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Review

# Voltage-dependant anion channels: Novel insights into isoform function through genetic models☆

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ABSTRACT

Voltage-dependant Anion Channels, also known as mitochondrial porins, are pore-forming proteins located in the mitochondrial outer membrane (MOM) that, in addition to forming complexes with other proteins that localize to the MOM, also function as the main conduit for transporting metabolites between the cytoplasm and mitochondria. VDACs are encoded by a multi-member gene family, and the number of isoforms and specific functions of VDACs varies between species. Translating the well-described *in vitro* characteristics of the VDAC isoforms into *in vivo* functions has been a challenge, with the generation of animal models of VDAC deficiency providing much of the available information about isoform-specific roles in biology. Here, we review the approaches used to create these insect and mammalian animal models, and the conclusions reached by studying the consequences of loss of function mutations on the genetic, physiologic, and biochemical properties of the resulting models. This article is part of a Special Issue entitled: VDAC structure, function, and regulation of mitochondrial metabolism.

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**Contents**



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## 1. Introduction

Voltage-dependent Anion Channels (VDACs) are the most abundant proteins in the mitochondrial outer membrane (MOM) [1], a key region of the organelle that plays a central role in various cellular processes, including metabolite flux, metabolic compartmentation, and apoptosis. Not surprisingly, VDACs have been implicated in all of these processes via differing mechanisms: facilitating metabolite flux by enabling transport of ATP, ADP, phosphocreatine and other small ions across the MOM [2,3], anchoring hexokinases to the MOM during glycolysis [4] and aiding release of cytochrome c from the intermembrane space, either directly regulation pore opening and closure [5,6] and/or indirectly by interacting with Bcl-2 family members [7] (reviewed in Ref. [8]). The diversity in function is matched by the evolution of different isoforms of the protein, each with distinct roles in mitochondrial biology, and comparing and contrasting the functional characteristics of these variants in different model systems will be the focus of this review. Since the first studies to isolate the relevant genes and characterize the functional roles of porins were conducted in yeast, and later studies were conducted in flies and mammals, we will analyze the models for porin deficiency in that order.

## 2. Yeast VDAC isoforms

VDACs have been characterized in a variety of eukaryotes, with the number of isoform variants ranging from one to perhaps five, depending on the species [1,9,10,11]. The yeast (*Saccharomyces cerevisiae*) variants of VDAC; YVDAC1 and YVDAC2, are encoded by the single copy genes POR1 and POR2, respectively, with the proteins having a 49% amino acid identity. POR1 is considered the major isoform in yeast, with the ability to form channels in phospholipid membranes [12]. The POR1 deletion strain (Δ*por1*) exhibited a delayed growth adaptation in non-fermentable glycerol at 30 °C, and a complete lack of growth at 37 °C [13]. In addition to the growth phenotypes, the deletion strain also demonstrated significantly reduced levels of mitochondrial cytochromes; cytochrome c,$c_1$,b and $aa_3$, along with COXIV, while containing normal levels of other mitochondrial proteins. The ability of the deletion strain Δ*por1* M22-2 to grow at lower temperature led to a multi-copy suppressor screen for a functional substitute for YVDAC1, resulting in the discovery of YVDAC2. The YVDAC2 isoform, however, was neither able to form channels in reconstituted *in vitro* systems nor rescue the growth phenotype, and its true function remains to be determined [12]. Yeast has been used extensively as a model system for various mitochondrial dependent processes including, amongst others, the release of cytochrome c during apoptosis. Conflicting reports have argued for [14,15,16,17] and against the necessity of YVDAC1 [18,19] for cytochrome c release during apoptosis. Other processes where a role for YVDAC1 has been proposed include as a channel for metabolites such as NADH, and as a conduit for reactive oxygen species (ROS) signaling via redox regulation and consequent regulation of mRNA and protein levels in the cell. [20,21,22,23]. YDAC1 has been hypothesized to control cellular redox states by the transport of ROS species such as the superoxide anion ($O_2^-$) across the MOM, and this in term is known to affect cellular mRNA and protein levels (reviewed in Ref. [21]). Over-expression or POR1 complementation in yeast of yeast, fruit fly and mammalian VDACs has also been an important tool in isolating abundant amounts of purified VDAC protein and examining the consequences of engineered point mutations with regard to structure and function [10,24,25].

