2
Review of Literature

Proteins are the main stakeholders of the cell and are responsible for several critical cellular functions like cell signalling, transport across the cell, cell division, nucleic acid organization and ATP generation [Bonora *et al.*, 2012, Casem, 2016, Spies and Smith, 2017]. To perform the above tasks, proteins are continuously synthesized by arduous working machinery of ribosomes [Liljas, 2001]. However, an over-production of proteins can burden the protein degradation machinery of the cell and may induce protein aggregation [Ross and Poirier, 2004]. Cells have developed a highly efficient system of cellular PQC to counter the abnormal inclusions of protein aggregates [Chen *et al.*, 2011]. The PQC has three components, i.e., chaperones, UPS, and autophagy [Amm *et al.*, 2014]. The primary role of PQC machinery is to properly fold or separate the misfolded proteins from the healthy protein molecules, as a failure of folding may induce protein degradation [Upadhyay *et al.*, 2016]. From a very nascent stage of their synthesis, polypeptide chains are highly prone to misfold and can form aggregates, but they are rescued from the event of misfolding by a particular type of protein called as chaperones [Hendrick and Hartl, 1995].

Proteins which are not correctly folded by the chaperones are deleterious to the cellular health and so must be removed by the machinery of autophagy and UPS [Wong and Cuervo, 2010]. Autophagy is bulk cellular waste degradatory machinery that regulates many cellular mechanisms such as cell division, survival, death, cell growth and immunity [Das *et al.*, 2012, Jacquin and Apetoh, 2018]. Autophagy is primarily categorized in three different types, i.e. macroautophagy (or in few individual cases also known as selective autophagy), microautophagy and chaperone mediated autophagy [Glick *et al.*, 2010]. During autophagy, a double membranous structure surrounds the cellular waste and is decorated with various proteins like microtubule associated protein 1 light chain 3 (LC3), autophagy related (ATG) proteins, uncoordinated 51-like kinase 1 (ULK1), Beclin1 proteins which helps it in targeting the cellular waste to lysosome for their degradation by the lysosomal hydrolases, a process known as macroautophagy [Kabeya *et al.*, 2000, Kuroyanagi *et al.*, 1998, Liang *et al.*, 1999, Matsuura *et al.*, 1997, Pyo *et al.*, 2012, Tsukada and Ohsumi, 1993]. However, in the past several years with the identification of molecules such as p62, ubiquitin in autophagy, the selective nature of autophagy in protein degradation is also confirmed [Kirkin *et al.*, 2009, Lamark *et al.*, 2009]. Autophagy is also observed to be modulated by chaperones, and with the help of specific receptors of the lysosomal membrane, it may target the substrate protein to lysosome for removal from the cell [Kaushik and Cuervo, 2018]. Defective autophagy can produce pathological changes in the cell that may finally lead to many types of disorders like infections, cancer, neurodegeneration, aging and maintaining healthy autophagy is crucial for survival [Levine and Kroemer, 2008].

UPS is the other crucial cellular protein degradatory machinery of the cell and was discovered while searching for answers on the theme of specific degradation of the protein [Ciechanover, 2005]. It is well known that UPS, in concerted action with chaperone machinery and autophagy pathway, continuously oversee proteomic content of the cell [Lindquist and Kelly, 2011]. The cellular proteome is a well-regulated interconnected network of proteins

where small perturbations in the functions of these regulator proteins may result in severe abnormalities or pathogenic conditions [Powers *et al.*, 2009, Vidal *et al.*, 2011]. UPS consists of an array of proteins forming a vast network that is directly or indirectly connected to most, if not all, of the other cellular pathways; and therefore, intricate implication of such proteins in the molecular pathomechanism of several types of diseases, from development to degeneration has been widely explored [Ciechanover and Brundin, 2003, Upadhyay *et al.*, 2017]. However, a tight regulation provided by UPS over the turnover and activity of several of its substrates avoids multiple such diseases to occur. Figure 2.1 provides a glimpse of how a broad spectrum of UPS regulated proteins is associated with onset or progression of a host of pathological conditions that will further help in identifying new interaction partners and substrates of UPS, which are involved in manifestation of these diseases [Mishra *et al.*, 2018].

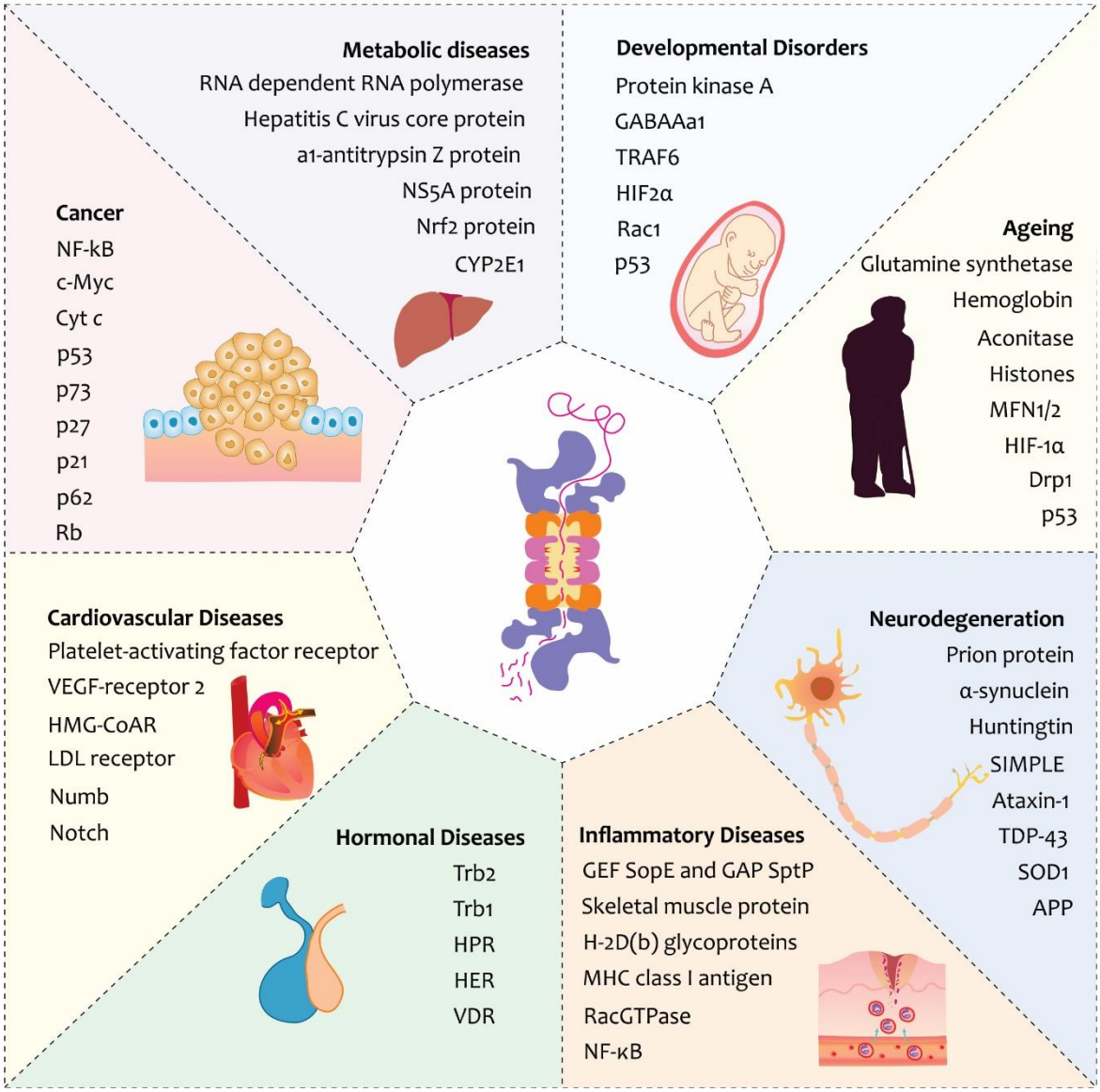


Figure 2.1 : *Functional deregulation of proteasomal degradation causes onset of multiple disease-like conditions*: Proteasome-mediated degradation of substrate proteins has an indispensable impact on cellular physiology and overall health. Any abnormality or irregularity in their efficient degradation may alter the expression and turnover of many critical protein molecules, which are important for several signalling and metabolic pathways, resulting in a number of pathological conditions. Many of these crucial proteins are identified and presented with the disease. **Source: Published As Ribhav Mishra and Amit Mishra et.al., 2018 in Medicinal Research Review. DOI: 10.1002/med.21502.**

The UPS system is responsible for targeting a more significant number of intracellular proteins for degradation [Ciechanover, 2006]; and it depends upon reaction between substrate protein, an approximately 8kDa protein, i.e. ubiquitin and enzymatic reactions performed by E1-Ub-activating, E2-Ub-conjugating and E3-Ub-ligating enzymes [Myung *et al.*, 2001]. The degradation mechanism of UPS involves first, the activation of ubiquitin molecules by ATP hydrolysis and its subsequent binding with E1 enzyme by thioester linkage. The charged ubiquitin then gets transported to E2-Ub-conjugating with the help of thioester bond and finally E2 shifts the charged ubiquitin directly or indirectly to the substrate protein; where the indirect transfer occurs with assistance from E3 ubiquitin ligases [Vilchez *et al.*, 2014]. The misfolded or aberrant protein is finally transferred to a complex protease of the cell, i.e., proteasome [Myung *et al.*, 2001]. The complete process of ubiquitin conjugation and degradation by proteasome needs protein recognition by E3 ubiquitin ligases [Willis *et al.*, 2008]. E3 enzymes of the category containing really interesting new gene (RING) finger domains recruit the substrate protein and helps in shifting of ubiquitin from the cysteine site of E2 to the misfolded protein or in other cases E3 ligases with Homologous to E6-AP Carboxyl Terminal (HECT) domain can lead to a complex formation with the activated ubiquitin and later transfers the same to the client protein [Ardley and Robinson, 2005]. Some reports also indicate that E2-Ub-conjugating enzyme can directly interact with the substrate protein and then shifts the ubiquitin, without the assistance of E3 ubiquitin ligases but that requires further verifications [David *et al.*, 2010, Stewart *et al.*, 2016].

