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Ubiquitin Regulation of Trk Receptor Trafficking and Degradation

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Abstract

The regulation of Trk receptors is critical for orchestrating multiple signalling pathways required for developing and maintaining neuronal networks. Activation of Trk receptors results in signalling, internalisation and subsequent degradation of the protein. Although ubiquitination of TrkA by Nedd4-2 has been identified as an important degradation pathway, much less is known about the pathways regulating the degradation of TrkB and TrkC. Critical to the interaction between TrkA and Nedd4-2 is a PPxY motif present within TrkA but absent in TrkB and TrkC. Given the absence of this interaction motif, it remains to be determined how TrkB and TrkC are ubiquitinated. Here we report that the adaptor protein Ndfip1 can interact with all three Trk receptors and show for TrkB the recruitment of Nedd4-2 through PPxY motifs present in Ndfip1. Ndfip1 mediates the ubiquitination of TrkB, resulting in receptor trafficking predominantly on Rab7 containing late endosomes, highlighting a pathway for TrkB degradation at the lysosome. In vitro, overexpression of Ndfip1 increased TrkB ubiquitination and decreased viability of BDNF-dependent primary neurons. In vivo, conditional genetic deletion of *Ndfip1* increased TrkB in the brain and resulted in enlargement of the granular cell layer of the dentate gyrus.

Keywords Ndfip1 · Neurotrophins · Ubiquitin · Rab · Dentate gyrus · TrkB

Introduction

The Trk family of receptor tyrosine kinases is known to regulate many aspects of neuronal development and function, including neuronal survival, differentiation and synapse formation [1]. The Trk family consists of three receptors which are activated by distinct neurotrophins; TrkA is the preferential receptor for nerve growth factor, TrkB for brain-derived neurotrophic factor and neurotrophin 4, and TrkC for neurotrophin 3 [1]. The classic view of neurotrophin signalling holds that the neurotrophins are synthesised and released by target tissues that act on innervating neurons to promote their survival and differentiation. Neurotrophin binding promotes Trk receptor dimerization and the activation of the intracellular tyrosine kinase domain; this results in the autotransphosphorylation of several tyrosine residues for activation of downstream signalling [2].

The activated receptors are subsequently endocytosed and ubiquitinated, allowing for either targeted degradation or retrograde transport on endosomes through the axon to the cell body. This transport of Trk receptors allows for trafficking to distinct compartments within the cell, resulting in differential activation of signalling pathways that are highly contextual and specific to each compartment [3–5]. Therefore, deciphering the mechanisms of Trk receptor ubiquitination that are involved in endocytosis, trafficking and degradation is of crucial relevance for understanding the regulation of signalling cascades initiated by neurotrophin signalling.

All Trk receptors are known to undergo ubiquitination [6], but our understanding of the influence that ubiquitination exerts upon neurotrophin receptors is currently limited to TrkA [7–9]. Both in vitro and in vivo studies have demonstrated that TrkA, but not TrkB or TrkC, can be recognised by the E3 ligase Nedd4-2 [10], resulting in ubiquitination and degradation of the receptor. Nedd4-2 specifically interacts with TrkA through a PPxY motif on the receptor that is not present on TrkB or TrkC receptors. As such, the mechanism required for the ubiquitination of both TrkB and TrkC remains to be determined. Here we report that TrkA, TrkB and TrkC can interact

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with Ndfip1, an adaptor protein for the Nedd4 family of ubiquitin ligases, and identify that for TrkB this interaction results in ubiquitination and endosomal transport of the receptor for degradation.

Results

Trk Receptors Interact with Ndfip1

TrkA has previously been shown to interact with and be ubiquitinated by Nedd4-2 [10], a member of the Nedd4 family of ubiquitin ligases. However, both TrkB and TrkC do not contain the PPxY motif required to bind to the WW domains of Nedd4 proteins, suggesting that these receptors may be ubiquitinated through a different mechanism. Ndfip1 is an adaptor protein that mediates the ubiquitination of proteins by the Nedd4 family of ubiquitin ligases [11]. Ndfip1 recognises target proteins and recruits and activates Nedd4 members through PPxY motifs, allowing for the subsequent ubiquitination of target substrates [12–14]. Given that both TrkB and TrkC do not contain PPxY motifs, we investigated whether Ndfip1 could interact with these Trk receptors. Immunoprecipitation assays were conducted in HEK-293T cells overexpressing each Trk receptor with Ndfip1. We observed that all three Trk receptors interact with Ndfip1 (Fig. 1a).

As Ndfip1 is an endosomal-bound protein, the N terminus is exposed to the cytosol, whereas the C terminus is exposed to the lumen of endosomes [15]. To determine the orientation for the interaction between TrkB and Ndfip1 in the cell, we performed bimolecular fluorescence complementation (BiFC) assays [15]. Fusion proteins were created using TrkB and a fragment of Venus fluorescent protein attached to the C terminus (TrkB-VC), as well as Ndfip1 with a complementary fragment of Venus attached to either the N terminus (VN-Ndfip1) or the C terminus (Ndfip1-VN). A fluorescent signal was observed with expression of TrkB-VC with VN-Ndfip1 (Fig. 1b), but not with Ndfip1-VN (Fig. 1c), indicating that the two proteins were orientated with the N terminus of Ndfip1 in close proximity to the C terminus of TrkB in the cytosol of the cell.

