# Differential control of retrovirus silencing in embryonic cells by proteasomal regulation of the ZFP809 retroviral repressor

Cheng Wang[a] and Stephen P. Goff[b,c,d,1]

[a]Department of Biological Sciences, Columbia University Medical Center, Columbia University, New York, NY 10032; [b]Department of Biochemistry and Molecular Biophysics, Columbia University Medical Center, Columbia University, New York, NY 10032; [c]Department of Microbiology and Immunology, Columbia University Medical Center, Columbia University, New York, NY 10032; and [d]Howard Hughes Medical Institute, Columbia University Medical Center, Columbia University, New York, NY 10032

Contributed by Stephen P. Goff, December 20, 2016 (sent for review July 15, 2016; reviewed by David Haussler and Frank J. Rauscher)

**Replication of the murine leukemia viruses is strongly suppressed in mouse embryonic stem (ES) cells. Proviral DNAs are formed normally but are then silenced by a large complex bound to DNA by the ES cell-specific zinc-finger protein ZFP809. We show here that ZFP809 expression is not regulated by transcription but rather by protein turnover: ZFP809 protein is stable in embryonic cells but highly unstable in differentiated cells. The protein is heavily modified by the accumulation of polyubiquitin chains in differentiated cells and stabilized by the proteasome inhibitor MG132. A short sequence of amino acids at the C terminus of ZFP809, including a single lysine residue (K391), is required for the rapid turnover of the protein. The silencing cofactor TRIM28 was found to promote the degradation of ZFP809 in differentiated cells. These findings suggest that the stem cell state is established not only by an unusual transcriptional profile but also by unusual regulation of protein levels through the proteasomal degradation pathway.**

embryonic stem cells | retrovirus | transcriptional repressor | proteasome

Transcription of retroviruses and retroelements in mammalian genomes is tightly controlled during embryonic development and in embryonic stem (ES) cells (1, 2). The complex transcriptional profile of these cells first came to light with the discovery that the replication of murine leukemia viruses (MLVs) is blocked in murine (ES) cells and other primitive stem cells (3–6). Infection of these cells by MLVs results in normal virus entry, reverse transcription of the viral RNA, and integration of viral DNA, but the proviral DNA is then transcriptionally silenced. A specific DNA sequence used to prime viral DNA synthesis, the primer binding site complementary to the 3′ portion of proline tRNA (PBSpro), is present on those viruses that are most strongly and rapidly silenced (7–10). A large protein complex, specifically expressed in ES cells, binds to the PBSpro sequence to mediate the transcriptional repression of proviral DNA (5, 9, 11, 12). This silencing complex contains the transcriptional repressor TRIM28, also known as KAP1 or TIF1β (6), and is tethered to the PBSpro DNA by ZFP809, a zinc-finger protein with sequence-specific DNA-binding activity (13). TRIM28 acts as a scaffold for a number of factors that mediate the silencing of the nearby DNA, including the NuRD histone deacetylase complex, histone H3K9 methyltransferase ESET, and heterochromatin protein 1 (14–17). TRIM28, a RING-domain protein, also plays an important role in the protein modification pathway. Like other members of the RING domain family, TRIM28 is an E3 ligase that mediates transfer of ubiquitin or the small ubiquitin-like modifier (SUMO) to target substrates (18, 19).

ZFP809 contains two domains, a KRAB box at the N terminus that is responsible for the interaction with TRIM28 and a zinc-finger domain containing seven zinc fingers that provide its sequence-specific DNA-binding activity (Fig. 1*A*). Whereas most of the components of the silencing complex are expressed ubiquitously, ZFP809 is expressed specifically in embryonic cells and not in differentiated cells (13, 20). ZFP809 is the key ES cell-specific component of the silencing complex. Ectopic expression of suitable forms of ZFP809 in differentiated cells is sufficient to induce the formation of the DNA-binding silencing complex and to establish the silencing in those cells (13), suggesting that the lack of restriction of MLVs in differentiated cells is due to the reduced amount of ZFP809 protein.

The basis of the cell type-specific expression of ZFP809 is not known. ES cells have unusual transcriptional profiles (21, 22), and a natural supposition would be that the ZFP809 gene is specifically transcribed in ES cells. However, the cell type-specific expression of proteins such as ZFP809 could be effected by regulation at any of several steps: at the level of transcription, posttranscriptional mRNA processing or stability, mRNA translation, or posttranslational protein modification or stability. To identify the mechanism of regulation of ZFP809, we have directly examined the levels of mRNA and the synthesis and stability of the protein. We find that the down-regulation of ZFP809 in differentiated cells is not due to the reduction of ZFP809 mRNA but to changes in protein stability. The full-length ZFP809 protein is relatively stable in ES cells but is degraded rapidly in differentiated cells. Mutagenesis and protein ubiquitinylation assays indicate that the degradation is mediated through the ubiquitin-dependent proteasomal pathway.

## Results

**Expression of mRNAs and Proteins of ZFP809 in Different Cell Types.** The ZFP809 gene is transcribed to form two mRNAs by alternative splicing: One mRNA encodes a full-length protein, and a second mRNA encodes a shorter, C-terminally truncated protein. Surprisingly, mRNA expression databases report that the levels of total ZFP809 mRNAs are roughly comparable among murine ES cells, induced pluripotent stem cells, and differentiated cells (23). ZFP809 mRNA levels were certainly

### Significance

**A key transcriptional silencer responsible for suppression of retroviral gene expression in embryonic stem cells is found to be regulated itself at the level of protein turnover, mediated by ubiquitinylation and proteasomal degradation. The implication is that the stem cell state is characterized not only by an unusual transcriptional profile but also by unusual posttranscriptional regulation.**

Author contributions: S.P.G. designed research; C.W. performed research; C.W. and S.P.G. analyzed data; and C.W. and S.P.G. wrote the paper.

Reviewers: D.H., University of California, Santa Cruz; and F.J.R., The Wistar Institute.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. Email: spg1@cumc.columbia.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620879114/-/DCSupplemental.

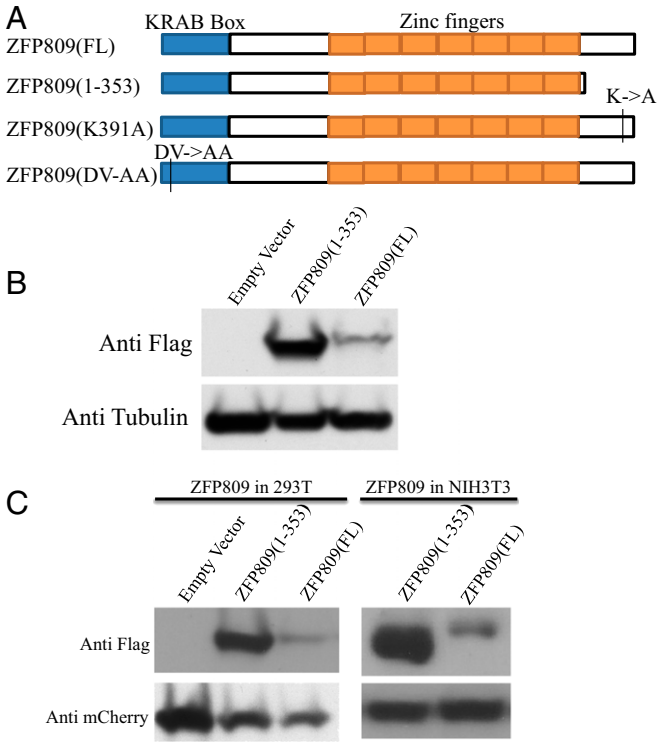


**Fig. 1.** Expression of ZFP809 proteins in different cell types. (*A*) Domain structure of the full-length form of ZFP809(FL) protein and various mutant constructs. KRAB box and zinc fingers are indicated. A C-terminally truncated form, ZFP809(1–353) is indicated, closely mimicking the natural short form. Mutants with a change of lysine residue near the C terminus to alanine, ZFP809(K391A), and change of aspartic acid and valine (DV) residues in the KRAB box to alanine, [ZFP809(DV-AA)], are indicated. (*B*) Western blot of proteins from 293T cells transfected with empty vector DNA or constructs expressing Flag-tagged versions of ZFP809(1–353) or ZFP809(FL). Blots were probed with anti-Flag or control anti-tubulin as indicated. (*C*) Western blot of 293T or NIH 3T3 cells transfected with empty vector or constructs encoding a single mRNA expressing both Flag-tagged ZFP809(1–353) or ZFP809(FL) and an IRES-driven mCherry. Blots were probed with anti-Flag or anti-mCherry antibodies as indicated.

not especially high in ES cells but rather in some experiments were even modestly lower in ES cells than in other tissues (24). Whole-genome RNA-sequence data indicated that the transcript encoding full-length ZFP809 is the major form in both embryonic cells and differentiated cells and that the levels of transcripts did not change significantly during differentiation (25) (Fig. S1).

