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COMMENTARY

The BRAG/IQSec family of Arf GEFs

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ABSTRACT

The IQSec/BRAG proteins are a subfamily of Arf-nucleotide exchange factors. Since their discovery almost 15 y ago, the BRAGs have been reported to be involved in diverse physiological processes from myoblast fusion, neuronal pathfinding and angiogenesis, to pathophysiological processes including X-linked intellectual disability and tumor metastasis. In this review we will address how, in each of these situations, the BRAGs are thought to regulate the surface levels of adhesive and signaling receptors. While in most cases BRAGs are thought to enhance the endocytosis of these receptors, how they achieve this remains unclear. Similarly, while all 3 BRAG proteins contain calmodulin-binding IQ motifs, little is known about how their activities might be regulated by calcium. These are some of the questions that are likely to form the basis of future research.

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Introduction

The ADP-Ribosylation factors or (Arfs) are a family of small G-proteins that control a variety of cellular events including carrier vesicle formation in the secretory and endocytic pathways, the docking and fusion of regulated secretory granules, and many intracellular signaling events.[1] The 6 mammalian Arfs are grouped into 3 classes based on sequence similarity, with class I comprised of Arf1, Arf2 and Arf3, Class II containing Arf4 and 5 and Class III containing only Arf6. Like most other GTPases, the Arfs cycle between an inactive GDP-bound and active GTP-bound state. Interconversion between the 2 states requires 2 classes of accessory proteins; guanine nucleotide exchange factors (GEFs), which catalyze removal of GDP and its exchange for GTP, and GTPase activating proteins (GAPs) that stimulate GTP hydrolysis. The human genome encodes 15 different GEFs that are specific for Arfs, referred to as the Sec7 family based on homology with the yeast Arf GEF Sec7p.[2] Although some of this diversity stems from tissue-specific expression, the large number of GEFs relative to the number of Arfs suggests that the Arfs are under extensive regulatory control. This review will focus on one sub-family of Arf GEFs referred to as the Brefeldin Resistant Arf GEFs or BRAGs.

Characteristics of the BRAG subfamily

The name BRAG is somewhat misleading, as other Arf GEFs, including the cytohesin, EFA6 and Fbx8 subfamilies are also resistant to BFA. The NCBI has therefore introduced a new nomenclature in which the BRAGs are referred to as the IQSec family, based on the presence of a characteristic calmodulin-binding IQ motif and the conserved catalytic domain referred to as the Sec7 domain. Mammalian genomes contain 3 BRAG/IQSec genes, BRAG1/IQSec2, BRAG2/IQSec1 (also known as GEP100) and BRAG3/IQSec3 (also known as synArfGEF) (Fig. 1). Each BRAG/IQSec gene encodes multiple splice variants that are generally expressed in a tissue-specific manner. Among the 3 family members, only BRAG2/IQSec1 is ubiquitously expressed.

As noted above, members of this sub-family are uniquely characterized by the presence of an IQ motif located in the N-terminal third of the protein in addition to the Sec7 and PH domain present in other members of Arf GEF family. Intriguingly, this non-canonical IQ motif preferentially binds calcium-free calmodulin, and releases it upon calcium binding (see below).[3] Although this has only been demonstrated directly for BRAG1/IQSec2, the flanking sequence is completely conserved across all 3 family members and is likely to function similarly in each case. Early studies using *in vitro* assays reported no role for this domain in the regulation of BRAG2 GEF activity.[4] However, recent work from our laboratory revealed that catalytic activity of BRAG1 is indeed regulated by calmodulin binding in hippocampal neurons.[3]

