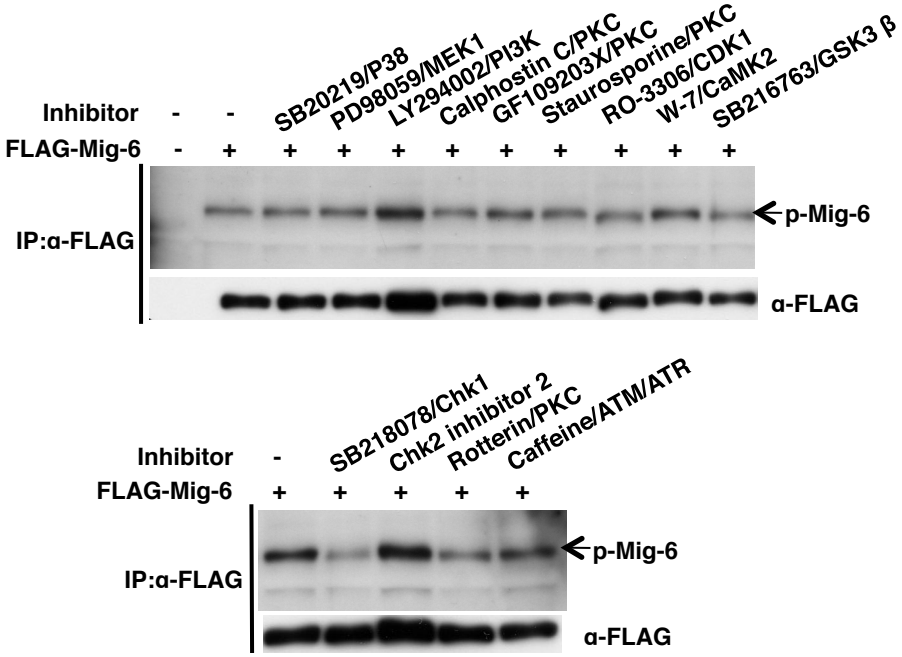
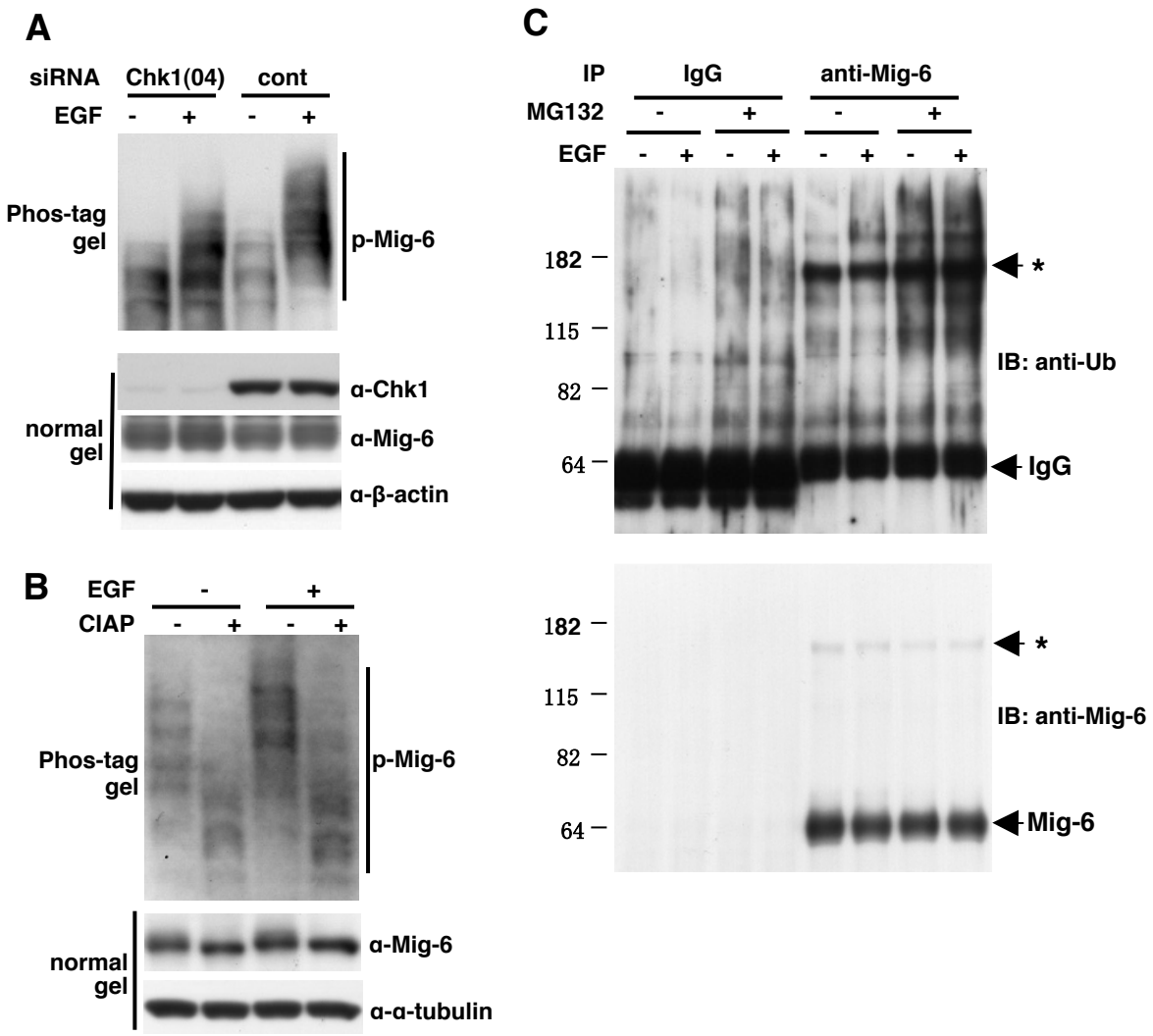
	Inhibitor	Target kinase	working concentration	Supplier
1	SB20219	p38	10μM	CALBIOCHEM
2	PD98059	MEK1	50μM	CALBIOCHEM
3	LY294002	PI3K	50μM	CALBIOCHEM
4	Calphostin C	PKC	0.25μM	CALBIOCHEM
5	GF 109203X	PKC	0.1μM	CALBIOCHEM
6	Staurosporine	PKC	0.1μM	CALBIOCHEM
7	RO-3306	Cdk1	20μM	CALBIOCHEM
8	W-7	CaMKII	50μM	CALBIOCHEM
9	SB216763	GSK3β	10μM	Tocris bioscience
10	SB218078	Chk1	10μM	CALBIOCHEM
11	Chk2 inhibitor II	Chk2	10μM	CALBIOCHEM
12	Rottlerin	PKC	5μM	CALBIOCHEM
13	Caffeine	ATM/ATR	5mM	Sigma

Supplementary Table S1. Protein kinase inhibitors used in this study.



