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REVIEW

Akirin proteins in development and disease: critical roles and mechanisms of action

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Abstract

The Akirin genes, which encode small, nuclear proteins, were first characterized in 2008 in *Drosophila* and rodents. Early studies demonstrated important roles in immune responses and tumorigenesis, which subsequent work found to be highly conserved. More recently, a multiplicity of Akirin functions, and the associated molecular mechanisms involved, have been uncovered. Here, we comprehensively review what is known about invertebrate Akirin and its two vertebrate homologues Akirin1 and Akirin2, highlighting their role in regulating gene expression changes across a number of biological systems. We detail essential roles for Akirin family proteins in the development of the brain, limb, and muscle, in meiosis, and in tumorigenesis, emphasizing associated signaling pathways. We describe data supporting the hypothesis that Akirins act as a "bridge" between a variety of transcription factors and major chromatin remodeling complexes, and discuss several important questions remaining to be addressed. In little more than a decade, Akirin proteins have gone from being completely unknown to being increasingly recognized as evolutionarily conserved mediators of gene expression programs essential for the formation and function of animals.

Keywords BAF · Cancer · Cell cycle · Embryonic · Myogenesis · Neurodevelopment · NFκB · Nucleus

Introduction

Akirins have recently emerged as critical regulators of multiple developmental processes by acting as modulators of gene expression patterns. Although these small, highly conserved, primarily nuclear proteins remain somewhat enigmatic, recent studies in a host of systems including *Caenorhabditis elegans* (*C. elegans*), *Drosophila melanogaster*, *Xenopus laevis,* mice, rats, and humans have resulted in a better understanding of their functional roles and some of the mechanisms through which they act. Here, we broadly review the field to summarize our current understanding of Akirin biology, including the genes they regulate and the protein–protein interactions in which they engage. We focus particularly on recent research demonstrating new Akirin family roles in brain development, limb formation, myogenesis, meiosis, and tumorigenesis (Fig. 1; Table 1). For further discussion of Akirin gene evolution and of Akirin family roles in the immune system, we refer the reader to recent helpful reviews on these particular topics [1–3].

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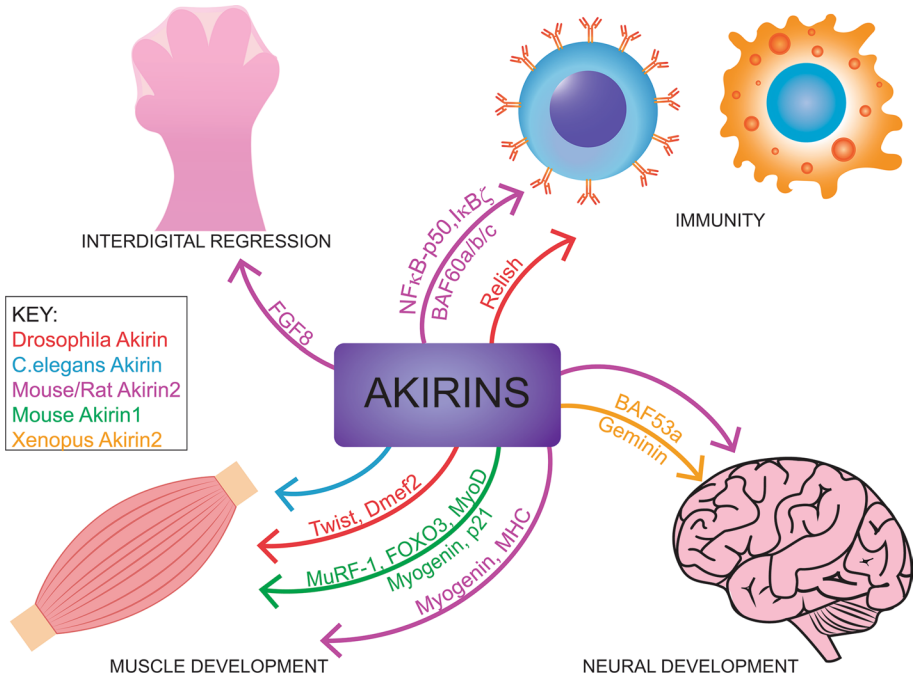

Fig. 1 Established roles for Akirins in developmental processes. Akirins have been shown to have essential roles in interdigital regression during limb development, in the immune system, during neural development and in muscle development and function. The colored arrows represent the organism studied and putative functional mechanisms are written above the relevant Akirin

The Akirin family

The earliest *Akirin* gene is found in non-animal eukaryotes prior to the division of plant, fungus, and animal lineages. *Akirin* genes are, however, rare in these organisms, being entirely lacking in plants and fungi; they are found consistently only in metazoan genomes [4]. In animals, the Akirin gene family consists of an ancestral single *Akirin* found in insects and worms, and its two vertebrate homologues, *Akirin1* and *Akirin2* [3, 4]. During evolution, birds and reptiles lost the *Akirin1* gene and have only *Akirin2*, while numerous gene duplication and loss events have led to 4 *Akirin* genes being present in several teleosts, and up to 8 *Akirin* genes reported in the *Salmonidae* family [5].

The single *Drosophila* Akirin was first characterized following a yeast-2-hybrid study as a mediator of the immune deficiency (Imd) immune system pathway. Its gene, originally called CG8580, was renamed *Akirin* from the Japanese phrase "akiraka ni suru" which means "making things clear", an allusion to the clear nuclear localization signal (NLS) present in this protein [6]. Another group had reported previously a role for a homologous gene in tick (*Ixodes scapularis*) reproduction and named it *subolesin* [7, 8]; a subsequent *Drosophila* embryogenesis study also referred to this gene *bhringi* [9]. Of the two vertebrate Akirins, *Akirin1* was initially referred to as *Mighty* [10] and *Akirin2* was alternatively referred to as *FBI1* in rats [11]. Each *Akirin* gene encodes a small protein of ~ 200 amino acids with a predicted molecular weight of ~ 22 kDa. Vertebrate *Akirin1* and *Akirin2* loci each consist of 5 exons and 4 introns of varying length [4], with both mouse genes on chromosome 4, human *Akirin1* at 1p34.3, and human *Akirin2* at 6q15.

Comparison between *Drosophila* Akirin and mammalian Akirins found 34% amino acid identity with Akirin1 and 39% amino acid identity with Akirin2 [6]. Phylogenetic comparisons of gene and protein structure revealed more non-synonymous changes and sites with higher rates of evolution in Akirin1 compared with Akirin2 [4], suggesting that Akirin2 is more similar to invertebrate Akirin. Consistent with this idea is the fact that constitutive knockout of *Akirin* in *Drosophila* is embryonic lethal, as is constitutive knockout of *Akirin2* in mice [6]. Additionally, while most *C. elegans Akirin* (*akir-1*) mutant animals survive, some are lost during embryogenesis [12, 13]. In contrast, *Akirin1* null mice survive and are outwardly normal [6]. In terms of the human genes, the GnomAD database (https://gnomad.broadinstitute.org/) indicates that *Akirin2* has a much higher probability of being intolerant to loss-of-function mutations than does *Akirin1*.

Initially, Akirin protein structures revealed little about function as the sequences have no homology to any recognizable catalytic domains, DNA binding domains, or any other proteins with known functions [4, 6, 11, 14]. However, predicted DNA-binding sites in the C-terminal half of Akirin and Akirin2 have been recently identified in silico, opening the possibility that Akirins may have a DNA-binding capability, though this has not been tested experimentally (see [3]). The N- and C-termini exhibit the strongest conservation across all species examined [4, 6]; however, whilst both Akirin1 and Akirin2 share a common N-terminal NLS, Akirin2 has a

