Cell death during crisis is mediated by mitotic telomere deprotection

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Dedicated to all the people affected by the crisis. Tumour formation is blocked by two barriers: replicative senescence and crisis1. Senescence is triggered by short telomeres and is bypassed by disruption of tumour-suppressive pathways. After senescence bypass, cells undergo crisis, during which almost all of the cells in the population die. Cells that escape crisis harbour unstable genomes and other parameters of transformation. The mechanism of cell death during crisis remains unexplained. Here we show that such fusions are the underlying cause of cell death. Mitotic arrest is bypassed by disruption of tumour-suppressive pathways. Telomere fusions triggered mitotic arrest in p53-compromised non-crisis cells, indicating that such fusions are the underlying cause of cell death.

Exacerbation of mitotic telomere deprotection by partial TRF2 (also known as TERF2) knockdown2 increased the ratio of cells that died during mitotic arrest and sensitized cancer cells to mitotic poisons. We propose a crisis pathway wherein chromosome fusions induce mitotic arrest, resulting in mitotic telomere deprotection and cell death, thereby eliminating precancerous cells from the population.

Replicative senescence is induced by partially deprotected telomeres, which activate a DNA damage response (DDR) without telomere fusions2. Crisis requires the bypass of senescence through loss of checkpoints and causes massive cell death concomitant with further telomere shortening and spontaneous telomere fusions. However, the mechanism of cell death was not understood. Mitotic arrest is induced by partial TRF2 knockdown2.

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associated with spindle assembly checkpoint (SAC)-independent telomere deprotection, and we therefore set out to test whether prolonged mitosis has a role in crisis.

We monitored mitotic duration using live-cell imaging. Mitosis in primary IMR-90 fibroblasts lasted <45 min. However, IMR-90 fibroblasts expressing HPV16 E6 and E7, which inhibit p53 and Rb, displayed variable mitotic duration upon senescence bypass (Fig. 1a, b). Prolonged mitosis, defined as mitosis of >2 h, became prominent in pre-crisis cells (Extended Data Fig. 1a). Spontaneous mitotic arrest also occurred in pre-crisis cells following expression of E6 or dominant-negative p53dd (Extended Data Fig. 1b), indicating that loss of p53 function was required (Fig. 1c, d and Extended Data Fig. 1c, d).

Overexpressing hTERT prevented senescence in IMR-90 cells (Fig. 1c, d and Extended Data Fig. 1c, d). Telomere elongation in IMR-90 E6E7 or p53dd cells also suppressed mitotic arrest (Fig. 1e, f and Extended Data Fig. 2a–c), confirming telomere shortening as the cause. Reversine inhibition of MPS1 (ref. 7) suppressed mitotic arrest (Extended Data Fig. 1e), indicating dependence on the SAC. Hesperadin, an Aurora B kinase inhibitor required for activation of the SAC upon tensionless kinetochore–microtubule attachment, suppressed mitotic arrest (Extended Data Fig. 1e), suggesting abnormal kinetochore–microtubule attachment.

To determine if telomere fusion causes mitotic arrest, we used two independent guide RNAs (sgTRF2-1 and sgTRF2-2), which efficiently reduced TRF2 expression and induced telomere fusions in young IMR-90 E6E7 cells (Fig. 2a and Extended Data Fig. 2d, e). These guide RNAs also led to mitotic arrest, comparable to IMR-90 E6E7 cells around population doubling (PD) 108 (Fig. 2b and Extended Data Fig. 2f, g, h). Telomere elongation in IMR-90 E6E7 cells (mean ± s.e.m.) e–g. Percentage of telomeric fusion (e), mitotic duration (f) and mean number of meta-TIF (g) in indicated cells (mean ± s.e.m.). h. Scatter plots with bars showing the mean percentage of cells with prolonged mitosis (two independent experiments). *P < 0.05, **P < 0.005, ***P < 0.0001. NS, not significant. Mann–Whitney test.
Suppression of both telomere fusion and mitotic arrest by a resistant TRF2 (TRF2°sgRNA) excludes off-target effects (Fig. 2c, d and Extended Data Fig. 3a–c).

