Supplementary Information

The NMD Pathway is Disrupted in Inflammatory Myofibroblastic Tumors

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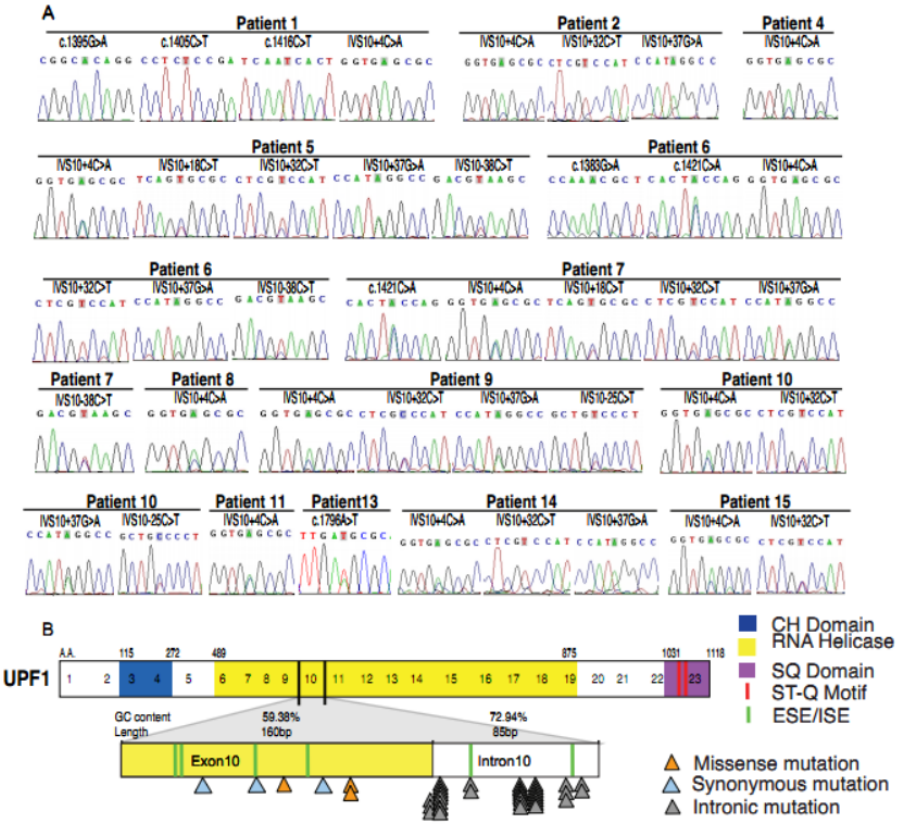
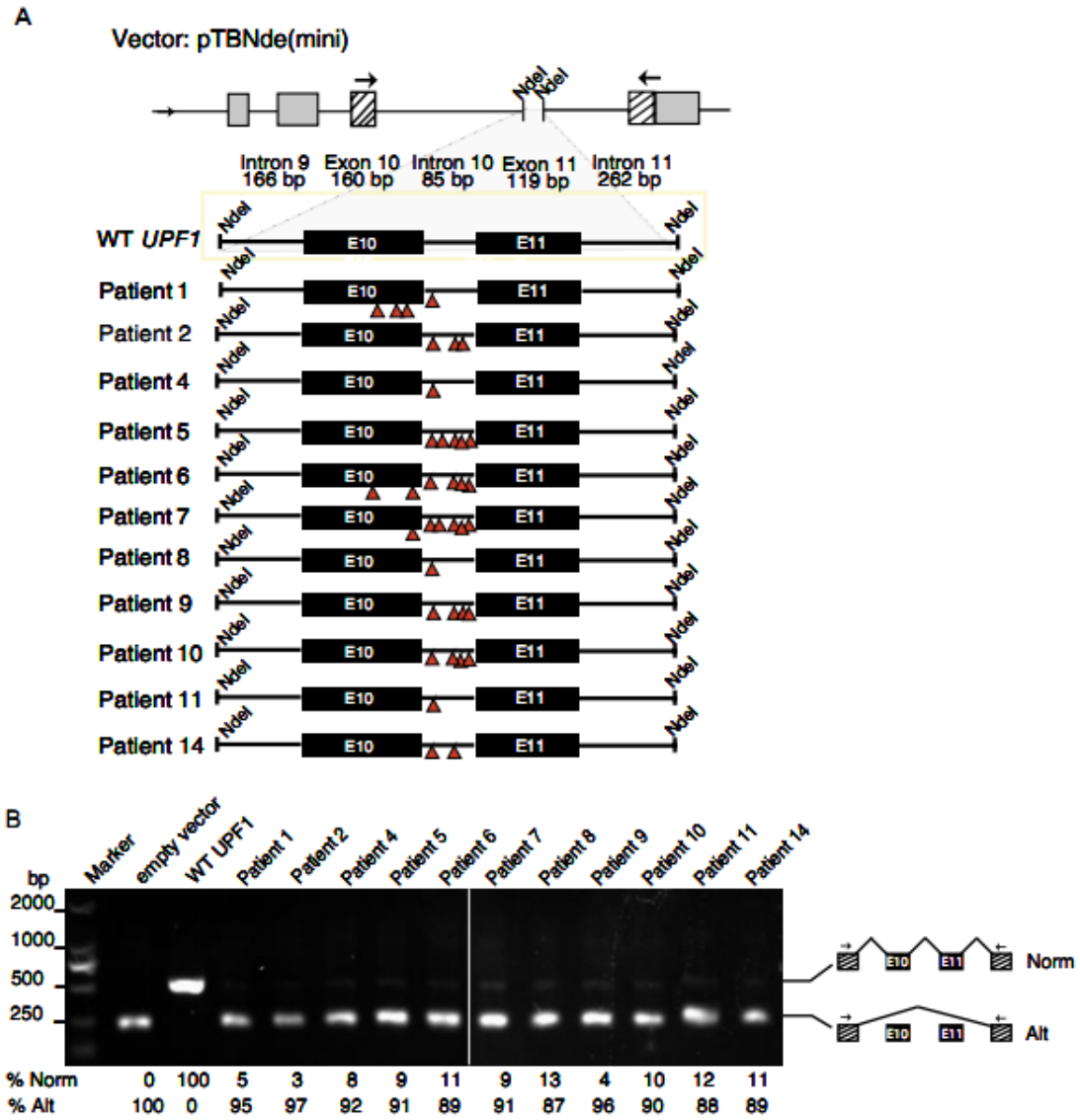
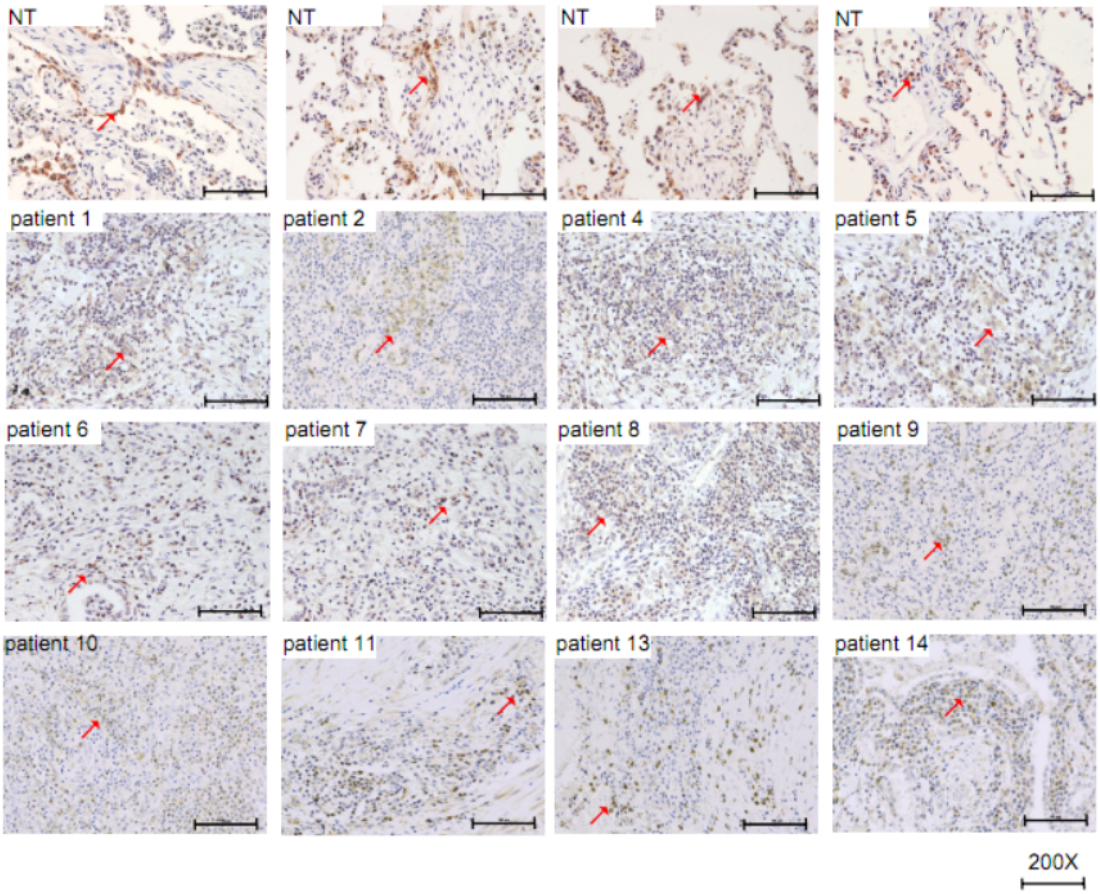


