Potent Delivery of Functional Proteins into Mammalian Cells *In Vitro* and *In Vivo* Using a Supercharged Protein

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SUPPORTING INFORMATION

Experimental Methods

Cell Lines and Cell Culture

All cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM glutamine, 5 I.U. penicillin, and 5 μg/mL streptomycin at 37 °C with 5% CO_2. HeLa and PC12 cell lines were purchased from ATCC. Paula Nunes (Harvard University), Steve Dowdy (UCSD), and Matthias Schnell (Thomas Jefferson University) kindly provided IMCD, 3T3.LNL.LacZ, and BSR cell lines, respectively.

Cloning

All overexpression plasmids were constructed on a pETDuet-1 backbone. Genes encoding mCherry and Cre were subcloned with a C-terminal His_6 tag installed using a PCR primer. Genes encoding Tat, Arg_{10}, penetratin, or +36 GFP and a $(GGS)_9$ linker were inserted N-terminal of mCherry and Cre by USER cloning. The genes encoding the $(GGS)_9$ linker, Tat, Arg_{10} and penetratin were designed by Gene Designer (DNA 2.0) and ordered as separate complementary DNA strands, phosphorylated using T4 PNK, and hybridized prior to cloning. Sequences encoding N-terminal His_6-tagged ubiquitin, and the corresponding G76V mutant, were assembled from a set of overlapping oligonucleotides[1] and ligated into *Nco*I and *Nhe*I restriction enzyme cleavage sites upstream of pET-+36 GFP[2] to create a fusion directly to the N-terminus of +36 GFP. Plasmids created in this work will be accessible through Addgene.

Protein Purification

Cells were grown in 1 L LB cultures at 37° C to OD_{600} = ~0.6 and induced with 1 mM IPTG at 30° C for 4 h. Cells were harvested by centrifugation and stored at -80° C. Frozen pellets were thawed in 40 mL PBS + 2 M NaCl and lysed by sonication. The lysate was cleared by centrifugation (10,000 *G*, 8 min) and the supernatant was mixed with 1 mL of Ni-NTA agarose resin for 30 min at 4

°C on a rotating drum. The resin was recovered by centrifugation (10,000 *G*, 8 min), resuspended in 20 mL PBS + 2 M NaCl, and packed into a 5 mL syringe containing a glass wool plug. The resin was washed with 15 mL of PBS containing 2 M NaCl and 20 mM imidazole. The protein fusion was eluted with 3 mL PBS containing 2 M NaCl and 500 mM imidazole. All Cre proteins were dialyzed against PBS + 0.5 M NaCl for 18 h at 4° C. All other proteins were dialyzed against PBS for 18 h at 4° C. Dialyzed proteins were centrifuged to remove precipitated protein and analyzed for purity by SDS-PAGE staining with Coomassie Blue. Further purification by cation exchange was necessary for Tat-mCherry, Arg_{10}-mCherry, Tat-Cre, and Arg_{10}-Cre to remove contaminants. These proteins were purified using a HiTrap Capto SP XL column on an AKTA Express FPLC and eluted using a linear gradient from PBS to PBS + 1 M NaCl. Tat-mCherry and Arg_{10}-mCherry were dialyzed back into PBS and Tat-Cre and Arg_{10}-Cre were dialyzed back into PBS + 0.5 M NaCl. Protein samples were stored in aliquots at -80° C in PBS (or PBS + 0.5 M NaCl for all Cre proteins). Arg_{20} and Arg_{30} mCherry and Cre constructs were also generated but did not express corresponding protein in significant quantities.

All +36 GFP-containing proteins were quantified by absorbance at 488 nm with an extinction coefficient[3] of 8.33 x 10^4 $M^{-1}cm^{-1}$. All mCherry-containing proteins (mCherry, Tat-mCherry, Arg_{10}-mCherry, and penetratin-mCherry) were quantified by absorbance at 587 nm with an extinction coefficient[4] of 7.2 x 10^4 $M^{-1}cm^{-1}$. All Cre-containing proteins (wild-type Cre, Tat-Cre, Arg_{10}-Cre, and penetratin-Cre) were quantified using a modified Lowry protein assay kit (Pierce). Cre-containing protein quantifications were also confirmed by *in vitro* activity assay. Briefly, various concentrations of Cre protein were incubated in 50 μL Cre reaction buffer (33 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5) and 500 ng of pCALNL-dsRed2 (linearized with *Pvu*I) at 37° C for 30 min and then at 70° C for 10 min. DNA was recovered by QIAQUICK spin column and half of the recovered DNA was analyzed on a 0.5% agarose gel containing ethidium bromide at 100 V for 30 min. DNA was visualized by ultraviolet light.

Deubiquitination Assay Western Blot

Cells were plated in a 48-well plate at a density of $1x10^5$ cells per well. After 18 h, cells were washed with cold PBS and incubated with 100 nM ubiquitin-+36 GFP or 100 nM mutant G76V ubiquitin-+36GFP in serum-free media for 1 h. Cells were washed three times with 20 U/mL heparin in PBS, and lysed directly in LDS sample buffer and sonicated.

Crude HeLa cytosolic extract was prepared by harvesting $5x10^6$ HeLa cells using a plate scraper into ice-cold PBS. Cells were pelleted at 200 *G* for 5 min and resuspended in 1 mL of 50 mM

Tris-HCl pH 7.5, 150 nM NaCl, 2 mM EDTA, 2 mM DTT, 1.7 μg/mL aprotinin, 10 μg/mL leupeptin, 10 mM PMSF, and 0.5% NP-40. Homogenized cells were incubated on ice for 10 min before centrifugation at 13,000 *G* for 15 min to remove nuclei and cell debris. Either wt or mutant ubiquitin-+36 GFP (5 pmol) was added to the lysate. The mixture was incubated with or without either 10 mM *N*-ethylmaleimide or 20 μg/mL ubiquitin-aldehyde for 1 hour at 37ºC.

Samples were analyzed on a 12% SDS-PAGE gel and transferred by electroblot onto a PVDF membrane (Millipore) pre-soaked in methanol. Membranes were blocked in 5% milk for 1 h and incubated in primary antibody in 3 % BSA for 30 min at room temperature. Anti-GFP antibody (1/10,000 dilution, ab290) was purchased from Abcam. The membrane was washed three times with PBS and treated with the secondary antibody IRDye 800CW Goat Anti-Mouse IgG (1/10,000 dilution, Li-COR Biosciences) in blocking buffer (Li-COR Biosciences) for 30 min. The membrane was washed three times with 50 mM Tris, pH 7.4 containing 150 mM NaCl and 0.05% Tween-20 and visualized using an Odyssey infrared imaging system (Li-COR Biosciences). Images were analyzed using Odyssey imaging software version 2.0.

