Supplementary Figures and Table

Supplementary Fig. S1. Knockdown of PCTAIRE1 in PPC1 cells.

(A) PPC1 cells transfected with scrambled RNA or one of three different siRNAs targeting PCTAIRE1 mRNA (siRNAs 1472, 1566, 1656). After 48 hours, the relative
levels of PCTAIRE1 mRNA were measured by qRT-PCR analysis.

(B) Cell lysates 48 hours after siRNA transfection were analyzed by immunoblotting.

(C) 72 hours after siRNA transfection, cellular ATP levels were measured as a surrogate indicator of relative number of viable cells, with data expressed as the ratio between values for cells transfected with PCTAIRE1 or control siRNAs (mean ± SD; n = 3).

*** p < 0.001 by t-test.

(D) To measure cell growth, 2.0 x 10^5 cells transfected with the indicated siRNAs were seeded onto 60 mm diameter plates. After 48 hours, the numbers of cells were counted.

*** p < 0.001

(E) PPC1 cells were transfected with the indicated siRNAs. The % annexin V cells was assessed by FACS analyses at indicated times.

(F) PPC1 cells stably containing inducible shRNAs targeting different sites on PCTAIRE1 mRNA (shRNA#1, #2) were cultured for 48 hours with various concentrations of doxycycline (Dox) ranging from 10 to 1000 ng/ml. Levels of PCTAIRE1 mRNA were measured by qRT-PCR, with normalization relative to GADPH (mean ± SD; n = 2).

(G) PPC1 containing Tet-inducible shRNAs were plated, and doxycycline (ON: 100 ng/ml, OFF: 0 ng/ml) was added to cultures after 24 hours. The % annexin V cells
was assessed by FACS analyses at indicated Dox-treatment hours (mean ± SD; n = 3).

(H) In vivo tumor xenograft analysis.  PPC1 cells stably containing shRNA #2 (5.0 x 10^6 cells resuspended in RPMI) were subcutaneously injected into the flanks of nu/nu mice.  When tumor volumes reached 150 ~ 200 mm³, the animals were provided water with or without 2% doxycycline ("Dox") (n = 6 mice per group).  Animals were sacrificed at 19 days post-injection for immunoblot analyses.  Tumor lysates were normalized for total protein content.  The degree of PCTAIRE1 expression was quantified by densitometry analysis and expressed as the ratio of the PCTAIRE1 level from tumors with and without doxycycline treatment (Dox (-)).  *** p < 0.001

(I) Tissue sections from recovered xenograft tumors analyzed for TUNEL or PARP cleavage.  Histological images were digitized and the percentages of positively stained tumor cells were quantified using imaging software (Aperio).  Data represent mean ± SEM (n = 6).  * p < 0.05, ** p < 0.01 by t-test.
**Supplementary Fig. S2. Knockdown of PCTAIRE1 inhibits growth of Du145, MDA-MB-468 and HeLa cells.**

(A, B) Du145(A) and MDA-MB-468(B) cells stably containing inducible shRNAs targeting different sites on PCTAIRE1 mRNA (shRNA#1, #2) cultured for 48 hours with (ON) or without (OFF) doxycycline (Dox) (100 ng/ml). Levels of PCTAIRE1 mRNA were measured by qRT-PCR (mean ± SD; n = 2).

(C, D) To measure growth rates, 1.0 x 10^5 cells were seeded onto 60 mm diameter plates, and after 24 hours Du145 (C) and MDA-MB-468 (D) cells stably containing inducible shRNAs were cultured with (ON) or without (OFF) 100 ng/ml doxycycline.
The number of cells was counted 1, 3, 5, and 7 days after seeding. *** p < 0.001 by t-test.

(E, F) Du145 (300 cells per well) (E) and MDA-MB468 (1,000 cells per well) (F) cells were plated, and after 24 hours doxycycline (ON: 100 ng/ml, OFF: 0 ng/ml) was added to cultures. The number of colonies was counted on day 10. All data represent mean ± SD (n = 3). *** p< 0.001 by t-test.

(G, H) FACS analysis was performed to assess the percentage of cells binding AnnexinV. Du145 (G) or MDA-MB-468 (H) cells were cultured with (ON: 100 ng/ml) or without (OFF) doxycycline. The % AnnexinV-positive cells was then assessed at the indicated times (mean ± SD; n = 3).

