Cell fitness screens reveal a conflict between LINE-1 retrotransposition and DNA replication

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LINE-1 retrotransposon overexpression is a hallmark of human cancers. We identified a colorectal cancer wherein a fast-growing tumor subclone downregulated LINE-1, prompting us to examine how LINE-1 expression affects cell growth. We find that nontransformed cells undergo a TP53-dependent growth arrest and activate interferon signaling in response to LINE-1. TP53 inhibition allows LINE-1+ cells to grow, and genome-wide-knockout screens show that these cells require replication-coupled DNA-repair pathways, replication-stress signaling and replication-fork restart factors. Our findings demonstrate that LINE-1 expression creates specific molecular vulnerabilities and reveal a retrotransposition-replication conflict that may be an important determinant of cancer growth.

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ectopic expression system in telomerase-immortalized retinal pigment epithelium-1 (RPE) cells, genetically stable diploid cells with intact p53 and DNA-damage responses (Fig. 2a,b). LINE-1 expression markedly inhibited RPE clonogenic growth by 98.2% compared with enhanced green fluorescent protein (eGFP) control (Fig. 2c).

**TP53** loss-of-function mutations clinically correlate with LINE-1 activity 12,25,27, so we compared clonogenic growth of RPE cells expressing LINE-1 or eGFP (measured as number of LINE-1 cells per 100 eGFP colonies) with and without **TP53** knockdown (Fig. 2d and Extended Data Fig. 2a). TP53 knockdown rescued LINE-1+ cell clonogenicity by 42.3-fold, but did not fully restore to LINE-1+ cells the clonogenic potential of eGFP-expressing cells. To test whether TP53 function affects retrotransposition efficiency in this system, we used a reporter assay to compare LINE-1 insertion frequencies in control and TP53-knockdown cells, but found no significant difference (Extended Data Fig. 2b). Thus, p53 restricts growth of these cells but not retrotransposition potential.

We next performed a genome-wide CRISPR knockout screen to identify knockouts that rescue growth of LINE-1+ cells (Fig. 2e and Methods). Single-guide RNAs (sgRNAs) targeting **TP53** were the only ones to significantly enhance cell fitness (Fig. 2f and Extended Data Fig. 2c). Guides targeting **CDKN1A** (p21), a **TP53**-dependent growth-arrest effector and retrotransposition suppressor28, were enriched but did not reach genome-wide significance (Fig. 2f and Extended Data Fig. 2c). Guide RNAs targeting other genes down-stream of **TP53** did not tolerate cells to LINE-1 expression. To validate these findings, we transduced two individual sgRNAs targeting **TP53** or **CDKN1A**, or non-targeting control (NTC), in RPE cells expressing Cas9, and found that each knockout rescued growth of LINE-1+ cells (Fig. 2g). These data demonstrate that LINE-1 expression causes a p53–p21-dependent growth arrest.

LINE-1 induces p53-mediated G1 arrest and an interferon response. To characterize this growth arrest further, we performed
RNA sequencing (RNA-seq) in RPE cells encoding a doxycycline-inducible (Tet-On) codon-optimized LINE-1 (ORFeus) or luciferase control. In total, 2,261 genes were differentially expressed by more than twofold and met Bonferroni-corrected significance (Fig. 3a). Gene set enrichment analysis revealed upregulation of the p53 pathway, and downregulation of cell-cycle progression genes (Fig. 3a, Extended Data Fig. 3a and Supplementary Table 1). Genes possessing p53 regulatory elements (‘direct targets’), including CDKN1A, were upregulated in LINE-1+ cells \((P < 2.2 \times 10^{-19})\), and genes repressed via p21 (‘indirect targets’) were downregulated \((P < 2.2 \times 10^{-19})\) (Fig. 3b). We confirmed by flow cytometry that LINE-1+ cells accumulated in G1 in a LINE-1- and TP53-dependent manner (Extended Data Fig. 3b).

LINE-1 expression induces expression of the apoptotic effectors phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1, NOXA) and BCL2 binding component 3 (BIRC3, PUMA), but does not activate caspase-3, as determined by western blot (data not shown); genes associated with the senescence-associated secretory phenotype (SASP)\(^{30–34}\) were not significantly upregulated (data not shown). These findings are consistent with LINE-1 inducing a p53-mediated G1 cell-cycle arrest.

Most (63.6%) of the gene sets upregulated by LINE-1 expression reflect interferon (IFN)-signaling (Fig. 3c and Supplementary Table 1) and IFN-stimulated genes (Extended Data Fig. 3c), consistent with prior reports\(^{30–31}\). This appears to be driven by IFN-β1 (IFNB1) and the double-stranded RNA (dsRNA)-sensing pathway involving...
Fig. 3 | LINE-1 activates a p53 and IFN response. a, Left: volcano plot of differentially expressed genes. Vertical dashed lines indicate log2(fold change) of -1 or +1, and the horizontal dashed line indicates a FWER-controlled P value of 0.05. Right: histograms of gene set enrichment analysis results. Gene set names are indicated above each plot. The number of genes is indicated on the y axis, and the x axis indicates differential expression bins. Individual genes comprising these datasets are highlighted in the volcano plot according to the colors of the bars in the histograms. Data are derived from n = 3 independent replicates. b, Violin plots illustrating differential expression of p53 transcriptional targets. Direct and indirect target genes are curated from refs. **7,8.** Horizontal bars mark median values. The number of genes in each group is indicated below the plot. c, Histogram of gene set enrichment results.

To identify these pathways, we conducted a knockout screen in TP53 reduced (TP53<sup>KO</sup>) RPE cells expressing Cas9 protein (RPE–Cas9) with Tet-On transgenes encoding codon-optimized LINE-1 or luciferase (Fig. 4a). We generated knockout-cell pools in triplicate and expressed LINE-1 or luciferase for 27 d, sampling the populations for sgRNA representation every 4–5 d. Knockouts that become more highly represented in LINE-1+ cells relative to luciferase<sup>+</sup> controls indicate a positive growth interaction, whereas those that are lost indicate a synthetic lethal interaction. Non-targeting control (NTC) sgRNAs and IFN<sup>+</sup> controls indicate expected growth effects are p53-dependent. As expected, sgRNAs targeting essential genes were depleted from both LINE-1+ and luciferase+ populations (Extended Data Fig. 4c). We found 1,390 gene knockouts with significant fitness interactions (Fig. 4b and Supplementary Table 2). Only 24 rescued LINE-1+ cell growth. Knockout of the APC tumor suppressor is among these (Extended Data Fig. 4d), which is notable since TP53 and APC mutations frequently co-occur in colorectal cancers<sup>7,18</sup>. IFNAR1 (IFN receptor) would be synthetic lethal with LINE-1 expression, and they would rely on specific pathways to suppress LINE-1 toxicity. Their loss of function may be potential therapeutic targets for LINE-1+ cancers.

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Toll-like receptor 3 (TLR3), DExD/H-box helicase 58 (DDX58, or RIG-I) and interferon induced with helicase C domain 1 (IFIH1, or MDA5) (Fig. 3d,e). cGAS–STING is not expressed in these cells. LINE-1 also induces nuclear factor-κB (NF-κB)—an immune signaling transcription factor that can be activated by the RNA-sensing pathway<sup>9,10</sup> and NF-κB transcriptional targets, including the proinflammatory cytokines interleukin-1β (IL-1β) and CXCL8 (Extended Data Fig. 3d). LINE-1 expression in TP53 knockdown cells similarly induces expression of IFNB1 and interferon-inducible genes, including TLR3, IFIT1 and IFIT2 (Extended Data Fig. 3e), indicating the response is p53-independent. In contrast, addition of nucleoside reverse-transcriptase inhibitors known to act on LINE-1, calcitabine (ddC) or didanosine (ddI)<sup>51</sup>, attenuated the IFN response (Extended Data Fig. 3f). Thus, LINE-1 expression induces an IFN response that might contribute to its inhibitory effects on cell growth independent of p53.