To confirm these whole-genome expression data, we assessed the mRNA levels of ZFP809 in different cell types by reverse transcriptase (RT)-PCR. PCR primer pairs were designed to amplify RT DNAs only from the alternatively spliced full-length and short-form mRNAs or from all ZFP809 mRNAs (Fig. S2*A*). Analysis of the RT products by agarose gel electrophoresis revealed products of the expected sizes. E14 ES cells and NIH 3T3 cells contained roughly equal levels of total ZFP809 mRNAs, and of the mRNA encoding the full-length form of ZFP809, whereas the mRNA encoding the short form was barely detectable (Fig. S2*B*). Measurements by quantitative (q)RT-PCR demonstrated that the levels of total ZFP809 mRNAs, normalized to the mRNA levels of the housekeeping gene GAPDH, were not markedly different between ES cells (E14), embryonic carcinoma (EC) cells (F9), and fibroblast cells (NIH 3T3) (Fig. S2*C*). The findings are fully consistent with the results in the databases. These experiments suggest that ES cell-specific ZFP809 expression is not determined by differential transcription nor differential stability of the mRNAs between the cell types.

ZFP809 expression, in principle, might be regulated by post-transcriptional or translational regulatory elements. To test whether selected sequences of ZFP809 mRNA could regulate the expression of a reporter transcript, we subcloned 5′ UTR, 3′ UTR, and coding sequences of ZFP809 mRNA, and a scrambled control sequence, into a Renilla luciferase expression construct (Fig. S3*A*). Equal amounts of these DNAs, along with a control plasmid encoding firefly luciferase, were introduced into a human transformed cell line (293T), mouse fibroblast line (NIH 3T3), and mouse embryonic carcinoma cell line (F9), and 48 h later the levels of both luciferases in lysates were measured. Levels of Renilla luciferase were unaffected by any of the ZFP809 sequences (Fig. S3*B*), providing no indication of regulation through effects on mRNA stability or translation.

Several lines of evidence previously suggested that ZFP809 might be regulated at the level of protein turnover. Our initial efforts to establish cell lines stably expressing the full-length ZFP809(FL) were not successful (13); transformation of differentiated cells with DNA constructs expressing the full-length ZFP809 cDNA resulted in a marked reduction in the recovery of transformants and the isolation of cell lines expressing only very low levels of the protein. Screening large numbers of colonies arising after transformation yielded a few that expressed high levels of ZFP809, but these rare lines were found to have lost a 3′ portion of the cDNA during the course of transfection and expressed a C-terminally truncated form of the protein, closely mimicking the short form expressed from the alternatively spliced mRNA. Tests of a construct expressing a variant lacking the C-terminal 50 amino acids but retaining the KRAB box and zinc fingers [ZFP809(1–353) (13)] revealed that this form of the protein was readily expressed at high levels. We proposed that expression of the full-length protein was toxic to differentiated cells, but another possible explanation is that the full-length protein was unstable.

To confirm these earlier observations, we transfected 293T cells with equal amounts of DNAs encoding epitope Flag-tagged versions of the full-length ZFP809(FL) or the truncated ZFP809(1–353) and examined the levels of expressed proteins by Western blot. The levels of the full-length protein were dramatically lower than those of the truncated form (Fig. 1*B*). To rule out the possibility that the difference in expression was due to different transfection efficiency, both forms of ZFP809 cDNA were cloned into a vector expressing a single bicistronic mRNA encoding both ZFP809 and an mCherry reporter translated from an IRES element. 293T or NIH 3T3 cells were transfected with the DNAs, and the levels of the expressed proteins were assessed by Western blot. ZFP809(FL) was expressed at much lower levels than ZFP809(1–353) in both 293T and NIH 3T3 cells, whereas the levels of mCherry protein were approximately the same (Fig. 1*C*). These results suggest that the full-length ZFP809 protein is significantly down-regulated in differentiated cells by posttranslational mechanisms.

**Rapid Degradation of Full-Length ZFP809 Protein in Differentiated Cells.** To test whether the low levels of full-length ZFP809 protein in differentiated cells were due to the degradation of protein, measurements of the protein half-life were made. Flag-tagged ZFP809(FL) and ZFP809(1–353) were expressed by transfection of 293T cells, and after 24 h cycloheximide (CHX) was added to block translation (26). Cells were lysed at various times after the addition of CHX, and the levels of ZFP809 were assessed by Western blotting. The levels of ZFP809(FL) protein dropped rapidly upon inhibition of translation, whereas the levels of ZFP809(1–353) remained relatively constant (Fig. 2 *A* and *B*). These data suggest that the C-terminal 50 residues contained a degron sequence responsible for the rapid degradation of the

