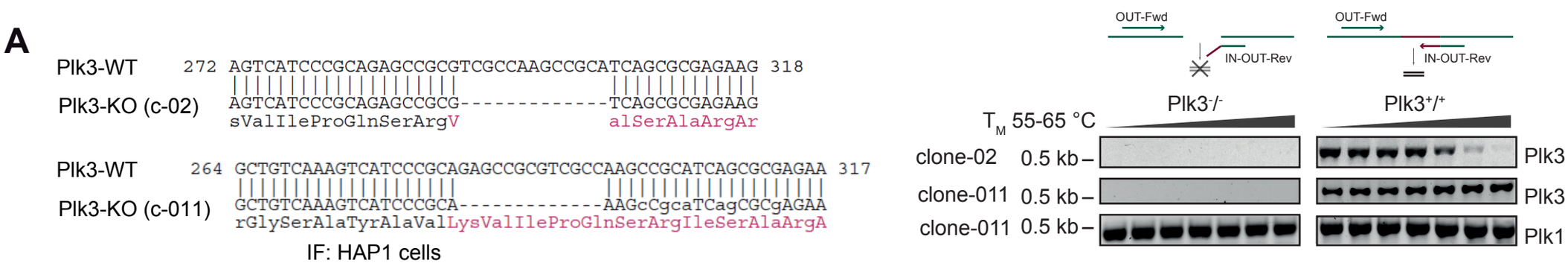
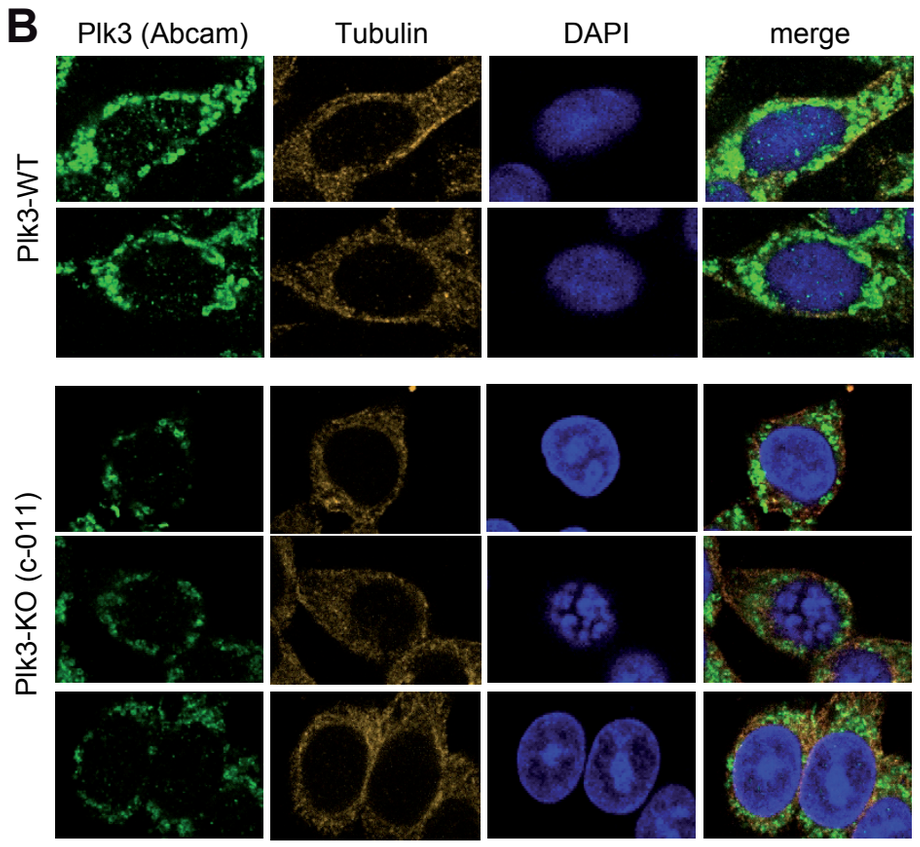
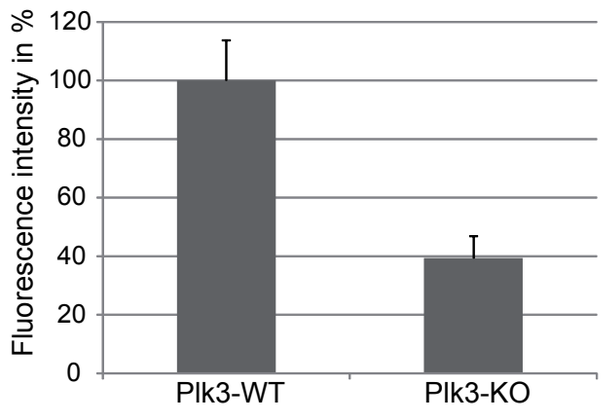