Mapping LINE-1 fitness interactions in TP53-deficient cells. We next hypothesized that TP53-deficient (TP53<sup>KO</sup>) LINE-1+ cells may rely on specific pathways to suppress LINE-1 toxicity. Their loss would be synthetic lethal with LINE-1 expression, and they would be potential therapeutic targets for LINE-1+ cancers.
knockout also enhanced cell growth (Extended Data Fig. 4e), highlighting that LINE-1-associated IFN activation suppresses cell growth independently of p53. In contrast, most genes identified in this screen (n = 1,366) demonstrate synthetic lethal interactions in LINE-1+ cells within 3 weeks of sustained expression (Fig. 4c).

We asked whether genes known to alter LINE-1 retrotransposition efficiency or that encode proteins that physically interact with ORF1p or ORF2p (ref. 39–42) were enriched for fitness interactions (Fig. 4d and Supplementary Table 3). Of these 239 genes, 59 (24.7%) were identified in our fitness screen, compared with 12.0% (1,390/11,564) of all genes tested, a 2.05-fold enrichment (\(\chi^2 = 8.4 \times 10^{-9}\)). The majority, 58 of 59 (98.3%), demonstrated synthetic lethal interactions. Of these 59 genes, 10 enhance retrotransposition, 26 suppress retrotransposition and 25 encode physical interactors. However, these 59 genes only account for 4.2% of genes identified in our study, indicating that most fitness interactors are distinct from host genes that regulate retrotransposition.

We performed an overrepresentation analysis on all significant fitness interactors and found a 1.4-fold enrichment of genes encoding nuclear proteins (\(\chi^2 = 6.61 \times 10^{-21}\); 50.1% of significant genes compared with 35.2% of genes in the library; Methods).

Fig. 4 | Mapping LINE-1 fitness interactions in TP53-deficient cells. a, TP53KD cells are RPE-Cas9 cells stably transduced with shRNA to knock down p53 and then engineered to express luciferase (pDA094) or codon-optimized LINE-1 (pDA095) in a doxycycline-inducible manner (Tet-On). Tet-On cells were transduced with the Brunello CRISPR knockout library at a multiplicity of infection of 0.3 and were puromycin-selected for 8 d before expression of LINE-1 or luciferase was induced for 27 d. Cell pools were sampled at 4- to 5-d intervals and analyzed for sgRNA representation with MAGECK. Count data are normalized to reads that align to 1,000 built-in NTC sgRNAs (black). b, Genes shown as rank-ordered plot of Stauffer Z scores (\(Z_s\)) with a FWER of 0.05. Inset indicates the number of 95% confidence interval overlaps over all time points between LINE-1 and luciferase groups among gene knockouts that meet the FWER threshold (red) versus those that do not (gray). c, Heat map of 1,390 significant genes depicting the Z scores over time, ranked by \(Z_s\). There are 1,366 synthetic lethal interactions and 24 rescue interactions. Most knockouts achieved detectable effects by 17–22 d into the screen, evidenced by increasing gene Z scores during these time points. d, Overlap of genes with LINE-1 fitness interactions observed in the present study with genes previously known to interact with LINE-1 proteins, physically or by modifying retrotransposition. Previously known LINE-1 interactors were identified by Liu et al., Goodier, Cheung & Kazazian, Taylor et al., and Moldovan and Moran.

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We found 41 gene ontology (GO) terms with a false-discovery rate (FDR) < 0.05 (Supplementary Table 4). The top enriched term was ‘mRNA processing’ (FDR = 2.29 × 10^-10); we also found terms related to maintenance of genome integrity, including ‘DNA repair’ (FDR = 4.47 × 10^-7) and ‘DNA replication’ (FDR = 0.01), and chromatin-related gene sets, including ‘histone modification’ (FDR = 3.07 × 10^-8) and ‘regulation of chromatin organization’ (FDR = 0.001).

**HUSH complex loss increases LINE-1 transgene expression.** Human silencing hub (HUSH) knockouts produced pronounced LINE-1 synthetic lethal interactions, which we validated by single-gene-knockout clonogenic growth studies (Extended Data Fig. 5a-c). HUSH is an epigenetic repressor complex that targets transgenic DNA sequences, including lentivirus insertions and endogenous LINE-1 loci. Thus, we tested whether HUSH loss increases LINE-1 expression, either from endogenous LINE-1 loci or from the codon-optimized transgene. We did not detect ORF1p or ORF2p in doxycycline controls (Extended Data Fig. 5d), indicating that HUSH-mutant RPE cells do not upregulate endogenous LINE-1 proteins. In doxycycline-treated cells with the LINE-1 transgene, ORF1p, ORF2p and transgene messenger RNA expression increased with HUSH knockout (Extended Data Fig. 5e,f), and ORF2p protein level linearly correlated with transgene mRNA level (two- to fourfold increase, Extended Data Fig. 5g). ORF2p expression could be similarly increased in HUSH-intact cells transfected with Tet-On LINE-1 plasmid treated with higher doses of doxycycline (Extended Data Fig. 5h), and this is highly cytotoxic. We conclude that the synthetic lethal effect of HUSH mutants is caused by enhanced expression of the LINE-1 transgene. We note that high levels of ORF2p expression overwhelm the survival advantage conferred by TP53 deficiency.

**RNA-processing gene knockouts sensitize cells to LINE-1 expression.** The GO term ‘mRNA processing’ encompasses 81 genes demonstrating fitness interactions in LINE-1 cells; these genes are enriched for spliceosome components (P = 2.24 × 10^-10), and knockouts of these are synthetic lethal in LINE-1 cells (Extended Data Fig. 6a,b). We validated this effect by treating cells with the splicing inhibitor pladienolide B (PLA-B), which acts on the essential gene SF3B1 (splicing factor 3b subunit 1), a component of the U2 small nuclear ribonucleoprotein (snRNP). At a PLA-B dose that reduced luciferase* clonogenic growth by 6.8%, LINE-1 cells grew 27.8% fewer colonies, a 4.1-fold increased sensitivity to PLA-B (P = 0.044, Extended Data Fig. 6c). We analyzed RNA-seq data from LINE-1 RPE and did not observe alternatively spliced isoforms of the LINE-1 transgene (data not shown), indicating that these gene knockouts probably affect cell growth through an indirect mechanism, rather than by directly processing the LINE-1 RNA. Notably, cells subjected to DNA damage also are sensitized to loss of spliceosome components.

We found pronounced synthetic lethal interactions caused by knockouts of genes encoding the nuclear exosome-targeting (NEXT) complex, which degrades intronic RNAs and processed transcripts. Two of the three complex members demonstrate synthetic lethal interactions (RBM7 and ZCCHC8), whereas the third (SKIV2L2) is encoded by an essential gene (Extended Data Fig. 6d). Similarly, RNASEH2 knockout is synthetic lethal in LINE-1 cells (Extended Data Fig. 6e). RNASEH2 facilitates retrotransposition by degrading LINE-1 RNA from RNA–DNA hybrids after reverse transcription occurs. Thus, when RNASEH2 is lost, this precludes LINE-1 retrotransposition and enhances toxicity.

Finally, we find that LINE-1 cells require the dsRNA adenosine (A) to inosine (I) editing enzyme ADAR1 (Extended Data Fig. 6f), as do cancer cell lines with high expression of interferon-stimulated genes.

