Supplementary Information

Genetic code expansion method for temporal labeling of endogenously expressed proteins

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Supplementary methods

Cell culture

HEK293 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and grown in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher) supplemented with 1% Penicillin-Streptomycin (Thermo Fisher) and 10% Fetal Bovine Serum (Thermo Fisher) at 37°C with 5% CO_2 and passaged every 2-3 days. For culturing HEK293 lines carrying a selection cassette, Geneticin (G418, Carl Roth) was added to a final concentration of 700 µg/mL.

Selection of sgRNAs

The human genomic sequences of the loci of interest were obtained from the website 'http://hek293genome.org/v2/' and used to design sgRNAs targeted to the 3' end of the open reading frames (ORFs) of *ACTB* (Exon3) and *TP53* (Exon10), respectively. Four different sgRNAs were selected for each target using the two web-based applications 'CHOPCHOP'[1] and 'sgRNA designer',[2] with all candidates displaying high efficiency and low off-target effects. Target sgRNAs were cloned into the CrispR/Cas9 vector px458dsR, a kind donation of D. Medgyesi (Ag Reth, Freiburg) and following the referenced protocol.[2] Plasmid px458 is a variant of Addgene vector Nr. 48138, in which GFP was replaced with dsRed. The effectiveness of the different sgRNAs was validated using the T7 endonuclease assay.[3] For this purpose, cells were transfected with the different Cas9/sgRNA-vectors for 48 h, followed by preparation of the genomic DNA with the Roti-Prep Genomic DNA MINI Kit (Carl Roth). A non-transfected control was treated equally. Subsequently, the loci of interest were PCR-amplified to yield fragments of 1100 bp each and Cas9-targeted and wild-type PCR products were mixed at a 1:1 ratio in 1x NEB buffer 2. After heating and re-annealing, 1 µL of T7 Endonuclease (New England Biolabs) was added to each reaction mixture of 20 µL and incubated for 1 h at 37°C. The analysis of cutting efficiencies on an agarose-gel indicated pX458dsR-actin-1 for *ACTB* and pX458dsR-tp53-4 for p53 as the most effective candidates, coinciding with the highest scores for both 'CHOPCHOP' and 'sgRNA designer'. Sequences are given in Supplementary Table 1.

Cloning of knock-in plasmids

In order to achieve high efficiency of stable tag-integration into HEK293 cells, we based our work on the COSMC vector kindly provided by Dr. Faustrup Kildegaard.[4] Four plasmids containing homology arms

(HoAs) were generated to create four different knock-in cell lines: pHoA_Actin-Amber-HA-pA, pHoA_p53(TAG)-HA-pA, and pHoA (TAG)-HA-pA, which fuse a conditional HA tag (Human influenza hemagglutinin tag) to the ORF of their respective target genes, as well as pHoA_Actin(TAG)-mC-pA and pHoA(TAG)-mC-pA, in which the HA tag is directly followed by the gene encoding the mCherry fluorescent protein. Homology arms corresponding to the targeted loci were ordered as gBlocks (Integrated DNA Technologies). Key features of these DNA sequences are mutated protospacer adjacent motif (PAM) sequences to prevent plasmid degradation, replacement of the natural stop codons with the amber stop codon, and incorporation the SCROL label as well as an Sv40 polyadenylation/termination motif. Sequences were designed to allow direct assembly with the PCR products of the COSMC vector via Gibson assembly (NEB). The assemblies were verified by sequencing to be free of unexpected mutations (GATC Biotech).

Cell transfection

Plasmid DNA was prepared either as Midipreps (JetStar) or Maxipreps (Qiagen) and diluted to 1 mg/mL stock solutions. Transfection master-mixes were prepared by thoroughly mixing 3.6 µg polyethylenimine (PEI) into 100 µL of serum-free DMEM without phenol red, followed by addition of 1.2 µg of plasmid(s) (i.e. 0.6 µg for each plasmid in two-plasmid transfections). The PEI/DNA mixture was then incubated at room temperature for 10-15 minutes and added dropwise to the cells unless indicated otherwise.

Amber stop codon suppression

Plasmids for transient suppression of the amber stop codon and incorporation of ncAAs were kindly provided by the Chin Lab.[5] The plasmid (U6-PylT*)$_4$/EF1α-PylRS contains the pyrrolysine amino-acyl tRNA synthetase (aaRS) as well as four copies of the corresponding tRNAs under control of the U6-promoter. As a control of transfection efficiency and amber codon suppression efficiency, the control plasmid (U6-PylT*)$_4$ / EF1α-sfGFP(TAG) was used, which contains sfGFP with an internal amber stop codon at position 150. At least 24 h after transfection, amber stop codon suppression was initiated by adding 1 mM of the unnatural amino acid Nε-Boc-L-lysine (Alfa Aeser).

Generation of stable cell lines

To generate stable cell lines, the general protocol of Lee et al. was followed.[4] Briefly, HEK293 cells were seeded into 6-well plates (Corning) and grown to a confluency of approximately 30% over 24 h. Cells were then transfected with the combination of pHoA_Actin(TAG)-HA-pA /pHoA_Actin(TAG)-mC-pA with pX458dsR-actin-1 or the combination of pHoA (TAG)-HA-pA/pHoA (TAG)-mC-pA and pX458dsR--4. After 24 h, media were replaced with DMEM containing 700 µg/mL G418. Selection was carried out over the course of two weeks while replacing the medium every 2-3 days and passaging the cells when confluency was reached. Four days prior to fluorescence-activated cell sorting (FACS), cell lines containing the mCherry-fusion constructs were transiently transfected with plasmids (U6-PylT*)$_4$ / EF1α-PylRS and (U6-PylT*)$_4$ / EF1α-sfGFP(TAG), alongside the addition of the ncAA. Single cells gated for positive mCherry fluorescence and low GFP fluorescence were selected with a FACS Aria Fusion cell sorter (BD Bioscience, Core facility Uni Freiburg). Selected cells were seeded into 96 well plates pre-loaded with 150 µL of conditioned DMEM. Single cell clones were grown and regularly observed for 2-3 weeks until single cell clones had grown into sizeable colonies. Subsequently, colonies were transferred into 96-well plates with G418 selection media and again grown to confluency. Cells were then passaged and 80% of the cells were harvested to obtain genomic DNA as described above. The genomic DNA was utilized for a range of screening polymerase chain reactions (PCRs). As indicated in Figure SI1, within a first round of ‘in-out’ PCRs, one primer was chosen to be positioned on the original genomic sequence and one on the insert sequence. In a second round of PCRs, primers were designed to bind on the first round PCR product, but shifted inwards. PCRs were carried out for both 5’ and 3’ ends of the inserts with the Q5 polymerase (New England Biolabs), according to the manufacturers guidelines. Finally, in cases where both rounds of PCR resulted in the correctly sized band for both 5’

