NETWORK ANALYSIS

Signaling to Transcription Networks in the Neuronal Retrograde Injury Response

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Retrograde signaling from axon to soma activates intrinsic regeneration mechanisms in lesioned peripheral sensory neurons; however, the links between axonal injury signaling and the cell body response are not well understood. Here, we used phosphoproteomics and microarrays to implicate ~900 phosphoproteins in retrograde injury signaling in rat sciatic nerve axons in vivo and ~4500 transcripts in the in vivo response to injury in the dorsal root ganglia. Computational analyses of these data sets identified ~400 redundant axonal signaling networks connected to 39 transcription factors implicated in the sensory neuron response to axonal injury. Experimental perturbation of individual overrepresented signaling hub proteins, including Abl, AKT, p38, and protein kinase C, affected neurite outgrowth in sensory neurons. Paradoxically, however, combined perturbation of Abl together with other hub proteins had a reduced effect relative to perturbation of individual proteins. Our data indicate that nerve injury responses are controlled by multiple regulatory components, and suggest that network redundancies provide robustness to the injury response.

INTRODUCTION

There are marked differences in the ability of different classes of neurons in the adult mammalian nervous system to regenerate after injury. Functional regeneration depends on the ability of injured neurons to integrate stimulatory and inhibitory signals from environmental cues (*1*, *2*) and to mobilize intrinsic neurite growth programs (*3*). Initiation of intrinsic repair mechanisms requires that the neuronal cell body changes growth patterns in response to an axonal injury. A well-described example of such a switch is the "conditioning lesion" phenomenon observed in peripheral sensory neurons, which switch from arborizing to elongating process growth if previously subjected to a conditioning injury in vivo (*4*, *5*). The conditioning lesion paradigm is widely used as a model to understand early events in the initiation of neuronal regeneration. Studies using this and other models have shown that information about the injury must be conveyed from the axonal lesion site to the cell body through retrograde transport mechanisms based on dynein-mediated trafficking of molecular signaling complexes (*6*, *7*). Such complexes can assemble on linkers consisting of importins (*8*, *9*), cytoskeletal components (*10*), or kinase signaling scaffolds (*11*, *12*), which allow trafficking of various phosphoprotein signals (*13*). For example, a number of mitogen-activated protein kinases (MAPKs) are involved in regeneration and repair mechanisms in affected neurons from various species (*14*–*17*), and MAPK family members are retrogradely transported after peripheral nerve injury (*10*, *18*–*20*).

Retrograde signals induce transcriptional reprogramming, thereby eliciting the regeneration response. Changes in the cell body that occur in response to retrograde injury signaling may include increased production of various molecules, including transcription factors (TFs), cytoskeletal proteins, cell adhesion and axon guidance molecules, and trophic factors and their receptors (*3*). A principal goal in the field has been to identify genes and proteins that regulate the outgrowth and regeneration of axons. Recent work has shown that the cell body response to injury can be reactivated by additional lesions weeks to months after the original injury (*21*, *22*), giving hope that detailed understanding of this process might lead to clinical approaches applicable for both newly injured and long-term patients. Thus, identification of the main molecular determinants of the injury response is of both basic and potential clinical interest. Straightforward comparisons of injured versus uninjured neurons in differential gene screens are complicated by the fact that it is difficult to identify the genes responsible for regeneration in the context of the many transcriptional events induced by the stress of injury or by the changed connectivity and signaling of affected cells. Furthermore, in vivo screens are necessarily carried out at the tissue level, hence on heterogeneous mixtures of responsive and nonresponsive cells (*23*). Consequently, screens of differential gene expression after nerve injury have mostly resulted in long lists of genes encoding proteins associated with regeneration, without a clear identification of genes that encode proteins that might induce regeneration (*3*).

The difficulty of teasing out the effects of individual genes and proteins from the massive changes that occur after neuronal injury has led to the hope that it may be possible to identify "master regulators" that coordinate the regenerative program in neurons. The hypothesis underlying these efforts is that a relatively small complement of TFs or proteins that function as critical nodes in signaling or transcription networks (or both) might control much of the functional response to nerve lesions. Although a number of candidate regulators have been suggested (*24*–*31*), thus far

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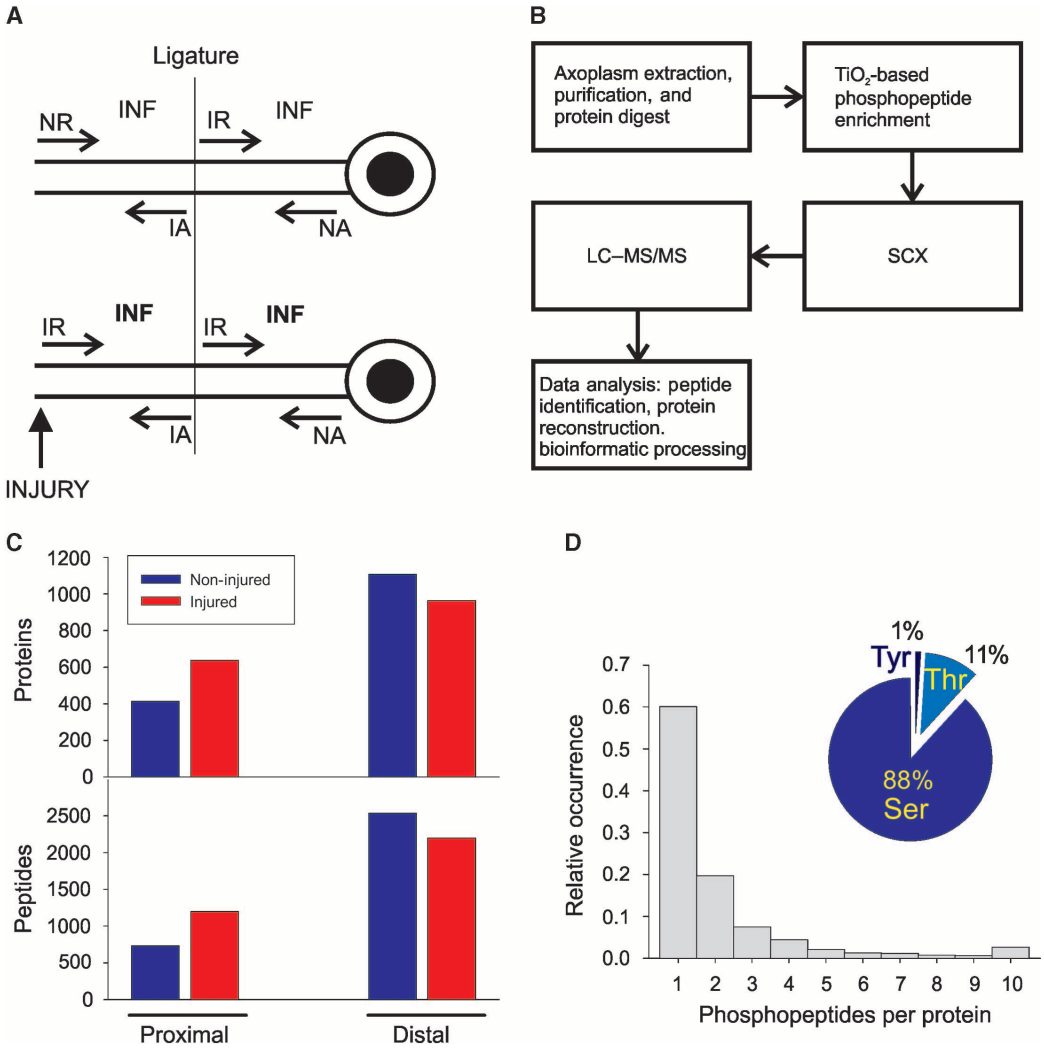

