Kalirin and Trio: RhoGEFs in synaptic transmission, plasticity, and complex brain disorders

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Summary

Changes in the actin cytoskeleton are a primary mechanism mediating the morphological and functional plasticity that underlies learning and memory. The synaptic RhoGEFs Kalirin and Trio have emerged as central regulators of actin dynamics at the synapse. The increased attention surrounding Kalirin and Trio stems from the growing evidence for their roles in the etiology of a wide range of neurodevelopmental and neurodegenerative disorders. In this review, we discuss recent findings revealing the unique and diverse functions of these paralog proteins in neurodevelopment, excitatory synaptic transmission, and plasticity. We additionally survey the growing literature implicating these proteins in various neurological disorders.

The Dbl RhoGEFs Kalirin and Trio

GTPases are enzymes that bind GTP, hydrolyzing it to GDP. Different conformational states act to limit GTPase activity [1]. These "molecular switches" function as signal transducers, regulating numerous cellular processes [2]. The Ras superfamily of small GTPases comprises more than 150 members functioning as monomeric G proteins [2]. The Ras homologous (Rho) family of small GTPases are a major branch of the Ras superfamily. Rho GTPases are regulators of actin dynamics, with Ras-related C3 botulinum toxin substrate 1 (Rac1), Ras homolog family member A (RhoA), and cell division control protein 42 homolog (Cdc42) being the most well-known [3, 4]. Structural changes in the actin

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cytoskeleton associated with synaptic plasticity involve Rho family GTPases through their regulation of actin polymerization, resulting in changes in spine morphology [5, 6].

GTPases are, in part, regulated by guanine nucleotide exchange factors (GEFs) that facilitate the exchange of GDP for GTP, opposing the action of GTPase-activating proteins (GAPs) [7]. RhoGEFs were discovered in the context of diffuse B-cell lymphoma (Dbl) [8]. A distinct feature of the Dbl family of RhoGEFs is the presence of a Dbl homology (DH) domain, which is recurrently followed by a regulatory C-terminal pleckstrin homology (PH) domain, together comprising the enzymatic GEF domain [9]. GEF domains are conserved across Dbl family members; however, the non-GEF sequences flanking the DH-PH domains are more divergent and act as additional regulators of intrinsic GEF function, both structurally and through facilitating protein-protein and protein-lipid interactions. This sequence diversity is a critical means by which various Dbl RhoGEF proteins display unique functions, through different subcellular targeting and sequestration, resulting in differing spatial-temporal activation of GTPases.

The **paralog** Dbl RhoGEFs Kalirin and Trio, the only RhoGEFs with tandem GEF domains, have been broadly investigated and have emerged as significant regulators of synaptic development, plasticity, and neurotransmission. Recent work has reinforced and expanded on these roles and has further positioned Kalirin and Trio as critical molecules in neurodevelopmental and neurodegenerative disorders. This review covers the recent advancements in understanding the relationship between Kalirin and Trio and highlights the current developments in their functions in synaptic physiology and, importantly, in disease.

Evolutionary Conservation and Proteomic Diversity

Kalirin and Trio are distinctive in that they are paralogous proteins evolving from a mutual ancestral gene via duplication. Orthologs are observed in *Drosophila* (dTrio) and in C. elegans (unc-73) (Figure 1). Kalirin and Trio are similarly divergent to both dTrio and to unc-73 [10]. Recent domain and **phylogenetic analyses** have shown that the duplication event occurred in **Urbilateria**, with the last common ancestor existing in **Prebilateria** [11]. The predicted core domain structure of the Kalirin/Trio-related protein in Prebilateria is thought to contain 2 spectrin repeats and dual GEF domains. The working model posits the single *Trio/Kalrn* ancestor acquired a kinase domain, resulting in the ancestral *Kalrn*, which was subsequently duplicated in Urbilateria to yield distinct *Kalrn* and *Trio* genes. Interestingly, the phylogenetic analysis found retention and loss events were unpredictable in invertebrates, with only a few invertebrate taxa having retained both proteins [11]. In vertebrates, Kalirin and Trio were found to contain multiple protein variants when the duplicate was present, suggesting an adaptability for novel protein function [11, 12]. This is consistent with understandings of gene duplication retention, in which gene copies are retained when they serve complementary functions (so-called **subfunctionalization**) [13]. Indeed, many Kalirin and Trio isoforms have been identified and the proteomic diversity of Kalirin and Trio mediates their specific functions, both in developmental and tissue-specific manners (Figure 1, Box 1). For instance, C-terminal variations in Kalirin affect its trafficking and regulate its function at excitatory synapses via the inclusion of a PDZ-ligand, which is not present in any Trio isoform [14, 15]. The emergence of a variety of Kalirin and

Trio isoforms may indicate a degree of functional separation, which may account for the evolutionary retention of complementary Kalirin and Trio isoforms that mediated functions once served by the ancestral Kalirin/Trio.

Kalirin and Trio are both present throughout development and into adulthood, with differing isoform-specific expressions and subcellular localizations. A distinct expression difference is that *Trio*, but not *Kalrn*, is expressed in migrating neural crest cells in *Xenopus laevis*, suggesting Trio isoforms may be more important in organization of neural tissue relative to Kalirin [11, 16]. This is consistent with their respective knockout phenotypes in mice [17]. In the CNS of vertebrates, larger Kalirin isoforms are expressed abundantly early in development and localize to growth cones, whereas the brain-specific isoform Kalirin-7 localizes to the **postsynaptic density (PSD)**, with expression paralleling that of synaptogenesis [18]. Kalirin-7 is expressed abundantly in the adult cortex and hippocampus, and persists as the dominant brain-specific isoform in the adult. However, there is an age-dependent increase in larger Kalirin isoform expression in orbitofrontal cortex [19]. Trio, likewise, displays dynamic expression patterns in embryonic and adult tissues, though the available isoform-specific data are less specific. Trio expression is apparent by embryonic day 10 in the neocortex and cerebellum, with the exception of Trio-8, which is limited to the cerebellar Purkinje neurons at P30 onward [20, 21]. While Trio is known to localize to growth cones, the presynaptic terminal, and the PSD, the exact temporal and isoform-specific expression patterns are not clearly delineated.

