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Review

Atrophins' emerging roles in development and neurodegenerative disease

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Abstract. The Atrophins are a widely expressed family of transcriptional co-regulators found in all metazoans. Atrophin1 was first identified as a neurodegenerative disease gene whereas Atrophin2 was identified based on homology. Phylogenetic studies indicate that the primordial Atrophin was an Atrophin2 type of gene and Atrophin2 has critical functions in normal mouse embryonic development whereas Atrophin1 is dispensable. Atrophins can interact with a wide range of proteins including membrane receptors, nuclear hormone receptors and other DNA binding transcription factors and can shuttle between the cytoplasm and the nucleus. In the nucleus, Atrophins can act as either co-repressors or co-activators and taken together this suggests that they are intermediaries in transcriptional responses to a diverse array of exogenous signals. Despite progress in understanding the normal role of Atrophins, the mechanism whereby mutations in Atrophin1 cause neurodegeneration has remained enigmatic, although most studies have focused on the idea that neurodegeneration is related to inappropriate transcriptional repression.

Keywords. Atrophin, neurodegenerative disease, polyglutamine expansion, embryonic development, *Fgf8*, Tailless, Tlx, transcription co-repressor.

The Atrophin Gene Family

The Atrophin genes first attracted notice because of the causative role that Atrophin1 plays in a devastating, dominantly inherited, neurodegenerative disease, DRPLA (Dentatorubral-pallidoluysian atrophy) [1, 2]. Disease-causing mutations are expansions of a trinucleotide repeat leading to the expression of a protein with an extended stretch of glutamine residues (Fig. 1A). Glutamine repeats of over 49 amino acids in the N-terminus of Atrophin1 causes neuronal death in the dentate nucleus of cerebellum, globus pallidus, caudate and putamen in brain. Symptoms include myoclonus, epilepsy, chorea, cerebellar ataxia and dementia [1].

There are two Atrophin genes in the human genome, Atrophin1 and Atrophin2 (Fig. 1A). Both Atrophin1 and Atrophin2 are widely expressed in various tissues including brain, heart, skeletal muscle and kidney [3, 4, 5, 6]. In brain, Atrophin1 is widely expressed in various regions including amygdala, caudate nucleus, hippocampus, and thalamus [3]. Atrophin1, located on chromosome 12p, encodes a protein of 1189 amino acids. Homology between Atrophin1 and Atrophin2 defines a bipartite Atrophin domain with unrelated amino and carboxy terminal halves interrupted by simple sequence elements that are not well con-

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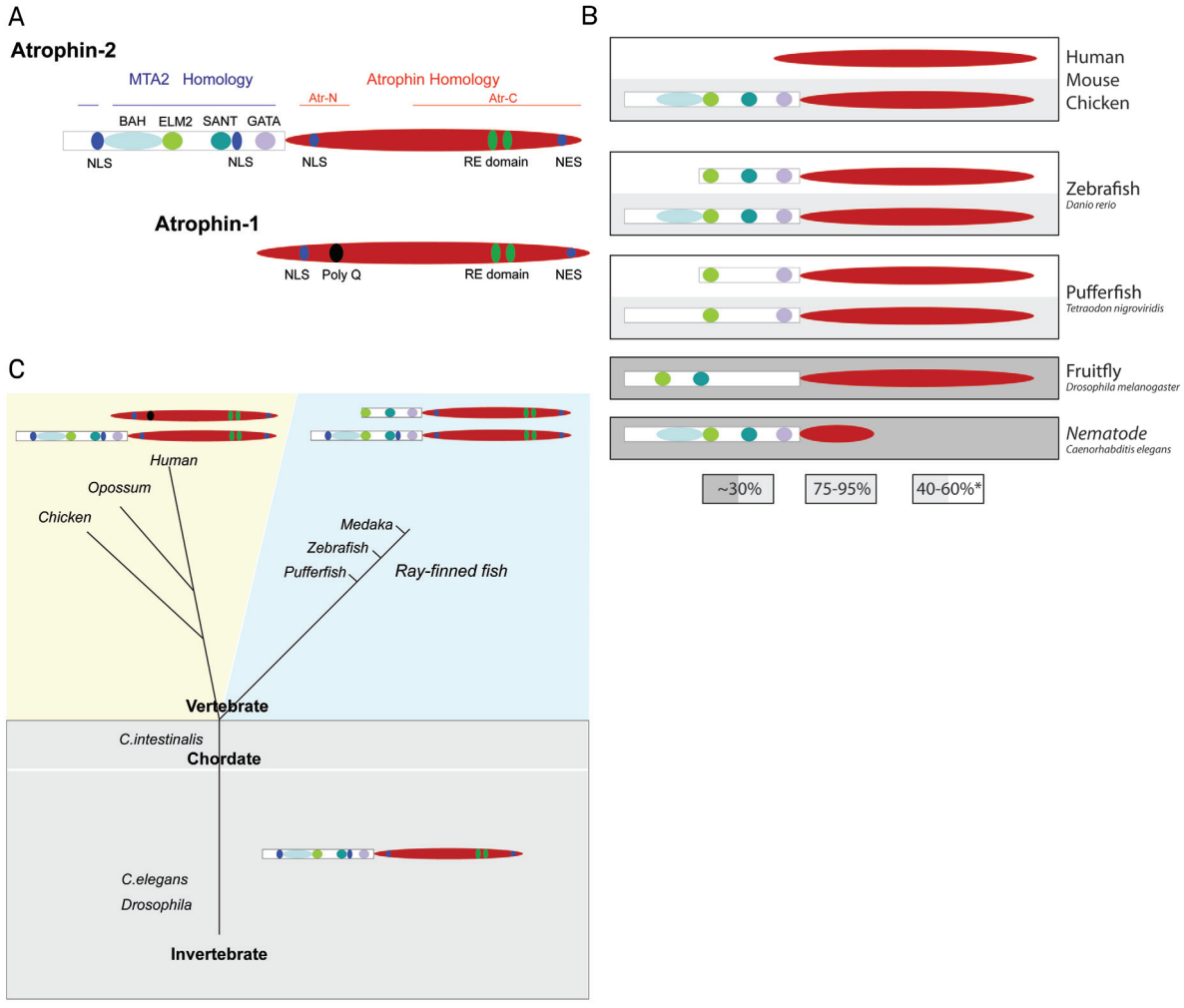

