Studies on Intracellular Logistics

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Chapter Six

SUMMARY AND DISCUSSION

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The definition of logistics reads as follows: The detailed organization and implementation of a complex operation. This does not only hold for Schiphol or the harbor of Rotterdam, but also to perhaps even more complex systems such as the functioning of cells. In fact, both activities appear unrelated but can inspire each other. The research presented in Studies on Intracellular Logistics also contains examples that show systems that are linked to each other which were previously not thought to be connected. Chapter Two discusses how nuclear envelope reconstruction as the final step in cell division restores the balance of soluble proteins between the nucleus and the cytoplasm. This was accomplished through the labelling of the nuclear envelope and DNA. Following this, the cells were tracked as they went through mitosis and changes in protein content and localization were quantified. Chapter Four presentes data pertaining to the relationship between the structure and mobility of the endoplasmic reticulum and the mobility of the late endosomal/lysosomal populations. These processes were considered rather unrelated. We showed differently by investigating the observation of a late endosome which appeared to be dragging an ER tubule and forming a novel junction in the network making up the endoplasmic reticulum. Finally, Chapter Five discusses data detailing the discovery of a novel protein involved in interactions between the ER and late endosomes and between the ER and the Golgi Apparatus. Interestingly, we found that these interactions have significant effects on the formation of proteins aggregates related to Parkinson's Disease pathology. Chapter One discusses the possibilities of the proteasome as a target for treatment of non-small cell lung carcinoma and Chapter Three summarizes the intracellular journey that the ligand epidermal growth factor (EGF) and its receptor (EGFR) undergo. This chapter detailed the arguably epic journey through space and time through the endolysosomal system and how that links to cellular activity.

Restoring the distribution of soluble proteins following mitosis.
When a cell progresses through mitosis, they copy their genomic material and divide it over two daughter cells. During mitosis, the cell undergoes major reorganizations. One important step is that the nuclear envelope (NE) that contains the DNA is fragmented and rebuild after the copied DNA is divided over the two daughter cells. The nuclear envelope fulfills an important role in maintaining the dichotomy between the nucleoplasm and the cytoplasm by keep proteins out of

the nucleus while actively allowing other proteins to enter the nucleus. It does so by regulating the passage of proteins of a certain size and shape through its nuclear pore complexes (NPC) (Wente and Rout, 2010, Kapinos et al., 2017). In addition, large proteins contain a nuclear location signal (NLS) that is recognized by the NPC for active nuclear import. The result is the sub-compartmentalization of nuclear versus cytosolic processes. When this barrier is removed, the soluble content of both compartments homogenizes. Once the two daughter cells are formed, the nuclear envelope must be reconstructed and its genetic material decondensed (Alberts, 2015). This raised the question as to how the cells restore the original distribution of nuclear and cytosolic protein and processes in the two daughter cells since the newly formed nuclear envelope could possibly encapsulate cytosolic proteins and processes ensure proper segregation of the homogenized cytoplasm and nucleoplasm. We show that once the nuclear envelope is deconstructed, the genetic material is packed so dense that even small (nuclear as well as cytosolic) proteins are excluded for the genetic material space. Once the genetic material is properly distributed to each daughter cell, the nuclear envelope is built around the condensed genetic material. As a consequence, most soluble proteins are automatically located outside the nucleus. Once the NE is sealed, the genetic material decondenses and the nucleus expands. Now (within a time frame of approx. 5 min) the nuclear proteins are reimported by virtue of their NLS or by diffusion when they are small enough for free diffusion. By treating cells with the compound analinoquinazoline 15 (Q15), the chromosomal condensation is inhibited through interaction with hGAP-G2 (a subunit belonging to Condensin II) (Shiheido et al., 2012). Q15 treatment allowed for cells to progress through mitosis with less condensed Chromosomes. We observed proteins in the nucleus, which had a size that would have caused them to be excluded by chromosomal condensation and reside in the cytoplasm. This data suggests that the state of nuclear condensation ensures exclusion of cytosolic material in the nucleus of dividing cells. This led to the question of how a cell would regulate a large protein complex that resides in both the cytoplasm and the nucleoplasm(Reits et al., 1997). The 20S proteasome is a 750 kDa protein complex. It is responsible for the removal of faulty and redundant proteins (Schweitzer et al., 2016). Due to its large size it is unable to freely diffuse through NPCs. This would imply that either cells contain no proteasomes early after mitosis or that another mechanism is at play. When the NE is reconstructed post mitosis, we observed a rapid import of 20S proteasome into the nucleus for a small period post NE sealing. This occurred at a faster rate than freely diffusing proteins. Then it abruptly stops

