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# Master regulators in development: views from the *Drosophila* retinal determination and mammalian pluripotency gene networks

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## Abstract

Among the mechanisms that steer cells to their correct fate during normal development, master regulatory networks are unique in their sufficiency to trigger a developmental program outside of its normal context. In this review we discuss the key features that underlie master regulatory potency during normal and ectopic development, focusing on two examples, the retinal determination gene network (RDGN) that directs eye development in the fruit fly and the pluripotency gene network (PGN) that maintains cell fate competency in the early mammalian embryo. In addition to the hierarchical transcriptional activation, extensive positive transcriptional feedback, and cooperative protein-protein interactions that enable master regulators to override competing cellular programs, recent evidence suggests that network topology must also be dynamic, with extensive rewiring of the interactions and feedback loops required to navigate the correct sequence of developmental transitions to reach a final fate. By synthesizing the *in vivo* evidence provided by the RDGN with the extensive mechanistic insight gleaned from the PGN, we highlight the unique regulatory capabilities that continual reorganization into new hierarchies confers on master control networks. We suggest that deeper understanding of such dynamics should be a priority, as accurate spatiotemporal remodeling of network topology will undoubtedly be essential for successful stem cell based therapeutic efforts.

## 1. Master regulatory networks in development

Each cell in a developing animal executes a defined sequence of events to reach its terminally differentiated state. Development is both progressive, in that the trajectory available to a cell narrows with each choice it makes, and deterministic, such that equivalent cells in different embryos take essentially the same path toward terminal differentiation. Waddington's epigenetic landscape, in which cells "roll" down a series of bifurcating valleys toward their ultimate fate, provides an intuitive model to explain these properties (Fig. 1A;

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Ferrell, 2012; Waddington, 1957). Hills between valleys stabilize trajectories and the landscape's downward slope limits retrograde motion. Meanwhile, at each fork in the path, cells can select either direction, but reliably roll left or right depending on their identity. Waddington's model raised a question that remains central to developmental biology research today: what are the mechanisms that instruct cells to follow reproducible trajectories appropriate and specific to their identities and spatiotemporal positions?

The concept of a "selector" gene, a term coined by Antonio García-Bellido to describe the deterministic partitioning of spatially distinct epithelial compartments by homeotic genes and later applied to the subdivision of the embryo by segment polarity genes, provided the first framework for considering how cells navigate Waddington's landscape (García-Bellido, 1975; Mann and Carroll, 2002; Mann and Morata, 2000). Selectors were defined as necessary and sufficient to confer positional information, but could not specify cellular identity, implying that additional genes with selector-like properties steer cells through downstream bifurcations as the accessible developmental paths narrow (Fig. 1A). This niche is occupied by "master regulators", transcription factors whose activities are necessary and sufficient to direct specific developmental trajectories (Allan and Thor, 2015; Mann and Carroll, 2002; Pradel and White, 1998). While the terms "master regulator" and "selector" have historically referred to genes or networks that operate at different stages of development, the overwhelming similarity between their properties and organizations suggests they are actually context-specific variants of a fundamental regulatory strategy. Therefore, while we use the term "master regulator" in this review, the concepts we discuss are equally relevant to the classic "selectors".

Important insight into the functional complexity inherent to master control genes began to emerge more than twenty years ago with the discovery that misexpression of Eyeless (Ey), a *Drosophila* Pax6 family transcription factor, could hijack the developmental programs of a limited subset of larval epithelial cells and convert them to retina (Halder et al., 1995). Based on its sufficiency for eye development, its discoverers proposed that Ey functions as a master regulator of organogenesis, sitting atop a hierarchy of genes whose ordered expression in response to even a transient burst of *ey* could initiate retinal development (Gehring, 1996).

Subsequent investigations revealed three members of this postulated hierarchy: *eyes absent* (*eya*), *sine oculis* (*so*), and *dachshund* (*dac*) (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997). However, these genes not only fulfill the prediction of operating downstream of *ey* (Gehring, 1996), but their ectopic expression also activates *ey* expression (Bonini et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997). Based on this latter finding, the field proposed that rather than acting as a simple linear pathway, positive transcriptional feedback organized these four master control genes into an interconnected retinal determination gene network (RDGN) (Desplan, 1997).

Organization into self-reinforcing collections of transcription factors is now known to constitute an essential feature of master regulators. Master control genes function in networks across kingdoms and in a variety of developmental contexts, ranging from establishment of mammalian embryonic pluripotency to plant organogenesis to myogenesis (Aziz et al., 2010; Chan and Kyba, 2013; Ciglar and Furlong, 2009; Desplan, 1997; Hamdi

et al., 1987; Jaenisch and Young, 2008; Kumar, 2009; Nambu et al., 1991; Ó'Maoiléidigh et al., 2014; Ohno, 1979; Silva et al., 2016; Siriwardana and Lamb, 2012; Takahashi and Yamanaka, 2015; Tapscott, 2005). Positive cross-regulation within these hierarchies has been proposed to assemble unique linear pathways tailored to the control of specific cellular events and to produce the bistable switch-like responses associated with cell fate decisions, consequently expanding the number of distinct instructions that master regulators provide in a manner that would be impossible for a single transcription factor (Desplan, 1997; Ferrell Jr, 2002; Ferrell Jr and Xiong, 2001; Mitrophanov and Groisman, 2008). Therefore, rather than dictating single decisions, master control networks act across time to negotiate the complex sequences of events that comprise developmental programs. The possible mechanisms that switch network behaviors provide a major point of discussion in this review.

The recent discovery that RDGN proteins not only promote, but also inhibit, the expression and activity of other network members (Atkins et al., 2013) has expanded our understanding of the regulatory potential afforded by organizing master control genes into networks. Specifically, by rearranging its component transcription factors into different regulatory hierarchies depending on context, the RDGN instructs and stabilizes developmental transitions. Thus, as retinal progenitor cells progress through development, the RDGN reconfigures its topology to propel them from proliferation to specification to differentiation. More broadly, rewiring of network relationships introduces flexibility and dynamics to master control activity and suggests that the networks themselves can break any given positive feedback loop to terminate one cellular behavior and initiate a new one, ensuring deterministic navigation of Waddington's landscape. While a single autoactivating master regulator would seem sufficient to initiate a particular developmental program, we argue that the coordinated termination of previous regimes, initiation of new trajectories, and antagonism of alternative paths means that robust negotiation of developmental transitions demands the more complex regulatory capabilities of a network. In this review, we examine the similarities and differences between the RDGN and the mammalian pluripotency gene network (PGN) with the goal of understanding how master regulatory networks dynamically rewire themselves to govern developmental transitions.

## 2. Introduction to *Drosophila* retinal development

RDGN transcription factors direct the progression of *Drosophila* eye development. During embryogenesis, Ey, together with its paralog Twin of Eyeless (Toy), marks a pool of about 80 cells that will become the progenitors of the larval eye-antennal imaginal disc (Czerny et al., 1999; Justin P. Kumar, 2011; Younossi-Hartenstein et al., 1993). Asynchronous proliferation expands this cell population more than fifty-fold during the first two larval instars (Justin P Kumar, 2011; Martin, 1982). In the second instar, elimination of Ey and Toy from the antennal portion of the disc establishes regional identity and is followed by induction of Eya, So, and Dac expression in the presumptive eye field (Fig. 1B) (Halder et al., 1998; Kenyon et al., 2003; Kumar and Moses, 2001; Wang and Sun, 2012; Younossi-Hartenstein et al., 1993). This sequence of events sets the stage for a burst of Decapentaplegic (Dpp) and Hedgehog (Hh) activity that halts proliferation and triggers photoreceptor specification and ommatidial assembly in a wave known as the morphogenetic

furrow (MF), which traverses the eye field over the next two days of development (Chanut and Heberlein, 1997; Curtiss and Mlodzik, 2000; Dominguez and Hafen, 1997; Greenwood and Struhl, 1999a; Ready et al., 1976; Tanya Wolff and Ready, 1991). These molecular and cellular activities have been reviewed extensively and will not be discussed here (Kumar, 2013; Treisman, 2013).

The regulatory relationships and interactions that define the RDGN as a master regulatory network occur in cells anterior to the MF, in a domain referred to as the preproneural region (Bessa et al., 2002; Greenwood and Struhl, 1999b). In this region, the core RDGN factors cooperate with Dpp and Hh signals to orchestrate the transition from proliferative progenitor to specified retinal cell type (Bessa et al., 2002; Curtiss and Mlodzik, 2000; Escudero and Freeman, 2007; Kango-Singh et al., 2003; Ready et al., 1976; Wolff and Ready, 1991). As will be discussed below, passage of the MF rewires the RDGN, leaving only Eya, So and Dac to contribute to the gene expression changes associated with ommatidial cell differentiation.

