are late endosomes having internal vesicles (intra-luminal membranes) and cargoes from endocytic pathways. MGRN1 knockdown resulted in accumulation of Alexa488-labeled EGF loaded vesicles which are regarded as late endosomes/MVBs (Kim et al, 2007). The result failed to address whether the fusion incompetency of MVBs in MGRN1 depleted conditions arose due to their improper biogenesis. To address this, GFP and MGRN1 siRNA treated HeLa cells were subjected to Alexa488-labeled
EGF chase for 40 min, fixed and stained with CD63 antibody, a marker for late endosomes and lysosomes. The chase period of 40 min allowed internalization of EGF-EGFR complex but degradation had not yet occurred. In all cells, CD63 positive Alexa-Fluor 488-labeled EGF-EGFR internalized MVBs of similar morphology were detected (Figure 4.1).

This result indicated that MGRN1 did not affect formation and maturation of MVBs, the fusion incompetency was due to some other effect (possibly downstream) exerted by MGRN1.

Figure 4.1: Functional MVBs are formed in MGRN1 depleted cells

HeLa cells were treated with irrelevant (GFP) or MGRN1 siRNAs and subjected to Alexa-Fluor 488 EGF uptake. Cells were washed, fixed at 40 min time point, immunostained for CD63 and imaged. Enlarged views of the areas within the white boxes (insets) are also shown. Insets reveal enlarged views of multiple Alexa-Fluor 488-labeled EGF positive puncta (green) on CD63 positive vesicles with intra-luminal membranes (red), shown by arrow. Scale bar, 5 μm.

4.02.02 Depletion of MGRN1 leads to formation of larger CD63 positive vesicles with intra-luminal membranes

Late endosomes often resemble MVBs – i.e. they display invaginations of their membrane and internal vesicles budded off the invaginations. Both control and MGRN1 depleted cells were fixed and stained for the late endosome marker CD63 and imaged. As expected, intra-luminal membrane containing CD63 positive vesicles of similar morphology were found all across the samples (Figure 4.2A). This further established unperturbed formation of MVBs.

In MGRN1 depleted cells the average diameter of vesicles was ~ 0.78 μm (~1.5 folds higher than control), where in GFP siRNA treated control cells vesicles diameters were ~ 0.51 μm. This
significant increase (p≤0.001, no of vesicles analyzed ~ 240 for both cells) in vesicles size was expected because of the initial observations where MGRN1 depletion resulted in highly fused structure of late endosome and lysosomes (Figure 4.2B).

Figure 4.2: Larger CD63 marked vesicles in MGRN1 depleted cells

A) HeLa cells were treated with GFP or MGRN1 siRNAs and fixed and immunostained with anti-CD63 antibody. Images are shown for 2X and 4X zoom. Enlarged views of the areas within the white boxes (insets) are also shown. Arrows indicate vesicles with inward budding. Scale bar, 5 μm.

B) Histogram shows significant increase (~1.5 folds) in average diameter of CD63 positive vesicles for MGRN1-depleted cells. Note the presence of intraluminal vesicles in both the samples, except that the ones with MGRN1 depletion are bigger in size. Approximately 240 vesicles were counted from three
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**4.02.03 Lysosomal competence remains unaltered upon depletion of MGRN1**

Blocked fusion in autophagic and endocytic pathways led to accumulation of proteins. As cargoes from both these pathways are degraded in lysosomes, it was obvious to ask if MGRN1 depletion had any alteration in activity of lysosomes. To evaluate that, the level as well as activity of Cathepsin D, a lysosomal aspartic protease was checked.

Cathepsin D is a soluble glycoprotein which is synthesized in rough endoplasmic reticulum as a precursor form and then undergoes several proteolytic cleavages and glycosylation to produce the active form (Richo et al., 1994). Glycosylated pro-Cathepsin D (immature form) is 52 KDa protein, that is marked for mannose-6-phosphate receptors (MRP) in golgi body and targeted to endosomes and lysosomes (Kornfeld S., 1990; Braulke and Bonifacino., 2009). Depending on the cell type, Cathepsin D (CTSD) may also be targeted to lysosomes in an M6P-independent manner (Rijnboutt et al., 1991; Glickman and Kornfeld, 1993; Capony et al., 1994). In lysosomes, the pro-Cathepsin D is cleaved by cysteine lysosomal and/or aspartic proteases, to generate an active intermediate 48 kDa single-chain molecule (Hentze et al., 1984; Samarel et al., 1989).

The intermediate 48 kDa single-chain species is then cleaved in lysosomes into a mature two-chain enzyme consisting of a light 14 kDa amino-terminal domain and a heavy 34 kDa carboxyl-terminal domain. The acidic environment helps generation of the mature forms (Gieselmann et al., 1985). The processing of 48 kDa requires participation of Cathepsin L and Cathepsin B, two lysosomal cysteine proteases (Laurent-Matha et al., 2006), though other proteases are also involved in this processing. Hence generation of mature Cathepsin D requires functionality of a number of lysosomal proteases, whose activities in turn depend on the acidic environment of lysosomes. Therefore by checking levels of mature and immature form of CTSD and activity of the CTSD, competence of lysosomes can be evaluated.

