Chapter 3: Mahogunin RING finger 1 affects fusion between amphisome and MVB with lysosomes
3.01 Introduction

Mahogunin Ring Finger-1 (MGRN1) is a ubiquitously expressed cytosolic E3 ubiquitin–protein ligase (He et al., 2003) protein that contains a C3HC4-type RING finger, a motif that gives rise to its the E3 ubiquitin–protein ligase activity (Phan et al., 2002; He et al., 2003). Null mutation in *Mgrn1* gene in mice causes phenotype similar to spongiform neurodegeneration as seen in prion diseases but without accumulation of protease resistant proteins (He et al., 2003). MGRN1 is reported to interact with aberrantly metabolized disease causing prion proteins (PrP) which are cytosolically exposed unlike the major isoform of cell surface localized PrP (Prusiner, 1998). These disease causing isoforms of PrP are generated either due to mutations in hydrophobic domains (HD) leading to enrichment of of a minor PrP isoform, CmPrP (Hegde et al., 1998; Hegde et al., 1999; Kim and Hegde, 2002), or due to inclusion of two premature stop codon resulting in reduced translocation into the ER, thereby yielding high level of CyPrP (Zanusso et al., 1999; Heske et al., 2004). The interaction between MGRN1 and PrP sequesters MGRN1 functions and causes phenotypic aberrations in lysosomes (Chakrabarti and Hegde, 2009).

In cell system siRNA mediated depletion of MGRN1 is reported to perturb endocytic trafficking pathway, therefore blocking degradation of epidermal growth factor receptor (EGFR) upon stimulation by epidermal growth factor (EGF) (Kim et al., 2007). Similar disruption in lysosomal trafficking of EGFR is observed in animal models system (Jiao et al, 2009). MGRN1 interact with and ubiquitinate ESCRT protein TSG101. Therefore either aberrant trafficking of receptors or accumulation of endocytic components or both may lead to sponiform neurodegeneration as seen in *Mgrn1* null mice, phenotypically similar to cause the prion disease affected brains (Kim et al., 2007).

Presence of loss of function mutations in ESCRT proteins like TSG101 (Doyotte et al., 2005) or Fab1 (endosomal phosphatidylinositol PtdIns(3)P 5-kinase) in Drosophila (Rusten et al., 2007) lead to aberrations in endo-lysosomal trafficking and resulting spongiform phenotype. ESCRT machinery components are involved in the sorting of ubiquitinated cargoes meant for degradation and formation of MVBs (Gruenberg and Stenmark, 2004; Katzmann et al., 2002, Raiborg and Stenmark, 2009). Along with endocytosis, autophagy is another heavily utilized arm of the lysosomal degradation pathway. Both these pathways are involved in a number of vesicular fusions process.
Depletion of various ESCRT subunits such as of HRS (ESCRT-0), TSG101 (ESCRT-1), VPS24 (ESCRT-III), CHMP2B (ESCRT-III) or VPS4 are reported to affect the structure and function of endo-lysosomal compartments as well as autophagy pathway by disrupting fusions events involving lysosomes, endosomes and autophagosomes (Tamai et al., 2007; Filimonenko et al., 2007; Lee et al., 2007; Nara et al., 2002; Rusten et al., 2007; Metcalf et al., 2010). In my work I have seen that, along with endocytic pathway MGRN1 regulates autophagic pathway. Role of autophagy in prion disease is still unclear and contradictory (Heiseke et al., 2009; Heiseke et al., 2010; Cortes et al., 2012). In this chapter, I discuss how MGRN1 controls vesicular fusion events essential for lysosomal degradation. These processes are also found to be perturbed when MGRN1 activity is compromised by expressing a catalytically inactive mutant of the ligase, RNAi mediated partial knowndown of its function or in the presence of aberrantly metabolized prion protein isoforms that interact with MGRN1 in the cytosol.

MGRN1 affects a central fusion event where both autophagic and endocytic pathways converge culminating to lysosomal degradation.

3.02 Observations

3.02.01 Depletion of MGRN1 affects endolysosomal morphology

In HeLa cells, LAMP2 positive late endosomes and lysosomes are enlarged and clustered in perinuclear region when MGRN1 is depleted by siRNA (Kim et al., 2007). In cells expressing mislocalised prion proteins that interact inappropriately with MGRN1 and inhibit its function, similar changes in lysosomal structure are found using acidophilic dye lysotracker (Chakrabarti and Hegde, 2009).

In accordance with these results, I observed in both HeLa and SHSY5Y cells, siRNA mediated depletion of MGRN1 altered the morphology of late endosomes and lysosomes. Lysosomes were found to form highly fused structure as seen by immunocytochemistry with CD63 and LAMP2, in HeLa (Figures 3.1A and B).
Figure 3.1A: Depletion of MGRN1 in HeLa cells lead to increase in size of endolysosomes

HeLa cells treated with irrelevant siRNAs (GFP siRNAs) or MGRN1 siRNAs, co-immunostained for CD63 and MGRN1 were imaged. There was a qualitative increase in size of vesicles with the functional depletion of MGRN1. The channels for acquiring the images are indicated. Enlarged views of the areas within the white boxes are also shown (insets). Scale bar, 5 μm. The immunoblot shows efficiency of siRNA-mediated MGRN1 knockdown.
Figure 3.1B: Depletion of MGRN1 in HeLa cells lead to increase in size of endolysosomes

HeLa cells were treated with irrelevant siRNAs (GFP siRNAs) or MGRN1 siRNAs, co-immunostained for LAMP2 and MGRN1 were imaged. There was a qualitative increase in size of vesicles with the functional depletion of MGRN1. The channels for acquiring the images are indicated. Enlarged views of the areas within the white boxes are also shown (insets). Scale bar, 5 μm. The immunoblot shows efficiency of siRNA-mediated MGRN1 knockdown.
Similar observations were made for SHSY5Y cells (Figures 3.2A and B).

**Figure 3.2A: Depletion of MGRN1 in SHSY5Y cells lead to increase in size of endolysosomes**

SHSY5Y cells were treated with irrelevant siRNAs (GFP siRNAs) or MGRN1 siRNAs, co-immunostained for CD63 and MGRN1 were imaged. There was a qualitative increase in size of vesicles with the functional depletion of MGRN1. The channels for acquiring the images are indicated. Enlarged views of the areas within the white boxes are also shown (insets). Scale bar, 5 μm. The immunoblot shows efficiency of siRNA-mediated MGRN1 knockdown.
Depletion of various ESCRT proteins like HRS, ALIX and TSG101 are reported to cause similar changes in endolysosomal morphology and found to be involved in MVB sorting machinery (Bache et al., 2003; Cabezas et al., 2005; Doyotte et al., 2005). Therefore it is justified to hypothesize that MGRN1 may involved in MVB formation and/or late endosome/lysosome biogenesis (Kim et al., 2007).
3.02.02 Lack of MGRN1 affects acidic vesicles (late endosomes/lysosomes) in melanocytes

To check if the complete loss of MGRN1 elicited a similar morphological alteration in late endosome and lysosomes as seen for RNAi technique, melanocytes obtained from mice were stained with acidophilic dye lysotracker. In melanocytes derived from Mgrn1 null mice (md1-nc cells) (Hida et al., 2009) size of the acidic vesicles was found to be increased as compared to melanocytes derived from control mice (a-6 cells). This indicated presence of aberrant lysosomes in md1-nc cells. In accordance with the results, the population of acidic vesicles of larger size were more for md1-nc cells than control a-6 cells (Figure 3.3A and B).

![Figure 3.3 : Lack of MGRN1 in melanocytes increases the size of acidic vesicles](image)

**A)** Melanocytes, melan a-6 and melan md1-nc cells were stained with lysotracker and imaged. Lack of MGRN1 causes enlargement of acidic vesicles. Two fields for each cell line are shown. Insets below a (melan a-6) and b (melan md1-nc) reveal enlarged views of the vesicles. Scale bar, 5μm.

**B)**

<table>
<thead>
<tr>
<th>Size range</th>
<th>&lt;0.3 μm</th>
<th>0.3-0.45 μm</th>
<th>0.45-0.55 μm</th>
<th>&gt;0.55 μm</th>
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![Box plots showing average diameter of vesicles](image)
B) Graph shows the size distribution of lysotracker positive vesicles imaged in panel A. Over 1000 vesicles from at least 25 cells are represented for each cell line. The horizontal line in each box shows the median value and the white square inside each box is the mean. The upper and lower boundaries of individual box show the upper and lower quartiles, the whiskers are SD (standard deviation). Outlier values are shown outside the whiskers.

The average diameter of the acidic vesicles in melan md1-nc cells was found to be ~0.63 µm which was significantly higher than that of melan a-6 cells having average diameter ~0.56 µm (p≤0.001, n=1000) (Figure 3.3C).

