Supplementary legends

Supplementary Figure 1 | Analyses of Q-KO ES cells. **a**, PCR analysis of two independent control (Ctrl1 and Ctrl2), cyclin $D1^{-/-}D2^{-/-}D3^{-/-}E1^{\Delta/\Delta}E2^{-/-}$ (Q-KO1 and Q-KO2) and one cyclin $E1^{\Delta/\Delta}E2^{-/-}$ (E-KO) ES cell lines. PCR primers (see Supplementary Table 2) were used to amplify the indicated exons of the *cyclin D1*, *D2*, *D3*, *E1* and *E2* genes. Note that cyclin $D1^{-/-}$, $D2^{-/-}$, and $D3^{-/-}$ alleles lack exons 1 and 2, but contain intact exon 5 (refs 54-56), cyclin $E1^{\Delta/\Delta}$ lack exons 6 and 7, but contain exon 3 (ref. 57), while the cyclin $E2^{-/-}$ alleles lack exons 1-3 and 6, but contain exon 11 (ref. 3). The sizes of the amplified fragments are shown on the left. **b**, Western blot analysis of control and Q-KO ES cell lines with the indicated antibodies. An arrow points to the cyclin E1-specific band and a star marks a cross-reacting non-specific band. GAPDH was used as a loading control. **c**, Western blot analysis of control and Q-KO MEFs with the indicated antibodies. GAPDH was used as a loading control. **a**, **b** and **c** are representative of n=3 independent experiments. **d**, Control (Ctrl), cyclin $D1^{-/-}D2^{-/-}D3^{-/-}$ (D-KO), cyclin $E1^{\Delta/\Delta}E2^{-/-}$ (E-KO) and cyclin $D1^{-/-}D2^{-/-}D3^{-/-}E1^{\Delta/\Delta}E2^{-/-}$ (Q-KO) ES cells were pulsed with bromodeoxyuridine (BrdU) for 1 hr, stained with an anti-BrdU antibody and propidium iodide and analyzed by flow cytometry. Shown are the percentages of cells in the indicated cell cycle phases, representative of n=4 independent experiments. **e**, Bars depicting the percentages of cells in the indicated cell cycle phases (determined as in panel **d**) in four independent Ctrl, D-KO, E-KO or Q-KO ES cell lines. The mean values (± s.d.) were [%]: Ctrl: G1, 15.1 ± 1.0; S, 75.7 ± 1.8; G2/M 9.2 ± 1.2; D-KO: G1, 19.7 ± 0.4; S, 72.1 ± 1.0; G2/M, 8.1 ± 0.7; E-KO: G1, 28.0 ± 1.6; S, 60.6 ± 2.6; G2/M 11.4 ± 2.6; Q-KO: G1, 43.9 ± 2.6; S, 44.7 ± 3.8; G2/M 11.4 ± 2.8. Analyses were done 3 times. Source data for **e** can be found in Supplementary Table 5.

Supplementary Figure 2 | Molecular analyses of ES cells. **a**, Reverse-transcription – quantitative PCR analysis of the indicated transcripts in control (Ctrl), cyclin $D1^{-/-}D2^{-/-}D3^{-/-}$ (D-KO), cyclin $E1^{\Delta/\Delta}E2^{-/-}$ (E-KO) and cyclin $D1^{-/-}D2^{-/-}D3^{-/-}E1^{\Delta/\Delta}E2^{-/-}$ (Q-KO) ES cells. Shown are mean ± s.d. of n=3 independent experiments. Mean expression levels observed in Ctrl ES cells were set as 1. **b**, Left panel: immunoblot analysis of Ctrl, E-KO, D-KO and Q-KO ES cells with antibodies against core pluripotency factors, representative of n=3 independent experiments. Tubulin was used as a loading control. Right panel: quantification of the densitometric scanning of band intensities from the left panel, mean ± s.d. of n=3 independent experiments. **c** and **d**, RNA-Seq analysis of ES cells. **c,** Number of transcripts downregulated (Down) or upregulated (Up) at least two-fold (> 2), or at least 4-fold (> 4) in Q-KO ES cells as compared to Ctrl ES cells. Three lines of Q-KO and Ctrl ES cells were used for analysis. **d**, Volcano plot of RNA-Seq results showing *p*-values and fold change. **e**, Q-KO ES cells were transduced with lentiviruses encoding Flag-tagged GFP or Flag-tagged Nanog 4E mutant containing phospho-mimicking glutamic acid substitutions within all four cyclin E-CDK2-dependent phosphoresidues. Lysates were immunoblotted with an anti-Flag antibody (to detect ectopically expressed proteins), and with antibodies against Nanog (to detect endogenous Nanog and ectopically expressed Nanog 4E mutant), Oct4 and Sox2, representative of n=3 independent experiments. Tubulin served as loading control. Two-tailed *t*-tests were used (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS). Source data for **b** and **c** can be found in Supplementary Table 5.