**Fanconi anemia proteins suppress LINE-1 toxicity.** DNA-repair genes that suppress LINE-1 toxicity were enriched for Fanconi anemia (FA)–BRCA1 pathway components (P = 7.65 × 10^-13, Fig. 5a). The FA pathway is critical for resolving DNA interstrand crosslinks and transcriptional R-loops that interfere with progression of DNA replication. Knockout of the majority (83%) of the genes known to cause FA and several related genes exhibited synthetic lethal interactions with LINE-1 (Fig. 5b and Extended Data Fig. 7a), including BRCA1 (FANCBS). We chose five genes to validate based on their functions in the pathway: FANCM, a helicase and branch translocationase that has high affinity for stalled replication forks and RNA–DNA hybrids; FANCA, which is required for FA ‘core complex’ assembly; FANCL, the E3 ubiquitin ligase that activates the downstream effectors of the ‘ID Complex’; and ID complex members FANCI and FANCD2. We confirmed knockout efficacy by measuring MMC-induced FANCD2 mono-ubiquitination (FANCD2-Ub) (Fig. 5c). MMC-induced FANCD2-Ub in NTC-treated cells, but not in the FA knockouts. These FA-deficient mutants were selectively sensitive to LINE-1 expression compared with NTCCs (Fig. 5d), and displayed slight increases in chromatin-bound γH2A.X, a marker of DNA damage, compared with NTC-treated LINE-1 cells (1.1- to 1.7-fold, Extended Data Fig. 7b). Expression of native LINE-1 sequence is also synthetic lethal in FANCD2-knockout cells compared with NTCs (Extended Data Fig. 7c).

On the basis of these data and reports that FA proteins suppress retrotransposition*, we hypothesized that the FA pathway is activated by LINE-1. To test this, we measured mono-ubiquitination of FA effector proteins FANCD2 and FANCI and found 1.6- and 1.5-fold increases, respectively, with LINE-1 expression (Fig. 5e). Importantly, LINE-1 cytotoxicity has been previously reported to depend on endonuclease (EN) and reverse transcriptase (RT) activities**, and we confirmed that expression of LINE-1 with inactivating EN and RT mutations is less toxic than wild-type (WT) LINE-1 (Extended Data Fig. 8). To dissect whether the enzymatic activities of LINE-1 are necessary for FA activation, we measured FANCD2 mono-ubiquitination in HeLa cells expressing wild-type LINE-1 or mutants lacking EN activity and/or RT activity. Whereas wild-type LINE-1 increased FANCD2-Ub (2.6-fold), both EN- and RT-inactivating mutations (H230A and D702Y, respectively)** did not (Fig. 5f). We next assessed FA activation by enumerating FANCD2 nuclear foci. We expressed WT or RT mutant LINE-1 and quantified FANCD2 nuclear foci in randomly imaged, EdU-labeled cells. Both hydroxyurea (HU) treatment and LINE-1 expression increased the number of FANCD2 foci in S phase (EdU+) cells (P = 1.7 × 10^-4 and 5.8 × 10^-11, respectively, Fig. 5g) but not those in G1/G2 (EdU-) phase (Extended Data Fig. 7d). The LINE-1 RT mutant did not induce FANCD2 foci formation. Together, these data demonstrate that LINE-1 activates the FA complex and replication-coupled DNA repair. By contrast, LINE-1 EN and RT mutants do not have this effect, suggesting that the LINE-1 retrotransposition intermediate is crucial to the process.

To evaluate DNA damage associated with LINE-1 expression, we measured γH2A.X and 53BP1 nuclear foci. We found that LINE-1 cells have transient increases in numbers of γH2A.X and 53BP1 foci as compared with control cells (P = 3.4 × 10^-4 and 1.7 × 10^-12, respectively, Fig. 5h). These increases are detectable in S phase and resolve by G2, whereas doxorubicin-induced DNA damage foci continue to accumulate (data not shown). This pattern is more consistent with LINE-1-induced replication stress*** than with a large burden of persistent, dsDNA breaks.

**Retrotransposition–replication conflict underpins LINE-1 toxicity.** We next explored interactions between LINE-1 retrotransposition and DNA replication using our fitness screen data. Stalled replication forks activate signaling pathways involving ataxia telangiectasia and Rad3-related (ATR) and ATR-interacting protein...
**Fig. 5 | The Fanconi anemia pathway is essential in p53-deficient cells.** **a.** A network of 75 DNA repair genes identified in the screen is enriched for Fanconi anemia genes (blue nodes). Edges indicate known physical interactions. **b.** Model of FA complexes responding to a DNA lesion (vertical line) encountered by a replication fork (blue line, genomic DNA; green line, nascent DNA). Genes are color-coded on the basis of the performance of their knockouts. Illustration generously provided by J. Fairman of the Department of Art as Applied to Medicine at Johns Hopkins University School of Medicine. **c.** Western blot of FANCD2 response to 24-h treatment with 1 µg ml⁻¹ MMC. Cells are treated with FA member sgRNAs or NTC. FANCD2 mono-ubiquitination assessed as the ratio of FANCD2-L (long) to FANCD2-S (short) band intensities (relative L/S ratio) graphed relative to NTC, MMC-treated cells. n.d., not determined. **d.** Clonogenic growth assay of LINE-1⁺ TP53⁰ cells with sgRNAs targeting the same genes as in c, n = 3 independent experiments. P value calculated with a one-sided t-test. **e.** Left: representative western blot of FANCD2 and FANCI following 72-h expression of LINE-1 or luciferase in RPE. MMC treatment reveals L (mono-ubiquitinated) and S (nonubiquitinated) protein bands. Right: quantification of FANCD2 foci. Number of cells per group: Lucif., n = 134; HU, n = 105; wild type, n = 109; RT (D702Y), n = 101. n.s., not significant. **f.** Left: representative images of FANCD2 foci (green) in EdU⁺ nuclei. Scale bar, 6 µm. Right: quantification of FANCD2 foci. Number of cells per group: untreated, n = 134; HU, n = 105; wild type, n = 109; RT (D702Y), n = 101. n.s., not significant. **h.** Left: γH2A.X and 53BP1 focus quantification in EdU⁺ TP53⁰ cells. Number of cells per group: Lucif., n = 326; LINE-1, n = 358; doxorubicin, n = 431. Two-sided t-tests were used for statistical comparisons in g and h. Right: representative images of γH2A.X (red), 53BP1 (green), EdU (cyan) and DAPI (blue). Scale bar, 12 µm. Uncropped blot images of c, e and f are shown in Supplementary Data 1.
**Fig. 6 | LINE-1 activity induces replication stress.**

**a.** Median count of sgRNAs targeting replication stress signaling genes ATRIP and the 9–1–1 complex (HUS1 and RAD1) during the screen. Error bars indicate 95% confidence intervals. 
**b.** Clonogenic assay of LINE-1–TP53 cells (induced with 1 μg ml⁻¹ doxycycline) with CRISPR knockout of ATRIP compared with NTC. Error bars indicate s.e.m.; n = 3 independent experiments. P value is calculated with an unpaired two-sided t-test. 
**c.** Clonogenic assay of LINE-1–TP53 cells (induced with 1 μg ml⁻¹ doxycycline) with drug inhibition of ATR kinase by 1 μM VE-821 compared with vehicle (DMSO). Error bars indicate s.e.m.; n = 3 independent experiments. P value is calculated with an unpaired two-sided t-test. 
**d.** Western blot of RPA2 occupancy on chromatin induced by LINE-1 compared with luciferase control after 72 h of expression in RPE. Chromatin-bound protein lysates were used. We used 1 μM MMC as a control to verify that these cells respond to replication stress. 
**e.** Western blot of p-RPA S4/S8 after 72 h of WT or mutant LINE-1 expression in HeLa cells. Relative signal intensity for n = 2 independent experiments ± s.e.m. is quantified. 1 μM MMC was used as a replication stress control and produces RPA2 hyperphosphorylation and a gel shift in total RPA2. WT LINE-1 expression has this effect to a lesser degree. Statistical significance is assessed by ANOVA (P = 0.0007). 
**f.** MMC dose-response clonogenic assay of LINE-1–cells or control. Molar concentration is indicated on the x axis. Data are plotted as the mean viability relative to 100 μM ± s.d.; n = 3 independent experiments. Two-sided t-tests were used to compare relative viability at each dose. 
**g.** Median count of sgRNAs targeting fork protection (RADX) and fork restart (BLM, WRN, WRNIP1) genes. Median values are depicted with 95% confidence intervals. Uncropped blot images of **d** and **e** are shown in Supplementary Data 1.

