Chapter 1: General Introduction
Prion protein is a cell surface glycoprotein, aberrant metabolism of which is implicated in various prion diseases characterized by spongiform neurodegeneration. These prion diseases are either transmissible (acquired via a misfolded isoform of the PrP termed PrP\textsuperscript{Sc}), or familial (inherited through PrP mutations that may increase generation of a minor PrP isoform, C\textsuperscript{mt}PrP). Autophagy is a cellular degradative process that involves delivery and degradation of long-lived, misfolded and accumulated proteins as well as damaged organelles in lysosomes. Defects in this are common in different neurodegenerative diseases where disruptions can occur at various stages in the maturation of the endosomes to multivesicular bodies to lysosomes or during the \textit{de novo} generation of autophagosomes with eventual blockage in degradation at the lysosomes.

Cytosolic E3 ligase, Mahogunin Ring Finger-1 (MGRN1) has been reported to interact with C\textsuperscript{mt}PrP, resulting in dysfunctional lysosomes. Here, we show that depletion of MGRN1 leads to an enlargement in the size of late-endosomes and lysosomes. There is an increase in the levels of autophagy markers, detected in cell lines and brain lysates from transgenic mice (with abundance of C\textsuperscript{mt}PrP). These alterations are due to blocked fusion between autophagosomes (via amphisomes) and lysosomes in cultured cells where MGRN1 is depleted either by RNA silencing or by expression of C\textsuperscript{mt}PrP. Autophagic flux and degradation competence are affected. MGRN1 inactivation simultaneously affects endocytic pathway and aids accumulation of amphisomes, without affecting their formation. Taken together these data suggest that MGRN1 plays a significant role in the fusion between amphisome/MVBs and lysosomes -- thus disrupting both autophagosomal-lysosomal and endo-lysosomal degradations. Interestingly, these phenotypes could be rescued by over-expression of ESCRT-I protein TSG101 and its monoubiquitination. We therefore conclude that the ESCRT-I protein, TSG101, a known modulator in the formation of endosomes, plays a crucial role in the generation of amphisome-lysosome and MVB-lysosome hybrid organelles. MGRN1 in turn regulates TSG101 through its monoubiquitination. This post-translational modification affects vesicular fusion events and clearance of cargo brought in by the two arms of lysosomal degradation (autophagy and endocytosis). This study for the first time shows that by regulating both the autophagosomal and endo-lysosomal degradation pathways, MGRN1 may govern spongiform neurodegeneration in some types of prion disease.
1.01 Lysosomal degradative pathway

Lysosomes are the terminal degradative organelles of cells. Efficient lysosomal degradation is indispensable for maintenance of cellular homeostasis and perturbations in this leads to several debilitating diseases. Lysosomes are specialized organelles that degrade macromolecules received from the secretory, endocytic, autophagic, and phagocytic pathways.

In the endocytic pathway, membrane bound proteins and growth factors are engulfed in early endosome, which mature into late endosomes that finally transfer their content to lysosome for degradation (Huotari and Helenius, 2011). Autophagy is another cellular degradative machinery where aged organelles and long lived proteins are engulfed in autophagosome which finally fuse with lysosome for degradation of its content (Eskelinen and Saftig, 2009). Perturbation in any one of these pathways is associated with neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington’s disease (HD), Nienmen-Pick type C disease (NPC), Frontotemporal dementia (FD) Amyotrophic lateral sclerosis (ALS) etc.

1.02 Endocytic pathway

The endocytic pathway is composed of a number of tubular- vesicular structures that control reutilization or degradation of cell surface components and regulates a number of fundamental processes inside the cell including nutrient uptake, immunity, development, membrane turnover and intra cellular signalling. It comprises a number of compartments, early endosomes, recycling endosomes and late endosomes/ multivesicular body (Huotari and Helenius, 2011), each having separate compositions at different stages of the pathway. Lysosome is the terminal component of this pathway where the degradation of cargoes take place. Early endosomes act as the first sorting station for segregation of cargoes. Housekeeping receptors and other proteins are recycled back to the plasma membrane directly or indirectly via the recycling endosome, while other molecules are routed towards the trans-Golgi network (TGN) or the lysosomes for degradation.

