Supplemental material

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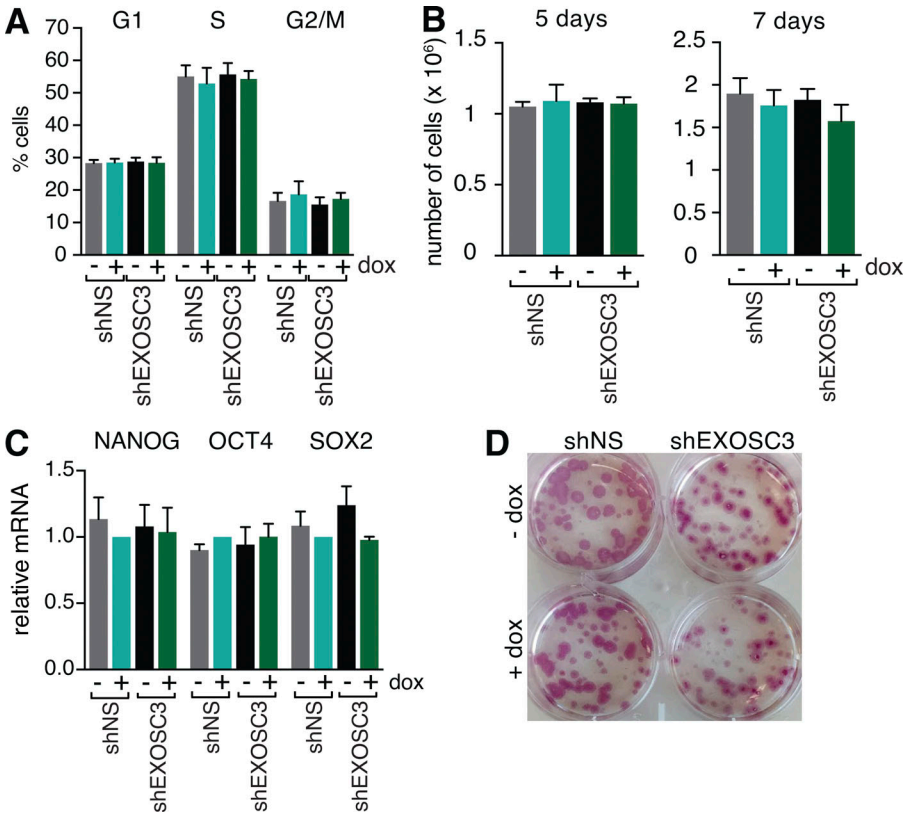

Figure S1. **Effects of EXOSC3 depletion on proliferation and pluripotency markers. (A)** After culturing shNS- and shEXOSC3-expressing cells with or without doxycycline for 7 d, BrdU incorporation was measured by flow cytometry. Data are presented as mean ± SEM (n = 3). **(B)** After culturing hESCs expressing the indicated shRNAs for 5 or 7 d, viable cells were counted. Data are presented as mean ± SEM (n = 3). **(C)** RT-qPCR analyses of OCT4, NANOG, and SOX2 expression in shNS- or shEXOSC3-expressing cells after culturing for 7 d in the presence or absence of doxycycline. Data are presented as mean ± SEM (n = 3). RNA levels are relative to shNS cells with doxycycline. **(D)** Alkaline phosphatase staining of shEXOSC3- and shNS-expressing cells after 7 d culture with and without doxycycline.