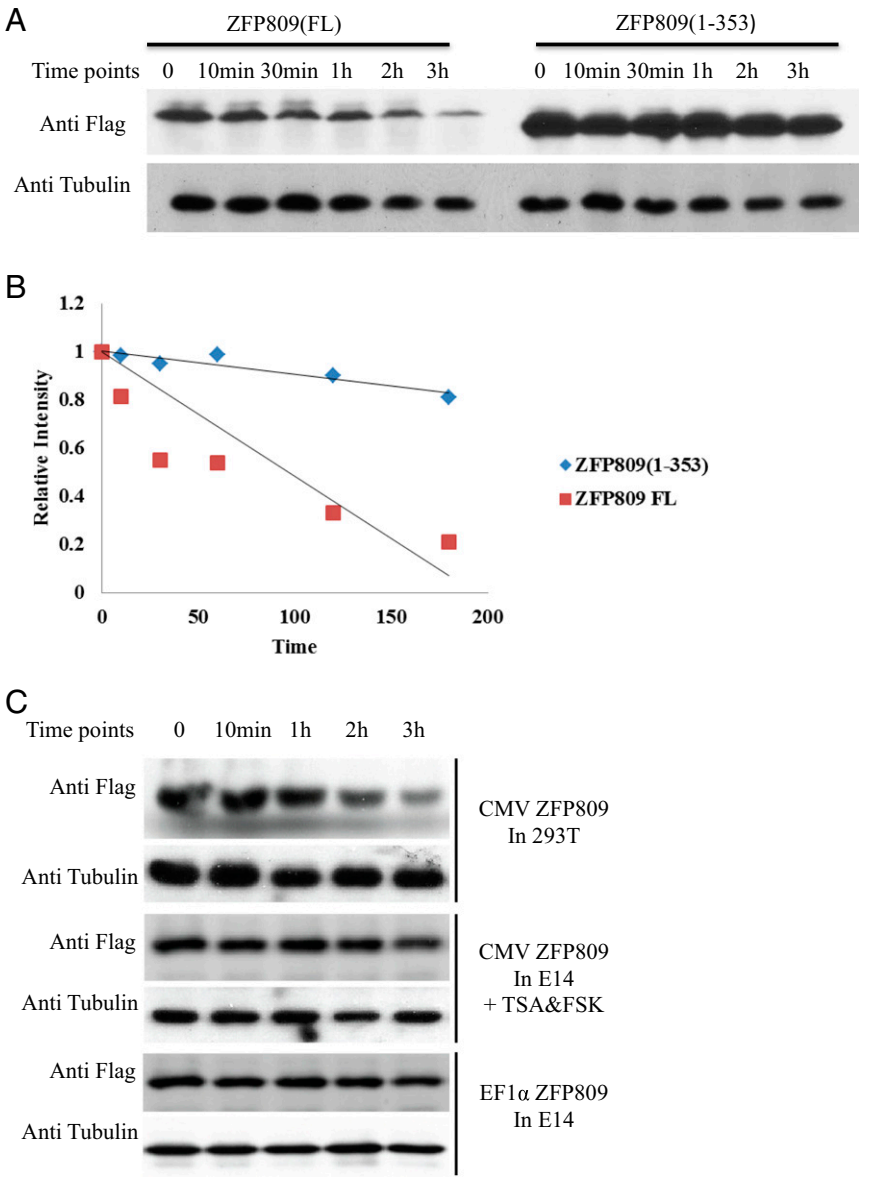


**Fig. 2.** Rapid degradation of full-length but not truncated ZFP809 in differentiated cells and not in stem cells. (*A*) 293T cells were transfected with plasmids expressing Flag-tagged versions of full-length ZFP809(FL) or truncated ZFP809(1–353). Twenty-four hours after transfection, CHX was added to stop translation. Cell lysates were collected 0, 10 min, 30 min, 1 h, 2 h, and 3 h after CHX addition, and ZFP809 levels were assessed by Western blotting and probed with anti-Flag or control anti-tubulin antibodies as indicated. Two exposures of the blot are shown. (*B*) Band intensities in *A* were quantified and are presented relative to the value at time 0. A decay curve for the ZFP809(FL) protein was calculated by the best fit to a linear regression. (*C*) E14 cells were transfected with plasmids expressing ZFP809 from the CMV promoter or EF1a promoter as indicated, CHX was added to stop translation, and lysates were taken at the indicated times and analyzed by Western blot probed with the indicated antibodies. E14 cells expressing proteins from the CMV promoter were treated with TSA and FSK for 4 h before addition of CHX to activate the promoter.

full-length protein in differentiated cells. We tested to see whether the fusion of the ZFP809 C terminus (C50) to reporter proteins could direct their degradation. 293T cells were transfected with constructs encoding EGFP or an EGFP–C50 fusion, or Renilla luciferase or a luciferase–C50 fusion, and the stability of the proteins after CHX arrest was measured by Western blots or luciferase assays. The addition of the ZFP809 C terminus had no effect on the lifetimes of the reporters, suggesting that the targeting of the C terminus is context-dependent and not recognized in the fusion proteins.

To examine the behavior of ZFP809 in ES cells, similar measurements of the protein half-life were made in E14 cells. Due to low transfection efficiency of mouse ES cells and rapid silencing of the CMV promoter in ES cells (27), however, the expression of ZFP809 driven by the CMV promoter was undetectable after transfection. Two approaches were taken to increase expression. One was to use the same CMV-driven construct but with addition of two drugs, trichostatin A (TSA) (28) and forskolin (FSK) (29), known to release the restriction of the CMV promoter in mouse ES cells. The other was to use the EF1α promoter to drive ZFP809 expression (30). In both cases, the full-length ZFP809 protein was expressed well in E14 ES cells and, most importantly, after CHX addition the protein was very stable (Fig. 2*C*). These results indicate that ZFP809 is degraded rapidly in differentiated cells and stable in ES cells.

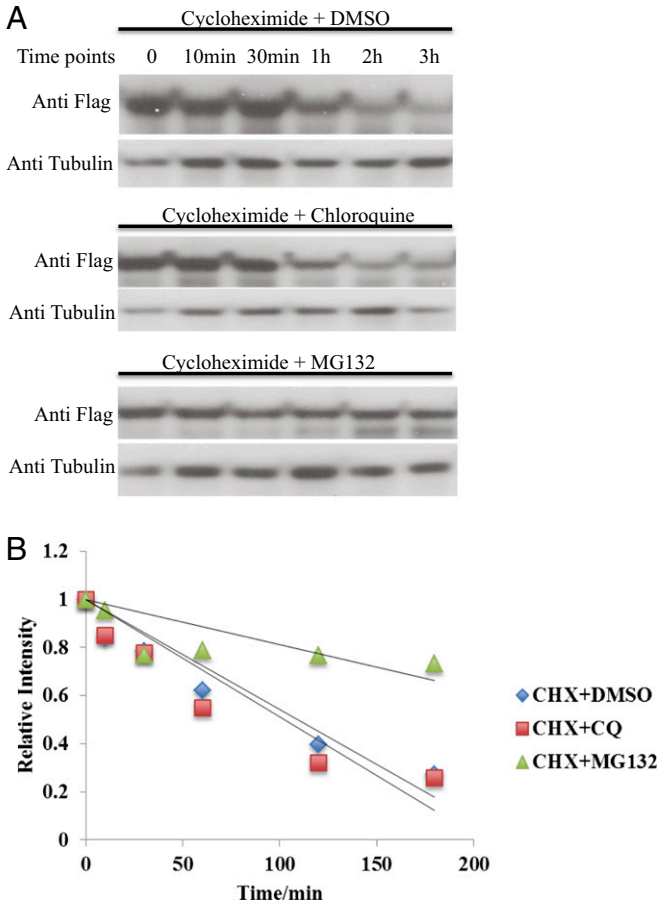


**Fig. 3.** ZFP809 stability in the presence of inhibitors of protein degradation. (*A*) Western blot of proteins from 293T cells transfected with a plasmid expressing Flag-tagged ZFP809. Forty-eight hours after transfection, cells were treated with CHX to stop translation, along with the inhibitors chloroquine or MG132 or vehicle DMSO control, and samples were taken at the indicated times. Blots were probed with anti-Flag or control anti-tubulin as indicated. (*B*) The intensity of each band in *A* was measured. Relative intensity was calculated by dividing the intensity of each band by the intensity of the band at time 0.

**Ubiquitin-Dependent Proteasomal Degradation of ZFP809.** Mammalian cells use two major systems for the intracellular degradation of proteins: the lysosomal and proteasomal pathways (31, 32). Chloroquine (CQ) is a lysosomotropic agent that inhibits lysosomal protein degradation (33), whereas MG132 is a potent, membrane-permeable proteasome inhibitor (34). To determine the route of ZFP809 degradation in differentiated cells, 293T cells expressing ZFP809 were treated with CHX to arrest translation as before, and MG132, CQ, or vehicle control (DMSO) was added and the levels of ZFP809 were monitored at different time points by Western blot. MG132, but not CQ or the vehicle DMSO, dramatically stabilized the full-length ZFP809 protein (Fig. 3). MG132 also stabilized ZFP809 in the rodent fibroblast Rat2 cell line (Fig. S4). These observations suggest that the full-length ZFP809 is rapidly turned over in many differentiated cells by proteasomal degradation.

Proteasomal degradation of substrate proteins can be either ubiquitin-dependent or ubiquitin-independent (35, 36). Proteins undergoing ubiquitin-mediated degradation are modified by the serial addition of multiple copies of ubiquitin, which form a ladder of bands when displayed on SDS gels (37). To test for ubiquitinylation of ZFP809, 293T cells were transfected with plasmids expressing His-tagged ubiquitin (38) and Flag-tagged ZFP809 or p53 (39). Twenty-four hours after transfection, MG132 was added for 4 h to stop degradation and allow accumulation of ubiquitinylated proteins. Lysates were prepared and ubiquitinylated proteins were isolated by binding to and elution from $Ni^{2+}$-NTA beads. Analysis of the eluted proteins by Western blot showed that both full-length ZFP809 and p53 were heavily ubiquitinylated (Fig. 4*A*).

