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Neuropathology. Author manuscript; available in PMC 2013 August 01.

Published in final edited form as:

Neuropathology. 2012 August ; 32(4): 420–431. doi:10.1111/j.1440-1789.2011.01280.x.

Isoform diversity and its importance for axon regeneration

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Abstract

Axon regeneration is a fundamental problem facing neuroscientists and clinicians. Failure of axon regeneration is caused by both extrinsic and intrinsic mechanisms. New techniques to exam gene expression such as Next Generation Sequencing of the Transcriptome (RNA-Seq) drastically increase our knowledge of both gene expression complexity (RNA isoforms) and gene expression regulation. By utilizing RNA-Seq, gene expression can now be defined at the level of isoforms, an essential step for understanding the mechanisms governing cell identity, growth and ultimately cellular responses to injury and disease.

Keywords

RNA-Seq; axon; regeneration; promoter; isoform; variant; untranslated region; coding DNA sequence; transcription start site

Adult central nervous system (CNS) neurons fail to regrow their axons and restore functional connections after an injury. When CNS axons are severed their distal portions undergo Wallarian degeneration--a process described by Santiago Ramon y Cajal nearly one hundred years ago[1]. Subsequently, the axonal endings proximal to cell bodies form dystrophic end bulbs that partially retract into highly dynamic structures[2] that persist in the lesion site for weeks to months[3] suggesting that severed axons retain motility but are inhibited in their attempts to regenerate. This view is supported by the finding that some CNS axons are able to extend long axons through permissive peripheral neuron grafts[4,5]. This and related findings led to the idea that the CNS environment present after an injury inhibits axon regeneration. Subsequently, major research efforts have focused on trying to understand the environmental influences that prohibit the axonal ends from growing across injury sites. These efforts identified key players that contribute to regenerative failure: immune cells including macrophages and microglia, reactive astrocytes which produce both physical and chemical barriers (reviewed in [6,7]), and the by-products of myelin degradation[8] (reviewed in [9]). Since the identification of these extrinsic inhibitory influences, much work has focused on neutralizing or overcoming their effects. Unfortunately elimination of the various inhibitory factors does not result in major improvements in axonal regeneration[10–13]. Considering these findings, it is likely that the majority of neurons themselves are not in a state in which they can successfully regrow an axon, even when presented with favorable environmental conditions.

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What evidence is there to suggest that adult CNS neurons need intrinsic modifications for axonal regeneration to succeed? First, there are substantial differences in the ability of embryonic versus adult CNS neurons to extend axons. A very simple observation is that culturing most adult CNS neurons is extremely difficult, if not impossible, whereas embryonic and early postnatal CNS neurons are easily cultured. This fundamental observation demonstrates that older CNS neurons are not capable of the plasticity and adaptability needed to survive in challenging conditions. In addition to this simple observation, there are clear differences in developmentally regulated gene expression changes that are associated with the growth properties of embryonic and early postnatal neurons suggesting differential gene expression changes contribute to the reduced axonal growth ability in mature CNS neurons[14]. Second, while axons from injured embryonic spinal cord can regenerate, if the same experimental lesion is performed in older spinal cords, regeneration fails[15–17]. Another piece of evidence stems from the observations that peripheral nervous system neurons, such as dorsal root ganglion (DRG) neurons, are capable of regenerating an axon[18]. DRG neurons exhibit robust growth in culture and grow axons into CNS white matter myelin tracts after injury[19]. Further, DRG neurons exhibit enhanced regeneration of both peripheral and central axons following injury so long there is a previous injury to the peripheral axon; this effect is known as a conditioning lesion[18,20]. If translation is pharmacologically blocked in DRG neurons, their ability to regenerate after injury is compromised[21]. These observations imply that failure of CNS neurons to regenerate axons is not solely due to the environment but that the pattern of neuronal gene expression is an important contributor to regenerative failure.

Several recent studies have identified genes important for axon regeneration such as the Krüppel-like transcription factors (KLFs) and cellular growth pathways involving mammalian target of rapamycin (mTOR) and the phosphatase and tensin homologue[22–25] (PTEN). Since the importance and relevance of developmentally regulated transcription factors, such as the KLF family, and intrinsic growth pathways like mTOR and PTEN are nicely summarized in two recent reviews[26,27], we will instead discuss efforts aimed at understanding how gene isoforms differ functionally and may be critical factors influencing the potential for axons to regenerate.

