Translational specialization in pluripotency by RBPMS poises future lineage-decisions

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SUMMARY

In mammals, translation is uniquely regulated at the exit of pluripotency to rapidly reprogram the proteome to enable lineage commitment. Yet, the developmental mediators of translational control and their mode-of-action remain elusive. Using human embryonic stem cells, we identified RBPMS as a vital translation specialization factor that allows selective translation of developmental regulators. RBPMS-driven translational control balances the abundance of cell-fate regulators to enable accurate lineage decisions upon receiving differentiation cues. RBPMS loss, without affecting pluripotency, specifically and severely impedes mesoderm specification and subsequent cardiogenesis. Mechanistically, the direct binding of RBPMS to 3'UTR allows selective translation of transcripts encoding developmental regulators including integral components of central morphogen signaling networks specifying mesoderm. RBPMS-loss results in aberrant retention of key translation initiation factors on ribosomal complexes. Our data unveil how emerging lineage choices from pluripotency are controlled by translational specialization via ribosomal platforms acting as a regulatory nexus for developmental cell fate decisions.

IN BRIEF:

Future lineage choices from pluripotency are controlled by translational specialization. The RNA binding protein RBPMS is a vital translational specialization factor that unlocks the mesoderm commitment potential of pluripotent stem cells by enabling selective translation of cell-fate regulators instructing lineage decisions.

HIGHLIGHTS:

- Lineage choices emerging from pluripotency are selectively controlled by translational specialization
- The RNA-binding protein RBPMS is a translation specialization factor dedicated to mesoderm commitment
- RBPMS-driven translational specialization enables accurate lineage commitment via balancing the availability of key morphogen signaling components
- RBPMS loss selectively impairs mesoderm commitment and subsequently impedes cardiogenesis
- RBPMS binds the 3'UTRs of target mRNAs to allow their selective translation; its depletion leads to aberrant retention of key translation initiation factors on ribosomal complexes

KEYWORDS

Selective translation, gastrulation, RNA binding proteins (RBP), translational regulation, germ layer specification, cellular identity, WNT signaling, pluripotency, hESC

INTRODUCTION

The protein synthesis machinery, the ribosome, directs how the information encoded in the genome is translated in time and space, and acts as a central regulatory hub in the communication of genetic information. In mammals, translational control is the primary determinant of protein abundance (Kristensen et al., 2013; Mauro and Edelman, 2002; Munoz et al., 2011; Schwanhausser et al., 2011). This is achieved by the rapid and dynamic regulation of global translation rates as well as by the selective translation of specific mRNA subsets (translational specialization)(Arnheim and

Southern, 1977; Dey et al., 2005; Genuth and Barna, 2018; Harding et al., 2003; Mauro and Edelman, 2002; Wolin and Walter, 1988). Translational specialization is highly context-specific and it is proposed to be vital for homeostasis(Hausser et al., 2019; Kong and Lasko, 2012; Shi et al., 2017), adaptive response to external stimuli such as stress, nutrient availability(Lamper et al., 2020; Lee et al., 2015; Lee et al., 2016; Spriggs et al., 2010) as well as in the regulation of cell-fate decisions during development (Lee et al., 2014). Although the phenomenon of translational specialization has been alluded for decades, its mediators and mechanisms remain elusive.

In mammals, translational platforms (translation initiation complexes, 40S, 60S, and assembled ribosomal complexes) are proposed to be modular hubs (Genuth and Barna, 2018; Shi et al., 2017). This modularity is built on the basis of diverse trans-acting factors such as RNA binding proteins (RBP) dynamically associating with the translational machinery (Chen et al., 2014; Darnell et al., 2011; Simsek et al., 2017). Only a fraction of the over ~2500 known RBPs, making up ~7.5% of the cellular proteome, is functionally and molecularly characterized (Hentze et al., 2018; Kwon et al., 2013; Luo et al., 2020; Trendel et al., 2019; Van Nostrand et al., 2020). Moreover the composition of the RNA interactomes of RBPs is context-dependent and dynamic (Sharma et al., 2021). Therefore, we reasoned that association of specific RBPs with ribosomal complexes could specialize their translational output by selectively translating specific groups of mRNAs. This could be achieved by their ability to associate with ribosomal platforms as well as with target mRNAs harboring their binding motifs. This would provide a rich regulatory landscape for decisions requiring dynamic, rapidly controlled and context-specific protein synthesis, for instance, lineage commitment during embryonic development.

Along these lines, during embryogenesis, pluripotent stem cells (PSC) are required to rapidly respond to differentiation signals to undergo a defined trajectory of cell-fate transitions, thereby generating the blueprint of the developing embryo. These cell-fate transitions require continuous rewiring of the translatome to synthesize factors that are essential for both fate decisions and the establishment of the newly acquired state. The mechanisms mediating this translational rewiring remain poorly understood. Analysis of RNAs associated with ribosomal complexes derived from mouse embryonic stem cells (mESC) in comparison to differentiated embryoid bodies revealed that mESCs are uncharacteristically dependent on monosomes for protein synthesis(Sampath et al., 2008). It was hypothesized that this could be due to the less complex cellular needs at the state of pluripotency reflected by the bigger nuclear to cytosol ratio

and simpler cellular components including ER and Golgi. However, upon induction of differentiation, there is an immediate and substantial increase in general translation followed by the synthesis of complex cellular structures including vast ER and Golgi networks and a smaller nuclear to cytosolic ratio, indicating a poorly understood systems-wide translational rewiring. At the exit of pluripotency, elegant in vivo studies using deep sequencing of ribosome-protected RNA fragments (ribo-seq) revealed that the mesoderm lineage is particularly dependent on translational control(Fujii et al., 2017). Importantly, apart from general translational control, key signaling cascades that drive mesoderm formation including WNT, SHH, Hippo, PI3K, and MAPK are proposed to be directly regulated by their selective translation. These reports along with ribo-seq studies (Ingolia et al., 2011) and intricate mass spectrometry-based studies analyzing ribosome composition in mouse ES cells(Shi et al., 2017; Simsek et al., 2017) established the extent of translational control at the state of pluripotency and proposed both the manifestation of 'translational code' built into the mRNAs of cell-fate regulators as well as the existence of RBP responsible for translational specialization (Fujii et al., 2017). However, the identity and mechanisms of these translation specialization factors as well as the extent of their direct influence on the developmental translatome represent a fundamental challenge in our understanding of the communication of genetic information during pivotal stages of embryonic cell-fate transitions, especially in humans.

In this study, we uncovered the RNA binding protein RBPMS as an embryonic translation specialization factor in human embryonic stem cells (hESC). RBPMS specializes the translation of developmental factors including central components of the mesoderm-specifying morphogen signaling networks. Translational specialization by RBPMS balances the availability of mesoderm-inductive signal transduction factors to enable accurate lineage decisions upon differentiation cues. Accordingly, the loss of RBPMS leads to translation inhibition in pluripotency and specifically impedes the ability of hESCs to efficiently commit to mesoderm. Mechanistically, binding of RBPMS to the 3'UTR of transcripts encoding developmental factors allows for their selective translation. RBPMS loss results in the aberrant retention of translation initiation factors on ribosomal complexes. Our findings reveal that the ribosome acts as a nexus for cell-fate-specific gene regulation in humans and that lineage choices are uniquely specialized by translational control.

RESULTS

RNA binding protein RBPMS is a translation machinery-associated protein in hESCs

We hypothesized that the translatome at the state of pluripotency is poised for rapidly responding to differentiation stimuli to revamp the proteome to enable embryonic cell-fate decisions. This could be achieved by the crosstalk between specific RNA-binding proteins and hidden RNA elements inbuilt into the transcripts of developmental regulators, together specializing translational output to endow this vital layer of developmental gene regulation. To identify such translation specialization factors, first, we build the translation machinery-associated proteome in hESCs by global analysis of proteins that differentially associate with the ribosomal platforms. To achieve this, we isolated ribosomal fractions from hESCs corresponding to 40S, 80S (monosome), and polysomes (light polysome fraction and heavy polysome fraction) at steady state conditions by polysome profiling and subjected them to liquid chromatography coupled mass spectrometry (LC-MS) to identify their proteomic composition (translation state mass spectrometry, TS-MS) (**Figure 1A, detailed protocol in STAR methods**).

To identify potential translation regulators that are associated with ribosomal complexes in each of the ribosomal fractions, we first assigned all robustly identified proteins to the fraction(s) for which they display the highest enrichment (log_2 label-free quantification (LFQ) ≥ 25, FDR $\leq$ 0.1, detailed methods in star methods). We next filtered out all ribosomal proteins and known translation factors and identified 1380 proteins in steady state. Next, we evaluated statistically significant enrichment of these proteins on the analyzed ribosomal fractions (fold change $\geq$2, p-value $\leq$0.05). In the steady state conditions, we identified 450 proteins to be significantly differentially enriched with the translational machinery, with a heavy bias towards the 40S fraction, which harbors approximately half of the differentially enriched proteins. (**Figure 1B, Table S1**). 930 proteins showed consistent enrichment in all ribosomal fractions without any bias for any particular fractions.

RBPs were the largest class of proteins (30-50% of the proteins differentially associating with the indicated fractions) in each of the ribosomal complexes (**Figure 1B, Table S1).** Additionally, chaperones, mediators of proteostasis as well as proteins involved in energy metabolism also associated with ribosomal platforms. Emerging evidence points to direct crosstalk between proteins involved in energy homeostasis and translational regulation (Millet et al., 2016; Simsek et al., 2017). Since the common unifying feature of these proteins was their association

with ribosomal complexes, we termed them translational machinery-associated proteins (TMAP). Next, we performed pathway enrichment analysis to evaluate the representative functional features of the TMAPs and grouped them based on their common functional features (**Figure 1C, Table S1**). Proteins in each fraction exhibited discrete functional signatures, and overlapped with TMAP signatures in the adjacent fractions, suggesting functional specialization and cooperativity. TMAPs in the steady state included regulators of protein and nucleic acid metabolism, ribosome biogenesis, translation, cellular metabolism including electron transport chain, translation and translation regulation as well as RNA transport, with major GO categories including RNA metabolic processes and mRNA processing and translational control.

Notably, in agreement with previous studies in mESCs, our data indicate that ribosomal platforms are frequented by numerous functionally diverse groups of proteins (Shi et al., 2017; Simsek et al., 2017). This makes the identification of a true translational specialization factor among them highly challenging. Challenging translation by transient translation stress is known to rapidly rewire the translatome to 'survival mode' where the majority of translation is significantly downregulated while proteins aiding survival and maintenance are selectively translated (Iserman et al., 2020; Kedersha and Anderson, 2002; Markmiller et al., 2018). Therefore, we reasoned that regulators of translational specialization could differentially enrich on ribosomal platforms upon translational challenge to enable this selective, specialized translational state. In agreement, the evaluation of polysome profile traces of hESCs grown in steady state in comparison to translational stress (2 h, 0.05M $NaAsO_2$) showed a pronounced decrease of the 80S and polysome peaks, denoting general translational inhibition (**Figures S1A, S1B**). Of note, hESCs recover back to steady state from such a challenge within 4 hours. Therefore, we hypothesized that translation stress-based dynamic enrichment of a given protein on ribosomal fractions could be used as a prioritization feature for a potential, currently unknown regulator of translation. We therefore performed TS-MS after challenging hESCs with $NaAsO_2$ and analyzed the enrichment of reliably identified proteins between steady state and translation challenge at each ribosomal fractions.

The reproducibility of such an approach is reflected by the low variance of the entire proteome in each of the biological replicates of ribosomal fractions upon principal component analysis (**Figure S1C**). Importantly while all independent ribosomal complexes clustered together, the corresponding complexes upon stress showed marked separation to that of steady state level. These findings indicate that a transient translational challenge remodels the ribosome-associated

proteome (**Table S1**). Nevertheless, the occurrence and dynamics of ribosomal proteins in expected fractions across all the samples further indicated that the isolated fractions indeed represent the indicated ribosomal complexes (**Figure S1D**).

