Molecular Paleobiology Of The Echinoderm Skeleton

Jeffrey R Thompson

Department of Earth Sciences, Natural History Museum, Cromwell Road, SW7 5BD London, UK

Abstract:

Molecular paleobiology provides a promising avenue to merge data from deep time, molecular biology and genomics, gaining insights into the evolutionary process at multiple levels. The echinoderm skeleton is a model for molecular paleobioloogical studies. I begin with an overview of the skeletogenic process in echinoderms, as well as a discussion of what gene regulatory networks are, and why they are of interest to paleobiologists. I then highlight recent advances in the evolution of the echinoderm skeleton from both paleobiological and molecular/functional genomic perspectives, highlighting examples where diverse approaches provide complementary insight and discussing potential of this field of research.

Key Words: Skeletogenesis, Development, Evo-Devo, Sea Urchins, Gene Regulatory Networks

1. Molecular Paleobiology

The fossil record provides invaluable insight into evolutionary transitions in morphology through deep time. Morphological evolution is the result of changes in organismal development and thus a holistic understanding of morphological evolution is not complete without an understanding of the evolution of the molecular and genomic mechanisms whose evolution underlie changes in *animal development*. While the fossil record provides the most direct record

of the history of life, the fossil record cannot provide direct insight into the molecular or genomic mechanisms which operate during development. Likewise, direct interrogation of *genomes, gene* function and regulation, and the molecular mechanisms operating during development is limited to extant taxa, or at least relatively geologically recent ones. Molecular paleobiology seeks to use the tools of molecular biology and genomics, including gene expression, functional genomics, molecular phylogenetics, and divergence time estimation, to address questions primarily formulated from paleobiology (Peterson et al., 2007, Wörheide et al., 2016). Molecular paleobiological approaches have shed light on diverse paleontological and evolutionary biological questions, from the origin and inter-relationships of paleobiologically important animal groups (Sperling et al., 2011, Vinther et al., 2012), to dating key transitions in the history of life (Lozano-Fernandez et al., 2016, Fleming et al., 2018, Schirrmeister et al., 2015, Howard et al., 2020). Furthermore, data from the fossil record are able to provide insight onto morphological evolution, not seen among extant lineages, and can be used to generate testable hypotheses about morphological character evolution, ontogenetic transitions, and homology (Garwood et al., 2014, Tweedt, 2017, Chipman and Edgecombe, 2019, Shubin et al., 1997, Shubin et al., 2009). The beauty of molecular palaeobiology is this two way transfer of information, fossils can inform on experiments to be carried out at the bench, and DNA sequence data can be used to understand the timing of evolutionary events and mechanisms of evolutionary change.

2. The Echinoderm Skeleton in Development and Evolution

The echinoderm skeleton provides a fantastic system with which to understand the evolution of a key morphological innovation from multiple perspectives spanning across

disciplines. From a palaeontological perspective, the $CaCO_3$ skeleton of echinoderms has provided most members of the phylum Echinodermata with an exceptional fossil record (Figure 1). This has facilitated their use to understand morphological, paleoecological, and trait-based evolution across long and deep time scales (Wright, 2017, Hopkins and Smith, 2015, Cole et al., 2019, Deline et al., 2020, Mongiardino Koch and Thompson, 2020b, Bauer, 2020, Syverson and Baumiller, 2014, Clark et al., 2020). In addition to this rich insight into paleobiological questions, the echinoderm skeleton is also a cutting-edge model system used to understand how the regulation, function, and expression of genes, directs the processes of animal development and evolution (Shashikant et al., 2018, Davidson et al., 2002a, Revilla-i-Domingo et al., 2007, Oliveri et al., 2008, Dylus et al., 2018).

With the publication of the sequenced genome of the purple sea urchin, *Strongylocentrotus purpuratus*, in 2006 (Sodergren et al., 2006), understanding the regulatory genomic mechanisms by which the echinoderm skeleton develops became more tractable than ever before. Analyses of the structure, arrangement, and number of genes and families of genes involved in skeletogenesis (the molecular and developmental processes that build the skeleton) laid the groundwork for the current, meticulous, understanding of the genetic regulatory mechanisms which orchestrate development of the larval skeleton in sea urchins (Oliveri et al., 2008, Rafiq et al., 2012, Rafiq et al., 2014, Shashikant et al., 2018, Ettensohn, 2009, Sharma and Ettensohn, 2010, Livingston et al., 2006). In addition to providing insight into how the regulatory genome directs the development of the larval skeleton, the publication of the sea urchin genome also allowed for novel, and creative, insight into the evolution of the echinoderm skeleton in deep time (Bottjer et al., 2006). With their manuscript entitled “Paleogenomics of Echinoderms”, which was published alongside the *S. purpuratus* genome in a special issue of *Science*, Bottjer et

al. (2006) made one of the first explicit attempts to link the evolution of genes to the initial appearance of a morphological feature, in this case the echinoderm skeleton, in the fossil record. A multitude of new comparative data from genomes and *transcriptomes* (all expressed genes in an organism or tissue determined through RNA sequencing) of other, non-echinoid, echinoderms has come to light in the 14 years since the publication of "Paleogenomics of Echinoderms". Nevertheless, there is little doubt that "Paleogenomics of Echinoderms" remains an influential paper in molecular, genomic and paleobiological studies of the echinoderm skeleton, and remains an important contribution highlighting multidisciplinary approaches to understand the evolution of a major evolutionary innovation underpinning the origin of a diverse phylum, the echinodermata.

What I would like to highlight in this contribution, are a number of novel insights into the deep-time evolution of the echinoderm skeleton, that have taken place since the publication of "Paleogenomics of Echinoderms*"* in 2006. To do this, I will first highlight the skeletons of echinoderms, and the cells that are responsible for building them. Next, I will then take some space to explain the conceptual and practical relevance of gene regulatory networks, and their various components, in development. Thereafter, I will briefly review what is known about the genetic regulatory networks that build the skeletons of larval and adult echinoderms. I will then focus on work using deep-time perspectives to understand the evolution of the echinoderm skeleton, focusing on examples from both the fossil record, and extant organisms. Finally, I would like to touch on a number of areas where I feel that future integration of paleontological data, and data from analyses of gene expression and gene function, can synergistically inform the evolution of the echinoderm skeleton, and the evolution of animal diversity more generally.

2.1 Echinoderm Skeletons

The activity of gene regulatory networks (GRNs) result in the development and growth of animal morphology, like the echinoderm skeleton. As skeletons make up the bulk of the animal fossil record, GRNs are the blueprints much of the data we find preserved in the fossil record. The exceptional fossil record of echinoderms is due to the abundance of their adult skeletons in the rock record. This $CaCO_3$ skeleton consists of numerous skeletal plates, or ossicles, which can be either abutting or imbricating (Smith, 1980), or sutured together with interlocking struts (Grun and Nebelsick, 2018, Smith, 1990). Much of the adult skeleton is embedded within the dermis, and is *mesodermal* in origin. It is the structure and arrangement of the skeleton which gives extant echinoderms their characteristic five-fold symmetry, and is responsible for the characteristic morphology of most classes (Figure 1). The skeleton of most adult echinoderms is characterized by a distinct trabecular microstructure, termed *stereom,* which contains an occluded *protein* matrix and sits surrounded within organic tissue termed the stroma (see Gorzelak (2020) this volume). Echinoderm skeletons can be highly variable in terms of their morphology, and this diversity of echinoderm skeletons forms the basis for much of the morphological diversity discussed in this volume.

In addition to their disparate adult skeletons, there are a diversity of larval skeletons found across the echinodermata (Figure 2a) (Mortensen, 1921). While these have a miniscule fossil record (Deflandre-Rigaud, 1946), they have formed the basis for a number of important studies attempting to infer larval ecology (Strathmann, 1971, Strathmann, 1975, Pennington and Strathmann, 1990), the effects of changes in ocean chemistry on larval mortality and vitality (Brennand et al., 2010, Byrne et al., 2013, Byrne et al., 2011) and attempts to infer echinoderm phylogeny (Smith, 1997, Wray, 1992, Strathmann and Eernisse, 1994). The larval skeleton of

echinoids, known as the echinopluteus, is an extensive structure consisting of between four and six skeletal elements which assist in orientation while in the water column (Figure 2a,c-d) (Pennington and Strathmann, 1990). The echinopluteus is characteristically referred to as easel-shaped, and most echinoplutei have pairs of elongate, ciliate, larval arms, which protrude from the body around the mouth (Mortensen, 1921). The skeletal elements of echinoplutei can be rod-like, or fenestrated, having the appearance of small ladders (Wray, 1992, Mortensen, 1921). While some direct developing echinoids have lost the echinopluteus larvae throughout the course of evolution, the presence of an echinopluteus has been demonstrated to be the ancestral state, at least amongst crown group echinoids (Wray, 1992).

Extensive larval skeletons are not unique to the echinoids. Many indirect-developing ophiuroids have planktonic ophioplutei larvae, which also have an elongate larval skeleton with up to four pairs of skeletal arms (Figure 2a) (Mortensen, 1921, Raff and Byrne, 2006, Gliznutsa and Dautov, 2011). Though not supported by recent phylogenomic analyses (Telford et al., 2014, Cannon et al., 2014, Mongiardino Koch et al., 2018), the gross morphological similarity between the ophiopluteus and echinopluteus had previously been taken as evidence for a close phylogenetic relationship of echinoids and ophiuroids; the so-called cryptosyringid hypothesis (Smith, 1997). Within the ophiuroids, the adult body plan either develops from a rudiment in the ophiopluteus, or through a distinct additional metamorphic stage known as the vittelaria (Mortensen, 1921, Byrne and Selvakumaraswamy, 2002). The ancestral condition within ophiuroids is still not well known, as the developmental mode is not well-known for most ophiuroids, however reduced ophiopluteal skeletal elements in vittelaria suggest ophioplutei are the ancestral condition (Byrne and Selvakumaraswamy, 2002). Furthermore, though these different larval types are distributed broadly across ophiuroid phylogeny (O'Hara et al., 2014,

McEdward and Miner, 2001), the morphological similarity of ophioplutei to echinoplutei also suggests that the ophiopluteus may be the ancestral condition within ophiuroids (Raff and Byrne, 2006), and analyses of the echinoderm skeletogenic cell suggest a single evolutionary origin for the *cell type* that builds both echinoid and ophiuroid larval skeletons (Erkenbrack and Thompson, 2019).

While the holothurians, or sea cucumbers, lack the extensive elongate skeletons found in echinoids, they too have larval skeletons (Figure 2a). In contrast to echinoids and ophiuroids, direct development is the most common developmental mode amongst holothurians (Sewell and McEuen, 2002). Indirect developing holothurian larvae are referred to as auricularia, and in many auricularia, small, spicules are found along the posterior end of the larvae (McCauley et al., 2012, Woodland, 1907b). Auricularia larvae of the synaptid holothurians are also known to possess skeletal wheels-like ossicles, similar to the wheel-shaped ossicles found in adult holothruians, and which are the ontogenetic outcomes of the aforementioned spicules (Stricker, 1986, Stricker, 1985, Woodland, 1907b). The larval skeleton of holothurians has often been overlooked relative to that of ophiuroids and echinoids, and many accounts of the holothurian larval skeleton predate the advent of scanning electron microscopy (Woodland, 1907b, Mortensen, 1921). While this is the case, understanding the growth and development of the holothurian larval skeleton has a crucial role to play in understanding the evolution and origin of echinoderm skeletons (Erkenbrack and Thompson, 2019, McCauley et al., 2012).

Asteroids are unique amongst the *eleutherozoans,* in that their larvae lack a larval skeleton (Figure 2a). While there exists a diversity of larvae amongst the asteroids, whose evolutionary histories provide an ideal model system to study the evolution of larval morphology (Hart et al., 1997, Carter et al., 2020), because none of these larvae have skeletons, they will not

be discussed in detail here. Though some larval asteroids have elongate larval arms, which may serve analogous purposes to those of echinoplutei and ophioplutei, these larval arms have no endoskeleton supporting them (Mortensen, 1921). In addition to the absence of a larval skeleton, asteroid larvae also lack skeletogenic cells, which give rise to the skeleton in the larvae of other eleutherozoans classes. It is this absence of a skeleton in asteroids, that has resulted in much of the controversy surrounding the homology or homoplasy of the echinoid or holothurian larval skeletons. All known crinoid larvae are direct developing, and as such lack a larval skeleton (McEdward and Miner, 2001). They thus do not factor into this discussion of larval skeletons.

2.2 Skeletogenic Cells In Echinoderms

The echinoderm skeleton is secreted by a distinct population of cells, known as skeletogenic cells or *sclerocytes*. Nascent biomineral is deposited in a *vacuole* within a cellular *syncytium* made up of the fusion of the cell processes and cytoplasm (Figure 3) (Gliznutsa and Dautov, 2011, Märkel et al., 1986, Smith, 1990). While both larval and adult echinoderms possess skeletons, much more is known about the molecular signature of larval skeletogenic cells in echinoderms than those which secrete the adult skeleton (Killian and Wilt, 2008). In particular, one population of embryonic/larval skeletogenic mesodermal cells of echinoids (Figure 3c-e), known alternatively as primary *mesenchyme cells* (PMCs), are exceptionally well characterized from a molecular perspective (Oliveri et al., 2008, Rafiq et al., 2012, Rafiq et al., 2014, Barsi et al., 2014, Barsi et al., 2015). In all known eleutherozoans, these embryonic and larval skeletogenic cells are mesenchymal, meaning in this case that they are loosely associated and mobile. Skeletogenic cells are first specified early in development, prior to *blastula stage embryos*, and go on to build the larval skeleton in indirect developing echinoids, ophiuroids, and

holothurians (McCauley et al., 2012, Dylus et al., 2016, Gliznutsa and Dautov, 2011, Okazaki, 1975). After their specification, the presumptive skeletogenic cells undergo an *epithelial* to mesenchymal transition, migrating from the wall of blastula or *gastrula* stage embryos, or the ingressing *archenteron* (dependent upon the taxon) into the *blastocoel* during development (Figures 2b, 3c-d) (Wray and McClay, 1988, Wu and McClay, 2007, Wu et al., 2007). Once in the blastocoel, they form a syncytium, in which the $CaCO_3$ of the larval spicule and skeleton is deposited (Figures 2c-d, 3e) (Gliznutsa and Dautov, 2011, Woodland, 1907b, Smith, 1990). Though the homology of these cells across echinoderm classes is debated (Erkenbrack and Thompson, 2019), the cellular movements and processes associated with larval skeletogenic cells are found across echinoderms with larval skeletons, and characterize larval skeletogenesis in echinoids, ophiuroids, and holothurians.

