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Early *Xenopus* gene regulatory programs, chromatin states, and the role of maternal transcription factors

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Abstract

For decades, the early development of the *Xenopus* embryo has been an essential model system to study the gene regulatory mechanisms that govern cellular specification. At the top of the hierarchy of gene regulatory networks, maternally-deposited transcriptions factors initiate this process and regulate the expression of zygotic genes that give rise to three distinctive germ layer cell types (ectoderm, mesoderm, and endoderm), and subsequent generation of organ precursors. The onset of germ layer specification is also closely coupled with changes associated with chromatin modifications. This review will examine the timing of maternal transcription factors initiating the zygotic genome activation, the epigenetic landscape of embryonic chromatin, and the network structure that governs the process.

Keywords

Maternal transcription factors; Enhancers; Promoters; Cis-regulatory modules; Gene regulatory network; Zygotic genome activation; *Xenopus* ; Germ layers; Feed forward loop

1. Introduction

After fertilization, the embryonic genome is inactive until transcription is initiated during the maternal-to-zygotic transition, whereby the onset of embryonic genome transcription is called zygotic genome activation (ZGA). At present, the functional relationship among TFs, co-regulators and the epigenetic landscape around ZGA is still poorly understood and several major questions remain. For instance, what combination of maternal transcription factors (TFs) contributes to the initiation of genome activation? How do the maternal TFs bind to the chromatin and coordinate the opening or closing of chromatin for their accessibility? How are the chromatin states in the form of histone and DNA modifications established during ZGA? What kinds of network structures are operational at the early stage of development to ensure dynamic changes in gene expression at different types of cells? The *Xenopus* species are model organisms well-suited to address these critical questions. They are highly tolerant toward RNA and DNA microinjection to obtain knockdown or overexpression phenotypes to uncover TF functions. The genomes of the traditionally used

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pseudotetraploid *Xenopus laevis* (Session et al., 2016) and the diploid cousin *Xenopus tropicalis* (Hellsten et al., 2010; Mitros et al., 2019) have been sequenced, which makes both amenable to a variety of high-throughput genomic approaches. In addition, the embryos of both species are amenable to CRISPR-cas9 mutagenesis-based approaches (Blitz et al., 2013; Nakayama et al., 2013; Wang et al., 2015; Shi et al., 2015; Blitz et al., 2016; Aslan et al., 2017). In this review, we will cover the essential players in the first network connections in the vertebrate gene regulatory network (GRN) occurring at the onset of ZGA. First, we will discuss the maternal TFs and signaling molecules that confer germ layer-specific GRNs. Second, we will highlight the cis-regulatory regions in the form of enhancers and promoters with particular focus on findings from the genomic data. Finally, we will approach the GRN from a systems biological perspective and discuss the network architecture of the early *Xenopus* GRN.

2. Roles of maternal TFs during germ layer specification

2.1. Localization of maternal gene products

After fertilization, the *Xenopus* zygote undergoes multiple rounds of division to give rise to smaller cells (blastomeres) without increasing the overall size of the embryo. During the early stages of this process, individual blastomeres are pluripotent and remain uncommitted to specific lineages (Heasman et al., 1984; Snape et al., 1987). Gradually, cells along the animal-vegetal axis acquire germ layer identities. Specifically, ectoderm is formed in the animal cap (top side of the embryo), endoderm is formed in the vegetal mass, and mesoderm is induced at the equatorial region between the animal and the vegetal poles of the *Xenopus* embryo. During this process, maternally deposited mRNAs and proteins are inherited into individual blastomeres. Some maternal products show localized expression, while the others are uniformly distributed along the animal vegetal polarity (Fig. 1). The localization of these maternal products control the germ layer cell identities by initiating their respective GRN programs. Multiple large-scale (Cuykendall et al., 2010; Flachsova et al., 2013) and genome-wide (De Domenico et al., 2015; Paraiso et al., 2019) screens identified hundreds of genes with localized expression, which includes a little over a dozen TFs and signaling molecules in both *Xenopus laevis* and *Xenopus tropicalis* embryos. Among these TFs are *otx1*, *vegt* and *sox7* gene products, which are enriched in the vegetal tissue, whereas the *foxi2* and *grhl1* gene products are enriched animally. Additionally, ubiquitously-expressed *foxh1*, *sox3*, *pou5f3* (*oct60*) TFs, and mediators of TGF-β (Smad2/3) signaling and Wnt (*ctnnb1/β-catenin* and *tcf7l1*) signaling play central roles in specifying the identities of germ layer specific gene regulatory programs. We will discuss the roles of these TFs in germ layer specification (Summarized in Table 1).

2.2. Endodermally-enriched TFs

Vegt: One of the most well-studied maternal determinants of germ layer specification in the *Xenopus* embryo is the vegetally localized T-box TF, Vegt (previously also called Xombi, Antipodean, or Brat) (Lustig et al., 1996; Zhang and King, 1996; Stennard et al., 1996; Horb and Thomsen, 1997). Gain-of-function of Vegt in the putative ectoderm exhibits strong endodermal-inductive properties, while loss-of-function of endogenous Vegt results in conversion of the putative endodermal cells into meso- and ectodermal cell fates

(Zhang et al., 1998; Kofron et al., 1999). This is consistent with the finding that Vegt regulates the expression of Nodal ligands (Kofron et al., 1999), which are one of the earliest transcribed zygotic genes necessary for the initiation of both the endoderm and mesoderm (reviewed by Schier, 2003). In addition to its role as an activator of transcription, Vegt is involved in repression of genes. Gain-of-function in ectodermal cells caused significant down-regulation of ectodermal genes (Taverner et al., 2005), while Vegt loss-of-function in endodermal cells caused up-regulation of meso- and ectodermal genes (Paraiso et al., 2019). The genome-wide binding of Vegt also supports the dual function of Vegt, as Vegt occupies both cis-regulatory modules (CRMs) of mesendoderm and ectodermal genes (Gentsch et al., 2013; Paraiso et al., 2019). Following the discovery of Vegt, the role of T-box TFs during early endoderm formation has become well appreciated in a variety of vertebrates including Axolotl (Pérez et al., 2007; Nath et al., 2007), zebrafish (Xu et al., 2004; Bjornson et al. 2005), and human (Teo et al., 2011).

