Supplementary Information

Title

Oncogenic RAS generates cancer stem cells in p53-deficient fibroblasts through SOX2 induced by CDK1-mediated protein O-GlcNAcylation

Authors

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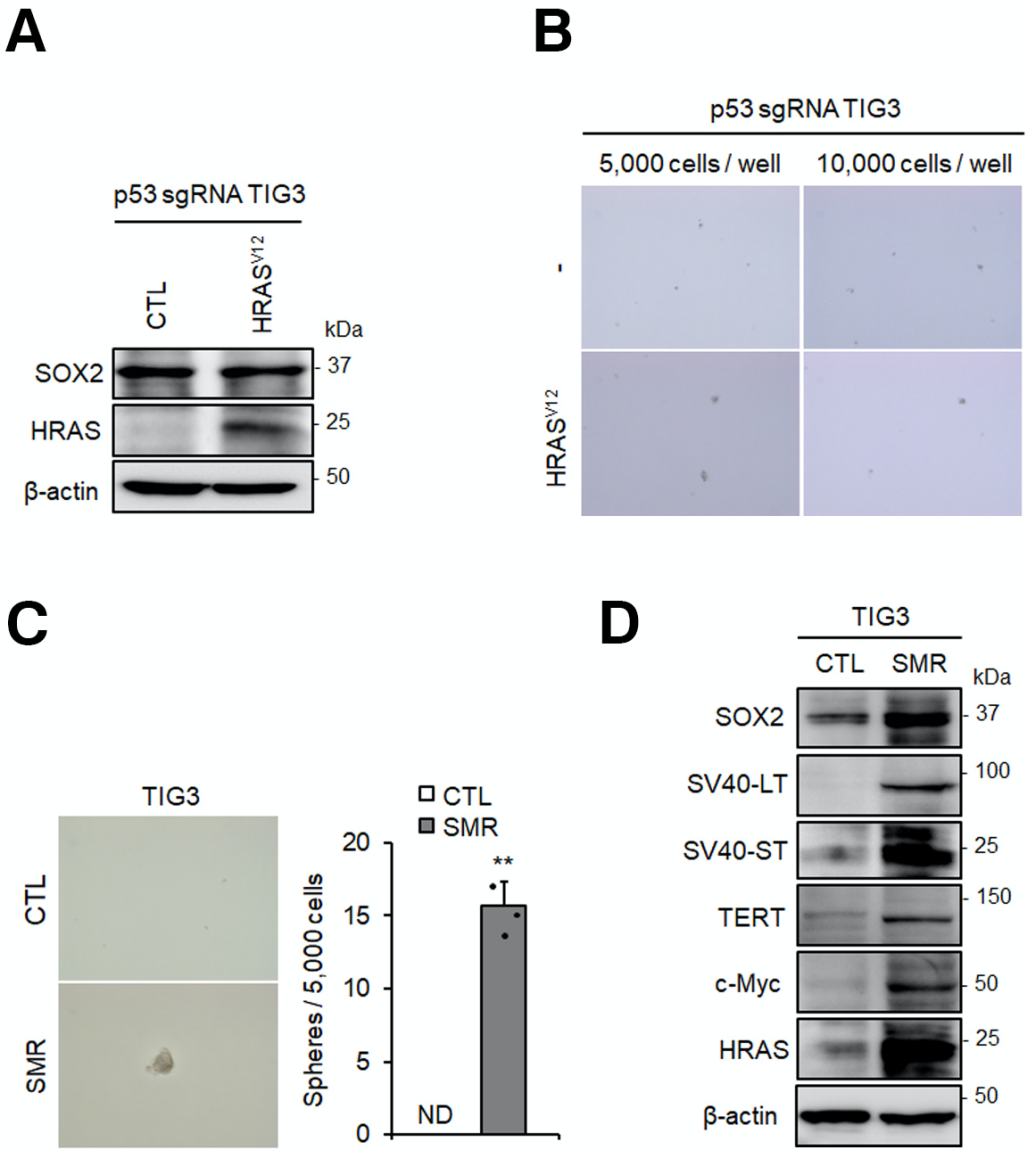
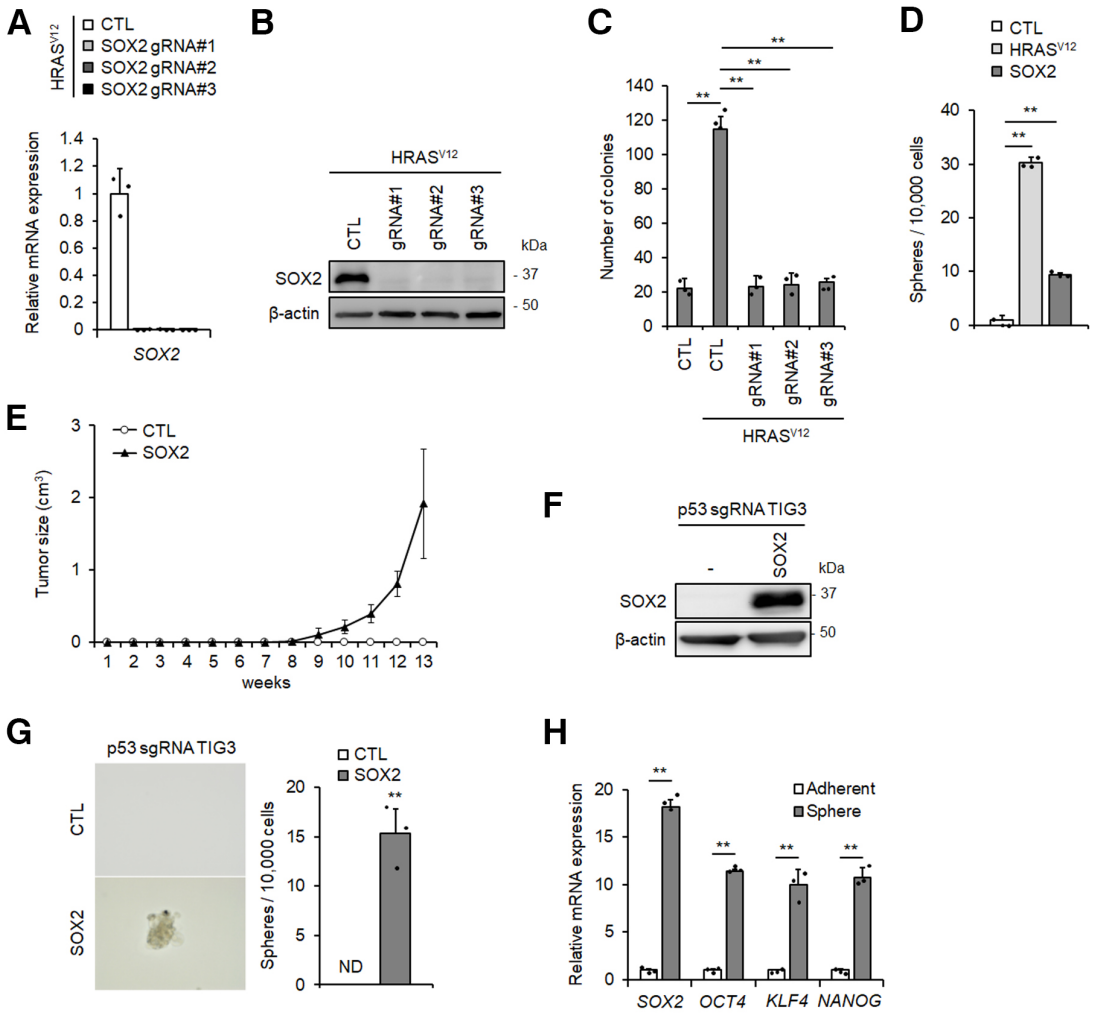
Supplementary Figures 1-5