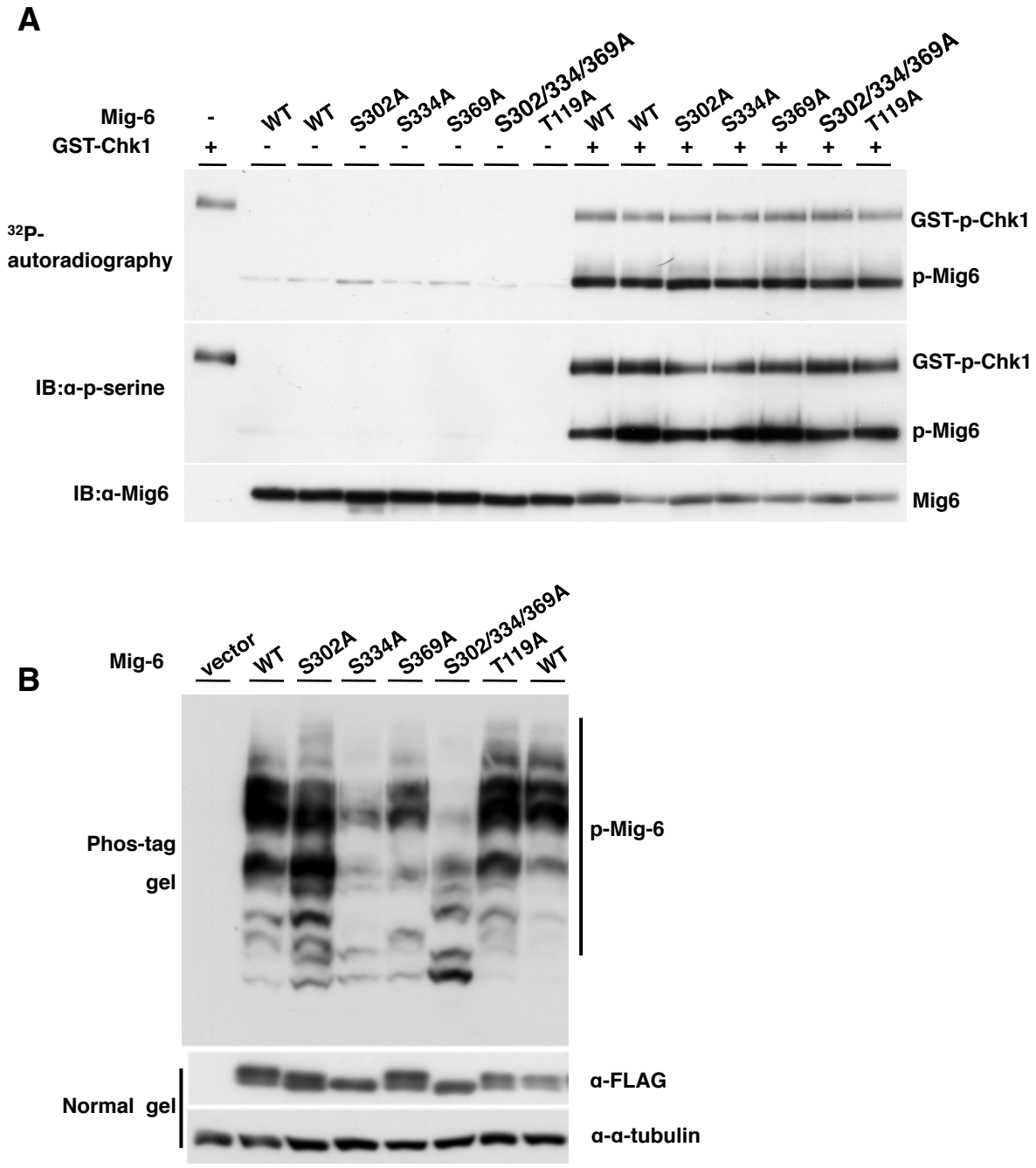
Supplementary Figure S1. Effects of protein kinase inhibitors on Mig-6 phosphorylation *in vivo*.

HEK293 cells were transfected with FLAG-Mig-6 and treated with the indicated kinase inhibitors for 3 h before harvesting. FLAG-Mig-6 was immunoprecipitated with anti-FLAG antibody from the cell lysates and p-Mig-6 was detected by the Phos-tag biotin system. Details of the kinase inhibitors are indicated in Supplementary Table 1.

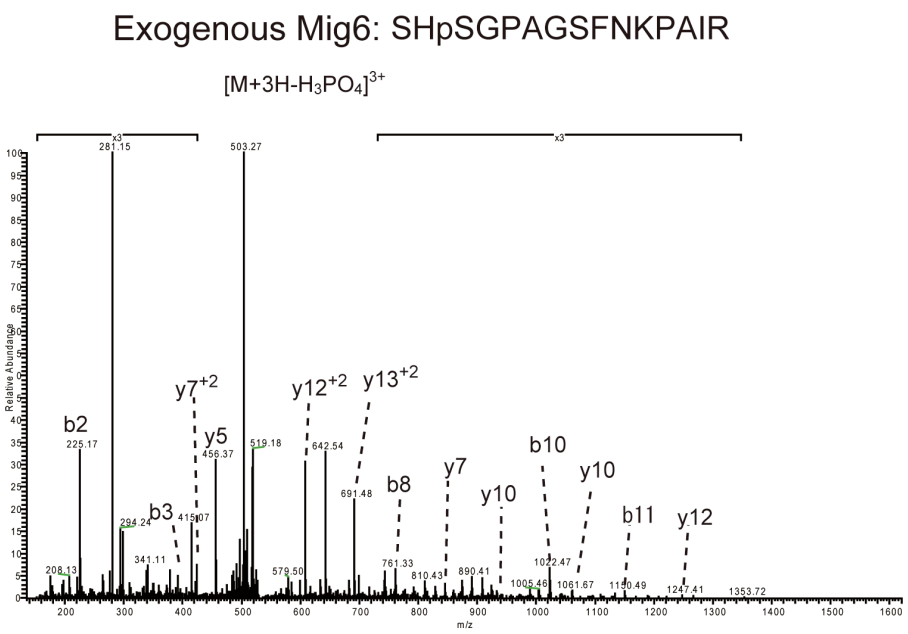


Supplementary Figure S2. EGF-promoted phosphorylation of Mig-6 is suppressed by Chk1 depletion.

