

Regulatory states in the developmental control of gene expression

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Abstract

A growing body of evidence shows that gene expression in multicellular organisms is controlled by the combinatorial function of multiple transcription factors. This indicates that not the individual transcription factors or signaling molecules, but the combination of expressed regulatory molecules, the regulatory state, should be viewed as the functional unit in gene regulation. Here, I discuss the concept of the regulatory state and its proposed role in the genome-wide control of gene expression. Recent analyses of regulatory gene expression in sea urchin embryos have been instrumental for solving the genomic control of cell fate specification in this system. Some of the approaches that were used to determine the expression of regulatory states during sea urchin embryogenesis are reviewed. Significant developmental changes in regulatory state expression leading to the distinct specification of cell fates are regulated by gene regulatory network circuits. How these regulatory state transitions are encoded in the genome is illuminated using the sea urchin endoderm–mesoderms cell fate decision circuit as an example. These observations highlight the importance of considering developmental gene regulation, and the function of individual transcription factors, in the context of regulatory states.

Key words: regulatory state; gene regulatory networks; sea urchin development; gene regulation

Introduction

Echinoderms have over the past decades served as a premier system for analysis of developmental gene regulatory networks (GRNs), and the insights deriving from this field have broad implications on many other branches of biology, including functional genomics [1, 2]. The premise of the GRN concept is that the system of regulatory interactions between regulatory genes, encoding transcription factors and signaling molecules, controls the developmental expression of all genes in the genome in every part of an organism and throughout its formation and subsequent lifetime. To experimentally address the complexity of information encoded in developmental GRNs is therefore not a simple task. Focusing on the regulatory states, the active states of a developmental GRN, proves to be an important step in illuminating the underlying GRN. Regulatory states are defined by the set of functionally active transcription factors expressed together within a nucleus at levels high enough to occupy relevant DNA-binding sites and execute regulatory functions. Furthermore, the specific functional status of

transcription factors regulated downstream of signaling pathways has to be taken into account. However, regulatory states are not just the product of developmental GRNs, they also represent important functional units in the control of gene expression. Thus, while individual transcription factors and signaling pathways may function in multiple developmental processes, context-specific gene regulation is achieved through the combinatorial function of transcription factors constituting a specific regulatory state.

Several observations highlight the importance of regulatory states in the control of developmental gene expression. First, countless *cis*-regulatory experiments show that the activation of *cis*-regulatory modules (CRMs) in multicellular animals requires the combinatorial function of multiple qualitatively distinct transcription factors, indicating that individual transcription factors, even when present at high levels, are not sufficient to activate specific gene expression [3–6]. Second, for most genes for which developmental regulation has been analyzed, specific CRMs that account for the specific gene expression pattern can be identified, demonstrating that spatial and

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temporal expression of genes during development is predominantly regulated at the transcriptional level [1]. In addition, once transcripts are expressed, further layers of regulation by post-transcriptional and posttranslational processes will affect the levels and function of expressed molecules. Third, many transcription factors are expressed in multiple developmental contexts, where they predominantly regulate the expression of context-specific sets of target genes, as shown, for example, for Pax6 in the mouse eye, brain and pancreas [7], which supports the idea that transcription factor binding as well as function is often determined by the context-specific regulatory state. The fourth type of evidence derives from developmental control mechanisms. Thus, when monitoring the mechanisms underlying the spatial specification of cell fate domains, such as, for example, the definition of the limb bud in a specific position of the embryo [8], novel expression of one or several transcription factors is usually a key step. These transcription factors are neither expressed during the developmental phase preceding the definition of the cell fate domain nor are they expressed in cells surrounding the domain. Thus, in the vast majority of cases, developmental functions depend on a qualitative change in the combination of transcription factors expressed together. Altogether, these and many other observations show that gene regulation is a function of regulatory states and not just of individual transcription factors.

Figure 1 summarizes the role of regulatory states in developmental gene regulation. The expression of regulatory states is directly controlled by upstream developmental GRNs in time and space. In turn, the set of transcription factors constituting the regulatory state controls the expression of all genes in the genome. In the following, I will first consider the control of gene expression downstream of a given regulatory state, before discussing approaches to identify regulatory state expression that have been successfully applied in sea urchin embryos, and finally turn to the developmental control of regulatory state expression in the differential specification of cell fates.

