Alterations in Ca^{2+} Signalling via ER-Mitochondria Contact Site Remodelling in Cancer

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Abstract

Inter-organellar contact sites establish microdomains for localised Ca^{2+}-signalling events. One of these microdomains is established between the ER and the mitochondria. Importantly, the so-called mitochondria-associated ER membranes (MAMs) contain, besides structural proteins and proteins involved in lipid exchange, several Ca^{2+}-transport systems, mediating efficient Ca^{2+} transfer from the ER to the mitochondria. These Ca^{2+} signals critically control several mitochondrial functions, thereby impacting cell metabolism, cell death and survival, proliferation and migration. Hence, the MAMs have emerged as critical signalling hubs in physiology, while their dysregulation is an important factor that drives or at least contributes to oncogenesis and tumour progression. In this book chapter, we will provide an overview of the role of the MAMs in cell function and how alterations in the MAM composition contribute to oncogenic features and behaviours.

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17.1 Introduction

Eukaryotic cells are faced with many challenges to sustain life (Chen and Silver 2012). To tackle these challenges, nature has come up with cellular compartmentalisation resulting in different organelles including the nucleus, endoplasmic reticulum (ER), mitochondria, peroxisomes and Golgi apparatus (Diekmann and Pereira-Leal 2013). The ability of eukaryotic cells to restrict processes to a subcellular localisation brings about numerous advantages (Chen and Silver 2012). However, to ensure the smooth orchestration of cellular processes, communication between the organelles is critical.

One way of inter-organellar communication is Ca^{2+} signalling. The fact that Ca^{2+} is not homogeneously distributed throughout the cell makes it possible to use Ca^{2+} as a messenger (Clapham 2007). Ca^{2+} signals typically arise from the ER and the lysosomes (Berridge et al. 2000; Berridge et al. 2003). These Ca^{2+} signals impact the other organelles via microdomains established by membrane contact sites (La Rovere et al. 2016; Raffaello et al. 2016). Important target organelles of ER-derived Ca^{2+} signals are the mitochondria, which help to maintain Ca^{2+} homeostasis in the cell (Rizzuto et al. 2012). Ca^{2+} uptake in the mitochondria is driven by the negative mitochondrial membrane potential (Rizzuto et al. 2000). The outer mitochondrial membrane (OMM) is freely permeable to Ca^{2+} due to the presence of porins, like the type 1 voltage-gated anion channel (VDAC1) (Gincel et al. 2001; Rapizzi et al. 2002). While Ca^{2+} easily reaches the intermembrane space, a more intriguing problem was the entry of Ca^{2+} into the mitochondrial matrix. Initial research pointed to the existence of a mitochondrial uniporter, albeit one with low affinity for Ca^{2+} (Carafoli 2012; Marchi and Pinton 2014). However, the low affinity of this uniporter could not easily be matched to observations that Ca^{2+} was dynamically and efficiently exchanged between the cytosol and the mitochondria, considering that cytosolic $[Ca^{2+}]$ is typically in the low-to-middle nanomolar range (Rizzuto et al. 1998; Csordás et al. 1999; Carafoli 2012).

The mechanism underlying this seemingly paradoxical observation is the presence of close contacts between ER and mitochondria, which favour quasi-synaptic Ca^{2+} transfer from the ER to the mitochondria (Rizzuto et al. 1998; Csordás et al. 1999). These sites can be isolated biochemically as mitochondria-associated ER membranes (MAMs). MAMs are parts of the ER membrane that are in close proximity to the mitochondrial membrane and are tethered to it (Szabadkai et al. 2006; van Vliet et al. 2014). At the MAMs, the distance between the ER and mitochondria is believed to be approximately 10 to 25 nm (Rizzuto et al. 1998; Csordás et al. 2006; Marchi et al. 2014b), allowing proteins situated on the ER membrane and OMM to interact and enabling efficient Ca^{2+}-based communication between the ER and mitochondria (Decuypere et al. 2011; Rowland and Voeltz 2012; Marchi et al. 2014b). In this way, MAMs provide a microdomain in which the $[Ca^{2+}]$ is several folds higher than in the bulk cytosol (estimated to be $>10\,\mu M$ at the ER-mitochondrial interface) (Csordás et al. 2010), alleviating the problem of the paradoxical low affinity of the mitochondrial Ca^{2+} uniporter (Rizzuto et al. 1993, 1998). Apart from Ca^{2+} homeostasis, MAMs are implicated in several processes critical to cell function, e.g. lipid transport, ER stress, apoptosis, autophagy, inflammation and anti-viral response (Vance 2014; van Vliet et al. 2014).

Due to their involvement in these specific functions, MAMs contain a select protein population. The inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) and VDAC1 channels are present in this sub-organellar domain (Várnai et al. 2005; Szabadkai et al. 2006), underlying the prominent role of the MAMs in ER-mitochondrial Ca^{2+} signalling. Furthermore, proteins indirectly involved in ER-mitochondrial Ca^{2+} flux can be found in and around the MAMs as well. These include glucose-regulated protein 75 (GRP75) (Szabadkai et al. 2006), mitofusin-2 (Mfn2) (de Brito and Scorrano 2008), phosphofurin

acidic cluster sorting protein-2 (PACS-2) (Myhill et al. 2008), promyelocytic leukaemia protein (PML) (Pinton et al. 2011), sigma-1 receptor (Sig-1R) (Hayashi and Su 2007) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) (Verfaillie et al. 2012), amongst others. Since Ca^{2+} signalling fulfils an important role in several cell physiological processes and considering its dysregulation in pathophysiological conditions, the expression of these proteins is often altered, and their functional activity is converted to promote tumour growth, proliferation, migration, apoptosis resistance and changes in cellular metabolism (Urra et al. 2016; Marchi and Pinton 2016). Furthermore, the MAMs harbour an increasing number of oncogenes and tumour suppressors that functionally impact ER-mitochondrial Ca^{2+} transfer and oncogenic features (Marchi et al. 2014a; Giorgi et al. 2015b; Bittremieux et al. 2016).

In this chapter, we will discuss (1) the MAM components, including the Ca^{2+}-transport systems, chaperones and structural proteins that are present, (2) how MAM components impact ER-mitochondrial Ca^{2+} transfer and their structural organisation and (3) how alterations in the function of these MAM components drive oncogenesis and tumour progression. An overview of the MAM components to be discussed in this chapter can be found in Fig. 17.1.

17.2 MAM Components

17.2.1 The Ca^{2+}-Signalling Machinery at the MAMs

17.2.1.1 IP$_3$R

The IP$_3$R is an intracellular Ca^{2+}-release channel, present in the ER membrane (Ashby and Tepikin 2001; Choe and Ehrlich 2006). IP$_3$Rs are opened by IP$_3$ (Foskett et al. 2007; Parys and De Smedt 2012; Fedorenko et al. 2014), a second messenger released into the cytosol after phosphatidylinositol 4,5-bisphosphate cleavage by phospholipase C. IP$_3$R activity is tightly controlled by cytosolic [Ca^{2+}] in a biphasic manner (Iino 1990; Finch et al. 1991; Bezprozvanny

et al. 1991; Parys et al. 1992). The Ca^{2+}-flux properties of the IP$_3$R are also regulated by other cellular factors, including ATP, regulatory proteins (Choe and Ehrlich 2006; Foskett et al. 2007; Parys and De Smedt 2012) and protein kinases and phosphatases (Vanderheyden et al. 2009). Structurally, the IP$_3$R consists of three domains: an N-terminal ligand-binding domain, which is subdivided in a suppressor region and the IP$_3$-binding core, a central modulatory domain and a pore-forming region in the C-terminal tail (Yoshikawa et al. 1999). The IP$_3$R comes in three different isoforms (IP$_3$R1, IP$_3$R2 and IP$_3$R3) which are encoded by different genes (*ITPR1*, *ITPR2* and *ITPR3*) and display 60–80% homology at the level of the amino acid sequence (Mikoshiba 2007; Foskett et al. 2007). Sensitivity towards their ligand IP$_3$ as well as regulation by Ca^{2+}, ATP and phosphorylation appears to be isoform specific (Newton et al. 1994; Miyakawa et al. 1999; Tu et al. 2005; Khan et al. 2006; Betzenhauser et al. 2008; Wagner et al. 2008; Vervloessem et al. 2015).

