Published in final edited form as:

Mol Cell Neurosci. 2018 October ; 92: 114–127. doi:10.1016/j.mcn.2018.08.001.

The effect of Jun dimerization on neurite outgrowth and motif binding

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Abstract

Axon regeneration is a necessary step toward functional recovery after spinal cord injury. The AP-1 transcription factor c-Jun has long been known to play an important role in directing the transcriptional response of Dorsal Root Ganglion (DRG) neurons to peripheral axotomy that results in successful axon regeneration. Here we performed ChIPseq for Jun in mouse DRG neurons after a sciatic nerve crush or sham surgery in order to measure the changes in Jun's DNA binding in response to peripheral axotomy. We found that the majority of Jun's injury-responsive changes in DNA binding occur at putative enhancer elements, rather than proximal to transcription start sites. We also used a series of single polypeptide chain tandem transcription factors to test the effects of different Jun-containing dimers on neurite outgrowth in DRG, cortical and hippocampal neurons. These experiments demonstrated that dimers composed of Jun and Atf3 promoted neurite outgrowth in rat CNS neurons as well as mouse DRG neurons. Our work provides new insight into the mechanisms underlying Jun's role in axon regeneration.

Keywords

Dorsal Root Ganglion; AP-1; chromatin immunoprecipitation; enhancer

Introduction

Spinal cord injury (SCI) is a devastating condition that affects approximately 17,000 people each year, with an estimated 286,000 people living with this condition in the United States (National Spinal Cord Injury Statistical Center). One necessary step toward functional

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Competing interests

Conflicts of interest: none

recovery after SCI is regeneration of severed axons. Dorsal Root Ganglion (DRG) neurons can be a useful model to study axon regeneration due to their intrinsic regenerative ability combined with their pseudo-unipolar morphology. The peripheral branch of their axons regenerates after certain injuries, while their central branch fails to regenerate after spinal cord injury. Injuries to the peripheral branches of DRG neurons allow for axon regeneration largely by promoting transcription-dependent changes in gene expression and axonal trafficking that increase the intrinsic axon regenerative capabilities of the neurons (Smith and Skene, 1997). These peripheral axotomy-induced changes to the DRG neurons can be exploited to promote regeneration of the central axon branches after spinal cord injury (Neumann and Woolf, 1999). For this reason, we have chosen to study one of the regulators of these peripheral axotomy-induced transcriptional changes, the AP-1 transcription factor c-Jun (Jun), a member of the bZIP family of transcription factors

After a peripheral axotomy, Jun is upregulated (Broude et al., 1997; Herdegen et al., 1992) and activated by phosphorylation (Herdegen et al., 1997; Lindwall et al., 2004) in DRG neurons. This activation persists until the completion of axon regeneration (Kenney and Kocsis, 1998). Jun-deficient mice exhibit impaired axon regeneration in facial motor neurons (Raivich et al., 2004). In cell culture, overexpression of Jun is sufficient to promote neurite outgrowth in PC12 cells and DRG neurons (Chandran et al., 2016; Dragunow et al., 2000; Pearson et al., 2003), but not in cortical neurons (Lerch et al., 2014; Venkatesh et al., 2016).

In order to be transcriptionally active, Jun needs to dimerize with another bZIP transcription factor. Jun readily forms dimers with members of the Jun, Fos, and ATF/CREB families (Hai and Curran, 1991), although under certain circumstances it can also form dimers with other proteins (Kageyama et al., 1991; Kerppola and Curran, 1994). Jun:Jun and Jun:Fos dimers bind to the heptanucleotide motif TGA [G/C] TCA, known as the TPA response element (TRE) (Angel et al., 1987; Angel and Karin, 1991; W Lee et al., 1987; Wes Lee et al., 1987). On the other hand, Jun:ATF dimers bind to the octanucleotide motif TGA CN TCA, known as the cAMP response element (CRE) (Chatton et al., 1994; Hai and Curran, 1991; Ivashkiv et al., 1990). Interestingly, Jun:Jun and Jun:Fos dimers have the ability to bind CRE motifs, but that is dependent on the flanking nucleotides (van Dam and Castellazzi, 2001). Additionally, some Jun:ATF dimers bind DNA sequences that are distinct by 1–3 base pairs from the canonical CRE motif: these sites are referred to as degenerated CRE sites (Benbrook and Jones, 1994, 1990). Different dimerization partners play a role in determining TRE/CRE preference, as well as degenerated TRE/CRE preference (Angel and Karin, 1991; Benbrook and Jones, 1994; Hai et al., 1989; Hai and Curran, 1991; Ivashkiv et al., 1990). Of all of these potential dimerization partners, the most studied in the context of axon regeneration is Atf3, which has been demonstrated to be an important component of the DRG neuronal response to peripheral axotomy (Gey et al., 2016; Seijffers et al., 2007)

In this manuscript, we investigate the genomic locations bound by Jun in DRG neurons following a peripheral axotomy. We observe that the majority of Jun's injury-responsive binding sites are on putative enhancer elements, while the majority of its injury-invariant binding sites are on promoter elements. We also measured changes in Jun's binding motif usage in response to injury in an attempt to gain better understanding of which binding

partners it may be utilizing. In a complementary set of experiments, we tested the sufficiency of single polypeptide chain Jun-containing dimers to promote neurite outgrowth of DRG, cortical and hippocampal neurons *in vitro*

Results

ChIPseq for Jun reveals changes in binding locations after peripheral injury, especially near putative enhancers

We selected the H-79X polyclonal anti-Jun antibody because it had been used by the ENCODE consortium to perform ChIPseq on Jun from the CH12.LX mouse lymphoblast cell line as well as several human cell lines. The ENCODE consortium performed rigorous quality checking on this antibody demonstrating its efficacy by immunoprecipitation and western blotting in three cell lines as well as sufficiently enriching for Jun-related motifs in their ChIPseq data sets. We validated this antibody for mouse DRG using ChIP-qPCR. We tested how many mice were needed to obtain effective enrichment for Jun-bound DNA by performing ChIP on the chromatin from the DRGs contributing to the sciatic nerve of 1, 3, or 5 mice pooled together. Each sample was immunoprecipitated with anti-Jun and with control IgG. Quantitative PCR was then performed that targeted two sites near the transcription start site (TSS) of the Jun gene. One site (positive control) is a well-studied target site of Jun-containing transcriptional complexes upstream of the Jun TSS (Angel et al., 1988). The other site was used as a negative control as it marks a region 5kb upstream of the Jun TSS, which is at least 1kb away from any known Jun binding site. qPCR performed on each of the eluates using each of these primer sets and quantified using the 2^{-Ct} method (Schmittgen and Livak, 2008) revealed that immunoprecipitation for c-Jun returned more signal at the positive locus than at the negative locus for each of the three pooled chromatin samples, while IgG gave virtually identical signals for the two sites (Supplemental Figure S1). Using a single mouse per sample yielded only a 4.4 fold enrichment of the positive over the negative control site, while the 3-mouse and 5-mouse samples gave 10.3 and 11.9 fold enrichments, respectively. This experiment suggests 1) that the H79X antibody is effective at immunoprecipitating c-Jun in our experimental paradigm, and 2) that the sciatic-related DRGs from 3 mice per biological replicate is sufficient for c-Jun ChIP experiments.