Protein Sequences

stGFP (Supporting Figs. 1 and 2):
MGHHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGK
LPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKF
EGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSVQ
LADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK

+36 GFP (Figs. 2a and 3e and Supporting Figs. 1-3, 7, and 8):
MGHHHHHHGGASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATRGKLTLKFI
CTTGKLPVPWPTLVTTLTYGVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKKDGK
YKTRAEVKFEGRTLVNRIKLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAK
FKIRHNVKDGSVQLADHYQQNTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEF
VTAAGIKHGRDERYK

Tat-stGFP (Supporting Figs. 1 and 2):
MGRKKRRQRRRGHMASMTGGQQMGRDPASKGEELFTGVVPILVELDGDVNGHKFSVRGEG
EGDATNGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQE
RTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQK
NGIKANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVL
LEFVTAAGITHGMDELYKAAALEHHHHHH

+36 GFP-mCherry (Fig. 1 and Supporting Figs. 1-6):
MASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATRGKLTLKFICTTGKLPVPWPTLV
TTLTYGVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKKDGKYKTRAEVKFEGRTLVNRI

KLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAKFKIRHNVKDGSVQLADHYQQ
NTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEFVTAAGIKHGRDERYKGGSGGSGGS
GGSGGSGGSGGSGGSGGSVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEG
TQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDG
GVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQ
RLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGG
MDELYKLEHHHHHH

Ubiquitin-+36 GFP (Fig. 2 and Supporting Figs. 1-3):
MGHHHHHHGGMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDG
RTLSDYNIQKESTLHLVLRLRGGASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATR
GKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKK
DGKYKTRAEVKFEGRTLVNRIKLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAK
FKIRHNVKDGSVQLADHYQQNTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEFVTA
AGIKHGRDERYK

Ubiquitin G76V-+36 GFP (Fig. 2):
MGHHHHHHGGMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDG
RTLSDYNIQKESTLHLVLRLRGVASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATR
GKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKK
DGKYKTRAEVKFEGRTLVNRIKLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAK
FKIRHNVKDGSVQLADHYQQNTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEFVTA
AGIKHGRDERYK

+36 GFP-Cre (Fig. 3 and Supporting Figs. 1-3, 7, and 8):
MASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATRGKLTLKFICTTGKLPVPWPTLV
TTLTYGVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKKDGKYKTRAEVKFEGRTLVNRI
KLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAKFKIRHNVKDGSVQLADHYQQ
NTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEFVTAAGIKHGRDERYKGGSGGSGGS
GGSGGSGGSGGSGGSGGSMASNLLTVHQNLPALPVDATSDEVRKNLMDMFRDRQAFSEHTW
KMLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQARGLAVKTIQQHLGQLNMLHRRS
GLPRPSDSNAVSLVMRRIRKENVDAGERAKQALAFERTDFDQVRSLMENSDRCQDIRNLAFL
GIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAGVEKALSLGVTKLVERWISVSG
VADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRLIYGAKDDSGQRYLAWSGHSA
RVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLDSETGAMVRLLEDGDHHHHHH

Tat-mCherry (Fig. 1 and Supporting Fig. 4):
MGRKKRRQRRRGGSGGSGGSGGSGGSGGSGGSGGSGGSVSKGEEDNMAIIKEFMRFKVHME
GSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDY
LKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGW
EASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHN
EDYTIVEQYERAEGRHSTGGMDELYKLEHHHHHH

Arg_{10}-mCherry (Fig. 1 and Supporting Fig. 4):
MRRRRRRRRRGGSGGSGGSGGSGGSGGSGGSGGSGGSVSKGEEDNMAIIKEFMRFKVHME
GSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDY
LKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGW

EASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHN
EDYTIVEQYERAEGRHSTGGMDELYKLEHHHHHH

penetratin-mCherry (Fig. 1 and Supporting Fig. 4):
MRQIKIWFQNRRMKWKKGGSGGSGGSGGSGGSGGSGGSGGSGGSVSKGEEDNMAIIKEFMR
FKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKH
PADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQ
KKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNI
KLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKLEHHHHHH

Tat-Cre (Fig. 3 and Supporting Fig. 7):
MGRKKRRQRRRGGSGGSGGSGGSGGSGGSGGSGGSGGSMASNLLTVHQNLPALPVDATSDE
VRKNLMDMFRDRQAFSEHTWKMLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQAR
GLAVKTIQQHLGQLNMLHRRSGLPRPSDSNAVSLVMRRIRKENVDAGERAKQALAFERTDFD
QVRSLMENSDRCQDIRNLAFLGIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAG
VEKALSLGVTKLVERWISVSGVADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRL
IYGAKDDSGQRYLAWSGHSARVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLDSET
GAMVRLLEDGDHHHHHH

Arg_{10}-Cre (Fig. 3 and Supporting Fig. 7):
MRRRRRRRRRRGGSGGSGGSGGSGGSGGSGGSGGSGGSMASNLLTVHQNLPALPVDATSDE
VRKNLMDMFRDRQAFSEHTWKMLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQAR
GLAVKTIQQHLGQLNMLHRRSGLPRPSDSNAVSLVMRRIRKENVDAGERAKQALAFERTDFD
QVRSLMENSDRCQDIRNLAFLGIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAG
VEKALSLGVTKLVERWISVSGVADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRL
IYGAKDDSGQRYLAWSGHSARVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLDSET
GAMVRLLEDGDHHHHHH

penetratin-Cre (Fig. 3 and Supporting Fig. 7):
MRQIKIWFQNRRMKWKKGGSGGSGGSGGSGGSGGSGGSGGSGGSMASNLLTVHQNLPALPV
DATSDEVRKNLMDMFRDRQAFSEHTWKMLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYL
LYLQARGLAVKTIQQHLGQLNMLHRRSGLPRPSDSNAVSLVMRRIRKENVDAGERAKQALAF
ERTDFDQVRSLMENSDRCQDIRNLAFLGIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKT
LVSTAGVEKALSLGVTKLVERWISVSGVADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIF
EATHRLIYGAKDDSGQRYLAWSGHSARVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIR
NLDSETGAMVRLLEDGDHHHHHH

+36 GFP-$(GGS)_4$-ALAL-$(GGS)_4$-mCherry (Supporting Fig. 9):
MASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATRGKLTLKFICTTGKLPVPWPTLV
TTLTYGVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKKDGKYKTRAEVKFEGRTLVNRI
KLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAKFKIRHNVKDGSVQLADHYQQ
NTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEFVTAAGIKHGRDERYKGGSGGSGGS
GGSALALGGSGGSGGSGGSVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYE
GTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFED
GGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIK
QRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTG
GMDELYKLEHHHHHH

+36 GFP-$(GGS)_4$-ALAL-$(GGS)_4$-Cre (Supporting Fig. 10):
MASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATRGKLTLKFICTTGKLPVPWPTLV
TTLTYGVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKKDGKYKTRAEVKFEGRTLVNRI
KLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAKFKIRHNVKDGSVQLADHYQQ
NTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEFVTAAGIKHGRDERYKGGSGGSGGS
GGSALALGGSGGSGGSGGSMASNLLTVHQNLPALPVDATSDEVRKNLMDMFRDRQAFSEHT
WKMLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQARGLAVKTIQQHLGQLNMLHR
RSGLPRPSDSNAVSLVMRRIRKENVDAGERAKQALAFERTDFDQVRSLMENSDRCQDIRNLA
FLGIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAGVEKALSLGVTKLVERWISV
SGVADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRLIYGAKDDSGQRYLAWSGH
SARVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLDSETGAMVRLLEDGDHHHHHH

Tat-T7 tag-mCherry (Supporting Fig. 9):
MGRKKRRQRRRGHMASMTGGQQMGRDPNSVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEF
EIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEG
FKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERM
YPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVE
QYERAEGRHSTGGMDELYKARGAAALEHHHHHH

mCherry-Arg_9 (Supporting Fig. 9):
MGHHHHHHGGASKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLK
VTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQ
DSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDG
GHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK
ARGGGSGGGSRRRRRRRRR

Tat-T7 tag-Cre (Supporting Fig. 10):
MGRKKRRQRRRGHMASMTGGQQMGRDPNSMSNLLTVHQNLPALPVDATSDEVRKNLMDM
FRDRQAFSEHTWKMLLSVCRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQARGLAVKTIQQ
HLGQLNMLHRRSGLPRPSDSNAVSLVMRRIRKENVDAGERAKQALAFERTDFDQVRSLMENS
DRCQDIRNLAFLGIAYNTLLRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAGVEKALSLGV
TKLVERWISVSGVADDPNNYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRLIYGAKDDSG
QRYLAWSGHSARVGAARDMARAGVSIPEIMQAGGWTNVNIVMNYIRNLDSETGAMVRLLE
DGDAAALEHHHHHH

