Chromobodies to Quantify Changes of Endogenous Protein Concentration in Living Cells

Authors
Bettina-Maria Keller, Julia Maier, Kathy-Ann Secker, Stefanie-Maria Egetemaier, Yana Parfyonova, Ulrich Rothbauer, and Bjoern Traenkle

Correspondence
bjoern.traenkle@nmi.de; ulrich.rothbauer@uni-tuebingen.de

In Brief
Understanding cellular processes requires the determination of dynamic changes in the concentration of endogenous proteins. We demonstrate the dependency of the intracellular level of chromobodies (CB, fluorescently labeled nanobodies) on the amount of their endogenous antigens and present a broadly applicable strategy how to employ turnover-accelerating CBs, to quantify dynamic changes of endogenous protein levels by quantitative live-cell imaging. This will enable unprecedented insights into the dynamic regulation of proteins, e.g. during cellular signaling, cell differentiation, or upon drug action.

Highlights
- Chromobodies are stabilized by antigen binding in live cells.
- Monitoring changes of endogenous protein levels in living cells with chromobodies.
- Broadly applicable system to generate turnover-accelerated chromobodies.
- Quantification of time- and dose-dependent compound effects.
Chromobodies to Quantify Changes of Endogenous Protein Concentration in Living Cells*

Bettina-Maria Keller‡, Julia Maier‡, Kathy-Ann Secker‡, Stefanie-Maria Egetemaier‡, Yana Parfyonova‡, Ulrich Rothbauer‡§, and Bjoern Traenkle‡¶

Understanding cellular processes requires the determination of dynamic changes in the concentration of genetically nonmodified, endogenous proteins, which, to date, is commonly accomplished by end-point assays in vitro. Molecular probes such as fluorescently labeled nanobodies (chromobodies, CBs) are powerful tools to visualize the dynamic subcellular localization of endogenous proteins in living cells. Here, we employed the dependence of intracellular levels of chromobodies on the amount of their endogenous antigens, a phenomenon, which we termed antigen-mediated CB stabilization (AMCBS), for simultaneous monitoring of time-resolved changes in the concentration and localization of native proteins. To improve the dynamic range of AMCBS we generated turnover-accelerated CBs and demonstrated their application in visualization and quantification of fast reversible changes in antigen concentration upon compound treatment by quantitative live-cell imaging. We expect that this broadly applicable strategy will enable unprecedented insights into the dynamic regulation of proteins, e.g., during cellular signaling, cell differentiation, or upon drug action. Molecular & Cellular Proteomics 17: 2518–2533, 2018. DOI: 10.1074/mcp.TIR118.000914.

Several methods are available to detect changes in the concentration of specific proteins in biological samples. The rise of mass spectrometry (MS)-based analysis has enabled relative and absolute quantification of proteins with unprecedented sensitivity and accuracy (1, 2). Considering that MS-based quantification requires expensive equipment and trained personnel, this technology is mostly applied for large-scale proteomic analyses. Because of the ever-growing availability of specific antibodies, antibody-based techniques such as enzyme-linked immunosorbent assay (ELISA) or immunoblotting are commonly used to analyze relative concentration changes of single proteins of interest (POIs)1. However, the informative value of these methods is limited because only average protein amounts are determined and no intercellular resolution is provided. In addition, tracing changes in protein concentration over time is very laborious and time-consuming. Alternatively, immunofluorescence (IF) can be used to assess the subcellular localization and the relative concentration of POIs on single-cell level. Like any immunodetection method, this can suffer from inaccuracies based on batch-to-batch antibody variability, epitope inaccessibility and cross reactivity (3). In addition, because of cell fixation and permeabilization procedures, IF allows no direct analysis of dynamic changes (4). Considering that most cellular processes are dynamic in nature and rely on the spatiotemporal orchestration under native conditions, the assessment of time-dependent changes of endogenous protein levels within the physiological environment of living cells is preferable.

With the rise of genome editing techniques, fluorescent protein (FP) tagging of endogenous proteins provides a straightforward approach to optically monitor the relative amount of a POI (5). However, as repeatedly described, FP tagging can interfere with crucial protein parameters such as turnover, subcellular localization, and participation in multi-protein complexes (6–8). During the last decade, intrabodies have emerged as beneficial tools to study the dynamic behavior of endogenous proteins in various cellular models. Because of their compact structure, small size, high stability and solubility, single-domain antibody fragments from camels (VHH, nanobodies) possess many advantageous properties to be employed within living cells (9–11). Acknowledging the potential of these binding molecules, numerous protocols and synthetic nanobody libraries for targeted selection of intracellularly functional nanobodies have been developed (12–14). By fusing nanobodies (NBs) to fluorescent proteins, so-called chromobodies (CBs) are generated. Upon cellular expression, they allow optical detection of endogenous proteins in live cells. With regard to imaging purposes transiently binding CBs addressing functionally inert epitopes are preferable to avoid unwanted effects on antigen mobility.
or by displacing natural interaction partners. To date, multiple target-specific CBs have been applied to visualize cellular processes in cultured cells and entire organisms without functional interference (15–20).

Recently, we have generated a CB (BC1-TagGFP2), which specifically targets the soluble, nonmembrane-associated fraction of endogenous β-catenin (CTNNB1) without affecting its transcriptional activity. By live-cell imaging of cells stably expressing BC1-TagGFP2 we observed an increased CB signal along with an elevation of intracellular CTNNB1 level upon compound-mediated induction of the WNT/β-catenin pathway (21). Notably, this was not because of altered transcription of the CB but attributed to a yet unexplained mechanism of antigen-mediated stabilization on protein level, which is in accordance to previous findings describing higher levels of bacterially injected NBs within the cytoplasm of mammalian cells in the presence of their cognate antigen (22).

Here, we demonstrate that antigen-mediated CB stabilization (AMCBS) is applicable for numerous CBs by showing this phenomenon for four different CBs targeting unrelated endogenous and nonendogenous antigens. To adapt CBs for monitoring changes in antigen concentration more precisely, we screened for N-terminal amino acids, which induce accelerated CB turnover. Based on our findings, we generated highly antigen-responsive CBs. As exemplarily shown for CTNNB1-specific CBs, stable chromobody cell lines allow visualization of rapid and reversible changes in the concentration of endogenous proteins upon compound treatment by quantitative live-cell imaging.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—All oligonucleotide sequences used in this study for DNA amplification are listed in supplemental Table S1. All expression constructs and cell lines used in this study are listed in supplemental Table S2.