**Figure 3.3C: Significant increase in the average diameter of acidic vesicles in melanocytes lacking MGRN1**

The average diameter of vesicles analyzed in panel 3.3B shows a minor but significant increase in size in melan md1-nc cells as compared with the control, melan a-6 cells. Over 1000 vesicles from at least 25 cells are analysed for each cell line *** p≤ 0.001, using Student’s t-test. Error bars, ±SEM.

### 3.02.03 Enlargement of acidic vesicles in melan md1-nc is specifically attributed upon its lack of MGRN1

To validate the specificity of lack of MGRN1 in the lysosomal phenotype aberration, both melanocytes were transfected with full length MGRN1 or catalytically inactive RING deleted MGRN1ΔR (Figure 3.4A). Over expression of MGRN1 partially reverted back the average size of vesicle (~0.57 µm) in melan md1-nc cells while average diameter of acidic vesicles upon overexpression of MGRN1ΔR were ~0.69 µm, almost like the untransfected melan md1-nc cells. In control a-6 cells, MGRN1ΔR led to a significant increase (p≤0.001) in the lysosomal size (~0.63 µm), while MGRN1 reduced this to ~0.43 µm, even lower than the untransfected melan a-6 cells (Figure 3.4B). I found significant change in the size of acidic vesicles in a-6 cell
overexpressing MGRN1ΔR when compared with the melan md1-nc overexpressing MGRN1. This suggests that MGRN1 is responsible for lysosomal phenotype.

![Image](image-url)

**A)**

- MGRN1
- MGRN1ΔR
- MGRN1
- MGRN1ΔR

**B)**

![Graph](graph-url)

**Average diameter of vesicles (µm)**

- MGRN1
- MGRN1ΔR
- MGRN1
- MGRN1ΔR

**a6**

**md1-nc**
Figure 3.4: Rescue in size of acidic vesicles in melanocytes lacking MGRN1

A) MGRN1 or MGRN1ΔR was ectopically expressed in melan a-6 and melan md1-nc cells. The samples were stained with lysotracker and imaged. Enlarged views of the areas within the white boxes (insets) are also shown. Scale bar, 5μm.

B) The effect of MGRN1 or MGRN1ΔR overexpression in melanocytes was plotted (mean ±SEM) for ~100 vesicles. Overexpression of MGRN1 partially rescues the enlarged lysosomal/vesicular phenotype, while MGRN1ΔR aggravates it. *** p≤ 0.001, ** p≤ 0.05 using Student’s t test. Error bars, ±SEM.

Taken together these results suggested that partial or complete loss of MGRN1 function led to aberration in late endosomal and lysosomal pathways by either affecting lysosomal biogenesis or by affecting endocytic and/or autophagic pathways.

3.02.04 Depletion of MGRN1 leads to altered level of proteins participating in autophagy

It was prudent to suspect that anomaly in lysosomal pathway could be associated with alterations in lysosome associated proteins. To check that, levels of various cytosolic and lysosomal proteins that are related to autophagic pathway were analysed for both control and MGRN1 siRNA treated cell lines. In HeLa cells, MGRN1 depletion enhanced level of Beclin1 (BECN1), LC3 II and p62 along with lysosomal resident protein LAMP2 as compared to GFP siRNA expressing cells (Figure 3.5A). Level of autophagic marker protein LC3-II was significantly higher (p≤0.05, n=3) in MGRN1 knockdown cells than control (Figure 3.5B).
Figure 3.5: Depletion of MGRN1 elevates level of autophagy related proteins

A) HeLa cells treated with GFP and MGRN1 siRNAs, were lysed and immunobblotted for autophagy and lysosomal proteins. The levels of GAPDH and β–tubulin serve as loading controls. The blots are representative of at least 3 experiments. Efficiency of knockdown was checked using anti-MGRN1 antibody. Note that the antibody used against LC3, detects only endogenous LC3 II.

B) The immunoblots shown in panel A were analyzed for the levels of LC3 II. Graph shows results from 3 independent experiments. ** p \leq 0.05, using Student’s t-test. Error bars, ±SEM.

Similar observations were obtained in SHSY5Y cells (Figures 3.6).

Figure 3.6: Depletion of MGRN1 elevates level of autophagy related proteins

SHSY5Y cells treated with GFP and MGRN1 siRNAs were lysed and immunobblotted for autophagy and lysosomal proteins. The blots are representative of at least 2 experiments. Efficiency of knockdown was shown using anti-MGRN1 antibody.
3.02.05 Mgrn1 null melanocytes have lower levels of autophagic related protein

Interestingly, level of LC3II in melanocytes derived from Mgrn1 null mice, melan md1-nc, was found to be significantly lower (p≤0.05, n=3) than as found in melan a-6 (control) cells. The level of p62 was however similar in both the cell lines (Figures 3.7A and B). This result apparently contradicted with the previous finding where level of LC3-II and p62 increased upon siRNA mediated depletion of MGRN1. As melanocytes derived cell lines propagate in culture over multiple passages (over 30), high levels of LC3 II (signifying abnormal autophagy) could be detrimental for these cells. Therefore a compensatory mechanism to maintain low levels of LC3II may be present in the null cells. The signaling adapter p62 protein is a multidomain and multipurpose protein implicated in activation of the transcription factor NFκB, linked to the extrinsic apoptosis pathway and known to modulate autophagy and also regulate tumorigenesis. Hence it is involved at critical decision making points that control cell death and survival (Moscat and Diaz-Meco, 2009). Dysregulation of p62, by either lowering or enhancing its levels would destabilize cellular homoeostasis, thus maintaining its basal levels would be essential for propagation of melan md1-nc cell lines in culture.

It cannot however be completely ruled out that the discrepancies observed in the LC3 II and p62 levels between siRNA treated and Mgrn1 null cells-derived samples could also reflect a situation arising due to incomplete knockdown and partial residual activity of MGRN1.
These observations indicated that MGRN1 might be involved in macroautophagic pathway.

3.02.06 Processing of exogenously expressed RFP-LC3 vesicles are altered upon depletion of MGRN1 function

For analysing the involvement of MGRN1 in macroautophagic pathway, cell lines were observed for processing of exogenously expressed RFP-LC3 (fluorescent tagged LC3) vesicles. HeLa cells, treated with Mock or MGRN1 siRNA and transfected with RFP-LC3, were imaged and quantified for the number of RFP-LC3 vesicles. There was a significant increase (p ≤ 0.001, number of cells analyzed, n=40) in average number of red fluorescent vesicles per cell when MGRN1 is functionally inactive. MGRN1 knockdown cells have ~16.1 while controls have ~5.5 vesicles per cell (Figures 3.8A and B).
Figure 3.8: Depletion of MGRN1 affects abundance of exogenously expressed LC3 vesicle

(A) HeLa cells treated with indicated siRNAs were transfected with RFP-LC3 and imaged.

(B) Graph shows significant increase in average number of red fluorescent vesicles per cell. MGRN1 knockdown cells have ~16.1 while controls have ~5.5 vesicles per cell. Number of cells analysed, n=40. *** p ≤ 0.001, using Student’s t-test. Error bars, ±SEM.

Similar result was obtained in siRNA treated SHSY5Y cells where average diameter of RFP-LC3 was measured. The average diameter of RFP-LC3 vesicles in MGRN1 depleted cells was ~0.56 μm, which is minor but significantly higher (p ≤ 0.001, number of cell analyzed, n= 15) of that of mock siRNA treated cells where the diameter was ~0.48 μm (Figures 3.9A and B).
Figure 3.9: Depletion of MGRN1 affects size of exogenously expressed LC3 vesicle

A) SHSY5Y cells treated with Mock or MGRN1 siRNAs were transfected with RFP-LC3 and imaged.

B) Histogram shows minor but significant increase in size of red vesicles in the presence of MGRN1 siRNA. 204 vesicles were analysed from 15 different cells for each condition. *** p ≤ 0.001, using Student’s t-test. Error bars, ±SEM.

MGRN1 depletion in Mouse embryonic fibroblast (MEF) cells marginally increased the size of RFP-LC3 vesicles from ~0.32 µm for mock siRNA treated cells to ~0.37 µm in MGRN1 knockdown cells. 80 vesicles were analysed for each data set (Figures 3.10A and B).
Figure 3.10: Depletion of MGRN1 affects exogenously expressed LC3 vesicle

(A) Mouse embryonic fibroblast cells (MEFs) were treated with Mock or MGRN1 siRNAs, transfected with RFP-LC3 and imaged.

(B) Quantification of this data shows that like cell lines, even in primary cells, MGRN1 depletion results in a marginal increase in size of RFP-LC3 vesicles. 80 vesicles were analysed for each data set. Error bars, +SEM.