Neurite Outgrowth and Axon Guidance

Actin cytoskeleton regulation by Rho GTPases is central for cell migration and polarization, with Trio and Kalirin participating as key players in these processes. In particular, studies have established the importance of Trio in neurite growth and axon guidance. Trio has been shown to function in neurite formation downstream of the neurotrophin nerve growth factor (NGF) via RhoG activation in PC12 cells [22]. These data are challenging to interpret, as extensions in PC12 cells are not neurites precisely. However, the NGF downstream target Kidins220 has been shown to interact with Trio and activate it, resulting in Rac1 activation and neurite extension both in PC12 cells and in hippocampal neurons, supporting the role of NGF in Trio-mediated neurite extension [23]. dTrio is also known to interact with the Netrin receptor Frazzled as part of a signaling network regulating CNS midline axon guidance in *Drosophila* [24]. Indeed, Netrin-1 mediated Rac1 activation in the mouse is dependent on Trio for normal axon guidance, as revealed by the reduction in Netrin-1-induced Rac1 activation and axon guidance in the absence of Trio [25, 26]. This mechanism also involves the interaction with heat shock cognate protein 70 (Hsc70) and the phosphorylation of Trio at Y2622 by the Src kinase Fyn [27, 28].

The bulk of developmental studies on Trio have focused on Rac1/RhoG regulation; indeed, Trio's GEF1 domain has been shown to be sufficient for neurite outgrowth [29]. Several studies have shown Trio's GEF2 domain to be natively inhibited, and its activation results in the restriction of neurite extension and dendritic branching [29–32]. In this regard, Trio's dual GEF domains provide dynamic regulation of neurite outgrowth and dendritic branching, with GEF1 mediating neurite extension and GEF2 destabilizing this process via

RhoA activation. Recent studies of Trio's activation of RhoA have suggested it is additionally involved in axon guidance. Slit homolog 2 protein (Slit2), a secreted glycoprotein involved in axon guidance and neuronal migration, induces RhoA activation through Trio, which is important for telencephalic wiring [33]. Slit2 is a ligand for the Roundabout (Robo/SAX-3) receptors, which are regulated by unc-73, possibly linking Slit2's regulation of Trio RhoA GTPases via this receptor [34]. It is evident that both Trio GEF domains are necessary for regulated neurite outgrowth and guidance; however, the role of additional Trio domains in this process has remained largely unknown. Interestingly, it has been demonstrated that Golgi-resident Trio regulates membrane trafficking in developing cerebellar granule cells via interactions of its N-terminal spectrin repeats with RABIN8, resulting in RAB8 and RAB10 activation [35]. Expression of active RAB8 and RAB10 is able to restore neurite outgrowth deficits induced by loss of Trio. RAB8 and RAB10 activation is critical for vesicle trafficking to the growth cone, suggesting that Trio activation of these proteins through its N-terminal domains is an additional factor important for neurite outgrowth. These new data, along with previous work, together establish both Trio GEF domains as regulators of axon development, while highlighting emerging roles of additional Trio domains in this process [36].

Kalirin is also involved in neurite and dendritic outgrowth and in dendritic arborization. Cortical neurons in Kalirin KO mice display simplified dendritic arborization and smaller neurite length [37]. Like Trio, distinct roles in this process are mediated by different Kalirin isoforms and functional domains [36]. Knocking out Kalirin in sympathetic neuronal cultures stunts preexisting axonal extension, whereas overexpression of Kalirin results in the prolific sprouting of new axonal fibers [38]. Interestingly, expression of the GEF1 domain alone increases growth cone size, whereas overexpression of the GEF2 domain results in over elongation of axons and increased neurite formation [15]. It has also been shown that Kalirin's kinase domain, which is unique to Kalirin-12, may mediate neurite formation and length [39]. Kalirin's Rac1 GEF1 domain was also found to be important for dendrite elaboration [39]. These data demonstrate the salience and isoform specific contributions of Kalirin in dendritic maturation and development. Nevertheless, upstream signals mediating Kalirin GEF activation in neurite outgrowth and guidance are not as well established as Trio's; however, work has shown that brain-derived neurotrophic factor (BDNF) activation of Rac1 in neurite outgrowth is mediated by Kalirin (Figure 2, Key Figure) [40].

Kalirin and Trio in Synaptic Physiology

Kalirin is a central signaling hub in excitatory synaptic transmission and plasticity; however, the role of Trio in these processes is less understood [18]. Trio interacts with the presynaptic LAR/Liprin complex and clusters with the scaffolding proteins Piccolo and Bassoon, likely through regulating synaptic membrane exocytosis protein 1 (RIM1) (Figure 2) [41, 42]. Trio is targeted to presynaptic terminals through association with Piccolo and Bassoon via spectrin repeats 3 and 4 [43]. Trio may influence the regulated assembly of actin in presynaptic terminals, perhaps through interactions with Piccolo and Bassoon (Figure 2). Indeed, Trio knockout and **haploinsufficiency** have been shown to increase paired-pulse ratios, indicating deficits in presynaptic release probability [44]. These findings are consistent with prior data showing Rac1 mediated synaptic release at the neuromuscular

junction is blocked with mutants in dTrio [45]. These data support a crucial role for Trio in presynaptic neurotransmitter release that is distinct from Kalirin. This idea is supported by data showing Trio to be a regulator of exocytosis in other cell types, such as pituitary endocrine cells [46]. Moreover, recent proteomic mapping of the Trio and Kalirin-7 interactomes has shown Trio, but not Kalirin-7, interacts with many presynaptic proteins known to regulate vesicle release and active zone dynamics [47].