## 3. Introduction to mammalian pre-implantation development

The pluripotency gene network (PGN), a more recently discovered master regulatory network comprising Octamer-binding transcription factor 4 (Oct4), Nanog, SRY-box 2 (Sox2), and Spalt-like transcription factor 4 (Sall4), maintains pluripotency during the pre-implantation stages of mammalian embryonic development. Maternally supplied mRNA encoding these proteins can be detected in single-celled zygotes, which give rise to all cells in the adult organism (Guo et al., 2010; Keramari et al., 2010; Pan and Schultz, 2011; Tan et al., 2013). Zygotic cell division generates two totipotent blastomeres that repeatedly divide over the first three days of development and continue to express PGN transcripts (Fig. 1C) (Boroviak and Nichols, 2014; Guo et al., 2010; Morgani and Brickman, 2014). Embryonic genome activation begins in the first pair of blastomeres and increases in a second wave by the eight cell stage (Li et al., 2010). Once eight cells are present, Nanog is highly expressed and blastomeres increase their surface area of contact in a process termed compaction, which forms the morula (Li et al., 2010). At this stage, the first fate specification events occur as asymmetric cell divisions generate two different cell types: the pluripotent inner cell mass (ICM), in which Oct4, Sox2, and Nanog are highly expressed, and the extra-embryonic trophectoderm precursors, which express Sox2 (Bedzhov et al., 2014; Chen et al., 2009; Keramari et al., 2010; Niwa et al., 2005; Ralston et al., 2010; Strumpf et al., 2005). Initially, the ICM contains a mixture of cells that express high or low Nanog, but this tissue subsequently sorts into the Nanog-positive, pluripotent epiblast and Nanog-negative primitive endoderm by the late blastocyst stage (Morgani and Brickman, 2014). Soon afterward, blastocysts hatch and implant into the uterine wall, completing pre-implantation development (Frum and Ralston, 2015).

## 4. Defining the members of master regulatory networks

Historically, the genes comprising the RDGN and PGN are loosely defined, with anywhere from one to dozens of proteins categorized as master regulators. Based on our idea that a high degree of connectivity is essential for master control network function, to identify

objectively the genes that participate in the cores of these networks we created a simple scoring scheme that emphasizes physical and regulatory links between transcription factors. Our first criterion was sufficiency to induce the target developmental program, as this ability is historically the benchmark that sets apart master control genes. Second, we only ranked proteins that control transcription, as other molecules, such as those that transduce intracellular signals, are generally deployed throughout the organism and therefore cannot confer the tissue specificity required of a master regulator. Third, we grounded our analysis in endogenous biology by requiring that candidates be necessary for and expressed in the developing retina or blastocyst, as appropriate. Finally, we counted nodes of interaction with other potential master regulators by separately scoring regulation of other candidates, regulation by other candidates, and protein-protein interactions. We envision that almost complete interconnectedness defines master control networks, such that each member directly governs the transcription of every other. Therefore, downstream ancillary sub-networks, which regulate gene expression to direct specific cellular activities, will share many features with master regulators but exhibit less extensive feedback. Tables 1 and 2 summarize our application of these criteria to the literature.

Our analysis of the RDGN considered all transcriptional regulators that can induce ectopic eyes when misexpressed in the fly: Ey, Eya, So, Dac, Toy, Teashirt (Tsh), Tiptop (Tio), Eyegone (Eyg), Twin of eyegone (Toe), Optix, Distal antenna (Dan), and Distal antenna-related (Danr) (Bessa et al., 2009; Bonini et al., 1997; Curtiss et al., 2007; Czerny et al., 1999; Datta et al., 2009; Halder et al., 1995; Jang et al., 2003; Li et al., 2013; Pan and Rubin, 1998; Seimiya and Gehring, 2000; Shen and Mardon, 1997; Weasner et al., 2007; Yao et al., 2008). The scoring scheme reaffirmed Ey, Eya, and So as core RDGN members, consistent with the prevailing opinion in the field (Table 1, Figs. 2–3). Assigning master regulatory status to Dac, Dan, and Danr, the next three highest ranked proteins, was more difficult. Although one option was to include or exclude them as a group, we noted that Dac, but not Dan or Danr, scored positively in all six categories. Given that Ey, Eya and So also scored positively in all categories, we set that pattern as the standard for inclusion in the network, and so for the purpose of this review we consider Ey, Eya, So and Dac to constitute the core of the RDGN. Undoubtedly, some of the lower scores simply reflect the currently incomplete understanding of molecular function and regulatory interactions, particularly with respect to transcriptional circuitries. Thus, as new data convert tentative regulatory relationships into defined mechanisms, genes like *dan* and *danr* that currently fall just below the bar may ultimately gain core membership

To define the core PGN master regulators, we performed a similar analysis to that described above for the RDGN. As a preface, we note that as opposed to the RDGN, where essentially all knowledge derives from experiments performed in the developing animal, much of the current understanding of pluripotency derives from work in cultured ESCs or induced pluripotent SCs (iPSCs) owing to the difficulty of experimentation in early mammalian embryos. While we incorporate numerous insights from cultured SCs throughout this review, we consider only genes whose genetic requirement for pluripotency has been demonstrated in the early embryo to be eligible for core PGN status. Also in contrast to the RDGN, for which the ability to induce ectopic retinal tissue provides a stringent test of sufficiency that defines a manageable number of candidate regulators, studies of mammalian cell

pluripotency have produced an extensive literature on the reprogramming of somatic cells by application of small molecule cocktails, signaling pathway inhibitors, expression of miRNAs, co-expression of competing lineage specifiers, or substitution of PGN proteins with downstream targets (Anokye-Danso et al., 2011; Buganim et al., 2012; Chen et al., 2011; Ichida et al., 2009; Li et al., 2011; Lyssiotis et al., 2009; Miyoshi et al., 2011; Montserrat et al., 2013; Moon et al., 2011; Redmer et al., 2011; Shu et al., 2013; Staerk et al., 2011). Because most of these strategies are unlikely to regulate pluripotency in the early embryo, we limited our analysis to transcription factors or cofactors that can induce pluripotency on their own or in combination with other transcriptional proteins: Oct4, Nanog, Sox2, Sall4, Estrogen-related receptor beta (Esrrb), Kruppel-like factor 4 (Klf4), Nuclear receptor subfamily 5 group A member 2 (Nr5a2), Geminin (Gmnn), c-Myc and GATA-binding protein 3 (Gata3) (Feng et al., 2009; Festuccia et al., 2012; Heng et al., 2010; Kim et al., 2009a, 2009b; Li et al., 2011; Montserrat et al., 2013; Shu et al., 2015, 2013; Silva et al., 2009; Stuart et al., 2014; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Thorold W Theunissen et al., 2011; Tsai et al., 2011; Tsubooka et al., 2009; Yu et al., 2007; Zhu et al., 2010).

Just as our rankings of RDGN factors highlighted the key roles of Eya, So, and Ey, so Oct4 and Nanog emerged atop the PGN list (Table 2, Figs. 4–5). We note some controversy in the literature regarding Nanog's sufficiency for pluripotency. In some reprogramming experiments Nanog appears expendable, while in others it is required to re-establish pluripotency (Carter et al., 2014; Schwarz et al., 2014; Silva et al., 2009; Stuart et al., 2014; Thorold W. Theunissen et al., 2011; Yu et al., 2007). Closer examination of these seemingly contradictory results suggests that Nanog is dispensable only under specific experimental conditions that mechanistically compensate for its loss, such as exogenous provision of its downstream transcriptional targets (Costa et al., 2013; Festuccia et al., 2012; Schwarz et al., 2014; Stuart et al., 2014). In light of these data, we consider Nanog a fully-fledged PGN member given its requirement for pluripotency in the embryo and its high connectivity score (Table 2).

As with our analysis of candidate RDGN master regulators, we awarded middle-ranked pluripotency factors core PGN status only if they received positive scores in all categories. Sall4 and Sox2 achieved that standard, while Esrrb and Klf4 did not (Table 2). Although Klf4 was a member of the original Yamanaka reprogramming quartet and inhibits differentiation in mouse ESCs, to date no studies have evaluated its requirement for pluripotency *in vivo* and so we do not consider it a core PGN factor (Li et al., 2005; Takahashi and Yamanaka, 2006; Zhang et al., 2010). However, based on its extensive connectivity with Oct4, Nanog, Sall4, and Sox2, Klf4 features prominently in the discussion below, and we suspect future studies will lead to its inclusion in that select group of master regulators. c-Myc, the fourth Yamanaka factor, has since been shown to be dispensable for iPSC induction, is only required for pluripotency in cultured ESCs, and has no known regulatory connections to other PGN members, eliminating it from further discussion (Cartwright et al., 2005; Nakagawa et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2008).

## 5. Self-reinforcing transcriptional feedback and cooperative protein-protein interactions are essential for master regulatory activity

Mutual potentiation of expression and activity of genes within core networks has long been considered an essential master regulatory feature (Bonini et al., 1997; Desplan, 1997; Pignoni et al., 1997; Shen and Mardon, 1997). For example, Ey initiates transcription of *eya* and *so*, whose translated protein products associate to form a bipartite transcription factor that positively feeds back to sustain *ey* expression (Atkins et al., 2013; Bonini et al., 1997; Halder et al., 1998; Niimi et al., 1999; Ostrin et al., 2006; Pauli et al., 2005; Pignoni et al., 1997). This regulatory structure appears critical to the RDGN's sufficiency to induce retinal tissue, as *ey* and *eya* mutually activate each other's transcription in misexpression experiments and neither gene can induce ectopic eyes if the other is mutated (Bonini et al., 1997).