Supplementary Figure 3 | Expression of G1 cyclins in wild-type trophoblast and neural progenitor cells. **a**, Human trophoblast cell lines HTR-8/SVneo (T1), JEG-3 (T2), and BeWo (T3), as well as a control human breast cancer cell line MDA-MB-436 (Ctrl), and **b**, A murine neural stem/progenitor cell line, a kind gift of Dr. C. Stiles (N1), mouse neural stem/progenitor cell lines NE-4C (N2) and NE-GFP-4C (N3), and rat fetal neural stem/progenitor cell line N7744100 (N4)

were analyzed by immunoblotting with the indicated antibodies. Tubulin served as a loading control. Note that wild-type trophoblast and neural stem/progenitor cells normally express G1 cyclins. Results are representative of n=3 independent experiments.

Supplementary Figure 4 | Analyses of Nanog, Oct4 and Sox2 degradation in Q-KO ES cells. a, Control and Q-KO ES cells were treated with 100 µg/ml cycloheximide (CHX, to block new protein synthesis) for the indicated times. The levels of Nanog, Oct4 and Sox2 proteins were determined by immunoblotting. Tubulin served as a loading control. **b**, Nanog band intensities from immunoblots as in **a** were quantified, normalized against tubulin and plotted. Each data-point represents a mean value derived from n=3 independent experiments. Right panel shows calculated mean half-life of Nanog in Ctrl and Q-KO ES cells, mean ± s.d. of n=3 independent experiments. *P*=0.008. **c**, Similar analysis as in **b** for Oct4 protein, mean ± s.d. of n=3 independent experiments. *P*=0.008. **d**, Similar analysis as in **b** for Sox2 protein, mean ± s.d. of n=3 independent experiments. *P*=0.014. **e**, Ctrl and Q-KO ES cells were treated with proteasome inhibitor MG132 (+), or left untreated (–), and the levels of Nanog, Oct4 and Sox2 proteins were determined by immunoblotting. Tubulin served as a loading control. Results are representatives of n=3 (**a-d**) or 4 (**e**) independent experiments. Two-tailed *t*-tests were used (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Source data for c, d and e can be found in Supplementary Table 5.

Supplementary Figure 5 | Analyses of Nanog, Oct4 and Sox2 phosphomutants. a, c and e, HeLa cells were transfected with plasmids encoding HA-tagged wild-type Nanog, Oct4 or Sox2 (WT) or encoding mutant proteins containing phospho-inactivating alanine (4A or 3A) or phospho-mimicking glutamic acid (4E or 3E) substitutions within all cyclin E-CDK2-dependent residues, together with CMV-GFP plasmid. Cells were treated with 100 µg/ml cycloheximide (CHX) for the indicated times, whole cell lysates were

