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Review

Urban planning of the endoplasmic reticulum (ER): How diverse mechanisms segregate the many functions of the ER

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ABSTRACT

The endoplasmic reticulum (ER) is the biggest organelle in most cell types, but its characterization as an organelle with a continuous membrane belies the fact that the ER is actually an assembly of several, distinct membrane domains that execute diverse functions. Almost 20 years ago, an essay by Sitia and Meldolesi first listed what was known at the time about domain formation within the ER. In the time that has passed since, additional ER domains have been discovered and characterized. These include the mitochondria-associated membrane (MAM), the ER quality control compartment (ERQC), where ER-associated degradation (ERAD) occurs, and the plasma membrane-associated membrane (PAM). Insight has been gained into the separation of nuclear envelope proteins from the remainder of the ER. Research has also shown that the biogenesis of peroxisomes and lipid droplets occurs on specialized membranes of the ER. Several studies have shown the existence of specific marker proteins found on all these domains and how they are targeted there. Moreover, a first set of cytosolic ER-associated sorting proteins, including phosphofurin acidic cluster sorting protein 2 (PACS-2) and Rab32 have been identified. Intra-ER targeting mechanisms appear to be superimposed onto ER retention mechanisms and rely on transmembrane and cytosolic sequences. The crucial roles of ER domain formation for cell physiology are highlighted with the specific targeting of the tumor metastasis regulator gp78 to ERAD-mediating membranes or of the promyelocytic leukemia protein to the MAM.

1. Introduction

The endoplasmic reticulum (ER) is a multi-functional organelle that distinguishes itself from other organelles by its size and the plethora of functions associated with it. The size of this organelle and the potential array of targeting mechanisms required to build this little intracellular city were first recognized almost 20 years ago [1]. Initial studies on the ER were done at the beginning of the 20th century by Santiago Ramon y Cajal in neurons [2]. Following its proper discovery in 1945 by Keith Porter and co-workers on electron micrographs [3] and its designation in 1952 [4], microscopic and biochemical observations led to the identification of two distinct domains of the ER: the rough ER, characterized by the presence of ribosomes, and the smooth ER that is devoid of ribosomes [5,6]. Both are found in biochemical preparations first generated in the early 1940s and termed microsomes by Albert Claude [7]. Quite early, it became also clear that the nuclear envelope is another domain of the ER [8]. Recent progress has highlighted how the same set of proteins that shape rough and smooth ER tubules also determine the formation of the nuclear pore complex, a decisive event in the biogenesis of the nucleus [9].

The presence of ribosomes and mRNA on rough ER preparations suggested that this domain of the ER mediates the synthesis of secretory and membrane proteins [5]. Consistent with this hypothesis, the machineries responsible for the translocation of proteins into the ER [10–13] and their glycosylation were found on the rough ER [14]. Sorting to this domain of the ER is thought to involve preferential targeting of rough ER proteins to sheet-like, low curvature domains of the ER that are decorated with ribosomes

Abbreviations: ACS4/FACL4, acyl-CoA synthase 4; ACAT/SOAT, acyl-CoA cholesteryl acyl transferase; AMFR, autocrine motility factor receptor; BAP31, B-cell receptor-associated protein of 31 kDa; BiP, immunoglobulin binding protein; CHOP, CCAAT/enhancer binding protein (C/EBP) homologous protein; Climp63, cytoskeleton-linking membrane protein of 63 kDa; COP, coat protein complex; DGAT acyl-CoA, diacylglycerol acyltransferase; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; ERES, endoplasmic reticulum exit sites; ERK, extracellular signal-regulated kinase; ERMES, endoplasmic reticulum-mitochondria encounter structure; ERQC, endoplasmic reticulum quality control compartment; GFP, green fluorescent protein; GRP, glucose regulated protein; IP3R, inositol 1,4,5-trisphosphate receptor; KDEL, lys-asp-glu-leu; LRP6, low-density lipoprotein receptor-related protein 6; MAM, mitochondria-associated membrane; NADPH, reduced nicotinamide adenine dinucleotide phosphate; OST, oligosaccharyl transferases; PACS-2, phospho-furin acidic cluster sorting protein 2; PAM, plasma membrane-associated membrane; PDI, protein disulfide isomerase; PERK, protein kinase (PKR)-like endoplasmic reticulum kinase; RFP, red fluorescent protein; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SNARE, soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor; SREBP, sterol-regulatory element binding protein 2; SRP, signal recognition particle; STIM1, stromal interaction molecule 1; TIP47, tail-interacting protein of 47 kDa (TIP47); TRAM, translocating chain-associated membrane protein; TRAP, translocon-associated protein; VAPB, vesicle-associated membrane, protein-associated protein B; XBP1, X-box binding protein 1

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[15]. However, the functions of the rough ER may not be restricted to secretory protein production, since recent studies have shown that autophagosome formation involves membranes of the rough ER [16,17], suggesting that this domain of the ER may exhibit heterogeneity under certain circumstances.

Contrary to these numerous breakthroughs in the understanding of the functions of the rough ER, progress on the smooth ER has been slower. Initially, this portion of the ER was identified by the absence of ribosomes on its surface [6]. The past two decades have seen the identification and characterization of subdomains of the smooth ER, including ER exit sites (ERES), the cortical ER (also known as peripheral ER or plasma membrane-associated ER, PAM), the mitochondria-associated membrane (MAM), and the ER quality control compartment (ERQC). Our image of the smooth ER as the lesser cousin of the rough ER is therefore changing quickly and the smooth ER emerges as a heterogeneous mix of highly specialized subdomains. Moreover, the existence of these membrane domains within the smooth ER supports the hypothesis that smooth ER proteins are actively sorted away from rough ER proteins [15], but the deciphering of such putative mechanisms is not yet very advanced.

Biochemical characterizations of the smooth ER determined that this domain is rich in enzymes involved in drug detoxification such as NADPH cytochrome c reductase [18] and epoxide hydrolase [19]. In the presence of high amounts of toxins such as ethanol, the smooth ER increases in size [20,21], concomitant with an increased production of smooth ER-associated detoxifying enzymes such as cytochrome P450 2E1 [22]. Similar expansion of the rough ER is observed when protein secretion increases, for example, during the differentiation of B cells into plasma cells, coinciding with an increase of proteins involved in the production of secretory proteins [23]. Together, these findings demonstrate not only the existence of rough and smooth ER-specific transcriptional responses, but also the ability of the cell to specifically expand either ER membrane system, including mechanisms that keep rough and smooth ER separate in a state of ER expansion. Interestingly, the site of lipid synthesis during ER expansion is the smooth ER [24], suggesting that ER expansion may first result in naked, smooth ER that can later be decorated with ribosomes [25].

