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Degradation of Stop Codon Read-through Mutant Proteins via the Ubiquitin-Proteasome System Causes Hereditary Disorders*[S]

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Background: 20 read-through mutations that produce C-terminally extended proteins are related to human hereditary disorders.
Results: The C-terminal extended proteins of mouse cFLIP-L (cellular FLICE-like apoptosisinhibitory protein) and human PNPO (pyridoxamine 5-phosphate oxidase) and HSD3B2 (3-hydroxysteroid dehydrogenase type II) are ubiquitylated and degraded, involving an E3 ligase, TRIM21, for cFLIP-L and PNPO degradation.
Conclusion: Read-through mutant cFLIP-L, PNPO, and HSD3B2 are degraded by the ubiquitin-proteasome system.
Significance: Degradation of read-through mutant proteins may cause hereditary disorders.

During translation, stop codon read-through occasionally happens when the stop codon is misread, skipped, or mutated, resulting in the production of aberrant proteins with C-terminal extension. These extended proteins are potentially deleterious, but their regulation is poorly understood. Here we show *in vitro* and *in vivo* evidence that mouse cFLIP-L with a 46-amino acid extension encoded by a read-through mutant gene is rapidly degraded by the ubiquitin-proteasome system, causing hepatocyte apoptosis during embryogenesis. The extended peptide interacts with an E3 ubiquitin ligase, TRIM21, to induce ubiquitylation of the mutant protein. In humans, 20 read-through mutations are related to hereditary disorders, and extended peptides found in human PNPO and HSD3B2 similarly destabilize these proteins, involving TRIM21 for PNPO degradation. Our findings indicate that degradation of aberrant proteins with C-terminal extension encoded by read-through mutant genes is a mechanism for loss of function resulting in hereditary disorders.

Translation from an mRNA to a protein is normally terminated at stop codons (UAA, UAG, and UGA). However, reading through a stop codon occasionally happens, the frequency of which is affected by the nucleotides around the stop codon (1, 2). When read-through of a stop codon occurs, the translation continues to the next in-frame stop codon or to the poly(A) tail at the 3′ terminus of the mRNA, resulting in the production of aberrant proteins with C-terminal extension. Similar C-terminally extended proteins are constitutively produced when a stop codon is mutated to code for an amino acid; in both cases, this can lead to potentially deleterious proteins.

Translation from mRNAs lacking a stop codon (nonstop mRNA) is suppressed by several mechanisms, including accelerated degradation of nonstop mRNA and translational repression triggered by stalled polysomes in the vicinity of the poly(A) tail (3–5). However, genome-wide bioinformatics analysis of seven vertebrate animal species indicated that a relatively small number of mRNAs lack a downstream stop codon and that most mRNAs contain additional in-frame stop codons in the 3′-untranslated region (3′-UTR) (Table 1). Therefore, even if a canonical stop codon is mutated to code for an amino acid, translation is terminated at a downstream stop codon in many cases. Currently, more than 400 read-through single nucleotide polymorphisms (SNPs) that result in encoding C-terminally extended proteins terminated at a downstream stop codon have been identified in humans (supplemental Table 1), and some of these SNPs are related to hereditary disorders. However, regulation of these C-terminally extended proteins is poorly understood.

As a model system to study the regulation of the read-through mutant genes, we found a read-through mutation in the long form of mouse cFLIP-L (cellular FLICE-like apoptosis-inhibitory protein) gene that results in a mutant cFLIP-L protein containing a C-terminal 46-amino acid extension (cFLIP-L+46). Structurally, cFLIP-L resembles caspase-8 but has no caspase activity, and therefore, it inhibits apoptosis signaling initiated by death receptor ligation (6). In this study, we demonstrate that homozygous *cFlip-l+46* mutant mice die at around 13.5 days postcoitum and undergo extensive apoptosis in the liver. Mechanistic analysis revealed that the extended

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[S] This article contains supplemental Table 1.
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TABLE 1

Number of stop mRNAs in seven vertebrate species

Full-length mRNA data were retrieved from the NCBI database. Stop mRNA, mRNAs that contain an in-frame termination codon in the 3′-UTR; Nonstop mRNA, mRNAs that lack an in-frame termination codon between the canonical termination and poly (A) tail; Unknown, mRNAs that cannot be categorized as stop or nonstop mRNAs because of incomplete sequence data in the 3′-UTR. Numbers of stop mRNAs, nonstop mRNAs, and unknowns are shown. aa, amino acids.

	Human	Mouse	Rat	Cow	Pig	Frog	Zebrafish
Total	72,110	77,951	60,136	70,360	38,382	28,813	47,797
Stop mRNA	66,861	70,014	51,784	61,064	30,171	23,913	42,528
Nonstop mRNA	552	117	31	11	9	26	7
Unknown	4697	7820	8321	9285	8202	4874	5262
Average length of extended amino acids (aa)	27.8	25.9	25.1	28.3	28.0	20.6	20.7

46-amino acid peptide functions as a degron to destabilize the cFLIP-L protein and that an E3 ubiquitin ligase, TRIM21 (tripartite motif-containing 21), mediates the ubiquitylation of cFLIP-L+46. We also show that C-terminally extended PNPO (pyridoxamine 5′-phosphate oxidase) and HSD3B2 (3β-hydroxysteroid dehydrogenase type II) proteins, encoded by read-through mutant genes found in patients of congenital diseases, are degraded by a similar mechanism. These findings indicate that degradation of aberrant C-terminally extended proteins that are encoded by genes with read-through mutations is a mechanism for loss of function and can lead to hereditary disorders.

Experimental Procedures

Reagents and Plasmids—Tissue culture plastics were purchased from Greiner Bio-One (Tokyo, Japan). Cycloheximide (CHX)[2] and anti-FLAG-agarose (M2) were from Sigma-Aldrich. MG132 was from Peptide Institute (Osaka, Japan). Anti-Myc-agarose (9E10) was from Santa Cruz Biotechnology, Inc. Cell Counting Kit-8 was from Dojindo Laboratories (Kumamoto, Japan). TNFα was provided by Prof. Tsujimoto (Teikyo Heisei University, Tokyo, Japan). cDNAs encoding mouse cFLIP-L, human PNPO, and HSD3B2 were amplified by PCR and cloned into the p3xFLAG-CMV-10 (Sigma-Aldrich) or pcDNA3 expression vectors (Invitrogen). cDNAs encoding extended peptides were PCR-amplified and inserted into the pEGFP-C2 expression vector (Clontech). pcDNA4/His/LacZ was purchased from Invitrogen. A human TRIM21 expression vector was provided by Prof. Hatakeyama (Hokkaido University, Sapporo, Japan). To generate siRNA-resistant TRIM21 expression vectors, synonymous nucleotide substitutions were introduced using *PfuUltra* high-fidelity DNA polymerase (Agilent Technologies) according to the manufacturer's protocol.

Mice and Histological Analysis—Animal experiments were approved by the Animal Experiment Committee of the National Institute of Health Sciences, Japan. *N*-Ethyl-*N*-nitrosourea (ENU)-mutagenized *cFlip-l+46* mutant mouse lines were originally generated by the RIKEN BioResource Center, as described previously (7). *cFlip*-deficient mice were provided by Dr. Yeh (Amgen Inc.) (8). All mice were housed under specific pathogen-free conditions.

Embryos at E12.5 were fixed in 10% neutral phosphate-buffered formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin-eosin. For TUNEL staining, sections were stained using an *in situ* apoptosis detection kit (Takara Bio, Shiga, Japan).