What are isoforms and why are they relevant to axon regeneration? Gene isoforms are messenger RNAs (mRNAs) that are produced from the same locus but are different in their transcription start sites (TSSs), protein coding DNA sequences (CDSs), and/or untranslated regions (UTRs), potentially altering gene function[28,29]. One mechanism of changing the expression of coregulated genes is through the use of alternative TSSs[30,31]. The idea that different binding partners confer different functional properties has been well studied in tissue specific gene regulation. For example, the same TF can direct gene expression in different tissues simply by binding with different TFs in each tissue[32]. This same idea may hold true for neuronal subtype specification and one way it could occur is through employment of alternative TSSs which direct different combinations of co-regulated genes (Figure 1). Alteration in CDSs can impart completely different protein functions, depending on what exons are ultimately expressed and what functional domains are included in the protein[33]. UTRs regulate the levels of primary transcript in numerous ways: transcript stability, folding, and turnover as well as translation efficiency[34,35]. In general, specific isoforms exhibit temporal or spatial differences in expression patterns, and it has been reported that alternatively expressed isoforms have different functions in the same cells. Exon usage has been shown to differ between tissues[36] and it is likely that exon usage differs between neuronal subtypes and in different pathological states. Defining these isoforms is important for understanding neuronal gene expression in both health and disease. Indeed, some progress has been made to characterize known isoforms of regeneration associated genes (Table 1). However, a comprehensive understanding of the roles of these

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genes requires identification of the full isoform repertoire of each neuronal cell type under different conditions (i.e. injured vs. non-injured).

How are different isoforms expressed in specific cell types/conditions identified? First the appropriate comparison has to be made to identify relevant isoforms. To do this it is necessary to compare gene expression in regenerating versus non-regenerating neurons. Isoforms identified this way are commonly referred to as ‘regeneration associated’ genes (RAGs). Second, the sequence of all mRNA species within a cell needs to be defined. New technologies such as Next Generation Sequencing of the Transcriptome (RNA-Seq) make this possible[29,37]. RNA-Seq allows detection of all gene isoforms expressed from a locus, a tremendous advantages over traditional microarray approaches. The functional importance of isoform diversity in the CNS is starting to be explored[38–40], but examples of the importance of specific isoforms to axon regeneration are limited even though the number of known isoforms produced from regeneration associated genes is high (Table 1). This knowledge gap is probably due to the technical challenges involved in studying the roles of individual isoforms in neurons before the use of RNA-Seq methodology. Not only was it difficult to identify all the mRNA isoforms expressed from a given gene locus in a given cell type, but the methods needed to determine the function of individual genes were and still are less than optimal. For example, overexpression studies rely on specific cDNAs, examining the function only of the protein coding region portion of the transcript, typically ignoring the function of 3′ or 5′ UTRs. Similarly troubling is the fact that loss of function studies typically disrupt the function of all or most isoforms. Here we highlight some examples of individual isoform function relevant to axon regeneration and outline some potential RNA-Seq analyses strategies to identify relevant isoforms and their regulation (Figure 2; Table 2).

Alternative transcription start sites

Cis-regulatory elements in the promoter contain sequences recognized by transcription factors and the basal transcription machinery; these are key elements and an important step in regulating isoform expression. Because of these cis-regulatory elements within the promoter, the location of the TSS is important for understanding the biogenesis of specific isoforms[30,41]. As mentioned, RNA-Seq enables the determination of individual isoform TSSs. Subsequently, the promoter DNA sequence can be isolated and then analyzed for cis-elements. Once this is accomplished for all the isoforms expressed in different cells types/states, such as a comparison between neurons capable or incapable of axon regeneration, it is possible to identify sets of isoforms undergoing similar patterns of gene expression regulation.

