Disabled-2: A modular scaffold protein with multifaceted functions in signaling

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Disabled-2 (Dab2) is a multimodal scaffold protein with signaling roles in the domains of cell growth, trafficking, differentiation, and homeostasis. Emerging evidences place Dab2 as a novel modulator of cell–cell interaction; however, its mode of action has remained largely elusive. In this review, we highlight the relevance of Dab2 function in cell signaling and development and provide the most recent and comprehensive analysis of Dab2’s action as a mediator of homotypical and heterotypical interactions. Accordingly, Dab-2 controls the extent of platelet aggregation through various motifs within its N-terminus. Dab2 interacts with the cytosolic tail of the integrin receptor blocking inside-out signaling, whereas extracellular Dab2 competes with fibrinogen for integrin α\textsubscript{Ib}β\textsubscript{3} receptor binding and, thus, modulates outside-in signaling. An additional level of regulation results from Dab2’s association with cell surface lipids, an event that defines the extent of cell–cell interactions. As a multifaceted regulator, Dab2 acts as a mediator of endocytosis through its association with the [FY]xNPx[YF] motifs of internalized cell surface receptors, phosphoinositides, and clathrin. Other emerging roles of Dab2 include its participation in developmental mechanisms required for tissue formation and in modulation of immune responses. This review highlights the various novel mechanisms by which Dab2 mediates an array of signaling events with vast physiological consequences.

Keywords:
- adaptor protein; Disabled-2; endocytosis; phosphatidylinositol 4,5-bisphosphate; platelets; sulfatides; tumor suppressor.

Introduction

Disabled-2 (Dab2), also known as differentially expressed in ovarian carcinoma (DOC-2), is a putative tumor suppressor that was first recognized because of its downregulation in ovarian tumors [1] and, later, in a variety of other tumors [2–4]. As an adaptor protein, Dab2 also plays roles in endocytosis [5,6], growth factor signaling [7], cytoskeletal reorganization [6], control of cell adhesion [8], differentiation of hematopoietic cells [9], and modulation of platelet aggregation [10]. Tissue-dependent expression analysis shows that Dab2 is mainly expressed in the brain [11], kidney [6], intestine [12], and ovary [13]. Interestingly, expression of Dab2 is induced in mouse mammary glands during pregnancy and lactation [14]. Remarkably, and despite the multifaceted roles attributed to Dab2 in cellular signaling, we still have a limited understanding of how Dab2 acts, what its interacting partners are, what role post-translational modification plays in its activity and in shuttling and, lastly, how Dab2 crosstalk influences disease development and progression.

The human DAB2 gene, located on chromosome 5p13, consists of 15 exons and encodes a protein consisting of 770 amino acids. Dab2 was first characterized as a protein with two alternatively spliced forms named p96 (also known as p82) and p67 (also known as p59), which lacks a central exon (Fig. 1) [15]. Historically, Dab2 was referred to as p96 because mouse Dab2 was observed as a 96 kDa phosphoprotein involved in colony-stimulating factor-1-mediated signaling in macrophages [15]. The spliced form p67 has been shown to be less efficient in endocytosis [16] as this isoform is unable to associate with clathrin and adaptor protein-2 (AP-2) [5,17]. Whereas p96 localizes in discrete intracellular spots, p67 exhibits an intracellular diffused distribution [5]. Both Dab-2 spliced forms share the conserved phosphotyrosine-binding (PTB) domain and, thus, show sequence homology with the Disabled gene found in Drosophila [18]. Consequently, the two mammalian orthologs of Drosophila Dab are named Dab1 [19], which is typically expressed in neurons, and the more ubiquitous counterpart, Dab2 (p67 and p96) [15]. In addition to the N-terminal PTB domain, Dab2 proteins also share the Asn-Pro-Phe (NPF) motifs and a proline-rich domain (PRD) located near their C-termini (Fig. 1), suggesting that they function primarily as adaptor molecules.

Disabled-2, a putative tumor suppressor

Immunohistochemical analyses show that Dab2’s expression is lost in ~90% of breast and ovarian cancer cells, as well as in colorectal [3], prostate [20], bladder [21], and esophageal squamous tumors [4] suggesting a role for Dab2 as a tumor suppressor.
suppressor [13,22]. Indeed, there is good correlation of the loss of Dab2 with the transition of ovarian surface epithelial cells to premalignant states [23]. Similarly, a correlation of the absence of Dab2 with epithelial disorganization has been observed in ovarian carcinomas and early embryos [24–26].