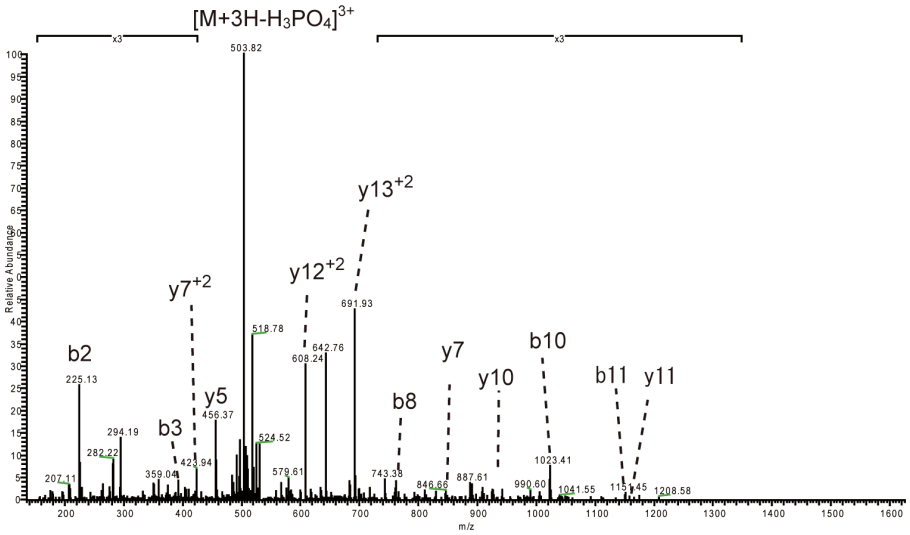
A. MDA-MB-231 cells were transfected with another siRNA oligo, *Chk1*(04). Quiescence was induced by 16 h serum starvation, and then the cells were stimulated with 20 ng/ml EGF for 15 min. Cell lysates were separated by 6% Phos-tag SDS-PAGE or normal SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. **B.** MDA-MB-231 cells were made quiescent by 16 h serum starvation, and then stimulated with 20 ng/ml EGF for 15 min. Cell lysates were incubated in 20 μl 0.05 mM of Tris-HCl (PH 8.0), with or without 1 unit Calf Intestine Alkaline Phosphatase (CIAP) at 37°C for 30 min. The reaction was stopped by the addition of SDS sample buffer and then the proteins were separated by Phos-tag SDS-PAGE or normal SDS-PAGE followed by immunoblotting. **C.** MDA-MB-231 cells were made quiescent by 16 h serum starvation, pretreated with or without 10 μM MG132 for 5 h, then stimulated with 20 ng/ml EGF for 15 min. Mig-6 was immunoprecipitated with anti-Mig-6 antibody and then immunoblotted with anti-ubiquitin antibody to detect ubiquitylation of Mig-6. (✱: nonspecific band.)


A
Mig-6
GST-Chk1
- WT WT S302A S334A S369A S302/334/369A T119A WT WT S302A S334A S369A S302/334/369A T119A
+ - - - - - - - + + + + + + + +
32P-autoradiography
GST-p-Chk1
p-Mig6
IB:α-p-serine
GST-p-Chk1
p-Mig6
IB:α-Mig6
Mig6
B
Mig-6
vector WT S302A S334A S369A S302/334/369A T119A WT
Phos-tag gel
p-Mig-6
Normal gel
α-FLAG
α-α-tubulin

A


Exogenous Mig6: SHpSGPAGSFNKPAIR
[M+3H-H3PO4]3+
Relative Abundance
m/z
b2
y5
y7+2
y12+2
y13+2
b3
b8
y7
y10
b10
y10
b11
y12

B

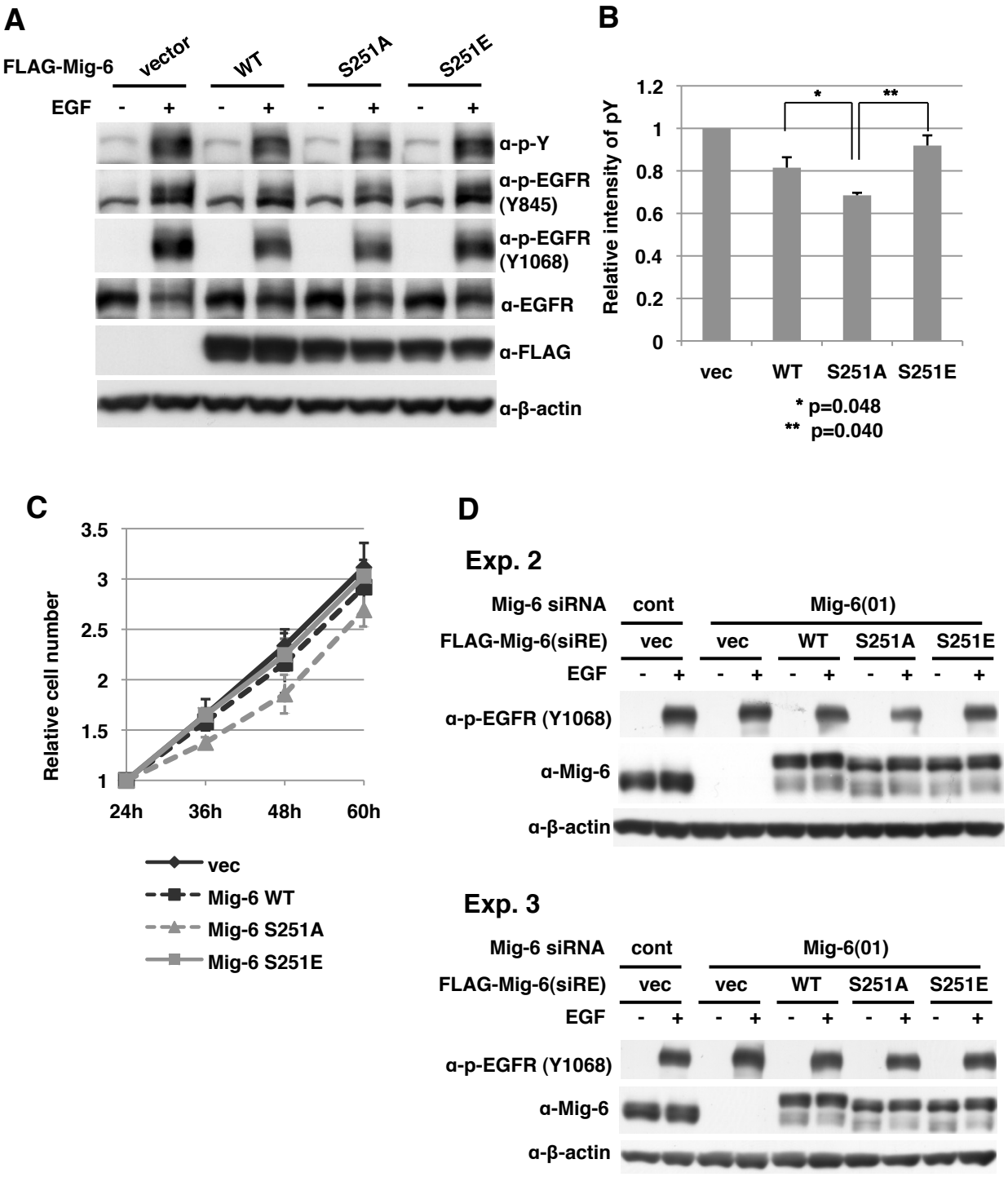

In vitro phosphorylation: SHpSGPAGSFNKPAIR
[M+3H-H3PO4]3+
Relative Abundance
m/z
b2
y5
y7+2
y12+2
y13+2
b3
b8
y7
y10
b10
b11
y11

Supplementary Figure S3. Phosphorylation status of wild-type and mutant Mig-6.