Fig. 1. Phosphopeptide enrichment scheme and overview of retrograde injury phosphoproteome. (**A**) Schematic of experimental lesion-ligation model using rat sciatic nerve. NR, noninjured axon, retrograde signal; IR, injured axon, retrograde signal; NA, noninjured axon, anterograde signal; IA, injured axon, anterograde signal; INF, inflammatory factor (potential contaminants from exudate). The relative INF contribution may change upon injury. (**B**) Preparation and enrichment scheme for phosphopeptides extracted from sciatic nerve axoplasm. LC–MS/MS, liquid chromatography–tandem mass spectrometry; SCX, strong cation exchange chromatography. (**C**) Number of phosphopeptides (lower panel) and corresponding phosphoproteins (upper panel) in each experimental group. Samples were generated by TiO_2-based enrichment with subsequent SCX fractionation, starting from 6 mg of protein per group. (**D**) Numbers of phosphopeptides per phosphoprotein. Pie chart inset shows relative distribution of the assigned phosphorylation sites. Additional supporting information is provided in fig. S1 and phosphoprotein lists in table S1.

there has been no truly comprehensive attempt to delineate the combined signaling and transcriptional networks governing the neuronal response to injury. Here, we investigated the response to lesion in peripheral sensory neurons—a well-established model for studies of neuronal development, growth, and injury (*6*, *32*)—to define networks linking axonal signaling to transcription in the cell body. Neuronal cell bodies in the L4 and L5 lumbar dorsal root ganglia (DRG) extend sensory axons to the periphery through the sciatic nerve. We combined analysis of the sciatic nerve axonal phosphoproteome with that of the L4 and L5 DRG transcriptome to identify transcriptional signatures enriched in injury-regulated genes and construct signaling networks connected to TFs identified from the transcriptome data. These combined signaling to transcription networks enabled us to identify candidate proteins involved in regulating the initiation of neurite regeneration.

RESULTS

Phosphoproteomics of axonal retrograde injury signaling in the sciatic nerve

We used an established and validated lesion-ligation model (*33*) to analyze the phosphoprotein composition of axoplasm accumulated at ligatures 24 hours after crush injury of rat sciatic nerve. The nerve was crushed 4 to 5 cm from the L4 and L5 DRG, and the ligation was placed ~2 cm proximal to the crush site. Comparison of ligature-enriched protein ensembles from uninjured and injured nerve allowed discrimination of four experimental groups: proximal and distal noninjured (NIP and NID, respectively) and proximal and distal injured (INP and IND, respectively). Each of these four groups is characterized by a distinct combination of injured versus uninjured anterograde and retrograde signals (Fig. 1A) [see (*33*)]. Phosphoproteins from these four groups were enriched and fractionated and then analyzed by tandem mass spectrometry (Fig. 1B and fig. S1). The analyses revealed ~5500 phosphopeptides altogether, constituting ~60% of all identified peptides and corresponding to a total of 1447 proteins (Fig. 1C), some of which are shared between groups. About 80% of the proteins were represented by one or two phosphopeptides, with the remainder showing up to 10 phosphopeptides per protein (Fig. 1D). Serine was by far the most prominent phosphorylation site represented in the data set, followed by threonine, with a small number of tyrosine phosphorylations (Fig. 1D and fig. S1).

This experimental model enables the identification of changes in protein ensembles undergoing retrograde transport after nerve injury through a comparison of distal ligation samples (*33*). Comparison of phosphopeptides in IND versus NID samples revealed 825 unique phosphorylations on 685 proteins, 417 of which were in NID and 268 in IND. Of these

differentially phosphorylated proteins, 164 contained two or more phosphorylation sites, and ~40% of the proteins in this group were phosphorylated on different sites in the injured and noninjured samples. Overall, ~40% of the assigned phosphorylation sites have been previously reported or could be inferred by similarity to records in databases, whereas the remaining ~60% had not previously been reported. A comprehensive list of the phosphoproteins and phosphorylation sites used for further analyses is shown in table S1. Gene Ontology (GO) analyses of phosphoproteins with mutually exclusive phosphorylation sites in the injured and noninjured groups showed the highest enrichment for proteins in the signal transduction, guanosine triphosphatase activity, microtubule-related transport, and metabolism GO subcategories (table S1).