To address whether telomeric DDR or telomere fusion induces mitotic arrest, we deleted TRF2 in young IMR-90 E6E7 cells lacking 53BP1 or ligase IV (Extended Data Fig. 3d, e). Suppression of 53BP1 or ligase IV strongly reduced fusion frequency (Fig. 2e) and prevented mitotic arrest (Fig. 2f, h), but did not reduce the number of deprotected telomeres (Fig. 2g and Extended Data Fig. 3f), thereby separating mitotic delay from DDR. Both telomere fusion and mitotic arrest phenotypes were suppressed by ATM inhibition12–14 (Extended Data Fig. 3g–i), again indicating that telomere fusion underlies mitotic arrest. ATM inhibition did not suppress mitotic arrest induced by Taxol15 (Extended Data Fig. 3k and l), confirming that the inhibitor does not perturb the SAC. Additionally, cells expressing a small hairpin RNA targeting TRF2 (shTRF2-F), which causes telomere deprotection in the absence of fusion16, did not undergo arrest (Fig. 2a, b and Extended Data Fig. 2c, f). These data are consistent with the observation that senescent cells, while harbouring a number of unfused deprotected telomeres17,18, do not display mitotic arrest (Fig. 1b and Extended Data Fig. 1a).

Deletion of TRF2 increased anaphase bridge frequency and pericentrin foci (Extended Data Fig. 4a, b) 7 days post infection, indicating multipolar mitosis when cells display telomere fusions and mitotic arrest. Accordingly, sgTRF2-2 cells exhibit unaligned metaphase chromosomes (Extended Data Fig. 4c), suggesting a chromosome congression defect. Tetraploidy did not increase as dramatically as the pericentrin foci (Extended Data Fig. 4d), ruling out tetraploidization as the cause of multipolarity. We conclude that multipolarity and centrosome amplification due to anaphase bridges and cytokinesis contribute to the mitotic arrest phenotype, although it is not clear how telomere fusions drive centrosome abnormality.

Mitotic arrest in young IMR-90 cells induces mitotic telomere deprotection16. To examine whether spontaneous mitotic arrest in pre-crisis cells also induces telomere deprotection we used premature sister separation, either cause or consequence of mitotic arrest17,18, as a marker of prolonged mitosis. Indeed, cells in pre-crisis displayed increased premature chromatid separation (Fig. 3a). Telomeric γ-H2AX foci analysis (metaphase telomere-dysfunction-induced foci, meta-TIF)19,20 revealed that metaphase spreads with separated chromatids displayed increased telomere deprotection (Fig. 3b, c and Extended Data Fig. 5a). Accordingly, suppression of TRF2 in young IMR90 E6E7 cells not only caused fusion and prolonged mitosis (Fig. 2a, b), but also increased premature separation (Extended Data Fig. 5b).

Cells exhibiting premature separation were mostly near-diploid, excluding the possibility that they have an increased number of telomeric ends (Extended Data Fig. 5c). We conclude that prolonged mitosis in pre-crisis is associated with a telomere DDR, and that mitotic arrest occurs in near-diploid cells. Consistently the ratio of tetraploid cells in pre-crisis cultures did not increase as prominently as the percentage of cells undergoing mitotic arrest (Extended Data Fig. 5d, e). We also rarely observed dipl chromosomes, which are a consequence of two rounds of DNA replication without mitosis (endoreplication) and thereby tetraploid (Fig. 3a).

Next we asked how prolonged mitotic arrest affects cellular fate in pre-crisis. Live cell imaging indicated three potential outcomes: cytokinesis, slippage (mitotic exit without cytokinesis), and cell death (Fig. 3d). Mitotic duration correlated with cellular fate, as cells that spent the least amount of time in mitosis underwent cytokinesis, and cells that resided longer in mitosis tended to slit or die (Fig. 3e). The cell death ratio increased significantly from 19% in cells arrested for 2–6 h to 50% in cells arrested for >6 h (Fig. 3f).