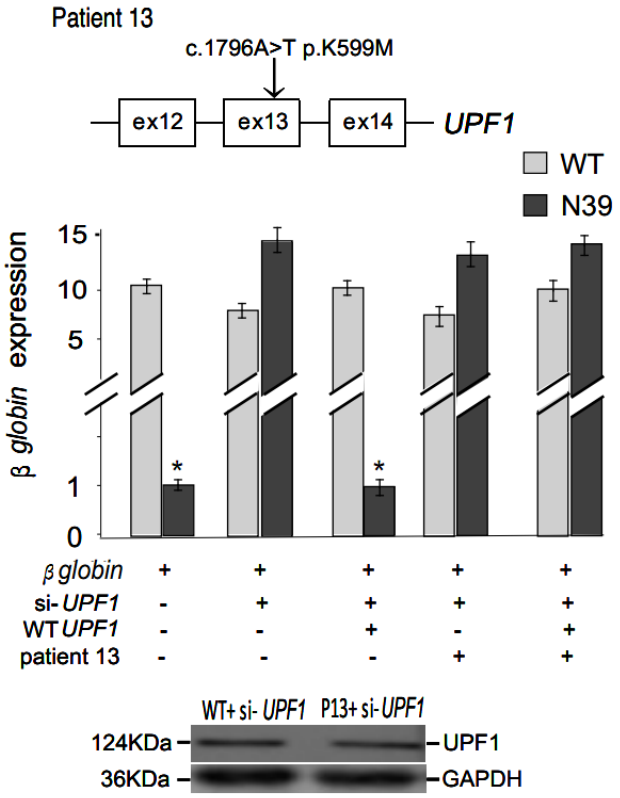
Supplementary Figure 1. Somatic *UPF1* mutations in pulmonary inflammatory myofibroblastic tumors (IMTs). (a) Chromatograms of *UPF1* DNA sequences from patient IMTs. A total of 41 single-base substitutions in genomic *UPF1* DNA were found in the IMTs of lung; each patient had between one to six point mutations. (b) Top: schematic of UPF1 protein domains. Bottom: location of the point mutations within the *UPF1* gene. ESEs and ISEs denote predicted exonic and intronic splicing enhancers, respectively.



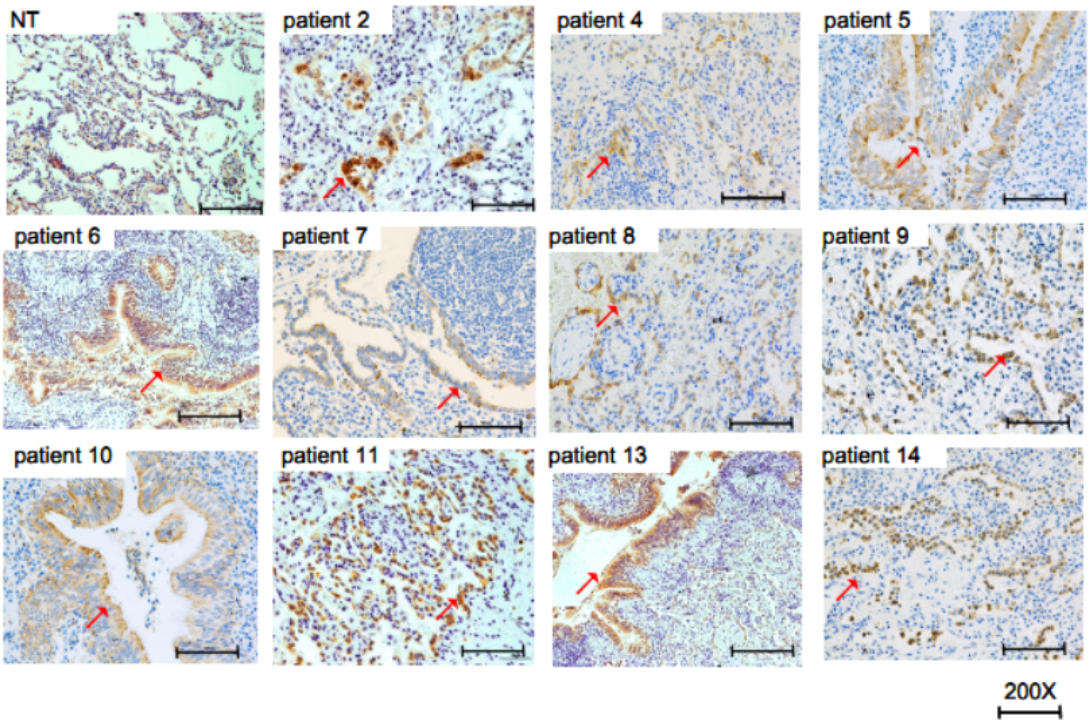
Supplementary Figure 2. *UPF1* mutations in patient IMTs trigger alternative *UPF1* splicing. (a) The indicated region of human *UPF1* (nt 22,779-23,570, RefSeq accession number NC_000019.9) was cloned into the *Nde*I site of the pTBNde minigene construct and mutations (denoted by the red arrows) were introduced to match those in patient 1, 2, 4-11 and 14. (b) RT-PCR analysis of HEK293 cells transfected with the constructs shown in panel a (primer locations are indicated by the arrows). Direct sequencing of the large (518 bp) and small (239 bp) bands indicated that they correspond to normally spliced and exon-skipped transcripts, respectively. The numbers below the gel are the average values (based on densitometry) from five independent transfections. The lanes were run on the same gel but were noncontiguous.



