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Supplementary Materials for

Tousled-like kinases stabilize replication forks and show synthetic lethality with checkpoint and PARP inhibitors

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Supplementary Materials and Methods

Oligo sequences for siRNA and CRISPR

We used MISSION® siRNA Universal Negative Control #1 from Sigma (SIC001), Silencer® Select Negative Control #2 from Thermo Fisher (cat. 4390846; Fig. 5C only) or siGFP: GGCUACGUCCAGGAGCGCCGCACC as a negative control for siRNA transfection unless otherwise indicated. siTLK1#1: GAAGCUCGGUCUAUUGUAA; siTLK1#2: GCAAUGACUUGGAUUUCUA; siTLK2#1: GGAGGGAAGAAUAGAUGAU; siTLK2#2: GGAAAGGAUAAAUUCACAG; siASF1a: AAGUGAAGAAUACGAUCAAGU; siASF1a (Fig. 5C only): GAGACAGAAUUAAGGGAAA; siASF1b: AACAACGAGUACCUCAACCCU; siASF1b (Fig. 5C only): CGGACGACCUGGAGUGGAA; sip53: GCAGUCAGAUCCUAGCGUC; siFLASH#1: CCGCAAGGAUGAAGAAAUA; siFLASH#2 (Fig. 5C only): AGAUAAAGACAGUAGGAAA (*5*).

For drug treatment we used: Roscovitine (Sigma), 50 μM for 2 hours; nocodazole (Sigma), 40 ng/ml for 10 hours; UCN-01 (Sigma), 0.3 μM for 2 hours (4 hours for PFGE and viability assays); MK-8776 (Selleck Chemicals), 1 μM for 4 hours; Gö-6976 (Millipore), 1 μM for 4 hours; AZ20 (Tocris), 1 μM for 4 hours; AZD7762 (Selleck Chemicals), 100 nM for 24 hours; Olaparib (Selleck Chemicals), 1 μM for 24 hours; ETP-46464 (Calbiochem), 5 μM for 24 hours.

Generation of knockout cell lines

Knockout MDA-MB-231 cell lines were generated using CRISPR/Cas9 genome editing. Guide RNA sequences targeting *TLK1* (guide#1 targeting exon 10, 5'-GAGGCGTAATCGATCTTGCA-3'; guide#2 targeting exon 10, 5'-AGGCGTAATCGATCTTGCAT-3') and *TLK2* (guide#4 targeting exon 7, 5'-CGACGAGTAGAACAGCCCCT-3') were designed using the Zhang Lab CRISPR Design tool (http://crispr.mit.edu/) and appropriate oligos (Sigma) were cloned into pX330-Cas9-EGFP. Cells were transfected with the pX330 plasmids and 48 hours following transfection, single cells positive for GFP were FACS-sorted at a ratio of 1 cell

per well in 96 well plates. For WT single cell clones, these were exposed to pX330-Cas9-EGFP without any targeting sequence cloned. Single cell clones were expanded and screened by Western Blot for protein levels of TLK1 and TLK2. Derivation of MEF cell lines used in fig. S3C, D was previously described (*36*).

SNAP-tag Histone based Pulse imaging

Cells were plated in Lab Tek II Chamber slides (Labclinics) 24 hours post-siRNA transfection. SNAP-labeling was initiated 48 hours post-transfection (24 h after plating). Cells were incubated with 1 uM TMR-Star (S9105S, NEB) for 30 min at 37ºC. After 2 PBS washes, cells were incubated in media for 30 min at 37ºC, followed by 2 extra PBS washes, and proceeded to pre-extraction for 5 min in 0,2% Triton/PBS on ice and fixation for 10 min in 4% PFA at room temperature. Cells were analyzed with standard immunofluorescence techniques by HTM (see above).

Short-term cell viability analysis

7,500 U-2-OS cells seeded onto 96-well plate and treated with siRNA using reverse transfection. After 24 hours, cells were treated with UCN-01 for 4 hours and allowed to recovered for 20 hours, followed by detection of cell viability using Cyto-X reagent (Tebu-bio). One representative experiment out of two biological replicates is shown, including the mean and SD from 6 technical replicates.

Antibodies

For Western blot: rabbit anti-TLK2 (kindly provided by Herman H.W. Sillje and A301-257A; Bethyl); rabbit anti-TLK1 (4125, Cell Signaling); mouse anti-H2AX pS139 (γH2AX; clone JBW301; 05-636; Millipore); mouse anti-p53 (clone Do-7; sc-47698; Santa Cruz); mouse anti-p21 (clone F-5; sc-6246; Santa Cruz); rabbit anti-H2AX (2595; Cell Signaling); mouse anti-Cdc45 (clone B01P; H00113130-B01P; Abnova); mouse anti-RPA (NA19L, Calbiochem); mouse anti-PCNA (clone PC-10; ab29; Abcam); rabbit-anti-RPA1 (clone EPR3472; ab79398; Abcam); mouse anti-RPA2 (clone 9H8/34A/αSSB34A; MS-691; Thermo Scientific); mouse anti-RPA3 (clone 11.1; ab588; Abcam); goat anti-MCM2 (A300-122A; Bethyl); rabbit anti-Orc1 (A301-892A; Bethyl);