The E3 ubiquitin ligases modulate several cellular mechanisms like cell division, differentiation, adhesion, migration, ATP production, apoptosis and are crucial for the overall cellular physiology and cell survival [Ardley and Robinson, 2005, Du *et al.*, 2015, Huang, 2010]. Dysfunctions of the E3 ubiquitin ligases are linked with different pathologies of cancer, neurodevelopment, neurodegenerative, and metabolic disorders [Upadhyay *et al.*, 2015, Upadhyay *et al.*, 2017]. The E3 ubiquitin ligases maintain the proteostasis state of the cell by selectively ubiquitinating and then further marking the misfolded aberrant proteins for removal, and thus they prevent the protein aggregation and the associated pathologies [Honda *et al.*, 1997]. Some of them which are identified and well characterized are E6-associated protein (E6-AP), carboxy-terminus of Hsc70 interacting protein (CHIP), mahogunin ring finger-1 (MGRN1) and HMG-CoA reductase degradation 1 homolog (HRD1); and they assist in maintaining cellular proteostasis [Chhangani *et al.*, 2014, Jana *et al.*, 2005, Mishra *et al.*, 2008, Yang *et al.*, 2007].

These E3 ubiquitin ligases are not explicitly restricted to use the machinery of proteasome for protein degradation but can also cooperate with chaperones and autophagy to remove the proteinaceous toxic inclusions from the cells [Joshi *et al.*, 2016]. Future research needs to focus on understanding the importance of several unexplored and novel E3 ubiquitin ligases to maintain the proteostasis condition of the cell for various types of cellular mechanisms like an immunological response, cellular transport which may elucidate their contributions into the pathologies of aging, neurodegeneration, and cancer. A clear representation of the PQC machinery for protein homeostasis maintenance in neurons is illustrated in figure 2.2. Along with it, the figure also shows how QC E3 ubiquitin ligases modulate cellular proteostasis and a lack of their functions results in different neurodegenerative and neurodevelopmental disorders [Mishra *et al.*, 2019].

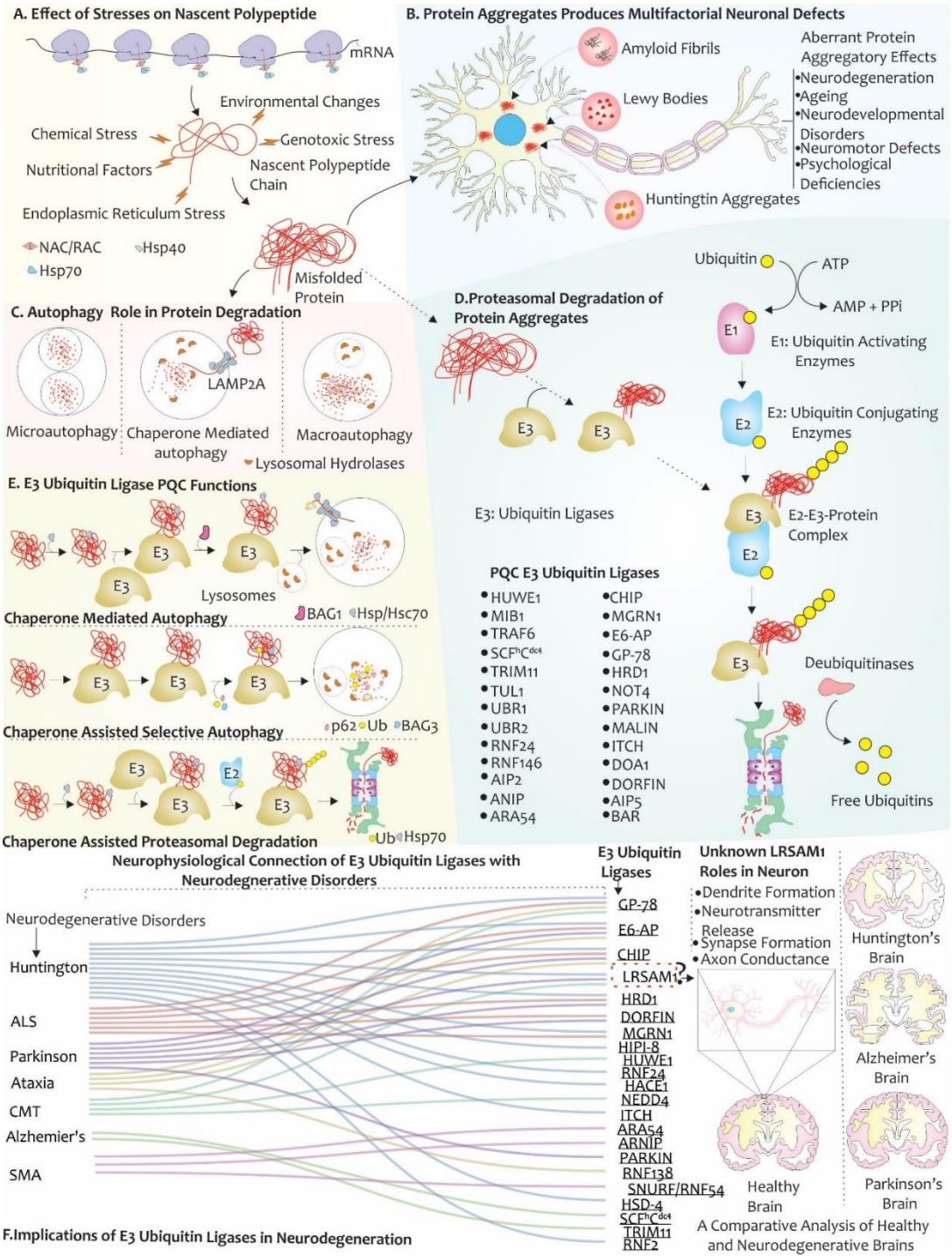


Figure. 2.2 : *Representation of E3 ubiquitin ligases in neuron*al PQC: (a) Nascent polypeptide chains are under constant threat of misfolding from various stresses and are prohibited from accumulation by a concerted action of multiple chaperones. (b) The misfolded polypeptides are highly prone to form aggregates in the neurons. The aggregate inclusions observed in Alzheimer's, Parkinson, and HD disturb the functions of the neuron and can generate several kinds of protein aggregatory effects. (c-d) Cells remove these toxic proteinaceous inclusions by two different components of PQC machinery, i.e., autophagy (c) and UPS (d). (e) E3 ubiquitin ligases were first observed to be central in the mechanism of UPS (d) for the removal of these misfolded proteins. However, later several studies suggest that E3 ubiquitin ligases with the assistance of chaperones such as Hsp70 and other co-chaperones can communicate with both autophagy and UPS to perform the task of removing misfolded protein aggregates from the cell. (f) LRSAM1 has shown potential to act as promising E3 ubiquitin ligase with the elucidation of its functions in several neurodegenerative disorders and in neuronal physiology. **Source: Published As Ribhav Mishra and Amit Mishra et.al., 2019 in Cellular and Molecular Life Sciences. DOI: 10.1007/s00018-019-03055-y.**

2.1 LRASM1 Molecular Nature: Gene Structure, Isoforms And Encoded Protein

LRSAM1 was first identified as a leucine rich repeat (LRR), and, zinc finger and nuclear localization signal (NLS) containing protein which modulates cell adhesion molecules [Li *et al.*, 2003]. The discovery of E3 ubiquitin ligase activity of LRSAM1 and elucidation of its different domains and functions is credited to Amit et al. in 2004. The team isolated LRSAM1 as an interacting partner of tumour suppressor gene 101 (TSG101) and named it as TSG101-associated ligase (TAL) which is associated with ubiquitylating TSG101 and inactivates its ability to direct endocytic cargoes for endo-lysosomal degradation [Amit *et al.*, 2004]. The same group further predicted the structure of the protein to consist of leucine rich repeats, coiled-coil (CC) region, ezrin-radixin-moesin (ERM), RING finger domain, sterile alpha motif (SAM) and two Pro-Thr-Ala-Pro (PTAP) sites [Amit *et al.*, 2004]. Along with TAL, LRSAM1 has another name, i.e., RING finger leucine repeat rich (RIFLE) [Amit *et al.*, 2004, Li *et al.*, 2003].

