Supplementary information：

Supplementary Figure legends:

Fig. S1: mRNA expression levels of *BRD4* and *PIN1* in various gastric cancer cells.

Fig. S2: Indirect immunofluorescence staining of the localizations of PIN1 (red) and BRD4 (green) in MKN28 cells. Staining of nucleus DNA by DAPI (blue) and a merge of all three stainings in the same cells are also shown.

Fig. S3: HEK293T cells were co-transfected with Flag-PIN1 and Myc-BRD4 or Myc-BRD4-T204A as indicated. Plasmid ratio (BRD4 vs PIN1: 2:1). Myc-BRD4 immunoprecipitates were immunoblotted for the associated PIN1.

Fig. S4: MEFs of *Pin1*$^{+/+}$, *Pin1*$^{-/-}$, *Pin1*$^{-/-}$*(Pin1)* or *Pin1*$^{-/-}$*(Pin1-C115A)* were treated with cycloheximide for the indicated time points and immunoblotted for the expression of Brd4 or Tubulin. A representative result from three independent experiments is shown in the left. Quantification of the results is shown in the right. Data represent the average of three independent experiments ± SD.

Fig. S5: HEK293T cells were transfected with WT or T204A mutant of BRD4. Twenty-four hours after transfection, cells were treated with CHX for indicated time points and immunoblotted for the expression of BRD4 or Tubulin. A representative result from three independent experiments is shown in the left. Quantification of the results is shown in the right. Data represent the average of three independent experiments ± SD.

Fig. S6: Expression levels of BRD4, PIN1 and Tubulin in AGS (left) or AZ-521 (right) transfected with control or *PIN1* siRNA.

Fig. S7: Endogenous BRD4 was immunoprecipitated from GES-1 cells and immunoblotted for associated PIN1. IgG was used as a control.

Fig. S8: BRD4 immunoprecipitates from *PIN1* knockdown AGS cells were immunoblotted with co-purified CDK9.

Fig. S9: Brd4 immunoprecipitates from WT or *Pin1*-deficient MEFs were immunoblotted with co-purified Cdk9.

Fig. S10: Brd4 immunoprecipitates from *Pin1*-deficient MEFs washed with high salt buffer were incubated with GST-PIN1 or GST-PIN1-W34A. After wash, BRD4 immunoprecipitates were used to pull-down BRD4-free Flag-CDK9 from transfected HEK293T cells and the associated CDK9 was detected with anti-CDK9 antibodies as in Fig. 5G.

Fig. S11: GST or GST-PIN1 conjugated agarose beads were incubated with MKN28 cell lysates. GST-PIN1 associated BRD2, BRD3 or BRD4 were detected with the indicated antibodies.

Fig. S12: Sequence alignment of PIN1-binding motif containing T204 BRD4 in different species.

Fig. S13: Cell proliferation for MKN28 cells expressing control shRNA, *BRD4* shRNA or sh*BRD4*/*BRD4-L* or sh*BRD4*/*BRD4-S*. Data represent the average of three independent experiments ± SD. (left). The cellular levels of endogenous or exogenous BRD4 in the indicated MKN28 cells (right).

Fig. S14: The relative mRNA expression levels of *BRD4*-L or *BRD4*-S isoform in various gastric cancer cells.

Fig. S15: BRD4 immunoprecipitates from the indicated gastric epithelial cells were immunoblotted with anti-pT204 BRD4 antibodies.

Fig. S16: BRD4 immunoprecipitates from G0 (serum starvation for 60 hr) or G1 phase (release from serum starvation for 8 hr) MKN28 cells were immunoblotted with anti-pT204 BRD4 antibodies.

Fig. S17: (A) Cell proliferation for AGS cells stably expressing sh*BRD4*/*BRD4* or sh*BRD4*/*BRD4-T204A*. Data represent the average of three independent experiments ± SD (left). The cellular levels of endogenous or exogenous BRD4 in the indicated AGS cells (right). (B) sh*BRD4*/*BRD4* or sh*BRD4*/*BRD4-T204A* AGS cells as in (A) were seeded in soft-agar and cultured for 28 days. Representative photographs were taken at day 28 (left). The relative percentage of colony numbers compared to shBRD4/BRD4 (set as 100%) cells from three independent experiments ± SD is shown in the right. (C) The wound-healing migration assays for various AGS cells as indicated. Representative photographs were taken at 0, 24 and 48 hr (left). The percentage of average speed of wound closure from three independent experiments ± SD is shown in the right. (D) Invasion assays for sh*BRD4*/*BRD4* or sh*BRD4*/*BRD4-T204A* AGS cells as indicated. Representative photographs of invading cells stained with crystal violet are shown in left. Quantification of cell invasion is shown in the rigth and data represent the average of three independent experiments ± SD.

