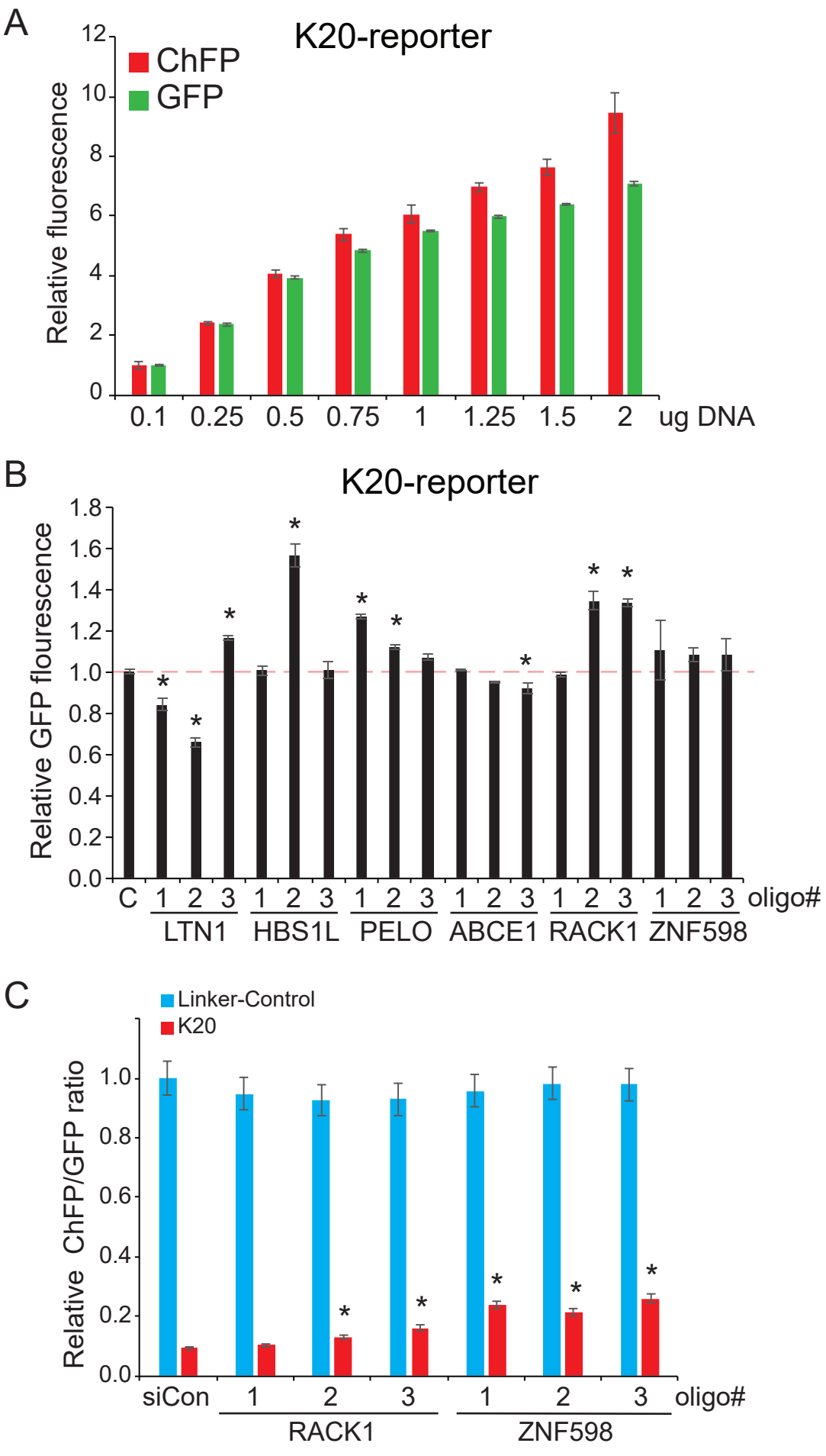
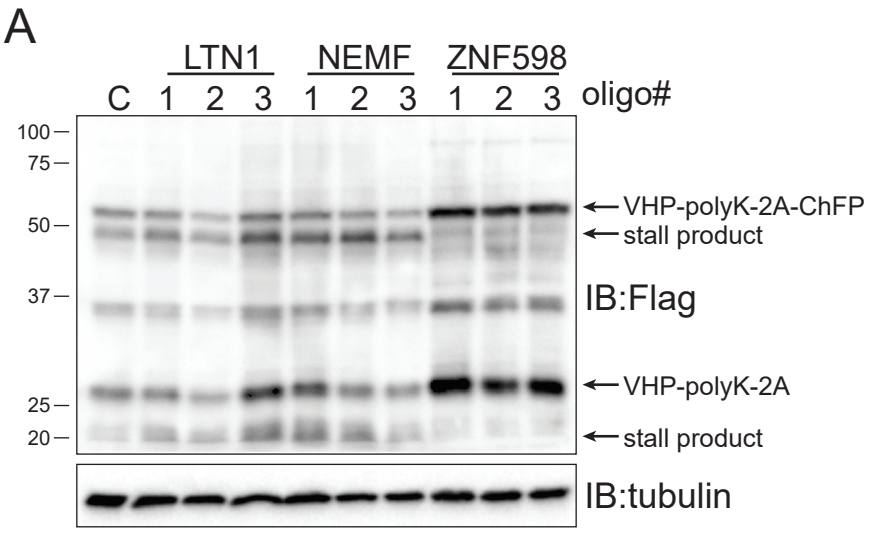
