The American Journal of Human Genetics, Volume *100*

Supplemental Data

A Ribosomopathy Reveals Decoding Defective Ribosomes Driving Human Dysmorphism

Nahuel A. Paolini, Martin Attwood, Samuel B. Sondalle, Carolina Marques dos Santos Vieira, Anita M. van Adrichem, Franca M. di Summa, Marie-Françoise O'Donohue, Pierre-Emmanuel Gleizes, Swaksha Rachuri, Joseph W. Briggs, Roman Fischer, Peter J. Ratcliffe, Marcin W. Wlodarski, Riekelt H. Houtkooper, Marieke von Lindern, Taco W. Kuijpers, Jonathan D. Dinman, Susan J. Baserga, Matthew E. Cockman, and Alyson W. MacInnes

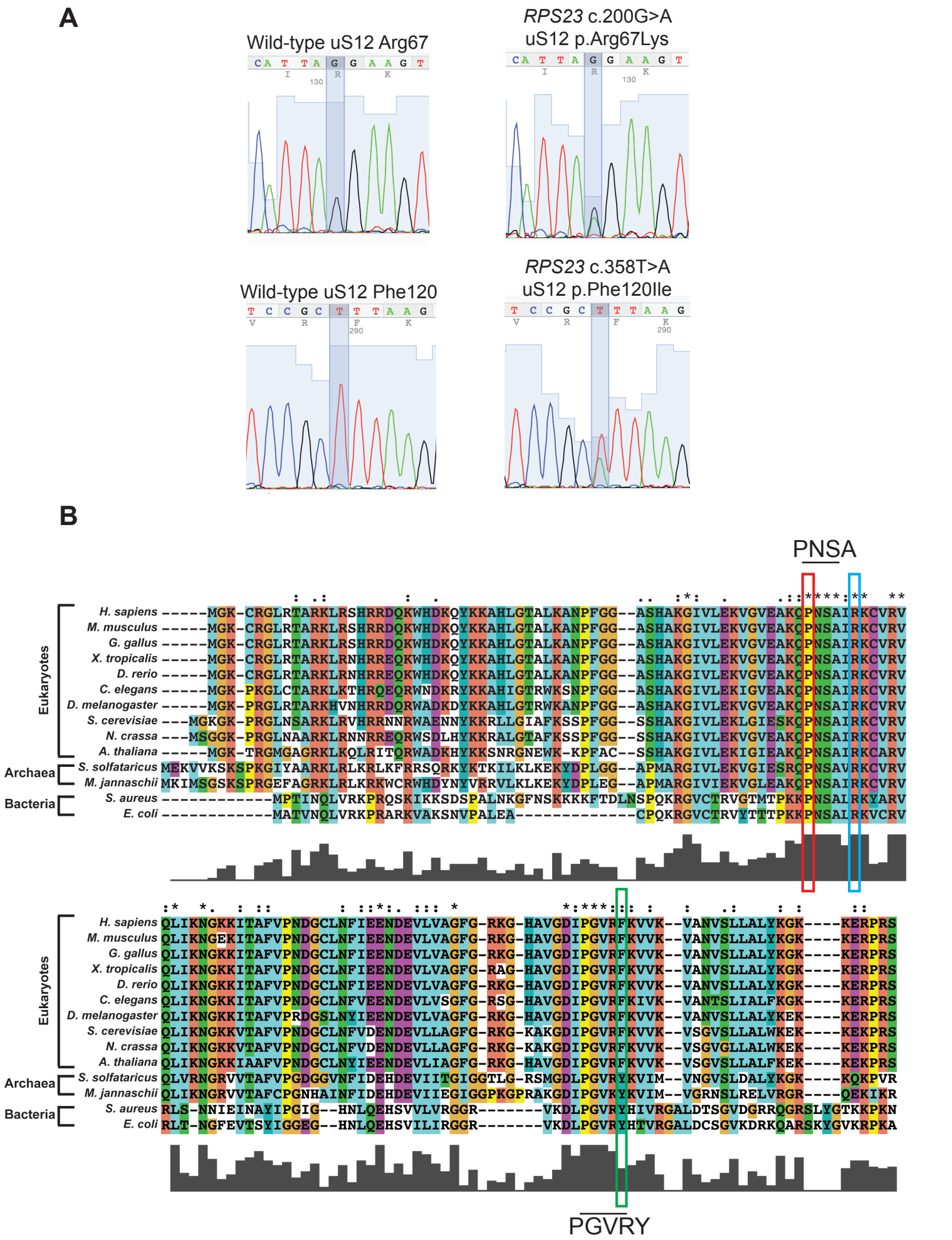
A
Wild-type uS12 Arg67
RPS23 c.200G>A
uS12 p.Arg67Lys
Wild-type uS12 Phe120
RPS23 c.358T>A
uS12 p.Phe120Ile
B
PNSA
PGVRY
Eukaryotes
Archaea
Bacteria
H. sapiens
M. musculus
G. gallus
X. tropicalis
D. rerio
C. elegans
D. melanogaster
S. cerevisiae
N. crassa
A. thaliana
S. solfataricus
M. jannaschii
S. aureus
E. coli

