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Axonal elongation triggered by stimulus-induced local translation of a polarity complex protein

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During development, axon growth rates are precisely regulated to provide temporal control over pathfinding1,2. The precise temporal regulation of axonal growth is a key step in the formation of functional synapses and the proper patterning of the nervous system. The rate of axonal elongation is increased by factors such as netrin-1 and NGF, which stimulate axon outgrowth by incompletely defined pathways. To clarify the mechanism of netrin-1 and NGF-stimulated axon growth, we explored the role of local translation. We find that intra-axonal protein translation is required for stimulated but not basal axon outgrowth. To identify the mechanism of translation-dependent outgrowth, we examined the PAR complex, a cytoskeleton regulator3. We find that the PAR complex, like local translation, is required only for stimulated but not basal growth. *PAR3* mRNA is localized to developing axons, and NGF and netrin-1 trigger its local translation. Selective ablation of *PAR3* mRNA from axons abolishes the outgrowth-promoting effects of NGF. These results identify a novel role for local translation and the PAR complex in axonal outgrowth.

An important aspect of axon pathfinding is the regulation of the rate of axonal elongation. At some stages in axon pathfinding, axons exhibit periods of reduced or absent growth, while at other periods, growth is markedly stimulated1,2,4. In animals harbouring a genomic deletion of the genes encoding either netrin-1 or nerve growth factor (NGF), signalling proteins that promote rapid axonal outgrowth of commissural and sensory axons, respectively, these axons continue to grow, but at a markedly reduced rate5,6. These findings suggest the existence of a basal independent axon growth rate and an NGF- or netrin-1-induced growth rate that is required to promote axonal elongation during development.

A distinguishing feature of developing axons, in contrast to mature axons, is their ability to translate proteins locally7,8, suggesting that local protein translation might have roles during

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axon guidance. Indeed, growth cone turning responses elicited by Semaphorin-3A and netrin-1 are blocked by selective axonal application of translation inhibitors9,10. Further, axonally localized β-*actin* mRNA is translated in Xenopus retinal growth cones in a manner consistent with a role in netrin-1-induced growth cone turning11. Additionally, NGF and netrin-1 induce a rapid increase in 4E-BP1 phosphorylation within growth cones, a marker for the induction of intra-axonal protein translation9,12. However, the repulsive turning effect of L-α-lysophosphatidic acid is independent of local protein synthesis9, and local protein synthesis is not required for axon growth in rat sympathetic neurons13. Therefore, intra-axonal protein synthesis is utilized by certain guidance cues and is a component of only selective aspects of growth cone function.

To characterize the involvement of local protein synthesis in axonal growth, we cultured rat embryonic dorsal root ganglia (DRG) in compartmentalized microfluidic chambers14 (Fig. 1a) allowing treatments to be selectively applied to axons14. The axons were kept for 24 h at a NGF concentration (5 ng ml^{-1}) that does not induce axon outgrowth (Supplementary Information, Fig. S1a). Consistent with previous reports15, application of 100 ng ml^{-1} NGF for 1 h to the axonal compartment resulted in a 1.37 ± 0.12-fold increase in axonal growth relative to vehicle (Fig. 1b). Selective axonal application of two mechanistically distinct translation inhibitors, cycloheximide and rapamycin, did not significantly affect basal axonal growth rates (Fig. 1b) as has been shown before for sympathetic neurons13. However, application of cycloheximide or rapamycin to axons completely inhibited NGF-stimulated axonal outgrowth (Fig. 1b), indicating that there are two modes of axonal elongation, translation-independent basal outgrowth and stimulated outgrowth, which utilizes local protein synthesis.

To identify transcripts whose translation is required for axon outgrowth we considered mRNAs that encode signalling proteins known to be present in growth cones and which may control stimulated but not basal axon outgrowth. The PAR complex has been found to be highly enriched in the growth cones of nascent axons of hippocampal neurons16. It comprises the evolutionarily conserved proteins PAR3, PAR6, and an atypical PKC, such as aPKCζ or aPKCλ,3 and functions in neurons to specify the axon from neurites during early neurodevelopment16. Although the PAR complex has been linked to membrane polarization and trafficking and has not been shown to affect axonal outgrowth, it can activate several signalling pathways that control cytoskeleton dynamics3. Interestingly, both NGF and netrin-1 promote the accumulation of active GTP-bound cdc4217, which can recruit and activate the PAR complex18.

Consistent with prior studies16, we find PAR3, PAR6, and aPKCζ localized to the tips of the presumptive axon in embryonic day 18 (E18) cultured rat hippocampal neurons at day *in vitro* 1 (DIV1) (Supplementary Information, Fig. S2a). At time points at which axon specification has been completed, we find each of the PAR complex proteins still present in growth cones and axons of DIV5 DRG and DSC neurons and DIV9 hippocampal neurons (Fig. 2a,b and Supplementary Information, Fig. S2b). The specificity of the immunofluorescence staining for PAR3 and PAR6 was established by the lack of staining in dissociated DRG neurons transfected with *PAR3* or *PAR6* siRNA (Fig. 2c–f). Together,

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these data indicate the PAR complex is present in axons at the same time that they are responsive to NGF and netrin-1.

To investigate the role of the PAR complex in developing axons, we examined the effect of siRNA-mediated knockdown of *PAR3* and *PAR6* on commissural axons in spinal cord explants. During the development of the spinal cord, axons of commissural neurons in the dorsal neural tube grow towards the floor plate at the ventral midline. Growth towards the midline is cause by two long-range chemoattractants secreted by the floor plate, netrin-15,19,20 and sonic hedgehog21. To visualize the length and direction of commissural axon projections in the developing spinal cord, we electroporated E11 rat spinal cord segments with siRNA and a GFP-expression plasmid, which results in selective labelling of DSC neurons22 (Fig. 3a). In untransfected or control siRNA-transfected spinal cord explants, most axons reach and cross the midline two days after electroporation (Fig. 3b,c,f). In explants transfected with *PAR3* or *PAR6* siRNAs, the axons grew markedly shorter (Fig. 3d–f) but without a change in direction (Fig. 3g,h). The specificity of the siRNA-mediated knockdown was established by rescue with an siRNA-resistant Cherry-PAR3 transcript (Supplementary Information, Fig. S3). Notably, stunted axons with preserved directionality is nearly identical to the phenotype observed in embryonic spinal cord of mice harbouring genomic deletions in the genes for netrin-1 and its receptor, DCC5,23. Together, these results indicate that PAR3 and PAR6 are required for the growth of axons towards the spinal midline.

