Expanded View Figures

Figure EV1. Transcriptome analysis in *Rif1* null ESCs and early-stage EBs.

A Volcano plot summarising the top differentially expressed genes in $Rif1^{-/-}$ ($Rif1^{F/F}$ +OHT) ESCs (left) and EBs (right), compared to $Rif1^{+/+}$ ($Rif1^{+/+}$+OHT). Two independent cell lines were analysed per genotype. In red, genes whose differential expression is statistically significant (FDR < 0.05). *Trim28* (*Kap1*) expression is not up-regulated in *Rif1* null cells, while *Xist* expression levels are significantly down-regulated in EBs.

B Heat maps summarising the logarithmically transformed, normalised expression levels of randomly chosen X-linked genes in $Rif1^{+/+}$ and $Rif1^{-/-}$ ESCs (brown) and 2 days EBs (blue). Failure to up-regulate *Xist* in $Rif1^{-/-}$ EBs is specific, as other X-linked genes do not show the same behaviour. *Rlim* (*Rnf12*) expression, for example, is not affected by *Rif1* deletion (highlighted, along with Xist, in the red box).

C Pie charts summarising the biological processes (GO enrichment analysis, GOrilla) most represented among the genes whose expression is significantly deregulated upon *Rif1* deletion in ESCs (top) and EBs (bottom). Differentially expressed genes were obtained from the DESeq2 (adjusted *P*-value, 0.05 and log2FCl > 0.5). Genes linked to developmental processes (GO5) represent only a small percentage of differentially expressed genes (about 50% up-regulated and 50% down-regulated).

