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The domesticated transposon protein L1TD1 associates with its ancestor L1 ORF1p to promote LINE-1 retrotransposition

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eLife Assessment

This **important** paper reports functional interactions between L1TD1, an RNA binding protein (RBP), and its ancestral LINE-1 retrotransposon which is not modulated at the translational level. The evidence for the association between L1TD1 and LINE-1 ORF1p is **solid**. The work implies that the a transposon-derived RNA binding protein in the human genome can interact with the ancestral transposable element from which this protein was initially derived. This work spurs interesting questions for cancer types, where LINE1 and L1TD1 are aberrantly expressed.

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Abstract

Repression of retrotransposition is crucial for the successful fitness of a mammalian organism. The domesticated transposon protein L1TD1, derived from LINE-1 (L1) ORF1p, is an RNA-binding protein that is expressed only in some cancers and early embryogenesis. In human embryonic stem cells, it is found to be essential for maintaining pluripotency. In cancer, L1TD1 expression is highly correlative with malignancy progression and as such considered a potential prognostic factor for tumors. However, its molecular role in cancer remains largely unknown. Our findings reveal that DNA hypomethylation induces the expression of L1TD1 in HAP1 human tumor cells. L1TD1 depletion significantly modulates both the proteome and transcriptome and thereby reduces cell viability. Notably, L1TD1 associates with L1 transcripts and interacts with L1 ORF1p protein, thereby facilitating L1 retrotransposition. Our data suggest that L1TD1 collaborates with its ancestral L1 ORF1p as an RNA chaperone, ensuring the efficient retrotransposition of L1 retrotransposons, rather

than directly impacting the abundance of L1TD1 targets. In this way, L1TD1 might have an important role not only during early development but also in tumorigenesis.

Introduction

Molecular domestication of transposable elements (TEs) or TE-derived sequences give rise to novel genes and regulatory sequences in the genome contributing to both genetic and epigenetic variation in an organism [1–3]. TEs are key drivers of genome instability, and thus are constantly exposed to silencing mechanisms by the host. Yet, co-option of TEs thorough evolution has created so-called domesticated genes beneficial to the gene network in a wide range of organisms [3, 4]. One example of such domesticated gene is LINE-1 type transposase containing domain 1 (*L1TD1*), which is the only domesticated protein-coding gene that originated from LINE-1 (long interspersed element 1 (L1)) [5]. Intact L1 retrotransposons are self-propagating by a "copy-and-paste" mechanism known as retrotransposition involving the function of two L1-encoded proteins L1 open reading frame 1 (L1 ORF1p) and L1 open reading frame 2 (L1 ORF2p) [6]. L1TD1 shares sequence resemblance with L1 ORF1p, a nucleic acid chaperone protein with binding capacity to both RNA and DNA [7]. L1 ORF1p contributes to the formation of L1 ribonucleoproteins (L1-RNPs), key factors for retrotransposition of L1 elements, but its co-factors are yet to be elucidated [8].

L1TD1, also named Embryonic Stem Cell Associated Transcript 11 (*ECAT11*), is expressed predominantly only in early embryogenesis and germ cells and gets rapidly downregulated upon the exit from pluripotency [9, 10]. Although it is dispensable for mouse development [9], L1TD1 is essential for the maintenance of human pluripotency [11, 12]. In the context of human embryonic stem cells (hESCs), L1TD1 regulates translation of a distinct set of mRNAs [13], including core pluripotency factors [12] and was recently shown to facilitate the dissolution of stress granules [13]. It has been proposed, that during mammalian evolution the *L1TD1* gene was under positive selection due to the function in the maintenance of pluripotency in some species [5]. Apart from early development, L1TD1 is expressed in in certain tumors including germ cell tumors and colorectal cancers. In this context, L1TD1 was identified as a possible prognostic marker for medulloblastoma [14] and colorectal tumors [15], where its expression is highly correlative with malignancy progression [16].

DNA methyltransferase (DNMT) inhibitors are successfully used as anti-cancer drugs and recent reports suggest that the lethal response of human tumor cells to these compounds is based to a large extent on the activation of an anti-viral immune response to endogenous retroviral transcripts [17, 18]. DNMT inhibitor treatment of non-small cell lung carcinoma (NSCLC) patient-derived cells resulted in reduced cell viability and up-regulation of L1TD1 indicating an epigenetic control of the *L1TD1* gene [19]. In addition, xenograft tumors with NSCLCs overexpressing L1TD1 showed decreased tumor growth suggesting a negative impact of L1TD1 expression on tumor viability. In contrast, L1TD1 was shown to be required for cell viability in medulloblastoma [14]. Hence, the exact mechanistic function of this domesticated transposon protein in human tumor cells is still unrevealed.

We have previously discovered that conditional deletion of the maintenance DNA methyltransferase DNMT1 in the murine epidermis results not only in the upregulation of mobile elements, such as Intracisternal A-particles (IAPs) but also in the induced expression of L1TD1 ([20], Suppl. Table 1). These findings are in accordance with the observation that inhibition of DNA methyltransferase activity by aza-deoxycytidine in human non-small cell lung cancer cells (NSCLCs) results in upregulation of L1TD1 [19]. Based on the potential role of L11TD1 as prognostic marker we aimed at elucidating the molecular function of the domesticated transposon protein and its potential role for the control of viability in human tumor cells. To this end, we activated L1TD1 expression by inducing DNA hypomethylation *via* deletion of DNMT1 in the

nearly haploid human cancer cell line HAP1 [21]. First, we found that L1TD1 expression and L1 activation correlate with local DNA hypomethylation. Next, we identified L1TD1-associated RNAs by RNA Immunoprecipitation sequencing (RIP-seq), which revealed transcripts and proteins with differential expression in the absence of L1TD1 by transcriptome and proteome analyses. We showed that L1TD1 protein binds to L1-RNPs. Importantly, L1TD1 facilitated L1 retrotransposition in HAP1 cells. Our results demonstrated that upon DNA hypomethylation, L1TD1 affects gene expression, cell viability, and cooperates with its ancestor protein L1 ORF1p in the control of L1 retrotransposition.

Results

L1TD1 expression is activated through DNA hypomethylation in HAP1 cells

To address the function of L1TD1 in human tumor cells, we generated a defined human tumor cell model. To this end, we used the nearly haploid tumor cell line HAP1 cells that allows efficient gene editing by CRISPR/Cas-9 technology [22]. A HAP1 DNMT1 knockout (KO) cell line has been previously validated for loss of DNMT1 protein and significant reduction in DNA methylation [23]. Using this cell line, we examined first the effect of DNMT1 ablation on gene expression. Transcriptome analysis revealed the deregulation of 2385 genes (log2FC>2, adjusted p-value< 0.05) (Suppl. Figure S1A and Suppl. Table S1). The majority of deregulated genes were up-regulated and included, in addition to L1TD1, genes with function in transcription and cell differentiation and genes encoding Melanoma Antigen Gene (MAGE) proteins and KRAB domain containing proteins (Suppl. Figures S1A-C). The expression of MAGE proteins is restricted to reproductive tissues by chromatin-associated mechanisms, including DNA methylation and was shown to be up-regulated by DNMT inhibitors [24, 25]. In the context of tumor development, MAGE proteins interact with the transcriptional master regulator and E3 ubiquitin ligase KAP1 (TRIM28), thereby inducing the degradation of tumor suppressor proteins [24, 25]. On the other hand, KAP1 interacts with KRAB domain zinc finger proteins to repress the expression of transposons by chromatin-mediated mechanisms [26, 27]. Interestingly, a recent report shows that deletion of the histone methyltransferase SETDB1 in HAP1 cells also results in DNA hypomethylation and upregulation of a similar set of zinc finger proteins [28]. In addition to these two gene classes, expression of the *de novo* methyltransferases DNMT3A and DNMT3B was significantly upregulated in the absence of DNMT1 suggesting a potential compensatory mechanism (Suppl. Figure S1A).

Next we analyzed the effect of DNMT1 ablation on DNA methylation of the *L1TD1* gene in HAP1 cells. As shown in **Figure 1A**, DNA methylation-specific PCR (MethyLight) analysis revealed significantly reduced DNA methylation at the *L1TD1* promoter in DNMT1 KO cells. DNA hypomethylation at the *L1TD1* gene correlated with a strong induction of L1TD1 mRNA and protein expression in DNMT1 KO cells (**Figure 1B-D**). Similarly, in the absence of DNMT1 the methylation of L1 transposons was reduced and expression of *L1 ORF1p*, the ancestor of L1TD1, was induced (Suppl. Figure S2A and S2B).

To analyze the role of L1TD1 in a DNMT1 null background, we ablated L1TD1 in DNMT1 KO cells by gene editing resulting in HAP1 DNMT1/L1TD1 double knockout (DKO) cells (**Figure 1C**). Based on the observations that L1TD1 is highly expressed in human germ cell tumors [11], we used the ovarian cancer cell line OV-90 as a positive control (**Figure 1D-E**). In accordance with previously published data with human embryonic stem cells (hESCs) [11], indirect immunofluorescence analysis revealed that L1TD1 was preferentially localized in the cytosol of HAP1 DNMT1 KO cells and OV-90 cells (**Figure 1E**). L1TD1 was reported to localize in P-bodies in hESCs [11]. We detected a similar granular localization of L1TD1 in HAP1 DNMT1 KO cells and OV-90 cells (**Figure 1E**).

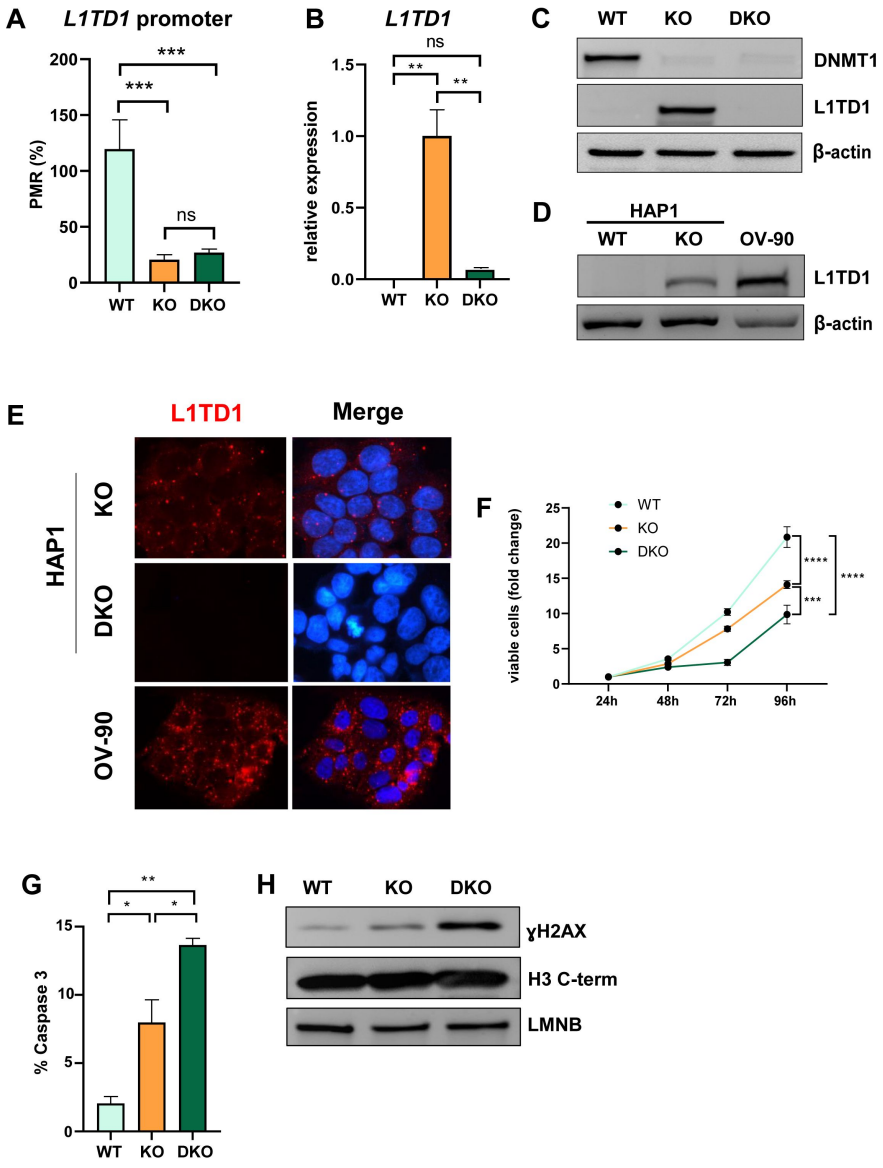

Figure 1.

DNA hypomethylation results in activation of L1TD1 expression and loss of L1TD1 affects cell viability in HAP cells.

(A) Quantification of DNA methylation levels at the *L1TD1* promoter in HAP1 wildtype (WT), DNMT1 KO and DNMT1/L1TD1 DKO cells using the MethyLight assay. DNA methylation is shown as percentage of methylation ratio (PMR). **(B)** qRT-PCR analysis of *L1TD1* mRNA expression in HAP1 wildtype (WT), DNMT1 KO and DNMT1/L1TD1 DKO cells. *GAPDH* was used as a normalization control and relative *L1TD1* mRNA levels in DNMT1 KO cells were set to 1. Data are shown as a mean of ± SD of 3 biological replicates. **(C)** Western blot analysis of L1TD1 levels in HAP1 WT, DNMT1 KO and DNMT1/L1TD1 DKO cells. β-actin was used as loading control. **(D)** Western blot analysis of L1TD1 protein expression in HAP1 WT and DNMT1 KO cells and OV-90 cells. β-actin was used as loading control. **(E)** Indirect immunofluorescence staining of L1TD1 (red) in HAP1 DNMT1 KO and DNMT1/L1TD1 DKO cells and OV-90 cells. In merged images nuclear DNA was stained with DAPI (blue). **(F)** Cell viability analysis of HAP1 WT, DNMT1 KO and DNMT1/L1TD1 DKO cells using the CellTiter-Glo assay measured over 96 h (n=6). **(G)** Bar graph representing the percentage of apoptotic cells of cultured cell lines quantified by flow cytometry analysis of cleaved caspase 3. **(H)** Western blot analysis of γH2AX levels in HAP1 WT, DNMT1 KO and DNMT1/L1TD1 DKO cells in nuclear extracts. Antibodies specific for histone H3 C-terminus and LAMIN B (LMNB) were used as loading controls. **(A-B, F-G)** Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison correction. * p≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001, ns = not significant.

Loss of DNMT1 protein in the human colorectal carcinoma cells results in chromosomal defects and apoptosis [29]. HAP1 cells with DNMT1 depletion are viable but showed significantly reduced cell proliferation and increased apoptosis (cleaved caspase 3) and DNA damage (γ-H2AX) (**Figure 1F-H**). Additional deletion of L1TD1 enhanced the antiproliferative effects of DNMT1 ablation. These results suggest that L1TD1 expression is regulated by DNA methylation and its depletion affects cell viability in DNMT1-deficient HAP1 cells.

