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Kalirin is Required for BDNF-TrkB Stimulated Neurite Outgrowth and Branching

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Abstract

Exogenous brain-derived neurotrophic factor (BDNF), acting through TrkB, is known to promote neurite formation and branching. This response to BDNF was eliminated by inhibition of TrkB kinase and by specific inhibition of the GEF1 domain of Kalirin, which activates Rac1. Neurons from *Kalrn* knockout mice were unable to activate Rac1 in response to BDNF. BDNF-triggered neurite outgrowth was abolished when *Kalrn* expression was reduced using shRNA that targets all of the major *Kalrn* isoforms, and reduced in neurons from *Kalrn* knockout mice. The *Kalrn* isoforms expressed early in development also include a GEF2 domain that activates RhoA. However, BDNF-stimulated neurite outgrowth in *Kalrn* knockout neurons was rescued by expression of Kalirin-7, which includes only the GEF1 domain but lacks the GEF2 domain. Dendritic morphogenesis, which requires spatially restricted, coordinated changes in the actin cytoskeleton and in the organization of microtubules, involves essential contributions from multiple Rho GEFs. Since Tiam1, another Rho GEF, is also required for BDNF-stimulated neurite outgrowth, an inhibitory fragment of Tiam1 (PHn-CC-EX) was tested and found to interfere with both Kalirin and Tiam1 GEF activity. The prolonged TrkB activation observed in response to BDNF in *Kalrn* knockout neurons and the altered time course and extent of ERK, CREB and Akt activation observed in the absence of *Kalrn* would be expected to alter the response of these neurons to other regulatory factors.

Keywords

Rho GEF; *Kalrn* knockout; TrkB kinase; TrkB phosphorylation; ERK; Akt; CREB

1. Introduction

The architectural and functional integrity of neural circuitry requires proper formation of dendritic arbors (Craig and Banker, 1994; Jan and Jan, 2010; Wong and Ghosh, 2002).

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Dendrite initiation and branching start with filopodial formation, a process that is regulated by actin organization/polymerization and microtubule organization (Galic et al., 2014; Sainath and Gallo, 2015). The early development of dendrites is regulated by intrinsic genetic programs, which define the competence of neurons in response to extrinsic cues (Barnes and Polleux, 2009; Craig and Banker, 1994). Extrinsic cues in dendritic morphogenesis include membrane-bound proteins and soluble components such as neurotrophins (Konur and Ghosh, 2005; Landgraf and Evers, 2005).

Brain derived neurotrophic factor (BDNF) plays crucial roles in regulating neuronal survival, differentiation and morphology (Huang and Reichardt, 2003; Park and Poo, 2013). BDNF promotes dendritic arbor growth (Ozdinler and Macklis, 2006; Reichardt, 2006; Wang et al., 2015) and axonal branching (Panagiotaki et al., 2010) by binding to TrkB (Bramham, 2008; Park and Poo, 2013), a receptor tyrosine kinase (Cohen-Cory and Fraser, 1995; McAllister et al., 1999). The binding of BDNF stimulates trans-phosphorylation of multiple Tyr residues, which triggers multiple downstream pathways, including activation of the small Rho family GTPase, Rac1 (Huang and Reichardt, 2003; Minichiello, 2009). A key feature of growth factor signaling is timing; receptor inactivation occurs after the internalized ligand-receptor complex is removed from the cell surface (Assaife-Lopes et al., 2014; Ji et al., 2010; Lai et al., 2012; Mitchell et al., 2012; Philippidou et al., 2011).

Rho family GTPases are critical regulators of actin cytoskeletal dynamics and neuronal morphogenesis (Nakayama et al., 2000; Tada and Sheng, 2006). Guanine-nucleotide exchange factors (GEFs) activate Rho proteins in a spatially and temporally controlled manner and are essential for neuronal process outgrowth and branching (Jaffe and Hall, 2005; Miller et al., 2013). Kalirin, a multifunctional Rho GEF, influences neuronal morphology in different ways at different stages of development (Ma et al., 2003; May et al., 2002; Shioda et al., 2011; Sommer and Budreck, 2009).

Kalirin has been shown to play a central role in signaling pathways triggered by several receptor tyrosine kinases, including EphB2, ErbB4, TrkA and PDGFR- β (Cahill et al., 2013; Chakrabarti et al., 2005; Penzes et al., 2003; Wu et al., 2013). In pheochromocytoma cells, an interaction between Kalirin and TrkA contributes to neurite outgrowth (Chakrabarti et al., 2005), suggesting a role for Kalirin in neurotrophin actions. However, whether Kalirin plays a role in BDNF/TrkB signaling in neurons is unknown.

Interestingly, Tiam1, another neuronal Rho GEF, is phosphorylated by TrkB *in vitro* and in cortical neurons; phosphorylation of Tiam1 is required for activation of Rac1 downstream of BDNF/TrkB signaling, and TrkB phosphorylation of Tiam1 is required for BDNF-induced neurite outgrowth in neurons (Lai et al., 2012; Miyamoto et al., 2006). In the current study, we set out to explore the hypothesis that endogenous Kalirin is also required for the ability of exogenous BDNF, acting through TrkB, to stimulate dendritic morphogenesis.

The *Kalrn* gene undergoes alternative splicing. Kalirin-9 (Kal9) and Kalirin-12 (Kal12) include two Rho GEF domains and are the only isoforms present in early development (Yan et al., 2015). Kal9 and Kal12 are required for normal dendritic development. Kalirin-7

(Kal7), which includes only one GEF domain and is absent in early development, becomes the major isoform in the adult brain [Fig. 1A].

In this study, we identified Kalirin as a key component of the downstream signaling by TrkB that leads to BDNF-induced neurite outgrowth and branching in hippocampal neurons. Kal GEF1 mediated Rac1 activation was required for BDNF-induced neuronal morphogenesis. The fact that Kal7, which lacks a GEF2 domain, stimulated neurite formation and rescued the ability of young KalSR^{KO} neurons to respond to BDNF, confirmed the essential role of Rac activation. Our findings demonstrate extensive crosstalk between two key players in the formation of dendrites early in development, Kalirin, a Rho GEF, and TrkB, a receptor tyrosine kinase.

