Novel regulators of endosome dynamics, MHCII antigen presentation and chemosensitivity

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Chapter 10:
Summary & Perspectives

Summary & Discussion

Understanding the fundamental organization of the cell, and to extent the collections of cells known as organisms, has marvelled scientists ever since Antoni van Leeuwenhoek. The last decades have seen a significant increase in the available tools and yielded an unprecedented view on the complexity of individual cells. Especially cell biological tools like microscopy have shifted biomedical science from a biochemical approach following a bulk of molecules to a more visual approach, allowing the analyses of biochemical processes in cellular time and space. Much is still to be discovered, but it becomes increasingly clear that different cellular processes are intimately linked, blurring the traditional boundaries between research fields. To get insight into the regulation of a process, roughly two approaches can be followed. The first is to focus on a cellular (or chemical) pathway or process and to delve into its details, using this as a means to discover novel proteins that fill a defined pathway. This approach was explored in Chapter 9, where a role for several novel proteins in sensitivity to Doxorubicin is described. Alternatively, one defined protein can be used as the centrepiece to investigate a complex biological process, as illustrated in Chapter 2. By focussing on the protein ORP1L we could demonstrate a role for late endosomes in regulating the rate of autophagosome maturation. Ideally, science combines both approaches, as shown in Chapter 3 and 5, where unstudied proteins were investigated to gain insight into endosome biology. In this Summary and Discussion chapter, the main conclusions from this dissertation will be summarized and put into perspective.

Regulation of and by the lysosome: positioning and function

Since its discovery by Christian de Duve (1, 2), the image of a lysosome (and its less acidic late endosomal counterpart) has slowly turned from an acidic bag full of proteolytic enzymes into a key regulator of a multitude of processes such as antigen presentation, autophagy and signal transduction (3). Execution of these functions requires the recruitment of a plethora of adaptor proteins, most notably to GTPases, leading to this diversity in function. A similar diversity is observed in the flavours in which lysosomes some. While classically detected with microscopy through staining for LAMP-1 or CD63, electron microscopy images expose a diverse range of endosome architectures associated with these markers, ranging from multi-vesicular bodies (MVBs) and multilamellar bodies to the traditional small, dense lysosome. There are also lysosome-related organelles specified by defined markers (such as tyrosines and melanosomes), whereas others are simply a merger between lysosomes and other compartments (eg. autolysosomes and amphisomes). However, a structure-function relationship is still poorly understood and will require coupling of structural data as obtained by EM to functional data from microscopy, for example by using a combination of correlative light electron microscopy (CLEM), biochemistry and cell biology.

Besides the structural differences, one of the functionally differentiating factors on LE/Lys are the GTPases, present in a somewhat mutually exclusive manner (4). Rab7 has emerged as the key GTPase on the LE/Ly, facilitating maturation of the originally Rab5 marked early endosome. Other LE/Ly are primarily marked by Arl8, Rab2a or Rab9 (5-7), although it is unclear whether these are temporary carriers

for bringing cargo to a different compartment or alternative branches (eg. maturation stages) of lysosomes. Given its predominant role in incoming traffic, Rab7 and its effectors impose important regulatory functions on the endolysosomal system as a whole, as well as on individual endosomes. Chapter 2 describes that by recruiting the cholesterol-binding and transporting protein ORP1L, Rab7 can toggle (or can be toggled if you will) decisions regarding incoming traffic of autophagosomes. Though at the cellular level this could be detrimental, since it leaves the cells with cargo destined for degradation, at the lysosomal level this can ensure fusion with individual lytic compartments capable of destructing the content of the enclosed cytosolic material. Our prediction is that subsets of Rab7-marked lysosomes that act as founding organelles for a series of lysosome-related organelles including melanosomes (8), lytic granules (9), and platelet-dense granules (10) could use this system to prevent their merger with other compartments, in order to maintain their integrity. Given the requirement for Rab7 and HOPS in the fusion of both early endosomes and autophagosomes with the LE/Ly, the ORP1L system probably does not ascertain specificity regarding incoming traffic. Spreading of cargo over different lysosomes by virtue of ORP1L could also aide in the distribution of nutrients and corresponding growth signals. For example, sufficiency of amino acids (predominantly arginine and leucine) in the lysosome leads to the activation of mTOR (11-13), which consequently inhibits TFEB-mediated transcription of LE/Ly and autophagy genes. On the other hand, ‘starving’ lysosomes signal amino acid deprivation to activate TFEB, leading to the opposite signal (14, 15). At the cellular level it would thus be advantageous to distribute nutrients evenly to cancel out both signals, which can be facilitated by ORP1L.
Interestingly, ORP1L has the ability to bind cholesterol. By placing Rab7 function under control of ORP1L, cholesterol as detected by the ORD domain of the latter orchestrates the timing and position of transport and fusion decisions. Cholesterol microdomains could hereby act as local transport and fusion hubs, where recruitment of dynein occurs, as well as HOPS and subsequently the SNARE proteins. This is corroborated by the notion that SNARE proteins on LE/Lys preferentially localize to cholesterol-enriched subdomains (16, 17), a phenotype similar for Rab7, ORP1L and dynein (18, 19). The latter suggests that by binding cholesterol, ORP1L might actually function to recruit Rab7 into these domains to facilitate transport and fusion. However, depletion of ORP1L does not impair minus-end transport or fusion, arguing against this. Our studies have indicated a crucial role for the ORD domain of ORP1L in the ability of Rab7 to recruit HOPS to organize late endosomal fusion. However, we did not observe impaired HOPS recruitment or fusion of autophagosomes with LE/Lys under low cholesterol conditions. Possibly cholesterol depletion was not complete, or alternatively, the function of the ORD domain extends beyond cholesterol and it interacts with other lipids or proteins, which can modulate its conformation and hereby the fusogenic capacity of its associated vesicle. Recently, a direct connection has been described between endosomal cholesterol levels and mTOR signalling (20), where it was shown that cholesterol drives activation of mTOR. Low endosomal cholesterol inactivates mTOR, leading to upregulation of autophagy. It will be interesting to investigate whether ORP1L and mTOR function on the same subsets of LE/Lys, and whether they are functionally connected.

