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Role of intramolecular and intermolecular interactions in ClC channel and transporter function

Received: 18 July 2005 / Accepted: 18 August 2005 / Published online: 16 September 2005
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Abstract The ClC family of chloride channels and transporters includes several members in which mutations have been associated with human disease. Clearly, an understanding of the structure–function relationships of these proteins will be critical in defining the molecular mechanisms underlying disease pathogenesis. The X-ray crystal structure of prokaryotic ClC proteins provides an exquisite template with which to model molecular aspects of eukaryotic ClC protein function. The dimeric structure of these proteins highlights the pivotal importance of intermolecular interactions in the modulation of channel/transporter activity, while mutagenesis studies implicate a crucial role for intrinsic interdomain interactions in regulated function. In this review, we will initially focus on the channel forming members of this family and discuss interactions within homodimeric channel complexes important for gating. Finally, with regard to both channel and transporter family members, we will discuss the multiple heteromeric interactions which occur with cytosolic proteins, and the putative functional impact of these interactions.

Keywords Dimeric interface · Domain–domain interaction · Channel gating · Post-translational modification · Regulated trafficking

Introduction

Based on primary sequence similarity, eukaryotic ClC proteins have been clustered into three main subgroups [1]. ClC-1, ClC-2, and ClC-Ka,b have been clustered with ClC-0, the prototypical eukaryotic ClC channel, to

form the first grouping. ClC-3 (A/B), ClC-4, and ClC-5 comprise a separate cluster, as do ClC-6 and ClC-7. Interestingly, members of the ClC-3 cluster, namely ClC-4 and ClC-5, have only recently been reported to function as chloride/proton antiporters upon exogenous expression in both *Xenopus* oocytes and mammalian tsA201 cells [2, 3]. Currently, the function of ClC-6 and ClC-7 as channels and/or transporters has not been resolved. Mutations in several family members have been linked to human disease (ClC-1, ClC-2, ClC-K, ClC-5, and ClC-7), highlighting their physiological significance [4–9]. In agreement with their localization, mutations in ClC-1, ClC-2, and ClC-Ka are thought to be pathogenic by altering chloride flux across the plasma membrane, resulting in changes in membrane potential or directional transepithelial transport. ClC-5 and its closely related family members ClC-4 and ClC-3, are predominantly expressed in intracellular organelles; consistent with this localization, mutations in ClC-5 are thought to cause disease by altering chloride flux and acidification of endosomes, in the proximal tubule of the kidney [10]. Finally, from the third grouping, mutations in ClC-7 are thought to cause osteopetrosis by disrupting acidification of the resorption lacuna [8].

An understanding of the structural basis for ClC protein function is key in defining the mechanism of action and molecular basis for disease. Except for ClC-K (which partners with the membrane protein Barttin [11]), it is thought that ClC proteins can confer function as a channel or transporter independently, without the participation of ancillary proteins. In fact, this was shown convincingly in studies of purified ClC-0, wherein native conduction and gating were conferred by reconstitution of purified ClC-0 into planar lipid bilayers [12]. The publication of the X-ray crystal structure of two prokaryotic members of this family galvanized the field by revealing a dimeric structure that possesses two pores through which chloride can permeate [13, 14].

Recently, functional analysis was conducted for the prokaryotic ClC for which the crystal structure was generated [15]. This prokaryotic family member

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functions as a chloride/proton antiporter, providing an elegant template for molecular modeling of eukaryotic CIC family members with similar function, CIC-4, CIC-5, and probably CIC-3 [2, 3, 15]. Interestingly, the crystal structure has proven its fidelity in predicting the molecular basis for key features of members of the CIC-0 cluster of chloride channels as well, i.e., the chloride permeation path and the outer and inner channel vestibules [16, 17]. Therefore, as initially suggested by Accardi and Miller, there are likely to be only subtle structural differences accounting for the different function of these CIC family members and there is value in using the prokaryotic protein structure to model certain molecular aspects of CIC channel function [15].