Fig. S18: (A) Cell proliferation of MKN28 cells incubated with different doses of PiB for 72 hr. (B) The MKN28 cells were seeded in soft-agar and cultured for 15 days with different doses of PiB. The relative percentage of colony numbers compared to cells without PiB treatment (set as 100%) from three independent experiments ± SD is shown. (C) The wound-healing migration assays for MKN28 cells treated with PiB (25 μM). The relative percentage of speed of wound

closure from three independent experiments ± SD is shown. (D) Quantification of cell invasion of MKN28 cells treated with 25 μM of PiB is shown and data represent the average of three independent experiments ± SD.

Fig. S19: Expression levels of PIN1 and BRD4 in MKN28 cells transfected with control or *BRD4* siRNA.

Supplementary Materials and Methods

Antibodies and peptides

Antibodies against Flag (sc-166355), HA (sc-805), Myc (sc-40), ubiquitin (sc-8017), GFP (sc-9996), and GST (sc-138) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against Tubulin (T0198) was from Sigma-Aldrich. Antibody against PIN1 (MAB2294) was from R&D systems (Minneapolis, MN, USA). Anti-BRD4 antibodies (A301) were from Bethyl Laboratories (Montgomery, TX, USA). Anti-p-Ser/Thr-Pro antibodies (05-368) were from EMD Millipore (Billerica, MA, USA). Anti-pT204-BRD4 antibodies were generated by Abmart(Shanghai, China). BRD4 and pBRD4 peptides were purchased from ChinaPeptides Co., Ltd. (Shanghai, China). BRD4-pNA peptides (Gln-Ala-Ser-(p-Thr)-Pro-Phe-paranitroanilide) were generated from GenicBio Limited (Shanghai, China).

Human tissue samples and immunohistochemical staining

Formalin-fixed and paraffin-embedded tissue microarrays of human gastric cancer tissues were purchased from Novus (IMH-316; Littleton, CO). Immunohistochemical staining for PIN1 and BRD4 was performed as previously described [4]. BRD4 staining intensity was graded as previously described with a score 0 to 3 [4]. Samples with a score 0 were graded as negative, samples with a score 1-3 were graded as positive (weak (1), moderate (2) and strong (3)). PIN1 staining intensity was graded the same as BRD4.

Generation of sh*BRD4*/*BRD4*- and sh*BRD4*/*BRD4-T204A*-MKN28 or AGS cells

sh*BRD4*/*BRD4* or sh*BRD4*/*BRD4-204A* MKN28 cells were generated by transfection of MKN28 cells with *BRD4* shRNA-resistant WT Myc-*BRD4* or Myc-*BRD4-T204A* expression vectors cloned in pcDNA3.1. Twenty-four hours after transfection, cells were transduced with

lentiviruses carrying pLKO.1-sh*BRD4*-1665 (Sigma, St. Louis, MO). The cells were then selected with 2.5 μg/ml of puromycin for the expression of shRNA and 500 μg/ml of G418 for the expression of shRNA-resistant *BRD4* and *BRD4-T204A*. Drug-resistant pools of cells were collected as stable cell lines and used in the experiments. The sh*BRD4*/*BRD4*-AGS and sh*BRD4*/*BRD4-T204A*-AGS cells were generated using the similar approaches.

Immunoprecipitation Assay

Cells were lysed with immunoprecipitation (IP) buffer (20 mM HEPES, pH7.9; 1.5 mM $MgCl_2$; 0.5 mM EDTA; 0.4M NaCl; 1% Nonidet P-40; 10% Glycerol; 1 mM DTT; 1 mM PMSF; 1X protease inhibitor cocktail (Roche)). Cell lysates were pre-cleared with protein A/G agarose beads, followed by immunoprecipitation with the appropriate antibodies at 4°C overnight in the presence of 15 μl of protein A/G agarose beads. The beads were washed three times with wash buffer (20 mM HEPES, pH 7.9; 0.5 mM EDTA; 0.15 M KCl; 0.5% Nonidet P-40; 1 mM DTT; 1 mM PMSF; 1X protease inhibitor cocktail (Roche)), resuspended in 60 μl of 1X SDS loading buffer and then subjected to immunoblot analysis.