These results indicated that MGRN1 depletion caused alteration in autophagic pathway in a cell line independent manner.

3.02.07 Functional depletion of MGRN1 reduces its ubiquitination activity

MGRN1 is a cytosolic ubiquitin ligase and reported to interact and monoubiquitinated TSG101 at multiple sites (Kim et al., 2007). Therefore perturbation of MGRN1 function by any means should block TSG101 ubiquitination. To access that, HeLa cells were transfected with MGRN1ΔR or CmpPrP mutant (A117V PrP). The deletion of the RING domain of MGRN1 rendered it inactive of its ubiquitin ligase property. CmpPrP is known to interact with MGRN1 and sequester its functions (Chakrabarti and Hegde, 2009). Ubiquitination of TSG101 was blocked in the presence of MGRN1ΔR or A117V PrP (Figure 3.11). When cells were transfected with either MGRN1 or WT-PrP (as control), ubiquitination of TSG101 took place. Cells transfected with MGRN1 and wild-type PrP (WT PrP) constructs were used as control for this experiment.
Figure 3.11: Ubiquitination of TSG101 is reduced when MGRN1 is nonfunctional

HeLa cells transiently co-transfected with HA-Ub and GFP-TSG101 constructs along with MGRN1 or MGRN1ΔR were lysed and immunoprecipitated with anti-GFP antibody. In vivo ubiquitination was detected by immunoblotting with anti-Ub antibody. Ubiquitination detected in the presence of MGRN1 is severely compromised when MGRN1ΔR is present. This reiterates previously published data to show that enzymatically active MGRN1 is required for TSG101 ubiquitination. In a similar experiment, cells were co-transfected with HA-Ub and GFP-TSG101 along with PrP or PrP(A117V), lysed, immunoprecipitated and analyzed for TSG101 ubiquitination. Note that the ubiquitination pattern seen in the presence of PrP is similar to that of MGRN1, while PrP(A117V) phenocopies MGRN1ΔR. The input levels of PrP, MGRN1 and TSG101 in the total lysates serve as loading control. From this experiment it may be inferred that expression of CmtPrP severely affects TSG101 ubiquitination, also reaffirming functional depletion of MGRN1 in the presence of enhanced levels of CmtPrP.
3.02.08 Partial depletion of MGRN1 increases level of autophagic marker in transgenic mice

Similar to cell lines and primary cells, I checked for the status of autophagy associated proteins in brain lysates from various transgenic mice, generated with PrP constructs that have previously been suggested to interact with and functionally sequester MGRN1 (Chakrabarti and Hegde, 2009; Rane et al., 2010). HuPrP(A117V) has previously been shown to generate enhanced levels of CmPrP in cells, in vitro and in vivo, compared to the other controls (Rane et al., 2010). Opn-HuPrP and Opn-HuPrP(A117V) was generated by replacing PrP signal sequence with a more efficient signal from Osteopontin (Opn). For Opn-HuPrP(A117V) the generation of CmPrP is almost reduced to the level seen for wild type, and no aberration in lysosomal phenotype is observed (Chakrabarti and Hedge, 2009).

I observed that level of LC3II was 1.4 fold more in the presence of HuPrP(A117V), a CmPrP than the non-transgenic control. Brain lysates from mice expressing Opn-HuPrP(A117V) or Opn-HuPrP had similar levels of LC3 II as the non-transgenic sample. p62 protein levels also corresponded with the LC3 II expression (Figures 3.12A and B).

Figure 3.12: Enhanced levels of autophagy markers in brain lysate of transgenic mice expressing CmPrP
A) Transgenic mice whole brain lysates were immunoblotted and analyzed for autophagy proteins/markers. The levels of GAPDH and β–tubulin serve as loading controls. Note similar levels of expression of PrP and MGRN1 across samples. The blots are representative of at least 3 independent experiments.

B) The immunoblots from panel A were analyzed for the levels of LC3 II. Graph shows fold change in LC3 II when normalized against corresponding GAPDH in the brain lysates. Results were analysed from three independent experiments. Error bars, SEM.

### 3.02.09 Overexpression of CyPrP and CtmPrP reduce degradation of GFP-LC3II

As siRNA mediated depletion of MGRN1 functions resulted in increase in the size and numbers of RFP-LC3 vesicles; I further wanted to check if expression of mislocalised prion proteins lead to alteration in degradation of GFP-LC3 proteins.

HeLa cells were co-transfected with GFP-LC3 and various PrP constructs, known to generate higher levels of CtmPrP and CyPrP. When analyzed in vitro, the PrP constructs having mutation in hydrophobic domain [PrP(AV3), PrP(A117V) and PrP(KHII)], generate increased CtmPrP relative to wild-type PrP (for wild type, secretory: transmembrane (Ctm) is 5.5 which diminishes to <1 for HD mutant) (Rane et al., 2010). I observed elevated level of GFP-LC3 II in cells expressing CyPrP and CtmPrP as compared to cells expressing wild type membrane-bound PrP (Figure 3.13). This result also indicated the role of MGRN1 in autophagy mediated lysosomal degradation.

Below is the table describing the various prion (PrP) constructs used:

<table>
<thead>
<tr>
<th>PrP construct</th>
<th>Detailed of the construct</th>
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<tbody>
<tr>
<td>WT PrP</td>
<td>This generates C terminal GPI (Glycosylphosphatidylinositol) linked Prion protein which remain anchored to cell surface (Prusiner, 1998).</td>
</tr>
<tr>
<td>PrP(AV3)</td>
<td>An artificial mutant in rodent PrP that contains three alanine-to-valine changes within the hydropphobic domain (HD), there by substantially increases HD hydrophobicity and</td>
</tr>
<tr>
<td><strong>PrP(A117V)</strong></td>
<td>Naturally occurring mutation in human PRNP (alanine to valine change at amino acid 117) that yields higher level $^{\text{Ctm}}$PrP and found to cause neurodegeneration when expressed in mice (Hegde et al., 1998, 1999).</td>
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<tr>
<td><strong>PrP(KHII)</strong></td>
<td>An artificial mutant in rodent PrP that lengthens the HD toward the N terminus and presumably shifts slightly the residues that would span the bilayer. This too causes neurodegeneration in transgenic mice (Hegde et al., 1998, 1999).</td>
</tr>
<tr>
<td><strong>Ifn-PrP</strong></td>
<td>PrP signal sequence is replaced with the signal sequence from Interferon-γ, therefore reduces translocation efficiency. It generates high level of cytosolic PrP, CyPrP (Rane et al., 2008).</td>
</tr>
<tr>
<td><strong>SA-PrP</strong></td>
<td>Artificial construct that does not possess N terminal signal sequence, therefore upon translocation to ER, the N-terminus of glycosylated SA-PrP was digested due to its exposure to the cytosol, leaving a protected C-terminal domain (‘Ctm’). It thus, generates $^{\text{Ctm}}$PrP exclusively. (Chakrabarti and Hedge, 2009)</td>
</tr>
</tbody>
</table>
With all these result taken together it was plausible to hypothesize that MGRN1 might play an important role in regulating of macroautophagy.

3.02.10 MGRN1 depletion leads to blocked fusion of autophagosomes with late endosomes and/or lysosomes

Enlarged size of late endosomes and/or lysosomes, along with increased levels of autophagy and lysosomal proteins, indicated two different possibilities. First, cargo destined to be degraded by macroautphagic pathway could get accumulated at lysosomes without its efficient degradation. Second, in a completely opposite scenario, if the rate of autophagy/proteolysis in lysosomes was high, it would demand increased synthesis of vesicle-associated proteins and hence result in elevated levels of autophagy and lysosomal proteins.

To understand which of the two hypothesized mechanism was actually playing, MGRN1 depleted cell lines (HeLa or SHSY5Y) or Mgrn1 null melanocytes were transfected with dual LC3B (mCherry-EGFP-LC3B) (Pankiv et al., 2007) and its maturation was studied.
**Mechanism of mCherry-EGFP-LC3B construct:**

In autophagosome, when pH is neutral this construct gives yellow color due to green fluorescence of GFP and red fluorescence of mCherry. When autophagosomes are matured to autolysosomes, fluorescence of acid labile GFP is quenched due to low pH. mCherry being acid-stable continues to fluoresce and thus gives rise to red color in autolysosomes. This construct thus helps to distinguish autophagosomes (green and red) from amphisomes/autolysosomes (red only) (Figure 3.14).

![Figure 3.14: Pictorial description of mCherry-EGFP-LC3B construct](image)

Autophagosome is yellow= red+green,
Autolysosome is red only.

In cell culture system MGRN1 was functionally deactivated either by using siRNA or overexpression of MGRN1ΔR and transfected with the dual LC3B (mCherry-EGFP-LC3B) construct.