To further investigate the protein interaction between Ndfip1 and TrkB, we deleted regions or mutated conserved residues in Ndfip1 and performed immunoprecipitation studies (Fig. 1d). Deletion of the final 18 residues at the C-terminal region of Ndfip1 (ΔTM3) was observed to limit the interaction of Ndfip1 with TrkB (Fig. 1d, lane 2). Altering the charge of the highly conserved arginine residue 146 in the first luminal loop region of Ndfip1 to glutamic acid resulted in a total loss of interaction between Ndfip1 and TrkB (Fig. 1d, lane 3). Conversely, mutation of the conserved Ndfip1 residue tyrosine 199 to alanine did not result in alteration of the interaction between Ndfip1 and TrkB (Fig. 1d, lane 4). Combined, these results indicate that there are critical interactions between the first luminal region and the C terminus of Ndfip1 that are required for binding TrkB.

Ndfip1 Mediates the Ubiquitination of TrkB

The above results identify that Ndfip1 interacts with TrkB receptors; next we investigated if this interaction could result in the ubiquitination of TrkB. We performed denaturing ubiquitination assays with TrkB in the presence of Ndfip1. Expression of TrkB alone resulted in limited ubiquitination of TrkB when expressed in HEK293 cells (which contain endogenous Ndfip1) (Fig. 2a, lane 1). In contrast, overexpression of Ndfip1 together with TrkB resulted in both mono- and poly-ubiquitination of TrkB (Fig. 2a, lane 3).

To determine which Nedd4 ubiquitin ligase was used for the ubiquitination of TrkB, we performed denaturing ubiquitin assays with both Nedd4-1 and Nedd4-2 in the presence of Ndfip1. We observed a higher abundance of ubiquitinated TrkB when Nedd4-2 was co-expressed with Ndfip1, compared with co-expression with Nedd4-1 (Fig. 2b, compare lane 2 with lane 3). These results indicate that Nedd4-2 is the preferred Nedd4 ligase for Ndfip1-mediated ubiquitination of TrkB. It should be noted that Nedd4-2 has also been found to be the preferred Nedd4 ligase for TrkA [10].

Next we investigated whether Ndfip1-mediated ubiquitination of TrkB was present in neurons. Using RNAi [12], we knocked down Ndfip1 in mouse cortical primary neurons and probed for both the abundance and ubiquitination of TrkB. Knockdown of Ndfip1 resulted in the loss of TrkB ubiquitination (Fig. 2c, top panel) and an increase in the abundance of the receptor (Fig. 2c, bottom panel). These findings support our overexpression studies that showed increased Ndfip1 expression results in ubiquitination of TrkB and the reduction in the abundance of the receptor. To determine whether Ndfip1-mediated ubiquitination of TrkB affects its turnover in vivo, we investigated TrkB abundance in embryonic mouse brains with and without Ndfip1. *Ndfip1*$^{flox/flox}$ mice were crossed with *Nestin-Cre* (*Ndfip1*$^{flox/flox}$; *Nestin-Cre*) to delete Ndfip1 from the majority of neurons in the brain. Western blotting for TrkB from the embryonic cortex showed that genetic deletion of *Ndfip1* resulted in the increased abundance of TrkB (Fig. 2d).

Ubiquitinated TrkB Is Trafficked on Early and Late Endosomal Pathways

A consequence of ubiquitination is the trafficking of the targeted protein to different compartments of the cell. To investigate the cellular location of ubiquitinated TrkB, we performed BiFC assays using labelled TrkB and ubiquitin. TrkB-VC and ubiquitin conjugated to a complementary fragment of Venus attached to the N terminus (VN-ubiquitin) were transfected into cells.

