Supplementary Information

Targeted protein degradation through cytosolic delivery of monobody binders using bacterial toxins

Nadine Eliane Schmit[1,3], Katyayanee Neopane[1,2,3] and Oliver Hantschel[1,*]

[1] Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, École polytechnique fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland

[2] Present address: Nestlé Institute of Health Sciences, EPFL Innovation Park, 1015 Lausanne, Switzerland

[3] These authors contributed equally to this study

*Correspondence and requests for materials should be addressed to O.H. (email: oliver.hantschel@epfl.ch).

Content

Supplementary Methods

Expression and purification of recombinant proteins

Since the chimeric toxin contains disulfide bonds, the *E. coli* Origami strain, deficient in thiol reductase was chosen for recombinant expression of all toxin-monobody fusion proteins in the pET21a vector. The proteins were expressed by growing the culture for 72 hours in Auto Induction Medium (AIM) at 20°C shaking at 200 rpm. For the proteins comprising the VHL, Terrific Broth (TB) was used instead of AIM, the cells were grown at 37°C until their OD_{595nm} reached between 0.8 and 1, and expression was induced with 0.5 mM IPTG prior to growing the cells at 18°C shaking at 200 rpm for 18 hours. The cells were harvested by centrifugation at 6000x*g* for 15 minutes and the pellet was resuspended using Tris-based Buffer A (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10% (w/v) glycerol, 20 mM Imidazole). For the constructs bearing a VHL, HEPES-based Buffer A was used (50 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 10% (w/v) glycerol, 0.1% (v/v) Triton-X-100, 20 mM Imidazole). Samples were kept on ice and DNAse I (Roche) was added to the homogenized cell suspension before lysis using the Avestin Emulsifex C3 homogenizer. The soluble protein was separated from cell debris by centrifugation at 14000x*g* for 45 minutes at 4°C. The supernatant containing protein was filtered through a 0.45 μm filter. The protein samples were incubated with 1.5 ml Ni-NTA agarose resin (Qiagen) equilibrated with the respective Buffer A for 1 hour at 4°C on a rotor. The lysates were then transferred into a 20mL plastic body column and the flow-through was allowed to pass completely. The column was washed with 50 column volumes of the respective Buffer A prior to elution with 6 mL of Buffer B (the same as the respective buffer A but containing 400 mM Imidazole). 50 μl each of the lysate, flow-through, wash and elution fractions were kept for SDS-PAGE analysis. Ni-NTA elution fractions were further purified by size-exclusion chromatography (SEC), using a Superdex 200 16/600 GL column (GE), pre-equilibrated with SEC buffer (25 mM Tris-HCL pH 7.5, 150 mM NaCl, and 5% (w/v) Glycerol) and the ÄKTA Avant system (GE). The elution fractions were collected and concentrated using Amicon Ultra centrifugal filters (Millipore). Protein concentrations were measured using the Nanodrop spectrophotometer.

For SDS-PAGE analysis, fractions of purification steps were mixed with 4X sample buffer (0.2 M Tris-HCL, 8% (w/v) SDS, 400 mM DTT, 40% (w/v) glycerol and 0.02% (w/v) bromophenol blue), boiled at 95°C, loaded in the pockets of a polyacrylamide gel and the gel was run at 100V constant in SDS running buffer (0.25 M Tris, 1.92 M glycine, 1% (w/v) SDS). SDS-PAGE gels were either used for immunoblotting or stained in coomassie staining solution (10% (v/v) acetic acid, 50% (v/v) absolute ethanol and 0.025% (w/v) coomassie brilliant blue G-250) for 1-2 hours at room temperature after boiling for few seconds, and destained using destaining solution (10% (v/v) acetic acid and 20% (v/v) ethanol).

Mammalian cell culture

K562 and Jurkat cells were purchased from DSMZ, HeLa cells were a kind gift from G. van der Goot (EPFL) and HEK293T were a kind gift from D. Trono (EPFL). HeLa and HEK293T cells were cultured in Dulbecco's Modified Eagle

Medium, DMEM (Gibco) and K562 cells and Jurkat cells were cultured in RPMI (Gibco), both media were supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1% (w/v) penicillin/streptomycin (Amimed). Cells were cultured in flat, round cell culture dishes of 10 or 15 cm diameter at 37°C in 5% (v/v) CO2. Adherent cells were split 3 times per week by washing with PBS, followed by incubation with 1-2 ml Trypsin-EDTA (Gibco) at 37°C, and resuspension by pipetting in growth medium. 10-20% of the cell suspension was added to a new dish containing growth medium. Suspension cells were split 3 times per week by centrifugation at 400x*g* for 3 minutes, resuspension in fresh growth medium and adjusting the concentration to $1x10^5$ cells/ml in a new culture dish. Blasticidin at the experimentally determined concentrations of 5 μg/ml for Jurkat cells and 7.5 μg/ml for K562 cells were continuously added to the growth medium of cells after lentiviral transduction with a pEM24 vector containing a blasticidin-resistance gene. (Figure S9 A) Doxycycline was used at 100 ng/ml final concentration to induce gene expression in cells transduced with pEM24 vectors.

T-cell stimulation and cell lysate preparation

Jurkat cells were stimulated with anti-TCR antibody clone C305 (Millipore) at a concentration of 100 ng/ml for 5 min at 37°C. The cells were cooled to 4°C immediately after stimulation by the addition of ice-cold PBS and centrifugation. Cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% (w/v) NP-40, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM orthovanadate, 1 mM PMSF (Sigma), 1 mg/ml TPCK (Applichem), 10 μg/ml protease inhibitor cocktail (Roche)), the lysate was cleared by centrifugation for 10 minutes at 20000x*g* at 4°C and the protein content was quantified using the Bradford reagent. Lysate was diluted in 4x Laemmli buffer to contain 100 μg total protein in 20 μl. Samples were analyzed by SDS-PAGE separation followed by immunoblotting using nitrocellulose membranes and the iBlot device (ThermoFisher), program 4.

Lentiviral transduction

Stable cell lines expressing the pEM24 vector with the A4GALT gene or with the VHL fused to the monobodies ML1, ML3 or HA4_YA were generated by lentiviral transduction. HEK293T were seeded in 10cm dishes to obtain 80% confluency the following day, when cells were co-transfected with plasmids pCMV-R8_74, encoding gag and pol proteins, pMD2_G, encoding VSV-G envelope and the pEM24 vector containing the respective gene of interest to produce lentivirus by calcium phosphate transfection. For one 10cm dish, 11.25 μg construct DNA, 3.95 μg envelope plasmid and 7.3 μg packaging plasmid DNA were mixed with 330 μl 0.1x Tris-EDTA, 55 μl 2.5 M $CaCl_2$ and with water to a total volume of 550 μl. 550 μl 2x Hepes Buffered Saline (HBS) were added dropwise while vortexing. The transfection complexes were incubated at room temperature for 20 minutes, then added dropwise to the HEK293T cells, which were incubated for 16 hours at 37°C in 5% (v/v) CO_2. The medium was replaced with 7 ml fresh growth medium and cells were incubated for 8 hours, before collecting the supernatant containing virus, which was transferred to a tube for overnight storage at 4°C and the cells were further incubated with 7 ml fresh growth medium. After 16 hours, the supernatant was pooled with the previously collected supernatant and filtered through a 0.22 μm filter. $5x10^5$ target cells (K562 or Jurkat) were pelleted by

centrifugation at 500x*g* for 3 minutes and resuspended in 5 ml supernatant containing virus. After incubation at 37°C in 5% (v/v) CO_2, a second infection was performed 24 hours later in the same way. 24 hours later, the medium was replaced by fresh growth medium by centrifugation and blasticidin (5 μg/ml for Jurkat cells and 7.5 μg/ml for K562 cells) was added to the culture medium 3 days after the second infection to select for cells having integrated the gene of interest. (Figure S9 B) Transduced cells were continuously cultured in presence of blasticidin and for at least 10 days before starting any experiments. To induce gene expression, 100 ng/ml doxycycline were added to the growth medium for at least 24 hours prior to analysis.

