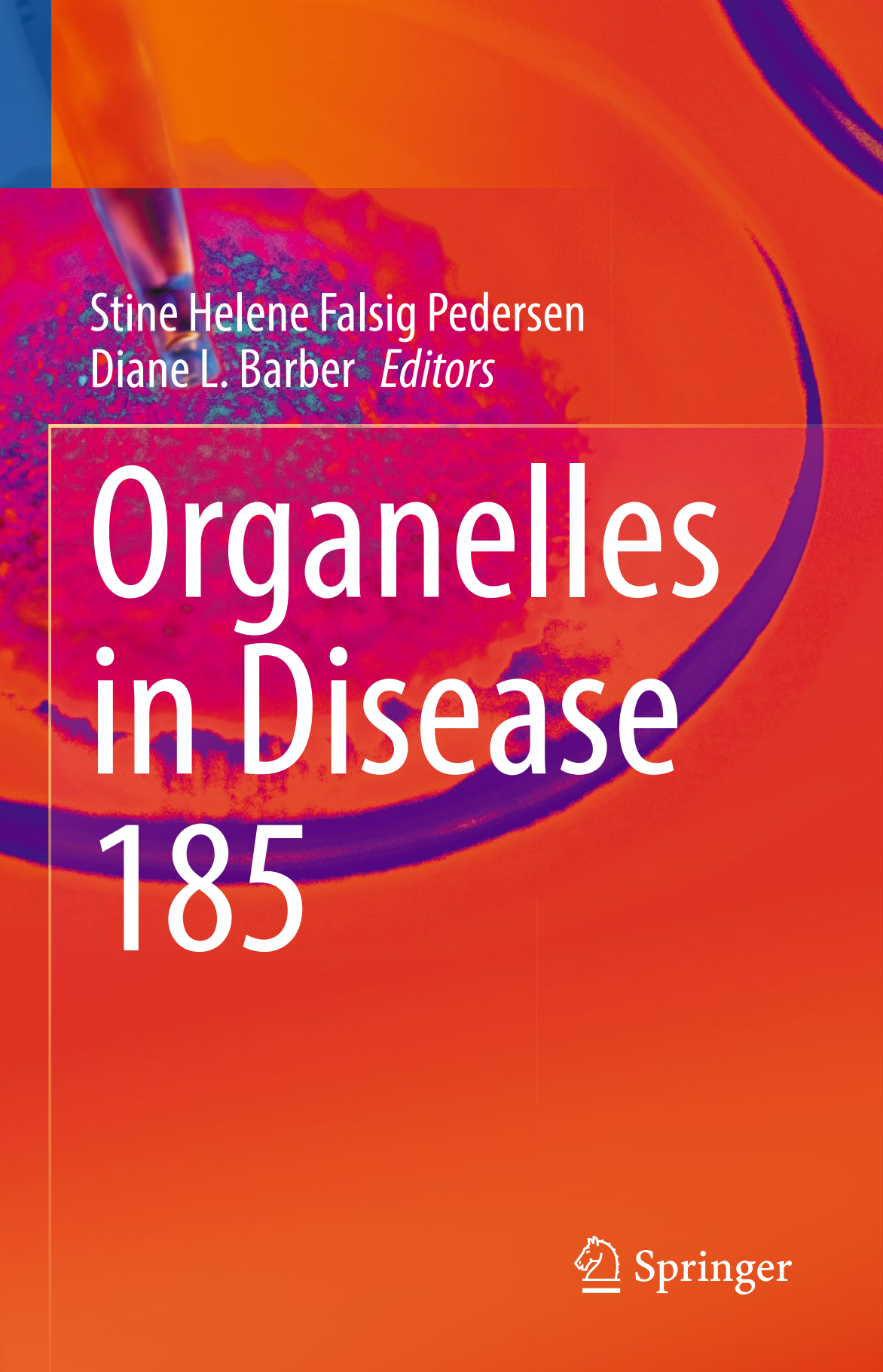
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Organelles in Disease

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Golgi pH and Ion Homeostasis in Health and Disease

Elham Khosrowabadi and Sakari Kellokumpu

Contents

Abstract Maintenance of the main Golgi functions, glycosylation and sorting, is dependent on the unique Golgi pH microenvironment that is thought to be set by the balance between the rates of V-ATPase-mediated proton pumping and its leakage back to the cytoplasm via an unknown pathway. The concentration of other ions, such as chloride, potassium, calcium, magnesium, and manganese, is also important for Golgi homeostasis and dependent on the transport activity of other ion transporters present in the Golgi membranes. During the last decade, several new

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disorders have been identified that are caused by, or are associated with, dysregulated Golgi pH and ion homeostasis. Here, we will provide an updated overview on these disorders and the proteins involved. We will also discuss other disorders for which the molecular defects remain currently uncertain but which potentially involve proteins that regulate Golgi pH or ion homeostasis.

Keywords Disease · Golgi dysfunction · Golgi homeostasis · Ion balance · Resting pH

1 Introduction

The pH of secretory and endocytic compartments is known to be uniquely acidic in each compartment and crucial for their efficient functioning in mammalian cells (Kim et al. 1996; Demaurex et al. 1998; Palokangas et al. 1998; Schapiro and Grinstein 2000; Wu et al. 2001; Paroutis et al. 2004, for a recent review, see also Kellokumpu 2019). In general, the acidity of the secretory compartments increases toward the plasma membrane, while the pH of endosomal compartments increases toward the cell center (late endosomes and lysosomes). Among all these compartments, the Golgi apparatus is the most enigmatic one, as it represents a converging point between the two pathways, receiving vesicular carriers from the endoplasmic reticulum (neutral) and the endosomal compartments (acidic) and sending them back to these destinations as part of its sorting functions. Moreover, Golgi resting pH decreases from the cis-Golgi cisternae (pH 6.7) to medial (pH 6.5) and trans-Golgi cisternae (pH 6.3) reaching pH 6.0 at the TGN. How this pH gradient along the cis-trans axis of Golgi cisternae is established is not completely clear, but it may involve two different mechanisms, which be need not be mutually exclusive: the first one may involve selective trafficking of vesicular carriers between the cis-Golgi and the neutral ER and between the trans-Golgi and the acidic endosomal compartments. The other one is the number of proton pumps or proton leak "channels" that may change along the cis-trans axis of the Golgi stack (Wu et al. 2001; Paroutis et al. 2004).

2 Maintenance of Golgi pH Homeostasis

The resting pH of the Golgi lumen is known to be established and maintained by three main ion transport systems that include the vacuolar (V)-ATPase, a counter ion (Cl^- or K^+) transport, and a proton "leak" pathway that shuttles protons from the Golgi lumen back to the cytoplasm (Wu et al. 2001; Demaurex 2002; Paroutis et al. 2004). Each of these three systems is briefly discussed below.

2.1 Golgi Acidification by the V-ATPase

The V-ATPase is the main acidifier of all secretory and endocytic compartments. It is a multimeric protein complex whose oligomeric structure varies between different compartments. Consistent with this, unique subunit isoforms are found in mammals, the expression of which is often tissue- and cell type-specific (Marshansky and Futai 2008). For example, multiple isoforms are found for the a-subunit which seems to dictate the V-ATPases into distinct intracellular localizations. In yeast the N-terminal cytosolic domains of two a-subunit isoforms (Vph1p and Stv1p) are responsible for the specific targeting of V-ATPase to vacuolar and Golgi compartments, respectively. In mice and humans, there are four distinct a-subunit isoforms (a1-a4) of which the a2-subunit seems to dictate the localization of the V-ATPase in the Golgi membranes. The other isoforms are found in V-ATPases that are expressed variably at the plasma membrane and/or endosomal-lysosomal compartments (Marshansky and Futai 2008; Flinck et al. 2020).

The V-ATPase uses ATP as an energy source to pump protons into the Golgi lumen (Fig. 1 top). Its activity is therefore dependent on cellular glucose or nutrient levels. Under normal conditions, it is assumed to be constantly active (Schapiro and Grinstein 2000; Wu et al. 2001). Consistent with this view, addition of excess (10 mM) ATP induces rapid Golgi acidification that equilibrates after few minutes at pH ~ 5 (Fig. 1 top), i.e., lower than the normal resting pH. This probably reflects experimental setup (high potassium bath solution without buffering, excess ATP). Likewise, Golgi lumen starts to alkalinize within few seconds equilibrating at neutral pH within few minutes after blocking the V-ATPase activity by using its specific inhibitor concanamycin A (Fig. 1, bottom).

2.2 Counter Ion Transport

Continuous proton pumping by the V-ATPase has the propensity to increase Golgi membrane potential (inside positive) that then would slow down the activity of the V-ATPase and thus Golgi acidity. Therefore, to keep membrane potential unaltered, proton pumping needs to be counteracted by Cl^- import or K^+ (Glickman et al. 1983; Schapiro and Grinstein 2000; Paroutis et al. 2004). In strong support of this, protein termed the GPHR (Golgi pH regulator), a Cl^- channel, was shown to be necessary for the maintenance of sufficiently acidic Golgi resting pH in the cells (Maeda et al. 2008). In addition, its mutagenesis was shown to alter glycosylation, to delay protein transport to the plasma membrane, and to induce Golgi fragmentation.

Other studies have suggested that passive K^+ efflux (instead of Cl^- influx) is used to counteract membrane potential increase brought about by proton pumping (Howell and Palade 1982). This may relate to the high permeability of the Golgi membranes to K^+ ions (Schapiro and Grinstein 2000) which could be mediated by Na^+ and K^+ conductive channels such as the Kv1.3 (Zhu et al. 2014) in conjunction

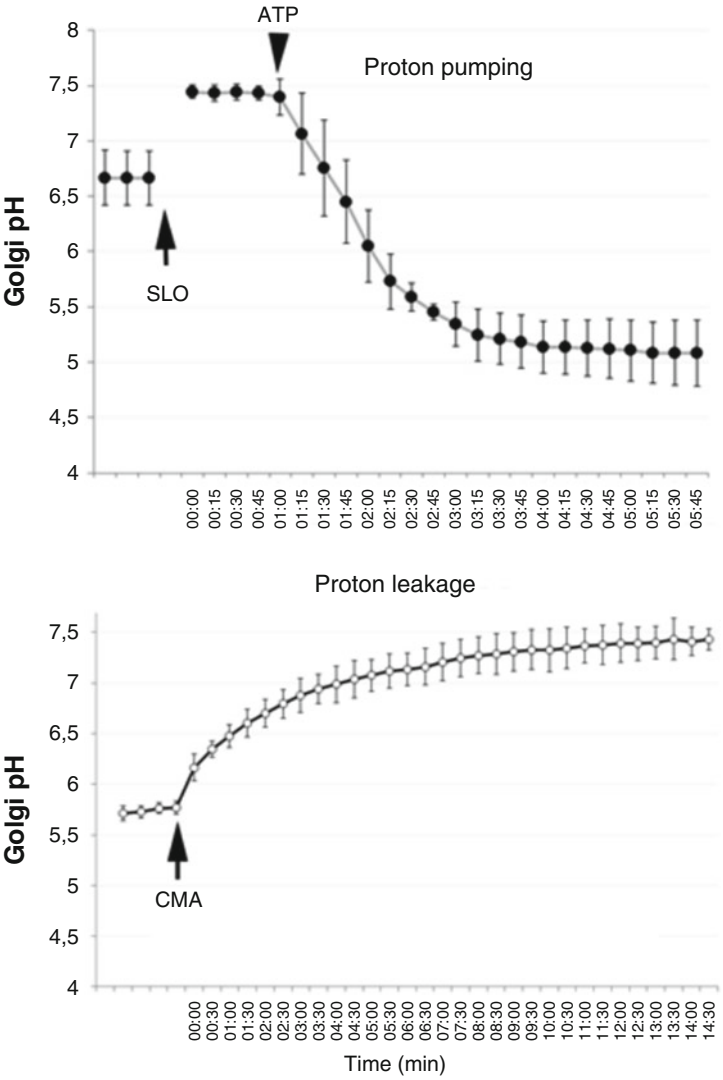

Fig. 1 Visualization of Golgi acidification by the V-ATPase and proton leakage across the Golgi membranes. Top: To visualize Golgi acidification by the V-ATPase, cells were transfected with ratiometric pHluorin (pH probe) carrying medial-trans-Golgi-targeting motif from B4GalT1). One day post-transfection, cells were ratio-imaged and permeabilized using streptolysin O (SLO) to allow controlled addition of ATP to the cytoplasm. Bottom: the V-ATPase inhibitor concanamycin A (CMA) was used to block proton pumping and to visualize proton leakage rate from the Golgi lumen back to cytoplasm

with the Na^+/K^+-ATPase (Poschet et al. 2001). Poschet et al. showed that dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) leads to hyperacidification of the trans-Golgi network (TGN) in CF lung epithelial cells, in contrast

to CFTR-corrected cells. In addition, treatment with the Na^+/K^+-ATPase inhibitor acetylstrophanthidin reduced the pH of CFTR-corrected cells to the level of CFTR mutant cells but had no effect on the latter. Based on the notion that sodium transport is under negative regulation by CFTR in CF cells, the authors suggested that in CFTR-corrected cells, Na^+/K^+-ATPase activity increases the interior positive membrane potential in the absence of active sodium channel, thereby counteracting acidification. In contrast, in CF cells, the sodium channel remains open allowing Na^+-efflux, which then can counteract positive charge build up brought about by proton pumping and by Na^+/K^+-ATPase-mediated net sodium influx (3 Na^+ vs. 2 K^+). This in turn facilitates continuous proton pumping and TGN acidification. Although interesting, further work is needed to confirm whether their model also applies to other mammalian cell types or cells with the wild-type CFTR gene.

2.3 Proton Leakage Pathway

Once the Golgi pH is sufficiently acidic (pH < 6.5), the "proton leakage" or efflux pathway starts to restrict further acidification by shuffling protons back to the cytoplasm. This leak (and its rate) can be easily visualized by shutting off the V-ATPase and following the rate of Golgi alkalinization with time (Fig. 1, bottom, see also Kellokumpu 2019). Thus, by counteracting proton pumping, it prevents over-acidification of the Golgi lumen. At resting pH (or pH set point), proton leakage rate matches that of proton pumping by the V-ATPase (Wu et al. 2001). Interestingly, the authors also showed that different compartments within the secretory pathway indeed display different leakage rates, which decrease from the endoplasmic reticulum to the Golgi apparatus and the secretory vesicles, consistent with their more acidic resting pH set points. Therefore, it seems that the proton leakage rate determines the pH set point of each compartment and perhaps the pH gradient along the cis-trans axis of the Golgi stacks. Yet, vesicular carriers that derive from the ER (neutral) or the endocytic compartments (acidic), respectively, may also contribute to the existing pH gradient along the Golgi stack.

Despite its functional importance, the identity of the "proton leak channel" has remained elusive. Physiological measurements have shown that proton efflux is sensitive to membrane potential and inhibited by Zn^{2+}, suggesting the involvement of a regulated channel (Cherny and DeCoursey 1999; Schapiro and Grinstein 2000). Yet, the two Golgi-localized Na^+/H^+ exchanger isoforms, NHE7 and NHE8, were considered as the best candidates for this "channel," as Na^+/H^+ exchange is driven by existing ion gradients, and high amounts of H^+ in the Golgi lumen can drive its exchange for cytoplasmic Na^+. In support of this view, forced overexpression of both NHE7 and NHE8 was found to increase Na^+ and K^+ influx as well as resting pH in the Golgi lumen (Numata and Orlowski 2001; Nakamura et al. 2005; Lawrence et al. 2010). However, other studies have shown that Golgi resting pH is not dependent on Na^+ ions (Demaurex et al. 1998). Recently, it was also demonstrated

that NHE7 functions as an acid loader, and not as an acid extruder, in both endosomes (Milosavljevic et al. 2014) and the Golgi compartment (Galenkamp et al. 2020). Therefore, it seems that these chloride/proton exchangers cannot mediate proton leakage across Golgi membranes.

An inherent property of proton leakage is its dependency on the concentration of protons in the compartment (Fig. 1), i.e., it is higher at low pH and decreases with increasing compartmental pH. Even though this type of behavior is consistent with the presence of a proton "channel," it is also compatible with the presence of a bicarbonate-dependent buffering system in the Golgi that works in the background, yet allowing acidification of the Golgi lumen until around pH 6, i.e., the pKa (pH 6.1) of the carbonic acid. In strong support of this view, an anion exchanger isoform (SLC4A2a) was identified in the Golgi membranes already decades ago (Kellokumpu et al. 1988; Holappa et al. 2001). Given that members of the SLC4A gene family are all known to mediate an electroneutral one-to-one exchange of bicarbonate for chloride (Romero et al. 2004; Alper 2006), it is likely that the Golgi-localized isoform regulates Golgi resting pH most likely by preventing its overacidification by the V-ATPase. In support of this view, our recent data shows that AE2a overexpression increases, and knockdown decreases, Golgi resting pH via modulating proton leakage rate in the cells (personal communication). These observations suggest that proton leakage across Golgi membranes likely involves a chemical buffering reaction, in which imported bicarbonate and luminal protons combine to form carbon dioxide and water via a short carbonic acid intermediate. The two end products are then expelled to the cytoplasm, a process that is helped by the flattened morphology of the Golgi cisternae as their shape provides an optimal surface-volume ratio for water and gas exchange.

In mammals, there are altogether nine members in the ClC chloride transporter family of which five (ClC-3 to ClC-7) function as Cl^-/H^+ exchangers, while the other four (ClC-1, ClC-2, ClC-Ka, and ClC-Kb) function as chloride channels and are strictly localized to the plasma membrane (Jentsch et al. 1999; Poroca et al. 2017). Only few of the various Cl^- transporters, including the mid-1-related chloride channel (MClC) and the Cl^-/H^+ exchanger variant ClC-3B showed strict localization in the Golgi membranes (Schwappach et al. 1998; Nagasawa et al. 2001; Gentzsch et al. 2003), suggesting that ClC-3B, due to its Cl^-/H^+ exchange activity, may in fact be responsible for proton leakage across Golgi membranes in exchange for cytoplasmic chloride. Yet, more recent evidence indicate that ClC-3B co-localizes in cells with LAMP1, a marker of late endosomes and lysosomes (Guzman et al. 2015; Poroca et al. 2017). Therefore, more studies are needed to proof or disproof whether the ClC-3B indeed mediates H^+ leakage across Golgi membranes or whether its main role is to regulate resting pH of the endo-lysosomal compartments.

2.4 Golgi Homeostasis of Other Ions

In addition to protons, other ions including Ca^{2+}, Mg^{2+}, and Mn^{2+} are present at high concentrations in the Golgi lumen (Van Baelen et al. 2004; Pizzo et al. 2010). They are known to be important for glycosylation (Breton et al. 2006), cargo concentration, and sorting (Chanat and Huttner 1991; Leach 1971; Vanoevelen et al. 2007) as well as for scavenging reactive oxygen species (ROS) in the cells (Coassin et al. 1992). These ions may also indirectly contribute to Golgi resting pH via affecting cation efflux (see above).

The presence of these ions at high concentrations in the Golgi lumen is maintained by a number of Golgi-localized channels and transporters, including pumps for Ca^{2+} and Mn^{2+} uptake (SERCA2, SPCA1/2), channels for Ca^{2+} release (IP3R, RyR) as well as luminal proteins that bind Ca^{2+} with high affinity (Van Baelen et al. 2004; Brini and Carafoli 2009; Lin et al. 2009; Vangheluwe et al. 2009; Zampese and Pizzo 2012). The protein, termed Golgi-associated anti-apoptotic protein (GAAP), has recently gained increasing attention due to its role in tumorigenesis (Rojas-Rivera and Hetz 2015; Carrara et al. 2017). It is a member of the transmembrane Bax inhibitor-1 motif containing (TMBIM) protein family that regulates Ca^{2+} fluxes between intracellular stores, confers resistance to apoptotic stimuli, and promotes cell adhesion and migration (Saraiva et al. 2013; Carrara et al. 2015). The Golgi also harbors several Ca^{2+}-binding proteins, including Cab45, CALNUC, p54/NEFA, and calumenin, all of which, except Cab45, are distinct from their ER counterparts (Lin et al. 1999, 2009). Of these, CALNUC, an EF-hand, and $Ca2^{+}$-binding protein is resident of the CGN and cis-Golgi cisternae, and plays an important role in Ca^{2+} buffering and secretion through the Golgi. Recent studies of Cab45 have demonstrated its importance in Ca^{2+}- and oligomerization-driven sorting that also involves actin cytoskeleton and the Ca^{2+}-ATPase SPCA1 (Pakdel and von Blume 2018). A central part of this system is the local synthesis of sphingomyelin that drives Ca^{2+} import by SPCA1 as well as protein sorting and export (Deng et al. 2018).

Golgi membranes also express transporters for zinc and copper cations. The former involve the SLC30 family of zinc transporters (ZnT 5–7) that import zinc into the Golgi lumen, while the SLC39 family of zinc transporters (ZIP 9 and 13) export it back to the cytoplasm (Xu et al. 2019). Copper transporters identified in the Golgi membranes also include ATPases such as the ATP7A and 7B that transfer copper from the cytosol into the Golgi lumen for incorporation into copper-dependent enzymes (Polishchuk and Lutsenko 2013). These ATPases are also redistributed in post-Golgi compartments and the plasma membrane to ensure copper balance and to avoid potentially toxic effects brought about by its accumulation in the cytoplasm.

3 Diseases Associated with Altered Golgi pH and Ion Homeostasis

Since 2012 (Rivinoja et al. 2012), several new disorders that are caused by dysregulated Golgi pH and ion homeostasis have been identified. In the next paragraphs, we will highlight the current knowledge on these disorders and their molecular causes when known. We will also discuss diseases for which the molecular defects remain uncertain but likely involve proteins that regulate Golgi pH or ion homeostasis.

3.1 Autosomal Recessive Cutis Laxa Type II

Cutis laxa represent rare, heterogeneous, and either inherited or acquired connective tissue disorders that are characterized by loose and inelastic skin and an aged appearance. The patients show variable phenotypes due to mutations in distinct genes that cause these disorders. The main classes are autosomal dominant (AD), autosomal recessive (AR), and X-linked recessive (XR) forms of cutis laxa. Individuals with autosomal recessive cutis laxa type II (ARCL2) have wrinkly skin over the entire body that can improve with age. Other features include an enlarged anterior fontanel, dislocation of the hips, hernias, and nearsightedness. Many children also suffer from severe developmental delay and seizures.

Even though the disease etiology is variable and not completely clear, some ARCL2 patients have mutations in the gene encoding the *a*2 subunit of the Golgi-localized V-ATPase (*ATP6V0A2*) (Kornak et al. 2007). Similar mutations in *ATP6V0A2* gene have also been identified in a closely related disorder, the "Wrinkly skin" syndrome (WSS), in which the patients show also connective tissues defects, yet without elastin deficiency (Morava et al. 2009). Although not directly measured yet, the mutations almost certainly affect the V-ATPase activity, Golgi acidification state, and Golgi resting pH, as patients' cells carrying these mutations show N- and O-linked glycosylation defects and altered membrane trafficking (Morava et al. 2005; Kornak et al. 2007; Hucthagowder et al. 2009), i.e., phenomena typically associated with dysregulated Golgi pH homeostasis (Kellokumpu 2019). Nevertheless, further studies are needed to unravel the role of an altered endosomal pH and its dysfunction in disease etiology, since the "*a*2" subunit seems to be expressed in addition to Golgi membranes also in early endosomes (Hurtado-Lorenzo et al. 2006).

3.2 Cancers

Direct Golgi pH measurements with fluorescent probes in breast and colorectal cancer cells (MCF-7, HT-29, and SW-48) suggested that the Golgi resting pH is more alkaline (0.2–0.4 pH units) than that of nonmalignant cells (Rivinoja et al. 2006). Since then, recent high-throughput Golgi pH measurements with ratiometric pHluorin have demonstrated that all cancer cell lines tested thus far (12 different) display markedly elevated Golgi resting pH (personal communication) when compared to a nonmalignant COS-7 control cell line. This and the fact that the AE2 protein is also overexpressed in some of these cell lines suggest that the observed Golgi pH increase is not a mere adaptation of cancer cells to grow in normal cell culture conditions.

Moreover, the observed Golgi pH increase also coincided with markedly elevated expression of cancer-associated Tn- and T-antigens in the same cells. The same antigens can also be induced in normal cells by using pH gradient dissipating drugs (Kellokumpu et al. 2002), suggesting that an altered Golgi resting pH may be the main cause for their increased expression in cancers. The fact that these antigens are commonly seen in cancers in vivo and that they promote cancer cell invasion and metastasis (Pinho and Reis 2015; Peixoto et al. 2019) emphasizes that an altered Golgi resting pH is an important factor that drives tumorigenesis and its progression. Confirmation of this must wait for new strategies that will allow Golgi pH measurements in situ.

In addition to altered glycosylation, another hallmark of cancers is the loss of cell polarity, a phenomenon that is intimately associated with epithelial-mesenchymal transition (Yeung and Yang 2017). Previous and recent data from others and us have indicated that an altered Golgi resting pH contributes to this polarity loss. Caplan et al. (1987) showed that pH gradient dissipating drugs were able to impair apical targeting of certain proteins in epithelial cells. Recently, we also demonstrated that apical targeting of the CEAMCAM5 (carcinoembryonic antigen, CEA), a well-known follow-up marker for colorectal cancer, is also a pH-dependent process (Kokkonen et al. 2019). CEA, a GPI-anchored protein, is present normally in the apical surface of colon epithelial cells but mistargeted both to the apical and basolateral domains in cancer tissues and cells, such as colon CaCo-2 cells. In these cells, we found that Golgi resting pH is 0.5 pH units higher than in nonmalignant control cells. Accordingly, when we treated polarized Madine-Derby Kidney cells (MDCK) with various pH gradient dissipating drugs such as concanamycin A (a proton pump inhibitor), stably expressed CEA was mistargeted also to basolateral surface without attenuating its trafficking to the cell surface. Other data presented in the paper (Kokkonen et al. 2019) suggested that this pH induced mistargeting is due to impaired association of the CEA-specific GPI anchor with lipid rafts. However, it should be noticed that mistargeting of some apical proteins might also be driven by altered N-glycosylation (Ho et al. 2016; Vagin et al. 2009).

Another important issue related to altered organelle pH homeostasis and cancers is multidrug resistance (MDR). In resistant cancer cell lines, chemotherapeutic drugs

(often weak bases) are taken up by both acidic secretory and endocytic compartments, where they become protonated and unable to escape back to the cytoplasm (Schindler et al. 1996; Altan et al. 1998). This sequestration in intracellular compartments helps resistant cells to reduce drug load in the cytoplasm. In contrast, drug-sensitive cells were shown to have defects in organelle acidification, whereby they fail to sequester these drugs in the same manner, thereby exposing cells and nuclei to high concentrations of these drugs. In further support of this, MDR transporters were shown to be expressed and to be active mainly in endo-lysosomal compartments (Rajagopal and Simon 2003), as the authors stated that the observed distribution of the MDR transporters does not preclude subcellular activity elsewhere in the endomembrane system depending on the physiological state of the cells. However, other mechanisms for MDR also exist (Simon 1999) including export from the cytoplasm via MDR transporters that reside in the plasma membrane. Recent evidence also highlights the involvement of abnormal glycosylation as an alternative multidrug resistance mechanism, since based on the current evidence (reviewed by Very et al. 2017), it has pleiotropic effects on various cellular functions such as apoptosis failure, signaling activation disruption, altered drug absorption and metabolism, and cell stemness acquisition.

Altogether, these findings suggest that dysregulated Golgi pH homeostasis causes glycosylation changes, which in turn drive phenotypic changes typically seen during cancer progression. Thus, there is a growing need to understand why Golgi pH homeostasis is dysregulated in cancer cells and why this dysregulation causes glycosylation defects. Potential causes may involve loss of the V-ATP activity, increased proton leakage or altered functioning of ion channel proteins in the Golgi as well as altered synthesis or unavailability of nucleotide sugars, altered expression of glycosyltransferases (Stanley 2011), enzyme mislocalization (Rivinoja et al. 2009), and loss of heteromeric glycosyltransferase complexes at elevated Golgi resting pH (Hassinen et al. 2011). The latter two are likely tightly coupled phenomena, given that oligomerization is important for Golgi retention (Nilsson et al. 1993, 1994, 2009) and that acidic Golgi luminal pH is needed for the formation of enzyme complexes (Hassinen and Kellokumpu 2014).

3.3 Viral Infections

Given the recent SARS and current corona virus (Covid-19) pandemics, better understanding of the molecular circuits needed for virus assembly and budding is a must, as they would help identify novel, more specific, and better antiviral therapeutics. One such shared but neglected feature that could be utilized in patient care is that during virus assembly, certain viral proteins are able to neutralize the secretory pathway compartments via increasing proton leak across organelle membranes. Today these proteins are termed as “viroporins” (Scott and Griffin 2015). They possess ion channel activity for monovalent ions and have dramatic effects on the secretory pathway organelles that can be mimicked by pH gradient dissipating

drugs. Viroporins include the influenza A virus M2 protein, the hepatitis C virus (HCV) p7 protein, and the Corona virus envelope (E) protein. Overexpression of the M2 in the cells alone has been shown to increase Golgi resting pH, slow down membrane trafficking, and alter Golgi morphology (Sakaguchi et al. 1996, Sugrue et al. 1990, Ciampor et al. 1992). An increase of Golgi resting pH is thought to be beneficial for the production of infectious virus particles and their escape from host cells, as higher pH would prevent premature activation of the fusion-competent hemagglutinin (needed for infectivity) as well as terminal sialylation of cell surface glycans (Rivinoja et al. 2009; Condon et al. 2013), i.e., sugar residues in host cell surface glycans to which both newly assembled avian and human influenza viruses remain bound until they are released by their own neuraminidases (Kimble et al. 2010). In the case of the hepatitis virus p7 protein, a pH increase is thought to protect egress of viral structural proteins through the secretory pathway, as the loss of p7 can be partially rescued by either neutralization of the secretory compartments with bafilomycin A1 or by ectopic expression of the influenza virus M2 protein (Wozniak et al. 2010; Bentham et al. 2013). The E protein of the infectious coronavirus causing bronchitis elicits a similar increase in Golgi pH and morphological changes in the secretory pathway compartments when overexpressed in mammalian cells (Machamer and Youn 2006; Westerbeck, and Machamer 2019). The benefit in this case appears to be that the elevated Golgi pH prevents premature cleavage of the viral S protein in the Golgi/TGN, thereby promoting release of fully infectious viruses from the cells.

3.4 Multigenerational Non-syndromic Intellectual Disability (ID)

Khayat et al. (2019) recently described a new pH homeostasis-associated disease termed the multigenerational non-syndromic intellectual disability (ID). The disease is associated with missense mutations in the sodium/proton exchanger NHE7 (SLC9A7). Despite the variant protein localized correctly in the TGN/post-Golgi vesicles and membrane trafficking was not abrogated, the synthesis of *N*-linked glycans was abnormal. This finding suggests that the main defect is the less acidic pH of the TGN/post-Golgi compartments in patient's cells. In further support, NHE7 act as an acid loader and thereby contributes to pH homeostasis of the TGN and endosomal system (Milosavljevic et al. 2014; Galenkamp et al. 2020). However, direct Golgi pH measurements with patient's cells are needed to verify that the mutation abrogates NHE7 activity.

3.5 Angelman Syndrome and Autism Spectrum Disorders

Angelman syndrome (AS) is a severe genetic disorder that primarily affects the nervous system. Characteristic features include delayed development, intellectual disability, severe speech impairment, and problems with movement and balance (ataxia). It is caused by the genetic defects in the UBE3A gene that lead to the loss of expression of the maternal allele encoding the E3 ubiquitin ligase E6-associated protein (E6AP/UBE3A) in nerve cells (Jiang et al. 1999; Kishino et al. 1997). Conversely, copy number variations that result in upregulation of the UBE3A/E6AP protein are strongly associated with the development of autism spectrum disorders (Khatri and Man 2019).

Although one role of the UBE3A/E6AP protein is to ubiquitinylate its substrates and target them for degradation by the proteasome, it is not clear how its loss causes the disease phenotype. Interestingly, Condon et al. (2013) have shown that UBE3A (m−/p+) knockout induces a profound disruption of the Golgi stacks and swelling of the Golgi cisternae in the cortex of UBE3A (m−/p+) mice. It also was associated with elevated Golgi resting pH and a concurrent reduction in protein sialylation, a process that is highly dependent on Golgi compartmental pH (Condon et al. 2013).

Interestingly, vesiculation or fragmentation of the Golgi apparatus are also common in many other neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Parkinson's, Alzheimer's, and Huntington's diseases (Fan et al. 2008; Haase and Rabouille 2015; Ayala and Colanzi 2017). This suggests that altered Golgi homeostasis may be an important player in these diseases as well. In support of this view, Sou et al. (2019) recently showed that Purkinje cells in GPHR conditional knockout mice also display vesiculated and fragmented Golgi apparatus, degenerated axon terminals, altered innervation, and selective loss of large amplitude responses in the cells. However, genetic screens have not yet revealed any mutations or expression level changes in proteins that regulate Golgi resting pH, leaving their involvement in these diseases to be confirmed. Other studies have also suggested that fragmentation of the Golgi apparatus in neurons takes place because of the dysregulated ER-Golgi transport (Huynh 2003; Joshi et al. 2014). Yet, considering that dysregulated Golgi pH can interfere with ER-Golgi transport (Palokangas et al. 1998), provides further support the role for dysregulated Golgi pH or ion homeostasis in these neurodegenerative diseases.

Consistent with dysregulated Golgi ion homeostasis, previous studies have revealed a list of key zinc transporters whose mRNA or protein levels are altered at different stages of Alzheimer's disease (for review, see Xu et al. 2019). Further support for this was obtained by modulating the expression in the central nervous system of some of these zinc transporters in animal models. Such manipulation either slowed down or prevented disease progression and significantly improved cognitive performance, movement, and the life span of the animals (Ritchie et al. 2003, Lannfelt et al. 2008, Adlard et al. 2008). Zinc transporters are divided into two major families. The SLC30 family of zinc transporters (ZnT1–10) export zinc out of the cytoplasm or into cellular organelles, thereby reducing zinc concentration in the

cytoplasm. In contrast, the SLC39 family of zinc transporters (ZIPs1–14) import zinc into the cytoplasm, either from the organelles or from the extracellular space (Xu et al. 2019). Of these, ZnT5–7 are Golgi-localized transporters that display increased expression levels in Alzheimer's disease patients (Kirschke and Huang 2002; Lovell et al. 2006; Xu et al. 2019). This suggests that more zinc accumulates in the Golgi lumen in AD patients. Yet, how this leads the extracellular accumulation of amyloid plaques rich in the β-amyloid (Aβ) peptide remains unclear.

3.6 Hailey-Hailey Disease

In humans, allelic mutations of the gene encoding the Golgi-localized Ca^{2+} ATPase SPCA1 (Vanoevelen et al. 2007; Brini and Carafoli 2009) are the cause of Hailey-Hailey disease, a skin disorder characterized by outbreaks of rashes and blisters that localize usually in the folds of the skin, but can cover large areas of the body. Typically, skin keratinocytes display increased levels of cytosolic Ca^{2+} ions, defects in protein sorting, and Ca^{2+} signaling (Missiaen et al. 2004; Ramos-Castaneda et al. 2005; Vanoevelen et al. 2007). The SPCA1 transports both Ca^{2+} and Mn^{2+} into the Golgi lumen and, therefore, plays an important role in Golgi cation homeostasis (Van Baelen et al. 2004; Missiaen et al. 2007). Lowered levels of Ca^{2+} and Mn^{2+} cations in the Golgi lumen in patients' cells lead to defects in protein folding, trafficking, and sorting or proteolytic cleavage of prohormones (Missiaen et al. 2004; Grice et al. 2010). These defects can explain why the cells in affected patients are unable to maintain structurally intact desmosomes and epidermis. The fact that Mn^{2+} is an important cofactor for many glycosyltransferases (Kaufman et al. 1994) suggests that glycosylation may also be altered in the affected cells and thus can contribute to the disease etiology as well.

3.7 Congenital Disorder of Glycosylation 2K (CDG2K)

CDG2K is an autosomal recessive disorder with a variable phenotype. Affected individuals generally show psychomotor retardation and growth dysmorphism that involve eye abnormalities, microcephaly, hepatomegaly, and skeletal dysplasia with hypotonia. The disease is manifested by displaying glycosylation defects in N-linked glycans and to be caused by mutations in TMEM165, a transporter believed to be involved in Golgi Mn^{2+} and Ca^{2+}/H^{+} transport (Foulquier et al. 2012; Potelle et al. 2016; Dulary et al. 2017, 2018; Thines et al. 2018). Yet, it is not yet fully clear what is its exact role in Golgi ion homeostasis, as unlike the Golgi-localized SPCA1, the ER-associated SERCA 2b Ca^{2+} pump was able to partially rescue TMEM165 knockout-induced Mn^{2+} depletion and concomitant defect in glycosylation (Houdou et al. 2019). Moreover, the authors also recently showed that the TMEM165

knockout can be rescued by galactose supplement in HEK293 cell culture media or when given to patients (Morelle et al. 2017).

3.8 *Menkes Disease and Related Syndromes*

Menkes disease (MD) is a lethal multisystemic disorder first described in 1962 by Menkes et al. (1962). Its main manifestations are progressive neurodegeneration, connective tissue defects, and the peculiar "kinky" hair. Serum copper and ceruloplasmin levels are also low. Severely affected MD patients die usually before the third year of life. A cure for the disease does not exist, but very early copper histidine treatment may correct some of the neurological symptoms (Tümer 2013).

Menkes disease (MNK) is inherited as an X-linked recessive trait, consistent with most patients being males. This disorder is known to be caused by intragenic mutations or partial deletions in the ATP7A gene that encodes the trans-Golgi network (TGN)-localized copper-ATPase (Polishchuk and Lutsenko 2013; Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993). As a consequence, patient cells deficient of this ATPase show poor intestinal absorption of copper and reduced transport of copper into organelles, including mitochondria (Dahmoush et al. 2016). However, the mechanistic details that cause the disease remain unclear.

In addition to Menkes disease, less severe disorders give rise to an allelic disorder termed the occipital horn syndrome, which is characterized by connective tissue malformations such as skin and joint laxity, tortuous blood vessels, and hernias. Wilson's disease (WD) is another related disorder and caused by mutations in the ATP7B gene that is expressed primarily in the liver where its main function is to excrete copper into the biliary tract. Therefore, affected individuals accumulate abnormal levels of copper in the liver. In vitro studies showed that ATP7B, located in the trans-Golgi network, re-localizes in unidentified vesicular compartment in response to increased copper load (Huster et al. 2003). Altered intracellular trafficking of ATP7B mutants has also been suggested to be among the disease-causing mechanisms (Skjørringe et al. 2017). However, further testing with human tissue samples is needed to clarify the underlying cause for the disease.

4 Perspectives and Key Questions

Maintenance of proper Golgi pH and ion homeostasis is now known to be a key factor regulating many Golgi functions such as glycosylation and protein sorting. In principle, a change in glycosylation can be regarded as a marker for altered functioning of the transporters including the V-ATPase, GPHR, the AE2 anion exchanger, or those that maintain high Ca^{2+} and Mn^{2+} concentrations in the Golgi lumen. The existing data also emphasizes that Golgi acidity, high cation concentration, and the redox state (Hassinen et al. 2019) are the key players in orchestrating

various membrane trafficking events, keeping Golgi resident enzymes correctly localized and active, and facilitating their cooperative interactions needed for glycosylation (Kellokumpu 2019). In addition, they are important for cargo selection into post-Golgi vesicular carriers and for protein sorting to the apical surface in polarized epithelial cells. Yet, it should be notified that there are other causes for altered glycosylation such as mutations or expression changes of glycosylation enzymes themselves (Freeze and Ng 2011) and changes in proteins that contribute to intra-Golgi trafficking and Golgi architecture (Bexiga and Simpson 2013) as well as in the nutritional status of cells (Ohtsubo and Marth 2006).

Dysregulated Golgi pH or ion homeostasis are currently known to cause a family of disorders that state back to the identification of cutis laxa and Hailey-Hailey disease already more than a decade ago (Rivinoja et al. 2012). Since then, new disorders of this family have come into light including congenital disorders of glycosylation 2K (CDG2K), several neurological disorders (multigenerational non-syndromic intellectual disability, Angelman syndrome), virus infections, and also recently, cancers in which glycosylation nearly always seems to be altered and promote cancer cell invasion and metastatic spread. A summary of affected proteins in these disorders is presented in Fig. 2. Yet, there are also other potential disorders for which definite proof for the involvement of an altered Golgi pH homeostasis is still missing. These include the major neurological disorders (Parkinson, Alzheimer, Huntington, and ALS). More disorders will inevitably follow, given the number of the Golgi-localized transporters known to regulate its pH and ion homeostasis in mammalian cells.

Even though glycosylation changes are nearly always associated with these disorders, they likely are similar despite the variable phenotypes of the patients. Therefore, the key questions for the future are to better understand what functional consequences mutated or dysregulated proteins have in the affected cells. Likewise, we do not yet understand whether the variable patient phenotypes result from different glycosylation patterns between patients or between individual proteins. More detailed understanding of these issues would help identification of novel diagnostic tools and development of knowledge-based therapies to reprogram Golgi pH or ion homeostasis in diseased cells. Such knowledge would also help us understand how glycosylation and sorting are executed at the level of the Golgi membranes and what molecular machineries are needed in the background to keep these main tasks ongoing.

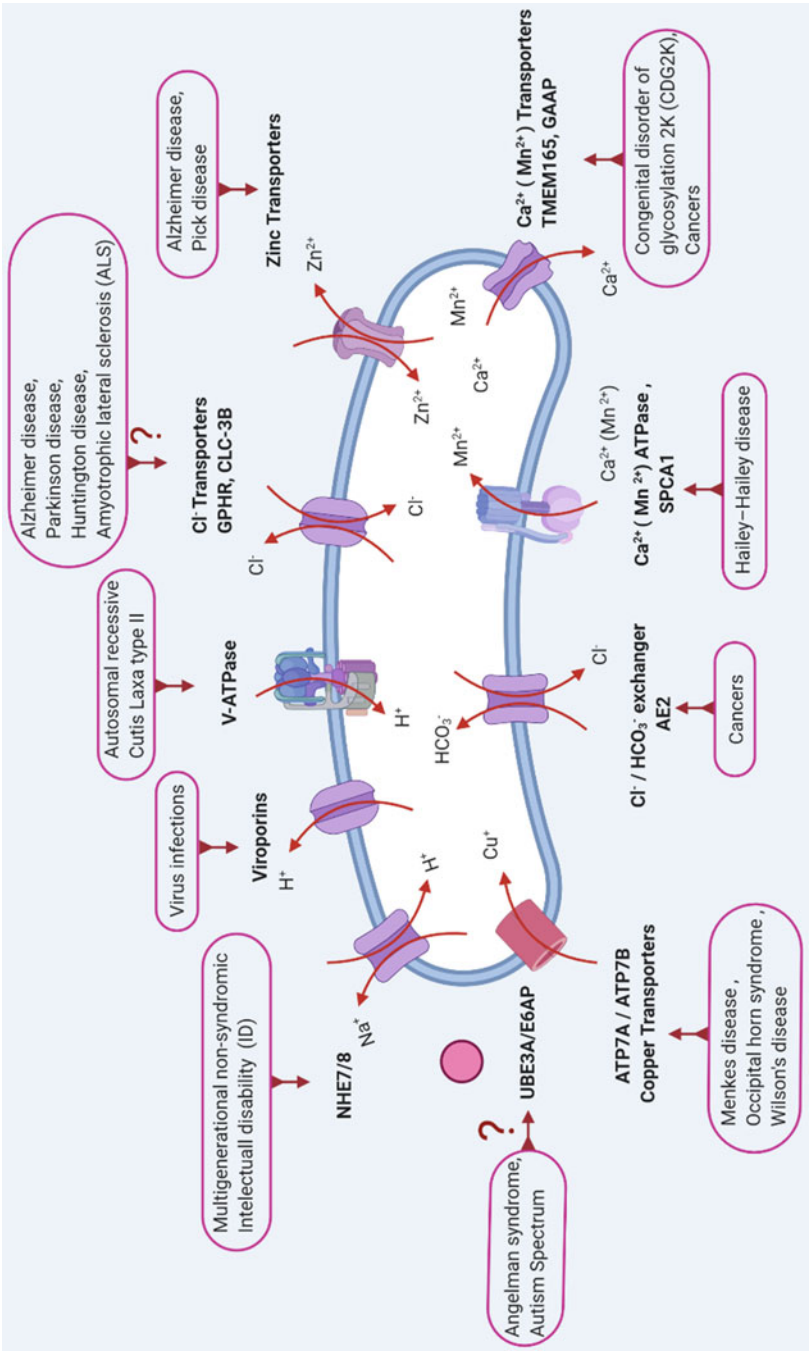

Fig. 2 Known human disorders associated with dysregulated Golgi pH and ion homeostasis. The depicted proteins affected in each disorder are shown. For more details, see the text

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Stress Granules in Cancer

Min-Seok Song and Elda Grabocka

Contents

Abstract The capacity of cells to organize complex biochemical reactions in intracellular space is a fundamental organizational principle of life. Key to this organization is the compartmentalization of the cytoplasm into distinct organelles, which is frequently achieved through intracellular membranes. Recent evidence, however, has added a new layer of flexibility to cellular compartmentalization. As such, in response to specific stimuli, liquid-liquid phase separations can lead to the rapid rearrangements of the cytoplasm to form membraneless organelles. Stress granules (SGs) are one such type of organelle that form specifically when cells are faced with stress stimuli, to aid cells in coping with stress. Inherently, altered SG formation has been linked to the pathogenesis of diseases associated with stress and inflammatory conditions, including cancer. Exciting discoveries have indicated an

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intimate link between SGs and tumorigenesis. Several pro-tumorigenic signaling molecules including the RAS oncogene, mTOR, and histone deacetylase 6 (HDAC6) have been shown to upregulate SG formation. Based on these studies, SGs have emerged as structures that can integrate oncogenic signaling and tumor-associated stress stimuli to enhance cancer cell fitness. In addition, growing evidence over the past decade suggests that SGs function not only to regulate the switch between survival and cell death, but also contribute to cancer cell proliferation, invasion, metastasis, and drug resistance. Although much remains to be learned about the role of SGs in tumorigenesis, these studies highlight SGs as a key regulatory hub in cancer and a promising therapeutic target.

Keywords Cancer · Membraneless organelles · Stress adaptation · Stress granules

1 Introduction

Stress granules (SGs) are non-membranous cytoplasmic organelles that assemble when cells are exposed to stress (Buchan and Parker 2009; Kedersha et al. 2013; Buchan 2014). They consist of a vast proteomic and transcriptomic network and range in size from tens of nanometers to several micrometers (Anderson and Kedersha 2008; Jain et al. 2016; Protter and Parker 2016; Khong et al. 2017; Namkoong et al. 2018). These structures are highly dynamic and can undergo fusion and fission. Furthermore, once stress subsides, SGs disassemble, and their components disperse back into the cytosol (Protter and Parker 2016; Wheeler et al. 2016). Earlier reports proposed that SGs function to store and target mRNA for degradation during stress (Anderson and Kedersha 2008). Studies over the past decade, however, have drastically expanded our understanding of their function. It is now well-established that SGs function as signaling hubs that regulate gene expression and signal transduction, and are critical to the cellular stress response and survival under adverse conditions.

In vivo, SGs have been associated with several pathologies, including cancer (Grabocka and Bar-Sagi 2016; Cruz et al. 2019; Herman et al. 2019; Wolozin and Ivanov 2019). Studies in cancer cells and animal models of tumorigenesis have established SGs as a stress-adaptive strategy hijacked by cancer cells to support tumorigenesis (Somasekharan et al. 2015; Grabocka and Bar-Sagi 2016; Protter and Parker 2016). Stress adaptation is emerging as an important property of cancer cells (Sharma et al. 2016; Truitt and Ruggero 2016; El-Naggar and Sorensen 2018). As oncogenic-driven hyperproliferation demands a high expenditure of cellular resources, cancer cells are often faced with stress conditions (Solimini et al. 2007; Stylianopoulos et al. 2012; Urra et al. 2016). As such, the increased demand for protein synthesis and flux in the endoplasmic reticulum results in proteotoxic stress and misfolded proteins, and hyper-replication of DNA leads to DNA damage and

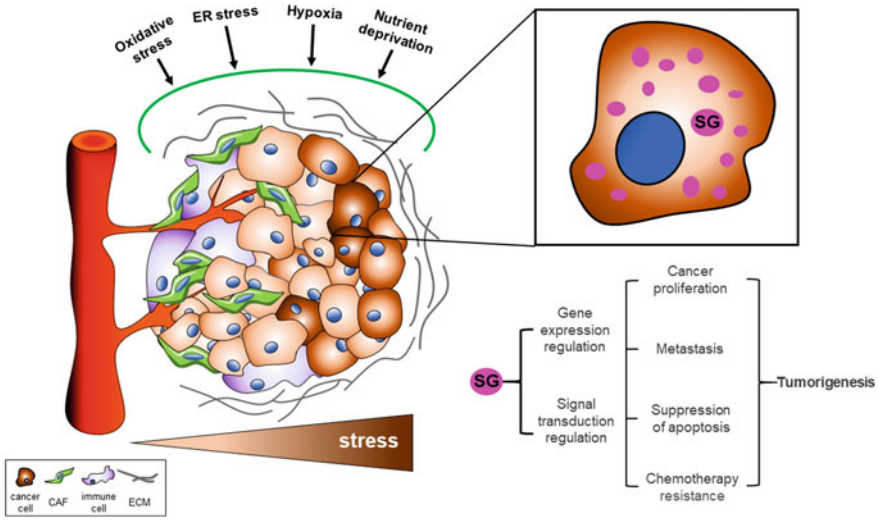

Fig. 1 SGs are key regulators of tumor stress adaptation. Cancer cells are located in a complex microenvironment that is marked by high levels of stress stimuli (oxidative stress, ER stress, hypoxia, and nutrient deprivation). In order to survive under such adverse conditions, cancer cells must adapt. SG formation is one of the key strategies for cancer cells to adapt to the stress conditions. Recent evidence indicates that SGs may contribute to tumorigenesis by modifying gene expression and signal transduction programs that regulate cancer cell proliferation, invasion and metastasis, suppression of apoptosis, and chemotherapy resistance

genotoxic stress (Joyce and Pollard 2009; Fiaschi and Chiarugi 2012; Yadav et al. 2014; Anastasiou 2017; Gouirand et al. 2018). The increased metabolic demand contributes to nutrient stress, reactive oxidant species (ROS), and pH imbalances (Vincent et al. 2015; Panieri and Santoro 2016). Furthermore, as tumors outgrow the local vascularization, the inadequate blood supply leads to reduced oxygen and nutrient levels (Fig. 1) (Wellen and Thompson 2010; Semenza 2012). Such levels of stress would normally lead to cell death, but cancer cells are able to quickly adapt and survive (Wellen and Thompson 2010; Gorrini et al. 2013; Wang and Kaufman 2014; Senft and Ronai 2016; Lee et al. 2020). Stress adaptation, therefore, can contribute to tumorigenesis by enhancing the cellular fitness and supporting the survival of cancer cells.

The stress adaptation of cancer cells is conferred, in large part, by the capacity of oncogenic molecules to elicit compensatory responses to tumor-associated stresses in order to promote tumor cell survival (Solimini et al. 2007; Commisso et al. 2013; Ruggero 2013; Easwaran et al. 2014; Eirew et al. 2015; Perera et al. 2015; Amaravadi et al. 2016; Lee et al. 2020). Such responses include alterations to the genetic, epigenetic, and transcriptomic landscape and, more recently, the hijacking of stress-coping cellular processes. Examples of the latter include oncogene-induced upregulation of macropinocytosis and autophagy, as well as modifications of lysosomes, which enable cancer cells to cope with nutritional stress (Commisso et al.

2013; Perera et al. 2015; Amaravadi et al. 2016). In addition, cancer cells upregulate the unfolded protein response (UPR) to cope with ER stress and misfolded proteins (Obacz et al. 2017). Whereas these processes are upregulated by cancer cells to cope with specific stress stimuli, SGs have been identified as a cancer cell stress-adaptive mechanism for a broad spectrum of tumor-associated stresses including oxidative-, proteotoxic-, osmotic- stress, as well as for nutrient deprivation (Fig. 1) (Somasekharan et al. 2015; Grabocka and Bar-Sagi 2016; Protter and Parker 2016).

Several studies have demonstrated that pro-tumorigenic signaling pathways that are hyperactivated in cancer stimulate the formation of SGs (Somasekharan et al. 2015; Grabocka and Bar-Sagi 2016; Protter and Parker 2016). This enhanced formation of SGs, in turn, may promote cancer development and progression. Evidence exists that SGs may support tumorigenesis not only through facilitating cancer cell survival but also through contributing to tumor cell proliferation and metastasis (Fig. 1). In addition, it has been shown that SGs may play an important role in the development of drug resistance (Fig. 1). These studies highlight that while SGs are important in the normal cellular stress response and may impact several diseases (reviewed in excellent detail elsewhere (Protter and Parker 2016; Mahboubi and Stochaj 2017; Cruz et al. 2019; Herman et al. 2019; Wolozin and Ivanov 2019)), the hijacking of this process by cancer cells may be critical for tumorigenesis and a promising therapeutic target. Here we review recent data illuminating the oncogenic signaling pathways that promote the formation of SGs in cancer cells and the mechanisms through which SGs may contribute to tumor progression and response to chemotherapy. In addition, we discuss how leveraging this knowledge may instruct the development of therapeutic strategies for the treatment of cancer and overcoming drug resistance.

2 Properties of Stress Granules

2.1 Formation of Stress Granules

Since the initial discovery of SGs in tomato cells exposed to heat shock, several studies have revealed SG formation as an evolutionary conserved response to stress produced by plants, protozoa, yeast, *C. elegans*, *Drosophila,* and mammalian cells (Nover et al. 1983; Arrigo et al. 1988; Collier et al. 1988; Buchan et al. 2008; Farny et al. 2009; Thomas et al. 2011; Gutierrez-Beltran et al. 2015). SG formation is induced by a variety of stress stimuli including oxidative stress, heat shock, ER stress, nutrient deprivation, UV irradiation, proteotoxic stress, and several chemotherapeutic agents (Kedersha et al. 1999; Kimball et al. 2003; Kwon et al. 2007; Mazroui et al. 2007; Fournier et al. 2010; Emara et al. 2012; Kaehler et al. 2014; Moutaoufik et al. 2014; Adjibade et al. 2015; Grabocka and Bar-Sagi 2016; Reineke et al. 2018; Lin et al. 2019).

The formation of SGs is closely linked to translation inhibition (Kedersha et al. 1999; Protter and Parker 2016). Cells respond to stress by blocking protein synthesis via the phosphorylation of the α subunit of the eukaryotic initiation factor 2 (eIF2). In mammalian cells, the phosphorylation of eIF2α is mediated by a family of four different serine/threonine kinases each of which is activated by specific forms of stress. These kinases include the general control non-derepressible 2 (GCN2) kinase which is activated by amino acid deprivation; the heme-regulated inhibitor (HRI) kinase which is activated by oxidative or osmotic stress; the double stranded RNA-dependent protein (PKR) kinase which is activated in response to viral infections; and the PKR-like endoplasmic reticulum kinase (PERK) which is activated by ER stress (Wek et al. 2006; Donnelly et al. 2013). eIF2α is one of the three subunits (α, β, γ) of eIF2 which mediates the binding of initiator methionyl-tRNA (Met-$tRNA_i^{Met}$) to the ribosome in a GTP-dependent manner (Donnelly et al. 2013). The eIF2-Met-$tRNA_i^{Met}$-GTP complex binds the 40S ribosomal subunit, as well as eIF1, eIF1A, eIF5, and eIF3, to form the 43S pre-initiation complex (PIC); PIC then associates with eIF4F on mRNA to form a new 48S complex which scans the mRNA for the start codon (AUG). Phosphorylation of eIF2α under stress prevents the formation of eIF2- Met-$tRNA_i^{Met}$-GTP, thus resulting in a translationally stalled, noncanonical 48S complex that is unable to recruit the 60S ribosomal subunit (Jackson et al. 2010). Consequently, ribosomes runoff the transcripts, causing a flux of messenger ribonucleoprotein complexes (mRNPs) and exposed RNA, which are critical for SG formation (Wheeler et al. 2016).

It is important to note that while translation inhibition is key for the formation of SGs, it can also occur independently of eIF2α phosphorylation (Jackson et al. 2010). For one, changes in the composition or activity of the eIF4F-cap binding complex (eIF4A, eIF4E, eIF4G) can inhibit translation and induce SG formation. As such, hydrogen peroxide initiates SG assembly by inhibiting translation initiation through disrupting the interaction of eIF4E with eIF4G (Emara et al. 2012; Fujimura et al. 2012). Also, chemicals such as hippuristanol and pateamine A interfere with translation initiation by blocking the eIF4A helicase, which is required for the ribosome recruitment phase of translation initiation. Lastly, the anti-inflammatory lipids 15-deoxy-Δ-12,14-prostaglandin J2 and prostaglandin A1, which are potent inducers of SG formation, inhibit translation by preventing the association of eIF4A with eIF4G (Bordeleau et al. 2006; Dang et al. 2006; Kim et al. 2007; Grabocka and Bar-Sagi 2016).

Regardless of the mode of translation inhibition, the resulting flux of mRNPs and exposed RNA are essential for the formation of SGs (Protter and Parker 2016; Wheeler et al. 2016; Ivanov et al. 2019). These molecules initiate the first steps of SG assembly by binding to RNA-binding proteins termed SG-nucleating proteins, which include the poly(A)-binding protein (PABP1) PABP1, the T-cell internal antigen 1 (TIA-1), TIA-1 related (TIAR), and Ras-GTPase-activating protein SH3-domain-binding protein1 (G3BP1). SG assembly is further aided by the ability of SG-nucleating proteins to phase-separate and coalesce in the cytoplasm, leading to the formation of nascent SGs. Further recruitment of proteins and transcripts via protein-protein, protein-RNA, and RNA-RNA interactions results in the formation

of mature SGs. The phase-separation capacity of SG-nucleating proteins is mediated by intrinsically disordered domains (IDD), which are a prominent feature of SG-nucleator proteins (Protter and Parker 2016; Mahboubi and Stochaj 2017). The role of these domains in the coalescence of SG-nucleator proteins is supported by several studies showing that, at high concentration, IDD domains can induce spontaneous phase separation. In agreement with this notion, one study showed that overexpression of SG nucleators, which would presumably increase the concentration of IDD domains, is sufficient to induce SG formation in vitro even in the absence of stress (Gilks et al. 2004; Matsuki et al. 2013; Lin et al. 2015; Molliex et al. 2015). In addition to IDD domains, phase separation of SG nucleators is regulated by posttranslational modifications which can enhance or weaken the multivalent interactions between these molecules (Kwon et al. 2007; Tsai et al. 2008; Carpio et al. 2010; Xie and Denman 2011; Owen and Shewmaker 2019). Shedding further light onto the macromolecular interactions that contribute to SG assembly, a recent study indicated that RNA-RNA interactions and ability to self-coalesce wherever there is a high concentration of RNA may also contribute to SG assembly (Van Treeck et al. 2018). Taken together, these features support a model where SGs are formed by the concerted action of phase separation, protein-RNA, protein-protein, and RNA-RNA interactions.

2.2 Stress Granule Structure

SGs are non-membranous structures of the cytoplasm that contain stalled mRNA transcripts, poly(A) mRNAs, microRNAs, translation initiation factors, large and small ribosomal subunit protein components, and a vast network of proteins (Jain et al. 2016; Khong et al. 2017; Markmiller et al. 2018; Namkoong et al. 2018). Recent studies suggest that SGs have a biphasic architecture consisting of a stable core, which is surrounded by a dynamic shell (Fujimura et al. 2009; Souquere et al. 2009; Jain et al. 2016; Wheeler et al. 2016; Markmiller et al. 2018). This architecture is thought to provide multiple levels of functionality within SGs whereby the shell provides a platform for an active exchange of transcripts and protein with the cytoplasm, whereas compartmentalization to the stable core by definition allows for more stable retention (Jain et al. 2016; Protter and Parker 2016; Wheeler et al. 2016; Van Treeck et al. 2018).

The dynamic nature of SG shells has rendered their full isolation and characterization intractable to date. However, stable SG cores have been purified and reveal a vast network of 411 proteins (Jain et al. 2016). These include several RNA-binding proteins, an array of signaling proteins including protein kinases, phosphatases, GTPases, ATPases, adaptor proteins, endoribonucleases, helicases, glycosyltransferases, ubiquitin modifying enzymes, and components of the RNAi machinery (Jain et al. 2016). Building on the methodology of Jain et al., characterization of the SG-core transcriptome revealed that 10–12% of the total mRNA molecules accumulate in SGs (Khong et al. 2017; Namkoong et al. 2018; Matheny

et al. 2019). This recruitment does not appear to be random. The ~185 gene mRNA transcripts that have been identified as most likely to find their way to SGs follow patterns of shared transcript length and translation efficiency and share a handful of specific RNA motifs (Khong et al. 2017; Namkoong et al. 2018; Matheny et al. 2019). Longer mRNAs and ncRNAs, transcripts with lower translation efficiency, and transcripts with RNA sequence motifs such as adenylate-uridylate (AU)-rich element, Pumilio-binding element, and guanylate-cytidylate (GC)-rich element are highly common in SGs (Lin et al. 2007; Khong et al. 2017; Namkoong et al. 2018; Van Treeck et al. 2018; Matheny et al. 2019; Moon et al. 2019). While SG cores induced by different stimuli shared several protein and transcript components, considerable differences were also observed, depending on the specific type of stress (Khong et al. 2017; Namkoong et al. 2018). Thus, the composition of SG cores is specific to the type of stress. Research has yet to illuminate exactly which proteins and transcripts associate with the SG shells, but it is likely that similar to SG cores, they will capture, modify, and exchange proteins and transcripts based on the specific kind of stress that the cell is experiencing.

3 Dysregulated Cancer Signaling and Stress Granule Formation

In vivo, SGs are found in cancer cells of osteosarcomas and tumors of the pancreas and colon but are absent in normal cells from the same tissues (Somasekharan et al. 2015; Grabocka and Bar-Sagi 2016). As previously mentioned, tumors are frequently faced with stress conditions, and perhaps not surprisingly, SGs are often detected in tumor regions experiencing stress. Evidence suggests, however, that the presence of SGs in tumors is not a sole consequence of heightened stress stimuli, but that dysregulation of several signaling pathways also contributes to SG formation. Dysregulated cancer signaling appears to facilitate SG formation in response to stress through promoting translation inhibition and protein-protein interactions important for SG assembly. This section discusses how dysregulated RAS, mTOR, HDAC, glycolytic, and hexosamine biosynthetic pathways can promote SG formation in cancer cells in vitro and in vivo.

3.1 RAS

Mutations in the *RAS* genes (*KRAS*, *NRAS*, and *HRAS*) constitute one of the largest oncogenic alterations in cancer and are present in approximately 30% of all human cancers (Pylayeva-Gupta et al. 2011). Mutant RAS proteins drive several cell functions that support cancer development and progression including cancer cell proliferation, apoptosis, metastasis, metabolism, immune modulation, cancer-

associated fibroblast modulation, and ECM composition and structure modification (Liu et al. 2011; Tao et al. 2014; Dias Carvalho et al. 2018; Yang et al. 2018). Furthermore, accumulating evidence indicates that mutant RAS proteins stimulate stress-adaptive responses, allowing the cell to resist tumor-associated stresses and chemotherapeutic agents (Commisso et al. 2013; Tao et al. 2014; Amaravadi et al. 2016; Yang et al. 2018).

SGs were observed in mutant KRAS pancreatic cancer cells but not in normal pancreas tissue, in both human samples and mouse models of pancreatic cancer (Grabocka and Bar-Sagi 2016). As SGs in this setting were detected in the absence of exogenous stress stimuli, but were present in hypoxic tumor regions, this study first linked SG formation with tumor-associated stresses in vivo. Moreover, the study showed that SGs were present in mutant KRAS pancreatic tumors but not in wild-type (WT) RAS tumors, despite similar levels of hypoxia. This observation indicates a cooperation between mutant KRAS signaling and tumor-associated stresses in SG formation. Of note, SGs were also detected in non-hypoxic regions of mutant KRAS pancreatic tumors, suggesting that mutant KRAS may cooperate with additional stresses in stimulating SG formation. Consistent with this model, mutant KRAS enhanced SG formation in cells exposed to various forms of stress in vitro. These included oxidative stress, proteotoxic stress, UV-C irradiation, and chemotherapeutic agent-induced stress. Mutant KRAS cells also showed a heightened dependence on SGs for survival under stress stimuli, when compared to KRAS-WT cancer cells (Grabocka and Bar-Sagi 2016). As such, inhibition of SG formation in KRAS mutant cells led to higher levels of cell death compared to KRAS-WT cells. Thus, higher cellular levels of SGs may indicate a heightened dependence on SGs for cancer cell survival. An earlier study reported that overexpression of mutant HRAS also stimulated SG formation thus suggesting that all mutant Ras isoforms may be able to stimulate SGs in vivo (Tourriere et al. 2003). With all of this in mind, SGs may be a unique vulnerability that can be exploited for the treatment of all *RAS* mutant tumors, the treatment options for which are currently limited.

Mechanistically, mutant KRAS was shown to stimulate SG formation by enhancing the levels of the prostaglandin 15-deoxy-delta 12,14-prostaglandin J2 (15-d-PGJ2) (Fig. 2) (Grabocka and Bar-Sagi 2016). 15-d-PGJ2 can induce SG formation by inhibiting translation through covalently binding to eIF4A to block its interaction with eIF4G, as well as stimulating eIF2α phosphorylation (Kim et al. 2007; Tauber and Parker 2019). Recently, the nuclear factor erythroid 2-related factor 2 (NRF2) was also implicated in 15-d-PGJ2-mediated SG formation in KRAS mutant pancreatic cancer cells (Mukhopadhyay et al. 2020). The mechanisms through which 15-d-PGJ2-stimulated NRF2 promotes SG formation remain unclear. However, NRF2 regulation of SGs was shown to rely on glutamine, thereby linking KRAS-mediated SG induction to glutamine metabolism.