HeLa cells were treated with mock or MGRN1 siRNA and transfected with the dual LC3B (mCherry-EGFP-LC3B) construct. MGRN1 knockdown significantly decreased (p≤0.001, number of cells analyzes, n=51) the percentage of red vesicles by ~2.0 folds (Figures 3.15 A and B) as compared to mock siRNA treated cells.
Figure 3.15: Altered processing of dual–tagged LC3B when MGRN1 function is compromised by siRNA treatment.

A) HeLa cells treated with mock siRNA or MGRN1 siRNA, were transfected with mCherry-EGFP-LC3B construct. 72 hours post transfection, cells were fixed and imaged. Yellow and red vesicles indicate autophagosomes and autolysosomes, respectively. Two fields for each condition are shown. Scale bar, 5μm.

B) Graph shows percentage of red vesicles analysed in panel A. The number of fluorescent bodies per cell among ~51 cells was calculated from 3 independent experiments. It shows a significant decrease in the percentage of red vesicles [calculated out of the total (yellow+red) vesicles for the entire sample set] using the given equation:

\[
\text{Percentage of red vesicles} = \left( \frac{\text{Average number of red vesicles in } n \text{ cells}}{\text{Average number of (yellow+red) vesicles}} \right) \times 100.
\]

*** p value ≤ 0.001 using Student’s t-test. Error bars, SEM.

When MGRN1 is functionally depleted by overexpression of catalytically inactive RING deleted mutant MGRN1ΔR, I observed decrease of ~2.5 folds (p≤0.001, total number of cells analyzed, n=55) in the percentage of red vesicles compared to MGRN1 control (Figures 3.16A and B).
Figure 3.16: Altered processing of dual–tagged LC3B upon expression of catalytically inactive MGRN1

A) HeLa cells transiently co-transfected with MGRN1 or MGRN1ΔR and mCherry-EGFP-LC3B were imaged. Note that expression of MGRN1ΔR closely phenocopies MGRN1 knockdown. Scale bar, 5μm. Two fields for each condition are shown.

B) Graph shows percentage of red vesicles analysed in panel A. 55 cells were analyzed. There is a significant decrease in the percentage of red vesicles [calculated out of the total (yellow+red) vesicles for the entire sample set] using the given equation:

\[
\text{Percentage of red vesicles} = \frac{\text{Average number of red vesicles in } n \text{ cells}}{\text{Average number of (yellow+red) vesicles}} \times 100.
\]

*** p ≤ 0.001 using Student’s t-test. Error bars, SEM.

Similarly, ~2.0 folds decrease (p≤0.001; total number of cells counted, n=26) in percentage of red vesicles for MGRN1 knocked down SHSY5Y cells was seen as compared to mock siRNA treated cells (Figures 3.17A and B).
**Figure 3.17: Altered processing of dual–tagged LC3B upon knockdown of MGRN1 in SHSY5Y**

A) SHSY5Y cells treated with mock siRNA or MGRN1 siRNA, were transfected with mCherry-EGFP-LC3B construct. 72 hours post transfection, cells were fixed and imaged. Two fields for each condition are shown. Scale bar, 5μm.

B) Graphical presentation of percentage of red vesicles from panel A shows comparable results between SHSY5Y and HeLa cells. 26 cells were analyzed. *** p ≤ 0.001, using Student’s t-test. Error bars, SEM.

When SHSY5Y cells were differentiated to neuronal fate by treatment with 10μM all- trans-retinoic acid (RA), for 4 days; decrease in the percentage of red autolysosomes in cells expressing MGRN1ΔR was observed (Figure 3.18).
Figure 3.18: Altered processing of dual–tagged LC3B in SHSY5Y cells differentiated to neuronal fate

A) SHSY5Y cells transiently co-transfected with MGRN1 or MGRN1ΔR and mCherry-EGFP-LC3B and treated with 10µM all-trans-retinoic acid for 4 day to differentiate. Cells were then imaged. Scale bar, 10µm. Enlarged views of the areas within the white boxes are also shown (insets).

B) The immunoblot shows expression MGRN1 and MGRN1ΔR.

In Mgrn1 null melan md1-nc cells ~4.1 folds decrease in the percentage of red vesicles was found when compared with the melan a6 cells (Total number of vesicles counted ~250) (Figure 3.19A and B).
Figure 3.19: Altered processing of dual–tagged LC3B in Mgrn1 lacking melanocytes

A) melan a-6 and melan md1-nc cells were transfected with mCherry-EGFP-LC3B and imaged 24 hours later. Note that total absence of MGRN1 (as in melan md1-nc cells) closely phenocopies the effects of its partial depletion in HeLa and SHSY5Y cells. Scale bar, 5μm. Two fields for each cell line are shown.

B) Graph plotting percentage of red vesicles from panel A. Histogram shows decrease in percentage of red vesicles in melan md1-nc cells, compared to the control melan a6 cells. 10 cells were analyzed. Error bars, SEM.

These results suggested that functional inactivation of MGRN1 (complete or even partial) compromised the maturation of autophagosomes (pH neutral vesicles) by their fusion with late endosomes and/or lysosomes (both of which are acidic vesicles), in a cell line independent manner. Therefore it is prudent to conclude that MGRN1 non functionality affect fusion of autophagosome with lysosomes. It is worth noting that a decrease in the percentage of acidic vesicles indicative of reduced lysosomal fusion was observed in melan md1-nc cells, despite a lack of increase in levels of LC3 II.
3.02.11 Impaired maturation of dual LC3B in HeLa upon overexpression of various disease associated C\textsuperscript{cm}PrP and CyPrP mutants

Similar to depletion or complete absence of MGRN1, ectopic expression of aberrant PrP mutants in HeLa also affects maturation of dual LC3B. HeLa cells were co-transfected with dual LC3B (mCherry-EGFP-LC3B) and various PrP constructs. These constructs generate increased levels of C\textsuperscript{cm}PrP or CyPrP and are reported to inappropriately interact with MGRN1, affect its cellular activity and phenocopy the effect on lysosomal morphology (Chakrabarti and Hegde, 2009). 24 hrs post transfection, cells were fixed and imaged. The percentage of red vesicles were quantified (Figures 3.20A and B). I found that expression of various C\textsuperscript{cm}PrP or CyPrP generating constructs led to at least ~2 folds decrease (p≤0.001; total number of cells counted, n=45) in the percentage of red vesicles compared to the controls (empty vector or wild-type PrP).

The result was tabulated as follows:

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Average percentage of red vesicles calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty vector</td>
<td>~57%</td>
</tr>
<tr>
<td>WT PrP</td>
<td>~62%</td>
</tr>
<tr>
<td>PrP(A117V)</td>
<td>~32%</td>
</tr>
<tr>
<td>PrP(KHII)</td>
<td>~28%</td>
</tr>
<tr>
<td>PrP(AV3)</td>
<td>~26%</td>
</tr>
<tr>
<td>SA-PrP</td>
<td>~32%</td>
</tr>
<tr>
<td>Ifn-PrP</td>
<td>~33%</td>
</tr>
</tbody>
</table>
Figure 3.20: Depletion of functional MGRN1 by ectopic expression of PrP mutants perturbs dual–tagged LC3B processing.
A) HeLa cells transiently co-transfected with various PrP constructs and mCherry-EGFP-LC3B were imaged 24 hours later. Scale bar, 5μm.

B) Histogram plotting the percentage of red vesicles (out of total yellow+red vesicles) in PrP expressing HeLa cells. 45 cells were analyzed for each PrP construct is indicated. *** p ≤ 0.001, using Student’s t-test. Error bars, SEM.

All these results indicated that partially or complete depletion of MGRN1 function affect maturation of dual LC3B vesicles. As this maturation required a number of vesicular fusion, I postulate that vesicular fusion is perturbed when MGRN1 is rendered non functional.

3.02.12 Impaired maturation of dual LCB vesicles can be rescued by overexpression of functional copy of MGRN1

I observed that absence of MGRN1 in melanocytes as well as depletion of MGRN1 activity by overexpression of disease causing PrP mutant (both CtmPrP and CyPrP) decreased percentage of red autolysosome as compared to control. Next was to ascertain whether this phenomenon was associated with the functionality of MGRN1.

Melanocytes (control a-6 and knockout md1-nc) were transfected with MGRN1 and MGRN1ΔR along with dual LC3B (mCherry-EGFP-LC3B). After 24 hrs, cells were fixed and number of yellow autophagosomes and red autolysosomes were quantified (Figures 3.21A). In a6 cells, or the percentage of red vesicles upon overexpression of MGRN1 was found to be ~45%, which reduced to ~32% upon expression of exogenous MGRN1ΔR, that blocked maturation of dual LC3B. In Mgrn1 knockout melanocytes, expression of functional copy of MGRN1 could salvage dual LC3B processing. Overexpression of MGRN1 in melan md1-nc cells, partially rescued and elevated the percentage of red vesicles to ~39% (that is >3 folds of untransfected cells in Figure 3.19), whereas MGRN1ΔR expression yielded percentage of red vesicles as ~19% (Figures 3. 21B). Overexpression of MGRN1ΔR in md1-nc did not aggravate the phenotype already exhibited in the untransfected melan md1-nc cells.
Figure 3.21: Maturation of mCherry-EGFP-LC3B is mediated via MGRN1 in melanocytes.