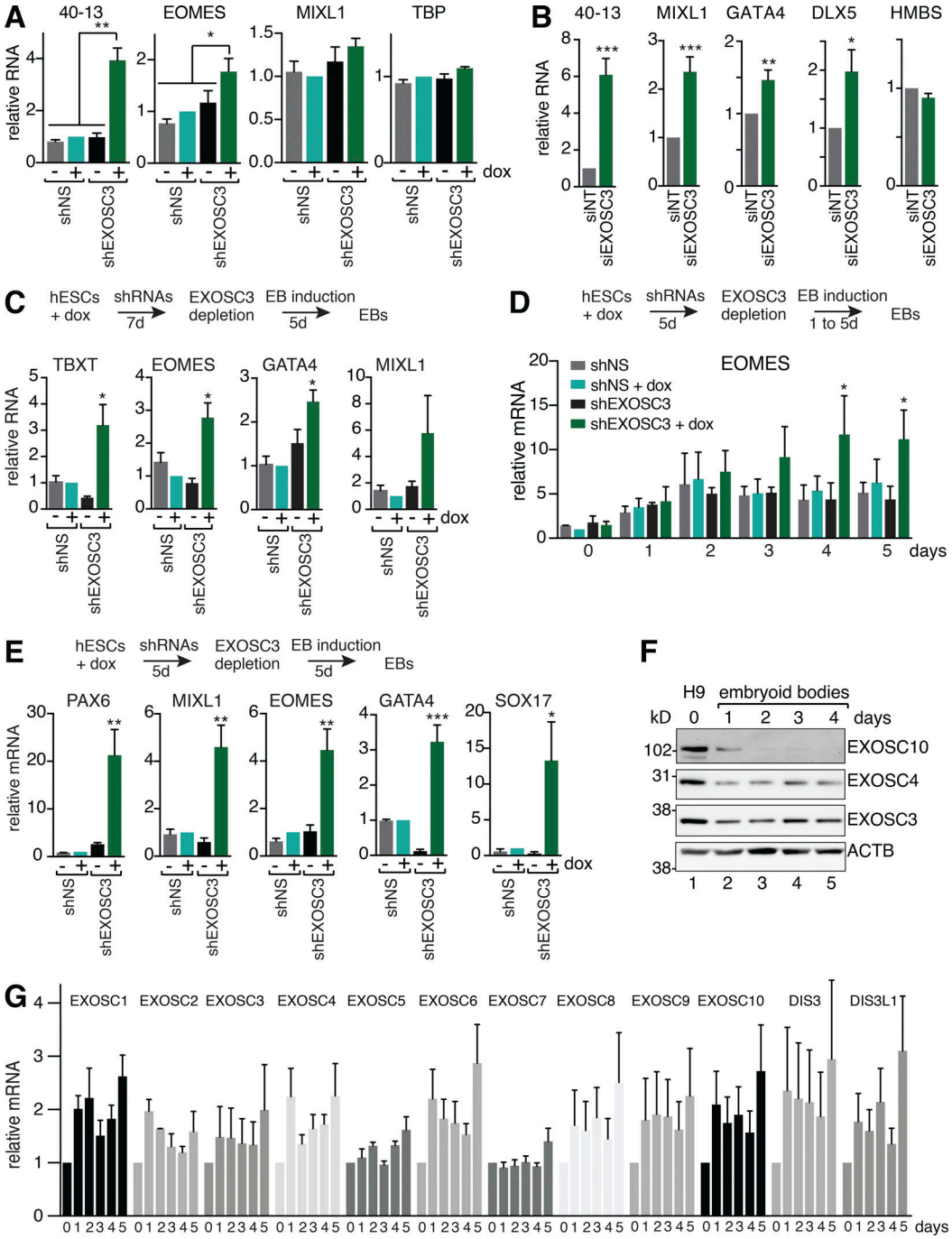

Figure S2. **The exosome is important for preventing differentiation. (A)** RT-qPCR analyses of 40–13 PROMPT, *EOMES*, *MIXL1*, and *TBP* RNA levels in shNS- or shEXOSC3-expressing cells after culturing for 7 d with or without doxycycline. Data are presented as mean ± SEM (n = 3). RNA levels are relative to shNS cells with doxycycline. **(B)** After treating hESCs with either nontarget (siNT) or an siRNA pool against *EXOSC3* mRNA, levels of the 40–13 PROMPT and mRNAs encoding MIXL1, GATA4, DLX5, and HMBS were measured with RT-qPCR. Data are presented as mean ± SEM (n = 7) relative to siNT-treated cells and normalized to *ACTB* mRNA. P values were calculated using two-tailed unpaired t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. **(C)** hESCs expressing shEXOSC3 or shNS RNAs were cultured for 7 d with or without doxycycline before inducing EB formation. After 5 additional days, EB RNA was assayed for the indicated markers using RT-qPCR. Data are presented as mean ± SEM (n = 3) of RNA levels relative to shNS cells cultured with doxycycline. *, P < 0.05. **(D)** Time course of *EOMES* mRNA induction. After culturing hESCs expressing shEXOSC3 or shNS RNAs for 5 d with or without doxycycline, EB formation was induced and the cells cultured for the indicated days. Extracted RNA was subjected to RT-qPCR. Data are presented as mean ± SEM (n = 3) of mRNA levels relative to shNS cells cultured with doxycycline at day 0. *, P < 0.05. **(E)** A second H1 hESC line expressing shEXOSC3 RNAs was cultured for 5 d with or without doxycycline. After inducing EB formation, cells were cultured for an additional 5 d. Afterward, RNA was extracted and qRT-qPCR performed to detect the indicated mRNAs. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data are presented as mean ± SEM (n = 3) of RNA levels relative to shNS cells cultured with doxycycline. **(F)** Levels of EXOSC10, EXOSC4, and EXOSC3 protein in WT H9 cells (lane 1) and EBs induced from these cells (lanes 2–5) were assessed by immunoblotting. ACTB, loading control. **(G)** After inducing H1 hESCs to differentiate to EBs (day 0), levels of mRNAs encoding the nine exosome core subunits and three catalytic subunits were measured using RT-qPCR during the 5 d of differentiation. Data are presented as mean ± SEM (n = 3) relative to hESCs at day 0.

