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A gene regulatory program controlling early *Xenopus* mesendoderm formation: network conservation and motifs

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Abstract

Germ layer formation is among the earliest differentiation events in metazoan embryos. In triploblasts, three germ layers are formed, among which the endoderm gives rise to the epithelial lining of the gut tube and associated organs including the liver, pancreas and lungs. In frogs (*Xenopus*), where early germ layer formation has been studied intensively, the process of endoderm specification involves the interplay of dozens of transcription factors. Here, we review the interactions between these factors, summarized in a transcriptional gene regulatory network (GRN). We highlight regulatory connections conserved between *Xenopus*, zebrafish, mouse, and human endodermal lineages. Especially prominent is the conserved role and regulatory targets of the Nodal signaling pathway and the T-box transcription factors, Vegt and Eomes. Additionally, we highlight some network topologies and motifs, and speculate on their possible roles in development.

Keywords

Xenopus; endoderm; gene regulatory network; network motifs; transcription factors; evolutionary conservation

1. Vertebrate mesendoderm formation

During early embryogenesis, cell fate specification proceeds through the combinatorial interactions of several signaling pathways and numerous transcription factors (TFs), which function within a broader chromatin landscape. The integration of these factors (‘inputs’) leads to a specific transcriptome profile (‘outputs’) that determines the identity of a particular cell. Critical genomic regions for this integration are cis-regulatory modules (CRMs) – combinations of regulatory elements, such as enhancers, where TFs bind to specific sequence motifs and recruit the necessary co-factors [1]. CRMs are critical for the proper implementation of gene regulatory programs in development, because they modulate

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the rate of gene transcription and control when a gene is turned “on” or “off” in both time and space. These complex programs can be organized into gene regulatory networks (GRNs) and visualized through logic maps [2,3]. Elucidating the GRNs will enhance our mechanistic understanding of developmental processes, and will enable comparisons across different organ systems, and across different species. These insights will also enhance our understanding of the causes of developmental defects.

Germ layer specification is one of the earliest developmental events in metazoan organisms, preceding the establishment of the organ and tissue primordia that form the complex adult organism. Cells of the three primary germ layers – the ectoderm, mesoderm, and endoderm – become further specified along distinct lineages. Ectodermal cells form the epidermis and nervous system; mesodermal cells become blood, muscle, kidneys, notochord, and connective tissue; and endodermal cells become the gastrointestinal and respiratory tracts. In amphibian development, the germ layers form along the animal to vegetal axis, which is established during oogenesis. The ectoderm develops from the animal region, while the endoderm arises from the opposite, vegetal pole. The mesoderm forms from the equatorial cells. During early stages, there is overlap between endodermal and mesodermal domains – and therefore it is common to refer to these jointly as the ‘mesendoderm.’ Two major advantages of the *Xenopus* system are the ease of obtaining thousands of synchronously developing embryos from a single clutch of eggs, and the ease of performing both gain- and loss-of-function studies to ascertain gene function. These advantages, combined with the relatively close evolutionary distance between *Xenopus* and other vertebrates, makes *Xenopus* a powerful model for elucidating the mechanisms underlying cell fate specification.

A comprehension of the complex GRN architecture that contributes to the specification of the germ layers *in vivo* is a critical unanswered question in developmental and evolutionary biology. Since germ layer formation, like most biological processes, is controlled by a hierarchy of regulatory steps, examining the earliest inputs in the regulation of germ layer development is important. This is underscored by the realization that germ layer specification is not programmed by molecules acting in a linear fashion, but instead is controlled by a set of transcription factors (TFs) acting in a complex network. In addition, the study of GRN in amphibian germ layer specification will enable powerful comparisons across different developmental systems, and across evolutionary taxa, to identify core GRN structures that are conserved, as well as subnetworks that were modified during evolution. Beyond animal development, numerous human congenital diseases result from abnormal formation of the mesoderm and endoderm [4,5]. Furthermore, recent advances in regenerative medicine and stem cell biology bring the promise of a new era of personalized medicine, aided by highly efficient *in vitro* differentiation techniques [5]. These goals can be significantly aided by a strong understanding of complex *in vivo* cellular differentiation programs, namely “GRN science.”

A little over a decade ago, efforts were made to compile the available molecular data into GRNs describing mesendoderm [6] and Spemann organizer [7] development. Since then, the widespread use of high-throughput technologies (e.g. microarrays, sequencing) provides us with the capacity to significantly broaden the number of network connections and, therefore,

our understanding of the structure of the mesendoderm GRN. For this review, we have utilized recent findings to update the *Xenopus* mesendoderm GRN through the beginning of gastrulation, linking together critical signaling pathways with transcriptional targets. We discuss the network's structure and its motifs, and review areas of conservation across vertebrates.

2. Generation of the mesendoderm gene regulatory network

2.1 Mesendoderm factors

We have assembled a *Xenopus* mesendoderm GRN that reflects data obtained from both *Xenopus laevis*, and the closely related diploid species *Xenopus tropicalis*. Each species greatly contributes to the GRN assembly, as *X. laevis* has been traditionally used in the study of mesendoderm formation, while *X. tropicalis* has been adapted more recently for genomic approaches. The 'inputs' in this GRN represent the TFs and signaling molecules (transduced via intracellular TFs) important for mesendoderm formation and early endoderm patterning, many of which have been elucidated [8,9]. Recently, genome-wide approaches have identified additional localized maternal and zygotic transcripts encoding transcription factors [10-16]. Based on a comprehensive catalogue of the *X. tropicalis* TFs [14], 130 TFs are found to be enriched vegetally (in comparison to the animal pole), and we have focused on the ~50 TFs expressed at relatively high abundance in the vegetal tissue (Transcripts Per Million values ≥ 50). This corresponds approximately to the expression level of *siamois1* – a critical and localized mesendoderm TF.

