

-Electronic Supplementary Information-

TuPPL: Tub-tag mediated C-terminal Protein-Protein-Ligation Using Complementary Click-Chemistry Handles

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Supplementary Figures and Tables

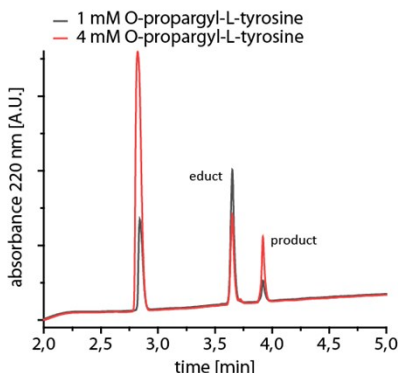


Figure S1. TTL mediated ligation of O-propargyl-L-tyrosine to the Tub-tag peptide (CF-VDSVEGEGEEEEGEE). Exemplary chromatogram recorded at 220 nm (black: 1 mM, red: 4 mM O-propargyl-L-tyrosine). The product peptide with O-propargyl-L-tyrosine elutes at 3.94 min and the sample with a substrate concentration of 4 mM showed an increased product-yield.

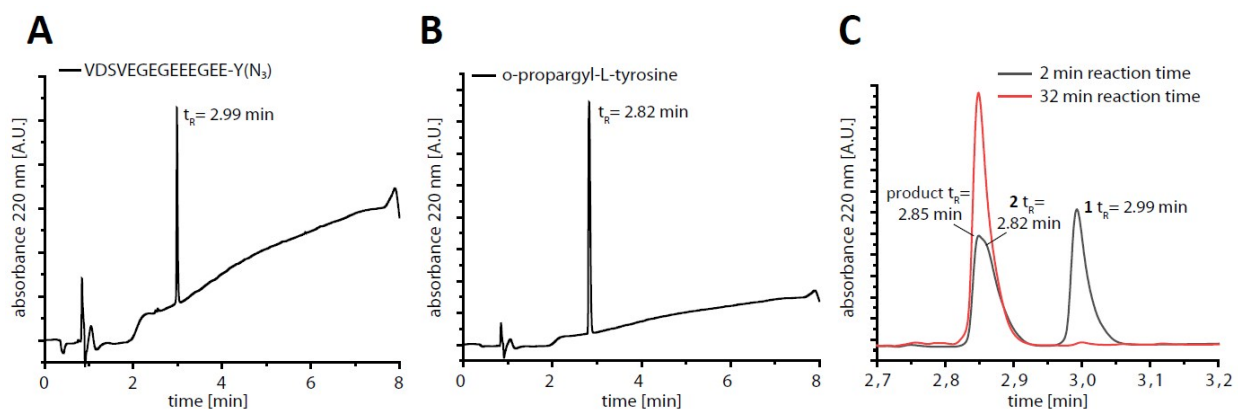


Figure S2. CuAAC model-reaction with Tub-tag peptide. 3-azido-L-tyrosine was incorporated C-terminally at the Tub-tag peptide (VDSVEGEGEEEEGEE) using previously described conditions (Schumacher et al. 2015). A) After purification with RP-HPLC a pure azide-containing peptide $t_R = 2.99$ min was isolated. B) O-propargyl-L-tyrosine has an elution time of $t_R = 2.82$ min. The progress of the CuAAC reaction of these two educts was analyzed with RP-UPLC. C) Chromatogram of the reaction after two (black) and 32 minutes (red). Full conversion of the azide-containing peptide was achieved after 32 minutes.

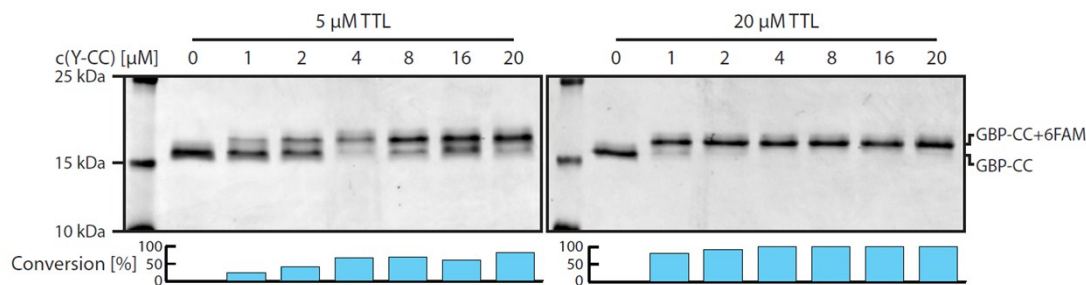


Figure S3. Concentration dependent, TTL catalyzed, ligation of O-propargyl-L-tyrosine to Tub-tagged GBP. Increasing O-propargyl-L-tyrosine concentration leads to increased incorporation efficacy using 5 μM TTL and 100 μM GBP at 30 °C after 3 h. Incorporation is demonstrated by CuAAC of 6FAM-azide and subsequent SDS-PAGE analysis. Complete conversion is reached at all tested O-propargyl-L-tyrosine concentrations when increasing the TTL concentration to 20 μM.