2. Materials and Methods

2.1 Antibodies

Commercial antibodies included: TrkB (#610101, mouse, BD Biosciences, San Jose, CA); phospho-TrkB Tyr515 (#9141, rabbit, Cell Signaling, Danvers, MA) and Tyr705/706 (#4621, rabbit, Cell Signaling), phospho-Akt (#4051, mouse, Cell Signaling), Akt (#4691, rabbit, Cell Signaling), phospho-CREB (#9198, rabbit, Cell Signaling), CREB (#9104, mouse, Cell Signaling), phospho-MAPK (Erk1/2) (#9106, mouse, Cell Signaling), ERK (#sc-154, rabbit, Santa Cruz, Santa Cruz, CA), FLAG (#F7425, rabbit, Sigma), GAPDH (MAB374, mouse, EMD Millipore) and MAP2 (AB15452, chicken, EMD Millipore). The Myc monoclonal 9E10 was from spent hybridoma medium (Borjigin and Nathans, 1994). Rabbit antibody to the Kalirin Sec14 domain has been described and validated (CT302) (Yan et al., 2015).

2.2 Plasmids

To reduce expression of endogenous Kalirin in rat hippocampal cultures, shRNA targeted to all major isoforms of rat and mouse Kalirin (Spec8 shRNA) was expressed using pSiren-DsRed (Francone et al., 2010). The sequences targeted were: Scramble shRNA, AATGCACGCTCAGCACAAGC, which corresponds to no rodent cDNAs in Genbank; Spec8 shRNA, GCAGGAACAATGTGAGCATG. The efficacy of the Spec8 shRNA was validated previously using the shorter isoform Kal7 (Yan et al., 2015). Further testing shRNA efficacy, pEAK.His-myc- Kal7, -Kal7, -Kal9 and -Kal12 were transfected into pEAK cells (hEK-293 derivative) (Ma et al., 2014; May et al., 2002) along with Spec8 shRNA, using TransIT-2020 (Mirus); cells were harvested into SDS lysis buffer (Yan et al., 2015) 24 h post transfection for western blot analysis. The Kal7-shRNA-rescue plasmid was produced by making seven silent mutations in the target sequence of the Spec8 shRNA and verified by DNA sequencing (CGC AAT AAC GTC TCC for RNNVS). PEAK-kGEF1 was described previously (Ma et al., 2014; May et al., 2002). Plasmids expressing two FLAG-tagged fragments of Tiam 1, pCMV5.C1199 (Tiam1[393–1591]) and pCMV5.PHnCCEx (Tiam1[393–841]) (Miyamoto et al., 2006), were kindly provided by Dr. Yuki Miyamoto (National Institute of Child Health Development, Tokyo). The Rac1 biosensor plasmid (Das et al., 2015a) was kindly provided by Dr. Yi Wu, UConn Health.

2.3 Experimental Treatment of Neuronal Cultures

Cultures of hippocampal neurons were prepared from embryonic day 18 Sprague Dawley rat embryos and cultures of hippocampal neurons were prepared from postnatal day 1 WT or KalSR^{KO} mice (Yan et al., 2015). To evaluate the effect of BDNF on neurite outgrowth, BDNF (10–50 ng/ml) (Alomone, Jerusalem, Israel) was added 24 h after plating and remained in the medium for the next 3 days; BDNF was reconstituted in H₂O as a sterile 10 µg/ml stock. For shRNA knock-down experiments, scramble or Kalirin shRNAs were introduced into hippocampal neurons by electroporation. For overexpression experiments, full length Kalirin vectors (7 µg DNA per 10⁶ neurons) were introduced into hippocampal neurons from rat or KalSR^{KO} animals by electroporation at the time of plating (Yan et al., 2015); transfection efficiency was around 10%. To examine the effects of K252a (Tocris) and ANA-12 (Tocris), stocks (10 mM K252a in DMSO; 25 mM ANA-12 in DMSO) were diluted into growth medium to yield 100 nM K252a and 10 µM ANA-12. DMSO was used in the vehicle group. K252a or ANA-12 was pre-incubated for 1h before BDNF was applied to the neurons and remained on the cells until harvest. To examine the effects of ITX3 (Sigma), a 1000x stock of ITX3 dissolved in DMSO (100 mM) was diluted into maintenance medium containing BDNF (final ITX3 concentration 100 µM) or not and kept on the cells until fixation. For the time course study, hippocampal cultures were plated into 12-well plates (10⁶ neurons per well); no growth factor or BDNF (50 ng/ml) was applied and cultures were harvested at the times indicated into 150 µl SDS-P lysis buffer [50 mM Tris pH7.6, 1% SDS, 130mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, P8340 (Sigma, 1:100), phosphatase cocktail inhibitor I (CalBiochem, 1:100), 1 mM orthovanadate and PhosSTOP (Roche, 1 tablet in 10 ml)]. The concentration of protein was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) and 20 µg of the total protein was loaded onto each lane as described (Yan et al., 2015). Samples were heated at 95°C for 5 min and centrifuged at 15,000 × g for 30 sec before loading onto gels.

2.4 Pull down assay

Hippocampal cultures from WT and KalSR^{KO} mice were maintained until DIV21, and 50 ng/ml BDNF was added in maintenance medium for 15 min. Neurons were extracted in 0.3 ml ice-cold MLB buffer (25 mM HEPES, pH7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA and 2% glycerol, 0.3 mg/ml PMSF, proteinase inhibitors (Schiller et al., 2008)). Cell lysates were incubated with GST-Pak-CRIB agarose beads slurry at 4°C for 1 hr with gentle agitation. Beads were washed 3 times and bound proteins were released by heating at 95°C in SDS-loading buffer for 5 min. Extracts (a pool from all samples, 100 µl) were incubated with 10 mM EDTA and 0.2 mM GTPγS (positive control) or 2 mM GDP (negative control) for 30 min at 30 °C, cooled and brought to 60 mM MgCl₂ prior to incubation with GST-Pak-CRIB beads.

2.5 Immunocytochemistry and Image Acquisition

Immunocytochemical staining of neuronal cultures was performed as described (Ma et al., 2014). Images were taken using a Zeiss LSM 510 Meta confocal microscope and a 40×

objective. A single image per neuron was taken to visualize the dendritic arbor. For each neuron, neurite number and branching were measured from the same image.

2.6 Western Blot Analysis

Cortices and hippocampi collected from P7 mice were sonicated into SDS lysis buffer, heated for 5 min at 95°C and centrifuged to remove insoluble debris. The concentration of protein in the supernatant was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin as a standard; 20 µg of total protein was loaded onto each lane. For analysis of the effect of BDNF on TrkB phosphorylation in neuronal cultures, phospho-specific antibodies were used first; PVDF membranes were stripped using low pH buffer and TrkB antibody was then applied.