Mutant KRAS was shown to stimulate 15-d-PGJ2 production through the downstream effector molecules RAF and RALGDS (Grabocka and Bar-Sagi 2016). Signaling from these KRAS effector molecules upregulates15-d-PGJ2 through two different paths. For one, mutant KRAS signaling upregulates cyclooxygenase 2 (COX2), which catalyzes 15-d-PGJ2 synthesis. Secondly, mutant KRAS

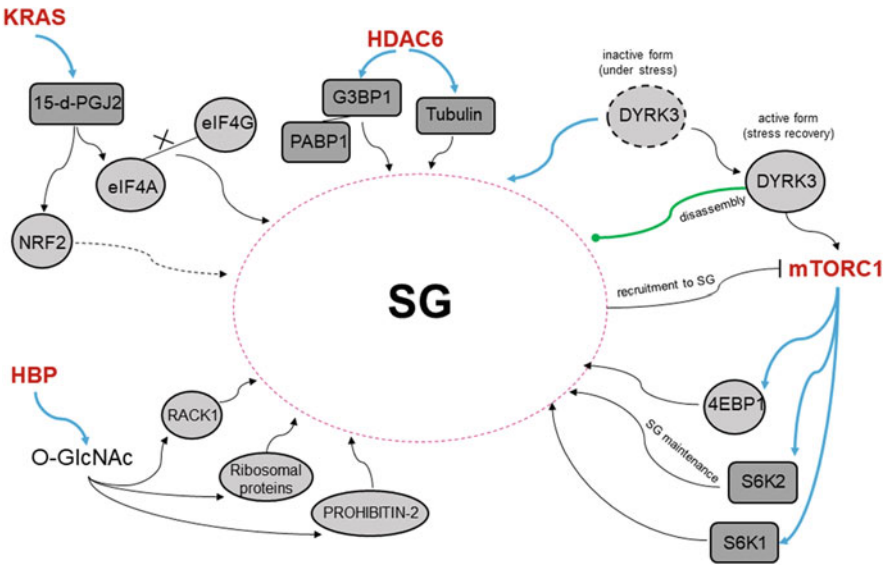

Fig. 2 SG regulation by pro-tumorigenic signaling. Oncogenes (mutant KRAS) and activation of pro-tumorigenic pathways (mTORC1, HDAC6, HBP) promote SG formation through multiple molecular mediators. The SG regulators are involved in induction (blue arrows) or disassembly (green lines) of SGs

downregulates the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (HPGD), which inhibits prostaglandin catabolism.

Interestingly, 15-d-PGJ2 is also secreted from mutant KRAS cancer cells to stimulate SG formation in a paracrine manner (Grabocka and Bar-Sagi 2016). In addition, paracrine stimulation of SG formation by mutant KRAS cancer cells promoted the survival of KRAS-WT cells when exposed to stress stimuli. The observation that mutant KRAS cells can promote survival of KRAS-WT cells through paracrine induction of SG formation is important; it raises the possibility that SG formation serves as a platform for mutant KRAS to promote the stress resistance and survival of the various cell types in the tumor stroma. Coupling this idea with the well-established role of tumor stroma in the development, growth, and drug resistance of mutant KRAS tumors, these findings also highlight the need for a better understanding of how SGs are integrated in KRAS-driven tumorigenesis.

3.2 mTORC1

The mammalian target of rapamycin (mTOR) is a crucial signaling node that regulates cell survival, proliferation, and metabolism (Saxton and Sabatini 2017; Mossmann et al. 2018). mTOR operates in two multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) which have distinct

compositions, functions, and substrate specificities. Both mTORC1 and mTORC2 are commonly hyperactivated in cancer; however, only mTORC1 activity has been linked to SG formation in cancer cells (Guertin and Sabatini 2007). mTORC1 is considered to be an essential factor for cancer metabolism reprogramming and adaptation to cellular stress (Chantranupong et al. 2016; Wolfson et al. 2016; Harachi et al. 2018; Lee et al. 2018). The mTORC1 catalytic complex consists of mTOR and co-factor molecules which include regulatory-associated protein of mTOR (RAPTOR), DEP domain containing mTOR interacting protein (DEPTOR) proline-rich AKT substrate 40 kDa (PRAS-40), FKBP38, and mammalian lethal with Sec13 protein 8 (mLST8). mTORC1 activity is both positively and negatively regulated by components of the catalytic complex.

Several studies have shown that mTORC1 activation in cancer cells can facilitate SG formation (Fig. 2). Inhibition of mTORC1, either genetically through shRNA-mediated downregulation or through pharmacological inhibition of its catalytic activity, impairs SG formation in cancer cells exposed to oxidative or proteotoxic stress (Fournier et al. 2013; Wippich et al. 2013). Similarly, inhibition of mTORC1 activity through the downregulation of RAPTOR also impaired SG formation (Fournier et al. 2013). Furthermore, mTORC1 inhibition impaired the activations of SG-mediated anti-apoptotic pathways under stress conditions (Fournier et al. 2013; Wippich et al. 2013).

While the mechanistic pathways through which mTORC1 facilitates SG formation have not been fully elucidated, two major downstream effector molecules, the ribosomal protein S6 kinase 1 and 2 (S6K1, S6K2) and the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1), have been implicated (Fournier et al. 2013; Sfakianos et al. 2018). Both S6K1 and S6K2 were shown to localize to SGs, and their kinase activity was required for SG formation under conditions of oxidative stress (Sfakianos et al. 2018). Interestingly, S6K1 and S6K2 appear to play distinct roles in SG formation. S6K1 was shown to promote the formation of SGs by regulating eIF2α phosphorylation, whereas S6K2 was required for SG maintenance after assembly (Sfakianos et al. 2018). mTORC1-stimulated 4EBP1 has also been implicated in SG formation under oxidative stress conditions (Fig. 2). In contrast to mTORC1-S6K1 mediated SG formation, the formation of SGs via mTORC1-4EBP1 appears to be peIF2α independent (Fournier et al. 2013). Instead, mTORC1-4EBP1 is thought to promote SGs by impinging on the eIF4E-eIF4GI interaction and translation initiation under stress.

It is somewhat paradoxical for a pathway that is best known for stimulating protein translation to be associated with translation inhibition. A potential explanation may be that under specific stress stimuli, mTORC1 promotes SG formation independent of its effect on protein synthesis. In this context, activation of stress kinases would counter mTORC1 signaling and inhibit translation; this would initiate SG formation which is then aided by the capacity of mTORC1 to promote protein interactions and modifications with roles in SG formation. Once components of the mTORC1 complex and effector molecules are recruited to SGs however, mTORC1 would be prevented from stimulating translation in the cytosolic compartment. In agreement with this model, SGs have been shown to inhibit mTORC1 activity

(Thedieck et al. 2013; Wippich et al. 2013). This is consistent with studies showing that while mTORC1 activation is required for cancer cell survival, chronic hyperactive mTORC1 can lead to apoptosis (Wippich et al. 2013). Thus, by recruiting mTORC1 and inhibiting its cytosolic function, SGs would contribute to cell survival by blunting chronic hyperactivation of mTORC1 (Wippich et al. 2013).

Mechanistically, SGs restrict mTORC1 hyperactivation through sequestering components of the catalytic complex including mTOR and RAPTOR and all three subunits (α-catalytic subunit; β and γ- regulatory subunits) of the upstream activator AMP-activated protein serine/threonine protein kinase (AMPK) (Hofmann et al. 2012; Takahara and Maeda 2012; Thedieck et al. 2013; Wippich et al. 2013). Distinct from mTOR and RAPTOR however, inhibition of AMPK does not impair SG formation in cancer cells. These studies have suggested that AMPK recruitment via interaction with G3BP1 occurs in the later stages of SG formation as a potential mechanism to restrain mTORC1 hyperactivation and promote survival.

Notably, the reactivation of mTORC1 in the recovery phase after stress has subsided has also been linked to SGs. As such, SG disassembly was shown to contribute to the activation of mTORC1 by the dual specificity tyrosine-phosphorylation-regulated kinase 3 (DYRK3) (Wippich et al. 2013). In its inactive state, DYRK3 promotes SG formation and, consequently, the recruitment of mTOR to SGs and inhibition of mTORC1. Stress recovery is associated with DYRK3 activation which stimulates SG dissolution to release mTORC1 components while simultaneously phosphorylating and inhibiting the mTORC1 inhibitor PRAS40. Altogether, these studies indicate that SG formation contributes to the inactivation of mTORC1 during oxidative stress, whereas SG dissolution contributes to the necessary reactivation during stress recovery.

3.3 Glycolysis and the Hexosamine Biosynthetic Pathway

It is well established that cancer cells alter their metabolism to derive energy from glycolysis instead of mitochondrial oxidative phosphorylation and utilize more glucose than normal cells (Ganapathy-Kanniappan and Geschwind 2013; Pavlova and Thompson 2016). Whereas the majority of glucose that enters the cell proceeds to glycolysis for ATP generation, the remaining glucose enters the hexosamine biosynthetic pathway (HBP) and along with glutamine, glucosamine, and acetyl-coenzyme A is utilized to generate the amino sugar uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) (Akella et al. 2019). Both glycolysis and the HBP pathway are known to promote SG formation, indicating that enhanced glycolytic and HBP flux may contribute to the formation of SGs observed in tumors (Fig. 2) (Jain et al. 2016).

A proteomic analysis of mammalian stress granule cores in cancer cells revealed a large number of proteins with ATPase activity as components of SGs (Jain et al. 2016). This study also showed that ATP is required for both SG assembly and dynamics. Whether specific ATPases are required for these processes remains to be

elucidated (Jain et al. 2016). However, given that cancer cells have a preferential reliance on glycolysis for ATP production, inhibition of glycolysis might be expected to impair SG formation in cancer cells. This prediction is supported by evidence that blocking the glycolytic pathway impaired SG formation in sarcoma cells (Jain et al. 2016). Glycolytic inhibitors, therefore, may be a promising strategy to inhibit SGs in vivo.

The final product of the HBP pathways, UDP-GlcNAc, is critical for the generation of metabolic intermediates as well as for the glycosylation of proteins; UDP aids in the N-linked and O-linked glycosylation of proteins in the ER and Golgi and in the O-Linked N-Acetylglucosamine (O-GlcNAc) modification of nuclear and cytoplasmic proteins by OGT (O-GlcNAc transferase) (Akella et al. 2019). Several studies support a role for glycosylation in tumorigenesis, and changes in protein glycosylation have been observed in several cancers including those of the pancreas, colon, melanoma, lung, liver, and prostate (Sharma et al. 2016). One proposed mechanism through which altered protein glycosylation contributes to cancer progression is through enabling cancer cells to cope with stress. Stress stimuli have been shown to enhance protein O-GlcNAc modifications, and oncogenic pathways such as PI3K-mTOR-MYC and MEK/ERK signaling pathways can stimulate OGT activity and O-GlcNAc modifications to enhance cell survival under stress (Sohn et al. 2004; Zachara et al. 2004; Taylor et al. 2008; Ferrer et al. 2014; Sodi et al. 2015; Zhang et al. 2015; Katai et al. 2016).

An RNA-mediated interference-based screen in osteosarcoma cells identified the HBP pathway and O-GlcNAc protein modifications as critical to SG formation, thereby implicating SGs as a potential mechanism through which enhanced HBP flux promotes tumorigenesis (Ohn et al. 2008). Knockdown of either sortilin (transmembrane protein that regulates the vesicular transport of the GLUT4 glucose transporter and glucose uptake), GFAT2 (glutamine: fructose 6 phosphate amidotransferase 2), or OGT inhibited SG formation in cancer cells but had no effect on eIF2α phosphorylation. Thus, the HBP pathway acts independently of peIF2α in inducing SGs in cancer cells. Instead, HBP-dependent SG formation is mediated by O-GlcNAc modification of receptor for activated C kinase 1 (RACK1), PROHIBITIN-2, and several ribosomal proteins, which promotes their aggregation into SGs (Ohn et al. 2008). In addition to components of the translational machinery, the HBP pathway regulates multiple proteins with established roles in SG formation (mTOR and AMPK) and can be manipulated by endogenous metabolites (glutamine) and oncogenic signaling pathways implicated in SG formation (PI3K and RAS/RAF). It remains to be elucidated how these signals integrate in vivo to promote SG upregulation. Nonetheless, given the role of the HBP pathway in SG formation, it is a promising therapeutic target for inhibiting SG formation in cancer.

3.4 HDAC6

Histone acetylation and deacetylation is an important posttranslational mechanism that regulates gene expression. Histone deacetylase (HDAC) proteins are key enzymes that regulate acetylation levels. Within the HDAC family, HDAC6 is unique as it determines the acetylation status of not only histones but also of several non-histone substrates such as dynein, α-tubulin, cortactin, and HSP90 (Li et al. 2018). HDAC6 is overexpressed in melanoma, lung, pancreas, breast, and bladder cancer and is thought to promote tumorigenesis by regulating cancer cell proliferation, metastasis, and immune regulators through both its histone and non-histone substrates (Lee et al. 2008; Wickstrom et al. 2010; Lafarga et al. 2012; Woan et al. 2015; Li et al. 2018). HDAC6 was shown to be a stable integral component of SGs and critical to SG formation in cancer cells under various stress stimuli including oxidative stress, UV irradiation, mitochondrial stress, or heat shock (Kwon et al. 2007). Mechanistically, HDAC6 is thought to promote SG formation through deacetylating G3BP1 which stimulates its RNA-dependent interaction with PABP1, a key component and regulator of SG assembly (Fig. 2) (Gal et al. 2019). In addition, the interaction of HDAC6 with dynein and microtubules has also been suggested to promote SG formation, potentially through mediating mRNP translocation to SGs (Kwon et al. 2007).

4 Stress Granules and Cancer Hallmarks

Significant amount of evidence supports a role for SGs in tumorigenesis. However, the cancer hallmarks impacted by SGs, and the mechanisms through which they do so, remain to be fully understood. This section discusses the role of SGs in the pathogenesis of cancer and the evidence linking SGs to cancer cell proliferation, metastasis, and survival and chemotherapy resistance.

4.1 Stress Granules and Proliferation

Accumulating evidence indicates that SG formation is linked to the proliferative status of cells. One such example is cellular senescence, which has been shown to have a profound effect on the cellular capacity to form SGs. Cellular senescence is a cytostatic program that can be triggered by multiple mechanisms. These include (a) telomere attrition that occurs with replicative "aging" of cells – known as replicative senescence – and (b) exposure to exogenous agents that induce DNA damage (oxidative stress, chemotherapy, UV light), which is referred to as stress-induced premature senescence and leads to cell-cycle arrest via the activation of the DNA damage response (DDR). Lastly, acquisition of oncogenic mutations can lead

to oncogene-induced senescence, which can also be mediated by DDR (Campisi and d'Adda di Fagagna 2007; Campisi 2013).

As a cell cycle-arrest program, senescence functions as a tumor-suppressive mechanism. However, senescent cells can also contribute to the generation of a tumor-promoting microenvironment via the secretion of several cytokines and chemokines (senescence-associated secretory phenotype; SASP) that promote cell cycle progression, de-differentiation, and metastasis. In in vitro models of stress-induced premature senescence, transition from the proliferative state to pre-senescence and senescence correlated with a progressive impairment of the cellular capacity to form SGs (Lian and Gallouzi 2009; Moujaber et al. 2017; Omer et al. 2018). These observations raised the possibility that SGs may play a role in preventing cells from exiting cell cycle and entering senescence. Mechanistically, senescence-dependent SG impairment was shown to be driven by the depletion of the transcription factor specific protein 1 (SP1) which regulates the expression of the SG-nucleating proteins G3BP1, TIA-1/TIAR, eIF4G, hnRNPK, and HuR (Moujaber et al. 2017). Consistent with a role for SGs in inhibiting senescence, a study showed SG inhibition via G3BP1 knockdown promoted stress-induced cellular senescence (Omer et al. 2018). SGs therefore appear to inhibit cellular senescence, while acquisition of the senescent phenotype on the other hand suppresses SGs. This is consistent with the tumor suppressive role of senescence and the function of SGs as a cytoprotective mechanism that can promote tumorigenesis. It should be noted, however, that PAI-1 – a marker of senescence as well as a downstream effector and key component of SASP – is recruited to SGs (Omer et al. 2018). As translocation to SGs would prevent the secretion of PAI-1, SGs in this setting could, in theory, function to suppress SASP and the associated tumor-promoting activity. This raises a note of caution regarding SG inhibition for the treatment of cancer; while inhibition of SGs could push cells toward senescence and, thus, halt tumor growth, the lack of SGs in senescent cells could also aid tumorigenesis by contributing to SASP.

Several proteins and mRNAs that carry out cell division localize to SGs in cancer cells; this observation supports the idea that SGs play a role in coordinating cell proliferation. RBFOX2 is a member of the RBFOX family of proteins that regulate alternative pre-mRNA splicing and mRNA stability (Jin et al. 2003; Lovci et al. 2013). Under stress conditions, RBFOX2 is recruited to SGs via its RNA-binding domain and preferentially binds cell cycle-related mRNAs including retinoblastoma 1 (*RB1*) mRNA (Park et al. 2017). RB1 is a negative cell cycle regulator, and excess RB1 arrests cells in G1. This study proposed that SGs promote cell cycle progression via RBFOX2-mediated recruitment and inhibition of *RB1* mRNA translation (Choi et al. 2019). Recruitment of mRNA transcripts encoding for proteins involved in proliferation appears to be a general theme of SGs, as gene enrichment analysis of mRNAs in SG cores demonstrated that proto-oncogene transcripts (e.g., *ABL2*, *PDGFRA*, *GSK3B*, *RUNX1*, *AKAP11)* were highly enriched (Namkoong et al. 2018). Importantly, this study showed that while distinct stresses showed differences in the variety of mRNAs that were preferentially recruited to SGs, enrichment of proto-oncogene transcripts was shared across stress types. Given that proto-

oncogene transcripts are rich in adenylate-uridylate (AU) sequences and consequently subject to mRNA processing and degradation, it has been suggested that recruitment to SGs may promote their stability, expression, and function to promote tumorigenesis (Namkoong et al. 2018). While several lines of evidence support a role for SGs in cancer cell proliferation under stress, studies also indicate that transcripts of both negative and positive regulators of proliferation are recruited to SGs. In addition, several of the protein components of SGs are involved in both negative and positive proliferation pathways. It is not clear how SGs would support cell proliferation by capturing proteins and mRNAs with seemingly opposite functions. It is possible that these components may be recruited at different levels relative to one another and that the sum of all parts ultimately favors proliferation.

In principle however, depending on cell intrinsic and extrinsic stimuli, the cellular levels of either positive or negative regulators of proliferation, as well as the levels at which they are recruited to SGs relative to one another, can shift. In addition, these transcripts could also be differentially modified through interactions with SG components with roles in mRNA processing and stability. As such, there is perhaps a context specific and dynamic balance between proliferative and anti-proliferative components of SGs. This would suggest that the impact of SGs on proliferation could also depend on context. With this in mind, it is interesting that primary osteosarcoma tumors where SGs were downregulated by shRNA-mediated knockdown of G3BP1 showed no difference in proliferation rate compared to control. Further studies are needed to understand whether this is specific to osteosarcoma or a shared phenotype of all tumors. However, this study may indicate that in the context of tumorigenesis, SGs may aid proliferation in later stages of tumor development or in specific cancer cell subclones, perhaps when a specific threshold of SG formation and SG signaling output is reached.

4.2 Stress Granules and Suppression of Cell Death

The relationship between stress granules and cell death is perhaps the earliest studied function of SGs. A number of in vitro studies demonstrate that SGs block the cellular apoptotic machinery that is triggered by stress stimuli in cancer cells, and several SG-mediated anti-apoptotic pathways have been defined. In addition, numerous studies have also shown that SGs are critical determinants of the sensitivity of cancer cells to chemotherapeutic agents.

4.2.1 Stress Granule-Mediated Suppression of Stress-Induced Apoptosis

SGs control live-or-die cell fate decisions along two broad paths. The first is through the sequestration of pro-apoptotic factors, limiting their activity at their target locations. Secondly, SGs curb the production of reactive oxygen species, limiting

apoptosis-inducing stress stimuli and cell damage. As discussed above, recruitment of components of the mTORC1 complex allows SGs to prevent mTORC1-hyperactivation-induced apoptosis in cancer cells. In addition, Arimoto et al. reported that SGs inhibit apoptosis by preventing p38 and JNK activation (Arimoto et al. 2008). Specifically, under oxidative stress conditions, SGs recruit RACK1 thus preventing its interaction with the MAPK kinase MTK1, which is required for p38/pJNK mediated apoptosis of cervical cancer cells (Arimoto et al. 2008). The coiled coil containing protein kinase (ROCK1) is another activator of JNK that is recruited to SGs (Tsai and Wei 2010). Sequestration of ROCK1 to SGs in cancer cells prevents the ROCK1-mediated phosphorylation of the JNK-interacting protein 3 (JIP)-3, thus inhibiting JNK activation and the induction of JNK-mediated apoptosis. In addition, translocation of TRAF2 to SGs inhibits TNF-mediated activation of nuclear factor (NF)-kB and apoptosis (Kim et al. 2005). Studies in non-cancerous cells show that SGs can recruit arginylated calreticutin to prevent its translocation to the plasma membrane and apoptotic function during stress; whether this mechanism also occurs in cancer cells remains to be determined (Lopez Sambrooks et al. 2012). More recently, it was demonstrated that macrophages utilize the SG translocation of DEAD-box helicase 3 X-linked (DDX3) to inhibit NLRP3 inflammasome activation (Samir et al. 2019). Activation of the NLRP3 inflammasome induces the secretion of pro-inflammatory cytokines and pyroptosis – a form of inflammatory cell death (Samir et al. 2019). Given the role of macrophages in driving tumor progression, it is tempting to speculate that SGs may promote tumorigenesis by preventing macrophage pyroptosis.

SGs have been shown to reduce ROS levels and ROS-dependent apoptosis; however, the mechanisms behind the antioxidant activity of SGs are not fully elucidated (Takahashi et al. 2013). One study identified an antioxidant function of the ubiquitin-specific peptidase 10 (USP10) and proposed that SGs may reduce ROS levels by facilitating the activation of USP10. It is currently unknown, however, how SGs may promote the antioxidant function of USP10 and how USP10 functions as an antioxidant (Takahashi et al. 2013). As discussed above, the NRF2 antioxidant pathway has been shown to promote SG formation. Given the role of SGs in regulating ROS levels, it would be interesting to determine whether SGs also impact the antioxidant activity of NRF2 (Mukhopadhyay et al. 2020).

4.2.2 Stress Granules and Chemotherapy Resistance

Studies in various in vitro models have explored the relationship between SGs and cancer cell resistance to chemotherapy. These studies have shown that chemotherapeutic agents including bortezomib, cisplatin, etoposide, oxaliplatin, paclitaxel, and sorafenib induce SG formation. A comprehensive review of these studies has been recently published (Gao et al. 2019). This section highlights the most salient aspects of SG-mediated drug resistance.

A shared feature of all chemotherapeutic agents that drive SG formation is that they do so by inducing the phosphorylation of eIF2α. The exact kinases responsible

for eIF2α phosphorylation, however, differ across agents. Sorafenib is a Raf1/Mek/Erk kinase inhibitor that is FDA approved for the treatment of patients with advanced hepatocarcinoma (HCC), renal carcinoma, and metastatic, progressive, and differentiated thyroid carcinoma refractory to iodine treatment. Sorafenib has been shown to induce SGs (Lin et al. 2012; Adjibade et al. 2015). Further studies showed that sorafenib treatment lead to the activation of the unfolded protein response (UPR) and induced SG formation via PERK-mediated phosphorylation of eIF2α (Adjibade et al. 2015; Pakos-Zebrucka et al. 2016; Feng et al. 2017).

Bortezomib is a proteasome inhibitor that is FDA approved for the treatment of multiple myeloma and mantle cell myeloma. Bortezomib treatment induced SGs in cancer cells of the colon, lung, cervix, head, and neck via HRI-mediated phosphorylation of eIF2α (Fournier et al. 2010; Kaehler et al. 2014; Burwick and Aktas 2017). Furthermore, bortezomib-induced SGs were shown to recruit and promote the degradation of transcripts of the cyclin-dependent kinase inhibitor p21 (WAF1/CIP1). As p21 is a protein that promotes cell cycle arrest and apoptosis, it was proposed that bortezomib-induced SGs lead to apoptosis inhibition and treatment resistance through downregulating p21.

Chemotherapeutic agents such as 5-fluorouracil (5-FU) cisplatin, etoposide, or oxaliplatin – which are used for the treatment of several cancers including colorectal, pancreas, breast, and head and neck – have been shown to induce SGs. 5-FU induces SG assembly by stimulating PKR-mediated phosphorylation of eIF2α (Kaehler et al. 2014). In all reported instances, SG formation in response to chemotherapeutic agents functioned as a mechanism of resistance to chemotherapy-induced cell death. In addition, inhibition of SGs, or of the kinases responsible for peIF2-α-mediated SG formation, sensitized cancer cells to chemotherapeutic agents.

Taken together these studies suggest that the blockage of SG formation would enhance chemotherapy cancer treatment. In addition, tumors driven by oncogenic pathways that stimulate SGs such as mutant RAS are well documented as refractory to chemotherapy. The capacity of these pathways to stimulate SGs therefore may also provide mechanistic insight into chemotherapy resistance and identify patients that could most benefit from anti-stress granule therapy.

4.3 Stress Granules and Tumor Metastasis

Invasion of local tissue and spread to distant sites to form metastases is a central feature of cancer and the primary cause of death for >90% of cancer patients (Hanahan and Weinberg 2011). Understanding the biological mechanisms of the metastatic process is crucial in finding successful therapeutic opportunities. The development of metastasis is a complex process that requires cancer cells to leave the local environment, circulate in the bloodstream, and acclimatize and survive the new environment of a secondary site. Consistent with the idea that highly metastatic cells utilize SGs for migration and survival, SGs have been observed in disseminated tumor cells isolated from the bone marrow specimens of breast cancer patients

(Bartkowiak et al. 2015). In addition, Somasekharan et al. demonstrated that the metastatic potential of osteosarcoma cells is linked to SG formation (Somasekharan et al. 2015). SG inhibition by shRNA-mediated knockdown of G3BP1 led to an impairment of the invasive and metastatic potential of sarcoma cells in vivo. Formation of SGs in this study was linked to the upregulation of YB-1, which can directly bind to the 5' UTR of G3BP1 mRNA to upregulate its translation. In agreement with these observations, a class I HDAC inhibitor suppressed sarcoma metastasis by enhancing YB-1 acetylation, which blocked the interaction of YB-1 with its mRNA target G3BP1, and downregulated G3BP1 levels and SG formation (El-Naggar et al. 2019). While the mechanisms through which SGs promote invasion and metastasis were unexplained in this study, the authors raised the possibility that SGs might sequester mRNAs encoding for proteins that inhibit invasion and metastasis. Sequestration of these mRNAs to SGs would prevent synthesis of the proteins they encode, thus enhancing the cellular capacity to invade and metastasize. In addition, based on the observation that G3BP1 knockdown reverted the growth pattern of primary tumors to noninvasive borders, this study proposed that SGs facilitate invasive capacity by selectively releasing mRNAs that encode matrix-degrading enzymes for translation.

Other studies suggest that SGs promote metastasis via inhibiting the ribonuclease inhibitor 1 (RNH1) which promotes metastasis through stimulating the activity of angiogenin (Pizzo et al. 2013). RNH1 is a component of SGs, and downregulation of RNH1 promoted migration and metastasis (Pizzo et al. 2013; Yao et al. 2013). Recruitment of RBFOX2 to SGs has also been shown to promote metastasis of melanoma cells to the lung as inhibiting the localization of RBFOX2 to SGs diminished lung metastasis in a mouse model (Choi et al. 2019). It is currently unknown how RBFOX2 recruitment to SGs may promote metastasis, but selective recruitment or exclusion of mRNAs encoding proteins, which inhibit or promote metastasis respectively, have been proposed as potential mechanisms (Choi et al. 2019). Another study in pancreatic cancer cells proposed that SGs may be implicated in the degradation of mRNA transcripts encoding for Binder of Arl Two (BART), which impairs cell invasion and metastasis by inhibiting ARL2-mediated activation of the RHO small GTPase, which is a key mediator of cell migration and metastasis (Taniuchi et al. 2011a, b). Although direct evidence that SGs contribute to *BART* downregulation is lacking, given that BART can be degraded by G3BP1, it is possible that SG formation enhances the interaction of *BART* mRNA with G3BP1 as well as *BART* degradation to facilitate cell invasion. Studies in noncancerous cells also showed that RHO is both a component and a mediator of SG formation, suggesting that a potential mechanism through which RHO promotes metastasis may be through SG formation (Tsai and Wei 2010). Taken together these studies indicate that while multiple lines of evidence point to a role of SGs in metastasis, further work is needed to identify and characterize the molecular mediators through which SGs may support this process.

5 Concluding Remarks

Stress adaptation, driven by dysregulated cancer signaling, is a fundamental property of cancer that has yet to be fully elucidated. As reviewed here, evidence from multiple in vitro and in vivo models indicates that oncogenic mutations and dysregulated signaling pathways in cancer modify the canonical molecules that regulate SG formation. By doing so, cancer cells take advantage of SG formation to enhance stress adaptation.

Oncogenic Ras mutations, hyperactivation of mTORC1 and HDAC, and dysregulation of glycolytic and hexosamine biosynthetic pathways have emerged as key pathways that stimulate SG formation in cancer cells. However, the full scope of oncogenic signaling pathways that may regulate SG formation remains to be established and may include a broader signaling network than is currently known. A recent study indicated that mutations in the E3 ubiquitin ligase binding adaptor SPOP1, which occur in ~15% of primary prostate cancers, led to enhanced SG formation in prostate cancer cells in vitro (Shi et al. 2019). As such, prostate cancers with SPOP1 mutations may be another example of tumors with enhanced SG formation and stress adaptation. Additional metabolic processes may also play an important role in SG formation in cancer. Glutamine deprivation was shown to impact SG formation in pancreatic cancer cells (Mukhopadhyay et al. 2020). However, cancer cells are depleted of several non-essential amino acids with roles in purine/pyrimidine synthesis, protein translation, and glutathione regulation which can impact translation inhibition and cellular stress and consequently SG formation. Lastly, protein levels of SG nucleators are upregulated in several tumors compared to normal tissue raising the possibility that higher levels of free SG-nucleator proteins in cancer may also amplify SG formation (French et al. 2002; Wang et al. 2018).

The initial view that SGs function solely to store RNA has been offset by a wealth of data that link SGs to several signal transduction and gene expression regulation pathways. In addition, it has been clearly demonstrated that the composition of SGs can vary significantly depending on the type of stress and tissue. The model that has emerged from these studies is that SG levels, composition, and dynamics determine their signaling output. As such, current studies aimed at understanding the role of SGs in cancer and their molecular mediators must address their context-dependent specificities and relevance.

Much remains to be learned about the cellular processes that SGs regulate in cancer and how they impact tumorigenesis. In addition, it still remains to be understood whether SGs are a feature of all tumors or only those of specific tissues (e.g., pancreatic cancer, osteosarcoma). As stress and the dysregulated signaling pathways described here are common in cancer, the expectation would be that SGs may also be a shared feature for most types of tumors. In the same vein, mTORC1, HDAC, and metabolic pathways are often dysregulated in the tumor stroma which is also exposed to stress stimuli. In addition, evidence that mutant KRAS can promote SG formation in a paracrine manner suggests that cancer cells may also instruct SG

formation in the tumor stroma (Grabocka and Bar-Sagi 2016). The question that inevitably arises is whether SGs are present in the tumor stroma and does this impact tumorigenesis. It is also important to note that SGs are part of a larger group of stress-adaptive organelles that are hijacked by cancer cells under stress including macropinosomes, autophagosomes, and lysosomes (Commisso et al. 2013; Perera et al. 2015; Amaravadi et al. 2016). In yeast, SGs are cleared by autophagy, and evidence suggests that targeting of SGs to degradative organelles by autophagy may be conserved in mammalian cells (Buchan et al. 2013; Ryu et al. 2014; Marrone et al. 2018). This raises the possibility that SGs may interact and integrate with other stress-adaptive organelles in cancer. Such interactions could have important implications for the stress adaptation of cancer cells and tumor progression. Future studies aimed at answering these questions can provide important insight into the role of SGs in tumorigenesis.

Given the evidence that SGs may play an important role in tumorigenesis, it will be essential to develop animal models that assess their tumor-relevant functions. Development of tools for in vivo imaging of SGs in such models may allow visualization of the context-dependent specificities of their formation. Another current challenge is the lack of specific SG inhibitors. Current pharmacological agents that inhibit SGs have broad effects. Additionally, genetic inhibition of SG formation is usually achieved through targeting one or more SG nucleators which, generally, have functions beyond SGs. The understanding of specific interactions or modifications that determine the SG-nucleating capacity of these molecules is critical for the development of tools that allow for the distinction of their SG-specific function and roles in tumorigenesis. The exciting work that lies ahead to fully elucidate the function of SGs in tumorigenesis also has promising therapeutic prospects. Given the well-documented roles of SGs in the chemotherapeutic response, the development of anti-SG therapies has the potential to provide efficacious treatment modalities for cancer patients.

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Lipid Droplets in Cancer

Toni Petan

Contents

Abstract Lipid droplets have a unique structure among organelles consisting of a dense hydrophobic core of neutral lipids surrounded by a single layer of phospholipids decorated with various proteins. Often labeled merely as passive fat storage repositories, they in fact have a remarkably dynamic life cycle. Being formed within the endoplasmic reticulum membrane, lipid droplets rapidly grow, shrink, traverse the cytosol, and engage in contacts with other organelles to exchange proteins and lipids. Their lipid and protein composition changes dynamically in response to cellular states and nutrient availability. Remarkably, their biogenesis is induced when cells experience various forms of nutrient, energy, and redox imbalances, including lipid excess and complete nutrient deprivation. Cancer cells are continuously exposed to nutrient and oxygen fluctuations and have the capacity to switch between alternative nutrient acquisition and metabolic pathways in order to strive

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even during severe stress. Their supply of lipids is ensured by a series of nutrient uptake and scavenging mechanisms, upregulation of de novo lipid synthesis, repurposing of their structural lipids via enzymatic remodeling, or lipid recycling through autophagy. Importantly, most of these pathways of lipid acquisition converge at lipid droplets, which combine different lipid fluxes and control their usage based on specific cellular needs. It is thus not surprising that lipid droplet breakdown is an elaborately regulated process that occurs via a complex interplay of neutral lipases and autophagic degradation. Cancer cells employ lipid droplets to ensure energy production and redox balance, modulate autophagy, drive membrane synthesis, and control its composition, thereby minimizing stress and fostering tumor progression. As regulators of (poly)unsaturated fatty acid trafficking, lipid droplets are also emerging as modulators of lipid peroxidation and sensitivity to ferroptosis. Clearly, dysregulated lipid droplet turnover may also be detrimental to cancer cells, which should provide potential therapeutic opportunities in the future. In this review, we explore how lipid droplets consolidate lipid acquisition and trafficking pathways in order to match lipid supply with the requirements for cancer cell survival, growth, and metastasis.

Keywords Autophagy · Cancer · Fatty acid · Ferroptosis · Lipid droplets · Metabolism · Stress

1 Introduction

The recently revived interest in cancer metabolism has resulted in the recognition of metabolic reprogramming as one of the major cancer hallmarks (Hanahan and Weinberg 2011). Moving forward from glucose and the classical Warburg effect, recent discoveries have shown that the metabolism of amino acids and lipids is also critical for tumorigenesis (Pavlova and Thompson 2016; Röhrig and Schulze 2016; Ward and Thompson 2012). Additionally, we are now aware that different tumors, and even cells within an individual tumor, display specific metabolic characteristics but also a remarkable metabolic plasticity that enables their adaptation to adverse conditions and drives their malignant potential (Hanahan and Weinberg 2011). However, even genetically distinct cancer types encounter similar stress conditions in the tumor microenvironment and may thus have common metabolic vulnerabilities that present unique therapeutic opportunities (Martinez-Outschoorn et al. 2017). In our quest for new cancer treatments, it is therefore imperative to discover the context-specific responses of cancer cells to nutrient and oxidative fluctuations and thereby expose their metabolic weaknesses.

The roles of lipids in cancer extend well beyond their typically ascribed roles in membrane biogenesis and energy production (Beloribi-Djefaflia et al. 2016; Röhrig and Schulze 2016). In fact, even these seemingly simple roles, as membrane building

blocks and energy-rich substrates, are far from being understood at the molecular and functional level. Moreover, we are only beginning to understand the distinct functions of individual species within the enormous variety of lipids and the intricacies of their collective effects in cell metabolism and signaling. The roles of individual lipids are intrinsically tied to the cooperative nature of lipid assemblies, whose function depends on their specific lipid composition and its dynamic changes at particular subcellular locations. Lipid droplets are emerging as novel regulators of many of these processes. These unique and remarkably dynamic organelles respond to nutrient fluctuations and various microenvironmental stress conditions to control the trafficking, storage, and use of lipids for a variety of purposes in the cell (Farese and Walther 2009; Jarc and Petan 2020; Koizume and Miyagi 2016; Krahmer et al. 2013; Olzmann and Carvalho 2019; Petan et al. 2018). They are readily available sources of fatty acids (FAs), sterols, and vitamins that are rapidly released on demand and under specific conditions. These lipids and their metabolites participate in and regulate multiple metabolic and signaling pathways within the cell and in the extracellular space, thereby affecting major cancer hallmarks, including cell growth, proliferation, metabolism, migration, inflammation, and immunity (Attané and Muller 2020; den Brok et al. 2018; Cruz et al. 2020; Currie et al. 2013; Koizume and Miyagi 2016; Petan et al. 2018; Tirinato et al. 2017). Moreover, lipid droplets also participate in the cellular trafficking and quality control of proteins, thereby affecting protein turnover, gene transcription, nuclear function, and various homeostatic and stress responses. Lipid droplets even manage the secretion of proteins that act as danger signals and activate immune cell responses and inflammatory pathways (Veglia et al. 2017; Jarc and Petan 2020). These fat-laden organelles also affect drug efficacy by altering the cellular distribution and activation of lipophilic anti-cancer agents (Dubey et al. 2020; Englinger et al. 2020).

Alterations in lipid droplet metabolism are emerging as important parts of cancer metabolic reprogramming. Their biogenesis and breakdown may either help cancer cells in their constant fight against stress or promote their demise. In this review, we focus on the mechanisms that govern lipid droplet function in response to nutrient and oxygen imbalances. We explore how these highly dynamic organelles consolidate lipid uptake, synthesis, recycling, distribution, and breakdown in order to match these entangled lipid fluxes with the requirements for cancer cell survival, growth, and metastasis.

2 Lipid Droplets Are Dynamic Organelles

2.1 Lipid Droplets Are Versatile Ensembles of Lipids and Proteins

Lipid droplets have a unique structure among organelles with a hydrophobic core consisting of neutral lipids surrounded by a single layer of phospholipids decorated

with various proteins (Henne et al. 2018; Olzmann and Carvalho 2019; Walther et al. 2017). Their neutral lipid core stores lipids primarily in their esterified, storage form, e.g., FAs as triacylglycerols (TAGs), cholesterol and other sterols in the form of sterol esters, retinoic acids as retinyl esters, and ceramides esterified into acyl ceramides (Jarc and Petan 2020; Molenaar et al. 2017; Senkal et al. 2017; Thiam and Beller 2017). Lipid droplets from different cells and tissues may display significant differences in the relative proportions of these major lipid species, often reflecting tissue-specific functions and storage requirements. By regulating the storage and release of these various lipids, lipid droplets have a direct impact on their involvement in processes essential for cell survival, growth, and proliferation, including energy production, membrane and organelle biogenesis, cell signaling, and gene transcription.

The lipid droplet proteome in mammalian cells contains approximately 150 proteins and includes proteins involved in lipid metabolism and signaling, redox metabolism, autophagy, gene transcription, ubiquitination, membrane trafficking, and immunity (Bersuker and Olzmann 2017; Bersuker et al. 2018). Many among these lipid droplet-associated proteins have unknown functions, whereas some have known roles in processes as yet unrelated to lipids or lipid droplets. In most cases, the functional importance of their lipid droplet localization is unknown. Furthermore, in some instances, the sequestration of proteins to the lipid droplet surface is a mechanism of control of their involvement in processes occurring at other cellular locations. For example, lipid droplets sequester histones, transcription factors (e.g., NFAT5), and chaperones (e.g., Hsc70 and calreticulin), thereby affecting gene transcription, protein quality control, and immune cell function (Cotte et al. 2018; Gallardo-Montejano et al. 2016; Johnson et al. 2018; Ueno et al. 2013; Veglia et al. 2017; Welte and Gould 2017).

Importantly, the lipid and protein composition of lipid droplets, as well as their size, number, localization, and mobility in the cell, change rapidly in response to cellular states and nutrient availability (Bosch et al. 2020; Herms et al. 2013, 2015; Thiam and Beller 2017). For example, a surge of FAs leads to a rapid activation of TAG synthesis and lipid droplet biogenesis in most cells. This process occurs with a remarkable efficiency within seconds to minutes following FA exposure, whereby the latter may be incorporated into both pre-existing lipid droplets and/or into newly emerging ones (Kassan et al. 2013; Kuerschner et al. 2008). On the contrary, FA and glucose depletion leads to rapid mobilization and redistribution of lipid droplets in the cell, thereby increasing their contacts with the mitochondrial network to couple lipolytic FA release from stored TAGs with mitochondrial FA intake and energy production (Herms et al. 2015; Rambold et al. 2015). However, paradoxically, mitochondria–lipid droplet contacts may also drive TAG synthesis and lipid droplet expansion (Benador et al. 2019). As discussed in this review, the highly dynamic nature of lipid droplet metabolism and its interactions with other organelles endows cells with multiple layers of flexibility, which is often exploited by cancer cells for protection against various stresses.

2.2 *Lipid Droplet Biogenesis Occurs at the Crossroads of Membrane and Neutral Lipid Metabolism*

The life cycle of the lipid droplet is tightly linked to its mother organelle, the endoplasmic reticulum (ER). TAG synthesis is a prerequisite for de novo lipid droplet formation and occurs between the two leaflets of the ER membrane by sequential addition of FAs to a glycerol backbone, catalyzed by a series of acyltransferase enzymes (Coleman and Mashek 2011). Importantly, the first several steps of the process are common to both phospholipid and TAG synthesis, enabling the cell to rapidly switch between phospholipid and neutral lipid production. This is essential for many aspects of the cellular stress response because it allows, for example, a shift from cell growth and proliferation during nutrient abundance, when the needs for membrane biogenesis are high, to quiescence during starvation, when lipids are syphoned into storage for later use (Bosch et al. 2020; Henne et al. 2018; Natter and Kohlwein 2013). The dephosphorylation of phosphatidic acid into diacylglycerol (DAG) by phosphatidate phosphatases, also called lipins (Zhang and Reue 2017), is the branching-off point between these two pathways and is immediately followed by the last step in TAG biosynthesis: the conversion of DAG into TAG catalyzed by diacylglycerol acyltransferases (DGATs). Cholesteryl ester synthesis also occurs within the ER membrane and is mediated by acyl-coenzyme A: cholesterol acyltransferase (ACAT) enzymes (Chang et al. 2009).

The newly synthesized neutral lipids accumulate in growing lipid "lenses" within the bilayer, eventually giving rise to nascent lipid droplets that bud from the ER membrane and are released into the cytosol (Salo and Ikonen 2019). The budding process is guided by proteins recruited to the nascent droplet, such as the ER membrane protein seipin that is essential for stabilization and growth of the droplet, and requires a particular rearrangement of membrane lipids that drives membrane bending and asymmetrical budding into the cytosol (Chorlay et al. 2019; Henne et al. 2018; Olzmann and Carvalho 2019; Thiam and Beller 2017). Several pathways of phospholipid synthesis and remodeling may contribute to these lipid rearrangements and enable membrane expansion to provide sufficient cover for the growing lipid droplet (Bosch et al. 2020; Penno et al. 2012). Remarkably, some components of the lipid droplet biogenesis machinery required for phospholipid and neutral lipid synthesis are transferred to the nascent lipid droplet and enable its growth independently of the ER (Krahmer et al. 2011; Wilfling et al. 2013). However, lipid droplets may also grow by fusion, and they form transient contacts with the ER, mitochondria, and other organelles, via protein tethers and membrane bridges, thereby allowing bidirectional lipid and protein transfer (Barbosa and Siniossoglou 2017; Bohnert 2020; Schuldiner and Bohnert 2017).

2.3 Lipid Droplet Breakdown Occurs via Lipolysis or Lipophagy

When cells are exposed to nutrient imbalances that lead to a deficit in lipids, lipid droplet breakdown is activated to provide lipids for essential processes (Bosch et al. 2020). At the organismal level, lipid droplet breakdown in adipocytes is hormonally regulated and provides FAs for mitochondrial energy production in non-adipose tissue during fasting and exercise (Haemmerle et al. 2011; Young and Zechner 2013; Zimmermann et al. 2004). However, lipid droplets in most tissues also undergo a dynamic cycle of biogenesis and breakdown in response to hormonal signals and nutrient cues from the environment (Bosch et al. 2020; Jarc and Petan 2019). Intriguingly, upon entry into target cells and tissues, adipose-derived FAs are incorporated into lipid droplets, which become the major platforms that regulate their subsequent use and distribution in the cell (Bosch et al. 2020; Zechner et al. 2012). For example, in the heart, liver, and most other tissues, lipid droplets provide FAs that not only drive mitochondrial energy production, but act as signals that activate transcriptional networks, such as the those mediated by the peroxisome proliferator-activated receptors (PPARs), that are necessary for proper coupling of FA supply with mitochondrial biogenesis, function, and oxidative capacity in the cell (Haemmerle et al. 2011).

Lipid droplet breakdown occurs via two major mechanisms: lipolysis and lipophagy (Currie et al. 2013; Petan et al. 2018; Schulze et al. 2017; Young and Zechner 2013; Zechner et al. 2017). Lipolysis is mediated by cytosolic (neutral) lipases that enable a highly regulated release of FAs from TAGs. Adipose triglyceride lipase (ATGL) is the major TAG lipase in most mammalian cells and catalyzes the first step in TAG lipolysis (Schreiber et al. 2019; Smirnova et al. 2005; Zimmermann et al. 2004), which is followed by the sequential action of hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) (Grabner et al. 2017). In certain conditions, lipid droplet breakdown also occurs by lipophagy, a form of selective (macro)autophagy that delivers parts of or whole lipid droplets to lysosomes for bulk degradation by hydrolytic enzymes, such as the TAG and cholesteryl ester hydrolase lysosomal acid lipase (LAL) (Schulze et al. 2017; Singh et al. 2009; Zechner et al. 2017).

In principle, while lipolysis generally leads to lipid droplet shrinkage, lipophagy provides a means of complete breakdown of all lipids and proteins within the droplet into basic building blocks, suggesting that each mechanism may serve a distinct purpose in the cell (Ogasawara et al. 2020; Petan et al. 2018; Schulze et al. 2017; Zechner et al. 2017). Lipolysis and lipophagy are regulated by common and complementary signaling pathways, and cells seem to preferentially use one or the other depending on cell type, nutrient status, and current requirements, although concurrent or sequential occurrence is also possible. Indeed, these two mechanisms of lipid droplet breakdown display a considerable crosstalk, whereby the activation of lipolysis may stimulate autophagy/lipophagy, but autophagy may also be activated in a compensatory manner upon inhibition of lipolysis (Goeritzer et al. 2015;

Ogasawara et al. 2020; Peng et al. 2016). In addition, chaperone-mediated autophagy may facilitate lipolysis by removing the lipid droplet-coating proteins perilipins 2 and 3 (Kaushik and Cuervo 2015). The main drivers and functions of this intricate interplay of lipid droplet breakdown mechanisms in various cell types and microenvironmental contexts are only beginning to be uncovered (Ogasawara et al. 2020).

3 Lipid Droplets Are at the Core of Cancer Metabolic Reprogramming

3.1 Cancer Cells Use Ingenious Ways of Lipid Acquisition That Converge at the Lipid Droplet

Some of the earliest studies implicating lipids in cancer have shown that aggressive cancers display elevated rates of de novo FA synthesis, revealing that tumors may satisfy their requirements for lipids independently of uptake from the circulation (Menendez and Lupu 2007; Röhrig and Schulze 2016). Ever since, numerous studies have also suggested the involvement of other branches of FA, cholesterol, phospholipid, and neutral lipid metabolism in neoplastic transformation, disease progression, and drug resistance (Carracedo et al. 2013; Currie et al. 2013; Hernández-Corbacho and Obeid 2018; Menendez and Lupu 2007; Petan et al. 2018; Snaebjornsson et al. 2019). Although the first inhibitors of FA synthesis have entered clinical development only recently, some intrinsic drawbacks of targeting this pathway have already been revealed (Röhrig and Schulze 2016).

Namely, cancer cells that have access to lipids from the circulation are resistant to inhibition of FA synthesis, since they may increase lipid uptake to compensate for the lack of endogenous lipogenesis (Martinez-Outschoorn et al. 2017; Röhrig and Schulze 2016; Snaebjornsson et al. 2019). Inhibitors of lipogenesis are also ineffective in cancer cells exposed to hypoxia and nutrient deprivation, because lipogenesis is already blocked under these conditions and cells switch to lipid acquisition from their immediate microenvironment (Ackerman and Simon 2014; Petan et al. 2018). Remarkably, cancer cells engage in opportunistic modes of extracellular nutrient acquisition to satisfy their needs for lipids, amino acids, and carbohydrates by scavenging exosomes, extracellular matrix proteins, and albumin and even engulfing necrotic cell debris and entire living cells (Commisso et al. 2013; Finicle et al. 2018; Jayashankar and Edinger 2020; Kamphorst et al. 2013; Kim et al. 2018; Michalopoulou et al. 2016).

Cancer cells also enter in symbiotic relationships with neighboring cells, including tumor-associated adipocytes, whereby lipid droplet lipolysis in adipocytes provides FAs for energy production in cancer cells (Attané and Muller 2020; Hoy et al. 2017; Nieman et al. 2010; Wang et al. 2017). Furthermore, recent studies have shown that even when extracellular sources of lipids are exhausted, stressed cells

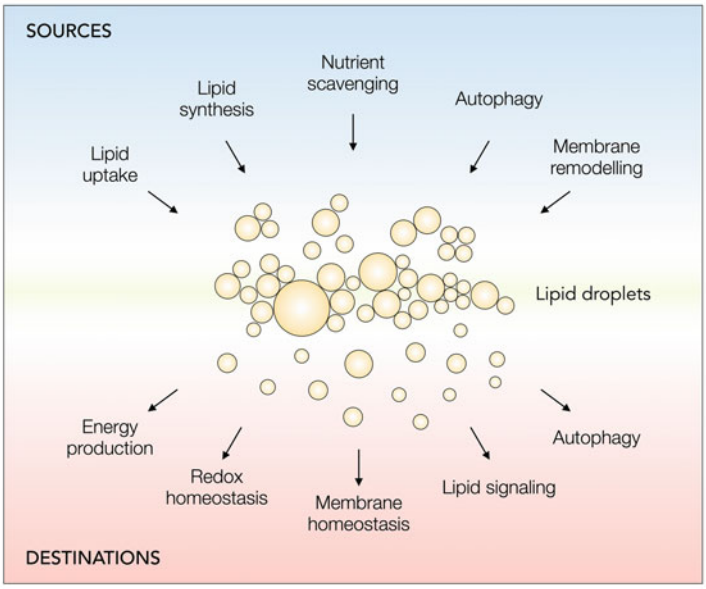

Fig. 1 Lipid droplets integrate lipid uptake and usage pathways in cancer cells. Based on the context and current conditions, cancer cells may use several lipid acquisition pathways, which all converge at the lipid droplet. Lipid droplets act as buffers that consolidate the various lipid fluxes and finely tune their release and distribution in the cell to drive essential processes that control cancer cell fate

may have access to additional endogenous lipid pools. These include lipids that can be recommissioned from their own structural and storage pools via several possible routes, including membrane phospholipid hydrolysis (e.g., by phospholipases A_2), autophagic degradation of organelles, and the breakdown of neutral lipids stored within cytosolic lipid droplets (Ackerman et al. 2018; Jarc et al. 2018; Lue et al. 2017; Nguyen et al. 2017; Petan et al. 2018; Pucer et al. 2013; Rambold et al. 2015).

Intriguingly, most if not all of these pathways of lipid acquisition converge at the lipid droplet (Fig. 1). Lipid droplets are perfectly positioned within the metabolic scheme of the cell to control both the acquisition of lipids (from the various internal or external sources mentioned above) and their utilization for various purposes and depending on specific cellular needs. Although lipid droplets are often regarded merely as transient repositories for the trafficking lipids on route to their final destination – and certainly there will be cases when this is true – the syphoning of various lipid fluxes into lipid droplets is in fact required for numerous homeostatic cell functions and, in particular, for the cellular stress response. One of the earliest and most notable examples was reported in cardiomyocytes (Haemmerle et al. 2011). Namely, while extracellular FAs may enter the cell in various ways and bind to different proteins in the cytosol, including nuclear transcription factors, they must first be incorporated into TAGs within lipid droplets and then released by lipolysis in order to bind to and activate PPAR-mediated gene transcription that

drives mitochondrial biogenesis and oxidative metabolism in these cells. This seemingly futile cycle of FA esterification and lipolytic release reveals one of the hallmark principles of lipid droplet biology, whereby the organelle acts as a focal point that coordinates lipid flux with metabolic and signaling pathways essential for cell function and resistance to stress (Fig. 1) (Jarc and Petan 2020; Khan et al. 2015; Mottillo et al. 2012; Ong et al. 2011; Zechner et al. 2012).

Similarly, cancer cells exposed to extracellular FAs form lipid droplets that in turn regulate mitochondrial redox metabolism to increase NADPH production and protect cancer cells from hypoxic damage (Bensaad et al. 2014). Lipid droplets are also formed in breast and ovarian cancer cells exposed to lipids derived from neighboring adipocytes and provide a consistent supply of FAs that drives FA oxidation, sustains metabolic reprogramming, and promotes tumor aggressiveness (Nieman et al. 2010; Wang et al. 2017). Moreover, lipid droplet biogenesis is also activated when exogenous lipids are limiting but endogenous lipids are present in excess, such as following autophagic breakdown of membranous organelles, in order to finely tune their uptake by mitochondria, thereby preventing mitochondrial damage and ensuring efficient energy production (Herms et al. 2015; Nguyen et al. 2017; Rambold et al. 2015). In this review, we discuss these and related studies that describe the various essential roles of lipid droplets in the response of cancer cells to stress and their ability to regulate downstream lipid fluxes depending on cellular requirements.

3.2 Lipid Droplets and Nutrient Scavenging

To ensure their survival and promote growth in a nutrient-poor environment, cancer cells use multiple nutrient scavenging strategies to obtain various macromolecules and break them down to their basic constituents in the lysosome, thereby ensuring the supply of energy substrates and anabolic building blocks (Finicle et al. 2018). Some cancer cell types, in particular those driven by oncogenic mutations in the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (Commisso et al. 2013; Jayashankar and Edinger 2020; Kamphorst et al. 2013; Kim et al. 2018; Palm et al. 2015), use macropinocytosis, a non-selective endocytotic uptake mechanism of different material, including extracellular fluid, proteins, vesicles, and cellular debris (Finicle et al. 2018; Jayashankar and Edinger 2020; Kim et al. 2018). Macropinocytosis is supported by activation of AMP-activated protein kinase (AMPK) and inhibition of mammalian target of rapamycin (mTOR) pathways; it promotes cancer cell proliferation and confers resistance to therapies that target cancer anabolism.

Remarkably, macropinocytosis enables the extraction of amino acids, nucleotides, and FAs even from dying cell corpses, a process termed necrocytosis (Jayashankar and Edinger 2020; Kim et al. 2018). Necrocytosis has been shown to help amino acid-deprived prostate cancer cells maintain lipid droplet levels, but it remained unknown if extracellular lipids are de facto scavenged from cell debris

(Kim et al. 2018). Indeed, some of the other types of acquired nutrients could provide energy and building blocks for essential cellular processes, thereby sparing existing lipid droplets. It was shown recently by tracing experiments that necrotic debris-derived FAs are indeed incorporated into breast cancer cells, thereby reducing their dependence on de novo FA synthesis and rendering them insensitive to inhibitors of FA synthase (Fig. 2a) (Jayashankar and Edinger 2020). These studies hint at the possibility that lipid droplets act as transient buffers for lipids taken up via macropinocytosis. It will be interesting to see in future studies whether FA release from lipid droplets is responsible for the observed reduced dependence on FA synthesis. Given their similar role in cells exposed to FA surges from lysosomal breakdown via autophagy (Nguyen et al. 2017; Rambold et al. 2015), it is possible that lipid droplets serve as central lipid buffering and distribution hubs that carefully balance lipid input with the requirements of these "voracious," macropinocytic cancer cells.

3.3 Lipid Droplets Maintain Membrane Unsaturation During Stress

Rapidly proliferating cancer cells rely on several oncogenic signal transduction pathways that activate mTOR signaling to maintain high levels of protein and lipid synthesis, which are prerequisites for cell growth and proliferation (Liu and Sabatini 2020). The mTOR pathway is activated in response to amino acid availability and drives cell growth by stimulating numerous anabolic pathways, including protein translation and nucleotide synthesis. It also promotes FA, cholesterol, and glycerolipid synthesis via the sterol regulatory element-binding protein (SREBP) transcription factors (Yecies and Manning 2011). This strong anabolic drive requires a coordination between nutrient availability, metabolic pathways, and the various oncogene-driven mitogenic signals. The survival of cancer cells is thus compromised when biosynthetic pathways, such as lipid and protein production, are not synchronized.

For example, in cancer cells exposed to limited oxygen availability, the conversion of palmitate, the principal product of de novo FA synthesis, into unsaturated FAs is compromised due to inactivation of the oxygen-dependent lipid desaturase stearoyl-coenzyme A desaturase 1 (SCD1) (Fig. 2b) (Kamphorst et al. 2013; Scaglia et al. 2009). Under these conditions, constitutive mTOR activity causes an imbalance between the elevated protein synthesis and the lagging membrane expansion, which ultimately leads to ER stress and cell death (Young et al. 2013). Consequently, these cells become dependent on the uptake of unsaturated FAs from extracellular sources in order to compensate for the diminished desaturase activity and restore the balance between protein and lipid synthesis (Ackerman and Simon 2014; Young et al. 2013). Even in normoxic conditions, elevated Ras oncogene signaling, which imposes a potent growth impetus to cancer cells by activating the

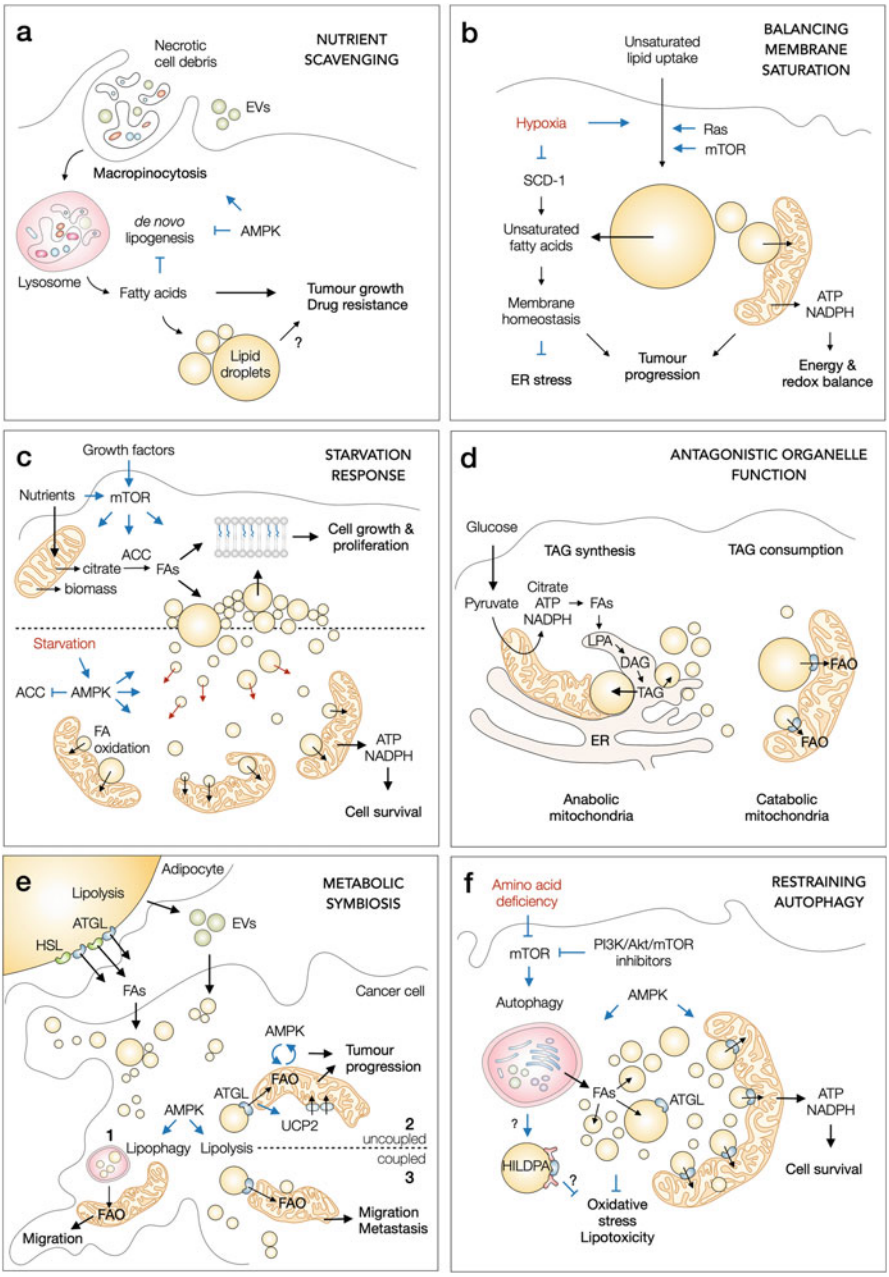

Fig. 2 Lipid droplets, lipid fluxes, and cancer cell fate. (**a**) Macropinocytosis of extracellular material, including necrotic cell debris and extracellular vesicles (EVs), provides amino acids, nucleotides, and lipids for cancer cell survival and resistance to drugs that target anabolic pathways, including inhibitors of FA synthesis; the macropinocytosis-derived FAs are incorporated into lipid droplets, whose role in mediating the effects of FAs is not yet clear. (**b**) Lipid droplets are important repositories of unsaturated FAs that are used by cancer cells to maintain proper membrane saturation and prevent endoplasmic reticulum (ER) stress, particularly when demands for lipids

MAPK pathway and mTOR complex 1 (mTORC1) signaling, drives the uptake of serum lysophospholipids as sources of unsaturated FAs to reduce the dependence of cancer cells on SCD1 activity (Kamphorst et al. 2013). Intriguingly, upregulated lysophospholipid uptake in cancer cells with Ras oncogenic mutations leads to increased lipid droplet storage (Fig. 2b) (Qiao et al. 2020). The latter is in turn coupled to elevated FA oxidation and improved redox metabolism that promotes tumor aggressiveness in vitro and in vivo, indicating that lipid droplets might mediate the effects of exogenous lysophospholipids in aggressive Ras-driven tumors.

Clearly, the provision of unsaturated FAs is critical for cancer cell survival and growth. There is accumulating evidence that lipid droplets are important sources and regulators of unsaturated FA trafficking. Indeed, recent studies in kidney cancer have found that lipid droplets play an important role in the maintenance of membrane unsaturation levels during hypoxia (Ackerman et al. 2018; Qiu et al. 2015). Constitutive hypoxia-inducible factor (HIF) signaling and abundant lipid storage are hallmarks of clear-cell renal cell carcinoma (ccRCC). It was found that

Fig. 2 (continued) are elevated, such as during Ras oncogene- and mTOR signaling-driven rapid cell growth, or when the synthesis of unsaturated lipids is compromised, e.g., due to hypoxia-induced inhibition of stearoyl-CoA desaturase-1 (SCD-1); during hypoxic stress, lipid droplets also drive mitochondrial oxidative metabolism to provide energy and reducing equivalents that reduce oxidative stress. (**c**) During nutrient replete conditions, when mTOR is active, lipid uptake and de novo FA synthesis drive both membrane synthesis and lipid droplet biogenesis; when lipids become limiting, lipid droplets support membrane synthesis, thereby sustaining cell growth and proliferation. Upon nutrient depletion, cells experience a fall in energy levels, leading to AMPK activation, which in turn blocks de novo lipogenesis and stimulates rapid lipid droplet dispersion to mitochondrial contact sites; AMPK also promotes the lipolytic release and transfer of FAs into mitochondria for oxidation, thereby restoring energy levels and the redox balance through ATP and NADPH production. (**d**) Distinct populations of mitochondria and lipid droplets may engage in opposing purposes in the same cell: mitochondria, tightly anchored to lipid droplets, provide citrate, ATP, and NADPH to support FA and TAG synthesis driving lipid droplet formation, whereas "free," cytosolic mitochondria dynamically interact with lipid droplets to take up and oxidize FAs. (**e**) In the tumor microenvironment, cancer cells take up FAs and EVs released by neighboring adipocytes and store them in lipid droplets, whose breakdown via (**1**) lipophagy or (**2**, **3**) lipolysis drives mitochondrial energy production, thereby promoting tumor growth and invasion. Under these lipid-rich conditions, AMPK supports lipolysis, lipophagy, and mitochondrial FA oxidation, which may be (**3**) coupled to or (**2**) uncoupled from ATP production via uncoupling protein 2 (UCP2); this uncoupling is instigated by the influx of lipid droplet-derived fatty acids and drives a feedback circuit that sustains AMPK activation. (**f**) In cells exposed to amino acid starvation or to inhibitors targeting the PI3K/Akt/mTOR pathway, mTORC1 is inhibited leading to activation of autophagy, which breaks down membranous organelles to release FAs that trigger lipid droplet biogenesis; rapid lipid droplet biogenesis protects mitochondria from excess FAs; lipid droplets provide an efficient way to gradually deliver FAs via ATGL-mediated lipolysis into fused mitochondria and enable cell survival during starvation; the process is supported by AMPK, which sustains autophagic flux and oxidative metabolism; the hypoxia-inducible lipid droplet-associated protein (HILPDA), an endogenous inhibitor of ATGL, is upregulated in response to autophagy-driven lipid droplet biogenesis, and it may participate in the fine regulation of lipolysis to prevent oxidative stress and lipotoxicity

HIF2α-dependent lipid droplet accumulation protects ccRCC cells from ER stress, thereby promoting cell proliferation and xenograft tumor growth (Qiu et al. 2015). Intriguingly, even in cells depleted of HIF2α, overexpression of the lipid droplet-coating protein PLIN2 is sufficient to restore lipid storage and protect from ER stress, which occurs at least in part due to mTOR-driven protein synthesis. Furthermore, it was found recently that lipid droplets formed in nutrient-replete ccRCC cells are rich in serum-derived unsaturated FAs and are gradually broken down when cells are exposed to low serum and oxygen stress (Ackerman et al. 2018). This delayed lipolytic release of unsaturated FAs is dependent on HSL activity and is responsible for replacing saturated acyl chains in cell membranes and prevention of ER stress (Fig. 2b). Concurrently, lipid droplets reduce the dependence on de novo FA synthesis, revealing that targeting lipid droplet biogenesis, e.g., via inhibition of DGATs, may be a more relevant therapeutic target than FA synthesis in ccRCC (Ackerman et al. 2018).