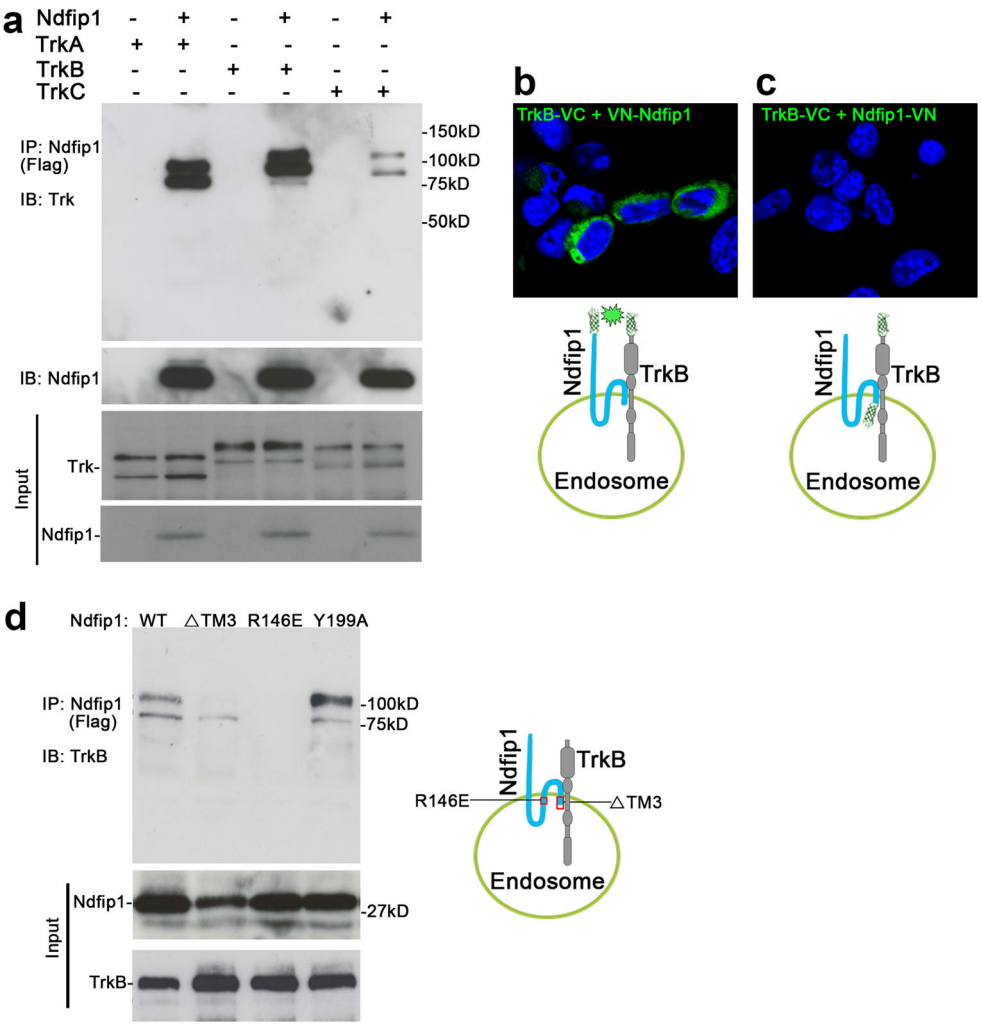

Fig. 1 Ndfip1 interacts with Trk receptors. **a** Immunoprecipitation of Ndfip1-Flag results in the co-precipitation of TrkA, TrkB and TrkC when expressed in HEK-293T cells. **b** BiFC was used to visualise the orientation for the interaction between Ndfip1 and TrkB; the N-terminal fragment of Venus was placed on either the cytoplasmic or luminal side of Ndfip1. A BiFC signal representing an interaction between Ndfip1 and TrkB was observed when the Venus fragment was placed on the cytoplasmic side of Ndfip1. **c** In contrast, no fluorescent signal was observed when the Venus fragment was placed on the luminal side of Ndfip1. **d** Immunoprecipitation experiments showed that removal of the C-terminal region of Ndfip1 (ΔTM3) resulted in a decrease in the interaction with TrkB. Mutation of the highly conserved residue arginine 146 to glutamic acid (R146E) in Ndfip1 resulted in a loss of binding to TrkB

Confocal imaging showed ubiquitinated TrkB was localised to all major endosomal pathways in the cell, including early endosomes (Rab5), late endosomes (Rab7 and 9) and recycling endosomes (Rab11) (Fig. 3a). Quantification of the localisation of ubiquitinated TrkB in the cell showed a significant increase in ubiquitinated TrkB in both Rab7 and Rab9 endosomes compared to Rab5 endosomes (Fig. 3b). These results indicate that late endosomes represent the major trafficking vesicles for ubiquitinated TrkB, a pathway known to result in the lysosomal degradation of proteins. Together our results suggest that approximately 70% of the ubiquitinated TrkB (as labelled with BiFC) in the cell were found on Rab5, 7, 9 or 11 endosomes (Fig. 3b). This highlights that ubiquitination of TrkB is a major protein modification step for cellular trafficking of the receptor.

Ndfip1 Abundance Regulates the Survival of BDNF-Dependent Neurons

To investigate whether Ndfip1 abundance alters the survival of neurons in a TrkB-dependent manner, we performed assays using an inducible Ndfip1 expression system. Mouse cortical primary neurons were cultured in two separate growth conditions, (i) control neurobasal media containing B27, a serum-free growth supplement or (ii) neurobasal media containing BDNF, a neurotrophin that signals through TrkB and can support neuronal growth. Initially, to determine if the BDNF growth conditions resulted in neurons that were BDNF-dependent for survival, we removed BDNF from the media and performed cell survival assays. We found a 50% reduction in neuronal survival 72 h after BDNF removal, indicating that the neurons were dependent on the BDNF/TrkB pathway for survival (Fig. 4a). In separate assays, neurons cultured in either B27 or BDNF-dependent conditions were infected with a lentivirus containing an inducible Ndfip1 construct under the control of 4-hydroxy tamoxifen (4HT) [12] (Fig. 4b). To determine if Ndfip1 expression reduces the survival of primary neurons, we induced Ndfip1 expression with 4HT in both the B27 and BDNF-dependent cultures and assayed for cell survival after 72 h. Control neurons (both