A subset of IP$_3$Rs is present at the MAMs, where it is responsible for ER-mitochondrial Ca^{2+} flux (Várnai et al. 2005; Mendes et al. 2005). By varying the spatio-temporal pattern of Ca^{2+} release from the ER, Ca^{2+} signalling can convey messages, which are differentially decoded at the subcellular level (Berridge et al. 2000, 2003). For example, constitutive low-level IP$_3$R-mediated ER-mitochondrial Ca^{2+} transfer maintains mitochondrial bioenergetics through stimulation of mitochondrial respiration and ATP production (Cárdenas et al. 2010; Kaufman and Malhotra 2014), whereas excessive Ca^{2+} release from the ER triggers apoptotic cell death due to mitochondrial Ca^{2+} overload (Giorgi et al. 2012; Morciano et al. 2015). Hence, IP$_3$Rs located at the MAMs play an important role in determining cell fate. Especially IP$_3$R3 seems to be abundant at the MAMs, since it has been proposed that pro-apoptotic ER-mitochondrial Ca^{2+} transfers preferentially occur via IP$_3$R3 (Blackshaw et al. 2000; Mendes et al. 2005). However, also the other IP$_3$R isoforms have been implicated in Ca^{2+}-mediated cell death (Gutstein and Marks 1997;
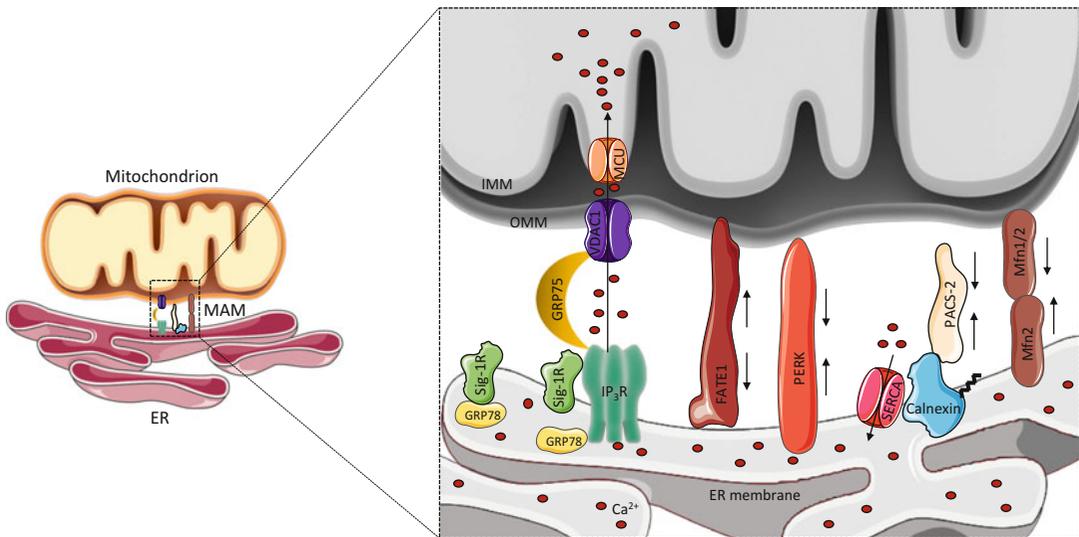
Fig. 17.1 MAM components playing a role in ER-mitochondrial Ca^{2+} signalling. The MAMs harbour a specific protein population consisting of Ca^{2+}-transport proteins and chaperones, as well as of proteins that enable their structural organisation. The principal components of the ER-mitochondrial Ca^{2+} exchange at the MAMs are the IP_3R and VDAC1, which are physically coupled by the chaperone protein GRP75. When Ca^{2+} is released from the ER by the IP_3R, it freely permeates the OMM via VDAC1, to be transported to the mitochondrial matrix by the MCU, located in the IMM. The chaperone Sig-1R is able to modify IP_3R-mediated Ca^{2+} signalling. Sig-1R is held inactive by binding to GRP78, but under ER stress binding to GRP78 is disrupted and Sig-1R interacts with the IP_3R, stabilising the IP_3R and enabling proficient Ca^{2+} signalling even under conditions of ER stress. The efficiency of Ca^{2+} exchange between ER and mitochondria is influenced by the presence and action of tethering proteins like PERK and Mfn2 and anti-tethering proteins like FATE1. The functional effect of tethering and anti-tethering proteins at the MAMs is indicated by *arrows* facing each other or *arrows pointing* in opposite directions, respectively. Besides its function as a tethering protein, PACS-2 also contributes to MAM organisation, while simultaneously having a role in the enrichment of the chaperone calnexin at the MAMs. Furthermore, calnexin is enriched at the MAMs by palmitoylation, a process that switches calnexin function from quality control/protein folding to ER Ca^{2+}-signalling control by enhancing SERCA activity. The interaction between calnexin and SERCA2b appears to be counteracted by the thiol reductase TMX1 (not shown for clarity reasons), which inhibits SERCA2b activity (For more details, please see text)

Jayaraman and Marks 1997; Assefa et al. 2004; Li et al. 2009; Akl et al. 2013) and thus may reside in the MAMs in certain cell types or systems.

17.2.1.2 VDAC1

As described above, Ca^{2+} released by the IP_3R is able to cross the OMM through VDAC1, a weakly anion-selective channel that is Ca^{2+} permeable and which is enriched at the MAMs (Hajnóczky et al. 2002; Shoshan-Barmatz and Gincel 2003; Colombini 2012). Apart from its role in mitochondrial Ca^{2+} transport, VDAC allows for substrates of the electron transport chain, like malate, succinate and nicotinamide adenine dinucleotide, to access the mitochondria (Shoshan-Barmatz et al. 2010). Moreover,

VDAC's channel properties permit ATP, produced by oxidative phosphorylation, as well as other mitochondrial products like reactive oxygen species (ROS) to diffuse into the cytosol (Shoshan-Barmatz et al. 2010). Additionally, VDAC1 oligomers have been implicated in the release of cytochrome c into the cytosol (Weisthal et al. 2014). As it were, VDAC functions as the channel that allows the mitochondria to communicate with their subcellular environment.

VDAC is able to switch between an open and a closed state in a voltage-dependent manner. While the channel is stable in the open state at low voltages, high voltages cause VDAC to switch to the closed state (Hodge and Colombini

1997; Gincel et al. 2000). Interestingly, the open state shows a weak selectivity towards anions, as opposed to the closed state, which blocks the passage of large anionic molecules, while it has been proposed to be selective for cations (Gincel et al., 2000; Schein et al., 1976; Shoshan-Barmatz et al., 2010). At the structural level, the N-terminus of the protein, which forms an α-helix, is important for its voltage-dependent gating (Abu-Hamad et al. 2009). Different mechanistic models have been proposed, albeit the exact mechanism has not been established yet (Shoshan-Barmatz et al. 2010), and this voltage-dependency has only been observed in vitro (Shoshan-Barmatz et al. 2010). Apart from voltage-dependent gating, the closed or open state of the channel is affected by modulators interacting with VDAC1. Examples include Bcl-XL (Vander Heiden et al. 2000, 2001), hexokinase (Azoulay-Zohar et al. 2004), tubulin (Rostovtseva et al. 2008), mitochondrial membrane lipids (Rostovtseva et al. 2006) and Ca^{2+} (Báthori et al. 2006).

In mammals, there are three known isoforms of VDAC: VDAC1, VDAC2 and VDAC3, with VDAC1 being the isoform that is expressed at the highest level and consequently has been studied most extensively (Messina et al. 2012). Recombinant expression of VDAC1 enhanced the Ca^{2+} transfer to the mitochondria (Rapizzi et al. 2002), yet this property seems not to be unique for VDAC1, as also the other isoforms display it (De Stefani et al. 2012). However, there is a unique role for VDAC1, but not for VDAC2 nor VDAC3, in conveying pro-apoptotic Ca^{2+} signals to the mitochondria (De Stefani et al. 2012).

17.2.2 The Chaperones

17.2.2.1 GRP75

GRP75 is a chaperone protein belonging to the heat shock 70 kDa (HSP70) protein family (Wadhwa et al. 2002a). GRP75 has been found at different subcellular localisations, e.g. the cytosol, the mitochondria, the ER and the Golgi apparatus (Wadhwa et al. 1995; Ran et al. 2000). Moreover, GRP75 is a pleiotropic protein. For example, GRP75 located in the mitochondrial matrix helps to import unfolded proteins into the matrix in an ATP-dependent manner in cooperation with Tim44 (Scherer et al. 1992; Kronidou et al. 1994; Schneider et al. 1996; Voos and Röttgers 2002). Furthermore, GRP75 is thought to play a role in endocytosis as well as exocytosis (Flachbartová and Kovacech 2013). At the MAMs, GRP75 plays an important role in Ca^{2+} signalling, as it forms a physical link between the IP$_3$R and VDAC1 (Szabadkai et al. 2006; Betz et al. 2013; Rieusset et al. 2016), thereby increasing the efficiency of ER-mitochondrial Ca^{2+} signalling.

17.2.2.2 Sig-1R

Sig-1R is a chaperone, which was first mistakenly identified as an opioid receptor subtype (Su 1982; Hayashi and Su 2007; Tagashira et al. 2014). Sig-1R mainly resides in the ER, specifically at the MAMs, under resting conditions (Hayashi and Su 2007). There, Sig-1R is bound to an ER lumen chaperone, glucose-regulated protein 78 (GRP78, also known as BiP) (Hayashi and Su 2007). Upon ER stress, however, this association is broken, and Sig-1R gains its chaperone function, targeting its client proteins like the ER stress sensor inositol-requiring enzyme 1 (IRE1) and the IP$_3$R, thereby regulating ER-mitochondrial Ca^{2+} signalling (cfr. infra) (Hayashi and Su 2007; Mori et al. 2013). Interestingly, Shioda et al. (2012) reported the existence of a truncated splice form of Sig-1R, which did not bind to the IP$_3$R. Overexpression of this splice variant decreased mitochondrial Ca^{2+} uptake, while promoting IP$_3$R degradation, as opposed to the non-truncated Sig-1R (Shioda et al. 2012). Furthermore, Sig-1R can undergo translocation to various subcellular localisations upon stimulation by agonists. These locations include the plasma membrane and the nuclear envelope (Su et al. 2010; Mavlyutov et al. 2015; Tsai et al. 2015; Chu and Ruoho 2016).