The dependence of cancer cells on the supply of unsaturated FAs from lipid droplets for long-term maintenance of membrane homeostasis and protection against ER stress is very likely not limited to kidney cancer. In rapidly proliferating yeast cells, lipid droplet turnover is essential for providing a balanced supply of saturated and unsaturated FAs for membrane synthesis (Natter and Kohlwein 2013; Petschnigg et al. 2009; Zanghellini et al. 2008), hinting at a conserved, essential function of lipid droplets across the eukaryotic kingdom. Lipid droplets are unique in their ability to consolidate different FA fluxes and regulate their input into phospholipid synthesis and remodeling pathways that are necessary for membrane homeostasis. Collectively, these studies suggest that lipid droplets are important repositories of unsaturated FAs that may be utilized by cancer cells to maintain membrane and organelle function particularly when demands for lipids are elevated, such as during oncogene-driven rapid cell growth, or when the synthesis of unsaturated lipids is compromised, e.g., due to hypoxia.

3.4 Lipid Droplets Match Nutrient Fluctuations with Cell Growth and Survival

Lipid droplet biogenesis and turnover are dynamically altered in response to changes in nutrient and energy status. Recent studies have significantly increased our understanding of the integration of lipid droplet turnover in the general cellular response to nutrient imbalances (Bosch et al. 2020), but new evidence is also emerging regarding their roles in the context of metabolic reprogramming in cancer. Cancer cells often have constitutively activated pathways of nutrient sensing and uptake and display oncogene-driven, growth factor-independent signaling that stimulates cell growth and survival irrespective of nutrient levels. AMPK and mTOR are two major intracellular kinases that reciprocally regulate adaptive cellular responses to nutrient stress and cell growth. They sense metabolite availability, energy and stress levels

and integrate these signals with those coming from growth factor and oncogene-driven pathways (González et al. 2020; Liu and Sabatini 2020; Palm and Thompson 2017). AMPK detects glucose and energy levels and responds to starvation by inhibiting anabolic pathways and cell growth and activating catabolic pathways to restore the energy balance. AMPK blocks de novo FA, cholesterol, and TAG synthesis; it activates lipolysis and FA oxidation and engages gene transcription programs responsible for mitochondrial biogenesis and oxidative metabolism (Hardie et al. 2012; Muoio et al. 1999; Wendel et al. 2009; Zechner et al. 2017). The amino acid-sensitive complex mTORC1 is positively regulated by the PI3K/Akt and MAPK pathways to promote cell growth and survival and is inactivated when amino acids are limiting. Because AMPK negatively regulates mTORC1, energy or glucose depletion also inhibits mTORC1 activity; however, amino acid deficiencies do not activate AMPK. Both kinases are often dysregulated in cancer, thereby allowing cancer cells to evade metabolic checkpoints and strive even in nutrient-limiting conditions. Emerging studies are beginning to reveal how lipid droplets respond to nutrient and energy fluctuations and how they are integrated in the sensing and regulatory networks that orchestrate the metabolic rewiring of stressed cancer cells.

3.4.1 Lipid Droplets Are Rapidly Mobilizable Energy Sources During Stress

Many of the hallmark changes in lipid metabolism in cancer cells are shared by rapidly proliferating, fermenting yeast cells (Natter and Kohlwein 2013). Both types of cells depend on lipogenic pathways for cell growth and viability. The synthesis of FAs and their incorporation into complex lipids, most notably phospholipids, drives membrane expansion, which is required for cell growth, cell cycle progression, and cell division. In yeast, TAG lipolysis has been linked with the cell cycle and provides FAs for membrane synthesis (Kurat et al. 2009; Zanghellini et al. 2008). Upon glucose depletion, the Snf1 protein kinase (the yeast orthologue of AMPK) is activated to engage a switch from glucose fermentation to FA oxidation as a primary source of energy. Intriguingly, this is accompanied by a shift from phospholipid to TAG synthesis resulting in elevated lipid droplet biogenesis (Bosch et al. 2020; Henne et al. 2018). This conserved mechanism of preservation of lipids that is activated at the onset of starvation prepares the cell for the possibility of prolonged periods of nutrient deficiency. Indeed, in starving yeast cells, lipid droplets are gradually consumed by microautophagy, a form of lipophagy involving the vacuole, and become essential for long-term survival (Seo et al. 2017).

Proliferating mammalian and cancer cells with access to nutrients mostly rely on glucose fermentation for energy production and use mitochondria as a biosynthetic organelle. Mitochondria provide building blocks and reducing equivalents for anabolic reactions, including FA synthesis, thereby ensuring a consistent supply of FAs for membrane biogenesis (Natter and Kohlwein 2013; Ward and Thompson 2012). In such nutrient- and lipid-rich conditions, mammalian cells also synthesize TAGs

and accumulate lipid droplets (Fig. 2c) (Herms et al. 2015). When extracellular lipids become limiting, lipid droplet-derived FAs may be used for phospholipid synthesis and drive cell proliferation (Herms et al. 2015). When both glucose and lipids are scarce, mammalian cells shut off phospholipid synthesis and turn on mitochondrial oxidative metabolism. Lipid droplet-derived FAs are then syphoned into mitochondria for oxidation and energy production. The decrease in energy levels is detected by AMPK, which not only activates FA oxidation and mitochondrial oxidative metabolism but also directly stimulates the rapid redistribution of lipid droplets along the microtubular network, thereby driving their recruitment to mitochondria and optimizing FA delivery (Herms et al. 2015; Zhu et al. 2019). AMPK activation and associated starvation responses, such as autophagy, mTORC1 inhibition, and protein kinase A (PKA) activation, also promote mitochondrial fusion, which is necessary for efficient FA intake and uniform distribution within the network of tubulated mitochondria (Gomes et al. 2011; Rambold and Pearce 2018; Rambold et al. 2015). Lipid droplets thus provide a rapidly mobilizable form of energy substrates for cell survival following a sudden glucose depletion and energy deficiency.

3.4.2 Cancer Cells Depend on the Long-Term Supply of Lipid Droplet-Derived Lipids

Cancer cells may be exposed to relatively long periods of nutrient deficiency due to insufficient vasculature and rapid tumor growth (Wellen and Thompson 2010). Their nutrient and oxygen supply may also be severely compromised following matrix detachment, migration, and invasion into neighboring tissue. Cancer cells having accumulated lipid droplets during nutrient (and oxygen) sufficiency rely on the long-term supply of lipid droplet-derived lipids not only to survive the immediate stress but also to migrate and resume growth at a new location (Clement et al. 2020; Wang et al. 2017). Indeed, lipid droplets, accumulated in nutrient-rich conditions, enable a prolonged protection from starvation by undergoing gradual lipid droplet breakdown (Jarc et al. 2018; Przybytkowski et al. 2007; Pucer et al. 2013). Aggressive breast cancer cells harboring Ras oncogenic mutations increase their lipid droplet storage upon exposure to even minute amounts of monounsaturated or polyunsaturated FAs when grown in nutrient replete conditions. When these cells are switched to lipid- and serum-free starvation medium, but still rich in glucose and amino acids, lipid droplets undergo gradual breakdown over several days in culture resulting in an increased resistance to cell death (Jarc et al. 2018; Przybytkowski et al. 2007; Pucer et al. 2013). In comparison with control cells without initial lipid loading, these cells also activate AMPK, decrease their dependence on de novo lipogenesis, and upregulate FA oxidation (Brglez et al. 2014; Jarc et al. 2018; Pucer et al. 2013). In fact, preloading aggressive breast cancer cells with lipid droplets suppresses the strong surge in lipogenic signaling that occurs at the onset of lipid and serum starvation. The activation of lipogenesis is driven by the major lipid sensor and transcription factor sterol regulatory element-binding protein-1c (SREBP-1c) and its target genes involved in FA and cholesterol synthesis, including FA synthase

(FASN), acetyl-coenzyme A carboxylase (ACC), SCD1, and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (Jarc et al. 2018; Pucer et al. 2013). The biosynthesis of FAs and other lipids consumes large amounts of ATP and reducing power in the form of NADPH (Natter and Kohlwein 2013). Therefore, the breakdown of pre-accumulated lipid droplets at the onset of starvation spares important cellular resources by reducing the need for de novo lipogenesis. The starving cancer cell may thus redirect the saved energy and redox equivalents to other essential processes that protect against starvation.

In addition, the concurrent increase in the levels of FA oxidation enzymes, including carnitine palmitoyltransferase 1A (CPT1A), whose inhibition is lethal to serum-starved breast cancer cells, suggests that the pre-accumulated lipid droplets provide a long-term supply of FAs for mitochondrial oxidation to support cell survival (Pucer et al. 2013). Indeed, a combined depletion of the major TAG lipase ATGL and pharmacological targeting of CPT1A abolished the protective effects of lipid droplets in breast cancer cells (Jarc et al. 2018). Moreover, the observed activation of AMPK and the ability of its activator AICAR to protect breast cancer cells from starvation-induced cell death (Pucer et al. 2013) are in line with the fact that AMPK supports cancer cell survival by stimulating FA oxidation, blocking lipogenesis, and driving both ATP and NADPH production (Buzzai et al. 2005; Carracedo et al. 2013; Jeon et al. 2012; Pike et al. 2011). Such changes in the metabolic landscape involving AMPK, mitochondria, and the lipid droplet may render cancer cells particularly well-equipped to handle prolonged periods of nutrient limitation. Collectively, these studies suggest that lipid droplets support Ras-driven cancer cell survival in lipid-limiting conditions by (1) reducing the need for energy-depleting de novo lipogenesis and (2) driving mitochondrial oxidative metabolism that replenishes cellular energy and redox capacity.

3.4.3 Devouring and Creating Fat: Metabolic Flexibility Driving Tumorigenesis

Recent studies suggest that the interactions between mitochondria and lipid droplets, besides optimizing FA transfer and rates of FA oxidation (Herms et al. 2015; Rambold et al. 2015), in fact enable the formation of complex metabolic and signaling "synapses." These are endowed with sophisticated feedback mechanisms that finely tune both lipid droplet and mitochondrial metabolism (Benador et al. 2019; Bohnert 2020; Bosch et al. 2020; Freyre et al. 2019; Jarc and Petan 2019). In fact, lipid droplet-mitochondria contacts may also reflect an essential role of mitochondria in the synthesis of TAG and lipid droplet biogenesis. Benador et al. have recently discovered that brown adipose tissue cells contain two segregated and functionally distinct subpopulations of mitochondria (Fig. 2d): peridroplet mitochondria, which are anchored to lipid droplets and are primarily involved in providing ATP and NADPH to support FA and TAG synthesis driving lipid droplet formation, and "free," cytosolic mitochondria that primarily take up and oxidize FAs (Benador et al. 2018, 2019). Furthermore, in white adipocytes, a tripartite lipid

droplet–mitochondria–ER interaction couples FA synthesis from glycolytic precursors processed in the citric acid cycle with their esterification into TAGs within the ER membrane and TAG storage in the growing lipid droplet (Freyre et al. 2019). Thus, overturning the classical biochemical dogma of the exclusively unidirectional mode of FA metabolism, cells may simultaneously engage in antagonistic biochemical processes, such as FA oxidation and synthesis, or lipid droplet expansion and breakdown, using distinct subpopulations of mitochondria and lipid droplets. Emerging studies hint at the possibility that such organelle and metabolic flexibility is also used by cancer cells to trigger and sustain metabolic reprogramming. Indeed, cancer cells grown in various nutrient- and lipid-rich conditions increase FA uptake and activate lipid droplet biogenesis in parallel with catabolic lipid droplet consumption and FA oxidation that drives cancer cell survival, growth, and metastasis (Clement et al. 2020; Lazar et al. 2016; Nieman et al. 2010; Pucer et al. 2013; Wang et al. 2017).

In the tumor microenvironment, cancer cells may "trick" neighboring adipocytes into releasing FAs from their large TAG stores, which are then taken up and used by cancer cells to form lipid droplets (Fig. 2e) (Attané and Muller 2020; Balaban et al. 2017; Clement et al. 2020; Nieman et al. 2010; Wang et al. 2017; Wen et al. 2017). These lipid droplets are broken down via lipolysis or lipophagy, thereby syphoning the adipocyte-derived FAs into mitochondria to be used for energy production and likely other purposes. Remarkably, in melanoma cells exposed to adipocyte-derived extracellular vesicles, mitochondria, lipid droplets, and lysosomes are redistributed and proximally located in cell protrusions to promote cancer cell migration via lipophagic lipid droplet breakdown and FA oxidation (Clement et al. 2020). Intriguingly, although typically sensing nutrient depletion, AMPK is activated in cancer cells co-cultured with adipocytes, most likely to promote and regulate the tight cooperation between lipid droplet consumption and FA oxidation, which may be coupled to or uncoupled from ATP production (Nieman et al. 2010; Wang et al. 2017; Wen et al. 2017; Zechner et al. 2017). Furthermore, upregulated ATGL-mediated lipid droplet lipolysis in breast cancer cells may lead to uncoupling of FA oxidation resulting in a drop in ATP levels and sustained AMPK activation, which promotes further FA uptake and mitochondrial biogenesis (Wang et al. 2017).

Another possibility that may explain the activation of AMPK in such lipid-rich conditions is a decrease in energy levels as a consequence of elevated FA/TAG cycling, whereby the influx of exogenous FAs stimulates a cycle of FA esterification into TAG and lipolysis at the expense of ATP (Prentki and Madiraju 2008; Przybytkowski et al. 2007). Namely, free FAs require ATP-dependent activation into FA-CoA by long-chain acyl-CoA synthetase (ACSL) enzymes before entering TAG synthesis or being transported into mitochondria following lipolysis (Cooper et al. 2015). In line with this, the ACSL inhibitor triacsin C suppresses both FA-induced lipid droplet biogenesis and AMPK activation in breast cancer cells during growth in nutrient-rich conditions (Pucer et al. 2013). Moreover, because inhibition of CPT1A with low concentrations of etomoxir (Raud et al. 2018) also reduces both AMPK activation and lipid droplet accumulation, it may be speculated that the exogenous FA supply stimulates FA oxidation that provides ATP and

NADPH to support the anabolic branch of FA/TAG cycling (Pucer et al. 2013). The elevated FA/TAG cycling may lead to ATP deficiency that promotes AMPK activation, which in turn further stimulates mitochondrial FA oxidation. AMPK may be required under these conditions to reduce unnecessary de novo lipogenesis, suppress excessive lipid droplet accumulation, activate lipolysis, and increase the mitochondrial capacity of the cell by stimulating gene expression programs responsible for mitochondrial biogenesis and oxidative metabolism.

Whether different subpopulations of mitochondria and lipid droplets enable these antagonistic processes in individual cancer cells remains to be confirmed. Moreover, the intracellular heterogeneity in mitochondrial and lipid droplet function is likely also influenced and combined with intercellular lipid trafficking and population dynamics, whereby individual cells preferentially specialize their lipid droplet function to serve specific roles, e.g., protect from bulk lipid influx or engage in anabolic vs. catabolic lipid metabolism (Herms et al. 2013; Thiam and Beller 2017).

3.5 When the Going Gets Tough, Lipid Droplets Team Up with Autophagy

When cells are exposed to prolonged nutrient deficiency, and in particular when amino acids become limiting, autophagy is typically strongly activated (Bosch et al. 2020; Galluzzi et al. 2017; Kroemer et al. 2010; Nguyen et al. 2017; Ogasawara et al. 2020; Rambold et al. 2015). Lipid droplets and autophagy engage in a complex relationship, which is currently poorly understood: (1) lipid droplets may be the target of autophagic degradation (Schulze et al. 2017), (2) they may be formed as a consequence of autophagic breakdown of other lipid-containing organelles (Lue et al. 2017; Nguyen et al. 2017; Rambold et al. 2015; VandeKopple et al. 2019), and (3) they may support the formation of autophagosomes by providing lipids (Bekbulat et al. 2019; Dupont et al. 2014; Shpilka et al. 2015) or supporting signaling that stimulates the expression of autophagy genes (Ogasawara et al. 2020; Petan et al. 2018; Zechner et al. 2017). Emerging studies suggest that changes in lipid droplet turnover are a conserved cellular response to high autophagic flux, occurring across the eukaryotic kingdom and playing various beneficial roles in cellular homeostatic and stress responses (Jaishy and Abel 2016; Petan et al. 2018; Wang 2016). The opposite is also true, since lipid overload and exogenous unsaturated FAs stimulate autophagy (Niso-Santano et al. 2015). Indeed, cells preloaded with (unsaturated) FA-induced lipid droplets display higher autophagic flux during starvation (Dupont et al. 2014). In accordance with this entangled relationship, it is not surprising that both lipid droplet turnover and autophagy are often simultaneously or sequentially activated by various kinds of stress.

In mouse embryonic fibroblasts (MEFs) exposed to acute amino acid starvation, mTORC1 is inactivated leading to the activation of autophagy, which in turn triggers

lipid droplet biogenesis (Fig. 2f) (Nguyen et al. 2017; Rambold et al. 2015). Lipids derived from membranous organelles are delivered into lysosomes by autophagy and broken down by acid phospholipases and lipases. The FAs released from lysosomes are rapidly esterified by DGAT1 into TAGs and stored in growing lipid droplets. Immediate lipid droplet biogenesis is required to avoid the accumulation of autophagy-derived free FAs that could overwhelm the mitochondrial FA transfer mechanism leading to piling up of toxic acylcarnitines at the mitochondrial "gates." Furthermore, the newly formed lipid droplets provide an efficient way to gradually deliver FAs into the network of fused mitochondria during the ongoing starvation. Indeed, under these conditions, free FAs are released from lipid droplets primarily by ATGL-mediated lipolysis, but not lipophagy (Rambold et al. 2015). Notably, ATGL may not only provide FAs but also stimulate signaling pathways that both activate mitochondrial oxidative metabolism and regulate autophagy/lipophagy (Zechner et al. 2017). Interestingly, rather than in the initiation of autophagy, AMPK seems to be involved in sustaining autophagic flux and oxidative metabolism during the starvation (Nguyen et al. 2017).

Surely, the fine regulation of lipolysis and its coordination with autophagy will be of critical importance for cell survival in starved cells. Indeed, the hypoxia-inducible lipid droplet-associated protein (HILPDA), an endogenous inhibitor of ATGL (Das et al. 2018), is upregulated in MEFs and in cancer cells during acute starvation (VandeKopple et al. 2019). Interestingly, HILPDA is activated in direct response to autophagy-driven lipid droplet biogenesis, thereby suppressing ATGL-mediated lipolysis. In accordance, ablation of HILPDA reduces lipid droplet accumulation and xenograft tumor growth in vivo, possibly by elevating oxidative stress, lipid peroxidation, and apoptosis due to excessive lipolysis (VandeKopple et al. 2019; Zhang et al. 2017). Although additional confirmation is clearly required, these results suggest that autophagy-driven lipid droplet turnover and the fine-tuning of lipolysis by HILPDA promote tumorigenesis.

While physiological levels of autophagy generally play a tumor suppressor role by preventing cell damage, maintaining cellular fitness, and restoring homeostasis, cancer cells may also subvert the autophagic machinery to enhance their resistance to stress. Lipid droplets and autophagy may play a complementary role in both contexts. For example, nutrient deficiency within cancer cells may be induced indirectly by exposing cells to drugs targeting major nutrient sensing and growth pathways, such as the PI3K/Akt/mTOR pathway (Lue et al. 2017). Intriguingly, although tumor growth is restricted by these drugs, cancer cells may circumvent therapeutic inhibition by activating autophagy. Importantly, this cancer treatment-induced autophagy stimulates lipid droplet biogenesis to sustain mitochondrial energy production and redox homeostasis, thereby reducing cancer cell death (Fig. 2f) (Lue et al. 2017). Intriguingly, the supply of FAs for lipid droplet biogenesis and oxidative metabolism is dependent on an unidentified member of the phospholipase A_2 family of enzymes, which release free FAs and lysophospholipids from membrane phospholipids (Lambeau and Gelb 2008; Murakami and Lambeau 2013;

Murakami et al. 2011). Several phospholipases A_2 have been implicated in lipid droplet metabolism and cancer cell survival (Cabodevilla et al. 2013; Guijas et al. 2014; Jarc et al. 2018; Pucer et al. 2013), but it is not clear how they cooperate with autophagy to stimulate lipid droplet and mitochondrial metabolism (Petan et al. 2018). The mechanisms and relevance of autophagy-driven lipid droplet turnover for tumor growth remain to be established.

Several in vitro studies have shown that lipophagy is typically activated under milder, albeit prolonged, starvation conditions than those activating bulk autophagy (Rambold et al. 2015; Wang 2016). For example, in contrast to amino acid-starved MEFs, autophagy-driven lipid droplet biogenesis does not occur in serum-starved MEFs, most likely because mTORC1 is not inhibited under these conditions, but instead lipophagy contributes to lipid droplet breakdown (Nguyen et al. 2017; Rambold et al. 2015). The activation of AMPK may drive lipophagy under such conditions, because it can bypass mTORC1 and activate lipophagy through direct activation of ULK1 even in nutrient-rich conditions (Kim et al. 2011; Li et al. 2019; Zechner et al. 2017). Moreover, AMPK phosphorylates PLIN2 and primes it for chaperone-mediated autophagy, which is an additional mechanism of AMPK-mediated regulation of both lipophagy and lipolysis (Kaushik and Cuervo 2016). AMPK also indirectly activates the deacetylase sirtuin 1 (SIRT1) and its target transcription factors peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α) and forkhead box protein O (FOXO), which regulate both neutral and acid lipolysis (Zechner et al. 2017).

A role for AMPK-driven lipophagy has been suggested in promoting cancer cell growth in the context of metabolic symbiosis between adipocytes and cancer cells (Wen et al. 2017). Adipocyte-derived FAs were found to stimulate AMPK-dependent lipophagy and mitochondrial energy production, which were required for the survival of neighboring cancer cells during starvation. On the contrary, in prostate cancer cells, the activation of lipophagy may occur in response to SIRT1-mediated acetylation of LAMP1 and lead to proliferative senescence, likely as a consequence of elevated oxidative stress (Panda et al. 2019). Accordingly, excessive lipophagy leads to an overflow of free FAs causing mitochondrial damage, ER stress, and cancer cell death in cervical cancer cells (Mukhopadhyay et al. 2017). Lipophagy has also been associated with reduced ccRCC tumor growth and increased patient survival (Xu et al. 2015). In line with these studies suggesting a tumor suppressor role for lipophagy, recent evidence has shown that LAL suppresses inflammation and metastasis in liver and lung cancer (Du et al. 2015; Zhao et al. 2016). With these mostly preliminary studies, we are only beginning to understand the role of lipophagy in cancer, which seems to play a dual, context-dependent role (Kounakis et al. 2019; Maan et al. 2018; Petan et al. 2018). In accordance with the opposing roles of neutral lipolysis in cancer, the role of lipophagy likely depends on the specific metabolic and oncogenic reprogramming of the cancer type in question and the microenvironmental conditions (Petan et al. 2018).

3.6 *Lipid Droplets, Lipid Peroxidation, and Ferroptosis in Cancer*

One of the primary functions of lipid droplets in most biological systems and conditions is the protection from various forms of lipotoxicity (Listenberger et al. 2003; Schaffer 2003). Lipid droplets have also recently been implicated in the regulation of the cellular distribution of unsaturated and polyunsaturated FAs (PUFAs) (Ackerman et al. 2018; Bailey et al. 2015; Jarc et al. 2018; Petan et al. 2018), which is essential for the maintenance of proper membrane saturation and redox balance. In fact, lipid droplets seem to act as antioxidant organelles by actively regulating the trafficking of PUFAs in order to prevent oxidative stress and cell death. Lipid droplets also regulate the release of PUFAs for their conversion by cyclooxygenases and lipoxygenases into a whole range of oxygenated mediators of inflammation in immune cells, adipocytes, and in cancer cells (Jarc and Petan 2020). The recent discovery of ferroptosis (Dixon et al. 2012), a type of programmed cell death driven by the oxidation of PUFAs in membrane phospholipids, has pinpointed the importance of lipid peroxidation for cellular well-being and protection from stress. Lipid droplets, being implicated in the regulation of PUFA lipotoxicity and trafficking, are thereby emerging as imminent regulators of ferroptotic sensitivity.

Ferroptosis is a form of programmed cell death that depends on the accumulation of lethal levels of oxidized lipids in cell membranes (Fig. 3) (Dixon and Stockwell 2019). Cells possess at least two major antioxidant mechanisms that act in parallel to protect from ferroptotic cell death: (1) the glutathione peroxidase 4 (GPX4) pathway and (2) the ubiquinol (coenzyme Q10) antioxidant system, which depends on the activity of ferroptosis-suppressor-protein 1 (FSP1; previously called AIFM2) (Bersuker et al. 2019; Doll et al. 2019). Currently, it is not clear whether any final executioner proteins of ferroptosis exist, since the process essentially depends on the propagation of lipid peroxidation chain reactions and the ultimate failure of protective antioxidant mechanisms, progressively leading to irreparable membrane and organelle dysfunction. Importantly, induction of ferroptosis by inhibition of GPX4 and/or FSP1 is effective at killing multiple types of cancers in vitro and in vivo (Badgley et al. 2020; Bersuker et al. 2019; Doll et al. 2019; Hangauer et al. 2017; Tousignant et al. 2020; Viswanathan et al. 2017; Zhang et al. 2019; Zou et al. 2019). Thus, the stimulation of ferroptosis in tumors may offer new opportunities for effective cancer treatment. However, certain types of cancer cells are resistant to known ferroptotic inducers suggesting that additional modulators of ferroptotic sensitivity exist.

Emerging studies point to a crosstalk between ferroptosis and lipid droplets. Diffuse large B cell lymphoma cancer cells treated with imidazole ketone erastin (IKE), which blocks cystine uptake and promotes ferroptosis by depleting glutathione, display a decrease in the levels of PUFA-containing phospholipids and TAGs, possibly as a consequence of a cell protective mechanism that removes oxidized PUFAs from these lipids (Zhang et al. 2019). The decrease in TAGs could be a consequence of elevated lipolysis, since IKE treatments led to a significant

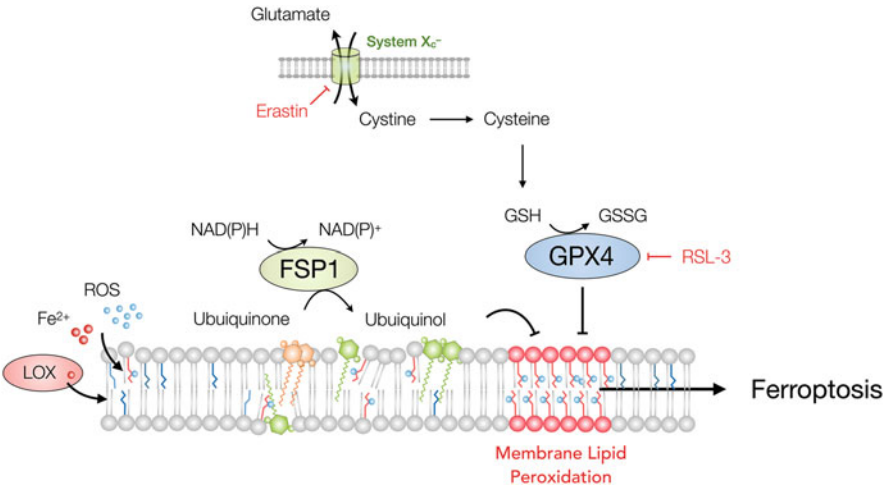

Fig. 3 Ferroptosis is a consequence of lethal membrane lipid peroxidation. Polyunsaturated fatty acids (PUFAs), mostly residing in membrane phospholipids, are particularly susceptible to oxidation by reactive oxygen species (ROS), non-enzymatic Fe^{2+}-mediated reactions, and lipoxygenase (LOX)-mediated peroxidation. The propagation of lipid peroxidation chain reactions along with a failure of antioxidant mechanisms leads to irreparable cell damage and cell death. Cells possess two complementary mechanisms of protection against ferroptosis. The first depends on cystine import, which is necessary for glutathione (GSH) synthesis, the main redox buffer in the cell, that is in turn required for the activity of glutathione peroxidase 4 (GPX4). GPX4 converts toxic PUFA peroxides into harmless lipid alcohols. The second mechanism depends on the activity of ferroptosis-suppressor-protein 1 (FSP1), which is necessary for the NAD(P)H-dependent regeneration of ubiquinol (coenzyme Q10), the major lipophilic antioxidant in cell membranes. Blocking cystine import by erastin or inhibition of GPX4 activity by RSL-3 results in a failure of the GPX4 antioxidant system, accumulation of lipid peroxides, and ferroptotic cell death

upregulation of ATGL expression, along with enzymes involved in de novo FA synthesis, phospholipid remodeling, and several lipoxygenases. This may indicate that PUFAs are first released from lipid droplets by ATGL and then incorporated in membrane phospholipids, thereby contributing to the lethal membrane lipid peroxidation caused by IKE (Fig. 4). In line with this idea, treatments with the lipophilic antioxidant ferrostatin prevented IKE toxicity and increased TAG accumulation in the cells. This is also in accordance with our studies in breast cancer cells showing that depletion of ATGL suppresses PUFA-induced oxidative stress and rescues cells from PUFA lipotoxicity, whereas lipid droplet biogenesis protects against PUFA lipotoxicity (Jarc et al. 2018). These findings suggest that in some cancer cells, lipid droplet breakdown via lipolysis may promote ferroptotic cell death.

Recent findings provide more support for the idea that lipid droplet breakdown regulates ferroptosis sensitivity. Several types of therapy-resistant cancer cells have been shown to be particularly sensitive to ferroptosis (Tousignant et al. 2020; Viswanathan et al. 2017). Namely, drug-resistant prostate cancer cells undergo an extensive metabolic reprogramming characterized by increased lipid uptake that drives lipid droplet accumulation and phospholipid remodeling. The latter results

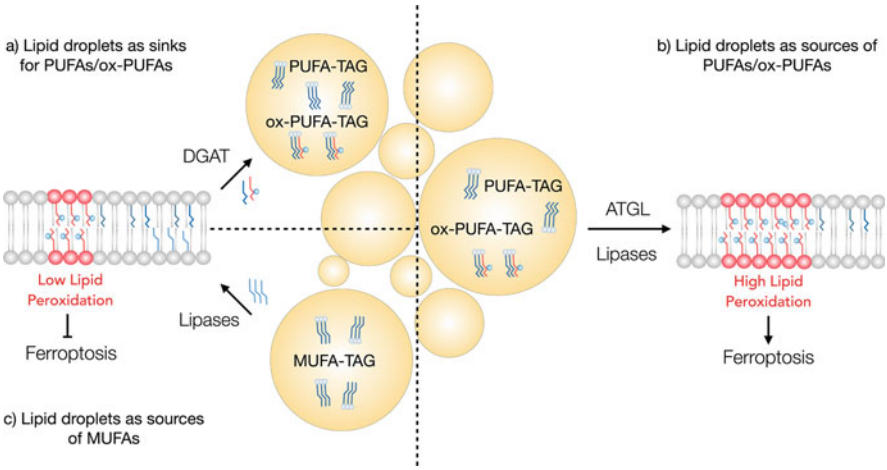

Fig. 4 Potential crosstalk between lipid droplets and ferroptosis. Lipid droplets may modulate ferroptosis by regulating polyunsaturated fatty acid (PUFA) trafficking. (**a**) Lipid droplet formation via DGAT-mediated triglyceride (TAG) synthesis may act as a sink for phospholipid-derived PUFAs, thus preventing their peroxidation; lipid droplet biogenesis may also restrict lipid peroxidation by sequestering already damaged, peroxidized PUFAs (ox-PUFAs) to suppress the propagation of lipid peroxidation. (**b**) ATGL-mediated TAG lipolysis may provide PUFAs for membrane synthesis, thus stimulating lipid peroxidation and sensitizing cells to ferroptosis. Other lipases and phospholipases may also release ox-PUFAs from TAGs or phospholipids. (**c**) ATGL may also provide monounsaturated fatty acids (MUFAs) that reduce the abundance of oxidizable PUFAs in membranes, thereby restricting lipid peroxidation

in elevated membrane PUFA content, thereby increasing lipid peroxidation and dependence on GPX4 activity (Tousignant et al. 2020). Counterintuitively, a depletion of TAGs and CEs was also observed, indicating the possibility that lipid droplet-derived lipids are consumed for phospholipid synthesis and thus mediate ferroptosis sensitivity. The study suggests that some other lipid species, such as acylceramides, concurrently drive the formation of a separate population of lipid droplets (Senkal et al. 2017; Tousignant et al. 2020). Interestingly, lipid droplets have also been suggested to sensitize breast cancer cells to ferroptosis via ATGL-mediated lipolysis in a cell density-dependent manner (Panzilius et al. 2018). Moreover, lipid droplet breakdown via lipophagy has recently been shown to promote GPX4 inhibition-induced ferroptotic cell death in hepatocytes (Bai et al. 2019). Finally, ferroptosis has been identified as a specific vulnerability of clear-cell carcinomas, whereby HILPDA, albeit acting in an ATGL-independent manner, mediates a HIF-2-α-dependent enrichment of PUFAs into TAGs and phospholipids (Zou et al. 2019). Collectively, these findings suggest that PUFA-TAGs stored within lipid droplets are drivers of ferroptotic sensitivity, most likely by providing PUFAs for phospholipid membrane synthesis (Fig. 4). Moreover, since TAGs stored within lipid droplets may also be oxidized, it is possible that lipid droplets themselves are sites of lipid peroxidation that promote ferroptosis if peroxidized lipids are not efficiently removed (Ramakrishnan et al. 2014; Veglia et al. 2017)**.** In line with

this idea, the Spastin/ABCD1/ESCRT-III lipid droplet-peroxisome tethering complex is necessary for the removal of peroxidized lipids from lipid droplets, which implicates both organelles in protecting cells against lipid peroxidation and possibly ferroptosis (Chang et al. 2019).

On the other hand, depending on the fatty acyl composition of lipid droplets and the predominantly released species, lipid droplet breakdown should also be able to protect from ferroptosis (Fig. 4). Accordingly, lipolysis of monounsaturated FA (MUFA)-enriched TAGs protects aggressive breast cancer cells from PUFA-induced oxidative stress and lipotoxicity, likely by reducing the relative abundance of membrane-resident PUFAs available for peroxidation (Ackerman et al. 2018; Jarc et al. 2018). In addition, the lipolytic release of MUFAs has been recently shown to promote mitochondrial biogenesis and oxidative metabolism via PLIN5-mediated allosteric activation of SIRT1 (Najt et al. 2019), which may additionally explain their beneficial effects on redox metabolism. However, lipid droplet biogenesis was not necessary for the ability of exogenous MUFAs to suppress erastin-induced ferroptosis (Magtanong et al. 2018). Instead, their ASCL3-dependent incorporation into plasma membrane phospholipids and displacement of PUFAs was found to be responsible for the effect in several cancer cell lines. The ability of lipid droplet biogenesis and/or breakdown to modulate ferroptotic sensitivity surely requires further exploration, particularly in the sense that combined targeting of lipid droplet turnover and the anti-ferroptotic redox machinery may prove to be a valid therapeutic strategy.

4 Conclusions and Perspectives

Given their central role as coordinators of lipid metabolism with cell growth and stress resistance, lipid droplets are emerging as potentially vulnerable hotspots in numerous cancers. However, we are only beginning to understand how lipid droplets respond to the various stressful conditions encountered by cancer cells and which are the essential tasks that these organelles perform to support the cellular stress response. We have to find out more about the particular mechanisms involved in order to use this knowledge in cancer treatment. Numerous points in their biogenesis and/or breakdown could potentially be targeted in order to either compromise the ability of lipid droplets to protect cancer cells from stress or to purposefully use lipid droplets to cause cell damage. For example, inhibiting lipid droplet biogenesis in starving cells dependent on autophagy for their survival could increase mitochondrial damage due to the build-up of cytosolic FAs and acylcarnitines (Nguyen et al. 2017). The inhibition of lipid droplet biogenesis in poorly vascularized tumors could abolish their function as long-term lipid reservoirs and compromise the ability of cancer cells to survive prolonged periods of starvation or resume growth upon reoxygenation (Bensaad et al. 2014; Jarc et al. 2018; Pucer et al. 2013). During the final stages of revision of this manuscript, two important papers were published showing that DGAT1-mediated lipid droplet biogenesis is a relevant target for the

treatment of melanoma and glioblastoma (Cheng et al. 2020; Wilcock et al. 2020). DGAT1 was even identified as a bona fide oncoprotein that enables enhanced lipid uptake and drives melanoma formation. Its ability to protect cancer cells from oxidative stress and membrane lipid peroxidation, which hints at protection from ferroptosis as well, was found pivotal for melanoma aggressiveness (Wilcock et al. 2020). Compromising the ability of cancer cells to form lipid droplets could also impair their chemoresistance and immune evasion (Cotte et al. 2018). Finally, recent studies have revealed that lipid droplets may also regulate drug efficacy by affecting the selective partitioning of lipophilic drugs in their hydrophobic core and even promote drug activation in situ (Dubey et al. 2020; Englinger et al. 2020).

In other cases, the activation of lipid droplet breakdown could be a beneficial strategy. For example, stimulation of lipolysis or lipophagy is detrimental for cancer cells under certain conditions, since it may increase the levels of oxidative and ER stress, elevate lipid peroxidation and even lead to ferroptotic cell death (Jarc et al. 2018; Mukhopadhyay et al. 2017; Zhang et al. 2019; Zou et al. 2019). However, caution should be exerted, because in many instances lipid droplet breakdown in fact promotes the resistance of cancer cells to stress, as discussed at length in this review. Clearly, the feasibility of targeting lipid droplets should be carefully examined in different tumor types and particular contexts. In summary, lipid droplets are highly dynamic compartments that consolidate lipid uptake, synthesis, recycling, distribution, and breakdown pathways in the cell and are emerging as promising targets either to (1) restrict the supply of essential lipids or to (2) promote the accumulation of damaging lipids in order to compromise cancer cell survival, growth, and metastasis.

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Patterns of Ciliation and Ciliary Signaling in Cancer

Anna A. Kiseleva, Anna S. Nikonova, and Erica A. Golemis

Contents

Abstract Among the factors that have been strongly implicated in regulating cancerous transformation, the primary monocilium (cilium) has gained increasing attention. The cilium is a small organelle extending from the plasma membrane, which provides a localized hub for concentration of transmembrane receptors. These receptors transmit signals from soluble factors (including Sonic hedgehog (SHH), platelet-derived growth factor (PDGF-AA), WNT, TGFβ, NOTCH, and others) that regulate cell growth, as well as mechanosensory cues provided by flow or extracellular matrix. Ciliation is regulated by cell cycle, with most cells that are in G0 (quiescent) or early G1 ciliation and cilia typically absent in G2/M cells. Notably, while most cells organized in solid tissues are ciliated, cancerous transformation induces significant changes in ciliation. Most cancer cells lose cilia; medulloblastomas and basal cell carcinomas, dependent on an active SHH pathway, rely on ciliary maintenance. Changes in cancer cell ciliation are driven by core oncogenic pathways (EGFR, KRAS, AURKA, PI3K), and importantly ciliation status regulates functionality of those pathways. Ciliation is both influenced by targeted cancer therapies and linked to therapeutic resistance; recent studies suggest ciliation may also influence cancer cell metabolism and stem cell identity. We review recent studies defining the relationship between cilia and cancer.

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1 Introduction

Although the primary monocilium (hereafter simply designated cilium) was first identified as a cellular organelle more than 120 years ago (Zimmerman 1898), it is only recently becoming appreciated as relevant to cancer. Structurally, the cilium arises from a "root," termed the basal body, which resides beneath the surface of the plasma membrane. From the basal body, nine tubulin doublets arranged as a hollow ring form a cytoskeletal structure termed the axoneme, which typically extends 3–10 μm from the cell surface and is coated by a ciliary membrane (Fig. 1). Early observational studies characterized populations of ciliated versus unciliated cells and established that ciliation varied during organismal development and that cells oscillated between ciliated and non-ciliated states in cycling cells (reviewed in Wheatley 2005). In developed organisms, many types of cell (including epithelial, endothelial, neuronal, and fibroblast) are ciliated. The presence of a cilium was established as unequivocally linked to a non-mitotic state, based on the observation that cellular centrioles function either as components of the centrosome, organizing a bipolar spindle in mitosis, or as components of the basal body; both functions cannot be performed simultaneously. However, the biological function of cilia was long elusive.

Two primary areas of ciliary function have been explored. First, based on the idea that ciliation typically coincides with the quiescent state (G0 or early G1), cilia have been proposed to function as checkpoints for cell cycle progression (Kim and Tsiokas 2011). This hypothesis has proven difficult to validate, in part because many proteins that influence ciliation also regulate cell cycle progression (Hua and Ferland 2018). Second, cilia were identified as functional "antennas," protruding from the cell surface into vesicular lumens (Wilson 2011) or contacting the extracellular matrix (Seeger-Nukpezah and Golemis 2012). Signals received and transduced in part or in sum by cilia include mechanical cues and a suite of soluble ligands including Sonic hedgehog (SHH), PDGF-AA, WNT, TGFβ, and NOTCH (Kiseleva et al. 2019). Compatible with their role as antennas, cilia concentrate receptors for these ligands on a specialized ciliary signaling membrane (Garcia et al. 2018), with activation of these receptors providing spatially and temporally localized intracellular signals that activate intracellular signaling pathways regulating cell polarization, differentiation, and proliferation.

Ciliary structural integrity and signaling were first identified as important for guiding embryonal morphogenesis and maintenance of tissue homeostasis in adults. Insight into these roles was provided from a study of naturally occurring mutations of genes encoding proteins important for ciliary structure and function and specific

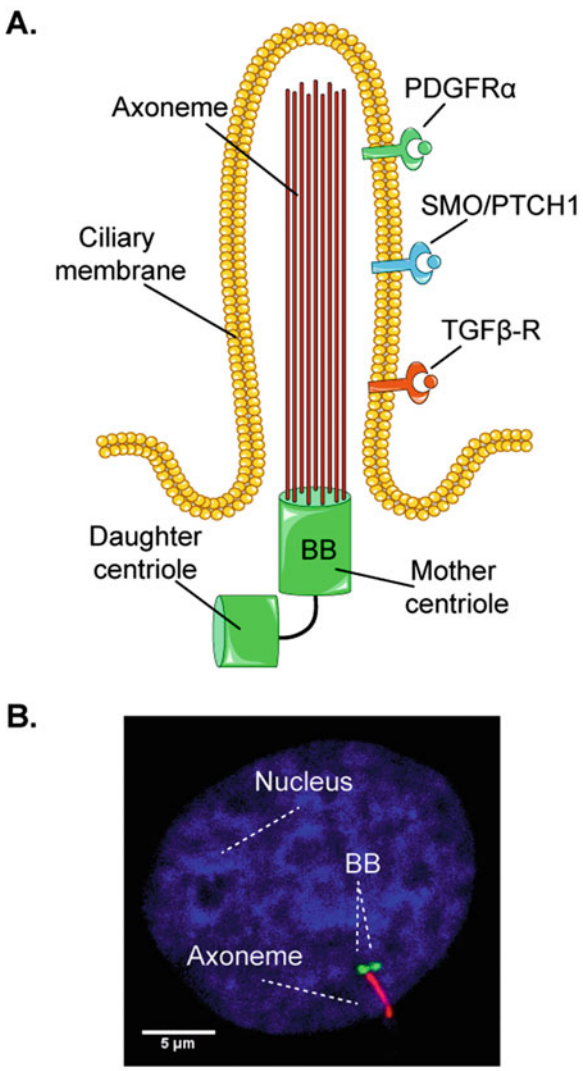

Fig. 1 The structure of the primary cilium. (**a**) The core of the primary cilium consists of a microtubule-based axoneme that is surrounded by a membrane bilayer continuous with the plasma membrane of the cell. Ciliary membrane contains different receptors that transduce signals through the primary cilium, including those that activate PDGFRα (PDGF-AA), the SMO/PTCH1 system (SHH), and TGFβ signaling pathways. The axoneme extends from the basal body (BB) – a structure that consists of the mother and daughter centrioles which anchor the cilium within the cytoplasm. (**b**) Immunofluorescence image of the primary cilium in the hTERT-RPE1 cell line. The cilia (red) is visualized with ARL13B, the basal body (green) with γ-tubulin, and the nucleus (blue) with DAPI. Scale bar, 5 μm

signaling proteins that predominantly localize to cilia. Such mutations cause a class of specialized developmental disorders termed ciliopathies, of which the most common are autosomal dominant polycystic kidney disease (ADPKD) and other forms of PKD, Meckel-Gruber Syndrome, Bardet-Biedl Syndrome, and Joubert Syndrome (Hildebrandt et al. 2011). The gross pathological manifestations of the ciliopathies reflect defects in polarization, proliferation, migration, and other molecular processes at the cellular level. Importantly, many of these processes are also distorted in cancer (Hanahan and Weinberg 2011; Seeger-Nukpezah et al. 2015), suggesting a possible relation between ciliation and cell transformation.

Although investigations in this area lag far behind studies of ciliopathies, a growing number of studies have documented changes in ciliation in the context of cancer initiation, progression, and therapeutic response. Notably, a number of studies have identified a significant reduction of ciliation in many types of solid tumor, potentially affecting cilia-related signaling. Signaling systems dependent on or influenced by ciliary receptors (including SHH, PDGF-AA, WNT, and NOTCH) play important roles in conditioning many aspects of cell growth, positioning loss of ciliation to have a significant biological action. Differences in ciliation between cancer cells and cells such as fibroblasts in the tumor microenvironment contribute to asymmetric exchange of signals that promote the disease. A growing number of protein-targeted therapies have unexpectedly been identified as affecting both the structural integrity of the primary cilium and cilia-associated signaling; such cryptic manipulation of cilia within tumors may alter cell-cell communication and contribute to therapeutic response. In this review, we will summarize recent findings bearing on the role of ciliation in cancer, highlighting priority areas for future research.

2 Ciliation and Cancer Cells

A feature of many types of solid tumor is loss of ciliation on cancer cells, which reduces or alters response to extracellular cues. However, cancer-associated changes in ciliation vary among different tissue and cell types within the tumor microenvironment (cancer cell, cancer-associated fibroblasts (CAFs), immune cells). First addressing cancer cells, cilia loss occurs during early stages of breast (Menzl et al. 2014; Yuan et al. 2010), pancreatic (Seeley et al. 2009, Schimmack et al. 2016), prostate (Hassounah et al. 2013), ovarian (Egeberg et al. 2012), and other cancer types. In some of these cancer types, experiments in which loss of ciliation was experimentally induced have demonstrated that this loss directly promotes tissue disorganization and oncogenesis and enhances cancer-related signaling (Hassounah et al. 2017; Cano et al. 2006). It has been proposed that changes in ciliation have a role in controlling signaling important for the pathogenesis of multiple endocrine-related cancers that are influenced by circulating hormones, given a number of hormones (somatostatin, melanin-concentrating hormone, insulin-like growth factor 1) have receptors on cilia, while other hormones (leptin, insulin, prolactin) influence ciliary length, thereby conditioning ciliary signaling activity (reviewed in O'toole and Chapple 2016). There is growing evidence that ciliation regulates specialized proteasomal activity, providing a general mechanism by which the presence or absence of this organelle can impact multiple downstream targets (Gerhardt et al. 2016).

For other types of cancer, the relationship between ciliation and transformation is either ambiguous or irrelevant. For glioblastoma multiforme (GBM), one study established cilia were retained in subpopulations of cells within 23 of 23 human tumors (Sarkisian et al. 2014). A second study described ciliogenesis as largely

disrupted at early stages of tumor development; a limited number of residual cilia in GBM cells had observable structural defects (Moser et al. 2014). There is some dispute as to whether the residual cilia promote or inhibit GBM pathogenesis (Sarkisian and Semple-Rowland 2019). Certainly, ciliation affects GBM-relevant signaling. For example, the lysophosphatidic acid receptor 1 (LPAR1) localizes to the primary cilia of human astrocytes (one of the cell types that can transform into GBM). LPAR1 is a G-protein-coupled receptor that works in a complex with Gα12/Gαq subunits, which are excluded from the cilia, inhibiting LPAR1 activation in normal cells. The loss of primary cilia relocalizes LPAR1 to the plasma membrane of cells, where it binds to Gα12/Gαq and stimulates cell proliferation. Treatment of mice bearing GBM patient-derived xenografts with a LPAR antagonist blocks tumor growth, indicating an important tumor-promoting function of at least one signaling system activated by loss of ciliation (Loskutov et al. 2018).

On the extreme end of ciliary irrelevance, neoplasms of the immune system (leukemias, lymphomas, myelomas) lack cilia. Conversely, some tumor types that require activity of the SHH/SMO/PTCH1/GLI2 signaling axis instead depend on retention of cilia. Medulloblastomas arising from constitutively active SMO, an SHH receptor which localizes to cilia, require the continued presence of cilia to support their growth; in contrast, medulloblastomas arising from constitutive activation of the transcription factor GLI2, a downstream transducer of SHH/SMO signals, require loss of cilia to eliminate signals that inhibit GLI2 (Han et al. 2009). Basal cell carcinomas are similar, with tumors that arise from common activating mutations of PTCH1 or SMO typically retaining cilia and requiring ciliation for active growth (Wong et al. 2009; Pellegrini et al. 2017).

There has been considerable interest in understanding how these changes in cancer cell ciliation are mediated. For many cases where cilia are known to be lost in cancer, this is ultimately associated with downregulation of genes required for ciliary formation (Cano et al. 2006). A number of upstream cues have been identified that induce loss of ciliation (Fig. 2). Importantly, and reflecting the fact that ciliation is fundamentally oscillatory during the cell cycle, many of these cues are directly linked to oncogenes that promote cell cycle progression and proliferative signaling and hence are commonly overexpressed or mutationally activated in cancer (Seeger-Nukpezah et al. 2013a). Most central among these, the mitotic kinase Aurora-A (AURKA) is a key and proximal regulator of ciliary disassembly (Pugacheva et al. 2007; Plotnikova et al. 2012a). AURKA activation is required to initiate axoneme destabilization and cilia resorption; AURKA is overexpressed in many cancer types, providing a common stimulus for deciliation. Further, AURKA activity is regulated by a number of associated proteins that are also known to function as oncogenes (e.g., PLK1 (Lee et al. 2012), NEDD9 (Nikonova et al. 2014), β-catenin (Dere et al. 2015)) or tumor suppressors (e.g., von Hippel-Lindau (VHL) (Xu et al. 2010; Thoma et al. 2007)) (reviewed in Korobeynikov et al. 2017). KRAS, commonly activated by mutations in many forms of cancer, was shown to efficiently induce deciliation in pancreatic cancer; among the major KRAS effectors, PI3K and MEK1/2 signaling are important mediators of this response (Seeley et al. 2009). Activation of KRAS helps to promote AURKA activation (Deng et al. 2018); in addition, PI3K activation

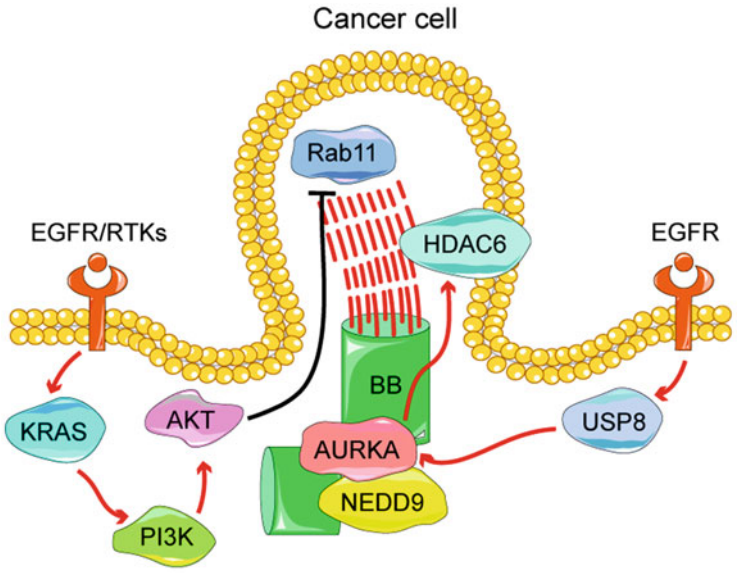

Fig. 2 Schematic representation of the processes underlying the loss of primary cilia in cancer cells. One of the key events determining ciliary loss is upregulation of the mitotic kinase Aurora-A (AURKA), activation of which in normal cells triggers ciliary disassembly. As cells enter G1 from quiescence, AURKA activation is promoted by stabilizing interactions with an upregulated scaffolding protein, NEDD9, and additional factors. Upstream, activation of AURKA is induced in normally cycling cells in part by transient mitogenic signals emerging from receptor tyrosine kinases such as EGFR, transmitted through a RAS/PI3K/AKT cascade. Deciliation of cancer cells is promoted by overexpression of EGFR, based on EGFR-associated activation of KRAS and PI3K/AKT, or activating mutations in KRAS. Besides inducing NEDD9, EGFR activation suppresses the AURKA inhibitor USP8; active PI3K and AKT inhibit RAB11, a key player controlling ciliary assembly. AURKA activation initiates axoneme destabilization and cilia resorption through phosphorylation of its downstream target histone deacetylase 6 (HDAC6) and additional targets

of its downstream effector, AKT, inhibits new ciliogenesis (Walia et al. 2019). The epidermal growth factor receptor (EGFR), a receptor tyrosine kinase overexpressed or mutationally activated in many solid tumors, activates KRAS and other factors that cause profound deciliation (Kasahara et al. 2018). Overall, multiple forms of oncogenic activation, or loss of VHL, particularly common in renal cell carcinoma, can contribute to loss of cilia by influencing activity of AURKA. In addition to overt oncogenes and tumor suppressors, a number of AURKA-interacting proteins have been identified that also influence AURKA activity (Korobeynikov et al. 2017); some of these, such as HDAC2, have been shown to contribute to loss of ciliation in some forms of cancer (Kobayashi et al. 2017). A summary of ciliation status as reported for multiple cancer types is provided in Table 1.

Table 1 Ciliation status in different cancer types

Cancer type	Ciliation status	Description	Relation to cilia-related genes/pathways
Skin/basal cell carcinoma (BCC)	Positive/ mixed	Cilia found in five of eight human basal cell carcinoma (BCC) biopsies (Wong et al. 2009)	Cilia supporting Hedgehog pathway activation (Kuonen et al. 2019; Wong et al. 2009). Removal of cilia in established tumors accelerated growth due to accumulation and overactivation of GLI2 (Wong et al. 2009). Loss of primary cilia in BCC leads to a switch from Hedgehog-dependent to KRAS-dependent tumor growth (Kuonen et al. 2019)
Brain/ medulloblastoma	Mixed phenotype	Some medulloblastomas have primary cilia; others do not (Han et al. 2009)	Cilia positively correlates with the activation of Hedgehog and WNT pathways and is important to maintain signaling transduction through these pathways (Han et al. 2009)
Gastric	Positive?	A small percentage of ciliated cells (approx. 5%) were found in gastrointestinal stromal tumors (GIST) based on an ultrastructural study (Castiella et al. 2013)	Unclear
Colorectal	Controversial	Colorectal adenocarcinoma cells have primary cilia, whereas normal colon epithelial cells do not (Senicourt et al. 2016)	The presence of cilia is important to maintain Hedgehog signaling in colorectal tumors (Senicourt et al. 2016). However, in another study, colorectal tumors were associated with ciliary loss connected to a deficiency of TTLL3 (Rocha et al. 2014)
Skin/melanoma	Negative	Primary cilium loss was observed in 16 cases of melanoma in situ, 16 primary invasive melanomas, and 8 metastatic melanomas (Kim et al. 2011). In a second study, human melanoma biopsies had a 4% ciliation rate, whereas 25% of melanocytes in nevi were ciliated (Snedecor et al. 2015)	Overexpression of EZH2, a transcriptional repressor of many cilia-associated genes; in addition, loss of cilia leads to the overactivation of protumorigenic Wnt/β-catenin signaling pathway (Zingg et al. 2018)

(continued)

Table 1 (continued)

Cancer type	Ciliation status	Description	Relation to cilia-related genes/pathways
Brain/glioblastoma multiforme (GBM)	Negative/ controversial	No normal cilia observed in GBM cells in human biopsies or cell lines; residual cilia have structural defects (Moser et al. 2014). In an independent study, some subpopulations of GBM tumor cells were reported as ciliated (Sarkisian et al. 2014)	PCM1 expression inhibits ciliogenesis and enhances proliferation (Hoang-Minh et al. 2016). Loss of cilia repositions the LPAR1 receptor, overactivating LPA signaling and increasing cell proliferation (Loskutov et al. 2018). Cilia release EV-containing growth factors, supporting tumor growth (Hoang-Minh et al. 2018)
Breast	Negative	Decrease (in basal subtype) or complete loss of cilia in breast cancer cell lines and breast cancer biopsies (Yuan et al. 2010). The loss of cilia in cancer cells and in cancer-associated stroma occurs gradually throughout the tumor development (Menzl et al. 2014)	Downregulation of ciliary genes *YNC2H1*, *IFT46*, *PKD2*, *NPHP3*, *BBS2*, *BBS4*, and *TTC8*, overexpression of *AURKA* (Ferchichi et al. 2013; Plotnikova et al. 2012b) and *NEK2* (Hayward et al. 2004), were associated with ciliary loss (Menzl et al. 2014)
Pancreas/pancreatic ductal adenocarcinoma (PDAC)	Negative	Ciliogenesis is repressed, based on examination of a panel of human PDAC biopsies (Seeley et al. 2009)	Oncogenic KRAS signaling induces ciliary loss (Seeley et al. 2009). Inhibition of histone deacetylase 2 (HDAC2) inhibits AURKA and restores ciliogenesis (Kobayashi et al. 2017)
Kidney/renal cell carcinoma (RCC)	Negative	RCC biopsies are characterized by ciliary loss, particularly in tumors with mutations of *VHL* (Basten et al. 2013)	Mutations in *VHL* gene impair ciliogenesis (Schermer et al. 2006). VHL null cells have abnormal activation of AURKA, driven in part by β-catenin-dependent transcription (Dere et al. 2015)
Prostate	Negative/ mixed	Ciliation rates were decreased in human biopsies representing different stages of prostate cancer (prostatic intraepithelial neoplasia and invasive prostate cancer) (Hassounah et al. 2013)	Cilia loss is associated with overactivated Wnt/β-catenin signaling pathway (Hassounah et al. 2013). Overexpressed centrosomal protein TACC3 downregulates cilia formation by disrupting filamin

(continued)

Table 1 (continued)

Cancer type	Ciliation status	Description	Relation to cilia-related genes/pathways
			A-meckelin interactions (Qie et al. 2020)
Ovarian	Negative/ mixed	Common ovarian cancer cell lines (SKOV3 and OVCAR3) are ciliated but have lower ciliation rates than normal ovarian epithelium (Egeberg et al. 2012). No data on ciliation rates in human biopsies available	Hedgehog, PDGFRα pathways and *AURKA*, are dysregulated (Egeberg et al. 2012). Overexpression of ASAP1 (a scaffold organizing protein regulating ciliary transport (Wang et al. 2012)) is associated with a poor prognosis (Hou et al. 2014)

3 Ciliation in the Tumor Microenvironment

It is now strongly appreciated that many behaviors of cancer cells in vivo depend on their interaction with multiple non-transformed cell types in the extracellular milieu (Maman and Witz 2018). These include tumor-infiltrating cells of the immune system, endothelial cells, and CAFs. Among these, myeloid and lymphoid cells lack cilia entirely, although some proteins associated with the ciliary basal body and targeted vesicular trafficking machinery are repurposed to form the immune synapse, a structure necessary for antigen recognition (Hua and Ferland 2018; Stephen et al. 2018; Finetti et al. 2015). Endothelial cells are typically ciliated, with the cilia coordinating mechanosensory signals provided by blood flow and influencing response to secreted factors such as bone morphogenetic proteins (BMPs) that maintain integrity of blood vessels (Vion et al. 2018). Given common hypoxia, tissue remodeling and vasculogenesis in tumors, and given that the cilia-regulating VHL/HIF1 system is an essential mediator of response to hypoxia, it is likely that ciliary function is altered, but to date, this topic has received surprisingly little study.

In contrast, much effort has gone into the investigation of ciliation in CAFs and pericytes (fibroblast-like cells that typically associated with blood vessels). These stromal cells act as a "feeding layer," producing growth factors that support cancer cell proliferation. Moreover, stromal cells protect tumor by forming a physical barrier and significantly decreasing the permeability of anticancer drugs. These cells typically are ciliated; notably, the presence of cilia on fibroblasts, but not cancer cells, establishes an asymmetric relationship in which these two types of cells respond differently to signaling cues such as SHH in the extracellular environment. One of the cancer types that is highly dependent on such an asymmetric signaling exchange between tumor and stroma is pancreatic ductal adenocarcinoma (PDAC), a type of cancer in which 90% of the tumor mass may comprise of stromal fibroblasts. In an elegant study, Tape et al. demonstrated that following KRAS transformation, PDAC cells produce and secrete extracellular SHH ligand. The PDAC cells themselves do not respond to this ligand, because KRAS causes ciliary resorption;

instead, SHH binds to its SMO receptor on the cilia of the stromal fibroblasts and stimulates them to produce a variety of growth factors, such as IGF1 and GAS6, that support tumor growth and are responsible for maintaining cancer cell signaling cascades involving AKT and other intermediates that promote tumor cell survival (Tape et al. 2016).

4 Targeted Cancer Therapies and Cilia

For decades, oncogenes such as EGFR (Chong and Janne 2013), AKT (Nitulescu et al. 2018), and AURKA (Nikonova et al. 2013) have been the subject of targeted drug development for control of cancer. Given these oncogenes influence ciliation, targeted inhibitors blocking oncogenic activity would also be expected to influence ciliation. There are a growing number of examples of cancer therapies inhibiting or increasing ciliation. For example, AURKA inhibitors such as alisertib cause striking stabilization of ciliation (Nikonova et al. 2014). The EGFR inhibitors gefitinib (Khan et al. 2016; Valencia-Gattas et al. 2016) and erlotinib (Kiseleva et al. 2019) cause restoration of ciliation. Conversely, inhibitors of heat shock protein 90 (HSP90), a chaperone for expression and activity of AKT, EGFR, and other oncogenic proteins, cause profound loss of ciliation (Nikonova et al. 2018).

It is likely that many additional drugs influence ciliation. For example, one study used engineered cell lines with GFP-tagged cilia (Zhang et al. 2019) to evaluate ~170 targeted kinase inhibitors for effects on ciliation. This identified inhibitors of PDGF and VEGF receptor family members (sunitinib, VEGFR inhibitors I and II, SU11652), GSK3β (GSK3β inhibitor XI) and CHK1 (SB218078) as compounds inducing ciliary disassembly, and inhibitors of interleukin-1 receptor-associated (IRAK) kinases (IRAK1/4 inhibitor), EGFR (Erlotinib, PD174265), MEK kinase (MEK 1/2 inhibitor), and a dual inhibitor of DNA-PK/PRKDC and PI3K/PIK3CB (DNA-PK inhibitor III) as proteins blocking disassembly (Kiseleva et al. 2019). In a broader screen, Khan et al. used a high-content analysis-based approach to screen a library of 1,600 compounds in PDAC cells to identify 118 drugs that stimulated cilia formation or elongation (Khan et al. 2016). This screen included not only drugs used for cancer but for other diseases, with examples of strongly active compounds including clofibrate, gefitinib, sirolimus, imexon, dexamethasone, and others. Surprisingly, some compounds with activity in promoting ciliation included ion channel modulators, DNA gyrase/topoisomerase inhibitors, antibacterial compounds, and COX inhibitors. For these compounds, given no known target relevant to direct control of ciliogenesis, it remains to be determined whether the ciliation phenotype was direct or a secondary consequence of the fact that most of the hits also reduced cell proliferation. Whatever the mechanism, ciliation is clearly affected; this implies that drugs used, for example, to target COX proteins, may have the unexpected consequence of systematically altering cellular responses to SHH and other proteins with ciliary signaling receptors.

In some ciliopathies, because of the essential involvement of defective ciliary signaling to the disease course, there has been considerable interest in directly targeting ciliation to ameliorate disease symptoms. For example, ADPKD arises from mutations in two genes, PKD1 or PKD2, which produce proteins that heterodimerize at cilia; in genetic studies combining mutation of genes leading to ciliary loss with mutation of PKD1 or PKD2, disease symptoms were much reduced (Ma et al. 2013). Similar results were seen applying drugs that target ciliation to mouse models of ADPKD; treatment with an AURKA inhibitor, which stabilizes cilia, worsened ADPKD symptoms (Nikonova et al. 2014), whereas treatment with inhibitors of EGFR (Nikonova et al. 2015) or HSP90 (Seeger-Nukpezah et al. 2013b, Smithline et al. 2014, Nikonova et al. 2018), which eliminate cilia, improved ADPKD symptoms. Although provocative, these studies are not conclusive as to the value of targeting ciliation, as the drugs involved also have significant non-ciliary biological effects. In cancer, whether targeting cilia with drugs would have beneficial effect or not is more complex, given the fact that solid tumors are comprised of multiple ciliated and non-ciliated cell types, coupled with the fact that ciliary signals can be cancer-promoting or cancer-restricting, depending on context. Some insight into whether targeting ciliary signaling might be beneficial or deleterious is provided indirectly from studies performed in PDAC, a cancer type that is highly dependent on tumor-fibroblast interactions mediated by SHH, which result in stromal activation (also known as desmoplasia), a process in which CAFs support tumor growth (Sahai et al. 2020; Tape et al. 2016). Hence, a rational approach to control PDAC was to explore the effect of inhibiting SHH, to limit desmoplastic response. Unfortunately, and in contrast to expectation, although inhibition of SHH reduced stromal growth and activation, such treatment increased metastasis and reduced survival, both in mouse models and clinical trials (Lee et al. 2014; Rosow et al. 2012). A similar result was seen in mouse models of bladder cancer (Shin et al. 2014). These results emphasize the role of the stroma in restraining tumor dispersion, as well as promoting tumor growth, and provide a strong caution against targeting ciliation in these systems.