Research during the past decade has shown that both rough and smooth ER enzymes rely on the precision of intra-ER targeting mechanisms. For example, triacylglycerol hydrolase becomes inefficient in the mobilization of lipids when its ER localization motif is mutated from a histidine-isoleucine-aspartic acid-leucine (HIEL) to a classical lysine-glutamic acid-aspartic acid-leucine (KDEL) sequence. Although both motifs allow the interaction with the KDEL receptor, only the HIEL motif leads to proper targeting of triacylglycerol hydrolase to the peripheral smooth ER [26]. Intra-ER targeting is also relevant for the study of the ER using fluorescent proteins in vivo. Studies had initially indicated that ER-targeted green fluorescent protein (GFP) is freely diffusible within the ER lumen, albeit at a slower speed than in aqueous solutions and three- to sixfold slower than in the cytoplasm [27]. However, red and green fluorescent proteins (RFP/GFP) when fused to the KDEL motif do not co-localize within the ER of neuronal cells [28]. This finding corroborates that intra-ER sorting is distinct from ER retention. Similarly, GFP fusions with presumed ER proteins have led to spurious results, such as in the case of the oxidoreductase ERp44, which targets to the rough ER when fused to GFP, although its endogenous form is found close to ER exit sites and the Golgi [29,30]. Another example is GFP-tagged Sec61β that loses its specific targeting to the rough ER seen for the endogenous protein [31]. This review aims to outline what is known about the composition of individual domains of the ER and the intracellular sorting mechanisms that give rise to the unique architecture of the ER. We will usually refer to what is known about ER domain enrichment in mammalian cells, unless noted otherwise. Individual domains discussed in the text and known bona fide markers are summarized in Fig. 1.

2. Targeting to rough ER domains

The distinctive feature of the rough ER is the presence of ribosomes and protein translocation channels, also called translocons. Together, these proteins mediate the production of secretory proteins and proteins to be inserted into the membranes of the secretory pathway. Translocons are made up of more than 20 polypeptides that span the membrane individually multiple times [32]; associated with them are the translocon-associated protein (TRAP) complex, translocating chain-associated membrane protein (TRAM), oligosaccharyltransferases (OST), signal peptidase, the signal recognition particle (SRP) receptor and accessory proteins [33,34]. Within the translocons, the Sec61 α, β, and γ proteins are thought to capture and bind ribosomes [32,35,36] that are also associated with translation initiation factors and mRNAs [37,38]. Formation of these interactions is thus a critical determinant of rough ER formation. Moreover, the interaction between the Sec61 proteins and ribosomes depends on the formation of a complex between the ER-associated signal receptor and the SRP that recognizes mRNAs giving rise to signal peptides [39,40]. However, secretory protein mRNA localization to the ER does not require the presence of SRPs, suggesting these mRNAs are additionally equipped with so far unknown targeting information [41,42]. A special case of ER targeting of mRNAs is observed with the mRNA for the yeast transcription factor Hac1, the substrate of the ER-stress activated endonuclease Ire1p. Here, the 3′ untranslated region element is sufficient for the targeting to localized accumulation of Ire1 activity [43].

Overall, association with the translocon depends frequently on direct protein–protein interactions with translocon and ribosome components. This mechanism is utilized in particular by the OST complex [44], composed of ribophorin I and II, OST48, STT3A, STT3B, and DAD1 in mammals [45,46], which can bind to the translocon in a ribosome-dependent or -independent manner [46,47]. These divergent OST targeting mechanisms stem from the sequence of events in the glycosylation process, which comprises ribosome-requiring cotranslational N-glycosylation, but also posttranslational N-glycosylation of newly synthesized proteins [48]. For some subunits of the OST complex, specific targeting information has been described: ribophorin I requires luminal domains for its retention within the ER, whereas cytosolic and transmembrane sequences are needed in the case of ribophorin II [49,50]. The rough ER-localized Hsp40 family protein ERj1 interacts with ribosomes that mediate its targeting to the rough ER via a positively charged motif in its cytosolic domain [51]. Additional translocon-associated proteins are the Sec62/Sec63 complex proteins [52]. The phosphorylation of Sec63p by protein kinase CK2 is required for the association of this complex with the protein translocation apparatus in yeast [53].

In addition to the directly involved translocation machinery, the rough ER also contains ER chaperones and oxidoreductases, which mediate the folding of newly synthesized polypeptides. Not all proteins of this group target tightly to the rough ER; some perform important and in some instances predominant roles on other parts of the ER [54]. Another caveat is that some chaperones and oxidoreductases associate with translocons that are not bound to ribosomes, suggesting they are preferentially associated with retro-translocation of misfolded proteins on domains of the smooth ER (see below). GRP78/BiP is among the chaperones that interact with active, importing translocons and serves to close the translocon during protein integration into the ER membrane [55–57]. In yeast, it has been shown that this interaction boosts the ATPase activity of GRP78/BiP and depends on the binding to Sec63p, a member of the DnaJ family [58]. ER oxidoreductases are also found on ribosome-associated translocons, among them in particular ERp57 [59]. Another example is the lectin chaperone calnexin, a transmembrane protein, which associates with incompletely translocated polypeptides at the translocon [60,61]. Like Sec63, calnexin is a substrate of protein kinase