The expression construct coding for Ubiquitin-Methionine-BC1-TagGFP2 (hereafter referred as Ub-M-BC1-TagGFP2, Fig. 3B) was generated by Gibson assembly cloning (23) of the following five fragments: fragment 1 - eGFP-N1 vector backbone digested with NheI and XbaI, fragment 2 - ubiquitin amplified from Ub-M-eGFP, a gift from Nico Dantuma (plasmid # 11938, Addgene, Cambridge, MA) (24), with the primer set ubiquitin-for and ubiquitin-rev, fragment 3 - BC1-NB amplified from the BC1-CB expression construct described in (21) with the primer set BC1-for and BC1-rev, fragment 4 - TagGFP2 amplified from the BC1-CB plasmid described in (21) with the primer set TagGFP2-for and TagGFP2-rev. Fragments were assembled using the Gibson-Assembly Master Mix (New England Biolabs GmbH, Frankfurt, Germany) according to the manufacturer’s protocol. The corresponding N-terminal mutants of the Ub-M-BC1-TagGFP2 expression construct were generated by site-directed mutagenesis of the Ub-M-BC1-TagGFP2 plasmid with the primer pair NB-N-term-X-mut-for (X represents the corresponding N-terminal amino acid) and NB-N-term-rev using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer’s protocol. The noncleavable mutant Ub_{G76V}-M-BC1-TagGFP2 was produced by site-directed mutagenesis using the primer set BC1-for and UB-G76V-mut-rev. Ub-M-BC1-TagGFP2 comprising a (G4S)_2 linker between BC1-NB and TagGFP2 was generated by Gibson assembly as described, with the following exception: TagGFP2 was amplified using the primer set TagGFP2-2GS-for and TagGFP2-rev. For molecular cloning of the Ub-M-BC1-TagGFP2 construct containing the longer linker (G4S)_2, Gibson assembly of the following five fragments was performed: fragment 1 - eGFP-N1 vector backbone, and fragment 2 - ubiquitin were amplified as described above. Fragment 3 - BC1-NB was amplified from the BC1-CB expression construct with the primer set BC1-for and BC1-4GS-rev, fragment 4 - TagGFP2 was amplified from the BC1-TagGFP2 plasmid with the primer set TagGFP2-4GS-for and TagGFP2-rev, fragment 5 - (G4S)_2 linker was produced by annealing of the two single stranded oligonucleotides 4GS-linker-oligo1 and 4GS-linker-oligo2 with complementary sequences. Fragments were assembled using the Gibson-Assembly Master Mix (New England Biolabs). To replace TagGFP2 within the Ub-X-BC1-TagGFP2 constructs by eGFP, TagRFP and mCherry, eGFP was amplified from the VB6-eGFP plasmid (19) with the primers eGFP-for and eGFP-rev, TagRFP was amplified from the POCA-CB-TagRFP plasmid (18) (kindly provided by ChromoTek GmbH, Planegg-Martinsried, Germany) using the primers TagRFP-for and TagRFP-rev. In case of mCherry, first a silent mutation was introduced to eliminate the PstI restriction site within the mCherry coding region in pmCherry-C1 plasmid (EGFP substituted by mCherry in pEGFP-C1, Clontech, Takara Bio Europe SAS, Saint-Germain-en-Laye, France) (25) using primer set mCherry-silent-mut-for and mCherry-silent-mut-rev. Subsequently, mCherry carrying a PstI silent mutation was amplified with the primers mCherry-for and mCherry-rev. The amplified DNA fragments encoding eGFP, TagRFP and mCherry were digested with BamH I and XbaI, and ligated into (1) Ub-M-BC1-TagGFP2, (2) Ub-F-BC1-TagGFP2, (3) Ub-A-BC1-TagGFP2, (4) Ub-R-BC1-TagGFP2, (5) Ub-S-BC1-TagGFP2 expression constructs by compatible sticky ends (Fig. 3B). PstI and BspEI restriction sites were used to replace the NC1-NB with eCA-NB or VB6-NB within the generated Ub-X-BC1-CB expression constructs (Fig. 3B). Corresponding nonbacteriophage sequences were amplified using the primers NB-univ-for and NB-univ-rev. VB6-NB was amplified from VB6-eGFP expression construct described in (19), eCA-NB was amplified from eCA-TagRFP described in (16). Destabilizing nanobody framework mutations BC1_{S70R}, BC1_{C92Y} and BC1_{S113F} were introduced by site-directed mutagenesis of the BC1-TagGFP2 expression construct using the primers BC1-NB-S70R-for and BC1-NB-S70R-rev, BC1-NB-C92Y-for and BC1-NB-C92Y-rev, BC1-NB-S113F-for and BC1-NB-S113F-rev, respectively. The fusion construct coding for N-terminally mCherry-tagged CTNNB1 (mCherry-CTNNB1) was generated by amplification of CTNNB1 from eGFP-CTNNB1 (21) using primer set mCherry-CTNNB1-for and mCherry-CTNNB1-rev. The amplified DNA fragment was purified and ligated into KpnI and BamHI restriction site of the empty backbone vector carrying mCherry (pm-
electrophoresis (SDS-PAGE) was performed according to standard
protein assay (Bio-Rad Laboratories GmbH, Munich, Germany)
concentrations of the soluble supernatant were determined by Brad-
gmbH, Wiesbaden-Nordenstadt, Germany) dissolved in ddH2O or
ersham Biosciences, GE Healthcare).
blotting, proteins were transferred onto nitrocellulose membrane (Am-
ethanol, 10% (v/v) glycerol, 0.02% bromphenole blue). For Western
Scientific).
CUA Att-3
AAA UUU UCU GCA Att-3
Schwerte, Germany) and cultivated for 24 h. In the following, cells
with the following sequences (one strand): 5′-GUC AUG ACC UUG AAC
GCA Att-3′ (sivM1), 5′-GGU UAG CC ACU CAA Att-3′ (sivM2), 5′-GAG GGA AAC UAU GCC GAA UTT-3′ (sivM3). For RNA
interference-mediated knockdown of PCNA we used siRNA duplexes
(TermoFisher Scientific) with the following sequences (one strand): 5′-CGU AUA UGC GCA GAU CUC ATT-3′ (siPCNA1), 5′-GGU GAG
AAA UUU UCU GCC GAA Att-3′ (siPCNA2), 5′-GGA GAU AUC ACA
CJA Att-3′ (siPCNA3). As control siRNAs we used Silencer™ Select
Negative Control No. 1 (siCTRL1) and No. 2 (siCTRL2) (TermoFisher
Scientific).

SDS-PAGE and Western Blotting—7.5 × 10⁵ - 2 × 10⁶ HeLa cells were seeded in 100 mm cell culture dishes (Corning, Fisher Scientific,
Schwerte, Germany) and cultivated for 24 h. In the following, cells
were either treated with 10 μM CHIR99021 (Tocris, Bio-Techne
GmbH, Wiesbaden-Nordenstadt, Germany) dissolved in ddH₂O or
subjected to plasmid DNA transfection. Subsequently, cells were
washed and harvested in PBS, snap-frozen in liquid nitrogen and
stored at −20 °C. Cell pellets were homogenized in 100 μl NP40 lysis
buffer (10 mM Tris/HCl, 150 mM NaCl, 0.5 mM EDTA; 0.5% NP40, 1
mM PMSF, 1 μg/ml DNasel, 2.5 mM MgCl₂, 1 protease inhibitor mix
M (SERVA Electrophoresis GmbH, Heidelberg, Germany)) by
repeated pipetting and vortexing for 60 min on ice. Lysates were
clarified by centrifugation for 15 min at 18,000 × g and 4 °C. Protein
concentrations of the soluble supernatant were determined by Brad-
ford Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany)
and adjusted to equal concentrations. Denaturing polyacrylamide
gel electrophoresis (SDS-PAGE) was performed according to standard
procedures. Protein samples were boiled in 2 x reducing SDS-sample
buffer (60 mM Tris/HCi, pH 6.8, 2% (v/v) SDS, 5% (v/v) 2-mercapto-
ethanol, 10% (v/v) glycerol, 0.02% bromphenol blue). For Western
blotting, proteins were transferred onto nitrocellulose membrane (Am-
erness Biosciences, GE Healthcare).

Intracellular Immunoprecipitation (IC-IP)—3 × 10⁶ HEK293T cells
were transiently transfected with equal amounts of expression vec-
tors encoding for GFP-CA, BC1-CB, destabilized versions of BC1-CB
or eGFP. In case of detection of endogenous CTNNB1 8 h after
plasmid transfection cells were treated with 10 μM CHIR99021 for
16 h. Transfection efficiency was controlled by fluorescence micro-
copy and cells were harvested 24 h after transfection. Cell pellets
were lysed as described and GFP-CA, BC1-CBs or eGFP were precip-
itated using the GFP-Trap (ChromoTek) according to the manufa-
ufacturer’s protocol. Input and bound fractions were subjected to
SDS-PAGE followed by Western blot analysis using anti-RFP, anti-
CTNNB1 and anti-GFP antibodies.

Cell Culture, Transfection, Stable Cell Line Generation and
Compound Treatment—The HeLa Kyoto cells (Cellosoaurus no.
CVCL_1922) were obtained from S. Narumiya (Kyoto University, Ja-
p), and the HEK293T, A549, U2OS cell lines were obtained from
ATCC, LGC Standards GmbH, Wesel, Germany (CRL3216, CCL-185,
HTB-96). All cell lines were tested negative for mycoplasma using the
PCR mycoplasma kit Venor GeM Classic (Minerva Bioslabs GmbH,
Berlin, Germany) and the TaqDNA polymerase (Minerva Bioslabs).
Because this study does not include cell line-specific analysis, all cell
lines were used without additional authentication. All cell lines were
cultivated according to standard protocols. Briefly, growth media
containing DMEM (high glucose, pyruvate, ThermoFisher Scientific)
for HeLa Kyoto, HEK293T and U2OS cells, or DMEM/F-12 (high
glucose, pyruvate, ThermoFisher Scientific) for A549 cells supple-
mented with 10% (v/v) fetal bovine serum (FCS, ThermoFisher Sci-
entific), l-glutamine (ThermoFisher Scientific) and penicillin-strepto-
mycin (ThermoFisher Scientific) were used for cultivation. Cells
were passaged using 0.05% trypsin-EDTA (ThermoFisher Scientific)
and were cultivated at 37 °C in a humidified chamber with a 5% CO₂
atmosphere. Plasmid DNA was transfected with Lipofectamine 2000
(ThermoFisher Scientific) in U2OS cells, Lipofectamine LTX (Thermo-
Fisher Scientific) for A549 cells, or polyethylenimine (PEI, Sigma Al-
drich Chemie GmbH, Munich, Germany) for HeLa Kyoto and
HEK293T cells. Prior to transfection, cells were allowed to grow to
70% confluency. To generate DNA/PEI complexes for transfection
in a 96-well plate format 100 - 200 ng DNA were mixed with 0.5 μg
PEI in 20 μl serum-free medium. Transfection mixture was incubated
for 15 min at room temperature and subsequently was added to the
adherent cells. Transfections using Lipofectamine 2000 or Lipoi-
fectamine LTX were carried out according to the manufacturer’s
protocol. RNA interference-mediated knockdown of PCNA and vi-
mentin was accomplished using Lipofectamine RNAiMax (Thermo-
Fisher Scientific) according to manufacturer’s instructions. Analysis
of siRNA-mediated knockdown occurred 72 h post transfection. Stable
HeLa BC1-TagGFP2 cell line (21) was maintained in medium supple-
mented with 3 μg/ml Blasticidin (Sigma) and stable A549, VB6-eGFP
(19) with 80 μg/ml Hygromycin (Carl Roth GmbH, Karlsruhe, Ger-
many). Stable HeLa CCC-TagRFP (18), stable HeLa_vGA-mCherry
(16), stable HeLa-Ub-R-BC1-eGFP were maintained in media supple-
mented with 0.5 mg/ml G418 (Roth). For the generation of the stable
cell lines HeLa_Ub-R-BC1-eGFP and U2OS_Ub-R-BC1-eGFP plas-
mid transfection of the indicated chromobody construct was per-
fomed using Lipofectamine 2000 (ThermoFisher Scientific) according
to the manufacturer’s protocol. 24 h post transfection cells were
subjected to a three-week selection period using 0.5 mg/ml G418
(Roth) followed by single cell separation. Single clones were analyzed
regarding the level of Ub-R-BC1-eGFP expression, respectively.
Compound treatment with 10 μM CHIR99021 (Tocris) was performed
for 16 h, with 100 μg/ml cycloheximide (Sigma) for 6 h, with 5 ng/ml
TGF-β (PeproTech Germany, Hamburg, Germany) for up to 72 h, with
10 μM MG132 (Calbiochem) and 10 μM NH₄Cl (Roth) for up to 10 h.
For dose-response experiments cells were treated with concentra-
tions from 0.1 μM to 10 μM CHIR99021 and 6-bromoindirubin-3′-oxime
(BIO, Calbiochem, Merck KGaA, Darmstadt, Germany) for up to 42 h.