To further explore the possibility that the PAR complex might mediate the axon outgrowth effects of NGF and netrin-1, we examined the effect of interfering with complex formation on basal and stimulated axonal outgrowth. Embryonic DRG neurons require NGF for survival, and were therefore cultured in media containing a minimal concentration of NGF (5 ng ml^{-1}) for 24 h, which results in basal levels of axonal growth24 (Supplementary Information, Fig. S1a). Application of 100 ng ml^{-1} NGF resulted in a 1.63 ± 0.19-fold increase in axonal outgrowth (Fig. 3i,k). Similarly, axons of cultured DSC neurons exhibit a basal axonal growth rate in the absence of netrin-1 that is increased 1.66 ± 0.14-fold upon application of netrin-1 (75 ng ml^{-1}) (Fig. 3j,l), consistent with previously described effects of netrin-1 on these neurons25.

To test the role of the PAR complex, the interactions between PAR3, PAR6, and aPKC, as well as aPKCζ activity, were disrupted by overexpressing well-characterized fragments that interfere with complex formation26,27 (see Supplementary Information, Methods). Expression of each fragment, or pharmacological inhibition of aPKCζ with a pseudosubstrate inhibitor, did not decrease basal axonal growth rates (Fig. 3i,j). However, each treatment inhibited, or even reversed, the growth-promoting effect of netrin-1 or NGF (Fig. 3k,l). These data indicate that complex formation between PAR3, PAR6, and aPKC, and aPKCζ activity are required specifically for the axon outgrowth-promoting effects of netrin-1 and NGF but not for axon growth under non-stimulated conditions.

The finding that the PAR complex is required for netrin-1- and NGF-stimulated axonal outgrowth, but not basal outgrowth, was reminiscent of the requirement for local translation in stimulated but not basal axon outgrowth (Fig. 1b). This similarity prompted us to examine

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whether mRNAs encoding PAR complex proteins were present in axons. A fluorescent *in situ* hybridization screen using riboprobes directed against mRNAs coding for constituents of the PAR complex revealed significant levels of *PAR3*, but not *PAR6* and *aPKCζ*, transcripts in the axons of DIV4 DRG and DSC neurons (Fig. 4a–c). The *PAR3* signal appears granular and dispersed as has been described for other axonal mRNAs28,29 and had an intensity similar to that of *RhoA*, an axonally localized transcript12,28,29 (Fig. 4b,c). To confirm the axonal localization of *PAR3* mRNA, we performed reverse transcription-PCR (RT-PCR) experiments on pure axonal preparations of DRG neurons cultured in microfluidic chambers14,30 (Supplementary Information, Fig. S4a). RTPCR using two distinct primer pairs resulted in amplification of *PAR3* and β-*actin* transcripts from axon preparations, whereas RT-PCR signals for all three isoforms of *PAR6* and *aPKCζ* were absent (Fig. 4d and Supplementary Information, Fig. S4). The purity of the axonal mRNA preparation was confirmed by absence of a signal for the cell body-restricted mRNA γ-*actin* (Fig. 4d and Supplementary Information, Fig. S4). Together, these two approaches indicate that developing axons contain *PAR3* mRNA, but not other transcripts encoding components of the PAR complex.

The presence of *PAR3* mRNA in axons raises the possibility that the level of PAR3 protein in axons is regulated by local translation of its transcript. To differentiate between PAR3 protein that is synthesized in the cell body and subsequently trafficked to axons from PAR3 protein synthesized directly within axons, axons of DRG explants were severed from their cell bodies. Isolated axons prepared in this manner continue to grow and respond to guidance cues for at least 4 h9,29. To test the effect of NGF on PAR3 levels in axons, DRG explants were cultured in low NGF (5 ng ml^{-1}) for 24 h, axons were then severed and treated with low or high (100 ng ml^{-1}) concentration of NGF in the absence or presence of cycloheximide for 1 h and then immunolabeled to quantify PAR3 protein levels (Fig. 4e,f). In axons treated for 1 h with low NGF, PAR3 levels were low, and were unaffected by cycloheximide treatment. However, severed axons exposed to high NGF exhibited a significant increase in PAR3 levels that could be totally blocked by cycloheximide (Fig. 4f).

To further examine the NGF-dependent increase in PAR3 levels in axons, we used a previously described reporter assay for axonal mRNA translation in which destabilized, membrane-anchored EGFP is expressed from an mRNA containing the 3'UTR of the axonal mRNA29,31 (see Methods). The appearance of puncta along axons thus indicates local translation events mediated by *cis*-acting elements in the 3'UTR. Reporter mRNAs containing the 3'UTR of *PAR3* or, as a control, the 3'UTR of histone *H1f0* mRNA, were detected by FISH at equal levels in the cell bodies of transduced DRG neurons; however, only the reporter mRNA containing the 3'UTR of *PAR3* was found in axons and growth cones (Supplementary Information, Fig. S5a). In DRG and DSC neurons expressing the *PAR3* reporter, very low levels of fluorescence were observed in unstimulated and cycloheximide-treated axons (Fig. 4g). However, treatment of these cultures with NGF or netrin-1 resulted in puncta throughout the axons and within growth cones (Fig. 4g and Supplementary Information, Fig. S5d,e,g). In contrast, axons expressing the control *H1f0* reporter exhibited no fluorescence in axons under any condition (Supplementary Information, Fig. S5c,d,f,g). Together with the data on endogenous *PAR3* translation in

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severed axons, these two lines of experimentation indicate that netrin-1 and NGF regulate axonal PAR3 levels through the local translation of *PAR3* mRNA.

