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Vav family exchange factors: an integrated regulatory and functional view

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Abbreviations: Ac, acidic; Ahr, aryl hydrocarbon receptor; cGMP, cyclic guanosine monophosphate; CH, calponin homology; CSH3, most C-terminal SH3 domain of Vav proteins; DAG, diacylglycerol; DH, Dbl-homology domain; GEF, guanosine nucleotide exchange factor; HIV, human immunodeficiency virus; IP_3, inositoltriphosphate; NFAT, nuclear factor of activated T-cells; NSH3, most N-terminal SH3 domain of Vav proteins; PH, plekstrin-homology domain; Phox, phagocyte oxidase; PI3K, phosphatidylinositol-3 kinase; PIP_3, phosphatidylinositol (3,4,5)-triphosphate; PKC, protein kinase C; PKD, protein kinase D; PLC-g, phospholipase C-g; PRR, proline-rich region; PTK, protein tyrosine kinase; SH2, Src homology 2; SH3, Src homology 3; SNP, single nucleotide polymorphism; TCR, T-cell receptor; ZF, zinc finger region.

The Vav family is a group of tyrosine phosphorylation-regulated signal transduction molecules hierarchically located downstream of protein tyrosine kinases. The main function of these proteins is to work as guanosine nucleotide exchange factors (GEFs) for members of the Rho GTPase family. In addition, they can exhibit a variety of catalysis-independent roles in specific signaling contexts. Vav proteins play essential signaling roles for both the development and/or effector functions of a large variety of cell lineages, including those belonging to the immune, nervous, and cardiovascular systems. They also contribute to pathological states such as cancer, immune-related dysfunctions, and atherosclerosis. Here, I will provide an integrated view about the evolution, regulation, and effector properties of these signaling molecules. In addition, I will discuss the pros and cons for their potential consideration as therapeutic targets.

Introduction

The first member of the Vav family was discovered due to an artifactual oncogenic activation of the *VAV1* gene during the course of focus formation assays conducted in M. Barbacid's lab.[1] Since it was the sixth human oncogene identified by that group, it was designated as the sixth letter of the Hebrew alphabet (*VAV*). Mouse and human *Vav2* and *Vav3* genes were isolated using standard cloning procedures in the 1995–1999 period.[2-4] The rest of family members were identified during the extensive characterization of genomes from multiple species that took place during this last decade. The isolation of the other family members led to the progressive designation of the founding member of the family as Vav1, a name that has become common currency in the field. Despite such a serendipitous discovery, we now know that Vav proteins play crucial signaling roles in a large variety of organisms and cell types. They also show a quite idiosyncratic activation by direct tyrosine phosphorylation, a property that makes them critical elements in protein tyrosine kinase (PTK)-regulated pathways. Consistent with the oncogenic activity shown by the founder member of the family, recent data have revealed that these proteins also play roles in tumorigenesis, metastasis, and many other diseases. In this review, I will provide a general overview about this family, including new data about its evolution, regulation, function, signaling specificity, and potential therapeutic value. Readers can find additional information about Vav proteins in previous review articles,[5-10] book chapters,[11] and online resources.[12-14]

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Phylogenetic Distribution and Structure

The earliest evidence of the presence of a Vav family protein in the phylogenetic tree is found in Choanoflaggelates, a group of unicellular aquatic protists considered to be the closest relatives to metazoans.[15] They are also the first known organisms that developed PTK- and Src homology 2 (SH2)-encoding genes,[15] thus emphasizing the close link that exists between Vav proteins and PTK-driven signal transduction events. Since then, members of this family have been found in all animal metazoans characterized so far. Single Vav family members are present in invertebrate species while vertebrates contain 3 family members. However, alternative splicing events yield different protein isoforms in all species. In mammals, Vav1 is primarily detected in haematopoietic cells whereas Vav2 and Vav3 display broader expression patterns.[2-4,16,17]

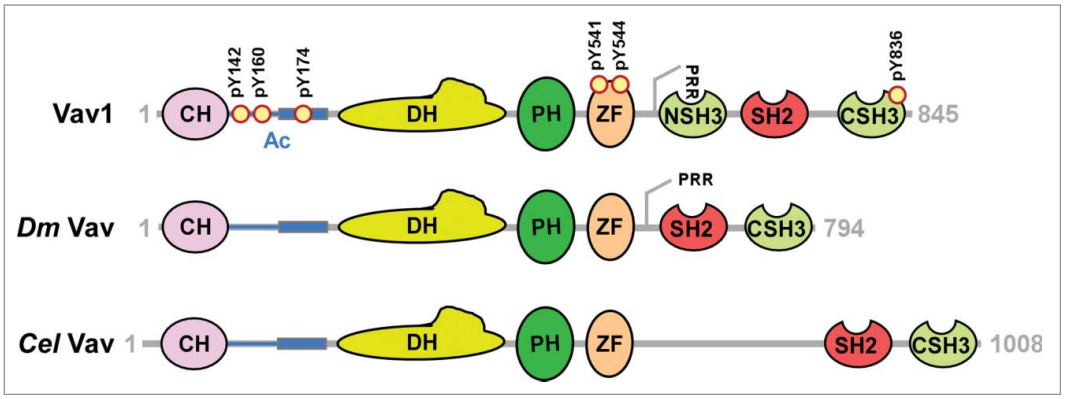

Figure 1. Examples of the multidomain structure of some Vav family proteins. Abbreviations for domains have been indicated in the main text. The shape of the DH tries to mimic the 3 dimensional structure of this domain. Phosphorylation sites are shown as yellow circles. Amino acid numbers correspond to the primary sequence of mouse Vav1. An Ac α helix involved in the stabilization of the autoinhibited structure of Vav proteins is shown as a blue box. Cel, *C. elegans*; Dm, *D. melanogaster*.