Supplementary Figure 3. UPF1 protein expression in patient IMTs. Immunohistochemical (IHC) analysis of IMTs from patients 1, 2, 4-11, and 13-14, and normal lung tissue (NT) from patients 1, 2, 4 and 5. Red arrows indicate selected areas of positive UPF1 antibody staining. The staining was mainly observed in the inflammatory cells, while lung epithelial cells exhibited weak or negative staining.



Supplementary Figure 4. The mutant UPF1 protein encoded by patient 13 has dominant-negative activity. Upper: RT-qPCR analysis of NMD activity, determined by comparing the level of wild-type (WT) *β-globin* mRNA with *β-globin* mRNA harboring a premature termination codon at codon position 39 (N39). N39 *β-globin* mRNA is degraded by NMD, while WT *β-globin* mRNA is not. Patient 13 has a missense mutation in *UPF1*, which we introduced by site-directed mutagenesis into a UPF1 expression vector. *β-globin* constructs expressing these mRNAs were transiently transfected into HEK293 cells, along with a WT UPF1 expression vector and/or a UPF1 vector expressing the K559M mutant (patient 13). UPF1 siRNA (si-UPF1) was co-transfected to deplete endogenous UPF1. Error bars indicate standard deviations from three independent experiments; the solid horizontal bar represents the median value for each group. *18S* mRNA was used as the endogenous control. Statistical analysis was performed using the Student's t test, *P < 0.05. Lower: Western blot analysis of UPF1 expression in HEK293 cells depleted of endogenous UPF1 by siRNA and transfected with WT or patient 13 (P13) UPF1 expression vectors. GAPDH is the endogenous control.



Supplementary Figure 5. NIK protein expression in patient IMTs. IHC analysis of IMTs from patients 2, 4-11, 13-14 and corresponding normal tissue (NT) of patient 2. Red arrows indicate selected areas of positive cytoplasmic NIK antibody staining. The staining was mainly observed in the lung epithelial cells while the inflammatory cells exhibited weak or negative staining for NIK.

Supplementary tables

Supplementary Table 1. Somatically Acquired *UPF1* Mutations in Patients with Pulmonary Inflammatory Myofibroblastic Tumors

Patient No.	Age (yr)	Sex	Location	No. of Mutations	Nucleotide Change	Predict Amino Acid Change	Mutation Type	Genotype
1	55	F	right upper	4	c.1395G>A		synonymous	homozygous
					c.1405C>T	p.P469S	missense	homozygous
					c.1416C>T		synonymous	homozygous
					IVS10+4C>A		intronic	homozygous
2	52	F	left lower	3	IVS10+4C>A		intronic	heterozygous
					IVS10+32C>T		intronic	homozygous
					IVS10+37G>A		intronic	heterozygous
3	45	M	left lower	0				
4	47	M	left lower	1	IVS10+4C>A		intronic	heterozygous
5	45	M	left upper	5	IVS10+4C>A		intronic	heterozygous
					IVS10+18C>T		intronic	heterozygous
					IVS10+32C>T		intronic	heterozygous
					IVS10+37G>A		intronic	heterozygous
					IVS10-38C>T		intronic	heterozygous
6	41	M	right lower	6	c.1383G>A		synonymous	heterozygous
					c.1421C>A	p.S474Y	missense	heterozygous
					IVS10+4C>A		intronic	heterozygous
					IVS10+32C>T		intronic	heterozygous
					IVS10+37G>A		intronic	heterozygous
					IVS10-38C>T		intronic	heterozygous
7	76	M	right upper	6	c.1421C>A	p.S474Y	missense	heterozygous
					IVS10+4C>A		intronic	heterozygous
					IVS10+18C>T		intronic	heterozygous
					IVS10+32C>T		intronic	heterozygous
					IVS10+37G>A		intronic	heterozygous
					IVS10-38C>T		intronic	heterozygous
8	73	M	left upper	1	IVS10+4C>A		intronic	heterozygous
9	66	M	right upper	4	IVS10+4C>A		intronic	heterozygous
					IVS10+32C>T		intronic	heterozygous
					IVS10+37G>A		intronic	heterozygous
					IVS10-25C>T		intronic	heterozygous
10	66	M	left lower	4	IVS10+4C>A		intronic	heterozygous
					IVS10+32C>T		intronic	heterozygous
					IVS10+37G>A		intronic	heterozygous
					IVS10-25C>T		intronic	heterozygous