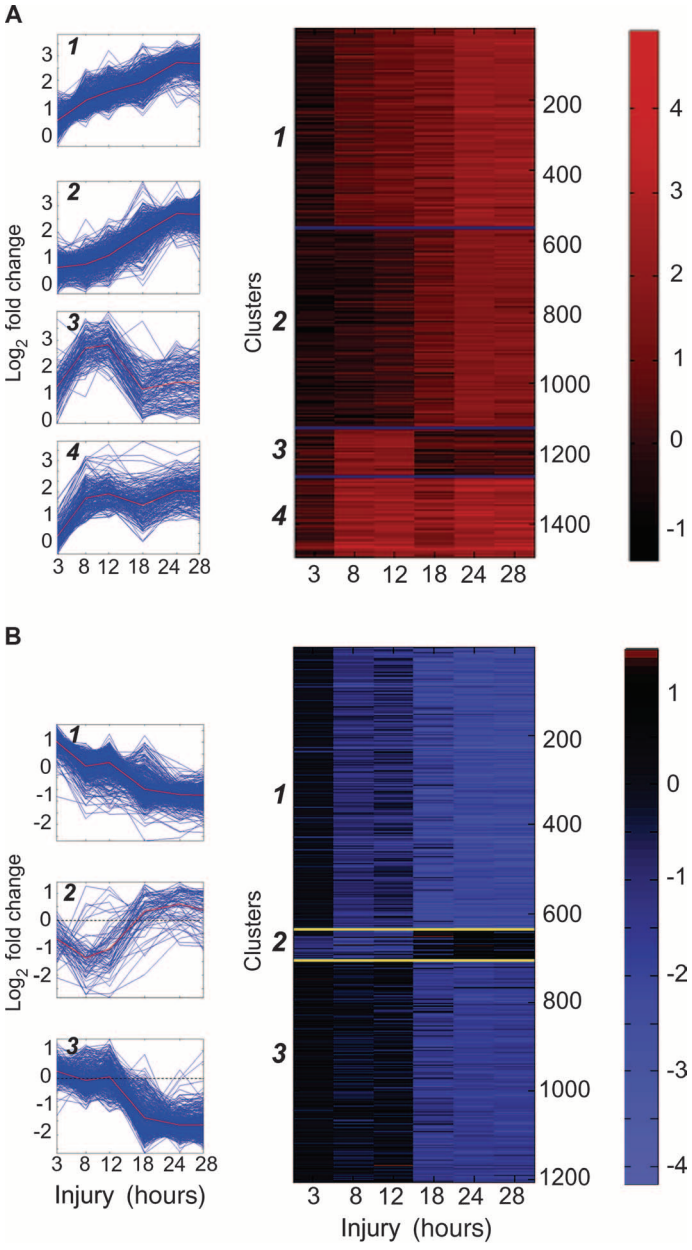

Fig. 2. Profiling of gene expression in DRG after sciatic nerve injury. (A) Heat map representation of *K*-means clustering of genes up-regulated in L4 and L5 DRG after sciatic nerve injury that passed ANOVA and FDR criteria as described in Materials and Methods. Plots of the temporal expression profiles for the four main up-regulated gene clusters are shown on the left. (B) Heat map representation of *K*-means clustering of genes down-regulated in L4 and L5 DRG after sciatic nerve injury that passed ANOVA and FDR criteria as described in Materials and Methods. Plots of the temporal expression profiles for the three main down-regulated gene clusters are shown on the left. Regulated gene lists are provided in table S2.

Transcriptomics of cell body responses in the DRG

We performed microarray analyses on adult rat L4 and L5 DRG after sciatic nerve lesion. In contrast to most previous differential gene expression studies of nerve injury, we examined a time frame of hours after injury to focus on transcriptional events that are a direct consequence of retrograde signals elicited by the lesion. We used Affymetrix Rat 230.2 arrays containing 31,100 probe sets to screen changes in gene expression in L4 and L5 DRG over a time course of 1 to 28 hours after a crush lesion of the sciatic nerve ~4 cm from the DRG. Array data were preprocessed and normalized, and differential expression was determined by analysis of variance (ANOVA) and false discovery rate (FDR), followed by *K*-means clustering. The 1-hour time point did not reveal significant differences in DRG gene expression relative to sham-operated controls and was subsequently used as a reference for comparison of gene expression over the course of the analysis. Analyses using *P* values up to 0.01 or FDR cutoffs below 15%, or both, showed that a widespread transcriptional response commenced 8 to 12 hours after injury, with the most robust responses apparent at 18 to 28 hours. This time lag might indicate a requirement for the arrival of dynein-driven retrograde signals to initiate the main transcriptional response. Heat maps of changes in gene expression that passed ANOVA and FDR tests at $q = 0.001$ are shown in Fig. 2. We subdivided the 1490 genes showing increased expression in response to injury into four clusters based on the kinetics of their up-regulation (Fig. 2A) and grouped the 1210 genes showing decreased expression into three clusters based on the kinetics of expression over the time course we evaluated (Fig. 2B). The corresponding gene lists are shown in table S2, and the annotated portions of these lists (~60% of the data sets) were used as the transcriptome input for subsequent computational analyses. Because of the high sensitivity of the ANOVA test, we chose a strict cutoff in *q* to avoid identification of a large number of false-positive genes. Thus, although the identified clusters contain the genes showing the most significant changes in expression, the total number of responding genes may actually be larger.

TF-binding site analyses

We began our identification of regulatory components in the system by analyzing the promoter regions of co-regulated genes in the identified clusters to investigate the possible involvement of specific TFs in response to injury. Querying TF-binding site (TFBS) databases with the promoter sequences of co-regulated genes allows the identification and prioritization of TFs as candidate control nodes in the ensemble of transcriptional interactions occurring in a given system, known as the transcriptional networks (*34*, *35*). The most widely used approach to reveal TFBS in the promoter regions of coexpressed genes is based on positional weight matrices (PWMs) constructed from collections of known binding sites for a given TF or TF family. TRANSFAC is the largest available collection of TFBSs and corresponding PWMs and has been used for analyses of regulatory regions in diverse gene classes (*35*, *36*). In addition to the ANOVA analysis described above, we also generated sets of the genes showing the greatest difference in expression (induced or re-

pressed) between injured and uninjured nerve for each individual time point by using two-sided *t* tests to compare replicates from each time point to the reference time point replicates, and FDR to control for multiple hypotheses. We scanned the gene sets thus obtained for overrepresentation of all available TFBS matrices curated in TRANSFAC 9 by comparison to equivalently sized control sets of nonresponding genes. This analysis revealed ~100 TFBS matrices that were significantly overrepresented in gene sets differentially regulated by injury. Because many of the matrices were highly redundant, these 100 TFBSs correspond to 453 TF candidates from 118 different families (fig. S2).

Intersecting signaling and transcriptional networks

We used the TRANSPATH database (*37*) as implemented in ExPlain to construct signaling networks consistent with the axoplasm phosphoproteomic data. TRANSPATH-generated networks such as that shown in Fig. 3A could be found for ~200 of the retrogradely transported phos-