Less is known about the molecular and cellular mechanisms operating in the skeletogenic cells of adult echinoderms, though their morphology and function during skeletogenesis has been characterized in a number of echinoderm groups. Adult echinoderm skeletogenic cells have been most thoroughly characterized in echinoids, where skeletal cells have been classified into at least two distinct groups, the sclerocytes and the odontoblasts (Märkel et al., 1986, Märkel et al., 1989). Additionally, sclerocytes have been identified and characterized in regeneration ophiuroid arms (Piovani et al., 2021). In echinoids, sclerocytes are the skeleton-secreting cells which secrete biomineral throughout the majority of the animal, while odontoblasts are those skeletogenic cells which are responsible for biomineralization of the continuously growing teeth of the Aristotle's lantern (Märkel et al., 1986, Märkel et al., 1989). Much of the adult echinoderm skeleton, including echinoid test plates and spines, asterozoan arm plates, and ossicles of the holothurian body wall, lie embedded within the dermis, and thus further discussion will focus on

the sclerocytes. In growing echinoderm skeletal elements, such as the margins of interambulacral plates of the echinoid test, there is a higher density of skeletal cells in areas of active skeletogenesis (Shimizu, 1997). Like the skeletogenic cells of the larvae, sclerocytes, are responsible for biomineralization within cytoplasmic sheaths or, when multiple cells have merged, a syncytial vacuole (Märkel et al., 1986, Smith, 1990, Märkel et al., 1989, Dubois and Jangoux, 1990, Ameye et al., 1999, Heatfield and Travis, 1975, Stricker, 1986, Piovani et al., 2021). Within the cytoplasmic sheath of this syncytial vacuole, the skeleton is surrounded by a matrix coat, comprised of polysaccharides and proteins (Märkel et al., 1989, Ameye et al., 1999). These sclerocytes have characteristic outgrowths which contact the stereom (Dubois and Jangoux, 1990, Ameye et al., 1999). During regeneration, the sheath of these skeletal cells surrounds the ends of growing stereom trabeculae, and new biomineral is deposited within the vacuole of the sheath (Dubois and Jangoux, 1990, Ameye et al., 1999, Heatfield and Travis, 1975).

While echinoderm skeletogenic cells were first identified and characterized based upon their function and morphology, most recent work has begun to understand these cells in the context of the suites genes they express (Rafiq et al., 2014, Piovani et al., 2021), and the role of these genes in building the skeleton. The expression and activity of these genes is the product of the molecular interactions encoded in the genome, and characterized by the skeletogenic gene regulatory network (Shashikant et al., 2018, Oliveri et al., 2008).

3. What is a developmental gene regulatory network?

In recent years (though see (Valentine and Campbell, 1975)), there has been interest from the palaeontological community in *developmental gene regulatory networks* (GRNs),

largely because of their potential explanatory power in understanding the evolution of animal body plans (Davidson and Erwin, 2006, Erwin and Davidson, 2009, Bottjer, 2017, Thompson et al., 2017, Thompson et al., 2015, Erwin, 2020) and homology of morphological characters (Wagner, 2007). Because of this interest, and because of the crucial role GRNs play in understanding the evolution and development of the echinoderm skeleton (Dylus et al., 2018, Dylus et al., 2016, Shashikant et al., 2018, Thompson et al., 2017), I feel it is worth taking some space to explain exactly what GRNs are, and why they are relevant in development and evolution.

Developmental gene regulatory networks describe the interactions of genes and gene products (e.g proteins) during the course of organismal development (Figure 4)(Peter and Davidson, 2015, Davidson et al., 2002a). Gene regulation is the process by which a gene or gene product, regulates the expression of another gene (Figure 4a-c). Genes encoding for proteins can regulate the expression of other genes both positively (Figure 4a; upregulation), or negatively (Figure 4c; downregulation), resulting in the fluctuating spatial and temporal patterns of gene expression across various cell and tissue types during development (Levine and Davidson, 2005, Davidson and Levine, 2008, Wray and Lowe, 2000). GRNs consist of regulatory interactions of hundreds of different genes and proteins, all of which interact directly or indirectly to ensure development proceeds correctly (Barsi et al., 2015, Peter and Davidson, 2015, Peter and Davidson, 2017, Khor et al., 2019). The structure of a GRN reflects the timing of gene expression and regulation, with more upstream components expressed earlier in development than those which are more downstream. This results in a hierarchical structure seen in many GRNS (Figure 4a-c), which allows for their constituent parts, or subcircuits, to be broken down and thought of as individual modules (Levine and Davidson, 2005, Peter and Davidson, 2016,

Peter and Davidson, 2017, Davidson and Erwin, 2006). GRNs are comprised of multiple different types of genes, including those which encode *transcription factors*, *signaling molecules*, and *differentiation proteins* (see below) all of which have different functions in development.

3.1 Transcription Factors: Regulating Gene Expression

Transcription factors are proteins, encoded for by genes, that regulate the expression of other genes through the physical process of binding to DNA sequences around the genes they regulate (Figure 4d-e) (Peter and Davidson, 2015, Wray et al., 2003, Gilbert, 2006). These sequences, called DNA binding sites, can be located in the non-coding regions flanking the *exons* where the *transcriptional machinery* (including the *RNA polymerase* and other aspects of the *transcription initiation complex*) binds, called the *promoter* (Watson et al., 2008). Additionally, DNA binding sites can be located in regulatory modules, consisting of numerous individual binding sites, that can be located thousands of nucleotides from the promoter, including in gene *introns*. These regulatory modules located far from the transcriptional start sites are called *enhancers* (Figure 4d-e).

When a transcription factor binds to a regulatory sequence of a gene, it interacts with proteins involved in RNA polymerase binding, *histone* modifying proteins and other transcription factors to stabilize the transcription initiation complex, open *nucleosomes* or otherwise facilitate or repress transcription of the target gene (Wray et al., 2003, Gilbert, 2006, Watson et al., 2008) (Figure 4d). Transcription factors are thus responsible for modulating both the amount and timing of expression of the genes that they regulate. They are responsible for both positive regulation, where their activity results in higher levels of expression of their

downstream target genes, and negative regulation or repression, where their binding to a regulatory region on their downstream target results in a reduction or silencing of its expression (Revilla-i-Domingo et al., 2007, Peter and Davidson, 2015, Watson et al., 2008).

Transcription factors act combinatorially to regulate the process of gene expression, and acting together with numerous other transcription factors, and *transcription co-factors*, during development (Figure 4d-e) (Wray et al., 2003, Gilbert, 2006, Watson et al., 2008, Davidson, 2006). Because transcription factors are responsible for gene regulation, they are amongst the most important components of a gene regulatory network (Peter and Davidson, 2015, Davidson, 2006, Davidson et al., 2002a). The sum of all transcription factors expressed in a particular cell or set of cells is called the regulatory state, and different regulatory states are responsible for the existence of differentially specified cell fates in development (Peter and Davidson, 2015). In this way, transcription factors are crucial components underlying the specification of the diverse cell types found throughout the course of animal development and evolution (Arendt et al., 2016, Arendt, 2008). Transcription factors are also amongst the more upstream members of GRNs, and can regulate many hundreds of downstream targets, including signaling molecules and differentiation genes (Khor et al., 2019, Rafiq et al., 2012, Rafiq et al., 2014).

3.2 Signaling Molecules: Communication Between Cells During Development

Cell to cell signaling provides a mean by which different tissues and organs are able to communicate during development. Cell-cell signaling typically involves a specific *ligand* sending the signal and binding to a specific receptor molecule on the surface of the cell that receives the signal. Signaling can take place through direct contact between two cells, through the diffusion of the ligand across small distances in the developing animal, or over long distances

via fluid, such as blood (Gilbert, 2006). The receptor protein spans the cell-membrane, with an extracellular portion outside the cell, a transmembrane region, and a cytoplasmic region inside of the cell. The extracellular portion receives a signal from other cells by binding to the ligand. This results in a change of shape of the receptor protein on the inside and outside of the cell, which in turn results in a series of enzymatic changes inside the cell, typically ending in the activation of a transcription factor (Gilbert, 2006). This series of interactions is called a *signal transduction cascade.*

During development, these signaling pathways operate between different cell and tissue types, and often provide spatial cues which result in the positioning of developing morphological structures, or the *induction* of new organs and tissue types (Duloquin et al., 2007, Gilbert, 2006). Signaling molecules play an important role in GRNs directing development, as they are responsible for activating particular GRN subcircuits in spatially distinct suites of cells during growth and development (Peter and Davidson, 2017, Peter and Davidson, 2015). Signaling molecules act as bridges during development, allowing for the output of a GRN in one cell to activate more downstream components in adjacent or nearby cells. Because of their role in cell to cell communication, signaling molecules help to confer modularity and hierarchy to the activity of GRNs (Levine and Davidson, 2005).

3.3 Differentiation Gene Batteries: The Interface With Morphology

Amongst the most downstream components of a gene regulatory network are the differentiation genes (Davidson et al., 2002b, Davidson et al., 2002a). Differentiation genes do not regulate the expression of other genes, and are so called because they are crucial players in the processes of *cellular differentiation*, the process by which cells change into more specific cell

types through the course of development. These differentiation genes, and the proteins for which they encode, are often expressed in specific cell types, including skeletal cells, photoreceptors, or neurons (Barsi et al., 2014, Barsi et al., 2015, Wilt et al., 2008, Garner et al., 2016). Differentiation proteins are expressed at the most peripheral portions of a GRN (Davidson et al., 2002a), building morphological structures at a molecular level, and encoding for proteins found in distinct tissue types such as skeletal tissue, nerves, and those that are responsible for pigmentation and color. Because of their position at the periphery of a network, they have also been proposed to undergo the highest rates of evolution within a GRN (Davidson and Erwin, 2006, Erwin and Davidson, 2009, Peter and Davidson, 2011).

4. The Echinoderm Skeletogenic Gene Regulatory Network

The gene regulatory network directing development of the embryonic and larval skeleton of echinoderms is one of the best known developmental GRNs in animal development (Figure 5). Though it has primarily been studied in echinoids, echinoderm larval skeletogenic cells express a distinct set of transcription factors, which interact together with signaling molecules and differentiation genes to build the larval skeleton. It is thus crucial to note, that most of the activity of the echinoderm skeletogenic GRN takes place specifically in the skeletogenic cells that build the skeleton (Figures 2b, 3). It would be impossible to discuss the role and function of all genes comprising the skeletogenic GRN in this contribution, so I herein choose to focus on some of the best characterized, and potentially most important, genes in the network. An important note moving forward concerns the nomenclature of genes and proteins. If the name of a molecule is shown in italics, it refers to the DNA sequence or *mRNA* transcript of the gene, e.g *Alx1*. If, the name is shown without italics, for instance, Alx1, the name refers to the protein that

has been translated from the mRNA transcript. As transcription factors are proteins, when discussed in the context of their regulatory function, typically the unitalicized version is used. If discussing the gene that encodes for the transcription factor, as is often the case when discussing gene expression, the name is italicized.

4.1 Transcription Factors In The Echinoderm Skeletogenic Gene Regulatory Network

The most upstream components of the skeletogenic GRN are regulatory genes that encode for transcription factors (Figure 5)., and one of the most extensively studied is the transcription factor *Alx1* (Ettensohn et al., 2003, Khor and Ettensohn, 2020). Alx1 has regulatory inputs into over 400 other genes that are differentially expressed in *S. purpuratus* skeletogenic cells (Rafiq et al., 2014, Khor et al., 2019) and *Alx1* expression has been identified in the skeletogenic cells of all larval echinoderms (with skeletogenic cells) thus far examined (McCauley et al., 2012, Dylus et al., 2016, Ettensohn et al., 2003, Yamazaki et al., 2010, Erkenbrack and Davidson, 2015, Yamazaki and Minokawa, 2015, Koga et al., 2016, Yamazaki et al., 2014, Khor and Ettensohn, 2017, Morgulis et al., 2019). Additionally, *Alx1* is expressed in sites of skeletogenesis in adult or post-metamorphic echinoids (Gao et al., 2015, Gao and Davidson, 2008), asteroids (Koga et al., 2016, Koga et al., 2014), and ophiuroids (Czarkwiani et al., 2013, Piovani et al., 2021).

In addition to Alx1, several other transcription factors have been implicated as crucial components of the skeletogenic GRN (Figure 5). The gene *Ets1/2* is expressed in the skeletogenic and non-skeletogenic mesodermal cells of embryonic and larval eleutherozoans (Rizzo et al., 2006). *Ets1/2* also has regulatory inputs into hundreds of genes expressed in skeletogenic cells (Rafiq et al., 2014) in *S. purpuratus* and *knockdown* (reduction or blocking of

gene expression) of this gene in multiple larval echinoderms results in a failure of mesodermal cells to properly differentiate, and thus failed skeletogenesis (Koga et al., 2010, Kurokawa et al., 1999). *Tbr,* a transcription factor gene expressed in skeletogenic cells and broader mesodermal tissues across embryonic and larval echinoderms is also necessary for larval skeletogenesis in *S. purpuratus* (Croce et al., 2001, McCauley et al., 2012, Erkenbrack and Davidson, 2015, Dylus et al., 2016, Yamazaki et al., 2014, Yamazaki and Minokawa, 2015, Oliveri et al., 2008). In contrast to *Alx1*, knockdown of *Tbr* in cidaroid echinoids does not result in a reduction of skeletogenic cells (Yamazaki et al., 2014). Furthermore, *Tbr* is not expressed in skeletogenic centers in adult echinoids or ophiuroids (Czarkwiani et al., 2013, Gao and Davidson, 2008), indicating it may be an evolutionarily later addition to the *S. purpuratus* skeletogenic GRN (Erkenbrack and Thompson, 2019).

There are numerous other transcription factors from the *S. purpuratus* skeletogenic GRN that are expressed in the skeletogenic cells of other echinoderms, including Erg, Hex, TGIF, and Jun (Figure 5). The spatial expression of these other transcription factors has not been surveyed at the same taxonomic breadth as for *Alx1*, *Ets1/2*, or *Tbr*, and thus the demonstrated extent of their involvement in skeletogenesis at broad taxonomic scales is lesser known. All of these genes have been shown to be expressed in the skeletogenic cells of *S. purpuartus*, however, and some have demonstrated expression in skeletogenic cells of other echinoids, ophiuroids, and holothurians (McCauley et al., 2012, Dylus et al., 2016, Erkenbrack et al., 2016, Russo et al., 2014, Piovani et al., 2021). While the spatial expression of these transcription factors has not been widely surveyed from a phylogenetic standpoint, their functional importance in the *S. purpuratus* skeletogenic GRN has been validated (Oliveri et al., 2008), and thus their role in

skeletogenesis outside of *S. purpuratus* remains a fruitful avenue of research for understanding the evolution of gene function in GRNs.

