StaPLs: versatile genetically encoded modules for engineering drug-inducible proteins

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Robust approaches for chemogenetic control of protein function would have many biological applications. We developed stabilizable polypeptide linkages (StaPLs) based on hepatitis C virus protease. StaPLs undergo autoproteolysis to cleave proteins by default, whereas protease inhibitors prevent cleavage and preserve protein function. We created StaPLs responsive to different clinically approved drugs to bidirectionally control transcription with zinc-finger-based effectors, and used StaPLs to create single-chain, drug-stabilizable variants of CRISPR–Cas9 and caspase-9.

Generalizable methods for pharmacological induction of protein function would be highly useful for gene- and cell-based therapies, or for investigating the temporal requirements of proteins in biological systems. Existing methods include the fusion of proteins to steroid-hormone-binding domains for sequestration in heat-shock complexes until drug application or to domains whose stability is enhanced by drug, and the fusion of complementary fragments to domains whose heterodimerization is induced by drug. However, regulation by hormone-binding domains is not broadly generalizable, destabilization domains often allow some protein function in the absence of drug, and protein fragments often require optimization to suppress spontaneous reconstitution or to provide satisfactory drug-induced reconstitution. Furthermore, fragment complementation requires two polypeptides for each activity to be regulated, which makes simultaneous regulation of multiple activities cumbersome.

We sought to develop single-chain, drug-controllable proteins whose function could be robustly activated by clinically available drugs that lack endogenous targets. Previously, we used the hepatitis C virus (HCV) nonstructural protein 3 protease domain (NS3 protease) and its inhibitors for drug-controlled protein tagging. In TimeSTAMP, a cis-cleaving NS3 protease removes a visualizable tag from a protein by default, but inhibitor application preserves the tag on newly synthesized copies. In SMASH, removal of a degron from a protein by a cis-cleaving NS3 protease can be inhibited by drug, thereby shutting off further protein production. Consequently, we reasoned that an internal autoproteolytic module comprising NS3 protease and a substrate sequence would constitute a StaPL that could control protein function in multiple ways (Fig. 1a). A StaPL module could link a protein to a functional domain so that function is removed in the absence of inhibitor but retained in its presence. Alternatively, a StaPL module could be inserted in a domain so that the domain is cleaved into two fragments by default but remains intact in the presence of inhibitor. Finally, a StaPL module could be placed between two copies of a dimerization-activated protein so that inhibitor preserves subsequently synthesized molecules as tandem dimers, thus leading to activation.

As the ability to control two processes in the same cell independently would be desirable, we first created two NS3 protease variants inhibited by different drugs, asunaprevir (ASV) and telaprevir (TPV), thereby diverging NS3 into two species defined by drug sensitivity (Supplementary Note 1, Supplementary Table 1, and Supplementary Fig. 1). These two variants, denoted by NS3AI (ASV inhibited) and NS3TI (TPV inhibited), were orthogonal in their drug sensitivity, with NS3AI being highly resistant to $1-10 \mu M$ ASV and NS3TI being highly resistant to $0.1-1 \mu M$ ASV (Supplementary Fig. 2a–c). SMASH tags containing NS3AI or NS3TI (Supplementary Fig. 2d) allowed ASV or TPV, respectively, to suppress accumulation of fused YFP (Fig. 1b). Together, they allowed for simultaneous independent suppression of two protein targets (Fig. 1c), which shows that NS3AI-ASV and NS3TI-TPV are indeed orthogonal protease-inhibitor pairs. We next explored the possibility of regulating the linkage of other functional domains in a synthetic fusion protein. We linked an NS3 protease cleavage site to either NS3AI or NS3TI, and referred to these sequences as StaPL modules (Supplementary Fig. 3a). We then used StaPLAI and StaPLTI modules to connect a nuclear localization sequence (NLS) to either tandem dimeric YFP (tdYFP) or tandem dimeric RFP (tdRFP). Preservation of each StaPL linkage occurred only with the cognate drug, whereas its downregulation can be useful for wet age-related macular degeneration and cancer.

We next used StaPL sequences to bidirectionally control gene expression. We chose to regulate vascular endothelial growth factor (VEGF), as VEGF upregulation can be beneficial in neuropathy or vascular insufficiency, whereas its downregulation can be useful for wet age-related macular degeneration and cancer. We first expressed in HEK293A cells a synthetic zinc-finger domain targeting the VEGFA promoter (ZF-VEGFA) fused either to StaPLAI followed by tdYFP and a p65 activation domain, or to StaPLTI followed by tdRFP and a KRAB repressor domain, while also testing two substrate sequences in the StaPL modules. As expected, full-length proteins were preserved only in the presence of the cognate drug (Supplementary Fig. 5a). We then tested whether these constructs could mediate pharmacological regulation of endogenous VEGFA by quantifying VEGF secreted from transfected cells (Supplementary Fig. 5b). Although TPV stabilization of the KRAB-based repressor downstreamregulated VEGF as desired, ASV stabilization of the p65-based activator had unclear effects. We therefore replaced p65 with the more potent VP64-p65-Rta (VPR) cassette and reconfirmed drug-dependent protein preservation using the more efficiently cleaved DEMEC/S substrate ( Supplementary Fig. 5a,c). Finally,
we tested the ability of the two final constructs, ZFVEGFA-StaPLAI-tdRFP-KRAB and ZFVEGFA-StaPLAI-YFP-VPR (Supplementary Fig. 6), to regulate VEGFA transcription (Fig. 1d). Individually, ZFVEGFA-StaPLAI-tdRFP-KRAB decreased VEGF secretion specifically in TPV, whereas ZFVEGFA-StaPLAI-YFP-VPR strongly increased VEGF secretion specifically in ASV. In cells coexpressing both constructs, VEGF was upregulated by ASV and downregulated by TPV, demonstrating orthogonal regulation (Fig. 1d). Thus, StaPL modules can regulate linkage between DNA-binding and regulatory domains in synthetic transcription factors, with two orthogonal StaPL–drug pairs enabling bidirectional control of an endogenous gene.