11	60	M	right lower	1	IVS10+4C>A		intronic	heterozygous
12	68	M	left	0				
13	73	M	right lower	1	c.1796A>T	p.K599M	missense	heterozygous
					IVS10+4C>A		intronic	heterozygous
14	61	M	left upper	3	IVS10+32C>T		intronic	homozygous
					IVS10+37G>A		intronic	homozygous
15	50	F	right upper	2	IVS10+4C>A		intronic	heterozygous
					IVS10+32C>T		intronic	heterozygous

Supplementary Table 2. UPF1 and NIK Protein Expression in IMTs and Inflammatory Cells

		UPF1 IHC signal		NIK IHC signal	
		inflammatory	lung epithelial	inflammatory	lung epithelial
Patient No.	*UPF1* status	cells	cells	cells	cells
1	splicing mutation	+	−	−	+
2	splicing mutation	+	−	−	+
3	WT	+	+	−	−
4	splicing mutation	+	−	−	+
5	splicing mutation	+	−	−	+
6	splicing mutation	+	−	−	+
7	splicing mutation	+	−	−	+
8	splicing mutation	+	−	−	+
9	splicing mutation	+	−	−	+
10	splicing mutation	+	−	−	+
11	splicing mutation	+	−	−	+
12	WT	+	+	−	−
13	missense mutation	+	+	−	+
14	splicing mutation	+	−	−	+
15	splicing mutation	+	−	−	+

IHC: immunological histological chemistry

+, antibody reactivity in >30% of cells

-, antibody reactivity in <10% of cells

Supplementary Table 3. Evidence that NMD is Disrupted in IMTs

Gene	Function	Putative NMD-inducing feature	Relative increase	Reference
Asparagine synthetase (ASNS)	Asparagine biosynthesis	uORF	4.6	9
Cystathionine-g-lyase (CTH)	Cysteine biosynthesis	Unknown	3.5	9
Pyrroline-5-carboxylate reductase 1 (PYCR1)	Proline biosynthesis	Unknown	7.1	9
Phosphoglycerate dehydrogenase (PHGDH)	Serine biosynthesis	uORF	5.9	9
Cysteinyl-tRNA synthetase (CARS)	tRNA charging	Unknown	3.8	9,10
Seryl-tRNA synthetase (SARS)	tRNA charging	Unknown	4.5	9,10
Leucyl-tRNA synthetase,mitochondrial (LARS2)	tRNA charging	uORFs	6.4	9
Nephropathic cystinosis (CTNS)	Amino acid transport	uORF	5.3	9,10
Glutamate/neutral amino acid transporter (SLC1A4)	Amino acid transport	Unknown	6.5	9
Activating transcription factor 3 (ATF3)	Transcription factor	Alt. exon with PTC	5.1	9
Activating transcription factor 4 (ATF4)	Transcription factor	uORFs	6.1	9,10

RT-qPCR analysis of the expression ratio of the indicated NMD substrates in patient 15 IMT vs. NT. These endogenous NMD substrates were previously defined on the basis of being stabilized upon depletion of the NMD factor UPF1 in HeLa cells (*ASNS, CARS, SARS, ATF3, CTH, PYCR1, PHGDH, LARS2, CTNS, SLC1A4,* and *ATF4*) (9) or upregulated upon depletion of UPF1 and CHX treatment in mouse ES cells (*Ctns, Atf4, Sars,* and *Cars*) (10). The values shown reflect three replicates of RT-qPCR, using 18S rRNA as a normalization control.