Flow cytometry

To assess the expression of Gb3 receptor (CD77) on the surface of different cells, HeLa cells were first trypsinized and resuspended in 5 ml fresh DMEM. $5x10^5$ cells per sample were centrifuged for 3 minutes at 500x*g* and washed once with PBS. The cell pellet was resuspended in 100 μl FACS buffer containing human FITC-conjugated anti-CD77 antibody diluted 1:20 and the tube was incubated at dark on ice for 20 minutes. The cells were washed with 3 ml PBS and the pellet was resuspended in 400 μl FACS buffer for analysis on the BD LSRII flow cytometer (Beckman-Dickinson) using the 488nm laser and a 525/40nm bandpass filter.

To assess the delivery of the proteins in suspension cells, $5x10^5$ K562 or Jurkat cells expressing the A4GALT gene were pelleted by centrifugation at 500x*g* for 3 minutes, washed once with PBS and resuspended in 1 ml Leibovitz medium containing 1 μM of the protein (unlabeled or labeled with Benzylguanine-AlexaFluor647) to be delivered. The cells were incubated in a 48-well plate for 30 minutes at 37°C and 5% (v/v) CO_2. The cells were collected in 15-ml tubes, centrifuged and washed three times with PBS. The cell pellets were then resuspended in 400 μl FACS buffer for analysis. To stain surface-bound proteins, cells were further incubated in 1 μM Benzylguanine-AlexaFluor647 for 30 minutes, washed 3 times with PBS and resuspended in 400 μl FACS buffer. The samples were analyzed on an LSRII flow cytometer (Beckman-Dickinson) using the 640nm laser and a 670/14nm bandpass filter for the detection of SNAP-tagged proteins.

Realtime-Glo (Promega) luminescent cell viability assay

Adherent cells were trypsinized, suspension cells were pelleted by centrifugation at 400x*g* for 3 minutes and the pellets were resuspended in Leibovitz's medium supplemented with 10% (v/v) FBS, 1% (w/v) P/S. After counting the cells using the CASYton cell counter, they were diluted to $1x10^5$ cells/ml and the NanoLuc enzyme and MT cell viability substrate (both Promega) were added to the cell suspension at a 1:500 dilution as indicated in the manufacturer's protocol. 2 times the appropriate blasticidin concentration for A4GALT expression and 200 ng/ml doxycycline (100 ng/ml end concentration) were added to the cell suspension prior to seeding the cells in an opaque white 96-well plate at a density of $5x10^3$ cells in 50μl per well. The proteins were diluted at 2 times the indicated end concentration in Leibovitz's medium supplemented with 10% (v/v) FBS and 1% (w/v) P/S, dilutions were sterile filtered and 50μl were added per

well. Alternatively, half-area 96-well plates were used and all the volumes were divided by 2. The plates were incubated at 37°C in 5% (v/v) CO_2. Luminescence was measured at the indicated time points using the M5 plate reader (Molecular devices).

Live cell microscopy

$8x10^4$ cells were seeded 24 hours before incubation with the protein in 35 mm glass-bottom dishes. Before protein incubation, the medium was aspirated and the cells were washed once with 1 ml sterile PBS. The protein was diluted in Leibovitz medium supplemented with 10% FBS (v/v) and 1% (w/v) P/S and the cells were incubated in 1 ml of the protein dilution at the indicated concentration for the indicated time. For incubation times longer than 1 hour, the protein dilutions were sterile filtered prior to addition to the cells. Before imaging, the cells were washed twice with PBS and fresh Leibovitz's medium was added. The dishes were imaged using a spinning disk confocal microscope (Yokogawa Spinning-Disk "CSU-W1") at the BIOP, EPFL with the 60x oil objective using the 488nm laser for AF488 labeled proteins and the 640nm laser for BG-Cy5 and BG-Sir labeled SNAP tagged proteins. For each sample, individual z-slices were imaged at the nuclear plane. The same laser settings were used for each sample of the same experiment.

Immunofluorescence

$8 x10^4$ cells were seeded 24 hours before incubation with the protein in a six-well plate with 18mm sterile round coverslips. Before protein incubation, the medium was aspirated and the cells were washed once with 1 ml PBS. The protein was diluted in Leibovitz medium supplemented with 10% (v/v) FBS and 1% (w/v) P/S and the cells were incubated in 1 ml of the protein dilution at the indicated concentration for the indicated time. The cells were washed once with PBS and then fixed in 4% (w/v) Paraformaldehyde (PFA) at room temperature for 15 minutes. The cells were rehydrated three times in PBS supplemented with 0.01% (v/v) Triton-X-100 (PBS-TX), prior to incubation in permeabilization solution (PBS, Saponin 0.1%, BSA 5%) for 1 hour at room temperature and 3 washes with PBS-TX. The coverslips were placed on the parafilm-coated and labeled lid of a 6-well plate and incubated with 50μl of the respective primary antibody diluted 1:500 in PBS-TX supplemented with 1% (w/v) BSA overnight at 4°C in a humidity chamber.

The coverslips were washed 3 times for 5 minutes with PBS-TX before incubating them with 50μl of the respective secondary antibody diluted 1:1000 in PBS-TX supplemented with 1% (w/v) BSA for 45 minutes in the humidity chamber at room temperature. The coverslips were washed 3 times for 5 minutes with PBS-TX and incubated with 100μl of Hoechst diluted in PBS for 10 minutes at room temperature. After 3 washes, coverslips were mounted on microscopy slides using Fluoromount-G mounting medium and imaged on the spinning disk confocal microscopy (Yokogawa Spinning-Disk "CSU-W1") at the BIOP, EPFL with the 60x oil objective using appropriate laser settings.