Disabled-2 is expressed in many types of epithelial cells and is required for the organization of the epithelium [26], a function that aligns with its putative tumor suppressor activity. Breast carcinoma cell lines, including MCF-7 and SK-Br-3, lack expression of Dab2 [13]. Dab2-transfected MCF-7 and SK-Br-3 cells grow more slowly in either low or high serum, show reduced ability to form colonies on agar plates, and SK-Br-3 cells grow more slowly in either low or high serum, lack expression of Dab2 [13]. Dab2-transfected MCF-7 and SK-Br-3 cells grow more slowly in either low or high serum, show reduced ability to form colonies on agar plates, and exhibit ~50% lower percentage of cells in S phase and ~25% lower percentage of cells in G2/M phase, suggesting an absence of Dab2 with epithelial disorganization has been observed in ovarian carcinomas and early embryos [24–26].

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Downregulation or loss of expression of Dab2 has been correlated with its abnormal promoter hypermethylation [31–36] and the presence of inhibitory microRNAs [31,37–39] in human cancers. Despite these findings, additional studies are necessary to better understand the correlation between Dab2 gene silencing and cancer development.

Disabled-2, an endocytic adaptor protein

Endocytosis of transmembrane cargo requires specific sorting signals that are identified by protein coat constituents. Depending on the structural protein that builds the outer layer, coats are grouped as either clathrin or nonclathrin coats. Adapter proteins constitute the clathrin coats and, consequently, are named clathrin-associated sorting proteins (CLASPs) [40]. The function of the CLASPs is to recognize transmembrane cargo to allow for their concentration and stabilization within the coats. Generally, CLASP folded regions bind to plasma membrane constituents, whereas disordered regions enable recruitment of clathrin and accessory proteins [41]. CLASPs are classified as monomeric (i.e., Dab2, Numb, clathrin assembly lymphoid myeloid leukemia protein, and epsin) or oligomeric (i.e., AP-2) proteins. Sorting signals consist of motifs, structural conformations, or covalent modifications (i.e., ubiquitylation and phosphorylation) located within the cytosolic interface of the cargo. Signal recognition mediated by CLASPs is particularly cooperative implicating other molecular interactions with phosphoinositides, other CLASPs, the ADP ribosylation factor family of guanosine triphosphatases, and clathrin. These cooperative associations compensate for the moderate to low affinities among the components of the clathrin coat and favor the local concentration of cargo within the clathrin-coated pits, which triggers cytosolic budding of a clathrin-coated vesicle [40]. These vesicles will then deliver cargo to the first intracellular sorting station, the endosomes.

Disabled-2 is an endocytic adaptor protein implicated in clathrin-mediated endocytosis and cargo trafficking in eukaryotes [40]. Dab2 knock-out mice studies demonstrate the role of the protein in megalin endocytosis, as the absence of Dab2 leads to a reduced number of clathrin-coated pits in kidney cells that impair the intracellular transport of megalin, and, consequently, amino acid and vitamin uptake [42]. Accordingly, subcellular localization studies in NIH 3T3 and HeLa cells show that endogenous Dab2 exhibits a punctate pattern in its steady state corresponding to ~200-nm clathrin-coated pit structures in which Dab2, clathrin, and AP-2 colocalized extensively [5]. The endocytic function of Dab2 has been expanded from studies using its homologue in Drosophila, dDab, which has been reported to trigger clathrin-mediated synaptic vesicle endocytosis and, consequently, a rapid clearance of neurotransmitter release [43].

Structural basis for Disabled-2 function in endocytosis

The ability of Dab2 to act as a scaffold protein results from its modular nature (Fig. 1). The N-terminal Dab2 PTB domain is able to simultaneously bind [FY]xNPx[YF] motifs, which are found in cargo transmembrane proteins, and phosphoinositides at opposite binding sites, thus, facilitating Dab2 anchoring [Fig. 2, [17,44]]. Among plasma membrane-bound cargo proteins containing [FY]xNPx[YF] motifs and recognized by the Dab2 PTB domain are integrins, megalin, amyloid precursor protein, LDL receptors,
Figure 2. Schematic representation of the role of the AP-2-independent Disabled-2 (Dab2) endocytic clathrin sorting complex formation. Dab2 simultaneously binds to cargo, by recognition of their [FY]xNPx[YF], and to the membrane-bound phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P₂). These associations facilitate recruitment of Eps15 homology (EH) domain-containing proteins, such as Eps15 and intersectin, which, in turn, bind to polyubiquitylated chains of cargo. Myosin VI also binds Dab2 and PtdIns(4,5)P₂, and these associations may contribute to stabilization of protein complexes, actin turnover, and recruitment of additional accessory proteins and clathrin.

and apolipoprotein E receptor 2 [45–49]. Curiously, and despite the fact that the low-density lipoprotein (LDL) receptor-related protein (LRP) exhibits two [FY]xNPx[YF] motifs, Dab2 does not act as an endocytic adaptor protein for this receptor, whose dominant endocytosis signal is a YxxΦ motif instead [50].