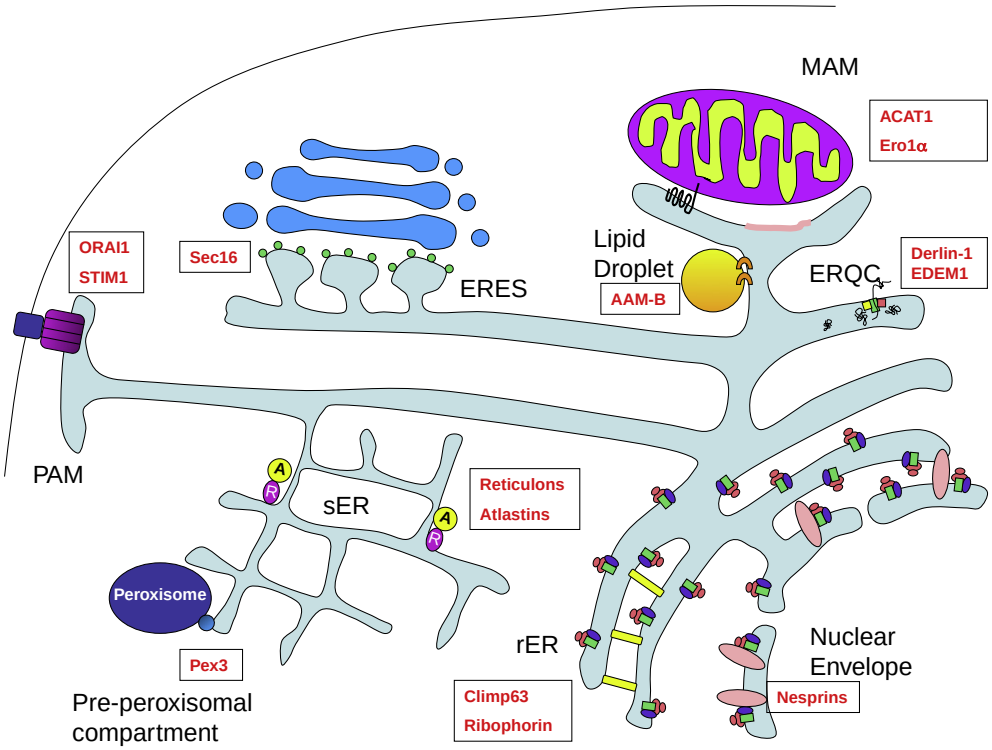

Fig. 1. ER subdomains, and selected markers. Rough Endoplasmic Reticulum (rER) sheets are characterized by ribosomes (pink ovals) which associate with translocon components (green rectangles) and ribophorins (purple ovals). Climp63 (yellow rectangle) is an important structural protein of the rER that also associates with microtubules. The ERQC (ER quality control compartment) is derived from the rER and also features translocon components (green rectangle) and associated proteins such as Derlin-1 (yellow square) and EDEM-1 (pink square) that facilitate the ubiquitination and retrotranslocation of misfolded proteins from the ER. Smooth Endoplasmic Reticulum (sER) tubule formation is in part mediated by the reticulons (purple circles) and atlastins (yellow circles). At the PAM (Plasma membrane-associated Membrane), STIM1 (purple rectangle) oligomerizes to form a pore which associates with the plasma membrane calcium channel ORAI1 (dark blue rectangle), mediating store operated calcium entry. The MAM (Mitochondria Associated Membrane) is a section of smooth ER that makes close contacts with mitochondria. ACAT1 (black squiggle) localizes to MAMs via a mitochondrial targeting sequence in its cytosolic tail, whereas other MAM proteins target to cholesterol-rich lipid domains within the MAM (pink membrane section). ER exit sites (ERES) at the transitional ER mark the point where COPII coated vesicles bud off from the ER en route to the ERGIC and Golgi compartments; ERES formation depends on Sec16 (small green circles), which associates with the ER membrane on the cytosolic face. Proteins destined for the peroxisomes sort into the pre-peroxisomal compartment, where they bud off into pre-peroxisomal vesicles in a Pex3 (small blue circle) -dependent manner. Lipid droplets are characterized by the presence of selected ER proteins including AAM-B. The nuclear envelope is equipped with ribosomes on the cytoplasmic face, where nesprins (pink ovals) are also found.

CK2, but also of extracellular signal-regulated kinase 1 (ERK1); when phosphorylated, calnexin interacts more strongly with ribosomes, suggesting that phosphorylated calnexin targets to the rough ER [62]. However, calnexin is one of many ER chaperones, which perform additional functions within the ER and which therefore is not restricted to the rough ER [63]. Consistent with this, a considerable portion of calnexin localizes to tubular, smooth ER [64], and in particular the MAM, to which it targets with the assistance of phosphofurin acidic cluster sorting protein 2 (PACS-2) [63].

A major determinant of the ribosome and translocon aggregation on the membranes of the rough ER appears to be the extent of ER membrane curvature. The rough ER is characterized by low curvature, resulting in sheet-like appearance [65,66]. Contrary to mechanisms that determine the formation of peripheral tubular ER, the formation of ER sheets is not well understood [67]. A mammalian coiled-coil protein, the cytoskeleton-linking membrane protein of 63 kDa (Climp63), actively promotes the formation of these ER sheets, to which ribosomes can attach [64], but also limits the diffusion of translocons within ER membranes [68]. The ER-localized, ribosome-interacting protein p180 plays a central role in the attachment of ribosomes to ER sheets in mammalian cells [69,70]. Expression levels of this protein determine the speed of ER-associated protein synthesis and the extent of ribosome attachment [70]. Interestingly, both Climp63 and p180 also tether the ER to microtubules [68,69], which could have implications into the intracellular distribution of the rough ER domains. This is evident in particular in yeast that lacks both Climp63 and p180 and whose ER does not fill the entire cell volume [64]. In addition, the abundance of tubule- and curvature-promoting smooth ER proteins also governs the abundance of rough ER sheets. Since mammalian cells are able to adapt their ER structure and function to particular demands, as seen for example during B cell development, ER domains are likely dynamically defined by their redox homeostasis and the extent of oxidative protein folding [23,71–73].

The nuclear envelope, which delineates the nucleus, is contiguous with the rough ER. This membrane is equipped with ribosomes on the cytoplasmic face, called the outer nuclear membrane, but lacks them on the nuclear face, the inner nuclear membrane [74]. The inner nuclear membrane distinguishes itself from ER membranes by an extensive, unique set of proteins. Many of these proteins are integral membrane proteins that connect the inner nuclear membrane to chromatin or the nuclear lamina, such as the lamin B receptor [75,76]. The sorting of these proteins to the inner nuclear membrane is thought to involve diverse mechanisms based on diffusion through the nuclear envelope membrane, translocation by vesicular transport from the inner nuclear envelope membrane and transport via the nuclear pore complex [77,78]. The outer nuclear contains a unique set of proteins that distinguishes it from the remainder of the rough ER. Among these proteins are members of the nesprin protein family [79,80]. The nesprins bridge nuclear lamina to the cytoskeleton with the help of inner nuclear membrane proteins, thus regulating nuclear migration and anchoring [81]. The outer nuclear membrane targeting

mechanism of nesprins relies on their interaction with inner nuclear membrane proteins with a nesprin klarsicht/ANC-1/syne homology (KASH) domain [82–85].