To determine the role of intra-axonal translation of *PAR3* mRNA in axon outgrowth, we used a recently described approach to selectively knockdown specific mRNAs from axons without affecting the cell body mRNA pool28. To generate neurons lacking *PAR3* mRNA from axons, E14 DRG neurons were grown in microfluidic chambers in order to fluidically isolate cell bodies and axons. The axonal compartment was selectively perfused with either of two *PAR3*-specific siRNAs and transfection reagent28. Axonal siRNA transfection resulted in knockdown of *PAR3* mRNA in axons without affecting *PAR3* mRNA in the cell bodies (Fig. 5a,b). In these axons, the NGF-induced increase in PAR3 was prevented, but PAR6 levels were unaffected (Fig. 5e and Supplementary Information, Fig. S6a–c), providing further evidence that local translation of *PAR3* is required for NGF-induced axon outgrowth. Furthermore, in axons depleted of *PAR3*, NGF-induced outgrowth was completely abolished, while it was unaffected in axons transfected with control siRNA (Fig. 5c,d).

A potential effector pathway of cdc42 and PAR6/aPKCζ is GSK3β.32. Phosphorylation of GSK3β at serine 9 leads to its inhibition, thereby inducing the binding of adenomatous polyposis coli (APC) protein to the plus ends of microtubules32. Consistent with previous studies15, selective application of NGF to axons leads to a significant increase in GSK3β phosphorylation (Supplementary Information, Fig. S6d,e). This effect is greatly reduced by axonal *PAR3* mRNA knockdown, indicating that locally translated PAR3 regulates GSK3β in response to NGF stimulation. Together, these data identify a requirement for the axonal pool of *PAR3* mRNA in NGF-induced axonal outgrowth.

The PAR complex regulates numerous signalling effectors besides GSK3β, including STEF/Tiam133 and Smurf134. STEF/Tiam1 are Rac-specific guanine nucleotide-exchange factors involved in neurite formation33, and Smurf1 is an E3-ubiquitin ligase that targets RhoA for degradation and promotes actin-rich filopodia-like protrusions in several cell types34. Therefore, the PAR complex may have a central role in axonal outgrowth by coordinating NGF and netrin-1 signalling with diverse pathways that control actin and microtubule dynamics (Supplementary Information, Fig. S7).

Previous studies linked the PAR complex to neuronal polarization, the process by which dendrites and axons are specified from the neurites16. Our findings add the regulation of axonal outgrowth to the known functions of the PAR complex. Similar to the requirement for local translation, the PAR complex is required only for stimulated but not basal outgrowth. We find that axons contain *PAR3* mRNA, and local PAR3 synthesis is part of the translation dependence of stimulated axonal outgrowth. Several mRNAs have been previously found to be present and translated in axons35; however, in very few cases has a functional role been found for these mRNAs. Using neurons that are selectively depleted of axonal *PAR3* mRNA, we find that axonal *PAR3* mRNA is not required for basal outgrowth, but is required for axonal outgrowth elicited by NGF and netrin-1. This study identifies a new mechanism regulating axonal elongation, in which factors such as NGF and netrin-1

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increase the synthesis and axonal levels of PAR3, triggering a switch to a rapid axonal growth mode.

Our study provides evidence for a novel mechanism for the regulation of the PAR complex. Activation of PAR complex signalling is thought to be regulated by selective recruitment to specific membrane domains3. Here we find that local translation of *PAR3* mRNA regulates the levels of PAR3 protein in growth cones. This unexpected mechanism of PAR complex regulation may be a recurrent feature of PAR complex signalling in diverse physiological processes and cell types.

METHODS

Cell culture and outgrowth assays

All reagents were from Invitrogen (Carlsbad, CA) unless otherwise indicated. E14 rat DRG and DSC explants and dissociated neurons were cultured as described25,29. For details see Supplementary Information, Methods. For axon outgrowth experiments, 24 h before the assay the medium of DRG cultures was exchanged to growth medium containing 5 ng ml^{-1} NGF. This concentration is sufficient to sustain cell survival but does not induce axon outgrowth (Supplementary Information, Fig. S1a). 100 ng ml^{-1} NGF or 75 ng ml^{-1} netrin-1 were bath applied to DRG or DSC explants, respectively, and phase-contrast images were taken of the same optical fields with a Nikon TE-2000-E microscope equipped with a CoolSnap HQ^2 CCD camera at 0 min and 60 min for DRG experiments and at 0 min and 180 min for DSC experiments. We previously found that this netrin-1 concentration is the optimal dose for filopodia formation in DSC axons25. One-way ANOVA with post-tests was used for the comparison of three or more groups, and unpaired, two-tailed *t*-test was used for the comparison of two groups. Error bars represent the standard error of the mean from a minimum of three independent experiments.

Compartmentalized culture in microfluidic chambers

To apply NGF, translation inhibitors, or siRNA specifically to distal axons and growth cones without affecting the cell bodies, E14 dissociated DRG neurons were grown in microfluidic chambers with 450-μm-long microgrooves14 (Xona Microfluidics, Temecula, CA). These chambers allow fluidic isolation of the axonal compartment from the cell body compartment14. The microfluidic chambers were coated with 100 μg ml^{-1} poly-L-lysine (Trevigen, Gaithersburg, MD). The growth medium was completely exchanged after 48 h. siRNA transfection in the axonal compartment was performed on DIV3, and outgrowth assays and FISH were done on DIV4.