From a structural perspective, the Dab1 PTB domain exhibits two central antiparallel β-sheets, which are capped with short N-terminal and long C-terminal α-helices [51,52]. Initially, PTB domains were proposed to recognize phosphorylated [FY]xNPx[YF] sequences in cargo; however, crystal structures of Dab1 and Dab2 PTB domains bound to [FY]xNPx[YF]-containing peptides were shown to associate with a β-turn conformation in which either the phenylalanine or tyrosine, but not the phosphotyrosine [5,53], within the C-terminal portion of the motif, is recognized by the PTB domain [51,52]. These observations were further supported by findings from another PTB domain-containing CLASP protein, the autosomal recessive hypercholesterolemia (ARH) protein, in which its PTB domain specifically recognized either tyrosine or phenylalanine within the [FY]xNPx[YF] motif located in the tail of the LDL receptor [54]. Taken together, these data suggest that PTB domains have a conserved recognition mechanism for transmembrane cargo proteins.

The PTB domain found in Dab proteins binds phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) [57,53], and possibly phosphatidylinositol 3,4,5-trisphosphate [44]. Specifically, the Dab2 PTB domain residues Lys53 and Lys90 are critical for PtdIns(4,5)P₂ recognition [17], with each of their side chains forming hydrogen bonds with the 4-phosphate and 5-phosphate groups of the lipid [51]. A model has been proposed in which interactions of adaptor proteins, such as Dab2, with PtdIns(4,5)P₂ head groups facilitate local destabilization and membrane deformation [55]. Thus, Dab2, for example, sorts LDL receptors into coated pits in a phosphoinositide-dependent manner, and this function does not require the presence of AP-2 or ARH [48]. However, Dab2 function requires the presence of other PtdIns(4,5)P₂-binding adaptor proteins to promote protein–protein interactions and to sustain endocytosis, as the affinity of the adaptor proteins for the lipid is typically low [56].

Post-translational modification of Dab2 in the form of phosphorylation is a common theme when considering membrane localization and its role in receptor internalization. Endocytosis of the vascular endothelial growth factor (VEGF) receptor, specifically VEGF2 and VEGF3, is partially mediated by Dab2 and occurs at a high rate in sprouting endothelial cells as observed in neonatal mouse retina using high-resolution imaging [57]. Dab2 is heavily phosphorylated primarily on serine residues in mammalian cells [15,58]. Specifically, atypical protein kinase C (aPKC) and aPKC-ζ phosphorylate the PTB domain of Dab2 and negatively regulate VEGF receptor-Dab2 association, VEGF internalization, and endothelial cell proliferation [57]. Therefore, during angiogenesis, phosphorylation of Dab2 could occur in endothelial cells of maturing vessels to favor their stabilization in a more mature tubular network. Dab2 is also phosphorylated in multiple sites by the cyclin-dependent kinase, cdc2, during mitosis, which facilitates Dab2 association to Pin1, a peptidyl-prolyl cis/trans isomerase that modulates post-phosphorylation events in Dab2 [59]. In this scenario, phosphorylation in serine and threonine residues downstream of the Dab2 PTB domain triggers its release from the membrane, loss of co-localization with clathrin, and subsequent reduction in receptor internalization during mitosis [60].

Whereas it is well-established that Reelin-induced Dab1 tyrosine phosphorylation leads to polyubiquitylation and degradation of Dab1 [61], little is known about the mechanisms that control the turnover of Dab2, given its role as a tumor suppressor. Recent studies, however, measured the dynamics of clathrin-coated associated proteins, including myosin VI, Dab2, and clathrin [62]. Using the microscopic technique of fluorescence recovery after photobleaching, the authors demonstrate that the tail insert-containing isoform of myosin VI exhibits a half-life (t½) of 13 ± 2 s on clathrin-coated structures in HeLa cells. Interestingly, Dab2 shows a similar
dynamic pattern with a $t_{1/2} = 10 \pm 1.5$, whereas clathrin displays a longer half-life ($t_{1/2} = 28 \pm 3.5$) in the same compartment. The shorter half-life of myosin VI and Dab2 may be associated with their transient roles in cargo clustering at the clathrin-coated pits.