Figure S1 (Related to Figure 1). Multiple sequence alignment for ribosomal protein uS12 in all domains of life reveals substitutions occur in highly conserved amino acids. (A) Sanger sequencing of human fibroblasts from individuals reported with *RPS23* gene mutations reveal double peaks corresponding to Arg67 and Phe120 (right panels) compared to single peaks in healthy control cells (left panels). **(B)** The multiple sequence alignment was generated using ClustalX 2.1. Below the alignment is the quality curve plotting the conservation score for each residue. The symbols above residues in the alignment are defined as follows: "*" indicates positions which have a single, fully conserved residue; ":" indicates a strongly conserved residue; "." indicates a weakly conserved residue. Strong and weak are defined as a score of > 0.5 for strong and < 0.5 for weak according to the Gonnet Pam250 matrix. A red box outlines the invariant proline residue hydroxylated by OGFOD1. A blue box outlines the invariant arginine that is mutated to a lysine in uS12 in Individual 1. The phenylalanine that is mutated to an isoleucine in uS12 in Individual 2 is outlined by a green box, is invariant in Eukaryotes, and strongly conserved in Archaea and Bacteria which have a tyrosine at this residue. The locations of the PNSA and PGVRY domains in bacteria are labeled.

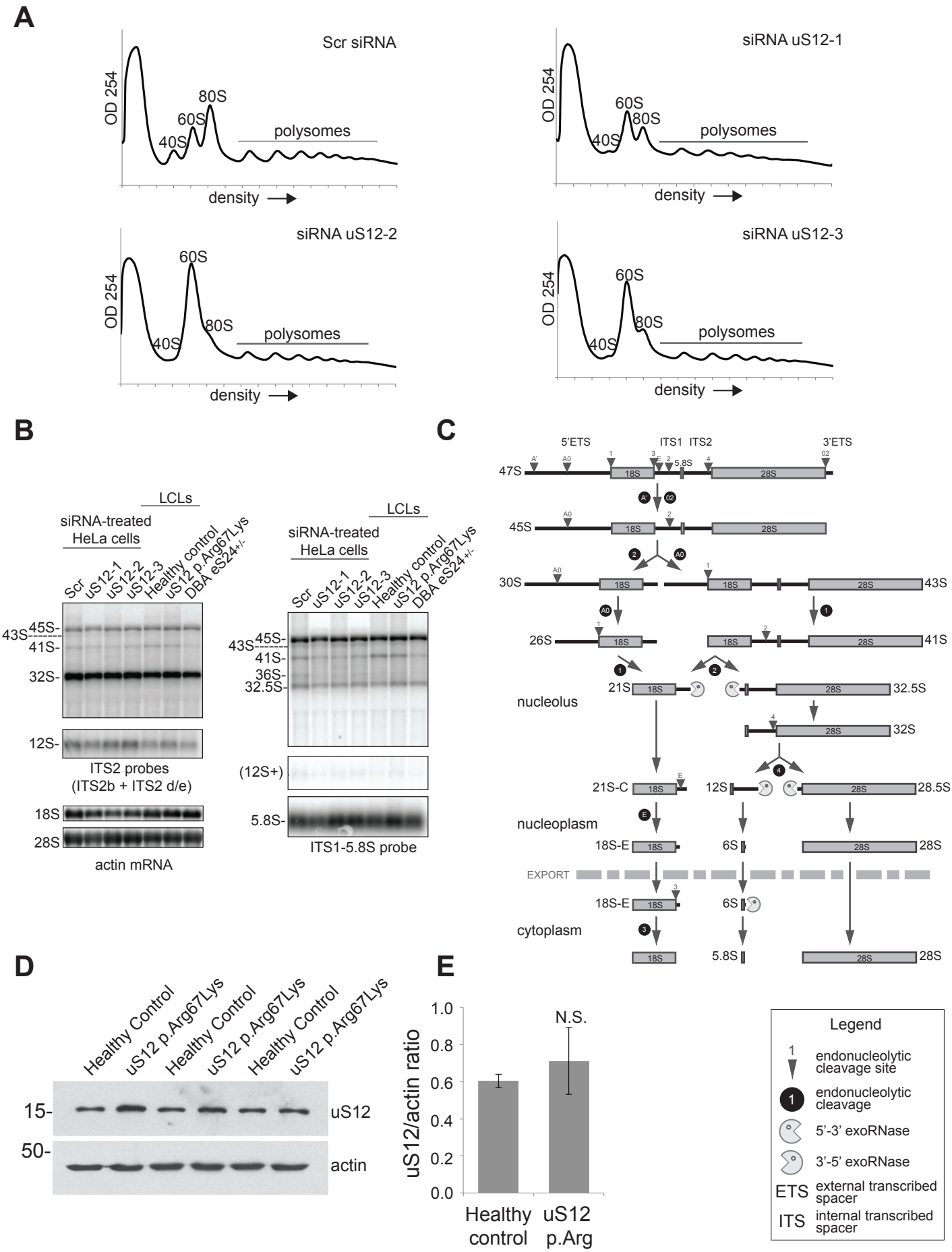
A
Scr siRNA
OD 254
40S
60S
80S
polysomes
density
siRNA uS12-1
siRNA uS12-2
siRNA uS12-3
B
LCLs
siRNA-treated HeLa cells
Scr
uS12-1
uS12-2
uS12-3
Healthy control
uS12 p.Arg67Lys
DBA eS24+/-
43S
45S-
41S-
32S-
12S-
ITS2 probes
(ITS2b + ITS2 d/e)
18S
28S
actin mRNA
36S-
32.5S-
(12S+)
5.8S-
ITS1-5.8S probe
C
5'ETS
ITS1
ITS2
3'ETS
47S
45S
30S
26S
21S
21S-C
18S-E
nucleolus
nucleoplasm
EXPORT
cytoplasm
12S
6S
5.8S
32.5S
32S
28.5S
D
Healthy Control
uS12 p.Arg67Lys
15-
50-
uS12
actin
E
uS12/actin ratio
1.0
0.8
0.6
0.4
0.2
0.0
N.S.
Healthy control
uS12 p.Arg 67Lys
Legend
endonucleolytic cleavage site
endonucleolytic cleavage
5'-3' exoRNase
3'-5' exoRNase
ETS external transcribed spacer
ITS internal transcribed spacer