More recent work affirms the relevance of this positive feedback loop to RDGN function in normal eye development, although with a few twists (Atkins et al., 2013). Briefly, loss of *so* in the preproneural region anterior to the MF reduced Ey expression, consistent with loss of positive feedback; direct transcriptional activation was confirmed using enhancer-reporter transgenes. Surprisingly, and contradicting predictions from the ectopic eye induction experiments mentioned above, this positive feedback loop appears to be Eya-independent, as *eya* loss-of-function clones in the same region did not reduce Ey expression. The finding that So operates independently of its usual co-activator to positively feed back onto *ey* during normal retinal development challenges the model of Eya-So as obligate partners within the RDGN hierarchy. Equally unexpected was the finding that co-overexpression of Eya and So, at levels that induce *ey* expression and ectopic eye induction in other imaginal discs, actually repressed Ey levels anterior to the MF; one possible explanation is that the absence of Dpp and Hh signaling that normally accompanies the onset of preproneural Eya and So expression determines the switch in direction of *ey* regulation. Together, these results suggest that RDGN topology is exquisitely sensitive to the levels of its core factors and signaling environment such that network relationships deduced from overexpression results may not precisely match the interactions and outputs used in the analogous context during normal retinal development.

Given the challenges in revealing the network topology of the genetically tractable *Drosophila* system, the obstacles to unraveling the PGN's internal regulatory structure in the early mammalian embryo are even greater. As mentioned above, most of the PGN is maternally deposited and expressed in pluripotent cells throughout pre-implantation development, making it difficult to determine which gene(s) reside atop the PGN hierarchy (Guo et al., 2010; Keramari et al., 2010; Pan and Schultz, 2011; Tan et al., 2013). Oct4 has been proposed to act as the most upstream PGN transcription factor that initiates the zygotic expression of the rest of the PGN, as it has been detected throughout pre-implantation development, regulates embryonic transcription by the two-cell stage when zygotic gene expression begins, can directly activate transcription of the core PGN factors, and helps control the maternal-zygotic transition (MZT) (Catena et al., 2004; Cauffman et al., 2005; Foygel et al., 2008; Jaenisch and Young, 2008; Kuroda et al., 2005; Okamoto et al., 1990;

Okumura-Nakanishi et al., 2005; Ovitt and Schöler, 1998; Palmieri et al., 1994; Rodda et al., 2005; Rosner et al., 1990). However, a rigorous analysis that deleted both maternal and zygotic *oct4 in vivo* found that neither *nanog* nor *sox2* expression was affected until well after the MZT was complete (Wu et al., 2013). Furthermore, the normal fertility of female mice used in these experiments, whose germlines lacked *oct4*, eliminates the hypothesis that *oct4* is an important MZT regulator.

While the PGN's topology during pre-implantation development remains unclear, positive reinforcement of transcription within the network is thought to maintain the appropriate zygotic levels of expression, similar to the RDGN (Niwa, 2007; Rizzino, 2009). Based on results from cell culture and transcriptional reporters, extensive direct transcriptional activation can occur within the PGN, but evidence that these relationships exist *in vivo* is indirect (Fig. 4) (Catena et al., 2004; Chan et al., 2009; Feng et al., 2009; Festuccia et al., 2012; Jiang et al., 2008; Kuroda et al., 2005; Lim et al., 2008; Masui et al., 2007; Okumura-Nakanishi et al., 2005; Rodda et al., 2005; van den Berg et al., 2010; Wei et al., 2013; Q. Wu et al., 2006; Yang et al., 2010, 2008, Zhang et al., 2013, 2010, 2008; J. Zhang et al., 2006). For example, Sox2 deletion in the epiblast strongly reduces *oct4* expression, but it is unclear to what extent the absence of direct *oct4* activation by Sox2 contributes to this result (Avilion, 2003). These questions reinforce the lesson introduced by the RDGN that evaluating transcriptional cross-regulation in the endogenous developmental context will be essential to elucidating true network topologies.

For both the RDGN and the PGN, the advent of CRISPR-based genome engineering technologies creates opportunities to evaluate the physiological relevance of positive feedback and to test specific hypotheses regarding the contributions of hierarchical regulation to normal network output. For instance, one unanswered question is to what extent positive cross-regulation is required for complete RDGN activation in the preproneural domain. Although both Ey and So directly activate *so* transcription, the relative contributions of these two inputs to *so* expression are not clear. If Ey initiates and So maintains *so* expression, then an Ey binding site mutation at the appropriate enhancer should eliminate expression, while a So binding site mutation should lead to a burst of *so* activation followed by decay once Ey is not longer expressed. Alternatively, if synergistic activation of *so* by both Ey and So is required for high expression levels, then mutating either binding site should significantly abrogate expression. Similar experiments can answer fundamental questions about transcriptional cross-regulation within the PGN.

## 6. Negative feedback attenuates master regulatory activities and rewires gene networks to permit developmental progression

The potency of master control networks to induce their target genetic program means that they must also be inactivated in contexts where they would be detrimental. For example, prolonging preproneural RDGN activity by maintaining *ey* expression posterior to the MF prevents retinal specification (Atkins et al., 2013). Extending the window of PGN activity would presumably similarly stall the progression of embryonic development. We propose that context-specific network rewiring, or the switching of regulatory relationships to

assemble new hierarchies, explains how master control networks can alter their behavior to activate a given cellular program in one context and attenuate it in others, and that such complexity is required to ensure precise and robust execution of an entire developmental progression. This mechanistic strategy explains how master regulatory hierarchies actively instruct a sequence of cellular decisions as cells negotiate bifurcations in Waddington's landscape.

The RDGN provides two clear examples where transcriptional activation switches to inhibition to direct a developmental transition. An abrupt change in RDGN expression dynamics marks the first. All four core RD proteins are co-expressed in the unspecified retinal precursors immediately anterior to the MF, but after the furrow passes, Ey is no longer detected, while levels of Eya, So, and Dac remain high (Bessa et al., 2002; Bras-Pereira et al., 2015; Halder et al., 1998). Switching off Ey expression is required for photoreceptor fate specification, since mitotic clones that inappropriately maintain high Ey levels fail to activate markers of neuronal differentiation (Atkins et al., 2013). To achieve this transition during normal development, the positive feedback loop that maintains anterior Ey expression must be interrupted. Recent work reveals that this inhibition results from rewiring the network such that Eya-So directly represses *ey* transcription in differentiating cells (Atkins et al., 2013).

How this activity switch is achieved is not yet understood molecularly, but the mechanism almost certainly results from different stoichiometry and composition of the transcriptional complexes that assemble at the *ey* locus anterior versus posterior to the MF. As mentioned above, Atkins *et al.* conclude that *eya* is not required for the positive feedback loop that maintains high *ey* levels anterior to the MF, though it is formally possible that sufficient residual Eya protein perdured in *eya* clones to assemble co-activating transcriptional complexes with So. In contrast, posterior to the MF, Eya and So cooperate, but to repress *ey*. The authors speculate that high levels of Eya, which are maximal posterior to the MF, are needed for repression, while lower levels may be used for activation. In support of this idea, they showed that increasing the levels of Eya and So anterior to the MF could reduce Ey expression. Dac is also required for *ey* repression posterior to the MF and can cooperate with *eya* and *so* to inhibit *ey* transcription in anterior overexpression clones, hinting that distinct RD protein complexes may underlie transcriptional switching at this locus (Atkins et al., 2013). One idea is that Dac joins Eya-So to confer repressive activity, as mammalian Dach1 can recruit co-repressors and directly repress target gene transcription, though analogous activities have not been confirmed in *Drosophila* (Chen et al., 2013; Chu et al., 2014; Li, 2002; K. Wu et al., 2006; Wu et al., 2011, 2009, 2008, 2007, 2003; Zhao et al., 2015). Furthermore, mammalian Eya, Six, and Dach proteins can assemble complexes that differentially regulate transcription according to their composition; in that system, addition of Eya is thought to convert repressive Six-Dach complexes to activating Eya-Six-Dach complexes (Li, 2002; Li et al., 2003). The observation by Atkins *et al.* that neither *eya* nor *dac* are required for *ey* activation anterior to the MF in the fly supports a different model in which posterior Eya-So-Dac complexes repress *ey* transcription while So activates *ey* in the anterior.

A second example of regulatory switching coordinates proliferation and specification in retinal progenitors. In these cells, Ey participates in transcriptional complexes with Homothorax (Hth) and Tsh to promote sustained proliferation and prevent premature transcription of downstream RD transcription factors (Bessa et al., 2002; Peng et al., 2009). While activation of *bantam* (*ban*) expression by Hth-Yorkie (Yki) complexes likely drives proliferation, how Ey is prevented from accessing and/or activating core RDGN loci prematurely is not known. Competency to switch from proliferation to specification is initiated when Ey activates transcription of *eya* and *so*, which in turn reinforces *ey* expression and promotes *dac* transcription (Anderson et al., 2006; Atkins et al., 2013; Bonini et al., 1997; Chen et al., 1997; Halder et al., 1998; Niimi et al., 1999; Ostrin et al., 2006; Pappu et al., 2005; Pignoni et al., 1997; Salzer and Kumar, 2009a). Dac then terminates the pro-proliferative of Hth-Yki complexes by inhibiting Hth expression and interfering with the ability of Hth-Yki to activate *ban* transcription (Brás-Pereira et al., 2015). Subsequently, Ey cooperates with Eya-So to activate *atonal* transcription, which specifies the first photoreceptor to initiate ommatidial assembly (Jemc and Rebay, 2007; T. Zhang et al., 2006; Zhou et al., 2014). Thus, mutual inhibition between the Ey-Hth-Tsh and Ey-Eya-So-Dac hierarchies shepherds precursors from asynchronous proliferation to coordinated differentiation. As in the first switching example, Dac appears to be a key player in the transcriptional repression events that drive this developmental transition. Whether Dac directly represses *hth* and *ban* or functions as a co-factor to recruit other transcriptional repressors in these contexts is an important question for the future.

