

# **$\beta$ -catenin phosphorylation by Neurotrophins and Hepatocyte Growth Factor signalling regulates axon morphogenesis**

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## Summary

Tyrosine phosphorylation of  $\beta$ -catenin, a component of adhesion complexes and the Wnt pathway, affects cell adhesion, migration and gene transcription. By reducing  $\beta$ -catenin availability using shRNA-mediated gene silencing or expression of intracellular N-cadherin, we show that  $\beta$ -catenin is required for axon growth downstream of Brain Derived Neurotrophic Factor (BDNF) and Hepatocyte Growth Factor (HGF) signalling. We demonstrate that receptor tyrosine kinases (RTK) Trk and Met interact with and phosphorylate  $\beta$ -catenin. Neurotrophins (NT) stimulation of Trk receptors results in phosphorylation of  $\beta$ -catenin at residue Y654 and increased axon growth and branching. Conversely, pharmacological inhibition of Trk or a Y654F mutant blocks these effects.  $\beta$ -catenin phospho(P)-Y654 colocalizes with the cytoskeleton at growth cones. However, HGF that also increases axon growth and branching, induces  $\beta$ -catenin phosphorylation at Y142 and a nuclear localization. Interestingly, dominant negative  $\Delta$ N-TCF4 abolishes the effects of HGF in axon growth and branching, but not of NT. We conclude that NT and HGF signalling differentially phosphorylate  $\beta$ -catenin, targeting  $\beta$ -catenin to distinct compartments to regulate axon morphogenesis by TCF4-transcription-dependent and independent mechanisms. These results place  $\beta$ -catenin downstream of growth factor/RTK signalling in axon differentiation.

## Introduction

The establishment of proper neuronal connections is crucial to brain physiology and requires the outgrowth and arborization of axons and dendrites, before synaptic contacts are stabilized. Both extracellular cues and an intrinsic cell program promote neuronal polarity and axon growth (Arimura and Kaibuchi, 2007). A variety of extracellular signals play a major role in neuronal differentiation and synapse formation (Arimura and Kaibuchi, 2007; Ciani and Salinas, 2005; Dalva et al., 2007; Huang and Reichardt, 2001; Takeichi, 2007). NT and their Trk receptors are among the best-known regulators of neuronal development and function (Chao, 2003; Huang and Reichardt, 2001; Huang and Reichardt, 2003; Zampieri and Chao, 2006). *TrkB* and *TrkC* are expressed in the hippocampus and their ligands BDNF, NT-3 and NT-4 regulate neurite outgrowth and branching in hippocampal neurons (Collazo et al., 1992; Labelle and Leclerc, 2000; Lindholm et al., 1996; Martinez et al., 1998; Vicario-Abejon et al., 1998). HGF and its RTK Met are also expressed in the hippocampus where HGF is a neurotrophic factor for hippocampal neurons (Honda et al., 1995; Korhonen et al., 2000). Furthermore, Wnt factors regulate neuronal differentiation through different signalling pathways (Ciani and Salinas, 2005). Despite the abundance of molecules controlling neuronal morphogenesis, the intracellular mechanisms that integrate these signals to define the morphology of the neuritic arbour are poorly understood.

$\beta$ -catenin is a cytoplasmatic protein that plays a critical role in calcium-dependent cell-cell adhesion and Wnt signalling (Lilien and Balsamo, 2005; Nelson and Nusse, 2004). As a component of the adhesion complex,  $\beta$ -catenin binds to the intracellular domain of cadherins and to  $\alpha$ -catenin, which in turn can bind to actin (Drees et al., 2005; Yamada et al., 2005). In the canonical Wnt pathway, Wnts inhibit Glycogen Synthase Kinase-3 $\beta$  (GSK-3 $\beta$ ) that targets  $\beta$ -catenin for degradation, resulting in  $\beta$ -catenin nuclear translocation and activation of T Cell Factor/Lymphoid Enhancing Factor (TCF/LEF)-driven gene transcription (Ciani et al., 2004; Ciani and Salinas, 2005; Logan and Nusse, 2004; Nelson and Nusse, 2004).

The regulation of cell adhesion underlies important processes such as neurite extension, synaptogenesis, cell migration and epithelial-mesenchymal transition in development

and metastasis (Brembeck et al., 2006; Dalva et al., 2007; Nelson and Nusse, 2004; Takeichi, 2007; Thiery, 2002). Tyrosine phosphorylation of  $\beta$ -catenin by RTK is known to alter cell adhesion (Nelson and Nusse, 2004; Lilien and Balsamo, 2005). For example, the EGF receptor (EGFR) associates with  $\beta$ -catenin in the core region which contains residue Y654 and induces  $\beta$ -catenin phosphorylation (Hoschuetzky et al., 1994; Takahashi et al., 1997), resulting in decreased affinity for cadherin (Hoschuetzky et al., 1994; Nelson and Nusse, 2004; Roura et al., 1999; Takahashi et al., 1997). Similarly, in epithelial cells phosphorylation of Y142 by non-receptor tyrosine kinases Fer, Fyn or RTK Met weakens the binding of  $\beta$ -catenin to  $\alpha$ -catenin (Brembeck et al., 2004; Lilien and Balsamo, 2005; Nelson and Nusse, 2004; Piedra et al., 2001). Activation of  $\beta$ -catenin signalling by Met and phosphorylation of  $\beta$ -catenin Y142 leads to its nuclear translocation and increased TCF-mediated gene transcription (Brembeck et al., 2004; Danilkovitch-Miagkova et al., 2001). In addition, Fer interacts with cadherin indirectly and phosphorylates PTP1B, regulating PTP1B binding to cadherin and the continuous dephosphorylation of  $\beta$ -catenin Y654 (Balsamo et al., 1998; Balsamo et al., 1996; Piedra et al., 2003; Xu et al., 2002; Xu et al., 2004). Together, the available data indicates that the tyrosine phosphorylation state of  $\beta$ -catenin, resulting from the balance of phosphatase and kinase activities, regulates cell migration by decreasing adhesion and activating  $\beta$ -catenin signalling in epithelial tissue.

In the nervous system, cadherin and  $\beta$ -catenin regulate axon extension (Riehl et al., 1996), dendritogenesis (Yu and Malenka, 2003), synaptic assembly and plasticity (Bamji, 2005; Bamji et al., 2006; Tai et al., 2007; Yu and Malenka, 2003). Interestingly, tyrosine phosphorylation of  $\beta$ -catenin also regulates synapse formation and function (Bamji et al., 2006; Murase et al., 2002) and the growth cone response to the guidance cue Slit (Rhee et al., 2007; Rhee et al., 2002).

In this paper we have investigated the relationship between  $\beta$ -catenin and growth factor/RTK signalling in axon morphogenesis. We find that  $\beta$ -catenin is required for axon extension. Stimulation of neuronal RTK, Trk and Met by NTs and HGF results in  $\beta$ -catenin tyrosine phosphorylation.  $\beta$ -catenin phosphorylation at Y654 by BDNF or NT-3 signalling increases axon growth and branching in hippocampal neurons, whereas the mutant Y654F or Trk inhibition abolishes the NT effects. We also demonstrate that HGF stimulates axon growth and branching by phosphorylating  $\beta$ -catenin at Y142.

Finally, we present evidence suggesting that the regulation of axon morphogenesis by NT and P-Y654  $\beta$ -catenin occurs independently of TCF4. In contrast, Met activation results in P-Y142  $\beta$ -catenin nuclear localization and TCF4-mediated gene transcription to promote axon growth and branching. Our results identify a crosstalk between  $\beta$ -catenin and growth factor signalling networks during axon morphogenesis.

## Results

### **Trk interacts with $\beta$ -catenin**

$\beta$ -catenin has been shown to associate with and to be a substrate of EGFR (Hoschuetzky et al., 1994; Takahashi et al., 1997). Here we explored whether it interacts with other RTK such as Trk in neuronal cell lines and primary neurons. PC12 6/15 cells, a clone of PC12 cells stably expressing the non-neuronal form of human TrkA (Hempstead et al., 1992; Llovera et al., 2004) were treated with Nerve Growth Factor (NGF). TrkA and  $\beta$ -catenin co-immunoprecipitated from PC12 6/15 lysates independently of NGF stimulation (Fig. 1A). To investigate whether this association extended to other Trk receptors we used hippocampal neurons, which express *TrkB* and *TrkC* (Martinez et al., 1998). Using anti-pan Trk antibodies we immunoprecipitated TrkB and TrkC from untreated and BDNF or NT-3-treated neurons.  $\beta$ -catenin co-immunoprecipitated with TrkB and TrkC from hippocampal neuron lysates (Fig. 1B). Thus,  $\beta$ -catenin interacts with Trk receptors in PC12 cells and developing neurons.

### **Trk phosphorylates $\beta$ -catenin at Y654**

The interaction of Trk receptors with  $\beta$ -catenin raises the possibility that  $\beta$ -catenin could be phosphorylated by these RTK. In *in vitro* phosphorylation assays, recombinant TrkA kinase or TrkA immunoprecipitated from PC12 6/15 cells was incubated with recombinant wild-type (WT) or mutant  $\beta$ -catenin in the presence or absence of the Trk kinase inhibitor, K252a. Following the addition of ATP, TrkA kinase produced a robust tyrosine phosphorylation of WT GST- $\beta$ -catenin (Fig. 2A). Preincubation of TrkA kinase with K252a reduced TrkA kinase activation to basal levels and blocked GST- $\beta$ -catenin phosphorylation (Fig. 2A). Interestingly, phosphorylation of mutant Y654F GST- $\beta$ -catenin by TrkA kinase was ~ 45% lower than that of WT GST- $\beta$ -catenin, while Y142F GST- $\beta$ -catenin phosphorylation was ~ 15% lower than phosphorylation of the WT protein (Fig. 2A). TrkA immunoprecipitated from PC12 cells also induced  $\beta$ -catenin tyrosine phosphorylation upon addition of ATP that was decreased by 30% by K252a (Fig. 2B). Furthermore, Y654F  $\beta$ -catenin phosphorylation was significantly lower (~ 40%) than that of WT  $\beta$ -catenin while the residual phosphorylation of Y654F  $\beta$ -catenin was not inhibited by K252a (Fig. 2B). Together, kinase assays indicate that TrkA phosphorylates  $\beta$ -catenin *in vitro* at Y654.

To study further the role of Trk in  $\beta$ -catenin phosphorylation,  $\beta$ -catenin immunoprecipitation was performed on lysates from 6/15 PC12 cells and hippocampal neurons in the presence or absence of pervanadate (to inhibit tyrosine phosphatases) for subsequent Western blot analysis using phosphospecific antibodies. Phosphorylated-Y654 (P-Y654)  $\beta$ -catenin was detected in both PC12 and hippocampal cell lysates and levels of P-Y654  $\beta$ -catenin increased following NGF, BDNF and NT-3 stimulation of PC12 cells and hippocampal neurons respectively, correlating with Trk phosphorylation (Fig. 2C, D). Stimulation with BDNF (50 ng/ml) for 10 minutes induced the phosphorylation of Y654, which peaks at 1 hour and was reduced after overnight treatment (Fig. 2E, F). In contrast, phosphorylation of Y142 was maintained at basal levels (Fig. 2E), indicating that NT signalling specifically phosphorylates  $\beta$ -catenin Y654. In addition to TrkB N-cadherin also co-immunoprecipitated with  $\beta$ -catenin (Fig. 2E), suggesting that  $\beta$ -catenin, N-cadherin and Trk interact in a big complex. Altogether these findings demonstrate that Trk receptors phosphorylate  $\beta$ -catenin Y654 in PC12 cells and in primary neurons.

### **Met interacts with $\beta$ -catenin and phosphorylates $\beta$ -catenin Y142**

*HGF* and *Met*, its RTK are both expressed in the hippocampus (Honda et al., 1995; Korhonen et al., 2000). This localization led us to examine whether *Met*, like *Trk*, interacts with and phosphorylates  $\beta$ -catenin. We found that *Met* and  $\beta$ -catenin co-immunoprecipitate from hippocampal neuron lysates and that this association decreases upon HGF treatment with or without pervanadate by  $87.6 \pm 3.3\%$  and  $85.6 \pm 5.1\%$ , compared to control ( $n \geq 3$ ) (Fig. 3B). This interaction suggested that  $\beta$ -catenin may be phosphorylated by *Met*. Using antibodies specific for P-Y142, recombinant *Met* kinase was found to induce the phosphorylation of WT GST- $\beta$ -catenin at Y142 upon addition of ATP. This decreased to basal levels when using mutant Y142F GST- $\beta$ -catenin (Fig. 3A). In contrast, phosphorylation of Y654F GST- $\beta$ -catenin was similar to that of the WT protein. Furthermore, prior treatment with HGF (50 ng/ml, 10 minutes) with or without pervanadate induced the phosphorylation of  $\beta$ -catenin Y142 in  $\beta$ -catenin immunoprecipitates isolated from hippocampal neurons (Fig. 3B). Following a peak phosphorylation at 10 minutes, P-Y142 was maintained up to 1 hour and started to decay slightly after overnight stimulation with HGF (Fig. 3C). Contrary to BDNF and NT-3 signaling, Y654 phosphorylation was not stimulated by HGF treatments (Fig. 3B)

indicating that NT and HGF signalling phosphorylate different tyrosine residues. Interestingly,  $\alpha$ -catenin (Fig. 3B) and N-cadherin (data not shown) co-immunoprecipitated with  $\beta$ -catenin and the phosphorylation of  $\beta$ -catenin at Y142 resulted in decreased amounts of  $\alpha$ -catenin in the  $\beta$ -catenin immunoprecipitates ( $88 \pm 5\%$  and  $77 \pm 3.4\%$  lower in HGF and HGF+pervanadate than in control;  $n \geq 3$ ; Fig. 3B). Together these findings indicate that Met interacts with  $\beta$ -catenin engaged in the adhesion complex and phosphorylates Y142, resulting in the detachment of  $\alpha$ -catenin from  $\beta$ -catenin.

