

## ***GAR-Driven Subcellular Localization of the Growth Controlling RNA Binding Protein Nucleolin***

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## **Abstract**

Nucleolin is a multifunctional RNA Binding Protein (RBP) with diverse subcellular localizations, including the nucleolus in all eukaryotic cells, the plasma membrane in tumor cells, and the axon in neurons. Here we show that the glycine arginine rich (GAR) domain of nucleolin drives subcellular localization via protein-protein interactions with a kinesin light chain in the kinesin motor complex in axons and plasma membrane interactions of GAR arginine rich sequences, in addition to its already reported involvement in liquid-liquid phase separation in the nucleolus. Nucleolin transport to axons requires the GAR domain, and heterozygous GAR deletion mice reveal reduced axonal localization of nucleolin cargo mRNAs and enhanced sensory neuron growth. Thus, the GAR domain governs axonal transport of a growth controlling RNA-RBP complex in neurons, and is a versatile localization determinant for different subcellular compartments. Localization determination by GAR domains may explain why GAR mutants in diverse RBPs are associated with neurodegenerative disease.

## **Keywords**

RNA Binding Protein, subcellular localization, glycine arginine rich (GAR), RGG domain, nucleolin, kinesin, axonal transport, cell size regulation, local translation, protein-membrane interaction.

## Introduction

Nucleolin is a multifunctional, highly-conserved, abundant RNA Binding Protein (RBP) that is found in multiple subcellular compartments, including a relatively stable major nucleolar pool, and more dynamic nucleoplasmic, cytoplasmic and plasma membrane complexes (Berger *et al*, 2015). The protein has been implicated in many cellular processes, including ribosome biogenesis, division and survival of cycling cells, oncogenesis and tumor growth, as well as size and length sensing in neurons and other large cells (Rishal & Fainzilber, 2019; Ugrinova *et al*, 2018). Although nucleolin is essential for cell viability (Storck *et al*, 2009; Ugrinova *et al*, 2007), and its cell-surface localization in tumor cells has been exploited in development of anti-cancer therapies (Berger *et al.*, 2015; Gilles *et al*, 2016; Romano *et al*, 2019), the mechanisms underlying subcellular localization of nucleolin are still unclear.

Nucleolin is composed of a number of functional domains, including an amino-terminal charged region, a central region comprising four RNA-binding domains, and a carboxy-terminal glycine/arginine-rich (GAR) domain (Fig. 1A). Early studies demonstrated that both RNA-binding and GAR domains are required for nucleolar localization of nucleolin (Creancier *et al*, 1993; Pellar & DiMario, 2003; Schmidt-Zachmann & Nigg, 1993), but the domain(s) involved in cytoplasmic, plasma membrane or neuronal process localization of nucleolin have not been determined. We recently found that nucleolin is required for axonal trafficking of mRNAs that regulate neuronal growth or survival (Perry *et al*, 2016; Terenzio *et al*, 2018), and proposed that this RBP is a key component in a cell size sensing mechanism based on transport of mRNA from cell center to periphery, and retrograde transport of locally synthesized proteins encoded by these mRNAs (Rishal & Fainzilber, 2019; Rishal *et al*, 2012). These findings stimulated efforts to understand how nucleolin is trafficked to and retained in different subcellular compartments.

We recently demonstrated axonal transport of nucleolin in association with the molecular motor kinesin 1 (Kif5a) (Perry *et al.*, 2016). We further showed that AS1411, a G-quadruplex forming nucleolin-targeted DNA aptamer (Bates *et al*, 2017), perturbs the association of nucleolin with the Kif5a motor complex (Perry *et al.*, 2016), suggesting that the aptamer-binding domain of nucleolin should also mediate its axonal transport. Interestingly, previous studies had shown that the RBPs FMRP and FUS interact with G-

quadruplex nucleic acid structures through GAR domains (Darnell *et al*, 2001; Ramos *et al*, 2003; Vasilyev *et al*, 2015; Yagi *et al*, 2018). We therefore examined whether the GAR domain of nucleolin can bind the AS1411 aptamer, and tested its role in axonal, plasma membrane and nucleolar localization of nucleolin. Here we show that the GAR domain drives nucleolin localization to all these subcellular compartments by distinct mechanisms, and present evidence from a gene-edited mouse model that the GAR domain is critical for axonal trafficking of mRNA by nucleolin and for its effects on neuronal growth.

## Results

### ***Nucleolin-kinesin interactions are mediated by the GAR domain***

We first tested whether the nucleolin GAR domain binds AS1411. Synthetic peptides corresponding to different segments of the nucleolin GAR domain (Fig. 1A and appendix Fig. S1A) were tested for AS1411 binding by ELISA (appendix Fig. S1B). The AS1411 aptamer, but not the control DNA, showed binding to 22-28 residue peptides derived from N-terminal, middle and C-terminal segments of the GAR domain (appendix Fig. S1C). We further narrowed our testing to sequences of 15 or 18 residue peptides (designated R3 or R4, respectively) and designed corresponding R- to-A (A3) or R-to-N (N4) control peptides. AS1411 bound to these shorter GAR-derived peptides but not the controls (Fig. 1B and appendix Fig. S1C). We then assessed the spectrum of GAR interactors by using biotinylated versions of the R3 versus A3 peptides as baits for pull-downs from mouse sciatic nerve axoplasm, identifying co-precipitating proteins by mass spectrometry (MS). The data were analyzed using SAINTexpress (Teo *et al*, 2014), and a total of 352 candidate interacting proteins were identified (Table EV1, Fig. EV1).

Interestingly, we identified 16 kinesin-complex components, comprising motors and adaptors from four kinesin subfamilies that transport a variety of axonal cargos (Fig. 1C). We chose to validate the interaction for Kif5a, a motor component of the kinesin 1 complex, since we had previously shown that the Kif5a complex associates with nucleolin, and that this association is reduced by the AS1411 aptamer (Perry *et al*, 2016). Indeed, affinity purification followed by automated capillary Western blotting showed that both R3 and R4 peptides associated with Kif5a, while their A3 and N4 controls did not (Fig. 1D-F). The requirement for the nucleolin GAR domain in kinesin binding was further confirmed by co-immunoprecipitation analyses of Kif5a with HA-Dendra2 tagged nucleolin constructs

expressed in HEK-293 cells. Both GAR domain deletion and substitution of all ten arginines in the GAR domain by asparagines (henceforth termed GAR(N)) significantly reduced co-precipitation of Kif5a with nucleolin (Fig. 2A, B). Moreover, the R4 peptide, but not its N4 control, acted as a dominant-negative by significantly perturbing endogenous Kif5a-nucleolin interaction in sciatic nerve axoplasm, as shown by reduction in their co-immunoprecipitation (Fig. 2C-E). We then set out to test direct binding of GAR-derived peptides with kinesin complex components, using surface plasmon resonance to assess binding of R4 versus N4 peptides with kinesin light chain 2 (KLC2) and with Kif5c. While there was no specific binding of the GAR-derived peptides to Kif5c; R4, but not the N4 control, bound KLC2 with a  $K_d$  of 57.9 nM (Fig. 2F). Recent work has linked transport of RBP-mRNA complexes in association with endosomes and lysosomes (Cioni *et al*, 2019; Liao *et al*, 2019). However, we do not find the lysosome/endosome marker LAMP1 in Kif5a-Ncl coprecipitates (appendix Fig. S2). Finally, we tested axonal localization of GAR domain constructs by monitoring appearance of axonal signal after photo-conversion of fluorescent dendra protein fusions in the cell bodies of cultured dorsal root ganglia (DRG) neurons. We observed a significant time-dependent increase of photoconverted dendra-GAR(WT), but not dendra-GAR(N), in distal axons of the transfected neurons (Fig. 2G). Taken together, these findings support a critical role for the GAR domain in nucleolin interaction with kinesin complexes and in axonal localization.

### ***Nucleolin localization to the plasma membrane is facilitated by the GAR domain***

Nucleolin has also been reported to reside at the plasma membrane (Bates *et al.*, 2017) and indeed, an appreciable fraction of the GAR protein interactors we identified are membranal (Fig. EV1). Although nucleolin lacks any obvious membrane interaction domains or motifs, arginine-rich peptides are known to penetrate cellular membranes (Allolio *et al*, 2018; Vazdar *et al*, 2018), raising the possibility that the arginine-rich GAR domain might enable nucleolin association with the plasma membrane. We performed molecular dynamics simulations to explore this possibility, examining the propensity of GAR-derived peptides to interact with an idealized phospholipid bilayer. Specifically, we compared simulated membrane association of the R4 GAR peptide with those of lysine (K4) or asparagine (N4) substitutions for the arginines in this sequence. As shown in Figures 3A and EV2, both the R4 and K4 peptide simulations showed robust membrane association, while

the N4 did not. Membrane contacts of the charged peptides were primarily driven by arginine and phenylalanine residues in the case of the R4 peptide, while phenylalanines predominated for the K4 (Fig. EV2C).

Experimental confirmation for membrane penetration by R4 and K4 peptides was obtained by FACS analyses of HEK-293 (Fig. 3B, C) and U937 (appendix Fig. S3A, B) cells incubated with fluorescently labelled peptides for one hour at 4°C. The R4 peptide entered the cells at higher efficiency than either K4 and N4 peptides. Similar results were obtained for R4 versus N4 peptides on DRG neuron cultures assessed by confocal microscopy (Fig. 3D, E). We then proceeded to assess plasma membrane association of endogenous nucleolin by cell surface biotinylation in HEK-293 cells, first validating the assay by demonstrating membrane expression of a G protein-coupled inwardly-rectifying potassium channel (appendix Fig. S3C). Endogenous nucleolin was readily detected in the biotinylated membrane protein pool (Fig. 3F). The role of the GAR domain in cell surface association of nucleolin was assessed in HEK-293 cells transfected with native nucleolin versus GAR domain deletion or GAR(N) mutants. Deletion or mutation of the GAR domain greatly reduced cell surface association of nucleolin (Fig. 3G, H). However, both wild-type GAR and the GAR(N) mutant showed comparable membrane localization when expressed as single domains (appendix Fig. S3D, E). Taken together, these experiments support a role for the GAR domain in plasma membrane interactions of nucleolin, and further suggest that membrane interactions can be influenced by the context of the domain in the protein.

### ***GAR and nucleolar localization of nucleolin***

The findings above support roles for the GAR domain in motor-driven transport of nucleolin and in plasma membrane interactions, but the main pool of nucleolin in cells is nucleolar, and previous reports had suggested that the GAR domain is required for nucleolar localization (Pellar & DiMario, 2003). We therefore asked to what degree the GAR domain influences accumulation or retention of nucleolin in the nucleolus. Transfection with Dendra2 fusions of full length or GAR deletion nucleolin revealed reduced partitioning of GAR deleted nucleolin to the nucleolus, albeit to different degrees in different cell types (Fig. 4A, B and appendix Fig. S4A-D). Disruption of liquid-liquid phase separation (LLPS) by hypotonic challenge with ddH<sub>2</sub>O (Nott *et al*, 2015) reduced nucleolin in nucleoli (Fig. 4C,D; appendix Fig. S4E-H), with very similar kinetics for full length and GAR deletion constructs in

both DRG neurons and N2a cells. Significant differences between full length and GAR deletion nucleolin were observed only in HEK-293 cells (appendix Fig. S4). Taken together these data indicate that GAR-deleted nucleolin is still found mainly in the nucleus, with somewhat less pronounced nucleolar segregation. Thus, the GAR domain contributes to nucleolin partitioning to nucleoli, but other domains of nucleolin such as the RNA recognition motifs (Okuwaki *et al.*, 2020) likely have a more dominant role in the LLPS dependent processes that form and stabilize nucleoli.

### ***GAR domain deletion perturbs mRNA localization***

In order to study the *in vivo* implications of perturbing GAR mediated nucleolin subcellular localization, we generated a GAR deletion mouse line by CRISPR/Cas9 gene editing, using two sgRNAs flanking the GAR domain (Fig. 5A). Four of the several founder mice generated were subsequently outbred on two genetic backgrounds, however, no F1 mice homozygous for the GAR deletion were identified from any of the founder lines. Genotyping of more than 30 E10.5 embryos from timed pregnant females revealed that there were no homozygous embryos, suggesting that biallelic deletion of the nucleolin GAR domain is lethal at early stages of development (appendix Fig. S5). Thus, subsequent analyses were conducted on animals heterozygous for the GAR deletion in nucleolin.

We first cultured DRG neurons from GAR<sup>+/-</sup> mice in modified Boyden chambers, allowing subsequent protein extraction from axons and cell bodies separately. Western blot quantification of nucleolin extracted from the cell soma compartment clearly showed that the mutant GAR deletion protein is expressed, while similar analysis of the axonal compartment revealed marked reduction of the deletion mutant band and a concomitant reduction of total nucleolin levels in the axon (Fig. 5B, C). Immunostaining of longitudinal sections from sciatic nerves of wild type and GAR<sup>+/-</sup> mice revealed a similar reduction of axonal nucleolin in the mutant animals (Fig. 6A, B). *Kpnb1* (Importin β1) and *mTOR* mRNAs are known cargos of nucleolin (Perry *et al.*, 2016; Terenzio *et al.*, 2018), hence we examined levels of these mRNAs by single molecule fluorescence *in situ* hybridization (FISH) on longitudinal sections of sciatic nerve from the same animals. These FISH analyses showed significant reductions in axonal levels of both *Kpnb1* and *mTOR* mRNAs in GAR<sup>+/-</sup> axons *in vivo* (Fig. 6C-F and Appendix Fig S6A). Thus, the GAR domain is required for axonal localization of nucleolin and its cargo mRNAs *in vivo*.

In order to further characterize the mRNA cargos that depend on nucleolin for axonal localization, we performed RNA-sequencing on pull-downs of nucleolin or Kif5a from sciatic nerve axoplasm, and on RNA extracted from isolated axons of wild type or GAR<sup>+/-</sup> sensory neurons cultured in Boyden chambers (Figs 7A and EV3). Of the close to 12,000 RNAs found in these datasets, 488 were enriched in nucleolin and Kif5a pull-downs and were also depleted from GAR<sup>+/-</sup> versus wild type axons (Figs 7A, B and EV3, Table EV2). From the latter, 45 mRNAs were enriched in the soma of GAR<sup>+/-</sup> neurons (purple cluster in Fig. 7B, C), hence are likely mislocalized due to subcellular changes in nucleolin localization. One of these mRNAs, *Inpp5f* (also known as *Sac2*), encodes a polyphosphoinositide phosphatase that was reported to regulate both cardiac cell and neuronal growth (Zhu *et al.*, 2009; Zou *et al.*, 2015), and was therefore selected for validation. We tested *Inpp5f* mRNA dependence on nucleolin for localization to axons by FISH on sensory neurons challenged with the AS1411 aptamer, which perturbs nucleolin localization to axons (Perry *et al.*, 2016). Indeed, AS1411-treated neurons revealed a significant reduction in axonal *Inpp5f* mRNA as compared to control aptamer treatments (Fig. 7D, E). Similar reduction of axonal *Inpp5f* mRNA was detected by qPCR analysis in sensory neurons grown in Boyden chambers (Fig. 7F).