and during the following steady state conditions, there is barely active nuclear import and export of 20S proteasome between the two compartments (Reits et al., 1997). Together our data indicated that the 20S proteasome must use an import mechanism that is temporarily active following mitosis. The nuclear import system is driven by a family of proteins termed karyopherins (Chook and Suel, 2011, Goldfarb et al., 2004, Kapinos et al., 2017, Kim et al., 2013). Following a screen to depleting all individual karyopherins from cells, we observed that reducing expression of Importin-5 (IPO5) resulted in a reduction of nucleus localized 20S proteasome. This was further confirmed by observing the location of IPO5 signals throughout mitosis. Its localization is predominantly cytoplasmic except for a short window following mitosis. This coincided with our observations of 20S proteasome nuclear import. As reducing IPO5 led to the reduction the nuclear 20S proteasome signal and an overexpression of IPO5 resulted in an increase of nuclear 20S proteasome signal, IPO5 is apparently critical for rapid distribution of proteasomes immediately following the reformation of the new nucleus in divided cells. These data support the existence of a novel import mechanism. This mechanism is only active for a short period of time and following mitosis. Furthermore, our data identifies a mechanism employed by cells in order to correctly distribute its soluble protein content. It would be of interest to investigate this mechanism with regards to its therapeutic potential. High degrees of mitosis are a hallmark of cancer and specific inhibition of such a mechanism may be of therapeutic interest. Through disruption of nuclear localizations of the 20S proteasome may be fatal for any cell as the proteasome is critical in the turn-over of proteins in the nuclear as well. Lacking the ability to dispose of transcription factors and other regulating proteins may prove to be too much to handle and dividing cells may be particularly vulnerable to such manipulations. The limited degree of cargo known to be imported by IPO5 also gives it specificity(Sutherland et al., 2015, Chao et al., 2012, Goto et al., 2013). Therefor targeting IPO5 for therapeutic purposes would be of interest.

Late endosomes play a role in maintaining the ER structure

The ER is an organelle found throughout the cell's cytoplasm (Alberts, 2015). This omnipresent organelle interacts with nearly every other organelle in the cell (Scorrano et al., 2019, Wu et al., 2018) and may affect many biochemical mechanisms in cells. These vary from lipid synthesis and distribution, to protein transport to the Golgi apparatus and even orchestrating the spatiotemporal regulation of the endosomal compartment (Friedman et al., 2013, Mesmin et al., 2019, Rocha et al., 2009, Jongsma et al., 2016). Movement of endosomes occurs

