nature methods

SUPPLEMENTARY INFORMATION

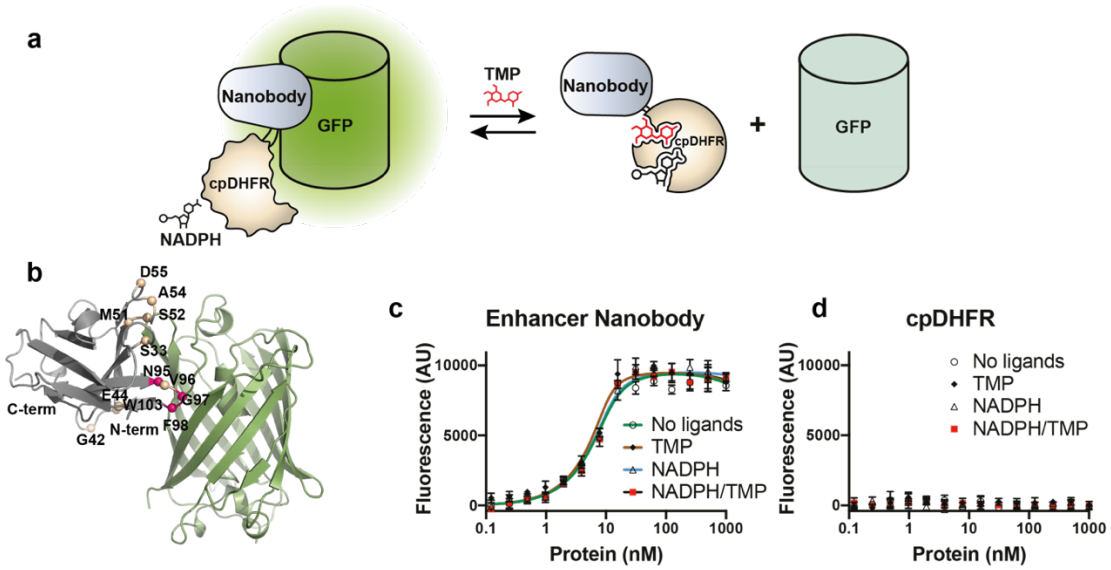
https://doi.org/10.1038/s41592-020-0746-7

In the format provided by the authors and unedited.

Chemogenetic Control of Nanobodies

Helen Farrants[1,2], Miroslaw Tarnawski[3], Thorsten G. Müller[4], Shotaro Otsuka[5,6], Julien Hiblot[1], Birgit Koch[1], Moritz Kueblbeck[5], Hans-Georg Kräusslich[4], Jan Ellenberg[5] and Kai Johnsson[1,2] ✉

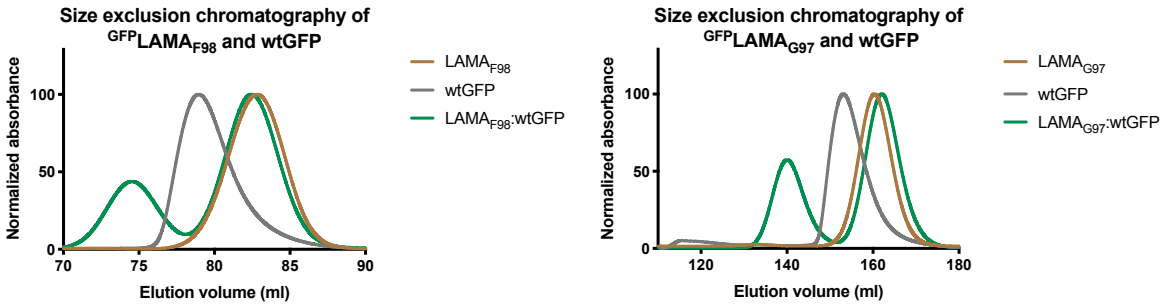
[1]Department of Chemical Biology, Max Planck Institute for Medical Research, Heidelberg, Germany. [2]Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland. [3]Protein Expression and Characterization Facility, Max Planck Institute for Medical Research, Heidelberg, Germany. [4]Department of Infectious Diseases, Virology, University Hospital Heidelberg, Heidelberg, Germany. [5]Cell Biology and Biophysics Unit, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany. [6]Present address: Max Perutz Labs, a joint venture of the University of Vienna and the Medical University of Vienna, Vienna Biocenter (VBC), Vienna, Austria. ✉e-mail: johnsson@mr.mpg.de



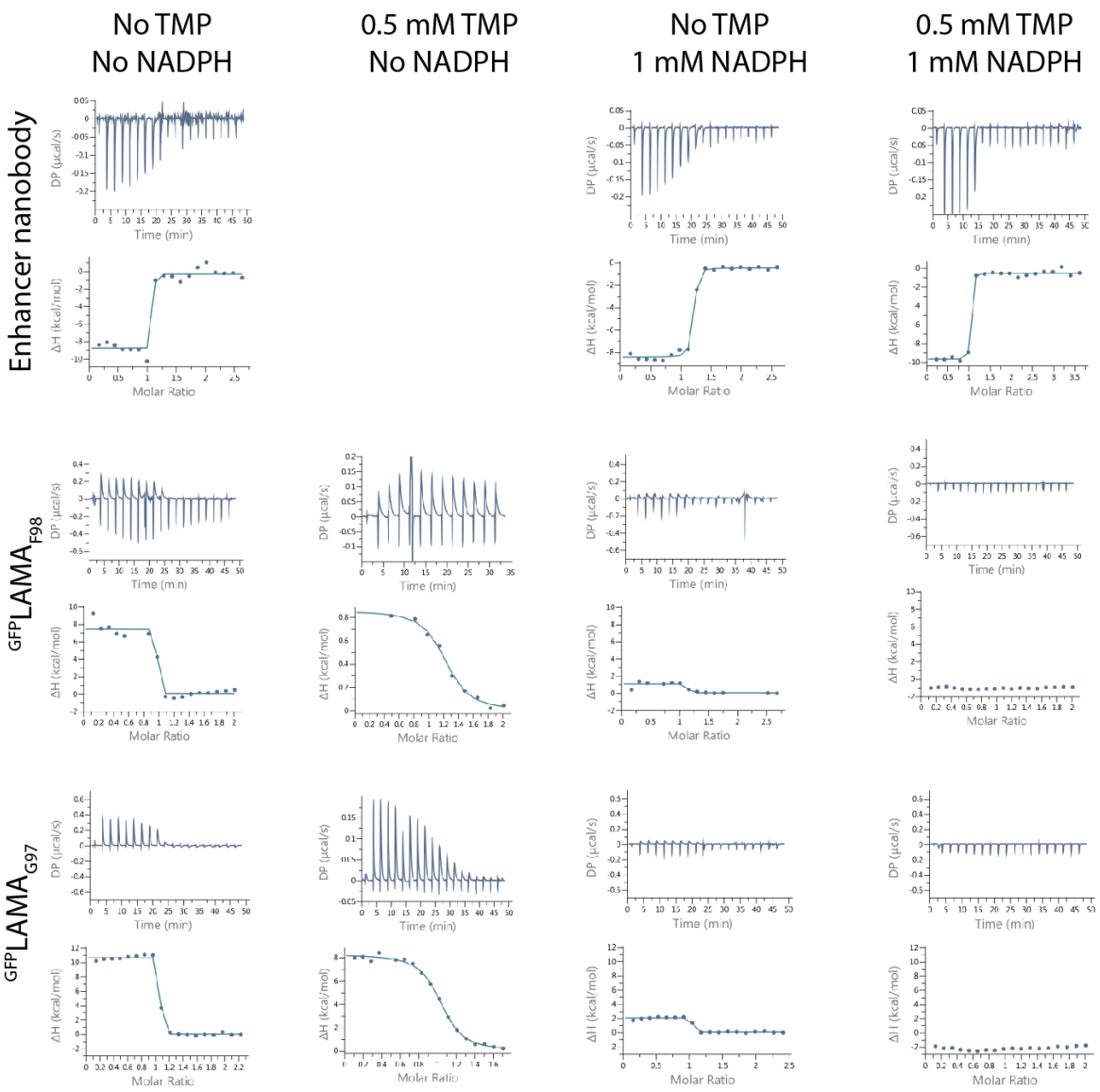
e

Insertion position	K_d NADPH (nM)	K_d NADPH/TMP (nM)	Region of nanobody	Switch
None	Below 1	Below 1	-	No
S33	20	39	CDR1	No
G42	45	49	FR2	No
E44	109	147	FR2	No
M51$_{GGS}$	1.6	50	CDR2	Slight
S52	40	170	CDR2	Slight
A54	1.7	2.3	CDR2	No
D55	Below 1	Below 1	CDR2	No
N95	3.3 ± 0.5	No fit	CDR3	Yes
V96	99 ± 14	No fit	CDR3	Yes
G97	6.0± 0.5	No fit	CDR3	Yes
F98	79 ± 7.3	No fit	CDR3	Yes
W103	Approx. 500	Approx. 500	FR4	No

Supplementary Fig. 1. Generation of LAMAs from the enhancer nanobody. (**a**) Schematic illustration of the ^{GFP}LAMAs. (**b**) Insertion sites of cpDHFR mapped onto the X-ray structure of the enhancer nanobody (grey) bound to GFP (green) (PDBID = 3K1K) as spheres. Numbering of residues is according to the the PDB file. Insertion positions with large potential for switching are highlighted in magenta. (**c,d**) Titration between recombinant enhancer nanobody (**c**) or cpDHFR (**d**) and wtGFP (10 nM), as quantified by fluorescent emission increase of wtGFP. Mean ± s.d. of triplicates, representative from three independent experiments with similar results. (**e**) Table of affinities from the titrations between the enhancer nanobody with cpDHFR insertions and wtGFP (10 nM). Screening was performed once in duplicates. Any proteins that scored "yes" in switching behavior was repeated in triplicates in three independent experiments with similar results. Data were fit the full equation of single site binding, accounting for the effect of nonspecific binding reported with s.e.m. of the fit for experiments performed in triplicates. Insertion position is represented as the last residue prior to cpDHFR insertion. NADPH = 100 µM, TMP = 500 µM for all experiments.