2.7 FRET assay of GEF activity

HEK-293 (pEAK) cells were transfected in a glass-bottom 24-well plate in duplicate. All wells except the non-transfected wells received the Rac1-biosensor vector (Das et al., 2015a). The total amount of vector per well was kept constant using empty pEAK10 vector. DNA was mixed with TransIT-2020 and fluorescence emission spectra were obtained in DMEM without phenol red, pyruvate or vitamins (US Biological) at 30 h after transfection (Das et al., 2015a), using an EnSpire 2300 Multilabel Plate Reader (Perkin Elmer). FRET signal was normalized to the 475 nm signal as described (Das et al., 2015a).

3. Results

3.1 BDNF mediated neurite outgrowth requires Kal9 and Kal12

BDNF is known to play a critical role in dendritic growth and morphological specialization. Well before birth, hippocampal and cortical neurons in the brain express BDNF and TrkB (Behar et al., 1997; Mioranza et al., 2014). Exogenous BDNF increases the number of primary dendrites in an activity dependent manner through TrkB in specific cortical layers (McAllister et al., 1997; McAllister et al., 1995). We first asked whether Kalirin is involved in BDNF-induced neurite outgrowth. During the first 7 days *in vitro* (DIV), Kal9 and Kal12 play an essential role in neurite formation and branching in hippocampal and cortical neurons (Yan et al., 2015). Based on the literature, we expected exogenous BDNF to increase the number of primary neurites and branch points produced by hippocampal neurons (Cheung et al., 2007; Ji et al., 2010). Recombinant BDNF was added to the culture medium 24 h after plating and remained in the medium until the cultures were fixed and examined at DIV4 [Fig.1A]. Using a control shRNA, and doses of BDNF ranging from 10 to 50 ng/ml, it was clear that exposure to exogenous BDNF produced the expected increase in the number of primary neurites and branch points [Fig.1A, B].

Expression of Kal9 and Kal12 was manipulated using an shRNA (Spec8 shRNA) targeted to the spectrin repeat region common to all major isoforms of Kalirin [Fig.1A]. As expected, neurite formation and branching were reduced when expression of Kal9/Kal12 was reduced [Fig.1A, B]. We next asked whether rat hippocampal neurons in which expression of Kal9/Kal12 is reduced could respond to exogenous BDNF in the same manner as neurons expressing the control shRNA. We found that attenuation of Kal9 and Kal12 expression

abrogated the ability of exogenous BDNF to stimulate neurite outgrowth; even the highest concentration of BDNF tested was unable to increase the number of primary neurites or branch points in hippocampal neurons expressing the Kalirin Spec8 shRNA [white boxes, Fig.1B].

We next turned to KalSR^{KO} hippocampal neurons as an independent means of determining whether *Kalrn* plays an essential role in the ability of these neurons to respond to exogenous BDNF. WT and KalSR^{KO} neurons were maintained without or with 50 ng/ml BDNF starting 24 hrs after plating, and quantification of primary neurites and branch points was performed on DIV5 [Fig.1C, D]. As reported previously, the number of neurites and branch points was reduced in KalSR^{KO} neurons (Yan et al., 2015). As expected, the addition of exogenous BDNF increased the number of primary neurites and branch points in WT neurons; in contrast, exogenous BDNF failed to enhance neurite outgrowth in KalSR^{KO} neurons [Fig. 1D]. The results obtained using KalSR^{KO} mouse hippocampal neurons were consistent with the results obtained using shRNA to reduce Kalirin expression in rat hippocampal neurons [Fig.1A, B]. The effectiveness of Spec8 shRNA was previously documented using a shorter Kalirin isoform (Yan et al., 2015); here we validated the knockdown efficiency (to 35–40% of control) for all isoforms: Kal7, Kal7, Kal9 and Kal12 [Fig.1E]. Taken together, our results on neurons from both rat and mouse demonstrate that Kal9/Kal12 play a crucial role in the ability of exogenous BDNF to stimulate neurite outgrowth and extension.

3.2 Kalirin is a major GEF activating Rac1 downstream BDNF/TrkB signaling

To determine whether the catalytic activity of the GEF1 domain of Kalirin plays an essential role in the morphological response of hippocampal neurons to exogenous BDNF, we took a pharmacological approach. ITX3, a cell permeant inhibitor of Kalirin (and Trio) GEF1-mediated Rac1 activation, specifically blocked the ability of BDNF to stimulate neurite growth and branching [Fig.2A]. Interestingly, ITX3 had no effect on neurite or branch point number in the absence of BDNF at DIV4. These results are consistent with previous observations that BDNF is not required for the development of basal neuronal architecture (Kellner et al., 2014). Inhibiting Kalirin GEF1 activity specifically blocked BDNF-stimulated neurite outgrowth. ITX3 does not inhibit the GEF activity of Tiam1, another Rac GEF known to play an essential role in the response of young neurons to BDNF (Bouquier et al., 2009).

To compare the effects of inhibiting Kalirin GEF1 to the effects of inhibiting TrkB kinase activity, we included K252a, a selective inhibitor of Trk kinases when used at an appropriate concentration (Ohmichi et al., 1992; Tapley et al., 1992), or ANA-12, a non-competitive TrkB-specific antagonist (Alder et al., 2013) in the culture medium from the time of plating [Fig.2A]. Both 100 nM K252a and 10 μ M ANA-12 completely eliminated the ability of exogenous BDNF to promote neurite outgrowth or increase the number of branch points [Fig.2A]. As observed for ITX3, incubation with K252a or ANA-12 did not alter the number of primary neurites or branch points under basal conditions [Fig.2A], suggesting that endogenous TrkB kinase activity is not required for basal neurite architecture development in primary hippocampal cultures during this early stage of development. Consistent with this conclusion, incubating primary hippocampal neurons in BDNF antibody or TrkB-Fc, to

remove endogenous secreted BDNF, did not affect the architecture of hippocampal neurites (Kellner et al., 2014). Since the response to exogenous BDNF was abolished in neurons lacking Kalirin or treated with an inhibitor of its first GEF domain [Fig.1], we concluded that this experimental system provides a valid means of evaluating the role of Kalirin GEF1/Rac1 in the response to BDNF.