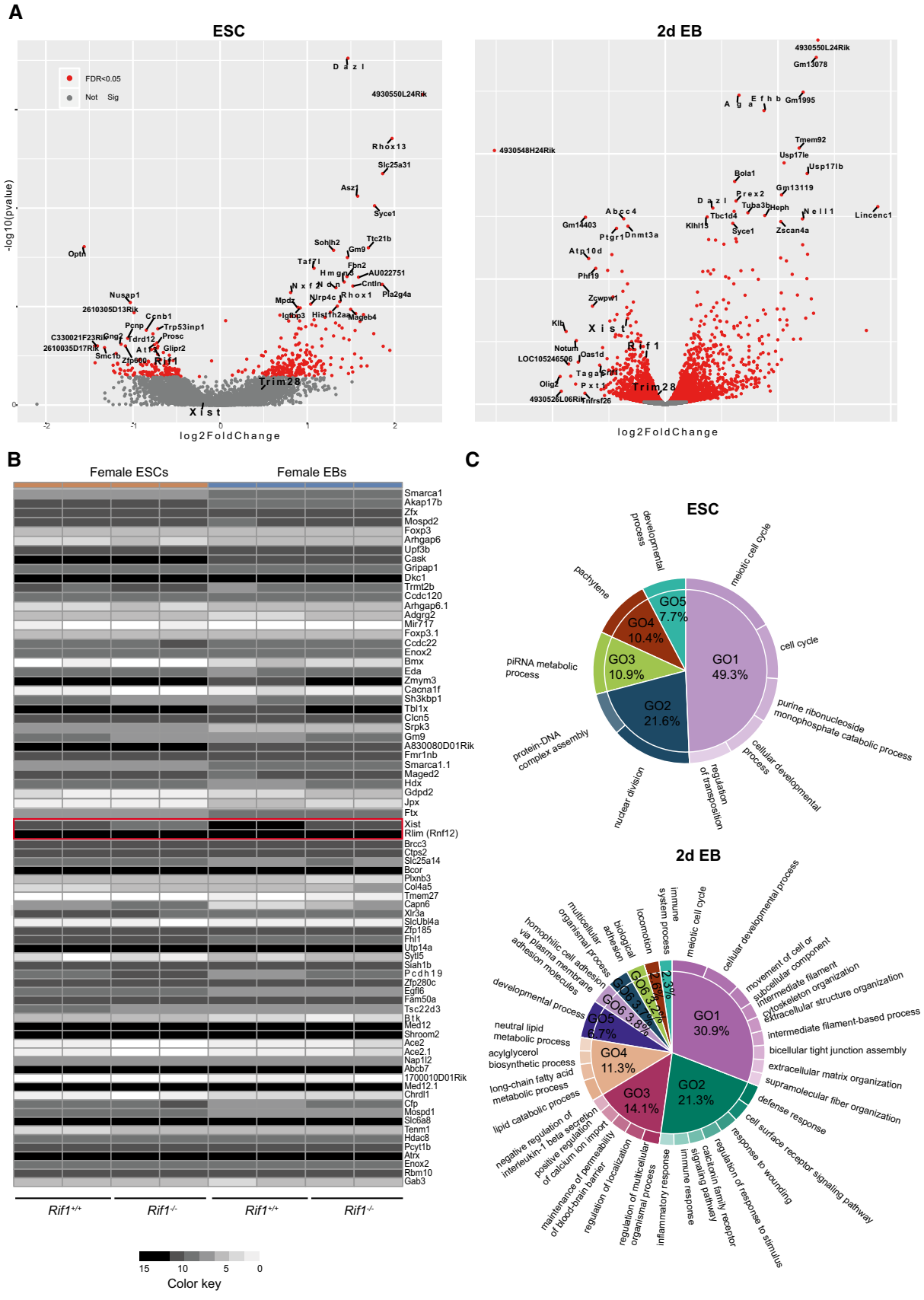

Figure EV1.