2.2 Criteria for identifying transcriptional targets

In order to build the mesendoderm GRN, we have taken the following rigorous approach to determine direct connections between the above TF 'inputs' and their downstream target genes [17]. This approach is similar to that previously taken by both Koide et al. [7] and Loose and Patient [6]. First, we enforced that there should be a strong correlation between perturbation of a regulatory TF and the expression changes of the suspected target genes. Regulation can be measured following gain- and/or loss-of-function experiments (e.g., injection of RNA encoding a TF, or a translation blocking antisense morpholino oligonucleotide, respectively) by analyzing changes in target RNA expression (e.g., RNA-seq, RT-PCR, northern blotting, *in situ* hybridization).

Second, we required that the 'inputs' and 'outputs' be expressed in a spatiotemporally consistent manner with regulation and the proposed direction (activation or repression). For a proposed activating ('positive') connection, the upstream TF and proposed target gene must be expressed in the same or overlapping regions, and during overlapping developmental time. Conversely, a proposed inhibitory ('negative') connection should find the target excluded from the spatiotemporal domain of the repressor. However, we note the possibility of finding some exceptions to this rule based on the known biological properties of TFs, and these knowledge-based connections were also included in the network. One example includes a negative autoregulatory feedback loop by *gsc* [18], where a gene modulates it's own expression.

Third, we required demonstration of a direct physical interaction between the TF and the regulatory region of the proposed target. As perturbation experiments alone are insufficient to distinguish between direct and indirect effects, we find this criterion essential. This criterion was satisfied experimentally through chromatin immunoprecipitation (ChIP), gel electromobility shift assay (EMSA), DNase footprinting, or reporter gene assays (containing appropriate mutations). Only connections that satisfied all three criteria were defined as direct. However, we note that DNA binding is only suggestive of functional regulation, and that the 'gold standard' evidence is to mutate the binding site and examine the effect on gene expression. While the rise in the use of ChIP-seq – ChIP coupled with high-throughput sequencing (HTS) – has produced large datasets of physical connections, the vast majority of these sites have not been subjected to laborious mutagenesis assays. In building the network, we therefore distinguished between functionally validated CRMs and physical interactions lacking this support. Finally, we have also looked to experiments in which protein synthesis is inhibited (e.g. cycloheximide) as a way to determine 'directness.' Regulatory connections proposed based on perturbation analyses that were validated in the presence of cycloheximide (CHX), although lacking in physical binding evidence, were identified as 'putative direct' targets.

2.3 Building network connections

To assemble the updated network, we have analyzed hundreds of manuscripts published over the approximately 25 year history of the investigation into the molecular basis of *Xenopus* mesendoderm formation. Building upon the networks presented by Koide et al. [7] and Loose and Patient [6] over a decade ago, we have made extensive use of recently published HTS data using *X. tropicalis* in multiple aspects. First, RNA-seq transcriptome profiling studies have revealed, in great detail, the timing of gene activation [19-22]. These data have allowed us to incorporate, to a greater extent, temporal information into a graphical organization of our network. Additionally, HTS data revealed vegetally enriched transcripts [14-16], which can be difficult to visualize by whole mount *in situ* hybridization.

Second, gain- and loss-of-function experiments, coupled with HTS or microarrays, have provided a wealth of regulatory connections. Additionally, ChIP-seq allows for the considerable improvement in the identification of direct target genes *in vivo*. Genome-wide binding of β-catenin [23,24], T-box TFs [25], Smad2/3 [26,27], Foxh1 [26], and several organizer-specific TFs [28] have all been investigated in *X. tropicalis.* Due to differences in data formatting across publications, we have mapped all available ChIP-seq datasets performed from blastula to early gastrula (Supplemental Table S1) to the version 9 *Xenopus tropicalis* genome (www.xenbase.org) using Bowtie2 [29], and identified peaks using the software MACS2 [30]. We then used Bedtools [31] to associate ChIP-seq peaks with TFs in our network, where peaks are filtered using a q-value of 0.01 and a peak distance of 10 kilobases (kb) from the gene body.

Using the criteria described above, we present a model of the GRN contributing to *Xenopus* mesendoderm development from fertilization through early gastrula (Nieuwkoop-Faber stage 10.5) (Figure 1). In total, this network includes 23 TFs and 12 growth factors. As we have focused on direct transcriptional responses, we have not included well-characterized

secreted signaling antagonists such as *chrd*, *nog* or *dkk1* in this network. However, we have chosen to include connections into the multi-signaling antagonist *cer1*, as the regulation of this gene has been extensively characterized [32,33]. We present the supporting evidence for these connections in Table 1, and in Supplemental Table S2 which includes additional putative connections between mesendodermal genes that did not satisfy our criteria. Thus, the directness of these connections is uncertain, and this represents an area of future investigation.

2.4 Organization of the network diagram

Using Biotapestry [34], we have built the GRN as a single bird's-eye view from the full genome [2] (Figure 1). In this visualization, all connections are displayed at once, regardless of time and space. We have made an effort to arrange the genes vertically based on approximate activation time [22], and horizontally from right to left across the dorsal to ventral axis [14]. At the top of the network, maternally inherited TFs (e.g., Vegt, Foxh1) and signaling ligands (e.g., Gdf1, Wnt11b) are shown. Maternally inherited TFs are displayed as diamonds, and ligands are shown as circles. All targets of signaling pathways are connected through chevrons indicating cell-surface receptors to their intracellular signal transducers. Zygotically activated growth factor ligands (i.e. Nodals, Bmp4) are connected back through the same signal transducer, so that all connections from a given pathway feed through a single TF node (e.g. Smad2/3).