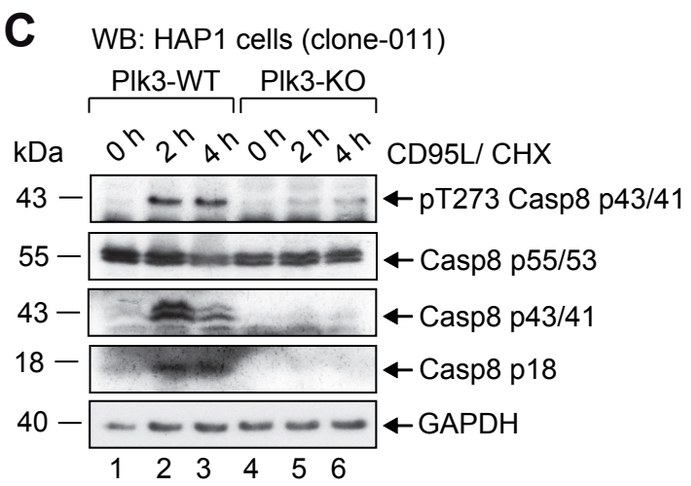
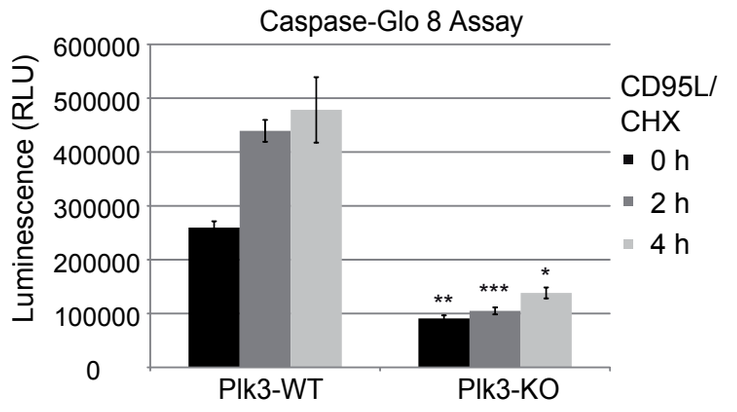
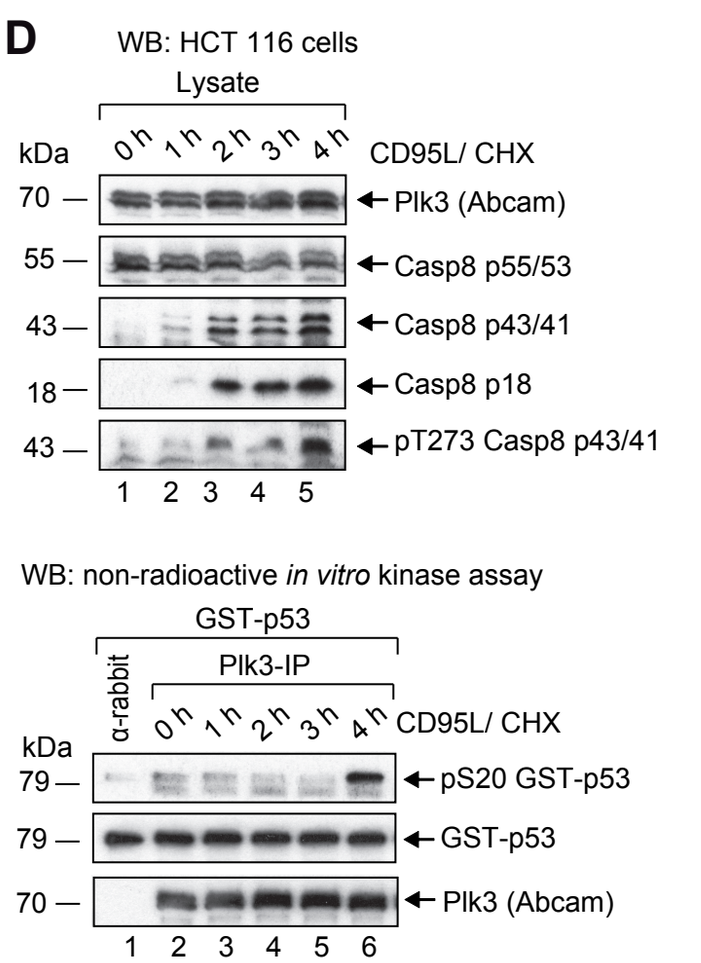