**Disabled-2 facilitates endocytosis by favoring multiple associations**

The role of Dab2 in clathrin assembly is facilitated by the presence of multiple NPF repeats in the protein [Fig. 1; [63]] that mediate association of Dab2 to Eps15 homology (EH) domain-containing proteins including Eps15 and intersectin [64]. However, the stability of the endocytic complex for receptor internalization depends on additional contacts between the ubiquitin-interacting motifs in the EH domain-containing proteins and the polyubiquitylated chains present in cargo [Fig. 2; [65]]. In addition, the Asp-Pro-Phe (DPF) motifs found in Dab2 (Fig. 1) facilitate direct binding to α-adaptin, thus, coordinately working with AP-2 in the formation of the clathrin-coated pits [5]. Flanking DPF motifs in Dab2 (in p96 only) are type I and type II sites for binding to the clathrin heavy chain (Fig. 1; [47]). The stability of the complex is further strengthened by association of the serine-rich and proline-rich regions of Dab2 with the actin-associated motor protein myosin VI [6,66], which also binds PtdIns(4,5)P2 in a calcium-dependent manner [67]. Phosphoinositide association, together with Dab2 binding, facilitates myosin VI recruitment to the plasma membrane where clathrin coats form [67]. In polarized epithelial cells, myosin VI associates with apical clathrin-coated vesicles, providing the driving force to move them towards the minus end of the actin filaments [68]. Myosin VI presents several spliced forms, with the larger one containing a region called tail insert, which is necessary for the protein to be recruited to clathrin-coated pits [69]. Interestingly, myosin VI spliced forms are recognized by Dab2 [6,66] and their association promotes myosin VI dimerization [70]; however, myosin VI does not localize to clathrin pits in the absence of the tail insert even if Dab2 is present [71]. Thus, it is possible that another protein/s, other than Dab2, facilitate(s) recruitment of the tail insert-containing form of myosin VI to apical-coated pits. Nonetheless, the interaction of Dab2 with myosin VI might be required to provide a link between the actin cytoskeleton and membrane receptors during endocytosis. In this model, Dab2 also serves as a bridge linking myosin VI while also binding to the transmembrane cargo proteins via their [FY]xNPFy[FY] motifs, thus, facilitating endocytosis and unidirectional trafficking [Fig. 2; [72]].

**Redundancy versus specificity: a common theme in endocytic signaling**

Questions arise as to whether Dab2 function is absolutely required for endocytosis to occur. Accordingly, knock down Dab2 has no effect on internalization of clathrin-dependent cargo such as the transforming growth factor β receptor (TGFβ-R), LDL receptor, and transferrin receptor [73]. Instead, depletion of Dab2 alters early endosome morphology, which consequently disables the traffic of cargo from these compartments to the recycling endosomal compartments. Curiously, depletion of myosin VI also leads to formation of enlarged endosomes resulting in reclusion of the transferrin receptor in swollen early endosomes [74]. Overall, these findings suggest that there might be a functional redundancy among adaptor proteins including Dab2 that may be cell type-dependent. Other evidence, instead, points to Dab2 being central to the endocytic process, at least for some cargo molecules and in specific cell types. Unlike transferrin receptor uptake, Dab2 is implicated in the internalization of the LDL receptor even in the presence of the AP-2 adaptor complex [17] as well as for the ATP binding cassette transporter known as the cystic fibrosis transmembrane conductance regulator in airway, but not intestinal [75], epithelial cells [76]. Likewise, Dab2 conserves its endocytic function in AP-2-deficient cells [48,76], and EH domain-containing protein-mediated integrin β1 internalization is Dab2-dependent but AP-2-independent [64]. Overall, Dab2 acts as a scaffold protein by interacting with many, but not all, cargo receptors, and by establishing simultaneous contacts at the site of endocytosis, favoring recruitment of other adaptor proteins that are essential for endocytosis to occur.

**Disabled-2, an obligatory intermediary in developmental processes**

Among Dab2 targets are players involved in TGFβ-R signaling and trafficking [32,77,78]. Dab2 links TGFβ-R activation to the Smad pathway by promoting TGFβ-R-mediated Smad2 phosphorylation, nuclear translocation of Smad2 and Smad3 proteins, and Smad-dependent transcriptional responses in NIH-3T3 murine fibroblasts and rat aortic smooth muscle cells [79]. This Dab2-mediated signaling occurs by direct interaction of Dab2 with TGFβ-R, as observed in other cell lines [80]. There are other lines of evidence that show Dab2’s positive regulation of the TGFβ-R pathway. For example, Dab2 is required for TGFβ-stimulated fibronectin expression, cell adhesion, and migration and it stimulates c-JUN N-terminal kinase (JNK) activity in NIH-3T3 and A10 cells [77]. Reciprocally, TGFβ stimulates Dab2 expression [81]. However, Ehrlich and colleagues showed that, in ES-2 human ovarian cancer cells, stably expressed Dab2 negatively modulates TGFβ-induced cholesterol-dependent JNK activation without affecting the JNK-associated Smad2 pathway [82]. Whereas, they also show that Dab2 directly interacts with TGFβ-R, the association is proposed to limit the lateral diffusion of TGFβ-R at the plasma membrane favoring clathrin-mediated-endocytosis of TGFβ-R [82]. The discrepancies between both groups may simply be the result of studying signaling events in different cell types.