Primarily, endocytosed proteins are internalized into clathrin coated pits that bud into vesicles that either fuse with each other or with pre existing early endosomes (Lakadamyali et al., 2006). Clathrin independent pathways such as caveolar-, GEEC-, and ARF6-dependent pathways (Mayor and Pagano, 2007) are also involved in transport of proteins to early endosomes. Rab5 is the key component of early endosomes. VPS34/p150, a phosphatidylinositol 3-kinase (PI(3)K
complex interacts with Rab5 and also present on early endosomes (Christoforidis et al., 1999; Zerial and McBride, 2001). The recycling back of receptors takes place at the early and recycling endosomes. The receptors for iron-bound transferrin (TfR) and low-density lipoprotein (LDL) are classic examples of such proteins which are recycled back to plasma membrane. The LDL receptors are uncoupled from their ligands at the mildly acidic pH of early endosome (pH ≈ 6.2) (Scott et al., 2014). The composition of early and recycling endosomes are different in terms of their acidification property and protein-lipid contents (Hsu and Prekeris, 2010; Huotari and Helenius, 2011). A number of different Rab proteins are associated in this pathway. Rapid recycling of receptor molecules are controlled by Rab4 (Li and DiFiglia, 2012; Hsu and Prekeris, 2010). Cargoes can recycled back via rab11 positive recycling endosomes (Galvez et al., 2012; Hsu and Prekeris, 2010). A fast and efficient recycling route may also function prior to early endosomes that involves Clathrin Independent Carriers (CLICs) (Howes et al., 2010).

Despite the difference in early and recycling endosomes, the distinction is often blurred at the molecular level and can form an extensive interconnected tubulo-cisternal exhibiting a highly reticular organization in some cell types (Scott et al., 2014).

From early endosomes, cargoes are destined to either trans golgi network (TNG) or to late endosome/MVB and lysosomes. Proteins destined for lysosomal degradations are tagged with multiple ubiquitins (multimonoubiquitination or polyubiquitination) at their Lys63 residues (Raiborg and Stenmark, 2009). The endosomal sorting complex required for transport (ESCRT) machinery aids sorting of these proteins to intralumenal vesicles (ILVs) that finally mature into multivesicular bodies (MVBs). The MVBs are late endosomes having a number of ILVs. Maturation of early endosome into late endosome is accompanied with switching of Rab5 on early endosomes to Rab7 on late endosomes. This switching is denoted as "Rab conversion" (Poteryaev et al., 2010). The early endosomes travel along microtubule and the trafficking is regulated by their interactions with dynein and kinesin (Huotari and Helenius, 2011). The pH of early endosomes and late endosomes are ≈6.2 and ≈5.5/5.0, respectively and the pH is maintained by V-ATPase, a multi-subunit proton pump (Scott and Gruenberg, 2011). Along the endosomal-lysosomal pathway, the concentration of acid hydrolases increases, while lumenal pH decreases. In most neuronal cells, the pH of the lysosomal lumen can be as low as 4.5 (Ishida, 2013). A rapid exchange of membrane components and solutes occurs between late endosomes and lysosomes, there by forming a hydrid organelle, referred as endo-lysosome, which is
converted into classical secondary or dense lysosomes (Huotari and Helenius, 2011). Both late endosomes and lysosomes contain LAMP1 and LAMP2 as membrane bound proteins (Huotari and Helenius, 2011, Bissig C, Gruenberg, 2014). Although similar at the molecular level, they vary in their physical properties and ultrastructure. The fusion of late endosomes/MVBs and lysosomes depends on intraluminal Ca2+ and calmodulin (Luzio et al., 2010). Upon fusion of MVBs with lysosomes their contents are degraded. Trafficking of epidermal growth factor receptor (EGFR) when stimulated by epidermal growth factor (EGF) is a classic example of this endocytic degradative pathway (Figure 1).

**Figure 1: the endocytic pathway.**
Membrane bound receptors are internalized in clathrin coated pits to early endosomes (Rab5 containing). From there they can recycle back to membrane via recycling endosomes (Rab11 positive). Or they can go to late endosomes (rab7 positive vesicles) to lysosomes for degradation.
1.03 Autophagic pathway

Autophagy is cellular degradation pathway for long lived proteins and damaged organelles, where cargoes are captured in double membrane autophagosomes. These subsequently fuse either directly with lysosomes or first with MVBs, then with lysosomes for degradation of the internalized components. Autophagy acts as survival pathway that helps to maintain cellular health by recyling cellular component during nutrient starvation and also by degrading protein aggregates which are otherwise detrimental for cells. It is involved in restricting pathogen invasion, regulation of cell death and plays an important role in diseases like cancer and neurodegeneration (Yang and Klionsky, 2010; Mizushima et al., 2008; Kroemer and Levine, 2008)

Autophagy refers to at least three processes by which intracellular constituents enter lysosomes for degradation: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy.

CMA involves transport of KFERQ (Lysine- Phenylalanine- Glutamate- Arginine- Glutamine) motif containing cytosolic protein to lysosomal membrane via the chaperone hsc70 and lysosomal membrane receptor LAMP-2A (Bejarano and Cuervo, 2010). In microautophagy, cytoplasmic contents enter lysosome when the lysosomal membrane invaginates and pinches off small vesicles for digestion within the lumen.

Macroautophagy is the major regulated catabolic mechanism in eukaryotic cells for degradation of long-lived proteins and aged organelles. Cytoplasmic cargoes are first sequestered in an isolation membrane (phagophore) which matures into the double membrane autophagosomes. Autophagosomes then fuse with lysosomes to form autolysosomes, where degradation of cargoes takes place (Figure 2).
Figure 2: The Macroautophagy pathway:

Autophagosome formation is initiated at isolation membrane. This then nucleates to form a complete double membrane autophagosome. The stages can be divided as induction, autophagosome nucleation, expansion and completion, followed by lysosome fusion, degradation and recycling.