To more directly test the requirement for nucleolin's GAR domain in axonal localization of cargo mRNAs, we asked if the loss of cargo mRNAs from axons with depletion of nucleolin can be reversed by nucleolin wild type vs. nucleolin $\Delta$ GAR protein expression. For this, endogenous nucleolin was depleted from adult DRG neurons using siRNA targeting the 3'UTR of nucleolin mRNA. These neurons were then transfected with siRNA-resistant nucleolin and nucleolin $\Delta$ GAR expression constructs and cell body and axonal levels of nucleolin cargo mRNAs were assessed by FISH. Endogenous nucleolin mRNA and protein were significantly reduced by siRNA knockdown (appendix Fig. S7). Nucleolin-depleted neurons transfected with si-resistant full length nucleolin showed comparable levels of *Kpnb1*, *mTor*, and *Inpp5f* mRNAs to the control siRNA transfected neurons, both in axons and cell body. In contrast, nucleolin-depleted neurons transfected with si-resistant nucleolin $\Delta$ GAR showed significantly lower *Kpnb1*, *mTor*, and *Inpp5f* mRNA levels in axons (Figs 8A-D, EV4, and EV5). Together, these data support an essential role of the GAR domain in nucleolin-dependent transport of axonal mRNAs.

### ***GAR mutant neurons exhibit increased growth***

The findings summarized above establish the GAR domain as a critical determinant of nucleolin localization to axons, and show that GAR domain deletion perturbs axonal localization of nucleolin cargo mRNAs important in growth regulation. Our previous studies have shown that axonal depletion of motor proteins, RNA binding proteins and mRNAs implicated in this growth controlling pathway have all caused acceleration of axonal growth (Perry *et al.*, 2016; Rishal *et al.*, 2012), as predicted by our previously proposed cell length sensing model (Albus *et al.*, 2013; Rishal & Fainzilber, 2019). Indeed, time-lapse imaging of sensory neuron growth in culture revealed accelerated growth of GAR<sup>+/-</sup> neurons as compared to their wild type counterparts (Fig. 9A, B). We sought to further corroborate this finding by expression of an HA-Dendra-GAR fusion construct as a dominant-negative that should compete with endogenous nucleolin for access to the axon. Indeed, expression of the wild-type GAR domain enhances axon growth as compared to expression of a mutant GAR wherein all arginines are substituted by asparagines (Fig. 9C, D). Thus, perturbation of GAR-mediated axonal localization of nucleolin enhances axon growth, as predicted by the length sensing model (Rishal & Fainzilber, 2019) and our previous findings (Perry *et al.*, 2016; Rishal *et al.*, 2012).

### **Discussion**

Our findings establish the GAR domain as a key determinant of nucleolin subcellular localization, most prominently directing its transport on kinesin motors to cytoplasmic extremities of the cell (Fig. 10). In addition to associating with kinesin motor complexes, the GAR domain also enables nucleolin association with the plasma membrane, and contributes to maintaining nucleolin within the nucleolus, a canonical membraneless organelle. Of note, we cannot rule out that the defects in nucleolar dynamics of nucleolin caused by GAR domain deletion contribute to the axonal localization and cell growth phenotypes, although the bulk of our evidence suggests that membrane and axonal localization roles are more prominent than nucleolar effects of GAR removal. The versatile roles of the GAR domain are reflected in the fact that deletion of this small domain in the mouse causes embryonic lethality. An early study had shown that a complete knockout of nucleolin was lethal to

chicken lymphoblast DT40 cells (Storck *et al.*, 2009), and it is striking that removal of such a small domain in this large protein is lethal *in vivo*.

GAR domains are found in numerous mammalian RBPs and their roles have been studied most extensively in RNA binding and in LLPS (Chong *et al.*, 2018; Thandapani *et al.*, 2013). Early work on nucleolin GAR focused on the contributions of this domain to nucleolar localization (Pellar & DiMario, 2003; Schmidt-Zachmann & Nigg, 1993), while more recent interest has focused on how the GAR domain influences binding of G-quadruplex RNA structures by RNA recognition motifs (RRM) in nucleolin (Masuzawa & Oyoshi, 2020; Saha *et al.*, 2020). The GAR domain in FMRP was also suggested to be important for localizing G-quadruplex RNAs (Goering *et al.*, 2020). Our results expand the spectrum of mechanisms employed by the GAR domain for additional versatility of functions, including plasma membrane association. Although nucleolin lacks transmembrane domains or canonical membrane anchoring motifs, substantial cell surface expression was reported in diverse cancer cells (Berger *et al.*, 2015), raising the question how nucleolin interacts with membranes. We have shown that the GAR domain fulfills this function, generating membrane contacts through arginine and phenylalanine residues, and that deletion or mutation of the GAR domain reduces cell surface nucleolin. Arginine-rich peptides are known to have the ability to passively penetrate cellular membranes (Takeuchi & Futaki, 2016). A number of mechanisms have been proposed for such translocation (Herce *et al.*, 2014; Lamaziere *et al.*, 2007; Mishra *et al.*, 2011; Pae *et al.*, 2014; Sun *et al.*, 2014), including the induction of membrane multilamellarity and fusion (Allolio *et al.*, 2018), with the first step being accumulation of arginine-rich sequence stretches at the membrane due to like-charge pairing of the guanidinium side-chain groups (Vazdar *et al.*, 2018). Our data suggests that GAR domains exploit these characteristics of arginine-rich sequences to provide a novel class of membrane anchoring or membrane traversing moiety for RBPs.

Other classes of RNA granules have been shown to use membrane linkers or adapters to hitchhike on endosomes, lysosomes or mitochondria for axonal transport (Cioni *et al.*, 2019; Gershoni-Emek *et al.*, 2018; Liao *et al.*, 2019). In contrast, GAR-mediated transport of nucleolin is likely dependent on direct interaction of the GAR domain with kinesin complexes. Since the RNA granule is in itself membraneless, this is a truly membrane-free axonal transport complex wherein the GAR domain provides an essential and direct link

between nucleolin containing RNA granules and the anterograde transport machinery. There is *in vitro* evidence for other membrane-free RBP-kinesin complexes (Baumann *et al*, 2020; Wu *et al*, 2020). Thus, mRNA transport to cellular extremities can be conducted by diverse carriers utilizing both membrane-associated and membrane-free transport complexes. *In vivo* evidence is therefore required to determine which of these mechanisms is physiologically important. Our gene editing approach provides such evidence for GAR mediated transport of nucleolin complexes, clearly identifying *Kpnb1*, *mTOR*, and *Inpp5f* mRNAs as being critically dependent on this specific transport complex for localization to sensory axons both in culture and *in vivo*. Our pull-downs and RNA-seq analyses have identified a host of additional mRNAs that are likely co-transported in the same complex, and complex stoichiometry and dynamics will be an intriguing topic for further investigation.

Generation of GAR mutant mice, albeit limited to heterozygotes, also allowed evaluation of physiological consequences of disruption of nucleolin complex transport to axons. Our previous work had proposed a size sensing mechanism dependent on microtubule motor-based localization of RNA encoding importin  $\beta$ 1 and mTOR, coupled with regulated local protein synthesis, to enable cytoskeleton length sensing for cell growth regulation (Rishal & Fainzilber, 2019). The model predicts that perturbation or reduction of the complex in the axon should lead to accelerated axonal growth, as previously shown by knockdown of molecular motors (Rishal *et al.*, 2012), 3'UTR deletion in *Kpnb1* (importin  $\beta$ 1) mRNA (Perry *et al.*, 2016), and AS1411 perturbation of kinesin-nucleolin interactions (Perry *et al.*, 2016). We have now shown that GAR<sup>+/-</sup> neurons also reveal accelerated axon growth, providing independent support for this growth control mechanism in a genetically engineered mouse model. Moreover, identification of *Inpp5f*, a known cell size regulator (Zhu *et al.*, 2009; Zou *et al.*, 2015), as an additional component of the RNA ensemble transported by the complex is an intriguing lead for future research.

We have shown that the diverse subcellular localizations of nucleolin are largely determined by its single small GAR domain, acting via multiple mechanisms – LLPS, protein-protein interaction, and protein-membrane association. This versatility suggests that different regulatory mechanisms must exist to allow the GAR domain to discriminate or translocate between these different options. Elucidating such regulation will be of great interest for the future, and one promising possibility is the activity of protein arginine

methyltransferases (PRMTs). PRMT methylation of arginines has been described in a spectrum of fundamental cellular processes, including RNA processing, nucleocytoplasmic distribution, signal transduction, and LLPS (Guccione & Richard, 2019; Tsang *et al*, 2019). A brain-enriched membrane-attached PRMT has been described (Lee *et al*, 2005; Park *et al*, 2019), thus it is intriguing to speculate that arginine methylation may be involved in translocation of GAR interactions between motors and membranes. Alternatively, local translation of nucleolin cargo mRNAs may generate local modifications of the cellular milieu that affect interactions of the GAR domain. For example, Inpp5f/Sac2 regulates phosphoinositides in the endocytic recycling pathway (Hsu *et al*, 2015), so its local translation in a restricted axonal subdomain might well change local membrane characteristics to modulate GAR association.

Finally, many axonal RBPs harbor a GAR domain and a number of known neurodegeneration-associated mutations in RBPs are found in their GAR domains. For example, mutations in the GAR domains of FUS, EWSR1, TAF15 are associated with amyotrophic lateral sclerosis (Kapeli *et al*, 2017), and methylation or citrullination within GAR domains affects FUS aggregation and activity (Hofweber *et al*, 2018; Tanikawa *et al*, 2018). The multiple roles of the GAR domain as a key localization determinant for nucleolin may be mirrored in many other RBPs, with implications for their cellular functions and for understanding the consequences of disease-associated mutations therein.

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### **Author Contributions**

E.D-M., I.K., and M.F. designed the study; E.D-M., I.K., O.A., I.R., J.K., S.A., E.E.Z., P.D-M., A.D-P., D-A.S., N.O. and D.G. planned and performed experiments, simulations, and data analyses; T.P.S., C.N.B. and P.K.S. conducted FISH analyses; S.B-D performed bioinformatics analyses, J.A.O-P. carried out mass spectrometry analyses; R.K. performed RNA-seq analyses; R.H-K generated the CRISPR engineered mice; G.C., A.L.B., P.J., J.L.T., and M.F. supervised research; E.D-M., I.K., and M.F. wrote the manuscript draft. All authors revised the manuscript and approved the final version.

### **Declaration of Interests**

The authors do not have any competing interests.

## Figure Legends

### Figure 1. The nucleolin GAR domain binds DNA aptamer AS1411 and kinesins.

**A.** Domain structure of nucleolin. RRM – RNA recognition motif; GAR – glycine- arginine rich domain. Amino acid sequence of the GAR domain is shown below. R3 and R4: peptides derived from the GAR domain C-terminus, efficiently binding AS1411 (see panel **B**). A3 designates the R3 peptide with three arginines substituted with alanines, N4 designates the R4 peptide with four arginines substituted with asparagines.

**B.** R3 and R4, but not the A3 and N4 peptides efficiently bind AS1411 in an ELISA assay with biotinylated peptides shown in **(A)** immobilized to streptavidin-coated plates. n = 4 technical replicates; means  $\pm$  SEM; \*\*\*\* p < 0.0001 in AS1411 vs. control aptamer, 2-way ANOVA with Sidak's post-test. a.u. - arbitrary units.

**C.** Kinesin protein family members affinity purified by biotinylated R3 peptide from mouse sciatic nerve axoplasm, identified by mass-spectrometry (schematics are based on (Hirokawa *et al*, 2009). Biotinylated A3 peptide served as a control. Mean spectral counts (normalized plus pseudocounts)  $\pm$  SEM, n = 3 independent biological repeats. All proteins shown were found to significantly purify with R3, but not A3, with probability > 0.95 and a mean fold change > 2 by SAINTexpress analysis (<http://crapome.org/>).

**D.** Schematic of kinesin pulldown from mouse sciatic nerve axoplasm, using biotinylated GAR peptides bound to streptavidin beads (STRP).

**E.** Automated capillary electrophoresis immunoassay traces of Kif5a pulled down by biotinylated R3 and R4 peptides. Biotinylated A3 and N4 peptides, respectively, served as controls. 4.5% of input used for pulldowns was loaded alongside the pulldown samples.

**F.** Quantification of **(D)**. a.u. - arbitrary units. n = 3 independent biological repeats; means  $\pm$  SEM; \*\* p < 0.01, paired Student's t-test.

*See also Fig. EV1 and Appendix Fig. S1.*

### Figure 2. Nucleolin-kinesin interaction is directly mediated by the GAR domain.

**A.** GAR domain deletion or mutation perturbs binding to Kif5a. Co-immunoprecipitation analysis of Kif5a and HA-Dendra2-tagged nucleolin (both overexpressed in HEK-293 cells). IP was performed with HA antibody and probed in Western blot with anti-Kif5a and anti-HA

antibodies. Ncl FL – HA-Dendra2- full length nucleolin; Ncl  $\Delta$ GAR – HA-Dendra2- nucleolin with a GAR domain deletion; Ncl GAR(N) – HA-Dendra2- nucleolin with all 10 arginines in the GAR domain mutated to asparagines; DEN – Dendra2. Note that HA input blots for HA-Dendra2 (Dendra) and the other three constructs are from the same membrane, but shown discontinuously owing to the different migration of these proteins in PAGE.

**B.** Quantification of **(A)**. n = 3 independent biological repeats; means  $\pm$  SEM; \*\*\*\* p < 0.0001, ANOVA with Tukey's post-test.

**C-E.** R4, but not N4 peptide reduces Kif5a co-immunoprecipitation with nucleolin from mouse sciatic nerve axoplasm, assayed by automated capillary electrophoresis immunoassay for Kif5a. Schematic of the assay is shown in **(C)**. Representative traces of the Kif5a immunoreactive peaks are shown in **(D)** and quantifications are shown in **(E)**. Immunoprecipitated Kif5a levels are normalized with input levels and expressed relative to N4. n = 3 independent biological repeats; means  $\pm$  SEM; \* p<0.05, paired Student's t-test.

**F.** Surface plasmon resonance analysis of KLC2 binding to biotinylated R4 peptide. Recombinant KLC2 was injected at different concentrations (5-160 nM) on biotinylated R4 or N4 peptides immobilized to a streptavidin sensor chip. The dissociation steady state constant KD, was determined by fitting the sensogram to a 1:1 model.

**G.** Kinetics analyses of photoconverted Dendra fusion proteins in cultured DRG neuron axons. Adult mouse DRG neurons were transfected by electroporation with constructs expressing HA-Dendra-GAR(WT) - wild-type nucleolin GAR domain N-terminally fused with HA-Dendra, or HA-Dendra-GAR(N) with 10 arginines in the GAR domain mutated to asparagines. 48 hr after plating and transfection, Dendra fluorescence was photoconverted using a DMD module to restrict conversion within the cell body. Time-lapse images were collected from the entire field of view, and red signal intensity was analyzed within axons at distances of 60-90  $\mu$ m from the cell body. Photoconverted signal sampled at 10 sec intervals is shown, normalized to signal intensity before photoconversion. N = 3 biological repeats comprising 7 cells each. Means  $\pm$  SEM; \*\*\*\* p<0.0001 (ANOVA).

*See also Appendix Fig. S2.*

**Figure 3. GAR domain interactions with phospholipid membranes.**

**A.** Molecular dynamics simulations of native (R4) or variant (K4 and N4) nucleolin GAR-derived peptides interacting with an idealized phospholipid bilayer (phosphatidylcholine (PC): phosphatidylserine (PS), 4:1) in water. Amino acid sequences of peptides are shown above. Shaded area – standard deviations of three independent runs, each 50 ns long. Mean values are indicated by colored lines and overlay of these is shown on the right.

**B.** Flow cytometry histograms of TAMRA-labeled peptide uptake into HEK-293 cells after 1h incubation at 4°C in growth medium.

**C.** Quantification of mean fluorescence intensities in **(B)**. n = 6 independent biological repeats; means ± SEM; \* p< 0.05, \*\*\* p< 0.001, \*\*\*\* p< 0.0001, ANOVA with Tukey's post-test.