4.2 Signaling Molecules In The Echinoderm Skeletogenic Gene Regulatory Network

Aside from transcription factors, signaling molecules play an important role in skeletogenic GRN for echinoderms (Figure 5). Though numerous signaling pathways are involved in regulation and development of the echinoderm skeleton (Adomako-Ankomah and Ettensohn, 2014), I will focus on the two which have been the most extensively studied in echinoderms, the VEGF and FGF signaling pathways. These two pathways are necessary components of normal skeletogenesis in echinoderms. The VEGF, or vascular endothelial growth factor pathway, is involved in vascularization and blood vessel growth and development in vertebrates, however, in echinoderms it underlies skeletogenesis (Morgulis et al., 2019). In both larval and adult skeletogenesis, the signaling ligand, *Vegf3* is expressed in *ectodermal* tissues overlying the skeletogenic mesodermal cells (Morgulis et al., 2019, Duloquin et al., 2007, Morino et al., 2012, Erkenbrack and Petsios, 2017, Adomako-Ankomah and Ettensohn, 2013, Adomako-Ankomah and Ettensohn, 2014, Czarkwiani et al., 2019). *VegfR-10-Ig,* which receives this signal from the overlying *Vegf3*-expressing ectodermal cells, is expressed specifically in these skeletogenic cells. Knockdown of *Vegf3* or *VegfR-10-Ig* results in reduced formation of skeletal biomineral and expression of skeletogenic genes, as well as incorrect spatial positioning of the skeletogenic mesodermal cells (Duloquin et al., 2007). This suggests that *Vegf3* sends spatial positioning cues to the skeletogenic mesodermal cells, and that *VegfR-10-Ig* expression in the skeletogenic cells regulates the expression downstream biomineralization genes involved in skeletogenesis (Duloquin et al., 2007, Morgulis et al., 2019). This pattern, with *VegfR-10-Ig*

414 expressed in the skeletogenic mesoderm and *Vegf3* expressed in the overlying ectoderm, has
415 been identified in larval regular euechinoid echinoids (Duloquin et al., 2007, Morgulis et al.,
416 2019, Adomako-Ankomah and Ettensohn, 2013), cidaroids (Erkenbrack and Petsios, 2017),
417 ophiuroids (Morino et al., 2012, Czarkwiani et al., 2019) as well as in skeletogenesis in adult or
418 metamorphic asteroids (Morino et al., 2012, Koga et al., 2014), ophiuroids (Czarkwiani et al.,
419 2019), and in the case of only *VegfR-10-Ig*, echinoids (Gao et al., 2015, Gao and Davidson,
420 2008). Also, importantly, neither *Vegf3* nor *VegfR-10-Ig* were identified in early development of
421 sea stars, which lack a larval skeletogenic cell (Morino et al., 2012). Taken together, this
422 evidence implicates the role of VEGF signaling in the spatial positioning of the skeleton, and
423 further regulation of skeletogenic genes, thus making it a crucial component of the skeletogenic
424 GRN in echinoderms.
425 In addition to VEGF signaling, the FGF, or fibroblast growth factor, pathway has also
426 been identified as an important part of the skeletogenic GRN. Like VEGF, the FGF ligand,
427 *Fgf9/16/20* (herein referred to as *Fgf*) is expressed in the ectoderm of larval sea urchins, though
428 in largely distinct territories from the expression of *Vegf3* (Adomako-Ankomah and Ettensohn,
429 2013, Röttinger et al., 2008). In contrast to *Vegf3*, however, *Fgf* is expressed not only in the
430 ectoderm, but also the skeletogenic mesodermal cells themselves. The FGF signaling receptor,
431 *FgfR2*, is expressed specifically in the skeletogenic mesodermal cells, as is the case with *VegfR*,
432 and other components of the skeletogenic GRN (Röttinger et al., 2008). Inhibition of FGF
433 signaling results in disrupted skeletogenesis and the downregulation of the biomineralization
434 genes SM30 and SM50 (Röttinger et al., 2008). Both VEGF and FGF signaling are thought to
435 underlie the branching, anastamozing, morphology seen in stereom. When larval spicules are
436 cultured from isolated skeletogenic mesodermal cells, and thus in the absence of ectodermal

signals, the triradiate spicules lack any branching (Okazaki, 1975). Furthermore, in regenerating asteroid spines, stereom at the spine margin, adjacent to the ectoderm, grows longitudinally via branching, suggesting that signals from the ectoderm modulate the direction and morphology of stereom growth (Dubois and Jangoux, 1990). Given that VEGF signaling is involved in patterning the anastomosing tubular morphology of blood vessels in vertebrates, it seems likely that it may fill a similar role in patterning the tubular, branching morphology of the echinoderm skeleton (Morgulis et al., 2019).

4.3 Differentiation Gene Batteries In The Echinoderm Skeletogenic Gene Regulatory Network

The most downstream components of the echinoderm skeletogenic GRN are the differentiation genes (Figure 5), which include biomineralization genes, as well as genes which assist in ion transport, and morphogenetic processes like cell fusion (Shashikant et al., 2018). These biomineralization genes are responsible for depositing nascent $CaCO_3$ and building the biomineral structure, and are thus very much the building blocks for the echinoderm biomineral skeleton. Though there are hundreds of downstream differentiation genes involved in echinoderm skeletogenesis, the most well-known of these biomineralization genes are those of the spicule matrix (SM) and MSP130 families (Figure 5). The SM genes, which include *SM30*, S*M37*, *SM50*, *C-lectin*, and *PM27* encode for c-lectin type *extracellular matrix* proteins and are expressed during sea urchin biomineral growth and (at least some of which) are occluded in the skeletal organic matrix (Mann et al., 2008b, Ameye et al., 1999, Livingston et al., 2006, Mann et al., 2008a, Mann et al., 2010). Crucially, the SM family of genes are specific to echinoids, and appear to be absent from the genomes and transcriptomes of other echinoderms (Dylus et al., 2018, Zhang et al., 2017). The SM genes are expressed in the skeletogenic cells of embryonic

and larval echinoids (Guss and Ettensohn, 1997b), and the proteins they encode for are occluded within the organic matrix of the larval skeleton (Mann et al., 2010). Knockdown of some SM genes in the larvae results in a failure of larval skeletal elements to form or elongate, though just how important particular SM genes are varies on a protein-by-protein basis (Wilt et al., 2008, Wilt et al., 2013). In addition to their role in larval skeletogenesis, SM30 and SM50 proteins have been identified specifically in the skeletogenic cells and occluded skeletal matrix of adult echinoid skeletal tissues (Ameye et al., 1999, Thompson et al., 2021). Though the SM genes are not present in non-echinoid echinoderms, it seems likely that there are analogous, yet non-homologous genes that fill a similar role in other echinoderm groups such as ophiuroids (Czarkwiani et al., 2019).

In addition to the SM genes, the MSP130 family of genes are a well-known component of echinoderm skeletogenesis (Figure 5). MSP130 is a cell-surface *glycoprotein* which is found in skeletogenic cells and skeletal tissues of echinoderms across classes and life history stages (Chiaramonte et al., 2020, Mann et al., 2008b, Livingston et al., 2006, Guss and Ettensohn, 1997a, Anstrom et al., 1987, Leaf et al., 1987, Minokawa et al., 1997, Mann et al., 2010, Mann et al., 2008a). Homologues of MSP130 genes have been identified across numerous biomineralizing metazoans (Ettensohn, 2014, Szabó and Ferrier, 2015, Cameron and Bishop, 2012, Marie et al., 2011), as well as in the genomes, transcriptomes, and *proteomes* of crinoids, echinoids, ophiuroids and holothurians (Dylus et al., 2018, Livingston et al., 2006, Davidson et al., 2020, Zhang et al., 2017, Li et al., 2020). Similarly to the SM genes, the MSP130 genes have undergone extensive *gene duplication* throughout their evolutionary history, with numerous *paralogues* and closely-related genes found in echinoids (Dylus et al., 2018, Davidson et al., 2020, Ettensohn, 2014, Livingston et al., 2006). Noteworthy, however, is the fact that

phylogenetic analyses support independent, homoplastic duplications of MSP130 and related genes in echinoids and ophiuroids, underpinning the crucial role of this gene in echinoderm biomineralization (Dylus et al., 2018).

Though they have been less extensively studied than the SM and MSP130 genes, additional differentiation genes include those that encode for extracellular matrix proteins, or those with roles in ion transport, cell-fusion, and biomineralization (Rafiq et al., 2012). A selection of these are *CAN* and *Caral7* which encode for carbonic anhydrases, a family of enzymes that catalyze the conversion of $C0_2$ and H_2O to H^+ and HCO_3^-ions for skeletogenesis and pH regulation (Mann et al., 2008b, Chow and Benson, 1979, Mitsunaga et al., 1986, Livingston et al., 2006), the adhesion protein, KirrelL, which is crucial for *filopodial fusion* of skeletogenic cells (Ettensohn and Dey, 2017), and P16 and P19, proteins with a poorly characterized function that are crucial for sea urchin larval skeleton elongation and have been implicated in skeletogenesis across echinoderms (Dylus et al., 2018, Cheers and Ettensohn, 2005, Costa et al., 2012). It is the downstream differentiation genes which are responsible for the process of *morphogenesis*, and in this way, they provide a direct link between the regulatory transcription factors and signaling molecules, and animal morphology.

Though there remains more work to be done characterizing the suites of genes expressed in echinoderm skeletogenic cells at wide phylogenetic scales, what has been done so far shows that the GRN contains hundreds of genes, many with shared and distinct functions (Shashikant et al., 2018). How the suite of expressed genes, and their functions, evolve, provides novel insight into how morphologies are likely to have evolved in both shallow and deep time.

5. Evolution of the echinoderm skeleton

5.1 What Can We Learn From The Fossil Record?

The exceptional echinoderm fossil record provides unparalleled insights into phenotypic evolution, providing clues as to the potential operation of GRNs in deep time. Though it is impossible to know with certainty the genomic regulatory networks and patterns of gene expression that were present in extinct taxa, the fossil record can provide insight into the phenotypic patterns of evolution that are inherently the morphological outcomes of the activity of gene expression and regulation. This in turn can provide insight into possible molecular scenarios underlying morphological evolution seen in deep time, and the fundamental evolutionary patterns used to generate hypotheses concerning GRN evolution.

5.1.1 Macroevolutionary Trends In The Evolution Of Echinoderm Body Plans

A recent example where the fossil record of echinoderms has been used to understand the macroevolutionary consequences of GRNs concerns the work of Deline et al. (2020) (Figure 6). GRNs have an inherently hierarchical structure, which has been proposed to underlie the differential evolvability of morphological characters whose development they direct (Erwin and Davidson, 2009, Peter and Davidson, 2015, Peter and Davidson, 2016, Peter and Davidson, 2017, Davidson and Erwin, 2006). Davidson and Erwin (2006) hypothesized that the hierarchical position of genes and GRN subcircuits within a developmental GRN may have corresponded to the morphological characters whose development they direct. They hypothesized that downstream differentiation genes at the periphery of a GRN are responsible for the evolution of species level characters, while the tightly and recursively wired subcircuits of transcription factors expressed earlier in development control phenotypic characters manifested at the phylum and class levels, such as symmetry, or the presence or absence of limbs. This hypothesis is an

extension of the work of Riedl (1977), who proposed the concept of differential and hierarchical evolvability of phenotypic characters underlain by a concept he termed “burden”. Reidl’s “burden” reflects the hierarchically arranged interdependence of organismal characters, and was invoked as an explanation for why some characters, those which define organismal body plans, were conserved across large animal groups, while others, those with less burden, appear to show higher rates of phenotypic evolution (Riedl, 1977, Schoch, 2010).

Using a large matrix of morphological characters from Cambrian and Ordovician echinoderms, Deline et al. (2020) analyzed morphological disparity of the echinoderm skeleton (Figure 6) and used estimates of phylogenetic signal to evaluate patterns of morphological evolution during the initial burst of echinoderm morphological diversification. To assess the relationship between character burden and evolvability, characters were coded based upon the number of morphological characters contingent upon their presence or absence in the character matrix. This value was then compared to the phylogenetic signal of each character, i.e. the phylogenetically based tendency for closely related taxa to have more similar traits to each other than to taxa they are less related to (Borges et al., 2019, Pagel, 1999). Their analyses revealed no clear relationship between phylogenetic signal and number of contingent characters, indicating that the phylogenetic distribution of characters does not seem to be directly related to the burden, or hierarchical rank of characters. This result countered the predicted model of Riedl (1977), and the extension to GRN theory proposed by Davidson and Erwin (2006), suggesting that characters with a high burden, which are thought to be conserved and relatively impervious to evolutionary change, are in fact not so. This implies not only that these body plan level characters are more evolvable than expected, but also that the hierarchical nature of the GRNs directing their development are not having a directly hierarchical effect on the evolution of morphology.

552

553 5.1.2 Reduction Of The Holothurian Skeleton

554 Extant holothurians are characterized by a highly reduced skeleton relative to other
555 echinoderms (Figure 7) (Smith and Reich, 2013). While most other extant and fossil
556 echinoderms have a robust calcium carbonate skeleton, the skeleton of most crown group
557 holothurians is comprised primarily of microscopic calcium carbonate spicules embedded in
558 their body wall (Stricker, 1986, Stricker, 1985, Woodland, 1906, Woodland, 1907b, Woodland,
559 1907a). The morphological transitions leading to the reduction of the holothurian skeleton are
560 well documented in the fossil record (Figure 7) (Smith and Reich, 2013, Rahman et al., 2019).
561 Phylogenetic analyses have consistently identified the ophiocistioids, echinozoans with a mix of
562 characters found in both crown group echinoids and crown group holothurians, as members of
563 the holothurian stem group (Smith, 1988, Smith and Reich, 2013, Rahman et al., 2019). Like
564 many echinoids, most known ophiocistioids in the fossil record have a skeleton composed of
565 large imbricating $CaC0_3$ plates. In addition to having large embedded plates in their body wall,
566 ophiocistioids also have a jaw apparatus not unlike that of an echinoid's Aristotle's lantern, and
567 large plated tube feet, similar to those found in bothriocidaroid echinoids and some somasteroid
568 asterozoans (Reich and Smith, 2009, Jell, 1983, Shackleton, 2005). In contrast to echinoids, and
569 similar to the crown group holothurians, the Rotasaccidae, a group of ophiocistioids, have
570 reduced much of their skeletons to small wheel-like spicules and have a predominantly soft body
571 wall (Figure 7) (Haude and Langenstrassen, 1976, Reich, 2010). More crown-ward stem group
572 holothurians, such as the Devonian *Palaeocucumaria*, also have reduced skeletons and largely
573 unplated bodies, while also having plated tube feet similar to those found in the ophiocistioids
574 (Smith and Reich, 2013).

The fossil record provides a clear record of the morphological transitions underlying the evolution of the holothurian body plan (Figure 7). This record also informs on testable hypotheses regarding the genomic and molecular basis for the reduction of the holothurian skeleton. Many of the transcription factors expressed during the development of the echinoderm skeleton seem to be largely conserved across classes and life history stages (Erkenbrack and Thompson, 2019, Dylus et al., 2018). For instance the transcription factor *Alx1*, known to be a key regulator of downstream biomineralization genes in *S. purpuratus* (Figure 5) (Rafiq et al., 2012), is expressed in the skeletogenic cells of the larval holothurian *A. parvimensis* (McCauley et al., 2012). Though the larval and adult skeletons are distinct, this suggests that at least some of the transcription factors at the core of the skeletogenic GRN in holothurians are conserved across other eleutherozoan clades. Conversely, comparative analyses of genome content across ambulacrarians have shown that while many of the signaling pathways and transcription factors are conserved, the holothurian *A. japonicus* has relatively fewer differentiation genes implicated in biomineralization, such as members of the MSP130, C-lectin, and carbonic anhydrase families, than *S. purpuratus*, the asteroid *A. planci* or the hemichordate *S. kowalevskii* (Zhang et al., 2017). This indicates that the reduction of the skeleton in holothurians, which took place along the holothurian stem lineage in the ophiocistioids (Rahman et al., 2019, Smith and Reich, 2013), may have been underpinned by a reduction in the number of downstream skeletogenic genes, and not associated with the loss or reduced expression of transcription factors.