All Vav family proteins contain a basic, evolutionarily conserved structural scaffold composed of an N-terminal calponin-homology (CH) domain, an acidic (Ac) region, a catalytic Dbl-homology (DH) domain involved in the guanosine nucleotide exchange reaction, a pleckstrin-homology (PH) region, a C1 subtype zinc finger (ZF) domain, an SH2 and a C-terminal SH3 (CSH3) (**Fig. 1**). Additional domains were sequentially acquired into the molecule during evolution, including a proline-rich region (PRR) and a more N-terminally-located SH3 (NSH3) (**Fig. 1**). The PRR was incorporated in the transition between nematodes and flies. The NSH3 was acquired at the level of tunicates and, since then, remained conserved in all species of the Chordate phyla. The annotation of recently sequenced genomes has revealed that the remnant structure of invertebrate Vav proteins can be found in some Chordate Vav proteins due to differential splicing events. Thus, it is likely that the incorporation of the NSH3 could represent a gain-of-function event not necessarily required for maintaining the basal activity of the full-length proteins. This is consistent with mutagenesis experiments indicating that the functional integrity of the NSH3 domain is not essential for the activation step and effector functions of Vav family proteins in most cell types.[18,19] An important structural property of Vav proteins is that some of their domains form superorder structures in the molecule. For example, the DH, PH, and ZF domains fold together into a common structural unit (referred hereafter as "catalytic core")[20-22] that, depending on the spatial distribution of the domains, has either positive or negative effects in the overall DH domain catalytic activity.[20] This central cassette also establishes phosphorylation-dependent interactions with the CH-Ac and the CSH3 regions,[20,23] a flip-flop mechanism essential for the control of the overall biological activity of these proteins during signal transduction.[4,18,20,23,24] The Vav PRR and NSH3 domain also form a single functional unit that facilitates the interaction with protein partners.[25,26] Vav proteins are the only Ras superfamily GEFs that harbor a DH-PH-ZF cassette and, consistent with their close connection to tyrosine phosphorylation events, the prototypical phosphotyrosine binding SH2 domain. As will be discussed latter, these structural idiosyncrasies have a significant impact in the regulation and function of these proteins.

Signaling Roles

Tyrosine-phosphorylated Vav proteins exhibit both catalysis-dependent and independent activities during cell signaling (**Fig. 2**). The enzyme activity involves the stimulation of GDP/GTP exchange on Rho subfamily GTPases, a catalytic step that favors the transition of these GTP-binding proteins from the inactive (GDP bound) to the active, GTP bound state.[4,24,27-29] The main in vitro substrates for Vav proteins include Rac1 and RhoA subfamily proteins.[4,22-24,27-31] Consistent with those catalytic functions, Vav protein can engage a large number of Rho family-dependent cytoskeletal, signaling, and biological responses in most cell types and species tested so far.[12-14] These functions are highly conserved, because active versions of *D. melanogaster* Vav can trigger cytoskeletal change and cell transformation when ectopically expressed in mouse cells.[31] By contrast, Vav proteins do not seem to effectively target Cdc42 under standard catalytic/substrate ratio conditions.[4,23,24,27-30] It should be noted, however, that a very limited number of biological processes regulated by Vav proteins have been attributed, at least partially to Cdc42-dependent mechanisms.[32-42] Whether this is due to catalytic or noncatalytic processes, remains to be determined. Interestingly, in the case of Vav1, the catalysis of nucleotide exchange on Rho proteins requires the cooperation of the DH, PH and ZF regions.[21,22] The requirement of the DH and ZF region seems to be also required in all Vav proteins tested so far. However, there is more flexibility regarding the involvement of the PH region, since constitutively active versions of both Vav2 and Vav3 with inactive PH domains are still catalytically active in vitro (Vav2, Vav3) and in vivo (Vav3).[21,22,43]

The most characteristic noncatalytic function of Vav proteins is the activation of the nuclear factor of activated T-cells (NFAT) in lymphocytes. Although not fully characterized yet, this route requires signals emanating from the Vav CH domain, the Vav-dependent activation of phospholipase C-γ (PLC-γ) and the downstream Ca^{2+}-dependent calcineurin phosphatase, and synergistic signals triggered by upstream antigenic receptors.[6,8,18,44-48] It probably involves other Vav family- and cell type-specific features as well, because Vav2 and Vav3, unlike the case of Vav1, can trigger NFAT activation in B- but not T-cells.[23,49] Noncatalytic roles for Vav1 in T lymphocytes have been also linked to other biological processes such as integrin-mediated spreading

and the stabilization of Slp-76 microclusters upon T-cell receptor (TCR) stimulation.[50,51] The effectors that mediate such functions are still unknown. The identification of ZF- and CSH3-binding proteins that are not obviously connected to the catalytic performance of Vav1 also suggests that additional catalysis-independent signaling routes might exist[5,6,8,11-14,52] (**Fig. 2**). Experimental evidence indicates that Vav2 and *D. melanogaster* Vav proteins can also trigger GTPase-independent routes in breast cancer cells and during eye development, respectively[53] (D. Martín-Bermudo, personal communication). Some studies have also described noncatalytic, nuclear roles for Vav proteins. Thus, Vav1 and Vav3 have been associated with transcriptional responses mediated by PU.1, NFAT, NFκB, heat shock transcription factor 1, and the androgen receptor.[54-60] In the case of Vav3, this transcriptional activity seems to rely on adaptor functions localized within the DH-PH-ZF cassette[57,58,60] (**Fig. 2**).

