# Translation on Demand as a post-transcriptional regulation mechanism of ES cell differentiation

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# **Declaration of originality**

I hereby declare that this thesis is my own work and that no other sources and tools than stated were used.

# Acknowledgement

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## **Abstract**

#### Introduction

#### Mouse embryonic stem cells

Mouse embryonic stem cells (mESCs) are defined by their self-renewal capabilities and pluripotency, allowing them to differentiate into any cell type of the three germ layers. While this differentiation process is limited in vivo, pluripotent stem cells (PSCs) cultivated in vitro can self-renew indefinitely under specific culture conditions (Nichols and Smith 2009). One such condition is the 2i+LIF (two inhibitors with leukemia inhibitory factor) medium, which maintains the naive pluripotent state of ESCs, closely mimicking the inner cell mass of pre-implantation mouse embryos.

The continuum of pluripotency is described by a progression from the naive state to a primed state, characterized by the priming for differentiation (Nichols and Smith 2009). Naive mESCs serve as a model for the inner cell mass of the pre-implantation embryo, exhibiting minimal lineage commitment. In contrast, epiblast stem cells (EpiSCs), which are isolated from later developmental stages, correspond to the post-implantation epiblast and are primed for differentiation (Boroviak et al. 2014).

During the transition from the naive to the primed states, sequential changes occur at multiple molecular levels, encompassing alterations in signaling pathways, epigenetic markers, and gene expression (Dejosez and Zwaka 2012). Specifically, early modifications in the phosphoproteome serve as indicators of these state transitions, initiating a cascade of changes that ultimately impact the transcriptome and proteome (Needham et al. 2019). This progression underscores the dynamic nature of pluripotency and highlights the intricate molecular mechanisms governing cell fate decisions in early development.

It is worth noting that early mESCs development differs from human embryonic stem cells (hESCs). While the molecular mechanisms controlling developmental processes are evolutionarily conserved, the speed at which these processes occur can vary significantly between species. For instance, the differentiation of motor neurons from embryonic stem cells is more than twice as fast in mice compared to humans (Rayon et al. 2020).

Regardless of the species, however, early developing embryonic stem cells need to undergo rapid changes in their gene expression profiles to ensure proper development and differentiation. Mechanisms such as ToD, can allow for swift adjustments in protein levels to aid these rapid changes. This ensures that cells can quickly respond to developmental cues and environmental changes, maintaining the necessary pace of development. Such mechanisms are crucial for adapting to the specific temporal requirements, ensuring that embryonic stem cells can effectively progress through the stages of development.

#### 2i Condition for maintaining naive pluripotency

Since serum contains undefined factors that can induce differentiation, the 2i medium was developed to provide a defined, chemically controlled environment that preserves mESCs in the naive pluripotent state (Ying et al. 2008). The 2i medium relies on the synergy between two inhibitors, PD0325901 and CHIR99021, to sustain mESCs in an undifferentiated state. PD0325901, a MEK inhibitor, blocks the MEK/ERK signaling pathway, suppressing differentiation signals from FGF and ERK that would otherwise prompt mESCs to exit pluripotency (see Figure 1). Meanwhile, CHIR99021, a GSK3 $\alpha$ / $\beta$  inhibitor, stabilizes  $\beta$ -catenin and activates Wnt/ $\beta$ -catenin signaling, promoting the expression of self-renewal genes and reinforcing pluripotency (Ying et al. 2008). Together, these inhibitors create a controlled environment that preserves mESCs in a ground state resembling the undifferentiated pre-implantation epiblast (Ying et al. 2008). Alongside leukemia inhibitory factor (LIF), which activates the JAK-STAT3 pathway to further inhibit differentiation, the 2i medium maintains mESCs in a naive pluripotent state, enabling them to retain full differentiation potential without lineage bias (Ying et al. 2008).

The removal of the 2i medium triggers the transition from the naive pluripotent state by reactivating the MEK/ERK and GSK3 pathways, allowing ERK signaling to promote differentiation and reducing Wnt signaling to enable lineage specification. This shift initiates a cascade of biological changes, including the downregulation of naive markers (such as Nanog and Esrrb) and upregulation of formative markers (such as Otx2), along with epigenetic remodeling, altered chromatin accessibility, and a metabolic shift from glycolysis to oxidative phosphorylation (see Figure 1) (Wray et al. 2011).