A full reconstruction of LRSAM1 gene with exons, the interspersed introns and different domains is given in figure 2.3 [Mishra *et al.*, 2019]. The LRSAM1 gene of *Homo Sapiens* is located on chromosome 9 at the position 9q33.3 and the protein is annotated to have three different isoforms differing at their N-terminus end, where its most highly expressed isoform I encodes a protein with molecular weight 84 kDa and have 26 exons (See figure 2.3(a)) [Guernsey *et al.*, 2010]. LRSAM1 of humans is expressed in a significant amount in the motor neurons and spinal cord [Engeholm *et al.*, 2014]. In mouse, LRSAM1 gene has 25 exons and is detected primarily in the motor neurons of the ventral horn, with a weight of 84 kDa and localizes just behind the Golgi Complex in fibroblast cell culture [Bogdanik *et al.*, 2013]. Further, LRSAM1 is present in a large proportion in the mouse dorsal ganglion, and spinal cord, and hence, LRSAM1 may have implications in various kinds of motor functions [Bogdanik *et al.*, 2013]. A complete description of different domains and motif of LRSAM1 such as LRR, NLS, CC, SAM, and RING are provided in figure 2.3 (a). The figure 2.3 (a) illustrates that LRSAM1 at its C-terminus contains leucine rich repeats, whereas other domain like CC, SAM (for protein-protein interaction) and RING (for ubiquitination) are present near the C-terminus of LRSAM1.

Leucine rich repeat (LRR) domains as observed in LRSAM1 are also present on innate immune activators and provides immune system ability to recognize a specific pattern of proteins or sequences on the surface of pathogenic microorganisms [Ng and Xavier, 2011]. Similarly, in LRSAM1, the LRR domain, helps it to recognize *Salmonella Typhimurium* and later build an immune response against the bacterial infection by activating autophagy [Huett *et al.*, 2012, Ng and Xavier, 2011]. Autophagy of bacteria also requires its recognition by other immune proteins such as nuclear domain 10 protein 52 (NDP52) which along with LC3 helps in the delivery of ubiquitinated bacteria to the autophagosome for further destruction [von Muhlinen *et al.*, 2010]. LRSAM1 interacts with NDP52 by its LRR domain, and the binding is independent of ubiquitin binding of NDP52 [Huett *et al.*, 2012]. The motif SAM1 is required for protein-protein interactions, self-association and to bind different types of non-SAM containing proteins; however, the full function of SAM1 in LRSAM1 is not fully understood [Kim and Bowie, 2003]. But the self-ubiquitination ability of LRSAM1 may be attributed to its SAM1 domain [Bian *et al.*, 2017, Huett *et al.*, 2012].

Domains like coiled coil and PTAP motif of LRSAM1 helps LRSAM1 to bind with its substrate, i.e., TSG101, where coiled coil domain is vital in specifying ubiquitination target in TSG101, and PTAP-PSAP motif is required for mediating this communication [Amit *et al.*, 2004, McDonald and Martin-Serrano, 2008]. The ubiquitination of TSG101 depends upon domain of RING at C'-terminus of LRSAM1 and mutations of the same not only affect the TSG101 ubiquitination, but it has shown to be the cause of pathological disorder like Charcot-Marie-Tooth [Amit *et al.*, 2004, Engeholm *et al.*, 2014, Hakonen *et al.*, 2017]. Other than ubiquitination,

the RING domain is also critical for regulating functions like transduction and transcription [Borden, 2000, Borden and Freemont, 1996, Lorick *et al.*, 1999]. More than 600 genes code RING domain-containing E3s and they participate in a variety of cellular pathways [Metzger *et al.*, 2012]. The same domain is characterized by a specific sequence of amino acids like $C\text{-}X_2\text{-}C\text{-}X_{(9\text{-}39)}\text{-}C\text{-}X_{(1\text{-}3)}\text{-}H\text{-}X_{(2\text{-}3)}\text{-}C\text{-}X_2\text{-}C\text{-}X_{(4\text{-}48)}\text{-}C\text{-}X_2\text{-}C$ (C: cysteine, H: histidine and X: amino acid) [Freemont, 2000]. Cysteine and Histidine are highly conserved amino acids of the RING domains, and they bind to two zinc atoms to maintain the structure of E3 ubiquitin ligases.

Along with LRR, the RING domain of LRSAM1 also helps it to recognize bacteria and induce ubiquitination without the assistance of other accessory proteins [Huett *et al.*, 2012]. The process of ubiquitination by LRSAM1 may involve its interaction with E1 enzyme, i.e., Ubiquitin-like modifier-activating enzyme 1 (UBA1), E2 enzymes: Ubiquitin-conjugating enzyme E2D2 (UBE2D2), UBE2D3, and K6 or K27 types of polyubiquitination [Huett *et al.*, 2012]. A copy of the LRSAM1 protein is present throughout the vertebrate genome but is not present in any of the non-chordate phylum, and many of the domains of the LRSAM1 is highly conserved in vertebrates indicating a vertebrate lineage for LRSAM1 [Amit *et al.*, 2004]. We performed a multiple sequence alignment for the leucine rich repeat domains of various types of annotated LRSAM1 for *Homo sapiens* (NP_001005373.1), *Mus musuclus (*NP_955006.1), *Gallus gallus* (XP_415540.3), *Xenopus tropicalis* (XP_012825245.1), *Danio rario* (NP_001093474.1) and *Anopheles gambiae* (XP_001230957.2). The multiple sequence alignment results are aligned in figure 2.3 with the help of Clustal omega and were visualized using Jalview software [Clamp *et al.*, 2004, Sievers *et al.*, 2011]. The results of the sequence alignment show conserved leucine amino acids of the LRR domains, which may play role in the generation of cellular immunological responses against pathological infections.

The discoveries of the several functions of LRSAM1 protein and its different domains that have been revealed in last few years shows the importance of LRSAM1 protein in maintaining cellular transport, cellular development, immunological responses, ubiquitination, neurological and motor disorders. However, more research to search for novel functions of LRSAM1 domains in cellular physiology will serve to better explain the relevance of these domains in LRSAM1 and provide clues for their functions in overall cellular health and fitness.