(ATRIP), as well as the tripartite RAD9, HUS1, RAD1 (9–1–1) complex. ATR and RAD9 are essential, but genes encoding all non-essential components of these complexes (ATRIP, HUS1 and RAD1) are synthetic lethal LINE-1 interactors (Fig. 6a). We validated that ATRIP-knockout cells exhibited heightened sensitivity to LINE-1 expression (Fig. 6b); they also failed to sufficiently activate FANC DNA-induced DNA damage (data not shown). Similarly, ATR inhibition with the compound VE-821 sensitized cells to LINE-1 (Fig. 6c) at a dose that had no effect on viability in luciferase+ cells (data not shown). Thus, compromising replication stress signaling is synthetic lethal in LINE-1+ cells, potentially related to the role of ATR–ATRIP signaling in activating the FA pathway⁴⁴,⁴⁵.

We next assayed for signs of replication-fork stall. Stalled replication forks accumulate single-stranded DNA (ssDNA) coated by replication protein A (RPA), a heterotrimer composed of RPA1, RPA2 and RPA3, to protect genomic DNA from nucleases⁶⁶. We isolated chromatin-bound protein fractions from cells treated with MMC or those expressing LINE-1 or luciferase and found that both MMC treatment and LINE-1 expression increased chromatin-bound RPA2 (Fig. 6d). These data show replication stress occurring in a LINE-1-dependent manner. We next asked whether LINE-1-associated replication stress depends on ORF2p enzymatic activity. We expressed wild-type or mutant LINE-1 from Tet-On plasmids in HeLa cells and measured p-RPA S4/S8, a phosphorylation modification placed on RPA2 during replication stress. Wild-type LINE-1 significantly
induced phosphorylation by 2.1-fold ($P = 0.0007$), whereas EN- and RT-inactive mutants did not (Fig. 6e). These data indicate that ORF2p must nick DNA and reverse transcribe in order to induce replication stress, highlighting the importance of the retrotransposition intermediate in these events. Moreover, LINE-1+ cells were 1.9-fold more sensitive to MMC as compared with luciferase-expressing controls (Fig. 6f). Together, these data indicate that LINE-1 retrotransposition induces replication stress and sensitizes cells to compounds that increase demands on replication-coupled DNA repair.

Several key processes occur downstream of replication stress signaling, including: (1) fork reversal (that is, translocation of the replication fork away from the lesion and resection by nucleases including ZRANB3, SMARCAL1 and HLTJ); (2) fork protection from excess degradation by nucleases; and (3) fork restart (Fig. 6g). Fork-reversal genes do not score in our screen, whereas the fork protection factor RADX and proteins that are important for fork restart—including Bloom helicase (BLM), Werner helicase (WRN) and WRN interacting protein 1 (WRNIP1)—are LINE-1 synthetic-lethal interactors (Fig. 6g). Fork restart additionally requires the removal of RPA from the ssDNA. To this end, we note that knockout of RFWD3, an FA member whose E3 ubiquitin ligase activity regulates RPA unloading from chromatin, produces synthetic lethality (Extended Data Fig. 7a). These findings indicate that replication-fork protection and restart, but not reversal, are essential for LINE-1 cell growth.

Taken together, these data are consistent with a model wherein LINE-1 retrotransposition intermediates cause replication stress (Fig. 7). LINE-1+ cells rely on FA-mediated DNA repair, replication-stress signaling and fork-restart pathways for growth.

**Discussion**

LINE-1 expression slows cell growth, yet it is a hallmark of many human cancers. Here, we used in vitro LINE-1 expression systems, gene-expression profiling and CRISPR–Cas9 gene-knockout screening to characterize cellular responses to LINE-1 expression. We find that LINE-1 expression in nontransformed cells triggers p53–p21 mediated G1 arrest. Along with studies that place p53 as an upstream repressor of LINE-1 expression, our findings explain associations between LINE-1 expression and TP53 mutations in human cancers. Interestingly, although TP53 loss promotes cell growth absent LINE-1 (ref. 19), we find LINE-1 enhances the relative growth advantage conferred by TP53 mutation, raising the possibility that LINE-1 expression early in tumorigenesis may select for TP53 mutations. This may be relevant in ovarian cancer, in which LINE-1 expression and fixation of TP53 mutations appear to be essentially concordant events in serous tubal intraepithelial carcinoma (STIC) precursor lesions. With implications for colon cancer development, we find LINE-1 enhances growth advantages conferred by APC mutation in p53-deficient cells. APC loss is an early event in these malignancies that can be antedated by LINE-1 expression and retrotransposition.

TP53 loss in turn tolerizes cells to LINE-1 expression. On the basis of a genome-wide CRISPR knockout screen, though, we find that LINE-1 expression confers specific molecular requirements for cell growth in a TP53-deficient background. LINE-1+ cells rely on RNA-processing machinery, including complexes that degrade RNA and splicesome components. The former may directly act on retrotransposition intermediates. Compromised splicing may lead to the accumulation of dsRNA and exacerbate interferon responses to LINE-1 expression, or to an excess of transcriptional R-loops on chromatin that pose barriers to DNA replication.

Most notably, our data indicate that retrotransposition conflicts with DNA replication. This model was suggested by the reliance of LINE-1+, p53-deficient cells on replication-coupled DNA-repair pathways mediated by the Fanconi anemia components.
All FA complex components show synthetic-lethal interactions with LINE-1 expression in our experimental system. Further, we demonstrate that the FA complex assembles in the S phase of the cell cycle in a manner that depends on LINE-1 enzymatic activity. In accordance with the importance of FA in reducing LINE-1 lesions, tumors that frequently express LINE-1 tend to amplify FA genes. Similarly, we find LINE-1+ cells have unique requirements for replication-stress signaling pathways (ATRIP, 9–1–1 complex components), replication-fork protection (RADX) and fork-restart factors (BLM and WRN helicases). We corroborate these genetic interactions biochemically by showing LINE-1 ORF2p enzymatic activities induce replication stress. Notably, both EN and RT activities are required to observe FA-pathway activation as well as replication-stress responses. Based on what is known about target-primed reverse transcription, this observation suggests that the branched LINE-1 insertion intermediate structures create physical blockades to replication-fork progression.

This model is further substantiated by independent, orthogonal observations in our field. In in vitro experimental systems, there is a predilection for de novo LINE-1 insertions to occur in S phase. Moreover, recent studies mapping LINE-1 insertion sites in vitro and in vivo in a wide variety of human cancers indicate nonrandom distributions of insertions with respect to DNA-replication timing. Finally, FA and BRCA1 inhibit LINE-1 retrotransposition, as has been shown by Liu et al., Mita et al., and Moran and Garcia-Perez (personal communication). These findings indicate that retrotransposition is occurring in association with DNA replication, and that replication-coupled DNA-repair pathways are likely reducing retrotransposition intermediates. Loss of these repair pathways enhances both retrotransposition and LINE-1-associated toxicity.

We propose that the most crucial retrotransposition intermediates are found in unreplicated dsDNA positioned to collide with replication forks. It is possible that multiple intermediates form in each cell, and that most are normally reduced by FA repair or other mechanisms rather than resolved into new genomic insertions. Considering that LINE-1 is aberrantly expressed in half of human cancers and many malignancies acquire between tens and thousands of somatic LINE-1 insertions, retrotransposition potentially represents an important source of endogenous replication stress and genomic instability in these malignancies.

Our findings underscore that limits on LINE-1 expression are required in order to preserve cell growth, and indeed we began our study after seeing a tumor subclone that lost LINE-1 expression and grew faster. Moreover, we provide the first evidence of unique molecular vulnerabilities in LINE-1+ cells, which has noteworthy implications for translational cancer research. From a therapeutic perspective, it is possible that LINE-1+ cancers will have characteristic drug sensitivities; for example, LINE-1 ORF2p expression and retrotransposition may prove a biomarker for tumors that respond to DNA-damaging agents, or inhibitors of ATR or WRN helicase. We also demonstrate that LINE-1 promotes a type I IFN response, suggesting roles for LINE-1 in sensitivities to immunotherapies or ADAR inhibition. Experiments in disease-specific model systems that recapitulate chronic LINE-1 exposure are needed to address these possibilities.