Otx1: Otx1 was previously shown to be maternally deposited and vegetally localized (Pannese et al. 2000; De Domenico et al. 2015), and the role of Otx1 in endoderm formation has been recently described (Paraiso et al., 2019). Otx1 is a critical collaborator of Vegt during endoderm formation. ChIP-seq analysis shows that Otx1 can bind to selected mesendoderm CRMs during cleavage stages in a manner whereby the majority of Otx1 binding sites overlap with Vegt binding (Paraiso et al., 2019). Interestingly, whereas Vegt can activate both mesoderm and endoderm gene expression, Otx1 only activates endoderm genes while concurrently repressing mesodermal genes. How these additive/synergistic and antagonistic interactions are differentiated at the level of CRMs is distinguished is currently unknown. Importantly, the maternal expression and vegetal localization of vertebrate Otx1 and invertebrate Otx1 homologs appear to be conserved across metazoans (Paraiso et al., 2019). The oocyte expression pattern, along with functional evidence in echinoderms (Hinman et al., 2003; Peter and Davidson 2010) and tunicates (Wada and Saiga, 1999) on the role of *otx* genes, suggest a deep conservation of *otx* function at least in bilaterian endodermal GRNs.

Sox7: The HMG-domain containing TF Sox7 is maternally expressed locally in the vegetal cells maternally (Zhang et al., 2005; Owens et al., 2016). Sox7 belongs to the F-type sub-family of Sox TFs, which include well known, zygotically-expressed, endodermal differentiation factor Sox17. Sox7 regulates the expression of Nodal ligands, endodermal markers such as *a2m* (formerly *endodermin*), and endodermal TFs *mixer* and *sox17b* (Zhang et al., 2005). Notably, the depletion of Sox7 does not cause major phenotypic disruption on endoderm formation, suggesting a combinatorial function of maternal TFs is needed during this process. Indeed, Sox7 appears to co-bind with other maternal TFs during early gastrula stages (Charney et al, 2017a), and is a predicted co-bound factor with Otx1 and Vegt (Paraiso et al., 2019). How Sox7 functionally co-regulates with Otx1, Vegt and other maternal TFs is unknown.

2.3. Ectodermally-enriched TFs

Foxi2: Maternally expressed forkhead domain TF Foxi2, is highly enriched in the animal region of the *Xenopus* embryo (Cha et al., 2012). Foxi2 is required for the expression of

ectodermal genes such as *lhx5* and *cdh1* (*e-cadherin*). Additionally, maternal Foxi2 has been shown to directly activate the zygotic expression of another Foxi TF, *foxi1*, by binding to the *foxi1* promoter. Like *foxi2*, *foxi1* is an important regulator of ectoderm formation (Mir et al., 2007). How the function of these related Fox TFs overlap is unknown and it will be interesting to dissect the genome-wide roles of *foxi2* and *foxi1*. Particularly, since Foxi2 acts high in the ectodermal gene regulatory hierarchy, Foxi2 ChIP-seq data will uncover important CRMs regulating the ectodermal GRN.

Grhl1: Grainyhead TFs are highly conserved across diverse animal species, and are responsible for epidermal barrier formation. Grh1 is maternally expressed and enriched animally (Paraiso et al., 2019). While not much is known about the role of maternal grhl1, zygotically-expressed Grhl1 is essential for epidermal ectoderm formation (Tao et al., 2005a). As one of the few ectodermally-localized maternal TFs, analysis of the *grhl1* downstream targets in the GRN will be critical in understanding the early ectoderm differentiation process.

2.4. Ubiquitously-expressed TFs

Foxh1: Foxh1, a member of the Forkhead family, is maternally supplied and expressed ubiquitously in the cleavage stage *Xenopus* embryo (Kofron et al., 2004). Foxh1 was initially identified as a Nodal signaling co-factor (Chen et al., 1996; Chen et al., 1997), which recruits co-effector Smad2/3 to activate downstream target gene expression. Early studies of Foxh1 function complemented by recent genomics approaches suggest transcriptional roles beyond collaboration with Nodal signaling during mesoderm induction. In the dorsal mesendoderm, Foxh1 collaborates with intracellular mediators of Wnt signaling, Tcf3 and Ctnnb1 (β-catenin), to regulate the expression of Nodal genes (Kofron et al., 2004; Reid et al., 2016; Charney et al., 2017a). Genome-wide approaches identified further roles of Foxh1 in regulating genes involved in formation of the endoderm such as *sox17a* (Chiu et al., 2014). Additionally, Foxh1 has been implicated as a repressor of gene expression (Chiu et al., 2014; Reid et al., 2016) to possibly inhibit precocious activation of ZGA. The mechanism by which Foxh1 toggles between these roles is unclear, although hypotheses can be inferred from genome-wide chromatin data (Fig. 2). For tissue-specific functions, Foxh1 appears to co-bind to different sets of TFs. Persistent Foxh1 binding through the blastula and gastrula stages co-localizes with Smad2/3 and Sox7, and this binding is enriched near dorsal mesendoderm genes (Charney et al., 2017a). In the endoderm, Foxh1 co-binds with endodermal TFs Vegt and Otx1 in the enhancers of target genes (Paraiso et al., 2019). Interestingly, the collaborative nature of Foxh1 and T-box TF interactions in mesendoderm formation has been documented in early zebrafish embryos whereby zebrafish Foxh1 collaborates with the Vegt paralog Eomes (Slagle et al., 2011; Nelson et al., 2014). For positive or negative regulatory functions, Foxh1 appears to switch partners whereby Foxh1 recruits the Groucho family of co-repressors Tle1/2/4 (Charney et al., 2017a) to mediate the switch to Foxh1's repressive roles (Reid et al., 2016).

Sox3: Sox3 is a member of the B group of the large Sox proteins and binds to variants of a common core consensus sequence AACAAT (Mertin et al., 1999; van Beest et al., 2000). Sox3 is expressed both maternally and zygotically in ectoderm, and is involved in neural

ectoderm specification (Rogers et al., 2008). Sox3's maternal role was inhibited using an affinity-purified antibody, which blocks Sox3 binding to DNA (Zhang et al., 2003), which indicated its primary function as a mesodermal suppressor in the ectoderm by negatively regulating Nodal signaling (Zhang and Klymkowsky, 2007). While maternal *sox3* mRNA is predominantly expressed in the ectoderm, it is also detected in the vegetal mass of early cleavage and blastula embryos (Blitz et al., 2017; Paraiso et al., 2019), suggesting it is possible it also plays roles in the mesoderm and the endoderm. Indeed, the role of Sox3 in *Xenopus* embryos has been expanded to pioneering roles in chromatin opening and mediating chromosome conformation to regulate early gene expression. Sox3 synergistically acts with Pou5f3 (an ortholog of mammalian Oct4/Pou5f1) to regulate ZGA (Gentsch et al., 2019) similar to what has been proposed in early zebrafish embryos (Lee et al., 2013). This functional collaboration by Sox3 and Pou5f3 in *Xenopus* is also reminiscent of the establishment of pluripotency in mammalian embryonic stem cells by these factors (Takahashi and Yamanaka, 2006).