A well-established function of TGFβ is to induce epithelial to mesenchymal transition (EMT) during both normal and pathological cellular states by promoting cell differentiation and migration [83]. The EMT is an essential mechanism required for tissue formation and organ development. During EMT, epithelial cells lose several of their polarized properties and take on highly motile features such as those observed in mesenchymal cells. While this transition is typically observed during embryonic development, it can be pathologically found in fibrosis and cancer. Knockout Dab2 causes early embryonic lethality [6,24,26] probably due to its role in the organization of the extra-embryonic endoderm epithelium. In fact, Dab2 mediates E-cadherin trafficking and, consequently, epithelial polarity and organization [26]. During TGFβ-mediated EMT, Dab2’s expression increases and the protein accumulates at their [FY]xNPFy[FY] motifs, thus, facilitating endocytosis and unidirectional trafficking [Fig. 2; [72]].
the membrane through binding to integrin β1 [78], a receptor whose Dab2-mediated activation is required for EMT [84]. An additional level of regulation results at the translational level, in which Dab2 expression is tightly regulated by the presence of a TGFβ-inducible post-transcriptional regulon, which modulates EMT in normal and pathological conditions [81]. Indeed, downregulation of Dab2 expression prevents TGFβ-mediated EMT and, instead, triggers apoptosis [78]. However, studies using mammary epithelial cells show that loss of Dab2 expression promotes the activation of the Ras/MAPK signaling and an increase in TGFβ, which in turn, triggers a stable EMT phenotype [85]. Thus, further studies are warranted to clarify discrepancies regarding the precise function of Dab2 in EMT.

The role of Dab2 as a tumor suppressor is highlighted by its antagonistic function in the canonical Wnt signaling pathway. This pathway, which leads to accumulation of the transcription factor β-catenin, requires the activation and membrane recruitment of Dishevelled (Dvl), a modular protein that associates with receptors and transmits signals to a variety of intracellular effectors. To exert this function, Dab2, through its PTB domain, associates with the Dishevelled, Egl-10, and Pleckstrin (DEP) domain of Dvl [86]. Overexpression of Dab2 inhibits Wnt-3A-mediated β-catenin/T-cell factor-dependent transcriptional activation without affecting the non-canonical Wnt-mediated JNK activation [86]. The DEP domain is required for the recruitment of Dvl proteins to the plasma membrane in noncanonical Wnt/planar cell polarity [87] and in canonical Wnt signaling [88]. Thus, Dab2 association with Dvl may impair Dvl binding to phosphatidic acid, an interaction that is required for its signaling function [90]. Dvl contributes to Axin recruitment to the membrane and its association with phosphorylated LRP5/6, a co-receptor that triggers canonical Wnt signaling in coordination with Frizzled receptors [90]. This association promotes Axin phosphorylation and degradation [91], which leads to stabilization of β-catenin levels. Interestingly, Dab2 interacts with Axin and blocks Axin interaction with the LRP5 co-receptor, resulting in β-catenin degradation [92]. Furthermore, the negative modulation of Dab2 in canonical Wnt signaling is also observed by its binding to the LRP6 co-receptor, an association that leads to LRP6 internalization through clathrin [93]. Binding to Dab2 requires Wnt-dependent activation of LRP6 by casein-kinase 2-mediated phosphorylation of LRP6. Because canonical Wnt signaling requires caveolin-dependent internalization of LRP6, Dab2 alters LRP6’s internalization fate by sequestering it in the clathrin-dependent endocytic route [93].

**Disabled-2, a modulator of platelet aggregation**

The generation of platelet plugs at the site of vascular injury requires a sequential process that depends not only on the presence of adhesive proteins and soluble platelet agonists but also on the predominant blood flow condition [94]. Interaction of adhesive proteins and agonists with their corresponding platelet receptors trigger the activation of multiple signaling pathways that favor changes in platelet shape and release of pro-aggregatory and anti-aggregatory molecules from α-granules, leading to inside-out signaling. Consequently, this signaling involves the activation of the integrin αIIbβ3 receptor, converting it from a low-affinity state to an activated state and favoring the binding of extracellular ligands, which trigger platelet adhesion and aggregation. Platelet receptors, such as those for Eph, Sema 4D, and Gas-6 proteins, also become activated in this phase [95]. Ligand-receptor interactions trigger signal transduction events that lead to platelet spreading, additional granule secretion, stabilization of platelet adhesion and aggregation, and clot retraction. This signaling mechanism is known as outside-in signaling [95]. The integrin αIIbβ3 receptor, a surface protein highly expressed in megakaryocytes and platelets [96], is activated by multimeric macromolecular ligands such as fibrinogen, which bind to the integrin αIIb subunit through their canonical Arg-Gly-Asp (RGD) motif [97]. Other RGD-containing agonists, such as von Willebrand factor and fibronectin, associate with the integrin receptor, stimulating cell spreading and aggregation on the vasculature [94].