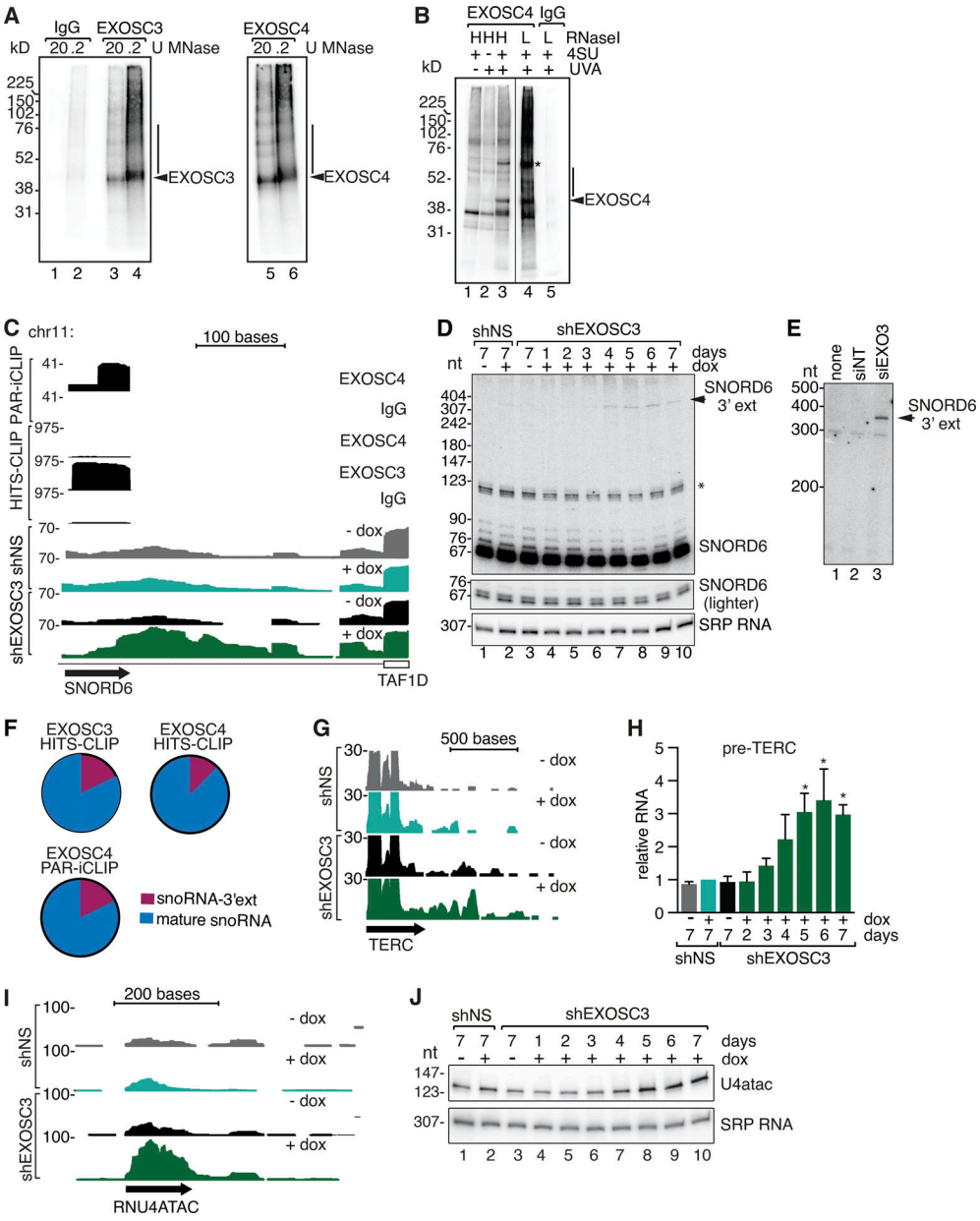

Figure S3. **Effects of exosome depletion on snoRNA, snRNA, and telomerase RNA targets. (A)** After UV cross-linking (254 nm) and immunoprecipitation, EXOSC3 (lanes 3 and 4) and EXOSC4 (lanes 5 and 6) RNPs were treated with the indicated units of micrococcal nuclease, 3′ linkers were ligated, and RNA was labeled with [γ-^{32}P]-ATP. After transferring to a membrane, RNPs were detected with autoradiography. Control reactions used nonimmune rabbit IgG (lanes 1 and 2). Bars show regions excised for cDNA. As the signals from EXOSC4 RNPs were more intense, lanes 5 and 6 were exposed separately. Both EXOSC3 (29.5 kD) and EXOSC4 (26 kD) migrate more slowly than expected due to the ∼7-kD 3′ linker and the fact that the exosome barrel protects ∼33 nt of RNA from nuclease digestion (Bonneau et al., 2009). **(B)** After labeling hESCs with 100 µM of 4-thiouridine for 7 h, PAR-iCLIP was performed and the cross-linked RNA fragments labeled with [γ-^{32}P]-ATP. Either 100 (L; lanes 4 and 5) or 500 units (H; lanes 1–3) of RNase I was added. A library was prepared from the region above the EXOSC4 band using 100 U RNase I (lane 4). A control library was prepared from the IgG lane (lane 5). Asterisk shows the band that may represent EXOSC9, which forms a stable dimer with EXOSC4 (Liu et al., 2006). **(C)** CLIP and RNA-seq reads aligning to the *SNORD6* locus were visualized on the University of Santa Cruz genome browser. RNA-seq libraries were prepared from cells grown with or without doxycycline for 