ChIPseq was performed for Jun using chromatin from whole mouse DRGs taken 7 days after a sciatic nerve crush or sham surgery. The 7 days post-surgery time point was chosen because the DRG neurons are hypothesized to be in the “extension phase” of axon regeneration at that time. Analysis of sequence read duplication rates revealed that the majority of reads in each sample were optical duplicates, indicating that the sequenced library had low complexity (Supplemental Figure S2A–B). This most likely stemmed from the small amount of starting DNA in the eluates, which led to use of a large number of PCR cycles during library preparation. To eliminate the biases that these PCR duplicates could introduce, all downstream quality checking and subsequent analysis was performed on the non-redundant fraction of the reads. Sample processing and quality checking indicated that the immunoprecipitation was successful according to ENCODE guidelines for the metrics Normalized Strand Coefficient (NSC), Relative Strand Correlation (RSC), and Fraction of Reads in Peaks (FRiP) (Landt et al., 2012) (Supplemental Figure S2C). See materials and

methods section for explanation of these quality checking terms. Sample clustering analysis revealed that replicate samples were more similar to each other than to the opposing condition (Supplemental Figure S2D); therefore replicate samples were pooled for downstream analysis

Differential peaks and peaks bound in both conditions were identified using the `bdgdiff` command in `macs2` (these peaks are provided in Supplemental Table 1), yielding a set of 1046 peaks. This differential binding analysis revealed 160 peaks with approximately equal signal both before and after injury (commonly bound), 185 peaks with significantly less signal (less bound) after injury, and 701 peaks with significantly greater signal (more bound) after injury (Figure 1A). This result is consistent with observations that Jun is activated and increases in expression following sciatic nerve injury (Herdegen et al., 1997; Kenney and Kocsis, 1998)

Historically, Jun binding sites have been studied near TSSs, where the majority of AP-1/ CREB binding has been thought to occur. However, ENCODE consortium datasets, along with other recent studies (Malik et al., 2014), suggest that the majority of Jun binding sites are actually in enhancer regions. To examine the relative distribution of binding sites from our ChIPseq data, we generated pie charts of the genomic locations of the peaks (Figure 1B–D). We found that 55.2% of the sites more bound after injury were within 1kb of a putative enhancer element (as defined in (Shen et al., 2012)) while only 25.8% of sites more bound after injury were within 2kb of a TSS (Figure 1B). A similar pattern was observed for the sites significantly less bound after injury: 50.3% were within 1kb of an enhancer, while only 19.5% were within 2kb of a TSS (Figure 1C). These results starkly contrast with the distribution of Jun sites bound regardless of injury – 81.3% of these were within 2kb of a TSS and only 8.8% were within 1kb of an enhancer (Figure 1D)

Since there are 2–4 times more putative enhancer elements in the mouse genome than TSSs (Shen et al., 2012; Yue et al., 2014), we examined whether the distribution of Jun binding sites we identified represented a statistical enrichment for enhancer elements compared to TSSs, by performing Fisher's exact tests to detect enrichment relative to genomic background. This approach revealed that differential Jun binding both near enhancers and near TSSs represented significant enrichments (Figure 1E), but that the enrichment for TSSs was significantly greater than for enhancers (TSS odds ratios of 9.5 and 6.9 for more bound and less bound peak sets, respectively, vs enhancer odds ratios of 3.1 and 2.75). No significant differences were observed between the distributions of more bound vs less bound sites. Additionally, these tests demonstrated a greater level of enrichment for TSSs for the commonly bound peaks than the differentially bound peaks (OR=120), but also a significant depletion of commonly bound peaks near enhancer elements (OR=0.25). These results suggest that a large proportion of Jun's injury response activities are carried out at enhancer elements. The majority of Jun's injury-responsive binding sites are on enhancer elements, while the majority of its injury-invariant binding sites are on promoter elements. However, when the relative numbers of these DNA elements are considered, Jun binding is more enriched for promoter regions than for enhancer regions in each of the conditions inspected

Differentially bound peaks near gene transcription start sites are not predictive of the differential expression of those genes

Since Jun is a transcription factor commonly associated with transcriptional activation, and its activity is necessary for efficient axon regeneration (Raivich et al., 2004), we hypothesized that the differential binding of Jun would track with differential expression of the genes associated with the bound DNA elements. As data on chromatin conformation in DRGs is not available, we had to restrict this analysis to the peaks proximal to TSSs. Using RNAseq data from (Motti et al., 2017), we examined the relationship between changes in Jun binding signal at sites within 2kb of TSSs and the changes in expression of the corresponding genes. We found that the target genes were equally likely to be upregulated or downregulated, regardless of whether Jun binding at their TSSs was increasing, decreasing, or unchanging (Figure 2A)

Examination of the Gene Ontology (GO) terms related to biological processes for the differentially expressed genes with nearby Jun binding sites revealed enrichment of neuronal and Jun-related GO terms such as transcription, apoptosis, development, differentiation, and axonogenesis (Supplemental Table 2). Interestingly, examination of the GO terms associated with the ‘concordantly’ upregulated genes (those whose expression goes up and that have a nearby site significantly more bound by Jun after injury) revealed even a stronger enrichment for the appropriate GO terms (Figure 2B). The set of upregulated genes with more Jun binding is related to axon and nerve development, axon regeneration, and neuronal apoptosis while the set of downregulated genes with less Jun binding has no terms related to neurons or axons at all (Figure 2C and Supplemental Table 2). The genes identified by this ‘concordant’ analysis do not seem to simply be a random subset of the differentially expressed genes, but instead seem enriched in major components of the neuronal response to injury

To test whether this GO term analysis could be reproduced with different data, we obtained a second RNAseq dataset examining DRG neurons 7 days after peripheral axonal injury (Tedeschi et al., 2016) and repeated the peak-to-gene assignment and GO term analyses. We again observed that target genes of differentially bound sites were equally likely to be upregulated or downregulated in these RNAseq data, regardless of whether Jun binding near their TSSs was increasing or decreasing. Similar patterns of results were also found for the GO term enrichment (data not shown)

Jun’s binding motif preferences change after peripheral nerve injury

We performed motif enrichment analysis using the program Homer (Heinz et al., 2010) on the sets of peaks called in the injured and uninjured conditions to inspect the overall motif patterns in the data. We found that both TRE and CRE DNA motifs were enriched within the DNA sequences contained in the peaks (relative to background) both before and after injury, although TRE was slightly more enriched overall. We observed that this pattern held when looking specifically at peaks within 2kb of TSSs as well as for peaks within 1kb of enhancers

Since Jun has the ability to bind both TRE and CRE DNA motifs, depending on its dimerization partner (Hai and Curran, 1991), and Atf3 is speculated to be an important Jun dimerization partner after peripheral axotomy, we hypothesized that CRE motifs would be preferentially enriched in the DNA sequences within the peaks more bound after injury. To test this, we performed motif enrichment analysis on each set of peaks returned by the differential binding analysis (more bound, less bound, and commonly bound) in each case using the two other sets of peaks as the background set. This analysis revealed that the TRE and CRE motifs were most significantly enriched within the commonly bound peaks (p-values of enrichment: TRE= 10^{-8} against less bound sites; CRE= 10^{-8} against less bound sites; TRE= 10^{-3} against more bound sites; CRE= 10^{-3} against more bound sites). The DNA sequences within sites more bound after injury still showed strong enrichment for the TRE and CRE motifs relative to the sites less bound after injury (p-values of enrichment: TRE= 10^{-12} ; CRE= 10^{-8}). The DNA sequences within sites more bound after injury additionally showed enrichment for a degenerated form of the CRE motif (relative to the less bound peaks and the commonly bound peaks) of the form TCA CG CAA (p-values of enrichment: 10^{-4} against less bound sites; 10^{-3} against commonly-bound sites). Since different Jun motifs are generally recognized by Jun dimerizing with different partners, we hypothesized that this change in motif usage might indicate Jun working preferentially with a particular binding partner after injury – one which might help Jun carry out pro-regenerative functions. To investigate this issue, we examined the relationships among different Jun-containing dimers, Jun motif usage, and effects on axon regeneration