A. *In vitro* phosphorylation assays of mutant recombinant Mig-6 proteins. Wild-type (WT) and the indicated mutant recombinant Mig-6 proteins (0.1 μg) were incubated in 20 μl of kinase buffer with 32P-labeled ATP and 0.1 μg of purified recombinant GST-Chk1 kinase at 30°C for 30 min. The reaction was stopped by the addition of SDS sample buffer and then the proteins were separated by SDS-PAGE. Phosphorylated Mig-6 was analyzed by 32P-autoradiography or immunoblotting (IB) with anti-phospho-serine antibody. **B**. Comparison of the phosphorylation status between wild-type and mutant Mig-6 in HEK293 cells. HEK293 cells were transfected with wild-type or the indicated mutant FLAG-Mig-6. After 48 h, cells were harvested, and the cell lysates were separated by 6% Phos-tag SDS-PAGE (upper panel) or normal SDS-PAGE and immunoblotted.

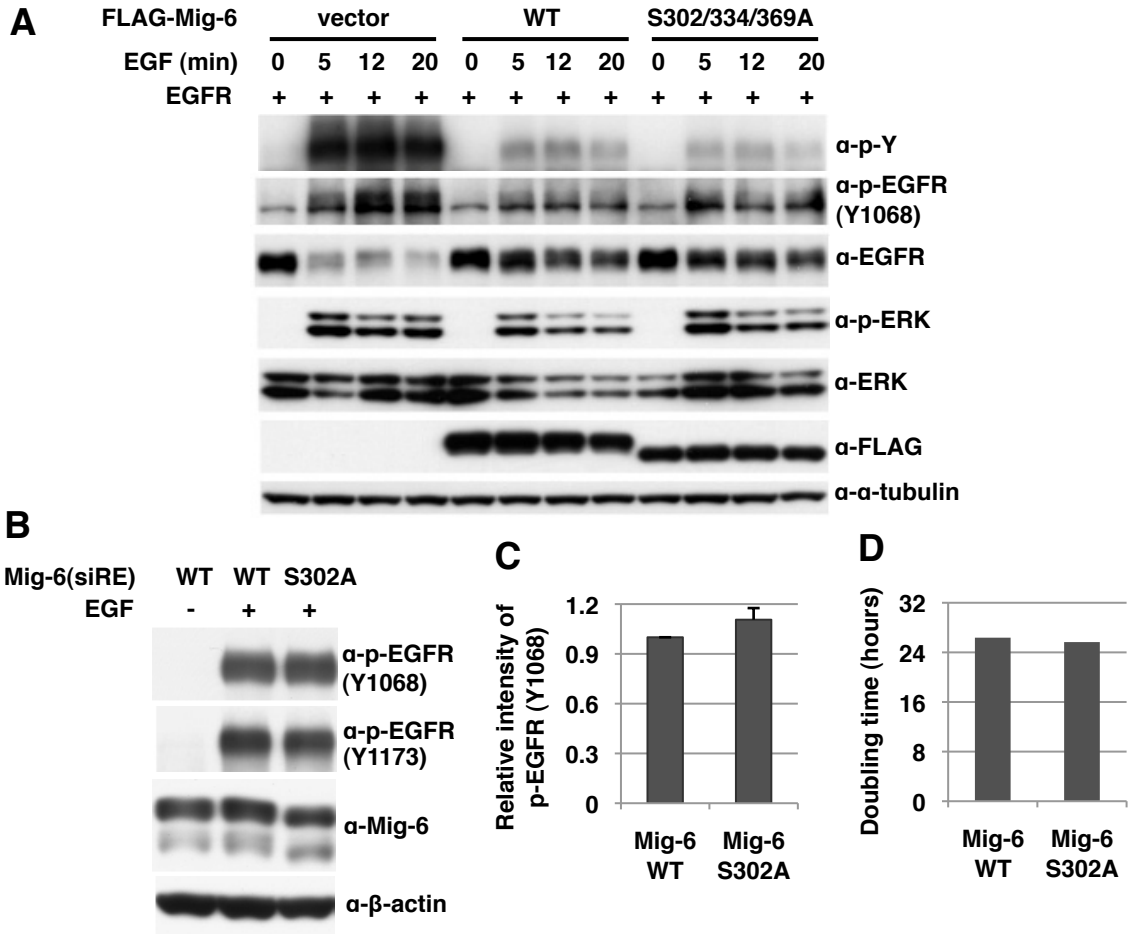
Supplementary Figure S4. Analysis of Mig-6 phosphorylation sites.

A. Identification of S251 phosphorylation of exogenous Mig-6 using MS. HEK293 cells were transfected with FLAG-Mig-6, quiescence was induced by 16 h serum starvation, then the cells were treated with 20 ng/ml EGF for 15 min. FLAG-Mig-6 was immunoprecipitated with anti-FLAG antibody from the lysate and was resolved by SDS-PAGE. After tryptic digestion of the FLAG-Mig-6 band, the phosphorylated peptides were analyzed by LC-MS. The spectrum of the charged ion (m/z 503.27) shows that S251 is phosphorylated in the indicated peptide. **B.** Identification of S251 phosphorylation of recombinant Mig-6 using MS. Recombinant Mig-6 protein (0.1 μg) was incubated in 20 μl of kinase buffer with ^{32}P-labeled ATP and 0.1 μg of purified recombinant GST-Chk1 kinase at 30°C for 30 min. The reaction was stopped by the addition of SDS sample buffer, then the proteins were separated by SDS-PAGE, followed by tryptic digestion and LC-MS. The spectrum of the charged ion (m/z 503.82) shows that S251 is phosphorylated in the indicated peptide. b ions, fragmentation ions containing the amino terminus of the peptide; y ions, fragmentation ions containing the carboxy terminus of the peptide.


A
FLAG-Mig-6
vector
WT
S251A
S251E
EGF
α-p-Y
α-p-EGFR (Y845)
α-p-EGFR (Y1068)
α-EGFR
α-FLAG
α-β-actin
B
Relative intensity of pY
vec
WT
S251A
S251E
* p=0.048
** p=0.040
C
Relative cell number
24h
36h
48h
60h
vec
Mig-6 WT
Mig-6 S251A
Mig-6 S251E
D
Exp. 2
Mig-6 siRNA
cont
Mig-6(01)
FLAG-Mig-6(siRE)
α-p-EGFR (Y1068)
α-Mig-6
α-β-actin
Exp. 3