Function of regulatory states in control of gene expression

Countless analyses demonstrate the expression of cell fate-specific combinations of transcription factors in different developmental contexts, as recently reviewed [1]. They confirm that although individual transcription factors may be expressed in multiple developmental contexts, regulatory states are expressed specifically in given embryonic domains at given developmental times. In most cases, cell fate-specific gene expression is therefore controlled by qualitatively different regulatory states, and not just by subtle quantitative differences in the relative abundance of transcription factors constituting a regulatory state. The importance of combinatorial gene regulation is perhaps best illustrated by considering the control of gene expression downstream of signaling pathways. Specific signal response transcription factors control the expression of a large set of genes in response to a given signaling interaction [1, 9, 10]. Yet, although the same signaling interactions are deployed repeatedly throughout development, the set of target genes controlled by these signal response transcription factors is specific to a given developmental context. Thus, other transcription factors within the specific regulatory states contribute to the selection of target genes and to the regulation of their expression. Regulatory states are therefore not just random collections of co-expressed individual transcription factors, but in respect to

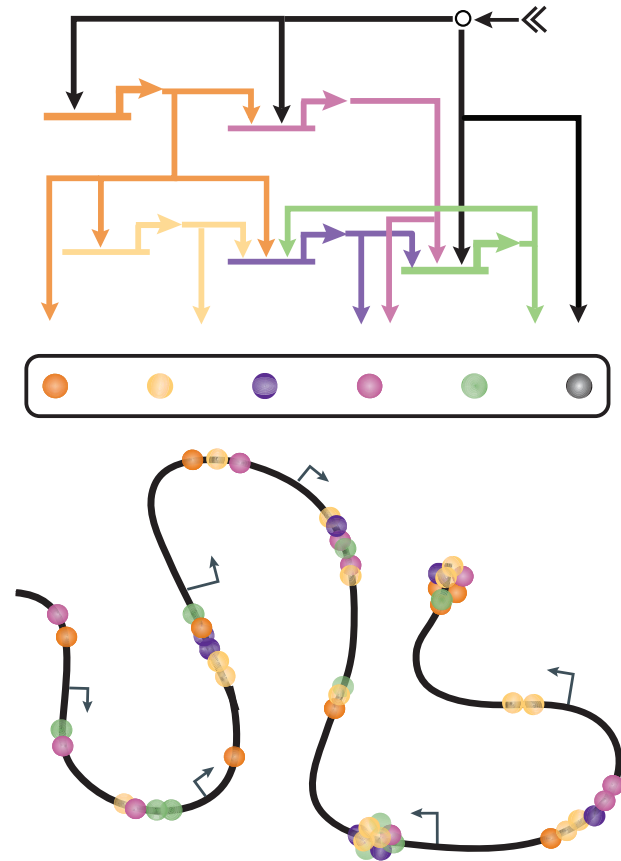


Figure 1. Regulatory states in developmental gene regulation. Scheme showing the expression of a cell fate-specific regulatory state (center) as controlled by the circuitry of a developmental GRN (top). The regulatory state consists of transcription factors co-expressed in a given nucleus, some of which are regulated by developmental signaling interactions (black). While the expression of all genes depends on the regulatory state, the specific combination of transcription factors regulating individual genes may vary (bottom). (A colour version of this figure is available online at: <https://academic.oup.com/bfg>)

the genes expressed in any given nucleus, the regulatory state has to be considered as a regulatory unit. When addressing the function of any given transcription factor, it will be in respect to the regulatory context for which these functions are described.