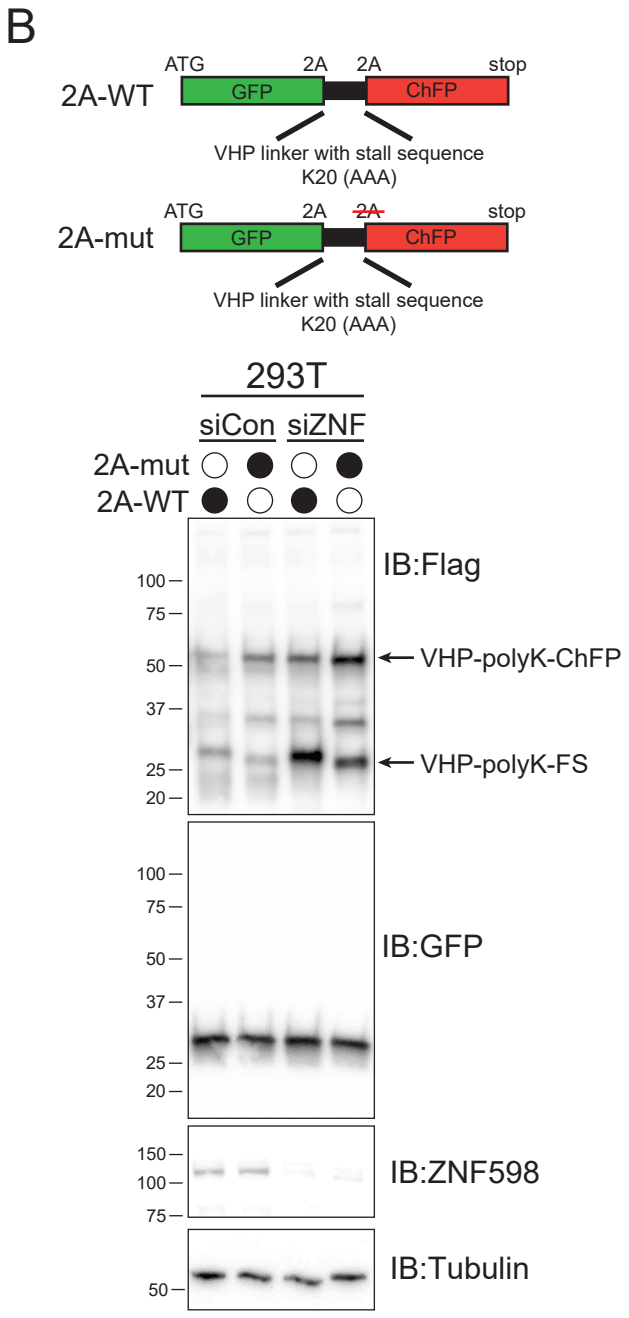
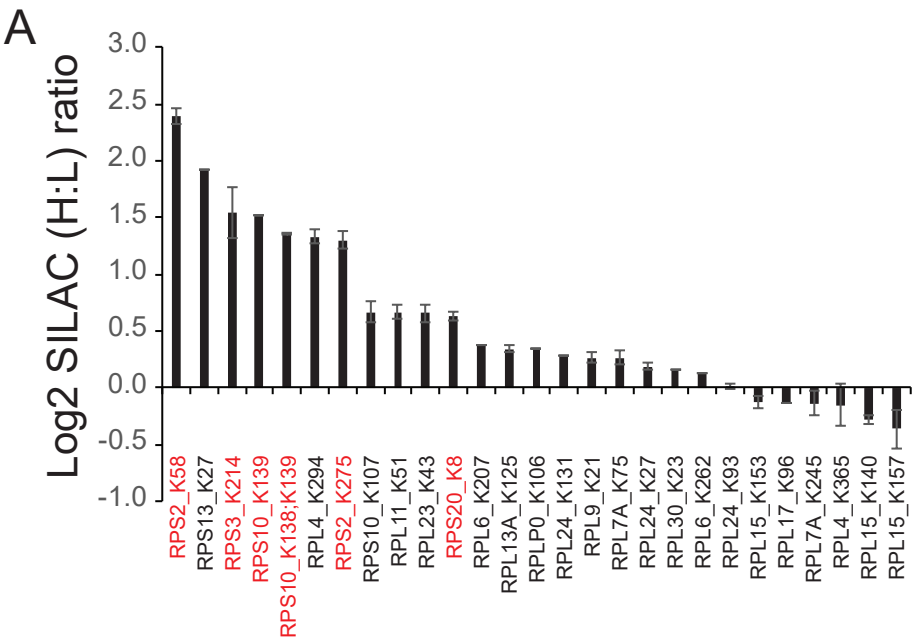