GST pull-down assay

Agarose beads containing GST, GST-PIN1 or its mutants were incubated with HEK293T cell lysates transfected with various Myc-tagged *BRD4* expression vectors in 600 μl IP buffer, followed by rotation at 4°C for overnight. Binding of the BRD4 to GTS-PIN1 was detected by immunoblotting with anti-Myc or BRD4 antibody.

Dot immunoblotting

Unphosphorylated and phosphorylated peptides were dissolved in 1X PBS at 25 μM. 1 μl of peptides solution was spotted on a nitrocellulose membrane. After drying, the membrane was blocked with 3% bovine serum albumin in PBS containing 0.1% Tween 20 for 1 hr at R.T., followed by incubation with GST-PIN1 (5 μg/mL) in 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Triton X-100 for 2 hr. After washing, the membrane associated PIN1 was detected by immunoblotting with anti-PIN1 antibodies.

Protein purification and crystallization of PIN1

The R14A mutant clone of PIN1 was used to express protein for crystallization as previously described [8]. PIN1 protein was concentrated to 20 mg/mL and used to produce crystals by mixing with a reservoir solution in 1:1 ration which is consist of 1.8 M ammonium sulfate, 100 mM HEPES pH 7.5, 1% (v/v) PEG400, and 1 mM DTT. The complex crystals of PIN1 with pT204-peptide (6aa) were obtained by soaking crystals in the reservoir solution with additional 8 mM of pT204-peptide (6aa) for 24 hr. Crystals were flash frozen in liquid nitrogen after being stabilized in 40% (v/v) PEG400, 50mM HEPES pH 7.5, and 1 mM DTT.

All diffraction data were collected at an insertion device synchrotron source (Sector 21 ID-D, Advanced Photon Source, Argonne National Labs, IL) using a Mar 300 CCD detector. Data were scaled and integrated using Autoproc[7]. Crystallographic phases were determined by molecular replacement, as implemented in the Phenix suite of packages [6]. Density for the peptide was apparent in initial electron density maps but was not modeled until the crystallographic R factor had dropped below 0.3. Crystallographic refinement using REFMAC5 [3] was interspersed with rounds of manual rebuilding using Coot [1], until the model converged. The refined coordinates have been deposited in the Protein Data Bank (access no.: 5UY9).

BRD4 isomerization assay

The proline isomerase assay was performed as described [42], withsome modifications. Briefly, 10 μL of a 20 μg/μl solution of GST, GST-PIN1-WT, or GST-PIN1-C115A were mixed with PIN1 activity buffer (50 mM Hepes, pH 7.8, 100 mM NaCl, 2 mM DTT, 0.04 mg/mL BSA) on ice for 10min, then 5 μl (50 ng/μl) of chemotrypsin solution were added. The reaction was startedby adding 30 μl (5 mM) of BRD4-pNA peptides, and pNA absorbance was followed at 405 nm.

Trypsin digestion of BRD4

sh*BRD4*/*BRD4* MKN28 cells were transduced with lentivirus expressing shRNA against *PIN1*. Myc-BRD4 immunoprecipitates from *PIN1* knockdown sh*BRD4*/*BRD4* MKN28 cells were then incubated with 0.5 μg/ml GST, GST-PIN1-WT, or GST-PIN1-C115A recombinant proteins for 30 min at room temperature (R.T.). After wash with washing buffer, BRD4 immunoprecipitates were digested with trypsin (10 ng/ml) for 3 min at room temperature. The digested fragments were visualized by western blot with anti-BRD4 antibody.

Purification of CDK9-free BRD4 or BRD4-free CDK9

BRD4 was affinity purified with anti-Myc antibody from Myc-BRD4 overexpressed HEK293T cells after 12 hr PiB (50 μM) treatment. Immunoprecipitated BRD4 was washed with high salt wash buffer (20 mM HEPES, pH7.9; 0.5 mM EDTA; 0.4 M KCl; 0.5% Nonidet P-40; 0.1% SDS; 1 mM DTT; 1 mM PMSF; 1X protease inhibitor cocktail (Roche)) to remove BRD4-associated CDK9, followed by incubation with GST, GST-PIN1, or GST-PIN1-C115A for 1 hr. CDK9 was affinity purified with anti-Flag antibody from Flag-CDK9 overexpressed HEK293T cells. Immunoprecipitated CDK9 was washed with high salt wash buffer to remove CDK9-associated BRD4. Flag-CDK9 was then eluted from protein G beads with Flag peptides and

incubated with BRD4 on beads for 2 hr at 4°C. The beads were washed three times with wash buffer (20 mM HEPES, pH7.9; 0.5 mM EDTA; 0.15 M KCl; 0.5% Nonidet P-40; 1 mM DTT; 1 mM PMSF; 1X protease inhibitor cocktail (Roche)), and BRD4-associated CDK9 was detected by western blot.