Table 1 Selected roles for Akirin proteins in various animal models

Biological process	Organism	Results	Selected references
Immunity	Drosophila	**Akirin** knockdown causes reduced Imd pathway activation in response to gram negative bacteria. **Akirin** was found to function downstream of, or in parallel with, the NFκB transcription factor, Relish	[6, 17]
	Mouse	**Akirin1** null mutant mice are outwardly normal, whereas **Akirin2** knockout is embryonically lethal	[6]
	Mouse	**Akirin2** interacts with BAF60a/b/c and IκBζ to upregulate IL-6 expression in response to immune effectors in macrophages	[14]
	Mouse	**Akirin2** is required for B cell proliferation and protection from apoptosis	[24]
Brain development	Mouse	Loss of **Akirin2** in telencephalic progenitor cells causes early cell-cycle exit, followed by massive apoptosis and extreme microcephaly	[25]
	Xenopus	In neural progenitor cells, **Akirin2** interacts with BAF53a and Geminin to restrict progenitor cell expansion. In neurons, **Akirin2** promotes neuronal differentiation at the level of Ngnr1	[22]
	Human	Deletions of the **AKIRIN2** gene (as one gene among many in 6q11–q15 deletions) were found to be associated with developmental delay	[72]
Muscle	Drosophila	**Akirin** is required for normal muscle development, by interacting with the transcription factor Twist at the Dmef2 promoter	[9]
	C. elegans	Mislocalization of muscle cell markers was observed in **akir-1** null worms; body-wall muscle structure deteriorates prematurely in adults	[13]
	Mouse	**Akirin2** knockout in myogenic progenitor cells causes loss of forelimb and intercostal muscle, as well as skeletal defects, craniofacial abnormalities and neonatal lethality	[26]
	Mouse C2C12 cells	**Akirin1** is regulated by myostatin and increases muscle markers MyoD, myogenin and p21 during myofiber production	[10]
	Mouse	**Akirin1** transcriptionally activates MuRF-1 and is required for normal phosphorylation of FOXO3	[54]
Limb development	Mouse	Loss of **Akirin2** blocked interdigital regression by indirectly causing persistent FGF8 expression in the apical ectodermal ridge	[27]
Meiosis	*C. elegans*	**akir-1** mutants have aggregated synaptonemal complex components in the germline, beginning at the earliest stages of meiosis	[12, 21]
Cancer	Rat	There is high expression of **Akirin2** in tumor cell lines compared with non-tumor cell lines. Knockdown of **Akirin2** mRNA in tumor cells led to reduced tumor size when injected into rats	[11]
	Human, mouse	High levels of **Akirin2** are associated with worsened prognosis for cholangiocarcinoma in humans. **Akirin2** knockdown in mouse reduces tumor size and inhibits vessel formation via an IL-6-STAT3-VEFGA pathway	[30]

second putative NLS that is not shared with either fly Akirin or Akirin1. Of these 2 putative NLSs in Akirin2, removal of the N-terminal one [residues 24–29 (P–K–R–R–R–C)] indicated that it is essential for nuclear localization [6]. Akirin2 contains two helical structures, one within the C-terminus and one within its central region, with the remainder of the sequence predicted to be intrinsically disordered [14]. Vertebrate Akirin2 proteins have five confirmed binding sites for 14–3-3β proteins (Fig. 2), some of which are also present in Akirin1 [4, 11].

Tissue expression of Akirins

In general, *Akirin* genes and/or the proteins they encode are widely expressed in a variety of tissues. According to the *Drosophila melanogaster* microarray expression databases, Flybase [15] and Flyatlas [16], Akirin is ubiquitously expressed at all stages of development as well as in the adult, with expression in some tissues further confirmed by immunostaining [9, 17]. Consistent with

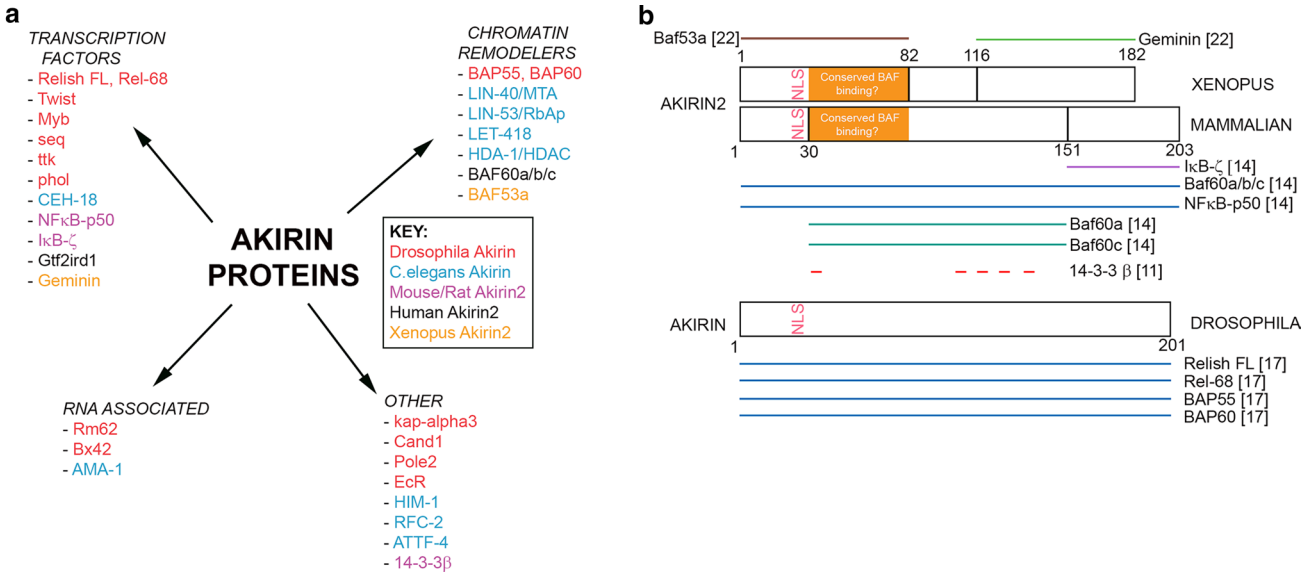

Fig. 2 Akirin interactors and binding sites. **a** Known Akirin interactors found in various studies from *Drosophila*, *C. elegans*, mammalian, and *Xenopus* Akirins. These interactors can be broadly categorized as transcription factors, chromatin remodelers, and RNA associated. **b** Direct binding sites discovered from Akirin-interacting proteins as elucidated by co-immunoprecipitation studies. The colored lines represent the region identified as essential for the denoted protein to interact with Akirins, shown above (for *Xenopus*) or below (mammalian and *Drosophila*) the representation of the Akirin protein structure

this, transcriptional reporter assays in *C. elegans* indicate Akirin promoter activity from late embryonic development to the adult hypodermal and neuronal cells [18, 19]. Germline expression of worm *Akirin* (*AKIR-1*) is enriched in oocytes, but it is also found at lower levels in all other germline cells [18, 20, 21]. In somatic cells, *C. elegans* Akirin (AKIR-1) protein is detectable in GFP-tagged transgenics in only a few epidermal cells during development and in the germline of the adult worm. Using immunofluorescence, AKIR-1 protein can also be seen in the nuclei of many tissues, including adult muscle nuclei, indicating that it is likely broadly expressed in most tissues, albeit mostly at low levels [13, 18, 20, 21].

In *Xenopus laevis*, both *Akirin1* and *Akirin2* mRNA are expressed throughout embryonic development, including in the egg, and in situ hybridization revealed an enrichment of both genes in the CNS [22]. Similarly, data from the mouse ENCODE transcriptome database indicate ubiquitous expression of both *Akirin1* and *Akirin2* in rodent tissues [23]. However, more detailed analysis suggests tissue-specific variation in expression levels. For example, adult rat testis, cerebrum, cerebellum, lung, ovary, thymus, muscle, and kidney exhibited robust *Akirin2* mRNA expression, whereas liver, heart, and spleen expressed only low levels [11]. Further work in the mouse has since shown strong Akirin2 protein expression in B lymphocytes of the spleen [24] and peritoneal macrophages [14], as well as in the embryonic brain [25], somites, muscle precursors [26], and limb bud [27]. In humans, both *Akirin1* and *Akirin2* were reported to be ubiquitously expressed [28], though levels vary [6]. *Akirin1* transcripts were also recently detected in human natural killer cells [29]. In our own studies, we have observed that high Akirin2 levels in blood leukocytes can interfere with accurate measurement of expression in vascularized tissues; therefore, we note that any expression data obtained from tissues not flushed by transcardial perfusion prior to collection may be confounded by blood contamination. With this caveat in mind, we nevertheless can conclude that the *Akirin* genes are likely expressed ubiquitously, though at varying levels, in most organisms. Given the apparent post-translational control of Akirin protein levels [20, 21, 26], immunostaining or western blot data should be gathered to confirm levels of Akirin proteins in tissue under study.

Cellular localization of Akirin proteins

Consistent with the NLS found in all Akirin homologues, immunostaining using antibodies against either endogenous or tagged Akirins has shown strong nuclear localization for *Drosophila* Akirin [6, 17], *C. elegans* [18, 20, 21], human Akirin1 and Akirin2 [6, 30], rat Akirin2 [11], and mouse Akirin2 [26]. However, recent research using subcellular fractionation, western blotting, and immunofluorescence staining for endogenous Akirin2 has also demonstrated an appreciable level of non-nuclear protein in mouse [26], and human [3, 30, 31]. There is currently no clear understanding of a cytoplasmic role for Akirins, although genetic and

physical interactions with nuclear *importin* genes such as importin-α2 (*C. elegans*) and karyopherin-α3 (*Drosophila*; a.k.a., importin-α3) have been reported [17, 21], suggesting that Akirins may have a role in the proper transport of proteins between nuclear and cytoplasmic compartments. This is likely to be a fruitful avenue of further research going forward.