17.2.2.3 Calnexin

Calnexin is another ER chaperone that is enriched at the MAMs and this in a palmitoylation-dependent manner (Lynes et al. 2012). This chaperone interacts with glycoproteins that are monoglucosylated (Hebert et al. 1995). Functionally, calnexin improves efficiency of ER protein folding and helps retaining misfolded proteins in the ER (Lamriben et al. 2016). Furthermore, calnexin modifies Ca^{2+} signalling in the cell through its functional interaction with sarco/endoplasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) (Roderick et al. 2000). When the cytosolic domain of calnexin is phosphorylated, the interaction inhibits SERCA2b activity, diminishing intracellular Ca^{2+} oscillations (Roderick et al. 2000). In further work, it was shown that calnexin interaction with SERCA was critically dependent on calnexin palmitoylation and was impaired upon ER stress induction (Lynes et al. 2013). The binding of calnexin to SERCA2b appeared to promote its activity, given the higher ER Ca^{2+}-store content of cells overexpressing wild-type calnexin, but not a palmitoylation-deficient calnexin mutant (Lynes et al. 2013). Interestingly, the binding of calnexin to SERCA2b is influenced by the thioredoxin-related transmembrane protein (TMX1), which is also targeted to the MAMs through palmitoylation (Lynes et al. 2012), since knockout of TMX1 improved binding of calnexin to SERCA2b (Krols et al. 2016; Raturi et al. 2016). TMX1 and calnexin thus may target overlapping binding sites in SERCA2b. As such, the stimulatory effect of calnexin on SERCA activity may be partially related to its ability to reduce the binding of TMX1, which inhibits SERCA activity, to SERCA2b (Krols et al. 2016; Raturi et al. 2016).

17.2.3 Proteins Defining MAM Structure

17.2.3.1 Mfn2

Mfn2, a GTPase protein situated in the OMM, was first studied for its function as a mitochondrial fusion protein, together with its homologue Mfn1 (Ranieri et al. 2013). Interestingly, in 2008 it was found that Mfn2 is also located on the ER membrane and enriched at the MAMs, where it acts as a tether that links mitochondria to the ER and supports efficient Ca^{2+} signalling between the two organelles (de Brito and Scorrano 2008). This tethering function of Mfn2 is realised by the interaction of Mfn2 in the ER membrane with Mfn1 or Mfn2 localised in the OMM. Recently, however, a discussion has risen about the role of Mfn2 as a mitochondrial tether in the MAMs. Ultrastructural analyses as well as functional, biochemical and genetic approaches showed that Mfn2 antagonised ER-mitochondrial tethering (Cosson et al. 2012; Filadi et al. 2015), since ablation of Mfn2 resulted in an increased inter-organellar proximity. It was proposed that Mfn2 functions as an anti-tether that maintains a correct, non-toxic distance between both the ER and the mitochondria (Filadi et al. 2015). However, very recently, the role of Mfn2 as a *bona fide* ER-mitochondrial tether has been confirmed in a series of experiments aiming to critically reappraise its function (Naon et al. 2016).

17.2.3.2 PACS-2

PACS-2 is an ER-associated protein involved in retrograde ER-Golgi trafficking of multiple proteins (Youker et al. 2009). This sorting protein was initially studied for its role in mitochondrial network and MAM organisation (Simmen et al. 2005). PACS-2 regulates communication between the ER and the mitochondria by controlling contact sites between the two organelles (Simmen et al. 2005). In this way, PACS-2 mediates apoptosis (cfr. infra) and ER homeostasis, while promoting transfer of lipids between the ER and mitochondria (Simmen et al. 2005). Interestingly, PACS-2 can also assist in calnexin enrichment at the MAMs in concert with the coat protein complex COPI (Myhill et al. 2008). Also mechanistic target of rapamycin complex 2 (mTORC2) can be found at the MAMs where it regulates MAM integrity via PACS-2 phosphorylation in a protein kinase B (PKB/Akt)-dependent way (Betz et al. 2013).

17.2.3.3 PERK

PERK, a kinase protein located in the ER membrane, functions as an ER stress sensor that controls the unfolded protein response (UPR), alongside IRE1 and activating transcription factor 6 (ATF6) (Sano and Reed 2013). The main effect of the UPR on the cell is the diminishment of mRNA translation to avoid further accumulation of improperly folded proteins, while at the same time, the transcription of chaperones is stimulated (Sano and Reed 2013). Furthermore, retrograde transport of unfolded proteins to the cytosol takes place, where they undergo ubiquitination and subsequent degradation (Sano and Reed 2013). PERK is mainly responsible for the halt of translation by virtue of phosphorylating eukaryotic translation initiation factor 2 (eIF2α), a protein needed for correct mRNA translation (Sano and Reed 2013). The phosphorylation disrupts recycling of eIF2α from its GTP-free form to its GTP-bound form (Sano and Reed 2013). Furthermore, eIF2α phosphorylation is responsible for the preferential translation of UPR-involved genes. In addition, PERK phosphorylates nuclear erythroid 2 p45-related factor 2 (Nrf2) inducing the expression of antioxidant genes to alleviate oxidative stress (Cullinan and Diehl 2004). When ER stress continues for a longer period, the cell will brace itself for apoptosis. In this process, PERK contributes by promoting the transcription of CCAAT-enhancer-binding protein homologous protein, a pro-apoptotic transcription factor (Oyadomari and Mori 2004; Sano and Reed 2013). Moreover, PERK was shown to be involved in autophagy regulation via ATF4-dependent transcription of autophagy-related (ATG) genes (Harding et al. 2000).

Additionally, PERK also serves as an ER-mitochondria tether at the MAMs, thereby facilitating the propagation of ROS signals between these two organelles (Verfaillie et al. 2012). Hence, PERK-knockout cells displayed significantly weaker ER-mitochondria contact sites, counteracting ROS-triggered apoptosis. This function of PERK to maintain ER-mitochondria juxtapositions was independent of its kinase activity but required its cytoplasmic domains (Verfaillie et al. 2012).

17.2.3.4 Foetal and Adult Testis Expressed 1 (FATE1)

FATE1 is a protein that belongs to the group of cancer-testis antigens (CTAs) (Dong et al. 2003), which is a heterogeneous group of proteins with limited expression in normal testis tissue. However, in several types of cancer, these CTAs are upregulated (Simpson et al. 2005; Whitehurst 2014). Recently, FATE1 was found to reside at the MAMs where it regulates ER-mitochondrial distance and ER-mitochondrial Ca^{2+} flux (cfr. infra) (Doghman-Bouguerra et al. 2016). Remarkably, silencing FATE1 led to an increased sensitivity towards paclitaxel, a chemotherapeutic drug, in non-small cell lung cancer cell lines (Whitehurst et al. 2007).

17.3 MAMs in Cancer

All types of cancer share certain stereotypical traits, called the hallmarks of cancer (Hanahan and Weinberg 2011; Giampazolias and Tait 2016). These features, acquired gradually during the development of tumours, include sustaining proliferative signalling, resisting cell death, activating invasion and metastasis, inducing angiogenesis and rewiring metabolism (Hanahan and Weinberg 2011; Giampazolias and Tait 2016). Importantly, MAMs and mitochondria play key roles in many cellular processes such as cell death, cell migration and energy production (Giampazolias and Tait 2016). Therefore, functioning of these cellular compartments is frequently altered and affected during acquisition of the hallmarks of cancer. In this section, we discuss the role of the proteins listed above in the various hallmarks of cancer.

17.3.1   Tumour Growth, Proliferation and Metastasis

17.3.1.1   The IP$_3$R

Since Ca^{2+} signalling controls a plethora of cellular functions that relate to cancer hallmarks, IP$_3$Rs have emerged as important regulators of tumour biology. A striking example of the importance of the receptor's function is the observation that lack of IP$_3$R-mediated Ca^{2+} signalling in thymocytes causes the development of malignancies in mice, resembling T-cell acute lymphoblastic leukaemia (Ouyang et al. 2014). Furthermore, there is a growing body of evidence that suggests IP$_3$R-mediated Ca^{2+} release plays a role in cancer cell migration (Wei et al. 2009, 2012; Huang et al. 2016). Also, migrating fibroblasts displayed cytosolic Ca^{2+} flickers mediated in part by IP$_3$R2 (Wei et al. 2009, 2012). Furthermore, IP$_3$R3 is overexpressed in glioblastoma cells, whereas reducing its expression via siRNA attenuated migration via inhibition of cytosolic Ca^{2+} signalling (Kang et al. 2010). Recently, it was also found that overexpression of ER protein 44, which negatively regulates Ca^{2+} release, prevented migration of A549 cells by suppressing IP$_3$R2-dependent Ca^{2+} release (Huang et al. 2016). Moreover, the IP$_3$R was shown to play a role in MCF-7 cell growth, since growth inhibition occurred upon blockage of the IP$_3$R (Szatkowski et al. 2010). Interestingly, 17-β-estradiol, which induces cell proliferation in MCF-7 cells, may do so by elevating IP$_3$R3 levels (Szatkowski et al. 2010). In the same cell line, a molecular and functional coupling between IP$_3$R3 and large-conductance Ca^{2+}- and voltage-dependent K$^+$ (BK$_{Ca}$) channels was responsible for ATP-induced proliferation in a cyclin-D1/cyclin-dependent kinase 4-dependent mechanism (Mound et al. 2013). Ablation of IP$_3$R3 or BK$_{Ca}$ resulted in attenuated proliferation (Mound et al. 2013). Interestingly, the IP$_3$R is also implied in senescence (Wiel et al. 2014), protecting cells from tumour onset and progression (Ben-Porath and Weinberg 2004; Collado and Serrano 2010; Kang et al. 2011). It was shown that loss of IP$_3$R2 allowed cells to avoid

oncogene-induced senescence (Wiel et al. 2014). This was also the case for the mitochondrial Ca^{2+} uniporter. This points to mitochondrial Ca^{2+} accumulation playing an important role in senescence through lowering the mitochondrial membrane potential and ROS (Wiel et al. 2014).