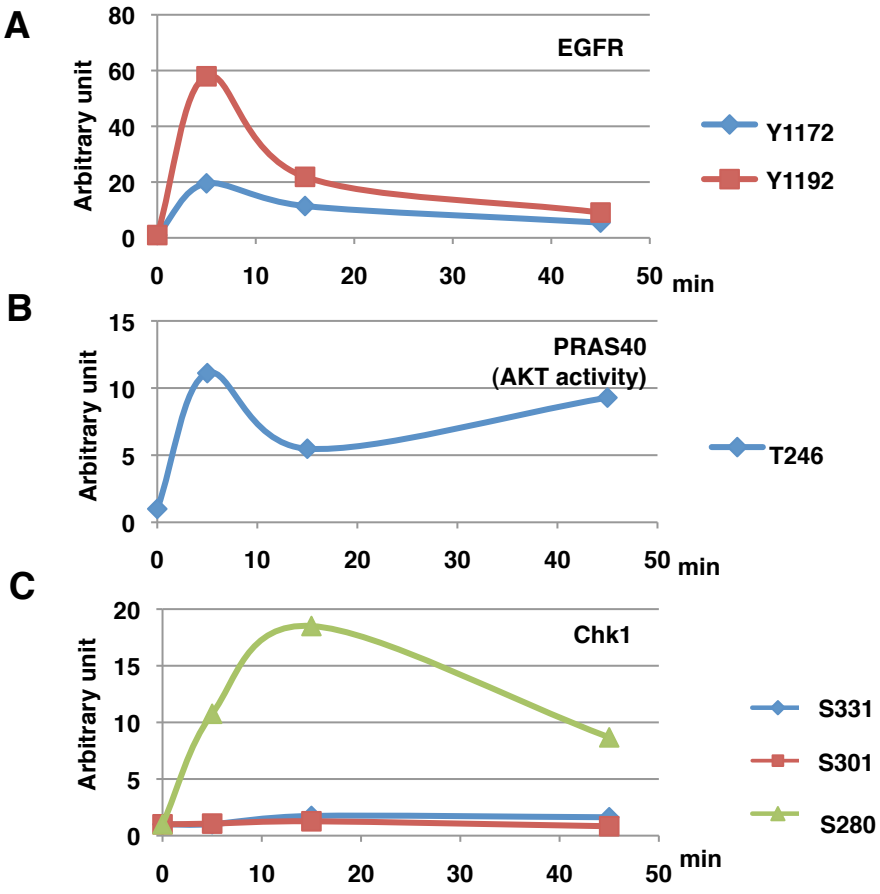
Supplementary Figure S5. Effect of Mig-6 S251 phosphorylation on EGF signaling.

A. Effect of Mig-6 S251 mutation on EGF signaling in HEK 293 cells. Wild type (WT), S251A, or S251E mutant FLAG-Mig-6 was expressed in HEK293 cells. After 16 h serum starvation, cells were stimulated with 20 ng/ml EGF, then harvested 15 min later. Cell lysates were separated using SDS-PAGE followed by immunoblotting with the indicated antibodies. **B.** Intensities of the EGF-induced phospho-tyrosine of EGFR (p-Y) in (A) (lane 2, lane 4, lane 6 vs. lane 8) were quantified by image analysis. The data from triplicate experiments were evaluated statistically and are shown graphically relative to the vector only (vec). **C.** Effect of S251 status on cell growth. Wild-type, S251A, or S251E mutant FLAG-Mig-6 was transfected into HEK293 cells. The cell growth was evaluated at the indicated times. Error bars indicate the standard deviation of three independent experiments. **D.** Effect of Mig-6 S251 mutation on EGF signaling in MDA-MB-231 cells. MDA-MB-231 cells were infected with retroviruses encoding Mig-6 wild type (WT), S251A, or S251E, and then transfected with an siRNA targeting *Mig-6*. After 16 h serum starvation, cells were stimulated with 20 ng/ml EGF for 15 min. Cell lysates were separated with SDS-PAGE followed by immunoblotting. The experiments were repeated 3 times and indicated in Fig 4A (Exp. 1) and the supplementary Fig S5D (Exp.2 and 3). The level of p-EGFR (Y1068) protein was measured and normalized to the level of β-actin. The triplicate data were statistically analyzed in Fig 4B.



Supplementary Figure S6. Effect of EGFR-binding domain mutations in Mig-6 on EGF signaling.

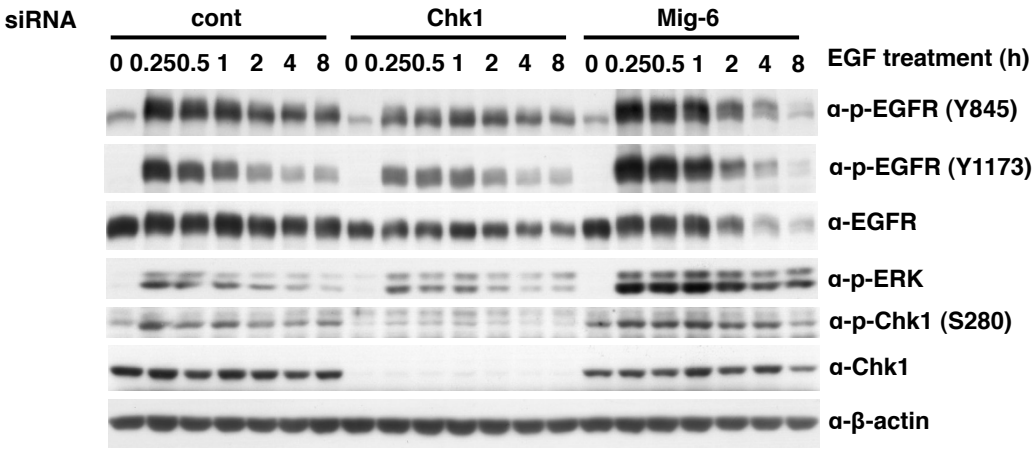
A. Wild-type or S302/334/369A mutant FLAG-Mig-6 was co-expressed with EGFR in HEK293 cells. After 16 h serum starvation, cells were stimulated with 20 ng/ml EGF, then harvested at the indicated times. Cell lysates were separated by SDS-PAGE followed by immunoblotting. **B.** Effect of Mig-6 S302A mutation on EGF signaling. MDA-MB-231 cells were infected with retroviruses encoding wild type (WT), or S302A mutant Mig-6, and then transfected with an siRNA targeting *Mig-6*. After 16 h serum starvation, cells were stimulated with 20 ng/ml EGF for 15 min. Cell lysates were separated with SDS-PAGE followed by immunoblotting. **C.** The intensity of p-EGFR (Y1068) protein in (B) was quantified by image analysis. The data from triplicate experiments were evaluated statistically and are shown graphically. **D.** Effect of S302 status on cell growth. MDA-MB-231 cells were infected with retroviruses encoding wild type (WT), or S302A mutant Mig-6, and then treated with an siRNA for *Mig-6*. Cell proliferation assays were performed. The doubling times are indicated graphically.