Online content
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Wild-type TP53 (Tet-On RPE generation). Cells were grown in DMEM (293, HeLa) or DMEM/F12 with 1.5% sodium bicarbonate (Licor) were added at a 1:10,000 concentration and imaged on a Licor Odyssey infrared imager. Western blotting.

Methods

Experimental model and subject details. Cell lines. We used Tet-On 3G HEK293 cells (ClonTech), Tet-On HEK293T (from D.B.), Tet-On 3G Hela (ClonTech), HEK293T (Tet-On, from A.H.), IHeLa-RPE2– (from A.H.) and IHeLa-RPE1–/- cells. Cells have been authenticated by STR profiling and were grown in DMEM (293, HeLa) or DMEM/F12 with 1.5% sodium bicarbonate (RPE) with 10% tetacycline-free FBS (Takara Bio USA). Cells were cultured at 37 °C, 5% CO2. Antibiotic selection was performed with puromycin (1 µg ml⁻¹), G418 (400 µg ml⁻¹), or blasticidin (10 µg ml⁻¹). Doxycycline was used at 1 µg ml⁻¹, unless otherwise stated. Cells were tested and were mycoplasma negative.

TP53 +/− Generation. For shRNA growth experiments, wild-type TP53–Cas9 cells were transduced with 2µ P-P53–shRNA–TagKIP (ref. 3) or PScor–mCh, empty, and then were transduced with LINE-1 or eGFP plasmids. To generate monovalent knockout cells, RPE–Cas9 cells were transduced with 2µ P-P53–shRNA–FlagRFP lentivirus and single red fluorescent protein (RFP) + cells were sorted by a FACS Aria into 96-well plates. Monoclonal cell lines were screened for p53 knockout by western blot in cells treated with 200 ng ml⁻¹ doxorubicin. Tet-On RPE generation. Wild-type TP53 (TP53 WT) or TP53ื cells were transduced with Sleeping Beauty transposase plasmid (pCMV(CAT)T7-S8100) and a donor plasmid containing Tet-inducible codon-optimized LINE-1 (ORFeus) or Luciferase (pDA091, pDA093, pDA094, pDA095) followed published guidelines. Cells were selected in G418 for 1 week, then sorted into 96-well plates by fluorescence. Monoclones were screened for luciferase induction with the ONE-Glo assay (Promega) or ORF1p protein induction by western blot.

Method Details. Viability Assessments. Viability was determined by clonogenic growth or CellTiter-Glo assay (Promega). Wild-type RPE cells were assessed by clonogenic growth by transfecting 1 × 10⁴ cells with 2 µg eGFP (pDA083) or 3 µg LINE-1 (pDA077) plasmid to achieve equimolar ratios. Cells were split to 10-cm growth-dishes and selected with G418 24 h after transfection. In Tet-On assays, 500 cells were plated and doxycycline was added to activate transgene expression. For MMC sensitivity experiments, cells were treated with 100 µM, 1 mM, 10 mM and 100 mM for 24 h on day 2 after plating. In VE-821 sensitivity, cells were treated with 1 µM drug or DMSO vehicle throughout the duration of the experiment. For assays in CRISPR knockout cells, knockout-cell pools were generated by infecting TP53–/− Tet-On RPE cells with lentivirus encoding either non-targeting control or a gene-targeting guide and selecting with puromycin for 1 week (see Supplementary Table 6 for guide sequences). For all assays, after 10–14 d of LINE-1 or control expression, colonies were washed with PBS and fixed (6% glutaraldehyde, 0.5% crystal violet) for 10 min. Plates were rinsed in water and air-dried, and then imaged on a flatbed scanner. Colonies with >50 cells were counted.

CellTiter-Glo assays (Promega) were performed in HEK293T cells transfected with LINE-1 (pDA007), LINE-1 ORF2 H230A (pDA025), LINE-1 ORF2 D702Y (pDA034), LINE-1 ORF2 H230A/D702Y (pDA027) or empty vector (pDA019). There were 8,000 cells plated per well and treated with doxycycline (10–1000 ng ml⁻¹) for 72 h. CellTiter reagents were then added, and luminescence was measured using a Glomax Multi + Detection System (Promega).

CRISPR knockout screening. We used the Brunello GPP pooled CRISPR knockout library packaged into lentivirus for screening. The library comprises 76,441 guide RNAs targeting 19,114 genes, with 4 sgRNAs per gene. Representative of these screens are curated from published reports. Cell-cycle phase genes were used log2(fold change) values to perform a preranked analysis. Direct and indirect gene knockouts with MAGeCK software v0.5.6 or v0.5.7 (ref. 79) with the Brunello reference index using Bowtie, allowing no mismatches. We restricted sequences. Bar-coded libraries were quantified using the NEB Library Quant Kit (cat. no. E7830) by sonication. PAGE was carried out with manufacturer-recommended buffers on 4–20% or 7.5% Mini TGX Gels (Biorad). Semi-dry transfers were carried out for Biorad gels or NuPAGE BisTris gels at 2.5 A for 5–15 min using the Trans-Blot Turbo (Biorad). Western transfers were carried out for Tris-Acetate gels at 30 V overnight at 4 °C. All blocking was performed with Odyssey Blocking Buffer (Licor). Primary antibodies were incubated with membranes overnight at 4 °C, then infrared-conjugated secondary antibodies (Licor) were added at a 1:10,000 concentration and imaged on a Licor Odyssey System. Western blots are generated using ImageJ and Westerns were stripped with Reblot Plus Strong Solution (Millipore Sigma). A list of antibodies used can be found in the Supplementary Methods Key Reagents table.

Cloning. Plasmids used in this study are listed in Supplementary Table 5. Several are available at https://www.addgene.org/Kathleen_Burns/. The mammalian expression vector PCEP4 (Invitrogen) was modified to possess a second- or third-generation Tet-inducible promoter (ClonTech) by Gibson assembly. LINE-1 sequences were inserted into the vector backbone by Gibson assembly with PCR amplicoms of endogenous LINE-1 sequence (LINE-1 RP) or ORFeus codon-optimized sequence. Control PCEP4 vectors encoded either eGFP or lacZ expression inserts. LINE-1 point-mutant constructs were also created by amplification and Gibson assembly. For Sleeping Beauty–induced LINE-1, ORFeus codon-optimized LINE-1 was cloned into the donor vector pSsBtet-NR or pSsBtet-GN (ref. 75) by Gibson assembly. Briefly, pSsBtet-NR or GN was digested with SfiI and DraII, gel-purified and assembled with PCR-amplified LINE-1 (primers SS-ORFeus5 and SS-ORFeus3 in Supplementary Table 6) using the HiFi 2× Assembly Master Mix (New England Biolabs).

Single-gene CRISPR knockout-cell generation. To validate screen hits, 20 bp CRISPR sgRNAs were cloned into the pLentiGuide-Puro vector digested with BstBI restriction enzyme as previously described, and the plasmids were packaged into lentivirus. We selected sgRNAs that were enriched in the screens. See Supplementary Table 6 for sgRNA sequences. Cells were incubated with lentiviral supernatants supplemented with 10 µg ml⁻¹ polybrene for 24 h, then selected with puromycin for 1 week, and used in downstream clonogenic assays and western blots.

Transfection. HEK293 and HeLa cells were transfected with Fugene HD reagent (Promega) following standard protocols. RPE cells were transfected using mid- or maxi-prepped plasmid DNA with Viafect reagent (Promega) at a DNA/Viafect ratio of 1/3.

Lentivirus packaging. HEK293FT cells were transfected with Fugene HD (Promega), following the manufacturer’s recommendations. Insert vector was added to packaging plasmids pMD.G and pSVAX2 at a ratio of 3/4 by mass. Medium was changed after 24 h and 48 h, and viral supernatants were collected and filtered through 0.45-µm filters. For screen libraries, complex lentivirus pools were packaged by a similar method by Applied Biological Materials.

