

Expanded View Figures

Figure EV1. Profiling and characterisation of small RNAs and tsRNAs in heterologous cell and tissue models of stem vs. differentiated states.

- A Expression of pluripotency genes in mESCs grown in stem or differentiating conditions ($n = 2$, error bars represent SD, and significance was calculated using Student's t -test).
- B Size distribution of the sequence reads from small RNA libraries that mapped to the genome.
- C Size distribution of the small RNA reads that map to the tRNA.
- D Correlation of tRNA gene copy number and tsRNA abundance. tRNA gene copy numbers for every anticodon are represented as percentages of the total tRNA copies on the genome.
- E Comparison of small RNA population changes in RA-induced differentiating cells treated with T4 PNK.
- F tsRNA profile of bulge stem cell ($CD34^+ \alpha 6^+$) or basal cell ($CD34^+ \alpha 6^+$) populations isolated from adult murine skin (see Appendix Fig S1).
- G Images of HMECs (Panels I–IV) engineered to represent stages of oncogenic transformation: I: parental HMECs immortalized with hTERT; II: immortalized HMECs with tamoxifen-inducible oncogenic HRAS (hTERT/ST/HRASG12V:ER/EV); III: HMEC as described in II with additional p53 knockdown (hTERT/ST/HRASG12V:ER/shP53); HMECs I–III were treated with 130 nM tamoxifen (5 days); and IV: HMEC II treated with tamoxifen (10 days).
- G' Quantification of 30–35 nt RNA levels in HMECs I–IV ($n = 3$, error bars represent SD, and significance was calculated using Student's t -test).
- H, I tsRNA profiles of stem ($CD44^+ CD24^-$) vs. differentiated ($CD24^+$) populations sorted from breast cancer cell lines: MDA-MB-231 (H) and HS578T (I) (see Appendix Figs S2 and S3).
- J Heat map of tsRNA reads mapped to parent tRNA residues across various samples sequenced in this study. Majority of the tsRNAs identified were processed specifically from the 5'-half of the mature tRNA.

Data information: * P value < 0.05, ** P value < 0.01; *** P value < 0.001; n.s, non-significant.