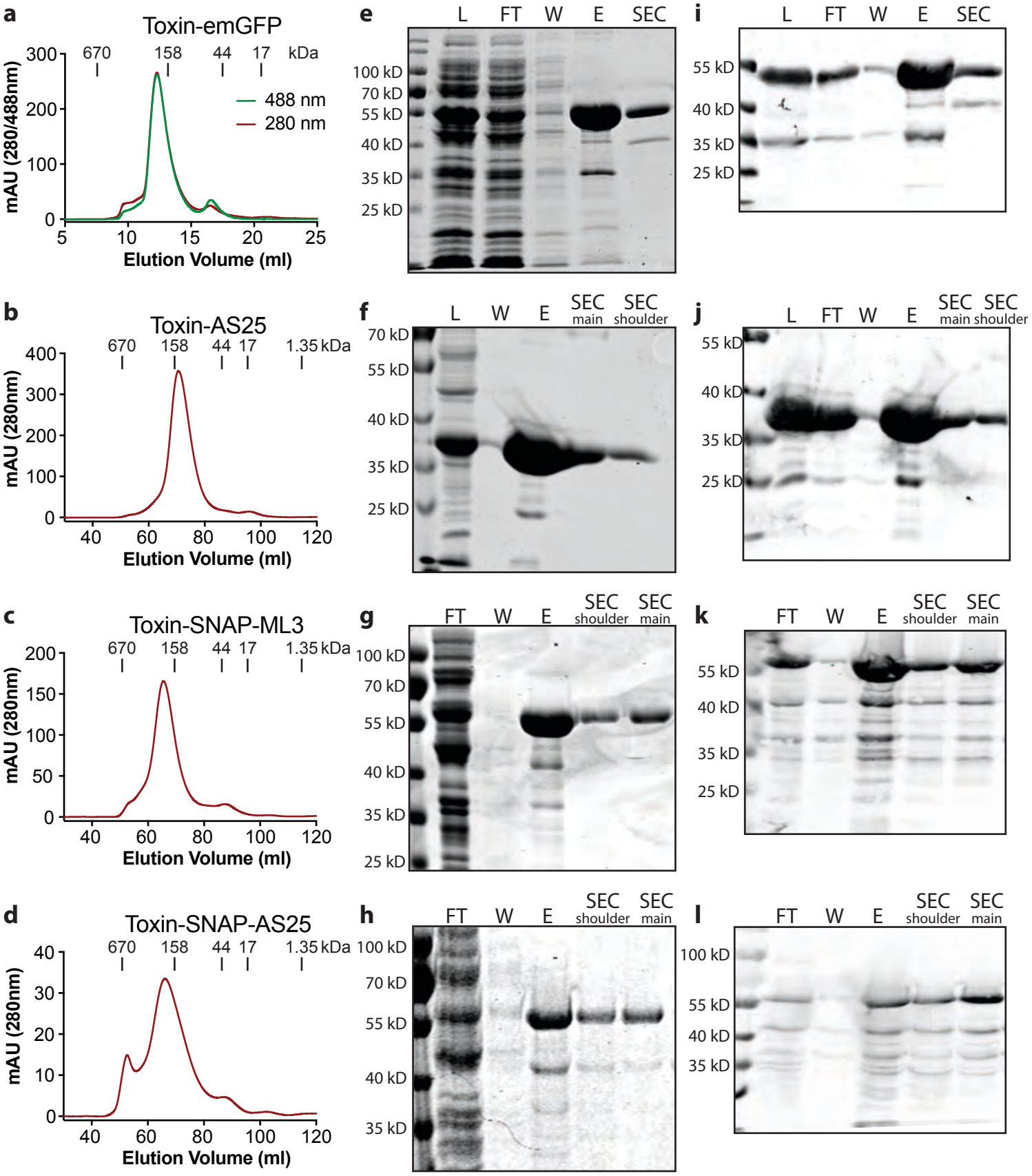

a
Toxin-emGFP
670 158 44 17 kDa
488 nm
280 nm
mAU (280/488nm)
Elution Volume (ml)
b
Toxin-AS25
670 158 44 17 1.35 kDa
mAU (280nm)
Elution Volume (ml)
c
Toxin-SNAP-ML3
670 158 44 17 1.35 kDa
mAU (280nm)
Elution Volume (ml)
d
Toxin-SNAP-AS25
670 158 44 17 1.35 kDa
mAU (280nm)
Elution Volume (ml)
e
L FT W E SEC
100 kD
70 kD
55 kD
40 kD
35 kD
25 kD
f
L W E SEC main SEC shoulder
70 kD
55 kD
40 kD
35 kD
25 kD
g
FT W E SEC shoulder SEC main
100 kD
70 kD
55 kD
40 kD
35 kD
25 kD
h
FT W E SEC shoulder SEC main
100 kD
70 kD
55 kD
40 kD
35 kD
i
L FT W E SEC
55 kD
40 kD
35 kD
25 kD
j
L FT W E SEC main SEC shoulder
55 kD
40 kD
35 kD
25 kD
k
FT W E SEC shoulder SEC main
55 kD
40 kD
35 kD
25 kD
l
FT W E SEC shoulder SEC main
100 kD
55 kD
40 kD
35 kD

Figure S1

Supplementary Figure S1. Expression and purification of recombinant fusion proteins. The crude bacteria lysate from *E. coli* Origami cells was first purified on a Ni-NTA column and then by SEC. (a-d): Size exclusion chromatograms; (e-h): Coomassie stained SDS-PAGE gels with the fractions from the Ni-NTA purification (L=crude lysate, FT=flow-through, W=wash, E=elution) and the main peak of the SEC after concentration, as well as in some gels the shoulder fraction from the SEC; (i-l): Corresponding immunoblots using an antibody recognizing penta-His of the following proteins: toxin-emGFP (a, e, i), toxin-AS25 (b, f, j), toxin-SNAP-ML3 (c, g, k) and toxin-SNAP-AS25 (d, h, l).

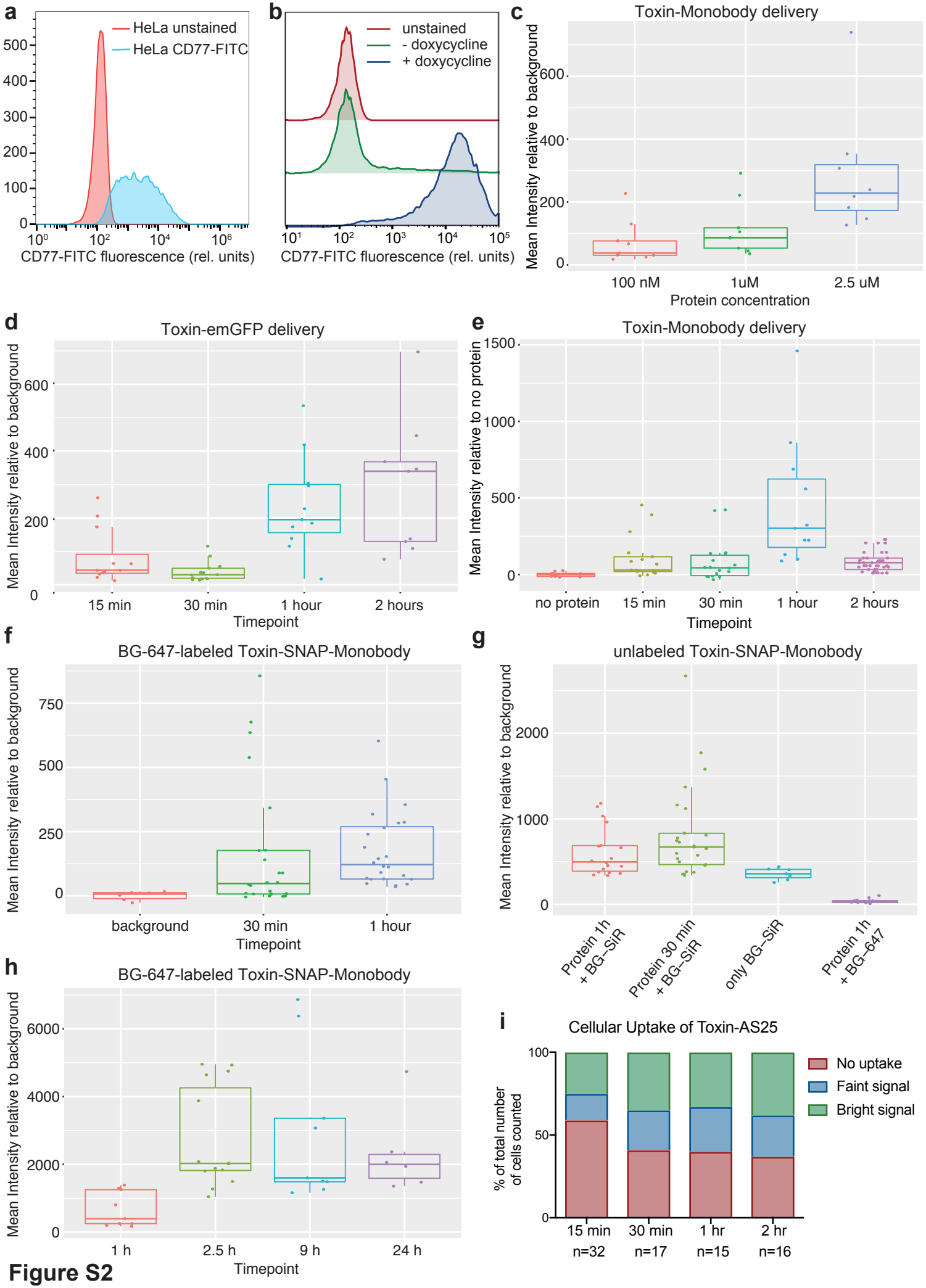

a
HeLa unstained
HeLa CD77-FITC
CD77-FITC fluorescence (rel. units)
b
unstained
- doxycycline
+ doxycycline
CD77-FITC fluorescence (rel. units)
c
Toxin-Monobody delivery
Mean Intensity relative to background
100 nM
1uM
2.5 uM
Protein concentration
d
Toxin-emGFP delivery
Mean Intensity relative to background
15 min
30 min
1 hour
2 hours
Timepoint
e
Toxin-Monobody delivery
Mean Intensity relative to no protein
no protein
15 min
30 min
1 hour
2 hours
Timepoint
f
BG-647-labeled Toxin-SNAP-Monobody
Mean Intensity relative to background
background
30 min
1 hour
Timepoint
g
unlabeled Toxin-SNAP-Monobody
Mean Intensity relative to background
Protein 1h + BG-SiR
Protein 30 min + BG-SiR
only BG-SiR
Protein 1h + BG-647
h
BG-647-labeled Toxin-SNAP-Monobody
Mean Intensity relative to background
1 h
2.5 h
9 h
24 h
Timepoint
i
Cellular Uptake of Toxin-AS25
% of total number of cells counted
No uptake
Faint signal
Bright signal
15 min n=32
30 min n=17
1 hr n=15
2 hr n=16