### **$\beta$ -catenin is involved in axon growth in hippocampal neurons**

Next we investigated the possible role of  $\beta$ -catenin and its interactions with Trk and Met in axon growth because both types of molecules have been implicated in axon morphogenesis (Honda et al., 1995; Huang and Reichardt, 2001; Huang and Reichardt, 2003; Riehl et al., 1996). First, we analyzed the involvement of  $\beta$ -catenin in axon growth in the absence of neurotrophic factor signalling. In order to do this we designed scrambled and  $\beta$ -catenin shRNA vectors for lentiviral infection of hippocampal neurons. Quantification of Western blots of neurons expressing  $\beta$ -catenin shRNA#1 and #2 indicate a decrease of  $\beta$ -catenin total levels of about 25 and 20% at three days of infection compared to neurons expressing scrambled shRNA (Fig. 4A). Anti- $\beta$ -catenin immunostaining indicates reduced  $\beta$ -catenin immunoreactivity, especially along the axon of GFP-positive neurons expressing  $\beta$ -catenin shRNA#1 (Fig. 4A). We measured axon length in neurons expressing scrambled or  $\beta$ -catenin shRNAs. In these experiments axon length was measured at 3 days in vitro (DIV) because this was the earliest time point at which we observed a significant reduction in the levels of  $\beta$ -catenin and axon length was still easily measured. Neurons expressing  $\beta$ -catenin shRNAs exhibit significantly shorter axons than neurons expressing scrambled shRNA (Fig. 4B, C). Furthermore, stimulation of TrkB and Met by BDNF and HGF respectively, increases axon length in neurons expressing scrambled shRNA (Fig. 4C). However, in neurons expressing  $\beta$ -catenin shRNA#1 and #2, BDNF or HGF treatment can not stimulate axon growth (Fig. 4C). These results demonstrate that  $\beta$ -catenin is required for axon growth downstream of BDNF and HGF signalling.

To further test the requirement of  $\beta$ -catenin in axon growth, we also expressed the intracellular domain of N-cadherin, which was previously used to study dendritogenesis

(Yu and Malenka, 2003). Intracellular N-cadherin sequesters  $\beta$ -catenin, inhibiting its degradation and transactivation capacity (Sadot et al., 1998). For these experiments neurons were co-transfected with EYFP and  $\beta$ -catenin or intracellular N-cadherin (1:4 ratio) (Yu and Malenka, 2003) and stimulated with either BDNF or HGF before axon length was measured. BDNF and HGF promoted axon growth in neurons expressing  $\beta$ -catenin (see below) but these effects were inhibited in neurons that express intracellular N-cadherin (Fig. 4D). Together with our results on  $\beta$ -catenin knockdown, these findings demonstrate that decreasing the availability of  $\beta$ -catenin inhibits the axon growth induced by BDNF and HGF signalling.

### **NTs regulate axon growth and branching by phosphorylating $\beta$ -catenin Y654**

We then looked at the physiological consequences of  $\beta$ -catenin phosphorylation by Trk, by studying axon growth induced by NTs in neurons co-expressing WT or mutant  $\beta$ -catenin and EYFP. Neurons expressing EYFP alone or together with  $\beta$ -catenin did not show axon length values that were significantly different (data not shown). Neurons were treated with BDNF, NT-3 or both and axon length was measured at 2 DIV. Stimulation with BDNF, NT-3 or both increased axon length in neurons expressing EYFP alone by 31%, 18% and 39% respectively (Fig. 5A, B). Neurons overexpressing  $\beta$ -catenin treated with BDNF, NT-3 or both exhibited axons 44%, 20%, and 29% longer respectively than untreated neurons (Fig. 5A, B). Thus, NT treatment increases axon growth in a similar manner in neurons overexpressing or not  $\beta$ -catenin. BDNF together with NT-3 did not increase axon length significantly more than BDNF or NT-3 alone, suggesting that signalling by only one NT was already maximal. Neurons expressing  $\beta$ -catenin treated with BDNF together with the pharmacological inhibitor of Trk activity K252a displayed axons with a length similar to that of controls (Fig. 5A, B). We found that in addition to increasing axon length, BDNF and NT-3 also increased axonal branching (measured as Total Axonal Branch Tip Number, TABTN) by a 35% and 37% respectively compared to control neurons. Furthermore, addition of K252a together with BDNF reduced branching to values similar to the control (Fig. 5A, C). K252a has recently been reported to inhibit Met (Morotti et al., 2002) as well as Trk. However, as BDNF binds to TrkB and K252a inhibited the effects of BDNF, our results strongly suggest that Trk receptors mediate the BDNF effects in axon growth and branching.

We also examined the role of  $\beta$ -catenin phosphorylation at Y654 in axon growth and branching, using mutant Y654F  $\beta$ -catenin which is not phosphorylatable at Y654. Untreated neurons expressing Y654F  $\beta$ -catenin exhibited axons of similar length to those of neurons expressing WT  $\beta$ -catenin (data not shown). Interestingly, we found that neurons expressing Y654F  $\beta$ -catenin treated with BDNF or NT-3 exhibited axon lengths similar to those of untreated neurons (Fig. 5A, B). We therefore concluded that expression of Y654F mutant blocks the NT-induced stimulation of axon growth. Expressing Y654F  $\beta$ -catenin also abolished the NT-induced axon branching (Fig. 5A, C). Conversely, expression of the Y142F  $\beta$ -catenin mutant did not affect the length or branching of the axon induced by NTs (Fig. 5). Altogether, these results demonstrate that phosphorylation at  $\beta$ -catenin Y654 (and not at Y142) is required for the axon extension and branching promoted by NTs.

#### **HGF regulates axon growth and branching by phosphorylating $\beta$ -catenin Y142**

Treatment with HGF also increased axon growth of hippocampal neurons expressing EYFP (18 %) or EYFP and WT  $\beta$ -catenin (35 % compared to untreated neurons; Fig. 6A, B). Remarkably, neurons expressing Y142F  $\beta$ -catenin showed no increase in axon length compared to control cells upon HGF treatment (Fig. 6A, B). In contrast, neurons expressing Y654F  $\beta$ -catenin treated with HGF exhibited axons longer than untreated neurons (41% compared to control; Fig. 6A, B). Treatment with BDNF and HGF did not increase axon length further than BDNF or HGF alone (data not shown). Moreover, HGF signalling increased axon branching in neurons expressing EYFP, EYFP and WT  $\beta$ -catenin or EYFP and Y654F  $\beta$ -catenin (Fig. 6A, C). However, the axon branching induced by HGF was blocked when expressing Y142F  $\beta$ -catenin. These results demonstrate that phosphorylation of Y142 is required for the regulation of axon growth and branching induced by HGF. Dendrite length and branching of EYFP-expressing neurons was not stimulated by BDNF or HGF treatments at 2 DIV suggesting that dendrite morphogenesis is not regulated by tyrosine phosphorylation of  $\beta$ -catenin at this stage of the hippocampal neuron development (Table 1). In summary, our data indicates that  $\beta$ -catenin is specifically phosphorylated at two different sites, Y654 and Y142, by NTs and HGF signalling to regulate axon growth and branching.

#### **P-Y654 $\beta$ -catenin colocalizes with the neuronal cytoskeleton, whereas P-Y142 is found in the nucleus**

To begin to elucidate the downstream events resulting from  $\beta$ -catenin tyrosine phosphorylation at Y654 and Y142 sites, we examined the subcellular localization of the phosphorylated forms, P-Y654 and P-Y142 in hippocampal neurons.  $\beta$ -catenin P-Y654 is present in the cytoplasm, along the axon and at neurite tips and growth cones, where it colocalizes with actin and  $\beta$ III-tubulin (Fig. 7A-C). In contrast,  $\beta$ -catenin P-Y142 localizes to the cytoplasm and nucleus of neurons (Fig. 7D). Anti-Met and anti- $\beta$ -catenin immunostainings also colocalize along neurites, at tips and in the cytoplasm (Suppl. Fig. 1). However, anti-Met and Hoestch staining do not significantly colocalize (Suppl. Fig. 1), suggesting that Met does not translocate to the nucleus to maintain Y142 phosphorylation. Thus, phosphorylation at Y654 and Y142 targets  $\beta$ -catenin to different subcellular compartments, suggesting that these two phosphorylated forms of  $\beta$ -catenin may involve distinct mechanisms to regulate axon morphogenesis.

#### **Dominant negative $\Delta$ N-TCF4 blocks the effect of HGF in axon growth**

In canonical Wnt signalling, nuclear  $\beta$ -catenin binds to the promoter of TCF/LEF to regulate gene transcription (Logan and Nusse, 2004). Consequently TCF factors are likely candidates in the response activated by nuclear  $\beta$ -catenin. Since P-Y142  $\beta$ -catenin was observed in the nucleus, we investigated whether dominant negative  $\Delta$ N-TCF4 that can not bind  $\beta$ -catenin (Roose et al., 1999) was able to modify the NT or HGF effects on axon growth and branching. As a positive control, we treated  $\Delta$ N-TCF4 expressing hippocampal neurons with Wnt-3a, which signals through the canonical Wnt pathway in dorsal root ganglia neurite outgrowth (Lu et al., 2004). Wnt-3a-treated neurons overexpressing EYFP alone or together with WT  $\beta$ -catenin exhibited axons 47 % or 46% longer than controls. Expression of  $\Delta$ N-TCF4 abolished the stimulation of axon growth induced by Wnt-3a (Fig. 8B), indicating that  $\Delta$ N-TCF4 blocks  $\beta$ -catenin nuclear signalling and that in hippocampal neurons Wnt-3a acts through the canonical pathway to increase axon growth. Interestingly, the HGF-induced axon growth and branching was also prevented in neurons expressing  $\Delta$ N-TCF4, whereas the BDNF and NT-3 effects were not altered (Fig. 8). These results indicate that HGF signals through TCF4 in axon growth and branching. In summary, our results indicate that HGF signalling phosphorylates  $\beta$ -catenin at Y142, inducing its nuclear translocation and TCF4-mediated gene transcription to regulate axon morphogenesis. In contrast, NT signalling promotes axon growth and branching by phosphorylating  $\beta$ -catenin Y654 independent of TCF4 function.

## Discussion

Brain function relies on the establishment of the correct pattern of neuronal connections. During development neurons extend their axons under the influence of many signalling molecules (Ayala et al., 2007; Ciani and Salinas, 2005; Dalva et al., 2007; Huang and Reichardt, 2001) that results in the unique morphology of axon arbors and the formation of synapses.  $\beta$ -catenin is a component of the cell adhesion complex and the canonical Wnt pathway that plays a prominent role in development, synapse formation and disease (Ciani and Salinas, 2005; Clevers, 2006; Goda, 2002; Logan and Nusse, 2004; Moon et al., 2004; Salinas and Price, 2005). In neurons,  $\beta$ -catenin tyrosine phosphorylation regulates synapse formation and function (Bamji et al., 2006; Murase et al., 2002). Here we have shown that differential  $\beta$ -catenin tyrosine phosphorylation by growth factor (NTs and HGF) signalling regulates axon morphogenesis. First, by expressing lentiviral-driven  $\beta$ -catenin shRNAs or sequestering  $\beta$ -catenin with intracellular N-cadherin, we demonstrate the requirement for  $\beta$ -catenin in axon outgrowth downstream of growth factor signalling. Second, we show that neuronal RTK Trk and Met phosphorylate  $\beta$ -catenin. We have identified Y654 and Y142 as the sites phosphorylated upon NT and HGF stimulation, respectively. We have also demonstrated the key role that Y654 and Y142 phosphorylations play in the regulation of axon growth and branching elicited by either family of growth factors. Our data indicates that although NT and HGF signalling converge on  $\beta$ -catenin, the pathways that control the growth and arborization of the axon diverge downstream of  $\beta$ -catenin Y654 and Y142 phosphorylations. The NT-phosphorylated form P-Y654  $\beta$ -catenin is found at growth cones, whereas P-Y142  $\beta$ -catenin translocates to the nucleus. Furthermore, the transcription factor TCF4, a central player of canonical Wnt signalling, is needed for HGF but not for NT signalling during axon growth and branching. Therefore, components of the Wnt pathway are shared by the signalling cascades activated by growth factors during axon morphogenesis. We have concluded that HGF and NTs, by activating their RTK, phosphorylate  $\beta$ -catenin at different sites, thus involving transcription-dependent and independent mechanisms that result in the fine tuning of axon morphogenesis.