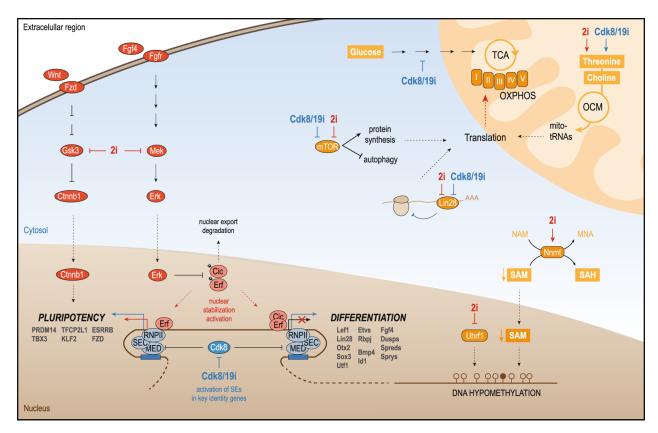


Figure 1: 2i mechanism of stabilizing naive pluripotency

The inhibition of Mek/Erk through 2i-dependent mechanisms prevents the phosphorylation of Erf and Cic, resulting in the stabilization of these factors within the nucleus. These transcriptional effectors may play a role in activating genes associated with naïve pluripotency while repressing those involved in primed/formative states. Cdk8/19i influences the phosphorylation of proteins that are part of the elongation and transcription machinery, including the super elongation complex (SEC), RNA polymerase II (RNPII), and Mediator (MED). This could lead to the rapid activation of genes regulated by super-enhancers, which include factors that stabilize the naïve state as well as certain factors from the primed/formative states that are also upregulated in response to Cdk8/19i. The activation of the naïve circuitry through 2i and Cdk8/19i may support the enhanced mitochondrial oxidative phosphorylation (OXPHOS) capacity of these cells. The degradation of Lin28a might alleviate its repressive effects on target mRNAs, leading to the upregulation of mitochondrial proteins among others. The balance between autophagy and protein synthesis may sustain the translation of these mitochondrial components. Additionally, the re-wiring of mitochondrial sources for one-carbon units may further support the translation of mitochondrial-encoded proteins. Furthermore, the increased availability of S-adenosylmethionine (SAM) levels, due to NNMT upregulation, combined with the impairment of DNA methylation caused by Uhrf1 degradation, contributes to the characteristic hypomethylated DNA state typical of 2i conditions (Martinez-Val et al. 2021).

#### ERK and mTOR signalling pathway and its effects on translation

ERK signaling influences translation primarily by modulating the activity of eukaryotic translation initiation factor 4E (eIF4E) (Lavoie, Gagnon, and Therrien 2020). ERK activates MAPK-Interacting Kinases (MNK)1 and MNK2 kinases, which phosphorylate eIF4E, enhancing its binding to the mRNA cap structure and promoting the formation of the eIF4F complex (see Figure 2). This complex, consisting of eIF4E, eIF4G, and eIF4A, is essential for recruiting the ribosome to mRNA, scanning for the start codon, and initiating protein synthesis. ERK signaling can selectively enhance the translation of specific mRNAs, particularly those with complex 5' UTRs, leading to the production of proteins involved in cell growth and survival. Additionally, ERK interacts with other pathways, such as mTOR, to coordinate translation regulation in response to various signals, thus optimizing cellular responses to environmental changes.

The mTOR pathway complements the role of ERK in translation regulation. It comprises two distinct complexes: mTORC1 and mTORC2. mTORC1 is primarily responsible for regulating protein synthesis, autophagy, and metabolism, while mTORC2 is involved in cell survival and cytoskeletal organization. Activation of mTORC1 occurs through nutrient availability, with amino acids serving as key activators, and through growth factors that activate the PI3K-AKT pathway (see Figure 2) (Ma and Blenis 2009). mTORC1 influences translation by phosphorylating 4E-BP1, which releases eIF4E to initiate cap-dependent translation (see Figure 2) (Ma and Blenis 2009). Furthermore, mTORC1 activates S6K1, which phosphorylates ribosomal protein S6, enhancing the translation of ribosomal proteins and elongation factors. These actions collectively increase the global protein synthesis capacity of the cell (see Figure 2) (Ma and Blenis 2009).

The ERK signaling pathway

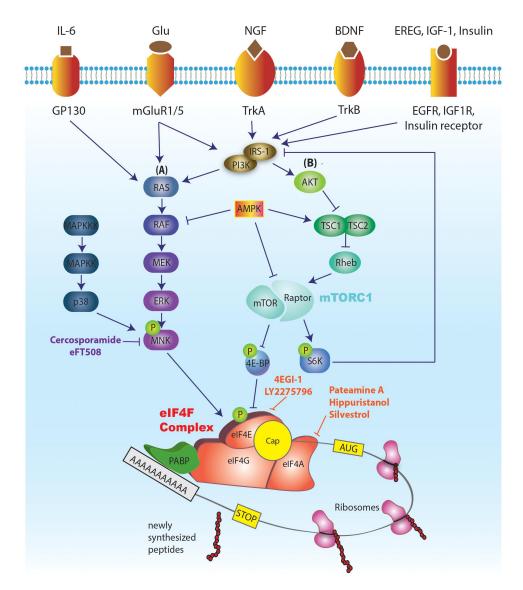


Figure 2: ERK and mTORs effects on elF4E regulation

The ability of eIF4E to bind to the mRNA cap makes it a key regulator of translation. A crucial step in translation initiation involves eIF4E binding to the mRNA cap, facilitating the formation of the eIF4F complex on the cap structure (comprising a 7mGp linked to the first nucleotide). The eIF4F complex includes eIF4E, eIF4G (a scaffolding protein), and eIF4A (a helicase). The successful assembly of the eIF4F complex on the mRNA cap promotes the recruitment of the pre-initiation complex (PIC), followed by scanning of the 5' UTR to locate the start codon AUG and the joining of the 60S ribosomal subunit, marking the completion of translation initiation. eIF4E acts as a downstream effector of both the mTORC1 pathway (via 4E-BP-dependent repression) and the ERK pathway (via eIF4E phosphorylation by MNK 1/2). The activities of mTORC1 and ERK signaling pathways are influenced by various external signals and internal cues (Uttam et al. 2018).