A
Plk3-WT 272 AGTCATCCCGCAGAGCCGCGTCGCCAAGCCGCATCAGCGCGAGAAG 318
Plk3-KO (c-02) AGTCATCCCGCAGAGCCGCG----------TCAGCGCGAGAAG
sValIleProGlnSerArgV alSerAlaArgAr
Plk3-WT 264 GCTGTCAAAGTCATCCCGCAGAGCCGCGTCGCCAAGCCGCATCAGCGCGAGAA 317
Plk3-KO (c-011) GCTGTCAAAGTCATCCCGCA----------AAGcCgcaTCagCGCgAGAA
rGlySerAlaTyrAlaValLysValIleProGlnSerArgIleSerAlaArgA
IF: HAP1 cells
OUT-Fwd
IN-OUT-Rev
OUT-Fwd
IN-OUT-Rev
Plk3-/-
Plk3+/+
T M 55-65 °C
clone-02 0.5 kb –
clone-011 0.5 kb –
clone-011 0.5 kb –
Plk3
Plk3
Plk1

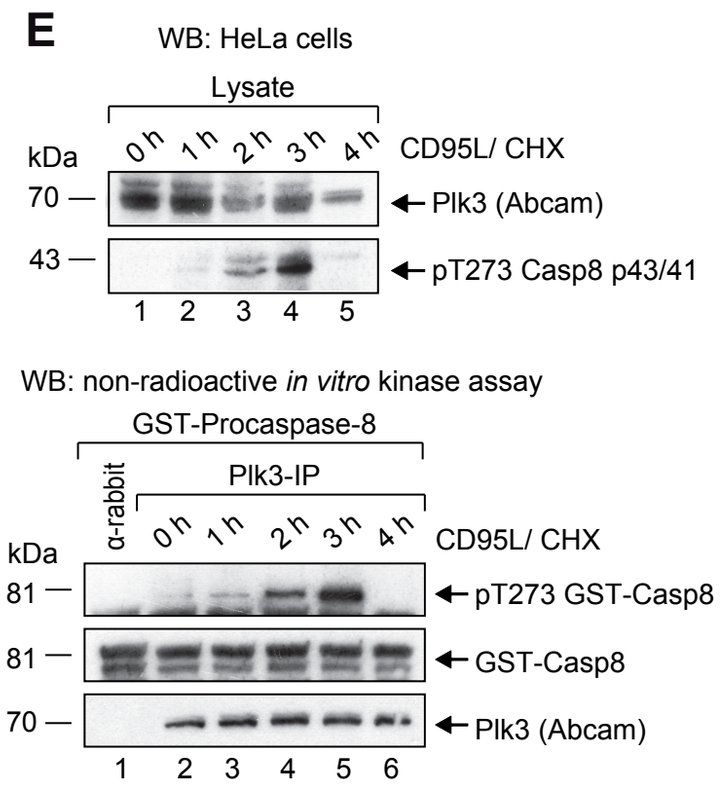

B
Plk3 (Abcam)
Tubulin
DAPI
merge
Plk3-WT
Plk3-KO (c-011)

