Transcriptional Regulation and Genes Involved in First Lineage Specification During Preimplantation Development

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Abstract The successful development from a single-cell zygote into a complex multicellular organism requires precise coordination of multiple cell-fate decisions. The very first of these is lineage specification into the inner cell mass (ICM) and trophectoderm (TE) during mammalian preimplantation development. In mouse embryos, transcription factors (TFs) such as Oct4, Sox2, and Nanog are enriched in cells of ICM, which gives rise to the fetus and yolk sac. Conversely, TFs such as Cdx2 and Eomes become highly upregulated in TE, which contribute to the placenta. Here, we review the current understanding of key transcriptional control mechanisms and genes responsible for these distinct differences during the first cell lineage specification. In particular, we highlight recent insights gained through advances in genome manipulation, live imaging, single-cell transcriptomics, and loss-of-function studies.

Mammalian Preimplantation Development

Preimplantation development refers to the period from fertilization to implantation, during which the fertilized oocyte progresses through a number of cleavage divisions and three major transcriptional and morphogenetic events that lead to the first cell-fate decision and development into a blastocyst capable of implantation (Fig. 1).

Maternal-to-Zygotic Transition

The first well-characterized event is the maternal-to-zygotic transition (MZT), which includes degradation of maternal mRNAs and replacement with zygotic

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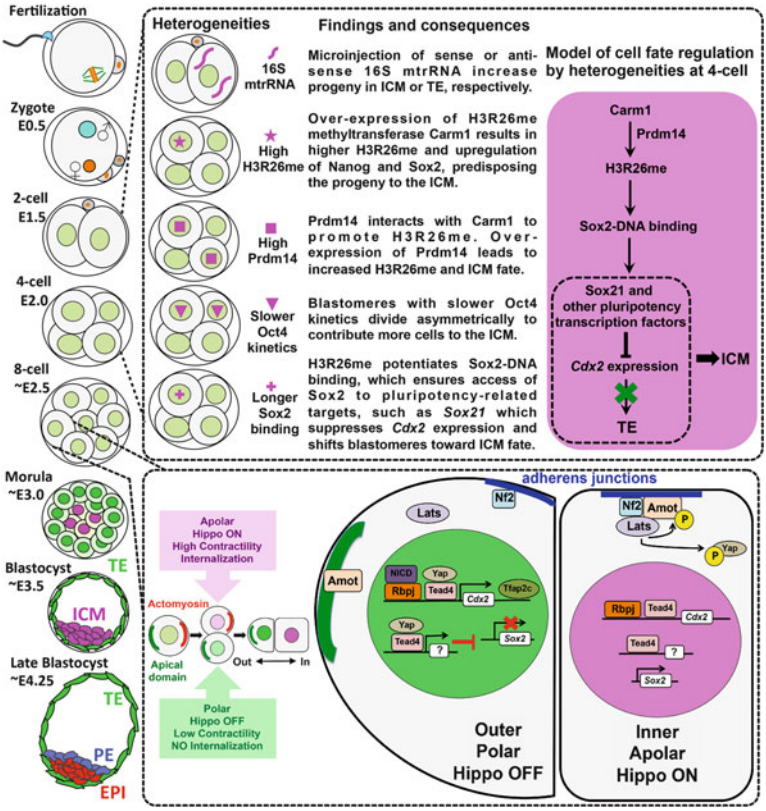