A) melan a-6 and melan md1-nc cells co-transfected with mCherry-EGFP-LC3B and either functional (MGRN1) or inactive (MGRN1ΔR) were imaged. Two fields for each cell line are shown. Scale bar, 5μm.

B) Graph plotting percentage of red vesicles (out of total yellow+red vesicles) shown in panel A. Approximately 10 cells were analyzed. Histogram denotes that overexpression of MGRN1 and MGRN1ΔR have exhibit opposite effects. Error bars, ±S.E.M.

Similar salvation of dual LC3B maturation was observed for various PrP transfected HeLa cells. CmPrP or CyPrP mediated perturbation of maturation of dual LC3B (mCherry-EGFP-LC3B) could be rescued by the overexpression of MGRN1. In presence of MGRN1, ~58% - ~68% percentage of fluorescent vesicles are red autolysosomes. The percentage of autolysosomes is significantly less (~20% - ~32%) in the presence of MGRN1ΔR and various PrP mutants (Figures 3.22 A and B). Results are tabulated below.
<table>
<thead>
<tr>
<th>PrP contract</th>
<th>MGRN1 or MGRN1ΔR</th>
<th>Percentage of red autolysosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT PrP</td>
<td>MGRN1</td>
<td>~68%</td>
</tr>
<tr>
<td></td>
<td>MGRN1ΔR</td>
<td>~38%</td>
</tr>
<tr>
<td>PrP(A117V)</td>
<td>MGRN1</td>
<td>~67%</td>
</tr>
<tr>
<td></td>
<td>MGRN1ΔR</td>
<td>~25%</td>
</tr>
<tr>
<td>PrP(KHII)</td>
<td>MGRN1</td>
<td>~58%</td>
</tr>
<tr>
<td></td>
<td>MGRN1ΔR</td>
<td>~20%</td>
</tr>
<tr>
<td>Ifn-PrP</td>
<td>MGRN1</td>
<td>~62%</td>
</tr>
<tr>
<td></td>
<td>MGRN1ΔR</td>
<td>~26%</td>
</tr>
</tbody>
</table>
Figure 3.22: Overexpression of functional copy of MGRN1 can salvage dual LC3B processing in presence of aberrant PrP constructs

A) HeLa cells co-transfected with the indicated PrP constructs, mCherry-EGFP-LC3B and either functional (MGRN1) or inactive (MGRN1ΔR) were imaged. Scale bar, 5μm.

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B) Histogram are plotted for the percentage of red vesicles. Number of cells analyzed for each PrP construct are indicated by n. The graph indicates that overexpression of MGRN1 partially rescues altered processing of mCherry-EGFP-LC3B, while MGRN1ΔR aggravates this phenomenon. Number of cells analyzed for each PrP construct are indicated. *** p ≤ 0.001, using Student’s t-test. Error bars, SEM.

Hence, when MGRN1 function was partially depleted (as by expression of CtmPrP or CyPrP mutant), presence of MGRN1ΔR further reduced the percentage of red vesicles. This was justified because expression of disease causing PrP could only partially reduce MGRN1 activity which could be further repressed by MGRN1ΔR expression.

These result validate that processing of dual LC3B is directly attributed to activity of MGRN1.

3.02.13 Live cell imaging of mCherry-EGFP-LC3B vesicle indicates the blocked vesicular fusion due to inhibition of MGRN1 function in real time

Depletion of MGRN1 functions resulted in reduction in number of red autolysosomes, although formation of yellow autophagosomes were found to normal. I observed that in MGRN1 knockdown cells, the size of yellow autophagosomes was arger than control cells. This was similar to the observations where RFP-LC3 vesicles were found to be bigger in size and higher in numbers. When I tried to look into the result closely, I postulated that less abundance of red vesicles in MGRN1 depleted cells could arise as consequence of two completely different situations:

First, blocked fusions of autophagosome with lysosomes.
Second, faster clearance of autolysosomes in MGRN1 depleted cells.

To check which one of these two was actually taking place, mock or MGRN1 siRNA treated and mCherry-EGFP-LC3B transfected. HeLa cells were imaged live (Ganley et al, 2011). A single yellow vesicle (autophagosome) was chased for both siRNA treated cells over indicated period of time.

In cells expressing mock siRNA, single yellow vesicles, expressing mCherry-EGFP-LC3B, turned completely red over a course of ~20-30 minutes (Figures 3.23 A and B), illustrating that the low pH in the lumen of the lysosome quenched the GFP signal, but not the mCherry.
A) HeLa cells treated with mock siRNAs, were transfected with mCherry-EGFP-LC3B construct. Live cells were imaged over indicated time periods. Entire cell is shown. Insets demarcates the vesicle which was tracked in real time. One representative field, out of 25 cells is shown. Scale bar, 5μm.

B) Fate of single vesicle tracked over indicated period of time. At 20 min time point yellow vesicle turns red due to quenching of green florescence.

Figure 3.23: Time-lapse images shows normal turnover of mCherry-EGFP-LC3B vesicles in control siRNA treated HeLa cell
In HeLa cells expressing MGRN1 siRNA, the GFP signal of autophagosomes was retained even after 60 minutes (Figures 3.24 A and B).

**Figure 3.24**: Time-lapse images shows blocked maturation of mCherry-EGFP-LC3B vesicles in MGRN1 siRNA treated HeLa cell

A) HeLa cells treated with MGRN1 siRNAs were transfected with mCherry-EGFP-LC3B construct. Live cells were imaged over indicated time periods. Insets demarcates the vesicle which was tracked in real time. One representative field, out of 25 cells is shown. Scale bar, 5μm.

B) Fate of single vesicle tracked. No change in yellow vesicle indicates that maturation of autophagosomes (yellow) to autolysosomes (red) is blocked.
In another set of experiments, when autophagosomes from MGRN1 knockdown cells were visualized over 150 minutes, GFP signal was retained in cells with functional depletion of MGRN1 – thus suggesting blocked fusion of autophagosomes with acidic vesicles (Figures 3.25A and B).

**Figure 3.25: Time-lapse images to follow maturation of mCherry-EGFP-LC3B vesicles.**

A) HeLa cells treated with MGRN1 siRNAs were transfected with mCherry-EGFP-LC3B and live cells were imaged. Images of the entire cell are shown over the indicated period of time. Scale bar, 5μm

B) Inset demarcates the vesicle which was tracked in real time. No change in yellow vesicle over 150 mins indicates that maturation of autophagosomes (yellow) to autolysosomes (red) is blocked.

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3.02.14 Bafilomycin treatment cannot further change the number of autolysosomes in cells that have perturbed mCherry-EGFP-LC3B maturation.

Bafilomycin A1 is a vacuolar H\(^+\) ATPase inhibitor that inhibits the acidification of organelles containing this enzyme, such as lysosomes and endosomes. It blocks fusions of autophagosomes with lysosomes (Yamamoto et al.,1998; Klionsky et al., 2008).

HeLa cells were transfected with functional and non functional copy of MGRN1 along with mCherry-EGFP-LC3B, followed by treatment with bafilomycin A1. Drug treatment showed no morphological changes in already enlarged yellow autophagosomes in cells expressing MGRN1ΔR. In control cells expressing MGRN1, treatment with bafilomycin A1, however, resulted in accumulation of neutral yellow vesicles in similar size range as the acidic red vesicles of the untreated controls (Figure 3.26A).

**Figure 3.26A: Bafilomycin A1 treatment shows no further effect on dual LC3B maturation**

HeLa cells transiently co-transfected with MGRN1 or MGRN1ΔR and mCherry-EGFP-LC3B and treated with vehicle (DMSO) or bafilomycin A1 and imaged. Bafilomycin A1 treatment resulted in accumulation of smaller yellow vesicles (autophagosomes). Insets are shown. Scale bar, 5μm.
Similar results were obtained when HeLa cells were partially depleted of MGRN1 function by overexpressing PrP(A117V), a CmPrP mutant. Wild type PrP (WT PrP) expressing cells were used as control (Figure 3.26B).

**Figure 3.26B: Bafilomycin A1 treatment shows no further effect on dual LC3B maturation**

HeLa cells transiently co-transfected with WT PrP or PrP(A117V) and mCherry-EGFP-LC3B and treated with DMSO (vehicle) or bafilomycin A1 and imaged. Bafilomycin A1 treatment resulted in accumulation of smaller yellow vesicles (autophagosomes). Insets are shown. Scale bar, 5μm.