Figure S2 (Related to Figure 2). uS12 p.Arg67Lys causes a mild and late ribosome biogenesis defect. (A) Polysome profiles of HeLa cells transfected with a scrambled siRNA control or three different siRNAs against uS12. 40S, 60S, 80S peaks and polysomes are labeled. Note the reduction of the 40S peaks and concomitant increase of 60S peaks in all samples with siRNAs against uS12. **(B)** Northern blot analysis of rRNA processing in HeLa cells transfected with a scrambled (Scr) siRNA or siRNAs against uS12, as well as LCLs derived from a healthy control, the uS12 p.Arg67Lys individual, or a DBA individual with an eS24 mutation. ITS2 and ITS1-5.8S probes are shown along with actin as a loading control. **(C)** A schematic and legend of the rRNA processing pathway in mammalian cells. **(D)** Western blot analysis of uS12 protein levels in healthy control and uS12 p.Arg67Lys LCLs. 3 independent experiments are shown. N.S. = not significant. **(E)** Quantification of uS12 protein levels from (D).

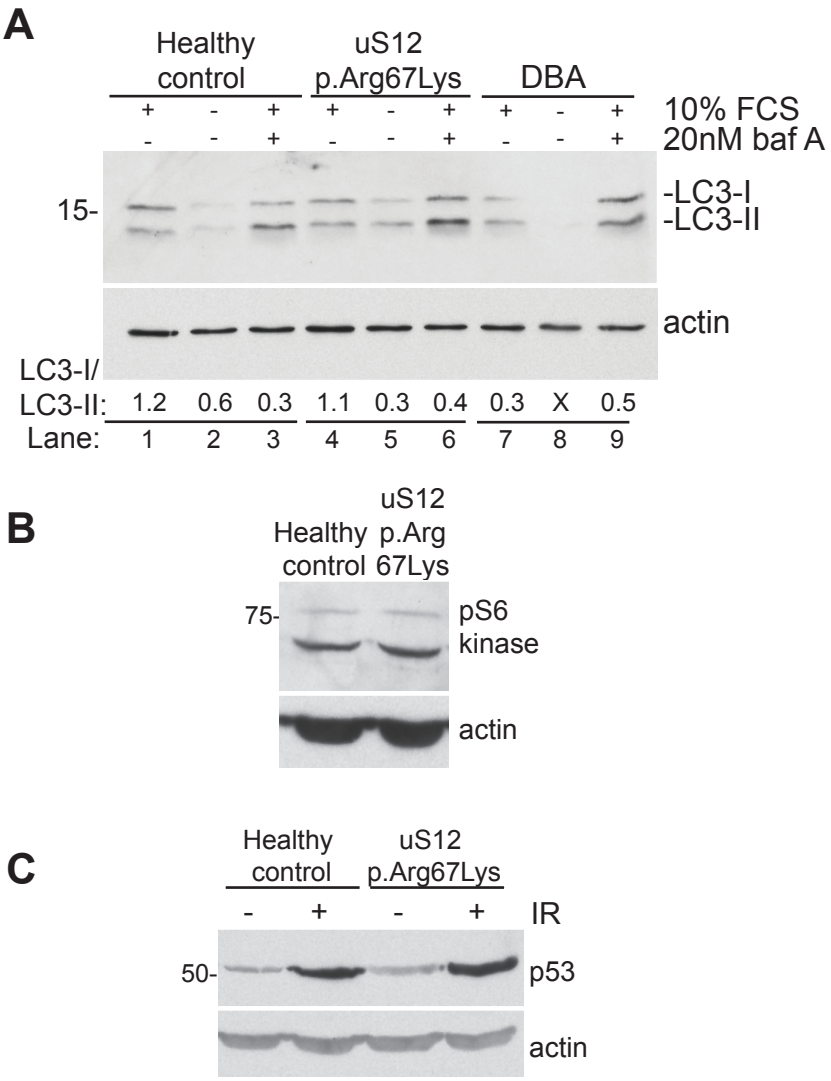

Figure S3 (Related to Figure 2). uS12 p.Arg67Lys does not induce autophagy, activate S6 kinase signaling, or stabilize p53. (A) Representative western blot analysis of LC3-I and LC3-II protein levels in fibroblasts derived from a healthy control, the uS12 p.Arg67Lys individual, or a DBA individual with a RP mutation. -10% FCS indicates samples starved of serum overnight. 20mM bafilomycin A was added for 6 hours to block autophagosome fusion with lysosomes and to provide a positive control for LC3-II accumulation. Ratios of LC3-I to LC3-II are calculated and listed. **(B)** Representative western blot analysis of S6 kinase phosphorylation in LCLs from the uS12 p.Arg67Lys individual or a healthy control. **(C)** Representative western blot analysis of p53 stabilization in LCLs with the uS12 p.Arg67Lys variant or from a healthy control either untreated or exposed to 25 Gy ionizing radiation (IR). In all cases biological triplicates were performed.