Rac1 activation downstream of BDNF is thought to mediate process outgrowth in developing neurons (Huang and Reichardt, 2003; Miyamoto et al., 2006); we asked whether Kalirin contributes to BDNF-mediated activation of endogenous Rac1. Hippocampal cultures from WT or KalSR^{KO} neurons (Yan et al., 2015), were exposed to BDNF for 15 min. Activated, GTP-bound Rac1 was detected by its ability to bind to the CRIB domain of Pak (Benard et al., 1999). As expected, BDNF treatment led to a significant increase in Rac1 activation in WT neurons. In contrast, BDNF was unable to increase the amount of activated Rac1 in KalSR^{KO} neurons [Fig.2B]. The simplest interpretation of this experiment is that Kalirin, one of multiple Rac1 GEFs expressed in neurons, catalyzes the activation of a small fraction of the Rac1 in hippocampal neurons exposed to BDNF.

Normal dendritic morphogenesis requires that Rac1 signaling pathways be regulated precisely in time and space (Sainath and Gallo, 2015). To establish the effects of simply increasing the activation of Rac1 in our system, a constitutively active variant [Rac1(Q61L)] was expressed in hippocampal neurons [Fig.2C, upper]. For comparison, the isolated GEF1 domain of Kalirin, which is a more active Rac1 GEF than Kal7, was also expressed [Fig.2C, lower]. Expression of Rac1(Q61L) yielded neurons with massive lamellipodial growth cones while expression of Kal-GEF1 produced lamellipodial extensions from the cell soma; in neither case was there any sign of additional neurite formation. These results are consistent with previous work (Allen et al., 2000; Fournier et al., 2003; Luo et al., 1996): Rac1 is essential for neurite outgrowth, but constitutively active Rac1 does not produce more neurites. The spatiotemporal activation of Rac1 is essential for proper morphogenesis.

3.3 Expression of Kal7 promotes neurite formation and rescues the ability of KalSR^{KO} neurons to respond to BDNF

Kal9 and Kal12 are complex proteins; based on the ability of ITX3, which inhibits only the first GEF domain of Kalirin, to block the actions of BDNF, we hypothesized that Kal7, with its single GEF domain, might be capable of controlling primary neurite formation in rat hippocampal neurons. Whether assessed at DIV2 or DIV4, rat hippocampal neurons expressing exogenous Kal7 had more primary neurites than neurons expressing GFP [Fig. 3A]. To verify that Kal9 and Kal12 were the only isoforms functioning this early in development (Yan et al., 2015), we expressed a previously validated Kal7-specific shRNA (Ma et al., 2008) in rat hippocampal neurons and examined their response to BDNF [Fig. 3B]. As expected, the Kal7 shRNA did not alter basal dendritic morphology or the ability of the neurons to respond to BDNF [Fig.3B]. To control for any off-target effects of our Spec8 shRNA construct, we created a Kal7 rescue plasmid, in which the coding sequence was mutated to make the rescue protein resistant to the Spec8 shRNA. We expressed Kal7 rescue with Spec8 shRNA in rat neurons in the absence or presence of BDNF [Fig.3C]. Neurons expressing Kal7 rescue and Spec8 shRNA were responsive to BDNF. As predicted by our

pharmacological data [Fig.2] (Yan et al., 2015), the actions of exogenous Kal7 mimicked those of endogenous Kal9 and Kal12 in early development (Yan et al., 2015).

We next asked whether exogenous Kal7 (introduced at the time of plating) could rescue the ability of KalSR^{KO} neurons to respond to BDNF, or whether there are permanent deficits from developing for nearly 3 weeks in the absence of Kalirin. Kal7 was expressed in KalSR^{KO} neurons, which were exposed to BDNF (20 ng/ml) 24 hrs after transfection and plating; neuronal morphology was analyzed at DIV5 [Fig.3D]. As expected, KalSR^{KO} neurons did not respond to BDNF [Fig.3D and Fig.1C, D]; consistent with the ability of Kal7 to stimulate neurite formation in rat hippocampal neurons, KalSR^{KO} neurons expressing Kal7 showed an increase in the number of primary neurites. Most importantly, KalSR^{KO} neurons expressing exogenous Kal7 regained the ability to respond to BDNF administration by producing more primary neurites. While multiple Rho GEFs are expected to contribute to BDNF mediated process outgrowth and branching, it is clear that Kalirin plays a major role in this response. The fact that exogenous Kal7 is capable of replacing endogenous Kal9/Kal12 focused our attention on the N-terminal regions common to all three isoforms (Sec14, spectrin repeats, GEF1) [Fig.1A].

3.4 TrkB activation by exogenous BDNF *in vitro* is altered in the absence of *Kalrn*

Next we explored whether TrkB activation is altered in the absence of Kalirin. We used hippocampal cultures from WT or KalSR^{KO} mice at DIV7 to compare the ability of exogenous BDNF to activate TrkB [Fig.4A, B]. TrkB dimerizes and the intracellular subunits phosphorylate each other at Tyr⁵¹⁵ and Tyr^{705/706} after binding BDNF (Huang and Reichardt, 2003; Minichiello, 2009). The time course over which TrkB activation is maintained is known to reflect the activity of phosphatases as well as the endocytic trafficking of TrkB and the essential creation of signaling endosomes (Assaife-Lopes et al., 2014; Mitchell et al., 2012; Philippidou et al., 2011). Antisera specific for the phosphorylation of TrkB at Tyr⁵¹⁵ and Tyr^{705/706} were used to evaluate the TrkB activation time course using neurons that had not previously been exposed to exogenous BDNF. The TrkB phospho-Tyr⁵¹⁵ antibody used recognizes the corresponding residue in TrkA and TrkC (Cell Signaling #9141), but by far the major Trk receptor in the hippocampus at this time is TrkB (Cheung et al., 2007; French et al., 1999; Fryer et al., 1996). In WT neurons, exogenous BDNF induced maximum phosphorylation at both sites in 5–15 min; phosphorylation at both sites decreased by 60 min [Fig.4A, B], but remained significantly higher than baseline. The time course observed for TrkB activation in WT hippocampal neurons was consistent with published studies monitoring the same sites (Ji et al., 2010; Lai et al., 2012). Retardation of TrkB recycling (via Rab11-positive endosomes) is also seen in Slitr5 knockout neurons (Song et al., 2015).