Cre-Arg_9 (Supporting Fig. 10):
MGHHHHHHGGASMSNLLTVHQNLPALPVDATSDEVRKNLMDMFRDRQAFSEHTWKMLLSV
CRSWAAWCKLNNRKWFPAEPEDVRDYLLYLQARGLAVKTIQQHLGQLNMLHRRSGLPRPSD
SNAVSLVMRRIRKENVDAGERAKQALAFERTDFDQVRSLMENSDRCQDIRNLAFLGIAYNTL
LRIAEIARIRVKDISRTDGGRMLIHIGRTKTLVSTAGVEKALSLGVTKLVERWISVSGVADDPN
NYLFCRVRKNGVAAPSATSQLSTRALEGIFEATHRLIYGAKDDSGQRYLAWSGHSARVGAAR
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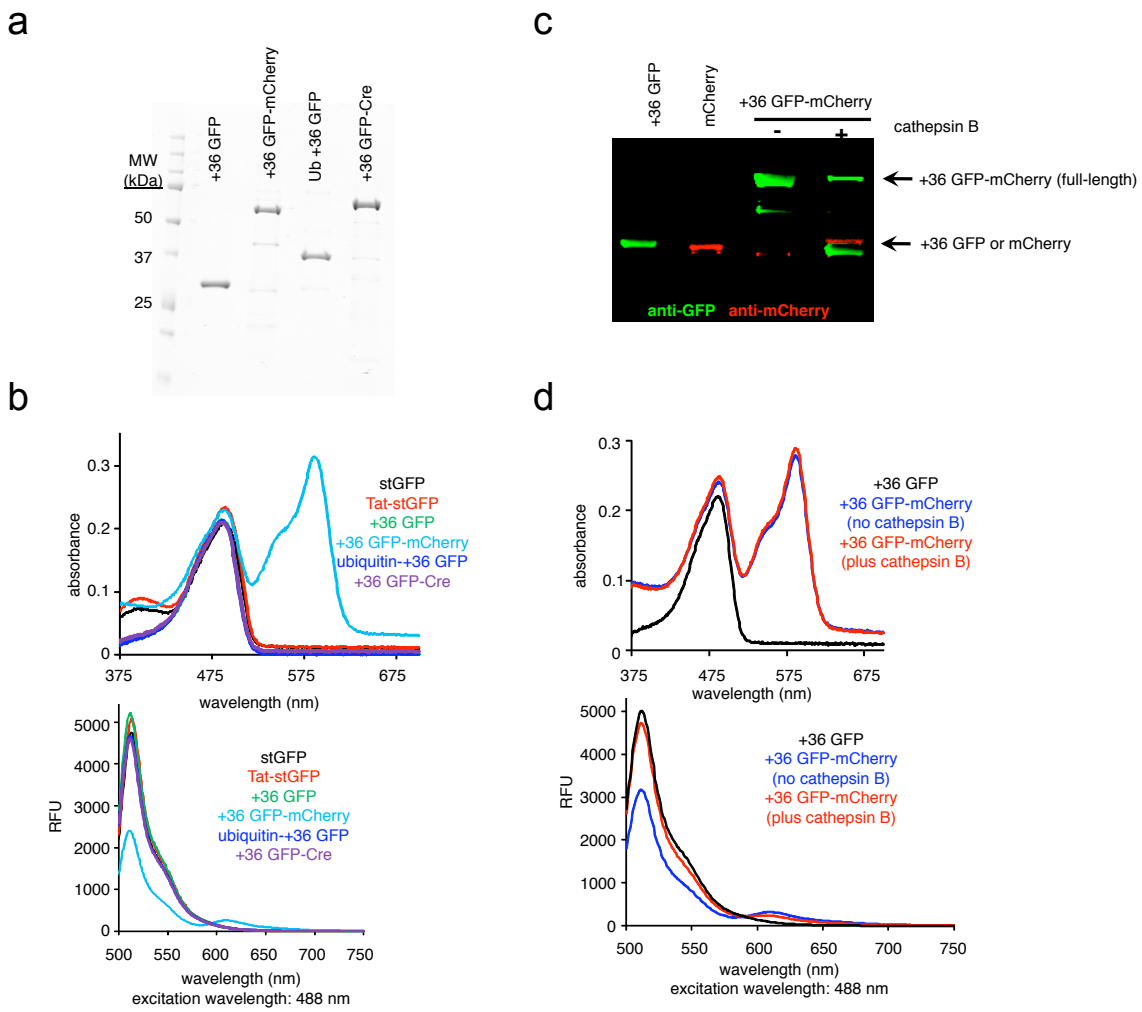
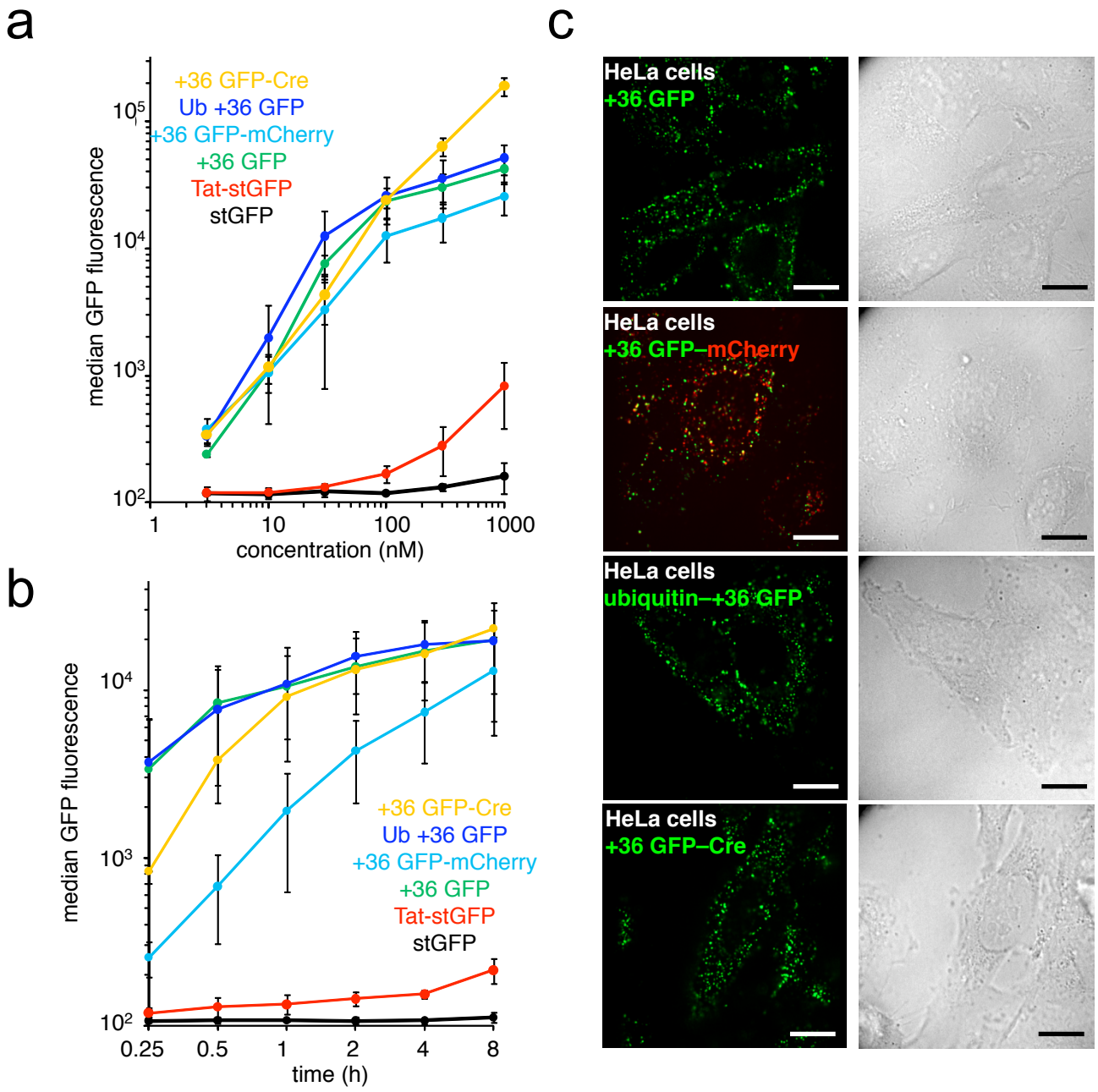
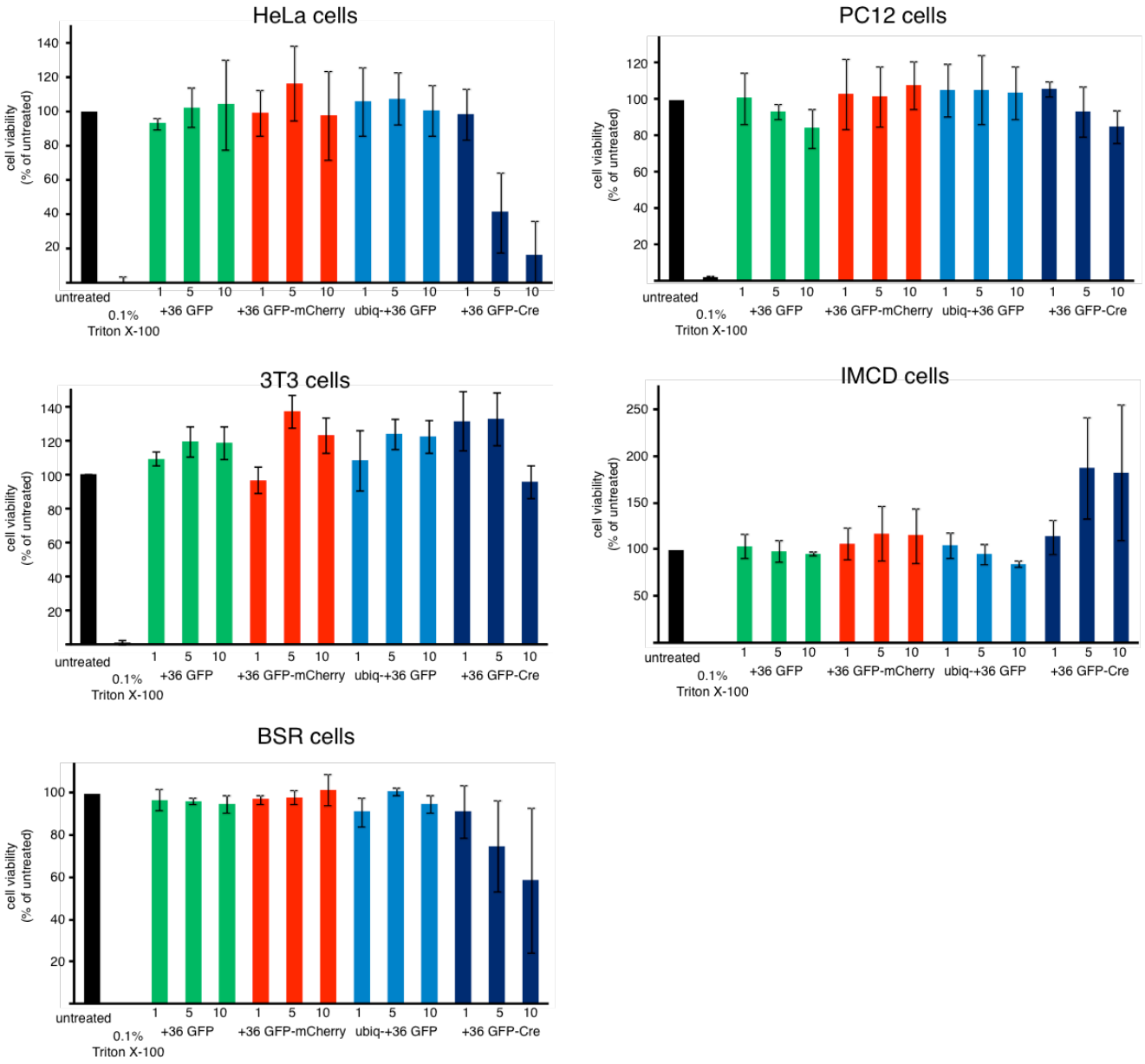


