Coronin-1 is a neurotrophin endosomal effector that is required for developmental competition for survival

Dong Suo1, Juyeon Park1, Anthony W Harrington2,4, Larry S Zweifel2,4, Stefan Mihalas3 & Christopher D Deppmann1

Retrograde communication from axonal targets to neuronal cell bodies is critical for both the development and function of the nervous system. Much progress has been made in recent years linking long-distance, retrograde signaling to a signaling endosome, yet the mechanisms governing the trafficking and signaling of these endosomes remain mostly uncharacterized. Here we report that in mouse sympathetic neurons, the target-derived nerve growth factor (NGF)–tropomyosin-related kinase type 1 (TrkA, also called Ntrk1) signaling endosome, on arrival at the cell body, induces the expression and recruitment of a new effector protein known as Coronin-1 (also called Coro1a). In the absence of Coronin-1, the NGF-TrkA signaling endosome fuses to lysosomes sixfold to tenfold faster than when Coronin-1 is intact. We also define a new Coronin-1–dependent trafficking event in which signaling endosomes recycle and re-internalize on arrival at the cell body. Beyond influencing endosomal trafficking, Coronin-1 is also required for several NGF-TrkA–dependent signaling events, including calcium release, calcineurin activation and phosphorylation of cAMP responsive element binding protein (CREB). These results establish Coronin-1 as an essential component of a feedback loop that mediates NGF-TrkA endosome stability, recycling and signaling as a critical mechanism governing developmental competition for survival.

Neurons are endowed with several features that distinguish them from unpolarized cells. One of the most obvious differences is their comparatively long length. With this extended distance comes several distinct challenges involving proper trafficking and maintenance of signal integrity. This form of communication is particularly important in the development and maintenance of the peripheral nervous system, where the assembly of neural circuits is coordinated by the target organs they innervate and control.

Among the best characterized of these long-distance signals are the structurally related family of target-derived growth factors, the neurotrophins. These factors convey their signal from the distal tip of the axon to the cell body and dendrites, which in turn coordinate the development of functional circuits1–2. Neurotrophins, including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NTF3) and neurotrophin-4/5 (NTF4/5), signal through two distinct receptor systems, the Trk family of receptor tyrosine kinases and the p75–NGF receptor (p75–NGFR)3. ‘Pro-building’ events such as synapse formation and survival are generally mediated by neurotrophin–Trk signaling endosomes that are formed at distal tips of axons in the periphery and travel back to neuronal cell bodies4–8.

In recent years, several effector proteins have been found to confer unique properties to long-distance, retrograde signaling endosomes. In particular, phospholipase C-γ (PLC-γ1), RAP1, pincher (pinocytic chaperone), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase 7 (ERK5, also called MAPK7) and cofilin (CFLI) have been shown to associate with the NGF-TrkA signaling endosome and are functionally important in the context of in vitro survival assays6,9–13. An emergent principle for endosomal-associated effectors is to have multiple roles in not only signaling to promote developmental events but also trafficking and maturation. For example, it was recently found that association of the actin-modifying protein cofilin is necessary for NGF-TrkA retrograde trafficking13. Several questions remain about this process, including which proteins and/or signaling pathways are essential for trafficking events such as internalization, recycling, long-distance transport or lysosomal fusion, and are there endosomally associated proteins that confer a unique signaling ability at a particular time and place?

In this study we identify Coronin-1 as a new effector protein for the NGF-TrkA signaling endosome. Coronin-1 is part of a family of structurally related proteins that are known for interacting with cytoskeletal proteins such as F-actin14–16 (Supplementary Fig. 1a–c). Although Coronin family members share similar structures and neuronal expression patterns, they do not appear to be functionally redundant. Although the most widely studied function of Coronin-1 is in the context of cytoskeletal dynamics, perhaps more relevant to the NGF-TrkA signaling endosome is its role in pathogen-host interactions. Previous reports concluded that Mycobacterium tuberculosis recruits Coronin-1 after engulfment by macrophages in order to avoid subsequent lysosomal fusion and evade phagocytic degradation17. In the absence of Coronin-1 or when the bacteria are killed

1Department of Biology, University of Virginia, Charlottesville, Virginia, USA. 2The Solomon Snyder Department of Neuroscience and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 3Allen Brain Institute, Seattle, Washington, USA. 4Present addresses: Janssen Pharmaceutical Companies of Johnson and Johnson, San Diego, California, USA (A.W.H.) and Department of Pharmacology and Department of Psychiatry and Behavioral Science, University of Washington, Seattle, Washington, USA (L.S.Z.). Correspondence should be addressed to C.D.D. (deppmann@virginia.edu).

Received 2 September; accepted 1 November; published online 24 November 2013; doi:10.1038/nn.3593
by heat, the pathogenic phagosome fuses rapidly to lysosomes. It was shown more recently that recruitment of Coronin-1 to the pathogenic endosome confers an ability to elicit calcium-calcineurin signaling, which also seems to be critical for preventing lysosomal fusion.\(^8\) We hypothesized that Coronin-1 could stabilize the NGF-TrkA signaling endosome in much the same way that it stabilizes the M. tuberculosis pathogenic endosome, thereby sustaining signaling integrity between the target organ and the neuronal cell body.

Here we find that the expression and association of Coronin-1 with the signaling endosome are induced by neuronal exposure to NGF. We find Coronin-1 to be necessary for NGF-dependent calcium release, which, through activation of calcineurin, allows the NGF-TrkA signaling endosome to evade lysosomal fusion and degradation at the cell body. We also identify a role for Coronin-1 in mediating signaling endosome transcytosis. We find that the majority of long-distance retrograde NGF-TrkA endosomes undergo Coronin-1-dependent recycling and re-internalization at the cell body. Coronin-1 loss of function uncouples neurotrophin signaling from calcium release and CREB activation, resulting in a destabilization of the signaling endosome and impaired survival signaling. We conclude that this newly identified NGF–Coronin-1 feedback loop is critical for competition for survival during development.

**RESULTS**

NGF is necessary and sufficient for Coronin-1 expression

We previously identified a positive feedback loop in which neuronal exposure to target-derived NGF enhanced its own signal duration during developmental competition for survival.\(^{19}\) Based on this finding, we speculated that NGF would induce a putative factor that is responsible for extending signal duration. To identify one of these factors, we analyzed a previously published microarray, which identified NGF-dependent genes in the superior cervical ganglia (SCG).\(^{19}\) Our criteria for this directed screen were that NGF have not only the capacity to induce the factor but also an ontology that is consistent with membrane or vesicle association. We found 77 genes that met this criteria displaying a more than twofold reduction in expression in SCGs from Ngf\(^{−/−}\); Bax\(^{−/−}\) mice compared to control Bax\(^{−/−}\) mice at postnatal day (P\(^0\)) (Fig. 1a and Supplementary Table 1). The Bax\(^{−/−}\) background is necessary in these experiments to circumvent the massive loss of neurons observed in the absence of NGF.\(^{20,21}\) Coronin-1 (encoded by Coro1a) met the criteria of being associated with vesicles, and its expression was reduced 7.34-fold in SCGs from NGF-null mice (Ngf\(^{−/−}\); Bax\(^{−/−}\) mice at P0) compared to control (Bax\(^{−/−}\)) mice (Fig. 1a).