As we predicted, TMAP signatures were notably different upon translational challenge. Mainly the proteostasis machinery (on the 40S) and the RNA degradation machinery (on polysomes) were enriched, while proteins involved in metabolism were depleted (on 80S and polysomes) (**Figure S1E, S1F**). Importantly, as we hypothesized, substantially more RBPs are associated with translational platforms (most on polysomes) upon such challenge (**Figure S1F**).

In line with our initial hypothesis, considering the direct role of RBPs in the regulation of translation, we focused on RBPs enriching on ribosomal complexes for investigating their role as translation specialization factors. In total, we identified 600 RBPs (excluding ribosomal proteins and known translation factors) to be significantly enriched on translational platforms when all conditions were combined (**Figure 1D, Table S1**). Interestingly, these constitute ~24 % of known RBPs and ~38% of the RBPs reported in the proteome of stress granules (Brannan et al., 2016; Hentze et al., 2018; Kwon et al., 2013; Markmiller et al., 2018). Importantly, 41 RBPs were depleted from the ribosomal complexes upon stress while 124 RBPs showed preferential association upon stress (**Table S1**). In line with our initial aim to identify factors that could play a central role in regulating translation, we reasoned that a potential candidate would show preferential, dynamic association with ribosomal complexes upon transient translational stress. We statistically evaluated the dynamic enrichment of these 600 RBPs with each of the evaluated ribosomal complexes between steady state and upon translational challenge and ranked them based on preferential enrichment upon stress (Hoteling's Two Sample T2) **Figure 1E, Table S1**). The top five enriched RBPs were BZW1 (Kozel et al., 2016), AARS(Negrutskii and Deutscher, 1991), HMGB2(Leppek et al., 2021), RBPMS, and KNOP1, of which all except RBPMS and KNOP1 (KNOP1 is reported to be in the nucleolus(Brecht et al., 2020)) are known regulators of translation, consolidating that our identification and prioritization criteria yield translational regulators. While RBPMS has never been reported as a regulator of translation, it has been suggested as a potential regulator of embryogenesis (Aguero et al., 2016; Gerber et al., 2002; Gerber et al., 1999). RBPMS is predominantly a cytosolic protein in hESCs (**Figure 1I**), enriched in 40S complexes in steady state, and moving into polysomal fractions upon translational challenge, suggesting its role in translational regulation (**Figure 1F, 1G**).

Because our method relies on density gradient separation of the protein complexes followed by mass spectrometry, the protein complexes with similar sedimentation profiles as those of the translational machinery could be present in our data set. Previous reports using mESCs reported membrane proteins, centrosomes, clathrin complexes as well as the vault complex to be present in TS-MS data sets(Simsek et al., 2017). However, components of these complexes were scarcely present in our data and were duly filtered out, although we cannot completely rule out transient interactions or shuttling of factors between these complexes and the translational platforms, which might be relevant **(Figure S1G, Table S1).** Alternative strategies do exist for the isolation of ribosome-associated proteomes (Shi et al., 2017). Regardless of the method of choice, a compendium of orthogonal experiments is warranted to confirm the direct association of newly identified proteins with translational platforms.

To confirm the association of RBPMS with ribosomal complexes, we used independent orthogonal approaches. We reasoned that if RBPMS associates with translational machinery in hESCs, then upon treatment with specific translation inhibitors, it would show a characteristic change in sedimentation profile corresponding to the inhibited step in translation.

First, we used the specific translation initiation inhibitor harringtonine (Ingolia et al., 2011). Following 2 µg/ml harringtonine treatment for 30 min, RBPMS was depleted from polysomes and concomitantly enriched in initiation fractions; changes in the enrichment of bona fide components of the translation machinery, EIF4G, PABP, and RPL13 serves as control (**Figure 1H,** *top two panels*). We next treated hESCs with an inhibitor of elongation, puromycin (1 µg/ml for 1h), to induce translational arrest. This led to the distribution of RBPMS across fractions (**Figure 1H,** *third panel*). Next, RNAse I (5U, 30 min) treatment which leads to the disruption of ribosomal complexes, results in the accumulation of RBPMS in the 40S complex (**Figure 1H**, *last panels*). To our knowledge, there are no other complexes that would show a similar sedimentation profile as ribosomal complexes and would simultaneously show the characteristic changes in the profile upon treatment with specific translation inhibitors. Considering its enrichment on the 40S complex in steady state and upon various treatments with translation inhibitors, notably upon RNAse I, we can rule out contamination with nascent RBPMS as it is highly unlikely. Finally, we performed direct immunoprecipitation of RBPMS followed by LC-MS from hESCs after prolonged RNAse I treatment to avoid transcript-dependent indirect associations (o/n on-bead RNAse I digestion) for an unbiased investigation of its associated factors (**Figure S1 H, I**). We

identified ribosomal subunits, ER-associated proteins, and in particular translation initiation factors and other translation-associated RBPs (**Figure 1J, S1J, Table S1**). Taken together, using orthogonal approaches, we identify RBPMS as a translation machinery associated protein in hESCs. Its preferential enrichment with the 40S subunit (steady state **Figure 1F, G, H**) and enrichment on polysomal fractions along with known regulators of translation as well as with key translation factors (**Figure 1J**) suggests a role for RBPMS in translational control.

RBPMS controls translation in hESC

To investigate the molecular and functional role of *RBPMS,* we targeted the exon-intron boundary of exon 1, using two guide RNAs employing CRISPR/Cas9-mediated genome engineering to generate a complete knockout in hESCs (hereafter *RBPMS*-KO) (**Figure 2A**). The homozygous deletion of the exon-intron boundary disrupted the natural open reading frame of RBPMS resulting in a complete loss of function, confirmed both at the RNA and protein levels in comparison to the isogenic wild type hESCs (WT) (**Figure S2A, Figure 2B, 2C**).

RBPMS-KO hESCs display significant polysome depletion, indicative of severe translational inhibition (**Figure 2D**). This leads to ~50% reduction in overall protein synthesis in hESCs, indicated by the significant reduction of newly synthesized proteins detected by short-term (10 minutes) labeling with puromycin (**Figure 2E, 2F, S2G**). Puromycin incorporation was evaluated either using an anti-puromycin antibody or fluorescent azide conjugated O-propargyl-puromycin, to avoid detection dependent bias, if any.

Nevertheless, translation inhibition due to the loss of RBPMS did not affect self-renewal/ or the cell cycle (**Figure S2B**), the overall mitochondrial integrity or metabolism (**Figures S2C, S2D, S2E**), while the global transcriptional output was minimally affected (**EU incorporation, Figure S2F**). Importantly, the loss of RBPMS did not alter the level of pluripotency markers or hESC homeostasis (**Figure 2G, 2H**) over more than 10 passages. Together, these results highlight the role of RBPMS as a regulator of translation in hESCs.

RBPMS is essential for mesoderm specification

During embryonic development, the blueprint of the body plan is established during lineage commitment of pluripotent stem cells, a process that also relies on the efficient rewiring of the proteome to support rapid cell identity changes upon instructive morphogen signaling. Because

RBPMS-loss strongly impaired protein synthesis in hESCs, we hypothesized that it might influence cell-fate decisions from pluripotency. To investigate the role of RBPMS in this process, we used a defined, directed and differentiation method towards three primary germ layers: ectoderm, mesoderm, and endoderm, recapitulating molecularly early embryonic cell fate decisions, as we previously reported (**Figure 3A**) (Frank et al., 2019).

The loss of RBPMS severely and specifically impaired mesoderm commitment without affecting endoderm and ectoderm differentiation (**Figures 3B, S3A, S3B, S3C, S3D**). Upon mesoderm induction, *RBPMS*-KO hESCs displayed defective induction of key mesoderm as well as cardiac mesoderm commitment factors *BRACHYURY* (*TBX-T*) and *MESP1* (**Figure 3B, 3C,3D**)(Bondue et al., 2008; Perea-Gomez et al., 2004; Tosic et al., 2019). Besides, upon mesoderm induction, *RBPMS*-KO cells aberrantly expressed pluripotency factors (**Figure S3E**). Comparative transcriptome analysis of *RBPMS*-KO and WT cells at the state of pluripotency and upon mesoderm induction further confirmed their inability to effectively induce key mesoderm markers. (**Figure 3E, S3H, Table S2**). Of note, as we previously observed that at the state of pluripotency, the transcriptional signature of core pluripotency factors was comparable between *RBPMS*-KO and WT cells (**Figure S3F, Table S2**). Strikingly, functional annotation of differentially expressed genes revealed that morphogen signaling central to mesoderm commitment including WNT as well as key processes involved in mesoderm differentiation failed to be upregulated in *RBPMS*-KO cells upon mesoderm induction, whereas genes involved in pluripotency and ectoderm differentiation were aberrantly upregulated (**Figure 3F, S3G, Table S2**). Expression of lineage markers undergoes rapid dynamics during the early stages of germ layer commitment. To confirm that the mesoderm commitment defects due to RBPMS-loss were not a result of disrupted timing, we analyzed their expression dynamics at close intervals (**Figure 3G**). In agreement with our previous observation, mesodermal markers as well as WNT signaling mediators failed to be properly activated upon *RBPMS*-KO cells over the course of 24 h of induction. Importantly, this goes hand in hand with reduced active beta-catenin levels in *RBPMS*-KO hESCs, indicating impaired WNT signaling, a central morphogen signaling network essential for mesoderm commitment (**Figure 3H**).

Next, we tested whether the impaired differentiation of *RBPMS*-KO hESCs to mesodermal lineage has functional consequences, detrimentally affecting terminal fate choices. In this regard, we chose defined differentiation to cardiomyocytes since it is a robust, high-efficiency method

allowing near-synchronous differentiation to a functional terminal fate (Rao et al., 2016a). Strikingly, *RBPMS*-KO cells failed to produce cardiomyocytes in contrast to WT cells that constantly yielded a homogenous population of cardiomyocytes (**Figure 3I**). Additionally, *RBPMS*-KO cells failed to produce cardiomyocytes across the cardiac corridor (**Figure S3I**), which is BMP/WNT concentration grid to test the ability of pluripotent stem cells to give rise to cardiomyocytes (Rao et al., 2016a). Thus, we conclude that RBPMS is essential for accurate cell-fate decisions enabling mesoderm commitment and cardiac differentiation.

RBPMS targets a broad network of mRNAs encoding central regulators of mesoderm-specifying signal transduction and morphogenesis

To comprehensively identify the network of mRNAs regulated by RBPMS in hESCs, we employed enhanced UV cross-linking and immunoprecipitation of ribonucleoprotein complex followed by massively parallel sequencing (eCLIP-seq) (**Figures 4A, S4A, S4B**)(Van Nostrand et al., 2016). Following removal of PCR duplicates and normalization relative to size-matched input controls from four independent replicates, we compiled transcriptome-wide, nucleotide-resolution and high confidence binding maps displaying >80% overlap of target mRNAs between replicates (**Figures 4B, S4C-E and Table S3**). Only the statistically significantly-enriched targets represented in all four replicates were considered for further analysis. RBPMS was predominantly found bound to 3'UTRs of mRNAs (**Figure 4C, 4D and Table S3**) on a bipartite CAC motif (**Figures 4E and S4F**) in hESCs.