5.2 What Can We Learn From Comparative Analyses In Extant Taxa?

Key to understanding the evolution of gene regulatory networks is to understand conserved and divergent aspects of their topology across differing phylogenetic distances. In

order to do this, comparative data on gene expression and gene function is necessary from a wide array of taxa. This is a serious roadblock in evolutionary developmental biology, where the generation of data from within a single taxon takes months and years of, often difficult, experiments. Because the GRN of *S. purpuratus* has been identified in such precise detail, however, comparative studies with other echinoderms were amongst the first to understand conservation and divergence in GRNs over vast evolutionary distances (Hinman et al., 2003), and echinoderms have become an ideal model system for evolutionary comparisons of gene regulatory networks. Gene expression data exists in embryonic or larval development for all five extant classes of echinoderms and comparisons of gene expression and function across and within these classes are providing a view of gene regulatory network evolution at multiple scales within the phylum echinodermata. As opposed to cross phylum comparisons, where homologous embryonic structures are difficult to pin-point, echinoderms fall in a sweet spot. Their embryos are evolutionarily divergent enough to show distinct differences in cell types, gene expression, and morphological structures, yet not too morphologically distinct that cell and tissue types cannot be easily recognized as homologous given multiple criteria. Below I will outline a number of cases where comparative analyses of the gene regulatory basis of echinoderm skeletogenesis have provided insight into the evolution of gene regulatory networks in deep time.

5.2.1 Evolution Of Divergent Mechanisms Of Cell Specification

Comparative analyses of the divergent gene regulatory networks across echinoids and other echinoderm outgroups have provided an unparalleled resource with respect to phylogenetic breadth for understanding the pace of, and mechanisms underpinning, the evolution of development. In euechinoid echinoids, the skeletogenic GRN is activated in the skeletogenic

cells through the activity of a GRN subcircuit called the double-negative gate (DNG). This molecular mechanism is so called because it involved the repression of one transcription factor acting as a repressor, by another, resulting in the activation of genes under the control of the second repressor. In particular, in well-studied euechinoids, the expression of key skeletogenic transcription factors is regulated by a double-repression mechanism (Figure 8a)(Revilla-i-Domingo et al., 2007, Oliveri et al., 2008). At the 16-cell stage embryo, the transcription factor *Pmar1*, a transcriptional repressor, is expressed in the micromeres, four cells located at the vegetal pole (bottom) of the embryo (Oliveri et al., 2002). Later in development, the transcription factor *HesC*, also a repressor, is expressed in all cells of the embryo, except for those cells where *Pmar1* was expressed earlier in development. Because Pmar1 is a repressor of *HesC*, in those cells where *Pmar1* was expressed earlier in development, *HesC* is not expressed at the later blastula stage. Also during the blastula stage, the key skeletogenic transcription factors *Alx1*, *Ets1*, and *Tbr*, and the signaling molecule *Delta* are all expressed in the same cells where *Pmar1* was expressed at the 16-cell stage (Revilla-i-Domingo et al., 2007, Oliveri et al., 2008). These genes (*Alx1*, *Ets1*, *Tbr* and *Delta*) are under repressive control of HesC, and thus the activity of Pmar1, which repressed *HesC*, results in their expression, and the specification of the skeletogenic cells that build the larval skeleton. In contrast to identified euechinoid embryos, aspects of the mechanism specifying skeletogenic cells in cidaroid echinoids are markedly different (Erkenbrack and Davidson, 2015, Yamazaki et al., 2014, Yamazaki et al., 2020). Crucially, HesC does not repress *Ets*, *Tbr*, or *Delta* (Figure 8b).

The double-negative gate is one of the best characterized GRN subcircuits with respect to breadth of phylogenetic sampling across the echinoderms, and thus provided an ideal model to determine how conserved the genetic regulatory mechanisms specifying the euechinoid

skeletogenic cell actually are, and to determine the antiquity of this molecular character. Thompson et al. (2017) coded the presence of the Pmar1-HesC double-negative gate for all echinoderm taxa where the data was available as of 2017 based on the presence or absence of gene expression, as well as inferences of regulatory interactions (e.g. a gene acting as a repressor is not likely to be co-expressed in the same cells as a gene it is repressing). Using time-calibrated phylogenetic trees of echinoids and other echinoderm outgroups, they then used ancestral state reconstruction to infer the probability that the Pmar1-HesC DNG was present or absent at particular ancestral nodes within the echinoids. These analyses showed that the DNG was likely responsible for specifying skeletogenic cells in the MRCA of euechinoid echinoids, and with a lesser probability at the MRCA of crown group echinoids. This work demonstrated that the Pmar1-HesC double-negative gate was probably present in the most recent common ancestor of crown group echinoids, and thus that this particular GRN subcircuit had an origin in at least the Late Palaeozoic, and has been a largely invariant character throughout the course of crown group echinoid evolution. Recent work by Yamazaki et al. (2020), has continued to build on our understanding of the evolution of skeletogenic cell specification. Using transcriptomic analyses, they were able to identify the *Pmar1* gene in the cidaroid *Prionocidaris baculosa* and carried out *over-expression* experiments (injection of excess mRNA into the egg) and knockdown experiments to determine its function. These experiments showed that Pmar1 did not repress *HesC* in cidaroids, however, when *Pmar1* mRNA was injected into *P. baculosa* embryos, *Alx1*, *Ets1* and *Tbr* all showed upregulation, indicative of a double-repression mechanism. Thus, while there is a double-repression mechanism in cidaroids, the second repressor is not *HesC* as is the case in *S. purpuratus* and other euechinoids, and its identity remains unknown (Figure 8b). Instead, *HesC* is positively regulated by *Delta*, which is itself under the control of the double-

667 repression mechanism (Yamazaki et al., 2020). In asteroids, where there is no *Pmar1*, or even
668 skeletogenic cell lineage, the regulatory topology present is even more different (Figure 8c)
669 (Cary et al., 2020, Yamazaki et al., 2020).
670 This case study concerning the evolution of the DNG informs on the mechanisms by
671 which GRNs underlying development evolve more generally. The divergence seen in
672 developmental mechanisms in cidaroids and euechinoids identified by Yamazaki et al. (2020) is
673 a clear example of the principle of *developmental systems drift* (True and Haag, 2001).
674 Developmental systems drift is the idea that two morphologically homologous structures can
675 develop via divergent molecular or regulatory mechanisms (True and Haag, 2001, Wang and
676 Sommer, 2011). The morphological features that comprise an organism's phenotype are those
677 that interact with the environment, and are thus under direct selective pressure. The genetic
678 regulatory networks and developmental pathways which encode for morphology, however, are
679 not. So long as the morphological structure remains the same, the molecular pathways expressed
680 during its development, and in particular the regulatory interactions between genes, can vary.
681 This is evident in cidaroids, where an unknown repressor regulates *Delta, Ets1, Tbr* and *Alx1* in
682 skeletogenic cell specification as opposed to HesC (Figure 8). Because the particular genes
683 which are expressed in development bear little relevance on the morphological outcome, they are
684 able to swap places during the course of evolution. So long as skeletogenic cells are specified, it
685 makes little difference whether HesC or the still unknown repressor, is the second repressor of
686 the double-repression mechanism. Within the context of these novel results from Yamazaki et al.
687 (2020) the ancestral state reconstructions of Thompson et al. (2017) also shed light on the
688 timescales over which the effects of developmental systems drift are visible. Comparisons of two
689 nematode species have identified evolutionary changes due to developmental systems drift on

timescales of 250–420 Ma (Wang and Sommer, 2011). The taxonomically more expansive analyses of the double-negative gate indicate that in echinoids, the drift from the cidaroid condition of Pmar1-unknown repressor to the euechinoid condition of a Pmar1-HesC double-repression mechanisms likely took place prior to the early Mesozoic, on par with the time scales suggested by nematodes. As comparative analyses in more taxa are carried out, the prevalence of developmental systems drift, and the timescales over which its effects are evident, will become clearer. Echinoderms, with their wealth of comparative data on gene expression and function, are well suited to play a part.

5.2.2 Skeletogenic Cell Evolution

Tightly tied to the concept of gene regulatory networks, is the concept of cell types. During development, numerous distinct cell types are specified and differentiate, giving rise to the multitude of tissues, including muscular, nervous, and skeletal, that are present across the body plans of animals. Throughout evolution, novel cell types evolve, giving rise to new tissues, morphological structures, and cellular functions, and leading to both increases and decreases in animal complexity (Arendt, 2008, Valentine et al., 1994, Arendt et al., 2016). While there are numerous definitions for exactly what defines a cell type, the definition used herein is that following Arendt et al. (2016), where a distinct set of transcription factors present in different cells are used to delineate different cell types.

Much controversy has surrounded the origin of the larval skeletons of echinoderms. In particular, the question as to whether or not the larval skeletons of echinoids and ophiuroids are homologous, or the product of convergent evolution, has pervaded the literature (Smith, 1997). The origin of this skeleton can be understood, however, by comparative analyses of the cells that

build it. Using a spatial dataset of transcription factor and signaling receptor expression, Erkenbrack and Thompson (2019) used ancestral state reconstructions to infer the likely ancestral states of skeletogenic gene expression in eleutherozoans. These analyses showed that the skeletogenic cells of ophiuroids and echinoids were, in fact, homologous features, based on reconstructed gene expression supporting the expression of *Alx1*, *Ets1*, and *VegfR* in skeletogenic cells in the eleutherozoan most recent common ancestor (Figure 9). Additionally, the analyses showed that *Tbr*, a transcription factor necessary for skeletogenesis in *S. purpuratus*, only became expressed specifically in skeletogenic cells recently in the evolutionary history of echinoids, in the MRCA of camarodont echinoids (Figure 9). Tbr has far fewer transcriptional inputs into skeletogenic differentiation genes than Ets1 and Alx1 in *S. purpuratus* (Rafiq et al., 2012), and functional knockdown of *Tbr* in non-camarodont echinoids does not appear to effect skeletogenic cell differentiation (Yamazaki et al., 2014). This suggests that the "shallower" regulation of skeletogenic genes by Tbr, as seen in *S. purpuratus*, may be due to its more evolutionarily recent role in skeletogenesis. This lays out testable predictions for analyses of GRNs in other animal groups; namely that some more evolutionarily recent additions to gene regulatory networks may have transcriptional inputs into fewer downstream genes than more evolutionarily ancient members of those GRNs. This would suggest that evolutionarily older genes in a network might have more time to accumulate new transcriptional targets due to mutations, selection, or drift. This might be expected to happen when functionally redundant or similar differentiation genes in the same network, such as two different genes both involved in skeletogenesis, come under the transcriptional regulation of the same upstream transcription factor due to a mutation in a regulatory element. These insights into the timescale of gene regulatory network and cell type evolution can only come about through comparative analyses of

multiple taxa spanning wide phylogenetic distances, making echinoderms the ideal model system for evolutionary studies of this kind.

6. Open Questions And Future Directions For Echinoderm Molecular Paleobiology

6.1 Adult Body Plan Development

A current limitation to molecular palaeobiological studies in echinoderms is the disconnect between the vast literature concerning molecular aspects of echinoderm development, and research on the echinoderm fossil record. This disconnect largely exists due to the biphasic lifestyle of echinoderms. While the echinoderm fossil record consists almost entirely of fossilized post-metamorphic or directly developed animals, the majority of studies of developmental gene expression, gene regulation and protein localization in echinoderms are focused on the embryonic and larval stages of indirect-developing echinoderms, which have virtually no fossil record, and a very limited preservation potential. Recent work is attempting to bridge this gap, and work on the development of post-metamorphic and juvenile echinoderms is shedding novel light onto the evolution of the adult body plan.

6.1.1 The Origin Of Symmetry

Perhaps the most obvious molecular palaeobiological question to still be answered within the echinoderms, is that concerning the developmental and genomic basis of the bizarre, enigmatic, pentaradial symmetry which characterizes members of the echinoderm stem and crown groups. The fossil record tells us that echinoderms have displayed varying forms of symmetry throughout their evolutionary history, from the bilaterally symmetrical *Ctenoimbricata* to the asymmetrical solutes, the triradial helicoplacoids and the pentaradial forms of more crown-

759 ward members (Figure 10a-e) (Zamora and Rahman, 2014). While, as bilaterians, the ancestral
760 symmetry was likely bilateral, all extant echinoderms display distinct five-fold symmetry.
761 Though the fossil record displays clear transitions in echinoderm body plans leading to the extant
762 pentaradial forms (Sumrall and Wray, 2007, Zamora and Rahman, 2014), there still remains little
763 understanding of molecular mechanisms underlying the development of the adult body plan in
764 echinoderms.

765 Crucial to understanding the evolution of echinoderm symmetry, is understanding the
766 identity of body axes in echinoderms. During extant echinoderm growth and development, the
767 first morphological structure to show the characteristic five-fold symmetry of echinoderms is the
768 *hydrocoel*, which forms as five lobed outgrowths from one of the coeloms (Morris et al., 2009,
769 Morris, 2011, Morris, 2012). In developing echinoids and asteroids, arrangement of the
770 hydrocoel and other coeloms relative to the mouth supports the idea that the adult oral-aboral (or
771 dorsal-ventral) axis may be equivalent to the anterior-posterior axis of other bilaterians (Morris,
772 2011, Morris, 2007, Morris et al., 2009, Peterson et al., 2000). During development of the adult
773 body plan, indirect developing echinoderms undergo a coelomic re-arrangement, which results in
774 a linear stacking of their coeloms in the adult body plan (Peterson et al., 2000). This stacking
775 results in the location of the left hydrocoel tissues (including the water vascular system)
776 surrounding the mouth, and stacked more adoral than the left and then right *somatocoels*. The
777 expression of posterior *Hox* genes in the somatocoels has been taken as evidence for their
778 posterior identity, which lead Peterson et al. (2000) to interpret the anterior-posterior axis as
779 passing from the mouth, through to the stacked coeloms. This model thus has the mouth,
780 hydrocoel, left somatocoel, and right somatocoel arranged from anterior to posterior, and

781 interprets the five rays of crown group echinoderms as outgrowths from the anterior-posterior
782 axis analogous to the limbs of arthropods and vertebrates.
783 Another hypothesis suggests that the metameric organization of echinoderm rays, such as
784 the arms of asterozoans (Czarkwiani et al., 2013) and the ambulacral and interambulacral tissues
785 and plating of echinoids (Morris, 2009, Morris and Byrne, 2005, Morris and Byrne, 2014), are
786 homologous with the single anterior-posterior axis of chordates and other bilaterians (Morris,
787 2012). This scenario thus implies that the pentameral arrangement of the echinoderm body plan
788 resulted from the duplication of a single anterior-posterior axis up to five times. This hypothesis
789 is rooted on the position of a growth zone of terminal addition, from which new axial tissues are
790 added in a metameric fashion during growth (Morris and Byrne, 2005, Morris et al., 2009,
791 Morris and Byrne, 2014). This is similar to the Ocular Plate Rule (OPR), which asserts that new
792 axial tissues grow via terminal addition from a growth zone (Mooi and David, 1994, Mooi et al.,
793 2005), though Mooi et al. (2005) explicitly did not consider manifestations of the OPR as
794 examples of metamerism. Building upon these interpretations, the framework of Minsuk et al.
795 (2009) interpreted echinoderm rays as five proximal-distal axes, as opposed to explicit
796 duplications of the anterior posterior axis, or outgrowths from a single anterior-posterior axis.
797 Gene expression patterns and analyses of genomic content and organization have
798 attempted to bring clarity to the question of the echinoderm anterior-posterior axis, and the origin
799 of the pentaradial body plan. Early analyses of the genome of *S. purpuratus* showed that the Hox
800 cluster, the set of closely related transcription factors that are known to specify axial identity
801 along the anterior-posterior axis across a wide array of animal groups (Mallo et al., 2010), had
802 undergone a *translocation* (re-arrangement of gene order within along a chromosome) with the
803 order of the Hox genes in the genome re-arranged relative to vertebrates, arthropods and other