Fig. 1 Schematic representation of morphological changes and cell lineage specification during mouse preimplantation development. Cell fates and cell types are color coded. Heterogeneities detected in early blastomeres, biased lineage segregation, and proposed transcriptional control mechanisms underlying the intrinsic heterogeneities are highlighted in the *upper box*. At late eight-cell stage, blastomere compaction and polarization occur. During 8-cell to 16-cell, two kinds of daughter cells are generated: polar cells with apical domain and low actomyosin contractility and apolar cell without apical domain but high actomyosin contractility. Initiation of Hippo signaling is regulated by polarity: Hippo is off in the polar cells and active in apolar cells. Internalization of outer apolar cells is mainly driven by higher actomyosin contractility. In the outer cells, Amot, Nf2, and Lats do not form an active complex, such that Yap will not be phosphorylated. Unphosphorylated Yap translocates into the nucleus to bind Tead4 and activate target genes such as *Cdx2*. Expression of *Cdx2* is also promoted by the transcription factor Tfap2c through an intronic enhancer, as well as by Notch signaling through Notch intracellular domain (NICD) and Rbpj. Yap and Tead4 also activate an unknown component, which represses *Sox2* expression. In inner apolar cells, Amot, Nf2, and Lats form an active complex; Yap will be phosphorylated and kept out of the nucleus preventing expression of *Cdx2* and allowing expression of *Sox2*. Meanwhile, Notch signaling is not active in inner cells, resulting in no activation of *Cdx2*. Cell polarity, internalization, and key transcriptional regulation signals are highlighted in the *lower box*. *E* embryonic day, *TE* trophectoderm, *ICM* inner cell mass, *PE* primitive endoderm, *EPI* epiblast

transcripts. This dramatic reprogramming of gene expression is necessary for the establishment of totipotency and embryo development (Latham et al. 1991; Li et al. 2013). In the mouse, this process is first detectable at the one-cell stage but occurs mostly during the two-cell stage (Schultz 2002; Zhou and Dean 2015). Recent studies taking advantage of high-resolution live imaging and single-cell transcriptomic assays have discovered heterogeneities in transcription factor (TF) binding and gene expression between blastomeres as early as the two-cell stage, which may bias the first cell-fate determination (Fig. 1, upper box, reviewed below).

Embryo Compaction and Polarization

The second major event is embryo compaction and polarization, which initiate during the eight-cell stage in mouse embryos. Blastomere morphology becomes flattened, and biochemical changes to cellular metabolism, ion transport, and cell–cell contacts result in early embryonic cells first resembling somatic cells (Fleming et al. 2001). In addition to E-cadherin and β-catenin (De Vries et al. 2004), E-cadherin-dependent filopodia (Fierro-Gonzalez et al. 2013) and actomyosin cortex-derived force (Maitre et al. 2015) have been recently found essential for compaction. Proper compaction is required for the accompanying cell polarization and following cell division-dependent repositioning (Cockburn and Rossant 2010; White et al. 2016b).

During compaction, blastomeres also initiate polarization, to establish apical domains (Fig. 1, lower box), where apical and basal associated proteins first become localized [examples are, Pard3, Pard6, F-actin, and atypical protein kinase C (aPKC) (Yamanaka et al. 2006; Rossant and Tam 2009)]. In addition, other factors like microtubule (Houliston et al. 1989) and Rho-GTPase (Clayton et al. 1999; Kono et al. 2014) are also actively involved in this process. Daughter cell inheritance of polarity in subsequent divisions has been recently confirmed as critical for solidification of first cell-fate acquisition (Leung et al. 2016).

Blastomere Allocation and ICM/TE Separation

Following compaction and polarization, the third critical event is blastomere allocation into ICM and TE cell fates. Two distinct types of divisions occur during 8- to 16-cell transition: symmetric divisions that give rise to two polar cells both inheriting an apical domain versus asymmetric division that gives rise to one polar cell with an apical domain and one apolar cell (Chazaud and Yamanaka 2016). Additionally, Korotkevich et al. have recently used both in vivo and in vitro manipulated blastomeres to suggest that acquisition of an apical domain is both required and sufficient for initiation of first lineage specification (Korotkevich et al.

2017). Importantly, studies have found that some daughter cells on the outside of the embryo after 8- to 16-cell division can internalize and adopt an inner position and ICM fate (Morris et al. 2010; Yamanaka et al. 2010; Watanabe et al. 2014), suggesting that division orientation of blastomeres alone cannot predict or regulate the fate of daughter cells. Notably, recent studies revealed that this important internalization process is driven by higher actomyosin contractility within these cells induced by asymmetric segregation of the apical domain (Fig. 1, lower box) (Anani et al. 2014; Samarage et al. 2015; Maitre et al. 2016). These findings have shed new light on the link between cell polarity, cell position, and the Hippo signaling—a key pathway involved in the establishment of the first cell lineage separation (discussed below).