#### Additional mechanisms influencing translation

Naturally, there are many other described mechanisms that can influence translation. RNA-binding proteins (RBPs) can bind mRNA through modular RNA-binding domains, which primary sequence of amino acids dictates the specificity of the RBP (Lunde, Moore, and Varani 2007). By binding the mRNA, these proteins can influence the turnover rate of their specific mRNA, stability of the mRNA as well as form complexes with other proteins to recruit the ribonucleoproteins (RNPs) of the translation machinery (Glisovic et al. 2008). Another prominent example of post-transcriptional regulation is the interaction of mircoRNAs (miRNAs) with mRNAs. miRNAs have been shown to bind targeted sequences at the 3' untranslated region (UTR) for blocking translation, deadenylation of the mRNA or for decapping (Huntzinger and Izaurralde 2011). In mESCs specifically, families of miRNAs have been shown to maintain pluripotency and suppressing genes that drive differentiation (Melton, Judson, and Blelloch 2010). The fact that, ERK signalling globally downregulates miRNAs, further highlights the role of miRNAs to maintain pluripotency and ERKs to exit the naive state (Sun et al. 2016).

Lastly epigenetic regulation, although indirectly, can influence translation. In mESCs, the incorporation of specific histone variants, such as H3.3, has been shown to modulate the chromatin environment. Regulatory complexes can modify chromatin to control gene expression during development by influencing the accessibility and stability of specific genomic regions (Banaszynski et al. 2013).

#### **Translation on demand**

We define ToD as an increase in protein abundance due to enhanced translation, occurring without any changes in mRNA levels of the corresponding gene. In other words, ToD is a regulatory mechanism where translation efficiency is selectively increased for transcripts of specific genes. This upregulation allows for rapid adjustments in protein levels, supporting cellular responses that need immediate protein synthesis without requiring new mRNA transcription. Such translation driven increases could be particularly useful in situations where quick adaptations are necessary, as it enables cells to respond to changes in the

environment or developmental cues by rapidly elevating the protein output from existing mRNA pools.

In vivo, there are already described mechanisms that facilitate forms of selective, rapid translation of mRNAs. For example, neurons need local translation at active synapses for quick cellular responses (Holt and Schuman 2013). In early embryos, maternal mRNA is stored and activated after fertilization, enabling the embryo to rapidly synthesize proteins necessary for early development without new mRNA synthesis (De Leon, Johnson, and Bachvarova 1983). In stress response, rapid and selective synthesis of stress proteins, such as heat shock proteins (HSPs), is facilitated by ToD, allowing cells to quickly adapt and protect themselves under stress conditions without increasing mRNA levels (Holcik and Sonenberg 2005).

#### Aim of this study

In this project, we aim to identify ToD candidates from a multi-omic dataset, which includes a time series of bulk RNA sequencing and proteomic data (Yang et al. 2019). These multiple omic layers enable us to explore how protein abundance responds to changes in mRNA levels and to identify genes that may be regulated through ToD mechanisms. Next to finding genes that are subject to ToD regulation, we want to illustrate why these specific genes are prioritized and to uncover the underlying mechanisms of this regulation. This involves exploring the biological roles and functional significance of these genes in cellular processes. Additionally, we are interested in understanding the gene regulatory network and proteome changes that occur as mESCs exit pluripotency, as these regulatory networks are most likely to be regulated by ToD.

By analyzing the temporal dynamics within these data layers, we can distinguish cases where protein levels increase independently of mRNA changes, a hallmark of ToD regulation. This approach provides a comprehensive view of the post-transcriptional mechanisms that contribute to protein abundance, offering insights into gene-specific regulatory processes. Understanding the more nuanced relationship between RNA and protein expression, as well as the timing of regulation, will provide valuable insights into the funda-

mental principles of molecular biology and enhance our comprehension of cellular regulatory networks as well as early development.

Furthermore, this study should serve as a framework to detect ToD candidates in by leveraging the use of transciptome and proteom data together. We also aim to validate our findings and approaches with two other datasets to check the validity of our methods and see if we obtain similar results. This validation will help ensure that the mechanisms identified are robust and applicable across different biological contexts.

#### Methods and tools

#### **Experimental Approach and Workflow**

To research ToD, we exploit the fact that protein translation is largely inhibited under 2i conditions. By sampling mouse embryonic stem cells under 2i conditions and then periodically after 2i removal, we can take snapshots of the early changes in the transcriptome and proteome (see Figure 3). Upon 2i removal, we trigger the transition from the naive pluripotent state to the primed formative state. This allows us to detect changes in the translational behavior of genes aiding this transition.

We connect transcriptomic and proteomic dynamics by comparing their Log Fold Change (LFC) between time points. This way, we can compare qualitative trends for transcript and protein abundance rather than comparing them by count values. After identifying genes that are likely ToD-regulated, we assess their biological function and place them in the context of early development (see Figure 3).

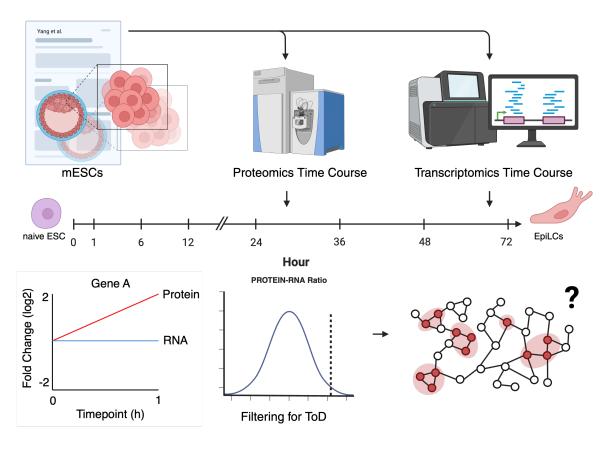


Figure 3: **Experimental Approach and Workflow** Exemplary workflow for the Yang dataset. Proteomic and transcriptomic time courses were used to examine LFC changes at different time intervals. Filtering criteria were applied to determine if a specific gene is likely to be ToD regulated. ToD candidates were tested for functional enrichment and other annotations methods to determine their role in early development.