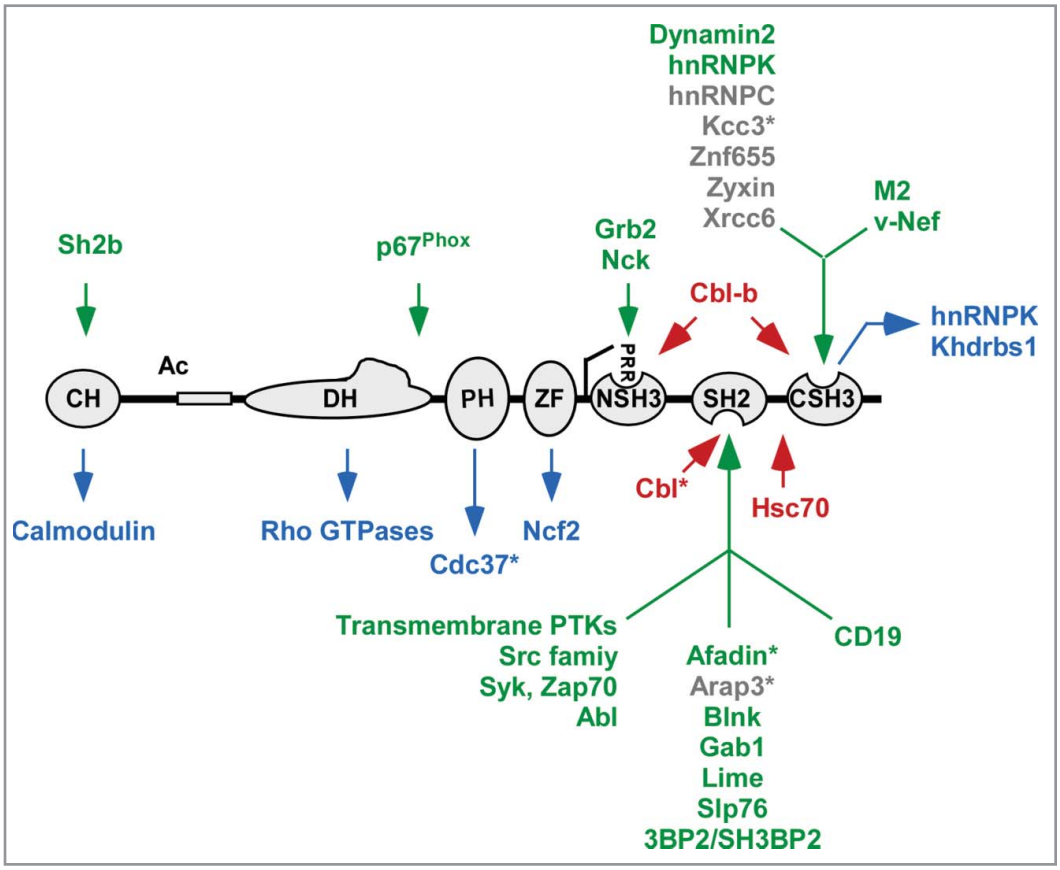

Figure 2. The Vav family interactome. Examples of some of the Vav family interacting proteins and domains involved. Effector molecules are shown in blue. Positive and negative regulators are shown in green and red, respectively. Proteins whose interaction was only described for Vav2 or Vav3 are indicated by asterisks. Please, note that some of these proteins may exert several functions in the context of Vav signaling.

Many of the pathways stimulated by cytoplasmic Vav proteins contribute to signaling diversification events via the generation of second messengers such as diacylglycerol (DAG), inositoltriphosphate (IP_3), Ca^{2+}, phoshadidylinositol (3,4,5)-triphosphate (PIP_3), phosphatidic acid, arachidonic acid, and cyclic guanosine monophosphate (cGMP). The best example for such an activity is seen in lymphocytes, where Vav1 favors the stepwise activation of PLC-γ, phosphatidylinositol-3 kinase (PI3K), protein kinase C (PKC), and PKD.[48,61,62] This leads to the stimulation of non-conventional routes for a Rho GEF such as, for example, the DAG-mediated stimulation of the RasGRP1-Ras-Raf-Erk axis in stimulated T- and B-cells.[8,62-66] Signaling diversification processes have been also found in other cell types such as mouse natural killer cells, mast cells, osteoclasts, vascular smooth muscle cells, and *C. elegans* tissues.[67-71] Depending on the cell type and signaling response involved, Vav proteins can trigger these signaling diversification events in either a catalysis-dependent (i.e., cGMP production in vascular smooth muscle cells, DAG and IP_3 production in B lymphocytes, IP_3 production in *C. elegans* tissues) or independent (i.e., DAG and IP_3 production in T lymphocytes) manner.[47,62,70,71]

A still poorly addressed issue is the level of functional redundancy among the 3 Vav family proteins present in Chordate species. However, cell biology and biochemical data suggest that Vav proteins play overlapping, but not identical functions. This evidence includes the different morphologies exhibited by Vav1 and Vav2 oncogene-transformed cells, the synergistic effects of Vav2 and Vav3 in the generation of the Vav2;Vav3-dependent transcriptomal program of breast cancer cells, the Vav1-specific activation of NFAT in T-lymphocytes, the PH requirement for biological activity, and differences in the spectrum of Vav CSH3 binding proteins.[3,4,18,49,53,72-74] It remains to be determined whether those nonredundant activities can be attributed to different subcellular localizations, activities, or spectra of binding partners. To date, nothing is known regarding the signaling specificity of protein isoforms generated by each Vav family gene by differential splicing in some tissues.

In vivo Roles

Genetic analyses have demonstrated that Vav proteins play key signaling roles in many animal metazoans. In the case of *C. elegans*, the depletion of Vav leads to larval lethality due to

defective rhythmic contractile responses in a number of tissues, including the pharynx, ovaries, and intestine. This phenotype has been associated to Rho- and IP_3-dependent Ca^{2+} generation defects.[71] In *D. melanogaster*, the elimination of Vav leads to defective neuronal axon guidance-related events and embryonic lethality. The few Vav-less flies that manage to hatch show extensive locomotor coordination defects caused by abnormal axon innervation of the ellipsoid body, a key locomotor center in Diptera. These defects are due to the normal implication of this protein in Rac1-dependent axon guidance and repulsion processes.[75,76] *D. melanogaster* Vav is also involved in the development of the eye using, as indicated above, catalysis-independent mechanisms (D. Martín-Bermudo, personal communication).