Proliferation assay and soft-agar colony formation assay

Proliferation assay and soft-agar assays were performed as previously described [2]. For cell proliferation assay, cells were seeded into 96-well plates at a density of $2x10^3$ cells/well. The numbers of cells were determined with MTS Cell Proliferation Assay Kit (Promega) at the indicated time points. For soft-agar assay, a total of 5000 MKN28 or AGS cells stably expressing shCtr, sh*BRD4*, sh*BRD4*/*BRD4* or sh*BRD4*/*BRD4*-T204A were suspended in DMEM containing 0.3% SELECT Agar® (Invitrogen) and then plated in 6-well plates coated with an initial underlay of 0.5% SELECT Agar® (Invitrogen) in culture medium. Colony growth was scored after 15 or 28 days of cell incubation at the normal condition. The size cut-off for counting is 50 μm. Representative photographs were taken at day 15 or 28 to show colonies.

Cell migration and invasion assays

Cell migration was analyzed by a wound-healing assay. Cells were grown to nearly 100% confluency and serum starved overnight before producing the scratch wound by dragging a 200 μl pipette tip across the layer. Detached cells were washed away with cell culture medium. Cells were cultured in DMEM with 0.5% FBS. The closure of the wound was monitored by microscopy at the indicated time points after inflicting the wound. Cell invasion assay was performed using Transwell invasion chambers (Becton, Dickinson and Company) according to manufacturer's instructions.

In Vivo Tumorigenicity Assays

Five-week-old female, athymic nude mice (n=3) (Harlan Laboratories, Indianapolis, IN, USA) were subcutaneously implanted with sh*BRD4*/*BRD4*- or sh*BRD4*/*BRD4*-T204A-MKN28 cells ($1x10^7$) for 7 months. Tumor volume or weight was measured after mice were killed. Tumor volume was calculated from caliper measurements by the formula: W^2 x L x 0.5. All experiments involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Illinois at Urbana-Champaign.

Immunofluorescence staining

Cells were grown on coverslips, fixed for 10 min in 2% formaldehyde, and permeabilized for 15 min in PBS containing 0.2% Triton X-100. Cells were then rinsed with PBS and blocked for 30 min at room temperature in PBS containing 0.2% Triton X-100 and 3% fetal bovine serum. Cells were the incubated with the anti-BRD4 (1:200), anti-PIN1 (1:200) antibodies at 4 ^{0}C overnight. After wash, FITC-conjugated anti-rabbit (1:200) or Texas Red-conjugated anti-mouse secondary antibodies (1:500) were added for 1 hr at room temperature. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) (5 μg/ml). Cells were mounted and images were captured by wide-field Fluorescence Microscope (Carl Zeiss Inc.).

Cell synchronized to G_1 phase

MKN28 cells synchronized by serum starvation was performed as previously described [5]. Briefly, cells were cultured in DMEM with 0.5% FBS for 60 hr and released into complete media for 8 hr to allow cell to progress to G_1 phase.

Primers for RT-PCR and ChIPs

	sense	anti-sense
Hs-*MET*-promoter-	TTGACCTTCACACACCCAGAT	TTCTGAGTTTGAGTGCCATGA

chip		
Hs-*MMP9*-promoter-chip	CTGGTCCTGGTGCTCCTG	CTGCCTGTCGGTGAGATTG
Hs-*MET*-RT	CCCCACCCTTTGTTCAG	TCAGCCTTGTCCCTCCT
Hs-*MMP9*-RT	AGACCTGGGCAGATTCCAAAC	CGGCAAGTCTTCCGAGTAGT

1 Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. Acta Crystallogr D Biol Crystallogr 2010; 66: 486-501.