Akirins in gene regulation

Given the primary cellular localization of Akirins in the nucleus, it is not surprising that the best-characterized functions for these proteins are in gene regulation. Research in a wide variety of systems has demonstrated that Akirins have a context-dependent role in modulating gene expression via both activation and repression. We thus begin our discussion of Akirin functions by focusing on this primary role, including the protein–protein interactions and signaling pathways involved. Following this, we will then delineate some of the tissue-specific functions of Akirin proteins that may, or may not in some cases, result from their nuclear role in gene regulation.

Initial studies in *Drosophila* identified a role for Akirin in gene activation of the Imd pathway [6] and in promoting Twist-regulated gene expression during muscle development [9]. *Akirin* knockdown using RNAi led to reduced Imd pathway response to Gram-negative bacteria, as measured by luciferase reporter assays driven by the promoters for Imd-dependent genes *Attacin* and *Diptericin* [6]. This study also demonstrated that Akirin functions in parallel with, or downstream of, the NFκB transcription factor, Relish [6]. Close examination using immunostaining indicated that Akirin localized to puffed regions of polytene chromosomes along with Ser10-phosphorylated Histone H3 and Ser7-phosphorylated RNA PolII, both markers of transcriptionally active loci [9]. Akirin protein was also separately shown to colocalize with the active transcriptional mark acetylated-H3K9 and to be mostly excluded from loci marked by the transcriptionally inactive H3K9me2 [17]. Because Akirin colocalized and functioned in parallel with Relish/NF-κB, Bonnay et al. performed microarray analyses following stimulation of the Imd pathway to assess the transcriptional effects of Akirin knockdown in *Drosophila* S2 cells. They found that among the 170 Relish-dependent genes analyzed, 17 were also Akirin-dependent; in addition, 31 Akirin-dependent, Relish-independent genes were identified. Studies in murine cells support a conserved function for Akirin2 in immune pathway gene regulation. Mouse Akirin2 was shown to be critical for the transcriptional upregulation of several lipopolysaccharide (LPS)-inducible genes in mouse embryonic fibroblasts (MEFs), including those encoding IL-6, IP-10, RANTES, and BCL3 [6]. This was further supported in mouse macrophages by microarray analysis of known LPS-inducible genes: 187 genes that are normally responsive to LPS were no longer so in the absence of Akirin2 [2, 14].

In contrast to such roles in transcriptional activation, Akirins have also been reported to influence gene repression. For example, the microarray study mentioned above [17] also found that Akirin knockdown in *Drosophila* resulted in the upregulation of 205 genes, suggesting that Akirin normally mediates their repression. Furthermore, studies using GAL4-luciferase assays in rat hepatoma K2 cells demonstrated a transcriptional repressor role for Akirin2 (referred to in this study as FBI1; [11]). Akirin2 overexpression was able to decrease activity of MAP kinase phosphatase-1 (MKP-1)-Luciferase [11] and basal cell adhesion molecule (BCAM)-Luciferase [32] constructs. Consistent with this, both MKP-1 and BCAM proteins were upregulated in cell clones stably expressing an Akirin2 antisense cDNA [11, 32]. Thus, at least in some contexts, Akirin proteins may act as transcriptional repressors rather than activators. It should be noted that in studies knocking out or knocking down Akirins, some alterations in gene expression may be indirect.

Association with chromatin remodeling complexes

Fly Akirin was found to be present at some, but not all, active transcriptional sites, suggesting that Akirin's regulation of gene expression is selective rather than ubiquitous [9]. An explanation of this selective regulation was provided by reported interactions between Akirins and components of chromatin remodeling complexes. One example is the evolutionarily conserved complex related to the *Saccharomyces cerevisiae* SWI/SNF (switch/sucrose non-fermentable) complex, referred to as the BAP (Brahma-associated protein) complex in *Drosophila* and BAF (Brg1-associated factor) complex in vertebrates [33, 34]. Eukaryotic cells typically contain two types of SWI/SNF-related complex: *Drosophila* contain BAP (Osa-containing) or PBAP (BAP180, BAP170-containing) and mammals have BAF and PBAF, which are determined based on subunit composition. These multi-subunit protein complexes regulate transcription by repositioning nucleosomes using an ATP-powered core helicase (Brahma/Brg1), to modify the access of transcriptional activators, repressors, and other components of transcriptional machinery [35]. Impairment of BAP/BAF machinery has been shown to have devastating effects on organismal development, demonstrating its critical importance [36, 37].

A complete *Drosophila* proteome interaction map generated using yeast-2-hybrid screening indicated a direct interaction between Akirin and an integral BAP/PBAP complex protein, BAP60 [38]. This was further confirmed in a study of *Drosophila* myogenesis, where Nowak and colleagues found that Akirin colocalized with the Brahma helicase and with Osa, a BAP-specific subunit [9]. Furthermore, they

showed that muscle development defects occurred in heterozygous crosses between Akirin and shared members of the BAP/PBAP complexes, including Brahma, Moira, Snr1, BAP60, as well as Osa, indicating that Akirin genetically interacts with both BAP and PBAP complexes [9]. This was further supported in *Drosophila* S2 cells, where Akirin colocalized and coimmunoprecipitated with BAP60 and BAP55 following immune challenge [17]. The interaction between Akirins and SWI/SNF-related chromatin remodeling complexes is conserved, as murine Akirin2 coimmunoprecipitated with all three isoforms of BAF60 (a, b, and c), the mammalian homologue of BAP60 [14], in vitro, and with BAF60a in peritoneal exudate cells [14]. Further assays using truncations of the N-terminus (Δ1–30) and C-terminus (Δ151–203) of human Akirin2 found that BAF60a binds to both truncated forms, suggesting that the central portion of the protein is critical for this interaction (Fig. 2). This central portion of Akirin2, which has a putative helical structure, was found to be conserved amongst Akirins in 50 species analyzed [14]. Finally, *Xenopus* Akirin2 was found to coimmunoprecipitate with another member of the BAF complex, BAF53a, (homologous to fly BAP55), an interaction that required residues 1–82 of the frog Akirin2 protein [22]. However, it is interesting to note that no association was found between *Xenopus* BAF60 and Akirin2 in the same study [22], though it is possible that this resulted from methodological differences with prior studies. Putting these results together, we can conjecture that a critical BAF complex-binding region of Akirin2 lies approximately between amino acids 30–82 (Fig. 2).

Worms lack NFκB proteins; nevertheless, the role of Akirins in bridging transcription factors and chromatin remodeling machinery appears to be conserved, as worm Akirin (AKIR-1) is required for antifungal resistance through the activation of genes encoding for antimicrobial peptide [18]. Unlike the fly and vertebrate proteins, worm Akirin does not act with the SWI/SNF complex, but rather with the distinct Nucleosome Remodeling and histone Deacetylase (NuRD) complex [13, 18]. Worm Akirin interacts physically with the core subunits of the NuRD complex—HDA-1/HDAC, LIN-40/MTA, LIN-53/RbAp—and with LET-418, one of the Mi-2 subunit orthologs [18]. Low-confidence interactions were also found for the Mi-2 subunit orthologs CHD-3 and the NuRD-related complex protein MEP-1/Kruppel-like protein [18]. *C. elegans Akirin (akir-1)* was also shown to interact genetically with components of the NuRD complex in processes regulating innate immunity and required for movement and body length determination [13, 18]. Since SWI/SNF components were identified as low-confidence interactions in a mass-spectrometry screen, it is still possible that worm Akirin may interact with SWI/SNF in some biological processes and/or in a small number of cells [18]. Since the interaction between Akirin and NuRD has not yet been extensively explored in other systems, it may be that the Akirin–NuRD interaction is also evolutionarily conserved: a potentially fruitful topic of study in the future. Overall, the findings in worms are consistent with an evolutionarily conserved role for Akirins in regulating gene expression through interaction with chromatin remodeling complexes.

Association with transcription factors

Interrogation of Akirin protein sequences has revealed intrinsically disordered regions that are highly conserved [3, 14]. As intrinsically disordered domains increase the diversity of partners to which the same protein can bind [39], conservation of these domains indicates they may be important for Akirin function by mediating numerous protein–protein interactions. This hypothesis has become increasingly likely as Akirins have been shown to interact with multiple transcription factors in a variety of cellular contexts. The current body of literature has not experimentally demonstrated any clear DNA binding domains within Akirin proteins; however, recent in silico modeling of tick subolesin and vertebrate Akirin2 structure has proposed putative DNA-binding regions, raising the intriguing possibility that Akirins themselves could act as co-transcription factors [3].

Drosophila Akirin has been shown to colocalize and coimmunoprecipitate with the NFκB transcription factor, Relish, with the interaction stabilized following immune challenge [6, 17]. Akirin was also shown to colocalize and interact genetically, functionally, and physically with the highly conserved developmental transcription factor, *Twist*, both in vivo and in vitro [9]. Coupled with the data discussed above demonstrating BAP/BAF subunit binding, this led to the hypothesis that Akirin acts as a "bridge" between transcription factors and chromatin remodeling machinery [1, 2, 40].