Different Jun-containing dimers prefer different DNA motifs

To test the relationship between each Jun-containing dimer and DNA motif recognition, we used Jun-containing ‘tethered dimer’ constructs (Bakiri et al., 2002). These are single polypeptide chains containing tandem transcription factors (Jun and another bZIP transcription factor), which fold correctly into dimers, are transcriptionally active, and accurately replicate the activities of naturally formed dimers of the two constituent monomers (Bakiri et al., 2002). These were used in a luciferase reporter gene assay to measure transcriptional activation, compared with activation by the individual constituent factors. In our discussion, we will use a ‘~’ symbol between two gene names to denote a tethered dimer, a ‘:’ symbol to denote a naturally occurring dimer, and a ‘+’ to indicate co-transfection of two monomers

We tested the transactivation abilities of eight Jun-containing tethered dimers on each of the three motifs identified in the ChIPseq dataset. Generally, Jun dimers with Fos and Jun family members are expected to bind the TRE motif, while Jun dimers with Atf family members are expected to bind the CRE motif. Jun~Atf3 promoted expression of luciferase when driven by the CRE motif, as predicted (Figure 3A). Interestingly, we also saw CRE-driven expression of luciferase when overexpressing Jun~Fos11. Jun~Fos11 promoted expression of luciferase when driven by the TRE motif, as expected (Figure 3B). Jun~Atf3 also induced strong, but statistically non-significant, expression of luciferase under the TRE motif, which was unexpected. This statistical insignificance was evidently caused by the large amount of variance introduced into the ANOVA by data from Jun~Fos11 and Jun~Fos12. When those two conditions were removed from the model, Jun~Atf3’s effect became statistically

significant (Supplemental Figure S3A). Jun~Jun and Jun~Fos gave surprisingly small signals in this assay using the TRE motif and were not statistically significant. Finally, we also saw activation using the degenerated CRE motif from Jun~Atf3 (Figure 3C). Jun~Atf3 was the most effective activator of transcription when utilizing the Degenerated CRE motif, whereas Jun~Fosl1 was the most effective activator of TRE-driven transcription. Jun~Atf3 and Jun~Fosl1 were of comparable efficacy when using the CRE motif. To test for non-specific effects of each tethered dimer on luciferase expression, we performed experiments using a version of the luciferase plasmid containing only a minimal promoter without a Jun response element. This revealed a small amount of non-specific luciferase signal induced by several of the dimers (Supplemental Figure S3B). This minimal promoter control was also used as part of the normalization process for the results presented in Figure 3 (see methods). The individual monomers did not significantly activate transcription using any of the 3 response elements tested (Supplemental Figure S3C–E)

Since several of these tethered dimers demonstrated the ability to bind two or more Jun-related motifs, we next sought to determine for which motif each tethered dimer has greatest preference. For this purpose, we used electrophoretic mobility shift assays (EMSAs) with fluorescently-labelled oligonucleotide probes containing CRE, TRE, or degenerated CRE motifs. Each tethered dimer-motif pair that gave significant induction in the luciferase assay produced a visible band in the EMSA experiments, suggesting that the luciferase results were not due to off-target effects. To evaluate motif preference, we performed competition experiments by using two different fluorescent probes simultaneously. These experiments demonstrated that Jun~Atf4 and Jun~Atf7 prefer the CRE motif over the TRE motif, whereas Jun~Fos, Jun~Fosl1 and Jun~Fosl2 prefer the TRE motif over the CRE motif. Jun~Atf3, Jun~Jun and Jun~JunB have no clear preference for one motif over the other (Supplemental Figure S4A). Furthermore, all tested dimers prefer the canonical CRE motif more than the degenerated CRE motif (Supplemental Figure S4B). These experiments confirmed the results seen in the luciferase assay and further demonstrated motif preference in a competitive setting.

Since Jun:Atf dimers are generally expected to prefer the CRE motif over the TRE motif (Hai and Curran, 1991), we performed an additional dose-response competition experiment to measure Jun~Atf3's motif preference. EMSA reactions were performed as before with lysate from Jun~Atf3-transfected cells and 50 fmol of CRE oligo. Varying quantities of TRE oligo were added to each reaction, ranging from 0 to 10,000 fmol (Figure 3D). The signal produced by the bound CRE oligo was quantified in each reaction and the amount of TRE oligo needed to reduce the CRE signal to half of its initial intensity (the IC_{50}) was calculated using the Hill equation (Figure 3E). This dose-response experiment indicated that Jun~Atf3 requires a 1.6 fold excess quantity of TRE oligo in order to reduce the signal from the CRE oligo by 50%. This small excess quantity value is consistent with the results of the luciferase and earlier EMSA experiments, and provides further evidence that Jun~Atf3 binds CRE and TRE motifs with similar affinity

The above experiments were performed using tethered dimers with Jun on the N-terminal side of the polypeptide and Jun's dimer partner forming the C-terminus. To test whether our results were influenced by steric considerations based on the relative positions of the two

polypeptides in the dimer, we repeated the experiments using constructs with Jun in the C-terminal position for dimers containing Atf3, Fos, and JunB. These ‘reverse’ orientation dimers replicated the results seen for their ‘forward’ orientation counterparts in the luciferase assay (Supplemental Figure S5)

We used Oxr1–2A-GFP as the baseline control in the luciferase and EMSA experiments described above. We used this control construct, rather than GFP alone, because Oxr1 is a transcription factor, has no known affinity for Jun binding sites, and does not contain a DNA binding domain similar to that of Jun (Oliver et al., 2011; Volkert et al., 2000). Notably, the same control is used in our neurite outgrowth experiments, and has been observed to have minimal effects on neurite outgrowth in cortical neurons (Lerch et al., 2014)

Jun~Atf3 tethered dimer consistently promotes neurite outgrowth

To demonstrate the effects of different Jun dimers on neurite outgrowth (an *in vitro* heuristic for axon regeneration) we transduced four different Jun-containing tethered dimer constructs into cultured, adult mouse DRG neurons, comparing these results to transfection of each of the monomers, and with co-transduction of Jun with each of the other monomers. Each of the individually transduced monomers yielded approximately double the baseline amount of neurite outgrowth, although this increase was not statistically significant (Figure 4A). Co-transduction of Atf3 with Jun led to an approximately additive increase in neurite outgrowth (Figure 4A). The Jun~Atf3 tethered dimer produced a slightly smaller increase in neurite outgrowth than did co-transduction of its constituent monomers (Figure 4A). In contrast, the Jun~Atf4 tethered dimer produced a large increase in neurite outgrowth while the co-transduction of its monomers had only a modest effect (Figure 4A). These patterns were similar when examining the length of the longest neurite (LOLN), with Jun~Atf3 producing a significant increase that was slightly less than that of the co-transduction of its monomers, and with Jun~Atf4 producing the largest effect (Figure 4B).

One reason for the decreased regenerative ability of CNS neurons compared to DRG neurons may be a failure to express pro-regenerative transcription factors such as Jun dimers. To test this idea in cultured CNS neurons, we transfected eight different Jun-containing tethered dimer constructs into early postnatal rat cortical neurons, comparing these results to transfection of each of four AP1-related monomers, and with co-transfection of Jun with each of the other monomers. None of the individually or co-transfected monomers yielded a significant increase in neurite outgrowth (Figure 4C). Despite this, the Jun~Atf3 tethered dimer significantly promoted neurite outgrowth, while Jun~Atf4 significantly repressed it (Figure 4D). Jun~Jun and Jun~JunB showed smaller, but statistically significant increases to neurite outgrowth in this assay as well (Figure 4E), but none of the Fos family members had any effect (Figure 4F). We saw a similar pattern when quantifying the length of the longest neurite (LOLN): none of the individually or co-transfected monomers significantly affected LOLN, but the Jun~Atf3 and Jun~Jun tethered dimers significantly promoted LOLN (Figure 4G–J). The Atf3~Jun ‘reverse orientation’ tethered dimer performed comparably to Jun~Atf3, promoting total neurite outgrowth and LOLN (Supplemental Figure S6A–B).