Retrotransposition reporter assay. We used an eGFP reporter assay to measure retrotransposition. We transfected 2 × 10⁵ RPE cells with 2 µg LINE-1 reporter plasmids (MT525, JM111) or 2 µg eGFP plasmid and selected with 1 µg ml⁻¹
puromycin for 12 d. Cells were trypsinized and resuspended in cryotube buffer (HBSS, no phenol red, 1% FBS, 1 mM EDTA) at a concentration of ~1 × 10^6 cells per ml, then analyzed on a BD Accuri C6 Flow Cytometer. Singlets were gated on SSC-A/SSC-H and FSC-A/PSC-H, then GFP thresholds were set such that untransfected cells showed 0.1% eGFP cells. We normalized the percentage of eGFP+ cells in experimental groups to the percentage of eGFP+ in eGFP- transfected controls.

**Nucleoside reverse transcriptase inhibitor treatments for qRT-PCR.** We plated 250,000 Tet-On TP5325 cells expressing luciferase or LINE-1 on T25 flasks with 1 ng ml⁻¹ doxycycline added, which were then treated with 5 µM zalcitabine (d3T) or 5 µM didanosine (ddI) for 72 h. Cells were lysed, and RNA was extracted using Quick-RNA MicroPrep kit (Zymo Research).

qRT-PCR. cDNA was generated using the Script kit (Biorad) following RNA extraction using the Quick-RNA MicroPrep kit (Zymo). Primers were designed using Primer3 and tested against cDNA to ensure single bands were generated in the PCR. Real-time PCR was performed for 40 cycles (98 °C for 15s, 60 °C for 30s) using SOAdvanced 2x Master mix (Biorad) on the MyIQ cycler (Biorad). Fold change expression was determined by the 2^ΔΔCt method. See Supplementary Table 6 for primer sequences.

**Immunofluorescence Imaging.** HEK293T cells were transfected with doxycycline-inducible LINE-1:1 plasmid (pDA055) and stably selected with hygromycin for 2 weeks. We plated 5,000 cells in a black 96-well, glass-bottom plate (Corning, cat. no. 3663), treated with doxycycline (0–5,000 ng ml⁻¹, 24 h), fixed (3% paraformaldehyde, 10 min), permeabilized (0.5% Triton X-100/PBS, 3 min) and blocked (1% BSA/PBS-glycine, 30 min). Cells were incubated with anti-ORF1p (1:500 dilution, Millipore Sigma) and anti-FLAG (1:500 dilution, Sigma) primary antibodies; the Hoechst 33342 (1:50 dilution, Sigma) DNA marker; and HCS CellMask deep red cytoplasmic stain (1:20,000 dilution, Invitrogen). After brief washing in TBST, cells were incubated with rabbit Alexa Fluor 488 (1:200 dilution, Invitrogen) and anti-mouse Alexa Fluor 568 (1:200 dilution, Invitrogen) secondary antibodies. Imaging was performed with a TE300 epifluorescence microscope (Nikon) with a motorized stage and excitation/emission filters (Prior). Images acquired with a DS-QiMc camera at low magnification (20x Plan Fluor lens, 0.285µm per pixel, Nikon) using Nikon Elements software (Nikon). Twenty-five images were acquired per sample in a 5 x 5 grid (1.88 mm²). Images were analyzed using a custom MATLAB software to segment single cells using the HCS CellMask stain and nuclei using Hoechst 33342. Aqueous cell segmentation was manually verified to create a subset of 100 single cells in which ORF1p and ORF2p signal strengths were measured as the total intensity within each segmented cell for each fluorescence channel.

**Nuclear foci quantification.** We used either Tet-On TP5325 cells expressing luciferase or LINE-1 or Tet-On 3G HeLa cells transfected with doxycycline-inducible LINE-1:1 plasmid (pDA007, pDA025, pDA027, pDA033, pDA019), and stably selected with puromycin for 1–2 weeks. Cells were transiently transfected with either 6mM hydroxurea for 4 h or 200 ng ml⁻¹ doxorubicin for 2 h. We plated 100,000 cells on cover slips, and treated them with 1,000 ng ml⁻¹ doxycycline for 72 h. EdU was added for 2 h, and cells were pre-treated with 0.5% Triton X-100 for 5 min, fixed with 3.7% paraformaldehyde for 10 min, then permeabilized with 0.3% NP-40 for 10 min. EdU Click-IT reaction (ThermoFisher) was performed following manufacturer’s instructions. Slides were blocked (1% BSA/PBS-glycine, 30 min) and incubated with polyclonal rabbit FANC2D1 (1:1,000, Novus Biologicals), rabbit 33BP1 (1:500, Novus Biologicals) or mouse γH2AX (1:1,000, Millipore) for 1 h at room temperature, and then anti-rabbit Alexa Fluor 488 for FANC2D1 (1:200, ThermoFisher) and anti-rabbit Alexa Fluor 488 (1:2,000, ThermoFisher) for 33BP1 and γH2AX, respectively. Slides were imaged at low magnification with the same equipment as described above for 200 cells. We normalized the percentage of foci greater than 0.2 pixels to the total number of nuclei. Foci were quantified using a custom MATLAB script (ref. 23) to segment single cells using the HCS CellMask stain and nuclei using Hoechst 33342. Aqueous cell segmentation was manually verified to create a subset of 100 single cells in which ORF1p and ORF2p signal strengths were measured as the total intensity within each segmented cell for each fluorescence channel.

**Transposon insertion sequencing and PCR validations.** We generated line R1 human 1 transposon knockout clones by viral transduction into A549 cells. We screened ~70,000 clones by qPCR for the presence of the transposon insertion site using a custom-designed 33-base primer. We sequenced selected clones by Sanger sequencing, and inserted transposon insertions were confirmed by Southern blotting and PCR imaging of genomic DNA. We sequenced the transposon insertion site by Sanger sequencing and confirmed the transposon insertion site by Southern blotting and PCR imaging of genomic DNA.

**Data availability**

MAGeCK-normalized sgRNA read counts from CRISPR knockout screens and RNA-seq data and differential expression values have been deposited in the GEO database under accession number GSE119999. Source data for Figs. 2b, 5c,e,f and 6d,e are available online. Requests for resources and reagents should be directed to and will be fulfilled by K.H.B. Select plasmids created in the Burns Lab can be accessed at Addgene (https://www.addgene.org/Kathleen_Burns/).

