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

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Abstract

Retrograde communication from axonal targets to neuronal cell bodies is critical for both 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 mainly uncharacterized. Here we report that in mouse sympathetic neurons the target-derived NGF-TrkA signaling endosome, upon arrival at the cell body, induces the expression and recruitment of a novel effector protein known as Coronin-1. In the absence of Coronin-1, the NGF-TrkA signaling endosome fuses to lysosomes 6–10 fold faster than when Coronin-1 is intact. We also define a novel Coronin-1-dependent trafficking event where signaling endosomes recycle and re-internalize upon 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 CREB phosphorylation. These results establish Coronin-1 as an essential component of a novel feedback loop mediating 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

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Author Contributions:

C.D.D. and D.S. designed experiments. D.S. performed: coronin-1 expression analysis by immunostaining and immunoblot, all immunocytochemistry, signaling analysis of AKT, CREB, and ERK, calcium signaling experiments, and *in vivo* neuron counts of the SCG. C.D.D. built plasmid constructs, performed *in situ* hybridization, RT-PCR, coimmunoprecipitation, luciferase assays, and chick electroporation. J.P. quantified Flag-TrkA accumulation/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.

signal integrity. This form of communication is particularly important in the development and maintenance of the peripheral nervous system (PNS) where the assembly of neural circuits is coordinated by the target organs they innervate and control.

Amongst 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 coordinates the development of functional circuits[1,2]. Neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5), signal through two distinct receptor systems, the Trk family of receptor tyrosine kinases (RTKs), and 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 axons/growth cones in the periphery and travel back to neuronal cell bodies[4–8].

In recent years several effector proteins have been found to confer unique properties to long-distance, retrograde signaling endosomes. In particular, phospholipase C-gamma (PLC-γ1), rap1, pincher, phosphatidylinositol 3-kinase (PI3K), ERK5, and cofilin have been shown to associate with the NGF-TrkA signaling endosome and that they are functionally significant in the context of *in vitro* survival assays[6,9–13]. An emergent principle for endosomal-associated effectors is to play multiple roles, not only in signaling to promote developmental events, but also in trafficking and maturation. For example, it has recently been found that association of the actin modifying protein, cofilin, is necessary for NGF-TrkA retrograde trafficking[13]. Several questions remain about this process including: Which proteins/signaling pathways are essential for trafficking events such as internalization, recycling, long-distance transport, or lysosomal fusion? 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 novel effector protein for the NGF-TrkA signaling endosome. Coronin-1 is part of a family of structurally related proteins known for interacting with cytoskeletal proteins, such as F-actin[14–16] (Supplementary Figs.1A–C). Although Coronin family members share similar structure and neuronal expression patterns, they do not appear to be functionally redundant. While 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 interaction. Previous reports concluded that *Mycobacterium tuberculosis* recruits Coronin-1 upon engulfment by macrophages, in order to avoid subsequent lysosomal fusion and evade phagocytic degradation[17]. In the absence of Coronin-1 or when the bacteria are heat killed, the pathogenic phagosome rapidly fuses to lysosomes. More recently it has been shown 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[18]. 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 target organ and neuronal cell body.

Here we find that Coronin-1 expression and association 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 novel 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 novel NGF-Coronin-1 feedback loop is critical for competition for survival during development.

Results

NGF is necessary and sufficient for Coronin-1 expression in developing sympathetic neurons

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, we speculated that NGF would induce a putative factor 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 not only that NGF have the capacity to induce the factor, but also that it have an ontology consistent with membrane or vesicle association. We found 77 genes that met this criteria displaying a more than 2-fold reduction in expression in SCGs from $NGF^{-/-};Bax^{-/-}$ compared to controls $Bax^{-/-}$ mice at postnatal day 0 (P0) (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 (Coro1a) met the criteria of being associated with vesicles and was identified as being reduced 7.34 fold in SCGs from NGF null mice ($NGF^{-/-};Bax^{-/-}$ P0 mice) compared to controls ($Bax^{-/-}$) (Fig. 1A).

The expression pattern of Coronin family members broadly overlaps in the peripheral nervous system. In particular, TrkA positive peripheral neurons of the dorsal root ganglion show expression of all Coronin family members examined (Supplementary Fig. 2A). In the CNS, it appears that, while Coronin family members are highly expressed (*e.g.* cortical plate, hippocampus) they are not highly expressed in structures that are classically and discretely TrkA positive (*e.g.* 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. Antibody specificity was verified by comparing immunostained cryosections from wild-type and $Coronin\text{-}1^{-/-}$ mice (Supplementary Fig. 1D). Consistent with microarray data, Coronin-1 mRNA and protein levels are dramatically reduced in P0 SCGs from $NGF^{-/-};Bax^{-/-}$ relative to $Bax^{-/-}$ (Fig. 1B and C). We next sought to determine whether NGF is sufficient to induce *Coronin-1* expression *in vitro*. Sympathetic neurons were deprived of NGF in the presence of the broad spectrum caspase

inhibitor, Boc-Asp-FMK (BAF), to prevent cell death; NGF (45 ng/mL) was reapplied for the indicated amounts of time and mRNA and protein levels were analyzed via RT-PCR and immunoblot, respectively (Fig. 1D–F). In the absence of NGF, *Coronin-1* transcript and protein levels were low or undetectable and rapidly induced upon NGF re-addition (Figs. 1D–F). We next defined the developmental timing of Coronin-1 expression via immunohistochemistry for Coronin-1 on cryosections containing SCGs from E15, E18, P0, and P5 mice. (Fig. 1G). Consistent with the notion that NGF is required for Coronin-1 expression, a dramatic increase in the level of Coronin-1 protein was observed after final target innervation (represented by P0) as compared to stages prior to, and at the beginning of, target innervation (represented by E15 and E18, respectively). These data indicate that final target innervation and exposure to NGF is necessary and sufficient for Coronin-1 expression in the sympathetic nervous system.

Coronin-1 associates with TrkA and the signaling endosome in an NGF-dependent manner

Previous studies on pathogen-host interactions demonstrated a physical association between Coronin-1 and the *M. tuberculosis* phagosome[17]. 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 $TrkA^{Flag}$ mice where a Flag epitope has been knocked in frame with the extracellular domain of TrkA[8]. These neurons were grown in microfluidic devices and distal axons were pulsed with anti-Flag antibody (M1) and NGF for 30 minutes at 37°C [23] (Figs. 2A, B). At different time points after the NGF/M1 pulse, neurons were fixed and Flag-TrkA localization was detected using immunofluorescence. Imaging the cell body compartment ensures that the punctae observed represent long-distance, retrogradely transported signaling endosomes.

We first used this assay to examine co-localization between the signaling endosome and Coronin-1. We observed that roughly 70% of signaling endosomes co-localized with Coronin-1 in cell bodies one hour after NGF/α-Flag treatment on distal axons (Figs. 2C, D). This persists for at least 6.5 hours followed by a gradual decrease to roughly 40% at 24 hours post-NGF/α-Flag pulse. Notably, at 2.5 hours post-NGF/M1 pulse, co-localization between endosomal TrkA and Coronin-1 is 3-fold higher in cell bodies (59.27% ± 6.66%) compared to axons(19.49% ± 5.01%) indicating that this may be a subcellular compartment-dependent interaction, A lower magnification visualization of Coronin-1 in both dissociated neurons and electroporated chick spinal cord/DRG reveals that Coronin-1 is also highly localized in axons and the growth cones (Supplementary Figs. 1E–F). Coronin-1 association with the signaling endosome was also tested 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 following BDNF treatment, but retains TrkA downstream signaling[13]. Upon ligand stimulation, Coronin-1 and TrkB/A are both shifted into fractions containing early endosomes. While a relatively small amount of TrkB/A is detected in late endosome fractions after BDNF treatment, Coronin-1 remains in early endosome fractions, suggesting a role for Coronin-1 at the early endosome stage (Fig. 2E).

To further explore whether Coronin-1 and TrkA exist on endosomes of a similar maturation stage, we magnetically enriched BDNF-containing endosomes as described previously[24]. Briefly, cultured TrkB/A PC12 cells were treated with a BDNF/ferrofluid mixture allowing BDNF-TrkB/A containing endosomes to be isolated by magnetic purification. TrkB/A, Coronin-1 and the early endosome marker, Rab5, were enriched in conditions where NGF and the ferrofluid were added together consistent with the notion that Coronin-1 is physically associated with the NGF-TrkA signaling endosome (Fig. 2F). These data demonstrate that in response to neurotrophin signaling, Coronin-1 associates with TrkA on internalized membranes similar to early endosomes. Next we examined whether Coronin-1 can interact with TrkA in conditions where endosomal integrity is disrupted with detergent. We performed a co-immounoprecipitation (co-IP) between myc-tagged Coronin-1 and TrkA from PC12 cells lysed in Triton X-100 buffer in the presence or absence of NGF. Coronin-1 co-purifies with TrkA in both of these conditions (Fig. 2G). Together with the gradient sedimentation, colocalization, and magnetic purification experiments, these data suggest that not only does NGF regulate *Coronin-1* expression, but it also drives Coronin-1 association with the NGF-TrkA signaling endosome.