In this paper we have presented evidence for the interaction of  $\beta$ -catenin with Trk and Met and demonstrate for the first time the involvement of  $\beta$ -catenin phosphorylation downstream of both RTK in the regulation of axon growth and branching. Previous studies illustrated the relationship between  $\beta$ -catenin phosphorylation and RTK such as EGFR, Met and PDGF receptor in epithelial and tumoral cell migration (Brembeck et al., 2004; Hiscox and Jiang, 1999; Hoschuetzky et al., 1994; Theisen et al., 2007). We have shown that  $\beta$ -catenin is associated with TrkA, TrkB and TrkC in PC12 cells and hippocampal neurons. The interaction of  $\beta$ -catenin with Trk was independent of ligand stimulation, as described for EGFR (Hoschuetzky et al., 1994). In addition,  $\beta$ -catenin is a direct substrate of TrkA kinase in vitro. Substitution of Y654 by a phenylalanine results in a significant reduction of  $\beta$ -catenin phosphorylation by TrkA, whereas an Y142F mutation affected  $\beta$ -catenin phosphorylation to a much lesser extent. These experiments demonstrate that TrkA phosphorylates Y654 in vitro. Using TrkA immunoprecipitated from PC12 cells, the residual tyrosine phosphorylation of Y654F  $\beta$ -catenin was not prevented by a Trk inhibitor. TrkB was reported to associate with the non-receptor tyrosine kinase Fyn (Iwasaki et al., 1998; Pereira and Chao, 2007), which phosphorylates  $\beta$ -catenin at Y142 (Piedra et al., 2003). Our results also suggest that TrkA or a kinase that co-immunoprecipitates with TrkA, possibly Fyn, phosphorylates Y654F  $\beta$ -catenin in vitro. More importantly,  $\beta$ -catenin is phosphorylated at Y654 following activation of all three Trk receptors in PC12 cells and primary neurons. Phosphorylation of Y142 was not induced by BDNF, suggesting that NT signalling specifically targets  $\beta$ -catenin Y654 in a cellular context. Moreover, we have shown that  $\beta$ -catenin also associates with neuronal Met.  $\beta$ -catenin was phosphorylated by Met at Y142 in vitro and in neurons following HGF stimulation. In contrast, phosphorylation of mutant Y654F was similar to that of WT  $\beta$ -catenin and HGF treatment did not stimulate phosphorylation of Y654 in neurons, indicating a selectivity of HGF/Met signalling for Y142. Finally, we observed a decreased association of Met and  $\beta$ -catenin upon Y142 phosphorylation. Together, these findings extend to a neuronal context our knowledge about HGF signalling in non-neural cells (Brembeck et al., 2004; Monga et al., 2002; Rasola et al., 2007).

BDNF, NT-3 and HGF all induce axon growth and branching in hippocampal neurons (Korhonen et al., 2000; Labelle and Leclerc, 2000; Vicario-Abejon et al., 1998). By expressing  $\beta$ -catenin single point mutants, we demonstrate that phosphorylation of  $\beta$ -

catenin Y654 is required for the regulation of axon growth and branching induced by BDNF or NT-3. Conversely, phosphorylation of  $\beta$ -catenin Y142 is required for HGF signalling in axon morphogenesis. BDNF and HGF effects on axon growth were not additive, suggesting maximal signalling by each growth factor alone. Our results demonstrate that different growth factors target  $\beta$ -catenin to partially stimulate the growth and arborization of the axon, thus contributing to the complex network of pathways that direct this process. In agreement with this conclusion, regulating the availability of  $\beta$ -catenin by either shRNA-mediated  $\beta$ -catenin silencing or by sequestering it by expressing intracellular N-cadherin (Yu and Malenka, 2003) affected axon growth both in the presence and absence of exogenous growth factors. The ability of intracellular N-cadherin to block  $\beta$ -catenin transactivation (Sadot et al., 1998) and to place  $\beta$ -catenin away from the RTK could explain the inhibition of the axon growth promoted by BDNF and HGF. Together, these results indicate that  $\beta$ -catenin is required for axon growth downstream of growth factor signalling. Dendritic morphogenesis was not stimulated by BDNF or HGF signalling, suggesting that it is not regulated by tyrosine phosphorylation of  $\beta$ -catenin at the initial stages of the hippocampal neuron development analyzed. These results do not rule out a possible role of  $\beta$ -catenin phosphorylation on the maturation of the dendritic tree, in agreement with the implication of  $\beta$ -catenin in this later process (Yu and Malenka, 2003).

What are the mechanisms by which NT and HGF signalling regulate axon extension and branching? Tyrosine phosphorylation of  $\beta$ -catenin was proposed to regulate cell migration and gene transcription by detaching  $\beta$ -catenin from the cadherin-catenins complex (Huber and Weis, 2001; Lilien and Balsamo, 2005; Nelson and Nusse, 2004; Roura et al., 1999). In keeping with this motion, Bamji et al. (2006) showed that tyrosine phosphorylation of  $\beta$ -catenin upon BDNF treatment reduces  $\beta$ -catenin affinity for N-cadherin and mobilizes synaptic vesicles during synapse formation. Likewise, Murase et al. (2002) reported that depolarization, which mimics the effect of a tyrosine kinase inhibitor on  $\beta$ -catenin redistribution to dendritic spines, increases  $\beta$ -catenin and cadherin association in mature hippocampal cultures. In contrast, we were able to observe the  $\beta$ -catenin detachment from N-cadherin in our younger neurons only using pervanadate (Ozawa and Kemler, 1998) (Fig. 2E and data not shown). This result indicates that it is necessary to inhibit tyrosine phosphatases, which are working in consonance with tyrosine kinases (Balsamo et al., 1998; Kypta et al., 1996; Nelson and

Nusse, 2004), to detect the detachment of  $\beta$ -catenin from N-cadherin in 2 DIV hippocampal neurons. This result also suggests that a phosphatase that is developmentally regulated may dephosphorylate Y654  $\beta$ -catenin. In addition,  $\beta$ -catenin and cadherins change their location before and after synapse formation, showing a synaptic distribution as neurons mature (Bamji et al., 2006; Bamji et al., 2003; Benson and Tanaka, 1998; Uchida et al., 1996). Thus, the dynamic localization of cadherin-catenin complexes may result in the immunoprecipitation of adhesion complexes predominantly assembled at “local pools” (i.e., synapses) in older neurons, where the phosphorylation and detachment of  $\beta$ -catenin could mainly take place.

P-Y654 and P-Y142  $\beta$ -catenin were found in different subcellular locations in hippocampal neurons. P-Y654  $\beta$ -catenin is present at neurite tips and growth cones where it colocalizes with cytoskeletal components. This observation suggests that NTs by phosphorylating Y654  $\beta$ -catenin stimulate axon growth and branching by locally regulating the cytoskeleton. Guidance cues regulate growth cone motility and behaviour by eliciting local synthesis of proteins (Piper and Holt, 2004) and extensively affecting the actin and microtubule cytoskeleton (Kalil and Dent, 2005). While pathways activated by NTs may involve signalling to the nucleus (Chao, 2003; Huang and Reichardt, 2001; Sole et al., 2004), NTs can also transduce their signals locally and affect the cytoskeleton to regulate motility, the neuronal morphology and axon growth (Chen et al., 2006; Gehler et al., 2004; Miyamoto et al., 2006; Ye et al., 2003; Zhou et al., 2004). NGF induces growth of dorsal root ganglia neuron axons through inactivation of GSK-3 $\beta$  and regulation of the microtubule plus end binding protein APC at neurite tips (Arevalo and Chao, 2005; Zhou et al., 2004). BDNF increases filopodial length and number, a structure considered the first step in the formation of axonal branches (Gallo and Letourneau, 2004), by activating Actin Depolymerizing Factor /Cofilin (Chen et al., 2006; Gehler et al., 2004) and decreasing RhoA signalling (Yamashita et al., 1999). Indeed, members of the Rho family of small GTPases are key regulators of neuronal morphogenesis (Govek et al., 2005; Luo, 2000). Furthermore, the HGF- or BDNF-induced motile phenotypes and neurite outgrowth are mediated by Rho GTPases (Bosse et al., 2007; Pante et al., 2005; Royal et al., 2000; Zhou et al., 2007). Constitutive active RhoA reduces dendrite branching, suggesting that interactions with the actin cytoskeleton are critical for the  $\beta$ -catenin effects in dendritic morphogenesis (Yu and Malenka, 2003). Future work should help to clarify the mechanisms mediating

$\beta$ -catenin phosphorylation and the growth cone in response to NTs in axon morphogenesis.

Phosphorylated Y142  $\beta$ -catenin was reported to decrease the interaction of  $\alpha$ -catenin- $\beta$ -catenin and to increase the interaction with BCL9-2/Legless allowing its nuclear translocation and transcription of  $\beta$ -catenin target genes (Brembeck et al., 2004). We found that in neurons  $\alpha$ -catenin- $\beta$ -catenin dissociate in response to the HGF-induced  $\beta$ -catenin phosphorylation at Y142. The  $\alpha$ -catenin- $\beta$ -catenin dissociation was observed on  $\beta$ -catenin immunocomplexes also containing Met, thus suggesting that the RTK interacts with and phosphorylates a pool of  $\beta$ -catenin bound to the adhesion complex. P-Y142  $\beta$ -catenin, contrary to P-Y654, was observed in the nucleus in neurons. Consistent with this finding, we have shown that the HGF-induced axon growth and branching requires TCF4-transcriptional activation. This dependence on TCF4 function is also shared by Wnt-3a signalling in axon growth and for the Robo-Abl-Cables-N-cadherin- $\beta$ -catenin regulation of axon guidance mediated by  $\beta$ -catenin Y489 phosphorylation (Rhee et al., 2007). In contrast, the activity-induced dendritic arborization does not require TCF-transcriptional activation but rather availability of the cadherin-catenin complex (Yu and Malenka, 2003). On the other hand, phosphorylation of Y654 stimulates the association of  $\beta$ -catenin to the basal transcription factor TATA-binding protein and increases TCF4-mediated transcription in non-neural cell lines (Hecht et al., 1999; Piedra et al., 2001). Furthermore, it has been suggested that Bcr-Abl phosphorylation of  $\beta$ -catenin Y86 and/or Y654 regulates nuclear  $\beta$ -catenin signalling in chronic myeloid leukemia (Coluccia et al., 2007). Our results in developing neurons however indicate that NTs modulate axon growth and branching by phosphorylating  $\beta$ -catenin Y654 independently of TCF4 function, possibly by affecting the cytoskeleton.

Because  $\beta$ -catenin null mice die during early development, the function of  $\beta$ -catenin in the embryonic period has not been tested in vivo. Using conditional knock-out animals, a role of  $\beta$ -catenin on dendrite development of postnatally born hippocampal dentate gyrus neurons was identified (Gao et al., 2007).  $\beta$ -catenin conditional knock-out animals also showed a mild phenotype in mood- and anxiety-related behavioral models (Gould et al., 2008). However axon morphogenesis of hippocampal neurons has not been analyzed during the embryonic development of these animals. Expression of  $\beta$ -catenin deletion mutants in retinal ganglion cells of live *Xenopus* tadpoles (Elul et al.,

2003) resulted in altered axonal arborization, suggesting that interactions with the N- and C-terminal domains of  $\beta$ -catenin (containing either Y142 or Y654) are required to shape these axons in vivo. Investigating the impact of  $\beta$ -catenin phosphorylation in axon morphogenesis during mouse development will require the generation of Y654F and Y142F knock-in mice.

Our results place  $\beta$ -catenin at a convergence point of both NT/Trk and HGF/Met signalling pathways in the regulation of axon growth and branching. Thus our findings illustrate the complex networks operating in axon morphogenesis, as stimulation of different RTK results in the activation of TCF4 transcription-dependent and independent mechanisms downstream of  $\beta$ -catenin (Fig. 9). It would be interesting to investigate which are the TCF4 target genes responsible for the axon growth and branching promoted by HGF. In summary, this work highlights the important role played by  $\beta$ -catenin in integrating trophic signals that emanate from RTK during axon morphogenesis.

## **Materials and Methods**

### **Materials**

BDNF and NT-3 were obtained from Alomone Labs, NGF from Sigma, HGF and K252a from Calbiochem and Wnt-3a from R&D. Antibodies were purchased from the following companies: Met from Santa Cruz, P-Y654 and P-Y142 from Abcam,  $\beta$ III-tubulin from Covance, P-Tyrosine (P-Tyr; 4G10), TrkB and TrkC from Upstate Biotechnology,  $\beta$ -actin from Sigma,  $\beta$ -catenin from BD Transduction Laboratories, N-cadherin from Zymed,  $\alpha$ -catenin from the Hybridoma Bank and EYFP/GFP from Acris Antibodies.