This result indicated that treatment with bafilomycin A1 did not elicit additional perturbation on already blocked fusion between autophagosomes with lysosomes.

**3.02.15 Blocked fusion affects autophagic flux**

Autophagic induction results in conversion of LC3-I to LC3-II. When cells are induced for autophagy for a short period of time, the detectable amount of LC3-II increases with respect to LC3-I. However, if cells are subjected to autophagic induction for a relatively longer period of...
time, both LC3- I and LC3- II disappear due to their degradation at the lysosomes. When cells are treated with bafilomycin A1 (Yamamoto et al., 1998), degradation of LC3- II was partially inhibited, whereas the level of LC3- I remained unaffected. The amount of LC3- II at a certain point in time does not indicate the total autophagic flux. Rather, autophagic flux represented by differences in the amount of LC3- II between samples in the presence and absence of lysosomal protease inhibitors (Mizushima and Yoshimori, 2007, Ganley et al, 2011) is more accurate. As an alternative method for detecting the autophagic flux, level of p62, another autophagic marker (Pankiv et al., 2007) may also similarly be checked.

Mock and MGRN1 siRNA transfected HeLa cells were treated with bafilomycin A1 or left untreated and checked for the levels of LC3-II and p62. In control siRNA treated cells, drug treatment significantly increased (p≤0.05, no of independent experiments performed n=3) the level of LC3-II as compared to untreated control. In MGRN1 siRNA treated cells LC3-II level were already significantly higher than of control cells and did not changed significantly (p = 0.18) after bafilomycin A1 treatment. Level of p62 was observed as similar to LC3-II (Figures 3.27A and B).

**Figure 3.27: Compromise in MGRN1 activity perturbs autophagic fusion events.**

A) Mock or MGRN1 siRNA transfected HeLa cells were lysed and immunoblotted to analyze the levels of endogenous LC3 II and p62 in the presence or absence of 300nM bafilomycin A1. 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 3 experiments.
B) The immunoblots from panel A were analyzed for the levels of LC3 II. Graph shows fold change in LC3 II when normalized against corresponding β–tubulin in the cell lysates, analyzed from 3 independent experiments. ** p ≤ 0.05, n.s. not significant (p = 0.18) using Student’s t-test. Error bars, SEM.

HeLa cells expressing catalytically inactive MGRN1 showed significantly high level of endogeneous LC3-II as compared to untreated cells expressing functional MGRN1 (p ≤0.05). The level of LC3-II did not further increase upon treatment with bafilomycinA1(p =0.63) when MGRN1 was depleted. Bafilomycin A1 treatment however significantly increased in the level of LC3-II (p ≤0.05, no of independent experiment, n =3) in cells with functional MGRN1. Similar changes were observed for protein levels of exogenously expressed GFP-LC3II and p62 (Figures 3.28A and B).
Figure 3.28: Perturbation in autophagic fusion events is seen for both endogenous and exogenously expressed proteins when MGRN1 activity is perturbed by expression of catalytically inactive mutant

A) HeLa cells were transiently co-transfected with MGRN1 (as control) or MGRN1∆R and GFP-LC3. These were either treated with 300nM bafilomycin A1 or left untreated, lysed and immunoblotted to check for the indicated proteins. Without drug treatment amounts of GFP-LC3 II in control cells are negligible as noted in faint and dark exposures of the blot against GFP. The blots are representative of

B) 

<table>
<thead>
<tr>
<th>Baf A1</th>
<th>MGRN1</th>
<th>MGRN1∆R</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**n.s.**

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at least 3 experiments. The levels of GAPDH serve as loading control. Expression of MGRN1 and MGRN1∆R was checked.

B) (I) Quantification of data from blots produced in panel A shows fold change of endogenous LC3 II level when normalized against GAPDH from 3 independent experiments. ** p≤ 0.05, n.s not significant (p = 0.63), using Student’s t-test. Error bars, +SEM.

(II) Quantification of data from blots produced in panel A shows fold change of GFP-LC3 II/ GFP-LC3 I level. This fold change follows similar pattern as endogeneous LC3 II.

SHSY5Y cells transfected with MGRN1 or MGRN1∆R also elicited similar alterations in the levels of LC3 II and p62 as observed in HeLa cells (Figures 3.29A and B).

**Figure 3.29: Compromise in MGRN1 activity perturbs autophagic fusion events in SHSY5Y**

A) SHSY5Y transiently expressing MGRN1 or MGRN1∆R were either treated with 60nM bafilomycin A1 or left untreated; these were lysed and immunoblotted to check for the indicated proteins. The blots are representative of at least 3 experiments. The levels of GAPDH serve as loading control. Expression of MGRN1 and MGRN1∆R was checked.

B) Quantification of data from panel A denotes fold change in endogenous LC3 II level when normalized against GAPDH from 3 independent experiments. ** p≤ 0.05, n.s. not significant (p = 0.5), using Student’s t-test. Error bars, +SEM.
As previously discussed, a-6 melanocytes have a functional copy of MGRN1. In mock siRNA treated a-6 cells, bafilomycin A1 treatment increased levels of LC3-II. In MGRN1 siRNA treated a-6 cells however, the level of LC3-II was generally higher and did not yield a discernable difference against similar siRNA expressing cells without drug treatment. Similar alterations in p62 levels could be detected. This further confirmed that depletion of MGRN1 leads to blocked fusion of autophagosomes with lysosomes (Figures 3.30A and B).

**Figure 3.30: Compromise in MGRN1 activity perturbs autophagic fusion events**

A) melan a-6 treated with the indicated siRNAs, lysed and immunoblotted to check for the levels of endogenous LC3 II and p62 in the presence or absence of 50nM bafilomycin A1. The levels of β–tubulin serve as loading control. Note increase in LC3 II levels after depletion of MGRN1 even in melan a-6 cells. Efficiency of knockdown was shown using anti-MGRN1 antibody. This is representative of two independent experiments.

B) Quantification of data from panel A denotes fold change in endogenous LC3 II level when normalized against β–tubulin.

I wanted to check if complete absense of MGRN1 in melanocytes also affected autophagic flux in similar manner. Control melan a6 cells and Mgrn1 knockout melanocytes md1-nc cells were treated with 50 nM bafilomycin A1 and checked for level of LC3-II. In control melan a6
cells, bafilomycin A1 treatment led to an increase in the LC3-II levels. In md1-nc cells however level of LC3-II was much lower than untreated a-6 cells (seen in Figure 3.7) and drug treatment did not elevate it (Figures 3.31 A and B). This result further confirmed that autophagic flux was affected when MGRN1 was non functional.

**Figure 3.31: Mgrn1 null melanocytes shows aberration in autphagic flux**

A) Melan a6 and melan md1-nc cells either treated with 50 nM bafilomycin A1 (for 4 h) or left untreated, were lysed and immunoblotted to check for the indicated proteins. β-tubulin serves as loading control. This is representative of two independent experiments.

B) Quantification of data from (A) denotes fold change in endogenous LC3-II level when normalized against β-tubulin.

These emphasized that the effect of MGRN1 on autophagic flux was cell line independent. Partial loss of functional MGRN1 resulted in increase in LC3 II levels, total depletion reduced the basal LC3II protein levels. In both cases, though bafilomycin A1 treatment did not elicit any further alteration in LC3 II levels.
Taken together it was established that depleting MGRN1 functions led to perturbation of autophagic flux by impairing vesicular fusion events in macroautophagic pathway.

3.02.16 Compromising autophagy affects degradation via the ubiquitin-proteasome system (UPS)

Inhibition of autophagy has been reported to block the degradation of p62 which leads to increase in the level of proteosomal substrate due to delayed delivery to the proteasome. This has been previously shown by using an artificial construct (Ub\(^{G76V}\)-GFP), a ubiquitin proteasome pathway activity reporter (Korolchuk et al., 2009). Ub\(^{G76V}\)-GFP normally turns over rapidly and is present at low levels, but accumulates quickly if proteasome activity is impaired (Dantuma et al., 2000). Ub\(^{G76V}\)-GFP accumulates upon impairment of autophagy (Korolchuk et al., 2009) and therefore can be used as a tool for checking if autophagic turnover is perturbed.

HeLa cells treated with mock or MGRN1 siRNAs were transfected with Ub\(^{G76V}\)-GFP construct and treated with proteosomal inhibitor MG123 or left untreated. I observed that siRNA knockdown of MGRN1 in these cells resulted in an increase in the protein levels of Ub\(^{G76V}\)-GFP (Figure 3.32A), similar to those when autophagy was inhibited by deficiency of autophagy-related gene Atg7, Atg12 or Atg5 (Korolchuk et al., 2009). The level of Ub\(^{G76V}\)-GFP in MGRN1 knockdown cells were comparable to its level in control cell treated with proteosome inhibitor MG132. Knocking down of MGRN1 increased level of p62 as seen in previous experiments however had no effect on the levels of β-catenin, a proteasomal substrate. MG132 treatment affected the p62 and β-catenin protein levels similarly (Figure 3.32A).