In contrast, in medulloblastoma and basal cell carcinoma, stromal contribution is much less important, and the cancer cell itself is ciliated and in many cases dependent on ciliation, as noted above. In this setting, genetic ablation of cilia blocked initiation and early growth of medulloblastoma (Han et al. 2009) and BCC (Wong et al. 2009) dependent on mutations targeting SMO and PTCH1. However, these inhibitors were not effective in medulloblastomas or BCCs associated with driver lesions in the downstream pathway effector GLI2. Further, treatment of medulloblastomas with the SMO inhibitor sonidegib stimulates ciliary loss. This was associated with recurrent mutation of the ciliary gene *OFD1*, promoting cilia resorption, and caused cells and to become resistant to SMO inhibitors and dependent on noncanonical Hedgehog signaling (Zhao et al. 2017). Similar results were obtained following treatment of BCCs with SMO inhibitors (Kuonen et al. 2019). BCCs resistant to SMO inhibitors, not only was there a profile of increased mutation in multiple genes required for forming cilia, but in addition, there was enhanced activation of the RAS/MEK/ERK signaling pathway. In these cases, further

exploration of ciliary targeting may be useful; but in all cases, care is needed to avoid paradoxical worsening of disease.

5 Cilia, Ectosomes, and Exosomes

In addition to their role in receipt of signals, some recent studies have suggested a broader role of cilia in bidirectional transmission of specific cues from the ciliated cell to the extracellular environment. Extracellular vesicles (EVs, which include exosomes) are produced generally from the plasma membrane and have been shown to carry a variety of bioactive molecules, including enzymes, proteins, DNA, and mRNA, allowing their transport from one cell to another (Xu et al. 2018). Secretion of EVs is elevated in cancer, with the EVs performing a variety of functions, including helping activate the tumor microenvironment and preconditioning of metastatic niches.

Notably, studies of EVs in ciliopathies have noted some characteristic changes. For example, in a mouse model of autosomal recessive polycystic kidney disease (ARPKD), EVs containing PKD1, PKD2, and other cilia-related proteins coated the surfaces of cilia in the renal collecting duct. Further, the protein composition of EVs in a mouse model of ARPKD differed significantly from that found in healthy control mice (Hogan et al. 2009), as did those in human ADPKD patients from human controls (Hogan et al. 2015). These differences suggest use of exosomes which may be useful in diagnosis and have led some to propose a system of "urocrine signaling," by which secreted EVs directly support disease pathogenesis. Notably, secretion of specific types of EV has been linked to ciliation status. The exocyst is a highly conserved complex of proteins required for polarized vesicular secretion and is also required for ciliogenesis; loss of exocyst complex component 5 (EXOC5) results in formation of a very short cilium coupled to a significant reduction of EVs and dramatic differences in the protein composition of the EVs produced (Zuo et al. 2019a), as well as normal tubulogenesis in in vitro models (Zuo et al. 2019b). While these might be considered entirely separate consequences of EXOC5 loss, notably, genetic ablation of the intraflagellar transport protein IFT88 – with a primary function solely within cilia – had a similar effect on expression and composition of EVs. This raised the possibility that primary cilia serve as a driver for EV production (Zuo et al. 2019a) (Fig. 3).

Reciprocally, the ability of cilia to form specialized EVs has been observed in many organisms, over considerable evolutionary distance. In studies of *Chlamydomonas* flagella – one of the longest established model systems for study of cilia – release of agglutinin-containing vesicles from the flagella surface is important for two individuals to exchange signals that determine their mating behavior (Wood and Rosenbaum 2015). In higher eukaryotes, specialized EVs released by cilia are typically termed ectosomes. Their function is not yet well understood but currently a topic of intense study. Phua et al. have described a "decapitation," in which an ectosome is released from the ciliary tip early in ciliary

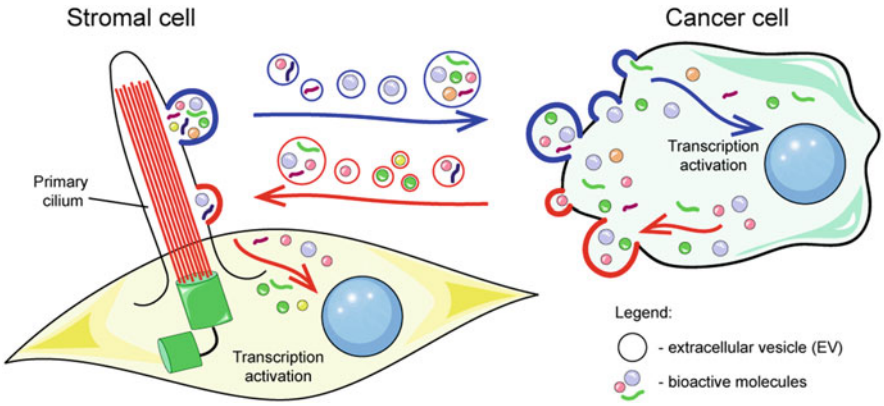

Fig. 3 Cilia take part in extracellular vesicular exchange between cancer cells and cells in the tumor microenvironment. Cell-cell signaling can be mediated by direct cell-cell interactions, by cell secretion of individual proteins, and by exchange of extracellular vesicles (EVs). In EVs, a lipid bilayer bearing a specific set of transmembrane proteins encompasses a small vesicle bearing additional proteins, nucleic acids, and other bioactive molecules. EVs play important roles in communication between cancer cells and stromal cells; the role of cilia in EV function is attracting considerable interest, based on the observations that cilia secrete EVs with specialized membrane composition, that some EVs selectively coat cilia and that some ciliary signaling proteins influence general cellular EV production

disassembly. The released vesicles are enriched for proteins that influence cell signaling and promote cilia growth; the authors suggested that decapitation helps remodel the composition of the cilium to prepare the structure for the disassembly (Phua et al. 2017). This process of decapitation, also known as ectocytosis, depends on the GTPase Rab7, which causes intraciliary actin polymerization in cycling cells (Wang et al. 2019); the factors regulating Rab7 in decapitation require further investigation.

Further, the fact that the ciliary membrane is distinct from the non-ciliary plasma membrane and contains many signaling receptors suggests that ectosomes may be useful in transmitting specific forms of transmembrane protein between cells. A study of the response to receipt of SHH signals identified secretion of a ciliary ectosome bearing G-protein-coupled receptors (GPCRs) from the ciliary tip (Nager et al. 2017). It is possible that vesicles produced by cilia may play an important role in progression of at least some forms of cancer. A recent study of GBM cell models found that quiescent GBM cells are ciliated and undergo decapitation (Hoang-Minh et al. 2018). Intriguingly, culture media collected from ciliated cell cultures stimulated the growth of proliferating GBM cells more than media collected from cells lacking cilia, suggesting that the ciliary vesicles may contain some mitogenic factors (Hoang-Minh et al. 2018). Much more work is required to address this important emerging field of study.

6 Summary

Two decades ago, almost nothing was known about the relevance of cilia to cancer. At present, the relation of ciliation and core cancer signaling processes is now well-established and is bidirectional. The role of ciliation as a cell cycle checkpoint remains less well defined, in part because of difficulty in designing experiments that cleanly separate removal or formation of cilia from additional effects on the cytoskeleton or cell cycle machinery. New ways in which ciliation affects cancer proliferation continue to arise. For example, signaling between TSPAN8, SMO, and PTCH1 at cilia in breast cancer stem cells has been found essential to maintain the expression of the stemness-promoting transcription factors NANOG, OCT4, and ALDHA1 (Zhu et al. 2019). This cilia-associated signaling is associated with therapeutic resistance, a common feature of stem and progenitor cells; in parallel, another recent study has suggested that many therapy resistant cells have upregulated cilia (Jenks et al. 2018). These linkages require more investigation, particularly addressing whether ciliation is causative or correlative of resistance in multiple settings.

Moving beyond growth and lineage controls, additional studies have begun to connect ciliation to core metabolism of fats and lipids, processes which are essential contributors to cancer pathogenesis (Font-Burgada et al. 2016; Hay 2016). For example, several reports demonstrated a role for cilia in maintaining normal glucose homeostasis and regulating insulin secretion in pancreatic β-cells, suggesting completely orthogonal mechanisms by which loss of cilia during PDAC progression may help promote cancerous transformation (Volta et al. 2019; Gerdes et al. 2014). Other work has identified availability of cholesterol specifically at the ciliary membrane as a factor critical for enabling SHH signaling (Kinnebrew et al. 2019). Conversely, loss of cilia in cancerous transformation was identified as activating the mevalonate pathway, essential for cholesterol synthesis; excitingly, disruption of cilia in a mouse model of PDAC promoted tumorigenesis, with this activity reversible by statin treatment (Deng et al. 2018). As lifestyle choices leading to increased obesity and deficient glucose metabolism become more prominent as sources of cancer risk (Golemis et al. 2018), understanding the relationship of ciliation to these metabolic defects assumes increasing importance.

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Targeting Cancer Lysosomes with Good Old Cationic Amphiphilic Drugs

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Abstract Being originally discovered as cellular recycling bins, lysosomes are today recognized as versatile signaling organelles that control a wide range of cellular functions that are essential not only for the well-being of normal cells but also for malignant transformation and cancer progression. In addition to their core functions in waste disposal and recycling of macromolecules and energy, lysosomes serve as an indispensable support system for malignant phenotype by promoting cell growth, cytoprotective autophagy, drug resistance, pH homeostasis, invasion, metastasis, and genomic integrity. On the other hand, malignant transformation reduces the stability of lysosomal membranes rendering cancer cells sensitive to lysosome-dependent cell death. Notably, many clinically approved cationic amphiphilic drugs widely used for the treatment of other diseases accumulate in lysosomes, interfere with their cancer-promoting and cancer-supporting functions and destabilize their membranes thereby opening intriguing possibilities for cancer therapy. Here, we review the emerging evidence that supports the supplementation of current cancer therapies with lysosome-targeting cationic amphiphilic drugs.

Keywords Cancer · Cathepsins · Cationic amphiphilic drugs · Cell death · Lysosome · pH · SMPD1

1 Introduction

Most of the conventional cancer therapeutics damage the cell division machinery while the more recent targeted therapies are predominantly directed against tumor-specific growth-promoting kinases and immune checkpoints. In spite of the steady improvements in response rates, the current treatments are frequently compromised by either intrinsic resistance, acquired resistance or severe side effects. Accumulating data suggest that the efficacy of the existing treatments could be improved by simultaneous targeting of unrelated cellular functions that support the malignant phenotype (Luo et al. 2009; Nagel et al. 2016; Anania et al. 2020). This concept is based on the realization that the persistent activity of oncogenic pathways triggers a number of cellular stresses, such as proteotoxic stress, replication stress, metabolic stress, and oxidative stress. As a consequence, most cancer cells become addicted to survival-promoting stress response pathways (e.g. heat shock response, DNA damage response, and autophagy) and to increased activity of homeostasis-maintaining cellular processes (e.g. glucose uptake, lysosomal degradation, and acid extraction) (Jäättelä 1999a; Dai et al. 2007; Høyer-Hansen and Jäättelä 2008; Porporato et al. 2011; Kallunki et al. 2013; O'Connor 2015; Levy et al. 2017; White et al. 2017).

Targeting such non-oncogene addictions of cancer cells may open new possibilities for therapeutic intervention.

The lysosomal compartment is among the promising non-oncogene cancer targets. In addition to providing cancer cells with energy and building blocks, it contributes directly to several common cancer traits, such as growth signaling, metastasis, angiogenesis, pH gradient reversal, cell division and drug resistance (Groth-Pedersen and Jäättelä 2013; Olson and Joyce 2015; Davidson and Vander Heiden 2017; Liu et al. 2018; Condon and Sabatini 2019; Hämälistö et al. 2020). To meet the altered metabolic and survival requirements brought about by malignant transformation, lysosomes undergo several changes during cancer progression (Kallunki et al. 2013; Davidson and Vander Heiden 2017; Hämälistö and Jäättelä 2016; Perera et al. 2019). Despite their obvious potential as therapeutic targets, lysosomes have been largely overlooked in cancer drug discovery. Below, we discuss in more detail how lysosomes contribute to cancer progression and how lysosome-targeting drugs could contribute to cancer treatment not only by inhibiting the cancer-promoting functions of lysosomes, but also by exploiting the hydrolytic power of lysosomal hydrolases to kill cancer cells from inside (Ostenfeld et al. 2005; Petersen et al. 2013; Rebecca et al. 2017; Amaravadi et al. 2019).

2 Lysosomal Functions: Beyond Nutrient Recycling

The lysosomal compartment consists of a set of single-membrane organelles, i.e. terminal lysosomes, late endosomes (also referred to as multivesicular bodies), endolysosomes, autolysosomes, phagolysosomes, and autophagolysosomes, which interconvert through continuous fusion and fission events to maintain the homeostasis and functionality of the compartment (Klionsky et al. 2014; Bright et al. 2016; de Araujo et al. 2020). All these organelles are characterized by an acidic lumen and an abundance of common markers, such as lysosomal-associated membrane proteins LAMP1 and LAMP2. For the sake of simplicity, we refer to the entire compartment here as lysosomes, if not otherwise stated.

Besides their conventional housekeeping function in the maintenance of cellular energy homeostasis (Fig. 1), lysosomes control numerous unrelated cellular processes such as metabolic adaptation, pH equilibrium, adhesion and motility, invasion, various cell death pathways, inflammation, plasma membrane repair and secretion (Fig. 2) (Holland et al. 2020). To accomplish these tasks that take place in different subcellular locations, most mammalian cells contain a few hundred (ranging from 50 to 1,000) lysosomes that can be roughly divided into two spatially distinct populations, one in the vicinity of nucleus, concentrated around the microtubule-organizing center and another residing in the cellular periphery. The lysosomal positioning is, however, not stationary. Instead, most lysosomes are in continuous movement either by passive diffusion or active transport along microtubule tracks or actin microfilament networks thereby coupling lysosomal position to lysosomal function (recently reviewed in (Oyarzun et al. 2019; Lie and Nixon

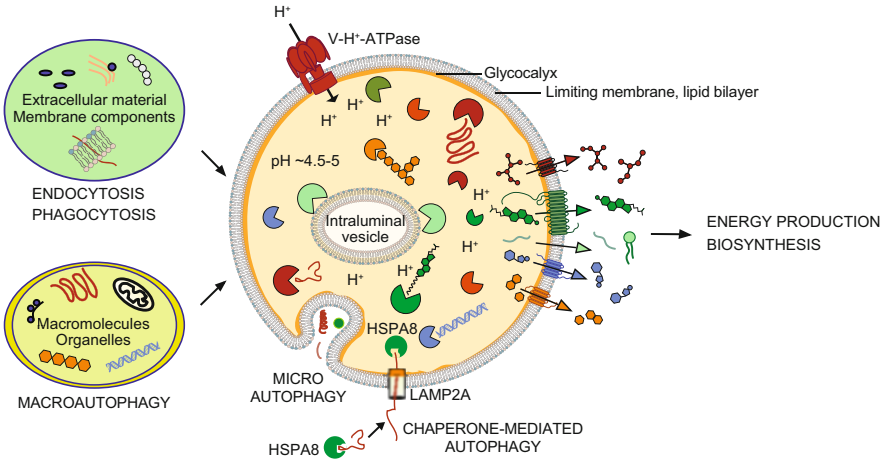

Fig. 1 Lysosomes as cellular recycling bins. Lysosomes receive mixed cargo through different forms of endocytosis, autophagy, and phagocytosis. In the lysosomal lumen, lysosomal hydrolases (proteases, lipases, glycosidases, DNases, RNases, phosphatases, and sulphatases) degrade the cargo into simple lipids, amino acids, saccharides, and nucleotides, which are recycled via diffusion or by specific transporters back to the cytoplasm, where they can be used for energy production or as building blocks for macromolecule biosynthesis. The activity of most lysosomal hydrolases depends on the acidic pH of the lumen, which is maintained by the proton pumping activity of the v-H^+-ATPase. For further details, see the text

2019)). In this chapter, we describe the main functions of lysosomes and their coordination in normal cells.

2.1 Recycling Centers with Acid Hydrolyses as Workhorses

As catabolic end stations of endocytic, autophagic, and phagocytic pathways, lysosomes take care of the degradation and recycling of diverse cargo, such as damaged and obsolete cellular material, extracellular matrix and pathogens (Xu and Ren 2015; Galluzzi et al. 2017; Niedergang and Grinstein 2018). For this purpose, they are filled with a mixture of hydrolytic enzymes that can digest most cellular material to metabolites, which are recycled back to the cytoplasm and used to produce energy and new macromolecules (Fig. 1). Most lysosomal hydrolases function optimally in an acidic environment, which is established and maintained by the vacuolar H^+-ATPase (v-H^+-ATPase), a multi-subunit protein complex consisting of a membrane-embedded domain in charge of the proton transport and a cytosolic ATPase domain that provides the energy required to transport protons against a concentration gradient (Forgac 2007; Hayek et al. 2019). Regardless their acidic pH optimum, many lysosomal hydrolases retain partial activity in neutral pH (Fonovic and Turk 2014). To prevent their uncontrolled leakage into the cytosol, the barrier

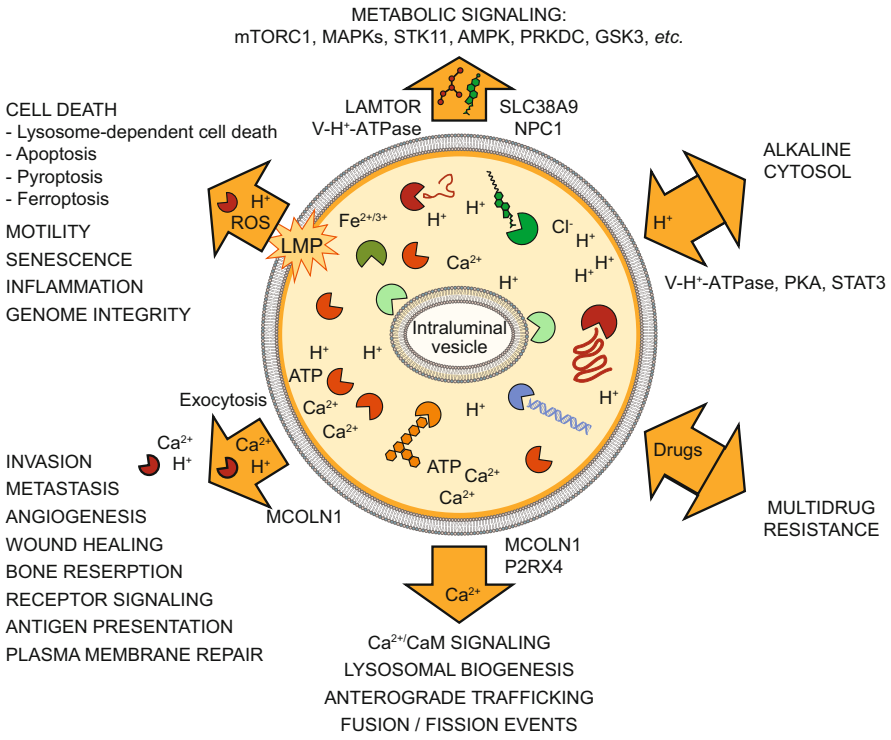

Fig. 2 Lysosomal functions beyond recycling. Clockwise from the top: On the lysosomal surface, the LAMTOR – v-H^+-ATPase complex senses the cellular nutrient status through the abundance of metabolites (e.g. amino acids and cholesterol) and regulates the activities of metabolic MTORC1, STK11-PRKDC-AMPK, MAPK, and GSK3 signaling pathways accordingly. In addition to maintaining lysosomes acidic, the v-H^+-ATPase complex plays an important role in the maintenance of alkaline cytosol. Upon cytosolic acidification, its activity can be enhanced by PKA and STAT3. Many basic drugs are trapped in the acidic lumen thereby contributing to drug resistance. Lysosomes serve also as an important cellular Ca^{2+} reservoir. The regulated Ca^{2+} release via lysosomal Ca^{2+} channels (e.g. MCOLN1 and P2RX4) activates Ca^{2+}/CaM-dependent signaling pathways (e.g. PPP3C and ADCY1), which control lysosomal biogenesis and membrane integrity, and regulates lysosome trafficking, fusion, and fission. Fusion of lysosomes with the plasma membrane leads to lysosomal exocytosis, which contributes to invasion and metastasis, regulation of cell surface receptors, and plasma membrane repair. Depending on the extent and cellular context, lysosomal membrane permeabilization (LMP) can trigger numerous cell death pathways or senescence, promote inflammation and motility and assist in mitotic chromosome segregation. For further details, see the text

function of the lysosomal perimeter membrane, a 7–10 nm lipid bilayer, is ensured by a protective glycocalyx formed by highly glycosylated luminal tails of LAMP1, LAMP2 and other integral membrane proteins, while the controlled flux of metabolites and ions across the membrane is achieved by numerous channel-forming proteins and protein complexes (Saftig and Klumperman 2009; Sterea et al. 2018).

2.2 *Metabolic Adaptation Governed by the LAMTOR Complex*

Lysosomes can be envisioned as the "stomach of the cell," whose contents reflect cellular nutrient status and guide the metabolic activity of the cell accordingly. Such metabolic adaptation is largely coordinated by a pentameric complex consisting of five late endosomal/lysosomal adaptor MAPK and MTOR activator (LAMTOR, also known as Ragulator) units that are anchored to the lysosomal membrane through myristoyl and palmitoyl modifications in its LAMTOR1 subunit (González et al. 2020; Nada et al. 2009). Being originally identified as a platform for the regulation of mitogen-activated protein kinase 3 (MAPK3, also known as ERK1) and lysosomal position (Wunderlich et al. 2001; Teis et al. 2006), LAMTOR is today best known for its crucial role in the regulation of cellular metabolism by mechanistic target of rapamycin complex 1 (mTORC1), the master regulator of cell growth. When both nutrients and growth factors are available, lysosomes promote anabolic pathways by enabling the activation of mTORC1, which promotes the synthesis of proteins, lipids, nucleotides, and ribosomes while inhibiting autophagy and lysosomal biogenesis (recently reviewed in (Condon and Sabatini 2019; Lawrence and Zoncu 2019; Kim and Guan 2019)). The activation of mTORC1 depends on both internal and external cues that are sensed by members of the RAS superfamily of small GTPases, RAGs, and RHEB, respectively. In the presence of essential metabolites, especially amino acids, two RAG heterodimers that associate with the lysosomal LAMTOR complex recruit inactive mTORC1 to the lysosomal membrane (Sancak et al. 2008; Kim et al. 2008), where RHEB can activate it pending that RHEB itself has been converted to an active, GTP-bound conformation by growth factor signaling (Menon et al. 2014; Dibble and Cantley 2015). Hitherto, arginine, and cholesterol are the only lysosomal metabolites verified as potent activators of LAMTOR-associated RAG GTPases. Their abundance in the lysosomal lumen is communicated to the LAMTOR-RAG complex in a v-H^+-ATPase-dependent manner by arginine- and cholesterol-sensitive interactions between the lysosomal amino acid transporter SLC38A9 and the LAMTOR-RAG complex (Castellano et al. 2017; Wang et al. 2015). Certain essential amino acids (e.g. arginine and leucine) that can originate either from the extracellular space or lysosomes can also activate lysosomal RAGs through a set of specific cytosolic nutrient sensors (Kim and Guan 2019). Additional nutrient control to mTORC1 activation is provided by RHEB, whose activation depends on purine nucleotides and glucose (González et al. 2020; Hoxhaj et al. 2017). Taken together, these strict requirements ensure that mTORC1 does not activate the anabolic phase unless required building blocks and energy are available to complete the process.

When the levels of essential nutrients and energy become limiting for growth, cellular metabolism is switched to a catabolic phase characterized by increased glycolysis, lipolysis, autophagy, and lysosomal degradation. In such conditions, mTORC1 is rapidly inactivated and released from the LAMTOR-RAG complex, while AMP-activated protein kinase (AMPK) is activated in order to restore the

energy homeostasis by promoting catabolic pathways and inhibiting dispensable ATP-consuming processes (Hardie et al. 2016). Notably, AMPK activity also ensures that mTORC1 remains inactive as long as the cellular energy status is compromised by directly phosphorylating a critical mTORC1 subunit RAPTOR and RHEB GTPase activating protein tuberous sclerosis complex 2 (Gwinn et al. 2008; Corradetti et al. 2004). Even though the canonical AMP-dependent AMPK activation does not require lysosomal membrane as an activation platform, a subset of trimeric AMPK complexes, which encompass an α-subunit with kinase activity and regulatory β and γ subunits, associates with the lysosomal membrane, through myristoylated β-subunits (Oakhill et al. 2010; Zhang et al. 2014). The recruitment of AMPK to the lysosomal membrane depends additionally on DNA-dependent protein kinase (PRKDC)-mediated phosphorylation of its γ subunit (Puustinen et al. 2020). It remains unclear whether this phosphorylation actually takes places on the lysosomal membrane, but PRKDC is in the growing list of lysosome-associated kinases (Puustinen et al. 2020). Recent data has revealed that the lysosomal localization of AMPK is a prerequisite for its fast activation in response to glucose deprivation, and that aldolase, a v-H^+-ATPase-associating glycolytic enzyme serves as a glucose sensor in this non-canonical, AMP-independent AMPK activation pathway (Zhang et al. 2017). Upon glucose deprivation, aldolase becomes rapidly unoccupied by its glycolytic substrate, fructose-1,6-biphosphate, and the v-H^+-ATPase complex undergoes conformational changes that allow AMPK activating serine/threonine kinase 11 (STK11, also known as LKB1) to dock to the lysosomal AMPK via interactions between AXIN1 scaffold protein, v-H^+-ATPase, and LAMTOR.

During prolonged starvation, the maintenance of lysosomal degradative pathways requires lysosomal biogenesis. This is to a large extent brought about by the microphthalmia/transcription factor (MiT/TFE) family of transcription factors, especially TFEB, TFE3, and MITF, which regulate the majority of genes necessary for lysosomal biogenesis and macroautophagy (Sardiello et al. 2009; Puertollano et al. 2018). In nutrient rich anabolic growth conditions, the nuclear localization of TFEB is effectively inhibited by several phosphorylation events, which either promote the binding of TFEB to cytoplasmic 14-3-3 proteins or guide it to the proteasomal degradation. mTORC1 is the major kinase responsible for keeping TFEB away from the nucleus, but several other kinases, such as MAPK1, glycogen synthase kinase 3 (GSK3), various protein kinase C (PKC) isoforms and AKT have also been implicated in the regulation of TFEB localization and stability. In order to be re-activated, TFEB has to be dephosphorylated. Hitherto, a Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase calcineurin is the only known TFEB phosphatase (Medina et al. 2015). In starved cells, its activation is triggered by Ca^{2+} release through a phosphoinositide-gated lysosomal transient receptor potential cation channel mucolipin 1 (MCOLN1). It remains to be studied whether other lysosomal Ca^{2+} channels could similarly activate calcineurin and TFEB. It is tempting to speculate that, purinenergic receptor P2RX4, which forms ATP-activated Ca^{2+} permeable channels on lysosomal membrane in response to increased lysosomal pH (Huang et al. 2014), could be involved in TFEB activation

upon lysosomal membrane damage or other stimuli that disturb lysosomal pH gradient.

2.3 *Position Matters: Plasma Membrane Repair and Exocytosis*

Lysosomes actively degrading autophagic or endocytic cargo reside typically near the microtubule-organizing center close to the nucleus. Some cells have, however, a subpopulation of lysosomes dispersed in cellular periphery, often delineating plasma membrane (Hämälistö and Jäättelä 2016). Such peripherally localized lysosomes are ideally positioned to rapidly respond to plasma membrane damage by either covering the damaged area in a "sandbag" fashion or resealing it in a "lipid patch" fashion via a Ca^{2+}-regulated lysosomal exocytosis (Andrews and Corrotte 2018; Cheng et al. 2015a). In the "lipid patch" model, a rapid influx of extracellular Ca^{2+} at the damage site activates MCOLN1-conducted lysosomal Ca^{2+} release from the plasma membrane proximal lysosomes. The local increase in Ca^{2+} then promotes the interaction between lysosomal synaptotagmin 7 (SYT7) and SNARE complex to trigger the fusion. Subsequently, the released sphingomyelin phosphodiesterase (SMPD1) catalyzes ceramide-dependent removal of the lesion by caveolar endocytosis (Andrews and Corrotte 2018). It should be noted here that the role of lysosomal exocytosis is plasma membrane healing is under debate and probably limited to the repair of very small wounds or merely their restructuring (Moe et al. 2015). Instead, it is clear that lysosomal exocytosis can serve as means to secrete lysosomal hydrolases, ATP, and exosomes (intraluminal vesicles of late endosomes) to the extracellular space, where they have distinct tissue specific functions, for example, in invasion and metastasis (discussed in more detail below), bone resorption, antigen presentation, immune responses, and cell-to-cell communication (recently reviewed in (Hessvik and Llorente 2018; Buratta et al. 2020)). Lysosomal exocytosis can be activated by TFEB, whose activity increases the pool of peripheral lysosomes and promotes their fusion with plasma membrane by a mechanism involving MCOLN1 expression and activity (Medina et al. 2011). Thus, TFEB activation could provide a therapeutic strategy for lysosomal storage diseases as means to clear undigested garbage from the lysosomes (Medina et al. 2011).

2.4 *Lysosomal Membrane Permeabilization: More Than a Cell Suicide Mechanism*

Lysosomes can be considered as ticking suicide bombs. Due to their massive degradative potential, a rupture of a few lysosomes is theoretically enough to kill the cell. The concept of such lysosome-dependent cell death was presented already

in the 1950s by Christian de Duve (de Duve 1983), but its existence and significance were substantiated only decades later when methods to distinguish lysosome-dependent cell death from autolytic processes in dead cells were developed (Foghsgaard et al. 2001; Leist and Jäättelä 2001). Eventually, further methodological improvements led to the realization that temporally and spatially controlled lysosomal leakage is a common, non-lethal phenomenon with putatively widespread physiological importance (Hämälistö et al. 2020; Thurston et al. 2012; Aits et al. 2015a).

2.4.1 Lysosome-Dependent Cell Death

Lysosome-dependent cell death is defined as an evolutionarily conserved subroutine of regulated cell death initiated by perturbations of intracellular homeostasis, demarcated by the permeabilization of lysosomal membranes and precipitated by cathepsins, with optional involvement of mitochondrial outer membrane permeabilization and caspases (Galluzzi et al. 2018). It contributes to several pathophysiological conditions, including but not limited to mammary gland involution (Kreuzaler et al. 2011), pancreatitis (Halangk et al. 2000), liver injury (Canbay et al. 2003), photoreceptor degeneration (Kinser and Dolph 2012), neurodegeneration (Syntichaki et al. 2005; Dehay et al. 2010), bacterial and viral infections (Matsuda et al. 2012; Laforge et al. 2007), and cellular aging (Loison et al. 2014). While the molecular mechanisms underlying lysosomal membrane permeabilization are under debate, the following examples reveal that several paths can lead to lysosomal leakiness. During post-lactational regression of murine mammary gland, mammary epithelial cells switch from secreting milk fat globules to phagocytosing them. As a result, their lysosomes accumulate high amounts of triglycerides and metabolize them to free fatty acids, especially oleic acid that is known to distort membranes and is the likely cause of lysosomal leakage in involuting breast (Sargeant et al. 2014). In a fruit fly model of retinal photoreceptor degeneration, the likely effector is insoluble rhodopsin that accumulates in lysosomes (Kinser and Dolph 2012), and in a murine model of Parkinson's disease, a mitochondrial oxidative attack (Dehay et al. 2010). And finally, pathogenic Vibrio parahaemolyticus has a distinct way of killing host cells by injecting a cytotoxic type III secretion effector VepA into their cytoplasm, where it targets v-H^+-ATPase thereby rupturing host cell lysosomes (Matsuda et al. 2012). Additional effector molecules implicated in lysosomal rupture include reactive oxygen species, proteases, various lipid metabolites, pro-apoptotic BCL2 family members, silica particles, and cholesterol crystals (reviewed in (Aits and Jäättelä 2013; Johansson et al. 2010)).

Leaky lysosomes are not detrimental to cells only because of the ensuing loss of essential lysosomal functions discussed above. Released reactive oxygen species and local acidification can harm cellular components directly, and cathepsins (especially cysteine cathepsins B and L and aspartyl cathepsin D) initiate degradative pathways leading to cell death with varying morphological features depending on the extent of lysosomal leakage and cellular context (Kågedal et al. 2001). Limited

lysosomal leakage activates the intrinsic caspase-dependent apoptosis pathway via cathepsin-mediated activation and inactivation of pro-apoptotic and anti-apoptotic BCL2-family members, respectively (Cesen et al. 2012), while high extralysosomal cathepsin activity, possibly assisted by cytosolic acidification and other lysosomal hydrolases, results in uncontrolled necrosis with explosive loss of plasma membrane integrity. Notably, the crosstalk between apoptosis and lysosomal cell death occurs also the other way around, apoptotic caspases triggering the permeabilization of lysosomes, which amplifies and ensures the caspase-initiated death process (Gyrd-Hansen et al. 2006; Oberle et al. 2010). Importantly, lysosome-dependent cell death does not depend on caspase activation, which is of great importance with regard to the treatment of apoptosis-resistant cancers (Groth-Pedersen and Jäättelä 2013).

In addition to cell death routines discussed above, lysosomal leakage can either directly contribute to other regulated cell death pathways, occur in parallel with them or be activated by them. For example, in specific forms of pyroptosis (an inflammatory cell death executed by gasdermin-mediated plasma membrane pore formation), lysosomal leakage can serve as the activator of the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome (reviewed in (Galluzzi et al. 2018; Wang et al. 2018)). In ferroptosis, an oxidative form of cell death defined for its dependence on iron and inhibition by glutathione peroxidase 4, lysosomal Fenton reactions contributes to the lipid peroxidation, which is often associated with lysosomal leakage (Mai et al. 2017; Kurz et al. 2010). Finally, the extralysosomal accumulation of a lysosomal galactosidase B1 in senescent cells strongly suggests that lysosomal membrane permeability increases during cellular senescence, a non-lethal process by which the cells permanently lose their proliferative capacity (Lee et al. 2006).

2.4.2 Lysosomal Leakage as an Intracellular Delivery System

Since the discovery of lysosomes and their degradative enzymes, it has been widely assumed, also by the authors of this review, that the intact lysosomal membrane is a prerequisite for cell viability (de Duve 1983; Aits and Jäättelä 2013; de Duve et al. 1955). This view has, however, changed recently owing to the development of highly sensitive methods to detect damaged lysosomes by exploiting the high affinity binding of cytosolic galectins to the β-galactoside-rich lysosomal glycocalyx (Thurston et al. 2012; Aits et al. 2015b). When the integrity of the lysosomal membrane is disturbed, cytosolic galectins readily enter lysosomal lumen and bind tightly to β-galactoside. In so doing, they mark leaky lysosomes with “galectin puncta” that can be visualized for several hours after the damage, thus enabling the detection of transient leakage events. The ability to detect minor lysosomal leakage has paved the way to the realization that cells can tolerate a low degree of lysosomal leakage and that endosomal sorting complex for transport (ESCRT) machinery is rapidly recruited to the site of damage to assist in the repair or reorganization of the membrane (Aits et al. 2015b; Skowyra et al. 2018; Radulovic et al. 2018). If the damage is more severe or the repair fails, lysosomes are tagged

with ubiquitin and cleared by a selective macroautophagy, called lysophagy (Papadopoulos et al. 2020).

The concept of non-lethal lysosomal leakage has opened an intriguing possibility that akin to the lysosomal exocytosis delivering lysosomal hydrolases to the extracellular space, controlled intracellular release of lysosomal enzymes inside the cell may carry non-lethal physiological functions (Hämälistö and Jäättelä 2016). Indeed, a minor release of cathepsin B (CTSB) from a few telomere-proximal lysosomes in prometaphase and subsequent CTSB-mediated cleavage of a subset of histone H3 are frequent events that contribute to the appropriate segregation of telomeres in cultured human cells and healthy murine tissues (Hämälistö et al. 2020). The exact molecular mechanisms governing mitotic lysosomal leakage are unknown, but inappropriately fused or entangled telomeres are putative triggers. This is supported by strictly telomere-proximal localization of leaky lysosomes, dramatic increase in the number of leaky lysosomes following induction of telomere end fusions, and accumulation of telomere-related chromosome segregation errors after inhibition of either lysosomal leakage or CTSB activity.

As mentioned above, lysosomal membrane permeabilization can serve as a trigger for the activation of the NLRP3 inflammasome in the context of pyroptosis. However, the activation of the NLRP3 inflammasome leads to pyroptosis only when it gets out of control, while it normally functions as an important arm of the innate immune system. NLRP3 serves as an intracellular sensor by detecting a broad range of exogenous and endogenous stimuli, including lysosomal rupture, that result in the formation the NLRP3 inflammasome and caspase 1-dependent release of pro-inflammatory cytokines IL-1β and IL-18 (recently reviewed in (Swanson et al. 2019)). The list of lysosome-rupturing stimuli that have been connected to NLRP3 inflammasome activation include phagocytosed cholesterol crystals, uric acid, silica particles, chemotherapeutics, asbestos, microbial toxins, and many more. Since the first report identifying CTSB as a mediator of the NLRP3 inflammasome in response to a microbial toxin, nigericin (Hentze et al. 2003), accumulating data have supported the role of cysteine cathepsins, especially CTSB, as well as lysosomal K^+ and Ca^{2+}-fluxes as NLRP3 inflammasome activators acting downstream of lysosomal membrane permeabilization (recently reviewed in (Campden and Zhang 2019)). Regarding the mechanism by which cytosolic cathepsins activate the NLRP3 inflammasome, CTSB has been shown to bind to NLRP3, and caspase-1 has been suggested as its substrate (Hentze et al. 2003; Bruchard et al. 2013). Hitherto, a model in which NLRP3-bound CTSB activates caspase-1 lacks, however, experimental validation. It remains also unclear how the cells of innate immune system survive the lysosomal leakage. Since they appear to do so, the activation of the NLRP3 inflammasome by lysosomal leakage can be considered as another example of cellular processes where lysosomal membrane permeabilization delivers enzymes to a specific subcellular location.

A third example where lysosomes deliver hydrolases to the cytosol in a controlled manner is related to cell adhesion. CTSZ regulates adhesion, migration, and endocytosis of immune and cancer cells by carboxy-terminal trimming of cytosolic proteins, such as β2 integrin (Jevnikar et al. 2009) and profilin 1 (Pecar Fonovic

et al. 2013; Pecar Fonovic and Kos 2015). Similarly, CTSH-mediated amino-terminal trimming of focal adhesion-associated talin promotes migration of cancer cells (Jevnikar et al. 2013). Since the activation of both CTSZ and CTSH normally occurs in the acidic lysosomal lumen, the observed cytosolic activities are likely to originate from enzymes escaping from lysosomes. The actual permeabilization of lysosomal membrane in these models remains, however, to be demonstrated.

2.4.3 Maintenance of Lysosomal Membrane Integrity

As is evident from the above, the control of lysosomal membrane integrity is not simply a question of maintaining the membrane integrity to sustain cell survival. Instead, a more dynamic regulation that allows controlled leakage without jeopardizing cell survival is required. Yet, the mechanisms that control lysosomal membrane integrity are only beginning to emerge. Among its well-established regulators in normal cells are LAMPs that form the protective glycocalyx, cysteine cathepsins that can cleave LAMPs, microtubule network along which lysosomes move, various sphingolipids, cholesterol, fatty acids, reactive oxygen species, and oxidized lipids as well as apolipoprotein D, a component of high-density lipoprotein that has potent antioxidant capacity (Aits and Jäättelä 2013; Appelqvist et al. 2013; Groth-Pedersen et al. 2012; Pascua-Maestro et al. 2017) (Fig. 3). How these regulatory mechanisms are affected by malignant transformation and cancer therapy are discussed in more detail below.

3 Metastatic Cancer: A Lysosomal Disease

Changes in the lysosomal compartment govern cancer development from cancer initiation to metastatic disease (Kallunki et al. 2013; Davidson and Vander Heiden 2017; Hämälistö and Jäättelä 2016; Perera et al. 2019). As a result, most aggressive cancers present with a dramatically enlarged and altered lysosomal compartment, on which they depend for their survival and invasiveness. It should, however, be noted that lysosomal changes vary considerable between different tumor types and even inside the same tumor, where the changes are normally most evident in the invasive edge. Typical changes include larger volume and more peripheral localization of the lysosomal compartment, increased activity and altered trafficking of a subset of lysosomal hydrolases, especially cysteine cathepsins, and altered lipid composition associated with multilamellar structures. Below, we discuss how these changes promote cancer growth and invasion and how they contribute to the pH equilibrium, multidrug resistance and the integrity of cellular membranes.

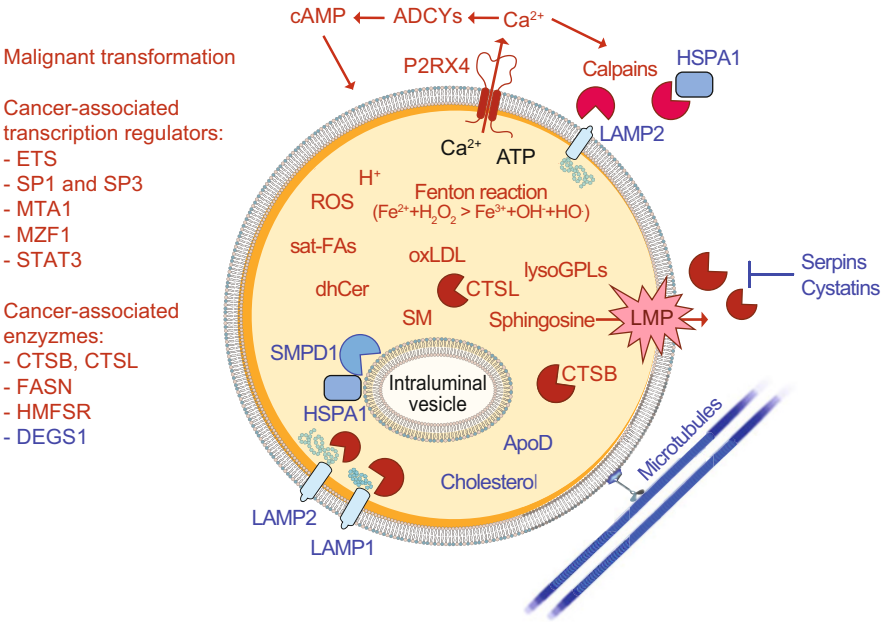

Fig. 3 Factors contributing to the lysosomal membrane integrity. Malignant transformation reduces the lysosomal membrane stability by multiple means. Through the activation of indicated transcription factors it increases the expression of cysteine cathepsins (CTSB and CTSL), which cleave membrane-stabilizing LAMP1 and LAMP2. Cancer-associated increase in FASN and HMGSR increases the levels of membrane-destabilizing (mono-)saturated fatty acids (sat-FAs) and membrane-stabilizing cholesterol, respectively, while decrease in DEGS1 increases levels of dihydroceramides (dhCer). Cancer-associated trafficking of HSPA1 into the lysosomal lumen promotes the hydrolysis of membrane-destabilizing sphingomyelins by SMPD1. P2RX4-mediated Ca^{2+} release followed by ADCY1-mediated cAMP production contributes to the lysosomal leakage induced by CADs. Ca^{2+} can also activate calpain, which contributes to lysosomal membrane destabilization by cleaving LAMP2 and HSPA1. Association of lysosomes with microtubules has a stabilizing effect. Finally, lysosomal membrane stability is generally reduced by reactive oxygen species that can be produced by iron-dependent Fenton reaction, oxidized low density lipoproteins (oxLDLs) and detergent-like lipids (e.g. sphingosine and lysoglycerophospholipids (lysoGPLs)) and increased by cholesterol and APOD. Following lysosomal membrane permeabilization, cytosolic cathepsins promote cell death, which can be inhibited/delayed by cytosolic protease inhibitors, serpins and cystatins. Factors promoting and inhibiting lysosomal membrane integrity are marked blue and red, respectively. For further details, see the text

3.1 How Do Proliferating Cells Maintain Lysosomal Biogenesis?

To fuel their active biosynthetic processes with building blocks, proliferating cancer cells switch their glucose metabolism from oxidative phosphorylation to lactate fermentation (often referred to as aerobic glycolysis or Warburg effect) (Vander Heiden et al. 2009). Additionally, they hitchhike the citric acid cycle for the

production of biosynthetic precursors, especially for lipid synthesis (DeBerardinis et al. 2008). The resulting anabolic metabolism is largely governed by the co-operation of the PI3K/AKT/mTORC1 pathway and MYC oncoprotein, which regulate complementary aspects of cancer cell metabolism from upregulation of nutrient transporters to slowing down the lysosomal catabolism. To achieve the latter, mTORC1 inhibits the nuclear localization of MiT/TFE3 factors, while MYC competes with them for the binding to their promotor elements that regulate lysosomal genes, including MiT/TFE factors themselves (Annunziata et al. 2019). Accordingly, most cancer cells thrive without MiT/TFE-mediated upregulation of lysosomal degradation, pending that they are supplied with sufficient nutrients via plasma membrane transporters. In some aggressively growing cancers, nutrient flux through plasma membrane is, however, not sufficient. In such cases, cancer cells can use KRAS-activated macropinocytosis as a nutrient salvage pathway (Commisso et al. 2013), and a subset of KRAS-driven pancreatic adenocarcinomas with high macropinocytotic flux are able to activate MiT/TFE-driven lysosomal biogenesis, while mTORC1 remains active, to ensure the degradation of the received cargo (Perera et al. 2015). Additionally, some rare cases of melanoma, renal cell carcinoma, and sarcoma have high lysosomal catabolism as a result of genomic amplifications or chromosomal translocations of genes encoding MiT/TFE factors (Perera et al. 2019). It remains, however, unclear how cancer cells with active mTORC1 and MYC manage to keep MiT/TFE factors active. One possibility is activation of protein kinase C, which can couple TFEB activation with inactivation of Zinc Finger with KRAB and SCAN domains 3 (ZKSCAN3), transcriptional TFEB repressor, through two parallel signaling cascades without compromising mTORC1 activity (Li et al. 2016). Notably, lysosomal biogenesis in response to starvation can also be activated through an MiT/TFE3-independent signaling pathway, involving AMPK and sirtulin 1 (SIRT1) histone deacetylase, that leads to the displacement of bromodomain protein 4 (BRD4)-mediated transcriptional repression from a broad range of lysosome- and autophagy-related genes (Sakamaki et al. 2017).

Taken together, these data suggest that except for acute stress situations that inactivate mTORC1 and cause a pause in proliferation, most cancers rely on MiT/TFE3-independent pathways that allow lysosomal biogenesis in the presence of active mTORC1 and MYC. As discussed below, such pathways may also be crucial for providing cancer cells with lysosomes tailored to perform cancer-specific tasks beyond nutrient recycling.

3.2 Specific Upregulation of Cysteine Cathepsins

Cancer-associated changes in lysosomal localization and content do not necessarily provide them with enhanced recycling capacity, but may rather promote migration, invasion, and metastasis. In line with this, malignant transformation does not result in a uniform upregulation of lysosomal genes. For example, the enlargement and more peripheral location of the lysosomal compartment in murine embryonic

fibroblasts following transformation with c-src^{Y527F} oncogene (Fehrenbacher et al. 2008) is associated with a significant ($P < 0.05$) over twofold increase in the expression of a very limited number of genes involved in lysosomal biogenesis, including only four lysosomal hydrolases, cysteine cathepsins Ctsl and Ctsb, galactosidase alpha (Gla) and hyaluronoglucosaminidase 1 (Hyal1), one v-H^+-ATPase subunit (Atp6v0e2) and one lysosomal transmembrane protein, battenin (Cln3), while the expression levels of Tfeb, Tfe3, and Mitf, Smpd1, and Mcoln1 are significantly reduced (Rohde and Jäättelä 2013). Similarly, ERBB2-induced invasive phenotype and peripheral localization of lysosomes in breast cancer cells correlates with upregulation of only a few selected cysteine cathepsins, whose expression is indispensable for the peripheral location of lysosomes and the invasive phenotype, while many other lysosomal proteins, including CTSD, are significantly downregulated (Rafn et al. 2012). Whereas the increased expression of cysteine cathepsins is emerging as a marker of invasive phenotype and poor prognosis in several cancers (Olson and Joyce 2015), the mechanisms regulating their expression vary between different tumors. Transcriptional regulators implicated in their metastasis-associated expression include SP1/SP3, ETS family, myeloid zinc finger-1 (MZF1), signal transducer and activator of transcription 3 (STAT3), and metastasis-associated 1 (MTA1) (Mohamed and Sloane 2006; Kumar et al. 2018; Brix et al. 2019; You et al. 2015). Additionally, gene amplifications, use of alternative promotors, epigenetic modifiers, and increased mRNA stability have been linked to high cysteine cathepsin expression (Mohamed and Sloane 2006).

3.3 Peripheral Lysosomes: Drivers of Metastatic Behavior and Cell Survival

Invasive cancer cells are characterized by a relatively large population of peripheral lysosomes that often accumulate in the tips of plasma membrane projections, such as lamellipodia and invadopodia (Hämälistö and Jäättelä 2016; Fehrenbacher et al. 2008). The bidirectional movement of lysosomes between perinuclear and peripheral locations occurs along microtubule tracks propelled by minus end–directed dynein and plus end–directed kinesin motors and regulated by lysosomal phosphoinositide levels, numerous small GTPases and adaptor proteins (Oyarzun et al. 2019; Lie and Nixon 2019). The hetero-octameric BORC (biogenesis of lysosome-related organelles complex 1 (BLOC1)–related) complex links lysosomes to kinesin motors in a LAMTOR-regulated manner. LAMTOR retains lysosomes in the perinuclear area by binding to a BORC subunit called lyspersin, which prevents BORC from associating with ADP ribosylation factor like GTPase 8B (ARL8B) and kinesin motors. In response to growth factors and nutrients, mTORC1 replaces BORC from the LAMTOR complex thereby allowing ARL8B to bind BORC and move lysosomes towards the cell periphery (Filipek et al. 2017; Pu et al. 2017). In line with this, several factors that promote mTORC1 activation, cellular motility, and invasion,

such as nutrients, growth factors, and oncogenes, increase the size of the peripheral lysosome population (Fehrenbacher et al. 2008; Rafn et al. 2012; Korolchuk et al. 2011), which promote the metastatic capacity, cell survival, and pH homeostasis of cancer cells.

Accumulating evidence supports the role of extracellular cathepsins in tumor progression. Cathepsins from tumor cells and tumor-associated stromal cells can reach the tumor microenvironment either via lysosomal exocytosis as active enzymes or via the secretory pathway as proenzymes that require proteolytic activation (Olson and Joyce 2015). In the extracellular space, proteolytically active cathepsins promote invasion, angiogenesis and growth by degrading components of the extracellular matrix, basement membrane, and cell junctions, by shedding adhesion molecules and cell surface receptors, by activating growth factors and cytokines and possibly by contributing to the activation of other invasion-promoting proteases, such as matrix metalloproteases and plasmin (reviewed in (Olson and Joyce 2015; Vasiljeva et al. 2019; Vidak et al. 2019)). CTSZ can also utilize a unique proteolysis-independent mechanism to activate growth and invasion by stimulating focal adhesion kinase (FAK)—SRC signaling through an integrin-binding Arg-Gly-Asp motif within its propeptide (Akkari et al. 2014; Kos et al. 2015). Besides cathepsins, secreted heparanase (HPSE) contributes to the disassembly of extracellular matrix and basal membranes by cleaving their heparane sulfate networks (Vlodavsky et al. 2002; Bartolini et al. 2020), and the acidification of extracellular space by secreted protons ensures the functionality of acid hydrolases in the extracellular space. Also, LAMP1 and LAMP2 trafficked to the cell surface as a result of exocytosis do not serve solely as markers of exocytosis activity but may also enhance the migratory potential of cancer cells (Alessandrini et al. 2017). As mentioned above, lysosomal exocytosis can also be used as a means to repair or restructure plasma membrane, which may be important for the survival of metastatic cancer cells exposed to mechanical stress when invading through matrix and basement membranes.

Based on their localization close to the plasma membrane and established role in invasion, it is often assumed that peripheral lysosomes promote invasion only through exocytosis. Peripheral localization of lysosomes does, however, not always correlate with increased exocytosis (Rafn et al. 2012; Johnson et al. 2016). Lysosomes can contribute to the invasion also inside the cell by degrading matrix components delivered to them through endocytosis (Nielsen et al. 2017; Lobert et al. 2010; Leonoudakis et al. 2014). Moreover, peripheral lysosomes are emerging as cytoplasmic regulators of the turnover and trafficking of actin-based cell adhesion structures, such as focal adhesions (reviewed recently in (Moreno-Layseca et al. 2019)), whose activity can be additionally regulated by cytosolic CTSZ and CTSH as described above.

Peripheral lysosomes can also provide a platform for mTORC1 activation by bringing lysosomes closer to upstream signaling molecules, such as plasma membrane-associated AKT kinases (Korolchuk et al. 2011). Somewhat surprisingly, the peripheral lysosomes have, however, decreased v-H^+-ATPase activity and increased H^+ leakage resulting in significantly higher luminal pH (~6) and lower

proteolytic capacity than their juxtanuclear counterparts with pH below 5 (Johnson et al. 2016). It is difficult to envision that the acidification defective peripheral lysosomes would be the preferred site for the mTORC1 activation, which depends on active v-H^+-ATPase and lysosomal proteolysis as described above. One solution to this dilemma is that the peripheral lysosome population consists of two distinct subpopulations, one with increased luminal pH preferentially fusing with the plasma membrane and the other actively degrading endocytosed material and supporting mTORC1 signaling. Supporting this view, inhibition of lysosomal acidification has been reported to promote exocytosis (Edgar et al. 2016; Latifkar et al. 2019).

3.4 Lysosomal Control of pH Gradient Reversal

It is often stated in the literature, that cancer cells have more acidic lysosomes than normal cells. The experimental evidence supporting this claim is, however, sparse. On the contrary, the peripheral lysosomes characteristic for cancer cells have relatively high pH (Johnson et al. 2016), and several cancer cell lines have higher lysosomal pH than normal cells (Altan et al. 1998; Gong et al. 2003). Instead of highly acidic lysosomes, malignant transformation is characterized by larger lysosomal compartment and disturbed pH regulation associated with the acidification of the extracellular space (White et al. 2017; Fehrenbacher et al. 2008; Zheng et al. 2020).

The metabolic switch of cancer cells to aerobic glycolysis presents cancer cells with a problem of excessive acid production. To avoid the acidification of their cytosol, cancer cells become dependent on the so-called pH gradient reversal, which denotes that they acidify their extracellular pH from 7.2–7.4 in normal cells to 6.2–6.8, while alkalizing the normally close to neutral cytosolic pH to values as high as 7.7 (White et al. 2017; Cardone et al. 2005). Mechanistically, pH gradient reversal has been mainly associated with increased activity of plasma membrane-localized ion transporters, including solute carrier family 9 member A1 (SLC9A1, also known as Na^+/H^+ exchanger 1), Na^+-dependent and -independent Cl^-/HCO_3^--exchangers and proton-linked monocarboxylate transporters as well as carbonic anhydrases that all contribute to net acid extrusion (Cardone et al. 2005; Stock and Pedersen 2017). Even though lysosomes comprise the major cellular proton store, knowledge about their role in cancer-associated pH gradient reversal in cancer cells is only beginning to emerge (Chen et al. 2020). The role of lysosomal exocytosis in this process is, however, supported by data demonstrating that both cytosolic and extracellular acidification triggers microtubule- and SLC9A1-dependent outward movement and exocytosis of lysosomes (Rozhin et al. 1994; Heuser 1989; Glunde et al. 2003; Steffan et al. 2009). Additionally, cytosolic acidification stimulates the proton scavenging capacity of lysosomes by enhancing the activity of lysosomal v-H^+-ATPase (Liu et al. 2018). This activation is brought about by a rapid recruitment of STAT3 to the lysosomes where it associates with v-H^+-ATPase and stimulates its ATPase activity. In support of the role of STAT3 in the maintenance

of alkaline cytosol of cancer cells, the cytosolic pH of HeLa cervix carcinoma cells drops from ~7.5 to ~7.1 upon STAT3 depletion, while lysosomal pH increases from ~4.6 to ~5.4. Thus, lysosomal acidification combined with lysosomal exocytosis contributes to the maintenance of alkaline cytosol and acidification of the extracellular space, which through a positive feedback loop further stimulates lysosomal exocytosis and thereby links pH regulation to the invasiveness.

3.5 Multidrug Resistance: Lysosomal Sequestration of Chemotherapeutics

Multidrug resistance (MDR) defines a phenotype where a cell is resistant to the effects of various structurally unrelated compounds. While the phenomenon is typically connected to over-expression of ATP binding cassette (ABC) subfamily B member 1 (ABCB1, also known as P-glycoprotein or MDR-1) and related ATP-dependent drug efflux pumps in the plasma membrane (Gottesman et al. 2002), accumulating evidence indicate that lysosomes contribute to the MDR phenotype by several mechanisms (Groth-Pedersen and Jäättelä 2013; Zhitomirsky and Assaraf 2016). In various cancer cells, functional ABC drug efflux pumps localize to lysosomes where they translocate their substrates, such as doxorubicin, into the lysosomal lumen thereby keeping them away from their cytoplasmic or nuclear targets (Rajagopal and Simon 2003; Yamagishi et al. 2013). Another lysosomal membrane protein, ATPase copper transporting β (ATP7B), has been implicated in resistance to cisplatin and other platin-based anti-cancer drugs by translocating them to lysosomes (Vyas et al. 2019). And finally, weakly basic hydrophobic chemotherapeutics, such as anthracyclines and vinca alkaloids, can diffuse passively across cellular membranes and sequester in acidic lysosomes as a result of ion trapping (Duvvuri et al. 2004). The subsequent drug accumulation in the lysosomes reduces lysosomal degradative capacity and activates TFEB-mediated lysosomal biogenesis, acidification, and exocytosis, resulting in more basic drugs getting trapped in the re-acidified lysosomes and eventually being secreted out of the cell via exocytosis (Zhitomirsky and Assaraf 2014, 2017).

3.6 Control of Lysosomal Membrane Integrity in Cancer Cells

Above, we have discussed the cancer-associated increase in cysteine cathepsin activity as a cancer-promoting event, but it can also pose a threat to cancer cell survival and an unexpected opportunity for cancer treatment. The transformation-associated increase in cysteine cathepsin activity sensitizes cells to lysosome-dependent cell death by at least two mechanisms (Fig. 3). In the lysosomal lumen,

cysteine cathepsins degrade the protective glycocalyx shield on the internal leaflet of the lysosomal membrane by hydrolyzing the heavily glycosylated membrane proteins LAMP-1 and LAMP-2 (Fehrenbacher et al. 2008). When released to cytosol, they serve as major effectors of lysosome-dependent cell death, and their increased activity further sensitizes cancer cells to the lethal consequences of lysosomal membrane permeabilization (Fehrenbacher et al. 2004). Another characteristic of cancer cells is that they express high levels of fatty acid synthase (FASN) and synthetize saturated and monounsaturated fatty acids at a very high rate (Rysman et al. 2010). This results in more saturated lipidome, which in turn may destabilize lysosomal membranes (Sargeant et al. 2014). Cancer cells have, however, various ways to protect themselves against potentially detrimental consequences of lysosomal leakage. To counteract the effects of cytosolic cathepsins, many cancers express high levels of cytosolic protease inhibitors, such as serpins (e.g. SERPINB3 and SERPINB4) and type I cystatins (CSTA and CSTB), that possess potent inhibitory activity against several cysteine cathepsins (Sun et al. 2017; Breznik et al. 2019). Moreover, the major stress-inducible member of the heat shock protein A family 1 (HSPA1, also known as HSP70), which effectively protects lysosomal membranes against permeabilization (Nylandsted et al. 2004), is highly expressed in many tumors correlating often with poor prognosis (Jäättelä 1999b; Ciocca and Calderwood 2005). In addition to its normal cytosolic location, ample amounts of HSPA1 translocate through the cell surface to cancer cell lysosomes (reviewed in (Balogi et al. 2019)). Contrary to most other proteins ending up in the lysosomal lumen, HSPA1 resists lysosomal hydrolases and remains functional in this harsh environment (Nylandsted et al. 2004). Its resistance to hydrolysis is likely due to its resilient, pH-dependent anchorage to the membranes of lysosomal intraluminal vesicles (ILVs) (Kirkegaard et al. 2010; Mahalka et al. 2014). This anchorage relies on a high-affinity binding between the positively charged amino-terminus of HSPA1 and an ILV-specific anionic phospholipid, bis (monoacylglycero)phosphate (BMP) (Fig. 4). ILVs serve as the sites of lysosomal lipid degradation and their high BMP content provides the negative charge required for the tethering of lysosomal lipases, such as SMPD1, to the membrane where their substrates are located (Breiden and Sandhoff 2019). The BMP-associated HSPA1 inhibits the dissociation of several sphingolipid-degrading enzymes from the membrane, thereby further enhancing their activity and inhibiting their degradation (Kirkegaard et al. 2010, 2016). HSPA1-mediated stabilization of lysosomal membranes in cancer cells has been largely ascribed to its ability to enhance SMPD1-mediated hydrolysis of sphingomyelin to ceramide, but its ability to regulate other lysosomal lipases is also likely to contribute to lysosomal membrane stability (Petersen et al. 2013).

Due to multiple metabolic and signaling aberrations, cancer cells generate elevated levels of reactive oxygen species. Iron and other chemically reactive metals can through Fenton-type chemical reactions in the lysosomes further amplify the oxidative load and generate reactive oxygen species leading to oxidization and destabilization of lysosomal membrane lipids (Kurz et al. 2010). Interestingly, lysosomal HSPA1 protects lysosomal membranes also against oxidative stress

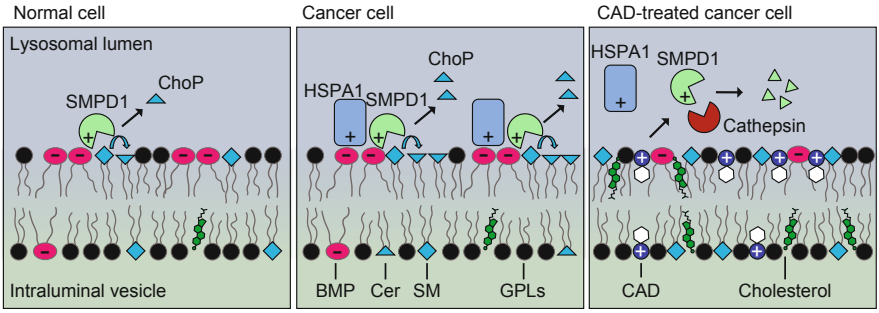

Fig. 4 HSPA1 and CADs are allosteric regulators of lysosomal SMPD1 activity. In normal cells, lysosomal intraluminal vesicles are enriched in bis(monoacyl)glycerophosphate (BMP). Negative charge of its head group recruits SMPD1 (and many other lysosomal lipases) to the membrane surface, where it hydrolyses sphingomyelin (SM) to ceramide (Cer) and phosphorylcholine (ChoP). In cancer cells, HSPA1 translocated to the lysosomal lumen is recruited to intraluminal vesicles by the negative charge of BMP, and inhibits the dissociation of SMPD1 from BMP thereby enhancing SM hydrolysis, which has a stabilizing effect on lysosomes. In CAD-treated cancer cells, positively charged CAD integrates to the membrane of intraluminal vesicles and neutralizes its negative charge. As a result, SMPD1 dissociates from the membrane and is degraded by cathepsins. The subsequent accumulation of SM destabilizes lysosomes. GPL, glyserophospholipid

(Nylandsted et al. 2004; Kirkegaard et al. 2010). It remains to be studied, whether this protection is attributed to a direct antioxidant effect of HSPA1 or whether it is due to indirect effects, such as changes in the lipid composition of the membranes.

4 Lysosomes as Targets for Cancer Therapy

Already in 1950's, de Duve suggested harnessing lysosomes for cancer therapy. The early efforts to develop lysosome-targeting anti-cancer drugs were, however, largely discontinued in the late 1970's due to the assumption that such drugs would kill all the lysosome-containing cells in the body (Firestone et al. 1979). It was first after the realization that malignant transformation is associated with substantial changes in lysosomal composition that lysosomes gained broader interest as putative cancer targets (Fehrenbacher et al. 2004, 2008; Kroemer and Jäättelä 2005). This line of research was further encouraged by accumulating data revealing that cancer cells frequently harbor acquired mutations and other changes that allow them to escape spontaneous and therapy-induced apoptosis, while gaining sensitivity to lysosome-dependent cell death (Groth-Pedersen and Jäättelä 2013; Hanahan and Weinberg 2011). In the meanwhile, it has become clear that while targeting individual lysosomal enzymes, such as cathepsins, heparanase, and V-H^+-ATPase, show promising results in experimental settings, a more comprehensive inhibition of lysosomal function may be required to achieve substantial responses in cancer therapy (Kallunki et al. 2013; Davidson and Vander Heiden 2017). Thus, we focus here in

therapeutic approaches that destabilize lysosomal membranes and thereby have detrimental effects on all lysosomal functions described above.

4.1 Clinically Relevant Inducers of Lysosomal Membrane Permeabilization

Dozens of clinically relevant drugs and cytokines induce lysosomal membrane permeabilization and lysosome-dependent cell death in cancer cells (reviewed in (Groth-Pedersen and Jäättelä 2013; Domagala et al. 2018)). The most promising drugs can be divided into three categories, (1) those altering lysosomal lipid composition, (2) those disturbing lysosome trafficking, and (3) those increasing reactive oxygen species (ROS).

Most of the lysosome-destabilizing drugs in the lipid targeting category are SMPD1-inhibiting cationic amphiphilic drugs (CADs). Their mechanism of action and emerging potential as cancer drugs is discussed in detail in the next chapter. Additionally, two interesting experimental cancer drugs induce lysosome-dependent cell death by SMPD1-independent mechanisms. Tetrahydrocannabinol, the main active component of *Cannabis sativa*, destabilizes lysosomes by inhibiting dihydroceramide desaturase (DEGS1), an enzyme that converts dihydroceramide to ceramide in the endoplasmatic reticulum (Hernandez-Tiedra et al. 2016). The subsequent accumulation of membrane-destabilizing dihydroceramides (and possible other dihydrosphingolipids) in lysosomal membranes results in lysosomal membrane damage and lysosome-dependent cell death. Notably, recent phase II clinical trials have indicated positive results regarding the survival of glioblastoma patients upon tetrahydrocannabinol treatment (Dumitru et al. 2018). The second membrane-disturbing drug is a complex of partially unfolded alpha-lactalbumin and oleic acid originally found in human milk and dubbed HAMLET for "Human Alpha-Lactalbumin Made LEthal to Tumor cells." HAMLET and its bovine counterpart BAMLET are effectively endocytosed by cancer cells and they deliver high amounts of oleate into their lysosomes, where its emulsifying effect destabilizes the limiting membrane (Rammer et al. 2010). These milk-derived complexes have broad tumoricidal activities in vitro and their therapeutic efficacy has been demonstrated in various animal cancer models as well as in human clinical trials for skin papillomas and bladder cancer (reviewed in (Ho et al. 2017)).