This raises the question of why individual transcription factors are not sufficient to control gene expression. A possible explanation for the requirement of combinatorial gene regulation is that transcription factors controlling a given CRM are not equivalent in their function. Although perhaps some of the transcriptional activators might be able to substitute for one another, some regulatory functions can only be executed by specifically dedicated transcription factors. The best described examples are pioneer factors that have the capacity to bind DNA even when wrapped around histones [11]. Thus, pioneer factors are thought to play a particular role in the initial identification of cis-regulatory sequences. Binding of pioneer factors is a requirement for other transcription factors to have access to the regulatory sequences and contribute to the control of gene expression. But pioneer factors are only part of the equation. Pioneer factors can be expressed broadly such as FoxA in the endoderm or Zelda in the early *Drosophila* embryo, and nevertheless contribute to the regulation of genes that are expressed

exclusively in only a subset of cells [4, 12–14]. For example, FoxA binding to the regulatory sequences of the liver gene *Alb1* will only lead to gene expression in the liver, while this gene remains silent in other foregut endoderm cells, despite the binding of FoxA to the *Alb1* locus [12]. Other transcription factors that control the expression of *Alb1* function together with FoxA to ensure liver-specific gene expression. Although our current understanding of specific transcription factor functions is limited, it is conceivable that other types of functional specialization exist among those transcription factors bound together to a specific CRM, accounting for the requirement for combinatorial function of different regulatory factors. For instance, some transcription factors are responsible for the looping of a CRM to its basal transcriptional promoter to initiate gene transcription [15, 16]. Other transcription factors recruit enzymes that enable various modifications of specific amino acid residues within associated histones. For example, in skeletal muscle cells, G9a, a lysine methyltransferase, is recruited to the *myogenin* promoter by the transcription factor Sharp-1, and contributes to the repression of *myogenin* ([17]; for a recent discussion see [1]). The recruitment of broadly expressed cofactors, including histone modification enzymes, to the regulatory sequences of genes that are expressed in a cell fate-specific manner is an important function of transcription factors, and may affect the ability of additional transcription factors to bind to regulatory sequences.

Division of labor among transcription factors is also indicated by the observation that although a combination of factors is required for the control of gene expression, not all factors necessarily do so by direct sequence-specific binding to DNA. A recent study demonstrates that transcription factors required for expression of genes in response to estrogen stimulation are recruited to the regulatory sequences of their target genes by binding to a transcription factor recognizing the CRMs [18]. Although both Gata3 and the retinoic acid receptor (RAR) are required for activation of gene expression, neither of them appeared to bind to CRMs through their cognate binding sites, but by binding to the estrogen receptor- α . These results indicate that the precise regulatory function of each transcription factor within the context of a given CRM may range from the initial sequence-specific identification of the CRM, the opening of the chromatin by recruitment of cofactors, the recruitment of additional transcription factors, to the looping of the CRM to the basal transcription start site, and most likely includes additional functions. In the absence of any of these functions, gene expression will not be supported. The function of the regulatory state therefore lies in reading regulatory DNA sequences, and thus in determining which genes will be expressed in a given nucleus. However, to initiate gene transcription, the function of additional cofactors and histone modification enzymes is required, and they are recruited to CRMs by sequence-specific transcription factors.

Although the regulatory state should be considered as a functional unit in the sense that it determines the set of expressed genes, not all genes expressed downstream of a given regulatory state are controlled in the same manner, as indicated in Figure 1. Thus, comparing the regulation of differentiation genes expressed within a given cell type shows that only a subset of the regulatory state will be used to drive the expression of each gene. Even for those CRMs active within the same regulatory context, the precise grammar can be different among these CRMs, in respect to the type of transcription factors contributing to gene regulation, and in respect to the number and position of binding sites for each regulatory factor. For example, the

expression of crystallin genes in the mouse lens epithelium is driven by Pax6, c-Maf, Six3, Sox2, RAR/RXR and other regulatory factors, but the precise combination of these factors binding to the enhancers and promoters of each crystallin gene varies [1, 19]. Similarly, genes expressed preferentially in sea urchin skeletogenic cells are differentially affected by the perturbation of two upstream transcription factors, Ets1 and Alx1 [20]. Some genes are affected by perturbation of Ets1, some by perturbation of Alx1, some by both perturbations and some were not affected at all, indicating regulatory inputs other than Ets1 and Alx1. Thus, even when exposed to the same regulatory state, individual genes are not controlled by exactly the same set of transcription factors, but different subsets of transcription factors within the same regulatory state will control the expression of individual genes within a nucleus. However, only the complete regulatory state will lead to the expression of the entire set of genes.