The X-ray crystal structure supported the “double-barreled” shot gun model of CIC channel gating originally proposed by Miller on the basis of the biophysical properties of the reconstituted CIC-0 channel activity [12]. This model proposes that conformational changes in a common gate, presumably conferred by a shared structural element of the dimer, precedes channel opening and subsequently each “barrel” or protopore can open and close independently. Together, the structure- and function-based models argue for a critical role for protein–protein interactions in gating of the channel. Not surprisingly, the physiological impact of CIC proteins also depends on their interaction with other proteins. Cell and tissue-based studies argue for an additional role in heteromeric interactions in the regulation of CIC activity and regulatory proteins have been identified which act to modify CIC protein localization and/or function.

In this review, we will focus on the role of intermolecular and intramolecular interactions in the regulation of CIC channels and transporters. Initially, we will discuss those interactions that contribute to chloride channel function within the CIC-0 cluster of CIC proteins. Finally, we will discuss examples of interactions with members throughout the CIC family of channels and transporters that form heteromeric complexes of functional significance.

Intermolecular and intramolecular interactions contribute to CIC channel gating

Protopore gating

The structural models of prokaryotic CIC proteins suggest that the minimal functional unit of eukaryotic CIC channels is dimeric [13] (Fig. 1). Together with previous biophysical studies, this structural model suggests that association of two CIC polypeptides confers function even though each individual monomer possesses a protopore through which chloride ion permeates. Dimeric association is thought to create a common gate, which undergoes “slow” conformational change to permit channel activation. Subsequent to the release of this common “slow” inactivation gate,

independent “fast” gating of each protopore occurs. Gating of each protopore has been modeled as a toggle mechanism involving subtle side chain movements of a single glutamate residue residing within the chloride permeation path [14]. Protopore gating is thought to be regulated by protonation of this key glutamate residue and the relative occupancy of binding sites for chloride in the permeation path and the channel vestibules. This residue is also implicated in the proton transport function of the antiporters [2, 3], suggesting that both chloride ions and protons share at least a limited region within same conduction pathway. However, there are suggestions in the literature that the molecular basis for “fast” gating in the microsecond to millisecond time frame may also involve structures in addition to the glutamate in the pore. Eukaryotic CICs possess extensive cytosolic termini for which no structural information exists [1]. Site-directed mutation of a residue close to the carboxy terminus of the CIC-0 (A783P), led to a change in voltage-dependent gating, although the relative contribution of the “fast” and common gate was not established in this study [18]. Similarly, Hebeisen and Fahlke reported that chloride currents mediated by CIC-1 variants lacking regions in the carboxy terminus, exhibited alterations in both the “fast” and common gate. These authors attribute these gating abnormalities to structural changes in the external channel vestibule and anion binding to this region [19]. These studies clearly suggest that gating may require complex interactions between the membrane domain and the carboxy terminus. This idea is reinforced in the discussion of the common gate in the following paragraphs.

The common gate

The common or “slow” gating mechanism, occurring on the millisecond to second time frame in CIC-0, exhibits voltage, chloride, and pH dependence. The molecular basis for the common gate remains unclear and is thought to involve relatively substantive conformational changes, as reflected in its large temperature dependence [20]. Conceptually, it is tempting to hypothesize that the structural basis for common gating requires conformational changes at interfacial regions within the channel dimer (see Fig. 1). As revealed in the crystal structure of prokaryotic CICs, there is an extensive interface between the two polypeptides of the dimer within the membrane. Further, there is empirical evidence to suggest that this interface may indeed play a key role in orchestrating the overall activity of the protein. In the eukaryotic proteins CIC-1 and CIC-5, disease-causing mutations can be mapped along the putative interface between the polypeptides in the membrane [21, 22]. Most of these mutations lead to severe diseases or exert a dominant effect in heterozygotes supporting the argument that these mutations perturb the common gating mechanism.

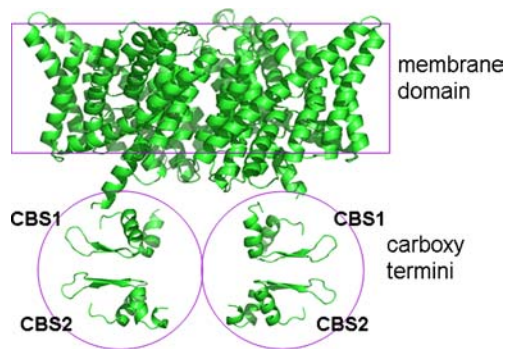


Fig. 1 Cartoon of the putative structure of eukaryotic CIC proteins. A *rectangle* has been drawn around the dimeric membrane domain based on the structure of ecCIC solved by Dutzler and colleagues [13]. The structures of the cytosolic regions of eukaryotic CIC channel/transporters are unknown. However, the carboxy terminus of each monomer within the dimer possesses two CBS domains and structures of proteins bearing a pair of CBS domains have been solved. Therefore, in this cartoon, we included two pictures of the structure of a hypothetical protein from *Thermoplasma acidophilum* (PDB code 1PMV) to represent the two carboxy termini of the CIC dimer. A *circle* encloses each carboxy terminus. This figure was generated using PYMOL