Both Kalirin and Trio are found at the PSD and have roles in postsynaptic plasticity (Figure 2) [47–49]. Knocking down Kalirin and Trio expression in neurons has a dramatic effect on postsynaptic structure and function [50, 51]. For example, knocking down Kalirin or Trio individually in postsynaptic CA1 pyramidal neurons produce similar reductions in AMPAR-**eEPSC** amplitude following Schaffer collateral stimulation [50]. However, knocking down both Kalirin and Trio simultaneously in CA1 pyramidal neurons resulted in a near complete loss of glutamatergic synapses. Recombinant expression of either Kalirin or Trio alone was able to restore glutamatergic synapse function in these neurons [50]. These findings suggest a post-development redundancy in postsynaptic function between these two RhoGEFs in synaptic transmission. The influence Kalirin and Trio have on postsynaptic glutamatergic synapse function is also bidirectional, given that biolistic overexpression of Kalirin-7 or Trio-9 in CA1 pyramidal neurons in organotypic hippocampal slice cultures produces nearly identical increases in AMPAR-eEPSC, but not NMDAR-eEPSC amplitude [50].

Kalirin-7 has been shown to interact with many proteins of the PSD, including the NMDAR subunit GluN2B and PSD-95 (Figure 2) [16, 49]. Prior studies in the Kalirin and Kalirin-7 specific knockout mice demonstrated impaired, but not eliminated, hippocampal LTP induction [16, 50, 52]. Interestingly, molecular replacement of Kalirin-7 with an N-terminal point mutation (T95A) of a purported CaMKII phosphorylation site is sufficient to block LTP induction- and overexpression-mediated increases in AMPAR-eEPSCs [50, 53]. This LTP block is also observed when making the homologous mutation in Trio (T66A), suggesting that the N-terminal domain of these proteins, like other GEFs, is sensitive and critical for protein function.

Phosphorylation of Kalirin and Trio

Kalirin and Trio, as large and complex multidomain proteins, are subject to extensive posttranslational modifications that regulate their functions. Despite having C-terminal kinase domains, Trio and Kalirin have not been shown (to our knowledge) to autophosphorylate [54]. Several studies have investigated the role of phosphorylation, with CaMKII being reported to phosphorylate Kalirin-7 at several sites. One of these residues, T95, is important for Kalirin-7 function by regulating GEF activity, linking NMDAR activation to that of small GTPases through CaMKII [53]. However, recent phosphoproteomic analysis of Kalirin-7 has proposed T79 and S83 as alternative N-terminal CaMKII phosphorylation sites [55, 56]. Nevertheless, mutations of T95, and the analogous residue on Trio, have been shown to modulate plasticity and GEF activity, demonstrating this residue to be critical to the synaptic function of Kalirin [50, 55]. How this N-terminal residue modulates distal GEF1 actively is unclear, though it likely involves dynamic changes

in protein folding that increase GEF1 activity through an allosteric process or perhaps facilitates greater Rac1 access to this catalytic domain.

Kalirin phosphorylation is additionally isoform- and brain region-specific [56]. For instance, tyrosine phosphorylation of Kalirin-7 modulates sensitivity to calpain, influencing its subsequent cleavage [56]. Kalirin-7 is also a substrate for CdK5, which targets Kalirin-7 at T1590. Cdk5 phosphorylation increases GEF activity and its regulation is involved in Kalirin-7 mediated spine formation and function [57, 58]. Phosphorylation of Trio in the context of synaptic function is less understood, though it has been shown that tyrosine phosphorylation via Fyn is an essential event in Trio activation of Rac1 in Netrin-1 mediated axon guidance [27].

Synaptic Adhesion Molecules

Kalirin-7 is a downstream effector for many postsynaptic adhesion molecules, such as N-cadherin and ephrinB-EphB (Figure 2) [59, 60]. These studies demonstrate Kalirin-7 function is required for N-cadherin and ephrin-induced spine development, morphology, and plasticity, providing mechanisms linking cellular adhesion to dynamic changes in the actin cytoskeleton. Trio is similarly thought to be involved in integrating cellular adhesion signals. Various cadherin-mediated processes have been shown to involve Trio as a downstream regulator of Rac1/RhoA actin dynamics in several model systems. For instance, Trio forms a complex with Cadherin-11 that is important for cranial neural crest cell migration and may control the mature organization of neuronal clusters in the hindbrain [61–63]. Additionally, Trio is an integral part of the N-cadherin adhesion complex in endothelial cells, though the function of this complex is unexplored in neurons [12]. Recently, Kalirin-7 was discovered to be an interactor of Neuroligin-1, and functions as a downstream regulator of Neuroligin-1-mediated synaptic enhancement in an interaction dependent manner (Figure 2) [47]. This is consistent with a recent study showing Neuroligin-1 promotes actin assembly associated with spine enlargement and plasticity [64]. Interestingly, it was shown that Neuroligin-1 interacts with the N-terminal domains of Kalirin-7 that are lacking in Kalirin-5 [47]. Analogous to other RhoGEFs, the non-enzymatic domains of Kalirin-7 regulate its subcellular localization and influence on synaptic structure and function. The GEF domains of Kalirin-7 and Kalirin-5 are equally active and both retain the PDZ-ligand that targets these proteins to the synapse, suggesting additional divergent functions of the N-terminal domains of Kalirin-7 in controlling dendritic morphology and synaptic plasticity [49]. These may be distinct from those of Trio at the PSD, as it was shown from proteomics that Trio does not interact with Neuroligin-1 as robustly, which highlights a particular divergence from Kalirin-7 [47]. The phenotypic overlap between Kalirin-7 and Neuroligin-1 suggests reciprocity in function, with Kalirin-7's ability to control cell morphology being dependent on Neuroligin-1. This is an exciting area for further study.