Analysis of developmental regulatory state expression

To reveal the mechanisms controlling developmental gene expression, identification of regulatory states is therefore essential. Once the sea urchin genome sequence became available, the echinoderm community collaborated in a large-scale effort to compile the spatial and temporal expression profiles of various functional classes of molecules, most prominently transcription factors and molecules involved in various signaling pathways, during early embryogenesis [21–31]. On the basis of these gene expression patterns, lists of candidate genes were assembled for each embryonic domain indicating the potential players of the GRN underlying specification of the domain. This systematic analysis of regulatory gene expression patterns was a crucial factor in the success of subsequent GRN analyses. Instead of working with just a small representation of expressed transcription factors, where the presence of additional layers of indirect regulatory interactions can never be ruled out, having a genome-wide survey of transcription factor expression allows for a comprehensive analysis of all regulatory functions of a regulatory state. As a result, developmental GRNs have been experimentally analyzed for the early cell fates of almost the entire embryo of the purple sea urchin *Strongylocentrotus purpuratus* [32–40]. These GRNs demonstrate how genomic programs operate the specification of diverse cell fates during early embryogenesis and they shed light on the function of regulatory factors in diverse developmental contexts.

The systematic analysis of developmental regulatory states provides an important starting point for the analysis of developmental GRNs. The initial analysis of regulatory gene expression in sea urchin embryos was based on the quantitative analysis of expression levels for all known regulatory genes in the sea urchin genome, identifying those regulatory genes expressed at levels that potentially support regulatory function. A previous computational simulation showed that in this embryo, developing at 15°C, about 10 transcripts per cell are sufficient to produce several hundred molecules of transcription factor molecules within a few hours, sufficient to activate downstream gene expression [41]. Assuming that in the early embryo, each cell fate domain consists of at least 16 cells, the whole embryo should show at least 160 copies of regulatory gene mRNA to produce functional levels of transcription factors [23]. Thus, regulatory genes expressed at levels of at least 150–300 transcripts/embryo were included in the spatial analysis of gene expression. The

most important data set showing the developmental expression of regulatory genes derives from whole-mount *in situ* hybridization experiments carried out at multiple stages of embryogenesis [42]. Although such data are not quantitative, they show at once the expression of an individual regulatory gene in all cells of the embryo.

A crucial step for using large sets of spatial gene expression data in GRN analysis is the transformation of qualitative descriptions of expression patterns to digitalized Boolean annotations. Thus, the spatial distribution of regulatory gene transcripts throughout the entire embryo is annotated as Boolean ON/OFF states of gene expression [32, 38]. Gene expression is considered ON where transcripts are detectable by *in situ* hybridization and thus possibly sufficiently prevalent to indicate regulatory function, or OFF where transcripts are not detectable. As a result of this annotation, the gene expression status is provided for all cells of the embryo, irrespective of the gene expression pattern, thus discriminating between genes not being expressed and genes for which no data are available. In the context of GRNs, absence of a transcription factor in a given cell fate domain can be just as important as its presence elsewhere, particularly in the case of transcription factors that function as repressors. The systematic digital annotation of regulatory gene expression patterns in echinoderms has generated an invaluable resource that allows for comparative analyses of large collections of spatial expression data, which would not be possible based on qualitative image data alone. For every regulatory gene that is part of a developmental GRN in sea urchin embryos, these data are publicly accessible through the BioTapestry GRN models, as can be seen, for example, at <http://gms.biotapestry.org/SpEndomes/> [43–45]. The Boolean annotation of regulatory gene expression also allows the direct analysis of regulatory state expression, making it possible to rapidly identify within a large set of spatial expression data those regulatory genes expressed in a given embryonic domain at a given time in development. Whole-mount *in situ* hybridization analyses have thus in echinoderms served an invaluable purpose to relatively quickly assess expression of individual genes throughout the embryo and at different developmental times. Despite the relative low throughput of this approach, the early definition of standards for the echinoderm community and the consistent annotation of gene expression patterns have over the years led to the accumulation of a precious data set. These expression data were initially mostly restricted to early development of the sea urchin embryo but is currently being extended to include the entire first 72 h of sea urchin development ([46, 47]; Valencia, J. & I.S.P, unpublished data).