The mammalian NFκB family of transcription factors contains 5 family members which are typically found as homo or heterodimers: p50 (NFκB1), p52 (NFκB2), p65/RelA, RelB, and c-Rel. Inactive NFκB transcription factors are sequestered in the cytoplasm by IκB proteins. Upon activation, IκB proteins are phosphorylated and degraded, allowing NFκB dimers to be released and translocate to the nucleus where they bind to κB binding sites, resulting in gene activation or repression [41]. Following immune system stimulation in mammalian cells, Akirin2 was found to interact with the NFκB subunit p50, but not p65/RelA [14]. In addition, the C-terminus of Akirin2 binds to ankyrin repeats within the C-terminus of the atypical IκB-like protein, IκBζ (zeta) [14]. This was proposed to generate a bridge complex consisting of p50-Akirin2-IκBζ, which functions to regulate gene transcription. The idea that these molecules form a cooperative transcription activating complex is supported by the fact that IκBζ facilitates the binding of p50 homodimers to κB DNA [42] and that both IκBζ

and Akirin2 are required for expression of the IL-6 and IL-12b genes [14].

Interestingly, Akirin2 was recovered in a yeast-2-hybrid screen for interactors of the transcription factor, Gtf2ird1 (GTF2 repeat domain containing protein 1). The GTF2IRD1 gene is implicated in Williams–Beuren syndrome, a developmental disorder presenting with intellectual disability and a variety of craniofacial and cardiovascular defects [43]. No further work has been done on this possible interaction, so it is unclear how significant Gtf2ird1 may be as an Akirin2 partner. However, as discussed in detail below, our work utilizing tissue-specific knockout of Akirin2 has uncovered phenotypes that could relate to Williams–Beuren syndrome, including agenesis of the cerebral cortex and craniofacial defects ([25, 26]; see sections below). Finally, *Xenopus* Akirin2 has been shown to interact with the N-terminus of the transcription factor, Geminin, via its own C-terminus (amino acids 116–182) [22]. It is important to note that *Xenopus* Akirin1 did not interact with either BAF53a or Geminin, indicating a non-redundant role for Akirin2 in these interactions [22].

Association with 14-3-3 proteins

One of the initial studies on Akirin2 isolated in a yeast-2-hybrid search for novel 14-3-3β-interacting proteins, leading to its alternative name, fourteen-three-three-beta interactant-1 (FBI1; [11]). Akirin2 has five putative 14-3-3β binding sites (some of which are conserved throughout vertebrate Akirins; [4, 44, 45]), and an Akirin2/14-3-3β interaction was further confirmed using GST pulldown assays and co-immunoprecipitation in K2 cells [11]. Interestingly, mutation of any of the five binding sites led to loss of this interaction, suggesting that multiple binding sites are required for the interaction to occur. Using an antibody raised against Akirin2, an Akirin2/14-3-3β complex was shown to bind to the *MKP1* [11] and *BCAM* [32] promoters via consensus sequences within promoters known as GC boxes, resulting in gene repression. The 14-3-3 proteins interact with a wide array of partners including transcription factors, enzymes, and cytoskeletal components. Through these interactions, 14-3-3 proteins are implicated in the regulation of many cellular processes including proliferation, apoptosis, and cell cycle regulation [46]. Given the possibility of partial non-nuclear localization of Akirins, it is interesting to speculate that 14-3-3 interactions might target Akirins to a variety of subcellular compartments.

Association at promoters of Akirin-dependent genes

The hypothesis that Akirins act as bridging proteins to stabilize the interaction of chromatin remodeling machinery and transcriptional regulators is further supported by studies finding that Akirins are critical for the recruitment of such regulators to specific promoter regions. As noted above, *Drosophila* Akirin interacts with both the NFκB transcription factor Relish and the chromatin remodeling complex subunit BAP60. Using Chromatin Immunoprecipitation (ChIP) experiments, Akirin, BAP60, and Relish were all found to be bound to promoter regions of the Akirin-dependent genes, *Attacin-C*, *Drososin,* and *Cecropin-A1*. The strength of this enrichment was increased with extended immune system activation via treatment with heat-killed *Escherichia coli* [17]. Consistent with Akirin regulating a limited set of genes, it was found with Twist and the BAP/PBAP member Moira at the promoter for one Twist target gene, *Dmef2*, but not at another, *even-skipped* [9]. Levels of Moira bound to the *Dmef2* promoter were Akirin-dependent, as 64% less Moira was bound in the absence of Akirin. In contrast, Twist binding to the *Dmef2* promoter did not require Akirin, suggesting that Twist may first bind to the Dmef2 promoter, then interact with Akirin, which subsequently recruits Moira [9].

Akirin2 knockout in murine macrophages prevents IL6 and IL12p40 expression following immune system stimulation, which can be explained by a vastly reduced recruitment of phospho-PolII at the *IL6* and *IL12b* promoters [6, 14]. Consistent with the interaction between Akirin2, the BAF complex and IκBζ, the deletion of either *Akirin2* or IκBζ caused reduced occupancy of the BAF core helicase, Brg1, at both the *IL6* and *IL12b* promoters. This suggests that the interaction of Akirin2 with these components at the gene promoters is essential for gene expression to commence [14]. Akirin2 was also found to be required for proper chromatin remodeling, as isolated chromatin exhibited decreased availability of the *IL6* promoter in the absence of *Akirin2* [14]. Together, this suggests a model of transcriptional regulation in which an IκBζ–Akirin2–BAF60a complex binds to DNA via IκBζ. In this model, Akirin2 recruits the BAF complex to the promoter through BAF60a. Consistent with this general model, *Akirin2* deletion in B cells led to reduced Brg1 recruitment at *myc* and *cyclin D2* promoters, together with reduced mRNA expression of both of these genes [24].

Genes that are regulated by chromatin remodeling complexes typically have a lower CpG content, as their main regulation is through movement of nucleosomes rather than histone modification or DNA methylation [47]. As noted above, Bonnay et al. showed in *Drosophila* that Relish target genes activated during immune stimulation could be divided into Akirin-dependent and -independent genes. Comparing gene promoters, it was found that Akirin-dependent genes contained fewer CpG rich regions than did Akirin-independent genes [17]. This observation holds true in vertebrates, as only 20% of Akirin2-dependent genes in mouse peritoneal macrophages were found to contain CpG islands in their

promoters, compared with 58% of Akirin2-independent genes [14]. Together, this evidence establishes an important function for Akirins in the recruitment of chromatin remodeling complexes to low CpG promoters.

Gene regulation through histone modifications

Consistent with the fact that Akirins interact with chromatin remodeling complexes containing histone acetyltransferases, several studies have reported a link between Akirins and histone modification. In *Drosophila*, Akirin-dependent gene promoters are rich in H3K4 acetylation, a mark of active transcription, in contrast to Akirin-independent gene promoters; furthermore, the abundance of these marks is reduced in the absence of Akirin or its binding partners, BAP60, or Relish [17]. Consistent with this, ChIP of mouse macrophages using antibodies for the active transcriptional markers trimethylated H3K4 and acetylated H3K9 revealed that in the absence of Akirin2, H3K4 methylation and H3K9 acetylation decreased on Akirin2-dependent gene promoters but not Akirin2-independent gene promoters [14]. Therefore, the presence of Akirins seems to be required for the acquisition of activating histone modifications at some promoters.

How might Akirins have such a function? DNA methyltransferase-associated protein-1 (DMAP1) interacts with *Drosophila* Akirin as well as two known Akirin interactors, BAP55 and Relish [48]. Similar to Akirin, DMAP1 is implicated in activation of Relish target genes in the Imd immune pathway [48]. DMAP1 also contains a SANT (SWI3-ADA2-NcoR-TFIIIB) domain that interacts with histone tails and functions as a component of a histone acetyltransferase complex that acetylates H4K16 to promote chromatin relaxation [49]. In addition, the Akirin2 binding protein, IκBζ, has been reported to facilitate H3K4 trimethylation [11]. Therefore, through its reported interactions with DMAP1 and IκBζ, Akirin2 may help modify gene expression via histone modification; however, further investigations are required to determine the extent to which Akirin2 directly alters histone modifications.