Coronin-1 stabilizes the signaling endosome by preventing lysosomal fusion

Coronin-1 is known to be a requisite host factor for *M. tuberculosis* survival in macrophages[17]. They concluded that this protection involves preventing engulfed *M. tuberculosis* from undergoing lysosomal fusion. These previous studies, along with our finding that Coronin-1 associates with NGF-TrkA, suggest that Coronin-1 might serve a novel non-pathogenic role in preventing the neurotrophin signaling endosome from lysosomal fusion. Consistent with this, in sympathetic neurons, we found less than 10% co-localization between Coronin-1 and lysosomes as measured by immunocytochemistry for Coronin-1 and Lysotracker staining (Fig. 3A).

To determine whether Coronin-1 can prevent NGF-TrkA signaling endosome degradation, we used an α-Flag antibody feeding assay in combination with Lysotracker staining to identify TrkA-containing lysosomes. Co-localization between these markers was performed following a 30-minute pulse of NGF/α-Flag on distal axons and a chase period for the indicated periods of time (Figs. 3B–C). In neurons isolated from $TrkA^{Flag}$ mice, co-localization remains below 10% until more than 24 hours post-NGF/α-Flag pulse (Fig. 3C). In contrast, neurons isolated from $TrkA^{Flag}$;*Coronin-1* $^{-/-}$ mice showed signaling endosomes rapidly fusing with lysosomes, as reflected by a co-localization in the cell body of 3–8 fold higher than in control neurons at all time-points post-pulse (Figs. 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, since loss of *Coronin-1* appears to have no effect on lysosomal fusion in axons (Fig 3D). Importantly, loss of *Coronin-1* does not influence the total number of lysosomes in cell bodies or axons in the context of these experiments. (Supplementary Fig. 3A).

To determine whether Coronin-1 broadly influences lysosomal fusion, we examined degradation of internalized EGF in the presence or absence of *Coronin-1*. Consistent with

previous reports fluorescent EGF does not undergo retrograde transport (data not shown)[26]. Therefore, these ligands were fed to sympathetic cell bodies for 30 minutes and internalized ligands and lysosomes were visualized 6 hours later. Consistent with previous observations, co-localization of EGF with lysosomes was 57.52% ± 3.8% in neurons isolated from wild type animals[27] (Fig. 3E). Moreover, in the absence of Coronin-1 internalized EGF fuses to lysosomes in a manner similar to wild type (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 an anti-Flag antibody feeding assay on neurons isolated from *TrkA*^{Flag} mice where NGF was applied to the cell body instead of distal axons for 6.5h. In the presence of Coronin-1, 7.3% ± 5.33% of lysosome puncta colocalized with Flag-TrkA whereas in the absence of Coronin-1 this colocalization is elevated to 40% ± 12.64%. Taken together, these data suggest that Coronin-1 may specifically influence the stability of a particular subset of internalized receptors, such as those involved in long-distance retrograde signaling.

We have previously reported a series of NGF-dependent positive feedback loops, which are critical for developmental competition for survival[19]. One of these feedback loops involves NGF-dependent extension of NGF's own downstream signal duration. Upon NGF deprivation, neurons that are grown in the presence of NGF for 4 days display prolonged phospho-Akt (P-Akt) signaling compared to neurons grown for 1 day in the presence of NGF[19]. To address whether this change in signal duration is due to Coronin-1 dependent stabilization of TrkA, we examined the decay of P-Akt upon NGF withdrawal in neurons isolated from wild type or *Coronin-1*$^{-/-}$ mice (Fig. 3F,G). P-Akt levels from wild type neurons remain stable for over 4 hours after NGF deprivation, whereas neurons from *Coronin-1*$^{-/-}$ mice display a much faster P-Akt decay rate. These data are consistent with the notion that Coronin-1 is required for a feedback loop where NGF modulates the persistence of its own signaling through up-regulation and recruitment of Coronin-1.

Coronin-1 facilitates recycling of retrogradely transported NGF-TrkA at the cell body

The above findings that retrogradely trafficked NGF-TrkA takes roughly 30 hours for significant lysosomal fusion to occur in the cell body, are inconsistent with previous findings demonstrating that PC12 cells downregulate TrkA within 2–3 hours[28]. This prompted us to examine NGF-TrkA endosome accumulation in the cell body as a function of time after anti-Flag antibody/NGF pulse on distal axons. Remarkably, we observed very little difference in the initial accumulation and clearance of endosomes after pulse, despite the roughly 6-fold difference in rate of lysosome fusion in the presence versus absence of *Coronin-1* (Fig. 4A). However, after the initial wave of Flag-TrkA accumulation at the cell body (>8hrs), a significant and sustained doubling of internalized Flag-TrkA was observed in wild-type over *Coronin-1*$^{-/-}$ neurons (Fig. 4A).

If not lysosomal fusion, what might be the mechanism for clearance of NGF-TrkA? NGF-TrkA signaling endosomes have been reported to correspond to early and recycling endosome compartments marked by Rab5 and Rab11, respectively[29,30]. Therefore, we examined Coronin-1 co-localization with these markers in different subcellular compartments. Notably, Coronin-1 preferentially associates with Rab11 over Rab5 in the

cell body, but no significant differences were observed in the proximal or distal axon compartments (Fig. 4B). Does Coronin-1 influence NGF-TrkA association with Rab11? To address this, we performed Flag feeding assays on neurons from *TrkA*^{Flag} or *TrkA*^{Flag}*;Coronin-1*$^{-/-}$ mice as described in Fig. 2A, and performed immunocytochemistry for internalized Flag-TrkA and Rab11. Remarkably, loss of *Coronin-1* displays a dramatic reduction in Flag-TrkA co-localization with Rab11 where 45.68% ± 6.75% of Flag-TrkA puncta are Rab11 positive in neurons from *TrkA*^{Flag} mice compared to 4.58% ± 3.68% in *TrkA*^{Flag}*;Coronin-1*$^{-/-}$ neurons (Fig. 4C). We next sought to determine whether Coronin-1 influences recycling of long-distance NGF-TrkA signaling endosomes. To this end, we took advantage of a derivation of the Flag feeding assay, where anti-Flag antibody/NGF are fed to distal axons and an anti-mouse Cy-3 antibody is applied to the cell body similar to an assay previously performed[31]. Cy-3 positive punctae only appeared if retrogradely transported Flag-TrkA traveled back to the Soma, recycled to the plasma membrane, and re-internalized (Supplementary Fig. 3B). Indeed, when Coronin-1 was intact, these long-distance endosomes efficiently recycled and reinternalized at 2.5 and 6 hours after feeding, whereas this did not appear to be the case when *Coronin-1* was absent (Figs. 4D, E). This appears to apply uniquely to endosomes that have arrived at the soma, since we observed very little recycling when the Cy-3 antibody was applied to axons. Taken together, these data suggest that *Coronin-1* is dispensable for internalization and retrograde transport of NGF-TrkA, but essential for recycling and evading lysosomal fusion.

Coronin-1 is required for NGF-TrkA dependent calcium mobilization and CREB activation

A generalized influence on NGF-TrkA dependent signals might be expected based on our finding that Coronin-1 is involved in signaling endosome maturation, however it remains unclear whether Coronin-1 represents a modulator for specific NGF-TrkA dependent pathways. Therefore, we next sought to determine whether Coronin-1 influences downstream NGF-TrkA pathways, independent of its role in NGF-TrkA signal duration. Coronin-1 has previously been reported to be important for calcium mobilization in the context of T-cell development and pathogen-host interaction[18,32]. Therefore, we hypothesized that Coronin-1 links the neurotrophin signaling endosome to calcium signaling. To test this possibility, we examined NGF-induced calcium release in sympathetic neurons grown in microfluidic devices isolated from P0 wild type or *Coronin-1*$^{-/-}$ mice. To visualize calcium, we loaded cells with Fluo-4 for 20 minutes followed by treatment of indicated NGF concentrations for 15 minutes. As expected, increasing concentrations of NGF led to increasing calcium levels in wild type neurons (Figs. 5A, B). In the absence of *Coronin-1*, NGF loses its ability to induce calcium release (Figs. 5A, B). NGF-TrkA signaling is also known to activate the pro-survival transcription factor, CREB, through a variety of upstream kinases including, PKA, CAMK2, and RSK[33]. To assess whether Coronin-1 also influences NGF-dependent CREB activation, Coronin-1 was knocked down in PC12 cells and NGF-dependent phosphorylation of CREB and ERK was assessed. Loss of Coronin-1 significantly dampened NGF induction of CREB but not ERK phosphorylation (Fig. 5C). Next, *Coronin-1* was knocked down and 3xCRE-luciferase reporter gene assays were performed in sympathetic neurons to assess CREB transcriptional activity (Fig. 5D and Supplementary Fig. 3C). Loss of *Coronin-1* completely abrogated NGF induction of 3xCRE luciferase activity, even at high NGF concentrations (Fig. 5D). Importantly, loss of

Coronin-1 does not universally disrupt CREB activation since forskolin-induced increase of cAMP was able to induce 3xCRE luciferase activity with and without knockdown. Taken together, these data indicate that Coronin-1 is an effector for not only NGF-TrkA trafficking and recycling, but independent of this, signaling through calcium and CREB.