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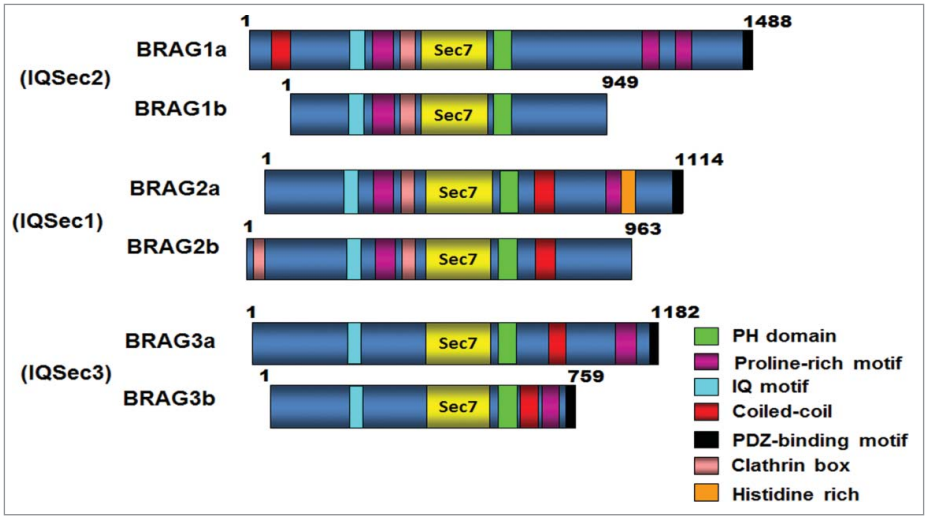

Figure 1. Domain organization of BRAG/IQsec proteins: BRAG1a (NP_001104595.1), BRAG1b (NP _055890.1), BRAG2a (NP_001127854.1), BRAG2b (NP_055684.3), BRAG3a (NP_001164209.1) and BRAG3b (NP_ 056047.1).

The central region of all 3 BRAG isoforms contains a highly conserved Sec7 catalytic domain in tandem with a PH domain. In the case of BRAG2 there have been conflicting reports on the ability of its PH domain to bind specific membrane lipids or proteins, and the role of these interactions in regulating GEF activity. An early study by Vaughan and colleagues suggested that the GEF activity of BRAG2 was insensitive to phospholipids;[4] however, more recent studies dispute this initial claim. Jian et al reported that the catalytic activity of both full length BRAG2 and a BRAG2 fragment containing only the Sec7-PH domain tandem was 15-20-fold higher in the presence of $PI(4,5)P_2$.[5] In contrast, Zeghouf and colleagues reported that the PH domain of BRAG2 potentiates its catalytic activity by 3 orders of magnitude in response to lipid binding, but exhibited little specificity in its requirement for negatively charged lipids; all phosphoinositide species, and even phosphatidylserine stimulated activity robustly and to a similar extent.[6]

Finally, the BRAG2 PH domain has also been reported to directly interact with several transmembrane receptors. Sabe and colleagues reported that BRAG2 is recruited to the phosphorylated EGF receptor by direct association of its PH domain with phosphorylated tyrosine residues 1068/1086 in the EGFR cytoplasmic tail.[7] In contrast, Kornau and colleagues demonstrated a direct interaction between BRAG2 and a non-phosphorylated tyrosine (Y876) in the cytoplasmic domain of the GluA2 subunit of neuronal AMPA receptors that required the PH domain.[8] In this case, phosphorylation of Y876 actually *inhibited* BRAG2 binding. Moreover, BRAG2 catalytic activity was stimulated by the cytoplasmic domain of GluA2 or a peptide derived from it.[8] Together these findings suggest that BRAG2 activity can be regulated by interactions with both lipids and proteins, perhaps simultaneously.

Biological functions of BRAGs

Myoblast fusion

The first evidence of a biological function for BRAGs came from work in *Drosophila*. The single *Drosophila* BRAG gene was identified in a screen for genes involved in muscle development, and was given the name LONER (also called *shizo*—see section on neuronal functions of BRAGs) based on the inability of mutant myoblast cells to fuse with surrounding cells.[9] In that study, the *loner* protein was found to localize to cell/cell fusion sites through interactions with the heterotypic adhesion proteins *dumbfounded (Duf)* and *roughest (rst)*.[9] A subsequent study identified a similar interaction between *loner* and the cytoplasmic domain of N-cadherin, which also localized to cell-cell contact sites. Interestingly, myoblast fusion defects in *loner* mutant flies could be partially rescued by a loss of function mutation in N-cadherin, suggesting that surface expression of N-cadherin inhibits fusion, and that *Loner* may promote fusion by driving N-cadherin endocytosis.[10] In this regard, BRAG2 has been reported to drive endocytosis of another cadherin, E-cadherin, in vertebrate cells in response to both EGF[7] and HGF.[11]

It should be noted that while *loner* was originally characterized as an Arf6 GEF, complete loss of Arf6 in flies does not inhibit myoblast fusion;[12] it is therefore possible that one or more other Arfs fulfill that role. However, vertebrate myoblasts (C2C12 cells) do appear to require Arf6 for fusion, at least in part through its

downstream activation of the lipid modifying enzymes phospholipase D and the PI4P5 kinase PIP5Kγ.[13,14]