Autophagosome formation occurs at phagophore assembly site (PAS) (Suzuki et al., 2001). It is initiated by activation of autophagy related gene -1 (ATG1) complex that contains ATG1, ATG13 and ATG17 and other accessory proteins (Chen and Klionsky, 2011). Vps34, a class III phosphatidylinositol-3-kinase (PI3K) generates phosphatidylinositol-3-phosphate (PI3-P) at the PAS. This allows the recruitment of other ATG proteins to form autophagy-regulating macromolecular complex (PI3K complex). PI3K complex contains VPS34, Beclin 1/ATG6, ATG 14/barkor and p150/Vps15, among other proteins (Itakura et al., 2008). Beclin1 enhances the activity of VPS34. AMBRA1, UVRAG and Bif-1 act as positive regulator or beclin1 where as anti-apoptotic proteins Bcl-2 and Bcl-XL act as negative regulators (Sinha and Levine, 2008). After initiation of the phagophore, it elongates. Elongation and expansion of the phagophore membrane requires involvement of two ubiquitin-like systems. In the first conjugation event,
ATG7 (E1-like) and ATG10 (E2-like) conjugate ATG12 to ATG5 (Mizushima et al. 1998). ATG12-ATG5 interacts non covalently with ATG16L to form a 800-kDa complex (Mizushima et al., 2003), which is necessary for autophagosome formation.

The second ubiquitination-like reaction involves modification of the microtubule-associated protein 1 light chain 3 (MAP1-LC3/LC3/ATG8) by the action of ATG7 (E1-like) and ATG3 (E2-like). LC3 is cleaved at its C terminus by ATG4 to form the cytosolic LC3-I, which by the action of ATG7-ATG3 is conjugated with phosphatidylethanolamine (PE) to form LC3-II (Tanida et al., 2004a). LC3-II specifically associates with autophagosomes, and this property makes it most widely used autophagy marker. LC3-II binds to both inner and outer layer of autophagosomes and the binding is mediated by ATG12-ATG5-ATG16L complex (Denton et al., 2012). Autophagosomes are transported along microtubule in a dynein-dependent manner to lysosomes, which are clustered around the microtubule organising center (MTOC) located near the nucleus (Jahreiss et al., 2008). After fusion of autophagosomes with lysosomes, the LC3-II moieties attached to the outer membrane are recycled back to the LC3-I form by the action of ATG4 (Tanida et al., 2004b). Following the fusion of autophagosomes with lysosomes, intra-autophagosomal LC3-II is degraded by lysosomal hydrolytic enzymes (Kabeya et al., 2000). The details of the autophagosome–lysosome fusion in mammalian autophagy are still unclear, although it is thought that the fusion step involves proteins such as ESCRT, SNAREs, Rab7, UVRAG, LAMP-2 and the class C Vps proteins (Rusten et al., 2007; Noda et al., 2009). Fusion of autophagosomes with lysosomes can be either direct or indirect. During indirect fusion, an autophagosome fuses with MVB to form an amphisome. The amphisome then fuses with a lysosome for degradation of its contents. Therefore at this point of vesicular fusion events, autophagy and endocytic pathway converge.

In neurons, autophagy is constitutively active and is required for survival (Nixon et al., 2008). Induction of autophagy is generally controlled by the mTOR kinase (mammalian Target of Rapamycin), which is regulated by growth factors (especially insulin) and nutrient levels (Levine and Klionsky, 2004, Lum et al., 2005). Autophagic pathway independent of mTOR also exists that involves Beclin 1 and the class III phosphatidylinositol 3-kinase hVps34 (Nixon et al., 2008).
1.04 Role of ESCRT proteins in sorting of cargoes in MVB

ESCRTs are multisubunit proteins that sort endocytosed ubiquitinated proteins such as misfolded plasma membrane proteins, activated growth factors, hormone and cytokine receptors in multivesicular bodies (MVBs). Multiple monoubiquitination or polyubiquitination through Lys63 acts as sorting signal for such proteins. The ESCRT machinery consists of four complexes, ESCRT-0, -I, -II and –III along with several accessory components (Hanson et al., 2009; Hurley and Emr, 2006; Williams and Urbe’, 2007). In addition to this canonical function, there are evidences showing the involvement of ESCRT proteins specially ESCRT-I, III and VPS4 in late fusion events of endolysosomal and autophagic pathway.

Ubiquitynated cargo is captured by ESCRT machinery in the endosomal membrane in a sequential manner, followed by deformation of endosomal membrane and breaking off of the endosomal invasion containing sorted cargoes. Ubiquitin dependant cargo sorting was first identified in vacuolar protein sorting (VPS) mutants of *Saccharomyces cerevisiae* (Katzmann et al., 2002). ESCRT proteins are conserved across species and their depletion causes impaired formation of ILVs, abnormal genesis of MVBs as well as inhibition of lysosomal degradation of protein.