Negative feedback also limits PGN activity to terminate the pluripotency-sustaining program and permit differentiation. The regulatory circuit centers on microRNA-145 (miR-145), which interacts with the 3’ UTRs of the *oct4, sox2*, and *klf4* to inhibit their translation and antagonize pluripotency (Xu et al., 2009). Consistent with this role, miR-145 expression is low in pluripotent cells and high in differentiating SCs. Furthermore, in ESCs, Oct4 binds the *miR-145* promoter and represses its transcription (Xu et al., 2009). Thus, the current model is that in pluripotent cells, low levels of miR-145 attenuate Oct4, Sox2, or Klf4 levels, thereby preventing runaway PGN activity. When differentiation begins, repression by Oct4 is relieved, though it is unclear whether this switch reflects a change in occupancy or transcriptional activity, and the resulting higher miR-145 levels block *oct4, sox2*, and *klf4* translation to terminate the pluripotency program.

In the three examples above, the switch in regulatory behavior most likely stems from differentially expressed binding partners that change the composition, activity, and target gene specificity of the transcriptional complexes. Evidence for such a combinatorial transcription factor code that dictates enhancer specificity has emerged from a study of the genome-wide occupancy dynamics of the PGN factors Oct4 and Sox2 (Aksoy et al., 2013). During the early stages of embryogenesis, when cells remain fully pluripotent, Oct4 and Sox2 occupy enhancers that regulate transcription at loci that promote pluripotency (Ambrosetti et al., 1997; Kuroda et al., 2005; Rodda et al., 2005; Wang et al., 2007; Yu et al., 2012). As cells transition out of pluripotency, both Oct4 and Sox2 pair with new binding partners, shifting enhancer specificity to activate lineage-specific programs of gene expression (Aksoy et al., 2013; Frum et al., 2013; Jin et al., 2009; Le Bin et al., 2014; Lodato et al., 2013; Niwa et al., 2000; Stefanovic et al., 2009; Thomson et al., 2011; Wang et

al., 2012). For example, in the contexts of primitive endoderm and cardiac specification, Oct4 replaces Sox2 with Sox17 (Aksoy et al., 2013; Stefanovic et al., 2009). The ensuing change in the Oct-Sox consensus binding site configurations recognized by Oct4-Sox17 globally alters Oct4 occupancy such that it now activates the endodermal program of gene expression (Aksoy et al., 2013). Analogously, Sox2 swaps Oct4 for Brn2, which alters enhancer specificity to initiate the regulatory switch toward neural specification (Jin et al., 2009; Lodato et al., 2013). While these regulatory transitions are clear, exactly how they are effected is not. Both examples predict that correct lineage specification requires cells to transition through unstable intermediate states in which Sox2 and Sox17 compete for Oct4, and Oct4 and Brn2 compete for Sox2. In support of this model, single blastomere expression analyses of 64-cell mouse embryos detected coexpression of Oct4, Sox2 and Sox17 (Aksoy et al., 2013; Guo et al., 2010). How the relative magnitude of these complexes' effects on transcription or additional components may contribute to these deterministic developmental transitions has not been explored.

Although changes in genome-wide occupancy and enhancer recognition specificity have not yet been examined for different RDGN complexes, such regulation undoubtedly contributes to developmental transitions. Central to such mechanisms is the combinatorial recognition of different patterns of binding motifs by different sets of transcription factors. However, not all RDGN factors are sequence-specific DNA binding proteins; in fact, Eya and Dac lack obvious sequence specific binding activity (Hammond et al., 1998; Mardon et al., 1994; Pignoni et al., 1997). Adding or subtracting these cofactors to different master regulatory complexes may not drive target gene specificity, but modulating the strength or direction of transcriptional regulation could enable as dramatic a developmental switch as regulating different genes. The conversion of Eya-So from activator to repressor of *ey* transcription is a prime example. In addition to the possibility that Dac confers repressive activity to Eya-So (see discussion above), other transcriptional regulators may contribute. The most obvious candidate co-repressor, Groucho (Gro), which can bind both Eya and So, was ruled out because *gro* mutant tissue posterior to the MF does not de-repress *ey* (Atkins et al., 2013; Goldstein et al., 2005; Kenyon et al., 2005; Silver et al., 2003). Another tantalizing but untested candidate is Sine oculis binding protein (Sbp), a So co-factor that is exclusively expressed in the cells where Eya-So represses *ey* transcription and whose overexpression in retinal precursors stalls their development (Kenyon et al., 2005).

PGN proteins may also switch their direction of transcriptional regulation to drive developmental transitions. Consistent with this idea, Oct4 and Nanog can both activate and repress target gene expression (Catena et al., 2004; Ezashi et al., 2001; Guo et al., 2002; Hammachi et al., 2012; Kuroda et al., 2005; Liang et al., 2008; Liu and Roberts, 1996; Navarro et al., 2012; Pan et al., 2002, 2006; Pan and Pei, 2005; Rodda et al., 2005; Torres and Watt, 2008; Zhang et al., 2008). A particularly intriguing observation is that depending on the cell line and reporter construct used, Oct4 directly activates or represses *nanog* expression (Kuroda et al., 2005; Pan et al., 2006; Rodda et al., 2005). Given that Oct4 both initiates differentiation and maintains pluripotency *in vivo*, perhaps repression of *nanog* helps terminate pluripotency and initiate tissue specification via a mechanism that resembles *ey* inhibition by Eya-So to allow photoreceptor differentiation in the *Drosophila* eye (Aksoy et al., 2013; Frum et al., 2013; Le Bin et al., 2014; Niwa et al., 2000; Stefanovic et al., 2009;

Thomson et al., 2011; Wang et al., 2012). Signaling pathways that initiate differentiation may regulate these switching behaviors; for example, FGF signaling appears dispensable for pluripotency but is required for primitive endoderm differentiation (Huang et al., 2015).

## 7. Master regulators promote developmental robustness by inhibiting competing gene regulatory networks

To ensure that cells reproducibly select the correct path down Waddington's landscape, each time a master control network makes a developmental choice, it both promotes transcription of genes that carry out the desired cellular activity and represses expression of genes that oppose that choice. This strategy prevents the instability that would be caused by simultaneous activation of multiple self-reinforcing genetic programs. Given the potency of positive feedback, prolonged antagonism of competing master regulatory networks and mutual negative feedback regulation between competing networks stabilizes cell fate decisions and makes developmental transitions irreversible.

RD proteins robustly generate eyes by inhibiting head cuticle and antennal fates. Early in larval *Drosophila* eye development, the initially overlapping expression patterns of Ey and the transcription factor Cut become restricted to the eye and antenna, respectively, marking the first determination of fates in this tissue (Kenyon et al., 2003). Molecularly, regional identity is actively maintained, as Cut and its cofactor Homothorax (Hth) directly repress transcription of *ey* in the antenna, while Ey inhibits Distalless (Dll) expression and Eya-So inhibits Hth, Tsh, Cut, Lim1, and Wingless expression in the retina (Bessa et al., 2002; Hazelett et al., 1998; Punzo et al., 2004; Salzer and Kumar, 2009b; Treisman and Rubin, 1995; Wang and Sun, 2012; Weasner and Kumar, 2013). Mutual repression of competing fates is critical for normal development, as retinal cells that do not express *eya* or *so* inappropriately take on head fate, while antennal cells lacking *cut* and *hth* turn on the RDGN and generate ectopic eyes (Salzer and Kumar, 2009b; Wang and Sun, 2012; Weasner and Kumar, 2013). The ability to suppress competing genetic programs may also contribute to the tissue-specific competence of ectopic RDGN expression to initiate retinal development outside the normal visual field (Salzer and Kumar, 2010).

PGN transcription factors also couple positive regulation of the desired developmental trajectory with active inhibition of alternate programs. Similar to the Ey-Cut relationship in the developing eye, Oct4 and Nanog are initially co-expressed with the transcription factor Cdx2 in early morulae (Chen et al., 2009; Niwa et al., 2005; Strumpf et al., 2005; Wu et al., 2013). By the blastocyst stage, Oct4 and Nanog are restricted to the inner cell mass, while Cdx2 is found in the outer cells that will form trophectoderm; this separation is required for differentiation to occur (Chen et al., 2009; Niwa et al., 2005; Strumpf et al., 2005; Wu et al., 2013). Mechanistically, and again reminiscent of the relationship between the RDGN and the head-antennal transcription factors, Oct4 and Nanog directly repress transcription of *cdx2* and *vice versa* (Chen et al., 2009; Niwa et al., 2005; Strumpf et al., 2005; Wu et al., 2013; Yeap et al., 2009). The PGN also utilizes mutual inhibition to stabilize the early pluripotency program. Oct4 and its target *miR-302* directly repress *nr2f2* to prevent neural differentiation in human ESCs, but when differentiation begins this regulation is reversed;

similarly, Oct4 directly represses *miR-145* in pluripotent cells, but *miR-145* prevents translation of *oct4, sox2*, and *klf4* mRNAs in differentiating cells (Rosa and Brivanlou, 2011; Xu et al., 2009). Thus, mutual repression between master regulators and competing factors stabilizes genetic programs and governs the transition between developmental states.