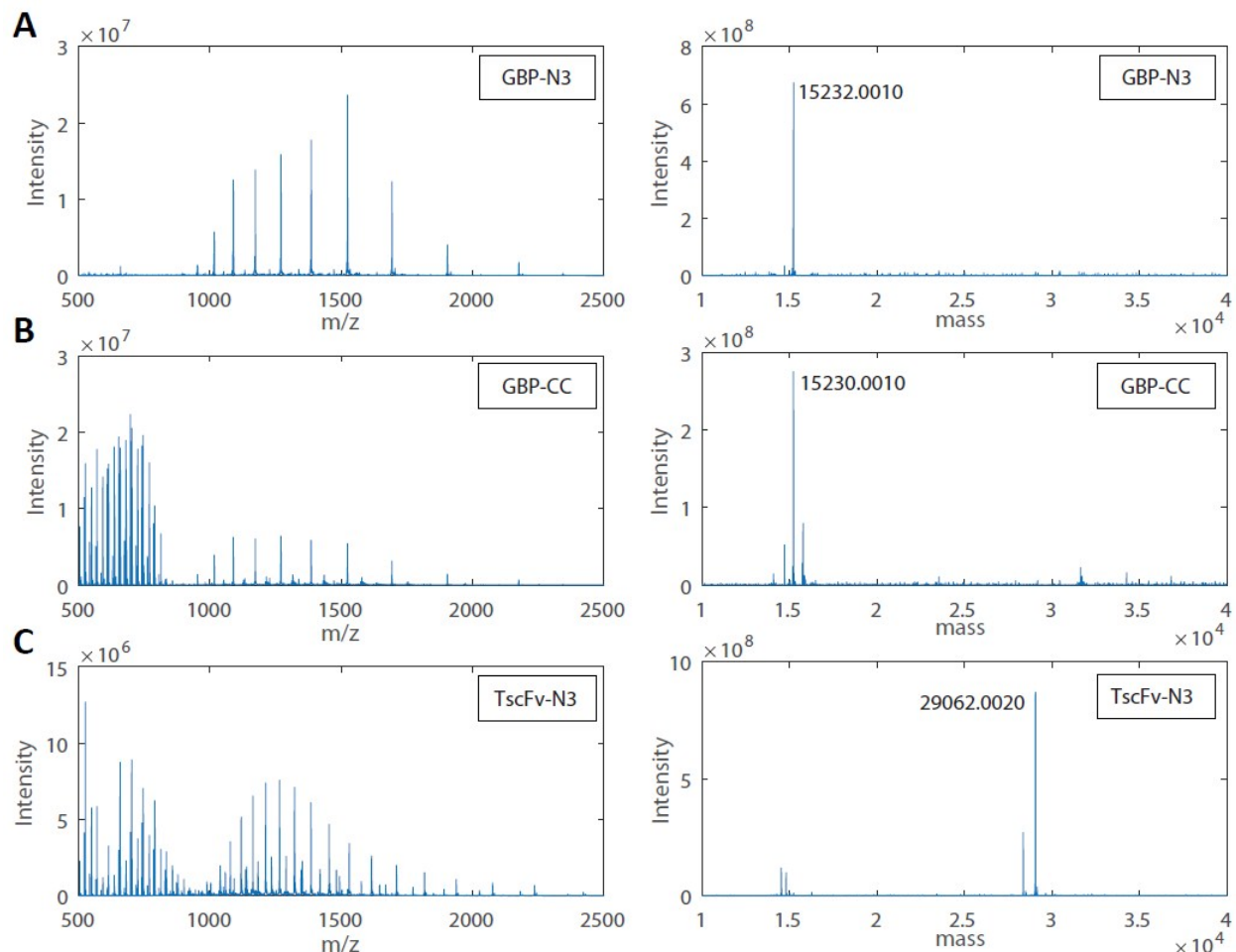


Figure S4. Intact MS analysis of tyrosine derivative modified proteins. Raw (left) and deconvoluted (right) MS spectra of GBP-Tub conjugated with 3-azido-L-tyrosine (GBP-N3) or O-propargyl-L-tyrosine (GBP-CC) and TscFv-Tub conjugated with 3-azido-L-tyrosine (TscFv-N3). GBP-N3 expected: 15232 Da = 15028 Da (GBP-Tub) + 222 Da (3-azido-L-tyrosine) – 18 Da