TrkB phosphorylation (activation) at both sites was also increased in KalSR^{KO} neurons exposed to exogenous BDNF for 5 min and 15 min. In contrast to WT neurons, TrkB phosphorylation in KalSR^{KO} neurons had not begun to decline 60 min after exposure to exogenous BDNF [Fig.4A, B]. Similar results were obtained using the same approach to evaluate TrkB activation by exogenous BDNF in DIV7 cortical cultures prepared from WT and KalSR^{KO} mice (data not shown). Although continuous exposure of KalSR^{KO} neurons to

exogenous BDNF did not increase neurite outgrowth or branching, acute exposure to BDNF resulted in the prolonged activation of TrkB. Total TrkB levels did not differ in DIV7 cultures prepared from WT and KalSR^{KO} mice and maintained in medium lacking exogenous BDNF [Fig.4C]. These results demonstrated that the kinetics of TrkB phosphorylation/dephosphorylation are altered in neurons lacking Kalirin after exposure to BDNF.

We next determined whether Trk activation *in vivo* differs in WT and KalSR^{KO} mice at this developmental stage. Total TrkB levels did not differ in hippocampal or cortical tissue from postnatal day 7 WT vs. KalSR^{KO} mice [Fig.4D, E]. Our data indicate that Trk family phosphorylation (activation) at Tyr⁵¹⁵ does not differ in the hippocampus or cortex of WT vs. KalSR^{KO} mice at this age [Fig.4D, E].

3.5 Activation of TrkB targets is altered in KalSR^{KO} hippocampus and cortex

In response to BDNF, phosphorylated TrkB stimulates the MAPK/ERK, GSK-3 β /PI3K/Akt and PLC- γ /CaMK/CREB signaling pathways to control a wide variety of downstream responses (Chao, 2003; Minichiello, 2009). The time course over which these pathways, which respond to many inputs in addition to BDNF, are activated and inactivated is critical to the outcomes observed (Ji et al., 2010; Lai et al., 2012; Marshall, 1995; Park and Poo, 2013). Since our cell culture studies revealed a role for Kalirin in determining the time course over which TrkB is activated in response to BDNF [Fig.4], we reasoned that activation of its downstream targets might be altered at this developmental stage. To test this hypothesis, we used phospho-specific antibodies to evaluate activation of the ERK, Akt and CREB pathways (Chao, 2003; Huang and Reichardt, 2003; Minichiello, 2009) in tissue from WT and KalSR^{KO} mice. Levels of phospho-ERK normalized to total ERK were five-fold lower in KalSR^{KO} cortex and hippocampus at P7 than in WT tissue [Fig.5A, B]. A lesser, but still significant, decrease was observed in Akt activation (phospho-Akt/total Akt) and in CREB activation (phospho-CREB/total CREB) in KalSR^{KO} vs. WT cortex and hippocampus [Fig.5A, B]. While altered TrkB signaling would be expected to contribute to the diminished phosphorylation of ERK, CREB and Akt in KalSR^{KO} neurons, altered signaling by other receptor tyrosine kinases and other signaling pathways would also be expected to contribute.

3.6 Activation of TrkB targets is altered in KalSR^{KO} hippocampal and cortical neurons

To investigate whether BDNF/TrkB signaling is altered due to the absence of Kalirin, we compared the effect of BDNF on activation of ERK, CREB and Akt in hippocampal cultures prepared from WT and KalSR^{KO} mice. ERK, CREB and Akt phosphorylation was evaluated after neurons were treated with BDNF for 5, 15 and 60 min. The time course over which pERK, pCREB and pAkt levels rose and fell in response to BDNF in WT cultures was consistent with the literature (Ji et al., 2010; Lai et al., 2012). The time course for activation of ERK, CREB and Akt was significantly altered in KalSR^{KO} cultures [Fig.6A, B]. Peak activation of ERK in response to BDNF (pERK/ERK) was delayed in KalSR^{KO} neurons. The extent to which CREB and Akt were activated in response to BDNF (pCREB/CREB and pAkt/Akt) was diminished in KalSR^{KO} neurons. TrkB downstream signaling is clearly altered in neurons lacking Kalirin. In addition, at baseline (time 0, before exogenous BDNF

was added), ERK, CREB and Akt activation was lower in KalSR^{KO} neuronal cultures than in WT cultures [Fig.6B, right panel]; this result was consistent with our *in vivo* observations and may reflect altered signaling by multiple growth factors [Fig.5A, B]. TrkB activation kinetics were prolonged in KalSR^{KO} neurons [Fig.4]; altered TrkB dephosphorylation may contribute to the alterations observed in downstream target activation. Decreased activation of these common downstream targets would be expected to contribute to the reduced ability of exogenous BDNF to stimulate the formation and elongation of neurites in KalSR^{KO} neurons [Fig.1C, D].

3.7 Tiam PHn-CC-EX inhibits Kalirin GEF1 activation of Rac1

BDNF-stimulated neuronal morphogenesis is known to require Rac1 activation by Tiam1 (Lai et al., 2012; Miyamoto et al., 2006). The PHn-CC-EX region of Tiam1 blocks the ability of Tiam1 to bind to membranes, an essential step in its activation of Rac1 (Stam et al., 1997), blunts its GEF activity in COS-7 cells transfected with TrkB and treated with BDNF and interferes with BDNF-stimulated morphogenesis in neurons (Lai et al., 2012; Miyamoto et al., 2006). We first explored the possibility that this fragment of Tiam1 could be used to distinguish the actions of Tiam1 and Kalirin by expressing PHn-CC-EX alone or with Kal7 at the time of plating. Neuronal morphology was altered significantly by expression of PHn-CC-EX; lamellipodial-like structures appeared at the distal tips of many short, thin processes [Fig.7A]. Co-expression of PHn-CC-EX and Kal7 produced neurons with multiple short processes and lamellipodia [Fig.7B], a notably different morphology from expression of Kal7 alone [Fig.3A].

To try to understand these responses, we turned to a FRET-based bioassay for Rac1 activation in non-neuronal cells (Das et al., 2015a). PHn-CC-EX was expressed alone or with Kal-GEF1, Kal7 or Tiam1 C1199 (a more stable form of Tiam1) (Miyamoto et al., 2006; Stam et al., 1997) and a Rac1 biosensor. Expression of PHn-CC-EX alone had no effect on background FRET [Fig.7C]. As expected, PHn-CC-EX suppressed the ability of Tiam1 C1199 to activate Rac1 [Fig.7C, D]. Importantly, PHn-CC-EX also decreased the ability of Kal-GEF1 and Kal7 to activate Rac1 in a dose-dependent manner. As levels of PHn-CC-EX rose, levels of Kal7 and Tiam1 C1199 proteins fell. Kal7 and Tiam1 share the ability to interact with phosphoinositides and with EphB2, NMDA receptors and spinophilin (Kiraly et al., 2011; Miller et al., 2015; Terawaki et al., 2010). The ability of PHn-CC-EX to bind phosphoinositides and multiple proteins (Joshi et al., 2013; Stam et al., 1997; Terawaki et al., 2010) may account for its ability to disrupt Rac1 activation by Kalirin. Expression of PHn-CC-EX cannot be used to distinguish the effects of these two Rac1 GEFs.