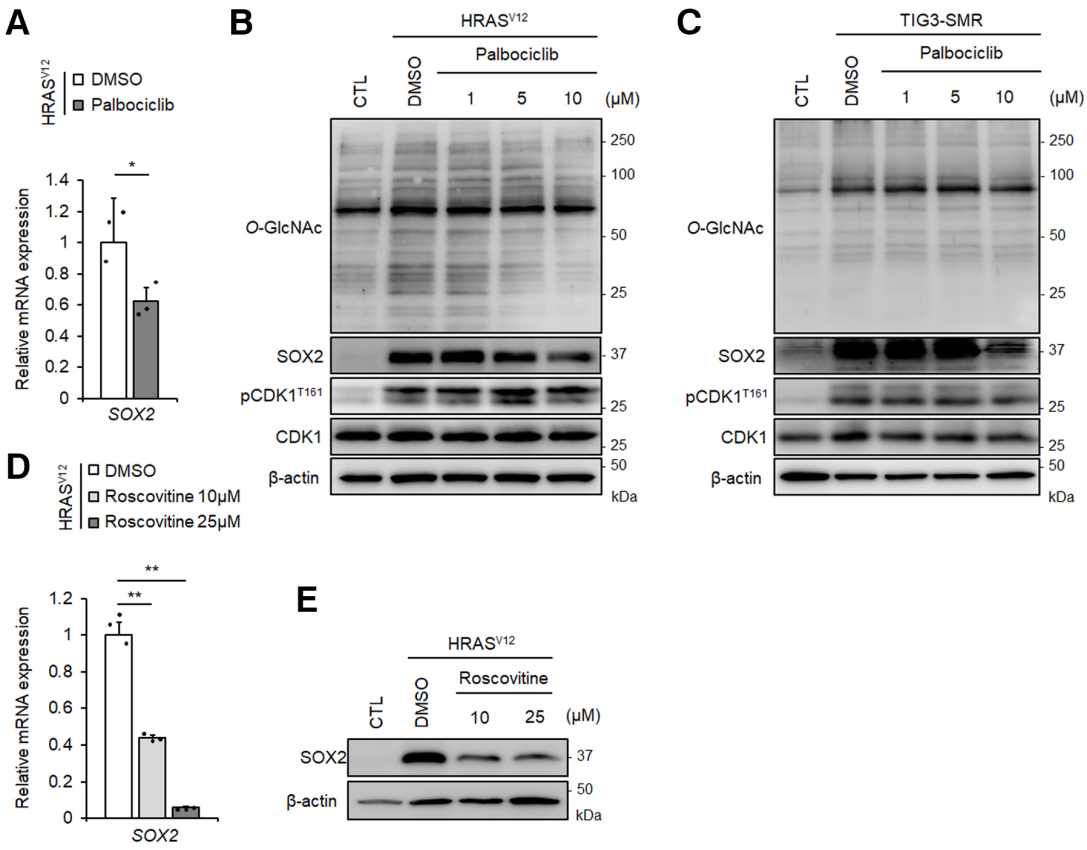
Supplementary Figure 1. Oncogenic mutations causing transformation are required for SOX2 expression and sphere formation in TIG-3 cells**. A**, p53-deficient human TIG-3 cells were generated by co-expressing p53 sgRNA with CRISPR and Cas9 protein using lentiviral infection for 2 days and selection with puromycin for 3 days. The $HRAS^{V12}$ mutant protein was stably expressed in these cells by retroviral infection for 2 days and selection with hygromycin for 3 days. Expression of HRAS and SOX2 was confirmed by immunoblot analysis of the *$HRAS^{V12}$*-expressing *p53* knockout TIG-3 cells. **B**, Sphere formation in *$HRAS^{V12}$*-expressing *p53* knockout TIG-3 cells. Representative images of spheres from 5,000 and 10,000 cells are shown. **C**, SV-40, c-Myc, and $HRAS^{V12}$ were stably expressed in TIG-3 (TIG-3–SMR) cells by retroviral infection for 2 days, and the cells were selected with blasticidin, neomycin, and hygromycin for 3 days.