Coronin-1 stabilizes the signaling endosome via calcium/calcineurin signaling

Having established that Coronin-1 is necessary for NGF to induce calcium release, we sought to determine how this NGF-dependent calcium signaling is related to NGF-TrkA endosomal stability. We used the cell permeable calcium chelator, BAPTA-AM, to determine if lowering intracellular calcium levels causes increased fusion of signaling endosomes to lysosomes. We were surprised to find that the ratio of signaling endosome/lysosomal fusion in BAPTA-AM treated neurons from $TrkA^{Flag}$ mice phenocopied that observed in the absence of *Coronin-1* (Fig. 5E). Moreover, increasing intracellular calcium levels with the calcium ionophore, calcimycin, partially rescued the *Coronin-1* null phenotype (Fig. 5E). Taken together these data suggest that NGF-TrkA endosome stability is directly related to its ability to induce calcium release.

Given that pathogens like *M. tuberculosis* use the calcium-dependent protein phosphatase, calcineurin, to avoid degradation, we speculated that the signaling endosome may use a similar mechanism. We used either cyclosporin A (CsA) or FK506 to assess whether inhibition of calcineurin on neuronal cell bodies resulted in an increase in the ratio of signaling endosome/lysosomal fusion. Similar to BAPTA-AM treatment, CsA or FK506 treatment of neurons from $TrkA^{Flag}$ mice resulted in elevated endosomal fusion to lysosomes, which phenocopied the fusion rates observed in $TrkA^{Flag};Coronin\text{-}1^{-/-}$ neurons (Fig. 5F). Consistent with Figure 3E, inhibition of calcium or calcneurin signaling does not influence EGF-lysosomal fusion in wild type or $Coronin\text{-}1^{-/-}$ 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 Coronin-1 protection of endosomal TrkA and its impact on competition for survival

We and others have previously built computational models describing competition for survival in developing neurons[19,34]. This model describes how neurons with similar responsiveness and access to target derived trophic factor can distinguish themselves from one another[1]. This requires several feedback loops that promote a ‘bistability’ resulting in either survival or apoptosis. Two of these feedback loops are required for competition to occur and include: (1) NGF regulating the expression of its own receptor, TrkA, resulting in an enhanced ability to take up NGF, as well as increased robustness of downstream signaling pathways in neurons that ‘win’ the competition and (2) NGF regulation of 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 Figure 3C[19]. We first fit a rate

of active TrkA degradation in the presence or absence of Coronin-1, which matches the observed sharp sigmoidal experimental values for signaling endosome-lysosome fusion (Fig. 3C, (Supplementary Figs. 4–6)). In the absence of Coronin-1 the rate of endosome-lysosome fusion hastens dramatically. 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 within 2 hours after internalization[35]. This suggests the existence of an additional, Coronin-1 independent, mechanism regulating NGF-TrkA stability. It stands to reason that this putative mechanism would 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 3 post-endocytic TrkA pools defined by their stability: (1) those with Coronin-1, which are very stable (protected), (2) those without Coronin-1 that are moderately stable (unprotected), and (3) those without Coronin-1 that are highly unstable (punished). We have modeled the impact of this putative Coronin-1-independent degradation pathway under conditions of low (Supplementary Fig. 4A–F) or high (Supplementary Fig. 4G–L) trophic factor availability. Notably, the only conditions that display multiple intersections representing a life/death bistable system are those that have 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).

Based on 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[19]. Although punishment of unprotected endosomes may occur via a different mechanism than punishment via 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 (Figs. 6A–B). Finally, 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[19] (Fig. 6B). These simulations are consistent with the target matching hypothesis where a drop to half of NGF production as observe in *NGF +/−* mice leads to loss of half of the neurons, respectively. 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 sympathetic neuron survival

Testing these predictions in sympathetic neurons, we first examined retrograde NGF-dependent survival in neurons from *Coronin-1*$^{-/-}$ and wild type mice. These neurons were established in microfluidic devices and the indicated NGF concentrations were applied to distal axons for 36 hours at which time neuronal death was assessed by Hoechst staining. Increasing the concentration of NGF exclusively on distal axons results in increased survival in wild type neurons. *Coronin-1*$^{-/-}$ neurons display low levels of survival relative to wild type controls even at 100 ng/mL of NGF indicating an essential role for Coronin-1 in long-distance survival signaling (Fig. 6C). Notably, 10 ng/mL NGF applied to the cell body of *Coronin1*$^{-/-}$ neurons is sufficient to support full survival relative to wild type controls. However, lower concentrations of NGF (0.1 and 1 ng/mL) applied to cell bodies fail 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[19]. Consistent with modeling data, in the absence of *Coronin-1* we observe a dramatic reduction in sympathetic neurons similar to that observed in SCGs from *NGF*$^{+/-}$ mice (Fig. 6D, E). Moreover in SCG neurons from *NGF*$^{+/-}$*;Coronin1*$^{-/-}$ P0 mice we observed a further reduction of neuron number compared to *NGF*$^{+/-}$ and *Coronin-1*$^{-/-}$ 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 significantly 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 a similar trophic state, which we suggest is a key feature of the target-matching hypothesis.

Discussion

We demonstrate that Coronin-1 participates in a novel NGF-TrkA feedback loop that is required for proper development of the sympathetic nervous system. Target-derived NGF is necessary and sufficient for *Coronin-1* 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 associated with endosome arrival at the cell body including: 1) a novel transcytosis

event and 2) avoidance of lysosomal fusion. Loss of Coronin-1 results in abnormal neurotrophin signaling and trafficking which results in a diminished capacity of neurons to compete with one another, manifesting in excess developmental neuron death (Supplementary Figs 4–6). This defines a novel 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. These questions include: 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 a novel 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[2]. 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 is established that sympathetic neuron survival requires long-distance endosomal transport and signaling from distal axons to the cell body[7,36]. Therefore it is not surprising that the endosome would associate with an effector like Coronin-1 upon arrival at the cell body, which would endow NGF-TrkA-containing endosomes with several specific pro-survival 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 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[8,37,38]. The recruitment of Coronin-1 to the NGF-TrkA signaling endosome seems to fit into this theme, since it regulates two key trafficking events at the cell body: transcytosis/recycling and avoidance of lysosomal fusion.

The requirement of recycling is becoming recognized as a critical component of neurotrophin signaling. Lee and colleagues demonstrated that in mass cultures, TrkA undergoes a relatively high rate of recycling and that by mutating the TrkA juxtamembrane region responsible for recycling, cell survival is impaired[31]. In future studies it will be interesting to determine 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/dendritic plasma membrane is considered transcytosis. While our study is the first report of retrograde transcytosis for NGF-TrkA, it has been previously found that as part of a maturation process, *de novo* synthesized TrkA must be recycled to the neuronal cell surface prior to being anterogradely transported to the growth cone[29]. Similar to our study, they 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[39,40].

A role for Coronin-1 in preventing lysosomal fusion has previously been described in the context of pathogen-host interaction[17]. However, this is the first example 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 duration of signals emanating from the NGF-TrkA endosome like PI3K or ERK (Figs. 3D, 6C).

NGF-dependent Coronin-1 expression represents 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 first described by Hamburger and Levi-Montalcini[41]. 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[19]. This study suggests that the Coronin-1 feedback loop may represent this critical element of competition regulating NGF-dependent signal duration. We have updated our mathematical model[19] 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 *Coronin-1*$^{-/-}$ animals (Fig. 6F, Supplementary Figs. 4–6). Future studies examining how the constituents of the signaling endosome change with each spatio-temporal trafficking step promise to lend insight into the logic underlying target-driven development of the peripheral nervous system.

Online Methods

Antibodies

Antibodies were previously validated for the applications used and dilutions/applications were as follows: Coronin 1a(Abcam ab53395 1:400IHC); Coronin 1A (Santa Cruz sc-100925 1:1000WB); Anti-Coronin-1A (N-terminal) antibody produced in rabbit(Sigma SAB4200078-25UL 1:1000WB); Goat anti-Mouse IgG H&L (Cy3 ®) secondary antibody (Abcam ab97035 1:1000WB); Phospho-CREB (Ser133) Antibody (Cell Signaling Technology 9191S 1:1000WB); M1 [ANTI-FLAG M1, CLONE M1] (mouse) (Sigma F3040 1:1000WB); Tubb3-antibody (Covance MMS-435P-250 1:1000WB); Anti-TrkA (rabbit) (Millipore 06-574 1:1000WB 1:500IP); myc (9E-10), concentrate (DHSB 9E-10 (myc) 1:1000WB); Phospho-Akt (Cell Signaling Technology 9271S 1:1000WB); pan Akt (Cell Signaling Technology 2920S 1:1000WB); Phospho-p44/42 MAPK (Cell Signaling Technology 9106S 1:1000WB); Anti-Rab5 antibody (Abcam ab18211 1:400 IHC 1:1000WB); Rab11 (D4F5) XP® Rabbit mAb (Cell signaling Technology 5589P 1:400 IHC).