Representative images of cortical neurons transfected with control transcription factor, Oxr1–2A-GFP, (left) and Jun~Atf3 (right) with neurite tracing overlay are shown in Figure 4K. We noticed that the Atf3 monomer, whether individually or co-transfected, generally had diffuse localization throughout the cell, whereas the Jun~Atf3 tethered dimer was primarily localized to the nucleus (not shown). This suggests that the Jun~Atf3 tethered dimer may be working as a transcription factor as intended, while a large proportion of the Atf3 monomer may not.

To test the robustness of these findings for CNS neurons, we performed experiments in embryonic rat hippocampal neurons using the dimers that promoted neurite outgrowth in the cortical neurons (and their constituent monomers). In these neurons, co-transfections of Jun and Atf3 significantly promoted NTL (Supplemental Figure S7A), but not LOLN (Supplemental Figure S7B). Jun~Atf3 promoted both neurite outgrowth (Supplemental Figure S7A) and LOLN (Supplemental Figure S7B) in this model, but the other tethered dimers did not increase either parameter. Interestingly, neurite outgrowth of transfected hippocampal neurons was positively correlated with the intensity of signal of the tagged TFs, for those constructs with a positive effect on neurite growth. Analysis of the data using a cutoff for high signal intensity (corresponding to high levels of expression) increased the effect size of the Jun~Atf3 dimer and the Jun + Atf3 co-transfection considerably (data not shown).

Discussion

In this work, we provide a useful resource for the axon regeneration community in the form of ChIPseq data for c-Jun in DRG neurons in response to peripheral axotomy. This dataset contains novel information about where and to which motifs Jun binds before and after axotomy, and can be used to better understand the TFs with which Jun cooperatively binds after injury. Our data reveal that a large percentage of Jun's injury responsive binding site changes occur distal to TSSs, at putative enhancer sites. When chromatin conformation data in these biological conditions becomes available, these ChIPseq data will help to reveal which genes Jun is regulating through such distal binding sites. In a complementary series of experiments, we used tethered dimers of Jun and its partners to demonstrate the effects of specific Jun-containing dimers on phenotypic changes in neurons.

The differential binding results from our ChIPseq data support the hypothesis that Jun binding activity is increased after peripheral axotomy, in line with the previously reported increase in Jun expression (Kenney and Kocsis, 1998; Motti et al., 2017; Stam et al., 2007; Tedeschi et al., 2016; Zou et al., 2009) and overrepresentation of Jun binding sites in the promoters of differentially expressed genes (Chandran et al., 2016; Michaelievski et al., 2010). However, our results suggest that the majority of changes in Jun activity after peripheral axotomy occur at enhancers rather than at promoters. Previous experiments with Jun deficiency in facial motor neurons (Raivich et al., 2004; Ruff et al., 2012) and retinal ganglion cells (Fernandes et al., 2013) have shown the expression of a number of regeneration-associated genes to be Jun-dependent. Many such genes do not have known Jun binding sites in their promoter regions, and we did not observe Jun binding to these

promoters in our ChIPseq data. These genes are presumably regulated by Jun either indirectly or through enhancer binding sites.

Surprisingly, we saw a lack of concordance between increases in Jun binding near promoters and increases in gene expression after injury. Similarly, Michaelevski et al. observed an overrepresentation of Jun binding sites in the promoter regions of both upregulated and downregulated genes after peripheral axotomy (Michaelevski et al., 2010). Our observations underline the complexity of the transcriptional response to injury, likely involving competition among transcription factors acting at individual promoter and enhancer regions, as well as differential involvement of coactivators and corepressors. In DRGs responding to peripheral axotomy, the signaling, epigenetic, and transcriptional states of the cells are such that this complex interplay results in axon regeneration.

Since Jun is important in many biological processes (proliferation, death, regeneration, etc), has many dimerization partners (Jun, Fos, and Atf families), and can bind multiple motifs (TRE, CRE, and some variants), our initial hypothesis was that different dimers would preferentially bind different motifs, and that this differential motif usage would mediate Jun's specific response to axonal injury. However, while we did observe some changes in motif usage after injury, the majority of post-injury binding sites had either TRE, CRE or no recognizable Jun-related motif – exactly the same as before injury. This suggests that changes in chromatin accessibility or overall Jun activity could be more crucial than changes in motif affinity for Jun's pro-regenerative functions. The tethered dimer overexpression experiments, however, demonstrate at least some dimerization partner specificity in Jun's ability to promote neurite outgrowth. The tethered dimer experiments agreed with previous results on which motif is preferred by each dimer, but also demonstrated a large amount of promiscuity in motif binding. Jun~Atf3 is the only dimer tested in our studies that consistently promoted neurite outgrowth. It also showed the greatest promiscuity in motif binding of the dimers tested. Jun~Fos1 showed the second most promiscuity in motif binding, but did not exert any measurable effect on neurite outgrowth. It is possible that the subtle differences in motif preference between these two dimers result in their large differences in effect on neurite outgrowth, but it seems more likely that there is an unknown factor, in addition to motif preference, that better explains the dimerization partner-specific effects of Jun on neurite outgrowth.

Our neurite outgrowth results both replicate results observed previously and clarify a major division in the field. It has been previously shown that Jun and Atf3 have at least additive effects on neurite outgrowth in DRG neurons and PC12 cells (Chandran et al., 2016; Pearson et al., 2003), but this effect fails to replicate in cortical neurons (Venkatesh et al., 2016). Our experiments with monomer co-transfections replicate the findings of Chandran and colleagues that Jun and Atf3 monomers have approximately additive effects on neurite outgrowth in DRG neurons (Chandran et al., 2016). Our experiments further show that the majority of this effect (about 80%) can be phenocopied by Jun~Atf3 tethered dimers, suggesting that Jun and Atf3 may accomplish much of their neurite growth-promoting effects in DRG neurons by forming a complex. In early postnatal rat cortical neurons, our results replicate the findings of Venkatesh and colleagues that Jun and Atf3 do not promote substantial neurite outgrowth or exhibit an additive effect (Venkatesh et al., 2016).

Intriguingly, we found that forced dimerization of Jun and Atf3 enhances neurite outgrowth in both cortical and hippocampal neurons. This indicates that the ability of Jun and Atf3 to promote neurite outgrowth most likely depends on their dimerization with each other. In hippocampal and DRG neurons, the signaling state of the cells evidently allows a sufficient percentage of the overexpressed monomers to form functional dimers and promote neurite outgrowth, whereas these dimers may not form in cortical neurons, even though the relevant chromatin is accessible once dimerization is forced in cortical neurons.

Our experiments help to shed light on the ability of Jun and Atf3 to co-operatively effect neurite outgrowth and axon regeneration. Our results suggest that Jun and Atf3 form functional dimers that act in a pro-regenerative manner, but that injured CNS neurons may not be in the appropriate signaling state to allow Jun and Atf3 to dimerize productively. Accordingly, we hypothesize that overexpression of Jun~Atf3 tethered dimers might more effectively promote axon regeneration *in vivo* than was observed when overexpressing a mixture of the two monomers (Fagoe et al., 2015).

Our experiments in DRG neurons identified Jun~Atf4 dimers as strong promoters of neurite outgrowth. This result was surprising for several reasons. First, we are not aware of previous data showing such a relationship. Second, Atf4 monomers had minimal effects on neurite outgrowth, and mixtures of Jun and Atf4 exhibited small additive effects in neurite outgrowth compared to the individual monomers. Third, Jun~Atf4 dimers *inhibited* neurite outgrowth in cortical neurons. Despite this, Jun~Atf4 dimers were the strongest promoters of NTL and LOLN in DRG neurons. The fact that Jun~Atf4 dimers promote neurite outgrowth in DRG neurons while inhibiting it in cortical neurons suggests that they may have different sets of accessible binding sites in the two types of neurons, leading to regulation of different sets of genes. Further studies examining the chromatin accessibility of DRG and cortical neurons will be needed in order to begin to understand this divergent relationship.