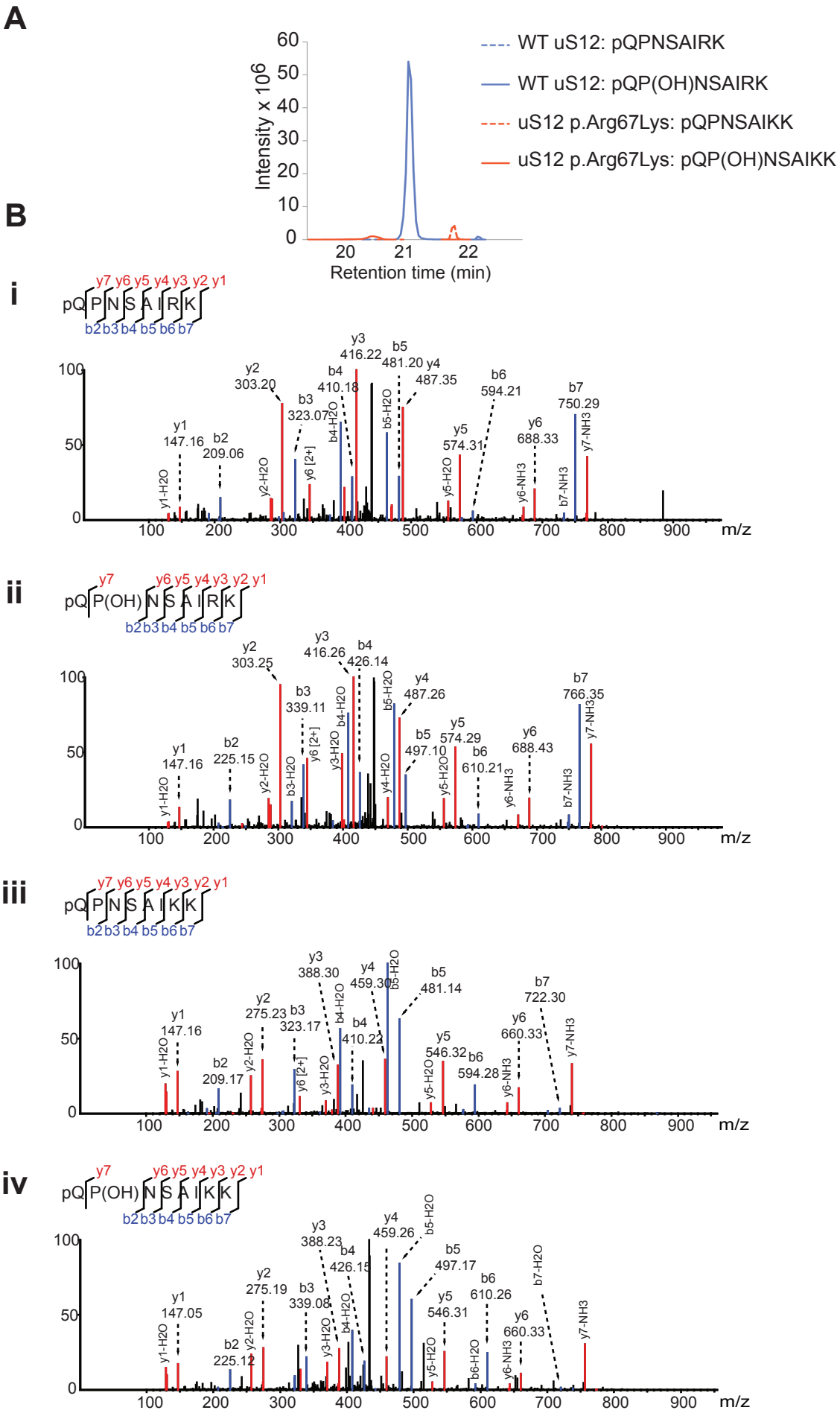
A
Intensity x 10^6
60
50
40
30
20
10
0
20
21
22
Retention time (min)
WT uS12: pQPNSAIRK
WT uS12: pQP(OH)NSAIRK
uS12 p.Arg67Lys: pQPNSAIKK
uS12 p.Arg67Lys: pQP(OH)NSAIKK
B
i
pQPNSAIRK
y7 y6 y5 y4 y3 y2 y1
b2 b3 b4 b5 b6 b7
y3 416.22
b5 481.20
y4 487.35
y2 303.20
b4 410.18
b6 594.21
b7 750.29
b3 323.07
y1 147.16
b2 209.06
y5 574.31
y6 688.33
y7-NH3
y1-H2O
y2-H2O
y6 [2+]
b4-H2O
b5-H2O
y5-H2O
y6-NH3
b7-NH3
100
50
0
100
200
300
400
500
600
700
800
900
m/z
ii
pQP(OH)NSAIRK
y7 y6 y5 y4 y3 y2 y1
b2 b3 b4 b5 b6 b7
y3 416.26
b4 426.14
y2 303.25
b3 339.11
y4 487.26
b7 766.35
y5 574.29
b5 497.10
y6 688.43
b6 610.21
y7-NH3
y1 147.16
b2 225.15
y1-H2O
y2-H2O
b3-H2O
y6 [2+]
y3-H2O
b4-H2O
b5-H2O
y4-H2O
y5-H2O
y6-NH3
b7-NH3
100
50
0
100
200
300
400
500
600
700
800
900
m/z
iii
pQPNSAIKK
y7 y6 y5 y4 y3 y2 y1
b2 b3 b4 b5 b6 b7
y3 388.30
y4 459.30
b5 481.14
b7 722.30
y2 275.23
b3 323.17
b4 410.22
y6 660.33
y1 147.16
y5 546.32
b6 594.28
y7-NH3
b2 209.17
y1-H2O
y2-H2O
y6 [2+]
y3-H2O
b5-H2O
y5-H2O
y6-NH3
100
50
0
100
200
300
400
500
600
700
800
900
m/z
iv
pQP(OH)NSAIKK
y7 y6 y5 y4 y3 y2 y1
b2 b3 b4 b5 b6 b7
y3 388.23
y4 459.26
b5-H2O
b4 426.15
b5 497.17
b7-H2O
y2 275.19
b3 339.08
b6 610.26
y5 546.31
y6 660.33
y7-NH3
y1 147.05
b2 225.12
y1-H2O
y2-H2O
y3-H2O
b4-H2O
y5-H2O
b6-H2O
y6-NH3
100
50
0
100
200
300
400
500
600
700
800
900
m/z