HeLa cells were treated with GFP or MGRN1 siRNA for 72 hrs. In MGRN1-depleted cell lysates, level of CTSD (both mature and immature form) was found to be 1.5-folds higher than GFP siRNA treated samples (Figures 4.3A and B). However, the ratio between the proenzyme and the mature forms was similar to the controls (non significant change, p=0.75) (Figure 4.3B...
iii). This indicated that processing of CTSD was similar for both the samples. Proper proteolytic cleavage of CTSD signified that the enzyme had reached its destination compartment where ambient pH is optimum for its processing. As this processing also involves other lysosomal cysteine proteases (Laurent-Matha et al., 2006) it is justified to state that MGRN1 did not alter lysosomal function.
A) 34 KDa - Mature form  CTSD (Low exp)
    52 KDa - Immature form
    34 KDa - Mature form  CTSD (High exp)
    51 KDa - β-Tubulin
    72 KDa - Mature form
    68 KDa - MGRN1

    siGFP  siMGRN1

B) i) **

    Normalized CTSD levels
    (mature form)

    siGFP  siMGRN1

    ii) **

    Normalized CTSD levels
    (immature form)

    siGFP  siMGRN1

    iii) n.s.

    CTSD fold change (mature form/immature form)

    siGFP  siMGRN1

1.0 1.2 1.4

0.0 0.2 0.4 0.6 0.8 1.0

Normalized CTSD levels (mature form)

Normalized CTSD levels (immature form)

CTSD fold change (mature form/immature form)
Figure 4.3: Unperturbed processing of Cathepsin D in MGRN1 depleted cells

A) HeLa cell treated with indicated siRNAs were analyzed for the levels of CTSD. The low and dark exposures of the CTSD blot indicate the different processed forms of the enzyme. The levels of β-tubulin serve as loading control. Efficiency of knockdown was confirmed by immunoblotting with anti-MGRN1. The blots are representative of at least three experiments.

B) Histograms plotting data show significant (~1.5 folds) but similar increase in the levels of mature and immature CTSD, as normalized against the protein levels of β-tubulin (i) and (ii) with the depletion of MGRN1. However, note that the fold change in the mature and immature forms of CTSD are comparable between control and MGRN1 siRNA-treated samples (iii). Graph representing three independent experiments. **P ≤ 0.05, n.s., not significant (P = 0.75) using Student’s t-test. Error bars, ±S.E.M.

The activity of CTSD was also verified by fluorometric analysis. Control or MGRN1 depleted cells were treated with vehicle control or pepstatin A. Pepstatin A is an aspartic peptidase inhibitor. Fluorometrically analysis further revealed that CTSD activity was unchanged upon depletion of MGRN1. Pepstatin A inhibited CTSD activity irrespective of MGRN1 functionality (Figure 4.4).

![Figure 4.4: Unchanged activity of Cathepsin D in MGRN1 depleted cells](image)
Histogram shows CTSD activity for cell lysates generated from control and MGRN1 siRNA treated cells. There was no change in CTSD activity for both the samples. To block CTSD activity, cells were either treated with vehicle control or pepstatin A, as indicated. Graph represents average of three independent experiments, performed in triplicate for each cell concentration. Error bars, ±S.E.M.; RFU, relative fluorescence units.

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**4.02.04 Cathepsin D accumulates in endocytic compartments upon MGRN1 depletion**

The levels of pre and pro Cathepsin D increase upon depletion of MGRN1 but the activity of CTSD remained unchanged. Immunocytochemistry revealed that CTSD positive vesicles in MGRN1 knockdown cells were significantly enlarged (~1.4 folds, p≤0.001, counted for 205 vesicles) than GFP siRNA treated cells. (Figures 4.5 A and B).

This result further suggested that a fraction of total cellular CTSD got accumulated in endocytic compartments (Braulke and Bonifacino, 2009) because of incompetent fusions of vesicles. This in turn resulted in an increase in the protein levels without proportional alteration in activity. The morphology of CTSD positive vesicles were similar throughout the sample as was observed for CD63 vesicles. This hinted that the fraction of the compartments where CSTD get accumulated might be late endosomes/MVBs.
Figure 4.5: Enlarged Cathepsin D positive vesicles in MGRN1 knockdown cells

A) HeLa cells treated with irrelevant (GFP) or MGRN1 siRNAs, fixed and immunostained with anti-CTSD antibody. Two fields for each condition are shown. There is a qualitative increase in CTSD vesicles in MGRN1 depleted cells. Enlarged views of the areas within the white boxes (insets) are also shown. Scale bar, 5 μm.

B) Graph plotting the average diameter of vesicles showed significant increase (~1.4 folds) when MGRN1 is depleted. Approximately 205 vesicles were counted from three independent experiments. ***p<0.001, using Student’s t-test. Error bars, ±S.E.M.
4.02.05 MGRN1 does not change acidic pH of lysosomes

Lysosomes must maintain their acidic pH between 4 and 5 which is needed for functionality of hydrolytic enzymes (Lloyd and Mason, 1996, Pillay et al., 2002). Vacuolar-type H+-ATPase (V-ATPase) creates the acidic environment of lysosome (Forgac, 2007).

