CHAPTER FIVE

Lessons from a transcription factor: Alx1 provides insights into gene regulatory networks, cellular reprogramming, and cell type evolution

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Abstract

The skeleton-forming cells of sea urchins and other echinoderms have been studied by developmental biologists as models of cell specification and morphogenesis for many decades. The gene regulatory network (GRN) deployed in the embryonic skeletogenic cells of euechinoid sea urchins is one of the best understood in any developing animal. Recent comparative studies have leveraged the information contained in this GRN, bringing renewed attention to the diverse patterns of skeletogenesis within the phylum and the evolutionary basis for this diversity. The homeodomain-containing transcription factor, Alx1, was originally shown to be a core component of the skeletogenic GRN of the sea urchin embryo. Alx1 has since been found to be key regulator of skeletal cell identity throughout the phylum. As such, Alx1 is currently serving as a lens through

Current Topics in Developmental Biology, Volume 146
ISSN 0070-2153
https://doi.org/10.1016/bs.ctdb.2021.10.005

which multiple developmental processes are being investigated. These include not only GRN organization and evolution, but also cell reprogramming, cell type evolution, and the gene regulatory control of morphogenesis. This review summarizes our current state of knowledge concerning Alx1 and highlights the insights it is yielding into these important developmental and evolutionary processes.

1. Introduction

The *alx1* gene was originally identified in a sea urchin (*Strongylocentrotus purpuratus*) expressed sequence tag (EST) study, which isolated a single, partial cDNA with a sequence similar to that of the Drosophila gene *aristaless* and reported that the cognate mRNA was selectively expressed in embryonic skeletal cells (primary mesenchyme cells, or PMCs) (Zhu et al., 2001). Subsequent knockdown of *alx1* in two sea urchin species, *S. purpuratus* and *Lytechinus variegatus*, revealed that the gene was essential for PMC differentiation and skeletogenesis in both species (Ettensohn, Illies, Oliveri, & De Jong, 2003). Since these initial studies, the structure, function, and regulation of *alx1* have been examined in multiple echinoderm taxa and developmental contexts. This work has revealed that *alx1* is a pivotal, conserved regulator of skeletal cell identity throughout the phylum. Analysis of *alx1* is currently providing important insights into diverse developmental processes, including (a) the architecture, function, and evolution of developmental gene regulatory networks (GRNs), (b) cellular reprogramming, and (c) cell type evolution.

2. The *alx1* gene and protein

2.1 Organization and evolution of the *alx1* gene in echinoderms

All echinoderms with well-annotated genomes contain unambiguous orthologs of Alx1 and a paralogous protein, Alx4 (also known as Calx) (Fig. 1). Both proteins are transcription factors of the homeodomain class, a large family of proteins in echinoderms and other animals (Bürglin & Affolter, 2016; Howard-Ashby et al., 2006). Alx1 contains a central, paired-type homeodomain that mediates DNA binding, a novel motif known as the D2 domain (discussed in detail below), and a C-terminal OAR (*otp/aristaless/rax*) domain. The latter is not well characterized but appears to modulate the transcriptional regulatory activity of the protein (Brouwer, ten Berge, Wiegerinck, & Meijlink, 2003; Fan et al., 2019; Tapie et al., 2017) (Fig. 2). Alx4 also contains a paired-type homeodomain

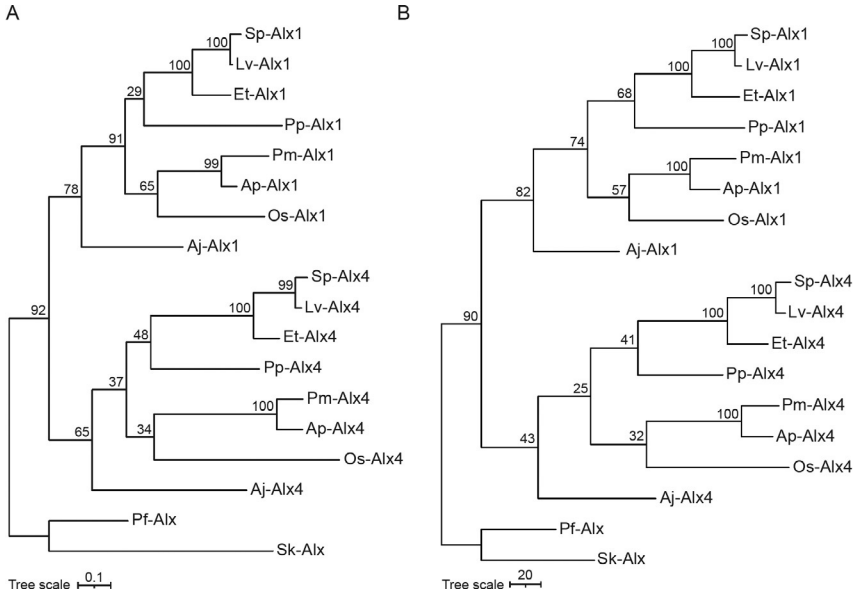

Fig. 1 Molecular phylogeny of Alx1 and Alx4 proteins. (A) Maximum likelihood (ML) method and JTT matrix-based model (Jones, Taylor, & Thornton, 1992). Initial trees for the heuristic search were obtained automatically by applying the Maximum Parsimony method and the tree with the highest log likelihood (−6934.06) is shown. Branch lengths reflect the number of substitutions per site. (B) Maximum parsimony (MP) method. Tree #1 of two equally parsimonious trees (length = 1263) is shown. The consistency index is (0.745735), the retention index is (0.649888), and the composite index is 0.488831 (0.484644) for all sites and parsimony-informative sites (in parentheses). The tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Branch lengths were calculated using the average pathway method (Kumar et al., 2018) and reflect the number of changes over the entire sequence. For both ML and MP trees, positions with less than 85% site coverage were eliminated, i.e., fewer than 15% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option), and evolutionary analyses were conducted using MEGA X (Stecher, Tamura, & Kumar, 2020). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Aj—*Anneissia japonica* (a crinoid); Ap—*Acanthaster planci* (a sea star); Lv—*Lytechinus variegatus* (a euechinoid sea urchin); Sp—*Strongylocentrotus purpuratus* (a euechinoid sea urchin); Et—*Eucidaris tribuloides* (a cidaroid sea urchin); Pp—*Parastichopus parvimensis* (a sea cucumber); Pm—*Patiria miniata* (a sea star); Os—*Ophiothrix spiculata* (a brittle star); Pf—*Ptychodera flava* (a hemichordate); Sk—*Saccoglossus kowalevskii* (a hemichordate).

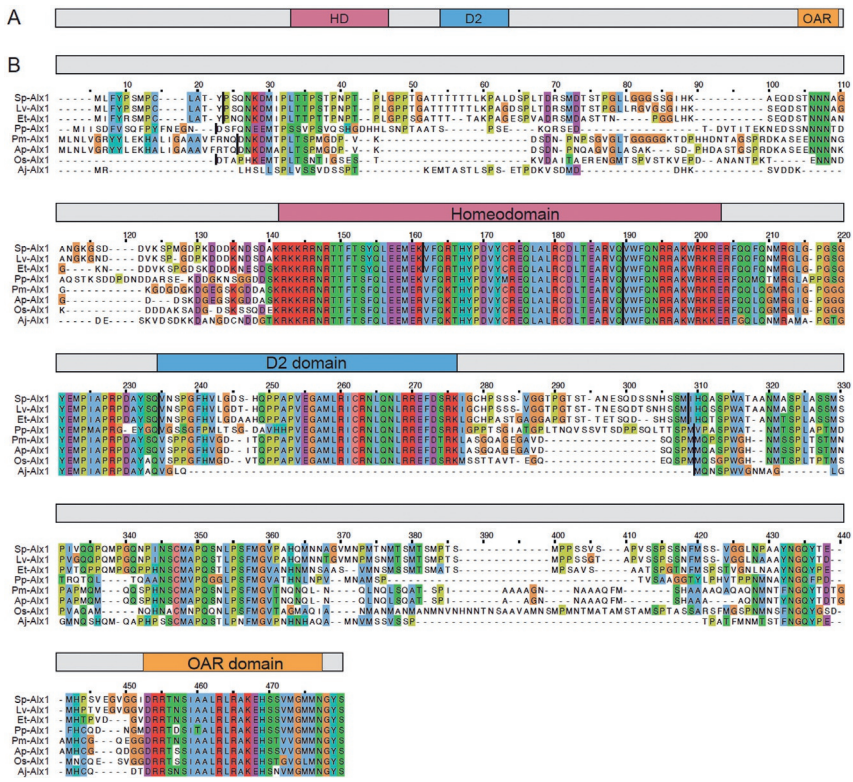

Fig. 2 (A) General domain architecture of Alx1. D2–D2 domain, HD—homeodomain, OAR—Otp/Aristaless/Rax domain. (B) Alignment of echinoderm Alx1 proteins, generated using Clustal Omega (Sievers et al., 2011) and Jalview (Waterhouse, Procter, Martin, Clamp, & Barton, 2009). Color scheme: Blue—hydrophobic; Cyan—aromatic; Green—polar; Magenta—negatively charged; Orange—glycine; Pink—cysteine; Red—positively charged; White—unconserved; Yellow—proline. Black lines between amino acids indicate positions of splice junctions.