**D.** Uptake of TAMRA-labeled R4 and N4 peptides into cultured DRG neurons. Cells were incubated with peptides dissolved to 5 μM in growth medium for 30 min at 4°C, washed, fixed and analyzed by confocal microscopy. PC – phase contrast. Scale bar – 10 μm. **E.** Quantification of **(D)**. n = 3 independent biological repeats; means ± SEM; \* p<0.05, paired Student's t-test a. u. – arbitrary units.

**F.** Analysis of cell surface nucleolin in HEK-293 cells. Cells were treated with 0.5 mM sulfo-NHS-SS-biotin for 30 min at 4°C, lysed and subjected to affinity pulldown of biotinylated proteins with streptavidin Dynabeads, eluted by 50 mM DTT and probed in Western blot with antibodies indicated on the left. Endogenous nucleolin (Ncl) was readily detected in the biotinylated membrane protein pool. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and importin β1 (Impβ1) served as negative controls.

**G.** HEK-293 cells were transfected with plasmids expressing HA-Dendra2- full length nucleolin (Ncl FL), HA-Dendra2-nucleolin with a GAR domain deletion (Ncl ΔGAR) or HA-Dendra2-nucleolin with all 10 arginines in the GAR domain mutated to asparagines (Ncl GAR(N)) and processed as in **(F)**.

**H.** Quantification of **(G)**. Levels of nucleolin in pulldowns were normalized to input levels and expressed as % relative to full-length HA-Dendra2-nucleolin. n = 4 independent biological repeats; means ± SEM; \*\*\*\* p<0.0001, ANOVA with Dunnett's post-test.

*See also Fig. EV2 and Appendix Fig. S3.*

**Figure 4. GAR domain and nucleolar localization of nucleolin.**

**A.** GAR deletion in nucleolin reduces partitioning to nucleoli in adult DRG neurons, transduced with the peripheral neuron specific AAV-PHP.S vector expressing full length HA-Dendra2-nucleolin (Ncl FL) or respective  $\Delta$ GAR mutant (Ncl  $\Delta$ GAR) upon seeding and analyzed by epifluorescence microscopy 9-10 days later. Shown are representative Dendra2 fluorescence images collected from the non-activated (green) emission line. Hoechst 33342 (10  $\mu$ M) was used to outline nuclei. Superimposed phase contrast and Dendra2 fluorescence images are shown on the right. Scale bar – 25  $\mu$ m.

**B.** Quantification of the nucleolar/nuclear intensity ratios in DRG cells shown in (A). n = 38-54 cells per sample in 3 independent biological repeats; means  $\pm$  SEM; \*\*\*\* p<0.0001, Mann-Whitney test.

**C.** Time-lapse live imaging of nuclei in DRG neurons transduced with AAV-PHP.S HA-Dendra2-nucleolin (full length and  $\Delta$ GAR mutant), after a hypotonic challenge with double-distilled water (ddH<sub>2</sub>O). Shown are epifluorescence images (Dendra2 green emission line) taken at indicated times after medium replacement with ddH<sub>2</sub>O. Scale bar – 10  $\mu$ m.

**D.** Quantification of nucleolar Dendra2 signal in (C) with intensity values immediately before medium replacement set as 100%. n = 17-24 cells per sample in 3 biological repeats; means  $\pm$  SEM; Two-way ANOVA with Sidák's post-test detected a significant time-dependent reduction of nucleolar signal (p < 0.0001) and no difference between full length and  $\Delta$ GAR mutant nucleolin (p > 0.05).

*See also Appendix Fig. A4.*

**Figure 5. Reduced levels of axonal nucleolin in nucleolin GAR<sup>+/-</sup> mice.**

**A.** Targeted deletion of nucleolin GAR domain by CRISPR-Cas9. Schematic shows mouse nucleolin exons targeted by single guide RNAs (sgRNAs) and resulting deletion in the GAR domain amino acid sequence.

**B.** Western blot analysis of nucleolin in DRG neurons from wildtype (WT) and GAR<sup>+/-</sup> mice cultured in Boyden chambers. Tuj1 was used as a loading control. The higher nucleolin band in GAR<sup>+/-</sup> corresponds to WT nucleolin and lower band to nucleolin with a 41 aa deletion (corresponding to about 4 kDa reduction in protein size).

C. Quantification of (B), total nucleolin levels (sum of both bands where applicable) were normalized to Tuj1 and expressed as  $GAR^{+/-}$  / WT ratios. n = 3 independent biological repeats; means  $\pm$  SEM; \* p < 0.05, 1 sample t-test.

See also Appendix Fig. S5.

**Figure 6. Reduced levels of nucleolin mRNA cargos in axons of nucleolin  $GAR^{+/-}$  mice.**

A, B. Representative maximum projections of exposure matched confocal images of immunofluorescence for nucleolin and neurofilament (NF) and the neuronal marker Tuj1 in sciatic nerve axons from WT and  $GAR^{+/-}$  mice shown in (A). Upper panels show total nucleolin stain. Middle panels show merged image of nucleolin (gray), NF and Tuj1 (magenta), and DAPI (blue). Lower panels show nucleolin overlaps with NF and Tuj1 signals as the “axon only” signal. B shows quantification of nucleolin immunofluorescence with approximately a 50% reduction in nucleolin in the axons of  $GAR^{+/-}$  mice *in vivo*. n = 3 WT, n = 4  $GAR^{+/-}$ ; means  $\pm$  SEM; \* p < 0.05, unpaired Student’s t-test. Scale bar – 10  $\mu$ m.

C-F. Representative, exposure matched maximum projection confocal images of FISH for *Kpnb1* (C) or *mTOR* (E) mRNA and NF plus Tuj1 immunostaining from sciatic nerve sections from WT (left) or  $GAR^{+/-}$  mice. Upper panels for each show total mRNA signal. Middle panels show mRNA (gray) signals merged with NF plus Tuj1 (magenta) and DAPI (blue). Lower panels show mRNA signal that overlap with NF plus Tuj1 signal (labeled “axon only” signal). Quantification of axonal *Kpnb1* (D) and *mTOR* (F) mRNA signals compared to the negative control, *DapB* mRNA, show a significant reduction in these axonal mRNAs in the  $GAR^{+/-}$  mice. n = 3 WT, n = 3  $GAR^{+/-}$ ; means  $\pm$  SEM; \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\*p < 0.0001 unpaired Student’s t-test. Scale bar – 10  $\mu$ m.

See also Appendix Fig. S6.

**Figure 7. Axonal transcripts associated with the Ncl-Kif5a complex.**

A. Workflow for profiling mRNAs bound by the Ncl-Kif5a complex. Nucleolin (Ncl) and Kif5a-binding RNA was isolated from wild type (WT) adult mouse sciatic nerve axoplasm by immunoprecipitation; in addition, DRG neurons from adult WT and  $GAR^{+/-}$  mice were cultured in modified Boyden chambers and RNA was isolated from cell body and axonal sides. RNA-seq analysis from the resulting four datasets yielded 11,771 overlapping

transcripts. The latter were further processed into a subset of 488 transcripts enriched in Ncl and Kif5a pulldown and depleted in axons of nucleolin  $GAR^{+/-}$  mice compared to WT (B). For a detailed workflow please see Fig. EV3.

**B.** Clustering of 488 Ncl/Kif5a-enriched transcripts reduced in  $GAR^{+/-}$  versus WT axons. Yellow cluster – transcripts not significantly enriched in the soma of  $GAR^{+/-}$  DRG neurons versus the WT control, Purple cluster – transcripts enriched in the soma of  $GAR^{+/-}$  DRG neurons versus the WT control. Heatmap shows mean log<sub>2</sub> fold changes across four datasets (see also Fig. EV3A), from left to right: 1) nucleolin-binding mRNAs in mouse sciatic nerve axoplasm; 2) Kif5a-binding mRNAs in mouse sciatic nerve axoplasm; 3) mRNA abundance in DRG neuron cell bodies of  $GAR^{+/-}$  mice relative to abundance in WT mice; 4) mRNA abundance in DRG neuron axons of  $GAR^{+/-}$  mice relative to abundance in wild type mice. All transcripts chosen for this cluster analysis showed significant enrichment in Ncl IP and reduction in  $GAR^{+/-}$  axons versus WT, as determined by Rank-Rank Hypergeometric Overlap (RRHO) as well as a fold-change in Kif5a IP versus control > 2.

**C.** Genes comprising the purple cluster - transcripts significantly enriched by both Ncl and Kif5a immunoprecipitation and showing a reduction in  $GAR^{+/-}$  axons concurrent with an enrichment in  $GAR^{+/-}$  soma (compared to the WT control).

**D.** Representative images for FISH analysis of *Inpp5f* in DRG neurons treated for 48h with 10  $\mu$ M AS1411 or 10  $\mu$ M control aptamer, replated and grown for 18h. FISH signal is shown in gray, cell somata (left) and axons (right) are visualized by neurofilament immunostaining (magenta). Scr – scrambled FISH probe served as a negative control. Scale bar – 10  $\mu$ m.

**E.** Quantification of axonal *Inpp5f* signal in (D) as density of RNA granules along axons. n = 11-19 cells per sample; means  $\pm$  SEM; \* p<0.05, unpaired Student's t-test.

**F.** RT-qPCR analysis of axonal *Inpp5f* mRNA levels in DRG neurons grown in Boyden chambers and treated for 48h with 10  $\mu$ M AS1411 or 10  $\mu$ M control aptamer. Axon/cell body ratios of *Inpp5f* normalized to *Gapdh* levels are shown. n = 4 independent biological repeats; means  $\pm$  SEM; \* p<0.05, unpaired Student's t-test.

See also Fig. EV3.

**Figure 8. Wild-type, but not  $\Delta$ GAR mutant nucleolin rescues defective axonal localization of its cargo mRNAs in DRG neurons treated with nucleolin siRNA.**

**A-B.** Representative exposure-matched FISH/IF images for DRG neurons co-transfected with control vs. nucleolin (Ncl) siRNA plus siRNA-resistant wild type (WT; **A**) or  $\Delta$ GAR (**B**) Ncl cDNAs. Axonal Kpnb1 mRNA is decreased with the Ncl knockdown, and this is not rescued by expression of  $\Delta$ GAR Ncl mutant (scale bars – 10  $\mu$ m).

**C-D,** Quantification of RNA signals from FISH/IF for *Kpnb1*, *mTOR*, and *Inpp5f* in cell bodies (**C**) and axons (**D**) for DRG cultures transfected as in **A** are shown. Axons of neurons cotransfected with Ncl siRNA plus  $\Delta$ GAR show significantly lower signals for each mRNA compared to Ncl siRNA plus WT and control siRNA plus WT or  $\Delta$ GAR ( $N \geq 10$  for cell bodies,  $N \geq 25$  for axons over 3 biological repeats; \*\*\*\*  $p \leq 0.001$  by one-way ANOVA with Tukey HSD post hoc).

See also Figs EV4 and EV5, and Appendix Fig. S7

**Figure 9. Reduced levels of full length axonal nucleolin increase axonal outgrowth in DRG neurons.**

**A.** Cultured DRG neurons from wild type (WT) Thy1-YFP mice and Thy1-YFP /  $GAR^{+/-}$  mice (YFP signal is shown in green). Cells were imaged every hour for a period of 48 h. Scale bar – 100  $\mu$ m.

**B.** Quantification of the time-lapse imaging experiment shown in (**A**). Growth rate of the longest neurite of each cell was calculated from the time point of starting growth.  $n = 4$  independent biological repeats; means  $\pm$  SEM, \*\*\*\*  $p < 0.0001$ , two-way ANOVA.

**C.** Fluorescence images of cultured DRG neurons from adult C57BL/6 mice infected with AAV-PHP.s expressing HA-Dendra fused with the wild type nucleolin GAR domain (GAR WT) or with the GAR domain with all 10 arginines substituted with asparagines, GAR(N). Neurons were re-plated 9-10 days after AAV infection. 24h after re-plating cells were fixed and stained with anti-HA (red) and anti-NFH (green) antibodies. Scale bar - 100  $\mu$ m.

**D.** Quantification of total axonal outgrowth and mean process length in HA-positive cells in (**C**). Outgrowth measurements were based on NFH staining.  $n = 6$  independent biological repeats; means  $\pm$  SEM; \*  $p < 0.05$ , \*\*\*  $p < 0.001$  (Student's-test).

**Figure 10. Essential roles for the GAR domain in subcellular localization of nucleolin.**

The nucleolin GAR domain binds a kinesin light chain, directly linking nucleolin-mRNA complexes to kinesin motors for axonal transport (**A**). The GAR domain further mediates membrane association of nucleolin. GAR-mediated subcellular targeting of nucleolin complexes enables export of key mRNAs to the axon, and the local translation of their encoded proteins for local functions in the axon, or for retrograde transport to the cell body. This reciprocal transport mechanism provides intrinsic regulation of axon length and growth, and indeed deletion or mutation of the GAR domain (**B**) perturbs mRNA localization to axons and increases axonal elongation.

**Figure EV1. Mass spectrometry of R3-interacting proteome in sciatic nerve axoplasm**  
(related to Fig. 1).

**A.** Venn diagram showing filtering of R3 peptide-binding proteins identified by mass spectrometry. A total of 2367 proteins were identified in the R3 and A3 pulldowns (at least one peptide in one out of the 6 samples). This set was filtered to a subset of 965 with at least one peptide in all three experimental replicates of R3, further narrowed down to 525 proteins with a SAINT probability score > 0.95 (equivalent of  $\alpha < 0.05$ ). Finally, the latter subset was filtered for R3/A3 fold change >2, yielding 352 proteins.

**B.** Top 15 canonical pathways represented in the 352 R3-binding proteins (Ingenuity Pathway Analysis), ranked by  $-\log_{10}(p\text{-values})$ .

**C.** Subcellular localization categories as defined by Ingenuity Pathway analysis (IPA) for the 352 R3-binding proteins (numbers indicate percentages of total).

**Figure EV2. Molecular simulations of GAR peptide interactions with phospholipid membranes** (related to Fig. 3).

**A.** Interaction of three R4 peptides with a POPC:POPS (4:1) membrane in water, 500 ns simulation time. POPC – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPS – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; 4:1 refers to molar ratio. Arginines are shown in blue, glycines in green and phenylalanines in light gray. Water is indicated in red, hydrophobic phospholipid tails in cyan and POPC/POPS polar groups in gray. Note the deep penetration of phenylalanine side chains into the hydrophobic bilayer core.

**B.** Distributions of mass densities of molecules in the simulation box shown in (A), with the center of symmetry in the middle of the POPC:POPS membrane. Enlargement of the region bounded by the dashed line is shown at the bottom.

**C.** Number of contacts between the membrane and individual residues in the peptides, at a distance of <0.6 nm from the membrane. Average number of contacts from three trials is shown. Error bars – SD.

**Figure EV3. RNA-seq to identify nucleolin interacting mRNAs (related to Fig. 7).**

**A.** Heatmaps showing genes depleted or enriched relative to controls in the four RNA-seq datasets generated in this study. Clusterograms show Z-scores for individual biological replicates (Rep). Controls used as a reference for finding differentially expressed genes were as follows: Nucleolin immunoprecipitation (IP): IP with nucleolin antibody pre-blocked by its target peptide; Kif5a IP: rabbit IgG; GAR<sup>+/-</sup> cell bodies and axons: wild type littermates.

**B.** The number of enriched and depleted genes in the four RNA-seq datasets shown in (A).

**C.** Workflow for identifying the 488-member transcript set shown in the clusterogram in Fig. 7B. Rank-Rank Hypergeometric Overlap (RRHO) analysis (Plaisier et al., 2010) was used to identify transcripts that are enriched in the nucleolin IP dataset and depleted from GAR<sup>+/-</sup> axons, as compared to WT. The resulting set was further narrowed down by using a cutoff fold enrichment > 2 in both nucleolin and Kif5a IPs. The final set, comprising 488 transcripts, was further clustered by the 'hclust' function in R, using the Pearson method.