Figure 1. Protein architecture and Phylogenetic history of Atrophins. (*A*) The N-terminus of mouse Atrophin2 is homologous to MTA2, containing BAH, ELM2, SANT, GATA domains (light blue, green, turquoise and light purple ellipses respectively) and two NLS (dark blue ellipses). The C-terminus is the Atrophin domain with separate blocks of homology with Atrophin-1 (Atr-N and Atr-C) separated by low complexity sequences. Atr-N contains one NLS, Atr-C contains one NES and two RE domains. (*B*) The domain architecture and primary sequence of Atrophins are highly conserved across species from *C. elegans* and *Drosophila* to vertebrates including teleosts, rodents, birds and primates. Homology between invertebrate Atrophins and veretebrate Atrophin2 type genes is ~30 % (comparison between molecules enclosed in dark and light grey boxes), homology between vertebrate Atrophin2 type proteins is ~75~95 % (comparisons between proteins enclosed in light grey boxes) Within species comparisons of Atrophin1 and Atrophin2 type proteins (between white and light grey enclosed proteins) indicate homologies of ~40–60 %. The asterisk indicates within-species homology comparison. (*C*) The Atrophin phylogenetic tree. Invertebrate genomes and the genome of the primitive chordate, *Ciona intestinalis* have one Atrophin gene. All of the domains present in the vertebrate Atrophin2 gene are found in one or more of the invertebrate or primitive chordate genes, indicating that the common ancestor to the invertebrates and vertebrates had an Atrophin2 type gene. All vertebrate genomes sequenced to date have two Atrophin genes, supporting the idea that a gene duplication event early in the vertebrate lineage produced two Atrophin2 type genes. In the lineage leading to mammals, birds and marsupials, one of the two genes has been truncated to produce the Atrophin1 gene. In fish, a less severe truncation, again only affecting one of the two genes, removed the BAH domain.

served. No other functional domains are apparent in Atrophin1. Atrophin2, located on chromosome 1p, encodes two isoforms, Atrophin2-L and Atrophin2-S [4, 5]. The Atrophin2-S isoform is 990 amino acids in length and has a domain structure that is co-linear with Atrophin1, with which it shares 50.8 % similarity. This region of the protein carries several Arg-Glu (RE) repeat motifs of unknown function, which gave the gene the alternative name RERE [6, 7]. The Atrophin2-L isoform, 1559 amino acids long, has a 569 amino acid amino-terminal extension to the Atrophin domain with significant homology to the MTA (Metastasis-associated Proteins) family of proteins. The domain structure of Atrophin2 is conserved from *C. elegans* [8], *Drosophila* [9], zebrafish [10] to primates [4, 6]; however, Atrophin1 can only be found in the genome of higher vertebrates. This suggests that the Atrophin1 gene arose during evolution as a truncated duplication of a primordial Atrophin2 type gene.

Genomic data across a range of species supports this general conclusion and suggests that the duplication occurred in the chordate ancestor of the vertebrate lineage. In invertebrates such as *Drosophila* or *C. elegans*, there is only one Atrophin gene. The *Drosophila* gene, DAtr [9], is located on chromosome 3L and encodes a protein of 1985 amino acids with an overall 38 % homology to human Atrophin2 (Fig. 1B). The N-terminal portion of DAtr contains an ELM2 domain and a SANT domain, involved in protein-protein interaction and implicated in DNA binding. The N-terminal of DAtr shares 20.7 % homology with N-terminal of human Atrophin2, the C-terminal of DAtr shares 25.3 % homology with human Atrophin1, shares 31.6 % with C-terminal of human Atrophin2. In *Anopheles gambiae*, the one Atrophin homolog, AGAP006669, encodes a protein of 2482 amino acid and has an overall 44.5 % homology with *Drosophila* Atrophin. The C-terminus of Anopheles Atrophin has a 27.3 % homology with human Atrophin1. In *C. elegans*, Egl-27 has an overall 28 % homology with human Atrophin2 [8]. Egl-27 encodes a protein of 1129 amino acid, contains BAH, ELM2, SANT and GATA domains in N-terminus, also has two RE-repeat motifs in its C-terminus (Fig. 1B). Thus Egl-27 is likely to be the only Atrophin ortholog in nematode in terms of conserved domains and protein sequences. The genome of a urochordate, *C. intestinalis* contains an Atrophin homolog that is similar in structural organization to the invertebrates.