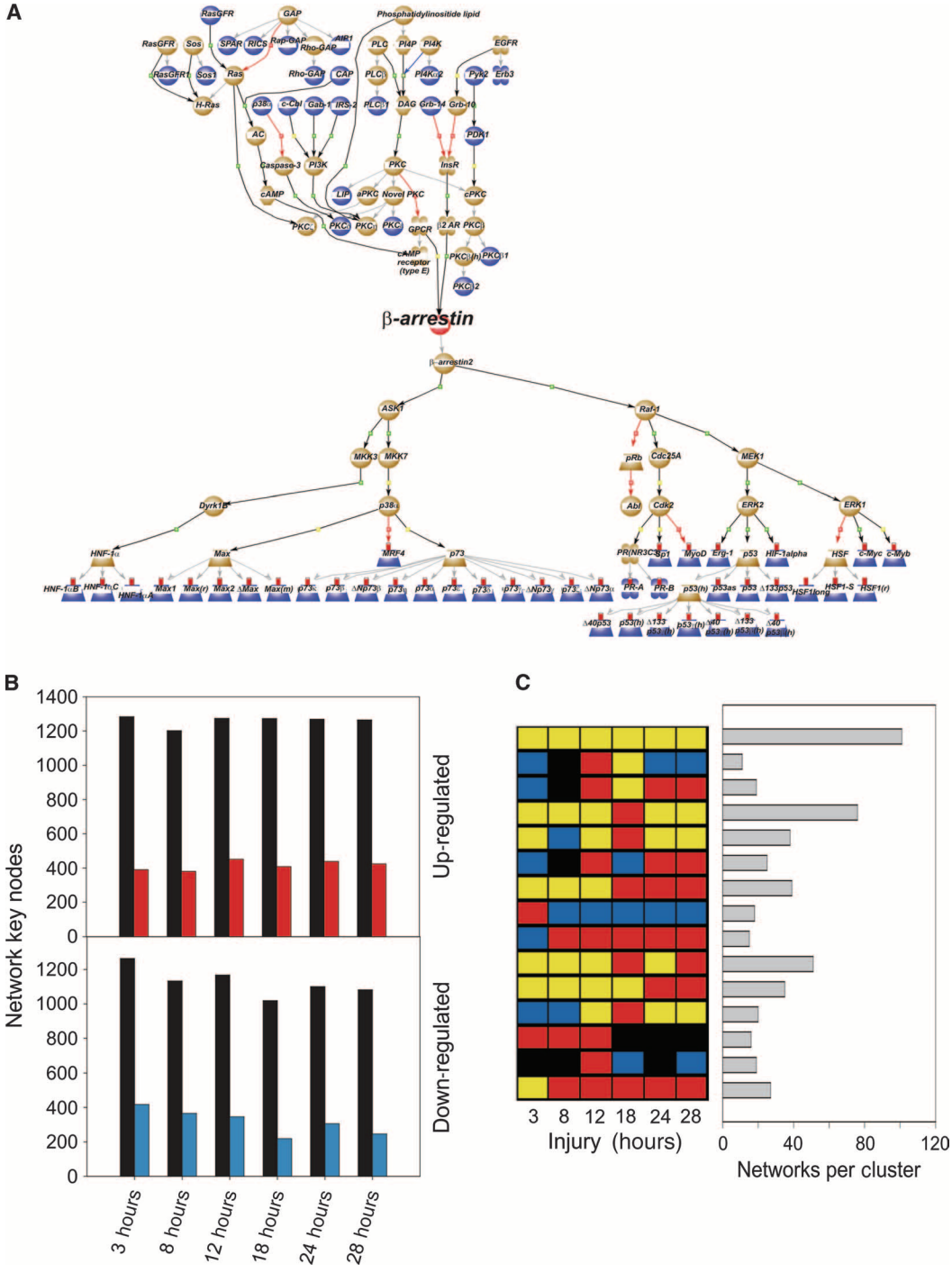

Fig. 3. Linking axonal phosphoproteomes to cell body transcriptomes. Overlap analyses linking cell signaling networks based on phosphoproteins correlated with retrograde injury with activation or repression pathways for TFs implicated in the ganglia response. (**A**) A representative network. Blue depicts proteins identified in the sciatic nerve phosphoprotein data set and TFs implicated from TFBS enriched in the microarray data. Key to shapes of the different elements and interactions is given in fig. S3. Red ellipse indicates a key node of the network. (**B**) Total number (black bars) of predicted signaling networks at indicated times after injury compared to numbers of networks identified by overlapping phosphoproteome and transcriptome data for transcriptional activation (red bars) or decreased transcription (blue bars). Networks were generated in ExPlain software 2.3 with restriction parameters as described in Materials and Methods. (**C**) Numbers of signaling networks (right) feeding into different groupings of TFs with indication of their net effects on gene expression (left panel; red, increased; blue, decreased; yellow, mixed; black, no effect) at the indicated time points after injury. Supporting information for this figure is provided in table S3 and fig. S3.

phoproteins in our data sets. The redundancy and extensive cross talk characteristic of signal transduction networks is reflected in the fact that >1800 networks passed statistical significance filters for ~200 phosphoproteins (Fig. 3B). At this stage of the analysis, we juxtaposed the axonal signaling networks and ganglia gene regulation data sets. This intersection reduced the number of candidate networks to ~500, covering a wide range of signaling pathways (Fig. 3B, fig. S3, and table S3). The analyses revealed that multiple networks are implicated in the ganglionic transcriptional response at any given time point (Fig. 3C and table S4). The multiplicity of gene regulation events associated with each network at the different time points does not permit assignment of specific networks solely to increases or decreases in overall gene expression in this system.

The intersecting signaling and transcription data sets reduced the number of statistically significant candidate TFs from 453 (fig. S2) to 39 (Fig. 4), corresponding to 26 TF families with 44 TFBS matrices. Time-dependent clustering of these signaling network–associated TFs showed that nearly all of them are downstream of multiple signaling networks (Fig. 4). Two TF clusters showed a high correlation with changes in gene expression at later time points (18 to 28 hours after injury); these clusters included TFs in the STAT (signal transducer and activator of transcription), HNF (hepatocyte nuclear factor), USF (upstream stimulatory factor), Jun, Smad, SRF (serum response factor), and ER-α (estrogen receptor–α) families. Each network cascade involved multiple TFs, ranging from a handful per network to >30. Overall, the intersecting signaling to transcription networks covered ~28% of the retrogradely transported phosphoprotein data and ~41% of the gene regulation data. Thus, cross-referencing the two data sets enabled us to focus on the proteins, genes, and pathways most likely to be relevant to the neuronal injury response.

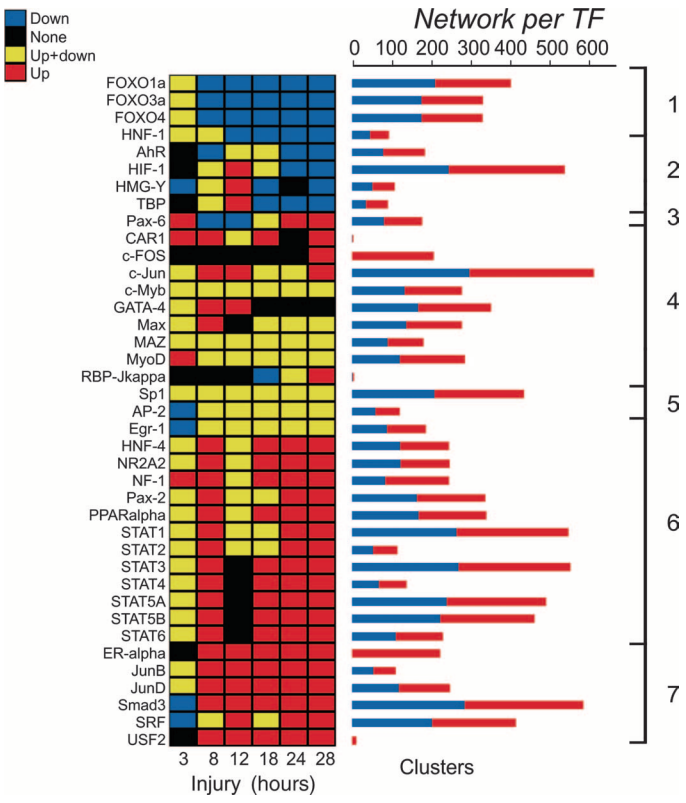

Fig. 4. TF involvement in signaling to transcription networks. TFs associated with key node–based networks at $P < 0.05$ at different time points after injury are depicted together with the net effect on gene expression on the left (red, up; green, down; yellow, mixed; black, no effect). The number of networks impinging on each TF is depicted on the right. TFs were clustered according to gene expression patterns at each time point and network linkages. Supporting information is presented in table S4.

