Chapter 5: Mahogunin RING finger 1 mediated fusion between amphisome and MVB with lysosomes is regulated by TSG101 ubiquitination
5.01 Introduction

In the previous chapters, I have shown that MGRN1 affects lysosomal degradative pathway by regulating fusion of lysosomes with late endosomes/MVBs and amphisomes. The blocked vesicular fusion however does not affect lysosomal competence as evaluated by the activity of lysosomal aspartic protease Cathepsin D; the pH of lysosomes also remains unchanged. In this chapter the mechanism behind regulation of vesicular fusion by MGRN1 will be discussed.

Reports show that MGRN1 interacts with TSG101, an ESCRT-I protein and monoubiquitinates it (Kim et al., 2007). ESCRT machinery is a multimeric protein complex that participates in sorting of ubiquitinated cargoes from cell membrane to endosomes and therefore helps in biogenesis of multivesicular bodies (Raiborg and Stenmark, 2009). Some ESCRT proteins are known to facilitate vesicle fusion with lysosomes. Inactivation of various ESCRT subunits are reported to affect the structure and function of endo-lysosomal compartments as well as the autophagy pathway (Nara et al., 2002; Lee et al., 2007; Filimonenko et al., 2007; Tamai et al., 2007; Metcalf et al., 2010).

TSG101 binds to ubiquitinated cargoes through its N-terminal UEV domain which are sorted into vesicles that finally forms internal vesicles in MVBs (Sundquist et al., 2004; Katzmann et al., 2001). Association of TSG101 to endosomal membrane occurs though its binding to the ESCRT-0 protein, HRS. This is followed by recruitment of other downstream ESCRT proteins needed for maturation and formation of MVB (Raiborg and Stenmark, 2009). Report shows that TSG101 aids formation of stable vacuolar domains within the early endosome that eventually form MVBs (Razi and Futter, 2006).

When TSG101 is depleted, structural rearrangement of the early endosome takes place, where the pattern of tubular and vacuolar domains is replaced by enlarged vacuoles lacking surface proteins notably clathrin (Doyotte et al., 2005). The formation of such multicisternal early endosome results in aberration in endo-lysosomal trafficking. Reports show that inactivation of TSG101 causes accumulation of ubiquitinated proteins on early endosomes and it also blocks degradation by autophagic pathway (Filimonenko et al., 2007).

MGRN1 and TSG101 interact in a bimodal manner. PSAP motif of MGRN1 interacts with N-terminal UEV (catalytically inactive ubiquitin E2 variant) domain of TSG101. Another interaction between these two proteins involves the residues 317-392 of MGRN1 and the COOH terminal residues 311-390 of TSG101 (Kim et al., 2007). Monoubiquitination of TSG101 by
MGRN1 is needed for proper endosomal trafficking of EGF-EGFR complexes to lysosomes as well as its degradation (Kim et al., 2007).

In my work I have seen that multi-monoubiquitination of TSG101 by MGRN1 is involved in vesicular fusion of late endosomes/MVB with lysosomes. The blockage in autophagic and endocytic fusion imparted by depletion of MGRN1 can be salvaged by overexpression of TSG101. At the same time monoubiquitination of TSG101 is also essential for its involvement in vesicular fusion events.

Interestingly, I report that when MGRN1 is inactive, formation of MVBs or autophagosomes is not altered. These indicate presence of other ubiquitin ligases modifying TSG101 enzymes. It is reported that TAL (TSG101 associated ligase), another RING finger motif is involved in multiple monoubiquitylation of TSG101 which eventually regulates cargo sorting in MVB (Amit et al., 2004). Therefore I can conclude that regulation of TSG101 by MGRN1 affects at the late stage of vesicular fusions.

5.02 Observations

5.02.01 Defects in autophagic flux caused by MGRN1 is mediated via TSG101

The ESCRT-I protein, TSG101 is the first identified substrate for MGRN1 (Kim et al., 2007). In chapter 3, I have established that MGRN1 depletion leads to blockage in autophagic flux by blocking fusion of amphisomes with lysosomes. As TSG101 act as a downstream protein of MGRN1, it was plausible to ask if exogenous expression of TSG101 could rescue autophagic flux by restoring vesicular fusion.