The expression patterns of Coronin family members broadly overlap in the peripheral nervous system. In particular, TrkA-positive peripheral neurons of the dorsal root ganglion show expression of all Coronin family members that we examined (Supplementary Fig. 2a). In the CNS, it appears that although Coronin family members are highly expressed (for example, in the cortical plate and hippocampus), they are not highly expressed in structures that are classically and discretely TrkA positive (for example, the basal forebrain) (Supplementary Fig. 2b).

To confirm that Coronin-1 expression is dependent on NGF, we performed in situ hybridization and immunohistochemistry on...
Cryosectioned SCGs isolated from P0 Ngf/−; Bax−/− or Bax−/− mice. We verified antibody specificity by comparing immunostained cryosections from wild-type and Coro1a−/− mice (Supplementary Fig. 1d). Consistent with microarray data, Coronin-1 mRNA and protein levels were markedly reduced in SCGs from P0 Ngf/−; Bax−/− mice relative to Bax−/− mice (Fig. 1b,c). We next sought to determine whether NGF is sufficient to induce Coro1a expression in vitro. We deprived sympathetic neurons of NGF in the presence of the broad-spectrum caspase inhibitor Boc-Asp-FMK to prevent cell death; we then reapplied NGF (45 ng ml−1) for the indicated amounts of time and analyzed mRNA and protein levels by RT-PCR and immunoblot, respectively (Fig. 1d–f). In the absence of NGF, Coro1a transcript and protein levels were low or undetectable and were rapidly induced after re-addition of NGF (Fig. 1d–f). We next defined the developmental timing of Coronin-1 expression by immunohistochemistry for Coronin-1 on cryosections containing SCGs from embryonic day (E) 15, E18, P0 and P5 mice. (Fig. 1g). Consistent with the notion that NGF is required for Coronin-1 expression, we observed a substantial increase in the level of Coronin-1 protein after final target innervation (represented by P0) as compared to stages before (represented by E15), and stages at the beginning of (represented by E18), target innervation. These data indicate that final target innervation and exposure to NGF are necessary and sufficient for Coronin-1 expression in the sympathetic nervous system.

Coronin-1 associates with the NGF-TrkA signaling endosome

Previous studies on pathogen-host interactions demonstrated a physical association between Coronin-1 and the M. tuberculosis phagososome17,22. We sought to determine whether there is an analogous interaction between Coronin-1 and the NGF-TrkA signaling endosome. In order to clearly visualize post-endocytic retrogradely transported NGF-TrkA, we used sympathetic neurons isolated from P0 Ntrk1Flag mice, in which a Flag epitope is knocked in frame with the extracellular domain of TrkA8. We grew these neurons in microfluidic devices and pulsed distal axons with a Flag-specific antibody (M1) and NGF for 30 min at 37 °C (ref. 23) (Fig. 2a,b). At different time points after the pulse with NGF and M1, we fixed the neurons and detected Flag-TrkA localization using immunofluorescence. Imaging the cell body compartment ensures that the puncta observed represent long-distance, retrogradely transported signaling endosomes.

We first used this assay to examine colocalization between the signaling endosome and Coronin-1. We observed that roughly 70% of signaling endosomes colocalized with Coronin-1 in cell bodies 1 h after treatment with NGF and the Flag antibody on distal axons (Fig. 2c,d). This colocalization persisted for at least 6.5 h and was followed by a gradual decrease to roughly 40% at 24 h after the NGF and Flag antibody pulse. Notably, at 2.5 h after the pulse, colocalization between endosomal TrkA and Coronin-1 was threefold higher in cell bodies (59.27 ± 6.66% (mean ± s.e.m.)) compared to axons (19.49 ± 5.01%), indicating that the interaction may be subcellular-compartment dependent. A lower magnification visualization of Coronin-1 in both dissociated neurons and an electroporated chick spinal cord and dorsal root ganglion revealed that Coronin-1 was also highly localized in axons and growth cones (Supplementary Fig. 1e,f). We also tested the association of Coronin-1 with the signaling endosome using PC12 cells stably expressing a chimeric Trk receptor containing the extracellular portion of TrkB and the intracellular domain of TrkA (TrkB/A). This receptor is activated and internalized after BDNF treatment but retains TrkA downstream signaling13. After ligand stimulation, Coronin-1 and TrkB/A were both shifted into fractions containing early endosomes. Although we detected a relatively small amount of TrkB/A in the late endosome fractions after BDNF treatment, Coronin-1 remained in the
Coronin-1 prevents signaling endosome fusion with lysosomes at the cell body. (a) Coronin-1 does not colocalize with lysosomes. Shown is immunostaining for Coronin-1 (green) and visualization of lysosomes using LysoTracker (red) in rat sympathetic neurons that were grown for 3 d in vitro (DIV). Colocalization was assessed manually (<10%) and was negligible. Scale bar, 10 μm. WT, wild type. (b) Signaling endosome colocalization with lysosomes in the presence or absence of Coro1a. Flag feeding was performed for 30 min, which was followed by a chase period for the indicated time in sympathetic neurons isolated from Ntrk1WT (WT) or Ntrk1KO; Coro1a−/− (KO) mice. Scale bar, 10 μm. (c) Quantification of the percentage of lysosomes positive for Flag as a function of time after feeding with Flag antibody and NGF (n = 5 for all groups). Curves were fit using variable slope regression parameters in GraphPad Prism. Error bars, s.e.m. (d) Quantification of colocalization between the signaling endosome and lysosomes in neurons isolated from Ntrk1WT or Ntrk1KO; Coro1a−/− mice (n = 5 for all groups). Cell bodies and axons were visualized at different times after feeding with Flag antibody and NGF, as described in b and c. Error bars, s.e.m. (e) EGFR lysosomal fusion in the presence or absence of Coro1a. The percentages of EGFR-positive lysosome puncta are shown on the merged images. Scale bar, 10 μm. (f) pAkt decay in the presence or absence of Coro1a in neurons cultured from wild-type or Coro1a−/− mice for 2–3 DIV, deprived of NGF (in the presence of NGF function-blocking antibody) for the indicated times, lysed and analyzed by immunoblot for pAkt and Akt. (g) Quantification of the data in e. Experiments were repeated at least three times and quantified with densitometry. pAkt signals were normalized to total Akt (n = 3). Error bars, s.e.m. *P < 0.05 using unpaired two-tailed Student’s t test. Uncropped gels and blots are shown in Supplementary Figure 7.