The ubiquitin-dependent pathway involves the covalent linkage of a polyubiquitin chain to a lysine residue of the substrate (40). The observation that the full-length ZFP809 is degraded, and the C-terminally truncated ZFP809(1–353) is not (Fig. 2*A*), suggests that the C terminus might contain a site for polyubiquitinylation. This region contains a single lysine residue, K391. To test for the importance of this residue in ZFP809 turnover, we generated a construct expressing a mutant protein with this single lysine residue changed to alanine (K391A). Tests of protein half-life showed that ZFP809(K391A) was significantly more stable

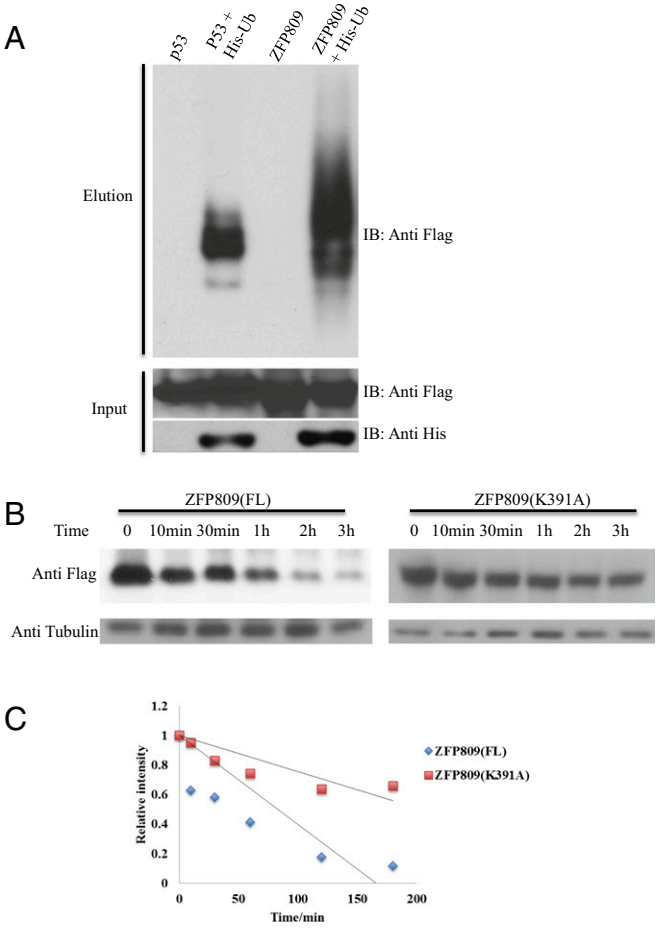


**Fig. 4.** Ubiquitin-dependent degradation of ZFP809. (*A*) In vivo ubiquitinylation assay. 293T cells were cotransfected with plasmids expressing Flag-tagged p53 or ZFP809(FL), with or without plasmid expressing His-tagged ubiquitin. Twenty-four hours after transfection, MG132 was added for 4 h to stop protein degradation and lysates were prepared. Lysates were incubated with $Ni^{2+}$-NTA agarose beads, and the bound His-tagged proteins were eluted and analyzed by Western blot, probed with anti-Flag antibodies. The total ZFP809 and ubiquitin protein levels in the lysates that were input into the binding reactions are shown below. IB, immunoblotting. (*B*) Western blot of proteins from 293T cells transfected with constructs expressing wild-type ZFP809 or mutant K391A. Cells were treated with CHX at time 0 and samples were taken at the indicated times. Blots were probed with anti-Flag or anti-tubulin antibodies as indicated. (*C*) Quantification of ZFP809 levels. The intensity of each band in *B* was measured, and the relative intensity was calculated as a fraction of the intensity of the band at time 0.

than the wild type in differentiated cells (Fig. 4 *B* and *C*). Further tests by selection for expression of a linked drug-resistance marker in transfected NIH 3T3 cells showed good expression of this mutant without toxicity, identical to the truncated ZFP809(1–353) and in marked contrast to the WT protein (data not shown).

**TRIM28 Promotes the Degradation of ZFP809.** The above results suggest that full-length ZFP809 protein in differentiated cells is a target for ubiquitin-dependent protein degradation. TRIM28, which interacts with ZFP809, is a RING domain-containing protein and known to serve as an E3 ligase for transfer of ubiquitin (41). RING domain proteins often bind to Cullins, a protein family serving as a scaffold for the assembly of a complex that mediates protein degradation (42). To test the role of TRIM28 in the degradation of ZFP809 in differentiated cells, we used siRNA to partially knock down TRIM28 expression in 293A cells (Fig. 5*A*).

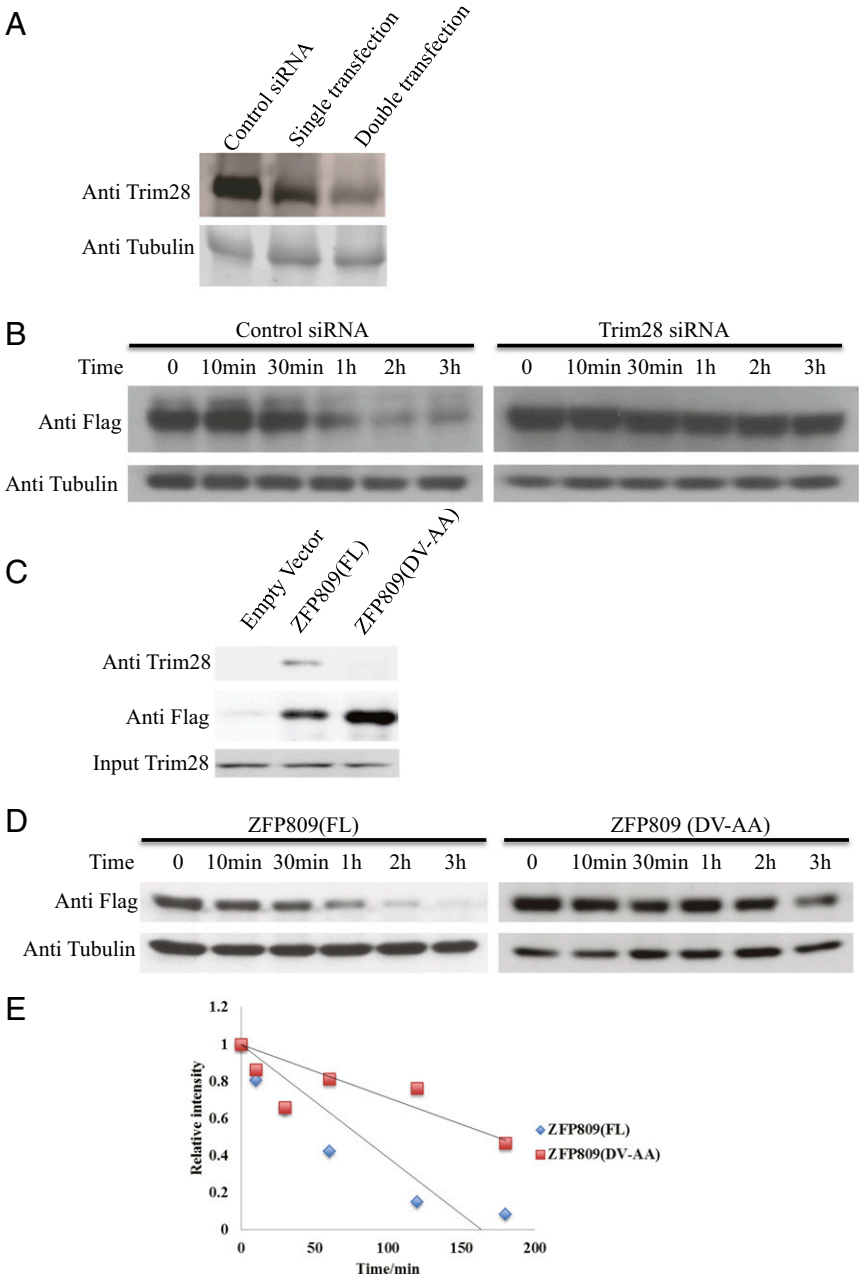


**Fig. 5.** TRIM28 promotes degradation of ZFP809. (*A*) Knockdown of TRIM28. 293A cells were transfected with control siRNA and TRIM28 siRNA twice. Lysates were prepared after each transfection and analyzed by immunoblotting with anti-TRIM28 and anti-tubulin antibodies. (*B*) Cells treated with control siRNA or TRIM28 siRNAs were transfected with DNA encoding Flag-tagged ZFP809, CHX was added to stop translation, and lysates were prepared at the indicated times and analyzed by Western blot. (*C*) Coimmunoprecipitation of TRIM28 with wild-type or mutant ZFP809 proteins. 293T cells were transfected with DNAs expressing Flag-tagged wild-type ZFP809(FL), mutant ZFP809(DV-AA), or empty vector, and after 24 h lysates were prepared. Anti-Flag magnetic beads were added to the cell lysates, and the bound proteins were recovered by elution, analyzed by Western blotting, and probed with anti-TRIM28 or anti-Flag antibodies. The total TRIM28 in the lysates input into the binding reactions is shown below. (*D*) Western blot of proteins from 293T cells transfected with constructs expressing wild-type ZFP809 or mutant ZFP809(DV-AA). Cells were treated with CHX at time 0 and samples were taken at the indicated times. Blots were probed with anti-Flag or anti-tubulin antibodies as indicated. (*E*) Quantification of ZFP809 levels. The intensity of each band in *D* was measured, and the relative intensity was calculated as a fraction of the intensity of the band at time 0.