In addition to a potential role for residues located at the dimer interface in mediating the common gate function, residues within each protopore and lying close to the permeation path have also been implicated in this activity. Mutation of a cysteine (C212 in CIC-0), which lies in close proximity to the protopore glutamate residue described above and potentially buried deep within the membrane, leads to disruption of the “slow” gate and abolition of zinc-mediated inhibition [23]. These findings suggest possible molecular and functional interactions between the common and protopore modes of CIC channel gating.

Heterodimers

The potential for CIC channels to form heteromeric complexes with other family members has yet to be fully explored. As previously mentioned, CIC proteins are dimeric with an extensive interface between monomers within the membrane [13]. However, to date, the minimal molecular requirements for dimerization are unknown. There is compelling evidence to suggest that heterodimeric association of different CIC channels can occur within the CIC-0 cluster of channels. Lorenz and colleagues were the first to report that the co-expression of two different family members CIC-1 and CIC-2, led to the appearance of a distinct chloride conductance, exhibiting hybrid gating properties [24]. Subsequently, this same research group showed that the construction of concatamers of CIC-0 and CIC-2 led to the appearance of double-barreled channel activity, with one protopore mediating a CIC-0 like unitary conductance of 10 pS and another protopore with a CIC-2 like unitary conductance of approximately 3 pS [25]. These findings

indicate that disparate family members within this cluster can functionally associate and further, this interaction may influence gating rather than protopore conductance.

Cytosolic domains

Electrophysiological studies of splice variants and truncated CIC channels lacking regions within the amino and/or carboxy termini support the notion that the cytoplasmic domains also contribute to the “slow” gate of these channels. Partial deletion (residues 16–61) of the amino terminus of CIC-2, a member of the CIC-0 cluster of CIC proteins, led to modification of the properties of the hyperpolarization activated “slow” gate of CIC-2 [26, 27]. Similarly, a splice variant of guinea pig CIC-2, lacking a small region within the amino terminus (residues 77–86) exhibits altered gating kinetics [28]. The chloride and pH dependency of the amino terminus truncation mutants are also altered relative to the wild-type proteins. These findings suggest that the amino terminus contributes to the molecular basis for gating. However, it is not clear whether the participation of the amino terminus is mediated through interactions with other regions of the channel protein [26] or with distinct, ancillary proteins [29].

A primary role for the carboxy terminus of CIC channels in mediating the common gate function has emerged in recent mutagenesis studies [30, 31]. As in the preceding analyses of the amino terminus, most experiments were conducted in members of the CIC-0 cluster of CIC channels. The carboxy terminus possesses a pair of putative cystathione beta-synthase (CBS) regions, CBS1 and CBS2. The duplication of CBS domains is found in most proteins bearing these regions and several crystal structures show that these regions associate to form a dimer [32]. As in other proteins possessing these domains, their dimerization is not essential for, but rather appears to enhance protein expression and function at the cell surface via an unknown mechanism [30, 32–35]. Maduke and colleagues showed that expression of the membrane domain of CIC-0 alone fails to confer channel function whereas native function can be conferred by co-expression of the membrane domain plus CBS1 with the carboxy terminal CBS2 domain [18]. Estevez and colleagues also employed a “split molecule” strategy, co-expressing the entire CIC-1 protein up to the carboxy terminus of CBS1 with the remaining full length carboxy terminus or truncated versions to determine which segments are required to confer voltage-dependent “slow” gating [30]. Co-expression of this truncated protein with the remaining carboxy terminus including CBS2 does enhance functional expression of CIC-1 [30]. The co-expressed carboxy terminus included an extensive intervening sequence, CBS2 and the extreme carboxy terminus. The second CBS domain (CBS2) appears dispensable for channel activity, as CBS1 can substitute for CBS2 in this study and an

in-frame deletion of CBS2 did not markedly affect functional expression. However, a short region immediately preceding CBS2 is required for channel activity [30]. These findings suggest that CBS domain dimerization may provide an optimal molecular platform for presentation of key regions in the intervening sequence between the CBS domains to complementary interacting regions in the channel protein. Consistent with this idea, single site mutations predicted to disrupt CBS domain structure impaired the voltage-dependent slow gate [30]. Although these studies suggest that the carboxy terminus exerts an important role in channel slow gating activity, future study is required to establish the molecular mechanism underlying this function.