#### Data overview and preprocessing

The data for this project is comprised of three datasets with our main focus being the Yang Data, due to its higher temporal resolution in early time points and more consistent time course in the proteomic data (see Table 1). This dataset was sourced from Supplementary Table S2 (temporal proteomic data) and Supplementary Table S3 (temporal transcriptomic data) in the publication Multi-omic Profiling Reveals Dynamics of the Phased Progression of Pluripotency (Yang et al. 2019).

The temporal proteomic data is in the form of Log2 Label-Free Quantification (LFQ) intensities and included the following time points: 0min, 30min, 1h, 6h, 12h, 24h, 36h, 48h,

and 72h. Each time point had four biological replicates, which were also pooled. The temporal transcriptomic data consisted of non-normalized transcript counts and included the following time points: 0min, 1h, 6h, 12h, 24h, 36h, 48h, and 72h. Each time point had two biological replicates, which were averaged. Since the transcriptomic dataset did not include a 30min time point, this time point was removed from the proteomic data for further analysis (see Table 1).

To increase our accuracy in calling meaningful ToD candidate genes, we realigned the transciptomic data with the GRCm38 reference genome using STAR (Mudge et al. 2025; Dobin et al. 2013).

The Leeb data has a temporal resolution from 0 to 32 hours with 4 hour intervals. Each time point has four biological replicates in the proteomic time course and no replicates in the transcriptomic time course (see Table 1). Gaussian Process Regression (GPR) was used to reduce noise in the transcriptomic time course data.

The Leeb MC data includes time course data for 0h, 4h and 8h for the purpose of calling ToD candidates. Additionally, it includes a time course with the same temporal resolution, where 2i conditions were kept while a medium change was still applied to the cells. Each time point has four biological replicates in the proteomic time course and three replicates in the transcriptomic time course (see Table 1).

Table 1: Datasets

	Temporal Resolution	Replicates	Replicates
Dataset	(h)	Transciptomics	Proteomics
Yang Data	0, 1, 6, 12, 24, 36,	2	4
	48, 72		
Leeb Data	0, 4, 8, 12, 24, 32	1	4
Leeb MC Data	0, 4, 8	3	4
(Medium Change)			

#### **Transcriptomic Data Normalization**

For the Yang dataset as well as the Leeb MC dataset we used DESeq2 to normalize the transciptomic data (Love, Huber, and Anders 2014). DESeq2's normalization procedure operates on the assumption that the majority of genes exhibit no differential expression. To normalize gene counts, DESeq2 uses the median of ratios method. This involves dividing the count of each gene in a sample by its geometric mean across all samples, resulting in a ratio (see Equation 1; Equation 2). The geometric mean serves as a measure of central tendency, representing the average gene count across samples, and provides a standard for comparing individual sample counts. The size factor for a given sample is then determined by calculating the median of these ratios (see Equation 3). This method is robust, accounting for sequencing depth and RNA composition, and effectively compensates for sample specific biases.

$$\operatorname{Geometric} \operatorname{Mean}_g = \left(\prod_{s=1}^n \operatorname{Count}_{gs}\right)^{1/n} \tag{1}$$

- $\operatorname{Geometric} \operatorname{Mean}_g:\operatorname{The geometric mean of counts for gene }g$
- $\mathsf{Count}_{gs}: \mathsf{The}\; \mathsf{count}\; \mathsf{of}\; \mathsf{gene}\; g \; \mathsf{in}\; \mathsf{sample}\; s$
- n: The total number of samples

$${\rm Ratio}_{gs} = \frac{{\rm Count}_{gs}}{{\rm Geometric\ Mean}_{g}} \tag{2}$$

$$\mbox{Size Factor}_s = \mbox{median}(\mbox{Ratio}_{qs} \mid \mbox{for all } g) \eqno(3)$$

#### **Calculation of Fold Changes and Ratios**

LFC values between consecutive time points for proteomic and transcriptomic time courses were calculated with Equation 4.

$$\mathsf{logFC}_{\mathsf{between \ time \ points}} = \mathsf{logFC}_2(\frac{\mathsf{Expression}_{\mathsf{time \ point}_{n+1}}}{\mathsf{Expression}_{\mathsf{time \ point}_n}}) \tag{4}$$

LFC values against the first time point (0h) for proteomic and transcriptomic time courses were calculated with Equation 5.

$$\mathsf{logFC}_{\mathsf{against\ time\ point\ 1}} = \mathsf{logFC}_2(\frac{\mathsf{Expression}_{\mathsf{time\ point}_n}}{\mathsf{Expression}_{\mathsf{time\ point}_1}}) \tag{5}$$

We calculated a Protein-to-RNA-LFC-Ratio (LFC-Ratio) to highlight genes with greater changes in protein than RNA, identifying possible ToD candidates (see Equation 6). A positive Protein-to-RNA FC Ratio indicates that protein expression has increased more than RNA expression, suggesting potential translational upregulation through ToD.

$$\mathsf{LFC\text{-}Ratio} = \mathsf{logFC}_{\mathsf{between\ time\ points\ protein}} - \mathsf{logFC}_{\mathsf{between\ time\ points\ RNA}} \tag{6}$$

#### Gene Overrepresentation Analysis with clusterProfiler

We performed Gene Overrepresentation Analysis (ORA) on potential ToD candidates at each time point using the R package clusterProfiler (Yu et al. 2012). This analysis aimed to identify overrepresented biological functions, molecular activities, or cellular localizations among ToD candidates compared to a background reference. Using a hypergeometric model, clusterProfiler evaluates the enrichment of gene set terms within each set of ToD candidates, generating p-values to quantify significance. To control for false discovery rates (FDR), we applied Benjamini-Hochberg correction to the resulting p-values (Benjamini and Hochberg 1995). This approach highlighted time-specific biological processes potentially regulated at the translational level among ToD candidates. The corresponding complete data set (genes both present in the proteomic and transcriptomic data) was used as a background (or universe) for the analysis.