Immunofluorescence—For immunofluorescence, HeLa cells (pa-
rental and stable CB-expressing cells) were seeded at 2000–4000
cells per well in 0.1 % Clear 96-well plates (Greiner Bio-One GmbH,
Frickenhausen, Germany) and cultured for 24–48 h. For fixation, cells
were washed twice with PBS and were fixed with 4% formaldehyde
(v/v, PFA, AppliChem GmbH, Darmstadt, Germany) in PBS. Subse-
sequently, cells were blocked and permeabilized using 5% bovine se-
rum albumin (w/v, BSA, Roth) and 0.3% Triton X-100 (v/v, Roth) in
PBS. Primary and fluorochrome-conjugated secondary antibodies
were diluted in 1% BSA and 0.3% Triton X-100 in PBS and antibody
staining was carried out according to standard procedures. Fluorescence images were acquired using MetaXpress Micro XL system (Molecular Devices, San Jose, CA) and 20× or 40× magnification.

**Image Segmentation and Analysis**—Depending on experimental setting and cell line, 1.5 × 10^5–1 × 10^4 cells were plated per well in a black μClear 96-well plate (Greiner). Images were acquired with an ImageXpress micro XL system (Molecular Devices) and analyzed by MetaXpress software (64 bit, 6.2.3.733, Molecular Devices). Fluorescence images comprising a statistically relevant number of cells were acquired for each condition. For quantitative fluorescence analyses, the mean fluorescence in a defined (segmented) area of interest (e.g., whole cell or nucleus) was determined. Using the Custom Module Editor (version 2.5.13.3) of the MetaXpress software, we established an image segmentation algorithm that identifies areas of interest based on parameters of size, shape, and fluorescence intensity above local background. For nuclear segmentation, nuclei in fixed cells were stained with 0.02 μg/ml 4’,6-diamidino-2-phenylindole (DAPI, Sigma) whereas live cells were continuously incubated with 2 μg/ml Hoechst33258 (Sigma). To segment the whole cell including the nucleus as well as the cytosolic compartment, the ectopically expressed antigen or its respective control was used to generate the corresponding segmentation mask (supplemental Fig. S1). The average fluorescence intensities in whole cells or nuclei were determined for each image following subtraction of background fluorescence. From these values the mean fluorescence and standard errors were calculated for three independent replicates and student’s t test was used for statistical analysis.

**RESULTS**

**Quantitative Image Analysis of Antigen-mediated Stabilization of a CTNNB1-specific CB**—Previously, we have shown that an increase of endogenous CTNNB1 upon treatment with the GSK3-β inhibitor CHIR99021 (CHIR) was accompanied by rising levels of a CTNNB1-specific CB stably integrated and constitutively expressed in HeLa cells (cell line hereafter referred to as HeLa_BC1-TagGFP2) using immunoblot analysis (see Figure 11 of (21)). Here, we focused on an imaging-based readout and analyzed the level of both proteins in situ by immunofluorescence staining with a CTNNB1-specific antibody in combination with the detection of BC1-TagGFP2 fluorescence. Treatment with CHIR resulted in a strong CTNNB1 signal increase in HeLa_BC1-TagGFP2 and parental HeLa cells. A corresponding increase in CB fluorescence was observed in HeLa_BC1-TagGFP2 (Fig. 1A). For a population-wide quantitative analysis encompassing hundreds of cells, we established an automated image segmentation algorithm (supplemental Fig. S1). Using this algorithm, we quantified fluorescence signals derived from the CTNNB1 antibody staining and BC1-TagGFP2 in individual cells. Quantification of the CTNNB1 signal in nontreated or CHIR-treated parental HeLa and HeLa_BC1-TagGFP2 cells revealed highly similar observed fluorescence signals derived from the CTNNB1 antibody staining and BC1-TagGFP2 in individual cells. Quantification of the CTNNB1 signal in nontreated or CHIR-treated parental HeLa and HeLa_BC1-TagGFP2 cells revealed highly similar

Next, we tested whether BC1-TagGFP2 stabilization is also evident in the case of ectopically expressed CTNNB1. We transiently co-expressed parental HeLa cells with plasmids coding for BC1-TagGFP2, mCherry-CTNNB1 or mCherry as control (Fig. 1E). Although the expression of CB and antigen differed substantially between individual cells, the overall effect of AMCBS was evident as shown by a 1.7-fold increase of mean BC1-TagGFP2 fluorescence detected in mCherry-CTNNB1-expressing cells (Fig. 1F).

**Observation of antigen-mediated stabilization of chromobodies targeting further antigens**—To investigate whether AMCBS is a general phenomenon of CBs, we made use of previously established chromobody cell lines: (1) HeLa cells expressing an HIV p24 capsid protein (CA)-specific chromobody (HeLa_αCA-mCherry), (2) HeLa cells expressing a proliferating cell nuclear antigen (PCNA)-specific chromobody (HeLa_CCC-TagRFP), and (3) A549 cells expressing a vimentin (VIM)-specific chromobody (A549_VB6-eGFP, A549_VB6-TagRFP) (16, 18, 19). We modulated cellular antigen levels by transient expression of ectopic antigen, siRNA-mediated antigen depletion, or pathway-induced expression of the endogenous antigen followed by microscopic analysis.

First, we transfected HeLa_αCA-mCherry cells with constructs encoding either GFP-labeled p24 capsid protein (processed from GFP-gag-pol (26), for simplicity referred to as GFP-CA), or GFP as control. Whereas GFP-CA-positive cells displayed a markedly increased αCA-mCherry signal compared with nontransfected cells, no differences for αCA-mCherry fluorescence was observed in cells co-expressing GFP (Fig. 2A). Moreover, in the absence of antigen, αCA-mCherry was homogenously distributed between cytoplasm and nucleus, whereas in GFP-CA-expressing cells the CB strongly co-localized with its target in the cytoplasm (Fig. 2A). That this colocalization is likely because of intracellular binding of the αCA-mCherry to GFP-CA is supported by intracellular co-immunoprecipitation (supplemental Fig. S2). Using software-assisted image segmentation, we quantified αCA-mCherry fluorescence in nuclear, cytoplasmic or whole-cell area of GFP- or GFP-CA-expressing cells and found a 2.5-, 6.0-, or 4.3-fold increase, respectively (Fig. 2B). Quantitative immunoblot analysis revealed a 2.2-fold enrichment of αCA-mCherry (supplemental Fig. S3A). Considering a transfection efficiency of ~50% this is comparable to the microscopically determined values.

To test whether the CB level also responds to antigen depletion, we transfected HeLa_CCC-TagRFP cells with siRNAs targeting PCNA or control siRNAs and analyzed the fluorescence intensity derived from the CCC-TagRFP 72 h post transfection. Compared with control siRNAs, PCNA knockdown led to a ~70% reduction of CCC-TagRFP fluorescence (Fig. 2C, 2D). Accordingly, immunoblot analysis showed a strong reduction of CCC-TagRFP and PCNA (sup-
**Fig. 1. Antigen-mediated stabilization of the CTNNB1-specific chromobody BC1-TagGFP2.**

A. Fluorescence images of parental HeLa cells and HeLa cells stably expressing the CTNNB1-specific chromobody (CB) BC1-TagGFP2 (HeLa_BC1-TagGFP2) either left nontreated (nt) or incubated with 10 μM CHIR99021 (hereafter referred as CHIR). Endogenous CTNNB1 was detected with a CTNNB1-specific antibody.

B. Scatter plot showing the normalized CTNNB1 signal (y-axis) vs. the normalized BC1-TagGFP2 signal (x-axis) for parental HeLa, HeLa_BC1-TagGFP2, HeLa_BC1-TagGFP2 + CHIR groups.