Cell Culture and Transfection—Mouse embryonic fibroblasts (MEFs) and HeLa cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco), and 50 μg/ml kanamycin (Sigma-Aldrich). Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNA transfections were carried out using Lipofectamine RNAi/MAX (Invitrogen) according to the manufacturer's instructions. Typically, cells were transfected, and 48 h later, the cells were treated with 10 μM MG132 for 6 h. Target sequences for mouse *cFlip-l*, human *TRIM21*, and mouse *Trim21* were as follows (5′ to 3′): mouse *cFlip-l*, GAATAGACTTGAACACAAA; human *TRIM21* 1, AAGCAGGAGTTGGCTGAGAAG; human *TRIM21* 2, GCTCCCTCATCTACTCCTT; mouse *Trim21* 1, CCTAAACCCTCATCTCTTT; mouse *Trim21* 2, CCTGGACACGTTAGATATT.

Immunoblot Analysis and Coimmunoprecipitation—For immunoblot analysis, cells were lysed in a lysis buffer containing 0.1 M Tris/HCl (pH 7.5), 1% SDS, 10% glycerol. For coimmunoprecipitation, cells were lysed in a lysis buffer containing 0.02 M Tris/HCl (pH 7.5), 0.2% Nonidet P-40, 0.15 M NaCl, 10% glycerol, and 1× complete protease inhibitor mixture (Roche Applied Science), and the lysates were immunoprecipitated with the indicated antibodies. Lysates or immunoprecipitates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The following antibodies were used to detect proteins: anti-cFLIP-L (Dave-2) (Enzo Life Sciences, Plymouth Meeting, PA); anti-HSP90 (catalog no. 610419) (BD Transduction); anti-cleaved caspase-3 (catalog no. 9664), anti-β-catenin (catalog no. 9562), and anti-Myc (catalog no. 2276) (Cell Signaling); anti-human TRIM21 (12108–1-AP) (Proteintech Group, Chicago, IL); anti-β-tubulin (ab6046) (Abcam); anti-FLAG (M2) (Sigma-Aldrich); anti-HA (3F10) (Roche Applied Science); and anti-GAPDH (FL-335), anti-β-actin (C-2), anti-GFP (B-2), anti-His (H-3), and anti-mouse TRIM21 (M-20) (Santa Cruz Biotechnology).

Measurement of Apoptosis by Flow Cytometer—Apoptosis was analyzed with an annexin V-FITC apoptosis detection kit (BioVision, Milpitas, CA), as described previously (9). Briefly, after treatment, cells were gently trypsinized and washed with serum-containing medium. Cells were collected by centrifugation, additionally washed with PBS, and resuspended in binding buffer. The cells were stained with annexin V-FITC and propidium iodide at room temperature for 5 min in the dark,

[2] The abbreviations used are: CHX, cycloheximide; UPS, ubiquitin-proteasome system; ENU, *N*-ethyl-*N*-nitrosourea; MEF, mouse embryonic fibroblast; VEGF-Ax, vascular endothelial growth factor A; E*n*, embryonic day *n*.

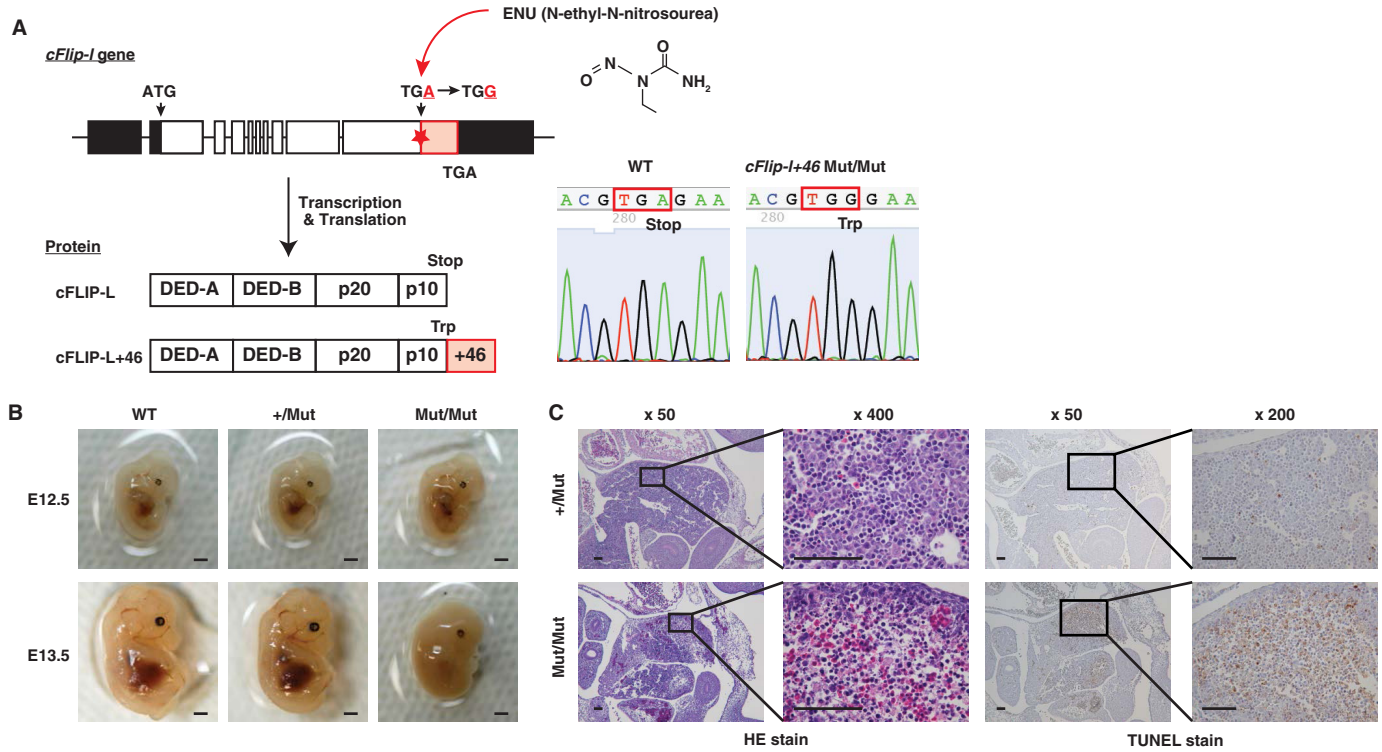

FIGURE 1. **A read-through mutation in the mouse *cFlip-l* gene is homozygous lethal with extensive apoptosis in liver.** *A*, in a G1 male mouse library of ENU-driven mutagenesis, we identified a stop codon read-through mutation (X482W) in *cFlip-l* gene that causes a C-terminal 46-amino acid extension (cFLIP-L+46). *B*, WT, heterozygous (+/Mut), and homozygous *cFlip-l+46* mutant (Mut/Mut) embryos at E12.5 and E13.5. *Bars*, 1 mm. *C*, histological analysis of *cFlip-l+46* mutant embryos at E12.5. Sections were stained with hematoxylin-eosin or TUNEL. *Bars*, 100 μm.

according to the manufacturer's instructions, and analyzed on a FACScan flow cytometer (BD Biosciences).

Ubiquitylation Experiments—HeLa cells transfected with the indicated vectors were treated with 10 μM MG132 and lysed in lysis buffer containing 0.1 M Tris/HCl (pH 7.5), 1% SDS, 10% glycerol. After heat denaturation, the lysates were diluted 10 times with 0.1 M Tris/HCl (pH 7.5). Proteins were immunoprecipitated with anti-FLAG or anti-Myc agarose-conjugated beads, and the immunoprecipitates were analyzed by Western blotting with anti-HA.