and a C-terminal OAR domain, but the protein is not highly similar to Alx1 outside these two regions and lacks a D2 domain. The *alx1* and *alx4* genes are arranged in tandem, strongly supporting the view that they arose from duplication. This gene duplication likely occurred in the stem lineage of all echinoderms, as the closest outgroup to echinoderms, hemichordates, possess a single, *alx4*-like gene (Figs. 1 and 5). The nearest chordate relatives of echinoderms, cephalochordates, have two *alx-4* like genes, but these paralogs were the products of an independent gene duplication event that occurred after the divergence of the chordate and echinoderm lineages. A parsimonious interpretation is that the most recent common ancestor

(MRCA) of ambulacrarians (hemichordates + echinoderms) possessed a single *alx4*-like gene which underwent duplication in the echinoderm lineage, while independent duplications in the chordates, including whole genome duplications, gave rise to several *alx1*-related genes. Notably, vertebrate members of the Alx1 family also play important roles in skeletal development (reviewed by Khor & Ettensohn, 2020).

Duplication of the ancestral *alx1/4* gene in the echinoderm lineage appears to have been followed relatively rapidly by neofunctionalization of the gene; viz., by the acquisition of robust, skeletogenic properties. Several lines of evidence support this view. First, *alx1* has a highly conserved role in skeletogenesis throughout the echinoderm phylum. In all echinoderms and at all life history stages that have been examined, *alx1* expression is restricted to skeletogenic cells (Figs. 3 and 4). In clades in which loss-of-function studies have been carried out (euechinoids, cidaroids, and holothuroids), *alx1* has been shown to play an essential role in skeletogenic specification (Erkenbrack & Davidson, 2015; Ettensohn et al., 2003; McCauley, Wright, Exner, Kitazawa, & Hinman, 2012; Pieplow et al., 2021). This conclusion has been further supported by gain-of-function studies in euechinoids and asteroids, which have shown that ectopic expression of Alx is sufficient to endow cells with skeletogenic properties (Ettensohn, Kitazawa, Cheers, Leonard, & Sharma, 2007; Koga et al., 2016). Significantly, Alx4 cannot substitute for Alx1 in supporting skeletal development during sea urchin embryogenesis, demonstrating a divergence in the functional properties of the two paralogous genes (Khor & Ettensohn, 2017).

In contrast to *alx1*, the function of *alx4* has not been examined in any echinoderm. Alx4 is expressed in the coelomic pouches of modern sea urchin and sea stars, suggesting that its ancestral role may have been related to the development of non-skeletogenic mesoderm (Koga et al., 2016). In sea urchins, *alx4* is transiently co-expressed with *alx1* in PMCs, and *alx1* provides direct, positive inputs into the *alx4 cis*-regulatory system (Khor, Guerrero-Santoro, & Ettensohn, 2019; Rafiq, Shashikant, McManus, & Ettensohn, 2014). Unfortunately, nothing is known concerning the developmental expression or function of the single *alx4*-like gene of hemichordates or the possible relationship between this gene and small, calcium carbonate-based biominerals found in adult hemichordates (Cameron & Bishop, 2012).

Alx1 provides a striking example of transcription factor evolution at the level of protein sequence. Although *cis*-regulatory changes are widely

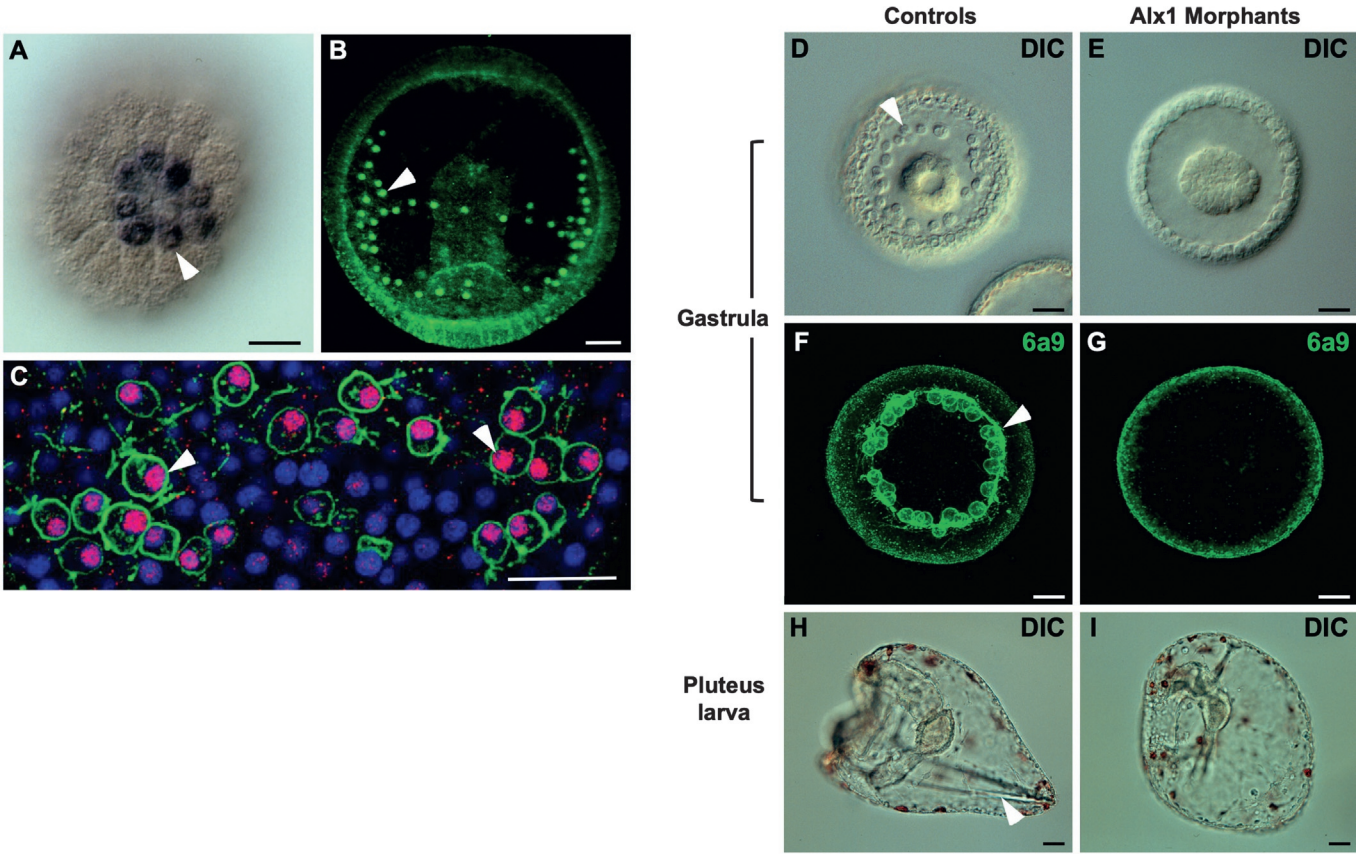

Fig. 3 See figure legend on opposite page.

considered to make a greater contribution to the evolution of genetic networks than changes in transcription factor sequence, due primarily to the likely pleiotropic effects of the latter, such effects can be bypassed through gene duplication and neofunctionalization (Lynch & Wagner, 2008). In the case of *alx1*, duplication of the ancestral gene was followed by the exonization of a novel, 41-amino acid motif known as the D2 domain, located between the homeodomain and the C-terminus (Khor & Ettensohn, 2017). The exonization of the D2 domain likely occurred in the MRCA all eleutherozoans (a group that comprises all echinoderms except crinoids), as this domain is present in the Alx1 proteins of all modern eleutherozoans but not in any crinoid Alx1 sequences that have been identified to date. (Figs. 5 and 6). The D2 domain is essential for Alx1 to exert its skeletogenic function in the sea urchin embryo. The endogenous Alx4 protein lacks a D2 domain but, strikingly, experimental insertion of the Alx1 D2 motif into Alx4 is sufficient to endow the protein with robust skeletogenic properties (Khor & Ettensohn, 2017). Moreover, the D2 domain is sufficiently highly conserved among eleutherozoans that the D2 motifs of sea stars and sea urchins, taxa that diverged >450 million years ago, are functionally interchangeable (Khor & Ettensohn, 2017).