HeLa cells were transfected with mock or MGRN1 siRNA along with control vector or HA-TSG101 and treated with bafilomycin A1. In mock siRNA treated cells, bafilomycin A1 treatment led to significant increase (p≤0.05, n=3) in the level of LC3-II irrespective of the presence or absense of HA-TSG101. In MGRN1 depleted cells, which were transfected with empty vector, basal level of LC3-II was higher than control and did not significantly elevate (p=0.2) upon bafilomycin A1 treatment. This indicated blocked autophagic flux in MGRN1 depleted samples, as seen earlier. However when HA-TSG101 was overexpressed in MGRN1
depleted cells, level of LC3-II rose significantly (p≤0.05, n=3). The change in the level of p62 was comparable to that of LC3-II (Figures 5.1A and B). These results indicated that overexpression of TSG101 could rescue the blockage in autophagic flux by restoring vesicular fusion events.

Figure 5.1: Functional TSG101 salvages blocked autophagic flux
A) HeLa cells treated with the indicated siRNAs were transfected with either empty vector or HA-TSG101. Cell lysates were immunoblotted to analyze the levels of endogenous LC3-II and p62 in the presence or absence of 300 nM bafilomycin A1. GAPDH was used as loading control. Efficiency of knockdown was shown by immunoblotting with anti-MGRN1. ▶ indicates HA-TSG101; ← endogenous TSG101. The blots are representative of at least three independent experiments. B) Graph shows fold change in LC3 II when normalized against corresponding GAPDH levels; analyzed from three independent experiments. **p≤0.05, n.s., not significant, (p= 0.2) using Student’s t-test. Error bars, ±S.E.M.

Similarly in SHSY5Y cells, when MGRN1 was rendered nonfunctional by expression of catalytically inactive RING deleted mutant of MGRN1 (MGRN1ΔR), exogenous expression of TSG101 could partially rescue the autophagic fusion events (Figures 5.2A and B).
Figure 5.2: MGRN1 mediates defects in autophagic flux is regulated via TSG101

A) SHSY5Y cells co-transfected with MGRN1 or MGRNΔR construct and either empty vector or HA-TSG101. Cell lysates were analyze for the levels of endogenous LC3 II in the presence or absence of 60 nM bafilomycin A1. GAPDH was used as loading control. Efficiency of all transfections was checked. The blots are representative of at least three independent experiments.

B) LC3-II fold change

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<tr>
<th>Condition</th>
<th>MGRN1</th>
<th>MGRNΔR</th>
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<tr>
<td>Baf A1</td>
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<tr>
<td>Vector control</td>
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<td>HA-TSG101</td>
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** p < 0.01; n.s. = not significant.

A) SHSY5Y cells co-transfected with MGRN1 or MGRNΔR construct and either empty vector or HA-TSG101. Cell lysates were analyzed for the levels of endogenous LC3 II in the presence or absence of 60 nM bafilomycin A1. GAPDH was used as loading control. Efficiency of all transfections was checked. The blots are representative of at least three independent experiments.
B) Graph shows fold change in LC3 II when normalized against corresponding GAPDH levels; analyzed from three independent experiments. **p≤ 0.05, n.s., not significant, (p=0.8) using Student’s t-test. Error bars, ± S.E.M.

It should be emphasized that though TSG101 is tightly regulated in cells (McDonald and Martin-Serrano, 2008), its ectopic expression reversed the MGRN1 depletion phenotype.

5.02.02 Restoration of EGFR degradation upon expression of TSG101

As depletion of MGRN1 was showed to block degradation of the epidermal growth factor receptor, it was obvious to ask if expression of TSG101 could rescue this phenotype.

To address this, HeLa cells expressing control or MGRN1 siRNAs were transfected with HA-TSG101 and stimulated with epidermal growth factor (EGF). Cells were chased at 37°C and samples were lysed at different time points for a total 3 hrs chase. The level of EGFR was checked by western blot analysis. In MGRN1 knockdown cells, overexpression of HA-TSG101 could induce normal degradation of EGFR at 3 hrs, similar to control cells. Hence, the dynamics of EGFR degradation was rescued by TSG101 (Figure 5.3).