RNF26: location, location, location

When cells are stained for endosomal markers, the bulk of all endosomal compartments, as well as the Golgi-network localize to a region close to the microtubule-organizing centre (MTOC), a phenomenon described in a wide range of cell types, including immune cells and neuronal cells (21-23). This is represented in nearly every cartoon about endocytosis, where more mature endosome stages are always depicted on the bottom, deeper into the cell. However, it has been elusive how this organization comes about and what the function of this compartment clustering is. Our data in Chapter 3 imply that all endosomal carriers are clustered at the MTOC by ubiquitin-dependent recruitment to ER-localized E3-ligase RNF26. Depletion of this enzyme lead to a scattering of all endosomal compartments, but not the Golgi. Furthermore, endosome fusion and maturation was perturbed upon RNF26 loss, indicating that the clustering of endosomes at the MTOC promotes cargo degradation and fusion. Mechanistically, RNF26 recruits different endosomal adaptor proteins via a p62-dependent ubiquitination step, hereby docking the vesicle to the ER and inducing its retention at the perinuclear region. De-ubiquitination organized by USP15 would release the vesicle and enable fast, microtubule-based transport, allowing the vesicle to travel to its desired location. Intriguingly, RNF26 was not found throughout the ER, but rather localized specifically to the perinuclear ER, though not completely overlapping with the here present ER-sheets (24). This localization depended on its RING-domain, implying other factors are involved in constricting the enzyme to the defined position. RNF26 thus facilitates a novel inter-organelle contact-site, between RNF26 and endosomal adaptor proteins. It will be interesting to investigate a potential interplay of RNF26 with endosomal transport decisions toggled by ORP1L/VAP-A (25) and Protrudin/Rab7 ER-LE contact sites (26). Together, our data from Chapter 3 suggest that organization of the endosomal system into a perinuclear cloud by RNF26 facilitates cargo exchange and serves to optimize efficient functioning of the endosomal system.

OTUD1: a novel regulator of endosome biology

The endocytic pathway is regulated by a collection of factors, which can roughly be divided into three interconnected groups. The first are the factors on the endosome itself, the hardware like the Rab7-RILP-ORP1L system, serving its own interest and safeguarding the integrity of the LE/Ly compartment. The second group is the cargo, which signals its preferred destination towards the endosome, hereby influencing the endocytic events. A third group, probably most sensitive to external (both intracellularly or extracellularly derived) stimuli, are the proteins that control the quality and quantity of the hardware, either at the cellular or endosomal level. These are the modifiers of post-translational modifications. A novel factor in the latter category described in chapter 4 of this thesis is OTUD1, a de-ubiquitinating enzyme (DUB) of the OTU-family. Loss of OTUD1 inflicted a scattering of the early and late endocytic compartment, as well as a decreased capacity of cells to degrade ligand-bound EGFR, implying a broad dysfunctioning of the early-late endocytic pathway. Given its cytosolic and actin-bound nature, OTUD1 most likely acts as an indirect regulator of endosome maturation. Whereas the phenotype of OTUD1 loss is evident, two fundamental questions are required for understanding