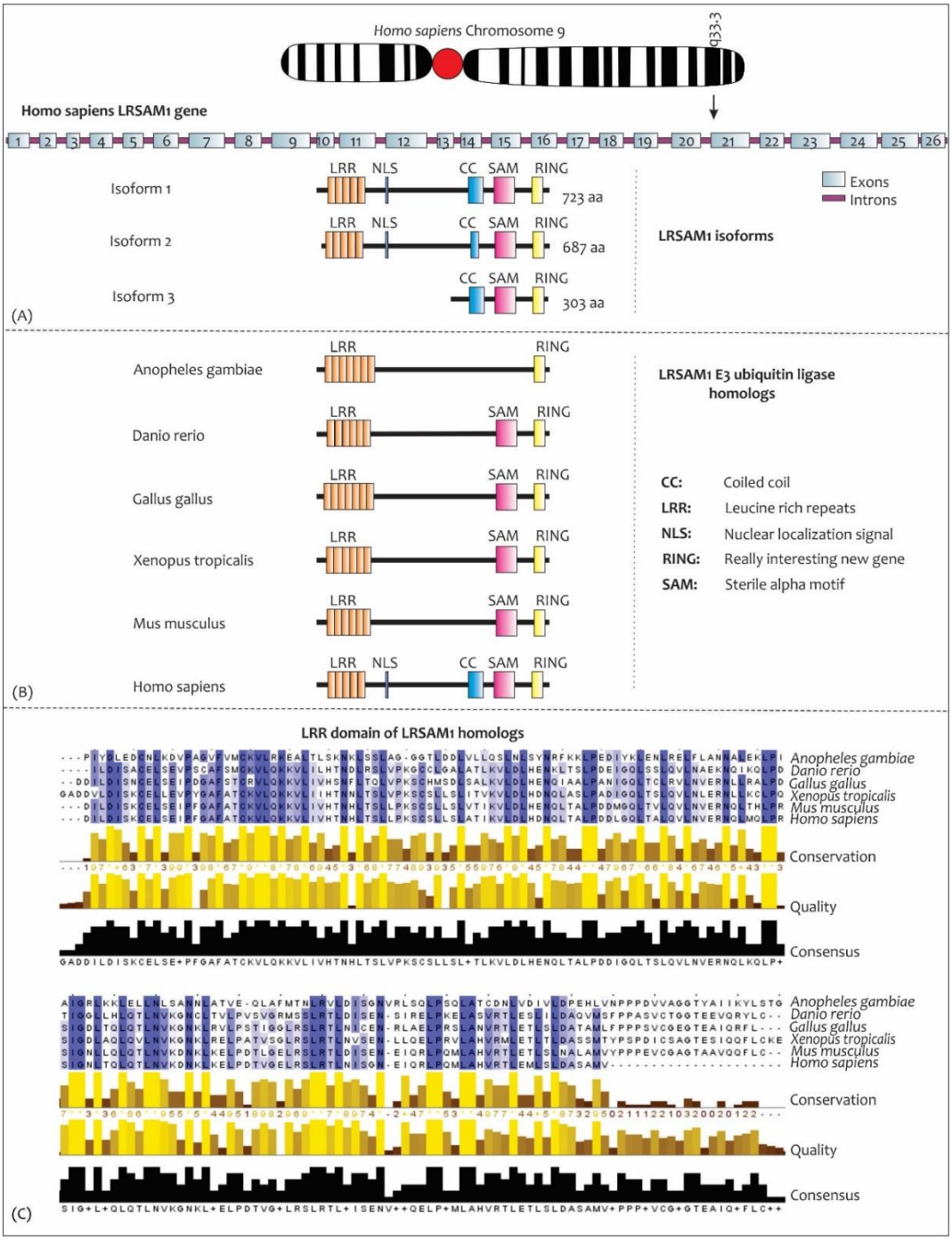


Figure 2.3 : *Schematic illustrations of Homo sapiens LRSAM1 gene, protein, domain organization, isoforms and the homologs in different vertebrates along with a sequence alignment performed for LRR domains*: (a) LRSAM1 gene in Homo sapiens is present on the q arm of chromosome 9, and its locus position is at 9q33.3. The gene is made of 26 exons in humans and encodes LRSAM1 protein. The LRSAM1 protein exists in three different isoforms, where the most commonly expressed form is 723 aa long. (b) LRSAM1 protein is not observed in non-vertebrates species. In various vertebrates such as *Danio rerio* (NP_001093474.1), *Anopheles gambiae* (XP_001230957.2), *Gallus gallus* (XP_415540.3), Xenopus tropicalis (XP_012825245.1), *Mus musuclus* (NP_955006.1) and Homo sapiens (NP_001005373.1), LRSAM1 protein domains are compared with each other to indicate the variations of the LRSAM1 from lower to higher vertebrates. (c) The LRR domains of LRSAM1 proteins from different vertebrates were used for sequence alignment using the tools of Clustal omega and the results of the same were then visualized by Jalview. A default colouring scheme was applied for Clustal omega results in Jalview software. The number 0–9 with plus sign shows the amino acid conservation and an asterisk sign denotes complete conservation of amino acids. **Source: Published As Ribhav Mishra and Amit Mishra et.al., 2019 in Cellular and Molecular Life Sciences. DOI: 10.1007/s00018-019-03055-y.**

2.2 Molecular Functions of LRSAM1 in Endosomal Sorting of Receptors

Endocytosis is a mechanism by which cell internalizes a majority of extracellular material, few ligands, and the lipid portion of the plasma membrane [Doherty and McMahon, 2009]. It also helps cells to degrade the cellular waste by lysosomal machinery and thus assist in ATP generation, signal transduction, cell division and cell adhesion [Grant and Donaldson, 2009, Piper and Katzmann, 2007]. Internalization of receptors by the endosomal mechanism is vital in maintaining a homeostatic state of receptor populations in the cell. The recycling of receptors by the cell regulates the responsiveness of cell to the ligand binding, i.e. either activation or re-sensitization in case of an over-exposure of the ligands [Irannejad *et al.*, 2015]. The sorting of the endosomes either for degradation or for recycling depends upon the ubiquitin labelling. The degradation by lysosomal machinery only occurs for the ubiquitinated endosome, the endosomes after ubiquitination is also known as multi-vesicular bodies that contain the vesicles made up of different proteins needs to be degraded by lysosomes [Henne *et al.*, 2011]. The ubiquitinated cargo of endosomes is recognized by special machinery known as endosomal sorting complex required for transport-I (ESCRT-I).

In yeast, proteins of the ESCRT-I complex are known as Vps family of proteins, while the mammalian homolog of the same is called as TSG101 [Babst *et al.*, 2000]. A TSG101 mutant lacks ability to sort lysosomal hydrolase, cathepsin D, mannose-6-phosphate and EGFR receptors that can have additional deleterious consequences in the cell-like prolonged signalling similar to the one present in tumour cells [Babst *et al.*, 2000]. TSG101 is also critical for modulating the budding of HIV-1, as it binds with the (PTAP) motif of the L-domain present in the p6 protein of HIV-1 by using its domain of ubiquitin E2 variant (UEV) and a depletion of TSG101 by siRNA knockdown causes arrest of HIV-1 budding [Garrus *et al.*, 2001]. The above work shows how efficiently the virus machinery overtakes the host mechanism of receptor endocytosis and degradation to exit from the cell. However, a separate report suggests how overexpression of an N-terminal domain, i.e. TSG-5' of TSG101 abolishes virus assembly and interferes with the exit of virus from the cell and thus causes downregulation of HIV-1 exit [Demirov *et al.*, 2002].

Hence TSG101 can self-modulate its level in the cell, and the same is observed in a study where overexpression of TSG101 downregulates the endogenous TSG101 level [Feng *et al.*, 2000]. LRSAM1 also governs TSG101 in the cell, as it polyubiquitinates lysine residues of an uncomplexed TSG101 at the C-terminus inducing its degradation by proteasomal machinery [McDonald and Martin-Serrano, 2008]. The binding of TSG101 and LRSAM1 is of bimodal type and the first of these interactions are present in amid the TSG101 UEV domain and PTAP-PSAP1 motif of LRSAM1 and the other one occurs in between LRSAM1 CC domain and TSG101 steadiness box [Amit *et al.*, 2004]. The RING domain and the E3 ubiquitin ligase activity of LRSAM1 is crucial for modulating amount of TSG101 in the cell, and its RING finger point mutations prevent the ubiquitination of TSG101 [Amit *et al.*, 2004, McDonald and Martin-Serrano, 2008]. The importance of UEV and RING finger domain for binding between TSG101 and LRSAM1 was further confirmed by TSG101 UEV and RING finger mutant, which lacks the ability to bind LRSAM1 [McDonald and Martin-Serrano, 2008].

TSG101 is monoubiquitinated multiple times by MGRN1 E3 ubiquitin ligase and is directed to the proteasomal machinery for degradation; however LRSAM1 performs both mono-ubiquitination and polyubiquitination of TSG101 and affects its endolysosomal trafficking and proteasomal degradation respectively [Amit *et al.*, 2004, Kim *et al.*, 2007, McDonald and Martin-Serrano, 2008]. LRSAM1 specifically ubiquitinates lysine residues present at the steadiness box C-terminus of the TSG101 and the same is affected by mutations of the lysine residues to the arginine in the steadiness box. Interestingly the binding is prevented by an ESCRTI complex protein, i.e. Vacuolar protein sorting-associated protein 28 (VPS28), as it masks the lysine residue of TSG101 from ubiquitination and further degradation [McDonald

and Martin-Serrano, 2008]. This indicates how steadiness box in TSG101 regulates the degradation of TSG101 [Amit *et al.*, 2004, McDonald and Martin-Serrano, 2008].

TSG101 controls the endosomal sorting of epidermal growth factor receptor (EGFR) receptors and the same is affected by the multiple time's ubiquitinations of TSG101 by LRSAM1 and not by its polyubiquitination [Amit *et al.*, 2004]. Multiple monoubiquitinations of TSG101 by LRSAM1 inactivates the functions of late ESCRTI complex to sort proteins [Amit *et al.*, 2004]. Deactivation of TSG101 by LRSAM1 prevents the endocytic degradation of EGFR by ESCRTI complex and leads to accumulation of EGFR. Catalytically RING inactive form of LRSAM1 induces the degradation of EGFR in the cell due to the stabilization of EGFR-TSG101 complex in the ESCRTI [Amit *et al.*, 2004]. In figure 2.4 (a), it is explained, how the LRSAM1 mediated ubiquitination of TSG101 interferes with endosomal sorting functions of TSG101 and may inhibit the endolysosomal degradation of EGFR proteins in the cell.

Viruses egress from the cell is a vital stage in the life of virus infection [Oldham *et al.*, 2015]. Several host proteins in the past are identified with their ability to assist the virus in the pathway of virus budding from the cell. TSG101 is involved in the exit of retrovirus HIV1 virus-like particles from the cell [Garrus *et al.*, 2001]. The virus PTAP motif of gag protein p6 region binds with the TSG101 UEV domain and recruits TSG101 and other proteins of ESCRTI at the location of virus exit [Martin-Serrano *et al.*, 2001, VerPlank *et al.*, 2001]. LRSAM1 co-localizes with the gag protein of the retrovirus HIV1 in a complex with TSG101, hence gag protein likely recruits the complex of TSG101 and LRSAM1 to the site of virus budding and may leads to insertion of LRSAM1 into the virus-like particles, which is already known for TSG101 [Amit *et al.*, 2004, Myers and Allen, 2002]. The egress of the virus from the cell was higher for an LRSAM1 ubiquitin ligase mutant, suggesting a deubiquitinated TSG101 has higher efficiency to get inserted into the virus-like particles than a ubiquitinated TSG101. Thus ubiquitination of TSG101 prevents the insertion of TSG101 into the complex of virus-like particles and blocks virus egress from the cell. In figure 2.4, a cycle of virus entry and budding from the infected cell is shown. Further, the same figure illustrates how LRSAM1 induced ubiquitination and inactivation of TSG101 causes a defective virus budding.