However, we noted that cells also died during interphase. Live cell imaging revealed that 32% (86/266) of deaths occurred after prolonged mitosis (>2 hours), 14% (38/266) of cells died during mitosis lasting <2 hours and 53% (142/266) of cells died in interphase (Fig. 3g). To address whether cells that died during a short mitosis or interphase were associated with prolonged mitosis in the previous cell cycle, we traced the cells in question to the mitosis before death. Of the cells that succumb in interphase, 46% of the traceable previous mitosis was prolonged (18/39) (Fig. 3g). Of the cells that died during a short mitosis, 29% of the previous mitosis...
was prolonged (4/14) (Fig. 3g), indicating that cell death during either short mitosis or in interphase follows prolonged mitosis in the previous cycle.

Partial knockdown of TRF2 exacerbates mitotic telomere deprotection in young IMR-90 E6E7 cells. To understand whether mitotic telomere deprotection is the cause of death in pre-crisis, we tested whether a partial knockdown of TRF2 would enhance cell death upon spontaneous mitotic arrest. IMR-90 E6E7 cells were transduced with shTRF2-F at PD45, PD70 or PD85 (Extended Data Fig. 6a). The resulting partial suppression of TRF2 did not affect cell growth dynamics in the younger populations, but slowed down growth in the population infected at PD85 (Fig. 4a–c). However, in all settings, the cells entered crisis prematurely (Fig. 4a–c). γ-H2AX foci analysis around PD100 revealed that shTRF2-F cells suffer from increased numbers of TIF, which was greatly enhanced on separated chromatids (Fig. 4d and Extended Data Fig. 6b, c). Accordingly, live-cell imaging revealed shTRF2-F expression increased the cell death ratio, especially after a short period of mitotic arrest (Fig. 4e). In contrast, TRF2 overexpression (Extended Data Fig. 6d), which partially suppresses mitotic telomere deprotection, reduced cell death after mitotic arrest, suppressed TIF and delayed crisis (Extended Data Fig. 6e–g). Neither shTRF2-F nor TRF2 overexpression affected mitotic duration (Extended Data Fig. 6h, i). shTRF2-F also did not increase fusion frequency (Extended Data Fig. 6i), thereby attributing the increased cell death to exaggerated loss of TRF2, not to increased fusion formation. Accordingly, loss of TRF2 in cells lacking 53BP1 significantly increased cell death and shortened the mitotic duration before cell death upon colcemid-induced mitotic arrest (Extended Data Figs 3d and 7a, b). Furthermore, inhibition of Aurora B kinase by hesperadin, which suppresses mitotic telomere deprotection (Extended Data Fig. 7c), greatly reduced cell death upon colcemid exposure (Extended Data Fig. 7d, e), supporting a model in which amplified telomere deprotection induced by mitotic arrest triggers cell death.

This model predicts that shTRF2-F would sensitize cells to drugs that induce mitotic arrest. Therefore HT1080 6TG cells expressing shScramble or shTRF2-F were exposed to Taxol, vinblastine, dimethyl-enastron, and as a control the topoisomerase I inhibitor camptothecin. Expression of shTRF2-F significantly sensitized HT1080 6TG cells to Taxol, vinblastine, and dimethyl-enastron, all of which induce mitotic arrest (Fig. 4f). No such effect was observed upon exposure to camptothecin (Fig. 4g), confirming that TRF2 levels affect cellular fate specifically upon mitotic arrest.

Here we show that chromosome end-to-end fusions during crisis cause spontaneous mitotic arrest, amplifying telomere deprotection, which determines cellular fate. We suggest that telomere deprotection upon spontaneous mitotic arrest is the underlying molecular signal that leads to cell death in crisis (Fig. 4g). While we cannot rule out a role of fusion breakage cycles and the resulting chromosomal

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**Figure 4 | Mitotic telomere deprotection dictates cellular fate upon mitotic arrest.** a–c, Growth curves of indicated cells. d, Scatter plots mean telomeric and non-telomeric γ-H2AX foci ± s.e.m. in individual indicated cells shown in a (n > 30). IF-FISH, as in Fig. 3b. Magenta, metaphases with separated chromatids. e, Ratio of indicated fates in pre-crisis IMR-90 E6E7 cells expressing shScramble and shTRF2-F infected at PD85, analysed as in Fig. 3f (Fisher’s exact test). f, Viability assay of HT1080 6TG expressing shScramble and shTRF2-F. Right, the ratio between logIC50 of shScramble and shTRF2-F from three independent experiments (two-tailed t-test). g, Model of mitotic cell death pathway during crisis. *P < 0.05, **P < 0.005, ***P < 0.0001. NS, not significant.