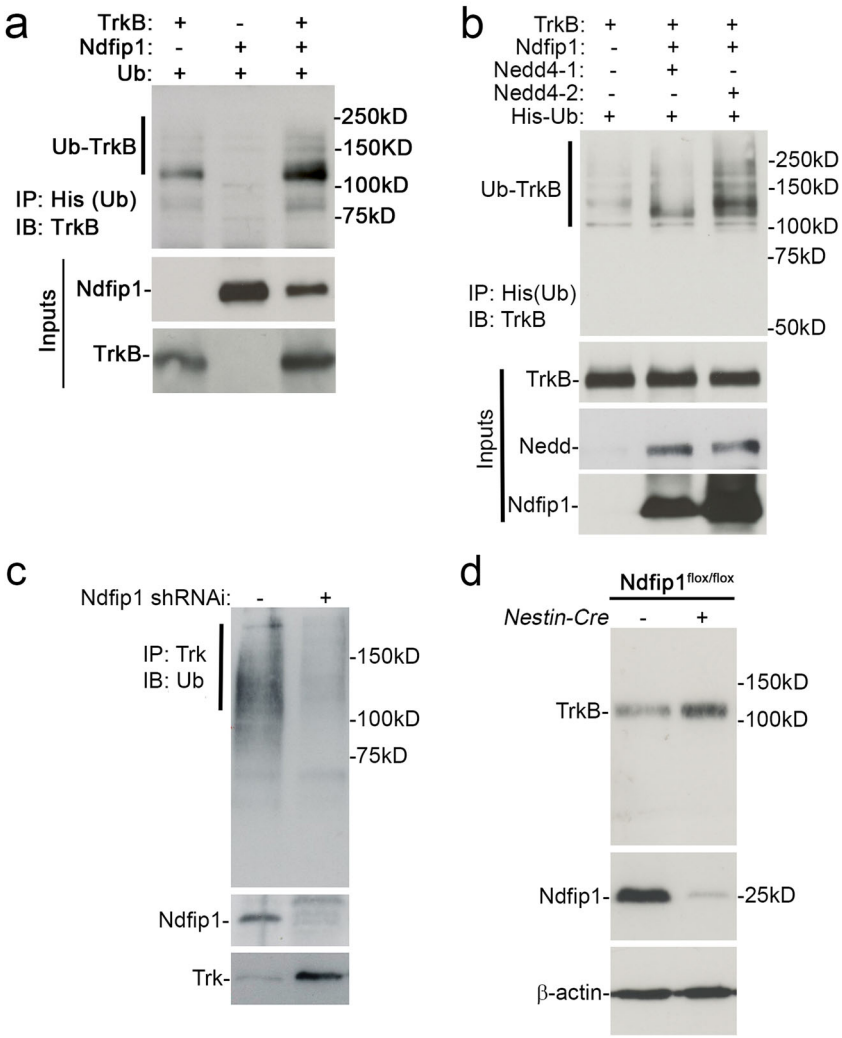

Fig. 2 Ndfip1 mediates the ubiquitination of TrkB. **a** Denaturing ubiquitination assays for TrkB show an increased amount of TrkB ubiquitination when Ndfip1 is co-expressed (lane 3). **b** Denaturing ubiquitination assays show increased ubiquitinated TrkB when co-expressed with Ndfip1 and Nedd4-2 (lane 3) compared to co-expression of Ndfip1 and Nedd4-1 (lane 2). **c** Knockdown of Ndfip1 using RNAi resulted in decreased ubiquitination of TrkB in primary cortical neurons. **d** TrkB abundance is increased in the cortex of E15.5 *Ndfip1*$^{flox/flox}$; *Nestin Cre* embryos compared to *Ndfip1*$^{flox/flox}$ embryos

B27 and BDNF-dependent) without 4HT induction or without lentiviral infection were also assayed for neuronal survival. No significant decrease in neuronal survival was observed in B27-dependent neurons after 4HT-induced expression of Ndfip1 (Fig. 4c). In contrast, BDNF-dependent neurons showed a significant decrease in neuronal survival after 4HT-induced expression of Ndfip1 when compared to control neurons without 4HT induction (Fig. 4d). These results indicate that increased expression of Ndfip1 renders BDNF-dependent neurons susceptible to death, suggesting a loss of TrkB receptors on these neurons that are required for BDNF signalling.

Ndfip1 Abundance Alters Granular Cell Layer Size in the Dentate Gyrus

TrkB has previously been shown to play a cell-autonomous role in hippocampal neurogenesis, with a loss of TrkB resulting in decreased neurogenesis and a reduction in the granular cell layer of the dentate gyrus [16]. So far, our experiments have shown that Ndfip1 mediates the ubiquitination of TrkB, resulting in a reduction of the receptor in the cell. Therefore, we investigated whether deletion of Ndfip1 in the mouse brain results in increased TrkB and alterations in dentate gyrus size. Measurement of the granular cell layer of the dentate gyrus in of both *Ndfip1*$^{flox/flox}$*;Nestin-Cre* and wild-type littermate control mice was conducted at a number of postnatal stages. We observed that from postnatal day 10 (P10), the granular cell layer of *Ndfip1*$^{flox/flox}$*;Nestin-Cre* mice exhibited a significant increase in size compared to wild-type littermate controls, and this difference was maintained into adulthood (Fig. 5a–c). The enlargement of the granular cell layer after deletion of *Ndfip1* was due to an increased cell number and not cell size (Fig. 5d). Immunostaining and Western blotting for TrkB in the dentate gyrus of P10 mice indicated increased TrkB in *Ndfip1*$^{flox/flox}$*;Nestin-Cre mice* compared to wild-type littermate controls (Fig. 5e, f). Together, our