(I) HeLa cells transfected with scrambled RNA or three different siRNAs targeting PCTAIRE1 (siRNAs: si-1472, si-1566, si-1656). At 72 hours after transfection, cell lysates were prepared and analyzed by immunoblotting using rabbit anti-PCTAIRE1 (top), or anti-beta-actin (bottom) antibodies.

(J) HeLa cells transfected with control RNA or three siRNAs targeting PCTAIRE1. After 72 hours, cellular ATP levels were measured using Cell Titer Glo reagents, with data expressed relative to cells transfected with control scramble RNA (mean ± SD; n = 3). *** p< 0.001 by t-test.
(K) HeLa cells stably containing inducible shRNAs targeting different sites on PCTAIRE1 mRNA (shRNA#1, #2) or scramble-control shRNA were cultured for 48 hours with 100 ng/ml doxycycline (Dox). Protein lysates were prepared, and analyzed by SDS-PAGE/immunoblotting using antibodies for PCTAIRE1 (top) and beta-actin (bottom).

(L) HeLa cells stably containing inducible shRNAs were seeded at 500 cells per well in 60 mm dishes. After 24 hours, doxycycline (ON: 100 ng/ml, OFF: 0 ng/ml) was added. Colonies consisting of > 50 cells were enumerated on day 10. All data represent mean ± SD (n = 3). *** p < 0.001 by t-test.

(M) HeLa containing Tet-inducible shRNAs (scramble or shRNA#2) were plated and doxycycline (ON: 100 ng/ml, OFF: 0 ng/ml) was added to cultures after 24 hours. The percentage of annexin V-positive cells was assessed by FACS analyses at indicated times after Dox treatment (mean ± SD; n = 3).
Supplementary Fig. S3. PCTAIRE1 regulates centrosome dynamics.

(A) HeLa cells transfected with siRNAs (scramble or si-PCTAIRE1-1656) were incubated for various times, then analyzed by FACS for DNA content (cell cycle analysis) and phospho-Histone H3 antibody staining. Data in upper panels represent DNA content (x-axis) versus cell number (y-axis). Lower panels represent DNA content (x-axis) versus phospho-H3 staining (y-axis), where each dot equates to a single cell analyzed. The red box indicates mitotic cells: 4n DNA content plus phospho-H3 positive.
(B) Time-lapse photomicroscopy was used to study PPC1 cells stably containing inducible shRNA (#2) that had been transfected with EGFP-tubulin and mCherry-histone H2B plasmids. Cells were treated with (Tet-ON) or without (Tet-OFF) 100 ng/ml doxycycline for 2 days. Representative photomicrographs are provided as merged images of EGFP-tubulin and mCherry-histone H2B (green and red, respectively), and the approximate time the cells remained at each phase.

(C, D, E, F) 267B1/K-ras and 267B1 cells transfected with control or siRNAs targeting PCTAIRE1 (Si-1472, 1566, 1656), and 72 hours later were fixed and stained using antibodies specific for the centrosome marker pericentrin (green), or alpha-tubulin (red), followed by DAPI for DNA detection (blue). (C, E) Representative photographs of cells transfected with siRNAs (C: 267B1/K-ras, E: 267B1). (D, F) The percentage of cells with failure to separate duplicated centrosomes among prometaphase cells was enumerated (D: 267B1/K-ras, F: 267B1). Columns, mean (n = 3, determinations based on examination of 100 prometaphase cells); bar, SD. ** p < 0.01, *** p < 0.001 by t-test.
Supplementary Fig. S4. PCTAIRE1 phosphorylates p27 at Ser10.

(A) HEK293T cells transfected with plasmids producing HA-tagged p27, Myc-tagged PCTAIRE1, PCTAIRE2, PCTAIRE3, or empty vector. Lysates were either loaded directly onto gels (“Input”) or subjected to immunoprecipitation (IP) using anti-HA antibody. Immune complexes were analyzed by SDS–PAGE/immunoblotting.