#### **Transciption Factor Acticity inference with decoupleR**

We used the R package decoupleR to infere acticity of Transciption Factors (TFs) that might be subject to ToD (Badia-i-Mompel et al. 2022). decoupleR utilizes CollecTRI, a comprehensive resource containing a curated collection of TFs and their transcriptional targets compiled from 12 different resources (Müller-Dott et al. 2023). Interactions are weighted according to their mode of regulation, either as activators or inhibitors. To infer TF activity, decoupleR uses the Univariate Linear Model (ULM) method to calculate TF enrichment scores. For each sample in our datasets and each TF in the CollecTRI network, it fits a linear model that predicts the observed gene expression based solely on the TF's TF-Gene interaction weights. Once fitted, the obtained t-value of the slope is used as the score. A positive t-value indicates that the TF is active, while a negative t-value suggests that the TF is inactive.

#### **Motif enrichment**

#### **Software**

#### Results

#### **Detecting Translation on Demand Candidates**

A hallmark of ToD regulation is a rapid increase in translation, while the rate of transcription stays stable. As a filtering criteria of ToD regulation, we selected only genes who showed RNA LFCs which stayed inside a stable range for each time interval. The stringency of this range depends on the biological question we want so answer with a specific list of ToD candidates. For example, for gene set enrichment it might make sense to broaden the range we define as stable to detect functional trends in a certain time interval which would not be detectable otherwise. If not stated otherwise we used a stable LFC RNA range from minus one to one. The next filtering step to determine if a gene might be subject to ToD regulation at a specific time interval was to filter for a LFC-Ratio of at least one to

ensure a substantial increase in protein levels while maintaining a relatively stable RNA LFC, which filters for robust protein level changes independent of RNA fluctuations (see Figure 4 A). Again, this LFC-Ratio threshold depends on the biological context and needs to be adapted for the dataset in question. If not stated otherwise, we used a LFC-Ratio threshold of one. This filtering step results in an initial ToD candidate list generated with the Yang Data of 113 ToD candidates for the first time interval (0h to 1h), 103 for the second (1h to 6h) and 78 for the third (6h to 12h) etc. (see Figure 4 B). Corrected for time, the number of ToD candidates are 103, 17 and 13 for the first three time intervals respectivly (see Figure 4 B). This trend continues in later time intervals. The fact, that we detect more ToD candidates per unit of time in early time intervals might be due to the relevance of ToD regulation during these early develompental time intervals, because rapid protein systhesis of specific transcipts is needed at that time to initiate priming for differentiation.

To make the ToD candidate calling less subject to noise, we implemented a third filtering step. After a gene passes the LFC-Ratio threshold at a given time interval, we look at its protein LFC trajectory normalized for the first time point (0h) and see if the subsequent LFC value after the threshold passing is within a certain range defined by the time interval where it passed the LFC-Ratio threshold. For example, if a gene passed the LFC-Ratio threshold at the 0h to 1h time interval, we look in the protein LFC trajectory of that gene for 1h vs 0h and the next LFC value at 6h vs 0h. The LFC value at the 6h vs 0h time interval is only allowed to go down by 30% compared to the previous value at 1h vs 0h to eliminate genes that showed higher levels of noise in their protein LFC trajectory. After applying this filtering method, the number of ToD candidates for the first three time intervals were 61, 52, and 34 respectively (see Figure 4 C). The trend of having more ToD candidates when correcting for time in the early time intervals remained evident after applying the filtering step.

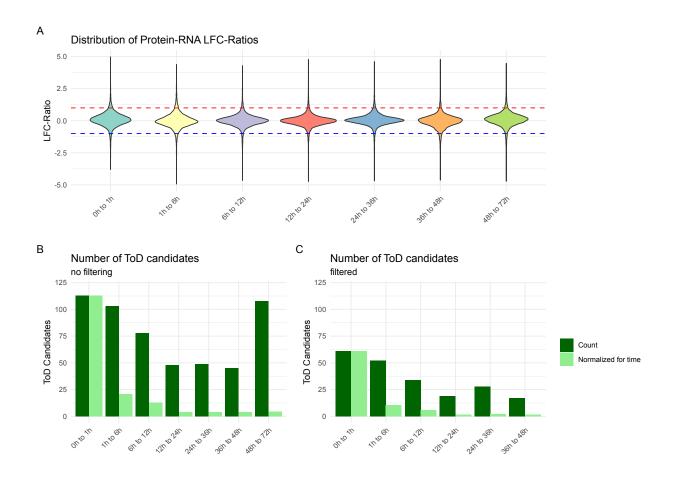


Figure 4: Filtering for ToD candidates in the Yang Data

- (A) Distribution of LFC-Ratios at different time intervals. The dashed red line (LFC-Ratio of 1) shows the LFC-Ratio threshold a gene needs to pass. The dashed blue line (LFC-Ratio of -1) shows the opposite behaviore where we see a decreased protein expression.
- (B) The barplot shows the number of ToD candidates at each time interval which passed the LFC-Ratio of one. The dark green bars show the absolute number of ToD candidates at a given time interval. The light green bars show the number of ToD candidates corrected for time.
- (C) The barplot shows the number of ToD candidates at each time interval which passed the LFC-Ratio and which passed the third filtering step. The dark green bars show the absolute number of ToD candidates at a given time interval. The light green bars show the number of ToD candidates corrected for time.