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abnormalities as a cause of death\textsuperscript{23}, several observations argue against it. Only few fusions are observed in crisis cells (Fig. 2a), which is enough to trigger prolonged mitosis (Fig. 2b). Disruption of TRF2 leads to the rapid onset of prolonged mitosis, arguing against long-term effects of fusion breakage cycles (Fig. 2a). Cells succumb to death in the first prolonged mitosis, or in the following cell cycle (Fig. 3f, g), ruling out a long-term process. Increasing damage signals without increasing fusion frequency causes more death (Fig. 4e and Extended Data Fig. 7a), arguing for signalling from deprotected telomeres as the cause for death. As most cell death during pre-crisis is associated with mitotic arrest, we propose that prolonged mitosis is the main mechanism that limits cellular lifespan upon bypass of senescence. These findings might also offer a clinical opportunity, since exacerbation of mitotic telomere deprotection sensitizes cancer cells to mitotic drugs. Mitotic arrest, however, has also been associated with tumorigenesis in checkpoint-compromised cells\textsuperscript{24}. Similarly, bone marrow failure and cancer in individuals with telomeropathies are frequent, which could potentially be explained by mitotic arrest resulting from overly short telomeres\textsuperscript{25,26}. Telomere-driven spontaneous mitotic arrest and the resulting mitotic telomere deprotection in the pre-crisis stage may thus function as a double-edged sword, explaining both cell death and chromosome instability upon bypass of senescence.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.K. (karlseder@salk.edu).
METHODS

Cell culture and treatment. Human IMR-90 primary lung fibroblasts (ATCC) and their derivatives were grown in Glutamax-DMEM (Gibco) supplemented with 0.1 mM nonessential amino acids and 15% fetal bovine serum. HT1080 6TG (ATCC) cells were grown in Glutamax-DMEM supplemented with 0.1 mM nonessential amino acids and 10% bovine growth serum. All cells were grown at 7.5% CO₂ and 3% O₂. Colcemid (Life Technologies), Taxol (A. G. Scientific), vinblastine (A. G. Scientific), dimethylestraon (A. G. Scientific), hesperadin (Selleck), revesine (Selleck) and camptothecin (Selleck) were used at the indicated concentrations. ATMD inhibitor (KU-55933) (Tocris) was used at 10 μM. FACS analysis was performed as described27. Cells were tested for mycoplasma contamination and found negative.

Live-cell imaging. Live-cell imaging was performed in 8-well microslide chambers (iBidi) on a Zeiss inverted fluorescent microscope with a 20× 0.8 NA oil objective at 37°C and 7.5% CO₂ (Xlmulti S1 module, Zeiss). Images were captured with an AxioCam MRm (Zeiss) using Axio Vision software (Zeiss) typically every 6 minutes for at least 48 h. Mitotic duration was defined as movie frames from nuclear envelope breakdown or a previous frame of cell rounding to a frame of cytokinesis, slippage or mitotic cell death. Prolonged mitosis was defined as a mitosis that continues for more than 2 h. Cells that escaped from a movie screen during prolonged mitosis were included in the mitotic duration analysis but excluded from the cell fate analysis.

For shTRF2-F, image capture was started 5 days after infection. For sgEMPTY (guide RNA without target), sgTRF2-1 and sgTRF2-2, image capture was started 7 days after infection. Where indicated, population doubling is the one at their seeding, typically 1 day before the starting date of image capture. Typically more than two independent movies were analysed to confirm reproducibility except for Fig. 1b, where one movie was analysed per data point. Where indicated, Taxol, colcemid and hesperadin were added to the culture right before starting live imaging.

Vectors and viral infections. Target sequences of CRISPR/Cas9 are as follows5: sgTRF2-1, 5′-ACTGCATAATCGCGGATTGA-(PAM)-3′; sgTRF2-2, 5′-TGTCTGGAGGGATTGAAGA-(PAM)-3′; sg53BP1, 5′-CAGAATCATCCTCTAGAACC-(PAM)-3′; and sgLIG4, 5′-TGCCGTCAAACTACTGAG-(PAM)-3′. Target sequences of sg353BP1 and sgLIG4 were first cloned into LentiCRISPR vectors (Addgene plasmid 49535), followed by re-cloning of the guide RNA expression cassette (U6 promoter, target sequence and gRNA scaffold) into NheI site of lentiviral vectors.