804 animals (Martinez et al., 1999, Arenas-Mena et al., 2000, Cameron et al., 2006). Subsequent
805 interpretations have postulated that this translocation of Hox genes relative to their ancestral
806 *collinearity* (matched location of genes on the chromosome relative to their axial expression
807 during development) may have been associated with the pentaradial symmetry seen in the
808 echinoderm crown group (Mooi and David, 2008, David and Mooi, 2014). One of the first
809 published expression patterns for a hox gene during the formation of the echinoderm adult body
810 plan, *Hox3*, was expressed in a pentaradial pattern in the dental sacs of the echinus rudiment in *S.*
811 *purpuratus* and the first high levels of multiple Hox gene expression during *S. purpuratus*
812 development coincide temporally with rudiment formation (Arenas-Mena et al., 1998). The
813 expression of the five posterior-most Hox genes in the somatocoels of *S. purpuratus* revealed a
814 collinear arrangement to their expression in the coelomic mesoderm, where the arrangement of
815 the expression patterns of these genes corresponds with their arrangement in the genome
816 (Arenas-Mena et al., 2000). In *S. purpuratus*, the posterior Hox genes are expressed in the
817 coelomic mesoderm of the left and right somatocoels. The expression patterns of these genes are
818 co-linear, with *Hox11/13b* being expressed in the most posterior tissues of the somatocoel and
819 *Hox7* in the most anterior, in a curved stripe which corresponds to the curvature of the larval gut
820 (Arenas-Mena et al., 2000). The co-linearity of *hox* expression was furthermore identified during
821 development of the crinoid *Metacrinus rotundus*, where *Hox5*, *Hox7*, *Hox8* and *Hox9/10* were
822 found to be expressed in a linear pattern along the length of the somatocoels (Hara et al., 2006).
823 Subsequent work in the direct developing echinoid *Holopneustes purpurescens* showed the
824 expression of the posterior-most Hox gene *Hox11/13* in the posterior tissues of the *vestibule*,
825 while the more anterior Hox genes *Hox5* and *Hox3* in more anterior tissues of the epineural folds
826 and coelomic mesoderm respectively (Morris and Byrne, 2005, Morris and Byrne, 2014). An

anterior to posterior expression of Hox genes has also been identified in the somatocoel of the direct developing sand dollar *Peronella japonica* (Tsuchimoto and Yamaguchi, 2014). More recently, the expression patterns of eight Hox genes were surveyed during the pentactula stage of the holothurian *Apostichopus japonicas*, and found to be expressed along the *endodermal* tissues of the digestive tract, albeit in a co-linear fashion as in echinoids and crinoids (Kikuchi et al., 2015).

Though Hox genes have helped to elucidate the orientation of the anterior-posterior axis in echinoderms, their hypothesized role in patterning the pentaradial body plan of echinoderms has been refuted by genomic data (Figure 10f). As genomic information has become more widespread across echinoderms, and advances in sequencing technology have made interrogating echinoderm Hox clusters easier, it has come to light that the translocation of the Hox cluster seen in *S. purpuratus* has not been found in any non-echinoid echinoderms (Zhang et al., 2017, Li et al., 2020, Davidson et al., 2020, Baughman et al., 2014). This may suggest that the translocation of the Hox cluster seen in echinoids may be a synapomorphy of the class, or at least the camarodont echinoids in which the hox translocation has been identified (Davidson et al., 2020). Given that most echinoderms surveyed have ancestrally ordered hox clusters, the hypothesis that Hox cluster translocation is associated with pentaradiality has confidently been refuted (Figure 10f) (Byrne et al., 2016, Li et al., 2020).

In addition to the Hox genes, the recent work surveying the spatial expression patterns of components of two signaling pathways, the BMP and Nodal pathways, have begun to inform on the development of the pentaradial echinoderm body plan. Nodal is involved in patterning dorsal-ventral axes and left-right asymmetry in numerous animal groups, including larval sea urchins (Molina et al., 2013). In development of the juvenile rudiment of *H. erythrogramma*,

Nodal is expressed in the right ectoderm, while *BMP2/4* was expressed in the left ectoderm in the presumptive vestibular ectoderm, which forms part of the rudiment (Koop et al., 2017). The downstream target of Nodal in embryonic sea urchin development, *BMP2/4*, is also expressed in the hyrdocoel lobes (Koop et al., 2017), which will become sheathed in the vestibule to form the primary podia. Additionally, the transcription factors *Msx*, *Dach*, *Six1/2*, *Six3/6* and *Pax6*, putative downstream targets of BMP signaling, are expressed in developing hydrocoel lobes, podia and spines, indicating a role in development of some metameric axial structures of the ambulacral system (Koop et al., 2017, Byrne et al., 2018). Some of these genes are also expressed in the tube feet of the post-metamorphic asteroid *Parvulastra exigua* (Byrne et al., 2020). Though the implication of particular genes in the growth and development of ambulacra is an exceptional step forward in understanding adult and juvenile sea urchin development these structures are formed though after the pentaradial body plan has already been patterned, thus they may not be involved in the process of establishing pentamery (Koop et al., 2017).

6.1.2 The Molecular Basis For Differential Evolvability

Because of their excellent fossil record and molecular resources, echinoderms provide an ideal group to examine differential morphological divergence and constraint in the fossil record, and to attempt to understand the molecular mechanisms underlying differential morphologies in extant taxa. As already mentioned, fossil echinoderms have been a classical model system for understanding morphological disparity, beginning with the work of Foote (1991), Foote (1992) and leading up to more recent treatments from Deline and Ausich (2011), Deline et al. (2020), Hopkins and Smith (2015) and Wright (2017). With the wide array of molecular and genomic tools readily available for echinoderms, including those which can be used to functionally

interrogate the development of the adult body, exciting opportunities are developing to understand not only patterns in morphological diversification within the echinoderms, but also the molecular mechanisms that underlie these morphological differences in body plans. An ideal model system for this work is the crown group echinoids. The crown group echinoids provide a prime example of differential morphological divergence and constraint. The regular echinoids, including (among others) cidaroids, camarodonts, and diadematoids, have exhibited striking morphological constraint throughout their evolutionary history, and the earliest regular echinoids in the fossil record appear very morphologically similar to cidaroids in the oceans today (Thompson et al., 2015). In contract, the irregular echinoids, which evolved from regular echinoid ancestors in the early Jurassic (Saucéde et al., 2007), have undergone extreme morphological diversification since their divergence from the regular echinoids exploring novel morphospace, evolving secondary bilateral symmetry, and exhibiting high morphological disparity (Hopkins and Smith, 2015, Mongiardino Koch and Thompson, 2020a, Boivin et al., 2018).

Crown group echinoids are the ideal group to examine the molecular underpinning of this differential morphological diversification because not only because of the striking differences in morphospace utilization between regular and irregular echinoids, but also because they are the most experimentally tractable model system for functional analyses of adult body plan growth and development. Understanding the differential genomic and cellular mechanisms involved in the development of regular and irregular echinoids will require careful choice and trade-offs between of phylogenetically informative taxa, and experimentally tractable animals. Recent experimental progress on the development of the direct developing echinoids with a short time (a matter of days) from fertilization to adult body plan formation such as *Heliocidaris*

erythrogramma (Edgar et al., 2019a, Edgar et al., 2019b, Wang et al., 2020, Koop et al., 2017) make these animals prime candidates for understanding the molecular mechanisms underpinning adult body plan growth in regular echinoids. Amongst the irregular echinoids, the facultatively direct developing clypeasteroid *Clypeaster rosaceus* is a potential ideal choice for experimental work in the post-metamorphic body plan of an irregular echinoid, with readily available transcriptomic resources (Armstrong and Grosberg, 2018), and a relatively short time between fertilization and adult body plan development. Recent work examining skeletogenic cell gene expression in regenerating ophiuroids has shown that different suits of skeletogenic genes are expressed in different skeletal elements of the regenerating arm (Piovani et al., 2021). Whether or not different suites of skeletal genes may also be expressed in differential skeletal elements of regular and irregular echinoids may thus also provide insight into their differential evolvability. With novel hypotheses and experimental organisms, paired with new, robust, functional techniques to examine gene function such as CRISPR Cas-9 genome editing (Wessel et al., 2020, Liu et al., 2019, Yaguchi et al., 2020), uncovering the differential molecular mechanisms underpinning regular and irregular echinoid development, and thus the vast morphological differences in their morphology, are not far off.

7. Concluding Thoughts

Much new data on echinoderm development and evolution has come to light in the fourteen years since Bottjer *et al.* published "Paleogenomics of Echinoderms". I hope that I've herein shown the utility of echinoderms as an ideal model group for the integration of the fossil record and deep time, with data about gene expression, development, and gene regulatory networks. Beyond being a tractable model system, novel work from echinoderms are providing

fundamental new insight into how cell types, gene regulatory networks, and organismal morphology evolve. As comparative approaches involving dense and wide taxonomic sampling, explicit phylogenetic frameworks, and genomic resources become more commonplace in the study of developmental evolution, the rich datasets provided by echinoderms will surely provide even more insight in the coming decades.

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Figures

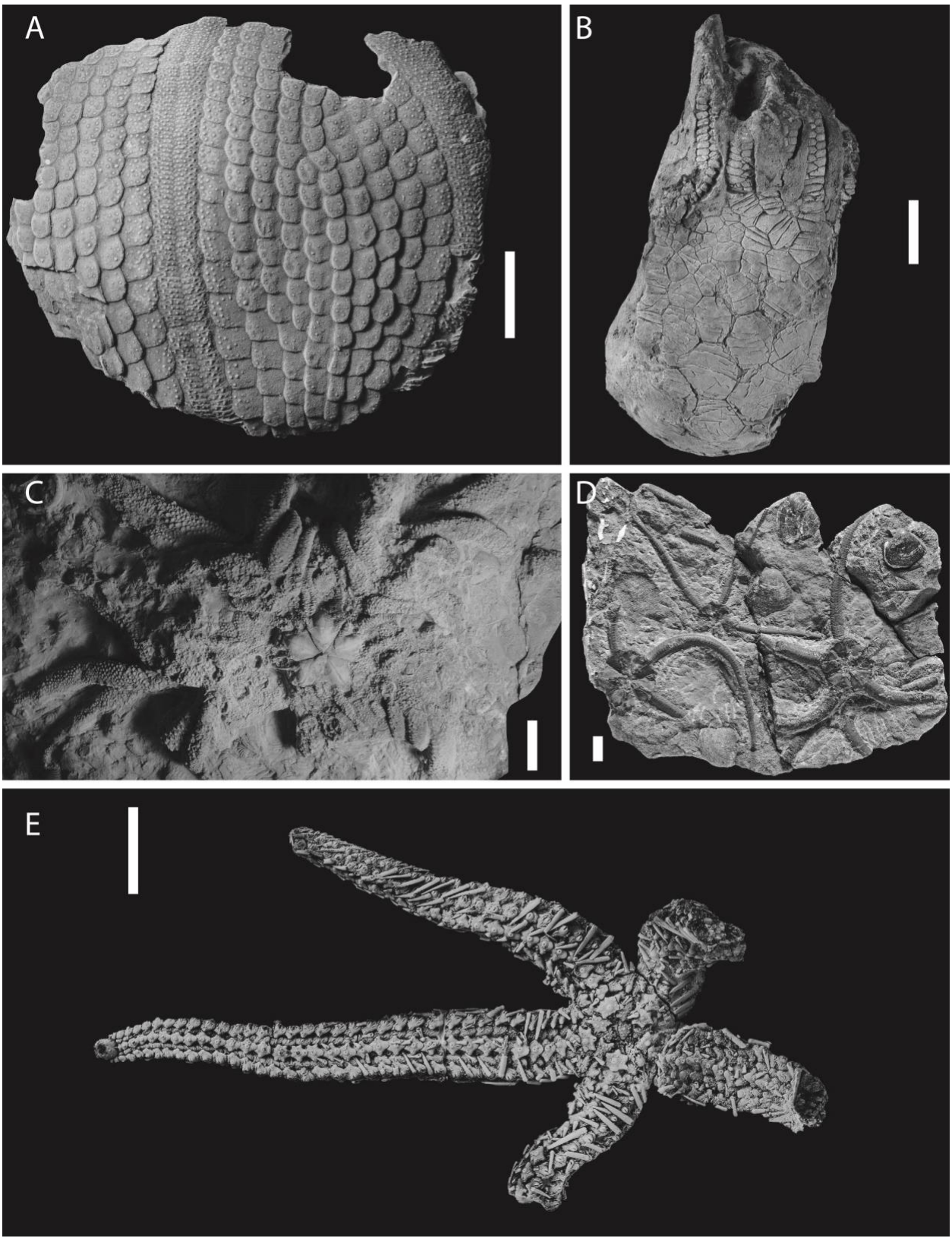

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Figure 1. Examples of the skeletons of adult echinoderms from the crown group classes. The biomineralized echinoderm skeleton is composed of numerous $CACO_3$ plates, which can be seen in fossil members of each of the extant classes. (A) the skeleton of the stem group echinoid *Pholodechinus brauni*. The test of stem group echinoids is made up of numerous columns of both ambulacral and interambulacral plates, most of which bore spines. (B) the stem group crinoid *Griphocrinus pirovanoi*. The crown of the animal includes the many calcified plates of the calyx, in addition to multiple arm plates. Modified from Thompson et al. (2013) (C) the biomineralized skeleton of the ophiocistioid stem group holothurian *Eucladia johnsoni*. Unlike crown group holothurians, this stem group member had a plated test consisting of numerous imbricating calcified plates, as well as plated tube feet and a central calcified jaw apparatus not unlike the Aristotle's lantern of echinoids. (D) Skeleton of the fossil ophiuroid *Palaeocoma milleri*. Modified from Ewin (2019). The fossil asteroid *Alkaidia sumralli* showing the many-plated morphology of the sea star skeleton. Courtesy of T. Ewin and A. Gale.