Functional annotation of 3'UTR-bound targets revealed that RBPMS binds mRNAs regulating gastrulation, tissue morphogenesis, as well as signal transduction – particularly WNT signaling, which are all essential for successful cell fate commitment during gastrulation (**Figure 4F, Table S3**). 3'UTR targets of RBPMS are factors operating in a variety of cellular compartments (**Figure S4G**). A curated set of such 3'UTR targets, selected for high signal-over-input enrichment and grouped on the basis of their molecular and developmental functions illustrates how RBPMS targets a broad network of mRNAs encoding crucial regulators of morphogen signal transduction and cell fate commitment that enables mesoderm development (**Figure 4G, 4H**). Together, we discovered that RBPMS directly targets a network of mRNAs that encode central regulators of mesoderm commitment.

Translational specialization of the regulators of mesoderm specification requires RBPMS

The impaired translation and the mesoderm commitment defect in *RBPMS*-KO along with its direct binding on the 3'UTR of transcripts encoding key mesoderm-instructive developmental regulators led us to hypothesize that, in hESCs, RBPMS could be regulating the association of mRNAs that are important for future lineage choice with the translational apparatus.

To test this hypothesis, we first applied translation state RNA sequencing (TS-Seq) to investigate transcriptome-wide changes in the occupancy of ribosomal complexes upon RBPMS loss in hESCs. To this end, transcripts associated with ribosomal complexes (the 40S, 80S, light, and heavy polysomes) were isolated after ribosome fractionation, enriched for poly-adenylated transcripts and subjected to transcriptome sequencing, in parallel with total RNA from *RBPMS*-KO hESCs and corresponding isogenic WT (**Figure 5A**). To correct for technical variability and allow data normalization, two different sets of spike-ins were added to each fraction of our three biological replicates, one after lysis and the other after polysome fractionation

The loss of RBPMS resulted in severe translational inhibition, which makes the evaluation of changes in ribosome occupancy on specific mRNAs cumbersome (**Figure 2**). Therefore, to identify meaningful changes in ribosome occupancy, after normalization with dual spike-in controls, a two-step regression-based clustering approach was used. This approach allows the identification of statistically significant clusters of transcripts that were substantially different in their pattern of ribosome occupancy across the translational platforms in the *RBPMS*-KO hESCs in comparison to isogenic WTs. Notably, we obtained 20-30 Million clean reads per ribosomal fraction per replicate.

We identified 8 mRNA clusters exhibiting a significant difference in the pattern of ribosome occupancy between RBPMS-KO and WT hESCs. Importantly, upon loss of RBPMS, ribosomal complexes were severely depleted in six of the clusters which harbored the majority of the translationally affected transcripts, while two clusters showed enrichment (**Figure 5B** and **Table S4**). Translationally repressed genes were crucial to developmental cell-fate commitment and embryogenesis, while translationally activated genes were involved in unrelated processes (**Figure 5C, S5D, Table S4**). Critically, integrative analysis of transcriptomics and TS-Seq data revealed that transcripts only changing transcriptionally are not directly implicated in morphogen signaling or mesoderm/cardiac-mesoderm development, the major processes affected by the loss of RBPMS (**Figure S5A** and **Table S4**).

We next investigated the status of direct RBPMS targets with respect to the translationally affected clusters. In line with our assumptions, most RBPMS-bound 3'UTRs were strongly depleted from ribosomal fractions (**Figure 5E, F, H** and **Table S4**). In agreement, metagene analysis illustrated ribosomal depletion relevant to the actual coordinates of RBPMS eCLIP peaks, further indicating that RBPMS binding at the 3'UTR of a given mRNA is a key determinant of its translational status (**Figure 5G**).

Functional annotation of the 3'UTR targets of RBPMS whose translation was severely impaired revealed that they control mesoderm specification, cell-fate commitment during development, and importantly, morphogen signaling including WNT signaling (**Figure 5I**). Since morphogen signaling, particularly by WNT, BMP/NODAL, and FGF defines mesoderm commitment from pluripotency, we then investigated the ribosome occupancy on genes known to be regulating these processes (Brade et al., 2013; Loh et al., 2016). Strikingly, a substantial number of transcripts involved in WNT signal transduction along with those of BMP/NODAL and FGF signaling were severely depleted from active translational compartments upon *RBPMS*-KO. This indicates that the presence of RBPMS is essential for their appropriate association with ribosomal complexes (**Figure 5H, Table S4**), implying the direct role of RBPMS-mediated translational specialization in embryonic signal transduction (**Figure 5D, S5C**). In line with these findings, the levels of active beta-catenin, a key WNT signaling component, were substantially low upon loss of RBPMS (**Figure 3G**). However, RBPMS does not affect transcript stability in general (assessed for a selection of pluripotency factors and direct RBPMS 3'UTR targets involved in WNT signaling, following actinomycin-D treatment to inhibit transcription) (**Figure S5E**). The loss of RBPMS severely impairs the ribosome occupancy of crucial developmental genes involved in the control of early embryonic fate determination at the state of pluripotency (illustrated by the translational status of the curated list of key developmental genes affected by its loss, including key 3'UTR targets **Figure 5J, S5B**), pointing to a central role played by RBPMS in the translational specialization of cell fate commitment from pluripotency. Together, our data indicate that the translation state of the regulators of cell-fate decisions including components of morphogen signal transduction crucial for lineage commitment are balanced in pluripotent stem cells by RBPMS-mediated translation specialization, thereby poising future lineage choices.

RBPMS-loss results in aberrant retention of translation initiation factors on ribosomal complexes

To understand how RBPMS regulates translation, we investigated its association with the translation initiation complexes. This was motivated by its (i) predominant association with the 40S complex **(Figures 1F,G**); (ii) further enrichment in the 40S fraction upon hESC treatment with translation inhibitors (**Figure 1H**); and (iii) co-purification with key translation initiation factors (**Figure 1J**). To this end, we interrogated the dynamics of key factors influencing translation initiation on ribosomal platforms upon loss of RBPMS with respect to isogenic WT. First, we specifically interrogated the enrichment of key initiation factor eIF4G (involved in cross-talk between 43S preinitiation complex and eIF4F complex), and poly(A)-binding protein PABP. Both displayed comparable levels indicating that RBPMS loss does not influence the predisposition of mRNAs to be translated (**Figure 6A,B**). The slight reduction of eIF4G from polysomal fractions in *RBPMS*-KO is indicative of translation inhibition. However, a key component of the 43S preinitiation complex and a regulatory hub for global translation, eIF2A(Wek, 2018), showed aberrant retention across ribosomal fractions following RBPMS loss (**Figure 6A,B**). Additionally, two vital eIF3 complex components, eIF3E (involved in selective translation)(Lin et al., 2020; Shah et al., 2016) and eIF3H (previously reported to be involved in the selective translation during embryonic development)(Choudhuri et al., 2013) were aberrantly retained in polysomal fractions and substantially depleted from the 40S complex in *RBPMS*-KO cells, respectively (**Figure 6A,B**). Strikingly, eIF5A, essential for proper elongation, for error resolution at ribosomal pause sites, and for termination (Schuller et al., 2017), was sequestered into the 40S ribosomal fraction in the absence of RBPMS (**Figure 6A,B**). Notably, the total levels of these factors do not reflect the change in their pattern of distribution in ribosomal fractions in *RBPMS*-KO hESCs **(Figure 6C**). Together our data reveal that loss of RBPMS results in aberrant retention of translation initiation factors on ribosomal complexes in hESCs.

Next, we asked whether binding by RBPMS to the 3'UTR of model mRNAs suffices for controlling their translation in hESCs. To this end, we generated a set of dual luciferase-based reporter constructs with RBPMS binding sites in the 3'UTR of SFRP1 or ACTB mRNAs. Loss of RBPMS lead to a significant decrease in luciferase activity selectively in SFRP1- 3'UTR fusions (with chicken ACTB and luciferase-only controls remaining unaffected compared to WT cells; **Figure 6D**). Since luciferase-based reporter assays are end-state readouts,

Since luciferase-based reporter assays are end-state readouts, we used time-lapse microscopy of fluorescent reporters harboring RBPMS binding sites at the 3'UTR along with indicated controls to independently measure the linear influence of RBPMS on reporter levels (and by end read out at 24hrs post-transfection). We observed a significant increase in the translation efficiency in the presence of RBPMS only when its binding sites were present in the 3'UTR (**Figure 6E, S6A, S6C**). Together, our data indicate that 3'UTR binding of RBPMS specializes the translation of its targets in hESCs.

Finally, to confirm the role of RBPMS as a translation specialization factor central to mesoderm specification and to rule out discrepancies in our findings caused by any potential aberration due to genome engineering in *RBPMS*-KO hESCs, we knocked-in an inducible copy of RBPMS using PiggyBac transposon-based genomic insertion (**Figure 6F**). Timely re-expression of the most prominent cytosolic isoform (**Figure 1I**, the mid band in RBPMS immunoblot) of RBPMS in *RBPMS*-KO cells fully restored both ribosome occupancy defects and protein synthesis, including the protein levels of SFRP1, one of the RBPMS 3'UTR targets, and a component of WNT signaling (**Figure 6G,H,I, S6B**)(Liang et al., 2019). Importantly, re-expression of RBPMS restored the mesoderm commitment capacity, reinstating the ability of *RBPMS*-KO cells to generate terminally differentiated cardiomyocytes (**Figure 6J**). Taken together, our data reveal that the competence of hESCs for mesoderm specification is translationally specialized by RBPMS through the selective translation of developmental factors such as central morphogen signaling components, thereby poising future lineage choices.

DISCUSSION

Translation presents a highly tunable, temporal and spatial avenue for regulating gene expression, especially in vertebrates (Genuth and Barna, 2018; Jackson et al., 2010). The mammalian translation apparatus has expanded substantially in comparison to its prokaryotic and yeast counterparts (Genuth and Barna, 2018), providing docking points for various translation machinery-associated proteins, especially RBPs. This would allow ribosomes to prioritize and selectively translate specific groups of mRNAs, considering the ability of RBPs to interact with transcripts carrying their binding motifs. However, our understanding of how such molecular regulatory modules are appended to ribosomal complexes as well as how they might induce functional specialization remains limited.

Pluripotent stem cells are known for having unique translational requirements, mainly dependent on monosomes for sustenance (Ingolia et al., 2011; Sampath et al., 2008). For instance, key pluripotency factors such as *NANOG* and *C-MYC* rely on upstream open reading frames (uORF) to regulate their expression and the potential regulation of their homeostasis by eIF4G2 driven internal ribosome entry site (IRES) dependent translation (Yoffe et al., 2016). Together, these observations motivated us to investigate the role and mediators of translational specialization in hESCs.

In an effort to identify potential translation specialization factors in pluripotency, we systematically probed the dynamics of translation machinery-associated proteins in hESCs. Ribosomal platforms showed a highly diverse and dynamic proteome. We identified an array of proteins involved in various primary cellular processes such as metabolism, protein homeostasis, and RNA metabolism to be associated with ribosomal complexes. In agreement, ribosomal platforms in mESCs were shown to be heterogeneous and customizable to allow translational specialization (Shi et al., 2017; Simsek et al., 2017). Notably, the largest class of proteins associating with ribosomal complexes were RBPs, in line with our initial hypothesis of RBPs being vital translation specialization factors.

RNA-binding protein with multiple splicing (RBPMS) is evolutionarily conserved across vertebrates carrying a single RNA recognition motif close to its N-terminus(Teplova et al., 2016). Loss of RBPMS leads to a reduction in polysome occupancy and protein synthesis indicating its central role in regulating translation in hESCs. Surprisingly, despite such strong translational inhibition, RBPMS loss does not influence the expression of pluripotency factors. Thus, RBPMS regulates translation in hESCs without affecting the state of pluripotency.