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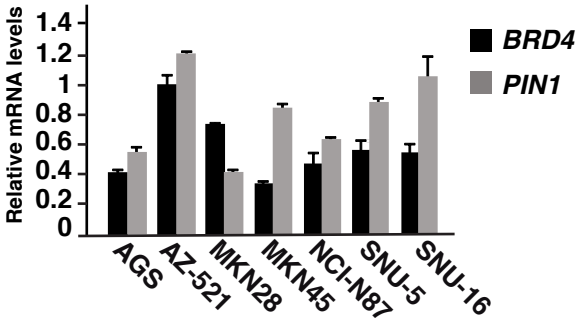

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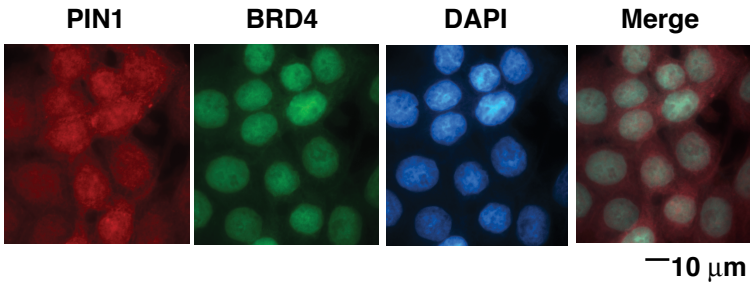

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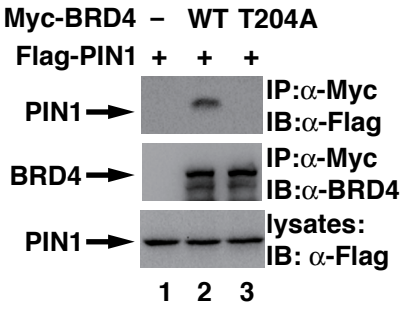

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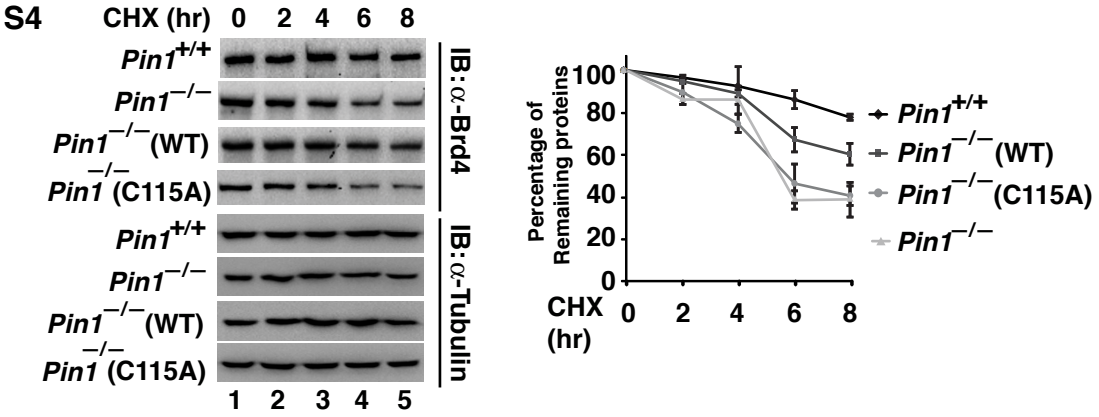

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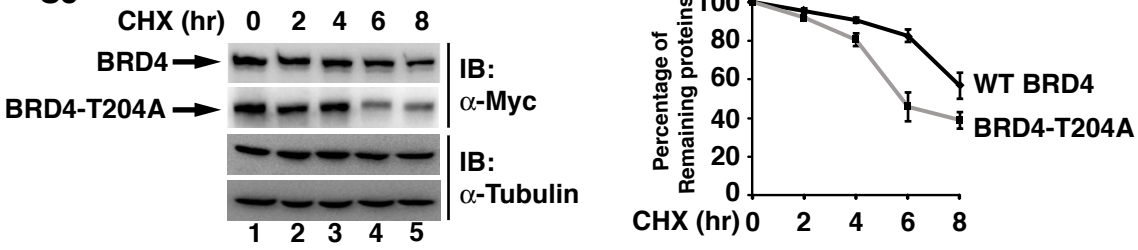

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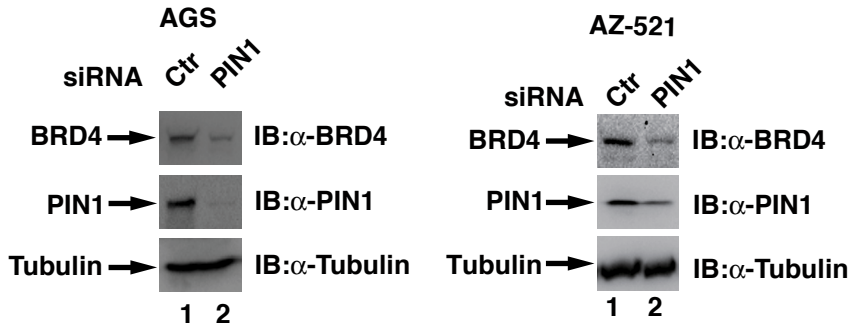