How can you predict the cis-elements in the promoters of individual isoforms? There are numerous freely available software programs capable of scanning nucleotide sequences and evaluating the frequency of transcription factor binding sites[42–45]. This enables the identification of candidate transcription factors regulating individual isoforms, which may be critically important if differential isoform expression arises from the TSSs, and not in the coding DNA sequence. Understanding gene expression regulation at the promoter level is not without precedent; using zebrafish RGCs as a model for developing and regenerating axons, two rat promoters for growth associated protein 43 (*Gap43*) were identified. One promoter is active when RGCs are growing to their targets in early development and a different promoter is active when they are regenerating[46]. While GAP43 is expressed in the developing and regenerating optic nerve, this study suggests that the genetic programs driving axon regeneration and axon path finding during development are different. Understanding the extent and importance of these differences is crucial to devising a plan to reactivate developmental gene programs that promote axon growth after injury.

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Do other regeneration associated genes utilize alternative TSSs? Activating transcription factor 3 (*Atf3*) is a known regeneration associated gene[47,48] associated with numerous promoters. *Atf3* expression increases after nerve injury and overexpression of a constitutively active form of *Atf3* increases the rate of peripheral nerve regeneration[47,49]. In cancer studies an alternative TSS was previously characterized[50]. Each promoter is active under different conditions. One *Atf3* isoform uses the P1 promoter (Figure 3) which is primarily activein response to stress and in numerous cancers[50], whereas the conventional *Atf3* promoter (P2) is typically activated in response to mitogenic stimuli[51]. While the CDS for these isoforms is the same, one way functional differences may occur is through differential promoter regulation resulting in functional differences resulting from changes in coregulated genes. Alternatively, these promoters may be driving varying levels of ATF3 expression. It is unclear which promoters are in use in regenerating DRG neurons and whether the same promoters are used during development when axons are first extending towards their targets.

The use of alternative TSSs is not limited to *Atf3*. Numerous regeneration associated genes have known alternative TSSs annotated in the UCSC Genome Browser (Figure 1; Table 1). Some examples are themitogen-activated protein kinase 8 interacting protein 1 (*Mapk8ip1*/ Jip1), protein tyrosine phosphatase receptor type S (*Ptprs*), and Rho-associated coiled-coil containing protein kinase 2[52–55] (*Rock2*). MAPK8IP1/JIP1, a scaffolding protein for the c-Jun N-terminal kinases (*Jnks*), is a newly identified regeneration associated gene with multiple TSSs[53] (Figure 3). *Mapk8ip1* null DRG neurons exhibit delayed neurite extension and reduced neurite length, possibly due to a reduction in JNK phosphorylation[53]. Which isoforms are expressed after peripheral nerve injury is currently unknown. If *Gap43* serves as an example, it will be interesting to determine if these alternative promoters are active in a particular combination during development and then in another combination during axon regeneration. Identifying the set of promoters in use during development and in regeneration is necessary if the goal is to define what pathways need to be reactivated to elicit an optimal intrinsic regeneration response in injured neurons.

Alternative protein coding DNA sequences (CDS)

Isoforms harboring changes in the CDS have been the most thoroughly characterized because they commonly give rise to proteins with different functional properties[33]. In neurons, some well characterized genes which produce isoforms with changes in CDS are members of the immunoglobulin superfamily, molecules which have well defined roles in facilitating axon growth and guidance. Three genes in this family, neural cell adhesion molecule 1 (*Ncam1*), L1 cell adhesion molecule (*L1cam*), and deleted in colorectal cancer (*Dcc*), all undergo alternative splicing giving rise to isoforms that exhibit cell type and developmental stage specific expression in the nervous system. For example, *Ncam1* has isoforms that produce proteins of 180kD, 140kD, or 120kD in size[56]. These isoforms show preferential expression in different cell types and contribute to different aspects of neuronal phenotype such as neuronal process branching and synaptic maturation (reviewed in[57]). In the injured spinal cord of rats treated with a monoclonal antibody to Nogo-A (IN-1), *Ncam* expression increased. This result suggests that *Ncam* could be a target to enhance axon regeneration[58]; however which *Ncam* isoforms were upregulated remains unknown. *L1cam* is another regeneration associated gene which undergoes alternative splicing yielding two alternative isoforms with different functions[59–61]. Alternative splicing that includes both exons 2 and 27 defines the predominantly neuronal, long L1CAM isoform[59,62]. Specifically exon 27 is involved in L1CAM targeting to neuronal growth cones and exon 2 is used in determining ligand specificity[63,64]. The L1CAM isoform without exons 2 and 27 is considered the short form and is predominantly expressed in glial and nonneuronal cells[62,65]. NCAM and L1CAM isoforms are examples of how the same gene can produce