the function of OTUD1. First, what is the substrate of this DUB, which has been shown to specifically cleave lysine-63 linked poly-ubiquitin chains (27), and what is the function of ubiquitination on this substrate? Given the importance for K63-chains in endosomal signaling (28), it seems reasonable to assume that OTUD1 de-ubiquitinates an endosomal adaptor or cargo protein, likely terminating their functionality. Ubiquitin-proteomics in combination with site-directed mutagenesis of the substrate will be required for studying this part of the OTUD1 cascade. Second, how is the activation or targeting of OTUD1 towards the substrate organized. This aspect is often underrepresented in studies focussing on DUBs. Especially for DUBs counteracting proteasomal substrate degradation this is often the most interesting part, since it provides information as to why the DUB has functionally evolved to reverse an complex energy consuming process. Simply counteracting an E3-ligase is inefficient, so DUBs are usually regulated by alternative pathways to introduce crosstalk between different signalling routes. Our studies described in this chapter show that the actin cross-linking Filamin proteins and oxidative stress sensor Keap1 bind OTUD1 at different sites. Both proteins were not substrates for OTUD1, arguing that they could serve as recruitment or regulatory factors for OTUD1, thus linking the actin cytoskeleton to regulation of endocytosis. This would be in agreement with the known role of Filamin A in the regulation of EGFR degradation and lysosome positioning (29, 30). It will be interesting to investigate the function of OTUD1 from this perspective, whereby OTUD1 possibly act as a spatial regulator of endosome maturation.

USP54 connects endosomes to cellular contact sites

Crosstalk between cellular compartments is essential for major re-organization programs such as cell migration and polarity instalment (31-33). Such communication can be of physical manner, such as transport of cargo, or of immaterial manner, like signal transduction pathways leading to activation of proteins at a different compartment. In Chapter 5 we identify another DUB acting on the endosomal system. We show that the DUB USP54 localizes to different compartments and travels between at least some of them, potentially serving as a messenger for crosstalk between compartments. USP54 is among the largest DUBs (187 kDa (34)) and proposed to be an inactive DUB. We show that besides the USP-domain it contains multiple additional domains that control localization to specific compartments in the cell. Mass spec analysis of the interactors of these different domains yielded proteins marking roughly four cellular compartments; tight/adherens junctions, cilia, endosomes and actin. USP54 traffics from endosomes to cell-cell junctions and is critical for the integrity of tight and adherens junctions, by controlling the recruitment of its interactor ZO-1, an integral junction member, to these sites of intercellular contact. On endosomes, USP54 was found to co-localize and interact with a select few members of the HOPS complex (VPS11, VPS18 and VPS39), but these endosomes were not positive for any marker known to co-localize with these VPS-proteins. Other HOPS members did not co-localize with USP54, arguing for a novel role for these VPS-proteins in the transport of USP54-positive carriers, possibly towards cell-cell contacts. Actin localization of USP54 is probably accounted for by myosin binding, as three myosin motors were found to interact with the

actin-localizing domain. Given the intimate coupling of both endosome recycling and tight junction assembly to actin polymerization (35, 36), USP54 could recruit these motors to perform critical functions such as endosome fusion with the plasma membrane, or could be recruited by these motors to catalyse de-ubiquitination of a potential substrate. Besides impaired membrane localization of ZO-1, USP54 depletion yielded cells with a more peripheral and stable microtubule network. A similar change in microtubule structure is observed in polarized cells, which are characterized by their presence of strong tight and adherens junctions (31, 37). In summary, we believe that by influencing cell-cell contact assembly, USP54 is in control of the establishment of cell polarity, which will be the focus of future research on this DUB.

USP54 has been described as an inactive DUB, based on a missing histidine near the catalytic site (38). However, we find that USP54 is capable of catalysing de-ubiquitination, albeit at a low rate. This catalytic action is required for its function, since overexpression of a catalytically incompetent USP54 mimics its depletion in terms of microtubule reorganization, suggesting DUB activity is essential for its function. In order to understand its full mechanism of action, it will thus be essential to identify the substrates of USP54.

The common denominator of many of the USP54-interactors is a function in plasma membrane trafficking of glucose receptor GLUT4 in adipocytes. This process is executed by endosomes labeling positive for the GTPase Rab4, which also controls recycling of many tight and adherens junction proteins (39, 40). It will be interesting to investigate whether the USP54-positive endosomes are indeed co-labeling with Rab4 and whether USP54 influences Rab4-mediated recycling of GLUT4. Interestingly, USP54 deficient mice are characterized by an increase in adipocyte mass (41), a phenotype similar to mice harbouring increased GLUT4 at the plasma membrane (42, 43). Thus, whereas the exact mechanism of action of USP54 is still unknown, our data support a model in which USP54 controls cell polarity by functioning at the interface of endosome recycling and tight junction formation.