**D.** Venn diagram showing the number of transcripts in subsets generated by the filtering workflow described in (B).

**E.** Ingenuity Pathway Analysis (top 10 canonical pathways) of the 488-member transcript set shown in Fig. 7B, ranked by  $-\log_{10}(\text{p-values})$ .

**Figure EV4. Rescue of axonal mTOR mRNA in DRG neurons treated with nucleolin siRNA (related to Fig. 8).**

**A-B.** Representative exposure-matched FISH/IF images for DRG neurons cotransfected with control vs. Ncl siRNA plus siRNA-resistant wild type (WT; A) or  $\Delta$ GAR (B) Ncl cDNAs. Axonal mTor mRNA is decreased with the Ncl knockdown, and this is not rescued by expression of  $\Delta$ GAR Ncl mutant. Scrambled Quasar RNA probe (Scr) (scale bars – 10  $\mu\text{m}$ ).

## **Figure EV5. Rescue of axonal *Inpp5f* mRNA in DRG neurons treated with nucleolin siRNA**

(related to Fig. 8).

**A-B.** Representative exposure-matched FISH/IF images for DRG neurons cotransfected with control vs. Ncl siRNA plus siRNA-resistant wild type (WT; **A**) or  $\Delta$ GAR (**B**) Ncl cDNAs. Axonal *Inpp5f* mRNA is decreased with the Ncl knockdown, and this is not rescued by expression of  $\Delta$ GAR Ncl mutant (scale bars – 10  $\mu$ m).

## **METHODS**

### **Mice**

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the Weizmann Institute of Science or the University of South Carolina. Adult C57BL6/OlaHSD and BALB/c mice were purchased from Harlan Laboratories (Envigo, Israel) and male Sprague Dawley rats (175-250 g) were purchased from Charles Rivers Laboratories. The C57BL/6YFP16 mice (Feng *et al*, 2000) were maintained at the Veterinary Resources of the Weizmann Institute. Nucleolin GAR<sup>+/-</sup> were generated and bred by the Weizmann Institute Veterinary Resources Department's core facility for transgenics and knockouts as described in the detailed methods section. All animals were housed in the Veterinary Resources Department of the Weizmann Institute or Animal Resource Facility of the University of South Carolina in a temperature-controlled room under a 12h light/dark cycle. Water and food were available *ad libitum*. Both female and male mice were used alternately in all experiments. Per biological replicate, mice for all conditions were sex and age matched, and littermates were used whenever possible. Tissue was extracted from animals 8-20 weeks of age.

### **Primary neuronal cultures**

Dorsal root ganglia (DRG) from 8-12 week old mice were dissected and dissociated for neuron cultures with 100 U of papain (P4762, Sigma) followed by treatment with 1 mg/ml collagenase-II (11179179001, Roche) and 1.2 mg/ml dispase-II (04942078001, Roche) for additional 25-30 minutes. The ganglia were mechanically triturated in HBSS supplemented with 10 mM Glucose, and 5 mM HEPES (pH 7.35), by aspiration in a glass Pasteur pipette whose opening was narrowed by fire polishing and was pre-covered in serum containing

media. Cells were then laid on a 20% Percoll cushion in Leibovitz L15 medium and recovered through centrifugation at 1000 g for 8 min. Cells were washed briefly in growth medium and plated on pre-coated glass coverslips or plates in growth medium (F12 supplemented with 10 % Fetal Bovine Serum and antibiotic formulation Primocin (Invivogen) according to manufacturer's instructions). Pre-coating included a first step of coating with poly-L-lysine (P4832, Sigma) followed by laminin (23017-015, Invitrogen). When cells were grown for >2 days, growth media was supplemented with 10 $\mu$ M Cytosine  $\beta$ -D-arabinofuranoside (AraC) starting at 24 hours after plating and replenished every 48 hours. For compartmentalized cultures (modified Boyden chambers), cells were plated on Millicell 1  $\mu$ m pore size inserts (MCRP06H48, Millipore) as previously described (Willis & Twiss, 2011) and allowed to grow for 3 days prior to extraction. Where indicated, cells were treated with 10  $\mu$ M AS1411 (GGTGGTGGTGGTTGTGGTGGTGGTGG) or 10  $\mu$ M control DNA (CCTCCTCCTCCTTCTCCTCCTCCTCC) oligonucleotides (Integrated DNA Technologies) as described before (Perry *et al.*, 2016). All cells were incubated at 37 $^{\circ}$  Celsius and 5% CO<sub>2</sub>. For rescue experiments as shown in Figs 7, EV4, and EV5, DRGs isolated from rats were transfected with HA-Dendra2-nucleolin-WT or HA-Dendra2-nucleolin- $\Delta$ GAR constructs (without the 3'UTR of Ncl) using Amaxa Nucleofactor after dissociation. Neurons were cultured and 3 h later transfected with 50 nM control siRNAs (Sahoo *et al.*, 2020) or two siRNAs targeting 3' UTR of *Ncl* mRNA (siRNA 1: GUUGAAUGACAGAGCCUUUUU; siRNA 2: GGACAUUCCAAGACAGUAAUU) using Dharmfect-3. Cultures were fixed 96 h later and FISH/IF was performed (see below). RTddPCR and immunoblotting for Ncl mRNA and protein, respectively, was performed on sister cultures to validate knockdown of endogenous mRNA.

### Cell lines

HEK-293 (human, female, RRID: CVCL\_0045), U937 (human, male, RRID: CVCL\_0007) and Neuro-2A (mouse, male, RRID: CVCL\_0470) cells were purchased from ATCC (Cat# CRL-1573, CRL-1593, CCL-131, respectively). HEK-293 and Neuro-2A cells were cultured in DMEM (Gibco), supplemented with 10% fetal bovine serum (Gibco), 100 U/mL *penicillin* and 100  $\mu$ g/mL streptomycin. U937 cells were grown in RPMI-1640 medium (Biological Industries) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL *penicillin* and 100  $\mu$ g/mL

streptomycin. All cells were incubated at 37° C (Farin *et al*, 2011) and 5% CO<sub>2</sub>. The cell lines used were not authenticated.

### **Plasmids and transfections**

Full-length mouse nucleolin ORF was subcloned into a pcDNA3.1-based mammalian expression vector from a plasmid kindly provided by Ronit Pinkas-Kramarski (Farin *et al*, 2011). The sequence was modified using restriction-free cloning to generate GAR deletion (amino acids 646-697 from UniProt sequence P09405) and GAR(N) variants, and to introduce N-terminal HA-Dendra2 tags, as well as generating a construct expressing HA-Dendra2 alone. Phusion polymerase (Thermo Fisher) was used for mutagenesis, according to manufacturer's instructions. Dendra2 sequence was cloned from pDendra2 construct (Evrogen). For AAV generation, HA-Dendra2 fusions with full length nucleolin, nucleolin with deleted GAR deletion and domain were subcloned under the human synapsin (hSyn1) promoter in a previously described AAV genomic vector (Mahn *et al*, 2018; Marvaldi *et al*, 2020), using PCR with primers tailed with *Ascl* and *EcoRV* restriction sites. Kif5a expression plasmid was obtained from Addgene (#31607). HEK-293 and N2a cells were purchased from ATCC (CCL-131) and cultured in DMEM (Gibco), supplemented with 10% fetal bovine serum (Gibco), 100 U/mL *penicillin* and 100 µg/mL streptomycin. U937 cells were purchased from ATCC (CRL-1593.2) and grown in RPMI-1640 medium (Biological Industries) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL *penicillin* and 100 µg/mL streptomycin. For transfection of HEK-293 cells, jetPEI (Polyplus-transfection) was used according to manufacturer's instructions. In experiments where HA-Dendra2-nucleolin or HA-Dendra2 constructs were co-transfected with Kif5a, a 4:1 expression ratio was used to compensate for differences in expression levels.

### **Antibodies**

Following primary antibodies were used for this study: rabbit anti-nucleolin (Abcam, ab50279, 1:1000 for WB), rabbit anti-nucleolin (Proteintech, 10556-1-AP, 1:100 for IF), rabbit anti-Kif5a (Abcam, ab5628, 1:1000 for WB, 1:100 for Wes), rabbit anti-HA (Sigma, H6908, 1:1000 for WB), rabbit anti-LAMP1 (Abcam, ab24170, 1:1000 for WB), mouse anti-GAPDH (Millipore, MAB374, 1:5000 for WB), mouse anti-β-III tubulin (R&D systems, MAB1195, 1:1000 for WB), mouse β-III tubulin (Tuj1) (Biolegend 801202, 1:500 for IF),

mouse anti-NFH (Developmental Studies Hybridoma Bank, RT97, 1:200 for IF), chicken anti-NFH (Abcam, ab72996, 1:1000 for IF) mouse anti-neurofilament (Biolegend 837904, 1:1000 for IF), sheep anti-digoxigenin (Roche, 11207733910, 1:2000 for ELISA). HRP-conjugated secondary antibodies for immunoblots were purchased from Bio-Rad Laboratories, Alexa Fluor Secondary antibodies were from Jackson ImmunoResearch and streptavidin-HRP from Abcam (ab7403, 1:10000).

### **ELISA assay for AS1411-binding peptides**

Streptavidin-coated 96 well ELISA plates (R&D systems, CP004) were washed twice in PBS-T (PBS 1x + 0.05% Tween-20), then incubated for 1 hour at room temperature with 0.025  $\mu$ M Biotinylated peptides, blocked by incubation with 1% BSA (Sigma, A9647) in PBS for 30 minutes at 37°C, then incubated with 0.04  $\mu$ M digoxigenin (3'-DIG) AS1411 or control aptamer (Integrated DNA Technologies) in PBS for 1 hour at room temperature. Then, plates were incubated with HRP-conjugated anti-DIG antibody (Roche, 11207733910) diluted 1/2000 in 1% BSA-PBS for 30 minutes at 37°C. Signal was detected using TMB ELISA substrate (Sigma, T0440). Plates were washed 3 times before each subsequent step with PBS-T and 5 times prior to signal detection. Signal was read using a Tecan5 plate reader reading absorbance at 360 nm wavelength.

### **Axoplasm isolation**

Mouse sciatic nerve axoplasm was isolated as previously described (Rishal et al., 2010). In brief, freshly dissected sciatic nerves were collected in Nuclear Transport buffer (TB) at a ratio of 1 SN/50  $\mu$ l buffer (20 mM HEPES, 110 mM KAc, 5 mM MgAc, pH 7.4 supplemented with Complete protease inhibitor EDTA free, (Roche 1187358000), phosphatase inhibitor cocktail 2 (1/1000, Sigma 5726), phosphatase inhibitor cocktail 3 (1/1000, Sigma P0044) and RNase inhibitors (200U/ml, RNaseIn, Promega N251B). Tissue was manually ground with a micropestle in a microtube until it lost its fibrous consistency. Lysates were centrifuged 10,000 g for 10 minutes at 4°C and pellet was discarded.

### **Immunoprecipitation**

Nucleolin immunoprecipitation from axoplasm was conducted as follows: approximately 200  $\mu$ g of axoplasm/protein lysates were incubated with 10 $\mu$ g antibody for 3 h, then

supplemented with 100  $\mu$ l Protein G magnetic beads (Dynabeads, Thermo Fisher Scientific 10004D), pre-blocked with salmon sperm DNA (10 $\mu$ l DNA per 100 $\mu$ l beads for 1 hour 4°C), for additional 1 hour. Incubation buffer was adjusted to final 0.1% NP40 to avoid beads aggregation. All incubation steps were done at 4°C with overhead rotation. Beads were washed with increasing NP40 concentration as follows: TB-0.1% NP40 for 3-5 minutes, TB-0.5% NP40 3-5 minutes, TB-1% NP40 1 minute, TB-no detergent 3-5 minutes, then transferred to a clean Eppendorf tube in TB without detergent. Elution from beads was conducted by denaturing the proteins from the beads with Laemmli sample buffer or WES sample buffer (see below) supplemented with DTT to a final concentration of 40mM for 5 minutes at 95°C.

#### **Axoplasm pulldown with biotinylated peptides**

Axoplasm lysates were incubated over-night at 4°C with streptavidin magnetic beads (Dynabeads M-280, Thermo Fisher Scientific 11205D) pre-conjugated to biotinylated peptides by incubation with 2  $\mu$ M peptides in PBST for 1 hour at room temperature. A pre-cleaning step was added, clearing the sample with beads pre-conjugated to control peptide (A3 or N4) for 3 h at 4°C prior to the over-night incubation with target or control peptides. Washes were as indicated for immunoprecipitation, followed by 2 washes with mass spectrometry grade water (Fluka analytical 39253) to remove any traces of detergents and salts.

#### **Mass-spectrometric analysis**

Sample-incubated streptavidin magnetic beads were resuspended in 20  $\mu$ l 5 mM DTT 100mM  $\text{NH}_4\text{HCO}_3$  and incubated for 30 min at room temperature. After this, iodoacetamide was added to a final concentration of 7.5 mM, and samples incubated for 30 additional minutes. 0.5  $\mu$ g of sequencing grade trypsin (Promega) was added to each sample and incubated at 37°C overnight. Supernatants of the beads were recovered, and beads digested again using 0.5  $\mu$ g trypsin in 10  $\mu$ l 100 mM  $\text{NH}_4\text{HCO}_3$  for 2 hrs. Peptides from both consecutive digestions were combined and recovered by solid phase extraction using C18 ZipTips (Millipore), eluted in 15  $\mu$ l 50% acetonitrile 0.1% formic acid, and resuspended in 5  $\mu$ l 0.1% formic acid for analysis by LC-MS/MS. Peptides resulting from trypsinization were analyzed on a QExactive Plus (Thermo Scientific), connected to a NanoAcquity™ Ultra

Performance UPLC system (Waters). A 15-cm EasySpray C18 column (Thermo Scientific) was used to resolve peptides (90-min 2-30% gradient with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B). MS was operated in data-dependent mode to automatically switch between MS and MS/MS. The top 10 precursor ions with a charge state of 2+ or higher were fragmented by HCD. Peak lists were generated using PAVA in-house software (Guan et al., 2011). All generated peak lists were searched against the mouse subset of the UniprotKB database (UniProtKB.2013.6.17) (plus the corresponding randomized sequences to calculate FRD on the searches), using Protein Prospector (Clauser et al., 1999). The database search was performed with the following parameters: a mass tolerance of 20 ppm for precursor masses; 30 ppm for MS/MS, cysteine carbamidomethylation as a fixed modification and acetylation of the N terminus of the protein, pyroglutamate formation from N terminal glutamine, and oxidation of methionine as variable modifications. All spectra identified as matches to peptides of a given protein were reported, and the number of spectra (Peptide Spectral Matches, PSMs) used for label free quantitation of protein abundance in the samples.

### **Western blot and Wes capillary immunoanalysis**

For Western blot, proteins were blotted on nitrocellulose membranes using a Trans-Blot Turbo™ Transfer System (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in TBST buffer for 1 hour. Primary antibodies were incubated for 1 hour at room temperature or overnight at 4°C with shaking. Secondary HRP-conjugated antibodies were diluted 1:10,000 in TBST and incubated for 1 hour at room temperature. Blots were developed using Radiance ECL substrate (Azure biosystems) or SuperSignal™ West Femto (Thermo Fisher Scientific) or Clarity western ECL (Biorad) substrates on an Amersham Imager 680 or Biorad Chemidoc. Automated capillary electrophoresis immuno-quantification runs were conducted on a WES instrument (ProteinSimple) according to manufacturer recommendations. The indicated MW are determined based on an internal standard of known MW spiked in to every capillary.