## 3. Fly VDAC/porin isoforms

As described earlier in this review, while VDAC has been classically viewed as the major determinant of MOM since the 1970s, studies over the past 15 years have clearly demonstrated that VDAC also interacts with both cytosolic and MOM proteins (reviewed in Ref. [26]). For example, VDAC integrates mitochondrial and cytoplasmic energy metabolism through its binding of hexokinases, effectively linking glycolysis and mitochondrial oxidative phosphorylation, in addition to being the possible mitochondrial docking site for glycerol kinase and creatine kinase [26]. The fruit fly provides a powerful genetic model system to identify and further characterize VDAC functions. *Drosophila melanogaster* contains a cluster of four genes (*porin*, *CG17137* [*Porin2*], *CG17139*, and *CG17140*) that encode proteins that are homologous to known VDACs [25]. *porin* exhibits the greatest homology to mammalian VDACs and is ubiquitously expressed in the fruit fly, while the other three fly VDACs have a more spatially restricted expression pattern, predominantly in the male reproductive tract [25]. A series of hypomorphic alleles has been generated and characterized using imprecise excision of a P element inserted in the 5′ untranslated region [27] of *porin* [28]. Flies lacking *porin* exhibit a variety of mutant phenotypes including partial developmental lethality, abnormal mitochondrial respiration, partial complex I deficiency, fertility defects, skeletal muscle abnormalities and neurological dysfunction. The fertility defects include reduced fertility and fecundity in females and infertility in males associated with sperm immotility. The muscle phenotype consists of abnormal mitochondrial morphology with unusual inclusions by electron microscopy as well as a functional deficit manifested as a defect in a climbing assay. The neurological dysfunction in porin-deficient flies is manifested by an increased sensitivity to mechanical stress ("bang sensitivity"), progressive retinal dysfunction demonstrated by an abnormal electroretinogram (ERG), and an aberrant electrophysiological response at the larval neuromuscular junction (NMJ). The neurological dysfunction at the larval NMJ is also characterized by an abnormal distribution of mitochondria within the motor neuron. Using a mitochondria-targeted GFP transgene expressed in the motor neuron, the quantity of mitochondria in the presynaptic termini and axons is significantly reduced, with concomitant accumulation of mitochondria in motor neuronal cell bodies [28]. Park et al. also recently reported phenotypic characterization of fly mutants deficient for porin which confirms the neurological and male infertility phenotypes, as well as demonstrating that the observed abnormal mitochondrial morphology can be rescued with overexpression of Drp1, a regulator of mitochondrial fission [29]. This observation suggests that porin may play an important role in the regulation of mitochondrial dynamics [29]. The phenotypes of defective energy metabolism with perturbed mitochondrial dynamics, male infertility with sperm immotility, and neurological dysfunction in porin-deficient fruit flies are reminiscent of abnormal phenotypes seen in mitochondrial diseases and suggest that porin deficiency in the fly is a valid model of mitochondrial dysfunction that is relevant both for rare primary mitochondrial diseases and for common adult neurological and metabolic diseases for which mitochondrial dysfunction has been implicated to play a pathogenic role.

## 4. Mammalian VDAC isoforms

### 4.1. Gene organization

A prerequisite to understanding the functional characterization of mammalian VDACs is a description of the organization of the three VDAC isoforms in mammals. In mammals, three VDAC isoforms; VDAC1, VDAC2 and VDAC3, have been characterized, with VDAC1 and VDAC3 shown to have 9 exons and VDAC2 having 10, the additional exon encoding part of the 5′-UTR region [30,31]. There is roughly 70% nucleotide sequence identity amongst the three VDAC isoforms and around 90% nucleotide identity between the human and mouse variants (henceforth referred to as mVDACs for mice and hVDACs for humans) [30]. All three isoforms in humans and mice have the start codon located in the second exon, span varying lengths of

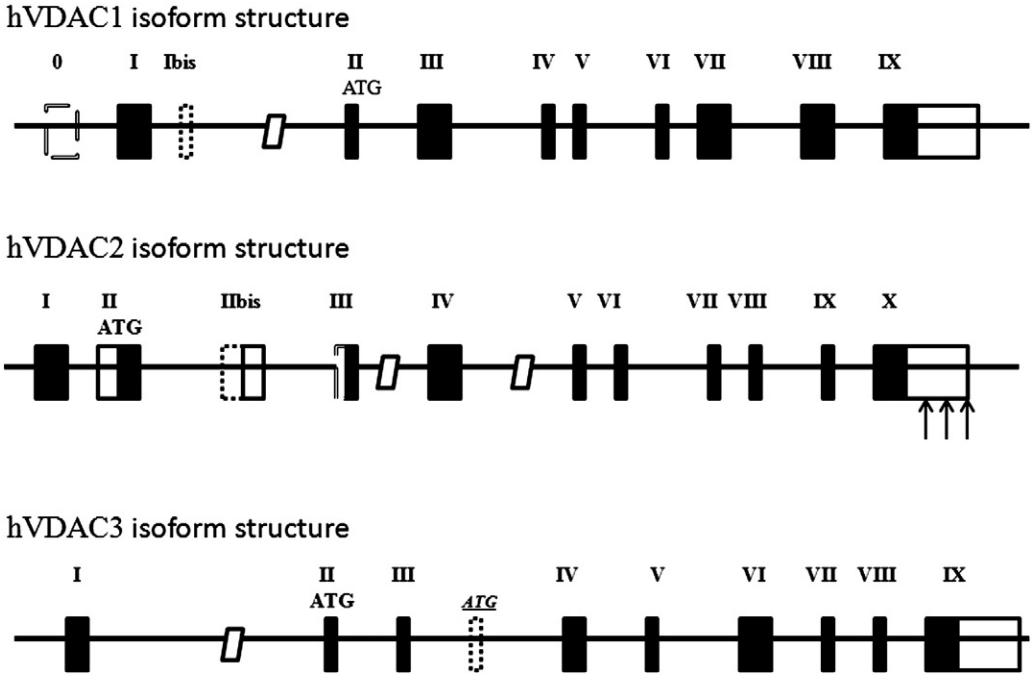


**Fig. 1.** Gene structures of human VDAC isoforms with their reported alternate spliced variants (not drawn to scale). Predicted putative exons are shown in dashed boxes. Alternate polyadenlyation sites are marked with arrows for hVDAC2. Translated regions are filled boxes, untranslated regions are not filled. The predicted start codons of the isoforms are shown, along with the ATG mini-exon for hVDAC3 (in italics). The vertical arrows represent the multiple polyadenylation sites found in the VDAC2 gene. See text for details.