Two types of Atrophin genes are found throughout the vertebrate lineage, consistent with the idea that a gene duplication event occurred very early in the vertebrate lineage. A second Atrophin gene first appears in the genome of fish where one of the two genes is co-linear with Atrophin2 and the other is partially truncated in its amino terminal domain (Fig. 1C). Birds, marsupials and mammals all have clearly distinct Atrophin2 and Atrophin1 type genes. In higher organisms such as *Xenopus*, zebrafish, *gallus* and primates, there are two Atrophin paralogs in the genome, named as Atrophin1 (DRPLA) [1] and Atrophin2 (RERE) [6] (Fig. 1C). Protein sequences of Atrophin orthologs in higher organisms are highly conserved, for example, the homology between Xenopus Atrophin2 and human Atrophin2 is 82.3 %. Atrophin1 is relatively shorter in length and has 50.8 % homology with the C-terminal of Atrophin2 (Fig. 1B). In addition to the Atrophin1 homology C-terminal, the amino terminal extension of full length Atrophin2 contains BAH, ELM2, SANT and GATA domains.

As outlined above, the idea that Atrophin1 is an incomplete copy of the Atrophin2 gene is well supported by the phylogenetic record and, importantly, it provides a framework for understanding the distinct functions of the two genes in vertebrates and for relating them to the invertebrate function. It is interesting in this regard that the Atrophin2-S isoform of mice is expressed from an internal promoter and is therefore essentially a pre-existing copy of an Atrophin1 type gene [4]. It will be very interesting to determine whether invertebrates or Urochordates express an Atrophin2-S isoform. If they do, it would imply that expression of an Atrophin1 type protein is more ancient than the gene duplication event that created a distinct gene. In either case, it is apparent that higher vertebrates have two evolutionarily divergent Atrophin genes that express two structurally distinct types of proteins, an observation that has implications for deciphering their biology.

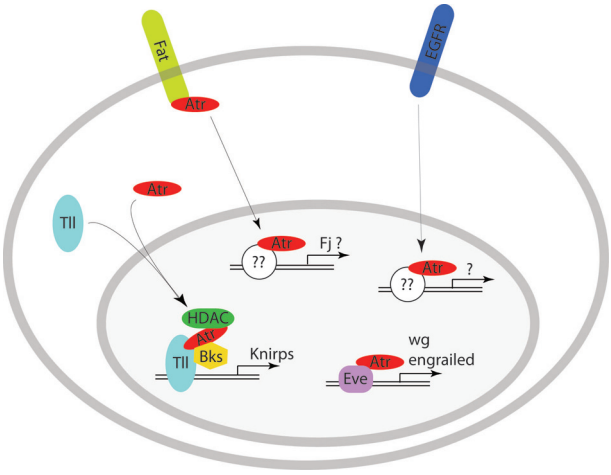

Figure 2. Atrophin functions as a transcriptional co-repressor that shuttles between nucleus and cytoplasm to transduce extracellular signals. Atrophin interacts with Fat at the plasma membrane and regulates *four-jointe*d (Fj) transcription in the nucleus; Atrophin genetically interact with EGFR pathway to affect downstream gene expression by mechanisms that are unclear; Atrophin interacts with hormone nuclear receptor Tll in the cytoplasm and co-operatively regulate *knirps* expression in nucleus; Atrophin directly interacts and represses transcription factor Eve activity in nucleus, regulates *engrailed* and *wg* expression. Atrophin is found associated with Histone Deacetylase (HDAC) in nucleus both in *Drosophila* and in mammals.

Domain Structure of the Atrophins. The function of Atrophins can be related to the distinct domains present in full length Atrophin2, the MTA homologous N-terminal domain and the Atrophin-like C-terminal domain. The Atrophin domain is bipartite, with conserved amino terminal (Atr-N) and carboxy terminal (Atr-C) portions interrupted by a simple sequence (Fig. 1A). In Atrophin1, Atr-N contains about 150 a.a. and Atr-C about 425 a.a. In Atrophin2, the homologies are more extensive, with an Atr-N segment of about 200 a.a. and Atr-C of about 550

amino acids. Atrophin1 also contains two arginine-glutamic acid dipeptide (RE) repeats and one putative Nuclear Export Signal (NES) within the Atr-C segment [11]. The Atr-N segment carries a putative Nuclear Localization Signal (NLS). The function of the Atr-N and Atr-C segments is not clear but it is worth mentioning that poorly conserved versions occur in several uncharacterized genes in the human genome in contexts that are not otherwise related to the Atrophin gene family.