While our work strongly suggests important roles for some Jun-containing dimers (e.g., Jun~Atf3), it does not rule out a contribution of other Jun-containing dimers to axon regeneration after peripheral axotomy. For example, both Atf5 and JunD are expressed in DRG neurons after injury (Motti et al., 2017; Tedeschi et al., 2016), and could be involved. Indeed, (Kenney and Kocsis, 1998) suggest that Jun:JunD dimers form and bind TRE motifs in DRGs after peripheral axotomy. Jun~Jun dimers promoted neurite outgrowth in DRG and cortical neurons, albeit to a lesser extent than Jun~Atf3. Since Jun~Jun had a different pattern of effects on luciferase expression driven by CRE and TRE motifs as compared to Jun~Atf3 dimers, we hypothesize that it could be promoting neurite outgrowth by a different mechanism that may be worth additional investigation. Additionally, our work does not examine the effects of dimers containing Atf, Fos or other Jun family members without Jun itself. For example, Atf3 homodimers, like Jun~Atf3 heterodimers, can bind both TRE and CRE sites (Hai and Curran, 1991) and could therefore affect the expression of many of the same genes.

In our neurite outgrowth experiments involving monomers, we used forms of the proteins containing the tether sequence as well as a flag or HA tag in order to control for the presence of those components in the tethered dimers since we wanted to be able to compare the

effects of the monomers on neurite outgrowth to that of the tethered dimers. It is possible that these non-native features have an effect on the experimental results, but we do not believe that the effect is large in comparison to the experimental effects that we measured since many of our experimental conditions replicated results found in other works (Chandran et al., 2016; Venkatesh et al., 2016).

Our results also suggest several interesting directions for future work. This work raises the question of whether overexpression of Jun~Atf3 tethered dimers would be more effective at promoting axon regeneration after a CNS injury than either monomer alone (Raivich et al., 2004; Seijffers et al., 2014) or a co-transduction of monomers (Fagoe et al., 2015). This work also suggests that there would be benefit from a better understanding of how the signaling state of the cell determines which Jun-containing dimers form and what effects these have on the cell. For example, Jun:Atf2 dimerization-mediated cell death, in response to signaling by Dual Leucine Zipper Kinase (DLK) and Leucine Zipper Kinase (LZK), was recently found to be part of the RGC response to axotomy (Welsbie et al., 2017). It will be important to determine how Jun's effects on cell death/degeneration, proliferation, and neuronal differentiation combine to signal axonal regeneration after injury.

Materials and Methods

Animal Care and Sciatic Nerve Crush Surgeries

Eighteen male C57BL/6 mice aged 6 to 8 weeks were used for sciatic nerve crush surgeries. All procedures were performed in accordance with the protocols approved by the Institutional Care and Use Committee at the University of Miami. Animal anesthesia and post-operative care were performed as described in (Danzi et al., 2016) with buprenorphine injections as the post-operative analgesic. The sciatic nerve was exposed at the level of the mid-thigh and then crushed with a pair of #5 forceps (Dumont; Switzerland) for 10 seconds before closing the incision. In the case of a sham surgery, the forceps were held around the nerve without crushing it for that same amount of time and then the incision was closed normally. This procedure was repeated on the other leg to obtain a bilateral sciatic nerve crush or sham. Eighteen mice were used because preliminary ChIP-qPCR experiments indicated that a minimum of three mice per biological replicate were necessary to obtain enough chromatin for a successful immunoprecipitation of Jun and we wanted to perform three biological replicates of the sham and injured conditions.

Chromatin Immunoprecipitation

Seven days after surgery, mice were sacrificed and the three DRGs innervating the sciatic nerve on each side (six ganglia in total) were extracted and placed in HibernateE (BrainBits). Once the DRGs from three mice had been extracted and pooled together, the samples were gently spun down at $80 \times g$ for 2 minutes. The pellet was then resuspended in 10mL of Complete Tissue Fixation Solution (Active Motif) containing 1% fresh formaldehyde and fixed at room temperature for ten minutes. The ActiveMotif ChIP-IT High Sensitivity Kit was then used to perform the remainder of the immunoprecipitation process with the following alterations: 1) TE + 1% SDS was used instead of ActiveMotif's Chromatin Prep Buffer in Section C step 9; 2) 400 μ L of ChIP Buffer was used for the

sonication, which was carried out in 15mL Bioruptor Pico Tubes with 250mg of sonication beads (Diagenode; Catalog # C01020031); and 3) the sonication was performed on a Bioruptor Pico sonicator for 7 cycles of 30 seconds on and 30 seconds off per cycle with the water bath maintained at 4C continuously (Diagenode). Each chromatin preparation made in this manner yielded enough material for an input and two immunoprecipitation reactions. Immunoprecipitations were carried out according to the ChIP-IT High Sensitivity kit instructions using 4µg of polyclonal rabbit anti-Jun (SantaCruz Biotechnology; Catalog # sc-1694X), or rabbit anti-IgG (Jackson Immuno Research; Catalog # 011-000-003) antibodies. The anti-IgG antibody was only used in the ChIP-qPCR experiment, not in the ChIPseq. For ChIP-qPCR experiments, the immunoprecipitated DNA was eluted in 200µL of TE. For ChIPseq, the eluates of each pair of IPs using the anti-Jun antibody from the same chromatin preparation were pooled together. Kapa Hyperprep was used for library preparation (Kapa). Samples were sequenced on an Illumina NextSeq 500 (Illumina) generating 75bp single-end reads.

ChIP-qPCR

Immunoprecipitated DNA was analyzed by quantitative Polymerase Chain Reaction (qPCR). 5µL of immunoprecipitated DNA, 5µL of primer mixture (2µM final concentration of each primer), and 10µL 2X Power Sybr Green PCR Master Mix (ThermoFisher) were used for each reaction. qPCR was carried out for 40 cycles on an ABI 7300 Real-Time PCR System, where each cycle consisted of 10 seconds at 95C, 30 seconds at 60C, and 30 seconds at 72C. A 10 minute 'hot start' at 95C was performed before the start of the first cycle, and a dissociation step was performed after the final cycle. Three technical replicates of each reaction were performed. The positive control primers generate an amplicon spanning from 100bp upstream to 50bp downstream of the transcription start site of the Jun gene – a well-known c-Jun binding site (Angel et al., 1988). The negative control primers generate a 167bp amplicon approximately 5kb upstream of the Jun TSS. This site does not have any predicted TRE or CRE binding motifs and was not observed to have any Jun binding within 1kb in the ENCODE Jun ChIPseq data set in mouse CH12.LX cells (ENCSR000ERO). The qPCR primers used are as follows: posCTL-FWD: 5'- atccagcctgagctcaacac-3', posCTL-REV: 5'- tcttctagggtgaggtctcca-3'; negCTL-FWD: 5'- tcattgctgagagcatggag-3', negCTL-REV: 5'- gagtggctgagagtccaagg-3'.

ChIPseq data processing

Bowtie2–2.2.3 was used to align the ChIPseq reads to the Ensembl GRCm38 version of the mouse genome (Langmead and Salzberg, 2012). Duplicate reads were marked with Picard tools 1.1.103 (<http://broadinstitute.github.io/picard>) and then removed with Samtools 1.2 (Li et al., 2009). Replicate inputs and eluates of each condition were then pooled. Macs2–2.1.10 (Feng et al., 2012) was used to call peaks on the pooled replicates using a fragment length of 190bp for each set. Macs2 bdgdiff command was then used on the bedgraph files of each sample set produced by the callpeaks algorithm. This produced the set of 1046 peaks used for all analyses presented here. The raw fastq files and fully-processed peak calls are available on GEO under accession GSE105442.