L1TD1 binds to L1 transcripts and a subset of mRNAs

L1TD1 was previously shown to act as RNA-binding protein by binding to its own transcript [11] and selected group of mRNAs under L1TD1 translational control [13]. To determine the L1TD1 binding repertoire in the context of L1TD1- and DNMT-dependent cell viability and proliferation control, we performed RNA immunoprecipitation (RIP) followed by sequencing (RIP-seq) (**Figure 2A**). L1TD1-containing RNP complexes (L1TD1-RNPs) were immunoprecipitated in DNMT1 KO cells by an L1TD1-specific antibody. We identified 597 transcripts enriched (log2FC>2, adjusted p-value < 0.05) in L1TD1-RNPs when compared to the input (**Figure 2B** and Suppl. Table S2). In addition, we used DNMT1/L1TD1 DKO cells as a negative control. Only transcripts enriched (log2FC>2, adjusted p-value < 0.05) in both assays (DNMT1 KO *versus* input and DNMT1 KO *versus* DNMT1/L1TD1 DKO) were considered as L1TD1-associated RNAs (Suppl. Figure S3A and Suppl. Table S2) resulting in the identification of 228 transcripts. Importantly, *L1TD1* mRNA was detected as one of the top transcripts in L1TD1-RNPs (**Figure 2B**). Of note, the transcript of *YY2*, a retrotransposon-derived paralogue of yin yang 1 (YY1) [30] was also enriched in L1TD1-RNPs.

To validate the RIP-seq results, selected individual transcripts were amplified by gene-specific qRT-PCR analysis after repeating the RIP. Transcripts of *L1TD1*, *YY2* and *ARMC1* (as one of the RIP top hits) were specifically enriched in L1TD1-RNPs obtained from DNMT1 KO cells when compared to DNMT1/L1TD1 DKO cells, whereas the negative control *GAPDH* did not show such enrichment (**Figure 2C**). Gene Ontology analyses of the 228 common hits indicated enriched groups in cell division and cell cycle regulation and, in accordance with a recent study [31], enriched transcripts encoding zinc finger and in particular KRAB domain proteins which have been implicated in the restriction of endogenous retroviruses [32] (Suppl. Figures S3B and S3C).

Analysis of RIP-Seq using TEtranscript designed for the analysis of transposon-derived sequences [33], in the differentially expressed sets indicated an association of L1TD1 with *L1* transcripts (**Figure 2D** and Suppl. Table S3), which was also validated by qRT-PCR analysis of L1TD1 RIP samples (**Figure 2E**). In addition, we also identified *ERV* and *AluY* elements as L1TD1-RNP-associated transcripts (Suppl. Figure S3D and Suppl. Table S3). These combined results suggest that L1TD1 interacts with a set of transcripts including RNAs with functions in cell division and cell cycle control. Furthermore, L1TD1 has kept the ability to associate with *L1* transcripts potentially regulating them similar to its ancestor protein L1 ORF1p [34].

Loss of L1TD1 modulates the proteome and transcriptome of HAP1 cells

To explore the cellular function of L1TD1, we analyzed the proteomes and transcriptomes of DNMT1 KO and DNMT1/L1TD1 DKO cells. Comparison of the proteomes revealed 98 upregulated and 131 downregulated proteins in DNMT1/L1TD1 DKO cells versus DNMT1 KO cells (log2FC ≥ 1, adj. p-value ≤ 0.05) (**Figure 3A** and Suppl. Table S4). Gene Ontology Enrichment Analysis revealed that differentially expressed proteins were mainly associated with regulation of cell junction and cysteine-type endopeptidase activity involved in apoptotic process (Suppl. Fig. S4A). To investigate whether the corresponding protein abundance of mRNAs contained in L1TD1-RNPs is affected by L1TD1 expression, we compared proteomic data with the RIP-seq data. Comparison of differentially expressed proteins with transcripts associated with L1TD1 identified only L1TD1

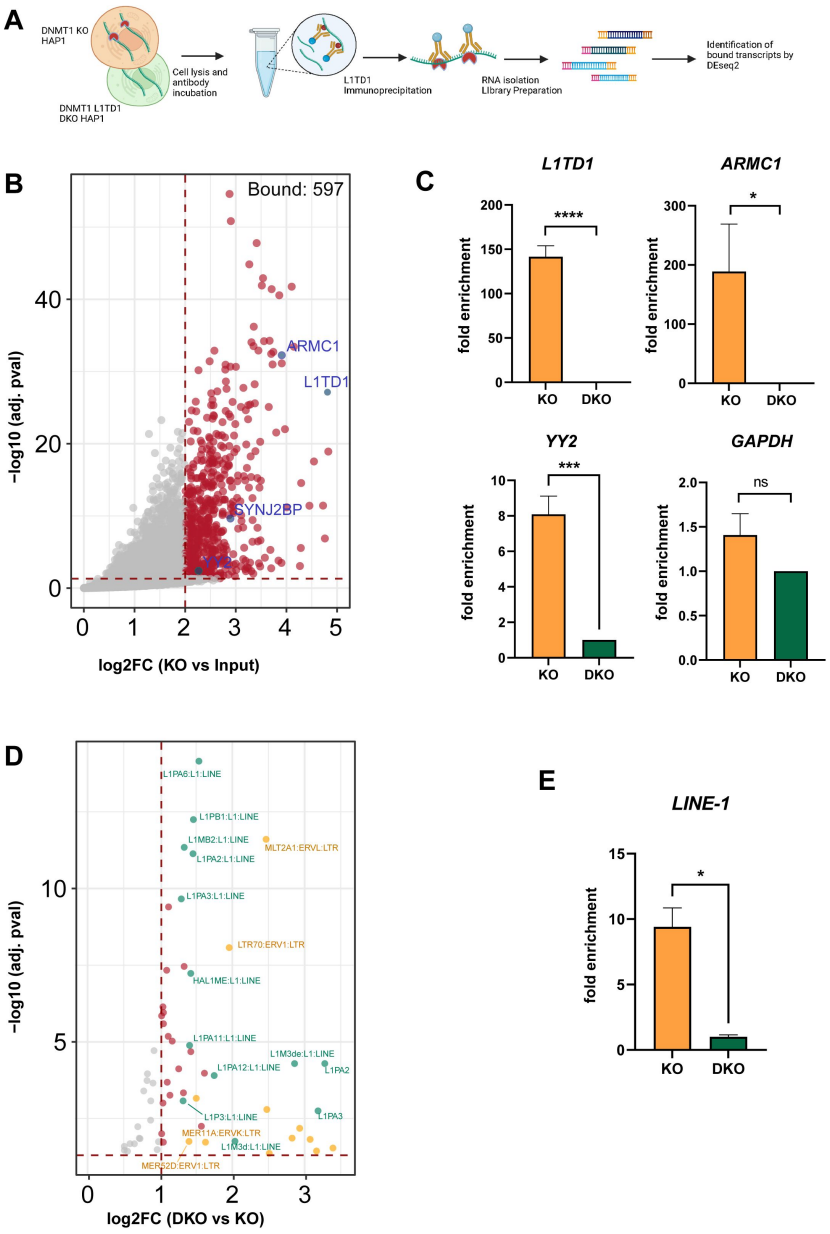

Figure 2.

RIP-seq identifies a set of RNAs and transposon transcripts associated with L1TD1.

(A) Schematic representation of the RNA immunoprecipitation sequencing (RIP-seq) method (panel created with *BioRender.com/x79t726*). L1TD1-RNA complexes were isolated from HAP1 KO cell extracts with an L1TD1-specific antibody, RNA was isolated from complexes and input and cDNA libraries were prepared using the Smart-seq3 protocol. Sequencing data was analyzed by DEseq2 and TEtranscript software, separately. **(B)** Volcano plot showing L1TD1-associated transcripts as a result of DESeq2 analysis (cut-off log2FC > 2 and adj. p-value < 0.05). Selected hits are highlighted in blue. **(C)** RIP-qPCR analysis confirms L1TD1 interaction with the transcripts *L1TD1*, *ARMC1*, *YY2*. The bar graphs represent the fold enrichments of the transcripts in the IP samples of DNMT1 KO relative to DNMT1/L1TD1 DKO cells (set to 1) and normalized to input samples in the indicated cells. *GAPDH* was used as a negative control for the RIP-qPCR analysis. **(D)** Volcano plot showing L1TD1-associated transposon transcripts as result of the TEtranscript analysis with a log2FC > 1 and adj. p-value < 0.05. LINE1 elements are highlighted in green, ERV elements in yellow and other associated transposon transcripts in red. **(E)** RIP-qPCR analysis confirms the association of L1TD1 with *L1* transcripts. Statistical significance was determined using paired two-tailed t-test. All data in the figure are shown as a mean of ± SD of 3 biological replicates. * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001, ns = not significant.

(Suppl. Fig. S4B). The lack of overlap between RIP-seq and proteomics data (except L1TD1) suggested that modulation of the abundance of proteins encoded by L1TD1-associated transcripts is not the main function of L1TD1.

Interestingly, we discovered that L1 ORF1p was upregulated in the absence of L1TD1 (**Figure 3A** and Suppl. Table S4). Western blot analysis confirmed the increased expression of L1 ORF1p in HAP1 DNMT1/L1TD1 DKO cells when compared to DNMT1 KO cells (**Figure 3C**), while L1 transcript levels showed no significant change (Suppl. Figure S5A). This indicates that L1TD1 attenuates the expression of L1 ORF1p suggesting a potential regulatory crosstalk between L1TD1 with its ancestor protein.

To investigate a potential effect of L1TD1 on the expression levels of associated RNAs, comparative RNA-seq analysis was performed with DNMT1 KO and DNMT1/L1TD1 DKO cells. Upon loss of L1TD1, we identified 323 upregulated and 321 downregulated transcripts, respectively (log2-fold change ≥ 1; adjusted p-value < 0.05) (**Figure 3B** and Suppl. Table S5). About 25% of the differentially expressed proteins (34% of upregulated and 22% of downregulated proteins) identified in the proteome analysis were also significantly deregulated at the RNA level (Suppl. Table S4). However, none of the deregulated transcripts, except *L1TD1*, was detected in L1TD1-RNPs (**Figure 3B** and Suppl. Tables S2 and S5). This finding was corroborated by qRT-PCR analysis of L1TD1-RNP-associated transcripts (Suppl. Figure S5B), suggesting that the cellular function of L1TD1 might be independent of a deregulation of its associated mRNAs. Based on our observations implying that L1TD1 does not exert its cellular function as regulator of RNA abundance, while being associated with transposon RNAs (**Figure 2D**), we used TEtranscript [33] to identify differentially expressed TE-derived sequences transcriptome-wide. Loss of L1TD1 resulted in upregulation of specific SINEs such as AluY elements (Suppl. Figure S5C and Suppl. Table 6). Taken together, these data suggest that L1TD1 affects the abundance of transposon transcripts in DNMT1-deficient HAP1 cells.

L1TD1 interacts with the L1 ORF1p protein

An interaction of L1TD1 with *L1* RNA (**Figure 2D**) and increased L1 ORF1p levels in L1TD1-depleted cells (**Figure 3B**) imply a direct association of L1TD1 with L1 ORF1p. We thus hypothesized that L1TD1 associates with L1-RNPs *via* L1 ORF1p. Therefore, we immunoprecipitated L1 ORF1p from HAP1 DNMT1 KO cells and OV-90 cells, and tested the IPs for the presence of endogenous L1TD1. Indeed, L1TD1 was co-precipitated with L1 ORF1p from DNMT1 KO cells and OV-90 cells (**Figures 3D** and S6B). Next, we asked whether the association of L1TD1 with L1 ORF1p is mediated by RNA. As shown in Suppl. Figures S6A and S6B, L1TD1 associates with L1 ORF1p in an RNA-independent manner. In agreement with their association in protein complexes, indirect immunofluorescence experiments revealed a partial co-localization of L1TD1 and L1 ORF1p in both cell lines (**Figure 3E** and Suppl. Figure S6C). In summary, our results suggest that L1TD1 associates with its ancestor ORF1p and this association does not depend on RNA intermediates.

L1TD1 has a positive impact on L1 retrotransposition

Since L1TD1 associates with *L1* RNA and modulates L1 ORF1p expression, we next asked whether the domesticated transposon protein can impact L1 retrotransposition in HAP1 cells. To this end, we carried out plasmid-based retrotransposition assays [35] in DNMT1 KO and DNMT1/L1TD1 DKO cells (**Figure 4A-B**). Following transfection and blasticidin selection, the rate of retrotransposition of the reporter construct was assessed through counting blasticidin-resistant colonies, transfected either with a retrotransposition-competent reporter construct or a retrotransposition-deficient control. To take into account differences in proliferation and blasticidin sensitivity DNMT1 KO and DNMT1/L1TD1 DKO cells were transfected in parallel with the blasticidin resistance gene vector pLenti6.2 and the numbers of clones from the retrotransposition assay were corrected for differences in transfection efficiency (Suppl. Figure

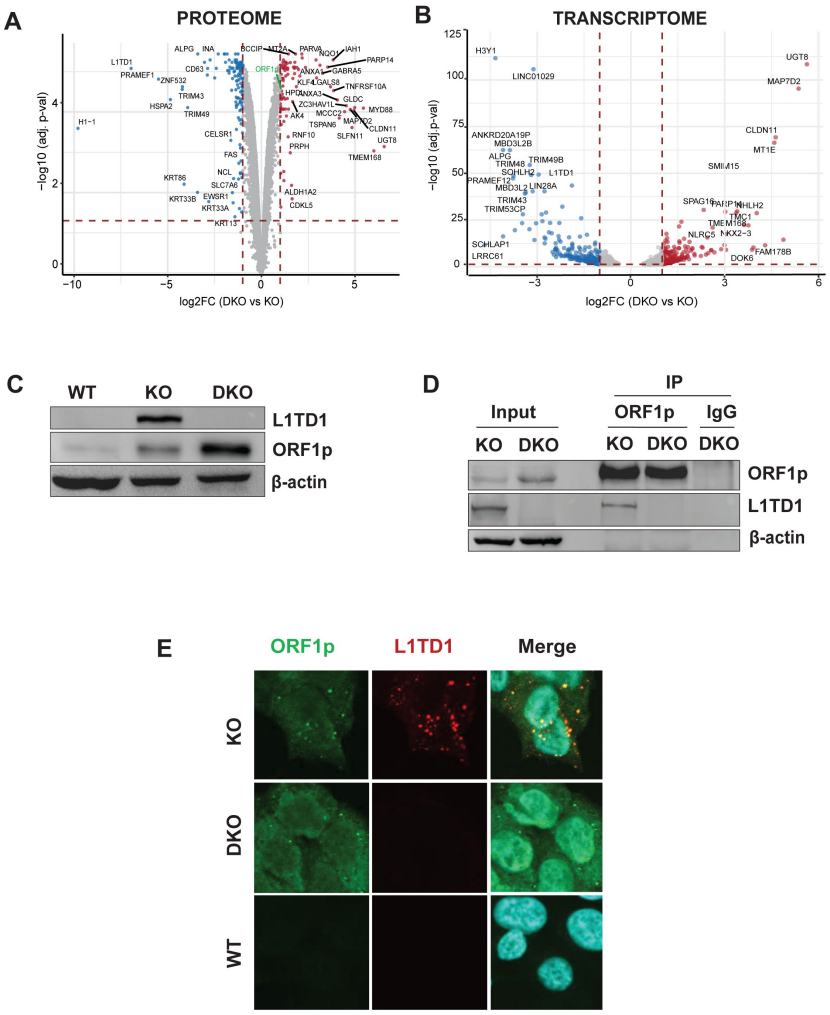

Figure 3.