Figure S2

Supplementary Figure S2. CD77 (Gb3) expression in HeLa and Jurkat cells (a) Flow cytometry plot of HeLa cells stained with CD77-FITC to stain for Gb3. (b) Jurkat cells were transduced with a doxycycline inducible A4GALT gene, to express Gb3 on their surface. The cells were treated with doxycycline for 24h, stained with an anti-Gb3 (CD77) antibody and analyzed by flow cytometry. (c) Quantification of the images shown in figure 2c. (d) Quantification of the experiment shown in figure 2a. (e) Quantification of the experiment shown in figure 2b. An additional timepoint of 15 minutes, which is not shown in the images has also been quantified. (f) Quantification of the experiment shown in figure 3a. (g) Quantification of the experiment shown in figure 3b. For panels (c)-(g) boxplots represent the median value, the first and third quartiles (lower and upper hinges) and the smallest and largest value within 1.5 times the interquartile range (lower and upper whiskers). (h) Quantification of the experiment shown in figure 3c. (i) HeLa cells were incubated with AF488-labeled Toxin-AS25 for the indicated time and live cell images were taken on a confocal microscope with a 20x objective. Cells were counted and categorized based on their fluorescence intensity into arbitrary categories. The number of images taken is indicated by the number n.

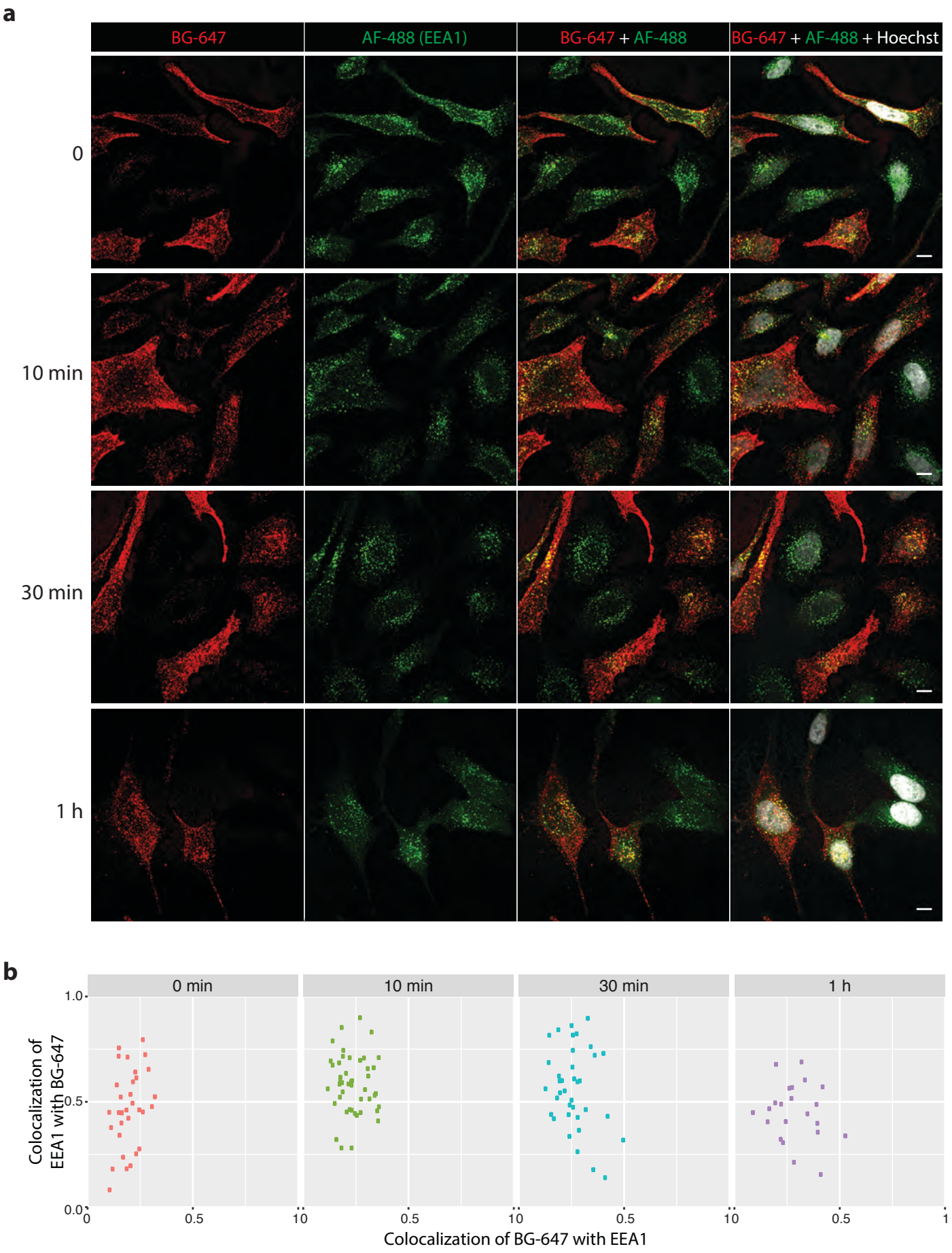

a
BG-647
AF-488 (EEA1)
BG-647 + AF-488
BG-647 + AF-488 + Hoechst
0
10 min
30 min
1 h
b
0 min
10 min
30 min
1 h
Colocalization of EEA1 with BG-647
Colocalization of BG-647 with EEA1
1.0
0.5
0.0
0
0.5
1

Figure S3

Supplementary Figure S3. Colocalization analysis of fixed BG-647 labeled toxin-SNAP-AS25 with early endosomes in HeLa cells. (a) HeLa cells were incubated with the BG-647-labeled protein for 10 minutes, washed, incubated in growth medium and fixed after 0, 10 or 30 min or 1h. Early endosomes were stained with an antibody against EEA1 and are shown in green and nuclei were stained with Hoechst. (b) The Mander's overlap coefficient 1 versus 2 between the antibody and the protein signals are plotted for each cell.