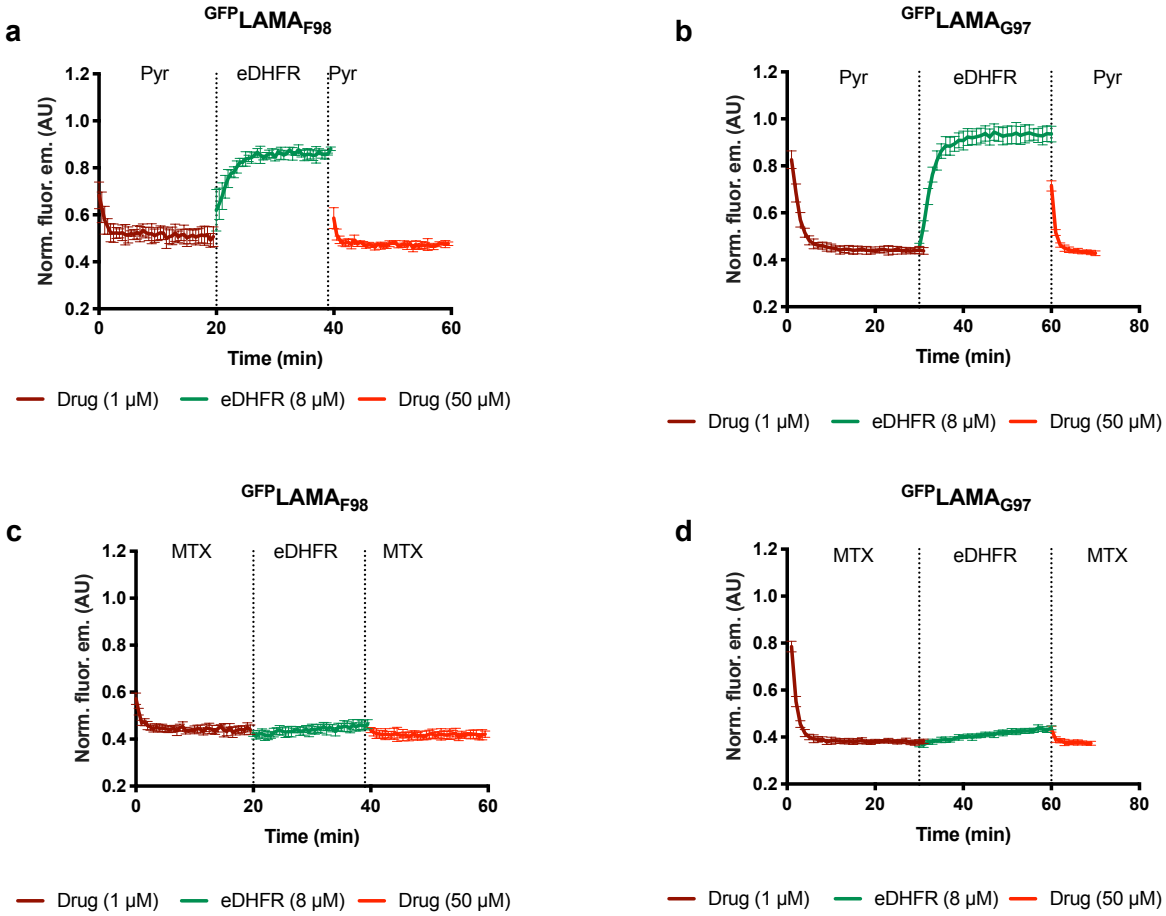


Supplementary Fig. 2. Complex formation between ^{GFP}LAMAs and wtGFP. Size exclusion chromatography was performed with LAMAs and wtGFP. Absorbances are normalized (%) to the maximum intensity values for each run. LAMAs and wtGFP were each injected separately on the column for calibration. An excess of $^{GFP}LAMA_{F98}$ or $^{GFP}LAMA_{G97}$ (2-5 fold) was premixed with wtGFP and incubated on ice for 10 minutes, before being separated on the same column. For $^{GFP}LAMA_{F98}$ an S200-16-600 (GE-Healthcare) was used. For $^{GFP}LAMA_{G97}$ an S75-26-600 (GE-Healthcare) was used. Representative of two independent experiments.

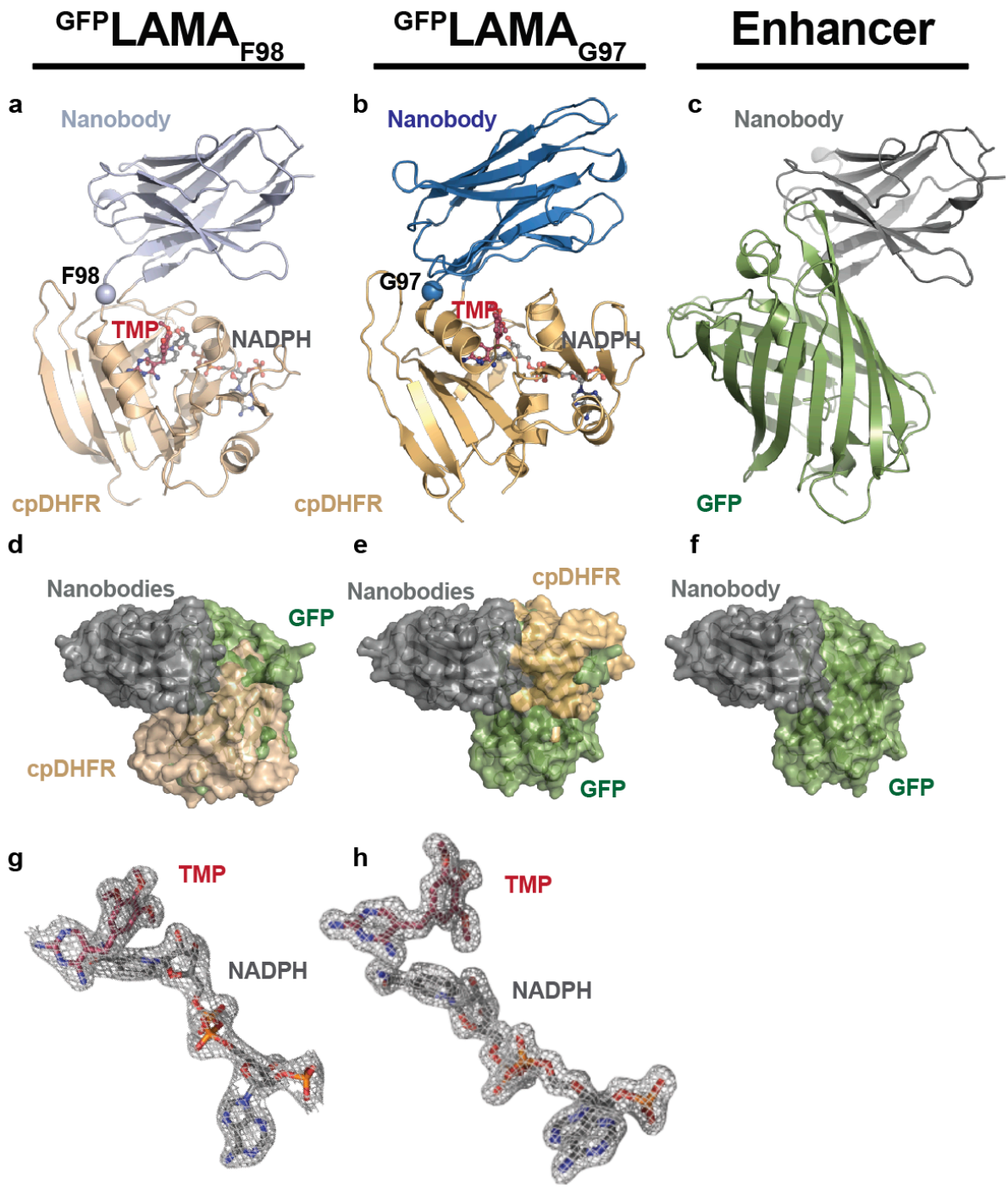


LAMA	K_d No Ligands (nM)	K_d TMP (nM)	K_d NADPH (nM)	K_d TMP/NADPH (nM)
Nanobody only	Below 1	Not Measured	Below 1	Below 1
F98	Below 25	1000 ± 350	Below 80	No fit
G97	Below 5	380 ± 60	Below 15	No fit

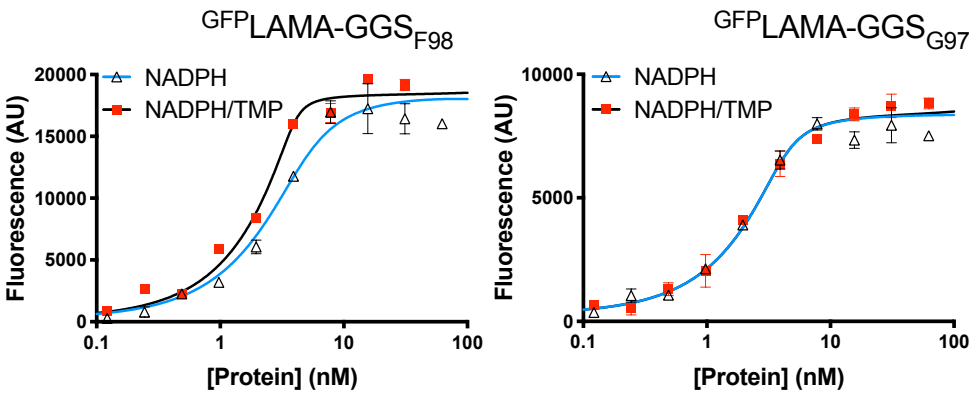
Supplementary Fig. 3. ITC measurements of the interaction between LAMAs and wtGFP. Proteins were dialysed against buffers containing NADPH (1 mM) and/or TMP (0.5 mM), before measurements. The data was fit using the Malvern PEAQ-ITC analysis software, fixing the binding as 1:1 and calculating a K_d ± s.d.. Representative traces of three independent experiments with similar results.



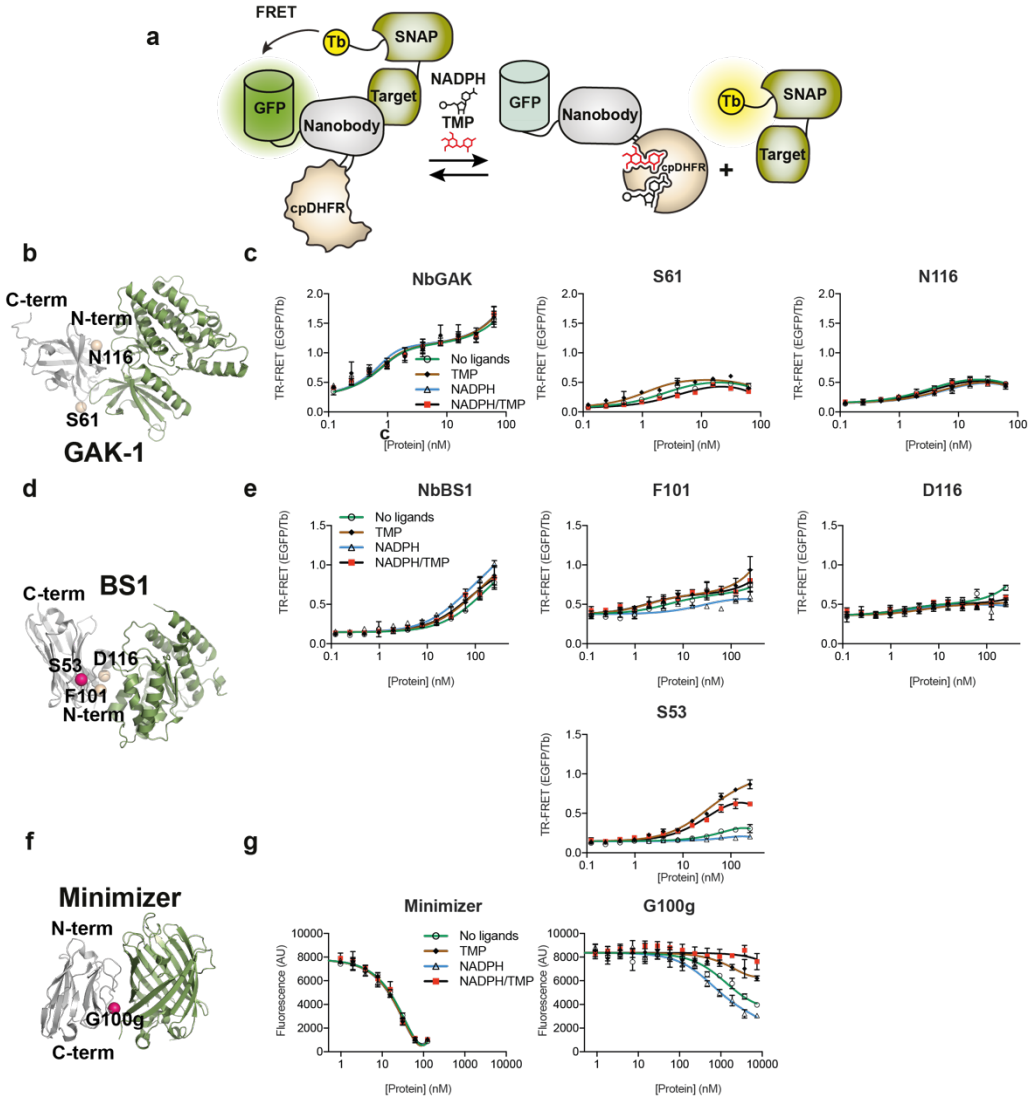
Supplementary Fig. 4. Kinetics of dissociation and association between LAMAs and wtGFP in a 1:1 complex at 200 nM. Addition of different dihydrofolate drugs (1 µM in first addition, 50 µM in second addition) was performed in the presence of NADPH (100 µM). eDHFR was added to sequester the drugs (8 µM). Dissociation was followed by the decrease in fluorescent intensity of wtGFP on the addition of pyrimethamine (Pyr) (**a**,**b**), and methotrexate (MTX) (**c**,**d**). Mean ± s.d. from triplicates, representative of two independent experiments with similar results.