We then investigated whether StaPL modules in internal loops can regulate protein function. We inserted StaPLA into nucleo-deficient Streptococcus pyogenes Cas9 (dSpCas9) with an N-terminal fusion to VPR (VPR–dCas9), choosing positions 573 and 1,246, in nonconserved loops of dSpCas9. To assay for function, we transfected these constructs and a single guide RNA (sgRNA) targeting a tet operon (tetO) sequence into HEK293 cells stably expressing mCherry from a TRE3G promoter containing seven tetO repeats16. We expected the dSpCas9 domain to be cleaved into nonfunctional fragments by default, but also to retain its ability to fold and function in TPV (Fig. 2a). Both constructs achieved RFP expression in TPV, with the variant at 1,246 performing better (Supplementary Fig. 7a,b). Cells expressing this protein, VPR–dSpCas9(StaPLA1) (Supplementary Fig. 8), increased RFP fluorescence 24-fold and RFP mRNA expression 6-fold after 24 h in TPV compared with vehicle (Fig. 2b,c). Thus, intradomain insertion of a StaPL module enables pharmacological regulation of VPR–dSpCas9 function.

Next, we characterized the kinetics, reversibility, and dose responsiveness of transcriptional activation by StaPL (Supplementary Note 2). Kinetics were consistent with the accumulation of new intact protein copies in the presence of drug (Supplementary Fig. 9a–c). Reversal was not observed 24 h after the removal of TPV from VPR–dSpCas9(StaPLA1), but was observed after the removal of ASV from ZFVEGFA-StaPLAI-YFP-VPR, consistent with the known covalent mechanism of action of TPV and noncovalent mechanism of action of ASV (Supplementary Fig 9d,e). The dose responsiveness of VPR–dSpCas9(StaPLA1) and ZFVEGFA-StaPLAI-YFP-VPR mirrored that of SMASHa and SMASHb, respectively (Supplementary Fig. 9f,g and Supplementary Tables 2 and 3), indicating consistent drug responsiveness of the NS3AI and NS3AI proteases in different contexts.

Finally, we postulated that StaPL sequences could enable drug-dependent preservation of a tandem dimer. This would allow proteins naturally activated by homodimerization to be activated by NS3 protease inhibitors. We tested this concept on caspase-9 (Cas9), whose natural activation involves dimerization of an N-terminal domain and which can be artificially activated by chemically induced dimerization15. We created a fusion protein consisting of, in order, a mini-GFP, a tet operator (tetO) sequence into HEK293 cells stably expressing mCherry from a TRE3G promoter containing seven tetO repeats15. We expected the dSpCas9 domain to be cleaved into nonfunctional fragments by default, but also to retain its ability to fold and function in TPV (Fig. 2a). Both constructs achieved RFP expression in TPV, with the variant at 1,246 performing better (Supplementary Fig. 11a). ASV-induced cell death was confirmed by live HeLa cells 24 h after transfection and drug addition. Scale bar, 20 μm. Data represent a single experiment. Corroborating results were obtained in an independent immuno blotting experiment (data not shown). c, SMASH tagging of PSD95 and Arc can be controlled independently in the same cells via orthogonal tags. HeLa cells were lysed 24 h after transfection and drug treatment for immunoblotting. Data represent a single experiment. d, Orthogonal StaPL modules allow bidirectional transcriptional control via synthetic transcription factors. Top, designs of ZFVEGFA-StaPLAI-YFP-VPR and ZFVEGFA-StaPLAI-tdRFP-KRAB. Bottom, bidirectional regulation of VEGF production by ZFVEGFA-StaPLAI-YFP-VPR and ZFVEGFA-StaPLAI-tdRFP-KRAB, 48 h after HEK293A transfection and treatment with DMSO, ASV, or TPV, VEGF in culture media was quantified by ELISA. Mean values from three independent experiments are graphed; gray dots represent individual values. Concentrations were calculated relative to those in similarly treated cells transfected with empty vector. Mean empty vector values were 716 pg/ml for DMSO, 673 pg/ml for ASV, and 930 pg/ml for TPV. Error bars represent s.e.m. For pairwise comparisons, *P < 0.01, ** P < 0.001 by one-way ANOVA (see Methods for exact P values).

Fig. 1 | StaPL concept and engineering of orthogonal NS3 proteases and StaPL effectors. a, A StaPL module comprising HCV NS3 protease and a cognate substrate sequence linked in cis can be used to link two functional domains in an artificial multidomain protein (left), to connect two fragments of a natural protein domain (center), or to connect two copies of a protein a tandem dimer (right). b, Proteases NS3a (asunaprevir inhibited) and NS3b (telaprevir inhibited) allowed orthogonal drug-induced degradation of YFP via preservation of degron-containing SMASH tag in live HeLa cells 24 h after transfection and drug addition. Scale bar, 20 μm. Data represent a single experiment. Corroborating results were obtained in an independent immunoblotting experiment (data not shown). c, SMASH tagging of PSD95 and Arc can be controlled independently in the same cells via orthogonal tags. HeLa cells were lysed 24 h after transfection and drug treatment for immunoblotting. Data represent a single experiment. d, Orthogonal StaPL modules allow bidirectional transcriptional control via synthetic transcription factors. Top, designs of ZFVEGFA-StaPLAI-YFP-VPR and ZFVEGFA-StaPLAI-tdRFP-KRAB. Bottom, bidirectional regulation of VEGF production by ZFVEGFA-StaPLAI-YFP-VPR and ZFVEGFA-StaPLAI-tdRFP-KRAB, 48 h after HEK293A transfection and treatment with DMSO, ASV, or TPV, VEGF in culture media was quantified by ELISA. Mean values from three independent experiments are graphed; gray dots represent individual values. Concentrations were calculated relative to those in similarly treated cells transfected with empty vector. Mean empty vector values were 716 pg/ml for DMSO, 673 pg/ml for ASV, and 930 pg/ml for TPV. Error bars represent s.e.m. For pairwise comparisons, *P < 0.01, ** P < 0.001 by one-way ANOVA (see Methods for exact P values).
being tested as a safety switch for cellular therapies such as chimeric antigen receptor T cells\(^{16,17}\). StaPLd-Casp9 could have similar applications as iCasp9, while using a clinically approved drug. We confirmed that StaPLd-Casp9 and iCasp9 were activated specifically by their respective drugs to induce comparable maximum levels of apoptosis after 24 h (Supplementary Fig. 13). The orthogonality of the two systems suggests that they could be used to control two populations of cells independently in vivo. Given that intact copies of StaPLd-Casp9 would need to accumulate in ASV to induce apoptosis, whereas preexisting copies of iCasp9 should induce apoptosis once a certain concentration of AP20187 is reached, the time course of apoptosis with StaPLd-Casp9 should be more gradual than that with iCasp9. Indeed, apoptosis was more gradual with ASV and StaPLd-Casp9 than with AP20187 and iCasp9 (time to maximum effect of 24 h versus 3 h; Supplementary Fig. 14a,b). This suggests that a StaPLd-Casp9-expressing cell population could be partially depleted by intermittent dosing of ASV, which could be useful for rheostatic control of cellular therapies.