Several recent studies have also suggested that the CBS domains may serve to regulate channel gating/function through nucleotide binding [32, 36]. Mutations predicted to impair ATP binding via the CBS domains resulted in alterations in the voltage-dependent gating of CIC-1 [36], such that open probability of the common gate would be enhanced at resting membrane potential. These authors suggest that nucleotide depletion and enhanced opening of CIC-1 channels may contribute to stabilizing the membrane potential of skeletal muscle cells during ischemia and metabolic stress. ATP depletion also results in significant activation of CIC-2 channel function [37], suggesting that both CIC-1 and CIC-2 may contribute to a cellular stress response. This idea was reinforced in a recent paper documenting a functional interaction between CIC-2 and Hsp90 [38]. Interestingly, a disease-causing mutation in the carboxy terminus of CIC-2 (G715E) leads to impaired ATP binding [32] and exhibits altered nucleotide regulation of voltage-dependent gating [39]. These findings stress the importance of developing an improved understanding of the molecular consequences of nucleotide interaction with the carboxy terminus of these proteins.

In summary, the common gating function of CIC channels appears to involve multiple domains within these dimeric proteins and likely requires their coordinated interaction. Several key questions will likely drive future research in this area. Namely, we need to determine how the cytosolic domains interact with each other and the functional consequences of this interaction on voltage-dependent slow gating. Furthermore, we need to identify the molecular basis for interaction of the cytosolic domains with the membrane domain and assess how such interaction engages the slow gate.

Heterodimerization may impact on CIC channel/transporter trafficking

Members of the CIC-3 cluster of CIC proteins exhibit an overlapping subcellular distribution. It is generally accepted that CIC-3A, CIC-4, and CIC-5 localize

to endosomal compartments, where they facilitate endosomal acidification [40–43] through an unresolved mechanism. As previously mentioned, it has been determined that at least CIC-4, CIC-5, and possibly CIC-3 function as Cl^-/H^+ antiporters, yet the exact role for this activity in endosomal function has yet to be determined [2, 3]. Two splice variant forms of human CIC-3 (CIC-3A and a longer CIC-3B) have recently been described [44]. CIC-3B localizes to the Golgi compartments [45] and interestingly, co-expression of CIC-3B with CIC-3A leads to the partial re-direction of CIC-3A to the Golgi, possibly because association with CIC-3B confers a PDZ motif and interaction with the Golgi scaffolding protein, GOPC (see following section for further detail). These findings argue that the association of distinct family members may have important consequences with respect to organellar targeting. We have determined that CIC-4 and CIC-5 are co-expressed in recycling endosomes and can be co-immunoprecipitated from native tissues, i.e., renal proximal tubule epithelia [43]. It will be important to test the hypothesis that their interaction may promote co-localization in recycling endosomes, as the carboxy terminal PPXY motif of CIC-5, predicted to be important for its endocytosis, is not conserved in CIC-4. In this case, heterodimerization may confer a dominant endosomal trafficking motif to the complex. Alternatively, there are multiple endosomal trafficking motifs in both proteins that may promote endocytosis of CIC-4 independent of CIC-5, despite the lack of the PY motif. Therefore, future studies are required to determine the functional consequences of the interaction between endosomal CIC transporters and the relative role of distinct endosomal targeting motifs.

Interactions with cytoskeletal/scaffolding and cytosolic proteins contribute to CIC channel/transporter function and trafficking

It is well known that channel/transporter proteins can form functional interactions with cytosolic and/or other membrane proteins and that these interactions can have a vital impact on their physiological activity. In the following paragraphs, we will discuss putative interactions between CIC and cytosolic proteins, including kinases, phosphatases, ubiquitin ligases, and cytoskeletal proteins.