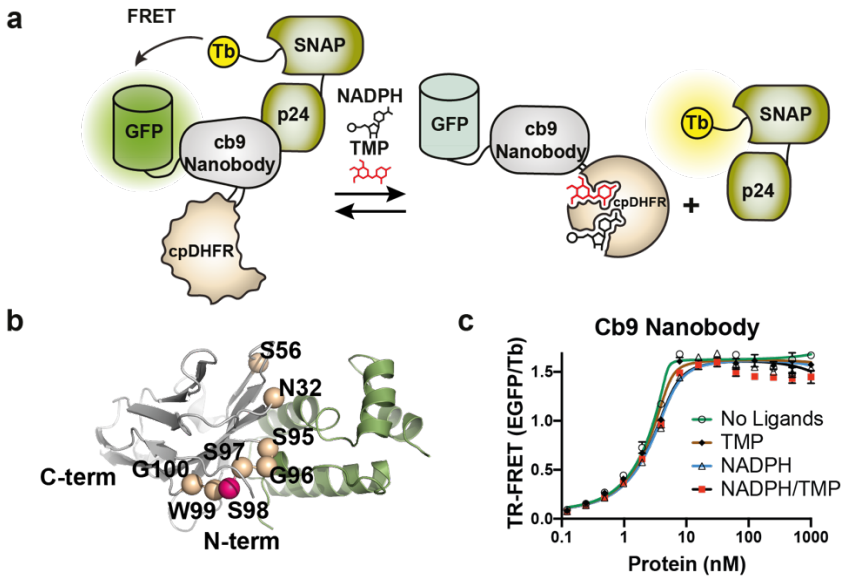
Supplementary Fig. 5. Structural analysis of ^{GFP}LAMAs. (**a-c**) Comparison between the X-ray structures of ^{GFP}LAMA$_{F98}$ (PDBID = 6RUL) (**a**), ^{GFP}LAMA$_{G97}$ (PDBID = 6RUM) (**b**), both in the presence of TMP (red), NADPH (grey), and enhancer nanobody bound to GFP (PDBID = 3K1K) (**c**). Structures are represented as cartoons, ligands as stickballs. The insertion positions of cpDHFR are highlighted on the LAMA structures. (**d-f**) Overlay of the nanobody domain of LAMA structures on the enhancer nanobody bound to GFP with ^{GFP}LAMA$_{F98}$ (**d**), and ^{GFP}LAMA$_{G97}$ (**e**). The enhancer nanobody bound to GFP (**f**) is shown for comparison. (**g,h**) The 2mFobs-DFcalc omit electron density maps showing the placement of TMP and NADPH in cpDHFR of ^{GFP}LAMA$_{F98}$ (**g**) and ^{GFP}LAMA$_{G97}$ (**h**) contoured at 1.0 σ.



Supplementary Fig. 6. Titrations of ^{GFP}LAMAs with GGS-linkers. Fluorescent emission from titration between cpDHFR inserted with GGS-linkers into the nanobody structure and wtGFP (10 nM). Mean ± s.d of triplicates, fit with the full equation of single site binding, accounting for the effect of nonspecific binding. Representative experiment from two independent trials with similar results. Fitted affinities correspond to single digit nanomolar affinities.



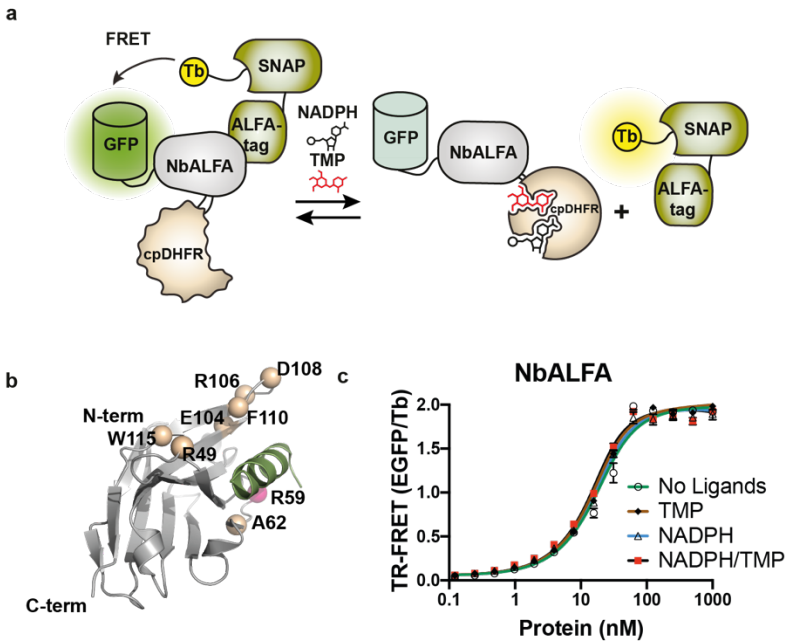
Supplementary Fig. 7. Insertion of cpDHFR into other nanobodies. (**a**) Schematic illustration of the TR-FRET assay to monitor binding between the nanobodies (Nb) and target protein. The Nb is expressed as an EGFP fusion (FRET acceptor) and the target expressed as a SNAP-tag fusion, labelled with Tb-cryptate (FRET donor). (**b, d, f**) X-ray structure of a nanobodies and targets. Numbering of residues is according to the PDB file. Tried insertions are mapped as beige spheres, with most promising position mapped in magenta. The N- and C- termini are also indicated. (**b**) NbGAK (grey) and target (green) (PDBID = 4C57). (**d**) BS1 nanobody and (green) in and target (PDBID = 4TVS). (**f**) minimizer nanobody for GFP (grey) and target (green) (PDBID = 3G9A). (**c**, **e**) TR-FRET assay between EGFP-fused Nb with or without cpDHFR insertion at indicated positions and Tb-labelled target. (**c**) Target for GAK Nb = 1 nM. (**d**) Target for BS1 Nb = 5 nM. (**g**) EGFP fluorescent modulation assay between minimizer with or without insertion of cpDHFR. EGFP = 10 nM. Titrations are mean ± s.d. from triplicates and representative from two independent experiments. Data were fit with the full equation of single site binding, accounting for the effect of nonspecific binding. Insertion position is represented as the last residue prior to cpDHFR insertion. For all titrations, NADPH = 100 µM, TMP = 500 µM.



d

Insertion position	K_d NADPH (nM)	K_d NADPH/TMP (nM)	Region of nanobody	Switch
None	1.1 ± 0.3	1.6 ± 0.3	-	No
N32	Below 1	Below 1	CDR1	No
S56	1.4	Below 1	CDR2	No
S95	3.5 ± 0.4	70 ± 18	CDR3	Yes
G96	330 ± 10	2220 ± 50	CDR3	Yes
S97	63 ± 2	1100 ± 100	CDR3	Yes
S98	7 ± 1	524 ± 34	CDR3	Yes
W99	22	50	CDR3	Limited
G100	14	18	CDR3	No

Supplementary Fig. 8. Generation of LAMAs from the p24 binding nanobody, cb9. (**a**) Schematic illustration of the TR-FRET assay to monitor binding between the cb9 nanobody and the p24 target protein. The ^{p24}LAMA is expressed as an EGFP fusion (FRET acceptor) and the p24 target expressed as a SNAP-tag fusion, labelled with Tb-cryptate (FRET donor). (**b**) X-ray structure of cb9 (grey) with the C-terminal region of p24 (green) represented as cartoon, with insertion sites of cpDHFR represented as spheres (PDBID = 2XV6). Numbering of residues is according to the PDB file. The insertion position with a large potential for switching is highlighted in magenta. (**c**) Titration between recombinant cb9 nanobody and Tb-labeled p24 (5 nM), measured by TR-FRET. Mean ± s.d from triplicates, representative from three independent experiments with similar results. (**d**) Table of affinities between the cb9 nanobody and Tb-labeled p24 (5 nM). Screening was performed once in duplicates. Any proteins that scored “yes” in switching behavior was repeated in triplicates in three independent experiments with similar results. Data were fit the full equation of single site binding, accounting for the effect of nonspecific binding reported with s.e.m. of the fit for experiments performed in triplicates. Insertion position is represented as the last residue prior to cpDHFR insertion. NADPH = 100 µM, TMP = 500 µM in all titrations.