Apolar cell internalization and outer/inner configuration is the first sign of two-cell lineage specification: inner cell mass (ICM) and trophectoderm (TE). The apolar blastomeres located inside of the morula give rise to the ICM from which the embryo proper and yolk sac tissue are derived, whereas the outer polar blastomeres differentiate exclusively into the TE from which placenta is derived (Arnold and Robertson 2009; Zernicka-Goetz et al. 2009). Well-defined gene expression patterns occur within these two distinct lineages. For example, in the mouse embryo, transcription factors Oct4 (also known as Pou5f1), Nanog, and Sox2 are enriched in ICM and function to promote pluripotency and inhibit differentiation, while Cdx2 and Eomes become highly expressed in TE to potentiate epithelial differentiation (Marikawa and Alarcon 2009; Burton and Torres-Padilla 2014; Marcho et al. 2015). Appropriate regulation and mutually exclusive localization of these TFs is critical for successful ICM/TE lineage separation and formation of a competent blastocyst (Rossant and Tam 2009; Paul and Knott 2014).

Key Transcriptional Regulation During ICM/TE Separation

Whereas distinct localizations of transcription factors within ICM/TE lineages have been well illustrated, their functions, interactions, and upstream regulatory networks are still not fully delineated. Previous studies and recent advances have shown that Hippo signaling, Notch signaling, transcription factor AP-2γ (Tfap2c)-dependent regulation, heterogeneities in early blastomeres, epigenetic regulation, as well as many newly discovered genes are involved in the expression of these master TFs and cell lineage specification.

Hippo Signaling and TE Specific Genes

The Hippo signaling pathway is conserved in both *Drosophila* and mammals, regulating cell proliferation, differentiation, and death. Hippo signaling activity can be influenced by multiple stimuli, including cell position and cell–cell adhesion

(Yu and Guan 2013). Interestingly, recent studies indicated that in mouse preimplantation embryos, initiation of Hippo signaling is linked to blastomere polarity rather than cell position (Anani et al. 2014). Core components of Hippo pathway include the protein kinase Lats, transcriptional coactivator Yap, transcription factor Tead, and other key members such as Nf2 and angiomotin (Amot). When this pathway is activated, Yap is phosphorylated by Lats and is excluded from nucleus. Without binding to Yap, Tead cannot be activated, therefore preventing transcription of its target genes. Conversely, inactivation of the pathway induces dephosphorylation of Yap, which can then enter the nucleus and activate Tead to promote transcription of its target genes (Manzanares and Rodriguez 2013; Sasaki 2015). In sum, Hippo activation suppresses Tead gene targets, while Hippo inhibition induces gene expression downstream of Tead.

The connection between the Hippo signaling cascade and cell lineage specification was first discovered through the analysis of mutant mouse embryos lacking transcription factor TEA domain family member 4 (Tead4) (Yagi et al. 2007; Nishioka et al. 2008). Yagi et al. found that *Tead4*-null embryos failed to make blastocoel cavity and significantly reduced Cdx2, and all blastomeres were shifted into ICM with *Oct4* and *Nanog* expression. These results suggested *Tead4* is upstream of *Cdx2* and required for TE formation. Further experiments showed that *Tead4*-null embryos exhibit a more severe phenotype than *Cdx2*-null embryos (Strumpf et al. 2005) and confirmed that *Tead4* is upstream of both *Cdx2* and Gata3 (Ralston et al. 2010), another TE-specific transcription factor.

Subsequent studies focusing on other core components of the Hippo pathway have revealed the important role in regulation of TE-specific genes. Nishioka and colleagues (Nishioka et al. 2009) found that in inner cells, Yap is phosphorylated (Hippo signaling on) and excluded from nucleus, leading to Tead4 inactivation, while in outer cells, Yap is unphosphorylated (Hippo signaling off) and localized in the nucleus to activate Tead4. Studies on angiomotin (Amot) (Hirate et al. 2013) and Nf2 (Cockburn et al. 2013) demonstrated that in inner cells, Amot localizes to adherens junctions, is phosphorylated by Lats, and combines Nf2 to form an active complex to phosphorylate Yap. In outer cells, Amot is sequestered away from adherens junctions to apical domains, resulting in dephosphorylated Yap that can translocate into the nucleus to bind Tead4 and activate target genes (Fig. 1, lower box).

