**Supplementary figures**

**Figure S1**

A RNase H2B transcript levels are greatly reduced in RNase H2ΔTam MEFs 3 d post 4-OHT, as quantified by qPCR. Error bars are SEM, \*\*\*=p<0.001, t-test (n=3).

B Flow cytometry analysis using α-γH2AX revealed increased DNA damage in RNase H2ΔTam MEFs compared to control MEFs.

C Confocal microscopy showing almost complete overlap of α-dsDNA and DAPI signals (scale bar, 10 µM).

D Cell cycle analysis by flow cytometry at 3 d post 4-OHT confirmed proliferative arrest of RNase H2ΔTam and control MEFs following serum starvation (0.5 % FCS for 3 d). Note the virtual absence of apoptotic sub-G1/G0 cells in all experimental groups. S-Phase cells were labeled with EdU. Error bars represent SEM, \*\*\*=p<0.001, \*=p<0.05, 2-way-ANOVA and Bonferroni’s post test (n=3).

**Figure S2**

A qPCR revealed strong downmodulation of cGAS and STING transcripts in both RNase H2ΔTam and control MEFs 3 d after siRNA transfection (simultaneous with 4-OHT treatment), compared to scrambled siRNAs. Error bars are SEM, \*\*\*=p<0.001, \*\*=p<0.01, 1-way-ANOVA (n=3).

B qPCR demonstrated efficient knockdown of Atg5 mRNA expression in RNase H2ΔTam and control MEFs 3 d after siRNA transfection (simultaneous with 4-OHT treatment). Error bars are SEM, \*\*\*=p<0.001, 1-way-ANOVA (n=3).

C Enzymatic activity of lysosomal β-hexosaminidase is not altered in RNase H2ΔTam MEFs 3 d post 4-OHT. Error bars are SD, n.s.=not significant, t-test (n=3).

D Confocal immunofluorescence analysis showed no major changes in lysosome distribution between RNase H2ΔTam and control MEFs 3 d post 4-OHT. Lysosomes were stained with α-LAMP-1, while DAPI served as nuclear counterstaining. Scale bar, 20 µM.

E Transcript levels of lysosomal genes Lamp1 and Ctsa were not significantly altered in RNase H2ΔTam MEFs compared to control MEFs 3 d post 4-OHT, as measured by qPCR. Error bars are SEM, n.s.=not significant, t-test (n=4).

**Figure S3**

A RNase H2ΔTam and control MEFs were treated with indicated doses of Rapamycin and Torin 1 for 3 d (in parallel with 4-OHT) and CXCL10 transcript levels were analyzed by qPCR. Solvent-treated cells served as controls (DMSO). Error bars represent SEM, \*\*\*=p<0.001, \*\*=p<0.01, 1-way-ANOVA and Tukey's multiple comparison test (n=3).

B The Rapamycin and Torin 1 regimen used in Fig. 4E+F and S3A led to efficient inhibition of mTOR. Phosphorylation of the mTOR substrate S6 kinase was assessed by Western Blotting, while actin served as loading control.

C Transcript levels of major retroelement classes in HEK293T and HeLa cells lacking RNase H2A were quantified by qPCR. Although a trend towards higher retroelement expression was observed in most RNase H2A-knockout clones compared to parental cells, this did not reach statistical significance. Error bars are SEM, n.s.=not significant, 1-way-ANOVA and Tukey's multiple comparison test (n=3).

**Figure S4**

A The LINE-1 reporter plasmids contain the two LINE-1 genes ORF1 and ORF2, which are under control of a strong promoter (dark blue, e.g. the CAG promoter for the dual-Luciferase LINE-1 reporter). In addition, the LINE-1 3’ UTR is interrupted by a reporter gene cassette, which has its own promoter (light blue) and and is expressed from the antisense strand relative to the L1 promoter (depicted upside down). The reporter gene in turn is disrupted by an intron (I) that is removed upon transcription and mRNA processing. Thus, the reporter gene (firefly luciferase or eGFP in this study) becomes functional only after one full cycle of transcription, reverse transcription and integration.