C. Bar graph showing the normalized mean CTNNB1 signal for HeLa parental and HeLa_BC1-TagGFP2 groups.

D. Bar graph showing the normalized mean BC1-TagGFP2 signal for untreated and CHIR treated groups.

E. Fluorescence images of parental HeLa cells expressing mCherry and mCherry-CTNNB1.

F. Bar graph showing the normalized mean BC1-TagGFP2 signal for mCherry and mCherry-CTNNB1 groups.

Endogenous CTNNB1 was detected with a CTNNB1-specific antibody.
pemental Fig. S3B). Finally, to test AMCBS in vimentin chromobodies-expressing A549 cell lines (A549_VB6-eGFP, A549_VB6-TagRFP) (19), we analyzed whether TGF-β-induced increase or siRNA-mediated depletion of VIM is accompanied by corresponding changes in CB fluorescence. Previously, we have shown by Western blotting that both treatments are highly effective to raise or deplete the cellular levels of endogenous vimentin in these cell models (see Fig. 4 of (19)). Here, quantitative live-cell imaging of A549_VB6-eGFP cells revealed a continuous increase of the CB signal upon TGF-β treatment with ∼4-fold elevation after 72 h (Fig. 2E, 2F). Conversely, we observed a reduction of the CB signal to ∼20% upon siRNA-mediated knockdown of VIM compared with control siRNAs in A549_VB6-TagRFP cells (Fig. 2G, 2H). These data strongly support the hypothesis that antigen-mediated stabilization is a general phenomenon of numerous CBs, as we showed an antigen dependence for CBs targeting different cellular antigens. Notably, we were not only able to detect an increase but also a depletion of the corresponding target structure by monitoring the CB signal over time.

**Optimization of CB Turnover**—In the above-described cell models, CB expression is constitutively driven from a strong promoter (CMV) resulting in medium-to-high cellular accumulation of CBs. Because excessive CB expression might prevent the detection of small changes in antigen concentration, a low basal antigen-independent CB amount is desired. In this context, a set of destabilizing nanobody framework mutations were recently described to reduce the cellular amount of nonbound chromobodies (27). Thus, we asked whether the effect of the most destabilizing mutations (S70R, C92Y and S113F, according to Kabat numbering, (28)) is transferable to BC1-TagGFP2 (supplemental Fig. S4A). We generated corresponding expression constructs (BC1S70R-TagGFP2, BC1C92Y-TagGFP2, BC1S113F-TagGFP2) and compared them to BC1-TagGFP2 upon co-expression with mCherry-CTNNB1 or mCherry (supplemental Fig. S4B). Analysis of CB fluorescence revealed a reduced mean fluorescence of the BC1S70R-TagGFP2 and BC1S113F-TagGFP2 mutants and a strong reduction for the BC1C92Y-TagGFP2 mutant close to background fluorescence (supplemental Fig. S4C). Further quantification of the CB signals in mCherry-CTNNB1- or mCherry-expressing cells showed that antigen-dependent stabilization measured for all mutations is moderately increased compared with the nonmutated version (supplemental Fig. S4C). However, when we tested antigen binding of these modified chromobodies by intracellular immunoprecipitation of CTNNB1 (21), we could not detect any CTNNB1 in the bound fraction of the low expressing BC1C92Y-TagGFP2 construct indicating that this framework mutation abolishes binding (supplemental Fig. S4D).

Although the framework mutation approach can improve antigen-dependent responsiveness, it also bears the risk of nonfunctional binding molecules. Therefore, we conceived a strategy to reduce ground levels of CBs, which is not expected to impair antigen binding. First, we tested whether CBs are degraded via the ubiquitin proteasome system (UPS) or lysosomal protein degradation. To analyze this in the absence of an antigen we chose the HeLa_aCA-mCherry cell line, which lacks an endogenous binding partner of the aCA-CB. Following treatment of the cells either with the proteasome inhibitor MG132, or NH₄Cl, which inhibits lysosomal degradation (29) we monitored CB fluorescence by time-lapse imaging for 10 h. The data revealed a clear increase in aCA-mCherry fluorescence after incubation with MG132, whereas NH₄Cl-treated cells showed no changes in the CB signal (supplemental Fig. S5A, S5B). Considering the high similarity of sequence and structure of CBs we conclude that most CBs are likely degraded via UPS.

According to the N-end rule, one of the key determinants of protein half-life in the UPS is the N-terminal amino acid (30, 31). This residue is recognized by E3 ubiquitin ligases, which initiate ubiquitylation of accessible nearby lysine residues priming the protein for proteasomal degradation (Fig. 3A). To screen for N-terminal amino acid residues that confer accelerated CB degradation we implemented the ubiquitin fusion technique, which employs co-translational cleavage of ubiquitin from a fusion protein composed of an N-terminal ubiquitin.
Fig. 2. Antigen-mediated stabilization of chromobodies. A, Fluorescence images of living HeLa cells stably expressing an HIV CA-specific CB (HeLaαCA-mCherry). To modulate antigen concentration, cells were transiently transfected with expression constructs encoding corresponding antigen GFP-gag-pol (GFP-CA) or GFP as control. Nontransfected cells are indicated by arrow heads. Nuclei were stained with Hoechst33258. Images of both conditions have identical exposure and contrast setting to allow for direct comparison of fluorescence intensities. Scale bar: 50 μm. B, Bar chart represents mean αCA-mCherry fluorescence detected in the cytoplasm, nuclei or whole-cell area of GFP-CA expressing cells normalized to the mean signal detected in corresponding areas of GFP-expressing cells. Mean fluorescence was calculated from three samples (n = 3; >200 cells). Error bars: S.D. For statistical analysis student’s t test was performed, *** p < 0.001. C, Fluorescence images of living HeLa cells stably expressing a PCNA-specific CB (HeLa_CCC-TagRFP) upon RNA interference-mediated reduction of Proliferating Cell Nuclear Antigen (PCNA). Shown are cells 72 h post transfection of two control siRNAs (siCTR 1–2) and three
uitin and any protein of interest (30, 32). This allowed us to generate CBs displaying any desired amino acid exposed at their N termini. To produce Ub-CB fusions, we designed an expression construct as outlined in Fig. 3B. To test whether this construct is processed as intended, we expressed the original BC1-TagGFP2, an ubiquitin fusion thereof comprising methionine (M) as the CB’s N-terminal residue (Ub-M-BC1-TagGFP2) and the noncleavable mutant UbG76V-M-BC1-TagGFP2 in HEK293T cells, and compared the apparent size of the matured protein by immunoblot. In line with efficient cleavage of Ub, we detected no size difference between BC1-TagGFP2 and the Ub-M-BC1-TagGFP2 construct, whereas UbG76V-M-BC1-TagGFP2 displayed a size shift toward a higher molecular weight, which corresponds to uncleaved Ub-CB fusion protein (supplemental Fig. S6). In the following, we generated a set of 20 expression constructs differing only in the N-terminal amino acid exposed after Ub cleavage. Upon transient expression in HeLa cells, we determined mean CB fluorescence by quantitative imaging. With phenylalanine (F), alanine (A), lysine (K), tryptophan (T), arginine (R), and tyrosine (Y), we identified residues, which led to a reduced CB fluorescence of 40–60% compared with methionine (M), whereas the expression construct carrying a serine (S) at its N terminus showed the highest fluorescence (Fig. 3C).

For further analyses, we focused on the amino acids Phe, Ala, and Arg, which conferred the strongest reduction of CB fluorescence and Ser as the most stabilizing residue. As the expression of ubiquitin ligases may vary between different cell lines, we additionally tested the levels of these modified CBs (Ub-F-BC1-TagGFP2, Ub-A-BC1-TagGFP2, Ub-R-BC1-TagGFP2, Ub-S-BC1-TagGFP2) in U2OS and A549 cells. Quantitative analysis of the fluorescence revealed similar CB signals as detected in HeLa cells (supplemental Fig. S7).

To test whether the effect of the identified amino acids is transferable to CBs targeting different antigens, we replaced the BC1 nanobody by αCA or VB6 in our chromobody expression constructs. For a comparative analysis we transiently expressed all modified CBs in HeLa cells in combination with mCherry as a transfection control and performed fluorescence imaging followed by automated quantification of the CB signals (supplemental Fig. S8A, S8B). Our data revealed a reduced fluorescence for all CB constructs carrying an N-terminal Phe and Arg residue as well as a stabilizing effect of Ser. Notably, the strong effect of Ala detected for BC1-TagGFP2 was not observable for Ub-A-VB6-TagGFP2 or Ub-A-αCA-TagGFP2 (supplemental Fig. S8A, S8B).

According to our experimental design, differences in CB expression and fluorescence should only arise from differences in the CB degradation velocity. Thus, we analyzed CB fluorescence in HeLa cells transiently expressing Ub-M-BC1-TagGFP2, Ub-F-BC1-TagGFP2, Ub-R-BC1-TagGFP2, and Ub-S-BC1-TagGFP2 over time upon inhibition of translation by cycloheximide (CHX) (Fig. 3D). Compared with Ub-M-BC1-TagGFP2 and Ub-S-BC1-TagGFP2, which displayed 69 and 80% of the initial fluorescence after six hours, fluorescence of CBs with N-terminally exposed Phe or Arg decreased more rapidly to about 28% of their respective initial fluorescence (Fig. 3E). From this we conclude that Phe and Arg are turn-over-accelerating amino acids, which confer rapid degradation of CBs.