We have compared the connections in this network with two previous GRNs [6,7] and find that a major improvement in the current network is the identification of more bone-fide direct transcriptional connections between TFs (Supplemental Table S3). Our network contains a total of 96 direct network connections - 82 positive and 14 negative. Direct connections are displayed in the GRN as a solid line connecting the upstream transcription factor to its downstream target. We also identify 36 putative direct interactions, which are displayed as dashed lines. Comparatively, Koide et al. [7] and Loose and Patient [6] previously reported a total of 41 and 60 direct network connections, respectively. Based on differing criteria from the current analysis, some of the direct connections identified by Loose and Patient [6] were here considered putative. Below, we review key features of the mesendoderm network.

3. Maternal factors and the initiation of the network

3.1 Vegetally-localized maternal transcription factors: Vegt and Sox7

Maternal factors play a critical role in the activation of the mesendoderm GRN. The best characterized TF important for the initiation of the *Xenopus* zygotic mesendoderm gene program is the T-box factor Vegt, which is asymmetrically localized vegetally [35-37]. The maternal knockdown of *vegt* in *Xenopus laevis* results in the loss of both mesoderm and endoderm [38,39]. Several studies have revealed Vegt to be a master regulator of the endoderm lineage through transcriptional regulation along two parallel routes: the zygotic activation of the Nodal genes, and activation of endodermal TFs [38-41]. Importantly, the Vegt LOF phenotype can be rescued by the injection of RNA encoding various Nodal ligands, indicating that a critical function of Vegt is the zygotic activation of the Nodal

signaling pathway [41]. Consistent with this, *nodal1*, *nodal5* and *gdf3* are direct targets of Vegt, and *nodal* is putatively direct based on protein synthesis inhibition. The expression of *nodal6* and *nodal2* are also regulated by Vegt, but it remains unclear whether these activations are direct.

In addition to regulating the pSmad2/3 signaling pathway, Vegt is important for the direct activation of core mesendodermal genes. TFs *sox17a*, *sox7*, and *gsc* are bona-fide Vegt direct targets; and *sox17b*, *mixer*, *mix1*, *hhex*, and *ventx1* are additional *putative* direct targets. The *cer1* gene, encoding a secreted Bmp/Wnt/Nodal antagonist [42], is also a direct target. At present, we have very little data to fully understand how these targets interact to establish the mesendoderm GRN.

In zebrafish and human, it appears that the T-box transcription factor Eomes plays a role similar to frog maternal Vegt in the activation of the endodermal gene regulatory program. Interestingly, functional studies of *Xenopus* Eomes add further support to the notion that this TF can perform an overlapping role with Vegt in specifying early mesendoderm. Eomes gain-of-function in naive animal caps results in expression of the mesendodermal genes *mix1*, *xbra*, *wnt8*, *sox17a*, *foxa4*, and *gsc* [43,44]. Furthermore, zygotic Vegt and Eomes cooperate in mesoderm formation in the late-gastrula embryo [25]. In zebrafish, perturbation analysis reveals that Eomesa is required for the activation of *sox17*, as well as the two nodal ligands *squint* and *cyclops* [45]. ChIP analyses confirm that Eomesa physically binds to the *squint* locus [46,47]. Eomesa also binds regulatory regions near *sox17*, and additional binding was observed for *mixl1*, *foxa*, *foxa3*, *vent*, and *gsc* [46].

Eomes ChIP-seq and shRNA knockdown, in combination with microarray analysis, during the *in vitro* differentiation of human embryonic stem (ES) cells to definitive endoderm (DE) reveals that EOMES regulates a similar set of target genes as *Xenopus* Vegt [48]. The genes *MIXL1*, *GDF3*, *CER1*, *SOX17*, *FOXA1*, *FOXA2*, *FOXA3*, AND *VENTX* (with the exception of *FOXA3*, which is not found in *Xenopus*) are likely direct EOMES targets based on the application of our criteria. Finally, the NODAL gene – a *Xenopus* Vegt target – appears to have a regulatory region associated with EOMES binding, but is unaffected by the knockdown [48]. Overall, the Vegt T-box transcription factor has a highly evolutionarily conserved relationship with Eomes orthologs in some other species, sitting at the top of the gene regulatory hierarchy to function as a master regulator of endoderm formation.

In *Xenopus*, Sox7, a maternal SoxF type TF, has been implicated in the activation of mesendoderm targets. Overexpression of Sox7 mRNA into naive animal caps reveals that *nodal, nodal5*, and *nodal6* are putative direct target genes [49]. Since Vegt also putatively activates zygotic *sox7* expression [49], this indicates the likelihood of the *nodal* genes to be co-regulated by maternal Vegt and both maternal and zygotic Sox7. This network structure illustrates the importance of understanding gene regulation as a network, instead of activation mechanisms at the single gene/activator level. We also note that recent transcriptome profiling of blastomeres from 8-cell stage embryos identified 65 genes reproducibly enriched in the vegetal pole [12]. Among these, o*tx1*, *pbx1*, *sox7* and *vegt* are highly enriched in the *Xenopus* vegetal tissue, most of them with poorly characterized roles in endoderm formation.

3.2 Nodal signaling and maternal Foxh1

It is clear that, in addition to directly activating endodermal TFs, a major function of Vegt is to activate expression of the *nodal* genes. The loss of Nodal signaling in *Xenopus* results in the disruption of endoderm and mesoderm formation, a severe delay or complete disruption of gastrulation movements, resulting in an embryo with a shortened anteroposterior (A-P) axis [26,50-54]. These findings are consistent with an analysis of Nodal-deficient zebrafish embryos (*sqt*;*cyc* double mutants) where mesodermal and endodermal markers are not expressed and gastrulation is abnormal [55]. Mice have a single *Nodal* gene, and *Nodal-null* embryos fail to form a primitive streak and do not undergo proper gastrulation [56]. Taken together, Nodal signaling is necessary for mesendoderm development in vertebrates.