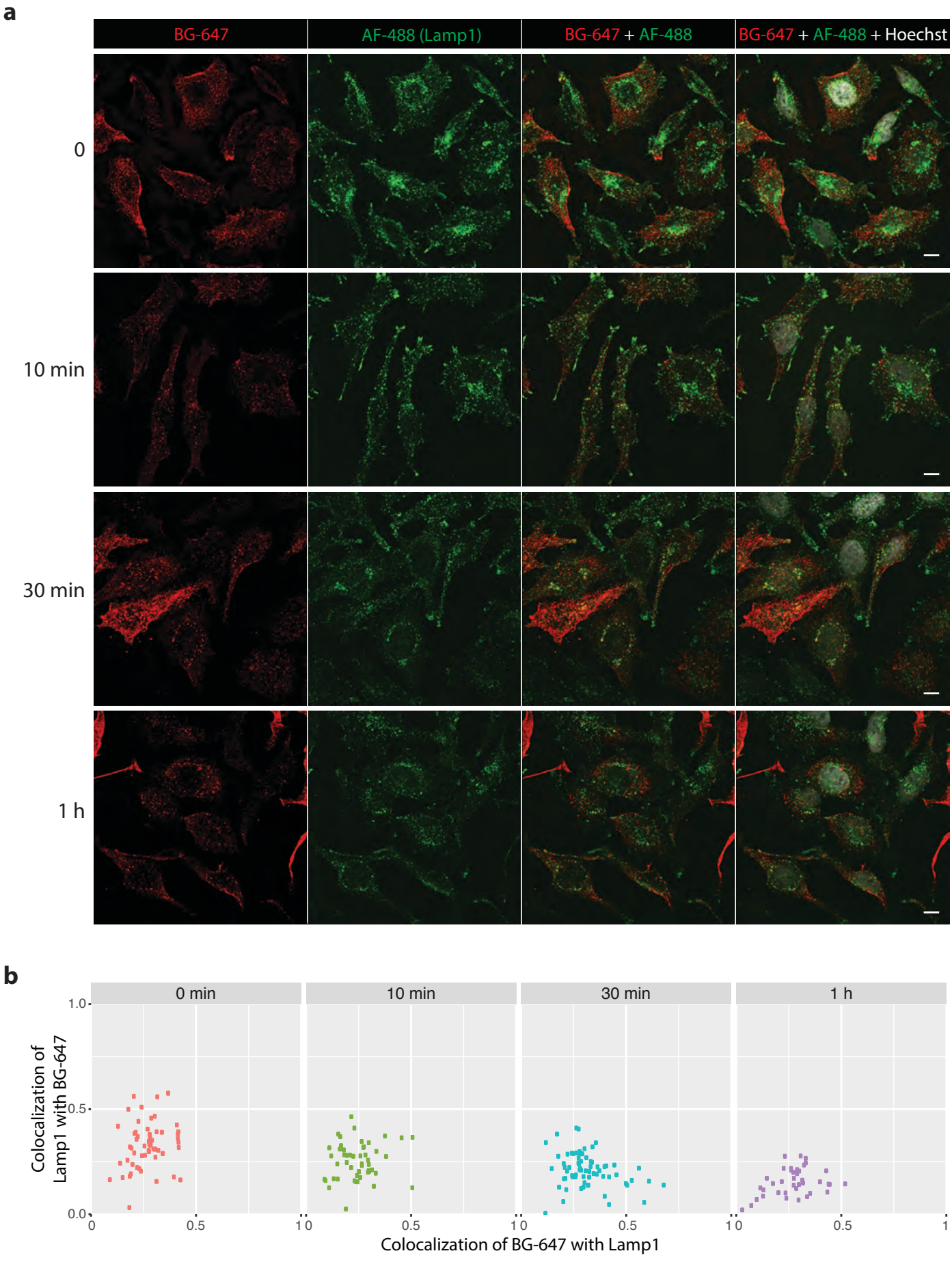
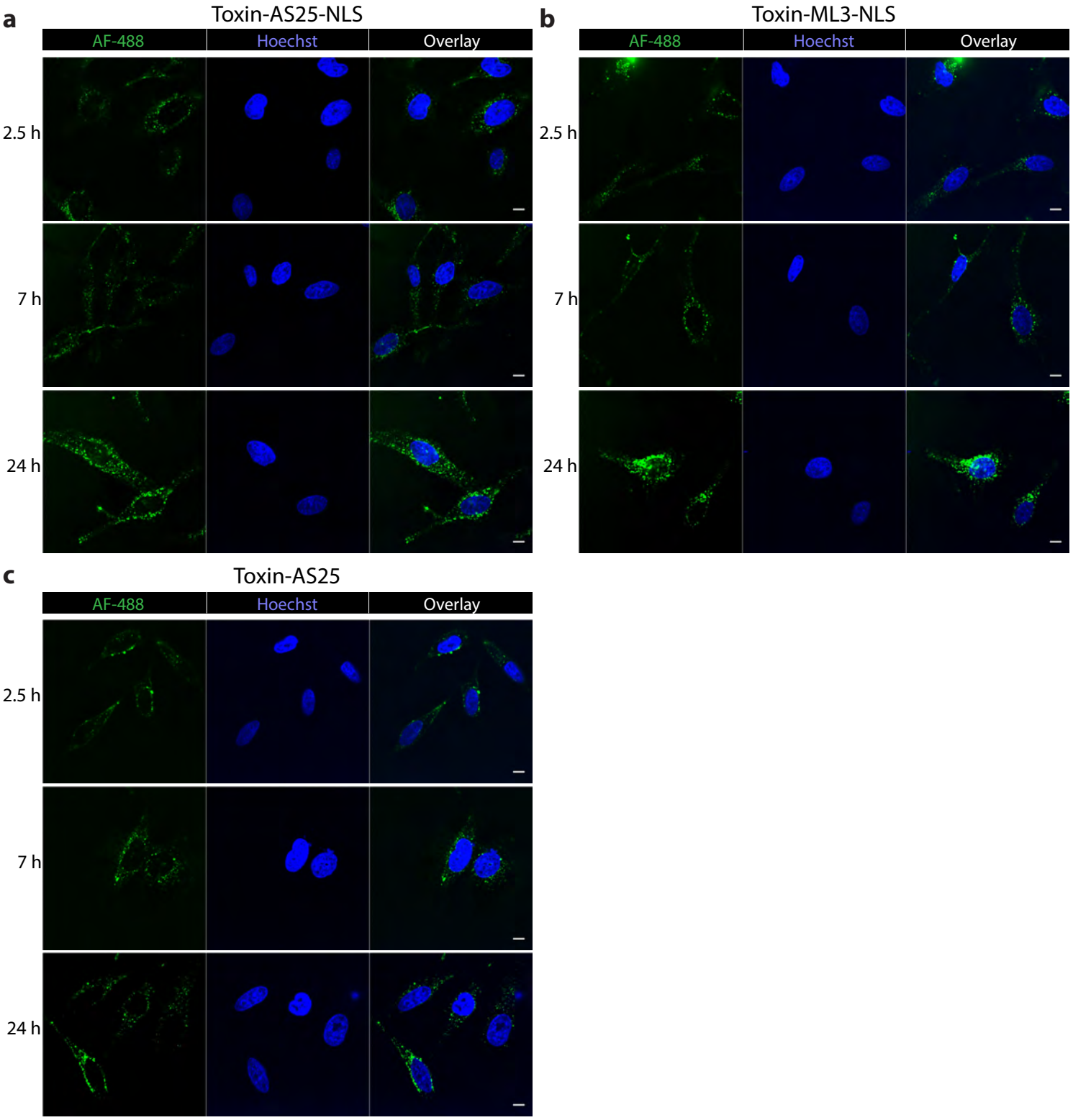

a
BG-647
AF-488 (Lamp1)
BG-647 + AF-488
BG-647 + AF-488 + Hoechst
0
10 min
30 min
1 h
b
0 min
10 min
30 min
1 h
Colocalization of Lamp1 with BG-647
Colocalization of BG-647 with Lamp1
1.0
0.5
0.0
0
0.5
1

Figure S4

Supplementary Figure S4. Colocalization analysis of BG-647 labeled toxin-SNAP-AS25 with lysosomes in HeLa cells. (a) HeLa cells were incubated with the BG-647-labeled protein for 10 minutes, washed, incubated in growth medium and fixed after 0, 10 or 30 min or 1h. Lysosomes were stained with an antibody against Lamp1 and are shown in green and nuclei were stained with Hoechst. (b) The Mander's overlap coefficient 1 versus 2 between the antibody and the protein signals are plotted for each cell.


a
Toxin-AS25-NLS
AF-488
Hoechst
Overlay
2.5 h
7 h
24 h
b
Toxin-ML3-NLS
AF-488
Hoechst
Overlay
2.5 h
7 h
24 h
c
Toxin-AS25
AF-488
Hoechst
Overlay
2.5 h
7 h
24 h