3. The smooth ER

In most cells, the sheet-like rough ER is interspersed with smooth ER tubules [15,86]. The over-expression of certain smooth ER proteins such as cytochrome b(5) or a mutant form of vesicle-associated membrane protein-associated protein B (VAPB) can induce the proliferation of smooth tubules, which then become stacked against each other [87,88]. While the mechanism behind these observations is not entirely clear, recent research has identified a group of proteins composed of reticulons and DP1/Yop1p that promotes the curvature of ER membranes by transforming sheet-like ER (as found on the rough ER or the nuclear envelope) into tubular ER. Conversely, the loss of these proteins leads to the loss of tubule-shaped ER in both yeast and mammalian cells [89]. Reticulons span the ER membrane at least twice with two hydrophobic domains, thus forming a hairpin-like structure, which is required for their function, but also for their targeting to the smooth ER [90,91]. Additionally, reticulon 4a also localizes to the *X. laevis* nuclear envelope, where it may promote nuclear envelope growth [92]. Similarly, the combined deletion of *S. cerevisiae* RTN1 and YOP1 leads to the clustering of nuclear pore complexes and a defect in nuclear import [9].

The atlastin protein family, related to dynamin and the mitofusins, interacts with reticulons and DP1/Yop1p and complements their function, likely promoting the homotypic fusion of ER tubules and catalyzing the branching of ER tubules at the expense of GTP [93,94]. The absence of atlastins leads to fragmented, unbranched smooth ER, whereas their over-expression promotes sheet-like ER. Interestingly, mitochondria structure may be maintained by a related and connected mechanism, since mitofusin-2, a GTPase closely related to the atlastins not only mediates the fusion of mitochondrial tubules, but also the attachment of mitochondria to the ER at the MAM [95]. At the moment, it is unclear if and how dynamin-related protein 1 (Drp1), the dynamin-related GTPase that mediates mitochondria fission, also influences the structure of the ER [96].

Other proteins with functions in intracellular sorting and trafficking have been associated with the formation and maintenance of the rough ER/smooth ER domain formation. For example, interference with the ER-Golgi SNARE protein syntaxin 18 specifically alters the distribution of smooth ER proteins, but not of rough ER proteins, although the exact mechanism for this observation is currently not known [97]. Moreover, some members of the Rab protein family can regulate the structure of the ER (Rab5) and its apposition to lipid droplets (Rab18) [98,99]. Although commonly associated with endosomal trafficking in mammalian cells, the absence of Rab5 in *C. elegans* recapitulates the phenotype of a reticulon knockout and results in a loss of peripheral tubules. Conversely, the over-expression of Rab18 leads to increased apposition of lipid droplets to the rough ER.

One of the major functions of the smooth ER is the storage of calcium [100,101]. Whereas luminal calcium binding proteins such as calreticulin [102] and SERCA calcium pumps appear to be widely distributed throughout the ER, IP3R and ryanodine receptor calcium channels often cluster within the smooth endoplasmic reticulum [103,104], not overlapping perfectly with SERCA calcium pumps [105]. The result of these distinct distributions is the formation of ER subcompartments with different calcium uptake and release abilities [106–108]. An ER domain termed MAM fulfills a central role in ER calcium release, suggesting that this domain could be an important location of ER calcium release channels [54,109]. However, IP3Rs are not particularly enriched on this domain [110,111] and rather show a pan-ER distribution that includes in particular ER domains close to the plasma membrane, as seen for example in confluent, polarized Madin-Darby canine kidney cells [112,113]. Regardless of the IP3R intra-ER distribution, the MAM has clearly been identified as a major calcium release site.

What mechanism could explain this apparent contradiction? One reason could be the interaction of calcium release channels and/or calcium pumps with regulatory proteins, which could be unevenly distributed throughout the ER and which would then be primarily responsible for the formation of these distinct calcium release domains. In this hypothesis, the locally distinct interaction of regulatory proteins with IP3R and SERCA would determine whether the ER takes up or releases calcium at specific points within the ER network. Both the IP3R and SERCA are known to interact with numerous such proteins, including the ER chaperones and oxidoreductases ERp44, Ero1α and the sigma-1 receptor (IP3) as well as ERp57 and calnexin (SERCA) [114–118]. Indeed, the IP3R1 activator Ero1α and the SERCA2b inhibitor calnexin target preferentially to the MAM [63,111,119]. Similar to these examples, presenilin-2, an aspartyl protease associated with Alzheimer's disease, localizes to the MAM and inhibits SERCA activity [120,121]. Another mechanism could rely on local accumulations of calcium storage proteins within the sarcoplasmic reticulum of muscle cells (calsequestrin and calreticulin) or the smooth ER of nonmuscle cells (calreticulin) [122,123]. Here, the availability of calcium for intracellular signaling would be determined by the distinct intra-ER localization of releasable calcium. Support for this idea comes from the finding that the location of ER calcium release largely depends on the calcium buffering capacity of the ER, which is chiefly determined by calreticulin and calsequestrin, respectively [124]. In the case of calsequestrin, intra-ER accumulations are mediated by a head-to-tail oligomerization mechanism [125,126]. We will next discuss what is known about the targeting of proteins to the domains of the smooth ER.

4. The mitochondria-associated membrane (MAM)

Many of the smooth ER domains form interfaces with other organelles, an idea that was first proposed by Alex Novikoff for contacts between the smooth ER and lysosomes as the GERL (Golgi-ER-lysosome) compartment, whose presumed role is the formation of special types of lysosomes directly from the ER [127,128]. The role of ER-organelle contacts is particularly well studied in the case of the ER-mitochondria interface, the MAM subdomain [54,109,129], a domain of the ER that lacks ribosomes [130,131]. Today, we know that as much as 20% of the mitochondria surface is in contact with the ER that itself encompasses close to 50% of the total membrane in liver cells, suggesting that the MAM is a major player in cell physiology [132–134]. The existence of the MAM was first postulated after close contacts were observed between ER and mitochondria on electron micrographs, in which the resolution is high enough to see structures and measure distances between the two organelles [135]. More recently, 3D electron tomography has been used to visualize the structure of MAM in fixed and live cells [134,136]. Since the resolution of light microscopy is not sufficient to resolve the distance between ER and mitochondria at the MAM (10–50 nm), biochemical fractionation has been the main tool to study the MAM, beginning with the development of a Percoll MAM fractionation protocol by the Vance laboratory [111,137,138], although it is not clear whether this protocol reliably separates MAM from other domains of the ER, in particular from the rough ER [111]. Instead, such a separation can be reliably achieved on Optiprep-based gradients [63,119,139,140].