prepared and immunoblotted with an anti-HA antibody, as well as with an anti-GFP (control of transfection efficiency) and an anti-tubulin antibody (loading control). **b**, **d** and **f,** HA band intensities were quantified, normalized with GFP, and plotted. **g–i**, 293T cells were transfected with empty vectors (EV), or with expression plasmids encoding (in panel **g**) wild-type HA-Nanog (WT) or mutant HA-Nanog containing alanine (4A) substitutions within cyclin E-CDK2-dependent residues; or (in panel **h**) wild-type HA-Oct4 (WT) or mutant HA-Oct4 containing alanine (3A) substitutions within cyclin E-CDK2-dependent residues; or (in panel **i**) wild-type HA-Sox2 (WT) or mutant HA-Sox2 containing alanine (4A) substitutions within cyclin E-CDK2-dependent residues, together with histidine-tagged ubiquitin (His-Ub) expression plasmids. Cells were treated with 15 μM MG132 overnight before protein extraction. Cell extracts were incubated with Ni-NTA matrices to capture ubiquitinated proteins via His-NTA interaction. Immobilized proteins were resolved by SDS–PAGE and immunoblotted with an anti-HA antibody (IB: HA, upper panels, Ni-NTA pull-down) to detect polyubiquitinated Nanog, Oct4 or Sox2. Whole cell lysates (WCL) were also resolved by SDS–PAGE and immunoblotted with an anti-HA antibody (IB: HA, lower panels). Note that phospho-inactivating (alanine) substitutions strongly increased ubiquitination of Nanog, Oct4 and Sox2 proteins. Results in **a-i** are representatives of n=3 independent experiments. Source data for **b**, **d** and **f** can be found in Supplementary Table 5.

Supplementary Figure 6 | Analyses of the effect of CVT-313 on Nanog, Oct4 and Sox2 protein levels in ES cells. **a**, Quantification of the results from the three ES cell lines shown in Fig. 8a. The levels of each protein in cells treated with vehicle only (VO) were set as 100%. Shown are mean ± s.d. of n=3 independent experiments. Two-tailed *t*-tests were used (**, $p < 0.01$). **b**, Treatment of Q-KO ES cells with CVT-313 leads to further decrease in the levels of pluripotency factors. Comparison of the levels of the indicated proteins between control ES cells treated with vehicle only (Ctrl, 0), Q-KO ES cells treated with vehicle only (Q-KO, 0), and Q-KO ES cells treated with 10 μm CVT-313,

which inhibits CDK2 and CDK1 (Q-KO, 10). Protein lysates were immunoblotted with the indicated antibodies. **c**, CDK1 can phosphorylate pluripotency factors. In-cell kinase reactions. 293T cells were transfected with constructs encoding cyclin B1 together with analog-sensitive CDK1 (AS), or with wild-type CDK1 (WT), and with Flag-tagged versions of Sox2, Nanog or Oct4 (+Flag-tagged) or with empty vectors (-). Cells were permeabilized and incubated with 6-PhEt-ATPγS. Only AS kinases can utilize this 'bulky' ATP version, and hence they transfer thiophosphate moieties to their substrates. Upper panels: following incubation with 6-PhEt-ATPγS, Flag-tagged Sox2, Nanog or Oct4 proteins were immunoprecipitated (IP) with an anti-Flag antibody, and immunoblots were probed (IB) with an anti-thiophosphate ester antibody (ThioP). Bands corresponding to Nanog and Oct4 proteins are marked by arrows, non-specific bands by stars. Middle panels: lysates were immunoblotted with an anti-Flag antibody (to detect transfected proteins). Lower panels: lysates were immunoblotted with an anti-thiophosphate ester antibody to confirm that AS CDK1-cyclin B1 kinase phosphorylates a large number of endogenous substrates, as expected. Results are representatives of n=5 (**b**) or 3 (**c**) independent experiments. Source data for **a** can be found in Supplementary Table 5.

Supplementary Figure 7 | Response of patient-derived glioblastoma tumor-initiating cells and human triple-negative breast cancer cells to CVT-313 treatment. The indicated cell lines were treated with vehicle only (VO) or with 10 μm CVT-313 for 48 hr. **a**, Cells were stained for Annexin V and analyzed by flow cytometry. Shown are percentages of apoptotic (Annexin V^+) cells, mean ± s.d. of n=3 independent experiments. **b** and **c**, glioblastoma (**b**) and breast cancer cells (**c**) were pulsed with BrdU, stained with an anti-BrdU antibody and propidium iodide and analyzed by flow cytometry. Shown are percentages of cells in the indicated cell cycle phases, mean ± s.d. of n=3 independent experiments. Two-tailed *t*-tests were used (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS). Source data can be found in Supplementary Table 5.