Loss of RBPMS impaired the ability of hESCs to undergo proper germ layer commitment, specifically ablating mesoderm specification and impeding terminal differentiation to cardiomyocytes. In accordance, many of the direct RBPMS targets are key regulators of mesoderm commitment, including pivotal components of morphogen signaling, including WNT; the central cardiac mesoderm specifier. RBPMS binds predominantly to the 3'UTR of its targets, and its loss leads to their marked depletion from ribosomal complexes. Importantly, this further impairs the translation status of vital mesoderm commitment factors. RBPMS was recently suggested to regulate splicing in smooth muscle cells (Nakagaki-Silva et al., 2019), extrapolated from the targets identified by overexpression of RBPMS in HEK293-T cells which naturally do not express

RBPMS (Farazi et al., 2014). A cursory analysis revealed that minimal overlap between the reported targets in HEK293-T cells and in hESCs, indicating cell type specificity of its endogenous targets. Additionally, we find no evidence for splicing being the primary RBPMS function in hESCs. In fact, previous studies have indicated that mesoderm commitment is particularly susceptible to translation perturbations, for example via tRNA pseudouridylation (Fujii et al., 2017; Guzzi et al., 2018; Yoffe et al., 2016). Now, we report the role of RBPMS as a translation specialization factor promoting mesoderm commitment in hESCs. This specificity can be attributed to the RBPMS-dependent ribosome occupancy on transcripts encoding morphogen signaling components, especially the WNT signaling network.

The combination of BMP/WNT signaling faced by pluripotent stem cells is a key determinant of mesodermal and cardiac fate in vitro (Kempf et al., 2016; Loh et al., 2016; Rao et al., 2016b), while cells exiting the primitive streak in vivo are exposed to a specific ratio of WNT to BMP signaling gradient, which defines the mesodermal lineages they will differentiate into (Bisson et al., 2015; Marvin et al., 2001; Peng et al., 2019). Our work identified RBPMS as a translation specialization factor enabling mesoderm specification, a finding that confirms the postulation of a translational module specialized for mesoderm development drawn in an elegant study *in vivo* (Fujii et al., 2017). Our functional characterization of RBPMS also explains how morpholino-mediated RBPMS silencing in Xenopus embryos caused severe cardiac development defects (Gerber et al., 2002; Gerber et al., 1999). Of course, given the molecular differences in the state of pluripotency between humans and other model organisms, like mice, more comparative work is warranted for describing the role of RBPMS therein (Ghimire et al., 2021).

Mechanistically, RBPMS predominantly associates with translation initiation complexes, and its loss leads to the aberrant retention of translation initiation factors eIF2A, -3H, and -3E, on ribosomal complexes. All these factors have been reported to be involved in translation specialization (Choudhuri et al., 2013; Lin et al., 2020; Shah et al., 2016; Wek, 2018). Importantly, RBPMS loss severely depletes the translation apparatus of the key ‘surveillance’ factor eIF5A. This points to a role for RBPMS in enabling proper translation initiation and elongation in hESCs.

Translational specialization by RBPMS is dependent on the presence of its mRNA binding motif and reinstating RBPMS levels restores the mesoderm specification capacity of hESCs. In fact, our reporter assays showed that the insertion of RBPMS recognition elements in the 3’UTR suffices for boosting translation. Notably, not all RBPMS targets are translationally repressed. This

is not uncommon for translation specialization factors. For instance, the loss of eIF3D, a translation factor enabling selective translation upon stress, leads to both activation and inhibition of mRNAs (Lee et al., 2015; Lee et al., 2016).

Nevertheless, the loss of RBPMS led to a global reduction in ribosome occupancy, not only on its direct mRNA targets. This leads to impaired WNT signaling, which has itself been implicated in regulating global translation (Madan et al., 2018). Furthermore, in regards to RBPMS, this could be additionally attributed to co-binding by other RBPs known to influence translation in pluripotent stem cells (Wilbert et al., 2012; Zeng et al., 2016). Thus, further mechanistic and functional investigation of the RBPMS-WNT axis in the translational control of cell-fate is an exciting prospect for future studies.

In summary, our data support a model whereby the state of pluripotency is translationally poised for differentiation into future lineages via the selective translation of the regulators of embryonic cell-fate. This is exemplified by translational specialization by RBPMS, which unlocks the mesoderm commitment potential of hESCs via selective translation of key developmental regulators, including morphogen and signal transduction components. Regulation of gene expression is paramount to developmental cell-fate transitions. In this regard, traditionally the focus has been mainly on delineating signaling pathways, transcriptional and epigenetic mechanisms contributing to developmental cell-fate decisions. We propose that RNA-binding proteins associate with ribosomal platforms to regulate translation of cell-fate regulatory mRNAs in time and space during embryonic cell-fate decisions, similar to how transcription factors are known to specify cell-fate commitments. Our catalog of translation machinery-associated proteins provides a rich resource for investigating additional translational specialization paradigms, and reveals a fertile regulatory space to be explored in the future. Collectively, we propose a pivotal role for translational specialization in sculpting cellular identity during early developmental lineage decisions.

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Data regarding this manuscript will be provided upon request.

AUTHOR CONTRIBUTIONS

L.K. conceived and supervised the projects. L.K and D.B designed all the experiments. G.A, and D.B conducted all the computational analysis. D. B and K. K performed the experiments. A.P and H.B assisted in various stages of the project. L.K and D.B analyzed and interpreted the data. L.K and D.B. drafted all illustrations, with assistance from G.A. L.K prepared the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1: RNA binding protein RBPMS is a translation machinery associated protein in hESCs

(A) A schematic outline of the underlying strategy employed for Translation state mass spectrometry (TS-MS).

(B) RNA binding proteins are the largest class of protein on ribosomal platforms in hESCs. n= total number for proteins/ribosomal complex, (only proteins detected in all three biological replicates above $\log_2$ LFQ ≥ 25, FDR ≤ 0.1 were considered). (LFQ: label-free quantification; LC-MS: liquid chromatography mass-spectrometry)

(C) Gene Ontology-based functional enrichment analysis for differentially enriched proteins on ribosomal platforms (p-value ≤ 0.001). (FC: fold change)

(**D**) Heatmap shows the enrichment of RNA binding protein on ribosomal platforms in steady state conditions and upon transient transitional stress (n=600).

(**E**) Hotelling's two-tailed T2-based significant dynamic enrichment of RBPs on ribosomal platforms upon stress, top 5 enriched RBPs in the inlet (Hotelling's T2 values for ranking translation machinery-associated proteins using MEBA implemented using MetaboAnalyst).

(F), (G) Dynamic enrichment of RBPMS on ribosomal platforms in steady state and upon translational stress in hESCs evaluated by Mass spectrometry (line graph) and by western blotting. RPL7A, RPS6, and G3BP1 serve as controls.

(H) RBPMS-enrichment on ribosomal platforms evaluated by indicated translational inhibitor treatment with accompanying ribosome traces. Protein levels of RBPMS and the indicated controls on ribosomal fractions were detected by Western blotting.

(I) RBPMS is predominantly a cytosolic protein, evaluated by Western blot analysis upon nuclear/cytosolic fractionation, G3BP1, and TUBA1B cytosolic control, LAMINB1 nuclear control.

(J) Circos plot depicting the enrichment of indicated proteins upon immunoprecipitation of RBPMS after extended treatment with RNAse I over the IgG control. Log2 LFQ ≥25 in all three biological replicates and $\log_2$ fold change ≥2, p-value ≤0.05). (n=3 unless otherwise indicated).

Error bars represent ±SEM; p-values calculated using Student's t-test, *≤0.05)

See also **Figure S1**.

Figure 2: RBPMS controls translation in human ES cells

(**A**) Schematic representation of RBPMS locus in humans and the CRISPR/Cas9-based targeting strategy used to generate homozygous *RBPMS*-KO, confirmed at the (**B**) RNA level by RT-qPCR and (**C**) at the protein level by western blotting.

(**D**) Loss of RBPMS impedes translation in hESCs, indicated by representative polysome profiles from sucrose gradient fractionation of *RBPMS*-KO hESCs w.r.t isogenic WTs along with quantification of the area under the indicated ribosomal fractions on the right.

Protein synthesis is inhibited in *RBPMS*-KO hESCs. De novo protein synthesis is evaluated by measuring puromycin in-cooperation on nascent proteins using (**E**) anti-puromycin antibody by western blotting and (**F**) by measuring uptake of OPP-puromycin, quantifications on the right.

(**G**) RBPMS-loss does not affect pluripotency. Representative images of WT and RBPMS-KO hESCs stained for OCT4, SOX2 and NANOG: Bar graph shows normalized expression levels of indicated pluripotency markers.

(**H)** RT-qPCR for pluripotency markers *OCT4*, *SOX2* and *NANOG*. Bar graphs show relative fold change to WT hESCs normalized to *RPL37A*.

Error bars represent ±SEM; p-values calculated using Student's t-test (n.s.>0.05, *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001, n = 3).

See also **Figure S2**.

Figure 3: RBPMS is essential for mesoderm specification

(A) Schematic of lineage differentiation approaches used to determine the competence of *RBPMS*-KO hESCs to undergo germline commitment.

Mesoderm commitment is impaired upon loss of RBPMS. (**B**) Representative images of MESP1 staining upon mesoderm induction of RBPMS-KOs hESCs for indicated mesodermal markers, quantification on the right. Impaired mesoderm commitment was confirmed by RT-qPCR **(C)** highlighting reduced activation of mesoderm markers *TBX-T* and *MESP1* RT-qPCR for mesoderm markers TBX-T and MESP1, **(D)** as well as by western blotting for TBX-T.

(E) Heatmap shows normalized expression levels of indicated mesoderm markers

(**F**) Gene Ontology-based functional enrichment analysis for differentially expressed genes in WT mesoderm and *RBPMS*-KO mesoderm (significance level indicated by color code).

(**G)** Kinetics of induction of mesodermal markers confirms mesoderm commitment defects upon *RBPMS*-KO in hESCs. Bar graphs represent gene expression kinetics of indicated markers.

(**H**) Loss of RBPMS reduces active BETA CATENIN levels in hESCs as detected by Western blotting.

(**I)** Loss of RBPMS detrimentally affects terminal fate choices of hESCs indicated by defined differentiation to cardiomyocytes. Representative images of indicated markers upon cardiac differentiation of WT hESCs w.r.t *RBPMS*-KO hESCs.

Error bars represent ±SEM; p-values calculated using Student's t-test (p-values : n.s.>0.05, *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001, n = 3).

See also **Figure S3**.

Figure 4: RBPMS targets a broad network of mRNAs encoding central regulators of mesoderm-specifying signal transduction and morphogenesis

(A) Schematic of the eCLIP-seq approach employed to faithfully generate an unbiased transcriptome-wide direct binding map for RBPMS at single-nucleotide resolution in hESCs.

(B) Biological quadruplicates of eCLIP show at least 80% overlap between each other, pie charts show the correlation of statistically significant uniquely mapped reads for each replicate over SMInput.

(C) RBPMS reliably binds predominantly the 3'UTR of its direct target transcripts, demonstrated here by the distribution of the significantly enriched eCLIP peaks against the paired SMInput (fold change ≥ 2; p-value ≤ 0.05 in all 4 replicates).

(D) Metagene plot visualizing the peak distribution of RBPMS over SMInput illustrating prominent 3'UTR binding.

(E) Top sequence motif bound significantly bound by RBPMS in the indicated category.

(F) 3'UTR targets of RBPMS regulate molecular processes central to mesoderm commitment including components of morphogen signal transduction network, depicted by significantly enriched GO terms.

(G) A curated set of RBPMS 3'UTR targets group based on their proven role in the indicated cellular, developmental and functional process, depicted as a heatmap of enrichment over SMInput.

(H) Representative read density tracks show read density for RBPMS across the gene body of *SFRP1*, a representative target (inlet shows a zoom into the 3'UTR of *SFRP1* in two independent replicates).

See also **Figure S4**.

Figure 5: Translational specialization of the regulators of mesoderm specification requires RBPMS

(A) Schematic of the TS-Seq employed to evaluate the role of RBPMS globally as well as specifically on its direct targets.