In summary, we describe a generalizable design for drug-inducible proteins in which proteins are rescued from StaPL-induced fragmentation by HCV NS3 protease inhibitors. As examples, we used StaPL modules to confer drug control on protein localization of apoptosis with StaPLd-Casp9 should be more gradual than that with iCasp9. Indeed, apoptosis was more gradual with ASV and StaPLd-Casp9 than with AP20187 and iCasp9 (time to maximum effect of 24 h versus 3 h; Supplementary Fig. 14a,b). This suggests that a StaPLd-Casp9-expressing cell population could be partially depleted by intermittent dosing of ASV, which could be useful for rheostatic control of cellular therapies.

In summary, we describe a generalizable design for drug-inducible proteins in which proteins are rescued from StaPL-induced fragmentation by HCV NS3 protease inhibitors. As examples, we used StaPL modules to confer drug control on protein localization.
and on the functional output of zinc-finger-based transcriptional regulators. We also demonstrated that internal insertion of a StaPL module can be used to regulate a CRISPR–Cas9-based transcriptional activator, and to regulate apoptosis through linkages of two copies of Cas9 with a StaPL module. Potential advantages of StaPL over existing techniques for drug control of protein function include simplicity, robustness, multiplexibility, and the use of clinically approved drugs (Supplementary Note 3).

We expect that StaPL modules can be used to create drug-stabilized forms of many other natural proteins. For example, loop insertion of StaPL modules could create drug-inducible forms of Cas9 proteins from various species. This will allow targeting of two different genes by two different sgRNAs and regulation by two different drugs while involving the expression of only two polypeptide chains (for example, SpCas9 with a StaPL insert and Staphylococcus aureus Cas9 with a StaPL insert), compared with four polypeptides when heterodimerizing systems are used\(^{15,16}\). More generally, many proteins tolerate the insertion of protein domains into exposed loops\(^{30}\), and should therefore be amenable to drug regulation via internal StaPL module insertion and linkage preservation by NS3 protease inhibitors.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41592-018-0041-z.

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Author contributions

M.Z.L. conceived of the study. C.L.J. designed orthogonal StaPL modules and StaPL-controlled proteins. R.K.B. and C.L.J. constructed plasmids and designed and carried out mammalian cell experiments. M.Z.L. and C.L.J. wrote the manuscript, with contributions from R.K.B.

Competing interests

M.Z.L., C.L.J., and R.K.B. have filed a provisional patent (Application 62/536307) with the US Patent and Trademark Office for compositions and methods for inducing controlled proteins. R.K.B. and C.L.J. constructed plasmids and designed and carried out mammalian cell experiments. M.Z.L. and C.L.J. wrote the manuscript, with contributions from R.K.B.

Additional information

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DNA plasmids and molecular cloning. Plasmids were constructed via standard molecular biology techniques: restriction enzyme digestion (Fermentas), PCR and overlap extension PCR with PrimeSTAR polymerase (Clontech), and assembly using either In-Fusion enzyme (Clontech) or T4 ligase (Thermo Fisher). DNA was transformed into XL10 Gold (Agilent) or Stellar (Clontech) competent Escherichia coli with ampicillin (100 µg/ml) selection. Plasmid DNA was isolated with the PureLink hiPure Plasmid Maxiprep kit (Thermo Fisher) or the Plasmid Plus Midi prep kit (Qiagen). Subcloned regions were verified by Sanger sequencing, assisted by Geneious (Biomarkers).

Hepatitis C virus sequences used in this study were derived from genotype 1a HCV (GenBank accession number NC_004102). Plasmids encoding PSD95-SMASH, Arc-SMASH, and (Venus) YFP-SMASH mutant variants were under the control of the CMV promoter in the pCMV-SORT6 backbone (Life Technologies), and were adapted from those described previously. The S. pyogenes Cas9 constructs, which carried nuclease-deactivating mutations D10A and H84A (dSpCas9), were under the control of the PGK promoter and consisted of a VP RNA-targeting vector domain linked to BFL, dSpCas9 (with or without a DEEMECF/C’s cleavage site and StaPL module) inserted into an internal loop, and a tandem NIS. In these constructs, the VP’s internal NIS was replaced with a GGSGGGS linker. The S. pyogenes sgRNA directed to the TRE3G locus (target sequence GTAGTGTGTCATACGATA on nontemplate strand).