Additional adhesion molecules have been suggested to interact with Kalirin-7. Proteomic analysis of pan-Kalirin interacting partners shows Kalirin clusters synaptic adhesion molecules [65]. Kalirin-7-specific proteomic analysis has identified additional adhesion molecules, including the ionotropic **glutamate receptor delta 2 (GluD2)** as a specific interactor [47]. GluD2 is unique in that it is predominantly expressed in Purkinje neurons of

the cerebellum [66]. GluD2 functions similarly to neuroligins, acting as an adhesion molecule and synaptic organizer interacting trans-synaptically with neurexin via the intermediary secreted protein cerebellin [67]. Further functional analysis of this interaction is needed. Given the diversity of adhesion molecules functioning through Kalirin-7, we propose Kalirin-7 as a fundamental downstream regulator of upstream synaptic adhesion (Figure 2). More work is needed to delineate further the role of other adhesion proteins in this process and the role of Trio in this pathway.

Kalirin and Trio RhoGEFs in Disease

Aberrant activity of RhoGEFs is implicated across human disease, mediating changes in the density and morphology of spines observed in neuropsychiatric, neurodegenerative, and neurodevelopmental disorders [68]. Pathological signaling involving Kalirin and Trio is associated with various disorders, specifically autism spectrum disorder (ASD), intellectual disability (ID), Alzheimer's disease (AD), Huntington's disease (HD), and schizophrenia [51, 69–71]. Most if not all of these disorders are characterized by disruptions in synaptic structure and function, with many sharing similar genetic etiologies, highlighting conservation in the underlying pathobiology and aberrant signaling. Therefore, it is not surprising that Trio and Kalirin dysfunction is associated with overlapping and distinct pathologies, reflective of their discrete and overlapping functions, expression patterns, and protein-protein interactions [11, 47, 50].

Autism spectrum disorder and intellectual disability

Convergent evidence has accumulated that abnormalities in the development and function of glutamatergic synapses are involved in many cognitive disorders, including ASD and ID [72, 73]. It is thought most forms of severe ID, and many cases of ASD, are genetic in origin [74, 75]. Many ASD risk factors identified in genetic screens are indeed key regulators of synaptic plasticity, and included genes that code for scaffolding molecules, glutamate receptors, and cell adhesion molecules [76]. Furthermore, dysregulation of synaptic actin organization, including dysregulation of Rac1, has been implicated in ASD-related phenotypes [75, 77, 78]. The role of Trio in regulating actin dynamics in synapse development and plasticity demonstrate it is particularly sensitive to disruptions associated with ASD/ID. Indeed, many **de novo mutations** and **rare variants** in *TRIO* have been identified in patients (Figure 3). Recent whole exome sequencing studies in ASD and public database mining have increased the number of disease-related *TRIO* mutations identified, and have confirmed that de novo mutations in *TRIO* are a significant risk factor for ASD/ID [71, 79–81]. Recent findings from various groups reveal that a large number of these ASD and ID-related de novo mutations are strikingly clustered in the GEF1 domain of Trio, with an even stronger association with ASD/ID for the Rac1 interacting DH1 subdomain (Figure 3) [71, 82–85]. This cluster of ASD-related mutations in Trio's GEF domain represents one of a very small number of ASD/ID mutation "hotspots" that have been identified to date. A majority of these disease-related *TRIO* mutations are **hypomorphic**, reducing Trio's ability to activate Rac1, potentially reflecting a phenotype similar to Trio haploinsufficiency [44]. When expressed in neurons, Trio harboring these mutations were found to decrease postsynaptic AMPA receptor function [71]. However, other mutations have been identified

that are **hypermorphic** [71]. One ASD/ID-related mutation identified in the GEF1 domain of Trio increases Rac1 activation, with neuronal expression resulting in dramatic increases in postsynaptic AMPA and NMDA receptor function [71]. This increase in glutamatergic neurotransmission was caused by this hypermorphic mutation, making Trio abnormally synaptogenic [71]. It is now apparent that mutations in either the GEF1 or GEF2 domains dysregulate GTPase activation, and the location of mutation and the resulting functional phenotype contribute to the ASD/ID diversity associated with Trio dysfunction. Indeed, recent work has suggested that opposite modulation of Rac1 via mutations in *TRIO* is associated with a distinct and domain-specific ASD/ID phenotype [85]. In particular, specific Trio-mediated Rac1 activation phenotypes correlate with head size and degree of ID, with the majority of mutations in GEF1 associated with microcephaly and a second cluster of mutations in the spectrin repeats associated with macrocephaly (Figure 3) [85]. Additionally, a recent report of a disease-related mutation associated with bipolar disorder, located in the GEF2 domain that was found to increase RhoA activation, demonstrating domain specific phenotypes across many disorders [84].

Given Trio's role in axon guidance and glutamatergic synapse regulation, we propose a model in which dysregulated Trio function results in a combination of disturbed synapse formation, developmental miswiring, and post-development aberrant glutamatergic transmission. Despite a number of previous large-scale human **exome sequencing** studies showing a genome-wide association of *TRIO* with ASD/ID, *TRIO* was not among the top 102 ASD-associated genes identified in a recent study [76]. In this study, of the ~17,500 genes analyzed, *TRIO* ranked 116th, putting it just below the gene identification threshold. It is important to note that *TRIO*'s association with ASD/ID is likely underestimated by human exome sequencing studies given that significance calculations are limited to entire genes rather than specific gene regions that encode functional protein domains. As discussed, ASD-related de novo mutations are largely clustered in Trio's GEF1/DH1 domain (Figure 3) [71]. The exomic DNA encoding Trio's DH1 domain exhibits a much higher degree of constraint relative to other coding regions of *TRIO*, and thus a higher level of genome-wide significance is observed when this region of the *TRIO* gene is considered independently [71].