WB-western blot, IP-immunoprecipitation, IHC-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 previously described[42].

All animals were maintained in a c57bl6 background. *NGF*$^{-/-}$[20] and *Bax*$^{-/-}$ [43] genotyping and generation of *Bax*$^{-/-}$*; NGF*$^{-/-}$ animals was described previously[44]. *TrkA*^{Flag} animals were a generous gift from David Ginty and genotyping of this allele was described previously[8]. *Coronin1*$^{-/-}$ mice were a generous gift from Jean Pieters and genotyping was performed as described previously[18]. *TrkA*^{Flag}*;Coronin-1*$^{-/-}$ mice could be generated at Mendelian ratios and bred in the double homozygous state. Mice were maintained in a BL6 background.

Tissue culture and transfection

Sympathetic neuron cultures were established as described previously[19]. Briefly, neurons were obtained by dissociation of P0–P3 rat or mouse superior cervical ganglia. These neurons were plated in mass or compartmentalized microfluidic devices in DMEM supplemented with 10% FBS, penicillin/streptomycin (1U/mL), and 50 ng/mL of NGF purified from mouse salivary glands [45,46]. Glial contamination was removed from cultures using either 5 μM cytosine arabinofuranoside (Ara-C) or aphidicolin for 48 hours and NGF concentrations were changed as indicated. For microfluidic devices, neurons were given time to project their axons to the outer chamber (2–4 DIV) and indicated treatments were performed for 36 hours. Sympathetic neurons were transfected using the amaxa nucleofector system (Lonza) according to manufacturer's protocol.

Normal PC12 cells or PC12 cells stably expressing the TrkB/A chimeric protein[13] were maintained in DMEM (Invitrogen), supplemented with 1 U/mL penicillin-streptomycin (Invitrogen) and 5% horse serum. PC12 cell were transfected using lipofectamine 2000 (Promega) according to manufacturer's instruction.

The siRNAs that were used were either pre-validated (LaminA/C) or custom designed. Both were purchased from Dharmacon/Invitrogen. The custom siRNA had the following sequence:

Coronin-1: Sense-5′ Th CCAUGACAGUGCCUAGAAAdT

Antisense-5′ UUUCUAGGCACUGUCAUGGdTdT

Reporter gene assays

3x-CRE reporter gene assays were performed as previously described[47] and according to manufacturer's instructions for the dual luciferase renilla kit (Promega). Briefly, sympathetic neurons or pc12 cells were transfected with 3x-CRE luciferase and pRenilla-null vectors as well as the indicated siRNAs. 24 hours after transfection cells were treated with the indicated concentrations of NGF or 10uM Forskolin. Cell extracts were prepared

using passive lysis buffer (Promega) and luminescence was measured. Luciferase activity was normalized to Renilla activity.

In ovo Electroporation

Chick electroporation was performed as previously described[48]. Briefly, myc-coronin-ires-GFP driven in the pCAGGS expression vector was injected into chick neural tube at Hamburger Hamiltpon stage 11–13. Half of the neural tube was electroporated using a BTX Electrosquare porator ECM 830 from BTX configured to deliver 5×50msec pulses of 30V through a pair of gold plated electrodes- 5mm (length), 0.5mM (diameter). Eggs were incubated for 2–3 days and chick spinal cords were sectioned and stained for GFP or myc.

Co-immunoprecipitations and Immunoblot analysis

Co-immunoprecipitation and immunoblot analysis was performed as previously described[49]. Briefly, sympathetic neurons or PC12 cells were harvested as previously described[50] in (Tris 50mM pH=7.5, NaCl 137 mM, 2mM EDTA, 10%glycerol, 1% Triton-X-100, 0.05% digitonin) containing protease and phosphatase inhibitor cocktails (Roche). Lysates were cleared by centrifugation at 14,000xg for 10 minutes at 4°C. Supernatants were then incubated with the indicated antibodies rocking at 4°C for 2 hours. Protein A/G sepharose was added and tubes were allowed to rock at 4°C for an additional 2 hours. Beads were washed 5 times with lysis buffer and protein was eluted by boiling in 2x Laemmli buffer for 10 minutes. These samples were subjected to SDS-PAGE followed by western blot analysis using the indicated antibodies and either ECL or Licor for visualization.

RT-PCR

RT-PCR was performed as described previously[19]. Briefly, RNA was isolated from mass sympathetic neuron cultures using Trizol (Invitrogen) as described by the manufacturer. First strand cDNA was synthesized using random hexamers and the Superscript III system (Invitrogen). Primers that were used for RT-PCR are as follows:

GAPDH: F- CCCATCACCATCTTCCAGGA, R- TTGTCATACCAGGAAATGAGC

Coronin-1: F: TTCCCTCAAGGATGGCTACGT R- CCTCCAGCCTTGACACGGTAT

In Situ Hybridization

Probe preparation and *In situ* hybridization was performed as described previously[19]. Briefly, trunks from P0 mice of the indicated genotypes were snap frozen in OCT and SCGs were cryo-sectioned at a thickness of 14 μm. Probes for Coronin-1, Coronin-2, and Coronin-3 were amplified from a cDNA library derived from SCGs and cloned into the pBK-CMV vector (Stratagene). The primers used to clone Coronin-1, 2, and 3 *in situ* probes are as follows:

Coronin-1

F: GGCCTCGAGATGGCTCTGATCTGTGAGGC

R: GGCTCTAGAACGCTGTAGATTGTGTCCGG

Coronin-2

F: GGCCTCGAGAGCAGGGAGAACGTATCAGC

R: GGCTCTAGAACTTGGGAATTTGCCTTGG

Coronin-3

F: GGCCTCGAGTCAGGGCAGAGCATAGTATGG

R: GGCTCTAGAAGTTCACTGTCTTCCTCCGG

Feeding and recycling assays

The TrkA-Flag feeding assay was performed as described previously[8]. Briefly, the Flag M1 antibody (Sigma) was used to label distal axons of neurons isolated from *TrkA*^{Flag} mice for 20 minutes at 4°C. Unbound antibody was removed by rinsing distal axon compartments with growth medium and then medium containing NGF (45 ng/mL). Cells were placed at 37°C 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 alpha-Flag immunostaining. A similar protocol was used to assess EGF internalization. In this case fluorescent EGF was used, which negated the need for further immunostaining. To assess lysosome localization, lysotracker was added 2 hours prior to fixation on the cell body side of microfluidic devices. Co-localization between endosomes and lysosomes was assessed via confocal microscopy and blinded, manual quantification.

For the recycling assay, we performed a protocol described by Lee and colleagues[31]. Initially Flag feeding assays were carried out as described above in neurons isolated from *TrkA*^{Flag} mice. 30 minutes prior to the end of the indicated chase period a goat anti-Mouse Cy3 antibody was added to cell body or axon compartments of microfluidic devices. Cells were then fixed with 4% paraformaldehyde and imaged. Cy3+ puncta represent Flag-TrkA+ endosomes that have undergone retrograde transport, recycled to the surface and reinternalized.

Microfluidic devices

Microfluidic devices were generated as described previously[23]. These chambers were afixed to coverglass coated with poly-D-lysine (50 μg/mL) and laminin (1μg/mL). 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 hours prior to scoring cell survival via Hoechst staining[19]. For experiments where NGF deprivation is called for alpha-NGF and BAF were used to neutralize remaining NGF and keep cells alive, respectively. For mass cultures, the same procedure is followed with no microspheres. Images of Hoechst staining are acquired and blinded for unbiased quantification. Dead versus alive neurons were scored as described previously [51]. The top concentration of NGF on wild type neurons is set to 1 and all other conditions are relative to that.

Immunocytochemistry

Immunocytochemistry and immunohistochemistry was performed as described previously [8]. Neurons or sections were fixed with 4% PFA and blocked/permeabilized (5% goat serum, 0.05% Triton-x-100 in PBS) for 30 minutes at room temperature. Cells were then incubated overnight at 4°C with primary antibody diluted in blocking buffer. Cells were then washed 3x with 1x PBS and incubated with fluorescent secondary antibody for 1 hour at room temperature followed by 3 washes in 1x PBS and mounting in fluoromount (vectashield). Fluorescence was visualized using inverted confocal microscopy and 3D reconstructed images were made in ImageJ.