For shTRF2-F, image capture was started 5 days after infection. For sgEMPTY (guide RNA without target), sgTRF2-1 and sgTRF2-2, image capture was started 7 days after infection. Where indicated, population doubling is the one at their seeding, typically 1 day before the starting date of image capture. Typically more than two independent movies were analysed to confirm reproducibility except for Fig. 1b, where one movie was analysed per data point. Where indicated, Taxol, colcemid and hesperadin were added to the culture right before starting live imaging.

Telomere analysis. Telomere analysis was performed as described20. For telomere and centromere double-staining, Alexa488-conjugated telomeric PNA probe (Celltrackers, Invitrogen) and Cy3-conjugated centromeric PNA probe (CEN6-Cy3, PNA Bio Inc.) were used. Percentage of chromosome ends with a telomeric PNA signal was determined by counting the number of chromosome ends with a signal at both telomeric and centromeric PNA probe. For chromosome alignment analysis, cells in which most H3S10-P-positive chromosomes align according to chromosome shape from MPM-2-positive cells. For chromosome alignment analysis, cells in which most H3S10-P-positive chromosomes align between (and do not overlap with) two γ-tubulin foci were selected as metaphase. Cells were analysed 7 days after infection or at indicated PD.

Telomere blots. Double-stranded telomere analysis was performed as described previously27.

Viability assay. Premixed WST-1 Cell Proliferation Reagent (Clontech) was used for viability assay according to manufacturer’s instruction. HT1080 6TG cells infected with either shScramble or shTRF2 were seeded in 96-well plates at 6 days post infection and exposed to Taxol, vinblastine, dimethyl-enastron, and camptothecin at 7 days post infection for 48 h. Triplicate wells were analysed for each drug concentration. The results were reproduced by three independent experiments. The log(C50) value was analysed by log(inhibitor) versus normalized response (variable slope) method using Prism 6 software.

Statistical methods. Each figure legend shows the number of samples per experiment and number of experiments that were analysed independently. No statistical methods were used to predetermine sample size. Two-tailed unpaired t-tests and two-tailed Mann–Whitney tests were used to compare two data sets, where Gaussian distribution is assumed and not assumed, respectively. To detect any trends among multiple data sets in Fig. 3a and Extended Data Figs 5a and 7c, one-way ANOVA was used. Mitotic duration data sets of IMR-90 E6E7 cells in Fig. 1b were analysed with Kruskal–Wallis tests, while two-tailed tests and two-tailed Mann–Whitney tests were used to compare two data sets, where Gaussian distribution is assumed and not assumed, respectively. To detect any trends among multiple data sets in Fig. 3a and Extended Data Figs 5a and 7c, one-way ANOVA was used. Mitotic duration data sets of IMR-90 E6E7 cells in Fig. 1b were analysed with Kruskal–Wallis tests to detect trends (P < 0.0001), in addition to Mann–Whitney tests, as described above. For statistical analysis of cellular fate after mitotic arrest, the ratio of death versus non-death (cytokinesis and slippage) was analysed with a two-tailed Fisher’s exact test. For Fig. 3f, data from short (2–6 h) and middle (6–10 h) mitotic arrest were combined and compared to that of long mitotic arrest (>10 h). The null hypothesis was rejected when P values were less than 0.05. No randomization was performed. The investigators were not blinded to allocation during experiments and outcome assessment. All statistical analysis was performed using Prism 6 software.