### **TrkA immunoprecipitation and in vitro kinase assays**

TrkA was isolated from a PC12 clone overexpressing human TrkA (PC12 6/15) following stimulation with NGF 100 ng/ml (7 minutes at 37°C). Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA pH 8, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5mM DTT) containing Complete protease inhibitor (Roche), 40 mM  $\beta$ -glycerophosphate, 1mM sodium orthovanadate and 25 mM sodium fluoride. Cell lysates were incubated with anti-pan Trk antibodies (Martin-Zanca et al., 1989) followed by Protein G-Sepharose (Sigma). GST- $\beta$ -catenin recombinant proteins (Roura et al., 1999) were produced in BL21 bacteria and cleaved from GST with thrombin where necessary (8U to cleave 1 mg protein, 80 minutes, 30°C). GST- $\beta$ -catenin (0.8  $\mu$ g; 6.7 pmols) phosphorylation by recombinant TrkA and Met kinases (Cell Signalling) was performed following manufacturer's instructions (15 minutes, 30°C).  $\beta$ -catenin (1.5  $\mu$ g; 17 pmols) phosphorylation by immunoprecipitated TrkA was assayed (20 minutes, 30°C) in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 0.5 mM EDTA, 1mM DTT, 0.1% NP-40, 1 mM sodium orthovanadate with or without 100 nM K252a (preincubated with the kinase 5 minutes, RT) and 0.1 mM ATP. Samples were analyzed by Western blot.

### **Hippocampal cultures and transfection**

Rat primary hippocampal neurons were isolated from 18-19 day embryos and cultured in DMEM medium supplemented with N2 and B27. Neurons were plated on poly-D-lysine coated glass coverslips either at 500-1000 cells/mm<sup>2</sup> for transfection or biochemical experiments or 70 cells/mm<sup>2</sup> for immunostaining following lentiviral

infection. WT, Y654F and Y142F  $\beta$ -catenin cDNAs were obtained and cloned into pcDNA3.1 as described (Piedra et al., 2003). Xenopus intracellular N-cadherin (containing part of the fifth extracellular domain, the transmembrane and the cytoplasmic domains) was kindly provided by Dr. X. Yu (Yu and Malenka, 2003). Cells were co-transfected at 1 day in vitro (DIV) with 1.2  $\mu$ g of  $\beta$ -catenin/cadherin constructs and 0.3  $\mu$ g of EYFP DNA using Lipofectamine 2000 (Invitrogen) (Espinet et al., 2000). After 3 hours the medium was changed and supplemented with appropriate growth factors, Wnt-3a (all at 50 ng/ml) and K252a (100 nM; preincubated 20 minutes before BDNF addition) when necessary. Neurons were fixed at 2 DIV and immunostained with anti-GFP antibodies.

### **Immunofluorescence and measurements**

For phosphospecific  $\beta$ -catenin immunostaining neurons were treated with pervanadate (1 mM, 15 minutes, 37°C) before fixation. Neurons were fixed with 4 % paraformaldehyde (PFA) for 20 minutes at RT. For cytoskeletal staining detergent fixation (3% formaldehyde, 0.2% glutaraldehyde, 0.2% Triton X100, 10 mM EGTA pH 7.2) was used (10 minutes, 37°C) where indicated. Binding of TRITC or FITC-Phalloidin (0.2  $\mu$ g/ml in PBS, 1 hour at RT; Sigma) to localize actin filaments was performed in cells permeabilized with 0.2% Triton X100 in PBS without blocking. Cells were washed with phosphate buffer saline (PBS) and blocked and permeabilized in PBS containing 5% Foetal calf serum, 5% Horse serum, 0.2% glycine and 0.1% Triton X100. Secondary antibodies were Alexa Fluor 488 and Fluor 594 (Molecular Probes). Micrographs were obtained using an inverted Olympus IX70 microscope (10x, 0.3 NA and 20x, 0.4 NA) equipped with epifluorescence optics and a camera (Olympus OM-4 Ti). Images were acquired using DPM Manager Software. Alternatively, images were obtained using an Olympus confocal IX70 microscope and the FluoView 500 Software and processed using MacBiophotonics ImageJ software ([www.macbiophotonics.ca](http://www.macbiophotonics.ca)). Axon length was measured using Adobe Photoshop software and the axon was identified as the longest neurite at this stage (2 DIV) of the hippocampal cell development. Branching was measured by counting Total Axonal Branch Tip Number (TABTN)(Yu and Malenka, 2004). Typically, 15-20 neurons were measured/condition in  $\geq$  three independent experiments. Axon length and branching plots represent values normalized to the corresponding untreated control, shown as average  $\pm$  s.e.m. Significance was calculated by the Student T test. Asterisk (\*)

indicates statistical significance compared to the corresponding untreated control and hash (#) compared to stimulated controls (see legends for details).

### **Immunoprecipitations and Western blotting**

Neurons were deprived of B27 overnight and stimulated with growth factors (50 ng/ml, 10 minutes, unless otherwise indicated) at 2 DIV. Cells were treated with pervanadate to inhibit tyrosine phosphatases (1mM final; 10 minutes together with the stimulus) where indicated. 6/15 PC12 cells were serum starved overnight before stimulation with NGF 100 ng/ml. PC12 cells and hippocampal neurons were lysed in NP-40 (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 10% glycerol and 1% NP-40) or RIPA buffer containing protease and phosphate inhibitors. RIPA buffer was used for all the experiments showing  $\beta$ -catenin phosphorylation in neurons. Cell lysates were incubated with antibodies and Protein-G-Sepharose overnight at 4°C prior to Western blot analysis. Densitometric analysis of the scanned film images was performed using Scion software.  $\beta$ -catenin phosphorylation was calculated by referring the intensity of the phosphospecific band to that of the untreated control and normalizing against total  $\beta$ -catenin (see legends).

### **ShRNA by lentiviral infection**

shRNA primers specific for rat  $\beta$ -catenin were designed chosen using algorithms available on Promega and Invitrogen websites. The primers selected correspond to GTTTGTGCAGTTGCTTTAT (shRNA#1) and GGGTTCCGATGATATAAAT (shRNA#2). Primers were designed for cloning into pSUPER plasmid using the BglII and HindIII sites. They were subcloned into the pLVTHM plasmid (from Dr. Trono, Geneva) together with the H1 promoter for the RNA pol III. shRNA vectors were transfected into HEK293T cells together with the plasmids psPAX2 and pMD2G (from Dr Trono) using the polyethyleneimine transfection method. Transfection efficiency was analyzed by GFP expression (driven by the pLVTHM plasmid). The HEK293T medium was collected after 48 hours of transfection and centrifuged at 50,000xg for 3 hours. The viral pellet was resuspended in sterile PBS plus 2% Bovine Serum Albumin. Hippocampal neurons were transduced 2 hours after plating. According to GFP expression driven by the lentiviral vector ~ 90-95% of neurons were transduced. Neurons were stimulated with growth factors at 2 DIV and fixed at 3 DIV for GFP immunostaining and measurement of axon length with at least 25 or more GFP-positive

transduced neurons measured/condition.

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## Figure legends

**Figure 1.  $\beta$ -catenin interacts with TrkA in PC12 cells and with TrkB and TrkC in hippocampal neurons.** (A) Control (Myc) or  $\beta$ -catenin immunoprecipitation from 6/15 PC12 cells starved or stimulated with NGF (100 ng/ml) for different times shows that TrkA (detected with panTrk antibodies: 140 kDa and 110 kDa forms) co-immunoprecipitates with  $\beta$ -catenin, independent of its phosphorylation state. (B) Control (GFP) or pan-Trk immunoprecipitation from hippocampal neurons untreated or treated with BDNF or NT-3 (50 ng/ml; 5 minutes) shows that TrkB and TrkC co-immunoprecipitate with  $\beta$ -catenin.

**Figure 2. Trk phosphorylates  $\beta$ -catenin at Y654.** (A) In vitro kinase assay using recombinant TrkA kinase and WT or mutant GST- $\beta$ -catenin. Anti-P-Tyr Western blot shows phosphorylated TrkA kinase and GST- $\beta$ -catenin. WT GST- $\beta$ -catenin is phosphorylated by TrkA kinase upon addition of ATP, while K252a inhibits the TrkA induced- $\beta$ -catenin phosphorylation. Phosphorylation of Y654F and Y142F GST- $\beta$ -catenin by TrkA kinase is ~ 45% and 15% lower than that of WT GST- $\beta$ -catenin. Panel below shows anti- $\beta$ -catenin Western blot for the GST- $\beta$ -catenin input in the kinase assay (0.8  $\mu$ g). (B) In vitro kinase assay using TrkA immunoprecipitated from 6/15 PC12 and recombinant WT and Y654F  $\beta$ -catenin. Anti-P-Tyr Western blot shows TrkA (140 kDa and 110 kDa bands) and the tyrosine phosphorylation of  $\beta$ -catenin. Phosphorylation of WT  $\beta$ -catenin is decreased by preincubation with K252a (100 nM), whereas phosphorylation of Y654F  $\beta$ -catenin is lower than that of WT  $\beta$ -catenin and not inhibited by K252a. In the first lane no recombinant  $\beta$ -catenin was added to control for phosphorylation of any endogenous  $\beta$ -catenin that may have been immunoprecipitated with TrkA. Quantification corresponds to % of the increase in the intensity of P-Tyr  $\beta$ -catenin relative to minus ATP/total  $\beta$ -catenin. (C) Control (Myc) or  $\beta$ -catenin immunoprecipitation from 6/15 PC12 cells untreated or treated with NGF 100 ng/ml and pervanadate for the last 10 minutes of stimulation. Phosphorylation of  $\beta$ -catenin Y654 increases after NGF treatment in parallel with TrkA stimulation as detected by anti-P-Tyr Western blot. (D) Control (His) or  $\beta$ -catenin immunoprecipitation from untreated hippocampal neurons or those treated for 10 minutes with BDNF or NT-3 50 ng/ml in the presence of pervanadate. Upon NT stimulation, phosphorylation of  $\beta$ -

catenin at Y654 increases parallel with phosphorylation of TrkB/C detected by anti-P-Tyr Western blot. (E) Control or  $\beta$ -catenin immunoprecipitation from hippocampal neurons untreated or treated for 10 minutes with BDNF 50 ng/ml with or without pervanadate.  $\beta$ -catenin P-Y654 increases with BDNF treatments both with and without pervanadate, while phosphorylation at Y142 is maintained at basal level increasing only slightly with pervanadate. TrkB and N-cadherin co-immunoprecipitate with  $\beta$ -catenin and N-cadherin association shows a small decrease after pervanadate treatment. (F)  $\beta$ -catenin immunoprecipitation from untreated neurons or those treated with BDNF 50 ng/ml for the indicated times in the presence of pervanadate for the last 10 minutes of stimulation.  $\beta$ -catenin P-Y654 peaks at 1 hour and is reduced after overnight stimulation. Quantifications in C, D, E and F correspond to % of the increase in the intensity of the phosphospecific  $\beta$ -catenin (P-Y654 or P-Y142) band relative to the control normalized to total  $\beta$ -catenin.

**Figure 3. Met phosphorylates  $\beta$ -catenin at Y142.** (A) In vitro kinase assay with recombinant Met kinase and WT or mutant GST- $\beta$ -catenin. Anti-P-Tyr Western blot shows phosphorylated Met kinase upon addition of ATP and its basal activation (-ATP condition). Anti-P-Y142 Western blot indicates that WT and Y654F GST- $\beta$ -catenin are phosphorylated by Met kinase at Y142, whereas phosphorylation of Y142F GST- $\beta$ -catenin is as low as in basal condition (-ATP). (B) Control (His) or  $\beta$ -catenin immunoprecipitation from untreated hippocampal neurons or those treated with HGF 50 ng/ml for 10 minutes with or without pervanadate.  $\beta$ -catenin P-Y142 increases with HGF treatments, whereas phosphorylation at Y654 is not stimulated by HGF signalling. Met and  $\alpha$ -catenin co-immunoprecipitate with  $\beta$ -catenin, and the recovery of both proteins decreases upon phosphorylation of  $\beta$ -catenin at Y142. (C)  $\beta$ -catenin immunoprecipitation from untreated neurons or those treated with HGF 50 ng/ml for the indicated times.  $\beta$ -catenin P-Y142 peaks at 10 minutes, is maintained at similar levels up to 1 hour and starts decaying slightly after overnight stimulation. Quantifications in B and C correspond to % of the increase in the intensity of the phosphospecific  $\beta$ -catenin band relative to the control normalized to total  $\beta$ -catenin.

