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NETWORK DESIGN PRINCIPLES FROM THE SEA URCHIN EMBRYO

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Abstract

As gene regulatory network models encompass more and more of the specification processes underlying sea urchin embryonic development, topological themes emerge that imply the existence of structural network "building blocks". These are subcircuits which perform given logic operations in the spatial control of gene expression. The various parts of the sea urchin gene regulatory networks offer instances of the same subcircuit topologies accomplishing the same developmental logic functions but using different genes. These subcircuits are dedicated to specific developmental functions, unlike simpler "motifs", and may indicate a repertoire of specific devices of which developmental gene regulatory networks are composed.

Developmental gene regulatory networks (GRNs) are composed of the interactions of regulatory genes, including signaling inputs. The function of these interactions is to produce the progressive sequence of spatial transcriptional regulatory states that generate the events of development. A GRN provides a direct translation of the genomic code for development, since its structure is literally encoded in the *cis*-regulatory sequences of the control systems determining regulatory gene expression in time and space. We now have a relatively advanced GRN model for portions of the early sea urchin embryo. This states explicitly the regulatory interactions responsible for the spatial specification of the skeletogenic and non-skeletogenic mesodermal territories, and of the endodermal territories up to gastrulation (Endomesoderm GRN, here En-GRN). By gastrulation the whole embryo has been spatially partitioned into domains or territories expressing distinct regulatory states, i.e., distinct sets of transcription factors, and each territory will produce a particular part of the post-gastrular embryo. The En-GRN has been validated by direct *cis*-regulatory experiment at a significant fraction of its key nodes. The skeletogenic lineage portion of the En-GRN is most nearly complete, and it provides satisfying causal explanations for all of the observed developmental functions and transitions that this lineage executes up to the point when the differentiated cells ingress into the blastocoel just prior to gastrulation [1]. A recent comprehensive review of the field of transcriptional regulatory circuits [2] described the sea urchin En-GRN as "the most comprehensive and successful model in animals".

Here, rather than delving further into developmentally oriented explications of process based on this GRN, I shall use the structure of the GRN for a "meta-analysis". Its focus is on the recurrence, in diverse contexts, of certain subcircuit topologies that are utilized for given developmental functions. An additional GRN is useful here, for comparison. This is a

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recently constructed GRN model for specification of the oral and aboral ectoderm of the sea urchin embryo (Ec-GRN; [3]). Together the En-GRN and the Ec-GRN encompass all of the pre-gastrular embryo except the neurogenic apical territory, which is under study elsewhere [4,5]. In the near future the sea urchin embryo is likely to become the first developmental system to be globally represented in a transcriptional regulatory network analysis.

A definition of GRN "building blocks"

GRNs are composed of modular subcircuits and their interconnections, where each such subcircuit executes a given developmental "job" (for discussion of this concept and review see ref.[6]). The modules of a given GRN evolve at different rates and may often arise in the genome at different times in the evolutionary history of each species. Thus the GRN (and the regulatory genome itself) is essentially a mosaic structure, in both evolutionary and functional terms. Each subcircuit has a given topology, i.e., its functional linkages describe a given form, and it produces a given developmental output, defined by some change or other effect that it mediates in respect to spatial gene expression. These outputs can be described in terms of logic functions, for instance setting up exclusive spatial domains of gene expression: if you express *x* you will not express *y*, and if you express *y* you will not express *x*. There is a one-to-one relation between subcircuit topology and output logic function. We may now ask ourselves whether there might exist in nature such subcircuits each defined by a topology and a spatial logic output. Were this the case, we could consider the set of these subcircuits as the "building blocks" from which animal GRNs are constructed.

How could such putative building blocks be defined, and how would we know one if we saw it? Two simple and essential criteria are (i) that the subcircuit topology and its logic output recur in diverse GRN contexts; but (ii) that the same topology and output can be generated by different constituent genes. Thus the unique subcircuit topology would be what counts, and function would depend on that, not on peculiar biochemical characteristics of the players. To at least some extent these players, i.e., given sets of regulatory genes, are interchangeable. In the following I extract from the sea urchin GRNs several examples which meet these criteria, and which, it can be claimed, provide evidence of the existence of GRN building blocks.