Calcium imaging

Calcium imaging was performed as previously described[52]. Briefly, neurons grown on cover glass were loaded with Fluo-4 calcium indicator (3 μM) for 20 minutes at 37°C (5% CO_2). The neurons were then moved to an environmental chamber (37°C, 5% CO_2) mounted on an inverted confocal microscope. Basal calcium levels were assessed by randomly imaging 6–10 cells per coverslip prior to treatment. Indicated concentrations of NGF were added to neurons and allowed to incubate for 15 minutes. The previous 6–10 neurons were imaged again to assess their calcium levels after treatment. We determined the change calcium levels by F/F0 to quantify the calcium intensity. F0 is the 0ng/mL intensity and F is the intensity of different NGF treatment. No neurotrophin treatment conditions were set to 1 and all other conditions are represented relative to that value.

In vivo sympathetic neuron counts

SCG neuron number was quantified as described previously[19]. Briefly, trunks from P0 mice were snap frozen in OCT and cryo-sectioned at a thickness of 10 μm. Every 5th section was processed for Nissl staining. Images were acquired, blinded, and quantified as previously described[53].

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 10nm (Liquids Research Ltd., Bangor, UK). Approximately 20 million TrkB/A cells were suspended in 9 mL of DMEM and 1 mL ferrofluid suspension. To one sample, 50ng/mL BDNF was added and cells were incubated at 37C for 15 minutes. Cells were then pelleted, homogenized and processed as described previously to obtain a crude endosomal fraction[13]. Samples were then placed adjacent to a magnet for 1 hour 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 was performed exactly as described previously[13].

Phylogeny and similarity analysis

Amino acid sequences of all TNFR family members were analyzed using phylogeny.fr. Amino acid similarity analysis was performed using ClustalW.

Statistical Analysis

No statistical method was used to predetermine sample size, however we chose sample sizes similar to those reported in previous publications[13, 19, 18]. Data were collected randomly and assessed blindly. Data distribution was assumed to be normal but this was not formally tested. Statistical analysis was based on at least 3 experiments and is described in figure legends. Error bars represent standard error of the mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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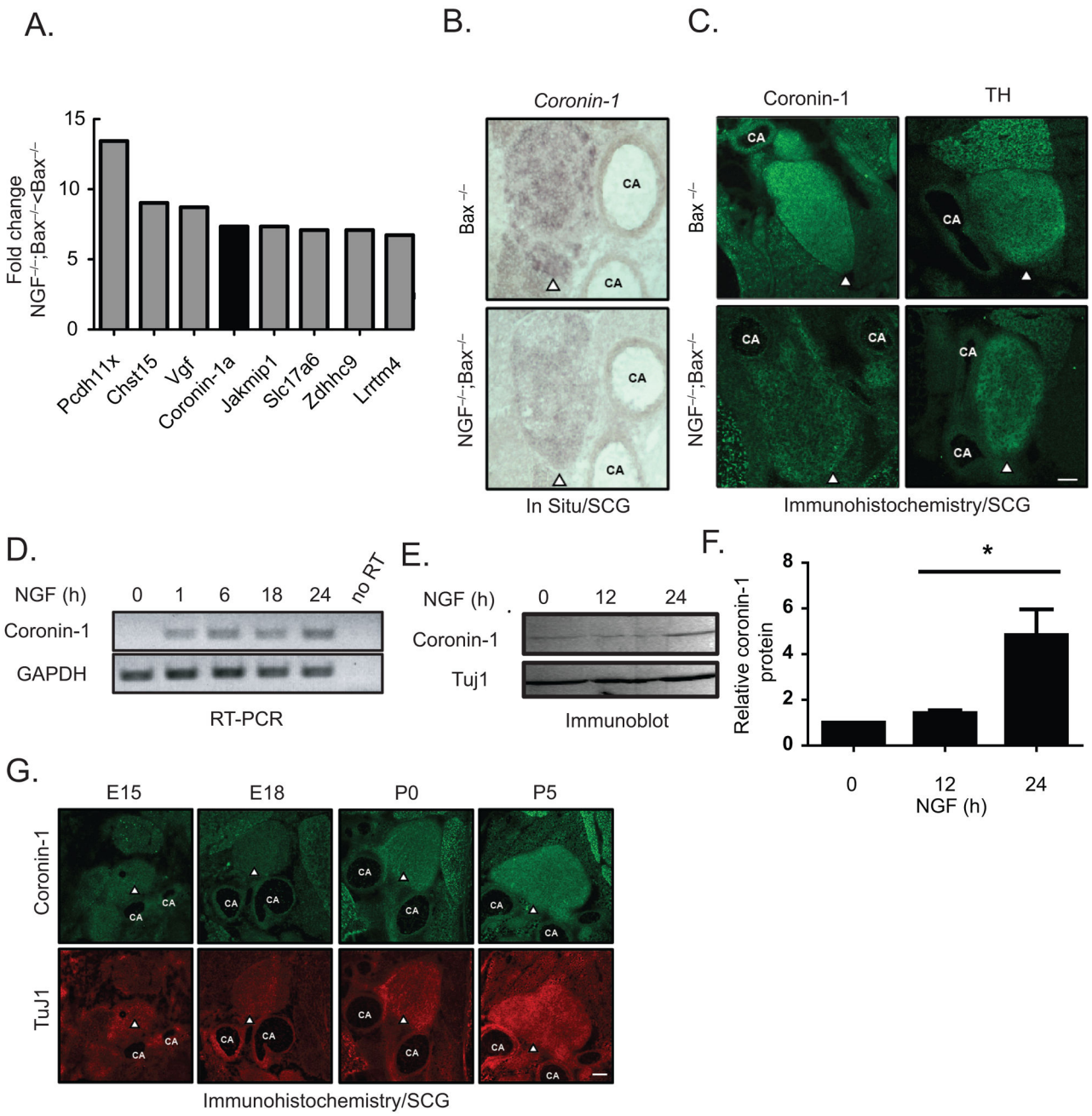

Figure 1. Coronin-1 expression is regulated by NGF in sympathetic neurons

(A) Candidates for the putative NGF signal duration enhancing factor. Top candidate genes with reduced expression in SCGs from *NGF*$^{-/-}$;*Bax*$^{-/-}$ P0 mice compared to *Bax*$^{-/-}$ controls and are known to associate with membrane and/or vesicles. All 76 genes are represented in Supplementary Table 1.

(B) NGF is necessary for *Coronin-1* mRNA expression. Trunks of P0 *Bax*$^{-/-}$ or *NGF*$^{-/-}$;*Bax*$^{-/-}$ mice were cryo-sectioned and Coronin-1 message was visualized in the SCG by *in situ* hybridization. Carotid artery is marked as CA and the white arrow indicates the position of the SCG.

(C) NGF is necessary for Coronin-1 protein expression. Trunks of P0 *Bax*$^{-/-}$ or *NGF*$^{-/-}$;*Bax*$^{-/-}$ mice were cryo-sectioned and Coronin-1 protein was visualized in the SCG by immunostaining. Carotid artery is marked as CA and the white arrow indicates the position of the SCG. Scale bar on right corner is 100 μm.

(D) NGF is sufficient for induction of Coronin-1 mRNA expression. RT-PCR for Coronin-1 and GAPDH. Sympathetic neurons from P0 rats were cultured for 3 days, deprived of NGF for 16 hours and treated with 45 ng/mL NGF for indicated times.

(E) NGF is sufficient for Coronin-1 protein expression. Sympathetic neurons from P0 rats were cultured for 1 day, deprived of NGF for 36 hours and treated with 45 ng/mL NGF for indicated times.

(F) Quantification of E by densitometry using ImageJ. Three experiments were averaged and standard error of the mean was calculated (n=3). Error bars, s.e.m.

(G) Coronin-1 protein expression during development. Trunks of E15, E18, P0 and P5 WT mice were cryosectioned and Coronin-1 or βIII tubulin proteins were visualized in the SCG by immunostaining. The carotid artery is used as a landmark and is marked as CA and the white arrow indicates the position of the SCG. Scale bar=100 μm.

*$p<0.05$ using unpaired two-tailed Student's t-test

Uncropped gels and blots are shown in Supplementary Figure 7.