Akirin roles in cell signaling pathways

Akirins have been proposed to act through several signaling pathways, in ways that are cell-type dependent. One is the mitogen-activated protein kinase (MAPK) cascade, in which activation of cell surface receptors triggers phosphorylation of downstream extracellular signal-regulated kinases ERK1/2 (a.k.a. MAPK3/1). The strength and duration of ERK activation (phosphorylation) controls the outcome, ultimately resulting in transcriptional changes controlling cell cycle progression, proliferation, and differentiation [50]. In both K2 hepatoma cells [11] and LLC1 cells [51], stable expression of an Akirin2 antisense construct shortened phosphorylated-ERK1/2 (p-ERK1/2) activation concurrent with increased mRNA expression of MAP kinase phosphatase-1 (MKP-1) [11]. In contrast, Akirin2 knockout studies utilizing *LysM-Cre* for myeloid lineage cells [14] and *CD19-Cre* for B cells [24] found no change in p-ERK1/2, p-p38/MAPK or p-c-Jun terminal kinases (JNKs) in response to LPS treatment (myeloid lineage) or anti-CD40 treatment (B cells), demonstrating the importance of cellular context when studying Akirin2 cell signaling. In addition, there is limited evidence in porcine muscle satellite cells that Akirin2 activates ERK1/2 and NFATc1 through a calcineurin signaling pathway [52] and regulates an AMP-activated protein kinase (AMPK) pathway [53]. This latter pathway may be shared by both vertebrate Akirins: *Akirin1* null mice exhibit reduced AMPKα in skeletal muscle, whilst overexpression of Akirin1 leads to increased AMPKα in the C2C12 muscle cell line [54].

In *C. elegans*, Akirin has been shown to act downstream of the transforming growth factor beta (TGFβ) signaling pathway [13]. Akirin mutant worms have reduced body size, similar to TGFβ Sma/Mab mutants, and double-mutant analysis indicates that Akirin acts in the same pathway as *sma-2*, *sma-3* (encoding R-Smad), and downstream of *dbl-1* (encoding the TGFβ ligand) [13, 56]. The TGFβ Sma/Mab pathway regulates multiple target genes through several transcription factors [56]. It is likely that Akirin regulates only some of these targets, as *akir-1* mutants exhibit approximately half of the magnitude of effect (in RAD-SMAD reporter activation and body size reduction assays) observed in TGFβ Sma/Mab ligand/ R-Smad mutants. Consistent with this, Akirin does not act via *sma-9*, one of the transcription factors in this pathway, indicating that it regulates body size through a different transcription factor [13]. Akirin mutants also share other phenotypes with mutants in the TGFβ Sma/Mab pathway: they have defects in male tail development, increased autophagy, and defects in lipid droplet formation [13, 57–59]. These studies are consistent with a transcriptional role for Akirin during development, in response to the activation of the TGFβ pathway. Akirin also has a conserved role in the innate immune response, which is not mediated by NFκB, since worms lack this pathway; rather, this requires PMK-1 (the MAPK14 ortholog), consistent with the mammalian results discussed above [18].

Akirin roles in the regulation of cell cycle, cell proliferation, and cell survival

A wide variety of evidence in multiple model systems supports a major functional role for Akirin proteins in the regulation of cell proliferation and cell survival. Cell cycle progression and proliferation are closely associated with

14-3-3 proteins and MAP kinase signaling cascades [46, 50]. As noted above, MKP1 suppression by an Akirin2/14-3-3β complex coincided with a shortened p-ERK1/2 activation period [11]. Insufficient ERK1/2 signal has been shown to decrease CyclinD1 expression, which in turn regulates progression through the G1 phase of the cell cycle [11, 60]. In contrast, other studies show a role for Akirin2 in the regulation of cell cycle progression independent of MAPK signaling. For example, restricted *Akirin2* knockout in B cells using *CD19-Cre;Akirin2*$^{fl/fl}$ mice resulted in reduced mRNA expression of *Cyclin D1, Cyclin D2*, and their regulator, *c-Myc* [24]. This was not due to disruption of the MAPK pathway, as levels of p-ERK1/2 and p-p38MAPK were unchanged in *Akirin2* null B cells; instead, ChIP using antibodies for the BAF complex helicase, Brg1, revealed that *Akirin2* deletion abolished the binding of chromatin remodeling complexes to the mouse *Myc* and *CyclinD2* gene promoters [24].

Consistent with a role in cell cycle progression, *Akirin2* null B cells failed to proliferate in response to mitogens, resulting in fewer cells in bone marrow, spleen, and inguinal lymph nodes and vastly smaller spleens in these *CD19-Cre;Akirin2*$^{fl/fl}$ mice [24]. Further experiments showed fewer B cells in S-phase and reduced [^{3}H]-thymidine uptake in response to mitogen activation in the absence of *Akirin2* [24]. Consistent with these findings, multiple studies have shown that *Akirin2* knockdown reduces colony formation of tumor cells, tumor volume, and/or tumor weight [11, 30, 51].

Regulation of balanced proliferation and apoptosis is vital for cells during both development and immune activation. *Akirin2* knockout splenic B cells failed to upregulate the anti-apoptotic genes *Bcl-2* and *Bcl-xl* and thus were unable to avoid apoptosis in response to anti-CD40 treatment [24]; similarly, the key apoptosis effectors cleaved-caspase 3 (CC3) and cleaved-caspase 7 (CC7) were found to increase following *Akirin2* knockdown in T98G glioma cells [31]. Our own work in mice utilizing *Akirin2*$^{fl/fl}$ conditional knockout mice and tissue-specific Cre drivers showed that knockout telencephalic cerebral cortex progenitors [25] and myogenic precursor cells [26] exhibit massive apoptosis during embryogenesis in vivo.

For the remainder of this review, we will turn our attention to roles recently discovered for Akirins in the development of multiple organ systems and cell types (Fig. 1). As discussed above, the initial function described for any Akirin protein was in innate immunity, a function conserved from *Drosophila* to *C. elegans* to mammalian cells. Because this has been covered in excellent detail in two recent reviews [1, 2] we will not discuss Akirin roles in the immune system any further than we have above. We will focus instead on functions in brain development, myogenesis, limb formation, meiosis and germline progression, and cancer.

The role of Akirin proteins in brain development

Early studies found expression of *Akirin1* and *Akirin2* in adult human brain [6] and rat cerebrum and cerebellum [11] using Northern blotting. More recently, we have detected *Akirin2* expression using RT-PCR in the developing mouse cerebral cortex as early as E11 and continuing through to adulthood [25]. Additional work in *Xenopus laevis* found expression of both *Akirin1* and *Akirin2* at all developmental stages examined (Stages 1–30, including the egg), with whole mount in situ hybridization indicating that *Akirin1* and *Akirin2* levels become enriched in the nervous system during development [22]. These expression data suggest that Akirins could play important roles during neural development; perhaps because *Akirin2* is an essential gene while *Akirin1* is not [6], work thus far has focused on Akirin2.

Our study focused on the developing mouse cerebral cortex was the first to examine Akirin functions in the brain of any organism [25]. Deletion of the conditional floxed *Akirin2* allele was restricted to the developing telencephalon using *Emx1-Cre* (hereafter referred to as *Emx1-Cre;Akirin2*$^{fl/fl}$), which resulted in a severe microcephalic phenotype, with complete loss of the dorsomedial cortex and near-complete loss of the ventrolateral cortex. Nearly all mutants died at birth, though a few survived for up to 4 weeks [25]. Shortly after *Emx1-Cre* becomes active (embryonic day (E)9.5), increased CC3 was detected in the mutant telencephalic wall, with apoptosis becoming massive by E12; by E13 the cortex was reduced to a thin layer of cells. Interestingly, the apoptosis in *Emx1-Cre;Akirin2*$^{fl/fl}$ telencephalon initially occurred near the pial surface at the emerging preplate containing the first post-mitotic neurons, subsequently followed by loss of Pax6-positive radial glia progenitors. We observed a reduced ratio of EdU/Ki67 double-positive cells in *Emx1-Cre;Akirin2*$^{fl/fl}$ telencephalon, suggesting increased cell-cycle exit at E10.5, prior to massive apoptosis [25]. This is consistent with the cell cycle changes reported in *Akirin2* knockout immune cells [24]. In addition, *Emx1-Cre;Akirin2*$^{fl/fl}$ embryos exhibited disruption of the ventricular zone apical surface associated with loss of N-cadherin, which is consistent with results in cholangiocarcinoma (CCA) tumor cells described by Leng et al., further discussed below. This work demonstrated a critical role for Akirin2 in neural development and suggested that it maintains cell cycle progression in neural progenitor cells, possibly through regulation of cell adhesion. In the absence of Akirin2, neural progenitors appear to prematurely differentiate, resulting in apoptosis and severe tissue disruption [25].

A subsequent study in *Xenopus* utilized morpholinos (MO) to knockdown *Akirin2* at the two-cell stage [22].