**Disabled-2-mediated inside-out and outside-in signaling**

As a modulator of platelet aggregation, Dab2 exhibits a dual role in fibrinogen-integrin αIIbβ3 binding by influencing both the inside-out and outside-in signaling pathways. The dual function of Dab2 is associated with its subcellular localization in platelets. Accordingly, Dab2 likely distributes in the cytosol and in α-granules in platelets as determined using sucrose-density-gradient centrifugation, immunofluorescence staining, and by immunoelectron microscopy [10,98]. Dab2 is secreted from α-granules and localizes at the platelet surface, where it modulates platelet aggregation transiently as the protein seems to be internalized back to α-granules in a clathrin-independent, actin-dependent fashion [98]. Likewise, megakaryocytes internalize fibrinogen in an integrin αIIbβ3-dependent manner, leading to fibrinogen transportation to secretory α-granules [99–101], and that activated platelets internalize the purinergic P2Y1 receptor in inner compartments, including α-granules, in a clathrin-independent manner [102]. These observations, albeit still debatable in the field, suggest that there is a continuous traffic of molecules to α-granules throughout the platelet life cycle as older platelets show a loss of α-granule content [103]. The expression of Dab2 first occurs in megakaryocytes [104], the platelet precursors. In platelets, α-granules are proposed to originate from multivesicular bodies and late endosomes from megakaryocytes [105]. Also, some Dab2 can be found in early endosomes [16]. Thus, it is likely that, in megakaryocytes, cytosolic Dab2 is transported to early endosomal compartments, from which α-granules are generated. In the inside-out signaling, the cytosolic pool of Dab2 is phosphorylated at Ser24 by PKC [58] increasing its affinity for the integrin β3 subunit, blocking integrin αIIbβ3 activation, and inside-out signaling and, thus, modulating fibrinogen-mediated adhesion [104]. However, as Ser24 phosphorylation is not required for integrin-mediated fibrinogen uptake [106], it is possible that the PTB domain of Dab2 mediates fibrinogen internalization as PTB associates with the [Fy]XP[N/P]Y motif of the integrin β3 subunit [Fig. 3; [107]]. In addition, we speculate that membrane recruitment of Dab2 to the receptor is likely enhanced by binding to PtdIns(4,5)P2. Not surprisingly, PtdIns(4,5)P2 influences many aspects of platelet function, including platelet shape...
[108] and spreading [109]. In fact, PtdIns(4,5)P₂ levels are increased in the inner leaflet membrane of activated platelets [110] and are believed to be required for secretion of Dab2-containing α-granules [111]. Likewise, the Dab2 homologue, Dab1, has also been shown to negatively modulate platelet activation and adhesion. Binding of dimeric [l2-glycoprotein I to the apolipoprotein E receptor 2’ (ApoER2’) and glycoprotein Ibα increase platelet activation [112]. Activation and tyrosine phosphorylation of ApoER2 result in Dab1 release from the cytosolic tail of ApoER2’ [113]. Because Dab1 associates with the nonphosphorylated [FY]xNPx[YF] motif of ApoER2’ [114], its [l2-glycoprotein I-ApoER2’-dependent tyrosine phosphorylation can occur on the tyrosine residue of the [FY]xNPx[YF] motif. Indeed, this motif is required for Dab2-mediated ApoER2’ internalization as demonstrated from studies carried out in mammalian cell lines [49]. Therefore, it is likely that Dab2 exerts a similar role to Dab1 in ApoER2’ internalization in platelets.

Disability-2 and the paradox of in vitro and in vivo studies

Further insights into the function of Dab2 in vivo resulted from phenotypic analyses of megakaryocyte lineage-restricted Dab2 knockout (Dab2–/–) mice [115]. These mice are normal in size but exhibit prolonged bleeding time and defective thrombus formation despite having normal platelet morphology and granules. Under stimulation with a low dose of thrombin, mouse Dab2-deficient platelets trigger a reduction in fibrinogen storage, thrombin-induced ADP release, and integrin αIIbβ3 receptor activation. Consequently, these platelets are defective for aggregation, spreading on fibrinogen, and clot retraction. This behavior is associated with the inability of mouse Dab2-deficient platelets to respond to thrombin-activated, G_{12/13} mediated RhoA-ROCKII and Akt-mTOR activation [115]. Consistent with these observations, Dab2 is also required for fibrinogen uptake in human megakaryocytes [106]. However, not all findings reported in Dab2-deficient murine platelets agree with those carried out in vitro using human platelets. For example, human Dab2 negatively modulates platelet-fibrinogen association, by blocking integrin receptor-fibrinogen association, and platelet aggregation events by blocking P-selectin-sulfatide interactions [10,98,104,116], whereas mice Dab2 stimulates platelet aggregation under low doses of thrombin, and favors thrombin-stimulated inside-out signaling and ADP release [115]. Dab2 has also been detected in human plasma [117], implying that it is possible that megakaryocyte Dab2+/– mice are still able to secrete Dab2 from other tissues. Nonetheless, the differences observed can be readily explained by the isoform-specific expression of Dab2 in these cells. Human platelets predominantly express the p96 isoform of Dab2, whereas the p67 isoform is exclusively present in mice platelets [115]. Each of the isoforms are associated with distinct functions [17,118,119], but the most evident difference is that, in contrast to p96, the p67 isoform exhibits protein trafficking defects as demonstrated in p67 isoform-specific knock-in mice studies [16]. These observations correlate well with the lack of DPF motifs and clathrin binding sites in p67 (Fig. 3). The relevance of these sites in the role of Dab2 in platelet function is unknown. Studies using a Dab2+/– mouse model expressing the human Dab2 p96 isoform would be more appropriate for explaining the observed discrepancies.
Disabled-2 modulates platelet aggregation via lipid recognition