The second category includes several commonly used microtubule-targeting anticancer drugs, such as vinca alkaloids and taxanes (Broker et al. 2004; Groth-Pedersen et al. 2007), as well as type II monoclonal antibodies against B cell surface antigen CD20 (tositumomab) that trigger a remodeling of actin cytoskeleton (Ivanov et al. 2009). These drugs induce dramatic lysosomal swelling and increased lysosomal cysteine cathepsin activity prior to lysosomal leakage suggesting a disturbance in trafficking-dependent vesicle fusion and fission events as an underlying mechanism.

An interesting example of the third category of ROS inducing agents is salinomycin, an antimicrobial drug that was identified as a potent cancer drug in a high-throughput screen for compounds killing breast cancer stem cells, which are typically refractory to conventional treatments (Gupta et al. 2009). Recently, it was shown that salinomycin, and it is more potent derivative ironomycin, effectively sequester iron in lysosomes resulting in high ROS production by iron-dependent Fenton reaction and ROS-mediated damage to lysosomal membranes (Mai et al. 2017).

In addition to drug-like molecules, advances in photodynamic therapies and nanoparticle research may provide additional ways to target cancer lysosomes in the future (Skupin-Mrugalska et al. 2014; Liu et al. 2020). For example, mixed-charge nanoparticles can be designed to selectively target cancer lysosomes, where they form nanoparticle assemblies and crystals, which mechanically disrupt the integrity of lysosomal membranes (Borkowska et al. 2020).

5 Targeting Cancer Lysosomes by SMPD1-Inhibiting CADs

The identification of sphingomyelin metabolism as an essential regulator or lysosomal membrane integrity originates from studies showing that the lysosome-stabilizing effect of HSPA1 is mediated by its ability to promote SMPD1 activity as described above. Whereas HSPA1-based therapies are emerging as effective treatment options to activate SMPD1 and improve lysosomal function in sphingolipidoses (Kirkegaard et al. 2016), the direct inhibition of HSPA1 in the lysosomal lumen is technically challenging. On the other hand, the inhibition of its target, SMPD1, can be easily accomplished since over one hundred clinically relevant CADs are functional inhibitors of this enzyme (Kornhuber et al. 2008, 2010a). Similar to HSPA1, CADs target the electrostatic interaction between SMPD1 and BMP-rich lysosomal vesicles, but with an exactly opposite outcome (Fig. 4). As positively charged lipid-like substances, CADs accumulate in lysosomal membranes and neutralize their BMP-mediated negative charge. In doing so, they release SMPD1 and other BMP-dependent lipases from the membrane and render them readily accessible to cathepsin-mediated degradation (Kornhuber et al. 2010a; Hurwitz et al. 1994; Alakoskela et al. 2009). The importance of SMPD1 inhibition in CAD-induced cell death is supported by the ability of non-lysosomal sphingomyelin phosphodiesterase to partially rescue cells from CAD cytotoxicity and the striking correlation between the ability of CADs to inhibit SMPD1 and to kill cancer cells (Petersen et al. 2013). Moreover, cancer cells are especially sensitive to the accumulation of sphingomyelin (Barcelo-Coblijn et al. 2011; Teres et al. 2012), which may contribute to the selective cytotoxicity of diverse CADs towards cancer cells observed both in vitro as well as in dozens of animal cancer models (Table 1).

Table 1 Examples of SMPD1-inhibiting CADs with proven anti-cancer activity in murine/rat cancer models

CAD	Drug category	Cancer type	Model	Ref.
Amiodarone	Antiarrhythmic	Hepatoma	N1-S1 allograft (rat)	Wu et al. (2018)
			Hep3B xenograft (mouse)	Wu et al. (2018)
Amlodipine	Antianginal, Ca^{2+} blocker	Epidermoid skin cancer	A431 xenograft (mouse)	Yoshida et al. (2004)
Amitriptyline	Antidepressant	Multiple myeloma	Sp2/O, JJN3 xenografts (mouse)	Zhang et al. (2013)
Astemizole	Antihistamine	Non-small cell lung cancer	H460 xenograft (mouse)	Peng et al. (2014)
		Prostate cancer	VCaP xenograft (mouse)	Liu et al. (2019)
Bepridil	Antianginal,	Chronic lymphocytic leukemia	Patient-derived xenograft (mouse)	Baldoni et al. (2018)
	Ca^{2+} blocker	Small cell lung cancer	Kp1 allografts (mouse)	Jahchan et al. (2013)
			H187 xenografts (mouse)	Jahchan et al. (2013)
			Patient-derived xenograft (mouse)	Jahchan et al. (2013)
Cepharanthine	Natural compound	Hepatoma	Hca/FAB allograft (mouse)	Li et al. (2011)
		Oral squamous cell carcinoma	B88 xenograft (mouse)	Harada et al. (2003)
Chloroquine	Antimalarial	Pancreatic adenocarcinoma	8988T xenograft (mouse)	Yang et al. (2011)
– DC661[a]		Colorectal cancer	HT-29 xenograft (mouse)	Rebecca et al. (2017)
– Lys05[a]		Colorectal cancer	HT-29 xenograft (mouse)	McAfee et al. (2012)
		Melanoma	1205Lu xenograft (mouse)	McAfee et al. (2012)
Citalopram	Antidepressant	Colon cancer	Chemically induced tumor (rat)	Tutton and Barkla (1982)
			HXM2, HXM4 allografts (mouse)	Tutton and Barkla (1982)
			HCT116 xenograft (mouse)	van Noort et al. (2014)
Clemastine	Antihistamine	Glioma	Patient-derived xenografts (mouse)	Le Joncour et al. (2019)
Clomiphene	Ovulation inducer	Fibrosarcoma	HT1080 xenograft (mouse)	Zheng et al. (2017)
Desipramine	Antidepressant	Breast cancer	MCF7 xenograft (mouse)	Petersen et al. (2013)
Diphenhydramine	Antihistamine	Melanoma	B16-F10 allograft (mouse)	Or et al. (2016)
Ebastine	Antihistamine	Breast cancer	MCF7 xenograft (mouse)	[b]

(continued)

Table 1 (continued)

CAD	Drug category	Cancer type	Model	Ref.
Emetine	Antiprotozoal	Glioma	GBM157 xenograft(mouse)	Visnyei et al. (2011)
Escitalopram	Antidepressant	Glioma	U87MG xenograft (mouse)	Chen et al. (2018)
Fluoxetine	Antidepressant	Colon cancer	Chemically induced (rat)	Tutton and Barkla (1982)
			HXM2, HXM4 allografts (mouse)	Tutton and Barkla (1982)
		Glioma	U87, GBM8401 xenografts (mouse)	Liu et al. (2015)
		Prostate cancer	PC3 xenografts (mouse)	Abdul et al. (1995)
Fluphenazine	Antipsychotic	Breast cancer	R3230AC allograft (rat)	Hilf et al. (1971)
			MDA-MB-231 xenograft (mouse)	Goyette et al. (2019)
		Melanoma	UACC903, 1203 Lu, A2058 xenografts (mouse)	Kuzu et al. (2017)
Imipramine	Antidepressant	Glioma	*GRLp53het* and *GRLp53fko* transgenic (mouse)	Shchors et al. (2015)
		Small cell lung cancer	Kp1 allografts (mouse)	Jahchan et al. (2013)
			H187 xenografts (mouse)	Jahchan et al. (2013)
			Patient-derived xenograft (mouse)	Jahchan et al. (2013)
Leelamine	Natural compound	Breast cancer	SUM159 xenograft (mouse)	Sehrawat et al. (2017)
		Melanoma	UACC 903 & 1203 Lu xenograft (mouse)	Gowda et al. (2014)
		Prostate cancer	22Rv1 xenograft (mouse)	Singh et al. (2018)
Loratadine	Antihistamine	Breast cancer	MCF7 xenograft (mouse)	[b]
		Gastrointestinal cancer	CT.26.WT xenograft (mouse)	Chen et al. (2017a)
Mefloquine	Antimalarial	Acute myeloid leukemia	OCI-AML2, K562, MDAY-D2 xenografts (mouse)	Sukhai et al. (2013)
			Patient-derived xenograft (mouse)	Sukhai et al. (2013)
		Colon cancer	Patient-derived xenograft (mouse)	Takeda et al. (2019)
		Gastric cancer	YCC1 xenograft (mouse)	Liu et al. (2016a)
		Hepatoma	HepG2 xenograft (mouse)	Li et al. (2018)

Nortriptyline	Antidepressant	Melanoma	UACC903 xenograft (mouse)	Kuzu et al. (2017)
Penfluridol	Antipsychotic	Epidermoid lung carcinoma	LL/2 allograft (mouse)	Wu et al. (2014)
		Bladder cancer	UM-UC-3	van der Horst et al. (2020)
		Breast cancer	4T-1 allograft (mouse)	Ranjan et al. (2016)
		Glioma	U87MG xenograft (mouse)	Ranjan et al. (2017), Ranjan and Srivastava (2017)
		Non-small cell lung cancer	A549 xenograft (mouse)	Xue et al. (2020)
		Pancreatic adenocarcinoma	UNKC-6141 allograft (mouse)	Dandawate et al. (2020)
			Patient-derived xenograft (mouse)	Dandawate et al. (2020)
			PANC-1, Bx-PC-3, AsPC-1 xenograft (mouse)	Dandawate et al. (2020), Ranjan and Srivastava (2016)
Perhexiline	Antianginal	Breast cancer	MDA-MB-468 xenograft (mouse)	Ren et al. (2015)
		Colorectal cancer	Patient-derived xenografts (mouse)	Wang et al. (2020)
		Chronic lymphocytic leukemia	*Tcl-1*^{Tg}*:p53*$^{-/-}$ transgenic (mouse)	Liu et al. (2016b)
		Gastric cancer	HGC27 xenograft (mouse)	Wang et al. (2020)
Perphenazine	Antipsychotic	Melanoma	UACC903, 1205 Lu, A2058 xenografts (mouse)	Kuzu et al. (2017)
Pimozide	Antipsychotic	Acute myeloid leukemia	Ba/f3 FLT3 ITD xenograft (mouse)	Nelson et al. (2012)
		Breast cancer	MDA-MB-231 xenograft (mouse)	Dakir et al. (2018)
			HC11-Cuzd1 xenograft (mouse)	Mapes et al. (2018)
		Colorectal cancer	HCT116, SW480 xenograft (mouse)	Ren et al. (2018)
		Hepatoma	MHCC-97L xenograft (mouse)	Chen et al. (2017b)
		Osteosarcoma	KHOS/NP xenograft (mouse)	Subramaniam et al. (2020)
		Prostate cancer	TRAMP transgenic (mouse)	Kim et al. (2019)
Promethazine	Antihistamine	Small cell lung cancer	Kp1 allografts (mouse)	Jahchan et al. (2013)
			H187 xenografts (mouse)	Jahchan et al. (2013)

(continued)

Table 1 (continued)

CAD	Drug category	Cancer type	Model	Ref.
			Patient-derived xenograft (mouse)	Jahchan et al. (2013)
Siramesine	Antidepressant	Fibrosarcoma	WEHI-164 allograft (mouse)	Ostenfeld et al. (2005)
		Breast cancer	MCF7 xenograft (mouse)	Ostenfeld et al. (2005), Petersen et al. (2013), Groth-Pedersen et al. (2007)
		Pancreatic β-cell cancer	*Rip1-Tag2* transgenic (mouse)	Petersen et al. (2013)
Sertaline	Antidepressant	Colon cancer	HT29 xenograft (mouse)	Gil-Ad et al. (2008)
		Non-small cell lung cancer	A549 xenograft (mouse)	Jiang et al. (2018)
		Melanoma	A375 xenograft (mouse)	Reddy et al. (2008)
Sertindole	Antipsychotic	Breast cancer	MDA-MB-231 xenograft (mouse)	Zhang et al. (2018)
		Gastric cancer	MGC803 xenograft (mouse)	Dai et al. (2020)
Thioridazine	Antipsychotic	Breast cancer	MDA-MB-231, 4T1 xenograft (mouse)	Goyette et al. (2019), Yin et al. (2015)
		Gastrointestinal cancer	CT.26.WT xenograft (mouse)	Chen et al. (2017a)
		Glioma	U87MG xenograft (mouse)	Cheng et al. (2015b)
		Non-small cell lung cancer	A549 xenograft (mouse)	Shen et al. (2017)
		Medulloblastoma	Vandy-MB-11 xenograft (mouse)	Huang et al. (2015)
		Ovarian cancer	2274 and SKOV3 xenografts (mouse)	Park et al. (2014), Yong et al. (2017)
		Prostate	LNCaP xenografts (mouse)	Singh et al. (2019)
Trifluoperazine	Antipsychotic	Breast cancer	MDA-MB-231 xenograft (mouse)	Goyette et al. (2019)
		Melanoma	H1_DL2 brain metastasis, xenograft (mouse)	Zhang et al. (2020)

[a]DC661 and Lys05 are dimeric forms of chloroquine
[b]Unpublished work by L. Groth-Pedersen and M.J

5.1 Chemical Characteristics of CADs and SMPD1-Inhibiting CADs

CADs include dozens of pharmacologic agents commonly used to treat a broad spectrum of diseases, including allergies, psychiatric disorders, heart diseases, and infections (Kornhuber et al. 2008, 2010a) (Table 1). Instead of their primary high affinity targets, such as histamine H1 receptor, dopamine D2 receptor or sodium-dependent serotonin transporters, CADs are defined by their chemical properties that make them lysosomotropic, signifying that they accumulate in lysosomes. CADs are small molecules featured by a hydrophobic tail with one or more aromatic or aliphatic ring structures and a hydrophilic moiety containing at least one basic functionality, typically an amine. This amine is protonated at physiological pH resulting in ion trapping of CADs inside lysosomes where they can reach up to 1,000-fold accumulation (Halliwell 1997; Trapp et al. 2008). In their non-protonated form, CADs are hydrophobic and penetrate biological membranes through passive diffusion, which is likely to be the main mechanism by which they enter the cell and lysosomes, even though endocytic uptake routes are also possible. Based on various models to predict the chemical characteristics that favor lysosomotropism, values of two parameters have proven to be essential, pK_a (negative logarithm of the acid dissociation constant) between 6.5 and 11 and logP (logarithm of the partition coefficient between *n*-octanol and water) above 2 (Nadanaciva et al. 2011).

Whereas lysosomotropism ensures lysosomal accumulation of a drug, it is not sufficient for the effective inhibition of SMPD1. The structure-property-activity-relation model introduced by Kornhuber and co-workers predicts that the functional inhibition of SMPD1 is favored when pK_a for the most basic nitrogen atom is higher than 8.45, logP is higher than 3.61 and the compound's cationic nitrogen atom is free of sterical hindrance (Kornhuber et al. 2008). Their second model with higher accuracy takes additionally into account the molecular weight of the compound, sum of van der Waals surface areas of polar atoms, pK_a for the second-most basic nitrogen atom and pK_a for the most acidic group. Based on this model, 135 out of 2028 (6.66%) drugs licensed for medical use are functional inhibitors of SMPD1 (Kornhuber et al. 2011). In spite of their high structural diversity, SMPD1 inhibiting drugs are enriched in a few Anatomical Therapeutic Chemical (ATC) drug classification system therapeutic groups, including A03 (drugs for functional gastrointestinal disorders), C08 (calcium channel blockers), N04 (anti-Parkinson drugs), N05 (psycholeptics), N06 (psychoanaleptics), and R06 (antihistamines). Additionally, many oncology drugs designed to target tyrosine kinases have CAD structure, and at least sunitinib has already been verified as a potent SMPD1 inhibitor experimentally (Nadanaciva et al. 2011; Ellegaard et al. 2013).

5.2 CAD-Induced Lysosome-Dependent Cell Death

The emerging interest in CADs as putative anti-cancer drugs is based on their cancer-specific toxicity and ability to kill a wide range of cancer cells of various origins, including all 60 cancer cell lines constituting National Cancer Institute's cancer cell panel as well as various apoptosis and therapy resistant cancer cells and cancer stem cells (Petersen et al. 2013; Kuzu et al. 2017; Takeda et al. 2019; Li et al. 2018; Ellegaard et al. 2016; NCI 2015; Bielecka-Wajdman et al. 2017). Regardless of the target cell, the cytotoxicity of CADs correlates very well with their ability to inhibit SMPD1 and to induce lysosomal membrane permeabilization (Petersen et al. 2013; Ellegaard et al. 2016; Pagliero et al. 2016). Accordingly, the order of efficacy of CADs is strikingly similar across different cancer cell lines, and the most potent CADs, including well known drugs such as penfluridol, sertraline, perhexiline, astemizole, sertindole, and mefloquine, induce growth inhibition and cell death regardless of the target cell at sub-micromolar and low micromolar concentrations, respectively (Ellegaard et al. 2016; NCI 2015). It should be noted here that the CADs presently receiving most attention as anti-cancer drugs, chloroquine and hydroxychloroquine (Verbaanderd et al. 2017; Xu et al. 2018), are relatively poor inhibitors of SMPD1 and accordingly weak inducers of cancer cell death. Thus, it is reasonable to expect that CADs with stronger SMPD1 inhibitory capacity would be more efficient in cancer treatment.

Even though CAD-induced cell death depends on SMPD1 inhibition and genetic depletion of *SMPD1* is sufficient to kill some cancer cells, SMPD1 inhibition does not fully explain the efficient CAD-induced cancer cell cytotoxicity (Petersen et al. 2013; Kuzu et al. 2017). The direct detergent activity of CADs as well as their ability to activate various signaling pathways and enhance the production of ROS, may be among the additional mechanisms that together with SMPD1 inhibition lead to lysosomal leakage. Supporting the role of Ca^{2+} signaling in this process, several CADs, with different structures and primary targets, induce a rapid lysosomal Ca^{2+} release through P2X purinergic receptor 4 (P2RX4) and subsequent Ca^{2+}- and adenylyl cyclase 1 (ADCY1)-dependent synthesis of cAMP as a signaling route contributing to CAD-induced lysosomal membrane permeabilization and cell death in breast and lung cancer cells (Anand et al. 2019). Ca^{2+} signaling may also contribute to lysosomal leakage through calpain-mediated cleavage of HSPA1 and LAMP2 (Sahara and Yamashima 2010; Villalpando Rodriguez and Torriglia 2013). Furthermore, CAD-induced SMPD1-independent changes in cellular lipidome are likely to alter lysosomal membrane stability. In this respect, the reported CAD-induced elevation in lysoglycerophospholipids (lysoGPLs) levels is especially interesting since these monoacyl variants of glycerophospholipids have detergent-like properties and are capable of damaging lysosomal membranes (Correa et al. 2019; Lecommandeur et al. 2017; Nielsen et al. 2020).

5.3 *Multiple Anti-cancer Activities of CADs*

In addition to their direct cytotoxicity, CADs have several other anti-cancer activities that support their repurposing in oncology. Many of these can be achieved with considerably lower drug concentrations than cell death and may thus be highly relevant in clinical settings. For example, the ability of CADs to act synergistically with classic chemotherapy requires 10–100 times lower CAD concentrations than their direct cytotoxic effect (Groth-Pedersen et al. 2007; Ellegaard et al. 2016). Below, we divide therapeutically relevant outcomes of CAD treatment into two categories depending on their dependence on the disturbance of lysosomal pH gradient or SMPD1 inhibition.

5.3.1 Consequences of Increased Lysosomal pH

CADs induce a relatively rapid increase in lysosomal pH possibly due to a combined effect of their own cationic nature and proton leakage (Ohkuma and Poole 1978; Ostenfeld et al. 2008). Because most cellular functions of lysosomes depend on acidic luminal pH, this has numerous deleterious effects in cancer cells. Firstly, increased lysosomal pH leads to the inhibition of mTORC1 activity and cell growth (Lawrence and Zoncu 2019; Yim and Mizushima 2020). mTORC1 inhibition then triggers autophagy, but due to the reduced activity of lysosomal hydrolases, both autophagic and endocytic macromolecule recycling pathways are eventually inhibited (Amaravadi et al. 2019; Farkas et al. 2009). Secondly, high lysosomal pH and inhibition of cysteine cathepsins results in juxtanuclear positioning of lysosomes and inhibition of exocytosis with subsequent inhibition of migration, invasion, and metastasis (Olson and Joyce 2015; Rafn et al. 2012; Ballabio and Bonifacino 2020). Thirdly, neutralization of lysosomal pH triggers lysosomal Ca^{2+} release through ATP-gated P2RX4 cation channel (Huang et al. 2014; Anand et al. 2019), which in turn may serve as a trigger for CAD-induced endoplasmatic reticulum stress response (Kuzu et al. 2017). Fourthly, reduced cysteine cathepsin activity interferes with proper mitotic chromosome segregation resulting in genomic instability (Hämälistö et al. 2020). And lastly, increased lysosomal pH contributes to the CAD-induced reversal of the MDR phenotype (Yamagishi et al. 2013). Importantly, this reversal can be induced by low sub-micromolar concentrations of CADs, and it has been demonstrated in various murine cancer models with structurally diverse CADs (Petersen et al. 2013; Ellegaard et al. 2016; Peer et al. 2004; Nguyen et al. 2014; Vella et al. 2015; Johannessen et al. 2019). The mechanism by which CADs revert MDR phenotype is, however, still under debate. Suggested mechanisms include reduced sequestration of basic chemotherapeutics in lysosomes due to increased lysosomal pH, general increase in membrane permeability, changes in physical properties of lysosomal membranes via chemically activated degradation of membrane phospholipids as well as direct and SMPD1-mediated effects on ABCB1

activity (Yamagishi et al. 2013; Jaffrezou et al. 1995; Kornhuber et al. 2010b; Casey et al. 2014).

While increasing lysosomal pH, CADs also induce a significant decrease in cytosolic pH, possibly via proton leakage (Ohkuma and Poole 1978). The acidification of the cytosol has many deleterious effects in cancer cells, such as inhibition of glycolysis, activation of apoptosis, and inhibition oncogenic STAT3 transcription factor (White et al. 2017; Liu et al. 2018; Gowda et al. 2014; Dai et al. 2020).

5.3.2 Consequences of SMPD1 Inhibition

As discussed above, inhibition of SMPD1 is essential for CAD-induced lysosomal membrane permeabilization and may contribute to the reversal of MDR phenotype. Additionally, the accumulation of sphingomyelin results in the inhibition of cholesterol efflux from lysosomes (Kuzu et al. 2017). The resulting accumulation of various lipids in lysosomes inhibits lysosomal fusion events thereby contributing to reduced endocytic and autophagic flux (Kuzu et al. 2017). The increase in cellular sphingomyelin disturbs also autophagosome maturation (Corcelle-Termeau et al. 2016), suggesting that CADs may inhibit autophagy already at the step before the closure of autophagosomes. From the cancer therapeutic point of view, this would be advantageous as the toxic material to be engulfed would remain in the cytoplasm instead of being sealed in the lumen of autophagosomes. It is also interesting to note here that in a murine model of lung adenocarcinoma, *Smpd1* deficiency reduces tumor development in a manner associated with significant enhancement of T-cell-mediated antitumor immunity (Kachler et al. 2017). Thus, SMPD1-inhibiting CADs may act by both tumor cell-intrinsic and -extrinsic mechanisms to suppress tumor growth in vivo.

5.4 Potential of CADs as Anti-cancer Agents

In line with the numerous anti-cancer effects assigned to them in vitro and their favorable pharmacokinetic profiles, CADs have proven effective also in numerous animal cancer models covering major cancer types (Table 1). The potential of CADs in cancer treatment is also supported by several pharmaco-epidemiological studies. A Danish nationwide cohort study demonstrates a statistically significant association between the post-diagnostic use of the most commonly prescribed CAD antihistamine in Denmark, loratadine, and reduced mortality among patients with any non-localized cancer, and in particular with non-localized non-small cell lung cancer, when compared with use of non-CAD antihistamines (Ellegaard et al. 2016). Of the less frequently described CAD antihistamines, astemizole use has a similar significant association with reduced mortality among patients with any non-localized cancer, and ebastine use shows a similar tendency. In line with the ability of CADs to potentiate the effects of chemotherapeutics, the association

between CAD antihistamine use and reduced cancer mortality is stronger among cancer patients with records of concurrent chemotherapy than among those without such records (Ellegaard et al. 2016). Also among Danish ovarian cancer patients, CAD antihistamine use associates with significantly reduced mortality compared to use of non-CAD antihistamines (Verdoodt et al. 2019a). Further supporting the anti-cancer activity of CAD antihistamines, similar Swedish studies reveal strong associations between the use of the most commonly prescribed CAD antihistamine in Sweden, desloratadine, and reduced cancer mortality among breast cancer and melanoma patients as well as the use of less prescribed ebastine and loratadine and reduced cancer mortality among breast cancer patients (Fritz et al. 2020a, 2020b). Combined, these studies strongly suggest that CAD antihistamines could have a beneficial effect in cancer therapy.

Population-based case control studies provide also some support for CADs reducing cancer risk by demonstrating significantly reduced incidences of colorectal cancer among users of tricyclic antidepressants and selective serotonin reuptake inhibitors (Walker et al. 2011; Xu et al. 2006), of glioma among users of tricyclic antidepressants (Walker et al. 2011), and of pre-menopausal, mucinous ovarian cancer among users of CAD antihistamines (Verdoodt et al. 2019b). It should be noted that the positive effects may be cancer-type specific since the similar use of tricyclic antidepressants that associates with reduced risk of colorectal cancer shows no association with the risk of breast, prostate or lung cancer (Walker et al. 2011).

Contrary to the promising associations between CADs and reduced cancer mortality and risk listed above, the use of selective serotonin reuptake inhibitors (SSRI), all of which are CADs, associates with faster disease progression among epithelial ovarian cancer patients (Christensen et al. 2016). The authors of the study speculate that the effect is due to altered serotonin levels in the tumor microenvironment and growth-promoting effect of serotonin in this cancer type. Supporting the cancer-type specific cancer-promoting effect in ovarian cancer, SSRIs citalopram, escitalopram, fluoxetine, and sertraline inhibit the growth of gliomas, melanomas as well as colon, prostate, and lung cancers in animal models (Tutton and Barkla 1982; van Noort et al. 2014; Chen et al. 2018; Liu et al. 2015; Abdul et al. 1995; Gil-Ad et al. 2008; Jiang et al. 2018; Reddy et al. 2008; Zhang et al. 2018; Dai et al. 2020) (Table 1). Similarly, pimozide, which shows potent anti-cancer activity in various mouse models, including triple negative breast cancer (Dakir et al. 2018; Ren et al. 2018; Chen et al. 2017b; Subramaniam et al. 2020; Kim et al. 2019) (Table 1), enhances the progression of ERBB2- and HRAS-induced mammary tumors presumably by inducing hyperprolactinemia that results in prolactin-induced anti-apoptotic signaling in cancer cells (Johnston et al. 2018). And finally, fluoxetine has been reported to increase the number of brain metastasis in a mouse model of brain metastatic variant of MDA-MB-231 breast cancer by increasing the permeability of blood–brain barrier and activating glial cells (Shapovalov et al. 2014). Thus, when choosing the optimal CAD for cancer therapy, one should carefully study the CAD unrelated effects of the drug in the cancer in question. Fortunately, there are plenty of drugs to choose from.

6 Perspectives

As the increasingly stressed budgets of our healthcare services are unlikely to be able to fund the current explosion in the cost of new cancer precision medicines for much longer, drug repositioning becomes an increasingly appealing strategy to improve cancer care (Bertolini et al. 2015). Taking into consideration the long list of well-documented anti-cancer effects assigned for lysosome-targeting CADs in vitro, rapidly growing evidence for their efficacy in preclinical cancer models, strongly supportive epidemiological data and clinical safety data seldom seen for any anti-cancer drugs, CADs emerge as highly attractive candidates for drug repositioning.

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Cancer-Related Increases and Decreases in Calcium Signaling at the Endoplasmic Reticulum-Mitochondria Interface (MAMs)

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Contents

Abstract Endoplasmic reticulum (ER)-mitochondria regions are specialized subdomains called also mitochondria-associated membranes (MAMs). MAMs allow regulation of lipid synthesis and represent hubs for ion and metabolite signaling. As these two organelles can module both the amplitude and the spatiotemporal patterns of calcium (Ca^{2+}) signals, this particular interaction controls several Ca^{2+}-dependent pathways well known for their contribution to

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tumorigenesis, such as metabolism, survival, sensitivity to cell death, and metastasis. Mitochondria-mediated apoptosis arises from mitochondrial Ca^{2+} overload, permeabilization of the mitochondrial outer membrane, and the release of mitochondrial apoptotic factors into the cytosol. Decreases in Ca^{2+} signaling at the ER-mitochondria interface are being studied in depth as failure of apoptotic-dependent cell death is one of the predominant characteristics of cancer cells. However, some recent papers that linked MAMs Ca^{2+} crosstalk-related upregulation to tumor onset and progression have aroused the interest of the scientific community.

In this review, we will describe how different MAMs-localized proteins modulate the effectiveness of Ca^{2+}-dependent apoptotic stimuli by causing both increases and decreases in the ER-mitochondria interplay and, specifically, by modulating Ca^{2+} signaling.

Keywords Calcium · Calcium signaling · Cancer · Downregulation · MAMs · Upregulation

1 Introduction

Ca^{2+} is the third most abundant metal in nature, and it was adopted as a regulator in the early evolutionary stages in prokaryotes (Cai et al. 2015). Ca^{2+} ions play a crucial role in countless biological processes, and one of their most important contributions is undoubtedly represented by Ca^{2+} signaling, a complex network of extra- and intracellular messenger systems that mediates a wide range of pathways (Rimessi et al. 2020). The characterization of the complex network involving Ca^{2+} signaling has been in progress for approximately 140 years since the first experiments examining the contraction of isolated rat hearts (Ringer 1883). Since then, extensive progress has been made in understanding the numerous molecular pathways involved, although many aspects are still being debated and still need to be defined. Evolutionarily, cells have developed systems to constantly maintain Ca^{2+} concentrations at very low background levels to avoid the precipitation of phosphate salts, making this ion the logical choice for the exchange of signals (Carafoli and Krebs 2016). The crucial role of Ca^{2+} in cell biology results from the ability of cells to shape Ca^{2+} signals in the dimensions of space, time, and amplitude (Alonso et al. 2009).

Ca^{2+} enters cells through an assortment of Ca^{2+}-permeable channels that respond to different stimuli or acts as a second messenger, e.g., in the phosphoinositol signaling pathway, in which inositol trisphosphate (IP3) binds to Ca^{2+} channels on the endoplasmic reticulum (ER), transporting Ca^{2+} into the cytoplasm. Once in the cell, the effects of Ca^{2+} can be mediated by direct binding to its effectors, such as the phosphatase calcineurin, or indirectly by activating the ubiquitous Ca^{2+}-binding protein calmodulin, leading to the regulation of target molecules such as the Ca^{2+}/

calmodulin-dependent kinases CaMKII and CaMKIV (Kerkhofs et al. 2017). Temporally and spatially defined Ca^{2+} changes in the cytoplasm or in well-defined organelles represent a highly versatile intracellular signal capable of regulating many different processes, including depolarization, hormonal secretion, contraction of smooth and striated muscles, and cellular replication and activation of cytoplasmic, mitochondrial, and nuclear enzymes (Giorgi et al. 2018a).

Proteins that participate in Ca^{2+} signaling are mostly ubiquitous, but their distribution is highly tissue-specific (Berridge et al. 2003). Cells that need rapid Ca^{2+} signals, such as myocytes, express many voltage-activated calcium channels to allow quick Ca^{2+} entry through the plasma membrane, which then, via ryanodine receptors (RyRs) on the sarcoplasmic reticulum, triggers further calcium release. However, nonexcitable cells display calcium oscillations that last for tens of seconds and preferentially use the phosphoinositol signaling pathway to control gene expression and metabolism (Cui et al. 2017).

Therefore, a lack of Ca^{2+} ions can lead to various issues, and excess Ca^{2+} ions have harmful effects. Indeed, a sustained rise in intracellular Ca^{2+} is considered the initial step of irreversible cellular injury, mediated by the activation of the intracellular self-destructive lysosomal enzymes responsible for breakdown of subcellular organelle membranes and increases in oxidative stress and for the hyperactivation of phospholipases and endonucleases, which, through DNA damage, participate in apoptosis (Danese et al. 2017). Intracellular Ca^{2+} signals are controlled by Ca^{2+} influx through the plasma membrane (PM) and Ca^{2+} release from intracellular stores, mainly the ER and Golgi. Intracellular Ca^{2+} stores are constantly refilled while cytosolic Ca^{2+} is extruded from the cell by the plasma membrane Ca^{2+} ATPase (PMCA) pump, to maintain the optimal cytosolic Ca^{2+} concentration (Marchi et al. 2018).

In the cell, one of the organelles in which changes in $[Ca^{2+}]$ are particularly important is the mitochondrion (Giorgi et al. 2018b), which decodes Ca^{2+} signals in very sensitive and specific inputs that regulate metabolism, energy production, autophagy, and apoptosis (Giorgi et al. 2018a).

Membrane juxtaposition of both the mitochondria and the ER leads to the highly specialized MAMs compartment, which can be defined as areas of close organelle apposition but that are biochemically distinct from pure mitochondria and pure ER (Morciano et al. 2018). These contact sites are part of abundant heterotypic contacts, which, especially in recent years, have been well characterized and which include the ER-plasma membrane, ER-Golgi, lipid droplets–peroxisomes, mitochondria-lipid droplets, mitochondria–vacuoles/endosomes/lysosomes, ER-lipid droplets, mitochondria-plasma membrane, mitochondria–peroxisomes, ER-lipid droplets, and mitochondrial inner and outer membranes (Eisenberg-Bord et al. 2016).

To witness the strong tethering between the ER and mitochondria, an isolated MAM fraction contains membrane fragments of the outer mitochondrial membrane, the ER, and some plasma membrane proteins (Poston et al. 2013). Tomography analysis has revealed the morphology of these ER-mitochondria-connecting tethers (Csordas et al. 2006). The maintenance of this delicate structural juxtaposition results strategic for the regulation of a huge number of biological processes,

essentially through Ca^{2+} exchange. Poston et al. reported that there are around 1,000 molecular components of the MAMs fraction (Poston et al. 2013) and their study led to an elucidation of the multiple roles played by this particular subcellular compartment. In particular, MAMs co-regulate and influence Ca^{2+} signaling/dynamics, synthesis/transport of lipids and lipid intermediates, autophagy, apoptosis, and energy metabolism.

Noteworthy is the fact that MAM structures are sensitive to physiological cell conditions and this reflects in a transient and highly variable MAM composition. The length of ER-mitochondria tethers is a determining factor, critical for an efficient Ca^{2+} transfer, and an ER-mitochondria physical distance modulation is a condition found in different pathophysiological situations. About that, these two organelles' interplay is also involved in mitochondrial shape and size, and MAM-regulated mitochondrial fusion/fission process undoubtedly covers a crucial role in governing mitochondrial dynamics. Dynamin-related protein 1 (Drp1) is responsible for mitochondrial fission; following its activation, Drp1 translocates from the cytosol to the mitochondria and oligomerizes and constricts this organelle until its division is achieved. Focusing on mitochondrial fusion, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) are responsible for the outer membrane fusion, while optic atrophy 1 (Opa1) mediates mitochondrial inner membrane fusion (Ponte et al. 2020).

MAMs are enriched in channels involved in calcium transfer, allowing perfect and synergistic signaling between the ER and mitochondria. Moreover, MAMs target many proteins with oncogenic/oncosuppressive functions that modulate cell signaling pathways involved in physiopathological processes (Danese et al. 2017).

As Ca^{2+} signaling-governed processes (such as energy production, metabolism, autophagy, and apoptosis) are dysregulated in cancer cells and play a key role in Ca^{2+} transfer and signaling in MAMs, the perturbation of these Ca^{2+} transport systems at the ER and the mitochondria in relation to tumor onset and progression has become a very hot topic, especially in recent times. In fact, the recent characterization of the many oncogenes and tumor suppressors residing at the MAMs has led many research groups to elucidate how these proteins mediate their functions by altering ER-mitochondrial Ca^{2+} transfer, thereby promoting or preventing cancer cell survival. Increases or decreases in calcium exchange through the MAMs interface can either exert protumorigenic effects (such as promoting metastatic transformations) or antitumorigenic effects (such as restoring sensitivity to apoptosis) in a cancer type- and cancer state-specific manner (Kerkhofs et al. 2018).

The aim of this review is to clarify how the perturbation of Ca^{2+} signaling at the ER-mitochondria interface can play a double-sided role in tumor pathology and progression. Modulation of calcium signaling at the MAMs, highly dynamic signaling hubs, could therefore represent a good therapeutic strategy in the future.

2 MAM-Localized Ca^{2+} Signaling Modulators in Cancer: Channels and Receptors

Ca^{2+} signaling represents an important tool that regulates many physiological cellular events from proliferation to cell death. Given the pivotal role it plays in such events, it is understandable why, over the past decades, remodeling of its shape has been demonstrated to be involved in the onset of many pathological conditions, such as tumor progression (Monteith et al. 2012; Prevarskaya et al. 2014; Marchi et al. 2020). Proteins involved in the maintenance of Ca^{2+} homeostasis consist of pumps, exchangers, and channels and have been described as part of the Ca^{2+} signaling "toolkit" (Berridge et al. 2003).

In resting conditions, the free cytosolic Ca^{2+} concentration is much lower than that in most extracellular fluids, and an ion concentration gradient is generated. Thus, when Ca^{2+}-permeable ion channels in the plasma membrane are open, Ca^{2+} flux into the cell increases (Carafoli 2002). However, as already mentioned, Ca^{2+} signaling can be generated by both external and internal cellular sources.

In the cell, the main ion reservoir from which Ca^{2+} can be transferred is the endoplasmic reticulum. On the one hand, the ER is the primary cell Ca^{2+} store; on the other hand, the main cellular Ca^{2+} signaling translators are the mitochondria.

Indeed, depletion of luminal ER Ca^{2+} levels is followed by a rapid increase in ion mitochondrial concentration. To ensure this interaction is effective, the ER and the mitochondria are juxtaposed on the MAMs at a short distance of approximately 10–25 nm (Csordas et al. 2006; Rizzuto et al. 1998; Marchi et al. 2014) in the smooth ER and at approximately 50 nm in the rough ER (Wang et al. 2015; Giacomello and Pellegrini 2016).

2.1 ER Side

Many ER-resident proteins involved in Ca^{2+} transfer have been found at the MAMs: the sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and inositol 1,4,5-trisphosphate receptors (IP3R), among others. SERCAs are members of the P-type ATPase superfamily of primary active transporters (a large family of membrane-embedded pumps (Wang et al. 2015)) and can maintain the correct cytosolic and reticular Ca^{2+} concentrations.

The 110 kDa SERCA protein has 10 helix intramembrane domains involved in the interaction with two Ca^{2+} ions transferred to the ER lumen at the expense of adenosine triphosphate (ATP). The Ca^{2+} flux is coupled to the exchange of two to three protons moved to the cytoplasm (Palmgren and Nissen 2011). In addition to transmembrane domains, SERCA has three cytoplasmic regions: the nucleotide-binding domain (N), designed for ATP binding; the phosphorylation (P) domain, which hosts the amino acid residue phosphorylated by ATP; and the actuator (A) domain at the N-terminus, which controls enzyme dephosphorylation. During

ATP hydrolysis, conformational changes in the protein domains occur, and as consequence, the intermembrane domains warp, enabling Ca^{2+} transport (Toyoshima et al. 2000; Moller et al. 2010).

To date, at least 12 isoforms of SERCA (SERCA1a-b, SERCA2a-d, SERCA3a-f) have been identified in vertebrates (Lipskaia et al. 2014), each characterized by tissue and developmental specificity. This diversity is because SERCAs are encoded by three different genes located on three chromosomes (ATP2A1, ATP2A2, and ATP2A3), each generating alternative splicing variants that differ mainly in the C-terminus of the protein.

The diversities in the coding sequencing of these proteins do not affect the protein tertiary structures, which are highly conserved among all isoforms, but instead lead to differences in Ca^{2+} affinity. Among all these proteins, ubiquitous SERCA2b is the isoform with the highest Ca^{2+} affinity and plays a crucial role in the regulation of ER Ca^{2+} uptake and Ca^{2+} homeostasis (Vandecaetsbeek et al. 2009). All SERCA isoforms are present along the entire ER membrane and are not particularly enriched in MAMs.

SERCA activity can be modulated by many proteins. Among them, the recently identified ER-luminal protein disulfide isomerase thioredoxin-related transmembrane protein 1 (TMX1) displays palmitoylation-dependent MAMs localization. TMX1 can directly interact with SERCA2b (Gutierrez and Simmen 2018; Lynes et al. 2012) and inhibit its activity, reducing Ca^{2+} transfer.

If SERCA activity is lowered by TMX1, its activity is enhanced by the redox active form of the redox-sensitive selenoprotein N (SEPN1) (Gutierrez and Simmen 2018). MAMs result particularly enriched in redox regulatory proteins, and TMX1 and SEPN1 are among them (Krols et al. 2016; Marino et al. 2015).

Calnexin is a chaperone protein that localizes at the ER-mitochondrial contact sites in a palmitoylation-dependent manner (Lynes et al. 2012). The primary function of this protein is to interact with misfolded proteins to improve the folding efficiency of ER proteins (Lamriben et al. 2016). Upon palmitoylation, calnexin moves to the MAMs, where it interacts with SERCA2b, increasing Ca^{2+} transfer from the cytosol to the ER (Lynes et al. 2013). Interestingly, the modulation of SERCA2b activity by calnexin is counteracted by TMX1 in a way that may suggest competition for the same binding site (Krols et al. 2016; Raturi et al. 2016).

IP3Rs are large-conductance nonselective cation channels that together with the RyRs, which is mainly expressed in sarcoplasmic reticulum, are major structures through which Ca^{2+} exits the ER (Ashby and Tepikin 2001).

IP3R channels are homo- or heterotetramers composed of four subunits of approximately 300 kDa each. The molecular structure of the IP3R monomer, determined by cryogenic electron microscopy, consists of three structural domains: an N-terminal ligand-binding domain, containing both the IP3-binding core and the suppressor region, a central modulatory domain, and a Ca^{2+} channel region at the C-terminus containing six intramembrane helices. The C-tails interact directly with the N-terminal domains of the other subunits (Fan et al. 2015).

In vertebrates, there are three different isoforms of IP3R (IP3R1, IP3R2, and IP3R3) encoded by three genes (ITPPR1, ITPR2, and ITPR3, in humans). Despite

the high homology in the amino acid sequences (60–80%), these isoforms have a different expression pattern, with IP3R1 mainly expressed in neuronal cells, IP3R2 in muscle and liver cells, and ubiquitous IP3R3 in most cultured cells (Mikoshiba 2007; Foskett et al. 2007). In addition, the different isoforms show differences in ligand-binding sensitivity and regulation by Ca^{2+} and ATP (Newton et al. 1994; Miyakawa et al. 1999; Tu et al. 2005; Khan et al. 2006; Betzenhauser et al. 2008; Wagner 2nd et al. 2008; Vervloessem et al. 2015).

IP3Rs are enriched at MAMs levels, where they also exert a structural role, being in close proximity with the mitochondrial voltage-dependent anion channel 1 (VDAC1) and by interacting with the chaperone glucose-regulated protein GRP75 which acts as a tether between the two proteins and organelles (Bernard-Marissal et al. 2018). It has also been recently highlighted that IP3R isoforms differently regulate ER-mitochondrial contacts and local calcium transfer, proving that IP3Rs structural role in MAM compartment is crucial (Bartok et al. 2019).

The activity of IP3R receptors is regulated primarily by inositol trisphosphate (IP3), released at the plasma membrane after the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC).

However, IP3Rs can also be modulated by ATP, post-translational modification (Mak and Foskett 2015; Bansaghi et al. 2014; Yule et al. 2010; Prole and Taylor 2016; Ivanova et al. 2014; Ramos-Franco et al. 1998), and Ca^{2+} ions, which act both from the luminal ER side, increasing the sensitivity to its ligand, and from the cytoplasmatic sides from which Ca^{2+} plays a dual role as an activator at low concentrations and an inhibitor if its concentration is higher than 300 nM (Table 1).

As noted earlier, there is a juxtaposition between the two MAM-forming organelles, and Ca^{2+} release from the ER is followed by uptake at the mitochondrial interface.

2.2 Mitochondrial Side

After being released from the ER, Ca^{2+} ions can first cross the outer mitochondrial membrane through VDAC and, once in the mitochondrial intramembrane space, enter the matrix through the mitochondrial Ca^{2+} uniporter (MCU).

VDAC is a 30-kDa protein existing in all eukaryotic cells in three different isoforms: VDAC1 and VDAC2 are expressed in most mammals, and VDAC3 is the isoform with the lowest expression (De Pinto et al. 2010; Huang et al. 2014; Maldonado et al. 2013). VDAC is the most abundant outer mitochondrial membrane protein, and due to its permeability not only to anions but also to respiratory substrates, ATP, reactive oxygen species (ROS), and cytochrome C can be considered master regulators of mitochondrial bioenergetics (Shoshan-Barmatz et al. 2010; Weisthal et al. 2014). The permeability of this channel is highly impacted by its two conformational states, opened and closed, since in the closed state, the channel is permeable only to small ions but not to anionic metabolites (Shoshan-Barmatz et al. 2010; Gincel et al. 2000; Schein et al. 1976). The switch between the

Table 1 Summary of Ca^{2+} signaling modulators founded at MAMs and implicated in cancer onset and progression

		Modulator	Ca^{2+}-related mechanism	Tumor
Downregulation of MAMs Ca^{2+} crosstalk	Low ER-Ca^{2+} release	*Akt*	IP3R3 phosphorylation	Thyroid, breast, cervical, ovarian, non-small cell lung, pancreatic, prostate, gastric, brain, and colon cancer; renal and hepatocellular carcinoma (Revathidevi and Munirajan 2019)
		BAP1	IP3R3 deubiquitylation and stabilization	Mesothelioma (Bononi et al. 2017), uveal and cutaneous melanoma, renal carcinoma (Rai et al. 2016)
		Bcl-2	Decreases ER Ca^{2+} efflux by targeting IP3R3	Lymphoma, small cell lung cancer (Bittremieux et al. 2019)
		Bcl-XL	Enhance IP3R-mediated Ca^{2+} signals	Multiple myeloma, melanoma, glioblastoma, and prostate, colorectal, non-small-cell lung, and pancreatic cancer (Trisciuoglio et al. 2017; Scherr et al. 2016; Zhang et al. 2014; Yoshimine et al. 2013)
		ERO1-α	Oxidizes IP3R1 promoting ER Ca^{2+} release	Breast and colon cancer (Takei et al. 2017; Tanaka et al. 2017)
		H-Ras	Decreases IP3R3 expression	Pancreatic carcinoma; colorectal and head and neck cancer; lung, hematopoietic, and dermatological cancers (Munoz-Maldonado et al. 2019)
		Mcl-1	Stimulates non-MAM-localized IP3R3 Ca^{2+} release increasing ER Ca^{2+} leak	Lung, breast, and cervical cancer (Chen et al. 2019; Campbell et al. 2018; Zhang et al. 2012)
		p53	Binds to SERCA pump	Almost all
		PACS-2	Player in MAMs integrity regulation	Colorectal cancer (Kveiborg and Thomas 2018)

(continued)

Table 1 (continued)

		Modulator	Ca^{2+}-related mechanism	Tumor
		PERK	Acts as MAMs structural tethering	Breast cancer (Feng et al. 2017)
		PML	Regulates the phosphorylation of IP3R3	Almost all
		PTEN	Antagonizes IP3R3 Akt-mediated phosphorylation	Lung, prostate, head, stomach, breast, and pancreatic cancer (Salmena et al. 2008)
		RyR2	ER Ca^{2+} release	Melanoma, breast cancer, lymphoma, prostate cancer, thyroid carcinoma (Xu et al. 2019; Carpi et al. 2018; Lu et al. 2017; McCarthy et al. 2003; Mariot et al. 2000)
		STAT3	Promotes IP3R3 degradation	Breast cancer (Yu et al. 2014)
	Low mitochondrial uptake	*Bcl-2*	Regulates mitochondrial Ca^{2+} uptake targeting VDAC1	Hematopoietic, lung, gastric, breast, and prostate cancer (Frenzel et al. 2009)
		Bcl-XL	Regulates mitochondrial Ca^{2+} uptake targeting VDAC1	Multiple myeloma, melanoma, glioblastoma, and prostate, colorectal, non-small-cell lung, and pancreatic cancer (Trisciuoglio et al. 2017; Scherr et al. 2016; Zhang et al. 2014; Yoshimine et al. 2013)
		EZH2	Its inhibition inactivates MICU1	Breast, prostate, and endometrial cancers; melanoma and head and neck squamous cell carcinoma (Kim and Roberts 2016)
		FATE1	Acts as a MAMs anti-tether agent	Hepatocellular carcinoma; colon and gastric cancer (Dong et al. 2003)
		Fhit	Increases mitochondrial Ca^{2+} hotspots number	Silenced in >50% of cancers (Kiss et al. 2017)

(continued)

Table 1 (continued)

		Modulator	Ca^{2+}-related mechanism	Tumor
		miR-25	Downregulates MCU	Prostate and in colon cancer (Marchi et al. 2013)
		miR-7	Reduce VDAC1 expression	Hepatocarcinoma and neuroblastoma (Chaudhuri et al. 2016a; Bargaje et al. 2012)
		TRPC3	Affects mitochondrial membrane potential	Breast cancer (Wang et al. 2019)
Upregulation of MAMs Ca^{2+} crosstalk	High ER-Ca^{2+} release	*ERO1-α*	Regulates Ca^{2+} efflux from the ER	Breast and colon (Takei et al. 2017; Tanaka et al. 2017)
		GRP78	Store ER Ca^{2+}	Epithelial ovarian and prostate cancer; diffuse large B cell lymphoma; renal cell, colorectal, endometrial gastric, and squamous cell carcinoma (Niu et al. 2015)
		IP3R3	Ca^{2+} release from the ER	Hepatocellular and kidney carcinoma; cholangiocarcinoma (Guerra et al. 2019; Ueasilamongkol et al. 2020; Rezuchova et al. 2019)
		Sig1R	Binds and activate IP3R3	Glioma and melanoma; prostate, lung, colon, and breast cancer (Crottes et al. 2013)
	High mitochondrial Ca^{2+} uptake	*MCU*	Mitochondrial Ca^{2+} uptake	Breast cancer; hepatocellular carcinoma (Vultur et al. 2018)
		MCUR1	Positive regulator of MCU	Hepatocellular carcinoma (Jin et al. 2019; Ren et al. 2018)
		MICU1	Regulates MCU gating	Renal, ovarian, breast, and lung cancer (Marchi et al. 2019a)
		RIPK1	Binds MCU to promote Ca^{2+} entry	Colorectal cancer (Zeng et al. 2018)

opened and closed states is regulated by many factors, including Bcl2 family members (Tsujimoto and Shimizu 2000), Ca^{2+} ions (Bathori et al. 2006), and voltage. Indeed, high mitochondrial voltages induce VDAC to close (Gincel et al. 2000) in a N-terminus-mediated manner (Abu-Hamad et al. 2009).

Among VDAC channels, the most frequently expressed and consequently studied isoform is VDAC1 (Messina et al. 2012), which has been shown to be targeted to the MAMs (Hajnoczky et al. 2002; Shoshan-Barmatz and Gincel 2003; Colombini 2012) and to regulate the Ca^{2+} flux through the mitochondria outer membrane (Rapizzi et al. 2002). If regulation of mitochondrial Ca^{2+} signaling is not a unique feature of VDAC1, the ability to transmit proapoptotic stimuli to the mitochondria seems to be an exclusive characteristic of this isoform (De Stefani et al. 2012).

To reach the mitochondrial matrix and regulate all the previously mentioned processes, Ca^{2+} entering the outer mitochondrial membrane has to permeate the inner mitochondrial membrane that, unlike the outer membrane, is not permeable to ions. The accumulation of Ca^{2+} inside the mitochondrial matrix follows an electrogenic gradient and is driven by the low Ca^{2+} affinity uniporter complex MCU. Due to the low Ca^{2+} affinity of this MCU complex, the rapid mitochondrial ion accumulation is difficult to explain without considering the presence of close contacts between the ER and the mitochondria, which create microdomains with a high Ca^{2+} concentration (Rizzuto et al. 1998).

MCU is a complex of approximately 480 kDa composed of the channel-forming subunits MCUa and MCUb, organized mainly in pentamers. MCUa and MCUb have opposite effects on Ca^{2+} ion transfer (allowing and inhibiting permeation, respectively), and their relative quantities in the complex regulate the Ca^{2+} transport capability of MCU itself. In addition to the channel-forming subunits, mitochondrial calcium uptake 1 and 2 (MICU1 and MICU2) and the essential MICU regulator (EMRE) are part of the uniporter complex and play a pivotal role in regulating the integrity of the complex itself (De Stefani et al. 2015; Oxenoid et al. 2016; Raffaello et al. 2013; Sancak et al. 2013). MCU complexes were enriched in MAMs, positioned more to the mitochondrial periphery, indicating high accessibility to cytoplasm-derived Ca^{2+} inputs (Marchi et al. 2017).

Among the mitochondrial Ca^{2+} uptake family of regulator proteins MICU1 and MICU2, the best characterized is MICU1, which functions as a gatekeeper that can sense the Ca^{2+} levels of the intermembrane space. Indeed, at low concentrations, the gate is closed, but as soon as the Ca^{2+} levels pass the [Ca^{2+}] threshold of 700 nM for MICU1-MICU2 heterodimers and 300 nM for MICU1 homodimers, the Ca^{2+}-binding EF hands of MICU1 bind the ion and undergo a conformational change that opens the channel (Csordas et al. 2013; Mallilankaraman et al. 2012a; Perocchi et al. 2010; Petrungaro et al. 2015; Park et al. 2020) (Table 1).

3 Decrease in ER-Mitochondria Ca^{2+} Crosstalk

3.1 Dysfunctional ER-Ca^{2+} Release

As described in the introductory section, in recent years, increasing evidence has shown that organelles communicate with each other through Ca^{2+} signaling. In particular, at the MAMs level, interorganellar Ca^{2+} signaling is profoundly spatiotemporally regulated. Interestingly, in the tumor setting, an alteration of Ca^{2+} signaling has been shown to affect malignant transformation and tumor progression through the control of cell death programs and metabolism (Rimessi et al. 2020; Monteith et al. 2007).

In this context, the ER not only plays a decisive role in Ca^{2+} signaling but also guarantees a control system for correct protein folding and stress sensing. Alterations in ER homeostasis, including substantial Ca^{2+} depletion, are associated with the pathophysiology of many diseases, including cancer (Mekahli et al. 2011).

The normal Ca^{2+} exchange between the ER and the mitochondria requires adequate filling of the ER Ca^{2+} stores. Thus, decreasing the ER Ca^{2+} levels will compromise ER-mitochondrial Ca^{2+} transfer. As a consequence, changes in the ER Ca^{2+} store content affect the Ca^{2+} efflux from the ER to the mitochondria and ultimately cell survival (Ivanova et al. 2017).

The maintenance of physiological low levels of mitochondrial Ca^{2+} uptake by IP3R is crucial to preserve cellular bioenergetics in normal and cancer cells by enabling the dehydrogenase activation of the tricarboxylic acid (TCA) cycle, strong ATP production and metabolic intermediates for the generation of building blocks, allowing the cells to enter the cell cycle and proliferate. In breast cancer cells but not in normal cells, Ca^{2+} release suppression mediated by the inhibition of IP3R activity caused cell death through a deregulated autophagic mechanism (Singh et al. 2017a) and mitotic disruption, as reported by Cárdenas C. et al. (2016).

Regarding type 3 IP3R, the depletion of IP3R3 or its pharmacological blocking increased the level of the autophagic marker microtubule-associated protein 1A/1B-light chain 3 (LC3)-II through the upregulation of autophagic protein 5 (Atg5) and ROS generation, leading to the blockage of tumor growth in a mouse model of breast cancer (Singh et al. 2017a). This finding is correlated with the high expression of IP3R3 in human malignant tissues and high concentrations of metabolites in the serum of patients (Singh et al. 2017b).

Moreover, it has been reported that the inhibition of IP3R with caffeine, a nonspecific inhibitor of these receptors, leads to a decreased migration of glioblastoma cells and a substantially increased mean survival in a mouse glioblastoma xenograft model (Kang et al. 2010). In the Caco-2 colon cancer cell line, IP3R3 silencing, or nonspecific pharmacological inhibition by 2-APB in gastric cancer cells, induced apoptosis, while overexpression protected cells from staurosporine-induced apoptotic death (Shibao et al. 2010).

Interestingly, various MAM-located oncosuppressors and oncogenes have been reported to interact with IP3Rs, including the oncogene protein kinase B (PKB), also

known as Akt, promyelocytic leukemia protein (PML), BRCA1 associated protein 1 (BAP1), phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and B-cell lymphoma 2 (Bcl-2) family proteins, modifying the Ca^{2+} release patterns and cell fate (Bononi et al. 2017; Akl and Bultynck 2013; Missiroli et al. 2017; Kuchay et al. 2017; Giorgi et al. 2010). Although the aforementioned proteins are all present at the ER-mitochondria interface, only PTEN and PML are particularly enriched on MAMs (Missiroli et al. 2016; Bononi et al. 2013).

Akt, as well as protein kinase C (PKC) isozymes (Pinton et al. 2004), is a key player in regulating multiple signaling pathways through calcium signaling tuning, such as cell metabolism, cell proliferation, and survival (Szado et al. 2008). Notably, in human breast cancers, the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway is frequently dysregulated (Gonzalez-Angulo et al. 2011; Stemke-Hale et al. 2008).

On the ER side, IP3R Akt-mediated phosphorylation results in a decreased magnitude of Ca^{2+} release and, as a result, reduced mitochondrial Ca^{2+} uptake. Moreover, this decrease in Ca^{2+} flux protected glioblastoma cell lines from the effects of apoptotic stimuli (Szado et al. 2008).

In 2012, our group demonstrated that Akt specifically phosphorylates type 3 IP3R, leading to diminished mitochondrial Ca^{2+} influx and, consequently, protecting cells from apoptosis (Marchi et al. 2012).

PML tumor suppressor protein has been implicated in diverse cellular processes ranging from tumor suppression to defense against virus infection (Bernardi and Pandolfi 2007; Everett and Chelbi-Alix 2007; Hsu and Kao 2018; Pinton et al. 2011). An extranuclear fraction of PML has been demonstrated to be targeted to the MAMs in a p53-dependent manner (Missiroli et al. 2016) and to form a multicomplex with type 3 IP3R, the serine threonine kinase Akt and protein phosphatase 2A (PP2A) (Giorgi et al. 2010).

It has been shown that PML regulates the phosphorylation of IP3R by controlling the activity of Akt through the recruitment of the PP2A phosphatase at the MAMs interface. Hence, PML can coordinate Ca^{2+} mobilization into the mitochondria, which then triggers the cell death program. Conversely, in the absence of PML, PP2A does not assemble with IP3R and Akt, resulting in a higher activation of Akt (phospho-Akt). Once activated, Akt can hyperphosphorylate IP3R, thereby suppressing ER Ca^{2+} release to the mitochondria (Giorgi et al. 2011).

BAP1 is a member of the ubiquitin C-terminal hydrolase (UCH) subfamily of deubiquitylating enzymes and has tumor suppressor activity, which has been mainly correlated with its nuclear localization (Lee et al. 2014; Ismail et al. 2014). When BAP1 localizes to the ER, it binds, deubiquitylates, and stabilizes the activity of the IP3R3 channel, modulating Ca^{2+} release from the ER to the cytosol and then to the mitochondria, promoting apoptosis. In $BAP1^{+/-}$ carriers, the reduced level of BAP1 resulted in a diminished IP3R3 quote with a subsequent Ca^{2+} transfer decrease from the ER to the mitochondria. This event caused a reduced propensity of $BAP1^{+/-}$ cells to undergo apoptosis following DNA damage induced by asbestos or UV light (Bononi et al. 2017).

PTEN is another Ca^{2+}-related tumor suppressor that has been shown to be mutated or suppressed in many tumors (Salmena et al. 2008). Bononi et al.

demonstrated that a fraction of cellular PTEN is localized at the MAMs, where it interacts with IP3R3, antagonizing its Akt-mediated phosphorylation and enhancing Ca^{2+} transfer from the ER to mitochondria. In this way, it reestablishes cellular sensitivity to Ca^{2+}-mediated proapoptotic stimuli. Conversely, PTEN knockdown reduced the Ca^{2+} release from the ER and decreased mitochondrial Ca^{2+} transients, thus preventing cell death activation (Bononi et al. 2013). Moreover, a novel role for PTEN has been proposed; it can compete with F-box and leucine-rich repeat protein 2 (FBXL2), an E3-ubiquitin ligase F-box protein, for binding to IP3R3 to prevent its degradation. It has been demonstrated that FBXL2 degradation of IP3R3 is enhanced in cancer cells in which PTEN expression is lowered, thereby resulting in the inhibition of apoptosis (Kuchay et al. 2017).

The Bcl-2 family of anti- and proapoptotic proteins is predominantly localized to the mitochondria, ER, and MAMs, and their activities strongly reflect their intracellular localization (Morciano et al. 2018). Bcl-2 is a proto-oncogene known for its involvement in inhibition of apoptosis through its interaction with the proapoptotic proteins BCL2 associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak) (Rimessi et al. 2020). Indeed, at the ER, Bcl-2 prevents excessive Ca^{2+} flux by directly targeting all three IP3R receptor isoforms, which would lead to mitochondrial Ca^{2+} overload and opening of the permeability transition pore (mPTP) (Chen et al. 2015; Bonora et al. 2017). Dysregulation of Bcl-2 expression has been highlighted in various cancers, including hematopoietic, lung, breast, and prostate tumors (Morciano et al. 2018).

Bcl-XL is another antiapoptotic member of the same family that is frequently overexpressed in many tumors, such as multiple myeloma, melanoma, glioblastoma, prostate cancer, colorectal cancer, non-small cell lung cancer, and pancreatic cancers (Trisciuoglio et al. 2017; Scherr et al. 2016; Zhang et al. 2014; Yoshimine et al. 2013). This protein is localized at the MAMs (Monaco et al. 2015), where it directly binds the IP3R channels, regulating IP3R-related Ca^{2+} release. Bcl-XL caused a strong sensitization of IP3R, promoting prosurvival Ca^{2+} oscillations (White et al. 2005).

Among the antiapoptotic proteins of the Bcl-2 family, myeloid cell leukemia 1 (Mcl-1) also lowers the calcium ER store content by stimulating IP3Rs outside of the MAMs, thereby increasing Ca^{2+} leakage from the ER, resulting in a decline in the basal ER Ca^{2+} levels (Eckenrode et al. 2010). In the presence of low [IP3], in Mcl-1-expressing cells, store depletion becomes more prominent, indicating that the sensitivity of IP3-dependent Ca^{2+} release is enhanced by Mcl-1. Mcl-1-mediated IP3R sensitization also contributes to low-level IP3R-mediated Ca^{2+} signaling from the ER to the mitochondria and thereby stimulates mitochondrial bioenergetics (Bittremieux et al. 2016).

At the MAMs, oncogenic H-Ras also affects Ca^{2+} transfer to the mitochondria to promote evasion from the apoptotic cascade (Rimessi et al. 2014). In colorectal cancer cells, oncogenic K-Ras modified the expression of IP3Rs, weakening the Ca^{2+} release from the ER to allow cells to escape Ca^{2+}-mediated apoptosis (Pierro et al. 2014). Indeed, Ras-driven mitochondrial dysfunction causes metabolic and redox changes that favor tumorigenesis (Hu et al. 2012). Hence, proper maintenance

of IP3R3 protein levels is crucial for preventing oncogenesis by strengthening tumor-suppressive ER-mitochondrial Ca^{2+} transfer.

Furthermore, MAMs are a molecular platform for the regulation of many oxidoreductases. In this context, endoplasmic reticulum oxidoreductin 1-α (ERO1-α) activity is broadly investigated for its enrichment at ER-mitochondria contact sites (Anelli et al. 2012) and its high expression in different tumor types (Kakihana et al. 2012). This oxidase impacts ER-Ca^{2+} storage and IP3-dependent fluxes. During ER stress, ERO1-α oxidizes type 1 IP3R, promoting the release of Ca^{2+} from the ER (Anelli et al. 2012). Furthermore, endoplasmic reticulum resident protein 44 (ERp44) (an ER luminal chaperone protein) binds to IP3R1 and inhibits its channel activity under reducing conditions, resulting in the blockade of Ca^{2+} transfer to the mitochondria (Higo et al. 2005). Oxidation of IP3R1 by ERO1-α causes the dissociation of ERp44, thus leading to the activation of Ca^{2+} release via IP3R1 (Li et al. 2009). ERO1-α silencing has been demonstrated to profoundly affect mitochondrial Ca^{2+} uptake, likely modifying MCU activity. Thus, ERO1-α links redox and Ca^{2+} homeostasis in MAMs (Anelli et al. 2012).

Recently, the oncogenic transcription factor signal transducer and activator of transcription 3 (STAT3), which mediates the signaling of cytokines, growth factors, and oncogenes (Yu et al. 2014), has been shown to localize only to MAMs (Su et al. 2020). At this location, it modulates ER-mitochondria Ca^{2+} release by interacting with the IP3R3 channel and promoting its degradation, resulting in greater cellular resistance to apoptotic stimuli (Avalle et al. 2019). In breast cancer cell lines, silencing STAT3 enhances the ER Ca^{2+} release and sensitivity to apoptosis following oxidative stress, correlating with increased IP3R3 levels. This evidence suggests that STAT3-mediated IP3R3 downregulation in the ER crucially contributes to its antiapoptotic functions via Ca^{2+} flux modulation.