4. Discussion

We have demonstrated an essential role for Kalirin in the ability of neurons to respond to BDNF. Neurite outgrowth is many steps removed from the initial interaction of BDNF with TrkB. Although we demonstrate a role for Kalirin in several intervening steps, we are far from understanding the pathway. Our observations provide compelling evidence that Kalirin, acting downstream of TrkB, is required early in development for neurite outgrowth and branching in response to exogenous BDNF. Kal GEF1 mediated Rac1 activation plays an

essential role in the ability of BDNF to stimulate neurite outgrowth, and Kal7, with its single GEF domain, can substitute for the longer isoforms, with their two GEF domains [Fig. 1, 2] (Yan et al., 2015). Kalirin contributes to activation of the ERK, Akt and CREB pathways *in vivo* and to the ability of hippocampal and cortical neurons to activate these pathways in response to exogenous BDNF *in vitro* [Fig. 5, 6]. Based on its phosphorylation state, TrkB is activated to a similar extent in tissue from wildtype and KalSR^{KO} tissue. However, the time course over which TrkB is activated and inactivated after exposure to BDNF differs in neurons lacking Kalirin [Fig. 4, 6]. The regulated endocytosis of many receptors, including TrkB, is crucial to normal cortical development (Song et al., 2015; Yap and Winckler, 2015). The role of the natural truncated variant TrkB.T1, which lacks the kinase domain and becomes the dominant TrkB protein in the adult (Fenner, 2012), has not been investigated here, nor have the changes in NMDA signaling that occur within 2 minutes of TrkB activation by BDNF (Levine et al., 1998).

4.1 TrkB signaling in the absence of Kalirin

As described previously, neurons lacking Kal9/Kal12 are unable to produce the normal number of neurites and branch points when maintained in culture (Yan et al., 2015). The current studies used the same approach to elucidate the role of Kalirin in neurite and branch point formation in response to exogenous BDNF. Neither the level of TrkB nor the phosphorylation of TrkB at Tyr⁵¹⁵ differed in KalSR^{KO} cortex or hippocampus at postnatal day 7 [Fig.4D, E]. The phosphorylation of TrkB may be different in KalSR^{KO} tissues in older animals, because at postnatal day 7 the brain is still developing. However, activation of three downstream targets of BDNF/TrkB signaling (P-ERK, P-CREB and P-Akt) was diminished in the KalSR^{KO} cortex and hippocampus [Fig.5A, B]. Whether altered *in vivo* TrkB signaling in the absence of Kalirin is the direct cause of these changes is not yet clear. Baseline activation of ERK, CREB and Akt in KalSR^{KO} neurons was diminished compared to wildtype neurons, and the ability of BDNF to activate Akt and CREB in KalSR^{KO} neurons was suppressed [Fig.6A, B]. Altered ERK, Akt and CREB activation would be expected to contribute to the blunted morphological effects of BDNF seen in the absence of Kalirin [Fig.1], but the exact molecular signaling pathway is not clear at present.

TrkB is rapidly activated by BDNF, even in the absence of Kalirin. The binding of BDNF to full length TrkB induces trans-phosphorylation of specific Tyr residues (Chao, 2003; Segal and Greenberg, 1996), a key step for activation of downstream signaling pathways (Huang and Reichardt, 2001; Minichiello, 2009). Increased phosphorylation of TrkB at Tyr⁵¹⁵ and Tyr^{705/706} was observed within 5 min in neurons expressing or lacking Kal9/Kal12. We documented a striking prolongation in the time course of TrkB phosphorylation at Tyr⁵¹⁵ and Tyr^{705/706} in KalSR^{KO} neurons [Fig.4A, B]. The effects of BDNF/TrkB on the activation of key downstream targets is dependent on sustained signaling from endosomes after internalization of BDNF/TrkB (Cohen et al., 2011; Lazo et al., 2013; Ouyang et al., 2013); normal signaling can be disrupted by prolonged phosphorylation or lack of internalization of TrkB (Assaife-Lopes et al., 2014; Ji et al., 2010; Mitchell et al., 2012). The prolonged time course of TrkB dephosphorylation in the absence of Kal9/Kal12 could reflect changes in endocytic trafficking (Ma et al., 2014; Schiller et al., 2008) or the phosphatases inactivating TrkB. Altered levels of cell surface NR2B in Kal7^{KO} neurons as

well as *in vitro* studies have suggested a role for Kalirin in endocytic trafficking (Kiraly et al., 2011; Schiller et al., 2008).

Several tyrosine phosphatases (LAR, MKP-1 and PTPN12) play an essential role in BDNF/TrkB dependent neurite outgrowth and branching (Ambjorn et al., 2013; Jeanneteau et al., 2010; Yang et al., 2006). Receptor protein tyrosine phosphatase type O (PTPRO) and protein tyrosine phosphatase σ (PTP σ) both dephosphorylate TrkB, providing a negative modulatory effect on the ability of BDNF to stimulate neurite outgrowth and spine morphogenesis (Faux et al., 2007; Gatto et al., 2013; Kurihara and Yamashita, 2012). Like KalSR^{KO} neurons, neurons lacking Slitrk5, a PTP σ interactor, are unable to respond to BDNF (Song et al., 2015). Levels of these phosphatases are controlled by BDNF (and hence also surface TrkB) and affect multiple signaling pathways, such as MAPK, Akt and PLC. The molecular features that determine phosphatase substrate specificity and their effects on TrkB signaling *in vivo* are not yet fully understood.