5 d. Arrow indicates snoRNA genomic locus and orientation. **(D)** RNA extracted from shEXOSC3- (lanes 3–10) or shNS-expressing (lanes 1 and 2) cells grown in the presence (lanes 2 and 4–10) or absence (lanes 1 and 3) of doxycycline for the indicated days was subjected to Northern analysis to detect SNORD6. Arrowhead points to 3′ extended RNA. SRP RNA was a loading control. Asterisk shows nonspecific hybridization to 5S rRNA. **(E)** After transfecting hESCs with the indicated siRNAs, SNORD6 was detected by Northern blotting. To better resolve the 3′ extension, the gel in E was run further than in D. Arrow, 3′ extended *SNORD6* RNA. **(F)** HITS-CLIP and PAR-iCLIP reads mapping to mature snoRNAs and their 3′extensions. **(G)** RNA-Seq reads mapping to the TERC (telomerase RNA component) locus encoding telomerase RNA. Libraries were prepared from cells grown with or without doxycycline for 7 d. Arrow shows the TERC locus. **(H)** RT-qPCR was used to quantitate pre-TERC RNA levels in shEXOSC3- or shNS-expressing cells cultured with or without doxycycline for the indicated days. Data are presented as mean ± SEM (n = 3), normalized to *ACTB* mRNA, relative to shNS cells with doxycycline. *, P < 0.05. **(I)** RNA-Seq reads mapping to the *RNU4ATAC* locus. Libraries were prepared from cells grown with or without doxycycline for 7 d. Arrow, mature U4atac. **(J)** RNA extracted from shEXOSC3 (lanes 3–10) and shNS-expressing (lanes 1 and 2) cells was subjected to Northern blotting to detect U4atac snRNA. SRP RNA is a loading control.