L1TD1 cross-talk with its ancestor L1 ORF1p.

(A) Volcano plot displaying the comparison of the proteomes of HAP1 DNMT1 KO and DNMT1/L1TD1 DKO cells determined by mass spectrometry. Differentially abundant proteins were plotted as DNMT1/L1TD1 DKO over DNMT1 KO (log2FC ≥ 1, adj. p-value < 0.05 (red) and log2FC ≤ −1, adj. p-value < 0.05 (blue)). **(B)** Volcano plot illustrating the DESeq2 analysis of RNA-seq performed with HAP1 DNMT1 KO and DNMT1/L1TD1 DKO cells. Differentially expressed genes are plotted as DNMT1/L1TD1 DKO over DNMT1 KO (log2FC ≥ 1, adj. p-value < 0.05 (red) and log2FC ≤ −1, adj. p-value < 0.05 (blue)). **(C)** Western blot analysis illustrating protein levels of L1TD1 and L1 ORF1p in HAP1 WT, DNMT1 KO and DNMT1/L1TD1 DKO cells. β-actin was used as loading control. **(D)** Physical interaction of L1 ORF1p and L1TD1. L1 ORF1p was immunoprecipitated with an L1 ORF1p-specific antibody from whole cell extracts prepared from DNMT1 KO and DNMT1/L1TD1 DKO cells. Precipitated L1 RNP complexes and inputs were analyzed on a Western blot. IgG was used as a negative IP control and β-actin was used as loading control. **(E)** Confocal microscopy images of indirect immunofluorescence co-stainings using mouse ORF1p (green) and rabbit L1TD1 (red) antibodies in DNMT1 KO, DNMT1/L1TD1 DKO and HAP1 WT cells. In merged images nuclear DNA was stained with DAPI.

S7B). An average of 506 retrotransposition events were observed in DNMT1 KO cells in three independent experiments whereas DNMT1/L1TD1 DKO cells yielded over 5-fold fewer colonies when transfected with the retrotransposition-competent reporter construct (86 retrotransposition events on average) (**Figures 4C-D** and Suppl. Figure S7A). No blasticidin resistant clones were obtained for the backbone vector pCEP4, but comparable retrotransposition rates were observed for retrotransposition-competent reporter constructs in three independent retrotransposition assays (Suppl. Figures S7A and S7B). Combined, these data suggest that L1TD1 enhances L1 retrotransposition in DNMT1-deficient HAP1 cells and this effect is mediated through the association with ORF1p and *L1* RNA.

Discussion

In this study we show that the domesticated transposon protein L1TD1 promotes L1 retrotransposition. Numerous cellular factors have been shown in the past to affect retrotransposition [36–39]. However, many of these proteins are part of host defense mechanisms and restrict retrotransposition, and it was speculated that L1TD1 also limits L1 retrotransposition [5]. Unexpectedly, we identified L1TD1 as one of the cellular factors that has a positive impact on L1 retrotransposition, most likely by interacting with L1 RNPs and acting as RNA chaperone. L1TD1 deletion resulted in reduced L1 retrotransposition despite upregulation of ORF1p indicating that the higher levels of the transposon protein cannot fully compensate the loss of L1TD1. Interestingly, a similar effect has been previously described for the nonsense-mediated decay factor UPF1 [40]. UPF1 knockdown increased the amount of L1 mRNA and proteins but simultaneously reduced the effectiveness of the retrotransposition. A positive effect of L1TD1 on retrotransposition was recently also observed upon L1TD1 overexpression in HeLa cells [31]. We show here that L1TD1 not only binds to L1 transcripts but also associates with L1 ORF1p in an RNA-independent manner. These observations are compatible with a model where L1TD1/ORF1p heteromultimers bind to *L1* RNA (Suppl. Figure S7C). The additional presence of L1TD1 might thereby enhance the RNA chaperone function of ORF1p.

L1TD1 was lost or pseudogenized multiple times during mammalian evolution and has evolved under positive selection during primate and mouse evolution suggesting an important function during development [5]. L1 elements can retrotranspose in the germline, in embryonic stem cells, and in the early embryo [36–39]. Mouse embryos with repressed L1 expression show impaired embryonic development, suggesting that L1 is part of the developmental program of early embryos [41]. The simultaneous L1TD1 expression during early embryogenesis might ensure activation of L1 retrotransposition at this developmental stage [9].

To gain insight into the regulatory function of L1TD1 in tumor cells, we performed RIP-seq, transcriptome and proteome analyses in HAP1 cells. The RIP-seq approach identified, in addition to the known associated *L1TD1* mRNA [11], a defined set of transcripts comprising mRNAs, lncRNAs and transposable elements including *L1* transcripts. These findings indicate that L1TD1 has not only inherited its function as RNA-binding protein but also its affinity for transposon transcripts. Recently, Jin *et al.* published a study on the impact of L1TD1 on the translation in hESCs and identified L1TD1-bound RNAs by CLIP-seq (cross-linking immunoprecipitation-high-throughput sequencing) [31]. Interestingly, the majority of L1TD1-associated transcripts in HAP1 cells (69%) identified in our study were also reported as L1TD1 targets in hESCs suggesting a conserved binding affinity of this domesticated transposon protein across different cell types. The same study [31] showed that L1TD1 localizes to high-density RNP condensates and enhances the translation of a subset of mRNAs enriched in the condensates. Our mass spectrometry data showed that L1TD1 ablation leads to a significantly changed proteome. None of the proteins encoded by the mRNAs associated with L1TD1 showed significantly changed steady state levels in the absence of L1TD1 suggesting that this is not a direct effect of L1TD1 association with the respective mRNAs. However, a subset of L1TD1-associated transcripts encode proteins involved in

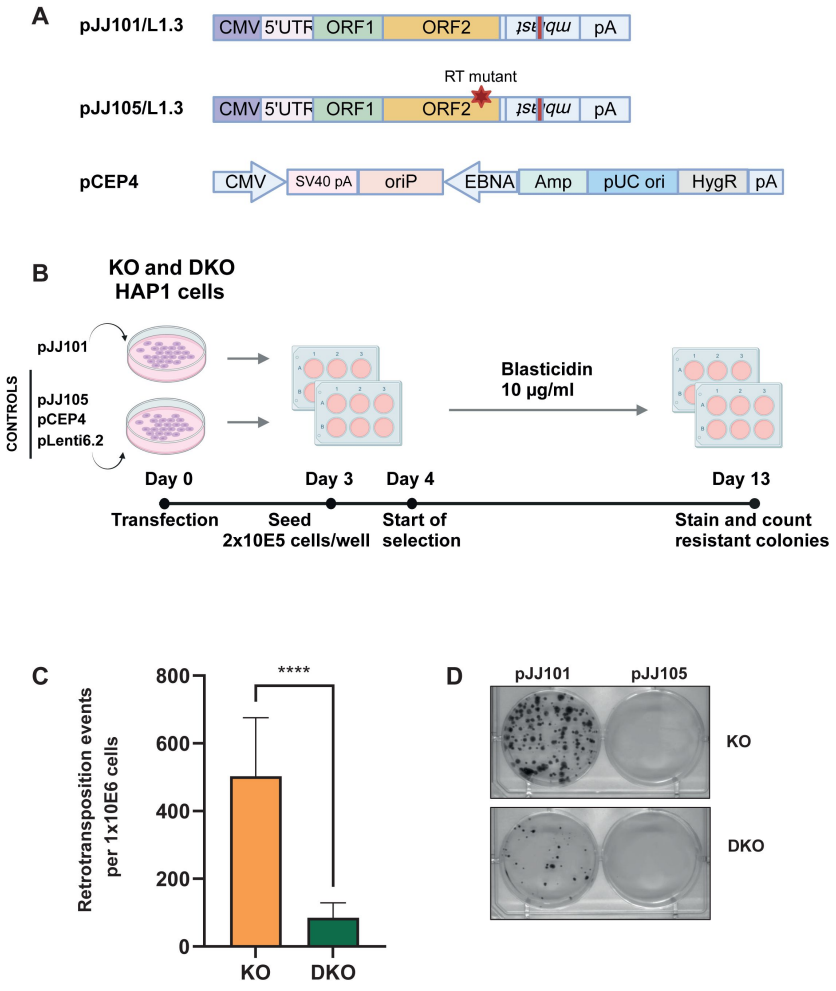

Figure 4.

L1TD1 promotes L1 retrotransposition.

(A) Schematic representation of plasmids used for retrotransposition (Figure modified from [35] and created with *BioRender.com/k03r500*). The pJJ101/L1.3 construct contains the full length human L1.3 element with a blasticidin deaminase gene (mblast) inserted in antisense within the 3'UTR. The *mblast* gene is disrupted by an intron and *mblast* expression occurs only when L1 transcript is expressed, reverse transcribed and inserted into the genome. The pJJ105/L1.3 plasmid contains a mutation in the reverse transcriptase (RT) resulting in defective retrotransposition. The backbone plasmid pCEP4 was used as additional negative control. The blasticidin deaminase gene containing plasmid pLenti6.2 was used as transfection/selection control. **(B)** Workflow of retrotransposition assay. DNMT1 KO and DNMT1/L1TD1 DKO cells were separately transfected with pJJ101 and control plasmids. Equal number of cells were seeded for each condition. Blasticidin selection (10 µg/ml) was started at day 4 and resistant colonies were counted on day 13. Panel created with *BioRender.com/e85e605*). **(C)** Bar graph showing the average number of retrotransposition events per 10^6 cells seeded in three independent experiments. Blasticidin resistant colonies in pLenti6.2 transfected cells were used for normalization. Statistical significance was determined using unpaired t-test. All data in the figure are shown as a mean of ± SD of three independent experiments, **** p ≤0.0001. **(D)** Representative pictures of bromophenol blue stainings of blasticidin resistant colonies for each genotype and each transfection.

the control of cell division and cell cycle. Thus, it is possible that subtle changes in the expression of these protein that were not detected in our mass spectrometry approach contribute to the antiproliferative effect of L1TD1 depletion (see below).

In accordance with the hESC study [31], we did not find an overlap between RNAs associated with L1TD1 and their deregulation at the RNA level. This suggests that in HAP1 cells L1TD1 does not have a global effect on RNA stability and translation of its associated RNAs. This is consistent with a recent study on L1 bodies, where RNA processing association of L1 body components did not correlate with RNA stability or translational control of the RNA targets of L1 bodies [42].

Of note, L1TD1 associated with *SINE* transcripts such as AluY and L1TD1 ablation positively affected AluY transposon expression. This relatively young subfamily of SINE elements is still retrotransposition-competent and their mobilization depends on the activity of LINE proteins [43, 44]. These observations suggest that L1TD1 has not only inherited its function as an RNA-binding protein but also the affinity to *L1* and *SINE* transcripts. L1TD1 association with *AluY* transcripts could potentially be connected to modulation of L1 and SINE-1 transposon activities. It would be interesting to check in future experiments the impact of L1TD1 in Alu retrotransposition reporter assays.

L1 is one of the few protein-coding transposons that is active in humans and L1 overexpression and retrotransposition are hallmarks of cancers [6, 45]. Similarly to the epigenetic control of the ancestral L1 elements [46, 47], L1TD1 expression in HAP1 cells is restricted by DNA methylation and DNA hypomethylation induced by DNMT1 ablation results in robust L1TD1 RNA and protein expression. DNMT1 ablation in human colorectal carcinoma cells results in mitotic catastrophe and loss of cell viability [48], whereas HAP1 DNMT1 KO cells show increased DNA damage response and apoptosis but are viable. The less severe phenotype of DNMT1 ablation in HAP1 cells might be due to the compensatory up-regulation of DNMT3A and DNMT3B.

We report here that L1TD1 deletion in DNMT1-deficient cells led to reduced cell viability and increased apoptosis in HAP1 cancer cells. The observation that loss of L1TD1 led to increased apoptosis and DNA damage, but decreased L1 retrotransposition is at first glance unexpected. The positive role of L1TD1 for proliferation might be due to a transposition-independent effects of L1TD1 on the expression of cell cycle regulators as discussed above. Potential deleterious effects of enhanced retrotransposition might be buffered by the up-regulation of KRAB domain proteins upon loss of DNMT1. Our findings are in contrast to the negative effect of L1TD1 on cell viability and tumor growth observed in the NSCLC xenograft model [19] but in accordance with the findings that L1TD1 has a positive impact on cell viability in seminoma [11] and medullablastoma [14] suggesting a cancer cell type-specific effect of L1TD1 that might be related to the DNA methylation state of the tumors.

Taken together we present here a novel cellular function for a domesticated transposon protein by showing that L1TD1 associates with L1-RNPs and promotes L1 retrotransposition. This function might have a beneficial effect during early development but could also impact on tumorigenesis.

Materials and methods

Cell lines and cell culture

HAP1 is a near-haploid human cell line derived from the KBM-7 chronic myelogenous leukemia (CML) cell line [21, 49]. HAP1 cells were cultured in Iscove's Modified Dulbecco Medium (IMDM, Sigma Aldrich, I3390) supplemented with 10% Fetal Bovine Serum (Sigma Aldrich, F7524) and 1% penicillin/streptomycin (Sigma Aldrich, P4333).

The human malignant papillary serous carcinoma cell line OV-90 [50] was cultured in MCDB 105 (Sigma Aldrich, 117) and Medium 199 (Sigma Aldrich, M4530) in a 1:1 ratio supplemented with 15% Fetal Bovine Serum (Sigma Aldrich, F7524) and 1% penicillin/streptomycin (Sigma Aldrich, P4333). The cells were kept in a humidified incubator at 37°C and 5% CO_2.

Gene editing

DNMT1 knockout (KO) HAP1 cells harboring a 20 bp deletion in the exon 4 of the *DNMT1* gene were generated using CRISPR/Cas9 gene editing. DNMT1/L1TD1 double knockout (DKO) HAP1 cells were generated by CRISPR/Cas9 gene editing resulting in a 13 bp deletion in exon 4 of the L1TD1 gene. CRISPR/Cas9 gene editing was performed by Horizon Genomics (now Horizon Discovery).