In mammals, the elimination of the haematopoietic -specific Vav1 protein, either alone or in combination with other Vav family proteins, leads to severe defects in lymphocyte development, selection, and effector functions. The signaling dissection of those defects indicate that Vav proteins are essential for T-cell and B-cell receptor responses that contribute to cytoskeletal rearrangement, formation of lymphocyte/antigen presenting cell contacts, PI3K- and PLC-γ-mediated signaling diversification events, activation of downstream transcriptional factors, expression of lymphocyte surface markers and cytokines, regulation of cell cycle-promoting proteins, and stimulation of survival pathways. The function of Vav proteins in lymphocytes has been extensively reviewed before.[7,8,12-14] It is likely that Vav1 would play additional roles in T-cells, because the lack of Vav1 unexpectedly leads to the long-term development of T-cell lymphomas, a process further aggravated when the *Rasgrf2* gene is simultaneously knocked out in mice.[77] The mechanistic basis for this "tumor suppressor"-like role remains unknown. Milder effects have been also observed in other haematopoietic cell types, including stem cells, natural killer cells, platelets, neutrophils, macrophages, foam cells, mast cells, and dendritic cells.[12-14]

Outside the haematopoietic system, Vav2 is important for the nitric oxide-mediated relaxation of blood vessels. Due to this, its depletion leads to hypertension and hypertension-associated diseases such as cardiovascular remodeling, cardiorenal fibrosis, and kidney function alterations.[70,78] Vav3 plays roles in bone remodeling, cerebellum development, and axon wiring events in the ventrolateral medulla. The former function is connected to the control of macrophage-colony stimulation factor- and $\alpha_v\beta_3$ integrin-dependent late osteoclast maturation stages.[69] In the cerebellum, Vav3 is involved in Purkinje cell dendritogenesis, the survival and migration of granule cells, and the formation of the intercrural fissure during the perinatal period. Due to these roles, the absence of Vav3 leads to motor coordination and gaiting defects in early postnatal periods of knockout mice.[79] Finally, Vav3 controls the migration of axons of GABAergic neurons from the caudal to the rostral region of the ventrolateral medulla,[80] a brainstem center that controls sympathetic, respiratory, heart, cardiovascular, and renal activities.[81] When Vav3 is absent, the lack of GABAergic wiring eliminates the tonic inhibition exerted by the caudal over the rostral ventrolateral medulla, leading to chronic sympathoexcitation and a large variety of sympathetic-mediated defects such as tachycardia, brachypnea, hypertension, and metabolic syndrome.[80,82,83] The analysis of double *Vav2*$^{-/-}$; *Vav3*$^{-/-}$ and triple *Vav1*$^{-/-}$;*Vav2*$^{-/-}$;*Vav3*$^{-/-}$ knockout mice has revealed defects in axon guidance, dendritic spine formation, and synaptic plasticity in retinal, cortical and hippocampal neurons.[84,85] Alterations in enterocytic differentiation, intestinal epithelial barrier integrity, and angiogenesis have been also observed.[86,87] It is not known as yet whether these functions depend on either the single or combined action of these 3 GEFs.

Genetic data indicate that Vav proteins may cooperate with other Rho GEFs in some biological responses. Those include the collaboration of *D. melanogaster* Vav and Trio during axon guidance processes, the cooperation shown by mouse Vav1 and RasGRF2 proteins in both immature and tumor mouse T-cells, and the functional interaction between Vav1 and P-Rex1 in mouse neutrophil responses.[75,77,88,89] However, despite the multiple Rho GEFs coexpressed with Vav proteins in normal tissues and disease conditions, the inactivation of Vav proteins is sufficient to generate a significant impact in many signaling contexts. This indicates that they regulate quite specific regulatory steps that cannot be redundantly stimulated by other Rho GEFs.

Regulatory Mechanisms

The biological activity of Vav proteins is controlled by phosphorylation-dependent changes in their intramolecular architecture. Nonphosphorylated Vav proteins are inactive due to a "closed" conformation mediated by extensive intramolecular interactions between the CH-Ac, the CSH3, and the catalytic core (**Fig. 3A–C**). These contacts occlude the GTPase binding site, favor a catalytically incompetent conformation of the DH-PH-ZF cassette, and limit the noncatalytic output of these proteins.[4,18,20,23,24,30] This inhibited structure is unleashed upon the phosphorylation of Vav proteins on a number of tyrosine residues located in the Ac (Tyr^{174} and, to a lower extent Tyr^{142}, Tyr^{160}), ZF (Tyr^{541} and Tyr^{544}), and CSH3 (Tyr^{836}; amino acid numbers correspond to the primary sequence of mouse Vav1)[20,23,30,90] (**Fig. 3A**), leading to the activation of both the catalytic and noncatalytic functions of these proteins.[4,18,20,23,24,30,90] The actual structure of this "open" conformation has not been elucidated as yet in the case of the full-length Vav proteins. Due to this, it remains to be determined whether the CH-Ac and CSH3 fully detach from the rest of the domains of the molecule during this activation step or, alternatively, remain in contact with other domains in a different spatial orientation. However, structural studies conducted with N-terminally truncated Vav1 indicate that the catalytic DH-PH-ZF core of Vav proteins probably undergoes a shift from a catalytically incompetent to a catalytically competent conformation upon the release of the inhibitory domains.[20] Likewise, the CSH3 seems to remain in contact with the catalytic core when Vav proteins are in the "open" state.[23] In addition to the exposure of effector sites, it is possible that this flip-flop activation could be used for other regulatory layers. For example, it can be speculated that the need of multiple phosphorylation events for