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proteins with functional and cell type dependant expression differences, but isoforms with CDS changes may also define specific populations of neurons. For example, a recent study which profiled the transcriptome of developing cortical layers using RNA-Seq identified over 1500 layer specific isoforms[66]. Interestingly, numerous regeneration associated genes were found to have layer specific isoforms (*Mapk8ip1*, *Ptprs*, *Rock2*, *Bex1*, and microtubule associated protein 1 a: *Mtap1a*) indicating that these genes have the potential to produce differentially regulated isoforms, and thus differ in their function as well. The brain expressed X-linked (*Bex1*) gene locus produces four different isoforms, each having different CDSs (Figure 3). Understanding the possible alterations in function and/or expression pattern is relevant to axon regeneration because BEX1 interacts with the p75 neurotrophin receptor ($p75^{NTR}$), is expressed in a variety of neuronal tissues during development, and regulates neuronal differentiation[67]. Recently, it has been shown that BEX1 is localized to neuronal processes, its expression substantially increases in both motor and dorsal root ganglion neurons after a peripheral nerve injury, and that *Bex1* null mice undergo delayed peripheral nerve regeneration[52].

The KLFs are zinc finger transcription factors, most of which are expressed in the mammalian CNS. The first study demonstrating the importance of two members of the Krüppel-like family of transcription factors (KLFs), KLF6 and KLF7, for optic nerve regeneration was performed in zebrafish[68]. Interestingly, several members of this TF family have opposing expression patterns throughout development. This pattern correlates with the age of the neuron and its ability to grow an axon: KLF4 and -9 are highly expressed in adult neurons which show reduced axon regeneration potential, overexpression of KLF4 or -9 suppresses neurite outgrowth in vitro, and conditional knockout of KLF4 in RGC neurons results in enhanced axon regeneration after optic nerve crush[22,69]. Conversely, KLF6 and -7 are highly expressed in embryonic neurons but downregulated in the adult, and overexpression in RGC, hippocampal, or cortical neurons in vitro increases neurite outgrowth[22,69].

Is there reason to believe that *Klfs* give rise to multiple KLF isoforms? A study examining zinc-finger transcription factors, which includes *Klfs*, found that over 50% of these genes produce multiple isoforms[70]. Indeed, new isoforms for *Klf13* were identified with changes in the number of zinc finger domains, which is likely to affect DNA binding ability and thus *Klf13* function[70]. The expression and identity of *Klf6* and *Klf7* isoforms in the CNS is unknown. It is known that *Klf7* expression is high in developing TrkA+ DRG neurons[71], but only *Klf6* has been reported to be upregulated after a sciatic nerve injury[72]. Currently, we do not know if different isoforms of *Klf6* or *-7* exist, but it is plausible that different isoforms would have different functions in the context of axon regeneration.

Alternative 5′ and 3′ Untranslated Regions

What are untranslated regions (UTRs) and what is their function? UTRs are regions of mRNAs that do not code for protein. They are interesting because they confer regulatory information to the primary transcript. UTRs can regulate mRNA stability, translation rate, harbor sequences that mediate subcellular targeting of mRNAs (zipcode sequences) and/or those that govern global gene expression such as microRNA (miRNA) Response Elements (MREs)[34,35,73,74].

Alternative splicing and polymorphisms in the 3′UTR have been shown to affect isoform function through regulation by miRNAs, which can modulate translation via regulation of the primary transcript[75]. MiRNAs typically downregulate transcript expression by triggering degradation or halting translation. For example, the homeobox (*Hox*) genes are responsible for patterning the anterior/posterior body axis and their expression along the body axis is