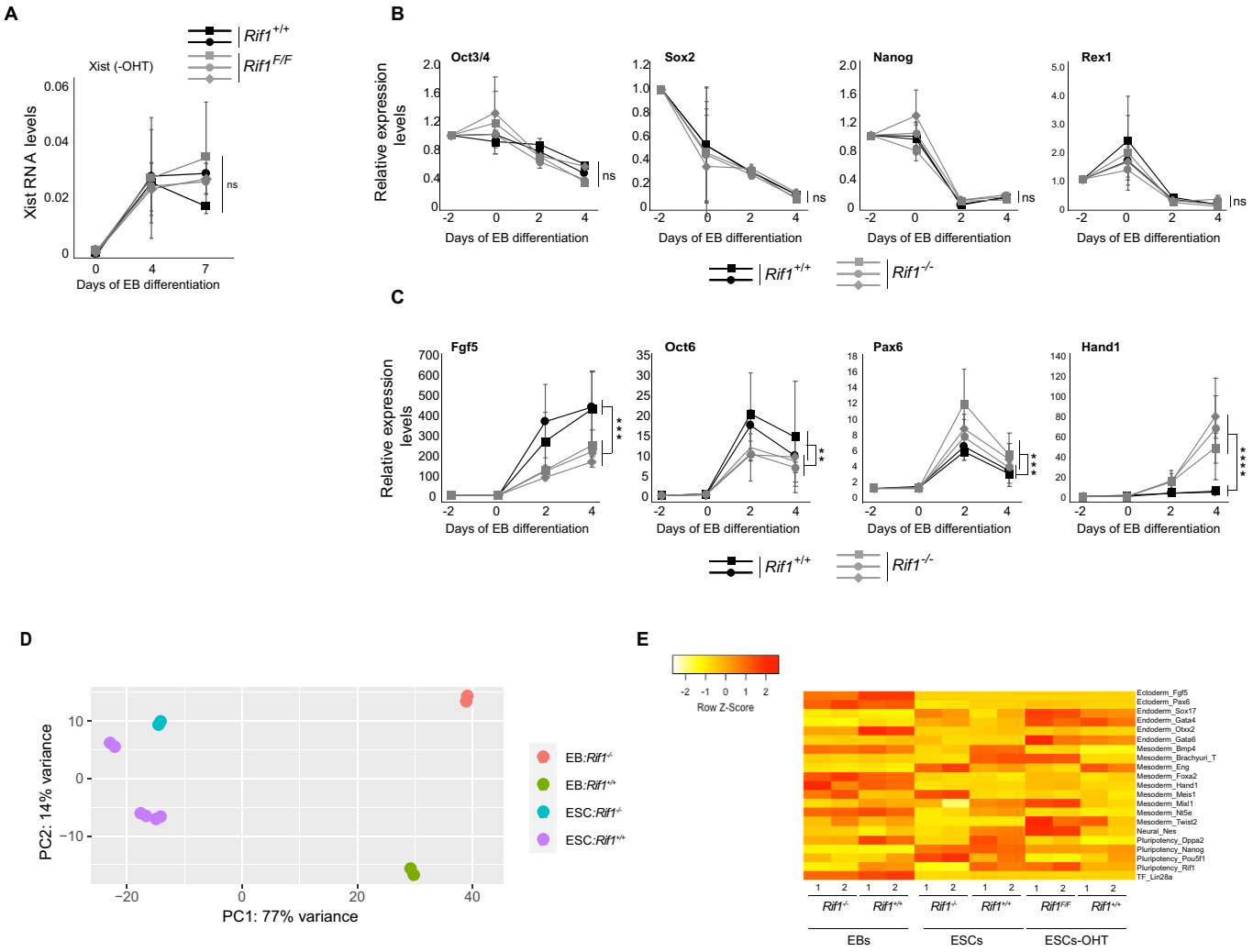

Figure EV2. *Rif1*$^{-/-}$ mESCs can exit pluripotency and commit to differentiation.

A Xist RNA levels monitored by RT-qPCR in two independent *Rif1*$^{+/+}$ (black) and three independent *Rif1*$^{F/F}$ (grey) female mESC lines, during EB differentiation, in the absence of OHT. Xist RT-primers Xist ex7 F and R were used. Values are normalised to a geometric mean consisting of the expression of *Gapdh*, *Ubiquitin* and *β-Actin*. Expression levels are plotted as mean ± standard deviation of a minimum of three individual experiments with statistical significance determined using two-way ANOVA. ns = not significant.

B, C Time course analysis of expression levels for the indicated genes, during EB differentiation of two individual *Rif1*$^{+/+}$ (*Rif1*$^{+/+}$ +OHT, black) and three individual *Rif1*$^{-/-}$ (*Rif1*$^{F/F}$ +OHT, grey) female mESC lines. Expression levels are first normalised to a geometric mean consisting of the expression of *Gapdh*, *Ubiquitin* and *β-Actin* and then plotted relative to pre-samples (day −2), as mean ± standard deviation of three or four individual experiments