Candidate hub proteins in signaling networks activated by injury

The high interconnectivity (Fig. 3) and redundancy in TF targeting (Fig. 4) by the signaling networks suggest that the system should be resistant to perturbations of single components. However, a closer examination of phosphoprotein representation in the different networks showed multiple appearances of various proteins in many of the networks (Fig. 5A). A number of these overrepresented proteins have already been implicated in nerve regeneration (*3*), although most have not been mechanistically linked to retrograde injury signaling (*6*, *7*). Proteins such as c-Abl, PDK1 (phosphoinositide-dependent protein kinase 1), different PKC (protein kinase C) isoforms, p38 (a MAPK), and others appear in >80% of the networks implicated in up-regulation (Fig. 5B) or down-regulation (Fig. 5C) of gene expression in DRG after sciatic nerve injury. Removal of these proteins in silico caused substantial reductions in the size and connectivity of the reconstructed networks, raising the possibility that such multiply connected proteins, hereby designated "hub proteins," might be critical for the system and that their perturbation might have far-reaching effects.

We therefore selected a few of the most highly represented hub proteins for targeting in a functional screen, focusing on candidates positioned to affect pathways upstream of multiple TFs (Fig. 6A). Many kinase inhibitors are nonspecific, and even those thought to be highly specific can have widespread secondary and tertiary effects (*38*). We therefore limited this screen to those kinases for which we could obtain well-characterized small-molecule inhibitors or activators. We applied these small molecules to freshly dissociated and triturated adult mouse DRG neurons for 3 hours, after which we removed the compounds by washing and monitored neuronal morphology and outgrowth parameters on a substrate permissive for outgrowth over a period of 72 hours (Fig. 6B). Such transient applications of perturbing agents were intended to induce a specific perturbation of the retrograde injury signal elicited by the triturating lesion without interfering with later signaling events or with neurite outgrowth mechanisms per se (*9*). Figure S4 shows a comparison of the neuronal outgrowth and branching response of neurons cultured with this protocol and neurons from animals that underwent a prior conditional crush lesion of the sciatic nerve in vivo. To ascertain the effectiveness of this transient drug application approach, we used the pan–serine-threonine kinase inhibitor staurosporine as a positive control. Indeed, staurosporine caused significant dose-dependent inhibition of neurite length (Fig. 6C) with a concomitant increase in branching frequency (fig. S4C) without affecting cell viability. Because long-term application of staurosporine is highly toxic, the specific effects observed with transient application are consistent with a temporally restricted perturbation of signaling events.

Robustness in retrograde injury signaling

Having demonstrated that it is possible to reduce neurite outgrowth by transient blockade of injury-induced phosphorylation, we examined whether transient perturbations might also enhance outgrowth. The

network analyses indicated that several isoforms of PKC are likely to be involved in injury-regulated gene expression, influencing at least 28 TFs (Fig. 6A), leading us to test the effects of the PKCα and PKCβ inhibitor bis(4-fluoroanilino)-phthalimide. This inhibitor increased overall neurite outgrowth, which reached four times that of controls after 48 hours in culture (Fig. 6D). In contrast, the general PKC activator PMA (phorbol 12-myristate 13-acetate) caused a factor of ~2 decrease in total neurite outgrowth at 48 hours after plating. Thus, transient pharmacological perturbations concomitant with injury can induce both decreases and increases in the neurite outgrowth capacity of DRG sensory neurons. However, attempts to modulate specific PKC isoforms indirectly through inhibition of diacylglycerol kinase pathways caused relatively minor effects.

We then tested the effects of inhibitors of Abl (Abelson proto-oncogene), AKT, and p38, all hub proteins highly overrepresented in signaling networks (Fig. 5) upstream of multiple TFs (Fig. 6A). Abl, a tyrosine kinase found in nearly all the different signaling networks, also acts as a key node in its own network. The net effect of the different phosphoproteins upstream of Abl should lead to Abl activation after injury (table S3), and the network analyses suggest that Abl activation should affect 25 TFs: activating 12, inhibiting another 12, and disinhibiting one (p53) (Fig. 6A). Application of imatinib, which specifically inhibits Abl through an interaction with its tyrosine kinase domain (*39*), within the 3-hour window after trituration reduced neurite outgrowth significantly at the 24-hour time point, with a less pronounced effect at later time points (Fig. 6E). Branching frequency increased concomitantly with inhibition of neurite outgrowth (fig. S4C).

AKT, another kinase represented in nearly all the networks (Fig. 5), acts as a prominent downstream factor in the insulin receptor substrate 2 (IRS2) network. The phosphoprotein network analyses predicted that AKT should also be activated by injury and in turn should affect the activity of 33 TFs: inhibiting 15, activating 5, disinhibiting 10, and having