The distinct, MTA-2 homologous, N-terminus of human Atrophin2 contains four conserved motifs: BAH, ELM2, SANT and GATA. These motifs often appear in proteins that function as transcription co-repressors and/or they are predicted to directly interact with other transcription regulators, or transcription factors that have DNA-binding activity (Fig. 2). This suggests that Atrophin2 may be involved in regulating transcription or may bind DNA.

The BAH (bromo-adjacent homology) module is frequently associated with proteins involve in DNA methylation, replication, and transcription regulation [12]. Its function is not well-understood; however yeast Origin Recognition Complex 1 protein (Orc1p) directly interacts with Silent Information Regulator (Sir1p) through its BAH domain and it has been suggested that the BAH domain may have a function that is specific to replication and transcription regulation [13].

The ELM2 (Egl-27 and MTA1 homology 2) domain is also found in the MTA-2 (Metastasis Associated) protein, a core component of the NuRD transcriptional co-repressor complex [8, 14]. The ELM2 domains of both MTA-2 and Atrophin-2 mediate association with HDACs (Histone Deacetylase) indicating a conserved role for this domain [15, 16].

The SANT domain is well studied in the context of chromatin-remodeling enzymes where it functions as a histone-binding module. SANT domains are found in many chromatin-remodeling complexes, such as Ada2, MTA2, SMRT, SWI [17, 18]. In SMRT, the SANT motif interacts directly with the HDAC3 as well as with the histone tail, and is required for HDAC3 activation [19]. Possible functions of the SANT module of Atrophin-2 include modification events of histone acetylation, deacetylation, and ATP-dependent chromatin remodeling and the SANT domain could play a role during either transcriptional activation or repression [17]. A recent report has shown that the Atrophin SANT domain can directly interact with histone methyltransferase G9a [16] and an Atrophin protein complex can exert histone methylation activity. Considering the ability of the SANT domain to interact with multiple nuclear receptors, the SANT domain could play a central role in multiple different functions of Atrophins.

The GATA domain, located at 501–552 a.a. in human Atrophin2, is a zinc finger type DNA binding module in GATA class transcription factors. In this context it recognizes the consensus DNA sequences (A/T)GATA(A/G) in the regulatory regions of genes [20]. GATA transcription factors normally contain two GATA domains and there is no evidence that the GATA domain in Atrophin2 has DNA-binding activity.

Atrophin1 and Neurodegeneration. DRPLA is a rare autosomal dominant neurodegenerative disease in which patients exhibit cerebellar ataxia, myoclonic epilepsy, chereoathetetosis and dementia. The underlying cause is progressive and significant neuronal loss in brain regions such as globus pallidus, subthalamic nucleus, dentate nucleus and spinal cord. The incidence of DRPLA is very low except in Japan. Nagafuchi et al. reported the cloning of the DRPLA mutation in 1994 and found variable expanded polyglutamine repeats (49–75) encoded in the Atrophin1 gene [1]. Atrophin1 is widely expressed in many tissues, highest in central nervous system, with expression across many brain regions including striatum, hippocampus, cerebral cortex, cerebellum, diencephalon and brain stem [21]. The function of Atrophin1 is not well understood and it is not known whether polyglutamine extensions cause neuronal death via a mechanism that is dependent or independent of the normal function of the protein. It has been suggested that Atrophin1 can interact with transcriptional regulators such as ETO1 [22], CBP [23] and can function as a transcriptional repressor. The Poly(Q) extension region is found within the simple-sequence rich segment separating the Atr-N and Atr-C segments and no specific function has been defined for this region. Since a null mutation in the mouse Atrophin1 gene has no apparent phenotype, it seems unlikely that loss-of-function is the cause of DRPLA [5].

In brain extracts from post-mortem DRPLA patients, full-length Atrophinl runs ~200KD on, but there is an additional Atrophinl band at ~120KD [24]. Two similar Atrophin1 bands at 200KD and 120KD are also seen in samples from poly(Q) extended Atrophin1 transgenic mice [25]. This suggests that Atrophinl exists in neurons in two forms: a full length form and a cleaved fragment. Nucifora et al. used C-terminal and N-terminal specific Atrophinl antibodies and found that the 120KD fragment lacks the C-terminal epitope, suggesting it is an N-terminal fragment [11]. There are several putative caspase cleavage sites in the Atrophin1 protein sequence [26, 27]. For example, Caspase 3 cleaves Atrophin at aspartic acid 109 in cultured cells, and mutating this residue

significantly decreases Atrophin1's cellular toxicity. However, in an *in vitro* assay, none of the caspases successfully produced the 120KD Atrophin1 fragment seen *in vivo* [11], leaving the *in vivo* cleavage site and the responsible proteinase undetermined.