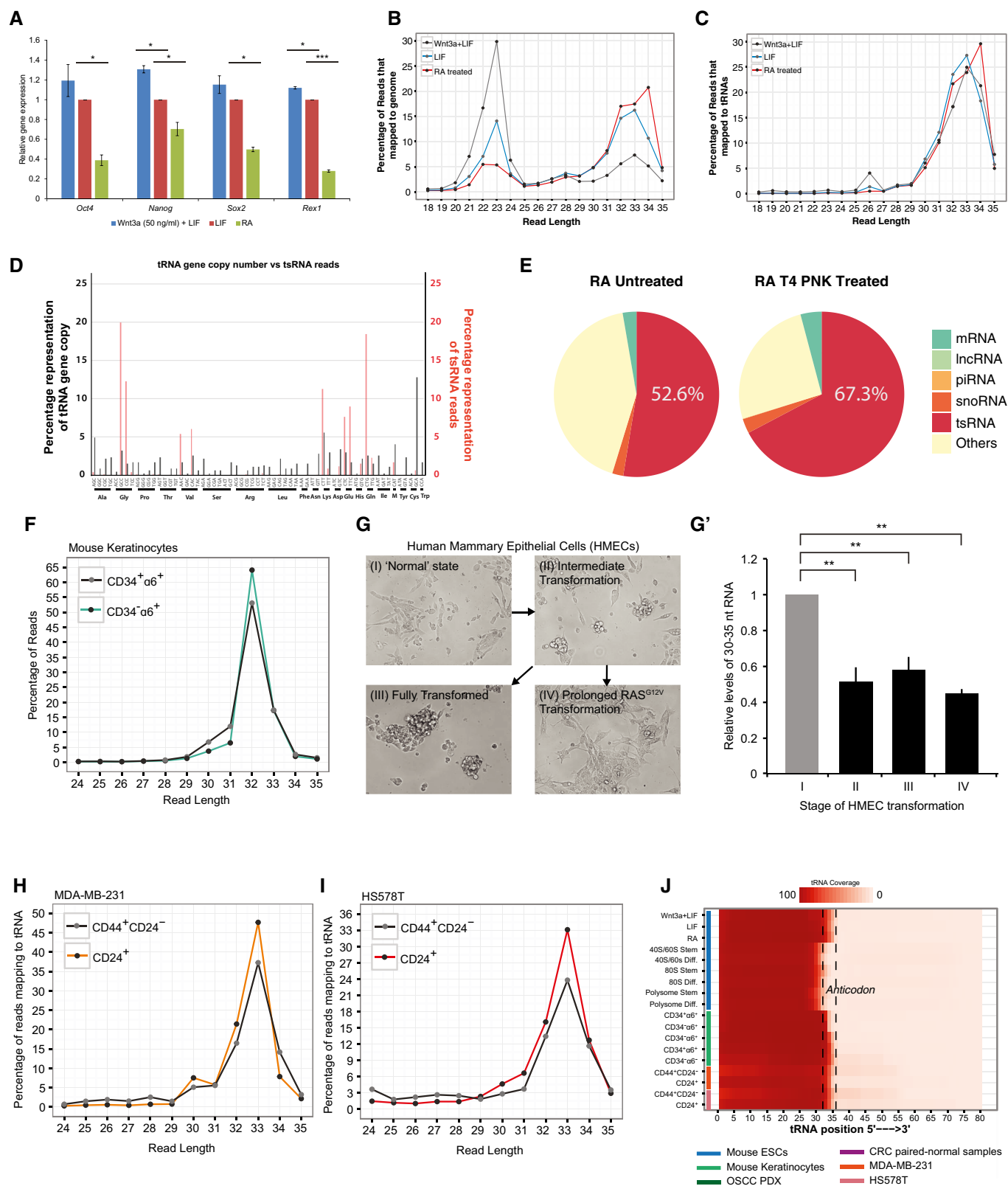


Figure EV1.