Identification of TRIM21 by Liquid Chromatography/Mass Spectrometry (LC/MS)—Lysates from HeLa cells expressing GFP+46 or GFP+22 were immunoprecipitated with antibody against GFP. Following SDS-PAGE, gels were stained with SYPRO ruby protein gel stain (Lonza, Rockland, ME), and protein bands that specifically bind to the GFP+46 were excised from the gel and subjected to in-gel trypsin digestion. The peptides extracted from the gel pieces were analyzed by LC/MS/MS using an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) connected to a Paradigm MS4 HPLC system (Michrom BioResources, Auburn, CA). The analytical column for LC/MS was a reversed-phase column (L-column 2 ODS; 150 × 0.075 mm, 3 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan). The mobile phase was 0.1% formic acid containing 2% acetonitrile and 0.1% formic acid containing 90% acetonitrile (A buffer). The peptides were eluted at a flow rate of 300 nl/min with a gradient of 2–65% of A buffer over 50 min. MS/MS conditions were as follows: an electrospray voltage of 2.5 kV in positive ion mode, a capillary temperature of 275 °C, and a collision energy of 35% for MS/MS. The spectra data obtained by MS/MS were subjected to database search analysis with the SEQUEST algorithm (Proteome Discoverer version 1.4, Thermo Fisher Scientific) using the UniProt database.

RNA Isolation and Quantitative PCR—Total RNA was prepared from MEFs with RNeasy (Qiagen). First strand cDNA was synthesized from 1 μg of total RNA with an oligo(dT) primer using the SuperScript first-strand synthesis system (Invitrogen). Quantitative real-time PCR was performed with an ABI Prism 7300 sequence detection system using TaqMan real-time PCR master mix (Applied Biosystems, Foster City, CA) with TaqMan probes/primers for mouse *cFlip-l* and 18S ribosome RNA (Applied Biosystems). The relative amounts of *cFlip-l* mRNAs were calculated by using the comparative *Ct* method. Mouse 18S ribosomal RNA was used as an invariant control.

Bioinformatics Analysis—Data for human, mice, rat, cow, pig, frog, and zebrafish full-length mRNAs were retrieved from the NCBI RefSeq database in January 2015. Read-through SNP data and associated full-length human mRNA data were retrieved from dbSNP and RefSeq in the NCBI database in March 2014. Stop mRNAs that contain an in-frame termination codon in the 3′-UTR were selected. 3′-UTR sequences were translated *in silico* using a Perl translate module.

Results

Homozygous cFlip-l Read-through Mutant Mice Show Extensive Apoptosis in Liver and Die during Embryogenesis—In a large scale archive of ENU-mutagenized mice (10), we found a read-through mutation (X482W) in the *cFlip-l* gene that results

in encoding a mutant cFLIP-L protein containing a C-terminal 46-amino acid extension (cFLIP-L+46) (Fig. 1*A*). To understand how the extended protein is regulated and the phenotypic consequences of the read-through mutation, we analyzed the mutant mice and the cFLIP-L+46 protein.

Heterozygous *cFlip-l+46* mutant (+/Mut) mice developed normally and were fertile, but homozygous mutant (Mut/Mut) mice died at around E13.5 (Fig. 1*B* and Table 2*A*). To exclude the possibility that abnormalities found in the mutant (Mut/Mut) mice are due to ENU-generated mutation of another, unknown gene that happened to be homozygous in the *cFlip-l+46* Mut/Mut mice, we crossed heterozygous *cFlip-l+46* mutant (+/Mut) mice onto heterozygous *cFlip*-deficient (+/−) mice. The resulting transheterozygote (−/Mut) mice died at around E11.5 (Table 2*B*), which is slightly later than the embryonic lethality observed in *cFlip* null (−/−) mice (Table 2*C*). Because any mutation except for *cFlip-l* is heterozygous in the transheterozygotes and gives no phenotypic changes, the lethality observed in the transheterozygote embryos is due to the complication in the *cFlip-l* gene. Accordingly, these results indicate that the homozygous read-through mutation of the *cFlip-l* gene is embryonic lethal. Histological analysis of *cFlip-l+46* Mut/Mut embryos at E12.5 showed hemorrhage with many condensed nuclei and TUNEL-positive cells in the liver (Fig. 1*C*). Thus, homozygous *cFlip-l* read-through mutant (Mut/Mut) embryos survived beyond E10.5 when *cFlip* null mice die because of impaired yolk sac vasculature development (8, 11) but eventually died at around E13.5, probably because of extensive apoptosis in the liver. Because the extended cFLIP-L+46 protein is destabilized (see below), these results suggest that a tiny amount of cFLIP-L+46 protein enables the embryos to survive beyond E10.5 but that it is not sufficient to suppress hepatocyte apoptosis at a later embryonic stage.

Reduction of cFLIP-L+46 Protein Levels Results in Higher Sensitivity to TNFα-induced Apoptosis—Next, we analyzed the behavior of the cFLIP-L+46 protein. Levels of cFLIP-L+46 protein were dramatically reduced in the Mut/Mut whole embryo (Fig. 2*A*) and liver (Fig. 2*B*) at E12.5. In the liver, the

TABLE 2

Viability of *cFlip-l* read-through mutant and *cFlip* deficient embryos

Embryos were obtained at the indicated gestation periods after crossing heterozygous *cFlip-l+46* mutant (+/Mut) mice (*A*), intercrossing heterozygous *cFlip* deficient (+/−) mice with heterozygous *cFlip-l+46* mutant (+/Mut) mice (*B*) or crossing heterozygous *cFlip* deficient (+/−) mice (*C*). Numbers of living embryos are shown. Numbers in parenthesis represent dead embryos.

A

	WT	+/Mut	Mut/Mut	Total
E11.5	3	5	2	10
E12.5	10	7	7	24
E13.5	7	16	4 (2)	29
E14.5	15	30	1 (5)	51
E15.5	3	9	0 (4)	16

B

	+/+	+/-	+/Mut	-/Mut	Total
E9.5	1	2	4	3	10
E10.5	16	9	6 (2)	10 (1)	44
E11.5	14	14	13	3 (7)	51
E12.5	1	5	6	0 (4)	16

C

	+/+	+/-	-/-	Total
E10.5	15	19(2)	5 (5)	46
E11.5	12	21	2 (10)	45
E12.5	2	8	0 (2)	12