In addition to transcription factors, intercellular signaling interactions are important components of developmental GRNs. The control of gene expression downstream of signaling interactions is executed by dedicated signal response transcription factors whose regulatory function is determined by the signaling pathway [10]. A complete description of the regulatory state therefore not only includes expression of transcription factors but also information about presence or absence of signaling interactions. For example, the function of Tcf, the transcription factor controlled by Wnt signaling, is decided by association with a cofactor. In the presence of Wnt signaling, Tcf binds to nuclearized β -catenin, a co-activator of gene expression, while in the absence of Wnt signaling and nuclear β -catenin, the co-repressor Groucho binds to Tcf and leads to repression of Tcf target genes [48]. System-level analyses of the spatial expression of signaling ligands and receptors and the functional assessment of given signaling interactions have

shown the contribution of diverse signaling interactions to the developmental specification of cell fates in the sea urchin embryo [49–51].

Spatial expression data provide critical information for GRN analysis, as they allow direct comparison of expression of either individual regulatory genes or entire regulatory states across the entire embryo or at least several cellular domains neighboring the gene expression domain. In addition, determining the quantitative levels of regulatory gene expression is also relevant, perhaps not so much for understanding the differential specification of cell fates as for understanding the kinetics of the developmental process. Thus, the rate of transcript accumulation will determine the temporal delay between onset of regulatory gene transcription and onset of target gene expression, and therefore the kinetic behavior of the GRN [41, 52]. Quantitative data of developmental gene expression have been obtained for early sea urchin embryogenesis [53–55]. Even though these quantitative data are often generated with whole embryos, and thus only reveal the total transcript level per embryo, when combined with spatial expression data that reveal the number of cells in which gene expression occurs, an estimate for transcript levels per cell can be obtained. Furthermore, transcript levels have also been determined for isolated cell types such as skeletogenic cells and pigment cells [20, 56], and eventually, single-cell transcriptomes will expand our current understanding of regulatory states in each embryonic domain.

Control of developmental regulatory state expression

During development, regulatory state expression changes both in time and in space to define the body plan and progressively specify its constituent cell fates. Thus, the identification of regulatory mechanisms for given developmental functions often starts with characterizing the change in regulatory state associated with the developmental process. A Boolean computational model of the endomesoderm GRN was able to reproduce the expression of regulatory states during sea urchin development, showing that developmental changes in regulatory state expression are indeed controlled by the underlying GRNs [57].

An example for how the developmental expression of regulatory states is controlled by GRN circuitry is shown in Figure 2. This circuit is a component of the sea urchin endomesoderm GRN that encodes the distinct specification of mesodermal and endodermal cell fates [37, 38, 58]. Both cell fates are specified in the descendants of a common cell lineage, the veg2 cells. Of all the regulatory genes involved in endodermal and mesodermal specification, only few are expressed during earliest developmental stages. Analyzing the spatial expression of these regulatory genes shows that endomesoderm progenitors express an ‘endomesodermal’ regulatory state, composed of transcription factors later associated with either endodermal (such as Hox11/13b and FoxA) or mesodermal (such as Gcm) specification [37]. However, distinct populations of cells can be identified a few hours later, expressing either endodermal or mesodermal regulatory states (Figure 2). The reason for mesodermal genes being expressed first in the endomesoderm precursors and later in the mesodermal progenitors is their regulation by Delta/Notch signaling, induced by the expression of Delta ligand in adjacent skeletogenic mesoderm cells [59]. In turn, endodermal genes are controlled by Tcf/ β -catenin that is present initially in all veg2 descendants because of maternal anisotropies [37, 60, 61]. Thus,