### **Surface plasmon resonance analysis**

KLC Binding to R4 GAR and N4 GAR derived peptides was monitored by surface plasmon resonance (SPR) on a Biacore S200 system using a biotinylated streptavidin chip (Series S SA). Recombinant KLC2 was injected different concentrations (5-160 nM) in a single cycle kinetics mode on biotinylated R4 or N4 GAR derived peptides, or free biotin. Before data collection, a normalization cycle followed by a priming cycle were run to stabilize the instrument. Binding assays were performed in triplicates in PBST buffer with a flow rate of 30  $\mu$ L/min at 25 °C. 120-s contact time was required to achieve steady-state binding, Regeneration of the chip surface was achieved by a 30-s injection of 2 mM NaOH in water. The dissociation equilibrium constant,  $K_D$ , was determined by fitting the sensogram to a build in global fitting routine, using a 1:1 model using S200 Evaluation Software 1.1.

### **Molecular simulations of peptide-phospholipid bilayer interactions**

Simulations in Fig. 3A. In two separate simulation setups, three previously equilibrated peptides of each sequence (R4, N4, K4) were placed either freely in a box with only water (500 ns for each sequence) or in a box with water and membrane composed of phosphatidylcholine and phosphatidylserine (PC:PS, 4:1) (500 ns for each sequence + 3 additional independent runs, each 50 ns long). Both systems contained 150 mM KCl including additional  $K^+$  ions to neutralize the negative charge of peptides (in case of R4 and K4). All MD simulations were performed using the Gromacs 5.1.2 package (Abraham *et al*, 2015). Calculations were done with charmm36-july forcefield (Klauda *et al*, 2010) and the membrane was prepared and solvated using Charmm-gui interface by employing the CHARMM-GUI server (Wu *et al*, 2014). The peptides were built in VMD (Humphrey *et al*, 1996) using its Protein builder tool.

Simulations in Fig. S3. Newton's equations of motion were integrated by employing the leap-frog algorithm (Hockney *et al*, 1974) with a time step of 2 fs. The trajectory frames were recorded every 10 ps and energy every 2 ps. For simulations with a membrane, energy was recorded every 2 ps. A cutoff of 1.2 nm was applied to short-range electrostatic interactions while long-range electrostatics was calculated with the use of the particle mesh Ewald method (Darden *et al*, 1993). Van der Waals interactions were truncated at 1.2 nm; in addition, for the membrane simulations, the van der Waals potentials were decreased using

Force-switch so that the forces went smoothly to zero between 1.0 and 1.2 nm. Bonds with hydrogen atoms, and all bonds for membrane setup, were constrained by the LINCS algorithm (Hess *et al*, 1997), and water molecules were kept rigid by the SETTLE algorithm (Miyamoto & Kollman, 1992). The temperature of the system was maintained at 310 K using the velocity rescaling thermostat with a stochastic term, and the Parrinello–Rahman barostat was utilized for isotropic, resp. semi-isotropic for membrane simulations, pressure coupling with a reference pressure of 1.01 bar. The time constants of the thermostat and barostat were 0.5 ps and 10 ps, respectively, and 1 ps and 5 ps for the membrane setup. The peptides were built in VMD Protein builder. Each structure was then equilibrated, minimized, and in order to provide enough time for the peptides to accommodate, a 100 ns long simulation was run. The simulation box was 6×6×6 nm big containing ~7000 water molecules together with 150 mM KCl including additional K<sup>+</sup> ions to neutralize the negative charge of peptides (in case of R4 and K4). In the case of simulations of peptide's triplets, box 7×7×7 nm containing ~11000 water and the same ionic strength strategy was used. Peptides were placed in the box randomly in a distance where they did not initially interact with each other. In all simulations, water molecules were described by the TIP3P water model (Jorgensen *et al*, 1983), and to account for electric polarizability, all charges of K<sup>+</sup> and Cl<sup>-</sup> ions were rescaled by a factor of 0.75 (Leontyev & Stuchebrukhov, 2011). The membrane was prepared and solvated using Charmm-gui interface by employing the CHARMM-GUI server. The bilayer was equilibrated and minimized following the standard minimization and equilibration protocol provided by CHARMM-GUI. The simulation box of size 8×8×14 nm contained a PC:PS (4:1) bilayer consisting of 160 PC lipids and 40 PS lipids, 150 mM KCl, including additional K<sup>+</sup> ions to neutralize the negative charge of the membrane and peptides (in case of R4 and K4), and ~21000 water molecules. Peptides were placed in the box randomly (and it was checked that it does not depend on orientation or the initial distance of peptides from the membrane, see Fig. 3A) in a distance where they did not initially interact with membrane leaflets or with each other. The simulations were performed with three peptides to be able to observe pair or cluster formation but at the same time to keep the simulations feasible and not costly. To ensure that our 500 ns long simulations are converged, the analysis were processed separately for the first 250 ns and the last 250 ns and the results were compared. There are no differences between the two analyses which means the simulation is converged. Also, to be sure that the initial distribution of peptides

in boxes does not affect the result, another three short 50 ns simulations for each N4, R4, and K4 sequences were performed yielding similar results.

### **Cellular peptide uptake assays**

DRG neurons expressing YFP under the Thy1 promoter (Feng *et al.*, 2000) were cultured on a 4 Well Glass Bottom slide chamber ( $\mu$ -Slide, ibidi 80427) for 24 h prior to treatment. For cold treatment (4°C), plates were taken out of the incubator and onto ice and taken in to the cold room (2-8°C) to cool for 20 minutes. Then, medium was replaced with cold (4°C) growth medium supplemented with 5  $\mu$ M R4 (FGGRGRGGFGGRGGFRGG) or N4 (FGGNGNGGFGGNGGFNGG) peptides N-terminally conjugated with TAMRA (Sigma). Following additional 30 minutes, cells were washed 3 times in cold PBS, fixed in cold 4% PFA for 20 minutes, and imaged in PBS using Olympus FV1000 Confocal laser-scanning microscope with a 60x water immersion objective (UPLSAPO 60X O NA:1.35). Average TAMRA intensity per pixel was measured using FIJI software. Cells were traced manually based on the phase contrast channel.

For analysis of peptide uptake into HEK-293 and U937 cells, 5  $\mu$ M TAMRA-conjugated peptides were added to the growth medium for 60 minutes. Then, cells were collected into 15 ml tubes for washing with cold PBS in pre-cooled centrifugation (2  $\times$  2 min, 1,000  $\times$  g). Cells then were suspended in 400  $\mu$ l cold PBS and filtered through a nylon mesh into 5 ml tube to obtain a uniform single-cell suspension. Cells were analyzed on a FACS-LSRII cytometer (BD Biosciences) using BD FACSDIVA software (BD Biosciences).

### **Cell surface biotinylation**

On the day following transfection, HEK-293 cells grown on 10 cm plates (one plate per sample) were washed twice with ice-cold PBS and treated with 0.5 mM sulfo-NHS-SS-biotin (Thermo Scientific 21331) in PBS for 30 min at 4°C with gentle rocking. Free sulfo-NHS-SS-biotin was removed and unreacted reagent was quenched by adding ice-cold 50 mM Tris-HCl in PBS pH 7.4. Cells were washed once with PBS and once with PBS + 0.1 mM oxidized glutathione (Sigma G4376). Proteins were extracted 30 min lysis in RIPA buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche 1187358000) and 0.1 mM oxidized glutathione. Lysates were centrifuged for 12000g to pellet debris and 1/20 of

lysates was used for input samples. The remainder of the lysate was used for pulldown of biotinylated proteins with 30  $\mu$ l of MyOne Streptavidin C1 Dynabeads (Thermo Scientific 65002) for 1h on a rotator at 4°C. Beads were washed for 4 x 5 min at 4°C and biotinylated peptides eluted by incubating with 50 mM DTT in PBS for 30 min at 50°C. Efficiency of cell surface labeling was assessed by Western blotting input samples with streptavidin-HRP.

### **Generation of AAV-PHP.S vectors and transduction of cultured DRG neurons**

ORFs of HA-tagged nucleolin full-length, nucleolin  $\Delta$ GAR, nucleolin GAR(N) and nucleolin-derived GAR domain were subcloned into an AAV plasmid under the human Synapsin1 (hSyn1) promoter to ensure neuronal specificity (Mahn *et al.*, 2018; Marvaldi *et al.*, 2020). For AAV production we used AAVpro HEK-293T cells and (Takara Bio 632273) AAVpro<sup>®</sup> Purification Kit for All Serotypes from Takara Bio (#6666) according to manufacturer's instructions. Four 15 cm plates were transfected with 20  $\mu$ g of DNA (AAV plasmid containing the expression cassette, pAAV-PHP.S (Addgene # 103006) and pAdDeltaF6 (University of Pennsylvania Vector Core) helper plasmids) in equimolar ratios using jetPEI<sup>®</sup> (Polyplus) in DMEM medium without serum or antibiotics. Medium (DMEM, 20 % FBS, 1 mM sodium pyruvate, 100 U/mL penicillin 100 mg/mL streptomycin) was added on the following day to a final concentration of 10% FBS and virus particles were harvested three days after transfection. Virus titers (as determined by RT-qPCR) were in the range of  $10^{12}$ - $10^{13}$  viral genomes/mL. For infection of cultured neurons, cells were transduced with the viral stock at a 1:200 dilution simultaneously with seeding. The cells were replated on glass bottom 35 mm MatTek dish for hypoosmotic treatment experiment or coverslips for evaluation of neurite outgrowth 8-9 days after transduction. Aforementioned experiments were conducted on the next day after replating.

### **Hypoosmotic treatment of cultured cells**

Adult mouse DRG neurons transduced with AAV-PHP.S (Chan *et al.*, 2017) encoding HA-Dendra2-nucleolin (full length and  $\Delta$ GAR), or N2a or HEK-293 cells transfected with HA-Dendra2-nucleolin (full length and  $\Delta$ GAR) expression plasmids were live imaged with the Nikon Ti-LAPP illumination system equipped with a temperature-controlled chamber and an Andor EMCCD camera. Cells were incubated with 10  $\mu$ M Hoechst 33342 in medium for 10 minutes prior acquisition. Images were acquired in DAPI, GFP, TRITC and DIC channels at X60

magnification with Nikon CFI plan apochromat 60X oil lambda objective. Medium was removed and the cells were immediately refocused and imaged or the t=0 image, followed by careful addition of 1 mL ddH<sub>2</sub>O by pipetting and image acquisition using a same setting with 42 seconds interval for 5 minutes. Images were analyzed by NIS-Elements software (Nikon).

### **Generation of nucleolin GAR knockout mice using CRISPR-Cas9**

GAR knockout lines were generated by using two sgRNAs to create a deletion in the nucleolin coding sequence. The sgRNAs were designed to minimize off target deletions in nonspecific areas in the genome and to maximize the probability of Cas9 cleavages at the targeted sites, using CHOPCHOP (v.2, [chopchop.cbu.uib.no](http://chopchop.cbu.uib.no)) and other tools detailed in the Benchling implementations ([www.benchling.com](http://www.benchling.com)), see also table S3. Guides were designed to cut in exon13 and exon14, flanking all RGG repeats in the GAR domain. Selected sgRNAs were prepared in-house as previously described (Ran et al, 2013). In brief, sgRNA were cloned into px459 plasmid. T7 promotor sequence was fused to sgRNA target sequence by PCR reaction, and sgRNAs were in vitro transcribed with MEGAscript (Ambion cat. AM1354) and cleaned with MEGAclear kit (Ambion cat. AM1908). sgRNAs ability to direct Cas9 recombinant protein to the appropriate DNA sites was tested in vitro using Guide-it sgRNA Screening Kit (Clontech Cat. No. 631440) with PCR product containing the relevant part of the gene (940 base pairs) amplified from genomic DNA as template (primers: Fwd- GCATGGAGAACTTGGGTCTG, Rev- ATGAAGCTGTCCCCACCAAT. Cas9 mRNA was in vitro transcribed with mMACHINE T7 Ultra Kit (Ambion cat. AM1345) from a linearized plasmid and cleaned with MEGAclear kit (Ambion cat. AM1908). Genome edited animals were generated by the Weizmann Institute Transgenic Unit by microinjection. Cas9 mRNA and the two sgRNAs (sgRNAa: GGCUCCGAGGCGGCAGAGGAGG, cuts at position 666 of NM\_010880.3 (Refseq sequence), end of GAR ; sgRNAb; GAAGGUGGCUUUGGUGGUCGAGG, cuts at position 898 of Refseq sequence, beginning of GAR) were microinjected into fertilized oocytes from superovulated CB6 F1 donor mice. Founders were crossed with WT C57BL/6J-OlaHsd for multiple generations. Genotyping was done by PCR reaction on genomic DNA extracted from 3-4 weeks pups' tails, using the screening primers indicated above. For each sgRNA, potential genomic

alterations in off-targets within genes were further screened by PCR on DNA from tails of F3 progeny of the founder further bred for experiments (table S3 for primer sequences).

### **Fluorescent In Situ Hybridization**

For FISH in *in vitro* cultured neurons, DRGs neurons were isolated from adult rats aged 2-3 months and cultured as described previously (Twiss *et al.*, 2000). Briefly, DRG neurons were harvested in Hibernate-A medium (BrainBits) and then dissociated as described. After centrifugation and washing in DMEM/F12 (Life Technologies), cells were resuspended in growth medium consisting of DMEM/F12, 1 x N1 supplement (Sigma), 10% fetal bovine serum (Hyclone), and 10  $\mu$ M cytosine arabinoside (Sigma). Dissociated DRGs were plated immediately on laminin/poly-L-lysine-coated coverslips in growth media containing 10  $\mu$ M of control or AS1411 aptamer. DRG neurons were let to grow for 48 h followed by replating and letting the cells grow for next 18 h. Further DRG cultures were fixed for 15 min in 2 % PFA in PBS and then processed for FISH as described (Sahoo *et al.*, 2018). RNA-FISH was performed using custom 5' Quasar 670-labeled 'Stellaris' probes against rat *Inpp5f* mRNA (BioSearch Technologies, Cat# SS561665-01-48), 5' Quasar 570-labeled 'Stellaris' probes against rat *mTor* mRNA (Terenzio *et. al.*, 2018), and 5' Quasar 570-labeled 'Stellaris' probes against rat *Kpnb1* mRNA (Perry *et. al.*, 2016). 5' Quasar 570- or Quasar 670-labeled scrambled probes were used as controls for specificity; samples processed without addition of primary antibody were used as control for antibody specificity. Primary antibody consisted of RT97 mouse anti-NF (1:200). FITC-conjugated donkey anti-mouse (1:200) was used as secondary. Samples were mounted as above and analyzed using a Leica DMI6000 epifluorescent microscope with ORCA Flash ER CCD camera (Hamamatsu).