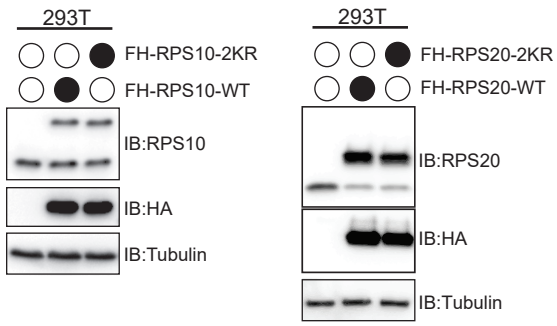
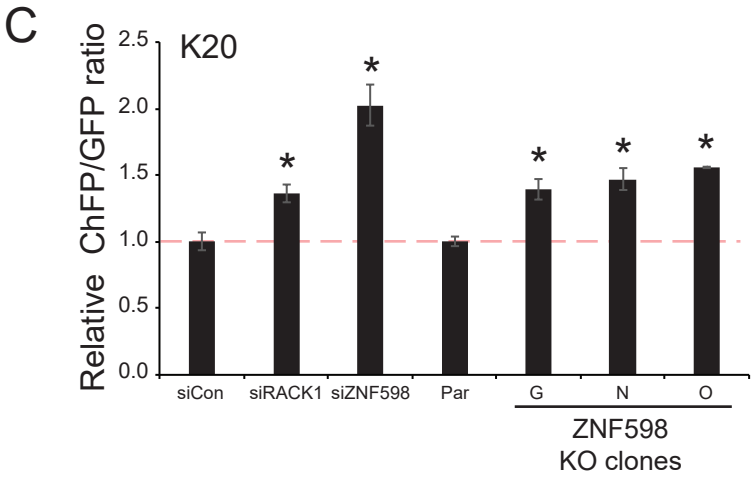