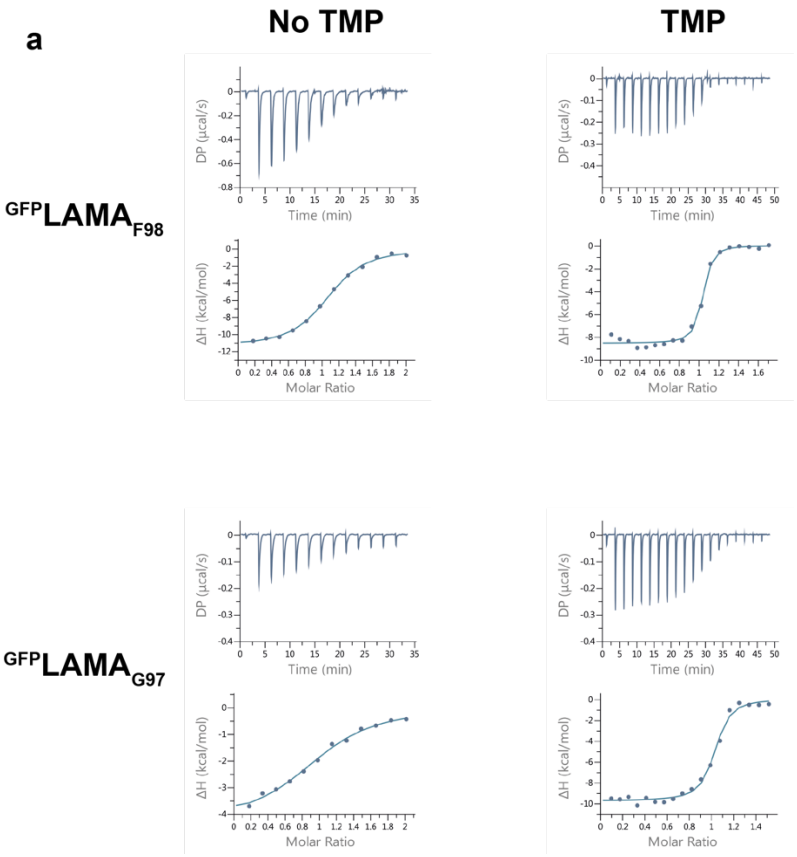


d

Insertion position	K_d No Ligands (nM)	K_d TMP (nM)	K_d NADPH (nM)	K_d NADPH and TMP (nM)	Region of nanobody	Switch
None	8.2 ± 0.8	5.6 ± 0.6	6.8 ± 0.7	4.9 ± 0.6	-	No
R49	81	250	6	103	FR2	Limited
R59	69 ± 8	Over 1000	532 ± 27	989 ± 60	CDR2	Yes
A62	Over 1000	232 ± 21	286 ± 25	Over 1000	CDR2	Yes
E104	34 ± 2	62 ± 4	66 ± 4	83 ± 5	CDR3	No
R106	26 ± 2	31 ± 3	25 ± 2	41 ± 4	CDR3	No
D108	10 ± 1	19 ± 1	29 ± 1	12 ± 1	CDR3	No
F110	47 ± 3	55 ± 4	58 ± 4	85 ± 6	CDR3	No
W115	Over 1000	Over 1000	346 ± 37	Over 1000	FR4	Limited

Supplementary Fig. 9. Generation of LAMAs from the ALFA-tag binding nanobody, NbALFA. (**a**) Schematic illustration of the TR-FRET assay to monitor binding between the NbALFA and the ALFA-tag-SNAP-tag target protein. The $^{ALFA\text{-}tag}$LAMA is expressed as an EGFP fusion (FRET acceptor) and the ALFA-tag target expressed as a SNAP-tag fusion, labelled with Tb-cryptate (FRET donor). (**b**) X-ray structure of NbALFA (grey) with the ALFA-tag (green) represented as cartoon, with insertion sites of cpDHFR represented as spheres (PDBID = 6I2G). Numbering of residues is according to the PDB file. The insertion positions with a large potential for switching is highlighted in magenta. (**c**) Titration between recombinant NbALFA nanobody and Tb-labeled ALFA-tag (5 nM), measured by TR-FRET. Mean ± s.d from triplicates, representative from three independent experiments with similar results. (**d**) Table of affinities between the NbALFA and Tb-labeled ALFA-tag-SNAP-tag (5 nM or 20 nM). Screening was performed in triplicates, with any switching positions verified in two independent experiments with similar results. Data were fit with the full equation of single site binding, accounting for the effect of nonspecific binding, with s.e.m. of the fit. Insertion position is represented as the last residue prior to cpDHFR insertion. NADPH = 100 µM, TMP = 500 µM in all titrations.

a

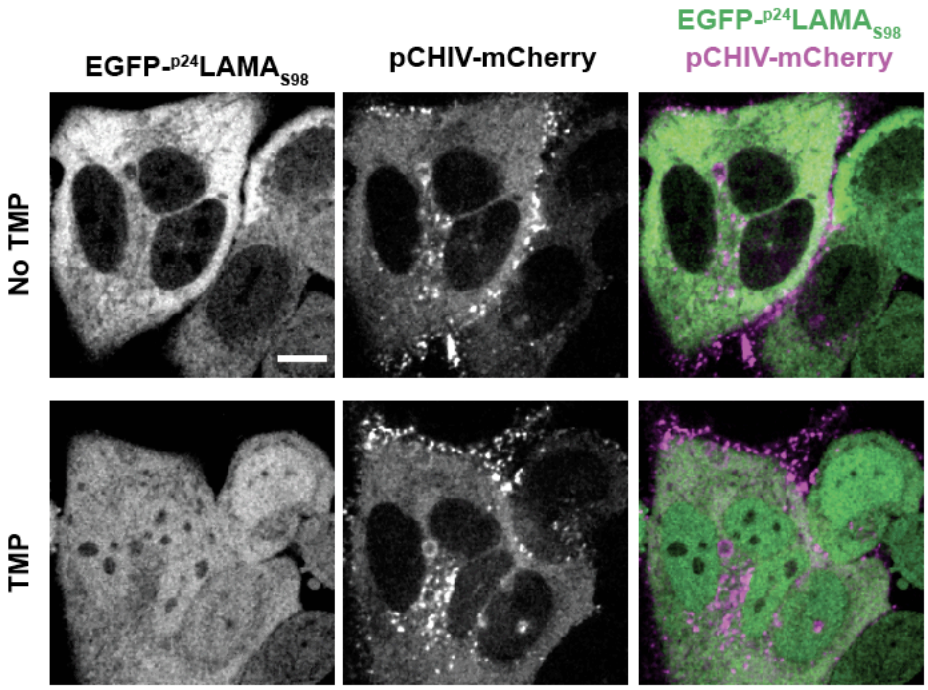


b

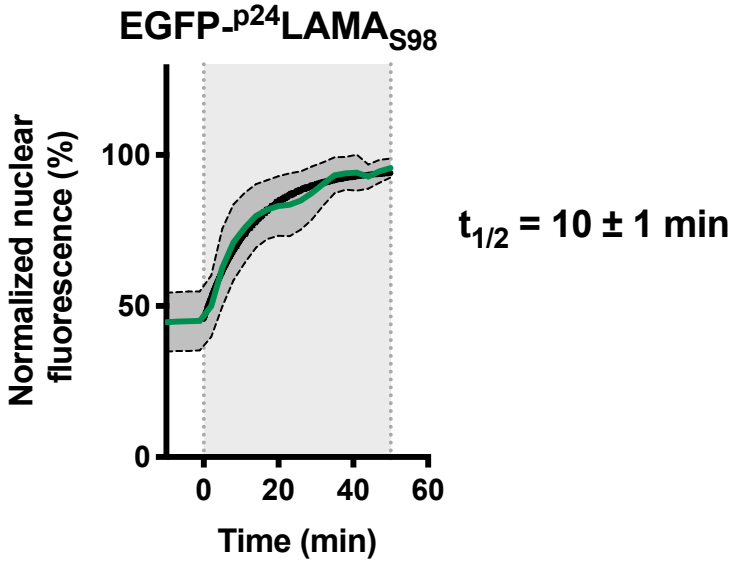
LAMA	K_d No TMP (nM)	K_d TMP (nM)
$^{GFP}LAMA_{F98}$	1900 ± 300	39 ± 13
$^{GFP}LAMA_{G97}$	4700 ± 1500	89 ± 24

Supplementary Fig. 10. Cooperative binding of NADPH and TMP to LAMAs. LAMAs were dialyzed against buffers containing ± TMP (500 µM), before measurements. The data was fit using the Malvern PEAQ-ITC analysis software, fixing the binding as 1:1 and calculating a K_d ± s.d.. Representative traces of three independent experiments with similar results. (**a**) top left panel: $^{GFP}LAMA_{F98}$ = 40 µM, NADPH = 400 µM, TMP = 500 µM. Top right panel: $^{GFP}LAMA_{F98}$ = 20 µM, NADPH = 170 µM. Bottom left panel: $^{GFP}LAMA_{G97}$ = 30 µM NADPH = 300 µM, TMP = 500 µM. Bottom right panel: $^{GFP}LAMA_{G97}$ = 30 µM NADPH = 300 µM. (**b**) Table of calculated affinities between NADPH and ^{GFP}LAMAs in presence of absence of TMP.