mouse anti-ATM pS1981 (560007; BD Pharmingen); rabbit anti-Chk2 pT68 (2661; Cell Signaling); rabbit anti-p53 pS15 (9284; Cell Signaling); rabbit anti-RPA2 pS4/S8 (A300-245A; Bethyl); rabbit anti-RPA2 pS33 (A300-246A; Bethyl); rabbit anti-KAP1 pS824 (ab70369, Abcam); rabbit anti-ASF1(*7*); rabbit anti-Histone H3 (ab1791, Abcam); mouse anti-Vinculin (clone hVIN-1; V9264; Sigma); mouse anti-HA (clone F-7; sc-7392; Santa Cruz). The corresponding secondary antibodies conjugated with HRP from Jackson ImmunoResearch were used.

For immunofluorescence analysis: mouse anti-H2AX pS139 (γH2AX; clone JBW301; 05-636; Millipore); rabbit anti-H2AX pS139 (γH2Ax; sc-101696; Santa Cruz); rabbit anti-H2AX pS139 (γH2AX; 2577; Cell Signaling); mouse anti-RPA (NA19L, Calbiochem); rabbit-anti-RPA1 (clone EPR3472; ab79398; Abcam); mouse anti-PCNA (clone PC-10, sc-56, Santa Cruz); rabbit anti-PCNA (clone FL-261; sc-7907; Santa Cruz). The corresponding secondary antibodies conjugated with Alexa Fluor 488, 546, or 647 from Invitrogen were used.

For DNA fiber and combing analysis: mouse anti-ssDNA IgG2a (poly dT; MAB2024; Chemicon), mouse anti-BrdU IgG1 (clone B44; 347580; Becton Dickinson) and rat anti-BrdU IgG (clone BU1/75; ABC117 7513; AbCys SA). The corresponding secondary antibodies conjugated with Alexa Fluor 488, 546, or 647 from Invitrogen were used.

Statistical analysis of TCGA datasets

The results presented here are in part based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov/). CGA data available as in February 2017 was downloaded from the cBioPortal (*44*) using the R programming language package "cgdsr" (http://www.R-project.org). GISTIC calls were used to estimate copy number variation and expression matrices from RNA-Seq z-scores were used for all available datasets. Mutation information was downloaded from the same portal. A gene was considered amplified if the GISTIC call was larger than 0. Mutual exclusivity of amplifications between gene pairs was computed using the Fisher's exact test. For each gene pair and dataset Pearson correlation coefficients and p-values of expression were computed using the function *cor.test*.

Association of expression and survival was performed using the “survival” package in R (https://CRAN.R-project.org/package=survival). For the univariate analysis p-values were computed using no covariates and expression as a continuous variable through the *coxph* function. Groups of samples for Kaplan-Meier plots were divided by the median expression. For multivariate analyses the clinical variables gender, stage, age and proliferation (Ki67 expression terciles) were taken into account except for those datasets where only female samples were present.

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset (*45*) was downloaded from the cbioportal (http://www.cbioportal.org/) for the genes of interest. Z-scores matrices were taken for expression analyses and clinical variables including the PAM50 classification were used as annotated in the clinical information associated with this dataset. The association of gene expression and disease free survival was computed on ER+ samples with no therapy and considering cohort, age and PAM50 subtype as covariates. All routines were implemented in R.