Human Cas9 (excluding its CARD caspase recruitment domain) was amplified from the pPC3Kas9-His plasmid (a gift from the G. Salvesen lab; Addgene plasmid # 11830) [9]. Dual copies of the Cas9 large and small subunits were linked together by an HA epitope tag, a DEEMECF/C’s cleavage site, and a StaPL module. This PlaPLd-Cas9 construct was subcloned into the pcDNA5/FRT shuttle vector (Thermo Fisher), which contains a CMV promoter. An internal ribosome entry sequence (RES) and a coding sequence for eRFP (a monomeric RFP from Yeast) were ligated downstream of the PlaPLd-Cas9 construct, yielding a StaPLd-Cas9 construct. The iCasp9 gene was amplified from the pMSCV-Fdel Casp9.IRES.GFP plasmid (a gift from the D. Spencer lab; Addgene plasmid # 15567) and subcloned into the pcDNA5/FRT shuttle vector via a flexible linker.

Gel staining and blotting. For SDS–PAGE analysis, cells were cultured in 24- or 12-well plates (Greiner Bio-One) for 8–48 h after transfection and drug addition. Culture media were removed, and then cells were lysed with 50 or 100 µl of hot (90°C) SDS lysis buffer (100 mM Tris-HCl, pH 6.0, 5% SDS, 20% glycerol, 0.2% bromophenol blue). Lysates were sonicated to shear DNA, heated briefly to 90°C, centrifuged, and loaded on either NuPAGE–4–12% Bis-Tris (Life Technologies) or 4–15% Criterion TGX (Bio-Rad) gels, along with Novex Sharp pre-stained protein standard (Life Technologies). Transfers onto PVDF membrane were performed using either the iBlot system (Life Technologies) or the Trans-Blot Turbo system (Bio-Rad).

Membranes were typically probed with primary and secondary antibodies using the iBlot system (Life Technologies). Alternatively, we carried out immunoprecipitation by blotting the membrane with 7.5% (w/v) nonfat dry milk in Tris-buffered saline (TBS) for 1 h at ambient temperature on an electric rocker, washing it three times with TBS with 0.1% Tween 20 (TBS-T), incubating it for 1 h with primary antibodies in 5% BSA in TBS-T, washing it three times in TBS-T, incubating it for 1 h with fluorescent secondary antibodies in 7.5% nonfat dry milk in TBS-T, and washing it a final three times in TBS-T.

For the immunoblotting experiment depicted in Supplementary Fig. 1b, membranes were cut into two sections and trncunted in order to stain all pre-transfections in parallel with the same anti-PDS95, and then cut into sections in parallel with rabbit anti-GAPDH. In all other cases, membranes were stained intact. Uncropped versions of non-truncated immunoblotting membranes are available (Supplementary Fig. 15).

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Western blotting quantifications were done in the Fiji distribution of ImageJ\(^\text{\textregistered}\). Integrated densities for bands of the same protein species were measured using a consistently sized rectangle, and background measurements from the same lanes were subtracted from these. We normalized protein-of-interest bands by dividing by loading control bands, which were quantified via the same method. Data analysis was done in Excel (Microsoft) and OpenOffice (Apache) software.

**Microscopy.** Brightfield microscopy of live StaPLD-Casp9-expressing HeLa cells (in 12-well plates; Greiner Bio-One) was done on an EVOS FL Cell Imaging System with a 4×0.1 numerical aperture (NA) objective. Fluorescence widefield microscopy of live transfected HeLa cells (in 35-mm glass-bottom four-chamber dishes; In Vitro Scientific), live or fixed transfected HEK293-TRE3G-mCherry stable cells (in plastic 12-well plates (Greiner Bio-One) or glass-bottom 12-well plates (In Vitro Scientific)), and staPLD-Casp9-expressing HeLa cells (mounted on glass slides with #1.5 coverslips) was performed on an Axiovert 200 M inverted microscope (Zeiss) with 40×/1.2 NA water-immersion, 5×/0.25-NA air, and 10×/0.5-NA air objectives, respectively. The following excitation (ex) and emission (em) filters were used: Green/Yellow (YFP, Alexa Fluor 488), ex 485/10 nm (Chroma), em 510 nm long-pass dichroic (Omega) and 525/40 nm (Chroma); Red (RFP, eqRFP), ex 568/20 nm (OmegA), em 585 long-pass dichroic (Chroma) and 620/60 nm (Chroma). Excitation was from an X-Cite 120 W metal-halide lamp with a 3–mm-core liquid light guide (Lumen Dynamics), and acquisition was performed by an ORCA-ER camera (Hamamatsu). The system was controlled by MetaMorph Pro software (A1261; Apple) running the MacOS 10.6.8 operating system (Apple) and Micro-Manager 1.4.10 software.\(^{\text{\textregistered}}\)

Confocal fluorescence microscopy of live transfected HEK293A cells was done in 35-mm glass-bottom four-chamber dishes (In Vitro Scientific) on an UltraVIEW VoX system (PerkinElmer) equipped with a CSU-X1 spinning disc (Yokogawa Electric) and a C9100-50 EM-CCD (electron-multiplying charge-coupled device) system (Hamamatsu). Imaging was performed using a Vysis DBC-16000 FL (v4.0) control and analysis manager software. In some cases, sample measurements exceeded those of the top standard (Tecan). Human VEGF protein dilution standards were used to calculate sample absorbances at 450 nm returned to fresh drug-containing DMEM. Live transfected HEK293A-TRE3G-mCherry cells, plates were coated with 0.5 mg/ml poly-d-lysine hydrobromide (Sigma-Aldrich) in water by incubation at 37 °C for 4 h to overnight, and were washed four times with water before cells were plated. Live StaPLD-Casp9 HeLa cells were imaged in DMEM supplemented as described above, and live transfected HeLa cells and HEK293-TRE3G-mCherry cells were imaged in Hank’s Balanced Salt Solution (HBSS; HyClone). In the case of the time course experiments, appropriate protease inhibitor drug was maintained in HBSS during imaging before cells were returned to fresh drug-containing DMEM. Live transfected HEK293A cells were imaged in FluoroBrite DMEM (Life Technologies) supplemented as described above. Fixed cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in phospho-buffered saline (PBS; HyClone) for 15 min at ambient temperature, washed twice with HBSS, and subsequently stored in HBSS. Image processing and analysis were done with Fiji. For stained/fixed StaPLD-Casp9-expressing HeLa cells, 10-µm-tall z-stacks were acquired in 1-µm intervals and were subsequently transformed into maximum-intensity projections and background-subtracted. For live transfected HEK293A cells, z-stacks were acquired in 0.5-µm intervals and ranges encompassing 4 µm were transformed into maximum-intensity projections. For live or fixed transfected HEK293-TRE3G-mCherry cells, background-subtracted images were quantified for RFP fluorescence using the integrated density function.