Coronin-1 prevents signaling endosome degradation

Coronin-1 is known to be a requisite host factor for M. tuberculosis survival in macrophages17. It has been shown that this protection involves preventing engulfed M. tuberculosis from undergoing lysosomal fusion. This previous study, along with our finding that Coronin-1 associates with NGF-TrkA, suggest that Coronin-1 might have a nonpathogenic role in preventing the neurotrophin signaling endosome from lysosomal fusion. Consistent with this idea, in sympathetic neurons, we found less than 10% colocalization between Coronin-1 and lysosomes as measured by immunocytochemistry for Coronin-1 and LysoTracker staining (Fig. 3a).

To determine whether Coronin-1 can prevent the degradation of NGF-TrkA signaling endosomes, we used a Flag antibody feeding assay in combination with LysoTracker staining to identify TrkA-containing lysosomes. We analyzed colocalization between these markers after a 30-min pulse with NGF and the Flag antibody on distal axons and a chase period for the indicated times (Fig. 3b,c). In neurons isolated from Ntrk1WT mice, colocalization remained below 10% until more than 24 h after the NGF and Flag antibody pulse (Fig. 3c). In contrast, neurons isolated from Ntrk1KO; Coro1a−/− mice showed signaling endosomes rapidly fusing with lysosomes, as reflected by colocalization in the cell body that was threefold to eightfold higher than that in control neurons at all time points after the pulse (Fig. 3b,c). This is consistent with the finding that NGF signaling is long lived and suggests that the mechanism for this is at least in part through Coronin-1-dependent evasion of lysosomal fusion. Notably, this phenomenon appears to be restricted to the cell body, as loss of Coro1a seems to have no effect on lysosomal fusion in axons (Fig. 3d). In contrast to extended NGF treatment, loss of Coro1a did not influence the total number of lysosomes in cell bodies or axons in the context of these experiments25 (Supplementary Fig. 3a).
Figure 4 Coronin-1 mediates recycling of the signaling endosome. (a) Accumulation of signaling endosomes in the presence or absence of Coronin-1. Flag feeding was performed for 30 min, which was followed by a chase period for the indicated time in sympathetic neurons isolated from Ntrk1<sup>Flag</sup> or Ntrk1<sup>Flag,Rab</sup>Coro1a<sup>−/−</sup> mice (as described for Fig. 3b). A three-dimensional model was generated from 2 stacks obtained for Figure 3b,c, and the total number of Flag-TrkA endosomes per neuron were counted (n = 5 for the groups at 1 h and 8 h; Ntrk1<sup>Flag</sup>, n = 7 for the 12 h group; Ntrk1<sup>Flag,Rab</sup>Coro1a<sup>−/−</sup>, n = 9 for the 12 h group; n = 9 for 24 h group). Error bars, s.e.m. (b) Coronin-1 colocalization with Rab5 or Rab11 in sympathetic neurons that were immunostained for Coronin-1 and Rab5 or Rab11. Colocalization was assessed and is shown as a percentage of Coronin-1 puncta positive for Rab5 or Rab11 in the cell bodies, proximal axons (PA) or distal axons (n = 5 for all groups). Error bars, s.e.m. (c) Coronin-1 is required for the recruitment of Rab11 to post-endocytic NGF-TrkA. Shown is visualization of Flag and Rab11 by immunostaining after NGF feeding was performed for 30 min followed by 2.5 h of chase in neurons isolated from Ntrk1<sup>Flag</sup> or Ntrk1<sup>Flag,Rab</sup>Coro1a<sup>−/−</sup> mice. (d) Flag-TrkA transcytosis and recycling in the presence or absence of Coronin-1. The recycling assay was performed as described in e on neurons isolated from Ntrk1<sup>Flag</sup> or Ntrk1<sup>Flag,Rab</sup>Coro1a<sup>−/−</sup> mice grown in microfluidic devices. Flag antibody was fed to distal axons for 30 min, which was followed by a chase period for the indicated amount of time. Recycled Flag-TrkA in cell bodies and axons was marked by Cy-3. A schematic for the Flag-TrkA recycling assay is shown in Supplementary Figure 3b. Dotted vertical lines represent axon tracts. Scale bars, 10 μm. (e) Quantification of the data in d (n = 5 for all groups). Error bars, s.e.m. *P < 0.05 using unpaired two-tailed Student’s t test.

To determine whether Coronin-1 broadly influences lysosomal fusion, we examined degradation of internalized epidermal growth factor (EGF) in the presence or absence of Coro1a. Consistent with previous reports, fluorescent EGF does not undergo retrograde transport (data not shown<sup>26</sup>). Therefore, we fed these ligands to sympathetic cell bodies for 30 min and visualized internalized ligands and lysosomes 6 h later. Consistent with previous observations, there was 57.52 ± 3.8% colocalization of EGF with lysosomes in neurons isolated from wild-type animals<sup>27</sup> (Fig. 3e). Moreover, in the absence of Coronin-1, internalized EGF fused to lysosomes in a manner similar to that in wild-type mice (55.87 ± 4.3%) (Fig. 3e). The fact that Coronin-1 had no impact on EGF lysosomal fusion suggests that it is not a general effector of membrane trafficking. For direct comparison, we performed a Flag antibody feeding assay on neurons isolated from Ntrk1<sup>Flag</sup> mice in which we applied NGF to the cell body instead of Rab5 or Rab11, and clearance of endosomes after the pulse despite the roughly sixfold difference in the rate of lysosome fusion in the presence compared to absence of Coro1a (Fig. 4a). However, after the initial wave of Flag-TrkA accumulation at the cell body (>8 h), we observed a significant and sustained doubling of internalized Flag-TrkA in wild-type as compared to Coro1a<sup>−/−</sup> neurons (Fig. 4a).

Coronin-1 facilitates recycling of the signaling endosome
Our finding that retrogradely trafficked NGF-TrkA takes roughly 30 h for substantial lysosomal fusion to occur in the cell body is inconsistent with previous findings demonstrating that PC12 cells downregulate TrkA within 2–3 h (ref. 28). This inconsistency prompted us to examine NGF-TrkA endosome accumulation in the cell body as a function of time after pulse with Flag antibody and NGF on distal axons. Notably, we observed very little difference in the initial accumulation and clearance of endosomes after the pulse despite the roughly sixfold difference in the rate of lysosome fusion in the presence compared to absence of Coro1a (Fig. 4a). However, after the initial wave of Flag-TrkA accumulation at the cell body (>8 h), we observed a significant and sustained doubling of internalized Flag-TrkA in wild-type as compared to Coro1a<sup>−/−</sup> neurons (Fig. 4a).