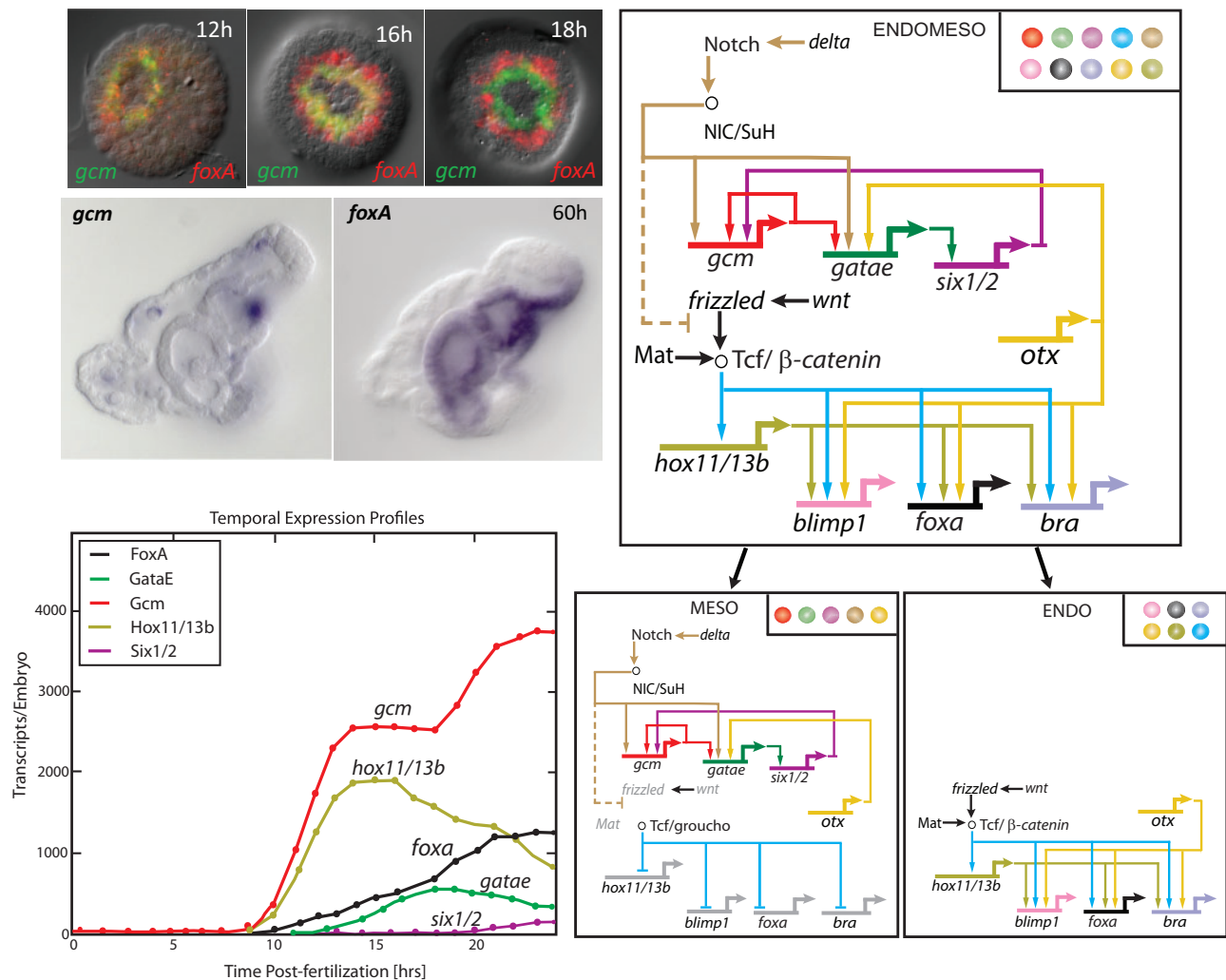


Figure 2. Control of regulatory state expression in the endoderm/mesoderm cell fate decision. (A) Early in development, endodermal and mesodermal regulatory genes are co-expressed in endomesoderm progenitor cells (12 h), but expression eventually resolves into two different spatial domains (18 h) giving rise to endoderm expressing *foxA* and mesoderm expressing *gcm* (60 h). (B) Temporal expression profiles of endodermal and mesodermal regulatory genes show that *gcm* (mesoderm) and *hox11/13b* (endoderm) are the earliest genes expressed in endomesoderm cells. Despite being controlled by different regulatory mechanisms, both genes are initially expressed with similar accumulation rates. (C) Developmental GRN controlling endoderm/mesoderm cell fate decision showing that *gcm* is controlled by Delta/Notch signaling and is upstream of a positive feedback circuit active after 18 h. Spatial expression of the endoderm GRN is controlled by Tcf/ β -catenin. Both endoderm and mesoderm GRNs are active in endomesoderm progenitors without cross-interaction, and are turned off in cells of the alternative cell fate. Regulatory states are shown in top-right corner. (A colour version of this figure is available online at: <https://academic.oup.com/bfg>)

in early endomesoderm progenitors, both Tcf/ β -catenin and SuH/NICD are available, leading to the co-expression of regulatory genes later specific to either endoderm or mesoderm. Interestingly, *gcm* and *hox11/13b* show similar kinetics in expression levels during early specification of endomesoderm progenitors, despite being controlled by different regulatory mechanisms.

Once the endomesoderm progenitors divide, about half of the daughter cells become physically separated from the Delta signaling source, terminating mesodermal gene expression. These cells continue to express endodermal regulatory genes and will give rise to foregut and midgut endoderm. Those daughter cells that continue to receive Delta/Notch signaling initially continue to express an endomesodermal regulatory state. However, within a couple of hours, these cells turn off endodermal regulatory genes by a Delta/Notch signaling-

dependent mechanism that leads to the clearance of nuclear β -catenin and the conversion of Tcf into a repressor by binding to Groucho [38]. Eventually, Delta/Notch signaling turns off also in mesodermal cells, and expression of mesodermal regulatory genes is maintained by a positive feedback circuit between *gcm*, *gatae* and *six1/2* [58]. The dramatic change in regulatory state expression during the endoderm/mesoderm cell fate decision in the sea