A
K20-reporter
ChFP
GFP
Relative fluorescence
0
2
4
6
8
10
12
0.1
0.25
0.5
0.75
1
1.25
1.5
2
ug DNA
B
K20-reporter
Relative GFP flourescence
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
C 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 oligo#
LTN1 HBS1L PELO ABCE1 RACK1 ZNF598
C
Linker-Control
K20
Relative ChFP/GFP ratio
0.0
0.2
0.4
0.6
0.8
1.0
siCon 1 2 3 1 2 3 oligo#
RACK1 ZNF598

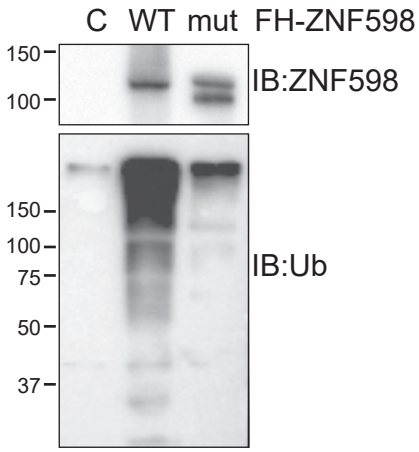

A
LTN1
NEMF
ZNF598
C 1 2 3 1 2 3 1 2 3 oligo#
100
75
50
37
25
20
VHP-polyK-2A-ChFP
stall product
IB:Flag
VHP-polyK-2A
stall product
IB:tubulin

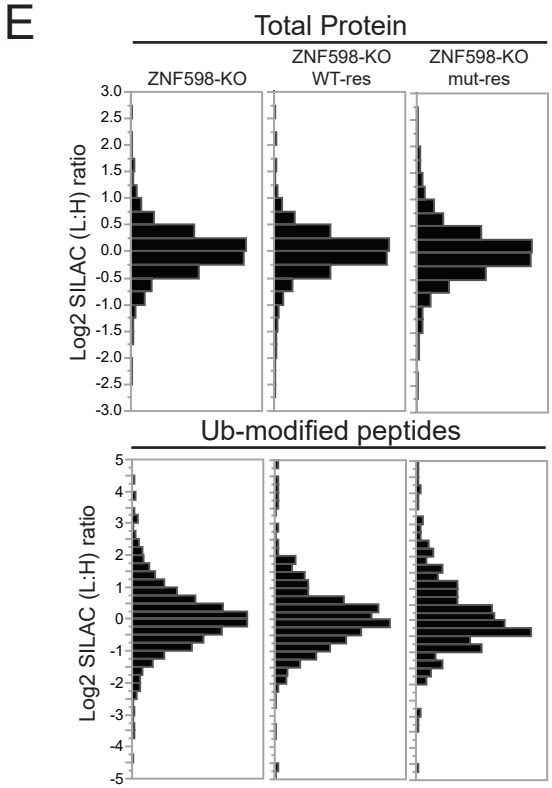

B
ATG 2A 2A stop
2A-WT
GFP
ChFP
VHP linker with stall sequence
K20 (AAA)
ATG 2A 2A stop
2A-mut
GFP
ChFP
VHP linker with stall sequence
K20 (AAA)
293T
siCon siZNF
2A-mut
2A-WT
100
75
50
37
25
20
IB:Flag
VHP-polyK-ChFP
VHP-polyK-FS
IB:GFP
150
100
75
50
IB:ZNF598
IB:Tubulin