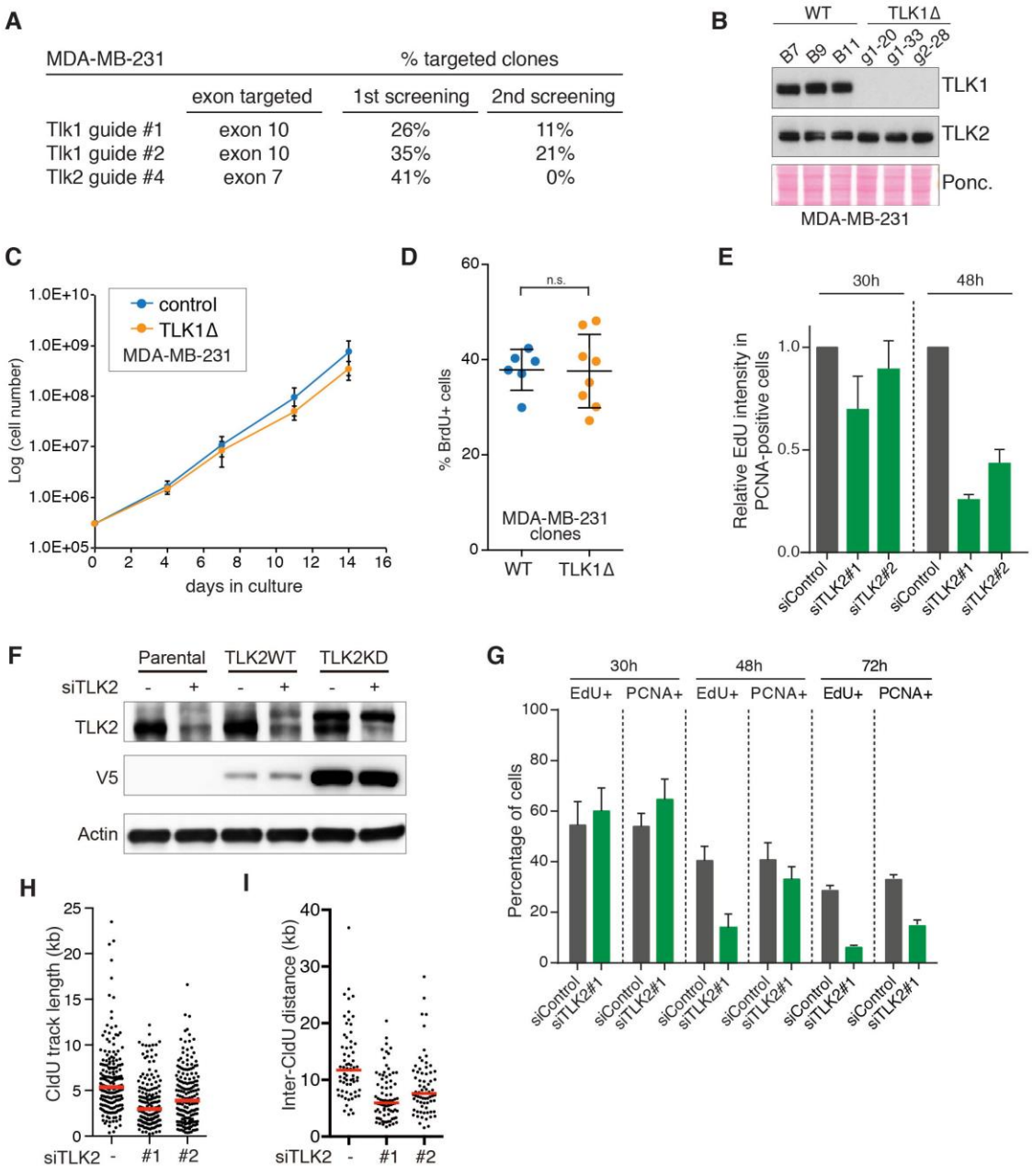

Fig. S1. TLK2 is required for efficient DNA replication. (**A**). Table of CRISPR knockout targeting efficiencies scored by western blot screening. 1st screening corresponds to 3rd passage. 2nd screening corresponds to approximately 6-10th passage. (**B**). Examples of western blot screening of WT single cell clones and TLK1-null single cell clones. Note that higher compensatory protein levels of TLK2 are not apparent. (**C**). Analysis of proliferation rates of MDA-MB-231 clones. Plots of the mean and SD of 3

independent clones performed in technical duplicate (n=3). (**D**). S-phase population analysis of WT and TLK1-null MDA-MB-231 single-cell clones. Cells were pulsed with BrdU, followed by FACS analysis of PI content and BrdU positive cells. Percentage of BrdU-positive cells is shown. Means and standard deviation from 6 independent WT clones and 8 TLK1-null clones are shown. Statistics: unpaired two-tailed *t* test. n.s.=non-significant. (**E**). Analysis of DNA synthesis by measuring EdU incorporation efficiency in U-2-OS cells 30h and 48hr after siRNA transfection. Cells were pulsed with EdU for 15 minutes and subjected to pre-extraction, followed by measurements of EdU intensity in S-phase cells identified by PCNA detection. Mean and standard deviation of relative EdU intensity to control cells from biological triplicate (n=3) are shown. (**F**). Western blot analysis of siRNA treated U-2-OS cells stably expressing siRNA-resistant wild-type (WT) or kinase-dead (KD) TLK2 as in Fig 1E. (**G**). S-phase population analysis in siRNA treated U-2-OS cells. S phase cells were identified by PCNA staining, while actively replicating cells were scored by EdU incorporation at the indicated time points. Means and standard deviation from biological triplicate (n=3) are shown. (**H**, **I**). DNA replication analyzed by DNA combing in U-2-OS cells treated with two independent TLK2 siRNAs. Length of CldU-labelled tracks (H, n>160) and inter-CldU distance (I, n>60) were measured. Median is indicated by a red line.