The normal length of the polyglutamine stretch in Atrophinl is 7 to 23. In DRPLA patients, the length of the polyglutamine repeat is extended to 49~75 [1]. Patients carrying poly(Q) extended Atrophinl develop ataxia, dementia, and myoclonic epilepsy as they become older, and the symptoms get progressively worse as they age. Loss of pallidal and nigral neurons is seen in post mortem examination of brain samples, suggesting that this is a neurodegenerative disease. Histology of post mortem brain slides shows that Atrophinl forms inclusions in these neurons [1, 2]. Atrophinl inclusions can be found both in cytoplasm and nucleus, and inclusions are increased in size and number in patients with severe symptoms. Yazawa I. et al. [28, 29, 30] reported that Atrophin1 is ubiquitinated and aberrantly phosphorylated in brain protein extract from DRPLA patient samples, and aberrant phosphorylation can also be detected in cytoplasmic inclusions and on nuclear membrane. Okamura-Oho Y et al., [31] reported that C-Jun NH2-terminal kinase can recognize a conserved serine site on Atrophin1 and can phosphorylate Atrophin1 in neuronal cells. An extended polyglutamine mutation reduced the interaction of Atrophin and c-JNK, suggesting that c-JNK could be the kinase that phosphorylates Atrophin1 in vivo. How ubiquitination and phosphorylation affect Atrophinl function or its aggregation, however, is not yet understood.

Given the lack of a clearly defined normal function for Atrophin1, functional studies have focused on pathological correlates. Several protein sequences have been shown to be critical for the ability of the human Atrophin1 protein to induce pathology *in vitro*; they are a nuclear localization signal (NLS) near the N-terminus and a nuclear export signal (NES) near the C-terminus. Fusion of the NLS of Atrophin1 to a poly(Q) extended version of Huntingtin caused the novel protein to accumulate in the nucleus and increased the severity of the disease phenotype produced in transgenic mice [32]. This suggests that the NLS in Atrophin1 is fully functional and may play an important role in phenotype progression. In cell culture systems, modeling the human disease by increasing the number of polyglutamine residues from 29 to 65 (AT-FL-29Q versus AT-FL-65Q) did not produce cellular toxicity. Cleavage or inactivation of the NES, however, caused AT-FL-65Q to become toxic to cells, consistent with the idea that toxicity requires nuclear localization and confirming that a 65Q extension carried within an N-terminal fragment of Atrophin1 produces cellular toxicity [11]. The NES in the Atr-C segment is also relevant to neuronal pathology *in vivo* as cleavage of the C-terminus in a 65Q version of Atrophin1 produces a 120KD fragment and increases cell neurodegeneration in a DRPLA transgenic mouse model [11, 24].

Studies designed to test the transcriptional activity of Atrophin1 have consistently found evidence for co-repressor activity. When human Atrophin1 fused to the GAL4 DNA binding domain is expressed in fly embryos, it represses transcription of a reporter gene containing a Gal4 binding site, suggesting that Atrophin1 functions as a co-repressor [9]. This is also the case in cell culture systems where human Atrophin1 with normal or extended poly(Q) exhibits co-repressor activities when tethered to DNA [22, 23]. It has also been reported that Atrophin1 can interact with transcriptional regulators such as ETO1, CBP and function as a transcriptional co-repressor when co-expressed in cultured cells. Finally, it was recently reported that histone deacetylase inhibitors could significantly alleviate the cellular toxicity of poly(Q) extended Atrophin1 in both cultured cells and transgenic mouse model [33, 34]. Taken together, these evidences suggest that poly(Q) extension may cause malfunction in Atrophin1 co-repressor activity. The difficulty in this interpretation is that no significant difference in co-repressor activity between normal and extended poly(Q) Atrophin1 has been apparent in the available assays, leaving the role of transcriptional repression in disease pathology uncertain.

Atrophin1, like many other polyQ expansion disease genes, forms nuclear inclusions in its pathological form [35, 36]. In all DRPLA patients and mouse models, inclusion is consistently observed and considered to be a marker of pathology [25, 37]. Some studies have indicated that increased inclusion decreases toxicity, whereas other studies have been interpreted to show that inclusions trigger the cellular responses that lead to apoptosis and neuronal loss [38, 39]. Although still somewhat controversial, recent data seems to indicate that inclusions are not pathogenic and may in fact be a cellular protective mechanism for sequestering the aberrant protein [40] as cellular toxicity does not correlate with the inclusion number or inclusion size *in vitro* [41].

Functional Motifs within Atrophin2. In contrast to Atrophin1, Atrophin2's localization is predominantly nuclear [5, 6, 9]. Cytoplasmic Atrophin2 is evenly distributed without obvious sublocalization, suggesting that its localization in the cytoplasm is not determined by localization signals. Nuclear localization, on the other hand, is highly regulated by at least five distinct signals; three NLS, one NES and a