Integrin trafficking

Integrins are also important in vertebrate myoblast fusion. Knockdown of α3 or β1 integrin, or inhibition of α9β1 integrin function inhibits myoblast fusion both *in vitro* and *in vivo*.[15,16,17] In parallel with studies in *Drosophila*, knockdown of BRAG2 in cultured mammalian myoblasts also impairs fusion, apparently by disrupting the organization of focal adhesions.[13]

Studies from our laboratory have shown that BRAG2 modulates the level of cell surface β1- integrin, at least in part by regulating its rate of endocytosis. Depletion of BRAG2 in Hela cells results in the accumulation of β1-integrin on the cell surface and a corresponding enhancement of attachment and spreading on fibronectin coated substrates.[18] This effect is selective for β1-integrin, as surface transferrin receptor levels were unaffected. Interestingly, Arf6-depleted cells do not accumulate β1-integrin on the cell surface and actually spread more slowly on fibronectin, in agreement with numerous reports that Arf6 regulates integrin recycling.[19] While BRAG2 does indeed activate Arf6 in cell-based assays, we found that it also activates the class II Arf, Arf5, and that knockdown of endogenous BRAG2 reduces the activity of both Arf5 and Arf6.[20] Surprisingly, depletion of Arf5 but not Arf6 phenocopies BRAG2 knockdown; it slows β1 integrin endocytosis and enhances cell spreading. Conversely, the effects of BRAG2 depletion on both surface β1-integrin levels and cell spreading are reversed by expression of a rapid cycling (constitutively active) Arf5T161A mutant, but not by rapid cycling Arf6T157A.[20] Similar findings were reported recently in endothelial cells, where BRAG2 depletion resulted in increased surface expression of α5β1 integrin and a corresponding decrease in surface αvβ3, both of which were rescued by expression of rapid cycling Arf5.[21] Together these observations suggest an unexpected role for Arf5 in the modulation of β1 integrin trafficking, however the nature of that role remains unclear. While endocytosis of β1 integrin is completely blocked by clathrin depletion, knockdown of Arf5 merely slows internalization, suggesting that it modulates, but is not essential for integrin endocytosis.[20] We hypothesize that BRAG2, via Arf5, enhances the disengagement of β1 integrins from focal adhesions, thereby freeing them for clathrin-mediated endocytosis.

Of the many factors that regulate focal adhesion disassembly, microtubules are thought to play a vital role in the delivery of 'disassembly factors' that mediate turnover.[22] A recent study in keratinocytes identified the serine/threonine protein kinase MAP4K4 as one such factor.[23] MAP4K4 is delivered to focal adhesions by binding to the microtubule tip-binding protein EB2. Importantly, MAP4K4 also interacts with BRAG2, which becomes phosphorylated in a MAP4K4-dependent manner. Keratinocytes lacking MAP4K4 exhibited reduced Arf6 activation, increased surface β1 integrin expression, enlarged focal adhesions and a reduced rate of β1 integrin internalization, essentially phenocopying BRAG2 depletion.[23] Although these data suggest that phosphorylation of BRAG2 by MAP4K4 may stimulate its catalytic activity, this remains to be tested directly. Interestingly, these authors found that rapid cycling Arf6T157A is capable of normalizing focal adhesion dynamics in keratinocytes lacking either MAP4K4 or BRAG2, suggesting that Arf6 is an essential effector of this pathway. Why Arf6 is sufficient in keratinocytes but not Hela cells [20] or other transformed cells (our unpublished results) remains a mystery and will require further study.