(B) Global impact of the loss of RBPMS on ribosome occupancy in hESCs, revealed by two-step regression analysis of the mRNAs enriching on ribosomal fractions.

(C) Functional analysis of translationally repressed transcripts in *RBPMS*-KOs versus isogenic WT hESCs illustrated as a significantly enriched curated list of GO terms (significance levels indicated in the key on the right).

(D) Loss of RBPMS severely impairs ribosome occupancy on mRNAs encoding components of WNT, BMP, NODAL, and FGF signaling, vital mesoderm specifying signal transduction networks.

(E) Translation status of direct RBPMS targets upon its loss in hESCs, grouped based on its binding coordinates, in the indicated translationally affected clusters identified by TS-Seq.

(F) Distribution (%) of direct RBPMS targets that are translationally affected in the indicated categories.

(G) Metagene plot indicating a 3'UTR binding bias for translationally repressed targets of RBPMS.

(H) Heatmap depicting ribosome occupancy of translationally repressed RBPMS 3'UTR targets.

(I) Functional analysis of translationally repressed 3'UTR targets of RBPMS in *RBPMS*-KO hESCs with respect to corresponding wildtypes illustrated as a significantly enriched curated list of GO terms.

(J) Translational status of the curated list of key developmental genes upon loss of RBPMS, illustrated as heatmap depicting the ratio of occupancy indicated ribosomal complexes on transcripts between *RBPMS*-KO and wildtype hESCs.

See also **Figure S5**.

Figure 6: RBPMS depletion results in aberrant retention of translation initiation factors on ribosomal complexes

(A),(B) Loss of RBPMS in hESCs results in impaired retention of translational initiation factors, involved in translational specialization, depicted by the distribution of the occupancy of indicated factors in ribosomal complexes, isolated by polysome fractionation. (n=2).

(C) Total levels of the indicated proteins in *RBPMS*-KO hESCs compared to the corresponding WT.

(D) eCLIP-seq motif-based 3'UTR binding of RBPMS specifically enhances translation of dual luciferase-based reporters in hESCs in the presence of RBPMS.

(E) RBPMS enhances the translation of fluorescence-based dual reporters specifically upon in the presence of its RNA binding motif. Indicated 3'UTRs were fused to GFP open reading frame, co-transfected with plasmids expressing RBPMS or mCherry in WT or *RBPMS*-KO hESCs and fluorescence was recorded continuously for 48h by live microscopy.

F) Timely reconstitution of RBPMS in *RBPMS*-KO hESCs rescues (**G)** ribosome occupancy, **(H)** general protein synthesis, **(I)** translation defect of 3'UTR targets of RBPMS as well as **(J)** lineage commitment defects.

Error bars represent ±SEM; p-values calculated using Student's t-test p-values : n.s.>0.05, *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001, n = 3).

See also **Figure S6**.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Translational challenge rewires the proteome of ribosomal complexes in hESCs

(A) Polysome traces from sucrose gradient fractionation of hESCs in steady state and upon transient translational stress.

(B) Transient translational stress leads to significant depletion of polysomal fractions, shown by bar plots of the area under the indicated fractions from polysome trances.

(C) Transient translation stress causes a substantial change in the total proteome of ribosomal complexes, indicated by the principal component analysis of the indicated fractions from TS-MS.

(D) Heatmap depicting the abundance of ribosomal proteins constituting small and large ribosomal subunits.

(E) The abundance of RNA binding proteins on polysomal fractions show a substantial increase upon transient translational stress. n= total number for protein/ribosomal complex, (only proteins detected in all three biological replicates above $\log_2$ LFQ $\geq$ 25, FDR $\leq$ 0.1were considered). **(F)** Gene Ontology-based functional enrichment analysis for differentially enriched proteins on ribosomal platforms upon transient translational challenge (p-value $\leq$ 0.001).

(**G**) The overlap between identified translation-associated factors with components of the indicated multi-protein complexes.

(H) Coomassie blue-stained gels depicting the indicated eluates from RBPMS and IgG (control) pull-downs, respectively. Size separated proteins were pooled independently, isotope-labeled, and quantified by LC-MS.

(I) Volcano plot showing specific enrichment of proteins upon RBPMS pull-down w.r.t IgG control, in triplicates.

(**J**) Enrichment of ribosomal proteins and translation factors upon RBPMS pull down.

Error bars represent ±SEM; p-values calculated using Student's t-test (n = 3).

Figure S2: RBPMS loss only affects protein synthesis in hESCs

(A) Sanger sequencing-based confirmation of genome editing in the generated *RBPMS*-KO hESCs.

(B) Evaluation of the effect of cell cycle upon *RBPMS*-KO in hESCs.

(C) Evaluation of mitochondrial distribution (mitofusin and mitotraker staining) and **(D)** mitochondrial integrity (live Mitotracker intake) in *RBPMS*-KO and wildtype hESCs.

(E) Mitochondrial oxygen consumption rates (OCR; pmol O2/min) in WT and RBPMS-deficient hESCs. Specific inhibitors used in the analysis are indicated.

(**F**) Representative micrographs depicting the nascent transcription in WT and *RBPMS*-KO hESCs, measured by EU (5-Ethynyl Uridine) labeling assay.

(**G**) De novo protein synthesis evaluated by measuring puromycin in cooperation of nascent proteins using anti-puromycin antibody between WT and *RBPMS*-KO hESCs, evaluated by fluorescence microscopy. Error bars represent ±SEM; p-values calculated using Student's t-test (p-values: *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001, n = 3).

Figure S3: Evaluation of the effects of RBPMS loss on lineage commitment by hESCs

Loss of RBPMS does not affect **(A)** & **(B)** neuroectoderm and **(C)** & **(D)** endoderm commitment potential of hESCs, evaluated by immune fluorescence microscopy and qPCR of the indicated markers.

(E) Loss of RBPMS results in residual expression of indicated pluripotency markers upon mesoderm induction, evaluated by RT-qPCR analysis.

(F) Loss of RBPMS does not affect the expression of pluripotency markers in hESCs.

(G) Induction of mesoderm markers is affected upon *RBPMS*-KO.

(H) *RBPMS*-KO hESCs show aberrant expression of indicated endodermal and ectodermal markers upon mesoderm induction.

(**I**) *RBPMS*-KO fail to differentiate to cardiomyocytes across the WNT-BMP gradient permissive for cardiac commitment, "cardiac corridor" as compared to WT hESCs.

Error bars represent ±SEM; p-values calculated using Student's t-test (p-values: n.s.>0.5, *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001, n = 3).

Figure S4: eCLIP-seq maps direct RBPMS targets in hESCs

(A) Representative western blot-based validation of immunoprecipitation performed during eCLIP of RBPMS, TUBA1B serves as the control.

(B) Representative eCLIP libraries, illustrated as tape-station readouts.

(**C**) Uniquely mapped reads for all eCLIP samples.

(**D**) Read density of eCLIP-Seq data indicating fold enrichment over respective SMIinputs.

(**E**) Correlation of enriched eCLIP-peaks over SMInput between each replicate.

(F) Top sequence motif significantly bound by RBPMS in the indicated category along with their distribution categorized based their binding on the mRNA coordinates for each replicate.

(G) Enriched GO terms (cellular component) found in RBPMS 3'UTR targets.

Figure S5: Transcriptional changes due to the loss of RBPMS do not explain the molecular and developmental defects of RBPMS-KO hESCs

(A) Functional annotation of genes affected transcriptionally alone upon loss of RBPMS does not suffice for the molecular and developmental defects due to the absence of RBPMS in hESCs.

(B) Ribosome occupancy of indicated 3'UTR targets of RBPMS between WT and *RBPMS*-KO hESCs. (**C**) Dynamics of indicated mRNAs on the indicated ribosomal platforms, bound by RBPMS on the 3'UTR. (**D**) Gene Ontology-based functional enrichment analysis for 3'UTR targets translationally activated in *RBPMS*-KO hESCs (significance level indicated by color code).

(**E**) Loss of RBPMS does not change the stability of indicated mRNAs, including RBPMS 3'UTR targets, revealed by time-course experiment upon Actinomycin B treatment.

Error bars represent ±SEM; p-values calculated using Student's t-test (n=3).

Figure S6: RBPMS loss leads to a depleted SFRP1 pool in hESCs

(**A**) Schematic depicting the fluorescence-based translation reporter constructs used. Bar graphs depicting GFP mean fluorescence intensity (MFI) of the translation reporters in indicated conditions measured 24 hours post transfection shown in lower panel.

(**B**) Levels of SFRP1 evaluated by western blot analysis in *RBPMS*-KO and WT hESCs, quantifications on the right.

(**C**) Loss of RBPMS does not influence the abundance of reporters lacking RBPMS binding sites serves as controls for translation reporters shown in Figure 6E.

Error bars represent ±SEM; p-values calculated using Student's t-test (n.s.>0.5, *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001, n = 3).

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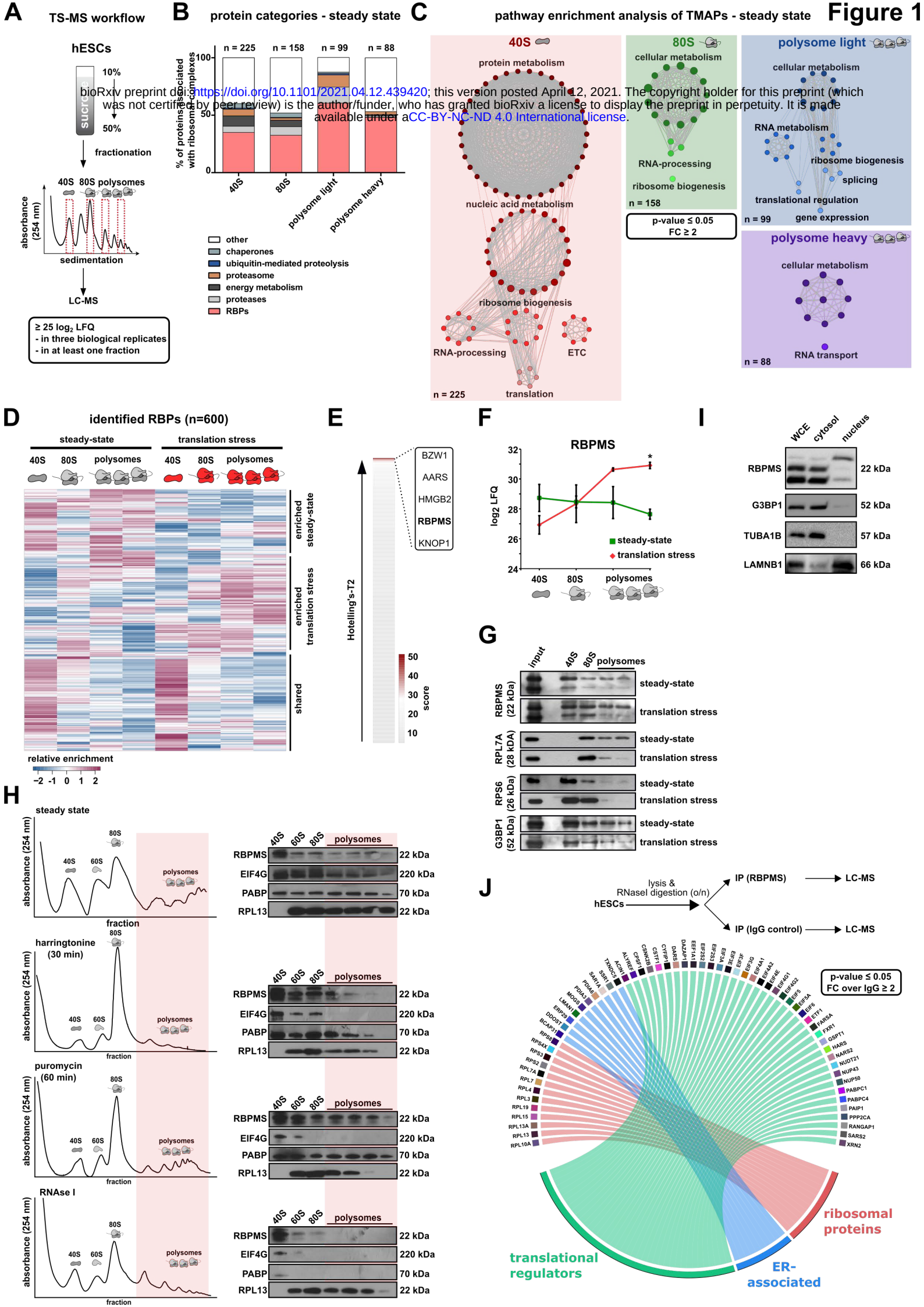
Figure 1