What might the mechanism for the clearance of NGF-TrkA be, if not lysosomal fusion? NGF-TrkA signaling endosomes have been reported to correspond to early and recycling endosome compartments marked by Rab5 and Rab11, respectively<sup>29,30</sup>. Therefore, we examined Coronin-1 colocalization with these markers in different subcellular compartments. Notably, Coronin-1 preferentially associated with Rab11 over Rab5 in the cell body, but we observed no significant differences in the proximal or distal axon compartments (Fig. 4b). Does Coronin-1 influence the association of NGF-TrkA with Rab11? To address this question, we performed Flag feeding assays on neurons from Ntrk1<sup>Flag</sup> or Ntrk1<sup>Flag,Rab</sup>Coro1a<sup>−/−</sup> mice
Coronin-1 mediates NGF-dependent signaling, calcium mobilization and CREB phosphorylation. (a) NGF induction of calcium is Coronin-1 dependent. Shown is calcium release, visualized with the calcium dye Fluo-4, from sympathetic neurons isolated from wild-type and Coro1a−/− mice that were established in culture. Images of single neuronal cell bodies were acquired 15 min after NGF treatment. Scale bar, 5 μm. (b) Quantification of the calcium release shown in a as a function of NGF concentration. All fluorescence intensities (F) are relative to those collected with 0 ng ml−1 NGF (F0) and are represented as F/F0 (n = 5 for all groups). Error bars, s.e.m. (c) Coronin-1 is required for NGF-dependent phosphorylation of CREB. Shown are pCREB, Coronin-1 and GAPDH protein levels, assessed by immunoblot, in PC12 cells that were transfected with siRNA targeting Coro1a or lamin, cultured for 24 h and treated with NGF for the indicated amount of time. (d) Requirement of Coronin-1 for CREB transcriptional activity. Coro1a or Lamin was knocked down in sympathetic neurons, which was followed by assessment of 3×Cre-luciferase reporter gene activity in the presence of the indicated concentrations of NGF or 5 μM forskolin (FSK). Data are represented as relative light units (n = 3 for all groups). Error bars, s.e.m. (e) Influence of calcium on signaling endosome stability. Signaling endosome colocalization with the lysosome was examined as described in Figure 3. Reducing intracellular calcium release by BAPTA-AM treatment of neurons from Ntrk1Ftag mice phenocopies the rate of lysosomal fusion observed in Ntrk1Ftag; Coro1a−/− mice. Likewise, increasing intracellular calcium with calcimycin (50 nM) treatment of neurons from Ntrk1Ftag; Coro1a−/− mice partially rescues the lysosome fusion phenotype (n = 5 for all groups). Error bars, s.e.m. (f) Influence of calcium on signaling endosome stability. The calciuminhibitors CsA (100 nM) and FK506 (500 nM) added to neurons from Ntrk1Ftag mice phenocopy the rate of lysosomal fusion observed in Ntrk1Ftag; Coro1a−/− mice (n = 10; CsA, n = 5; FK506, n = 5). Error bars, s.e.m. (g) EGFP-positive lysosome puncta (%) are not influenced by calcimycin (calcium ionophore) or CsA and FK506 (calcineurin inhibitors). Error bars, s.e.m. *P < 0.05 using unpaired two-tailed Student’s t test and one-way analysis of variance nonparametric test. Uncropped gels and blots are shown in Supplementary Figure 7.
Coronin-1 stabilizes the signaling endosome via calcium and calcineurin signaling

Having established that Coronin-1 is necessary for the induction of calcium release by NGF, we sought to determine how this NGF-dependent calcium signaling is related to NGF-TrkA endosomal stability. We used the cell-permeable calcium chelator 1,2-bis(o-aminophenoxy)-ethane-N,N,N,N-tetraacetic acid, tetraacetoxyethyl ester (BAPTA-AM) to determine whether lowering intracellular calcium levels causes increased fusion of signaling endosomes to lysosomes. We were surprised to find that the ratio of signaling endosome to lysosomal fusion. We were surprised to find that the ratio of signaling endosome to lysosomal fusion.

In the absence of Coronin-1, the rate of endosome-lysosome fusion 

which phenocopied the fusion rates observed in Ntrk1Flag; Coro1a−/− neurons (Fig. 5f). Consistent with our results described above (Fig. 3e), inhibition of calcium or calcineurin signaling did not influence EGF-lysosomal fusion in wild-type or Coro1a−/− neurons (Fig. 5g). From these data, we conclude that NGF-TrkA recruitment of Coronin-1 leads to calcium release and calcineurin activation, both of which are required to maintain signaling endosome stability.

Modeling the role of Coronin-1 in competition

We and others have previously built computational models that describe competition for survival in developing neurons. Our previous model describes how neurons with similar responsiveness and access to target-derived trophic factor can distinguish themselves from one another. This distinction requires several feedback loops that promote a ‘bi-stability’ that results in either survival or apoptosis. Two of these feedback loops are required for competition to occur and include (i) NGF regulating the expression of its own receptor, TrkA, which results in an enhanced ability to take up NGF, as well as an increased robustness of downstream signaling pathways in neurons that ‘win’ the competition; and (ii) NGF regulating its own signal duration, which we related to the degradation rate of active TrkA. We next sought to examine whether this second critical feedback loop corresponds to NGF-dependent Coronin-1 expression.

In order to predict the impact of losing Coronin-1 on neuronal competition for survival, we updated our previous model with empirical observations from this study (Fig. 3e)19. We first fit a rate of active TrkA degradation in the presence or absence of Coronin-1, which matched the observed sharp sigmoidal experimental values for signaling endosome-lysosome fusion (Fig. 3c and Supplementary Figs. 4–6). In the absence of Coronin-1, the rate of endosome-lysosome fusion hastens substantially. Yet even in the absence of Coronin-1, NGF-TrkA endosomes persist much longer than other growth factor signaling endosomes. For example, post-endocytic EGF degradation peaks hastens substantially. Yet even in the absence of Coronin-1, NGF-TrkA endosomes persist much longer than other growth factor signaling endosomes.
be employed in the axon as the signaling endosome approaches the cell body, which would be required for engagement of the aforementioned NGF-dependent competition feedback loops.

We predict the existence of at least three post-endocytic TrkA pools defined by their stability: (i) those with Coronin-1, which are very stable (protected), (ii) those without Coronin-1 that are moderately stable (unprotected) and (iii) those without Coronin-1 that are highly unstable (punished). We modeled the impact of this putative Coronin-1-independent degradation pathway under conditions of low (Supplementary Fig. 4a–f) or high (Supplementary Fig. 4g–i) trophic factor availability. Notably, the only conditions that displayed multiple intersections representing a life-death bistable system were those that had Coronin-1-induced protection of endosomes and, independent of this, another mechanism representing a moderate probability of unprotected endosome being converted to punished endosomes, resulting in rapid fusion with lysosomes (Supplementary Fig. 4).

On the basis of these observations, we sought to further improve our simulation of developmental competition by building in a third feedback loop representing the conversion of unprotected endosomes to punished endosomes. In order for this parameter to be useful in the context of developmental competition, this probability must begin at a low level and rise to moderate levels by the end of competition.

This is reminiscent of the BDNF-p75-NGFR punishment feedback loop described previously, which was found to be essential to expedite competition. Although punishment of unprotected endosomes may occur through a different mechanism than punishment by BDNF-p75-NGFR, their feedback loops are likely to increase on a similar time scale. For this reason, the parameter corresponding to the unprotected endosome punishment pathway is coupled with the previously described p75-NGFR–dependent punishment pathway.