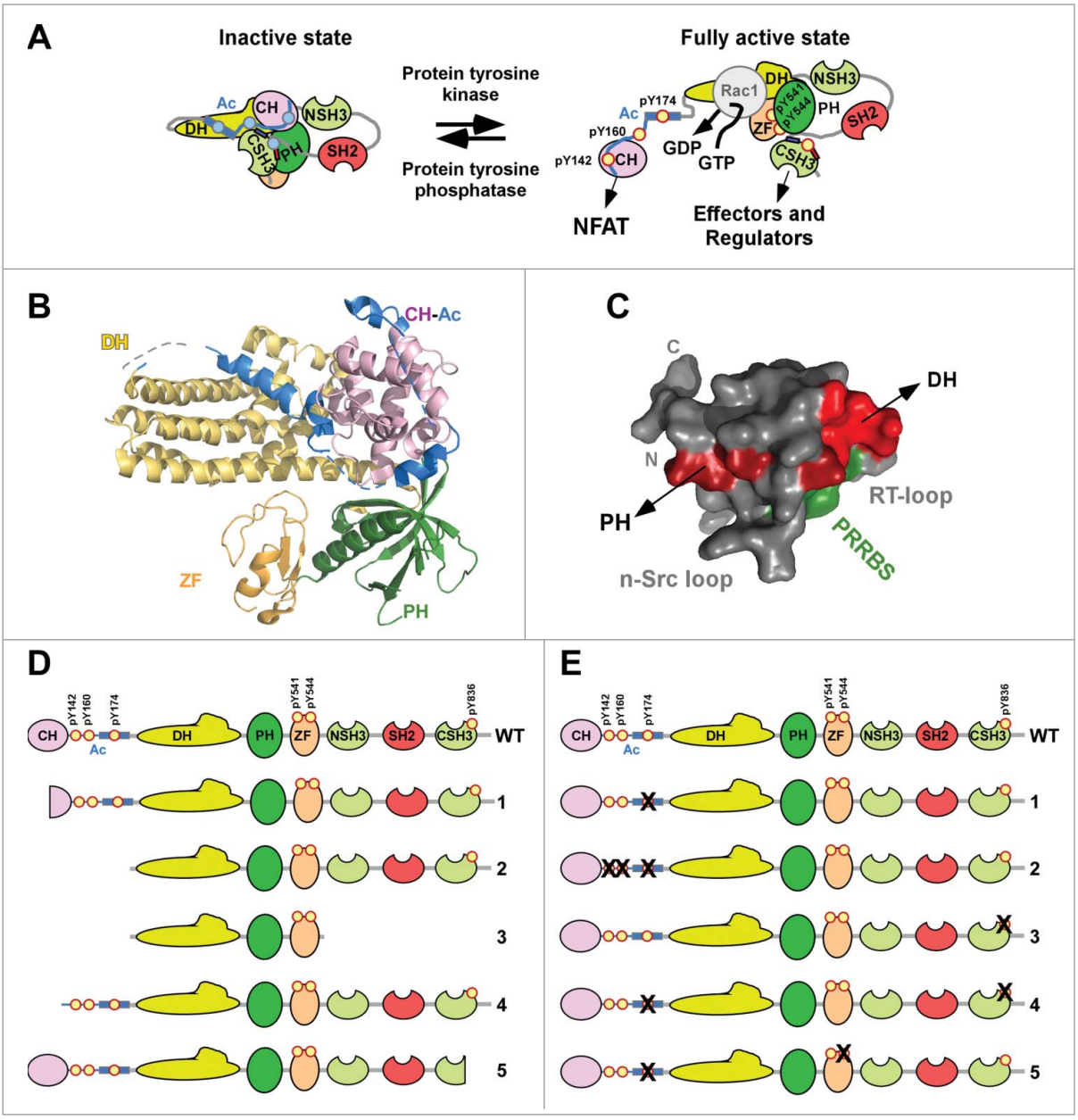

Figure 3. Mechanism of activation of Vav family proteins. (**A**) A model for the phosphorylation-mediated activation of Vav proteins based on recently described structural and biochemical data. The autoinhibited state of nonphosphorylated Vav proteins is stabilized by extensive contacts of the CH domain and 2 tyrosine residues of the Ac region (Tyr^{142}, Tyr^{160}) with the DH and PH domains[20]; an α helix present in the Vav Ac domain (which contains the Tyr^{174} phosphosite) with amino acids located in the GTPase binding interface of the DH domain[20,30]; and additional residues of the Ac with the PH domain.[20] In addition, 2 independent amino acid stretches of the CSH3, which do not include the canonical PRR binding site, establish interactions with both the DH and PH domains (see also panel **C** below).[23] Upon phosphorylation of indicated residues, the autoinhibited structure is released. Note that the 3 dimensional structure of inactive and active full-length Vav proteins is unknown, so the conformations shown for the 2 functional states of Vav proteins are hypothetical. Nonphosphorylated and phosphorylated tyrosine residues are shown as blue and yellow circles, respectively. The 2 sites of the CSH3 domain of Vav proteins involved in formation of the autoinhibited structure are depicted as dark blue and red boxes. Color codes for Vav domains are those used in **Figure 1**. (**B**) Crystal structure of the autoinhibited Vav1 CH-Ac-DH-PH-ZF region.[20] Stretches of the Ac region that could not be crystallized are shown as broken lanes. (**C**) The Vav1 CSH3 structure showing the areas that establish the intramolecular interaction with indicated domains (red color).[23] C, domain C-terminus; N, domain N-terminus; PRRBS, PRR binding site. The area corresponding to the canonical CSH3 PRR binding site is shown in green. (**D and E**) Examples of some hyperactive Vav mutants (proteins 1 to 5) generated by either domain truncation (**D**) or amino acid mutations (**E**). The full-length protein (WT) is included in both panels to facilitate an easy understanding of the type of mutations made. In **E**, the specific amino acid residues that had been mutated have been highlighted using a cross sign. Mutants are ordered from top to bottom following the order of discovery. The first mutation ever found in the Vav family is indicated in **D** (mutant 1).[1,91] The truncated mutant proteins labeled as 2, 3, 4, and 5 shown in panel **D** were first described by Schuebel et al. (1996),[3] Schuebel et al. (1998),[24] Movilla et al. (1999),[4] and Barreira et al. (2014),[23] respectively. In **E**, mutant proteins labeled as 1–2 and 3–5 were first described by Lopez-Lago et al. (2000)[90] and Barreira et al. (2014),[23] respectively.