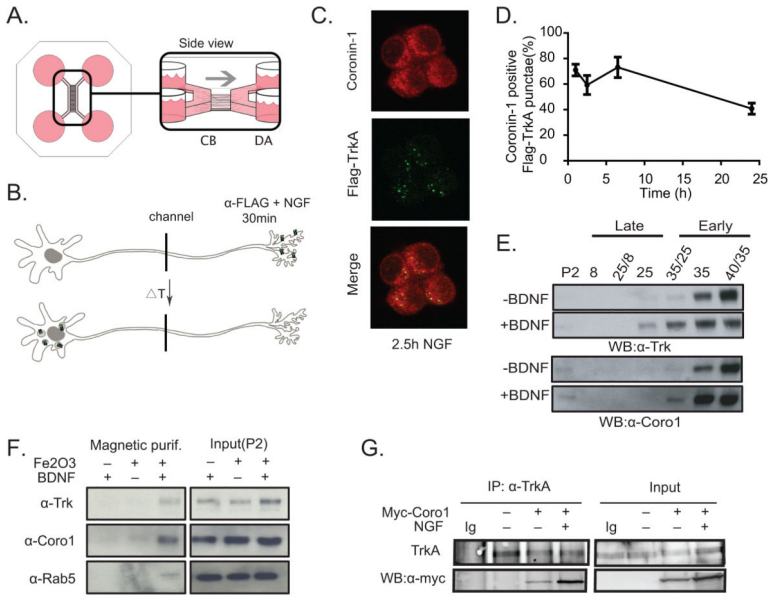

Figure 2. Coronin-1 associates with TrkA and the signaling endosome

(A) Schematic of the microfluidic device, which allows separation of cell bodies and axons.

(B) Schematic of retrograde Flag-TrkA feeding assay. Sympathetic neurons from P0 *TrkA*^{Flag} mice were established in microfluidic devices. Distal axons were labeled with α-Flag antibody at 4°C for 30 minutes followed by NGF treatment and incubation at 37°C for the indicated period of time.

(C) Coronin-1 co-localization with the NGF-TrkA signaling endosome. The Flag feeding assay was performed followed by a 1 hour NGF treatment. Coronin-1 and signaling endosomes were then visualized by immunostaining with anti-Coronin-1 antibody followed by application of fluorescent secondary antibodies to detect Coronin-1 and internalized Flag antibody. Scale bar = 10 μm.

(D) Quantification of co-localization between Coronin-1 and the signaling endosome. This represents the percentage of signaling endosomes positive for Coronin-1 as a function of time after NGF feeding. Error bars, s.e.m.

(E) Following a 15 minute BDNF treatment of TrkB/A stable cells, both TrkB/A and Coronin-1 are enriched in the early endosome fraction (25/35) of a discontinuous sucrose gradient.

(F) Co-purification of Coronin-1 on TrkA positive endosomes. Magnetic purification of endosomes present after BDNF treatment of TrkB/A PC12 cells. Western blot analysis of TrkB/A, Coronin-1 and the early endosome marker Rab5. Right panels represent input material and left panels represent magnetic purification.

(G) Myc-Coronin-1 co-immunoprecipitates with TrkA in an NGF-dependent manner. Co-immunoprecipitation between endogenous TrkA and overexpressed myc-Coronin-1 in PC12 cells treated with or without NGF.

*$p<0.05$ using unpaired two-tailed Student's t-test

Uncropped gels and blots are shown in Supplementary Figure 7.

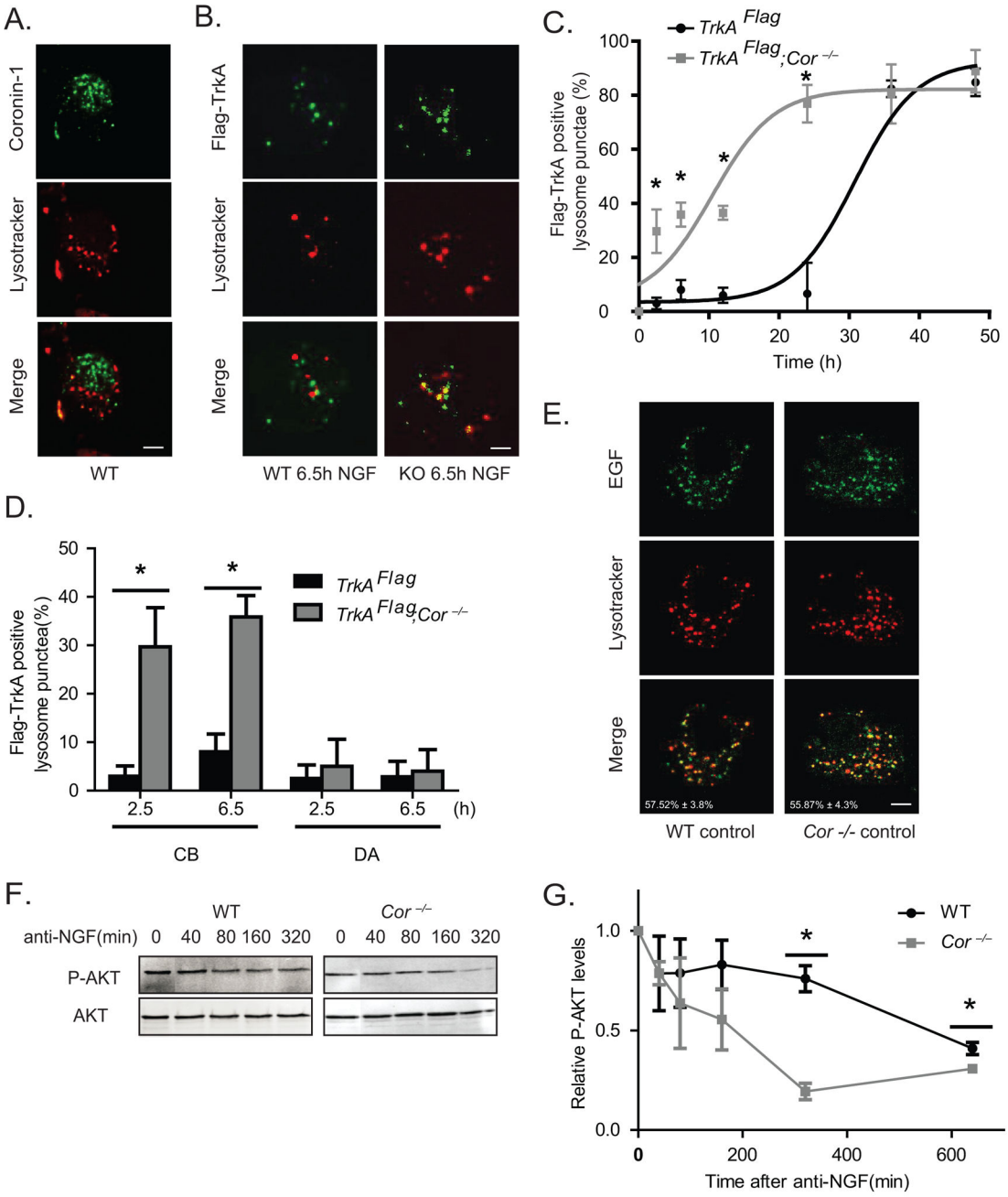

Figure 3. Coronin-1 prevents signaling endosome fusion with lysosomes at the cell body

(A) Coronin-1 does not co-localize with lysosomes. Rat sympathetic neurons were grown 3 days *in vitro* (DIV), immunostained for Coronin-1 (green) and lysosomes were visualized using Lysotracker (red). Co-localization was assessed manually (<10%) and is negligible. Scale bar = 10μm.

(B) Signaling endosome co-localization with lysosomes in the presence or absence of *Coronin-1*. Flag feeding was performed for 30 minutes followed by chase period for the indicated time in sympathetic neurons isolated from $TrkA^{Flag}$ or $TrkA^{Flag}$;*Coronin-1*$^{-/-}$

mice. Internalized Flag antibody is detected using a fluorescently conjugated anti-mouse secondary antibody and lysosomes were stained with Lysotracker. Scale bar is 10 μm.

(C) Quantification of the percentage of lysosomes positive for Flag, as a function of time after anti-Flag/NGF feeding (n=5 for all groups). Experiments were performed as described for panel (B). Curves were fit using variable slope regression parameters in graph pad prism. Error bars, s.e.m.

(D) Quantification of co-localization between the signaling endosome and lysosomes in neurons isolated from $TrkA^{Flag}$ or $TrkA^{Flag}$;*Coronin-1*$^{-/-}$ mice (n=5 all groups). Cell bodies and axons were visualized at different times after anti-flag antibody/NGF feeding as described in panels B–C. Error bars, s.e.m.

(E) EGF lysosomal fusion in the presence or absence of *Coronin-1*. Scale bar is 10 μm.

(F) P-Akt decay in the presence or absence of *Coronin-1*. Neurons were cultured from WT or *Coronin-1*$^{-/-}$ mice for 2–3 DIV, deprived of NGF (in the presence of anti-NGF function blocking antibody) for the indicated times, lysed and analyzed by immunoblot for P-Akt, Akt, and Tuj1.

(G) Quantification of E. Experiments were repeated at least three times and quantified with densitometry. P-Akt 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.