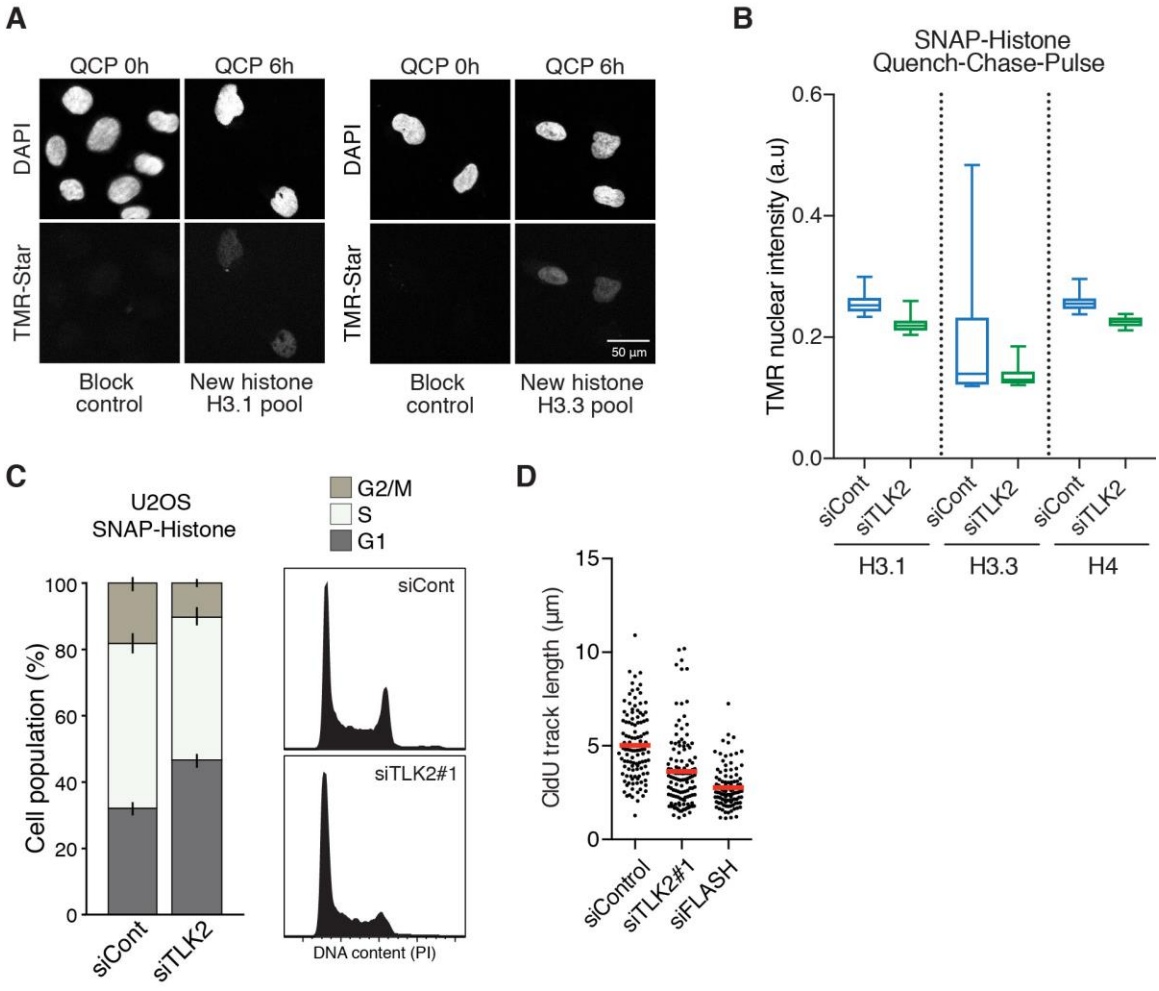

Fig. S2. TLK2 is required for replication-coupled chromatin assembly. (**A**). Immunofluorescence analysis of SNAP-tagged histone labeling. Quench-chase-pulse (QCP) experiments were performed as described in Fig. 2A and the Materials and Methods. U-2-OS cells were pulsed with SNAP-Block, and after a chase period of 6 hours (QCP 6h), pulsed with TMR-Star for 30 minutes. Representative images are shown for SNAP-HA-H3.1 and SNAP-HA-H3.3 cell lines. QPC 0h indicates the quenching control. (**B**). Representative measurements from one biological replicate of chromatin assembly analysis using SNAP-tagged histones. Number of nuclei analyzed is n>700. Median is shown with boxes and whiskers: 25-75 and 5-95 percentile ranges. (**C**). Cell cycle analysis of U-2-OS cells stably expressing SNAP-tag histones 48 hours after siRNA transfection. The mean(SD) of n=4 independent experiments are shown. (**D**). Replication elongation analyzed by DNA fiber analysis. U-2-OS cells were treated with

TLK2 or FLASH siRNAs for 30 hours and sequentially pulsed with IdU and CldU, followed by DNA fiber analysis. The length of CldU-labeled tracks (n>100) was measured. One representative experiment out of two biological replicates is shown with the median highlighted.