Simulations reveal that when all previously reported feedback loops are in play, including the newly revised NGF-dependent signal duration loop mediated by Coronin-1, 61% of neurons gain enough trophic signal strength to survive (Fig. 6a,b). In the absence of the Coronin-1 signal duration feedback loop, or when only 50% of NGF is available, only 30% of starting neurons gain sufficient trophic signal strength to survive (Fig. 6a,b). In addition, under conditions simulating loss of Coronin-1 and availability of 50% of endogenous NGF, only 17% of starting neurons survive (Fig. 6b). Notably, because the onset of the previously described p75-NGFR–dependent paracrine punishment signaling is linked to trophic signaling, weakening trophic signaling by removing a percentage of NGF results in a delay in competition. These simulations are consistent with the target-matching hypothesis in which a drop to half NGF production, as is observed in Ngf+/− mice, leads to loss of half of the neurons. Scaling endosome protection as a function of NGF-TrkA uptake is essential in order to recapitulate the linear quality of target matching. This requirement allows a range of TrkA concentrations over which the NGF uptake from the target tissue is similar for each winning neuron, even though different percentages of neurons may ultimately survive as a function of the rate of NGF production.

Coronin-1 is required for NGF-dependent survival

To test these predictions in sympathetic neurons, we first examined retrograde NGF-dependent survival in neurons from Coro1a+/− and wild-type mice. We established these neurons in microfluidic devices and applied the indicated concentrations of NGF to distal axons for 36 h, at which time we assessed neuronal death by Hoechst staining. Increasing the concentration of NGF exclusively on distal axons resulted in increased survival in wild-type neurons. Coro1a−/− neurons displayed low levels of survival relative to wild-type controls even at 100 ng ml−1 of NGF, indicating an essential role for Coronin-1 in long-distance survival signaling (Fig. 6c). Notably, 10 ng ml−1 NGF applied to the cell body of Coro1a−/− neurons was sufficient to support full survival relative to wild-type controls. However, lower concentrations of NGF (0.1 and 1 ng ml−1) applied to cell bodies failed to support the same levels of survival as in wild-type controls (Supplementary Fig. 3d). These data point to an essential role for Coronin-1 in mediating long-distance NGF-dependent survival.

We next sought to determine whether Coronin-1 also influences sympathetic neuron survival in vivo by counting the number of neurons in P0 SCGs from the indicated genotypes as described previously. Consistent with the modeling data, in the absence of Coro1a, we observed a marked reduction in the number of sympathetic neurons, which was similar to that observed in SCGs from Ngf+/− mice (Fig. 6d,e). Moreover in SCG neurons from Ngf+/−;Coro1a−/− mice at P0, we observed a further reduction of neuron numbers as compared to Ngf+/− and Coro1a−/− neurons. These findings correlate very well with the survival predicted by simulations (Fig. 6f) and suggest that Coronin-1 is a critical NGF-TrkA effector protein that is required for developmental competition for survival in vivo. Modeling suggests that although these genotypes yield substantially different outcomes with respect to number of surviving neurons, at the conclusion of the competition, the neurons that survive have comparable abundances of protected, unprotected and punished endosomes. This indicates that although different numbers of neurons win in the presence of different concentrations of NGF, the winners ultimately have similar trophic states, which we suggest is a key feature of the target-matching hypothesis.

DISCUSSION

We demonstrate that Coronin-1 participates in a new NGF-TrkA feedback loop that is required for proper development of the sympathetic nervous system. Target-derived NGF is necessary and sufficient for Coro1a expression in sympathetic neurons. We also find that Coronin-1 directly interacts with the signaling endosome and is essential for several core NGF-dependent signaling events, such as calcium release, calcineurin activation and CREB phosphorylation. Coronin-1 is also critical for at least two NGF-TrkA endosomal trafficking events that are associated with endosome arrival at the cell body: a newly identified transcytosis event and avoidance of lysosomal fusion. Loss of Coronin-1 results in abnormal neurotrophin signaling and trafficking, which leads to a diminished capacity of neurons to compete with one another, manifesting in excess developmental neuron death (Supplementary Figs. 4–6). This defines a new framework for modulating neurotrophin signaling in the context of developmental competition for survival (Supplementary Fig. 3e).

The notion of a long-distance signaling endosome sparks several questions related to how this form of signaling differs from signaling at the plasma membrane. Among these questions are does the nature of endosomal signaling change as a function of subcellular locale, how do endosome-derived signals influence endosome trafficking, and what are the functional consequences of these trafficking events? The discovery that Coronin-1 is an endocytic TrkA effector protein provides a foothold for answering these questions.

Over the past decade, several NGF-TrkA signaling endosome effector proteins have been identified. To our knowledge, none of these effectors has been found to preferentially associate with the endosome in a particular subcellular compartment. Here we find that Coronin-1 preferentially associates with endosomal NGF-TrkA in the cell body (Fig. 2d). It has been established that sympathetic neuron survival requires long-distance endosomal transport and signaling from distal
Axons to the cell body. Therefore, it is not surprising that the endosome would associate with an effector such as Coronin-1 on arrival at the cell body, which would endow NGF- TrkA-containing endosomes with several specific prosurvival signaling properties, including modulation of calcium release, calcineurin activation and CREB-dependent transcription.

An emergent property of the NGF-TrkA endosome is that its trafficking is directed by its own signaling. This mechanism has been shown for several aspects of trafficking, including TrkA phosphorylation of dynamin, which is required for internalization; NGF-TrkA-dependent PI3K activity, which is required for retrograde transport but not internalization; and NGF-TrkA-dependent MAPK activity, which is required for endosomal trafficking into dendrites. The recruitment of Coronin-1 to the NGF-TrkA signaling endosome seems to fit into this theme, as it regulates two key trafficking events at the cell body: transcytosis-recycling and avoidance of lysosomal fusion.

The requirement of recycling is being increasingly recognized as a critical component of neurotrophin signaling. Researchers in a previous study demonstrated that in mass cultures, TrkA undergoes a relatively high rate of recycling and that by mutating the TrkA juxtamembrane region that is responsible for recycling, cell survival is impaired. It will be interesting to determine in future studies whether Coronin-1 associates with this juxtamembrane region to facilitate TrkA recycling.

By definition, internalization of the signaling endosome at the distal axon followed by recycling at the somatic or dendritic plasma membrane is considered to be transcytosis. Although our study is the first report, to our knowledge, of retrograde transcytosis for NGF-TrkA, it has been found previously that as part of a maturation process, de novo–synthesized TrkA must be recycled to the neuronal cell surface before being anterogradely transported to the growth cone. Similarly to our study, the previous study found that these recycled endosomes are associated with Rab11 and expression of dominant-negative Rab11 blocked recycling and transcytosis, resulting in defects in axon growth. Rab11-dependent recycling has also been implicated in BDNF-TrkB-dependent dendrite formation and synaptic plasticity.