Protein degradation assays showed that ZFP809(FL) was dramatically stabilized in TRIM28-depleted cells (Fig. 5*B*), suggesting that TRIM28 plays a role in ZFP809 turnover.

It has been previously shown that substitution of two conserved amino acids (DV) with alanine residues (AA) in the KRAB box of zinc-finger proteins abolishes the interaction between the KRAB box and TRIM28 (43). To investigate the importance of the ZFP809–TRIM28 interaction for ZFP809 degradation, we mutated the DV residues to AA in the KRAB box of ZFP809 to make the mutant ZFP809(DV-AA). 293T cells were transfected with DNAs expressing Flag-tagged versions of the wild type or ZFP809(DV-AA), and after 48 h lysates were prepared. The ZFP809 proteins were immunoprecipitated with anti-Flag antibodies, and their association with TRIM28 was assessed by Western blots probed with anti-TRIM28 antibodies. The wild-type ZFP809 but not ZFP809(DV-AA) bound TRIM28 (Fig. 5*C*), confirming that the mutation blocked the interaction between TRIM28 and ZFP809. To determine the effects on the stability of ZFP809, cells expressing the wild type and mutant were treated with CHX and the levels of ZFP809 were monitored by Western blot as before. Whereas the wild type showed the usual rapid decay, the mutant ZFP809(DV-AA) was degraded at a much slower rate (Fig. 5 *D* and *E*). These observations suggest that the interaction between ZFP809 and TRIM28 plays a significant role in the degradation of ZFP809.

**Mutations of the C Terminus of ZFP809 Result in Less Ubiquitinylation.** The ZFP809 mutant proteins ZFP809(K391A) and ZFP809(DV-AA) were relatively stable in differentiated cells, suggesting that the lysine residue K391 and the interaction between TRIM28 and ZFP809 are important for the turnover of ZFP809 (Figs. 4*B* and 5*D*). Full-length ZFP809 is heavily ubiquitinylated in differentiated cells (Fig. 4*A*). To assess the ubiquitinylation status of ZFP809 (FL) and mutants, assays for ubiquitinylation in vivo were performed with vectors expressing ZFP809(FL), ZFP809(DV-AA), ZFP809(K391A), and ZFP809(1–353). Western blotting showed that ZFP809(DV-AA) and ZFP809(K391A) were also polyubiquitinylated, but the ubiquitinylation level was markedly lower than that of ZFP809(FL), even though overall protein levels were much higher (Fig. 6). Furthermore, the truncated ZFP809(1–353) was mainly monoubiquitinylated rather than polyubiquitinylated (Fig. 6). Monoubiquitinylation is not a signal for protein degradation, consistent with the observation that ZFP809(1–353) was very stable in differentiated cells.

**Neddylation Inhibitor MLN4924 Stabilizes ZFP809.** TRIM28 is a RING domain-containing protein, a member of a large family of proteins that assemble with the Cullin proteins to form multisubunit Cullin–RING E3 ubiquitin ligase (CRL) complexes (44). Cullin proteins require activation by a specific covalent modification, the addition of Nedd4 (neddylation) (45). Neddylation of one or more subunits activates the CRL complexes and promotes their protein degradation activities (46). A small-molecule inhibitor, MLN4924, blocks the neddylation activity of Nedd8, which is required for the activation of Cullin proteins (47,48). To test whether neddylation is important for ZFP809 degradation, we examined the half-life of ZFP809 in the presence of MLN4924. Addition of MLN4924, and not the vehicle control DMSO, dramatically stabilized the full-length ZFP809 in differentiated cells (Fig. S5). This finding suggests that neddylation, and likely the CRL complex, are important for the degradation of ZFP809.

## Discussion

ES cells and other primitive cell types exhibit unusual mRNA profiles, and much of their specific patterns of gene expression can be attributed to unusual chromatin modifications and transcriptional activity of their genomic DNAs. However, these cells also exhibit unusual patterns of global protein modifications including ubiquitinylation (49), and indeed the very maintenance of their pluripotency requires unusual profiles of ubiquitinylation and proteasomal degradation (50–52) (for a review, see ref. 53). In this study, we found that the ZFP809 protein, the key component of a retroviral silencing complex, is regulated by the proteasomal protein degradation system.

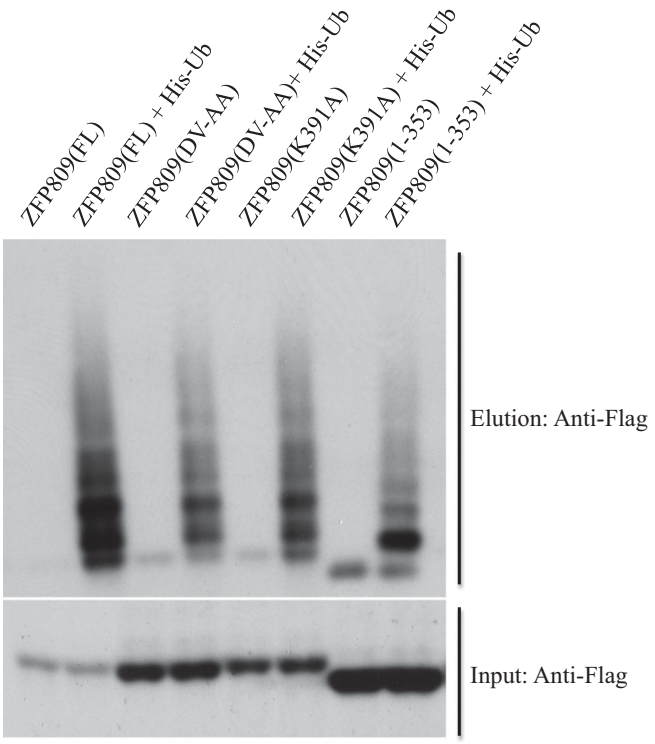


**Fig. 6.** Ubiquitinylation of wild-type and mutant forms of ZFP809. 293T cells were transfected with plasmids expressing ZFP809(FL), ZFP809(DV-AA), ZFP809(K391A), or ZFP809(1–353), either alone or with a plasmid expressing His-ubiquitin, as indicated. Twenty-four hours after transfection, MG132 was added to stop protein degradation for 4 h and lysates were prepared. Lysates were incubated with $Ni^{2+}$-NTA agarose beads, and the bound His-tagged proteins were eluted and analyzed by Western blot, probed with anti-Flag antibodies. The total ZFP809 protein levels in the lysates input into the binding reactions are shown below.

ZFP809 protein is expressed in ES cells but not in most differentiated cells, and is responsible for the rapid and efficient silencing of exogenous (13) and endogenous (20) retroviral DNAs in ES cells. Ectopic expression of stable forms of ZFP809 in murine or human differentiated cells is sufficient to establish silencing of MLV proviruses, suggesting that it is the limiting factor and that all other components of the silencing machinery are present in most cells. Surprisingly, the levels of ZFP809 mRNAs are comparable in ES cells and differentiated cells. We found that the full-length ZFP809 protein is subject to ubiquitin-mediated degradation in most cells but is uniquely stable in ES and EC cells. Thus, unlike many targets of ubiquitinylation and degradation in ES cells, ZFP809 is protected from or resistant to such regulatory events in these cells.