## 8. Interactions with epigenetic machinery expand the regulatory repertoire of master control networks

As cells progress through Waddington's landscape, epigenetic regulation of gene expression maintains choices that were made earlier in development and prevents retrograde motion away from their target fate. Chromatin modification can also augment and maintain the results of direct transcriptional regulation as cells switch from activating to repressing target loci during developmental transitions. Thus, while mechanistic understanding is still limited, master control networks likely diversify their regulatory capability by modulating cells' epigenetic states.

Connections between chromatin modifying proteins and the transcription factors of the RDGN and PGN underscore the need for communication between master regulators and the epigenome. For example, in a phenotype reminiscent of *eya, so*, or *dac* loss, deletion of repressive Polycomb Group (PcG) genes leads to ectopic Hth and Tsh expression posterior to the MF (Janody, 2004). Based on these results, one idea is that RD transcription factors recruit PcG proteins to aid in switching cells from unspecified, proliferative precursors to differentiating retinal cells. Dac is the best candidate to interface with this chromatin-remodeling complex, as it terminates the asynchronous proliferation program in the preproneural domain by turning off Hth expression and recruits co-repressors in mammals (Brás-Pereira et al., 2015). Focusing on the PGN, proteome-wide interaction studies have identified association of Oct4, Sox2, or Nanog with at least five distinct chromatin modifying complexes (Ding et al., 2012; Gagliardi et al., 2013; Gao et al., 2012; Pardo et al., 2010; van den Berg et al., 2010). The NuRD repressor and MLL activator complexes, both of which interact with Oct4, are required for ESC pluripotency and reprogramming, suggesting that these relationships are functional in the context of pluripotency (Ang et al., 2011; Dos Santos et al., 2014; Kaji et al., 2007, 2006; Yang et al., 2014; Zhu et al., 2009). Mechanistically, at least one component of the NuRD complex, the helicase Chd4, depends on Oct4 for the expected genomic localization in reprogramming experiments (Esch et al., 2013). Specific functions for Oct4-NuRD or Oct4-MLL complexes *in vivo* or in ectopic development have not yet been revealed but are tantalizing targets for future work.

It is interesting to consider how chromatin modification might amplify and stabilize the gene expression switches initiated by master regulatory networks. While molecular interactions between the *Drosophila* RDGN and chromatin remodeling factors are not yet linked to the control of gene expression, insights from mammalian RD proteins suggest that such regulation will prove important (Goldstein et al., 2005; Kenyon et al., 2005; Kobayashi et al., 2001; López-Ríos et al., 2003; Patel et al., 2012; Silver et al., 2003). Eya1 and Six1 recruit the SWI/SNF complex to activate downstream target transcription that drives cochlear neurogenesis, while Dach1 primarily associates with co-repressors, as discussed

above (Ahmed et al., 2012; Chen et al., 2013; Chu et al., 2014; Li, 2002; K. Wu et al., 2006; Wu et al., 2011, 2009, 2008, 2007, 2003; Zhao et al., 2015). The fact that both Eya and Dach proteins can associate with Six transcription factors raises the possibility that Six family members may switch RD binding partners to reverse the direction of epigenetic regulation during developmental transitions (Li et al., 2003). Pax proteins, on the other hand, can directly recruit both activating and repressing chromatin-remodeling complexes and swap these epigenetic partners at individual target loci, hinting that regulatory switching by RD proteins may not be confined to the Eya, Six, and Dach families (Abraham et al., 2015; Blake and Ziman, 2014; Budry et al., 2012; Diao et al., 2012; Kim et al., 2012; Mayran et al., 2015; Patel et al., 2014, 2012; Yang et al., 2006). Another intriguing observation is that mutation of *skuld* (*skd*) or *kohtalo* (*kto*), two Trithorax Group (TrxG) genes, leads to inappropriate maintenance of Ey posterior to the MF (Janody, 2004). Setting aside the obvious caveat that TrxG activity typically promotes gene expression, this phenotype may hint that interactions with TrxG proteins help switch Eya-So from activating to repressing *ey* in differentiating cells.

## 9. How efficiently can master regulators hijack developmental trajectories?

Although significant progress has been made in defining the developmental transitions driven by master regulators, the full temporal dynamics of the cellular behaviors and regulation that accompany the initiation of organ development or the return of somatic cells to pluripotency remain obscure. This opacity is glaringly apparent in ectopic contexts. For example, neither the biochemical function nor the signaling regulation of Eya in misexpression experiments matches observations made in loss-of-function analyses (Hsiao et al., 2001; Jin et al., 2013; Jusiak et al., 2012; Morillo et al., 2012; Rayapureddi et al., 2003; Tootle et al., 2003; Xiong et al., 2009). Coupled with the notorious inefficiency of reprogramming, such biological discrepancies significantly limit the field's ability to harness the therapeutic potential of master regulators for regenerative medicine.

Yamanaka proposed two models to grapple with the question of what cellular conditions limit the efficiency of reprogramming by the PGN (Yamanaka, 2009). The first model posits that stable genetic and epigenetic heterogeneity predisposes a fixed subset of cells to return to pluripotency (the "Elite Model"). Alternatively, stochastic fluctuations in chromatin states may constantly change the subset of cells susceptible to reprogramming (the "Stochastic Model"). Both models are supported by evidence. Careful analysis of a nearly homogenous population of B cells after PGN induction found that virtually all cells could be reprogrammed, albeit at different rates, lending support to the Stochastic Model (Hanna et al., 2009). However, a subsequent retrospective single cell-tracking experiment contradicted this result by measuring a consistent time to reprogramming for a smaller fraction of cells (Smith et al., 2010). More recent studies found that specific cellular characteristics, such as a rapid cell cycle, mark a subset of cultured granulocyte monocyte progenitors as reprogrammable prior to PGN expression, and that reprogramming susceptibility is heritable, strongly supporting the elite model (Guo et al., 2014; Pour et al., 2015). Finally, it is possible that neither model completely describes the sequence of ectopic establishment of pluripotency, as cells may undergo initial stochastic and later deterministic phases during reprogramming (Buganim et al., 2013, 2012).

The specific conditions that permit imaginal disc cells to respond to ectopic RDGN expression are unknown. Most cells refract RDGN activity, and only a subset of those that ectopically express the complete network activate neuronal differentiation markers and ultimately form adult eye structures, reminiscent of the inefficiency noted with PGN-mediated reprogramming (Chen et al., 1999; Kango-Singh et al., 2003; Salzer and Kumar, 2010). While the global pattern of open chromatin is similar between late larval imaginal discs (McKay and Lieb, 2013), and thought to aid RD proteins' ability to activate downstream target transcription, no experiments have analyzed chromatin in more restricted pools of cells and correlated that information with ectopic eye induction efficiency. Signaling pathway activity also cannot yet predict which cells will respond to the misexpressed RDGN. For example, two pathways that potentiate RD transcription factor activity, Decapentaplegic (Dpp) and Hedgehog (Hh), were hypothesized to confer competence to larval cells that generate ectopic eyes (Chen et al., 1999; Kango-Singh et al., 2003), but were later shown to be neither required nor sufficient for a responsive state (Salzer and Kumar, 2010).

To distinguish whether a predictable pool of cells in regions with the correct genetic and signaling context responds to RDGN misexpression by adopting retinal fate, or stochasticity makes different sub-populations eligible over time instead, experiments similar to those employed by stem cell biologists are needed. Specifically, live imaging that couples a reporter of neuronal specification with lineage tracing or retrospective cell tracking could describe the timing and dynamics of individual cell trajectories during ectopic eye development. Under the Elite Model, induction of neuronal markers should happen in a burst at the same time and place whereas the Stochastic Model predicts multiple independent initiations over a longer developmental window. Such experiments could also distinguish scenarios in which single transdetermined cells proliferate to generate clonal ectopic retinas or many cells independently adopt the new fate and later contribute to the same eye. Once these basic observations are made, a fascinating future step will be to analyze the temporal sequence of gene expression and cellular behaviors that creates an eye from non-retinal tissue.

Comparing the RDGN and PGN reveals significant gaps in our understanding of how master regulatory networks control endogenous and ectopic organogenesis. Perhaps nowhere are these limitations more evident than in iPSC-based cell therapies, in which somatic cells are returned to pluripotency, genetically altered, re-differentiated, and introduced into patients to treat disease (Hotta and Yamanaka, 2015; Papapetrou, 2016). Specifically, unacceptable rates of tumorigenesis, discrepancies in the degree of differentiation between endogenous versus artificially generated cells, and genetic and epigenetic "memories" of the reprogramming process all limit therapeutic success and reinforce the urgency of continuing to improve our understanding of the full repertoire of strategies by which master regulatory networks alter cells' developmental trajectories (Robinton and Daley, 2012).