Supplementary Figure 8 | Analyses of human triple-negative breast cancer cell lines ectopically expressing phosphomimicking Sox2 or Oct4 mutants. **a**, MDA-MB-436 cells were engineered to ectopically express Flag-tagged Sox2 mutant containing phospho-mimicking glutamic acid substitutions within all four cyclin E-CDK2-dependent phosphoresidues (Sox2 4E). Left panel, cells were treated with vehicle only (0) or with 10 μm CVT-313 (10) for 48 hr. Protein lysates were immunoblotted for Sox2 4E (using an anti-Flag antibody) or for the endogenous Sox2. Tubulin served as a loading control. Note that Sox2 4E mutant was resistant to CVT-313 treatment, i.e. its levels did not decrease upon treatment of cells with the inhibitor, in contrast to the endogenous Sox2. Middle panel, cells stably expressing Sox2 4E, or control cells transduced with an empty vector (Ctrl) were treated with vehicle only (VO) or with 10 μm CVT-313 for 48 hr, stained for Annexin V and analyzed by flow cytometry. Shown are percentages of apoptotic (Annexin V^+) cells, mean ± s.d. of n=3 independent experiments. Right panel, cells stably expressing Sox2 4E, or control cells transduced with an empty vector (Ctrl vector) were treated with as above, pulsed with BrdU, stained with an anti-BrdU antibody and propidium iodide and analyzed by flow cytometry. Shown are mean ± s.d. of n=3 independent experiments. Note that expression of Sox2 4E did not affect the apoptotic and cell cycle response of MDA-MB-436 cells to CVT-313 (also compare with Supplementary Fig. 7a, c). **b**, Similar analysis as in **a** using a human triple-negative breast cancer cell line HCC38 engineered to ectopically express Sox2 4E mutant. Shown are mean ± s.d. of n=3 independent experiments. **c**, Similar analysis as in **a** using a human triple-negative breast cancer cell line CAL51 engineered to ectopically express Flag-tagged Oct4 mutant containing phospho-mimicking glutamic acid substitutions within all three cyclin E-CDK2-dependent phosphoresidues (Oct4 3E). Shown are mean ± s.d. of n=3 independent experiments. Two-tailed *t*-tests were used (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Source data can be found in Supplementary Table 5. Western blots shown are representatives of n=3 independent experiments.

Supplementary Figure 9 | Unprocessed original scans of blots.

Supplementary Table 1 | Antibody information.

Supplementary Table 2 | Sequences of PCR primers.

Supplementary Table 3 | Comparisons of transcript abundance between Ctrl and Q-KO ES cells. The Table has three sheets. The first sheet (Q-KO vs Ctrl) compares the abundance of the indicated transcripts between Q-KO and Ctrl ES cells. Columns list: (A) gene ID (from UCSC hg19 reference); (B) mean normalized counts (baseMean); (C) $\log_2$ fold change; (D) the standard error of the $\log_2$ fold change (lfcSE); (E) the $\log_2$ fold change divided by lfcSE (stat); (F) two-tailed *p*-value obtained using the Wald test; (G) adjusted *p*-value (Benjamini-Hochberg test). DESeq2 performs independent filtering by default using the mean of normalized counts as a filter statistic. The adjusted *p*-values for the genes which do not pass the filter threshold are marked as NA (such as Prg3). Genes upregulated at least 4-fold in Q-KO are highlighted in red, genes downregulated at least 4-fold in Q-KO are highlighted in blue. The second sheet lists genes upregulated at least 4-fold in Q-KO. Trophectodermal/trophoblast genes are marked in column (H), and references listed in column (I). The third sheet lists genes downregulated at least 4-fold in Q-KO.

Supplementary Table 4 | Mapping of cyclin E-CDK2-dependent phosphosites on Nanog, Oct4 and Sox2.

Supplementary Table 5 | Statistics source data.