and 3' ends, PCR products of the second round of PCR were purified (Qiagen) and sequenced (GATC Biotech). Only clones that displayed 100% correct base pair alignments with designed sequences were used for further experiments.

Western Blotting

Cells were grown in 6-well plates, transfected for at least 24 h, and subjected to varying ncAA pulses as indicated within the main manuscript. In the case of ncAA chase experiments, cells were carefully washed three times in pre-warmed DMEM to remove trace amounts of ncAA. For harvesting, HEK293 cells were gently detached by washing with DMEM and spun down at 300 × *g*. If analysis was conducted at a later time, cell pellets were frozen at -20°C. Cells were then lysed in 100 µL of ice-cold RIPA buffer (Thermo Fisher) supplemented with 100x Protease Inhibitor Cocktail (Cell Signaling) for 30 min on ice with gentle shaking. Cell debris was removed by centrifuging the lysate in a pre-cooled centrifuge for 30 min at 16000× *g*. Next, the protein concentration of each sample was determined with a bicinchoninic acid assay (BCA kit, Novagen) according to the manufacturer's instructions. For each sample, the lysate was adjusted to a maximum amount of 30 µg in 30 µL RIPA buffer, which was then mixed with 10 µL Biorad Laemmli Buffer (Biorad) and boiled at 95°C for 5 min. As a ladder, 7 µL of 'Precision Plus Protein Dual Color Standard' (Biorad) was utilized. Samples were then separated by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) on Any kD Mini-PROTEAN TGX precast protein gels (Biorad) at 200V for approximately 30 min. Size-separated proteins were transferred onto 0.2 µm nitrocellulose membranes using the Trans-Blot Turbo system (Biorad). After transferring, membranes were incubated in blocking buffer TBS-T (50 mM Tris, 150 mM NaCl, 0.05 % (v/v) Tween-20 at pH 7.4) supplemented with 5% (w/v) BSA (Sigma-Aldrich) for 1 h with gentle shaking. The primary antibody anti-HA (Cell Signaling, C29F4) was diluted 1:1000 in Blocking Buffer and immunostaining was conducted at 4°C overnight. Next, membranes were washed 3 times with TBS-T, followed by incubation with an anti-rabbit secondary antibody (Thermo Fisher**,** 32260) coupled to horseradish peroxidase in blocking buffer for 2 h at RT. After three further washing steps in TBS-T, ECL solution (ThermoFisher, 32260) was spread over the membranes and chemiluminescence was detected with an ImageQuant LAS-4000 minisystem (GE Healthcare). Band intensities were determined by densitometry with Fiji.[6] Values were fitted with exponential decay formulas $N(t) = N_0\left(1 - e^{-\lambda t}\right)$ and $N(t) = N_0 e^{-\lambda t}$, in which λ denotes the decay rate in 95% confidence interval.

Cell culture on 384-well plates

For PLA-assays on 384-well plates (Corning), wells were first washed twice with phosphate-buffered saline (PBS, Thermo Fisher). Next, 10 µL of 10 µg/mL fibronectin (Antikoerper online, ABIN377072) diluted in PBS were added and incubated for at least 30 min at 37°C, followed by two more washes with PBS. Subsequently, wells were preloaded with a mixture of 40 µL DMEM and 6 µL transfection mixture (as described above) containing the plasmids. Immediately after, 200–1000 freshly harvested HEK293 cells were added per well and spun down to the glass surface at 300× *g*. Cells were grown for 24 h before adding the ncAA.

Proximity ligation assay

Cells were fixed with 4% PFA in PBS (v/v) at room temperature for 15 min, washed three times with PBS, then permeabilized with 0.5% Triton in 1% BSA/PBS for 30 min at 37°C and again washed three times with PBS. At all times, a liquid film of about 5 µL was left to cover the cells to prevent drying of the sample. The PLA assay was carried out using the Duolink Far-Red PLA kit and reaction mixtures were prepared according to the manufacturer's instructions and previously described.[7] First, the primary anti-HA antibody was diluted 1:1600 in Duolink Antibody diluent and 50 µL were added to each well for incubation of the samples overnight at 4°C. The wells were then washed 4 times with WashA for a total of 1 h. Secondary antibodies labeled with oligonucleotides (Rabbit plus and Rabbit minus, Sigma-Aldrich, DUO92002 and DUO92005) were diluted in Antibody diluent 1:10. For each well, 10 µL of diluted secondary antibody solution were added, followed by sample incubation for 1 h at 37°C. After again

thoroughly washing the samples for 1 h with WashA, each well was incubated in 10 µL ligation mixture for 1 h, followed by incubation in 10 µL amplification mixture for 90 minutes after a brief wash with WashA. Finally, cells were washed twice with WashB, counterstained with WashB + 1 µg/mL DAPI (4',6-diamidino-2-phenylindole dihydrochloride) and 25 nM Phalloidin-Atto 488 for 15 min, and again washed twice with WashB. For the PLA, the 610 nm channel was omitted, since stable cell lines express mCherry continuously.