17.3.1.2   VDAC1

The expression levels of VDAC1 are correlated with tumour growth in different types of cancer. Zhang et al. showed that a decrease in miRNA-320a allowed for a high VDAC1 expression in non-small cell lung cancer cells and that this was correlated with the initiation and progression of cancer (Zhang et al. 2016b). Furthermore, cervical cancer tissues positive for VDAC1 showed an increased tumour size and deep stromal invasion compared to tissues negative for VDAC1 (Wu et al., 2016a). In the same study, VDAC1 knockdown inhibited cell proliferation and migration (Wu et al. 2016a), which was also shown in human papilloma virus-related cervical cancers (Zhang et al. 2016a). This evidence suggests that VDAC1 promotes tumour survival and invasion. Interestingly, knockout of VDAC1 in MEF cells increased proliferation rates under hypoxic conditions through activation of the extracellular signal-regulated protein kinase (ERK) 1/2 pathway (Brahimi-Horn et al. 2015).

Moreover, VDAC1's role in Ca^{2+} signalling has been linked to cell migration. Myeloid cell leukaemia sequence 1 (Mcl-1), an anti-apoptotic protein from the B-cell lymphoma 2 (Bcl-2) protein family, is able to bind VDAC1 with high affinity, thereby seemingly promoting mitochondrial Ca^{2+} uptake. Mcl-1 binding to VDAC1 promoted cell migration without affecting cell proliferation. The pro-migration effect of Mcl-1 could be antagonised by VDAC-based peptides that interfere with VDAC1/Mcl-1-complex formation (Huang et al. 2014). Also other anti-apoptotic Bcl-2 proteins, including Bcl-XL, inhibit VDAC1-mediated Ca^{2+} uptake in the mitochondria (Arbel et al. 2012; Monaco et al. 2015; Vervliet et al. 2016). The mechanism involved Bcl-XL's Bcl-2 homology 4 (BH4) domain and VDAC1's N-terminus (Monaco et al. 2015). The inhibitory impact of Bcl-2

proteins on VDAC1-mediated Ca^{2+} uptake in the mitochondria is consistent with the original papers that describe Bcl-2 proteins as negative regulators of VDAC1-mediated apoptosis (Shimizu et al. 1999, 2000).

17.3.1.3 The Chaperones

A study by Vilner et al. (1995) demonstrated that Sig-1R was overexpressed in a large range of cancer cell lines, both human and rodent. Later studies added that there might be a link between Sig-1R overexpression and metastasis. This was proposed by Aydar et al. since the highest expression levels were found in metastatic cell lines (Aydar et al. 2006). Sig-1R mRNA levels were also found to be higher in invasive breast cancer tissue derived from patients, compared to normal breast tissue (Wang et al. 2004); Sig-1R mRNA was overexpressed in colorectal cancer and colorectal cancer liver metastases (Skrzycki and Czeczot 2013), and overexpression of Sig-1R in hilar cholangiocarcinoma was linked to poor differentiation, lymph node metastasis and advanced disease stage (Xu et al. 2014).

Concerning the molecular mechanisms that are possibly involved, the ability of Sig-1R to interact with several ion channels seems to be important for various oncogenic features (Crottès et al. 2013). In K562 myeloid leukaemia cells, the link between Sig-1R and expression of the potassium ion channel human ether-à-go-g-related gene (hERG), which controls several processes like migration and adhesion (Pillozzi et al. 2007, 2011), was investigated (Crottès et al. 2011). It was observed that Sig-1R is important for hERG maturation by improving maturation efficiency and stabilisation of the α-subunit (Crottès et al. 2011). Other ion channels that interact with Sig-1R and that play a role in cancer include L-type voltage-gated Ca^{2+} channels, voltage-gated Na^+ channels and Ca^{2+}-activated K^+ channels (for extensive review, see Crottès et al. 2013). However, the interaction of Sig-1R with these ion channels is not necessarily related to its role at the MAMs.

Not only by directly interacting with other proteins, but also indirectly, Sig-1R is able to modify properties of ion channels: Palmer et al. (2007) found that Sig-1R can bind cholesterol and stabilise lipid rafts via the insertion of cholesterol. In turn, the cholesterol level of lipid rafts can impact the signalling molecules present in these domains (Gniadecki 2004; Palmer et al. 2007), thereby altering the activity of ion channels nearby.

In addition, GRP75 overexpression is correlated with tumour growth and invasion (Kaul et al. 1998; Yi et al. 2008; Jin et al. 2016). Also, in K562 cells high expression levels of GRP75, as well as other chaperone proteins, coincided with resistance towards the proteasome inhibitor bortezomib (Klíková et al. 2015), and inhibition of GRP75 reduced cisplatin resistance in ovarian cancer (Yang et al. 2013).

Also calnexin, as a chaperone, may play a role in tumoural growth in response to growth factors. Lakkaraju and van der Goot (2013) found that in squamous carcinoma cells, caspase-8-mediated cleavage of calnexin occurs upon stimulation of the cells with epidermal growth factor. This yields a calnexin fragment that inhibits protein inhibitor of activated STAT3 (PIAS3), an inhibitor of signal transducer and activator of transcription 3 (STAT3), which functions as an oncogenic transcription factor (Lakkaraju and van der Goot 2013). This, in turn, promotes STAT3-dependent transcription and possibly tumour growth (Lakkaraju and van der Goot 2013).

17.3.1.4 Proteins Defining MAM Structure

The importance of Mfn2 for cell proliferation is suggested by findings in vascular smooth muscle cells: overexpression of Mfn2 in cultured vascular smooth muscle cells inhibited proliferation by blocking the mitogen-activated protein kinase (MAPK)/ERK signalling pathway (Chen et al. 2004). This mechanism was found to be independent of its role in mitochondrial fusion (Chen et al. 2004; Guo et al. 2007). In concert with observations of Mfn2 acting in an anti-proliferative way, Zhang et al. (2013) showed that Mfn2 expression was lower in gastric tumours than in normal mucosal tissue and that expression levels were negatively correlated with tumour size, while Wu et al. (2016b) observed that poor

overall survival in hepatocellular carcinoma patients correlated with low Mfn2 expression levels. Also in primary breast cancer, a loss of Mfn2 was detected (Kannan et al. 2016). Strikingly, knockdown of TMX1 in HeLa and A375P melanoma cells generates a similar phenotype as low-level Mfn2 expression (Raturi et al. 2016). This includes increased SERCA activity and altered MAM structure (Raturi et al. 2016). In the case of TMX1, Raturi et al. propose that the stimulatory effect on tumour growth upon TMX1 knockdown is due to an elevated Ca^{2+} retention capacity at the ER combined with an increased ER-mitochondrial distance (Raturi et al. 2016). This, in turn, leads to reduced ER-mitochondrial Ca^{2+} flux and impairment of mitochondrial metabolism, possibly contributing to the Warburg effect (cfr. infra) (Raturi et al. 2016).

Interestingly, another study revealed that Mfn2 deficiency decreased proliferation by blocking autophagy in HeLa cells (Ding et al. 2015). Similarly, A549 human lung adenocarcinoma cells showed disturbed cell proliferation and invasion upon Mfn2 knockdown (Lou et al. 2015). A recent bioinformatics study conducted on the same cell line revealed that Mfn2 knockdown resulted in repression of genes implicated in cell-cycle progression as well as DNA replication and MAPK signalling pathway (Lou et al. 2016). These opposing data suggest that Mfn2's role in cancer is highly context dependent. It may however also relate to a critical window for proper ER-mitochondria distance, in which too close apposition results in excessive apoptosis sensitivity, whereas too far apposition results in defective energetic and metabolic features, as discussed elsewhere (Naon and Scorrano 2014).

PERK, the ER stress sensor, also fulfils this double-edged function in relation to tumour growth. For example, PERK activity has been linked to cell-cycle arrest (Brewer and Diehl 2000; Hamanaka et al. 2005). Since PERK is involved in a general slowdown of the translation process, several proteins are impacted including the drivers of the cell cycle, which comprise the cyclins (Brewer and Diehl 2000; Hamanaka et al. 2005). In this case, cyclin D1's expression is

severely altered due to its short half-life (Brewer and Diehl 2000; Hamanaka et al. 2005). This brings about a redistribution of p21, which blocks cyclin-dependent kinase 2, resulting in cell-cycle arrest in the G1 phase (Brewer and Diehl 2000; Hamanaka et al. 2005).

In addition, PERK causes an increase in p53 through decreased E3 ubiquitin ligase human double minute 2 (Hdm2)-dependent removal, promoting apoptosis when a certain threshold is reached (Li et al. 2006; Zhang et al. 2006). Another feature of this p53 accumulation is the induction of p21 and subsequent cell-cycle arrest, as stated above (Ono et al. 1997). Furthermore, PERK is involved in the upregulation of p47, an N-terminal truncated analogue of p53, which mediates cell-cycle arrest in the G2 phase (Bourougaa et al. 2010). Conversely, PERK has been proposed to improve the degradation of p53 in a glycogen synthase kinase 3 β (GSK3β)-dependent manner (Qu et al. 2004; Pluquet et al. 2005).