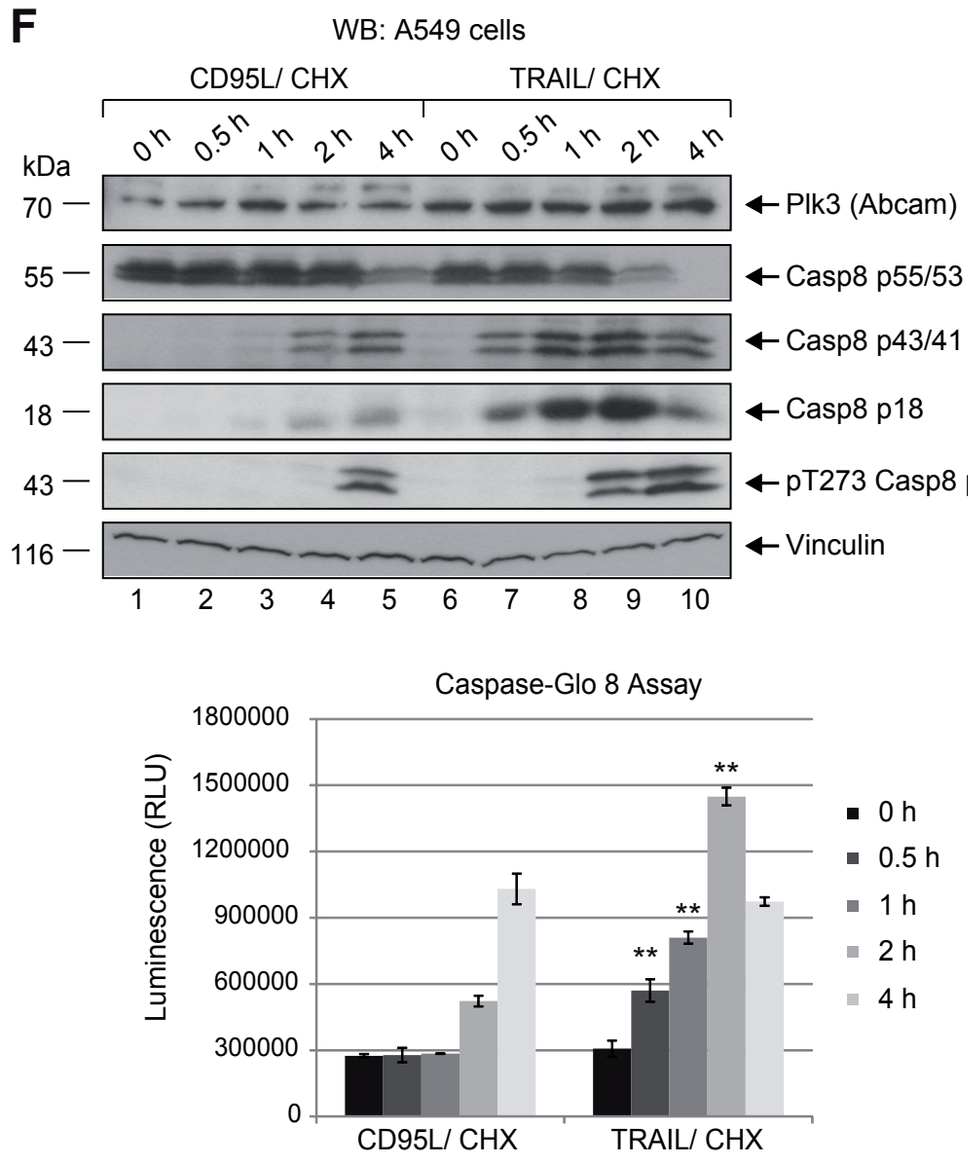

Fluorescence intensity in %
120
100
80
60
40
20
0
Plk3-WT
Plk3-KO

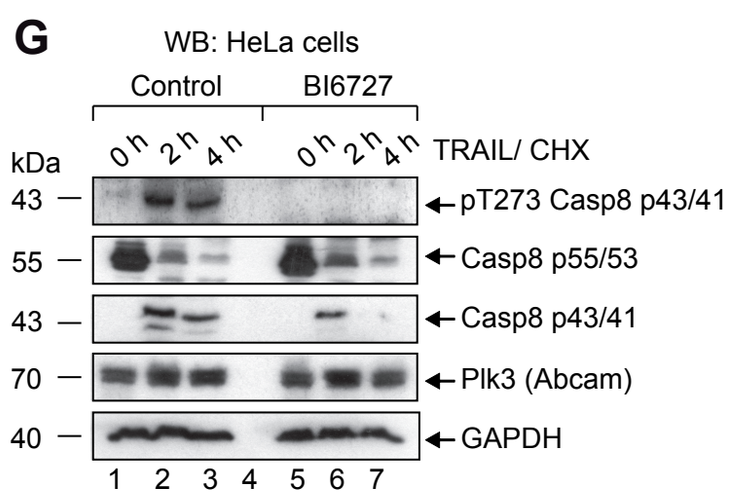

C
WB: HAP1 cells (clone-011)
Plk3-WT
Plk3-KO
kDa
0 h 2 h 4 h 0 h 2 h 4 h
CD95L/ CHX
43 — ← pT273 Casp8 p43/41
55 — ← Casp8 p55/53
43 — ← Casp8 p43/41
18 — ← Casp8 p18
40 — ← GAPDH
1 2 3 4 5 6


Caspase-Glo 8 Assay
Luminescence (RLU)
600000
500000
400000
300000
200000
100000
0
CD95L/ CHX
0 h
2 h
4 h
**
***
*
Plk3-WT
Plk3-KO


D
WB: HCT 116 cells
Lysate
kDa
0 h 1 h 2 h 3 h 4 h
CD95L/ CHX
70 — ← Plk3 (Abcam)
55 — ← Casp8 p55/53
43 — ← Casp8 p43/41
18 — ← Casp8 p18
43 — ← pT273 Casp8 p43/41
1 2 3 4 5
WB: non-radioactive in vitro kinase assay
GST-p53
Plk3-IP
α-rabbit
0 h 1 h 2 h 3 h 4 h
CD95L/ CHX
kDa
79 — ← pS20 GST-p53
79 — ← GST-p53
70 — ← Plk3 (Abcam)
1 2 3 4 5 6


E
WB: HeLa cells
Lysate
kDa
0 h 1 h 2 h 3 h 4 h