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## Highlights

- Organization into self-reinforcing networks defines master regulators.
- Negative feedback allows master regulators to propel developmental transitions.
- An objective scoring scheme identifies master control transcription factors.
- Comparing mammalian and fly master regulators reveals shared regulatory strategies.

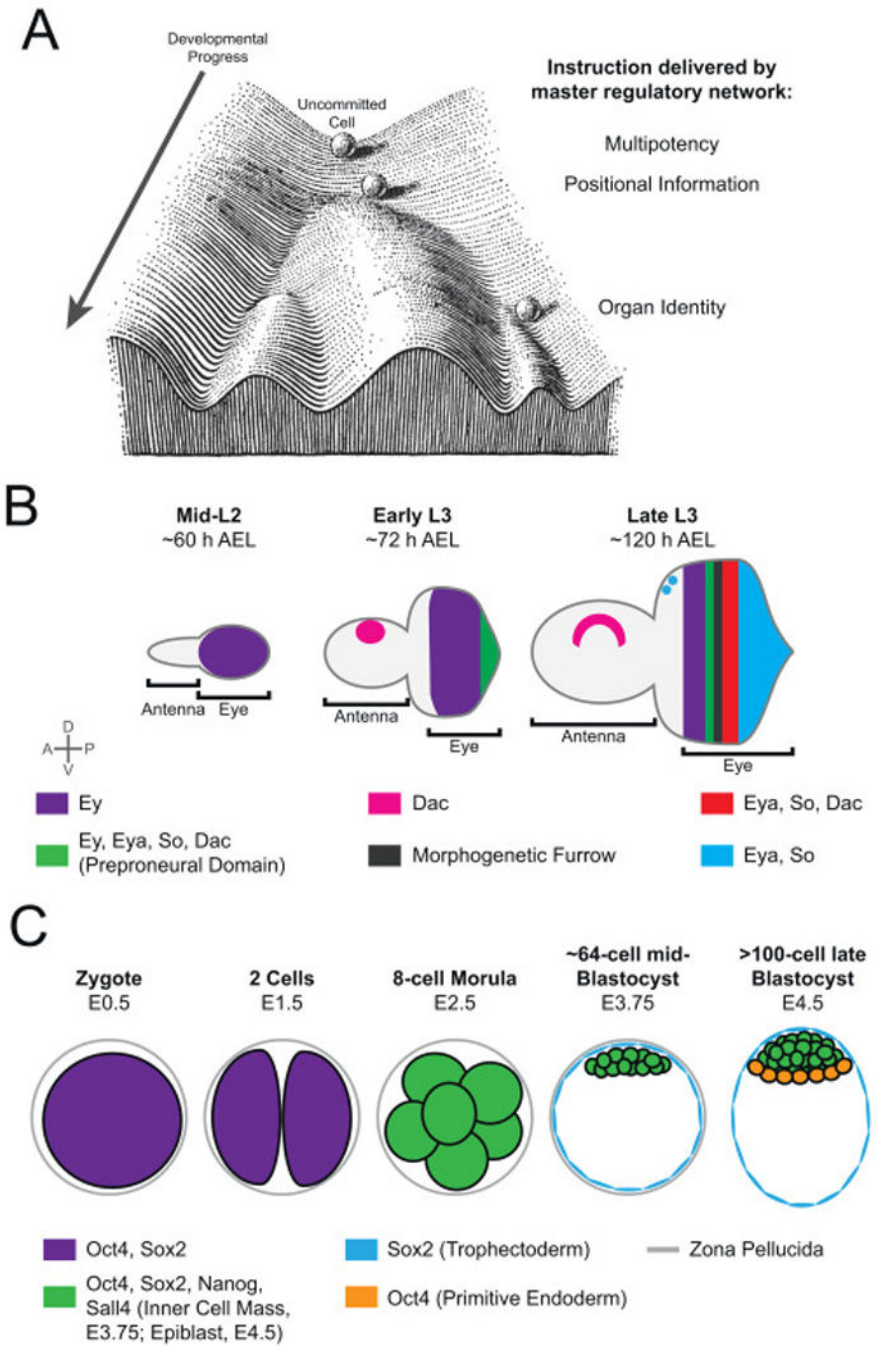


**Figure 1.**

Master regulatory instructions in Waddington's landscape and the expression patterns of core RDGN and PGN proteins in their endogenous developmental contexts. Color-coding indicates combinations of proteins present, irrespective of levels. (A) Master regulatory inputs direct cellular decisions at bifurcations in Waddington's landscape. Adapted from Waddington, 1957. (B) Core RDGN transcription factors. Diagrams represent larval eye-antennal imaginal discs oriented anterior to the left and dorsal up. Developmental time at 25 °C is presented as the number of hours after egg laying (AEL), L2 denotes the second

larval instar, and L3 denotes the third larval instar. (C) Core PGN transcription factors. Diagrams represent the pre-implantation mammalian embryo, staged according to developmental time measured in embryonic (E) days.

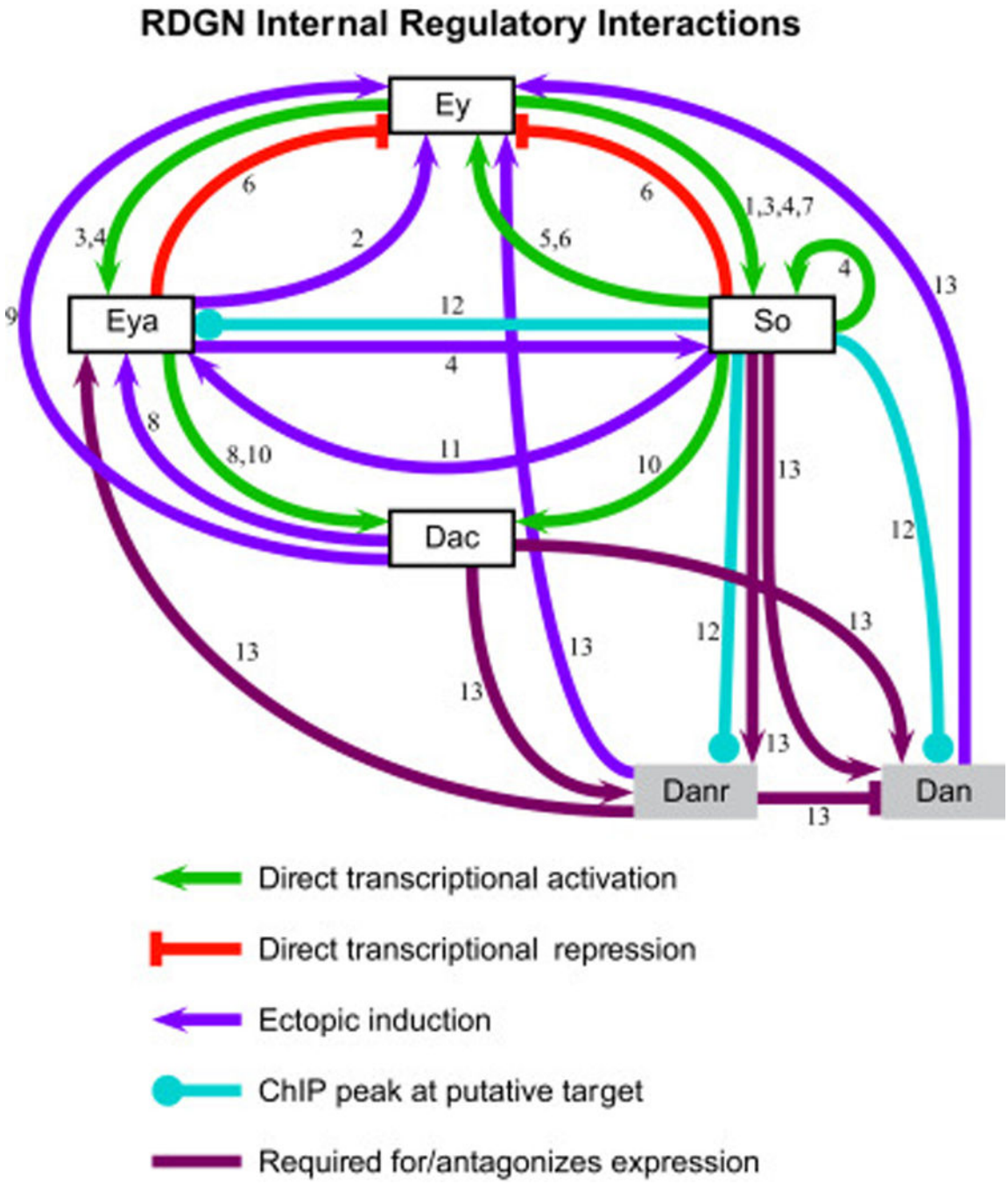


**Figure 2.**

A summary of RDGN regulatory interactions. Core members are highlighted with white boxes and the next two highest scoring transcription factors from Table 1 with grey boxes. Line color signifies the type of evidence considered and numbers refer to the literature used. The category Required for/antagonizes expression (maroon) refers to altered expression of a gene in the mutant background of another, but without mechanism; this category on its own was not considered sufficient proof of direct regulation to inform Table 1. The presence of an appropriate ChIP peak (turquoise) suggests but is considered insufficient to prove

transcriptional regulation. When direct transcriptional regulation has been demonstrated (green and red), ChIP peaks and genetic requirements are not also indicated. Because interactions between only the top six genes from Table 1 are shown, the number of connections depicted may be lower than the interaction scores.