Hippo Signaling Promotes ICM Fate Acquisition

Cell lineage separation is controlled by expression of specific TFs in each lineage. Sox2 is the first known factor selectively located in inner cells, prior to other TFs such as Oct4 and Nanog (Guo et al. 2010). During the exploration of how Sox2 is regulated and restricted to ICM progenitors, Wicklow and colleagues (Wicklow et al. 2014) discovered an essential role of Hippo pathway in restriction of Sox2 to the inner cells that promotes ICM fate acquisition. As Cdx2 restricts expression of *Oct4* and *Nanog* to ICM by inhibiting their expressions in outer TE cell (Strumpf

et al. 2005), Cdx2 may also restrict Sox2 to ICM. However, Sox2 is still restricted to ICM in *Cdx2*-null embryos, indicating that restriction of Sox2 to ICM is Cdx2 independent (Wicklow et al. 2014) and necessitating additional unknown regulatory mechanisms.

It has been shown that Tead4 is activated in outer TE cells, and to promote transcription of its target genes (Yagi et al. 2007; Nishioka et al. 2008, 2009), these results together support a hypothesis that in outer cells, activated Ted4 upregulates an unknown factor that represses *Sox2* expression and that conversely, in inner cells, inhibition of Sox2 is not established (Fig. 1, lower box). Indeed, overexpression of *Lats2* prevents Yap nuclear localization and results in ectopic *Sox2* expression in outer cells (Wicklow et al. 2014). Thus, Hippo signaling cascade regulates not only TE-specific TFs but also ICM-restricted expression of *Sox2*, to establish complementary expression patterns and ICM/TE segregation.

Notch Signaling and TE Specific Genes

Interestingly, *Tead4*-null embryos can express *Cdx2* and form blastocoels with normal lineage commitment when cultured at 5% oxygen condition, which reduces oxidative stress (Kaneko and DePamphilis 2013). Experiments also confirmed that Tead4 regulates energy homeostasis and prevents accumulation of excess reactive oxygen species (ROS) (Kaneko and DePamphilis 2013).

Considering previous studies that demonstrated the central role of Tead4 in TE development and lineage specification (Yagi et al. 2007; Nishioka et al. 2008, 2009; Ralston et al. 2010), as well as recent findings that Tead4 can directly regulate *Cdx2* (Home et al. 2012; Rayon et al. 2014), a possible explanation is that under low oxygen conditions, other Tead proteins or other parallel signaling pathways functionally compensate for loss of *Tead4*. Indeed, an earlier study had reported that overexpression of activator-modified Tead1 also increased *Cdx2* expression (Nishioka et al. 2009). Recently, involvement of Notch signaling in TE lineage specification was also uncovered (Rayon et al. 2014). During a search for *cis*-regulatory elements responsible for TE-restricted expression of *Cdx2*, a TE-specific enhancer was identified that contains functional binding sites for both Tead and Rbpj, the transcriptional effector of the Notch signaling pathway (Tun et al. 1994; Koch et al. 2013). Experiments confirmed that Notch signaling is active at eight-cell stage and then gradually restricted to outer cells of the morula. Forced expression of Notch can drive cells to the outer position and TE cell fate (Rayon et al. 2014). Taking advantage of double mutants for *Tead4* and the Notch effector *Rbpj*, Rayon et al. demonstrated that Hippo and Notch signals converge on *Cdx2* to cooperatively promote TE lineage specification. Notably, they also reported that inhibition of Notch signaling only reduced *Cdx2* expression but had no effect on other TE-specific TF genes such as *Gata3* and *Eomes* (Rayon et al. 2014), which may explain why Notch signaling itself is not strictly required for TE development and blastocyst formation (Souilhol et al. 2006).

Transcription Factor AP-2γ (Tfap2c) Promotes TE

AP-2 family members have been demonstrated to be involved in multiple cellular events such as cell proliferation, morphogenesis, and tumor progression. In mammals, AP-2 family includes four transcription factors, Tfap2a, -b, -c, and -d (Bosher et al. 1995; Hilger-Eversheim et al. 2000). Previous studies have confirmed the essential role of *Tfap2c* in proliferation and differentiation of trophoblast cells, and *Tfap2c* mutant embryos exhibited malformed development in extraembryonic tissue (Auman et al. 2002; Werling and Schorle 2002). In addition, forced expression of *Tfap2c* in embryonic stem cells induced expression of trophoblast stem cell markers and trophectoderm cell fate (Kuckenberg et al. 2010). However, role of *Tfap2c* in preimplantation embryos was not defined.