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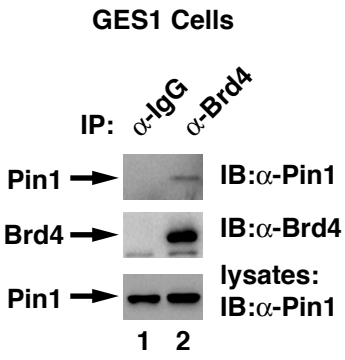

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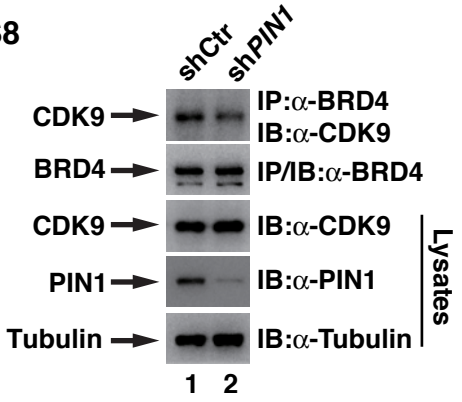

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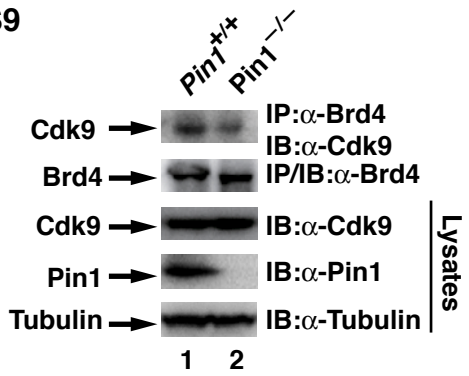

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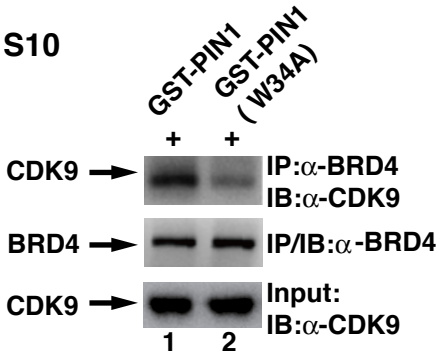

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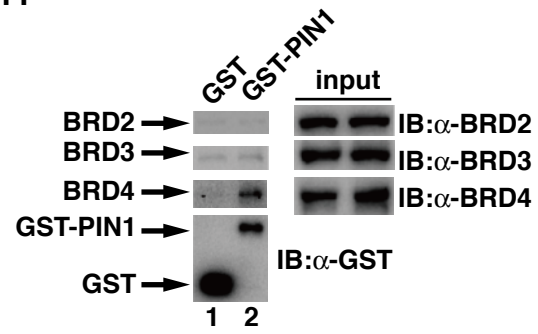

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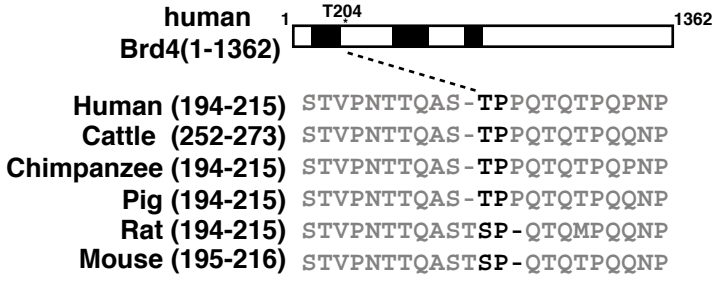