Figure S4 (Related to Figure 4). Comparative assessment of wild-type and variant uS12 hydroxylation in LCLs. (A) Exemplar chromatogram indicating elution time and intensity of extracted ion masses corresponding to the following pyro-Glu (pQ) modified $[M+H]^{2+}$ $uS12_{61-68}$ species: non-hydroxylated wild type (dashed blue line; m/z 448.751); hydroxylated wild type (solid blue line; m/z 456.749); non-hydroxylated p.Arg67Lys (dashed red line; m/z 434.748); hydroxylated p.Arg67Lys (solid red line; m/z 442.746). See Table S1 for summary of peptide level counts used for relative quantitation in Figure 4. **(B)** Corresponding uS12 fragmentation spectra **(i)** wild type non-hydroxylated; **(ii)** wild type hydroxylated; **(iii)** p.Arg67Lys non-hydroxylated; **(iv)** p.Arg67Lys hydroxylated.

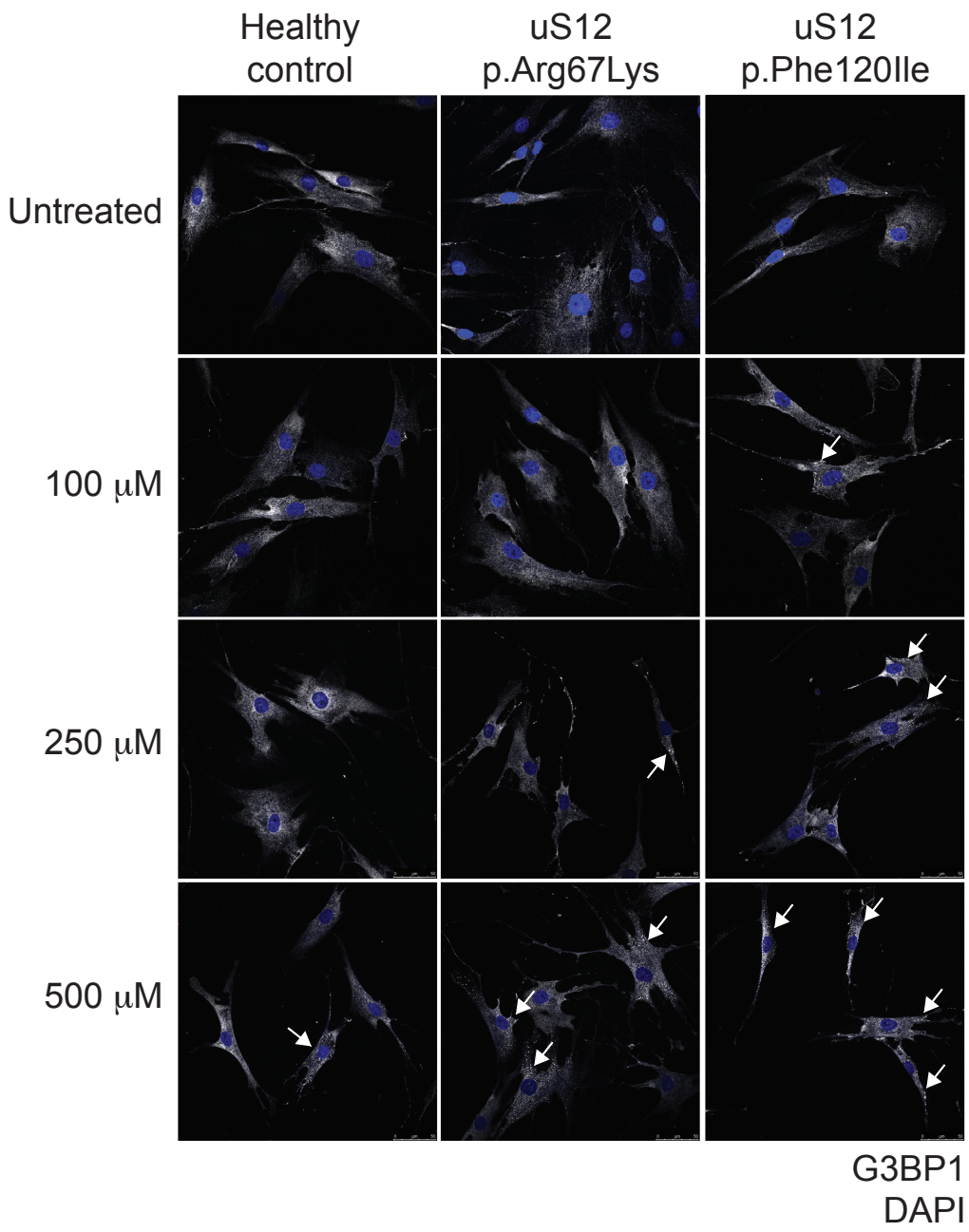

Figure S5 (Related to Figure 5). The uS12 variants increase stress granule formation in fibroblasts. Confocal microscopy of fibroblasts either untreated or treated with 100, 250, or 500 μM arsenite for 30 minutes, stained with antibodies against G3BP1 (grey), and labeled with DAPI (blue). Arrows indicate stress granule positive cells.

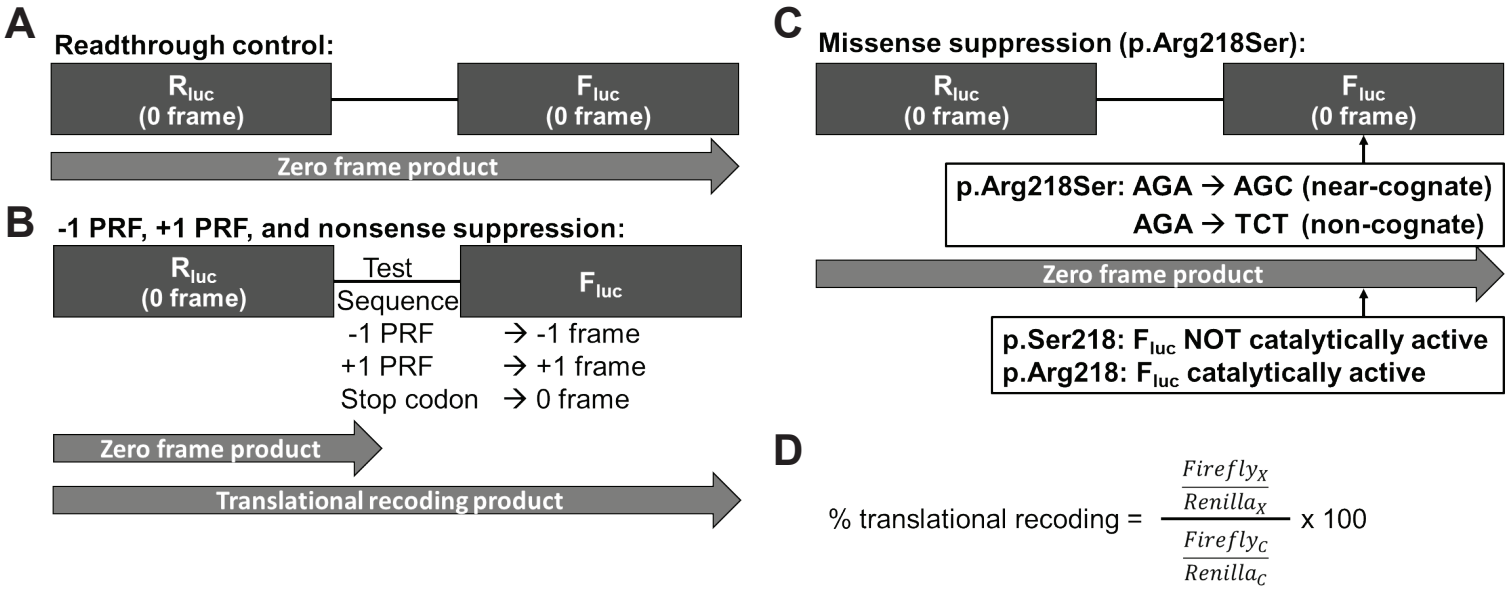

Figure S6 (Related to Figure 5). Translational fidelity dual luciferase reporters used to assay: (A) Readthrough control; **(B**) -1 PRF, +1 PRF, and nonsense suppression; and **(C)** missense suppression. **(D)** Formula to calculate translational recoding efficiency. x stands for experimental and c for control.