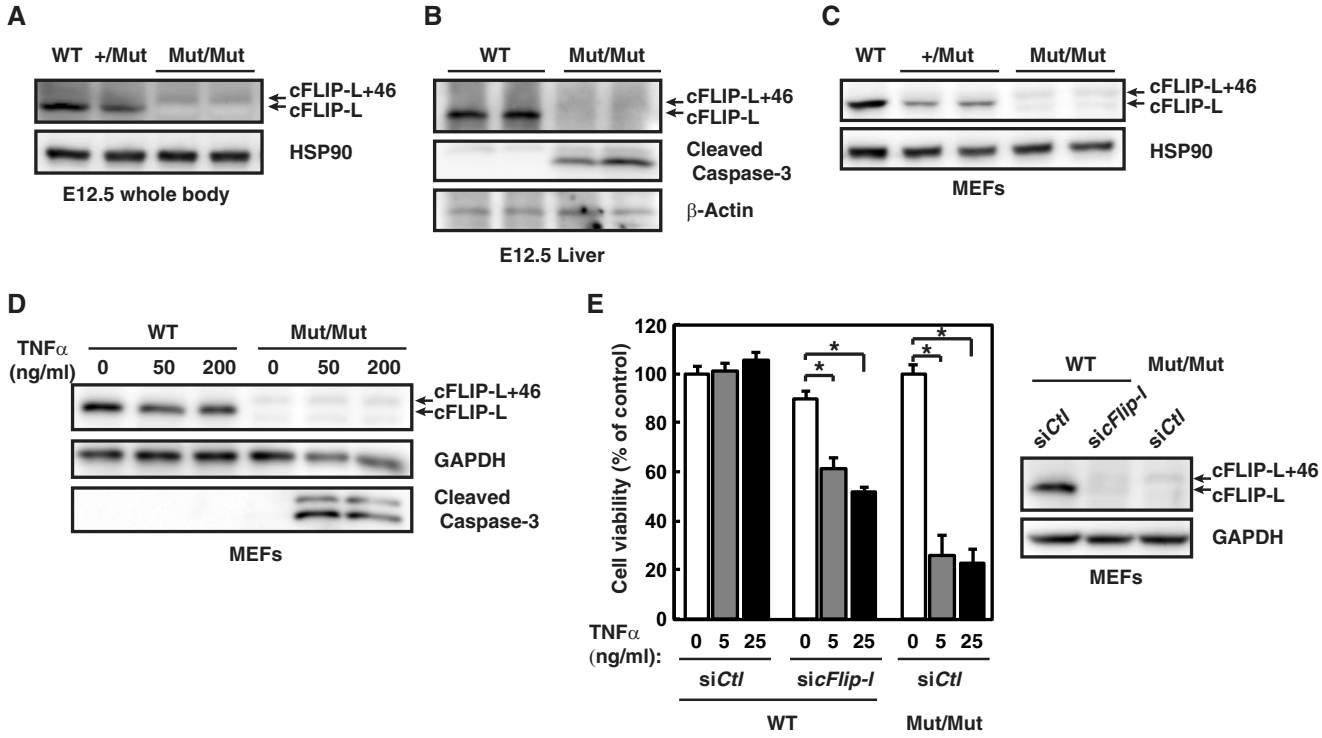

FIGURE 2. **Severe reduction of cFLIP-L+46 protein levels results in higher sensitivity to TNFα-induced apoptosis.** *A–D*, expression of WT and C-terminally extended cFLIP-L proteins in whole E12.5 embryos (*A*), in embryonic liver at E12.5 (*B*), and in MEFs (*C* and *D*). *D*, MEFs were treated with 50 or 200 ng/ml TNFα for 5 h. *E*, MEFs were transfected with control (si*Ctl*) or mouse *cFlip*-specific (si*cFlip-l*) siRNAs and treated with 5 or 25 ng/ml TNFα for 24 h. Cell viability was determined using the Cell Counting Kit-8 (*left*). *Error bars*, S.D. ($n = 3$). *, $p < 0.01$ by Student's *t* test. cFLIP-L and cFLIP-L+46 proteins in MEFs were analyzed by Western blotting (*right*).

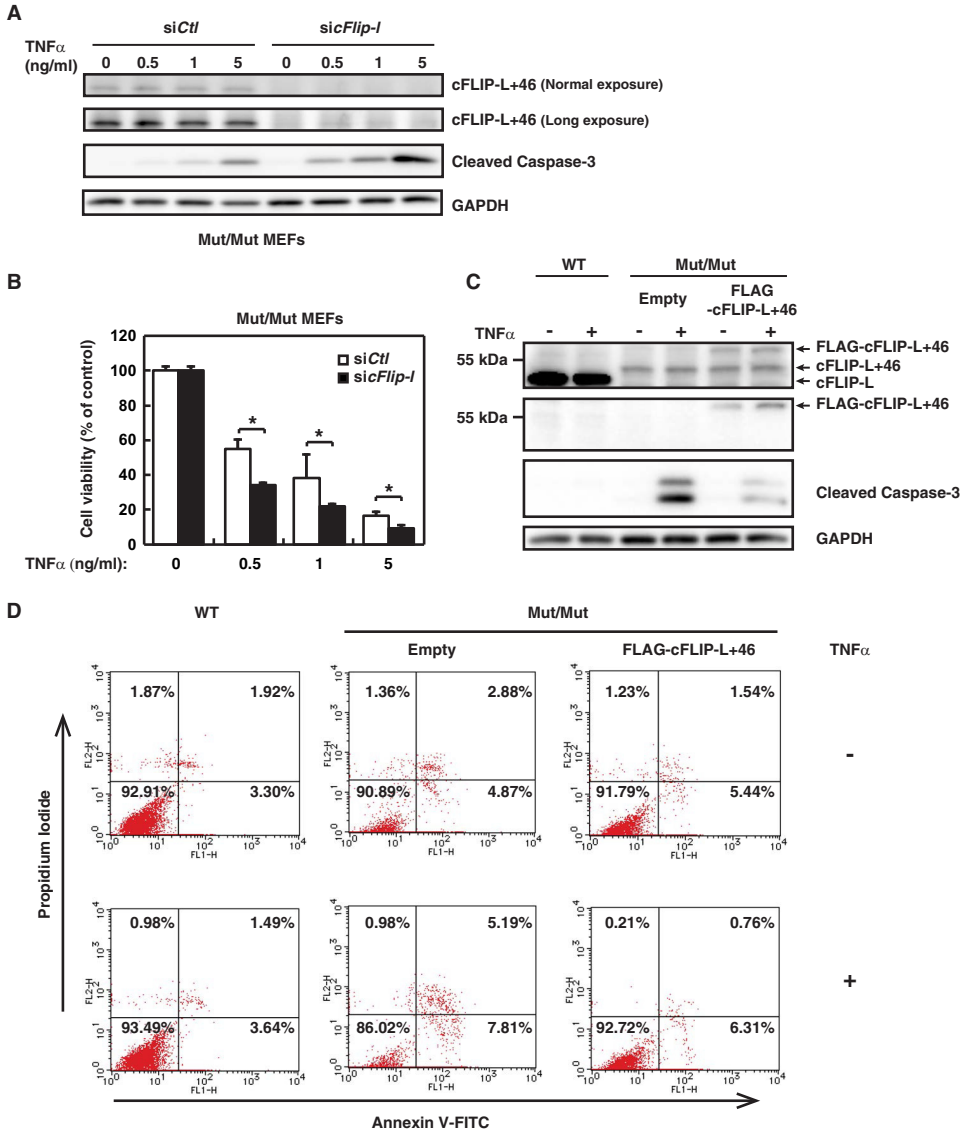

FIGURE 3. **cFLIP-L+46 protein has the ability to inhibit apoptosis.** *A* and *B*, Mut/Mut MEFs were transfected with mouse si*Flip-l* and treated with 0, 0.5, 1, 5 ng/ml TNFα for 6 h (*A*) or 24 h (*B*). Levels of cFLIP-L+46 and cleaved caspase-3 were analyzed by Western blotting (*A*), and cell viability was determined using Cell Counting Kit-8 (*B*). *Error bars*, S.D. ($n = 3$). *, $p < 0.05$ by Student's *t* test. *C* and *D*, Mut/Mut MEFs expressing FLAG-cFLIP-L+46 were treated with 5 ng/ml TNFα for 6 h (*C*) or 48 h (*D*). Levels of cFLIP-L+46, FLAG-cFLIP-L+46, and cleaved caspase-3 were analyzed by Western blotting (*C*). Cell death by TNFα was determined using annexin V and propidium iodide staining. Double negative cells (*bottom left*) represent living cells, whereas annexin V-positive (*bottom right*), propidium iodide-positive (*top left*), or double positive (*top right*) cells represent dead cells (*D*).

active form of caspase-3 was observed (Fig. 2*B*), which is in good agreement with the observed hepatocyte apoptosis (Fig. 1*C*). Reduced cFLIP-L+46 protein levels were similarly observed in Mut/Mut MEFs (Fig. 2*C*). Consistent with the reduced levels of cFLIP-L+46 protein, the Mut/Mut MEFs were highly sensitive to TNFα-induced apoptosis (Fig. 2*D*) as were wild-type MEFs depleted of cFLIP-L using siRNA (Fig. 2*E*).