Cytoskeletal/scaffolding proteins

Several CIC family members have been found to associate with cytoskeletal elements. Investigation of the functional consequences of these interactions has provided considerable insight into the functional modulation of these channels. For example, disruption of the actin cytoskeleton following treatment with cytochalasin D or latrunculin results in significant activation of CIC-2

currents [46], supporting a role for actin remodeling in modulating the functional properties of the CIC-2 channel. Biochemical studies further revealed that the N-terminus of CIC-2 (residues 31–74) can associate directly with the actin cytoskeleton via electrostatic interactions [46]. This finding is significant, in light of the evidence implicating a functional role for CIC-2 in cell-cycle-dependent events, which are usually accompanied by major cytoskeletal rearrangement and actin remodeling [47–49]. Interestingly, the endosome-associated CIC-5 was also reported to interact directly with a cytoskeletal protein, cofilin, via its carboxy terminal tail [50]. Cofilin, which is an actin-binding protein, promotes disassembly of actin filaments, and has been implicated in actin remodeling [51]. Phosphorylation of cofilin leads to actin stabilization and a decrease in albumin trafficking, a function thought to be modulated by CIC-5 [50]. These findings suggest that the function and/or trafficking of CIC-5 may be mediated through associations with the actin cytoskeleton.

CIC-2 is also thought to interact directly or indirectly with the microtubule-associated dynein motor complex [52]. This association was initially defined in biochemical studies employing purified CIC-2 protein as bait for interacting proteins in detergent-solubilized rodent brain lysate. Dynein was identified in the column eluant by mass spectrometry. Both CIC-2 and dynein are likely to interact *in vivo* as they could be co-immunoprecipitated from hippocampal membranes. The functional significance of this interaction was demonstrated by the finding that the subcellular distribution of endogenous CIC-2 in a fibroblastic cell line was dependent on the integrity of the dynein motor complex [52]. This finding provides the first direct evidence to suggest that the functional expression of CIC-2 at the cell surface is regulated through endosomal trafficking.

Extensive investigations have proven that a protein's subcellular localization is highly dependent on "localization motifs", which dictate interactions with specific organelle-resident proteins. This has also been shown for members of the CIC family. CIC-3A localizes to synaptic vesicles and late endosomal compartments and plays a vital role in acidification of these compartments, as revealed in studies of vesicles obtained from CIC-3 knockout mice [40]. CIC-3B, on the other hand, localizes to the Golgi compartment [45]. This protein has an extended C-terminal cytosolic domain that contains a unique PDZ (PSD95/Dlg/ZO-1) domain-binding motif, which is implicated in CIC-3B's interaction with the second PDZ-binding domain motif of several scaffolding proteins including EBP50 and the Golgi-associated protein, GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein) [44]. The interaction of CIC-3B with GOPC via its carboxy terminus possibly accounts for its Golgi localization. CIC-3A lacks this PDZ domain-binding motif and this may contribute to its distinct localization in the late endosomal compartment [45].

Interactions with kinases, phosphatases and ubiquitin ligases alter CIC channel/transporter activity and trafficking

Electrophysiological studies suggest that kinase- and phosphatase-mediated reactions significantly modulate the functional properties of several members of the CIC family, including CIC-1, CIC-2, and CIC-3. In general, although there are some exceptions [53], agonists that enhance cellular kinase activity suppress chloride flux through these proteins, whereas those that promote phosphatase activity increase CIC function at the cell surface [37, 54–56]. Several kinases have been implicated in the regulation of CIC-2, including PKA, PKC, the M-phase-specific p34^{cdc2}/cyclin B kinase and serum and glucocorticoid inducible kinase isoform 3 [48, 54–57]. Type 1 protein phosphatases have been implicated in the functional regulation not only of mammalian CIC-2 (i.e. PP1, PP2A phosphatases), but also the related *Caenorhabditis elegans* CLH-3 (i.e. the *C. elegans*-specific CeGLC 7 α , 7 β phosphatases) [37, 54]. As these phosphatases have been implicated in controlling *C. elegans* mitotic and meiotic cell cycle events [58–60], it has been suggested that CLH-3 may be functionally important in cell-cycle-dependent events. As CLH-3 exhibits biophysical and regulatory properties that are similar to CIC-2 [37, 47, 61] an analogous functional role may be ascribed to CIC-2 in mammals. Indeed, a single C-terminal serine residue in rabbit CIC-2 is directly phosphorylated by the M-phase specific p34^{cdc2}/cyclin B kinase [48, 49].