2.2 DNA binding properties of Alx1

Alx1 contains a paired-class homeodomain of the glutamine-50 type (Galliot, de Vargas, & Miller, 1999). In vitro binding studies have shown that paired-class homeodomain proteins, including vertebrate members of the Alx1 family, bind preferentially to palindromic sites that contain two

Fig. 3 Alx1 expression (left panels) and function (right panels) in a euechinoid sea urchin (*Strongylocentrotus purpuratus*). (A) Whole mount in situ hybridization showing expression of *alx1* at the early blastula stage. Expression is restricted to the eight large micromere (LM) descendants (arrow), which give rise exclusively to PMCs. (B) Gastrula stage embryo immunostained with an anti-Alx1 antibody. Nuclear staining is observed specifically in primary mesenchyme cells (PMCs) (arrow). (C) High magnification view of a gastrula stage embryo co-stained with monoclonal antibody 6a9 (green), which recognizes a PMC-specific cell surface protein, and anti-Alx1 antibody (pink). Nuclei (blue) are stained with DAPI. Alx1 is restricted to PMC nuclei (arrows). (D–G) Gastrula stage embryos (vegetal views) examined with differential interference contrast (DIC) optics (live embryos) or immunostained with 6a9. Control embryos (D, F) have PMCs (arrows) but these cells are absent when Alx1 expression is blocked with a morpholino (E, G). (H, I) Pluteus larvae (lateral views) 4 days post-fertilization. Control embryos have extensive skeletal elements (arrow, H) but Alx1 morphants (I) completely lack skeletons. Scale bars = 25 μm.

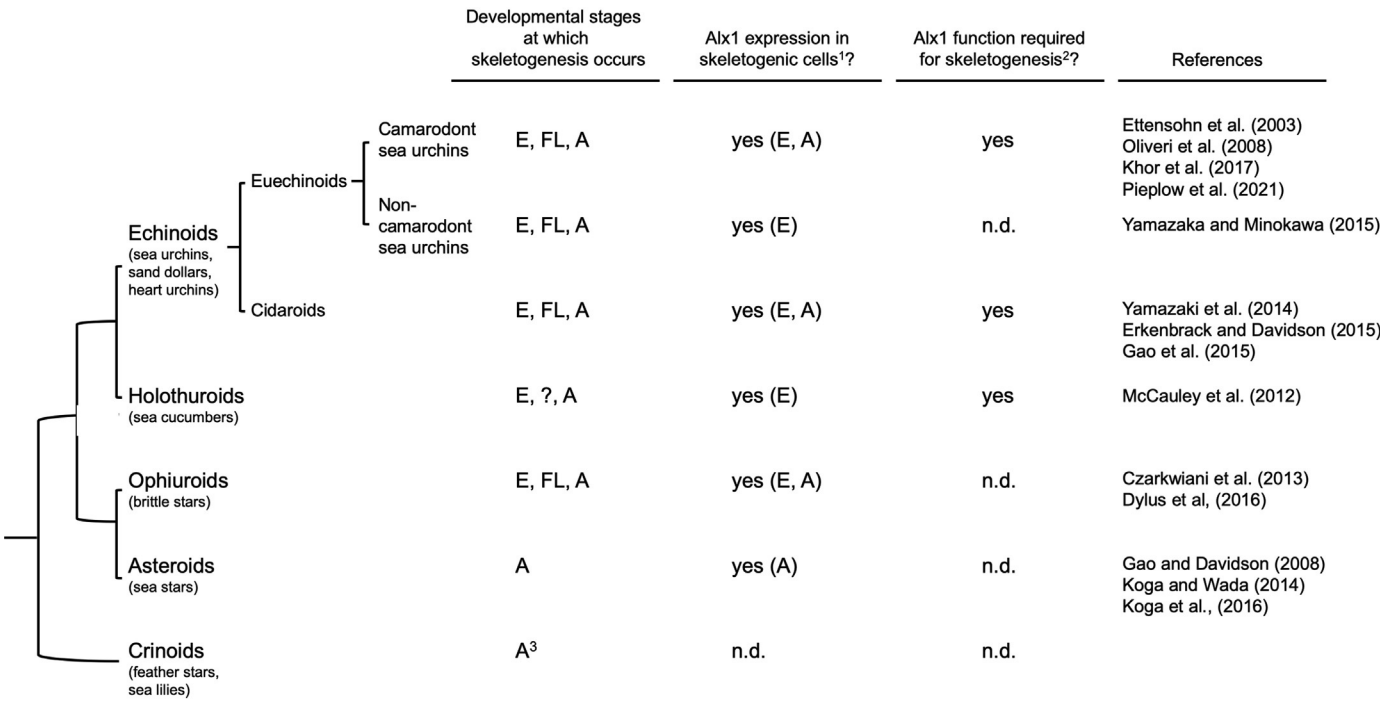

Fig. 4 Conservation of *alx1* expression and skeletogenic function among echinoderms. The phylogenetic relationships among modern echinoderms are indicated at left (note that branch lengths do not reflect evolutionary time). [1]Developmental stages missing in this column indicate only that *alx1* expression has not been examined in that taxon at that particular stage (i.e., equivalent to n.d.). [2]The function of *alx1* has been tested only at embryonic stages, using gene knockdowns and CRISPR/Cas9-mediated gene editing. [3]All crinoids that have been described exhibit direct development. A—adult, *E*—embryo, FL—feeding larva, n.d.—not determined.

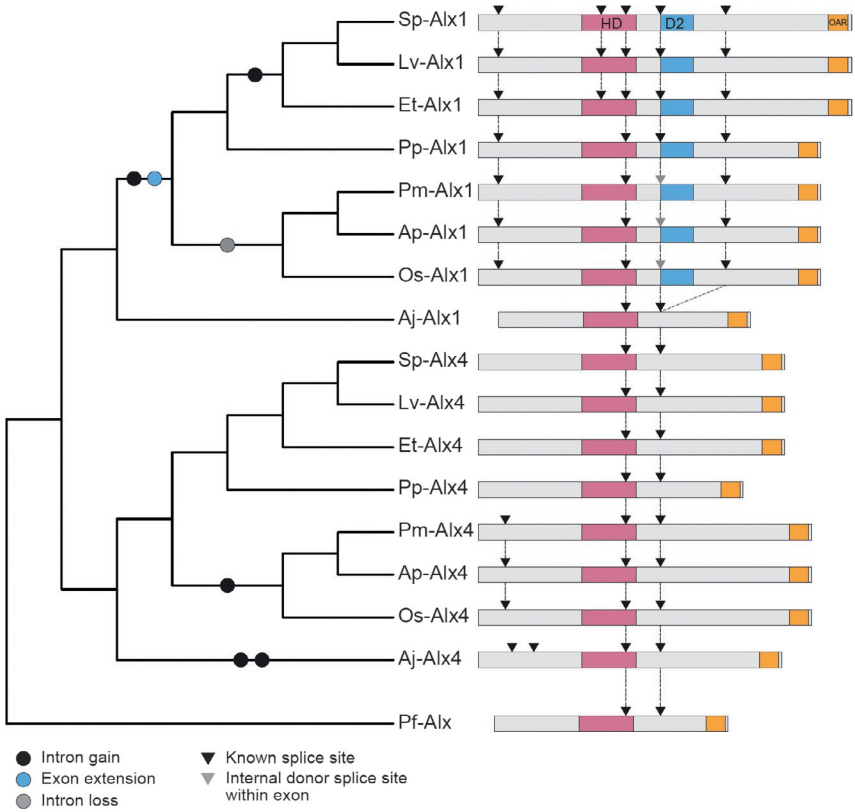

Fig. 5 The evolution of echinoderm *alx1* gene structure. Left: The molecular phylogeny of echinoderm Alx1 and Alx4 proteins is shown as in Fig. 1, with a hemichordate (Pf) as an outgroup. Intron/exon losses and gains are indicated, including the exonization of the D2 domain (blue circle). Branch lengths are arbitrary. Right: The intron-exon organization of ambulacrarian (echinoderm + hemichordate) *alx1* and *alx4* genes, drawn to scale. Species abbreviations are the same as those show in Fig. 1 Arrowheads mark positions of introns. Dotted lines indicate conserved intron positions. HD—homeodomain (pink box); D2—D2 domain (blue box); OAR—OAR (*otp/aristaless/rax*) domain (orange box).

inverted TAAT sequences (so-called half-sites) separated by 3 base pairs (Cai, 1998; Qu, Tucker, Zhao, De Crombrugghe, & Wisdom, 1999; Wilson, Guenther, Desplan, & Kuriyan, 1995; Wilson, Sheng, Lecuit, Dostatni, & Desplan, 1993). Binding to palindromic sites involves the cooperative binding of two protein molecules and the formation of a trimeric protein-DNA complex. Artificial dimerization of Alx1 via a flexible linker has provided evidence that dimerization enhances Alx1 transcriptional activity in vivo (Damle & Davidson, 2011).