Canonical subcircuits and their spatial logic functions

Our first example is what we call the **double negative gate.** The topology is illustrated in in Fig. 1A, B. It consists of a gene encoding a repressor which is activated in a confined spatial domain, the target of which is a gene encoding a second repressor which is broadly activated except where the first gene silences it. The double negative gate is a global spatial control system. It ensures that the target genes of the second repressor will be specifically transcribed in the same spatial domain where the initial component of the gate operates, but that these same genes will be specifically repressed everywhere except in that domain: the output of the double negative gate is [*x* and *1-x*] spatial control logic. The double negative gate in Fig.1A was discovered as a spatial specification device in the skeletogenic mesoderm portion of the En-GRN [1, 7]. Its ultimate function is to activate a small set of regulatory genes that set up the skeletogenic regulatory state, shown in Fig. 1A, i.e., the *tbr, alx1, tel, ets, soxC* and *delta* genes. The *es (early signal)* gene is an unknown gene responsible for modification of a TGFβ ligand [8], activation of which is apparently also a target of the double negative gate. This double negative gate works as follows: When the skeletogenic lineage is born the founder cells inherit two transcriptional inputs, Otx and Tcf-β catenin, and these are used to activate the *pmar1* gene. This gene encodes a canonical repressor [9, 10], and it acts to prevent transcription of the *hesC* gene, as shown in the Figure. HesC encodes a second repressor that is soon zygotically activated globally, i.e., everywhere

except where *pmar1* is being expressed. The double negative gate target genes are all driven by ubiquitously present activators but are subject to dominant transcriptional repression by HesC. Thus they can only be expressed in the skeletogenic lineage in consequence of *pmar1* expression there. Though it cannot be reviewed here, note that there is a great deal of both *cis*- and *trans*-evidence specifically demonstrating this modus operandi [1, 7, 9-11], and unpublished *cis*-regulatory studies on *pmar1* and the double negative gate target genes).

Fig.1B shows a network subcircuit of exactly the same topology, redrawn from the Ec-GRN. This double negative gate, however, is composed of entirely different players. An early and potent specification input in the oral ectoderm is Nodal signaling [12]. The regulatory gene *gsc* is an immediate target of the Nodal signal transduction system in the oral ectoderm [3], and like *pmar1, gsc* encodes a repressor [13, 14]. Its target is the *sip1* gene, encoding another repressor. This double negative gate is thus unlocked in the oral ectoderm by expression of the Gsc repressor, and thereby target genes such as *foxG* are transcribed there while being repressed in the aboral ectoderm by Sip1. There is apparently an additional linkage in this double negative gate, for it is essentially held open by positive *foxG* feedback into the *gsc* gene (Fig.1B).

The double negative gate is not an uncommon subcircuit. An excellent example can be found in the specification of the *sim* domain in the early *Drosophila* embryo [15]. Double negative gates are encountered elsewhere in the sea urchin GRNs as well. For example, *blimp1* repression of *hesC* and *hesC* repression of *delta* ensures by the same kind of logic that *delta* is expressed where repressive *blimp1* had earlier been expressed, while *delta* is specifically prevented from expression elsewhere by HesC repression [11].

Fig. 1C, D illustrates two examples of **dynamic feedback lockdown** subcircuits, again drawn respectively from the En-GRN [1] and the Ec-GRN [3], and again constructed of entirely different genes. Here also, the topology bears a one to one relationship with the function. The topology here consists of an interlocking cluster of three positive feedback loops with additional feedback to a gene upstream of those included in the three loops. The output is to cause the territorial regulatory state to become independent of its prior specification inputs by stabilizing its current state; thus it is a progressive function. The subcircuits in Fig. 1C, D have the function of ensuring that the spatial regulatory state is maintained even though the specific specification inputs that set them up are transitory. In the skeletogenic territory, e.g., *pmar1* is only transiently expressed [7. 9. 10], and the three gene feedback (the linkages among the *erg, hex*, and *tgif* genes in Fig. 1C) suffices to stabilize expression of these genes once it is activated by inputs from the double negative gate target genes. The wiring of this subcircuit also includes an additional feedback from *tgif* to the upstream double negative gate target gene *alx1*. All of these genes later provide direct feeds into the differentiation genes downstream, so it can be said that the feedback lockdown subcircuit stabilizes the definitive differentiation regulatory state as well. Indeed if expression of any of the components of this subcircuit is interrupted, failure of skeletogenesis results [1]. The wiring of the three-gene aboral ectoderm feedback lockdown subcircuit (the linkages among *irxa, dlx*, and *lhx2.9* in Fig. 1D) is uncannily similar. Again many other examples can be adduced. Among them are a dynamic feedback lockdown subcircuit utilized in endomesoderm specification [16], in which a transient *blimp1* input gives rise to a long term dynamic feedback between the *gatae* gene and an *otx* cis-regulatory module [17], and a further feedback into the *otx* gene from one of its targets, the *bra* gene [18]. Again, this is a widely utilized design feature: to cite an example from a very different type of developmental process, the GRN subcircuit controlling pluripotency in mammalian ES cells also consists of three regulatory genes, *sox2, oct4*, and *nanog*, linked together by recursive mutual feedbacks [19]. As reviewed elsewhere these kinds of lockdown subcircuits are now a predictable feature of developmental specification systems [6].