ChIPseq Quality Checking

Cross-correlation analysis was performed as described in (Landt et al., 2012) using version 2.0 of phantompeakqualtools (Kundaje, 2010) which runs on spp version 1.10.1 (Kharchenko et al., 2008). Read duplication analysis was performed according to the ENCODE3 specifications as described in (https://docs.google.com/document/d/11G_Rd7fnYgRpSIqrIfuVIAz2dW1VaSQThzk836Db99c/edit#). Briefly, after duplicate reads were marked with Picard tools but not yet removed, the fraction of reads that were non-redundant was calculated along with the fraction of reads that occurred exactly once. The ratio of reads that occurred exactly once to the total number of non-redundant reads gives us the PCR Bottleneck Coefficient (#1) reported in Supplementary Figure S1c. The Fraction of Reads in Peaks (FRiP) was calculated by using bedtools intersect (Quinlan and Hall, 2010) to find the reads from each pooled sample (with duplicates removed) that overlap with any of the peaks called by Macs2 on default settings (the peak set produced as an intermediate in the differential peak identification analysis) and then dividing those overlapping reads by the total number of non-redundant reads in the pooled sample. FRiP is a measure of the signal-to-noise ratio of the data, with scores of at least 1% being considered sufficiently high quality for transcription factor ChIPseq data sets (Landt et al., 2012). DiffBind 2.4.7 (Ross-Innes et al., 2012; Stark and Brown, 2011) running in R 3.4.0 was used to generate the sample clustering heatmap using the set of peaks produced by Macs2 bdgcmp. Each tile in the heatmap represents the correlation between a pair of samples. The correlation is calculated by counting the number of reads in each peak for each sample.

Genomic Distribution analysis

The set of transcription start sites was resolved from Ensembl annotation version 81 of GRCm38, taking the region within 2kb of each site, and then merging overlapping regions with bedtools merge (Quinlan and Hall, 2010). The set of enhancer regions was created by combining the enhancer sites from the 19 different tissues examined in (Shen et al., 2012), taking the region within 1kb of each site, and then merging overlapping regions with bedtools merge. This enhancer region set was then shifted from mm9 coordinates to mm10 coordinates using the UCSC liftOver tool. Bedtools intersect was then used to remove any enhancer regions that overlapped with TSS regions. Bedtools intersect was then used to identify the peaks that overlapped TSS-proximal regions. The remaining peaks that overlapped with enhancer regions were then extracted with another call to bedtools intersect. Homer version 4.8.3 annotatePeaks.pl script (Heinz et al., 2010) was used for annotation assignments of the remaining non-TSS-proximal and non-enhancer peaks into the categories of exon (at least 2kb away from TSS), intron (at least 2kb away from TSS), intergenic, or Transcription Termination Site. Finally, bedtools fisher was used to calculate the p-values and odds ratios of each set of peak overlaps with TSS or enhancer regions. Specifically, bedtools fisher uses the size of the entire mouse genome, along with the spans of the genome covered by each of the two feature sets being compared (peaks, TSS regions, and enhancer regions) in order to calculate a 2x2 contingency table from which it performs a fisher's exact test (Quinlan and Hall, 2010).

ChIPseq differential motif analysis

Motif analysis was performed using Homer 4.8.3 findMotifsGenome.pl script (Heinz et al., 2010). Using the sets of peaks more bound, less bound, and commonly bound after injury, each pair of peak sets were analyzed for differential motif enrichment by using one set as the foreground and the other as the background set of genomic regions for analysis. Each pair was tested in both orientations of this analysis to ensure that enrichment and depletion findings were consistent.

RNAseq data processing

The RNAseq data from (Motti et al., 2017) were downloaded from GEO GSE59547. The RNAseq data from (Tedeschi et al., 2016) were downloaded from GEO GSE66128. Both datasets were analyzed in the same way. Reads were aligned to the mm10 genome using Ensembl version 81 as the annotation. Alignment was performed using Tophat 2.0.12 running Bowtie2–2.2.3 (Kim et al., 2013; Langmead and Salzberg, 2012). Reads aligning to each gene were counted using HTSeq-count (Planet et al., 2012). Differential gene expression analysis was performed using edgeR 3.18.1 in R 3.4.0 (Robinson et al., 2010).

Differential Expression and ChIPseq analysis

To compare ChIP peaks with differentially expressed genes, peak-to-gene assignment was performed using the annotatePeaks.pl script from Homer 4.8.3 (Heinz et al., 2010). The output was then filtered using the ‘Distance to TSS’ column to select only the peaks within 2kb of TSSs. The sets of differentially expressed genes from the RNAseq analysis were then filtered to only include those genes with an associated peak and divided into the six pairwise comparison sets: upregulated genes with more bound peaks, upregulated genes with less bound peaks, upregulated genes with commonly bound peaks, downregulated genes with more bound peaks, downregulated genes with less bound peaks, and downregulated genes with commonly bound peaks. Each of these six sets of genes were then given as input to Panther 11 (Mi et al., 2017) for gene set enrichment analysis of Gene Ontology Biological Processes.

Tethered Dimer Construct Cloning

We received Jun~Jun, Jun~JunB, and Jun~Fos tethered dimer constructs as gifts from Dr. Erwin Wagner and Dr. Latifa Bakiri. We moved these coding regions into a pAAV vector which uses a CMV promoter and appended a 3x Flag tag to the N-terminus of each polypeptide. Using this same vector and Flag tag, we then cloned Jun~Atf3, Jun~Atf4, Jun~Atf7, Jun~Fosl1, and Jun~Fosl2 dimers using the mouse CDS for each gene. We then created monomer forms of each construct by excising Jun from the N-terminal position but leaving the Flag tag and the flexible tether. We additionally made a version of the Jun monomer with an HA tag in place of the Flag tag for use in co-transfection experiments. Finally, we used the Jun~Jun plasmid to replace the N-terminal Jun with various other dimer partners to create the Atf3~Jun, Fos~Jun, and JunB~Jun ‘reverse-orientation’ tethered dimers which still contain a Flag tag at the N-terminus.

HEK cell culturing and transfection

HEK293T cells were cultured in T75 tissue culture flasks (ThermoFisher) using DMEM (ThermoFisher) supplemented with 10% Fetal Bovine Serum (HyClone), Penicillin/Streptomycin, and Glutamax (ThermoFisher). Transfections were performed using Eugene 6 or Eugene HD reagents (Promega) with Opti-MEM (ThermoFisher) according to the manufacturer's protocol using a 3:1 ratio of transfection reagent to DNA. For luciferase assays, 10,000 cells per well were plated in clear bottomed, white-walled 96-well plates (Corning). These cells were grown in the media described above without phenol red to avoid interference with the assay. For the live/dead assays, 5,000 cells per well were plated in clear 96-well plates (Falcon) and were also grown in the media without phenol red for consistency with the luciferase assay. The reduction from 10,000 cells to 5,000 cells for the live/dead assay was necessary to achieve accurate quantification in that assay. For both the luciferase and live/dead assays, each well was then transfected with 5 μ L of transfection solution (containing a total of 100ng of DNA) 24 hours after plating. Experiments were then performed 48 hours after transfection. The same timeline was used for preparing the protein lysates for EMSAs, but 300,000 cells were plated in a 6-well plate instead. Consequently, the transfection reaction was scaled 30X as well.