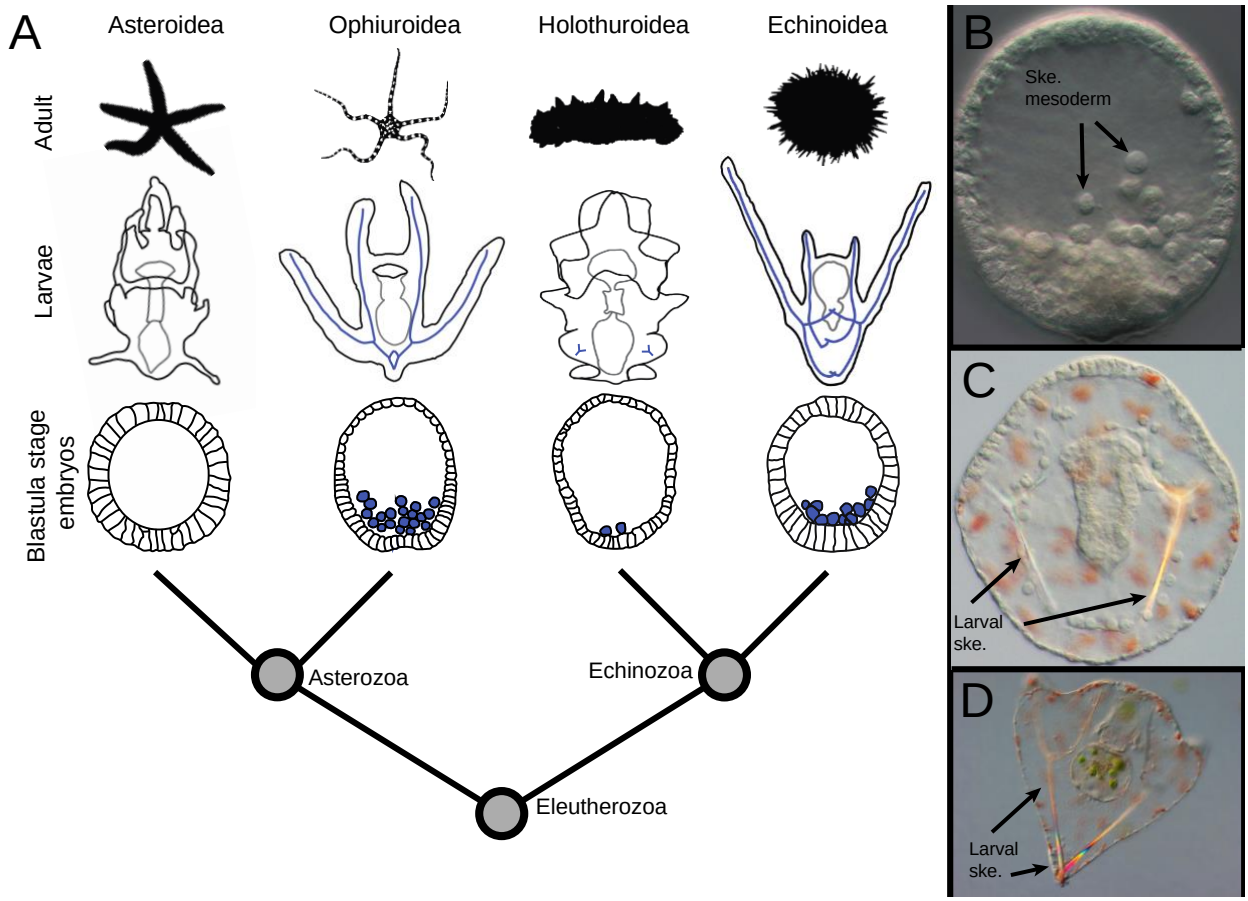


Figure 2. Eleutherozoan echinoderm larval skeletons. (A) shows the phylogenetic distribution of eleutherozoans echinoderm larvae and blastula stage embryos with skeletal mesodermal cells. Larval skeletons and skeletal mesodermal cells are shown in blue. Extensive larval skeletons are found in the ophiopluteus larvae of ophiuroids and the echinopluteus larvae of echinoids. Auricularia larvae of holothurians have a miniscule skeleton consisting of two small spicules found in posterior end of the larvae. The bipinnaria larvae of asteroids lack a larval skeleton, and a mesodermally derived skeletogenic cell lineage. (B) Skeletogenic mesodermal cells in mesenchyme blastula stage embryo of the echinoid *Strongylocentrotus purpuratus*. (C) Larval skeleton in prism stage embryo of *S. purpuratus*. There are two bilaterally arranged skeletal elements which are derived from triradiate spicules. (D) Larval skeleton in the pluteus larvae of

S. purpuratus. All larvae are found in indirect developing larvae. No known crinoid larvae are indirect developers, thus crinoids are excluded from the diagram. Ske; skeleton.

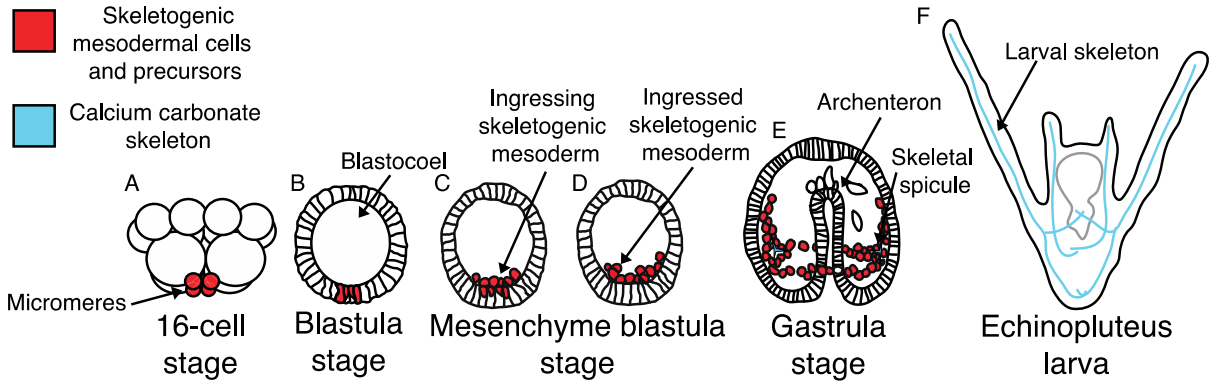
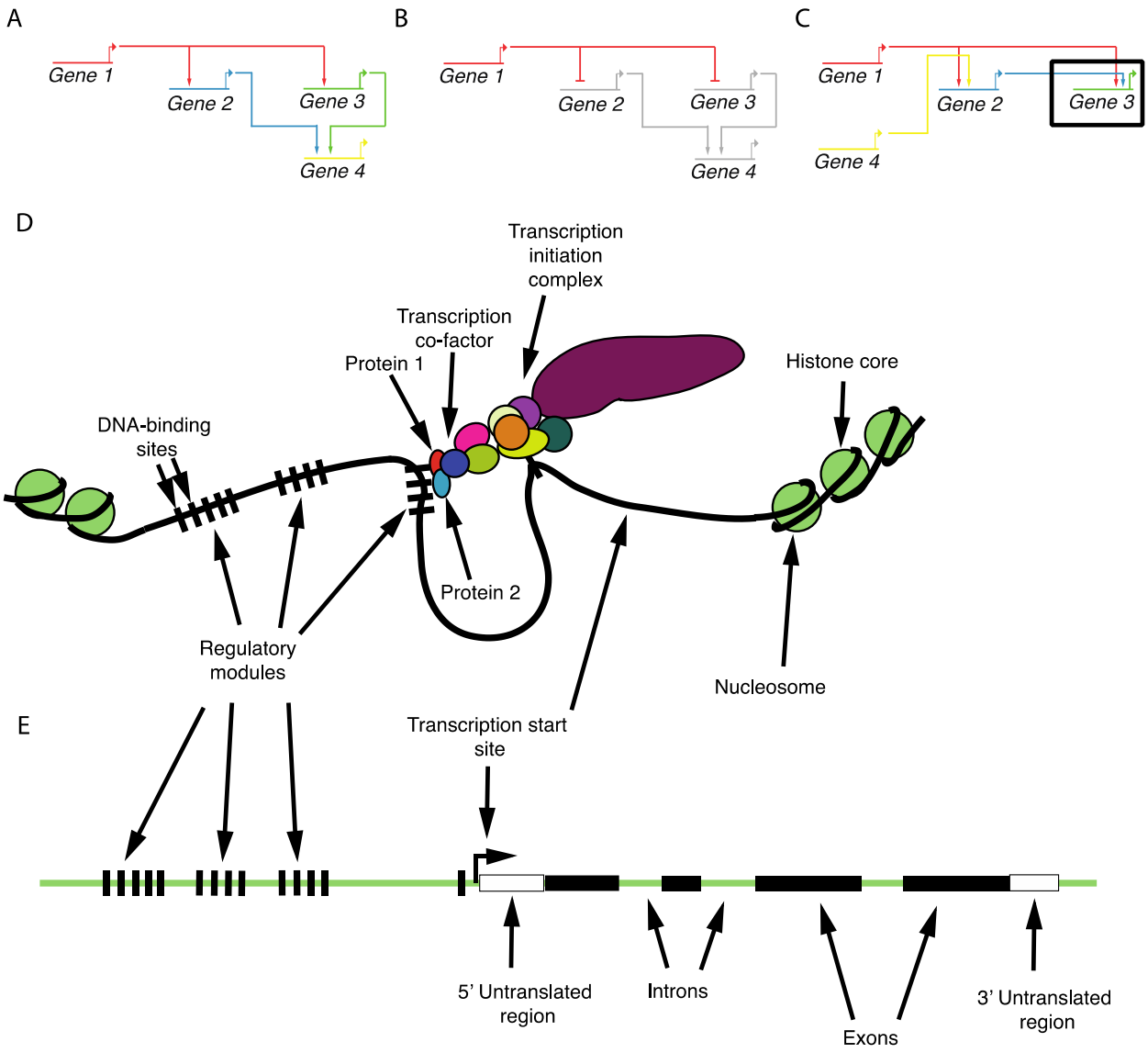


Figure 3. Schematic diagram showing select developmental stages of an indirect developing echinoderm larva. This diagram is based on that of a camarodont echinoid, and is, in many ways, representative of other skeleton-bearing echinoderm larvae. (A) shows the sixteen-cell stage, during which, in camarodont echinoids and most other known euechinoid echinoids, the cell lineage which will become the skeletogenic cells is first specified. The skeletogenic cells are derived from the four micromeres (shown in red), which are present at the vegetal pole (bottom) of the embryo. Later in development, (B) shows the blastula stage embryo which begins at the approximately 128 cell stage in the camarodont *Strongylocentrotus purpuratus*. The blastula consists of a sphere of cells surrounding an open cavity, called the blastocoel. In blastula-stage embryos, the cells which will give rise to the skeletogenic cells, and which have descended from the micromeres, are located in the vegetal pole of the embryo as part of the epithelium of the blastula wall (shown in red). (C) During the mesenchyme blastula stage, the skeletogenic cells migrate from the epithelial wall of the blastula into the blastocoel. This migration is known as an epithelial to mesenchymal transition, as the skeletogenic cells (red) which were part of the epithelium of the blastula, are now loose and mobile. All of the skeletogenic cells migrate into the blastocoel (D), where they will later secrete the biomineralized tri-radiate spicules of the skeleton. Later in development, (E) shows a gastrula stage embryo, at which point the

archenteron, which will eventually attach to the wall of the ectoderm to form the gut, has formed from an invagination in the vegetal pole of the embryo (the blastopore). During gastrulation, the skeletogenic cells (red) have arranged themselves into bilaterally symmetrical ventro-lateral clusters on either side of the embryo, and begun to secrete the tri-radiate spicules which will grow to form the larval skeleton (blue). (F) shows a simplified echinopluteus larvae. The skeletogenic cells are not shown, but the larval skeleton, which now comprises four elongate skeletal elements, can be seen in blue. Some differences, such as the presence of four distinct micromeres, do exist between camarodont echinoids and other echinoderms, such as cidaroid echinoids, which have a variable number of micromeres, and ophiuroids and holothurians, which lack any micromeres at all. Furthermore, as shown in Figure 2, there are also differences in the morphologies of the larvae and skeletons of other echinoderms. Diagrams in B-D are modified from Erkenbrack and Thompson (2019) and E is modified from McClay et al. (2020).



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1656 **Figure 4. Diagrammatic representation of gene regulatory networks (GRNs).** A-C show

1657 wiring diagrams showing simple gene regulatory networks. In both (A) and (B) there are four

1658 genes: Genes 1, 2, 3, and 4. The arrows indicate physical regulatory interactions, where a

1659 protein encoded for by each gene binds to DNA in a regulatory region of a downstream target

1660 gene. In (A), gene regulation is positive, where the binding of gene 1 to its downstream targets, 2

1661 and 3, results in an increase in their expression (upregulation). Genes 2 and 3 also positively

1662 regulate their downstream target, Gene 4. In (B), Gene 1 acts as a repressor, and results in the

repression of its downstream targets, 2, and 3, and in turn their target Gene 4. In (C) genes 1 and 4 both regulate gene 2, and gene two and gene 1 both regulate gene 3. This scenario is more representative of biological reality, as numerous transcription factors interact combinatorially to regulate gene expression during animal development. (D) shows a simplified diagram of the cis-regulatory region of gene 3 from (C) with the binding of the transcription factors encoded for by genes A and B binding to DNA-binding sites in their respective regulatory modules upstream of (before) the transcription initiation complex and the transcription start site. (E) shows the organization of gene 3. The cis-regulatory region, as shown in (D) is located upstream of the transcription start site and the transcribed portions of the gene. Exons are transcribed while introns are not. The 5' and 3' untranslated regions (so-named because of their position relative to the gene) will not be translated into the protein, but are transcribed. (E) an (D) are simplified from Wray et al. (2003) to which I refer the reader for a more in-depth discussion of transcriptional regulation.