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tightly controlled during development. In *Drosophila*, several isoforms of the *Hox* gene family differ in the lengths of their 3′UTRs, imparting differences in the MREs within those transcripts[76]. Thus these isoforms undergo developmental regulation and could have specific function in setting up anteroposterior body axis[76]. In addition to development, different sequences in the UTRs of isoforms may affect pathological states. For example, polymorphisms within the 3′UTR of amyloid precursor protein (*App*) are associated with altered miRNA regulation of the primary transcript and could be linked to Alzheimer's disease progression[77]. MiRNA activity in neurons can regulate activity dependent dendritic spine formation[78,79]. While these are examples of individual isoform regulation, miRNAs have the potential to simultaneously regulate hundreds of transcripts, also making them global regulators of gene expression[75]. A role for miRNAs in axon regeneration is just starting to be explored[80] but understanding which miRNAs regulate genes that are prohibitive to axon growth could provide an important new mechanism to globally alter gene expression in ways that would activate intrinsic axon growth pathways in injured neurons.

One of the most well characterized examples of a UTR effecting function and mRNA targeting is the alternative transcripts produced from the brain derived neurotrophic factor (*Bdnf*) gene. *Bdnf* produces two isoforms that differ in the lengths of their 3′ UTRs while coding for the same protein[81]. The isoform with the shorter 3′ UTR is sequestered in the neuronal cell body while the transcript with the longer 3′ UTR is targeted to dendrites. This differential targeting enables activity dependent translation in the dendrites where BDNF has an essential role in regulating dendritic arborizations and long term potentiation[81]. The glucocorticoid receptor (GR; gene name: nuclear receptor subfamily 3, group C, member 1: *Nr3c1*) is another example of a gene with enormous diversity in both the 5′ and 3′ UTRs. Because GR is ubiquitously expressed, this diversity is thought to enable tissue specific isoform expression (reviewed in[82,83]).

Of the isoform regulatory mechanisms conferred by UTRs, one most relevant to axon regeneration may be targeting of mRNAs to the axon via zipcode motifs. For example, *L1cam* and beta-1-integrin (*Itgb1*)promote axonal growth in CNS injury models. However, gene therapy strategies using them might fail because the transcript in question does not target appropriately within the neuron to promote axon growth[60,84]. How are transcripts targeted to specific subcellular locations in neurons? Transcripts are targeted by 'zipcodes' which are sequences found in the 3′ UTR that enable mRNA trafficking proteins, such as zipcode binding protein 1 (*Zbp1*) and poly(rC) binding protein 1 (*Pcbp1* aka: heterogeneous nuclear ribonucleoproteinE1; *hnRNP*), which bind and shuttle the transcript to the area of the cell where it will be translated[73,85]. One of the first characterized zipcode motifs and mRNA binding proteins involved in neuronal process transcript targeting was demonstrated in the cytoskeletal protein component beta actin (*Actb*)[86,87]. *Actb* targeting relies on a relatively short nucleotide motif (~40–55 base pairs in length) and disruption of transcript targeting by mutating the nucleotide motif alters cytoskeletal organization[87]. Identifying additional zipcodes is challenging because methods have relied primarily on alignment, examination of conserved sequences, and mutation or deletion analysis. This is where using RNA-Seq becomes advantageous because if the targeting of individual isoforms is to be understood, then the sequence identity has to be recovered[88]. Predicting motifs that can target a transcript to the axon will allow for future gene therapy approaches to appropriately target growth promoting transcripts to the growth cone of injured axons and perhaps facilitate meaningful regeneration.

In summary, to fully understand neuronal specific gene expression and the mechanisms that regulate differential isoform expression between neuronal subtypes, we need to understand the full repertoire of transcripts expressed from each gene; a new technology, RNA-Seq,

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makes this possible. With this technology we now have the ability to precisely define the isoforms expressed within specific neuronal populations, examine their sequences for changes in the 5′ or 3′ UTRs which may alter TSS, transcript expression regulation, and examine changes in the CDS which may affect functional domains in the protein. Since these alterations in mRNAs generated from the same gene loci may affect function, they are necessary to consider during the elucidation of cell type specific isoform function. Applying RNA-Seq to the question of axon regeneration will undoubtedly increase our ability to define the isoforms relevant to axonal growth and our ability to understand the regulation of 'regenerating' versus 'non-regenerating' neurons.

Acknowledgments

This work was supported by National Institutes of Health grants HD057632 and NS059866, U.S. Army (W81XWH-05-1-0061), the Walter G. Ross foundation and the Buoniconti Foundation. We thank Dr. Murray Blackmore for helpful discussions, Dr. Dario Motti, Dr. Kunie Sakurai and Samuel Beckerman for helpful edits of the manuscript.