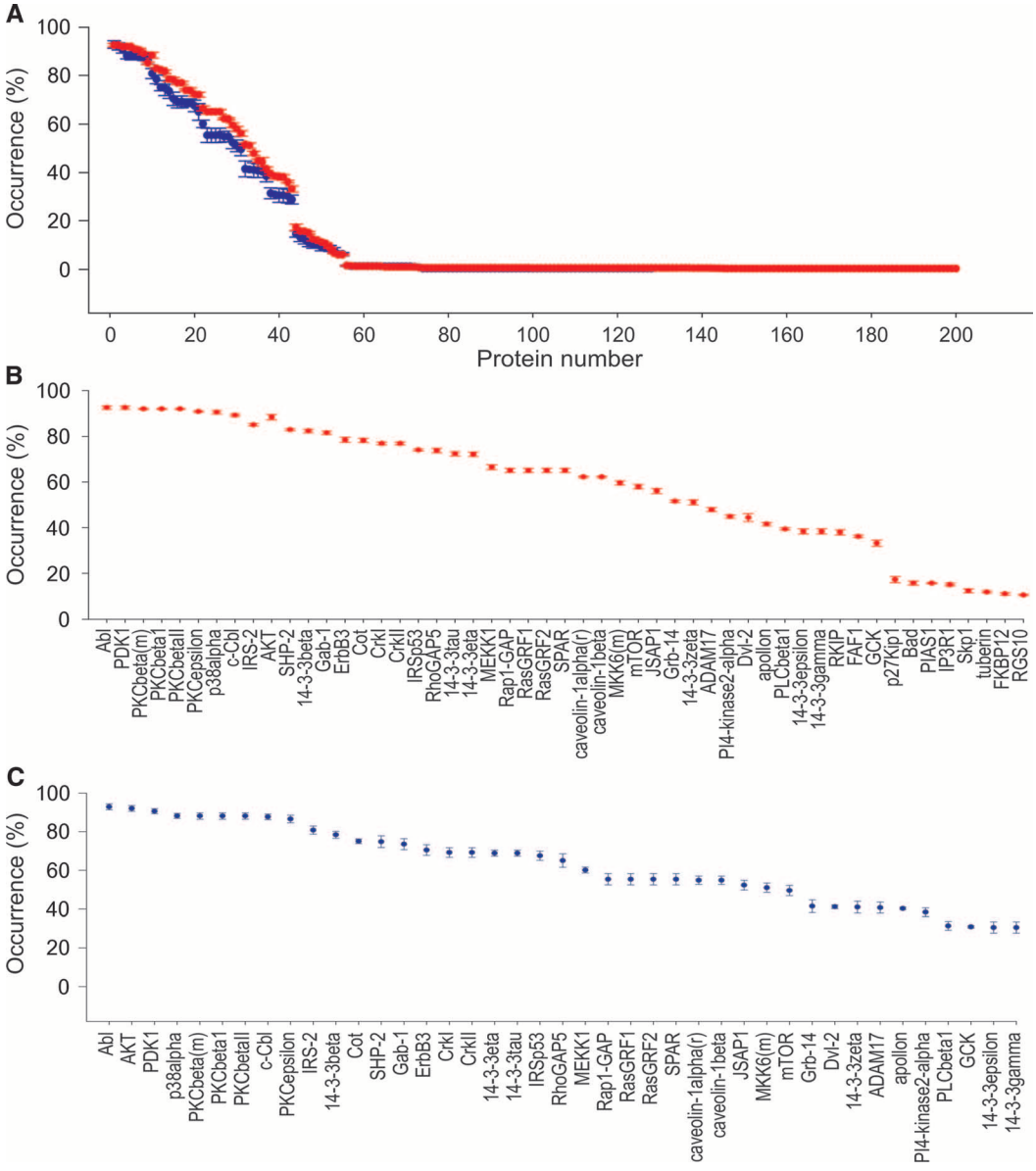

Fig. 5. Hub proteins involved in multiple networks. (**A**) Occurrence of identified phosphoproteins in signaling networks implicated in up-regulation (red) or down-regulation (blue) of gene expression. (**B** and **C**) Note the relative overrepresentation of ~50 proteins, detailed for up-regulation networks in (B) and for down-regulation networks in (C).

mixed effects on 3 (Fig. 6A). LY-294002, which was used to specifically inhibit AKT, caused a marked decrease in total neurite outgrowth by 72 hours after plating (Fig. 6E) without a parallel effect on branching frequency (fig. S4C).

Analysis of p38 key node–related networks showed that activation of p38 should activate 10 TFs, inhibit 14, disinhibit 5, and have mixed effects on different isoforms of CREB (cyclic adenosine 5′-monophosphate response element–binding protein) (Fig. 6A). Inhibition of p38 caused a decrease in total neurite outgrowth (Fig. 6E), along with an increase in neurite branching frequency (fig. S4C), at 24 hours after plating. However, the effects of p38 inhibition were transient and did not persist at later times (Fig. 6E).

The effects of the individual compounds on neurite length lead us to examine the consequences of combining them, thereby simultaneously inhibiting two different hub kinases in the 3 hours immediately after injury. Combinations of Abl and AKT inhibitors at various concentrations