P*ou5f3*: *Xenopus* and zebrafish PouV family TFs are evolutionarily closely related to mammalian Pou5f1/Oct4 (Frankenberg et al. 2010; Hellsten et al. 2010), which plays crucial roles during early mammalian embryogenesis and embryonic stem cell pluripotency. In *Xenopus,* the Pou V genes *pou5f3.1, pou5f3.2 and pou5f3.3* (previously *oct91, oct25,* and *oct60,* respectively) are expressed ubiquitously in the early embryo (Chiu et al., 2014). Temporally, *pou5f3.2* and *pou5f3.3* are both expressed maternally, but *pou5f3.3* RNA is more abundant than *pou5f3.2* before the blastula stage (Hinkley et al. 1992). Injection of a cocktail of *pou5f3.1, pou5f3.2 and pou5f3.3* MOs into *Xenopus* embryos caused axial defects including gross head abnormalities and shortening of the trunk and tail (Morrison and Brickman 2006; Chiu et al., 2014). Interestingly, Pou motifs are enriched in regions of Foxh1 ChIP-seq peaks, and PouV knockdown showed up-regulation of Foxh1 target genes such as (*cer1, foxa4, gata4, gsc, nodal2, snai1,* and *vegt*) (Chiu et al., 2014). These results suggest that PouV proteins negatively regulate the expression of a subset of Nodal target genes. In *Xenopus*, Pou5f3 was shown to interact with Sox3 and initiates local chromatin remodeling to facilitate poised or active transcription during ZGA (Gentsch et al., 2019). Sox3 and PouV interaction may be additive or redundant as loss of function of Sox3 and PouV individually is ineffective, whereas simultaneous depletion led to much stronger phenotypes. Interestingly, the maternal PouV TFs was also shown to inhibit the function of the TFs Vegt and Ctnnb1 (Cao et al., 2007) and Nodal signaling (Cao et al., 2005) in ectoderm, implying their diverse functions during early embryogenesis.

2.5. Intracellular mediators of signaling pathways

Gdf1/Nodal/Smad2,3: Maternally expressed *gdf1* (formerly *vg1*) transcripts encoding for a Nodal ligand is expressed in the vegetal mass along with *vegt* transcripts. Gdf1 loss-of-function results in reduction in Smad2/3 phosphorylation (Birsoy et al., 2006) and results in shortened axial elongation indicative of convergent extension defects. The activated Smad2/3-Smad4 complex regulates target genes, which include *foxh1, eomes, foxh1.2, gtf2i, gtf2ird1, mixer, tcf3* (also known as *e2a*) and *tp53*. The role of Gdf1 appears to be specific to the mesoderm-inducing function of endodermal cells as Gdf1 loss-of-function specifically affects the organizer (dorsal mesoderm) gene expression, including

BMP antagonists *chrd* and *nog*, and the zygotic Nodal gene *xnr1*, but not the endodermal gene *sox17a* (Birsoy et al., 2006). During cleavage stages, *nodal5* and *nodal6* are activated by Vegt, and these ligands contribute to the earliest zygotic activation of the Nodal signaling pathway. This role of Nodal signaling during endoderm formation has been well documented in a variety of vertebrates (reviewed by Schier, 2003).

Wnt/TCF/Ctnnb1 (β-catenin): Maternal *wnt11b*, is localized to the vegetal pole in the egg, relocated to the dorsal vegetal cells following cortical rotation, and activates a canonical Wnt signaling pathway to specify the dorsal fate (Ku and Melton, 1993; Tao et al., 2005b). Molecularly, nuclear β-catenin protein accumulates dorsally, forms a complex with Lef/Tcf TFs, and directly regulates *sia1* and *sia2* homeobox gene expression (Brannon et al., 1995; Laurent et al., 1997). At the same time, dorsally enriched maternal Wnt/β-catenin signaling activates the *nodal5* and *nodal6* genes (Yang et al., 2002; Xanthos et al., 2002). Co-occurrence between Foxh1 (a major Smad2/3 co-factor) and β-catenin ChIP-seq peaks supports the model that Nodal and Wnt signaling pathways crosstalk and co-regulate the expression of dorsal mesoderm genes, including *hhex*, *lhx1*, *otx2*, *cer1* and *gsc* (Chiu et al., 2014; Nakamura et al., 2016).

2.6. Other TFs

Hundreds of TFs are detectably expressed at the RNA level maternally and dozens are expressed in animally or vegetally localized manner (Fig. 3). Among these TFs are those previously discussed and many more have unknown functions. Examples include *snai1*, *pbx1* and *gbpb1* which are vegetally-localized. Particularly interesting TFs among the ubiquitously-expressed may be members of the Zic family as Zic motifs are often enriched within ChIP-seq peaks of the above mentioned maternal TFs (Paraiso et al., 2019). Currently, the role of Zic family members in ZGA and germ layer specification is not well understood. It will be interesting to see how the role of these maternal TFs integrate to initiate GRNs of the three germ layers.

3. Enhancers, Promoters and Chromatin States

3.1. Genomic approaches to identifying enhancers

Much work has been performed in order to understand the function of CRMs during early *Xenopus* embryogenesis. The CRMs upstream of genes such as *gsc* (reviewed by Koide et al., 2005) and *hhex* (Rankin et al., 2011) have been functionally dissected. However, the depth of understanding of the regulatory regions of these two genes are the exception in our understanding of the CRMs in the genome. In addition, recent genomic datasets have uncovered putative enhancers downstream the *hhex* gene body (Fig. 4), outside the 6 kb upstream region of *hhex* that has been analyzed (Rankin et al., 2011). This downstream region is bound by TFs such as Foxh1, Otx1 and Vegt; and is contained within the *hhex* endodermal super-enhancer (Paraiso et al., 2019). Due to these reasons, the use of ChIP-seq, ATAC-seq, DNAse-seq, etc. to identify the genomic coordinates of epigenetically marked histones and open chromatin have been attractive approaches in identifying putative CRMs in the genome. Promoter regions have been found to be associated with H3K4me3 marking (Heintzman et al., 2007). Enhancers on the other hand have been associated with a variety

of features, in addition to binding of multiple TFs (Fig. 5). Chromatin marks such as H3K4me1 (Heintzman et al., 2007), H3K27ac (Creyghton et al., 2010), Ep300 (Heintzman et al., 2007), and DNA accessibility (Boyle et al., 2008) have been used to identify active enhancers. Additionally, extragenic binding of RNA Polymerase II (De Santa et al., 2010) and transcripts (enhancer RNAs) (Kim et al., 2010) have been associated with marks of active enhancers, genome-wide. For the next section, we highlight the chromatin states of the early embryo as from the view of promoter and enhancer epigenetic marks.