Click chemistry

For direct detection of the incorporated ncAA via click chemistry, transfected cells (HEK293 Actin(TAG)-mC-pA) in 384-well plates were incubated for 48 h with 1 mM N-Propargyl-Lysine (Sichem) followed by fixation and permeabilization as described above. Click chemistry in the form of copper(I)-catalyzed azide-alkyne cycloaddition was performed by joining 5 µM Atto488 azide (Atto Tec, AD 488-101), 1 mM $CuSo_4$, and 100 mM L-ascorbic acid in 100 mM Tris pH 8.5. Cells were incubated in 50 µL staining mixture for 2 h at 37°C, followed by extensive washing in Tris-Buffer and counterstaining with DAPI as described above.

Image acquisition and processing

Images were acquired with a Zeiss Observer.Z1 epifluorescence microscope. An LED Colibri.2 (Zeiss) was used for illumination with excitation wavelengths of 400 nm (DAPI, 20 ms), 505 nm (GFP/A488, 200 ms), 565 nm (mCherry, 2000 ms), and 635 nm (PLA-signal, 2000 ms) in combination with the Zeiss emission filter 81HE. Images were recorded with a Plan Apochromat 20x (NA 0.8) objective from Zeiss and a Hamamatsu Orca 2 12-bit camera. For the PLA signal and due to the high profile of HEK293 cells, typically 12-15 focal planes were imaged at a step-size of 0.5–0.75µm. Z-stack images were then combined to the projected image *P* by calculating

$$\forall\, x, y;\ P_{x,y} = \max(I_{x,y}(z))$$

where *x* and *y* are the row and column pixel positions of image *I*. This allowed us to get one image, in which all PLA dots are in focus. PLA dot detection was then performed by finding local maxima that stand out from the local background pixels by a certain threshold *T. T* was manually chosen for independent experiments depending on the fluorescence background of the image and was generally between 10-30 intensity units of the 12 bit image. This allowed us to avoid a fix intensity threshold that would have removed dimer PLA dots from the analysis. To avoid PLA signal overlaps, the antibody concentrations were titrated to a concertation at which PLA signals at a ncAA pulse of 96 h (maximum expected intensity) were still resolvable as single point source. To further compensate for possible fused PLA dots as a result of the maximum intensity projection a watershed algorithm was applied to separate these dots.[8] Cytoplasmic foreground areas without the identification of the nucleus in the DAPI channel were discarded. Standard thresholds and watershed algorithms were used to segment the cytoplasmic areas and in order to achieve single cell resolution.[9] A representative projection image is shown in Figure SI14. PLA dots within the images have a diameter of <1.0 µm. All image processing was carried out in matlab (Mathworks).