through the recruitment of motor proteins. This movement ensures directionality in the form of plus-end transport and minus-end transport facilitated by the kinesin motor family and dynein-dynactin motor, respectively (Jordens et al., 2001). We have observed that when a late endosome travels through the cytoplasm, it may be accompanied by the ER. This phenomenon has been described as ER hitchhiking. A previously observed yet hardly described phenomenon. In order to quantify this type of movement, we developed an algorithm that can quantify the percentage of moving labelled membranes. Using this tool, we observed that the late endosomal compartment is able to generate ER movement. Our data indicated that this is a novel type of movement which occurs independently from known ER movement mechanisms facilitated by growing microtubules, termed TAC mediated ER movement and direct motor protein to ER recruitment, termed ER sliding (Bola and Allan, 2009). Furthermore, this movement can be facilitated by various flavors of endosomes such as late endosomes, early endosomes, recycling endosomes and even ER to Golgi traveling endosomes. We tested the individual contribution of all the structures to show that late endosomes/lysosomes generate the bulk of ER dynamics. This is further confirmed by the reduction of movement caused by locking late endosomal/lysosomal movement by various means such as starvation or overexpression of RILP or TMEM55B, which sequesters late endosomes around the microtubule organizing center (MTOC). Not only did our data show that late endosomes/lysosomes generate movement, it also showed that the hitchhiking ER tubules may fuse with existing tubules to generate a brand-new ER junction. Following this observation, we investigated whether a reduction in late endosomal movement would also result in a reduction of peripheral ER junctions per μm2. To answer this question, we develop another algorithm which enabled us to accurately quantify the number of ER junctions per μm2 of a given region of interest. Our data showed a clear reduction in peripheral ER junctions when compared to control samples following late endosomal/lysosomal locking for blocking of transport. This showed a clear connection between late endosomal movement and maintaining the intricate ER structure. There are many interactions found between the ER and late endosomes/lysosomes and many of these interactions are facilitated by MSP-domain containing proteins. This family has always been believed to contain only two members, VAPA and VAPB (Murphy and Levine, 2016). However automatic annotations have predicted at least three other proteins that contain MSP domains. One of these proteins, MOSPD2, has been shown to maintain interactions with similar partners as VAPA, proteins such as ORP1L, STARD3 and STARD3NL (Di Mattia et al., 2018). We in

vestigated whether these are the type of MCSs assist in facilitating ER hitchhiking by depleting VAPA, VAPB and/or MOSPD2. Our observations suggest that depletion of one or two of the three proteins did not result in any observed changes in cells. However, depleting all three proteins simultaneously showed significant reduction in ER movement generated by late endosomes/lysosomes as well as a reduction in peripheral ER junctions. Together, these data show that late endosomal/lysosomal movement and the connections made between them and the ER are involved in maintaining the ER network architecture and movement. These data further solidify ER hitchhiking as a true form of ER movement. Interestingly, this suggests that late endosomes/lysosomes also have some control over the ER in addition of various process where the ER control late endosomes/lysosoes. This shows a dynamic exchange and equilibrium between the two compartments. One example of such a symbiosis is that ER restructuring could be used by late endosomes / lysosomes to gain access to large calcium stores needed for formation of protrusions and neurite outgrowth (Raiborg et al., 2015). For example, during protrusion formation LEs/Lys are directed to the site of membrane expansion to provide membrane materials. At the same time, the ER is required here for calcium supply and ER hitchhiking could help to couple both processes. Together, the data described in Chapter 4 strongly implies that ER hitchhiking is a significant novel addition to to ER mobility and architecture generation.

MOSPD2 is an MSP-domain containing protein which is involved in transporting Ceramide from the ER to the cis-Golgi in a non-vesicular manner.

As Chapter four above discussed, membrane contact site (MCSs) are found throughout the cell, between many organelles and fulfill many different functions. Many of these MCSs are between the endosomal compartment and the ER. For example, ER proteins Protrudin and VAPA interact together with late endosomal protein FYCO, to facilitate plus end movement and protrusion outgrowth. Similarly, ER protein VAPA interacts with endosomal cholesterol sensor ORP1L RILP to control minus end transport towards the microtubule-organization center (MTOC). ORP1L can have multiple conformational states dependent on late endosomal cholesterol levels and the low cholesterol state results in an interaction with the ER protein VAP and the formation of contact sites between these compartments. This situation can be mimicked by overexpression of a truncated form of ORP1L (ORP1LΔORD) that also results in intense contact sites between the ER and late endosomes (Rocha et al., 2009). By combining this phenotype with a form of a proximity