Together with the IP3R receptors, RyRs and SERCA are the major Ca^{2+} players in the ER (Berridge 2012). In general, RyRs regulate melanocyte and T cell proliferation (Hakamata et al. 1994; Kang et al. 2000) and astrocyte migration (Matyash et al. 2002). Ryanodine receptor type 2 (RyR2), a member of the RyR family, controls the Ca^{2+} release from the sarcoplasmic reticulum into the cytosol (Ding et al. 2017). Different studies have confirmed the association of RyR2 with several cancer types, including melanoma (Carpi et al. 2018), breast cancer (Lu et al. 2017), lymphoma (McCarthy et al. 2003), and prostate cancer (Mariot et al. 2000). Recently, it has been reported that RyR2 is downregulated in thyroid carcinoma tissues and that low expression levels of RyR2 are closely associated with poor prognosis in thyroid carcinoma patients (Xu et al. 2019).

Over the past years, the tumor suppressor p53 has been shown to be altered in many human cancer tissues, including colon, breast, lung, brain, bladder, pancreatic, stomach, and esophageal cancer (Vogelstein et al. 2000). Some of p53 fraction is located at the MAMs, where it directly binds to the SERCA pump, changing its oxidative state and thus leading to an increased Ca^{2+} load, followed by an enhanced flux to the mitochondria. Consequently, during apoptotic stimulation, more Ca^{2+} can be released from the ER into the mitochondria, enhancing mitochondrial Ca^{2+} overload, opening of the mitochondrial mPTP, release of caspase cofactors, and

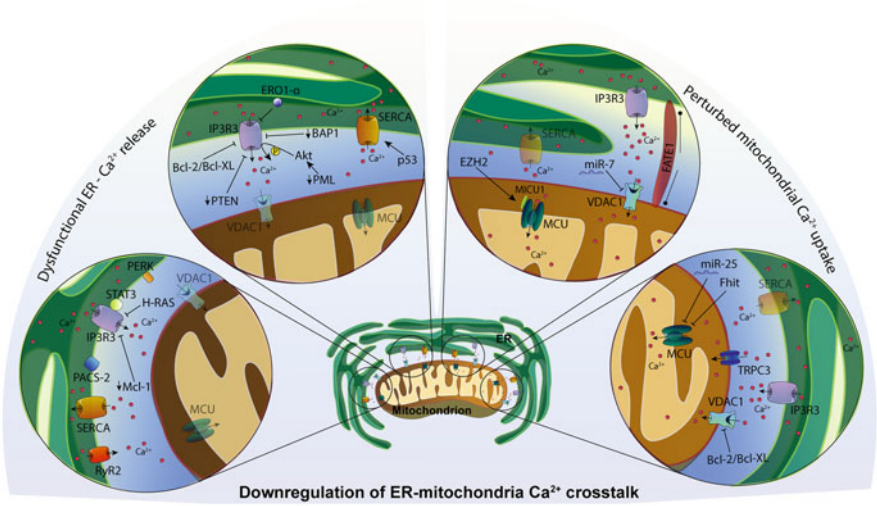

Fig. 1 Downregulation of MAMs Ca^{2+} crosstalk in cancer: graphical representation of the calcium signaling regulators involved in a cancer-related decreased Ca^{2+} crosstalk state. See text for further details. Ca^{2+}, calcium; ER, endoplasmic reticulum

ultimately induction of the intrinsic apoptosis pathway (Morciano et al. 2018). Dysregulation of p53-dependent Ca^{2+} homeostasis led to reduced ER Ca^{2+} release, resulting in a low responsiveness to apoptotic stimulation (Giorgi et al. 2015).

We must also note the phosphofurin acid cluster sorting 2 protein (PACS-2) and PKR-like ER kinase (PERK). PACS-2 is a multifunctional protein involved in retrograde ER-Golgi trafficking of multiple proteins (Youker et al. 2009). Although it is unclear whether a direct interaction of PACS-2 at the MAMs occurs, it was demonstrated that depletion of PACS-2 reduces mitochondrial-ER contact sites and mediates apoptosis (Simmen et al. 2005). PACS-2 was also demonstrated to be a fundamental player in rapamycin complex 2 (mTORC2)-dependent regulation of MAMs integrity (Betz et al. 2013). PERK is a protein kinase that, together with inositol-requiring enzyme 1 (IRE1) and transcription factor 6 (ATF6), acts as an ER stress sensor from the ER membrane, controlling UPR functioning. The function of this protein in the MAMs is independent of its role as an ER stress sensor and transcriptional regulator of redox homeostasis. Indeed, PERK maintains, through its cytoplasmic domains, the juxtaposition of the ER and the mitochondria, acting as a structural tether and permitting the transmission of ROS-mediated signals (Verfaillie et al. 2012).

In conclusion, changes in the ER Ca^{2+}-store content would perturb Ca^{2+} transfer from the ER to the mitochondria and ultimately influence cell death or survival. A reduction in intracellular store Ca^{2+} release is certainly the main mechanism adopted by cancer cells to escape mitochondria-mediated apoptosis (Fig. 1).

3.2 Perturbed Mitochondrial Ca^{2+} Uptake

Cancer-derived modifications in cellular physiology could be related to impairment of the Ca^{2+} signaling network, which is frequently associated with the dysregulation of several Ca^{2+} channels and pumps (Prevarskaya et al. 2014; Hanahan and Weinberg 2000).

In addition to limiting the excessive release of Ca^{2+} from the ER, cancer cells can effectively prevent mitochondrial Ca^{2+} overload by limiting mitochondrial Ca^{2+} uptake.

Among the proteins responsible for limitation of mitochondrial calcium influx are Bcl-2 and Bcl-XL, the antiapoptotic Bcl-2-family proteins discussed in the previous paragraph; Bcl-2 and Bcl-XL are partially localized at the mitochondrial outer membrane and, similar to other antiapoptotic proteins, are frequently upregulated in cancer; these proteins can regulate mitochondrial Ca^{2+} uptake through VDAC1 (Shoshan-Barmatz et al. 2010).

Considering that VDAC1 is involved in death and cell survival, it is not surprising that this channel could be a target for Bcl-2 family proteins (De Stefani et al. 2012). These proteins target the N-terminal region of VDAC1 (Abu-Hamad et al. 2009; Arbel and Shoshan-Barmatz 2010), and it has been demonstrated that only the Bcl-XL BH4 domain is essential to bind VDAC1 and inhibit cell death (Monaco et al. 2015). Several studies demonstrated that the interaction between Bcl-XL and VDAC1 suppresses proapoptotic Ca^{2+} uptake, preventing the dissipation of the mitochondrial potential and the release of cytochrome c and apoptosis-inducing factor (AIF) through the outer membrane.

Indeed, studies concerning mitochondrial Ca^{2+} uptake that compare Bcl-XL-overexpressing versus Bcl-XL-deficient cells have demonstrated that this protein may be involved in MAMs microdomain reorganization and results in an alteration of the capacity of mitochondrial Ca^{2+} uptake, proving that Bcl-XL inhibits VDAC1 (Monaco et al. 2015; Bittremieux et al. 2016; Shimizu et al. 2000; Li et al. 2008).

Nevertheless, VDAC1 in hepatocarcinoma tissues can be downregulated by the small noncoding RNA miR-7, influencing tumor proliferation and metastasis (Chaudhuri et al. 2016a; Bargaje et al. 2012). Chaudhuri et al. showed that in human neuroblastoma cells and in mouse primary cortical neurons, miR-7 can reduce VDAC1 expression, with consequent inhibition of mitochondrial Ca^{2+} uptake, membrane depolarization, mitochondrial fragmentation, cytochrome c release, and ROS production, promoting cancer cell survival (Chaudhuri et al. 2016a).

MCU allows calcium ion permeation into the mitochondrial matrix, and its overexpression leads to an increase in mitochondrial Ca^{2+} entry and ROS production, influencing the migration, invasion, and size of different tumor types (Yu et al. 2017; Tang et al. 2015; Wang et al. 2007). However, a reduction in MCU expression decreases mitochondrial Ca^{2+} uptake, the opening of the mPTP and the release of proapoptotic factors, thus having a protective effect on apoptosis (Marchi

et al. 2019b; Sebag et al. 2018; Oropeza-Almazan et al. 2017; Yuan et al. 2017; Liao et al. 2015; Qiu et al. 2013; Penston and Wormsley 1986).

Marchi et al. showed that, through MCU downregulation, the miR-25 MCU-targeting microRNA could perturb Ca^{2+} homeostasis, reducing the concentration of mitochondrial Ca^{2+} levels in HeLa cells. However, high levels of miR-25 have been observed both in prostate and colon cancer. The miR-25-dependent reduction in mitochondrial Ca^{2+} uptake correlates with resistance to proapoptotic stimuli and can be reversed by anti-miR-25 overexpression. Treatment with anti-miR-25 can restore the MCU expression levels and reverse the pathophysiology, thus suggesting a novel therapeutic target for prostate and colon cancer (Marchi et al. 2013).

One gene that is frequently deleted in many human cancers, principally in those caused by environmental carcinogens, is fragile histidine triad (FHIT). Consequently, its product, the Fhit protein, is absent or reduced in most cancers (Huebner and Croce 2003). The Fhit protein is localized in the mitochondria and the cytosol and acts as a tumor suppressor, increasing susceptibility to apoptosis (Siprashvili et al. 1997). Reintroduction of Fhit to the highly carcinogen-susceptible $Fhit^{-/-}$ mouse model reduced tumor sizes by activating apoptotic cell death (Zanesi et al. 2005). The Fhit protein generates ROS and enhances mitochondrial Ca^{2+} uptake by increasing mitochondrial Ca^{2+} hotspots. Therefore, Fhit acts as a tumor suppressor by modulating MCU opening and enhancing the susceptibility of cells to apoptosis, thus potentiating the effect of apoptotic agents (Rimessi et al. 2009).

Transient receptor potential cation channel subfamily C member 3 (TRPC3) belongs to a group of nonselective cation channels that are involved in different cellular mechanisms. TRPC3 channels can influence the mitochondrial membrane potential following their up- and downregulation. The activation of Ca^{2+}-sensitive downstream pathways occurs through the influx of calcium from transient receptor potential channels (TRP channels), which act as apoptotic regulators (Wang et al. 2019; Takahashi et al. 2018; Raphael et al. 2014; Monet et al. 2010). However, Shengjie Feng et al. have shown that a fraction of the TRPC3 protein is localized to the mitochondria and mediates mitochondrial Ca^{2+} uptake when the cytosolic calcium concentration is elevated. Since, as we previously noted, mitochondrial membrane potential seems to be affected by TRPC3 channels and because mitochondrial Ca^{2+} uptake is not abolished when MCU expression is downregulated (De Stefani et al. 2011), TRPC3 might be another channel that allows the entry of calcium into the mitochondria, in addition to MCU (Kirichok et al. 2004). In particular, resistance to apoptosis and the proliferation of some tumors could be related to its downregulation, which results in reduced mitochondrial calcium uptake (Feng et al. 2013).

Fetal and adult testis-expressed 1 protein (FATE1) is a 21-kDa protein that belongs to the cancer-testis antigen proteins that are mainly expressed in the testis under physiological conditions and are upregulated in different cancer types (Dong et al. 2003; Whitehurst 2014; Simpson et al. 2005). This molecule, being a member of the mitochondrial fission factor (Miff) protein family, shares some structural

similarities with Mff (Gandre-Babbe and van der Bliek 2008). The oncoprotein FATE1, which is located on the mitochondrial outer membrane preferentially in the MAMs compartment, is implicated in the regulation of Ca^{2+}-dependent apoptosis in cancer cells, acting as an anti-tether agent through the modulation of the distance between the ER and the mitochondria (Doghman-Bouguerra et al. 2016), being a direct connection between its increased expression and MAMs morphology in adrenocortical carcinoma (AAC) patients with a poor prognosis (Doghman-Bouguerra et al. 2016). Overexpression of FATE1 in adenoid cystic carcinoma (ACC) was related to a decrease in mitochondrial Ca^{2+} uptake that confers resistance to proapoptotic stimuli and chemotherapeutic drugs (Doghman-Bouguerra et al. 2016).

In most human cancer types, including head and neck squamous cell carcinoma (HNSCC), high levels of enhancer of zeste homolog 2 (EZH2) have been detected. EZH2 is the enzymatic subunit of the PRC2 complex (polycomb repressive complex 2), which methylates lysine 9 and lysine 27 of histone H3, and is fundamental for transcriptional repression (Kim and Roberts 2016; Schuettengruber et al. 2007; Boyer et al. 2006). EZH2 acts as an oncogene, and its high expression levels are associated with tumor cell proliferation and migration (Zhou et al. 2015a; Ning et al. 2015). Furthermore, it has been shown that inhibition of EZH2 in HNSCC cells in vitro and in vivo induces loss of mitochondrial membrane potential ($\Delta\Psi_m$) with consequent activation of cell death pathways. Inhibition of EZH2 involves accumulation of Ca^{2+} into the mitochondria, induced by inactivation of MICU1 (Zhou et al. 2015b; Cosentino and Garcia-Saez 2014) (Fig. 1).

4 Upregulation of ER-Mitochondria Ca^{2+} Crosstalk

4.1 New Insights into Ca^{2+} Signaling Perturbation in the MAMs

The numerous molecular pathways described thus far all involve a decreased uptake of Ca^{2+} to the mitochondria, resulting from decreased ER release or mitochondrial defects. Historically, reports that have assessed the remodeling of MAMs Ca^{2+} signaling associated with tumorigenesis, invasion, and metastasis all led to the conclusion that cancer cells undergo minor mitochondria-dependent apoptosis because of decreases in the Ca^{2+} release from the ER. Recently, the characterization of new MAM-localized proteins and the finding of new mechanisms of action led the scientific community to consider that even an upregulation of Ca^{2+} signaling at the MAMs level could be harmful and drive tumor onset and progression. In the following paragraphs, we will describe how this condition, hitherto described as the cause of apoptotic cell death, can lead to the onset and development of tumor diseases.

4.2 Increased ER-Ca^{2+} Release

The endoplasmic reticulum is an organelle that contains a network of tubules and flattened sacs and is mainly known for its major role in the production, processing, and transport of proteins and lipids. The ER also represents the major intracellular store of Ca^{2+}, an ion that is necessary on its lumen for second-messenger-induced Ca^{2+} release, the control of capacitative Ca^{2+} influx, and intra-ER chaperone activities such as polypeptide translocation, protein folding, and ER-associated degradation (Buck et al. 2007). In normal tissue cells, a sustained Ca^{2+} flux from the ER to the mitochondria can enhance the sensitivity of mitochondria to apoptotic stimuli; however, in some cases, an increase in Ca^{2+} ion leakage from the ER to the MAMs can promote tumor formation, especially in specific tissues and organs. For ER-mitochondria interorganellar Ca^{2+} signaling and, in particular, increased ER Ca^{2+} release, the recent revelation of the mechanisms by which IP3R3 upregulation drives oncogenesis via ER-mitochondrial Ca^{2+} crosstalk is particularly important. This statement is particularly strong because until last year, IP3R3 was well characterized as a Ca^{2+}-related proapoptotic protein. In fact, the tumor suppressors BAP1 and PTEN have a stabilizing effect on IP3R3 in the ER, promoting susceptibility to cell death (Bononi et al. 2017; Kuchay et al. 2017), and in contrast, the oncogene K-Ras^{G13D} downregulates IP3R3, preventing the apoptotic death of cancer cells (Pierro et al. 2014). Three recent works by Guerra et al. (2019), Rezuchova et al. (2019), and Ueasilamongkol et al. (2020), for the first time, have deviated from the idea that IP3Rs only have an anti-oncogenic potential by driving proapoptotic Ca^{2+} signals to mitochondria but attributed an oncogenic potential to ER-mitochondria Ca^{2+} crosstalk. In an analysis of tumor tissues, the IP3R3-protein levels were elevated in hepatocellular carcinoma biopsies compared to healthy liver biopsies (Guerra et al. 2019), in clear cell renal cell carcinoma kidney biopsies compared to healthy regions (Rezuchova et al. 2019) and in cholangiocarcinoma cancer biopsies and cancer cell lines compared to normal tissues and normal cholangiocyte cell models (Ueasilamongkol et al. 2020). In all cases, only type 3 IP3Rs were found to be overexpressed in tumor tissues, with no changes or slight downregulation of type 1 and type 2. In particular, IP3R3 seems to be completely absent in normal human hepatocytes but is clearly present in biopsies from individuals with hepatitis B virus, hepatitis C virus (HCV), non-alcoholic fatty liver disease (NAFLD), and alcoholic liver disease (ALD), which are the four most common predisposing factors to the development of hepatocellular carcinoma (Guerra et al. 2019). This increase was more pronounced in the late stages of hepatocellular carcinoma.

Notably, in cholangiocarcinoma cells, most IP3R3 is localized to the MAMs, while in normal cholangiocytes, it resides in the ER subapical pole. In these cells, MAM localization promotes basal respiration by increasing mitochondrial Ca^{2+} signaling, and thus, depletion of this channel in these cells is deleterious for nuclear and mitochondrial functionality (Ueasilamongkol et al. 2020). In HepG2 cells, IP3R3 upregulation promotes cell death, but its chronic overexpression can increase

the resistance of these cells to cell death inducers, enhancing malignant cell survival (Guerra et al. 2019).

The common key in all these cases is the extreme adaptation ability that drives oncogenesis and malignant cell transformation. These cancer cells became addicted to high IP3R3 levels at the MAM compartment for their survival, to maintain sustained cell metabolism and to obtain malignant features such as increased motility, migration, and invasion.

We want to include in this section the already mentioned ERO1-α, an extensively studied protein due to its ability to regulate many processes. ERO1-α is particularly enriched at the ER-mitochondria interface, controlling ER redox homeostasis and oxidative folding and regulating Ca^{2+} efflux from the ER and, consequently, mitochondrial Ca^{2+} accumulation (Anelli et al. 2012). ERO1-α is highly expressed in different tumor types and is associated with a poor prognosis in breast cancer (Kutomi et al. 2013). In fact, the expression of ERO1-α in triple-negative breast cancer cells is correlated with that of programmed cell death-ligand 1 (PD-L1), both at the protein and mRNA levels, via hypoxia-inducible factor 1-α (HIF-1α). Depletion of ERO1-α led to a significant reduction in PD-L1-mediated T-cell apoptosis, suggesting that ERO1-α has a key role in tumor-mediated immunosuppression (Tanaka et al. 2017).

Another MAMs Ca^{2+}- and tumor-related protein that acts at the ER level is the receptor chaperone stress-activated chaperone sigma-1 receptor (Sig1R), which senses ER Ca^{2+} concentrations and regulates cell survival. This protein could be considered "borderline" in this section considering its mechanism of action; in fact, Sig1R is an ER-localized protein that favors the efflux of calcium ions from the endoplasmic reticulum and has been described as being overexpressed in breast cancer, especially in cancer cells with metastatic potential (Gueguinou et al. 2017). ER chaperones are important in maintaining proper intracellular Ca^{2+} levels, protein folding, and the unfolded protein response (UPR) under ER stress conditions (Bartoszewska and Collawn 2020).

Two MAM-localized chaperones that belong to the heat shock 70 kDa (HSP70) protein family are of considerable importance in Ca^{2+} signaling: chaperone glucose-regulated protein GRP75 and glucose-regulated protein 78 (GRP78, also known as immunoglobulin heavy-chain-binding protein BiP) (Brocchieri et al. 2008; Wadhwa et al. 2002).

GRP75 ensures the juxtaposition between IP3R and VDAC1 in the mitochondrial outer membrane (Szabadkai et al. 2006). Its localization is mainly mitochondrial, but it is also present at low levels in the cytoplasm, nucleus, ER, and Golgi apparatus (Ran et al. 2000; Wadhwa et al. 1995), where it exerts many different functions from the import of unfolded proteins into the mitochondrial matrix to modulation of exocytosis and endocytosis (Flachbartova and Kovacech 2013; Voos and Rottgers 2002; Schneider et al. 1996; Kronidou et al. 1994; Scherer et al. 1992). Sig1Rs are particularly enriched at the MAMs and in normal tissues form a complex with GRP78, another MAM-localized chaperone. GRP78 can bind to misfolded proteins and to unassembled complexes and modulates ER-associated degradation (ERAD), which regulates the UPR (Pfaffenbach and Lee 2011; Wang et al. 2009; Little et al.

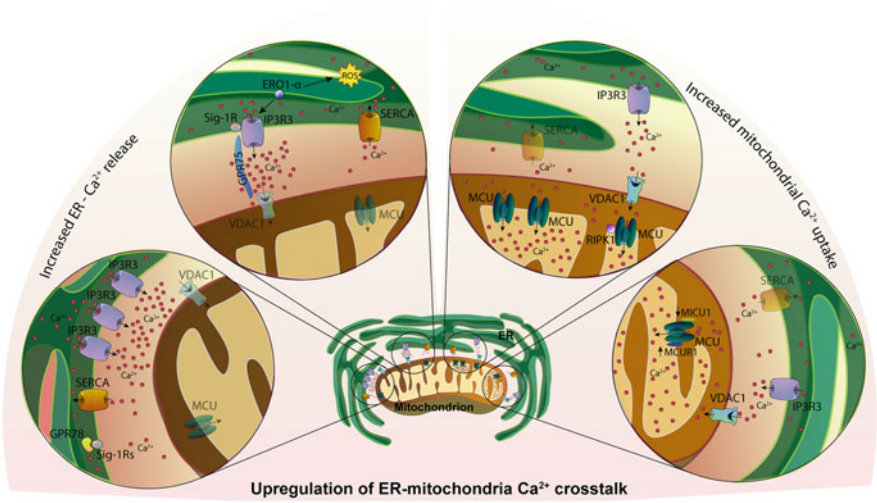

Fig. 2 Upregulation of MAMs Ca^{2+} crosstalk in cancer: graphical representation of the calcium signaling regulators involved in a cancer-related increased Ca^{2+} crosstalk state. See text for further details. Ca^{2+}, calcium; ER, endoplasmic reticulum

1994). Its molecular structure displays two domains: the substrate-binding domain (SBD), involved in binding unfolded peptides, and the nucleotide-binding domain (NBD), which binds ATP to be hydrolyzed to obtain energy to prevent unfolded protein aggregation at the N-terminus (Luo et al. 2006; Lindquist and Craig 1988). GRP78, like almost all other chaperones, is useful for storing ER Ca^{2+} as a high-capacity Ca^{2+}-binding protein under physiological conditions (Hendershot 2004).

Szabadkai et al. highlighted the mechanism by which Sig1R, dissociating from BiP, binds IP3R3 following the activation of IP3Rs. This event leads to IP3R3 stabilization at the MAMs and to an enhancement of IP3R3-mediated Ca^{2+} fluxes to the mitochondria (Szabadkai et al. 2006). Although BiP is an excellent target to consider for neuroprotective therapeutic strategies (Enogieru et al. 2019), it also influences how tumor cells survive, proliferate, and develop chemoresistance. During chronic ER stress conditions that involve prolonged ER Ca^{2+} depletion, Sig1R localization changes from the MAMs to the peripheral ER, reducing cellular damage and thus preventing cell death. Another mechanism of Ca^{2+} homeostasis perturbation implemented by Sig1R that has direct consequences on cell invasiveness in breast cancer has been described by Gueguinou et al. (2017). Sig1R favors the migration of cancer cells by forming a functional molecular platform with the calcium-activated K^+ channels SK3 and ORAI calcium release-activated calcium modulator 1 (Orai1) (Gueguinou et al. 2017) (Fig. 2).

4.3 Increased Mitochondrial Ca^{2+} Uptake

Before the identification of the molecular players forming the MCU complex, the role of mitochondrial Ca^{2+} in cancer progression was simply confined to receiving Ca^{2+} from the ER, thereby regulating the apoptotic response. Low ER Ca^{2+} release results in reduced mitochondrial [Ca^{2+}], mPTP inhibition, and resistance to chemotherapeutic-induced cell death. Consistent with this view, many oncogenic factors act at the MAMs to limit ER-mitochondria Ca^{2+} transfer (see the "Downregulation of ER-mitochondria calcium crosstalk" section). However, many mitochondrial Ca^{2+} channels that are responsible for favoring Ca^{2+} accumulation, such as VDACs, are overexpressed, rather than reduced, in cancer (Mazure 2017). These observations suggest that an increased intrinsic capacity of the mitochondrial compartment to accumulate Ca^{2+} could contribute to sustained malignant progression, although, at least theoretically, it predisposes cells to Ca^{2+}-induced cell death. The oncogenic mechanisms regulated by mitochondrial Ca^{2+} mainly rely on the association between Ca^{2+} and the formation of mitogenic ROS, as well as pure stimulation of mitochondrial metabolism. Ca^{2+} accumulation activates four mitochondrial dehydrogenases, which in turn stimulate the respiratory chain and hence ATP production (Denton 2009). Thus, as a consequence of increased metabolic activity, ROS are generated inside the matrix, but they fail to trigger cell death, probably due to the superior antioxidant defense that often distinguishes the malignant phenotype (Gorrini et al. 2013).

The correlation between augmented mitochondrial Ca^{2+} entry, ROS production, and cancer growth appears evident for tumors overexpressing the uniporter complex pore-forming subunit MCU. Indeed, increased levels of MCU have been reported in different tumors, including breast and hepatocellular carcinomas (Vultur et al. 2018). In breast cancer, MCU-dependent mitochondrial Ca^{2+} entry is associated with ROS overproduction and higher metastatic potential through a mechanism that involves the downstream activation of HIF1-α transcriptional activity (Tosatto et al. 2016). Consistent with these observations, upregulation of MCU in triple-negative breast cancer cells promoted metastasis in an in vivo mouse model by enhancing glycolysis, a series of neoplastic events that is counteracted by the tumor-suppressor activity of miRNA-340 (Yu et al. 2017). Moreover, receptor-interacting protein kinase 1 (RIPK1) binds MCU to promote Ca^{2+} entry and colorectal cancer progression through stimulation of mitochondrial bioenergetics (Zeng et al. 2018). In hepatocellular carcinomas, the Ca^{2+}-ROS axis orchestrated by MCU resulted in activation of metalloproteinase-2 (MMP2) (Ren et al. 2017), a zinc-dependent endopeptidase associated with extracellular matrix degradation and metastasis (Shay et al. 2015).

The link between Ca^{2+} and ROS overproduction is also relevant for the cancer-related functions of MICU1, the principal member of the MCU complex that regulates the gating of the channel (Kamer and Mootha 2015). Our group recently showed that MICU1 downregulation, as a result of higher AKT activity, could sustain cancer progression through Ca^{2+}-dependent ROS generation (Marchi et al.

2019a). Indeed, loss of MICU1 disinhibits MCU, leading to Ca^{2+} permeation under resting (nonstimulated) conditions and increased mitochondrial ROS levels (Csordas et al. 2013), which could ultimately result in cell death (Mallilankaraman et al. 2012a; Liu et al. 2016). This finding implies that malignant cells showing low MICU1 levels predispose concomitant mechanisms to minimize the detrimental effects induced by ROS. Consistent with this view, MICU1 depletion in normal hepatocytes triggered extensive cell death, but upon pharmacological inhibition of mPTP opening, the loss of MICU1 conferred a strong proliferative advantage (Antony et al. 2016). Moreover, a combination of high mitochondrial Ca^{2+} entry through genetic manipulation of the MCU complex and mPTP closure exacerbated the tumorigenic potential of different cancer cells (Marchi et al. 2019b). Taken together, these observations suggest that variations in the composition of the MCU complex are a key event that cooperates with other oncogenic pathways to favor cancer growth.

Further evidence that supports this scenario derives from the protumorigenic role of MCU regulator 1 (MCUR1), which has been described as a matrix-located, positive regulator of the uniporter complex (Mallilankaraman et al. 2012b). In hepatocellular carcinomas, MCUR1 was strongly upregulated, and ROS production was augmented, leading to ROS-dependent degradation of p53 and consequent resistance to apoptosis (Ren et al. 2018). Notably, the cancer cell detoxification capacity was also increased due to activation of nuclear factor erythroid 2-related factor 2 (NRF2) (Jin et al. 2019), a master gene in the orchestration of the cellular antioxidant response (Cuadrado et al. 2019). Thus, MCUR1 can regulate two cancer hallmarks at once: Ca^{2+}-mediated metastatic potential and resistance to apoptosis. However, the expression of MCUR1 correlates with the permeability transition and reduced cell survival (Chaudhuri et al. 2016b), indicating that MCUR1 oncogenic activities might be solely due to the concomitant inhibition of the functions of the mPTP through a superior mechanism. Nevertheless, it has been proposed that MCUR1 could act as a complex IV assembly factor rather than as an MCU interactor (Paupe et al. 2015). In this context, variations in mitochondrial Ca^{2+} uptake and ROS levels are side products of respiratory chain defects; therefore, the active role of Ca^{2+} in MCUR1-mediated oncogenesis should be completely reevaluated.

Overall, these observations indicate that increased mitochondrial Ca^{2+} uptake acts with other oncogenic mechanisms (e.g., mPTP inhibition or activation of antioxidant systems) to sustain cancer growth and dissemination. The protumorigenic role of mitochondrial Ca^{2+} signaling involves other pathways in addition to ROS production and excess malignant cell bioenergetics, including the MCU-dependent control of cytosolic Ca^{2+} through store-operated Ca^{2+} entry (SOCE). The activity of the MCU complex sustains cytosolic Ca^{2+} fluxes through SOCE, which in turn regulates cytoskeletal dynamics and cellular migration (Prudent et al. 2016). Moreover, recent findings suggest that spontaneous mitochondrial Ca^{2+} oscillations through the MCU complex are essential for mitotic entry and cell cycle progression (Koval et al. 2019; Zhao et al. 2019), thus revealing another mechanism that could account for the aberrant proliferation of cancer cells with an altered composition of the MCU complex (Fig. 2).

5 Conclusions

The importance of the multiple and complex signaling pathways generated by the displacement of Ca^{2+} ions and, specifically, the Ca^{2+}-dependent communication between structurally and functionally interconnected intracellular organelles has been increasingly highlighted and described, especially in recent years. Evidence of this phenomenon is the dramatic effects on cell health that derive from perturbation of the MAMs morphology and modification of the ER-mitochondria tethering distance. Moreover, alterations in the MAMs protein pool and functionality have been connected with several pathological conditions, including diabetes, neurodegeneration, infection, and antiviral response and cancer (Pinton 2018). Tumor cells, in fact, could modify the systems that maintain cellular Ca^{2+} homeostasis to promote their survival and metastasis. The crucial role of the regulation of spatiotemporal Ca^{2+} signaling in the MAMs in cancer is confirmed by evidence that different oncogenes and tumor suppressors reside at the ER-mitochondria interface.

As shown previously, both an increase and a decrease of calcium ion exchange between these two organelles can, in a nonexclusive way, lead to the promotion or suppression of tumor behaviors in many tissues. This phenomenon is an indication of how the equilibrium that rules calcium homeostasis in this subcellular compartment is delicate, complex, and intimate. Specifically, although Ca^{2+} oscillations are essential at MAMs to feed mitochondrial metabolism, a persistent increase in mitochondrial $[Ca^{2+}]$ can lead to cell death. In this scenario, by limiting mitochondrial calcium uptake, many cancer cells develop resistance to death. On the other hand, it was also highlighted that an increased mitochondrial ability to accumulate Ca^{2+} supports malignant progression, by boosting mitochondrial metabolism and sustaining mitogenic ROS production. Thus, depending on the tumor context, MAM-localized Ca^{2+} signaling can exert different functions, also according to the different oncogenic paths involved.

Several questions have yet to be answered, many aspects remain to be clarified, and molecular pathways must be described to reach a good understanding of the complex mechanisms that stem from calcium signaling at the MAMs, knowledge that will be very useful in the development of novel therapeutic strategies for several tumors.

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Endosomal Acid-Base Homeostasis in Neurodegenerative Diseases

Hari Prasad and Rajini Rao

Contents

Abstract Neurodegenerative disorders are debilitating and largely untreatable conditions that pose a significant burden to affected individuals and caregivers. Overwhelming evidence supports a crucial preclinical role for endosomal dysfunction as an upstream pathogenic hub and driver in Alzheimer's disease (AD) and related neurodegenerative disorders. We present recent advances on the role of endosomal acid-base homeostasis in neurodegeneration and discuss evidence for converging

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mechanisms. The strongest genetic risk factor in sporadic AD is the ε4 allele of Apolipoprotein E (ApoE4), which potentiates pre-symptomatic endosomal dysfunction and prominent amyloid beta (Aβ) pathology, although how these pathways are linked mechanistically has remained unclear. There is emerging evidence that the Christianson syndrome protein NHE6 is a prominent ApoE4 effector linking endosomal function to Aβ pathologies. By functioning as a dominant leak pathway for protons, the Na^+/H^+ exchanger activity of NHE6 limits endosomal acidification and regulates β-secretase (BACE)-mediated Aβ production and LRP1 receptor-mediated Aβ clearance. Pathological endosomal acidification may impact both Aβ generation and clearance mechanisms and emerges as a promising therapeutic target in AD. We also offer our perspective on the complex role of endosomal acid-base homeostasis in the pathogenesis of neurodegeneration and its therapeutic implications for neuronal rescue and repair strategies.

Keywords Alzheimer's disease · Amyloid · ApoE4 · Endosomal pH · Na^+/H^+ exchanger · NHE6

Abbreviations

AD	Alzheimer's disease
ApoE4	Apolipoprotein E4
APP	Amyloid precursor protein
eNHE	Endosomal Na^+/H^+ exchanger
HDAC	Histone deacetylase
V-ATPase	Vacuolar H^+-ATPase

1 A Balancing Act: pH Regulation in the Endosome

The endosome is a busy station handling cargo traffic at the degradation-recycling crossroads. There is widespread interest in deciphering basic mechanisms of endosomal traffic not only to understand a fundamental cellular process but also to identify new diagnostic and therapeutic targets in disease (Afghah et al. 2019; Casey et al. 2010; Maxfield 2014). Endosomes play important roles in membrane and protein turnover, nutrient uptake, receptor-ligand uncoupling, antigen presentation, enzyme activation, vesicle budding and exosome formation, and as crucial regulators of cellular signaling pathways (Afghah et al. 2019; Casey et al. 2010; Maxfield 2014). Despite this multipartite function of the endosome, relatively few studies have addressed the contribution of endosomes to human health and disease. However, recent studies have begun to shed light on the mechanistic roles of endosomes in a host of developmental and degenerative disorders of the brain, including autism and Alzheimer's disease, and in oncogenesis, including glioblastoma (Kondapalli et al. 2014, 2015; Nixon 2005, 2017; Ko et al. 2020). Although very diverse at first

sight, pathophysiologic commonalities between these disorders emerge upon closer analysis. Compelling evidence points to vesicle trafficking defects as a unifying mechanism underlying these disorders, which in turn critically depend on luminal pH within the endosomal-lysosomal compartment (Kondapalli et al. 2014; Prasad and Rao 2015a).

A precise balance of luminal pH and endosomal ion flux is required for endosomal trafficking, and a thorough dissection of pathways regulating endosomal pH may be a promising strategy to reveal pathophysiological mechanisms (Kondapalli et al. 2014; Prasad and Rao 2015a). Traditionally, research on endosomal acidification in human health and disease has focused on the vacuolar-type ATPase (V-ATPase) that pumps protons into the endosomes. Altered V-ATPase activity and defective lysosomal acidification have been linked to cellular aging and neurodegenerative disorders including Parkinson's and Alzheimer's disease (Colacurcio and Nixon 2016). However, a growing number of studies are highlighting the role of multiple ion transporters that act in concert with the V-ATPase for luminal pH regulation (Scott and Gruenberg 2011) (Fig. 1a). The compensatory movement of Cl^- ions is critical to allow acidification of the endolysosomal lumen by dissipating the membrane potential resulting from unidirectional pumping of H^+ by the V-ATPase. The CLC family includes plasma membrane Cl^- channels as well as vesicular transporters, CLC3 through CLC7 in mammals, which function as Cl^-/H^+ exchangers. The stoichiometry of exchange, $2Cl^-/H^+$, results in three negative charges introduced into the vesicle lumen per transport cycle, albeit with loss of one H^+, to counter the V-ATPase. Gene disruptions in mouse models result in severe neurodegeneration, including loss of hippocampus in *Clcn3*$^{-/-}$ null mouse and pathological similarity to lysosomal storage disorders such as neuronal ceroid lipofuscinosis in various targeted *Clcn7*$^{-/-}$ models, reviewed elsewhere (Jentsch and Pusch 2018; Poroca et al. 2017). Potential players in regulating endosomal pH also include the mucolipin subfamily of transient receptor potential (TRPML) calcium channels found on endosomes, particularly the pH-regulated TRPML3 isoform, overexpression of which is known to increase endosomal pH (Martina et al. 2009). Activation of two-pore Ca^{2+} channels (TPCs) residing in endosomal-lysosomal compartments may also regulate luminal pH (Morgan and Galione 2007) (Fig. 1a).

Recent clinical reports and genetic studies have underscored the contribution of endosomal Na^+/H^+ exchangers in the pathophysiology of a plethora of human disorders (Kondapalli et al. 2014; Prasad and Rao 2015a; Zhao et al. 2016). Encoded by the *SLC9* gene family of solute carriers, Na^+/H^+ exchangers (NHE) are grouped into three subfamilies: (1) *SLC9A* that has nine isoforms, *SLC9A1–9*, encoding NHE1–9 proteins; (2) *SLC9B* that has two isoforms, *SLC9B1–2*, encoding NHA1–2 proteins; and (3) *SLC9C* that has two isoforms, *SLC9C1–2*, encoding NHE10–11 proteins (Brett et al. 2005a; Donowitz et al. 2013; Fuster and Alexander 2014; Pedersen and Counillon 2019) (Fig. 1b). The *SLC9A* subfamily consists of electroneutral Na^+/H^+ exchangers that reside on the plasma membrane (NHE1, NHE2 and NHE4), recycle between plasma membrane and endosomal compartments (NHE3 and NHE5), or function within intracellular compartments, including

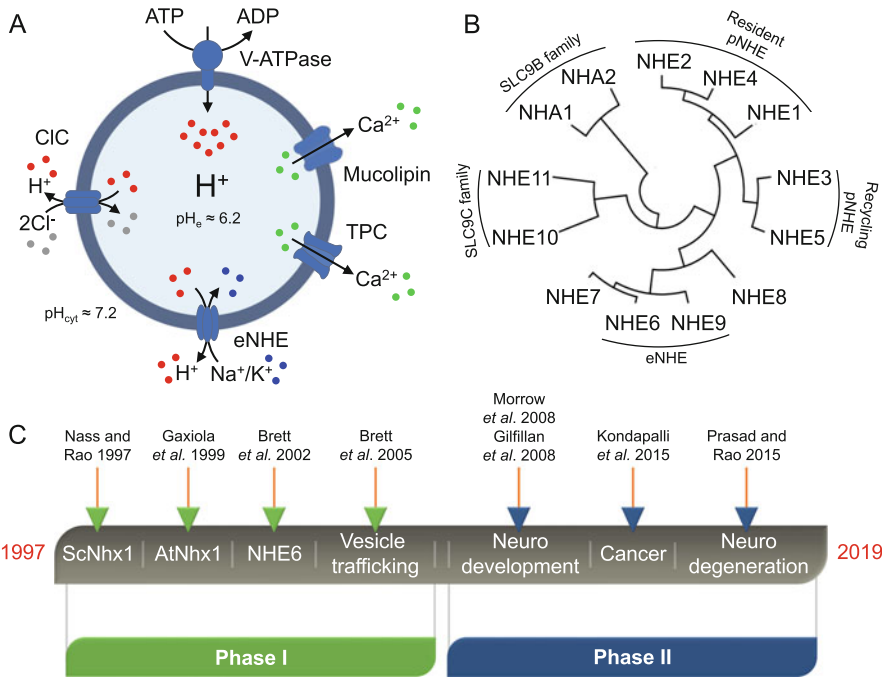

Fig. 1 Endosomal pH regulation by Na^+/H^+ exchangers. (**a**) Under physiological conditions, the endosomal pH is acidic (≈6.2) as compared to the cytoplasmic pH (≈7.2). According to the "pump-leak" model, conserved from yeast to plants and mammals, the exchange of luminal protons for Na^+ or K^+ ions by eNHE counters the activity of the H^+ pumping V-ATPase to precisely tune the endosomal pH. Also depicted in the figure are ClC transporters and Ca^{2+} channels (mucolipins and TPC) also reported to regulate endosomal pH. The pH values indicated in the figure were obtained from experimental observations (Prasad and Rao 2018a). (**b**) *SLC9* phylogenetic tree constructed using the Mega5 program depicting three major subclasses of *SLC9A*, *SLC9B*, and *SLC9C* in mammals. There are nine NHE isoforms within the *SLC9A* subclass, which are further subdivided as resident or recycling plasma membrane (pNHE), and intracellular including the endosomal NHE (eNHE) isoforms, NHE6 and NHE9. (**c**) Timeline of two decades of research on eNHE highlighting some relevant publications. *eNHE* endosomal Na^+/H^+ exchangers, *ClC* Cl^-/H^+ antiporters, *TPC* two-pore channels

the Golgi (NHE8), *trans* Golgi network (NHE7), and the early and recycling endosomes (NHE6 and NHE9) (Brett et al. 2005a; Donowitz et al. 2013; Fuster and Alexander 2014; Pedersen and Counillon 2019) (Fig. 1b). Cation selectivity of the NHE transporters reflects the availability of ions in the surrounding milieu: whereas plasma membrane isoforms are restricted to Na^+ (and Li^+), intracellular isoforms can additionally transport K^+ ions (Pedersen and Counillon 2019). As a result, cation/H^+ exchange can be driven by both proton and cation gradients. All NHE members have a cytoplasmic C-terminal domain, which is significantly unstructured and binds to a number of regulatory factors, including calcineurin homology protein (CHP) and the PDZ-domain scaffold protein NHERF (Pedersen and Counillon 2019). The C-terminal tail mediates scaffolding to the cytoskeleton

and activation of transport in response to signaling cascades, although much less is known about regulation and trafficking of endosomal NHE isoforms (Kondapalli et al. 2014; Kagami et al. 2008).

Endosomal NHE (eNHE) activity was originally described in the tonoplast membranes of plant vacuoles (Barkla and Blumwald 1991; Blumwald and Poole 1985). The gene was first cloned in yeast, where it was recognized as an evolutionarily ancient subtype, distinct from the well-characterized plasma membrane NHE (Nass et al. 1997; Nass and Rao 1998; Brett et al. 2005a), and subsequently multiple isoforms were identified in plants, mammals, and other organisms (Gaxiola et al. 1999; Brett et al. 2002). Early research on eNHE (Fig. 1c) focused on fundamental questions relating to their role in vesicle trafficking using model organisms, first in yeast and soon after in plants and metazoans, including mammalian cells (Brett et al. 2002, 2005b; Gaxiola et al. 1999; Nass et al. 1997; Nass and Rao 1998; Bowers et al. 2000). In the last decade (Fig. 1c), there has been an exponential rise in interest due to the emerging links between eNHE and a growing list of human diseases, including neurodevelopmental disorders such as autism and intellectual disability (Christianson Syndrome), attention deficit hyperactivity disorder, addiction, several types of cancers (Ko et al. 2020), and more recently to neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Gilfillan et al. 2008; Kerner-Rossi et al. 2019; Kondapalli et al. 2014, 2015; Morrow et al. 2008; Prasad and Rao 2015b, 2018a; Ullman et al. 2018). While a fraction of eNHE patient variants reported in the literature may be benign polymorphisms, a significant proportion is deleterious and causal to disease phenotypes (Ilie et al. 2016; Kondapalli et al. 2013; Prasad et al. 2017). As a major source of proton leak activity in endosomes, the eNHE functions as an endosomal gatekeeper that must be tightly regulated and controlled (Kondapalli et al. 2014). Given that Na^+/H^+ exchangers are estimated to have exceptionally high transport rates of ~1,500 ions/s, even small perturbations in expression or activity may result in dramatic consequences within the limited confines of endosomal space (Lee et al. 2013). Dysregulation of endosomal pH could potentially impact a plethora of downstream events including alterations in cargo sorting and trafficking, protein processing, and receptor turnover and thus represents a previously unidentified pathogenic mechanism in neurodegeneration. Here, we review evidence linking endosomal pH and eNHE function to Alzheimer's disease and discuss how this knowledge might help us understand the broader role for endosomal acid-base homeostasis in neurodegenerative diseases. We also briefly address the potential role of endosomal pH in other neurodegenerative disorders and discuss translational prospects.

2 Lessons from Baker's Yeast

Simple organisms such as yeast can powerfully inform our understanding of genetic players in neurological disorders such as Alzheimer's disease and aid in discovery of new therapies (Treusch et al. 2011). Much of what we know about specific functions

and raison d'etre of eNHE in humans has come from rigorous studies on the yeast ortholog Nhx1. Beginning in the mid-1990s, Nhx1 was cloned, localized to the prevacuolar compartment in yeast, and defined as the founding member of an evolutionarily conserved, phylogenetic cluster of endosomal $Na^{+/}H^{+}$ exchangers with proposed roles in vesicle trafficking, ion homeostasis, and biogenesis of lysosomes (Bowers et al. 2000; Brett et al. 2005a; Nass et al. 1997; Nass and Rao 1998). Extension of these studies to plants also revealed a critical role in determining flower color and salt tolerance (Gaxiola et al. 1999; Kondapalli et al. 2014). The "pump-leak" model of pH regulation is conserved from yeast to plants and humans, with the exchange of luminal protons for sodium or potassium ions by eNHE acting as a brake that counters H^{+} pumping activity of the V-ATPase to precisely tune the endosomal pH (Kondapalli et al. 2014) (Fig. 1a). Loss of this leak pathway leads to over-acidification of early and recycling endosomes, missorting of cargo, defects in protein processing, and receptor turnover (Kondapalli et al. 2014). Importantly, trafficking defects in Nhx1-null mutants could be corrected with weak base or exacerbated by weak acids (Brett et al. 2005b). Furthermore, a genome-wide analysis of vacuolar pH in ~4,600 yeast null mutants revealed a reciprocal link between luminal pH and vesicle trafficking (Brett et al. 2011). Hyperacidification of the endolysosomal compartment was observed in the null mutant of Ncr1, the yeast ortholog of the human gene linked to Niemann-Pick type C (NPC) disease, resulting in the endosomal trapping of unesterified cholesterol and establishing a link between dysregulation of luminal pH and neurodegeneration (Brett et al. 2011). Deletion of Nhx1 (also known as Vps44) results in enlarged endosomes, hyperacidic luminal pH, enhanced proteolysis of the chaperone protein Vps10 (an ortholog of the AD susceptibility factor SORL1/sortilin-related receptor 1), mistrafficking of lysosomal hydrolases, and profound deficiencies in lysosomal cargo sorting and delivery (Bowers et al. 2000; Brett et al. 2005b; Nass and Rao 1998). Interestingly, these cellular phenotypes are strikingly reminiscent of preclinical stages of Alzheimer disease, as discussed below, and point to a role for eNHE in AD. Although the contribution of the V-ATPase in acidification of endosomal-lysosomal compartments in well established, the role of eNHE is remains largely unexplored in mammalian cells, including neurons. Much of the early evidence concerning endosomal NHE involvement in vesicle trafficking is based on studies performed using simple yeast models and non-neuronal mammalian systems; the direct extrapolation of such observations to explain complex events such as synapse formation and turnover, however, is not straightforward and further research is necessary to elucidate mechanisms that link disrupted neuronal function to changes in endosomal pH in AD.

3 Endosomopathy Is a Preclinical Hallmark of Alzheimer's Disease

The number of people living with dementia worldwide is currently estimated at 47 million and is projected to triple by 2050. Alzheimer's disease (AD) is the leading cause of dementia and contributes up to 70% of case (Baumgart et al. 2015; Tarawneh and Holtzman 2012). A majority of AD cases occur sporadically, with 40–65% of patients carrying at least one copy of the E4 allele of Apolipoprotein E (ApoE4), the strongest known genetic risk factor for AD. Indeed, the presence of two copies of the E4 allele is known to increase risk of AD by ~12-times as compared to E3 isoform (Corder et al. 1993; Verghese et al. 2013; Yamazaki et al. 2016). Unfortunately, the underlying cellular pathology that leads to neurodegeneration occurs long before clinical symptoms emerge, resulting in abysmal clinical failure (up to 99.6%) of drugs to reverse advanced pathology of amyloid plaques, neurofibrillary tangles, and neuronal death (Cummings et al. 2014). Therefore, early detection and intervention in preclinical stages may be key to treatment of this devastating disease. In this context, it is critical to identify preclinical AD features in high-risk populations and cognitively normal seniors that are associated with future cognitive decline and mortality.

Endosomal abnormalities, as early features of AD that precede the clinical onset of the disease by several decades, were proposed as early as 1998 by Troncoso et al. (1998). More recently, there have been several studies documenting endosomal dysfunction and trafficking defects in AD; however, the underlying molecular mechanisms and cellular consequences of endosomal deficits largely remains to be elucidated. Notably, in 2017, Nixon coined the term "endosomopathy" in relation to AD, and Petsko and colleagues proposed endosomal "traffic jams" as an upstream pathogenic hub in AD, suggesting that interventions designed to "unjam" the endosome may have far-reaching therapeutic implications (Nixon 2017; Small et al. 2017). Indeed, the earliest and most prominent brain pathology known to precede cognitive impairment is the marked enlargement and amplification of the endosomal pool, pointing to underlying dysfunction of the endosomal-lysosomal pathway (Nixon 2005, 2017). More importantly, inheritance of ApoE4 is known to potentiate prominent pre-symptomatic endosomopathy in AD brains (Cataldo et al. 2000). Although this observation was made around two decades ago, the absence of known molecular effectors and druggable targets has precluded translation from bench to bedside.

The importance of endosomal function in AD is strengthened by several recently identified genes in genome-wide association studies (GWAS) of AD risk, including BIN1, PICALM, CD2AP, EPHA1, and SORL1, that are known to regulate endosomal mechanisms (Karch and Goate 2015). Understanding the abnormalities in the endosomal-lysosomal system in AD could potentially be a new "lamp post" that lights the path to new promising therapeutic targets. AD-related endosomal dysfunction manifests functionally as abnormal endocytic activity, mistrafficking of lysosomal hydrolases, and abnormal accumulation of unesterified cholesterol (Nixon

2005, 2017). One well-studied endosomal molecular player is Rab5, a regulatory guanosine triphosphatase (GTPase) that is associated with the early or sorting endosome and participates in early endosomal biogenesis and cellular signaling reactions. The family of Rab proteins regulates multiple steps in membrane trafficking, from vesicle formation to fusion. Abnormal activation of Rab5 is observed in AD and Down syndrome where it is thought to contribute to prominent early endosomal dysfunction (Jiang et al. 2010; Kim et al. 2016; Nixon 2005, 2017). Several AD-related risk factors, including amyloid precursor protein (APP) dosage, increased β-secretase BACE1 (beta-site APP cleaving enzyme 1) expression, accumulation of β-carboxyl-terminal fragment of APP (β-CTF), inheritance of the ApoE4 allele, reduced retromer/Vps35 expression, and abnormal cellular accumulation of cholesterol, potentiate Rab5-mediated dysfunction of early endosomes (Cataldo et al. 2000; Jiang et al. 2010; Kim et al. 2016).

In comparison, the role of recycling endosomes is relatively understudied and underappreciated as a component of AD. Synaptic function critically depends on recycling endosomal function and surface proteostasis of membrane proteins, which includes the mechanisms by which synaptic proteins are endocytosed and recycled back to the plasma membrane to regulate their surface expression (Park et al. 2004; Schmidt and Haucke 2007). Rab11 is a member of the Rab family of proteins that localizes to recycling endosomes where it mediates membrane traffic. Recently, β-CTF-mediated Rab11 impairment was reported in AD, leading to the dysfunction of recycling endosomes that could contribute to synapse dysfunction and defective long-term potentiation (LTP) seen in this disorder (Park et al. 2004; Woodruff et al. 2016). Other studies have documented impaired endocytic recycling of glutamate and insulin receptors in brains of ApoE4 carriers (Chen et al. 2010; Zhao et al. 2017). The pan-functional role of endosomal pH in plasma membrane protein recycling and turnover has recently received much attention in the context on autism and brain cancer, with the accumulation of evidence showing that recycling endosomal alkalization promotes receptor recycling and prolonged oncogenic signaling, whereas endosomal hyperacidification enhances turnover and thereby reduces lifetime of membrane proteins (Kondapalli et al. 2013, 2015; Prasad and Rao 2015a). These and many other observations have been consolidated into a unifying hypothesis centering on vesicle trafficking dysfunction that is particularly germane to our understanding of AD pathogenesis.

4 Protons to Patients: Linking Endosomal pH and Neurodegeneration

We hypothesize that endosomal pH is central to the mechanism of AD. Notably, APP, its proteolytic fragments (Aβ and β-CTF), and key clipping enzymes (β and γ-secretases) localize to endosomes. Pathological endosomal acidification promotes amyloid pathology at all key steps: production, aggregation, and deposition (Fig. 2).

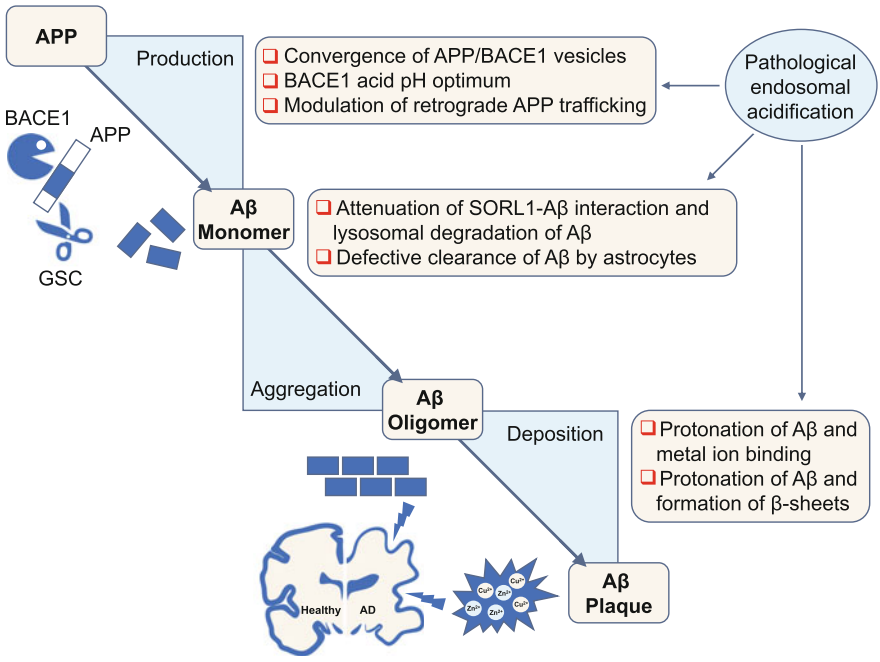

Fig. 2 Acidic endosomal pH promotes Alzheimer's disease pathology. Pathological endosomal acidification promotes amyloid pathology at all key steps of amyloidogenesis, including cleavage of APP to produce Aβ, monomer aggregation, and oligomer deposition, culminating in plaques complexed with metal ions. *APP* amyloid precursor protein, *BACE1* β-secretase, *GSC* gamma secretase complex, *SORL1* Sortilin Related Receptor 1, *Aβ* amyloid β

The processing of APP and production of Aβ itself is linked to endosomal pH and function at multiple levels: First, the endosomal lumen must be acidic for the obligatory and rate-limiting convergence of APP/BACE1 (substrate/enzyme) vesicles in neurons (Das et al. 2013). Second, BACE1 protease has an acid pH optimum at pH 4.5 and its active site is in the lumen of the vesicle (Vassar et al. 1999; Vassar and Kandalepas 2011). Notably, there exists a roughly bell-shaped relationship between pH and BACE activity with optimum at pH 4.5, and both very low levels and very high levels depress enzyme activity. Importantly, biochemical studies determining the pH dependence of BACE activity around the functional pH range of endosomes showed roughly linear relationship between acidity and BACE activity. At pH 6, which broadly corresponds to early endosomal pH, the BACE activity is low, i.e., relative activity below 20% that increases markedly in conditions resembling pathological endosomal acidification to ~50% at pH 5.5 and ~80% at pH 5.0 (Vassar et al. 1999). The prominent pH dependence of the BACE enzyme predicts that conditions that hyperacidify endosomes could promote BACE1 mediated clipping of APP. Notably, reducing BACE1 levels ameliorates neuropathology and behavioral defects in AD and Down syndrome mouse models, pointing to its importance as a promising therapeutic target (Cai et al. 2001; Jiang et al. 2016).

Third, endosomal pH regulates retrograde APP trafficking from endosomes and *trans*-Golgi network to aid in physical approximation of APP and BACE1 (Prasad and Rao 2015b). Furthermore, APP shows an acidic pH-dependent conformational switch, although the functional consequence remains to be determined (Hoefgen et al. 2015). Next, defective Aβ cellular clearance and degradation processes can lead to the accumulation and aggregation of Aβ to form neurotoxic oligomers. Hyperacidic endosomal pH could impact this aggregation step of Aβ by at least two distinct mechanisms. First, acidic endosomal pH disrupts SORL1-Aβ interaction and attenuates lysosomal targeting of Aβ for degradation in neurons (Caglayan et al. 2014). Second, pathological endosomal acidification reduces the capacity of astrocytes to clear Aβ (Prasad and Rao 2018a). Finally, acidic endosomal pH could aggravate Aβ deposition into neurotoxic plaques by enhancing protonation of three histidine residues (His6, His13 and His14) in Aβ to promote metal ion binding and formation of β-sheets and fibril assembly (Olubiyi and Strodel 2012) (Fig. 2).

In parallel to these observations, it has been recognized for over two decades that drugs causing endosomal alkalization including V-ATPase inhibitors (e.g., bafilomycin and concanamycin), alkalizing drugs (e.g., chloroquine and ammonium chloride), and ionophore drugs that mediate $Na^+(K^+)/H^+$ exchange (e.g., monensin and nigericin) have beneficial roles in reducing APP processing and Aβ production (Caporaso et al. 1992; Haass et al. 1995; Lahiri 1994; Schrader-Fischer and Paganetti 1996; Urmoneit et al. 1998). However, V-ATPase inhibitors, alkalinizing drugs and ionophores have potent and multiple compartmental effects. As a result, they cause undesired changes in vesicle trafficking, Golgi and lysosomal function, and impact the mammalian target of rapamycin (mTOR) and autophagy pathways. Therefore, they have therefore not been exploited for managing AD (Mauvezin and Neufeld 2015; Zoncu et al. 2011). Thus far, there are no clinical agents in AD therapy available to specifically and effectively target endosomal pH. In this context, the discovery of endosomal Na^+/H^+ exchangers and recognition of their key roles in precisely tuning endosomal pH and vesicle trafficking provides a unique opportunity for compartment-specific targeting of early and recycling endosomal pathology in AD.

5 Acid Indigestion in the Endosome: The Role of ApoE4

Key cellular pathologies associated with ApoE4 include endosomal dysfunction, lysosomal leakage, translocation of lysosomal cathepsin D, and neuronal cell death (Cataldo et al. 2000; Ji et al. 2002; Persson et al. 2017). As discussed earlier, the endolysosomal network is central to the recycling and turnover of cellular components, and the pH within this network plays a critical role in receptor-mediated endocytosis and vesicular trafficking (Casey et al. 2010). Thus, reliable measurement of intracellular pH is increasingly important in studies of disease mechanisms. To gain a comprehensive understanding of the cellular "pH-stat," more recently, flow-cytometry and confocal microscopy-based tools have been developed for precise, ratiometric fluorescence-based quantification of endosomal, lysosomal,

and cytoplasmic pH. Using these techniques on ApoE genotyped patient fibroblasts and murine astrocytes with human ApoE variants, we demonstrated that endosomes were hyperacidic (∼1 pH unit lower), whereas lysosomes were hyperalkaline (∼1 pH unit higher), in disease-associated ApoE4, compared to normal ApoE3 cells (Prasad and Rao 2018a). Evidence from lower eukaryotes, e.g., yeast, shows that acidic endosomes also result in more acidic lysosomes (Brett et al. 2005b). However, only a single eNHE isoform (Nhx1) and single Cl^-/H^+ transporter (Gef1) exist in yeast, whereas mammalian cells have multiple isoforms with discrete localizations to various endosomal and lysosomal compartments. As a result, pH homeostasis in each organelle may be regulated independently and uniquely adapted to specific needs, and alterations in luminal pH in endosomes might not extend to lysosomes. This might also explain why ectopic expression of NHE6 does not alter lysosomal pH (Prasad and Rao 2018a). Significant downregulation of expression of V-ATPase might underlie lysosomal alkalinization in ApoE4 astrocytes (Prasad and Rao 2018a). Previously, abnormal alkalization of lysosomal pH was also reported in a genetic model of presenilin 1-deficiency in AD, in cell culture and neurons (Lee et al. 2010). A comprehensive overview of lysosomal pH dysregulation in AD is available elsewhere, so we will focus on the regulation of endosomal pH in AD. The recent identification of intracellular NHE isoforms in genome-wide association studies of risk factors in AD further strengthens the link with endosomal pH (Martinelli-Boneschi et al. 2013; Meda et al. 2012; Perez-Palma et al. 2014). We suggest that defective pH regulation in the endosomal-lysosomal system may be a broad, unifying mechanism and a new paradigm for understanding the pathogenesis of familial and sporadic late onset AD.

6 NHE6 Is an ApoE4 Effector

Although several genetic studies have identified links between eNHE to a host of neurological disorders, including autism, intellectual disability, attention deficit hyperactivity disorder, epilepsy, Parkinson's disease, brain cancer, multiple sclerosis, and more recently to late-onset AD, a mechanistic basis for the contribution of Na^+/H^+ exchange to these disorders is still emerging (Kondapalli et al. 2014; Prasad and Rao 2015a). Mutations in *SLC9A6*, encoding NHE6, are causal to Christianson syndrome, a monogenic X-linked disorder with neurodevelopmental hallmarks of syndromic autism and intellectual disability. Although ubiquitous in tissue distribution, NHE6 is highly expressed in the brain, particularly in the hippocampus and Purkinje cell layer of the cerebellum, where it has been implicated in neuronal spine dynamics, dendritic arborization, and synaptic strength (Deane et al. 2013; Gilfillan et al. 2008; Kondapalli et al. 2014; Ouyang et al. 2013). As summarized in Table 1, patients with loss-of-function mutations in NHE6 also present with striking and progressive neurodegenerative pathology including (1) early-onset, severe degeneration of cortex and cerebellum, (2) loss of neurons, (3) gliosis, and (4) deposition of hyperphosphorylated tau in neurons and astrocytes (Garbern et al. 2010; Mignot

Table 1 Summary of genetic evidence supporting the role of NHE6 and NHE9 in neurodegenerative disorders

Gene name	Study type	Nucleotide change	Protein change	Phenotype	Reference
SLC9A6/ NHE6	Linkage analysis, electron microscopy, histopathological and biochemical investigations	c.1109_1117delGG AGTACCT	p. W370_T372del	Corticobasal degeneration and tau deposition	Garbern et al. (2010)
	Serial brain MRI, clinical and mutational analysis	c.916C > T	p.Q306X	Progressive degeneration of cortex and cerebellum	Mignot et al. (2013)
	Brain MRI, clinical and mutational analysis	c.1560dupT	p.T521YfsX23	Parkinsonism in female carriers	Riess et al. (2013)[a]
	Clinical and mutational analysis	c.190G > T	p.E64X	Corticobasal degeneration syndrome and parkinsonism in female carriers	Sinajon et al. (2016)
SLC9A9/ NHE9	GWAS	Multiple SNPs		Alzheimer's disease	Perez-Palma et al. (2014)
	GWAS	rs17636071		Alzheimer's disease, response to cholinesterase inhibitors	Martinelli-Boneschi et al. (2013)
	GWAS	rs9828519		Multiple sclerosis, response to interferon-ß	Esposito et al. (2015)

GWAS genome-wide association studies, *MRI* magnetic resonance imaging, *del* deletion, *dup* duplication, *fs* frameshift, *X* stop codon, *SNPs* single nucleotide polymorphisms; Amino acids are represented by their single letter codes in protein (p) sequence

[a]Mutation referred in relation to longer NHE6.1 isoform (NP_001036002.1)

et al. 2013). Notably, NHE6 deletion in mice leads to cellular phenotypes reminiscent of AD, including endosomal hyperacidification, endolysosomal dysfunction, accumulation of unesterified cholesterol in endosomes, and neurodegeneration (Ouyang et al. 2013; Stromme et al. 2011). These observations point to a more widespread role for NHE6 in neurodegenerative disorders. Given the link between ApoE genotype and endosomal pathology, we hypothesized that the Christianson syndrome protein NHE6 is a potential ApoE effector and that dysfunction of NHE6 in disease-associated ApoE4 variants is causal to a subset of pathologies and phenotypes associated with AD.

6.1 NHE6 Is Downregulated in Alzheimer's Disease

Our analysis of publicly available patient databases revealed that NHE6 is one of the top 100 downregulated genes in late-onset AD, predictive of endosomal hyperacidification (Prasad and Rao 2018a). Furthermore, NHE6 downregulation was proportional to disease severity as determined by cognitive and pathological scores (Prasad and Rao 2015b). Importantly, NHE6 expression strongly correlated with average expression of set of synapse genes found to be downregulated in AD. These correlations suggest that reduced NHE6 levels and, by extrapolation, pathological endosomal acidification may underlie synaptic pathology in AD. Indeed, NHE6 protein was low in sporadic AD brains relative to control (Prasad and Rao 2015b). Female carriers of loss-of-function NHE6 mutations have learning difficulties and behavioral problems and some present in late adulthood with low Mini-Mental Status Exam (MMSE) scores indicating early cognitive decline and susceptibility to neurodegeneration (Sinajon et al. 2016). Consistent with these clinical data, transcriptomic analysis of brains areas involved in cognition revealed that NHE6 is among the most highly downregulated genes (up to sixfold) in old (70 years) brain, compared to adult (40 years) (Naumova et al. 2012). NHE6 was also identified as a top hub transcript in AD with 202 network connections that control a plethora of downstream effects (Webster et al. 2009). Another study employing knowledge-based algorithms predicted NHE6 as a molecular player contributing to early-stage AD (Mayburd and Baranova 2013). A network analysis of the metastable subproteome associated with AD identified NHE6 as a top hub gene regulating trafficking and clearance mechanisms necessary for safeguarding cellular proteostasis (Kundra et al. 2017). Although not identified in GWAS studies on AD risk, a more recent deep-learning approach that involved construction of an integrated heterogeneous omics profile using gene expression and methylation data identified NHE6 as one of the 35 genes that improved the accuracy of AD prediction (Park et al. 2019). Together, these observations provide compelling evidence supporting a more widespread role for NHE6 and endosomal acid-base homeostasis in AD. Central to our hypothesis, ApoE4-associated downregulation of NHE6 expression was observed in post-mortem brains and cellular models (Prasad and Rao 2018a; Xu et al. 2006).