Recently, a detailed study by Denton et al. provided evidence for a direct protein interaction between CLH-3 and GCK-3 (germinal center kinase-3) [61]. Yeast two-hybrid studies first identified GCK-3 as a binding partner for the C-terminus of the CLH-3 isoform, CLH-3b. Interestingly, heterologous expression of GCK-3 elicited a dramatic inhibition of recombinant CLH-3b in HEK293 cells. Conversely, disruption of endogenous GCK-3 gene expression in worm oocytes by siRNA led to constitutive activation of CLH-3 currents, supporting the biological significance of this interaction [61]. Denton and colleagues suggested that there may be a key role for this kinase in the cell-cycle-dependent modulation of CLH-3b. As the mammalian homolog of GCK-3, PASK (proline alanine-rich STE-20 related kinase) is a cytoskeletal-associated kinase that is highly expressed in neuronal and epithelial cells [62], it is possible that CIC-2 may be modulated in a similar manner, though future studies are required to validate this claim.

Several investigations have pointed to an additional role for kinase activity in modulating the cell surface stability of CIC-2. First, phosphoinositide (PI) 3-kinase, which has been implicated in the endocytic trafficking of other membrane proteins, such as the transferrin receptor [63] has been proposed to regulate CIC-2 function [64]. Furukawa and colleagues provided evidence to suggest that phosphorylation of the CIC-2 on its carboxy terminus by p34^{cdc2}/cyclin B kinase leads to enhanced

targeting to the proteasomal degradation pathway [48, 49]. These findings suggest that the cell surface expression of CIC-2 channels, i.e., channel number, may be regulated in a phosphorylation-dependent manner and future studies are required to identify the protein-binding partners which mediate regulated endosomal trafficking. We have already alluded to the role of dynein in the trafficking of CIC-2. The function of dynein is modified by phosphorylation [65] and this regulation may potentially account for phosphorylation-dependent changes in the cell surface expression of CIC-2.

A distinct type of post-translational modification, ubiquitination, may also modify trafficking of the endosomal CIC protein, CIC-5 [66, 67]. CIC-5 possesses a proline-rich motif in an intervening region between the two CBS domains of its carboxy terminus, similar, but not identical to the PPXY motif utilized by ENaC [66]. In vitro binding studies led to the hypothesis that this region of the CIC-5 protein binds the E3 ubiquitin ligase Nedd 4.2, or the Nedd 4-like protein WWP2, possibly leading to its ubiquitination, entry into the endosomal pathway and/or degradation. Consistent with this hypothesis, mutation of this proline-rich motif results in an increase in the relative cell surface expression of CIC-5 in in-vivo studies [66, 67]. It has been proposed that Nedd 4.2 also modulates the cell surface expression and function of CIC-K, through direct action on Barttin, a β subunit which possesses a PPXY motif [11, 68]. However, the role of ubiquitination in CIC-5 trafficking and degradation remains vague and future research is required to determine the role of this modification in intracellular trafficking in the context of multiple additional trafficking motifs. For example, alternative internalization motifs may be utilized in the case of CIC-3 and CIC-4, as neither of these endosome-localized transporters possesses a PPXY motif.

Conclusions

In this review, we have highlighted the importance of intramolecular and intermolecular interactions in mediating the intrinsic channel/transporter function of CIC proteins. However, an understanding of the molecular mechanisms linking domain-domain interactions to protein function remains to be elucidated, awaiting the generation of further structural models. Finally, we discussed the role of intermolecular interactions in the context of post-translational modification of CIC proteins and subcellular trafficking. Even though this field is in its infancy, there are tantalizing suggestions of the impact of these interactions on the function of CIC channels/transporters at the cell surface and in different subcellular compartments.

Acknowledgements The authors would like to acknowledge the contributions of Winnie Luong, Dr. Raha Mohammad-Panah, Dr. Canhui Li, and Dr. Mohabir Ramjeesingh to the ideas and several of the experiments discussed in this review. Our research

was supported by the Heart and Stroke Foundation of Canada and the Canadian Institutes of Health.

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