**Enzyme-linked immunosorbent assay.** HEK293A cells were cultured in 12-well plates (Greiner Bio-One) with 1 ml of DMEM (supplemented as previously described) in each well and were transfected 48 h before harvesting of media supernatants (transfection reagent volume: 200 µl). Media were not changed after transfection. Appropriate protease-inhibitor drugs were applied concurrently with transfection. Cells transfected with empty vector and given identical protease-inhibitor treatments served as controls. From each well, 900 µl of media supernatant was collected for ELISA analysis and frozen at −20 °C until it was processed. Samples were thawed to ambient temperature 1 h before use, and were cleared by centrifugation at 12,000g for 10 min at 4 °C.

We carried out sandwich ELISA with the Human VEGF Quantikine ELISA kit (R&D Systems) according to the manufacturer’s protocol. Absorbances at 450 nm (with reference wavelength 540 nm) were measured thrice and averaged, using an Infinite M1000 PRO plate reader (Tecan) under the control of i-control software (Tecan). Human VEGFA mRNA dilution standards were used to quantify relative VEGFA levels by four-parameter logistic regression (http://www.elsaanalysis.com/). In some cases, sample measurements exceeded those of the top standard value, and were diluted twofold and rescanned for interpolation. The calculated [VEGF] for such samples was thus multiplied by two. Because each well of HEK293A contained 1.2 ml of media post-transfection, [VEGF] values were adjusted by a factor of 1.2 to obtain true concentrations in picograms per milliliter. VEGF measurements are expressed as differences from cells transfected with empty vector and incubated in similar drug conditions.

**Quantitative real-time PCR.** To measure mRNA levels in HEK293A or HEK293-TRE3G-mCherry cells, we first plated cells on plastic 12-well plates (Greiner Bio-One or Thermo Scientific Nunclon Delta Surface). For HEK293-TRE3G-mCherry cells from were coated with 0.5 mg/ml poly-d-lysine hydrobromide (Sigma-Aldrich) in water by incubation at 37 °C for 4 h to overnight, and were washed four times with water before plating. We harvested cells by aspirating media and lysing with 500 µl of RNA lysis buffer (Zymo). Lysates were frozen at −20 °C before processing of 150 µl of each sample with molecular-biology-grade ethanol (Sigma). RNA (Micro) was quantified from a NanoDrop spectrophotometer (Thermo Fisher); otherwise they were re-extracted.

RNAs were reverse-transcribed with Superscript IV VILO Master Mix (Thermo Fisher) according to the manufacturer’s instructions. Prior to reverse transcription, samples were normalized by dilution of some samples with RNase-free water. Each RNA sample was reverse-transcribed in duplicate. cDNAs were used as templates in PCR reactions using the Applied Biosystems Taqman Gene Expression Master Mix (Fisher Scientific). PCR reactions (10 µl) were performed in Hard-Shell thin-wall 384-well PCR plates (Bio-Rad) sealed with Microseal B adhesive (Bio-Rad) and were analyzed on a Bio-Rad CFX384 thermocycler. Each PCR reaction for a given cDNA sample was performed in duplicate. All steps were performed using barrier pipette tips.

PCR primers and Taqman probes were synthesized by Integrated DNA Technologies and used at final concentrations of 500 nM for primers and 250 nM for probes. Sequences of primer pairs and probes were as follows:

- **mCherry RFP forward primer:** GAGGCTGAACGTGAAAGGAC
- **mCherry RFP reverse primer:** GATGTTGTAGCTCCGTGTGGTG
- **mCherry PCR probe:** 56-FAM-CAACATTGTAZEN/3IABkFQ
- **Human VEGFA forward primer:** CCATGAACCTTCTCGTCTGCTG
- **Human VEGFA reverse primer** (exon-spanning): TGACCTCCACATCCTGGTAT
- **Human GAPDH forward primer:** CCATGCTTGCATCAGGGTGA
- **Human GAPDH forward primer** (exon-spanning): CACACTGCTGTCAATGA

Using dilution series, we verified that primer/probe sets had efficiencies of >90%, and that pairs of primer/probe sets (RFP and GAPDH, VEGFA and GAPDH) had efficiencies within 5% of another one. We calculated Cq values from amplification curves using the Single Threshold method in CFX Manager software (Bio-Rad). Data were analyzed in Excel. We obtained relative gene expression values by the ΔΔCq method: first, Cq values for genes of interest were normalized by the housekeeping gene GAPDH, and then the resultant values were normalized to drug- and/or time-matched empty-vector-transfected controls.

**Cell staining.** To assess the activity of engineered suicide genes, we stained StaPLD-Casp9-expressing or iCas9-expressing HeLa cells after drug incubation. Media were collected to harvest dead/lifted cells, adherent cells were trypsinized to harvest living cells, and the two were pooled and pelleted at 500g for 5 min. Cells were washed once with HBSS, resuspended in either HBSS or annexin V binding buffer (Biotium), and stained with the NucView 488 caspase-3 assay kit for live cells (Biotium) or the annexin V CF488A conjugate kit (Biotium), respectively, according to the manufacturer’s instructions. In some instances, cells of the parent cell line (Flp-In HeLa) were annexin-V-stained in parallel. For medium-term preservation, cells were fixed in 4% PFA in PBS for 15 min at ambient temperature after staining, centrifuged at 10,000g, and resuspended in HBSS after aspiration of the PFA. For microscopy, a 25-µl droplet of each sample was then placed on a SuperFrost Plus glass slide (Fisher Scientific) and allowed to dry partially before the addition of Vectashield mounting medium with DAPI (Vector Labs) and a #1.5 coverslip (Fisher Scientific). Slides were sealed with clear nail polish and kept at 4 °C.