B *De-novo* LINE-1 retrotransposition is strongly diminished in RNase H2A-deficient HEK293T cell clones. Cells were transfected with an eGFP-tagged LINE-1 reporter plasmid and fluorescence was measured 3 d later using flow cytometry. eGFP fluorescence was normalized to a retrotransposition incompetent LINE-1 plasmid. Transfection efficiency among parental HEK293T cells and RNase H2A knockout clones was comparable (data not shown). Fluorescence is only generated after one completed round of LINE-1 retrotransposition consisting of transcription, splicing, reverse transcription and genomic integration. Error bars are SEM, \*\*\*=p<0.001, \*\*=p<0.01, 1-way-ANOVA and Tukey's multiple comparison test (n=4).

**Supplementary tables**

**S1 Genotyping primers & PCR program**

|  |  |
| --- | --- |
| H2B\_flox\_for | TCCTGAGCTTGAAAAGTCACTTCG |
| H2B\_flox\_rev | TTTATGAAGCCCATCACTACACGC |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| initiation | 95 | °C | 3 | min |  |
| denaturation | 95  | °C | 30  | s | 35 x |
| annealing | 53  | °C | 30 | s |
| elongation | 72  | °C | 30  | s |
| final elongation | 72  | °C | 10  | min |  |

|  |  |
| --- | --- |
| Cre\_ERT2\_for | GCGGTCTGGCAGTAAAAACTATC |
| Cre\_ERT2\_rev | GTGAAACAGCATTGCTGTCACTT |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| initiation | 95  | °C | 3 | min |  |
| denaturation | 95  | °C | 30  | s | 35 x |
| annealing | 51,7  | °C | 1 | min |
| elongation | 72  | °C | 30  | s |
| final elongation | 72  | °C | 10  | min |  |

**S2 Target specific qPCR primer pairs**

|  |  |
| --- | --- |
| mGAPDH\_fw | TTCACCACCATGGAGAAGGC |
| mGAPDH\_rev | GGCATCGACTGTGGTCATGA |
| mRNaseH2B\_fw | AGGTTTCCAGGGACAAGGAAGAGGA |
| mRNaseH2B\_rev | GTCAATGAAGCTGGAGGTTCTGGAAG |
| mIRF7\_fw | ATGCACAGATCTTCAAGGCCTGGGC |
| mIRF7\_rev | GTGCTGTGGAGTGCACAGCGGAAGT |
| mIFIT1\_fw | GAACCCATTGGGGATGCACAACCT |
| mIFIT1\_rev | CTTGTCCAGGTAGATCTGGGCTTCT |
| mIFIT2\_fw | ATGAGTTTCAGAACAGTGAGTTTAA |
| mIFIT2\_rev | AACTGGCCCATGTGATAGTAGACCC |
| mCXCL10\_fw | GCCGTCATTTTCTGCCTCA |
| mCXCL10\_rev | CGTCCTTGCGAGAGGGATC |
| mIFIT3\_fw | TGGCCTACATAAAGCACCTAGATGG |
| mIFIT3\_rev | CGCAAACTTTTGGCAAACTTGTCT |
| mAtg5\_fw | AGCCAGGTGATGATTCACGG |
| mAtg5\_rev | GGCTGGGGGACAATGCTAA |
| mCtsa\_for | GACTCCAAGCACTTCCACTACTGGT |
| mCtsa\_rev | CTGGCTGGATCAGAAAGGGGCCGTG |
| mLamp1\_for | TAATGGCCAGCTTCTCTGCCTCCTT |
| mLamp1\_rev | AGGCTGGGGTCAGAAACATTTTCTT |
| ALU-F | GAGGCTGAGGCAGGAGAATCG |
| ALU-R  | GTCGCCCAGGCTGGAGTG |
| HERV-K\_pro-F | GCCGATGAAAAAGCCCGTAAGG |
| HERV-K\_pro-R | TTGACACTCAGGATTGGCGTTTTC |
| LINE-1-ORF1-F | GGTTACCCTCAAAGGAAAGCC |
| LINE-1-ORF1-R | GCCTGGTGGTGACAAAATCTC |
| h GAPDH-F | GATCATCAGCAATGCCTCCT |
| h GAPDH-R | TGTGGTCATGAGTCCTTCCA |
| mSTING\_fw | AAATAACTGCCGCCTCATTG |
| mSTING\_rev | ACAGTACGGAGGGAGGAGG |
| mcGAS\_fw | GAGGCGCGGAAAGTCGTAA |
| mcGAS\_rev | TTGTCCGGTTCCTTCCTGGA |
|  |  |