ESCRT-0 is the first complex to interact with ubiquitinated cargoes and associate with endosomal membrane. It is composed of HRS (Hepatocyte growth factor receptor substrate) and STAM (signal-transducing adaptor molecule) subunits. HRS binds to endosomal lipid phosphatidylinositol 3-phosphate (PtdIns(3)P) and is thereby recruited to endosomal membrane (Raiborg et al., 2001). ESCRT-0 binds to multiple ubiquitin molecules (through ubiquitin-interacting motifs) and also has a clathrin binding domain at its C-terminus. This helps to increase the local concentration of ESCRT-0 proteins in clathrin coats at the endosomal membrane (Raiborg et al., 2001; Raiborg and Stenmark, 2009).

ESCRT-I consists of four subunits TSG101 (VPS23 in yeast), VPS28, VPS37 and MVB12 (Morita et al., 2007; Chu et al., 2006). It contains a headpiece (that binds to ESCRT-II), a rigid 13-nm stalk and an endpiece that contains the ubiquitin- and ESCRT-0-binding UEV (Ubiquitin E2 variant) domain. The UEV domains of TSG101 and VPS23 bind to PSAP (Proline-Serine/Threonine-Alanine-Proline) -like motif in HRS, and together with additional interactions these motifs contribute to the endosomal recruitment of ESCRT-I. The ESCRT-I proteins bind to ubiquitin also through their UEV domain (Raiborg and Stenmark, 2009).
ESCRT-II is composed of one EAP45 (VPS36 in yeast), one EAP30 (VPS22 in yeast) and two EAP20 (VPS25 in yeast) subunits (Im and Hurley, 2008; Hierro et al., 2004). The N terminus GLUE domain (GRAM-like ubiquitin-binding) in EAP45 binds to ubiquitin (Alam et al., 2006), as well as 3-phosphorylated phosphoinositides at the endosomal membrane. ESCRT-II also binds to the ESCRT-I VPS28 carboxy-terminal domain subunit through a helix immediately C-terminal to the GLUE domain.

ESCRT-III is composed of CHMP6 (VPS20 in yeast), CHMP4A, B, C (VPS32/Snf7 in yeast) CHMP3 (VPS24 in yeast) and CHMP2A, B (VPS2 in yeast). VPS20 interacts with VPS25 subunit of ESCRT-II and thus get recruited to endosomal membrane (Teo et al., 2004). VPS20 then interacts with VPS32 and mediates the assembly of other ESCRT-III subunits (Teis et al., 2008). Finally, VPS2 associates with the VPS24 cap to mediate recruitment of the ATPase VPS4 (Teis et al., 2008; Sakse na et al., 2009). VPS4 catalyzes the dissociation of all three ESCRT complexes from the endosome (Obita et al., 2007; Babst et al., 1998) and thus play a critical role in the MVB sorting pathway. The enzyme DOA4 deubiquitinates MVB cargoes prior to sorting into MVB vesicles also is recruited by ESCRT-III and Bro1 (Amerik et al., 2000) (Figure 3).

![Figure 3: Organization of ESCRT machinery](image)

ESCRT-0 is first required onto endosomal membrane. Association of ESCRT-I, II and III takes place. VPS4 aids dissociation of ESCRT complex from endosomes and thus helps MVB sorting.
1.05 ESCRT proteins in regulating endocytic and autophagic pathway

Autophagy and endocytosis are two important pathways involving lysosomal degradation of internalized or accumulated proteins. MVBs act as the junctional point for both the pathways. There are emerging evidences depicting the association of ESCRT proteins in various steps of both the pathways.

As the definition goes, lack of various ESCRT subunits hampers formation of MVB and thus affect endocytic pathway. Of late, few ESCRT proteins have been reported to play key role in fusion events of autophagic pathways. Inactivation of these proteins is associated with various diseases where significant aberrations are detected in the autophagic pathways (Lee et al., 2007; Rusten and Simonsen, 2008; Rusten and Stenmark, 2009). Recent evidences suggest that members of the ESCRT machinery can take part in various stages of autophagic pathways ranging from the formation of autophagosomes, their maturation to autolysosome through fusion with various endocytic vesicles and final degradation at lysosomes (Figure 4).