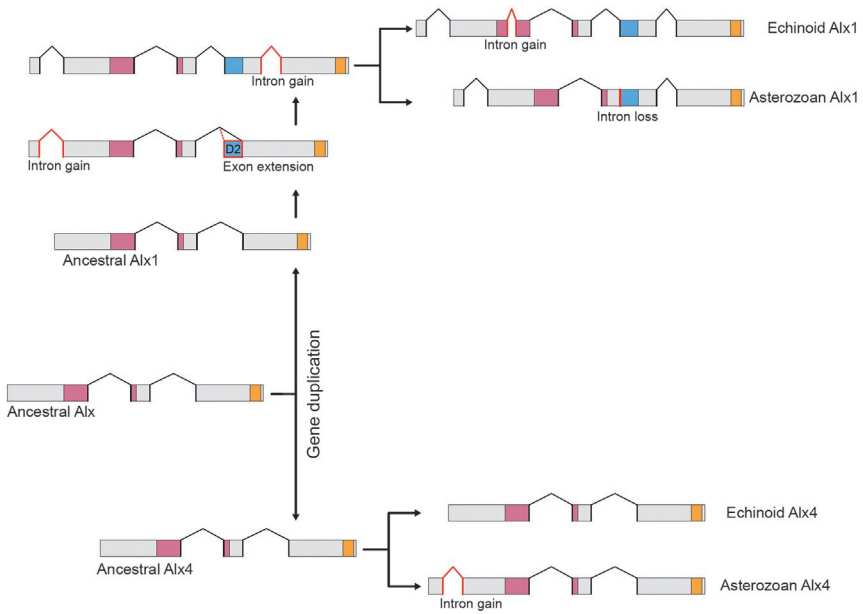

Fig. 6 Intron losses/gains and the exonization of the D2 domain during *alx1* evolution. Following gene duplication in the MRCA of echinoderms, the ancestral *alx1* gene underwent rapid evolution through multiple intron gains and more importantly, acquired the D2 domain through exonization of previously non-coding sequences. By contrast, *alx4* appears to have retained an intron-exon structure similar to that of the ancestral *alx1/4* gene. Red lines—introns gains; Red bar—intron loss; Dotted red line—postulated shift in 3′ splice site that resulted in the exonization of the D2 domain. Exons and introns are not drawn to scale.

Analysis of Alx1 binding sites in vivo by ChIP-seq has identified hundreds of palindromic binding sites in the sea urchin genome but also, unexpectedly, large numbers of half-sites (Khor et al., 2019). Gel-shift assays have recently confirmed that Alx1 binds to half-sites and have revealed that dimeric complexes also form on such sites, but by a mechanism distinct from the well-known mechanism of cooperative dimerization that occurs at palindromic sites (Guerrero-Santoro, Khor, Açıkbaş, Jaynes, & Ettensohn, 2021). Experimental dissection of a *cis*-regulatory element (CRE) associated with *Sp-mtmmpb*, a gene that encodes a PMC-specific matrix metalloprotease, showed that two Alx1 half-sites, acting independently and redundantly, were responsible for PMC-specific reporter expression. These same studies also showed that Alx1 and Alx4 form heterodimeric complexes in vitro (Guerrero-Santoro et al., 2021). During development, however, the onset of *alx1* expression precedes that of *alx4* by several hours,

and at the time of PMC differentiation the level of *alx1* mRNA in PMCs is >20-fold higher than that of *alx4* mRNA, suggesting that heterodimeric complexes are of low prevalence. As noted above, Alx4 cannot substitute for Alx1 in vivo when expressed at similar levels (Khor & Ettensohn, 2017), but it remains possible that heterodimeric complexes, if they are present in vivo, might be active in supporting transcription.

The proximity of the D2 domain to the homeodomain suggests that it might play some role in modulating DNA binding. Strong support for this hypothesis has come from the recent demonstration that deletion of the D2 domain reduces the ability of Alx1 to engage in cooperative binding on palindromic target sites in vitro (Guerrero-Santoro et al., 2021). This finding supports the hypothesis that evolutionary recruitment of the D2 domain modified the intrinsic DNA binding properties of Alx1, thereby allowing the protein to acquire new transcriptional targets and adopt a novel developmental function. The D2 domain may have other effects on Alx1 function that have yet to be discovered; for example, it might modulate interactions with hypothetical protein partners.

3. Alx1 and gene regulatory network (GRN) architecture

3.1 Upstream regulators of *alx1*

3.1.1 Early zygotic activation

In euechinoid sea urchins, expression of *alx1* is first detectable during cleavage, when the gene is selectively activated in the large micromere lineage, which will give rise exclusively to PMCs (Fig. 3A). In *S. purpuratus*, the species that has been best studied in this regard, specific expression in is evident by whole mount in situ hybridization (WMISH) in the large micromeres in the first interphase after these cells are born (Ettensohn et al., 2003; Sharma & Ettensohn, 2010). More sensitive methods (Nanostring analysis and QPCR) show that *alx1* is activated even earlier, at the 16-cell stage (5 h post-fertilization), when expression appears to be restricted to the micromeres, the progenitors of the large micromeres (Cavalieri, Geraci, & Spinelli, 2017). Thus, any model of *alx1* activation in sea urchins must account for the early, spatially restricted expression of the gene.

The activation of *alx1*, like that of all genes selectively expressed in the endomesoderm of echinoderm embryos, is dependent on maternally entrained mechanisms that stabilize β-catenin in the vegetal region during early cleavage (Ettensohn et al., 2003) (Fig. 7). In euechinoid micromeres, a pivotal gene directly downstream of β-catenin is *pmar1* (also known as

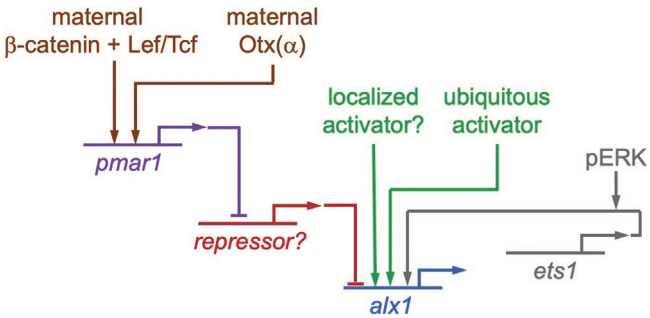

Fig. 7 Activation of *alx1* in the large micromere lineage of euechinoid sea urchins. Pmar1, which acts as a repressor, is a key activator of several early regulatory genes in the PMC GRN, including *alx1*. Although the effects of *pmar1* on *alx1* activation are usually considered to be mediated through derepression of *hesC*, several lines of evidence indicate this is not the case (see text for details). Thus, there may be a different repressor (shown here as "repressor?") downstream of *pmar1*, and/or a localized activator ("localized activator?") may function independently of *pmar1*. Because misexpression of Pmar1 activates *alx1* throughout the embryo, one or more ubiquitous activators must also exist. Note that the positive input from *ets1* (shown in gray), which requires direct phosphorylation of Ets1 by phospho-ERK (pERK), is not required for the earliest phase of *alx1* expression during cleavage, but is required for the maintenance of *alx1* expression later in development, at the blastula stage.

micro1) (Kitamura, Nishimura, Kubotera, Higuchi, & Yamaguchi, 2002; Nishimura et al., 2004; Oliveri, Carrick, & Davidson, 2002; Oliveri, Davidson, & McClay, 2003). Over-expression of Pmar1 results in a dramatic increase in *alx1* expression throughout the embryo; this ectopic activation requires repressor motifs in Pmar1 and bypasses the requirement for β-catenin (Cheng, Lyons, Socolar, & McClay, 2014; Ettensohn et al., 2003; Oliveri, Tu, & Davidson, 2008; Yamazaki, Ki, Kokubo, & Yamaguchi, 2009). The mechanism by which *pmar1* activates *alx1*, however, has not been established. A model based on a combination of: 1) Pmar1-mediated repression of a second repressor, HesC, and 2) positive regulation by zygotic Ets1 has been proposed (Damle & Davidson, 2011; Oliveri et al., 2008) but other evidence indicates that these mechanisms cannot account for the activation of *alx1* (reviewed by Shashikant, Khor, & Ettensohn, 2018a). One key issue is that the lineage-specific expression of *alx1* is initiated too early in development to be explained by these mechanisms (Sharma & Ettensohn, 2010). In addition, Yamazaki et al. (2009) described a mutant form of Pmar1/micro1 (N-HD-A-C) that globally

represses *hesC*, yet fails to produce ectopic expression of *alx1*. Based on these and other findings, it seems clear that other, unidentified factors play a role in *alx1* activation.