How this pathway functions in the early formation of the mesendoderm has been the focus of intense study. The maternal TGFβ ligand Gdf1 (previously known as Vg1) is also localized to the vegetal cells [57], and likely plays a role in anterior mesendoderm formation [58]; however, the endogenous role of Gdf1 has remained understudied, in part due to the inefficient conversion of the ligand precursor into its mature form [59]. Importantly, expression of *nodal5* and *nodal6* prior to the mid-blastula transition indicate that these ligands contribute to the earliest activation of the Nodal signaling pathway [60,61], and the early onset of *gdf3* suggests that it also contributes to the early phosphorylation of Smad2/3. The Nodal signaling cascade activates transcription in the blastula through Smad2/3 (in complex with the maternal partner to all R-Smad signaling, Smad4). Overall, the vegetal localization of Nodals and Gdf1 is consistent with the model in which high levels of Nodal promote endoderm and low levels promote mesoderm, which has been observed in *Xenopus* explant experiments [40].

As it is difficult to distinguish between the output of different Nodal and TGFβ ligands, we have therefore fed all 7 ligands through a single Smad2/3 node. The mesendoderm GRN presented here contains 24 direct targets activated by Nodal signaling (Figure 1). These targets include core endoderm TFs such as *gata4, gata6, mix1, mixer*, and *foxa4*, dorsal endoderm genes such as *hhex* and *cer1*, the organizer gene *gsc* and the pan-mesodermal gene *t* (also known as *brachyury*), among others. These targets validate the notion that Nodal signaling contributes broadly in gene activation in the mesoderm and endoderm germ layers. Additionally, positive autoregulation of *nodal1* and *nodal2* promotes further enhanced expression of the signal [62].

The activated Smad2/3-Smad4 complex regulates target genes in concert with co-TFs, and to date, identified Smad2/3 co-factors include Foxh1, Eomes, Foxh1.2, Gtf2i, Gtf2ird1, Mixer, Tcf3 (also known as E2a) and Tp53 [48,63-71]. Of these, the transcriptional regulation via Smad2/3 interactions with maternal Foxh1 has been extensively investigated in *Xenopus* [26,66,72], zebrafish [70,73,74], mouse [75,76], and differentiated human ES cells [71]. Our *Xenopus* GRN contains 9 direct targets activated via Smad/Foxh1, including the growth factors, *nodal1*, *nodal2*, and *wnt8a*; the BMP/Wnt/Nodal antagonist *cer1*; and the TFs *gsc*, *otx2*, *mix1*, *hhex*, and *pitx2*. Among these, to date, *gsc* and *pitx2* regulation via Foxh1/Smad2 is conserved across mouse, fish and frog [62,77]. Finally, in human definitive endoderm differentiation *CER1*, *PITX2*, *GSC*, and *MIX1* are also induced by FOXH1/SMAD2 [78].

It has been well-characterized in mouse, fish and frog that the loss of Foxh1 does not fully recapitulate the loss of Nodal signaling – indicating the necessity for additional Smad2/3 binding partners. To this extent, in zebrafish, Eomes has been implicated as the Smad2/3 co-factor responsible for the remaining Nodal-mediated regulation that occurred in the Foxh1-null [70]. Our network suggests *gata4*, *gata6*, *eomes*, and *foxa2* are also regulated by Nodal-signaling via a Foxh1-independent mechanism. It will be necessary to investigate whether Eomes and Mixer regulate these targets in a Foxh1-indepenent fashion.

3.3 Wnt/β-catenin signaling

In addition to germ layer specification along the animal-vegetal axis, Nodal signaling is critical in patterning the embryo along the dorsal-ventral axis. While *vegt* mRNA appears to be distributed across the vegetal tissue, Nodal signaling is higher in the dorsal mesendoderm of the *Xenopus* blastula [52,79]. This pattern is attributed to high levels of Wnt/β-catenin signaling on the dorsal side of the embryo. While a detailed discussion of dorsal-ventral patterning of mesendoderm is beyond the scope of this review, it is useful to discuss in brief the role of maternal Wnt/β-catenin signaling. Maternal *wnt11b*, is localized to the vegetal pole in the egg, relocated to the dorsal vegetal cells following cortical rotation, and activates Wnt signaling to specific dorsal fate [80-82]. Dorsal nuclear β-catenin directly regulates *sia1* and *sia2*, two homeobox genes that control dorso-anterior specification [83,84] and many other genes, via the canonical Wnt cascade feeding through β-catenin-Lef/Tcf complexes. Maternal Wnt/β-catenin signaling also activates the expression of all Nodal genes in the dorsal mesendoderm, in particular the early activation of *nodal5* and *nodal6* [61,85]. Both Nodal and Wnt signaling are critical for the formation of the Nieuwkoop center and Spemann organizer [84,86,87], and the network reveals substantial overlap in the regulation of dorsal mesendoderm target genes such as *hhex, lhx1, otx2, cer1* and *gsc*. Consistent with this crosstalk model, there is a substantial co-occurrence between Foxh1 (a major Smad2/3 co-factor) and β-catenin ChIP-seq peaks [24,26]. It should be noted that recent β-catenin ChIP-seq performed by Nakamura et al. [24] reveals β-catenin binding associated with target genes previously thought to be indirectly regulated by Wnt/β-catenin via Sia, such as *hhex* and *gsc* [84,166]. While the biological activities of the putative enhancers remain to be validated, this finding suggests that dorsal mesendoderm targets are regulated through complex network motifs (see section 5). Taken together, the mesendoderm GRN is highly controlled by maternal Vegt, and the signaling inputs from Nodal and Wnt signaling pathways, the activation of which coincide with the onset of zygotic gene transcription.

4. Core zygotic mesendoderm transcription factors

A number of zygotic TFs have been identified as critical for the formation of the mesendoderm. Here, we discuss the roles of the Mix family, Gata4/5/6, Foxa and Sox17.

4.1 The Mix family

The critical role of the Mix family TFs in mesendoderm development has been investigated across numerous model systems [88]. In the two *Xenopus* species, the single mammalian gene encoding Mix-like 1 (Mixl1) is represented by *mix1, mixer*, and species-specific expansions and losses of genes referred to as *bix* [89].