**Figure 5.3: Defects in EGFR degradation caused by MGRN1 are mediated via TSG101**

HeLa Cells treated with the control or MGRN1 siRNAs and transfected with HA-TSG101 were subjected to EGF uptake. Lysates were analyzed for the levels of EGFR at specified time intervals. β-tubulin was used as loading control. ◀ indicates HA-TSG101; ← endogenous TSG101. Efficiencies of knockdown and transfection were also checked.
5.02.03 TSG101 acts downstream of MGRN1

It was further verified if overexpression of MGRN1 could also rescue the phenotypes generated due to depletion of TSG101.

HeLa cells treated with control or TSG101 siRNAs were transfected with MGRN1 (full length) or MGRN1ΔR (catalytically inactive). Bafilomycin A1 treatment increased the levels of LC3-II and p62 in mock siRNA treated cells transfected with MGRN1. When MGRN1ΔR was expressed in mock siRNA treated cells, there was an elevation in the basal level of LC3-II and p62. Drug treatment did not alter this. In TSG101 siRNA treated cells, expressing either MGRN1 or MGRN1ΔR, bafilomycin A1 could not elicit an increase in the levels of LC3 II and p62 beyond their basal expression levels without the drug. Functional inactivation of TSG101 caused a detectable elevation in LC3 II and p62. Over-expression of MGRN1 or MGRN1ΔR did not affect this further, even if cells were treated with bafilomycin A1 (Figure 5.4). In summary, depletion of TSG101 caused autophagic flux aberration which could not be overcome by exogenous MGRN1. The reverse was, however, possible. This experiment hence proves that TSG101 acts downstream of MGRN1.

**Figure 5.4: Defects in autophagic flux caused by TSG101 depletion cannot be salvaged by MGRN1 expression**

In a reverse experiment, HeLa cells were treated with mock or TSG101 siRNAs, followed by transfection of MGRN1 or MGRN1ΔR. Cell lysates were immunoblotted to analyze the levels of endogenous LC3 II and p62 in the presence or absence of 300 nM bafilomycin A1. GAPDH was used as loading control. Efficiency of knockdown was checked by immunoblotting with anti-TSG101. Expression of
MGRN1 or MGRN1ΔR was checked. Note that the expression of MGRN1ΔR phenocopies TSG101 depletion; also MGRN1 cannot rescue the effects mediated by TSG101.

5.02.04 Blocked endocytic trafficking in TSG101 depleted cells

TSG101 is reported to be involved in the formation of endosomes and MVBs (Katzmann et al, 2001). Depletion of TSG101 led to blockage in endocytic degradation of EGF-EGFR complex (Doyotte et al., 2005, Razi and Futter, 2006). As perturbation in autophagic flux by TSG101 depletion could not be rescued by overexpression of MGRN1, it was prudent to check if the endocytic pathway was also similarly affected.

HeLa cells were treated with GFP or TSG101 siRNAs were transfected with MGRN1 or MGRN1ΔR. They were then stimulated with Alexa-488 tagged EGF and imaged for the kinetics of EGF-induced EGFR uptake, trafficking and degradation. As expected, in GFP siRNA treated cells, overexpression of MGRN1ΔR blocked clearance of vesicular cargo. Depletion of TSG101 blocked degradation of EGF-EGFR complex which could not be rescued by over expression of MGRN1. In the presence of TSG101 siRNA, overexpression of MGRN1ΔR generated a more severe qualitative phenotype than MGRN1 (Figure 5.5).

Figure 5.5: Defects in endocytic pathway by TSG101 depletion cannot be rescued by MGRN1ΔR

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<th>MGRN1</th>
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![Image](image7.png)
Cells treated with mock or TSG101 siRNAs and transiently transfected with MGRN1 or MGRN1ΔR were subjected to Alexa-Fluor 488 EGF uptake. They were washed, fixed at indicated time points and imaged. The disruption of endo-lysosomal pathway by TSG101 depletion could not be salvaged by MGRN1 overexpression. Scale bar, 5 μm. In the presence of TSG101 siRNA, overexpression of MGRN1ΔR generated a more severe qualitative phenotype than MGRN1.