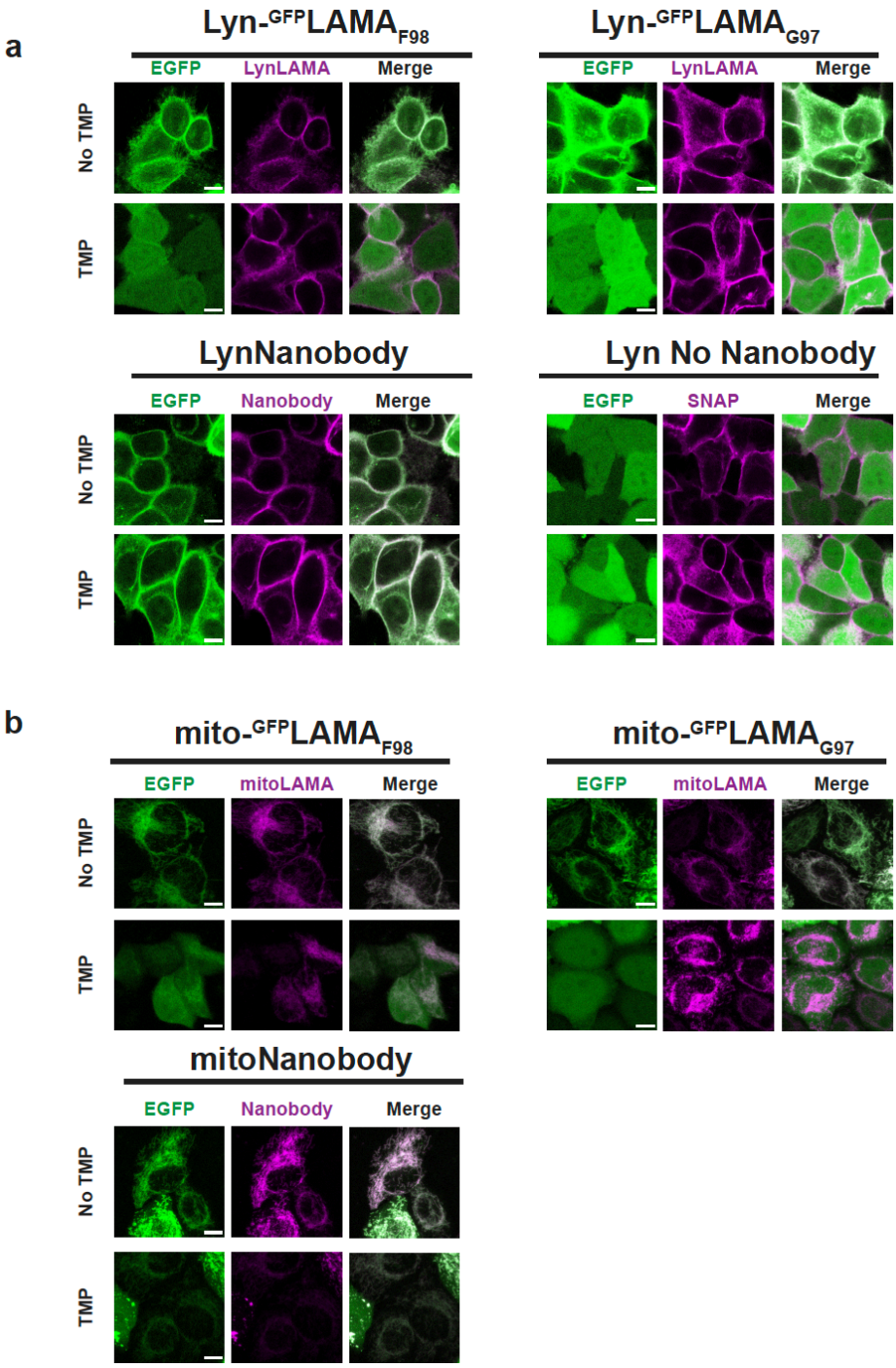
a



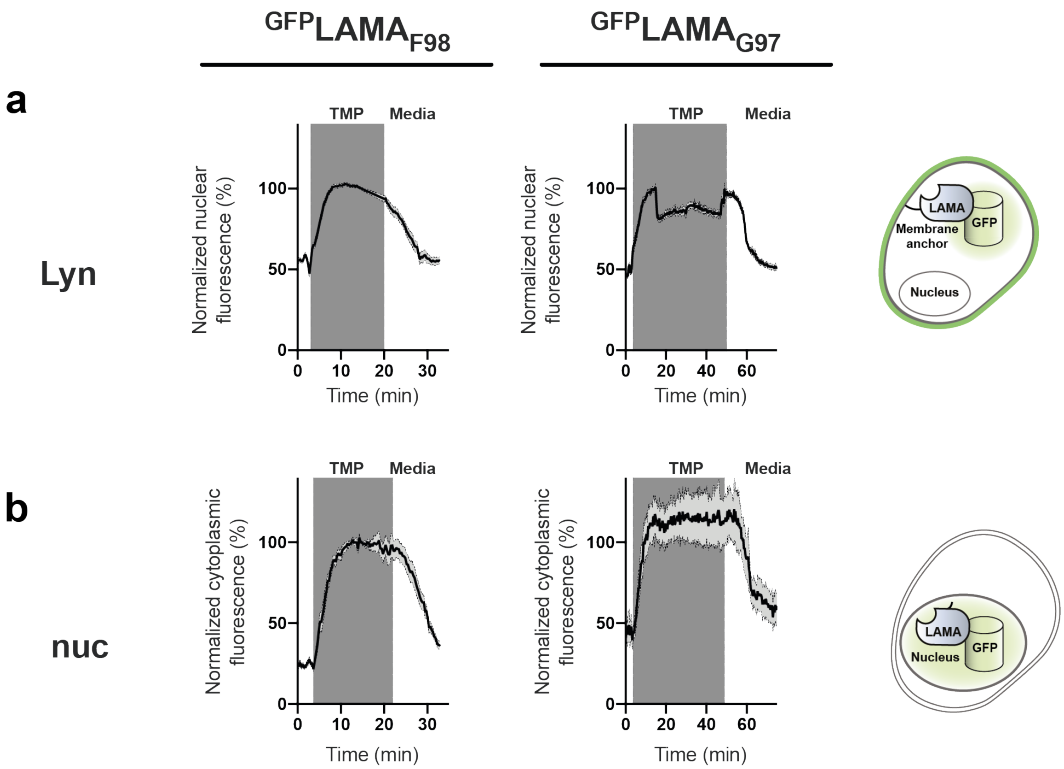
b



Supplementary Fig. 11. Characterization of $^{p24}LAMA_{S98}$ in live cells. (**a**) Full panel of micrographs from before and after addition of TMP (10 µM) to HeLa TZM-bl cells transfected with pCHIV-mCherry. EGFP-$^{p24}LAMA_{S98}$, pCHIV-mCherry and merge are shown. Viral assembly sites of HIV are visualized as bright puncta in the mCherry channel. Representative images from three independent cell cultures, which gave similar results. Scale bar, 10 µm. (**b**) Kinetics of nuclear EGFP-fluorescence after addition of TMP (10 µM, grey background), TMP was added at time point 0, mean ± s.d. are shown as green line and dashed bar with dark grey fill, from N = 46 cells from three independent cell cultures. Data was fit with a one-phase association curve, shown in black. $t_{1/2}$ is calculated from the 46 individual traces and stated as the mean ± s.e.m..



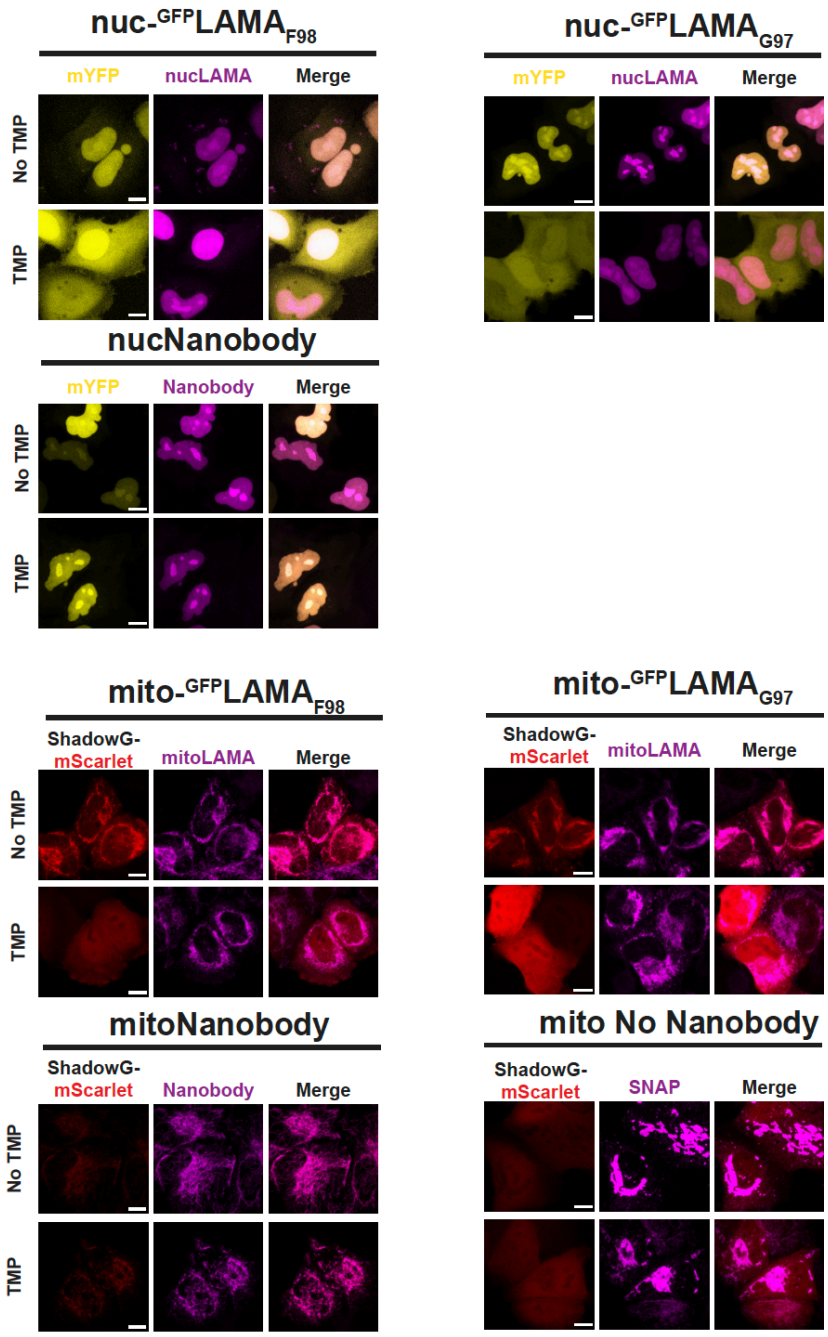
Supplementary Fig. 12. Live-cell imaging of localized GFPLAMAs with EGFP. (**a**) LAMAs anchored to the inner plasma membrane through a Lyn-sequence. EGFP was coexpressed with an IRES with no cellular localization. (**b**) LAMAs anchored to the outer mitochondrial membrane using an Ntom20 sequence. Lyn-and mitoNanobody indicates the enhancer nanobody with no cpDHFR insertion (positive control), but expressed as a SNAP-tag fusion. No Nanobody indicates SNAP-tag anchored with no nanobody or LAMA (negative control). SNAP-tag was imaged after labelling with BG-SiR (500 nM) for visualization purposes. TMP = 10 µM. Scale bar, 10 µm. Representative images from at least 3 independent cell cultures, with similar results.



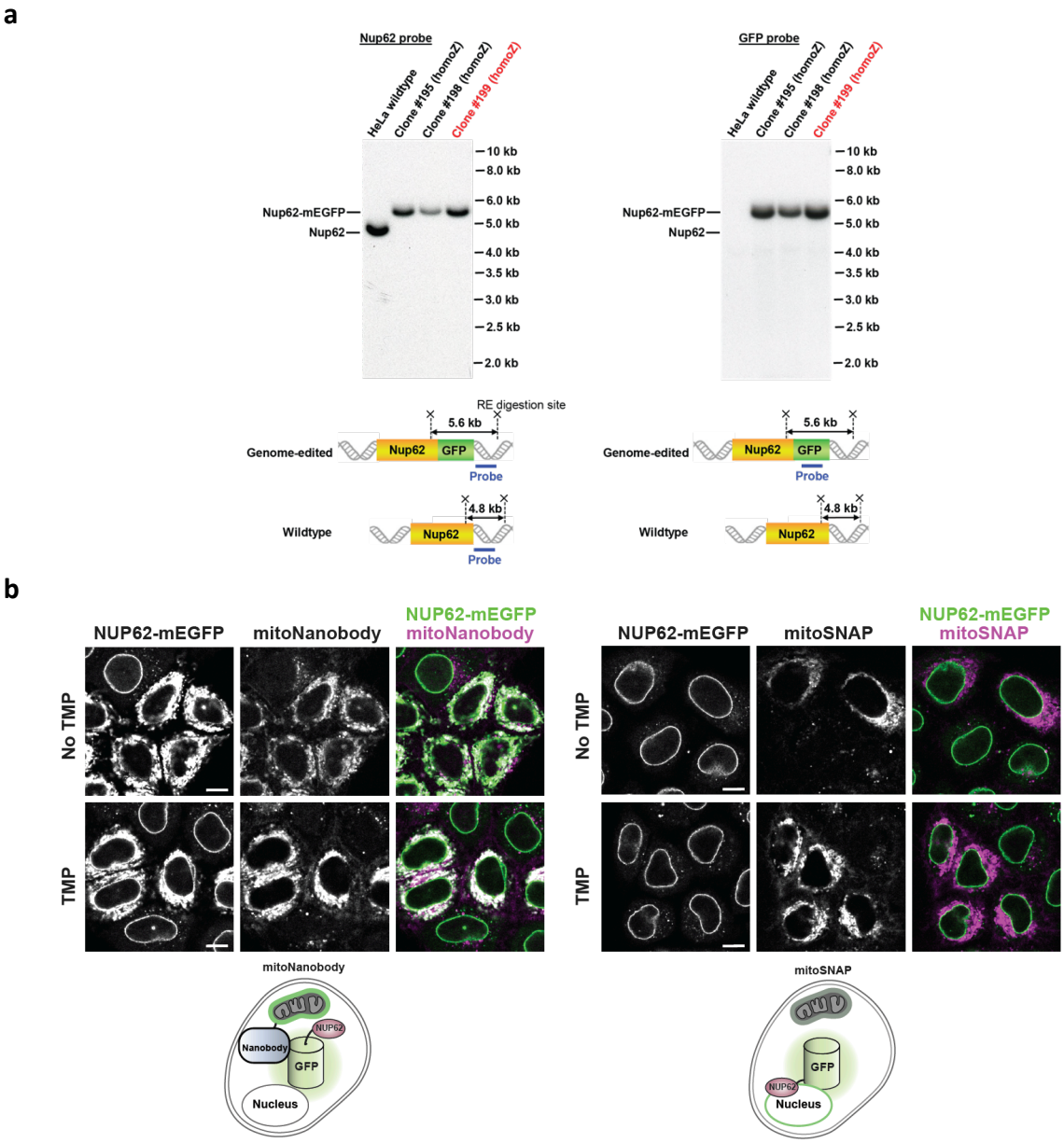
c

	^{GFP}LAMA$_{F98}$		^{GFP}LAMA$_{G97}$	
	$t_{1/2}$ **TMP** (min)	$t_{1/2}$ **Media** (min)	$t_{1/2}$ **TMP** (min)	$t_{1/2}$ **Media** (min)
mito	1.5 ± 0.5	3.4 ± 2.0	3.8 ± 1.3	3.7 ± 1.0
Lyn	1.8 ± 0.6	2.2 ± 0.6	3.5 ± 1.2	3.9 ± 1.1
nuc	1.7 ± 1.2	3.0 ± 0.6	3.0 ± 1.1	4.1 ±1.4