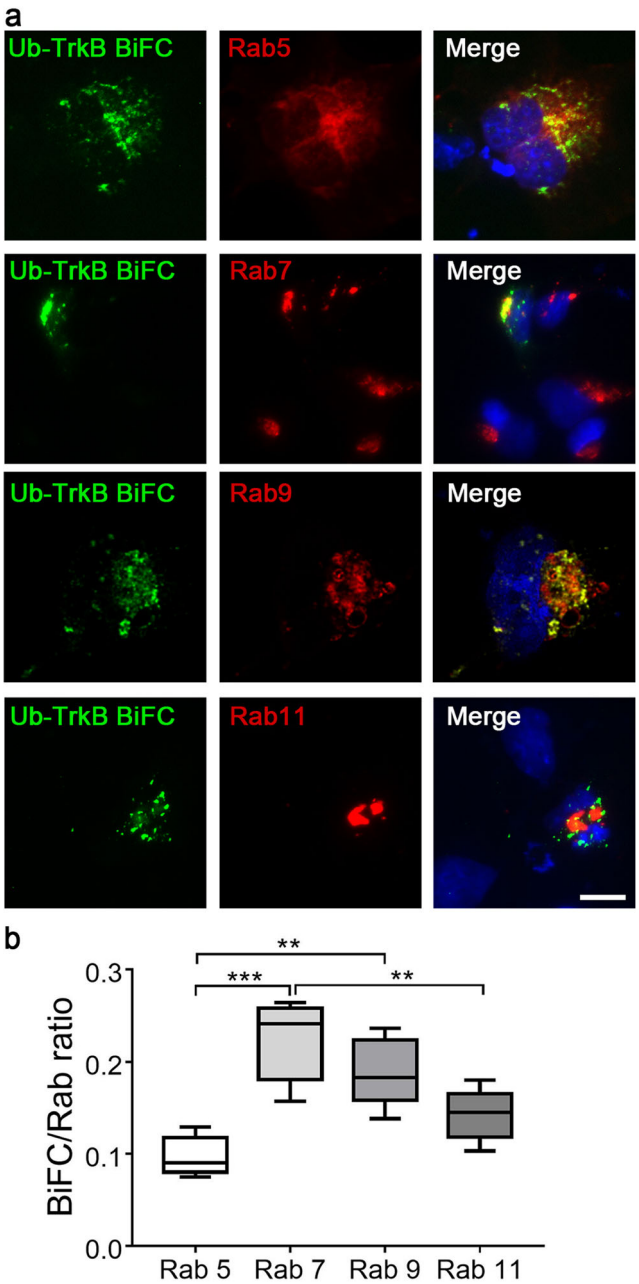

Fig. 3 Ubiquitinated TrkB is trafficked on endosomal pathways in the cell. **a** BiFC was used to visualise the transport of ubiquitinated TrkB in combination with Rab-GTPases used as endosomal markers. Ubiquitinated TrkB was found on both early (Rab5 positive), late (Rab7 and 9 positive) and recycling endosomes (Rab11 positive). **b** To quantify the colocalisation of ubiquitinated TrkB with specific Rabs, Manders coefficients were calculated between ubiquitinated TrkB and each of the Rab proteins following image deconvolution. Manders analysis revealed ubiquitinated TrkB was significantly increased in late endosomal pathways (Rab7 and 9) that are directed towards the lysosome. $N = 5$ for each Rab analysed (**$P < 0.01$ and ***$P < 0.001$, ± SEM). Scale bar, 10 μm

data suggests that loss of Ndfip1 leads to increased TrkB in the dentate gyrus resulting in the enlargement of the granular cell layer.

Discussion

Understanding the ubiquitin-mediated regulation of neurotrophin receptor trafficking and degradation is critical for understanding neuronal architecture and signalling. Here we identify Ndfip1 as an important component for the ubiquitination of TrkB receptors. Ndfip1 is known to have dual functions in both recruiting target proteins for ubiquitination and activating members of the Nedd4 ligase family [17]. Ndfip1 can function with a number of Nedd4 family ubiquitin ligases; however, in the brain, Ndfip1 has been found to predominantly interact with Nedd4-2 [18], which has been identified as a ubiquitin ligase for TrkA degradation. Here we also find that Ndfip1-mediated ubiquitination of TrkB predominantly requires Nedd4-2. Importantly, Ndfip1 can interact with all three Trk receptors, suggesting a conserved mechanism for regulating neurotrophic trafficking involving a complex between Trk receptors, Ndfip1 and Nedd4-2. Although TrkA can directly interact with Nedd4-2 [10], our results suggest that Ndfip1 may still be required to act as a scaffold to both recruit and activate Nedd4-2 on endosomes for both complex formation and TrkA ubiquitination.

Ubiquitination is an important signalling mechanism involved in the trafficking of proteins in the cell. Proteins that are poly-ubiquitinated are commonly found to be trafficked to the lysosome for degradation, whereas mono- and multi-monoubiquitination can result in trafficking to other locations in the cell. Here we observed that Ndfip1 could mediate both mono- and poly-ubiquitination of TrkB. Using BiFC to track the location of the ubiquitinated receptor, we observed ubiquitinated TrkB on all major endosomal pathways in the cell including early, recycling and late endosomes, indicating the importance of ubiquitin-mediated transport of TrkB. Previous findings have shown that neurotrophin signalling through Trk receptors can occur at both the cell surface and intracellular compartments within the cell [19]. We observed ubiquitinated TrkB on multiple endosomal pathways, allowing for prolonged signalling after receptor activation, although we have not distinguished between the different ubiquitin chains required for each trafficking pathway. Whilst different ubiquitin patterns may account for why TrkB was observed in multiple endosomal pathways, we importantly observed significantly increased ubiquitinated TrkB in pathways required for the degradation of the receptor.