**Turnover-accelerated CBs Show Improved Antigen-mediated Stabilization**—To analyze whether the N-terminally modified CBs show an improved antigen-dependent enrichment, we compared the stabilization effect of CTNNB1 on Ub-R-BC1-TagGFP2, Ub-F-BC1-TagGFP2, and Ub-S-BC1-TagGFP2 to Ub-M-BC1-TagGFP2. Upon co-expression of the modified constructs either with mCherry-CTNNB1 or mCherry as a control in HeLa cells (Fig. 4A), we determined the average CB fluorescence within co-transfected cells. With respect to the degree of CB stabilization, N-terminal Met and Ser behave similarly with stabilization factors of 1.8 and 1.9, respectively. For Phe and Arg we detected the strongest stabilization effects with factors of ~2.3 (Fig. 4B). Taken together, the Arg-modified BC1-CB construct showed the most rapid turnover (Fig. 3E), lowest fluorescence in the absence and greatest stabilization in the presence of the antigen (Fig. 4B). In addition we tested the N-terminal Arg modification in the context of the αCA-CB (αCA-TagRFP). Upon transitory expression in HeLa cells we observed an increased stabilization of the Ub-αCA-TagRFP construct in the presence of antigen compared with the nonmodified version (supplemental Fig. S9A, S9B).

We additionally analyzed a potential impact of the linker length between NB and FP moiety and the FP moiety itself on AMCBS. Hence, we first substituted the (G4S)2 linker in the Ub-M-BC1-TagGFP2 expression construct by the shorter (G4S)4 or the longer (G4S)6 linker sequence. When we analyzed mean CB fluorescence upon transient co-expression

---

**Antigen-mediated Chromobody Stabilization (AMCBS)**

PCNA-specific siRNAs (siPCNA 1–3). Scale bar: 50 μm. D, Quantification of mean nuclear CCC-TagRFP fluorescence normalized to fluorescence of cells transfected with siCTR1 from three samples (n = 3; >200 cells) depicted as bar charts. Error bars: S.D. For statistical analysis student’s t test was performed, **p < 0.001. E, Fluorescence images of living A549 cells stably expressing a vimentin (VIM)-specific CB (AS549_VB6-eGFP) either left nontreated (0 h) or stimulated with 5 ng/ml TGF-β for indicated time points. Scale bar: 50 μm. F, Quantification of mean VB6-eGFP cellular fluorescence derived from time-lapse imaging. Fluorescence values were normalized to fluorescence detected in nontreated cells (n = 3; >200 cells) each depicted as bar charts. Error bars: S.D. G, Fluorescence images of AS549 cells stably expressing a vimentin (VIM)-specific CB (AS549_VB6-TagRFP) upon RNA interference-mediated reduction of VIM. Shown are fixed cells 72 h post transfection of two control siRNAs (siCTR 1–2) and three VIM-specific siRNAs (siVIM 1–3). Nuclei stained with DAPI. Scale bar: 50 μm. H, Quantification of mean VB6-TagRFP fluorescence normalized to fluorescence of cells transfected with siCTR1 from three samples (n = 3; >200 cells) depicted as bar charts. Error bars: S.D. For statistical analysis student’s t test was performed, *p < 0.05.
Antigen-mediated Chromobody Stabilization (AMCBS)

A

B

C

D

E

M D E F G A C L N H I K T W V Q P S R Y

N-terminal amino acid

Ub-M-BC1-TagGFP2 Ub-F-BC1-TagGFP2 Ub-A-BC1-TagGFP2 Ub-R-BC1-TagGFP2 Ub-S-BC1-TagGFP2

nt C5 DAPI C5 DAPI C5 DAPI C5 DAPI C5 DAPI

1 h 1 h 1 h 1 h 1 h

2 h 2 h 2 h 2 h 2 h

3 h 3 h 3 h 3 h 3 h

4 h 4 h 4 h 4 h 4 h

6 h 6 h 6 h 6 h 6 h

E

norm. mean Ub-X-BC1-TagGFP2 [A.U.]

time [h]

M D F A R S
with mCherry or mCherry-CTNNB1 in HeLa cells, we found no significant differences between the linker lengths irrespective of the overexpression of the antigen (supplemental Fig. S10). To evaluate a potential impact of the FP on AMCBS, we substituted TagGFP2 either by eGFP, mCherry, or TagRFP in our N-terminally modified BC1-CB constructs and monitored degradation velocities in the presence of CHX. Within six hours we observed the most rapid degradation to 26% for Ub-R-BC1-eGFP (supplemental Fig. S11A), whereas corresponding CB constructs comprising either mCherry or TagRFP are less degraded to 43% or 57%, respectively (supplemental Fig. S11B, S11C). Additionally, we analyzed antigen-dependent stabilization as described above. Here, we detected a 2.9-fold fluorescence increase of Ub-R-BC1-eGFP in antigen-expressing cells compared with Ub-M-BC1-eGFP (supplemental Fig. S12A), which exceeds the stabilization

**Fig. 3. Identification of CB turnover-accelerating N-terminal amino acids.** A, Schematic overview of protein degradation via the N-end rule. The N-terminal amino acid of a target protein is recognized by E3 ubiquitin ligases, which initiate ubiquitylation of accessible nearby lysine residues through E2 ubiquitin ligases priming the protein for proteasomal degradation. B, Illustration of the expression construct used to screen for turnover accelerating N-terminal residues in the context of Ub-X-BC1-TagGFP2. C, Ub-X-BC1-TagGFP2 constructs exposing all possible amino acids at their N termini were transiently expressed in HeLa cells. Bar chart represents mean CB fluorescence, normalized to Ub-M-BC1-TagGFP2 (upper panel). Representative images depict CB fluorescence for each modified N terminus. Scale bar: 200 μm (lower panel). For statistical analysis student’s t test was performed, ***p < 0.001. D, To determine turnover velocities of modified CBs, HeLa cells were transfected with the indicated Ub-X-BC1-TagGFP2 constructs, 24 h post transfection cells were either left nontreated (nt) or were treated with 0.1 mg/ml cycloheximide (CHX). At indicated time points cells were fixed and nuclei stained with DAPI. Shown are representative images of three biological replicates. Scale bar: 200 μm. E, Quantification of Ub-X-BC1-TagGFP2 fluorescence intensity after CHX treatment. Mean fluorescence of each CB variant was normalized to the respective nontreated control and plotted against time (n = 3, >200 cells each). Error bars: S.D.
factor of 2.3 observed for Ub-R-BC1-TagGFP2 (Fig. 4B). Similar stabilization factors were observed for Ub-R-BC1-mCherry (supplemental Fig. S12B) and Ub-R-BC1-TagRFP (supplemental Fig. S12C). Finally, to verify antigen binding of the modified CB constructs, we performed intracellular immunoprecipitations from HEK293T cells expressing Ub-M-BC1-eGFP, Ub-R-BC1-eGFP, the original BC1-TagGFP2 (21) as positive control, or GFP as negative control. Immunoblot analysis revealed that all CB constructs precipitate endogenous CTNNB1 in comparable amounts whereas no binding to GFP was observed (supplemental Fig. S13). From that we conclude, that intracellular antigen binding is neither affected by ubiquitin fusion nor by the introduction of turnover-accelerating amino acids.

**Monitoring Rapid Changes in CTNNB1 Level in Living Cells With Turnover-accelerated Chromobodies**—A major benefit of the AMCBS approach compared with end-point assays is its applicability to continuously monitor time-dependent changes in POI concentration in living cells. Previously, we demonstrated that an increase of endogenous CTNNB1 upon compound treatment can be visualized by real-time imaging of the CB signal in HeLa_BC1-TagGFP2 (21). For decreasing antigen concentrations, we supposed that CBs with accelerated degradation would reflect these changes more precisely. Thus, we tested, whether turnover-accelerated CBs can track reversible changes in POI concentration in live cells. Because transient expression of CBs displays a substantial heterogeneity of intracellular CB levels, we generated monoclonal HeLa and U2OS cell lines stably expressing the turnover-accelerated CTNNB1 chromobody (HeLa_Ub-R-BC1-eGFP, U2OS_Ub-R-BC1-eGFP). Quantitative fluorescence imaging of HeLa_Ub-R-BC1-eGFP cells revealed that in the absence of experimentally modulated levels of CTNNB1, CB fluorescence is nearly indistinguishable from autofluorescence of parental HeLa cells, whereas elevating endogenous CTNNB1 levels by CHIR treatment strongly increased CB fluorescence. Under both conditions, in situ detection of CTNNB1 showed that the mean CTNNB1 concentration was highly similar in parental HeLa and HeLa_Ub-R-BC1-eGFP cells (supplemental Fig. S14A and S14B), indicating that expression of the turnover-accelerated BC1-CB has no effect on the amount of endogenous CTNNB1. To test whether the Arg-modified CB construct retains its binding properties, we performed intracellular immunoprecipitation in HeLa_Ub-R-BC1-eGFP cells compared with HeLa_BC1-TagGFP2 cells. Our immunoblot analysis revealed that the modified BC1-CB can precipitate endogenous CTNNB1 upon induction with CHIR (supplemental Fig. S14C).