Luciferase Assays

Luciferase assays were performed in HEK293T cells using the Dual Glo Luciferase Assay System (Promega) according to manufacturer's instructions using the pGL4.74 Renilla Luciferase under a Thymidine Kinase promoter as the internal control. Experiments were performed by transfecting 50ng of experimental plasmid, 25ng of Firefly luciferase, and 25ng of Renilla luciferase into each well. Three technical replicate wells were performed per condition per biological replicate. At least three biological replicates were performed for each experimental construct-luciferase pair. The data presented in Figure 3A–C, Supplemental Figure S3A,C–E, and Supplemental Figure S5 gives the Firefly / Renilla luciferase signal ratio for each experimental construct using the stated motif in the Firefly luciferase vector minus the average Firefly / Renilla luciferase signal ratio produced by the same experimental construct against the control construct which contained only a minimal promoter without an experimental motif. This approach was employed to remove the variance produced by non-specific effects of some experimental constructs on the minimal promoter luciferase construct, as shown in Supplemental Figure S3B. The pGL4.24 luc2P vector was used as the minimal promoter control construct. Experimental firefly luciferase vectors were made from the pGL4.29 luc2P/CRE/Hygro vector. This vector was digested with NheI and HindIII (NEB) to excise the three CRE sites initially upstream of the minimal promoter. Primers were synthesized containing one copy of the TRE, CRE and Degenerated CRE motifs along with their reverse complementary sequences. These pairs were annealed to create double stranded DNA with the appropriate pre-existing sticky ends. TRE sense oligo sequence: 5'-CTAGCCGCTTGATGACTCAGCCGAAA-3'. TRE anti-sense oligo sequence: 5'-AGCTTTTCCGGCTGAGTCATCAAGCGG-3'. CRE sense oligo sequence: 5'-CTAGCCGCTTGATGACGTCAGCCGAAA-3'. CRE anti-sense oligo sequence: 5'-AGCTTTTCCGGCTGACGTCATCAAGCGG-3'. Degenerated CRE sense oligo sequence:

5'-CTAGCCGCTTGATGACGCAAGCCGGAAA-3'. Degenerated CRE anti-sense oligo sequence: 5'-AGCTTTTCCGGCTTGCGTCATCAAGCGG-3'.

Electrophoretic Mobility Shift Assays

Forty-eight hours after transfection, nuclei were isolated from HEK293T cells and lysed in 1mL of a Tris buffer containing 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine, 1mM DTT, protease inhibitor cocktail and PMSF (ActiveMotif). BCA of these samples identified their total protein concentration as being between 100–200ng/uL. Western blot of the samples probed with an anti-flag antibody (Sigma F1804) demonstrated that each of the tethered dimer constructs produced a single polypeptide strand product of approximately the correct molecular weight (data not shown). EMSAs were performed by mixing 1μL of the nuclear protein lysate with 50fmol of each labelled oligo in a total volume of 20μL using the binding reaction solution described the Odyssey Infrared EMSA Kit (LI-COR). Jun~Atf3, Jun~Atf4, Jun~Fos, Jun~Fos1, and Jun~Fos2 bound such a large fraction of the labelled oligos in their reactions that they were diluted 1:10 further before being added to the binding reaction. Binding reactions were incubated in the dark for 40 minutes while 4.5% non-denaturing Tris-borate polyacrylamide gel was pre-run at 70V. One microliter of Orange Loading Dye (LI-COR) was then added to each reaction and samples were loaded and run on gel at 70V for 60 minutes. Gels were then imaged on an Odyssey Infrared Imaging System (LI-COR) using both the 700nm and 800nm fluorescent channels. Oligonucleotides with the 700nm or 800nm fluorophore labelling on the 5' end of each strand were purchased from IDT and then annealed to produce double stranded labelled DNA oligos. TRE oligo sense sequence: 5'-IR700-CGCTTGATGACTCAGCCGGAA-3'; anti-sense: 5'-IR700-TTCCGGCTGAGTCATCAAGCG-3'. CRE oligo sense sequence: 5'-IR800-CGCTTGATGACGTCAGCCGGAA-3'; anti-sense: 5'-IR800-TTCCGGCTGACGTCATCAAGCG-3'. Degenerated CRE oligo sense sequence: 5'-IR700-CGCTTGATGACGCAAGCCGGAA-3'; anti-sense: 5'-IR700-TTCCGGCTTGCGTCATCAAGCG-3'.

Dorsal Root Ganglion Culturing and Transduction

AAV8 particles were prepared by the University of Miami Viral Vector Core from the following subset of the constructs described above: Jun (with HA tag), Jun (with Flag tag), Fos, Atf3, Atf4, Jun~Jun, Jun~Atf3, Jun~Atf4, and Jun~Fos. AAV8-GFP was purchased from the University of Miami Viral Vector Core and used as the control condition in these experiments, as Osr1-2A-GFP was too large to package into AAV. Approximately 4.3×10^{11} genomic copies of virus were added to each well for each condition. For mixtures of two viruses, both were added in equal proportions so that the total amount of virus added was still 4.3×10^{11} genomic copies. Adult C57BL/6 mice were sacrificed and their DRGs dissected as described in the Chromatin Immunoprecipitation section above. Neurons were plated at a concentration of 1,000 cells per well in 24-well plates (Falcon) coated with 0.5mg/mL poly-D-lysine (Sigma). Two technical replicate wells were used per condition per biological replicate. Cells were cultured in DMEM F12 media. DRG neurons were grown for 2 days in culture at 37°C with 5% CO₂ before fixation and immunofluorescent staining. Wells containing non-transfected cells in each plate were used as negative controls for the staining.

Cortical Neuron Culturing and Transfection

Postnatal day 3 Sprague-Dawley rat pups (Charles River) were sacrificed and their cortices dissected as described in (Mehta et al., 2016). 150,000 cells were then resuspended in 20 μ L of P3 Primary Cell Nucleofector Solution + Supplement (Lonza; Catalog #V4SP-3096) with 1.7 μ g of plasmid DNA and transfected using the AMAXA Nucleofector 96-well Shuttle System (program: Neurons, High Viability; Lonza). Cells were then plated at a concentration of 15,000 cells per well in 24-well plates (Falcon) coated with 0.5mg/mL poly-D-lysine (Sigma). Three technical replicate wells were used per condition per biological replicate. Cells were cultured in Proneural Growth Medium supplemented with PNGM SingleQuots (Lonza; Catalog # CC4461) conditioned overnight using cultured P5 rat glia. Cortical neurons were grown for 2 days in culture at 37°C with 5% CO₂ before fixation and immunofluorescent staining. The bottom row of each plate contained non-transfected cells in each well as a negative control for the staining.

Hippocampal Neuron Culturing and Transfection

Embryonic day 18 Sprague-Dawley rat pups (Charles River) were sacrificed and their hippocampi dissected as described in (Buchser et al., 2010). Transfection of these neurons was performed identically to cortical neurons as described above. Cells were then plated at a concentration of 3,000 cells per well in 96-well plates (Falcon) coated with 0.5mg/mL poly-D-lysine (Sigma). Eighteen technical replicate wells were used per condition per biological replicate. Cells were cultured in NBActive4 medium (BrainBits) at 37°C with 5% CO₂ for 2 days before fixation and immunofluorescent staining. The bottom row of each plate contained non-transfected cells in each well as a negative control for the staining.