For FISH in tissue sections, sciatic nerves from wild type and nucleolin *GAR*<sup>+/-</sup> mice were dissected from adult mice aged 8-10 weeks, both sexes were used) and fixed for 4 h at room temperature in 2% paraformaldehyde in PBS, washed 3 x 5 min with PBS and cryoprotected overnight in 30% buffered sucrose at 4°C. Custom designed Quasar 570 labeled Stellaris probes (BioResearch Technologies) were used to detect *mTor* and *Kpnb1* mRNA in sciatic nerve axons from wildtype or *GAR* +/- mice. FISH was performed on 10  $\mu$ m thick sciatic nerve sections as previously described (Terenzio *et al.*, 2018) with some modifications. Briefly, tissue sections were washed three times in 20 mM glycine (in 1X PBS) for 5 minutes

each followed by three 5 minute washes in 0.25 M NaBH<sub>4</sub> (in 1X PBS). This was followed by a quick wash in 0.1 M triethanolamine then a 10 minute wash in 0.1M triethanolamine + 0.25% acetic anhydride. After a quick wash in 2X SSC, tissue sections were dehydrated in a series of increasing concentrations of ethanol for 3 minutes each (70 to 95, to 100%) followed by delipidation in chloroform for 5 minutes. After delipidation, tissue sections were immediately put in 100% ethanol and then 95% ethanol for 3 minutes each. After two washes in 2X SSC, tissues were incubated in 0.2 M HCl for 10 minutes. Tissue sections were permeabilized in 1% Triton X-100 in PBS for 2 minutes, then washed three times in PBS. Sections were then equilibrated in 2X SSC supplemented with 10% formamide. Sections were incubated with Stellaris probes (0.25 μM), mouse SMI-312 anti-phospho-NF (1:1000; Biolegend 837904), Tuj1 (1:500; Biolegend 801202), and mouse RT97 (1:200; DHSB) overnight at 37°C in a humidified chamber. After hybridization, sections were washed twice for 30 minutes each time in 2X SSC supplemented with 10% formamide followed by three washes in 2X SSC. Sections were washed with 0.3% Triton X-100 in 1X PBS then incubated in donkey anti-mouse FITC secondary antibody (1:200; Jackson Immuno-Res) in 0.3% Triton X-100 in PBS with 10X blocking buffer (1:100; Roche) for 1 hour at room temperature. Sections were then washed three times for 5 minutes each time in PBS, rinsed in DEPC water, then coverslipped with Prolong Gold Antifade with DAPI (Invitrogen).

### **Immunofluorescence analysis**

Tissue sections were washed in 20mM glycine in PBS three times 10 minutes each time, washed in 0.25 M NaBH<sub>4</sub> three times 5 minutes each time, then permeabilized in 0.2% Triton X-100 in PBS. After three rinses in PBS, tissue sections were blocked in blocking buffer (10% donkey serum, 0.1% Tween-20, 20 mM glycine, PBS). Sections were incubated in rabbit nucleolin, mouse SMI-312, mouse Tuj1, and mouse RT97 primary antibodies overnight at 4°C. After three 5 minute washes in PBS, sections were incubated in donkey anti-rabbit Cy5 and anti-mouse FITC secondary antibodies (1:200 each; Jackson Immuno-Res) for 1 hour at room temperature. After three 5 minute washes in PBS, sections were rinsed in ddH<sub>2</sub>O then coverslipped with Prolong Gold Antifade with DAPI.

### **Dendra conversion time-lapse imaging**

Adult mouse DRG neurons transfected by Amaxa Nucleofector II with HA-Dendra2-GAR(WT) or with HA-Dendra2-GAR(N) expression plasmids were live imaged with the Nikon Ti-LAPP illumination system equipped with a temperature-controlled chamber, Digital Mirror Device (DMD) and an Andor EMCCD camera. Cells were grown 48 hours in complete F12 medium prior acquisition. Images were acquired in GFP, TRITC and DIC channels at X60 magnification with Nikon CFI plan apochromat 60X oil lambda objective. The photoconversion was performed in the cell body area by DAPI channel (50% of maximum led intensity for 20 seconds) using DMD module. The images were collected using GFP and TRITC channels at 10s interval for 20 minutes. DIC channel was used prior to photoconversion and in last four time points. Images were analyzed by NIS-Elements software (Nikon). A neurite segment located at a distance of 60-90  $\mu\text{m}$  from the cell body was selected for the kinetic intensity analysis.

### **Image analysis**

FISH/IF images were captured using a Leica SP8X confocal microscope with HyD detectors. For nucleolin IF, Leica SP8X with Lightning detection was used to capture images. Scrambled probes were used to set the image acquisition parameters to limit acquiring non-specific signals. For IF, the no primary antibody control was used to set the image acquisition parameters. For each nerve, xyz scans of three randomly chosen regions of interest (123.29 x 123.29  $\mu\text{m}$ ) with a z depth of 4.5  $\mu\text{m}$  (15 optical planes) were scanned with a 63X oil immersion objective (1.4 NA). The colocalization plugin in ImageJ (<https://imagej.nih.gov/ij/plugins/colocalization.html>) was used to extract the mRNA or protein signal in each optical plane that overlapped with axonal markers (NF and Tuj1). The axon only mean intensity values were normalized to the NF and Tuj1 signals in each XY plane. For analysis, the values of each optical plane were averaged for each ROI. The lowest and highest value for each animal (n = 4 or 5) was discarded. The average axon only scramble signal was subtracted from the axon only mRNA signals from each ROI average.

### **RNA-seq analysis**

RNA-sequencing libraries were prepared using the SMARTSeq v4 RNA Ultra Low Input (10 pg) + Nextera XT library kit. Libraries were indexed and sequenced by HiSeq4000 with 50 bp paired-end reads and at least 41 M reads were obtained for each sample. Quality control was performed on base qualities and nucleotide composition of sequences, mismatch rate,

mapping rate to the whole genome, repeats, chromosomes, key transcriptomic regions (exons, introns, UTRs, genes), insert sizes, AT/GC dropout, transcript coverage and GC bias to identify problems in library preparation or sequencing. Reads were aligned to the mouse mm10 reference genome (GRCm38.75) using the STAR spliced read aligner (ver. 2.4.0). Average input read counts were 66.0 M and average percentage of uniquely aligned reads were 72.6 %. Total counts of read fragments aligned to known gene regions within the mouse (mm10) ensembl (GRCm38.80) transcript reference annotation are used as the basis for quantification of gene expression. Fragment counts were derived using HTSeq program (ver. 0.6.0). Genes with minimum of 5 counts for at least one condition (all replicates) were selected and differentially expressed transcripts were determined by Bioconductor package EdgeR (ver. 3.14.0). Scripts used in the RNA sequencing analyses are available at <https://github.com/icnn/RNAseq-PIPELINE.git>. The Rank-Rank Hypergeometric Overlap (RRHO) algorithm (Plaisier *et al*, 2010) was used to determine the overlap between transcripts enriched in the nucleolin IP dataset, and transcripts depleted in the GAR+/- axons, as compared to their WT counterparts. Transcripts were sorted by directional p-values, followed by RRHO with a sliding window of 50. The overlap transcript lists were obtained for the lowest adjusted p-values. The resulting overlap transcript list was further filtered using a threshold of fold change >2 in IP over control, for both Ncl and Kif5a IPs, yielding a list of 488 mutually enriched in Ncl and Kif5a IPs and downregulated in GAR+/- vs WT dataset. These 488 transcripts were clustered using the 'hclust' function in R, using the Pearson method. Enrichment analysis was done on overlapping and unique gene lists using Ingenuity Pathway Analysis (IPA, version 42012434, QIAGEN).

### **RT-qPCR and RT-ddPCR analyses**

For RT-qPCR RNA was extracted from DRG neurons cultured in Boyden chambers as previously described (Willis & Twiss, 2011). cDNA was prepared from 500 ng of RNA, using SuperScript™ III First-Strand Synthesis System (Thermo Scientific, 18080051). Quantitative real-time PCR (qPCR) was performed using the PerfeCTa SYBR green FastMix (Quanta Biosciences) and gene-specific primers for Inpp5f and Gapdh, on the ViiA-7 system (Thermo Fisher Scientific). For RT-ddPCR RNA was isolated from DRG neurons, using RNeasy microisolation kit (QIAGEN). Fluorimetry with Ribogreen (Life Technologies) was used for RNA quantification; 20 ng of RNA were used for reverse transcription (RT) with SensiFAST

cDNA synthesis kit (Bioline) according to the manufacturer's protocol. ddPCR was performed using custom *Nci* mRNA specific primer sets (IDT; Forward primer: 5'CGGAAGAGGCGGATTTGG3'; Reverse Primer: 5'GGAAAGAATGGGATGGAAGGA3') and detected with Evagreen using a QX200TM droplet reader (Biorad).

### **Axon outgrowth analysis**

For outgrowth analysis nucleolin GAR<sup>+/-</sup> and wild-type mice, were crossed with Thy1-YFP mice (Feng *et al.*, 2000). DRG neurons from adult mice were plated on 35 mm glass bottom dishes (MatTek) coated with poly-L-lysine and laminin. After seeding, neurons were imaged every hour for 48 hours using Fluoview (FV10), a fully automated confocal laser-scanning microscope with a built-in CO<sub>2</sub> incubator, at a 60X magnification. 3x3 neighbouring sites were montaged and analyzed using ImageJ, as previously described (Perry *et al.*, 2016).

For outgrowth analysis in GAR-overexpressing neurons, cultured DRG neurons from adult C57/Bl6 mice were infected with AAV-PHP.s encoding HA-Dendra2-GAR(WT) or HA-Dendra2-GAR(N) on seeding. At 8-10 days in vitro cells were replated onto new coverslips for an additional 24h, fixed and stained for NFH and HA. Images were captured at 10x magnification on ImageXpress Micro (Molecular Devices) and infected cells were analyzed using the Metamorph software (Molecular Devices). The total outgrowth parameter was defined as the sum of all process lengths.

### **Quantification and statistical analysis**

Data shown represent mean  $\pm$  SEM, unless otherwise noted. N represents number of independent biological repeats, unless otherwise mentioned. Statistical analyses and graphs were generated using GraphPad Prism 8 software. Pairwise analyses were conducted by paired two-tailed t-test (Figs 1F, 2E, 3E, 5C) or unpaired two-tailed t-test with homoscedasticity (Figs 6B, D, F, 7E, F), depending on experimental design. Mann-Whitney non-parametric test was used for Fig. 4B and appendix Fig S4B,D since the data did not fit a Gaussian distribution. Groupwise analyses were conducted by one-way ANOVA for one factor analyses assuming equal variances (appendix Fig S3B, E, Figs 3C, H, 8C-D) or two-way ANOVA where >1 factor was analysed (Figs 1B, appendix fig S1C, 2B, 2G, 4D, appendix fig S4F, H, 9B, D). Shapiro-Wilk test was used to test for data normality. Linear binomial test was used in appendix Fig S5C. Statistically significant p-values are shown as \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and \*\*\*\*p < 0.0001.

## Data and Code Availability

1. Gene expression analysis (RNA-seq) data generated from this paper have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO series accession number: GSE142576 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142576>).
2. Scripts used in the RNA sequencing analyses are available at <https://github.com/icnn/RNAseq-PIPELINE.git>.
3. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al*, 2019) partner repository with the dataset identifier PXD020804 (<http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PX020804>).