**Figure 4.  $\beta$ -catenin is involved in axon growth downstream of BDNF and HGF signalling.** (A) Western blot from hippocampal neurons infected with lentiviral-driven scrambled or  $\beta$ -catenin shRNA#1 and #2 shows the decrease in  $\beta$ -catenin expression by

$\beta$ -catenin shRNAs compared to scrambled shRNA (scr) or non-infected (ni) neurons. Actin was used as a loading control. Plot represents quantification of  $\beta$ -catenin corrected against the loading control ( $n \geq 4$  experiments). Panels show confocal images for anti- $\beta$ -catenin and anti-GFP (indicating transduced neurons) immunostainings in neurons expressing scrambled or  $\beta$ -catenin shRNA#1. Note the lower levels of  $\beta$ -catenin especially along the axon in  $\beta$ -catenin shRNA#1-expressing neurons. Arrows indicate colocalization between GFP and  $\beta$ -catenin immunostainings in neurons expressing scrambled shRNA that is reduced in neurons expressing  $\beta$ -catenin shRNA#1. Bar= 20  $\mu$ m. (B) Hippocampal neurons expressing scrambled or  $\beta$ -catenin shRNA#1 fixed and immunostained against GFP (driven by the lentiviral vector) at 3 DIV show the decrease in axon length obtained by expressing shRNA#1. Bar = 40  $\mu$ m. (C) Quantification of axon length of scrambled and  $\beta$ -catenin shRNA neurons, untreated or treated with BDNF or HGF (normalized to untreated neurons expressing scrambled shRNA). Neurons expressing  $\beta$ -catenin shRNA#1 and #2 display axons shorter than controls. Stimulation with BDNF or HGF (50 ng/ml) promotes axon growth in neurons expressing scrambled shRNA, but not in neurons expressing  $\beta$ -catenin shRNA#1 and #2 ( $n = 4-8$  experiments). \* $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$  when comparing to the untreated neurons expressing scrambled shRNA; # $p \leq 0.05$ , ##  $p \leq 0.01$  when comparing to the BDNF or HGF-treated neurons expressing scrambled shRNA. (D) Quantification of axon length in neurons expressing N-cadherin intracellular or  $\beta$ -catenin, either untreated or stimulated with BDNF or HGF (normalized to the respective untreated neurons). Neurons expressing  $\beta$ -catenin display axons longer than controls upon BDNF or HGF (50 ng/ml) stimulation. In contrast, in neurons expressing intracellular N-cadherin, BDNF or HGF can not stimulate axon growth ( $n = 4$  experiments). \* $p \leq 0.05$ , \*\*\*  $p \leq 0.001$  when comparing to the corresponding untreated control; # $p \leq 0.05$ , ##  $p \leq 0.01$  when comparing to the BDNF or HGF-stimulated  $\beta$ -catenin-expressing neurons.

**Figure 5.  $\beta$ -catenin phosphorylation at Y654 regulates axon growth and branching induced by NTs.** (A) Hippocampal neurons transfected with EYFP alone (control) or with EYFP and WT or mutant (Y654F or Y142F)  $\beta$ -catenin. BDNF and NT-3 (50 ng/ml) induce axon growth and branching in control neurons and in neurons overexpressing WT or Y142F  $\beta$ -catenin, but the NT-induced axon growth is inhibited in neurons expressing the mutation Y654F. Treatment with BDNF plus K252a results in axons with a length and branching values similar to those of untreated neurons and of

Y654F-expressing cells. Bar= 80  $\mu$ m. (B) Quantification of axon length normalized to the respective control. BDNF and NT-3 increase axon length in control EYFP neurons and in neurons expressing WT or Y142F  $\beta$ -catenin, but not in neurons expressing the Y654F mutant. Treatment with BDNF together with NT-3 does not result in a significant further increase. Treatment with K252a (100 nM) and BDNF abolishes the increase in axon length induced by BDNF. (C) Quantification of axon branching (TABTN) shows that BDNF and NT-3 increase branching of axons expressing WT and Y142F  $\beta$ -catenin, but expression of Y654F abolishes the branching induced by NTs. K252a (100 nM) together with BDNF abolishes the BDNF-promoted branching. (\* $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ ; n = 3-8 experiments).

**Figure 6.  $\beta$ -catenin phosphorylation at Y142 regulates axon growth induced by HGF.** (A) Hippocampal neurons transfected with EYFP alone (control) or with EYFP and WT or mutant (Y654F or Y142F)  $\beta$ -catenin. HGF (50 ng/ml) induces axon growth in neurons expressing WT or Y654F  $\beta$ -catenin, but the HGF-induced axon growth is inhibited in neurons expressing the mutation Y142F. Bar = 40  $\mu$ m. (B) Quantification of axon length normalized to the respective control shows that HGF increases axon length in neurons expressing WT and Y654F  $\beta$ -catenin, but not in neurons expressing Y142F. (C) Quantification of axon branching shows that HGF treatment induces axon branching that is blocked by the Y142F mutation (\*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ ; n = 4-7 experiments).

**Figure 7. P-Y654  $\beta$ -catenin colocalizes with the neuronal cytoskeleton and P-Y142 is found in the nucleus.** (A) Double immunostaining for endogenous  $\beta$ -catenin and F-actin (phalloidin) in hippocampal neurons shows that  $\beta$ -catenin and F-actin partially colocalize (arrows) at neurite tips and lamella (inset). (B) Confocal image of hippocampal neurons fixed using detergent fixation shows the double immunostaining for P-Y654 and  $\beta$ III-tubulin colocalizing at enlarged growth cones where microtubules unbundle and along the axon (arrows). P-Y654 immunostaining can also be observed along filopodial-like processes and axonal spread areas devoid of  $\beta$ III-tubulin (arrowheads). (C) Confocal image of neurons fixed by detergent fixation shows the double immunostaining for P-Y654 and F-actin (phalloidin). Similar to anti- $\beta$ -catenin antibodies, anti-P-Y654 antibodies stain the cell body and neurite tips where P-Y654 and F-actin colocalize (arrows). (D)  $\beta$ -catenin and P-Y142 colocalize within the cytoplasm of neurons (arrows), whereas colocalization of Hoestch and anti-P-Y142

immunostaining shows that P-Y142  $\beta$ -catenin is found in the nucleus (arrowheads). (E) Anti-P-Y654 antibodies stain the cell membrane at cell contacts and the cytoplasm, but the nucleus (stained by Hoestch) is negative. Bars = 15  $\mu$ m (A), 20  $\mu$ m (B and C) and 45  $\mu$ m (D and E).

**Figure 8.  $\Delta$ N-TCF4 abolishes the effect of HGF in axon growth and branching.** (A)

Hippocampal neurons transfected with EYFP and  $\Delta$ N-TCF4, untreated or treated with BDNF, NT-3 and HGF (50 ng/ml).  $\Delta$ N-TCF4 abolishes the axon growth induced by HGF, but not by BDNF or NT-3. Bar = 40  $\mu$ m. (B) Quantification of axon length in EYFP- or EYFP plus  $\Delta$ N-TCF4-expressing neurons normalized to the respective untreated control shows that HGF and Wnt-3a (used as positive control; 50 ng/ml) do not increase axon length in neurons expressing  $\Delta$ N-TCF4, whereas NTs similarly stimulate axon growth in  $\Delta$ N-TCF4-expressing or control neurons. (C) Quantification of axon branching in neurons expressing EYFP alone or EYFP and  $\Delta$ N-TCF4 shows that the HGF-induced branching is inhibited by  $\Delta$ N-TCF4 expression, whereas the BDNF and NT-3-induced effects are not affected. (n=5-7 experiments) \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , when comparing to the corresponding untreated controls; #  $P \leq 0.05$ , ##  $P \leq 0.01$ , ### $P \leq 0.001$ , when comparing to the corresponding stimulated control.

**Figure 9. Model for the role of  $\beta$ -catenin tyrosine phosphorylation in axon growth and branching.**

Adhesion complexes are assembled at the plasma membrane of the cell body, axon and growth cone.  $\beta$ -catenin residue Y654 is found at the region that binds to N-cadherin, whereas  $\beta$ -catenin Y142 is in the  $\alpha$ -catenin binding region (Aberle et al., 1996; Lilien and Balsamo, 2005).  $\alpha$ -catenin also binds to actin (Drees et al., 2005; Yamada et al., 2005). Trk and Met associate to a pool of  $\beta$ -catenin bound to N-cadherin and  $\alpha$ -catenin.  $\beta$ -catenin phosphorylation by HGF/Met signalling at  $\beta$ -catenin Y142 targets  $\beta$ -catenin to the nucleus, where it activates transcription by TCF4 to increase axon growth and branching. The detachment of P-Y142  $\beta$ -catenin from the adhesion complex may imply dynamic regulation of Y654 phosphorylation, perhaps by Src family tyrosine kinases (Roura et al., 1999) and its dephosphorylation before nuclear translocation of P-Y142  $\beta$ -catenin (according to the lack of nuclear immunostaining for P-Y654).  $\beta$ -catenin phosphorylation by NT/Trk signalling at  $\beta$ -catenin Y654 detaches  $\beta$ -catenin from N-cadherin (Bamji et al., 2006; Huber and Weis, 2001; Lilien and Balsamo, 2005; Roura et al., 1999) and P-Y654  $\beta$ -catenin associates with actin and

microtubules at the growth cone, possibly regulating the cytoskeleton to promote axon growth and branching. Note that whereas we demonstrate that  $\beta$ -catenin/ $\alpha$ -catenin dissociate upon phosphorylation of  $\beta$ -catenin Y142, the depicted  $\beta$ -catenin dissociation from N-cadherin upon Y654 phosphorylation is based on the references above. For simplicity, Met has been located at the cell body and Trk at the growth cone plasma membrane (N, N-cadherin;  $\beta$ ,  $\beta$ -catenin;  $\alpha$ ,  $\alpha$ -catenin; --, F-actin; "", microtubules).

**Supplemental Figure 1.** Met (A) and  $\beta$ -catenin (B) immunostainings colocalize intracellularly and at neurite tips (C; arrows). (D) Met immunostaining does not significantly colocalize with Hoestch nuclear staining (arrowheads). Bar = 45  $\mu$ m.