Figure 4: ESCRT complex regulates both autophagic and endocytic pathway
Depletion of Hepatocyte growth factor receptor substrate (HRS), an ESCRT-0 protein results in accumulation of ubiquitinated proteins on early endosomes (Filimonenko et al., 2007). HRS is also required for the accumulation of internal vesicles within MVBs but it does not interfere with the formation of functional MVB that can fuse with lysosome. Its absence moderately inhibits EGF degradation (Razi and Futter, 2006). Loss of HRS protein, further, impairs maturation of autophagosome without affecting the formation of these intracellular structures. In HRS depleted HeLa cells, colocalisation of GFP-LC3-positive structures surrounding group A Streptococcus (GAS) with lysosomal marker LAMP1 is reported to be reduced. Similar observation is made upon nutrient starvation when HRS is nonfunctional (Tamai et al., 2007). In transgenic mice neurons, deletion of Hrs gene shows accumulation of ubiquitinated proteins, such as glutamate receptors and autophagy-regulating protein, p62. These molecules are particularly prominent in the hippocampal CA3 neurons and cerebral cortex with advancing age. Homeostatic control of glutamate receptors in neurons needs proper ubiquitination and it plays important role in several neurodegenerative diseases (Tamai et al., 2008).

In mammalian cells, EGF-stimulated MVB formation requires the tumor susceptibility gene, TSG101, a component of the ESCRT-I. TSG101 is needed for the formation of stable vacuolar domains within the early endosome that develop into MVBs. Inactivation of this leads to tubulation of the vacuolar domain of early endosomes and also perturbs MVB formation. Thus it potentially inhibits degradation of EGF (Razi and Futter, 2006). Depletion of TSG101 causes accumulation of ubiquitinated proteins on early endosomes and in close proximity to lysosomal maker LAMP2. Evidences also suggest a parallel increase in p62 and LC3 positive autophagosomes and reduction in the formation of autolysosomes – indicating a blockage in autophagic degradation (Filimonenko et al., 2007). In Drosophila melanogaster null mutations in the ESCRT-I protein, VPS28 causes accumulation of autophagosomes as seen by confocal and electron microscopy. This is due to blockage in fusion between autophagosomes and endolysosomes (Rusten et al., 2007).

Involvement of ESCRT-III proteins in autophagic pathways are well studied in cell lines and fly models. Expression of mutant forms of the ESCRT-III protein, CHMP2B or depletion of CHMP4B in primary neurons leads to accumulation of autophagosomes as seen in Frontotemporal dementia, FTD (Lee et al., 2007). Similarly, loss of function of the ESCRT-III components, mSnf7-2 or CHMP2B causes neuronal cell loss due to extensive accumulation of
autophagosomes and multilamellar bodies. Phenotypically this resembles depletion of ESCRT-I components in cell culture systems (Doyotte et al., 2005). This suggests that ESCRT-III dysfunction interferes with fusion of autophagosomes with vesicles of the endocytic pathway (Lee et al., 2007). Further, recently, it has been reported that CHMP4B helps autophagosomal degradation of extranuclear chromatin during cytokinesis. CHMP4B is seen to colocalise with both chromosome bridges and micronuclei. During cytokinesis, autophagosomes and lysosomes accumulate around CHMP4B-positive micronuclei. Cataract associated mutation in CHMP4B abolishes this phenotype and results in impaired autophagic degradation (Sagona et al., 2014). In *Drosophila*, inactivation of ESCRT-III by overexpression of mutant CHMP2B also results in accumulation of autophagosomes. In flies, null mutations in the CHMP4B homolog, VPS32 causes accumulation of autophagosomes. This is due to blocked fusion between autophagosomes and endolysosomes (Rusten et al., 2007). In the fly system, similar disruption of clearance of autophagosomes is reported by knocking down of ESCRT-I, II and III proteins (Lee et al., 2007; Rusten et al., 2007).

1.06 ESCRT regulatory proteins in regulating endocytic and autophagic pathway

ESCRT regulatory proteins are shown to have a key role in autophagosomal maturation and endolysosomal fusions.

**Fab1:**

The endosomal PtdIns(3)P 5-kinase Fab1 participates in the maturation of autolysosomes. Lack of Fab1 does not perturb formation of amphisomes; rather it results in the accumulation of these vesicles owing to blockage in later fusion events as seen in *Drosophila melanogaster*. The lack of progression to autolysosomes is hypothesized to be due to inefficient acidification of the endosomal compartments (Rusten et al., 2006). This is in line with role of Fab1 in regulating the maturation of late endosomes into lysosomes (Rusten et al., 2006; Nicot et al., 2006).

**VPS4:**

The AAA ATPase VPS4 (vacuolar protein sorting) interacts with ESCRT-III components and is necessary for ESCRT-III function, probably by disassembling multimeric ESCRT-III complexes on the endosomal membrane (Lata et al., 2008). ATPase deficient VPS4 acts as dominant
negative mutant in endosomal sorting and transport (Babast et al., 1998). In *Drosophila*, loss of VPS4 function causes the accumulation of autophagosomes due to inhibited fusion with the endolysosomal system. The severity of the phenotype caused by dominant negative mutant of VPS4 is milder than that caused by null mutations in the ESCRT-I and II proteins, VPS28 and VPS25. Similarly, expression of the dominant negative mammalian homolog of VPS4, SKD1DN (referred to as SKD1E235Q mutant) in cell lines perturbs endo-lysosomal and endosome-autophagosomal transport. As a secondary effect, generation of autophagosomes is elevated possibly due to a feedback control loop (Nara et al., 2002).