Taken together all these results show that functional TSG101 is able to rescue the effects of MGRN1 inactivation, however, the reverse does not happen.

4.02.05 Monoubiquitination of TSG101 affects vesicular fusion in autophagic pathway

MGRN1 is reported to multi-monoubiquitinate TSG101 (Kim et al, 2007). In vivo analyses shows that the pattern of ubiquitination and as well the solubility of TSG101 differs between wild-type and Mgrn1 null mutant mice and the pattern also changes with time (Jiao et al, 2009). The change in solubility of TSG101 is hypothesized to play a key role in the pathogenesis of spongiform encephalopathy in Mgrn1 null mice.

Induction of autophagic flux was used as a readout to test the significance of MGRN1-mediated monoubiquitination of TSG101 in regulating vesicular fusion with lysosomes. HeLa cells were transfected with MGRN1 or MGRN1ΔR along with HA-TSG101 and two different ubiquitin mutants. K0 Ub is a lysineless ubiquitin mutant that promotes only monoubiquitination (Tan et al, 2008). ΔG75/76 Ub in another mutant which cannot be conjugated to substrates rather binds noncovalently to ubiquitin interacting domains and acts as a competitive inhibitor of Ub binding (Mattera and Bonifacino, 2008). The cells were treated with bafilomycin A1 or vehicle controls and level of LC3- II and p62 were checked. Over-expression of TSG101 in the presence of K0 Ub, elicited significant increase (p≤ 0.05) in the levels of LC3 II in the presence of bafilomycin A1, irrespective of the catalytic activity of MGRN1. No change in LC3 II levels could be detected upon drug treatment in the presence of ΔG75/76 Ub. As expected, functional depletion of MGRN1 led to elevated levels of LC3 II (Figures 5.6A and B). Therefore monoubiquitination of TSG101 in presence of MGRN1 is required for autophagic fusion events.
Figure 5.6: MGRN1-mediated monoubiquitination of TSG101 is required for degradation of cargo in autophagic pathway

A) HeLa cells co-transfected with MGRN1 or MGRN1ΔR, HA-TSG101 along with K0 Ub or ΔG75/76 Ub were lysed and immunoblotted to analyze the levels of endogenous LC3 II in the presence or absence of 300 nM bafilomycin A1. GAPDH was used as loading control. Efficiencies of all transfections were checked. The blots are representative of at least three experiments. Note that overexpression of TSG101, without its monoubiquitination cannot rescue autophagosomal degradation. ◄ indicates HA-TSG101; ← endogenous TSG101.

B) LC3-II fold change

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<th>Condition</th>
<th>MGRN1</th>
<th>MGRN1ΔR</th>
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** n.s.
B) Graph shows fold change in endogenous LC3 II level when normalized against GAPDH from three independent experiments. **p≤0.05, n.s., not significant (p=0.3 and 0.6 in the presence of MGRN1 and MGRN1ΔR, respectively) using Student’s t-test. Error bars, ± S.E.M.

4.02.06 Biomodal interaction between MGRN1 and TSG101 is needed for vesicular fusion

MGRN1 interacts with TSG101 in a bimodal manner. One of those involves the interaction of UEV (ubiquitin E2 variant) domain of TSG101 and the PSAP motif MGRN1 that facilitates TSG101 monoubiquitination (Kim et al, 2007). If one of these motifs get mutated or deleted, the interaction and the consequent ubiquitination perturbs resulting in blocked fusion between amphisome/MVB and lysosomes.