ligation co-precipitation method termed BioID, we generated a list of proteins found between the late endosomal compartment and the ER (Roux et al., 2013). From this list, we found a protein that shared many similarities to VAPA. MOSPD2 is a single transmembrane domain with an MSP domain and one critical additional domain termed CRAL/TRIO. Our data shows that MOSPD2 is a resident ER protein that interacts with ORP1L in an FFAT-MSP dependent manner. The CRAL/TRIO domain has been previously described to interact with small lipophilic molecules (Panagabko et al., 2003), which suggested that MOSPD2 could have additional functions in lipid transport, analogous to ORP1L that transfers cholesterol. We silenced MOSPD2 through siRNAs and analyzed the cells for lipid content by lipidomic analysis and due to the high similarities and capacity to dimerize with each other, we also depleted VAPA, VAPB, MOSPD1 and MOSPD3. Lipidomic analysis showed a reduction in Glucosylceramides and an increase in its precursor, ceramide, in cells with reduced MOSPD2 levels unlike the situation where the homologues were silenced. This suggested that MOSPD2 appeared to be involved in the synthesis or transport of glucosylceramide from the ER to cis-Golgi. Of note, MOSPD2 is the only family member with a CRAL-TRIO domain and this can be critical for this effect. Using these data, we determined that the affected type of ceramide was glucosylceramides. Furthermore, our observations confirmed Glucosylceramide to be the affected lipid species by observing ceramide to glucosylceramide conversion by thin layer chromatography. By shearing cells and generating microsomes, we created conditions where conversion of ceramide to glucosylceramide would only occur in a non-vesicular manner. By depleting cells of MOSPD2, we observed a significant reduction in glucosylceramide conversion. Next, we again depleted cells of MOSPD2 and subsequently treated the cells with BFA, which causes fusion of the Golgi with the ER. This should eliminate the need for ER to Golgi transport to allow ER ceramide to get to the UDP glucose ceramide glucosyl glucosyltransferase (UGCG) in the Golgi, as they are now in the same compartment. Indeed, MOSPD2 depletion now no longer reduced the amount of glucosylceramide. These data further support the hypothesis that MOSPD2 fulfills a role in transporting ceramides from the ER to the cis-Golgi. We then investigated whether MOSPD2 may interact with the enzyme responsible for glycosylating ceramides, UDP glucose ceramide glucosyl glucosyltransferase (UGCG) (Wu et al., 1999). This revealed that MOSPD2 indeed interacts with UGCG in a non-MSP-FFAT dependent manner. Furthermore, after modulating UGCGs activity we observed stronger interactions between the two proteins. This was further increased by the combination of MOSPD2ΔCRALTRIO and UGCG

mutant D92A. The latter combination also yielded a clear increase in colocalization of MOSPD2 towards the cis-Golgi, where UGCG resides. When we probed this interaction in vitro we noticed that the interaction increased depending on the availability of components needed for UGCGs activity. This implied that there is a functional interaction between the two proteins. In order to further characterize MOSPD2, we sought to implicate it in physiology and disease. Since glucosylceramide is the start of glycolipid synthesis and these products are related to various lysosomal storage diseases, we decided to study the involvement of MOSPD2 in the generation of alpha-synuclein aggregates, which are part of the pathological phenotype of Parkinson's Diseases (PD). Recent studies have shown that these aggregates may form as a result of skewed ceramide concentration (Zunke et al., 2018). We observed that MOSPD2 overexpression resulted in an increase in cells containing alpha-synuclein aggregates and depletion resulted in the opposite. Reduction in the overexpression phenotype was achieved by inhibiting UGCG with a well described UGCG inhibitor D-threo-1-phenyl-2-decanoylamino-3-morpholinopropanol (PDMP). Treatment with this inhibitor also showed a decrease in cells containing alpha-synuclein aggregates. These data further suggest that MOSPD2 is involved in the ceramide pathway. However, additional experiments will be needed to provide additional data the involvement of MOSPD2 in glucosylceramide synthesis and the serious pathology of Parkinson's Disease. Considering that MOSPD2 is just one of the pathways available to the cell for transporting ceramides, this indeed may be an interesting candidate for reducing the number of glucosylceramides in a cell, without disrupting the entire synthesis. Its CRAL-TRIO domain could be an excellent target for manipulation.

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