Supplementary Fig. 13. Reversibility of ^{GFP}LAMA in live cells. TMP (10 µM) in complete media (no phenol red) was perfused over the cells in the dark gray indicated time-span. Complete media (no phenol red) was perfused over the cells in the light gray indicated area. Mean (solid black line) ± s.e.m. (grey dashed area) from three independent experiments with similar results. (**a**) ^{GFP}LAMAs anchored to the inner plasma membrane with a Lyn$_{11}$ sequence, and EGFP expressed via an IRES. Fluorescence in the nucleus was measured and normalized to the fluorescent value after perfusion of TMP. For Lyn-^{GFP}LAMA$_{F98}$ N= 13 cells. For Lyn-^{GFP}LAMA$_{G97}$ N= 12 cells. (**b**) ^{GFP}LAMAs with nuclear localization sequence NLS, and EGFP expressed via an IRES. Fluorescence in the cytoplasm was measured and normalized to the fluorescent value after perfusion of TMP. For nuc-^{GFP}LAMA$_{F98}$ N= 11 cells. For nuc-^{GFP}LAMA$_{G97}$ N= 10 cells. (**c**) half time of max nuclear or cytoplasmic fluorescence calculated from perfusions. Each induvial trace was fit and reported as means ± s.d. from all traces three independent experiments. For mito-^{GFP}LAMA$_{F98}$ N=14 cells. For mito-^{GFP}LAMA$_{G97}$ N=9 cells. for other values of N see (**a**) and (**b**).

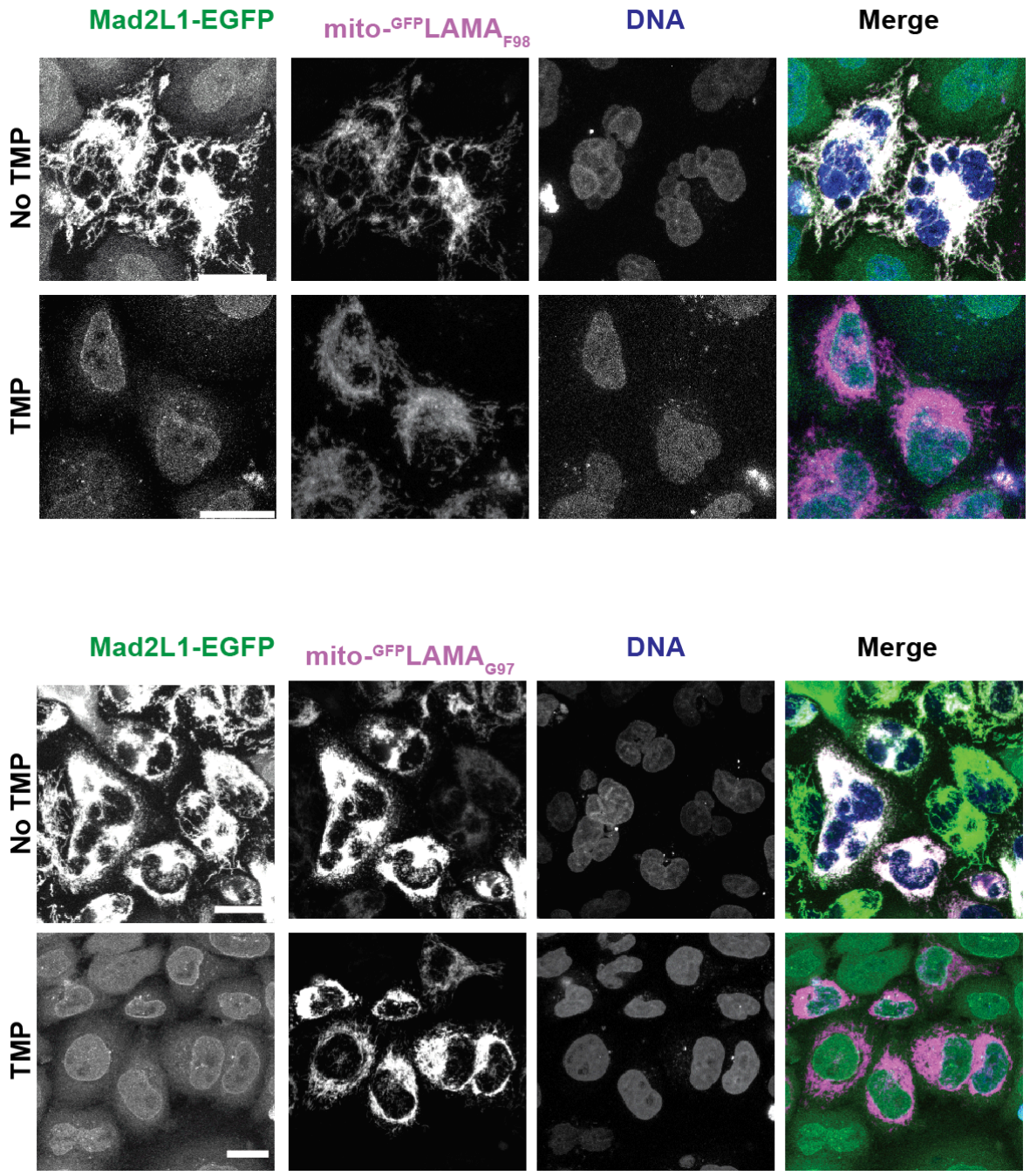


Supplementary Fig. 14. Live cell imaging of GFP-LAMAs extended to YFP and ShadowG-mScarlet. GFPLAMAs with nuclear and mitochondrial localization were co-expressed with YFP or mScarlet-ShadowG. mScarlet was fused to ShadowG for a marker in imaging. nuc- and mitoNanobody indicates the enhancer nanobody with no cpDHFR insertion (positive control)., but expressed as a SNAP-tag fusion. No Nanobody indicates SNAP-tag anchored with no nanobody or LAMA (negative control). SNAP-tag was imaged by labelling with BG-SiR (500 nM) for visualization purposes. TMP = 10 µM. Scale bar, 10 µm. Representative images from at least three independent cell cultures with similar results.

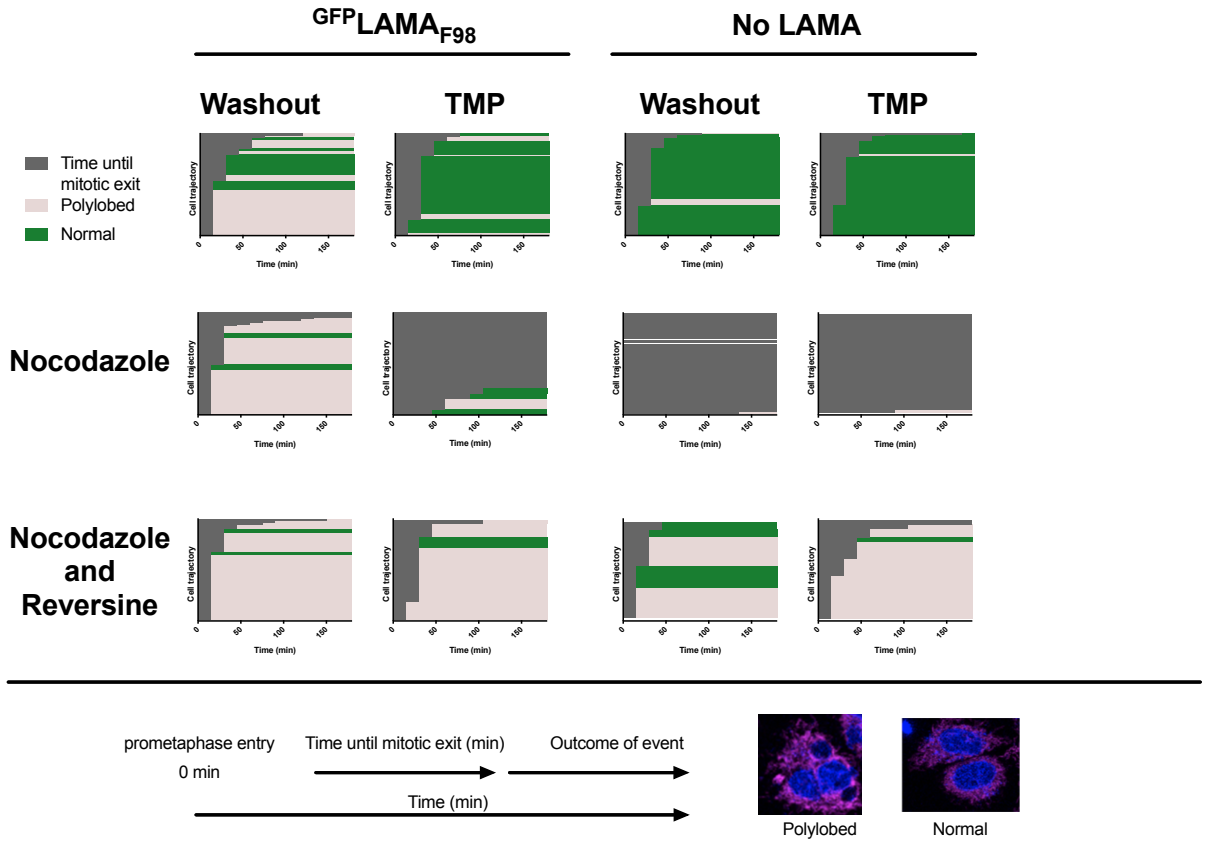


Supplementary Fig. 15. Use of ^{GFP}LAMAs to mislocalize NUP62-mEGFP. (**a**) Homozygousity of NUP62-mEGFP genome edited HeLa Kyoto cell lines were examined by Southern blotting, as previously described by *Koch et al., 2018.* The Southern blotting was performed once . Clone 199 was used for this study. (**b**) NUP62-mEGFP cells transfected with enhancer nanobody only anchored to the outer membrane of the mitochondria (mitoNanobody), or transfected with SNAP-tag anchored to the outer membrane of the mitochondria (mitoSNAP). Labeled with BG-TMR (10 µM) for visualization purposes. Experiments were performed twice independently and the sequestering was reliably reproduced. TMP = 10 µM. Scale bar, 10 µm.

Koch, B. *et al.* Generation and validation of homozygous fluorescent knock-in cells using CRISPR–Cas9 genome editing. *Nat. Protoc.* **13**, 1465-1487 (2018).