Immunofluorescence

Neurons grown on 12-mm-diameter glass cover slips were fixed in 4% paraformaldehyde in cytoskeletal buffer (60 mM PIPES, pH 7.0, 27 mM HEPES, 10 mM EGTA, 4 mM $MgSO_4$) for 20 min at room temperature. The cover slips were washed three times in TBST (0.1% triton X-100 in TBS), blocked for 30 min in 3% bovine serum albumin (BSA) in TBST and labelled with primary antibodies in blocking solution. Antibodies used were: PAR3 (1:1,000, Millipore, Temecula, CA), PAR6 (1:1,000)18, cdc42 (1:250, Santa Cruz

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Biotechnology, Santa Cruz, CA), aPKCζ (1:250, Santa Cruz Biotechnology), phosphoGSK3β (Ser9) (1:250, Abcam, Cambridge, MA), GSK3β (1:500, Thermo Fisher Scientific, Rockford, IL), GAP43 (1:2,000, Millipore), and tau-1 (1:1,000, Millipore). Alexa 488 and 568 conjugated secondary antibodies were used. Stacks of images were acquired and three-dimensional deconvolution was done with AutoDeblur X (Media Cybernetics, Bethesda, MA).

siRNA transfection

siRNA-mediated knockdown of *PAR3* mRNA was achieved by transfecting siRNA oligonucleotides using Genesilencer transfection reagent (Genlantis, San Diego, CA) as previously described25,28. Experiments were performed 24 h after siRNA transfection. The sequences for the siRNAs used are given in Supplementary Information, Methods.

Electroporation of embryonic spinal cords

Electroporation of spinal cords explants was performed as described previously22, with minor modifications (for details see Supplementary Information, Methods). Spinal cords were electroporated at a developmental stage (E11) at which commissural axons have formed and are growing towards, but have not yet reached, the midline22. Therefore, we can exclude the possibility that experimental treatments affect axon specification. Explants were electroporated with siRNA and a GFP expressing plasmid, cultured for 48 h, removed from the collagen matrix, fixed in 4% paraformaldehyde in TBS and mounted on coverslips using ProLong Gold. For the quantification of commissural axon growth, lines parallel to the ventral border of the dorsal root entry zone were drawn in 50 μm intervals and the number of axons crossing these lines was determined along a 150-μm-long segment of the spinal cord.

Generation and transduction of Sindbis virus

A Sindbis virus vector containing the P76S point mutation in the nsP2 protein leading to reduced neuronal cytotoxicity29 and the helper plasmid DH-BB (S. Schlesinger, Washington University) were used to generate Sindbis pseudovirions according to the manufacturer's instructions (Invitrogen). The pseudovirus particles were purified over a sucrose gradient, concentrated and titered using BHK-21 cells. For axonal overexpression of dominant-negative proteins a modified viral vector was used containing an encephalomyocarditis virus internal ribosome entry site (IRES)29. This virus allows the overexpression of proteins directly off the viral genome in axons without requiring cell body infection12. Reporter experiments utilized the myr-dEGFP system31 that involves expressing a transcript encoding destabilized, EGFP with a cellular half-life of 1 h that enables dynamic changes in translational activity to be reflected by changes in fluorescence intensity31. Additionally, the reporter contains a myristoylation consensus sequence, resulting in a myristoylated, destabilized EGFP (myr-d1EGFP) that exhibits negligible diffusion in the plasma membrane29,31. As a result of these two features, translation of the reporter leads to the appearance of fluorescent puncta, which remain localized near the site of translation. Colabeling with ribosomal markers identified these puncta as ribosome-enriched hotspots of translation12,31. pSinRep5-myr-d1EGFP$^{3'UTR\ PAR3}$ and pSinRep5-myr-d1EGFP$^{3'UTR\ H1f0}$ contained the full length 3'UTR of the rat *PAR3* mRNA (Genbank

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accession number NM_031235) or rat histone *H1f0* mRNA (BC061842) fused to the viral 3' conserved sequence element.

Fluorescence *in situ* hybridization

Antisense riboprobes were transcribed *in vitro* from sense oligonucleotides using the MEGAshortscript *in vitro* transcription kit (Ambion) with digoxigenin-conjugated UTP (Roche, Basel, Switzerland). For the detection of each mRNA a mix of five non-overlapping riboprobes was used. The riboprobes are matched in their GC content. For details of the FISH procedure and sequences of the oligonucleotides used to generate riboprobes see Supplementary Information, Methods.

RT-PCR

Isolation of pure axonal mRNA and RT-PCR was performed as described30. 30,000–50,000 E14 DRG neurons were grown in a tripartite microfluidic chamber, in which the distal axons are separated from the cell body compartment by an additional compartment (Supplementary Information, Fig. S4a). The use of these chambers essentially eliminates the possibility that neuronal or glial cell bodies migrate to the axonal side. On DIV5, chambers were inspected for the presence of cell bodies on the axonal sides to confirm complete partitioning of the cell bodies and axons. Pure distal axons and cell bodies were prepared by perfusing Trizol reagent in the corresponding compartment, and the RNA in the lysates was extracted using the Trizol protocol. The RNA was resuspended in 10 µl H_2O. cDNA was transcribed using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR using oligo$(dT)_{20}$ primers and random hexamers in a 20 µl reaction using the entire RNA preparation. cDNAs were amplified using Platinum *Taq* DNA polymerase in 20 µl reactions: 4 U *Taq* polymerase, 2.5 µl cDNA, 1x DNA polymerase buffer, 0.2 µM primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$. For the amplification the following protocol was used: 94°C for 2 min; 42 cycles: 94°C for 30 sec, 55°C for 30 sec, 72°C for 17 sec; 72°C for 3 min. The sequences of the oligonucleotides used as primers in the PCR reactions are given in Supplementary Information, Methods.