[1]Punzo C, Seimiya M, Flister S, Gehring WJ, Plaza S: Differential interactions of eyeless and twin of eyeless with the sine oculis enhancer. *Development* 2002, 129:625–34. [2]Bonini NM, Bui QT, Gray-Board GL, Warrick JM: The Drosophila eyes absent gene directs ectopic eye formation in a pathway conserved between flies and vertebrates. *Development* 1997, 124:4819–26. [3]Ostrin EJ, Li Y, Hoffman K, Liu J, Wang K, Zhang L, Mardon G, Chen R: Genome-wide identification of direct targets of the Drosophila retinal determination protein Eyeless. *Genome Res*. 2006, 16:466–76. [4]Halder G, Callaerts P, Flister S, Walldorf U, Kloter U, Gehring WJ: Eyeless initiates the expression of both sine oculis and eyes absent during Drosophila compound eye development. *Development* 1998, 125:2181–91. [5]Pauli T, Seimiya M, Blanco J, Gehring WJ: Identification of functional sine oculis motifs in the autoregulatory element of its own gene, in the eyeless enhancer and in the signalling gene hedgehog. *Development* 2005, 132:2771–82. [6]Atkins M, Jiang Y, Sansores-Garcia L, Jusiak B, Halder G, Mardon G: Dynamic rewiring of the Drosophila retinal determination network switches its function from selector to differentiation. *PLoS Genet*. 2013, 9:e1003731. [7]Niimi T, Seimiya M, Kloter U, Flister S, Gehring WJ: Direct regulatory interaction of the eyeless protein with an eye-specific enhancer in the sine oculis gene during eye induction in Drosophila. *Development* 1999, 126:2253–60. [8]Chen R, Amoui M, Zhang Z, Mardon G: Dachshund and eyes absent proteins form a complex and function synergistically to induce ectopic eye development in Drosophila. *Cell* 1997, 91:893–903. [9]Shen W, Mardon G: Ectopic eye development in Drosophila induced by directed dachshund expression. *Development* 1997, 124:45–52. [10]Pappu KS, Ostrin EJ, Middlebrooks BW, Sili BT, Chen R, Atkins MR, Gibbs R, Mardon G: Dual regulation and redundant function of two eye-specific enhancers of the Drosophila retinal determination gene dachshund. *Development* 2005, 132:2895–905. [11]Weasner B, Salzer C, Kumar JP: Sine oculis, a member of the SIX family of transcription factors, directs eye formation. *Dev. Biol*. 2007, 303:756–71. [12]Jusiak B, Karandikar UC, Kwak S-J, Wang F, Wang H, Chen R, Mardon G: Regulation of Drosophila Eye Development by the Transcription Factor Sine oculis. *PLoS One* 2014, 9:e89695. [13]Curtiss J, Burnett M, Mlodzik M: distal antenna and distal antenna-related function in the retinal determination network during eye development in Drosophila. *Dev. Biol*. 2007, 306:685–702.

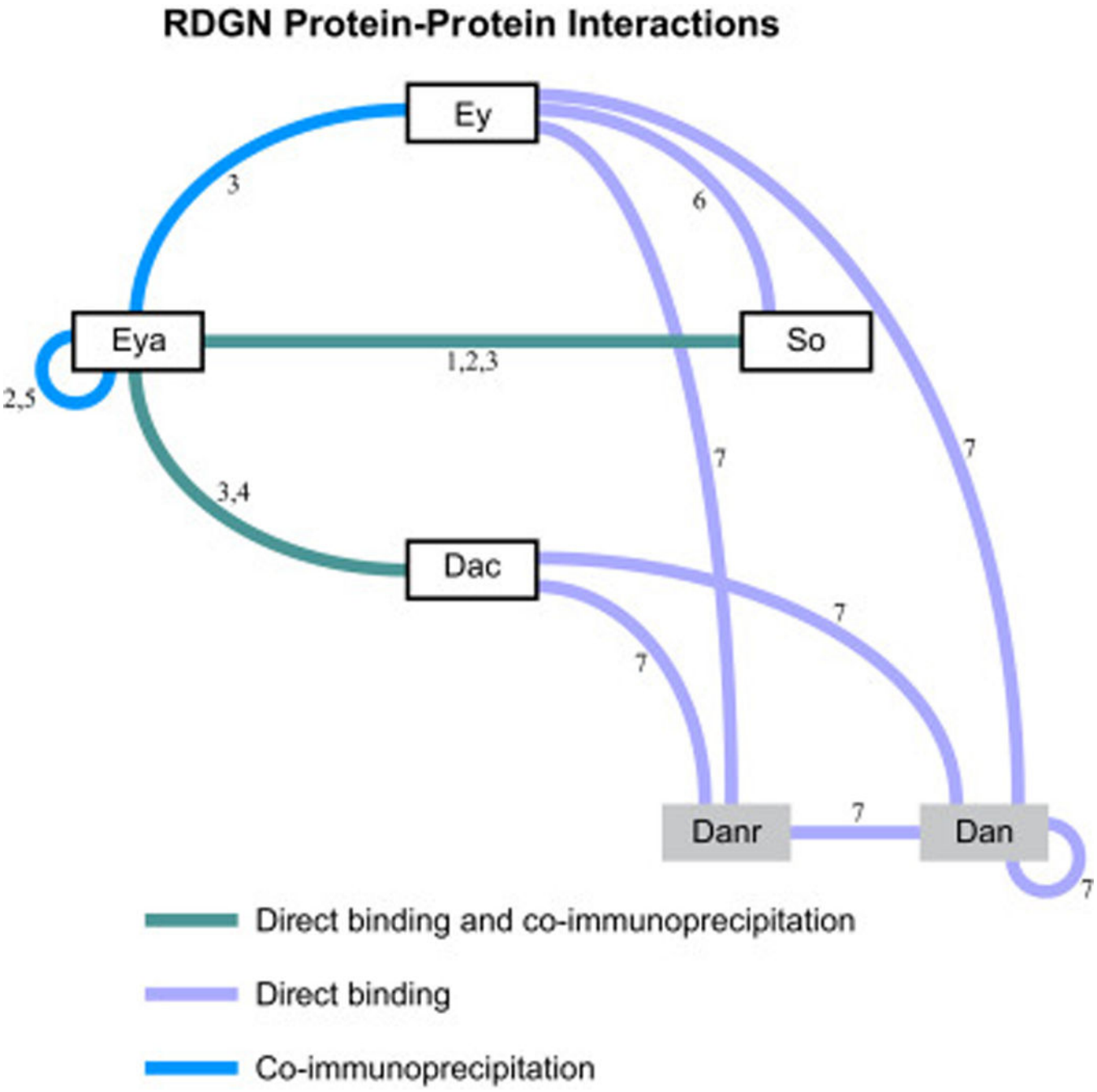


**Figure 3.**

A summary of RDGN protein-protein interactions. Core members are highlighted with white boxes and the next two highest scoring transcription factors from Table 1 with grey boxes. Line color signifies the type of evidence considered and numbers refer to the literature used. Because interactions between only the top six genes from Table 1 are shown, the number of connections depicted may be lower than the interaction scores.

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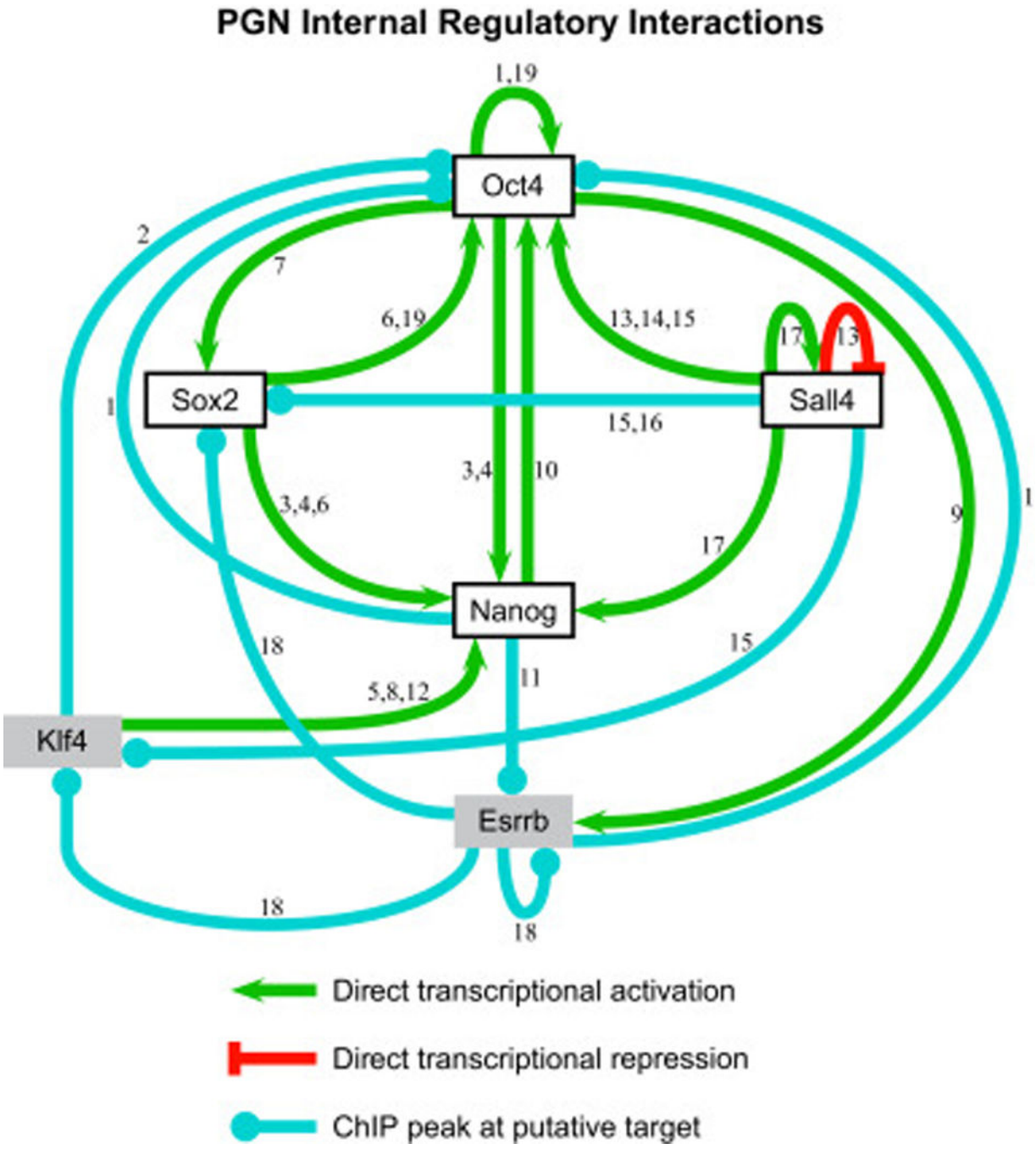