Also, PERK and its downstream signalling axis have been implicated in metastasis of several cancers, e.g. cervix cancer, breast cancer and head and neck squamous cell carcinoma (Nagelkerke et al. 2013, 2015; Mujcic et al. 2013). Epithelia to mesenchymal transition, which is an indication of the level of invasiveness, is also correlated with PERK signalling, which is underpinned by observations in primary breast cancer, colon cancer, gastric cancer and lung cancer (Feng et al. 2014). Furthermore, the human epidermal growth factor receptor 2 (HER2)/Neu protein is able to induce PERK activity, which allows for redox homeostasis via Nrf2 (Bobrovnikova-Marjon et al. 2010). Subsequently, loss of PERK in HER2/Neu-dependent mammary adenocarcinoma was responsible for growth attenuation and decreased metastasis (Bobrovnikova-Marjon et al. 2010).

About the role of PACS-2 in tumour progression, not much is known. However, PACS-2 was shown to be a regulator of ADAM17, a metalloproteinase that is involved in epithelial development, growth and tumour progression (Dombernowsky et al. 2015). More specifically, loss of PACS-2 diminished ADAM17 levels at

the cell surface due to increased degradation (Dombernowsky et al. 2015).

17.3.2 Apoptosis

Another major hallmark of cancer cells is their ability to evade apoptosis (Hanahan and Weinberg 2011). As briefly indicated before, Ca^{2+} signalling is able to regulate the apoptotic process. While Ca^{2+} oscillations were found to be pro-survival signals due to stimulation of critical enzymes of the tricarboxylic acid cycle (TCA), high-amplitude Ca^{2+} signals that last for a longer time can cause apoptosis through mitochondrial Ca^{2+} overload (Hajnóczky et al. 1995; Orrenius et al. 2003; Joseph and Hajnóczky 2007; Roderick and Cook 2008; Denton 2009). This, in turn, causes the opening of the mitochondrial permeability transition pore, mitochondrial swelling and eventually the release of pro-apoptotic factors like cytochrome c in the cytosol (Halestrap 2014; Morciano et al. 2015; Jonas et al. 2015). In order to prevent Ca^{2+}-induced apoptosis and/or to promote Ca^{2+}-dependent bioenergetics, cells may rewire their Ca^{2+}-signalling toolkit (Capiod et al. 2007; Chen et al. 2013; Stewart et al. 2015).

17.3.2.1 The IP_3R

The IP_3R exerts a central role in ER-mitochondrial Ca^{2+} signalling, making it prone to the electrical rewiring of the cancer cell. IP_3R expression levels are altered in various cancers, supporting the critical role of the IP_3R in Ca^{2+} signalling from the ER. For instance, hormone-refractory prostate tumour cells showed increased levels of IP_3R1 (Boutin et al. 2015). This is thought to increase Ca^{2+} leakage from the ER, so that less Ca^{2+} is available for the induction of apoptosis by mitochondrial Ca^{2+} overload (Boutin et al. 2015). Interestingly, bladder cancer cells evade cell death by doing the opposite: treatment with cisplatin diminished IP_3R1 levels, provoking cisplatin resistance (Tsunoda et al. 2005). By lowering IP_3R expression levels, cancer cells prevent the event of toxic mitochondrial Ca^{2+} overload (Prevarskaya et al.

2014). Furthermore, some diffuse large B-cell lymphomas (DLBCLs) express high levels of IP_3R2 (Akl et al. 2013). The reason for this IP_3R2 elevation remains elusive, but one hypothesis is that in metabolically stressed cancer cells, low levels of ATP, a positive regulator of the IP_3R, are insufficient to provide the basal Ca^{2+} signalling needed to fuel mitochondrial bioenergetics (Akl et al. 2013; Akl et al. 2014). Hence, by upregulation of the IP_3R2, which is the IP_3R isoform most sensitive to IP_3, these cancer cells are able to survive (Akl et al. 2013, 2014).

In cancer cells, the process of apoptosis is not only influenced by modifying the expression levels of the IP_3R, but also by altering its Ca^{2+}-release properties. For instance, phosphorylation of the IP_3R dramatically changes its function (Vanderheyden et al. 2009). PKB/Akt is a serine-threonine kinase that phosphorylates the IP_3R C terminally via a substrate motive that is conserved in all isoforms (Khan et al. 2006). While ER Ca^{2+} levels remain unaffected in HeLa cells overexpressing PKB/Akt, IP_3R-dependent ER Ca^{2+} release was shown to be negatively affected (Szado et al. 2008; Marchi et al. 2008). Again, this mechanism may protect against mitochondrial Ca^{2+} overload and subsequent apoptosis (Marchi et al. 2008). Furthermore, it was shown that the protective effect of PKB/Akt overexpression was isoform specific: in COS7 cells, almost completely lacking IP_3R1, PKB/Akt activation led to a decreased IP_3-induced Ca^{2+} release and conferred a protective effect against apoptosis (Marchi et al. 2012). However, in SH-SY 5Y cells, lacking IP_3R3, ER Ca^{2+} release was not modified, while expressing the type 3 isoform in these cells restored the protective effect (Marchi et al. 2012). This suggests that the anti-apoptotic effect of PKB/Akt is mediated in an IP_3R3-dependent way (Marchi et al. 2012). The effect of PKB/Akt-mediated phosphorylation is thought to be directly counteracted by phosphatase and tensin homolog (PTEN), which also localises at the MAMs and dephosphorylates the IP_3R, thereby increasing again the IP_3R-mediated Ca^{2+} release (Bononi et al. 2013).

A recent study showed that extra-nuclear PML contributes to protection against Ca^{2+}-mediated apoptotic cell death via interaction with the IP_3R (Giorgi et al. 2010). PML located at the MAMs physically interacts with the IP_3R3. In cells expressing ER-targeted PML, apoptotic stimuli induced a higher cytosolic and mitochondrial Ca^{2+} response (Giorgi et al. 2010). Furthermore, it was revealed that PML-expressing cells displayed lower levels of phosphorylated IP_3R3 and phosphorylated, active PKB/Akt and higher levels of the phosphatase 2A (PP2A) compared to PML-negative cells (Giorgi et al. 2010). PML stimulates pro-apoptotic Ca^{2+} signalling at the MAMs by recruiting PP2A to IP_3R3-PKB/Akt complexes (Giorgi et al. 2010), resulting in a suppressed PKB/Akt-mediated IP_3R3 phosphorylation, since PP2A negatively regulates the activity of PKB/Akt at the ER (Pinton et al. 2011; Bittremieux et al. 2016).

IP_3R function can also be stimulated by phosphorylation (Gomez et al. 2016). During reperfusion injury of the heart, a fraction of the protein kinase GSK3β is localised at the sarco/endoplasmic reticulum (SR/ER) and the MAMs. There, it interacts with the IP_3R Ca^{2+}-channelling complex, regulating its protein composition and modulating Ca^{2+} transfer between the SR/ER and mitochondria. During hypoxia reoxygenation, GSK3β activity is augmented, resulting in increased IP_3R phosphorylation and IP_3R hyperactivity. Consequently, increased IP_3R-mediated SR/ER-mitochondria Ca^{2+} transfer leads to cardiomyocyte cell death. Therefore, inhibition of GSK3β may protect the heart from lethal reperfusion injury by cellular Ca^{2+} overload (Gomez et al. 2016).

The critical role of IP_3R3 underlying cellular apoptosis sensitivity has also been confirmed independently in two isogenic cell lines, one of which expresses oncogenic K-Ras and one in which this oncogenic allele was deleted via homologous recombination (Pierro et al. 2014). The presence of oncogenic K-Ras caused a lowering in the ER Ca^{2+}-store content, thereby decreasing the likelihood of pro-apoptotic Ca^{2+} transfer and thus decreasing apoptotic sensitivity (Pierro et al. 2014). This was due to the increased

expression level of IP_3R1 relative to that of IP_3R3 in the cell line expressing oncogenic K-Ras, augmenting basal Ca^{2+} leak via IP_3R1 and suppressing pro-apoptotic Ca^{2+} transfer into the mitochondria via the IP_3R3 (Pierro et al. 2014).

Apart from phosphorylation and expression regulation, IP_3R-mediated Ca^{2+} release can be modulated directly by the binding of an increasing number of oncogenes and tumour suppressors (Akl and Bultynck 2013; Bittremieux et al. 2016). Notably, several members of the Bcl-2-protein family are known to interact with the IP_3R. First, there is the anti-apoptotic protein Bcl-2 itself, whereas its canonical function comprises the sequestration of pro-apoptotic Bcl-2-protein family members like Bik and Bid via its BH3 domain (Youle and Strasser 2008; Czabotar et al. 2014) evidence was found that Bcl-2 interacts via its BH4 domain with 20 amino acids in the central, modulatory part of the IP_3R (Rong et al. 2008, 2009; Monaco et al. 2012). This interaction protects cells against Ca^{2+}-mediated apoptotic cell death (Hanson et al. 2008; Rong et al. 2008). The importance of Bcl-2's complex formation with the IP_3R became clear in Bcl-2-dependent chronic lymphocytic leukaemia and in DLBCL cell lines (Zhong et al. 2011; Akl et al. 2013). Some subtypes of DLBCL display high levels of IP_3R2, the most sensitive isoform with respect to IP_3 (Akl et al. 2013). Therefore, Bcl-2 overexpression is needed in these cells to avoid Ca^{2+}-induced apoptosis triggered by the high expression levels of IP_3R2, making these cells balancing on the edge of apoptosis, a state that was coined "primed-for-death at the ER" (Akl et al. 2013; Akl et al. 2014). Following this concept, TAT-IDP, a peptide mimicking the IP_3R-binding site for Bcl-2, and its derivative BIRD-2, induced apoptosis by disrupting IP_3R/Bcl-2 interaction and eliciting spontaneous toxic Ca^{2+} signalling (Zhong et al. 2011; Akl et al. 2013; Akl et al. 2015; Lavik et al. 2015; Greenberg et al. 2015).