(B) PPC1 cells stably containing inducible shRNA targeting PCTAIRE1 (shRNA#2)
cultured for 2 days with (ON) or without (OFF) 100 ng/ml doxycycline (Dox). Cells were fractionated to yield cytosolic and nuclear compartments, which were normalized for protein concentration and analyzed by SDS-PAGE/immunoblotting. Protein loading consisted of 5% of total cytosolic proteins and 20% of total nuclear proteins.

(C) Phosphorylation of p27 by PCTAIRE1 assessed by phos-tag SDS-PAGE. PPC1 cells were co-transfected with the indicated plasmids. After 48 hours, cells were treated with MG132 (10 µM) for 4 hours. Cell lysates were immunoprecipitated with anti-Myc antibody, and subjected to phos-tag SDS-PAGE, followed by immunoblotting with anti-p27 (Top, short exposure; Bottom, long exposure).

(D) (Top) PPC1 cells co-transfected with the indicated plasmids. After 48 hours, cells were treated with MG132 (10 µM) for 4 hours. Cell lysates were immunoprecipitated with Myc antibody, and assessed by immunoblotting with the indicated antibodies. Samples were also loaded directly onto gels without IP (“Input”). (Bottom) The degree of phosphorylated p27 (Ser10) expression was quantified by densitometry and normalized by Myc expression. Data are expressed relative to the amount of phosphorylated p27 (Ser10) in lane 1 where Myc-p27 was overexpressed without PCTAIRE1. * p < 0.05 by t-test.

(E) (Top) PPC1 cells with Tet-inducible shRNA#2 targeting PCTAIRE1 transfected...
with Myc-p27 (wild type p27 and phospo-site mutants). After 6 hours, the culture medium was replaced by media with or without 100 ng/ml doxycycline (Dox). After 48 hours, cells were treated with MG132 (10 µM) for 4 hours. Cell lysates were harvested and immunoprecipitated with anti-Myc antibody, followed by immunoblotting with the indicated antibodies. (Bottom) The degree of phosphorylated p27 (Ser10) expression was quantified and expressed relative to the amount of phosphorylated p27 (Ser10) in lane 1 where Myc-p27 was overexpressed without PCTAIRE1 knockdown. * p < 0.05

(F) PPC1 cells co-transfected with Myc-p27 (wild type) and siRNAs targeting PCTAIRE1. After 48 hours, cells were treated with MG132 (10 µM) for 4 hours. Cell lysates were harvested and immunoprecipitated with anti-Myc antibody and assessed by the indicated antibodies.

(G) In vitro kinase assays performed with purified p27 or p27-S10A substrate proteins, and immunoprecipitated HA-tagged PCTAIRE1 or PCTAIRE1 kinase dead mutant (K/M mutant) proteins. Samples were analyzed by immunoblotting as indicated.

(H) In vitro kinase assays conducted with purified p27 substrate protein and immunoprecipitated HA-tagged PCTAIRE1, PCTAIRE2, and PCTAIRE3 proteins. Samples were analyzed by immunoblotting as described above.
(I) *In vitro* kinase assay using purified p27 protein substrate and immunoprecipitated HA-tagged PCTAIRE1, with or without treatment with the cyclin-dependent kinase inhibitor SNS-032 (0, 1, 10, 100 nM) or the receptor tyrosine kinase inhibitor ABT-869 (0, 1, 10, 100 nM).
Supplementary Fig. S5. PCTAIRE1 knockdown leads to accumulation of p27.
(A) PPC1 cells were treated with 25 μg/ml cyclohexamide (CHX) only (left) or CHX + MG132 (10 μM) (right), and cell lysates were harvested at the indicated times. An equal amount of protein from each lysate was analyzed by immunoblotting using mouse anti-p27 antibody. Relative p27 expression was quantified by densitometry analysis, and the results expressed as the percentage of p27 expression at 0 hour.

(B) PPC1 cells transfected with control RNA or three different siRNAs targeting PCTAIRE1. After 2 days, cell lysates were collected in 1x Laemmli buffer and subjected to immunoblotting analysis for p27 (top), beta-actin (middle), and PCTAIRE1 (bottom).