As discussed in this text and widely accepted, LRSAM1 mediated ubiquitination and inactivation of TSG101 disrupts the endosomal trafficking of protein like EGFR and may have implications in the pathology of cancer, where EGFR level is deregulated [Tomas *et al.*, 2014]. The regulatory functions of LRSAM1 in endosomal trafficking may have further consequences in the mechanism of a disruptive endosomal trafficking disease like Charcot-Marie-Tooth type 2, where LRSAM1 ubiquitination functions are severely affected [Engeholm *et al.*, 2014, Hakonen *et al.*, 2017]. We have briefly explained in the above text that LRSAM1 mediated ubiquitination of endosomal sorting pathway also modulates the egress of virus-like Human immunodeficiency virus 1 (HIV1) from the infected cells. Thus, further exploring the role of LRSAM1 in the budding mechanism of HIV1 virus may have a usage in the development of different types of anti-retroviral therapies.

Future work must focus on characterizing the molecular functions of LRSAM1 in the machinery of endosomal trafficking, ESCRT-I complex, and endosomal trafficking-related diseases. LRSAM1 assisted HIV1 virus budding from the cell, and its significance in virus assembly is a field which can be better explored to identify the possibility of using LRSAM1 as a drug target against the virus egress and viral assembly in pathologies of HIV1 infections. As we have discussed and is well known, LRSAM1 as an E3 ubiquitin ligase degrade TSG101 by utilizing the proteolytic machinery of the proteasome. However, the association of LRSAM1 with the other crucial proteolytic machinery of the cell, i.e. autophagy will need to be further investigated.

2.3 Dynamic Role of LRSAM1 in Xenophagy

The bulk of cellular waste is removed from the cell by the machinery of autophagy; it is a degradatory machinery which is conserved in many aspects of its functions from yeasts to higher mammals [Mizushima *et al.*, 2010]. The substrates of autophagy may range from old and defective organelles such as endoplasmic reticulum, mitochondria, ribosomes, nucleus to misfolded or aberrant proteins and infectious agents [Bento *et al.*, 2016]. These wastes and toxic materials are packaged into a double membrane vesicular structure known as autophagosome, which is transported to the lysosomes for final removal [Bento *et al.*, 2016, Yu *et al.*, 2018]. The mechanism of autophagy seems to get activated under various situations of stresses like proteotoxicity, heat, pH, nutrient, microbial infections and helps to protects the cells against these deleterious conditions [Dikic and Elazar, 2018].

A wide array of proteins till now are discovered which orchestrate the process of autophagy. ATG proteins are the major contributors in the process of autophagy and by genetic screening, approximately 40 ATG genes are characterized and recognized in the yeasts [Mochida *et al.*, 2015]. The ATG proteins are classified into four different groups, i.e. ATG9-ATG18 complex, ULK complex, two types of ubiquitin-like protein (UBL) conjugation mechanisms and class III phosphatidylinositol 3-kinase (PI3K) complex [Wang *et al.*, 2015]. The ULK complex modulates the early stages in autophagosome development by employing the complex of ATG9-ATG18-ATG2 at the site of phagophore to form a structure called a phagophore assembly site (PAS). ATG9, along with ATG18 and ATG2, helps in the synthesis of autophagosome structure and initiates the growth and production of autophagosome [Tanida, 2011]. The other class of ATG proteins, i.e. class III PI3K complex mediates next phase of autophagy, i.e. nucleation. One of the members of this complex, i.e. ATG14 facilitates this mechanism by recruiting other proteins like Beclin1, Vps34, and Vps15 and then direct the complex of PI3K to the place of autophagosome formation and to perform further functions in autophagy [Obara and Ohsumi, 2011].

In the past few decades, several reports have confirmed the selective nature of autophagy. Many proteins are characterized like p62, NDP52 that can regulate or execute the process of selective autophagy. Proteins selectively degraded by autophagy are recognized with the help of receptors like Neighbor of BRCA1 gene 1 (NBR1), p62, NDP52, TOLLIP1, which recognizes a specific signal like ubiquitin chain present on the substrate protein and sort them for the degradation by autophagy [Khaminets *et al.*, 2016, Zaffagnini and Martens, 2016]. The entry of the cargo receptor and the ubiquitinated proteins into the autophagic cascade is promoted by a specific class of proteins known as ATG 8 family proteins [Abdollahzadeh *et al.*, 2017]. There are two sub-families present in the ATG8 family proteins, i.e. LC3 or MAP1LC3 family made up of microtubule-associated protein 1A/1B-light chain 3 (MA1LC3A, MAP1LC3B and MAP1LC3C) and Gamma-aminobutyric acid receptor-associated protein (GABARAP) group that consists of GABARAPL1, GABARAPL2 and GABARAPL3 [Abdollahzadeh *et al.*, 2017]. ATG8 family proteins bind with the autophagy cargo receptor proteins by their LC3 interacting region (LIR) and thus modulates the mechanism of selective autophagy [Noda and Inagaki, 2014].

Ubiquitination is key to selective autophagic degradation of proteins, performed with the help of lysosome. Several types of ubiquitination of the proteins are characterized, but poly-ubiquitination is one of the crucial mechanisms for the recognition and later degradation by selective autophagy. Different E3 ubiquitin ligases like CHIP, MGRN1, and E6-AP are implicated in the process of protein ubiquitination and later in their degradation [Chhangani and Mishra, 2013, Zhou *et al.*, 2014]. These E3 ubiquitin ligases degrade many of the disease-causing misfolded proteins which if starts to aggregate may produce harmful consequences. LRSAM1 is involved in the process of autophagy and eliminates the bacterial cells by the

process of selective autophagy known as xenophagy. Similarly, E3 ubiquitin ligases such as Smad ubiquitin regulatory factor 1 (smurf1), parkin, RING-finger protein 5 (RNF5), neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) are also associated with the process of xenophagy and modulate host cell response against bacterial infection [Franco *et al.*, 2017, Kuang *et al.*, 2012, Lin *et al.*, 2017, Manzanillo *et al.*, 2013]. Xenophagy is a poorly understood mechanism; the knowledge garnered from several years of research indicates that the mechanism is useful for the innate immunity system to remove bacterial pathogens, which can subvert the primary immunological response of the host.

S. Typhimurium is an intracellular pathogen, which causes gut infections, diarrhea, fever, and inflammation in the intestine [Gart *et al.*, 2016]. The bacteria can evade the response of the extracellular immune system and replicates within the autophagic vacuole of the host cells [Yu *et al.*, 2014]. The bacteria from the vacuole travels into the cytoplasm of the cell and can spread its pathology [Gart *et al.*, 2016, Tattoli *et al.*, 2012]. However, the autophagic system of the host cell in cytoplasm can capture *S. Typhimurium* in a double-layered membranous assembly known as autophagosome that finally fuses with the lysosome for the degradation (see figure 2.4 (b)). The autophagic capturing of the bacteria depends upon the coat of a polyubiquitin chain on the *S. Typhimurium*, recruitment of different autophagic adaptor proteins like NDP52, p62 and activation of autophagy (see figure 2.4 (b) [Shahnazari *et al.*, 2010, Thurston *et al.*, 2012]. LRSAM1 can effectively ubiquitinate the *S. Typhimurium* cell wall coat and directs the bacteria for autophagic degradation [Huett *et al.*, 2012]. The work of Huett et al. also showed that LRSAM1 gets co-localized with the *S. Typhimurium* post-infection and a knockdown of LRSAM1 induces *S. Typhimurium* number in the host cell, indicating a higher risk of bacterial infection.

LRSAM1 gets co-localized to the bacteria along with ubiquitin and generates a K6 or K27 type of ubiquitin signals with the help of E1-UBA1 and E2-UBE2D2 or UBE2D3. The localization and ubiquitination require the assistance of LRR and RING domains of LRSAM1, respectively [Huett *et al.*, 2012]. The event of localization is followed by the recruitment of autophagic receptor protein NDP52, which interacts with LRSAM1 via its SKIP carboxyl homology (SKICH) domain; however this binding does not affect LRSAM1 localization to bacterial cell [Huett *et al.*, 2012]. But, the recruitment of autophagic receptors like NDP52, p62 at the bacterial infection site depends upon the ubiquitination of *S.Typhimurium* by LRSAM1, and a knockdown of LRSAM1 leads to the failure of autophagic receptors to get localized at bacterial infection site [Huett *et al.*, 2012]. Different *in vitro* experiments performed during the above-described study also shows a co-localization of LRSAM1 to the bacterial pellets of *Shigella*, *Listeria,* and *AEIC.* The anti-bacterial property of LRSAM1 is further confirmed by finding that in Charcot-Marie-Tooth disease, LRSAM1 null patients derived lymphoblasts are unable to mount an anti-bacterial response after exposure to a strain of *S. Typhimurium* bacteria [Huett *et al.*, 2012]. The above findings indicate that CMT patients are more susceptible to *S. Typhimurium* infection, as they lack a functional LRSAM1 protein. A complete illustration of LRSAM1 role in bacterial recognition and in targeting the *S. Typhimurium* to autophagy is explained in figure 2.4 (b) [Mishra *et al.*, 2019].