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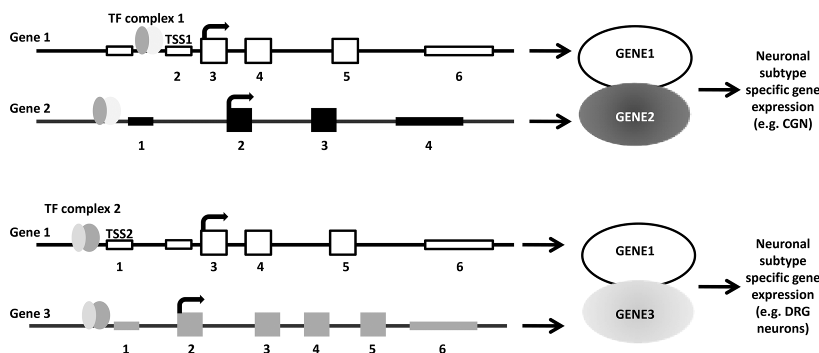

Figure 1. Example of how alternative TSSs can impart functional changes to isoforms from the same gene loci

Gene 1 has two TSSs (TSS1 and TSS2), one CDS (exons 3–5) and one 3′ UTR (exon 6). In this example the only difference in the two isoforms expressed from the Gene 1 locus is in the TSS. Transcription factor complex 1 (TF complex 1) simultaneously regulates Gene 1 and 2 by binding to cis-elements upstream of TSS1 and the TSS in Gene 2. A different TF complex (TF complex 2) regulates the expression of Gene 1 and Gene 3. In this example, GENE2 or GENE3 co expression with GENE1 results in different functional outcomes: specification of sensory neurons or cerebellar granular neurons (CGNs). Genomic DNA is indicated by the black line, UTRs are thinner boxes and CDS encoding exons are thick boxes.

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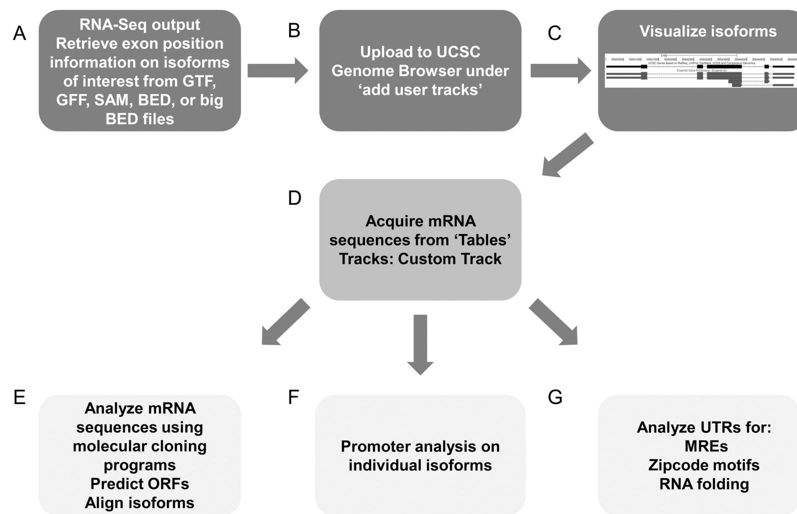

Figure 2. One approach to the downstream analysis of RNA-Seq data to identify and analyze mRNA isoforms

Algorithms that assemble the small sequence fragments produced by Next Generation Sequencing will produce a file type (GFF, GTF, BED, big BED) that can be uploaded to the UCSC Genome Browser to visualize isoforms in the context of the reference genome (A, B, C). Once transcripts are visualized in the browser, the Tables tab will allow retrieval of the sequences in the user defined track, in this case, isoforms (C). After individual isoform sequences are in FASTA format they can be easily input into molecular cloning and analysis programs to predict open reading frames (ORFs) and align isoform sequences (E) as an example. TSS position information also facilitates isolation of the genomic promoter DNA sequence (F). UTR sequences can be uploaded to various programs for downstream analysis such as zipcode motifs, which target mRNAs within cells, and for microRNA Response Elements (MREs; G).