To examine whether the residual cFLIP-L+46 can inhibit apoptosis signaling in Mut/Mut MEFs, we down-regulated cFLIP-L+46 in Mut/Mut MEFs using siRNA. As shown in Fig. 3, *A* and *B*, depletion of the residual cFLIP-L+46 protein significantly sensitized the cells to TNFα-induced apoptosis. Conversely, exogenous expression of cFLIP-L+46 protein in Mut/Mut MEFs suppressed TNFα-induced caspase activation (Fig. 3*C*) and increase of annexin V-positive apoptotic cells (Fig. 3*D*). These results indicate that cFLIP-L+46 protein has an activity to inhibit apoptosis but that the amount of cFLIP-L+46 protein is severely reduced, resulting in higher sensitivity to apoptosis in the Mut/Mut MEFs and hepatocytes.

C-terminally Extended cFLIP-L+46 Protein Is Rapidly Degraded by UPS—We next investigated why levels of cFLIP-L+46 protein are severely reduced. In MEFs, the turnover of cFLIP-L+46 protein was much faster than that of wild-type cFLIP-L protein. When the MEFs were treated with CHX, cFLIP-L+46 protein disappeared within 1 h, whereas wild-type cFLIP-L protein was retained for over 12 h (Fig. 4*A*). Similarly, cFLIP-L+46 exogenously expressed in HeLa cells turned over more rapidly than wild-type cFLIP-L (Fig. 4*B*). Treatment with a proteasome inhibitor, MG132, increased the accumulation of cFLIP-L+46 in Mut/Mut MEFs (Fig. 4*C*). In addition, ubiquitylation of cFLIP-L+46 exogenously expressed

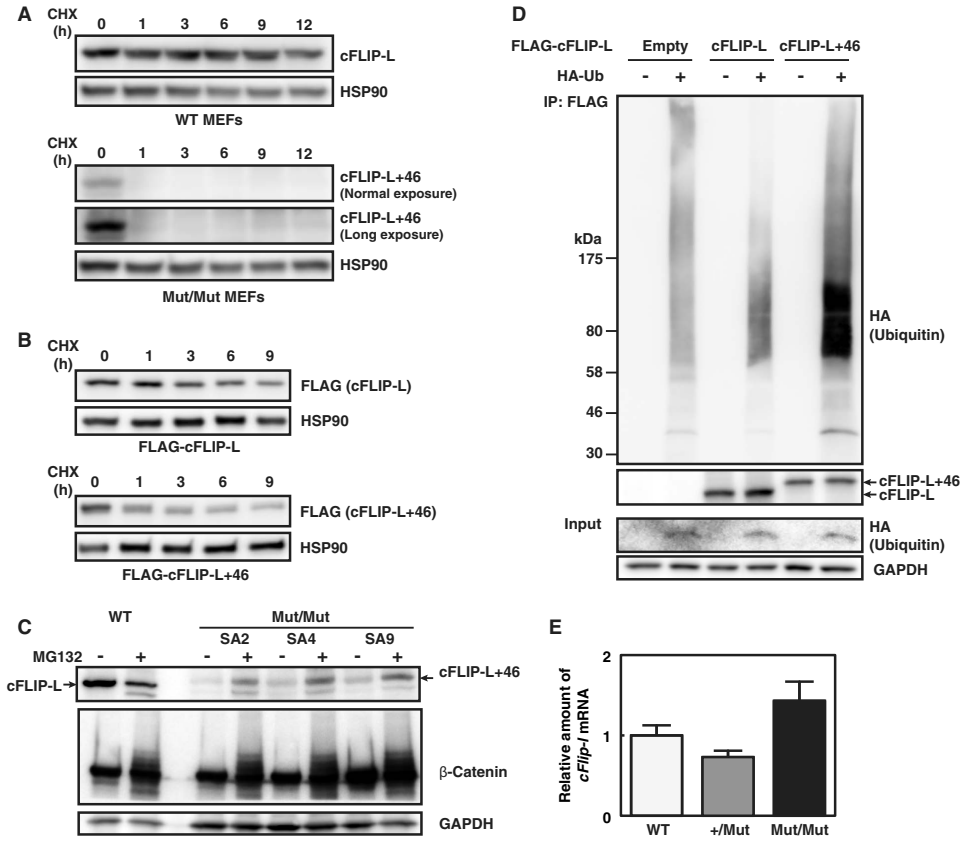

FIGURE 4. **The extended peptide facilitates degradation of cFLIP-L+46 through the UPS.** *A* and *B*, turnover of cFLIP-L or cFLIP-L+46 proteins in MEFs (*A*), and HeLa cells constitutively expressing wild-type FLAG-cFLIP-L or extended FLAG-cFLIP-L+46 proteins (*B*). MEFs and HeLa cells were treated with 30 μg/ml CHX for the indicated times. *C*, proteasome inhibitor increases the level of cFLIP-L+46 protein in Mut/Mut MEFs. MEFs were treated with 10 μM MG132 for 6 h. *D*, enhanced ubiquitylation of cFLIP-L+46 protein. HeLa cells were transfected with FLAG-tagged cFLIP-L or cFLIP-L+46 and HA-tagged ubiquitin (HA-Ub) for 48 h and treated with 10 μM MG132 for 6 h. Ubiquitylation was analyzed as described under "Experimental Procedures." *E*, levels of *cFlip-l* mRNA in MEFs were measured by quantitative PCR and normalized against 18S ribosomal RNA expression. *Error bars*, S.D. ($n = 4$). *IP*, immunoprecipitation.

in HeLa cells was highly enhanced compared with that of wild-type cFLIP-L (Fig. 4*D*). On the other hand, levels of *cFlip-l+46* mRNA in Mut/Mut MEFs were not reduced compared with *cFlip-l* mRNA in wild-type (WT) MEFs (Fig. 4*E*). These results collectively indicate that the expression of cFLIP-L+46 protein is severely reduced because of the rapid degradation by the UPS.

TRIM21 Is Responsible for Ubiquitylation of cFLIP-L+46 Protein—To examine whether the extended peptide can promote proteasomal degradation when grafted onto a heterologous protein, we genetically fused the extended peptide to the C terminus of GFP and expressed it in HeLa cells. GFP+46 levels were much lower than those of GFP, and this difference was reduced by proteasome inhibitor MG132 (Fig. 5*A*), indicating that the extended peptide functions as a degradation signal. Trimming 10 C-terminal amino acids from the extended peptide to form GFP+36 did not affect the degradation signal activity, whereas deletion of 24 C-terminal amino acids to form GFP+22 abolished the activity (Fig. 5*A*).