genomic DNA and are located on different chromosomes in a syntenic relationship between the two species [32]. There is also a conservation of coding exon–intron boundaries and a splice acceptor boundary for all three mVDACs, indicative of a gene family that arose by gene duplications and divergence. The most significant sequence difference amongst the isoforms is the presence of an 11–12 amino acid amino terminal extension that is limited to VDAC2, the functional significance of which remains undetermined.

### 4.2. Alternative splicing

Based on searches of expressed sequence tag (EST) and sequence databases, it was uncovered that there are alternate spliced exon variants for hVDAC1 and hVDAC2, although the functional roles of these variants, if any, remain unexplained [26,33]. The hVDAC1 splice variant has a 157 bp alternative exon (referred to in Ref. ([26] as exon1bis) located downstream of exon1 in the 5′-UTR region. It appears to be a true exon with a 3′ consensus splicing sequence and encodes for a fusion transcript of exon1bis with exons 2–9. The authors also reported another possible longer alternate exon (referred to as exon 0) in the 5′-UTR region, with several ESTs and a whole transcript listed in EST collections and GenBank, respectively. It follows from the locations of the exons (in the 5′-UTR region) that neither variant alters the resulting protein sequence; hence the significance of these transcripts is not clear. The hVDAC2 gene, in contrast, was reported to have an alternate exon (referred to as exon2 bis) between exons 2 and 3 corresponding to a 26 amino acid extension at the N-terminal region instead of the 11–12 amino acid sequence. This variant corresponded to a VDAC2 protein termed Humpor that already been reported by Ha et al. in an earlier publication [34]. The functional consequences of this extension, likewise, are unclear [34]. The human VDAC3 gene has an additional alternatively spliced internal 3-base (ATG) mini-exon between the exon 3 and exon 4 splice junctions, 1.9 Kb downstream of exon 3 and surrounded by splice enhancer elements. It is predicted to result in the insertion of a methionine at amino acid position 39 [26]. An identical variant has also been described in the rat and mouse VDAC3 genes [26,35,36] (A map of the gene structure of the three isoforms and the splice variants is shown in Fig. 1). Since the VDAC3 mini-exon is a start codon and appears to have a stronger Kozak consensus sequence than the annotated 5′ start site, it was proposed that this could initiate the translation of a truncated protein downstream of the native site [36].

A complementation analysis of the yeast porin-deletion mutant with the full-length and truncated mVDAC3 cDNA provided an interesting outcome, where the full-length construct failed to complement the growth deficiency in the mutant (a previously observed result), while the truncated cDNA rescued the mutant phenotype. While trivial explanations such as translation efficiency could explain this difference, it was proposed that the deletion of the first 39 residues could result in a constitutively open channel. Amino acid substitutions of the inserted methionine residue with neutral residues that do not disrupt protein structure failed to complement the growth phenotype. On the other hand, substitutions with charged amino acids produced a full rescue, potentially highlighting the importance of the N-terminal region and its structural integrity in VDAC3 function. Despite the above experiments, investigation by Western blotting of this truncated protein using epitope-tagged constructs and highly specific antibodies failed to demonstrate expression of this truncated isoform in mammalian cells, bringing into question its biological relevance [36,37]. The alternatively spliced VDAC3 mini-exon is expressed in brain, heart and skeletal muscle to varying levels, with the heart showing a higher expression of this variant compared to the unspliced isoform, and most other tissues having minimal or no expression of the alternate variant. While the expression of this alternate variant in tissues with high metabolic potential raises interesting possibilities about its functional implications *in vivo*, there remain many unanswered questions about the potential post-translational functions of this splice variant.

### 4.3. Alternative polyadenylation

Another possible mechanism of post-transcriptional regulation observed for the VDAC genes is the alternate polyadenylation of the transcripts, resulting in varying lengths of the 3′-UTR region. While

the mVDAC1 and mVDAC3 genes encode only one strong primary polyadenylation site, the mVDAC2 gene encodes multiple transcripts of varying lengths, most likely because of its alternate polyadenylation sites [10]. Based on 3′-RACE experiments, the 3′-UTR region was shown to have one aberrant and two canonical polyadenylation sites, with, based on Northern blot analysis, the shortest product being the most abundantly expressed. The differing lengths of the 3′-UTR regions of the mVDAC2 transcript could have functional consequences for mRNA turnover, translation efficiency, the subcellular location of transcripts [38,39,40], and, perhaps more interestingly, miRNA binding that could regulate tissue-specific expression of transcripts- areas which are open for further investigation [41,42,43]. A similar set of transcripts of varying sizes based on the polyadenylation sites has also been reported for hVDAC2 [44].