Supplementary Table 4. *UPF1* DNA Sequencing Primers

Target	Sequence		Sequence	
exon1	f	GCAGTTCCTGCTCTAGGCTG	F	GCAGTTCCTGCTCTAGGCTG
	r	GATTGCAGGACCTGGGGCAG	R	TAAGCTCAGGCCGAGCCAGAG
exon2	f	GGCATGGACATGGCTCTGTG	F	GGTTAGACCAGCTGTGTGGG
	r	TCAGGGTGTCGGAGGTTGCT	R	CACCTCCTGACCGGTAACAG
exon3	f	CAGCAGGACTCTCCTTGGAG	F	TGCTCCTTATCCCCTCGGAG
	r	AGATCAGCGGGTGTGCCAAG	R	ACCCAGGTTTGTTAGCGTG
exon4	f	GGATGAGGTGTGACTGCCTC	F	ATGGACCGTGAACGGTACCG
	r	AACGCTCTCATGCCTCACCC	R	TGGAGGACAACTCCCTGGAG
exon5	f	CAGAGCTCAAGTGCACAGGG	F	ATGCAGGGCATGCCCCTTTG
	r	TCTCACTGGGTCAAGCCGTC	R	TCCCCGCAACCAGAAACCAC
exon6	f	CCTGTGTGGCATGGAGTTCC	F	AGGGTTCTCCTTGCAGGTGG
	r	ACATCAGCTCCCACAGCCTG	R	CTCTGGAAGCCTGAGGAGAG
exon7-8	f	ACGGCAGAGGCTTGCTGTAG	F	CTGTGCTGGAGGCTAACC
	r	AAGAGCAGTGGAGGGCGATG	R	AGCATGAGATGCTGGCCCAG
exon9	f	CGGCTCATGGTGAGGTAGAG	F	AGCGTTTGGTGCAGAGCCAG
	r	GAACCCCACTCCACAGTGAC	R	AGATGCCACAGGTGGCAACC
exon10-11	f	GTCACTGTGGAGTGGGGTTC	F	ACTCAGGATGTCGGAGAGGC
	r	AGAGAGCGGTAGGCACCATG	R	CACGTGCTCTCTTCGGTGTG
exon12-13	f	AGCCCAGGATGTTGAGGCTG	F	TCACACCACTGCACTCCAGC
	r	TTCCAGGCAGCGAGATGTCC	R	ACAACTGAGCGCAGAGAGGC
exon14-15	f	TCACCACAGCCTGGACCATG	F	TGTCTGGGAGGGACAGCTTG
	r	TGCCCTGACCTCAAGCAGTG	R	GAAGCTGATGGGCCAGTTCC
exon16-17	f	AGGACCTGCAGCACTGTAGC	F	TTGCCCTGTGTCTGAACTCA
	r	TCCTGGGCCATTCTGAGCTC	R	AGGAGACCAAGTGTGCCCGAG
exon18	f	TCTAGCCTGGGTGACAGAGC	F	GCCTGCTGGCTGATAGTGAC
	r	CTGCTCAGAGTCCTCTGACC	R	GGAAGTGAGGACCGATGAGC
exon19-20	f	GTGTGCAGGGTCAGTGGCTTG	F	TCTTGGACCGTCCTGTGAGA
	r	AAGCTGCAGCCATGGGAACG	R	ATCCCGGTGTGAGGACAAGG
exon21-23	f	ATCTTCAGCCTGGGCAGAGC	F	CAGGACAGATGTGCAGCTCC
	r	ATACCACCCCTCCAGTGCAG	R	CCCATCCTTCTCCTTGAGGC
exon24	f	GTACCTGTGGGGCTCAGGTC	F	TGCCCTTCTCCCTCCTGACAG
	r	GGGTTTGTCAAGGCTGCTGC	R	TACCCGGTGCATGCCTCTAC

Supplementary Table 5. RT-PCR Primers

Target	Sequence	
UPF1 cDNA E7-12	f	TCACTGTCAG GTGGGACCTG
	r	GATCTGGTTGTGCAGGGCCA
	F	CGCCTACTTC ACTTTGCCCA
	R	GTCCACGGCGATGTTGCTC
Minigene expression	F	CAACTTCAAGCTCCTAAGCCACTGC
	R	TAGGATCCGGTCACCAGGAAGTTGGTTAAATCA
Germline Cε transcripts	F	CGTCTCGGGTGCGTGGGC
	R	CAGGTGATCGTGGGCGACT