BRAGs and angiogenesis

Angiogenesis is another physiological process that requires tight regulatory control of cell adhesion. Formation of new vessels from existing ones involves the directed migration of vascular tip cells, which direct vessel patterning in response to local environmental cues.[24] Among these cues are the semaphorins, a family of secreted proteins that also function in axon guidance to control neurogenesis (see also section below on neuronal functions of BRAGs). The semaphorins actually inhibit angiogenesis by binding to a family of receptors (plexins) expressed by endothelial tip cells, and act as repulsive cues during vessel development. Gutkind and colleagues have shown that Semaphorin 3E signals through a cognate plexin, Plexin D1, to induce the rapid disassembly of integrin-mediated adhesions in an Arf6- and BRAG2-dependent manner.[25,26] In this context, BRAG2 does not interact directly with Plexin D1, but appears to be activated by PtdIns(4,5)P_2 generated in response to plexin ligation. Chavakis and colleagues have reported a similar effect of BRAG2 depletion on vascular sprouting, which could be reversed by expressing either rapid cycling Arf5T161A or Arf6 T157A mutants.[21] Importantly, these authors also showed that BRAG2 depletion *in vivo* impaired vascular development in a zebrafish model, as well as in a mouse model of post-ischemic neoangiogenesis.[21]

Interestingly, MAP4K4 has also been shown to affect angiogenic sprouting.[27] In 3D endothelial cell cultures, control cells formed well defined sprouts whereas MAP4K4 deficiency resulted in short aberrant sprouts with long cellular protrusions, attributed to the inability

of endothelial cells to retract membrane during cell migration. In mice, the head skin of MAP4K4 knockout embryos exhibited large avascular areas relative to controls, suggesting delayed vascular development. At the molecular level, MAP4K4 phosphorylates moesin, which then displaces talin from β1-integrin-containing focal adhesions, eventually leading to focal adhesion disassembly.[27] Together with the work by Yue et al.[23] this suggests a model in which MAP4K4 is delivered to focal adhesions by microtubules where it cooperates with BRAG2 and Arf5 (or Arf6) to release β1-integrins from focal adhesions. Future work will be required to dissect out the precise roles of each component in adhesion turnover.

Neuronal function of BRAGs

The single *Drosophila* BRAG was also identified in a screen for factors that modulate neuronal pathfinding, where it was given the name *schizo*. During embryonic development neurons send out axons that are guided by local cues to their appropriate destination. One such guidance cue is *Slit*, a secreted extra-cellular matrix protein produced by midline glial cells in *Drosophila*. Through interaction with the axonal receptor Roundabout (*Robo)*, *Slit* prevents migrating axons from crossing the embryonic midline (analogous to the repulsive Semaphonin/Plexin system in vascular development). *Slit/Robo* signaling is reduced at points where axons need to cross the midline (commissure formation) and overexpression of *Slit* in midline glia prevents formation of normal commissures. In *shizo* mutant flies axons fail to cross the midline, a phenotype attributed to their inability to counteract repulsive signaling mediated by *Slit*. A similar phenotype was observed upon inhibition of endocytosis in midline glia, by expression of mutant *shibire* (the fly ortholog of dynamin). This latter observation suggests that *shizo* functions to reduce the surface level of *Slit* on midline glia, by promoting its endocytosis.[28] It should be noted however that *slit* is a secreted protein and lacks a transmembrane domain, but remains closely associated with membranes,[29] and it is not yet clear how it is endocytosed.

BRAGs in the brain

All three BRAG proteins are abundantly expressed in the mammalian brain, where they localize predominantly to post synaptic densities (PSDs). Proteomic analysis of purified PSDs indicated that BRAGs are among the most abundant proteins in these structures.[30] Interestingly, both BRAG1 and BRAG2 are abundant in the PSDs of excitatory synapses, while BRAG3 localizes exclusively to inhibitory GABAergic synapses.[31,32] Consistent with their localization, both BRAG1 and BRAG2 bind the excitatory synapse scaffolding protein PSD95 via their C-terminal PDZ motifs[33,34] while BRAG3 interacts with the inhibitory synapse scaffold gephyrin.[35]

Mutations in BRAG1 have been discovered in families with non-syndromic X-linked intellectual disability (XLID). These include multiple instances of truncations either N-terminal to or within the Sec7 domain, and a mis-sense mutation leading to altered splicing of exon 7 (S861T), at the C-terminus of the Sec7 domain.[36,37,38] Importantly, 2 studies have identified point mutations in families with XLID, 4 of which mapped to the Sec7 domain (R758Q, A789V, Q801P and R863W) and reduce catalytic activity.[39,40] One mutation mapped to the IQ domain (R359C), implying that calmodulin binding is critical for BRAG1 function in the context of the PSD.[39]