### 4.4. Tissue-specific expression

It has been observed that there is differential expression of both VDAC1 and VDAC2 across different tissues in mice, with the highest levels for both isoforms observed in heart, liver, skeletal muscle and brain as measured by Northern blotting. Interestingly, while mVDAC1 is expressed in very low levels in the testes, there is a high level of expression of mVDAC2 in the same tissue [30]. The prototypic mVDAC3 isoform is expressed in testes, liver, ovary, adrenal, lung, spleen, and kidney, as measured by RT-PCR [37].

### 4.5. Functional conservation of VDACS

As an additional demonstration of functional conservation, and to characterize the translational and functional nature of the proteins encoded by mVDAC isoforms, rescue experiments were carried out by transfecting the mouse cDNA orthologues flanked by the yeast VDAC promoter, 5′-UTR, and 3′-UTR, into the yeast porin mutant Δ*por1*. It was found that while mVDAC1 and mVDAC2 cDNAs completely rescued the temperature-sensitive growth deficiency of the yeast mutant, mVDAC3 cDNA facilitated only a partial rescue, with comparatively far fewer colonies growing at the restrictive temperature of 37 °C [10]. A similar experiment also showed that while each isoform increased the MOM permeability of the deletion strain to NADH, the order of decreasing permeability was mVDAC1 > mVDAC2 > mVDAC3. The electrophysiological properties of each isoform, characterized by planar lipid reconstitution experiments, also showed significant variation, with mVDAC1 acting as the canonical VDAC, showing normal membrane insertion, conductivity and selectivity, while mVDAC2 behaved as two populations of molecules, with one having lower conductance and selectivity compared to prototypical porin. mVDAC3 showed normal insertion into planar lipid membranes but did not exhibit any clear voltage-dependency in conductance and selectivity. All three isoforms were able to confer polyethylene glycol permeability to liposomes, with similar molecular weight cut-offs of between 3400 and 6800 Da [45]. The functional reconstitution of VDACs in a yeast deletion background was also performed with hVDAC1 and hVDAC2 cDNAs, with similar results [33]. hVDAC1 was ascribed to be the canonical VDAC in terms of voltage-dependence and conductance, while hVDAC2 appeared to be present in a sub-conductance state, losing its voltage-dependence with time. Both constructs managed to rescue the temperature-sensitive growth phenotype of the POR1 deletion strain, and hVDAC1 was able to bind to hexokinase when expressed in yeast mitochondria, providing additional support for the functional conservation of mitochondrial porins. A summary of the differences in gene organization between the three isoforms is tabulated in Table 1.

## 5. VDAC deficiency: Embryonic stem cells

The first report describing a deletion model for mammalian VDACs used homologous recombination to knockout each of the three VDAC isoforms in mouse embryonic stem (ES) cells [46]. This approach was used to delete exons 2–5 for VDAC1, the promoter and exons 1 and 2 (including the start codon) for VDAC2 and exons 6–9 for VDAC3, and homologous recombination strategies were used to disrupt both alleles present in the ES cells, generating isoform-specific and completely deficient cells. The deletions provided evidence for another distinction between the three isoforms, in that while ES cells harboring each of the three deletions showed a significant reduction in oxygen consumption, VDAC3$^{-/-}$ cells did not show a statistically significant difference from wildtype ES cells in uncoupled respiration, reminiscent of the previously mentioned yeast rescue results (in the section on *Yeast VDAC isoforms*). In addition, while cells lacking VDAC1 showed a significant increase in citrate synthase activity, reflecting a possible compensatory proliferative effect on mitochondria, a similar phenotype was not observed for VDAC2$^{-/-}$ and VDAC3$^{-/-}$ cells. When the COX activity of the three deletions cell types was measured, after normalization to mitochondrial content based on the citrate synthase activity, VDAC1$^{-/-}$ and VDAC2$^{-/-}$ cells showed a significant reduction of COX activity, potentially explained by the possible impairment of outer membrane transport concomitantly coupled to reduced inner membrane respiration. Also observed was a reduction in hexokinase activity in all three cell types, reminiscent of results later observed in mutant mouse tissue. A potentially compensatory increase in VDAC1 protein levels in the VDAC2$^{-/-}$ and VDAC3$^{-/-}$ cells was also observed, providing a possible explanation of the lack of mitochondrial proliferation in these cell types. While there are clear limitations in approaching isoform function in a single cell type such as ES cells, it is apparent from the divergent range of bioenergetic phenotypes between the three

**Table 1**
Comparison of the genomic organization of the mammalian VDAC isoforms.

| | VDAC1 | VDAC2 | VDAC3 | Ref. |
|---|---|---|---|---|
| ▪ Number of exons | 9 | 10 | 9 | [30,31] |
| ▪ Alternate spliced isoform | 2 | 1 | 1 | [26,34,37] |
| ▪ Alternate protein sequence | No | Yes | Yes | [26,34,37] |
| ▪ Percentage cDNA sequence identity between mouse and human isoforms[a] | 90% | 90% | 68% with hVDAC1<br>73% with hVDAC2 | [30,31] |
| ▪ Percentage cDNA identity between mouse isoforms | With mVDAC2-71%<br>With mVDAC3-68% | With mVDAC1-71%<br>With mVDAC3-73% | With mVDAC1-68%<br>With mVDAC2-73% | NCBI database |
| ▪ Chromosomal location | mVDAC1-Chr11<br>hVDAC1-Chr5 | mVDAC2-Chr14<br>hVDAC2-Chr10 | mVDAC3-Chr8 | [30,32] |
| ▪ Tissues showing highest expression in mice | Heart, liver, skeletal muscle and brain | Heart, liver, skeletal muscle and brain | Testes, liver, ovary, adrenal, lung, spleen, and kidney | [30],[36] |

[a] Comparison with the canonical splice variant.

isoform knockouts that there likely are distinct functional roles for each isoform in the cell, and this is reflected in the deletion mouse models elaborated upon in the following sections.