Supplementary Table 6. RT-qPCR Primers

Target	Sequence	
β-globin-NS39	F	TTTTCTCGAGACACCATGGTGCACCTGACTCCTG
	R	CTTAGGGTTGCCCATAACAG
WT β-globin	F	GCAACCCTAAGGTGAAGGCT
	R	GGACAGCAAGAAAGCGAGC
UPF1	F	GACG CAGGGCTACA TCTCCA
	R	CGTTGCTTAGCTCTTCCGCC
NIK	F	TTCATCGCTGGGTCCAAACA
	R	CAACACACACGGGCCATTTT
	R	GCACTTCTGTTCCTCGCAGA
IL-8	F	CACTGCGCCAACACAGAAAT
	R	GCTTGAAGTTTCACTGGCATC
CCL20	F	CTGCTACTCCACCTCTGCG
	R	TGCGCACACAGACAACTTTT
CXCL1	F	CTTGCCTCAATCCTGCATCCC
	R	CTCTGCAGCTGTGTCTCTCTT
CTH	F	TGCTTCAGGTTTAGCAGCCA
	R	TCAGATGCCACTTGCCTGAA
PYCR1	F	CAAGGGCTTCACAGCAGCA
	R	CCTTGTTGTGGGGTGTCAAC
PHGDH	F	TGCAAATCTGCGGAAAGTGC
	R	GATGACATCAGCGGTCACCT
CARS	F	CTGAAGATCTTTGGGGCCGT
	R	AGGGACTTTTTGCTCTCGGG
SARS	F	TGCAGCAAGACAATCGGAGA
	R	CGTCACACTTCAGGATGGCT
LARS2	F	AGCCTGTGAGCAGATCCAGA
	R	TGGCCCACCATTTAGCTGTC
CTNS	F	CGCTTTCTTGTGATCCGCAG
	R	GTCAGGTTCAGAGCCACGAA
ASNS	F	CATGGAAGACAGCCCCGATT
	R	CATCCAGAGCCTGAATGCCT
SLC1A4	F	GTGGCCTTGGCGTTCATCAT
	R	AGCTGCAACCACAAGATTGG
ATF3	F	TGATGCTTCAACACCCAGGC
	R	CCTGGCCTGGGTGTTGAAG

ATF4	F	AACCTCATGGGTTCTCCAGC
	R	GGTCATCTATACCCAACAGG
TRAF2	F	GCGCTGCGACCGTTGG
	R	CAGGCAGAAGGAGCAGTACC
18S	F	AGTCTAGAGCCACCGTCCAG
	R	CCTACCTAGAATGTGGCTG
NIK-uORF	F	TCTACAAGCTTGAGGCCGTG
	R	GGACCCAGCGATGAAAATGC

Supplementary Table 7. Construct Primers

Target	**Sequence**	
E10-11 Hybrid Mini-gene	F	GGAATTCCATATGCGTCTTCTCCCATCACTGCC
	R	GGAATTCCATATGACACGGAACTCACGCTGCTG
***NIK uORF* Hybrid**	F	AAGCTTGGGGCGACCACGGGCCGGGA
PGL3-control	R	CTTTATGTTTTTGGCGTCTTCCATGG

Supplementary Table 8. Primers for Site-directed Mutagenesis

Target	Sequence	
Mutagenic minigene	F	GGGAGGCCCTGTGCCGTGAAGCG
c.1395G>A	R	CGCTTCACGGCACAGGGCCTCCC
Mutagenic minigene	F	GGTTGAGGTCGGAGAGGCCCTGCGC
c.1405C>T	R	GCGCAGGGCCTCTCCGACCTCAACC
Mutagenic minigene	F	CCTGGGAGTGATTGAGGTCGGGGAGG
c.1416C>T	R	CCTCCCCGACCTCAATCACTCCCAGG
Mutagenic minigene	F	CACTCCCAGGTGAGCGCCGTCCTCA
IVS10+4C>A	R	TGAGGACGGCGCTCACCTGGGAGTG
Mutagenic minigene	F	CCGGCCCATGGACGAGGCCCCGC
IVS10+32C>T	R	GCGGGGCCTCGTCCATGGGCCGG
Mutagenic minigene	F	AGGCCCCGCGCACTGAGGACGGC
IVS10+18C>T	R	GCCGTCCTCAGTGCGCGGGGCCT
Mutagenic minigene	F	TCCCGGCCTATGGGCGAGGCCCG
IVS10+37G>A	R	CGGGGCCTCGCCCATAGGCCGGGA
Mutagenic minigene	F	CACGCACAGCTATGGCCGCCTCTCCG
IVS10-38C>T	R	CGGAGAGGCGGCCATAGCTGTGCGTG
Mutagenic minigene	F	GCCGTGAAGCGTTTGGGCAGCTGGCAC
c.1383G>A	R	GTGCCAGCTGCCCAAACGCTTCACGGC
Mutagenic minigene	F	CATAAACCTGGTAGTGGTTGAGGTCGGGGAGG
c.1421C>A	R	CCTCCCCGACCTCAACCACTACCAGGTTTATG
Mutagenic minigene	F	GGGGTTGGCAACACGCACAGCTGTGGC
IVS10-25C>T	R	GCCACAGCTGTGCGTGTTGCCAACCCC
Mutagenic *UPF1* plasmid	F	GGTACCGGGCCTTGATGCGCACCG
c.1796A>T	R	CGGTGCGCATCAAGGCCCGGTACC
Mutagenic *UPF1* plasmid	F	GGTTGAGGTCGGAGAGGCCCTGCGC
c.1405C>T	R	GCGCAGGGCCTCTCCGACCTCAACC
Mutagenic *UPF1* plasmid	F	CATAAACCTGGTAGTGGTTGAGGTCGGGGAGG
c.1421C>A	R	CCTCCCCGACCTCAACCACTACCAGGTTTATG
Mutagenic *NIK* plasmid	F	GCGCTTGGCCGGTTGAGCACAAGCC
c.-19A>T	R	GGCTTGTGCTCAACCGGCCAAGCGC