Next, we compared CB performance in the original HeLa_BC1-TagGFP2 (21) and the newly generated HeLa_Ub-R-BC1-eGFP cell line to monitor reversible changes of endogenous CTNNB1 levels. To that end, cells were cultivated in the presence of CHIR for 16 h. Subsequently, cells were washed and continuously cultivated. One, two, three, four, six, eight, and 24 h post removal of CHIR, cells were fixed and immunostained for CTNNB1 followed by fluorescence imaging (Fig. 5A). In all cell lines we found a ~12-fold increase of endogenous CTNNB1 upon CHIR treatment, which rapidly returned to base level within four hours after compound removal (Fig. 5B). Concurrently, we also observed elevated CB signals after 16 h of CHIR treatment in both CB cell lines. Notably, in cells expressing turnover-accelerated Ub-R-BC1-eGFP, relative mean CB fluorescence not only increased more strongly compared with the original nonmodified BC1-TagGFP2, but also decreased more rapidly after CHIR removal. In contrast, in cells expressing the nonmodified BC1-TagGFP2 construct the CB signal remained at higher levels after removal of CHIR (Fig. 5B). A similar rapid decrease of the CB signal was observed in U2OS_Ub-R-BC1-eGFP cells upon removal of CHIR (supplemental Fig. S15). For a better illustration of the reversible changes in BC1-CB fluorescence we performed continuous live-cell imaging of HeLa_BC1-TagGFP2, HeLa_Ub-R-BC1-eGFP, and U2OS_Ub-R-BC1-eGFP cells upon addition and subsequent removal of CHIR (supplemental videos SV1–3).

To assess the accuracy of CTNNB1 estimation by BC1-CB fluorescence, we plotted the ratio of normalized mean fluorescence (CTNNB1/BC1-CB) against time (supplemental Fig. S16). In an ideal model, CB fluorescence would be proportional to the amount of CTNNB1 at any given time. Consequently, the ratio of the normalized fluorescence values would always be 1. Comparing the overall deviation from the ideal model, the newly generated cell line comprising the turnover-accelerated BC1-CB clearly outperforms the original cell line. For additional validation, we generated whole-cell lysates from CHIR-treated parental HeLa, HeLa_BC1-TagGFP2 and HeLa_Ub-R-BC1-eGFP cells at indicated time points and monitored CTNNB1 and CB levels by immunoblot analysis (supplemental Fig. S17, Fig. 5C). These results are in line with our microscopically obtained data, confirming a more rapid turnover of the Ub-R-BC1-eGFP upon removal of CHIR. In summary, these data show that the turnover-accelerated CBs substantially improve the ability to monitor reversible changes in the amount of endogenous CTNNB1 by AMCBS.

**Determination of Compound Effects Using Turnover-accelerated Chromobodies**—In preclinical drug development the use of cellular in vitro models has strongly increased over the last years. These models serve as powerful tools to screen for novel candidates as well as to evaluate cellular compound efficacy. However, to our knowledge there are no methods available to continuously monitor dose-response and kinetics of drug action on the level of endogenous target proteins. Here, we applied our turnover-accelerated BC1-CB-expressing cell line U2OS_Ub-R-BC1-eGFP to monitor the effect of two well established GSK3-β kinase inhibitors CHIR and 6-bromoindirubin-3-oxime (BIO) on endogenous CTNNB1 in a quantitative live-cell imaging setup. We continuously imaged U2OS_Ub-R-BC1-eGFP upon incubation with different inhibitor concentrations for 42 h (Fig. 6A) and quantified nuclear fluorescence every 3 h (Fig. 6B). For both inhibitors an overall
Antigen-mediated Chromobody Stabilization (AMCBS)

A

|          | nt | 16 h | 16 + 1 h | 16 + 2 h | 16 + 3 h | 16 + 4 h | 16 + 6 h | 16 + 8 h | 16 + 24 h |
|----------|----|------|----------|----------|----------|----------|----------|----------|-----------|
| CTNNB1   |    |      |          |          |          |          |          |          |           |
| parental |    |      |          |          |          |          |          |          |           |
| DAPI     |    |      |          |          |          |          |          |          |           |
| CTNNB1   |    |      |          |          |          |          |          |          |           |
| CB       |    |      |          |          |          |          |          |          |           |
| DAPI     |    |      |          |          |          |          |          |          |           |
| WT-BC1-TagGFP2 | | | | | | | | | |
| DAPI     |    |      |          |          |          |          |          |          |           |

B

- parental
- HeLa-BC1-TagGFP2
- HeLa-Ub-R-BC1-eGFP

C

- HeLa_BC1-TagGFP2
- HeLa_Ub-R-BC1-eGFP

Molecular & Cellular Proteomics 17.12

2529
dose-dependence of the effect was obvious. Prolonged time-lapse image analysis revealed that higher inhibitor concentrations (2.5 $\mu$M for CHIR and 5 $\mu$M for BIO) induced onset of cell death after 30–36 h. Notably, we also identified differences in the kinetics of drug action. Although for every concentration of CHIR the corresponding maximum effect is observed after 22 h and gradually declines thereafter, the effect of BIO reaches a plateau at about 24 h for nontoxic concentrations. Interestingly, the maximum tolerated concentration of both inhibitors (1 $\mu$M CHIR and 2.5 $\mu$M BIO) led to a similar increase in BC1-CB fluorescence of about 3.5-fold (Fig. 6).

ANTIGEN-MEDIATED CHROMOBODY STABILIZATION (AMCBS)

Fig. 5. Monitoring reversible changes of endogenous CTNNB1 levels in HeLa Ub-R-BC1-eGFP cells using CB fluorescence. A, Parental HeLa cells, HeLa_BC1-TagGFP2 and HeLa_Ub-R-BC1-eGFP cells were either left nontreated (nt) or incubated with 10 $\mu$M CHIR for 16 h. Subsequently, CHIR was removed and cells were continuously cultivated for additional 24 h. At indicated time points cells were fixed and immuno-stained using a CTNNB1-specific antibody. Shown are representative fluorescence images displaying the CTNNB1 antibody and the chromobody (CB) signal. Nuclei were stained with DAPI, scale bar: 50 $\mu$m. B, Immunoblot analysis of whole-cell lysates derived from individual cell lines at indicated time points as described in (A). Shown are signals for endogenous CTNNB1, chromobodies (BC1-TagGFP2, Ub-R-BC1-eGFP) and GAPDH as loading control detected with CTNNB1-, GFP- or GAPDH-specific antibodies.

Fig. 6. Monitoring kinetics of drug action in U2OS_Ub-R-BC1-eGFP. A, Representative images of living U2OS_Ub-R-BC1-eGFP cells at indicated time points upon compound treatment. Cells were treated with the indicated concentrations of CHIR (left panel) or 6-bromodirubin-3-oxime (BIO) (right panel), or with respective controls (H2O in case of CHIR and 0.01% DMSO for BIO). For image segmentation, live cells were stained with Hoechst33258. B, Nuclear CB-signal was imaged and quantified in three hour intervals as described in supplemental Fig. S1.

Error bars: S.E.
might have multiple reasons including off-target effects, it is also possible that U2OS cells only tolerate a certain elevation of CTNNB1 for a defined period. Taken together these data demonstrate that turnover-accelerated chromobodies in combination with quantitative imaging allows a precise determination of dose- and time-dependent compound effects on the level of individual endogenous proteins in living cells.

DISCUSSION

Chromobodies (CBs) comprising nanobody (NB)-derived binding moieties genetically linked to fluorescent proteins have become valuable tools to visualize endogenous antigens within living cells (10, 14). Recently, we and others observed that NBs are stabilized in the presence of their antigen (21, 22, 27). Originally observed for a CB targeting hypo-phosphorylated CTNNB1, here we show antigen-mediated stabilization (AMCBS) for four different CBs targeting soluble (cytoplasmic/nuclear CTNNB1), structural (vimentin), nuclear (PCNA) and virus-derived (p24-CA) antigens. Considering similar observations reported by others, it is conceivable that AMCBS is a general phenomenon common to numerous CBs. Notably, by monitoring CB signals in different stable cell systems, we not only visualized elevation but also, for the first time, depletion of cellular antigens over time. Although substantial changes in protein levels over longer periods can be sufficiently visualized using CBs in their original format, it has to be considered that specific signals of bound CBs in response to smaller and/or more rapid changes in protein levels can be obscured by the diffuse signal of nonbound CBs. To cope with this issue, recently, several approaches have been described to modify such intracellular nanoprobe accordingly.