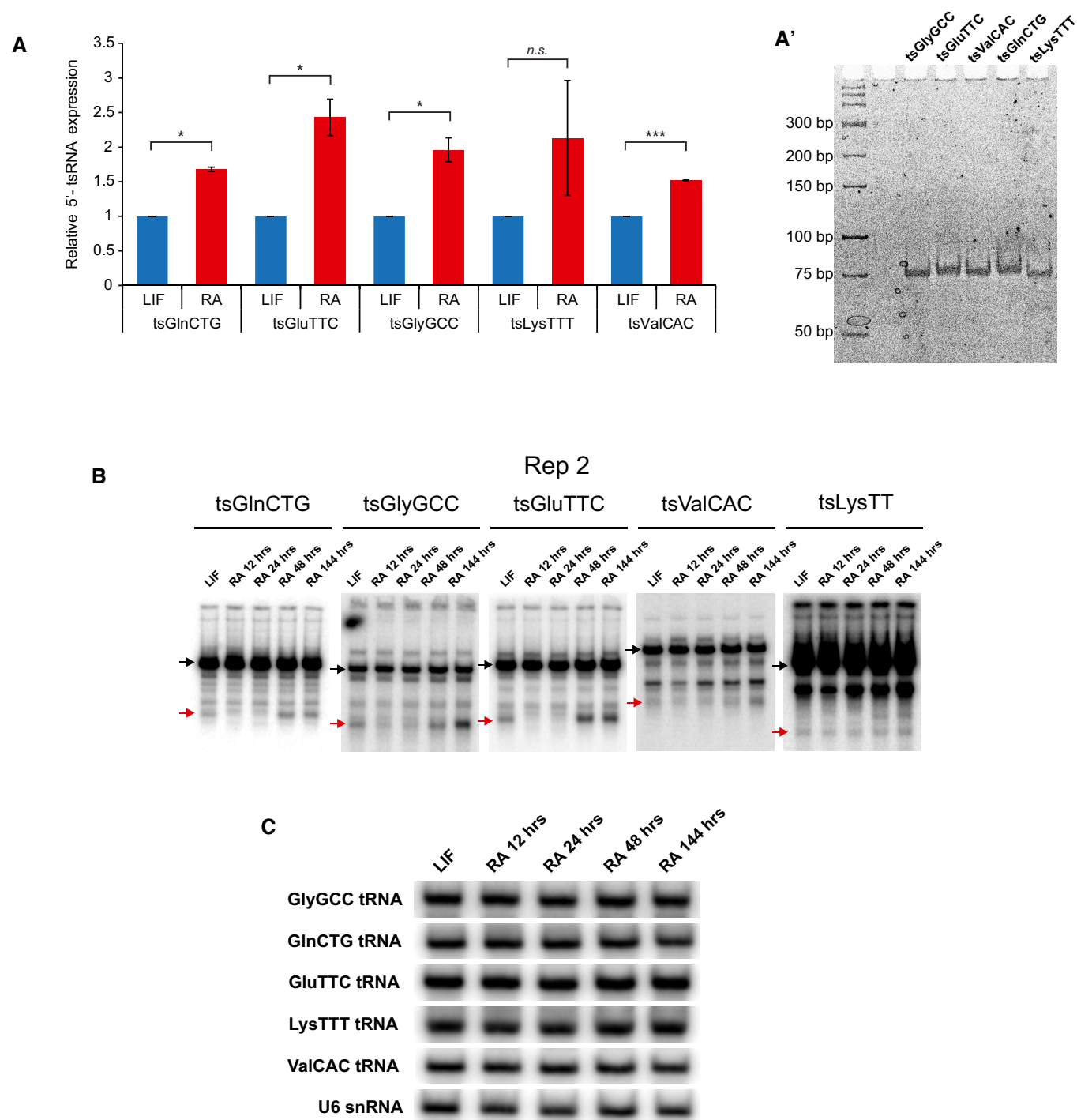


Figure EV2. Northern blot and qPCR validation of tsRNA expression in LIF and RA treated mESCs.

A qPCR validation of tsRNAs between LIF and RA at 48 h of differentiation ($n = 2$, error bars represent SD, and significance was calculated using Student's t -test).

A' Gel showing the tsRNA band amplified in the qPCRs.

B Replicate of northern blots of candidate tRNA and 5'-tsRNAs across time points of RA differentiation. Black arrows indicate tRNA species, and the red arrows indicate tsRNA species.

C Northern blot of parent tRNA across various time points of RA-induced differentiation.