3.2. Chromatin states and ZGA

In most animals, the early embryonic genome is transcriptionally silent and is programmed into a pluripotent state after the union of the egg and the sperm genome. This development is initially under the control of maternal products, including TFs that are loaded into the female gamete during oogenesis. These maternal TFs play central roles in coordinating the initiation of zygotic GRNs by binding to the CRMs of the genome to regulate the transcriptional responses of target genes. In addition, the presence of other transcriptional regulators such as co-activators/repressors and the chromatin state surrounding the CRMs influence gene expression. Significant efforts have been placed to uncover the chromatin state of these TF-bound CRMs during ZGA to comprehend the relationship between epigenetic regulation and gene expression during the earliest cell fate decision process.

The onset of zygotic genome activation (ZGA) is one of the first major milestones in embryonic development, the timing varies significantly among different animals (reviewed by Jukam et al., 2017). For example, in mice, this process begins right after the first cleavage cycle (24 hours post-fertilization) while in *Drosophila melanogaster*, ZGA occurs at the 14th nuclear cycle (2.5 hours post-fertilization) at ~6,000 nuclei stage. In *Xenopus laevis*, ZGA generally occurs after the first 12 cleavage divisions during the early blastula stage, which is also known as the mid-blastula transition (MBT). However, recent high-resolution transcriptome profiling (Collart et al., 2014; Owens et al., 2016) has revealed that zygotic transcripts of *pre-mir427* are detected as early as the 8-cell stage (at the third cleavage), which is significantly earlier than the classically defined MBT (Newport and Kirschner, 1982). Additionally, from the same transcriptomic data, dozens of zygotic transcripts are first detected at the 128- and 256-cell stages, including *nodal5* and *nodal6* (Yang et al., 2002). These data indicate that *Xenopus* ZGA is not a single switch-like temporal event but instead occurs broadly during a time window where new transcription gradually begins.

Evidence from *Xenopus* suggests that gene promoter marking in the form of histone H3 lysine 4 trimethylation (H3K4me3) are largely established around the period of zygotic genome activation (Akkers et al., 2009; Hontelez et al., 2015). Loss of transcription through α-amanitin treatment does not affect H3K4me3 marking, suggesting that the establishment of this mark is controlled by maternal factors (Hontelez et al., 2015). However, the mechanism of regulating the timing of the methyltransferase activity is unknown. Interestingly, the appearance of the H3K4me3 mark during early development varies across species. Zebrafish and *Drosophila* H3K4me3 marking occurs largely during ZGA (Li et al., 2014; Vastenhouw et al., 2010), similar to *Xenopus*. While in mice, unusually broad, non-canonical H3K4me3 domains (wider than 5kb) were observed in

matured oocytes (Dahl et al., 2016; Liu et al., 2016; Zhang et al., 2016). During ZGA, these broad domains disappear and H3K4me3 marking become restricted to the conventional TSSs of transcriptionally active genes during ZGA

Early embryonic enhancers are labeled with a variety of histone marks. These include the general enhancer histone mark H3K4me1, active enhancer histone mark H3K27ac (Histone H3 lysine 27 trimethylation) and the co-activator Ep300 (Gupta et al., 2014; Hontelez et al., 2015). Just like H3K4me3, these enhancer marks appear to be largely established during ZGA, consistent with findings in *Drosophila* (Li et al., 2014). At present, how H3K4me1 and H3K27ac accumulation on CRMs in *Xenopus* embryos is regulated is unknown, although numerous histone methyl- and acetyl-transferases that regulate their deposition are maternally expressed in the early *Xenopus* embryo (Collart et al., 2014; Owens et al., 2016). Importantly, the presence of these enhancer marks is highly correlated with maternal TF binding to CRMs (Charney et al., 2017a; Paraiso et al., 2019; Gentsch et al., 2019). It is therefore tempting to speculate that maternal TFs are somehow involved in the deposition of these histone marks, perhaps by recruiting specific histone modifying complexes to CRMs. While a Wnt signaling co-activator, Ctnnb1 (β-catenin), was shown to be required for the deposition of H3R8me2 mark through recruitment of the methyltransferase Prmt2 (Blythe et al., 2010) in the promoter region of organizer genes, it is currently unclear whether the H3R8 mark is a critical regulator of ZGA.

3.3. Repressive chromatin marking

The question of how a given gene is dynamically modified, sometimes with active histone marks in one cell type, but with repressive states in another cell type, is a central question to understanding the germ layer gene regulatory program. The PRC2 complex deposits H3K27me3 and represses target gene expression (Bannister and Kouzarides, 2011). In most species, the increase of H3K27me3 begins to emerge after ZGA (Vastenhouw et al., 2010; Li et al., 2014). This implies that transcriptional quiescence before ZGA is not imposed by H3K27me3-marked repression. In mammalian embryonic stem cells (ESCs), the co-occurrence of active H3K4me3 and repressive H3K27me3 chromatin modifications has been described as a bivalent mode on promoters of poised developmental genes (Bernstein et al., 2006; Mikkelsen et al., 2007). However, this bivalent mode has not been detected during early embryogenesis of mouse, fly and frog embryos (Akkers et al., 2009; Liu et al., 2016; Zhang et al., 2016), suggesting that the bivalency marking is not a common state of the vertebrate embryonic genome. For instance, sequential ChIP-seq experiments carried out using *Xenopus* embryos reveal that the bivalent marking (H3K4me3 and H3K27me3) of genes is not a prevalent configuration in *Xenopus* embryos (Akkers et al., 2009). Another interesting finding is the spatially regulated activity of H3K27me3 marking. When histone marking of endodermally expressed genes was examined, the genes were specifically marked by H3K27me3 repressive mark in ectodermal (animally located) cells. This suggests that H3K27me3 participates in repressing unwanted endodermal gene expression in ectoderm, but not in endoderm, thus contributing to the spatially distinct chromatin states in different cell types.