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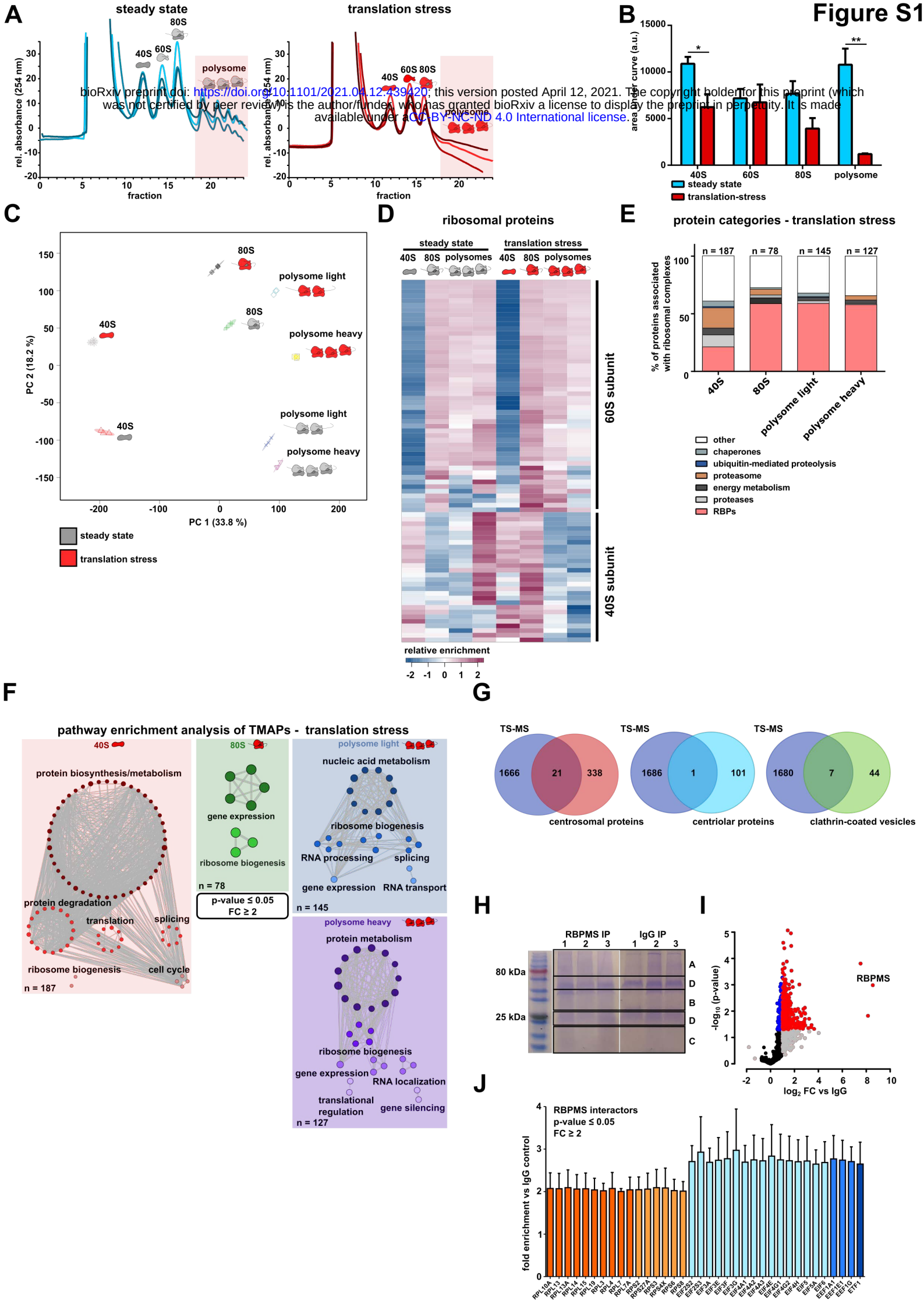

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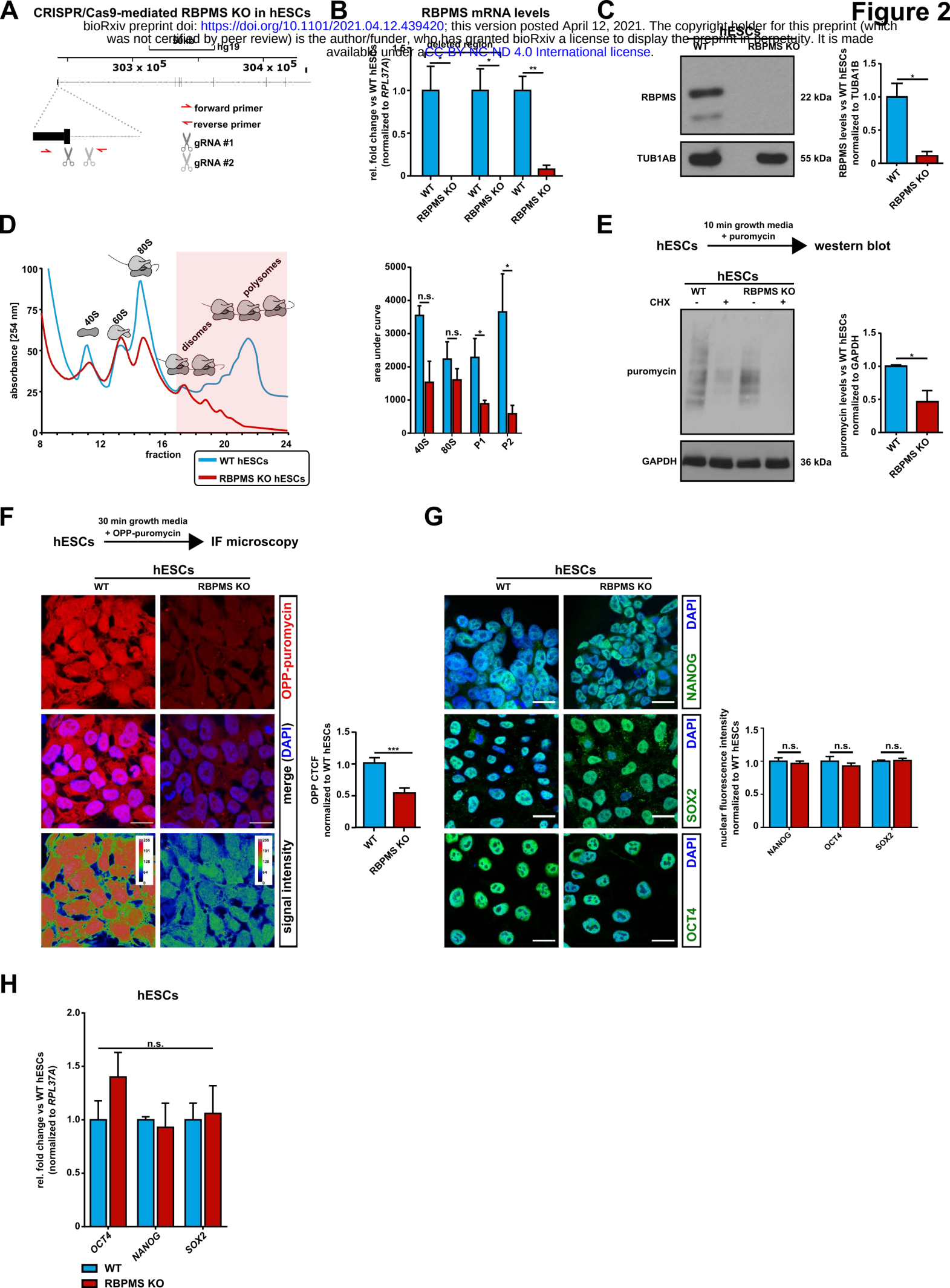

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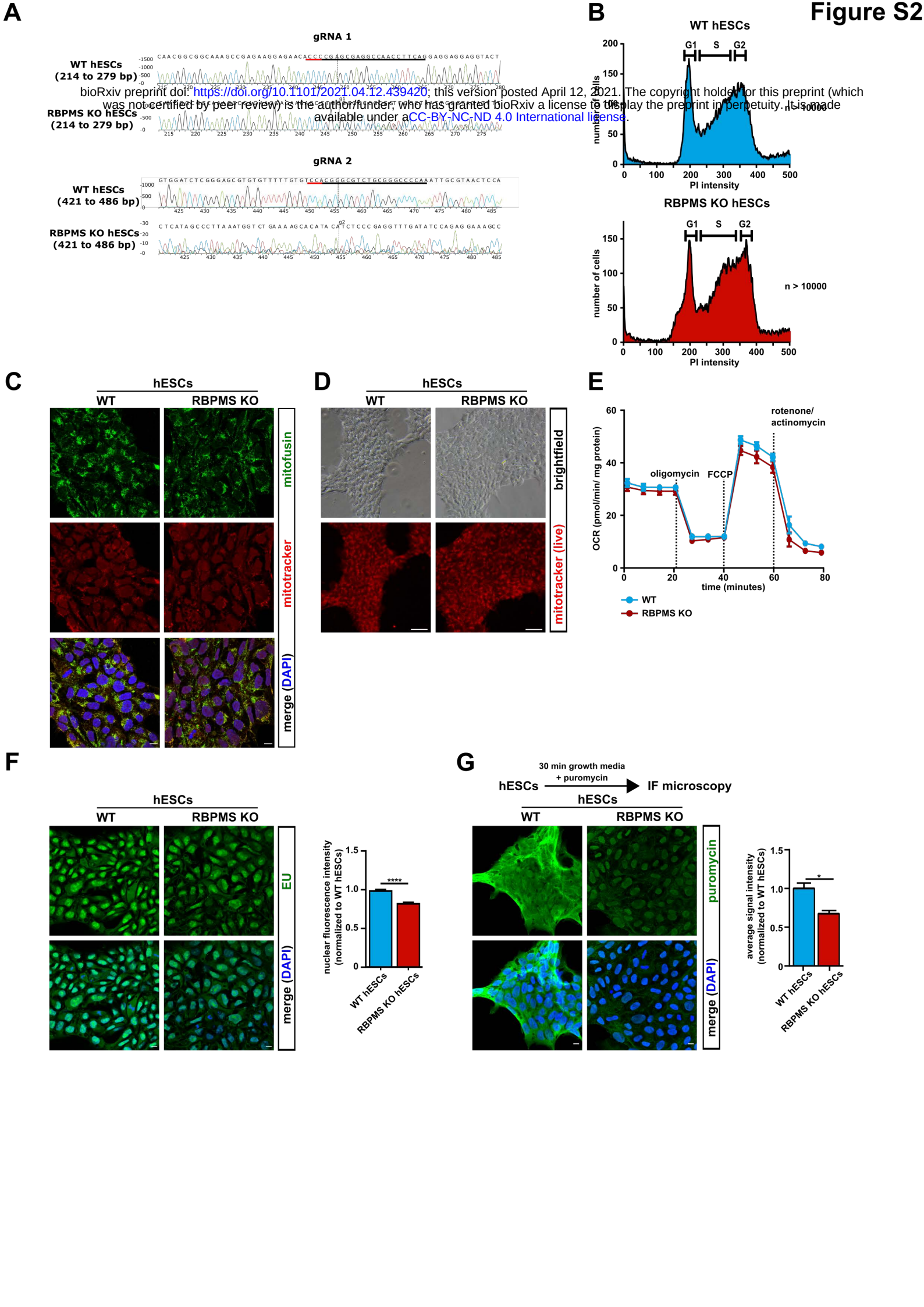