A role for Coronin-1 in preventing lysosomal fusion has been described previously in the context of pathogen-host interactions. However, this is the first example, to our knowledge, of Coronin-1 protecting a growth factor signaling endosome from lysosomal fusion. Beyond the specific calcium-dependent signaling pathways that Coronin-1 mediates, we suggest that it more generally influences the duration of signals emanating from the NGF-TrkA endosome, such as PI3K or ERK (Figs. 3d and 6c).

NGF-dependent Coronin-1 expression is a classic development feedback loop. What are the implications of this feedback loop on competition for survival? From our previous work we know that modulation of neurotrophin signal duration is critical during the developmental competition for survival described previously. Mathematical modeling suggests that a feedback loop consisting of an NGF-dependent change in NGF-TrkA signal duration is required for developmental competition for survival. This study suggests that the Coronin-1 feedback loop may be this critical element of competition that regulates NGF-dependent signal duration. We have updated our mathematical model to include new parameters from the empirical observations made in this study (Supplementary Figs. 4–6). Indeed, a Coronin-1 feedback loop fits well with the previous model and predicts in vivo survival phenotypes in CreoLa−/− animals (Fig. 6f and Supplementary Figs. 4–6). Future studies examining how the constituents of the signaling endosome change with each spatiotemporal trafficking step will lend insight into the logic underlying target-driven development of the peripheral nervous system.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We are grateful to D. Ginty (Harvard Medical School), in whose lab the initial phases of this work were conveyed, conducted and supported (US National Institutes of Health (NIH) grant 5RO1NS034814 and the Howard Hughes Medical Institute) and for providing Nrk1−/− mice and other mouse lines. We thank J. Pieters (Biozentrum, University of Basel) for providing CreoLa−/− mice. We also thank P. Neff, J.S. Cauley and the Keck Center for Biological Imaging for technical support. We are grateful to B. Condon, D. Ginty, R. Kuruvilla, N. Sharma, N. Watson, B. Winckler, M. Wheeler and the members of the Deppmann laboratory for helpful discussion. This work was supported by the Sloan Foundation, the University of Virginia Fund for Excellence in Science and Technology and the NIH National Institute of Neurological Disorders and Stroke (1R01NS072388).

**AUTHOR CONTRIBUTIONS**

C.D.D. and D.S. designed experiments. D.S. performed Coronin-1 expression analysis by immunostaining and immunoblot, all immunocytochemistry analyses, signaling analysis of AKT, CREB and ERK, calcium signaling experiments and in vivo neuron counts of the SCG. C.D.D. built plasmid constructs and performed in situ hybridization, RT-PCR, coimmunoprecipitation, luciferase assays and chick electropropora. J.P. quantified Flag-TrkA accumulation and disappearance and performed in vitro neuron death assays. L.S.Z. and A.W.H. performed endosomal fractionation biochemistry experiments. S.M. performed computational modeling. C.D.D., S.M. and D.S. wrote the manuscript. C.D.D. supervised the project.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Reprints and permissions information is available online at [http://www.nature.com/reprints/index.html](http://www.nature.com/reprints/index.html).

1. Dabrowski, A. & Umemori, H. Orchestrating the synaptic network by tyrosine phosphorylation signaling. J. Biochem. 149, 641–653 (2011).
2. Harrington, A.W. & Ginty, D.D. Long-distance retrograde neurotrophic factor signalling in neurons. Nat. Rev. Neurosci. 14, 177–187 (2013).
3. Lewin, G.R. & Barde, Y.A. Physiology of the neurotrophins. Annu. Rev. Neurosci. 19, 289–317 (1996).
4. Hendry, I.A., Stichel, K., Thoenen, H. & Iversen, L.L. The retrograde axonal transport of nerve growth factor. Brain Res. 68, 103–121 (1974).
5. Ehlers, M.D., Kaplan, D.R., Price, D.L. & Koliatsos, V.E. NGF-stimulated retrograde transport of trk in the mammalian nervous system. J. Cell Biol. 130, 149–156 (1995).
6. Delcroix, J.-D. et al. NGF signaling in sensory neurons: evidence that early endosomes carry NGF retrograde signals. Neuron 39, 69–84 (2003).
7. Ye, H., Kuruvilla, R., Zweifel, L.S. & Ginty, D.D. Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. Neuron 39, 57–68 (2003).
8. Sharma, N. et al. Long-distance control of synapse assembly by target-derived NGF. Neuron 67, 422–434 (2010).
9. Grimes, M.L. et al. Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. J. Neurosci. 16, 7950–7964 (1996).
10. Wu, C., Lai, C.F. & Mobley, W.C. Nerve growth factor activates persistent Rap1 signaling in endosomes. J. Neurosci. 21, 5406–5416 (2001).
11. Valdez, G. et al. Pincher-mediated macroendocytosis underlies retrograde signaling by neurotrophin receptors. J. Neurosci. 25, 5236–5247 (2005).
12. Watson, F.L. et al. Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat. Neurosci. 4, 981–988 (2001).
13. Harrington, A.W. et al. Recruitment of actin modifiers to TrkA endosomes governs retrograde NGF signaling and survival. Cell 146, 421–434 (2011).
14. Suzuki, K. et al. Molecular cloning of a novel actin-binding protein, p57, with a WD repeat and a leucine zipper motif. FEBS Lett. 364, 283–288 (1995).
15. Chan, K.T., Roadcap, D.W., Holoweckyj, N. & Bear, J.E. Coronin 1C harbours a second actin-binding site that confers co-operative binding to F-actin. J. Biochem. 144, 89–96 (2012).
16. Gafetdill, J., Albrecht, I., Zanolari, B., Steinmetz, M.O. & Pieters, J. Association of the leukocyte plasma membrane with the actin cytoskeleton through coiled-coiled trimeric coronin 1 molecules. Mol. Biol. Cell 16, 2786–2798 (2005).
17. Ferrari, G., Langen, H., Naito, M. & Pieters, J. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell 97, 435–447 (1999).
18. Jayachandran, R. et al. Survival of mycobacteria in macrophages is mediated by coronin 1-dependent activation of calcineurin. Cell 97, 435–447 (1999).
19. Deppmann, C.D. et al. A model for neuronal competition during development. Science 320, 369–373 (2008).
20. Crowley, C. et al. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76, 1001–1011 (1994).
21. Deckwerth, T.L. et al. BAX is required for neuronal death after trophic factor deprivation and during development. Neuron 17, 401–411 (1996).
22. Nguyen, L. & Pieters, J. The Trojan horse: survival tactics of pathogenic mycobacteria in macrophages. Trends Cell Biol. 15, 269–276 (2005).
23. Park, J.W., Vahidi, B., Taylor, A.M., Rhee, S.W. & Jeon, N.L. Microfluidic culture platform for neuroscience research. Nat. Protoc. 1, 2128–2136 (2006).
24. Glebov, O.O., Bright, N.A & Nichols, B.J. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat. Cell Biol. 8, 46–54 (2006).
25. Frampton, J.P., Guo, C. & Pierchala, B.A. Expression of axonal protein degradation machinery in sympathetic neurons is regulated by nerve growth factor. J. Neurosci. Res. 90, 1533–1546 (2012).
26. Ferguson, I.A., Schweitzer, J.B., Bartlett, P.F. & Johnson, E.M. Receptor-mediated retrograde transport in CNS neurons after intraventricular administration of NGF and growth factors. J. Comp. Neurol. 313, 680–692 (1991).
27. Philippidou, P. et al. Trk retrograde signaling requires persistent, Pincher-directed endosomes. Proc. Natl. Acad. Sci. USA 108, 852–857 (2011).
28. Julien, J., Guili, V., Reichardt, L.F. & Rudkin, B.B. Molecular kinetics of nerve growth factor receptor trafficking and activation. J. Biol. Chem. 277, 38700–38708 (2002).
29. Ascaño, M., Richmond, A., Borden, P. & Kuruvilla, R. Axonal targeting of Trk receptors via transcytosis regulates sensitivity to neurotrophin responses. J. Neurosci. 29, 11674–11685 (2009).
30. Cui, B. et al. One at a time, live tracking of NGF axonal transport using quantum dots. Proc. Natl. Acad. Sci. USA 104, 13666–13671 (2007).
31. Chen, Z.Y., Ieraci, A., Tanowitz, M. & Lee, F.S. A novel endocytic recycling signal distinguishes biological responses of Trk neurotrophin receptors. Mol. Biol. Cell 16, 5761–5772 (2005).
32. Fläger, N., Rangel, L., Danilenko, D.M. & Chan, A.C. Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. Science 313, 839–842 (2006).
33. Lonze, B.E. & Ginty, D.D. Function and regulation of CREB family transcription factors in the nervous system. Neuron 35, 605–623 (2002).
34. Leslie, M. Lost in translation: the signal hypothesis. J. Cell Biol. 170, 338 (2005).
35. Carpenter, G. & Cohen, S. 125I-labeled human epidermal growth factor. Binding, internalization, and degradation in human fibroblasts. J. Cell Biol. 71, 159–171 (1976).
36. Kuruvilla, R. et al. A neurotrophin signaling cascade coordinates synaptic neuron development through differential control of TrkB trafficking and retrograde signaling. Cell 118, 243–255 (2004).
37. Bodmer, D., Ascaño, M. & Kuruvilla, R. Isoform-specific dephosphorylation of dynamin1 by calcineurin couples neurotrophin receptor endocytosis to axonal growth. Neurotrophin 70, 1085–1099 (2011).
38. Kuruvilla, R., Ye, H. & Ginty, D.D. Spatially and functionally distinct roles of the PI3-K effector pathway during NGF signaling in sympathetic neurons. Neuron 27, 499–512 (2000).
39. Huang, S.-H. et al. BDNF-dependent recycling facilitates TrkB translocation to postsynaptic density during LTP via a Rab11-dependent pathway. J. Neurosci. 33, 9214–9230 (2013).
40. Lazo, O.M. et al. BDNF regulates Rab11-mediated recycling endosome dynamics to induce dendritic branching. J. Neurosci. 33, 6112–6122 (2013).
41. Levi-Montalcini, R. & Hamburger, V. Proλiferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. Exp. Zool. 111, 457–502 (1949).
Online methods

