Supplementary Figure Legends

Figure S1. IR induces ATM-dependent cellular senescence, also in proliferating cells. a. IR induces cellular senescence as assessed by SA-β-gal staining (left) and BrdU incorporation rates (right) in IR-induced senescent human fibroblasts (IrrSen). As IrrSen were contact-inhibited, cells were replated more sparsely before BrdU incorporation assays. Early passage quiescent (contact-inhibited) non-irradiated cells (Quie) were used as control. (For the quantification shown, around 50 cells for SA-β-gal staining and 500 for BrdU staining per sample were analyzed; scale bar = 100 μm) **b.** Persistent DDR is constantly and actively maintained. IrrSen BJ hTERT cells were treated with the ATM inhibitor KU55933 (ATMi; 10 μM) or vehicle alone (DMSO). Left, bar graph shows the percentage of BrdU-positive cells after 24 hours BrdU pulses at the indicated time points after treatment (** p-value < 0.01, *** p-value < 0.001; for the quantification shown, around 600 cells per sample were analyzed; n = 3). Right, triplicate qPCR reactions show an increase of KI-67 mRNA levels in ATMi-treated cells. **c.** Low doses and fractionated IR generate persistent DDR. BJ hTERT cells were irradiated with 2 Gy, 2 Gy per day for 10 days, and 20 Gy and stained 30 days later. Bar graphs show the percentage of 53BP1-positive cells (± s.e.m.) and the number of 53BP1 foci per cell. (For the quantification shown, around 200 cells per sample were analyzed). **d**. IR induces persistent DDR activation also in proliferating cells. Bar graphs shows BrdU incorporation rate (left) and the percentage of SA-β-gal-positive cells (right) (± s.e.m.) before and at different time points after irradiation (20 Gy) (For the quantification shown, around 50 cells for SA-β-gal staining and 200 for BrdU staining per time point were analyzed). **e.** Bar graphs show the percentages of pATM foci-positive cells (left) and the average number of pATM foci per cell (right) (± s.e.m.), at different time points in proliferating cells. More than

100 discrete foci cannot be counted accurately due to their proximity (1 hour time point) (For the quantification shown, around 100 cells per time point were analyzed).

Figure S2. Irradiation induces persistent DDR also in another type of HDF strain (MRC5), in cells expressing telomerase and in cells maintained at low oxygen tension. a. IR induces persistent DDR activation in MRC5 HDFs. Bar graphs show the percentages of 53BP1 foci-positive cells (left) and the average number of 53BP1 foci per cell (right) (± s.e.m.), at the indicated time points following irradiation (20 Gy). **b.** IR induces persistent DDR activation in telomerized BJ HDFs (IrrSen BJ hTERT). Top, bar graphs show the average number of 53BP1 foci per cell (± s.e.m.) and bottom, the percentages (± s.e.m.) of 53BP1 foci-positive cells in BJ hTERT and BJ cells, at different time points after 20 Gy IR. **c.** IR induces persistent DDR activation also under low oxygen conditions. Top, bar graphs show the average number of 53BP1 foci per cell and bottom, the percentages of 53BP1 foci-positive cells (± s.e.m.), at different time points, under 3% or 20% oxygen culture condition. More than 100 discrete foci cannot be counted accurately due to their proximity (1 hour time point). (For the quantification shown, around 100 cells per time point were analyzed).

Figure S3. Bleomycin induces persistent DDR activation and CHK2 pT68 accumulates in foci in IrrSen cells. a. Bleomycin induces persistent DDR activation. Bar graphs show the percentages of BrdU-positive cells, the percentages of pS/TQ foci-positive cells and the average number of pS/TQ foci per cell (± s.e.m.), at the indicated time points following bleomycin treatment. (For the quantification shown, 100-1000 cells per time point were analyzed). **b.** CHK2 pT68 forms discrete nuclear

foci that colocalize with persistent γH2AX in IrrSen cells. Representative images of CHK2 pT68 and γH2AX immunostaining. Twenty minutes after IR (1 Gy), CHK2 pT68 shows a diffuse staining, while in IrrSen BJ hTERT cells, it forms foci colocalizing with γH2AX. The percentage of colocalization (± s.e.m.) is indicated. (Scale bar = 50 μm)

Figure S4. Telomeres colocalize with DDR foci upon irradiation in different HDF strains, upon bleomycin treatment and upon low or fractionated IR. a. Persistent γH2AX foci colocalize with telomeres in IrrSen HDFs. Graphs show the percentages of colocalizations (± s.e.m.) between γH2AX foci and telomeric PNA probe (Telo) in MRC5 (left) and BJ (right) cells at the indicated time points after 20 Gy IR. (For the quantifications shown, 50-200 cells per time point were analysed). **b.** Persistent DDR foci localize at telomeres in bleomycin-treated cells. Left, representative images of colocalizations between DDR, detected as 53BP1 foci, and telomeric DNA, detected using a telomeric PNA probe (Telo), at the indicated time points after bleomycin treatment. Arrows indicate telomeric signals colocalizing with 53BP1 foci. (Scale bar = 10 μm). Right, graph shows the percentages of colocalizations (± s.e.m.) between 53BP1 foci and telomeric PNA probe (Telo), and the number of pS/TQ foci per cell (± s.e.m.) in bleomycin-treated BJ cells, at the indicated time points after treatment. (For the quantification shown, around 50 cells per time point were analyzed). **c.** Persistent DDR foci localize at telomeres in cells irradiated with low dose or fractionated IR. BJ hTERT were irradiated with the indicated dose and stained 30 days later. Left, representative images of colocalizations between DDR, detected as 53BP1 foci, and telomeres, detected using a telomeric PNA probe (Telo). Right, bar graph shows the percentage of 53BP1 foci colocalizing with telomeres at the different