Immunofluorescent Staining of neurons

DRG, cortical and hippocampal neurons were fixed and stained the same way, with the only difference being the volumes adjusted for 24-well vs 96-well plates. Media was removed from the neurons and replaced with 4% formaldehyde in PBS for 20 minutes. Cells were washed four times with PBS to remove the formaldehyde, then treated with 0.5% Triton X-100 in PBS for 10 minutes to make the cellular membrane permeable. This Triton solution was then removed with four washes of PBS. The cells were then incubated in blocking solution (PBS with 5% Normal Goat Serum, 0.5% BSA, and 0.03% Triton X-100) for 1 hour. Fresh blocking solution was then added containing the primary antibodies. Mouse anti-flag (Sigma; Catalog # F1804) was used at 1:1000, Chicken anti-GFP (Abcam; Catalog # ab13970) was used at 1:2000, and Rat anti-HA (Sigma; Catalog # 11867423001) was used at 1:1000, and Rabbit anti-BIII-tubulin (Sigma; Catalog # T2200) was used at 1:2000. Cells were incubated overnight at 4°C in the primary antibody solution. This solution was then removed with four PBS washes. Cells were then incubated in blocking buffer containing secondary antibodies and Hoechst at room temperature for 1 hour. Goat anti-mouse 488 (ThermoFisher), goat anti-mouse 546 (ThermoFisher), goat anti-rat 488 (ThermoFisher), goat anti-chicken 488 (ThermoFisher), and goat anti-rabbit 647 (ThermoFisher) were used at a dilution of 1:1000. Hoescht was used at a final concentration of 4 μ g/mL. The secondary antibody solution was removed with four PBS washes.

High Content Analysis of DRG, Cortical and Hippocampal Neurons

DRG, cortical and hippocampal neurons were scanned with a Cellomics ArrayScan VTI automated microscope (Cellomics). Images were automatically traced using the Neuronal Profiling Bioapplication (version 3.5). Only cells with at least one 10µm long BIII-tubulin-positive process were used in the ensuing analyses. The non-transfected row of neurons in each plate were used to sample the background distribution of fluorescent intensities. This was done separately for each staining condition because different combinations of primary and secondary antibodies exhibit differing levels of background fluorescence. The 99th percentile of this distribution of background signal was then used as the cutoff for declaring a cell with that staining condition as transfected or transduced. Typical transfection/transduction rates ranged from 10–50%, depending on the construct. Transfected or transduced neurons of each condition were then compared to Oxr1–2A-GFP neurons (GFP-transduced neurons in the case of DRGs) from the same plate in order to calculate their relative effect on neurite total length (NTL) and length of the longest neurite (LOLN). For a biological replicate of a condition to be considered successful and included in downstream analysis, at least 5% of the traced neurons needed to be transfected/transduced with a total of at least 50 neurons for the individual transfections/transductions or 25 neurons for the co-transfections/transductions. Using these criteria, one replicate of the JunB monomer, and two replicates of the Jun~Jun and Jun~JunB tethered dimers were excluded from analysis for the hippocampal experiments out of the five replicates performed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We would like to thank present and past members of the LemBix Lab for discussions and assistance as well as Yan Shi and the U.M. High Content Screening Core. This work was supported by National Institutes of Health HD057632 (JLB and VPL), the Buoniconti Fund (JLB and VPL), the Miami Project to Cure Paralysis (JLB and VPL), and the Walter G. Ross Foundation (VPL).

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Highlights

- The majority of Jun's axotomy-induced changes in DNA binding occur at enhancers
- Changes in Jun binding near genes does not predict changes in their expression
- Single polypeptide chain dimers of Jun and Atf3 promote CNS neurite outgrowth
- Single polypeptide chain dimers containing Jun behave differently than a mixture of the corresponding monomers

Figure 1:

Peripheral axotomy results in a change to Jun's binding localization. A) Venn Diagram of sites bound by Jun. 701 sites are significantly more bound after injury, 185 sites are significantly less bound after injury, and 160 sites are bound to a similar extent both before and after injury. B-D) Distribution of peaks more bound (B), less bound (C), and commonly bound (D) after injury by genomic regulatory annotation demonstrates that the majority of Jun's injury-responsive binding site changes occur at putative enhancer elements while the majority of Jun's injury-invariant binding sites occur proximal to TSSs. Non-TSS and non-enhancer proximal intronic, exonic, intergenic, and transcription termination sites (TTS) are also shown. E) Bar plot of odds ratios (error bars correspond to 95% confidence intervals) obtained by Fisher's exact test on the overlap of each peak set with each regulatory element set. All bars plotted are significant relative to random genomic binding (Odds Ratio of 1, shown by horizontal line). All pairwise comparisons of bars are significant except for the

two pairs specifically marked as non-significant (n.s.). This means that the distribution of differentially bound peaks across enhancer and TSS-proximal regions is different from that of the commonly bound peaks, but the distribution of more bound peaks is not different from that of less bound peaks.

Figure 2: Differential Jun binding near transcription start sites. A) Bar plot showing the proportion of differentially- and commonly-bound Jun sites within 2kb of a TSS whose associated genes are upregulated (left) or downregulated (right) 7 days after sciatic nerve axotomy. This plot indicates that differentially bound peaks near gene transcription start sites are not predictive of the differential expression of those genes. B) Bar plot of enriched Biological Process GO terms from the set of significantly upregulated genes with Jun peaks within 2kb of their TSS which are significantly more bound after injury. This list is a sampling of the complete list found in Supplemental Table S2 and shows a strong enrichment for neuronal and axon growth-related terms. C) Bar plot of enriched Biological Process GO terms from the set of significantly downregulated genes with Jun peaks within 2kb of their TSS which are

significantly less bound after injury. This list is a sampling of the complete list found in Supplemental Table S2 and does not show any enrichment for neuronal terms at all.

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Figure 3: The DNA motif transactivation potential of eight Jun-containing dimers. A-C) Bar plot of the minimal promoter-normalized ratio of Firefly luciferase signal to Renilla luciferase signal for Oxr1 and each of the eight tethered dimers when using a Firefly luciferase plasmid containing a CRE (A), TRE (B), or Degenerated CRE (C) motif upstream of a minimal promoter. In each panel, * indicates p-value <0.05, ** indicates p-value <0.01, *** indicates p-value <0.001 in a one-way ANOVA with a post-hoc Dunnett's test. N=6 in panels A and C. N=3 in panel B. Horizontal dashed line in each panel indicates average signal produced by Oxr1 in that condition. Error bars represent standard error of the mean among biological replicates. Jun~Atf3 significantly promotes luciferase signal when exposed to the CRE or Degenerated CRE motifs. Jun~Fosl1 significantly promotes luciferase signal when exposed to the CRE or TRE motifs. D) EMSA dose-response experiment showing the binding of

Jun~Atf3 to 50fmol of CRE oligo in each reaction and increasing quantities of TRE oligo, with quantities shown at the top. E) Quantification of the CRE oligo signal expressed as a percentage of the signal obtained when no TRE oligo is added, plotted as a function of increasing concentration of TRE oligo. This sigmoidal plot was fit using the Hill equation to predict the concentration of TRE oligo necessary to decrease the CRE signal to half of its initial value. This IC_{50} value was found to be 82 fmol of TRE.

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Figure 4: Jun~Atf3 promotes neurite outgrowth in DRG and cortical neurons. In each panel, * indicates p-value <0.05, ** indicates p-value <0.01, *** indicates p-value <0.001 in a one-way ANOVA with a post-hoc Dunnett's test. N=3 for each condition. Error bars represent standard error of the mean among biological replicates. A-B) DRG neuron results. A) Effect of monomers, co-transduced monomers and tethered dimers on Neurite Total Length (NTL). B) Effect of monomers, co-transduced monomers and tethered dimers on the Length of the Longest Neurite (LOLN). C-J) Cortical neuron results. C) Effect of monomers and co-transfected monomers on NTL. D-F) Effect of tethered dimers consisting of Jun and an Atf (D), Jun (E), or Fos (F) family member on NTL. G) Effect of monomers and co-transfected monomers on LOLN. H-J) Effect of tethered dimers consisting of Jun and an Atf (H), Jun (I), or Fos (J) family member on LOLN. K) Representative images of a cortical neuron transfected with Oxr1-2A-GFP (left) and another transfected with Jun~Atf3 (right). βIII-

tubulin staining is shown in white and GFP (left) or Flag (right) staining is shown in green. Cellomics tracing of neurites is overlaid in white to improve visibility.

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