Viability and Apoptosis Assay

Both assays were performed as described previously [51]. For the viability assay 2×10^3 cells of each cell line were seeded in triplicates in solid white 96-well plates and allowed to attach to the plates overnight. Cell viability was measured using CellTiter-Glo Luminiscent Cell Viability Assay (Promega, G7571) and Glomax Discover plate reader (Promega). The measurement was performed every 24 hours starting from the day after seeding (24h, 48h, 72h).

For the apoptosis assay 5×10^6 cells were collected 48 hours after seeding, fixed for 20 min 2% paraformaldehyde (Merck Life Science) and for 30 min in 75% ethanol. Cells were permeabilized with 0.1% TritonX100 (Merck Life Science) for 10 min and blocked in 10% donkey serum (Merck Life Science) for 30 min and then incubated with 1:50 diluted anti-cleaved caspase 3 antibody in phosphate-buffered saline (PBS) (Cell Signaling, 9661) for 1 hour. Cells were washed and incubated with 1:400 Alexa 488 anti-rabbit secondary antibody (Jackson Immuno Research, 751-545-152). Samples were acquired using FACSCelesta flow cytometer (BD Bioscience) and the data were analyzed using FlowJo software 10.6.1 (BD Bioscience)

Immunoprecipitation

HAP1 cells pellets harvested from 15-cm culture plates were lysed in Hunt Buffer (20 mM Tris/HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with Complete Protease inhibitor cocktail (Roche, 11697498001), Complete Phosphatase inhibitor cocktail (Roche, 11836145001), 10 mM sodium fluoride, 10 mM β-glycerophosphate, 0.1 mM sodium molybdate and 0.1 mM PMSF. The lysis was achieved by repeating freeze-thaw three times followed by centrifugation 12,000g for 15 min at 4°C. Cell lysate was collected and the protein concentration was measured using Bradford assay. 40 µl of Dynabeads^{TM} Protein G beads (ThermoFisher Scientific, 10004D) was blocked with 10% BSA in a rotor at 4°C for 1 hour. 1 mg of cell lysate was incubated with blocked Protein G beads and monoclonal L1 ORF1p antibody (EMD Millipore, MABC1152) at 12 rpm at 4°C overnight. The next day, the beads were washed three times with Hunt buffer.

Western Blot Analysis

Immunoprecipitated complex was eluted from the beads with 1x SDS loading dye followed by heat-incubation at 95 °C for 5 min for detection of proteins. For input samples, 20 µg whole cell lysate was similarly denatured as IP samples. Proteins were separated in SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Amersham Protran, GE10600001, Sigma Aldrich) by wet transfer method. The membrane was blocked with the blocking solution (1x PBS, 1% polyvinylpyrrolidone, 3% non-fat dried milk, 0.1% tween-20, 0.01% sodium azide, pH 7.4) and incubated with the corresponding antibodies listed in Supplementary Table 8. To detect the protein of interest, ECL Western blotting detection reagents were used with a FUSION FX chemiluminescence imaging system.

Mass spectrometry analysis

Proteomes of DNMT1 KO and DNMT1/L1TD1 DKO cells were analyzed by quantitative TMT (Tandem Mass Tag) multiplex mass spectrometry analysis. Proteins were precipitated from cell extracts with acetone. After reduction and alkylation of Cys under denaturing conditions, proteins were digested with LysC/trypsin overnight. Peptides were cleaned-up using Oasis MCX (Waters) and labeled with TMT6plex (Thermo Fisher) according to manufacturer's protocol. Equal amounts of each channel were mixed, desalted and fractionated by neutral pH reversed phase chromatography. Equal interval fractions were pooled and analyzed by LC-MS3 on an Ultimate 3000 RSLCnano LC coupled to an Orbitrap Eclipse™ Tribrid™ mass spectrometer via a FAIMS Pro ion mobility interface (all Thermo Fisher). Data were analyzed with MaxQuant 1.6.7.0. [52]. Differentially enriched proteins were determined with LIMMA [53]. Workflow and analysis are described in detail in the Supplementary Table 4.

RNA Immunoprecipitation and RIP-qRT-PCR

The native RIP protocol was followed as previously described in [54]. In brief, cells were resuspended in 1 ml polysomal lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.0), 0.5% NP-40, 1 mM DTT) supplemented with Protector RNase inhibitor (Sigma Aldrich, 3335399001) and protease inhibitor cocktail (Roche, 11697498001). Cell lysis was facilitated with 27G needle and syringe at least seven times. The lysates were centrifuged at 12,000 g for 15 min at 4°C. 1% input was taken from the cell lysate for RIP-seq experiment. The supernatant was cleared via pre-incubation with Dynabeads^{TM} Protein G beads (ThermoFisher Scientific, 10004D) rotating for 1h at 4°C. For immunoprecipitation 1 mg of pre-cleaned cell lysate was incubated with 4µg mouse monoclonal L1TD1 antibody (R&D systems, MAB8317) at 4°C at 20 rpm on the rotor overnight. 40 µl Dynabeads^{TM} Protein G beads per IP was blocked with 10% bovine serum albumin (BSA) for 1 h at 4°C. Blocked beads were added to the lysate and antibody mixture rotating for 1 h at 4°C. Next, the beads were pelleted and washed three times with polysomal lysis buffer. 20% of the RIP was used for Western blot analysis to confirm the presence of immunoprecipitated L1TD1. 1% of the RIP was saved as input. All experiments were performed in biological triplicates.

Validation of the RIP experiments by RIP-qRT-PCR was performed using the Sigma Aldrich RIP-qRT-PCR: Data Analysis Calculation Shell. Fold enrichment of transcripts in the IP samples was calculated as ratio of qRT-PCR values of KO cells representing the specific association of the transcript to L1TD1 relative the qRT-PCR values of DKO cells representing unspecific binding in the absence of L1TD1 (arbitrarily set to 1) and normalized to the qRT-values of input samples in the indicated cells. For L1 primers specific for the L1.2 subfamily were used.

RNA isolation

To extract total RNA, immunoprecipitated RNA and 1% input RNA Monarch RNA Cleanup Kit (New England Biolabs, T2047L) was used according to the manufacturers' instructions. In the case of RIP samples, the beads were first treated with DNase I (New England Biolabs, M0303S) at 37°C for 15 min and 1 ml TRIzol (Thermo Fischer Scientific, 15596018) was directly added on. Upon addition of 150 µl chloroform the mixture was vortexed vigorously for 15 sec and centrifuged for at 12,000 g for 15 min at 4°C. Aqueous phase was transferred to a new Eppendorf tube and mixed with 1 volume of EtOH (>95%). The mixture was loaded on the RNA columns and spun for 1 min. The columns were washed two times with the washing buffer (supplemented with the kit) and eluted in 20 µl nuclease free water. RNA concentration was measured using the Qubit RNA High Sensitivity kit (Thermo Fischer Scientific, Q32852).

cDNA library preparation

The cDNA libraries for the RIP-seq experiment were prepared as described in Smart-seq3 protocol by Hagemann-Jensen *et al.*, 2020 [55].

cDNA synthesis and qRT-PCR

1 µg of total RNA was reverse transcribed using iScript cDNA synthesis Kit (Bio-Rad, 1708891). Reverse transcription reaction was performed as follows: 25°C for 5 min, 46°C for 30 min, 95°C for 5 min. The resulting cDNA was diluted to 1:10 with nuclease free water. 5 µl of the cDNA was used for SYBR Green qPCR Master mix (Bio-Rad 1725275) together with the primers used in this study which were listed in Supplementary Table 7.

Methylight Assay

Genomic DNA was extracted from DNMT1 KO, DNMT1/L1TD1 DKO and WT HAP1 cells using the Wizard Genomic DNA isolation kit (Promega) following the manufacturer's protocol. Next, 1 µg of genomic DNA per sample was subjected to bisulfite conversion using the EZ DNA Methylation Kit (Zymo research Cat. no. D5001) following the manufacturer's instructions. Bisulfite-treated DNA was eluted in ddH_2O to a final concentration of 10 ng/µL and stored at −20°C until further use. The MethyLight method was performed as previously described by Campan *et al.* [56] using primers for *L1TD1* and *L1*; *ALU* was used as a reference [57]. The primer sequences are provided in Supplementary Table 7. The MethyLight reactions were done in technical triplicates. Each reaction contained 7.5 µL of 2X TaqMan Universal PCR Master Mix (Thermo Fisher Cat. No. 4324018), 300 nM of each primer and 100 nM of probe. For the *L1TD1* reactions 50 ng and for the *L1* reactions 20 ng of bisulfite-converted DNA were used in a total volume of 15 µL. The reactions were performed on a BioRad CFX96 thermocycler with an initial incubation at 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. Each run included the individual samples, a serial dilution of fully methylated DNA, a non-methylated DNA control (Human Methylated & Non-Methylated (WGA) DNA Set D5013 Zymo research), and a non-template control. The PMR (percentage of methylation reference) of each individual sample was calculated using the following formula: 100 × [(GENE-X mean value) sample/ (ALU mean value) sample] / [(GENE-X mean value)100% methylated / (ALU mean value)100% methylated].

RNA-seq and RIP-seq Analyses

RNA sequencing data were analyzed using the RNA-Seq pipeline of IMP/IMBA Bioinfo core facility (ii-bioinfo@imp.ac.at). The pipeline is based on the nf-core/rnaseq pipeline [doi: 10.5281/zenodo.1400710] and is built with nextflow [58].The RNA-seq analysis was performed as described in the following: Adapters were clipped with trimgalore [59]. Abundant sequence fractions (rRNA) were removed using (bowtie2) [60]. Cleaned raw reads were mapped against the reference genome (hg38,Homo_sapiens.GRCh38.107.gtf) with STAR (reverse_stranded, STAR) [61]. Mapped reads were assigned to corresponding genes using featureCounts [62]. Abundances were estimated through Kallisto [63] and Salmon [64]. Analysis of differentially expressed genes was performed using DESeq2 [65]. TEtranscript software [33] was used to identify differentially expressed TE-derived sequences. In the case of RIP-seq, differential enrichment of transcripts relative to input as well as to the negative control (HAP1 DNMT1/L1TD1 DKO) was calculated using DEseq2.

L1 Retrotransposition Assay

Transfection of HAP1 cells with pJJ101, pJJ105, EGFP, pLenti6.2, pCEP4 was performed using polyethyleneimine (PEI)/NaCl solution. Briefly, 0.2×10^6 HAP1 cells/well were seeded into 6-well plates. 24 hours later and at 70% confluence, medium was replaced by fresh IMDM without penicillin/streptavidin followed by transfection. For each transfection, 100 µl of 150 mM NaCl, 7 µl of PEI were mixed with DNA/NaCl solution (100 µl of 150 mM NaCl, 4 µg of plasmid DNA), incubated at room temperature for 30 min, followed by adding the mixture in a drop-wise fashion onto cells. 18 hours post-transfection, the media was replaced with complete IMDM including 1% penicillin/streptavidin. Transfection efficiency was monitored 48 hours after transfection using fluorescent microscopy.

The retrotransposition assay was performed as described in [35] with the following modification. To measure retrotransposition efficiency, 2×10^5 cells per well were seeded in 6-well culture plates and cultured at 37°°C overnight. On day 0.4 ug of pJJ101/L1.3, pJJ105/L1.3, pLenti6.2, pCEP4 vectors described in Kopera *et al.* [35] were transfected separately using PEI/NaCl in DNMT1 KO and DNMT1/L1TD1 DKO cells. To correct for potential differences in transfection efficiency and susceptibility to blasticidin in DNMT1 KO and DNMT1/L1TD1 DKO cells, we transfected DNMT1 KO and DNMT1/L1TD1 DKO cells in parallel with the blasticidin deaminase containing vector pLenti6.2. The following day (d1) the media was replaced with IMDM medium containing 1% penicillin/streptavidin. On day 3, 2×10^5 cells per well seeded into 6-well culture dishes for each genotype and condition. Blasticidin treatment 10 µg/ml was started on day 4 and the cells were cultured 37°°C for 9 days without medium change. Blasticidin resistant colonies were fixed on day 13 with 4% methanol-free formaldehyde (Thermo Fischer Scientific, 28908) at room temperature for 20 min and stained with 0.1% bromophenol blue (w/v) at room temperature for 1 hour. The pictures of the wells containing the colonies were taken using the FX chemiluminescence imaging system. The pictures were further processed in Fiji Software following the Analyze Particles function [66]. The colony counts were obtained from three technical replicates per transfection. Mean colony counts were calculated and adjusted retrotransposition mean was calculated by adjusting for blasticidin resistant colonies in in blasticidin vector (pLenti6.2) transfected cells.

Indirect Immunofluorescence Staining

For immunostaining the protocol from Sharma *et al.* [67] was followed with minor modifications. Briefly, 75×10^3 HAP1 cells per well were plated on cover slips in 12 well plates. Next day, the cells were washed with 1x PBS and fixed with 4% methanol-free formaldehyde at room temperature for 10 min. The cells were washed with 1x PBS/Glycine pH 7.4 and permeabilized with 0.1% Triton-X in 1x PBS/Glycine for 3 min. The samples were blocked with 1x PBS/Glycine/1% BSA at room temperature for 1 hour. After blocking, the cells were stained primary antibodies in blocking solution at 4°C overnight. On the following day, the cells were washed three times with 1x PBS/Glycine/1% BSA for 5 min and stained with secondary antibody in blocking solution for 2 hours in the dark. After a wash with PBS/Glycine pH 7.4, cells were incubated with DAPI (Sigma Aldrich, D9542, 1:10000) in PBS/Glycine at room temperature for 10 min and washed three times with 1x PBS/Glycine pH 7.4. The slides were mounted with ProLong Gold Mountant (Thermo Fischer Scientific, P36930) and dried. The staining was analyzed using an Olympus Confocal Microscope.