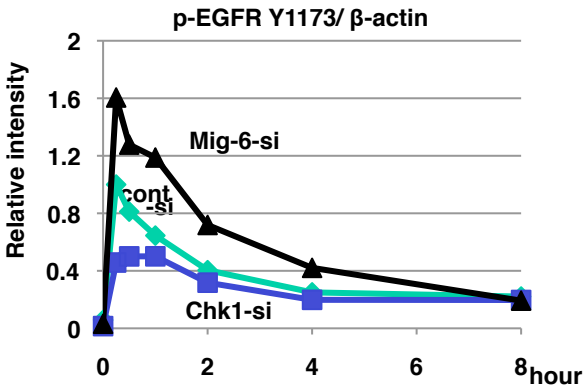


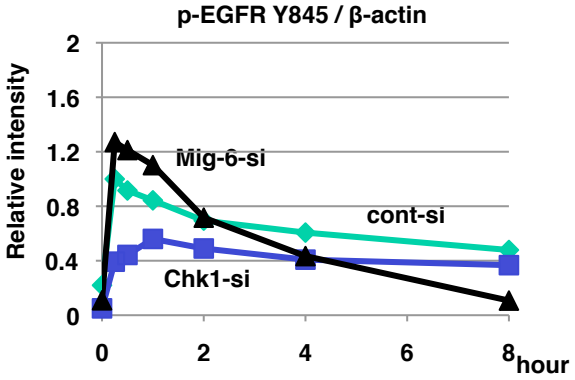
Supplementary Figure S7. EGF-promoted phosphorylation of Chk1 at S280.

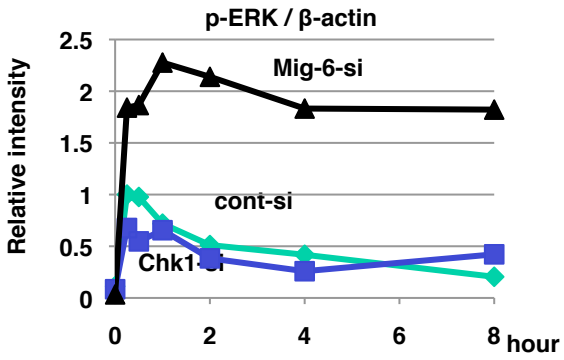
A–C. EGF signaling promotes activation of PI3K/Akt and phosphorylation of Chk1 at S280. Quiescence was induced in HeLa cells by 16 h serum starvation, the cells were then treated with EGF and harvested at the indicated times. The whole cell lysates were subjected to phospho-iTRAQ (see Materials and Methods). Oscillations in the phosphorylation of EGFR (Y1172, Y1192), PRAS40 (T246), and Chk1 (S280, S301, S331) are indicated.

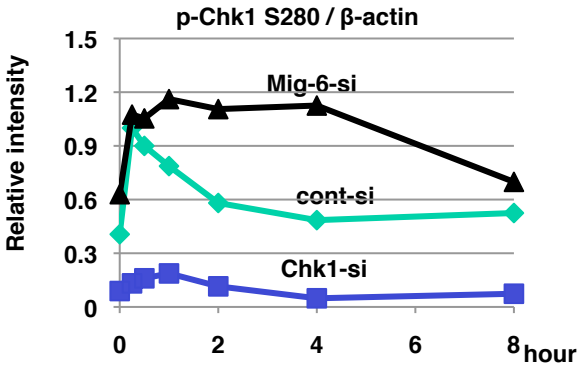
Exp. 2

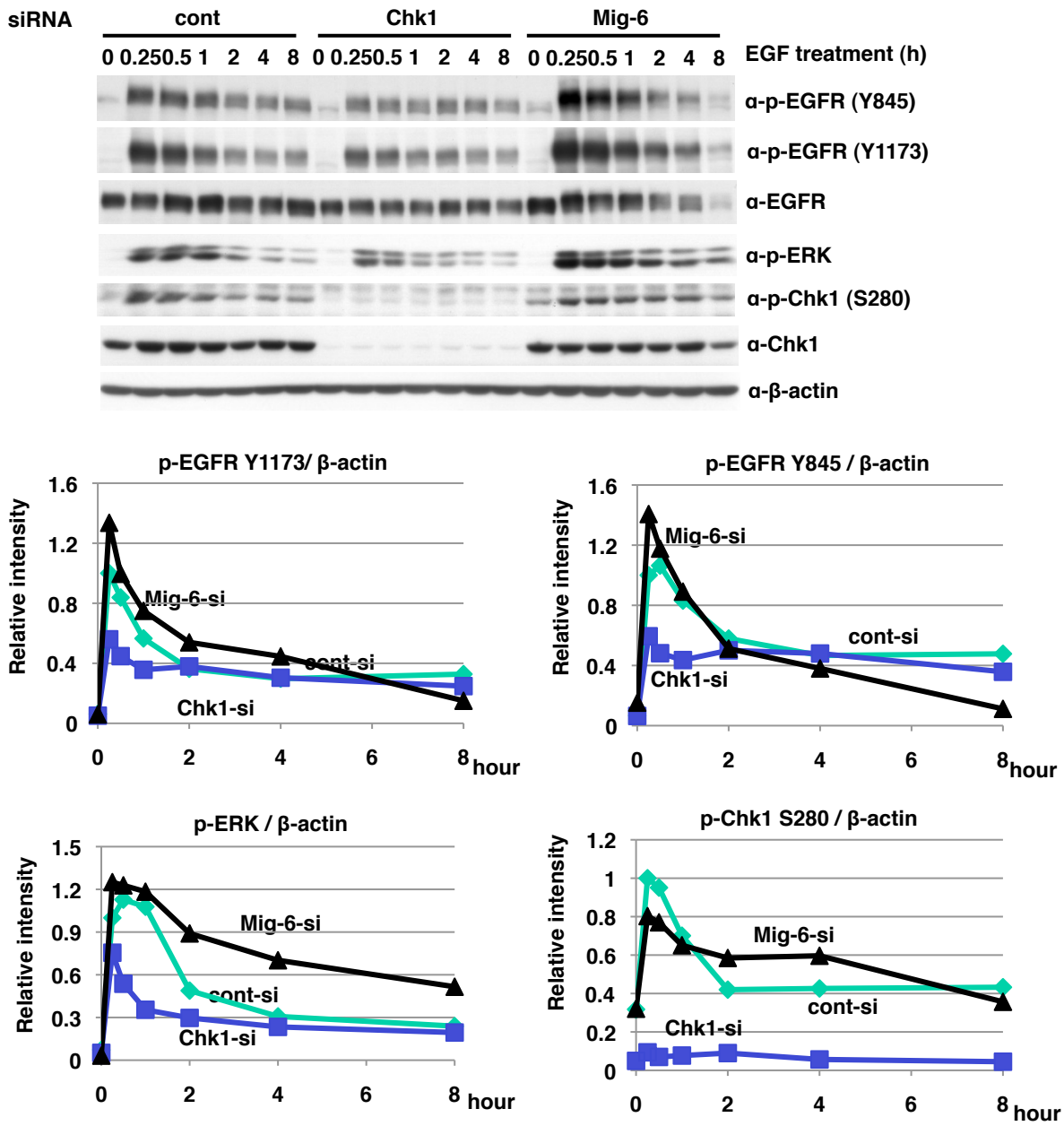

siRNA
cont
Chk1
Mig-6
0 0.250.5 1 2 4 8 0 0.250.5 1 2 4 8 0 0.250.5 1 2 4 8
EGF treatment (h)
α-p-EGFR (Y845)
α-p-EGFR (Y1173)
α-EGFR
α-p-ERK
α-p-Chk1 (S280)
α-Chk1
α-β-actin