Historically, the MAM has been associated with lipid exchange between the ER and mitochondria [141]. This is reflected by the enrichment of lipid synthesis and transfer proteins on the MAM, which give the MAM a characteristic composition that distinguishes it from the remainder of the smooth ER [137,138]. Enzymes that are particularly enriched on the MAM include acyl-CoA synthase 4 (ACS4/

FACL4), acyl-CoA cholesteryl acyl transferase (ACAT/SOAT), and Acyl-CoA:diacylglycerol acyltransferase (DGAT) [138,140]. For the proteins of this group, a MAM targeting signal has only been detected for DGAT2 in the form of a cytosolic 67 amino acid sequence that is sufficient to target RFP to mitochondria [142].

Lipid transfer between the ER and mitochondria requires proteinaceous links, since it becomes inefficient after the treatment of MAM with proteases [143,144]. The identity of the links has been partially elucidated during the past decade. In yeast, the endoplasmic reticulum–mitochondria encounter structure (ERMES) forms a protein complex that tethers the ER to mitochondria and mediates mitochondria inheritance and movement. The ERMES complex is formed between the integral ER membrane protein Mmm1p, the peripheral ER membrane protein Mdm12p, and the two mitochondrial outer membrane proteins Mdm10p and Mdm34p [145,146]. Mdm12p and Mmm1p are members of the synaptotagmin-like, mitochondrial and lipid-binding protein (SMP) family, whose members are widespread among eukaryotic species and play roles ranging from endocytosis to lipid metabolism and mitochondrial inheritance [147]. We currently do not know which mammalian proteins fulfill the function of the ERMES complex.

In mammalian cell systems, the MAM has revealed itself as a major cellular signaling hub that controls cellular metabolism and death via the controlled exchange of calcium between the ER and mitochondria [148,149]. Whereas the IP3R-mediated calcium release during resting conditions directly boosts the mitochondrial metabolism, apoptosis triggers the release of cytochrome c, which binds to ER IP3Rs, thus potentiating and accelerating apoptosis progression. Consistent with these functions, the disruption of ER-mitochondria contacts by depleting tethering complexes leads to delayed apoptosis progression and altered calcium homeostasis in both the ER and mitochondria [95,110,150]. The link between MAM formation and the maintenance of cellular homeostasis as well as the sensing of cellular stress is highlighted by the observations that the extent of the MAM and the proximity of the ER and mitochondria increase under conditions of ER stress [136,151]. The full significance of this observation is currently not clear, but juxtaposed mitochondria could in principle serve to alleviate ER stress by providing additional ATP for oxidative protein folding [54]. Together, these observations highlight the significance of the MAM for cellular physiology and stress the importance of identifying protein tethers between the two organelles. One of these tethers is the cytosolic chaperone Grp75, which interacts with both the IP3R on the ER and the voltage-dependent anion channel on mitochondria, thus facilitating calcium transfer between the two organelles [110]. However, Grp75 bridging does not influence the proximity between the ER and mitochondria, suggesting that the role of this protein is restricted to calcium signaling. Another protein complex at the MAM is formed when mitochondrial Fis1 and the smooth ER protein BAP31 (see also Section 5) interact with each during the onset of apoptosis. The formation of this complex leads to recruitment of procaspase-8 to the MAM, but how this is connected to pro-apoptotic, IP3R-mediated calcium release is currently not clear [152]. Another example is the formation of complexes of ER- and mitochondria-localized mitofusin-2, a dynamin-related GTPase. Mitofusin-2 complexes are required for the formation of the MAM and the efficient transfer of calcium between the ER and mitochondria in mammalian cells [95]. Moreover, the complete absence of mitofusin-2 leads to the formation of swollen and aggregated ER that shows reduced branching, whereas the mitochondria undergo fragmentation [95]. Since ER-targeted mitofusin-2 can rescue the ER, but not the mitochondria or the MAM phenotype, it has been hypothesized that the altered ER morphology in mitofusin-2 knockout cells stems from a so far uncharacterized function of mitofusin-2 on the ER. Interestingly, such a function appears to mirror the impact of atlastins on the equilibrium between the smooth and the rough ER, since the absence of atlastins also leads to tubulated, poorly branched ER [67,93]. Despite these parallels, it is currently not known whether the atlastins and mitofusins functionally or physically interact with each other. The mechanism that determines ER or mitochondria targeting of mitofusin-2 is also unclear at this point.

To date, four MAM targeting mechanisms are known. The first described MAM targeting mechanism requires the cytosolic sorting protein PACS-2. PACS-2 had initially been identified as a possible MAM-organizing protein, since its knockdown leads to a disruption of the MAM, whose consequences on ER-mitochondria calcium and apoptosis signaling are similar to what is known about mitofusin-2 knockout [150]. However, in cooperation with the cytosolic protein coat COPI, PACS-2 also contributes to the MAM-enrichment of the chaperone calnexin [63], a repressor of SERCA2b [117]. PACS-2 interacts with calnexin on two serines that are substrates for protein kinase CK2 [63]. Since COPI mediates retrieval from the cis-Golgi to the ER [153], these findings suggest an involvement of ER-to-Golgi trafficking in the distribution of ER proteins to specific smooth ER domains and the MAM in particular. Rab32 is another trafficking molecule that regulates the composition of the MAM [139]. Upon its activation, select MAM-localized ER proteins are relocated to the cellular periphery, concomitant with altered ER calcium handling.

A second MAM targeting mechanism has been demonstrated for the ER oxidoreductase Ero1α, which requires oxidizing conditions to target to the MAM [119]. This enzyme can recharge PDI to allow for efficient production of disulfide bonds in secretory proteins [154], but also is a target of the CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP), an ER-stress-triggered transcription factor. Increased amounts of Ero1α after extended periods of ER stress promote ER calcium release through a direct interaction with IP3R1 [115]. However, we currently do not know where this interaction occurs, since Ero1α quickly relocates from the MAM upon redox or oxygen stress [119].

A small number of MAM proteins utilize a mitochondria targeting sequence in their cytosolic domains to target to the MAM, for example DGAT2 [142]. DGAT2 does not target exclusively to the MAM, as shown by its relocation to lipid droplets in cells loaded with oleic acid [142].