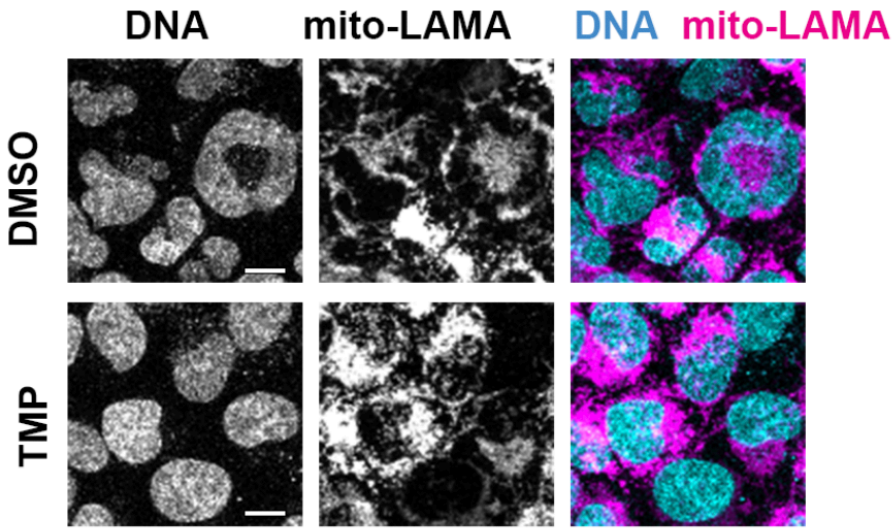


Supplementary Fig. 16. Mislocalization of Mad2L1-EGFP using ^{GFP}LAMAs. Localization of Mad2L1-EGFP in cells transiently transfected with ^{GFP}LAMAs, in the presence of TMP (10 µM), or after 24 hours washout with media containing DMSO. The LAMAs were labelled with BG-SiR (500nM) for visualization purposes, and the DNA stained with Hoechst 33342, to score nuclear morphology. Some non-transfected cells are also seen in the image. TMP = 10 µM. Scale bar, 20 µm. Representative images from 3 independent cell cultures, with similar results.

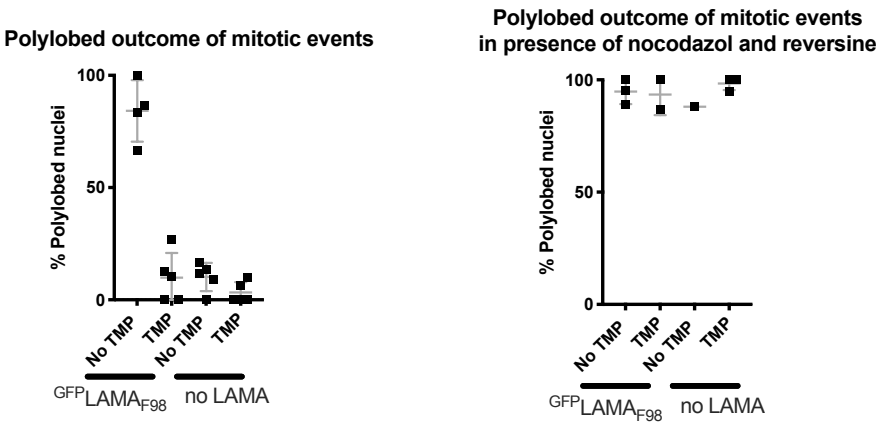


Supplementary Fig. 17. Cellular trajectories of live-cell imaging of Mad2L1-GFP cells stably expressing ^{GFP}mitoLAMA$_{F98}$. Cells were imaged every 15 minutes for 20 hours, and aligned at the point at which prometaphase starts (time point 0). Each event was followed until end of mitosis, either by cytokinesis, or decondensation of DNA. After the mitotic event, the nuclear morphology was scored as either polylobed or normal. The mito-^{GPF}LAMA$_{F98}$ was fused to SNAP-tag and labelled with BG-SiR (100 nM) for visualization purposes. Reversine is an inhibitor of MPS1, which is known to override the mitotic checkpoint complex and mitotic arrest. TMP = 50 µM, nocodazole = 330 nM, reversine = 5 µM. No drug treatment total number of cells are: N = 47, N = 76, N = 79, N =89 cells. Nocodazole treatment total number of cells are: N = 59, N = 19, N = 50, N =27 cells. Nocodazole and reversine treatment: N = 53, N = 29, N = 13, N =23 cells.

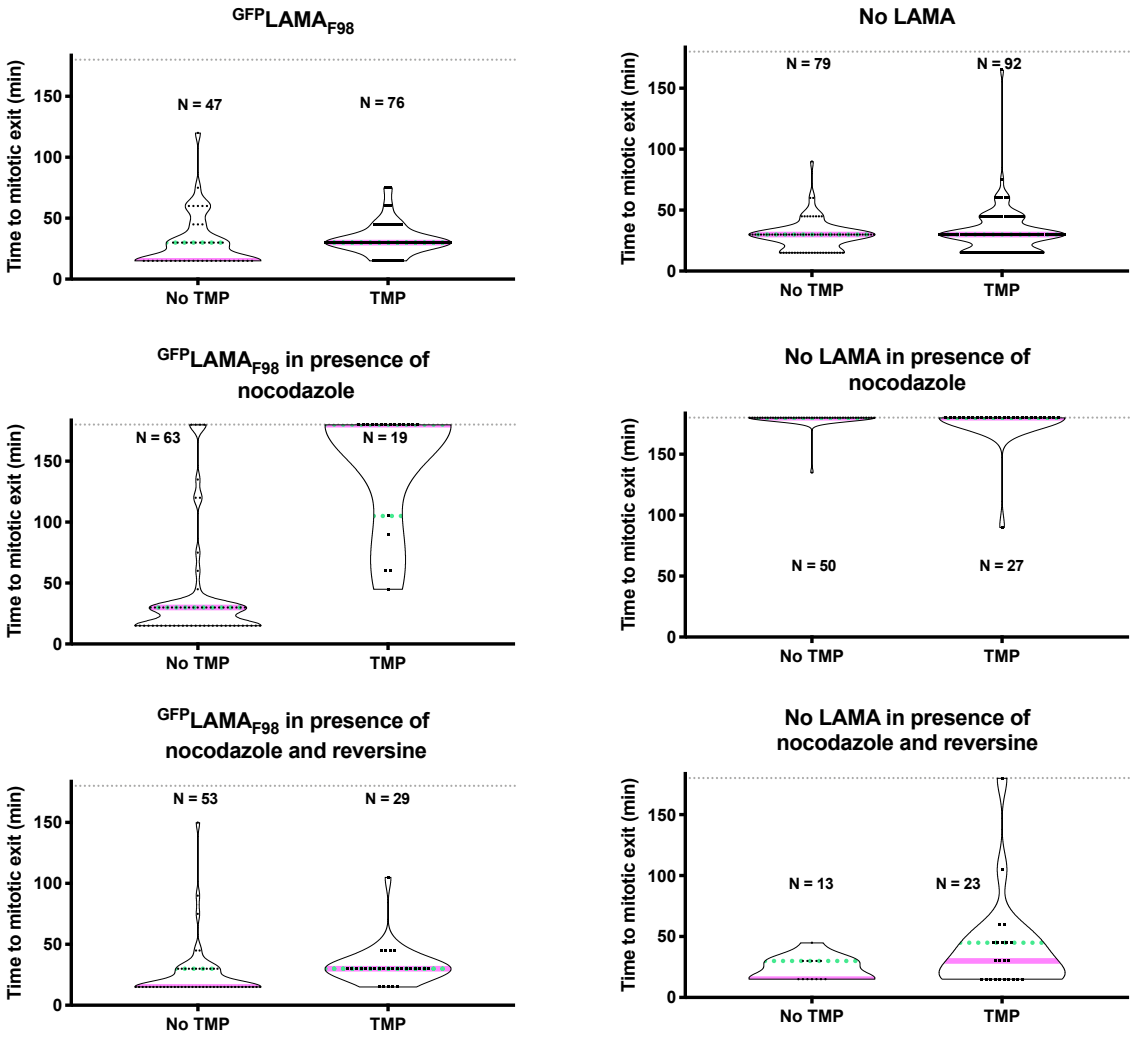
a



b



Supplementary Fig. 18. Nuclear morphology of Mad2L1-EGFP after mislocalization by a ^{GFP}LAMA. (**a**) Nuclear morphology of Mad2L1-EGFP cells stably expressing mito-^{GFP}LAMA$_{F98}$ after 20 hours of live-cell imaging after washout of TMP with media containing DMSO, or cells proliferated in TMP. mito-^{GPF}LAMA$_{F98}$ was labelled on the SNAP-tag with BG-SiR (100 nM) for visualization purposes. Scale bar, 10 µm. (**b**) Nuclear morphology manually scored as percentage of cells with polylobed nuclei after mitotic events during 20 hours of live-cell imaging. Mean ± s.d. from five (in the absence of nocodazole or reversine) or three (in the presence of nocodazole and reversine) independent cell cultures. For cells imaged in the absence of nocodazole and reversine the total number of analyzed cells over five independent experiments was: N = 46, N = 75, N = 47, N=92. For cells imaged in nocodazole and reversine the total number of analyzed cells over five independent experiments was: N = 52, N = 30, N = 13 and N= 23 cells. TMP = 50 µM, nocodazole = 330 nM, reversine = 5 µM.



Supplementary Fig. 19. Duration of mitosis in HeLa Kyoto Mad2L1-EGFP cells stably expressing mito-$^{GFP}LAMA_{F98}$. Cells were kept either in media containing 50 µM TMP (TMP) or without TMP (no TMP) in the presence of the indicated drugs (330 nM nocodazole, 5 µM reversine) for 20 hours. Time lapse imaging was performed every 15 min. Time from prometaphase until mitotic exit in Mad2L1-EGFP cells with mito-GFPLAMAF98 or no LAMA was manually scored. The time of mitosis was cut at 180 min (indicated by grey dotted line). Violin plots of median (magenta) ± interquartile range (green) of time spent in mitosis. Data collated in five (absence of nocodazole or reversine) or three (presence of nocodazole only, or presence of nocodazole and reversine) independent experiments. Total number of cells in each analysis are indicated on each plot.

Supplementary Videos 1-4. Reversibility of ^{GFP}LAMAs in live cells.

Addition and wash-out of TMP in live HeLa Kyoto cells expressing EGFP (green) and ^{GFP}LAMAs in different cellular locations. HeLa Kyoto cells were transiently transfected with pcDNA3.0 constructs expressing $^{GFP}LAMA_{F98}$ or $^{GFP}LAMA_{G97}$ with Ntom20 (outer mitochondrial membrane anchor), Lyn_{11} (first peptide sequence of Lyn, localizing in inner plasma membrane), or NLS (nuclear localization). EGFP was expressed bicistronically using an IRES sequence. TMP (10 µM) was perfused over the cells in complete media (no phenol red) using a syringe pump during continuous acquisition. Complete media (no phenol red) was then perfused over the cells to wash out TMP.