Following *Akirin2* knockdown, the investigators initially found an expansion of dorsal structures in tadpoles, with an enlarged area of expression of neural progenitor markers Sox2 (pan-neural progenitors) and Nkx6.2 (ventral neural progenitors). Furthermore, this study determined that Akirin2 interacts via its N-terminus with BAF53a and via its C-terminus with Geminin. These interactions are hypothesized to antagonize the Sox2-promoting Geminin function in neural progenitor cells of *Xenopus* embryos [22]. Downregulation of Sox2 is a major initiator in a cascade in which cells transition from the neural progenitor state to neurons. However, knockdown of *Akirin2* or *Baf53a* in *Xenopus* expanded the Sox2 expression pattern, as did Geminin overexpression, suggesting that in frogs Akirin2 restricts progenitor expansion [22], in contrast to results in the mouse cortex [25]. Previous studies established that Geminin maintains progenitor state in *Xenopus* embryos through binding to Brg1 and disrupting its association with pro-neuronal transcription factors, Ngnr1 and NeuroD [61]. Furthermore, these authors examined the role of Akirin2 during neurogenesis and found reduced expression of *NeuroD* and *N-tubulin* but not *Ngnr1* in *Akirin2* morphants, suggesting that Akirin2 acts at the level of Ngnr1 to activate N-tubulin expression and promote neuronal differentiation [22]. Despite the differences due to species, CNS region, and approach, this study and ours [25] can be reconciled with a general hypothesis that Akirin2 has a dual function in neurogenesis depending on the differentiation stage of neural cells: in neural progenitor cells, Akirin2 is required to maintain the correct number of progenitor cells, whilst in neurons Akirin2 is required for the progression of neuronal maturation.

The role of Akirin2 in coordinating gene expression via interactions with BAF chromatin remodeling machinery is likely to be relevant to the mammalian cortical phenotypes observed [25]. Numerous recent studies have uncovered a critical role for the BAF complex in mammalian neurogenesis. In mice, BAF53a is a necessary and sufficient component of the neural progenitor BAF (npBAF) complex, which keeps cells in a progenitor state prior to the switch to the neuronal BAF (nBAF) complex [62]. The npBAF and nBAF differ in specific subunits that are swapped at the onset of neurogenesis [63]. This switch in subunits is vital for the transition from progenitor to neuron, as forced expression of BAF53a prevents differentiation of progenitor cells into neurons [62, 63]. Potentially, Akirin2 interacting with BAF53a is an important factor in the switch from the npBAF to the nBAF complexes, a hypothesis that will be interesting to explore in future studies utilizing mammalian models.

Several questions remain to be examined: for example, with no currently identified Baf53b homologue in *Xenopus*, does frog Akirin2 interact with the nBAF via a different component? The *Xenopus* work [22] contrasts with our results in the mouse cortex [25], in which loss of Akirin2 led to premature neuronal differentiation and death, rather than expansion of the progenitor pool. As the C-terminal region of Akirin2 is highly conserved between frogs and mice, the Geminin interaction is likely to also be maintained in mice. We suggest different outcomes are likely explained by different timing of Akirin2 perturbance (neurula stage in frogs vs. early corticogenesis in mice) and the very different progression of neurogenesis between rodents and frogs. Given Akirin2's suggested function via BAF-dependent mechanisms, it is interesting that loss of Brg1, an essential ATPase subunit of the BAF complex, results in expansion of neural stem cells in *Xenopus* but a loss of neural stem cells in mice [68]. This is entirely consistent with the results of both Liu et al. [22] and Bosch et al. [25].

Mutations in a number of BAF subunit genes have been associated with Coffin–Siris Syndrome, a rare autosomal dominant disorder in which microcephaly is observed [69, 70]. Consistent with this, knockout of the Brg1 gene in progenitors (using the broadly active *Nestin-Cre*) leads to a smaller cortex; importantly, however, nothing like the complete loss of cortex seen in *Akirin2* mutant mice [25, 62, 68]. This latter finding indicates that the phenotypes observed in the *Emx1-Cre;Akirin2*$^{fl/fl}$ cortex reflect disruption of both BAF complex gene regulation and other BAF-independent roles of Akirin2. If Akirin2 is important for the switch to neuron-specific BAF subunits, its loss may lead to apoptosis of neurons that have exited the cell cycle but have not correctly initiated a differentiation program. Clearly, elucidating further the molecular mechanisms through which Akirin2 regulates corticogenesis will require identifying gene expression patterns that are disrupted in its absence, as well as the mammalian cortex-specific transcription factors with which it partners.

Expansion of the cortex is a hallmark of human evolution: it makes up ~ 80% of human brain mass, and this increased size is due to alterations in the number, type, and temporal regulation of cortical progenitor cells [71]. Therefore, the demonstrated roles for Akirin2 in the control of neural progenitor proliferation and neuronal maturation could be relevant to human disease. Though Akirins have not yet been directly associated in any neurological disorders, one recent study examined neurodevelopmental phenotypes in children with rare deletions of proximal 6q (6q11–q15), a chromosomal region containing the AKIRIN2 gene [72]. Interestingly, the authors found that 6 of their cohort of 20 individuals had a deletion in AKIRIN2, and these children had moderate to severe developmental delay. This result tentatively links Akirin2 to a measurable result in humans and provides the impetus for further in-depth study in the role of Akirin2 during cortical development.

Akirin roles in myogenesis

Because myogenesis is, perhaps, the developmental process for which the most data exist across a broad range of organisms, we will discuss roles for Akirin, Akirin1, and Akirin2 separately in the sections that follow.

Akirin

As noted above, a role for *Drosophila* Akirin in myogenesis was first discovered after it was isolated in a yeast-2-hybrid assay for protein interactors of Twist [9]. Due to the known role of Twist in the development of muscle, these researchers investigated the effect of *akirin* mutations on *Drosophila* embryos during development. They found that *akirin* mutants exhibited a wide array of somatic muscular defects, including missing muscles, mis-attached muscles, and duplicated muscles [9]. Further work determined that *akirin* interacts both genetically and physically with Twist, and that *akirin* co-occupied the promoter of *Dmef,* a Twist-regulated gene critical for embryonic somatic myogenesis, with the SWI/SNF components Brahma, Snr1 and Osa. As noted above, *akirin* was not present at the *even-skipped* promoter, another Twist target, suggesting that *akirin* functions at selective Twist target genes during muscle development [9, 40].

In contrast to flies, *C. elegans Akirin* is not an essential gene, but we recently showed that it plays a similar, but distinct, role in muscle development and maintenance. Akirin protein is detected at low levels in muscle nuclei, as in other nuclei throughout the worm soma [13]. In more than half of *akir-1* null mutant worms, staining for muscle cell markers revealed mislocalization. In the adult worm, vulvar muscles exhibit functional defects manifested in egg retention in the uterus, vulvar bursts, and mislocalization of a transcription factor required for vulvar development [13]. In light of the *Drosophila* studies detailed above, it is important to note that worm vulva tissues are known to require gene regulation via the worm Twist orthologue, HLH-8 [73]. Finally, loss of Akirin results in the premature deterioration of body-wall muscle structure in adult worms [13]. Together, these results support an evolutionarily conserved role in muscle development and maintenance for Akirin.

Akirin2

Consistent with its critical role during mammalian embryonic development [6] and the work discussed above, we also recently found *Akirin2* to be a critical gene in mouse myogenesis [26]. Strong Akirin2 protein expression was detected in developing mouse somites at E10.5, and *Akirin2* knockout in somites and their derivatives using a *Sim1-Cre* transgenic line resulted in devastating effects leading to neonatal lethality. The mutant embryos contained no intercostal or forelimb muscles, and exhibited defects of the skeleton, including non-parallel and fused ribs, shorter forelimbs, and craniofacial abnormalities. This was found to be due to extensive apoptosis of early myogenic precursor cells, which never developed into the Pax3-expressing cells that enter the limb bud and give rise to skeletal muscle [26]. Similar to what we observed in *Akirin2*-null telencephalon, dividing muscle precursor cells appear unable to tolerate the loss of *Akirin2* during embryogenesis [25, 26].

Given the early death of *Akirin2*-null muscle precursors in vivo, we utilized C2C12 myoblasts to look for later roles of Akirin2 [26]. Upon induction of myotube differentiation by switching to a low-serum media, Akirin2 protein levels increase, peaking after 12 h and then rapidly dropping to a very low level concurrent with upregulation of the differentiation proteins myogenin and myosin heavy chain (MHC). The pattern of Akirin2 expression thus resembles that of the transcription factor MyoD, which transcribes myogenesis-promoting target genes at the onset of differentiation [74]. Interestingly, the mRNA expression level of Akirin2 did not replicate this protein pattern. Instead, *Akirin2* mRNA maintained steady expression during differentiation, suggesting a dynamic regulation of Akirin2 protein via a currently unknown mechanism presumably involving post-translational modifications. Furthermore, shRNA knockdown of *Akirin2* in C2C12 myoblasts led to significant decreases in myogenin and MHC, but not MyoD, proteins during subsequent myotube differentiation, supporting a role for Akirin2 in the progression of myogenesis gene expression [26]. Intriguingly, when Akirin2 was overexpressed in C2C12 cells, MHC protein again decreased, but neither MyoD nor myogenin levels changed. We suggest that Akirin2 may be necessary for cells to reach an intermediate (myogenin-positive) stage of differentiation, following which it must be downregulated for efficient up-regulation of MHC [26]. One other study using C2C12 reported that Akirin2 overexpression led to increases in the mRNA for MHCI, MHCIIa, MEF2C, NFATc1, and MCIP1.4, as well as higher immunofluorescence staining for MHC [75], differing somewhat from our results. We note that altered mRNA levels do not always correspond to protein levels, as we found for Akirin2 itself [26]. In any case, it is now clear that Akirin2 plays important roles in multiple stages of myogenesis in mammals, and thus the role of Akirin in *Drosophila* and *C. elegans* is well conserved.