**Figure 4.**

A summary of PGN regulatory interactions. Core members are highlighted with white boxes and the next two highest scoring transcription factors from Table 2 with grey boxes. Line color signifies the type of evidence considered and numbers refer to the literature used. The presence of an appropriate ChIP peak (turquoise) suggests but is considered insufficient to prove transcriptional regulation. When direct transcriptional regulation has been demonstrated (green and red), ChIP peaks and genetic requirements are not also indicated.

Because interactions between only the top six genes from Table 2 are shown, the number of connections depicted may be lower than the interaction scores.

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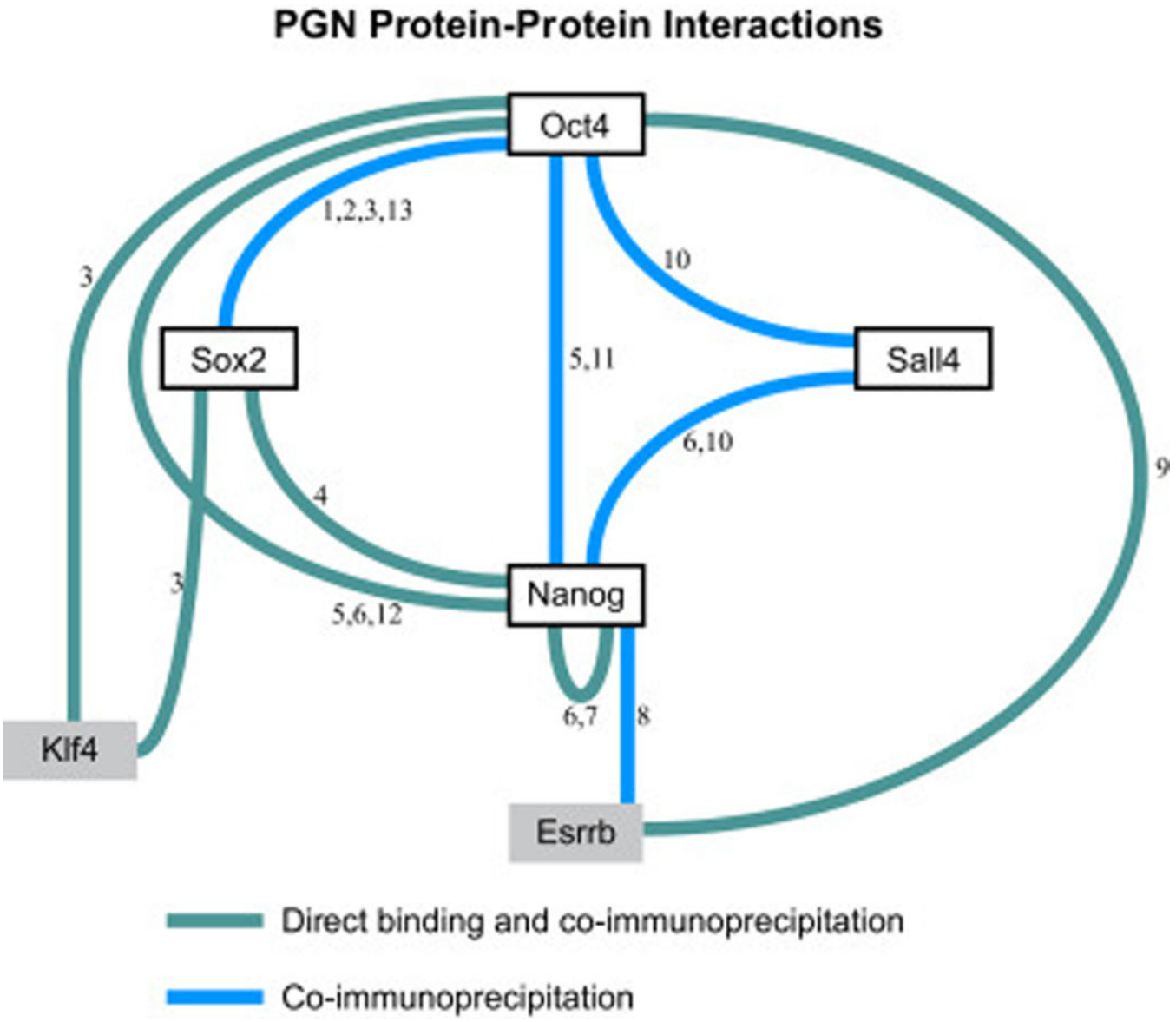


**Figure 5.**

A summary of PGN protein-protein interactions. Core members are highlighted with white boxes and the next two highest scoring transcription factors from Table 2 with grey boxes. Line color signifies the type of evidence considered and numbers refer to the literature used. Because interactions between only the top six genes from Table 2 are shown, the number of connections depicted may be lower than the interaction scores.

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**Table 1**

**Scoring scheme to define core members of the RDGN**

Using the published literature, we assigned one point for each of the first three criteria satisfied, and then to emphasize the importance of network connectivity to master regulatory potency, each interaction node was eligible for one point. Interactions between the top six scoring genes are summarized in Figs. 2 and 3.

| Candidate Master Regulator | Sufficient | Required | Expressed | Regulates expression of another candidate | Expression regulated by another candidate | Binds another candidate | Total Score |
|---|---|---|---|---|---|---|---|
| **So** | | 1 | | 5 | 4 | 2 | 14 |
| **Eya** | | 1 | | 3 | 3 | 4 | 13 |
| **Ey** | | 1 | | 2 | 4 | 2 | 11 |
| **Dac** | | 1 | | 2 | 2 | 1 | 8 |
| **Dan** | | 1 | | 1 | 0 | 4 | 8 |
| **Danr** | | 1 | | 1 | 0 | 3 | 7 |
| **Optix** | | 1 | | 3 | 0 | 0 | 6 |
| **Toy** | | 1 | | 2 | 1 | 0 | 6 |
| **Tsh** | | 0 | | 4 | 0 | 0 | 6 |
| **Tio** | | 0 | | 2 | 0 | 0 | 4 |
| **Eyg** | | 1 | | 0 | 0 | 0 | 3 |
| **Toe** | | 1 | | 0 | 0 | 0 | 3 |

**Table 2**

**Scoring scheme to define core members of the PGN**

Using the published literature, we assigned one point for each of the first three criteria satisfied, and then to emphasize the importance of network connectivity to master regulatory potency, each interaction node was eligible for one point. “Required” and “Expressed” considered only data derived from pre-implantation embryonic tissue and disregarded experiments in cultured cells. Interactions between the top six scoring genes are summarized in Figs. 4 and 5.

| Candidate Master Regulator | Sufficient | Required | Expressed | Regulates expression of another candidate | Expression regulated by another candidate | Binds another candidate | Total Score |
|---|---|---|---|---|---|---|---|
| **Oct4** | | 1 | | 4 | 7 | 7 | 21 |
| **Nanog** | | 1 | | 3 | 4 | 6 | 16 |
| **Esrrb** | | 0 | | 4 | 3 | 2 | 11 |
| **Sall4** | | 1 | | 5 | 1 | 2 | 11 |
| **Sox2** | | 1 | | 2 | 3 | 3 | 11 |
| **Klf4** | | 0 | | 2 | 2 | 2 | 8 |
| **Nr5a2** | | 0 | | 3 | 0 | 0 | 5 |
| **Gmnn** | | 1 | | 0 | 0 | 0 | 3 |
| **c-Myc** | | 0 | | 0 | 0 | 0 | 2 |
| **Gata3** | | 0 | 0 | 0 | 0 | 0 | 1 |