**Flow cytometry.** Flow cytometry was performed on live or fixed cells (for annexin-V-stained cells) or fixed cells (for the caspase-3 sensor) prepared as described above, using a Digital Vantage instrument (Becton Dickinson) under the control of CytExpert software (Becton Dickinson) and flow cytometric data were analyzed with the Stanford Shared FACS Facility. A 488-nm laser was used to excite Alexa Fluor 488 stain, and a 594-nm laser was used to excite eqRFP. The parent Flp-In HeLa cell line, which did not express eqRFP, was used to confirm eqRFP-positive monoclonal populations for the stable cell lines. For each sample, 10⁶ events were collected. Gates were constructed inside forward scatter and side scatter plots (FSC-A versus SSC-A and FSC-H versus FSC-A) to side debris and non-singlet events, respectively (Supplementary Fig. 11c). Cytometry data were analyzed and processed with FlowJo software (FlowJo LLC).
Statistical analyses. Statistical analyses were carried out with Excel (Microsoft) and Prism (Graphpad). To test for significant pairwise differences between drug responsiveness of SMASHwt, SMASHAI, and SMASHTI variants for a given ASV or TPV drug concentration, normality of the data were confirmed by the Shapiro–Wilk normality test, then data were subjected to two-way ANOVA, with multiple comparisons between each variant's mean normalized NS3 activity for each concentration of ASV and TPV. Data for ASV and TPV were analyzed separately. Corrected P values from multiple comparisons were corrected post hoc by Tukey test.

Corrected P values are as follows:

- 0.01 μM ASV: NS3wt versus NS3AI, P = 0.2476; NS3wt versus NS3TI, P = 0.9568; NS3AI versus NS3TI, P = 0.1530
- 0.1 μM ASV: NS3wt versus NS3AI, P = 0.1404; NS3wt versus NS3TI, P = 0.0017; NS3AI versus NS3TI, P = <0.0001
- 1 μM ASV: NS3wt versus NS3AI, P = <0.9999; NS3wt versus NS3TI, P = <0.0001; NS3AI versus NS3TI, P = <0.0001
- 10 μM ASV: NS3wt versus NS3AI, P = 0.9963; NS3wt versus NS3TI, P = 0.0001; NS3AI versus NS3TI, P = <0.0001
- 0.1 μM TPV: NS3wt versus NS3AI, P = 0.9174; NS3wt versus NS3TI, P = 0.2797; NS3AI versus NS3TI, P = 0.4779
- 1 μM TPV: NS3wt versus NS3AI, P = 0.3484; NS3wt versus NS3TI, P = 0.0088; NS3AI versus NS3TI, P = 0.1513
- 10 μM TPV: NS3wt versus NS3AI, P = 0.2116; NS3wt versus NS3TI, P = 0.0088; NS3AI versus NS3TI, P = 0.0002

To test for significant pairwise differences between VEGF secretion in different drug conditions for the given zinc-finger construct or constructs, we confirmed normality of the data by the Shapiro–Wilk normality test, then subjected the data to one-way ANOVA, with multiple comparisons between mean normalized VEGF concentration for each drug condition. Data for each expressed zinc-finger construct were analyzed separately. P values from multiple comparisons were corrected post hoc by Tukey test. Corrected P values are as follows:

- ZFVEGFA-StaPL7-tdRFP-KRAB: DMSO versus ASV, P = 0.9969
- ZFVEGFA-StaPL7-tdRFP-KRAB: DMSO versus TPV, P = 0.0009

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Datasets supporting the findings of the current study are available from the corresponding author upon request. Mammalian expression plasmids with complete sequence information are available at http://www.addgene.org for YFP-SMAShAI (111500), YFP-SMAShTI (111501), ZFVEGFA-StaPLAI-YFP-VPR (111502), ZFVEGFA-StaPLTI-tdRFP-KRAB (111504), VPR-dSpCas9(StaPLTI) (111508), and StaPLd-Casp9 (111511).

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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- n/a Confirmed
- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

- Microscopy data was acquired using Micro-Manager for Mac OS X version 1.4.10 (UCSF) or Volocity for Windows version 5 (Improvion).
- ELISA data was acquired using i-control for Windows version 3.4.2 (Tecan). RT-qPCR data was acquired using CFX Manager for Windows version 3.1 (Bio-Rad). Western blot membrane scans were typically acquired using ImageStudio for Mac OS X version 3.1.4 (Li-COR).
- Some were acquired using Odyssey Software for Windows (Li-COR). Flow cytometry data was acquired using CellQuest software (Becton Dickinson).

Data analysis

- FlowJo version 10.2 for Mac OS X (Flowjo LLC) was used to analyze flow cytometry data. Various numerical data were analyzed with Excel 2008 for Mac version 12.0 (Microsoft) and OpenOffice for Mac version 4.0.1 (Apache Software Foundation). DNA sequencing data were analyzed with Geneious for Mac version 7.0.3 (Biomatters). Molecular modeling of protein crystal structures was performed with UCSF Chimera for Mac version 1.11.2 (UCSF RBVI) and MacPyMol version 1.8.6.0 (Schrödinger). Statistical calculations were performed using Prism 7 for Mac OS X version 7.0c (Graphpad). Image analysis was performed with ImageJ/Fiji for Mac version 1.48 (NIH). Analysis of ELISA data was performed using the publicly available tool http://elisaanalysis.com/.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
**Data**

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Mammalian expression plasmids with complete sequence information are available at addgene.org for YFP-SMASHAI (111500), YFP-SMASHTI (111501), ZFVEGFA-StaPLAI-YFP-VPR (111502), ZFVEGA-StaPLTI-tcrFP-KRAB (111504), VPR-dSpCas9(StaPLTI) (111508), and StaPLd-Casp9 (111511). Datasets supporting the findings of the current study are available from the corresponding author upon reasonable request.