In euechinoids, the progenitors of the PMC lineage, the LMs, arise as a consequence of two rounds of unequal cell division. Low concentrations of several detergents, including SDS, equalize these divisions, delaying or preventing the formation of micromeres and blocking PMC specification (Langelan & Whiteley, 1985). The expression of *alx1* is also suppressed in such embryos, although *pmar1* expression is not (Sharma & Ettensohn, 2010). These findings suggest that unequal cleavage might be required for a regulatory step between *pmar1* activation and *alx1* expression. Recently, however, it has been reported that SDS also blocks skeletogenic specification in a direct developing, equally-cleaving sea urchin, suggesting that SDS might inhibit PMC specification by mechanisms other than by equalizing cleavage; e.g., by perturbing membrane-associated molecular determinants (Edgar, 2019). In this context, it is also noteworthy that ophiuroids (brittle stars) and holothuroids (sea cucumbers) ordinarily exhibit equal cleavage yet produce *alx1*-expressing skeletogenic cells; *alx1* activation is therefore not linked to unequal cell division in these taxa (Dylus et al., 2016; McCauley et al., 2012; Primus, 2005; Tominaga, Nakamura, & Komatsu, 2004; Vaughn, Garnhart, Garey, Thomas, & Livingston, 2012).

3.1.2 Later regulatory inputs

At the blastula stage, *alx1* comes under the positive regulatory control of Ets1, a maternally and zygotically expressed transcription factor of the ETS family (Damle & Davidson, 2011; Oliveri et al., 2008; Rafiq et al., 2014) (Fig. 7). Zygotic expression of Ets1 is initially restricted to the large micromere-PMC lineage, but during gastrulation the gene is also expressed by non-skeletogenic mesoderm cells (Flynn et al., 2011; Kurokawa et al., 1999; Rizzo, Fernandez-Serra, Squarzoni, Archimandritis, & Arnone, 2006). Ets1 is activated by direct, ERK-mediated phosphorylation (Röttinger, Besnardeau, & Lepage, 2004). The acquisition of the Ets1 regulatory input into *alx1* coincides with a striking, transient elevation of p-ERK in presumptive PMCs at the blastula stage and a concomitant nuclear accumulation of Ets1 protein (Fernandez-Serra, Consales, Livigni, & Arnone, 2004: Röttinger et al., 2004; Sharma & Ettensohn, 2010; Yajima et al., 2010). ERK activity, functioning directly in the presumptive PMCs and acting through Ets1, is required for the maintenance (but not the initial activation) of *alx1* expression (Rafiq et al., 2014; Rafiq,

Cheers, & Ettensohn, 2012; Röttinger et al., 2004; Sharma & Ettensohn, 2010). Alx1 like Ets1, contains a consensus MAPK phosphorylation site, but this site is dispensable for the skeletogenic function of Alx1 (Khor & Ettensohn, 2017). The mechanism by which ERK activity is selectively activated in the LM lineage is an important, unresolved question. This activation does not require signaling from other cell types but does depend upon unidentified, zygotic transcriptional inputs downstream of β-catenin (Fernandez-Serra et al., 2004; Röttinger et al., 2004).

3.2 Downstream targets of *alx1*

3.2.1 Regulatory genes

Because the expression of *alx1* declines after gastrulation while skeletogenesis continues, it seems plausible that the regulatory functions of the gene are handed off to downstream transcription factors. Consistent with this hypothesis, the gene targets of Alx1 in *S. purpuratus* include a small handful of regulatory (i.e., transcription factor-encoding) genes, including *alx4*, *dri*, *fos*, *nk7*, and *foxB* (Oliveri et al., 2008; Rafiq et al., 2014) (Fig. 8). The developmental functions of most of these genes have not been explored. Some downstream targets of *dri* and *foxB* have been identified in sea urchins (Oliveri et al., 2008), but *dri* and *foxB* are not expressed at detectable levels in the skeletogenic cells of brittle stars, suggesting that they do not have highly conserved roles in echinoderm skeletogenesis (Czarkwiani, Dylus, & Oliveri, 2013; Dylus et al., 2016). In a different sea urchin species, *L. variegatus*, *alx1* regulates *snail and twist*, two transcriptional repressors that have been implicated in PMC ingression, as discussed below.

3.2.2 Linking a GRN to morphogenesis: Control of PMC behavior by alx1

PMCs have long been of special interest because of their striking morphogenetic behaviors, which include epithelial-mesenchymal transition (EMT), directional cell migration, and cell-cell fusion (Ettensohn, 2020; McIntyre, Lyons, Martik, & McClay, 2014). With a growing understanding of the transcriptional network deployed in the LM-PMC lineage, including the sub-circuitry controlled by Alx1, there is an opportunity to develop a comprehensive understanding of the gene regulatory control of these cell behaviors.

Alx1 regulates all three of the most prominent cell behaviors exhibited by PMCs. In Alx1 morphants, LM descendants fail to undergo EMT and instead remain within the vegetal plate epithelium (Ettensohn et al., 2003). Saunders and McClay (2014) examined the behavior of LM progeny

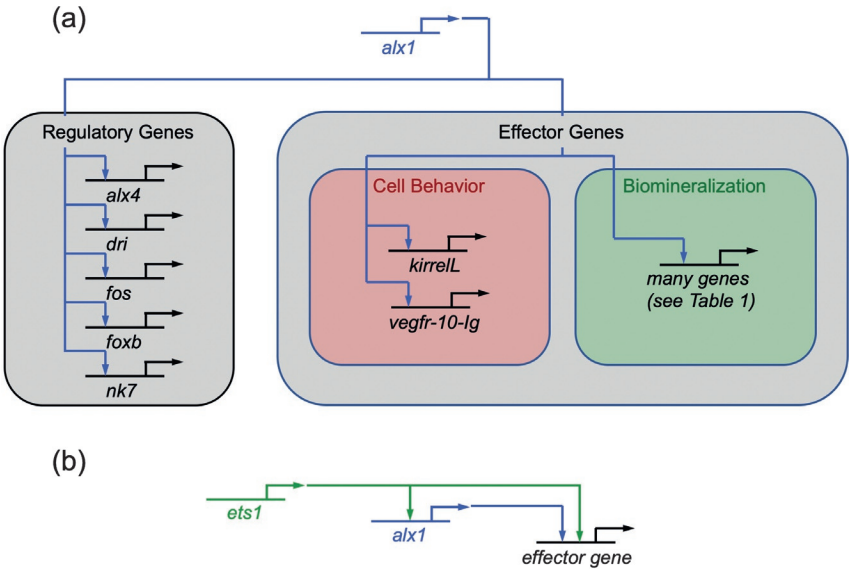

Fig. 8 Direct targets of *alx1* in euechinoid sea urchins. (A) Major classes of genes regulated by *alx1* are shown, along with examples of target genes in each class. Each gene shown is predicted to be a direct target of Alx1 based on A) reduced expression of the cognate mRNA in Alx1 morphants (Rafiq et al., 2014) and 2) the presence of one or more Alx1 binding sites near the gene (Khor et al., 2019). See Table 1 for a list of direct Alx1 targets with functions related to biomineralization. (B) Diagram illustrating the direct, feedforward co-regulation of target genes by *ets1* and *alx1*, a circuitry that regulates the expression of many effector genes expressed specifically by PMCs.

in such embryos by time-lapse imaging and found that Alx1 was not required for several cell behaviors typically associated with EMT (apical-basal polarization, apical narrowing, and basal lamina remodeling), but was required for the de-adhesion of presumptive PMCs from neighboring cells. In *L. variegatus*, this effect may be mediated through the positive regulation of *snail* and *twist*, two repressors that are required for PMC ingression (Wu & McClay, 2007; Wu, Yang, & McClay, 2008). The direct downstream targets of these repressors are unknown, although *snail* positively regulates E-cadherin expression in sea urchins as it does in vertebrates (Wu & McClay, 2007). Notably, *alx1* is not the only regulatory gene that affects PMC EMT, as several others (most of which lack known regulatory connections to *alx1*) also contribute (Kurokawa et al., 1999; Saunders & McClay, 2014). Following ingression, PMCs are guided to specific target sites along the blastocoel wall by VEGF3, which is secreted by the ectoderm and signals through a PMC-specific receptor, VEGFR-10-Ig (Duloquin

et al., 2007). Alx1 provides direct, positive regulatory inputs into *vegfr-10-Ig* (Khor et al., 2019; Oliveri et al., 2008; Rafiq et al., 2014), thereby regulating the directionality of PMC migration.