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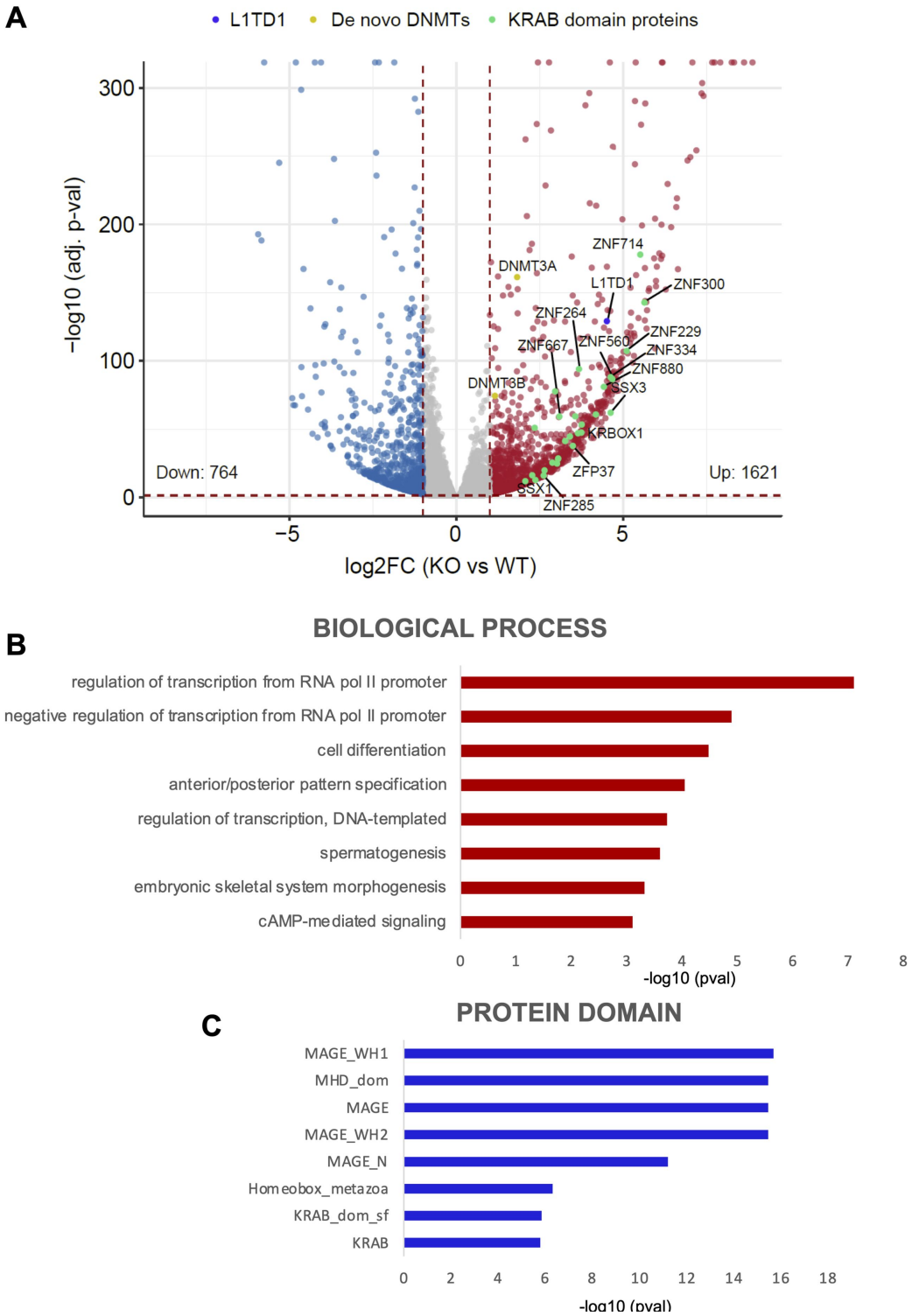
Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [68] partner repository with the dataset identifier PXD047402. RNA-seq and RIP-seq data were submitted to GEO under accession numbers GSE169614, GSE254459 and GSE254460.

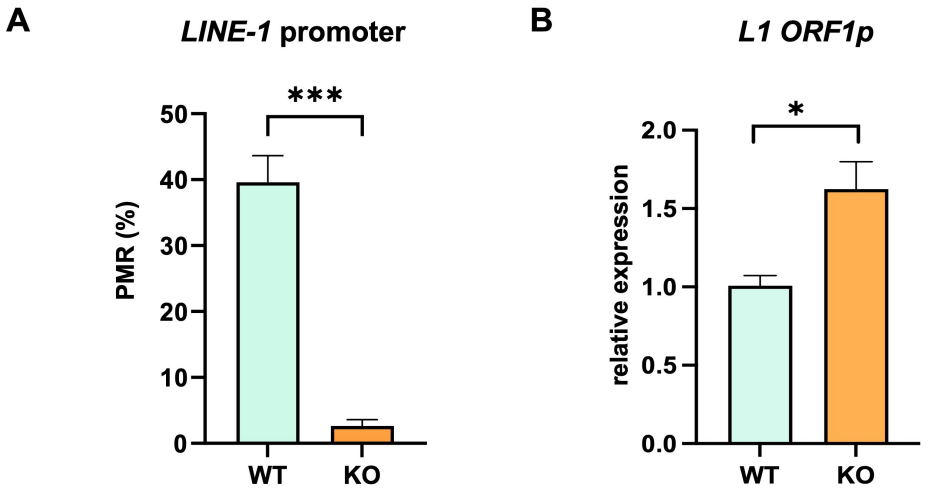
Supplementary figures



Supplementary Figure S1

Deregulation of the HAP1 transcriptome upon loss of DNMT1.

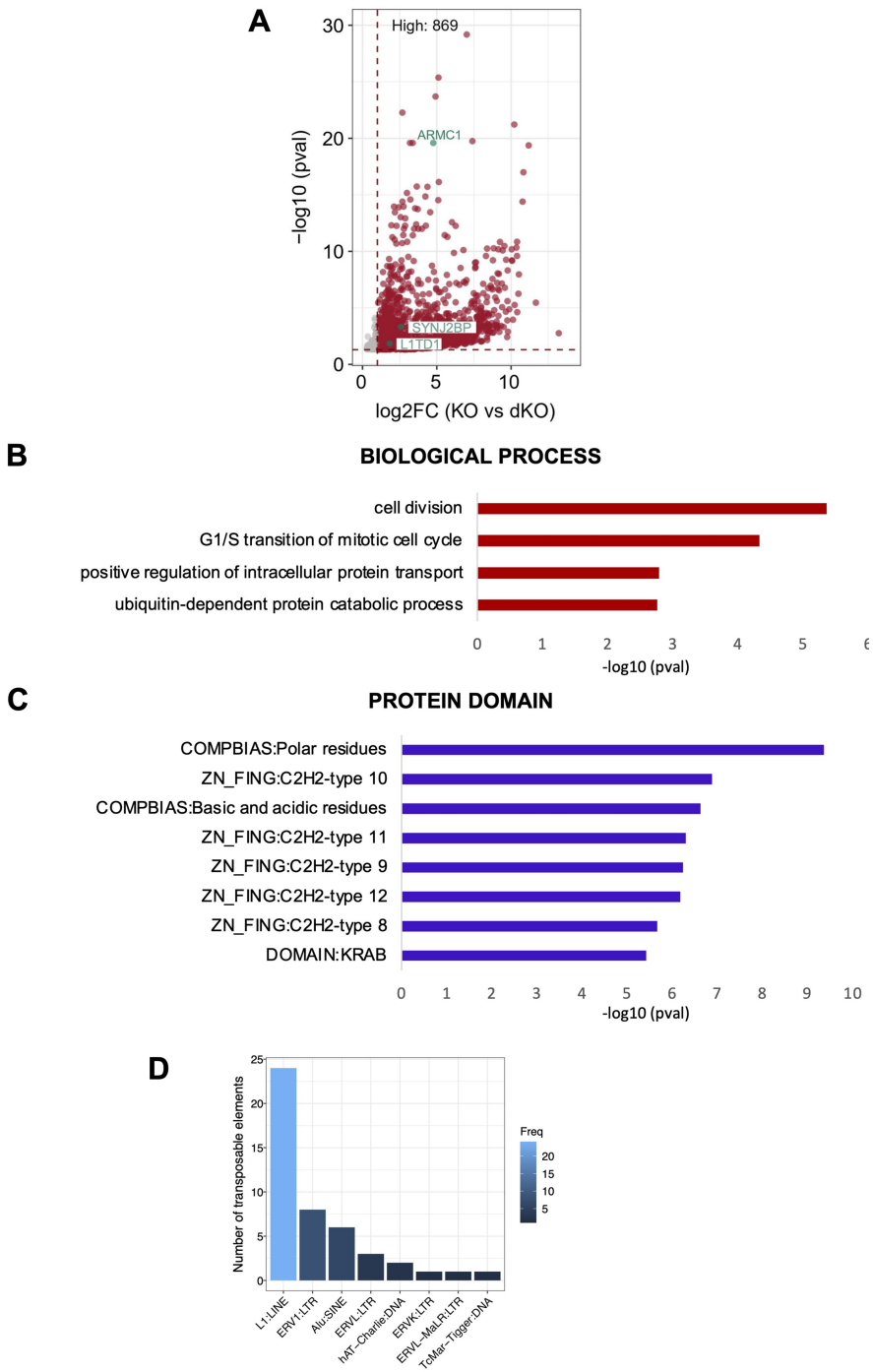
(A) Volcano plot illustrating the DESeq2 analysis of RNA-seq of HAP1 DNMT1 KO and wildtype (WT) cells. Differentially expressed genes are plotted as DNMT1 KO over WT (log2FC ≥ 1, adj. p-value < 0.05 (red) and log2FC ≤ −1, adj. p-value < 0.05 (blue). L1TD1 is highlighted in blue, KRAB- containing zinc finger proteins in green and *de novo* DNMTs in yellow. **(B and C)** DAVID Gene Ontology enrichment analysis of top upregulated transcripts DESeq2 comparisons (DNMT1 KO *versus* input and DNMT1 KO *versus* WT HAP1, log2FC ≥ 2, adj. p-value < 0.05) in terms of biological processes **(B)** and protein domains **(C)**.



Supplementary Figure S2

DNMT1 ablation results both in DNA hypomethylation at L1 elements and expression of L1 ORF1p transcripts.

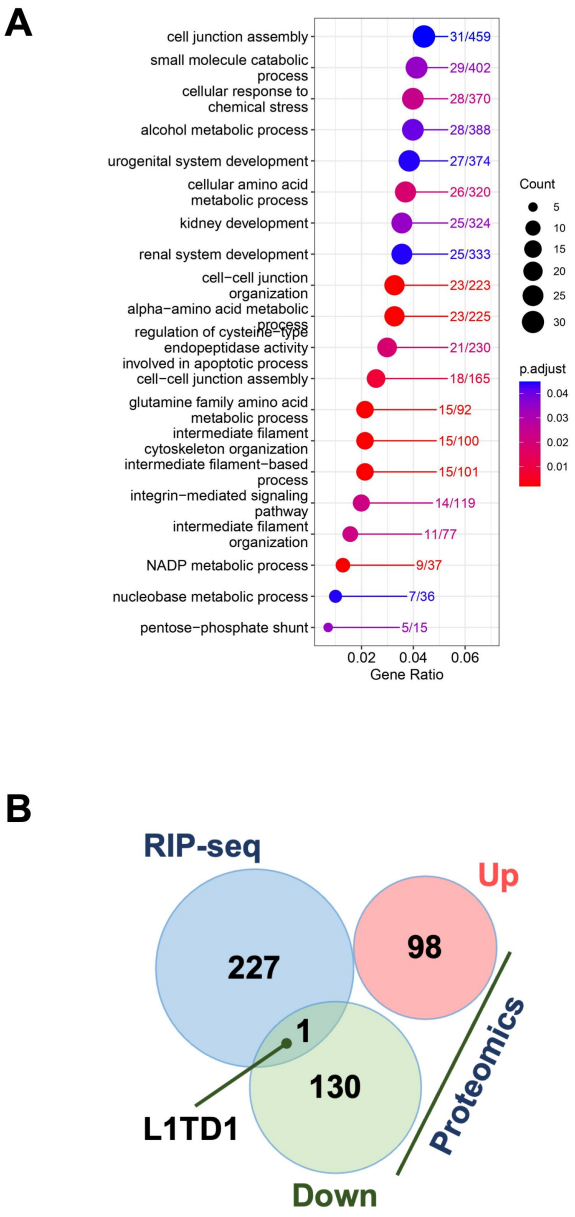
(A) Quantification of DNA methylation levels at the *L1TD1* promoter in HAP1 wildtype (WT) and DNMT1 KO cells using the MethyLight assay. DNA methylation is shown as percentage of methylation ratio (PMR). **(B)** qRT-PCR analysis of *L1* mRNA expression in HAP1 wildtype (WT) and DNMT1 KO cells. *GAPDH* was used as a normalization control. All data in the figure are shown as a mean of ± SD of 3 biological replicates. * p ≤0.05, *** p ≤0.001.



Supplementary Figure S3

L1TD1 interacts with a specific set of mRNAs and transposon transcripts.

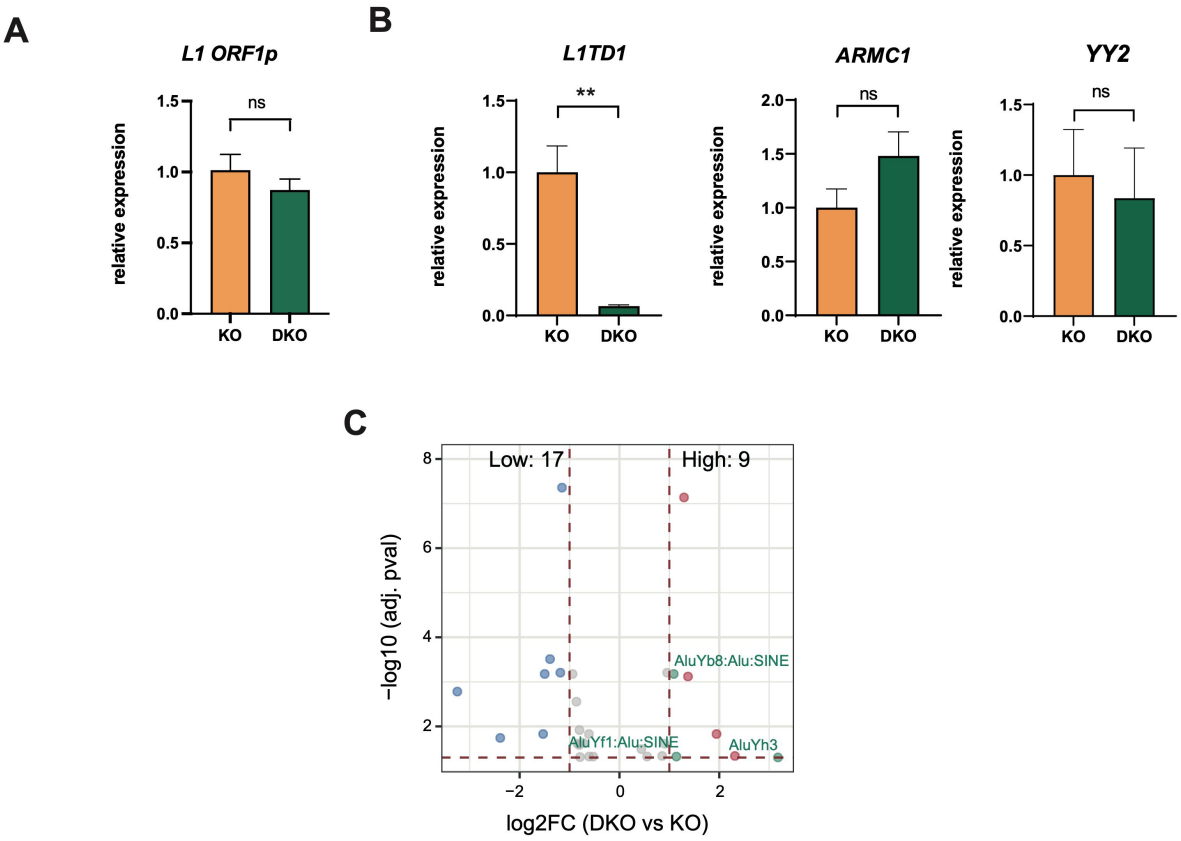
(A) Volcano plot showing the L1TD1-associated transcripts enriched in DNMT1 KO IP over DNMT1/L1TD1 DKO IP (log2FC ≥ 2 and adj. p-val < 0.05). The transcripts of *L1TD1*, *SYNJ2BP*, *ARMC1* are highlighted in green. **(B)** and **(C)** DAVID Gene Ontology enrichment analysis of 228 common transcripts identified as L1TD1 targets in both DESeq2 comparisons (DNMT1 KO *versus* input and DNMT1 KO *versus* DNMT1/L1TD1 DKO) in terms of biological processes **(B)** and protein domains **(C)**. **(D)** Classification of TEs associated with L1TD1 (log2FC > 1, adj. p-val < 0.05) according to their frequency (taken from Suppl. Table S3).