## 6. VDAC deficiency in mice

### 6.1. Survival and growth

In this section, observations made in mice lacking the VDAC1 and VDAC3 isoforms will be reviewed, and brief mention will be made about as yet unpublished findings in VDAC1 and VDAC2 deficient mice. VDAC1$^{+/-}$ and VDAC3$^{+/-}$ ES cells were used to obtain mouse chimeras, and subsequent heterozygous mice tested for Mendelian transmission of the deletion mutations [47]. While the mutant and wildtype alleles of the VDAC3 locus were transmitted in the expected Mendelian ratios, VDAC1$^{-/-}$ mice were born in less than expected numbers, indicative of partial embryonic lethality. This lethality was shown to occur at 10.5–11.5 days of embryogenesis and was influenced by the strain background. While the VDAC1 mutation bred onto an inbred C57Bl6 background showed almost complete lethality, outcrossing to the mixed CD-1 background increased the survival rate to around 70% of the expected frequency [48]. The surviving VDAC1$^{-/-}$ mice were fertile and showed mild growth retardation, while the VDAC3$^{-/-}$ male mice were found to be infertile, and the VDAC1$^{-/-}$/VDAC3$^{-/-}$ double knockout offspring similarly showed reduced Mendelian ratios, infertility, and more severe growth retardation.

### 6.2. Regulating synaptic plasticity through the MPTP

VDACs have long been proposed to be a part of the Mitochondrial Permeability Transition Pore complex (MPTP) [49,50,51], although the evidence for this has been debated (reviewed in Ref. [52]). Amongst a wide range of purported functions, the MPTP has been implicated in learning and synaptic plasticity based upon its role in mitochondrial calcium buffering, ATP production, and metabolite flux at the synapse [53], [54]. With both VDAC1 and VDAC3 protein present in cells of the hippocampus, the VDAC1$^{-/-}$, VDAC3$^{-/-}$ and VDAC1$^{-/-}$/VDAC3$^{-/-}$ mice were investigated for learning and stable memory phenotypes, since both processes require synaptic plasticity; the experience-related changes in synaptic strength [47]. Although none of the three mutant strains exhibited hippocampal structural alterations, both VDAC3$^{-/-}$ and VDAC1$^{-/-}$/VDAC3$^{-/-}$ mice demonstrated a significant reduction in contextual fear conditioning. This test involves pairing an aversive stimulus such as a mild foot shock with an auditory-conditioned stimulus such as white noise that is provided in a particular visual context, with the expected result being fear and freezing in response to the visual training context. This was not observed for the VDAC1$^{-/-}$ mice, specifically implicating VDAC3 in hippocampus-dependant contextual fear conditioning. However, all three genotypes showed a deficit in cued fear conditioning, where the same conditioned fear experiment is performed with the re-presentation of the auditory cue in a different visual context. A possible explanation for this phenotypic difference is varying contributions of VDACs to different regions of the amygdaloid nuclei, which are known to play a role in contextual and cued fear conditioning. The three strains were then subject to a spatial learning paradigm using the Morris water maze, where the mice are tested on their ability to remember and find the location of a hidden platform when placed in opaque water. All three mutants showed a significant defect in spatial learning, another hippocampus-dependant process that reflects a higher order cognitive function than that of fear conditioning. To rule out sensory or motor deficits as confounding factors in these tests, hot-plate, open-field activity, and locomotor tests were conducted and the mutant mice performed identically to wildtype controls, indicating that the defects reflected a specific abnormality in synaptic plasticity and not simply sensory deficits or a global impairment in brain function [48].

To investigate which parameters of synaptic plasticity could account for the observed associative and spatial learning and memory phenotypes, hippocampal slices were subject to electrophysiologic testing paradigms and analyzed for short-term and long-term synaptic plasticity. Paired pulse facilitation (PPF) is a measure of presynaptic plasticity where two temporally close-spaced depolarizations augment synaptic transmission, and is thought to reflect the effect of residual calcium caused by the first depolarization. In contrast, the post-synaptic response to a train of depolarizations can be to either enhance or diminish subsequent signal transmission, the former being long-term depression and the latter long-term potentiation (LTP). Using *ex vivo* tissue sections, both presynaptic plasticity, as measured by short-term PPF, and post-synaptic plasticity, as measured by LTP, were deficient to different degrees in the knockout strains. VDAC1$^{-/-}$ hippocampal slices exhibited a significant deficit in LTP, while VDAC3$^{-/-}$ slices exhibited a significant deficit of PPF. VDAC1$^{-/-}$/VDAC3$^{-/-}$ deficient slices were impaired in both pre- and post-synaptic plasticity. Based upon VDAC1$^{-/-}$/VDAC3$^{-/-}$ mice showing synaptic plasticity phenotypes similar to those observed in wildtype mice treated with the MPTP inhibitor CyclosporinA, it was proposed that VDACs could play a role in calcium buffering or calcium-induced calcium release in the presynaptic terminal as part of the MPTP. Such a general regulation of calcium levels could possibly affect kinases involved in synaptic plasticity such as $Ca^{2+}$/calmodulin-dependent protein kinase II, and a later study showed that calcium-induced calcium release was indeed enhanced in VDAC1$^{-/-}$ isolated brain mitochondria, providing a possible mechanism for the observed alterations in plasticity [55]. However, while the MPTP may well be perturbed by the absence of VDACs in the brain, additional studies of MPTP function in other tissues and cell types lacking each VDAC isoform failed to support a role for VDACs in the MPTP [56].