In Fig. 1E, F, we consider the **community effect subcircuit.** This is an intra-territorial signaling subcircuit utilized in specification of territories composed of adjacent, similarly functioning cells. The key topological feature of this subcircuit is an intercellular feedback which is powered by a *cis*-regulatory apparatus controlling transcription of the signaling ligand. The output is coordination of regulatory state across the territory. In the case of Fig. 1e the ligand gene is *nodal*. Its particular *cis*-regulatory feature is that it responds to the same signal transduction system that it activates in recipient cells [20, 21]. Thus each cell of the territory both receives and expresses the Nodal signal. The Nodal ligand is diffusible. As confirmed by a model calculation [22], the consequence of this together with the feedback dependence of *nodal* gene expression is to smooth out local differences, and ultimately to force all the interconnected cells of the territory to generate similar levels of intracellular signaling (activated Smad in the case of Fig. 1E). The effect is to make the levels of transcription of downstream genes responding to the signal transduction system similar, i.e. to homogenize levels of expression of the regulatory state within the territory. There are additional features of this feedback circuitry not shown in the Figure. The oral ectoderm system in Fig. 1E runs at a steady state far below kinetic saturation, and given the intercellular feedback, this requires a damper on the system. In the oral ectoderm this is provided by one of a downstream genes also responding to the Nodal signal transduction apparatus, which encodes the Nodal antagonist Lefty [12, 22, 23]. In Fig. 1F is another example of exactly the same community effect "wiring," but this time the ligand gene is *wnt8* and the territory is the endomesoderm. Again the ligand gene responds to the same signal transduction system it stimulates, here Tcf-βCatenin [24,25], and again the cells of the territory both express and receive the signal. As with the other subcircuits considered here, community effect circuitry is not rare: several instances of the same or similar topology are to be found in a *Xenopus* GRN for early embryonic specification of endomesoderm [6, 26].

Additional canonical subcircuits that meet our criteria for GRN building block topologies have been discussed earlier. Among these is the very commonly encountered **exclusion effect subcircuit**. Many examples have been cited [27] in which specification of a given territory results in transcription of a gene encoding a repressor that targets a key regulatory gene required to generate an alternative regulatory state. This is an often cryptic device revealed when expression of the repressor is blocked, resulting in a fate switch to the alternative regulatory state. For instance, in the sea urchin embryo, endoderm is converted into pigment cells when repression in prospective endoderm cells of the mesodermal pigment cell regulator *gcm* by the endoderm factor FoxA is blocked [28]. The spatial logic function here is simply that cited above, if *x* then not *y* and if *y* then not *x*, where *x* and *y* are regulatory states. Finally, **differentiation gene battery subcircuits** coordinately control the spatial and temporal expression of sometimes very large sets of structural genes. These account for the specialized functions of the cell. The topological features of differentiation gene batteries are unique, as discussed extensively over the years [1, 6, 29]. Thus the many downstream genes of these batteries are similarly wired, in parallel, all responding to the same few regulators. This is rarely seen in the interior of GRNs, which consist of interconnections among regulatory genes most of which utilize a unique set of inputs. Furthermore, the drivers of differentiation gene batteries are frequently hooked up to the target genes sets in feed forward arrangements (e.g., see 1, 6, 30).

Discussion

Each of the types of subcircuit topology considered here is utilized for a particular biological purpose in the process of embryological development. In this basic feature they differ from conventional network "motifs" [31], such as simple feed forward or feed back loops, which are used ubiquitously in GRN circuitry for every different kind of biological purpose. The

subcircuit types discussed here sometimes include these very general motifs, as for example in the feed forward wiring of individual genes of differentiation gene batteries. But the structure of this motif does not capture the structure of the battery as a whole, our focus here. Similarly, the feedback lockdown circuits of Figs. 1C, D include but are not equivalent to simple feedback loops because they each contain several such. It is interesting that in both of the feedback lockdown cases figured here, as well as in the endomesoderm lockdown subcircuit discussed in text, not only are there three or more genes dynamically locked together, not just two, but they share an additional feature worth noting: a return linkage from what was initially a downstream gene to what was initially an upstream gene in the network. For instance in Fig, 1C, *tgif* feeds back to *alx1*; in Fig. 1D, *irx* feeds back to *tbx2/3*; in the endomesoderm loop, *bra* feeds back to *otx*.