Peptide		Patient #1		Patient #2		Patient #3		Patient #4		Patient: #5		Control #1		Control #2		Control #3	
		XIC	% OH	XIC	% OH	XIC	% OH	XIC	% OH	XIC	%OH	XIC	%OH	XIC	%OH	XIC	%OH
Arg67Lys$_{(61-67)}$: QPNSAIK	P62	**4.4E+07**	23.76	1.7E+07	7.75	**4.3E+07**	16.22	3.4E+06	56.18	2.8E+06	30.00	ND		ND		ND	
	P62(OH)	1.4E+07		1.4E+06		8.2E+06		4.4E+06		1.2E+06		ND		ND		ND	
WT$_{(61-67)}$: QPNSAIR	P62	**1.6E+08**	97.62	**1.8E+08**	91.74	**1.1E+08**	93.64	**8.9E+06**	96.43	**4.0E+06**	95.42	**7.4E+06**	99.57	**2.1E+07**	99.37	**5.0E+06**	99.36
	P62(OH)	**6.4E+09**		**2.0E+09**		**1.6E+09**		**2.4E+08**		8.4E+07		**1.7E+09**		**3.3E+09**		**7.7E+08**	
Arg67Lys$_{(61-67)}$: QPN(+.98)SAIK	P62	**8.5E+06**		1.1E+06		4.1E+06		<LOD		<LOD		ND		ND		ND	
	P62(OH)	<LOD		<LOD		<LOD		<LOD		<LOD		ND		ND		ND	
WT$_{(61-67)}$: QPN(+.98)SAIR	P62	**1.2E+07**	98.66	**1.6E+07**	93.92	**1.2E+07**	95.22	**1.1E+05**	99.59	<LOD		**2.4E+05**	99.83	**5.1E+05**	99.84	**1.3E+05**	99.89
	P62(OH)	**9.2E+08**		**2.4E+08**		**2.4E+08**		**2.8E+07**		<LOD		**1.4E+08**		**3.2E+08**		**1.2E+08**	
Arg67Lys$_{(61-68)}$: QPNSAIKK	P62	**1.8E+07**	51.36	6.4E+06	32.62	**2.7E+07**	33.91	3.3E+06	49.19	4.1E+06	29.85	ND		ND		ND	
	P62(OH)	1.9E+07		3.1E+06		**1.4E+07**		**3.2E+06**		**1.7E+06**		ND		ND		ND	
WT$_{(61-68)}$: QPNSAIRK	P62	2.0E+06	99.41	**2.2E+07**	96.19	**1.3E+07**	97.01	1.1E+06	98.75	1.3E+06	97.25	1.1E+05	99.72	1.0E+06	99.60	<LOD	
	P62(OH)	**3.4E+08**		5.4E+08		**4.1E+08**		**8.5E+07**		**4.7E+07**		**4.0E+07**		**2.5E+08**		**7.7E+07**	
Arg67Lys$_{(61-68)}$: QPN(+.98)SAIKK	P62	6.9E+06	43.93	1.5E+06	17.70	**7.0E+06**	24.06	7.3E+05	34.45	<LOD		ND		ND		ND	
	P62(OH)	5.4E+06		3.2E+05		2.2E+06		3.8E+05		<LOD		ND		ND		ND	
WT$_{(61-68)}$: QPN(+.98)SAIRK	P62	5.4E+05	99.64	**3.9E+06**	94.70	2.7E+06	94.80	2.2E+05	98.07	1.6E+05	98.04	1.1E+05	97.56	2.5E+05	99.63		
	P62(OH)	1.5E+08		6.9E+07		**4.9E+07**		1.1E+07		7.9E+06		4.2E+06		**6.8E+07**		1.9E+07	
Arg67Lys$_{(61-68)}$: Q(-17.03)PNSAIKK	P62	**5.6E+07**	45.27	**2.6E+07**	48.09	**3.6E+08**	18.21	**2.0E+07**	40.68	**2.8E+07**	19.18	ND		ND		ND	
	P62(OH)	**4.7E+07**		**2.4E+07**		**8.1E+07**		**1.4E+07**		**6.6E+06**		ND		ND		ND	
WT$_{(61-68)}$: Q(-17.03)PNSAIRK	P62	4.0E+06	99.56	**4.8E+07**	96.42	**8.0E+07**	96.76	**4.2E+06**	98.91	**6.7E+06**	98.57	1.5E+06	99.23	3.5E+06	99.57	6.7E+05	99.77