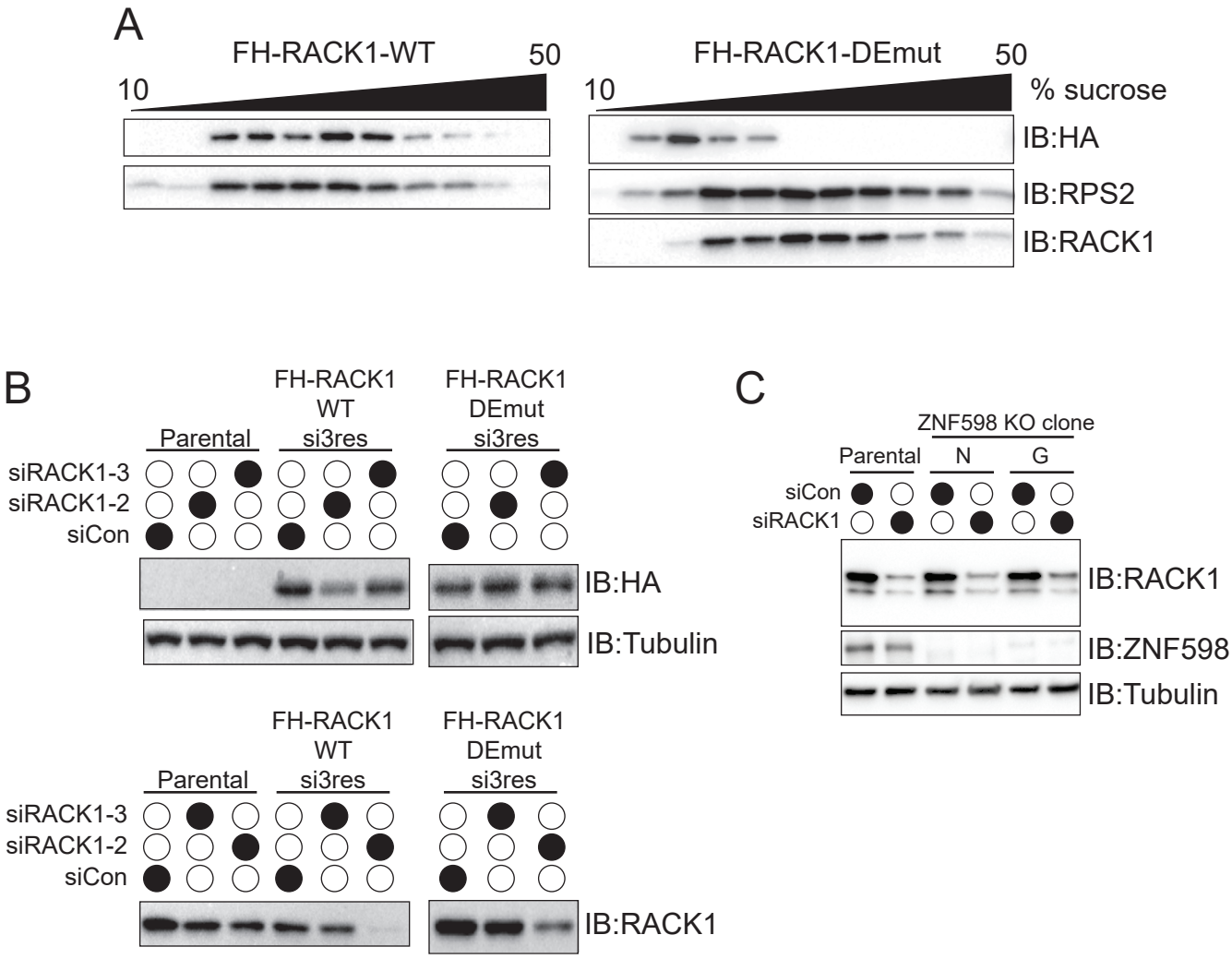

A
Log2 SILAC (H:L) ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
RPS2_K58
RPS13_K27
RPS3_K214
RPS10_K139
RPS10_K138;K139
RPL4_K294
RPS2_K275
RPS10_K107
RPL11_K51
RPL23_K43
RPS20_K8
RPL6_K207
RPL13A_K125
RPLP0_K106
RPL24_K131
RPL9_K21
RPL7A_K75
RPL24_K27
RPL30_K23
RPL6_K262
RPL24_K93
RPL15_K153
RPL17_K96
RPL7A_K245
RPL4_K365
RPL15_K140
RPL15_K157


B
293T
FH-RPS10-2KR
FH-RPS10-WT
IB:RPS10
IB:HA
IB:Tubulin
293T
FH-RPS20-2KR
FH-RPS20-WT
IB:RPS20
IB:HA
IB:Tubulin


C
K20
Relative ChFP/GFP ratio
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
*
siCon
siRACK1
siZNF598
Par
G
N
O
ZNF598
KO clones