Studies using satellite cells isolated from porcine muscles are also consistent with a role for Akirin2 in the proliferation and differentiation of these cells [52, 76]. For example, siRNA knockdown of Akirin2 led to reductions in the mitotic marker phospho-histone H3 (PH3) and incorporation

of EdU whilst overexpression of Akirin2 had the opposite effects [52]. Additionally, overexpression of Akirin2 led to increases in protein levels of myogenesis differentiation factors, MHCI, MCIP1.4, NFATc1 and calcineurin activity, whilst knockdown leads to decreases in these same mRNA and proteins [52, 76]. The function of Akirin2 has thus been linked to calcineurin signaling, ERK1/2 signaling, and AMPK signaling [52, 53, 76]; however, further studies are necessary to allow firm conclusions to be drawn about the Akirin2-dependent cell signaling pathways in satellite cells.

Intriguingly, we note that mutations in *Akirin2* have been associated with meat quality in cattle [77–79] and pigs [80]. A single nucleotide polymorphism (SNP; G → A) was found in the 3′UTR of the *Akirin2* gene in Japanese Black cattle with high-marbling of the *musculus longissimus* muscle [77–79]. Furthermore, whole genome sequencing of Berkshire pigs, which have superior meat quality due to neutral fatty lipid deposits and marbling fat, revealed that *Akirin2* was among a group of genes selected for in this breed [80]. Taken together, Akirin2 appears to be an important indicator of meat quality and may be a useful marker for selective breeding programs: a gastronomic confirmation of its critical role in myogenesis.

Akirin1

A role for Akirin1 (initially named Mighty) during myogenesis was first uncovered due to its regulation by the negative growth factor, Myostatin [10]. In *myostatin* null mice, *Akirin1* mRNA expression was among the genes significantly increased; conversely, *Akirin1* was repressed by exogenous myostatin treatment [10]. This result was further supported by examining double-muscled cattle, which have a non-functional *myostatin* gene: myoblasts derived from these cattle express higher levels of Akirin1 protein, in addition to increased MyoD, myogenin, and p21 protein during differentiation [10]. Injections of a virus encoding Akirin1 into the tibialis anterior (TA) muscle of mdx mice, a model of Duchenne muscular dystrophy, resulted in an increase in muscle weight and fiber size [10]. As mentioned earlier, however, *Akirin1* null mice displayed no obvious phenotypic differences compared to wild-type littermates [6]. Subsequent work specifically examining muscle found no difference in *Akirin1* null mice in TA myofiber number or quadriceps weight [54]. However, *Akirin1* null mice did show a switch in muscle fiber type, from oxidative to fast oxidative fibers. *Akirin1* null mice exhibited muscle succinate dehydrogenase activity, consistent with a reduced oxidative capacity of skeletal muscle in these mice [54].

Akirin1 has a protein expression pattern similar to that of Akirin2 during C2C12 proliferation and differentiation [10]. Akirin1 levels increase and peak at 12–24 h after initiation of differentiation, followed by a decrease in protein expression concurrent with the upregulation of the pro-myogenic proteins, MyoD, myogenin, p21, and MHC. In support of in vivo phenotypes, Akirin1-overexpressing C2C12 cells displayed upregulation of p21, MyoD, and myogenin as well as generating an increased number of MHC-positive myotubes that were larger [10, 81]. Consistent with this, siRNA targeted against *Akirin1* led to decreased MyoD, myogenin and p21 expression [10] and reduced total myotube area in differentiating C2C12 cells [82].

Myostatin is known to maintain quiescence of satellite cells, which are activated following muscle tissue injury and can then proliferate and differentiate into myotubes. Salerno et al. found that mouse TA muscle injured by notexin, a myotoxin derived from snake venom, increased Akirin1 at both the mRNA and protein levels; however, it must be noted that the western blot band for Akirin1 appeared at 49 kDa in this study [55], much higher than the predicted molecular weight of the protein and higher than reported in a subsequent study [83], so we must cautiously interpret this result. Immunocytochemistry of regenerating TA muscle demonstrated upregulated Akirin1 at days 5 and 7 following both notexin and mechanical (cut) injuries, further supporting an important role in muscle regeneration. Consistent with this proposed role, dexamethasone (Dex) was shown to impair satellite cell proliferation and differentiation by increasing myostatin levels and thus decreasing Akirin1 protein expression [81]. Furthermore, shRNA knockdown of *myostatin* prevented Dex treatment from downregulating Akirin1 protein. As Dex treatment is associated with muscle atrophy, this study supports a mechanism whereby Dex inhibits Akirin1 via myostatin, thus reducing muscle cell growth [81]. Finally, cryolesions of TA muscle showed that Akirin1 levels are increased at 10 days post-lesion and that this upregulation is even greater in β2-adrenergic receptor knockout mice, further supporting a role for Akirin1 in muscle tissue injury recovery [83].

Progress has been made regarding the mechanism of action of Akirin1 in muscle tissue, although more work is required for a full picture. Akirin1 has been shown to activate the E3-ubiquitin ligase, muscle-specific RING finger-1 (MuRF-1) at the transcriptional level [54]. Akirin1 knockout quadriceps muscle has lower MuRF-1 protein and mRNA levels, and overexpression of Akirin1 in C2C12 leads to increased MuRF-1 protein and mRNA levels. In addition to this, Akirin1 overexpression in C2C12 cells activates the MuRF-1 promoter via a luciferase assay, confirming an effect of Akirin1 on activation of this gene [54]. Furthermore, Akirin1 was shown to be required for normal phosphorylation state of transcription factor FOXO3, for which the MuRF-1 promoter has binding sites. Akirin1 knockout mice exhibited increased p-FOXO3 (Ser253) while Akirin1 overexpression in C212 cells led to reduced phosphorylation. Dephosphorylation of FOXO3 triggers its migration

to the nucleus, thus leading to activation of MuRF-1 transcription [54]. The authors raise the interesting possibility that Akirin1 could directly interact with FOXO3, though this remains speculative. In addition, Akirin1 knockout mice have reduced CREB-1 protein levels and thus reduced CREB-1 binding to the MuRF-1 promoter; however, this mechanism is currently thought to be an indirect one [54]. Finally, clues to how Akirin1 is transcriptionally regulated utilized luciferase assays to show that myostatin inhibits the *Akirin1* promoter via Smad, ERK/p38MAPK and PI3 kinase pathways [10], and cryolesion experiments showed a reduction in Smad3 and Smad4 protein levels concomitant with increased Akirin1 protein levels; this suggests an inverse relationship between Smad and Akirin1 protein levels [83]. This link to Smad signaling is intriguing in light of the recent results in *C. elegans* discussed above, showing that Akirin acts through the homologous TGF-β-Sma/Mab pathway to control body size [13].

Akirin roles in interdigital web regression

Though further studies are needed to understand the mechanisms involved, one recent study identified a role for Akirin2 in the process of interdigital web regression during murine limb development [27]. In the *Emx1-Cre;Akirin2*$^{fl/fl}$ restricted knockout mouse model utilized primarily to study cortical development [25], we noticed a consistent fusion of digits 2 and 3 in the forelimb and digits 2, 3, and 4 in the hindlimb. We discovered that this was due to *Emx1-Cre* activity in the ectoderm of developing limb buds, leading to deletion of *Akirin2*. This led to soft-tissue syndactyly caused by retention of FGF8 signal in the ectoderm, increased cell proliferation, and reduced apoptosis in the mesenchymal tissue adjacent to the Akirin2-null limb ectoderm [27]. These data indicate an effect of Akirin2 on the expression of diffusible morphogens from the ectoderm during limb development, although the complete signaling pathway that leads to syndactyly in its absence remains to be resolved.

Akirin roles in the germline

Akirins have also been found to be important for germline development and gametogenesis, though our understanding is limited to *C. elegans* thus far. The germline refers to the tissue that generates eggs and sperm through a specialized cell division termed meiosis [84]. The formation of gametes requires association of homologous chromosomes though the synaptonemal complex, a protein complex responsible for the association of homologous chromosomes in meiotic prophase I [84]. We identified Akirin in a *C. elegans* screen for genes involved in the disassembly of the synaptonemal complex; *akir-1* mutants exhibited aggregation of synaptonemal complex subunits that led to defects in chromosome structure [12]. Surprisingly, when *akir-1* is simultaneously deleted with *ima-2* (which encodes importin-α2), this phenotype is exacerbated, leading to synaptonemal complex protein aggregates almost throughout the germline, starting in the earliest stages of meiosis [21]. These defects stem from the inability to properly import and deposit the sister-chromatid cohesion complex [21]. Thus, aggregation of synaptonemal complex proteins is likely an outcome of cohesin loading defects, and not a direct function of Akirin.