**MGRN1:**
Mahogunin RING finger 1 (MGRN1) interacts with and post-translationaly modifies ESCRT-I protein, TSG101. TSG101 cannot be multimono-ubiquitinated when MGRN1 is depleted. This further leads to blocked fusion of lysosome with amphisomes and MVBs. A simultaneous increase in the number of autophagosomes is also detected. My research has suggested a role for this ESCRT-I protein in late fusion events with lysosomes (Majumder and Chakrabarti, 2015).

**1.07 MGRN1 in spongiform neurodegeneration**

**1.07.01 MGRN1 as aE3 ubiquitin ligase:**

MGRN1 is a ubiquitously expressed E3 ubiquitin ligase that shows E2-dependent autoubiquitylation activity *in vitro* (He et al., 2003). It is a coat color mutant where loss of function in mahoganoid (md) results in defect in pigment-type switching that prevents them from producing yellow pigment and thus darkens coat color of mice (Miller et al., 1997). The phenotypic effect is similar to another coat color mutant Attractin (Atrn) (formerly called Mahogany), a cell surface receptor implicated in melanocortin signalling (Phan et al., 2002). Mice lacking *Mgrn1* show aberrant patterning of the left right body axis during development that causes lethal congenital heart defects in ~50% of animals (Cota et al., 2006). The null mice also exhibit mitochondrial dysfunction and elevated oxidative stress (Sun et al., 2007).

Majority of mice with null mutation in *Mgrn1* have late-onset neuropathology (seen at 6-12 month old mice) in multiple regions of the brain showing vacuolation associated with neuronal
loss similar to prion mediated spongiform neurodegeneration. Interestingly, accumulation of protease resistant proteins is not seen in *Mgrn1* null mice brain and therefore it is not identical to Prion disease PrPSc (Prusiner, 1998).

*Mgrn1* encodes a novel 494-amino acid protein containing a C3HC4 RING (really interesting new gene) domain that may function as an E3 ubiquitin ligase (Phan et al., 2002; He et al., 2003). There are four mRNA isoforms of MGRN1 of which the central RING domain is only recognizable protein motif; however, regions flanking the RING domain and the amino-terminal region of the protein are also conserved between vertebrate and invertebrate genomes (He et al., 2003).

MGRN1 has been first reported to ubiquitinate ESCRT protein TSG101 (Kim et al., 2007). Off late cytoskeleton protein α-tubulin (Srivastava and Chakrabarti, 2014), melanocortin 2 receptor MC2R (Cooray et al., 2011) and the ER (endoplasmic reticulum) ubiquitin E3 ligase, GP78 (Mukherjee and Chakrabarti, 2016) have been identified as other substrate of MGRN1.

Depletion of MGRN1 function by overexpression of mislocalized prion proteins leads to lysosomal phenotype aberration. Lysosomes appears highly fused structures and the size of the acidic vesicles are also increased (Chakrabarti and Hegde, 2009).

### 1.07.02 MGRN1 interacts with aberrant metabolized of prion proteins CmPrP and CyPrP:

Mammalian prion proteins are highly conserved ~35 KDa cell surface glycoprotein having a C-terminal glycosylphosphatidylinositol (GPI) linkage. Mutations in prion proteins are implicated in various neurodegenerative diseases including scrapie, bovine spongiform encephalopathy (BSE), Creutzfeldt-Jakob disease (CJD), and Gerstmann-Straussler-Scheinker disease (GSS) (Aguzzi et al., 2007; Collinge and Clarke, 2007). The pathogenesis of prion disease often involves accumulation of less soluble misfolded and protease resistant PrPSc. PrPSc is capable of propagating by itself in the host by stimulating the conversion of normal cellular PrP (termed PrPc) to PrPSc (Prusiner et al., 1990; Kocisko et al., 1994). Although accumulation of PrPSc, is the key event for the transmission of prion disease, there are evidence of involvement of other mutations. These mutations result in changes in the PrP expression, folding, and trafficking which lead to prion disease pathology. Several familial PrP mutations cause neurodegeneration with little or no generation of PrPSc as the transmissible agent (Tateishi and
Kitamoto, 1995; Tateishi et al., 1996; Chiesa et al., 2003), rather, these result in the generation of various metabolic isoforms of PrP (Hetz and Soto, 2006; Chakrabarti et al., 2009).

One class of mutations leading to increase in the hydrophobicity of the central hydrophobic domain (P105I, G114V, A117V, G131V, S132I, and A133V) enhances generation of C-terminal membrane bound CmPrP, a minor PrP isoform (as judged by *in vitro* assays; Hegde et al., 1998; Kim and Hegde, 2002). CmPrP is identified as a key component of GSS (Hegde et al., 1998). It is localized as membrane bound form by a hydrophobic domain (HD, between residues 112-135) and the N-terminal domain exposed to cytosol (Hegde et al., 1998). Slight increase in generation of CmPrP (5–20% of total PrP) is known to cause neurodegeneration in transgenic mice (Hegde et al., 1998; Hegde et al., 1999). Furthermore, several familial diseases in humans are associated with hydrophobicity-increasing mutations in the HD (e.g., A117V; Hsiao et al., 1991) that may increase CmPrP generation (Hegde et al., 1998). CmPrP level is also suggested to be elevated by accumulation of PrPSc (either due to increased generation or stabilized against degradation) (Hegde et al., 1999).