4.2 Roles for Multiple Rho GEFs and neurotrophins in neurite outgrowth

Activation of Rho GTPases by neurotrophins is known to play a role in the establishment of dendritic and axonal morphology. Both Rac1 and RhoA are involved in BDNF stimulated dendritic growth (Cheung et al., 2007). Dual Rho GEFs like Kal9 and Kal12 could play a unique role in coordinating the activation of Rac1/RhoG and RhoA, which signal to distinctly different effectors (May et al., 2002; Penzes et al., 2001). Recent studies have demonstrated important roles for additional Dbl family members, PDZ-GEF1, and Dock3, a non-Dbl Rho GEF, in the actin rearrangement and microtubule assembly changes needed for neurite outgrowth downstream of BDNF (Hisata et al., 2007; Namekata et al., 2012). The distinctive features of the different Rho GEFs that link neurotrophin receptors to activation of Rho GTPases may be needed to confer cell type and developmental specificity to the responses. Although Rac1 is essential for proper dendritic morphology, it is clear that expression of constitutively active Rac1 or exogenous KalGEF1 fails to stimulate normal dendritic development [Fig.2C], while expression of dominant negative Rac1 reduces dendritic complexity (Allen et al., 2000; Fournier et al., 2003; Luo et al., 1996). Normal neurite initiation clearly requires spatiotemporal coordination of Rho GTPase activation and inactivation by multiple GEFs and GAPs (Das et al., 2015b; Galic et al., 2014).

Tiam1 is clearly required for the effects of BDNF on dendritic arborization (Miyamoto et al., 2006; Tolia et al., 2007). TrkB both binds to and phosphorylates Tiam1; strikingly, mutation of the Tyr residue in Tiam1 that is phosphorylated by TrkB eliminates the ability of BDNF to affect dendritic morphology. We tried to use the PHn-CC-EX fragment of Tiam1 to distinguish the roles of Tiam1 and Kalirin in BDNF-dependent neurite formation [Fig.7]. The subcellular localization of Tiam1 and Kalirin, which reflects their interaction with phosphoinositides and with multiple different proteins, governs their access to Rho GTPases. Our cell based assay for Rac1 activation demonstrated that PHn-CC-EX diminished the ability of both Tiam and Kalirin to activate Rac1, precluding its use as a tool to distinguish their roles. This result is consistent with the crystal and solution structures of PHn-CC-EX (Joshi et al., 2013; Subramanian et al., 2013; Terawaki et al., 2010), which identified

binding sites for phosphoinositides and multiple protein partners shared by these two Rho GEFs.

Earlier studies in PC12 cells demonstrated a role for Kalirin in the ability of NGF/TrkA to promote neurite formation (Chakrabarti et al., 2005). Signaling by other receptor tyrosine kinases (EphB2, ErbB4, PDGFR- β) has also been shown to involve Kalirin (Penzes et al., 2003; Wu et al., 2013). The ability of BDNF, acting through TrkB, to promote neurite outgrowth in early development requires Tiam1 (Tolias et al., 2007; Um et al., 2014), Slitrk5 (Song et al., 2015) and Kalirin. A direct interaction between TrkB and Tiam1 and the essential phosphorylation of a single Tyr in Tiam1 by TrkB place Tiam1 early in this signaling pathway. Although Tiam1 has a PDZ domain and Kal7 terminates with a PDZ binding motif, this type of direct interaction is unlikely to play an important role; Kal9 and Kal12, the isoforms expressed early in development, do not have a PDZ binding motif. In addition, the sequence of the Kal7 PDZ binding motif (–STYV) does not match the established specificity of the PDZ domain in Tiam1 (Shepherd et al., 2011). Additional studies are needed to define the roles of the many other proteins that play essential roles in the complex process of dendrite formation and branching.

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1. Kalirin is required for BDNF-stimulated dendritic outgrowth and branching
2. BDNF/TrkB activation of Rac1 in neurons is largely mediated by Kalirin
3. The Tiam1 fragment PHn-CC-EX blocks Tiam1 and Kalirin Rac1 activation
4. Kalirin isoforms lacking the second GEF domain can rescue BDNF responsiveness
5. Kalirin affects the time course of BDNF/TrkB pathway activation in neurons

Fig 1. Exogenous BDNF mediated neurite outgrowth requires *Kalrn*

A. Top - diagram showing major Kalirin isoforms and site targeted by Spec8 shRNA. Rat hippocampal neurons transfected at the time of plating with control or Spec8 shRNA were maintained in the absence or presence of 50 ng/ml BDNF from the time of plating until they were fixed at DIV4. Morphological measurements of transfected neurons used DsRed; representative neurons are shown. Scale bar, 50 μ m. **B.** The concentration of BDNF added to the culture medium was varied from 10 to 50 ng/ml; cultures were fixed at DIV2 or DIV4 for quantification of primary neurites and branch points. Data shown are for DIV4; 10 ng/ml

BDNF gave a maximal response. Spec8 shRNA had the expected effect on neurite number and branch points. $**p<0.01$ between Con and Spec8 shRNAs. **C.** Hippocampal neurons from P0 WT or KalSR^{KO} animals were maintained in culture medium lacking or containing 50 ng/ml BDNF from 24 hr until DIV5; fixed cells were stained with antibody to MAP2 for quantification. Representative cells are shown. Scale bar, 50 μ m. **D.** Quantification of effect of exogenous BDNF on number of primary neurites and branch points at DIV5. Exogenous BDNF failed to enhance neurite outgrowth or branching in KalSR^{KO} neurons. $**p<0.01$. $##p<0.05$ between WT and KalSR^{KO} groups. Three separate experiments were performed using rat hippocampal neurons; two separate experiments were performed using mouse hippocampal neurons. For each experiment, 12–18 neurons per group were analyzed. **E.** HEK-293 (PEAK) cells were transfected with the four indicated myc-tagged Kalirin plasmids plus two amounts of Spec8 shRNA vector, and harvested 24 h later. Western was developed using myc and GAPDH antibodies. The experiment was repeated 5 separate times with similar results.

Fig 2. Kalirin GEF1 mediated Rac1 activation is required for BDNF effects

A. Rat hippocampal neurons were grown in the absence (Control) or presence of exogenous BDNF until DIV4. Before the addition of BDNF, neurons were pre-incubated for 1 h with DMSO (diluted 2500-fold), K252a (100 nM), ANA-12 (10 μ M) or ITX-3 (100 μ M). Primary neurites and branch points were quantified as described above; data were compared by 2-way ANOVA using drug and BDNF as independent variables. ** $p < 0.001$; * $p < 0.05$. **B.** BDNF (50 ng/ml for 15 min) activated endogenous Rac1 in WT hippocampal cultures at DIV21 but was unable to activate Rac1 in KalSR^{KO} neurons. Four separate experiments were performed; data were compared by 2-way ANOVA using genotype and BDNF as independent variables. ** $p < 0.001$. **C.** Examples of DIV4 hippocampal neurons transfected at plating with GFP-Rac1 Q61L or GFP-Kal GEF1 and stained for MAP2 (Yan et al., 2015). Lamellipodial extensions were indicated by arrows. Non-transfected neurons were indicated by arrowheads. Scale bar: 20 μ m.