The main general point is that the subcircuits considered in this paper are examples of canonical network devices specifically utilized to establish and mobilize the spatial regulatory states that drive the developmental process. They are functional components from which the GRN is assembled, and in this sense they can be regarded as building blocks of the overall GRN topology. They recur in diverse contexts because the problems they solve are common to developmental specification processes; these problems themselves recur in diverse contexts as each new regulatory state domain is set up.

A precise evolutionary corollary to the concept of developmental GRN building blocks is that the building blocks originated independently, at evolutionary stages preceding the complex, mosaic GRNs that we see in modern organisms. An interesting theoretical project would be to consider the diversity and progressivity of regulatory state patterns that could be generated just by these simple subcircuits (combined with inductive signaling). Of course we may be looking at only a tiny fraction of the whole repertoire of developmental GRN building blocks. Only extensive comparative analysis of additional GRNs across bilaterian development will reveal the actual dimensions of this repertoire. For one thing the most extensive developmental GRNs we have so far, those from sea urchin [32] and *Drosophila* [33] embryos, each represent certain types of embryological process [6, 34]. Postembryonic development and development from stem cells will most certainly operate with somewhat different devices, less dependant on fixed prior spatial regulatory states (see for example [35-37]). Perhaps each form of developmental process will be constructed out of its own set of building blocks. Or, to invert this argument, perhaps our understanding of the mechanisms of diverse forms of development will eventually be formalized in terms of these building block repertoires. The further consequences, were this to be the case, are not solely philosophical, as they would lead directly to rational and general practical concepts for physically re-creating or re-engineering any form of developmental process.

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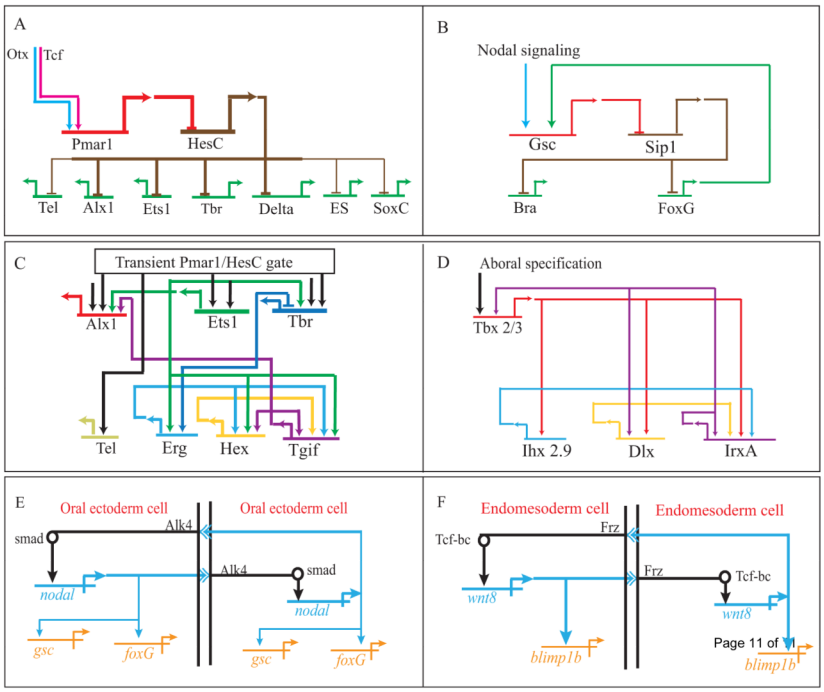

Fig. 1. Putative "building block" subcircuits of the sea urchin GRNs

(A, B) Double negative gate subcircuits. The first gene of the double negative gate is shown in red and the second in brown; target gene outputs are in green. In this and the following panels, thick lined linkages are backed by both trans-perturbation analysis and by direct published or unpublished cis-regulatory analysis, and thin lines are linkages deduced from trans-perturbation analysis. (A) Subcircuit from the skeletogenic mesoderm lineage (1); (B) Subcircuit from the oral ectoderm GRN (3). **(C, D) Dynamic feedback lockdown subcircuits.** The genes participating in the triple feedback subcircuit are colored in blue, yellow, and purple, and in each case an upstream gene receiving an additional feedback input from the triple loop is in red. (C) Subcircuit from the skeletogenic mesoderm lineage (1); (D) Subcircuit from the aboral ectoderm GRN (3). **(E, F) Community effect subcircuits.** The ligand genes are in blue, the signal transduction system is black, and downstream genes are in orange and red .(E) Subcircuit from the oral ectoderm GRN (22); (F) Subcircuit from the endomesoderm GRN (25).