The other class involves synthesis of cytosolic PrP (CyPrP), synthesis of which in sufficient amount appears to be a contributing factor in prion mediated neurodegeneration (Rane et al., 2008). Two separate mutations are discovered that cause inclusion of premature stop codon mutants (at residues 145 and 160) in PrP and thus display reduced translocation into endoplasmic reticulum (ER) and increase generation of CyPrP. (Zanusso et al., 1999; Heske et al., 2004). Combined effect of a weak PrP signal sequence, ER stress induced reduced PrP translocation (Kang et al., 2006; Orsi et al., 2006, Hetz and Soto, 2006), and decrease in proteasome activity upon PrPSc accumulation (Kristiansen et al., 2007) plays important role in the generation and accumulation of CyPrP. Reduction in translocation of PrP also can cause age dependant neurological dysfunction, despite the degradation of CyPrP (Rane et al., 2008).

Cytosolically expressed mislocalized forms of PrP (CmPrP and CyPrP) interact inappropriately with MGRN1 and thus inhibits its functions (Chakrabarti and Hegde, 2009), as seen in cell culture as well as mice models. Similar effects are seen by forced expression of PrPSc in cytosol (Kristiansen et al., 2007). In cell culture system depletion of MGRN1 also shows lysosomal phenotype aberration (Chakrabarti and Hegde, 2009; Majumder and Chakrabarti, 2015), it phenocopies CmPrP and CyPrP expression, leading to region-selective neurodegeneration.
The interaction between MGRN1 and PrP involves octapeptide repeats (OR) on PrP (Chakrabarti and Hegde, 2009). Mice expressing PrP lacking ORs do not show typical spongiform pathology in the CNS upon prion infection (Flechsig et al., 2000).

In cultured cells that lack MGRN1 expression, neither CyPrP aggregates nor CtmPrP lead to alterations in lysosomal morphology. Therefore expression of CyPrP and CtmPrP are not intrinsically cytotoxic, it rather depends on the cellular environment and presence of interacting partners. It is noteworthy that MGRN1 does not ubiquitinates PrP. Although PrP mediated neurodegeneration in mice can be significantly more severe than simply knocking out MGRN1 (He et al., 2003), region selectivity of prion disease pathology might be imparted by MGRN1. The influence of depletion of MGRN1 on the lysosomal phenotype indicates that MGRN1 interaction plays one of the central roles leading to prion mediated neurodegeneration (Figure 5).

**Figure 5: MGRN1 interacts with CtmPrP and CyPrP**

PrPC are synthesized at ER and then transported to cell surface. If mislocalized, it forms CtmPrP and CyPrP which interacts with MGRN1 and sequesters its function. MGRN1 when functional interacts with TSG101.
1.07.03 MGRN1 interacts with and ubiquinates ESCRT-I protein TSG101:

MGRN1 is an E3 ubiquitin ligase that ubiquinates the ESCRT-I protein TSG101 (Kim et al., 2007). TSG101 is a key component of the ESCRT machinery involved in endo-lysosomal trafficking (Hurley, 2008). Ubiquitination is a multistep process in which the small polypeptide ubiquitin is activated in an ATP-dependent manner and then covalently attached to target proteins through the concerted actions of ubiquitin activating (E1), conjugating (E2) and ligase (E3) proteins (Hershko and Ciechanover, 1998).

The interaction between MGRN1 and TSG101 happens in a bimodal manner, one of which involves the highly conserved P(S/T)AP motif (Proline-Serine/Threonine-Alanine) at C-terminal region of MGRN1 and the UEV domain (catalytically inactive ubiquitin E2 variant) at N-terminal region of TSG101. Another interaction takes place via the residues 317-392 of MGRN1 and the COOH terminal residues 311-390 of TSG101 (Kim et al., 2007).

MGRN1 promotes multimonoubiquitination of TSG101 in cooperation with its cognate E2 enzyme Ubc5a (Kim et al., 2007). In Mgrn1 null mice, pattern of ubiquitination of TSG101 is age dependant, -- less ubiquitinated and more soluble TSG101 is detected in young mice while more ubiquitinated and insoluble TSG101 is detected in adult mice as compared to wild type age matched controls (Jiao et al, 2009). The presence of ubiquitinated TSG101 in adult mice indicates that upon prolonged absence of MGRN1, other ubiquitine ligases may act on TSG101.