(H₂O). GBP-CC expected: 15229 Da = 15028 Da (GBP-Tub) + 219 Da (O-propargyl-L-tyrosine) – 18 Da (H₂O). TscFv-N3 expected: 29066 Da

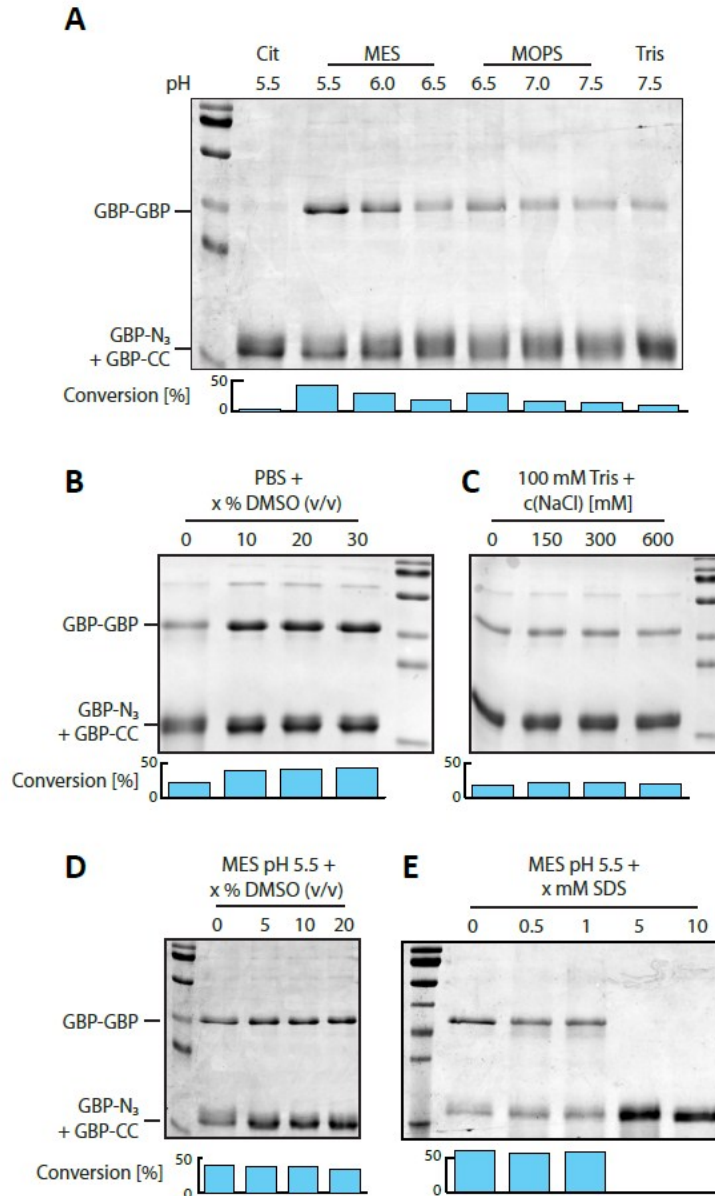


Figure S5. Optimization of CuAAC reaction conditions for protein-protein conjugation. SDS-PAGE analysis of GBP-homodimer formation under various conditions. Conversion efficacy was estimated by densitometric analysis. **A)** We observed a clear effect of buffer component as well as pH value on conjugation efficacy. Citrate almost completely abolished conjugation most probably due to its Cu ion complexation potential. Tris also has been shown to complex Cu ions which might explain the observed low conjugation efficacy in our experiment. When using MES or MOPS (two non-chelating compounds) we observe an increase in conjugation efficacy and in addition a clear pH dependence. Lower pH values are favored with

pH 5.5 giving the best results of all tested conditions. However, we observed slight aggregation at low pH this effect was reversible upon pH neutralization. **B)** Since this low pH might not be tolerated by all proteins we also assessed the effect of the miscible organic solvent DMSO under physiological buffer conditions (1x PBS, pH 7.4). Addition of 10 % (v/v) DMSO doubled the conjugation efficacy whereas higher concentrations did not further increase efficacy. **C)** NaCl concentration did not significantly influence the conjugation in the tested range and also **D)** the combination of the two beneficial conditions, MES pH 5.5 and 10 % (v/v) DMSO did not have an additive effect. **E)** We also could not observe a further beneficial effect of low concentrations of SDS which has been shown to increase protein protein conjugation involving ubiquitin (Schneider, Schneider et al. 2016). 5 mM and 10 mM SDS seemed to completely prevent conjugation.