**Field-specific reporting**

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- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

**Life sciences study design**

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample/replicate sizes were selected to comport with current standards in the field. For key experiments this meant attaining n=3 or more. |
|-------------|----------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | In one independent VEGF ELISA experiment (For Figure 1d), the measured [VEGF] for empty vector-transfected, TPV-treated cells was over 3 times higher than that of empty vector-transfected cells treated with ASV or DMSO. Since drug-matched empty vector control wells were used to normalize data, the anomalous empty vector value made data difficult to confidently interpret, and so these data were excluded from analysis. |
| | In the immunoblot experiment used to compare NS3 mutant variants (Supplementary Figure 1b-c, Supplementary Figure 2a), several of the 8-sample series were difficult to quantify due to handling artefacts (creasing, smudging, uneven staining) and were thus excluded from analysis. Retained lysates were used to perform reruns of SDS-PAGE and immunoblotting and generate usable data for quantification. |
| | In several microscopy and RT-qPCR experiments used to assess the performance of VPR-dCas9(StaPLTI) (Figure 2b-c, Supplementary Figure 9b,d,f), TPV induction of RFP fluorescence or mRNA was found to be suboptimal. This was found to be attributable to poor transfection efficiency due to cell culture conditions and transfection method, and these data were excluded. Using a different serum source, using plastic growth substrates instead of glass, and using Cal Phos instead of Lipofectamine improved transfection efficiency and reproducibility of findings. |
| | In several RT-qPCR experiments used to assess the performance of ZFVEGFA-StaPLAI-YFP-VPR (Supplementary Figure 9c,e,g), ASV induction of VEGFA mRNA was found to be suboptimal. This was attributed to poor transfection efficiency due to cell culture conditions, and these data were excluded. Using a different serum source and high-purity plasmids for transfection improved transfection efficiency and reproducibility of findings. |
| | In initial flow cytometry experiments used to query the dosage-dependence and timecourse of apoptosis due to StaPLd-Casp9, and to compare StaPLd-Casp9 to iCasp9 (Supplementary Figures 12, 13, 14), the peak StaPLd-Casp9-triggered apoptosis in 1 μM ASV was found to be suboptimal, which was likely attributable to using an older passage of the stable cell line. Earlier data were excluded in favor of data obtained from younger passage cells. |
| Replication | For key findings, at least 3 independent experiments were performed in order to gauge the reproducibility of results. In some cases, findings were corroborated through the use of different assays to generate converging lines of evidence (e.g. by immunoblot and fluorescence microscopy, or by fluorescence microscopy and RT-qPCR). As the outcome of cell culture experiments was highly contingent upon expression of protein constructs, reproducibility was ensured by using low-passage cells, high-quality serum, high-purity plasmids (if applicable), and optimizing growth substrate and transfection method (if applicable). |
| Randomization | No randomization was performed, as the study did not involve discrete human or animal subjects. |
| Blinding | Experimenters were not blinded, as most datasets were gathered in an automatic fashion by machines (plate spectrophotometer, real-time thermocycler). |

**Reporting for specific materials, systems and methods**
Materials & experimental systems

| Involved in the study | n/a |
|-----------------------|-----|
| □ □ Unique biological materials | □ |
| □ □ Antibodies | □ |
| □ □ Eukaryotic cell lines | □ |
| □ □ Palaeontology | □ |
| □ □ Animals and other organisms | □ |
| □ □ Human research participants | □ |

Methods

| Involved in the study | n/a |
|-----------------------|-----|
| □ □ ChIP-seq | □ |
| □ □ Flow cytometry | □ |
| □ □ MRI-based neuroimaging | □ |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Unique materials consist of plasmids that will be made available at addgene.org.

Antibodies

- **Antibodies used**
  - Mouse anti-PSD95, NeuroMabs (Antibodies Inc.), Cat. No. 75-028, clone K28/43, 1:1000
  - Rabbit anti-β-actin, GeneTex, Cat. No. GTX124214, polyclonal, 1:3333
  - Rabbit anti-GAPDH, Santa Cruz, Cat. No. sc-25778, polyclonal, 1:500
  - Rabbit anti-Arc, Synaptic Systems, Cat. No. 156 003, polyclonal, 1:200
  - Mouse anti-GFP, Pierce, Cat. No. MA5-15256, clone GF28R, 1:1000
  - Rabbit anti-ttdTomato, OriGene, Cat. No. TA150128, polyclonal, 1:2000
  - Rabbit anti-HA, Cell Signaling, Cat. No. 3724S, clone C29F4, 1:1000
  - Mouse anti-GFP, Pierce, Cat. No. MA5-15256, clone GF28R, 1:1000
  - Rabbit anti-HA, Santa Cruz, Cat. No. sc-7392, clone F-7, 1:1000
  - Rabbit anti-eqRFP, Evrogen, Cat. No. AB233, polyclonal, 1:1000
  - Mouse anti-β-actin, Santa Cruz, Cat. No. sc-81178, clone ACTBD11B7, 1:1000
  - 680RD-conjugated goat-anti-mouse, LI-COR, Cat. No. 926-68070, 1:3333
  - 800CW-conjugated goat-anti-rabbit, LI-COR, Cat. No. 926-32211, 1:3333
  - 680RD-conjugated goat-anti-rabbit, LI-COR, Cat. No. 926-68071, 1:3333
  - 800CW-conjugated goat-anti-mouse, LI-COR, Cat. No. 926-32210, 1:3333