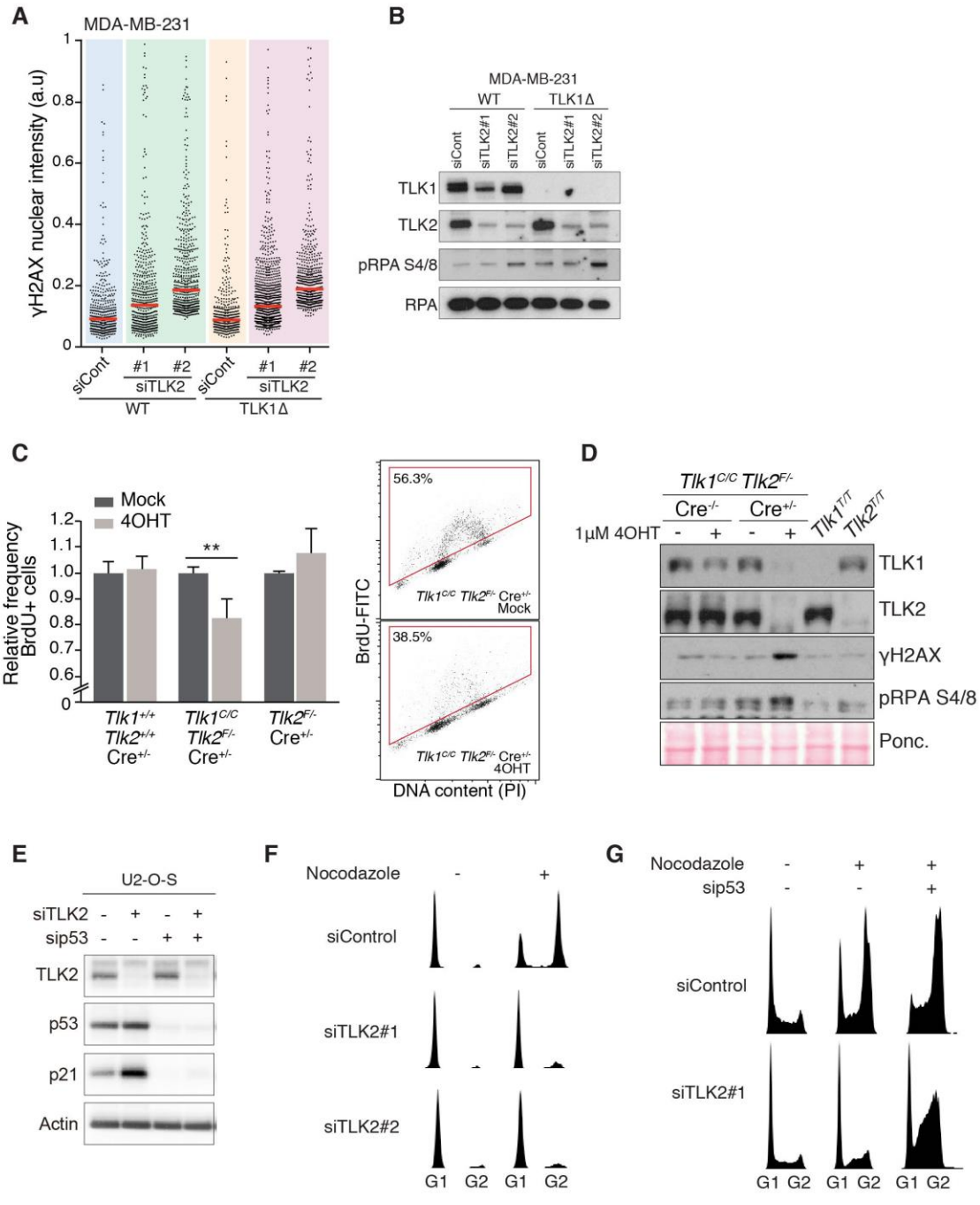
A
MDA-MB-231
γH2AX nuclear intensity (a.u)
siCont
#1
#2
siTLK2
WT
TLK1Δ
B
MDA-MB-231
WT
TLK1Δ
siCont
siTLK2#1
siTLK2#2
TLK1
TLK2
pRPA S4/8
RPA
C
Mock
4OHT
Relative frequency BrdU+ cells
**
Tlk1+/+ Tlk2+/+ Cre+/-
Tlk1C/C Tlk2F/- Cre+/-
Tlk2F/- Cre+/-
56.3%
38.5%
BrdU-FITC
DNA content (PI)
Mock
4OHT
D
Tlk1C/C Tlk2F/-
Cre-/-
Cre+/-
Tlk1T/T
Tlk2T/T
1μM 4OHT
TLK1
TLK2
γH2AX
pRPA S4/8
Ponc.
E
U2-O-S
siTLK2
sip53
TLK2
p53
p21
Actin
F
Nocodazole
siControl
siTLK2#1
siTLK2#2
G1
G2
G
Nocodazole
sip53
siControl
siTLK2#1

Fig. S3. Sustained depletion of TLK activity leads to DNA damage and checkpoint-induced G_1 arrest. (**A**). High-throughput microscopy (HTM)-mediated quantification of the nuclear intensity of γH2AX in MDA-MB-231 WT or TLK1-null cells 72 hours after siRNA transfection. One representative experiment is shown out of three independent experiments. Red bars indicate the median. (**B**). Western blot analysis of MDA-MB-231 cells 72 hours after TLK2 siRNA transfection. (**C**). BrdU incorporation is reduced following the Cre-mediated deletion of TLK1 and TLK2 (middle bars), but not TLK2 depletion alone (right bars). MEFs were mock treated or treated with 4OHT for 72h, washed, plated and cultured for 24h. Relative percent of positive cells to mock treated cells is displayed. $Tlk1^{+/+}$ $Tlk2^{+/+}$ $Cre^{+/-}$ n= 3; $Tlk1^{C/C}$ $Tlk2^{F/-}$ $Cre^{+/-}$ n=5; $Tlk2^{F/-}$ $Cre^{+/-}$ n=5. Results are shown as mean± SEM. Experiment was performed in technical duplicates. Examples of flow cytometry data are shown (right panels). (**D**). Replication stress and DNA damage are elevated in MEFs following Cre-mediated deletion of both TLK1 and TLK2 (4^{th} lane), but not in TLK1 or 2 depleted cell cultures (right 2 lanes). (**E)**. Western blot analysis of U-2-OS cells 48 hours after transfection with TLK2 siRNA alone or together with p53 siRNA. (**F**). Cell cycle analysis as in Fig. 3H. TIG3 primary human fibroblasts were transfected with siRNA for 40 hours and treated with or without nocodazole followed by PI staining and flow cytometry analysis. One representative experiment out of three biological replicates is shown. (**G**). Cell cycle analysis of U-2-OS cells transfected with indicated siRNA and treated as in (F). One representative experiment out of two biological replicates is shown.