To reestablish that the degradation of Ub\(^{G76V}\)-GFP took place via proteasomal pathway, cells were left untreated and treated with bafilomycin A1 (autophagy degradation inhibitor) or MG132 (proteasomal inhibitor). Elevated levels of Ub\(^{G76V}\)-GFP were seen in the presence of the proteasome inhibitor MG132 but not the autophagy blocker bafilomycin A1. The same assay also showed that p62 accumulated more upon bafilomycin A1 treatment, where as β-catenin was accumulated by MG132 treatment (Figure 3.32B).

Depletion of MGRN1 functions thus affected autophagic degradation which in turn block degradation of the artificial Ub\(^{G76V}\)-GFP construct. This experiment further provided evidence that autophagic degradation was blocked upon inactivation of MGRN1 function.
**Figure 3.32: Degradation via the ubiquitin-proteasome system is affected when autophagy is compromised**

A) HeLa cells treated with mock or MGRN1 siRNAs were transiently transfected with Ub$^{G76V}$-GFP construct (denoted as Ub-GFP) and treated with 10μM MG132 or vehicle control for 6 hours before harvesting. Cell lysates were immunoblotted with the indicated antibodies. Faint and dark exposures show accumulation of Ub-GFP with MGRN1 depletion. Ponceau-S stained membrane shows equal loading of total protein across samples. RNAi efficiency was shown using MGRN1 antibody.
B) UbG76V-GFP transfected cells were either left untreated or treated with 300nM bafilomycin A1 or 10μM MG132. Samples were probed with GFP, p62 and β-catenin antibodies. Ponceau S stained membrane was used as loading control.

3.02.17 Depletion of MGRN1 irreversibly compromised autophagic degradation

For studying autophagy, it is a common practice to stimulate the onset of this process by nutrient deprivation or treatment with autophagy inducing drugs. So far I observed that depletion of MGRN1 blocked fusion of autophagic vesicles without any external stimulus. It was worth exploring what happened to the marker proteins when such cells were pushed towards autophagy. For this HeLa cells were treated with rapamycin, a commonly used autophagy inducing drug. Autophagy is negatively regulated by the mammalian target of rapamycin (mTOR) and therefore can be induced in a diverse range of cells (yeast through mammals) by the mTOR inhibitor rapamycin (Sarkar et al., 2009). In cells with normal autophagic turnover, treatment with rapamycin within a limited time range increased levels of LC3-II. This went back to basal levels when rapamycin was removed. But if autophagic flux is perturbed, rapamycin treatment yields different results, as observed in MGRN1 depleted cells.

HeLa cells were transfected with vector control or MGRN1 or MGRN1ΔR along with GFP-LC3 and treated with rapamycin for inducing autophagy. After 24 hrs of rapamycin treatment, cells were subject to rapamycin removal. In control and MGRN1 expressing cells, removal of rapamycin significantly decreased (p≤0.05, number of independent experiment, n=3) level of transiently expressing GFP-LC3 II from its elevated level seen in presence of rapamycin. However the level of GFP-LC3 II remained high in MGRN1ΔR expressing cells even after drug removal. This indicated that degradation via autophagic flux was blocked in MGRN1ΔR expressing cells (Figures 3.33A and B).

These showed that autophagic degradation was irreversibly compromised when MGRN1 was non functional.
Figure 3.33: Compromise in MGRN1 activity blocks rapamycin induced autophagic degradation

A) HeLa cells transiently co-transfected with GFP-LC3 and MGRN1 or MGRN1ΔR were either left untreated or treated with rapamycin for 24 hours. Cells transfected with empty vector and GFP-LC3 were used as controls. Following this, cells were either immediately lysed or allowed to recover from the drug treatment for 12 hours and then harvested. All samples were immunoblotted using anti-GFP and anti-MGRN1 antibodies. GAPDH was used as loading control. The blots are representative of at least 3 experiments.

B) Histogram shows fold change in the ratio of GFP-LC3 II/I when normalized against GAPDH from panel C. Graph was generated from 3 independent experiments. **P≤0.05, n.s., not significant (P =0.67) using Student’s t-test. Error bars, ±S.E.M.
A similar result was obtained in imaging study where the number of GFP-LC3 vesicles was counted in HeLa cells that were similarly treated with rapamycin to induce autophagy and then withdrawn from the drug. There was an increase in GFP-LC3 vesicles numbers upon rapamycin treatment irrespective of the status of MGRN1. However, drug withdrawal had remarkably different effects when MGRN1 was nonfunctional -- the total number of vesicles remained high as the treated sample, while in the presence of functionally active MGRN1, the vesicle numbers decreased (Figures 3.34A and B). Full length MGRN1 expressing cells phenocopied the untransfected HeLa cells.
Figure 3.34: Compromise in MGRN1 activity blocks rapamycin induced autophagic degradation of GFP-LC3II vesicles
A) HeLa cells transiently co-transfected with GFP-LC3 and MGRN1 or MGRN1∆R were either left untreated or treated with rapamycin for 24 hours. Cells transfected with empty vector and GFP-LC3 were used as controls. Following this, cells were either immediately fixed or allowed to recover from the drug treatment for 12 hours and then fixed and were imaged for the presence of green fluorescent structures. Rapamycin treatment increases no of green vesicles irrespective of status of MGRN1. However, upon withdrawal of rapamycin, no of green vesicles reduces in empty vector and MGRN1 expressing cells. Cells expressing MGRN1∆R have the GFP-LC3II vesicles in higher numbers. Scale bar, 5 μm.

B) Cells imaged in (A) were scored for total number of GFP-LC3 positive vesicles and average number of such vesicles was calculated. n, total number of cells for the various treatments are indicated in the figure. Error bars, ±S.E.M.

These results clearly indicated that degradation via autophagic pathway needed functional MGRN1. When MGRN1 was non functional, autophagy was stimulated (as seen by elevation in the level of LC3-II/LC3-I), but the downstream processes of autophagic degradation were perturbed.

Thus the results so far suggested that affecting MGRN1 activity severely affected autophagic flux and its degradative competence by affecting vesicular fusions.

3.02.18 Depletion of MGRN1 affects endocytic pathway

Endocytic trafficking is one of the key events for cellular homeostasis as well as protein degradation inside cells. Internalization of cargo takes place at early endosomes which then matures in MVB and further fuses with lysosome for degradation (Mullins and Bonifacino, 2001; Katzmann et al., 2002; Gruenberg et al., 2004). Depletion of MGRN1 using siRNA was reported to block degradation of EGFR (epidermal growth factor receptor) by disrupting endosome to lysosome trafficking and the downstream signaling cascade (Kim et al., 2007).

My observations re-iterated that epidermal growth factor (EGF)-induced EGFR degradation and clearance required functional MGRN1. HeLa cells were treated with GFP or MGRN1 siRNAs and then first allowed to internalize Alexa488-labeled EGF and chased at 37°C with unlabeled medium for a number of different time point up to 3 hr. The fate of the internalized EGF was monitored by confocal immunofluorescence microscopy. In MGRN1 knock-down cells accumulation of Alexa-Fluor 488 labelled EGF indicated that compromising MGRN1 function blocked clearance of cargo brought in via the receptor-mediated endocytosis pathway (Figure
3.35A). In a similar experiment, level of EGFR was checked by western blot analyses. EGF induced EGFR degradation was found to be blocked in MGRN1 siRNA treated cells (Figure 3.35B).

Figure 3.35: Functional depletion of MGRN1 perturbs the endo-lysosomal pathway, and subsequent clearance of cargo.

A) HeLa cells treated with GFP or MGRN1 siRNAs were subjected to Alexa-Fluor 488 EGF uptake. Cells were then washed, fixed at indicated time points and imaged to monitor the fate of the fluorescent EGF-EGFR complex. Scale bar, 5μm.

B) Cells were similarly treated as in panel A, except untagged EGF was used to stimulate EGFR-mediated uptake, trafficking and degradation. β-tubulin was used as loading control. Efficiency of knockdown was confirmed by immunoblotting with anti-MGRN1. The blots are representative of at least 3 experiments.
These results clearly showed that along with autophagic degradation, MGRN1 has a role in endocytic degradation of lysosomal degradative pathway.