Apart from Bcl-2, the closely related anti-apoptotic Bcl-XL protein is also able to interact with the IP_3R, albeit not via its BH4 domain

(White et al. 2005; Monaco et al. 2012). Bcl-XL, through its hydrophobic cleft, binds the IP$_3$R at its C-terminal region (Eckenrode et al. 2010) by targeting two BH3-domain-like sequences (Yang et al. 2016). The binding between Bcl-XL and the IP$_3$R results in a reduction in ER [Ca^{2+}], increased Ca^{2+} oscillations in the cytosol and protection against apoptosis by sensitising the IP$_3$R to low basal levels of IP$_3$ (Li et al. 2007).

Bcl-XL does not only alter Ca^{2+} signalling in the cell via direct modulation of the IP$_3$R, but also via a nuclear factor of activated T-cells (NFAT)-dependent pathway, which modifies IP$_3$R expression levels (Li et al. 2007). Furthermore, Mcl-1, another Bcl-2-protein family member, was shown to interact directly with the IP$_3$R via the last transmembrane domain in its C-terminal tail (Eckenrode et al. 2010). Like Bcl-XL, Mcl-1 was found to sensitise the IP$_3$R, thereby increasing the frequency of Ca^{2+} oscillations in the cell and the number of oscillating cells (Eckenrode et al. 2010). Figure 17.2 displays the proteins that regulate ER-mitochondrial Ca^{2+} signalling via the IP$_3$R and VDAC1. For reasons of clarity, a distinction was made between the regulation of pro-survival and pro-apoptotic Ca^{2+} signalling.

Finally, inhibition of Bcl-2-family members has emerged as an attractive anticancer strategy, particularly by preventing the complex formation between the anti- and pro-apoptotic Bcl-2-family members (Davids and Letai 2012). However, the function of anti-apoptotic Bcl-2-family members appears to be involved in ER-mitochondrial contact sites as well. Indeed, recent studies showed that targeting the hydrophobic cleft using BH3 mimetics like ABT-737, a non-selective Bcl-2/Bcl-XL inhibitor, enhance anticancer treatments by increasing ER-mitochondria contact sites and stimulating ER-mitochondrial Ca^{2+} transfer (Fan et al. 2015; Xie et al. 2016). As such, ABT-737 could restore the sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin treatment. This correlated with the ability of cisplatin to induce mitochondrial Ca^{2+} overload, an important feature of the successful induction of cell death by anticancer treatments (Bittremieux and Bultynck 2015; Bonora et al. 2015; Fan et al. 2015; Giorgi et al. 2015a; Xie et al. 2016).

17.3.2.2 VDAC1

As VDAC1 is the gateway for Ca^{2+} entry in the mitochondria, this protein's function may also be influenced in cancer cells to ensure their survival. Bcl-XL was found to bind to and block VDAC1 with its BH4 domain, thereby inhibiting Ca^{2+}-mediated apoptosis (Monaco et al. 2015). Intriguingly, cytosolic Ca^{2+} levels impact VDAC1 expression levels (Weisthal et al. 2014). An increase in cytosolic [Ca^{2+}], elicited by, for example, H$_2$O$_2$, induces a rise in VDAC1 expression, which at the same time correlates with the ability to form oligomers in the OMM, through which the pro-apoptotic protein cytochrome c is released from the mitochondria (Weisthal et al. 2014).

VDAC1 does not only contribute to Ca^{2+}-mediated apoptotic cell death, but also influences apoptosis occurring independently of Ca^{2+}. For instance, in human glioma cells subjected to hypoxia, VDAC1 has been implicated in the activation of mitophagy (Qiao et al. 2016). In these cells, the mitochondrial deacetylase sirtuin-3 (Sirt3) stimulates the association between VDAC1 and parkin, an E3 ubiquitin ligase, stimulating mitophagy (Qiao et al. 2016; Bernardini et al. 2017). Consequently, knockdown of Sirt3 inhibited mitophagy, rendering the cells prone to apoptotic cell death (Qiao et al. 2016). Thus, VDAC1 in concert with Sirt3 plays a role in protecting cancer cells through mitophagy. Interestingly, parkin also seems to play a role in the regulation of mitochondrial homeostasis and energy metabolism (Calì et al. 2013). Overexpression of parkin in HeLa and SH-SY5Y neuroblastoma cells increased physical as well as functional interactions between the ER and the mitochondria, whereas parkin silencing caused mitochondrial fragmentation and compromised mitochondrial Ca^{2+} transients due to reduced ER-mitochondria tethering (Calì et al. 2013).
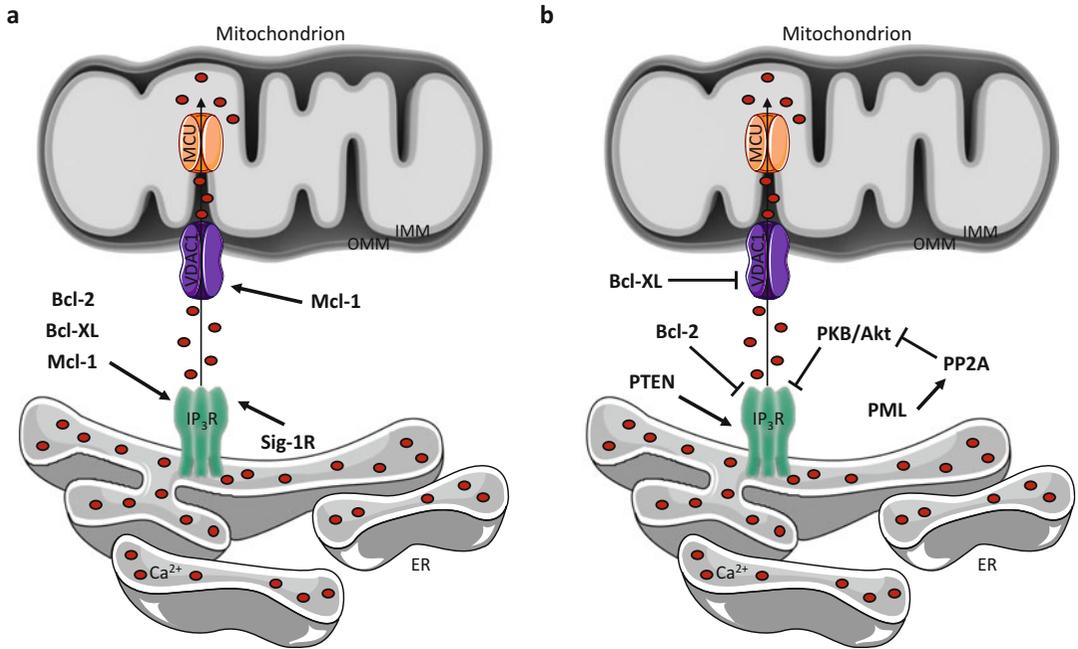
Fig. 17.2 Regulation of pro-survival and pro-apoptotic Ca²⁺ signalling at the MAMs. *Arrow-headed lines* indicate a stimulatory interaction, *while bar-headed lines* indicate an inhibitory interaction. (**a**) Regulation of pro-survival Ca²⁺ signalling at the MAMs. Bcl-2, Bcl-XL and Mcl-1 increase pro-survival Ca²⁺ oscillations and stimulate cell metabolism by interacting with the C-terminus of the IP₃R, which results in a sensitisation of the channel to basal IP₃ levels. Mcl-1 also enhances cell survival through binding to VDAC1, thereby increasing its activity and thus mitochondrial Ca²⁺ uptake. While these proteins directly impact the Ca²⁺-flux properties of the IP₃R or VDAC1, Sig-1R indirectly promotes cell survival. Under conditions of ER stress, Sig-1R becomes active and stabilises the IP₃R, ensuring the transmission of pro-survival Ca²⁺ signalling into the mitochondria. (**b**) Regulation of pro-apoptotic Ca²⁺ signalling at the MAMs. Bcl-2 and Bcl-XL do not only support pro-survival Ca²⁺ signalling, but also inhibit pro-apoptotic Ca²⁺ signalling. Binding of Bcl-2 via its BH4 domain to the central region of the IP₃R diminishes the Ca²⁺ flux through the IP₃R, while Bcl-XL inhibits VDAC1 via its BH4 domain that targets the N-terminus of VDAC1. Ca²⁺ release from the ER is also decreased by PKB/Akt, which inhibits IP₃R function by phosphorylation. The phosphatase PTEN counteracts the function of PKB/Akt at the MAMs by dephosphorylating the IP₃R. PML indirectly influences the phosphorylation state of the IP₃R by recruiting the phosphatase PP2A, which negatively regulates PKB/Akt activity in the MAMs and counteracts PKB/Akt-mediated phosphorylation of IP₃R in the MAMs. Thus, PML alleviates the suppression of IP₃R-mediated Ca²⁺ flux from the ER to the mitochondria imposed by PKB/Akt (For more details, please see text)

Furthermore, VDAC1 provides a link between apoptosis and differentiation of cancer cells. In glioblastoma tumour cells, in which VDAC1 expression was silenced, a shift from pro-apoptotic proteins linked to cell proliferation, including avian myelocytomatosis virus oncogene cellular homolog (c-Myc) and nuclear factor κB (NF-κB), to pro-apoptotic proteins regulating cell differentiation, including p53, was observed (Arif et al. 2016). This led to differentiation of the glioblastoma cells into astrocyte- and neuron-like cells. Additionally, several studies showed that hexokinase (HK), the enzyme catalysing the first step of glycolysis (Wilson 2003), bound to VDAC1, confers protection from apoptosis in HEK and HeLa cells (Bryson et al. 2002). This protection is proposed to result from the inhibition of the interaction between VDAC1 and the pro-apoptotic Bcl-2 family member Bax by HK (Bryson et al. 2002). Interestingly, for HK to execute its anti-apoptotic effect, its binding to VDAC1 is needed (Arzoine et al. 2009; Abu-Hamad et al. 2009). Furthermore, evidence also suggests that the

binding of HK to VDAC1 reduced mitochondrial ROS generation (da-Silva et al. 2004; Sun et al. 2008). Since ROS production is often elevated in cancer cells (Liou and Storz 2010; Panieri and Santoro 2016), this may be another mechanism by which HK protects tumour cells against cell death, as both the HK-I and -II isoforms conveyed protection towards apoptosis-inducing oxidants through their association with VDAC1 (Bryson et al. 2002; Ahmad et al. 2002).