### 6.3. A role in fertility

While mitochondrial function appears important for human fertility [57] and in particular male fertility, there are limited examples of this in mouse models. The previously mentioned infertility phenotype of VDAC3$^{-/-}$ male mice is one such example [58]. While VDAC3$^{-/-}$ males exhibit normal copulatory activity, they failed to impregnate females in over 100 matings, while VDAC3$^{-/-}$ females exhibit normal fertility. The male infertility parallels that seen in male *D. malanogaster* discussed earlier. To identify the cause, fertility assays examining changes in sperm count, testes size, weight, histology and cellular apoptotic levels were performed, with VDAC3$^{-/-}$ males showing no difference from control in any of these characteristics. The VDAC3$^{-/-}$ males did show reduced sperm motility (17% of the sperm were motile compared to 66% in control males) and most of the sperm were not actively swimming after addition of various energy substrates. There were also remarkable ultrastructural abnormalities in the sperm, with most of the sperm axonemes in cross-section showing the loss of one microtubule outer doublet from the normal 9 + 2 arrangement. The loss was highly specific when the axoneme were bisected and observed in a particular orientation, with the same single doublet (referenced as position #7) either missing or at intermediate stages of loss, and the loss was only observed in sperm from the cauda epididymis, not the testes, indicating that the loss is a consequence of sperm maturation and not development. Ectopically placed doublets, partial axoneme duplications, and loss of half the doublets were also observed, but far less frequently. There was no structural defect in the mitochondria observed in the midpiece of sperm, but given the location of VDAC3 in mitochondria, these observations suggest that the microtubule architecture of the axoneme is reliant on the presence or function of sperm MOM proteins.

### 6.4. Mitochondrial phenotypes

Studies using VDAC1$^{-/-}$ mice confirmed the importance of this protein as a carrier of metabolites across the MOM. It was found that in detergent "skinned" muscle fibers lacking VDAC1, where mitochondria are preserved *in situ*, there is a reduction in ADP-stimulated oxygen consumption, suggesting reduced transport of ADP across the outer and/or inner mitochondrial membranes in both cardiac and skeletal muscle types [2]. Abnormal mitochondrial morphology in the subsarcolemmal population of mitochondria of both the soleus (oxidative) and the gastrocnemius (mixed oxidative/glycolytic) muscle types was observed, with compact cristae and grossly enlarged mitochondria. A follow-up report examining the role of VDAC3 in MOM permeability and mitochondrial morphology using VDAC3$^{-/-}$ mice was recently published [59], and demonstrated a similar effect on ADP-stimulated respiration, but with the deficiency limited to cardiac muscle fibers, emphasizing that different VDAC isoforms are performing different roles depending on their tissue location. Enlarged mitochondria and irregular cristae structure, as was observed in the VDAC1$^{-/-}$ mice, were present in the intermyofibrillar and subsarcolemmal regions of the heart but not in gastrocnemius muscle in the VDAC3$^{-/-}$ mice. Similar to VDAC1$^{-/-}$ mice, the absence of one VDAC isoform did not alter the protein levels of the other isoforms in the muscle. Defects in the electron transport chain (ETC) complex activities in the muscles of both VDAC1$^{-/-}$ and VDAC3$^{-/-}$ mice were also observed. There was a statistically significant reduction in Complex I + III, II + III and IV activities in VDAC1$^{-/-}$ muscles, and a similar defect in Complex II activity in the gastrocnemius. There was no defect in the Complex I and II activities in the heart of the VDAC1$^{-/-}$ mice, while the VDAC3$^{-/-}$ heart showed a reduction in Complex IV activity. Thus, absence of different VDAC isoforms leads to diminished respiratory chain activities, however the biophysical basis for these changes remains unknown; whether it reflects perturbations in super-molecular respiratory complexes is an area currently under investigation.