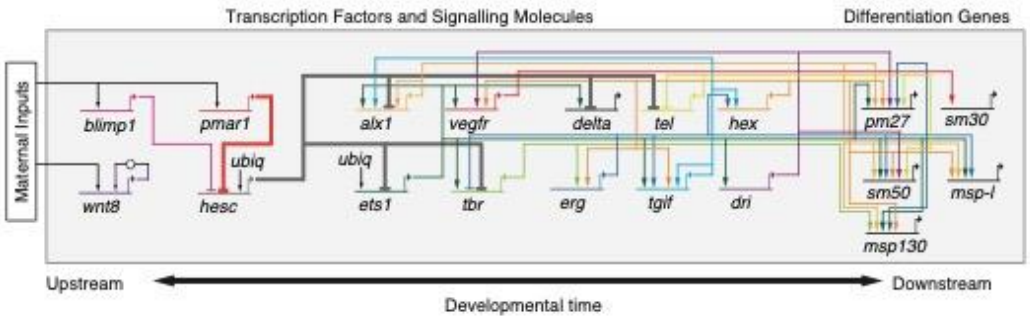
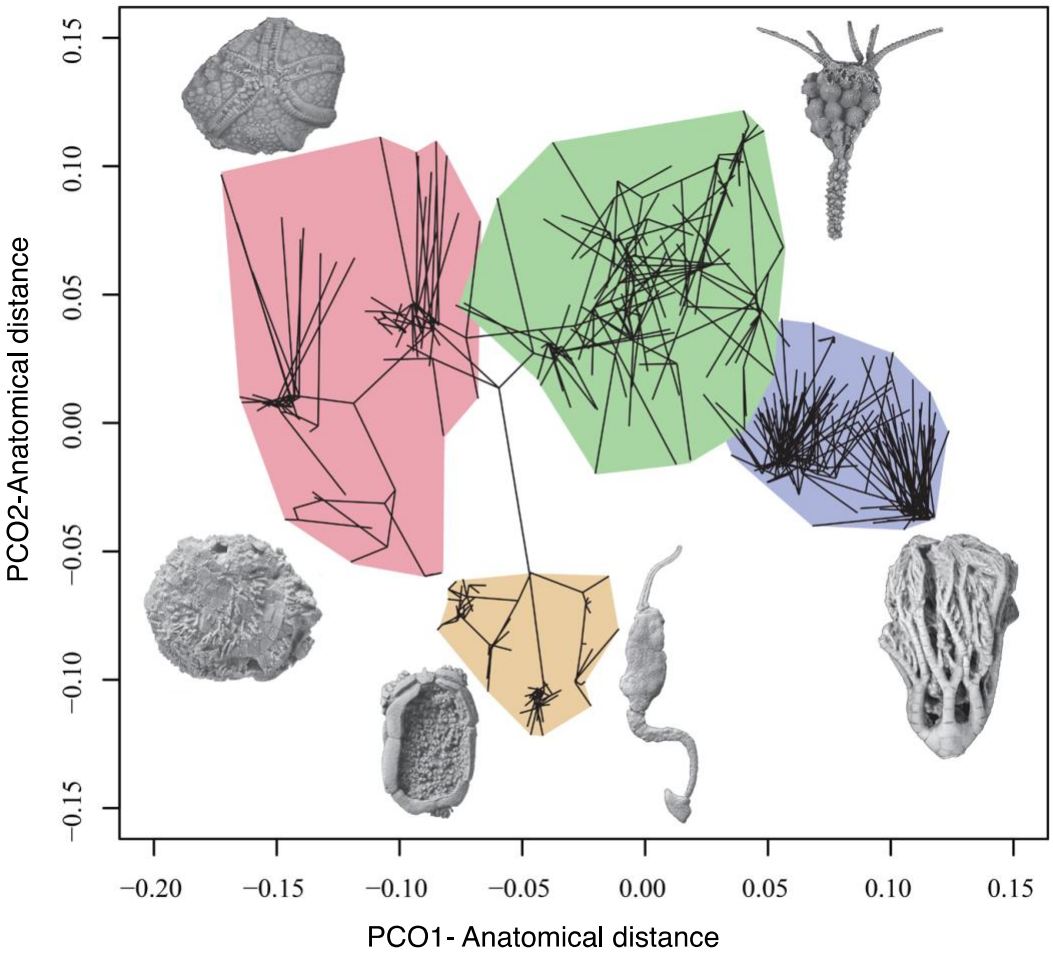


Figure 5. Simplified skeletogenic gene regulatory network from embryonic and larval *Strongylocentrotus purpuratus*. To the left are more upstream transcription factors and signaling molecules such as *Pmar1 and HesC* which are involved in skeletogenic cell specification and operate earlier in development. Towards the center of the diagram are transcription factors such as *Alx1, Ets1/2,* and *Tbr* all of which are crucial components responsible for conferring skeletogenic cell identity in larval *S. purpuratus*, and which regulate the expression of up to hundreds of differentiation genes involved in skeletal growth and biomineral deposition. Also towards the center is the signaling molecule VegfR, which has a crucial role in positioning of the larval spicule during skeletogenesis. Additional genes, such as *Hex, Dri, Erg,* and *Tel* are transcription factors with roles in skeletogenesis, who regulate the expression of downstream differentiation genes. At the right are differentiation genes such as the spicule matrix genes *SM30, SM50,* and *MSP130* whose expression is necessary for normal biomineral deposition and growth. Black arrow depicts more upstream or downstream components of the GRN. Arrows in wiring diagram indicate positive regulatory interactions, while plungers represent repressive regulatory interactions. Components of the Double-Negative Gate have been shown depicted with bold lines.



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1706 **Figure 6. Phylomorphospace showing the distribution of Cambrian and Ordovician**

1707 **echinoderms based on their morphological disparity.** The morphospace shows two axes from

1708 a principal coordinate analysis based on Gower's similarity metric. Line's represent the

1709 phylogenetic relationships of all taxa included in the morphospace. Analyses of morphological

1710 disparity resulted in four main echinoderm body plans (highlighted in color). The characters used

1711 to create this morphospace show no hierarchical signal in evolution, casting doubt on the

1712 relationship between character burden and the genetic regulatory underpinning of morphological

1713 characters. Modified from Deline et al. (2020) and courtesy of Brad Deline.

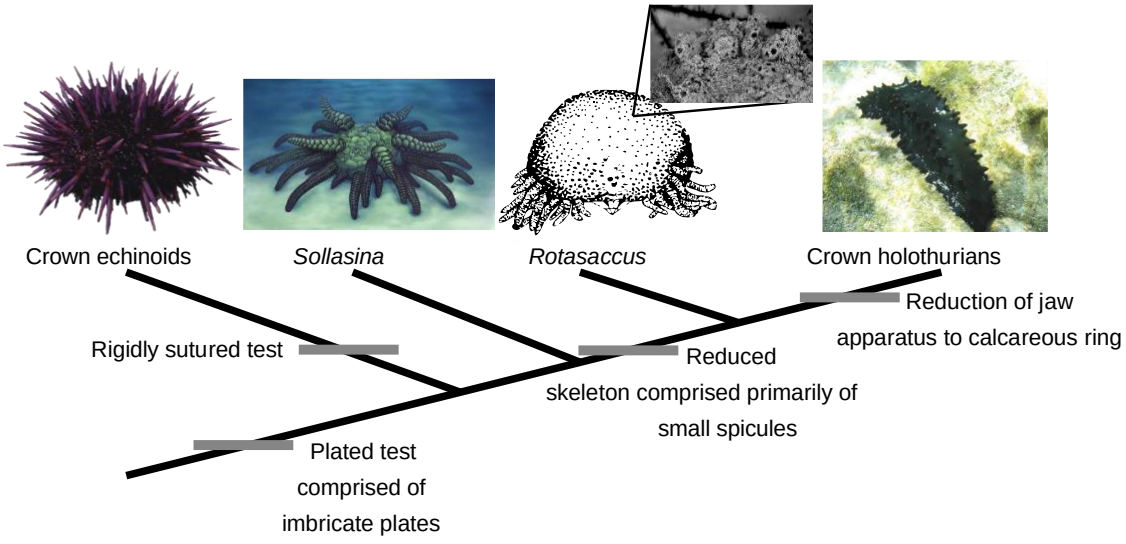


Figure 7. Phylogenetic Tree showing the reduction of the adult skeleton in holothurians throughout the course of echinozoan evolution. Extant and extinct echinoids have a test composed of multiple robust $CaCO_3$ plates, which became rigidly sutured near the transition from the echinoid stem group to crown group (Thompson et al., 2020). Most stem group echinoids, however, had tests composed of multiple imbricate plates. Like stem group echinoids, stem group holothurians like the ophiucistioid *Sollasina* also had skeletons comprised of multiple imbricate plates and calcified jaw apparatuses (Rahman et al., 2019). Along the lineage leading to crown group holothurians, however, a reduction of the skeleton took place to tiny, ossicles embedded in the dermis. This is first seen in ophiocistioids like *Rotasaccus* (which have jaw apparatuses), and even more extensively realized in crown group holothurians, many of which only have skeleton consisting of small wheel-like ossicles and jaws reduced to a calcareous ring (Smith and Reich, 2013). Scanning electron micrograph of *Rotasaccus* and *Stichopus chloronotus* courtesy of Mike Reich. Drawing of *Rotasaccus* is modified from Smith (1988) and *Sollasina cthulhu* from Elizabeth Martin in Rahman et al. (2019)

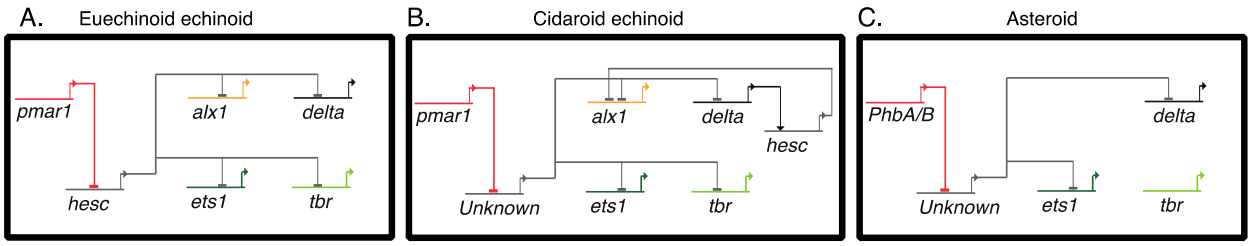


Figure 8. Differences in cell specification mechanisms in the early development of eleutherozoans. Simplified wiring diagrams showing cell specification mechanisms in euechinoids, cidaroids, and asteroids. (A) The double-negative gate, the gene regulatory network subcircuit through which the skeletogenic cells of numerous euechinoid echinoids are specified. The repressor Pmar1 represses *HesC*, also a repressor. HesC represses *Alx1*, *Ets1*, *Tbr*, and *Delta*, so the repression of *HesC*, by Pmar1 results in the expression of *Alx1*, *Ets1*, *Tbr*, and *Delta* later in development (Oliveri et al., 2008, Revilla-i-Domingo et al., 2007). (B) In cidaroid echinoids, a double-repression mechanism is still present, though *HesC* does not act as the second repressor. Instead, an unknown gene acts as the second repressor, repressing *Delta*, *Ets1*, and *Tbr* (Yamazaki et al., 2020, Erkenbrack and Davidson, 2015). This difference between cidaroids and euechinoids is an example of developmental systems drift (True and Haag, 2001). Asteroids lack embryonic or larval skeletogenic cell lineage, though some aspects of this regulatory circuitry are still present in mesodermal tissue of asteroids. Notably, *Pmar1* is not present in the genome of asteroids, and its repressive role is fulfilled by PhbA/B (Yamazaki et al., 2020).

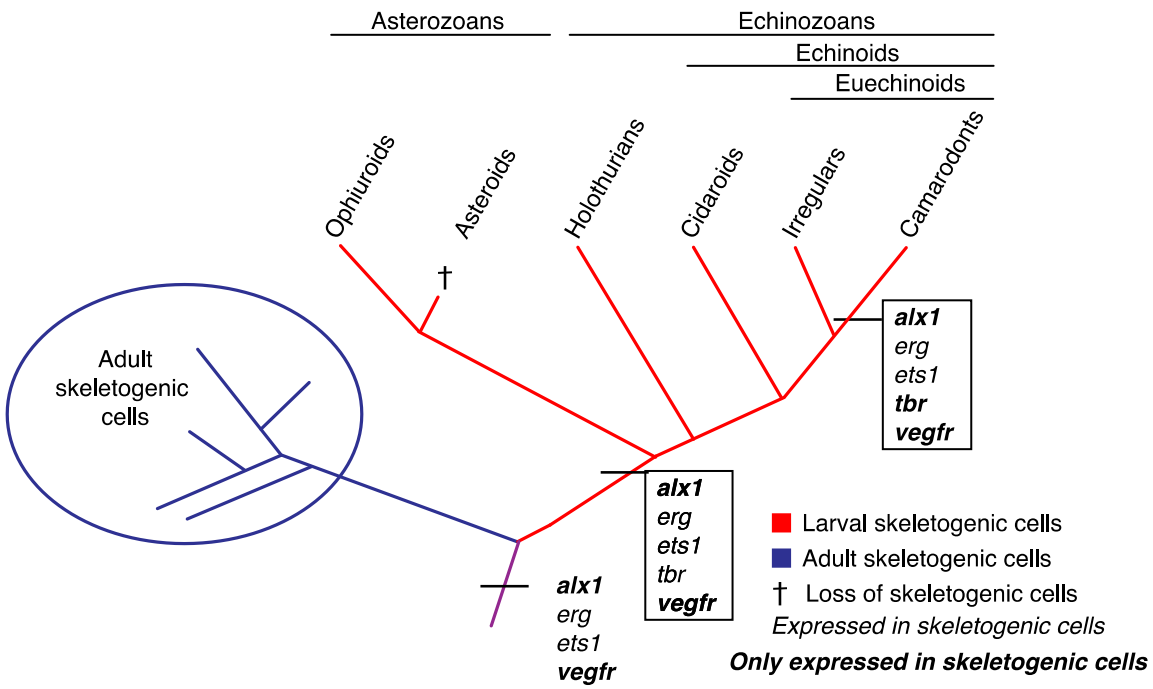
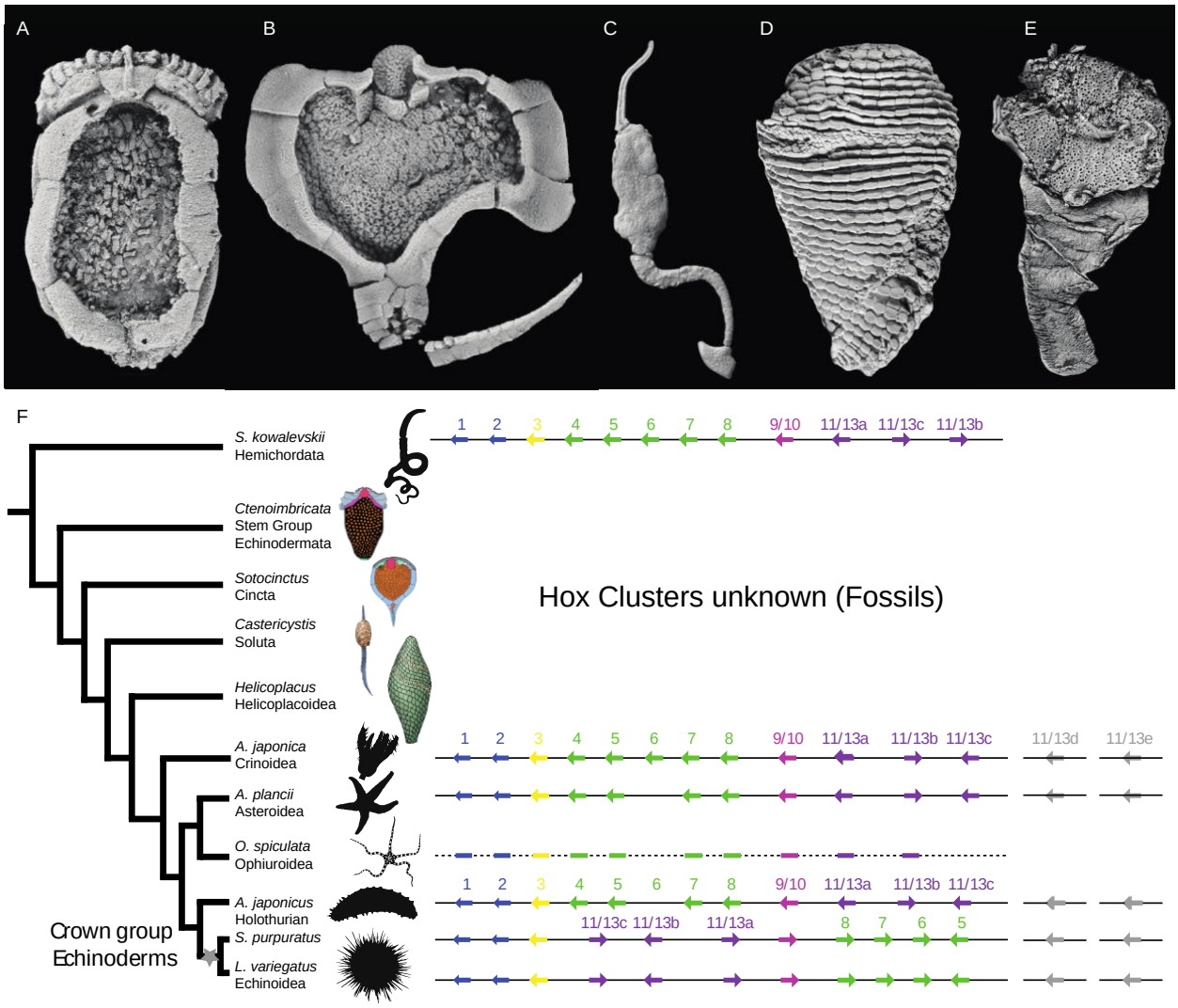