To repress the expression of nontarget-bound intrabodies a DNA-binding KRAB domain was fused to an intrabody, thereby establishing a negative transcriptional feed-back mechanism (33). However, like any regulatory circuit involving transcription, this approach reacts sluggishly to rapid changes of cellular POI levels. Moreover, because of DNA-binding, KRAB domain-containing intrabodies accumulate in the nucleus even in the absence of the target protein (33). When we added the KRAB domain to our VIM- and CTNNB1-specific CBs, we observed a strong enrichment of both CBs in the nucleus, which impedes target concentration analysis by AMCBS (data not shown). Consequently, this system is not suitable to monitor nuclear proteins and rapid changes in protein levels. Another approach to lower the concentration of unbound intrabodies was reported for constructs comprising a PEST domain, which promotes rapid ubiquitin-independent proteasomal degradation (34). Upon introduction of PEST-modified CB expression constructs in live cells, we observed a substantial decrease in CB fluorescence. However, this was accompanied by a rapid onset of cell death irrespective of the addressed antigen (data not shown).

Recently distinct point mutations within the framework regions were described to destabilize, and accordingly lower the amount of intracellular NBs. Such modified NBs were shown to be re-stabilized in the presence of overexpressed antigen and thus are functional to detect recombinant or viral antigens e.g. by flow cytometry (27). Although it was stated that only NBs comprising framework mutations are stabilized in the presence of the antigen, our analysis revealed substantial antigen responsiveness even for nonmodified CBs. This indicates that antigen-mediated stabilization is inherent to CBs per se and does not depend on mutational destabilization. Moreover, as shown for the BC1CH2TAGFP2 version, the introduction of mutations within the framework regions bears the risk to lose functional binding molecules. Notably, this is in line with previous reports of multiple NBs that show a participation of the framework regions in antigen binding (35–38).

Here, we conceived a strategy to reduce base levels of nonbound CBs, which do not affect antigen binding. We focused on the N terminus of CBs, which has never been reported to participate in antigen binding and employed the rather old concept of the N-end rule (30, 39). With either Met or Ala at the N terminus, CBs are subjected to the Ac/N-end rule pathway that involves N-terminal acetylation, which presumably results in a long half-life of the protein (30). The other major degradation pathway of the ubiquitin proteasome system is the Arg/N-end rule pathway. To screen for CB turnover accelerating N-terminal amino acid residues we implemented the ubiquitin fusion technique (40) and identified Arg and Phe, which, when exposed at the N terminus of all tested constructs, mediates the fastest CB turnover. The identification of these representatives of basic or bulky hydrophobic residues is in accordance with short half-lives described for other proteins displaying those residues at their N termini (31).

Additionally, we observed significant differences in the degradation velocities of CBs comprising different fluorescent proteins. We assume that variations in position and number of lysine residues accessible for ubiquitylation within the fluorescent moiety have an impact on turnover rates of the corresponding CBs.

Although our findings provide strong evidence that CBs are degraded via the ubiquitin proteasomal system, the precise molecular and structural mechanisms, which are responsible for the stabilization of CBs upon antigen binding within living cells remain to be elucidated. E3 ubiquitin ligases recognize the N-terminal amino acid and initiate ubiquitylation of accessible nearby lysine residues (31). Notably, such ubiquitylated proteins are stable within living cells unless they also expose an unstructured region, which is needed to initiate degradation (41). CBs have two tightly folded domains: the binding moiety, which shows a typical immunoglobulin fold and the cylindrical FP structure. Consequently, unstructured regions are restricted to the complementarity determining regions (CDRs) of the NB, the interconnecting linker, and to the short alpha helices forming the caps on the ends of the FP beta-barrel. Forming the paratope, the CDRs are in close
contact with the antigen and thus it can be speculated that antigen binding masks these potential initiation sites and thus prevents degradation of the CB. Additionally, it is conceivable that CB molecules become partially immobilized in the cell upon antigen binding and are therefore less likely to interact with proteasomes compared with freely diffusible CBs. The possible reduced mobility of the bound CBs is in agreement with findings showing that larger protein complexes have a limited and/or reduced diffusion coefficient (42). Finally, competition of antigen and ubiquitin ligases for CBs, which would also facilitate the escape of antigen-bound CBs from ubiquitylation could also contribute to the observed phenomenon of AMCBS.

For monitoring protein levels optically, FP tagging of endogenous proteins is a straightforward approach. However, FP tagging can interfere with crucial protein parameters such as turnover, subcellular localization, and participation in multiprotein complexes (6–8). To avoid a permanent FP fusion, as turnover, subcellular localization, and participation in multi-FP tagging can interfere with crucial protein parameters such

AMCBS.

nylation could also contribute to the observed phenomenon of AMCBS.

The herein described turnover-accelerated chromobodies substantially expand the possibilities of these multifunctional nanoprobes. AMCBS with highly antigen-responsive CBs combines for the first time visualization of subcellular localization and redistribution of endogenous proteins with monitoring and quantification of rapid changes of protein levels by quantitative live-cell imaging. Like for any molecular probe applied in quantitative live-cell imaging, a potential influence of CB binding on antigen levels has to be carefully evaluated. Here, we demonstrated that the level and the dynamics of endogenous CTNNB1 is not affected by the presence of CTNNB1-specific CBs. Notably, similar observations were made for the PCNA-CB (CCC-TagRFP) and VIM-CB (VB6-eGFP) as reported previously (18, 19). Moreover, the generation of organisms stably expressing CBs also strongly indicates that CB binding in trans does not affect the levels of tightly regulated antigens (18, 44). From a technical perspective, this AMCBS approach is readily applicable, as fluorescence microscopy instrumentation is widely available in cell biology laboratories. Because of continuous improvements of nano-/chromobody screening protocols (45, 46), the number of available chromobodies is constantly growing (14) and will enable time-resolved quantification of many further proteins of interest in the near future. AMCBS in combination with target-specific chromobodies could be further adapted to detect post-translational modifications or the presence and abundance of specific splice variants, which cannot be detected with conventional FP fusions.

Acknowledgment—We thank Marion Jung (ChromoTek GmbH) for providing reagents and the HeLa_CCC-TagRFP cell line.

* This work was supported by the Ministry of Science, Research and Arts of Baden-Württemberg (https://mwk.baden-wuerttemberg. de) (V.1.4.-H0-1403-74).

[5] This article contains supplemental material. U.R. is shareholder of the commercial company ChromoTek GmbH.

1 To whom correspondence may be addressed: Natural and Medical Sciences Institute at the University of Tuebingen, Markwiesenstr. 55, 72770 Reutlingen, Germany. Tel.: +49 7121 51530-415, Fax: +49 7121 51530-816; E-mail: ulrich.rothbauer@uni-tuebingen.de.

** To whom correspondence may be addressed: Department of Pharmaceutical Biotechnology, Eberhard Karls University Tuebingen, 72770 Reutlingen, Germany. Tel. +49-7121-51530-478; E-mail: bjoern.traenkle@nmi.de.

These authors contributed equally to this work.

Author contributions: B.T., U.R., and B.K. conceived the study. B.K., B.T., J.M., S.E., K.S., and Y.P. performed all experiments. B.K., B.T., and U.R. analyzed the data. B.K., U.R., and B.T. wrote the manuscript.

REFERENCES

1. Li, H., Han, J., Pan, J., Liu, T., Parker, C. E., and Borchers, C. H. (2017) Current trends in quantitative proteomics - an update. J. Mass Spectrometry 52, 319–341

2. Lindemann, C., Thomanek, N., Hundt, F., Lerari, T., Meyer, H. E., Wolters, D., and Marcus, K. (2017) Strategies in relative and absolute quantitative mass spectrometry based proteomics. Biol. Chem. 398, 687–699

3. Walker, J. M. (2009) The Protein Protocols Handbook, Third Edition Ed., Humana Press

4. Schnell, U., Dijk, F., Sjollem, K. A., and Giepmans, B. N. (2012) Immunolabeling artifacts and the need for live-cell imaging. Nat. Methods 9, 152–158

5. Leonetti, M. D., Sekine, S., Kamiyama, D., Weissman, J. S., and Huang, B. (2016) A scalable strategy for high-throughput GFP tagging of endogenous human proteins. Proc. Natl. Acad. Sci. U.S.A. 113, E3501–E3508

6. Snapp, E. L. (2009) Fluorescent proteins: a cell biologist’s user guide. Trends Cell Biol. 19, 649–655

7. Stadler, C., Rexhepaj, E., Singan, V. R., Murphy, R. F., Peppercok, R., Uhlen, M., Simpson, J. C., and Lundberg, E. (2013) Immunofluorescence and fluorescent-protein tagging show high correlation for protein localization in mammalian cells. Nat. methods 10, 315–323

8. Virant, D., Traenkle, B., Maier, J., Kaiser, P. D., Bodenhofer, M., Schmees, C., Vojnovic, I., Pitsak-Lukats, B., Endesfelder, U., and Rothbauer, U. (2018) A peptide tag-specific nanobody enables high-quality labeling for dSTORM imaging. Nat. Communications 9, 930

9. Kaiser, P. D., Maier, J., Traenkle, B., Emel, F., and Rothbauer, U. (2014) Recent progress in generating intracellular functional antibody fragments to target and trace cellular components in living cells. Biochim. Biophys. Acta 1844, 1933–1942