3.02.19 Depletion of MGRN1 affects fusion of vesicles with lysosome

In summary, the results so far have established a role for MGRN1 in regulating both autophagic and endocytic pathway. MGRN1 was reported to ubiquitinat the ESCRT-I protein TSG101 (Kim et al., 2007). ESCRT proteins are classically involved in biogenesis of multivesicular bodies (MVB) (Katzmann et al., 2001; Babst et al., 2002; Babst et al., 2002a; Raiborg and Stenmark, 2009). Recent reports showed that they are also involved in fusion of MVBs with lysosomes and also autophagosomes with lysosomes (Filimonenko et al., 2007; Lee et al., 2007). Based on these reports as well as the results I obtained, it was prudent to postulate that MGRN1 modulates lysosomal degradation by blocking one or more following vesicular fusion events (i) MVB and lysosome, (ii) autophagosome and lysosome, (iii) autophagosome and MVB [fusion between these generates an amphisome] or (iv) amphisome and lysosome (Metcalf et al., 2010).

To distinguish between the different fusion events, HeLa cells treated with mock siRNA or MGRN1 siRNA were transfected with RFP-LC3 and monitored for EGF uptake and degradation over a period of time. RFP-LC3 vesicles were utilized as marker for autophagosomes and EGF labelled vesicles marked endosomes. In both control and MGRN1 knockdown cells, presence of RFP-LC3 vesicles and loading with Alexa-Fluor 488 labelled EGF on the cell surface werecomparable. After 30 mins of chase both the cell had green (Alexa-Fluor 488 labelled EGF-EGFR internalized endosomes), red (RFP-LC3 autophagosomes) and yellow (amphisomes) vesicles. After 180 minutes, in control cells almost all the Alexa-Fluor 488 labelled EGF-EGFR internalized vesicles were degraded and only puncta positive for RFP-LC3 remained. The result reconfirmed that lysosomal degradation was normal for control cells as seen in the previous experiments. In MGRN1 depleted cells however the green, red and yellow vesicles could be detected even after 180 minutes. This indicated that fusion of MVB (green vesicles) with lysosome and that of amphisome (yellow) and lysosome were compromised (Figures 3.36A and B). Since yellow vesicles could be seen both at 30 and 180 minutes, it was justified to extrapolate that amphisome formation was not affected in the presence of MGRN1.
Figure 3.36: Functional aberration of MGRN1 blocks fusion of vesicles with lysosomes.
A) HeLa cells treated with mock or MGRN1 siRNAs and transiently transfected with RFP-LC3 construct were subjected to Alexa-Fluor 488 EGF uptake. Cells were washed, fixed at indicated time points and imaged. Insets reveal enlarged views of green (Alexa-Fluor 488 labelled EGF), red (RFP-LC3) and yellow (Alexa-Fluor 488 EGF and RFP-LC3 colocalised) vesicles as shown by respective coloured arrow-heads. Scale bar, 5μm.

B) Graph represents average number of red, green and yellow fluorescent vesicles analyzed over 5 fields. Error bars, SEM.

Similar experiments were recapitulated in SHSY5Y cells co expressing RFP-LC3 subjected to EGF uptake and degradation, using Alexa-Fluor 488 labelled EGF. Functional inactivation of MGRN1 resulted in blocked degradation of EGF-EGFR complex by compromising fusion between MVB (green vesicles) with lysosome and that of amphisome (yellow) and lysosomes (Figure 3.37). However the kinetics was found to be much slower for SHSY5Y cells, complete degradation of EGF-EGFR complex required 360 min for control cells.

Figure 3.37: In SHSY5Y cells, vesicular fusion with lysosome is blocked when MGRN1 is non functional
SHSY5Y cells transiently transfected with MGRN1 or MGRN1ΔR and RFP-LC3 construct were subjected to Alexa-Fluor 488 EGF uptake. The time points at which fixed cells were imaged are indicated. Insets reveal enlarged views of green (Alexa-Fluor 488-labeled EGF), red (RFP-LC3) and yellow (Alexa-Fluor 488 EGF and RFP-LC3 colocalized) vesicles as shown by respective colored arrow heads. Scale bar, 5 μm.

3.03 Conclusion:

Mahogunin RING Finger 1 is a ubiquitously present cytosolic ubiquitin ligase (He et al., 2003). Reports showed that inactivation of MGRN1 in a subset of prion diseases (where CyPrP and CmPrP, aberrantly metabolised isoform of prions, are accumulated inside cells), resulted in lysosomal phenotypic aberration (Chakrabarti and Hedge, 2009). Along with these, degradation via endocytic pathway was found to be blocked due to perturbation in endosome to lysosome trafficking and downstream signaling cascade (Kim et al., 2007).

In my work I have established that MGRN1 participates in the regulation of both autophagic and endocytic pathways leading to lysosomal degradations. MGRN1 regulates vesicular fusion events comprising both the pathways, and inactivation of MGRN1 functions thus results in blockage in transport of cargoes to lysosomes.

Melanocytes obtained from Mgrn1 null mice show accumulation of bigger acidophilic vesicles similar to those observed previously when MGRN1 is functionally sequestered in prion mediated neurodegeneration (Chakrabarti and Hedge, 2009). Depletion of MGRN1 by siRNA in cultured cells causes clustering and enlargement of late endosome and lysosomes. These lysosomal phenotypes indicate either (i) improper biogenesis of lysosomes. and/or (ii) accumulation of cargoes in lysosomes without efficient degradation.

Autophagy and endocytosis are two different lysosomal degradative pathways. Perturbations in any or both these pathways could lead to aberrations in functions as well as phenotypes of lysosomes. Depletion in MGRN1 functions blocks maturation of dual LC3B (mCherry-GFP-LC3B) vesicles (from autolysophagosomes to autolysosomes) that indicates hindrance in autophagic fusion events. Blocked maturation of dual LC3B vesicles is observed for both partial (CmPrP and CyPrP mediated MGRN1 functional sequestration) or complete (RNAi technique and in melanocytes obtained from Mgrn null mice) inactivation of MGRN1. On similar lines,
autophagic flux is found to be affected when MGRN1 was nonfunctional. As a support of these observations, I find that accumulation of both endogenous LC3-II and p62 as well as ectopically expressed proteins (GFP-LC3, RFP-LC3) occur due to inefficient autophagic degradation when MGRN1 is functionally inactive. Taken together all these data suggest that when MGRN1 is rendered nonfunctional (either by overexpression of mislocalized prion proteins, or overexpression of catalytically inactive mutant of MGRN1 or by RNAi technique), or absent (in Mgrn1 null melanocytes) initiation of autophagy is normal, but its downstream degradative pathway is blocked. It is hence prudent to conclude that when MGRN1 is nonfunctional, accumulation of cargoes at late endosomes and lysosomes takes place that lead to formation of highly fused acidophilic vesicles.

Endocytic pathway is also reported to be blocked when MGRN1 is non functional (Kim et al., 2007). Therefore, both arms of lysosomal degradation are found to be impeded when MGRN1 was depleted. It may be speculated that though both these pathways originate differently, they finally converge at later stages for degradation of cargo at lysosomes. As expected, when MGRN1 is nonfunctional, fusion of MVB (multivesicular body/ late endosomes) with lysosomes is arrested, thus affecting degradation via endocytic pathway. At the same time, although formation of autophagosomes and their maturation to amphisomes (fused structure of autophagosome and MBV/ late endosomes) is normal; fusion of amphisomes with lysosomes is impeded. This also explained why in MGRN1 knockdown cells autolysosomes are so few in numbers as compared to control cells. These results clearly indicated that MGRN1 participated in late fusion events which are common for autophagy and endocytosis in lysosomal degradative pathways.

MGRN1 mediated regulation of vesicular fusions may be mediated by its interaction with the ESCRT protein, TSG101. (Kim et al., 2007). ESCRT proteins are involved in sorting of ubiquitinated cargoes from cell membrane to endosomes and biogenesis of multivesicular bodies (Raiborg and Stenmark, 2009). Inactivation of various ESCRT subunits are reported to affect the structure and function of endo-lysosomal compartments as well as autophagy pathway by disrupting fusions events involving lysosomes, endosomes and autophagosomes (Nara et al., 2002; Lee et al., 2007; Filimonenko et al., 2007; Tamai et al., 2007; Metcalf et al., 2010). Interestingly, although there are defects in autophagis and endocytic pathway, Mgrn1 null mice can survive and melanocytes obtained from them can propagate in cell culture for 30 cycles.
suspect the presence of a yet unexplored compensatory mechanism that enables \textit{Mgrn} null melanocytes to survive. It may be pointed out that while MGRN1 seems to play a very important role in maintaining cellular homeostasis, \textit{Mgrn1} null mutants are not all embryonic lethal. This is plausible because, not all the fusion processes leading to lysosomal degradation are blocked when MGRN1 is inactive. In this study, I have established only a subset of autophagic and endocytic fusion events that are affected when MGRN1 is depleted.