### 6.5. Regulating hexokinase interactions with mitochondria

Glycolysis is in part controlled through glucose phosphorylation by the various isoforms of hexokinase. A large number of studies have examined the binding of hexokinase to the MOM, where it presumably benefits from the close proximity to the primary source of ATP, and the proteins involved in the regulation of this binding have all been under investigation for a few decades now [60]. The initial identification of VDAC1 as an interacting partner for binding to the MOM was carried out by Nakashima et al. in 1986 [61], and has since been supported by a number of other studies [4,62,63,64]. There is preferential glucose phosphorylation by ATP derived from the mitochondrial matrix in comparison to ATP from the cytosol ([65]), and hexokinase binding to VDAC1 is reversible and is inhibited by the product of the hexokinase reaction, glucose-6-phosphate. This mitochondrial-bound hexokinase is an important aspect of the Warburg effect; the increase in glycolysis in cancer cells even in the presence of oxygen [66]. The VDAC1$^{-/-}$, VDAC3$^{-/-}$ and VDAC1$^{-/-}$ VDAC3$^{-/-}$ mice were therefore tested for levels of hexokinase in the mitochondria of energy-demanding heart and muscle tissue [67]. VDAC1$^{-/-}$ mice, but not VDAC3$^{-/-}$ mice, showed a significant reduction of mitochondria-associated hexokinase activity in heart and soleus, but otherwise normal total tissue activity. To correlate this result with the binding of HK2, the major hexokinase isoform in heart and skeletal muscle, protein levels were measured from skinned fiber mitochondrial preparations by Western blotting, and there was a marked reduction of HK2 associated with VDAC1$^{-/-}$ muscle mitochondria. To investigate the possibility of a physiological regulatory role for HK-VDAC binding, glucose tolerance was examined, with VDAC1$^{-/-}$ and VDAC1$^{-/-}$/VDAC3$^{-/-}$ mice showing significantly impaired glucose tolerance, whereas none was observed in VDAC3$^{-/-}$ mice. The defective glucose clearance in VDAC1$^{-/-}$ mice could be attributed to multiple factors, including a defect in insulin secretion, excess gluconeogenesis, impaired glycogen synthesis, or insulin resistance. Our lab has attempted to further characterize the glucose intolerance using a hyperinsulinemic euglycemic clamp experimental protocol. This approach has shown clear skeletal muscle insulin resistance coupled with altered triglyceride synthesis and transcriptional alterations in lipogenesis pathways in the VDAC1$^{-/-}$ mice (unpublished results).

A signaling pathway connecting AKT signaling, HK-VDAC binding, and apoptosis has been proposed, with AKT hypothesized to prevent VDAC closure by phosphorylating and inhibiting mitochondria-associated GSK3-β, which in turn inhibits HK-VDAC binding by phosphorylating VDAC and disassociating HK2 [64,68,69]. HK2 has been proposed to inhibit apoptosis by inhibiting Bax-mediated cytochrome c release and downstream apoptotic initiation [70,71,72], although a specific mechanism for this has yet to be delineated. AKT signaling is also directly implicated in glucose regulation by being downstream of insulin receptor-mediated signaling and upstream of GLUT4 recruitment to the cell surface and cellular glucose uptake [73,74,75]. Based on the observed skeletal muscle insulin resistance, we have also investigated potential disruption of AKT signaling in VDAC1$^{-/-}$ mice, and preliminary data indicate that there are changes in the levels and phosphorylation status of the AKT signaling components (unpublished results). By playing a potentially important role in cellular AKT signaling and hexokinase activation, pathways that have been implicated in glucose regulation and apoptosis, both of which in turn have an important role in cancer biology, VDAC1, in addition to the more-exhaustively investigated VDAC2, has been proposed to be an attractive target for anti-cancer therapeutics (reviewed in Ref. [76]).

### 6.6. Roles in apoptosis

There has been considerable interest in the role of VDACs in apoptosis, beginning with a report implicating VDAC1 in the release of cytochrome c [14]. VDAC1 has been the most studied isoform in cell death processes (reviewed in Refs. [77] and [26]). However, the fact that VDAC1 and VDAC3 deficient animal models do not exhibit alterations in apoptosis suggests that the role of these isoforms in cell death as minor. Since the discovery that the pro-apoptotic multi-domain protein BAK directly interacts with VDAC2 [78], a primary focus has been on dissecting this relationship.

VDAC2, while genetically and structurally related to other two VDAC isoforms, has distinct and unique properties. Studies of the *in vitro* electrophysiological properties of mVDAC2 revealed that there are two populations of channels that varied in conductance states [45]. The presence of two populations of VDAC2 channels might reflect cellular regulatory processes attributed specifically to VDAC2. Mammalian VDAC2 also has additional 11–12 amino acids at the amino terminus [10,30] that most likely influences its channel properties [79]. Studies of mutant mouse VDAC2 with substitutions of five highly conserved amino acids further detailed its electrophysiological properties. This VDAC2 mutant is constitutively "open" and neutralizes the voltage sensor, exhibits normal flux of anions, yet, unlike wildtype VDAC2, fails to rescue the conditionally growth-restrictive phenotype at 37 °C [80]. Hence, VDAC2 presents multiple sub-states with different ionic selectivity and permeability relative to the canonical VDAC1.