A curious feature of the ZFP809 gene is that its transcription results in the formation of two alternatively spliced mRNAs that encode two forms of the protein, a full-length and a C-terminally truncated form (Fig. 1*B*). Both mRNAs are expressed in ES and differentiated cells, with the mRNA for the full-length protein being by far the more abundant in both cases. Expression of cDNAs revealed that the full-length protein is the regulated form, selectively stable in ES cells and degraded elsewhere. The

C-terminal 50 residues, and lysine 391 in this sequence in particular, are required for degradation. It is unclear why the two forms are made; both forms of the protein retain the KRAB domain for interaction with TRIM28 and the zinc fingers required for DNA binding, and the short form seems to have full functionality in silencing. Why low levels of the truncated form would be expressed constitutively is not apparent.

Another surprising aspect of the system is the involvement of TRIM28 in the regulation of the levels of ZFP809. The major function of the interaction between ZFP809 and TRIM28 is to bring TRIM28 and all its associated factors to the target DNA to mediate the silencing through chromatin and DNA modification. The interaction would be expected to result in the stabilization of ZFP809. However, in differentiated cells, the interaction is actually involved in the destabilization of ZFP809 (Fig. 6*D*). The results suggest that TRIM28 is playing a major role in keeping ZFP809 levels low in differentiated cells. TRIM28 may be acting as the E3 ligase directing the ubiquitinylation of ZFP809, or it may be acting indirectly, but depleting TRIM28 or preventing the interaction with ZFP809 significantly reduces the rate of ZFP809 degradation (Fig. 6 *B* and *D*). Although TRIM28 binding to ZFP809 is needed, we do not yet know whether the E3 ligase activity of TRIM28 is also essential for its stimulated degradation of ZFP809. How its destabilizing activity is restricted to differentiated cells, and is prevented from functioning in ES cells, remains unclear. One intriguing possibility would be the cell type-specific SUMOylation of the critical K391 lysine, blocking its ubiquitinylation, which could toggle the cells between embryonic and differentiated states.

The addition of MG132 stabilizes ZFP809 (Fig. 5*B*) and results in the accumulation of heavily polyubiquitinylated protein (Fig. 5*A*), strongly suggesting regulation by ubiquitin-dependent proteasomal degradation. Tests of mutant forms of the protein for ubiquitin content suggest that eliminating the interaction with TRIM28 by mutation [in ZFP809(DV-AA)], removing lysine 391 by mutation (in K391A), or removing the C-terminal region [in ZFP809(1–353)] all reduce the levels of polyubiquitin many fold, although some of the modification remains (Fig. 6). The continued modification of K391A and the truncated ZFP809(1–353) indicates that other lysines in ZFP809 or even in the Flag tag are serving as secondary targets for ubiquitinylation when K391 is not present. The truncated protein seems to be no more than monoubiquitinylated (Fig. 6), and as such would not be recognized by the proteasome for degradation.

Many RING-finger proteins such as TRIM28 function as E3 ubiquitin ligases in concert with one of the Cullin proteins to target proteins for degradation. The Cullins in turn often require modification by addition of Nedd proteins (neddylation), for their functionality (46). The stabilization of ZFP809 by addition of the neddylation inhibitor MLN4924 suggests that a Cullin protein is likely involved in TRIM28-mediated degradation of ZFP809. It is not known which Cullin or Cullins are involved.

ZFP809 is not the only protein involved in MLV silencing that is tightly regulated in ES cells, and indeed the levels and activity of TRIM28 are also subject to complex regulation. The PHD domain of TRIM28 functions as an intramolecular E3 ligase that SUMOylates the adjacent TRIM28 bromodomain, and this modification is required to recruit the SETDB1 histone methylase to effect transcriptional silencing (54). Phosphorylation of TRIM28 is also required for its function in transcription repression (55). The active TRIM28, once phosphorylated and SUMOylated, has a shorter half-life and is targeted for degradation by RNF4 (56). This pattern of behaviors suggests that after silencing a specific chromatin region, TRIM28 function for that region is subsequently turned off. The continued presence of TRIM28 may not be required to maintain the silencing of MLVs in stem cells. Once silenced, the proviruses presumably cannot be activated during differentiation because the restriction complex has already placed many silencing marks on the associated chromatin, or on the proviral DNA (57).

Retroviral DNAs are subject to profound regulation in ES cells and during their differentiation. This may be in large part to prevent genotoxic expression of exogenous and endogenous proviruses, but it is also likely a reflection of the fact that viral transcriptional elements have been co-opted by the host for the regulation of cellular genes important for maintenance of the pluripotent state and for appropriately timed gene expression during their differentiation (58, 59). Although much of this regulation is at the level of transcription, the observations of ZFP809 here suggest that some of this regulation may be controlled at a higher level by protein turnover. A full characterization of "stemness"—the pluripotent state—will thus likely require a detailed knowledge of the ubiquitin ligase substrates, and more generally the machinery that adds and reads out posttranslational protein modifications.

## Materials and Methods

**Cell Culture and Transfection.** The 293A cell line (human embryonic kidney cells), 293T cell line [human embryonic kidney cells expressing simian virus 40 (SV40) large T antigen], NIH 3T3 cells (mouse fibroblast cell line), MEFs (mouse embryonic fibroblasts), F9 cells (an embryonic carcinoma cell line), and Rat2 cells (a rat fibroblast cell line) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. E14 cells, a mouse ES cell line, were cultured on a layer of MEF cells in DMEM with 20% (vol/vol) FBS, supplemented with nonessential amino acids, 2 μM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1,000 U/mL leukemia inhibitory factor (Stem Cell Technologies). E14 cells were transferred to plates coated with 0.1% gelatin for transfections. DNAs were introduced into cells by transfection using polyethylenimine reagent (60) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. All cells were cultured at 37 °C in 5% $CO_2$.

**Plasmids.** The plasmids pcDNA3.1-3X-Flag-ZFP809(FL) and -ZFP809(1–353) were as described (13). The coding regions from these plasmids were transferred into the pLVX IRES mCherry vector using SnaBI and XhoI restriction sites. The 5′ UTR, cDNA, and 3′ UTR sequences of ZFP809 mRNA were inserted after the stop codon of the Renilla gene ORF in the pcDNA3.1 plasmid expressing Renilla luciferase to test for effects on reporter expression. Plasmids pcDNA3-Flag-p53 and pCI-His-ubiquitin were purchased from Addgene. Mutations of ZFP809 changing lysine residue 391 to alanine (K391A), or the aminoproximal aspartic acid and valine to alanines (D8V9 to AA), were introduced using overlap extension PCR (61) and SLIC cloning (62).

**Analysis of ZFP809 mRNA Levels.** E14, F9, and NIH 3T3 cells were harvested and total RNA was extracted using TRIzol reagent (Invitrogen). Four micrograms of total RNA per cell line was used to produce cDNA using random hexamers and a SuperScript III Kit (Invitrogen). Two microliters of each cDNA was used for quantitative PCR analysis of ZFP809 and GAPDH transcript levels. The expression of ZFP809 was normalized to the level of GAPDH. One microgram of each cloned cDNA was used for PCR, with primers detecting generic or different transcripts of ZFP809 or GAPDH as a control. Amplified DNA was displayed by agarose gel electrophoresis and stained by ethidium bromide.

The PCR primers are as follows. qRT-PCR primers: GAPDH-F (forward), 5′-ACC TTT AGC CTT GCC CTT T-3′; GAPDH-R (reverse), 5′-ACA TCA CCC CCA TCA CTC AT-3′; ZFP809-F, 5′-TGGTGGCGGAGACCGAGTCA-3′; ZFP809-R, 5′-TCCTCTGAG-CAGCGTCCAGGT-3′. PCR primers for generic or different isoforms: 809 exon 4/5, 5′-GACACGATTGAAGCAGAACTAAAG-3′; 809 generic, 5′-ATTGAGGTGAGACTT-GCGGA-3′; 809-short1, 5′-TCAGCTATCCCCATACTGACCT-3′; 809-short2, 5′-TCCC-CATACTGACCTGTGTGT-3′; 809-long1, 5′-TCAGAGCCCACCCCCAAT-3′; 809-long2, 5′-CCATATGCCCAAGTCCATGT-3′.