CD95L/ CHX
70 — ← Plk3 (Abcam)
43 — ← pT273 Casp8 p43/41
1 2 3 4 5
WB: non-radioactive in vitro kinase assay
GST-Procaspase-8
Plk3-IP
α-rabbit
0 h 1 h 2 h 3 h 4 h
CD95L/ CHX
kDa
81 — ← pT273 GST-Casp8
81 — ← GST-Casp8
70 — ← Plk3 (Abcam)
1 2 3 4 5 6


F
WB: A549 cells
CD95L/ CHX
TRAIL/ CHX
0 h 0.5 h 1 h 2 h 4 h 0 h 0.5 h 1 h 2 h 4 h
kDa
70 — ← Plk3 (Abcam)
55 — ← Casp8 p55/53
43 — ← Casp8 p43/41
18 — ← Casp8 p18
43 — ← pT273 Casp8 p43/41
116 — ← Vinculin
1 2 3 4 5 6 7 8 9 10
Caspase-Glo 8 Assay
Luminescence (RLU)
1800000
1500000
1200000
900000
600000
300000
0
0 h
0.5 h
1 h
2 h
4 h
**
**
**
CD95L/ CHX
TRAIL/ CHX


G
WB: HeLa cells
Control
BI6727
0 h 2 h 4 h 0 h 2 h 4 h
TRAIL/ CHX
kDa
43 — ← pT273 Casp8 p43/41
55 — ← Casp8 p55/53
43 — ← Casp8 p43/41
70 — ← Plk3 (Abcam)
40 — ← GAPDH
1 2 3 4 5 6 7

Supplementary Information, Figure S5. Phosphorylation of caspase-8 by Plk3.