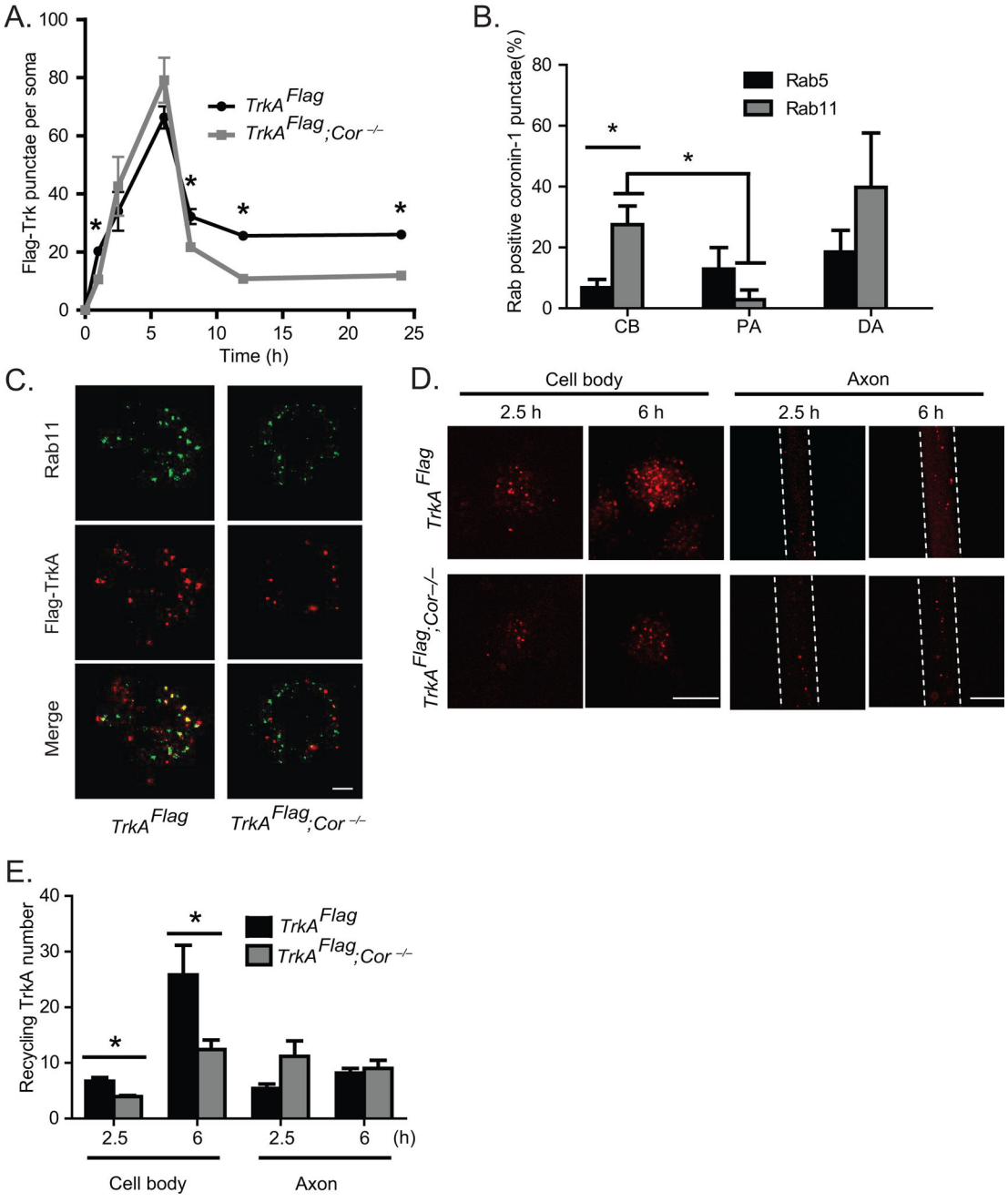

Figure 4. Coronin-1 mediates recycling of the signaling endosome

(A) Accumulation of signaling endosomes in the presence or absence of Coronin-1. Flag feeding was performed for 30 minutes followed by chase period for the indicated time in sympathetic neurons isolated from *TrkA*^{Flag} or *TrkA*^{Flag}*;Coronin-1*$^{-/-}$ mice as described for Fig. 3B. A 3-dimensional model was generated from z-stacks obtained for Figure 3B–C and the total number of Flag-TrkA endosomes per neuron, were counted (n=5 for 1hour group and 8 hour group; *TrkA*^{Flag} n=7 for 12 hour group; *TrkA*^{Flag}*;Coronin-1*$^{-/-}$ n=9 for 12 hour group; n=9 for 24 hour group).

(B) Coronin-1 co-localization with Rab5 or Rab11. Sympathetic neurons were immunostained for Coronin-1 and Rab5 or Rab11 and co-localization was assessed and is represented as a percentage of Coronin-1 punctae positive for Rab5 or Rab11 in the cell bodies (CB), proximal axons (PA) or distal axons (DA) (n=5 for all groups). Error bars, s.e.m.

(C) Coronin-1 is required for recruitment of Rab11 to post-endocytic NGF-TrkA. NGF feeding was performed for 30 minutes followed by 2.5h chase in neurons isolated from $TrkA^{Flag}$ or $TrkA^{Flag}$;$Coronin\text{-}1^{-/-}$ mice. Flag and Rab11 were visualized via immunostaining.

(D) Flag-TrkA transcytosis/recycling in the presence or absence of Coronin-1. The recycling assay was performed as described in Panel E on neurons isolated from $TrkA^{Flag}$ or $TrkA^{Flag}$;$Coronin\text{-}1^{-/-}$ mice grown in microfluidic devices. Anti-Flag antibody was fed to distal axons for 30 minutes followed by a chase period for the indicated amount of time. Recycled Flag-TrkA in cell bodies and axons was marked by Cy3. Schematic for Flag-TrkA recycling assay is presented in Supplementary Fig. 3B. Scale bar = 10 μm.

(E) Quantification of D (n=5 for all groups). Error bars, s.e.m.

*$p<0.05$ using unpaired two-tailed Student's t-test

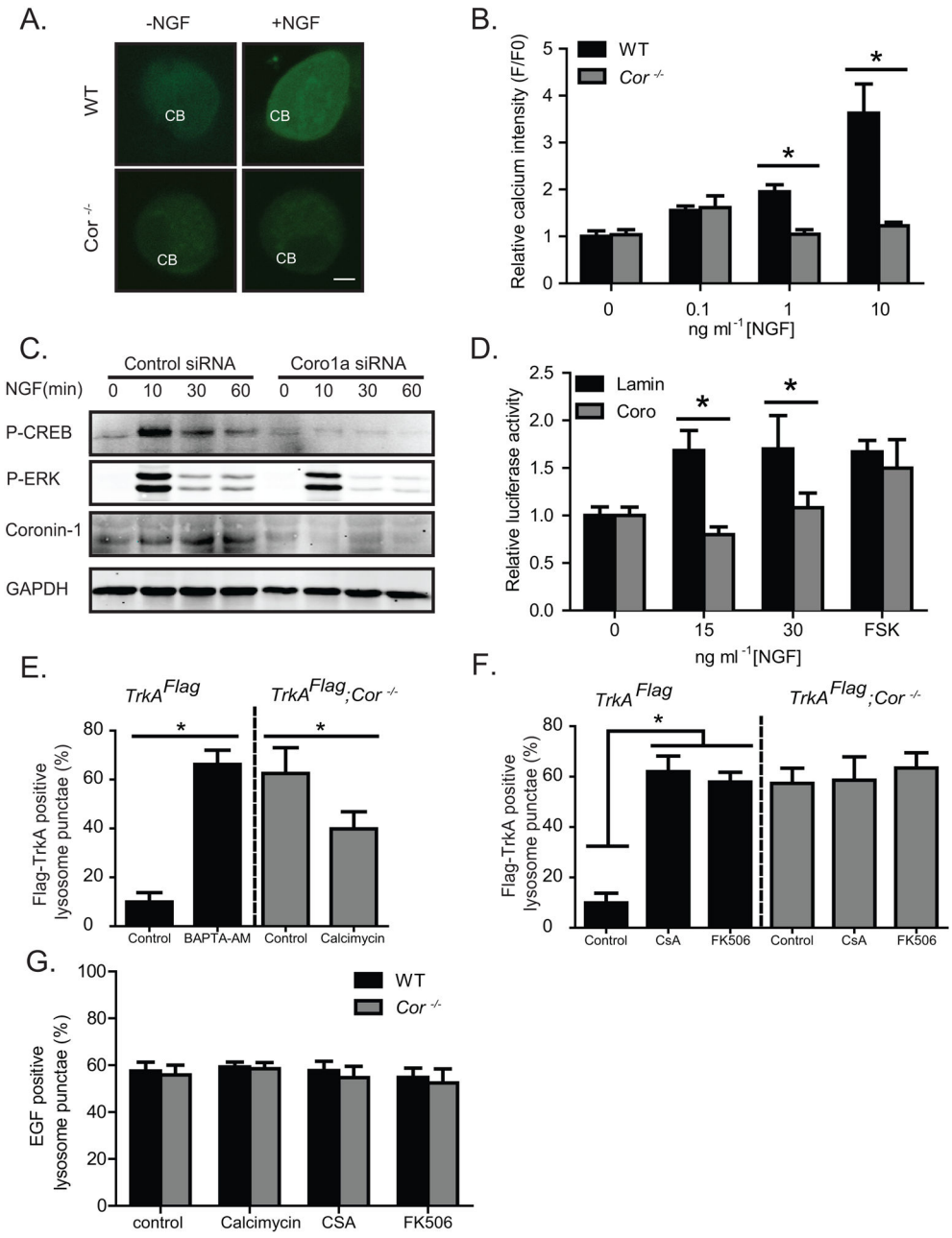

Figure 5. Coronin-1 mediates NGF-dependent signaling: calcium mobilization and CREB phosphorylation

(A) NGF induction of calcium is Coronin-1 dependent. Sympathetic neurons isolated from wild type and *Coronin-1*$^{-/-}$ mice were established in culture and calcium release was visualized with the calcium dye, Fluo-4. Images of single neuronal cell bodies (CB) were acquired 15 minutes after NGF treatment. Scale bar = 5μm.