Unlike the viability of VDAC1, VDAC3, and double deficient VDAC1/VDAC3 mice, global elimination of VDAC2 gene results in early stage mouse embryonic death [78]. The demonstration that VDAC2 forms a specific protein complex with BAK and suppresses BAK pro-apoptotic functions was a surprising discovery, yet is supported by further *in vitro* studies. Roy et al. confirmed that VDAC2

**Table 2**
Summary of mouse model systems available to investigate VDAC deficiency.

| | nVDAC1 | nVDAC2 | nVDAC3 | Ref |
|---|---|---|---|---|
| ▪ Mouse models available | Whole body knockout | Heart and Thymus conditional knockouts | Whole body knockout | [47,83], Unpublished data |
| ▪ Survival and growth | Pups born with less than Mendelian frequency, Mild growth retardation | No survivors with whole-body knockout Conditional knockouts show shortened lifespan | Normal growth and survival | [47,78], Unpublished data |
| ▪ Mouse model deficiency phenotypes | Glucose intolerance, insulin resistance. Defects in spatial learning, cued fear conditioning, long-term potentiation | Defects in apoptotic pathway affecting survival | Infertility, reduced sperm motility. Defects in contextual fear conditioning, spatial learning, paired pulse facilitation | [47,58,67,83] Unpublished data |
| ▪ Knockout cell lines | ES cells, MEFs, Epithelial cells | ES cells, Cardiomyocytes, Thymocytes | ES cells, MEFs | [46,47,58,67,78,83], Unpublished data |
| ▪ Cellular phenotypes | Reduced ADP diffusion, defects in ETC complexes, larger mitochondria and more compact cristae in certain muscle types. Reduced Hexokinase binding to MOM and possible altered Akt signaling | Altered apoptotic response to ER stress, abnormal Bak activity | Reduced ADP diffusion, defects in ETC complexes, larger mitochondria and more compact cristae in cardiac muscle | [46,47,58,67,78,83] Unpublished data |

recruits BAK to mitochondria and this is necessary for tBID-induced MOM permeabilization and cell death [81]. It has since been shown that inactive BAK exists in large complexes with VDAC2; these complexes constitutively localize to the MOM, and the native transmembrane anchor of BAK is required for its association with VDAC2 [82].

The necessity of VDAC2 for normal development has slowed progress in uncovering the *in vivo* role of VDAC2. Using conditional deletion of VDAC2 in lymphocytes, Cheng and colleagues showed that there was rapid cell death that could be rescued by the parallel deletion of BAK [83]. Using a similar conditional mutation strategy, we have also analyzed the consequences of the loss of VDAC2 *in vivo*. Deletion of VDAC2 from the mouse heart leads to the postnatal onset of progressive fibrosis and cardiomyopathy, resulting in early mortality. Similarly, concomitant deletion of BAK reverses the cardiac dysfunction. In cells lacking VDAC2, BAK is displaced from mitochondria and re-locates to the endoplasmic reticulum (ER). This alters the expression of a number of ER stress-related genes, and inhibits pharmacologically provoked ER stress-related apoptosis, that again can be rescued by elimination of BAK (unpublished results). We hypothesize that the accumulation of BAK in the ER leads to a novel gain of function and aberrant stress responses due to perturbations in ER calcium homeostasis.

VDAC2 is expressed in all mammalian tissues at varied levels [10]. It will be interesting to study the consequences of VDAC2 elimination in tissues such as the brain and testes where it is expressed the most, and conversely over-express VDAC2 in tissues such as the liver and kidney where the protein is normally found at low levels.

### 6.7. Other phenotypes

In addition to serving as a model system for studying direct observable phenotypes resulting from the absence of VDACs, the effects of VDAC loss of function mutations in conjunction with other mutant loci has begun to be addressed. An example of such an investigation was the study by Isrealson et al., where the interaction between Superoxide Dismutase 1 (SOD1) and the cytosol-facing domain of VDAC1 was elucidated using VDAC1$^{+/-}$ mice [84]. Mutations in SOD1 has been implicated as a cause of at least 20% of familial Amyotrophic Lateral Sclerosis, a progressive neurodegenerative disorder caused by selective loss of both the afferent cortical neurons in the brain and the ventral horn neurons in the spinal cord [85]. Since there is increased accumulation of mutant SOD1 on the MOM in animal models and patient samples, the authors investigated a possible role for VDAC1 in ALS, by crossing VDAC1$^{+/-}$ mice with mice harboring the SOD1$^{G37R}$ mutation [86]. The SOD1$^{G37R}$ mice exhibit axial tremors, asymmetric weakness and progressive paralysis of limbs, all of which are associated with extensive vacuolization and mitochondrial degradation in the motor neurons. The VDAC1$^{+/-}$/SOD1$^{G37R}$ mice showed accelerated onset, progression and attainment of end-stage disease when compared to the SOD1$^{G37R}$ mice. This phenotype was proposed to be a result of the loss of VDAC leading to reduced ATP/ADP/metabolite permeability, a drop in inner membrane $\Delta\psi_m$, accelerated mitochondrial dysfunction, increased ROS, and increased misfolding of SOD1.

The VDAC deficient mice have also been used to isolate primary cell lines that can be used for *in vitro* experimentation. The study by Okada et al., for example, isolated primary fibroblasts and respiratory epithelial cells from VDAC1$^{-/-}$ mice to characterize the role of VDAC1 in ATP release across the plasma membrane and the consequent release-dependant volume changes in response to hypotonic challenge [87]. Other studies using primary VDAC1$^{-/-}$, VDAC2$^{-/-}$, VDAC3$^{-/-}$ cell lines for *in vitro* experiments addressing mechanisms of apoptosis are underway.

In summary, animal models provide a level of biological complexity not present in more simple organisms and have been instrumental in uncovering interesting biological functions for the individual isoforms (Table 2). There are numerous areas of physiology, biochemistry, and genetics that remain to be explored. Based upon both their function and location, VDAC isoforms may be novel targets in the development of new therapeutics.

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