Neurotrophin signalling plays a critical role in determining neuronal morphology and subsequently the correct wiring patterns in the brain [20]. Both increased BDNF signalling through TrkB receptors [21] and the overexpression of TrkB result in increased dendritic branching in the brain [20]. Consistent with these prior studies, we have also observed that Ndfip1 knockout neurons display increased dendritic branching [22]. Here we find that Ndfip1 regulates the

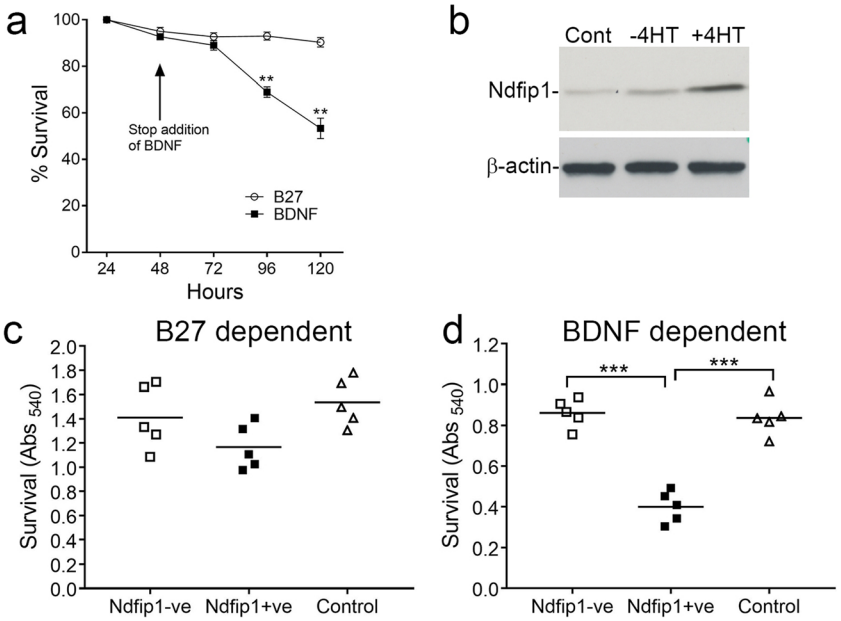

Fig. 4 Ndfip1 abundance can regulate the survival of BDNF-dependent neurons. **a** Primary cortical neurons were shown to be BDNF-dependent as removal of BDNF from the culture media resulted in neuronal death. $N = 3$ for each condition at each time point. **b** Inducible expression of Ndfip1 in neurons after the addition of 4HT (Cont is control neurons not infected with inducible Ndfip1 construct). **c** The survival of B27-dependent primary cortical neuron cultures was not altered upon induced expression of Ndfip1, using 4HT (Ndfip1+ve). **d** In contrast, there was a decrease in the survival of BDNF-dependent primary cortical neuron cultures when Ndfip1 expression was induced with 4HT (Ndfip1+ve). Control neurons were not infected with a lentivirus containing the inducible Ndfip1 expression construct. $N = 5$ for each condition, from three separate experiments (three litters). $^{**}P < 0.01$ and $^{***}P < 0.001$, ± SEM

ubiquitin-mediated degradation of TrkB, and loss of Ndfip1 results in increased TrkB in the brain. Therefore, a loss of Ndfip1 results in the dysregulation of TrkB degradation, allowing for amplified neurotrophin signalling and the increased dendritic branching phenotype observed in Ndfip1 knockout neurons. The expression profile of Ndfip1 in the developing mouse brain correlates well with a role in regulating neuronal patterning, with increasing Ndfip1 expression observed from E15 through to maximal levels from P7 to P28 [22, 23].

Outside of neuronal dendritic patterning, BDNF signalling through TrkB has also been shown to be important for neurogenesis in the dentate gyrus of the hippocampus, with links to antidepressant function in the brain [16]. Increased BDNF through direct infusion [24] increases dentate gyrus neurogenesis, whereas deletion of TrkB reduces neurogenesis in the dentate gyrus with an accompanying reduction in the size of the granular cell layer [16]. Here we found that loss of Ndfip1 increased TrkB abundance in the brain and this resulted in increased dentate gyrus size in the rodent hippocampus. Our findings suggest that in the dentate gyrus of the postnatal brain, Ndfip1 plays a role in modulating BDNF signalling through the ubiquitination of TrkB receptors.

In summary, we report that all three Trk receptors can interact with Ndfip1, an important adaptor/activator of the Nedd4 family of ubiquitin ligases. Ndfip1 can mediate the ubiquitination of TrkB by Nedd4-2, altering the trafficking and degradation of the protein, leading to changes to both neuronal patterning during development and dentate gyrus size in the postnatal brain.

Materials and Methods

Cell Culture, Plasmid Constructs and Transfection

HEK293T and COS cells were cultured in DMEM (Invitrogen), 10% FCS, 4 mM L-glutamine and 50 μg/mL PenStrep at 37 °C with 5% CO_2. Transfection of plasmids was performed using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. For BiFC studies, the coding region of TrkB was amplified by PCR and cloned into EcoRI and XhoI sites of pBiFC-VC155 (Addgene Plasmid 22011) to generate TrkB-VC (with the BiFC tag at the C terminus of TrkB). BiFC constructs for both Ndfip1 and ubiquitin have been described previously [15]. Constructs for the knockdown of Ndfip1 using RNAi have been reported previously [12].