Supplementary Figures

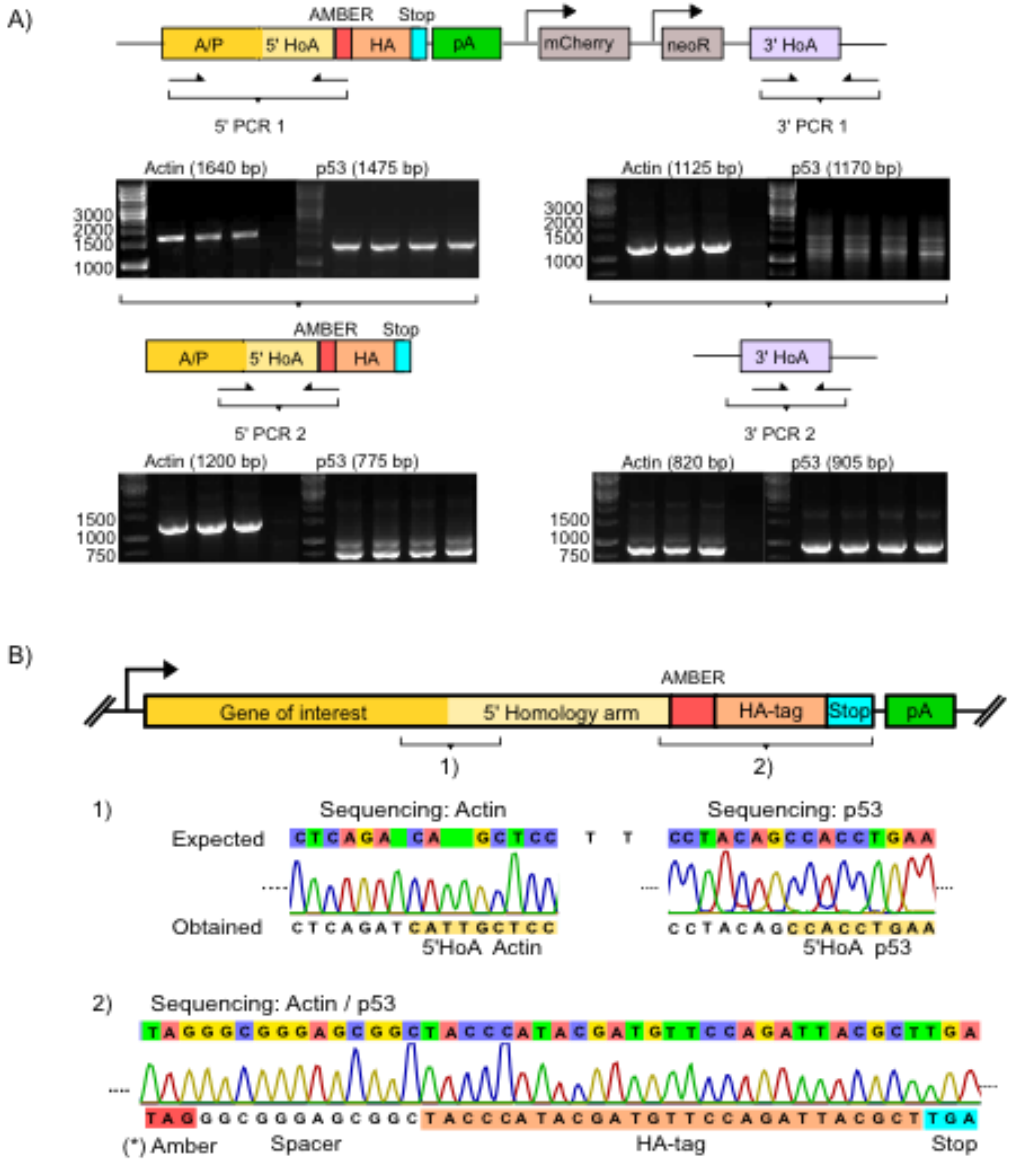

Figure SI1. Results of gene editing for incorporating the protein labeling tag at the 3' end of the β-actin and p53 loci in HEK293 cells. **A)** Design of the CRISPR/Cas9 gene fragment incorporated to the gene of interest. HoA, Amber, HA, pA, and Stop denote for up- (5'HoA) and down- (3'HoA) stream homology arms for homology-directed repair after CRISPR/Cas9 mediated restriction, the amber stop codon, the human influenza hemagglutinin tag sequence, polyadenylation site, and an alternative stop codon (Opal), respectively. For double positive selection of gene editing events mCherry and the neomycin resistance genes (NeoR) under the control of EF1a and Sv40 promoters were included within the inserted cassette. **B)** Confirmation of CRISPR/Cas9 protein labeling cassette insertion at the 3' end of the β-actin and p53 loci in HEK293 cells by PCR after a double-positive selection process. Variation of the primer sites confirmed the insertion of the entire protein labeling cassette (brackets). **C)** Sanger sequencing results confirming the insertion of the protein labeling cassette at the 3' end of the β-actin

and p53 gene loci. The transition of the GOI into the SCROL cassette and amber stop codon, spacer, HA-tag and opal stop codon regions are highlighted.

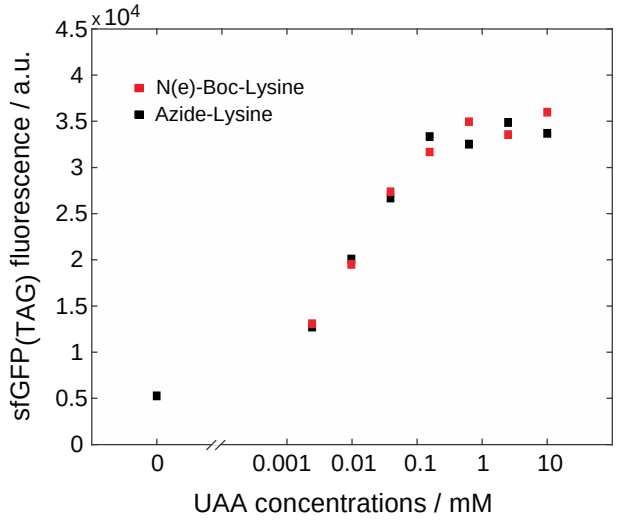

Figure SI2. Amber codon suppression efficiency at different ncAA concentrations. HEK293 cells were co-transfected with the PylRS vector and a vector carrying the $sfGFP_{(TAG)}$ sequence with an internal Amber stop codon at amino acid position 150. Twenty-four hours after transfection, HEK293 cells were exposed to either an (N(ε)-Boc-L-Lysine) or Azide-Lysine pulse at various concentrations for 2 days.

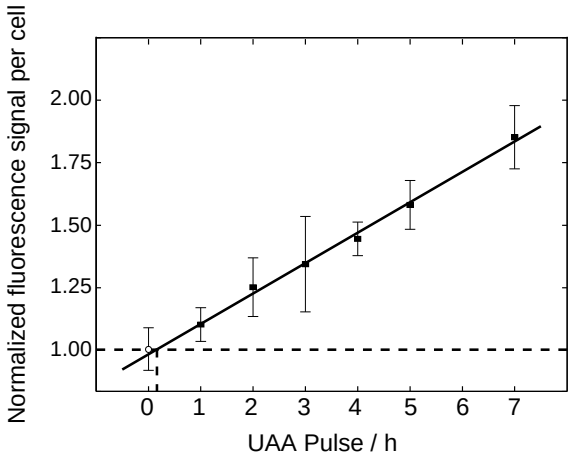

Figure SI3. Time dependence of the stop codon suppression after adding the ncAA. For this measurement, HEK293 cells were co-transfected with the vectors encoding the PylRS and the $sfGFP_{(TAG)}$ sequence with an internal Amber stop codon at amino acid position 150. Upon ncAA addition, (N(ε)-Boc-L-Lysine) leads to stop codon suppression and synthesis of fully fluorescent sfGFP.

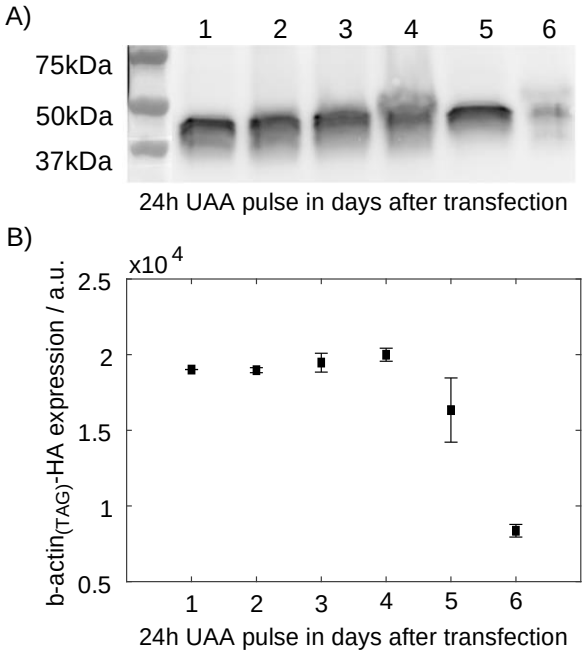

Figure SI4. Continuity of the Amber stop codon suppression efficiency after transient transfection. HEK293 cells with the β-actin$_{(TAG)}$-HA gene modification were transfected with the MbPyl vector. The stop codon suppression stability was tested by applying a 24-h pulse with N(ε)-Boc-L-lysine after different transfection times. **A)** Western blot (WB) analysis of β-actin$_{(TAG)}$-HA expression. **B)** WB band intensities obtained from analyzing the image in A. Error bars are calculated from three repeats of the experiment.