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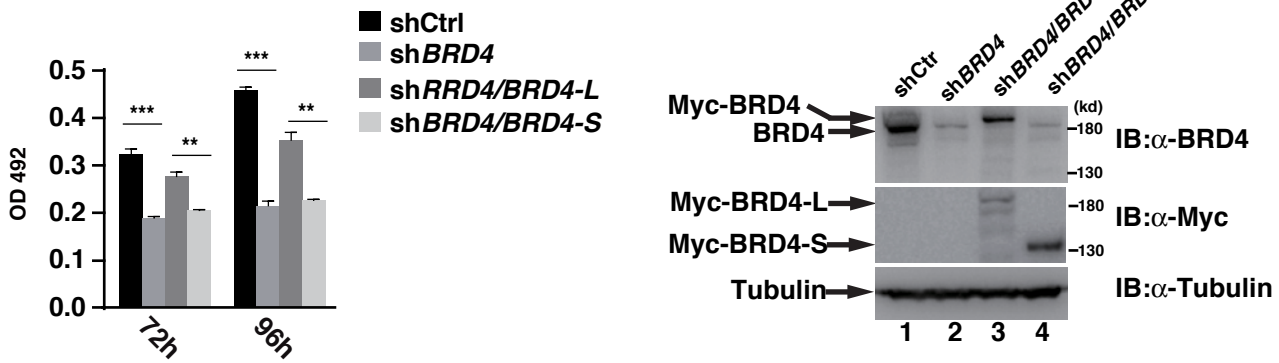

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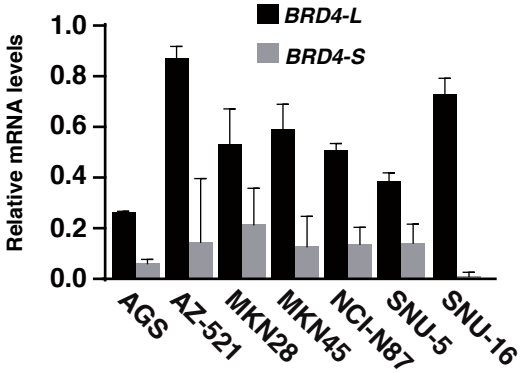

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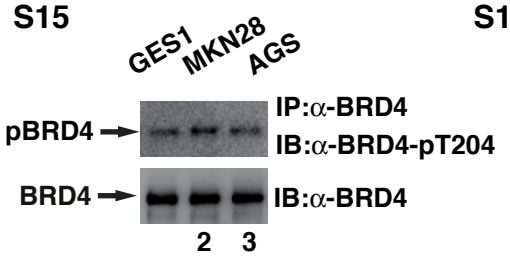

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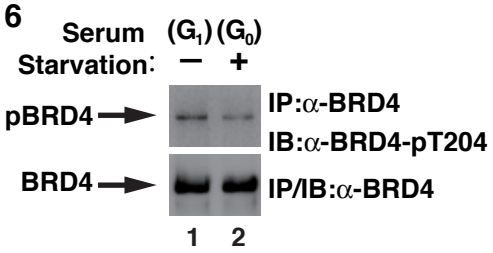