Kalirin is not associated with ASD or ID as strongly as Trio, which is perhaps surprising given their shared genetic ancestry and parallel functions. Only a few mutations identified in Kalirin have been thought to cause ID. One such mutation, found in the GEF1 domain of Kalirin, was predicted to be pathogenic in a pair of siblings with ID and musculoskeletal abnormalities [86]. Moreover, Kalirin has come out as an ASD-associated gene in a study from individuals from the Faroe Islands [87]. Interestingly, the male carrying the de novo Kalirin mutation (N2024D) in the RhoA GEF2 domain did not have ID (Figure 3). It might be that Kalirin ASD mutations are underrepresented due to a milder phenotype, resulting in patients not seeking clinical assistance inclusive of genetic testing. It may also be that the absence of Kalirin expression in neural crest cells does not render it as sensitive to disruptions relative to Trio. Another intriguing hypothesis, however, is that Trio, but not Kalirin, may be part of a larger ASD/ID interaction network. Indeed, a protein interaction network analysis has identified converging molecular pathways among heterogenous

ASD/ID [88]. Proteomic mapping of Trio and Kalirin-7 interactomes has revealed Trio, but not Kalirin-7, to be an interacting partner of many ASD/ID associated genes, such as DPYSL2 [47, 76].

Schizophrenia

The genetic architecture of schizophrenia is complex, involving both common and rare copy variants and single nucleotide mutations. Kalirin transcript levels and protein levels have been shown to be reduced in the dorsolateral prefrontal cortex of schizophrenia patients, with a loss of Kalirin protein correlating with spine loss [89, 90]. Interestingly, other brain regions in schizophrenia patients appear to have a dysregulation in Kalirin-9, which was found to be upregulated, but not in Kalirin-7 [91]. A genome-wide association study in a Japanese cohort found an association signal in the region of *KALRN* that did not reach genome-wide significance [92]. An additional resequencing and association study found that rare missense mutations in *KALRN* may be genetic risk factors for schizophrenia, adding further evidence that Kalirin may be involved in the etiology of schizophrenia [93]. In addition, a recent study found SZ patients in the South African Xhosa were more likely to have mutations in proteins involved in neurotransmission, including mutations in *KALRN* [94]. How schizophrenia-related variants directly relate to disease phenotypes is not entirely understood. However, characterization of several *KALRN* sequence variants have shown that single amino acid changes in *KALRN* result in impaired transcript level, protein function, neuronal morphology and cortical thickness, recapitulating many of the observations in Kalirin-mediated SZ [70, 95].

Interestingly, the largest whole exome sequencing study of schizophrenia parent-proband trios to date has identified *TRIO* as one of the strongest genes associated with schizophrenia, with a number of de novo loss of function mutations observed [96]. While no single gene reached genome wide significance in this study, *TRIO* is one of a small number of genes that has now reached genome wide significance following a recent meta-analysis of exome sequencing data taken from 24,248 schizophrenia cases, 97,322 controls, and 3,444 parent-proband trios (www.schema.broadinstitute.org). Moreover, recent assessments of transcriptomic organization have identified differential splicing changes in *TRIO* and *KALRN* in schizophrenia disease brains [97]. Kalirin-7 and Trio are known to interact with several proteins that have been implicated in SZ, including DISC1 (disrupted in schizophrenia) [98]. DISC1 is thought to function as a scaffold for Kalirin-7, thereby directly regulating activation of Rac1 [99]. Trio also has been shown to interact with DISC1 via its spectrin repeats and is also thought to facilitate GEF1 activation of Rac1 [100]. However, advances in sequencing technology and larger data sets have questioned the genetic association of DISC1 with SZ [101, 102]. DISC1 fails to reach significance in larger association and de novo mutation studies [96, 103, 104]. Nevertheless, this does not preclude its involvement in schizophrenia through very rare and penetrant variants [101, 105]. Other known interactors or effectors of Kalirin and Trio are also thought to be schizophrenia risk genes, including CDC42 and PAK [106].

Neurodegeneration

It has been speculated that functional decrease or loss of Kalirin could precipitate spine collapse, leading to synapse loss and cognitive dysfunction in AD [106]. Indeed, postmortem analyses of AD patient brains have found reduced levels of Kalirin transcripts and protein, as well as decreased levels and activation of Kalirin targets, such as PAK, in the hippocampus and prefrontal cortex [107–109]. These findings are supported by studies demonstrating Kalirin-7 overexpression prevents dendritic spine loss induced by amyloid oligomers and work showing that the restorative effects of the transcription factor XBP1 on memory and plasticity in AD models is mediated through control of Kalirin-7 [110, 111]. Amyloid-β (Aβ) may directly lead to Kalirin-7 dysregulation or loss through interaction with EphB2, an important upstream regulator of Kalirin-7 (Figure 2) [59]. EphB2 levels are decreased in AD patient brains and Aβ has been shown to directly interact with EphB2 [112, 113]. Depletion of EphB2 is critical in Aβ-mediated neuronal dysfunction, and rescuing EphB2 levels improves cognition in AD models, perhaps through spine stabilization via restoration of Kalirin-7 function and PAK activation [113]. Spine loss in AD may also involve dysregulation of other GTPase-regulatory proteins, such as Trio. Recent proteomic analysis of the Tau-P301S mouse identified depletion of GTPase-regulatory proteins as a salient feature of actin dysregulation and spine collapse observed in tauopathies [51]. Both Kalirin and Trio were prominently dysregulated in this study, pointing to a conserved role of these dual RhoGEFs in the pathophysiology of tauopathies. It is not known, however, what upstream factors mediate Trio dysregulation, and whether they are distinct from those of Kalirin-7.

Concluding Remarks

Trio and Kalirin are critical synaptic RhoGEFs underlying many essential processes of synaptic plasticity and development. Renewed interest in their biology has arisen from their strong association with neurodevelopmental, neuropsychiatric, and neurodegenerative disorders. In particular, Trio has emerged as an important gene in SZ and ASD/ID, whereas both Kalirin and Trio have now both been associated with neurodegenerative disorders. It is becoming more evident that disruptions in the regulation of GTPase activity is a pathogenic mechanism in many neurological disorders. Indeed, mutations in GAPs, specifically SynGAP, are also strongly associated with ASD and ID [76]. The diversity of Kalirin and Trio disease associations is reflective of their varied roles in axon guidance, synapse structure, and synaptic plasticity, which are mediated through precise isoform-specific expression and function. Deeper characterization of their distinct and overlapping biology will assist in better understandings of their pathobiology. Many fundamental questions still remain unanswered, including what the isoform-specific functions of Trio are and how they relate to Kalirin's (see Outstanding Questions). Additionally, most studies on the synaptic functions of Trio and Kalirin were performed in young animals (or in vitro models likely to be relevant to early developmental stages), and accordingly, the presynaptic and postsynaptic function of Trio in the adult remains relatively unexplored. This developing field will prove important in the evolving understanding of synapse biology, but also for the development of better therapeutic targets and interventions.