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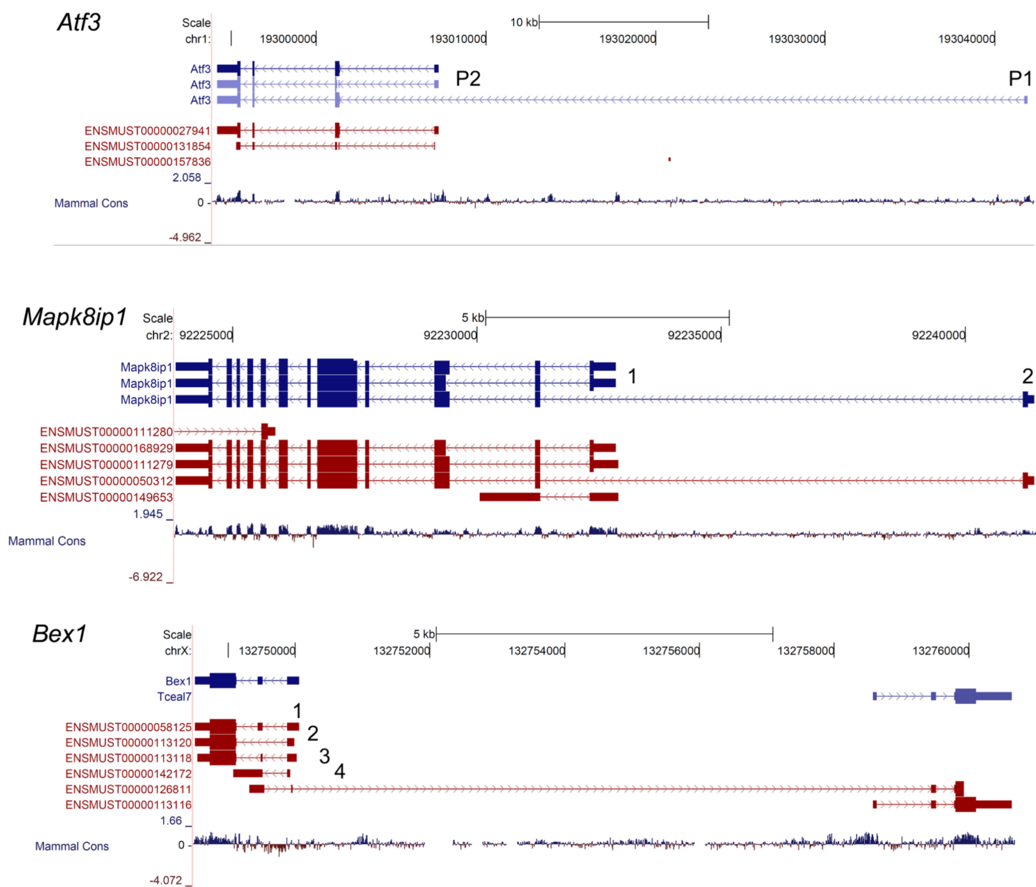

Figure 3. UCSC Genome Browser tracks for Atf3, Mapk8ip1, and Bex1

Mouse isoforms in the UCSC known genes database are listed at the top (in blue) and isoforms in the Ensemble database are below (in dark red). Bars indicate exons and the CDS is indicated with a thicker bar. The arrows on the lines connecting the boxes indicate strand. Arrows pointing to the right indicate (+) strand, arrows pointing left indicate (–) strand. For example, Atf3 is in the (–) strand and so the 5′ end of the transcript is on the right. The mammalian conservation at the DNA level is shown in the bottom track. For Atf3, two TSSs have been identified and the associated P1 and P2 promoter are labeled. Two TSSs for Mapk8ip1 have been identified and are labeled. Bex1 produces four isoforms which have different CDS.

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Table 1

Regeneration associated gene isoforms

The number of TSS, CDS, and 3′ UTR for each gene found in the UCSC Known Genes, Ensemble, and RefSeq mouse genome combined annotation. The relevant reference indicating a role for the gene in axon growth and regeneration is listed.