indicated irradiation treatments (For the quantification shown, around 20 cells per sample were analyzed; scale bar = 20 μm). **d.** DDR markers are physically associated preferentially with chromosome end. Upon telomere uncapping following the expression of a dominant negative allele of TRF2, γH2AX was enriched on telomere 12p, as assessed by ChIP and triplicate qPCR using primers at increasing distances from chromosome end (n = 2).

Figure S5. TRF2 colocalizes with persistent pATM foci and its overexpression does not prevent induction of persistent DDR at telomeres and cellular senescence establishment. a. Persistent pATM foci colocalize with TRF2 in IrrSen cells. Representative pictures of colocalizations between TRF2 and DDR, detected as pATM foci in IrrSen BJ hTERT cells. The percentage of colocalization (± s.e.m.) is indicated. (Scale bar = 20 μm) **b.** TRF2 overexpression does not significantly affect the number of persistent DDR foci and their colocalization with telomeres. TRF2 and GFP overexpressing BJ hTERT cells were irradiated (20 Gy) and analyzed 30 days later. Bar graphs show the number of 53BP1 foci per cell, (**c.**) the percentage of 53BP1 foci colocalizing with telomeres, (**d.**) the number of these events per cell and **(e.)** the percentage of SA-β-gal-positive cells (± s.e.m.), compared with non-irradiated control (For the quantification shown, around 50 cells per sample were analyzed).

Figure S6. Ectopic localization of TRF2 next to a DSB, acting locally *in cis*, induces a more protracted site-specific DDR activation and this is not due to steric hindrance. a. Site-specific DDR detection in NIH 2/4 cells upon I-SceI activation. Top, representative images of immunostaining show γH2AX focus colocalizing with LacI and YFP-Tet signals upon I-SceI activation in both LacI and

LacI-TRF2 expressing cells. Bottom, after I-SceI inactivation, a persistent site-specific γH2AX focus is detected only in LacI-TRF2 expressing cell. (Scale bar = 20 μm) **b.** Quantification of I-SceI-locus γH2AX-positive cells (± s.e.m.) expressing LacI, LacI-CFP or LacI-TRF2, as detected by immunofluorescence and confocal microscopy. I-SceI ON corresponds to 3 hours after RFP-I-SceI-GR induction, I-SceI OFF corresponds to 24 additional hours after removal of inducing agent. I-SceI site was detected as a distinct focus doubly positive for YFP-Tet and anti-LacI antibody signals (For the quantification shown, around 50 cells per sample were analyzed). **c.** Bar graph shows the average number of γH2AX foci per cell (± s.e.m.) upon IR (2 Gy). NIH 2/4 cells were transfected with LacI or LacI-TRF2 and irradiated 24 hours later. Immunofluorescence was performed at the indicated time points, after irradiation. (For the quantification shown, around 30 cells per sample were analyzed).

Figure S7. Exogenous telomeric DNA ends associate with telomeric proteins in the cell, they are not degraded and trigger protracted DDR signalling a. Schematic of microinjection experiments. Linearized plasmid DNAs with scrambled or telomeric tandem repeats at their 3' end, or in their circular form, were microinjected into the nuclei of HDFs. **b.** Exogenous linear telomeric DNA binds endogenous telomeric proteins. HeLa cells were transfected with linearized plasmid DNA carrying scrambled or telomeric tandem repeats. ChIP using an anti-TRF2 antibody, followed by qPCR, shows TRF2 binding to the transfected DNA, expressed as percentage of input. Anti-FLAG antibody was used as a negative control. Bar graph shows the ratio between each IP and its input (± s.e.m.) from triplicate qPCR reactions (n = 2). **c.** Cell cycle re-entry is not due to linear plasmid degradation. Top, schematic of the circular plasmid used for microinjection experiments. qPCR was

performed using a proximal pair of primers (approximately 200 bp from the DNA end) and a distal pair of primers (approximately 1500 bp from the DNA end) as indicated by the arrows. Bottom, 48 hours after microinjection, total DNA was extracted and triplicate qPCR results were normalized on non-transfected plasmidic DNA. Amplification of the distal region was normalized to 1. **d.** Exogenous linear telomeric DNA causes a more protracted DDR than scrambled DNA. Percentages of cells (± s.e.m.) showing p53 pSer15 induction were calculated at 24h and 48h post-microinjection of telomeric linear or scrambled linear DNA (For the quantification shown, around 300 cells per time point, per DNA type, were analyzed: n = 2).

Figure S8. Baboons accumulate persistent DDR foci during ageing. Bar graphs shows the percentages (± s.e.m.) of 53BP1 foci-positive cells in **(a.)** hippocampal neurons of four old baboons, compared to two young ones (* p-value < 0.05) and **(b.)** liver hepatocytes of ten old baboons, compared to twelve young ones (*** p-value < 0.001).