Primary Neuronal Cultures and Survival Assays

The cortex of C57BL/6J embryos was removed at embryonic day 13 (E13) and the tissue digested with the Papain Dissociation Kit (Worthington Biochemical Corporation,

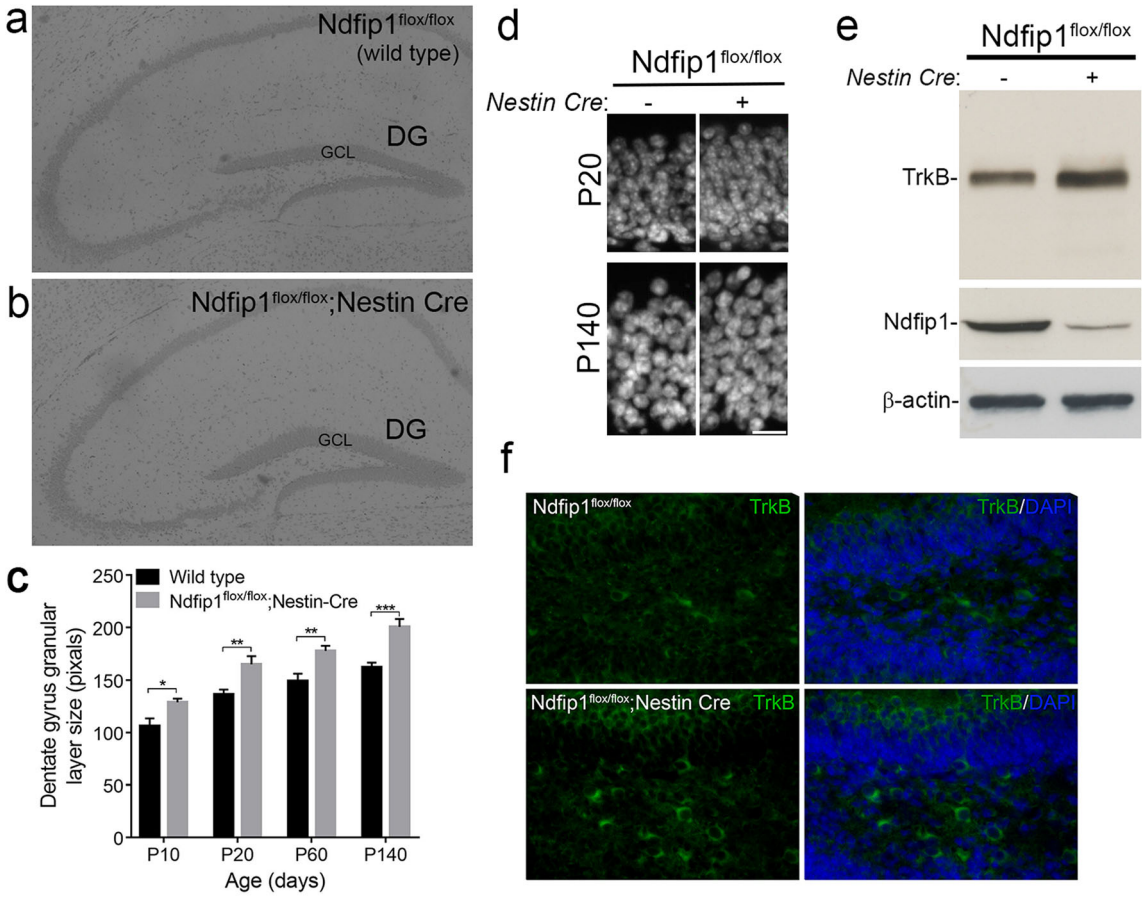

Fig. 5 Ndfip1 knockout mice have an enlarged granular cell layer of the dentate gyrus and increased TrkB abundance. **a**, **b** Images of the dentate gyrus from both *Ndfip1*$^{flox/flox}$ (wild type) and *Ndfip1*$^{flox/flox}$;*Nestin Cre* mice at P60 showing an increase in the granular cell layer (GCL) when *Ndfip1* is deleted. **c** Quantification of the granular cell layer size over time between *Ndfip1*$^{flox/flox}$ and *Ndfip1*$^{flox/flox}$;*Nestin Cre* mice. $N = 3$ per genotype at each age ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, ± SEM). **d** Image of granular cell layer at P20 and P140 showing an increase in cell number in *Ndfip1*$^{flox/flox}$;*Nestin Cre* mice. Scale bar 20 μm. **e** Western blot for the abundance of TrkB in the hippocampus of both *Ndfip1*$^{flox/flox}$ and *Ndfip1*$^{flox/flox}$;*Nestin Cre* mice at P10 shows an increase in TrkB when *Ndfip1* is deleted. **f** Immunohistochemistry for TrkB in the dentate gyrus of both *Ndfip1*$^{flox/flox}$ and *Ndfip1*$^{flox/flox}$;*Nestin Cre* mice at P10

Lakewood, NJ, USA) and mechanically dissociated using a flamed-tipped Pasteur pipette. Cells were plated at a density of 100,000 cells per 35-mm microwell plate precoated with 0.5 mg/mL poly-D-lysine. Neurons were maintained in two different types of media, the first containing B27 supplement (2% *v*/v, Invitrogen) in serum-free Neurobasal Medium with 0.5 mM glutamine and antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin). The second contained BDNF 100 ng/mL (added daily) in serum-free Neurobasal Medium with 0.5 mM glutamine and antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin). After 5 days in culture, the neurons were infected with an inducible Ndfip1-Flag construct using lentivirus. Briefly, lentiviral particles were made through transfection of HEK293T cells with packaging constructs pCMV δR8.2 and VSVg and an inducible Ndfip1-Flag [12] lentiviral plasmid using Effectene (Qiagen). Supernatants were harvested and primary neuronal cultures were infected with virus supernatant for 24 h. At 7 days in vitro, 100 nM 4-hydroxy tamoxifen was added to neuronal cultures to induce the expression of Ndfip1-Flag. Three days after induction of Ndfip1-Flag, neuronal survival was assayed using Thiazolyl Blue Tetrazolium Blue (MTT) assay.