Supplementary Figure S4

GSEA analysis of proteins upon loss of L1TD1.

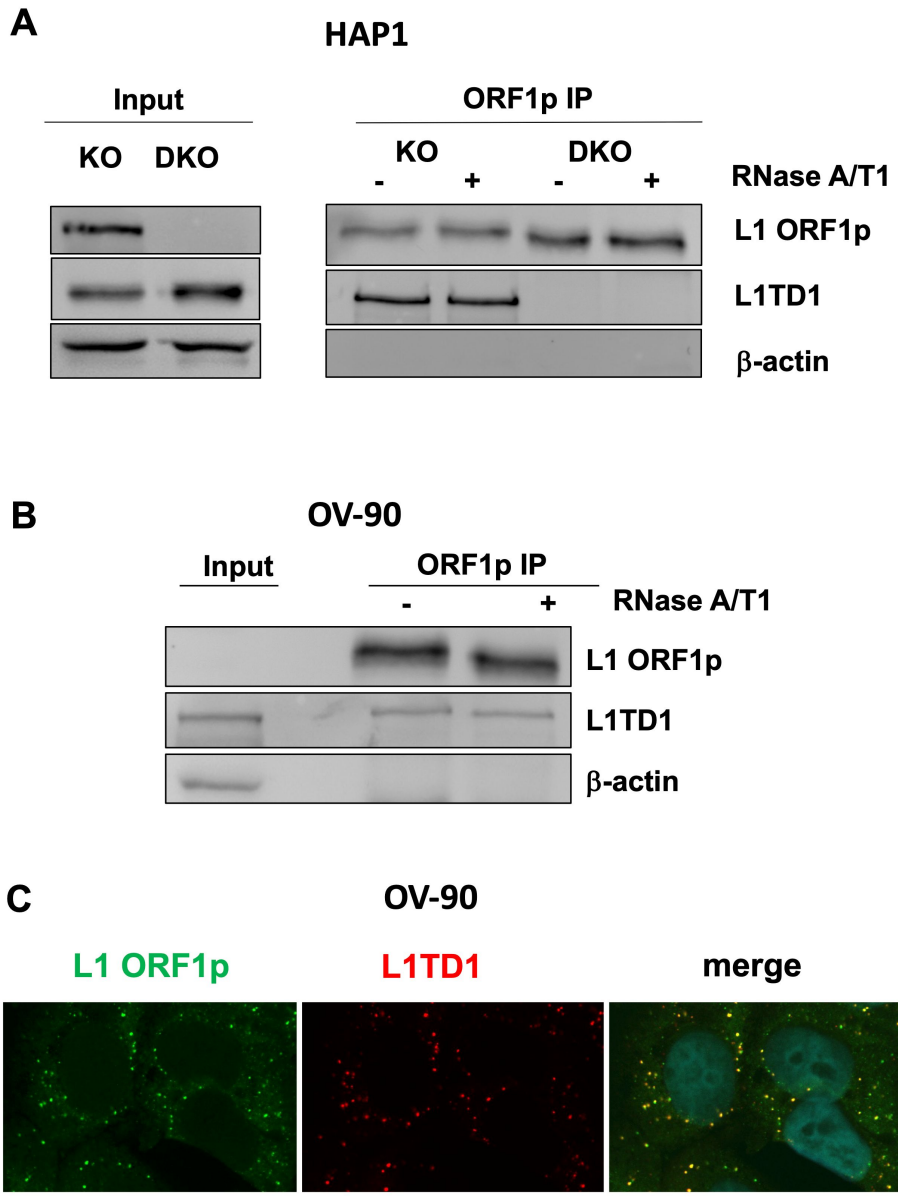
(A) Gene ontology enrichment analysis of proteins differentially expressed in HAP1 DNMT1/L1TD1 DKO *versus* DNMT1 KO cells. **(B)** Venn plot of the overlap of mRNAs identified in the RIP-seq analysis and differentially expressed proteins (up and down) identified in the mass spectrometry analysis of HAP1 DNMT1/L1TD1 DKO and DNMT1 KO cells. The single common mRNA is L1TD1.



Supplementary Figure S5

Ablation of L1TD1 leads to changes in the transcriptome of HAP1 cells.

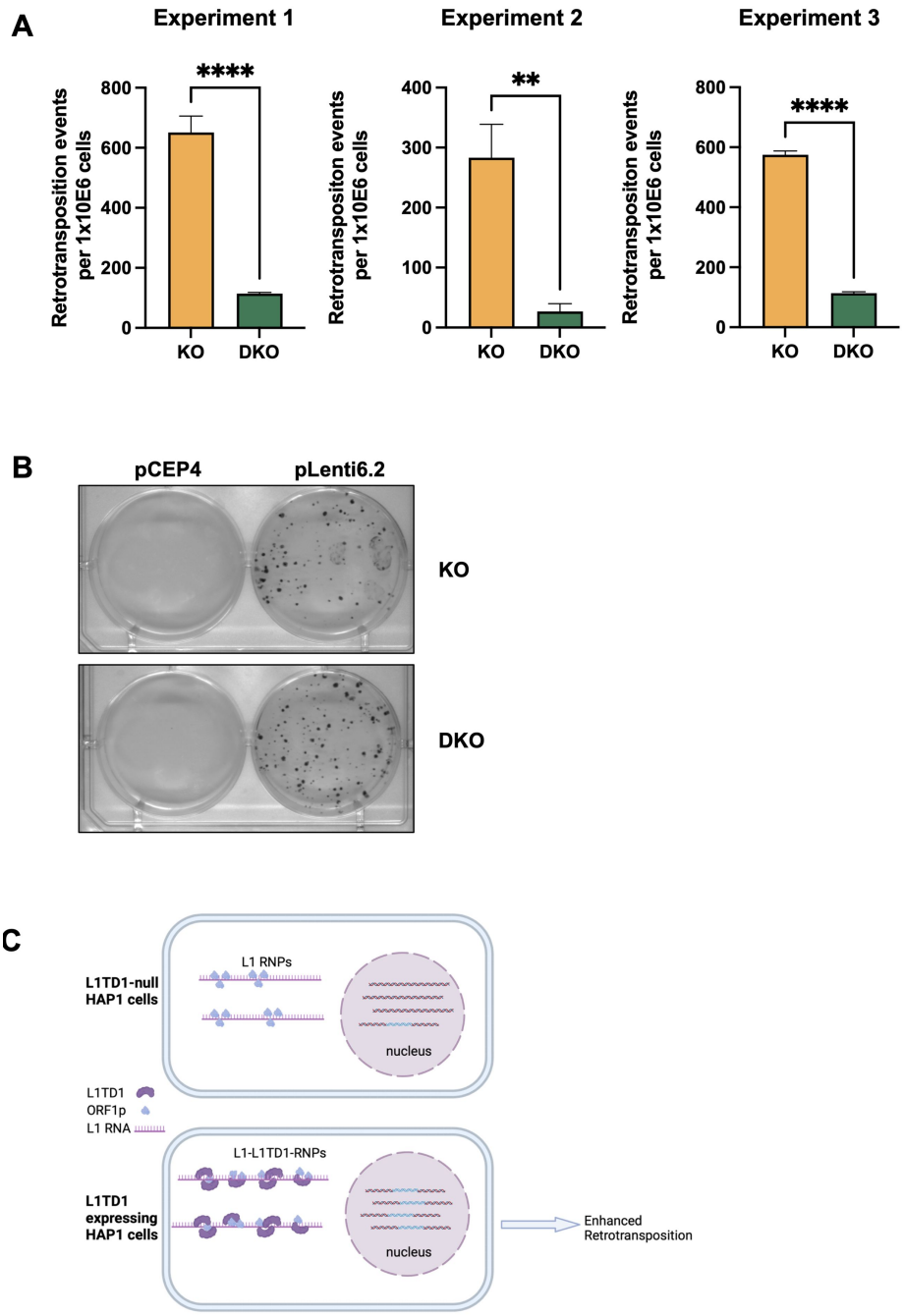
(A and **B)** qRT-PCR analyses of selected L1TD1-associated transcripts. Statistical significance was determined using unpaired two-tailed t-test. Data are shown as mean of ± SD of 3 biological replicates. ** p ≤0.01, ns not significant. **(C)** Volcano plot illustrating TEtranscript analysis of RNA-seq performed in HAP1 DNMT1 KO and DNMT1/L1TD1 DKO cells. Positively enriched TEs are shown in red and negatively enriched TEs are shown in blue. Alu elements are highlighted in green.



Supplementary Figure S6

RNA-independent interaction of L1TD1 and L1 ORF1p in HAP1 and OV-90 cells.

(A) Western blot analysis of L1 ORF1p IPs with and without RNase A/T_1 digestion in DNMT1 KO HAP1 cells using antibodies specific for L1 ORF1p and L1TD1 together with input samples. DNMT1/L1TD1 DKO HAP1 cells were used as a negative IP control and β-actin was used as Western blot loading control. **(B)** Western blot analysis of ORF1p IPs with and without RNase A/T_1 digestion in OV-90 cells using antibodies specific for L1 ORF1p and L1TD1 in OV-90 cells. Mouse IgG was used as a negative control for the IP and β-actin was used as Western blot loading control. **(C)** Indirect immunofluorescence analysis of ORF1p (green) and L1TD1 (red) in OV-90 cells. In merged images nuclear DNA was stained with DAPI (blue).



Supplementary Figure S7

L1TD1 enhances L1 retrotransposition.

(A) The bar graphs separately show the number of retrotransposition events per 10^6 cells seeded for three independent biological replicates. Blasticidin resistant colonies in pLenti6.2 transfected cells were used for normalization. Statistical significance was determined using unpaired t-test. All data in the figure are shown as a mean of ± SD of 3 technical replicates. ** p ≤0.01, **** p ≤0.0001. **(B)** Representative pictures of bromophenol blue stainings of blasticidin resistant colonies for transfection with the backbone plasmid pCEP4 used as negative control and the blasticidin deaminase gene containing plasmid pLenti6.2 used as transfection/selection control. **(C)** Potential mechanism of facilitated L1 retrotransposition by L1TD1. By association with L1-RNPs L1TD1 might enhance the chaperone function of L1ORF1p resulting in more efficient L1 retrotransposition. Panel C created with *BioRender.com/r80m690*.

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Reviewer #1 (Public review):

Summary:

In their manuscript entitled 'The domesticated transposon protein L1TD1 associates with its ancestor L1 ORF1p to promote LINE-1 retrotransposition', Kavaklıoğlu and colleagues delve into the role of L1TD1, an RNA binding protein (RBP) derived from a LINE1 transposon. L1TD1 proves crucial for maintaining pluripotency in embryonic stem cells and is linked to cancer progression in germ cell tumors, yet its precise molecular function remains elusive. Here, the authors uncover an intriguing interaction between L1TD1 and its ancestral LINE-1 retrotransposon.

The authors delete the DNA methyltransferase DNMT1 in a haploid human cell line (HAP1), inducing widespread DNA hypo-methylation. This hypomethylation prompts abnormal expression of L1TD1. To scrutinize L1TD1's function in a DNMT1 knock-out setting, the authors create DNMT1/L1TD1 double knock-out cell lines (DKO). Curiously, while the loss of global DNA methylation doesn't impede proliferation, additional depletion of L1TD1 leads to DNA damage and apoptosis.

To unravel the molecular mechanism underpinning L1TD1's protective role in the absence of DNA methylation, the authors dissect L1TD1 complexes in terms of protein and RNA composition. They unveil an association with the LINE-1 transposon protein L1-ORF1 and LINE-1 transcripts, among others.

Surprisingly, the authors note fewer LINE-1 retro-transposition events in DKO cells compared to DNMT1 KO alone.

Strengths:

The authors present compelling data suggesting the interplay of a transposon-derived human RNA binding protein with its ancestral transposable element. Their findings spur interesting questions for cancer types, where LINE1 and L1TD1 are aberrantly expressed.

Weaknesses:

Suggestions for refinement:

The initial experiment, inducing global hypo-methylation by eliminating DNMT1 in HAP1 cells, is intriguing and warrants more detailed description. How many genes experience mis-regulation or aberrant expression? What phenotypic changes occur in these cells? Why did the authors focus on L1TD1? Providing some of this data would be helpful to understand the rationale behind the thorough analysis of L1TD1.

The finding that L1TD1/DNMT1 DKO cells exhibit increased apoptosis and DNA damage but decreased L1 retro-transposition is unexpected. Considering the DNA damage associated with retro-transposition and the DNA damage and apoptosis observed in L1TD1/DNMT1 DKO cells, one would anticipate the opposite outcome. Could it be that the observation of fewer transposition-positive colonies stems from the demise of the most transposition-positive colonies? Further exploration of this phenomenon would be intriguing.

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Reviewer #2 (Public review):

In this study, Kavaklıoğlu et al. investigated and presented evidence for a role for domesticated transposon protein L1TD1 in enabling its ancestral relative, L1 ORF1p, to retrotranspose in HAP1 human tumor cells. The authors provided insight into the molecular function of L1TD1 and shed some clarifying light on previous studies that showed somewhat contradictory outcomes surrounding L1TD1 expression. Here, L1TD1 expression was correlated with L1 activation in a hypomethylation dependent manner, due to DNMT1 deletion in HAP1 cell line. The authors then identified L1TD1 associated RNAs using RIP-Seq, which display a disconnect between transcript and protein abundance (via Tandem Mass Tag multiplex mass spectrometry analysis). The one exception was for L1TD1 itself, is consistent with a model in which the RNA transcripts associated with L1TD1 are not directly regulated at the translation level. Instead, the authors found L1TD1 protein associated with L1-RNPs and this interaction is associated with increased L1 retrotransposition, at least in the contexts of HAP1 cells. Overall, these results support a model in which L1TD1 is restrained by DNA methylation, but in the absence of this repressive mark, L1TD1 is expression, and collaborates with L1 ORF1p (either directly or through interaction with L1 RNA, which remains unclear based on current results), leads to enhances L1 retrotransposition. These results establish feasibility of this relationship existing in vivo in either development or disease, or both.