Supplementary Material

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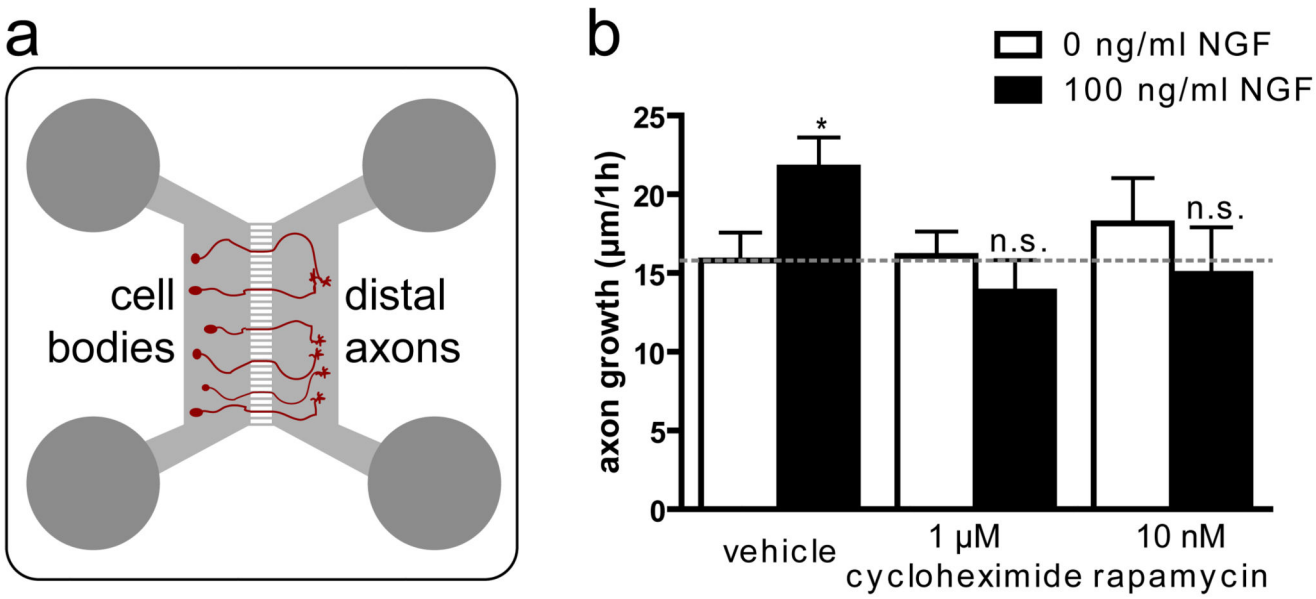

Figure 1. Local protein translation is required for NGF signalling

(**a**) Schematic representation of a microfluidic chamber. Neurons are plated into the left compartment (cell body compartment) and extend their axons through 450-µm-long microgrooves into the right compartment (axonal compartment). DRG neurons were used in these experiments since their axons are long enough to grow through the grooves, while dorsal spinal commissural (DSC) axons typically are too short to cross into the axonal compartment (U.H., S.R.J., data not shown). (**b**) Dissociated DRGs were cultured for 4 days in microfluidic chambers. Application of the protein translation inhibitors cycloheximide or rapamycin to the axonal compartment does not significantly change the basal growth rates (one-way ANOVA, P=0.7544), but completely abolishes NGF-induced axon growth. *P<0.05, n = 50 to 120 axons from 4 independent experiments per condition.

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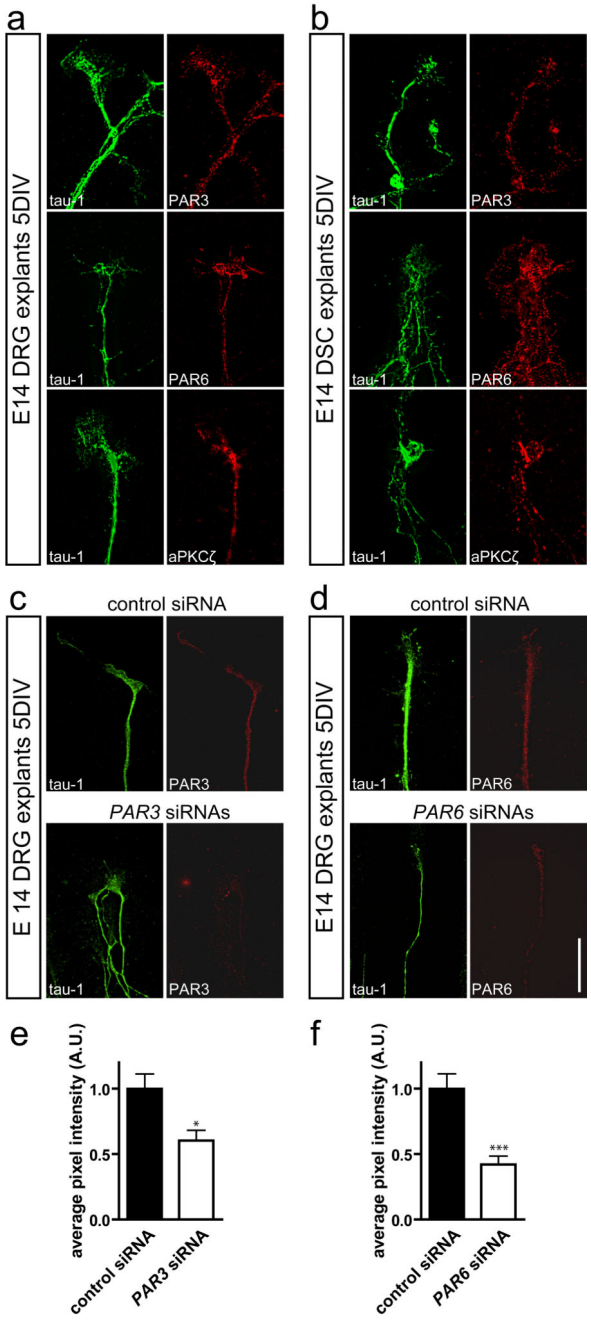

Figure 2. Proteins constituting the polarity complex are localized to growth cones of DRG and DSC neurons after axon formation

(**a, b**) Immunolabelling of DRG (**b**) and DSC (**c**) explants at 5 DIV for polarity complex proteins shows localization of PAR3, PAR6 and aPKCζ to growth cones. (**c, d**) Dissociated DRG neurons were transfected with siRNAs targeting *PAR3* or *PAR6* mRNA or a non-tageting control siRNA and immunolabelling against PAR3 (**c**) or PAR6 (**d**) was performed 48 h later. (**e, f**) Quantification of the average staining intensity for PAR3 and PAR6 normalized to tau-1 staining reveals that transfection with targeting siRNAs causes a

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significant decrease in staining intensities demonstrating the specificity of the PAR3 and PAR6 signal observed in axons and growth cones. *t*-test, PAR3, **P*=0.0104; PAR6, ****P*=0.005. n = 13 to 21 axons per condition. Scale bar, 20 μm.