To check that, HeLa cells were transfected with TSG101 or TSG101ΔUEV and K0 Ub along with MGRN1 or MGRN1 (SRAP) (MGRN1 mutated at its PTAP motif) in the various indicated combinations and were either treated with bafilomycin A1 or left untreated. In presence of K0-Ub, when both MGRN1 and TSG101 were present, a significant increase (p≤0.05, n=3) in the level of LC3-II was observed upon bafilomycin A1 treatment. However, the no such significant change in LC3 II levels were seen when either TSG101ΔUEV or MGRN1 (SRAP) was used (Figures 5.7A and B). Therefore the lack of interaction between MGRN1 and TSG101 (when either MGRN1 (SRAP) or HA-TSG101ΔUEV was used) disrupts autophagosomal degradation.
Figure 5.7: Interaction of MGRN1 and TSG101 is required for their monoubiquitination and subsequent vesicular fusion events

A) HeLa cells co-transfected with MGRN1 or MGRN1 (SRAP) along with HA-TSG101 or HA-TSG101ΔUEV and K0 Ub, as indicated, were lysed and immunoblotted to analyze the levels of endogenous LC3 II in the presence or absence of 300 nM bafilomycin A1. GAPDH was used as loading control.

B) Quantification of LC3 II levels from the Western blots in A) using densitometry. The data are expressed as fold change relative to the control condition and represent the mean ± SEM of three independent experiments. Statistical significance was determined using a t-test. **p < 0.01, n.s. = not significant.
control. Efficiencies of all transfections were checked. ▲ indicates HA-TSG101; ← indicates endogenous TSG101, → indicates HA-TSG101ΔUEV.

B) Graphical representation of LC3 II fold change as normalized against loading control. **p≤0.05, n.s., not significant (p= 0.22 and 0.77 in the presence of MGRN1 (SRAP) and HA-TSG101ΔUEV, respectively) using Student’s t-test. Error bars, ±S.E.M.

These further confirmed that MGRN1-dependent monoubiquitination of TSG101 affected vesicular fusion.

Similar results were observed in SHSY5Y cells, confirming the cell line independence of MGRN1 dependent TSG101 ubiquitination mediated vesicular fusion (Figures 5.8A and B).
Figure 5.8: MGRN1-mediated monoubiquitination of TSG101 is needed for vesicular fusion in autophagic pathway

A) SHSY5Y cells co-transfected with MGRN1 or MGRN1ΔR, HA-TSG101 or HA-TSG101ΔUEV along with K0 Ub or ΔG75/76 Ub were lysed and immunoblotted to analyze the levels of endogenous LC3 II in

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B) LC3-II fold change

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LC3-II fold change

** n.s. n.s. n.s.
the presence or absence of 60 nM bafilomycin A1. GAPDH was used as loading control. Efficiencies of all transfections were checked. The blots are representative of at least three experiments. Note that overexpression of TSG101, without its monoubiquitination cannot rescue autophagosomal degradation.

\[ \text{\textcircled{\textup{a}}} \text{ indicates HA-TSG101; \textup{←} indicates HA-TSG101}_{\DeltaUEV} \]

B) Graphical representation of LC3 II fold change as normalized against loading control. **p ≤ 0.05, n.s., not significant (p = 0.74, 0.45 and 0.92 in the presence of ΔG75/76 Ub, MGRN1 (SRAP) and HA-TSG101ΔUEV, respectively) using Student’s t-test. Error bars, ± S.E.M.

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**5.02.07 Monoubiquitination of TSG101 regulates endocytic trafficking**

Next, I addressed if TSG101 monoubiquitination could affect vesicular fusion events in the endocytic pathway as well.

For this, cells treated with mock or MGRN1 siRNA were transfected with HA-TSG101 along with either K0 Ub or ΔG75/76 Ub mutant. Cells were then stimulated with Alexa-488 tagged EGF and imaged at different points to check the kinetics of EGF-induced EGFR uptake, trafficking and degradation. At 180 min of chase, in presence of HA-TSG101 and K0-Ub mutants, endocytic degradation of EGF-EGFR complex was seen; irrespective of the functionality of MGRN1. Degradation at the lysosomes was found to be interrupted when cells were transfected with ΔG75/76 Ub mutants (Figure 5.9), irrespective of the presence of the siRNA used or the presence of HA-TSG101.