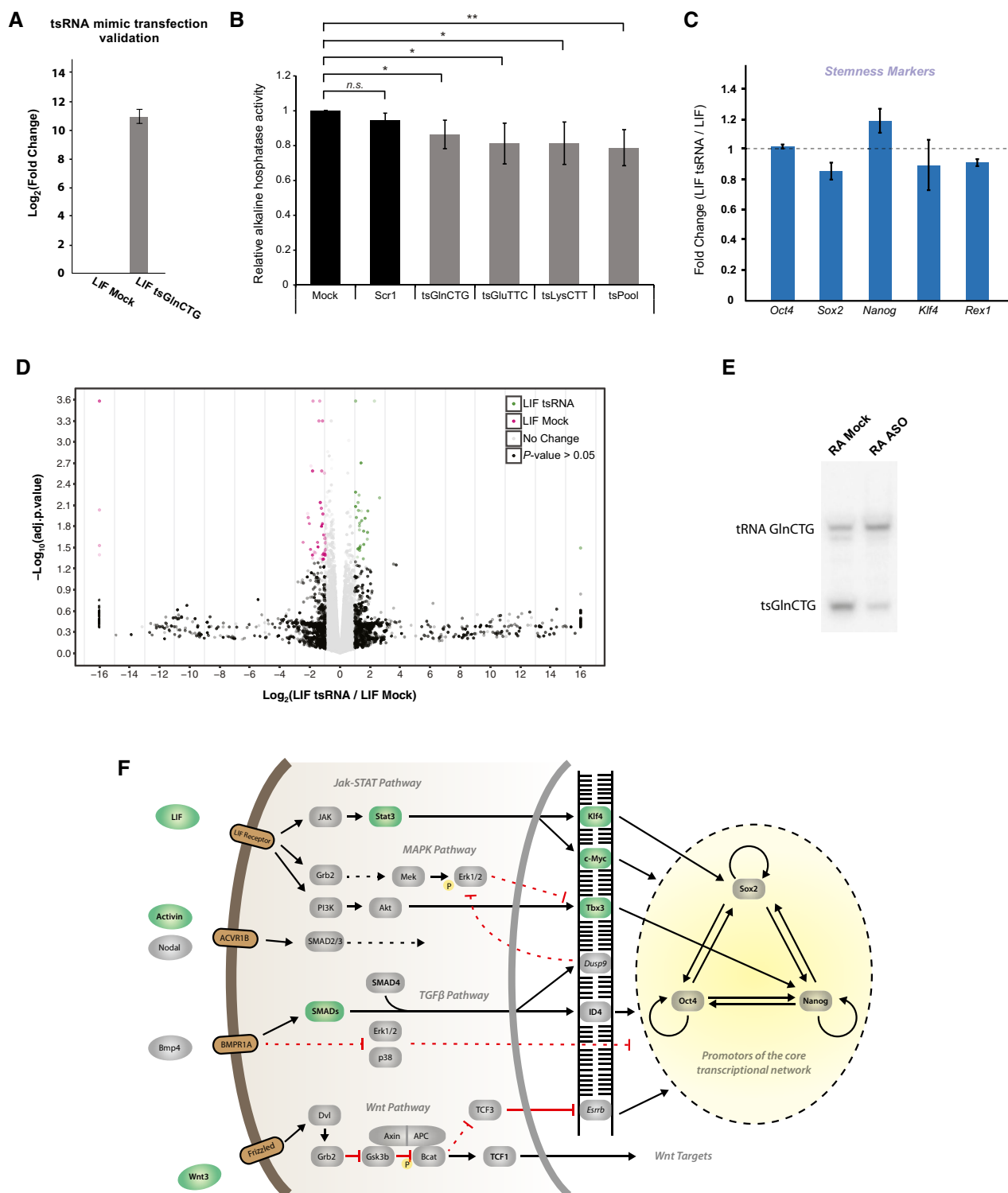


Figure EV3. Functional interrogation of tsRNAs in the modulation of stem and differentiated states in mESCs.

A qPCR quantitation of tsGlnCTG, transfected in LIF conditions ($n = 3$, error bar represent SEM, and significance was calculated using unpaired t -test).
 B Alkaline phosphatase assays of tsRNA mimic overexpression in LIF condition ($n = 3$, error bar represent SD, and significance was calculated using unpaired t -test).
 C Relative expression of stemness marker in tsRNA transfected in LIF conditions ($n = 2$, error bars represent SEM, and significance was calculated using unpaired t -test).
 D Volcano plot showing transcripts upregulated (in green) and downregulated (in pink) in LIF samples transfected with tsRNA mimics compared with mock controls.
 E Northern blot of tsGlnCTG in RA- vs. ASO-transfected RA cells.
 F Components of various stemness pathways upregulated (in green) in ASO-treated RA conditions.

Data information: * P value < 0.05 , *** P value < 0.001 , **** P value < 0.0001 .