The presence of large quantities of lipid synthesizing enzymes on the MAM might be at the basis of ER lipid microdomains [155–157]. For example, the MAM contains glycosphingolipid-enriched microdomains [158,159]. Moreover, staining of the ER with filipin that binds to cholesterol rafts, shows the presence of isolated such structures [160]. In contrast to the rough ER, which contains little raft-associated cholesterol compared to the plasma membrane [161–163], the MAM is apparently enriched for it, despite the absence of cholesterol-synthesizing enzymes on the MAM [164]. Interestingly, the sigma-1 receptor, a regulator of the IP3R3, localizes to lipid rafts within the ER in a cholesterol-dependent manner [59,165]. Normally found on the MAM in a complex with the chaperone BiP/GRP78, the sigma-1 receptor interacts with the IP3R3 upon IP3R stimulation or ER stress to boost this calcium channel's activity [114]. Moreover, the sigma-1 receptor, through its ability to bind cholesterol, regulates the distribution of cholesterol within the ER; cholesterol rafts within the ER dissipate upon transfection of sigma-1 receptor mutants unable to bind to cholesterol [160]. Interestingly, increased free ER cholesterol also inhibits the import of calcium via SERCA2b, suggesting that ER cholesterol homeostasis is a powerful control mechanism for ER calcium content [166]. Together, these observations suggest important connections between a modulation of ER cholesterol content and ER calcium homeostasis.

5. Other smooth ER domains: ER exit sites, plasma membrane-associated ER, ER quality control compartment

Less information is available on targeting mechanisms to other smooth ER domains, including ER exit sites (ERES), the PAM, which is

apposed to the plasma membrane, and the ER Quality Control Compartment (ERQC). ERES mediate the bulk of secretory protein export from the ER and generate the ER-Golgi intermediate compartment (ERGIC) [167,168]. ER exit sites are part of the transitional ER, which is characterized by its overlap with the rough ER and its juxtaposition to the Golgi complex [169]. It is thought that the transitional ER mediates the enrichment of fully folded, export-competent proteins [170]. Targeting to and formation of ERES is thought to depend on an interactor of COPII family proteins [167], the peripherally membrane associated protein Sec16, in mammals present as Sec16A and Sec16B, which can form a heteromeric complex in mammalian cells [171]. RNAi-mediated knockdown of this protein results in the disruption of ERES [171,172]. Sec16A localizes to ERES without the help of other COPII components in an area that is spatially distinct from the location of the COPII proteins Sec24C and Sec31A [173]. Targeting of Sec16 to ERES requires the presence of a central conserved domain, which contains a positively charged stretch, a potential interaction site with polar phospholipids or a yet-to-be-identified receptor protein [171,173,174]. Following the targeting of Sec16 to ERES, assembly of the COPII coat drives vesicle budding and export from the ER toward the ERGIC and Golgi complex [175,176].

For a long time, it was thought that this export and protein secretion in general occurs by bulk flow, even though it had been known for quite a while that the lack of anterograde signals can lead to the retention of mutant proteins along the secretory pathway [177]. The properties of secretory protein transmembrane domains have recently emerged as one determinant of membrane protein sorting to ERES and subsequent incorporation into transport vesicles. For example, a 22 residue transmembrane domain leads to COPII interaction and ER export of a fluorescent fusion chimeric protein, but it was shown that a 17 residue transmembrane domain did not give such an effect [178]. Furthermore, lipid modifications may lead to ER domain sorting, since the presence of a palmitoylation acceptor site on the Wnt signaling protein LRP6 mediates its targeting to the secretory pathway via ERES [179]. In yeast, chitin synthase Chs3 also requires palmitoylation for its export from the ER [180]. Whether these signals correspond to bona fide anterograde transport signals or whether they are based on different mechanisms remains to be tested.

Interestingly, the control of ER cholesterol content takes advantage of export from the transitional ER as well. Sterol-regulatory element-binding protein-2 (SREBP-2) is exported from the ER in a complex with the polytopic transmembrane protein Scap through interaction with COPII at ERES as long as the ER cholesterol content is below 5% [162]. This export from the ER subsequently allows proteolytic cleavage of SREBP-2 at the level of Golgi complex [181]. The proteolytic fragments of SREBP then boost production of cholesterol by promoting transcription of the cholesterol metabolism genes. Conversely, if ER cholesterol content rises above 5%, Scap binds cholesterol and undergoes a conformational change that allows Scap to bind the ER resident protein insulin-induced gene (Insig), precluding interaction of the Scap/SREBP complex with COPII at ERES [182]. A similar mechanism utilizes the translocation in renal cancer from chromosome 8 protein (TRC8), which also hampers COPII interaction with the Scap/SREBP complex [183].

Recent research has demonstrated that the ER is not only the point of origin for classical secretory pathway protein traffic to the cell surface, but also influences the plasma membrane in other, unexpected ways. During the past decade, a domain of the smooth ER that is in close contact with the plasma membrane has been designated as PAM. The PAM interacts with the plasma membrane extensively on as many as 1100 contact sites [184]. Most of the research on this domain has so far been done in yeast, where this domain is known to mediate direct sterol trafficking to the plasma membrane [185,186]. The interaction between translocons and components of the exocyst complex on this domain of the ER also mediates the inheritance of the cortical ER in yeast [187,188], as well as the targeting of secretory and basolateral proteins in polarized mammalian cells [189]. In this cell system, the proximity of the ER at contact sites has been estimated at 10–25 nm [190]. In yeast and mammalian cells, the PAM is a major site of synthesis for phosphatidylserine. This prominent plasma membrane lipid is predominantly found on the cytoplasmic leaflet of the plasma membrane [184,191]. Phosphatidylserine plays an important role during apoptosis, when it becomes exposed on the outer leaflet of the plasma membrane [192,193]. This exposure enables phagocytes to recognize apoptotic cells and subsequently engulf them [194,195]. Similar to the MAM, the PAM also plays an important role in cellular calcium homeostasis. This became evident, when ER-localized stromal interaction molecule 1 (STIM1) and Orai1, a plasma membrane calcium channel, two key players of store-operated calcium exchange were discovered to interact with each other [196,197]. The interaction between the two calcium handling proteins is triggered when ER calcium stores have been depleted [198,199]. The translocation of STIM1 to the PAM coincides with its multimerization [200,201] and requires the cytosolic domain of STIM1, which can interact with Orai1 to promote calcium funneling to the ER [202–204]. Another parallel to the MAM is that the PAM undergoes massive remodeling upon interference with ER calcium homeostasis. Upon calcium depletion, preformed precortical ER containing STIM1 translocates to the plasma membrane, where it loses its ribosomes and becomes smooth PAM that is sometimes stacked close to the plasma membrane [205].