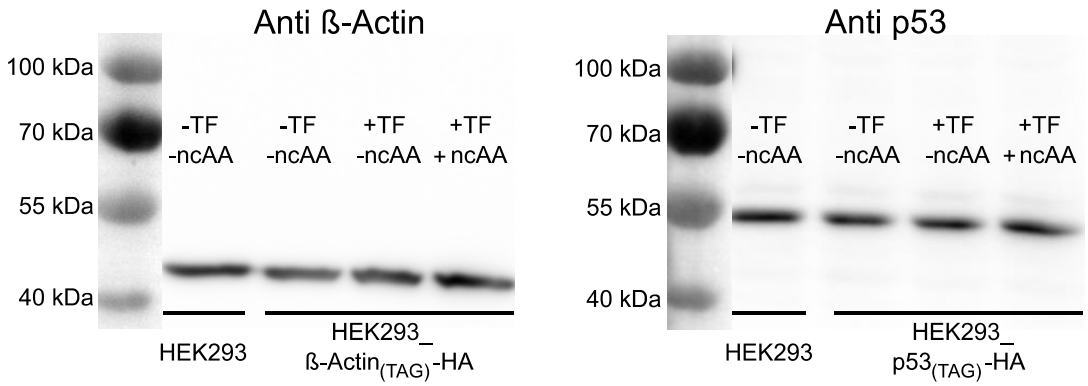

Figure SI5. Protein abundance controls. Comparison of β-actin (left) and p53 (right) protein abundance levels in unedited HEK293 cells in comparison to edited cells measured by western blot. Abbreviations: -TF: untransfected cells, +TF: transfected cells with the MbPyl vector, -ncAA: non canonical amino acid were omitted, +ncAA: non canonical amino acid were supplemented.

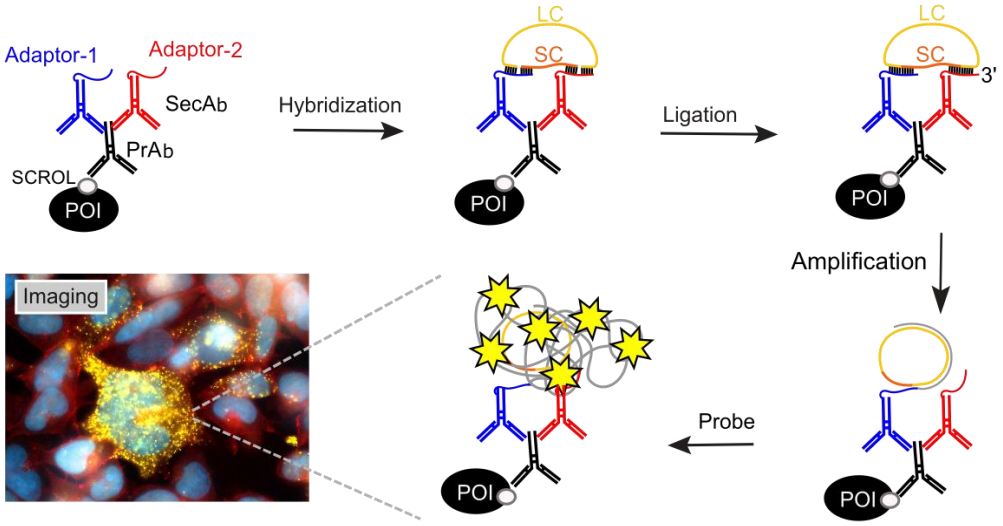

Figure SI6. Experimental set up of the SCROL-PLA for subcellular protein analytics. The protein of interest (POI) was targeted with a primary antibody (PrAb) specific for the SCROL label. Two secondary antibodies (SecAb) labeled with different oligonucleotide labels (Adaptors-1, and 2) were used to bind PrAb. In case an adaptor 1 is in proximity to adaptor 2, the strands can be bridged in a hybridization reaction with a short (SC) and long (LC) connector DNA strand. Upon ligation of the SC and LC, a circular amplification template is formed. In a rolling circle amplification (RCA), the proximity signal is amplified. The amplification product (PLA dot) was stained with a DNA fluorescence probe labeled with Cy3 (yellow dots). For a more detailed description, see reference.[8] The nucleus and cytoplasm are counterstained with DAPI and phalloidin-cy5.