Representative images of sphere formation in TIG-3–SMR cells are shown on the left; the graph on the right shows quantification of the numbers of spheres counted per 5,000 cells. Significance was confirmed by comparisons between the number of spheres in TIG-3 (CTL) and TIG-3–SMR cells. ND, none detected. $^{**}P < 0.01$. **D**, Western blot analysis of the indicated proteins in TIG-3–SMR cells. Uncropped blot images are presented in Supplementary Fig. S5.

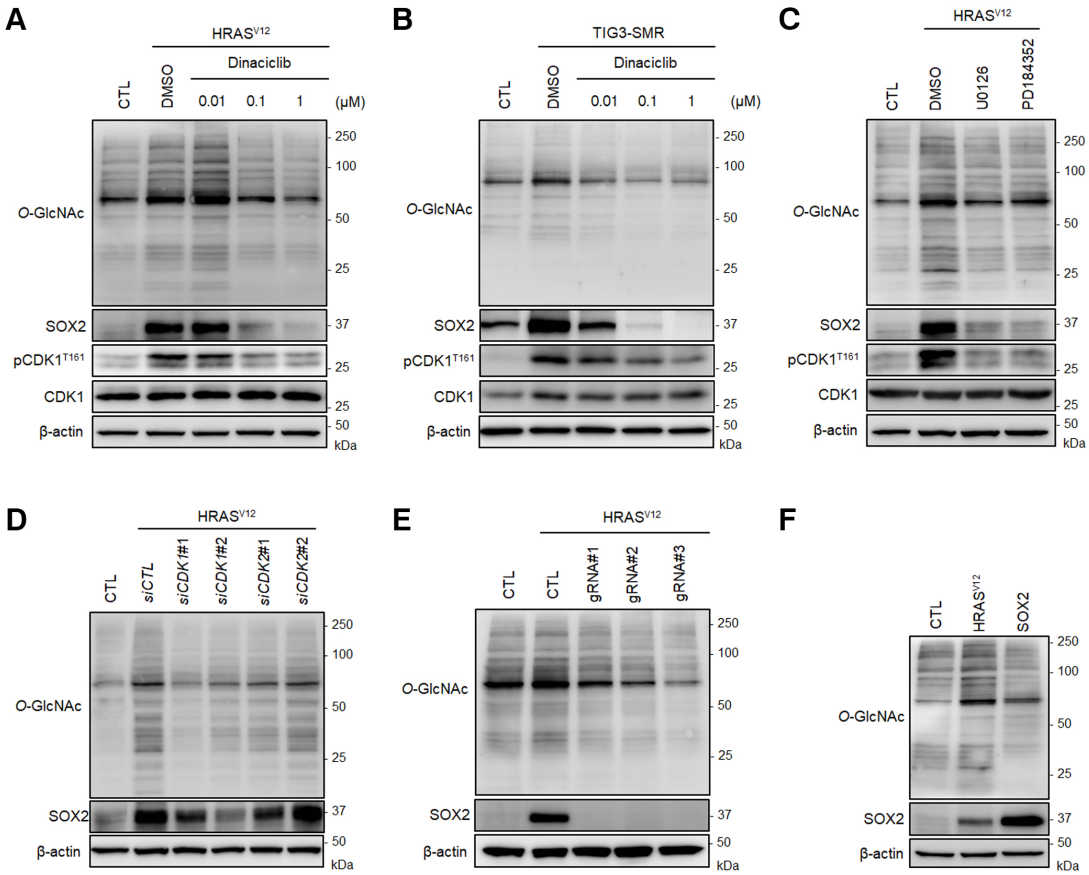


Supplementary Figure 2. SOX2 expression is required for colony, sphere, and tumour formation. **A** and **B**, *SOX2* sgRNAs were co-expressed with CRISPR and Cas9 proteins in *HRAS*^{V12}-expressing *p53*$^{-/-}$ mouse embryonic fibroblasts (MEFs). Expression of SOX2 was confirmed by qPCR analysis (A) and immunoblotting (B). **C**, Colony formation assay of *HRAS*^{V12}-expressing *p53*$^{-/-}$ MEFs containing each SOX2 sgRNA. $^{**}P < 0.01$. **D**, SOX2 was stably expressed in *p53*$^{-/-}$ MEFs by retroviral infection for 2 days, and cells were selected with puromycin for 3 days. The numbers of spheres formed from *HRAS*^{V12}- or *SOX2*-expressing *p53*$^{-/-}$ MEFs were counted after 7 days of culture. Significance was confirmed by comparison with the number of spheres from *p53*$^{-/-}$ MEFs expressing only the vector (CTL). $^{**}P < 0.01$. **E**, *p53*$^{-/-}$ MEFs (CTL) or *SOX2*-expressing *p53*$^{-/-}$ MEFs (1×10^6) were subcutaneously injected into immunodeficient mice (n = 5 per group). Tumour sizes were measured each week. **F**, SOX2 was stably expressed in *p53* knockout TIG-3 cells. SOX2 expression was confirmed by immunoblotting. **G**, Sphere formation of cells indicated in (F) after 7 days of culture. Representative images are shown on the left, and quantification

is shown on the right. ND, none detected. **$P < 0.01$. **H**, qPCR analysis of the stem cell marker genes *SOX2*, *OCT4*, *KLF4*, and *NANOG* in adherent and sphere-forming cells indicated in (G). **$P < 0.01$. A, C, D, G and H: Data are presented as the means ± SD of three independent experiments. Statistical analysis was performed with Student's t-tests. Uncropped blot images are presented in Supplementary Fig. S5.