Acknowledgements

Glossary

Paralog

Separate gene created by a duplication event within the same genome.

Phylogenetic analysis

Means of analyzing the evolutionary relationships between genes across species.

Urbilateria

A hypothetical last common ancestor of all organisms with bilateral symmetry (bilaterian clade).

Prebilateria

Non-bilaterally symmetric clade (i.e. sponges and placozoans)

Subfunctionalization

Process in which each paralog retains a subset of its original ancestral function, resulting in the preservation of duplicated genes in the same genome.

Postsynaptic density (PSD)

An electron-dense structure, 30–60 nm thick, situated subjacent to the postsynaptic membrane. The PSD is composed of neurotransmitter receptors, scaffolding proteins, signaling elements, and trans-synaptic adhesion molecules. Thousands of different proteins are known to be contained in the PSD, all of which coordinate together in the means of synaptic transmission, and the dynamic modulation of these PSD components is an essential feature of synaptic plasticity.

Haploinsufficiency

Model in which the functional copy of a gene (wild-type allele) in combination with the variant allele is unable to produce a normal or standard phenotype.

eEPSC

Evoked excitatory postsynaptic currents are postsynaptic currents induced through stimulation of presynaptic neurons.

GluD2

Glutamate receptor, ionotropic, delta 2. This protein is an ionotropic glutamate receptor expressed primarily in the Purkinje neurons of the cerebellum. Unlike other glutamate receptors, GluD2 does not bind glutamate, and functions largely as an adhesion molecule. Mutations or deletions in the GluD2 result in cerebellar ataxia.

De novo mutations

A genetic mutation that is not inherited and is present for the first time in a certain progeny. These mutations are present in the offspring, but not in the parents.

Rare variants

A genetic variant which occurs at very low (<1%) frequency in the population. While infrequent in the normal populations, these variants can be involved in the etiology of many disorders.

Hypomorphic

A mutation that results in a loss of gene function.

Hypermorphic

A mutation that results in a gain of gene function.

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Outstanding Questions

- Though much work has been done exploring Kalirin as a PSD signaling hub, the role of Trio in this process remains largely unknown. What is the postsynaptic role of Trio in plasticity and excitatory neurotransmission?

- How do Kalirin and Trio regulate presynaptic development, and do they function in presynaptic release?

- How do mutations in various domains of Trio and Kalirin contribute to the pathophysiology of various neurodevelopmental disorders?

- What is the mechanism by which Kalirin and Trio contribute to spine loss in neurodegenerative disorders?

- How can Kalirin and Trio be targeted for drug development?

- What are the functions of various Trio isoforms, such as Trio-8?

Highlights

- The synaptic RhoGEFs Kalirin and Trio are paralog proteins that have shared and distinct functions in neuronal development and in synaptic plasticity. Recent phylogenetic analyses have provided resolution surrounding the evolution of these related genes. The retention of both *Trio* and *Kalrn* in an organism is concomitant with an increase in Kalirin and Trio isoforms. This proteomic diversity and differential expression is an essential feature of their sub-functionalization, which mediates their distinct and conserved functions.

- Kalirin and Trio are present at the pre- and post-synaptic sites, and exert their effects on glutamatergic neurotransmission through their ability to regulate the actin cytoskeleton at these synapses.

- Kalirin-7 is a downstream effector of many postsynaptic adhesion molecules. Such findings implicate Kalirin and possibly Trio as major signaling hubs that regulate glutamatergic synapse development and function.

- Human genetics studies have identified *TRIO* as a risk gene in both Autism Spectrum Disorder (ASD) and Schizophrenia. In animal models, ASD-related mutations in Trio have been found to produce marked alterations in glutamatergic synapse function.

Box 1. Multiple Promoters and Alternative Splicing: Trio and Kalirin Proteomic Complexity

The *Kalrn* and *Trio* genes each give rise to numerous isoforms, due to pronounced alternative splicing and promoter usage (Figure 1) [14, 20, 21, 114]. Both proteins, in their full-length form, are defined by two GEF domains (GEF1 and GEF2) and a C-terminal serine/threonine kinase domain, a unique feature of these Dbl family members. Kalirin and Trio each have an N-terminal Sec14p domain trailed by numerous repeating spectrin-like repeats, with two Src-homology 3 (SH3) domains following the GEF sequences, and a single immunoglobulin-like (Ig) domain. Kalirin is defined further by the addition of a fibronectin-like (FN3) domain leading the C-terminal kinase domain (Figure 1).

C-terminal sequence variations are the most common variants among brain-specific Trio isoforms. Alternative splicing of Trio exon 48 results in two distinct Trio isoforms, Trio-9S and Trio-9L, which are considered the dominant brain-specific species [20, 21]. Trio-9S and Trio-9L most resemble full-length UNC-73 and dTrio in that they do not contain the C-terminal kinase domain. Moreover, a cerebellum-specific isoform is also observed after postnatal day 30 (P30) (in mice), termed Trio-8, which is specific to Purkinje neurons [20]. Trio-8 is unique in that it contains only the first Rac1/RhoG GEF domain.