Recently, Choi et al uncovered an essential role of *Tfap2c* in the regulation of tight junction biogenesis and cavity formation during mouse blastocyst development (Choi et al. 2012). Follow-up studies identified significant functions of *Tfap2c* in TE lineage specification during first cell-fate determination (Cao et al. 2015) and showed that Tfap2c directly regulates *Cdx2* expression through an enhancer in intron 1 during early cleavage stages (Fig. 1, lower box). Tfap2c also potentiates apical polarity via regulation of *Pard6b* expression, which is a key regulator for the establishment of cell polarity (Alarcon 2010). Importantly, these results suggest that Tfap2c also acts upstream of Rho-associated protein kinase (ROCK); thus, Tfap2c can repress position-dependent Hippo signaling in outer blastomeres through Pard6b and ROCK signaling, to promote TE formation during preimplantation development.

Heterogeneities in Early Blastomere Bias Cell Fate

Although it has been generally accepted that initiation of first cell-fate determination occurs during 8-cell to 16-cell transition in mouse embryos, many studies suggest that blastomeres are predetermined or biased at earlier stages. The link between oocyte polarities and blastocyst patterning is controversial (Hiiragi and Solter 2004; Plusa et al. 2005; Hiiragi et al. 2006), but studies have demonstrated heterogeneities in two-cell and four-cell blastomeres that may predict cell fate.

Taking advantage of single-cell RNA sequencing, different groups have confirmed the transcriptional heterogeneities between two-cell blastomeres that contribute to cell lineage separation (Biase et al. 2014; Piras et al. 2014; Shi et al. 2015). A recent study also reported differential distribution of 16S mitochondrial ribosomal RNA (mtrRNA) at two-cell stage in mouse embryos (Zheng et al. 2016). In situ hybridization results showed that while early two-cell blastomeres contain similar amount of 16S mtrRNA, late two-cell stage blastomeres exhibit apparent difference in 16s quantity. Furthermore, from the four-cell stage and onward, 16S mtrRNA is enriched in basal–lateral regions and is mainly detected in the ICM at

the blastocyst stage. Interestingly, microinjection of sense 16S mtrRNA significantly increased ICM progeny, while injection of antisense16S mtrRNA increased TE cells. These findings suggest that 16S mtrRNA may have an important role in promoting ICM lineage through an unknown mechanism (Zheng et al. 2016).

Compared with two-cell blastomeres, more heterogeneous properties have been discovered among four-cell blastomeres. Torres-Padilla and colleagues revealed arginine 26 residue of histone H3 (H3R26me), and its methyltransferase Carm1 levels vary among four-cell blastomeres. Overexpression of *Carm1* leads to higher H3R26me and a significant upregulation of Nanog and Sox2, biasing the progeny to ICM lineage (Torres-Padilla et al. 2007). Explaining these observations it has been shown that Prdm14 expression is also heterogeneous at four-cell stage and that Prdm14 can interact with Carm1 to promote H3R26me inducing ICM fate (Burton et al. 2013).

Using the fluorescence decay after photoactivation (FDAP) method, Plachta and colleagues demonstrated that blastomeres with slower Oct4 kinetics divide asymmetrically to produce more cells to ICM, while those with faster Oct4 kinetics contribute mostly to the TE through symmetric divisions (Plachta et al. 2011). Recently, two studies have shed new light on the transcriptional control mechanisms underlying the intrinsic heterogeneities that predict cell fate (Goolam et al. 2016; White et al. 2016a). They demonstrate that H3R26me potentiates long-lived Sox2-DNA binding, which ensures more access of Sox2 to its pluripotency-related targets, such as *Sox21*. Sox21 then suppresses *Cdx2* expression and biases blastomeres toward ICM (Goolam et al. 2016; White et al. 2016a). More importantly, this model may explain the early heterogeneities (Fig. 1, upper box) and strongly supports the possibility of nonrandom cell-fate determination during early mammalian development.