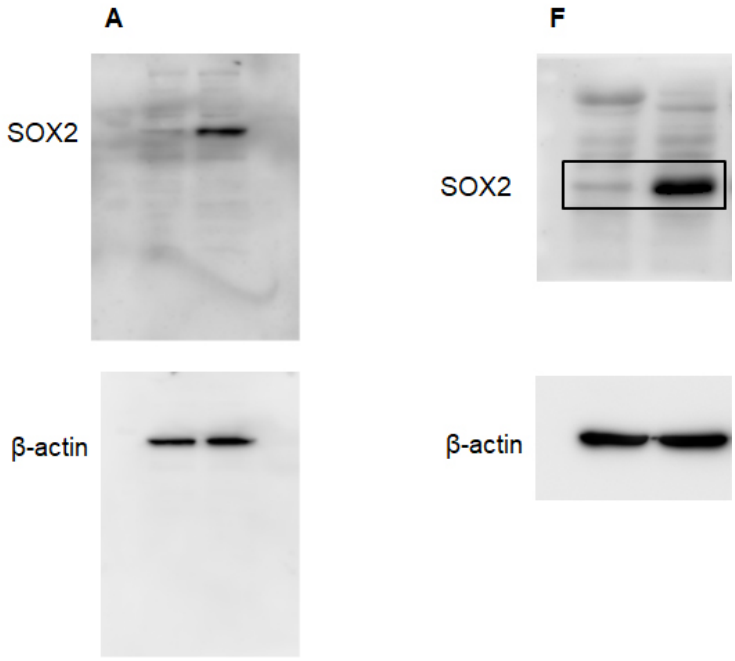


Supplementary Figure 3. CDK inhibitors suppress SOX2 expression.**A**, Expression levels of *SOX2* mRNA in *HRAS*^{V12}-expressing *p53*$^{-/-}$ mouse embryonic fibroblasts (MEFs) treated with palbociclib (10 μM) for 24 h. **P* < 0.05. **B** and **C**, Immunoblotting analysis of *O*-GlcNAc-modified proteins, SOX2 expression, and active pCDK1 in *HRAS*^{V12}-expressing *p53*$^{-/-}$ MEFs (B) and human TIG-3–SMR cells (C) treated with vehicle (DMSO) or palbociclib (1, 5, 10 μM) for 24 h. **D** and **E**, qPCR analysis (D) and immunoblotting (E) of SOX2 in *HRAS*^{V12}-expressing *p53*$^{-/-}$ MEFs treated with the CDK inhibitor roscovitine (10, 25 μM) for 24 h. ***P* < 0.01. A and D: Data are presented as the means ± SD of three independent experiments. Statistical analysis was performed with Student's t-tests. Uncropped blot images are presented in Supplementary Fig. S5.



Supplementary Figure 4. *O*-GlcNAc levels correlate with SOX2 expression. **A** and **B**, Western blot analysis of *O*-GlcNAc-modified proteins, SOX2 expression, and active pCDK1 in *HRAS*^{V12}-expressing *p53*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (A) and human TIG-3–SMR cells (B) treated with vehicle (DMSO) or dinaciclib (0.01, 0.1, 1 μM) for 24 h. **C**, Immunoblotting of *O*-GlcNAc-modified proteins, SOX2, and pCDK1 in *HRAS*^{V12}-expressing *p53*$^{-/-}$ MEFs treated with vehicle, U0126 (10 μM), or PD184352 (1 μM) for 24 h. **D**, Expression levels of *O*-GlcNAc-modified proteins and SOX2 in *HRAS*^{V12}-expressing *p53*$^{-/-}$ MEFs following knockdown of CDK1 or CDK2 by siRNAs. **E**, Immunoblotting assay of *O*-GlcNAcylation levels in *HRAS*^{V12}-expressing *p53*$^{-/-}$ MEFs containing each *SOX2* sgRNA. **F**, Expression levels of *O*-GlcNAc-modified proteins in *HRAS*^{V12}-expressing *p53*$^{-/-}$ MEFs and *SOX2*-expressing *p53*$^{-/-}$ MEFs. Uncropped blot images are presented in Supplementary Fig. S5.

Supplementary Figure 5. The original images in all Figures and Supplementary Figures.



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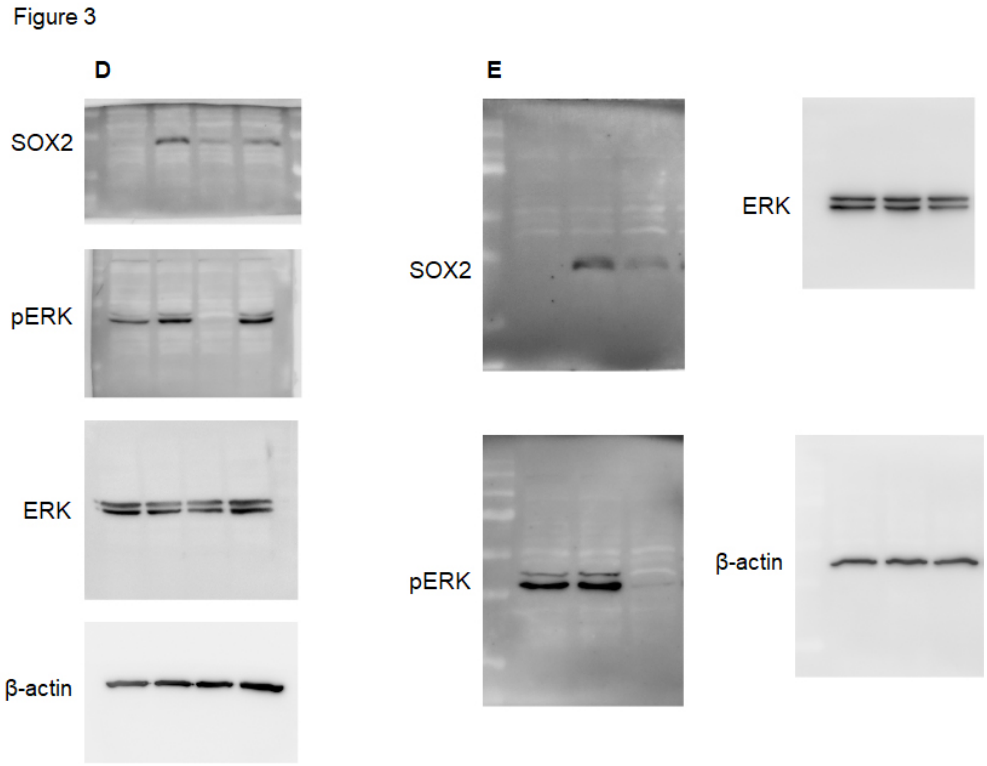