p-EGFR Y1173/ β-actin
Relative intensity
Mig-6-si
cont-si
Chk1-si
hour


p-EGFR Y845 / β-actin
Relative intensity
Mig-6-si
cont-si
Chk1-si
hour

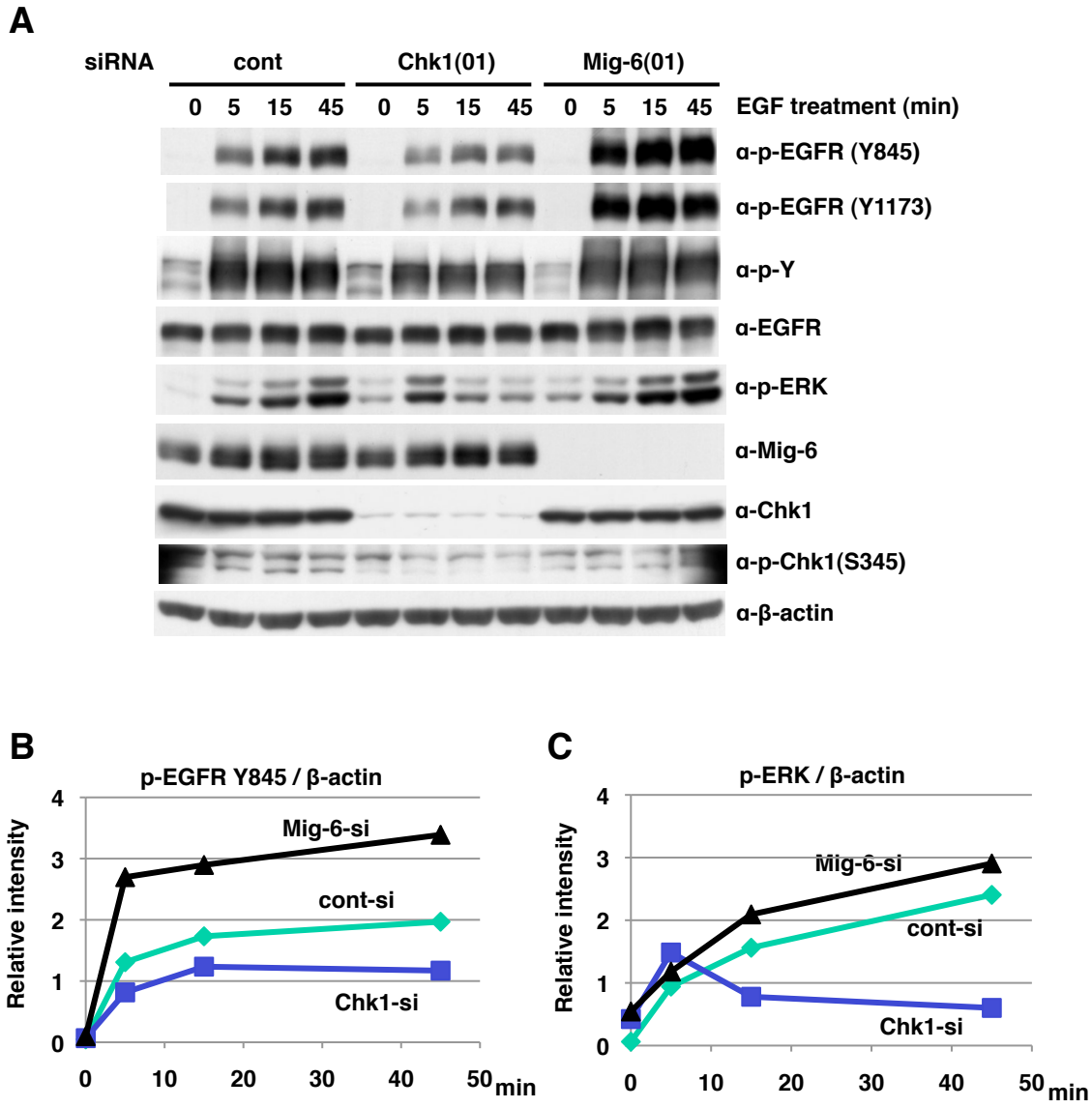

p-ERK / β-actin
Relative intensity
Mig-6-si
cont-si
Chk1-si
hour


p-Chk1 S280 / β-actin
Relative intensity
Mig-6-si
cont-si
Chk1-si
hour



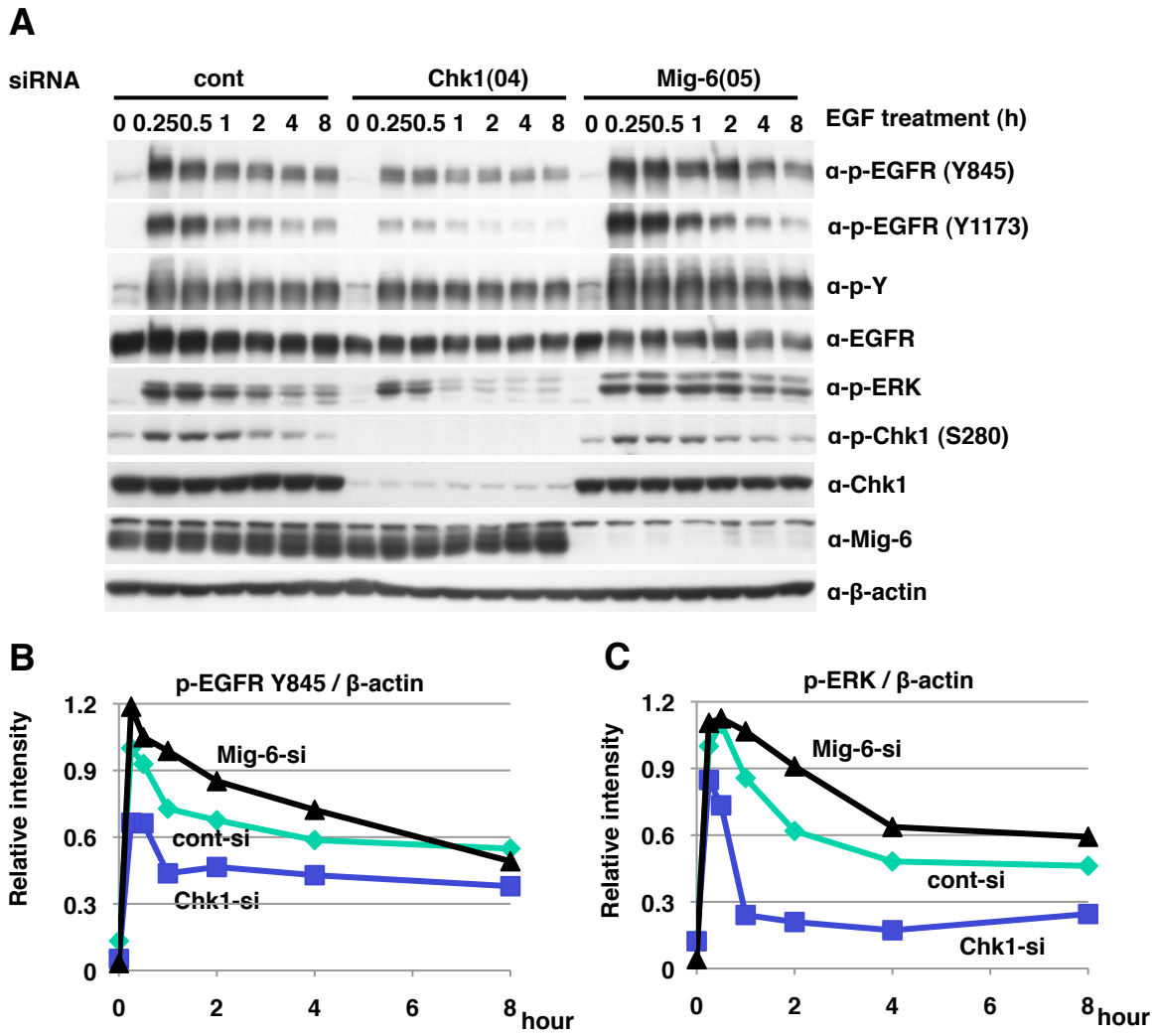
Supplementary Figure S8. Modulation of EGFR phosphorylation in MDA-MB-231 cells depleted of Chk1.