with statistical significance determined using two-way ANOVA (**$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$ and ns = not significant) comparing *Rif1*$^{+/+}$ to *Rif1*$^{-/-}$ cells. (B). Pluripotency-associated genes and (C) genes expressed during early stages of differentiation.

D The analysis of gene expression in *Rif1* wild-type and null female ESCs and EBs shows that the data cluster first according to the cell type (ESCs versus EBs, PC1) and then according to the genotype (*Rif1*$^{+/+}$ versus *Rif1*$^{-/-}$, PC2). The wider distance between the gene expression data of wild-type and *Rif1* null EBs as compared to ESCs is in agreement with the mixture of cell types that is characteristic of EBs.

E The analysis of the expression of key lineage markers in *Rif1* wild-type and null female cells, both ESCs and EBs, shows that *Rif1* null cells differentiate in all three lineages, with some individual genes displaying some degree of variable level of expression.

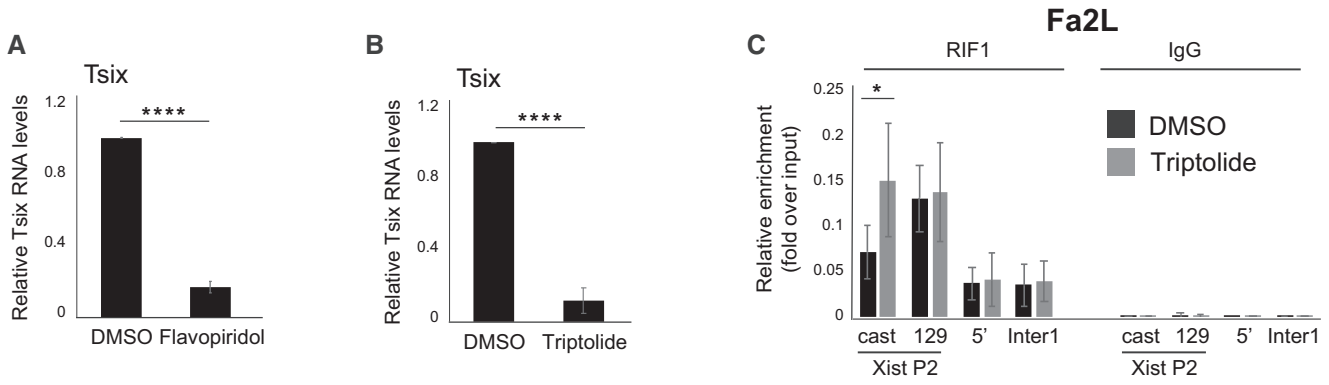

Figure EV3. Inhibiting Tsix transcription in Fa2L cells reverts RIF1 association with P2 from asymmetric to symmetric.