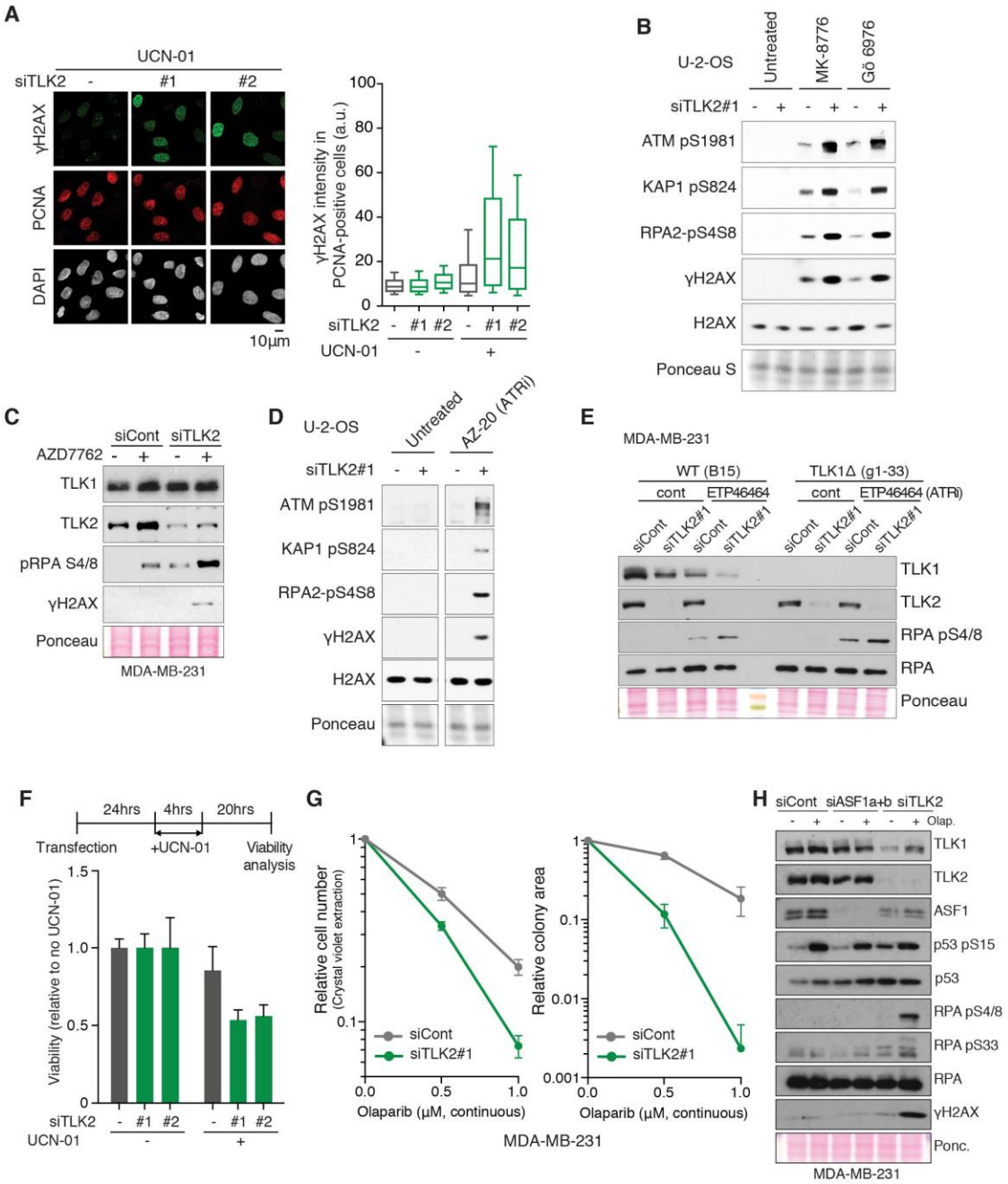
A
UCN-01
siTLK2 - #1 #2
γH2AX
PCNA
DAPI
10μm
γH2AX intensity in PCNA-positive cells (a.u.)
siTLK2 - #1 #2 - #1 #2
UCN-01 - +
B
U-2-OS
Untreated
MK-8776
Gö 6976
siTLK2#1 - + - + - +
ATM pS1981
KAP1 pS824
RPA2-pS4S8
γH2AX
H2AX
Ponceau S
C
siCont siTLK2
AZD7762 - + - +
TLK1
TLK2
pRPA S4/8
γH2AX
Ponceau
MDA-MB-231
D
U-2-OS
Untreated
AZ-20 (ATRi)
siTLK2#1 - + - +
ATM pS1981
KAP1 pS824
RPA2-pS4S8
γH2AX
H2AX
Ponceau
E
MDA-MB-231
WT (B15)
TLK1Δ (g1-33)
cont ETP46464
cont ETP46464 (ATRi)
siCont siTLK2#1 siCont siTLK2#1 siCont siTLK2#1 siCont siTLK2#1
TLK1
TLK2
RPA pS4/8
RPA
Ponceau
F
24hrs 4hrs 20hrs
Transfection +UCN-01 Viability analysis
Viability (relative to no UCN-01)
siTLK2 - #1 #2 - #1 #2
UCN-01 - +
G
Relative cell number (Crystal violet extraction)
siCont
siTLK2#1
Olaparib (μM, continuous)
Relative colony area
siCont
siTLK2#1
Olaparib (μM, continuous)
MDA-MB-231
H
siCont siASF1a+b siTLK2
- + - + - + Olap.
TLK1
TLK2
ASF1
p53 pS15
p53
RPA pS4/8
RPA pS33
RPA
γH2AX
Ponc.
MDA-MB-231