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Figure 5.9: Regulation of endocytic degradation by TSG101 monoubiquitination

HeLa cells treated with the indicated siRNAs, transiently co-transfected with HA-TSG101 along with K0 Ub or ΔG75/76 Ub was subjected to Alexa-Fluor 488 EGF uptake. Cells were washed, fixed at indicated time points and imaged. Note that overexpression of TSG101 and its monoubiquitination are required together to restore endo-lysosomal pathway. Scale bar, 5 μm

To summarize, monoubiquitination of TSG101 affects vesicular fusion events in both autophagic and endocytic pathways. This requires interaction between MGRN1 and TSG101.

5.03 Conclusion

Inactivation of MGRN1 leads to blockage in vesicular fusions between autophagosomes and lysosomes as well late endosomes/MVBs and lysosomes. This blockage in fusion although adversely affects autophagic and endocytic degradation of macromolecules, it does not alter lysosomal competence. The activity of lysosomal proteases as well as their pH remains unchanged. MGRN1 interacts with TSG101, an ESCRT-I protein to monoubiquitinate it. The blocked vesicular fusion in the absence of MGRN1 activity could be rescued by TSG101 overexpression.

TSG101 primarily binds to ubiquitinated cargoes and participates in sorting of those proteins in MVBs (Katzmann et al, 2001; Doyotte et al., 2005). Null mutation in Mgrn1 causes prion like spongiform neurodegenation phenotype (He et al., 2003). In Mgrn1 null mice brain tissue, the pattern of ubiquitination of TSG101 is altered -- less ubiquitinated TSG101 is detected in young mice while more ubiquitinated and insoluble TSG101 in adult mice (Jiao et al, 2009). The altered ubiquitination of TSG101 when MGRN1 is depleted leads to pertubation in endocytic pathway In TSG101 knockout cells, presence of enlarged lysosomes enriched with the autophagy-related protein LC3 suggests that autophagy is utilized as a survival mechanism prior to their ultimate death when TSG101 is non functional (Morris et al., 2012). Taken together these reports suggest that MGRN1 dependant ubiquitination of TSG101 is important for vesicular trafficking as is established in the present chapter.
TSG101 ubiquitination is indeed essential for vesicular fusion events for both autophagic and endocytic pathways downstream of MVB/amphisome formation. Overexpression of TSG101 can rescue the blocked autophagic flux when MGRN1 is nonfunctional. Endocytic degradation of EGFR which is normally perturbed upon MGRN1 depletion can also be rescued by TSG101 expression. TSG101 being substrate for MGRN1, acts downstream of it. Therefore if TSG101 is depleted, there occurs perturbation of autophagic and endocytic pathways which cannot be overcame by MGRN1 overexpression. When TSG101 is depleted, the vesicular distribution of EFG-EGFR complex seems to be affected.

This is expected as TSG101 depletion alters structure of early endosomes and formation of the MVBs (Doyotte et al., 2005; Razi and Futter, 2006). Depletion of MGRN1 does not affect the internalization of EGF-EGFR, or their localization at the early endosomes or MVBs, it only blocks lysosomal degradation. When MGRN1 is inactive, TSG101 is rendered nonfunctional due to lack of monoubiquitination., This effect can be seen in a subset of functions performed by TSG101. MGRN1 dependant monoubiquitination of TSG101 is one of the key events of governing fusion of amphisomes/ MVB with lysosomes in the autophagic and endocytic pathways. If monoubiquitination of TSG101 is blocked, both the autophagic and endocytic pathway are perturbed, even if functional MGRN1 is present. The PSAP motif of MGRN1 and UEV motif TSG101 are needed for their interaction and absence of any of these two motifs can perturb vesicular fusion.

Therefore I can conclude that the lack of monoubiquitination of TSG101 by MGRN1 causes blocked vesicular fusion leading to perturbation in the lysosomal degradative pathways. Depletion of MGRN1 does not affect proper formation of MVBs; it blocks only the downstream cascades.

As depletion of MGRN1 cannot affect formation of MVB, it is possible that ubiquitination of TSG101 by other E3 ligases takes place that promote early events of cargo sorting and MVB formation (Amit et al., 2004; Jiao et al., 2009). Here, for the first time I show that TSG101 is involved in vesicular fusion at the level of late endosomes; further, this is controlled by the functionality of MGRN1. This can be one of the key events in the pathogenesis of spongiform neurodegeneration caused by prion diseases.