How can the CRM of a given gene be marked by an active histone mark, while in other tissues the same CRM is marked by a repressive mark? Through the examination of various maternal TF ChIP-seq data, it was noted that maternal TFs co-binding to CRMs are associated with H3K4me1 or H3K27ac marks in later developmental stages (Paraiso et al., 2019). Co-binding of maternal TFs is particularly enriched in clusters of endodermal enhancers with high-levels of H3K4me1 marking or super-enhancers, which are associated with key cell identity genes (Lovén et al., 2013; Whyte et al., 2013). Current tissue-specific perturbation data show that maternal TFs regulate the RNA Polymerase II occupancy and enhancer RNA transcription in these co-bound CRMs (Paraiso et al., 2019). Possibly, these maternal TFs also regulate deposition of H3K4me1, similar to what has been shown in whole embryo datasets where Sox3 and Pou5f3 perform this function (Gentsch et al., 2019). Further study examining the biochemistry of the interactions between these maternal TFs and histone modifiers should provide useful insights to the dynamic epigenetic regulation occurring during ZGA and germ layer specification.

4. Network structure analysis of the early *Xenopus* GRN

We previously curated the endodermal gene regulatory network during early *Xenopus* development from fertilization through early gastrulation (Koide et al, 2005; Charney et al., 2017b). We used a bipartite criteria in identifying regulatory targets. First, a candidate target gene has to be perturbed by loss-of-function and/or gain-of-function experiments performed on the TFs. Second, there has to be evidence of directness in the regulatory interaction. We called an interaction 'putatively direct' of the activator is capable of inducing target gene transcription even in the presence of protein synthesis inhibitors. We called an interaction 'direct' if there is an identifiable cis-regulatory region, which can be implicated to the activating TF. This can be in the form of reporter gene assays, chromatin immunoprecipitation experiments, DNAse footprinting, and/or electrophoretic mobility shift assays. The resultant network describes the first few hours of *Xenopus* development starting from the control of maternal TFs in establishing the germ layers and ends with the role of early activated zygotic TFs during early gastrulation. Presented in Fig. 6 is a simplified GRN showing how combinatorial interactions of maternal TFs regulate spatially distinct expression patterns of zygotic targets, accounting for recently published evidences of regulatory interactions.

The *Xenopus* GRN provides the opportunity to dissect and understand the underlying network of interactions between TFs and their target genes by examining network motifs, or smaller network structures that appear frequently within the GRN. Network motifs can be classified based on the number of involved nodes: single node, two nodes and three node motifs. The singe node motifs (a single gene regulated by its own protein product) represent a positive or a negative auto-regulation. Two node network motifs (two genes, X and Y, mutually regulate each other) can involve with a positive, negative or double negative feedback loops. Lastly, the feedforward loops of network motifs are made of three nodes (two genes regulate a third downstream gene) are made of three nodes. Although the various types of network motifs present in a GRN were previously identified, the frequency of each type of network motifs present in GRN architecture have not been fully explored. In addition, how their presence is relevant to the function of individual TFs is uncertain.

We analyzed the entire literature of published *Xenopus* mesendoderm GRN structures (Charney et al., 2017b) and reported on the frequency of network motifs present among 23 TFs. We found 4 single-node motifs (2 positive and 2 negative regulation), 5 two-node motifs (3 positive feedbacks, 1 negative feedbacks, 1 double negative feedbacks), and 88 three-node motifs. Of these, 63 are feed forward loop (FFL) type I, representing ~70% of all three-node motifs. More specifically, in the *Xenopus* endodermal GRN, what appears to be common is the formation of feedforward loops whereby the product of gene A activates gene B, and both factors A and B activate the expression of gene C. This network motif appears to form whereby A is a maternal TF, B is an early expressed zygotic TF and C is a later expressed zygotic gene. In the majority of cases, the initial activator appears to be either Ctnnb1 (β-catenin), Foxh1, Smad2/3, or Vegt (gene A). 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 B), which, in turn, activate the expression a larger number of later expressed mesendodermal genes (gene C). A notable feature of coherent feedforward loops is that they may be useful in refining temporally the regulation of a cascade of gene expression (Mangan and Alon, 2003). This network structure could have implications in the differences in timing of gene induction from the embryonic genome, as seen in the broad window by which the timing of ZGA occurs. Interestingly, the relative abundance of this motif has been noted in the GRNs *E. coli* and *S. cerevisiae* (Mangan and Alon, 2003), as well as the developmental GRN of sea urchin (Peter and Davidson, 2017). Further meta-analysis of other established developmental GRNs in vertebrates suggest that the abundance of this feedforward loop is a staple of GRNs, as seen in the network structure of the *C. elegans* endoderm (Maduro, 2017), the mammalian T-cell (Kueh and Rothenberg, 2012), the mammalian pancreatic (Servitja and Ferrer, 2004) and the vertebrate neural crest (Simões-Costa and Bronner, 2015) GRNs. How this network motif and other motifs are functionally relevant is yet to be tested in the *Xenopus* system.

5. Summary and Prospects

The current *Xenopus* GRN is based on hand curated data accumulated over decades of work (Charney et al., 2017b). In the future, with the accumulation of more genomic data such as RNA-seq, ChIP-seq and ATAC-seq, we expect this GRNs to be built through integration of these datasets. A major challenge of such an approach is to determine which predicted interactions between TF and CRMs are functional as hundreds and thousands of such sites are predicted by bioinformatic approaches. Thus, what is needed in GRN science in the future is high throughput approaches to validate the predicted TF-CRM interactions *in vivo*. Advances in the use of CRISPR/Cas9 mediated deletions (Blitz et al., 2013; Nakayama et al., 2013; Wang et al., 2015; Blitz et al., 2016) and knock-ins (Shi et al., 2015; Aslan et al., 2017), along with high throughput reporter genes assays such as STARR-seq (self-transcribing active regulatory region sequencing) (Arnold et al., 2013) are likely to provide the opportunity to fill in this gap. We therefore expect the *Xenopus* and other animal model systems to facilitate significant advances in gene regulatory biology.