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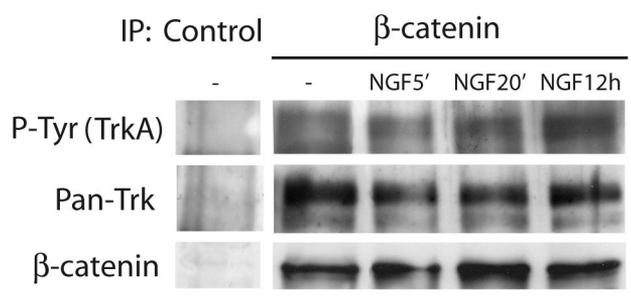
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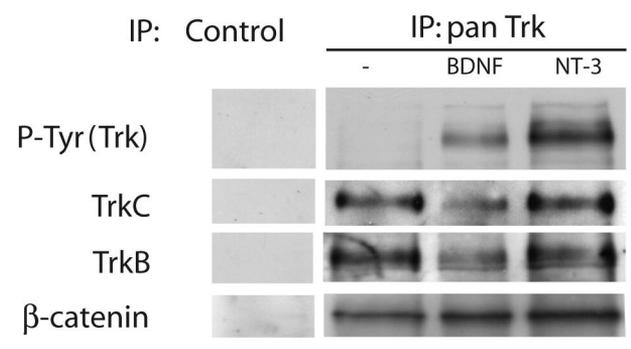
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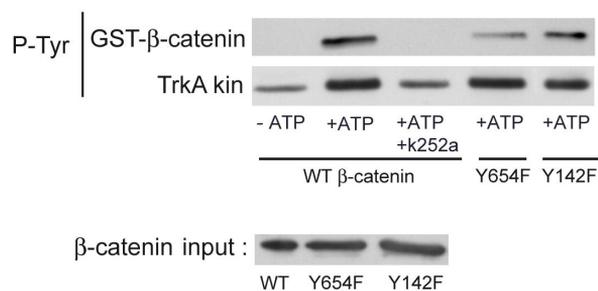
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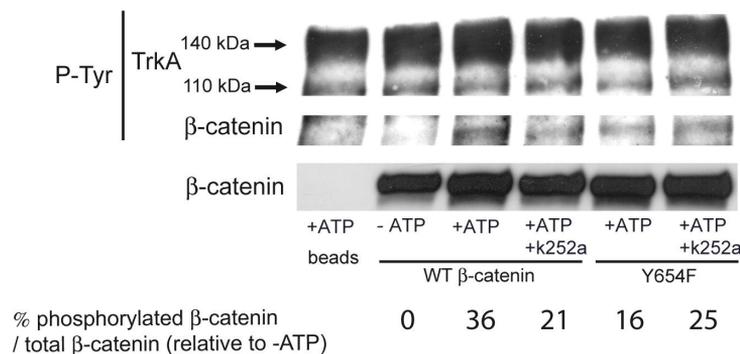
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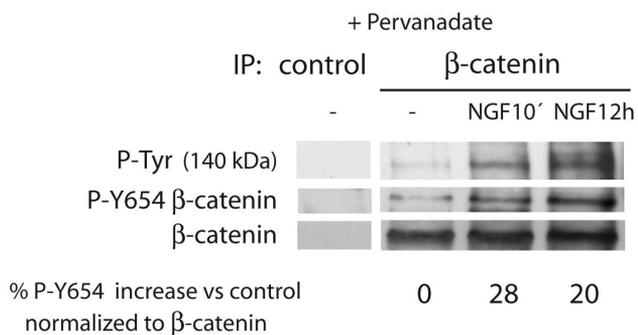
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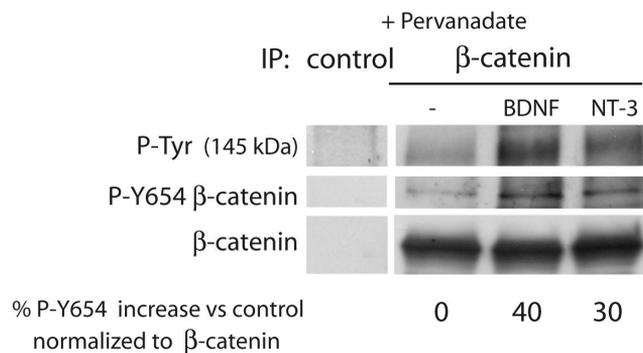
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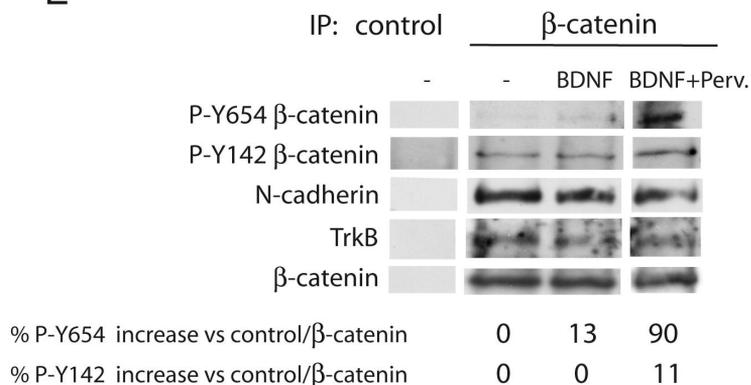
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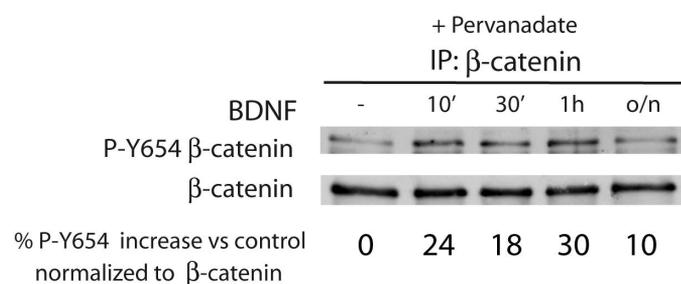
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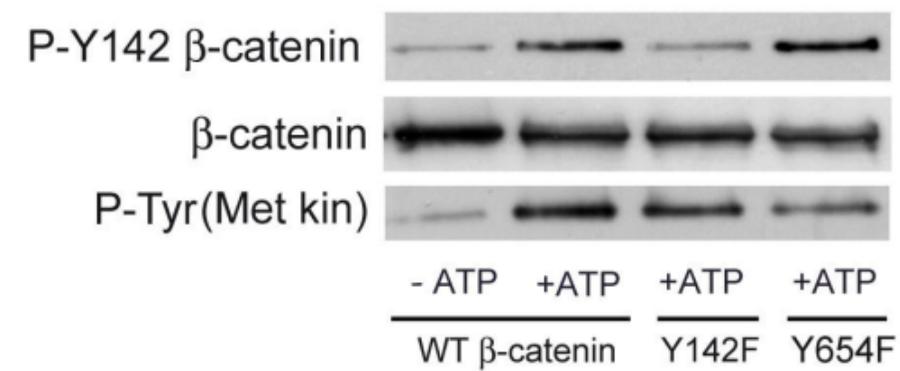
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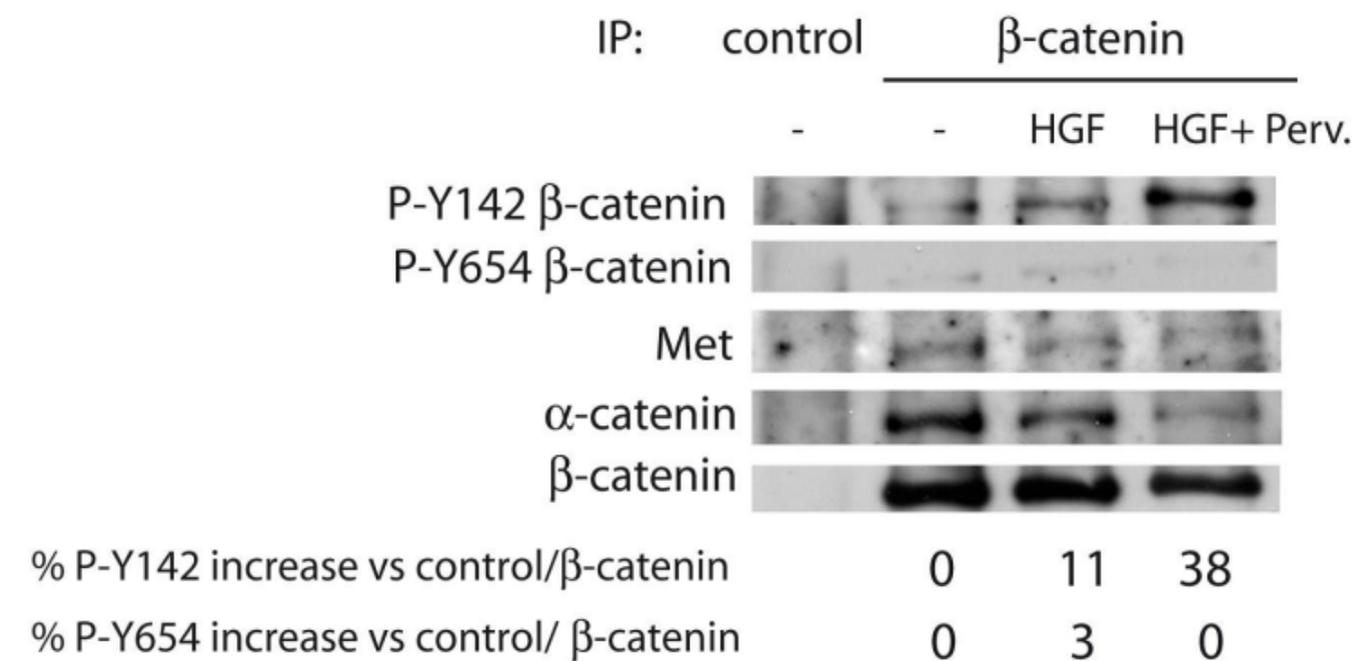
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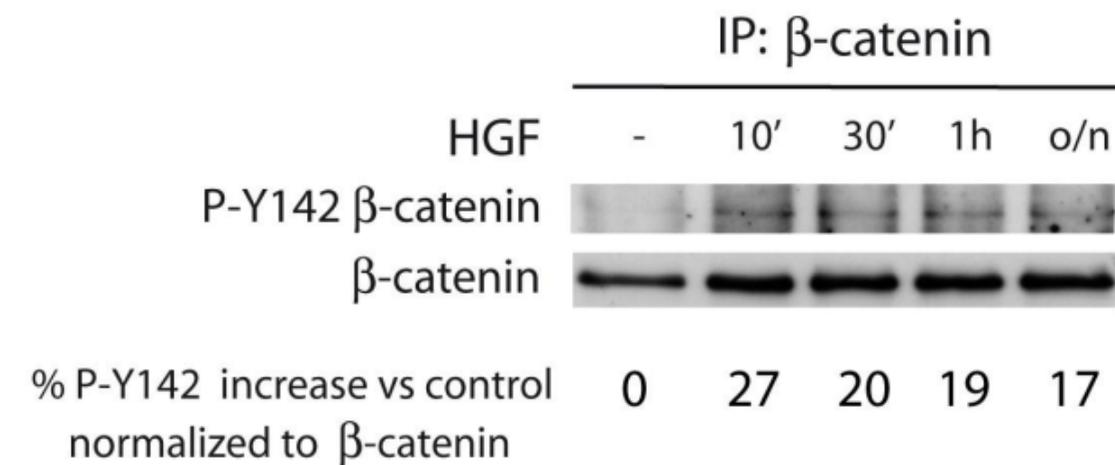
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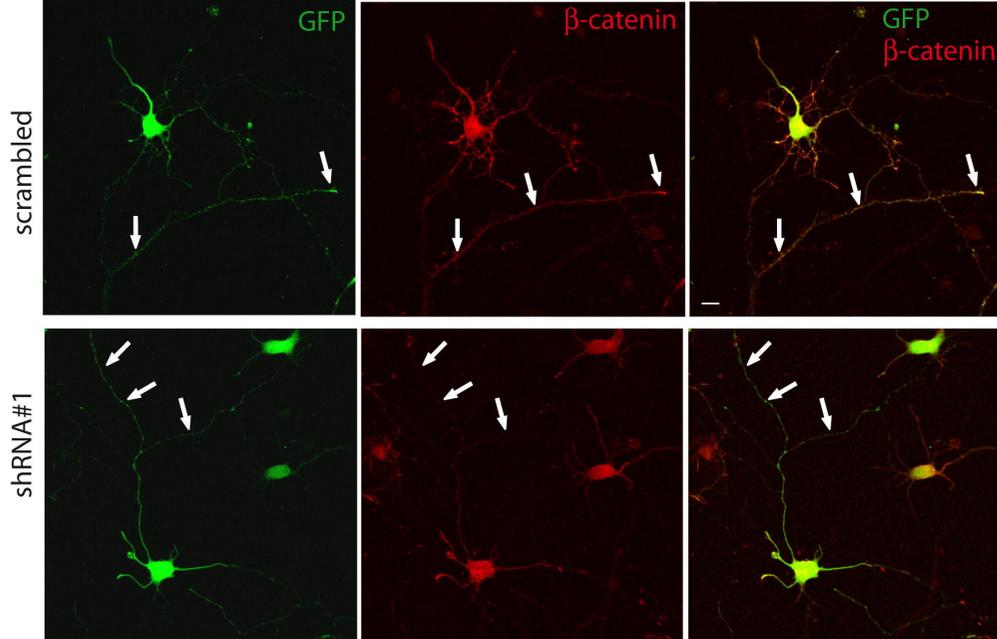
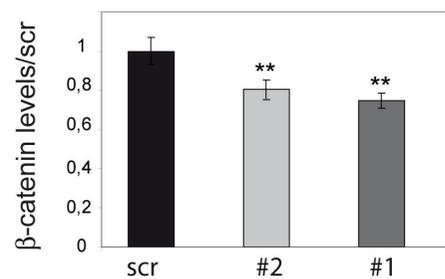
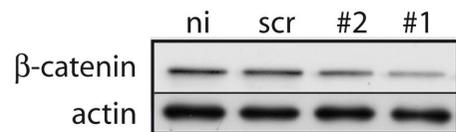
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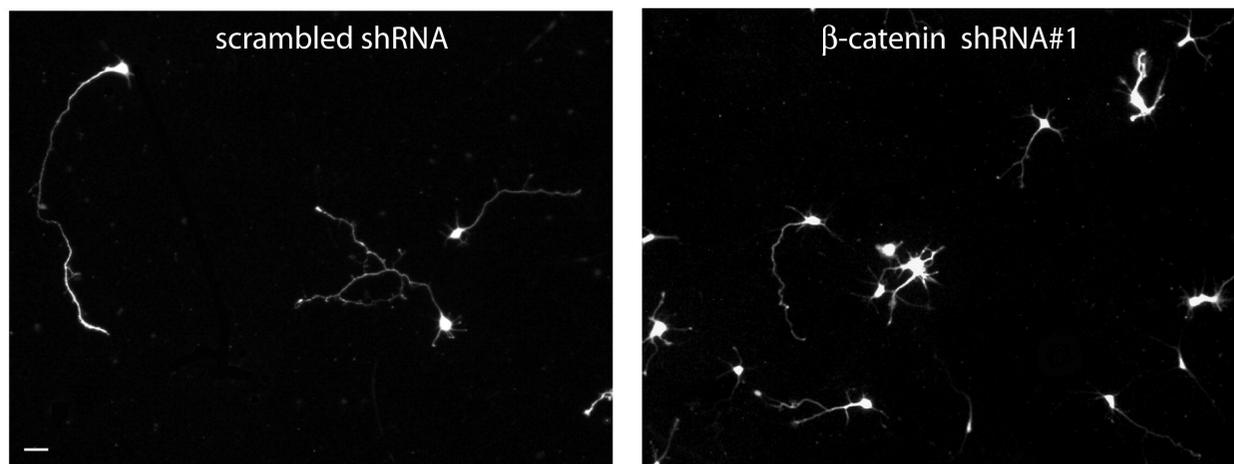
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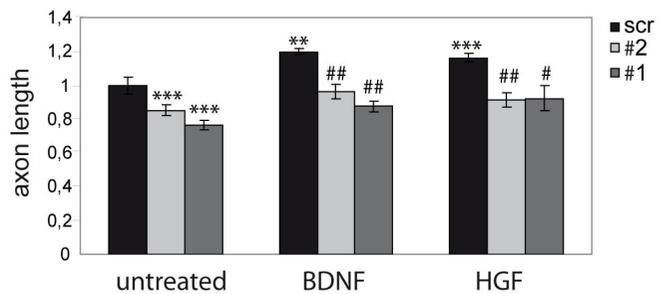
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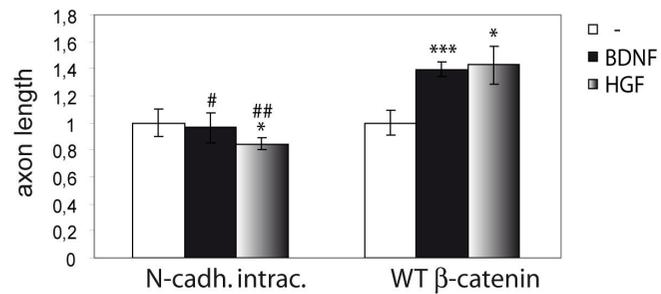
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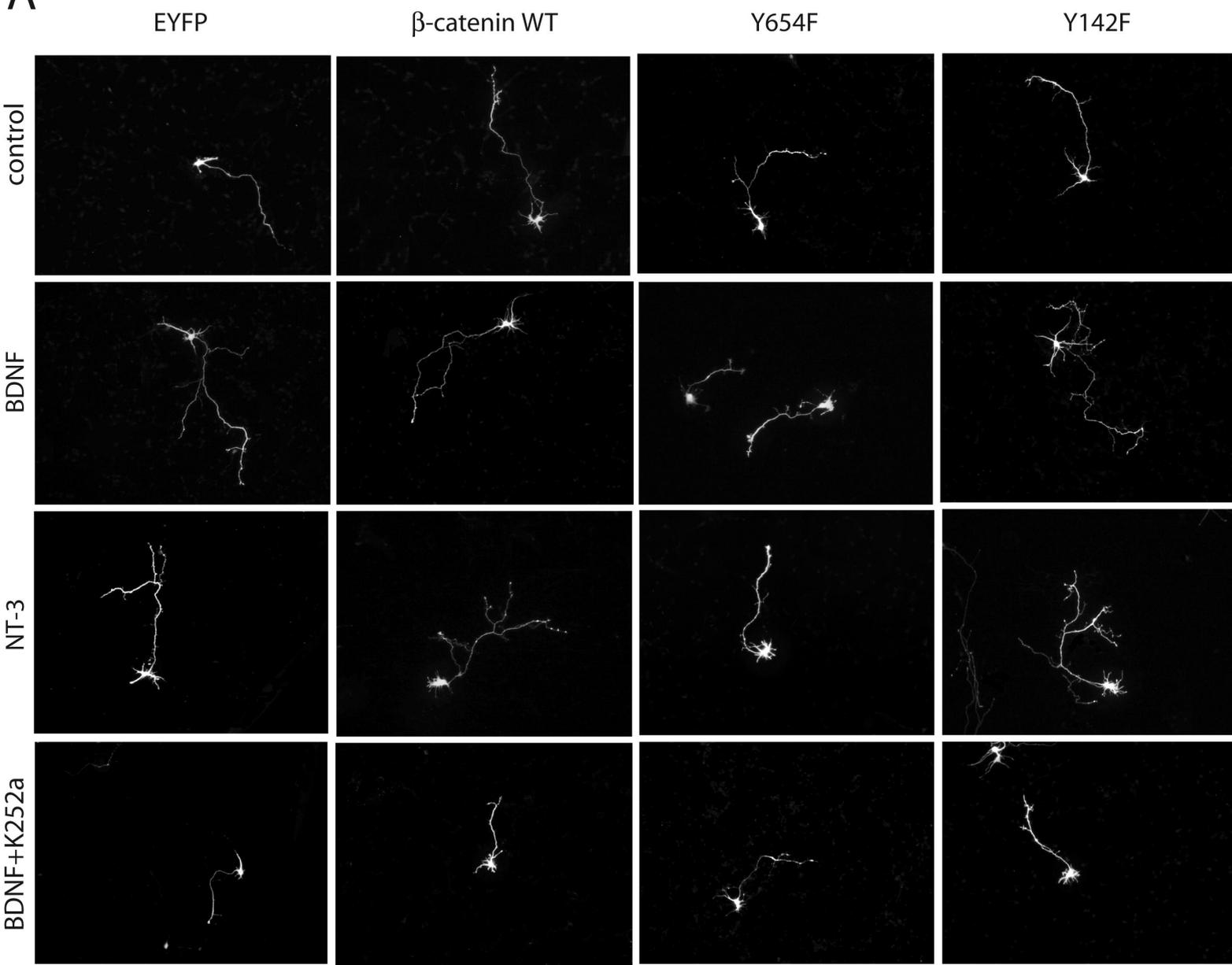
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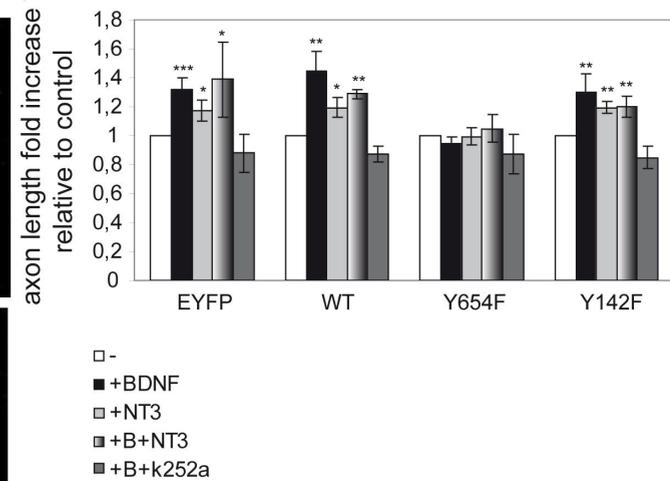
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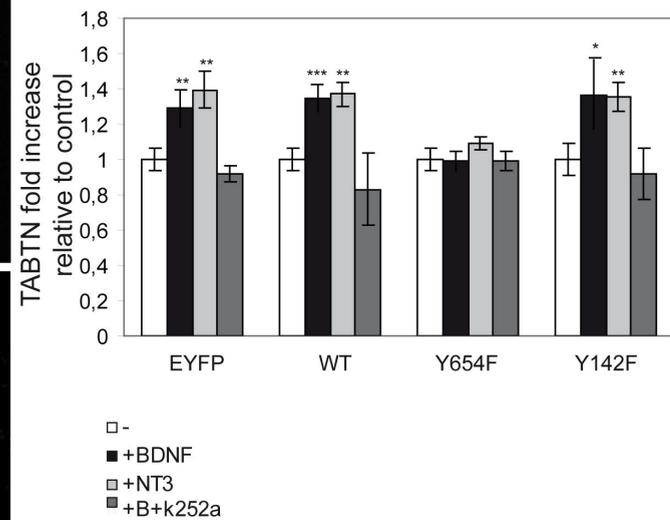
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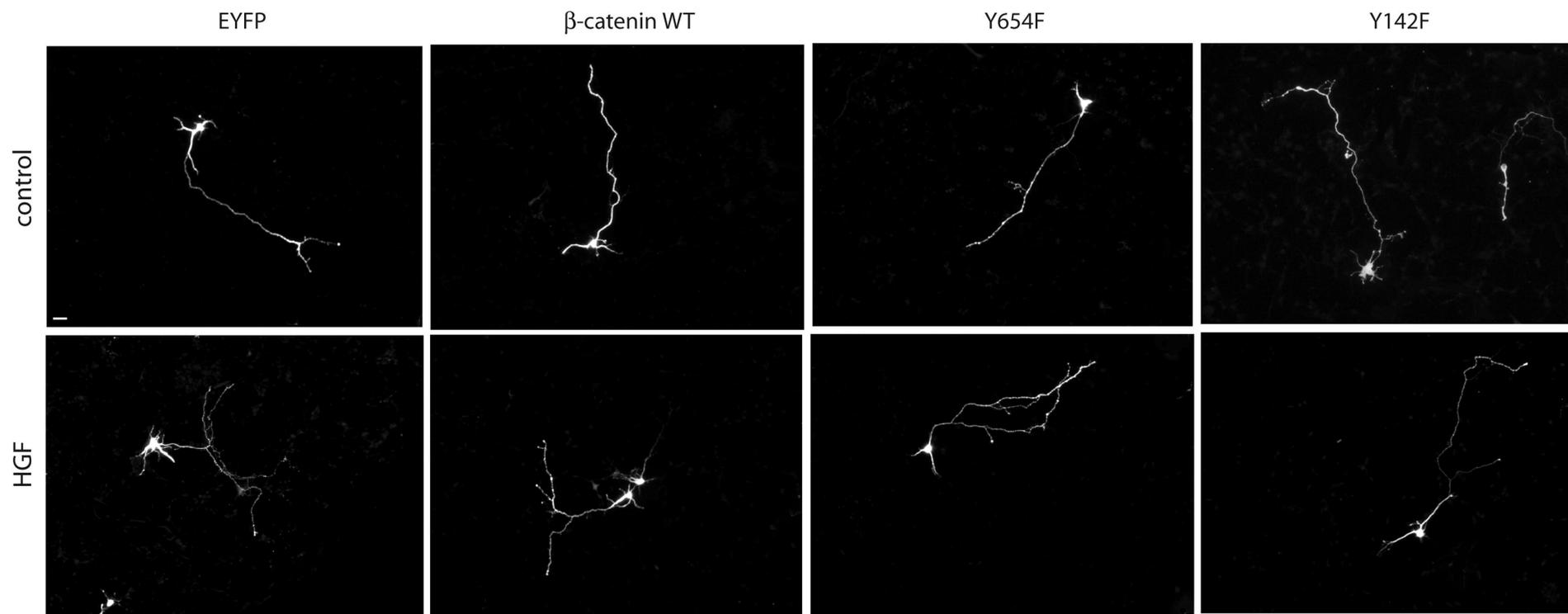
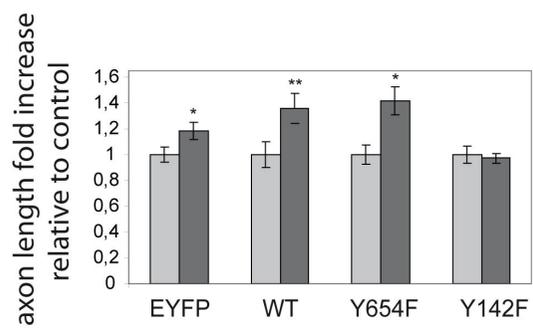
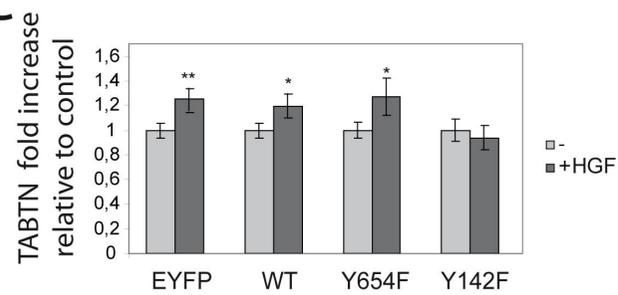