Epigenetic Control on Transcription During Lineage Specification

Besides key signaling pathways and crucial TFs, epigenetic control of gene transcription also plays an important role in cell differentiation and lineage specification (Paul and Knott 2014; Marcho et al. 2015). As DNA methylation has been shown to be dispensable for growth and differentiation of extraembryonic lineages (Sakaue et al. 2010), studies have focused on histone modifications during lineage specification. A link between histone modifications and gene expression patterns has also been illustrated. For example, histone lysine acetylation is normally considered as an active mark that correlates with chromatin accessibility and active transcription, whereas lysine methylation can be either active or repressive depending on the particular lysine residue which is modified (Tsukada et al. 2006; Bernstein et al. 2007).

During TE and ICM commitment, they exhibit asymmetries in specific histone modifications, and the last several years have seen a plethora of findings in this regard. For example, H4- and H2AS1P are increased in TE (Sarmento et al. 2004), while H3K27me3 is enriched in ICM (Erhardt et al. 2003). High level of H3R26me promotes DNA accessibility and biases cells to ICM fate (Torres-Padilla et al. 2007; Goolam et al. 2016; White et al. 2016a). Studies also demonstrate that H3K9me3 at *Cdx2* promoter is important for maintaining pluripotency and that loss of ESET in early embryos results in ICM failure (Yeap et al. 2009). Additionally, Suv39h methyltransferase mediates repressive H3K9me3 at ICM-specific gene promoters specifically in the TE lineage (Alder et al. 2010; Rugg-Gunn et al. 2010). H3K4me3 and H3K27me3 are enriched at promoters of genes exclusively expressed in ICM or TE in both murine and bovine embryos (Dahl et al. 2010; Herrmann et al. 2013). Loss of repressive H3K27me3 participation at TE-specific genes is essential for TE lineage development and embryo implantation (Saha et al. 2013; Paul and Knott 2014). In addition to methylation of histone H3 residues, acetylation of histone H4, such as H4K8ac and H4K12ac, also functions in early lineage specification (VerMilyea et al. 2009; Zhang et al. 2013a). It is likely that many more histone posttranslational modifications play similar roles but have yet to be defined during preimplantation lineage commitment.

Identification of Genes Essential for Lineage Specification

With readily available transcriptome-wide data, understanding the role of each expressed gene is an essential next step for elucidating developmental networks at play. Although RNAi technologies allow for genome-wide screens in cultured cells, these approaches cannot replace strategies for discovery in the embryo. Our lab has adopted a pooling strategy to allow for efficient RNAi-mediated forward genetic knockdown screen to identify genes required during preimplantation lineage specification. We recently accomplished a large-scale RNAi screen in mouse early embryos where 712 genes were screened and 53 genes were found to be required for successful lineage development and/or specification (example in Fig. 2), including *Suds3* (Zhang et al. 2013a), *Ctr9* (Zhang et al. 2013b), *Nop2* (Cui et al. 2016b), and a battery of genes without known early functions (Cui et al. 2016a). Interestingly, our results highlight that during the morula to blastocyst transition, TE lineage is more critical and/or more vulnerable as the majority of phenotypes that fail to form a blastocyst have TE defects rather than ICM defects. However, knockdown phenotypes that form a blastocyst but fail to hatch or outgrow have predominant defects in the ICM lineage. This finding suggests that while both lineages are essential during early embryo development, there are specific windows when proper function/specification of each is essential (Cui et al. 2016a).

To interpret the relationship between the genes identified in our screen and known pathways, we performed induced network module analysis (Kamburov et al. 2013) which showed ten of our genes (seeds in Fig. 3) form a network with