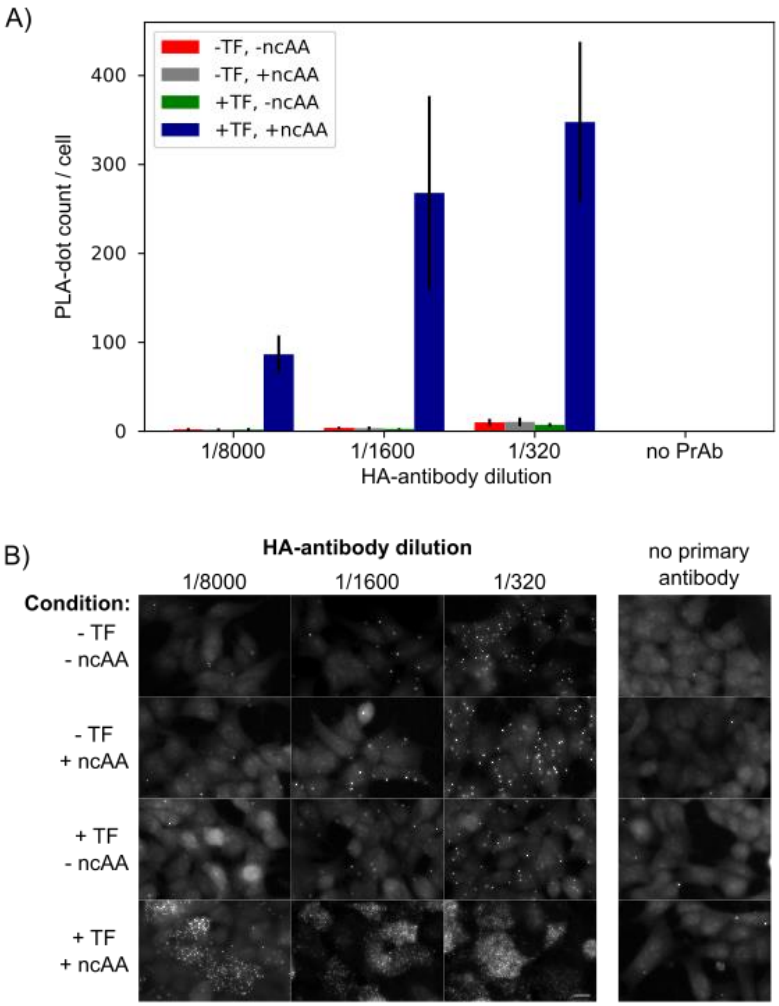

Figure SI7. Specificity test of the HA-antibody for the *in situ* detection of β-actin$_{(TAG)}$-HA proteins labelled with SCROL in HEK293 cells. (A) False positive RCA events were determined by comparing PLA dot counts within single cells (i) without MbPyl vector transfection cells in absence of ncAA (red bars), (ii) without MbPyl vector transfection in presence of the ncAA (grey bars), (iii) with MbPyl vector transfection in absence of ncAA (green bars), and (iv) with MbPyl vector transfection in presence of the ncAA (blue bars). B) Representative PLA images corresponding to the tested conditions in A. Abbreviations: -TF: untransfected, +TF: transfected, -ncAA: ncAA omitted, +ncAA: ncAA supplemented. The scale bar denotes for 20µm.

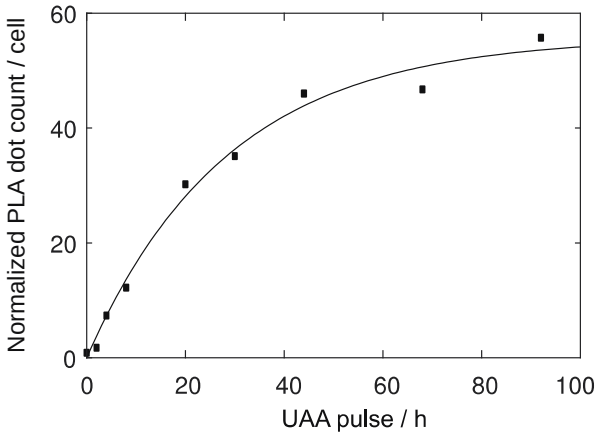

Figure SI8. Single cell PLA analysis of pulse SCROL labeled β-actin-HA in HEK293 cells. The data set used here is the same as that shown in Figure 3 of the main text. In difference to Figure 3 here, the quantitative PLA dot counts were normalized to all cells evaluated in the experiment, including non-transfected cells. The solid line represents a fit of the data to a first-order reaction rate equation. The rate constant for protein synthesis and half life time is 0.035 ± 0.001 h^{-1} and 19.8 ± 2 h, respectively.

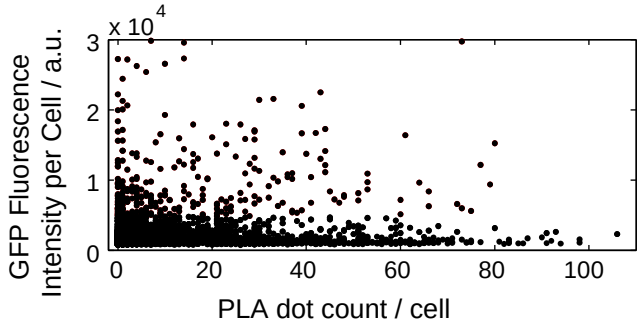

Figure SI9. Single cell correlation between suppression of the stop codon in $sfGFP_{(TAG)}$ and β-$actin_{(TAG)}$-HA. The single cell sfGFP fluorescence was detected by fluorescence microscopy, and the β-actin-$HA_{(TAG)}$ by PLA.

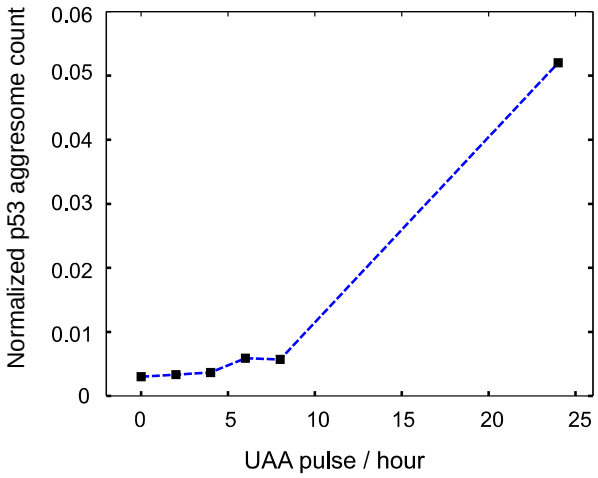

Figure SI10. Quantitation of aggresome accumulation of p53-(TAG)HA in HEK293 cells. Cells were pulsed with the ncAA for the indicated times, followed by sample fixation and PLA against HA-tagged P53. Aggresomes were identified identical to PLA dots, yet with a minimal size threshold of 20 pixels (see material and methods). Aggresome counts were normalized to the absolute cell count.