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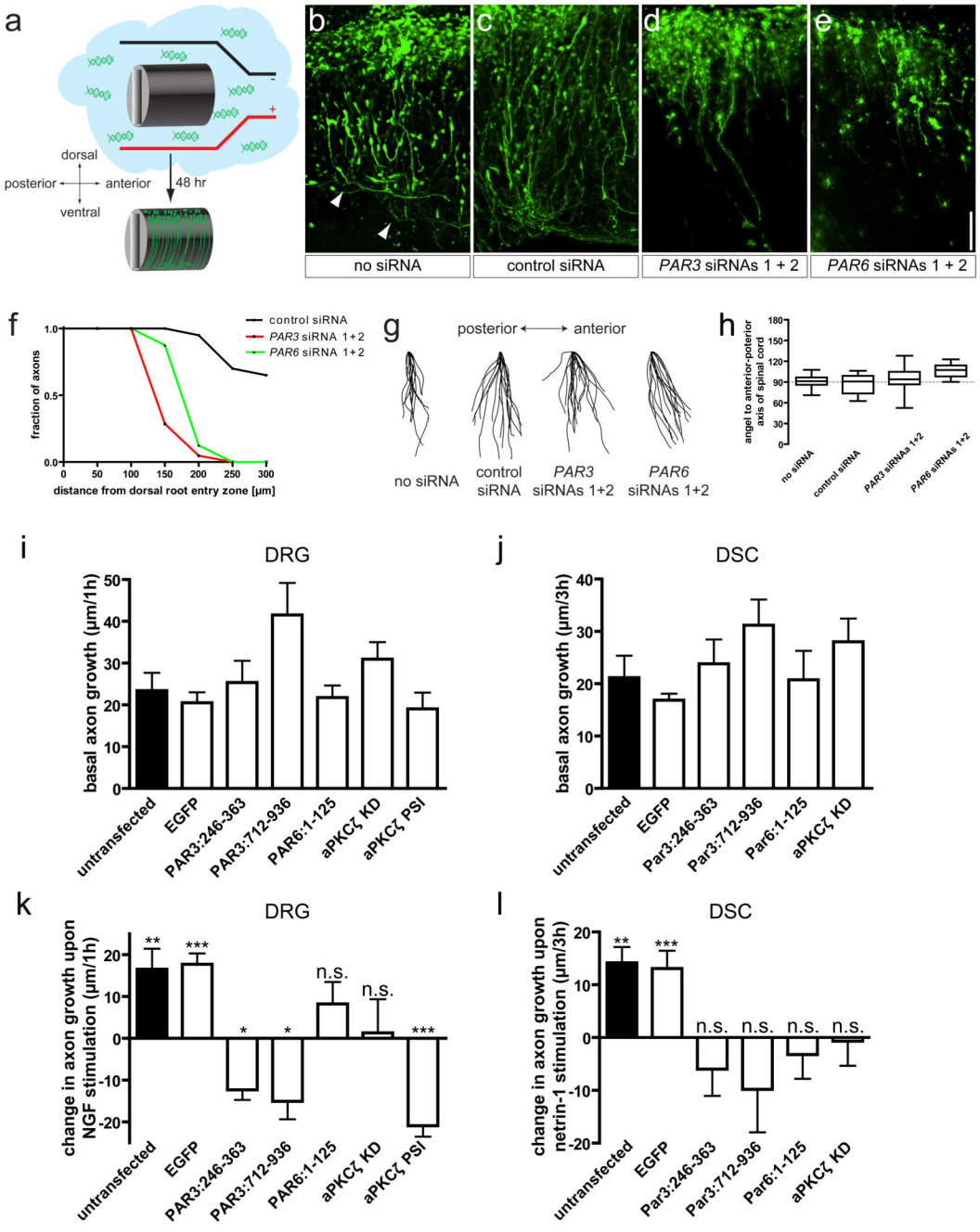

Figure 3. Signalling through the PAR complex is required for stimulated axon growth

(**a**) Schematic representation of the spinal cord electroporation. A segment of E11 rat spinal cord is placed between the electrodes with the dorsal part facing the anode. Commissural neurons in the dorsal spinal cord are preferentially transfected. (**b** and **c**) E11 spinal cords 48 h after electroporation with pUB-GFP alone (**b**) or pUb-GFP with control siRNA (**c**). Most axons have reached the midline and turned anteriorly (arrow heads). (**d** and **e**) Knockdown of *PAR3* and *PAR6* mRNA greatly reduces the number of axons reaching the midline. Scale bar, 100 μm. (**f**) Quantification of the commissural axon growth reveals significantly stunted

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growth upon *PAR3* or *PAR6* siRNA transfection (Chi-square, $P<0.001$ and $P=0.0311$, respectively). (**g** and **h**) Initial segments of transfected axons were traced and their angle to the longitudinal axis of the spinal cord measured. siRNAs transfection does not change directionality as measured by a comparison of the variances of the angles (Bartlett's test, $P=0.1157$). (**i–l**) Formation of the polarity complex is required for NGF and netrin-1 signalling. DRG (**i** and **k**) and DSC (**j** and **l**) explants were transduced on DIV3 and an axon outgrowth assay was performed 24 h later. (**i, j**) Axonal overexpression of EGFP or protein fragments that interrupt binding of PAR3 to PAR6 (PAR3: 246–363), PAR3 to aPKCζ (PAR3: 712–936), or PAR6 to aPKCζ (PAR6: 1–125), or overexpression of a kinase dead version of aPKCζ (aPKCζ KD), or pharmacological inhibition of aPKCζ (aPKCζ PSI) does not significantly decrease the basal axonal growth rate (one-way ANOVA, DRG: $P=0.085$, DSC: $P=0.3764$). (**k, l**) Change in axon growth rates relative to basal growth after stimulation with NGF or netrin-1. Overexpression of the dominant-negative proteins or inhibition of aPKCζ significantly inhibits or even reverses NGF- or netrin-1-induced axon growth while. The reversal of the growth-stimulating effect of NGF seen with some of the dominant-negatives might be due to the unmasking of parallel, NGF-dependent pathway. $*P<0.05$, $**P<0.01$, $***P<0.001$. n = 28 to 88 individual axons from three independent experiments per condition.