#### Early development is shaped by ToD candidates

Upon examining the filtered ToD candidates across various temporal intervals, it is evident that these candidates include numerous genes crucial for early developmental processes. Specifically, during the initial interval from 0 to 1 hour, a significant proportion of the

ToD candidates are associated with developmental functions. Notably, genes such as Agap1, Dnajc14, Emc7, Rab27a, Vti1a and Napb play roles in protein trafficking and vesicular transport. This observation suggests that protein trafficking is likely subjected to substantial demand during this initial period, coinciding with the onset of protein translation following the removal of the 2i condition. The increased load on protein trafficking mechanisms may reflect the cellular necessity to rapidly establish the proteomic landscape required for subsequent developmental stages.

Not suprisingly then, genes asscotiated with cytosceletal regulation like Cdc42bpa, Cep95, Plekhh1 and Stil are represented among ToD candidates in the first time interval.

Another group of genes, such as Celf2, Rex2, and Rsrp1, are involved in post-transcriptional regulation. Specifically, Celf2 encodes an RNA-binding protein that plays a critical role in regulating several post-transcriptional events. It is involved in pre-mRNA alternative splicing, mRNA translation, and stability. Celf2 mediates exon inclusion and/or exclusion in pre-mRNA, which is subject to tissue specific and developmentally regulated alternative splicing (Ladd, Charlet-B., and Cooper 2001). Lasty, another group of genes like Axin1, Csnk1g2, Jag1, Rap2c and Rgs19 contributes to regulatory networks that govern developmental processes.

ToD candidats contributing to the mentioned processes can also be found in subsequent time intervals, like the splicing factor Srsf11 or developmental regulators like Epha2 in time interval 1h to 6h among others (see Table 2).

These findings illustrate that the ToD candidates identified in the Yang dataset play a crucial role in facilitating the transition from the naive pluripotent state to the primed formative state. By contributing to essential processes such as protein trafficking, cytoskeletal regulation, post-transcriptional modification, and developmental signaling, these genes help orchestrate the complex molecular changes necessary for early development.

# Gene over-representation analysis reveals time dependent pathway regulation of developmental terms by ToD candidates

Gene ontology (GO) as well as Hallmark ORA provided further validation of these observations. In the 0h to 1h time interval, we identified a nuanced overrepresentation of Notch signaling as well as Wnt/β-catenin signaling (see Figure 5 A). The prominence of these pathways during the initial time interval suggests that they are among the first to respond to the removal of the 2i conditions. This response likely initiates a cascade of molecular events that prepare the cells for subsequent differentiation, highlighting their importance in the early phases of transitioning from a naive to a primed state. Both pathways are well known for their involvement in embryonic development (Zhou et al. 2022; Liu et al. 2022). Additionally, in the 1h to 6h time interval ToD candidates associated with TNFA signaling and TGF beta signaling where significantly over-represented (see Figure 5 B). During embryogenesis and organogenesis, TNFA signaling plays a crucial role in mediating inflammatory responses and regulating apoptosis, which can influence tissue remodeling and development (You et al. 2021). TNFA's involvement in these processes underscores its importance in maintaining cellular homeostasis and promoting the appropriate differentiation pathways necessary for proper tissue formation. Similarly, TGF-beta signaling is pivotal in embryonic development, as it regulates cell proliferation, differentiation, and migration. TGF-beta signaling is essential for the establishment of the body plan and the development of various tissues and organs (Deng et al. 2024). The overrepresentation of TGF-beta signaling in this interval suggests that ToD candidates may be actively contributing to the cellular transitions required for mESCs to exit the naive state and progress towards lineage specification.

Next to these signalling Hallmark terms, we saw a significant over-representation of GO biological processes associated with development in that time interval (see Figure 5 C). Tissue specific developmental terms such as tube morphogenesis, vasculature development, epithelial cell proliferation, skeletal system development, ossification and response to growth factor show the progression toward priming for lineage commitment. This priming requires sophisticated and rapid reprogramming of the epigenetic, gene

expression, and protein landscapes of ESCs. ToD might be a central mechanism enabling cells to make these necessary rapid changes.

Lastly, ToD candidates of the 6h to 12h time interval displayed a significant over-representation in GO molecular functions, specifically in calcium ion binding and calcium dependent protein binding as well as a nuanced over-representation in calcium dependent phospholipid binding (see Figure 5 D). Calcium-dependent signaling is a central and conserved mechanism essential for development by orchestrating key processes such as cell proliferation, differentiation, and migration. It plays a crucial role in various stages of embryogenesis, including the establishment of body plans, organogenesis, and tissue morphogenesis. The ability of calcium to act as a versatile second messenger allows it to integrate and decode diverse extracellular signals, thereby regulating the dynamic cellular environments necessary for proper development (Stewart and Davis 2019).