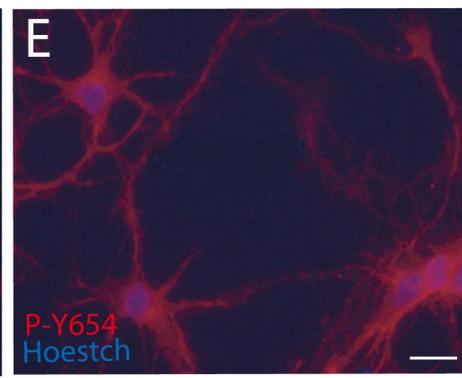
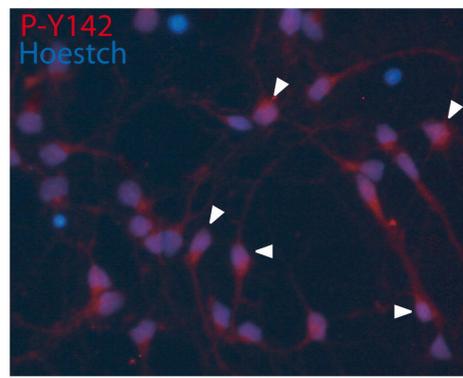
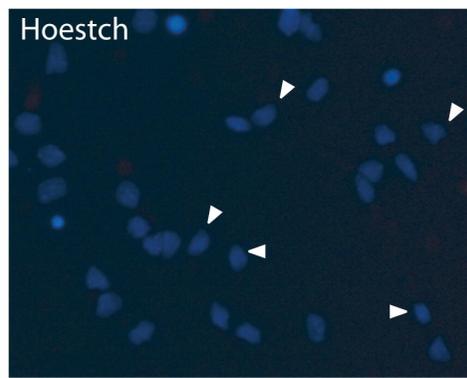
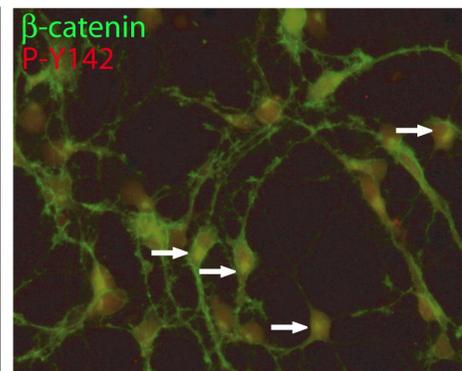
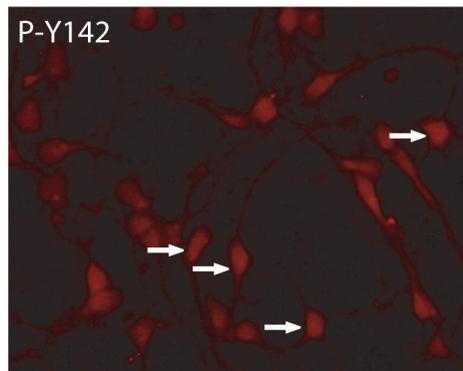
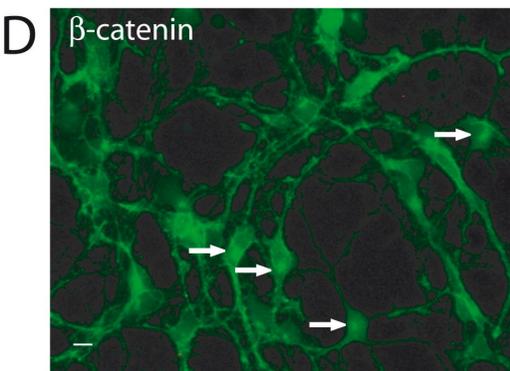
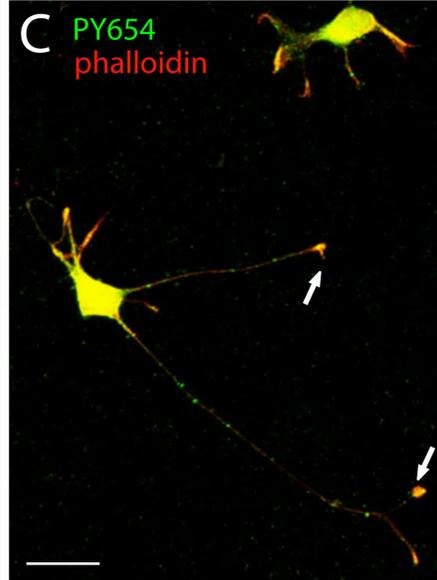
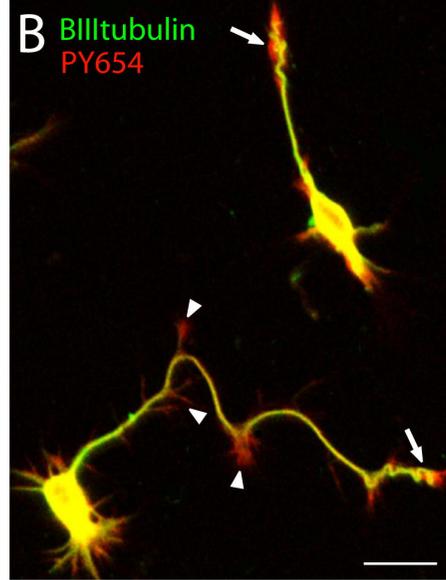
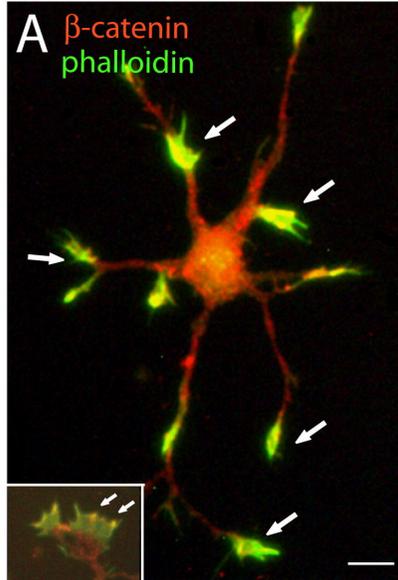
B