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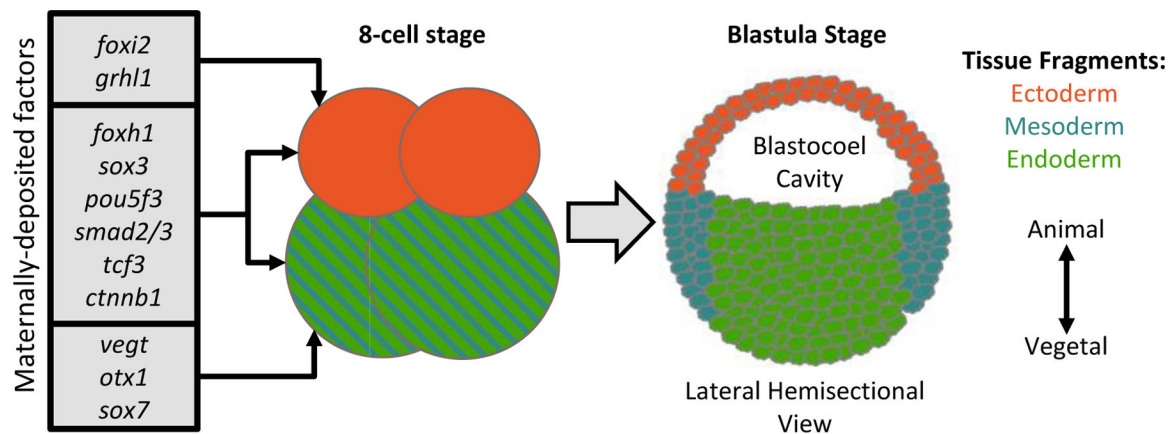
Fig. 1.

Maternal transcription factors are differentially inherited by blastomeres during cleavage stages. Gene products in the animal blastomeres are inherited by ectodermal cells, while those in the vegetal blastomeres are inherited by mesendodermal cells. Gene products such as foxh1 are ubiquitously-expressed in the oocyte and are inherited by blastomeres across the animal-vegetal axis.
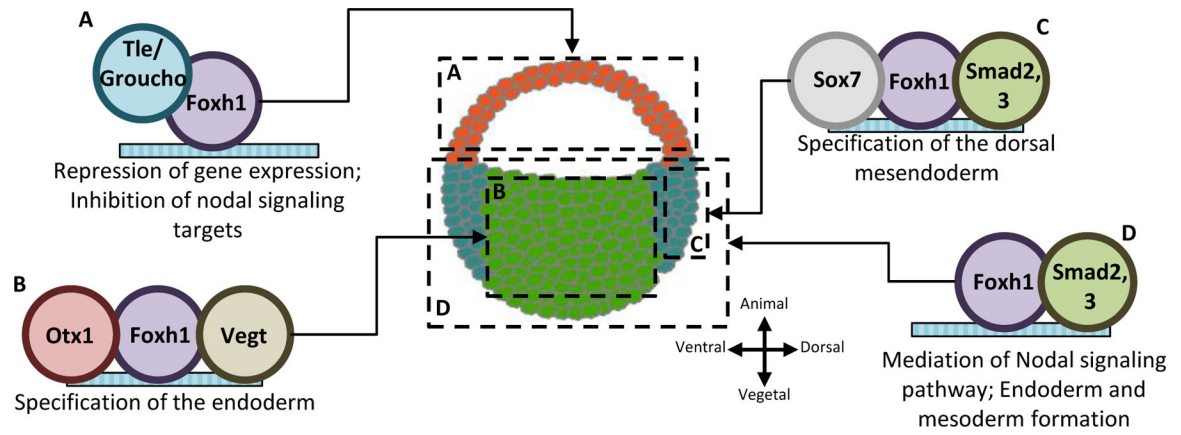
Fig. 2.