optimal Vav activation could be used to establish minimal, digital-like thresholds for the activation of these proteins, setting up gradients of activity, or engaging different downstream routes depending on the strength of incoming extracellular stimuli and the total number of phosphosites engaged at a given time. Conversely, they can represent a safeguard mechanism to avoid the spurious stimulation of Vav proteins by the stochastic phosphorylation of single phosphosites in nonstimulated cells. Interactions of the Vav CSH3 with other proteins in the "close" state may also induce conformational changes that could facilitate the access of the hidden tyrosine hydroxyl groups for the phosphorylation step. Alternatively, they could delay the return of the CSH3 back to the "close" state when taking place in the active conformation, thus facilitating either the time- or subcellular location-specific extension of active Vav signaling output in cells. Regardless of all its potential regulatory roles, the relevance of this intramolecular mechanism is underscored by its phylogenetic conservation and, in addition, by the hyperactivation observed in Vav proteins upon the mutation of either domains or residues involved in maintaining the autoinhibited conformation[3,4,18,20,23,24,31,71,90] (**Fig. 3D and E**). Indeed, a 66 amino acid-long deletion of the Vav1 CH domain was the genetic alteration that allowed the initial discovery of the *VAV1* oncogene[1,91] (**Fig. 3D**). It is worth noting that phosphorylation-independent activation steps for the Vav family have been observed upon the interaction of the p67 phagocyte oxidase subunit ($p67^{Phox}$) and the human immunodeficiency virus (HIV)-encoded v-Nef protein with the Vav1 catalytic core and CSH3, respectively[41,92] (**Fig. 2**). The basis for such activation remains unknown, although it may involve domain displacement mechanisms that could favor the spurious "opening" of the autoinhibited state of Vav proteins. This type of domain displacement-based activation has been found before in other signaling molecules, such as the Src kinase family.[93]

The Vav phosphorylation step is mediated by either transmembrane or cytosolic PTKs. The former subclass encompasses a large variety of receptors for both soluble growth factors and ephrins. The phosphorylation of Vav proteins by these receptors usually involves a direct, SH2-mediated interaction of Vav proteins with the autophosphorylated cytoplasmic tails of the kinases.[5,12-14] Historically, this was actually the first connection ever described between Vav proteins and PTKs.[94,95] Cytosolic Src, Syk, Janus, Tec, and Abl family kinases are mainly involved in the phosphorylation of Vav proteins downstream of receptors lacking intrinsic tyrosine kinase domains such as those for antigens, cytokines, and extracellular matrix proteins (i.e., integrins).[5,12-14] Src kinases are also in charge of phosphorylating Vav proteins in other signaling cascades, including those initiated by nitric oxide, Wnt, tumor necrosis factor, diacylglycerol-regulated protein kinases, and some transmembrane PTKs.[70,96-99] The close connection of Src PTKs with Vav proteins probably extends far in evolution, because these molecules are one of the few kinase families present in Choanoflaggelates.[15] Although the interaction of Vav proteins with cytosolic kinases may be direct as in the case of the transmembrane PTKs (**Fig. 4**, point a), it usually entails the prior orchestration of either 2 or 3-step plasma membrane translocation mechanisms. In the former case, Vav proteins establish SH2-mediated interactions with phosphorylated coreceptors (i.e., CD19, Lck interacting membrane protein)[49,100-102] (**Fig. 4**, point b). In the latter case, Vav proteins first interact with cytosolic adaptor molecules using the SH2 (Slp76, Blnk), PRR-NSH3 (Grb2, Nck) or the CSH3 (dynamin 2) domains[51,74,103-109] and, subsequently, utilize those adaptors to dock onto phosphorylated proteins localized at the plasma membrane (**Fig. 4**, point c). These docking proteins include coreceptors (CD28), membrane-anchored scaffolding proteins (i.e., Lat), and TCR-localized adaptor molecules (Shb).[106,110,111] Regardless of the translocation mechanism involved, the endpoint result is the phosphorylation of Vav proteins by the upstream cytoplasmic PTKs. This latter step involves transient physical interactions with the kinase, since the Vav SH2 domains are always essential for the effective phosphorylation of these proteins.[18,19] It is expected that Vav proteins will return to the inactive, "close" state at the end of the stimulation cycle via tyrosine dephosphorylation (see above, **Fig. 3A**). However, this regulatory step remains poorly characterized.

The translocation of Vav1 to the nucleus depends on a nuclear localization signal present in the PH region and, in addition, by uncharacterized cytoplasmic sequestering functions of the CSH3.[55] However, the mechanism that mediates the translocation step and subsequent activity of Vav1 in the nucleus still remains obscure. Posttranslational modifications could be involved, as inferred from the nucleus-specific arginine methylation of Vav1.[112] The cotranscriptional activity of Vav3 is regulated by the PH-mediated interaction with the cell division cycle 37 cochaperone[60] (**Fig. 2**).

Vav proteins can be potentially controlled by lysine acetylation, caspase-mediated cleavage, proteolysis, microRNAs, gene expression regulation, and epigenetic mechanisms.[33,113-122] The functional significance of most of these regulatory steps is still unknown. However, there is strong genetic evidence in favor of the microRNA-dependent regulation *C. elegans* Vav during Notch signaling.[117] The concerted action of demethylation and transcriptional factors (i.e., c-Myb) has been shown to be involved in the ectopic expression of human *VAV1* gene in nonhematopoietic cancer cells.[120,122] The mouse *Vav3* gene is also transcriptionally activated by the aryl hydrocarbon receptor (Ahr) under normal physiological conditions.[121,123] Early studies showed that the catalytic activity of Vav proteins could be activated upon the binding of DAG and PIP_3 to the ZF and PH domains, respectively.[124,125] However, such regulatory action seems at odds with the known inability of those domains to directly bind those second messengers.[126-129] Consistent with this, it has been shown that these 2 Vav domains do not undergo the expected DAG- and PIP_3-dependent changes in subcellular localization when expressed in cells.[127,128] In the case of the ZF, the reason for such lack of binding has been comprehensively analyzed at the structural and biochemical level.[126,127] In the case of the PH, the lack of PIP_3 binding is probably due to the absence of consensus amino acid sequences that typically mediate the high affinity interaction of other PH domains with this second messenger.[128,129] Extensive signaling and genetic evidence