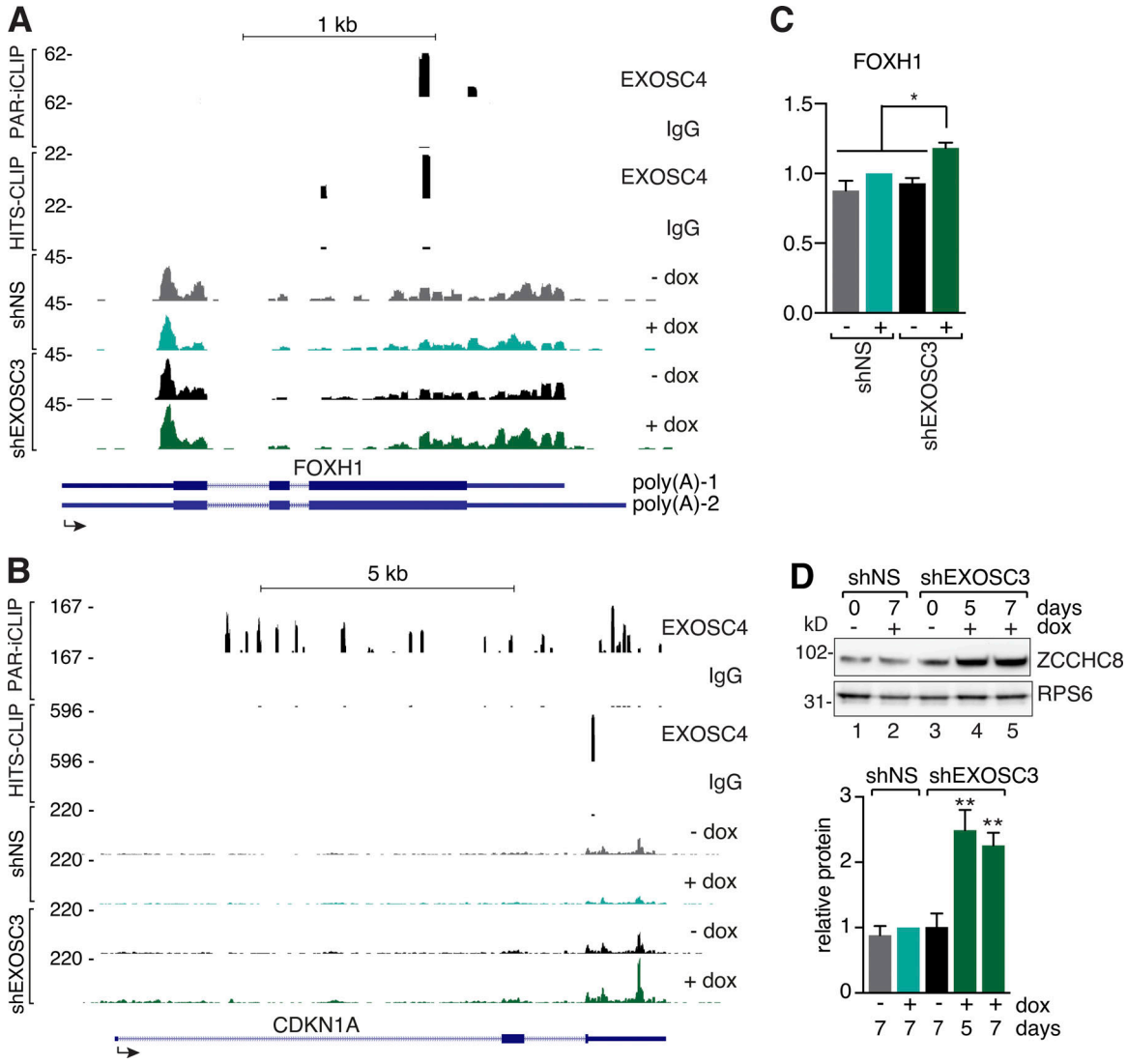

Figure S4. **The exosome regulates the levels of several mRNAs with roles in development and gene regulation. (A and B)** HITS-CLIP, PAR-iCLIP, and RNA-Seq reads aligning to *FOXH1* (A) and *CDKN1A* (B) were visualized on the UCSC genome browser. RNA-Seq libraries were prepared from cells grown with or without doxycycline for 7 d. Arrows indicate TSSs. **(C)** RT-qPCR quantitation of *FOXH1* mRNA in shNS- or shEXOSC3-expressing cells after culturing for 7 d with or without doxycycline. Data are presented as mean ± SEM (n = 3). RNA levels are relative to shNS-expressing hESCs with doxycycline. *, P < 0.05. **(D)** Western blotting was performed to detect ZCCHC8 in hESCs expressing shEXOSC3 (lanes 3–5) or nonsilencing (lanes 1 and 2) shRNAs grown with (lanes 2, 4, and 5) or without (lanes 1 and 3) doxycyline for 5 or 7 d. RPS6, loading control. Bottom: Quantitation of this blot and two biological replicates. Data are presented as mean ± SEM (n = 3). ZCCHC8 was normalized to RPS6 and compared with shNS-expressing hESCs with doxycycline. **, P < 0.01.