	P62(OH)	**9.0E+08**		**1.3E+09**		**2.4E+09**		**3.8E+08**		**4.6E+08**		**1.9E+08**		**8.2E+08**		**3.0E+08**	
Arg67Lys$_{(61-68)}$: Q(-17.03)PN(+.98)SAIKK	P62	**1.1E+07**	23.28	5.1E+06	46.59	**5.2E+07**	18.08	3.8E+06	33.58	4.4E+06	14.92	ND		ND		ND	
	P62(OH)	**3.2E+06**		**4.4E+06**		**1.2E+07**		**1.9E+06**		7.8E+05		ND		ND		ND	
WT$_{(61-68)}$: Q(-17.03)PN(+.98)SAIRK	P62	**1.8E+06**	99.36	**1.4E+07**	95.42	**1.4E+07**	96.60	**8.6E+05**	98.54	9.9E+05	97.96	2.5E+05	99.53	**1.4E+06**	99.33	4.0E+05	99.36
	P62(OH)	**2.7E+08**		**2.9E+08**		**3.9E+08**		**5.8E+07**		**4.8E+07**		**5.2E+07**		**2.1E+08**		**6.3E+07**	
Arg67Lys$_{(55-67)}$: VGVEAKQPNSAIK	P62	**1.1E+06**		<LOD		**7.6E+05**		<LOD		<LOD		ND		ND		ND	
	P62(OH)	<LOD		<LOD		<LOD		<LOD		<LOD		ND		ND		ND	
WT$_{(55-67)}$: VGVEAKQPNSAIR	P62	**3.2E+05**	98.17	**9.9E+05**	95.47	**1.4E+06**	95.51	6.6E+04	98.68	5.9E+04	99.37	2.9E+05	99.36	5.7E+04	99.62	5.9E+04	99.44
	P62(OH)	**1.7E+07**		2.1E+07		**3.0E+07**		4.9E+06		9.3E+06		**4.4E+07**		1.5E+07		1.1E+07	
Arg67Lys$_{(55-67)}$: VGVEAK(+43.01)QPNSAIK	P62	**3.2E+06**	44.90	<LOD		<LOD		<LOD		<LOD		ND		ND		ND	
	P62(OH)	**2.6E+06**		<LOD		<LOD		<LOD		<LOD		ND		ND		ND	
WT$_{(55-67)}$: VGVEAK(+43.01)QPNSAIR	P62	**7.1E+06**	97.64	**1.6E+06**	92.92	**1.7E+05**	95.47	<LOD		<LOD		**7.5E+05**	99.56	**2.3E+05**	99.27	5.0E+04	96.38
	P62(OH)	**2.9E+08**		**2.1E+07**		**3.6E+06**		**4.6E+05**		1.3E+06		**1.7E+08**		**3.1E+07**		**1.3E+06**	
Arg67Lys$_{(55-67)}$: VGVEAK(+57.02)QPNSAIK	P62	**7.8E+05**		<LOD		**7.6E+05**		<LOD		<LOD		ND		ND		ND	
	P62(OH)	<LOD		<LOD		<LOD		<LOD		<LOD		ND		ND		ND	
WT$_{(55-67)}$: VGVEAK(+57.02)QPNSAIR	P62	**5.4E+05**	97.16	**1.8E+06**	59.20	**2.4E+05**	95.93	**2.5E+05**	82.59	1.2E+05	74.80	**4.8E+05**	93.85	**9.0E+05**	71.39	2.2E+05	90.94
	P62(OH)	**1.8E+07**		**2.6E+06**		**5.8E+06**		**1.2E+06**		**3.5E+05**		**7.3E+06**		**2.2E+06**		**2.2E+06**	
Arg67Lys	TOTAL XIC P62	1.5E+08	37.75	5.6E+07	36.99	5.0E+08	19.00	3.2E+07	43.00	3.9E+07	20.87						
	TOTAL XIC P62(OH)	9.0E+07		3.3E+07		1.2E+08		2.4E+07		1.0E+07							
WT	TOTAL XIC P62	1.8E+08	98.06	2.9E+08	93.97	2.3E+08	95.67	1.6E+07	98.10	1.3E+07	98.01	1.1E+07	99.53	2.9E+07	99.43	6.5E+06	99.52
	TOTAL XIC P62(OH)	9.3E+09		4.5E+09		5.2E+09		8.1E+08		6.6E+08		2.4E+09		5.0E+09		1.4E+09	
	% Arg67Lys Incorporation	2.47		1.85		10.20		6.29		6.84							