(C, D) PCTAIRE1 knockdown does not modulate p27 mRNA levels. (C) PPC1 cells were transfected with scrambled RNA or three different siRNAs targeting PCTAIRE1 (siRNAs 1472, 1566, 1656). After 48 hours, relative levels of p27 mRNA were measured by qRT-PCR analysis. (D) PPC1 cells stably containing inducible shRNAs targeting PCTAIRE1 (shRNA #1, #2) in 60 mm dishes cultured with (Tet-ON) or without (Tet-OFF) 100 ng/ml doxycycline (Dox). After 48 hours, relative levels of p27 mRNA were measured by qRT-PCR analysis.

(E, F) PPC1 cells transfected with scrambled RNA or siRNAs targeting Cdk1, Cdk5, PCTAIRE1 (si-1472). (E) At 2 days after transfection, cell lysates were prepared and
analyzed by immunoblotting using mouse anti-p27 (top), anti-beta-actin (middle), or rabbit anti-PCTAIRE1 (bottom) antibodies.  (F) Two days after transfection, the relative levels of mRNA (Cdk1, Cdk5, PCTAIRE1) were measured by qRT-PCR analysis.

(G-O) MDA-MB-468 (G), Du145 (I), T47D (J), MCF7 (K), 267B1, 267B1/K-ras (N), and IMR-90 (O) cells were transfected with control RNA or different siRNAs targeting PCTAIRE1 mRNA.  After 2 or 3 days, cell lysates were prepared, normalized for total protein content, and analyzed by immunoblotting using antibodies for p27, beta-actin, and/or PCTAIRE1.  (H, L, M) MDA-MB-468 (H), T47D (L), and MCF7 (M) cells stably containing inducible shRNAs targeting PCTAIRE1 (shRNA#1, #2) were cultured for 3 days with (ON) or without (OFF) 100 ng/ml doxycycline (Dox).  Protein lysates were generated, normalized for total protein concentration, and analyzed by SDS-PAGE/immunoblotting.

(P) 267B1 cells were co-transfected with combinations of the indicated plasmids.  After 48 hours, MG132 was added to media and incubated for 4 hours.  Cell lysates were immunoprecipitated with Myc antibody, and assessed by SDS-PAGE/immunoblotting with the indicated antibodies.

(Q) 267B1 cells were co-transfected with combinations of the indicated plasmids.
After 48 hours, cells were cultured with or without MG132 for 4 hours. Then, cell lysates were generated, normalized for total protein concentration, and analyzed by SDS-PAGE/immunoblotting with the indicated antibodies.
Supplementary Fig. S6. Apoptosis induced by PCTAIRE1 knockdown, and subcellular localization of Eg5 in PCTAIRE1 knockdown cancer cells.

(A) HeLa cells were synchronized by a double thymidine block. Cell cycle progression was monitored by flow cytometric analysis with anti-phospho Histone H3 antibody (M phase).
(B) HeLa cells stably containing inducible shRNAs targeting PCTAIRE1 (shRNA#2) were cultured for 2 days with (ON) or without (OFF) 100 ng/ml doxycycline (Dox). Lysates from cells synchronized in G0/G1, early S, M phase, or asynchronous cell were subjected to immunoblot analysis with antibodies recognizing cleaved caspase-3, PCTAIRE1, or actin.

(C) HeLa cells stably containing inducible shRNAs targeting PCTAIRE1 (shRNA#2) were cultured for 2 days with (ON) or without (OFF) doxycycline (Dox). Also, cells were synchronized by a double thymidine block. Cell lysates were subjected to immunoblot analysis with indicated antibodies.

(D) PPC1 cells transfected with scrambled RNA or siRNAs targeting PCTAIRE1. At 48 hours after transfection, cell lysates were prepared and analyzed by immunoblotting using anti-Eg5 (top), anti-alpha tubulin (middle), or anti-PCTAIRE1 (bottom) antibodies.

(E) PPC1 cells containing shRNA (#2) were incubated with (100 ng/ml) or without doxycycline (Dox). After 48 hours, cells were lysed in buffer containing 1% NP-40, and cell lysates were subjected to immunoprecipitation (IP) with antibodies to p27, and the resulting precipitates were subjected to immunoblot analysis with antibodies for the indicated proteins. A portion (5%) of the lysates (“input”) was also subjected directly
to immunoblot analysis with the same antibodies.