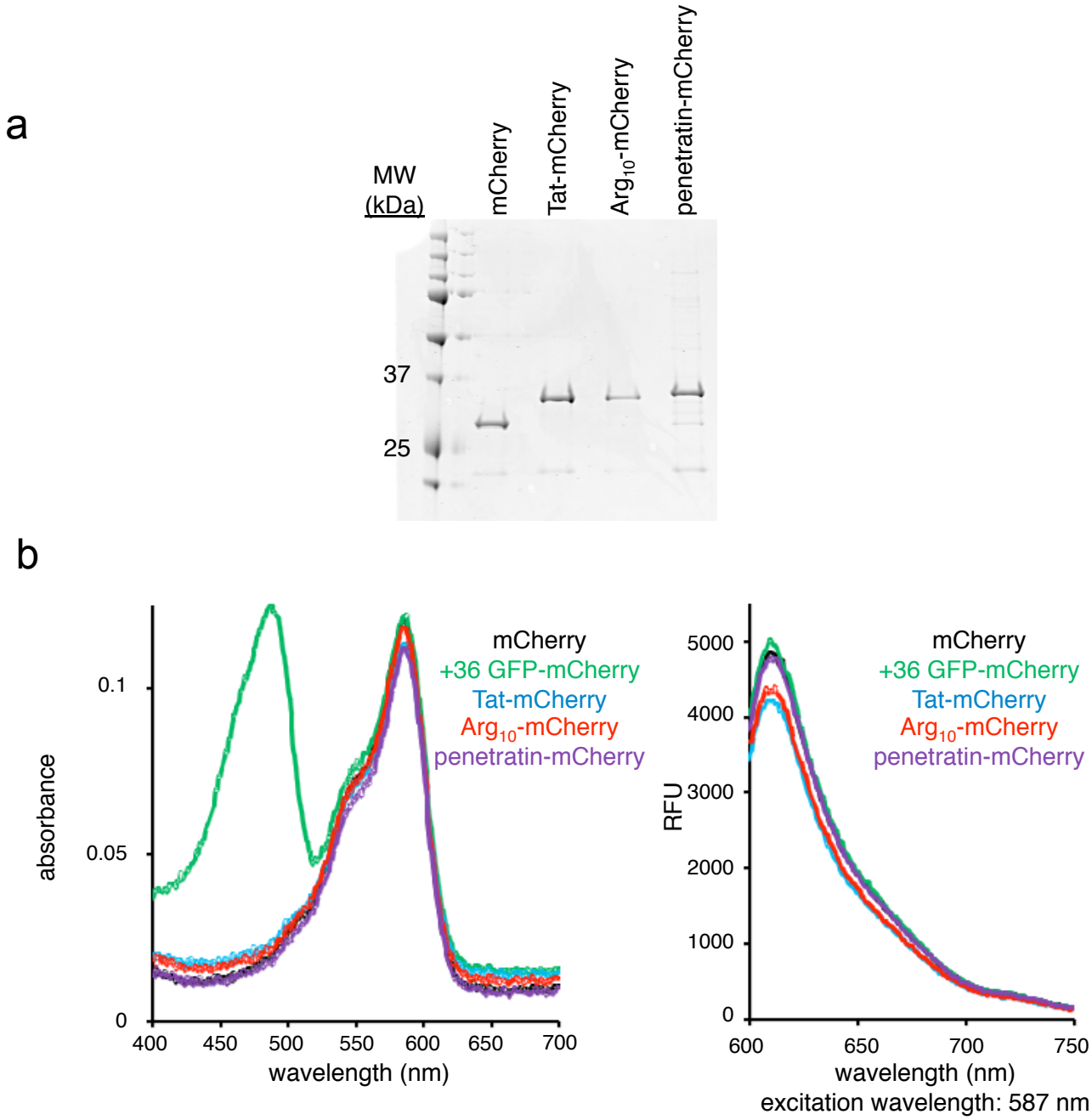
Supporting Figure 1. Characterization of +36 GFP fusion proteins. **(a)** Analysis of +36 GFP fusion proteins by SDS-PAGE. **(b)** Absorbance and emission spectra of GFP fusion proteins shows equal fluorescence except for +36 GFP mCherry which shows reduced fluorescence at 515 nm and also an extra emission peak at 615 nm, presumably due to FRET with the attached mCherry fluorophore. **(c)** Western blot of +36 GFP-mCherry incubated with or without cathepsin B. Incubation of 1 nanomole +36 GFP-mCherry with 0.25 μg cathepsin B (Sigma) in 100 μL of 50 mM MES, pH 5.5 at 37° C for 45 min results in cleavage yielding separate mCherry and +36 GFP protein domains. **(d)** Absorbance and emission spectra of +36 GFP-mCherry after incubation with cathepsin B shows recovery of fluorescence at 515 nm, presumably due to loss of FRET upon +36 GFP-mCherry cleavage.