In our network, we have examined connections into and from *mix1* and *mixer* based on integrated data from *X. laevis* and *X. tropicalis*. Of these, *mix1* is the earliest to be activated at the mid-blastula transition via direct regulation by Smad/Foxh1, and putative regulation by Vegt. To date, evidence also supports the direct activation of *gsc* and *cer1*, and the repression of *t* by Mix1. This network supports the notion that Mix1 is critical for the activation of dorsoanterior mesendoderm, and the exclusion of *t* from the dorsal organizer [90-92]. This places Mix1 at the top of a negative feed-forward loop, together with Gsc [93], which also represses *t* expression (see section 5.2). This loop may be conserved in mammals, as differentiating Mixl1-null mouse ES cells reveal a down-regulation of *gsc* and an up-regulation of *t* [94]. It is conceivable that Mixer – which also directly activates *gsc* – functions in a similar capacity to repress ventrolateral mesoderm. Supporting this, Mixer deficient *Xenopus* embryos showed up-regulation of mesoderm genes including *eomes*, *fgf3*, *fgf8*, *not*, and *gata2* [95]. Similarly, *t* expression was mildly reduced in Mixer morphants. Finally, while the relationship between *X. laevis* and *X. tropicalis* Bix TFs is unclear, current evidence suggests that *bix1*, in *X. laevis*, is directly activated by T [96], and in *X. tropicalis*, is activated by Nodal/Smad2 [26]. In *X. laevis*, *bix2* (previously known as *milk*) is putatively activated by Nodal/Smad2 [97], and in turn directly activates *gsc* [65,97]. Further elucidating the direct targets of Mix TFs in amphibians, fish, and mammals will be crucial to teasing out these subnetworks.

4.2 Gata family

The Gata transcription factors are highly conserved regulators of endoderm formation across metazoan model systems. In invertebrates, Gata transcription factors play crucial roles in the formation of *Drosophila* midgut [98,99], in the E lineage during *C. elegans* germ layer patterning [100-102], and the sea urchin mesendoderm [2,103]. In vertebrates, the Gata4/5/6 subfamily of Gata factors play a role in the formation of mouse extraembryonic endoderm [104,105], and in the formation of *Xenopus* [106,107] and zebrafish [108,109] endoderm.

Despite their importance, little is known about their molecular targets. In *Xenopus*, the putative direct targets of Gata4/5/6 are endodermal genes *hnf1b* and *sox17a*. Evidence in mouse ES cells also suggests that both *hnf1b* and *sox17a* are Gata4/6 targets [104,105], and zebrafish *gata5* mutants show a reduction in *sox17* expression [110]. While the directness of these interactions is unclear in the mouse and zebrafish, conservation of gene activation suggests similar network topologies are likely operating between frog, zebrafish and mouse.

A common feature of the Gata factors is the extensive mutual regulation among these three genes. In *Xenopus*, *gata5* gain-of-function upregulates *gata4* expression, while *gata4* gain-of-function upregulates *gata6* expression [107]. In zebrafish, loss of *gata5* downregulates the expression of *gata6* and vice versa [109]. Overexpression of Gata4, Gata5, or Gata6 in mouse ES cells results in the upregulation of all three factors [104,105,111], and *Gata4*-null mice show reduced *Gata6* expression [112].

Presently, it is unclear whether these connections are direct; however, functional analyses of a *Gata4 cis*-regulatory module in mouse supports direct co-regulation. Gata4 was shown to bind to an enhancer controlling foregut and midgut expression, suggesting an autoregulatory loop [113]. A second enhancer that controls *Gata4* expression in the septum transversum and

the mesenchyme surrounding the liver are bound by all three Gata factors [114]. These data suggest a direct positive relationship between the three factors.

4.3 Sox17

Sox17 is a highly conserved endodermal transcription factor across vertebrates. Sox17 plays a role in *Xenopus* [115] and zebrafish [116] endoderm formation, and in both mouse extraembryonic and definitive endoderm formation [117], and in the definitive endoderm in human ES cell assays [118]. In *Xenopus*, little is known about the direct targets of Sox17, but putative direct targets include *foxa1*, *foxa2*, and *hnf1b* [119-121]. Sox17 also directly regulates the expression of *foxa2* orthologs in mouse and human extraembryonic and definitive endoderm [118,122]. In addition, Sox17 targets the extraembryonic endoderm marker *Hnf1b* in mouse ES cells [122]. However, it's not known if either of these targets are direct. Finally, functional evidence shows that Sox17 genes are subjected to a positive feedback loop [119,121].

4.4 Foxa family

Foxa TFs are critical for endoderm development across diverse organisms [123,124]. Of the three Foxa TFs in mouse (*Foxa1*, *Foxa2*, and *Foxa3*), Foxa2 is required for early development. Foxa2-null mice display defects in the node (the equivalent of the Spemann organizer) and later gut tube [125]. Both Foxa1 and 2 bind to liver-specific enhancers in mouse pluripotent gut endoderm, well before these genes become transcriptionally active [126], and genetic analyses indicated that these TFs function together in hepatic development [127]. Since they have the capacity to bind to and open compact chromatin [128], Foxa TFs have been deemed ‘pioneer factors’ for gut development [129].

The *Xenopus tropicalis* genome encodes three Foxa TFs (*foxa1*, *foxa2*, and *foxa4*), which are zygotically transcribed. Gain-of-function analyses indicate that Foxa2 inhibits mesoderm and anterior endoderm formation in the gastrula embryo [130]. Loss-of-function analyses in sea urchin also support the conservation of this mechanism [131]. However, as the overexpression of VP16-Foxa2 fusion protein phenocopied the overexpression of Foxa2, Foxa2 presumably functions as an activator [130], and the authors postulate that Foxa2 activates a key repressor of mesodermal cell fate. At present, the direct targets of Foxa in the early embryo are unclear. Finally, in *Xenopus*, *foxa4* is the earliest and most abundantly expressed *foxa* gene during early mesendoderm specification. However, Foxa4's role in early mesendoderm development is not known, although by early neurula stages it promotes notochord formation and inhibits prechordal and paraxial mesoderm [132]. Our network analysis reveals that *foxa4* is activated by Smad2/3, via a Foxh1-independent mechanism [26]. Since *foxa4* expression is repressed directly by the Smad1 target *ventx1* [133], these connections support exclusion of *foxa4* expression from the ventrolateral mesoderm.