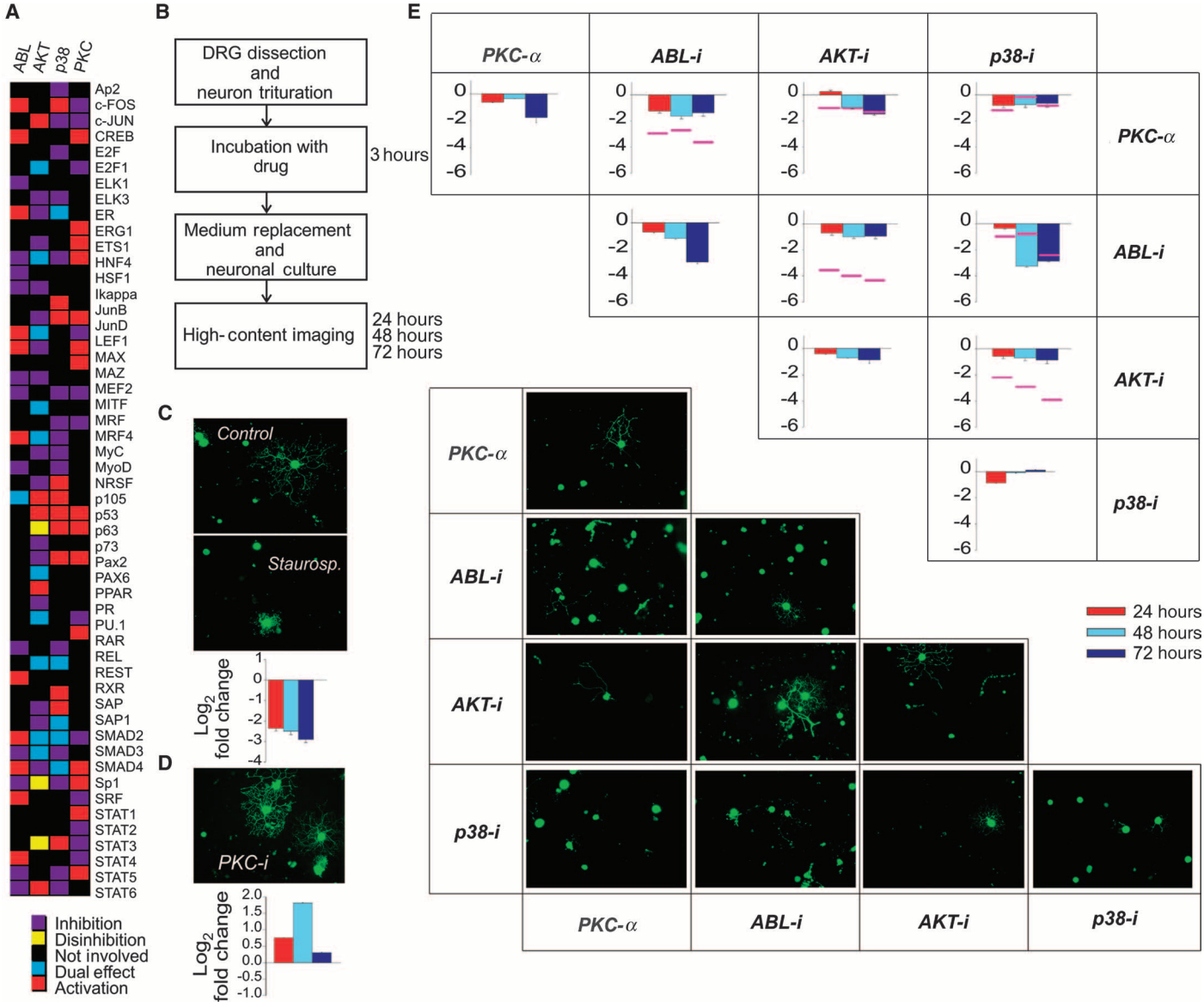

Fig. 6. Effects of perturbation of selected hub proteins. (A) Network analysis prediction of TF regulation for signaling pathways incorporating the four indicated hub proteins. (B) Flow chart of the experimental procedure for transient perturbation of injury signaling in DRG neurons. (C to E) Representative images and neurite outgrowth quantification in control cultures versus cultures treated with the indicated drugs or combinations thereof. Each quantification is based on three to six independent repeats, with at least 50 independent neurons measured per individual repeat. Data are in $\log_2$ ratio of the value for the indicated drug- versus vehicle-treated neurons. Error bars are SEM. Color codes are time points of observation after plating: red, 24 hours; cyan, 48 hours; and blue, 72 hours, respectively. Horizontal magenta lines in (E) denote the predicted effect of a combination of agents based on simple addition of previously observed effects for the separate compounds. Doses used are detailed in the Supplementary Material on the basis of dose-response curves generated for six different concentrations of each agent. Supporting information is provided in fig. S4.

failed to significantly affect total neurite outgrowth and, in fact, were much less efficacious than either inhibitor alone (Fig. 6E), although there was an effect of the combination on branching frequency (fig. S4C). We observed similar results on neurite outgrowth for Abl inhibition combined with PKC activation (Fig. 6E). Combinations of p38 inhibition with PKC activation or AKT inhibition did not show any synergism (Fig. 6E and fig. S4C). Only the combination of p38 and Abl inhibition revealed significant synergism in reducing neurite outgrowth at 48 hours after plating (Fig. 6E). Together, these data show that it is difficult to obtain efficient combinatorial perturbation of retrograde injury signaling, indicating that the system is highly robust.

DISCUSSION

Here, we examined the link between axonal injury signaling and the ensuing cell body response by using phosphoproteomics to characterize axonal signaling ensembles in rat sciatic nerve and microarrays to characterize the transcriptional response in DRG. The primary data sets were dauntingly large, comprising 879 proteins and 2465 phosphorylation sites on one hand and ~4500 transcripts on the other. This may be a consequence of the major changes caused by injury, whereby disruption of subcellular compartmentalization may cause mixing of kinases to generate a massive phosphorylation response (*40*, *41*). The extent and complexity of these data could also reflect that they were obtained from multicellular tissues challenged in vivo. Computational analyses and juxtaposition of the data sets provided clarification and insight, indicating that ~400 highly redundant signaling networks connect to 39 TFs to control a substantial part of the response to nerve injury in sensory neurons. Experimental perturbation of individual overrepresented signaling hub proteins affected neurite outgrowth in sensory neurons, but, paradoxically, in some cases, simultaneous application of inhibitors for two targets had a lesser effect than did each inhibitor alone. These data indicate that nerve injury responses are controlled by multiple regulatory components, and suggest that network redundancy provides robustness to the injury response system.

Despite the large size of the data sets and the number of candidate hub proteins and transcriptional regulators implicated by our analysis, additional transcriptional regulators and hub proteins may yet be identified because of lacunae in data collection and the limitations of the currently available computational approaches. A single phosphoproteomic screen as done here cannot provide saturating and quantitative coverage of all the proteins undergoing phosphorylation at different time points after sciatic nerve injury (*42*), and current coverage of gene annotation and validated TFBS predictions are far short of being genome-wide (*43*). Nonetheless, the intersecting networks account for ~28% of the retrogradely transported phosphoprotein data and ~41% of the gene regulation data. Given that both data sets must include confounding data from nonneuronal components in the source tissues and from other physiological influences, the approach of juxtaposing retrograde signaling data from nerve with ganglia transcriptomes allows rapid and efficient focus on those components of the data most relevant for retrograde injury signaling.

Our findings show that the major cell body transcriptional response to axonal injury occurs after a distinct temporal delay. Although rapid electrical signals may play a priming role in the cell body response to injury (*44*, *45*), relatively few genes show substantial changes in expression 3 hours after injury, which suggests that slower motor-driven signals are required to elicit the main transcriptional response. These may be de novo signals activated by the injury or by the loss of trophic or other signals present before injury (*7*). Complexes linked to molecular motors were indeed shown to be involved in retrograde injury signaling (*9*–*12*), and the temporal pattern observed in our data is consistent with the time frame needed for arrival of motor-dependent signals from the injury site. Indeed, proteomic profiling of retrogradely transported components in ligature-concentrated nerve axoplasm revealed numerous phosphoproteins specific to injured nerve. The number of these candidate signals is orders of magnitude larger than those previously reported (*6*, *7*). This was unexpected because most axonal retrograde signaling mechanisms reported to date have been focused on the transport of a limited number of molecules (*46*, *47*). These mechanisms include trapping of a ligand-receptor complex in an endosome to ensure continuous activation of the receptor by the trapped ligand en route (*48*–*50*) or binding of an activated kinase to a chaperone linker that protects it from dephosphorylation en route (*10*, *20*). In contrast, our data suggest that numerous components of a signaling network might travel together in the axon, perhaps linking to the retrograde transport machinery through a hub component that binds directly to molecular motors or importin subunits. Such mass movement of signaling molecules could maintain the network in an activated state through reciprocal activation cycles or wavelike propagation mechanisms (*51*). Indeed, it seems that retrogradely transported signaling ensembles might rival the complexity of synaptic phosphoproteomes, also thought to function by kinase cascades through a multiplicity of molecules (*52*, *53*).