D
C
WT
mut
FH-ZNF598
150
100
IB:ZNF598
150
100
75
50
37
IB:Ub


E
Total Protein
ZNF598-KO
ZNF598-KO WT-res
ZNF598-KO mut-res
Log2 SILAC (L:H) ratio
Ub-modified peptides
Log2 SILAC (L:H) ratio


A
FH-RACK1-WT
10
50
FH-RACK1-DEmut
10
50
% sucrose
IB:HA
IB:RPS2
IB:RACK1
B
FH-RACK1 WT si3res
FH-RACK1 DEmut si3res
Parental
siRACK1-3
siRACK1-2
siCon
IB:HA
IB:Tubulin
FH-RACK1 WT si3res
FH-RACK1 DEmut si3res
Parental
siRACK1-3
siRACK1-2
siCon
IB:RACK1
C
ZNF598 KO clone
Parental
N
G
siCon
siRACK1
IB:RACK1
IB:ZNF598
IB:Tubulin

Supplemental Figure Legends

Supplemental Figure S1 – GFP expression from the K20 reporter is minimally effected by loss of ribosome splitting factors. (Related to Figure 1)

A) 293T cells were transfected with increasing amounts of the K20-containing dual fluorescence reporter plasmid. The relative GFP (green bars) or ChFP (red bars) fluorescence is indicated.

B) 293T cells were transfected with control siRNA or three separate siRNA oligos targeting the indicated genes and subsequently transfected with either a no-stall linker control (blue bars) or the K20-containing (red bars) dual fluorescence reporter. The relative ChFP:GFP ratio is depicted with all data relative to the linker-control sample with control siRNA. Error bars represent SEM for three separate transfections and flow cytometry measurements. *= p-value of < 0.01 using Student's t-test comparing K20 ChFP:GFP ratios in the siRNA control to siRNAs targeting RACK1 or ZNF598.

C) 293T cells were transfected with three separate siRNA oligos targeting the indicated genes and subsequently transfected with the K20-containing dual fluorescence reporter. The relative GFP fluorescence is depicted. C = control scrambled siRNA. Error bars represent SEM for three separate transfections and flow cytometry measurements. *= p-value of < 0.01 using Student's t-test compared to control transfections.

Supplemental Figure S2 – Loss of ZNF598 allows for read-through of polyA-induced ribosome stalls in multiple reading frames (Related to Figure 1)

A) Whole cell lysates from 293T cells were transfected with three separate siRNA oligos targeting the indicated genes and subsequently transfected with the K20-containing dual fluorescence reporter were immunoblotted with the indicated antibodies. Arrows depict various translation products from the K20 stall reporter plasmid. C = control scrambled siRNA.

B) 293T cells were transfected with control siRNA or an siRNA oligo targeting ZNF598 (oligo #2) and subsequently transfected with the wild type K20-containing dual fluorescence reporter (2A-WT) or one in which the second 2A skipping sequence that separates the VHP-polyA sequence from the cherry fluorescent protein coding sequence was mutated (2A-mut). Whole cell lysates were immunoblotted with the indicated antibodies. Translation products arising from frameshifting (FS) events are indicated.

Supplemental Figure S3 – Regulatory ubiquitylation of RPS20 and RPS10 is mediated by ZNF598 and facilitates ribosome-associated QC events. (Related to Figure 2)

A) HCT116 cells were either untreated (light label) or treated with anisomycin for 4hrs (heavy). After harvesting, cells were mixed and ubiquitin-modified peptides were isolated from tryptic-digests of whole cell extracts. The Log2 SILAC ratio (H:L) for selected ubiquitin-modified peptides from ribosomal proteins is depicted. 40S ribosomal ubiquitylation events interrogated in this study are shown in red. The site of ubiquitin-modification is indicated. Error bars represent SEM from multiple peptide MS quantifications. This data was extracted from a previously published study (Higgins et al., 2015).