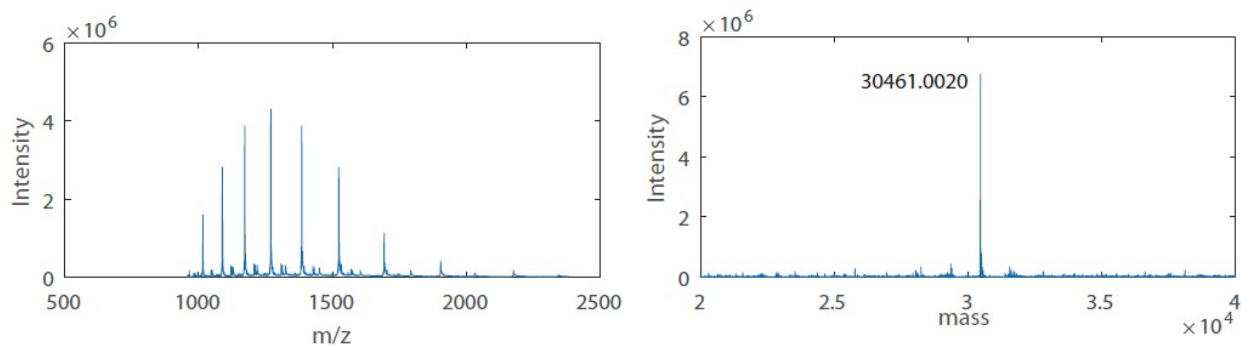


Figure S6. Raw (left) and deconvoluted (right) MS spectrum of GBP-GBP. Calculated Mass: 30461 Da = 15232 Da (GBP-N3) + 15229 Da (GBP-CC)

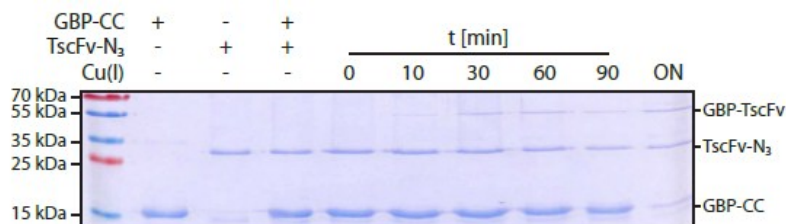


Figure S7. GBP-TscFv heterodimer formation by CuAAC at 4 °C. SDS-PAGE time course analysis of GBP-TscFv heterodimer formation. The monomeric substrates, alkynyl-GBP and azido-TscFv, of approx. 17 kDa and 30 kDa respectively, form a dimeric product (GBP-TscFv) in CuAAC.

	Cu 224.7 nm µg/ml	Cu 324.8 nm µg/ml
GBP-TscFv	<LOD	<LOD
GBP-TscFv + 0.25 mM CuSO₄	17.38	17.22
LOD(µg/ml)	0.01	0.06

Table S1. Copper content analysis by ICP-OES. Samples were diluted 1:5 in dialysis buffer. Samples were prepared by addition of 5 volumes 3 % HNO₃ and copper emission was recorded at two wavelengths (224.7 nm and 324.8 nm) on a Varian Vista-PRO CCD Simultaneous ICP-OES instrument. No residual copper was detected in the dialyzed sample. The same sample spiked with 0.25 mM CuSO₄ (equals CuSO₄ concentration in click reaction) served as a positive control. Measurements were performed by J. Obel (Dept. of Chemistry, LMU Munich)

Experimental Section

Chemical synthesis

The Carboxyfluorescein–Tub-tag peptide (CF-VDSVEGEGEEEGEE) was synthesized as previously described¹ using standard Fmoc based SPPS. The synthesis of O-propargyl-L-tyrosine was carried out according to a known procedure in literature.²

Cell lines

HER2 overexpression cell line SKBR3 (ATCC HTB30) and a control cell line with neglectable HER2 expression levels (1000 fold less than SKBR3), MDA-MB-468 (ATCC HTB-132), were cultured in DMEM/F12 + Glutamax-I (Gibco) supplemented with 10 % FCS at 37 °C, 5% CO₂.