Protein Lysate Preparation and Immunoprecipitation Assays

Mouse brain cortices from Ndfip1$^{flox/flox}$ or Ndfip1$^{flox/flox}$; *Nestin Cre* embryos at E17 or HEK-293T cells were lysed in ice-cold RIPA buffer (50 mM Tris, pH 7.2, 0.15 M NaCl, 2 mM EDTA, 1% NP40, 0.1% SDS) with complete Mini Protease inhibitor cocktail (Roche Diagnostics) for 20 min at 4 °C. Brain homogenates and cell lysates were cleared of insoluble debris by centrifugation at 13,000 rpm for 15 min at 4 °C. Protein concentration of lysates was measured using the BIO-RAD DC protein assay according to the manufacturer's instructions (Biorad). For precipitation experiments, Protein G beads (Pierce) were used for the precipitation of antibodies. Other beads used include Flag beads (Sigma) for

interaction studies and HisLink beads (Promega) for the ubiquitination assays. For each experiment, beads were washed four times with RIPA buffer before elution using the manufacturer's instructions. For the ubiquitination assays, HEK-293T cells were transfected with His-ubiquitin, Ndfip1-Flag and TrkB. Forty-eight hours after transfection, the ubiquitination assay was performed. Lysates were immunoprecipitated with HisLink under denaturing condition using 6 M Guarnidine HCl and NEM for inhibiting the deubiquitinase enzymes. Beads were washed three times before ubiquitinated proteins were eluted using 300 mM imidazole. Eluted fractions were suspended in Laemmli buffer for SDS-PAGE and analysed by Western blotting.

Western Blotting

Lysates or immunoprecipitates were resolved on 10% SDS-PAGE gels followed by transfer onto Hybond C nitrocellulose membrane (Amersham). Membranes were blocked for 1 h at RT in 5% non-fat milk in TBS, and 0.05% tween-20 (TBST). Blots were incubated overnight with primary antibodies at 4 °C followed by appropriate HRP-conjugated secondaries for 1 h at RT. Proteins were detected using Amersham enhanced chemical luminescence reagent as per the manufacturer's instructions (GE Healthcare) and visualised by exposure to x-ray film. Primary antibodies were rat monoclonal anti-Ndfip1 clone 1G5 (1:2000), TrkB polyclonal (H181 Santa Cruz), Trk polyclonal (C14 Santa Cruz), and mouse monoclonal anti-β-actin clone AC-40 (1:5000, Sigma). HRP-conjugated secondary antibodies were goat polyclonal anti-rat (1:5000 or 1:10,000, Millipore), goat polyclonal anti-rabbit (1:10,000, Millipore), and goat polyclonal anti-mouse (1:15000, Millipore).

Animals

All procedures were approved by the Florey Neuroscience Institutes Animal Ethics Committee. C57BL/6J mice were obtained from ARC, Australia. Ndfip1 conditional knockout mice on a C57BL/6J background have been reported previously [25]. *Ndfip1*$^{flox/flox}$ mice used in this study were crossed with *Nestin Cre* to delete Ndfip1 from the majority of neurons in the brain (Ndfip1$^{flox/flox}$; *Nestin Cre* mice).

Immunohistochemistry and Imaging

For postnatal immunostaining of TrkB in the hippocampus, mice were killed under deep anaesthesia by transcardial perfusion of PBS (pH 7.4) followed by 4% PFA in 0.1 M phosphate buffer (PB). Brains for TrkB staining were post-fixed for 1 h in 4% PFA in 0.1 M PB and then cryoprotected for 24 h in 20% sucrose in 0.1 M PB at 4 °C before coronal sectioning. Perfusion fixed sections (14 μm) were permeabilized with 0.3% Triton X-100 in 0.1 M PB and blocked with 10% FBS in 0.1% Triton X-100 with 0.1 M PB. Tissue was then washed in PBST (0.1 M PBS, 0.1% Triton X-100) and blocked with 10% normal horse serum in PBST. Sections were incubated with TrkB antibody overnight at 4 °C, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody for 1 h (1:500, Molecular Probes). Sections were counterstained with DAPI (1:10,000, Dako) before mounting under glass cover slips with anti-fade mounting reagent.

For BiFC studies, fluorescent images of ubiquitinated TrkB and Rab-GTPases were obtained using confocal microscopy (Leica TCS SP8). For colocalisation analysis, the Manders overlap coefficient was used to quantify colocalisation. The Manders coefficient describes the fraction of red (M1) or green (M2) channel pixels that colocalise with pixels from the other channel based on intensity. For the colocalisation analyses, the M1 coefficient was used to normalise for the differential expression levels between the Rab-GTPases. Prior to analysis, deconvolution was performed on each z-stack using the Huygens Remote Manager to enhance the resolution and contrast for greater accuracy during analysis. A consistent region representing a cell from a deconvolved confocal z-stack was applied to all z-stacks prior to analysis to ensure consistency in area of analysis. A threshold level, under which pixel values were considered background, was manually set and the same threshold applied to all stacks. To calculate the Manders overlap coefficient, the JACoP plugin for ImageJ was used (Bolte and Cordelieres, 2006).

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Compliance with Ethical Standards

All procedures were approved by the Florey Neuroscience Institutes Animal Ethics Committee.

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