Figure 9. Cell type tree showing hypothesized evolutionary relationships of skeletogenic cells in eleutherozoans. This phylogenetic tree depicts proposed evolutionary relationships amongst the skeletogenic cells (not taxa) of larval and adult echinoderms. It is presumed that both adult and larval skeletogenic cells of extant echinoderms are descended from the adult skeletogenic cells of extinct echinoderms that expressed skeletogenic genes such as *Alx1*, *Erg*, *Ets1* and the signaling molecule *VegfR*. Through the course of evolution, the transcription factor *Tbr* became co-opted into the skeletogenic process in larval echinoderms, being expressed in skeletogenic cells and other mesodermal tissues. Along the lineage leading to camarodont euechinoids, *Tbr* came to be expressed specifically in the skeletogenic cells, and also became necessary for the normal formation of the larval skeleton. This gradual process leading to ever-more specific expression sheds light onto the processes of gene expression evolution which underlie cell type evolution across the animal kingdom. Figure is modified from Erkenbrack and Thompson (2019).



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1765 **Figure 10. Echinoderm Symmetry and the Hox cluster.** Various Cambrian echinoderms

1766 displaying the varying symmetries found in fossil forms and the organization of the echinoderm

1767 Hox cluster. (A) The ctenocystoid *Ctenocystis utahensis*; (B) the asymmetrical cinctan

1768 *Lignanicystis barriosensis;* (C) an asymmetrical solute; (D) the triradiate helicoplacoid

1769 *Helicoplacus gilbert;* (E) the pentaradial *Kinzercystis durhami.* (F) The organization of Hox

1770 clusters found in echinoderms and a hemichordate plotted alongside the hypothesized

1771 phylogenetic relationships of ambulacrarians with different symmetries (Zamora and Rahman,

1772 2014). The translocation of the Hox cluster first identified in *S. purpuratus* appears to be an

apomorphy unique to echinoids, and thus cannot be associated with the transition to pentaradial symmetry, which happened earlier in the evolutionary history of echinoids (Byrne et al., 2016). At present this translocation has only been identified in the genomes of regular euechinoids from the order Camarodonta (the grey star on the phylogeny), further work may show it to be found only within this clade, or another subclade within Echinoidea. Hox clusters modified from (Li et al., 2020) and images and drawings of Palaeozoic echinoderms courtesy of Samuel Zamora.

Appendix 1. Glossary

Animal Development-The process by which a fertilized egg grows and undergoes molecular, cellular, and morphological changes throughout the lifetime of the animal.

Archenteron-The invaginated region at the vegetal pole (bottom) of gastrula-stage echinoderm embryos. The archenteron forms during gastrulation. The archenteron is the primitive gut, and the opening of the archenteron, the blastopore, will become the anus.

Blastocoel- The open cavity surrounded by cells in a blastula stage embryo.

Blastula stage embryos-A hollow ball of cells that forms following the cleavage stages of embryonic development. In the sea urchin *Strongylocentrotus purpuratus*, the blastula stage begins when there are 128 cells in the embryo.

Cell type-A classification used to distinguish different cells from one another in an organism. Cell types can be distinguished based upon morphology, spatial location or position in the anatomy of the species, or based upon molecular characteristics. Recent definitions distinguish different cell types based upon the complement of transcription factors that are expressed within the cell.

Cellular differentiation-The process by which a cell changes from one cell type to another.

Chromatin-DNA and its associated proteins. DNA is stored in the cell as chromatin, which helps to compact it from its total length. DNA is compacted as chromatin in the cell.

Collinearity-Spatial and temporal expression of genes corresponding with the location of these genes on the chromosome and within a genome.

Developmental Gene Regulatory Network-A hierarchical model that represents the numerous regulatory interactions amongst genes and their products in different spatial and temporal contexts during animal development.

Developmental systems drift-The principle that the genetic networks directing the development of two or more homologous morphological characters can change through the course of evolution, without effecting the morphology of the character. This thus implies a somewhat indirect relationship with genotype and phenotype.

Differentiation genes/proteins- Genes, or their resultant proteins that respond to a common set of cell-type specific regulators, including transcription factors, and are responsible for functional and structural characteristics of the cell type. In sea urchins, the SM, or spicule matrix genes, are prime examples of differentiation genes involved in skeletogenesis, as they encode for proteins which are involved in the occluded protein matrix of the skeleton.

Ectoderm-The germ layer located on the outer layer of the embruo. Ectoderm gives rise to tissues of the nervous system, as well as the skin.

Eleutherozoan-The clade consisting of echinoids, holothuroids, ophiuroids, and asteroids.

Endoderm- The endoderm is the innermost germ layer of the embryo. Endodermal derivatives include the epithelium of the gut.

Enhancer-A regulatory module consisting of up to several DNA-binding domains to which transcription factors bind to effect transcription of genes. Enhancers can be located thousands of nucleotides away from the transcription start site and promoter. They are able to effect translation through the activity of DNA looping, whereby the DNA sequences in between the enhancer and the promoter loop, so that the enhancer, and any bound transcription factors, are close to the transcriptional machinery, including the RNA polymerase and other components of the transcription initiation complex.

Epithelial cell-Sheets or tubes of connected cells, originating from any germ layer.

Exon-Exons are the portions of a gene which comprise the final mRNA product during the process of gene expression. They are thus what's left of an mRNA following removal of introns after RNA splicing. It is sometimes said that exons are the portions of the gene which are expressed as they are the portions of the gene which are protein-coding.

Extracellular matrix-Secreted macromolecules immediately surrounding cells. These are secreted by the cells themselves and are useful for cell adhesion and migration.

Filopodial fusion-Fusion of the filopodia, which are long, thin processes extending from the extracellular matrix of cells.

Gastrula stage embryos-Embryos which are undergoing the process of gastrulation, in which multiple layers of the body plan are established. Mesodermal and endodermal cells enter the embryo, while the cells of the ectoderm constitute the outside surface. In indirect developing echinoderm larvae, the archenteron invaginates and the blastopore is formed during the gastrula stage. The gastrula stage follows the blastula stage in indirect-developing echinoderm embryos

Gene-A sequence of nucleotides in dna or rna that encodes for a gene product such as an RNA or protein. Gene products have numerous functions throughout animal growth and development, including regulating the expression of other genes, maintaining and carrying out cellular or biochemical roles, and synthesizing structures.

Gene Duplication-A mutation taking place during the process of DNA replication which results in the duplication of a segment of DNA along a chromosome. Gene duplication is the process by which paralogues are generated during the course of evolution.

Genome-A genome is the sum of all dna in an organism. This includes genes which are expressed, but also introns (intragenic regions) and large regions of duplicated and non-coding dna, which comprise the majority of the genome. It is through differential regulation and expression of portions of the genome that animal development proceeds. Genomes are typically analytically determined through the process of DNA-sequencing.

Glycoprotein-Proteins with glycan chains attached via covale nt bonds to amino-acid side chains. Many extracellular proteins or proteins spanning cell membranes are glycoproteins.

Histone-Small proteins around which DNA is wrapped to make nucleosomes while DNA is compacted in chromatin.

Hydrocoel-One of the coelomic cavaties formed during the development of the adult and post-metamorphic echinoderm body plan. It is from the hydrocoel that the five-fold arrangement of the water vascular system develops.

Induction-An interaction during development by which one set of cells is able to alter the behaviour of an adjacent set of cells. This results in changes in cell division rate, morphology, or cell fate. Induction is carried out via cell-cell signalling at close range.

Intron-Introns, short for intragenic regions, are portions of a gene which are non-coding. Introns are removed from mRNA after transcription, a process known as RNA splicing. Introns can be both short, and long, and genes can have no introns, or many. In the human genome, most genes are comprised mostly of introns, with the average gene being made up of 95% non-coding intragenic regions.

Knockdown-An experimental technique in which the expression of a gene is reduced. By examining the effects on other genes, it is possible to establish evidence for positive or negative gene regulation between the gene that is knocked down, and the other genes. In echinoderms, knockdowns are usually accomplished through the use of morpholino antisense oligonucleotides (MASOs), which interfere with translation of the protein at the mRNA level.

Ligand-A molecule which binds to a site on a target protein. Ligands are a crucial component of cell-cell signalling pathways. Some are capable of diffusing small distances to bind with receptor proteins during cell-cell communication.

Mesenchyme Cell-Loosely packed, unconnected cells, capable of movement and often derived from the mesoderm.

Mesoderm-Mesodermal tissues in echinoderms include muscles, skeleton, gonads, and connective tissues. The mesoderm is located between the ectoderm and endoderm.

Morphogenesis-The process by which cells, tissues, or morphological features of an organism are shaped and arranged into structures during development.

mRNA-Messanger RNA. mRNA is a single-stranded molecule of RNA, which is produced during the process of transcription, during which mRNA is produced from DNA through the action of an RNA polymerase enzyme and other transcriptional machinery. mRNA

will typically be translated into a protein, and is thus crucial for monitoring and understanding gene expression during development.

Nucleosome-The coiled structure consisting of DNA, and the histones around which it is wrapped in chromatin. Each nucleosome in eukaryotic cells consists of a core of eight histones, around which DNA is coiled. Nucleosomes are important for the process of gene regulation, as DNA stored as a nucleosomes must be made more (or less) accessible for access by transcription factors.

Over-expression experiments-An experiment when mRNA transcripts corresponding to a particular gene are injected or otherwise introduced into a developing embryo to increase mRNA abundance. Over-expression experiments can be used to understand regulatory interactions. Genes that are upregulated following over-expression of a downstream gene are likely positively regulated by it, while genes whose expression decreases following over-expression of a downstream gene may be under negative regulation by this gene.

Paralog-Homologous genes with shared ancestry resulting from a gene duplication event.

Promoter-A promoter is the site where RNA polymerases and the other transcriptional machinery (including transcription factors) assemble and bind to the DNA during the process of transcription. The promotor is usually upstream of the transcription start site.

Protein-Proteins are macromolecules consisting of chains of amino acids. They are gene products resulting from the translation of mRNA molecules. During development, proteins perform a multiplicity of functions, such as enabling DNA replication and RNA synthesis, regulating the expression of other genes (transcription factors), providing structure, and catalyzing metabolic or other biochemical reactions.

Proteome-The sum of all proteins present in a given organism or tissue.

RNA Polymerase-An enzyme capable of synthesizing RNA. In eukaryotes, there are three RNA polymerases, though the one involved transcribing most coding genes is the Polymerase II (Pol II) enzyme. RNA polymerases bind to the promoter of a DNA sequence along with other transcription factors during the process of transcription.

Sclerocytes-Skeletal cells of echinoderms, particularly those not associated with the growth of echinoid teeth.

Signal transduction cascade-Enzymatic reactions taking place within a cell after it receives a signal from another cell. These enzymatic changes can include the phosphorylation of proteins, changes in protein structure, or dimerization (binding of two proteins). The end result of signal transduction cascades is usually the regulation of a transcription factor, which in turn regulates the expression of another gene.

Signaling Molecule-Signaling molecules are proteins involved in cell-cell communication. They include ligands and cell-surface proteins which are responsible for sending signals, as well as receptor proteins which receive the signal on another cell. The signal can be sent via direct contact between transmembrane proteins on adjacent cells, or the signal can diffuse over small distances, or be sent via fluids such as blood to travel longer distances. It is through the action of signalling molecules, that regulatory action in one cell is capable of inducing changes in gene regulation in other cells.

Somatocoel-Coelomic cavity formed during the development of the adult and post-metamorphic echinoderm body plan. There are two somatocoels, the left somatocoel and right somatocoel. In indirect developing echinoderms, the adult body plan develops, in part, from tissues of the left somatocoel.

Stereom-The porous, $CaCO_3$ microstructural meshwork which comprises the echinoderm skeleton.

Syncytium-A cell or cytoplasmic mass containing multiple nuclei formed by the fusion of several cells.

Transcription co-factor-A protein that acts in conjunction with another transcription factor, or transcription factors, to regulate the expression of another gene. Transcription co-factors, unlike transcription factors, do not bind directly to DNA, but rather work with other proteins to mediate transcription.

Transcription Factor-A transcription factor is a protein, encoded for by a gene, that binds to regulatory sequences on a piece of DNA to regulate the expression of the gene it has bound to. Transcription factors are crucial components of gene regulatory networks, as they are responsible for regulating the expression of the genes that they bind to. Transcription factors act combinatorially, and it is the combinatorial activity of transcription factors that results in precise spatial and temporal differences in gene expression during development.

Transcription Initiation Complex-The set of RNA polymerase and general transcription factors which are bound together at the promotor regulatory sequence of the DNA sequence during transcription of RNA from DNA.

Transcriptional machinery-All of the proteins, enzymes, and other molecules involved in the process of RNA transcription from DNA. This includes any RNA polymerases, general transcription factors, co-factors, co -activators such as the mediator complex, and other proteins which facilitate the process of transcription.

Transcriptome-A transcriptome is the sum of all expressed genes in an organism or tissue. It thus excludes portions of genes which are not expressed (introns) and genes which are not expressed in particular tissues or during certain stages of development. Transcriptomes can thus vary spatially and temporally through the course of animal growth and development. Transcriptomes are typically analytically determined through the process of RNA-sequencing (RNA-seq).

Translocation-A chromosomal mutation in which part of a chromosome breaks and re-attaches, resulting in a re-arrangement of gene order and location within the genome. The Hox cluster within echinoids has undergone a translocation through the course of their evolutionary history.

Vacuole-Closed membrane-bound sacs within cells filled with water and organic or inorganic molecules in solution.

Vestibule-An ectodermally-derived sac that forms over the left hydrocoel of echinoderms prior to metamorphosis. During the development of the adult body plan, the five lobes of the hydrocoel, which will form the ambulacra of the juvenile, elongate through the growing vestibule, and the vestibule forms the ectodermal epithelium of the juvenile's primary podia (tube feet).