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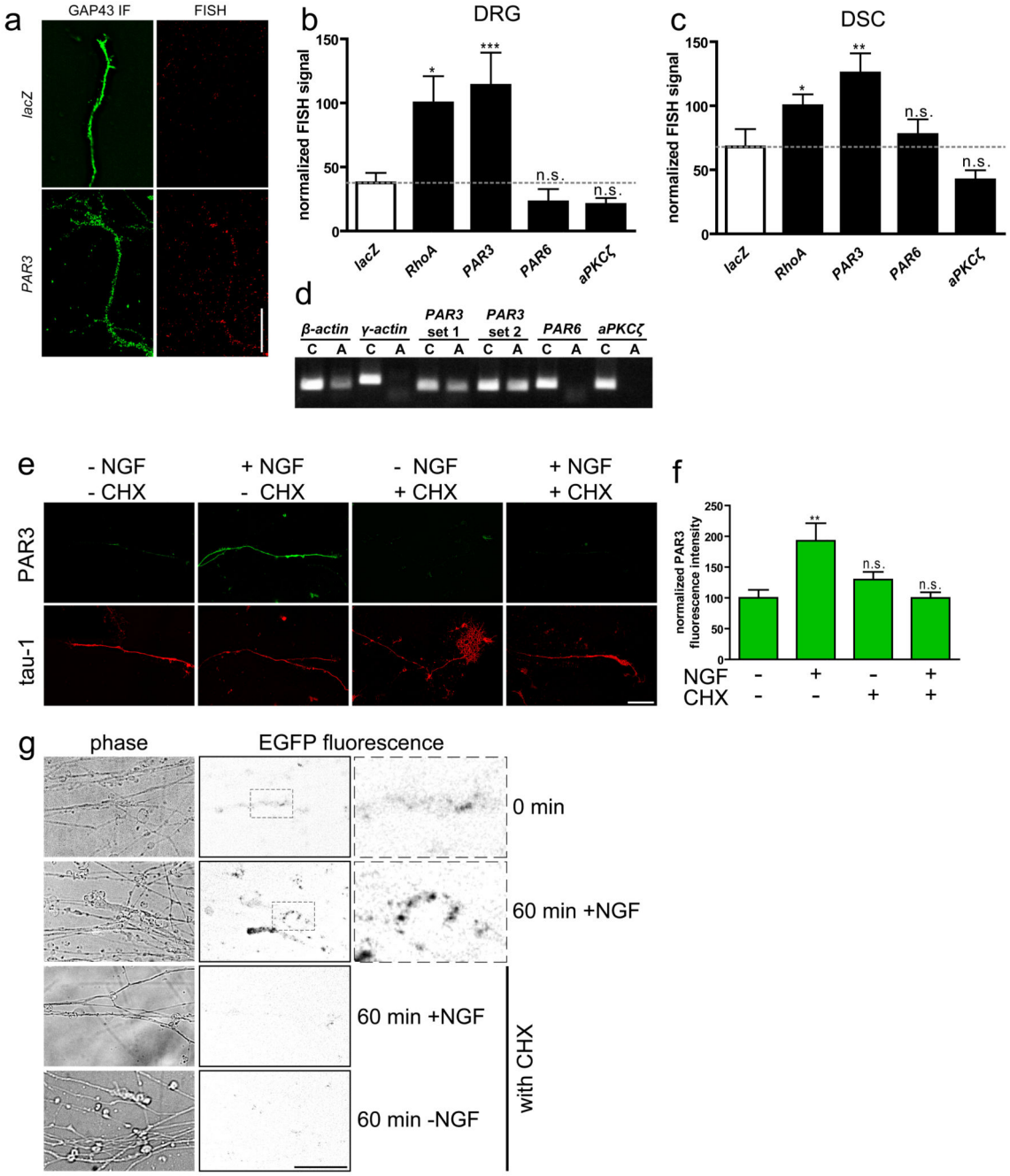

Figure 4. The *PAR3* mRNA is localized to axons and growth cones and locally translated in response to NGF

(**a**) FISH using digoxigenin-labelled riboprobes directed against *lacZ* and *PAR3* transcripts. *PAR3* transcripts are detected in axons and growth cones of DRG neurons. (**b, c**) Quantification of FISH signals for *lacZ*, *RhoA*, *PAR3*, *PAR6*, and *aPKCζ* transcripts in axons and growth cones of both DRG (**b**) and DSC (**c**) explants. The FISH signal for *RhoA* and *PAR3* mRNA is significantly stronger than the inherent background defined by the intensity of labelling for *lacZ*. **P*<0.05, ***P*<0.01, ****P*<0.001. *n* = 5 optical fields with

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several axons each per condition. (**d**) β-*actin* and *PAR3* mRNAs were detected by RT-PCR in DRG axonal lysates of harvested from microfluidic chambers while γ-*actin*, *PAR6*, and *aPKCζ* transcripts were not detected in axonal preparations. All transcripts were detected in cell body lysates. A, axonal lysate; C, cell body lysate. (**e**) Axons of DRG explants were severed from their cell bodies and incubated for 1 h with vehicle or 1 μM cycloheximide (CHX) and 100 ng ml^{-1} NGF. The axons were fixed and immunolabeled with antibodies against PAR3 and tau-1. Application of NGF to severed axons leads to a translation-dependent increase in staining intensity for PAR3. (**f**) Quantification of the PAR3 staining intensity in **a** reveals a significant, cycloheximide-sensitive increase upon NGF stimulation (one-way ANOVA with Dunett's post-test, $^{**}P<0.01$. n = 5 to 10 axons per condition), while tau-1 staining intensity is not changed ($P=0.1557$). (**g**) DRG explants were transduced with *PAR3* reporter pseudovirus on DIV3, and phase contrast and fluorescent images were taken after 24 h. Fluorescence images are shown in inverted contrast to more readily visualize puncta. Few puncta representing translation of the reporter are seen in axons of neurons expressing the *PAR3* reporter at the beginning of the experiment. NGF stimulation (100 ng ml^{-1}) greatly increases the number and intensity of puncta. Application of cycloheximide leads to the complete absence of puncta both under baseline conditions and after NGF stimulation. Scale bars, 25 μm.