Because the extended peptide does not contain a lysine residue that can be ubiquitylated, we reasoned that the peptide functions as a degron that interacts with a ubiquitin ligase to destabilize cFLIP-L protein. To identify the ubiquitin ligase involved in the destabilization of cFLIP-L+46 protein, we searched for proteins that interact with GFP+46 but not with GFP+22 by liquid chromatography-mass spectrometry and identified TRIM21, a RING-finger E3 ubiquitin ligase involved in innate and acquired immunity (12–14). Fig. 5*B* shows that GFP+46, but not GFP+22, co-precipitates TRIM21 protein in the cells. In addition, cFLIP-L+46 strongly, but cFLIP-L minimally, co-precipitates TRIM21 in the cells (Fig. 5*C*). These results confirmed the selective interaction of TRIM21 with the extended peptide harboring the degron activity.

We next investigated whether TRIM21 is required for the degradation of GFP+46 and cFLIP-L+46 proteins. siRNA-mediated down-regulation of human TRIM21 in HeLa cells increased the levels of GFP+46, which was suppressed by restoring TRIM21 levels by co-transfecting an siRNA-resistant FLAG-tagged human TRIM21 construct (Fig. 5*D*). In addition, knockdown of mouse *Trim21* in *cFlip-l+46* Mut/Mut MEFs significantly increased the level of cFLIP-L+46 (Fig. 5*E*). The rapid protein turnover of cFLIP-L+46 exogenously expressed in HeLa cells was suppressed by knockdown of human *TRIM21* (Fig. 5*F*). Furthermore, the ubiquitylation of cFLIP-L+46 protein was suppressed in cells depleted of TRIM21 and restored by co-transfection of the siRNA-resistant *Trim21* gene (Fig. 5*G*). These results indicate that TRIM21 is a ubiquitin ligase responsible for the destabilization of cFLIP-L+46 and GFP+46 proteins.

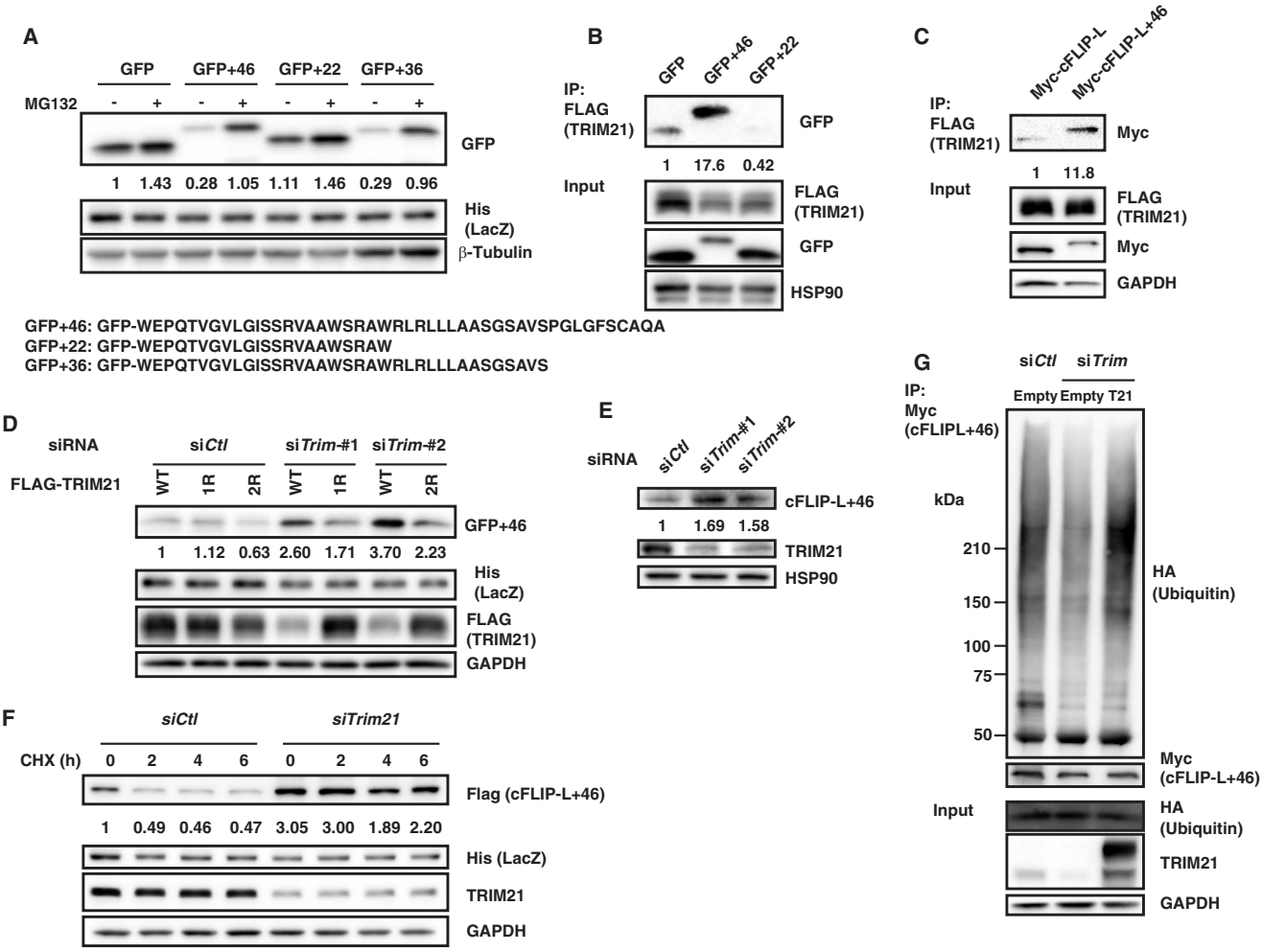

FIGURE 5. **TRIM21 mediates the ubiquitylation and degradation of cFLIP-L+46 protein.** *A*, expression of GFP fused to the extended peptide of cFLIP-L. HeLa cells were transfected with the indicated GFP fusions and His-LacZ as an internal control for transfection efficiency for 48 h, and then cells were treated with 10 μM MG132 for 6 h. *Numbers* at the *bottom* represent relative expression levels normalized against His-LacZ expression. *B* and *C*, TRIM21 interacts with the extended peptide of mouse cFLIP-L. HeLa cells were transfected with FLAG-tagged TRIM21 and the indicated GFP fusions (*B*) or transfected with FLAG-tagged TRIM21 together with Myc-tagged cFLIP-L or cFLIP-L+46 (*C*) and were treated with 10 μM MG132 for 6 h. Lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were analyzed by immunoblotting with an antibody against GFP (*B*) or Myc (*C*). *Numbers* at the *bottom* represent relative amounts in the immunoprecipitates normalized against FLAG-TRIM21 expression together with indicated GFP fusions (*B*) or with cFLIP-L or cFLIP-L+46 (*C*) expression in lysates. *D*, TRIM21 mediates the down-regulation of GFP+46 protein. HeLa cells were transfected with GFP+46 and His-LacZ together with indicated siRNAs and siRNA-sensitive (*WT*) or siRNA-resistant mutants (*1R* and *2R*) of FLAG-tagged TRIM21. *Numbers* at the *bottom* represent relative expression levels normalized against His-LacZ expression. *E*, depletion of TRIM21 increases cFLIP-L+46 protein levels in Mut/Mut MEFs. *Numbers* at the *bottom* represent relative intensity of cFLIP-L+46 protein normalized against HSP90 expression. *F*, HeLa cells were transfected with FLAG-cFLIP-L+46 and His-LacZ as an internal control for transfection efficiency together with siRNAs against *Trim21* and were treated with 10 μg/ml CHX for the indicated times. *Numbers* at the *bottom* represent relative expression levels normalized against His-LacZ expression. *G*, ubiquitylation of cFLIP-L+46 protein by TRIM21. HeLa cells were transfected with Myc-cFLIP-L+46, HA-Ub, siRNA against *Trim21*, and a siRNA-resistant FLAG-TRIM21 (*T21*) and treated with 10 μM MG132 for 2 h. Ubiquitylation was analyzed as described under "Experimental Procedures." *IP*, immunoprecipitation.