subnuclear localization signal. The three NLS are N-terminal, two of them reside within the MTA homology region unique to the Atrophin2-L isoform, and the third resides within the AtrN segment, near the amino terminus of the Atrophin2-L isoform. Within the nucleus, Atrophin2 is highly concentrated in a specific subnuclear domain, the PML oncogenic domain (POD) [5, 7] and a localization determinant has been mapped to a C-terminal location common to both Atrophin2-S and Atrophin2-L [5]. PODs are defined by the tight co-localization of PML and sp100 and they can contain many transcriptional regulators such as CBP, c-Myc, Rb, P300, and Atrophin2, depending upon the cellular context [42, 43]. Localization of the PML protein itself is triggered by sumoylation; however, repeated attempts have found no evidence for sumoylation of Atrophin2 [44, 45], suggesting that its POD localization signal in Atrophin2 is a protein-protein interaction motif responsible for binding to an as yet unidentified POD partner. The function of PODs is enigmatic, it is clear that they are not major sites of active transcription in the nucleus but they may be involved in transcriptional repression or they may serve as depots to sequester regulatory factors [42, 43]. The function of the two RE-repeat domains in the Atr-C motif is not very clear. Yanagisawa et al. reported that heterodimer formation by Atrophin1 and Atrophin2 is mediated by the proximate RE-repeat domain, and extended polyglutamine in Atrophin1 enhances the binding [6]. Thus, it is plausible that polyglutamine extended Atrophin1 may sequester Atrophin2 from its normal localization into nuclear inclusions, and loss of Atrophin2 activity in some neurons may contribute to the pathology of DRPLA.

Developmental Roles for Atrophins in Invertebrates

Cell polarity and cell migration are fundamental processes during early embryogenesis. In *C. elegans*, Herman et al. [8] found that mutation in Egl-27, (Egg-laying defect mutant 27), disrupted asymmetric division in a specific precursor (T-cell) lineage and migration in a neuroblast (QL) lineage. In the tail of developing C. *elegans* larvae, the precursor T-cell asymmetrically divides into Ta and Tp cells. Ta and Tp cells further differentiate into hypodermal cells, neuron or neuroblast cells, respectively. In *Egl-27* mutants, all T cells divided into hypodermal cells [8]. Egl-27 mutants also have defects in cell migration; QL neuroblasts migrate anteriorly rather than towards the posterior as in wild type animals. Since both T-cell asymmetric division and QL neuroblast migration are controlled by the wnt signalling pathway, it was suggested that Egl-27 might genetically interact with the wnt pathway. Based on its sequence homology with Atrophin2 and the *Drosophila* Atrophin homolog, DAtr, it seems likely that the *C. elegans* gene also functions as a co-repressor in regulating gene transcription during embryogenesis.

In contrast to the relatively descriptive characterization of C. *elegans* Atrophin phenotypes, clear evidence that Atrophins function as transcriptional regulators has emerged from studies of the role of the DAtr gene in fly development. Mutant embryos lacking both maternal and zygotic Atrophin fail to develop [9]. Mutant embryos lacking only maternal Atrophin display a strong segmentation defect phenotype, and the ventral border of the embryos are broader than wild type. *Twist*, a marker for the ventral-most region, expands its expression and covers most of the ventral half of mutant embryos. This ventralizing phenotype appears again in mouse Atrophin2 mutant embryos [4], and Atrophin2 plays an important role in dorsoventral patterning during embryogenesis, although it is not clear that the regulatory mechanisms and signaling pathways involved are similar and the evolutionary significance of this observation is unknown.

Flies lacking zygotic expression of DAtro also display segmentation defects. Establishment of the *Drosophila* segmentation pattern is controlled by a hierarchical cascade of transcription factors: maternal→gap→pair-rule→segment polarity. The expression pattern of the segment polarity gene *En* was disrupted in embryos with reduced and fused strips [9]. Zhang et al. analyzed the expression of both gap and pair-rule genes and found that the gap gene expression patterns, including *Hb* and *Kr,* remain intact. Pair-rule gene expression patterns on the other hand become ambiguous at the boundary, resulting in less-defined, expanded boundaries of pair-rule genes such as *Eve*, *Ftz*, *hairy.* Biochemical studies show that DAtr can physically interact with both maternal gene product Hunchback (*Hb*) and pair-rule gene product Even-skipped (*Eve*), and that DAtr functions as a co-repressor to repress Eve transcription activity. Taken together, this suggests that DAtr acts in a complex fashion, both directly and indirectly, in the regulation of segment polarity genes.

Atrophin is widely expressed in all metazoans and the idea that it functions as a transcriptional co-regulator is consistent with the idea that its function in different tissues or different developmental stages depends on the transcription factors that it interacts with. Zhang et al. [9] provided evidence that DAtr acts as a transcriptional co-repressor with the Even skipped (Eve) transcription factor during fly embryo segmentation. DAtr physically interacts with Eve and parti-

cipates in its transcriptional repressor activity (Fig. 2). Down-regulation of Eve activity at the boundaries of its expression contributes to maintenance of the stripe pattern. In *DAtr* embryos, the loss of *DAtr* leads to an expansion of the *Eve* expression domains.