Official Gene Symbol	Number of: Isoforms	TSS	CDS	3′UTR	Reference
Adcyap1 (Pacap)	2	2	1	1	Neuroscience, **151**:63–73
Atf3	1	1	1	1	J Neurosci, **27**:7911–7920
Atrn (attractin)	1	1	1	1	FASEB J, 19:153–4
Bex1	1	1	1	1	J Neurochem, **115**:910–920
Cd44	7	1	7	2	Neuron, **43**:57–67
Cdkn1a (p21)	2	2	2	1	J.Neurosci, **22**:1303–1315
Gal (galanin)	1	1	1	1	Neuron, **43**:57–67
Gap43	1	1	1	1	Development, **128**:1175–82
IL-6	3	2	3	2	J Neurosci, **24**:4432–43; J Biol Chem, **283**:416–26
Il6st (Gp130)	2	1	2	2	Neuron, **64**:617–623
Itga1 (integrin alpha7)	4	1	4	4	Neuron, **43**:57–67
Itgb1 (integrin beta1)	3	2	3	1	Neuron, **43**:57–67
Jun	1	1	1	1	Neuron, **43**:57–67
Klf4	1	1	1	1	Science, **326**:298–301
Klf6	1	1	1	1	Science, **326**:298–301
Klf7	3	2	3	2	Science, **326**:298–301
L1cam	2	1	2	2	PNAS, **102**:14883–14888
Lif	2	2	2	1	J Neurosci, **21**:7161–70
Mapk8ip1 (JIP1)	3	2	2	1	J Neurosci, **30**:7804–7816.
Matn2	4	1	4	4	J Cell Sci, **122**:995–1004
Mdk	4	4	2	1	J Neurosci Res, **87**:2908–2915
Mtap1a (Map1a)	2	2	2	2	FASEB J, 19:153–4
Mtap1b	3	2	3	3	J Neurosci, **24**:7204–7213
Nosip	2	1	2	1	J Neuropathol Exp Neurol, **60**:411–21

Official Gene Symbol	Number of: Isoforms	TSS	CDS	3′UTR	Reference
Npr2	4	4	3	1	J Neurosci Res, **86**:3163–9
Nrn1 (neurtitin)	1	1	1	1	FASEB J,19:153–4
Pten	2	1	2	2	Science, **322**:963–966; J Neurosci, **30**:9306–15
Ptprs (PtpSigma)	7	2	6	4	J Neurosci, **22**:5481–91; Science, 326:592–596
RhoA	1	1	1	1	J.Neurosci, **29**:15266–76
Rock2	4	3	4	4	J.Neurosci, **29**:15266–76
Smad1	2	1	2	2	J Neurosci, **29**:7116–23
Socs3	1	1	1	1	Neuron, **64**:617–623
Sprr1a	1	1	1	1	J.Neurosci, **22**:1303–1315
Stat3	3	1	3	1	J Neurosci, **26**:9512–9
Stk25 (Mst3b)	1	1	1	1	Nat Neurosci, **12**:1407–14
Tnfrsf12a (Fn14)	1	1	1	1	J Neurosci, **23**:9675–86
Tnfrsf19 (TROY)	5	2	3	3	Neuron, **45**:353–359
Trp53 (p53)	2	1	1	2	EMBO J, 25:4084–96
Trpc4ap	3	2	3	2	J Biol Chem, **283**:416–426

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Table 2

A list (not comprehensive) of frequently used programs for downstream RNA-Seq data analysis.

Analysis	Software	Reference	PMID
Fragment/read mapping and transcript assembly	Cufflinks	Trapnell et al., 2010	20436464
	Tophat	Langmead et al., 2009	19261174
	Bowtie	Trapnell et al., 2009	19289445
	MapSplice	Wang et al., 2010	20802226
	SOAP2	Li et al., 2009	19497933
	SpliceMap	Au et al., 2010	20371516
Promoter Analysis	MAPPER	Marinescu et al., 2004	15799782
	JASPAR	Bryne et al., 2008	18006571
	oPOSSUM	Ho Sui et al., 2007	17576675
	TRANSFAC	Matys et al., 2006	16381825
miRNA Response Element	TargetScan	Friedman et al., 2009	18955434
	MiRscan	Lim et al., 2003	12624257
zipcode motifs	REPFIND	Andken et al., 2007	17663765