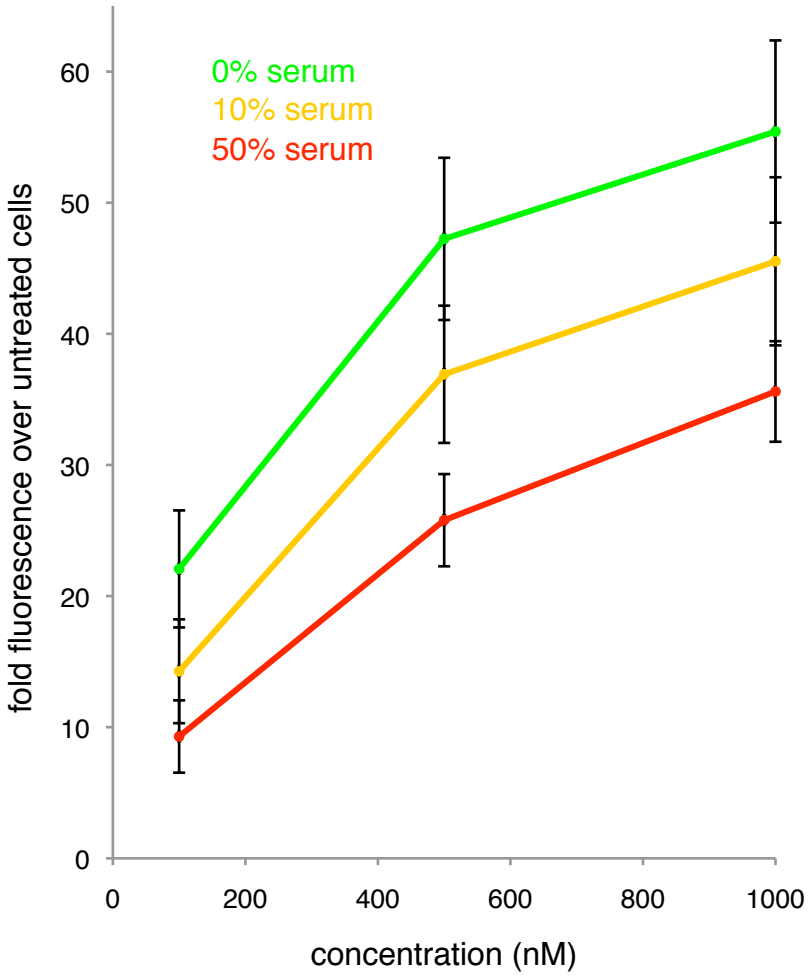
Supporting Figure 2. +36 GFP protein fusions penetrate cells rapidly and potently. **(a)** Flow cytometry of HeLa cells incubated at the concentrations shown in the presence of +36 GFP fusions for 4 hours at 37° C. Cells were washed three times with 20 U/mL heparin in PBS to remove membrane-bound protein prior to analysis. Untreated cells resulted in median GFP fluorescence values of 107 ± 5. Error bars represent the standard deviation of three independent biological replicates. **(b)** Flow cytometry of HeLa cells incubated in the presence of 100 nM of each +36 GFP fusion at 37° C for the specified time. Untreated cells resulted in median GFP fluorescence values of 100 ± 6. Error bars represent the standard deviation of three independent biological replicates. **(c)** Confocal fluorescence microscopy (left) and brightfield (right) of live HeLa cells incubated in the presence of +36 GFP fusions at 100 nM for 30 min at 37° C and washed with heparin. The scale bar represents 15 μm.