MDA-MB-231 cells were transfected with an siRNA for human *Chk1*(01*)* or *Mig-6*(01), or a control siRNA (cont). Quiescence was induced by 16 h serum starvation, then the cells were stimulated with 20 ng/ml EGF for the indicated times. Cell lysates were separated by SDS-PAGE and subjected to immunoblotting with the indicated antibodies. The experiments were repeated 3 times and indicated in Fig 6A (Exp.1) and the supplementary Fig S8 (Exp.2 and 3). The levels of phosphorylated proteins were measured and normalized to the level of β-actin.



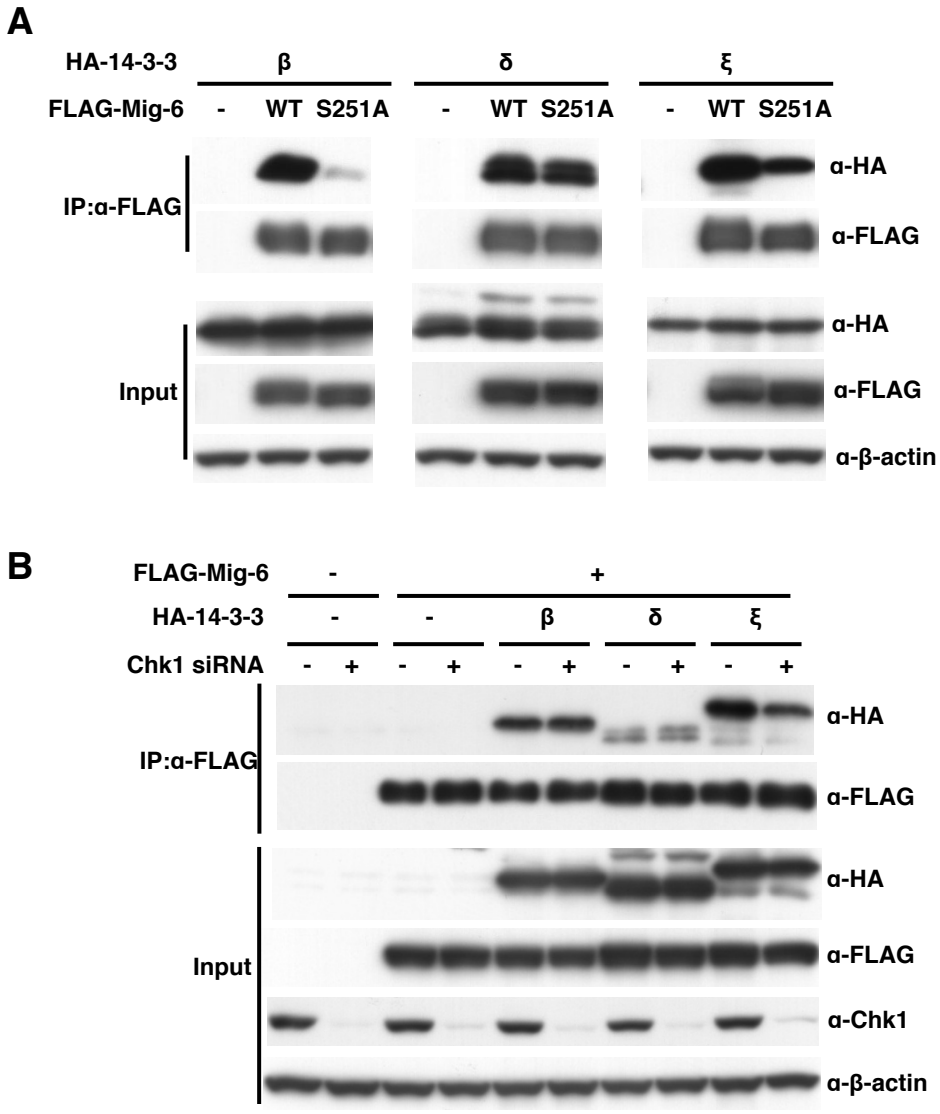
Supplementary Figure S9. Modulation of EGFR phosphorylation in MDA-MB-231 cells depleted of Chk1.

A. MDA-MB-231 cells were transfected with an siRNA for human *Chk1*(01) or *Mig-6*(01), or a control siRNA (cont). Quiescence was induced by 16 h serum starvation, then the cells were stimulated with 20 ng/ml EGF for the indicated times. Cell lysates were separated by SDS-PAGE and subjected to immunoblotting with the indicated antibodies. **B, C.** Quantification of the levels of phosphorylated proteins in (A) were normalized to the level of β-actin.



Supplementary Figure S10. Modulation of EGFR phosphorylation in MDA-MB-231 cells in early stage of EGF stimulation.

A. MDA-MB-231 cells were transfected with an siRNA for human *Chk1*(04) or *Mig-6*(05), or a control siRNA (cont) , serum starved for 16 h, then stimulated with 20 ng/ml EGF for 5 min, 15 min, or 45 min. The cell lysates were analyzed by immunoblotting with the indicated antibodies. **B, C.** Quantification of the levels of phosphorylated proteins in (A) were normalized to the level of β-actin.



Supplementary Figure S11. Phosphorylation of Mig-6 at S251 affects on binding of Mig-6 to 14-3-3.

A. HA-14-3-3 ξ, β, or δ, were co-expressed with FLAG-Mig-6 in HEK293 cells. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with anti-HA antibody. **B.** Depletion of Chk1 inhibited binding of Mig-6 to 14-3-3 ξ. HA-14-3-3 ξ, β, or δ were co-expressed with FLAG-Mig-6 in HEK293 cells. Then Chk1 was depleted by siRNA transfection. The cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with the indicated antibodies.