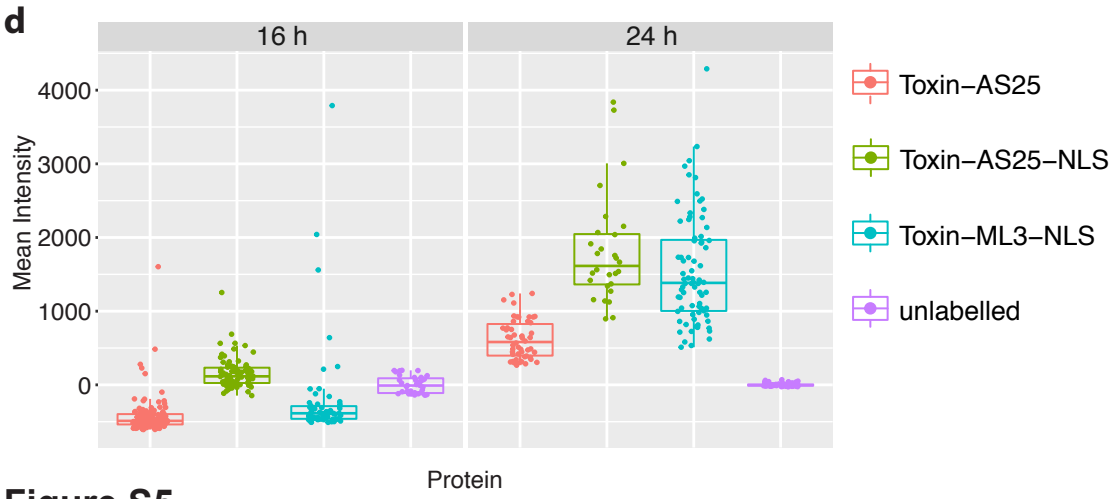

d
16 h
24 h
Mean Intensity
4000
3000
2000
1000
0
Protein
Toxin–AS25
Toxin–AS25–NLS
Toxin–ML3–NLS
unlabelled

Figure S5

Supplementary Figure S5. Uptake of NLS-tagged toxin-monobody proteins in the nucleus. HeLa cells were incubated with AF-488 labeled toxin-AS25-NLS (a) or toxin-ML3-NLS (b) or toxin-AS25 without NLS (c) for 2.5h, washed and incubated in growth medium for the indicated total times. The nuclei were stained with Hoechst. (d) Second experiment confirming the data shown in figure 4a and S5a-c. HeLa cells were incubated with AF-488 labeled toxin-AS25-NLS or toxin-ML3-NLS or toxin-AS25 without NLS for 2.5h, washed and incubated in growth medium for the indicated total times. The fluorescence intensity of the 488 nm signal in the nucleus stained with Hoechst was quantified from confocal microscopy images of live cells. Each dot represents the mean 488 nm fluorescence in the nucleus of a single cell, normalized to the mean of the control cells incubated with unlabeled toxin-AS25-NLS. Boxplots represent the median value, the first and third quartiles (lower and upper hinges) and the smallest and largest value within 1.5 times the interquartile range (lower and upper whiskers).

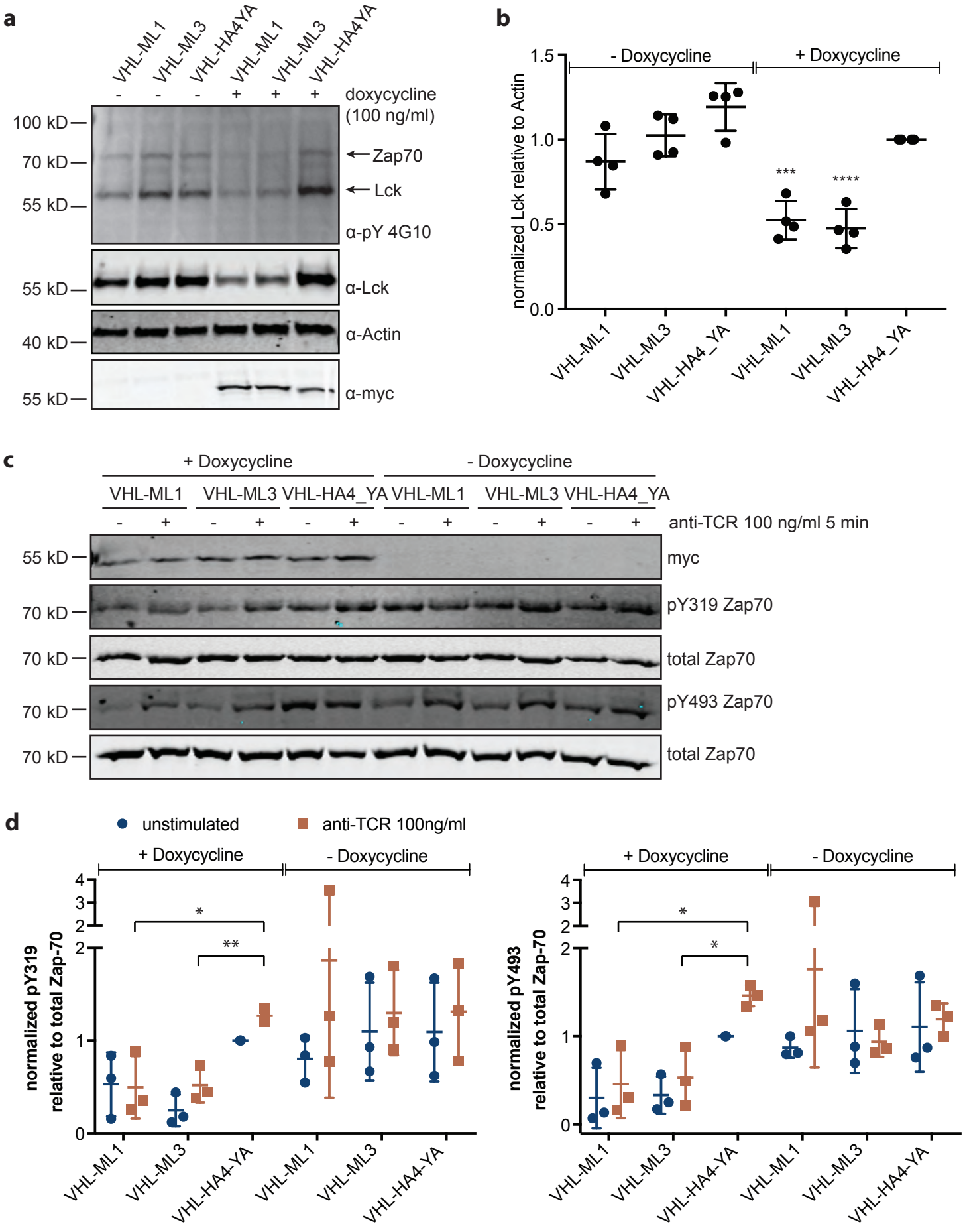

a
VHL-ML1
VHL-ML3
VHL-HA4YA
VHL-ML1
VHL-ML3
VHL-HA4YA
- - - + + +
doxycycline (100 ng/ml)
100 kD
70 kD
55 kD
Zap70
Lck
α-pY 4G10
55 kD
α-Lck
40 kD
α-Actin
55 kD
α-myc
b
- Doxycycline
+ Doxycycline
normalized Lck relative to Actin
1.5
1.0
0.5
0.0
***
****
VHL-ML1
VHL-ML3
VHL-HA4_YA
VHL-ML1
VHL-ML3
VHL-HA4_YA
c
+ Doxycycline
- Doxycycline
VHL-ML1
VHL-ML3
VHL-HA4_YA
VHL-ML1
VHL-ML3
VHL-HA4_YA
- + - + - + - + - + - +
anti-TCR 100 ng/ml 5 min
55 kD
myc
70 kD
pY319 Zap70
70 kD
total Zap70
70 kD
pY493 Zap70
70 kD
total Zap70
d
unstimulated
anti-TCR 100ng/ml
+ Doxycycline
- Doxycycline
normalized pY319 relative to total Zap-70
4
3
2
2
1
0
*
**
VHL-ML1
VHL-ML3
VHL-HA4-YA
VHL-ML1
VHL-ML3
VHL-HA4-YA
+ Doxycycline
- Doxycycline
normalized pY493 relative to total Zap-70
4
3
2
2
1
0
*
*
VHL-ML1
VHL-ML3
VHL-HA4-YA
VHL-ML1
VHL-ML3
VHL-HA4-YA