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**Author contributions**

D.A. and K.H.B. conceptualized this work and wrote the manuscript. Experiments were performed by D.A., J.P.S., C.L., P.-H.W., J.S.S., M.G. and Z.L. All data were primarily analyzed by D.A. Key resources were provided by A.I.H. (RPE cells), A.S. (FANCI mAb), and M.S.T. and V.D. (deidentified colorectal cancer samples). All authors participated in manuscript revisions. Funding was acquired by D.A., J.D.B. and K.H.B.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Extended Data Fig. 1 | LINE-1 heterogeneity in colon cancer. (a) Tissues collected for transposon insertion profiling by sequencing (TIP-seq) mapping of tumor-specific LINE insertions. Fresh frozen tissue was collected from two sites in the primary tumor (P1, P2) in the colon and one site in the metastatic tumor (M) in the liver. Normal tissue was collected from the liver. The liver metastasis exhibited ORF1p immunoreactivity as well (data not shown). (b) Circos plot detailing TIP-seq results and whether insertions were found in the primary (P only), metastasis (M only) or in both (P & M). In the validation process, we identified 11 3′ transduction events, 6 of which mapped to two LINE-1 sequences on Xp22.2 and one on 3q21.1 that are known to be highly active tumor alleles. As expected, the majority of this tumor’s de novo insertions were intronic or intergenic and not near known tumor suppressors or oncogenes. (c) We genotyped the insertions using hemi-specific PCR in genomic DNA obtained from dissected histology slides and compared to the allele’s presence in bulk frozen tissue used for TIP-seq. In all samples, we detected an inherited LINE-1 on 1q42.3, indicating that our PCR conditions were sufficient to genotype LINE-1 alleles. An early de novo insertion on 10q26.3 was found in all frozen tissue samples (primary and metastasis) and both CDX2high and CDX2dim slide-dissected samples. An insertion on 3q22.2 is present in the primary tumor subclonally and in the metastasis and therefore occurred before metastasis but after dedifferentiation of the CDX2dim clone. An insertion on 18q22.1 occurred late, after metastasis to the liver had occurred, since it was found in the primary CDX2high clone and not in the metastasis.
**Extended Data Fig. 2 | TP53 effects on LINE-1+ cell growth and retrotransposition.** (a) Demonstration of effective TP53 knockdown. RPE cells were treated with TP53 shRNA lentivirus (pDA079) or control lentivirus (pDA081). The Western blot shows the p53 response to treatment with the DNA intercalator doxorubicin (200 ng ml⁻¹ for 24 h). (b) Left, the retrotransposition reporter assay. LINE-1 is expressed from a plasmid with an antisense eGFP in the 3' UTR that is interrupted by a sense intron. During transcription, the intron is spliced, reconstituting the coding potential of the eGFP reporter. The eGFP reporter carries with it a CMV promoter and is inserted into the genome by LINE-1. Expression of eGFP from the genome allows for fluorescence-based quantification of retrotransposition rate by flow cytometry. Right, reporter assay performed in RPE with TP53 knockdown or control ± s.e.m., n = 3 independent experiments. P value was calculated by two-sided t-test. (c) Normalized median read counts of sgRNAs targeting TP53 and CDKN1A in cells expressing either LINE-1 (navy blue) or eGFP (green) control compared to non-targeting-controls (NTC). Individual sgRNAs are indicated by circles or triangles. Results from two biological replicates are depicted.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | LINE-1 RNAseq analysis. (a) Genes regulated by cell cycle were curated from CycleBase v3.0\(^7\) and differential expression values were plotted. S, G2, and M phase genes were significantly downregulated in LINE-1\(^+\) cells. Unpaired two-sided \(t\)-tests were used for statistical testing. N/A = not applicable. *p-values vs. N/A: G1 = not significant (n.s.), G1/S = 1.7e-9, S = 1.5e-2, G2 = 2.1e-13, G2/M = 5.2e-6, M = 3.4e-10. (b) Flow cytometry was used to assess cell cycle by quantifying DNA content using a PI DNA stain in Tet-On LINE-1 or Tet-On luciferase cells induced with 1 \(\mu\)g ml\(^{-1}\) doxycycline for 48 h. LINE-1\(^+\) cells with wild-type (WT) p53 accumulated in G1 phase (2n DNA copy number), whereas TP53\(^{KD}\) resulted in more even cell cycle proportions. These data are from one experiment. (c) Relative fold-change of interferon-stimulated genes in LINE-1 compared to luciferase-expressing cells measured by RNAseq. Error bars indicate s.e.m. (d) RNAseq analysis revealed upregulation of NF-\(\kappa\)B and several target genes in LINE-1\(^+\) cells. Error bars indicate s.e.m. (e) Differential expression of IFNB1 (right) and interferon-stimulated genes (left) in p53-knockdown cells expressing LINE-1 or luciferase for 72 h. Measured by qRT-PCR. Error bars indicate s.d., \(n\) = 3 biological replicates. * \(p < 0.05\), ** \(p < 0.001\). (f) Differential expression of TLR3, IFIT1, and IFIT2 with the addition of 5 \(\mu\)M zalcitabine (ddC) or 5 \(\mu\)M didanosine (ddI) in p53-knockdown cells expressing LINE-1 or luciferase for 72 h. Measured by qRT-PCR, \(n\) = 3 independent experiments. \(P\) values indicated within the plots.
Extended Data Fig. 4 | TP53-Knockdown Screen Supplement. (a) Behavior of non-targeting-control sgRNAs in the screen over time. Data points indicate the median sgRNA count per replicate and error bars the 95% confidence interval. (b) Behavior of TP53- and CDKN1A-targeting sgRNAs. Median values are depicted with 95% Confidence Intervals. There is no appreciable change in TP53 sgRNA representation between LINE-1+ and luciferase control cells, indicating loss of p53 function due to the shRNA. CDKN1A sgRNAs do not differ between groups as well, suggesting that CDKN1A effects are contingent on p53 function. (c) Examples of essential gene knockouts that deplete from both LINE-1+ and luciferase+ cells. Median values are depicted with 95% Confidence Intervals. (d) Knockout of APC provides a growth advantage to LINE-1+ cells. Median values are depicted with 95% Confidence Intervals. (e) Knockout of the interferon alpha and beta receptor subunit 1 (IFNAR1) but not subunit 2 (IFNAR2) provides a growth advantage in LINE-1+ cells. Median values are depicted with 95% Confidence Intervals.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | HUSH knockout is synthetic lethal due to derepression of the LINE-1 transgene. (a) Gene screen ranks by Z-scores. HUSH genes are in blue. (b) HUSH complex sgRNA performance during the screen. All knockouts drop out early from LINE-1+ cells (red) and do not affect growth of luciferase+ cells (black). Median values are depicted with 95% Confidence Intervals. (c) 12 d clonogenic growth assay in cells expressing LINE-1 (doxycycline-induced) with targeted knockouts of HUSH components compared to non-targeting-control (NTC). n = 3 independent experiments. Error bars indicate ± s.e.m. P values calculated by one-sided t-test. (d) Western blot comparing ORF1p and ORF2p expression in HUSH knockout cells or non-target-controls (NTC) that have not been treated with doxycycline compared to NTC with 24 h of 1 µg ml⁻¹ doxycycline treatment. ORF1p and ORF2p expression are only detected in NTC-treated cells with doxycycline added to the culture media. The double banding pattern for ORF1p is consistently seen with codon-optimized LINE-1. (e) Western blot comparing ORF1p and ORF2p expression 24 h after 1 µg ml⁻¹ doxycycline treatment in HUSH knockouts compared to NTC. The ORF2p antibody cannot distinguish between endogenous or transgenic LINE-1 expression. (f) qRT-PCR analysis of LINE-1 transgene expression in HUSH knockouts compared to NTC (induced with 1 µg ml⁻¹ doxycycline). Because the LINE-1 transgene is codon-optimized, qRT-PCR is specific for the transgene and does not amplify endogenous LINE-1 sequences. *p < 0.001. (g) Linear regression plot of LINE-1 transgene expression and ORF1p and ORF2p expression in HUSH knockouts compared to NTC. Shaded area indicates 95% confidence interval for regression line. Both ORF1p and ORF2p increase in expression with higher transgene mRNA expression, although the increase in ORF1p is minimal compared to that observed with ORF2p. (h) Heatmap of immunofluorescence imaging depicting the proportion of cells expressing ORF1p and ORF2p at different levels in HEK293T cells expressing Tet-On LINE-1 (pDA055) at increasing doses of doxycycline.
Extended Data Fig. 6 | RNA processing gene knockouts sensitize cells to LINE-1. (a) StringDB network plot of the 81 mRNA processing genes identified by this screen. Edges indicate known protein–protein interactions. This network is enriched for spliceosome machinery (green nodes). (b) Screen behavior of significant genes belonging to the spliceosome KEGG GO term. Median sgRNA counts are depicted with 95% Confidence Intervals. (c) Clonogenic assay (12 d) comparing growth of luciferase+ and LINE-1+ cells induced with 1 µg ml–1 doxycycline treated with 1 nM pladienolide B (PLA-B) or vehicle (DMSO). n = 3 independent experiments. Error bars indicate s.e.m. P value calculated by unpaired one-sided t-test. (d) Behavior of nuclear exosome complex genes in the screen. Median values are depicted with 95% Confidence Intervals. (e) Behavior of RNASEH2 component sgRNAs in the screen. Median values are depicted with 95% Confidence Intervals. (f) Behavior of ADAR1 sgRNAs in the screen. Median values are depicted with 95% Confidence Intervals.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | The Fanconi Anemia Pathway is required for growth of LINE-1+ cells. (a) Behavior of sgRNAs targeting Fanconi Anemia pathway genes in the screen. Median values are depicted with 95% Confidence Intervals. (b) Western blot of DNA damage marker γH2A.X in chromatin-bound protein fractions of LINE-1+ cells with or without perturbations to the FA pathway. H3 was used as loading control. γH2A.X levels were quantified and graphed relative to NTC-treated, LINE-1+ cells. (c) Clonogenic assay (10 d). TP53KD cells constitutively expressing Cas9 are treated with lentivirus encoding non-targeting-control (NTC) or FANCD2 sgRNA and then transfected with eGFP (pDA083) or the native LINE-1 sequence L1RP (pDA077). Left, representative images of colonies. Scale bar = 1 cm. Right, data are presented as the rate of LINE-1 per 100 eGFP colonies ± s.d. to control for transfection efficiency across samples, n = 3 independent experiments. P value obtained by unpaired two-sided t-test. (d) Quantification of FANCD2 foci in G1 and G2 phase (EdU-) HeLa cells. Number of cells per group: G1 untreated (n = 104), G1 HU (n = 352), G1 wildtype LINE-1 (n = 186), G1 RT (D702Y) (n = 138), G2 untreated (n = 60), G2 HU (n = 58), G2 wildtype LINE-1 (n = 42), G2 RT (D702Y) (n = 32). Two-sided t-tests were used for statistical comparisons. HU = hydroxyurea. RT = reverse transcriptase. ns = not significant.
Extended Data Fig. 8 | Viability assays with LINE-1 mutants. (a) Tet-On constructs for wild-type and mutant LINE-1 expression. (b) Viability of HEK293T cells after 4 days expressing wild-type or a mutant at increasing doxycycline doses. A multivariate ANOVA (Viability ~ ORF2 * doxycycline) was performed in R to calculate p values for ORF2 mutant status and doxycycline dose. Tests of viability differences among ORF2 mutants were further performed using two-sided t-tests at the 1000 ng ml⁻¹ doxycycline dose. N = 6 replicates per doxycycline dose. (c) Western blot of ORF1p and ORF2p 24 hours after inducing protein expression with 1000 ng ml⁻¹ doxycycline.
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Software and code