Fig 3. Kal7 rescues BDNF responsiveness

A. Representative images of myc-tagged Kal7 transfected DIV4 neurons. Kal7 was electroporated into rat hippocampal neurons during plating; neurons were fixed at DIV4 and Kal7 was visualized using a myc monoclonal antibody (Ma et al., 2014). Scale bar: 20 μ m. Quantification of primary neurite number in GFP- and Kal7-expressing rat neurons at DIV2 and DIV4; GFP-transfected neurons served as the control. Three separate experiments were performed using rat hippocampal neurons. Between 8 and 11 neurons per group per experiment were analyzed. **B.** Examples of Kal7 shRNA transfected rat hippocampal neurons at DIV4; 20 ng/ml BDNF was added to the medium 24h after plating and was not removed. 13–14 neurons per group per experiment were analyzed. Scale bar: 20 μ m. $**p < 0.001$. Four separate experiments were performed. **C.** Examples of Kal7-rescue and Spec8 co-transfected rat hippocampal neurons at DIV4. 20 ng/ml BDNF was added to the medium 24 h after plating and was not removed. Scale bar: 20 μ m. Three separate experiments were performed. **D.** Representative images of Kal7 transfected KalSR^{KO} neurons at DIV5; 20 ng/ml BDNF was added to the medium 24 hrs after plating and was not removed. Scale bar: 20 μ m. Quantification of primary neurite number at DIV5 in the presence/absence of BDNF. Two separate experiments were performed using KalSR^{KO} hippocampal neurons. Between 12 and 18 neurons per group per experiment were analyzed; data were compared by 2-way ANOVA using genotype and BDNF as independent variables. $**p < 0.001$.

Fig 4. BDNF activation of TrkB is altered in KalSR^{KO} neurons

A. Time course for exogenous BDNF treatment of DIV7 cultures from WT or KalSR^{KO} hippocampi; pTrk515, pTrk705/706, TrkB and βIII tubulin were visualized in lysates prepared after the indicated treatment times. The pTrk515 and 705/706 antibodies crossreacts with TrkA, which are quantitatively minor compared to TrkB, so the blots are labeled Trk515 and Trk705/706. **B.** The signal for phosphorylated Trk or TrkB was normalized to the total TrkB signal at each time point; the WT ratio for the 5 min sample was set to 100%. \$\$ $p=0.011$; ## $p=0.025$; 2-way ANOVA analysis was performed using time and genotype as independent variables, phosphorylation level as the dependent variable. Six separate experiments were performed for the TrkB activation time course. **C.** TrkB levels in WT and KalSR^{KO} cultures were normalized to βIII-tubulin levels; no significant difference in expression level was observed. **D.** Cortices and hippocampi from P7 wildtype and KalSR^{KO} mice were blotted for pTrk515 and total TrkB. **E.** Quantification of the ratio of pTrk515 to total TrkB. Quantification of TrkB to βIII tubulin ratio; no significant difference in expression level was observed. Six WT animals and 8 KalSR^{KO} animals were analyzed.

Fig 5. Activation of downstream targets of TrkB differs in KalSR^{KO} tissue

A. Activation of ERK, CREB and Akt in the same P7 wildtype and KalSR^{KO} mouse tissue samples analyzed in Fig.4D was evaluated by Western blot analysis using the indicated antisera. Representative blots are shown (n = 7 per genotype). **B.** Quantification of phosphorylated protein to total protein; ratios for WT were set to 1.00. ** $p < 0.01$; * $p < 0.05$.

Fig 6. Activation of downstream targets of TrkB differs in KalSR^{KO} neuronal cultures
A. Time course for exogenous BDNF treatment of DIV7 cultures prepared from WT or KalSR^{KO} hippocampi; pERK, ERK, pAkt, Akt, pCREB, and CREB were visualized in lysates prepared after the indicated treatment times. **B.** The signal for phosphorylated protein was normalized to the total protein signal at each time point; the WT ratio for the highest value was set to 100%. ## $p < 0.03$, * $p < 0.05$; 2-way ANOVA was performed using time and genotype as independent variables, with phosphorylation level as the dependent variable. **C.** Quantification of phosphorylated protein to total protein before BDNF was added (time 0);

ratios for WT were set to 1.00. ** $p<0.01$; * $p<0.05$. Six independent cultures were analyzed (n=3 per genotype) for BDNF time course experiments.

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Fig 7. Evaluation of PHn-CC-EX effects

Examples of rat hippocampal neurons transfected at the time of plating with PHn-CC-EX alone (**A**) or with PHn-CC-EX and Kal7 (**B**) and fixed at DIV4. PHn-CC-EX was visualized using a FLAG antibody and Kal7 with a myc antibody; β III tubulin staining (**A**) and MAP2 staining (**B**) were used to demonstrate the neuronal identity of the cells. Scale bar: 20 μ m. Three separate experiments were performed; arrowheads indicate lamellipodial extensions. **C** and **D**. Rac1 activation was assessed using HEK-293 (pEAK) cells transfected in a glass-bottom 24-well plate in duplicate. All wells except the non-transfected received 0.1 μ g of Rac1-biosensor vector (Das et al., 2015b). Vector amounts were adjusted to maintain cell viability: 0.015 μ g pEAK-kGEF1, 0.3 μ g pEAK-aKal7, 0.3 μ g C1199 and 0.05 and 0.3 μ g PHn-CC-EX. The total amount of vector per well was made up to 0.6 μ g DNA using empty pEAK10 vector. DNA was mixed with 0.9 μ l TransIT-2020 and spectra were obtained 30 h after transfection (Das et al., 2015b). **C**. Cells were extracted by boiling into SDS lysis buffer (Yan et al., 2015), and 1/10 of each sample was analyzed by SDS-PAGE. PVDF membranes were cut as indicated to enable detection of kGEF1 and Kal7 (myc epitope tagged) and C1199 and PHn-CC-EX (FLAG tagged) on the same membrane (**D**). **C** and **D** are representative of three experiments with hEK-293 cells and two experiments with COS-7 cells, all with similar results.