Modes of Foxh1 co-binding in the chromatin. In combination with different partner proteins, Foxh1 co-binds to putative CRMs and activate target genes in a spatially regulated manner.
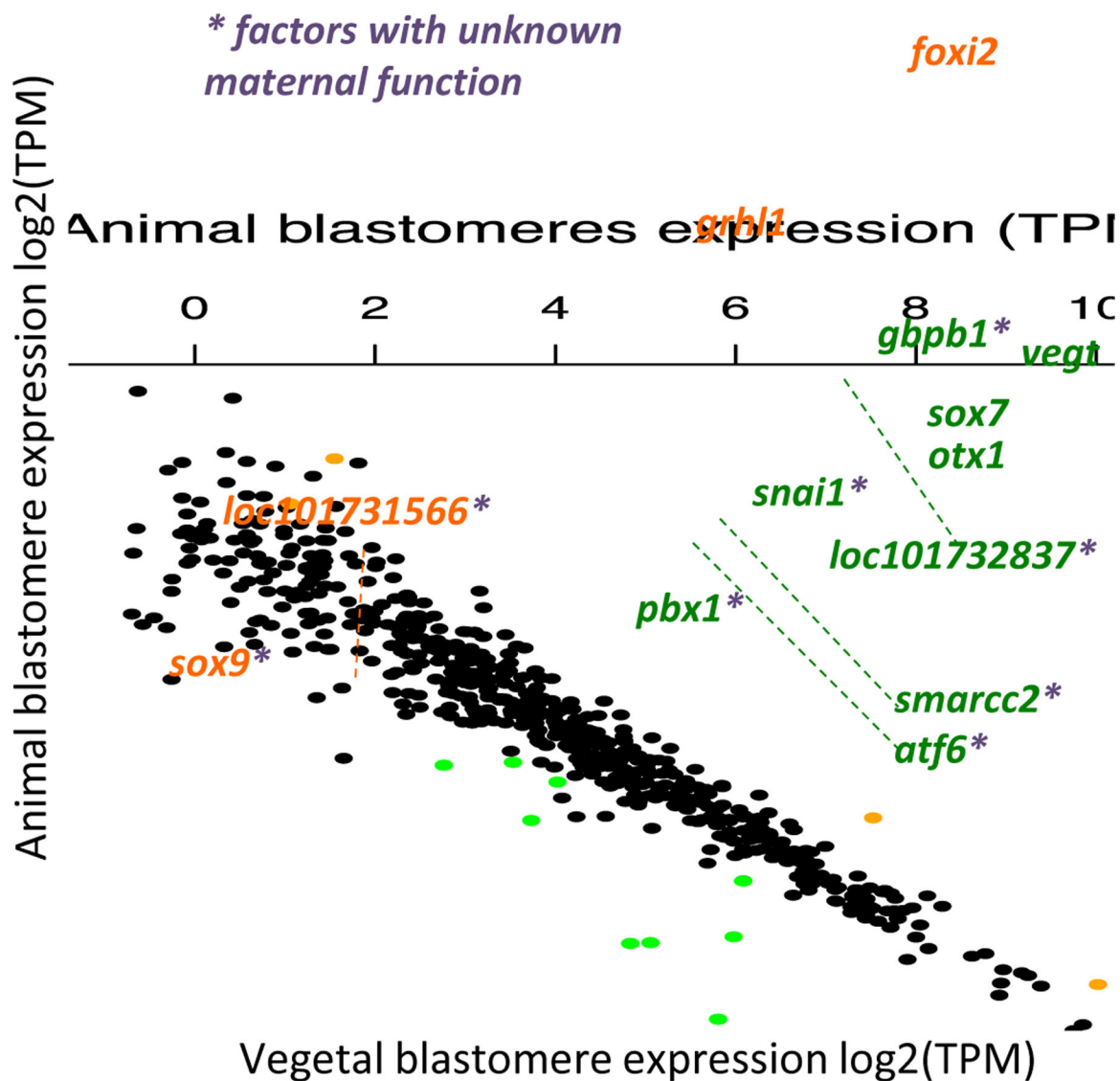
Fig. 3.
Expression of transcription factors at the 8-cell stage in the animal and vegetal blastomeres from RNA sequencing. Hundreds of transcription factors are expressed, while only a little over a dozen show localized expression. Figure adapted from Paraiso et al., 2019.
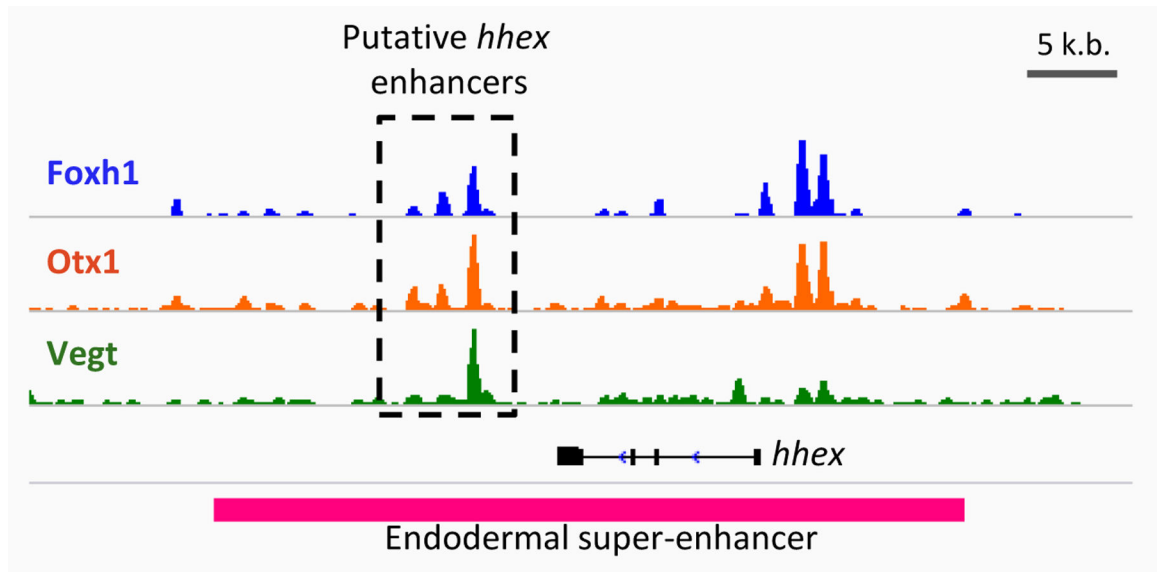
Fig. 4.
Genome browser showing the *hhex* loci with Foxh1, Otx1 and Vegt ChIP-seq signal, and the endodermal super-enhancer associated with this loci. Boxed are putative enhancers bound by maternal TFs that are previously untested for enhancer activity.
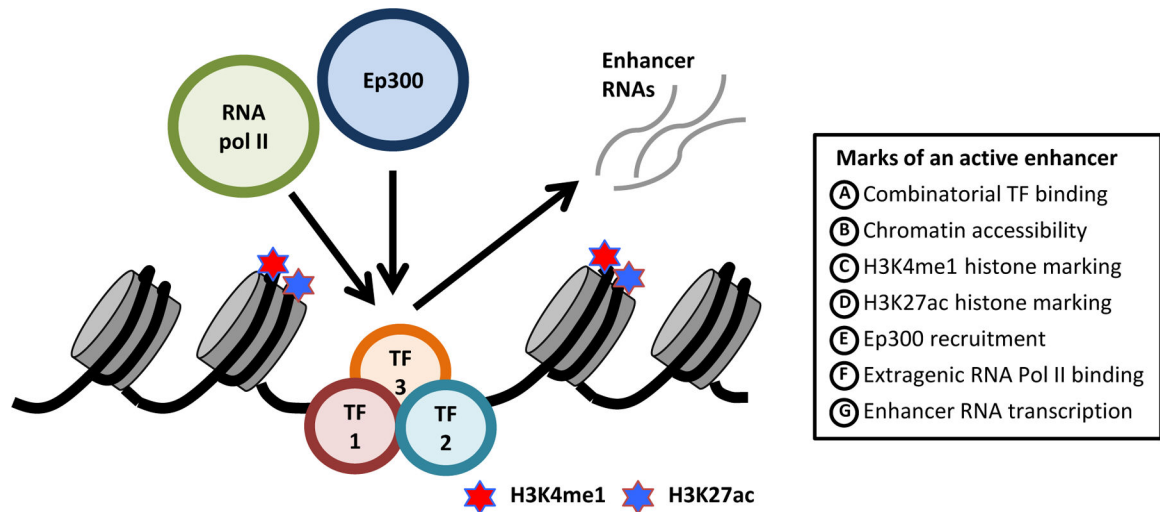
Fig. 5.
Marks of an active enhancer. Genome-wide approaches have used chromatin marks such as H3K4me1, H3K27ac, and Ep300 binding; extragenic RNA Polymerase II binding; transcription factor binding; chromatin accessibility and enhancer RNA transcription to identify putative active enhancers.
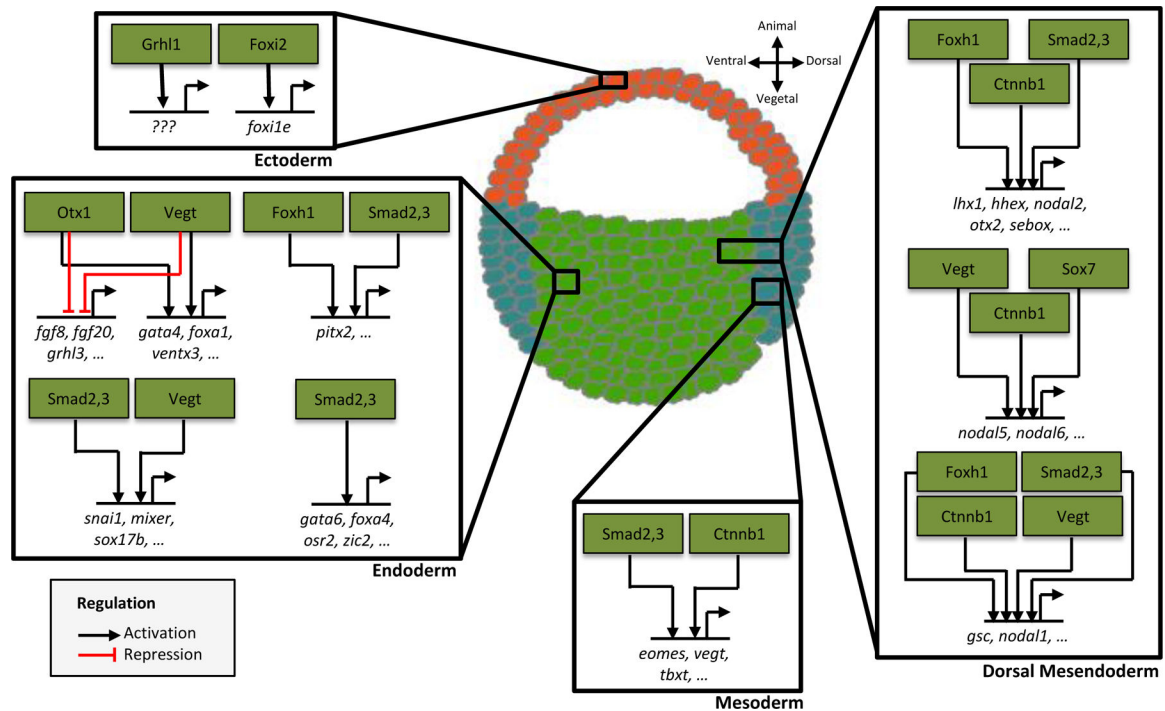
Fig. 6.