5. Network motifs in the *Xenopus* mesendodermal GRN

Network motifs are a subgroup of patterns found in GRN architectures. Here we analyzed the network motifs – representing autoregulatory, feedback, and feedforward loops [134] – found in the network presented here, as well as those previously reported [6,7]. Due to the

increase in the number of direct connections presented in this review over previous networks, we identified significantly more motifs (Figure 2 and Supplemental Table S3), which we will discuss below.

5.1 Autoregulatory and feedback loops

Autoregulatory loops involve the self-regulation of a transcription factor or a signaling pathway (Figure 2A and Supplemental Table S4). Based on our criteria, current evidence supports 4 direct autoregulatory loops. These include the positive autoregulation of Nodal signaling in the endoderm and dorsal mesoderm [26,62,135]; the positive autoregulation of *ventx2* on the ventral side of the embryo [136]; the negative autoregulation of *gsc* [18,28,137]; and the exclusion of *wnt8a* expression in the dorsal mesendoderm through the action of Tcf1/Ctnnb1 [24,138].

We next computationally interrogated our network for feedback loops – a motif that involves mutual regulation between two genes X and Y (Figure 2B and Supplemental Table S4). In a negative feedback loop, gene X positively regulates gene Y, while gene Y negatively regulates gene X; a double negative feedback loop is defined by mutual inhibition between the two genes. A positive feedback loop involves a mutual activation between genes X and Y. We identified five feedback loops in our network – one negative feedback, one double negative feedback and three positive feedback loops.

Based on gain- and loss-of-function analyses, a double negative feedback loop between the dorsal organizer gene *gsc* and the ventral gene *ventx2* has been proposed [139]. While reporter assays and EMSA experiments had confirmed *gsc* as a direct target of Ventx2 [140], it was only recently via ChIP-seq that Gsc binding to *ventx2* cis-regulatory regions has been confirmed [28]. This type of feedback loop enables the formation of sharp expression boundaries between cell lineages, as computationally demonstrated in modeling a double negative feedback between *gsc* and *t* ([141,142].

The network reveals a negative feedback loop between Gsc and Wnt/β-catenin (Ctnnb1), whereby *gsc* – activated by maternal Wnt11 b/β-catenin [82,85,138,143] – represses the expression of zygotic *wnt8a* from the dorsal organizer [28,137]. This type of feedback loop can be useful in cases where the initial activator becomes unnecessary in the control of later gene expression.

Positive feedback loops enable continuous expression of two genes that are important for the same lineage. On the ventral side of the embryo, Smad1 mediating BMP signaling binds the regulatory region of *ventx2* and activates its expression, which in turn appears to regulate the expression of *bmp4* and increases the production of Bmp4 ligand [144]. Finally, in the mesendoderm, the Nodal and Wnt/β-catenin signaling pathways positively feedback into each other [24,26,85,119,145-147]. In this motif, maternal Wnt11b/β-catenin activates the expression of all *nodal* genes in the dorsal mesendoderm. In turn, Smad2/3 (mediated by Foxh1), activates zygotic *wnt8a*, which is excluded from the dorsal organizer via the *gsc* gene as discussed above.

5.2 Feedforward loops

One important feature found in the network presented is the prominent presence of feedforward loops in three-gene network motifs. Of the eight different types of feedforward loops [148] (Figure 2C, Supplemental Table S4), the coherent feedfoward type I is the most abundant. The relative abundance of coherent feedforward type I loops compared to other types of feedforward loops is a feature found in a variety of transcriptional GRNs, including *E. coli* [148], *P. aeruginosa* [149], *S. cerevisiae* [148], and *D. melanogaster* embryogenesis [150,151]. In our network, approximately three-quarters of identified feedforward loops were coherent type I (Supplemental Table S4). In this type of loop, a positive regulator (gene X) and its target (gene Y) both positively regulate the expression of a common downstream gene (gene Z). In the *Xenopus* mesendoderm GRN, this loop appears frequently where gene X is maternal, gene Y is a primary activated zygotic gene and gene Z is a secondary activated zygotic gene (Figure 2D). In the majority of cases, the initial activator appears to be either β-catenin, Foxh1, Smad2/3, or Vegt (gene X). These maternal factors activate the expression of early and mid-blastula zygotic genes such as *wnt8a, sia1*, *sia2*, *mix1*, *gsc*, and the *nodal* genes (gene Y), which, in turn, activate the expression a larger number of later expressed mesendodermal genes (gene Z). Some examples of gene Z include *cer1*, *eomes*, *ventx1*, *hhex*, and *pitx2*. The benefits of this type of loop depend on whether the co-regulation of Z by both X and Y is an 'AND-gate' (where both factors are required to activate factor Z) or an 'OR-gate' (where either factor can activate gene Z) [134]. An 'AND-gate' can be beneficial in the tight control of factor Z expression, as factor Z is only activated once factor Y is expressed. On the other hand, an 'OR-gate' enables the sustained expression of factor Z, despite the loss of the initial factor X [134]. Such would be a critical motif functioning in early *Xenopus* embryogenesis, where maternal factors that act as the initial activators are degraded during blastula stages. Then, their direct, primary, activated zygotic targets can function to maintain the expression of later, secondary, activated genes. It's not clear as to whether the coherent type I feedforward loops we identified are controlled by AND-gates or OR-gates. It will be important to address this type of question as we further refine our understanding of gene regulation in the early *Xenopus* mesendoderm.