Comments on revised version:

In general, the authors did an acceptable job addressing the major concerns throughout the manuscript. This revision is much clearer and has improved in terms of logical progression.

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Author response:

The following is the authors' response to the original reviews.

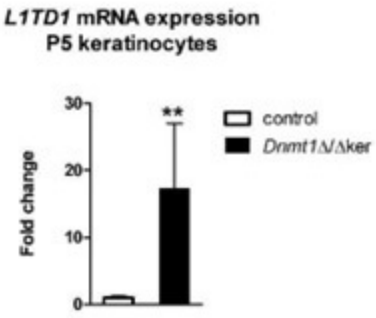
> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *In their manuscript entitled 'The domesticated transposon protein L1TD1 associates with its ancestor L1 ORF1p to promote LINE-1 retrotransposition', Kavaklıoğlu and colleagues delve into the role of L1TD1, an RNA binding protein (RBP) derived from a LINE1 transposon. L1TD1 proves crucial for maintaining pluripotency in embryonic stem cells and is linked to cancer progression in germ cell tumors, yet its precise molecular function remains elusive. Here, the authors uncover an intriguing interaction between L1TD1 and its ancestral LINE-1 retrotransposon.*
>
> *The authors delete the DNA methyltransferase DNMT1 in a haploid human cell line (HAP1), inducing widespread DNA hypo-methylation. This hypomethylation prompts abnormal expression of L1TD1. To scrutinize L1TD1's function in a DNMT1 knock-out setting, the authors create DNMT1/L1TD1 double knock-out cell lines (DKO). Curiously, while the loss of global DNA methylation doesn't impede proliferation, additional depletion of L1TD1 leads to DNA damage and apoptosis.*
>
> *To unravel the molecular mechanism underpinning L1TD1's protective role in the absence of DNA methylation, the authors dissect L1TD1 complexes in terms of protein and RNA composition. They unveil an association with the LINE-1 transposon protein L1-ORF1 and LINE-1 transcripts, among others.*
>
> *Surprisingly, the authors note fewer LINE-1 retro-transposition events in DKO cells than in DNMT1 KO alone.*
>
> *Strengths:*
>
> *The authors present compelling data suggesting the interplay of a transposon-derived human RNA binding protein with its ancestral transposable element. Their findings spur interesting questions for cancer types, where LINE1 and L1TD1 are aberrantly expressed.*
>
> *Weaknesses:*
>
> *Suggestions for refinement:*
>
> *The initial experiment, inducing global hypo-methylation by eliminating DNMT1 in HAP1 cells, is intriguing and warrants a more detailed description. How many genes experience misregulation or aberrant expression? What phenotypic changes occur in these cells?*

This is an excellent suggestion. We have gene expression data on WT versus DNMT1 KO HAP1 cells and have included them now as Suppl. Figure S1. The transcriptome analysis of DNMT1 KO cells showed hundreds of deregulated genes upon DNMT1 ablation. As expected, the majority were up-regulated and gene ontology analysis revealed that among the strongest up-regulated genes were gene clusters with functions in "regulation of transcription from RNA polymerase II promoter" and "cell differentiation" and genes encoding proteins with KRAB domains. In addition, the *de novo* methyltransferases DNMT3A and DNMT3B were up-regulated in DNMT1 KO cells suggesting the set-up of compensatory mechanisms in these cells.

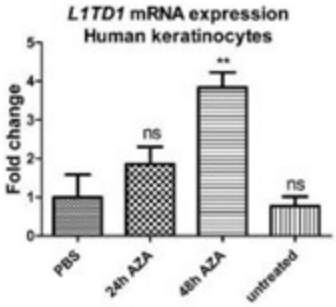
> *Why did the authors focus on L1TD1? Providing some of this data would be helpful to understand the rationale behind the thorough analysis of L1TD1.*

We have previously discovered that conditional deletion of the maintenance DNA methyltransferase DNMT1 in the murine epidermis results not only in the up-regulation of mobile elements, such as IAPs but also the induced expression of L1TD1 ([1], Suppl. Table 1 and Author response image 1). Similary, L1TD1 expression was induced by treatment of primary human keratinocytes or squamous cell carcinoma cells with the DNMT inhibitor azadeoxycytidine (Author response images 2 and 3). These findings are in accordance with the observation that inhibition of DNA methyltransferase activity by aza-deoxycytidine in human non-small cell lung cancer cells (NSCLCs) results in up-regulation of L1TD1 [2]. Our interest in L1TD1 was further fueled by reports on a potential function of L1TD1 as prognostic tumor marker. We have included this information in the last paragraph of the Introduction in the revised manuscript.

Author response image 1. RT-qPCR of L1TD1 expression in cultured murine control and *Dnmt1* Δ/Δker keratinocytes. mRNA levels of *L1td1* were analyzed in keratinocytes isolated at P5 from conditional *Dnmt1* knockout mice [1]. *Hprt* expression was used for normalization of mRNA levels and wildtype control was set to 1. Data represent means ±s.d. with n=4. **P < 0.01 (paired t-test).

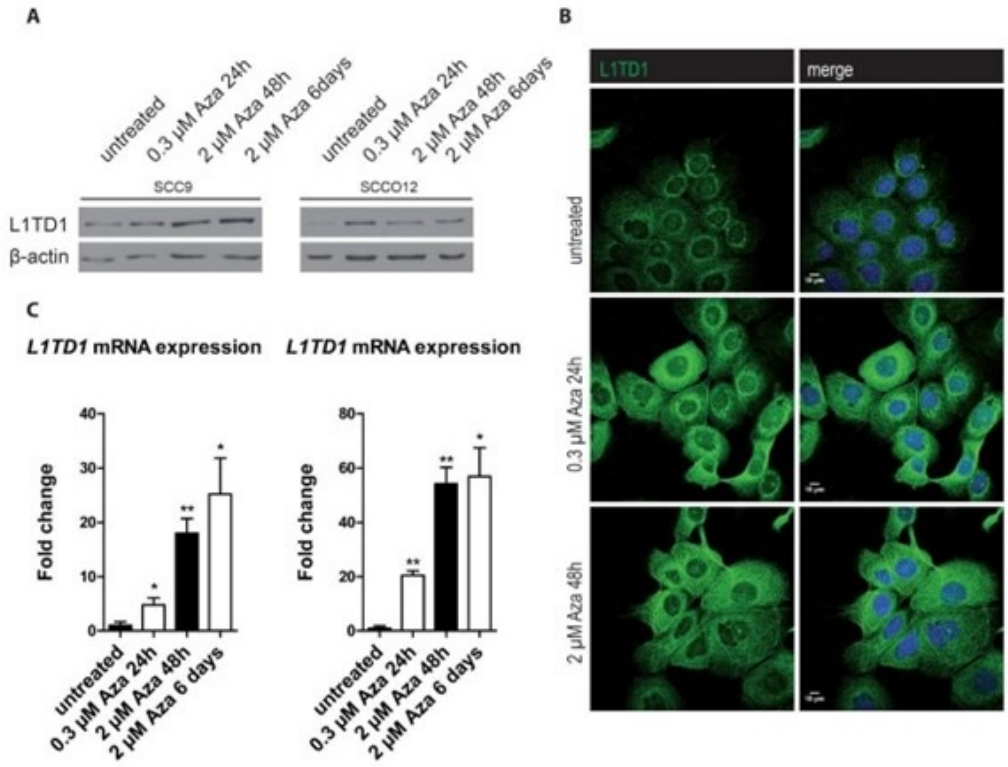


Author response image 2. RT-qPCR analysis of L1TD1 expression in primary human keratinocytes. Cells were treated with 5-aza-2-deoxycidine for 24 hours or 48 hours, with PBS for 48 hours or were left untreated. *18S rRNA* expression was used for normalization of mRNA levels and PBS control was set to 1. Data represent means ±s.d. with n=3. **P < 0.01 (paired t-test).



Author response image 3. Induced L1TD1 expression upon DNMT inhibition in squamous cell carcinoma cell lines SCC9 and SCCO12. Cells were treated with 5-aza-2-deoxycidine for 24

hours, 48 hours or 6 days. (A) Western blot analysis of L1TD1 protein levels using beta-actin as loading control. (B) Indirect immunofluorescence microscopy analysis of L1TD1 expression in SCC9 cells. Nuclear DNA was stained with DAPI. Scale bar: 10 µm. (C) RT-qPCR analysis of *L1TD1* expression in primary human keratinocytes. Cells were treated with 5-aza-2deoxycidine for 24 hours or 48 hours, with PBS for 48 hours or were left untreated. *18S rRNA* expression was used for normalization of mRNA levels and PBS control was set to 1. Data represent means ±s.d. with n=3. *P < 0.05, **P < 0.01 (paired t-test).



> *The finding that L1TD1/DNMT1 DKO cells exhibit increased apoptosis and DNA damage but decreased L1 retro-transposition is unexpected. Considering the DNA damage associated with retro-transposition and the DNA damage and apoptosis observed in L1TD1/DNMT1 DKO cells, one would anticipate the opposite outcome. Could it be that the observation of fewer transposition-positive colonies stems from the demise of the most transposition-positive colonies? Further exploration of this phenomenon would be intriguing.*

This is an important point and we were aware of this potential problem. Therefore, we calibrated the retrotransposition assay by transfection with a blasticidin resistance gene vector to take into account potential differences in cell viability and blasticidin sensitivity. Thus, the observed reduction in L1 retrotransposition efficiency is not an indirect effect of reduced cell viability. We have added a corresponding clarification in the Results section on page 8, last paragraph.

Based on previous studies with hESCs and germ cell tumors [3], it is likely that, in addition to its role in retrotransposition, L1TD1 has further functions in the regulation of cell proliferation and differentiation. L1TD1 might therefore attenuate the effect of DNMT1 loss in KO cells generating an intermediate phenotype (as pointed out by Reviewer 2) and simultaneous loss of both L1TD1 and DNMT1 results in more pronounced effects on cell viability. This is in agreement with the observation that a subset of L1TD1 associated

transcripts encode proteins involved in the control of cell division and cell cycle. It is possible that subtle changes in the expression of these protein that were not detected in our mass spectrometry approach contribute to the antiproliferative effect of L1TD1 depletion as discussed in the Discussion section of the revised manuscript.

> ***Reviewer #2 (Public Review):***
>
> *In this study, Kavaklıoğlu et al. investigated and presented evidence for the role of domesticated transposon protein L1TD1 in enabling its ancestral relative, L1 ORF1p, to retrotranspose in HAP1 human tumor cells. The authors provided insight into the molecular function of L1TD1 and shed some clarifying light on previous studies that showed somewhat contradictory outcomes surrounding L1TD1 expression. Here, L1TD1 expression was correlated with L1 activation in a hypomethylation-dependent manner, due to DNMT1 deletion in the HAP1 cell line. The authors then identified L1TD1-associated RNAs using RIP-Seq, which displays a disconnect between transcript and protein abundance (via Tandem Mass Tag multiplex mass spectrometry analysis). The one exception was for L1TD1 itself, which is consistent with a model in which the RNA transcripts associated with L1TD1 are not directly regulated at the translation level. Instead, the authors found the L1TD1 protein associated with L1-RNPs, and this interaction is associated with increased L1 retrotransposition, at least in the contexts of HAP1 cells. Overall, these results support a model in which L1TD1 is restrained by DNA methylation, but in the absence of this repressive mark, L1TD1 is expressed and collaborates with L1 ORF1p (either directly or through interaction with L1 RNA, which remains unclear based on current results), leads to enhances L1 retrotransposition. These results establish the feasibility of this relationship existing in vivo in either development, disease, or both.*
>
> ***Recommendations for the authors:***
>
> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Major*
>
> *(1) The study only used one knockout (KO) cell line generated by CRISPR/Cas9. Considering the possibility of an off-target effect, I suggest the authors attempt one or both of these suggestions.*
>
> *A) Generate or acquire a similar DMNT1 deletion that uses distinct sgRNAs, so that the likelihood of off-targets is negligible. A few simple experiments such as qRT-PCR would be sufficient to suggest the same phenotype.*
>
> *B) Confirm the DNMT1 depletion also by siRNA/ASO KD to phenocopy the KO effect. (2) In addition to the strategies to demonstrate reproducibility, a rescue experiment restoring DNMT1 to the KO or KD cells would be more convincing. (Partial rescue would suffice in this case, as exact endogenous expression levels may be hard to replicate).*

We have undertook several approaches to study the effect of DNMT1 loss or inactivation: As described above, we have generated a conditional KO mouse with ablation of DNMT1 in the epidermis. DNMT1-deficient keratinocytes isolated from these mice show a significant increase in L1TD1 expression. In addition, treatment of primary human keratinocytes and two squamous cell carcinoma cell lines with the DNMT inhibitor aza-deoxycytidine led to upregulation of L1TD1 expression. Thus, the derepression of L1TD1 upon loss of DNMT1 expression or activity is not a clonal effect. Also, the spectrum of RNAs identified in RIP experiments as L1TD1-associated transcripts in HAP1 DNMT1 KO cells showed a strong overlap with the RNAs isolated by a related yet different method in human embryonic stem

cells. When it comes to the effect of L1TD1 on L1-1 retrotranspostion, a recent study has reported a similar effect of L1TD1 upon overexpression in HeLa cells [4].

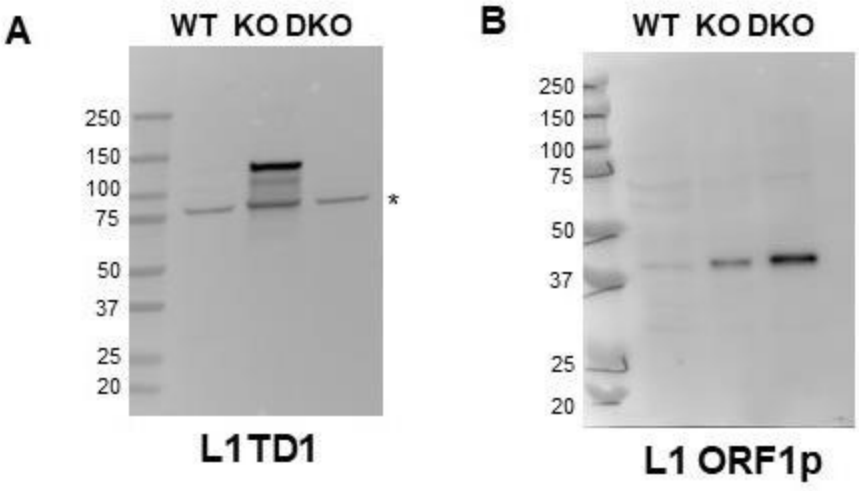
All of these points together help to convince us that our findings with HAP1 DNMT KO are in agreement with results obtained in various other cell systems and are therefore not due to off-target effects. With that in mind, we would pursue the suggestion of Reviewer 1 to analyze the effects of DNA hypomethylation upon DNMT1 ablation.