Cohesins are members of a protein complex that stabilizes interactions between sister chromatids following replication and prior to division. The proper assembly of the sister-chromatid cohesion complex is crucial to meiosis, and similar protein complexes act via similar mechanisms in mitosis. How *C. elegans* Akirin collaborates with IMA-2 to support this function is as yet unknown. This role in cohesion complex import and loading could be specific to Akirin's function in the germline; however, it is also reasonable to speculate that it is a manifestation of Akirin's role in chromatin remodeling. For example, sister-chromatid cohesion complex subunits were found in a mass-spectrometry based screen for AKIR-1 protein interactors [18]. We suggest that Akirin's chromatin remodeling role facilitates the loading of sister-chromatid cohesion protein complexes to chromosomes at the proper time. In addition, our studies also propose a role of Akirin in nuclear import, although this may not be direct [21]. Given the hints that Akirin proteins may exist in both cytoplasmic and nuclear compartments, it is tempting to speculate that one of their roles could be to assist in the nucleocytoplasmic shuttling of other protein complexes. Both Akirin1 and Akirin2 are expressed in the mouse testis (unpublished data, JW and SS), suggesting that Akirin may have an evolutionarily conserved function in meiosis that is worthy of further exploration.

Akirin regulation of tumorigenesis and cancer

In addition to the many important developmental roles outlined above, several studies have shown Akirin2 to be of importance to cancer biology. Early studies identified stronger expression of Akirin2 in tumor cell lines (rat hepatoma, glioblastoma, and adrenal pheochromocytoma) vs. non-tumor cell lines [11] and it was subsequently shown that cholangiocarcinoma (CCA; bile duct cancer) tumor tissue exhibits higher Akirin2 expression than adjacent non-tumor tissues in vivo [30]. Akirin2 mRNA expression has also been detected in solid glioblastoma (GBM) tissue and in GBM cell lines [31]. Furthermore, high Akirin2 levels are associated with a worse prognosis in CCA [30]. Knockdown

of Akirin2 transcripts via antisense plasmids or siRNA in K2 cells [11], Lewis lung cell carcinoma [51], and CCA [30] leads to reduced size of the tumors they generate when injected into mice. In addition, common cancer cell characteristics such as colony formation, anchorage independent growth, metastases, and cell migration are substantially reduced upon Akirin2 knockdown [11, 30, 51]. Further experiments have shown that Akirin2 knockdown reduces wound closure and inhibits vessel formation via reductions in vascular density, VEGFA supernatant concentration, microvessels, and hemoglobin content of injected Matrigel plugs in mice [30].

Intriguingly, Akirin2 has been shown to be negatively regulated by microRNAs (miRs) during CCA development. Examination of the 3′UTR of Akirin2 identified a number of putative miR binding sites; however, only one, miR-490-3p, was shown to substantially reduce Akirin2 levels [30]. Consistent with this, in CCA clinical samples, Akirin2 expression is inversely related to miR-490-3p levels. Interrogation of the Akirin2 3′UTR using a luciferase reporter assay showed that miR-490-3p reduces activity of the Akirin2 3′UTR-luciferase and a number of cancer phenotypes such as migration, wound closure, and microvessel formation are all inhibited by miR-490-3p addition. Also, the IL-6-STAT3-VEGF pathway is inhibited following miR-490-3p treatment, and all these effects are reversed with forced Akirin2 expression, suggesting that the function of miR-490-3p is through inhibition of Akirin2. These exciting recent advances in Akirin2 cancer biology function represent a promising avenue of research for the treatment of a number of tumor types which is likely to further inform us of a detailed mechanism of action.

Conclusions and future directions

This review of the literature on Akirins demonstrates their critical roles as essential, evolutionarily conserved proteins in a variety of biological processes during development, in mature function, and in the progression of disease. Although the “Akirin” nomenclature was originally chosen based on a Japanese phrase meaning “making things clear”, several aspects of their biology remain somewhat obscure. In conclusion, we discuss some potentially fruitful topics for future studies.

First, given the pleiotropic roles for Akirins in many tissues, and their central role in coordinating gene expression programs along with ubiquitous chromatin remodeling machinery, how is specificity achieved across tissues and developmental stages? Indeed, transcription of Akirin genes appears to be ubiquitous across tissues and relatively stable during normal development, suggesting that Akirins may frequently behave as housekeeping genes. An offshoot of this topic is the dynamic regulation of Akirin protein. We found that during muscle cell differentiation, Akirin2 protein levels fall precipitously, despite fairly constant transcript levels [26]. Additionally, another study identified miRs that bind to the 3′ UTR of the *Akirin2* transcript and regulate its translation [30]. It is important to address how widespread dynamic regulation of Akirin protein levels is, and the mechanisms that might underlie it, including post-translational modifications such as SUMOylation and ubiquitination. Nevertheless, it seems likely that in most healthy tissues and conditions, Akirin levels are fairly constitutive. Thus, specificity of Akirin roles is likely determined by the cellular context, which is controlled by other genes. Although many studies have reported individual genes regulated by Akirins in particular cell types, for the most part we do not yet have complete transcriptomes from multiple types of Akirin-null tissues. As these are collected, they could be analyzed to identify any core gene sets regulated by Akirin generally, as well as the unique signatures in brain, muscle, germline, and immune cells. Along with these data, comprehensive proteomic analyses could identify a more complete set of Akirin-interacting proteins, including the transcription factor partners that likely bring tissue-specificity to Akirin gene regulation. The development of better Akirin antibodies, and/or transgenic organisms expressing tagged Akirin proteins from the endogenous locus, would allow ChIP-Seq analyses as well, which could identify which of these genes (if any) are directly regulated by Akirins. In this light, it will also be important to follow up the recent in silico imputation of a possible DNA-binding domain within tick Subolesin and rat Akirin2 [3] with direct biochemical evidence.

Second, although Akirins are thought of primarily as nuclear proteins, we note that there is some evidence that both Akirin1 and Akirin2 may be found, at least in some cell types, in the cytoplasm as well. Given this, the interaction of invertebrate Akirin with nuclear importins is suggestive of the possibility that Akirins may in some contexts be shuttled between the cytoplasm and the nucleus (as are 14-3-3 proteins, with which Akirins are known to interact). If Akirins can sometimes act in the cytoplasm, it is not at all clear how. We note, however, that their predicted intrinsically disordered domains are suggestive of a role in mediating diverse protein–protein interactions. Further subcellular fractionation experiments in a variety of cell types, as well as in vivo tagging of Akirin proteins to monitor dynamic localization, should help elucidate this remaining question.

Third, to what extent are the functions and molecular mechanisms of Akirins conserved between organisms and between homologous proteins? While we know that roles in the immune system and myogenesis are well-conserved from flies to worms to mammals, this remains unknown for others. Meiosis, in particular, should be examined in Akirin1 and Akirin2 conditional knockout mice using germline-specific

Cre drivers, as any role for mammalian Akirins here would be of potential importance to human reproduction and embryonic health. Although it is clear from knockout phenotypes that Akirin1 cannot compensate for the loss of Akirin2 in embryonic development, the former does function in myogenesis. Despite the normal outward appearance of Akirin1 null mutant mice, these animals were not examined in detail for mild defects in most tissues, including the nervous system in which Akirin1 is expressed. Similarly, we need to address whether there are evolutionarily conserved signaling pathways linked to Akirin functions. Akirin1's link to Smad signaling in mouse muscle and the role of an Akirin-TGFβ/Sma/Mab pathway regulating worm body size is one potential example, but others remain to be examined.

Finally, it will be important to directly address the extent to which Akirins truly act as "bridge" proteins linking transcription factors to chromatin remodeling machinery. While there is some good evidence to indicate such a mechanism, Akirin proteins' small size (especially compared to the massive BAF/BAP complexes) and our lack of understanding about their structure make it difficult to conceptualize a bridging role. One related question is whether Akirins form dimers or higher-order multimers in the cell, which is possible given that *Drosophila* Akirin can interact with itself in a yeast-2-hybrid assay [17]. While the relative lack of identifiable domains and the predicted intrinsic disorder of Akirin protein structure might make it impossible, it seems worth trying to obtain crystal structure data that could elucidate the protein–protein and/or protein–DNA interactions of Akirins. We hope that in providing this summary of the roles played by this intriguing protein family, we might encourage other researchers to undertake new studies that will allow Akirins to live up to their name, making things clear(er) in development, gene expression, and disease.

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