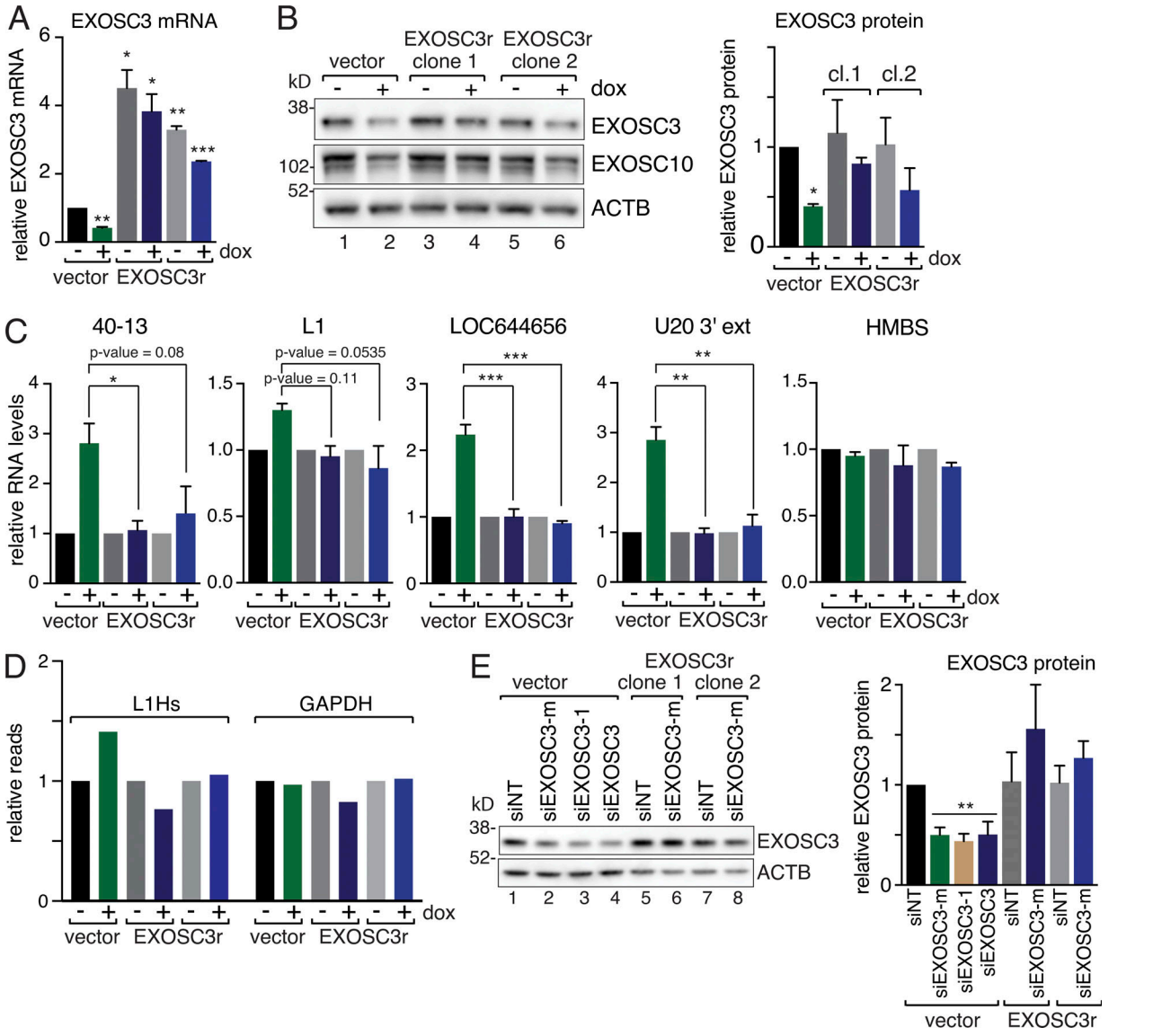

Figure S5. **Generation of hESCs expressing shRNA-resistant *EXOSC3* mRNA. (A)** *EXOSC3* mRNA was measured by RT-qPCR in H1 hESCs carrying either the empty vector or two independent clonal cell lines carrying the shRNA-resistant *EXOSC3* transgene (EXOSC3r) after 5 d of culture with or without doxycycline. Data are presented as mean ± SEM (n = 3). RNAs were normalized to *ACTB* mRNA, relative to hESCs carrying the empty vector without doxycycline. *, P < 0.05; **, P < 0.01; ***, P < 0.001. **(B)** Levels of *EXOSC3* and *EXOSC10* proteins were evaluated by immunoblotting in shEXOSC3-expressing H1 hESCs carrying an empty vector (lanes 1 and 2) or two shEXOSC3-expressing H1 hESCs clonal cell lines carrying the shRNA-resistant *EXOSC3* (EXOSC3r) transgene (lanes 3–6) after 5 d of culture with (lanes 2, 4, and 6) or without doxycycline (lanes 1, 3, and 5). ACTB, loading control. Right: Quantitation of three biological replicates. Data are presented as mean ± SEM (n = 3) compared with hESCs carrying the empty vector without doxycycline. *, P < 0.05. **(C)** Levels of the indicated RNAs in shEXOSC3-expressing H1 hESCs carrying an empty vector or two independent shEXOSC3-expressing H1 hESC clonal cell lines carrying the shRNA-resistant *EXOSC3* (EXOSC3r) transgene after 5 d of culture with or without doxycycline. Data are presented as mean ± SEM (n = 3). RNAs were normalized to *ACTB* mRNA, relative to the same cell line grown in the absence of doxycycline. *, P < 0.05; **, P < 0.01; ***, P < 0.001. **(D)** RNA-seq reads for L1Hs elements from shEXOSC3-expressing H1 hESCs carrying an empty vector or two independent shEXOSC3-expressing H1 hESC clonal cell lines carrying the shRNA-resistant EXOSC3 (EXOSC3r) transgene grown with or without doxycycline for 7 d. Read counts are normalized by the total reads in each library and are relative to the without doxycycline condition for each cell line. **(E)** shEXOSC3-expressing H1 hESCs carrying an empty vector (lanes 1–4) or an shRNA-resistant *EXOSC3* (EXOSC3r) transgene (lanes 5–8) were transfected with nontarget siRNAs (siNT, lanes 1, 5, and 7), an siRNA that mimics the mature shEXOSC3 RNA sequence (siEXOSC3-m, lanes 2, 6, and 8), the siEXOSC3-1 used previously (lane 3), or the pooled siRNAs against EXOSC3 (siEXOSC3, lane 4). *EXOSC3* protein was detected by immunoblotting. ACTB, loading control. Right: Quantitation of three biological replicates. **, P < 0.01.

Provided online are two tables in Excel. Table S1 reports the RNA-seq data for mRNAs and ncRNAs, HITS-CLIP and PAR-iCLIP tag counts, and PAR-iCLIP clusters. Table S2 lists the primers, oligonucleotide probes, antibodies, and siRNAs used in this study.

References

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