B) Whole cell extracts from either parental 293T cells or cells with stable expression of Flag-HA (FH) tagged wild type (WT) RPS20 or RPS10 or versions containing the indicated lysine to arginine mutations were immunoblotted with the indicated antibodies.
RPS20-2KR=K4R;K8R, RPS10-2KR=K138R;K139R.

C) HCT116 cells transfected with control siRNA (siCon) or siRNA targeting RACK1 or ZNF598 were subsequently transfected with the K20-containing dual fluorescence reporter and the resulting relative ChFP:GFP ratio is depicted. Parental HCT116 cells or three separate ZNF598 knockout (KO) clones were transfected with the K20-containing dual fluorescence reporter and the resulting relative ChFP:GFP ratio is depicted. Error bars represent SEM for three separate

transfections and flow cytometry measurements. *= p-value of < 0.01 using Student's t-test comparing siControl treated cells to siRNAs targeting RACK1 or ZNF598 or ZNF598-KO clones.

D) Wild type or RING-mutant (C29A) Flag-HA-tagged (FH) ZNF598 was isolated from whole cell lysates from stable expression cell lines using anti-Flag agarose. Eluted proteins were added to *in vitro* ubiquitylation reactions. Reaction products were separated by SDS-PAGE and visualized by immunoblotting with the indicated antibodies. C indicates control reactions with no added ZNF598.

E) Distribution of Log2 SILAC ratios (L:H) for all quantified proteins (top) or ubiquitin-modified peptides (bottom) from the indicated cells mixed with heavy-labeled parental cells prior to sample preparation and subsequent analysis by mass spectrometry.

Supplemental Figure S4 – Ribosome-associated RACK1 facilitates RRub and ribosome-associated QC. (Related to Figure 4)

A) Native whole cell lysates form cells expressing siRNA-resistant versions of Flag-HA-tagged wild type RACK1 or DEmut RACK1 (R36D;K38E) were separated on a 10-50% linear sucrose gradient and collected fractions were immunoblotted with the indicated antibodies.

B) Parental 293T cells or cells expressing siRNA-resistant versions (resistant to siRNA #3) of Flag-HA-tagged wild type RACK1 or DEmut RACK1 were transfected with control siRNA or two separate RACK1 targeting siRNAs. Whole cell extracts were immunoblotted as indicated. Note that the samples blotted for RACK1 were loaded in a different order.

C) Parental HCT116 cells or two separate ZNF598-KO clones (G, N) were transfected with control siRNA or siRNA targeting RACK1 (oligo#3). Whole cell extracts were immunoblotted with the indicated antibodies.

Supplemental Table 1 – Ub-modified peptides altered upon ZNF598 perturbation. (Related to Figure 2)

Description of column headers

Protein ID – Uniprot accession matching to ub-modified peptide identified

Gene_Symbol - Uniprot gene symbol matching to ub-modified peptide identified

prot_description – Description of the matching protein

Site position – position of ub-modified lysine residue based on the given protein accession

Sequence – sequence of the identified ub-modified peptide; #-signifies ub-modified lysine; *-signifies oxidized methionine

ZNFKO_TSCs – total spectral counts for the listed ub-modified peptide in SILAC samples from ZNF598 knockout cell lines.

ZNFKO_WTres_TSCs – total spectral counts for the listed ub-modified peptide in SILAC samples from ZNF598 knockout cell lines with rescued expression of wild type ZNF598.

ZNFKO_Mutres_TSCs – total spectral counts for the listed ub-modified peptide in SILAC samples from ZNF598 knockout cell lines with rescued expression of mutant ZNF598.

median_Light_Heavy – the median value for all SILAC ratios (log2 of the L:H ratio) quantified for the corresponding ub-modified peptide

standard_deviation - the standard deviation for all SILAC ratios (log2 of the L:H ratio) quantified for the corresponding ub-modified peptide

num_quant – the number of quantified peptides used to determine SILAC ratios (log2 of the L:H ratio) for the corresponding ub-modified peptide

Supplemental Table 2 – Table of sequence-based reagents. (Related to Star Methods)