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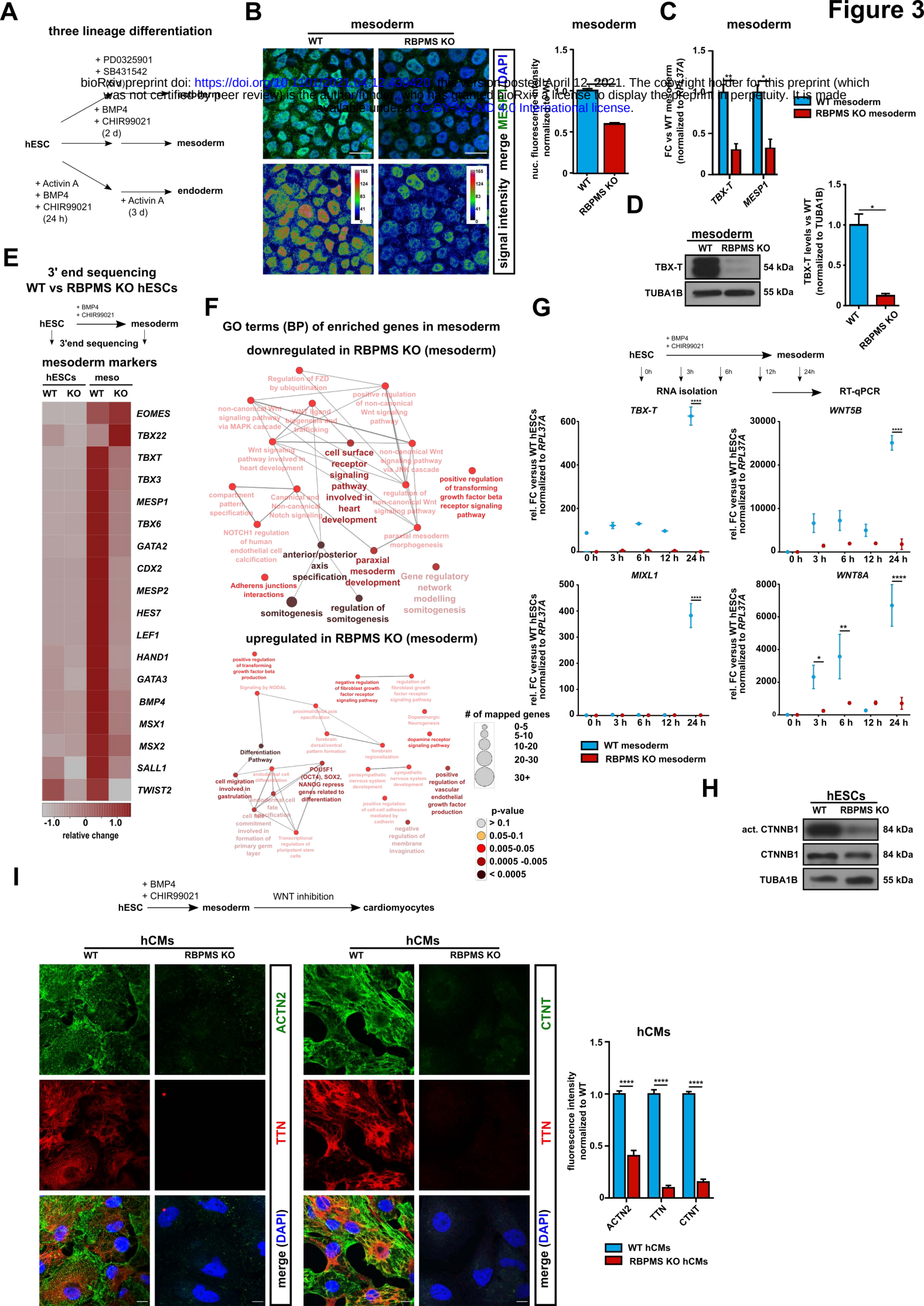

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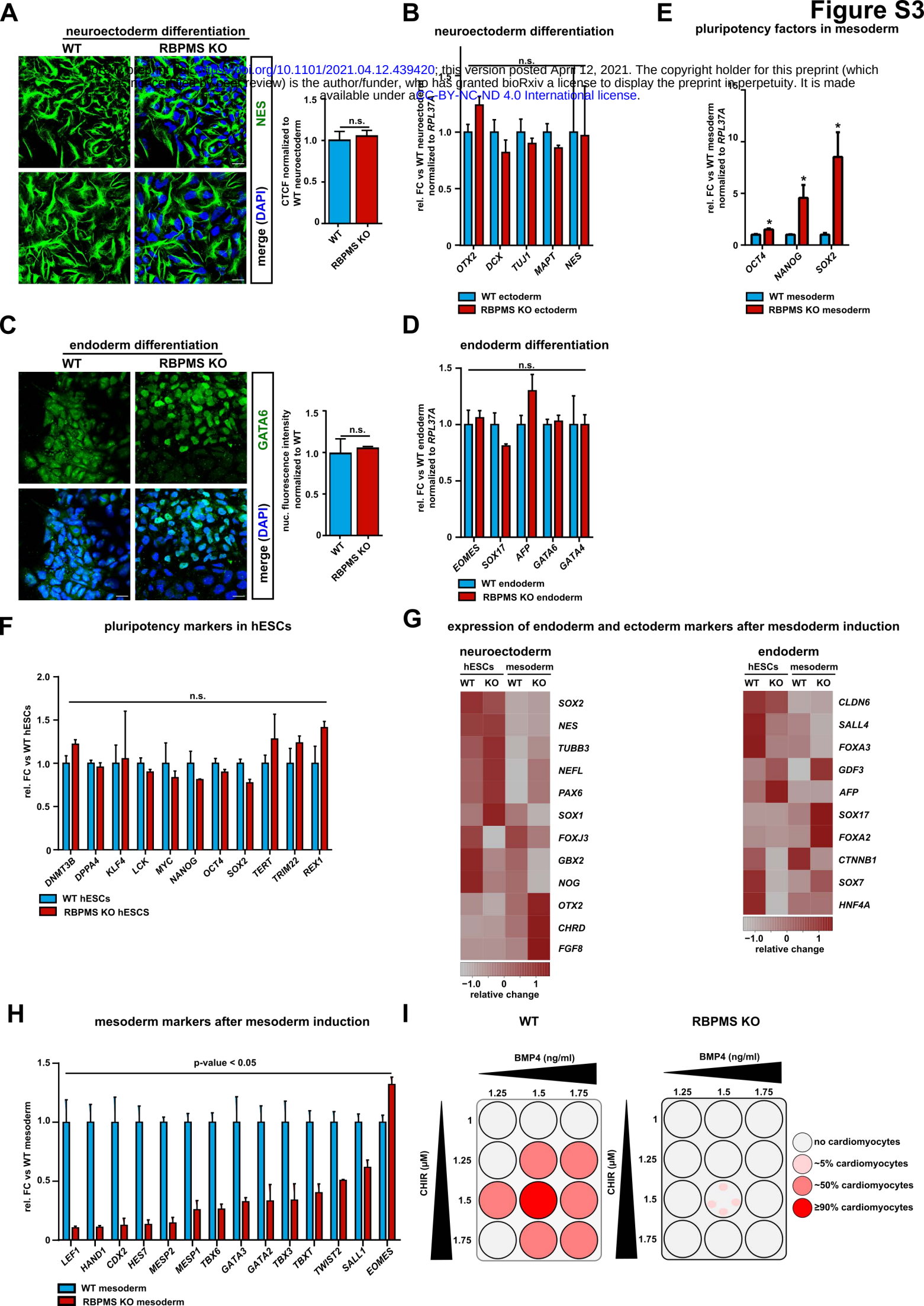

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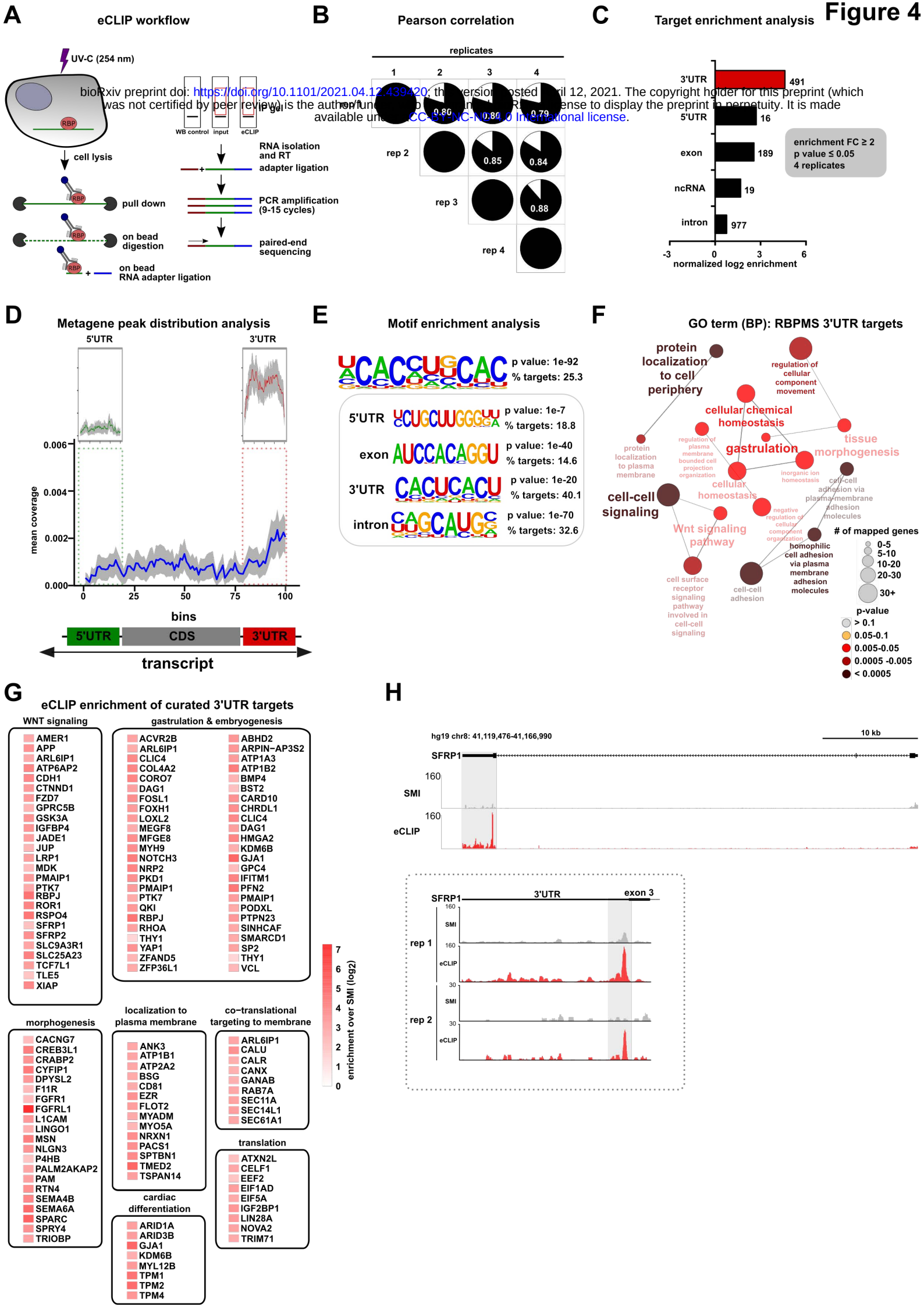