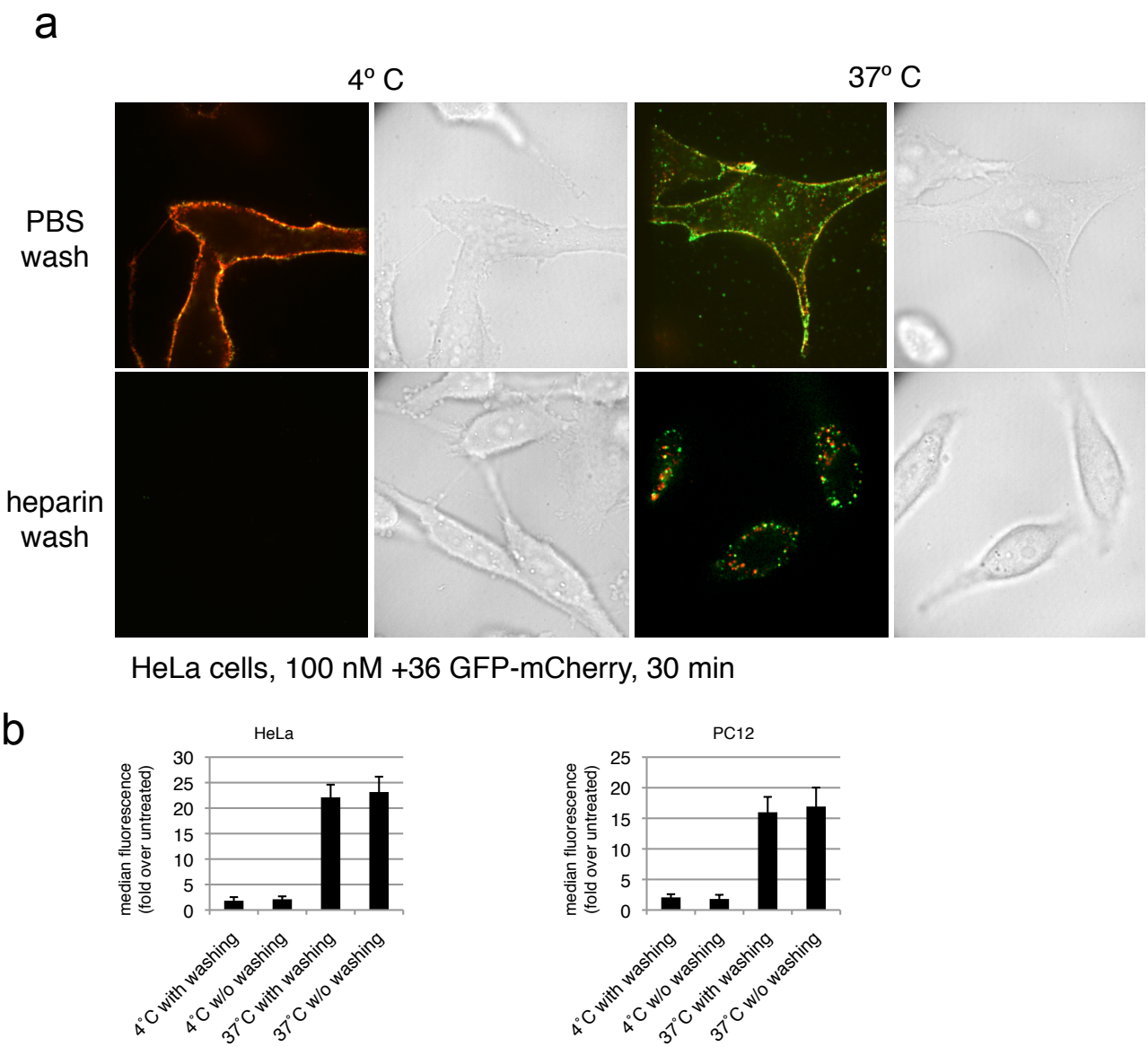
Supporting Figure 3. +36 GFP and +36 GFP fusions are not toxic at concentrations effective for protein delivery. At concentrations ≥ ~10 to 100 times the effective concentration for protein delivery in this work, +36 GFP-Cre (but not other +36 GFP fusion proteins or +36 GFP itself) reduced the viability of some cell lines and possibly stimulated IMCD cells. Values and error bars represent the average of and standard deviation, respectively, of three independent biological replicates.



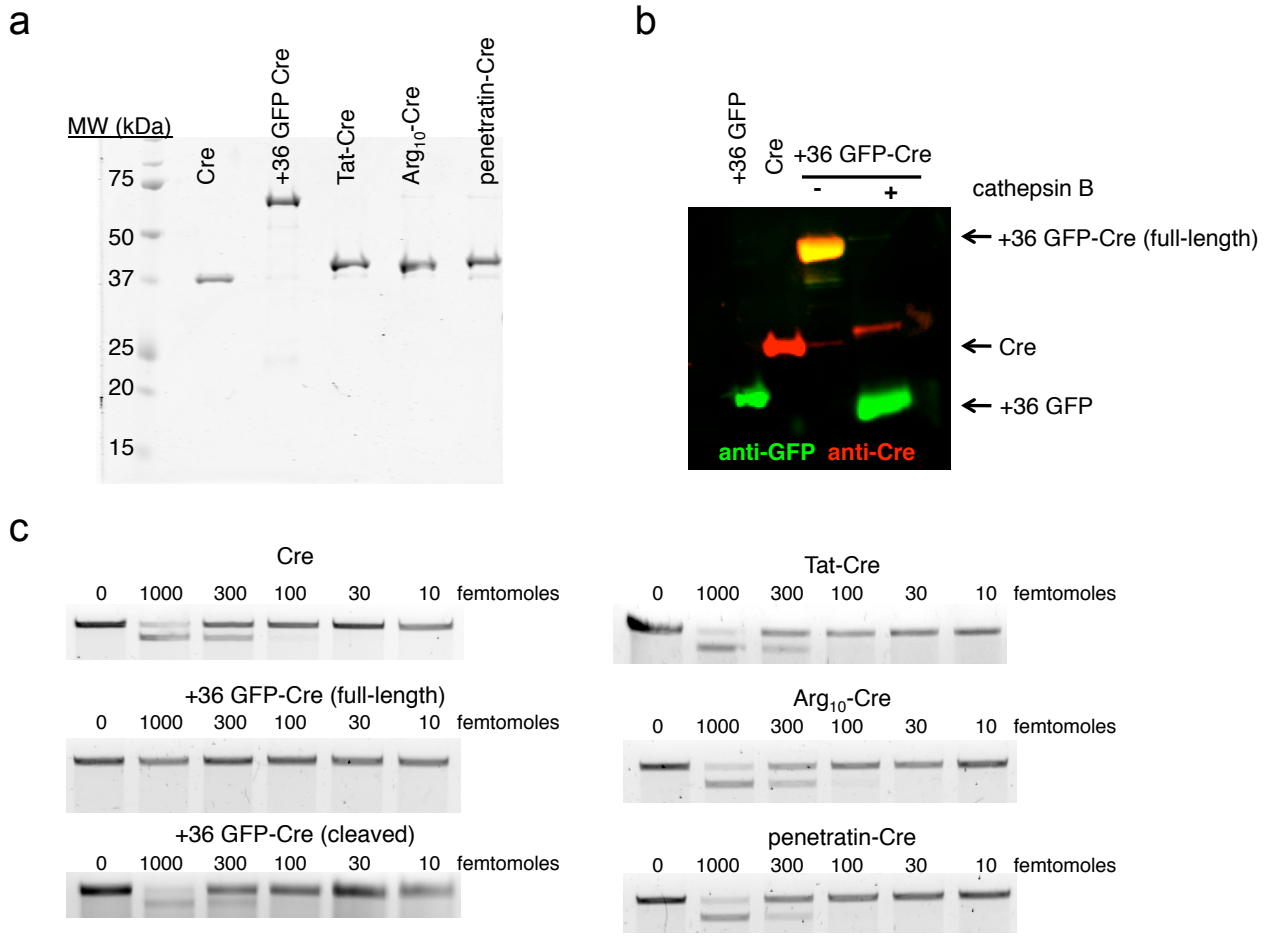
Supporting Figure 4. Characterization of mCherry fusion proteins. **(a)** Analysis of mCherry fusions to Tat, Arg_{10}, and penetratin by SDS-PAGE. **(b)** Absorbance and emission spectra of mCherry fusion proteins.