Fig. S4. TLK2 depletion causes genomic instability that is amplified by checkpoint and PARP inactivation. (**A**). Immunofluorescence analysis of γH2AX intensity in U-2-OS cells 24 hours after siRNA transfection and treatment with UCN-01 for 2 hours. Images (left) and quantification (right) of one representative experiment out of two are shown. Median with boxes and whiskers: 25–75 and 10-90 percentile ranges (n>2000). (**B**). Analysis of DNA damage signaling in U-2-OS cells treated 24 hours post-transfection with the CHK1 inhibitors MK-8776 (1 μM) or Gö-6976 (1 μM). Representative blots of two independent experiments are shown. (**C**). Analysis of DNA damage signaling in MDA-MB-231 cells treated 72 hours post-transfection with AZD7762 for 24 hours, followed by western blot and survival analysis (Fig. 4D). Representative blots of two independent experiments are shown. (**D**). Analysis of DNA damage signaling in U-2-OS cells treated with the ATR inhibitor AZ-20 (1 μM) for 4 hours, followed by western blot analysis for indicated proteins. Representative blots of two independent experiments are shown. (**E**). Analysis of DNA damage signaling in MDA-MB-231 cells treated 48 hours post-transfection with ATR inhibitor ETP-46464 (5 μM) for 24 hours, followed by western blotting. (**F**). Short-term cell viability analysis. Experimental design (top) and representative result of two biological replicates (bottom) is shown. Viability relative to the corresponding cells without UCN-01 treatment from six technical replicates is displayed with the mean and range. (**G**). Survival assays following treatment with the indicated doses of Olaparib in siRNA treated MDA-MB-231 cells as in Fig. 4E. Representative data from two independent experiments are shown. (**H**). Analysis of DNA damage signalling in MDA-MB-231 cells treated 24 hours post-siRNA transfection with Olaparib (1 μM) for 24 hours, followed by Western blotting.