Read-through PNPO and HSD3B2 Mutant Proteins Are Degraded through the UPS—In humans, more than 400 read-through SNPs result in encoding C-terminally extended proteins that terminate at downstream in-frame stop codons (supplemental Table 1). Among them, 20 are related to hereditary disorders (Table 3). We hypothesized that some of the read-through mutant proteins could be regulated by a mechanism similar to that which degrades cFLIP-L+46 protein. To test this hypothesis, we examined in HeLa cells the expression of GFP proteins fused to the extended peptides encoded by the 20 genes involved with hereditary disorders. We found that the extended peptides from PNPO (28 amino acids) and HSD3B2 (95 amino acids) destabilized the GFP protein, which was abrogated by MG132 (Fig. 6*A*).

Consistent with this, levels of PNPO+28 expressed in HeLa cells were suppressed compared with wild-type PNPO, and this suppression was abrogated by MG132 (Fig. 6*B*, *left panels*). PNPO+28 protein turned over more rapidly than wild-type PNPO (Fig. 6*C*, *left panels*), and PNPO+28 was more heavily ubiquitylated compared with wild-type PNPO (Fig. 6*D*, *left panels*). Similar results were obtained with HSD3B2+95 and the corresponding wild-type HSD3B2 proteins (Fig. 6, *B–D*, *right panels*). These results indicate that C-terminal extension of PNPO and HSD3B2 proteins caused by read-through mutations results in protein instability through the UPS.

Finally, we examined whether TRIM21 is involved in the destabilization of the extended PNPO+28 and HSD3B2+95

TABLE 3

Read-through SNPs related to human hereditary disorders

Column headings are as follows. Symbol, gene symbol; dbSNP ID, SNP database identifier; OMIM, online Mendelian inheritance in man identifier; Disease, description of genetic disease caused by read-through SNPs; Length, length of extended peptides encoded by read-through mutant genes. aa, amino acids.

Symbol	dbSNP ID	OMIM	Disease	Length
				aa
CRYM	104894509	123740	Deafness, autosomal dominant nonsyndromic	5
DBT	121965000	248610	Maple syrup urine disease, intermediate, type II	7
ITM2B	104894417	603904	Dementia, familial British	11
SH2D1A	111033625	300490	Lymphoproliferative syndrome, X-linked, 1	12
PAX6	121907922	607108	Aniridia	14
MOCS2	121908609	603708	Molybdenum cofactor deficiency B	18
CTSK	74315301	601105	Pycnodysostosis	19
FKRP	104894682	606596	Muscular dystrophy-dystroglycanopathy (limb-girdle), type C, 5	21
RUNX2	104893994	600211	Cleidocranial dysplasia	23
IKBKG	137853321	300301	Ectodermal dysplasia, anhidrotic, with immunodeficiency, osteopetrosis, and lymphedema	27
PNPO	104894631	603287	Pyridoxamine 5′-phosphate oxidase deficiency	28
HBA2	41321345	141850	Hemoglobin H disease, nondeletional	31
SHOX	137852559	312865	Leri-Weill dyschondrosteosis	48
NHP2	121908091	606470	Dyskeratosis congenita, autosomal recessive 2	51
FHL1	122459148	300163	Emery-Dreifuss muscular dystrophy 6, X-linked	52
RAD50	121912629	604040	Nijmegen breakage syndrome-like disorder	66
FOXF1	121909337	601089	Alveolar capillary dysplasia with misalignment of pulmonary veins	72
HSD3B2	80358218	201810	3-β-Hydroxysteroid dehydrogenase, type II, deficiency	95
FGFR3	121913101	134934	Thanatophoric dysplasia, type I	101
CLCF1	137853935	610313	Cold-induced sweating syndrome 1	170

proteins. siRNA-mediated knockdown of human TRIM21 increased the levels of PNPO+28, but not of HSD3B2+95 protein (Fig. 6*E*). Furthermore, the rapid turnover of PNPO+28 protein was suppressed by knockdown of human *TRIM21* (Fig. 6*F*), and TRIM21 strongly interacted with PNPO+28 in the cells (Fig. 6*G*). These results suggest that TRIM21 plays a role in the ubiquitylation of PNPO+28 but that another E3 ligase is responsible for the ubiquitylation of HSD3B2+95 protein.

Discussion

Currently, 20 stop codon read-through mutations are related to hereditary disorders in humans; however, it is not understood how the C-terminally extended read-through mutant proteins are regulated. In this study, we analyzed a read-through mutant of mouse *cFlip-l* as a model system and showed that the homozygous read-through mutant embryos undergo extensive apoptosis in the liver. The C-terminally extended peptide interacts with a ubiquitin ligase TRIM21 and promotes ubiquitylation and proteasomal degradation of cFLIP-L+46 protein. We also showed that the C-terminally extended proteins of human PNPO and HSD3B2 encoded by read-through mutant genes found in patients are degraded by a similar mechanism involving TRIM21 for PNPO degradation.

PNPO plays an essential role in brain metabolism, catalyzing the conversion of pyridoxine 5′-phosphate and pyridoxamine 5′-phosphate to pyridoxal 5′-phosphate, a metabolically active form of vitamin B6 (15). Patients with homozygous read-through mutation in the *PNPO* gene (X262Q) suffer from neonatal epileptic encephalopathy (16) and show no PNPO activity. The null PNPO activity can be explained by degradation of the extended PNPO+28 protein via the UPS. Because TRIM21 plays an important role in the degradation of the extended PNPO+28 protein (Fig. 6, *E* and *F*), inhibition and/or suppression of TRIM21 could be a strategy to restore PNPO protein levels in these cases.

HSD3B2 catalyzes the oxidation and isomerization of Δ^5-3β-hydroxysteroid precursors into Δ^4-ketosteroids and is essential for the formation of progesterone, a precursor for all classes of steroid hormones. Deficiency in the activity of HSD3B2 in the adrenal cortex causes congenital adrenal hyperplasia (17). A read-through mutation in *HSD3B2* (X373C) was found in patients with congenital adrenal hyperplasia, resulting in dramatic reduction of HSD3B2 protein levels and activity (18). It is likely that the mutant protein is degraded by the UPS, although the E3 ligase involved is currently unknown.

Among 20 read-through mutant proteins that are related to hereditary disorders in humans, C-terminally extended PNPO+28 and HSD3B2+95 proteins are degraded by the UPS as well as mouse cFLIP-L+46 protein. Interestingly, the extended peptides of PNPO+28, HSD3B2+95, and cFLIP-L+46 share the Leu-Xaa-Xaa-Leu-Leu (L*XX*LL) sequence, whereas other extended peptides encoded by the mutant genes in Table 3 do not have the sequence. The L*XX*LL sequence is well known as a protein recognition motif widely used in transcriptional regulation (19). To investigate whether the L*XX*LL sequence is involved in the degradation of C-terminally extended proteins, we generated point or deletion mutants of the sequence in cFLIP-L+46, but the mutations did not affect the protein degradation of cFLIP-L+46 (data not shown). Therefore, the L*XX*LL sequence in the C-terminally extended proteins is not likely to play an important role in regulating the protein stability.