Migrating PMCs extend long filopodia that contact one another and fuse, gradually giving rise to a cable-like structure that connects the entire population of PMCs in a single, syncytial network (Hodor & Ettensohn, 1998; Okazaki, 1965). The biomineralized rods that comprise the endoskeleton are secreted within this syncytial cable. KirrelL, a PMC-specific member of the Ig-domain superfamily of cell adhesion proteins, is required for filopodial contacts between PMCs to result in membrane fusion (Ettensohn & Dey, 2017). Although the expression and function of KirrelL were first examined in sea urchins, the protein is also selectively expressed in the skeletogenic cells of adult brittle stars (Piovani, Czarkwiani, Ferrario, Sugni, & Oliveri, 2021) and sea stars (Khor & Ettensohn, 2021), pointing to a conserved role in skeletogenesis throughout the phylum. Detailed analysis of the *cis*-regulatory control system of the *Sp-kirrelL* gene has recently elucidated its modular architecture and shown that both Alx1 and Ets1 provide positive inputs into two key regulatory modules (elements C and G) (Khor & Ettensohn, 2021). These findings establish a direct link between Alx1 and PMC fusion and point to the role of Alx1 in integrating the major morphogenetic behaviors of PMCs.

3.2.3 alx1 *as a terminal selector gene*

Alx1 is an example of a "terminal selector" protein (Arendt et al., 2016; Hobert, 2008; Hobert & Kratsios, 2019); that is, a lineage-specific transcription factor that initiates and maintains a terminal cell identity program through the direct regulation of cell type-specific effector genes. In support of this view, RNA-seq analysis of morphant *S. purpuratus* embryos has revealed that Alx1 provides positive inputs into almost half of the ~400 genes selectively expressed by PMCs and an even larger fraction of such genes that are highly expressed, demonstrating the pivotal role of Alx1 in controlling PMC identity (Rafiq et al., 2012, 2014). Many of these effector genes are direct targets as shown by a marked enrichment of Alx1 binding sites, identified both computationally and by ChIP-seq, in enhancers located near these genes (Khor et al., 2019; Khor & Ettensohn, 2021; Shashikant, Khor, & Ettensohn, 2018b). In several cases, mutational analysis of reporter gene constructs has confirmed that Alx1 binding sites regulate the PMC-specific activity of enhancers associated with effector genes (Guerrero-Santoro et al., 2021; Khor et al., 2019, Khor & Ettensohn, 2021).

The majority of the effector gene targets of Alx1 are associated with biomineralization, the primary function of fully differentiated PMCs (Fig. 8 and Table 1). Many of the biomineralization gene targets of Alx1 encode secreted or membrane-associated proteins that co-purify with biomineral isolated from larvae or adults (Karakostis et al., 2016; Mann et al., 2008, 2010). In addition, many are members of rapidly evolving families of biomineralization genes that have expanded in echinoderms, or in echinoderm sub-lineages, by gene duplication; examples include the spicule matrix gene family, MSP130-related genes, and P16-related genes (Livingston et al., 2006). These proteins have diverse and essential functions in biomineral formation; they encode proteins that regulate calcium uptake, proton transport, bicarbonate synthesis, phase transitions of calcium carbonate, and many other functions (Table 1). The multiple control points at which Alx1 impinges on biomineralization highlight the critical importance of this transcription factor for the terminal function of skeletal cells.

3.2.4 Co-regulation of effector genes by alx1 *and* ets1

Gene knockdown studies have shown that positive co-regulation of downstream genes by Alx1 and Ets1 is remarkably common; 85% of Ets1 targets are also regulated by Alx1, and 73% of all Alx1 targets are also regulated by Ets1 (Rafiq et al., 2014). More than a third of all effector genes, and almost 2/3 of the most highly expressed PMC effector genes, are co-regulated by these two transcription factors. One component of this co-regulation is a coherent feed-forward loop (Mangan & Alon, 2003) of the structure: Ets1 > Alx1, Ets1 + Alx1 > effector gene, as first identified by Oliveri et al. (2008). Consistent with this model, Ets1 positively regulates Alx1 at post-blastula stages (Ettensohn et al., 2003; Oliveri et al., 2008; Rafiq et al., 2014). Detailed analysis of *cis*-regulatory elements (CREs) that control *Sp-Kirrel* transcription has revealed direct inputs from both Alx1 and Ets1, indicating that in this case the feed-forward loop is a very simple one that involves the binding of both proteins to the transcriptional control system of the effector gene, without requiring intermediary transcription factors (Khor & Ettensohn, 2021) (Fig. 8B). Several recent studies have documented a marked enrichment of predicted Ets1 and Alx1 binding sites in PMC enhancers, suggesting that direct co-regulation by both TFs is very common (Khor et al., 2019, Khor & Ettensohn, 2021; Shashikant et al., 2018b).

Table 1 Gene targets of Alx1 that encode biomineralization proteins with known functions.

Gene	Nature of gene product	Function of gene product	Direct target of Alx1?	Selected references
Sp_Colf_13	Collagen	PMC substrate	Yes	Wessel, Etkin, and Benson (1991), Rafiq et al. (2014), Khor et al. (2019)
Sp-C-lectin	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Yes	Gong et al. (2012), Rafiq et al. (2014), and Khor et al. (2019)
Sp-Clect_13 (sm21)	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Yes	Gong et al. (2012), Rafiq et al. (2014), and Khor et al. (2019)
Sp-Clect_14 (sm20)	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Yes	Gong et al. (2012), Rafiq et al. (2014), and Khor et al. (2019)
Clect_25	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Yes	Gong et al. (2012), Rafiq et al. (2014), and Khor et al. (2019)
Sp-Sm29	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Yes	Gong et al. (2012), Rafiq et al. (2014), and Khor et al. (2019)
Sp-Sm30E	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Yes	Gong et al. (2012), Rafiq et al. (2014), and Khor et al. (2019)

Sp-C-lectin/ PMC1 (sm49)	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Yes	Gong et al. (2012), Rafiq et al. (2014), and Khor et al. (2019)
Sp-Fam20c	Secretory pathway kinase	Phosphorylation of biomineralization proteins	Yes	Rafiq et al. (2014), Khor et al. (2019), and Worby, Mayfield, Pollak, Dixon, and Banerjee (2021)
Sp-Hypp3152 (p16rel1)	Glycine-rich protein with signal sequence and transmembrane domain	Biomineral growth	Yes	Cheers and Ettensohn (2005), Rafiq et al. (2014), and Khor et al. (2019)
Sp-Hypp3153 (p16rel2)	Glycine-rich protein with signal sequence and transmembrane domain	Biomineral growth	Yes	Cheers and Ettensohn (2005), Rafiq et al. (2014), Khor et al. (2019)
Sp-P16	Glycine-rich protein with signal sequence and transmembrane domain	Biomineral growth	Yes	Cheers and Ettensohn (2005), Rafiq et al. (2014), and Khor et al. (2019)
Sp-Otop2L	Otopetrin	Proton transport, pH regulation	Yes	Rafiq et al. (2014), Tu et al. (2018), Khor et al. (2019) and (Chang et al., 2021)
Sp-Msp130	GPI-anchored cell surface glycoprotein	Calcium uptake	Yes	Carson, Farach, Earles, Decker, and Lennarz (1985), Rafiq et al. (2014), Killian and Wilt (2017), and Khor et al. (2019)

Continued

Table 1 Gene targets of Alx1 that encode biomineralization proteins with known functions.—cont'd

Gene	Nature of gene product	Function of gene product	Direct target of Alx1?	Selected references
Sp-Msp130r1	GPI-anchored cell surface glycoprotein	Calcium uptake	Yes	Carson et al. (1985), Rafiq et al. (2014), Killian and Wilt (2017), and Khor et al. (2019)
Sp-Msp130r2	GPI-anchored cell surface glycoprotein	Calcium uptake	Yes	Carson et al. (1985), Rafiq et al. (2014), Killian and Wilt (2017), and Khor et al. (2019)
Sp-Msp130r3	GPI-anchored cell surface glycoprotein	Calcium uptake	Yes	Carson et al. (1985), Rafiq et al. (2014), Killian and Wilt (2017), and Khor et al. (2019)
Sp-MmpL2	Matrix metalloprotease	Biomineral growth	Yes	Ingersoll and Wilt (1998), Rafiq et al. (2014), Khor et al. (2019), and Morgulis, Winter, Shternhell, and Gildor (2021)
Sp-MmpL5	Matrix metalloprotease	Biomineral growth	Yes	Ingersoll and Wilt (1998), Rafiq et al. (2014), Khor et al. (2019), and Morgulis et al. (2021)
Sp-p58-a	Basic protein with signal sequence and transmembrane domain	Biomineral growth	Yes	Adomako-Ankomah and Ettensohn (2011), Rafiq et al. (2014), and Khor et al. (2019)
Sp-Tgfbr2	Transforming growth factor beta (TGFβ) type II receptor	Biomineral growth	Yes	Rafiq et al. (2014), Sun and Ettensohn (2017), and Khor et al. (2019)