These findings underscore the critical role that ToD candidate genes play in early development. The proteins encoded by these genes are essential for facilitating the transition from a naive to a formative primed state. This aligns with our initial hypothesis that ToD may serve as a cellular mechanism that enables swift alterations in the proteomic state of stem cells during development. By integrating cellular signaling cues, ToD effectively regulates gene networks to ensure a proper developmental transition.

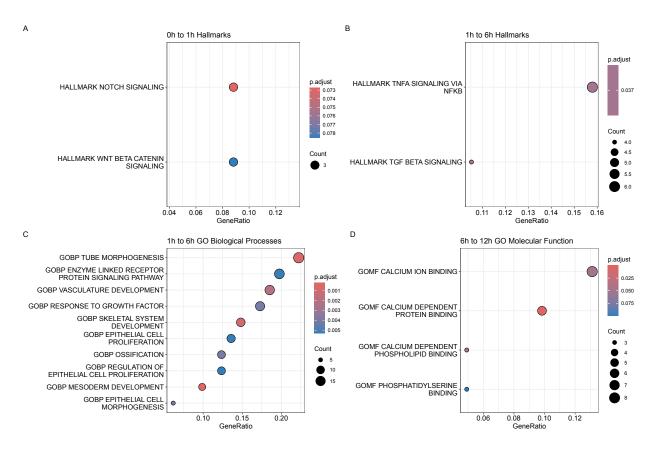


Figure 5: **Gene overrepresentation analysis of early ToD candidates in Yang Data** (A) Hallmark term over-representation analysis at the 0h to 1h time interval. Adjusted p-value threshold of 0.1. Stable transcript range for ToD call of -2 to 2.

- (B) Hallmark term over-representation analysis at the 1h to 6h time interval. Adjusted p-value threshold of 0.05. Stable transcript range for ToD call of -2 to 2.
- (C) GO term over-representation analysis for biological processes (BP) at the 1h to 6h time interval. Adjusted p-value threshold of 0.05. Stable transcript range for ToD call of -2 to 2. (D) GO term over-representation analysis for molecular function (MF) at the 6h to 12h time interval. Adjusted p-value threshold of 0.05. Stable transcript range for ToD call of -2 to 2. The color gradient represents the adjusted p-value, with red indicating a lower adjusted p-value and blue indicating a higher adjusted p-value. The size of each dot corresponds to the number of genes present in the respective gene set from the dataset.

#### ToD candidates regulate gene expression as transcription factors

As we identified a part of ToD candidate genes as transcription factors (TFs), we wanted to investigate if these TFs display their activity upon potential ToD regulation or with a temporal offset after upregulation through ToD. About 10% (20) of the filtered ToD candidates were identified as TFs (see Table 2). From those 20 TFs, five had target annoation in

CollecTRI (see Figure 6). Notably Hsf2, Nr2c1 and Nfe2l2 showed a possible ToD regulation during the 1h to 6h time interval. During that time interval their activity as TFs is still moderate. After ToD regualtion however the show elevated activity during intermediate time points from 24h to 36h. During early development, ESCs are under increased cellular stress, and the selective upregulation of Hsf2 protein production could enhance the cell's ability to cope with these stressors by increasing the expression of heat shock proteins (Åkerfelt, Morimoto, and Sistonen 2010). Similar to Hsf2, Nfe2l2 is part of the cellular stress response, specifically in sensing environmental and endogenous oxidative stress. Deficiency of Nrf2 (TF protein encoded by Nfe2l2) can lead to early embryonic lethality (Leung et al. 2003). As part of the highly conserved nuclear hormone receptors (NHRs) super-family, Nr2c1 is involved in many fundamental biological processes. Next to developmental regulation and cell fate regulation, NHRs influence reproduction, metabolism, circadian cycle as well as and immunological functions ("The Nuclear Hormone Receptor Gene Nr2c1 (Tr2) Is a Critical Regulator of Early Retina Cell Patterning" 2017).

While Pbx1 is also a ToD candidate during the 1h to 6h time interval, it shows its highest activity during the early time points from 0h to 6h (see Figure 6). It's worth mentioning that the filtering criteria for ToD rely solely on the ratio of protein to RNA. This means that although Pbx1 is a ToD candidate during a different time interval when it is less active, the protein abundance can still be high during the early time points. This temporal activity behavior of Pbx1 agrees with the established relationship to NANOG, a naive marker. Pbx1 upregulates Nanog expression to sustain self-renewal and maintaining pluripotency (Chan et al. 2009). Similarly, the expression pattern of Hmga1 aligns with that of Pbx1 (see Figure 6), as it is also involved in various aspects of development and differentiation, including cell proliferation and the regulation and maintenance of pluripotency (Vignali and Marracci 2020).

In summary, these results again show that ToD candidats fill critical roles in early ESC development. Hsf2, Nr2c1, and Nfe2l2 demonstrate selective upregulation, enhancing cellular stress responses and developmental processes. Hsf2 and Nfe2l2 are particularly crucial for managing stress through heat shock and oxidative stress responses, respectively. These findings underscore the importance of ToD in fine tuning gene expression to

support critical transition machanisms during early development.