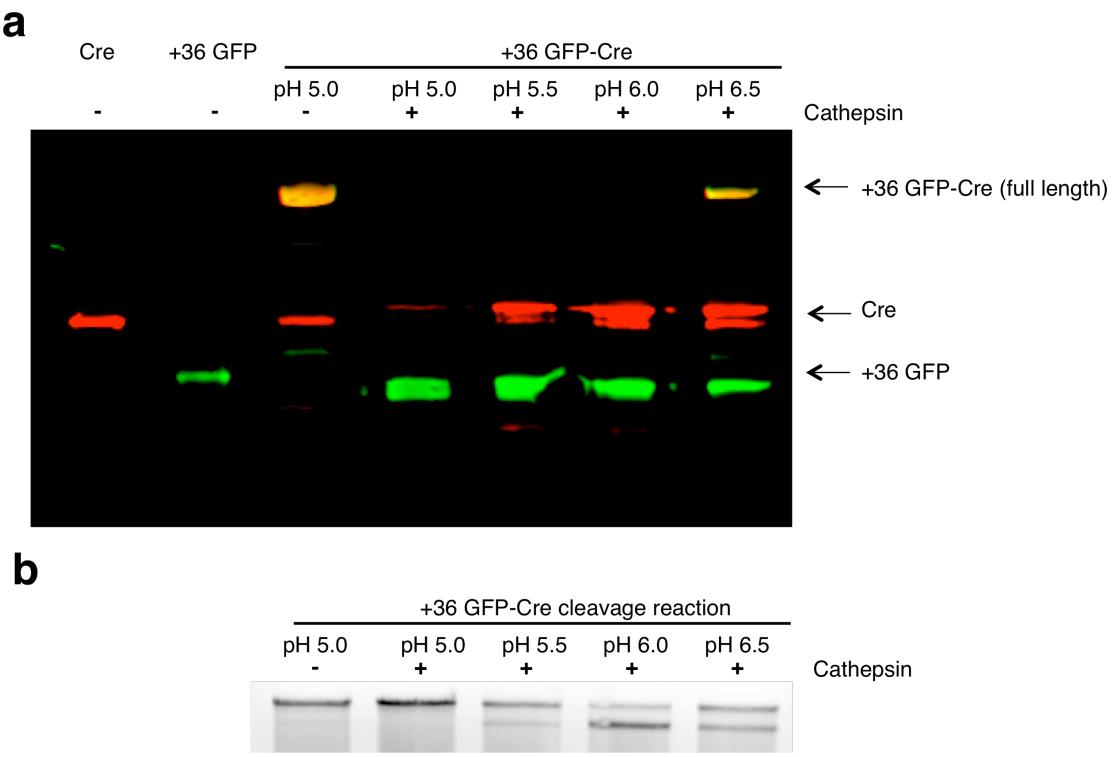
Supporting Figure 5. The effect of serum on +36 GFP-mCherry internalization. HeLa cells in DMEM supplemented with 0%, 10%, or 50% fetal bovine serum (sterile-filtered, Sigma) were incubated with the indicated concentrations of +36 GFP-mCherry for 1 h at 37° C. Cells were washed three times with 20 U/mL heparin in PBS to remove membrane-bound protein prior to flow cytometry analysis. Error bars represent the standard error of three independent biological replicates.



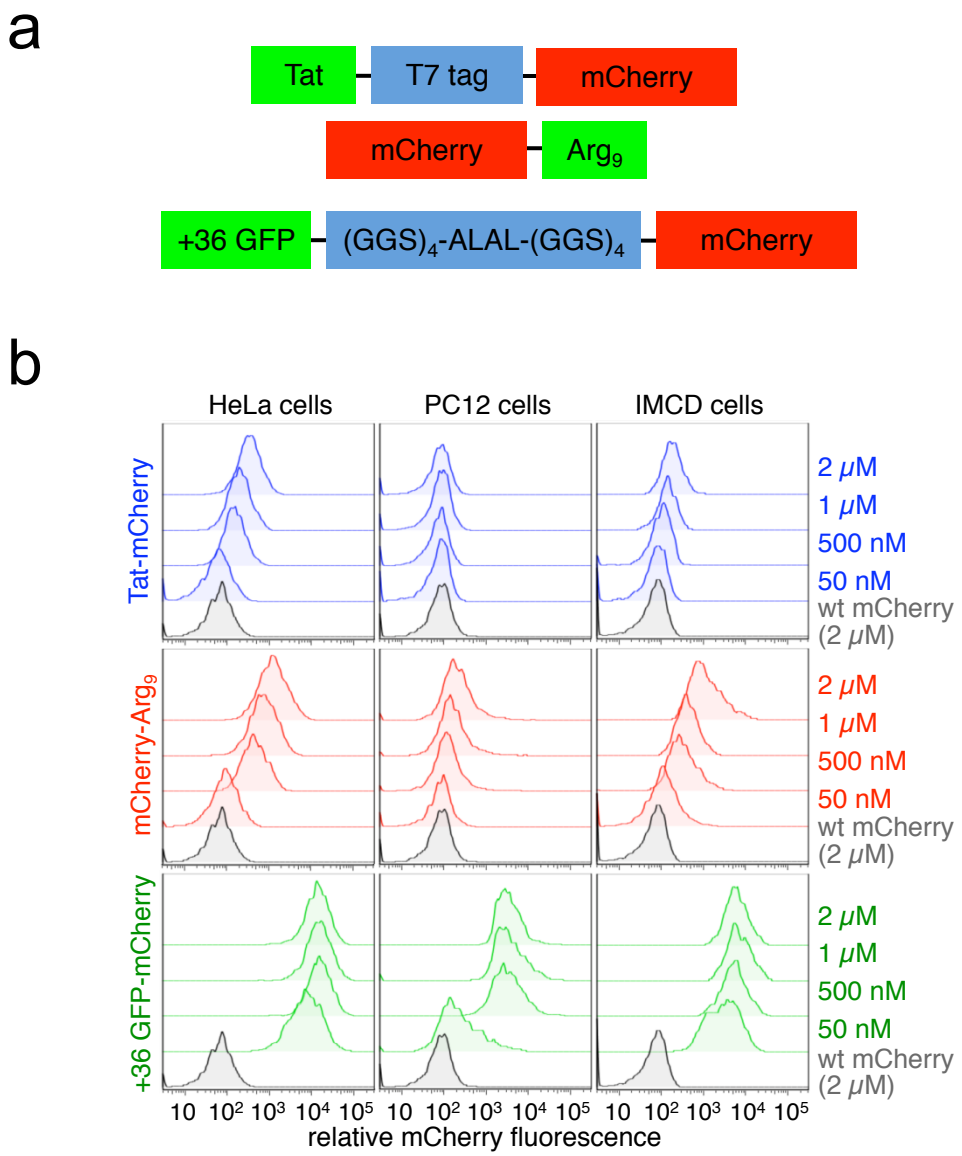
Supporting Figure 6. Membrane-bound protein is removed by heparin washing conditions. **(a)** Live-cell fluorescence microscopy indicates that at 4 °C +36 GFP-mCherry is membrane-bound but not internalized. After washing with heparin (but not after washing with PBS), this +36 GFP-mCherry signal is largely removed. At 37 °C, most of +36 GFP-mCherry signal remains even after heparin washing, consistent with internalization of +36 GFP-mCherry. **(b)** HeLa and PC12 cells subjected to the conditions described in **(a)** were trypsinized (which destroys surface-bound mCherry) then analyzed by flow cytometry. Cells incubated with +36 GFP-mCherry at 4 °C do not show significant mCherry fluorescence compared to cells incubated at 37 °C, further suggesting that the signal at 37 °C represents internalized protein signal, and that internalization at 4 °C is inefficient.