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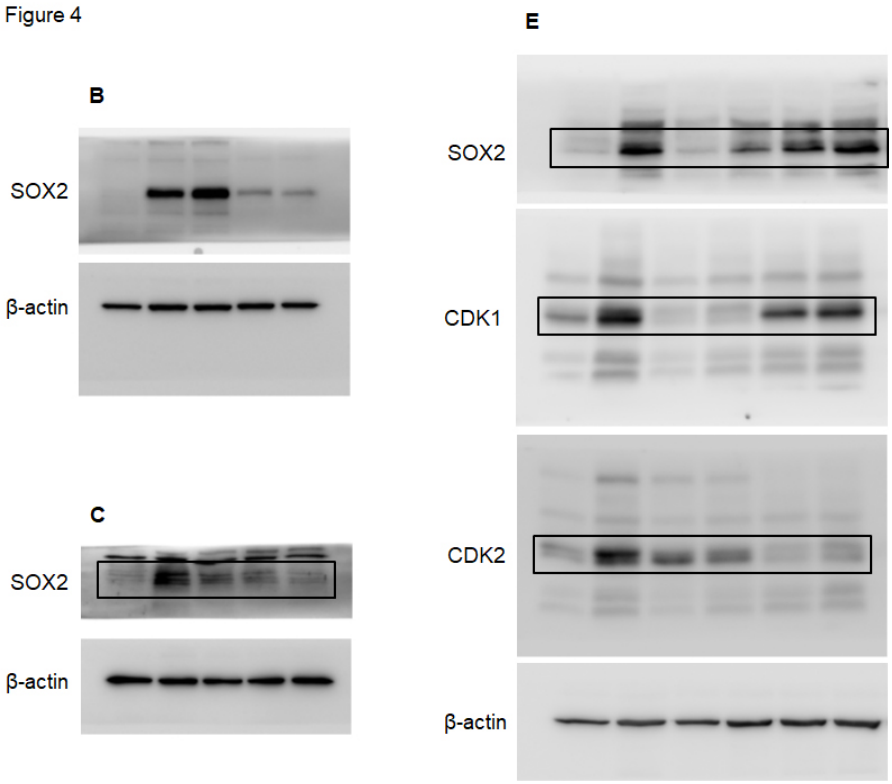