Subsequent work from our laboratory showed that overexpression of BRAG1 in rat hippocampal slices led to increased Arf6 activation and reduced AMPA receptor signaling, both of which were dependent on catalytic activity.[3] AMPA receptors cycle continuously between the plasma membrane and endosomal compartments in a manner that is regulated by upstream signals. One such signal is calcium influx mediated by NMDA receptors. Interestingly, we found that blockade of NMDA receptors abrogated the suppressive effect of BRAG1 on AMPA-R signaling, indicating that BRAG2 activity requires calcium influx. Moreover, expression of an IQ domain mutant that cannot bind calmodulin led to constitutive, NMDA-R-independent depression of AMPA-R signaling. The IQ domains in the 3 mammalian BRAGs fit the consensus for non-canonical, calcium-independent calmodulin binding.[41] We verified that BRAG1 does indeed bind calcium-free calmodulin and releases it upon calcium binding, suggesting that it is calmodulin dissociation that triggers BRAG1 activity in neurons.[3] Curiously, the IQ motif does not appear to regulate BRAG1 activity in non-neuronal (Hela) cells[3] raising the possibility that neuron-specific binding partners may be important for this layer of regulation.

BRAG2 has also been reported to regulate AMPA-R trafficking in hippocampal neurons via Arf6 activation.[8] These authors found that BRAG2 binds directly to the GluA2, GluA3 and GluA4(short) subunits of AMPA-R in a manner that enhances BRAG2 catalytic activity. Interestingly, binding to GluA2 was demonstrated for constructs containing both the Sec7 and PH domains of BRAG2, but not either domain alone, raising the possibility that the linker connecting the 2 domains could mediate this interaction. Conversely, BRAG2 binding required Y876 in the cytoplasmic domain of GluA2, and

is regulated by the phosphorylation status of this residue. However, in contrast to the binding of BRAG2 to the EGFR, which required phosphorylation of adjacent tyrosines,[7] binding to GluA2 required *de-phosphorylation* of Y876. Importantly, this site is actively de-phosphorylated in response to activation of metabotropic glutamate receptors (mGluRs), which induces AMPA-R internalization.[8]

A more recent study from the same laboratory found that BRAG1 and BRAG2 also interact with NMDA receptor subunits, in a calcium-dependent and isoform-specific manner. In this context, BRAG1 binds to the C-terminal cytoplasmic tail of GluN2B, while BRAG2 binds to a similar region of GluN2A.[42] Importantly, expression of these subunits is developmentally regulated, such that GluN2B is more abundant during embryogenesis and GluN2A predominates postnatally. In agreement with this, BRAG1-mediated Arf6 activation predominates in the developing brain, while BRAG2 activity increases with age after birth. The authors concluded that BRAG1 activity is important for the initial establishment of synapses, while BRAG2 is important for the fine tuning of synaptic strength that accompanies learning and memory after birth.

BRAG proteins in cancer metastasis

Metastasis is the dissemination of cancer cells from solid tumors to anatomically distant organ sites and their subsequent adaptation to the foreign tissue microenvironment.[43] While a role for Arf6 in tumor metastasis had been appreciated for some time, the earliest report of BRAG2 involvement in metastasis came from studies by Sabe and colleagues, who showed that knockdown of BRAG2 potently inhibited the migration of breast cancer (MDA-MB-231) cells through matrigel.[7] Moreover, upon EGF stimulation of these cells BRAG2 bound directly to the EGF receptor through association of its PH domain with phosphorylated tyrosine residues (Y1068/Y1086) in the receptor. Mutation of these residues impaired Arf6 activation in response to EGF, suggesting that this interaction stimulates BRAG2 catalytic activity. Interestingly, while overexpression of BRAG2 alone was insufficient to induce matrigel invasion by non-invasive MCF7 cells, co-expression with Arf6 rendered them invasive in an EGF dependent manner.[7] Conversely, depletion of BRAG2 in the mouse mammary tumor cell line 4t/Luc severely inhibited their ability to form metastatic nodules in the lung after injection into mammary fat pads. Immunohistological analysis of human breast tumor specimens revealed that a high percentage of both non-invasive (DCIS) and invasive tumors were positive for both BRAG2 and EGFR, suggesting that co-expression may be predictive of malignant phenotype.

BRAG2 has also been implicated in the metastasis of melanomas, although the signaling pathway in which it functions appears to be different from breast cancer cells. In the melanoma cell line LOX, BRAG2 acts downstream of Wnt5A and its co-receptors Frizzled4 and LRP6 to activate Arf6. In this context, active Arf6 stimulates the release of β-catenin from N-cadherin, allowing it to enter the nucleus where it mediates the transcription of genes required for invasion.[44] However, it remains unclear how Wnt5A-LRP6 signaling recruits or activates BRAG2 to mediate nucleotide exchange on Arf6.