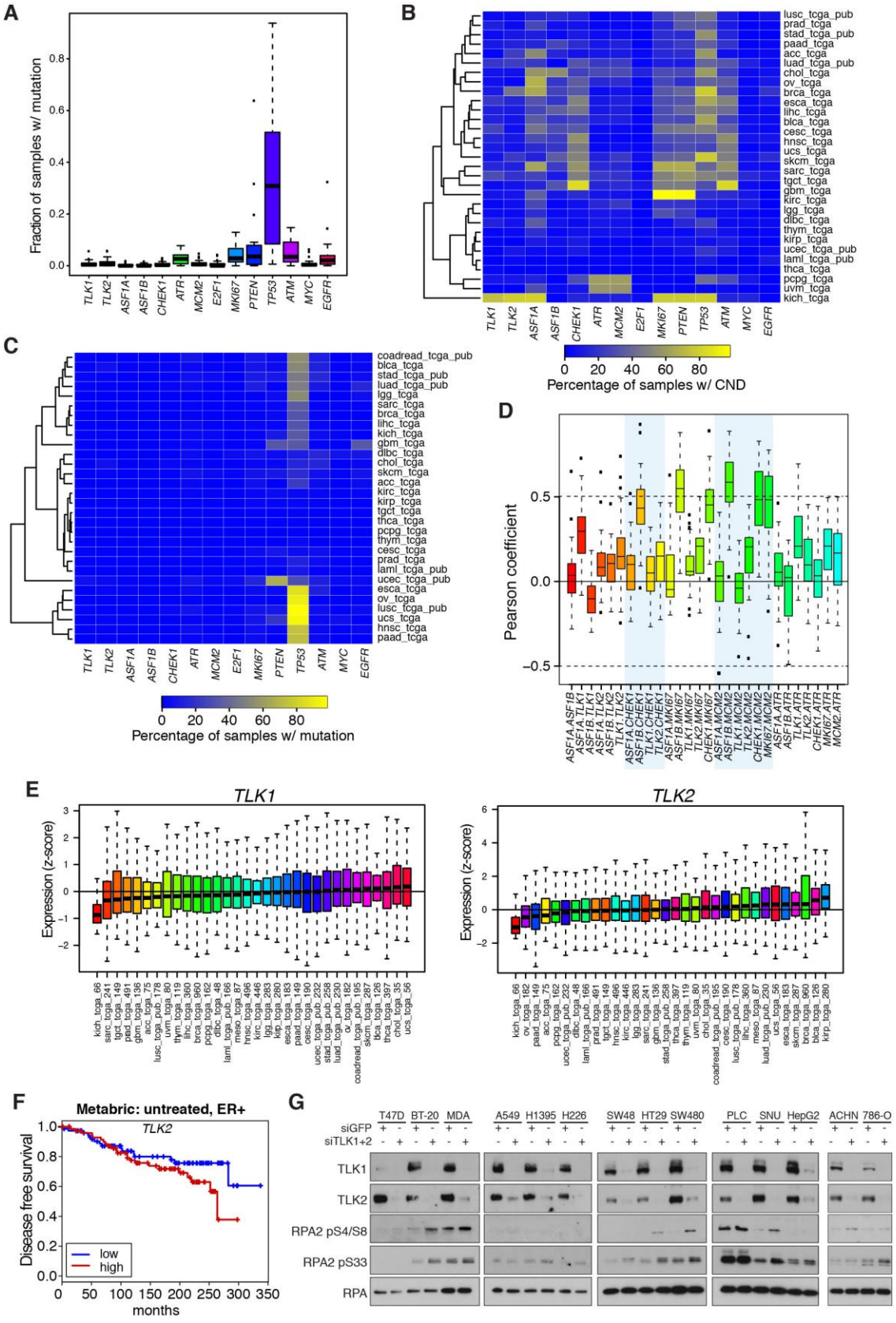
A
Fraction of samples w/ mutation
TLK1
TLK2
ASF1A
ASF1B
CHEK1
ATR
MCM2
E2F1
MKI67
PTEN
TP53
ATM
MYC
EGFR
B
Percentage of samples w/ CND
C
Percentage of samples w/ mutation
D
Pearson coefficient
E
TLK1
TLK2
Expression (z-score)
F
Metabric: untreated, ER+
TLK2
Disease free survival
low
high
months
G
T47D
BT-20
MDA
A549
H1395
H226
SW48
HT29
SW480
PLC
SNU
HepG2
ACHN
786-O
siGFP
siTLK1+2
TLK1
TLK2
RPA2 pS4/S8
RPA2 pS33
RPA

Fig. S5. Analysis of TLK status in cancer. (**A**). Boxplots of the percentage of samples with mutations identified for the indicated gene across TCGA datasets. Boxplots show 1st to 3rd quartiles with whiskers at 3rd Q +1.5*IQR and 1st Q -1.5*IQR. (**B**). Heatmap of percentage of samples with CND events in the indicated gene across TCGA datasets (corresponds to Fig. 6A, B). (**C**). Heatmap percentage of samples with mutation events in the indicated gene in the indicated dataset (corresponds to Fig. 6A, B). (**D**). Correlation between the expression of the indicated gene pairs within each TCGA cohort with expression data (n for each cohort are given in table S1 and fig. S5-E). Individual Pearson coefficients and log rank test (LRT) p-values for the expression of each gene pair and each cohort are provided in table S3. (**E**). Boxplots of gene expression (z-score) for the indicated genes in the indicated TCGA datasets. n for each cohort indicated in the x-axis labels. Outliers were removed from the plot for visualization purposes. (**F**). Kaplan Meier plot of disease free survival in ER+ untreated breast cancer patients in the Metabric cohort ((http://www.cbioportal.org/study?id=brca_metabric#summary) based on *TLK2* expression. The Hazard ratio (HR) and (LRT) p-value calculated using expression as a continuous variable is indicated for each plot. (**G**). Western blotting 48 hours post siRNA treatment in the indicated cell line.

Table S1. TCGA cohort designations. Cohort abbreviations used in Fig. 6 and fig. S5.

Table S2. Chromosome locations of genes analyzed. Chromosome location of all genes analyzed in Fig. 6 and fig. S5.

Table S3. Analysis of correlated gene expression. P-values and Pearson correlation coefficients for the expression of indicated gene pairs in the indicated TCGA cohort. Datasets discussed in Fig. 6 are highlighted in blue.

Table S4. Survival analysis of TCGA patient data. Log rank test-p-values for disease free survival (DFS) or overall survival (OS) from univariate analysis of expression of the indicated gene in the indicated TCGA cohort. Datasets discussed in Fig. 6 are highlighted in blue.

Table S5. Multivariate survival analysis of TCGA patient data. Multivariate analysis of selected TCGA cohorts. Disease free (DF) or overall survival (OS) are indicated.