Like Trio, Kalirin is expressed ubiquitously and can be found in cardiac and skeletal muscle, as well as in the liver and in endocrine tissue [115]. Alterative 3' splicing gives rise to the major Kalirin isoforms, Kalirin-12, Kalirin-9, and Kalirin-7. Kalirin-12 retains both GEF1 and GEF2 domains, as well as the kinase domain. Kalirin-9, however, lacks the kinase domain and is most similar to Trio-9 [14]. Kalirin-7 is the brain specific isoform of Kalirin, as well as the dominant species in the adult [116]. Kalirin-7 is most similar to Trio-8 in that it contains only the Rac1/RhoG GEF. It is, however, unique in that it contains a putative PDZ (postsynaptic density protein, *Drosophila* disc large tumor suppressor (Dlg1), and zonula occludens-1 protein) interacting motif at its C-terminal end (Figure 1) [116]. Moreover, recent work has identified additional alternative promotor usage that gives rise to further Kalirin-7 species with differing N-terminal sequences; with bKalirin-7 and cKalirin-7 being most prevalent, differing in only 25 amino acids (Figure 1) [114]. These N-terminal differences are thought to mediate unique phosphoinositide-dependent interactions that control Kalirin-7 protein localization and function [114]. An additional Kalirin-7 variant, termed Kalirin-7 or −5, lacks the N-terminal Sec14 domain and first four spectrin repeats, but retains the PDZ-interacting motif [14].
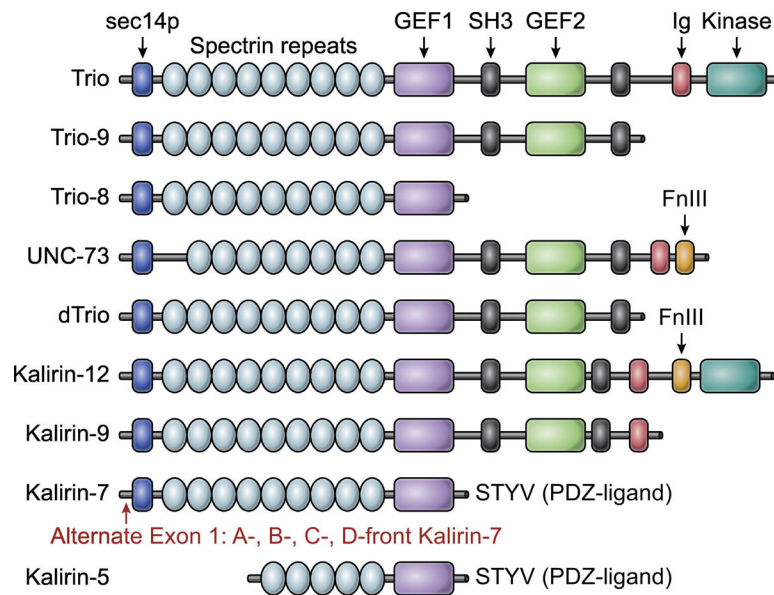
Figure 1. Major Trio and Kalirin isoforms.
Alternative splicing of Kalirin and Trio genes gives rise to numerous isoforms. This alignment displays the dominant Trio and Kalirin species, along with the Drosophila (dTrio, row 5) and C. elegans orthologs (UNC-73, row 4). Kalirin and Trio contain two guanine nucleotide exchange factors (GEFs) that are composed of a catalytic Dbl homology domain, and a pleckstrin homology domain that functions allosterically. GEF1 activates Rac1 and RhoG, whereas GEF2 activates RhoA. Kalirin and Trio GEFs are well conserved, with the greatest sequence diversity observed in their non-GEF flanking sequences. Kalirin-7 and Trio-9 are the dominant species in the brain. Kalirin-7 is unique in that it harbors a C-terminal PDZ-ligand. Alternative start sites in exon 1 of *Kalrn* generate Kalirin-7 with various N-terminal sequences (so called a, b, c, and d-front Kalirin-7). For further structural highlights, see Box 1. Abbreviations: SH3, Src homology 3; FnIII, Fibronectin type III; Ig, Immunoglobulin; GEF, Guanine nucleotide exchange factor; PDZ, Postsynaptic density-95/ discs large/zona occludens.