**Measurements of Protein Stability.** In various experiments, cells were incubated with inhibitors of translation (CHX, at 50 μg/mL) (26), the proteasome (MG132, at 10 μM) (63), lysosome functionality (CQ, at 100 μM) (64), and neddylation (MLN4924, at 1 mM) (47). ES cell cultures were treated with TSA (100 ng/mL) and FSK (10 μM) to release the silencing of the CMV promoter (28, 29). To monitor the rate of protein degradation, CHX was added to the cultures and cells were lysed by addition of Laemmli SDS sample buffer at 0 min, 10 min, 30 min, 1 h, 2 h, and 3 h after translation arrest.

Samples were analyzed by SDS/PAGE and immunoblotting with anti-Flag antibody to determine the levels of ZFP809 proteins.

**In Vivo Ubiquitinylation Assay.** 293T cells were transfected in 6-cm plates with 2 μg of the indicated expression plasmid alone or along with 4 μg of plasmid expressing His-tagged ubiquitin. Twenty-four hours after transfection, MG132 was added to stop the protein degradation for 4 h. Cells were lysed in buffer A (6 M guanidinium·HCl, 0.1 M $Na_2HPO_4/NaH_2PO_4$, 0.01 M Tris·HCl, pH 8.0, 5 mM imidazole, 10 mM β-mercaptoethanol) and the lysates were incubated with $Ni^{2+}$-NTA beads (Qiagen) for 4 h at room temperature. The beads were washed with buffer A, buffer B (8 M urea, 0.1 M $Na_2HPO_4/NaH_2PO_4$, 0.01 M Tris·HCl, pH 8.0, 10 mM β-mercaptoethanol), and buffer C (8 M urea, 0.1 M $Na_2HPO_4/NaH_2PO_4$, 0.01 M Tris·HCl, pH 6.3, 10 mM β-mercaptoethanol), and the bound proteins were eluted with Laemmli buffer (modified as in ref. 65). Samples were analyzed by Western blotting with anti-Flag antibody (Sigma).

**Western Blotting.** Proteins were resolved by SDS/PAGE. After transfer to nitrocellulose membranes, the blots were probed with mouse anti-Flag monoclonal antibody (Sigma), mouse anti-mCherry monoclonal antibody (Abcam), mouse anti-TRIM28 monoclonal antibody (Abcam), mouse anti-tubulin monoclonal antibody (Abcam), or mouse anti-beta actin monoclonal antibody. Secondary antibodies were ECL-based anti-mouse IgG and horseradish peroxidase-linked whole antibody (GE Healthcare). Membranes were developed using an ECL Kit (Life Technologies).

**Luciferase Assay.** 293T cells seeded in a six-well plate were transfected with pcDNA3-Rluc plasmid encoding Renilla luciferase and derivative plasmids with inserted ZFP809 mRNA sequence elements. Transfection was carried out using a polyethylenimine protocol in which the constructs were added at a ratio of 3 (Renilla luciferase) to 1 (firefly luciferase, serving as transfection efficiency control). Cells were lysed 48 h posttransfection with Passive Lysis Buffer (Promega), and luciferase activity was measured using a Dual-Glo Luciferase Assay System (Promega) in a Veritas Microplate Luminometer. Renilla luciferase data were normalized to firefly luciferase readings in each well.

**Knockdown of TRIM28.** For the transient knockdown of TRIM28, 293A cells were transfected with siRNA specifically targeting TRIM28 or a nontargeting siRNA (Thermo Scientific). Cells were transfected with Lipofectamine 2000 (Invitrogen) using a 2-d transfection protocol followed by 24 or 48 h of rest to gain maximum knockdown efficiency (66). Cells were collected following the resting period and analyzed by Western blotting.

**Protein Interaction Assay.** Equal amounts of pcDNA3-3X-Flag, pcDNA3-3X-Flag-ZFP809, or pcDNA3-3X-Flag-ZFP809(DV-AA) plasmids were transfected into 293T cells. Twenty-four hours after transfection, cells were lysed in IPH buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA; 0.5% NP-40) (6). Cell lysates were incubated with anti-Flag magnetic beads (Sigma). After 4 h of incubation, beads were washed with modified IPH buffer (the concentration of detergent was increased to 1%) and eluted with Laemmli buffer. The eluted proteins were analyzed by Western blotting.

**ACKNOWLEDGMENTS.** This work was supported by Grant R01 CA 30488 from the National Cancer Institute. S.P.G. is an Investigator of the Howard Hughes Medical Institute.