Supplementary Video 1. Reversibility of mito-$^{GFP}LAMA_{F98}$ with EGFP in live cells.
HeLa Kyoto cells expressing mito-$^{GFP}LAMA_{F98}$ IRES EGFP. TMP (10 µM) in complete media was perfused over the cells at 3:38-17:19 min:sec. Complete media was perfused over the cells at 23:24-33:42 min:sec. TMP was again perfused over the cells at 39:49-49:51 min:sec. Complete media was again perfused over the cells at 00:55:56-1:08:05 h:min:sec. Fluorescence of EGFP was imaged (green). Scale bar, 10 µm.

Supplementary Video 2. Reversibility of mito- $^{GFP}LAMA_{G97}$ with EGFP in live cells.
HeLa Kyoto cells expressing mito-$^{GFP}LAMA_{G97}$ IRES EGFP. TMP (10 µM) in complete media was perfused over the cells at 3:38-17:19 min:sec. Complete media was perfused over the cells at 48:01-58:03 min:sec. Fluorescence of EGFP was imaged (green). Scale bar, 10 µm.

Supplementary Video 3. Reversibility of Lyn-$^{GFP}LAMA_{F98}$ with EGFP in live cells.
HeLa Kyoto cells expressing Lyn-$^{GFP}LAMA_{F98}$ IRES EGFP. TMP (10 µM) in complete media was perfused over the cells at 3:38-15:30 min:sec. Complete media was perfused over the cells at 20:40-28:34 min:sec. Fluorescence of EGFP was imaged (green). Scale bar, 10 µm.

Supplementary Video 4. Reversibility of nuc-$^{GFP}LAMA_{F98}$ with EGFP in live cells.
HeLa Kyoto cells expressing nuc-$^{GFP}LAMA_{F98}$ IRES EGFP. TMP (10 µM) in complete media was perfused over the cells at 3:38-17:01 min:sec. Complete media was perfused over the cells at 22:11-31:36 min:sec. Fluorescence of EGFP was imaged (green). Scale bar, 10 µm.

Supplementary Video 5-8
Live-cell imaging of Mad2L1-GFP expressing a mito-LAMA.
Live cell imaging of a HeLa Kyoto genome edited to Mad2L1-EGFP cell line, and stably expressing mito-$^{GFP}LAMA_{F98}$ (Video 5, 6) or no LAMA, but expressing mito-SNAP-tag (Video 7, 8).The mitochondrially anchored protein constructs were expressed as a SNAP-tag fusion, continuously labeled with BG-SiR (100 nM) (magenta) to follow expressing cells and for autofocusing purposes. Nuclear morphology was visualized with Hoechst 33342 (blue). Transmission images were also recorded to visualize overall cell morphology (grey). The cells had been grown in the presence of TMP (50 µM), which was washed out at time point 0, or kept during the experiment.

Supplementary Video 5. Mitosis in a genome edited Mad2L1-EGFP cell line with mito-$^{GFP}LAMA_{F98}$ in the absence of TMP.
Genome edited Mad2L1-EGFP cells stably expressing SNAP-tagged mito-$^{GFP}LAMA_{F98}$. TMP (50 µM) was washed out at time point 0. Nucleus (blue), mito-$^{GFP}LAMA_{F98}$ (magenta) and transmission image (grey). Scale bar, 20 µm.

Supplementary Video 6. Mitosis in a genome edited Mad2L1-EGFP cell line with mito-$^{GFP}LAMA_{F98}$ in the presence of TMP.
Genome edited Mad2L1-EGFP cells stably expressing SNAP-tagged mito-$^{GFP}LAMA_{F98}$ and kept in TMP (50 µM) during imaging. Nucleus (blue), mito-$^{GFP}LAMA_{F98}$ (magenta) and transmission image (grey). Scale bar, 20 µm.

Supplementary Video 7. Mitosis in a genome edited Mad2L1-EGFP cell line with mito-SNAP-tag in the absence of TMP.
Genome edited Mad2L1-EGFP cells stably expressing SNAP-tag on the outer membrane of the mitochondria. TMP (50 µM) was washed out at time point 0. Nucleus (blue), mito-SNAP-tag (magenta) and transmission image (grey). Scale bar, 20 µm.

Supplementary Video 8. Mitosis in a genome edited Mad2L1-EGFP cell line with mito-SNAP-tag in the presence of TMP.
Genome edited Mad2L1-EGFP cells stably expressing SNAP-tag on the outer membrane of the mitochondria and kept in TMP (50 µM) during imaging. Nucleus (blue), mito-SNAP-tag (magenta) and transmission image (grey). Scale bar, 20 µm.

Supplementary Table 1. X-ray data collection and refinement statistics

	^{GFP}LAMA$_{F98}$ (PDB ID: 6RUL)	^{GFP}LAMA$_{G97}$ (PDB ID: 6RUM)
Data collection		
Space group	$P6_2$	$P12_11$
Cell dimensions		
a, b, c (Å)	86.6, 86.6, 73.0	47.9, 56.6, 53.3
α, β, γ (°)	90.00, 90.00, 120.00	90.00, 94.91, 90.00
Resolution (Å)	50-2.2 (2.3-2.2)*	50-1.6 (1.7-1.6)*
No. unique reflections	15911 (1970)*	37239 (6123)*
R_{merge}	0.058 (0.453)*	0.054 (0.490)*
$I/\sigma I$	22.6 (5.3)*	13.2 (2.4)*
Completeness (%)	99.9 (99.9)*	99.1 (98.6)*
Redundancy	10.1 (10.4)*	3.3 (3.2)*
Wilson B (Å^2)	50.7	26.1
Refinement		
Molecules per a.u.	1	1
Resolution (Å)	43.3-2.2	47.7-1.6
No. unique reflections	15910	37239
R_{work}/R_{free}	0.1747/0.2195	0.1830/0.2275
No. atoms	2298	2574
Protein	2167	2187
Ligand/ion	79	92
Water	52	295
B-factors	46.3	23.1
Protein	46.5	21.8
Ligand/ion	39.1	20.9
Water	46.6	33.8
R.m.s. deviations		
Bond lengths (Å)	0.008	0.006
Bond angles (°)	1.068	0.957
Ramachandran		
Favoured (%)	98.5	99.3
Outliers (%)	0	0
Clashscore	5.7	5.6

*Values in parentheses are for highest-resolution shell.

Supplementary Table 2. List of transfection plasmids used in main figures

Name	Expressed construct	Vector
wtGFP	His(x10)-TEVsite-wtGFP	pET51b(+)
^{GFP}LAMA$_{N95}$	His(x10)-TEVsite-^{GFP}LAMA$_{N95}$	pET51b(+)
^{GFP}LAMA$_{F98}$	His(x10)-TEVsite-^{GFP}LAMA$_{F98}$	pET51b(+)
^{GFP}LAMA$_{G97}$	His(x10)-TEVsite-^{GFP}LAMA$_{G97}$	pET51b(+)
SNAP-tag-p24	Strep-tag®II-SNAP-tag-p24(full)-His(x10)	pET51b(+)
EGFP-^{p24}LAMA$_{S98}$	Strep-tag®II-EGFP-^{p24}LAMA$_{S98}$-His(x10)	pET51b(+)
ALFA-tag-SNAP-tag	Strep-tag®II-ALFA-tag-SNAP-tag-His(x10)	pET51b(+)
$^{ALFA\text{-}tag}$LAMA$_{R59}$	Strep-tag®II-EGFP-$^{ALFA\text{-}tag}$LAMA$_{S98}$-His(x10)	pET51b(+)
EGFP-^{p24}LAMA$_{S98}$	EGFP-^{p24}LAMA$_{S98}$ IRES Puro	pWPI
pCHIV	HIV gag-mCherry without LTRs, Nef and Env	pcDNA3.1(Zeo)
pCHIV	HIV without LTRs, Nef and Env	pcDNA3.1(Zeo)
mito^{GFP}LAMA$_{F98}$ IRES EGFP	Ntom20-SNAP-tag-^{GFP}LAMA$_{F98}$-IRES EGFP	pcDNA3.0
mito^{GFP}LAMA$_{G97}$ IRES EGFP	Ntom20-SNAP-tag-^{GFP}LAMA$_{G97}$ IRES EGFP	pcDNA3.0
Lyn^{GFP}LAMA$_{F98}$ IRES EGFP	Lyn$_{11}$-SNAP-tag-^{GFP}LAMA$_{F98}$ IRES EGFP	pcDNA3.0
Lyn^{GFP}LAMA$_{G97}$ IRES EGFP	Lyn$_{11}$-SNAP-tag-^{GFP}LAMA$_{G97}$-IRES EGFP	pcDNA3.0
nuc^{GFP}LAMA$_{F98}$ IRES EGFP	SNAP-tag-^{GFP}LAMA$_{F98}$-NLS IRES EGFP	pcDNA3.0
nuc^{GFP}LAMA$_{G97}$ IRES EGFP	SNAP-tag-^{GFP}LAMA$_{G97}$-NLS IRES EGFP	pcDNA3.0
mito^{GFP}LAMA$_{F98}$	Ntom20-SNAP-tag-^{GFP}LAMA$_{F98}$	pcDNA3.0
mito^{GFP}LAMA$_{G97}$	Ntom20-SNAP-tag-^{GFP}LAMA$_{G97}$	pcDNA3.0

Amino acid sequences of LAMAs used:

Color guide:
Enhancer nanobody
cpDHFRL24G5
cb9 nanobody
NbALFA

^{GFP}LAMA$_{F98}$
MAQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREWVAGMSSAGDRSSYEDSV
KGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFLPADLAWFKRNTLNKPVIMGRHTWESIGRP
LPGRKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGDT
HFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERRGGGGGMISLIAALAVDRVIGMENAMPWNEYW
GQGTQVTVSS

^{GFP}LAMA$_{G97}$
MAQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREWVAGMSSAGDRSSYEDSV
KGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGLPADLAWFKRNTLNKPVIMGRHTWESIGRPL
PGRKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGDTH
FPDYEPDDWESVFSEFHDADAQNSHSYCFEILERRGGGGGMISLIAALAVDRVIGMENAMPWNFEYW
GQGTQVTVSS

^{GFP}LAMA$_{N95}$
MAQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREWVAGMSSAGDRSSYEDSV
KGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNLPADLAWFKRNTLNKPVIMGRHTWESIGRPLPG
RKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGDTHFP
DYEPDDWESVFSEFHDADAQNSHSYCFEILERRGGGGGMISLIAALAVDRVIGMENAMPWNVGFEYW
GQGTQVTVSS

^{P24}LAMA$_{S98}$
MAQVQLVESGGGLVQAGGSLRLSCAASGSFFMSNVMAWYRQAPGKARELIAAIRGGDMSTVYDDSV
KGRFTITRDDDKNILYLQMNDLKPEDTAMYYCKASGSSLPADLAWFKRNTLNKPVIMGRHTWESIGRPL
PGRKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGDTH
FPDYEPDDWESVFSEFHDADAQNSHSYCFEILERRGGGGGMISLIAALAVDRVIGMENAMPWNWGQ
GTQVTVSS

$^{ALFA\text{-}tag}$LAMA$_{R59}$
EVQLQESGGGLVQPGGSLRLSCTASGVTISALNAMAMGWYRQAPGERRVMVAAVSERLPADLAWFK
RNTLNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMVIGGGRVYE
QFLPKAQKLYLTHIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERRGGGGGMISLIAA
LAVDRVIGMENAMPWNGNAMYRESVQGRFTVTRDFTNKMVSLQMDNLKPEDTAVYYCHVLEDRVD
SFHDYWGQGTQVTVSS