Sp-Vegfr10	Vascular endothelial growth factor (VEGF) receptor	Regulation of biomineralization genes and biomineral growth	Yes	Duloquin, Lhomond, and Gache (2007), Knapp, Wu, Mobilia, and Joester (2012), Khor et al. (2019), and Morgulis et al. (2019)
Sp-Ig/TM	Protein with 3 Ig domains, signal sequence, and transmembrane domain	Branching of spicule rudiment	Unknown	Rafiq et al. (2014) and Ettensohn and Dey (2017)
Sp-Sm30B	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Unknown	Gong et al. (2012) and Rafiq et al. (2014)
Sp-Sm30C	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Unknown	Gong et al. (2012) and Rafiq et al. (2014)
Sp-Sm50/ Sm32	Secreted, C-lectin domain-containing spicule matrix protein	Regulation of mineral phase transitions	Unknown	Gong et al. (2012) and Rafiq et al. (2014)
Sp-MmpL7	Matrix metalloprotease	Biomineral growth	Unknown	Ingersoll and Wilt (1998), Rafiq et al. (2014), and Morgulis et al. (2021)
Sp-Cara7LA (can1)	Secreted carbonic anhydrase	Bicarbonate production	Unknown	Mitsunaga et al. (1986) and Rafiq et al. (2014)

Note that many other targets of Alx1 encode novel, secreted or membrane-associated proteins that co-purify with biomineral isolated from larvae or adults (Karakostis et al., 2016; Mann, Poustka, & Mann, 2008; Mann, Wilt, & Poustka, 2010), suggesting that these proteins also support biomineralization.

3.2.5 Signal-dependent regulation of effector genes at late stages of embryogenesis

Most genes regulated by *alx1*, including many biomineralization genes, are activated at the blastula stage, prior to PMC EMT and several hours before the onset of biomineral deposition. This activation occurs cell-autonomously in the LM lineage through the maternally entrained, β-catenin/Pmar1-dependent molecular program described above (Section 3.1). During gastrulation, however, the expression of most of these same PMC effector genes comes under the control of local, ectoderm-derived signals. One of the most important and best characterized of these signals is VEGF3, a ligand produced specifically by ectoderm cells that overlie sites of active skeletal growth (Adomako-Ankomah & Ettensohn, 2013; Duloquin et al., 2007). Ectodermal cues like VEGF3 act locally to regulate the expression of effector genes, thereby creating complex, non-uniform, patterns of gene expression within the PMC syncytium that likely control the stereotypical growth patterns of skeletal rods (Guss & Ettensohn, 1997; Harkey, Whiteley, & Whiteley, 1992; Knapp et al., 2012; Morgulis et al., 2019; Morgulis et al., 2021; Sun & Ettensohn, 2014). The molecular mechanisms that underlie this second, signal-dependent phase of effector gene expression, and the potential role of Alx1 in this process, are major unresolved questions. Because the expression of *alx1* declines during post-gastrula development while skeletogenesis continues, it seems plausible that *alx1* transfers its function to one or more of its regulatory gene targets (Section 3.2.1). Heretofore it has not been possible to test directly whether Alx1 continues to exert regulatory control at late embryonic stages, as silencing of the gene by conventional methods, viz., microinjection of morpholinos into fertilized eggs, completely suppresses PMC formation. This question could now be addressed experimentally, however, by blocking Alx1 function at late developmental stages using caged morpholinos (Bardhan, Deiters, & Ettensohn, 2021).

3.3 Competition between GRNs: Repression of alternative fates by Alx1

An important consequence of the deployment of the Alx1 subcircuit in the LM-PMC lineage is the repression of alternative transcriptional programs. The LM territory is surrounded by a torus-shaped region composed of non-skeletogenic mesoderm cells, including prospective pigment and blastocoelar cells. In Alx1 morphants, the domain of expression of pigment cell markers, including the key regulatory gene *gcm*, expands into the large micromere territory (Oliveri et al., 2008). In addition, gene expression

profiling of Alx1 morphants reveals increases in the levels of expression of several regulatory and effector genes associated with blastocoelar cell fate (Rafiq et al., 2014), strongly suggesting that Alx1 also represses this regulatory state in the LM-PMC lineage. It will be of considerable interest to determine the molecular mechanism(s) of this repression, as competition between alternative transcriptional programs is observed in other developmental contexts (Delás & Briscoe, 2020).

4. Alx1 and other developmental and evolutionary processes

4.1 Alx1 and cellular reprogramming

Cells of the LM-PMC lineage are committed to a skeletal cell fate early in development through the activity of localized maternal factors and the cell-autonomous deployment of the skeletogenic GRN (Section 3.1.1). Other mesodermal and endodermal cell types remain multipotent, however, at least through gastrulation, and some have the capacity deploy the skeletogenic GRN under appropriate experimental conditions. Blastocoelar cells (BCs) ordinarily give rise to a heterogeneous population of migratory, immunocyte-like cells (Solek et al., 2013), but if PMCs are ablated early in gastrulation, BCs adopt the PMC fate and produce a correctly patterned skeleton. This striking transfating process is associated with the molecular reprogramming of BCs, which ectopically deploy the skeletogenic GRN while extinguishing the expression of regulatory genes (*scl* and *gatac*) associated with an immunocyte fate (Ettensohn et al., 2007; Sharma & Ettensohn, 2011). One of the earliest steps in BC reprogramming is the activation of *alx1*, which is both necessary and sufficient for transfating (Ettensohn et al., 2007). Repression of the pre-existing regulatory state in transfating BCs may involve the same mechanisms by which *alx1* represses the BC program in LMs during normal development, although this has not been tested. Like presumptive BCs, endoderm cells also have the capacity to express a skeletogenic fate and they activate *alx1* in the process, apparently by first transitioning through a BC-like regulatory state (McClay & Logan, 1996; Sharma & Ettensohn, 2011).

A key difference between the activation of *alx1* during normal development and its ectopic expression during cell reprogramming is that the former is controlled by cell-autonomous mechanisms while the latter is signal-dependent. It was recently shown that the activation and maintenance of *alx1* expression in transfating BCs require VEGF3, a signaling ligand

produced by the ectoderm (Ettensohn & Adomako-Ankomah, 2019). PMCs control BC fate by sequestering VEGF3, thereby preventing activation of *alx1* and the downstream skeletogenic network in BCs (Ettensohn & Adomako-Ankomah, 2019). The molecular steps between the reception of the VEGF signal and *alx1* activation are unknown, but *alx1* expression also requires MEK activity (Ettensohn et al., 2007), suggesting that VEGF might act through the MAPK cascade as it does in other cell types (Simons, Gordon, & Claesson-Welsh, 2016). One candidate mediator is Ets1, an ERK-dependent, positive regulator of *alx1* in the LM-PM lineage (Section 3.1.2) which is also expressed by BCs during normal development (Flynn et al., 2011; Rizzo et al., 2006).

4.2 Alx1 and cell type evolution

Echinoderms that exhibit indirect development (that is, those that develop via a feeding larva) produce calcified endoskeletal elements during one or more of three different phases of their life cycle: embryogenesis, the feeding period of larval development, and the adult phase (Fig. 4). In three of the five classes of modern echinoderms, echinoids (sea urchins and sand dollars), ophiuroids (brittle stars), holothuroids (sea cucumbers), skeletogenic cells (PMCs) first appear early in embryogenesis and produce skeletal elements before the onset of larval feeding. In at least two taxa, echinoids and ophiuroids, additional skeletal cells arise after the larva begins to feed and produce skeletal structures that are physically separate from the elements produced by PMCs (Smith, Cruz Smith, Cameron, & Urry, 2008; Tominaga et al., 2004). Skeletal elements also form in the doliolaria of crinoids (Comeau, Bishop, & Cameron, 2017), but a comparison with other taxa is complicated by the fact that all crinoids described to date exhibit direct development (that is, they lack a feeding larva). Lastly, skeletal cells arise within the rudiment of the adult body that forms within the late feeding larva and secrete the test, teeth, and spines of the juvenile. These biomineralized structures grow continuously during adult life and regenerate following injury.

The lineage relationships among the populations of cells that produce these skeletal structures are poorly understood. In indirect developing euechinoids, the best studied taxon, the embryonic lineage of PMCs is known completely. At least some, and perhaps all, of the post-feeding larval skeletal elements arise from macromere-derived mesoderm rather than PMCs (Yajima, 2007). PMCs also do not contribute to adult skeletal cells, but the lineage of these cells is otherwise undefined (Yajima, 2007). One

possibility is that they arise from the coelomic mesoderm, which makes a major contribution to the adult body and which, like post-feeding skeletal cells, is derived from the macromeres of the cleavage stage embryo. Indeed, recent evidence strongly supports the view that the adult skeletogenic cells of sea stars arise from the posterior coelom (Yamazaki et al., 2021).