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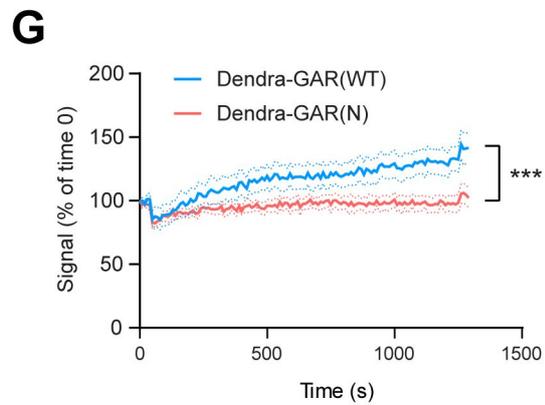
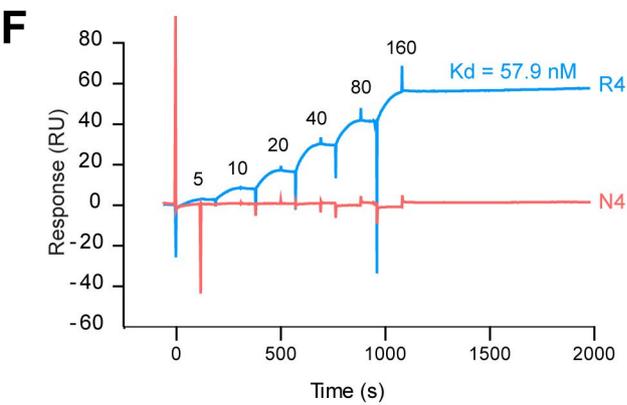
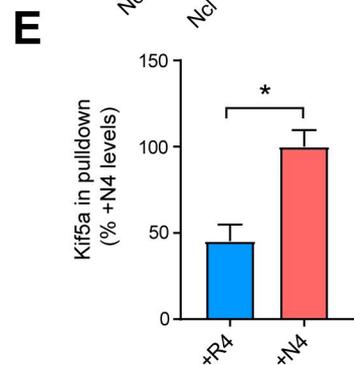
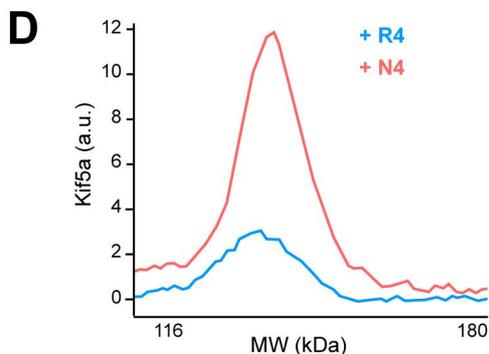
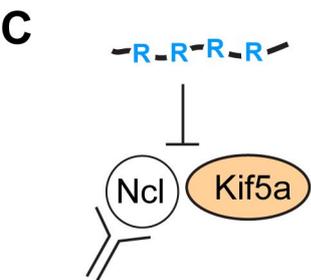
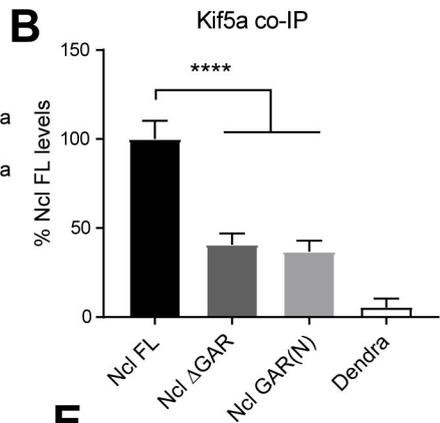
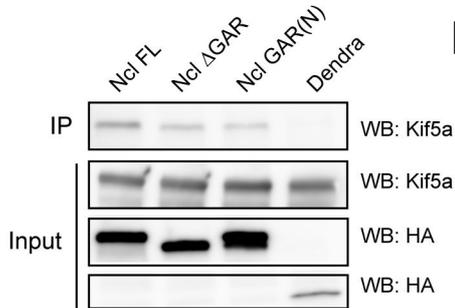
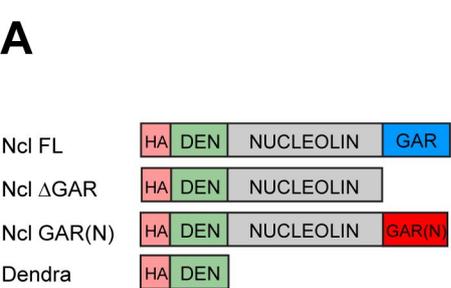
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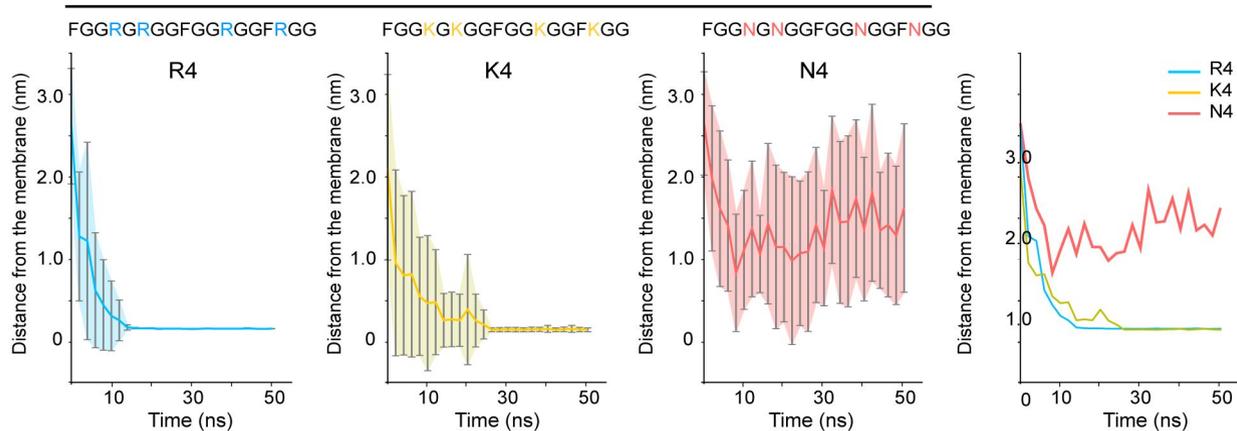
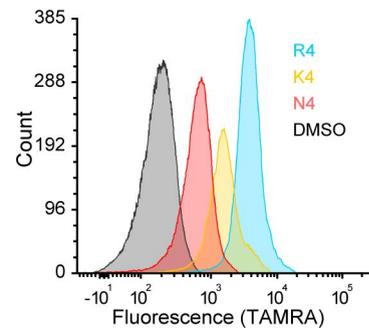
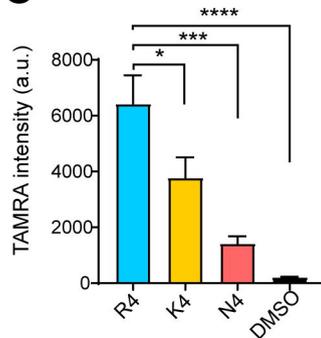
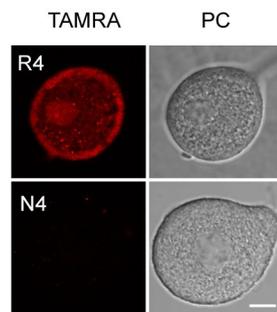
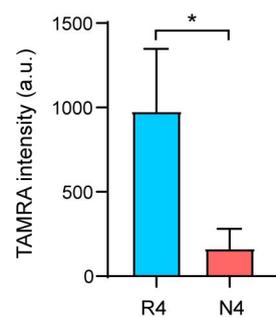
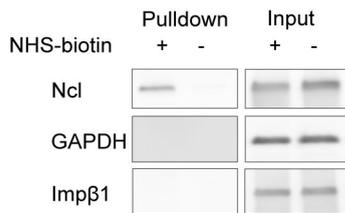
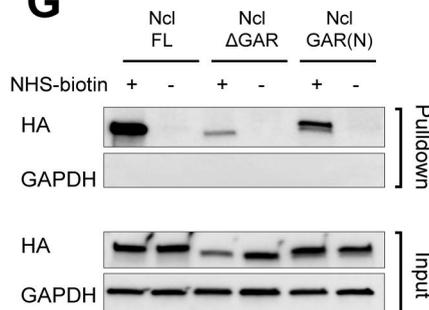
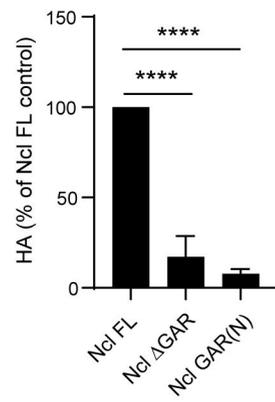
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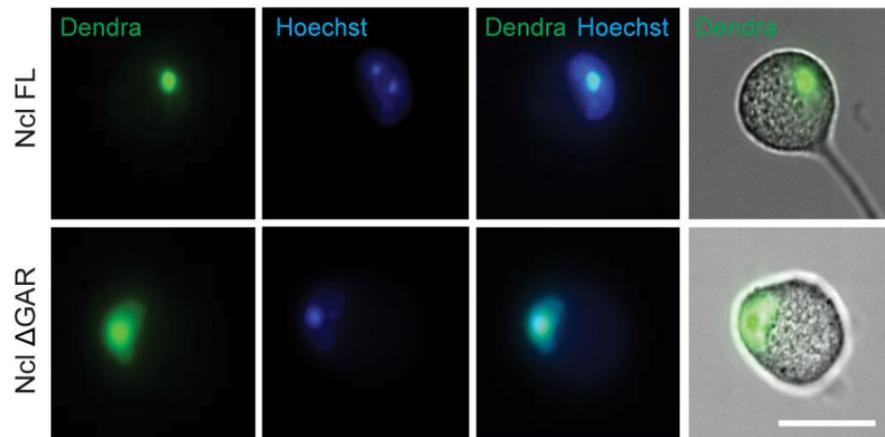
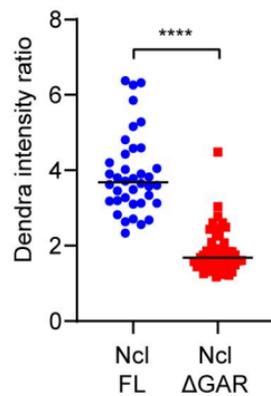
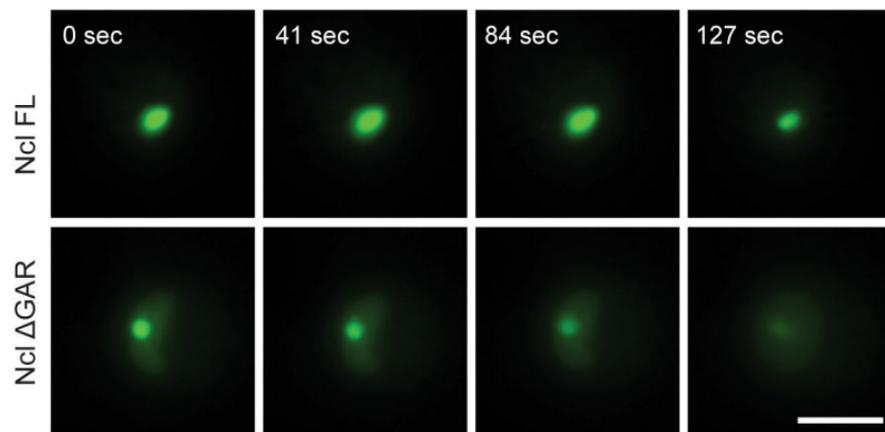
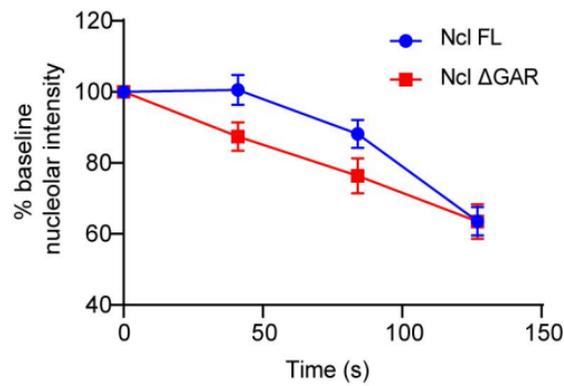


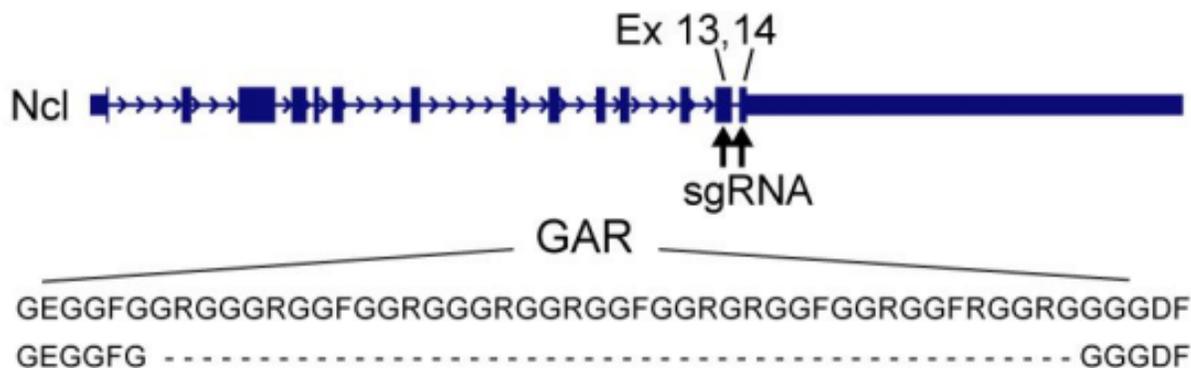
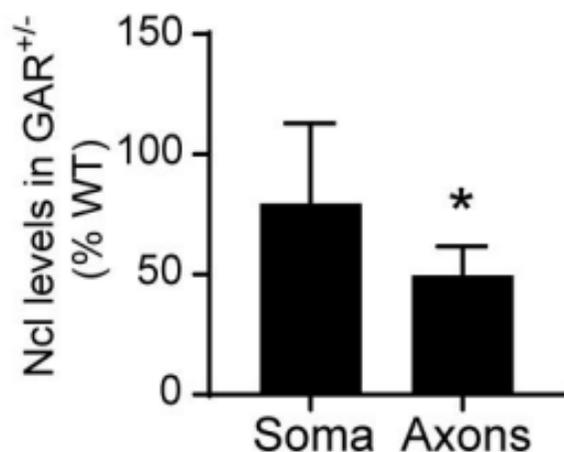


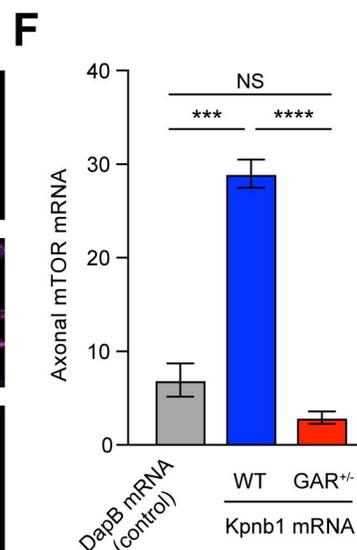
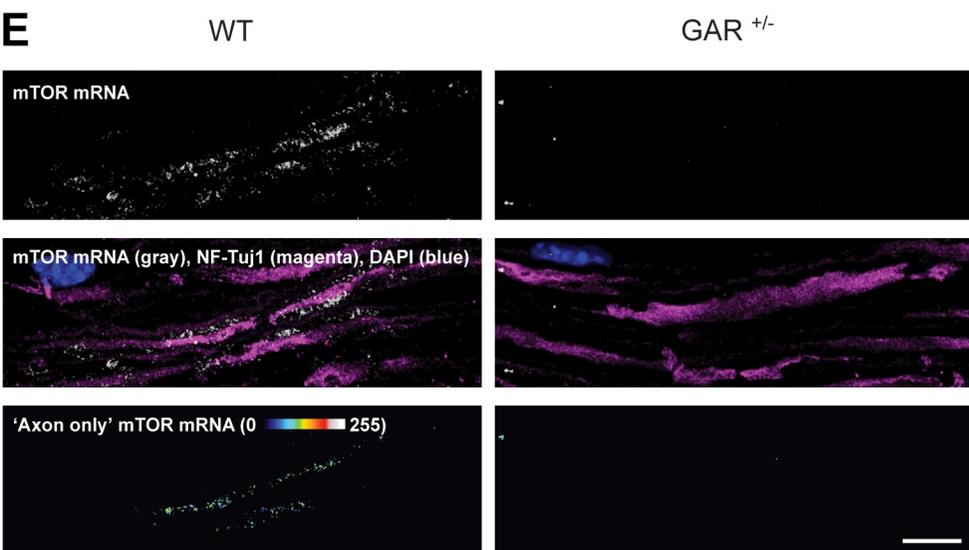
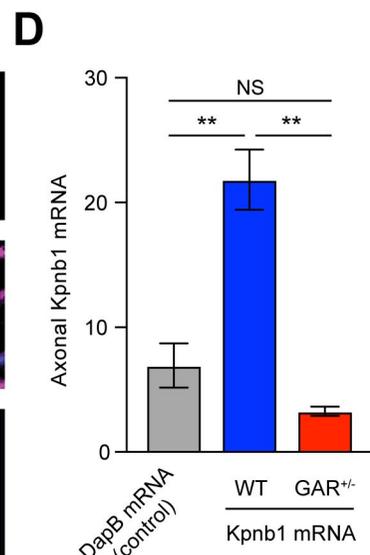
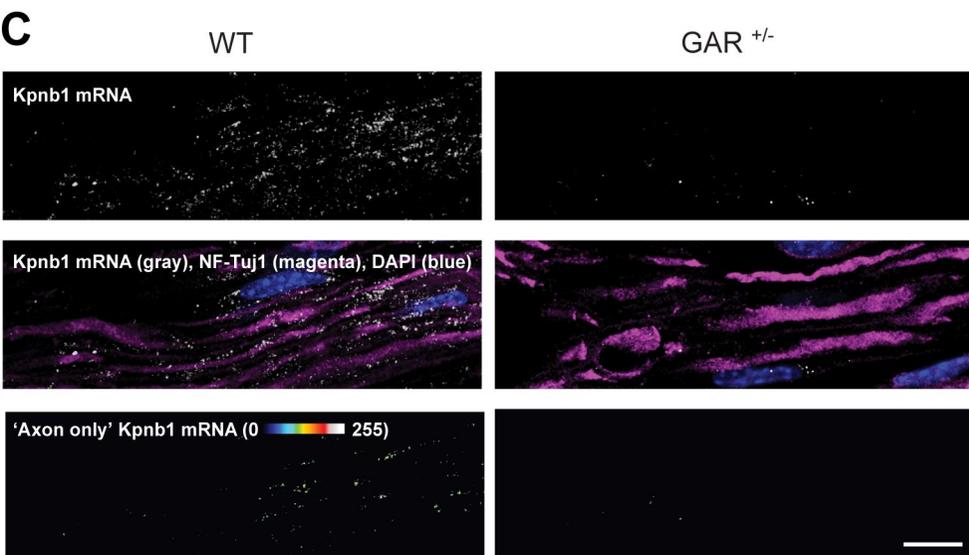
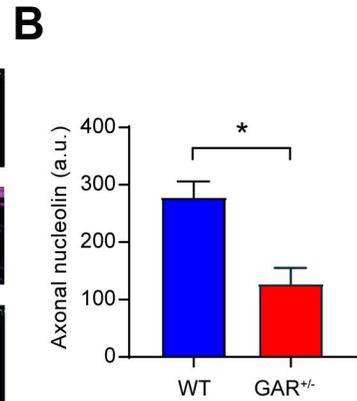
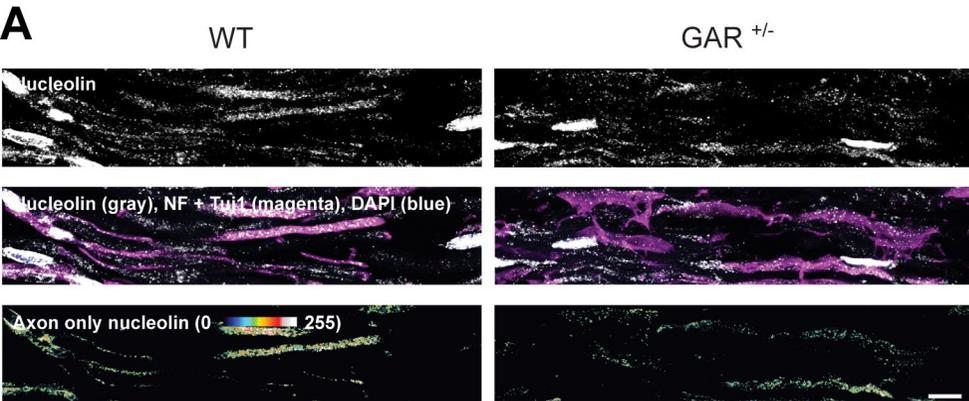
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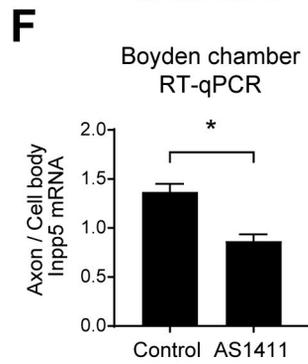
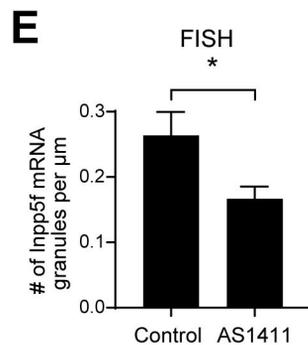
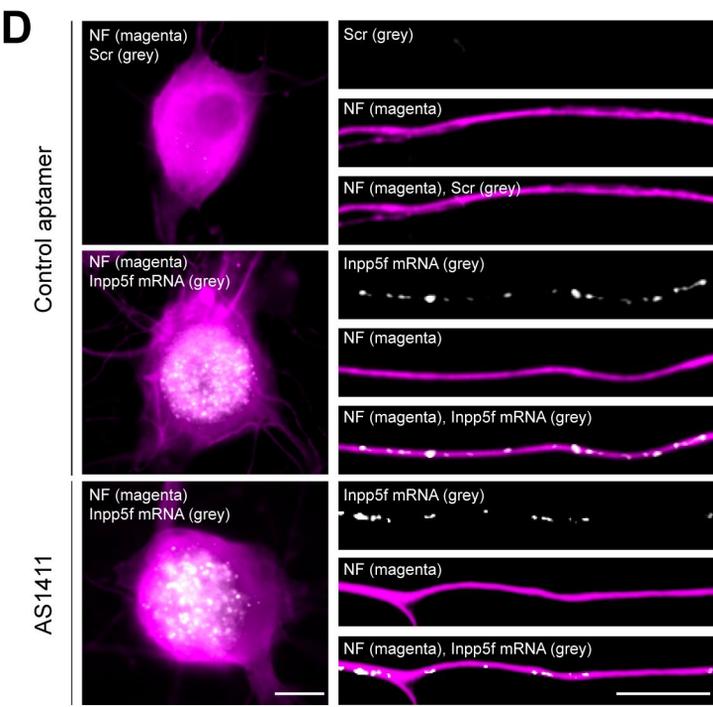
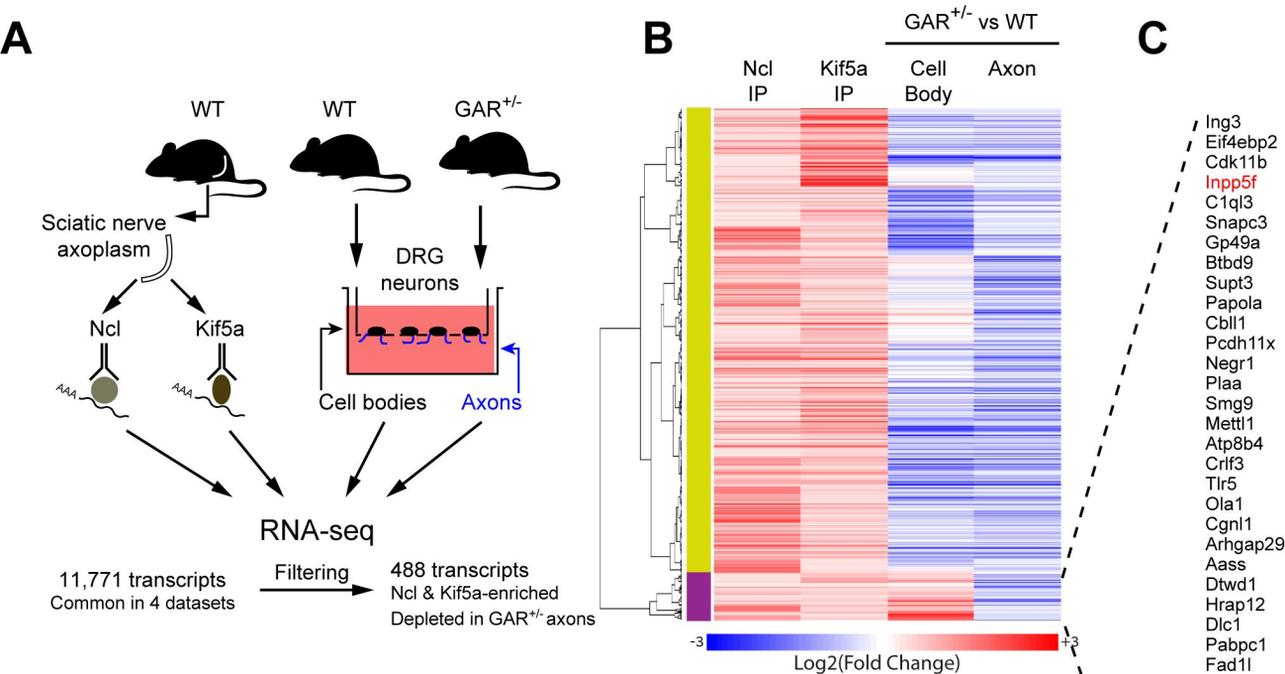
## Nucleolin GAR peptides