DAtr is found in both the cytoplasm and nucleus and the nuclear export and nuclear localization sequences of DAtr can shift its localization. This is likely to be important for its role as a transcriptional co-regulator with nuclear receptors that undergo a similar transition from the cytoplasm to the nucleus in order to carry out their function as DNA binding transcriptional regulators. DAtr interacts with the nuclear receptor Tll (*tailless*) [15] to repress *Knirps* expression. The interaction occurs through the highly conserved C-terminus of Atrophin and the highly conserved Ligand Binding Domain (LBD) of Tll. A DAtr molecule that is artificially tethered to the *Knirps* promoter region represses transcription, providing further support for the idea that Datr has a physiologically important role as a co-repressor. The *Drosophila* nuclear protein Brakeless can interact with both Tll and with Datr and function as a co-repressor [46]. This three-way relationship has been conserved during evolution, as evidenced by data showing that the vertebrate Tailless homolog, Tlx [47], and Brakeless homolog, ZNF608, can functionally interact with vertebrate Atrophins (Fig. 2) to mediate repression. A broader role for Atrophins as co-repressors for nuclear receptors is suggested by interactions with other nuclear receptors, such as COUP-TF and SVPI [47] through their conserved LBD.

Nucleocytoplasmic shuttling is also likely to be important for DAtr regulation of transcriptional events downstream of the cell adhesion molecule Fat [48]. DAtr interacts with Fat and fine-tunes *four jointed* expression in R3 cells, suggesting that it can function as a signal messenger to regulate gene expression in response to events at the cell membrane by interacting with intracellular part of membrane proteins (Fig. 2).

Developmental Roles for Atrophins in Vertebrates

The function of Atrophin1 and the Atrophin2-L isoform of the Atrophin2 gene have been studied with loss of function mutations in the mouse. An Atrophin1 null mouse is healthy and viable with no apparent defects [5]. No mutations have been reported that disrupt the function of the Atrophin2-S isoform. Given the domain similarity between Atrophin1 and Atrophin2-S, it seems plausible that these two proteins have redundant function, so the lack of an Atrophin2-S mutation must be considered as a significant gap in our understanding of Atrophin function in mammals.

The Atrophin2-L isoform is required for early embryogenesis and death occurs at E9.5 as a result of defects in heart development [4]. Atrophin2-L homozygous mutant embryos display defects in various other aspects of early patterning and morphogensis including failure of anterior neural tube closure, fusion of telencephalic and optic vesicles, reduced first branchial arches, irregular morphology of somites and, finally, the failure of heart tube looping around E9.5. Investigation of neural tube development at very early stages in Atrophin2 mutant revealed mis-regulation of gene expression in two critical signaling pathways, *Shh* and *Fgf8*. *Shh* is expressed in the notochord beginning on about day E7.5 and induces its own expression and that of other target genes, such as *Gli1 and Nkx2–1*, in the overlying ventral neural tube. The induction of Shh target genes by the notochord is a central event in establishing dorsoventral identity in the developing spinal cord. In Atrophin2-L mutant embryos, *Shh* expression is largely absent from the anterior notochord and the overlying neural tube, thus dorsalizing the neuroectoderm. *Fgf8* has a similar central role in patterning of the anterior neural plate. *Fgf8* is expressed at an early embryonic stage in a tight boundary domain between the epidermal ectoderm and the neuroectoderm, the Anterior Neuronal Ridge (ANR). *Fgf8* restricts the expression of *Emx2* to more caudal and lateral regions by repressing its expression in the neuroectoderm adjacent to the ANR. In Atrophin2-L mutants, *Fgf8* is down regulated and delocalized from the ANR with patchy expression in the surrounding epithelial and neural ectoderm [4]. Decreased *Fgf8* expression results in an expansion of *Emx2* across the anterior neural plate. The transcriptional regulation of Fgf8 is not understood and the molecular mechanism in which Atrophin2-L is involved is not known; however, the role appears to be conserved. In zebrafish similar disruptions of *Fgf8* expression were also observed in *babyface (bab)* mutants that carry nonsense mutations in an Atrophin2 homolog [10]. Interestingly, both *bab* alleles truncate Atrophin2 in the SANT domain and are thus apparently similar to the *open-mind (om*) and PT026 alleles in disrupting only the Atrophin2-L isoform [4]. An Atrophin2-S isoform, if present in fish, would not be disrupted by the *bab* mutations.

Atrophins are transcription co-regulators

The N-terminal homology to MTA proteins suggests that Atrophin2 may function as a transcriptional co-