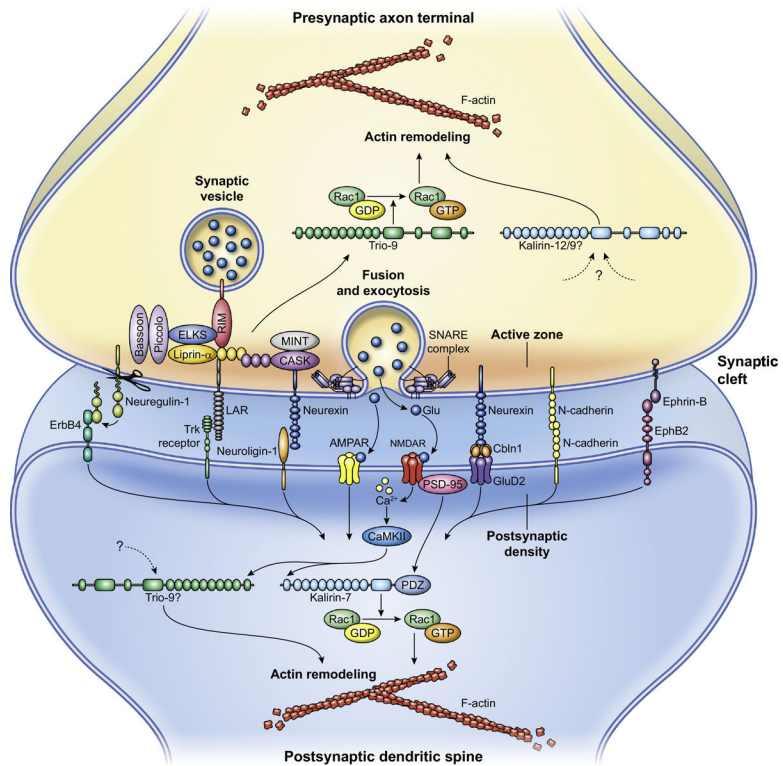
Figure 2, Key Figure. Kalirin and Trio serve postsynaptic and presynaptic functions.
Kalirin and Trio interact with multiple proteins at the pre- and post-synaptic sites. These interactions integrate upstream signals into dynamic changes in the actin cytoskeleton that work to regulate spine formation, dendritic morphology, and presynaptic release. Kalirin-7 interacts with several neurotransmitter receptors and adhesion molecules, translating glutamate-mediated signals and synaptic adhesion into changes in the actin cytoskeleton. Presynaptically, Trio interacts with many proteins involved in vesicle release and trafficking, regulating the actin dynamics facilitating these processes. Upstream signaling regulating Trio at postsynaptic spines and Kalirin at the presynaptic site is less understood. Abbreviations: Glu, Glutamate; Cbln1, Cerebellin-1; NMDAR, N-methyl-D-aspartate receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; LAR, LCA-related protein tyrosine phosphatase; PDZ, Postsynaptic density-95/discs large/zona occludens; CaMKII, Ca2+/calmodulin-dependent protein kinase II; EphB2, EPH receptor B2; CASK, calcium/calmodulin dependent serine protein kinase; RIM, Rab interacting molecule; ELKS, ERC/RAB6-interacting/CAST; MINT, Munc18 interacting protein; Trk, Tropomyosin receptor kinase; Rac1, Ras-related C3 botulinum toxin substrate 1; GTP, Guanosine triphosphate; GDP, Guanosine diphosphate.
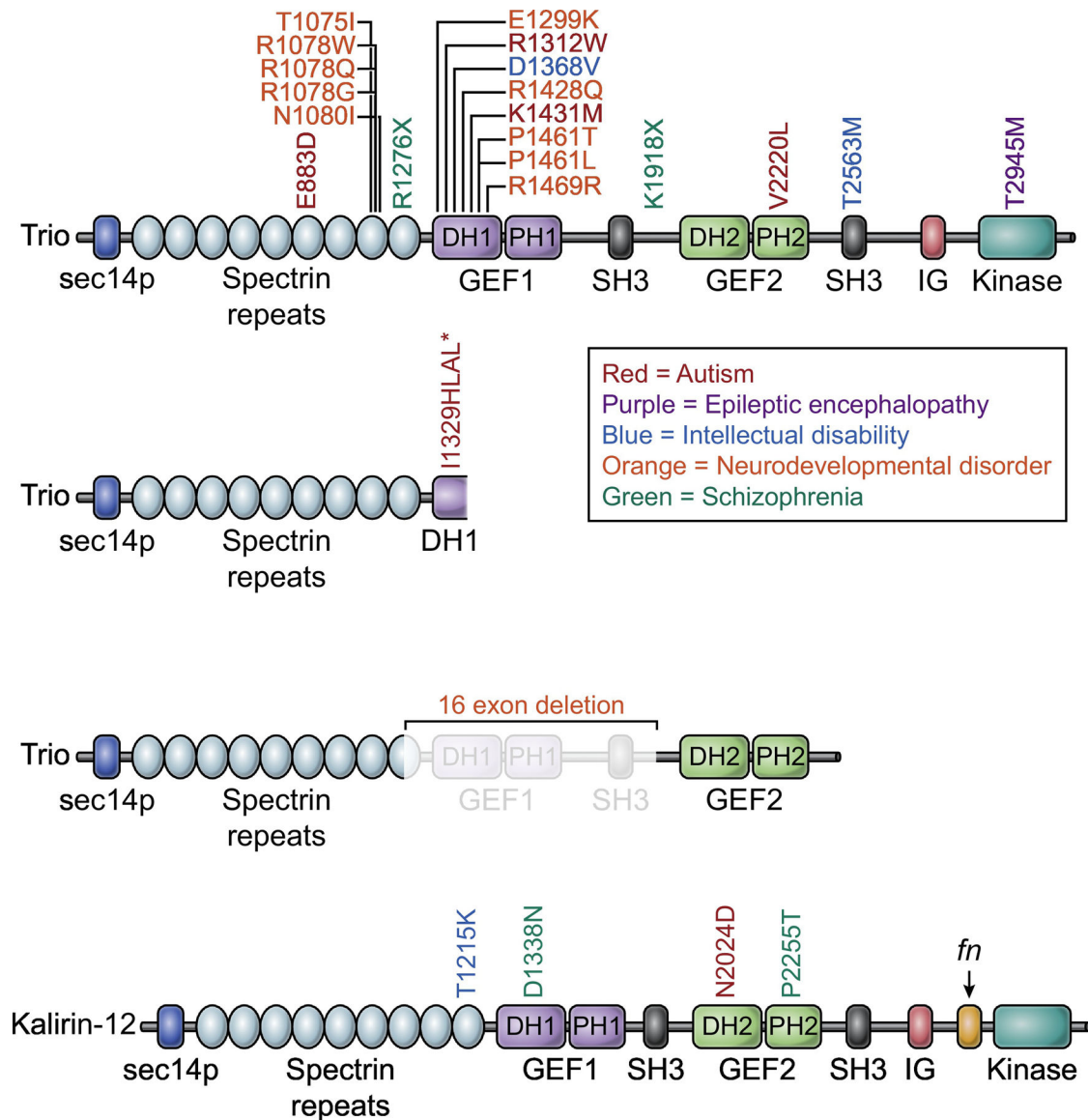
Figure 3: Disease-related mutations in Trio and Kalirin.
Mutations in Trio are associated with several neurological disorders. A majority of ASD/ID mutations occur in the first GEF domain, with an additional hotspot located in the second to last spectrin repeat. The top Trio row depicts missense mutations, the second and third rows depict nonsense mutations and copy number variants. The Asterix indicates a premature stop codon. Fewer disease-associated mutations have been identified in Kalirin. Abbreviations: DH, Dbl homology; PH, Pleckstrin homology; SH3, Src homology 3; FnIII, Fibronectin type III; IG, Immunoglobulin; GEF, Guanine nucleotide exchange factor; PDZ, Postsynaptic density-95/discs large/zona occludens.