> *(3) As stated in the introduction, L1TD1 and ORF1p share "sequence resemblance" (Martin 2006). Is the L1TD1 antibody specific or do we see L1 ORF1p if Fig 1C were uncropped? (6) Is it possible the L1TD1 antibody binds L1 ORF1p? This could make Figure 2D somewhat difficult to interpret. Some validation of the specificity of the L1TD1 antibody would remove this concern (see minor concern below).*

This is a relevant question. We are convinced that the L1TD1 antibody does not crossreact with L1 ORF1p for the following reasons: Firstly, the antibody does not recognize L1 ORF1p (40 kDa) in the uncropped Western blot for Figure 1C (Author response image 4A). Secondly, the L1TD1 antibody gives only background signals in DKO cells in the indirect immunofluorescence experiment shown in Figure 1E of the manuscript.

Thirdly, the immunogene sequence of L1TD1 that determines the specificity of the antibody was checked in the antibody data sheet from Sigma Aldrich. The corresponding epitope is not present in the L1 ORF1p sequence. Finally, we have shown that the ORF1p antibody does not cross-react with L1TD1 (Author response image 4B).

Author response image 4. (A) Uncropped L1TD1 Western blot shown in Figure 1C. An unspecific band is indicated by an asterisk. (B) Westernblot analysis of WT, KO and DKO cells with L1 ORF1p antibody.



> *(4) In abstract (P2), the authors mentioned that L1TD1 works as an RNA chaperone, but in the result section (P13), they showed that L1TD1 associates with L1 ORF1p in an RNAindependent manner. Those conclusions appear contradictory. Clarification or revision is required.*

Our findings that both proteins bind L1 RNA, and that L1TD1 interacts with ORF1p are compatible with a scenario where L1TD1/ORF1p heteromultimers bind to L1 RNA. The

additional presence of L1TD1 might thereby enhance the RNA chaperone function of ORF1p. This model is visualized now in Suppl. Figure S7C.

> *(5) Figure 2C fold enrichment for L1TD1 and ARMC1 is a bit difficult to fully appreciate. A 100 to 200-fold enrichment does not seem physiological. This appears to be a "divide by zero" type of result, as the CT for these genes was likely near 40 or undetectable. Another qRT-PCRbased approach (absolute quantification) would be a more revealing experiment.*

This is the validation of the RIP experiments and the presentation mode is specifically developed for quantification of RIP assays (Sigma Aldrich RIP-qRT-PCR: Data Analysis Calculation Shell). The unspecific binding of the transcript in the absence of L1TD1 in DNMT1/L1TD1 DKO cells is set to 1 and the value in KO cells represents the specific binding relative the unspecific binding. The calculation also corrects for potential differences in the abundance of the respective transcript in the two cell lines. This is not a physiological value but the quantification of specific binding of transcripts to L1TD1. GAPDH as negative control shows no enrichment, whereas specifically associated transcripts show strong enrichement. We have explained the details of RIPqRT-PCR evaluation in Materials and Methods (page 14) and the legend of Figure 2C in the revised manuscript.

> *(6) Is it possible the L1TD1 antibody binds L1 ORF1p? This could make Figure 2D somewhat difficult to interpret. Some validation of the specificity of the L1TD1 antibody would remove this concern (see minor concern below).*

See response to (3).

> *(7) Figure S4A and S4B: There appear to be a few unusual aspects of these figures that should be pointed out and addressed. First, there doesn't seem to be any ORF1p in the Input (if there is, the exposure is too low). Second, there might be some L1TD1 in the DKO (lane 2) and lane 3. This could be non-specific, but the size is concerning. Overexposure would help see this.*

The ORF1p IP gives rise to strong ORF1p signals in the immunoprecipitated complexes even after short exposure. Under these contions ORF1p is hardly detectable in the input. Regarding the faint band in DKO HAP1 cells, this might be due to a technical problem during Western blot loading. Therefore, the input samples were loaded again on a Western blot and analyzed for the presence of ORF1p, L1TD1 and beta-actin (as loading control) and shown as separate panel in Suppl. Figure S4A.

> *(8) Figure S4C: This is related to our previous concerns involving antibody cross-reactivity. Figure 3E partially addresses this, where it looks like the L1TD1 "speckles" outnumber the ORF1p puncta, but overlap with all of them. This might be consistent with the antibody crossreacting. The western blot (Figure 3C) suggests an upregulation of ORF1p by at least 2-3x in the DKO, but the IF image in 3E is hard to tell if this is the case (slightly more signal, but fewer foci). Can you return to the images and confirm the contrast are comparable? Can you massively overexpose the red channel in 3E to see if there is residual overlap?*

In Figure 3E the L1TD1 antibody gives no signal in DNMT1/L1TD1 DKO cells confirming that it does not recognize ORF1p. In agreement with the Western blot in Figure 3C the L1 ORF1p signal in Figure 3E is stronger in DKO cells. In DNMT1 KO cells the L1 ORF1p antibody does not recognize all L1TD1 speckles. This result is in agreement with the Western blot shown above in Figure R4B and indicates that the L1 ORF1p antibody does not recognize the L1TD1

protein. The contrast is comparable and after overexposure there are still L1TD1 specific speckles. This might be due to differences in abundance of the two proteins.

> *(9) The choice of ARMC1 and YY2 is unclear. What are the criteria for the selection?*

ARMC1 was one of the top hits in a pilot RIP-seq experiment (IP versus input and IP versus IgG IP). In the actual RIP-seq experiment with DKO HAP1 cells instead of IgG IP as a negative control, we found ARMC1 as an enriched hit, although it was not among the top 5 hits. The results from the 2nd RIP-seq further confirmed the validity of ARMC1 as an L1TD1-interacting transcript. YY2 was of potential biological relevance as an L1TD1 target due to the fact that it is a processed pseudogene originating from YY1 mRNA as a result of retrotransposition. This is mentioned on page 6 of the revised manuscript.

> *(10) (P16) L1 is the only protein-coding transposon that is active in humans. This is perhaps too generalized of a statement as written. Other examples are readily found in the literature. Please clarify.*

We will tone down this statement in the revised manuscript.

> *(11) In both the abstract and last sentence in the discussion section (P17), embryogenesis is mentioned, but this is not addressed at all in the manuscript. Please refrain from implying normal biological functions based on the results of this study unless appropriate samples are used to support them.*

Much of the published data on L1TD1 function are related to embryonic stem cells [3-7]. Therefore, it is important to discuss our findings in the context of previous reports.

> *(12) Figure 3E: The format of Figures 1A and 3E are internally inconsistent. Please present similar data/images in a cohesive way throughout the manuscript.*

We show now consistent IF Figures in the revised manuscript.

> *Minor:*
>
> *(1) Intro:*
>
> *- Is L1Td1 in mice and Humans? How "conserved" is it and does this suggest function?*

Murine and human L1TD1 proteins share 44% identity on the amino acid level and it was suggested that the corresponding genes were under positive selection during evolution with functions in transposon control and maintenance of pluripotency [8].

> *- Why HAP1? (Haploid?) The importance of this cell line is not clear.*

HAP1 is a nearly haploid human cancer cell line derived from the KBM-7 chronic myelogenous leukemia (CML) cell line [9, 10]. Due to its haploidy is perfectly suited and widely used for loss-of-function screens and gene editing. After gene editing cells can be used in the nearly haploid or in the diploid state. We usually perform all experiments with diploid HAP1 cell lines. Importantly, in contrast to other human tumor cell lines, this cell line tolerates ablation of DNMT1. We have included a corresponding explanation in the revised manuscript on page 5, first paragraph.

> *- Global methylation status in DNMT1 KO? (Methylations near L1 insertions, for example?)*

The HAP1 DNMT1 KO cell line with a 20 bp deletion in exon 4 used in our study was validated in the study by Smits *et al.* [11]. The authors report a significant reduction in overall DNA methylation. However, we are not aware of a DNA methylome study on this cell line. We show now data on the methylation of L1 elements in HAP1 cells and upon DNMT1 deletion in the revised manuscript in Suppl. Figure S1B.

> *(2) Figure 1:*
>
> *- Figure 1C. Why is LMNB used instead of Actin (Fig1D)?*

We show now beta-actin as loading control in the revised manuscript.

> *- Figure 1G shows increased Caspase 3 in KO, while the matching sentence in the result section skips over this. It might be more accurate to mention this and suggest that the single KO has perhaps an intermediate phenotype (Figure 1F shows a slight but not significant trend).*

We fully agree with the reviewer and have changed the sentence on page 6, 2nd paragraph accordingly.

> *- Would 96 hrs trend closer to significance? An interpretation is that L1TD1 loss could speed up this negative consequence.*

We thank the reviewer for the suggestion. We have performed a time course experiment with 6 biological replicas for each time point up to 96 hours and found significant changes in the viability upon loss of DNMT1 and again significant reduction in viability upon additional loss of L1TD1 (shown in Figure 1F). These data suggest that as expexted loss of DNMT1 leads to significant reduction viability and that additional ablation of L1TD1 further enhances this effect.

> *- What are the "stringent conditions" used to remove non-specific binders and artifacts (negative control subtraction?)*

Yes, we considered only hits from both analyses, L1TD1 IP in KO versus input and L1TD1 IP in KO versus L1TD1 IP in DKO. This is now explained in more detail in the revised manuscript on page 6, 3rd paragraph.

> *(3) Figure 2:*
>
> *- Figure 2A is a bit too small to read when printed.*

We have changed this in the revised manuscript.

> *- Since WT and DKO lack detectable L1TD1, would you expect any difference in RIP-Seq results between these two?*

Due to the lack of DNMT1 and the resulting DNA hypomethylation, DKO cells are more similar to KO cells than WT cells with respect to the expressed transcripts.

> *- Legend says selected dots are in green (it appears blue to me).*

We have changed this in the revised manuscript.

> *- Would you recover L1 ORF1p and its binding partners in the KO? (Is the antibody specific in the absence of L1TD1 or can it recognize L1?) I noticed an increase in ORF1p in the KO in Figure 3C.*

Thank you for the suggestion. Yes, L1 ORF1p shows slightly increased expression in the proteome analysis and we have marked the corresponding dot in the Volcano plot (Figure 3A).

> *- Should the figure panel reference near the (Rosspopoff & Trono) reference instead be Sup S1C as well? Otherwise, I don't think S1C is mentioned at all.*
>
> *- What are the red vs. green dots in 2D? Can you highlight ERV and ALU with different colors?*

We added the reference to Suppl. Figure S1C (now S3C) in the revised manuscript. In Figure 2D L1 elements are highlighted in green, ERV elements in yellow, and other associated transposon transcripts in red.

> *- Which L1 subfamily from Figure 2D is represented in the qRT-PCR in 2E "LINE-1"? Do the primers match a specific L1 subfamily? If so, which?*

We used primers specific for the human L1.2 subfamily.

> *- Pulling down SINE element transcripts makes some sense, as many insertions "borrow" L1 sequences for non-autonomous retro transposition, but can you speculate as to why ERVs are recovered? There should be essentially no overlap in sequence.*

In the L1TD1 evolution paper [8], a potential link between L1TD1 and ERV elements was discussed:

"Alternatively, L1TD1 in sigmodonts could play a role in genome defense against another element active in these genomes. Indeed, the sigmodontine rodents have a highly active family of ERVs, the mysTR elements [46]. Expansion of this family preceded the death of L1s, but these elements are very active, with 3500 to 7000 species-specific insertions in the L1-extinct species examined [47]. This recent ERV amplification in Sigmodontinae contrasts with the megabats (where L1TD1 has been lost in many species); there are apparently no highly active DNA or RNA elements in megabats [48]. If L1TD1 can suppress retroelements other than L1s, this could explain why the gene is retained in sigmodontine rodents but not in megabats."

Furthermore, Jin et al. report the binding of L1TD1 to repetitive sequences in transcripts [12]. It is possible that some of these sequences are also present in ERV RNAs.

> *- Is S2B a screenshot? (the red underline).*

No, it is a Powerpoint figure, and we have removed the red underline.

> *(4) Figure 3:*
>
> *- Text refers to Figure 3B as a western blot. Figure 3B shows a volcano plot. This is likely 3C but would still be out of order (3A>3C>3B referencing). I think this error is repeated in the last result section.*
>
> *- Figure and legends fail to mention what gene was used for ddCT method (actin, gapdh, etc.).*

> *- In general, the supplemental legends feel underwritten and could benefit from additional explanations. (Main figures are appropriate but please double-check that all statistical tests have been mentioned correctly).*

Thank you for pointing this out. We have corrected these errors in the revised manuscript.

> *(5) Discussion:*
>
> *-Aluy connection is interesting. Is there an "Alu retrotransposition reporter assay" to test whether L1TD1 enhances this as well?*

Thank you for the suggestion. There is indeed an Alu retrotransposition reporter assay reported be Dewannieux *et al.* [13]. The assay is based on a Neo selection marker. We have previously tested a Neo selection-based L1 retrotransposition reporter assay, but this system failed to properly work in HAP1 cells, therefore we switched to a blasticidinbased L1 retrotransposition reporter assay. A corresponding blasticidin-based Alu retrotransposition reporter assay might be interesting for future studies (mentioned in the Discussion, page 11 paragraph 4 of the revised manuscript.

> *(6) Material and Methods :*
>
> *- The number of typos in the materials and methods is too numerous to list. Instead, please refer to the next section that broadly describes the issues seen throughout the manuscript.*
>
> *Writing style*
>
> *(1) Keep a consistent style throughout the manuscript: for example, L1 or LINE-1 (also L1 ORF1p or LINE-1 ORF1p); per or "/"; knockout or knock-out; min or minute; 3 times or three times; media or medium. Additionally, as TE naming conventions are not uniform, it is important to maintain internal consistency so as to not accidentally establish an imprecise version.*
>
> *(2) There's a period between "et al" and the comma, and "et al." should be italic.*
>
> *(3) The authors should explain what the key jargon is when it is first used in the manuscript, such as "retrotransposon" and "retrotransposition".*
>
> *(4) The authors should show the full spelling of some acronyms when they use it for the first time, such as RNA Immunoprecipitation (RIP).*
>
> *(5) Use a space between numbers and alphabets, such as 5 µg.*
>
> *(6) 2.0 × 105 cells, that's not an "x".*
>
> *(7) Numbers in the reference section are lacking (hard to parse).*
>
> *(8) In general, there are a significant number of typos in this draft which at times becomes distracting. For example, (P3) Introduction: Yet, co-option of TEs thorough (not thorough, it should be through) evolution has created so-called domesticated genes beneficial to the gene network in a wide range of organisms. Please carefully revise the entire manuscript for these minor issues that collectively erode the quality of this submission.*

Thank you for pointing out these mistakes. We have corrected them in the revised manuscript. A native speaker from our research group has carefully checked the paper. In

summary, we have added Supplementary Figure S7C and have changed Figures 1C, 1E, 1F, 2A, 2D, 3A, 4B, S3A-D, S4B and S6A based on these comments.

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