A, B Tsix RNA levels following flavopiridol (A) and triptolide (B) treatment, relative to DMSO-treated Fa2L cells. RNA levels were first normalised to *18S* ribosomal RNA and plotted as mean from three individual experiments ± standard deviations. Statistical significance was determined using Student's two-tailed unpaired *t* test (****$P \leq 0.0001$).

C Allele-specific RIF1 association with *Xist* P2 in Fa2L cells following treatment with DMSO only (black) or triptolide (grey). *cast* indicates association with the *castaneus Xist* P2 promoter and *129* indicates association with the *129 Xist* P2 promoter. Enrichments are presented relative to input DNA. Mean ± standard deviation of three independent experiments. *P* calculated by Student's two-tailed paired *t* test. (*$P \leq 0.05$).

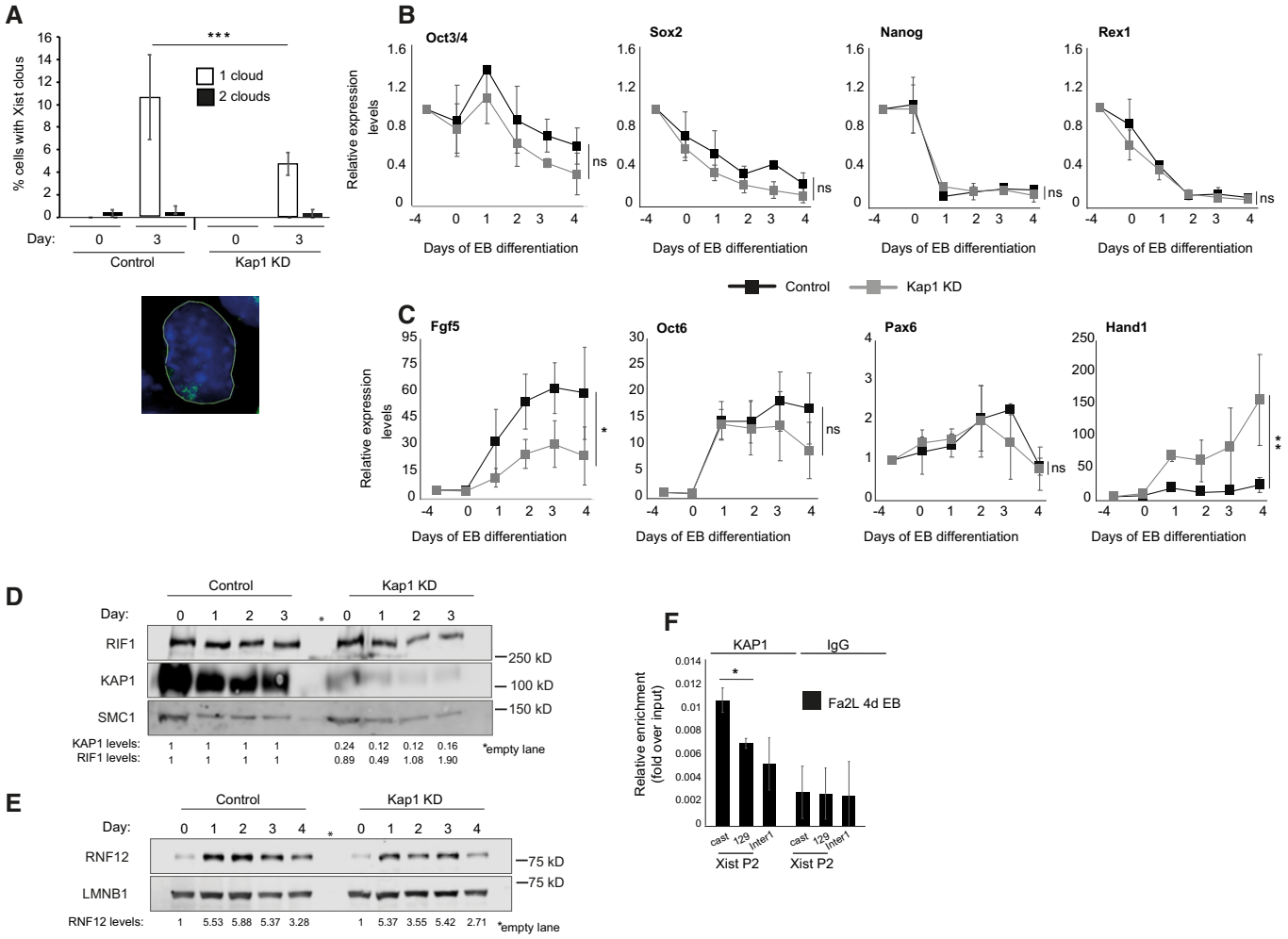

Figure EV4. Defective *Xist* up-regulation in KAP1 knock down cells is not due to lack of differentiation.

A Quantification of Xist cloud formation (one or two clouds, open or filled bars respectively) by RNA FISH in Control and Kap1 KD female mESCs, differentiated into EBs for 3 days. The average of two experiments is shown. $N > 400$ cells. The error bars represent ± standard deviation. P was calculated by χ^2 (***$P \leq 0.001$). Below is an example of Xist cloud RNA FISH signal. Green = Xist, blue = DAPI.

B, C Time course analysis of gene expression levels quantified by RT-qPCR during EB differentiation of female mESCs infected with shRNA directed against Luciferase (control, black) and Kap1 (grey), at the indicated timepoints. Values have first been normalised to a geometric mean consisting of the expression of *Rplp0*, *Ubiquitin* and *Sdha* and then presented relative to pre-samples (day: −4) Mean ± standard deviation from three independent experiments are shown. Statistical significance was determined using two-way ANOVA (*$P \leq 0.05$, **$P \leq 0.01$ and ns = not significant). (B). pluripotency-associated genes and (C) genes expressed during early stages of differentiation.

D Representative western blot analysis of RIF1 and KAP1 levels in Kap1 KD female mESCs. SMC1: loading control. Quantification of RIF1 and KAP1 protein levels normalised to SMC1 and relative to control cells are shown below.

E Representative western blot analysis of RNF12 levels in proteins extracted from female mESC infected with shRNA directed against Luciferase (control) and Kap1, at the indicated timepoints during EB differentiation. LAMIN B1 (LMNB1): loading control. Below is the quantification of RNF12 protein levels relative to day 0 of control and Kap1 KD cells respectively. Values normalised to LMNB1.

F Using allele-specific primers, ChIP-qPCR was used to analyse the association of KAP1 with *Xist* P2 in differentiating Fa2L cells. *cast* indicates association with the *castaneus Xist* P2 and *129* indicates association with the *129 Xist* P2. Enrichments are presented relative to input DNA. Mean ± standard deviation from a minimum of three independent experiments. P was calculated by Student's two-tailed paired t test (*$P \leq 0.05$).