(B) Quantification of calcium release described in panel A as a function NGF concentration. All fluorescence intensities (F) are relative to those collected for 0 ng/mL 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. PC12 cells were transfected with siRNA targeting Coronin-1 or lamin, cultured for 24 hours, treated with NGF for the indicated amount of time and P-CREB, Coronin-1, and GAPDH protein levels were assessed by immunoblot.

(D) Requirement of Coronin-1 for CREB transcriptional activity. Sympathetic neurons transfected with siRNA targeting Coronin-1 or lamin as well as a 3xCRE-luciferase reporter were cultured for 1 DIV and treated with the indicated concentrations of NGF or 5μM forskolin (FSK) for an additional 24 hours followed by a luminescence assay. 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 co-localization with the lysosome was examined as described in Fig. 3. Reducing intracellular calcium release by BAPTA-AM treatment of neurons from *TrkA*^{Flag} mice phenocopies the rate of lysosomal fusion observed in *TrkA*^{Flag}*;Coronin-1*$^{-/-}$ mice. Likewise, increasing intracellular calcium with Calcimycin (50 nM) treatment of neurons from *TrkA*^{Flag}*;Coronin-1*$^{-/-}$ mice partially rescues the lysosome fusion phenotype(n=5 for all groups). Error bars, s.e.m.

(F) Influence of calcineurin on signaling endosome stability. Calcineurin inhibitors, CsA (100 nM) and FK506 (500 nM) added to neurons from *TrkA*^{Flag} mice phenocopy the rate of lysosomal fusion observed in *TrkA*^{Flag}*;Coronin-1* $^{-/-}$ (in *TrkA*^{Flag} mice, control n=10; CSA, Fk506 n=5). Error bars, s.e.m.

(G) EGF positive lysosome puncta (%) are not influenced by calcimycin (calcium ionophore) or cyclosporinA (CSA) and FK506 (calcineurin inhibitors). Fluorescent EGF was fed to neurons from wild type or *Coronin-1*$^{-/-}$ mice for 30 minutes followed by a chase period of 6.5 hours. Error bars, s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test and one-way ANOVA non parametric test

Uncropped gels and blots are shown in Supplementary Figure 7.

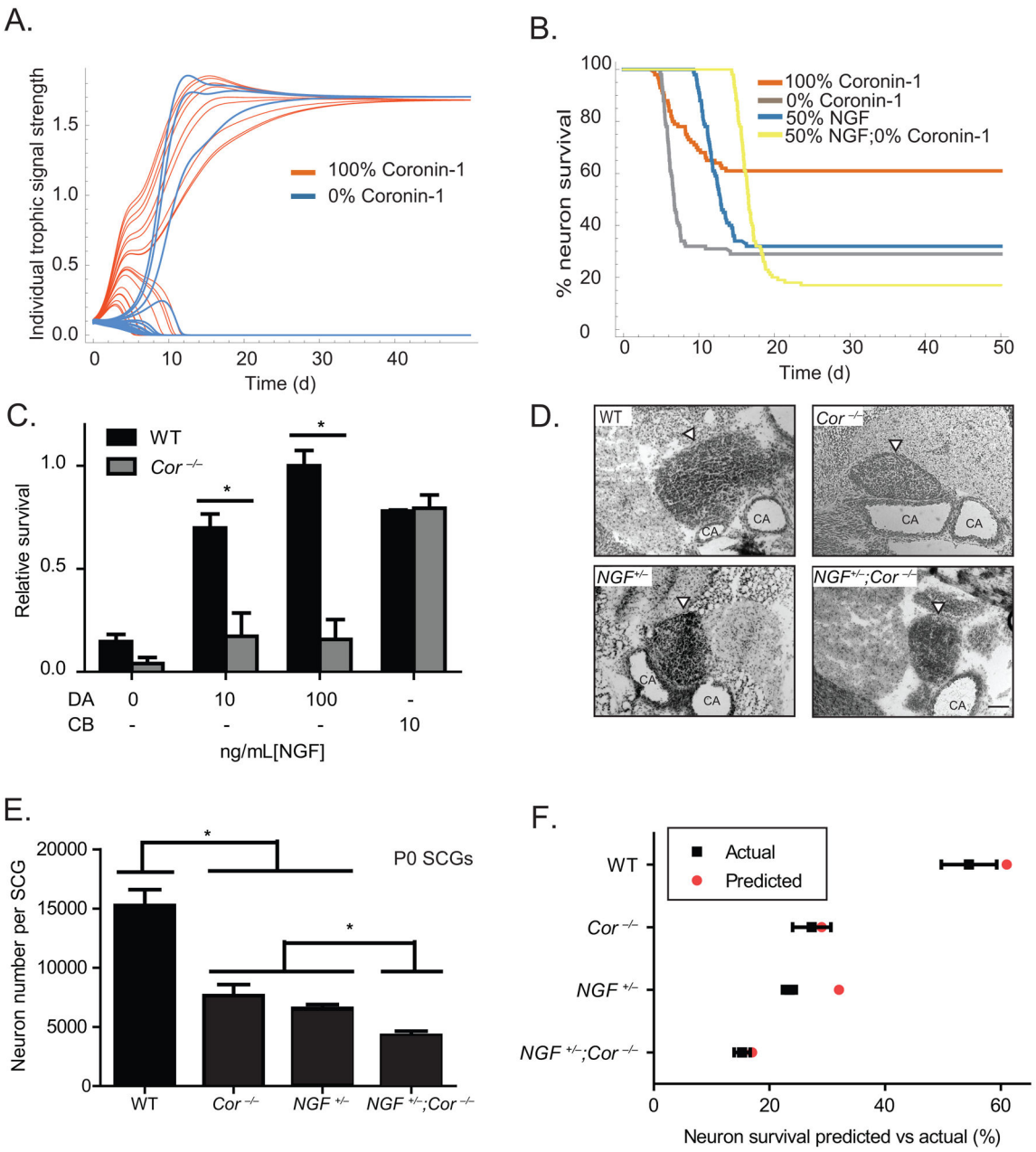

Figure 6. Coronin-1 is required for competition for survival and target matching

(A) Simulations showing the normalized TrkA concentration during development in a random set of 20 neurons (out of 100 simulated) for WT and *Coronin-1*$^{-/-}$. Parameters derived from *in vitro* experiments predict that in the absence of Coronin-1, fewer neurons survive the competition relative to WT neurons.

(B) Simulations of the time dependence of the surviving neurons for four genotypes: wild type, *Coronin-1*$^{-/-}$, *NGF*$^{+/-}$ and *NGF*$^{+/-}$;*Coronin-1*$^{-/-}$.

(C) Coronin-1 is required for long-distance retrograde NGF-dependent survival *in vitro*. P0 sympathetic neurons from wild type and *Coronin-1*$^{-/-}$ mice were grown in microfluidic chambers. The indicated concentrations of NGF were applied to cell bodies (CB) or distal

axons (DA) and cell survival was determined via Hoechst stain. Chambers that did not receive NGF received anti-NGF function blocking antibody. Each experiment was performed at least 3 times and survival is expressed relative to 100 ng/mL NGF on distal axons of wild type neurons(n=6 for all groups). Error bars, s.e.m.

(D) Coronin-1 is required for sympathetic neuron survival *in vivo*. SCGs were taken from at least 4 different P0 mice of the indicated genotypes. Trunks were cryo-sectioned and stained using the Nissl protocol. The carotid artery is marked as CA and the white arrow indicates the position of the SCG. Scale bar=100 μm.

(E) Quantification of total number of neurons per ganglia from panel D (WT n=4; $NGF^{+/-}$ n=6; $Cor^{-/-}$ n=4; $NGF^{+/-}$;$Cor^{-/-}$ n=7). Error bars, s.e.m.

(F) A comparison between predicted versus experimental competition for survival outcomes of following conditions: wild type, $Coronin\text{-}1^{-/-}$, $NGF^{+/-}$ and $NGF^{+/-}$;$Coronin\text{-}1^{-/-}$.

*p<0.05 using unpaired two-tailed Student's t-test