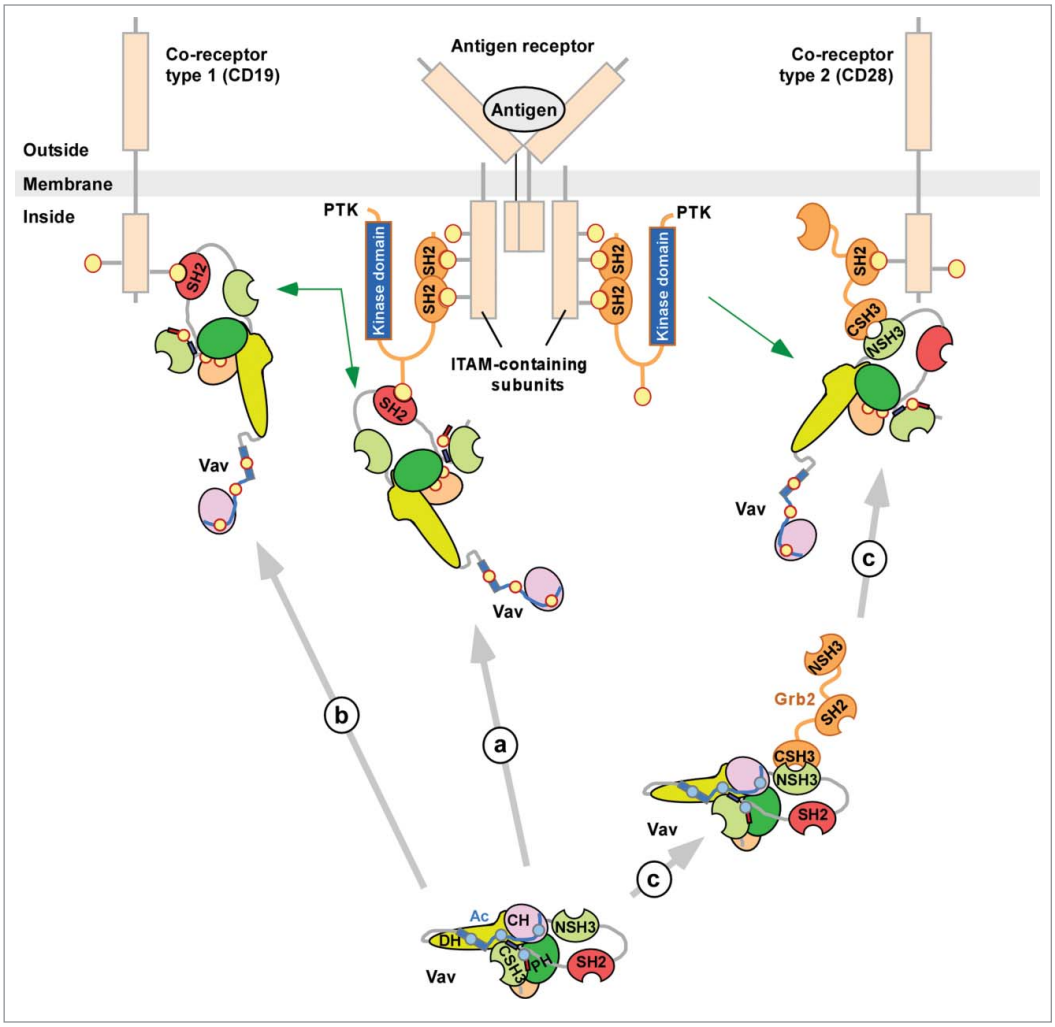

Figure 4. Signaling steps for the stimulation of Vav proteins in lymphocytes. For simplicity, we only show the phosphorylation step mediated by a Syk family member. Gray and green arrows represent translocation and phosphorylation steps, respectively. This step should include SH2-mediated interactions with the kinase (not shown in steps b and c). ITAM, immunoreceptor tyrosine-based activation motif.

also indicate that Vav proteins act upstream rather than downstream of both PLC-γ and PI3K family proteins.[8,47,62,130]

Pathological Roles

There are no consistent examples of either gain- or loss-of-function mutations for *VAV* family genes in human disease. However, the regulatory cycle of Vav proteins can be altered in some diseases by increased abundance or upon ectopic expression in tissues that do not normally express them.[53,120,122] In other cases, Vav proteins can become hyperphosphorylated due to the presence of oncogenic PTKs, mitogen-driven autocrine loops, increased abundance of Vav-binding adaptors, and the CSH3-mediated interaction with the γ-herpesvirus M2 adaptor protein.[35,73,131-136] Alternatively, the activation step can take place without apparent changes in the phosphorylation status of Vav proteins, as previously discussed in the case of the HIV v-Nef protein.[41] Genomic studies have revealed that single nucleotide polymorphisms (SNPs) in *VAV* genes seem to be linked to multiple sclerosis (*VAV1*), schyzophrenia (*VAV3*), and hypothyroidism (*VAV3*).[137-139] It is still unknown whether there is a cause-effect relationship between most of those genetic linkages and the evolution of those diseases. The only exception is multiple sclerosis, since patients containing specific SNPs in the first intron of the *VAV1* gene show elevated abundance of the *VAV1* mRNA and, in addition, increased production of proinflammatory cytokines by leukocytes present in both peripheral blood and the cerobrospinal fluid.[137] Conversely, the presence of a specific SNP in the first exon of the rat *Vav1* gene that promotes the expression of a poorly expressed Vav1 mutant version (R63W) leads to reduced production of proinflammatory factors and increased resistance to autoimmune encephalitis in this rodent species.[137] Specific *VAV2* and *VAV3* SNPs have been also associated with glaucoma development,[140] although this linkage has not been corroborated by independent studies using other patient cohorts.[141,142]

Experiments with knockout mice indicate that endogenous Vav proteins do play important roles in the evolution and/or progression of some of the above diseases. For example, the Vav1-Rac1;Rac2-Pak1 pathway has been shown to be important for the development and maintenance of c-Kit-positive acute myeloid leukemia through a cancer cell survival mechanism.[143] Vav3 is important for the fitness of $p190^{Brc\text{-}Abl}$-driven acute lymphoblastic leukemia, an effect attributed to the implication of this family member in the Rac2- and Pak1-dependent regulation of both pro- and antiapoptotic Bcl family members.[144] Vav2 and Vav3 proteins are both required for breast tumorigenesis as well as for the initiation and promotion phases of skin tumors.[53,97] In the case of breast, these proteins promote distal transcriptional programs affecting the proliferation, survival, and neoangiogenesis of cells in the primary tumor. In addition, they favor the

extravasation and subsequent fitness of breast cancer cells in the lung parenchyma.[53] In the skin, they make it possible the expression of large autocrine and paracrine programs that facilitate the keratinotyce survival to DNA damaging insults, keratinocyte G_1-S cell cycle transitions during the tumor promotion phase, and the generation of a proinflammatory environment.[97] Interestingly, the lack of Vav2 and Vav3 does not induce any overt defects in the normal development, function, and long-term homeostasis of both mammary glands and skin.[53,97] This suggests that Vav proteins may exert disease-specific tasks not directly connected to their normal function in healthy tissues. The contribution of Vav proteins to some of those tumors seems to be seminal, as inferred from the detection of Vav2;Vav3-dependent breast cancer gene signatures that can predict disease outcome in breast cancer patients.[53]