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Extended Data Figure 1 | Mitotic duration in pre-crisis cells is elongated.

a, Percentage of cells that spend more than 2 h in mitosis (prolonged mitosis), shown in Fig. 1b. b, Effect of indicated oncogenes on p53 expression. c, Scatter plots show mean mitotic duration ± s.e.m. of individual IMR-90 derivative cells analysed in Fig. 1d. d, Scatter plots with bars show mean percentage of prolonged mitosis analysed in Fig. 1d (1–4 independent experiments). The dots represent independent experiments and the bars the mean. e, Scatter plots show mean (± s.e.m.) mitotic duration of IMR-90 E6E7 PD95 cells exposed to reversine and hesperadin (**P < 0.0001, Mann–Whitney test). The result was reproduced in two independent experiments.
Extended Data Figure 2 | Telomere elongation abrogates extended mitotic duration. a, Telomere elongation by hTert in IMR-90 E6E7 and p53dd cells shown in Fig. 1e, f. IMR-90 E6E7 and p53dd cells were infected at PD73 and PD82, respectively, and analysed at the indicated PD. b, c, Scatter plots show mean mitotic duration ± s.e.m. of individual IMR-90 E6E7 (b) and p53dd (c) cells expressing hTert at indicated PD shown in Fig. 1e, f. d, Effect of sgTRF2 on TRF2 expression 7 days after infection. e, f, Scatter plots with bars show mean percentage of cells with telomeric fusion (e) and prolonged mitosis (f) in IMR-90 E6E7 derivatives shown in Fig. 2a, b (1–3 independent experiments). ***p < 0.0001. Mann–Whitney test.
Extended Data Figure 3 | Extended mitotic duration depends on telomere fusion. a, Effect of sgTRF2 on TRF2 expression in cells expressing sgTRF2-resistant TRF2 (TRF2^sgRNA^) 9 days after infection with CRISPR/Cas9. b, c, Scatter plots with bars show mean percentage of cells with telomeric fusion (b) and prolonged mitosis (c) in IMR-90 E6E7 expressing sgTRF2 in the presence of TRF2^sgRNA^ shown in Fig. 2c, d (two independent experiments). d, Schematic of 53BP1 or ligase IV suppression experiment in the presence of sgEMPTY or sgTRF2-2. e, Western analysis of IMR90 E6E7 cells expressing Cas9, Cas9-sg53BP1 or Cas9-sgLig4 in the background of sgEMPTY or sgTRF2-2. f, Representative meta-TIF images of cells suppressed for 53BP1 or LIG4 in the presence of sgEMPTY or sgTRF2-2 as described in d. g, h, Percentage of telomeric fusion (g) and mitotic duration (h) in IMR-90 E6E7 expressing sgEMPTY and sgTRF2 in the presence of DMSO or ATM inhibitor (mean ± s.e.m.). i, j, Scatter plots with bars showing mean percentage of cells with telomeric fusion (i) and prolonged mitosis (j) in IMR-90 E6E7 expressing sgTRF2 in the presence of ATM inhibitor shown in g, h (two independent experiments). k, Mean mitotic duration ± s.e.m. of IMR-90 E6E7 sgEMPTY cells exposed to 500 nM Taxol in the presence of DMSO or ATMi. l, Scatter plots show mean mitotic duration of individual cells shown in j. NS, not significant; *P < 0.05, **P < 0.005, ***P < 0.0001, Mann–Whitney tests. Results were reproduced in at least two independent experiments.
Extended Data Figure 4 | Telomere fusions lead to multipolar mitosis.

a–c, Ratio of anaphase chromosome with or without anaphase bridge (a), pericentrin foci in pro-, prometa- and metaphase (b) and metaphase chromosome with or without unaligned chromosome (c) in IMR-90 E6E7 expressing sgEMPTY and sgTRF2-2 7 days after infection (Fisher’s exact test, for pericentrin foci, 1 and 2 foci versus ≥3 foci). Representative images from sgEMPTY cells are shown below (b, c). Results were reproduced in two independent experiments. d, Scatter plots with bars show mean percentage of cells that possess tetraploidy (FACS analysis) and multipolarity (≥3 pericentrin foci as in j) in IMR-90 E6E7 cells expressing sgEMPTY and sgTRF2-2 7 days after infection (two independent experiments). **P < 0.005, ***P < 0.0001. Fisher’s exact test. Scale bars, 10 μm.
Extended Data Figure 5 | Mitotic arrest occurs in near-diploid cells. a, Bars show mean of three independent experiments of average number (± s.e.m.) of telomeric γ-H2AX foci in IMR-90 E6E7 at PD42, 72 and 105 as analysed in Fig. 4d (n = 25 per experiment, P = 0.0008, one-way ANOVA). b, Bars show the percentage of young IMR90 E6E7 cells with separated sister chromatids expressing sgEMPTY or sgTRF2-1/2 6 or 7 days post infection with CRISPR/Cas9. Results from two independent experiments except for sgEMPTY 6 days post infection (at least 31 metaphases per experiment) are shown. c, Ratio of ploidy of mitotic IMR-90 E6E7 cells at PD106. Cells with attached and separated sister chromatids are plotted separately. d, FACS analysis of cells shown in Fig. 1b. e, Bars show percentage of prolonged mitosis and tetraploid cells (4N) in IMR-90 E6E7 cells at indicated PD. Percentage of prolonged mitosis are the same data sets as Extended Data Fig. 1a.
Extended Data Figure 6 | TRF2 suppression and overexpression affects cell fate. 