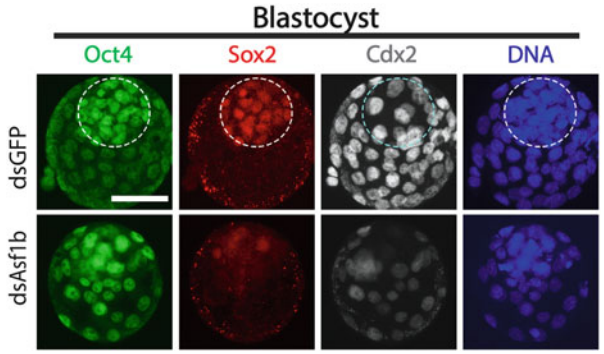

Fig. 2 Specific lineage markers of ICM (Oct4, Sox2) and TE (Cdx2) were characterized in both dsGFP control and dsAsf1b KD blastocysts by immunofluorescence. ICM cells (*circled*) in dsGFP control blastocysts are tightly arranged with robust expression of *Oct4* and *Sox2*, and TE cells are uniformly arranged with specific expression of *Cdx2*. Most dsAsf1b KD blastocysts exhibit ubiquitous Oct4 signal and with severely damaged *Sox2* and *Cdx2* expression, indicating impaired lineage specification. Oct4 (*green*), Sox2 (*red*), Cdx2 (*white*), and DAPI (*blue*). Scale bar, 50 μm

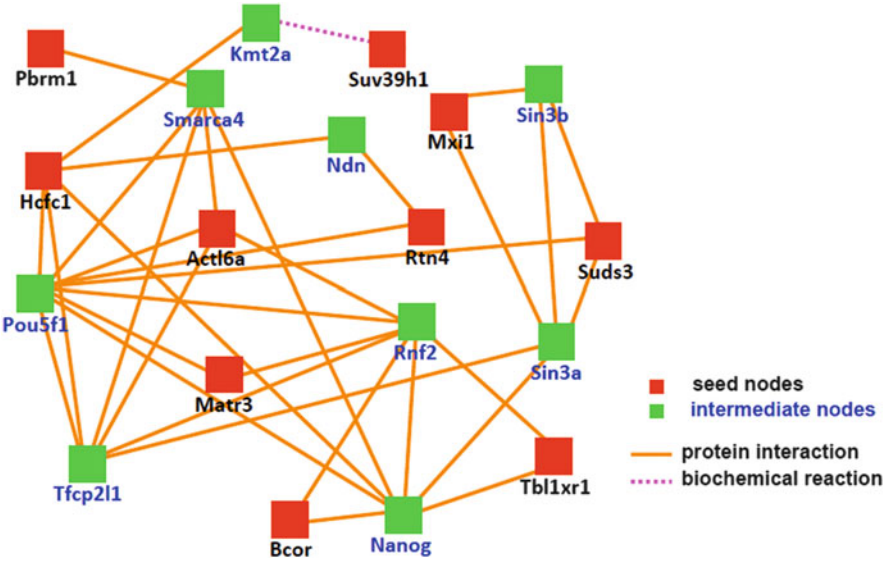

Fig. 3 In our recent screen, 712 genes were knocked down and 53 phenotypes were identified. Induced network module analysis showed 10 of our novel phenotypes (seed nodes in *red*) are connected with nine other genes (intermediate nodes in *green*), indicating that this network is essential for lineage specification and embryo development

nine other known genes (intermediates in Fig. 3). Importantly, these nine known genes already have knockout models that all confirm essential roles during early embryonic development (Cui et al. 2016a). These data suggest not only that this developmental network is essential for embryo development—but that this

screening strategy does not need to reach genome saturation to identify other/all pathways required.

Our screen selected genes to target based solely on expression during preimplantation and resulted in 7.4% of genes (53/712) with phenotypes. If there are ~11,000 genes expressed during preimplantation (Stanton and Green 2001), our results suggest that ~800 genes are required for lineage development and/or specification during preimplantation—the majority of which have yet to be discovered. We predict that screening one-fifth of all expressed genes (threefold more than we have finished) may be sufficient to identify the vast majority of networks/pathways required for early lineage events during preimplantation.

Conclusions

Here, we have reviewed recent advances in understanding transcriptional control mechanisms and crucial genes involved in first cell lineage specification, in particular, recent insights into Hippo signaling, Notch signaling, TF AP-2γ (Tfap2c) function, early heterogeneities, and epigenetic regulation. The first cell lineage decision is determined by many distinct mechanisms: some that act in parallel and some that act in networks. Although many dozen genes and pathways have been identified and—omics technologies have advanced in recent years, a comprehensive understanding of the genes required for the first lineage specification remains elusive.

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