Keap1: a versitile regulator of sorts

Cells are exposed to different forms of stresses, each activating one or more pathways that enable the cell to cope, for example autophagy to counteract nutrient stress. Another stressor is the increased presence of oxygen radicals, which cause damage by reacting with proteins, DNA and lipids (44). To quench these radicals, cells express enzymes catalyzing reducing reactions such as NQO-1 and GST, under the control of transcription factor NRF2. The latter is kept inactive and targeted for degradation by Keap1, which contains exposed cysteines prone to oxidation and hereby functions as an oxidative stress sensor. Besides targeting NRF2, Keap1 functions in multiple pathways such as the DNA damage response, autophagosome degradation, and TGFβ- and NFκB signalling (45-48). In Chapter 7 we report another function for Keap1, namely to control the level of histone acetylation in the cell. Decreased histone acetylation was correlated with impaired expression of MHCII induced by the cytokine IFNγ, arguing that the absence of Keap1 reduced MHCII antigen presentation. Interestingly, in Chapter 9 we describe a novel role for Keap1 in the expression of Topoisomerase IIα, without having established the

molecular mechanism. Given the regulation of Topoisomerase IIα expression by histone acetylation (49), it is tempting to speculate that this hampered expression created by loss of Keap1 is a result of an altered epigenetic landscape. Indeed, HDAC inhibitors have been shown to increase Topoisomerase IIα expression in several tumours (50-52), potentially counteracting the negative effect instigated by loss of Keap1.
Keap1 interacts with and regulates a large number of proteins, mostly via binding to their putative ETGE motif. Given the global de-regulation of Keap1 function by oxidative stress, it might be most interesting to analyse the common denominators of the pathways regulated by Keap1. This is most notably the stress response. Loss of Keap1 induces expression of oxidative stress response genes, NFκB signaling, inhibits PGAM5/Bcl-xL mediated apoptosis, and impairs epithelial-mesenchymal transition (EMT), all programs leading to or activated by stress control. In this context, decreased histone acetylation could help to negate expression of factors unrelated to stress control, providing resources for the processes of primary interest to the cell. Under homeostatic conditions, Keap1 thus acts as an inhibitor of the oxidative stress response, ensuring that cells can utilize their energy for things other than stress control.

Novel factors controlling resistance to doxorubicin

Chemotherapy is one of the cornerstones of cancer treatment, but its effectivity is often hampered by drug resistance in the tumour. This is also the case for treatment with topoisomerase II (TopoII) poisons such as doxorubicin, which is among the most frequently used anti-cancer drugs. To identify novel factors that control resistance to doxorubicin, we performed a genome-wide haploid genetics screen (Chapter 7). Using short incubations with doxorubicin to mimic the clinical condition, loss of Keap1, the SWI/SNF complex and CAAP1 rendered cells more resistant to this anthracycline. Keap1 and SWI/SNF controlled the extent to which TopoII poisons generate DNA damage, whereas CAAP1 influenced the resolution of the DNA damage response, as measured by DNA damage marker γ-H2AX. Given the known role for SWI/SNF in the loading of TopoII onto the DNA (53), we concluded that loss of the SWI/SNF complex decreased TopoII activity and hereby DNA double strand break formation. Several tumours harbour mutations in SWI/SNF complex members (54), suggesting a potential for treatment selection. This is supported by the finding that rhabdoid tumours, which are characterized by a nearly full penetrance SMARCB1 deletion (55, 56), respond poorly to doxorubicin (57). Also, epithelioid sarcoma patients harbouring deletions for SMARCB1 have a higher chance of relapse following treatment protocols that usually includes Topo II poisons (58). Furthermore, a dataset of triple negative breast cancer patients treated with a doxorubicin containing regimen showed a correlation between treatment response and expression of SWI/SNF subunits SMARCB1 and SMARCA4. Whereas often viewed as a single complex, mutations in SWI/SNF subunits occur at very distinct rates in different tumour types. For example, while rhabdoid tumours have a nearly full deletion of SMARCB1, small cell carcinoma of the ovary is characterized by deletion of the SMARCA4 subunit (59), and endometrial tumours have deletions in ARID1A and ARID5B (60). Thus, the SWI/SNF complex members

execute different functions and it will be interesting to determine the contribution of the individual members to the processes it has been linked to.
In summary, this thesis describes novel functions for several proteins and pathways controlling or feeding MHC class II presentation, as well as responses to an anti-cancer drugs. Along the way, we identified three nearly unstudied proteins, the E3-ligase RNF26 and the DUBs OTUD1 and USP54, that control the endosomal pathway in a distinct fashion.

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