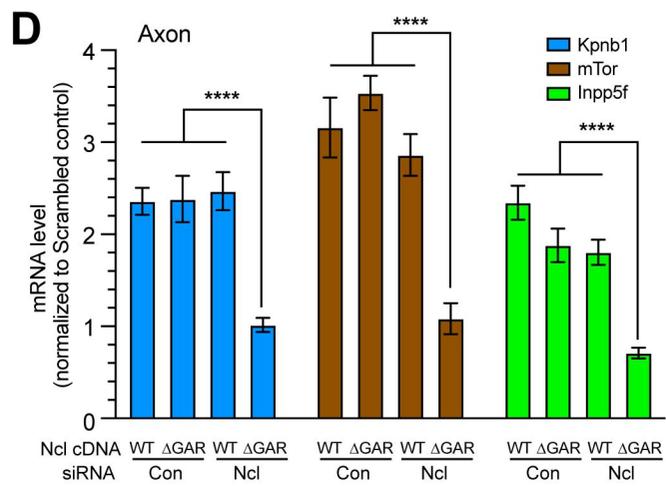
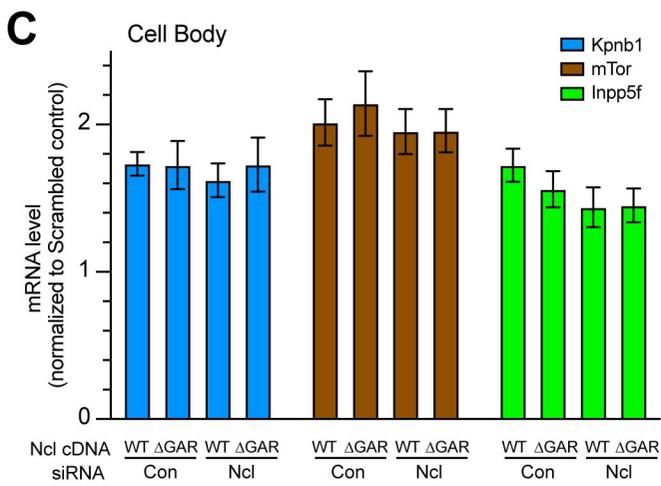
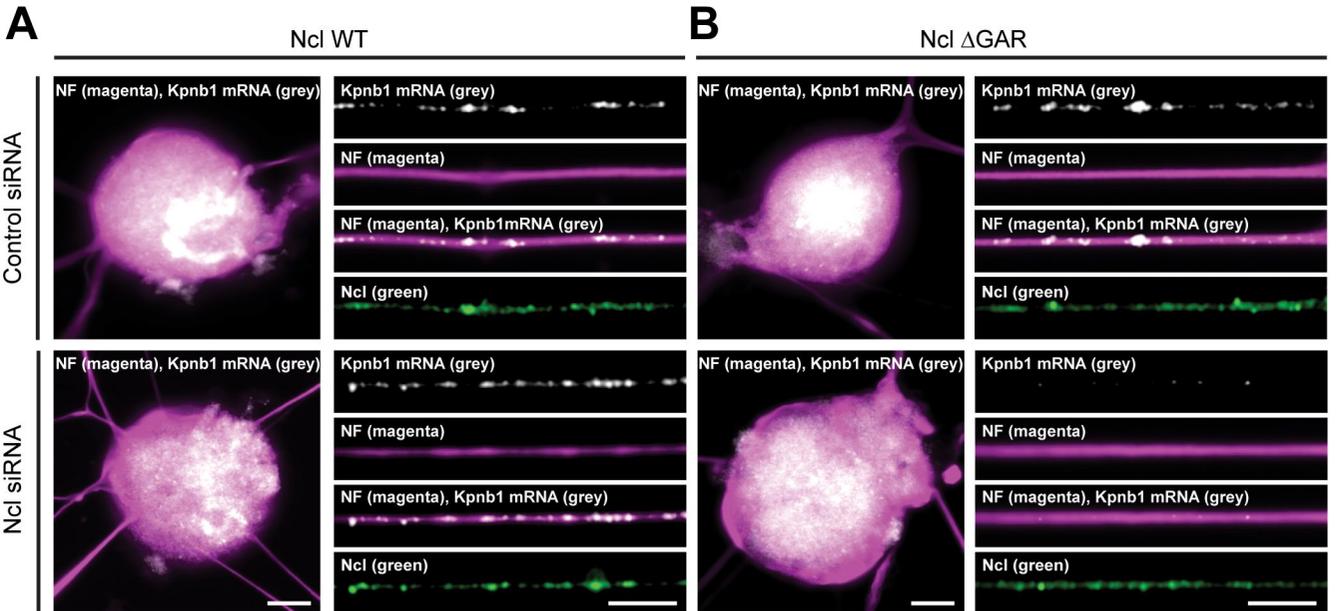
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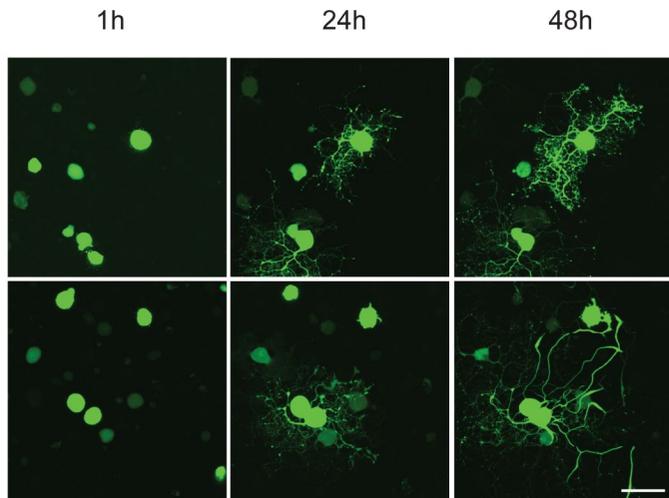
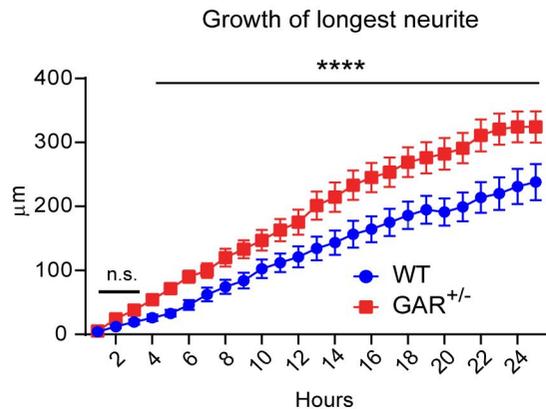
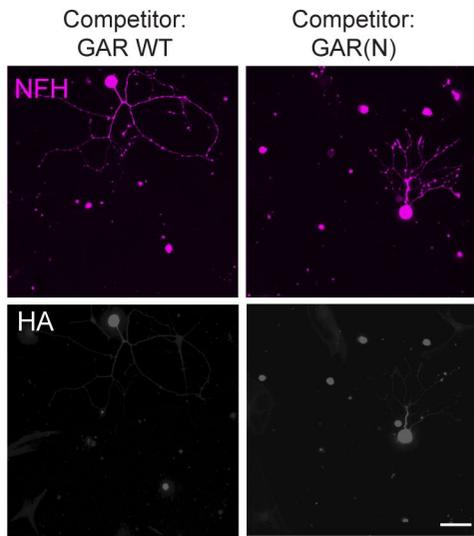
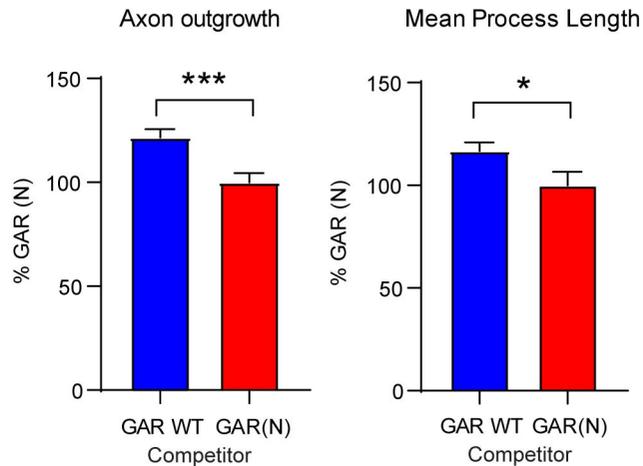
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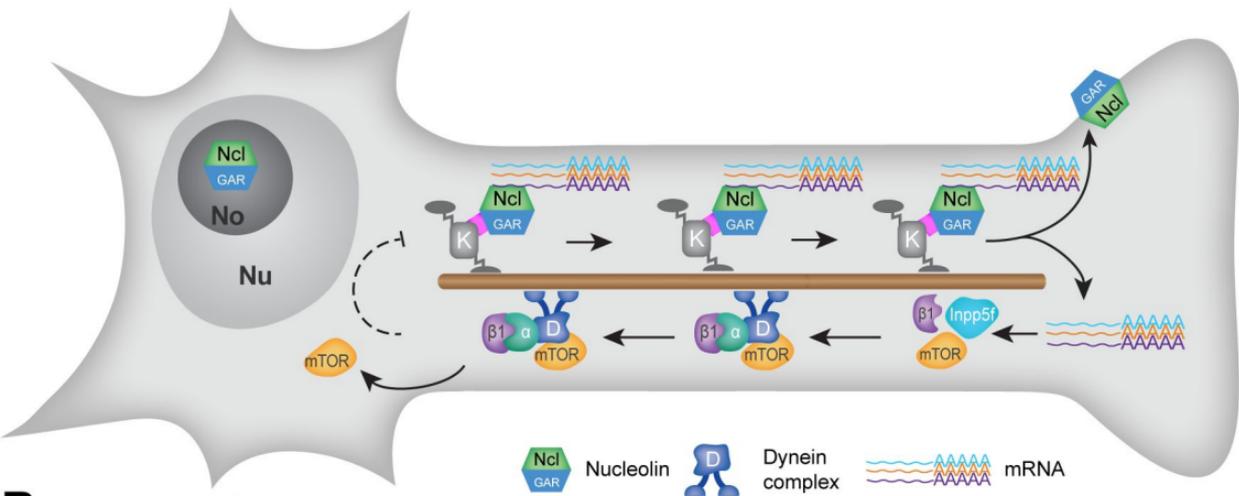
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