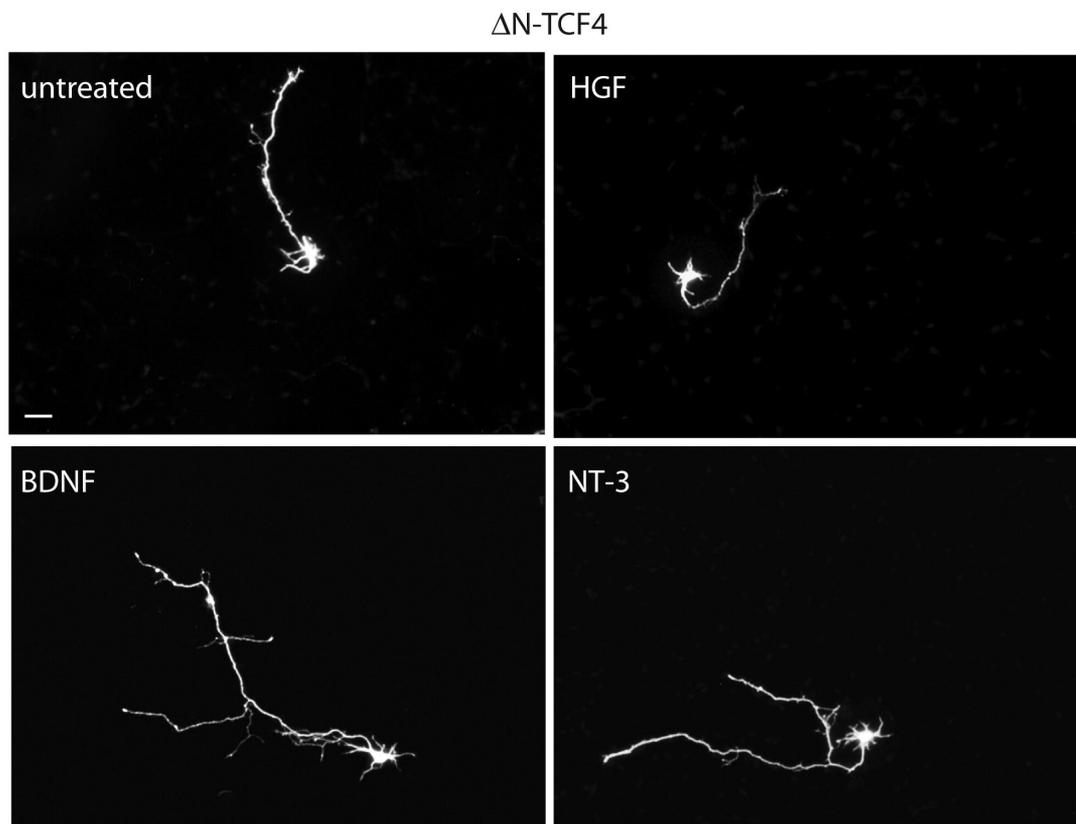
C



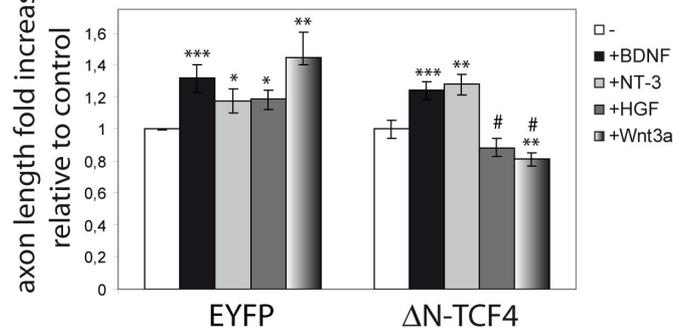
**A****B****C**



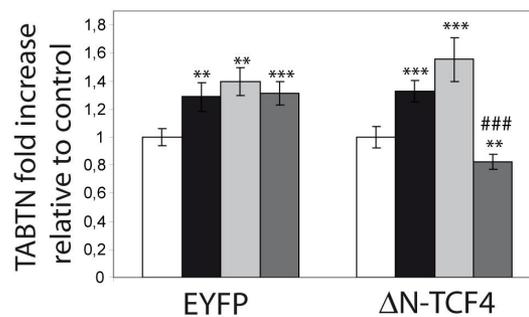
A

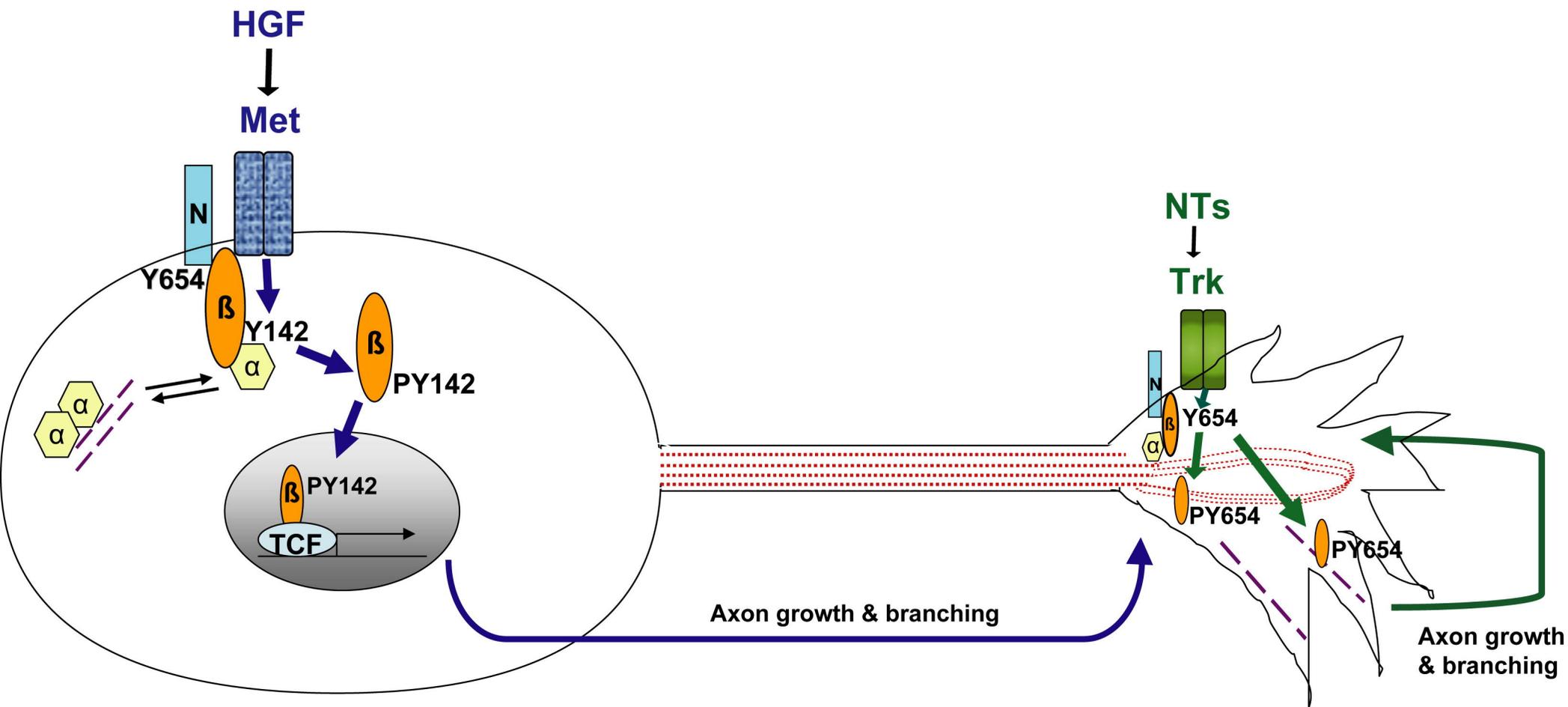


B



C





**Table 1. Dendrite morphogenesis is not regulated by tyrosine phosphorylation of  $\beta$ -catenin in 2 DIV hippocampal neurons.**

Dendrite length fold increase compared to control

<i>Treatment</i>	<i>Average<math>\pm</math>s.e.m.</i>	<i>Significance(n)</i>
Control	1 $\pm$ 0.12	N.S.* (n=11)
BDNF	1.16 $\pm$ 0.13	N.S. (n=9)
HGF	1.00 $\pm$ 0.07	N.S (n=6)

Dendrite branching (Total Dendrite Branch Tip Number, TDBTN) compared to control

<i>Treatment</i>	<i>Average<math>\pm</math>s.e.m.</i>	<i>Significance(n)</i>
Control	3.37 $\pm$ 0.29	N.S. (n=11)
BDNF	3.51 $\pm$ 0.48	N.S. (n=9)
HGF	3.49 $\pm$ 0.79	N.S (n=6)

\*N.S. = not significant