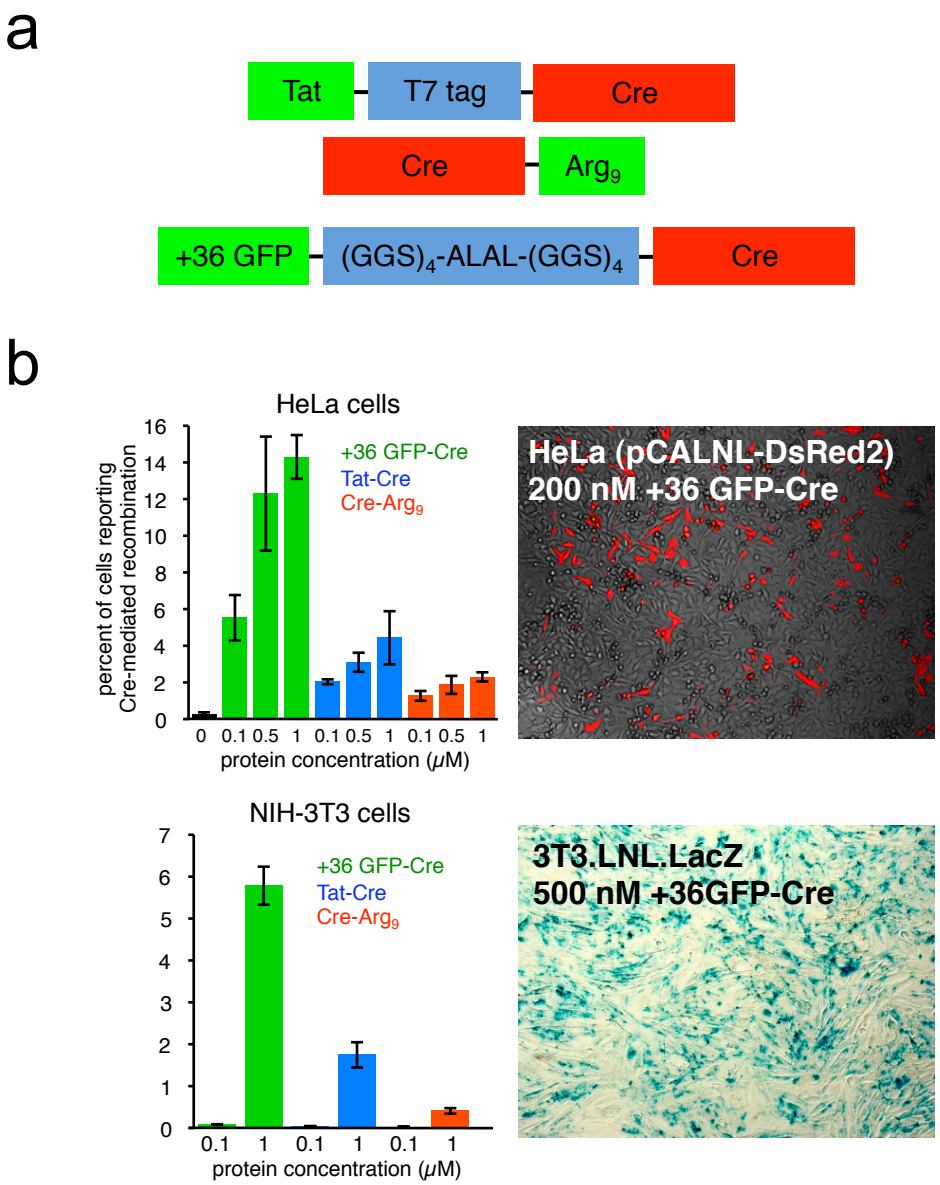
Supporting Figure 7. Characterization of Cre fusion proteins. **(a)** Analysis of Cre proteins by SDS-PAGE. **(b)** Western blot of +36 GFP-Cre incubated with or without cathepsin B. Incubation of 50 picomoles of +36 GFP-Cre with 0.25 μg cathepsin B (Sigma) in 10 μL of 50 mM MES, pH 5.5 at 37° C for 45 min results in cleavage yielding separate Cre and +36 GFP domains. **(c)** The concentration of Cre proteins was verified explicitly by *in vitro* activity assay. All Cre proteins showed roughly 50% cleavage activity at 300 femtomoles/reaction and nearly quantitative activity at 1000 femtomoles/reaction. Note that +36 GFP-Cre did not exhibit activity as the fusion protein but exhibited wild-type-like activity once cleaved by cathepsin B.



Supporting Figure 8. Cleavage and activity of +36 GFP-Cre incubated with cathepsin B in buffer at various pH values. **(a)** Western blot of +36 GFP-Cre incubated with or without cathepsin B. Incubation of 50 picomoles of +36 GFP-Cre with or without 0.5 μg cathepsin B (Sigma) in 20 μL of 50 mM MES, pH 5.0-6.5 at 37° C for 45 min results in variable amounts of full-length +36 GFP-Cre, Cre, and +36 GFP. Note that at pH 5.0, Cre is largely degraded by cathepsin B, while at higher pH values, Cre is cleaved from +36 GFP but is not degraded to a significant extent. **(b)** The *in vitro* recombinase activity of the products of +36 GFP-Cre incubation with cathepsin B at the pH values indicated demonstrate that +36 GFP-Cre is not active as the full-length fusion or after incubation (degradation) with cathepsin B at pH 5.0. The upper band is Cre substrate DNA, while the lower band is recombination product, as in Supporting Fig. 7.



Supporting Figure 9. mCherry delivery experiments using fusions with +36 GFP, Tat, and oligoarginine containing different linkers or fusion orientations. **(a)** The mCherry delivery experiments presented in **Fig. 1** were performed with proteins constructed as [protein delivery peptide/protein]-$[GGS]_9$-mCherry. We generated an alternative Tat-mCherry construct based upon a previously published Tat protein delivery expression vector.[7] We generated a mCherry-Arg_9 construct based upon a previously published oligoarginine protein delivery platform.[8] To facilitate intracellular cleavage of +36 GFP from mCherry, we included a known substrate[9] (ALAL) of the ubiquitous endosomal protease, cathepsin B, within the +36 GFP-mCherry linker. This linker, however, did not alter the cleavage susceptibility of the fusion protein (data not shown). **(b)** We incubated these three fusion proteins with HeLa cells, rat inner medullary collecting duct (IMCD) cells, and rat pheochromocytoma PC12 cells in serum-free media at various concentrations for 4 hours at 37 °C. After incubation, cells were washed under conditions that remove surface-bound protein, trypsinized, and assayed for internalized mCherry by flow cytometry. For all cell lines and at all concentrations tested, +36 GFP delivered ~10- to 100-fold more mCherry than Tat or Arg_9, consistent with **Fig. 1** results.



Supporting Figure 10. Cre delivery experiments using fusions with +36 GFP, Tat, and oligoarginine containing different linkers or fusion orientations. **(a)** The Cre delivery experiments presented in **Fig. 3** were performed with proteins constructed as [protein delivery peptide/protein]-[GGS]$_9$-Cre. We generated an alternative Tat-Cre construct based upon a previously published Tat protein delivery expression vector,[7] and an alternative Cre-Arg$_9$ construct based upon a previously published oligoarginine protein delivery platform.[7] To facilitate intracellular cleavage of +36 GFP from Cre, we included a known substrate[9] (ALAL) of the ubiquitous endosomal protease, cathepsin B, within the +36 GFP-Cre linker. This linker, however, did not alter the cleavage susceptibility of the fusion protein (data not shown). **(b)** We tested the ability of these three proteins to effect recombination in HeLa cells transiently transfected with pCALNL-dsRed, a DsRed2-based Cre activity reporter plasmid. After incubation with 100-1000 nM of each Cre fusion protein for 4 hours in serum-free media, cells were washed to remove surface bound protein and incubated in full media for 48 hours. Delivery of Cre was assayed by following DsRed2 expression using flow cytometry and fluorescence microscopy. Typically, +36 GFP-Cre generated 3- to 5-fold more recombinants than the

corresponding fusions with Tat, or Arg_9, consistent with **Fig. 3** results. **(c)** Cre delivery was further evaluated in a NIH-3T3 cell line harboring an integrated lacZ-based Cre-reporter. After incubation, treatment, and washing as described above, these cells were stained with X-Gal to identify recombinants. Consistent with the HeLa cell results and with **Fig. 3** results, +36 GFP-Cre resulted in 3- to 10-fold more efficient generation of recombinants than Tat or Arg_9.

Supporting References

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