The other types of feedforward loops involve a negative regulation between genes X, Y and Z [148] (Figure 2C, Supplemental Table S4). In the *Xenopus* system, these types of loops appear enriched among genes required for dorsal-ventral patterning of the mesendoderm. For example, Smad2/3 signaling promotes both *t* and *gsc* expression [26,43,87,93,152]. In turn, *gsc* represses *t* from the dorsal organizer. In a similar loop, *otx2* also functions in the restriction of *t* expression [90,153]. These loops, along with negative and double negative feedback loops, appear to be particularly useful in regionalizing the dorsal and ventral sides of the mesendoderm. It is likely that more of these types of loops will be identified in the future, as we better define the expression domains of more transcription factors during *Xenopus* gastrulation [14-16]. Also, as this review focused primary on mesendodermal genes, the molecular mechanism for the regionalization of the embryo between mesendoderm and prospective ectoderm is not reflected on this network. However, as some animal pole factors repressing Nodal signaling have been identified (e.g., maternal Sox3) [154], similar network motifs might be used in animal-vegetal patterning.

6. Prospects

We have generated a comprehensive gene regulatory network governing *Xenopus* mesendoderm development. New findings, and the increased accessibility of HTS technologies, have contributed greatly to the number of direct regulatory interactions between critical factors, and have revealed many more possible players whose functions remain unclear. We predict that the network will exponentially increase as more datasets are generated. In addition, advances in the use of CRISPR/Cas9 in *Xenopus* provides the opportunity to modify the endogenous interactions between TFs and their target CRMs. This will enable in-depth investigations into the role of CRMs in gene regulation, ultimately aiding in addressing one of the most critical questions in biology: how mutations in regulatory regions influence overall gene expression levels.

The mesendoderm network is initiated in the blastula embryo at the onset of zygotic gene activation, and the network presented here – which extends through the beginning of gastrulation – covers a timespan of approximately 3 hours. During this time of rapid developmental transitions, as maternal factors – important for the initiation of the network – are degraded, and zygotic transcription ramps up, we expect the network to be highly dynamic. While single-stage analyses may be sufficient in identifying direct target genes, the investigation of TF targets over time provides valuable kinetic information to uncover the complexities of the dynamic regulatory network.

In addition to transcription factors and signaling molecules, screens for non-coding RNAs, including microRNAs [155-159], and long non-coding RNAs [160,161], have identified many more potential regulators of gene expression. One example is through the negative regulation by mir-427 of the Nodal ligands *nodal5* and *nodal6*, and the nodal antagonist *lefty* [162]. Loss of function of mir-427 leads to mesodermal patterning defects. Interestingly, the interaction between Nodal signaling, Lefty and mir-427 generates an incoherent type II feedforward loop. As we learn more about the roles of these non-coding RNAs, we will have to integrate their regulatory roles into the GRN diagram.

As the GRN increases in connectivity and becomes more complete, this will enable future researchers to investigate the GRN from a systems level perspective. Identification of various network motifs can provide some new and interesting hypotheses based on the theoretical properties of these motifs [134], which can be experimentally tested *in vivo*. Additionally, quantitative modeling of these network can delve deeper into the nature of the regulatory relationships between transcription factors, as has been done in smaller networks [141,142,163]. Some of the challenging questions in the future are to parse which mesendodermal factors play major roles in regulating network function and maintaining network output robustness; as well as the role of redundancy in network regulation, and feedback/feedforward loop regulation. The *Xenopus* mesendoderm GRN, with its rich history and amenability to modern genomic tools, presents itself as one of the best systems to study these types of network questions *in vivo*. We hope that this network will provide a useful framework in moving towards a greater understanding of the complex GRN controlling early mesendoderm development, and as well as the formation of later endodermal derivatives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Comprehensive *Xenopus* mesendoderm gene regulatory network (GRN)
- Conserved regulatory connections in frog, fish, mouse, and human endoderm are revealed
- Coherent feedforward loops are the most common network motif in the GRN

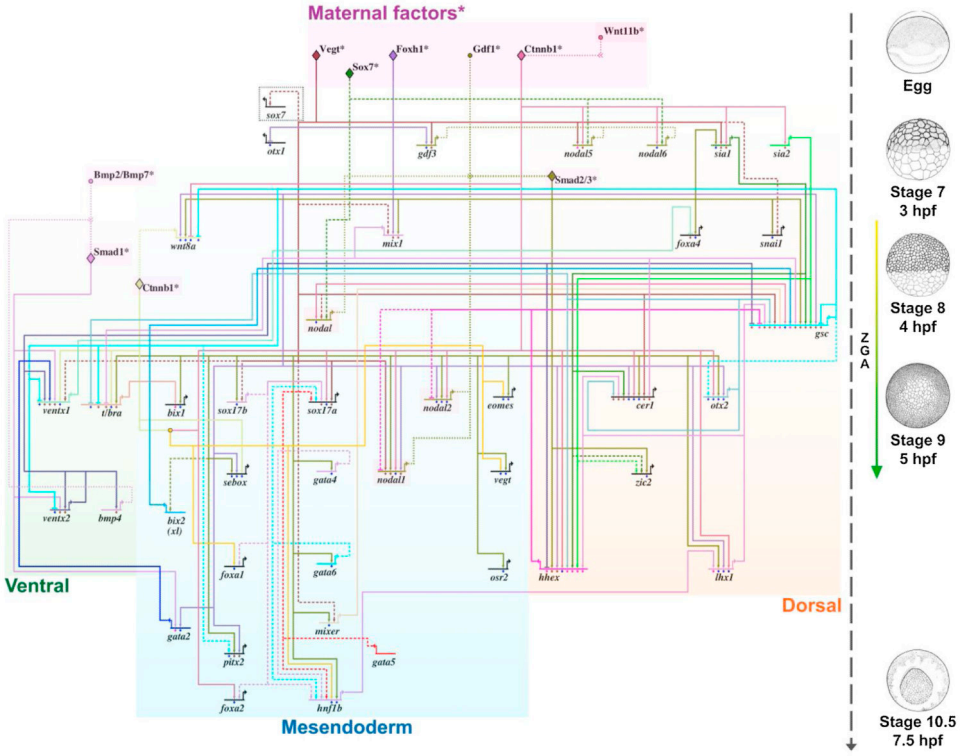

Figure 1. Xenopus mesendoderm gene regulatory network from fertilization through early-gastrula