One form of melanoma, uveal melanoma, is often driven by oncogenic mutations in the heterotrimeric G protein α subunits Gαq/11.[45] While it has been known for many years that Arf6 can be activated downstream of Gαq/11, this has largely been attributed to members of the cytohesin family, which interact directly with Gαq.[46,47] A recent study indicates that, like the cytohesins, BRAG2 can also interact with Gαq, although it is not yet clear that this interaction is direct.[48] Surprisingly, knockdown of BRAG2, but not cytohesin-2/ARNO substantially attenuates Arf6 activation in 2 uveal melanoma cell lines expressing oncogenic Gαq, and inhibits their anchorage-independent growth *in vitro*. Knockdown of Arf6 in these cells attenuates a large number of signaling pathways downstream of Gαq, including the activation of PLC, ERK, p38 and JNK, Rac, RhoA and Hippo/YAP.[48] Interestingly, Arf6 depletion appears to cause retention of Gαq at the plasma membrane and its depletion from endosomes, suggesting that Gαq signaling from endosomes is important for oncogenic transformation.

BRAG2 has also been reported to impact invasiveness and metastasis in pancreatic cancer cell lines.[49] In this setting, knockdown of BRAG2 led to increased expression of E-cadherin and its accumulation at intercellular junctions. Stable cell lines depleted of BRAG2 exhibited significantly attenuated invasion through matrigel, and reduced lung metastasis in a nude mouse model. Together with the studies described above, these findings indicate that BRAG2 is an important intermediate in oncogenic signaling pathways that control cell growth, cell-cell adhesion and migration.

Concluding remarks

Although each of the BRAG isoforms have specific functions related to the cells/tissues in which they are expressed, a common theme is that they exert these functions by regulating the levels of a subset of receptors (cadherins, integrins, neurotransmitter receptors)

at the cell surface. In most cases, depletion of BRAGs results in increased surface expression. While this suggests a role for BRAGs in endocytosis, how they function in this capacity is unclear. As noted above, BRAG2 depletion has no effect on endocytosis of other proteins such as transferrin receptor[20] indicating that it does not regulate the core endocytic machinery. A more likely interpretation is that BRAGs act, through Arfs (in most cases Arf6 and Arf5 for focal adhesion turnover), to liberate receptors that are anchored to the actin cytoskeleton (cadherins) or to other proteins that stabilize their surface expression (e.g. focal adhesion components or scaffolding proteins in the post-synaptic density), thereby allowing their internalization. Although, BRAG2 does interact with clathrin,[20] it is not yet clear whether this is important to its ability to modulate the rate of receptor endocytosis. It also remains unclear how Arfs regulate endocytosis in the situations described above. While it can be speculated that Arf6 regulates internalization by modulating $PI(4,5)P_2$ levels required for endocytosis, the contributions of Arf5 to this process remain unknown. While Arf5 is largely associated with the ER-Golgi intermediate compartment (ERGIC) elements of the ER do extend into focal adhesions, and presumably into cadherin-mediated adhesions as well. Future studies are clearly warranted to dissect the role of Arf5 and its effectors in endocytosis.

Another major unanswered question is the extent to which BRAG function is regulated by calcium/calmodulin. Consistent with the presence of a non-canonical IQ motif, we found that both BRAG1[3] and BRAG2 (unpublished data) bind calcium-free calmodulin and release it upon calcium binding. Calmodulin is clearly not required for BRAG2 catalytic activity in *in vitro* assays,[4,5,6] nor does it modulate activity *in vitro* when added in the presence or absence of calcium.[4] Similarly, the *Loner* IQ motif was shown to be unnecessary for myoblast fusion.[9] However, the genetic evidence that mutation of the BRAG1 IQ motif leads to X-linked intellectual disability,[39] and our data showing that the IQ motif modulates calcium-dependent activity of BRAG1 in neurons[3] suggest that calmodulin binding is indeed regulatory in some contexts. Considering that the IQ motif is a defining feature of the BRAG family, it is surprising that so few studies have examined its role in BRAG function. This should therefore be a priority for future studies.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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