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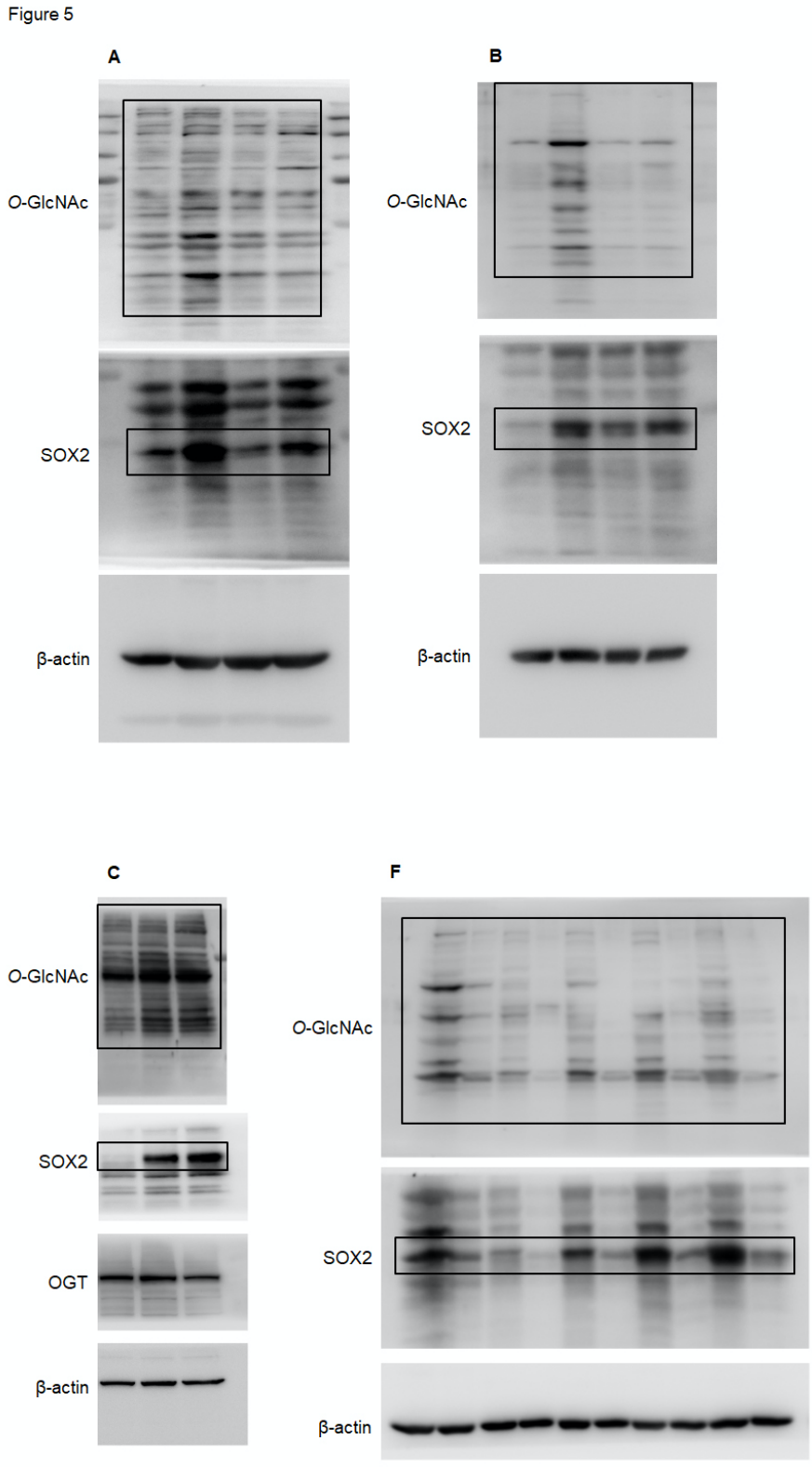

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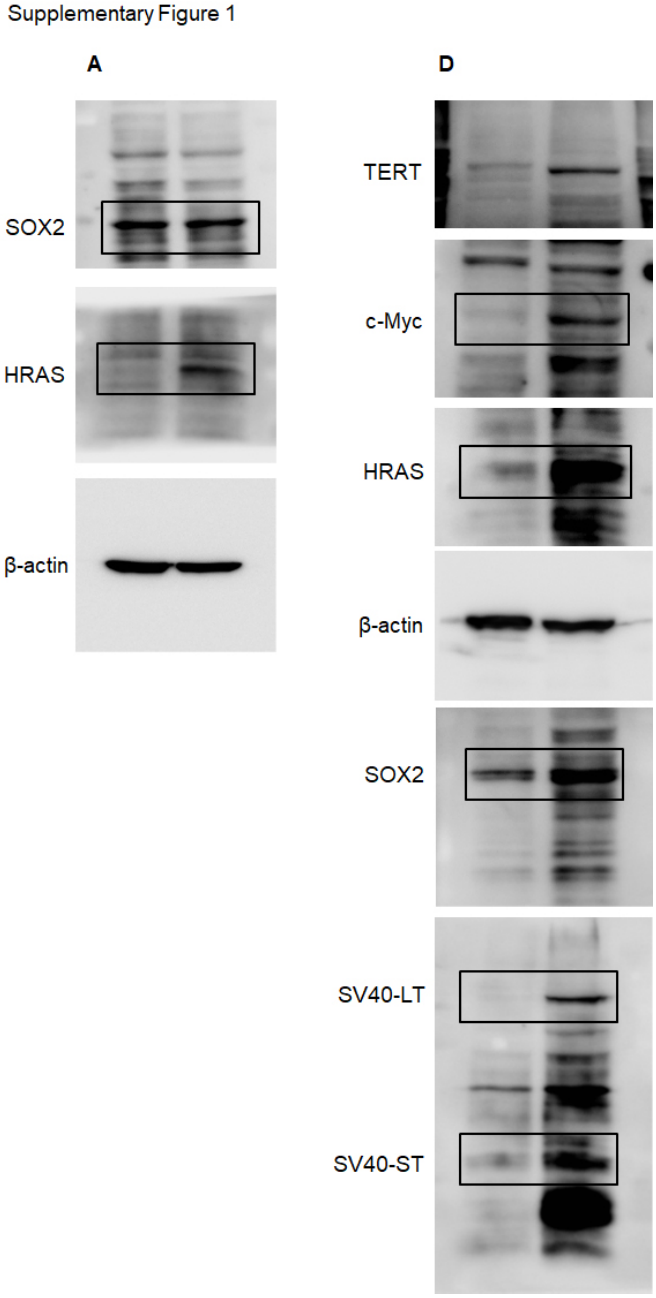