The network is comprised of 23 transcription factors and 12 growth factors. Maternal proteins are represented as diamonds, and signaling ligands as circles. Connections are drawn from the transcriptional regulator to the cis-regulatory region of the target gene. Direct connections are indicated as solid lines, and putative connections as dashed lines. Activating connections are represented by arrowheads, and repressive connections as a flat line. Connections from secreted ligands pass through a chevron, and are mediated by their respective intracellular transcription factors (e.g. Smad2/3, β-catenin). Approximate spatiotemporal information is provided from top to bottom (egg through early-gastrula) and from right to left (dorsal to ventral), with some exceptions (e.g. *xbra/t*). The activation time of zygotic *sox7* (boxed) is unknown. All direct connections are annotated for TF binding (blue diamond), reporter assay (pink diamond), and TF binding plus functional validation (maroon diamond). For additional connection details, including experimental evidence and references, see Table 1 and Table S2. Zygotic genome activation, ZGA.

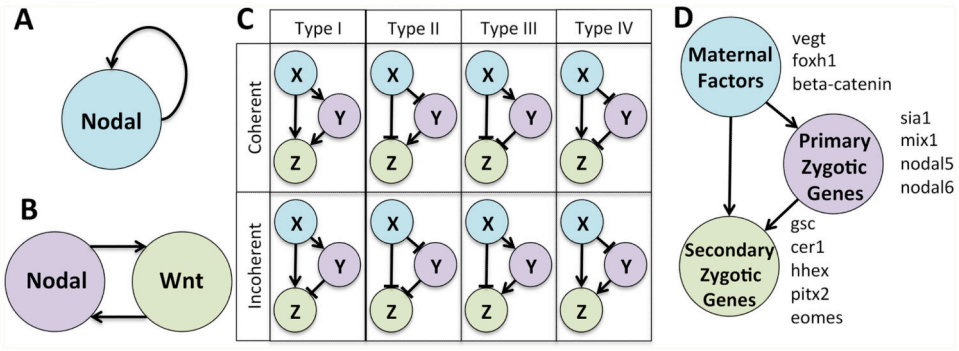

Figure 2. Network motifs found in the Xenopus mesendoderm GRN

(A) Autoregulatory loop, for example by nodal signaling (B) Positive feedback loop, for example between ventral genes Nodal and Wnt signaling (C) Coherent and incoherent feedforward loops and their regulatory structure (D) Type I feedforward loop, which appears to be the most common type of feedforward loop, frequently appears in the structure such that X is a maternal factor, Y is an early zygotic gene, and Z is either an early or late zygotic gene.

Table 1
Summary of direct and putative connections between network transcription factors

See also Table S2 for additional connection details. Putative direct targets are denoted as [P].

ctnnb1/β-catenin (Wnt)	cer1, eomes, foxa1, foxa2, gsc, hhex, hnf1b, lhx1, nodal1, nodal2, nodal, nodal5, nodal6, otx2, sebox, sia1, sia2, sox17a, t, vegt, ventx1, wnt8a	[23,24,82-85,119,138,145,147,164-166]
vegt	cer1, gdf3, gsc, mix1 [P], mixer [P], nodal1, nodal [P], nodal5, sia1, snai1 [P], sox17a, sox17b1 [P], sox7 [P], ventx1 [P]	[33,35,36,38,41,85,121,165,167-172]
foxh1	cer1, gata2, gdf3, gsc, hhex, lhx1, mix1, nodal1, nodal2, otx1, otx2, pitx2, sebox, wnt8a	[26,62,63,66,72,166,173-175]
sox7	nodal [P], nodal5 [P], nodal6 [P]	[49,171]
smad2/3 (Nodal)	bix1, bix2, cer1, eomes, foxa4, gata4, gata6, gsc, hhex, hnf1b, lhx1, mix1, mixer, nodal1, nodal2, osr2, otx2, pitx2, sia1, snai1, sox17b, t, vegt, wnt8a, zic2	[26,27,62,63,72,87,90,166,176,177]
smad1 (BMP)	gata2, ventx1, ventx2	[61,178-188]
sia1	cer1, gsc, hhex, zic2 [P]	[33,84,91,92,152,189,190]
sia2	gsc, hhex, zic2 [P]	[84,166,191,192]
mix1	cer1, gsc, t	[33,90-92,152,190,193]
mixer	gsc	[65,194,195]
bix2 (laevis only)	gsc	[65,97]
gsc	gsc, otx2 [P], pitx2 [P], t, ventx1, ventx2, wnt8a	[18,28,90,137,196]
otx2	cer1, gsc, hhex, t	[32,90,153,166,197]
lhx1	cer1, gsc, hhex	[166,190,197]
hhex	gsc, nodal1 [P], nodal2 [P]	[198-200]
gata4	hnf1b [P]	[106,107,201]
gata5	hnf1b [P], sox17a [P]	[106,107]
gata6	hnf1b [P], sox17a [P]	[107,201]
gata2	ventx1	[202]
sox17b	foxa1 [P], foxa2 [P], hnf1b [P], sox17a	[119-121,176]
hnf1b	lhx1	[203]
t	bix1	[96]
ventx1	foxa4	[133]
ventx2	bmp4, gsc, hhex, ventx1, ventx2	[136,140,166,202,204,205]