Autophagy is essential in maintaining cellular proteostasis and the same is involved in protecting the cells from the pathogenesis of bacterial infection [Bah and Vergne, 2017]. Many E3 ubiquitin ligases in the past are identified in inducing autophagy of the invading bacterial cells by selectively producing ubiquitin signals around the bacteria and thus guiding it for bacterial autophagy. The work of Huett et al. showed how LRSAM1 effectively utilizes autophagy in eliminating bacteria from the cell. More research on the questions of functions of LRSAM1 in xenophagy will add knowledge on how LRSAM1 can recognize several kinds of bacteria and thus consequently induce an autophagic response. LRSAM1 possess LRR domain which provides its ability to recognize specific patterns and signals displayed by different pathogens and allergens and thus may subsequently regulate the innate immunity body

response. Identifying more such functions of LRSAM1 will help us to better understand the LRSAM1 contributions in immunological responses.

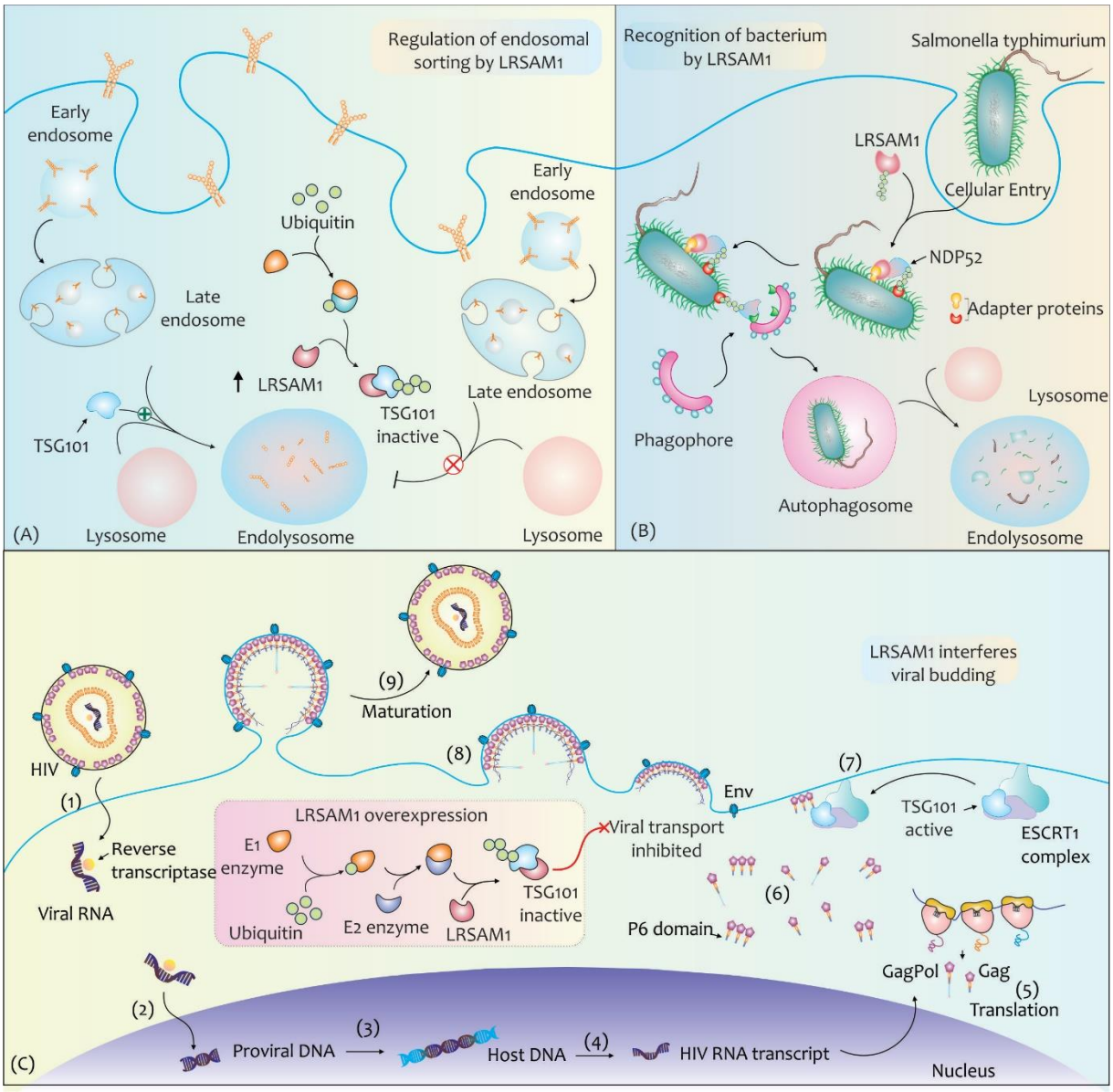


Figure 2.4 : *LRSAM1 modulates endosomal protein sorting, xenophagy and virus budding:* (a) LRSAM1 regulates the sorting of receptor proteins by selectively ubiquitinating an endosomal complex protein, i.e., TSG101. Ubiquitination of TSG101 reduces its ability to sort the endosomal complex of receptors proteins and that results in a failure of endolysosomal degradation of receptors. (b) LRSAM1 generates ubiquitin signals around the bacteria Salmonella typhimurium and targets the bacteria for autophagic degradation (or xenophagy). Different adaptor proteins like NDP52 and also p62 are attracted towards the ubiquitin coat of the bacterial cell and assist in the process of selective autophagic degradation of bacteria. (c) The HIV virus binds to TSG101 protein of the host cell using the p6 domain of Gag proteins and thus utilizes the host cell endosomal machinery to egress from the cell. Overexpression of LRSAM1 leads to the inactivation of TSG101 and abolishes the formation of ESCRT complex I which effectively reduces HIV budding from the infected cell. **Source: Published As Ribhav Mishra and Amit Mishra et.al., 2019 in Cellular and Molecular Life Sciences. DOI: 10.1007/s00018-019-03055-y.**

2.4 LRSAM1 Linked Molecular Pathways in Neurodegenerative Diseases

E3 ubiquitin ligases are characterized for their functions in development, maintenance and degrading different components of the cell. They participate in many developmental mechanisms like in differentiation and in different stages of organogenesis in neural stem cell [Stegmuller and Bonni, 2010, Yokomizo and Dzierzak, 2008]. During the maintenance of cellular functions, E3 ubiquitin ligases modulate various cellular mechanisms like cell sorting, cell signalling, metabolism and transcriptions [Acconcia *et al.*, 2009, Huang, 2010, Weake and Workman, 2008]. Finally, the significance of E3 ubiquitin ligases in the elimination of toxic proteinaceous aggregates from the cell over past several decades have made them an excellent therapeutic and possible option to treat many types of protein aggregatory diseases like Parkinson's, HD and ALS [Chhangani *et al.*, 2012, Ciechanover and Kwon, 2015].

Development, maintenance and modulation of components of brain is critical and a tedious process and is important for the normal health. E3 ubiquitin ligases are involved in these mentioned mechanisms and are often associated with neurodevelopmental and neurodegenerative disorders [Upadhyay *et al.*, 2017]. Finding out novel E3 ubiquitin ligases with functions in development, maintenance and modulation of brain components will help to develop an understanding of these E3 ubiquitin ligases molecular functions for a healthy brain. LRSAM1 has shown potential to be really important in many of the above facets of brain development and later in normal functioning of the brain.

LRSAM1 is one of the fundamental genes that were found to be mutated in the patients of Charcot-Marie-Tooth (CMT) disease [Guernsey *et al.*, 2010]. CMT is a hereditary polyneuropathy, which is often characterized by the sensory loss in the peripheral and distal muscles, muscle loss [Hoyle *et al.*, 2015]. CMT is further classified as axonal, demyelinating and intermediate (CMT1, CMT2, CMT4 respectively) [Nicolaou *et al.*, 2013]. CMT2 mutations are mostly inherited as autosomal dominant type and are of CMT1 and CMT2 types, whereas CMT4 type mutations are transmitted as autosomal recessive types [Tazir *et al.*, 2013, Vallat *et al.*, 2013]. Many reports in last few years from various parts of the world have identified mutations in LRSAM1 for CMT 2 phenotype and associated axonal degeneration (Figure 2.5)[Mishra *et al.*, 2019] [Engeholm *et al.*, 2014]. Peripheral muscles are the worst affected organs in the CMT2 patients because of the axonal degeneration. The disease is also known as CMT2P because of the wasting of peripheral muscles in CMT patients.