Table S1 (Related to Figure 4). List of uS12 peptides identified and quantitated from individual and control LCLs by mass spectrometry. Key: XIC: Extracted ion chromatogram counts; ND: Not Detected – p.Arg67Lys Peptide not assigned by MS/MS in healthy control; <LOD: Below limit of detection or confounding co-eluting peptide interfering with quantitation; Ion intensities in bold assigned by MS/MS; Posttranslationallly modified peptides: N(+.98) deamidation, Q(-17.03) pyroGlu, K(+43.01) carbamylation, K(+57.02) carbamidomethylation.

Oligonucleotide name	Sequence (5’ to 3’)
F-deltaB	CTTACATGTTTAATATTGTAGATTCCAGAAGCAGGTAAGAACAACAAAC TACAAGAAAAGCGGATCCCCGGGTTAATTAA
R-deltaB	CTTAAACATATACATACACAGTAGAATTTTTCGAATTATGAAATCGTAA TATAACTCTGCGAATTCGAGCTCGTTTAAAC
F-5’rps23b	GCCTAGGTAGGTGGGCTAGC
R-inhis5	GCCAGATCTGTTTAGCTTGCC
F-inhis5	CAATCGTATGTGAATGCTG
R-3’rps23b	GTACAAGAGGTTGACTTCAAG
F-GAL	GTAATATCACAATGTTTCGACTAACGGTTACAGTACGTTAAATTAGATA CTGCCTATGAATTGACATATTGAATTCGAGCTCGTTTAAAC
R-GAL	CATACTTGTTTCTTCTGTGGACACGTAGCTTTCTAGCAGAGTTCAAAC CTCTTGGCTTACCTTTACCCATTTTGAGATCCGGGTTTT
F-inGAL	GGTAATTAATCAGCGAAGC
R-inrpS23a	CCTCTTGGCTTACCTTTAC
MutArg69Lys	CAAGCAACCTAACTCTGCTATCAAAAAGTGTGTTAGAGTTCAATTAA
MutArg69Glu	GAATCCAAGCAACCTAACTCTGCTATCGAGAAGTGTGTTAGAGTTCAA TTAATCAAG
MutArg69His	GAATCCAAGCAACCTAACTCTGCTATCCATAAGTGTGTTAGAGTTCAA TTAATCAAG

MutLys62Arg	GAAAAATTGGGTATCGAATCCAGGCAACCTAACTCTGCTATC
nested-F	CAGAAGGTCTGCTCTTGTGGCTGG
nested-R	GTCCTCGAGAGGATAACTTTCGTGC
GWF	GGGGACAAGTTTGTACAAAAAAGCAGGCTACAAAATGGGTAAAGGTAAGCCAAGAG
GWR	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATGATCTTGGCTTTTCCTTCTTT

Table S2 (related to Materials and Methods). Oligonucleotides used for yeast experiments.