Figure S6

Figure S6. VHL-monobody mediated Lck degradation leads to reduced Zap70 phosphorylation. (a) Immunoblot analysis of the lysate of Jurkat cells transduced with myc-tagged VHL-monobody and grown in presence and absence of doxycycline with antibodies against tyrosine-phosphorylated proteins (4G10), Lck, Actin and the myc-tag. (Order as displayed from top to bottom) (b) Quantification of the ratio between the Lck and Actin signals. The data was transformed by setting the Lck:Actin ratio of doxycycline induced VHL-HA4_YA transduced cells to 1.0 for each experiment. Average values and standard deviations from 4 independent experiments were used, and *p* values were calculated from the untransformed data using a ratio paired t-test. (***$p < 0.001$, ****$p < 0.0001$) (c) Jurkat cells expressing VHL-ML1, VHL-ML3 or VHL-HA4_YA and induced with doxycycline or grown in doxycycline-free medium were stimulated with an anti-TCR antibody for 5 minutes and lysed. Immunoblot analysis of the cell lysate with antibodies against the myc-tag (upper blot), phosphorylated Y319 residue of Zap70 and total Zap70 on the same blot (blots 2 and 3 from top) and against phosphorylated Y493 residue of Zap70 and total Zap70 on the same blot (blots 4 and 5 from top). (d) Quantification of the ratio between the Y319-phosphorylated Zap70 and total Zap70 signals (left) and of the ratio between the Y493-phosphorylated Zap70 and total Zap70 signals (right). The data was transformed by setting the pZap70:Zap70 ratio of the unstimulated (doxycycline induced) HA4-Y87A control cells to 1.0 for each experiment. Average values and standard deviations from 3 independent experiments were used, and *p* values were calculated using a paired t-test. *$p < 0.05$, **$p < 0.01$.

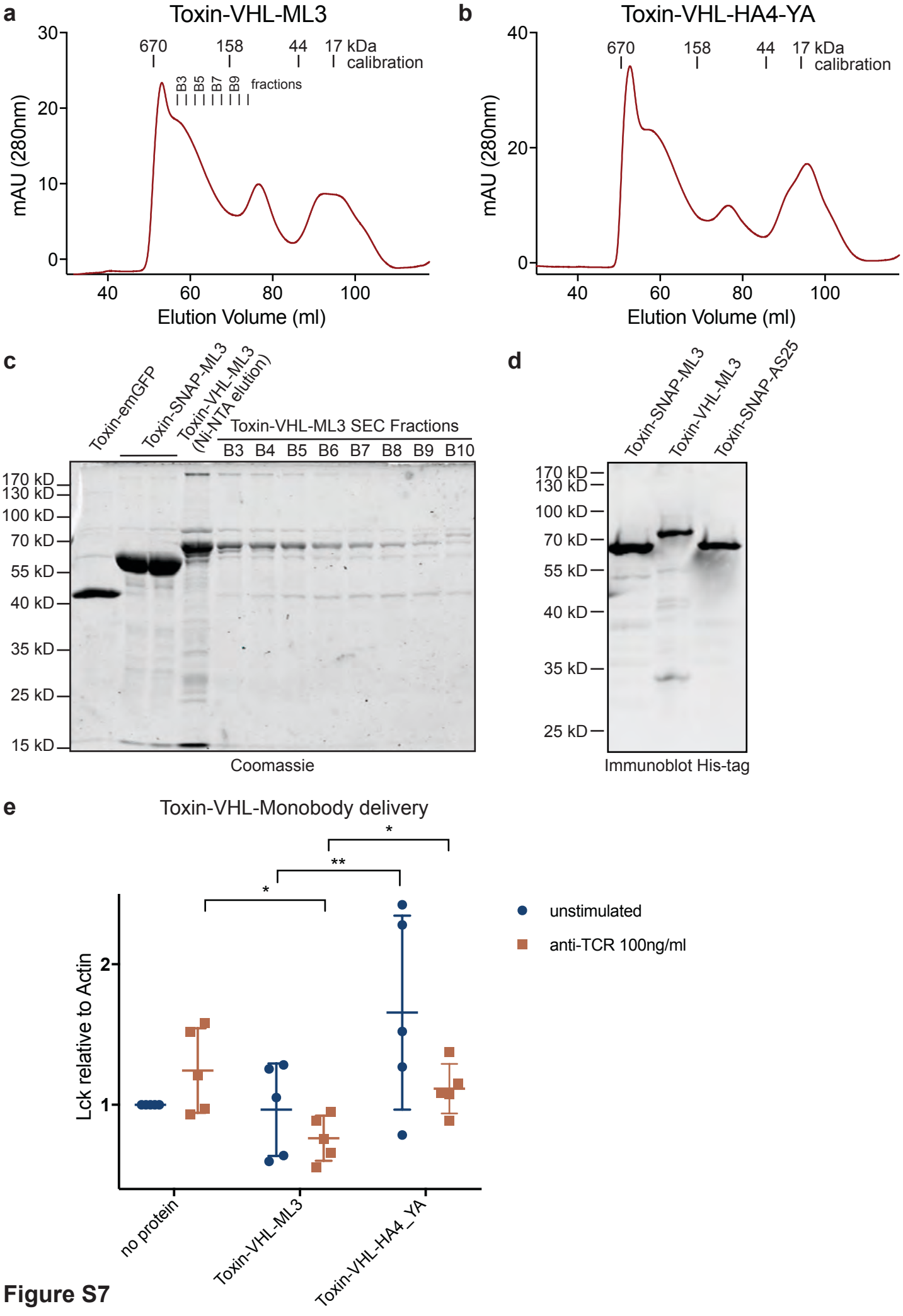

a
Toxin-VHL-ML3
mAU (280nm)
Elution Volume (ml)
670
158
44
17 kDa calibration
B3
B5
B7
B9
fractions
b
Toxin-VHL-HA4-YA
c
Toxin-emGFP
Toxin-SNAP-ML3
Toxin-VHL-ML3 (Ni-NTA elution)
Toxin-VHL-ML3 SEC Fractions
B3 B4 B5 B6 B7 B8 B9 B10
170 kD
130 kD
100 kD
70 kD
55 kD
40 kD
35 kD
25 kD
15 kD
Coomassie
d
Toxin-SNAP-ML3
Toxin-VHL-ML3
Toxin-SNAP-AS25
Immunoblot His-tag
e
Toxin-VHL-Monobody delivery
Lck relative to Actin
unstimulated
anti-TCR 100ng/ml
no protein
Toxin-VHL-ML3
Toxin-VHL-HA4_YA

Figure S7

Supplementary Figure S7. Expression and purification of recombinant toxin-VHL-monobody fusion proteins. The crude bacteria lysate from *E. coli* Origami cells was first purified on a Ni-NTA column and then by SEC. A and (b) Size exclusion chromatograms of toxin-VHL-ML3 (A) and toxin-VHL-HA4_YA (B) (c) Coomassie stained SDS-PAGE gel with Ni-NTA- and SEC-purified toxin-emGFP and toxin-SNAP-ML3 (2 lanes), Ni-NTA-purified toxin -VHL-ML3 and different fractions of the SEC purification loaded from left to right. SEC fractions B4 to B6 were pooled and concentrated from the SEC purification of toxin-VHL-ML3. Toxin-VHL-HA4_YA yielded similar results. (d) Immunoblot using an antibody recognizing penta-His of the SEC purified and concentrated proteins toxin-SNAP-ML3, toxin-VHL-ML3 and Stoxin-SNAP-AS25 (loaded from left to right). (e) Quantification of the ratio between the Lck and Actin signals in figure 5d. The data was transformed by setting the Lck:Actin ratio of uninduced, untreated cells to 1.0 for each experiment. Average values and standard deviations from 5 independent experiments were used, and *p* values were calculated from the untransformed data using a ratio paired t-test.