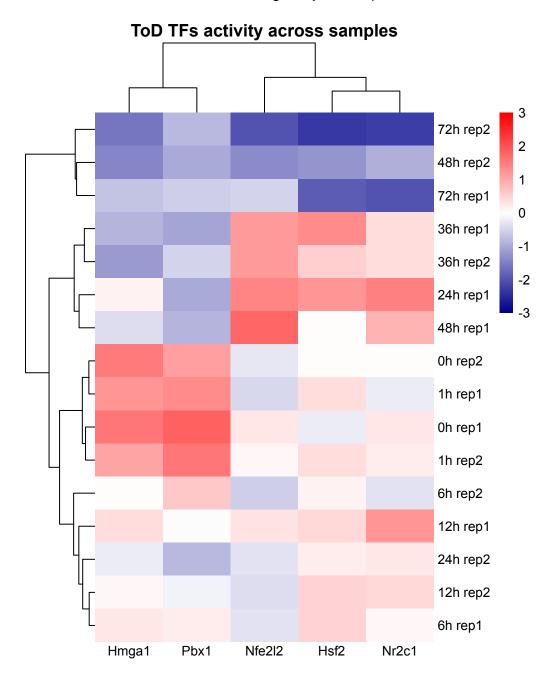


Figure 6: **ToD TFs activity across samples** Activity scores of ToD candidate TFs present in CollecTRI network. Positiv activity scores are colored in red. Negativ activity scores are colored blue.

**ToD candidates share RNA binding proteins target motifs** 

**Discussion** 

Conclusion

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# **Appendices**

# **Supplementary figures**

# **Supplementary tables**

Table 2: ToD candidate genes from the Yang Data

Time Interval	ToD candidate genes
Oh to 1h	Agap1, Al987944, Arhgap27, Axin1, Bbs5, Bbs9, Bex4,
	Btbd9, Cacna1a, Cbx6, Ccdc91, Ccnf, Cdc42bpa, Celf2,
	Cep95, Cgn, Csnk1g2, Csrnp2, Dcbld1, Dnajc14, Ebpl,
	Emc7, Enox2, Epha1, Fbxo9, Gba2, Gm5141, Gstp2,
	Hps4, Jag1, Napb, Nudt22, Odc1, Otud5, Plch1,
	Plekhh1, Rab27a, Rap2c, Rassf1, Rex2, Rgs19,
	Rhbdd3, Rnf187, Rsrp1, Slc38a2, Smim20, Sowahb, Stil,
	Taf11, Tex15, Tm2d3, Tmc6, Tmub2, Tnks, Tpst2,
	Vangl1, Vti1a, Zcchc2, Zfp850, Zik1, Zmym6
1h to 6h	Agtrap, Alg3, Arhgap30, Avpi1, Bmpr1a, Capn3,
	Ccdc112, Cdh1, Coro2b, Ctdsp2, Dip2c, Epha2,
	Fam168b, Fat1, Fzr1, G2e3, Hsf2, Ifrd1, Irs2, Itgb3,
	Jak1, Kifc5b, Lgals8, Lin28b, Maml1, Mapkapk2, Micall1,
	Nfe2l2, Nptx2, Nr2c1, Pbx1, Pde8a, Pecam1, Rimklb,
	Rnf187, Rps6ka6, Slc19a2, Slc9a8, Sptb, Sqstm1,
	Srsf11, Ss18l2, Sulf1, Suv39h1, Syde2, Tex15,
	Tmem106b, Traip, Trim37, Trmt44, Zfand5, Zfp84
6h to 12h	Adck2, Akap11, Bbx, Bcl2l11, Bex4, Birc2, Bnc2, Cenpj,
	Cers5, Cox7a2, Dzip1l, Eif1b, Enox2, Gpc4, Htra1,
	Kctd9, Klhdc10, Lnx2, Mpped2, Myo1d, Ormdl3, Parp9,
	Pnp, Rab40c, Rapgef2, Rbm44, Rims2, S100a10, Scd1,
	Sdc1, Slc25a31, Tmem17, Ttll4, Ube2q2

Time Interval	ToD candidate genes
12h to 24h	Alox15, Arhgef25, Bbc3, Camkv, Dapk1, Dgkd, Mapk6,
	Mief1, Mthfsd, Nuak2, Phf6, Pop5, Rab27a, Sh3kbp1,
	Slc35b1, Spop, Tktl2, Trim23, Zfp644
24h to 36h	Actn3, Asnsd1, Capn3, Chchd7, Cnn2, Dgkd, Enpp3,
	Fam83b, Fbxw17, Foxp1, Gng3, Gstm4, Hmga1, Hmox1,
	Lnx2, Mrpl52, Nr2c1, Otub2, Peli1, Plce1, Pop5,
	Rsl24d1, Siva1, Spta1, Srgap1, Sumo3, Syne1, Zfp644
36h to 48h	B4galt1, Clip2, Cthrc1, Fam222b, Flnc, Flywch1, Glt1d1,
	Irs2, Mlxip, Mutyh, Phykpl, Ptpn13, Rbm47, Rragd,
	Tmem245, Trio, Wwp1

# **Code and Data availability**

#### **Abbreviations**

Table 3: List of abbreviations

Abbreviation	Definition
ToD	Translation on demand
mESCs	Mouse embryonic stem cells
PSCs	Pluripotent stem cells
EpiSCs	Epiblast stem cells
hESCs	Human embryonic stem cells
LIF	Leukemia inhibitory factor
HSPs	Heat shock proteins
elF4E	Eukaryotic translation initiation factor 4E
MNKs	MAPK-Interacting Kinases
RBPs	RNA-binding proteins
RNPs	Ribonucleoproteins
miRNAs	mircoRNAs
UTR	Untranslated region
LFC	Log Fold Change
GPR	Gaussian Process Regression
LFC-Ratio	Protein-to-RNA-LFC-Ratio
GO	Gene ontology
BP	Biological processes
MF	Molecular function
TFs	Transcription factors
ULM	Univariate Linear Model
NHRs	Nuclear Hormone Receptors