Supplementary Table 9. Small Interfering RNAs

Target Gene	Sequence
UPF1	GATGCAGTTCCGCTCCATT
	GAGAATCGCCTACTTCACT
	GCTCGCAGACTCTCACTTT
NIK	GTGTGAAAGTCCAAATACA
	CAGGCTGAGTGTGAGAATA
	CGCCAAATCAAGCCAATTA
con	GUANGZHOU RIBOBIO CO.,LTD

Supplemental Methods

DNA/RNA sequencing and analysis

Genomic DNA from the FFPE samples was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen). All *UPF1* coding regions and exon-intron junctions (RefSeq accession number NC_000019.9) were amplified by nested PCR using the primers described in Supplementary Table 4. Total RNA from the patient's samples and cell lines was extracted using Trizol (Invitrogen). To prevent contamination with genomic DNA, the samples were treated with RNase-free DNase I (Fermentas). The synthesis of complementary DNA (cDNA) was performed using the PrimeScriptTM RT reagent Kit (Takara). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with primers in Supplementary Table 5. Quantitative real-time reverse transcription PCR (RT-qPCR) analysis was performed using the relative quantification method (ΔΔCT) in a RotorGene RG-3000 thermal cycler system (Corbett Research). *18S* mRNA was used as the endogenous control. Primers are described in Supplementary Table 6. All samples were evaluated in triplicate.

Constructs

Human *UPF1* expression vector was previously described (5). Human *NIK* corresponding to the 5' untranslated region (5' UTR, RefSeq accession number NM_003954.4) was amplified with the primers described in

Supplementary Table 7. The PCR products were cloned into the HindIII/NcoI site of the PGL3-control plasmid encoding firefly luciferase (Promega), and the NdeI site of the vector pTBNde (min), a gift from Dr. Francisco E. Baralle (International Centre for Genetic Engineering and Biotechnology, Italy). Site Directed Mutagenesis or point mutations were introduced by using the KOD-Plus-Neo kit (Toyobo) and the DpnI enzyme (Takara). Primer sequences are provided in Supplementary Table 8. Wild type *β-globin* construct and mutated *β-globin* construct with a nonsense codon at position 39 were gifts from Dr. Andreas E. Kulozik, University of Heidelberg, Germany.

Cell culture and transfections

HEK 293 (human kidney cells, ATCC, CRL-1573) and BEAS-2B (human bronchial cells, ATCC, CRL-9609) were maintained in DMEM containing 10% FBS. The cells were transfected using Lipofectamine 2000 (Invitrogen) and harvested for protein or RNA extraction 48h after transfection, or luciferase activity 24h after transfection. The relative luciferase activity was measured in passive lysis buffer (Promega) using the Dual-Luciferase Assay System and a GloMax 20/20 luminometer (Promega) according to the manufacturer's instructions. Data were normalized against the activity of the co-transfected pRL-TK plasmid encoding Renilla luciferase (Promega). The small interfering RNAs (siRNAs) transfected to deplete *UPF1* or *NIK* are described in Supplementary Table 9.

Protein analysis

Tissues were prepared with RIPA lysis buffer (Beyotime). The protein content was determined using the Bradford calorimetric assay (Shenergy Biocolor) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted on a membrane, and probed with antibodies (UPF1: rabbit monoclonal, abcam, ab109363 and ab92655, NIK: rabbit polyclonal, Santa Cruz, sc-7211 and GAPDH: mouse monoclonal, Beyotime, AG019) at 1:500 dilution. Conjugated affinity anti-mouse, or anti-rabbit IgG IRDye 800 (Rockland Immunochemicals), was used for secondary detection and imaged with Odyssey Infrared Imaging System (LI-COR Biosciences).

IHC

Sections were deparaffinized two times in xylene, followed by serial dilutions of ethanol. After heat-induced antigen retrieval in antigen unmasking solution (Vector Laboratory), the internal peroxidase activity was quenched by incubation with 3% hydroperoxide in methanol for 15min. Sections were blocked in Avidin solution for 15min, incubated in Biotin solution for 15min (Vector Laboratory), incubated in 5% bovine serum albumin and 20% goat serum in PBS for 1h. Sections were then incubated overnight at 4℃ with primary antibodies against UPF1 (rabbit monoclonal, Abcam, ab109363 and ab92655), IgE (mouse monoclonal, Santa Cruz, sc-52335) or NIK (rabbit polyclonal, Santa Cruz, sc-7211) at a 1:100 dilution.