The regulatory junctions identified here comprise ~50 hub phosphoproteins and 39 TFs, underscoring the complexity of the injury response process. The nerve injury response seems to be channeled through multiple and parallel pathways, integrating diverse inputs and controlling a complex transcriptional output. Such a plethora of signals may assist the neuronal cell body in assessing the extent of damage and distance of the injury site, ensuring an appropriate response (*54*). This differs from the more familiar kinase signaling networks identified in cancer, which appear to depend on a limited number of critical hubs (*55*). Thus, the multifaceted regulation we observe for intrinsic injury responses in lesioned peripheral neurons seems to have more in common with complex immunological responses or multigenic disorders than to such systems as oncogene-driven proliferating cells or diseases arising from monogenetic lesions. The multiplicity of networks affecting the activity of each TF suggests that the mechanism underlying the injury response is highly robust, and indeed, we did not observe complete block of neurite outgrowth response after pharmacological perturbation of the individual hub proteins we tested, perhaps also reflecting redundancy of critical signaling proteins (*56*). Moreover, combinations of pharmacological agents did not necessarily produce additive or synergic effects. Specifically, in the case of combinations with c-Abl inhibitor, the effects were weaker than for each compound alone; this finding indicates the existence of unpredicted modes of cross talk and a high degree of interconnectivity in the signaling networks, leading to robustness in the retrograde response.

Given the recent interest in combinatorial approaches to the development of therapies for nerve injury (*1*, *22*, *57*), our findings have potentially broad implications. Although many of the candidate hub proteins we identified are considered druggable targets by the pharmaceutical industry (*58*), the hope that modulation of a handful of master regulators might suffice to elicit regeneration may be overly naïve. Similarly, early optimism upon identification of initial candidates for extrinsic influences on regeneration (*59*) has largely been replaced with a more nuanced view as the true number of parallel pathways became known (*60*, *61*). Nonetheless, although our data suggest that it will not be trivial to identify useful cocktails that elicit robust intrinsic regenerative responses, we hope that the approach outlined in this study will enable future efforts to focus on a relatively tractable number of regulators. It is likely that such efforts will have to include dynamic in vivo measurements of signal flux through the system to characterize candidate modulators and drug prototypes (*62*). We

hope that the present effort to outline a comprehensive characterization of the retrograde injury response will provide a useful mechanistic basis for future efforts to understand and modulate this process.

MATERIALS AND METHODS

For complete details of experimental procedures, please see the Supplementary Materials.

Axoplasm and mass spectrometry

Eight- to 12-week-old Wistar rats were subjected to crush lesion, ligation, and axoplasm preparation as described (*33*, *63*). Axoplasm samples (in quadruplicate for each treatment group—injured and uninjured proximal and distal, totaling 16 phosphoproteome analyses) were denatured, reduced and alkylated, and digested with trypsin before phosphopeptide enrichment on 5-mm TiO_2 beads (GL Sciences), followed by strong cation exchange chromatography on a PolyLC column. Eluted fractions were desalted and analyzed as described (*64*). Initial automated analysis of spectra was with ProteinProspector 4.25, using the UniProt.2006.04.21 databases. Sequences and phosphorylation site assignments reported in table S1 were verified by manual inspection of the relevant spectra.

Microarrays and TFBS analyses

L4/L5 DRG were dissected from crush-lesioned or control sham-operated animals at the indicated time points. DRG total RNA was extracted with Qiagen RNeasy, 8 to 10 μg of RNA per sample were reverse-transcribed, and biotinylated complementary RNA was then hybridized to rat genome 230.2 arrays (Affymetrix). Each set was performed in triplicate over seven time points for a total of 39 arrays including controls. Array data were preprocessed with the robust multichip analysis algorithm (*65*), and data quality was assessed by principal components analysis. Log_2-transformed data were zero-transformed to the first time point. For ANOVA, gene profiles were standardized such that all genes would have the same SD. TFBS enrichment was evaluated in ExPlain 2.3 (Biobase) with gene sets passing an FDR threshold of 0.1 and fold change of 2 or more for each time point and their corresponding background sets. Promoter sequences were scanned from 600 base pairs (bp) upstream to 100 bp downstream of the predicted transcriptional start site for each such gene, and TFBSs were identified on the basis of TRANSFAC version 9. TFBS enrichment in test versus background sets was assessed by *t* test with a *P* value threshold of 0.05.

Network analyses

Phosphoproteomic data were used to generate protein signaling networks with TRANSPATH (*37*) in ExPlain 2.3. Key node–based networks as defined by ExPlain were built with the following search parameters: distance threshold, 4; connectivity penalty, 8; persistence reward, 0.5, with inclusion of superfamilies and transregulation reactions. Network score threshold was set to 10, *P* value to 0.001, and FDR to 0.05. Networks derived from microarray and TFBS analyses were cross-referenced with those derived from the phosphoproteins to generate combined networks for each time point.

High-content imaging screen

Eight compounds were tested for effects on neurite outgrowth on yellow fluorescent protein–expressing mouse DRG neurons (*66*) cultured in 96-well plates. Culture media and outgrowth-permissive substrates were as described (*9*). A dose-response profile was established for a 3-hour application of each agent before selection of the final test concentration. Neuronal cultures were imaged on an ImageXpress Micro System (Molecular Devices), and morphometric parameters were quantified with MetaXpress software (Molecular Devices) after subtracting results with vehicle. Treatment/control ratios were log-transformed and data were evaluated by one-way ANOVA ($P < 0.01$).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/3/130/ra53/DC1
Materials and Methods
Fig. S1. Enrichment of injury-related phosphopeptides.
Fig. S2. Initial analyses of TFBSs.
Fig. S3. Network structure of the 45 top-ranked networks. Color-coding as detailed in the legend to Fig. 3.
Fig. S4. Drug effects on morphometric parameters of the injury response in sensory neurons.
Table S1. Phosphoproteins identified in this study.
Table S2. Differentially expressed genes from the microarray data.
Table S3. Phosphoprotein representation in networks linking axonal signaling to the cell body transcriptional response.
Table S4. Linkage of signaling networks to gene regulation profiles at designated time points.
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Science Signaling

Signaling to Transcription Networks in the Neuronal Retrograde Injury Response

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Calling In the Repair Crew

The ability of a damaged neuron to regenerate depends on the initiation of a repair program in the cell body, so that the injured neuron switches from a "growth-as-normal" mode to an "injury-response" mode. Initiation of such a repair program depends in turn on the receipt by the cell body of injury signals from the lesion. Michaelevski *et al*. combined phosphoproteomic analyses of injured and uninjured rat sciatic nerve with microarray analyses of transcripts in the dorsal root ganglia to identify retrograde signaling networks implicated in activating the transcriptional response to axonal injury. Pharmacological manipulation of various protein kinases that appeared in many of these networks and were predicted to play a key role in affecting signaling network size and connectivity affected neurite outgrowth of cultured sensory neurons. Paradoxically, the combined manipulation of pairs of these kinases was sometimes less effective at affecting neurite outgrowth than manipulation of either alone—an observation that has substantial implications for development of appropriate therapies for treating nerve injury.