- **Validation**
  - Mouse anti-PSD95 has been used extensively in our lab for western blotting. Diminished intensity or absence of a PSD95 immunopositive band in conditions where PSD95 is expected to be degraded by SMASH serve as validation (Supplementary Figure 1a-b, Figure 1c).
  - Rabbit anti-β-actin has been used extensively in our lab for western blotting. The manufacturer has validated the antibody, noting decreased intensity of the β-actin immunopositive band in cells transfected with shRNA against β-actin.
  - Rabbit anti-GAPDH has been used extensively in our lab for western blotting. The manufacturer has validated the antibody by testing it against a GAPDH transfected 293T cell control lysate (sc-159909).
  - Rabbit anti-Arc has been used extensively in our lab for western blotting. Absence of an Arc immunopositive band in conditions where Arc is expected to be degraded by SMASH serve as validation (Figure 1c).
  - Mouse anti-GFP has been used extensively in our lab for western blotting against YFP. The manufacturer has verified it does not produce a band in untransfected cell lysates. We independently validate the antibody by demonstrating it does not produce a band for cells that were not transfected with a YFP-containing construct (Supplementary Figure 3).
  - Rabbit anti-ttdTomato was verified by the manufacturer not to produce a western blot band in untransfected cell lysates. We independently validate the antibody by demonstrating it does not produce a band for cells that were not transfected with an RFP-containing construct (Supplementary Figure 3).
  - Rabbit anti-HA has been used extensively in our lab for western blotting against the HA epitope. To validate the antibody, the manufacturer has verified that no band is produced in untransfected cell lysates.
  - Mouse anti-GAPDH has been used extensively in our lab for western blotting, and has been cited extensively in the literature.
  - Mouse anti-HA was validated by the manufacturer, who demonstrated that a western blot band is not produced for cells that do not express an HA-tagged protein.
  - Rabbit anti-eqRFP was verified by the manufacturer not to produce a western blot band in cells transfected with fluorescent proteins belonging to other families.
  - Mouse anti-β-actin has been used extensively in our lab for western blotting. The manufacturer validated the antibody by demonstrating that the intensity of a β-actin immunopositive band is strengthened in lysates of cells transfected with β-actin.
  - The LI-COR secondary antibodies have been used extensively in our lab for western blotting, are designed to have minimal cross-reactivity, and are extensively cited in the literature.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

- HEK293A cells, Life Technologies; HeLa cells, ATCC; HEK293-TRE3G-mCherry cells, Stanley Qi lab; Flp-In T-REx HeLa cells, Stephen Taylor lab.
**Authentication**

Cell lines were not stringently authenticated, but visual inspections of cells comported with expected morphologies.

**Mycoplasma contamination**

Cell lines were not tested for mycoplasma.

**Commonly misidentified lines** *(See ICLAC register)*

HEK293 cell lines were used in this study. HEK are listed in Version 8 of the ICLAC database of commonly misidentified or cross-contaminated lines, with HeLa listed as the contaminating cell line. Despite the risk for misidentification/contamination, we deem the use of HEK293 cells to be justifiable since this cell line has been typically utilized for the types of experiments we needed to perform (VEGF ELISA, dCas9 transfection and RT-qPCR). Further, we deem the risk of misidentification to be low. The morphologies of HEK and HeLa are visually discriminable and cell lines were regularly visually inspected (2-3 times per week).

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**Flow Cytometry**

**Plots**

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

**Sample preparation**

StaPLd-Casp9-expressing or iCasp9-expressing HeLa cells were stained after drug incubation. Media were collected to harvest dead/lifted cells, adherent cells were trypsinized to harvest living cells, and the two were pooled and pelleted at 500g for 5 min. Cells were washed once with HBSS, resuspended in either HBSS or Annexin V Binding Buffer (Biotium), and stained with the NucView 488 Caspase-3 Assay Kit for Live Cells (Biotium) or the Annexin V CF488A Conjugate Kit (Biotium), respectively, according to manufacturer’s instructions. In some instances, cells of the parent cell line (Flp-In HeLa) were annexin V-stained in parallel. For medium-term preservation, cells were fixed in 4% PFA in PBS for 15 min at ambient temperature following staining, centrifuged at 10,000g, and resuspended in HBSS after aspirating the PFA.

**Instrument**

Digital Vantage (Becton Dickinson)

**Software**

Data collection was performed under the control of CellQuest software (Becton Dickinson). Data were analyzed by FlowJo software (FlowJo LLC).

**Cell population abundance**

Flow cytometry data was collected but no sorting was performed. For each sample, 10,000 events were collected.

**Gating strategy**

Gates were constructed inside forward scatter and side scatter plots (SSC-A vs. FSC-A and FSC-A vs. FSC-H) to exclude debris and non-singlet events, respectively. (See Supplementary Figure 11c).

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.

StaPLd-Casp9-expressing or iCasp9-expressing HeLa cells were stained after drug incubation. Media were collected to harvest dead/lifted cells, adherent cells were trypsinized to harvest living cells, and the two were pooled and pelleted at 500g for 5 min. Cells were washed once with HBSS, resuspended in either HBSS or Annexin V Binding Buffer (Biotium), and stained with the NucView 488 Caspase-3 Assay Kit for Live Cells (Biotium) or the Annexin V CF488A Conjugate kit (Biotium), respectively, according to manufacturer’s instructions. In some instances, cells of the parent cell line (Flp-In HeLa) were annexin V-stained in parallel. For medium-term preservation, cells were fixed in 4% PFA in PBS for 15 min at ambient temperature following staining, centrifuged at 10,000g, and resuspended in HBSS after aspirating the PFA.

6. Identify the instrument used for data collection.

Digital Vantage (Becton Dickinson)

7. Describe the software used to collect and analyze the flow cytometry data.

Data collection was performed under the control of CellQuest software (Becton Dickinson). Data were analyzed by FlowJo software (FlowJo LLC).

8. Describe the abundance of the relevant cell populations within post-sort fractions.

Flow cytometry data was collected but no sorting was performed For each sample, 10,000 events were collected.

9. Describe the gating strategy used.

Gates were constructed inside forward scatter and side scatter plots (SSC-A vs. FSC-A and FSC-A vs. FSC-H) to exclude debris and non-singlet events, respectively. (See Supplementary Figure 11c).

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☑