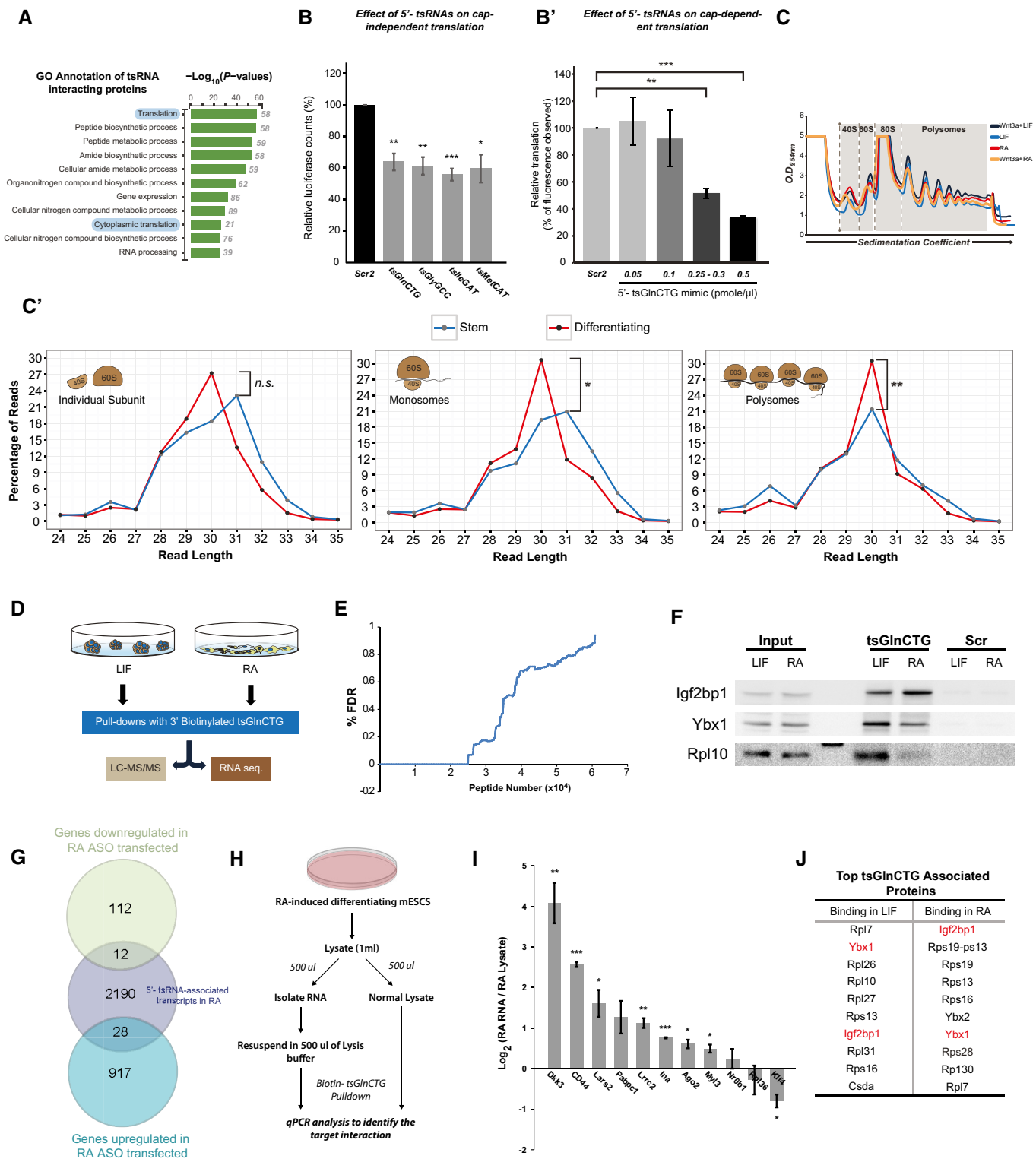


Figure EV4.

Figure EV4. Characterization of mechanism and molecular function of candidate tsRNAs in pluripotent vs. differentiating mESCs.

- A GO annotation of 109 common tsRNA-interacting proteins.
- B Effects of candidate 5'-tsRNAs: tsGlnCTG, tsGlyGCC, tsMetCAT, and tsIleGAT on uncapped luciferase mRNA in an *in vitro* translation system ($n = 2-3$, error bars represent SEM, and significance was calculated using unpaired *t*-test).
- B' Effects of tsGlnCTG on capped GFP mRNA in an *in vitro* translation system ($n = 2-3$, error bars represent SD, and significance was calculated using unpaired *t*-test).
- C Polysome profiles of different stem vs. differentiating states tested in this study. Gray areas indicate the fractions collected for sequencing.
- C' tsRNA distribution among the polysome fractions from indicated cell states.
- D Workflow to identify proteomic and transcriptomic interactions of tsGlnCTG in pluripotency vs. differentiating mESCs.
- E False discovery rate (FDR) of peptides detected in LC-MS/MS of biotinylated tsGlnCTG pulldown.
- F Western blot showing differential interaction of Igf2bp1, Ybx1, and Rpl10 with tsGlnCTG between LIF and RA conditions.
- G Overlap between transcripts that are associated with tsRNAs in RA condition and the transcripts that are affected upon ASO transfection in RA conditions.
- H Schematic of the experimental setup to identify protein-mediated and complementarity-based tsRNA target interaction.
- I qPCR analysis showing the enrichment of transcripts pulled down by tsGlnCTG in lysates devoid of proteins compared with normal lysate ($n = 2$, error bars represent SEM, and significance was calculated using unpaired *t*-test).
- J Top 10 tsGlnCTG-associated proteins in indicated cell states.

Data information: **P* value < 0.05, ***P* value < 0.01; ****P* value < 0.001; n.s., non-significant.