A maternal TF-centric GRN. Shown are a subset of known maternal TFs and their target zygotic genes highlighting the area of activity in the early embryo. Neither shown are target genes with more complex expression patterns, nor regulatory connections with other types of combinations of maternal TF input. For a more comprehensive GRN, see Charney et al., 2017b.

Table 1.

Maternal TFs and their gene regulatory function during ZGA and germ layer formation.

Maternal TF	Maternal expression in the animal-vegetal axis	Gene regulatory function	References
Vegt	Vegetal Pole	• Direct activator of endodermal genes • Direct repressor of ectodermal and mesodermal genes • Regulates RNA Polymerase II binding and transcription in enhancers • Co-binds with Foxh1 and Otx1 to site-select endodermal enhancers and super-enhancers	Lustig et al., 1996; Zhang and King, 1996; Stennard et al., 1996; Horb and Thomsen, 1997; Zhang et al., 1998; Kofron et al., 1999; Taverner et al., 2005; Paraiso et al., 2019
Otx1	Vegetal Pole	• Direct activator of endodermal genes • Direct repressor of ectodermal and mesodermal genes • Synergizes/Antagonizes with Vegt to regulate genes • Regulates RNA Polymerase II binding and transcription in enhancers • Co-binds with Foxh1 and Vegt to site-select endodermal enhancers and super-enhancers	Pannese et al. 2000; Paraiso et al., 2019
Sox7	Vegetal Pole	• Activates endodermal genes • Co-binds with Foxh1 and Smad2,3 near dorsal mesendodermal genes	Zhang et al., 2005; Charney et al, 2017a
Foxi2	Animal Pole	• Activates ectodermal genes	Cha et al., 2012
Grhl1	Animal Pole	• Unknown (maternally) • Zygotic Grhl1 regulates epidermal ectoderm formation	Tao et al., 2005a
Foxh1	Ubiquitous	• TF for the Nodal signaling pathway • Directly activates mesendodermal genes • Acts as dual TF to regulate gene expression • Regulates RNA Polymerase II binding and transcription in enhancers • Co-regulates with a variety of TFs in diverse spatial coordinates in the early embryo including Vegt, Otx1, Sox7 and Smad2,3 to site-select enhancers and super-enhancers	Chen et al., 1996; Chen et al., 1997; Kofron et al., 2004; Chiu et al., 2014; Reid et al., 2016; Charney et al., 2017a; Paraiso et al., 2019
Sox3	Ubiquitous	• Suppresses Nodal signaling in the ectoderm • Co-regulates gene expression with PouV TFs • Regulates zygotic TF binding • Mediates chromosome conformation and accessibility changes during ZGA	Zhang et al., 2003; Zhang and Klymkowsky, 2007; Gentsch et al., 2019
Pou5f3	Ubiquitous	• Suppresses Nodal signaling • Suppresses the function of tissue-specific factors such as Vegt and Ctnnb1 • Co-regulates gene expression with Sox3 • Regulates zygotic TF binding • Mediates chromosome conformation and accessibility changes during ZGA	Cao et al., 2005; Cao et al., 2007; Chiu et al., 2014; Gentsch et al., 2019
Smad2,3	Ubiquitous	• Mediates Nodal signaling pathway with Foxh1 • Activates endoderm gene expression • Induces mesoderm formation	Birsoy et al., 2006; Chiu et al., 2014
Tcf/Ctnnb1	Ubiquitous	• Specifies the dorsal identities in the embryo • Co-regulates with Nodal signaling to specify the dorsal mesendodermal fates	Ku and Melton, 1993; Tao et al., 2005b; Brannon et al., 1995; Laurent et al., 1997; Nakamura et al., 2016