For evaluating vesicular pH, cells transfected with GFP or MGRN1 siRNA were treated with LysoSensor Yellow/Blue dextran. This dextran-linked dye is known to accumulate in endocytic vesicles (endosomes and lysosomes) through endocytosis and exhibits pH-dependent dual-emission spectra – with an emission maximum of 530 nm at acidic pH, while at higher pH the emission maximum is at 450 nm. The pKa of LysoSensor Yellow/Blue dextran is ~3.9. pH of acidic vesicles was checked by both fluorometric technique and confocal analysis. The detailed procedure of this experiment is described in material and methods section.

Fluorometric analysis showed insignificant change of pH (p=0.9, no of independent experiments = 3) between control (4.62±0.02) and MGRN1-depleted samples (4.66±0.01) (Figure 4.6).

Figure 4.6: pH of acidic vesicles

Lysosomal pH values were measured ratiometrically using LysoSensor yellow/blue DND-160–Dextran. In GFP siRNA treated cells, the average lysosomal pH was detected as 4.62±0.02, while in cells treated with MGRN1 siRNA the pH was 4.66±0.01; n.s., not significant (p=0.9), using Student’s t-test. Graph represents average of three independent experiments. Error bars, ±S.E.M.

Confocal experiment data also supported the result obtained from fluorometric analysis (Figures 4.7A, B and C).
Figure 4.7: Lysosomal pH measurement using confocal microscopy.

A) pH calibration curve for the LysoSensor yellow/blue DND-160–Dextran indicator dye. The 530/440nm fluorescence ratio (R/G) of dextran loaded vesicles was measured using pH as x axis.

B) Histogram showing the average pH of lysosomes in HeLa cells treated with GFP or MGRN1 siRNAs, as analyzed from around 100 vesicles in the acidic pH range (4.0-5.5). Average lysosomal pH in GFP and MGRN1 siRNA treated cells were 4.71±0.04 and 4.77±0.03, respectively. n.s non significant (p value=0.1), using Student’s t-test. Error bar, ±SEM.

C) Confocal images representing lysosomal pH as indicated by LysoSensor yellow/blue DND-160–Dextran indicator dye in HeLa cells treated with GFP or MGRN1 siRNA. The emission at 530 and 440 nm were assigned magenta and blue colors, respectively.
These results implicated that the physiological nature of neither the late endosomes/MVBs nor lysosomes is perturbed upon MGRN1 inactivation.

4.03 Conclusion

Inactivation of MGRN1 leads to blockage of vesicular fusions between autophagosomes and lysosomes as well late endosomes/MVBs and lysosomes. This blockage in fusion although adversely affects lysosomal degradation of macromolecules, it does not alter lysosomal competence. Further, it is showed that upon depletion of MGRN1 while the size of late endosomes/ MVBs may be enlarged, their formation is unaffected.

The morphology of MVBs (containing Alexa- 488 EGF) is found to be similar in both control and MGRN1 knockdown conditions. The increased diameter of CD63 positive vesicles (late endosome) in knockdown cells supports the initial observations that highly fused late endolysosomal structures are formed.

Time dependant increase in Cathepsin D staining is shown in mice cells which are expressing disease causing CtmPrP and CyPrP mutant and thus having altered MGRN1 expression (Chakrabarti and Hegde, 2009). As expected; in cultured cells, the levels of Cathepsin D (both mature and immature form) shot up when MGRN1 is depleted by RNAi technique. The similar ratio of pro and matured form of Cathepsin D (CTSD) in both control and MGRN1 depleted cells suggests proper maturation of CTSD. Interestingly, the activity of CTSD as seen by fluorometric analysis is similar for both samples. This indicates that although the generation of CTSD is higher in knockdown cells than control; the amounts of CTSD reaching lysosomes are comparable for both samples. CTSD is possibly sequestered in late endosomal vesicles (Braulke and Bonifacino,2009) where they are acted on by other acid hydrolyses and matured forms are generated. The bigger and highly accumulated CTSD positive vesicles in knockdown cells further strengthen this hypothesis.

Treatment of cells with LysoSensor Yellow/Blue dextran reveals no alteration in acidic pH of lysosomes (acidic vesicles) in MGRN1 depleted cells. Therefore the normal acidic environment of late endosomes and lysosomes is unperturbed and so is the lysosomal competency. Taken together, I can conclude that the formation of MVBs is normal in MGRN1 knockdown cells. At the same time lysosomal biogenesis is unperturbed and its resident enzymes reach their destination. Furthermore, MGRN1 depletion has no effect on the acidic pH of lysosomes. As the
competency of lysosomes is unaltered, it may be emphasized that the blocked degradation of cargoes from autophagic and endocytic pathways is caused exclusively by blocked vesicular fusions.