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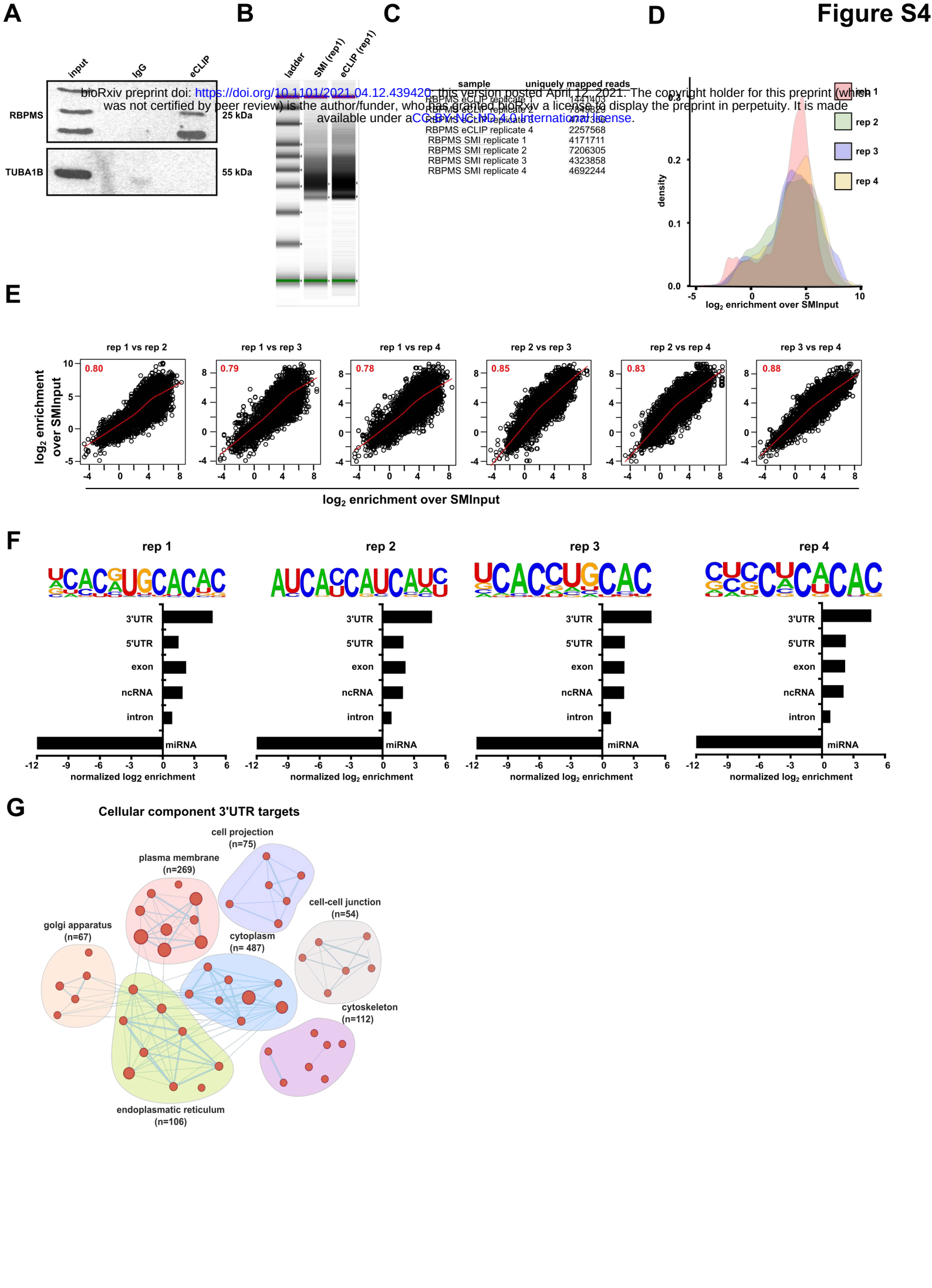

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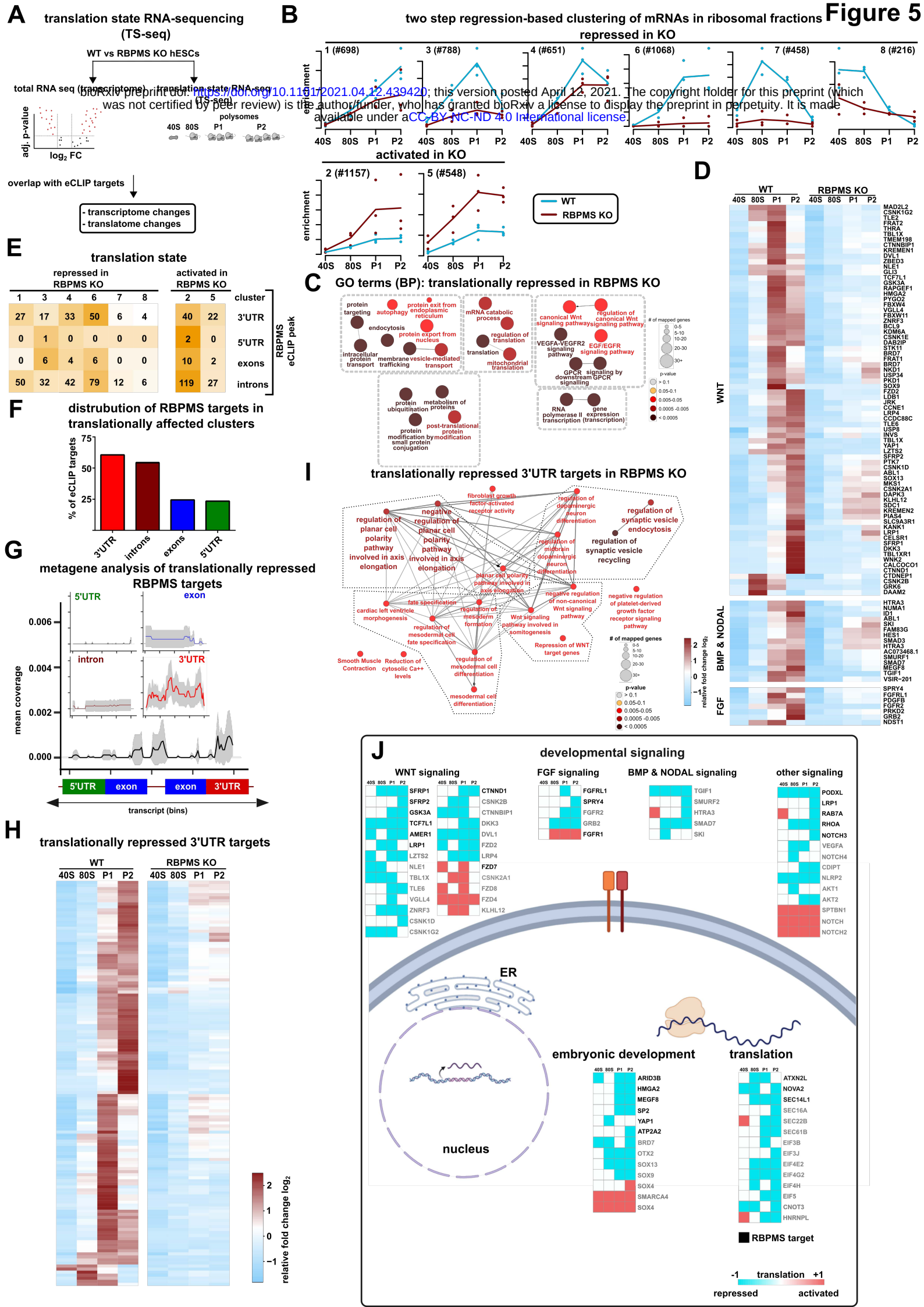

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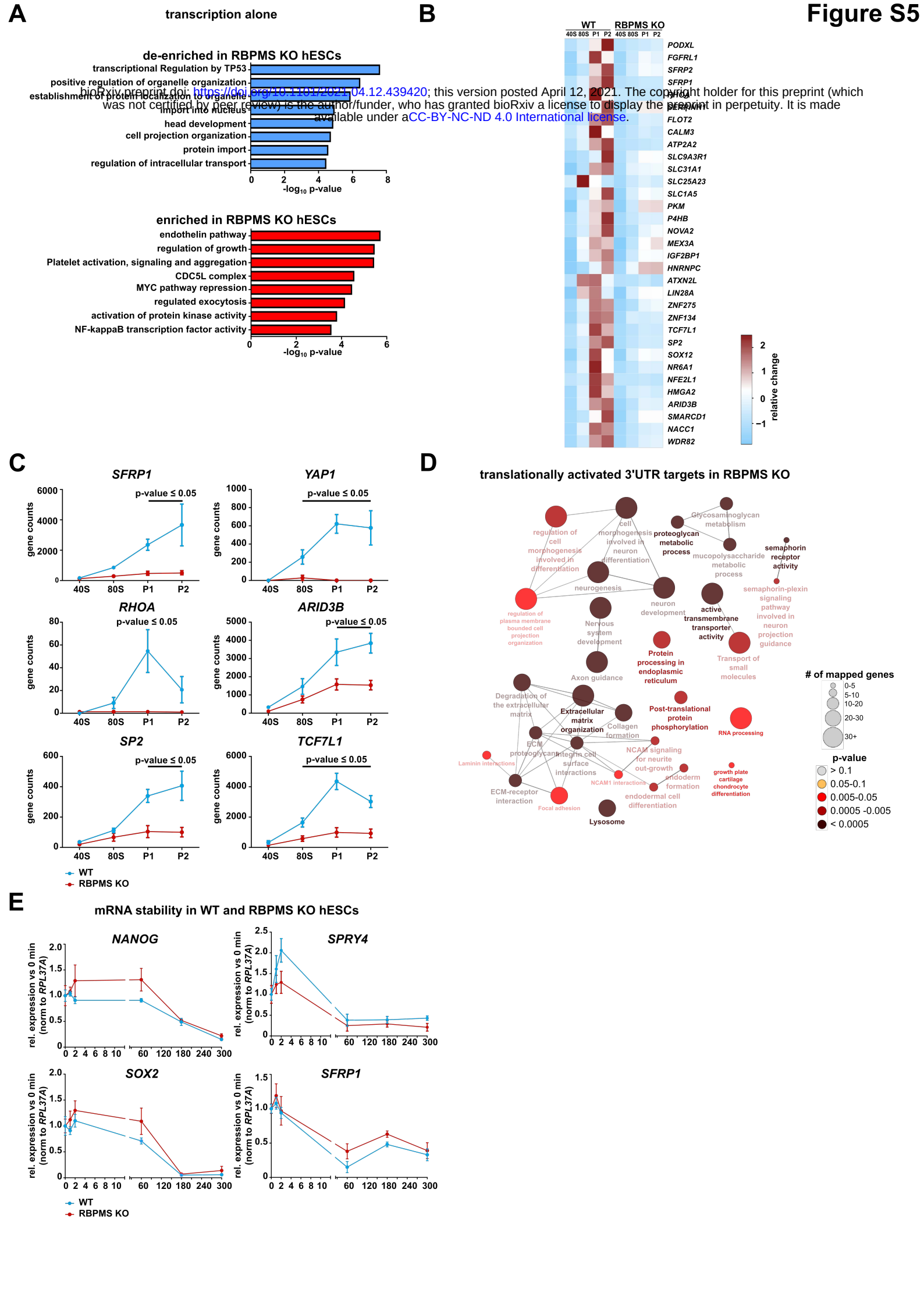

Figure S6