repressor to regulate target genes. When fused with Gal4 DNA binding domain in transgenic flies, DAtr and human Atrophin can repress the downstream reporter gene. The N-terminus of Atrophin2 itself can also function as repressor in cultured cells when artificially bound to a promoter [5]. The MTA-2 protein, the best characterized member of the MTA family, functions as an obligate component of the Mi-2/NuRD complex, a transcriptional co-repressor that contains MBD3, HDAC1, HDAC2, RbAp46 and RbAp48 in addition to MTA-2 [48]. MTA-2 modulates HDAC activity and the N-terminal, MTA-2 homologous, region of Atrophin2 can directly interact with HDAC and recruit HDAC1 into PML bodies [4, 5, 15]. A similar repressive mechanism appears to operate in zebrafish, as Plaster et al. [10] showed that the zebrafish Atrophin2 homolog, together with HDAC, could independently or cooperatively interact with the *Fgf8* signal pathway in zebrafish early embryo development. *In vivo* studies, where they can be interpreted as such, have indicated that Atrophin family proteins function as transcriptional co-repressors, inhibiting specific genes via interactions with DNA binding transcription factors and recruitment of histone deacetylase (Fig. 2). Many of the *in vivo* effects, though, could just as easily be explained by Atrophins acting as transcriptional co-regulators, and *in vitro* studies indicate that this is likely to be the case. The Atrophin domains are unique within the vertebrate proteome. Besides the nuclear localization/export signals, RE-repeats and proline-rich region, there is a highly conserved 94-a.a. motif in the Atrophin domain that can function as a strong transcriptional activator [5]. With the N-terminal MTA homology domain, Atrophin2 has both transcriptional activator and repressor activity *in vitro.* The bulk of the *in vivo* data support that Atrophins function as co-repressors and HDAC and G9a may be recruited to exert the repression activity. This implies that the functions of Atrophin are carried out in a protein complex in which different components are recruited and assembled in order to execute proper transcriptional regulation of downstream target genes in a tissue- and developmental stage-specific window. This may be accomplished by transcriptional regulators that selectively interact with the surrounding amino acid sequence such as proline-rich motif or SANT domain in the C-terminus and N-terminus [16, 22, 23, 46, 50, 51]. Given the balance between repressor and activator function that is implied, it would not be surprising if Poly(Q) extensions affect Atrophin1 by tipping the balance in one direction. A number of studies have demonstrated transcriptional repressor activity for Poly(Q) extended Atrophin1 and, in this model, the effect of Poly(Q) extension would be to favor the conformation or state of the transcriptional repressor [12, 22]. Substantial support for a similar model has been described recently in the case of another Poly(Q) extension gene, Sca1 [52].

In the nucleus, Atrophin2 is found in PML bodies and it interacts with other transcriptional regulators found in PML bodies, such as CBP and HDAC1, and regulates their activities [4, 5, 15, 23]. Atrophin interacts with HDACs through the N-terminal ELM2 and SANT modules and activates HDAC activity. Besides physically interacting with other transcriptional regulators, Atrophin can also genetically interact with transcriptional factors and transcription regulators (Fig. 2). Groucho, a known co-repressor, has strong genetic interaction with Atrophin [53], they share the same downstream transcription factors such as *engrailed, C15.* In *Drosophila,* Atrophin can genetically interact with nuclear repressor Yan to inhibit EGFR signaling activity [54]. In zebrafish, Atrophin can genetically interact with *Fgf8* to regulate early embryonic development [10].

Atrophin function is critical to early embryonic development; the expression level and locus thus needs to be precisely regulated during embryogenesis. It has recently been reported that in Drosophila, MicroRNA-8 can regulate Atrophin expression level both spatially and temporally [55]. DAtr is a direct target of *miR-8*, mutation of *miR-8* results in increased DAtr expression, which leads to apoptosis and behavior defect; whereas down-regulated DAtr expression is also detrimental to *Drosophila* development.

Summary

Although dominant mutations causing neurodegeneration first focused attention on the Atrophin gene family, more recent work has highlighted the importance of these transcriptional co-regulators for normal development. The dominant, gain-of-function nature of the disease-causing mutations has meant that the pathological role of Atrophin1 can not easily be related to the normal function of the gene family. The increasingly detailed picture of Atrophin biology that is emerging, though, has begun to allow us to see how a better understanding of the normal role of Atrophins will allow us to determine how pathological function relates to normal development. The Atrophin gene family is extant throughout the metazoa and roles in regulating a diverse set of transcriptional events have been found. Despite the diversity of transcriptional regulatory contexts, conserved themes have begun to emerge. Thus, Atrophins collaborate with the nuclear hormone receptor Tail-less in *Drosophila* and its vertebrate relative Tlx in humans [15, 47]. Equally well conserved apparently is the upstream regulation of

Atrophins by micro RNAs [55]. In both fish and mice, Atrophin2 is required for the proper regulation of *Fgf8* expression during embryogenesis [4, 10] and, while the molecular partners of Atrophin2 in this regulation have not been identified, the similarity in the pattern of defects is clear evidence of a conserved mechanism. A number of other observations are beginning to be evaluated, such as the interaction between DAtro and the membrane protein Fat [44] to determine whether or not this interaction is similarly conserved. The affected signal pathways and interacting partners of Atrophins may vary in different tissue at different stages, creating a large profile of target genes whose transcriptional regulation is controlled by Atrophins. On the other hand, the Atrophin transcript levels are themselves fine tuned by microRNAs. Taken together it is clear that the Atrophin family is at the center of a complex gene regulation network that governs cell fate in development, disease and other processes (Fig. 2). Major questions remain, the answers to which could dramatically change how we view Atrophin function and how we interpret studies in flies for their relevance to human biology. For example, what is the relationship between Atrophin1 and Atrophin2? The two proteins are physically associated *in vivo;* however, the functional significance of this interaction is not known. Similarly, what is the relationship between the function of Atrophin1 and the short, Atrophin1-like, isoform of Atrophin2? We can look forward to the answers to these and other questions illuminating the fascinating biology of the Atrophins over the coming few months and years.

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