LRSAM1 mutation such as a splice mutation produces a premature codon, which stops 20bp prior to the final exon and produces a truncated protein that has a loss in function and leads to a phenotype of autosomal recessive axonal CMT or AR-CMT2 [Guernsey *et al.*, 2010]. A separate report showed frameshift mutation in LRSAM1 RING domain is inherited as an autosomal dominant type and cause CMT2P pathology. The results of the study also proved that LRSAM1 is needed for the neurodevelopment, tail formation and movement in embryo of *Drosophila melanogaster* (see Figure 2.5) [Weterman *et al.*, 2012]. Similarly in an another work, a autosomal dominant pattern of inheritance was observed for LRSAM1 mutation which leads to frameshift, generates a stop codon and produces a premature LRSAM1 protein in CMT phenotypes [Nicolaou *et al.*, 2013]. The results of LRSAM1 mutations and their consequences for CMT are validated in CMT mouse model also, where exposure to a neurotoxic agent such as acrylamide causes progressive axonal degeneration [Bogdanik *et al.*, 2013].

Additionally a novel mutation of LRSAM1 was identified by Engelholm et al to be the cause of CMT2P where extra base pairs were observed in the LRSAM1 transcript and a subsequent disruption occurred in the LRSAM1E3 ligase activity [Engeholm *et al.*, 2014]. Recently, a novel missense mutation of Cys694Arg of LRSAM1 was found to be a causative factor of the CMT2P in a family [Zhu *et al.*, 2015]. The cysteine is highly preserved amino acid in the RING domain of most of E3 ubiquitin ligases and any alteration in this amino acid can have

significant impact on the ubiquitination activity of LRSAM1 and may lead to pathology of CMT2P [Zhu *et al.*, 2015]. A missense mutation in the LRSAM1, which replaces the conserved cysteine residue is responsible for CMT 2G and leads to reclassification of CMT2G as CMT2P [Peeters *et al.*, 2016]. In another interesting recent study done in a Chinese family for CMT2P pathological analysis, LRSAM1 CMT2P mutation was observed to be present near to the RING finger domain of LRSAM1 and may affect E3 ligase activity of LRSAM1 [Zhao *et al.*, 2018].

All the above reports indicates that most of the LRSAM1 mutations either, frameshift or missense in the pathology of the CMT2P is predominantly located in the RING domain or in its vicinity. The importance of RING domain of LRSAM1 in the pathogenesis of CMT2P is described by a study explaining loss of ubiquitination function after a RING domain mutation [Hakonen *et al.*, 2017]. LRSAM1 RING domain is critical for TSG101 ubiquitination and has implications in the process of endosomal protein trafficking and this can affect endosomal protein trafficking and consequently lead to CMT2P disease. Thus, LRSAM1 may cause CMT2P pathology by disturbing the mechanism of enodosomal protein trafficking [Bucci *et al.*, 2012].

LRSAM1 is also found to be involved in several other neurobiological disorders mechanism triggered by protein aggregation or protein misfolding. A study indicates that LRSAM1 overexpression can protect neuronal cells from the proteotoxicity of expanded polyglutamine proteins of HD by an unknown mechanism and can be a future research theme to be studied [Tang *et al.*, 2011]. The same work also showed an elevated level of LRSAM1 in the transgenic mouse of HD and can also be crucial in the pathology of HD, as many E3 ubiquitin ligases in past like E6-AP and MGRN1 have altered expression in HD transgenic mice [Chhangani *et al.*, 2014, Mishra *et al.*, 2008].

A very recent publication shows how the ubiquitin ligase function of LRSAM1 is vital for healthy functioning of transcriptional machinery of the cell and LRSAM1 mediated ubiquitination alters DNA binding protein fused-in sarcoma (FUS) functions [Hu *et al.*, 2016]. The results of the work describe how interaction between LRSAM1 and FUS is needed for modulating the level of FUS in the cell and regulates the cellular transcriptional machinery function. The RING domain in LRSAM1 is required for this interaction and a mutation in the RING domain disrupts the binding [Hu *et al.*, 2016]. Since, FUS is observed to be mutated in ALS, binding of LRSAM1 with FUS may have significance in the pathology of ALS and can be an area of future research work [Hill *et al.*, 2016]. In a very current work, patients of CMT2P with LRSAM1 mutations have shown to develop symptoms of Parkinson's disease at a late stage of their life [Aerts *et al.*, 2016]. Thus, It will be very interesting to check the association of LRSAM1 function with Parkinson's pathology, as LRSAM1 is present primarily in the motor neurons and in spinal cord while the pathology of Parkinson has been basically associated with the defects of the substantia nigra region of the brain [Aerts *et al.*, 2016].

The various discussed research so far clearly suggests the involvement of LRSAM1 with the pathological mechanism of some of the detrimental neurodegenerative disorders like HD, Parkinson's, CMT and ALS. One of these studies explained that the functions of LRSAM1 are needed for neurodevelopment, head and tail movement in *Drosophila melanogaster* embryo [Weterman *et al.*, 2012]. This indicates to us that LRSAM1 can also be essential for neurodevelopment and must be investigated for its involvement in neurodevelopmental disorders of humans. In a separate report, LRSAM1 was observed to modulate cellular proliferation in a neuronal cell model and thus is critical for neuronal division [Minaidou *et al.*, 2018]. These studies performed with LRSAM1 E3 ubiquitin ligase functions provide enough evidence to probe the importance of LRSAM1 in regulation of different neurobiological mechanisms like axonal transport, synapse formation, neurogenesis, neuronal growth, neurotransmitter release and neurodevelopment [Minaidou *et al.*, 2018, Przedborski *et al.*, 2003, Santiago *et al.*, 2017]. As many of these mechanisms are severely affected in neurodegenerative

disorders of HD, Parkinson's, ALS, hence the future research of LRSAM1 importance for these neurodegenerative disorders will make LRSAM1 as a viable molecular therapeutic agent.

In our text, we have comprehended that LRSAM1 is essential for maintenance of few crucial cellular functions like endosomal protein sorting or in generating autophagic response against bacterial infection and for regulating several neurobiological disorders. All these identified roles of LRSAM1 indicate that it is very useful for cell especially neuronal cell in protecting it from different stresses produced internally or externally. LRSAM1 communicates with both UPS and autophagy and hence can modulate the PQC of the cell using either of these mechanisms. The contribution of LRSAM1 to the PQC mechanism should be examined in future studies. Many such facets of LRSAM1 should be critically analysed.