An additional role for Dab2 as a negative regulator of outside-in signaling has been proposed and results from its direct interaction with both integrin receptor and sulfatides, sphingolipids found at the surface of most of eukaryotic cells [120]. Under resting conditions, Dab2 is found in the cytosol as well as in α-granules of megakaryocytes [104] and platelets [10,98]. Upon activation by thrombin, collagen, 12-O-tetradecanoylphorbol-13-acetate, or the thromboxane A2 receptor agonist U46619, platelets secrete Dab2 from α-granules in a PKC-dependent manner [10]. At the platelet surface, Dab2 binds to the extracellular domain of the αib subunit of the integrin receptor blocking integrin-fibrinogen interactions [10]. Whereas association of Dab2 to the integrin receptor is mediated by the Dab2 RGD motif (residues 64–66 in human Dab2) located within the PTB domain (Fig. 1), binding of Dab2 to sulfatides is required to effectively prevent platelet adhesion and aggregation [Fig. 3; [98]]. Two polybasic regions in the N-terminal region of Dab2 upstream of PTB (named N-PTB; amino acids 1–232) were initially identified as being responsible for sulfatide recognition [98]. One motif precedes the PTB domain (residues 24–29) and the second follows closely (residues 49–54). Interestingly, the first sulfatide-binding motif (SBM) in Dab2 has also been functional for endocytosis, as this region influences the number of Dab2-clathrin assemblies and facilitates cell spreading [121]. As occurs with Dab2, polybasic motifs found in other adhesive proteins, including selectins, laminins, and thrombospondins also mediate sulfatide interactions [122]. Remarkably, sulfatide and PtdIns(4,5)P2 binding sites overlap in Dab2 [123]; however, it is unlikely that these ligands compete for protein binding as they are located in different compartments in most eukaryotic cells.

Thrombin, a platelet agonist, cleaves and inactivates Dab2 [10]. The N-terminus of Dab2 (amino acids 1–234) has been shown to be cleaved by thrombin into several products [10]. A putative thrombin cleavage site (Gly30-Lys31) is predicted to be located upstream of the most representative fragment obtained from thrombin proteolysis [98]. Interestingly, binding of Dab2 to sulfatides protects Dab2 from proteolysis by thrombin [98], facilitating the modulatory role of Dab2 in platelet aggregation. Moreover, Dab2 association to sulfatides blocks P-selectin-sulfatide interaction, thus, inhibiting homotypic and heterotypic cell interactions [116]. The inhibitory role of Dab2 on platelet adhesion and aggregation is transient as the N-PTB region is internalized back to α-granules [98]. Recently, a Dab2 peptide containing the two SBMs has been structurally and functionally characterized [124]. This peptide (amino acids 24–58 in human Dab2) adopts a helical conformation when embedded in dodecylphosphocholine micelles [125], a membrane mimetic commonly used to study membrane proteins by solution NMR spectroscopy. Whereas the N-terminal region of Dab2 SBM is disordered, the C-terminal half of the peptide contains two helices. Structural changes in Dab2 may occur upon membrane binding as the second helix of lipid-embedded SBM of Dab2 overlaps with the longest β strand of the natively folded lipid-free PTB domain of the protein. Interestingly, the helical region drives Dab2 SBM insertion into micelles with the majority of the sulfatide-interacting residues mapping within the second sulfatide-binding motif (Lys49, Lys51, and Lys53) [124]. As a promising therapeutic peptide (Fig. 4), Dab2 SBM exhibits an anti-aggregatory platelet activity comparable with that described for Arg-Gly-Asp-Ser (RGDS), a fibrinogen-derived peptide [124].

The activity of Dab2 SBM can be exploited beyond its anti-aggregatory function by targeting platelet-mediated metastasis. Platelets represent the first circulating cells that interact to and aggregate around cancer cells (reviewed in [126]). Through this process, platelets increase the extravasation efficiency of cancer cells by improving their resistance to shear stress, which masks them from the immune system and favors their interaction with endothelial cells (reviewed in [126]). Interestingly, sulfatide levels are increased in a wide variety of tumors (reviewed in [120]) and in activated platelets [127]. Also, an increase of lung and liver metastasis is mediated by platelet-derived P-selectin [128], a sulfatide-binding protein that helps platelets adhere to endothelial cells, which in turn, promote the activation of leukocyte integrins, stabilizing cancer cell adhesion to endothelial cells [126]. Leukocytes release cytokines, which contribute to cancer cell extravasation [129]. Therefore, Dab2 SBM, or second generation-derived peptides, can potentially be employed in circulation to block sulfatide-dependent cancer cell-platelet interactions (Fig. 4).