In addition to export of proteins and lipids, the ER can also segregate folding intermediates. This is the function of the ER quality control compartment (ERQC), where incompletely folded proteins await ER-associated degradation (ERAD) [206]. The ER chaperone calnexin was the first protein found to localize to this ER domain upon ER stress that results in the accumulation of unfolded proteins [207,208]. The presence of calnexin on the MAM and its absence from the ERQC under resting conditions, suggests that this lectin can shuttle from the MAM to the ERQC upon ER stress [63]. On the MAM, calnexin likely modulates calcium handling, whereas on the ERQC, this chaperone either promotes or inhibits ERAD in a substrate-dependent manner [209]. Analogous to calnexin, the smooth ER protein BAP31 can interact with mitochondrial Fis1 on the MAM [152], but is also a component of the ERQC and an interactor of calnexin [210]. Within the ERQC, BAP31 promotes retrotranslocation of misfolded proteins through an interaction with the translocon components Sec61β and TRAM, as well as the Derlin-1 ERAD translocation complex [211]. Import of BAP31 into the ERQC requires microtubules and depends on Arf1 and COPII, suggesting that an intra-ER vesicular transport step may regulate transition from the rough ER to the ERQC [212]. Additional enzymes of the ERQC include the oxidoreductases PDI and ERp57, which promote retro-translocation of ERAD substrates [207,213]. Whether these two proteins are enriched on or specifically targeted to the ERQC is currently not known. Moreover, it is unclear, whether there is a relationship between the ERQC and the so-called EDEMosomes, where the ER disposes of unused ERAD components [214,215]. EDEMosomes are decorated with non-lipidated LC3 and are capable to fuse with the endosomal/lysosomal compartment, suggesting a direct pathway and connection from the smooth ER to lysosomes.

6. The smooth ER as a point of origin for Russell body, peroxisome and lipid droplet biogenesis

The functional differentiation of the ER is now known to go one step further by providing the point of origin for entire organelles. This is exemplified with Russell bodies, which were first described at the end of the 19th century as a characteristic of cancer cells, and were mistakenly thought to be a fungal parasite [216]. Indeed, we now know that plasma cells of multiple myeloma patients frequently exhibit Russell bodies [217]. However, over a hundred years of

research have shown that Russell bodies represent a differentiation of the ER and are used to store mutant, detergent-insoluble immunoglobulin that can neither be secreted nor degraded [218]. These condensations are generated by covalent disulfide bonds between multiple immunoglobulins, which explain why mild reducing agents can lead to the dissolution of Russell bodies [219]. Dependent on the type of immunoglobulin present, Russell bodies form either at the rough or at the smooth ER, suggesting that both domains are equipped to remove unwanted waste [220]. Their formation requires the recognition of immunoglobulins by the ERES-associated ER proteins ERGIC-53 and ERp44, which recognize the immunoglobulin sugar structure and unpaired disulfide bonds, respectively [220,221]. These two chaperones thus serve as Russell body sorting molecules at the level of ERES and ERGIC. Future research will have to examine whether there is a relationship between the ERQC and Russell bodies and whether there are any distinguishing characteristics of the Russell body membrane.

The smooth ER is also involved in the formation of peroxisomes, the organelles that mediate the degradation of fatty acids and the synthesis of ether lipids such as plasmalogens [222,223]. Although cell biologists first classified peroxisomes as endosymbionts, not unlike what had initially been suspected about Russell bodies, these organelles actually derive from a special domain of the ER, the pre-peroxisomal compartment [224,225]. Here, a subset of Pex proteins, a group of proteins that are required for peroxisome biogenesis, target to peroxisome-generating domains of the ER and are then incorporated into the nascent organelle [224]. The Pex proteins that have such a function are Pex3, Pex16 and Pex19. The correct targeting of Pex3p requires the formation of a Sec20p, Sec39p and Dsl1p protein complex at the ER in yeast [226]. Similar to Russell body biogenesis, peroxisome biogenesis might be influenced by the secretory pathway [227]. Specifically, proteins of the coatomer coat complex and members of the Arf protein family might be involved in the anterograde and retrograde transport to and from peroxisomes [228,229]. The role of Sec20p and Sec39p in ER-Golgi trafficking again highlights the potential connection between protein secretion and peroxisome biogenesis [230,231]. However, peroxisome biogenesis at the level of the ER does not depend on ongoing protein secretion, since peroxisome targeting is maintained in the presence of COPII transport inhibitors [232].

Yet another complex relationship between the ER, protein secretion and organelle biogenesis exists in the case of lipid droplets. In this case, trafficking between the ER and the Golgi mediated by COPI coated vesicles is essential for lipid droplet biogenesis [233]. Lipid droplets have been well known to be closely associated with ER membranes since the early 1970s [234]. Proteomic analysis of their content showed that the ER oxidoreductases ERp29 and peroxiredoxin 4 as well as the chaperones BiP/GRP78 and calnexin are found on lipid droplet membranes [235,236]. Proteins found in the lipid droplet membrane such as 17β-hydroxysteroid dehydrogenase type 11 and methyltransferase-like 7B (also known as AAM-B) target to the lipid droplet membrane from the ER using N-terminal hydrophobic sequences [237,238]. Interestingly, this targeting is reversible, suggesting the existence of modifiable lipid droplet targeting signals and mechanisms [239]. Lipid droplet biogenesis also appears to require the spatial confinement of enzymes that synthesize their lipid contents, mostly triacylglycerides and cholesterol esters [240]. DGAT1 and DGAT2 catalyze the final step in the synthesis of triacylglycerides, but only DGAT2 can translocate from the MAM to sites of lipid droplet biogenesis upon oleate treatment of cells [142]. At these sites, the DGAT2 enzymatic activity may channel triacylglycerides into nascent cytosolic lipid droplets [241]. Studies using mistargeted DGAT2 have shown that the intracellular localization of the DGAT2 enzymatic activity determines the site of lipid droplet formation [242]. Subsequently, lipid droplets become coated with proteins of the perilipin, adipose differentiation-related protein, tail-interacting protein of 47 kDa (PAT) family [243]. PAT proteins such as tail-interacting protein of 47 kDa (TIP47) target to lipid droplets using multiple amphipathic helices as targeting information to influence the structure of lipid droplets [244]. The trafficking to lipid droplets of the proteins of this family also requires COPI and COPII-based mechanisms, suggesting that lipid droplets like Russell bodies are in close contact with ERES and ERGIC [245]. PAT proteins regulate the metabolism of triacylglycerides contained in lipid droplets. For example, protein kinase A (PKA) phosphorylates perilipin on multiple sites [243], which leads to the activation of lipid droplet-associated lipid hydrolases and the translocation of lipid hydrolases to lipid droplets [246].

Together, the findings on Russell bodies, peroxisomes and lipid droplets demonstrate how the ER functions as a point of origin for organelle biogenesis. The fact that all three organelles originate from ER domains at or close to ERES underscores the ability of the ER to execute very tight control of membrane domain formation.