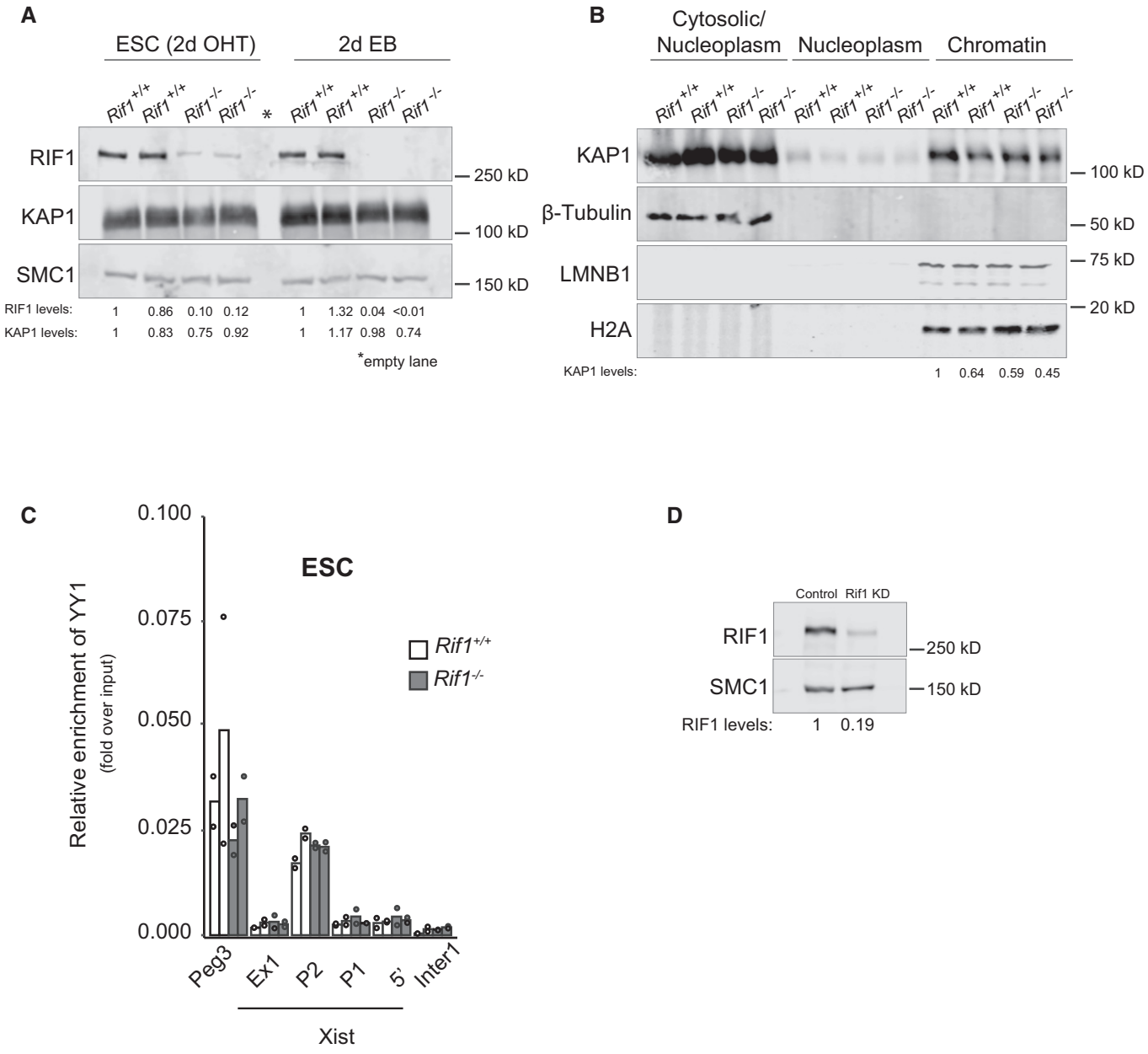

Figure EV5. The overall binding of KAP1 to chromatin is unaffected in *Rif1*$^{-/-}$ mESCs.

A RIF1 and KAP1 levels analysed by western blot in protein extracts from two *Rif1*$^{+/+}$ (*Rif1*$^{+/+}$ +OHT) and two *Rif1*$^{-/-}$ (*Rif1*$^{F/F}$ +OHT) independent female mESC lines, following 2 days of OHT treatment and at 2 days EB. SMC1: loading control. Below are the quantifications of RIF1 and KAP1 protein levels shown as relative levels compared to one of the *Rif1*$^{+/+}$ cells. Values normalised to SMC1.

B Western blot analysis of KAP1 levels in protein extracts from the indicated cell fractions, from two *Rif1*$^{+/+}$ (*Rif1*$^{+/+}$ +OHT) *and* two *Rif1*$^{-/-}$ (*Rif1*$^{F/F}$ +OHT) independent female mESC lines, following 2 days of OHT treatment. β-TUBULIN: marker for the cytosolic fraction. LMNB1 and histone H2A: markers for the chromatin/insoluble fraction. Below are the quantifications of KAP1 protein levels detected in the chromatin fractions shown as relative levels compared to one of the control cells. Values normalised to H2A.

C YY1 association with the *Xist* promoter in two independent *Rif1*$^{+/+}$ (*Rif1*$^{+/+}$ +OHT, black) and two *Rif1*$^{-/-}$ (*Rif1*$^{F/F}$ +OHT, grey) female mESC lines, analysed by ChIP-qPCR. Ex1 indicates a region within Xist exon 1, 2.5 kb downstream of the *Xist* transcriptional start site (TSS), P2 indicates *Xist* promoter P2 spanning the YY1 consensus motif. Peg3 indicates a known YY1-associated site on the *Peg3* gene. Data from two independent experiments are presented.

D Western blot analysis of RIF1 levels in protein extracts from Fa2L cells after Rif1 knock down. SMC1: loading control. Below is the quantification of RIF1 protein levels compared to control cells.