10. Helma, J., Cardoso, M. C., Muyldermans, S., and Leonhardt, H. (2015) Nanobodies and recombinant binders in cell biology. J. Cell Biol. 209, 633–644

11. Ingram, J. R., Schmidt, F. L., and Ploegh, H. L. (2018) Exploiting Nanobodies’ Singular Traits. Ann. Rev. Immunol. 36, 695–715

12. Pellis, M., Pardon, E., Zolghadr, K., Rothbauer, U., Vincke, C., Kinne, J., Dierynck, I., Hertogs, K., Leonhardt, H., Messens, J., Muyldermans, S., and Conrath, K. (2012) A bacterial-two-hybrid selection system for one-
step isolation of intracellularly functional Nanobodies. Arch. Biochem. Biophys. 526, 114–123

13. Moutel, S., Nizak, C., and Perez, F. (2012) Selection and use of intracellular antibodies (intrabodies). Methods Mol. Biol. 907, 667–679

14. Traenkle, B., and Rothbauer, U. (2017) Under the Microscope: single-domain antibodies for live-cell imaging and super-resolution microscopy. Front. Immunol. 8, 1030

15. Rothbauer, U., Zolghadr, K., Tillib, S., Nowak, D., Schemmellieh, L., Gahl, A., Backmann, N., Conrath, K., Muyldermans, S., Cardoso, M. C., and Leonhardt, H. (2006) Targeting and tracing antigens in live cells with fluorescent nanobodies. Nat. Methods 3, 887–899

16. Helma, J., Schmidtthals, K., Lux, V., Nuske, S., Scholz, A. M., Krausslich, H. G., Rothbauer, U., and Leonhardt, H. (2012) Direct and dynamic detection of HIV-1 in living cells. PloS One 7, e50026

17. Irannejad, R., Tomshine, J. C., Tomshine, J. R., Chevalier, M., Mahoney, J. P., Steyaert, J., Rasmussen, S. G., Sunahara, R. K., El-Samad, H., Huang, B., and von Zastrow, M. (2013) Conformational biosensors reveal GPCR signalling from endosomes. Nature 495, 534–538

18. Panza, P., Maier, J., Schmees, C., Rothbauer, U., and Sollner, C. (2015) Live imaging of endogenous protein dynamics in zebrafish using chromobodies. Development 142, 1879–1884

19. Maier, J., Traenkle, B., and Rothbauer, U. (2015) Real-time analysis of epithelial-mesenchymal transition using fluorescence single-domain antibodies. Sci. Reports 5, 13402

20. Schorpp, K., Rothenaigner, I., Maier, J., Traenkle, B., Rothbauer, U., and Hadkan, K. (2016) A multiplexed high-content screening approach using the chromobody technology to identify cell cycle modulators in living cells. J. Biomol. Screening 21, 965–977

21. Traenkle, B., Emele, F., Anton, R., Poetz, O., Haeussler, R. S., Maier, J., Kaiser, P. D., Scholz, A. M., Nueske, S., Buchteliner, A., Roter, M., and Rothbauer, U. (2015) Monitoring interactions and dynamics of endogenous beta-catenin with intracellular nanobodies in living cells. Mol. Cell. Proteomics 14, 707–723

22. Bianco-Toribio, A., Muyldermans, S., Frankel, G., and Fernandez, L. A. (2010) Direct injection of functional single-domain antibodies from E. coli into human cells. PloS One 5, e13227

23. Gibson, D. G., Young, L., Chuan, R. Y., Venter, J. C., Hutchison, C. A., 3rd, and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–345

24. Dantuma, N. P., Lindsten, K., Glas, R., Jeline, M., and Masucci, M. G. (2000) Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteinolysis in living cells. Nat. Biotechnol. 18, 538–543

25. Toulany, M., Maier, J., Iida, M., Rebholz, S., Holler, M., Grottky, A., Juker, M., Wheeler, D. L., Rothbauer, U., and Rodemann, H. P. (2017) Akt1 and Akt3 but not Akt2 through interaction with DNA-PKcs stimulate proliferative and post-irradiation cell survival of K-RAS-mutated cancer cells. Cell Death Discovery 3, 10702

26. Lampe, M., Briggs, J. A., Endres, T., Glass, B., Riegelsberger, S., Krausslich, H. G., Lamb, D. C., Brauchle, C., and Muller, B. (2007) Double-labelled HIV-1 particles for study of virus-cell interaction. Virology 360, 92–104

27. Tang, J. C., Drokhlyansky, E., Etemad, B., Rudolph, S., Guo, B., Wang, S., Ellis, E. G., Li, J. Z., and Cepko, C. L. (2016) Detection and manipulation of live antigen-expressing cells using conditionally stable nanobodies. eLife 5

28. Kabat, E. A., and Wu, T. T. (1991) Identical V region amino acid sequences and segments of sequences in antibodies of different specificities. J. Immunol. 147, 1709–1719

29. Seglen, P. O., and Reith, A. (1976) Ammonia inhibition of protein degradation in isolated rat hepatocytes. Quantitative ultrastructural alterations in the lysosomal system. Exp. Cell Res. 100, 276–280

30. Bachmair, A., Finley, D., and Varshavsky, A. (1986) In vivo half-life of a protein is a function of its amino-terminal residue. Science 234, 179–186

31. Varshavsky, A. (2011) The N-end rule pathway and regulation by proteolysis. Protein Sci. 20, 1298–1345

32. Varshavsky, A. (2000) Ubiquitin fusion technique and its descendants. Methods Enzymol. 327, 578–593

33. Gross, G. G., Junge, J. A., Mora, R. J., Kwon, H. B., Olson, C. A., Takahashi, T. T., Liman, E. R., Ellis-Davies, G. C., McGee, A. W., Sabattini, B. L., Roberts, R. W., and Arnold, D. B. (2013) Recombinant probes for visualizing endogenous synaptic proteins in living neurons. Neuron 78, 971–985

34. Sibler, A. P., Courtete, J., Muller, C. D., Zeder-Lutz, G., and Weiss, E. (2005) Extended half-life upon binding of destabilized intrabodies allows specific detection of antigen in mammalian cells. FEBS J. 272, 2878–2891

35. Kirchhofer, A., Helma, J., Schmidtthals, K., Frauer, C., Cui, S., Karcher, A., Pelli, M., Muyldermans, S., Casas-Delucchi, C. S., Cardoso, M. C., Leonhardt, H., Hopfner, K. P., and Rothbauer, U. (2010) Modulation of protein properties in living cells using nanobodies. Nat. Structural Mol. Biol. 17, 133–138

36. Schmidt, F. I., Hanke, L., Morin, B., Brewer, R., Brusci, V., Whelan, S. P., and Ploegh, H. L. (2016) Phenotypic lentivirus screens to identify functional single domain antibodies. Nat. Microbiol. 1, 16080

37. Fanning, S. W., and Horn, J. R. (2011) An anti-hapten camelid antibody reveals a cryptic binding site with significant energetic contributions from a nonhypervariable loop. Protein Sci. 20, 1196–1207

38. Noel, F., Malpertuy, A., and de Brevern, A. G. (2016) Global analysis of VHVs framework regions with a structural alphabet. Biochimie 131, 11–19

39. Gonda, D. K., Bachmair, A., Wunning, I., Tobias, J. W., Lane, W. S., and Varshavsky, A. (1989) Universality and structure of the N-end rule. J. Biol. Chem. 264, 16700–16712

40. Varshavsky, A. (2005) Ubiquitin fusion technique and related methods. Methods Enzymol. 399, 777–799

41. Prakash, S., Tian, L., Ratliff, K. S., Lehotzky, R. E., and Matouschek, A. (2004) An unstructured initiation site is required for efficient proteasome-mediated degradation. Nat. Structural Mol. Biol. 11, 830–837

42. Verkman, A. S. (2003) Diffusion in cells measured by fluorescence recovery after photobleaching. Methods Enzymol. 360, 635–648

43. Lo, C. A., Kays, I., Emran, F., Lin, T. J., Cvetkovska, V., and Chen, B. E. (2015) Quantification of protein levels in single living cells. Cell Reports 13, 2634–2644

44. Juillien, D., Vignard, J., Fedor, Y., Bery, N., Olichon, A., Crozatier, M., Erard, M., Cassard, H., Ducommun, B., Sales, B., and Mirey, G. (2016) Chromobody, a novel non-invasive molecular tool to explore and manipulate chromatin in living cells. J. Cell Sci. 129, 2673–2683

45. McMahon, C., Baier, A. S., Pascolutti, R., Wegrecki, M., Zheng, S., Ong, J. X., Erlandson, S. C., Hilger, D., Rasmussen, S. G. F., Ring, A. M., Manglik, A., and Kruse, A. C. (2018) Yeast surface display platform for rapid discovery of conformationally selective nanobodies. Nat. Structural Mol. Biol. 25, 289–296

46. Rothbauer, U. (2018) Speed up to find the right ones: rapid discovery of conformationally selective nanobodies. Nat. Structural Mol. Biol. 25, 198–201