Antibodies. Antibodies were previously validated for the applications used, and the dilutions and applications were as follows: Coronin-1a (Abcam, ab53395, 1:400 for immunohistochemistry); Coronin-1a (Santa Cruz, sc-100925, 1:1,000 for western blotting); Coronin-1a (N-terminal) antibody produced in rabbit (Sigma, SAB4200072-25UL, 1:3,000 for western blotting); goat anti-mouse IgG (Cy-3) secondary antibody (Abcam, ab97035, 1:1,000 for western blots); pCREB (Ser133) (Cell Signaling Technology, 9191S, 1:1,000 for western blotting); M1 (anti-Flag M1, clone M1) (mouse) (Sigma, F3040, 1:1,000 for western blotting); Tubb3 (Covance, MMS-435P-250, 1:1,000 for western blotting); TrkA (rabbit) (Millipore, 06-574, 1:1,000 for western blotting, 1:500 for immunoprecipitation); Myc (9E-10), concentrate (DHBS 9E-10 (Myc) 1:1,000 for western blotting); pAkt (Cell Signaling Technology, 9271S, 1:1,000 for western blotting); pan Akt (Cell Signaling Technology, 2920S, 1:1,000 for western blotting); phospho-p44/42 MAPK (Cell Signaling Technology, 9106S, 1:1,000 for western blotting); Rab5 (Abcam, ab18211, 1:400 for immunohistochemistry, 1:1,000 for western blotting); and Rab11 (D4F5) XP rabbit monoclonal antibody (Cell Signaling Technology, 5898P, 1:400 for immunohistochemistry).

Animals. All experiments were carried out in compliance with the Association for Assessment of Laboratory Animal Care Policies and approved by the University of Virginia Animal Care and Use Committee. Sprague Dawley rats were purchased from Harlan. Sympathetic rat neurons were isolated from P0–P2 rat pups as described previously. All animals were used.

Tissue culture and transfection. Sympathetic neuron cultures were established as described previously. Briefly, neurons were obtained by dissociation of P0–P3 rat or mouse SCGs. These neurons were plated in mass or compartmentalized single cell cultures in supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin (1 U ml⁻¹) and 50 ng ml⁻¹ of NGF purified from mouse salivary glands. Glial contamination was removed from cultures using 5 µM cytosine arabinofuranoside or aphidicolin for 48 h, and NGF concentrations were changed as indicated. For microfluidic devices, neurons were given time to project their axons to the side or aphidicolin for 48 h, and NGF concentrations were changed as indicated.

RT-PCR. RT-PCR was performed as described previously. Briefly, RNA was isolated from mass sympathetic neuron cultures using TRIzol (Invitrogen) as the manufacturer's instructions. First-strand cDNA was synthesized using random hexamers and the Superscript III system (Invitrogen). The primers used for RT-PCR are as follows: GAPDH forward, ACCACTACCATCTCCAGGA, reverse, TTGTCTATACGGAAATGACC; Coronin-1: forward, TTTTCCATCAAGGAATGACC, reverse, GTCTCGAGATGGCTCTGATCTGTGAGGC, reverse, GGCTCTAGA AGCGCTGTTAGATTGTGTCGC; Coronin-2: forward, GGCGCTCAGAGGCGCACTACG; reverse, GCCGCTGAGCTCGAGCGAGCATAGTGG; Coronin-3: forward, GGCGCTCAGAGGCGCACTACG; reverse, GCCGCTGAGCTCGAGCGAGCATAGTGG.