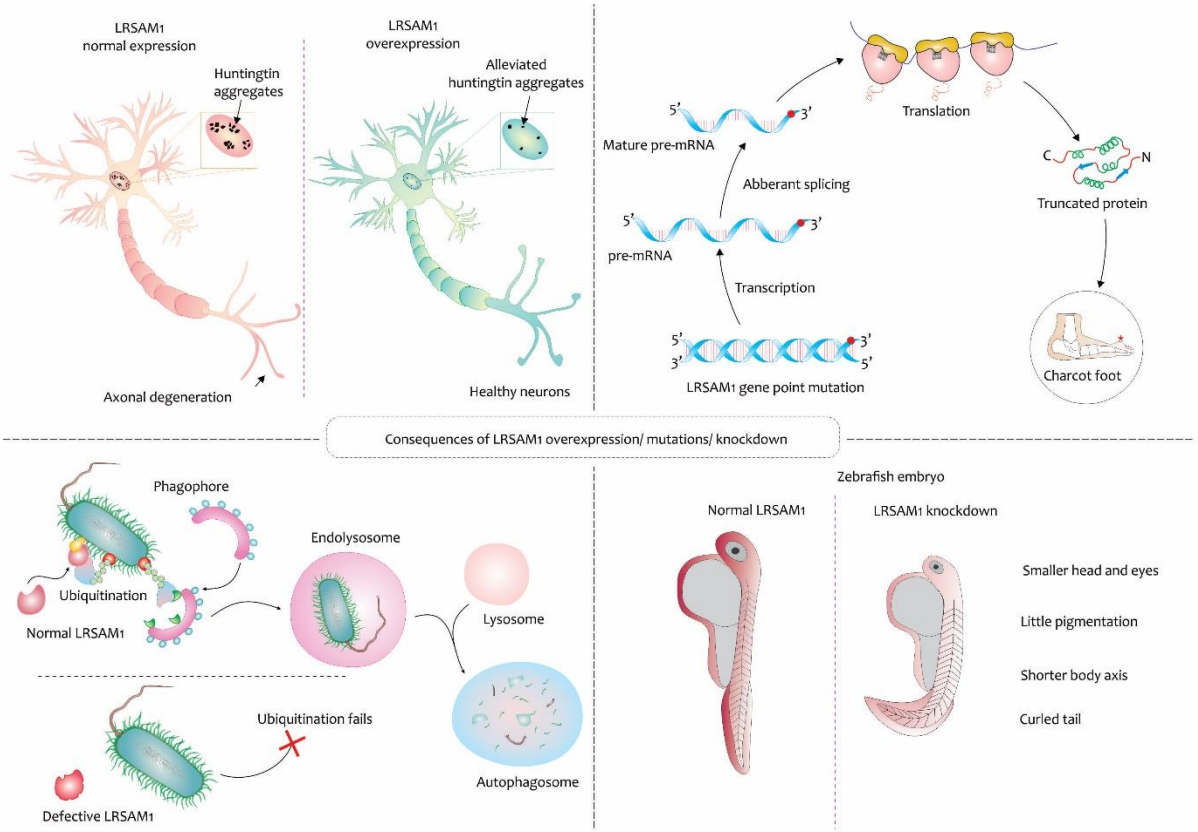


Figure. 2.5 : *Effect of LRSAM1 overexpression and lack of functions on cellular physiology*: (a) Neurons are highly susceptible to the toxic proteinaceous inclusions such as of huntingtin protein and that can lead to axonal degeneration. Ectopic expression of LRSAM1 has shown to alleviate neuronal cells from the toxic proteinaceous inclusions of expanded polyglutamine huntingtin protein. (b) Studies have suggested how a point mutation in LRSAM1 produces an abnormal protein and causes peripheral myopathies such as Charcot–Marie–Tooth type 2P (CMT2P) disease, a pathology of motor neurons that shows degeneration of axons. (c) LRSAM1-mediated production of ubiquitin signals around the bacterial cell is required for the removal of bacteria from the cells by the mechanism of autophagy. (d) LRSAM1 can have critical functions in nervous system development and other organs of the body. A morpholino oligonucleotide-induced knockdown of LRSAM1 of Zebrafish leads to abnormally small head and tails, produces little pigmentation, results in shorter body axis and a curved tail. **Source: Published As Ribhav Mishra and Amit Mishra et.al., 2019 in Cellular and Molecular Life Sciences. DOI: 10.1007/s00018-019-03055-y.**

2.5 Key Questions, Challenges and Future Prospective

LRSAM1 has shown to participate in several cellular physiological mechanisms, which are needed for cellular transport, defence, and proteostasis. Identifying such more novel functions of LRSAM1 in cells can be helpful in selectively targeting LRSAM1 for therapeutic interventions in the neurological and immunological disorders. The problems associated with the use of LRSAM1 as a therapeutic strategy should be characterized and discussed to develop a detailed molecular understanding of LRSAM1 functions in various cellular mechanisms. In this section of the text, few of these questions that are linked with the LRSAM1 study are systemically discussed.

The study of Amit et al. clearly suggests how LRSAM1 regulates endosomal trafficking of receptors and thus provide direction in identifying importance of LRSAM1 in mediating the endolysosomal degradation of other cellular receptors like G-protein coupled receptors (GPCR), fibroblasts growth factor receptors (FGFR), insulin and insulin-like growth factor receptors (IR and IGFR) of the cell [Goh and Sorkin, 2013, Irannejad and von Zastrow, 2014]. The proposed hypothesis can be fruitful in understanding how LRSAM may affect endosomal trafficking of receptors and consequently modulate their expression level in the cell. The findings from these studies will be useful in understanding the pathologies of several cancers where a continued receptor activation is observed [Ciardiello, 2002, Regad, 2015].

Accumulating evidence over the last few years of research clearly shows that LRSAM1 is one of the key protein that loses its function of ubiquitylation in the pathology of CMT2P disease [Hakonen *et al.*, 2017]. The patients of CMT2P shows polyneuropathy with degeneration of peripheral neurons, and since LRSAM1 localization pattern is specific to the peripheral neurons axons, the study of LRSAM1 in the pathology of CMT2P may focus on identifying novel LRSAM1 target proteins that can be linked with the CMT2P disorder [Hu *et al.*, 2016]. Another alternative route of LRSAM1 mediated CMT2P pathogenesis may involve a deregulation of the endosomal trafficking process that needs the attention of the scientific community to further elaborate on the effect of loss in LRSAM1 functions in CMT2P patients [Bogdanik *et al.*, 2013]. Before using LRSAM1 as a possible therapeutic target for the patients of CMT2P, the strategy needs its validation in cell culture, different animal models, and then later in clinical studies.

Various neurodegenerative diseases are shown to be initiated by the result of protein aggregation. The cellular system has developed a productive clearance system to remove these toxic protein inclusions from the cells. Several QC E3 ubiquitin ligases like E6-AP, MGRN1, CHIP in the past are observed to be a central component of this clearance system [Chhangani *et al.*, 2012, Upadhyay *et al.*, 2015]. LRSAM1 may behave as a QC E3 ubiquitin ligase and may modulate the turnover of some of the essential proteins implicated in the pathogenesis of the neurodegenerative disease. Overexpression of LRSAM1 has neuroprotective functions to protect the neural cell population against the pathogenicity of the expanded huntingtin polyglutamine proteins in cell culture model [Tang *et al.*, 2011]. The same neuroprotective effect of LRSAM1 in pathologies of Parkinson's, ALS and Alzheimer's must be checked for the QC efficacy of LRSAM1 in protein aggregatory diseases.

LRSAM1 degrades TSG101 by the machinery of proteasome and is also involved in autophagy for bacterial autophagy, this clearly suggests that LRSAM1 is capable of utilizing both the cellular protein degradatory machinery for different functions and thus may modulate cellular proteostasis by both the cellular mechanisms [Huett *et al.*, 2012, McDonald and Martin-Serrano, 2008]. Identification and characterization of novel protein substrates of LRSAM1 and their degradation either by the usage of proteasome or autophagy in the cells will assist us to comprehend LRSAM1 functions in several cellular mechanisms better and gain further knowledge on the pathologies associated with LRSAM1 lack of functions.

Involvement of LRSAM1 in the mechanism of neurodevelopment indicates that LRSAM1 has potential to modulate the proteome of the neurons, as, LRSAM1 loss in function severely impairs the formation of tail and vertebrate in the zebrafish model [Weterman *et al.*, 2012]. There are several possibilities where LRSAM1 may interact with different proteins of a developing neuron and hence regulate their cellular level. In previous research, various E3 ubiquitin ligases are observed to modulate the neuronal growth and hence, development by controlling the level of few critical growth and development related proteins [Upadhyay *et al.*, 2017]. In one report, Fbw7, a type of Skp, Cullin, F-box containing complex (SCF) E3 ubiquitin ligase modulates neuronal stem cell differentiation by regulating the levels of transcription factors, i.e. Notch1and c-Jun [Hoeck *et al.*, 2010]. Similarly, in another work, E3 ubiquitin ligase msel-10 was observe to decrease Notch signalling and thus may affect the developmental process of neural system, where Notch signalling is involved [Louvi and Artavanis-Tsakonas, 2006, Oberg *et al.*, 2001]. Likewise, other studies have identified E3 ubiquitin ligases like Mind bomb, ligand of numb protein-X (LNX), Suppressor of deltex gene, which selectively target Notch pathway and hence affect the developmental mechanism of the nervous system [Cornell *et al.*, 1999, Itoh *et al.*, 2003, Nie *et al.*, 2002]. All these studies indicate how E3 ubiquitin ligases play a central role in the nervous system development by controlling various protein levels in the cell; hence similarly LRSAM1 may have an interesting contribution in vertebrate development as it may target few of the developmental proteins of the nervous system.

Several works in the past have indicated that E3 ubiquitin ligase can serve as a potential target for drugs in treating pathologies. E3 ubiquitin ligase like mouse double minute 2 homolog (MDM2) is targeted by using small molecule antagonists Nutlin-3a which effectively bind MDM2 and causes a transcriptional upregulation of MDM2 substrate p53 and leads to arrest of cell cycle, cell death and apoptosis in cancerous cells [Vassilev *et al.*, 2004]. In a few other reports, many members of cullin-RING E3 ubiquitin ligases which are involved in critical cellular mechanism like cellular proliferation and are thus crucial in pathologies of cancer, is modulated by using regulators like Probe 8, VL111 and VH298 [Buckley *et al.*, 2012, Bulatov and Ciulli, 2015, Soares *et al.*, 2018].

Similarly, variety of regulators, i.e., either inhibitor or activator for LRSAM1 can be characterized in future studies, which can be useful in controlling LRSAM1 activity and consequently in modifying dysregulated pathways such as endosomal cell sorting, PQC mechanism. But previous research has shown that correctly identifying modulators of E3 ubiquitin ligases family such as MDM2 and CUL3 is mostly based upon the result of the ligand-enzyme structural studies derived from the crystal structure of these E3 ubiquitin ligases [Bulatov *et al.*, 2018, Vassilev *et al.*, 2004]. Thus to characterize LRSAM1 modulators, it is also necessary to first elucidate the crystal structure of LRSAM1 E3 ubiquitin ligase. More information on elucidating the crystal structure of LRSAM1 can be obtained from purifying the LRSAM1 protein and in one study the group of Guo Y et al., have successfully purified recombinant LRSAM1 for research purpose and that can be used in future studies to elucidate the crystal structure of LRSAM1 and thus in identifying its modulator [Guo *et al.*, 2017].

The participation of LRSAM1 in PQC has to be clearly defined and should be additionally examined. LRSAM1 may retain the ability to interact with both degradatory machinery of the cell, i.e., autophagy and proteasome and also has the potential to modulate cellular proteostasis making it interesting to investigate the functions of LRSAM1 in cellular PQC. Further other molecular functions of LRSAM1 in the PQC, especially in interacting with different members of the PQC like chaperone should be explored in future research.