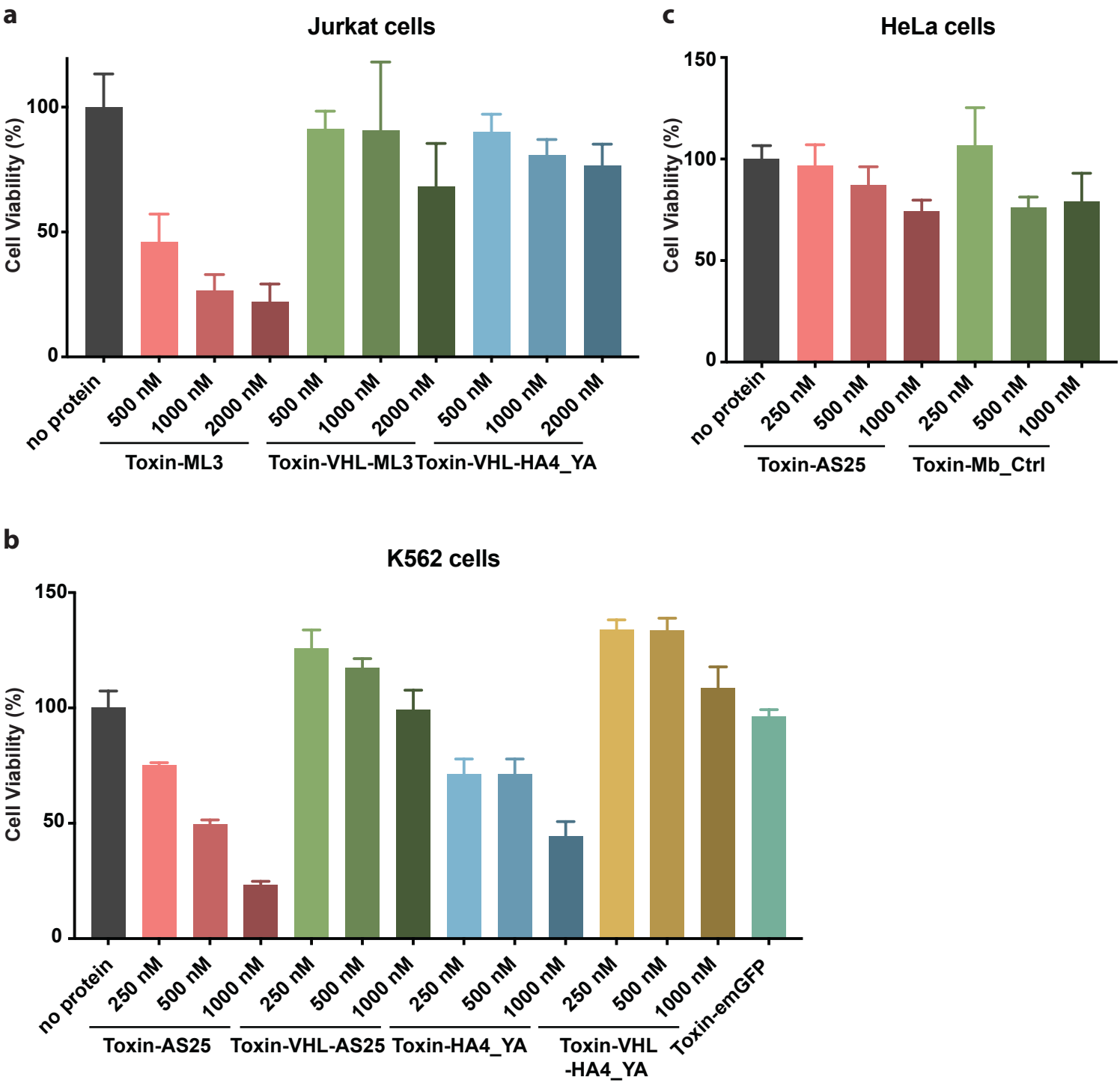

a
Jurkat cells
Cell Viability (%)
100
50
0
no protein
500 nM
1000 nM
2000 nM
500 nM
1000 nM
2000 nM
500 nM
1000 nM
2000 nM
Toxin-ML3
Toxin-VHL-ML3
Toxin-VHL-HA4_YA
c
HeLa cells
Cell Viability (%)
150
100
50
0
no protein
250 nM
500 nM
1000 nM
250 nM
500 nM
1000 nM
Toxin-AS25
Toxin-Mb_Ctrl
b
K562 cells
Cell Viability (%)
150
100
50
0
no protein
250 nM
500 nM
1000 nM
250 nM
500 nM
1000 nM
250 nM
500 nM
1000 nM
250 nM
500 nM
1000 nM
Toxin-emGFP
Toxin-AS25
Toxin-VHL-AS25
Toxin-HA4_YA
Toxin-VHL
-HA4_YA

Figure S8

Supplementary Figure S8. Cell viability measurement using the Realtime Glo assay upon protein delivery in different cell lines. Jurkat (a), K562 (b) or HeLa (c) cells were incubated with the indicated concentrations of proteins and Realtime Glo reagents for 48h. Mb_Ctrl designates an unrelated non-binding control monobody. Luminescent signal was measured and the control without proteins was set to 100% cell viability. Error bars indicate the SD of 3 technical replicates.

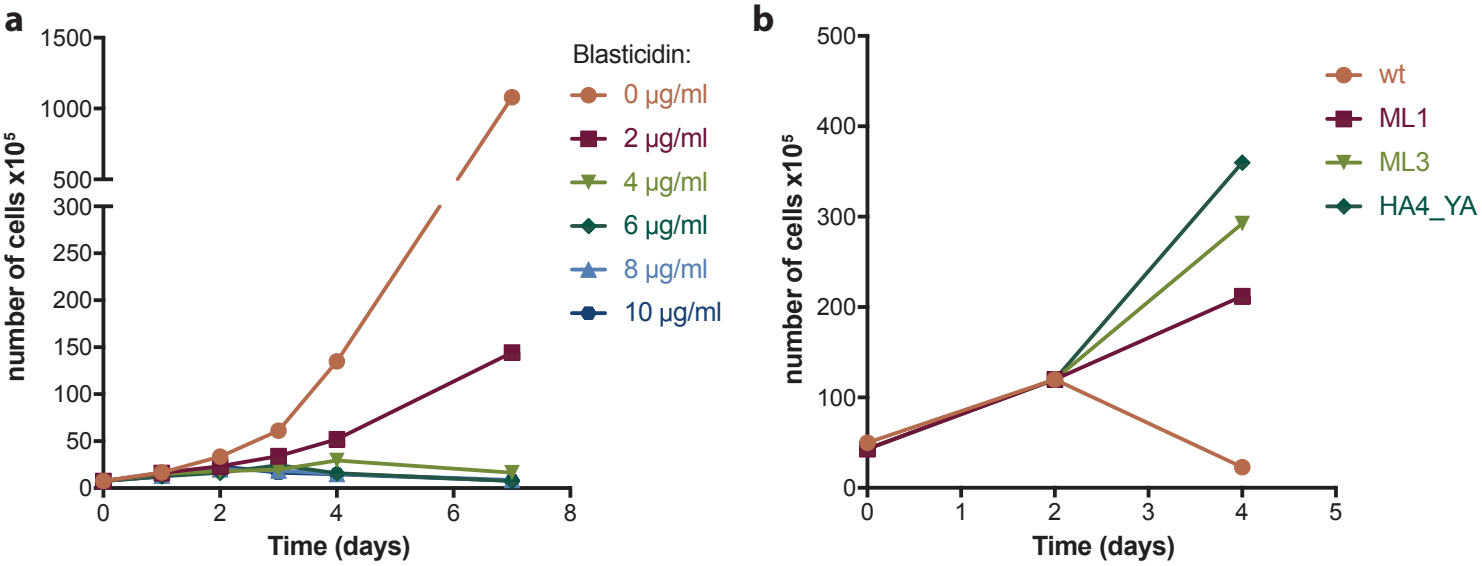

a
number of cells x10⁵
1500
1000
500
300
250
200
150
100
50
0
0
2
4
6
8
Time (days)
Blasticidin:
0 µg/ml
2 µg/ml
4 µg/ml
6 µg/ml
8 µg/ml
10 µg/ml
b
number of cells x10⁵
500
400
300
200
100
0
0
1
2
3
4
5
Time (days)
wt
ML1
ML3
HA4_YA

Figure S9

Supplementary Figure S9. Blasticidin selection following lentiviral transduction. (a) Wild type Jurkat cells were supplemented with blasticidin at the indicated concentrations and the cell number was measured for 7 days to determine the minimal lethal concentration. (b) Jurkat cells were transduced with the pEM24 vector containing the indicated VHL-monobody constructs or left untransduced and 5μg/ml blasticidin was added to the growth medium 3 days after the second transduction (day 0) and cell numbers were measured on day 2 and 4.