6.2 *Co-expression Clues in Alzheimer's Disease*

Genetic architecture and gene co-expression networks have been beneficial in understanding mechanisms of disease pathogenesis and progression in AD (Zhang et al. 2013). Clustering analysis of RNA-seq data from 524 normal human brains from the Allen Brain Atlas was used to gather functional insights from the inherent patterns of co-expression (Miller et al. 2014). This approach, also used in cancer research, is based on the premise that genes that are expressed together are likely to function together in a common pathway (Dang et al. 2019; Prasad and Rao 2015b; Zhang et al. 2013). Gene expression clustering works on the principle of "guilt by association" and could provide clues to functional interactions and form the basis for new hypothesis-driven research as evidenced by the close association of enzyme-substrate-receptor group (BACE1, APP, SORL1), receptor-ligand group (TREM2, TYROBP), and members of same family such as MS4A/CD20 proteins (MS4A4A, MS4A6A), and apolipoproteins (APOE, APOJ/clusterin) (Karch and Goate 2015) (Fig. 3). Recent studies have validated functional links between other close associations observed from this clustering analysis: (TYROBP, CD33, TREM2; Haure-Mirande et al. 2017), (ADAM10, SIRT1; Theendakara et al. 2013), (CD2AP,

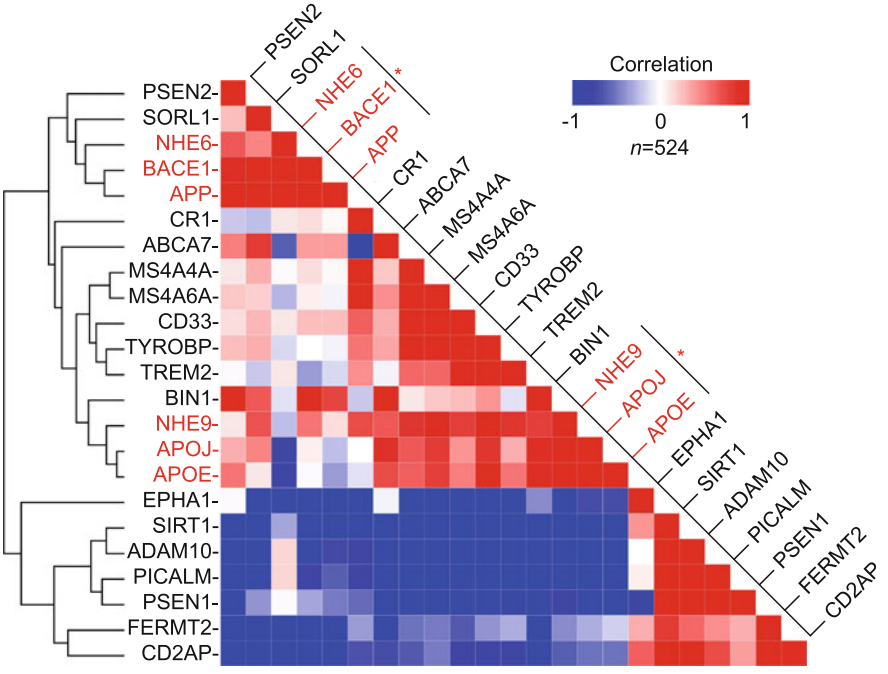

Fig. 3 Co-expression of eNHE isoforms with Alzheimer disease genes. Clustering analysis of RNA-seq data from 524 normal human brains from the Allen Brain Atlas (low correlation, blue; high correlation, red). Note the strong correlation of NHE6 with APP and BACE1 and of NHE9 with APOE and APOJ (red asterisks)

FERMT2; Shulman et al. 2014), (BIN1, APOJ/clusterin; Zhou et al. 2014), and (PICALM, PSEN1; Kanatsu et al. 2014). Thus, gene co-expression analysis may not only identify novel disease-related genes but also may point to previously unrecognized gene functions (Fig. 3).

NHE6 expression was tightly linked with amyloid precursor protein APP and β-secretase enzyme BACE1, with a prominent linear correlation for APP and NHE6 (Pearson correlation 0.75; $n = 524$; $p = 7.0 \times 10^{-96}$). Indeed, NHE6 ranked among the top 0.5% of human brain transcriptome transcripts (of 52,376) whose expression was most correlated with APP. This observation prompted an investigation of the role of NHE6 in APP metabolism and also uncovered a significant association of the closely related endosomal isoform NHE9 with APOE and APOJ, two lipid carrier proteins that are central to cholesterol homeostasis in brain. The APOE-NHE9 cluster also had a prominent linear correlation (Pearson correlation: 0.78, n: 524, p value: 1.7×10^{-108}), suggesting a functional link that warrants future investigation. Intriguingly, NHE9 has been associated with late-onset AD and poor response to cholinesterase inhibitor treatment in AD (Martinelli-Boneschi et al. 2013; Perez-Palma et al. 2014). Furthermore, NHE9 is among the top 1% of human brain transcriptome (out of 52,376 transcripts) whose expression was most correlated with the expression of APOE across 524 samples. Another interesting observation from our analysis is the differential clustering of presenilin proteins (PSEN1 and PSEN2) with the two sheddases, α- and β-secretase. Presenilin proteins constitute the catalytic subunits of the gamma-secretase complex, which participates in both non-amyloidogenic and amyloidogenic APP processing pathways, in addition to its essential role in processing of Notch and other substrates (Karch and Goate 2015). Intriguingly, during normal brain development, PSEN2 clustered with β-secretase (BACE1), and, in contrast, PSEN1 clustered with α-secretase (ADAM10), pointing at potential hitherto unnoticed differences in functions of the two presenilin proteins that could form the basis for new hypothesis-driven research (Fig. 3).

6.3 NHE6 Blocks Amyloid Buildup

Tripartite synapses are sites of cell-cell contact specialized to transmit and compute information in the brain, which, as the name suggests, consists of three essential components: presynaptic neuron, postsynaptic neuron, and the surrounding astrocyte process. Synaptic transmission involves translating the presynaptic information in form of neurotransmitters into various postsynaptic events, ranging from alterations in resting membrane potential, downstream biochemical cascades, and gene expression (Pereda 2014) (Fig. 4). Growing evidence points to deficits in synaptic transmission and loss of synapses in Aβ toxicity that occurs early in AD pathogenesis and precedes neuronal loss by several decades (Masliah et al. 2001; Sheng et al. 2012). Aβ production at the synapses is mediated by clathrin-dependent endocytosis of surface APP at presynaptic terminals into endosomes followed by physical

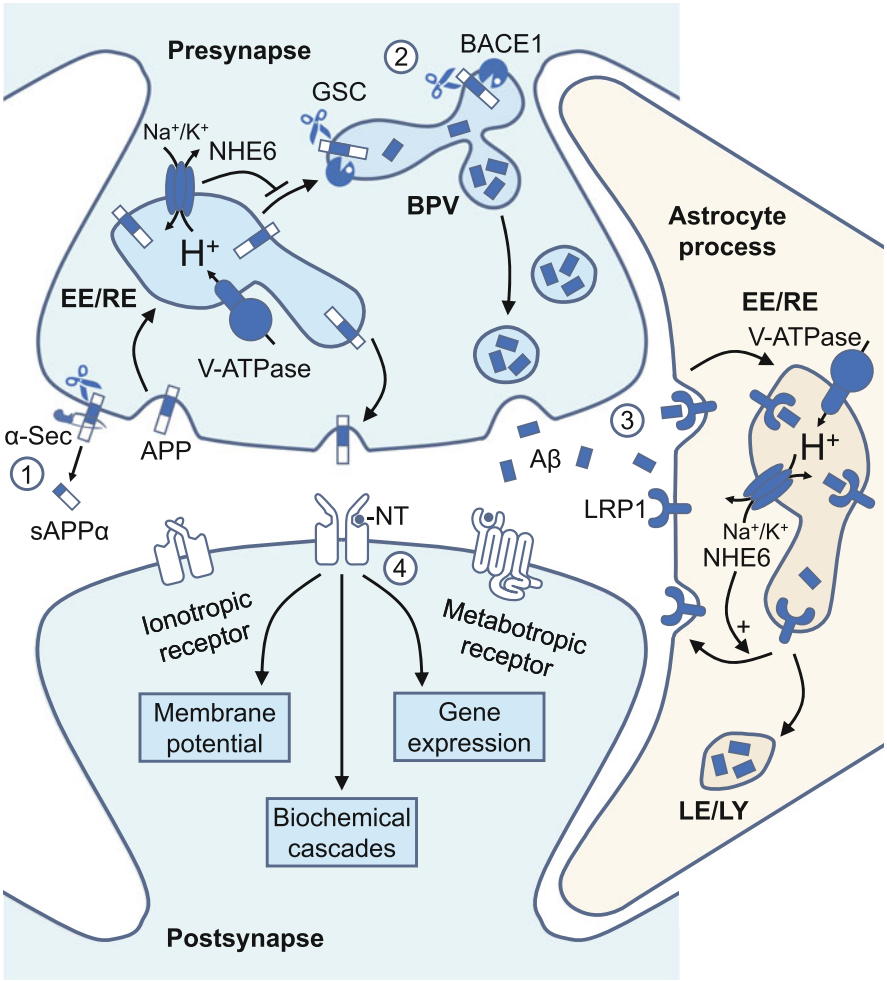

Fig. 4 Model for endosomal pH regulation of synaptic Aβ production and clearance. Endosomes are involved at multiple steps in the production and clearance of Aβ. At the cell surface (Step 1), APP can be cleaved by α- and γ-secretases (non-amyloidogenic pathway). Alternatively, APP is internalized by clathrin-dependent endocytosis at presynaptic terminals, where it undergoes amyloidogenic processing by β- and γ-secretases (Step 2). NHE6 alkalinizes the endosomal lumen, blocks trafficking of APP from the endosome to BACE1 positive vesicles, and limits Aβ production by neurons. Astrocytes clear Aβ peptides from the synaptic cleft by LRP1 receptor-mediated endocytosis (Step 3). NHE6 activity stabilizes surface expression of LRP1 and promotes Aβ clearance by astrocytes. An imbalance between Aβ production and clearance results in pathological accumulations of Aβ that could disrupt synaptic transmission and cause synapse loss (Step 4). *APP* amyloid precursor protein, *α-Sec* α-secretase, *BACE1* β-secretase, *GSC* γ-secretase complex, *BPV* BACE1-positive vesicles, *Aβ* amyloid β, *LRP1* LDL receptor-related protein 1, *EE/RE* early and recycling endosomes, *LE/LY* late endosome and lysosome, *NT* neurotransmitter

approximation of APP and BACE1 positive vesicles, proteolytic cleavage of APP, and release of Aβ into the synaptic cleft (Cirrito et al. 2005; Das et al. 2013) (Fig. 4). One way enlarged and amplified endosomes seen in AD brains could impact amyloidogenesis is by providing increased surface area for BACE1-mediated APP cleavage. Here, we discuss how hyperacidic pH within these dysfunctional endosomes in neurons could regulate the BACE1 function. Specific targeting of microenvironment within endosomes has the advantage of dampening BACE1-mediated cleavage of APP and not of non-amyloid substrates that are processed in an endocytosis-independent manner, thus preventing mechanism-based toxicities and adverse events (Ben Halima et al. 2016).

Using neuronal and non-neuronal cell models, NHE6 was shown to regulate trafficking and BACE1-mediated processing of APP (Prasad and Rao 2015b). First, endogenous APP was co-localized with NHE6 in Rab11 positive endosomal compartments. Next, in a well-characterized HEK293-derived cell line stably expressing human APP, NHE6 activity blocked retrograde trafficking of APP from endosomes to the *trans*-Golgi network. These changes were correlated to luminal pH of sorting endosomes, and causality established using monensin, an ionophore and Na^+/H^+ exchange mimetic (Prasad and Rao 2015b). It is important to note that neurons have developed an efficient mechanism of restricting substrate (APP) and enzyme (BACE1) to separate organelles and the APP-BACE1 convergence, intriguingly, is known to occur in acidic microdomains (Das et al. 2013). We therefore hypothesized that by limiting excess endosomal acidification, NHE6 in neurons blocks trafficking and physical approximation of APP from endosome and BACE1-positive vesicles, thus limiting biogenesis of Aβ under normal physiology (Fig. 4). This idea was validated in cell culture model by documenting significant reduction in substrate-enzyme (APP-BACE1) overlap and diminished Aβ production with NHE6 expression. In contrast, BACE1-mediated APP processing and Aβ production was elevated upon NHE6 depletion (Prasad and Rao 2015b). These observations linking NHE6 dysregulation to Aβ pathology were supported by (1) significantly lower brain weight and (2) elevated Aβ levels in brain homogenates from 7-month old $NHE6^{KO}$ mice, relative to wild-type mice (Prasad and Rao 2018a).

6.4 NHE6 Promotes Amyloid Clearance

Astrocytes are integral functional elements of the tripartite synapse, responding to neuronal activity and regulating overall brain homeostasis and housekeeping. Astrocytes are specifically dysfunctional in neurodegenerative diseases, including AD (Phatnani and Maniatis 2015). It is increasingly evident from studies in cell culture, humans, and mouse models that ApoE4 genotype negatively affects uptake and clearance of secreted Aβ by astrocytes, a major central nervous system cell type (Castellano et al. 2011; Mawuenyega et al. 2010; Verghese et al. 2013). Using murine astrocytes that produce, lipidate, package, and secrete human ApoE variants in a brain-relevant physiological fashion, significant time-dependent deficit in Aβ

clearance was observed in E4 variants, compared to E3. Consistent with data from human brain transcriptome, NHE6 transcript and protein expression was downregulated in ApoE4 astrocytes, correlating with endosomal hyperacidification and ~50% reduced surface levels of Aβ receptor LRP1 (low-density lipoprotein receptor-related protein 1) (Prasad and Rao 2018a). These findings were independently recapitulated by AD patient-derived ApoE4/4 fibroblasts compared with ApoE3/3 fibroblasts from age-matched control (Prasad and Rao 2018a).

Loss-of-function mutation or downregulation of eNHE hyperacidifies endosomes and reduces surface expression of membrane proteins, and, conversely, overexpression of eNHE alkalinizes endosomes and stabilizes membrane proteins; both mechanisms have been previously shown to play causal roles in human diseases, including autism and brain cancer (Kondapalli et al. 2014; Prasad and Rao 2015a). Therefore, hyperacidification of early and recycling endosomes in ApoE4 astrocytes was proposed to cause loss of LRP1 surface expression and Aβ clearance. Consistent with this hypothesis, correction of endosomal pH by ectopic, lentiviral-mediated expression of NHE6 resulted in robust restoration of surface LRP1 levels and correction of defective Aβ clearance by ApoE4 cells. Similar findings were observed with monensin, establishing causality. Thus, NHE6, by localizing to early and recycling endosomes, significantly alkalinizes luminal pH, stabilizes surface expression of LRP1, and elevates clearance of Aβ peptides (Prasad and Rao 2018a) (Fig. 4). Furthermore, given that LRP1 is a receptor for multiple ligands, these observations, if extended to other ligands and receptors, have the potential to explain number of ApoE4 defects besides Aβ clearance (Prasad and Rao 2018a). Taken together, the findings from these studies, summarized in Table 2, support the following unifying hypothesis: loss of NHE6 function in AD is central to the endosomal pathology observed in pre-symptomatic AD brains and results in over-acidification of early and recycling endosomes, which creates an imbalance between Aβ production and clearance, culminating in Aβ plaques and neurodegeneration (Fig. 5).

Given the pan-functional role of eNHE on recycling of endocytosed proteins to the plasma membrane (Kondapalli et al. 2014; Prasad and Rao 2015a), we suggest that loss of NHE6 and pathological luminal acidification may be an upstream event causal to dysfunction of recycling endosomes and defective recycling of multiple proteins, in addition to LRP1, including glutamate and insulin receptors known to be reduced in brains of ApoE4 carriers (Chen et al. 2010; Zhao et al. 2017). Further experimentation will determine the precise mechanism of pH-sensitive regulation of vesicular traffic. A slight alteration in the pH even as little as 0.2 pH units can have profound sequelae (Musgrove et al. 1987). Notably, under physiological conditions, each compartment within the endosomal network has a unique pH range that is one of the defining characteristics of compartmental identity and function (Casey et al. 2010; Pedersen and Counillon 2019). Modulation of acid-base homeostasis within these endosomal compartments may affect cargo fate, redirecting content to or from the degradation pathway. Thus, it is attractive to speculate that changes in luminal pH alone are sufficient to drive endosomal recycling, with modest alkalization of the

Table 2 Summary of experimental studies documenting the role of endosomal acid-base homeostasis in neurodegenerative diseases

Experimental system	Key findings	Reference
Post-mortem human brain tissue	Significantly lower NHE6 transcript and protein levels in AD brains relative to control	Prasad and Rao (2015b)
	NHE6 downregulation in AD correlated with disease severity as determined by cognitive and pathological scores	
	NHE6 expression strongly correlated with synapse genes down regulated in AD	
$NHE6^{KO}$ mice	Endolysosomal dysfunction, accumulation of unesterified cholesterol in endosomes and neurodegeneration	Stromme et al. (2011)
	Endosomal hyperacidification, diminished neuronal arborization and synapse number	Ouyang et al. (2013)
	Lower brain weight and elevated Aβ levels in the brain	Prasad and Rao (2018a)
Cell culture model	NHE6 blocks trafficking of APP from endosome to trans-Golgi network	Prasad and Rao (2015b)
	Expression of NHE6 regulates physical approximation of substrate (APP) and enzyme (BACE1)	
	BACE1-mediated APP processing and Aβ production was elevated upon NHE6 depletion	
Astrocytes	Endosomal hyperacidification and reduced surface levels of Aβ receptor LRP1 in ApoE4 astrocytes relative to ApoE3 astrocytes	Prasad and Rao (2018a)
	Significantly lower NHE6 transcript and protein levels in ApoE4 astrocytes relative to ApoE3 astrocytes	
	Restoring NHE6 expression by lentiviral expression or HDACi treatment corrected surface LRP1 levels and defective Aβ clearance deficit in ApoE4 astrocytes	
Neurons	Reduced surface levels of LRP8 in neurons treated with ApoE4	Xian et al. (2018)
	NHE6 lentiviral knockdown restores normal trafficking of LRP8 in the presence of ApoE4	
	Non-selective NHE inhibitor EMD87580 restores normal trafficking of LRP8 in the presence of ApoE4	
PBMCs	Significantly lower NHE9 transcript levels in PBMCs in MS patients with an active disease course	Esposito et al. (2015)
	NHE9 expression regulates polarization and differentiation of T cells	
	Proinflammatory IFNγ expression was elevated upon NHE9 depletion	

AD Alzheimer's disease, *MS* multiple sclerosis, *HDACi* histone deacetylase inhibitor, *PBMCs* peripheral blood mononuclear cells, *LRP1* LDL receptor related protein 1, *LRP8* LDL receptor related protein 8, *IFNγ* interferon gamma

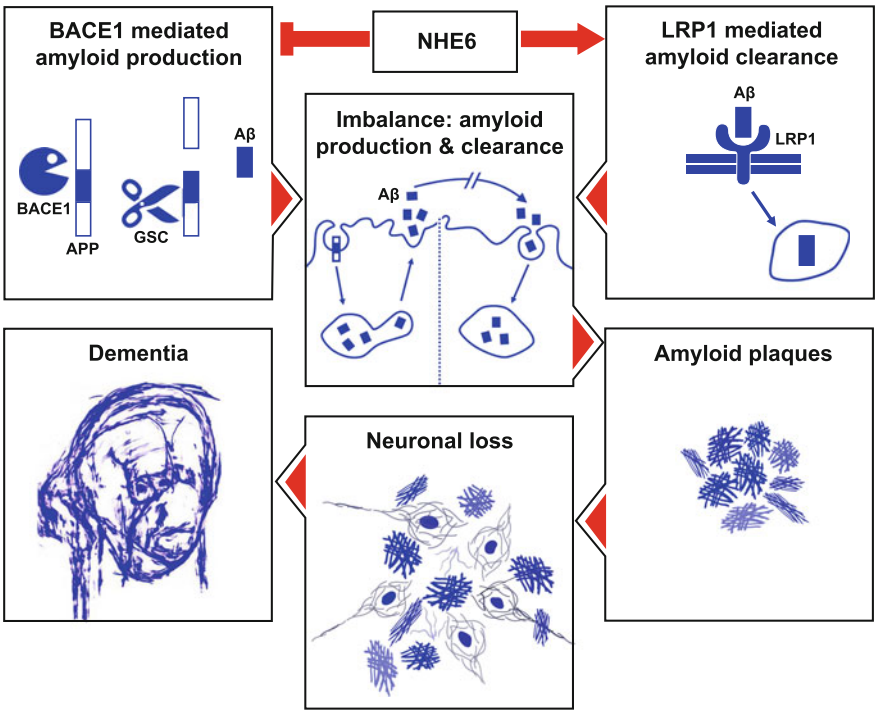

Fig. 5 Loss of NHE6 function is central to the endosomal pathology in Alzheimer's disease. NHE6 blocks BACE1-mediated amyloid production and promotes LRP1-mediated Aβ clearance. Downregulation of NHE6 expression or activity contributes to the endosomal pathology observed in pre-symptomatic AD brains by causing over-acidification of early and recycling endosomes, which creates an imbalance between Aβ production and clearance pathways, culminating in Aβ plaques, neurodegeneration, and dementia. Depiction of dementia is adapted from the self-portrait of Mr. William Utermohlen, an artist and AD patient. *APP* amyloid precursor protein, *BACE1* β-secretase, *GSC* gamma secretase complex, *LRP1* LDL receptor-related protein 1, *Aβ* amyloid β

endosomal pH favoring the recycling pathway, rather than sorting cargo for degradation, and vice versa. This hypothesis, however, requires experimental validation.

6.5 The Goldilocks Scenario: Just the Right pH?

Humans with loss-of-function mutations in NHE6 and mouse lines for NHE6 deletion show progressive neurodegeneration, and this aspect of their phenotype provides crucial evidence to support the role for NHE6 inhibition/downregulation and endosomal hyperacidification in AD (Tables 1 and 2) (Garbern et al. 2010; Mignot et al. 2013; Stromme et al. 2011). The phenotypes of lower brain weight and elevated Aβ levels in the brain observed in knockout mice suggest a protective role for NHE6 activity in Aβ metabolism, neuronal health, and function (Prasad and Rao

2018a). Supporting this notion, studies have demonstrated that NHE6 deletion and endosomal hyperacidification diminish neuronal arborization and can cause synapse loss in mice (Ouyang et al. 2013). Progressive changes in pathology of postnatal brain in two separate mouse models of NHE6 deletion revealed mixed neurodevelopmental and neurodegenerative changes (Xu et al. 2017). Furthermore, disruption of Ca^{2+} entry, a known ApoE4-related pathology, has been recently described in NHE9 null neurons, which show endosomal hyperacidification and impairment in neurotransmitter release (Chen et al. 2010; Ullman et al. 2018).

In contrast, studies by Xian et al. have reported neuroprotective effects of NHE6 knockdown and NHE inhibitor EMD87580 that reversed Apoer2/LRP8 surface trafficking deficits in neurons treated with ApoE4, proposed to result from a molten globule-like state of ApoE4 induced in low pH (Xian et al. 2018). More recent studies, however, showed enhanced stability at acidic pH relative to neutral pH for all ApoE isoforms, contradicting earlier reports of molten-globule confirmation of ApoE4 in a low pH environment (Garai et al. 2011). Because ApoE4 aggregates and shows enhanced binding to lipids in a low pH environment, it seems probable from a biophysical perspective that pathological aggregation of ApoE4 should be exacerbated in hyperacidic endosomal pH (Garai et al. 2011). Another biophysical property for consideration is that protonation of three histidine residues in Aβ under conditions of hyperacidic endosomal pH drives formation of β-sheets and metal ion binding, simulating the process of amyloid aggregation and plaque deposition (Olubiyi and Strodel 2012). Similarly, protonation of glutamate or aspartate resides which are buried in the four-helix bundle of the amino-terminal domain is thought to provide enhanced stability for ApoE in acidic environment (Garai et al. 2011). Several key questions should be considered to resolve the conflicting conclusions. Use of a non-selective NHE inhibitor EMD87580 could have confounding effects by acting on multiple NHE isoforms, including the recycling NHE5 isoform, which is enriched in neuronal endosomes and works to acidify, rather than alkalinize endosomal lumen (Diering et al. 2013); thus inhibition of NHE5 will lead to endosomal alkalization. Furthermore, it is known that depletion of NHE6 results in compensatory increase in NHE9 isoform, which shows overlapping endosomal distribution with NHE6, to delay or diminish endosomal acidification processes (Kondapalli et al. 2013; Prasad and Rao 2015b). Similarly, downregulation of NHE6 levels associated with reciprocal NHE9 upregulation was found in brains of autistic patients (Schwede et al. 2014). In the absence of direct endosomal pH measurements to confirm pH changes in response to gene knockdowns or inhibitor treatment, it is difficult to conclude how these manipulations lead to the observed cellular phenotypes.

Although similar in function, NHE6 and NHE9 have distinct and non-redundant roles as evidenced by disease pathologies in patients and cell phenotypes (Kondapalli et al. 2014). A key unresolved question in the field is whether eNHE isoforms have differential effects on the trafficking of specific membrane proteins. Answering this question will be critical to predict the effects of changes in endosomal pH on surface expression of different membrane proteins. For instance, the evidence in the literature suggests that NHE6, but not NHE9, regulates LRP1

trafficking, and on the other hand, epidermal growth factor receptor (EGFR) trafficking was altered by NHE9 expression and not by NHE6, pointing to isoform-specific functions in receptor trafficking (Ilie et al. 2016; Kondapalli et al. 2015; Prasad and Rao 2018a). It is important to note that NHE9 is also downregulated in ApoE4 models and has been previously linked to AD (Martinelli-Boneschi et al. 2013; Perez-Palma et al. 2014; Prasad and Rao 2018a). Thus, one potential mechanism, although it remains to be characterized, to explain beneficial effects observed with NHE6 knockdown could be that concurrent upregulation of NHE9 isoform enhances recycling of a subset of receptors, such as LRP8 reported in ApoE4 neurons.

Nevertheless, the intriguing observations made by Xian et al. may highlight an emerging Goldilocks scenario in biology, wherein cellular functions depend on an optimal condition, with higher or lower extremes potentially hampering them. It is possible that there exists a bell-shaped relationship between endosomal pH and vesicle trafficking in which intermediate levels of endosomal acidification potentiate endocytic recycling; both very low levels and very high levels depress surface expression. This paradigm could explain the protective effects of NHE6 activation and inhibition in ApoE4 cells, reported in the literature. This seemingly conflicting role for endosomal acid-base homeostasis emerging in AD could add an additional layer of complexity to this dauntingly complex syndrome.

7 Big Data Could Fuel Big Progress

There has been an effort to identify disease mechanisms and facilitate repurposing of drugs to restore gene expression in a given disease by trawling through transcriptome databases (Pessetto et al. 2017; Prasad et al. 2019). This in silico prediction approach has the advantage of being quick and inexpensive. Yeast microarray datasets remains a valuable, yet relatively untapped resource as compared to mammalian data for discovery-driven mining efforts. An unbiased bioinformatics approach, based on data from 284 microarray studies comprising a wide range of experimental conditions, was used to identify evolutionarily conserved mechanism for regulation of eNHE expression and to identify candidate molecules to enhance/restore NHE6 levels in AD (Prasad and Rao 2018b). A predictive model for regulation of Nhx1 expression via the histone deacetylase (HDAC) Rpd3 acting on transcription factor Abf1 to regulate Nhx1 expression was derived from top hits in gene expression (both up- and downregulation) that was then experimentally validated in yeast. The Rpd3 inhibitor, trichostatin A (TSA), increased Nhx1 expression by approximately threefold and alkalinized luminal pH, consistent with the in silico derived model (Prasad and Rao 2018b).

To extend these findings to mammalian cells, a panel of HDAC inhibitors was tested on ApoE4 astrocytes to identify candidates, including TSA and vorinostat that restored NHE6 expression but not that of closely related NHE isoforms. These HDAC inhibitors also normalized endosomal pH and corrected Aβ clearance

defects. In contrast, HDAC inhibitors that resulted in minimal changes in NHE6 expression (MC1568, tubacin) failed to correct Aβ clearance defects (Prasad and Rao 2018a). As predicted by this model, increased nuclear translocation of HDAC4 was observed in ApoE4 astrocytes, resulting in suppression of CREB (cAMP response element-binding protein) and NHE6 expression (Prasad and Rao 2018a, b). A translatable finding from these studies is that pharmacological HDAC inhibition (by TSA, vorinostat) or CREB activation (by Forskolin, Rolipram) selectively elevate endosomal pH and have the potential to correct human pathologies such as AD and autism resulting from aberrant endosomal hyperacidification (Prasad and Rao 2018a, b). Apart from CREB, HDACs have been previously shown to interact and inhibit myocyte enhancer factor 2A (MEF2A) transcription factor activity in neurons (Li et al. 2012). MEF2A is a key regulator of activity-dependent gene program that controls synapse development and function. Intriguingly, NHE6 is a known downstream target of MEF2A in neurons (Flavell et al. 2008). Thus, as an additional/alternative mechanism, we propose that ApoE4-mediated nuclear translocation of HDACs could potentially downregulate MEF2A-NHE6 axis resulting in endosomal hyperacidification that favors amyloidogenic processing of APP and production of Aβ in neurons.

8 Extending the Endosomal Acid-Base Paradigm Beyond Alzheimer's Disease

Evidence for an ever-expanding role for endosomes in neurodegeneration has led to the notion that the endosomal dysfunction could trigger multiple effects affecting diverse pathways. Thus, it appears likely that the disturbances in endosomal pH, by inducing complex patterns of synaptic dysfunction and alterations in neural circuitry, could play a role in multiple neurodegenerative disorders beyond AD. Similar to what has been shown in AD, endosomal aberrations and enlarged endosomes are also the earliest neuronal pathology described in Niemann-Pick type C (NPC), Parkinson's disease, Down syndrome, and other neurodegenerative disorders (Nixon 2005, 2017). NPC is an inherited lysosomal storage disorder associated with impairment in cholesterol trafficking and excessive glycosphingolipid storage (Alam et al. 2016). We have previously observed a direct link between hyper-acidic endosomal-lysosomal pH and disruption of cholesterol trafficking and accumulation of free cholesterol, quantified by filipin staining, in yeast and fibroblast models of NPC disease (Brett et al. 2011). This link between pathological endosomal acidification and cholesterol mistrafficking is strengthened by in vivo studies on $NHE6^{KO}$ mice, showing hyperacidic endosomes and positive labeling with filipin in neurons (Ouyang et al. 2013; Stromme et al. 2011). In both yeast and mammalian cell culture models of NPC disease, we showed that these trafficking phenotypes could be rescued by mild alkalization of endosomal-lysosomal compartments using low concentrations (10 nm) nigericin, a K^+/H^+ ionophore (Brett et al. 2011). Similarly,

endosomal alkalization might explain reduced cholesterol accumulation reported in NPC patient fibroblasts treated with alexidine dihydrochloride, an antimicrobial compound with V-ATPase inhibitory activity (Chan et al. 2012; Pugach et al. 2018). Given our observation of increased NHE6 expression and endosomal alkalization with HDAC inhibition, we suggest that correction of endosomal hyperacidification pathology could potentially contribute to well-documented therapeutic effects of HDAC inhibitor drugs in Niemann-Pick type C disease (Alam et al. 2016; Prasad and Rao 2018a).

Recent studies suggest that endosomal-lysosomal dysfunction may be a primary defect in Parkinson's disease (PD). For instance, mutations in Vps35/retromer, a master regulator of endosome sorting, have been linked to familial PD, highlighting the importance of endosomal pathway in the pathogenesis (Kett and Dauer 2016). More recently, deficiency of Vps35 has been also reported in primary tauopathies, including progressive supra-nuclear palsy and Picks' disease, suggesting a mechanistic overlap with PD (Vagnozzi et al. 2019). A genome-wide analysis of vacuolar pH in ~4,600 yeast null mutants identified dysregulation of luminal pH in Vps35 deletion yeast, although the extrapolation of these findings to humans remain to be validated (Brett et al. 2011). More compelling evidence to link endosomal acid-base dysregulation in PD emerged from recent clinical reports of patients with NHE6 mutations, summarized in Table 1. Riess et al. reported a family with NHE6 mutation where affected males had Christianson syndrome and obligate carrier females showed signs of Parkinsonism (Riess et al. 2013). Similar observations have been reported more recently wherein female carriers of NHE6 mutations showed corticobasal degeneration syndrome (CBDS) and atypical parkinsonism (Sinajon et al. 2016). These observations are further strengthened by a clinical report of a ΔWST^{372} in-frame deletion of three amino acids in NHE6 that was described by Garbern et al. in patients with corticobasal degeneration, severe intellectual disability, autistic symptoms accompanied by deposition of hyperphosphorylated tau in the brain (Garbern et al. 2010). Loss of function was demonstrated by structure-function evaluation of this mutation (Prasad and Rao 2015b). NHE6 inactivation is associated with tauopathy, characterized by neuronal and glial tau inclusions and a preponderance of pathological 4R tau isoform (Garbern et al. 2010). Consistent with this observation, our analysis of postmortem brains from normal and AD dataset revealed that decreased NHE6 expression was correlated with greater tau pathology assessed via neurofibrillary tangle scores (Prasad and Rao 2015b). Furthermore, NHE6 expression was found downregulated in substantia nigra in patients with Parkinson's disease (Hauser et al. 2005). Taken together, these observations provide compelling clues for the links between NHE6 function in Parkinson's disease and tauopathy, and future studies are awaited to define this broader role for endosomal pH in neurodegeneration.

More recently, a pharmacogenetic study by Esposito et al. identified an intriguing link between multiple sclerosis (MS) and endosomal pH (Esposito et al. 2015). MS is a chronic inflammatory and demyelinating condition of the central nervous system characterized with prominent neurodegeneration, and this newly discovered link may lead to new therapies. Intriguingly, chloroquine, a weakly basic anti-malarial

drug that accumulates in acidic organelles such as endosomes and alkalinizes luminal pH, was long found to have therapeutic effect in MS (Thome et al. 2013). Furthermore, polymorphisms in NHE9 are linked to N-glycosylation alterations, an important molecular mechanism in MS (Huffman et al. 2011; Mkhikian et al. 2011). Moreover, NHE9 downregulation was found in a model of Down syndrome, a developmental disorder with Alzheimer's-related endosome dysfunction and early-onset neurodegeneration (Hibaoui et al. 2014; Jiang et al. 2010). By using an approach that integrated GWAS with experimental studies, Esposito et al. showed a significant association between an intronic variant in NHE9 (rs9828519) and non-response to interferon-β (IFNβ) therapy in MS patients (Table 1). Downregulation of NHE9 levels was observed in peripheral blood mononuclear cells (PBMCs) in MS patients with an active disease course. Furthermore, NHE9 expression was induced in PBMCs with IFNβ treatment. NHE9 was found to influence polarization and differentiation of T cells and knockdown of this protein showed increased expression of proinflammatory molecule interferon gamma (IFNγ) (Esposito et al. 2015) (Table 2). A follow-up study Liu et al. identified significant association between rs9828519 variant and dysregulation of NHE9 expression in three brain regions, including occipital cortex, intralobular white matter, and substantia nigra (Liu et al. 2017). Further detailed mechanistic studies in well-defined animal models are warranted to understand the link between NHE9 and MS.

Taken together, available literature reviewed here and elsewhere raises the possibility that a significant proportion of deficits in patients with neurodegenerative disorders are due to endosomal trafficking defects that act as an upstream pathogenic hub. The precise mechanisms by which disturbances in endosomal pH cause neuronal dysfunction and ultimately death in different neurodegenerative disorders remain to be defined and potential therapeutic interventions aimed at endosomal pH need to be determined. This concept has far-reaching therapeutic implications. While the reversal of structural defects in endosomes such as amplifications in its size and number remains a challenge, the correction of endosomal pH deficits, the re-establishment of vesicle trafficking and an improvement of network function appear to be within plausible reach.

9 Summary, Key Questions, and Translational Prospects

What are the translational prospects of the complex endosomal acid-base alterations in neurodegenerative diseases reviewed herein? Most well-known genetic risks for sporadic AD are endosomal proteins, yet targeting these has not provided effective treatments. Notably, an earliest preclinical hallmark of the AD and other neurodegenerative disorders is dysfunction of endolysosomal system that manifests morphologically as enlarged and amplified compartments, biochemically as hyperacidic luminal pH, and functionally as trafficking defects that promotes amyloid pathology (Nixon 2017; Prasad and Rao 2018a; Small et al. 2017). There is a clear potential for development of interventions to exploit the disease-modifying benefit of endosomal

pH. An integrated approach involving data mining and in silico co-expression analysis combined with wet lab studies using $NHE6^{KO}$ mice, post-mortem brains, patient fibroblasts, cultured neuronal and astrocyte lines, and simple yeast models demonstrated a novel ApoE-regulated cellular mechanism and identified a druggable target in AD. By regulating endosomal pH in amyloid pathology, NHE6 is a prominent effector of ApoE4 (Prasad and Rao 2015b, 2018a, b). The fact that acidic endosomal pH is linked to AD at multiple levels makes endosomal pH an attractive target for "disease-modifying" drugs for AD therapy. Significant mechanism-based toxicity has been reported with strong inhibition of APP processing enzymes (β- and γ-secretases), which has directed efforts to identify novel regulators/modulators of APP processing (Ben Halima et al. 2016). Moderate secretase inhibition coupled with novel therapies targeting cellular microenvironment hold promise to benefit in CNS and limit mechanism-based toxicities.

Endosomal Na^+/H^+ exchangers bring new focus to endosome biology, a neglected area in neurodegenerative disorders, and forge a new link between autism/intellectual disability and AD. Endosomal pH emerges as a critical mechanistic link between neurodevelopmental and neurodegenerative disorders. We suggest that in both genetic and sporadic AD, there is disease-initiated perturbation of endosomal-lysosomal pH that may represent the mechanism for relentless disease progression. Consistent with this idea, amphipathic drugs such as bepridil and amiodarone which partition into acidic compartments and alkalinize endosomes also correct Aβ pathology in cell culture and animal models (Mitterreiter et al. 2010). Similarly, endosomal alkalization might explain APP redistribution and reduced Aβ production reported in APP stable cells treated with destruxin E, a natural cyclic hexadepsipeptide with V-ATPase inhibitory activity (Itoh et al. 2009). Our research provides a rational basis for such screening and repurposing of existing US Food and Drug Administration (FDA)-approved drugs and natural product derivatives, known to have off-label activity of endosomal alkalization, as "disease-modifying" drugs to target the cellular micro-environment in AD. Membrane transporters like NHE6 are generally useful "druggable" targets of therapeutic potential. A compelling example is of cystic fibrosis transmembrane conductance regulator (CFTR), one of the most widely studied membrane transporters that has been successfully targeted using small molecule potentiators and correctors as a therapeutic approach to cystic fibrosis. Development of small molecular activators of NHE6 to enhance endosomal pH therefore has the potential to reduce Aβ pathology. Targeting NHE6 has the advantage of specific β-secretase inhibition within endosomes, enhancing their potential as AD therapeutics without undesired mechanism-based toxicities. In addition to regulating luminal pH, endosomal NHE could modulate ionic composition of the endosomal lumen (e.g., K^+ and Na^+) that may directly impact inter- and intra-endosomal functions and dynamics, including osmotic effects and altering membrane curvature and microdomains (Scott and Gruenberg 2011). Further experiments are needed to determine if any such non-pH roles are perturbed in AD.

An emerging pathophysiology of AD is transsynaptic dissemination of the pathological proteins by altering the content of exosomes (Rajendran et al. 2006).

The luminal pH within endosomal-lysosomal compartment might play a role in exosome content, biogenesis, and release. In this context, it is important to note that the ionophore monensin that mediates Na^+/H^+ exchange, alkalinize luminal pH, and mimics constitutively activated NHE6 is known to stimulate the endosomal recycling pathway and exosome secretion (Muro et al. 2006; Savina et al. 2003). We speculate that NHE6 downregulation in AD might disrupt vesicular traffic and alter the number of intraluminal vesicles in the multivesicular body and/or alter the content of exosomes. Further studies are needed to characterize and validate this mechanism. Pathological events, such as epileptiform activity, and normal physiological processes, such as the sleep-wake cycle, are known to regulate Aβ production and if such diverse stimuli also regulate expression/activity of NHE6, a known epilepsy linked protein, remain to be determined (Cirrito et al. 2005; Kang et al. 2009; Kondapalli et al. 2014). Previously, we have shown that endosomal hyperacidification in astrocytes could result in reduced surface levels of glutamate uptake transporter, resulting in aberrant increase in levels of glutamate in and around the synaptic cleft, which could cause epileptiform phenotype and neurodegeneration seen in autism (Kondapalli et al. 2013). Similar mechanism could also explain, in part, AD-related excitotoxicity. While the evidence to link NHE6 downregulation and synapse dysfunction is compelling, it must be acknowledged that the overall contribution of endosomal pH and endosomal NHE to synaptic protein expression, in the context of normal physiology and in pathologies such as AD, remains unclear and more research is warranted. Thus, for example, although enhanced recruitment of NHE6 into dendritic spines during N-methyl D-aspartate (NMDA)-dependent LTP has been reported in the literature, the accumulation of NHE6 at dendritic spines and presynaptic terminals alone cannot be taken as unequivocal evidence for its role in synapse formation and maintenance (Deane et al. 2013). Given the emerging role for recycling endosomal dysfunction in AD (Woodruff et al. 2016), therapeutic enhancement of pH of recycling endosomes might result in better endocytic recycling of membrane proteins, including adhesion molecules and neurotransmitter receptors and transporters, and provide neuroprotection independently of protection against plaque formation. Targeting this inside-out control of surface proteostasis by eNHE therefore may provide new treatments for sporadic AD and related disorders.

Lastly, based on our data, we propose that amyloid pathologies may contribute to autism and intellectual disability phenotypes seen in patients with NHE6 mutations that could have important prognostic implications for early intervention to limit regression and marked neurodegeneration seen in Christianson syndrome patients (Mignot et al. 2013). Notably, alterations in APP processing and Aβ production have been reported in autism, Fragile X syndrome and 15q duplication in patients and animal models (Wegiel et al. 2012). An understanding of the mechanistic overlap between developmental and aging disorders is urgently needed to expand therapeutic options for these disorders. Consistent to our reports of lower NHE6 levels in AD, downregulation of NHE6 gene expression has been documented in post-mortem autism brains, marking the possibility of a mechanistic overlap between autism and AD pathologies (Prasad and Rao 2015b, 2018a; Schwede et al. 2014). A long-term goal of our research is to determine if a subset of autism patients with dysregulated

NHE6 activity, either by loss-of-function mutations or by downregulated gene expression, have a higher risk of premature aging and developing neurodegenerative disorders, thereby providing a rational basis to stratify patients for targeted therapies. In summary, studies on NHE6 have proven to be instrumental for appreciating the role of endosomal acid-base homeostasis in neurodegenerative diseases and have provided us an opportunity through which we can begin to study and understand the role of other ion transporters regulating endosomal pH in human pathologies. Restoring endosomal acid-base disturbance that fuels the progression of AD holds promise to slow and ultimately even prevent cognitive impairment in this syndrome. Future research will hopefully further define additional molecular players and pathways regulating critical endosomal acidification process and in doing so may identify promising targets for therapies for Alzheimer's disease and related neurodegenerative disorders.

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Endolysosomal Disorders Affecting the Proximal Tubule of the Kidney: New Mechanistic Insights and Therapeutics

Beatrice Paola Festa, Marine Berquez, Daniela Nieri, and Alessandro Luciani

Contents

Abstract Epithelial cells that line the proximal tubule of the kidney rely on an intertwined ecosystem of vesicular membrane trafficking pathways to ensure the reabsorption of essential nutrients. To function effectively and to achieve homeostasis, these specialized cells require the sorting and recycling of a wide array of cell surface proteins within the endolysosomal network, including signaling receptors, nutrient transporters, ion channels, and polarity markers. The dysregulation of the endolysosomal system can lead to a generalized proximal tubule dysfunction, ultimately causing severe metabolic complications and kidney disease.

In this chapter, we highlight the biological functions of the genes that code endolysosomal proteins from the perspective of understanding – and potentially reversing – the pathophysiology of endolysosomal disorders affecting the proximal tubule of the kidney. These insights might ultimately lead to potential treatments for currently intractable diseases and transform our ability to regulate kidney homeostasis and health.

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Abbreviations

AMN	Protein Amnionless
BM	Bone marrow
Ccnd1	Cyclin D1
CKD	Chronic kidney disease
ClC-5	H^+/Cl^- exchange transporter *5*
CMA	chaperone-mediated autophagy
Ctns	Cystinosin
Cubn	Cubilin
EM	Electron microscopy
GAP	GTP-activating protein
GLA	α-galactosidase
GNA12	Guanine nucleotide-binding protein subunit alpha-12
GWAS	Genome-wide association studies
INPP5B	Type II inositol-1,4,5-trisphosphate 5-phosphatase
iPSCs	Induced pluripotent stem cells
KI	Knock-in
KO	Knock-out
LMWPs	Low-molecular weight proteins
LRP2	Lipoprotein receptor-related protein 2
LSD	Lysosomal storage disease
map1lc3	Microtubule-associated protein 1A/1B light chain 3
mTOR	Mammalian Target of Rapamycin
NaPi-IIa	Sodium/phosphate cotransporter (isoform IIa)
OCRL	Inositol Polyphosphate-5-Phosphatase
Pcna	Proliferating cell nuclear antigen
PCT	Proximal convoluted tubule
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PQLC2	Cationic amino acid transporter 2
PT	Proximal tubule
PtdIns	Phosphatidylinositol
ROS	Reactive Oxygen Species
SGLT2	Sodium/glucose cotransporter 2
SQSTM1	Sequestosome 1
TFEB	Transcription factor EB
TFE3	Transcription factor E3
TJP1	Tight junction protein 1
TNTs	Tunneling nanotubes
v-ATPase	Vacuolar ATPase
YBX3	Y-box-binding protein 3

1 Introduction

Epithelial cells that form the proximal tubule (PT) of the kidney reabsorb a large variety of filtered low-molecular mass-nutrients and macromolecules through a particularly well-developed and efficient endolysosomal system. Given the pathway's central role in sustaining homeostasis, the disruption of the endolysosomal network causes a generalized dysfunction of PT cells. These alterations might drastically trigger massive losses of solutes and low-molecular weight proteins into the urine (a clinically relevant entity denoted as renal Fanconi syndrome), ultimately underwriting severe metabolic complications and chronic kidney disease (CKD) (Eckardt et al. 2013).

Over the last two decades, studies of inherited kidney diseases targeting the endolysosomal pathway, together with the advances in technology and foundational genomic resources, and analytical approaches, have provided novel insights into fundamental principles governing homeostasis and the physiology of proximal tubule cells (Devuyst et al. 2014). Through converging mechanisms, these pathway paradigms can aid the holistic understanding of the pathogenesis in more common kidney diseases. For instance, abnormally filtered proteins – such as immunoglobulin free light chains (Luciani et al. 2016) – might accumulate within the endolysosome system and disrupt subsequently its dynamics and degradative functions, triggering complex signaling cascades that drive epithelial dedifferentiation and PT dysfunction (Luciani et al. 2016). Furthermore, genome-wide association studies (GWAS) have identified the association between common sequence variants within or close to the genes encoding core components of the endolysosomal machinery and kidney-related traits (i.e., glomerular filtration rate and kidney function; Qiu et al. 2018; Sullivan and Susztak 2020), and different disease states, including albuminuria and CKD (Teumer et al. 2019; Sullivan and Susztak 2020). These large scale genome approaches support thus the fundamental role of the endolysosome system in regulating kidney homeostasis and health, and its contribution to kidney disease risk within the general population.

In this chapter, we chart selected highlights of the past decade of research on biological functions of the endolysosomal pathway, primarily from a perspective of understanding – and potentially treating – the human diseases affecting the proximal tubule of the kidney. In particular, drawing on recent insights into the endolysosomal system architecture and function, we examine how the endomembrane network sustains the absorptive capacity of specialized epithelial cells. We then discuss cellular pathways and the molecular mechanisms whereby deficiencies in various genes encoding endolysosomal proteins trigger proximal tubule dysfunction and potential targets for therapeutically treating these life-threatening diseases.

2 Endolysosomal System and the Kidney Proximal Tubule

PT of the kidney plays a crucial role in reabsorbing a large variety of essential nutrients, including ions and solutes, and hence maintaining body fluid and electrolyte homeostasis (Kriz and Kaissling 2013; Devuyst and Luciani 2015; van der Wijst et al. 2019). The wide array of these homeostatic processes critically relies on the expression and trafficking of specific transport systems that operate at the apical and basolateral domains facing the plasma membrane (Kriz and Kaissling 2013; Devuyst and Luciani 2015; van der Wijst et al. 2019). These transport processes require an electrochemical gradient, which is maintained by the activity of the Na^+–K^+–ATPase, and they impose a high energy demand that is provided by the mitochondrial network (Kriz and Kaissling 2013).

Proximal tubular cells also retrieve a considerable amount of plasma proteins that are continuously filtered through the glomerulus apparatus. These proteins include albumin (~66.5 kDa) and transferrin (~74 kDa) as well as low-molecular weight proteins (LWMPs) such as hormones, vitamin carrier proteins, enzymes, lipoproteins and cell surface antigen components, and immunoglobulin light chains or drugs and toxins (Christensen and Birn 2002). Many of these filtered proteins are processed and recycled by proximal tubular cells, preventing the urinary waste of plasma proteins under physiological conditions (Nielsen et al. 2016). This creates an almost protein-free luminal environment for the downstream kidney tubular cells and it contributes to the maintenance of physiological processes essential for the clearance of drug metabolites, signal transduction, and immune system homeostasis (Nielsen et al. 2016).

The retrieval of albumin and LMWPs across the apical proximal tubule cell membrane (Fig. 1) occurs via either specific receptor-mediated, clathrin-driven endocytosis (Christensen et al. 2009; Eshbach and Weisz 2017) or (potentially) through nonspecific fluid-phase endocytosis (Dickson et al. 2014). Owing to differences in cell ultrastructure and function, including the endolysosomal system, the proximal tubule is classically subdivided into three segments (S1-3; Fig. 1a), with S1 located in the first part of the proximal convoluted tubule (PCT), S2 in the later PCT, and S3 in the straight portion (Christensen et al. 2012). Recent advance in technology, which combines structural and functional imaging of the kidney, suggests that the reabsorption of LMWPs through receptor-mediated endocytosis occurs almost exclusively within S1 cells of the proximal tubule, which have a very high capacity for reabsorption and a far higher expression of endolysosomal system machinery, while nonspecific fluid-phase endocytosis of dextrans taking place in both S1 and S2 (Schuh et al. 2018).

PT endocytosis involves two multiligand receptors, such as the lipoprotein receptor-related protein 2 (LRP2)/megalin (hereafter referred to as megalin) and cubilin (also denoted as the intrinsic factor vitamin B_{12} receptor; Seetharam et al. 1981), and the cooperating protein amnionless (AMN; Fig. 1a). These proteins are located at the brush border of proximal tubular epithelial cells, with megalin displaying a maximal expression in the S1 versus S2 and S3 segments (Schuh

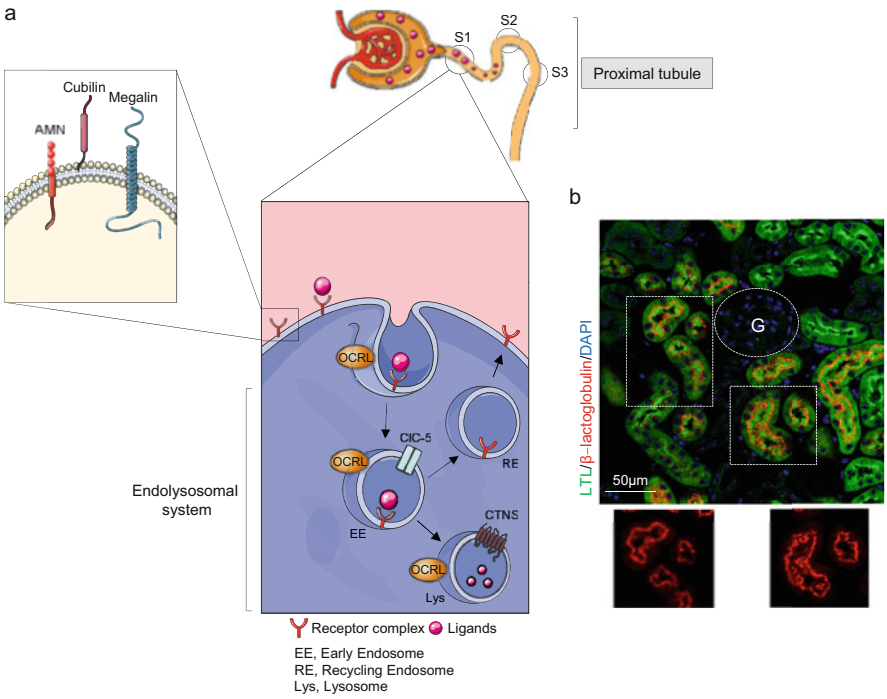

Fig. 1 Receptor-mediated endocytosis and endolysosomal system within proximal tubule of the kidney. (**a**) Filtered ligands, such as albumin and LMWPs, are retrieved and metabolized by epithelial cells lining the S1 segment of the proximal tubule, which expresses high levels of endolysosomal system proteins. Relatively little uptake of LMWPs takes place within S2 cells, which exhibit a much lower expression of endolysosomal system proteins. The LMW ligands interact with the megalin/cubilin/AMN receptor complex at the apical membrane of PT cells. After internalization, the receptor-ligand complex undergoes uncoating and progression along clathrin-dependent endocytic pathway. Once within the endosomal compartment, LMW ligands dissociate from their receptor complex, with the endocytic receptors being trafficked back to the apical membrane for new cycles of binding and internalization, while the ligands are transported to the endolysosome for degradation and recycling processes. Other possible pathways for albumin handling by PT cells, including fluid-phase endocytosis and transcytosis back to the blood circulation, are not detailed here. AMN, protein amnionless; ClC-5, H+/Cl- exchange transporter 5; OCRL, inositol polyphosphate 5-phosphatase; and CTNS, cystinosin. (**b**) Representative confocal micrographs showing the uptake of the fluorescent (Cy5)-labeled LMWP lactoglobulin (red) by LTL (*Lotus Tetragonolobus* Lectin, green)-proximal tubules of the mouse kidney. Dotted white squares represent regions of the respective panels magnified. G indicates glomerulus

et al. 2018). In contrast to megalin, which belongs to low-density lipoprotein (LDL) receptor family, cubilin is a highly conserved membrane-associated protein, with little structural homology to conventional endocytic receptors and characterized by the absence of transmembrane domain. Accumulating evidence indicates that the apical sorting of cubilin and its involvement in the proximal tubule endocytosis rely on the interaction with AMN (Fyfe et al. 2004) – a transmembrane protein that participates in the membrane anchorage, biosynthetic processing, and recycling of

receptor complexes from the endosomal compartments to the plasma membrane (Coudroy et al. 2005). Reinforcing this notion, epistasis-based animal studies have documented an abnormal compartmentalization of the multiligand endocytic receptors megalin and cubilin that accumulate within the endosomal organelles in the kidney proximal tubule of *Amn*-deficient mouse (Strope et al. 2004).

The binding of filtered ligands to, and the subsequent interactions between the endocytic receptors cubilin and megalin, induces internalization into clathrin-coated vesicles and their transport towards the endolysosomal compartments (Fig. 1b; Mishra et al. 2002; Gekle et al. 2005; Kaksonen and Roux 2018). An essential component in this pathway is the apical endosomal compartment where the ligands opportunely dissociate from the receptors through a process that requires sustained vesicular acidification (Faundez and Hartzell 2004) by the electrogenic vacuolar H^+-ATPase (v-ATPase) proton pump (Abbas et al. 2020). Once dissociated from their ligands, the endocytic receptors efficiently traffic to subapical Rab11-positive apical recycling endosomes and reach successively the apical membrane in a microtubule-dependent manner (Perez Bay et al. 2016), sustaining new cycles of ligand binding and internalization (Fig. 1a). Moreover, the acidification of endosomes not only drives the dissociation of ligands from their endocytic receptors, but it also controls the maturation of early endosomes to late endosomes, favoring the progression of the cargo-filled vesicles along the endocytic pathway, and hence feeding the endolysosomal network for degradation and recycling (Hurtado-Lorenzo et al. 2006).

The resulting breakdown products generated by the endolysosomal degradation are eventually exported to the cytoplasm through dedicated nutrient transporters that span the membrane of the endolysosome (Jézégou et al. 2012; Liu et al. 2012; Verdon et al. 2017), and further utilized to immediately support energy production and metabolism (Lim and Zoncu 2016). Furthermore, through an evolutionary conserved and "self-eating" catabolic process called autophagy (Levine and Kroemer 2019; Pohl and Dikic 2019; Ballabio and Bonifacino 2020), the endolysosome recycles malfunctioning proteins and/or superfluous subcellular structures confined within a double membrane structure called an autophagosome to preserve proximal tubule function, integrity, and homeostasis (Fougeray and Pallet 2015; Festa et al. 2018; Chengyuan et al. 2020). In addition to processes that eliminate toxic or damaged cellular components, the endolysosomal system sits at the center of complex regulatory circuitries that control the activation of the mammalian Target of Rapamycin (mTOR) and its associated regulatory complex 1 (mTORC1) – which controls cell metabolism and growth in response to a diverse set of environmental cues, such as growth factors and nutritional status (Liu and Sabatini 2020). Intriguingly, in the proximal tubule of the kidney, this intimate association between mTOR and endolysosomes integrates information about the availability of energy and nutrients to coordinate receptor-mediated endocytosis and transport processes (Grahammer et al. 2017). Therefore, the control of apical endocytosis by turning mTOR signaling landscape on and off at the membrane of the endolysosome might sustain nutrient transport, thereby ensuring the minimal loss of vital substrates into the urine.

Taking together, these recent insights are now putting the endolysosomal system into the spotlight, as it plays a key part in safeguarding the homeostasis and physiology of the proximal tubule. Further details on the role of the endolysosome within other kidney cell types can be found in other reviews (Surendran et al. 2014) and are not discussed here due to space constraints.

3 Diseases Targeting the Kidney Proximal Tubule

Given the endolysosomal pathway's central role in maintaining proximal tubule homeostasis, the dysregulation of the endolysosomal system leads to a generalized proximal tubule dysfunction, causing dehydration and electrolyte imbalance, rickets, muscular weakness, growth retardation, and culminating in the onset and progression of CKD (Klootwijk et al. 2015; Bökenkamp and Ludwig 2011; Devuyst et al. 2014; van der Wijst et al. 2019). The clinical entity of generalized proximal tubule dysfunction is referred to as renal Fanconi syndrome (or de Toni-Debré-Fanconi syndrome) to acknowledge the first description of such a case by Guido Fanconi at the University Hospital in Zurich (Fanconi 1931). Such proximal tubule dysfunction and renal Fanconi syndrome (Table 1) can stem from inherited disorders targeting the endocytic receptors (i.e., Donnai–Barrow and Imerslund–Grasbeck diseases; Nielsen et al. 2016) or components of the endolysosomal system, or affecting various aspects of metabolism (i.e., Fanconi–Bickel syndrome, Wilson disease, tyrosinemia, galactosemia, and congenital fructose intolerance; De Matteis et al. 2017; van der Wijst et al. 2019), or the homeostasis and function of the mitochondrial network (Emma et al. 2016). These disorders can be also acquired (Table 1), for instance following the exposure to toxins or drugs or heavy metals, or secondary to autoimmune diseases (Igarashi 2009), such as Sjögren syndrome and autoimmune interstitial nephritis.

Proximal tubular cells play also a central role in the pathogenesis of proteinuric conditions as abnormally filtered proteins such as albumin or immunoglobulin free light chains are internalized by the endocytic receptor megalin and cubilin, triggering various signaling cascades that drive tubulointerstitial damage and kidney failure (Bökenkamp and Ludwig 2011; Luciani et al. 2016). The storage of κ light chains within the endolysosome might alter its dynamics and proteolytic function owing to defects in acidification (Luciani et al. 2016), leading to dedifferentiation and loss of absorptive capacity of proximal tubular cells; hence, causing proximal tubule dysfunction and renal Fanconi syndrome.

The below sections analyze three clinically relevant entities (i.e., Dent disease, Lowe syndrome, and cystinosis) that are caused by inactivating mutations in genes encoding a set of proteins that regulate various aspects of the endolysosomal homeostasis and characterized by variable forms of proximal tubule dysfunction and renal Fanconi syndrome. Dysfunction of the endolysosomal system is also a crucial determinant of Anderson-Fabry disease – a disorder caused by loss-of-function mutations in the endolysosomal enzyme α-galactosidase (GLA) affecting

Table 1 Inherited and acquired causes of proximal tubule dysfunction and renal Fanconi syndrome

Causes of proximal tubule dysfunction and renal Fanconi syndrome
Congenital
• Arthrogryposis, renal dysfunction and cholestasis 1; ARCS1 (*VPS33B*; MIM #208085)
• Arthrogryposis, renal dysfunction, and cholestasis 2; ARCS2 *(VIPAS39;* MIM #613404)
• Cystinosis *(CTNS;* MIM #219800)
• Cystinuria (SLC3A1. *SLC7A9;* MIM #220100)
• Dent disease 1 (*CLCN5*; MIM #300009)
• Dent disease 2 *(OCRL;* MIM #300555)
• Donnai–Barrow syndrome *(LRP2;* MIM #222448)
• Fanconi renotubular syndrome 1: FRTS1 (MIM #134600)
• Fanconi renotubular syndrome 2; FRTS2 (*SLC34A1*; MIM #613388)
• Fanconi renotubular syndrome 3; FRTS3 (*EHHADH*; MIM #615605)
• Fanconi renotubular syndrome 4; FRTS4 *(HNF4A;* MIM #616026)
• Fanconi–Bickel syndrome *(SLC2A2,* MIM #227810)
• Galactosemia *(GALT.* MIM #230400)
• Glycogen storage disease type I (von Gierke disease) (*G6PC*; MIM #232200)
• Hereditary fructose intolerance (*ALDOB*; MIM #229600)
• Imerslund–Grasbeck syndrome 1 (*CUBN*; MIM #261100)
• Imerslund–Grasbeck syndrome 2 (AMN; MIM #618882)
• Iminoglycinuria (*SLC6A20. SLC6A19. SLC36A2.* MIM #242600)
• Lowe oculocerebrorenal syndrome *(OCRL:* MIM #309000)
• Maturity-onset diabetes of the young type 3 *(HNF1A:* MIM #600496)
• Renal tubular acidosis proximal, autosomal recessive *(SLC4A4:* MIM #604278)
• Tyrosinaemia type I (FAH; MIM #276700)
• Wilson disease *(ATP7B:* MIM #277900)
• Mitochondriopathies
Acquired
• Myeloma
• Sjögron syndrome
• Renal transplantation
• Acute tubulointerstitial nephritis with uveitis (TINU) syndrome
• Autoimmune interstitial nephritis and membranous nephropathy
• Primary biliary cirrhosis
• Renal haemosiderosis
Exogenous substances
• Drugs: aminoglycosides, salicylate, valproic acid, Chinese herbs, ifosfamide, cisplatin, imatinib, mesylate, adefovir, cidofovir, Tenofovir, zoledronic acid, deferasirox
• Chemical compounds: paraquat, bismus, methyl-3-chromone, 6-mercaptopurine, toluene
• Heavy metals: lead, cadmium, mercury, chromium, platinum
• Honeybee stings: melittin

primarily the integrity and physiology of the podocyte and other glomerular cells (Alroy et al. 2002; Christensen et al. 2007; Surendran et al. 2014). Additional details on the pathogenic mechanisms linking GLA deficiency, endolysosomal abnormalities, glomerular proteinuria, and kidney disease can be found in other reviews (Surendran et al. 2014; Miller et al. 2020). The detrimental effects induced by inherited dysfunctions of the endocytic receptor megalin and cubilin on proximal

tubule were nicely reviewed recently (Nielsen et al. 2016) and are not discussed here due to space constraints.

4 Inherited Endolysosomal Disorders Causing Proximal Tubule Dysfunction

4.1 Dent Disease and Lowe Syndrome

Dent disease is a rare X-linked kidney tubulopathy that was firstly described by Dent and Friedman in two unrelated English boys, who displayed rickets and kidney tubule dysfunction associated with hypercalciuria, hyperphosphaturia, LMW proteinuria, and aminoaciduria (Dent and Friedman 1964). This disease is clinically characterized by LMW proteinuria associated with hypercalciuria, which might lead to nephrolithiasis, nephrocalcinosis, and kidney failure (Blanchard et al. 2016).

Dent disease 1 (MIM no. 300009) is caused by loss-of-function mutations in the *CLCN5* gene (Xp11.22) encoding the 746-amino acid electrogenic $2Cl^-/H^+$ exchanger ClC-5 (Lloyd et al. 1996; Picollo and Pusch 2005; Scheel et al. 2005). Many of the reported mutations (i.e., missense and nonsense) result in a truncated or absence of ClC-5 protein, which would trigger a complete loss of its antiporter function (Lloyd et al. 1996). The clinical presentation of Dent disease type 1 reflects the predominant expression of ClC-5 within proximal tubule cells, where it co-distributes with the v-ATPase in the apical endosomal compartment. Genetic studies using *Clcn5* knockout (KO) and knock-in (KI)-based mouse models (Christensen et al. 2003; Novarino et al. 2010) have demonstrated that *Clcn5* deficiency leads to proximal tubule dysfunction by slowing down receptor-mediated endocytosis of ligands and their trafficking along the endolysosomal pathway. These cellular alterations were reflected by the loss, or reduced expression, of the endocytic receptor megalin and cubilin at the brush border (Christensen et al. 2003; Novarino et al. 2010). Of note, no ultrastructural changes were observed in the architecture of the endocytic machinery and formation of the endocytic vesicles as indicated by electron microscopy (EM) studies in mouse kidneys lacking ClC-5 or in kidney biopsy samples from patients harboring established mutations in *CLCN5* (Moulin et al. 2003).