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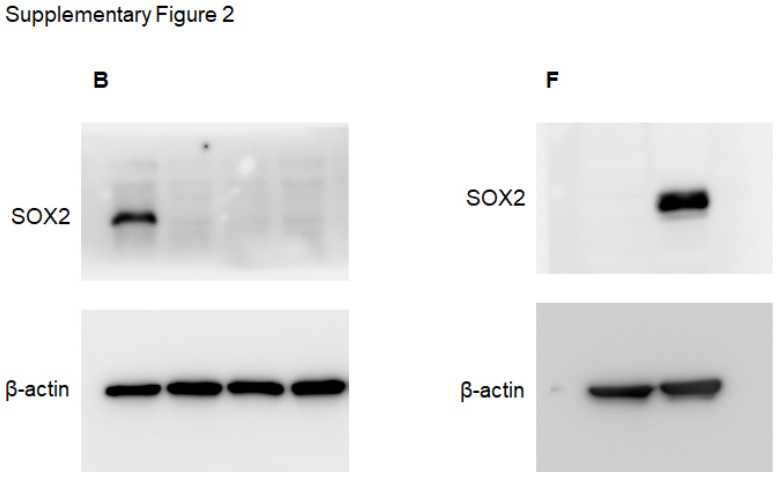

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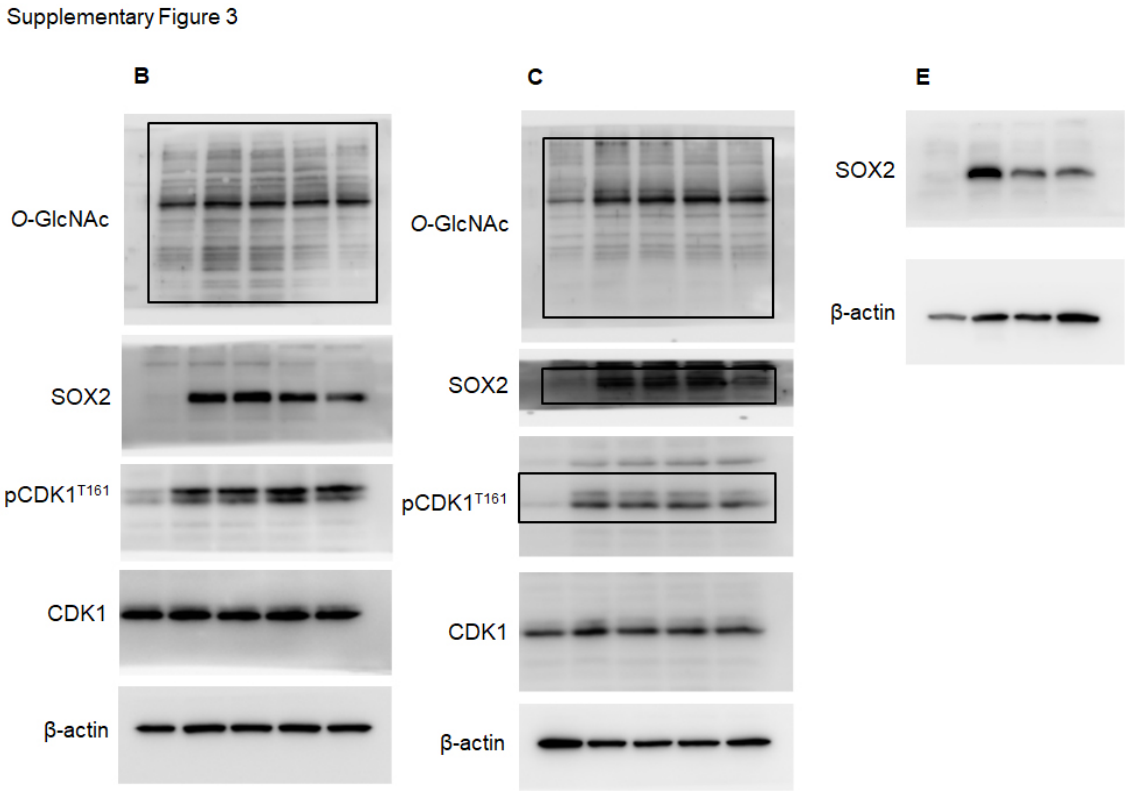

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