17.3.2.3   The Chaperones

Also the chaperone proteins at the MAMs are able to modify the cell's apoptotic pathways. Similar to VDAC1, Sig-1R acts in a Ca^{2+}-dependent and Ca^{2+}-independent way. Apoptotic Ca^{2+} signalling is impacted by Sig-1R's ability to bind to the IP$_3$R. Knockdown of Sig-1R resulted in increased degradation of the IP$_3$R3 via the proteasome, suggesting that it has a stabilising function (Hayashi and Su 2007). Furthermore, in conditions of physiologically normal ER [Ca^{2+}], Sig-1R is in a resting state, bound to GRP78 (Hayashi and Su 2007). However, under conditions of depletion of the ER Ca^{2+} stores, the Sig-1R/GRP78 complex is disrupted, and Sig-1R obtains its chaperone activity (Hayashi and Su 2007). It targets the IP$_3$R to ensure that IP$_3$-mediated pro-survival Ca^{2+} signalling to the mitochondria occurs properly. Interestingly, while during short periods of ER Ca^{2+} depletion, Sig-1R remains localised at the MAMs it is redistributed throughout the ER upon longer ER Ca^{2+} depletion, again as a pro-survival mechanism under continued ER stress, which is often present in cancer cells (Hayashi and Su 2007). By stabilising the IP$_3$R, it sustains pro-survival Ca^{2+} signalling during ER stress. Alternatively, it was reported that Sig-1R supports cell survival during ER stress via another client protein: IRE1 (Mori et al. 2013). Activation of IRE1 triggers its endonuclease activity, needed for splicing X-box binding protein-1 (XBP1) mRNA. This transcription factor then promotes the transcription of various ER chaperones (Yoshida et al. 2001). It is thought that Sig-1R's stabilisation of IRE1 contributes to prolonged signalling along the IRE1-XBP1 axis, thereby supporting cell survival under conditions of augmented and prolonged ER stress (Mori et al. 2013).

GRP75, on the other hand, impacts apoptosis in cancer cells primarily through its alteration of MAPK/ERK signalling and influence on p53. In medullary thyroid carcinoma cells, apoptosis and inhibition of cell growth were caused by a depletion of GRP75 (Starenki et al. 2015). Investigations into the pathways involved revealed that a temporary activation of MAPK/ERK signalling was responsible for the growth arrest, while apoptosis was induced through mitochondrial dysfunction. This consisted of loss of the mitochondrial membrane potential, lowered oxygen consumption and an elevation of ROS levels. Furthermore, it was shown that these mitochondrial effects were linked to a decrease in Bcl-2 expression (Starenki et al. 2015). Similar results in different cancer cell lines confirmed GRP75 as a negative regulator of MAPK/ERK signalling (Wu et al. 2013). An additional contribution to the anti-apoptotic function of GRP75 is its capacity to bind the tumour suppressor p53 (Wadhwa et al. 1998; Kaul et al. 2001; Wadhwa et al. 2002b). The interaction prevents nuclear translocation of p53, abrogating its function as a transcription factor (Wadhwa et al. 2002b). Moreover, keeping p53 in the cytoplasm speeds up its proteasomal degradation (Kaul et al. 2005). Strikingly, applying the HSP90 inhibitor 17-AAG, which blocks other heat shock proteins but not GRP75, stimulated GRP75 expression and reinforced its binding to p53, weakening the effect of the HSP90 inhibitor in hepatocellular carcinoma (Guo et al. 2014).

Also calnexin has been implicated in the regulation of apoptotic cell death. However, it seems that calnexin may play an anti-apoptotic as well as a pro-apoptotic role dependently on the circumstances. Caspase-3 and Caspase-7 have both been shown to cleave calnexin in vitro, while overexpression of the cleavage product partially inhibited apoptosis (Takizawa et al. 2004). Indirectly, calnexin also fulfils a pro-apoptotic role: its cytosolic tail is able to recruit caspase-8, which is responsible for the cleavage of Bap31 (Breckenridge et al. 2002;

Delom et al. 2007). Bap31's cleaved form stimulates Ca^{2+} release from the ER and apoptosis ensues (Breckenridge et al. 2003). In the MCF-7 breast cancer cell line, which is resistant to tunicamycin-induced cell death, calnexin is able to sensitise the cells to tunicamycin, independently of its chaperone function (Delom et al. 2007).

17.3.2.4 Proteins Defining MAM Structure

In MCF-7 cells, Mfn2 mediated apoptosis via the phosphoinositide 3-kinase (PI3K)/PKB/Akt signalling pathway (Ma et al. 2015). These results mimic the findings in vascular smooth muscle cells. There, Mfn2 was shown to trigger mitochondrial apoptosis by inhibiting the GTPase Ras, resulting in decreased PKB/Akt signalling along the Ras-PI3K-PKB/Akt axis (Guo et al. 2007). However, it was not established whether lowered PKB/Akt signalling was linked to an increased Ca^{2+} signalling through reduced phosphorylation of the IP_3R. In this regard, it is interesting to note that Wang et al. (2015) found that overexpression of Mfn2 induced Ca^{2+}-dependent apoptosis in hepatocellular carcinoma.

Additionally, several studies have linked PACS-2 with the apoptotic process, both via the intrinsic pathway as through the extrinsic one. For example, PACS-2 has been shown to interact with the Bcl-2-family protein Bid (Simmen et al. 2005): upon the addition of apoptotic stimuli, PACS-2 is responsible for the translocation of Bid to the mitochondria, where cytochrome c release and caspase activation ensue (Simmen et al. 2005). Furthermore, PACS-2 is involved in tumour necrosis factor-related apoptosis inducing ligand (TRAIL)-triggered apoptosis, more specifically in lysosomal permeabilisation (Werneburg et al. 2012). PACS-2 recruits Bim and Bax, two other members of the Bcl-2 family, to the lysosomal membrane to bring about cathepsin B release and subsequent apoptosis (Werneburg et al. 2012). On this note, it is interesting that cellular inhibitor of apoptosis protein-1 and -2 (cIAP-1/cIAP-2) repress expression levels of PACS-2 by promoting its ubiquitinylation (Guicciardi et al. 2014). In this way,

these cIAPs confer resistance to TRAIL-induced apoptosis in hepatobiliary cancer cell lines (Guicciardi et al. 2014). Curiously, PKB/Akt-mediated phosphorylation of Ser437 serves as a switch to shift from PACS-2's trafficking function to its function as a promoter of apoptosis (Aslan et al. 2009). When phosphorylated, PACS-2 is bound to the 14-3-3 scaffold protein, which inhibits its role in apoptosis (Aslan et al. 2009), in a similar way as the Bcl-2-protein family Bad (Zha et al. 1996). Interestingly, phosphorylation of PACS-2 does not merely serve to repress apoptosis, but is also required for polycystin-2 localisation to the ER (Aslan et al. 2009).

Apart from engaging Bcl-2-protein family members, PACS-2 also influences the regulation of p53. Sirt1 deacetylates p53, but upon DNA damage, PACS-2 is shuttled to the nucleus, where it interacts with Sirt1, preventing the deacetylation of p53 and inducing p21-dependent cell-cycle arrest (Atkins et al. 2014). Contrasting with its pro-apoptotic function, however, a recent study has found that PACS-2 was necessary for NF-κB-dependent Bcl-XL induction in response to DNA damage (Barroso-González et al. 2016).

Lastly, PERK has been shown to play a role in the survival of c-Myc-dependent cancer cells via its involvement in autophagy (Hart et al. 2012). As an oncogene, c-Myc regulates ribosome expression and biogenesis, increasing protein synthesis (van Riggelen et al. 2010). This increased synthesis load is accompanied by elevated PERK activity, which promotes autophagy as a survival mechanism through the upregulation of unc-51-like kinase 1 and ATG5 (Hart et al. 2012). This is underpinned by the observation that upon loss of PERK in these cancer cells, the balance tips from survival to apoptosis due to decreased autophagy (Hart et al. 2012).

Furthermore, PERK seems to play an important role in tumour cell survival under hypoxic conditions. Hypoxia was shown to trigger PERK signalling in xenograft models, while a dominant-negative PERK or eIF2α was linked with an increase in apoptotic cells in hypoxic regions of the tumour (Bi et al. 2005). Delving

into the molecular mechanisms responsible for this protection against hypoxia, an increase in ATG5 expression was found, indicating that the induction of autophagy provides the protection for tumour cells (Kouroku et al. 2007; Rouschop et al. 2010).