a. Effect of shTRF2-F on TRF2 expression 7 days after infection.

b, c. Telomeric and non-telomeric γ-H2AX foci in individual pre-crisis IMR-90 E6E7 cells expressing shScramble and shTRF2-F shown in Fig. 4b, c. Data in b and c were analysed as in Fig. 4d (n = 16, mean ± s.e.m.). Metaphases with separated chromatids are shown in magenta.

d. Effect of Myc–TRF2 on TRF2 expression 7 days after infection.

e. Ratio of mitotic cell fate in pre-crisis IMR-90 E6E7-expressing TRF2 cells, analysed as in Fig. 3f (Fisher’s exact test, death versus non-death).

f. Meta-TIF analysis of pre-crisis IMR-90 E6E7 cells expressing a control vector or Myc–TRF2. Bars show mean (± s.e.m.) telomeric and non-telomeric γ-H2AX foci (n = 25, Mann–Whitney tests).

g. Growth curve of IMR-90 E6E7 cells expressing a control vector or infected with Myc–TRF2-F at PD90.

h, i. Mean (± s.e.m.) mitotic duration of IMR-90 E6E7 cells expressing shScramble and shTRF2-F (h) and Myc–TRF2 (i) analysed in Fig. 4e and Extended Data Fig. 6e, respectively (Mann–Whitney test).

j. Mean (± s.e.m.) percentage of telomere fusion of pre-crisis IMR-90 E6E7 expressing shScramble and shTRF2-F analysed in Fig. 4a–c (Mann–Whitney test). *P < 0.05, **P < 0.005, ***P < 0.0001. NS, not significant.
Extended Data Figure 7 | Amplified telomere deprotection during mitotic arrest in crisis causes cell death.  

**a**, Ratio of mitotic slippage and cell death in IMR-90 E6E7 Cas9-sg53BP1 cells expressing sgEMPTY and sgTRF2-2 in the presence of colcemid, analysed as in Fig. 3f (Fisher’s exact test, death versus slippage). 

**b**, Scatter plots show mean mitotic duration ± s.e.m. before cell death of individual IMR-90 E6E7 Cas9-sg53BP1 cells expressing sgEMPTY and sgTRF2-2 in the presence of colcemid (Mann–Whitney test). 

**c**, Bars show mean (± s.d.) of three independent experiments of average telomeric and non-telomeric γ-H2AX foci in IMR-90 E6E7 cells at PD45 exposed to colcemid in the presence of DMSO or hesperadin at indicated concentrations for 24 h analysed as in Fig. 4d (50 metaphase per experiment). For one-way ANOVA telomeric foci, $P < 0.0001$; non-telomeric foci, not significant. 

**d**, Scatter plots show mean (± s.e.m.) mitotic duration of IMR-90 E6E7 cells at PD27 exposed to 100 ng ml$^{-1}$ colcemid in the presence of DMSO or 40 nM hesperadin (Mann–Whitney test). 

**e**, Ratio of mitotic cell fate in IMR-90 E6E7 cells around PD45 exposed to 100 ng ml$^{-1}$ colcemid in the presence of DMSO or 40 ng ml$^{-1}$ hesperadin (Fisher’s exact test, death versus slippage). **$P < 0.005$, ***$P < 0.0001$. NS, not significant.