Policy information about availability of computer code

Data collection: No software was used to collect data.

Data analysis: Software to analyze RNAseq data include STAR v2.4.5, R (HTseq and DESeq2 packages), and GSEA software v2.0 from the Broad Institute. Screen data were analyzed using MAgeCK, StringDB, and Webgestalt.

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MAgeCK-normalized sgRNA read counts from CRISPR KO screens and RNAseq counts and differential expression values are included in the GEO database under accession number GSE119999. Source data for 2, 5, and 6 are provided.
Field-specific reporting

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☐ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
For screens, sample size was determined based on the design as a positive or synthetic lethal screen. Higher library representation (500x starting compared to 100x for positive selection) and increased KO library replicate (triplicate as opposed to duplicate) numbers were chosen for synthetic lethal screens, as is described in the literature describing factors to consider in powering of genome-wide knockout screens. For all other assays, experiments were performed in duplicate, triplicate, or with higher replicate numbers, which is indicated throughout the text.

Data exclusions
Data were not excluded.

Replication
To follow up on screen hits, we used a variety of assays, including generation of individual (as opposed to pooled) CRISPR knockout cell lines and we confirmed knockout using functional assays (i.e., MMC response of FANCD2-Ub in Fanconi Anemia knockout cells).

Randomization
During screen growth periods, plates were held in a 37°C 95% CO2 incubator and cells were samples every 4-5 days. It took approximately 8 hours to perform the required cell culture procedures on such days. Plates were split up randomly into 4 batches and thus rotated throughout the incubator over the course of the entire experiment. Otherwise, randomization was not an issue in the experiments described in this manuscript.

Blinding
The most relevant assay wherein blinding was necessary was in quantifying clonogenic growth. To address this, the investigator who set up the experiments and fixed and stained the colonies (D.A.) printed out de-identified images of the plates and asked a blinded investigator (J.P.S., C.L.) to count the colony numbers, in order to verify that the counts were reflective of the true data and not the investigators’ bias.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
| ☐ Involved in the study         | ☐ Involved in the study |
| ☐ Antibodies                    | ☐ ChIP-seq |
| ☐ Eukaryotic cell lines         | ☐ Flow cytometry |
| ☐ Palaeontology                 | ☐ MRI-based neuroimaging |
| ☐ Animals and other organisms   |         |
| ☐ Human research participants   |         |
| ☐ Clinical data                 |         |

Antibodies

Antibodies used

- anti-mouse Alexa Fluor 568, ThermoFisher, cat# A-11004
- anti-rabbit Alexa Fluor 488, ThermoFisher, cat# A-11034
- IRDye 680RD Goat anti-mouse IgG, LI-COR, cat# 925-68070
- IRDye 680RD Goat anti-rabbit IgG, LI-COR, cat# 925-68071
- IRDye 800CW goat anti-rabbit IgG, LI-COR, cat# 925-32211
- IRDye 800CW goat anti-mouse IgG, LI-COR, cat# 925-32210
- mouse anti-flag, Sigma, F1804
- mouse anti-human FANCD2 Clone F17, Santa Cruz
- Mouse anti-human ORF1p Clone 4H1, Millipore Sigma, MAB3152
- mouse anti-human p53 Clone DO-1, CalBiochem, cat# OP43
- mouse anti-human vH2AX, Clone JBW301, EMD Millipore, cat# 05-636
- mouse anti-RPA2, Clone 9H8, Abcam, Cat# ab2275
- rabbit anti-p-RPA S4/S8, Bethyl, cat# A300-245A
- rabbit anti-H3, abcam cat# 1791
- rabbit anti-human b-tubulin clone S9F3, Cell Signalling Technology, cat# 2128
- rabbit anti-human FANCD2, Novus Bio, cat# N8100-182
Validation
All commercially available antibodies have been previously validated. The FANCI clone 589 antibody has been validated by the Smogorzewska lab and detects a band at the proper size that is responsive to positive control MMC. The ORF2p MT49 antibody produced by the Burns lab has been extensively validated by multiple conventional assays and is described in more detail here: biorxiv.org/content/10.1101/74425v1.

Eukaryotic cell lines
Policy information about cell lines

Cell line source(s)
- Tot-On 3G HEK93 Cells (ClonTech)
- Tot-On HEK93T LD cells (MD Boeke Lab)
- HEK293FT (AI Holland Lab)
- hTERT-RPE-1, puromycin sensitive [RPE] (AI Holland lab)
- RPE-Cas9 (AI Holland lab)
- RPE with p53 knockdown - produced in this study
- RPE-Cas9 with p53 knockdown - produced in this study
- RPE-Cas9, p53 knockdown, Tet-On LINE-1 (ORFeus) - produced in this study
- RPE-Cas9, p53 knockdown, Tet-On Luciferase - produced in this study
- RPE, Tet-On LINE-1 (ORFeus) - produced in this study
- RPE, Tet-On Luciferase - produced in this study

Authentication
RPE cells obtained from AI Holland underwent STR profiling and were confirmed as RPE. The remaining cell lines were either purchased commercially or not tested.

Mycoplasma contamination
Cells were periodically tested with the MycoAlert Lonza assay and confirmed negative.

Commonly misidentified lines
Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

Flow Cytometry
Plots
- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology
Sample preparation
The flow data presented do not include traditional flow plots. The data were collected in one dimension to assess retrotransposition efficiency using a field-specific reporter assay described in extended data figure 2. Single cells are first selected based on FSC/SSC parameters. Then cells are analyzed for GFP (FITC). First, a GFP-negative control is gated such that 0.1% are considered GFP+. Next, a GFP-positive control is analyzed to ensure that >90% of the cells fall into the GFP+ gate. Finally, the experimental groups are analyzed to assess GFP-content. We also assessed cell cycle stage based on DNA content.

Instrument
BD Accuri C6

Software
BD Accuri C6 software

Cell population abundance
Retrotransposition events occur in up to 20% of cells, making these an abundant target.

Gating strategy
n/a

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.