TSG101 is the ESCRT-I protein that binds to ubiquitinated cargoes and participates in sorting of such proteins in MVBs (Katzmann et al, 2001; Doyotte et al., 2005). The monoubiquitination of TSG101 modulates its solubility as well as ability to interact with cargo proteins and other ESCRT proteins. In cell culture systems, siRNA mediated depletion of MGRN1 disrupts endolysosomal trafficking of epidermal growth factor receptor (EGFR) (Kim et al, 2007). Lack of ubiquitination of TSG101 plays a crucial role in the EGFR trafficking leading to lysosomal degradation. Similarly in Mgrn1 null mice brain, level of EGFR is much higher than wild type mice brains (Jiao et al., 2009). Therefore, altered ubiquitination of TSG101 when MGRN1 is depleted leads to perturbation in endocytic pathway.

TSG101 may also be involved in autophagic 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).
1.07.04 MGRN1 regulates macroautophagy by modulating TSG101:

Depletion of MGRN1 in cell culture as well as mice models are shown to alters the morphology of acidic vesicles (Chakrabarti and Hedge, 2009; Majumder and Chakrabarti, 2015). Late endosomes and lysosomes are found as highly fused structures in MGRN1 depleted cells. During the course of my research, I have observed that along with the endolysosomal pathway, MGRN1 also regulates the autophagic pathway. 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), it leads to blocked fusion of vesicles at late stage of autophagic pathway. Accumulation of autophagic cargoes takes place inside cells. The initiation of autophagic pathway however remains unaltered. MGRN1 depletion also blocks degradation of EGFR (Kim et al., 2007). When MGRN1 is non functional, it perturbs the fusion of lysosomes with amphisosomes (vesicles formed by fusion of autophagosomes and late endosomes) and MVBs. MGRN1 mediated monoubiquitination of TSG101 is the central to the regulation of the autophagic and endocytic pathway. As MGRN1 acts upstream of TSG101, overexpression of functional copy of TSG101 can salvage the perturbed autophagic flux as well as endo-lysosomal degradation of EGFR.

The level of Cathepsin D increases in MGRN1 knockdown cells and they seem to accumulate in late endosomes along with lysosomes. Depletion of MGRN1, however, does not exert any effect on the lysosomal competence as evaluated by the activity of Cathepsin D. The acidic pH of lysosomes also remains unchanged. Interestingly, MGRN1 inactivation does not affect formation of functional MVBs.

Being an ESCRT-I protein, TSG101 primarily functions in sorting of cargoes in MVBs. Formation of functional MVBs upon MGRN1 depletion indicates presence of other E3 ubiquitin ligases for TSG101 to regulate the early events of cargo sorting and MVB formation. The result also supports the observation made by Jiao et al, where in Mgrn1 null adult mice brain, accumulation of multiubiquitinated TSG101 was seen (Jiao et al., 2009). Moreover, in Mgrn1 null young mice brain, ubiquinated TSG101 is present although in reduced amounts than that of the wild type brain. Two E3 ligases MDM2 and TAL, have been reported to ubiquitinate TSG101 (Amit et al., 2004; McDonald and Martin-Serrano, 2008). TAL-dependent ubiquitination of TSG101 has previously been reported to increase TSG101 solubility (Amit et al., 2004). TAL multimonoubiquitinates TSG101 to regulate its cargo-sorting and viral budding
functions (Amit et al., 2004). TAL also polyubiquitinates TSG101 and targets it for proteasomal degradation when it is not part of the ESCRT complex (McDonald and Martin-Serrano, 2008).

My work shows that MGRN1 is one of the prominent candidates to determine neuropathology in a subtype of prion diseases mediated \( \text{Cm} \text{PrP} \). Depletion of MGRN1 affects normal lysosomal degradative pathway. Abnormal lysosomal pathway is a hallmark for many neurodegenerative diseases like AD, PD, NPC, ALS etc. Unlike other neurodegenerative diseases, the role of autophagy remains unclear in prion diseases (Heiseke et al., 2010). Reports suggest the presence of autophagic vacuoles in neuronal cell models (Schätzl et al., 1997) as well as in human samples (Sikorska et al., 2004). In infectious prion diseases, autophagic flux is found to be enhanced (Liberski et al., 2008). There are conflicting evidences regarding the effectiveness of pharmacological induction of autophagy to alleviate \( \text{PrP}^{\text{Sc}} \) burden and prolong survival (Marzo et al., 2013; Heiseke et al., 2009). On the contrary, a recent report suggests that chemical induction of autophagy can mitigate the development of disease in mice models of genetic prion disease (Cortes et al., 2012). My results for the first time show that blockage in autophagic pathway can lead to lysosomal phenotype aberration similar to spongiform neurodegeneration which is a hallmark of prion disease. At the same time, the role of TSG101 in regulating fusion events at the level of late endosomes is reported for the first time. MGRN1 mediated monoubiquitination of TSG101 is needed for vesicular fusions in autophagy as well as endocytosis at the converging point of both the pathways leading to lysosomal degradation.