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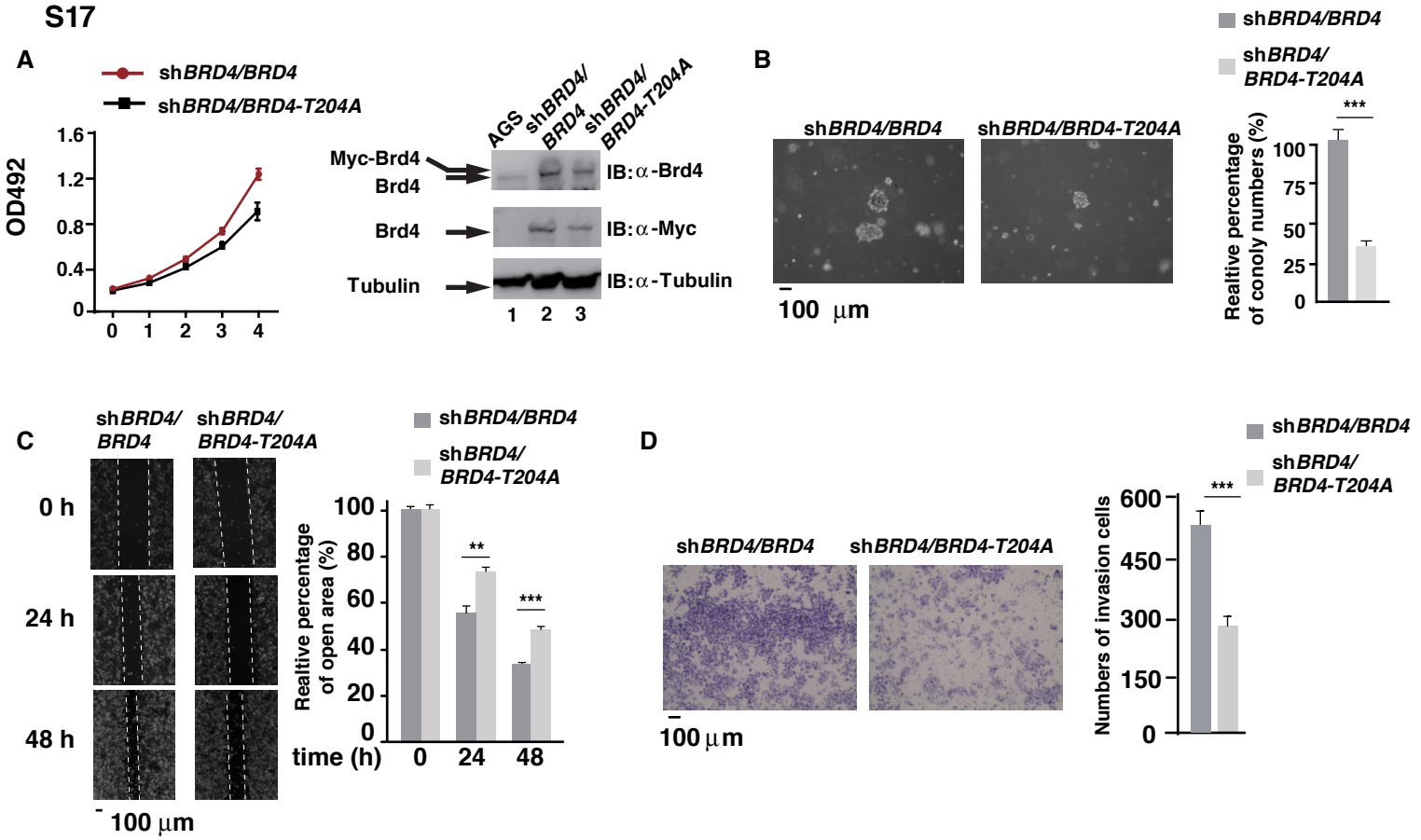
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shBRD4/BRD4-T204A
OD492
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1.2
0.8
0.4
0
0 1 2 3 4
AGS
shBRD4/BRD4
shBRD4/BRD4-T204A
Myc-Brd4
Brd4
IB:α-Brd4
Brd4
IB:α-Myc
Tubulin
IB:α-Tubulin
1 2 3
B
shBRD4/BRD4
shBRD4/BRD4-T204A
100 μm
shBRD4/BRD4
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***
Realtive percentage of conoly numbers (%)
100
75
50
25
0
C
shBRD4/BRD4
shBRD4/BRD4-T204A
0 h
24 h
48 h
100 μm
shBRD4/BRD4
shBRD4/BRD4-T204A
Realtive percentage of open area (%)
100
80
60
40
20
0
**
***
time (h) 0 24 48
D
shBRD4/BRD4
shBRD4/BRD4-T204A
100 μm
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shBRD4/BRD4-T204A
***
Numbers of invasion cells
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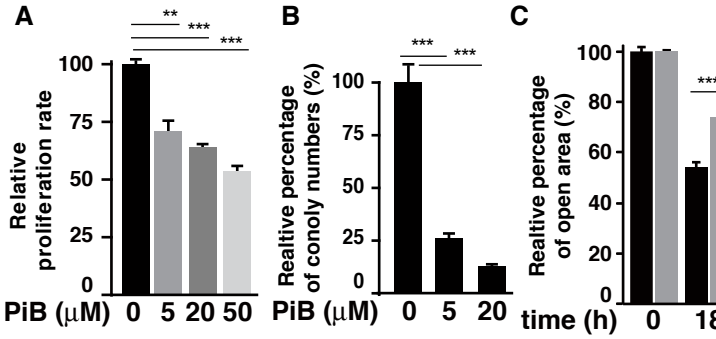
450
300
150
0

S18


A
**
***
***
Relative proliferation rate
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75
50
25
0
PiB (μM) 0 5 20 50
B
***
***
Realtive percentage of conoly numbers (%)
100
75
50
25
0
PiB (μM) 0 5 20
C
Realtive percentage of open area (%)
100
80
60
40
20
0
***
***
DMSO
PiB
time (h) 0 18 36
D
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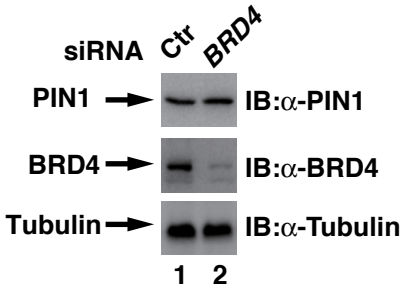
Numbers of invasion cells
800
600
400
200
0
PiB – +

S19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