The 3′-UTR is known to regulate the stability of mRNAs and the translation (20) but is rarely considered to encode functional protein sequence. However, in some cases, functional peptides are encoded in the 3′-UTR, downstream of the canonical termination codon, and they can influence the function of the mother protein. Examples include an isoform of vascular endothelial growth factor A (VEGF-Ax) and a read-through mutant of CRYM. VEGF-Ax with a C-terminal 22-amino acid extension is generated by a programmed translational read-through mechanism depending on heterogeneous nuclear ribonucleoprotein A2/B1. The extended

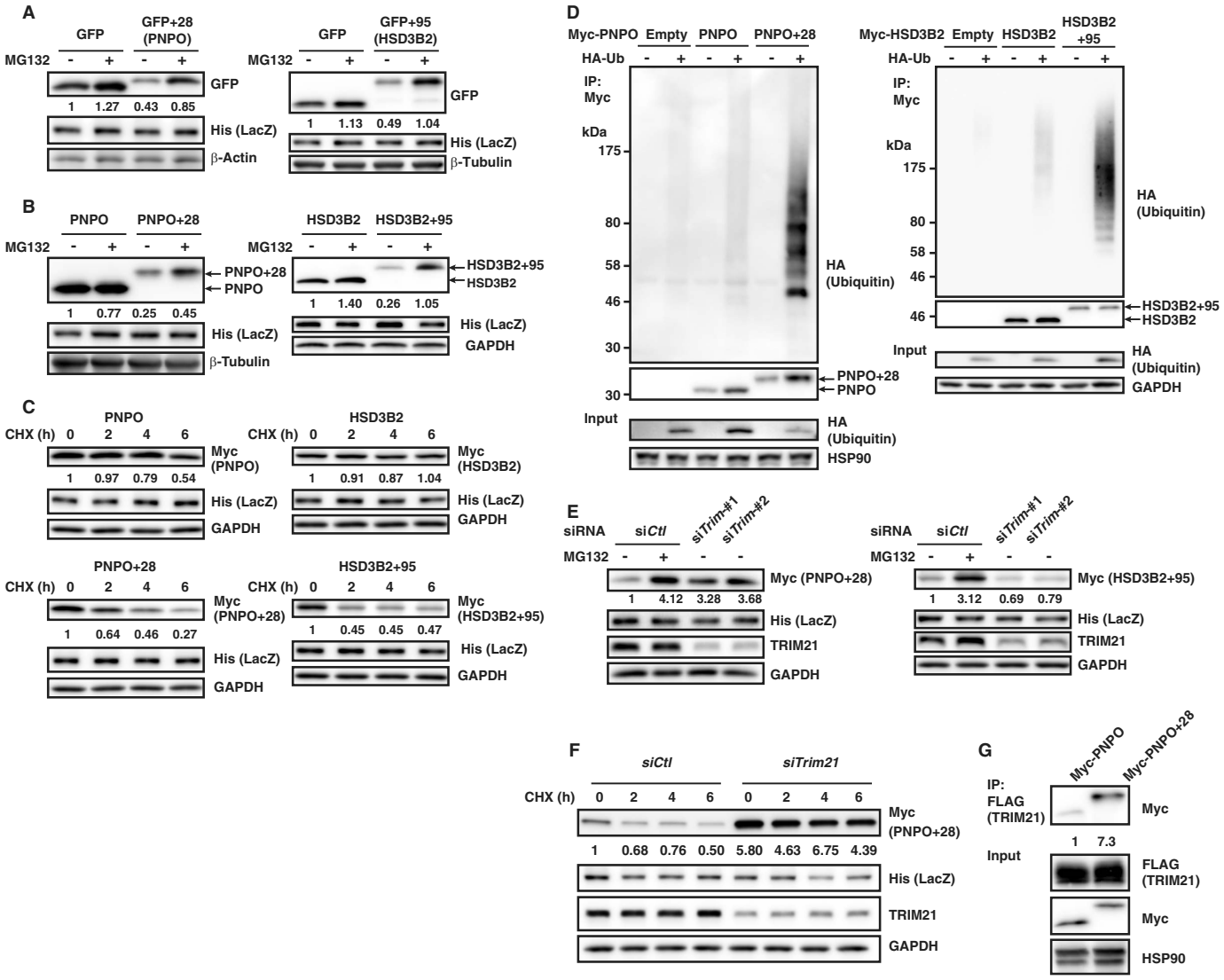

FIGURE 6. **Read-through PNPO and HSD3B2 mutant proteins are degraded through the UPS.** *A*, wild-type GFP or GFP fused to the extended peptides of PNPO (GFP+28) or HSD3B2 (GFP+95) were transfected into HeLa cells, and the cells were treated with 10 μM MG132 for 6 h. *B* and *C*, Myc-tagged wild-type (PNPO or HSD3B2) or read-through mutant (PNPO+28 or HSD3B2+95) genes were transfected into HeLa cells, and the cells were treated with 10 μM MG132 for 6 h (*B*) or were treated with 10 μg/ml CHX for the indicated times (*C*). *D*, enhanced ubiquitylation of the read-through mutant proteins. HeLa cells were transfected with Myc-tagged wild-type or read-through mutant genes together with HA-tagged ubiquitin (HA-Ub) and 48 h later were treated with 10 μM MG132 for 6 h. Ubiquitylation was analyzed as described under "Experimental Procedures." *E*, effect of *Trim21* knockdown on PNPO+28 and HSD3B2+95 levels. HeLa cells were transfected with Myc-PNPO+28 or Myc-HSD3B2+95 together with siRNAs against *Trim21*. *F*, effect of *Trim21* knockdown on PNPO+28 protein stability. HeLa cells were transfected with Myc-PNPO+28 together with siRNAs against *Trim21* and were treated with 10 μg/ml CHX for the indicated times. The cells (*A–C*, *E*, and *F*) were also co-transfected with His-LacZ, and the expression was measured as an internal control for transfection efficiency. *Numbers below* the *panels* represent relative expression levels normalized against His-LacZ expression. *G*, TRIM21 interacts with the extended peptide of human PNPO. HeLa cells were transfected with FLAG-tagged TRIM21 together with Myc-tagged PNPO or PNPO+28 and were treated with 10 μM MG132 for 6 h. Lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were analyzed by immunoblotting with an antibody against Myc. *Numbers* at the *bottom* represent relative amounts in the immunoprecipitates normalized against FLAG-TRIM21 expression together with PNPO or PNPO+28 expression in lysates. *IP*, immunoprecipitation.

peptide converts a proangiogenic VEGF-A into an anti-angiogenic VEGF-Ax protein (21). A read-through mutation in the *CRYM* gene is found in patients with non-syndromic deafness, and subcellular localization of CRYM protein is altered by the C-terminal extension (22). In addition to these observations, our study provides evidence for the destabilization of proteins by C-terminally extended peptides encoded in the 3′-UTRs of human *PNPO* and *HSD3B2*, which result in congenital diseases, and in mouse *cFlip-l*, which causes embryonic lethality. Because there are more read-through mutations known to be involved in human hereditary disorders (Table 3), further study will clarify the function of the extended peptides and how the C-terminally extended proteins are regulated.

Author Contributions—N. S. and M. N. designed the experiments and wrote the manuscript. Y. Sugaki performed bioinformatics analysis. N. S., N. O., Y. Sugaki, C. O., M. I., and M. N. performed the experiments. Y. Sakuraba and Y. G. generated cFlip-l+46 mutant mice. D. T., N. H., and N. K. performed LC/MS experiments. M. N. supervised all research. All authors approved the final version of the manuscript.

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Degradation of Stop Codon Read-through Mutant Proteins via the Ubiquitin-Proteasome System Causes Hereditary Disorders

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