7. ER domain enrichment and disease

Given the importance of ER membrane domain sorting for critical functions of the ER and organelle biogenesis of Russell bodies, peroxisomes and lipid droplets, a main area of research aims to understand which mechanisms are needed to implement the blueprint of the ER's urban planning department. A better understanding of these functions is expected to give insight into diseases that are associated with malfunctioning ER, but also ER-derived organelles such as the Golgi complex, lipid droplets and peroxisomes. While numerous diseases are separately tied to those organelles, historically, ER-associated diseases were summarized as ER storage diseases and are characterized by a decreased or increased extent of ER folding or retention [247]. Examples include cystic fibrosis, low density lipoprotein receptor defects and coagulation factor V and VIII deficiencies. Conceivably, the mislocalization of the ER retention and export machinery, such as the coatomer and COPII coat or the KDEL receptor could lead to such diseases. Malfunctioning ERAD could also lead to such diseases. In this context, the ubiquitin ligase RNF45, also known as gp78 or autocrine motility factor receptor (AMFR) is of interest. The intracellular localization of this protein has not been conclusively determined, since it has been found on the MAM and the plasma membrane [131,248]. The knockdown of this ER protein leads to a block of sarcoma metastasis in primary tumor, due to RNF45/gp78/AMFR promoting the degradation of the metastasis suppressor KAI1/CD82 by the proteasome [249,250]. At the same time, cells expressing low levels of gp78 are more susceptible to ERAD-mediated apoptosis, again dependent on its relationship with KAI1/CD82. Another connection of ER protein folding and degradation with cancer is the role of the unfolded protein response in tumor cell metabolism. Ire1, a type I transmembrane endonuclease, cleaves the XBP-1 mRNA, but also activates apoptosis signal regulating kinase 1 (ASK1) [251,252]. Similarly, protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) induces the production of the pro-apoptotic transcription factor CHOP [253,254]. Together, Ire1 and PERK promote cancer cell survival under hypoxic conditions [255,256]. Moreover, ER stress sensor proteins have been implicated in the development of diabetes [257,258]. Although the intra-ER localization of the two ER stress sensor proteins Ire1 and PERK is not known, their sorting and enrichment could be envisaged as critical for their signaling function in cancer and diabetes.

The center of the recent attention lies however on the links between the MAM and human disease, since this smooth ER domain is the calcium and apoptosis signaling hub of the ER [54,109,259]. Here, the ER is enriched for numerous disease-associated proteins, including presenilins (Alzheimer's disease [120]), mitofusin-2 (Charcot-Marie-Tooth disease type 2A in knockout models [260]), calnexin (multiple sclerosis-like symptoms in knockout models, [261]) and the

Table 1
ER subcompartments, their main functions and marker proteins. Summary of the main points presented in this review. References and details are found in the text.

ER subcompartment	Main functions	Marker proteins	Known sorting signals or mechanisms
Rough ER	• Secretory and membrane protein synthesis, folding	RibophorinI/II, Climp63	Interaction with ribosomes (Sec61), CK2 phosphorylation (Sec63, calnexin), cytosolic and transmembrane sequences (Ribophorin II),
Nuclear envelope	• Delineates nucleus	Nesprins	Interaction with inner nuclear membrane proteins using KASH domain
Smooth ER	• Lipid synthesis • Calcium storage	Reticulons, Atlastins	?
MAM	• ER/mitochondria calcium homeostasis • Apoptotic calcium signaling • Lipid transfer	ACAT1, FACL4, Erolα	Cryptic mitochondria interaction signal (UL37, DGAT2), oxidizing conditions (Ero1〈), lipid raft association (Sigma-1 receptor), resting conditions (calnexin, BAP31)
ERQC	• Export of unfolded proteins to the proteasome	Derlin-1, EDEM-1	ER stress (calnexin, BAP31)
Russell bodies	• Segregation of protein aggregates	Condensed Immunoglobulins	Sugar structure and unpaired cysteines
ERES	• Export of proteins from ER to Golgi	Sec16A/B	Interaction with COPII coat (positive charges in Sec16), ER export signal of cargo proteins
PAM	• Calcium import from extracellular space (Store Operated Calcium Exchange—SOCE) • Sterol trafficking to plasma membrane	STIM1, ORAI1	Multimerization (STIM1)
Pre-peroxisomal compartment	• Peroxisome formation	Pex3	?
Lipid droplets	• Storage of triacylglycerides	AAM-B	N-terminal hydrophobic sequences (AAM-B)

promyelocytic leukemia protein (PML), a tumor suppressor [262,263]. The common denominator of these diseases is a misregulation of apoptosis onset, likely connected to dysfunctional ER-mitochondria calcium signaling. Since the extent of MAM targeting for these signaling regulators varies and can depend on ER and cellular homeostasis, future research will have to elucidate the targeting mechanisms of these proteins in order to gain more insight into their role in human disease. Interestingly, since the formation of cholesterol-dependent rafts is involved in the formation of the MAM and the targeting to this domain of the smooth ER, one such mechanism may already have been described: cholesterol-induced cytotoxicity in macrophages leads to accelerated death of this cell type in advanced atherosclerotic lesions [264]. Cholesterol overloading may alter the composition and functioning of the MAM, resulting in the observed accelerated apoptosis progression.

ER domain formation is also a mechanism that is exploited by viruses. For example, Coronaviruses utilize EDEMosomes to generate double membrane vesicles that accommodate viral replication [215]. The human cytomegalovirus UL37 exon 1 protein, also known as viral mitochondrion-localized inhibitor of apoptosis (vMIA) targets to the MAM after its synthesis within the ER. From here it is able to translocate to mitochondria to exert its anti-apoptotic role by sequestering Bax [265]. Similar to DGAT2, its targeting and role in apoptosis requires a mitochondrial targeting signal, but not the association with lipid rafts [266]. Thus, these studies corroborate that precise intra-ER targeting dictates the functioning of cellular and viral proteins (Table 1).

8. Conclusions

Research during the past 2 decades has revealed that the ER resembles a city with multiple functions assigned to designated areas more than a village that specializes into one function. B cell to plasma cell development is just one example that shows the dynamic nature of ER domain formation and targeting. Together, multiple findings highlight the importance of understanding intra-ER targeting for human disease. Given that this area of cell biology is in some ways the final frontier of intracellular sorting, great progress is expected to occur during the next decade with important insight into the biogenesis of the ER as a complex assembly of membrane domains and into the significance of this organelle for human life and death. An important question for future research will be to elucidate the connection of vesicular trafficking to ER domain formation, given the multiple involvements of COP coats, Rab proteins and Arf GTPases in this cell biological mechanism.

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