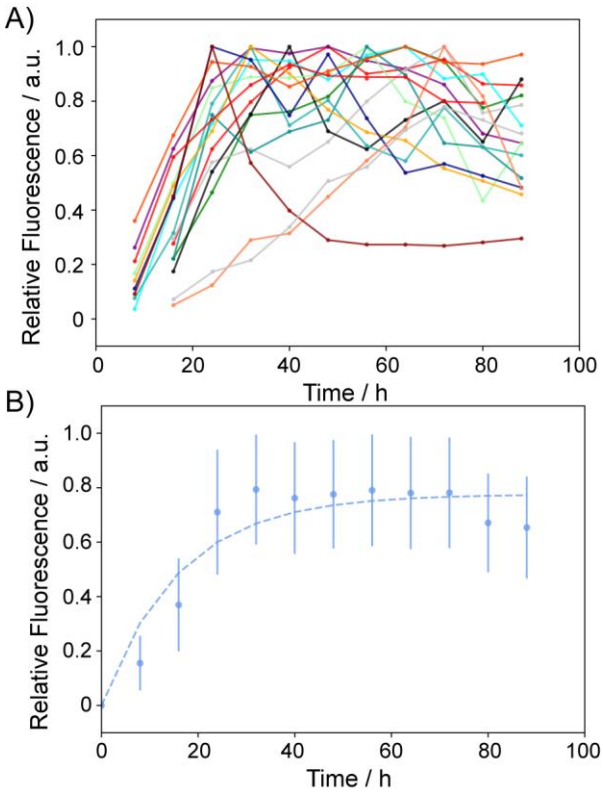

Figure SI11. Fluorescence time traces of single β-actin$_{(TAG)}$-mCherry cells. A) Fluorescence intensity values of 15 single cells β-actin$_{(TAG)}$-mCherry traced upon pulsing with N(ε)-Boc-L-lysine. B) Mean

fluorescence time trace of the 15 β-actin(TAG)-mCherry cells in A. The protein synthesis (τ_S) and half maximum consolidation time ($T_{1/2}$) was 0.067 ± 0.036 h^{-1} and 10.3 h ± 5 h, respectively.

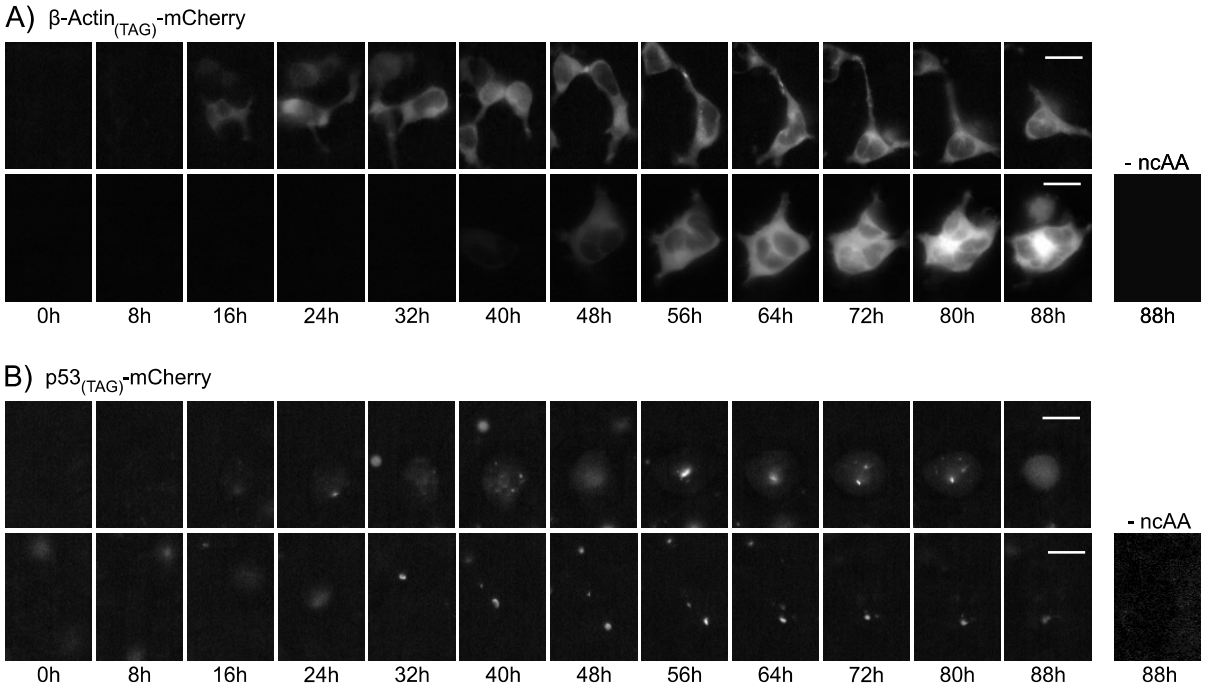

Figure SI12. Real-time fluorescence imaging of pulse SCROL labeled β-actin(TAG)-mCherry. Twenty-four hours after MbPyl transfection, the HEK293 cells were exposed to a ncAA (N(ε)-Boc-L-lysine) pulse. **A)** Representative images of a time trajectory for pulse labeling β-actin(TAG)-mCherry. Note the staggered onset of mCherry fluorescence for different cells during the ncAA pulse, reflecting expression bursts of β-actin in non-synchronized, dividing cells. **B)** Representative images of a time trajectory for pulse labeling p53(TAG)-mCherry. Only the fluorescence signal of p53 in the cytoplasmic aggresome could be detected. Negative control images show transfected cells that were cultured in absence of the ncAA (-ncAA).