Outside the cancer field, the systemic elimination of Vav1 blocks hyperlipidemia-linked prothrombotic states as well formation of atherosclerotic lesions. These therapeutic effects are due to the specific function of Vav1 in platelets and macrophage-derived foam cells, respectively.[145-147] The specific elimination of Vav1 catalytic activity has been shown to be sufficient for eliminating other clinically relevant problems such as allograft tissue rejection and graft versus host disease.[148] Unlike the case of breast and skin tumors, these latter results probably reflect the intrinsic role of Vav1 in normal T-lymphocyte signaling. Vav2 is also important for the development of hyperhomocysteinemia-induced renal glomerulosclerosis,[149] a pathophysiological event commonly associated to end-stage renal disease in hypertensive patients. This effect has been linked to defective reactive oxygen species production in the kidney during those conditions.[149] Finally, a number of genetic and cell biology approaches have demonstrated the implication of Vav proteins, namely Vav1, in the pathogenic cycles of both HIV and γ-herpesviruses.[41,73]

Therapeutic Value of Vav Proteins: Pros and Cons

The above data indicate that Vav proteins could represent potentially interesting therapeutic targets. However, this is far from being an open and shut case. Thus, a devil's advocate would probably dismiss these knockout-based data because they do not formally prove that the inactivation of Vav proteins is effective in already developed diseases and, even if that were the case, whether the simple inhibition of their catalytic activities would be sufficient to generate a noticeable therapeutic impact. To tackle this issue, it will be important to generate "second generation" knock-in mice capable of expressing catalytically inactive Vav proteins in an inducible manner at clinically relevant disease progression stages. The devil's advocate could also argue that the collateral effects induced by the inhibition of Vav proteins in healthy tissues might hamper the implementation of such anti-Vav therapies. According to the data generated with Vav family knockout mice, such side effects could include hypertension, metabolic syndrome, infection-associated endotoxemia, and immunosuppression.[8,54,78,82,83] Some of these problems stem from roles of Vav3 in the embryonic period or, in the case of endotoxemia, in noncatalytic functions of Vav1.[54,80] It is predictable therefore that they will not show when using catalytic inhibitors for Vav proteins in adult patients. However, the inactivation of Vav2 in postnatal periods should promote hypertension and associated diseases.[70] This will actually represent a common problem for most Rac1-based therapies, because the inducible smooth muscle cell-specific inactivation of the Rac1 gene in adult mice triggers the same defects.[150,151] However, these side effects can be prevented, or cured if already developed, using standard antihypertensive treatments currently available in the clinic[70,78,82] (S. Fabbiano and X.R.B., manuscript submitted). The systemic inhibition of Vav proteins will also lead to immunosuppression and, possibly, minor defects in other haematopoietic lineages.[8,12-14] However, we do not know yet whether such defects would arise upon the inducible inhibition of Vav1 or, alternatively, in adulthood periods when thymi have already regressed. It is also possible that, as in many other targeted therapies, clinical benefits could be obtained without dire side effects due to the high signaling and metabolic demands for cells in pathological settings. Evidence for such therapeutic windows does exist in the case of Vav2 and Vav3 in both the mammary gland and skin.[53,97]

Another important issue is whether we could successfully isolate pharmacologically effective chemical inhibitors for these proteins. Indeed, it is widely assumed that the isolation of high affinity inhibitors to the catalytic site of Rho GEFs is rather difficult due to the shallow nature of the GEF/GTPase binding interface.[152] However, the idiosyncratic nature of the catalytic core of Vav proteins and its mechanism of activation can offer alternative ways to achieve such inhibition without touching the catalytic site. For example, the DH-PH-ZF structure contains additional pockets for effective inhibition, a strategy that has been proven fruitful before in the case of other Rho GEF interdomain linker regions.[153] Alternatively, we could generate drugs that could mimic the inhibitory CH-Ac and CSH3 interactions with the catalytic core. Finally, Vav-dependent diseases could be probably treated using indirect approaches, such as either the single or combined inhibition of therapeutic targets encoded by Vav-regulated genes. Experimental evidence supporting this alternative approach has been recently obtained in Vav-dependent breast cancer cells.[53] In any case, and as discussed above, the success of this type of therapies will ultimately depend on the relative contribution of the catalytic function of these proteins to the fitness of already developed diseases. Although intrinsic to any type of target-directed therapies, all the aforementioned problems underscore the need of deepening our understanding of the role of Vav proteins, and especially of their catalysis-dependent pathways, in both normal physiological and disease conditions.

Final Remarks

Despite significant advances made in the understanding of the regulatory and effector properties of Vav proteins, there are still significant open questions in this field. Thus, we have still to get a holistic view of the regulatory landscape of these proteins, the contribution of GTPase-dependent and independent routes to

the overall biological program of each Vav protein, and the level of overlap/signaling specificity that exists among them. A better understanding of Vav2 and Vav3 is still required because, up to now, most of the characterization of the functional properties of this family has been Vav1-based. Likewise, the function of Vav proteins in many model organisms is still unknown. We also need to complete the spectrum of Vav-dependent diseases and the minimal number of Vav proteins that have to be inhibited to get optimal therapeutic effects. In this context, it would be of paramount importance to generate new animal models to assess the therapeutic value of targeting the catalytic activity of these proteins and, in addition, the side effects derived from such inhibition. Finally, we have to devise ways to generate high affinity, Vav family-specific inhibitors. This future work will give us a holistic understanding of these proteins and, perhaps more importantly, yield innovative tools to both diagnose and treat human diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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