1. Rowe HM, Trono D (2011) Dynamic control of endogenous retroviruses during development. *Virology* 411(2):273–287.
2. Jacobs FM, et al. (2014) An evolutionary arms race between KRAB zinc-finger genes ZNF91/93 and SVA/L1 retrotransposons. *Nature* 516(7530):242–245.
3. Teich NM, Weiss RA, Martin GR, Lowy DR (1977) Virus infection of murine teratocarcinoma stem cell lines. *Cell* 12(4):973–982.
4. Stewart CL, Stuhlmann H, Jähner D, Jaenisch R (1982) De novo methylation, expression, and infectivity of retroviral genomes introduced into embryonal carcinoma cells. *Proc Natl Acad Sci USA* 79(13):4098–4102.
5. Barklis E, Mulligan RC, Jaenisch R (1986) Chromosomal position or virus mutation permits retrovirus expression in embryonal carcinoma cells. *Cell* 47(3):391–399.
6. Wolf D, Goff SP (2007) TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. *Cell* 131(1):46–57.
7. Loh TP, Sievert LL, Scott RW (1987) Proviral sequences that restrict retroviral expression in mouse embryonal carcinoma cells. *Mol Cell Biol* 7(10):3775–3784.
8. Loh TP, Sievert LL, Scott RW (1988) Negative regulation of retrovirus expression in embryonal carcinoma cells mediated by an intragenic domain. *J Virol* 62(11): 4086–4095.
9. Petersen R, Kempler G, Barklis E (1991) A stem cell-specific silencer in the primer-binding site of a retrovirus. *Mol Cell Biol* 11(3):1214–1221.
10. Kempler G, Freitag B, Berwin B, Nanassy O, Barklis E (1993) Characterization of the Moloney murine leukemia virus stem cell-specific repressor binding site. *Virology* 193(2):690–699.
11. Wolf D, Cammas F, Losson R, Goff SP (2008) Primer binding site-dependent restriction of murine leukemia virus requires HP1 binding by TRIM28. *J Virol* 82(9):4675–4679.
12. Yamauchi M, Freitag B, Khan C, Berwin B, Barklis E (1995) Stem cell factor binding to retrovirus primer binding site silencers. *J Virol* 69(2):1142–1149.
13. Wolf D, Goff SP (2009) Embryonic stem cells use ZFP809 to silence retroviral DNAs. *Nature* 458(7242):1201–1204.
14. Le Douarin B, et al. (1996) A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. *EMBO J* 15(23):6701–6715.
15. Schultz DC, Friedman JR, Rauscher FJ, III (2001) Targeting histone deacetylase complexes via KRAB-zinc finger proteins: The PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. *Genes Dev* 15(4):428–443.
16. Schultz DC, Ayyanathan K, Negorev D, Maul GG, Rauscher FJ, III (2002) SETDB1: A novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev* 16(8):919–932.
17. Matsui T, et al. (2010) Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* 464(7290):927–931.
18. Saurin AJ, Borden KL, Boddy MN, Freemont PS (1996) Does this have a familiar RING? *Trends Biochem Sci* 21(6):208–214.
19. Deshaies RJ, Joazeiro CA (2009) RING domain E3 ubiquitin ligases. *Annu Rev Biochem* 78:399–434.
20. Wolf G, et al. (2015) The KRAB zinc finger protein ZFP809 is required to initiate epigenetic silencing of endogenous retroviruses. *Genes Dev* 29(5):538–554.
21. Yeo JC, Ng HH (2013) The transcriptional regulation of pluripotency. *Cell Res* 23(1): 20–32.
22. Fong YW, Cattoglio C, Yamaguchi T, Tjian R (2012) Transcriptional regulation by coactivators in embryonic stem cells. *Trends Cell Biol* 22(6):292–298.
23. Mikkelsen TS, et al. (2008) Dissecting direct reprogramming through integrative genomic analysis. *Nature* 454(7200):49–55.
24. Wu C, et al. (2009) BioGPS: An extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol* 10(11):R130.
25. Xiao S, et al. (2012) Comparative epigenomic annotation of regulatory DNA. *Cell* 149(6):1381–1392.
26. Schneider-Poetsch T, et al. (2010) Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat Chem Biol* 6(3):209–217.
27. Chung S, et al. (2002) Analysis of different promoter systems for efficient transgene expression in mouse embryonic stem cell lines. *Stem Cells* 20(2):139–145.
28. Spenger A, Ernst W, Condreay JP, Kost TA, Grabherr R (2004) Influence of promoter choice and trichostatin A treatment on expression of baculovirus delivered genes in mammalian cells. *Protein Expr Purif* 38(1):17–23.
29. Keller MJ, et al. (2007) Reversal of human cytomegalovirus major immediate-early enhancer/promoter silencing in quiescently infected cells via the cyclic AMP signaling pathway. *J Virol* 81(12):6669–6681.
30. Hong S, et al. (2007) Functional analysis of various promoters in lentiviral vectors at different stages of in vitro differentiation of mouse embryonic stem cells. *Mol Ther* 15(9):1630–1639.
31. Settembre C, Fraldi A, Medina DL, Ballabio A (2013) Signals from the lysosome: A control centre for cellular clearance and energy metabolism. *Nat Rev Mol Cell Biol* 14(5):283–296.
32. Peters JM, Franke WW, Kleinschmidt JA (1994) Distinct 19 S and 20 S subcomplexes of the 26 S proteasome and their distribution in the nucleus and the cytoplasm. *J Biol Chem* 269(10):7709–7718.
33. Shintani T, Klionsky DJ (2004) Autophagy in health and disease: A double-edged sword. *Science* 306(5698):990–995.
34. Rock KL, et al. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78(5):761–771.
35. Thrower JS, Hoffman L, Rechsteiner M, Pickart CM (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J* 19(1):94–102.
36. Rape M, Jentsch S (2002) Taking a bite: Proteasomal protein processing. *Nat Cell Biol* 4(5):E113–E116.
37. Maki CG, Huibregtse JM, Howley PM (1996) In vivo ubiquitination and proteasome-mediated degradation of p53(1). *Cancer Res* 56(11):2649–2654.
38. Young JA, et al. (2011) Fas-associated death domain (FADD) and the E3 ubiquitin-protein ligase TRIM21 interact to negatively regulate virus-induced interferon production. *J Biol Chem* 286(8):6521–6531.
39. Yu J, et al. (1999) Identification and classification of p53-regulated genes. *Proc Natl Acad Sci USA* 96(25):14517–14522.
40. Risseeuw EP, et al. (2003) Protein interaction analysis of SCF ubiquitin E3 ligase subunits from *Arabidopsis*. *Plant J* 34(6):753–767.
41. Liang Q, et al. (2011) Tripartite motif-containing protein 28 is a small ubiquitin-related modifier E3 ligase and negative regulator of IFN regulatory factor 7. *J Immunol* 187(9): 4754–4763.
42. Bennett EJ, Rush J, Gygi SP, Harper JW (2010) Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. *Cell* 143(6):951–965.
43. Peng H, et al. (2000) Biochemical analysis of the Kruppel-associated box (KRAB) transcriptional repression domain. *J Biol Chem* 275(24):18000–18010.
44. Sarikas A, Hartmann T, Pan ZQ (2011) The cullin protein family. *Genome Biol* 12(4):220.

45. Rabut G, Peter M (2008) Function and regulation of protein neddylation. 'Protein modifications: Beyond the usual suspects' review series. *EMBO Rep* 9(10):969–976.
46. Merlet J, Burger J, Gomes JE, Pintard L (2009) Regulation of cullin-RING E3 ubiquitin-ligases by neddylation and dimerization. *Cell Mol Life Sci* 66(11–12):1924–1938.
47. Garcia K, et al. (2014) Nedd8-activating enzyme inhibitor MLN4924 provides synergy with mitomycin C through interactions with ATR, BRCA1/BRCA2, and chromatin dynamics pathways. *Mol Cancer Ther* 13(6):1625–1635.
48. Godbersen JC, et al. (2014) The Nedd8-activating enzyme inhibitor MLN4924 thwarts microenvironment-driven NF-κB activation and induces apoptosis in chronic lymphocytic leukemia B cells. *Clin Cancer Res* 20(6):1576–1589.
49. Sutovsky P, et al. (2001) Accumulation of the proteolytic marker peptide ubiquitin in the trophoblast of mammalian blastocysts. *Cloning Stem Cells* 3(3):157–161.
50. Vilchez D, et al. (2012) Increased proteasome activity in human embryonic stem cells is regulated by PSMD11. *Nature* 489(7415):304–308.
51. Buckley SM, et al. (2012) Regulation of pluripotency and cellular reprogramming by the ubiquitin-proteasome system. *Cell Stem Cell* 11(6):783–798.
52. van der Stoop P, et al. (2008) Ubiquitin E3 ligase Ring1b/Rnf2 of polycomb repressive complex 1 contributes to stable maintenance of mouse embryonic stem cells. *PLoS One* 3(5):e2235.
53. Naujokat C, Sarić T (2007) Concise review: Role and function of the ubiquitin-proteasome system in mammalian stem and progenitor cells. *Stem Cells* 25(10): 2408–2418.
54. Ivanov AV, et al. (2007) PHD domain-mediated E3 ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing. *Mol Cell* 28(5): 823–837.
55. Li X, et al. (2007) Role for KAP1 serine 824 phosphorylation and sumoylation/desumoylation switch in regulating KAP1-mediated transcriptional repression. *J Biol Chem* 282(50):36177–36189.
56. Kuo CY, et al. (2014) An arginine-rich motif of ring finger protein 4 (RNF4) oversees the recruitment and degradation of the phosphorylated and SUMOylated Krüppel-associated box domain-associated protein 1 (KAP1)/TRIM28 protein during genotoxic stress. *J Biol Chem* 289(30):20757–20772.
57. Yao S, et al. (2004) Retrovirus silencing, variegation, extinction, and memory are controlled by a dynamic interplay of multiple epigenetic modifications. *Mol Ther* 10(1):27–36.
58. Robbez-Masson L, Rowe HM (2015) Retrotransposons shape species-specific embryonic stem cell gene expression. *Retrovirology* 12:45.
59. Rowe HM, et al. (2013) TRIM28 repression of retrotransposon-based enhancers is necessary to preserve transcriptional dynamics in embryonic stem cells. *Genome Res* 23(3):452–461.
60. Akinc A, Thomas M, Klibanov AM, Langer R (2005) Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J Gene Med* 7(5): 657–663.
61. Higuchi R, Krummel B, Saiki RK (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. *Nucleic Acids Res* 16(15):7351–7367.
62. Li MZ, Elledge SJ (2012) SLIC: A method for sequence- and ligation-independent cloning. *Methods Mol Biol* 852:51–59.
63. Han YH, Moon HJ, You BR, Park WH (2009) The effect of MG132, a proteasome inhibitor on HeLa cells in relation to cell growth, reactive oxygen species and GSH. *Oncol Rep* 22(1):215–221.
64. Qin H, Shao Q, Igdoura SA, Alaoui-Jamali MA, Laird DW (2003) Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and -competent breast tumor cells. *J Biol Chem* 278(32):30005–30014.
65. Wang C, et al. (2005) MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. *EMBO J* 24(18):3279–3290.
66. Henning MS, Morham SG, Goff SP, Naghavi MH (2010) PDZD8 is a novel Gag-interacting factor that promotes retroviral infection. *J Virol* 84(17):8990–8995.