Recently, the CTA FATE1 has been discovered at the MAMs, where it controls ER-mitochondrial distance (Doghman-Bouguerra et al. 2016). In fact, FATE1 functions as an anti-tether: it diminishes ER-mitochondrial contact sites and decreases mitochondrial Ca^{2+} uptake. Hence, it regulates the sensitivity towards pro-apoptotic stimuli that elicit apoptosis via Ca^{2+} signalling (Doghman-Bouguerra et al. 2016). Another anti-apoptotic function of FATE1 is its role in the prevention of accumulation of Bik, a pro-apoptotic Bcl-2-protein family member (Maxfield et al. 2015). The underlying molecular mechanism consists of the recruitment of the E3 ligase RNF183 by FATE1 and subsequent stimulation of Bik degradation (Maxfield et al. 2015), allowing cells to survive even in the presence of apoptotic stimuli.

17.3.3 Cellular Energetics and Biochemical Pathways

17.3.3.1 The IP$_3$R
Ca^{2+} plays an important role in controlling mitochondrial bioenergetics, since it stimulates ATP production and mitochondrial respiration as the α-ketoglutarate, isocitrate and pyruvate dehydrogenases are Ca^{2+}-dependent rate-limiting enzymes of the TCA (Cárdenas et al. 2010; Kaufman and Malhotra 2014). These dehydrogenases are inhibited in the absence of constitutive low-level Ca^{2+} transfer from ER to mitochondria, which turns on AMP-activated kinase (AMPK). This results in an increase in basal autophagic flux that is independent of mTOR (Cárdenas et al. 2010; Cárdenas and Foskett 2012). The pro-survival low-level IP$_3$R-mediated Ca^{2+} signalling can be modified by the activity of several anti-apoptotic Bcl-2-family proteins, which are often upregulated in cancer (Bittremieux et al. 2016). Bcl-2, Bcl-XL as well

as Mcl-1 have been reported to interact with IP$_3$Rs (White et al. 2005; Li et al. 2007; Eckenrode et al. 2010). The anti-apoptotic proteins target the C-terminal region of the IP$_3$R (a.a. 2570-2749) (White et al. 2005), resulting in a sensitisation of the IP$_3$Rs to basal IP$_3$ levels, thereby enhancing IP$_3$R-dependent Ca^{2+} oscillations and stimulating mitochondrial bioenergetics. Bcl-XL is also present in the MAMs, targeting and stimulating IP$_3$Rs and driving mitochondrial metabolism (Williams et al. 2016). Bcl-XL is recruited to the MAMs during non-apoptotic ER stress induction, augmenting mitochondrial bioenergetics through interaction with IP$_3$Rs. IP$_3$R sensitisation by Bcl-XL occurs via its hydrophobic cleft, which binds two BH3-like domains in the C-terminus of IP$_3$Rs, although the BH4 domain of Bcl-XL also contributes by targeting the central, modulatory domain of the IP$_3$R (Yang et al. 2016; Williams et al. 2016). Therefore, BH3 mimetic drugs that target Bcl-XL may also antagonise Bcl-XL's ability to sensitise IP$_3$Rs and thus may suppress Ca^{2+}-driven mitochondrial metabolism. This is very important, since cancer cells are particularly addicted to these basal Ca^{2+}-signalling events to sustain adequate TCA cycling (Cárdenas et al. 2016; Bultynck 2016). This process provides mitochondrial substrates, like nucleosides, that are essential for proper cell-cycle progression and cell division. In the absence of these Ca^{2+} fluxes, non-tumorigenic cells tune down their cell cycle, while tumorigenic cells progress through the cell cycle irrespective of their energetic state, resulting in a mitotic catastrophe (Cárdenas et al. 2016; Bultynck 2016).

As described above, the phosphorylation state of the IP$_3$R has a determining role in its Ca^{2+}-release properties, and PML is able to alter this phosphorylation state. Recently, it has been discovered that MAM-localised PML inhibits autophagy via the control of ER-mitochondrial Ca^{2+} signalling (Missiroli et al. 2016). By stimulating ER-mitochondrial Ca^{2+} flux, the cell's metabolism is being stimulated (Cárdenas et al. 2010; Kaufman and Malhotra 2014). p53 is acting as a molecular bridge to keep PML at its place in the MAMs (Missiroli et al. 2016). Upon

PML loss, however, metabolic stimulation via Ca^{2+} is not present anymore, and this turns on AMPK signalling and subsequent autophagy (Missiroli et al. 2016). Additionally, also mTORC2 may control metabolism via IP$_3$R3 phosphorylation, in a similar way it contributes to MAM integrity via PACS-2 phosphorylation (Betz et al. 2013). Activation of PKB/Akt in an mTORC2-dependent manner reduces IP$_3$R-mediated Ca^{2+} signalling and hence might impact the rate of oxidative phosphorylation (Betz et al. 2013).

17.3.3.2 VDAC1

As a gateway connecting the mitochondria to their environment, VDAC1 is not only a regulator of apoptosis: its localisation at the OMM of the mitochondrion also allows for regulation of cellular bioenergetics. For a start, it has been reported that the Ca^{2+}-flux properties of VDAC1 are stimulated by Mcl-1, which binds with high affinity to the anion channel in the OMM (Huang et al. 2014). This results in an increased mitochondrial Ca^{2+} uptake, thereby promoting ATP production and stimulating cell survival, as described above (Huang et al. 2014).

Furthermore, VDAC1 is thought to play a major role in constituting the Warburg effect through its interaction with HK (Bustamante and Pedersen 1977; Azoulay-Zohar et al. 2004; Pedersen 2008). A common characteristic of cancer cells is that they show high levels of glycolysis, even though they are oxygenated (Vander Heiden et al. 2009; Liberti and Locasale 2016). This aerobic glycolysis is commonly referred to as the Warburg effect (Vander Heiden et al. 2009; Liberti and Locasale 2016). It is proposed that, by binding VDAC1, ATP produced by oxidative phosphorylation is readily accessible for HK-I and -II to fuel the conversion of glucose to glucose-6-phosphate (G-6-P) (Pedersen 2008). This provides a functional coupling of glycolysis with the TCA. Interestingly, evidence suggests that HK is less sensitive to product inhibition by G-6-P through its interaction with VDAC1 (Bustamante and Pedersen 1977; Azoulay-Zohar et al. 2004). Another clue to VDAC1's role in the Warburg effect is that especially the

VDAC1-bound isoforms, HK-I and HK-II were found to show higher expression levels in several types of cancer, e.g. lymphoma, prostate and breast cancer (Pedersen 2008). Recently, mTORC2 was found to impact the binding of HK-II to VDAC1, again through PKB/Akt-dependent phosphorylation of HK-II, which stabilises its binding to VDAC1 (Betz et al. 2013).

The Warburg effect also comprises the suppression of oxidative phosphorylation in the mitochondria (Zheng 2012; Lu et al. 2015). A long unidentified player in this suppression is dimeric tubulin (Rostovtseva et al. 2008). Dimeric tubulin at concentrations in the nanomolar range was shown to reversibly block VDAC reconstituted into planar phospholipid membranes (Rostovtseva et al. 2008). Furthermore, this block of VDAC was demonstrated to decrease oxygen consumption in isolated mitochondria (Rostovtseva et al. 2008). In addition to this, it was shown that in HepG2 cells, an increase in dimeric tubulin resulted in mitochondrial depolarisation, while a decrease in dimeric tubulin was associated with mitochondrial hyperpolarisation (Maldonado et al. 2010). These results suggest that mitochondrial metabolism in cancer cells is attenuated by the tubulin-mediated blockage of VDAC (Maldonado et al. 2010). It is noteworthy that the effect of free tubulin on cellular metabolism was not found in primary hepatocytes: microtubule depolymerisation decreased the mitochondrial membrane potential, as in cancer cells, but inducing polymerisation did not increase it (Maldonado et al. 2010). This leaves the question whether the observations concerning free tubulin blocking VDAC are specific for cancer cells (Maldonado et al. 2010; Rostovtseva and Bezrukov 2012).

Apart from a shift towards aerobic glycolysis, other biochemical pathways like cholesterologenesis may be altered in cancer cells. Also in this process, a link to VDAC1 can be found: the channel is part of the polyprotein complex called the transduceosome, which is responsible for import of cholesterol into the mitochondria (McEnery et al. 1992; Liu et al. 2006; Rone

et al. 2012). In this complex, VDAC1 is proposed to interact with the translocator protein, anchoring the transduceosome to the OMM and facilitating the binding and import of the steroidogenic acute regulatory protein (Hauet et al. 2005). It is proposed that HK bound to VDAC1 may influence the amount of cholesterol synthesis and its import in mitochondria in cancer cells (Campbell and Chan 2007), while at the same time, the channel properties of VDAC1 itself may be influenced by the augmented levels of cholesterol in the OMM (Pastorino and Hoek 2008).

17.4 Conclusions

The MAMs and their various components, including Ca²⁺-transport systems, chaperones and structural components, establish an important Ca²⁺-signalling domain between the ER, the main intracellular Ca²⁺-storage organelle and the mitochondria, the main organelle controlling cell death and survival processes, including cellular bioenergetics and autophagy, apoptosis sensitivity, growth and proliferation. Dysregulation of these processes is a hallmark of cancer. Hence, alterations and perturbations in the structural organisation and functional properties of the MAMs have emerged as an important nexus that underlies oncogenesis, tumour growth and metastasis and responses to chemotherapy. Moreover, several oncogenes and tumour suppressors are localised at the MAMs. Thus, changes in MAMs can drive oncogenesis, while cancer cells at a later stage could remodel MAMs to favour tumour growth, proliferation and metastatic behaviour.

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