Disabled-2, an emerging player in immunity

Dab2 function in immunological processes is emerging, particularly in responses that depend on the action of dendritic cells (DC), which activate the T-cell-mediated antigen-specific
adaptive immunity (reviewed in [130]). In vitro studies showed that the granulocyte-macrophage colony-stimulating factor triggers differentiation of monocytes into DCs (reviewed in [131]), and that these differentiated DCs exhibit an increment in Dab2 expression [132]. Dab2 expression in differentiated DC cells not only inhibits T-cell stimulation but also negatively modulates DC immunogenicity [132], suggesting that Dab2 regulates the extent of immune responses. Interestingly, Dab2 is strictly expressed in Forkhead box 3-expressing regulatory T cells and that the presence of Dab2 is required for their immune function, as its deficiency leads to severe colitis in mice [133]. Dab2 is considered a neuroinflammatory mediator during autoimmune processes. Expression of Dab2 in microglia/macrophages, astrocytes, and oligodendrocytes exacerbates the experimental mice autoimmune encephalomyelitis disease in the early phase [134]. Dab2 expression in these cells is correlated with the expression of nitric oxide synthase [134], which has been associated with oligodendrocyte cell death [135]. Also, Dab2 is highly expressed in macrophages found in early acute human multiple sclerosis lesions, which relates to axonal injury [134]. Collectively, Dab2 modulates many aspects of immune responses, but the mechanism/s of action of the protein in several immune cells remains to be investigated.

Conclusions

Since its discovery in 1994, Dab2 has been involved in a wide variety of signaling processes, most of which have been described in endocytosis and platelet function. During endocytosis, Dab2 exploits its modular nature of associating with cargo and other adaptor proteins as well as facilitating clathrin recruitment, which is essential for cargo vesicular transport to endosomal compartments. Thus, Dab2 can also be considered as a scaffold protein that recruits proteins and lipids at the site of endocytosis. The major question to be addressed is whether Dab2 associations can occur simultaneously, as occurs with phosphoinositides and [FY]xNPx[YG] motifs, and whether they are cooperative. Extracellular Dab2 has been linked to the regulation of platelet function by its association with the integrin receptor and cell surface sulfatides. Intracellular Dab2 also modulates integrin receptor function. These observations raise several questions: Are these mechanisms independent from each other? What mechanism and under what physiological conditions does Dab2 predominate in the modulation of platelet aggregation? Is the extracellular pool of Dab2 secreted with a mixed population of pro-aggregatory and anti-aggregatory molecules or is Dab2 differentially released? How is the extracellular pool of Dab2 partitioned in integrin-associated and sulfatide-associated complexes? If Dab2 is recycled back to α-granules, as occurs with other proteins, can Dab2 undergo recurrent cycles of release and recovery? While it is evident that Dab2 can block P-selectin function, this observation raises the possibility that Dab2 can also modulate the function of other sulfatide-binding proteins engaged in platelet function such as that for the von Willebrand factor. More importantly, a Dab2-derived peptide has been shown to exhibit an anti-aggregatory platelet function. Interestingly, this peptide, or its derivative forms, could be employed to interfere with unwanted platelet-mediated processes such as extravasation of cancer cells. Synthetic peptides exhibit several advantages over small anti-cancer molecules as they can act transiently, be metabolically cleaved and cleared from circulation, and they do not accumulate in specific organs, all of which are key factors for a Dab2-derived peptide to participate transiently in platelet-mediated processes. Also, conflicting reports indicate that phosphoinositides can be present at the cell surface and, thus, can also be potential targets of Dab2. Likewise, Dab2 could reduce the availability of intracellular sulfatides, which have been observed in the cytoplasm of neuronal cells. As the lipid-binding properties of Dab2 are well established, it remains to be determined whether these associations play a role in Dab2 regulation of the TGF-β and Wnt signaling pathways or even in the emerging role of the protein in immune responses. Due to the observed, yet debated, outcomes of Dab2 function in different cell lines, it is possible that the function/s of Dab2 is cell type specific. Dab2 knockout is embryonic lethal, but the phenotype of a conditionally Dab2−/− mice correlates well with the role of the protein in epithelial organization as well as with defects in endocytosis as animals show a reduced number of clathrin coat pits in kidney proximal tube cells and a reduced transport mediated by the lipoprotein receptor megalin. However, conflicting reports exist between human in vitro and murine Dab2 knockout studies regarding the Dab2 function in platelet physiology. There is no doubt that the multiple Dab2 functions remain to be linked, and there is a hope that future investigations will clarify the multifaceted role of Dab2 in correlation with its tumor suppressor activity.

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