(**A**) Plk3-KO (HZGHC003037c002 and HZGHC003037c011) HAP1 cells were purchased from Horizon Genomics. HAP1 Plk3-KO clones were engineered using CRISPR/Cas9. Exon 2 (NM_004073) was selected for guide RNA design (GCTGATGCGGCTTGGCGACG). Both HAP1 clones contain a 13 bp-deletion in exon 2 of the Plk3 gene locus causing a frameshift. The frameshift in Plk3-KO HAP1 cells was verified by sequencing (left panel) and by PCR (right panel). The translation of the coding region of the HAP1 Plk3-KO clones leads to a premature stop codon (left panel). Schematic representation of the in/out micro-deletion PCR is shown in upper right panel. The forward primer binds 5' outside (out) the deletion. The reverse primer spans the wildtype (out) and the deleted (in) region reflecting the deletion of the knockout clone. Two HAP1 clones were conducted to the in/out micro-deletion PCR (lower right panel). To obtain a high primer specificity a temperature gradient between 55 - 65 °C was used. A Plk1-specific primer pair served as a control. (**B**) Parental Plk3-WT and Plk3-KO (clone-c011) HAP1 cells were tested using immunofluorescence microscopy. Cells were stained for Plk3 (Abcam), Tubulin and DNA. The quantification of fluorescence intensity for Plk3 is shown on the right. (**C**) Plk3-WT and Plk3-KO (clone-011) HAP1 cells were treated with CD95L and CHX for the indicated time periods. The lysates were blotted against pT273 Casp8, Casp8, cleaved Casp8 and β-Actin (left panel). Caspase-8 activity was determined using a Caspase-Glo 8 assay (right panel). Each bar represents the mean value ± s.d. (n=3). The differences between Plk3-WT cells and Plk3-KO cells were statistically significant by *Student's t-test* (*P≤0.05; **P≤0.005; ***P≤0.0005). (**D**) HCT 116 cells were treated with CD95L and CHX for the indicated time periods. Endogenous Plk3 was immunoprecipitated and incubated with recombinant GST-fused p53. Cell lysates (upper panel) were immunoblotted against Plk3, Casp8, cleaved Casp8 and pT273 Casp8. Samples from *in vitro* kinase assays (lower panel) were immunoblotted against pS20 p53, GST and Plk3. (**E**) HeLa cells were treated with CD95L and CHX for the indicated time periods. Endogenous Plk3 was immunoprecipitated and incubated with recombinant GST-fused Casp8-WT. Cell lysates (upper panel) and samples from *in vitro* kinase assays (lower panel) were immunoblotted against pT273 Casp8, GST and Plk3. (**F**) A549 were treated with CD95L or TRAIL and CHX for the

indicated time periods. The lysates were immunoblotted against Plk3, Casp8, cleaved Casp8, pT273 Casp8 and Vinculin (upper panel). Caspase-8 activity was determined by Caspase-Glo 8 Assay (lower panel). Each bar represents the mean value ± s.d. (n=3). The differences between CD95L and TRAIL treatment were statistically significant by *Student's t-test* (**P≤0.005). (**G**) HeLa cells were pretreated with 2 µM BI6727 for 2 h. Untreated cells were used as a control (Control). Subsequently cells were treated with 50 ng/mL TRAIL and CHX in the absence (Control) or in the presence of 2 µM BI6727 for the indicated time periods. The lysates were immunoblotted against Plk3, Casp8, cleaved Casp8, pT273 Casp8 and GAPDH.