The link between *Clcn5* deficiency, receptor-mediated endocytosis, and PT dysfunction was further substantiated by a proof-of-concept study investigating the effect of bone marrow (BM) transplantation in *Clcn5* KO mice (Gabriel et al. 2017). Transplantation of wild-type bone marrow (BM) in *Clcn5* KO mice significantly ameliorated the PT dysfunction, with bone marrow-derived wild-type cells engrafting the kidney proximal tubule, which rescues the apical expression of ClC-5 and megalin-mediated endocytosis (Gabriel et al. 2017). In line with these in vivo studies, coculture of proximal tubule cells derived from *Clcn5* KO mouse kidneys with BM-derived wild-type cells confirmed the rescue of ClC-5-driven

receptor-mediated endocytosis (Gabriel et al. 2017). The latter observations surmise the involvement of physical transfer of macromolecular constituents between engrafted and host kidney tubule cells through cellular projections called tunneling nanotubes (TNTs; Rustom et al. 2004). Given the morphological features of TNTs and their roles in intercellular communication, these thin membrane tubes might enable the transfer of the wild-type activity into the mutant cells of the proximal tubule. In favor of this idea, the application of an actin-depolymerizing agent in the coculture model blocked the formation of cell-to-cell contacts, ultimately preventing the rescue of ClC-5 expression in mutant PT cells by BM-derived wild-type cells (Gabriel et al. 2017).

A considerable genetic heterogeneity has been reported for Dent disease, with ~50–60% of patients carrying *CLCN5* mutations, ~15% with *OCRL* mutations, and the remaining 25–35% of patients harboring neither *CLCN5* nor *OCRL* mutations. Dent disease II (MIM no. 300555) defines patients with Dent disease who manifest extrarenal features and share mutations (Hoopes Jr et al. 2005; Shrimpton et al. 2009) in the *OCRL* gene with the oculocerebrorenal syndrome of Lowe (MIM no. 309000). Intriguingly, both patients with Dent disease type 1 (i.e., *CLCN5* mutations) and Dent disease type 2 (i.e., *OCRL* mutations) display proximal tubule dysfunction characterized by LMW proteinuria and ~ 90% hypercalciuria (Bökenkamp et al. 2009). In contrast to patients with Dent disease 1, nearly all patients with Dent disease 2 manifest biological evidence of muscle involvement, as indicated by above-normal levels of lactate dehydrogenase and creatine-phosphokinase (Bökenkamp et al. 2009; Park et al. 2014). Some patients with Dent disease 2 might also suffer from intellectual disability and development delay (Bökenkamp et al. 2009), though the severity of these manifestations is likely milder than those observed in patients with Lowe syndrome.

The *OCRL* gene encodes the inositol polyphosphate 5-phosphatase OCRL – an enzyme that acts on phosphoinositides, in particular showing the highest efficacy in removing the phosphate in position 5 of the inositol rings of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] and inositol 1,4,5 triphosphate [Ins(1,4,5)P_3] (Mao et al. 2009). Beyond its phosphatase enzyme activity, the OCRL protein contains also the pleckstrin homology (PH) domain, the ASPM, SPD-2, Hydin (ASH) domain, and the Rho GTP activating protein (GAP)-like domain (Ponting 2006; Peck et al. 2002; Faucherre et al. 2003; De Matteis et al. 2017). These motifs enable OCRL to interact with a wide array of proteins that facilitate its targeting to different cell compartments, including plasma membrane, clathrin-coated endocytic vesicles, trans-Golgi network and the endolysosomes (De Matteis et al. 2017), or to other binding partners involved in cytoskeleton-based remodeling such as Cdc42 and Rac1 (Faucherre et al. 2003; Jefferson and Majerus 1995).

Disease-causing mutations in *OCRL* disrupt the 5-phosphatase activity of the enzyme, promoting the accumulation of PtdIns(4,5)P_2 along the endolysosomal system, and hence triggering the onset and progression of life-threatening manifestations that restrictedly affect brain, eyes, and the proximal tubule. The direct impact of OCRL on transport events leading to renal Fanconi syndrome cannot be reliably

assessed on dedifferentiated cell systems, including non-kidney or non-epithelial cell types, or clonal cells isolated from urine (Devuyst and Luciani 2015; Luciani et al. 2016). Renal biopsy material, usually obtained at an advanced disease stage, is of limited value. The first *Ocrl* KO mouse showed no kidney, nor eye nor brain defects (Jänne et al. 1998) due to compensation by inositol polyphosphate 5-phosphatase (INPP5B), the closest paralogue of OCRL in mice and humans. Deficiency of *Inpp5b* alone leads to sterility in mice, while double knockout for *Inpp5b* and *Ocrl* is embryonically lethal (Bothwell et al. 2011).

Contrasting with a previous kidney tubular conditional *Ocrl* and *Inpp5b* KO mouse model (Inoue et al. 2017), where the phenotype reflects the combined loss of both Ocrl and Inpp5b proteins, the generation of new mouse model (*Ocrl* $^{Y/-}$ *Inpp5b*$^{-/-}$ *INPP5B* mice; hereinafter referred to as *Ocrl* $^{Y/-}$), in which a humanized *INPP5B* is expressed in the whole body of *Ocrl* $^{Y/-}$ *Inpp5b*$^{-/-}$ mouse helps us investigate the functional consequences associated with the specific loss of the phosphatase activity of OCRL (Bothwell et al. 2011; Festa et al. 2019). The absence of OCRL in the mouse kidney triggers proximal tubule dysfunction characterized by LMW proteinuria and aminoaciduria, muscular defects with dysfunctional locomotricity, and slightly reduced growth, as encountered in patients with Lowe syndrome (Bothwell et al. 2011; Festa et al. 2019). Mechanistically, PT dysfunction and LMW proteinuria were reflected by defective receptor-mediated endocytosis induced by the reduced expression of the endocytic receptor megalin, occurring early, in absence of kidney damage and failure (Festa et al. 2019). The mice lacking *Ocrl* did not show glycosuria, phosphaturia, and calciuria even at older age (Bothwell et al. 2011; Festa et al. 2019), mimicking the partial renal Fanconi syndrome encountered in the majority of patients carrying mutations in *OCRL*. Interestingly, *Clcn5*$^{Y/-}$ mice showed a similar defect in receptor-mediated endocytosis, causing a more severe PT dysfunction and LMW proteinuria, reflecting likely the specific roles played by OCRL and ClC-5 towards the endolysosomal system. The presence of a more complete form of renal Fanconi syndrome in the *Clcn5*$^{Y/-}$ mouse model was substantiated by the presence of hypercalciuria, phosphaturia, and glycosuria detected at 8 weeks, reflecting the decreased expression of sodium-glucose cotransporter-2 (SGLT2) and Na^+-P_i cotransporter 2 (NaPi-IIa) – beyond the dysregulated expression of the endocytic receptor megalin (Christensen et al. 2003; Festa et al. 2019).

By establishing differentiated and polarized proximal tubular cell cultures from *Ocrl*$^{Y/-}$mouse kidneys, we were able to reconstitute critical aspects of the disease in vitro. Compelling evidence suggests that OCRL-driven maintenance of PtdIns(4,5)P_2 homeostasis spatiotemporally coordinates the progression of endocytosed cargo along the endolysosomal pathway (De Matteis et al. 2017). For instance, through its interaction with endosomal Rab5, OCRL immediately translocates to early endosomes, where it acts as a switch that finely regulates the abundance of PtdIns(4,5)P_2, which is instrumental for the proper trafficking of internalized vesicles along the endocytic pathway (Vicinanza et al. 2011). The loss-of-function of the enzyme OCRL promotes an increase in the endosomal pool of PtdIns(4,5)P_2, which might subsequently fuel Rac-dependent stimulation of actin-based polymerization

(Vicinanza et al. 2011; Festa et al. 2019). The uncontrolled dynamics of the actin-based cytoskeleton impairs consequently the trafficking of various classes of receptors, including those that pass through the early endosomes to be rapidly recycled back to plasma membrane such as the endocytic receptors cubilin and megalin (Vicinanza et al. 2011; Festa et al. 2019). The trapping of megalin in early endosomes, and hence its defective endosomal trafficking, might partly link the *OCRL* deficiency to kidney tubule dysfunction associated with Lowe syndrome.

Recent exciting discoveries have dramatically overturned the narrow view of the cellular role of the enzyme OCRL, placing it at the center of complex regulatory signaling cascades that regulate endolysosomal system homeostasis and function. In the absence of OCRL, PtdIns(4,5)P_2 accumulates on autolysosome membranes and impairs autophagosome-lysosome fusion, ultimately leading to the accumulation of autophagosomes in patient-derived kidney cells and in the proximal tubule of patients with Lowe syndrome (De Leo et al. 2016). These studies suggest that endolysosomal alterations and impaired autophagy flux might thus drive kidney tubule dysfunction, and, seemingly, the onset of central nervous system-related symptoms in Lowe syndrome patients.

Similar to cellular alterations associated with *OCRL* deficiency, the loss of the function of the channel activity of ClC-5 skews the homeostasis and function of the endolysosomal system, as documented by the defective processing of LMW ligands (Gailly et al. 2008). In this context, it is thus tempting to speculate that endolysosomal abnormalities might compromise the autophagy-mediated quality control of the mitochondrial network, leading to the accumulation of damaged and/or dysfunctional, reactive oxygen species (ROS-producing) mitochondria and oxidative stress as observed in *Clcn5* KO mouse kidneys and their derived proximal tubule cells (Gailly et al. 2008). The pathophysiological crosstalk bridging mitochondrial oxidative stress and proximal tubule dysfunction associated with endolysosomal diseases will be elaborated in the section dealing with cystinosis.

4.2 Cystinosis

Cystinosis (MIM no. 219800) is a rare lysosomal storage disease (LSD, incidence: 1:100,000–1:200,000 live births; Platt 2018) caused by inactivating mutations in the gene *CTNS* encoding the lysosomal cystine-proton cotransporter cystinosin (CTNS) that exports cystine out of endolysosomes (Kalatzis et al. 2001). Given that the low abundance of the cystinosin at the lysosomal membrane is the rate-limiting step for cystine transport, the deficiency of cystinosin leads to the accumulation of cystine within virtually every cell in the body, thereby affecting the integrity and function of any given tissue and organ systems.

The renal Fanconi syndrome is often the first manifestation of cystinosis, usually presenting within the first year of life and characterized by the early and severe dysfunction of proximal tubule cells, highlighting the unique vulnerability of these

kidney cell types (Cherqui and Courtoy 2017). In addition, children with cystinosis display early deposition of cystine crystals in the cornea, thereby causing photophobia and painful corneal erosions (Gahl et al. 2002). In their second to third decade of life, patients with cystinosis can also develop hypothyroidism, hypogonadism, diabetes, myopathy, deterioration of fine vision, and decline of the central nervous system (Nesterova and Gahl 2008; Trauner et al. 2010; Viltz and Trauner 2013).

The availability of animal models has recently shed new light on the disease mechanisms and cellular pathways that underlie the proximal tubule dysfunction and renal Fanconi syndrome in cystinosis, contributing to the development and translation of potential preclinical drug candidates into clinical success. On certain genetic background (i.e., C57BL6), the loss of the cystinosin function, which leads to the accumulation of cystine crystals within mouse proximal tubular cells, causes a progressive PT dysfunction characterized by LMW proteinuria, glucosuria, and phosphaturia, before any structural damage and in the absence of renal failure (Nevo et al. 2010; Raggi et al. 2014; Gaide Chevronnay et al. 2014). However, the PT dysfunction in mice is less severe than that encountered in humans (Nevo et al. 2010); nevertheless, these mice might eventually develop CKD and end-stage renal disease (Nevo et al. 2010). Cystine accumulation and signs of kidney tubule dysfunction have been also reported in a mutant zebrafish model with a homozygous nonsense mutation (c.706 C > T; p.Q236X) in exon 8 of *ctns* (Elmonem et al. 2017) and in a novel congenic rat strain harboring the loss-of-function mutation in the *Ctns* within a recessively acting locus responsible for urinary glucose excretion ($Ctns^{ugl}$; Shimizu et al. 2019). Further investigations warrant for a more comprehensive characterization of the proximal tubule dysfunction in these new animal models of cystinosis.

Insights from mouse and cellular studies suggest that the pathological mechanisms of Fanconi syndrome in cystinosis are multifactorial, involving impaired vesicular trafficking (Raggi et al. 2014; Ivanova et al. 2015; Gaide Chevronnay et al. 2014), defective clearance of endocytic cargos (Raggi et al. 2014; Ivanova et al. 2015; Gaide Chevronnay et al. 2014), impaired proteolysis and limited amino acid and cysteine availability in the cytosol (Chol et al. 2004; Levtchenko et al. 2005; Wilmer et al. 2005), nutrient signaling (Andrzejewska et al. 2016; Ivanova et al. 2016) and autophagy defects (Sansanwal et al. 2010; Napolitano et al. 2015), energy imbalance (Levtchenko et al. 2006; Bellomo et al. 2018) and activation of the inflammasome (Prencipe et al. 2014; Veys et al. 2020), and apoptosis (Park et al. 2002, 2006; Taranta et al. 2017), which can eventually culminate in cell atrophy and irreversible tissue damage. Moreover, compelling evidence indicates that loss of cystinosin induced a phenotype switch associating abnormal proliferation and apical dedifferentiation, leading to defective receptor-mediated endocytosis and urinary loss of LMWPs in vivo (Raggi et al. 2014; Gaide Chevronnay et al. 2014). Despite the identification of cellular defects associated with cystinosis in different models and cell-based systems, a unifying framework linking cystinosin deficiency, endolysosomal dysfunction, and epithelial transport defects has remained incompletely understood.

In most mammalian cells, including those lining the proximal tubule, the endolysosomal system captures and degrades intracellular worn-out constituents through autophagy – an evolutionarily conserved and "self-eating" catabolic process by which cytoplasmic materials are delivered to endolysosome for degradation (Mizushima and Komatsu 2011; Levine and Kroemer 2019). This homeostatic process is particularly active in proximal tubule cells whose intense absorptive and transport properties require the maintenance of the mitochondrial network (Fougeray and Pallet 2015). The autophagy-mediated quality control and turnover of damaged mitochondria are required for protecting proximal tubule cells from acute tubular injury (Isaka et al. 2011); conversely, deletion of essential autophagy genes damages proximal tubule cells (Yamamoto et al. 2016; Luciani et al. 2020; Luciani and Devuyst 2020; Chen et al. 2020) through defective mitochondrial clearance and increased ROS production. Of note, accumulation of distorted mitochondria and of autophagy receptor sequestosome 1/SQSTM1 has been described in kidney biopsies and urinary cells from cystinosis patients (Sansanwal and Sarwal 2010; Sansanwal and Sarwal 2012), suggesting an involvement of autophagy-mediated quality control and surveillance systems in cystinosis. In line with this concept, mouse and cell-based model systems have demonstrated that the deletion of cystinosin impairs the autophagy-mediated clearance, both in vitro and in vivo, and these cellular alterations are induced by the defective endolysosome-driven degradation capacity (Festa et al. 2018; Luciani et al. 2018). Similarly, abnormal endolysosomes and microtubule-associated protein 1A/1B light chain 3 (map1lc3) – marking autophagosomes heighten in cystine – accumulating pronephric tubules in *ctns*–deficient zebrafish, demonstrating the evolutionary conservation of this connection (Festa et al. 2018; Luciani et al. 2018).

The defective autophagy clearance triggers the subsequent accumulation of damaged and/or dysfunctional mitochondria in cells, overproducing mitochondrial-derived ROS. Similarly, genetic or pharmacologic blockage of basal autophagy resulted in accumulation of ubiquitinated proteins and damaged and/or dysfunctional mitochondria, leading to abnormal cell proliferation and apical dedifferentiation reflected by decreased uptake capacities, and hence recapitulating the epithelial phenotype switch encountered in cystinosis cells (Festa et al. 2018; Luciani et al. 2018). Therefore, the lack of autophagy completion, due to impaired lysosomal degradation capacity or through inactivation of essential genes, leads to the persistence of ubiquitinated proteins and dysfunctional, ROS-overproducing mitochondria, slowing down the function and homeostasis of proximal tubule cells. These findings highlight the crucial role of autophagy-mediated quality control systems in preserving the repertoire of the mitochondrial network, whose homeostasis is key to sustaining the endomembrane transport within the proximal tubule.

A signaling cascade connecting excessive generation of mitochondrial ROS, oxidative stress, and epithelial dysfunction was deciphered, involving the tight junctions (Fig. 2). In differentiated epithelial cells, tight junctions repress the nuclear translocation of Y-box protein 3 (YBX3) – a transcriptional factor that promotes the activation of genes involved in cell proliferation, while repressing gene signatures that preserve proximal tubule differentiation during kidney development (Lima et al.

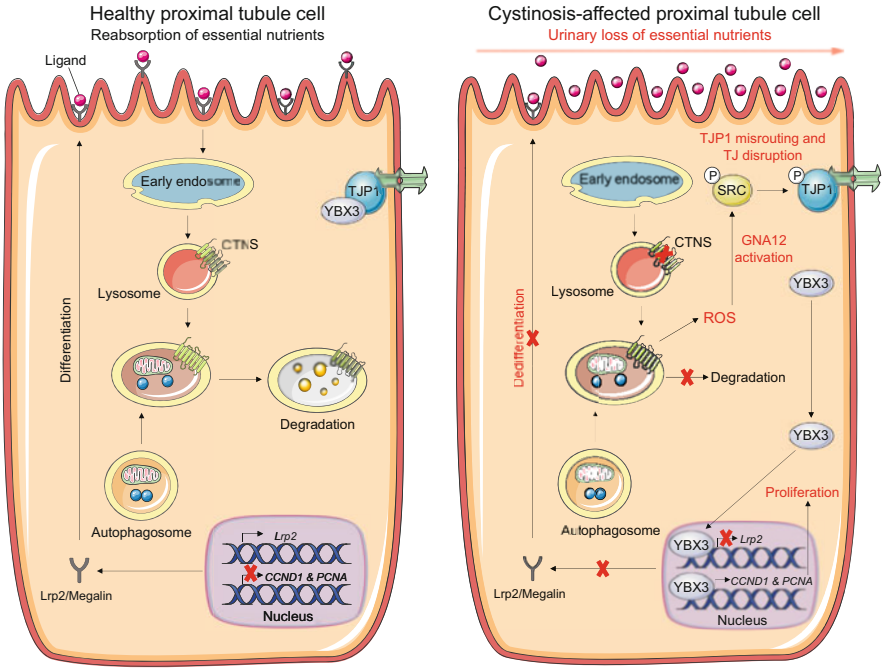

Fig. 2 Impaired autophagy links endolysosomal disease to epithelial dysfunction in the kidney. In wild-type proximal tubule cells (left), the maintenance of autophagy-endolysosomal degradation systems is key to sustaining the reabsorptive processes that account for the uptake of LMW ligands, which are continuously filtered through the glomerulus, and hence ensuring cellular and physiological homeostasis. By contrast, in cystinosis-affected proximal tubule cells (right), the loss of the cystinosin triggers endolysosomal dysfunction, resulting in defective autophagy-mediated clearance of damaged mitochondria. The mitochondrial abnormalities promote the generation of oxidative stress that stimulates GNA12/SRC-mediated phosphorylation of TJP1 and triggers a signaling cascade involving TJP1-associated YBX3. This leads to the activation of transcriptional programs involved in cell proliferation (*Ccdn1* and *Pcna*, respectively) while disabling those regulating apical differentiation (*Lrp2*), ultimately causing epithelial dysfunction in cystinosis cells

2010). Substantiating the key role of tight junction integrity in the maintenance of epithelial phenotype, the overproduction of mitochondrial ROS enhanced G protein subunit α (GNA12)–SRC-mediated phosphorylation of the tight junction protein 1 (TJP1), thereby favoring its dislodgment from cell boundaries and mistrafficking to enlarged, non-degradative endolysosomes (Festa et al. 2018; Luciani et al. 2018). The disruption of tight junction integrity promotes YBX3 signaling, with increased proliferation (i.e., *Ccnd1* and *Pcna*, respectively) and decreased apical differentiation (i.e., *Lrp2*), ultimately resulting in defective endocytosis and epithelial dysfunction in cystinosis (Fig. 2). The biological relevance of the tight junction-associated signaling was confirmed by the gain- and loss-of-function approaches targeting GNA12 or TJP1/YBX3, or by pharmacological interventions impeding activation of Gα12/SRC signaling axis in cystinosin-deficient proximal tubule cells (Festa et al.

2018; Luciani et al. 2018). Consistent with this, treatment of *Ctns* KO mice and their derived proximal tubule cells with the mitochondria-targeted oxygen scavenger Mito–TEMPO (Wang et al. 2016), which is being clinically tested in various mitochondrial diseases, repairs mitochondrial dysfunction and rescues the integrity of tight junction barrier, epithelial cell differentiation, and transport properties (Festa et al. 2018; Luciani et al. 2018). The identification of this signaling cascade highlights the key role of the endolysosome system as a signaling hub that preserves the autophagy-mediated quality control of the mitochondrial network, and hence the health and physiology of the proximal tubule.

5 Current Treatments and Potential Therapeutics

Genetic studies of endolysosomal diseases affecting the PT of the kidney have been key in dissecting interactions in cellular and molecular pathways that regulate the homeostasis and function of the endolysosomal system. The emerging insights from genetics might therefore have direct implications for nominating candidate targets for the development of novel therapeutic interventions. Despite recognized differences in clinical and molecular features, the endolysosomal diseases of the proximal tubule – including Dent disease, Lowe syndrome, and cystinosis – share similar supportive care, focusing on substantially reducing and/or attenuating the life-threatening episodes and clinical manifestations associated with renal Fanconi syndrome (Devuyst and Thakker 2010; De Matteis et al. 2017; Cherqui and Courtoy 2017). For instance, oral supplementations with water, bicarbonate, citrate, phosphate, salts, and vitamin D should rapidly avoid the metabolic complications and maintain body fluid and electrolyte homeostasis, substantially decreasing mortality and the overall morbidity (Devuyst and Thakker 2010; De Matteis et al. 2017; Cherqui and Courtoy 2017). Beyond supportive care, patients with cystinosis can benefit from the treatment with cysteamine (Gahl et al. 2002) – an oral drug that depletes endolysosomal cystine storage by cleaving cystine into free cysteine and cysteamine-cysteine mixed sulfide. These metabolites are subsequently exported from the endolysosome to the cytoplasm through the cationic amino acid transporter 2 (PQLC2) that spans the endolysosomal membrane (Jezegou et al. 2012). Despite remarkably emptying the endolysosomal storage, cysteamine therapy does neither correct nor prevent the established renal Fanconi syndrome (Brodin-Sartorius et al. 2012). Thus, there is an urgent need to identify new therapeutic avenues for these devastating diseases.

Mechanistic studies in different models and cell systems indicate that biological alterations linked to renal Fanconi syndrome involve impaired endomembrane dynamics, defective autophagy, energy depletion and mitochondrial oxidative stress, and endolysosomal signaling and trafficking (Devuyst and Luciani 2015; Cherqui and Courtoy 2017; De Matteis et al. 2017; Eshbach and Weisz 2017; van der Wijst et al. 2019). Interventions that ameliorate any steps in these cascades might thus reverse the proximal tubule dysfunction. For instance, small molecules that either

activate chaperone-mediated autophagy (CMA; Anguiano et al. 2013) or boost the excretion (McNeill et al. 2014) of cystine-loaded endolysosomes might ameliorate the clinical outcomes in patients with cystinosis if they are used concomitantly with cysteamine (Napolitano et al. 2015; Johnson et al. 2013). In line with this idea, recent studies suggest that the combination therapy with a cystine-depleting drug such as cysteamine and mTOR signaling pathway inhibitor (such as everolimus) might have the therapeutic potential to improve the treatment success rate of individuals with cystinosis (Hollywood et al. 2020). Using human models of cystinosis in the form of patient-specific pluripotent stem cells (iPSC) and kidney organoids, the combo treatment with cysteamine and everolimus rescues the defects in autophagy-lysosome degradation systems induced by cystinosin deficiency (Hollywood et al. 2020). Although the mechanism of this rescue remains incompletely understood, a potential mediator is the transcription factor EB (TFEB) – a master controller of endolysosomal biogenesis and autophagy (Sardiello et al. 2009; Settembre et al. 2011). Recent work showing that cystinosin is a member of the endolysosomal machinery that controls mTOR signaling (Andrzejewska et al. 2016; Ivanova et al. 2016), whose activity negatively regulates the translocation of both TFEB and the related transcription factor E3 (TFE3), and hence suppressing genes for endolysosomal biogenesis and autophagy (Liu and Sabatini 2020; Ballabio and Bonifacino 2020). Indeed, the induction of TFEB expression, nuclear translocation, and activation of TFEB-dependent signaling landscape by genistein promoted the clearance of endolysosomal storage of cystine, stimulated the endocytic cargo processing, and reduced both the number and size of endolysosomes in cystinosis cells (Rega et al. 2016). This supports a number of implications, including that pharmacological targeting of the endolysosomal homeostasis and signaling might potentially reverse proximal tubule dysfunction and renal Fanconi syndrome. Indeed, genistein has been reported to reduce the lysosomal storage of glycosaminoglycans (Piotrowska et al. 2006) that are caused by the absence or malfunctioning of lysosomal enzymes involved in the breakdown of these large sugar molecules, ultimately resulting in a cognition improvement (Piotrowska et al. 2008). These observations indicate that genistein-directed effects on the endolysosomal system might benefit not only cystinosis but also other endolysosomal storage diseases.

Throughout the past half-century, the paradigm shift in drug discovery strategies, together with improved knowledge of cell biology-disease signatures, novel preclinical disease models, and drug-based phenotypic screening, has prompted the development of targeted therapies that might deliver "first in class" drugs into the clinics (Tambuyzer et al. 2020). In particular, drug repurposing and the advent of chemogenomic library screening are increasingly becoming an attractive proposition because it involves the use of de-risked compounds, with potentially lower overall development costs and shorter development timelines (Moffat et al. 2017). With this lag in mind, De Leo and colleagues have recently identified small-molecule drug candidates (De Leo et al. 2020) that decrease the accumulation of the autophagy substrate SQSTM1 and restore the autophagy-lysosomal degradative pathway, which is compromised in cystinosis cells (De Leo et al. 2020). Among several positive hits, luteolin – a natural flavonoid that is present in various fruits and

vegetables – has emerged as the most interesting candidate. This compound has a good safety profile, owing to its similarity to genistein, and improves the endolysosome-mediated degradation of the autophagy cargoes, including damaged and dysfunctional (ROS-producing) mitochondria (De Leo et al. 2020). In addition, treating kidney cells from patients, or mouse and zebrafish models of cystinosis, with luteolin not only repaired endolysosome system and mitochondrial redox homeostasis and oxidative stress but also restored megalin expression to the plasma membrane and stimulated protein absorption (De Leo et al. 2020). These findings extend previous observations demonstrating that structural and functional deformities of the proximal tubule could be delayed in *Ctns*-knockout mice by administrating mitochondria-targeted ROS scavengers such as mitoquinone (Galarreta et al. 2015) or mito-TEMPO (Festa et al. 2018).

Repurposing existing drugs offers a potentially rapid mechanism to deployment in human clinical trials, with an example being alpelisib – a small-molecule drug that targets phosphoinositide 3-kinase (PI3K) subunit p110α and has been approved for clinical use in cancer therapy (Andre et al. 2019). Moreover, alpelisib has shown clinical benefit in children with PROS/CLOVES syndrome (Venot et al. 2018), a rare overgrowth syndrome resulting from somatic, mosaic gain-of-function mutations of the *PIK3CA* gene. Taking advantage of this drug repositioning strategy, Berquez and colleagues have recently anticipated additional evidence that supports the use of the PI3K inhibitor for therapeutically treating low-molecular weight proteinuria associated with Lowe syndrome and Dent disease type 2 (Berquez et al. 2020). Alpelisib reduces the phosphoinositide-directed actin polymerization, which blocks membrane trafficking through the endocytic and endolysosomal pathway, and improves the recycling of the multiligand receptor megalin to the apical membrane, restoring endocytosis-mediated uptake of LMWPs through the endolysosomal pathway in proximal tubule cells from humanized $Ocrl^{Y/-}$ mice (Berquez et al. 2020). As alpelisib does not completely block PI3K activity, and hence maintaining functions of the signaling pathway, it may be particularly suitable for long-term use and the hyperglycemia arising from alpelisib treatment may be manageable by dietary changes (Venot et al. 2018). These druggable interventions might therefore slow down the onset and progression of CKD, ultimately ameliorating the quality of the life for Lowe syndrome/Dent disease type 2 patients and for their families.

Beyond promising candidate targets for drug development, cell-based therapy including the direct replacement of defective CTNS or ClC-5 through the administration of functional CTNS or ClC-5-producing progenitor cells has been tested in animal models of cystinosis and Dent disease type 1 (Yeagy et al. 2011; Gabriel et al. 2017). Hematopoietic stem cells carrying functional *Ctns* or *Clcn5* were transplanted into irradiated *Ctns* or *Clcn5* knockout mice, resulting in a long-term improvement of kidney structure and function, including the renal Fanconi syndrome (Yeagy et al. 2011; Gabriel et al. 2017). However, the intriguing conceptual advance provided by these studies concerns the elucidation of cellular pathways and molecular mechanisms through which bone marrow transplantation rescues the resorptive properties of the proximal tubule in renal Fanconi syndrome. The existence of cell protrusions

consistent with tunneling nanotubes (Rustom et al. 2004) between engrafted wild-type and mutant proximal tubule cells might stimulate the direct exchange of cellular material including the transfer of the functional ClC-5 protein. As with other cellular paradigms of TNT assembly and formation, further studies will be required for deciphering the signals that trigger the formation of cellular protrusions and the nature of biomolecules transferred from wild-type macrophages to mutant proximal tubule cells. Taking together this evidence, the race is now on to determine whether these cell-based strategies will benefit individuals affected by these devastating diseases.

6 Conclusions

The maintenance of a healthy endolysosomal system is crucial for preserving the homeostasis and physiology of specialized epithelial cells that line the proximal tubule of the kidney, and loss-of-function mutations that impair the function of the endolysosomal network can invariably cause proximal tubule dysfunction and kidney disease. Indeed, the conjugation of impaired endolysosomal dynamics and altered endolysosome-based degradative capacity in cystinosis cells is strikingly similar to the cellular changes encountered in Lowe syndrome patients, disabling the autophagy flux and causing ultimately autophagosome accumulation. Furthermore, anomalies in the chloride transport that result from the loss-of-function of the activity of the ClC-5 channel/transporter trigger endolysosomal dysfunctions, mitochondrial oxidative stress, and epithelial phenotype switch, thereby promoting proliferation and apical dedifferentiation, and hence proximal tubule dysfunction and kidney failure in patients with Dent disease. Taken together, these observations undoubtedly indicate that the disruption of the endolysosomal system homeostasis might have similar functional consequences on the epithelial phenotype and identity, emphasizing its crucial role as a signaling and sensing hub that safeguards cellular and organismal homeostasis.

The convergence towards similar clinical phenotypes among patients with mutations in *OCRL* and in those with mutations in genes encoding transporters functioning in endosomes (i.e., *CLCN5*) and lysosomes (i.e., *CTNS*) raises the question as to whether these gene products coordinate the same cellular and/or molecular signatures. For instance, it is incompletely understood whether and how the OCRL deficiency might lead to mistrafficking and/or functional impairment of ClC-5 or CTNS. An intriguing scenario might be that local increase in PtdIns(4,5)P_2 could skew the channel activity of ClC-5, as demonstrated for other channels (for instance, TRPML1) that are regulated by phosphoinositides. Beyond serving as dynamic regulators of channel/transporter activities, the spatiotemporally coordinated production of phosphoinositides and turnover might also regulate the dynamics and plasticity of endolysosomal membranes; hence, ensuring the reformation of endolysosomal system and its homeostasis. Intriguingly, it is tempting to speculate that inherited defects in renal Fanconi genes (i.e., *CLCN5*, *CTNS*, and *OCRL*) might

disable the endolysosomal phosphoinositide switches, thereby skewing the biogenesis of the endolysosomal system, and hence its homeostasis and function. These questions are just examples of all the exciting work that lies ahead to comprehensively dissect the fundamental roles of the endolysosome network within specialized epithelial cells. A current challenge is to translate knowledge gained from fundamental studies of the endolysosomal system to understand the pathogenesis in more common diseases associated with metabolic disorders, cancer, and pathologies related to aging. Further insights into this crucial pathway might ultimately lead to the development of novel therapeutic avenues for the treatment of currently intractable diseases and transform our ability to regulate kidney health and homeostasis.

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Endo-Lysosomal Cation Channels and Infectious Diseases

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Contents

Abstract Among the infectious diseases caused by pathogenic microorganisms such as bacteria, viruses, parasites, or fungi, the most prevalent ones today are malaria, tuberculosis, influenza, HIV/AIDS, Ebola, dengue fever, and methicillin-resistant *Staphylococcus aureus* (MRSA) infection, and most recently Covid-19 (SARS-CoV2). Others with a rather devastating history and high fatality rates such as plague, cholera, or typhus seem less threatening today but have not been eradicated, and with a declining efficacy of current antibiotics they ought to be watched carefully. Another emerging issue in this context is health-care associated infection. About 100,000 hospitalized patients in the USA (www.cdc.gov) and 33,000 in Europe (https://www.ecdc.europa.eu) die each year as a direct consequence of an infection caused by bacteria resistant to antibiotics. Among viral infections, influenza is responsible for about 3–5 million cases of severe illness, and about 250,000

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to 500,000 deaths annually (www.who.int). About 37 million people are currently living with HIV infection and about one million die from it each year. Coronaviruses such as MERS-CoV, SARS-CoV, but in particular the recent outbreak of Covid-19 (caused by SARS-CoV2) have resulted in large numbers of infections worldwide with an estimated several hundred thousand deaths (anticipated fatality rate: <5%). With a comparatively low mortality rate dengue virus causes between 50 and 100 million infections every year, leading to 50,000 deaths. In contrast, Ebola virus is the causative agent for one of the deadliest viral diseases. The Ebola outbreak in West Africa in 2014 is considered the largest outbreak in history with more than 11,000 deaths. Many of the deadliest pathogens such as Ebola virus, influenza virus, *mycobacterium tuberculosis*, dengue virus, and cholera exploit the endo-lysosomal trafficking system of host cells for penetration into the cytosol and replication. Defects in endo-lysosomal maturation, trafficking, fusion, or pH homeostasis can efficiently reduce the cytotoxicity caused by these pathogens. Most of these functions critically depend on endo-lysosomal membrane proteins such as transporters and ion channels. In particular, cation channels such as the mucolipins (TRPMLs) or the two-pore channels (TPCs) are involved in all of these aspects of endo-lysosomal integrity. In this review we will discuss the correlations between pathogen toxicity and endo-lysosomal cation channel function, and their potential as drug targets for infectious disease therapy.

Keywords Endosome · Lysosome · TPC1 · TPC2 · TRPML1 · TRPML2 · TRPML3

1 Introduction

The 1918 influenza pandemic was the most severe pandemic in recent history. It was caused by the 1918 "Spanish" influenza virus, an influenza H1N1 virus containing genes derived from avian-like influenza virus strains. It is estimated that about 500 million people or one-third of the world's population at the time became infected with this virus. The number of deaths was estimated to be at least 50 million worldwide with about 675,000 occurring in the USA. In 2005, Tumpey and colleagues reconstructed the 1918 "Spanish" influenza virus and found that it was more lethal to mice than any other influenza virus strains tested before (Tumpey et al. 2005). Tumpey et al. determined that the viral proteins hemagglutinin (HA) and the polymerase complex PB1 played particularly important roles in its infectivity and severity. Nevertheless, it was not any single gene of the 1918 "Spanish" influenza virus but instead the unique combination of all of its genes together that made it so particularly dangerous. The "Spanish" influenza pandemic of 1918 was followed by three additional pandemics, in 1957, 1968, and most recently in 2009. These subsequent pandemics were less severe and caused considerably lower mortality rates than the 1918 pandemic. The 1957 H2N2 pandemic and the 1968 H3N2

pandemic each resulted in an estimated one million global deaths, while the 2009 H1N1 pandemic resulted in fewer than 0.3 million deaths. Nevertheless, the question remains whether a high severity pandemic on the scale of 1918 could occur again. Certainly, in 1918 no flu vaccine or antiviral drugs were available, neither were antibiotics such as penicillin for the treatment of secondary bacterial infections. However, current vaccines have variable effectiveness and they might not be readily available in the amounts needed during a pandemic outbreak. The emergence of Oseltamivir-resistant influenza viruses was also reported in treatment-naïve and treatment failure patients. Therefore, novel and effective small molecule drugs that can be produced rapidly and in large quantities are needed.

A viral disease with broad media coverage albeit with much lower total death rates compared to influenza is Ebola which is a viral hemorrhagic fever caused by the Ebola virus (EBOV). Ebola first appeared in 1976 in two simultaneous outbreaks, one in South Sudan, the other in the Democratic Republic of the Congo (DRC). The latter occurred in a village near the Ebola River, from which the disease takes its name. Although case numbers were very low, this outbreak was devastating with 318 cases, 218 deaths, and a fatality rate of 88%, one of the deadliest outbreaks in history. The outbreak in Sudan in 1976 resulted in 284 cases with 151 deaths and a fatality rate of 53%. Since 1976, 26 outbreaks of Ebola virus have occurred in ten African countries, including the Democratic Republic of the Congo, Sudan, Gabon, Cote d'Ivoire, South Africa, Uganda, Congo, Guinea, Sierra Leone, and Liberia. During the 2014 outbreak there were also cases in the USA and in Europe. While the 2014 outbreak resulted in approximately 11,310 deaths with a fatality rate of about 40%, the 2018 outbreak, the second largest Ebola epidemic so far, taking place again in the Democratic Republic of the Congo has sickened 3,714 people (including confirmed cases in neighboring Uganda) and killed 2,200 (fatality rate: 60%) despite medical specialist teams and effective vaccine being available. In August of 2019 two novel drugs against Ebola were tested in the DRC. One of the drugs, REGN-EB3, is a cocktail of three monoclonal antibodies against Ebola; the second, mAB114, is derived from a single antibody recovered from the blood of a person who survived Ebola in the DRC in 1995 (Mulangu et al. 2019). Both drugs showed high survival rates in the first trials. Nevertheless, Ebola remains one of the most fatal viruses known.

In contrast to influenza or Ebola, tuberculosis (TB) is receiving little media attention albeit being also one of the deadliest infectious diseases today. TB causes severe clinical outcomes especially in patients with immunological deficiencies and thus is known as a leading killer of HIV patients before the combined antiretroviral therapy (cART) era. TB disease is currently treated by taking several antibiotics for 6 to 9 months. The most common medications used include isoniazid, rifampicin, ethambutol, pyrazinamide. For treatment of drug-susceptible TB disease, two antibiotics (isoniazid and rifampicin) are taken for 6 months, with two additional antibiotics (pyrazinamide and ethambutol) taken for the first 2 months of the 6-month treatment period. For drug-resistant TB disease a combination of fluoroquinolones and injectable medications, such as amikacin or capreomycin are generally used for 20 to 30 months. Some types of TB are however developing

resistance to all of these medications. Close monitoring of treatment responses in patients harboring resistant TB is therefore critical.

From these few examples it becomes evident how urgently on the one hand novel medications, antibiotics, and antiviral drugs are needed and how important it is on the other hand to understand in more detail the mechanisms by which bacteria and viruses infect their host cells and successfully survive the cellular defense systems.

2 Examples of Pathogens Hijacking the Endo-Lysosomal System

A unifying principle of many pathogens is their interaction with the endo-lysosomal trafficking machinery of the host cell (Figs. 1 and 2). Endo-lysosomes comprise the following organelles: early endosomes (EE), late endosomes (LE), lysosomes (LY), recycling endosomes (RE) as well as autophagosomes and lysosome-related organelles (LRO). Endocytosed material may traffic either through the EE pathway (EE and RE) back to the plasma membrane or the LE/LY pathway where it is degraded, released into the cytosol, or exocytosed. The endo-lysosomal system thus controls not only endocytosis and intracellular cargo trafficking but also exocytosis and cargo release. In the following a few examples of pathogens shall be highlighted that efficiently hijack this system for their own purposes.

2.1 Viruses

During the influenza A virus (IAV) replication process, the hemagglutinin (HA) receptor-binding site attaches the virus to the cell. After binding to the host cell, HA triggers endocytosis of the virus particle which is further transported inside endosomal vesicles (Roy et al. 2000; Sieczkarski and Whittaker 2002; Rust et al. 2004; Chen and Zhuang 2008; de Vries et al. 2011). The environment inside late endosomes has a low acidic pH, which activates the influenza virus M2 ion channel (Lakadamyali et al. 2003; Pinto and Lamb 2006) and induces a conformational change of HA to expose the fusion peptide (Bullough et al. 1994). The activated M2 ion channel acidifies the inside of the viral particle, thus facilitating the release of packaged vRNPs (viral ribonucleoproteins) into the cytoplasm (Bui et al. 1996; Tumpey et al. 2007; Bottcher-Friebertshauser et al. 2014). After successful replication using the translation machinery of the host cell newly formed vRNPs are trafficked towards the plasma membrane for viral assembly which is facilitated by the recycling endosome protein Rab11 with different theories about the exact mechanism of this process (Amorim et al. 2011; Eisfeld et al. 2011; Momose et al. 2011; de Castro Martin et al. 2017).

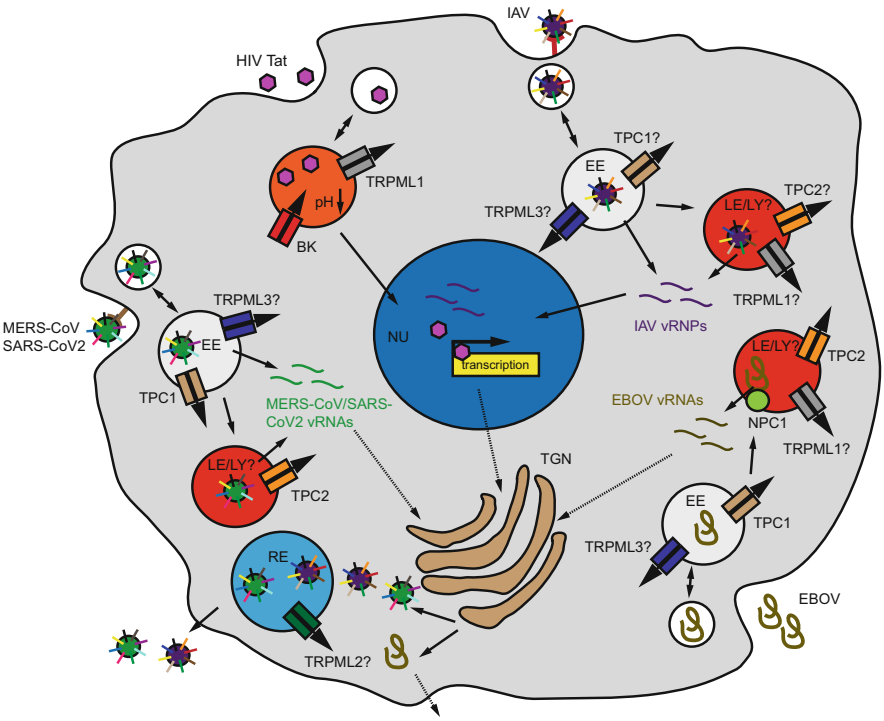

Fig. 1 Schematic showing the intracellular trafficking pathways of viruses, and the involvement and function of endo-lysosomal ion channels in the life cycle of different viruses. In particular, the onward trafficking and release of the respective viruses depends on TRPML/TPC function, i.e., fusion/fission and pH regulation of endo-lysosomal organelles as well as endo-lysosomal exocytosis. *LY* lysosome, *EE* early endosome, *RE* recycling endosome, *NU* nucleus, *TGN* trans-Golgi network, *IAV* influenza A virus, *EBOV* Ebola virus, *MERS-CoV* Middle East respiratory syndrome coronavirus, *SARS-CoV2* severe acute respiratory syndrome coronavirus 2, *vRNP* viral ribonucleoprotein, *BK* Ca^{2+}-activated potassium channel

The Middle East respiratory syndrome coronavirus (MERS-CoV), SARS-CoV, and SARS-CoV2 (Fig. 1) enter host cells by hijacking different human receptors. In all cases the viral spike or S protein plays the most important role in viral attachment, fusion, and entry. The S protein mediates viral entry into host cells by first binding to the host receptor through the receptor-binding domain (RBD) in the S1 subunit and then fusing the viral and host membranes through the S2 subunit. SARS-CoV and SARS-CoV2 recognize angiotensin-converting enzyme 2 (ACE2) as their receptor while MERS-CoV recognizes dipeptidyl peptidase 4 (DPP4) as its receptor (Tai et al., 2020). After successful endocytosis, coronaviruses are escaping the endo-lysosomal system via endosomes.

The Ebola virus (Fig. 1) is also trafficked through the endo-lysosomal system. Ebola virions are internalized into EE and subsequently trafficked to LE/LY in a Rab5 and Rab7 GTPase-dependent manner, where the cathepsins B and L process

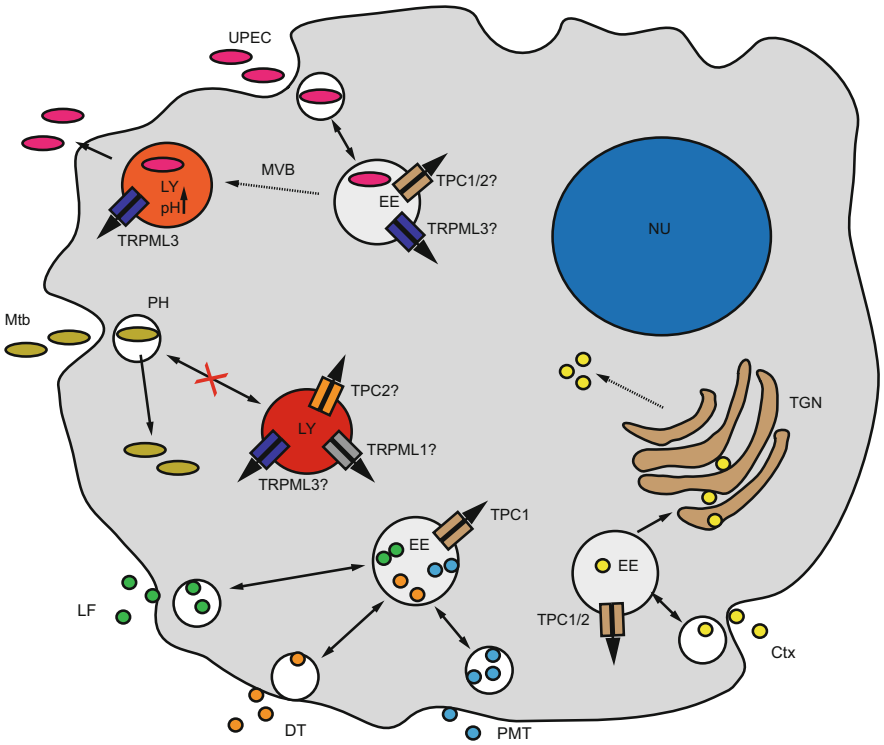

Fig. 2 Schematic showing the intracellular trafficking pathways of bacteria and bacterial toxins, and the involvement and function of endo-lysosomal ion channels in the life cycle of these pathogens. *LF* lethal factor (anthrax), *DT* diphtheria toxin, *PMT Pasteurella multocida* toxin, *Ctx* cholera toxin, *UPEC* uropathogenic *E. coli*, *Mtb Mycobacterium tuberculosis*, *PH* phagosome, *LY* lysosome, *MVB* multivesicular body, *EE* early endosome, *NU* nucleus, *TGN* trans-Golgi network

the virus glycoprotein 1 (GP1) into a fusogenic form. Subsequently, the interaction between the processed GP1 and the late endosomal/lysosomal protein Niemann-Pick C1 (NPC1) leads to GP2-dependent fusion of the viral membrane with the endosomal limiting membrane. The viral nucleocapsid is then released into the cytosol for subsequent transcription and replication of the viral genome (Salata et al. 2019). After replication, newly assembled EBOV are released from the host cell, either by lysis of host cells or by budding off from cell membranes. It seems that Niemann-Pick type C1 protein (NPC1) which when mutated or lost causes a lysosomal storage disease plays a role in viral entry. Thus, $Npc1^{-/-}$ mice are entirely free from viral replication and completely protected from EBOV infection (Carette et al. 2011; Herbert et al. 2015).

2.2 *Bacteria and Bacterial Toxins*

Bacteria and bacterial toxins also hijack the endo-lysosomal system in order to complete their replication in host cells. For example, cholera toxin (Ctx), produced by Vibrio cholerae, is composed of two distinct non-covalently linked peptides, the A and B subunits (Bharati and Ganguly, 2011; De Haan and Hirst, 2004). Both subunits are required for Ctx to function. Ctx can be endocytosed by clathrin-dependent and by clathrin-independent mechanisms. Ctx then traffics retrograde from endosomes to the trans-Golgi Network (TGN) and ultimately reaches the cytosol (Fig. 2) where it reacts with adenosine diphosphate ribosylation factor 6 (ARF6) which enhances the activity of the toxin. Activated toxin eventually leads to an elevation of cAMP which in turn activates the cAMP-dependent chloride channel CFTR (Cystic Fibrosis Transmembrane conductance Regulator). These processes lead to a massive secretion of water and electrolytes into the intestine lumen, eventually resulting in severe diarrhea.

Mycobacterium tuberculosis (Mtb; Fig. 2) can escape lysosomal degradation by phagosome modulation. It interferes with Rab-controlled membrane trafficking and arrests phagosome maturation (Vergne et al. 2004). Mtb^+ phagosomes recruit and maintain Rab5 and Rab22a to avoid Rab7 acquisition and thus inhibit phagolysosome biogenesis (Roberts et al. 2006). The V-ATPase is specifically excluded from the Mtb^+ phagosomes and lysosomal hydrolase levels were found to be reduced (Sturgill-Koszycki et al. 1994). Mtb finally disrupts the phagosomal membrane and escapes to the cytosol. Precisely how Mtb damages phagosomal membranes remains however largely unclear (Bussi and Gutierrez 2019). It further remains poorly understood how different cell type environments (e.g., in macrophages, neutrophils, dendritic cells, or non-phagocytotic cells) affect Mtb localization and survival.

Taken together, these examples illustrate how pathogens have developed sophisticated mechanisms to circumvent the immune responses of their host cells by hijacking the endo-lysosomal system. Their strategies are often highly complex and far from being completely understood, but in recent years it has become more and more evident that endo-lysosomal cation channels are highly relevant for pathogen uptake, trafficking, and ultimately their efficient replication.

3 Endo-Lysosomal Cation Channels in Pathogen Uptake, Trafficking, and Replication

The functional integrity of endosomes and lysosomes is critically dependent on both soluble and endo-lysosomal membrane proteins such as ion channels and transporters. Cation channels found in the endo-lysosomal system include members of the Transient Receptor Potential (TRP) channel superfamily, namely TRPML channels (mucolipins) as well as TPCs (two-pore channels), both of which are non-selective

Table 1 Role of endo-lysosomal ion channels in virus or viral protein trafficking

Family	Virus	Subcellular site involved	Trigger	Ion channels involved	References
Retroviridae	HIV	PM	Receptor	TRPML1/ BK	Khan et al. (2019)
Coronaviridae	MERS-CoV	PM or LE	Receptor and protease	TPC1/ TPC2	Gunaratne et al. (2018)
Arterviridae	EAV	EE	Low pH	TRPML2	Rinkenberger and Schoggins (2018)
Flaviviridae	YFV, ZIKV	Endosome	Low pH	TRPML2	Rinkenberger and Schoggins (2018)
Orthomyxoviridae	IAV	LE	Low pH	TRPML2	Rinkenberger and Schoggins (2018)
Filoviridae	EBOV	Endo-lysosome	Low pH	TPC1/ TPC2	Sakurai et al. (2015)

EE early endosome, *LE* late endosome, *PM* plasma membrane, *HIV* human immunodeficiency virus, *MERS* Middle East respiratory syndrome coronavirus, *EAV* equine arteritis virus, *YFV* yellow fever virus, *ZIKV* Zika virus, *IAV* influenza A virus, *EBOV* Ebola virus

cation channels permeable for sodium and calcium. TRPML channels and TPCs are particularly highly expressed in macrophages, but are also found in neutrophils, mast cells, dendritic cells, NK cells, T- and B-cells (Lindvall et al. 2005; Song et al. 2006; Cang et al. 2013; Kim et al. 2014; Dayam et al. 2015; Li et al. 2015; Sun et al. 2015; Cuajungco et al. 2016; Bretou et al. 2017; Dayam et al. 2017; Goodridge et al. 2019). TPCs as well as TRPMLs can interfere on several levels with pathogen uptake, trafficking, and replication (Tables 1 and 2). Previously established or postulated functional roles of these ion channels in the endo-lysosomal system include trafficking, fusion/fission, exocytosis, autophagy, and pH regulation (Dong et al. 2010; Ruas et al. 2010; Grimm et al. 2012; Kiselyov et al. 2012; Shen et al. 2012; Cang et al. 2013; Bae et al. 2014; Chen et al. 2014; Favia et al. 2014; Grimm et al. 2014; Lin-Moshier et al. 2014; Lin et al. 2015; Sakurai et al. 2015; Ambrosio et al. 2016; Bellono et al. 2016) and in several studies TRPMLs and TPCs have been found to play direct roles in virus and bacterial toxin trafficking or bacteria exocytosis. Other cation channels with claimed expression in endo-lysosomes are TRPM2 (Lange et al. 2009; Sumoza-Toledo et al. 2011), TRPA1 (Shang et al. 2016), P2X4 (Qureshi et al. 2007; Huang et al. 2014; Cao et al. 2015a, b; Murrell-Lagnado and Frick 2019), BK (Cao et al. 2015a, b), and TMEM175 (Cang et al. 2015; Lee et al. 2017). TRPM2 and TRPA1 have been mostly described as plasma membrane resident calcium-permeable cation channels. More recently, functional activity in lysosomes has also been postulated for these two channels (Lange et al. 2009; Sumoza-Toledo et al. 2011; Shang et al. 2016); however, direct endo-lysosomal patch-clamp evidence has not been provided so far. P2X4 and the potassium channels BK and TMEM175 have been confirmed to be active in endo-lysosomal membranes using the endo-lysosomal patch-clamp technique (Huang et al. 2014; Cang et al. 2015; Cao et al. 2015a, b; Lee

Table 2 Role of endo-lysosomal ion channels in bacterial pathogen and toxin trafficking

Bacterial pathogens and toxins	Organelle involved	Ion channels involved	Effect of ion channels on pathogen trafficking	References
Anthrax toxin lethal factor (LF)	EE and LE	TPC1	Deletion of TPC1 inhibits activation of LF	Castonguay et al. (2017)
Diphtheria toxin (DT)	EE and LE	TPC1	Deletion of TPC1 inhibits activation of DT	Castonguay et al. (2017)
Pasteurella multocida toxin (PMT)	EE and LE	TPC1	Deletion of TPC1 reduces Pasteurella multocida toxin uptake	Castonguay et al. (2017)
Cholera toxin (Ctx)	EE to Golgi to ER	TPC1/ TPC2	Both overexpression and knockout of TPC1/2 impair Ctx trafficking	Ruas et al. (2010, 2014)
H. pylori vacuolating cytotoxin A (VacA)	EE and LE	TRPML1	VacA inhibits TRPML1 to disrupt endo-lysosomal trafficking and to escape autophagic killing	Capurro et al. (2019)
Uropathogenic E. coli (UPEC)	$Rab27^{+}$ vesicles	TRPML3	TRPML3 senses UPEC-mediated lysosomal pH neutralization and induces lysosomal exocytosis to expel the bacteria	Miao et al. (2015)

EE early endosome, *LE* late endosome, *ER* endoplasmic reticulum

et al. 2017). While there is no infectious disease relevance reported for TMEM175, BK has been found to regulate extracellular Tat-mediated HIV-1 LTR transactivation in cooperation with TRPML1 (see Sect. 3.2). P2X4 is present on the plasma membrane and intracellular compartments such as lamellar bodies (LBs) and lysosomes. P2X4 is pH and ATP regulated and its activity in lamellar bodies where the pulmonary surfactant is stored has been reported to promote fusion pore opening and exocytotic content release from pneumocytes (Miklavc et al. 2011). In macrophages P2X4 may play a role in augmenting the killing of phagocytosed bacteria (Pettengill et al. 2012; Csoka et al. 2018). In studies using P2X4-deficient mice subjected to sepsis a role of P2X4 in killing bacteria has been confirmed but has been claimed to be independent of phagolysosome fusion or phagosome/lysosome acidification, and evoked by extracellular ATP. P2X4 in lysosomes becomes only active when the luminal pH increases to 7.4 (Huang et al. 2014), suggesting that P2X4 in contrast to, e.g., TRPML1 will only be active in lysosomes in exceptional circumstances.

In this review we will focus on current knowledge about the roles TPCs (TPC1 and TPC2) and TRPMLs (TRPML1, 2, and 3) play in infectious disease pathophysiology.

3.1 Two-Pore Channels: TPCs

While TPC1 is found to be highly abundant in early and recycling endosomes (Castonguay et al. 2017), TPC2 is predominantly expressed in late endosomes and lysosomes (Calcraft et al. 2009; Grimm et al. 2014; Arredouani et al. 2015). TPCs have been shown to play a role in trafficking and release of EBOV, MERS-CoV, and SARS-CoV2 from the endo-lysosomal system into the host cell cytoplasm (Sakurai et al. 2015; Gunaratne et al. 2018; Ou et al., 2020). Disruption of TPC function affects the escape of EBOV and coronaviruses from the endo-lysosomal network into the cytosol and thus affects disease progression. Sakurai et al. (2015) found that knockdown or knockout of either TPC1 or TPC2 prevents Ebola infection in vitro. Overexpression of the TPC2 "pore dead" mutant $TPC2^{L265P}$ also inhibited infection, so did voltage-gated Ca^{2+} channel blockers such as tetrandrine, verapamil, and the NAADP antagonist Ned-19. Tetrandrine showed the most efficient inhibitory effect among these antagonists against EBOV infection (Penny et al. 2019). Tetrandrine blocked both NAADP- and $PI(3,5)P_2$-stimulated currents through TPC1 and TPC2. Blocking TPCs thus provided an unexpected and novel strategy to combat Ebola infection.

Gunaratne et al. (2018) demonstrated that knockdown of TPCs impairs infectivity of MERS-CoV in MERS-CoV spike pseudovirus particle translocation assays. In contrast to TPC2 neither knockdown nor overexpression of TRPML1 showed an effect in blocking MERS-CoV infection. Interestingly, overexpression of both TPC1 and TPC2 also showed inhibitory effects on MERS-CoV infection, possibly due to dysregulation of endo-lysosomal trafficking pathways by the overexpressed protein. Besides, pharmacological inhibition of NAADP-evoked Ca^{2+} release by bisbenzylisoquinoline alkaloids was also found to block MERS pseudovirus translocation. Surprisingly however, the NAADP antagonist Ned-19, which blocks TPCs in EBOV infection, showed no effect in inhibiting MERS-CoV translocation. Overall, these data suggest a role for TPCs within acidic organelles to support MERS-CoV translocation (Gunaratne et al. 2018). Ou et al. (2020) recently claimed that tetrandrine decreases entry of SARS-CoV2 S pseudovirions. Ou et al. attributed this effect to TPC2 which is mainly expressed in LE/LY. While mentioning that they found no effect with a TRPML1 blocker, they did not mention TPC1. However, tetrandrine blocks both TPC1 and TPC2 (Sakurai et al., 2015). Furthermore, Ou et al. did not present any TPC knockout data to further corroborate their hypothesis. In future studies it would be necessary to include TPC1 and TPC2 knockout experiments and to confirm that tetrandrine blocks SARS-CoV2 entry in a TPC dependent manner.

TPC1 knockout and pharmacological blockage were also found to disrupt diphtheria toxin (DT) trafficking as well as the trafficking of other bacterial toxins such as *Pasteurella multocida* toxin (PMT) and Anthrax toxin, specifically one of its enzyme components called lethal factor (LF) (Castonguay et al. 2017). Castonguay et al. (2017) could however not demonstrate that the activity of cholera toxin (Ctx) depends on TPC1. This is contrary to results by Ruas et al. (2010, 2014) who

found an effect on Ctx trafficking in TPC1 and TPC2 knockout cells with the strongest effect in TPC1 knockout cells.

Taken together, the data on EBOV, coronaviruses, DT, Anthrax toxin, and other bacterial toxins suggest that TPC activity is critical for endo-lysosome bound pathogens and it is likely that other viral and bacterial pathogens such as yellow fever virus, influenza, or Mtb will also be impacted by modulation of TPC activity.

3.2 Mucolipins: TRPMLs

TRPML1 is a ubiquitously expressed lysosomal cation release channel. Its two relatives, TRPML2 and TRPML3 are less ubiquitously expressed and reside, in contrast to TRPML1 also in early and recycling endosomes (Venkatachalam et al. 2006; Karacsonyi et al. 2007; Kim et al. 2009; Martina et al. 2009; Grimm et al. 2012; Sun et al. 2015; Chen et al. 2017; Plesch et al. 2018).

TRPML1 has recently been found to play a role in Helicobacter pylori (HP) infection (Capurro et al. 2019). HP infection is a proven risk factor for gastric cancer. Its virulence factor vacuolating cytotoxin A (VacA) promotes more severe disease and gastric colonization. VacA interferes, by an unknown mechanism with lysosomal and autophagy pathways to generate a protected reservoir for HP that confers bacterial survival in vitro. Capurro and colleagues found that VacA targets TRPML1 to disrupt endo-lysosomal trafficking. A small molecule agonist (ML-SA1) of TRPML1 reversed the toxic effects of VacA on endo-lysosomal trafficking, leading to the clearance of intracellular bacteria. The authors further found that HP which lack VacA colonize enlarged dysfunctional lysosomes in the gastric epithelium of Trpml1$^{-/-}$ mice, where they are protected from eradication therapy (Capurro et al. 2019).

Besides bacterial infections, TRPML1 has also been shown to be involved in viral infections such as HIV, in particular HIV-1 which is an HIV subtype that is responsible for the majority of HIV infections worldwide (McCutchan 2006). Secreted from HIV-1 infected cells, the Tat protein can be taken up into host cells by receptor-mediated endocytosis and internalized into endo-lysosomes. To reach the nucleus where it facilitates HIV-1 viral replication, exogenous Tat must escape degradation by endo-lysosomes. HIV-1 Tat is essential for HIV-1 replication and plays an important role in latent HIV-1 infection, HIV-1 associated neurological complication, and other HIV-1 comorbidities. It is essential for viral transcription from the LTR (long terminal repeat) promoter. Khan and colleagues have recently reported on the involvement of both TRPML1 and the BK Ca^{2+}-activated potassium channel in regulating endo-lysosome pH as well as Tat-mediated HIV-1 LTR transactivation (Khan et al. 2019). The authors proposed that TRPML1 and BK activation lead to a further acidification of endo-lysosomes, resulting in an increased degradation of Tat protein and reduced LTR transactivation. TRPML1 and BK thus appear to provide therapeutic benefit against latent HIV-1 infection and HIV-1 comorbidities (Khan et al. 2019).

The TRPML1-related channel TRPML2 which is predominantly expressed in recycling endosomes (RE) has recently been shown to modulate viral entry. Thus, TRPML2 enhances infection of several RNA viruses, e.g., yellow fever virus, influenza A virus, and equine arteritis virus. And a rare TRPML2 variant in humans, K370Q, was found to show a loss-of-function (LOF) phenotype with respect to viral enhancement (Rinkenberger and Schoggins 2018).

Activation of TRPML3 was observed in lysosomes when the luminal pH is increased upon infection of bladder epithelial cells (BEC) by uropathogenic *E. coli* (UPEC) (Miao et al. 2015). Although UPEC are targeted by autophagy in infected BEC, they can avoid degradation by neutralizing the lysosomal pH. Based on their findings Miao et al. proposed the model that UPEC are transported sequentially through multiple intracellular compartments, but they are not expelled from BEC until they reach the lysosomes and only when the lysosomal pH is neutralized by UPEC. By pretreating BEC with BAPTA-AM, an intracellular calcium chelator, the authors showed that BAPTA-AM, but not BAPTA (which cannot penetrate BEC), significantly suppressed bacterial expulsion, indicating that an intracellular calcium store was important for initiating bacterial expulsion. The authors subsequently examined the involvement of the calcium releasing TRPML channels in this process. When testing the effect of the TRPML3 activator SN2 (Grimm et al. 2010) they found that it drastically increased bacterial expulsion while the TRPML channel blocker ML-SI1 (Samie et al. 2013) blocked the effect. Through TRPML KD experiments the authors eventually confirmed involvement of TRPML3. TRPML3 KD BEC failed to expel intra-lysosomal UPEC, resulting in marked increase of the intracellular bacterial load.

These examples demonstrate that TRPMLs like TPCs are critical for the toxicity and the survival of viral and bacterial pathogens that rely on a functional endo-lysosomal system within the host cell. However, it remains unclear whether simultaneous inactivation of different members of the two endo-lysosomal cation channel families would result in synergistic effects on pathogen trafficking, toxicity, and survival or whether their effects may be redundant. Certainly, it remains to be established how efficient and safe infectious disease treatment by interfering with the activity of TRPML channels or TPCs will ultimately be.

4 Summary

Undoubtedly, we need new antibiotics and antiviral drugs. Yet, it is remarkable that most pharmaceutical companies have recently pulled out of antibiotics research despite the fact that major health organizations continue to warn that bacterial resistance is one of the biggest global threats with hundreds of thousands dying every year from drug-resistant diseases with an estimated ten million deaths per year by 2050. For purely economic reasons – it is much harder to make profit with new antibiotics than with anticancer drugs or drugs for the treatment of chronic diseases – the pharmaceutical industry is largely ignoring the warnings of major health

organizations and clinicians. Increasing resistance to antibiotics being one urgent problem that needs to be addressed, the other thread comes from viral diseases for which we have currently no efficacious treatment or medications. Whether endo-lysosomal ion channels may be suitable targets for the treatment of infectious diseases remains to be established. Potential side effects must also be taken into consideration. Blocking, e.g., TRPML1 may be neurotoxic as non-functional TRPML1 or loss of TRPML1 causes the lysosomal storage disorder mucolipidosis type IV symptoms of which include psychomotor retardation and severe neurodegeneration. These effects might, however, only occur after long term or chronic treatment. Certainly, blocking TRPMLs and TPCs may not only affect virus and bacterial toxin trafficking through the endo-lysosomal system but will also affect any other cargo trafficking, transport, and degradation in endo-lysosomes as well as autophagy processes. Without animal trials it is hard to predict which effects may occur or may be critical during short term treatments as in the case of infectious diseases. Certainly, with pharmaceutical industry stepping back from its responsibility, academic research must fill this gap and ought to explore every promising novel strategy.

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