urchin embryo is therefore the result of two independent GRNs, activated together in endomesoderm progenitors, and the subsequent inactivation of either the mesodermal or endodermal GRNs in endodermal or mesodermal progenitors, respectively [38]. Even when both GRNs are co-expressed, no interaction between the two cell fate GRNs is observed. Thus, the mixed endomesodermal regulatory state is only the result of the combined activity of two GRNs, indicating that regulatory states themselves can be just as modular as their underlying

GRNs. Ultimately, changes in the geometry of the embryo, differential properties of signaling molecules and developmental time all contribute to the differential readout of the regulatory genome in progenitors versus descendants and endoderm versus mesoderm.

What this small circuit for separation of endoderm and mesoderm in the sea urchin embryo demonstrates, is that ultimately the DNA sequences responsible for given developmental functions such as the endoderm–mesoderm cell fate decision may be just a few binding sites, a tiny fraction of the regulatory genome, active for just a short time and thus basically undetectable based on genome-scale approaches without prior knowledge of the responsible transcription factors and the precise developmental timing [62]. However, where the developmental expression of regulatory states and the changes in regulatory states associated with a developmental function are identified, finding the underlying control mechanism within the regulatory genome becomes feasible. In the sea urchin, the consistent analysis and annotation of regulatory gene expression profiles, and the regulatory states that were deduced from these data, have provided a significant contribution to the analysis of GRNs. Similar data sets are available also for other animals, although often presented at the level of single genes and not in the form of regulatory states. However, the approaches used for identification of developmental regulatory states in the sea urchin embryo are to a large extent applicable to other systems as well and should enhance the identification of developmental mechanisms and their origin within the regulatory genome.

Key Points

- Regulatory states are the set of co-expressed transcription factors.
- Regulatory states determine the combinatorial control of gene expression.
- Extensive analysis of developmental regulatory state expression enables the identification of GRNs.
- Developmental changes in regulatory states determine the differential specification of cell fates and are controlled by developmental GRNs.

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