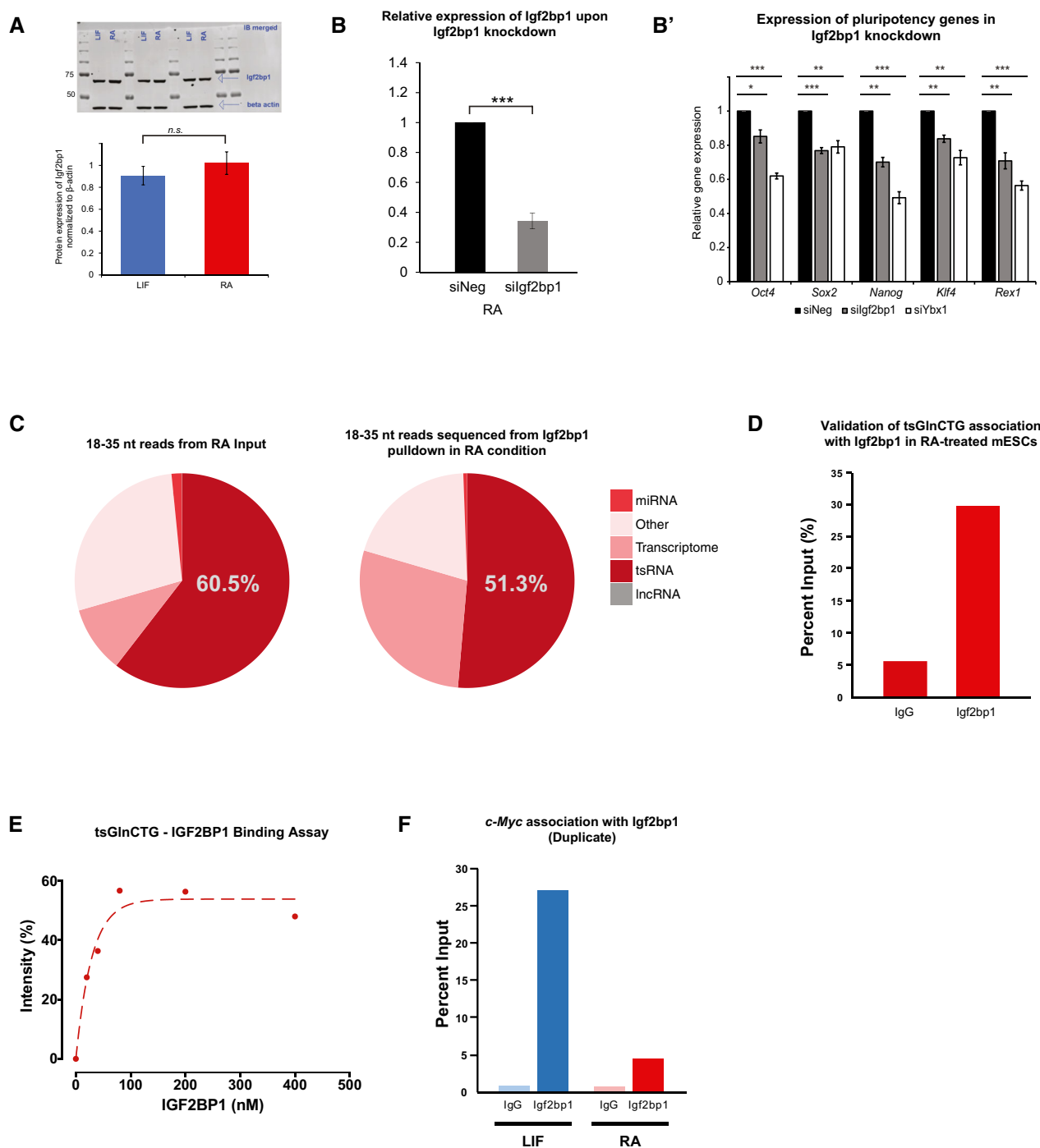


Figure EV5. Functional characterization of tsGlnCTG in the regulation of IGF2BP1 and c-Myc.

- A Merged triplicate Western immunoblots of Igf2bp1 and β -actin in mESCs grown under LIF or RA conditions (top) and densitometric quantitation of the same (below) ($n = 3$, error bars represent SD, and significance was calculated using Student's t -test).
- B Validation of siRNA-mediated knockdowns of Igf2bp1 in RA-treated mESCs ($n = 3$, error bars represent SD, and significance was calculated using Student's t -test).
- B' Expression of pluripotency genes in Igf2bp1 knockdown cells ($n = 3$, error bars represent SD, and significance was calculated using Student's t -test).
- C Small RNAs identified (18–35 nt) from input and Igf2bp1 pulldown in RA-treated mESCs.
- D qPCR validation of the tsGlnCTG enrichment upon Igf2bp1 pulldown in RA-treated mESCs.
- E Binding curve showing the interaction of tsGlnCTG and Igf2bp1 ($K_d = 33$ nM). The intensities of the Igf2bp1 band were normalized to band intensity of the total Igf2bp1 protein used for pulldown.
- F Biological duplicate for c-Myc quantitation from Igf2bp1 pulldown between LIF vs. RA conditions ($n = 2$, for duplicate check Fig 5B).

Data information: * P value < 0.05, ** P value < 0.01; *** P value < 0.001; n.s., non-significant.