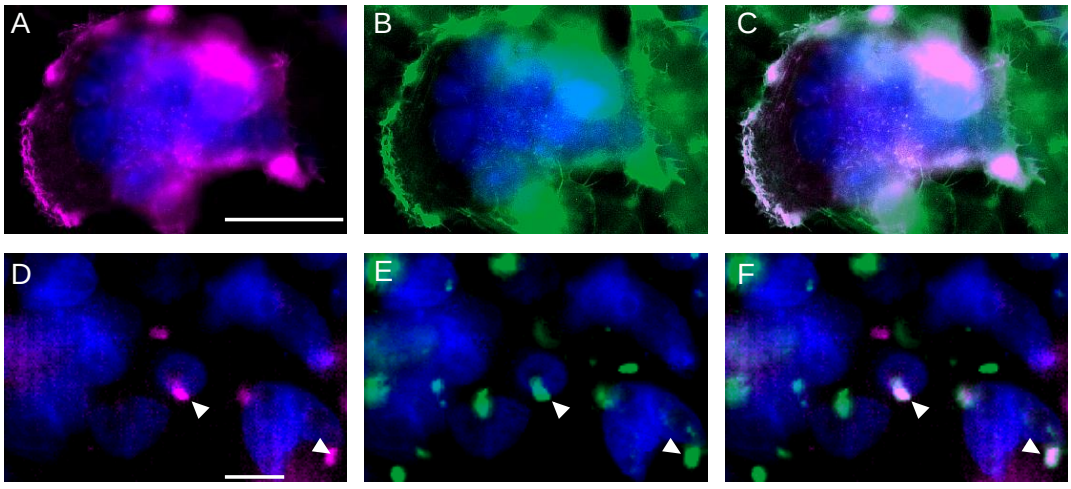

Figure SI13. Co-localization of newly synthesized SCROL labeled proteins and pre-existing proteins. The fluorescence images A–C and D-F show a β-actin$_{(TAG)}$-mCherry and p53$_{(TAG)}$-mCherry cell after a 6 hour pulse with the ncAA N(ε)-Boc-L-lysine, respectively. **A**) mCherry fluorescence of newly synthesized SCROL labeled β-actin, **B**) Cy3 labeled Phalloidine indicating total β-actin, and **C**) fluorescence overlay images of A and B. **D**) mCherry fluorescence of newly synthesized SCROL labeled p53, **E**) Cy3 labeled ant-p53 antibody indicating total p53, and **F**) fluorescence overlay images of D and E. The scale bars represent 10 μm.

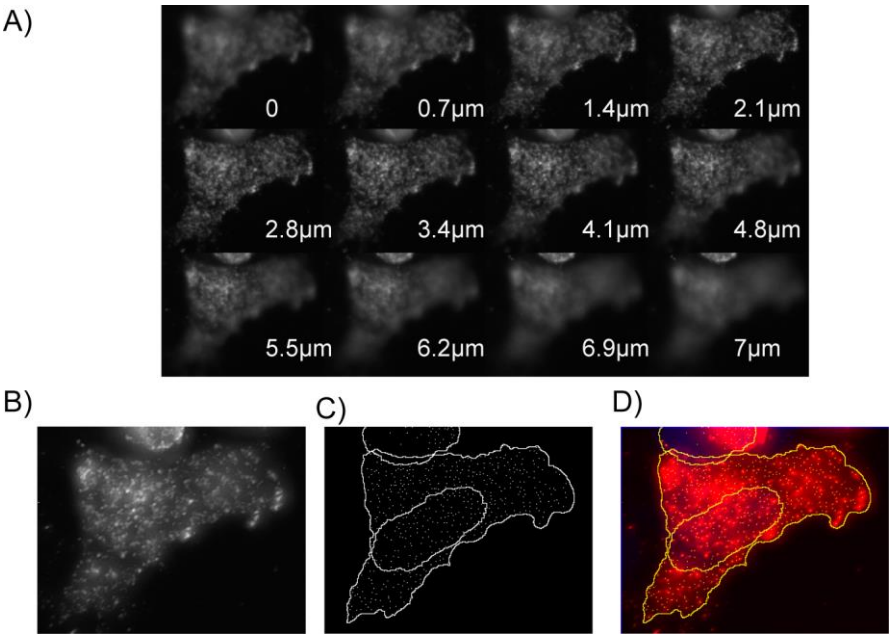

Figure SI14: PLA image processing. **A**) PLA image stack of a β-actin$_{(TAG)}$HA cell. The z-offset is indicated. **B**) Maximum intensity projection of the z-stack from A. **C**) PLA dots within single cells were then identified as local maxima. **D**) Overlay image with contrast adjustment of the PLA fluorescence channel for illustration.

Supplementary Table

Table SI1. Oligonucleotide sequences employed in this work. 1-4: sequences of oligonucleotides encoding sgRNAs (capital letters) for targeting endogenous loci of β–actin and P53, respectively. 5-15: oligonucleotides employed for PCR-screening of SCROL-labeled endogenous loci. In-out PCRs were conducted for the 5' and 3' ends of the inserts in two rounds, as depicted in Figure SI1.

ID Number	Oligonucleotide name	Sequence
1	tp53-sgRNA4F	caccgTGTCAGTGGGGAACAAGAAG

2	tp53-sgRNA4R	aaacCTTCTTGTTCCCCACTGACAc
3	actin-sgRNA1F	caccgCCACCGCAAATGCTTCTAGG
4	actin-sgRNA1R	AaacCCTAGAAGCATTTGCGGTGG
5	Act_5'_R1_FW	CCAGATGAGCTCTTTTTCTGGTGTTTGTCTCTC
6	EF1a_5'_R1_RV	GGGACTGTGGGCGATGTGCGC
7	Act_5'_R2_FW	GGCTCGTGTGACAAGGCCATGAGG
8	HA_5'_R2_RV	CATCGTATGGGTAGCCGCTCCCGC
9	SV40pA_3'_R1_FW	CCGCCGCCTTCTATGAAAGGTTGGG
10	Act_3'_R1_RV	GCAGCTAGGAACACCCACTACACCC
11	Act_3'_R2_RV	GGATCTCCACACACCTGCACTCTGG
12	P53_5'_R1_FW	GTTCCGAGAGCTGAATGAGGCCTTGG
13	P53_5'_R2_FW	CCCAGCCCAGAGCTGGAGGG
14	P53_3'_R1_RV	GGTGGATCGCCTGAGCCCAGG
15	P53_3'_R2_RV	GACCCTGAGCATAAAACAAGTCTTGGTGG

References

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