It is widely accepted that the skeletal cells of embryos and adults are homologous, based on many striking similarities in the gene expression programs of these cells, including the deployment of a core gene regulatory network consisting of *alx1*, *ets1*, *erg*, and *vegfr-10-Ig* (Erkenbrack & Thompson, 2019; Gao & Davidson, 2008; Gao et al., 2015; Shashikant et al., 2018a). In addition, proteomic studies reveal that many of the same biomineralization proteins are produced by embryonic and adult skeletal cells (Mann et al., 2008, 2010). Much less is known concerning the gene regulatory program of the post-feeding skeletal cells, but what little information is available is consistent with the hypothesis that the three cell types are homologous.

The complex patterns of skeletogenesis exhibited by modern echinoderms reveal striking evolutionary plasticity in the developmental program that specifies skeletal cells. Because the adult forms of all fossil and modern echinoderms possess skeletons, while embryonic skeletogenic cells are not found in all sub-lineages, it is widely believed that adult skeletal cells evolved first. Support for this view comes from hemichordates, the nearest outgroup to echinoderms, which lack an embryonic or larval skeleton but produce small, biomineralized elements as adults (Cameron & Bishop, 2012; Gonzalez, Jiang, & Lowe, 2018). A more contentious issue is whether the elaborate larval skeletons of indirect developing echinoids and ophiuroids are homologous or evolved independently. The discovery of *alx1*-expressing PMCs in a third echinoderm class, holothuroids (McCauley et al., 2012), and the many similarities in the molecular programs of echinoid and ophiuroid skeletal cells (Czarkwiani et al., 2013; Dylus et al., 2016; Morino et al., 2012; Morino, Koga, & Wada, 2016; Seaver & Livingston, 2015), point to homology as the simplest hypothesis, with an implied loss of embryonic skeletal cells in the asteroid lineage (see also Erkenbrack & Thompson, 2019). Recently, it was shown that a CRE upstream of the *kirrelL* gene of a crinoid responds to the regulatory environment of sea urchin embryonic cells and drives reporter gene expression selectively in PMCs, a result that highlights the remarkable conservation of skeletogenic gene regulatory circuitry across all echinoderms (Khor & Ettensohn, 2021). Thus, a reasonable working model is that the ancestral echinoderm possessed

skeletal cells as an adult and that a heterochronic shift occurred in the MRCA of eleutherozoans that deployed this ancestral skeletogenic program in the embryo.

How has *alx1* contributed to the origin and evolutionary diversification of echinoderm skeletal cells? Changes in the expression patterns of transcription factors are often considered to make a greater contribution to the evolution of developmental processes than changes in transcription factor sequence, as the latter may have pleiotropic effects (Lynch & Wagner, 2008). Alx1, however, provides a good example of both mechanisms at work. As discussed above (Section 2), the duplication of the ancestral *alx1/alx4* gene in the MRCA of echinoderms made possible the subsequent exonization of the D2 domain and the neofunctionalization of the gene. The exonization of the D2 domain likely occurred in the MRCA all eleutherozoans, as all modern eleutherozoan Alx1 sequences contain a highly conserved D2 domain, while this domain has not been found in crinoid Alx1 sequences. The incorporation of the D2 domain altered the DNA binding properties (and perhaps other biochemical properties) of Alx1. One can speculate that these biochemical changes may have been associated in some way with the heterochronic deployment of the skeletogenic GRN that occurred in eleutherozoans, as the D2 domain is essential for Alx1 to exert its embryonic function (Khor & Ettensohn, 2017). Whether this domain has a specific role in embryonic skeletogenesis is not known, however, as its function in the adult has not been explored.

Given the central importance of *alx1* as a skeletogenic selector gene, changes in its developmental expression must also have played a crucial role in the heterochronic shifts in skeletogenesis that occurred in the echinoderm lineage. These certainly involved changes in the timing of *alx1* expression as well as shifts in the location (embryonic cell lineage) of expression. The temporal shifts in *alx1* expression that took place during echinoderm evolution may have involved (a) *cis*-regulatory changes in *alx1* that placed the gene under the control of new regulatory inputs which were already present at earlier developmental stages or (b) heterochronic shifts in the developmental expression of pre-existing *alx1* regulatory inputs. To evaluate these models, it will be important to know more about the regulatory inputs that control *alx1* expression in different developmental contexts, including the feeding larva and the adult. Unfortunately in this regard, our present knowledge of the upstream regulatory control of *alx1* is limited to the most evolutionarily derived case; viz., euechinoid micromeres (Section 3.1.1).

Although changes in *alx1* sequence and expression have been central to the evolution of echinoderm skeletogenesis, there were other important events. As discussed above (Section 3.2.5), ectoderm-derived VEFG3 is essential for the expression of a large subset of biomineralization genes and for overt skeletogenesis. Moreover, evolutionary changes in VEGF3 expression have accompanied heterochronic shifts in skeletogenic cell specification (Erkenbrack & Petsios, 2017; Morino et al., 2012). The receptor for VEGF3, VEGFR-10-Ig, is expressed selectively by skeletogenic cells (Duloquin et al., 2007) and was likely a component of the ancestral, adult skeletogenic network in the MRCA of at least eleutherozoans and perhaps all echinoderms (Erkenbrack & Thompson, 2019; Gao & Davidson, 2008).

Given the central importance of *alx1* and *vegf3* in driving skeletogenesis, did heterochronic shifts in these two regulators, which are expressed in different tissues, occur in a coordinated fashion? The two might have occurred independently (see Koga, Morino, & Wada, 2014), but if the expression of *alx1* in the mesoderm and *vegf3* in the ectoderm were integrated through developmental interactions between the two tissues, then an evolutionary shift in the expression of only one of the two genes might have been sufficient to transfer skeletogenesis to a new developmental address. There is indeed some evidence that links VEGF signaling in the ectoderm to *alx1* expression in mesoderm cells: (a) VEGF signaling positively regulates VEGFR-10-Ig expression in PMCs (Adomako-Ankomah & Ettensohn, 2013; Duloquin et al., 2007; Morgulis et al., 2019) and (b) *alx1* expression is strongly dependent upon VEGF/VEGFR signaling in transfating BCs, (Ettensohn & Adomako-Ankomah, 2019), although not in PMCs (Adomako-Ankomah & Ettensohn, 2013; Morgulis et al., 2019). Teasing apart possible regulatory interactions that link *vegf3* and *alx1* expression in the ectoderm and mesoderm, respectively, will likely shed light on whether evolutionary shifts in the expression of these two key skeletogenic regulators occurred independently or in a coordinated manner.

5. Conclusions

Alx1 was originally identified in a search for gene products that endow embryonic skeletal cells of sea urchins with their unique identity. More recently, comparative studies have highlighted the conserved role of *alx1* as a skeletogenic selector gene throughout the phylum and at all life history

stages. Duplication of *alx1* early in echinoderm evolution and subsequent exonization of the D2 domain were central to the evolution of an elaborate, calcified endoskeleton. Subsequent heterochronic shifts in *alx1* expression were important in the evolution of the diverse patterns of skeletogenesis exhibited by modern echinoderms. In modern sea urchins, the best studied taxon, Alx1 plays a unique role in skeletal development by integrating the morphogenetic behaviors (EMT, directional cell migration, and cell-cell fusion) of skeletogenic cells with their terminal biomineralization function. Analysis of *alx1* in this clade is therefore providing a paradigm for establishing direct linkages between GRNs and morphogenesis—a key to understanding the developmental transformation of genotype into phenotype.

Although these are significant findings, many questions remain to be addressed. It will be very important to identify upstream regulatory inputs that control *alx1* expression in contexts other than the micromeres of euechinoids. Even in that well-studied case, many features of GRN circuitry both upstream and downstream of *alx1* remain obscure, including the mechanism by which *alx1* suppresses potential, alternative regulatory states in the LM-PMC lineage. Biochemical functions of the critically important D2 domain aside from its role in DNA binding also remain to be explored. From these selected examples, it seems clear that Alx1 will continue to be a valuable lens through which to view a diverse set of developmental and evolutionary processes. We can therefore anticipate many new and exciting lessons from this transcription factor in the future.

Acknowledgments

The preparation of this article was supported by grants to C.A.E. from the National Science Foundation (IOS-2004952) and the National Institutes of Health (R24OD023046). The authors are grateful to Dr. Brooke McCartney for many valuable suggestions regarding the manuscript.

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