(F, G) Subcellular localization of endogenous Eg5 in PPC1 cells. PPC1 cells were transfected with siRNAs targeting PCTAIRE1 (si-1656) or scramble control. After 48 hours, cells were fixed and processed for immunofluorescence staining with anti-Eg5 (green), anti-pericentrin (red), and DAPI (blue) (F). (G) PPC1 cells transfected with siRNAs (si-PCTAIRE1 or scramble) were stained with anti-Eg5 (green), anti-phospho-histone H3 (red), and DAPI (blue).
Supplementary Fig. S7. Characterization of rabbit anti-PCTAIREE1 antibody and correlation of PCTAIREE1 and p27 expression.

(A) Cell lysates from HEK293T cells transfected with plasmids encoding various Myc-tagged PCTAIREE family kinases (PCTAIREE1, PCTAIREE2, PCTAIREE3) analyzed by immunoblotting using rabbit anti-PCTAIREE1 antibody (Top). The blot was
reprobed with anti-Myc antibody (Bottom). Antibody detection was accomplished using an enhanced chemiluminescence method.

(B) PPC1 cell lysates 2 days after siRNA transfection were prepared, normalized for total protein content, and aliquots were analyzed by immunoblotting using rabbit anti-PCTAIRE1 (top) or anti-beta-actin (bottom) antibodies.

(C) PPC1 cells stably containing inducible shRNAs targeting different sites on PCTAIRE1 mRNA (shRNA#1, #2) were cultured for 2 days with (ON) or without (OFF) 100 ng/ml doxycycline (Dox). Protein lysates were prepared, normalized for total protein concentration, and analyzed by SDS-PAGE/immunoblotting using antibodies for PCTAIRE1 (top) and beta-actin (bottom).

(D) Representative examples of PCTAIRE1 nuclear immunostaining results are provided. Scale Bar = 100 μm.

(E) Correlation analysis of expression levels of PCTAIRE1 and p27 in prostate cancers. Representative immunohistochemical staining using rabbit anti-PCTAIRE1 and mouse anti-p27 antibodies is shown. Scale bar = 100 μm.

(F) Relationship between expression levels of PCTAIRE1 and p27 (n = 100, prostate cancers). Expression levels of p27 were inversely correlated with the PCTAIRE1 levels (rs = -0.43, p < 0.001).
(G) Protein expression of PCTAIRE1 and p27 was examined by immunoblot analysis.

(H) Total cell extracts were prepared from the indicated human cell lines and mouse testis and probed for PCTAIRE1 (top) and alpha-tubulin (bottom). Extracts from PPC1 cells with PCTAIRE1 knockdown (right three lanes: si-1472, 1566, 1656) were also loaded as negative controls. Black arrow indicates human PCTAIRE1 band, while two red arrowheads indicate nonspecific bands.
Supplementary Fig.S8. *PCTAIRE1* expression is upregulated in cancers, and high *PCTAIRE1* levels correlate with poor patient prognosis.

(A) Microarray datasets were accessed using the Oncomine database (http://www.oncomine.com). The median PCTAIRE1 rank was assessed across 15 analyses comparing normal tissue to each cancer group. The P value is given for the median-rank analysis.

(B) PCTAIRE1 copy number was assessed across 5 analyses comparing the normal tissue group to each cancer group.

(C, D) Kaplan-Meier survival plots were obtained using Kaplan-Meier Plotter.
(heep://www.kmplot.com) to display the probability of overall survival of 1,405 patients with lung cancer (C) or 1,115 patients with breast cancer (D) grouped according to PCTAIRE1 dichotomized into high versus low expression median mRNA expression levels. Hazard Ratio (HR) with 95% confidence intervals and P value for the comparison of the low and high PCTAIRE1 groups are shown.
### Supplementary Table S1. Specific interaction of PCTAIRE1 with p27.

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Three micrograms of plasmids producing LexA DNA-binding domain fusion proteins (listed at left) were co-transformed with 3 µg of pJG4-5 plasmid producing B42 transactivation domain fusion proteins (listed at right) into the EGY48 yeast strain.
Transformed cells were grown on semi-solid media lacking leucine or containing leucine as a control, which resulted in equivalent amounts of growth for all transformants. Plasmid combinations that resulted in growth on leucine-deficient media within 4 days were scored as positive (+). The beta-galactosidase activity of each colony was tested by filter assay and scored as blue (+) or white (-) after 90 minutes.