Feeding and recycling assays. The TrkA-Flag feeding assay was performed as described previously. Briefly, the Flag M1 antibody (Sigma) was used to label distal axons of neurons isolated from Ntrk1Flag mice for 20 min at 4 °C. Unbound antibody was removed by rinsing the distal axon compartments with growth medium and then medium containing NGF (45 ng ml⁻¹). Cells were placed at 37 °C for 30 min and excess Flag antibody was washed away, followed by continued incubation for the indicated amounts of time to induce internalization and retrograde transport. Cells were then fixed with 4% paraformaldehyde, and internalized TrkA was visualized by anti-Flag immunostaining. A similar protocol was used to assess EGF internalization. In that case, fluorescent EGF was used, which negated the need for further immunostaining. To assess lysosome localization, Lysotracker was added 2 h before fixation on the cell body side of the microfluidic devices. Colocalization between endosomes and lysosomes was assessed by confocal microscopy and blinded, manual quantification.

For the recycling assay, we followed a protocol described by Lee and colleagues. Initially, Flag feeding assays were carried out as described above in neurons isolated from Ntrk1FLAG mice. 30 min before the end of the indicated chase period, a goat anti-mouse Cy-3 antibody was added to cell body or axon compartments of microfluidic devices. Cells were then fixed with 4% paraformaldehyde and imaged. Cy-3 puncta represent Flag TrkA endosomes that have undergone retrograde transport, recycled to the surface and re-internalized.
(50 µg ml⁻¹) and laminin (1 µg ml⁻¹). Total volume differential between the two compartments was maintained at 100 µl to ensure fluidic isolation.

**In vitro survival assays.** For cell survival assays in chambers, fluorescent microspheres (Invitrogen) were added to distal axons 24 h before scoring cell survival with Hoechst staining. For experiments in which NGF deprivation is called for, anti-NGF and Boc-Asp-FMK were used to neutralize any remaining NGF and keep cells alive, respectively. For mass cultures, the same procedure was followed without microspheres. Images of Hoechst staining were acquired and blinded for unbiased quantification. Dead and alive neurons were scored as described previously. The top concentration of NGF on wild-type neurons was set to 1, and all other conditions are indicated relative to that.

**Immunocytochemistry.** Immunocytochemistry and immunohistochemistry were performed as described previously. Neurons or sections were fixed with 4% paraformaldehyde and blocked and permeabilized (5% goat serum and 0.05% Triton X-100 in PBS) for 30 min at room temperature. Cells were then incubated overnight at 4 °C with primary antibody diluted in blocking buffer. Cells were then washed three times with 1× PBS and incubated with fluorescent secondary antibody for 1 h at room temperature followed by three washes in 1× PBS and mounting in fluoromount (Vectorshield). Fluorescence was visualized using inverted confocal microscopy; and three-dimensional reconstructed images were made in ImageJ.

**Calcium imaging.** Calcium imaging was performed as previously described. Briefly, neurons grown on cover glass were loaded with Fluo-4 calcium indicator (3 µM) for 20 min at 37 °C (5% CO₂). The neurons were then moved to an environmental chamber (37 °C, 5% CO₂) mounted on an inverted confocal microscope. Basal calcium levels were assessed by randomly imaging six to ten cells per coverslip before treatment. The indicated concentrations of NGF were added to the neurons and allowed to incubate for 15 min. Randomly chosen neurons per coverslip before treatment. The indicated concentrations of NGF were added to the neurons and allowed to incubate for 15 min. Randomly chosen neurons per coverslip before treatment. The indicated concentrations of NGF were added to the neurons and allowed to incubate for 15 min. Randomly chosen neurons per coverslip before treatment. The indicated concentrations of NGF were added to the neurons and allowed to incubate for 15 min.

**In vivo sympathetic neuron counts.** SCG neuron number was quantified as described previously. Briefly, trunks from P0 mice were snap frozen in OCT and cryosectioned at a thickness of 10 µm. Every fifth section was processed for Nissl staining. Images were acquired, blinded and quantified as previously described.

**Biochemical analysis of endosomes.** Magnetic isolation of newly internalized vesicles was performed using a colloidal suspension of ferric oxide particles having an average diameter of 10 nm (Liquids Research Ltd., Bangor, UK). Approximately 20 million TrkB/A cells were suspended in 9 ml of DMEM and 1 ml of ferrofluid suspension. To one sample, 50 ng ml⁻¹ BDNF was added, and cells were incubated at 37 °C for 15 min. Cells were then pelleted, homogenized and processed as described previously to obtain a crude endosomal fraction. Samples were then placed adjacent to a magnet for 1 h, and ferrofluid-containing endosomes were isolated and collected from the sides of the sample tubes by the addition of Laemmli sample buffer. Discontinuous sucrose gradients used to separate early and late endosomal compartments were performed exactly as described previously.

**Phylogeny and similarity analysis.** Amino acid sequences of Coronin family members were analyzed using phylogeny.fr. Amino acid similarity analysis was performed using ClustalW.

**Statistical analyses.** No statistical method was used to predetermine sample size; however, we chose sample sizes similar to those reported in previous publications. Data were collected randomly and assessed blindly. The data distribution was assumed to be normal, but this was not formally tested. Statistical analyses were based on at least three experiments and are described in the figure legends. All error bars represent the s.e.m.

42. Zareen, N. & Greene, L.A. Protocol for culturing sympathetic neurons from rat superior cervical ganglia (SCG). J. Vis. Exp. doi:10.3791/988 (30 January 2009).
43. Knudson, C.M., Tung, K.S.K., Tourellotte, W.G., Brown, G.A.J. & Korsmeyer, S.J. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270, 96–99 (1995).
44. Glebova, N.O. & Ginty, D.D. Heterogeneous requirement of NGF for sympathetic target innervation in vivo. J. Neurosci. 24, 743–751 (2004).
45. Kuruvilla, R. et al. A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkB trafficking and retrograde signaling. Cell 118, 413–423 (2003).
46. Park, J.W., Vahidi, B., Tayloy, A.M., Rhee, S.W. & Jeon, N.L. Microfluidic culture platform for neuroscience research. Nat. Protoc. 1, 2128–2136 (2006).
47. Conkright, M.D. et al. TORCs: transducers of regulated CREB activity. Mol. Cell 12, 243–255 (2004).
48. Rao, M., Baraban, J.H., Rajaii, F. & Sockanathan, S. In vivo comparative study of RNAi methodologies by in ovo electroporation in the chick embryo. Dev. Dyn. 231, 592–600 (2004).
49. Mandai, K. et al. LIG family receptor tyrosine kinase-associated proteins modulate growth factor signals during neural development. Neuron 63, 614–627 (2009).
50. Mueller, P. et al. Regulation of T cell survival through coronin-1–mediated generation of inositol-1,4,5-trisphosphate and calcium mobilization after T cell receptor triggering. Nat. Immunol. 9, 424–431 (2008).
51. Deckwerth, T.L. & Johnson, E.M. Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. J. Cell Biol. 123, 1207–1222 (1993).
52. Gomez, T.M., Robles, E., Poo, M. & Spitzer, N.C. Filopodial calcium transients promote substrate-dependent growth cone turning. Science 291, 1983–1987 (2001).
53. Chen, X. et al. A chemical-genetic approach to studying neurotrophin signaling. Neuron 46, 13–21 (2005).