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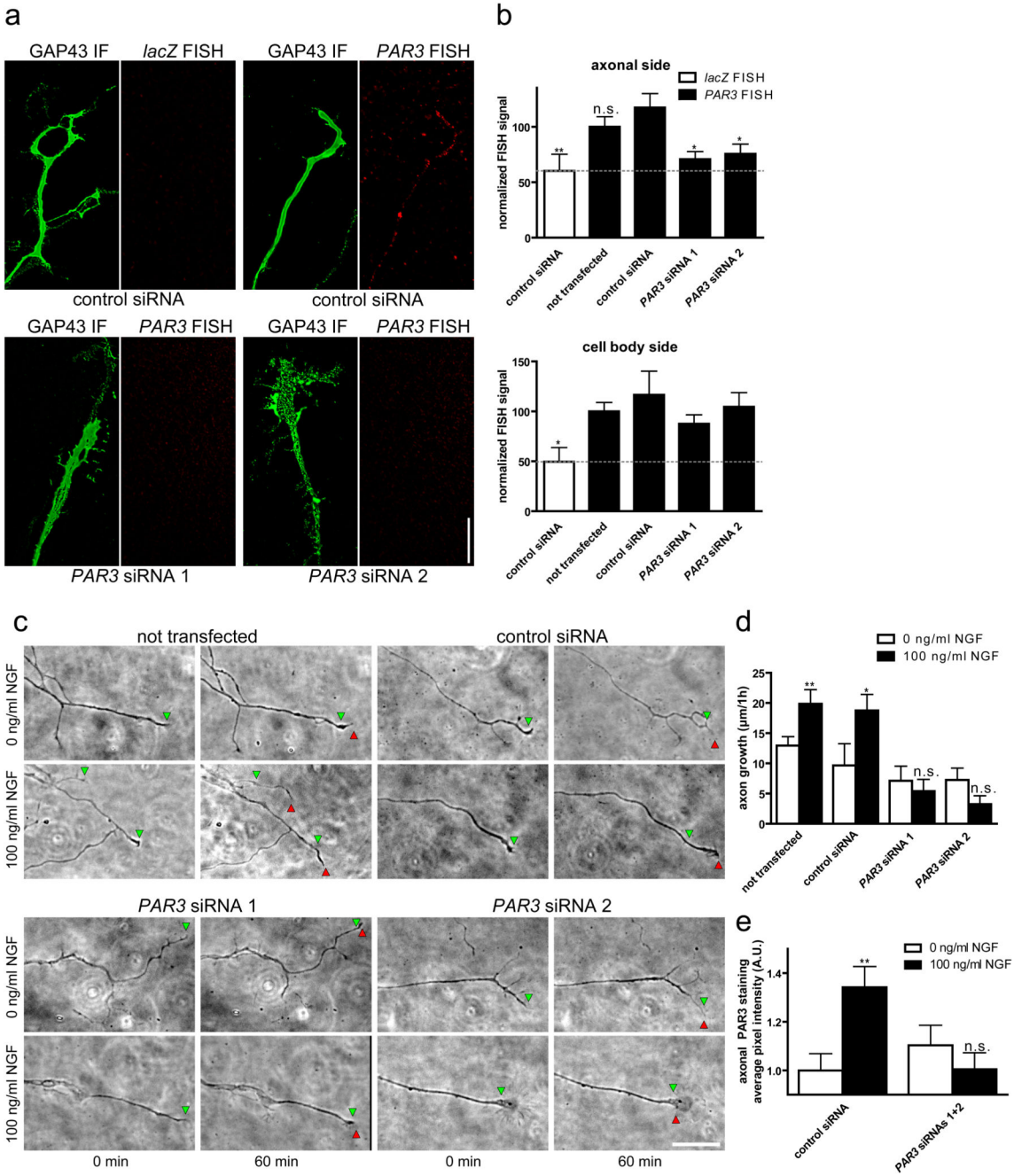

Figure 5. Local translation of PAR3 is required for NGF signalling

(**a**) FISH on axons transfected with siRNAs. Dissociated DRG neurons were grown in microfluidic chambers, and siRNAs were transfected in the axonal compartment only. Cells were fixed 24 h after transfection and labelled for *PAR3* and *lacZ* mRNA. (**b**) Quantification of the FISH signal reveals that transfection of *PAR3* siRNAs into axons does not affect the *PAR3* mRNA levels in the cell body compartment (one-way ANOVA, P=0.6224) but significantly reduces axonal *PAR3* mRNA signals compared to transfection with control siRNA. Labelling for *lacZ* on control siRNA transfected axons is used to define the intensity

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of background labelling. *P<0.05, **P<0.01. n = 5 optical fields with 10 axonal segments each. (**c**) Axon outgrowth assays on axons transfected with *PAR3* siRNA. DRG neurons were grown in microfluidic chambers and transfected with siRNA as above. Application of NGF to the axonal compartment induced axons growth in not transfected and control siRNA transfected axons while transfection with either *PAR3* siRNA completely abolished the growth effect of NGF. Locations of the growth cones at 0 min and 60 min are indicated by green down (▼) and red up triangles (▲), respectively. (**d**) Quantification of experiment in (**c**). The basal growth rate is not significantly affected by siRNA transfection (one-way ANOVA, P=0.0691). **P<0.01, *P<0.05. n = 20 to 115 axons per condition. Scale bars, 20 μm. (**e**) Application of NGF causes an increase in immunofluorescence staining intensity for PAR3 in distal axons and growth cones. SiRNA-mediated knockdown of axonal *PAR3* mRNA abolishes the effect of NGF on PAR3 protein levels within distal axons and growth cones. **P<0.01. n = 17 to 30 axons from three experiments per condition.