Protein expression, purification and SDS-PAGE analysis

TTL was expressed and purified according to a published protocol as follows¹. TTL was expressed from a pET28 vector in *E. coli* BL21(DE3) as Sumo-TTL fusion protein with an N-terminal His-Tag. Cells were induced with 0.5 mM IPTG and incubated at 18°C for 18 h. Lysis was performed in presence of Lysozyme (100 µg/ml), DNase (25 µg/ml) and PMSF (2 mM) followed by sonification (Branson® Sonifier; 5 times 7 x 8 sec, 40 % amplitude) and debris centrifugation at 20.000 g for 30 min. His-SUMO-TTL was purified using a 5 ml His-Trap (GE Healthcare). Purified protein was desalted on a PD10 column (GE Healthcare) by buffer exchange to MES/K pH 7.0 (20 mM MES, 100 mM KCl, 10 mM MgCl₂) supplemented with 50 mM L-glutamate, 50 mM L-arginine and 3 mM β-mercaptoethanol. Protein aliquots were shock-frozen and stored at -80°C.

Tub-tagged **GBP** (GFP binding protein) nanobody was expressed with a N-terminal His-tag in *E.coli* JM109 as described previously.¹

N-terminally His-tagged **eGFP** (enhanced green fluorescent protein) was expressed from a pRSET5D expression vector in *E.coli* JM109 following the GBP expression and purification protocol.

Tub-tagged **TscFv**, a single chain Fragment variable of the variable domains of the Her2 binding antibody Trastuzumab, was assembled from VL and VH coding PCR fragments and cloned into a pNE phagmid vector suitable for periplasmic expression³ (kindly provided by Andreas Ernst) with standard molecular cloning techniques adopted from⁴. The construct has the following topology: SS---His-tag---T(V_L-(G₄S)₄-V_H)---Tub-tag with an N-terminal signal sequence (SS) for periplasmic expression. Expression in *E. coli* BL21 (DE3) was induced at OD₆₀₀ = 0.6 - 0.8 with 1 mM IPTG. Cells were incubated at 30 °C overnight. For periplasmic extraction cells were pelleted, resuspended in periplasmic extraction buffer I (20 % w/v Sucrose, 100 mM Tris.HCl, 1mM EDTA, pH 8.0) and incubated for 10 min at 4 °C. Cells were pelleted again, resuspended in periplasmic extraction buffer II (5 mM MgCl₂ in H₂O) and incubated for 20 min at 4 °C. Both extraction fractions were pooled and His-tagged TscFv-tub was purified using a 1 ml His-Trap column (GE Healthcare) followed by gel filtration on a Superdex 200 Increase 10/300 GL (GE Healthcare) using PBS as a running buffer. Protein preparation was concentrated with Amicon ultra filter units (MWCO 10 kDa, Merck Millipore) and stored at 4 °C.

Intact protein MS

Intact proteins were analyzed using a Waters H-class instrument equipped with a quaternary solvent manager, a Waters sample manager-FTN, a Waters PDA detector and a Waters column manager with an Acquity UPLC protein BEH C4 column (300 Å, 1.7 µm, 2.1 mm x 50 mm). Proteins were eluted with a flow rate of 0.3 mL/min and a column temperature of 80°C. The following gradient was used: A: 0.01% FA in H₂O; B: 0.01% FA in MeCN. 5-95% B 0-6 min. Mass analysis was conducted with a Waters XEVO G2-XS QToF analyzer. Proteins were ionized in positive ion mode applying a cone voltage of 80 kV. Raw data was analyzed with MaxEnt 1 and deconvoluted between 10.000 and 40.000 Da with an accuracy of 1 Da/channel and 0.1 Da/channel for the GBP dimer.

Chemoenzymatic labeling of Tub-tag peptide with O-propargyl-L-tyrosine or 3-azido-L-tyrosine

TTL reactions with the substrate peptide CF-VDSVEGEGEEEGEE (0.2 mM) were performed in MOPS/K buffer (20 mM MOPS/K, 100 mM KCl, 10 mM MgCl₂, 2.5 mM ATP, 5 mM reduced GSH) at pH 7. The concentration of the respective tyrosine derivative was varied from 1 mM to 20 mM. To start the reaction 1 µM TTL was added and the reaction was incubated at 37°C. Aliquots (25 µL) were taken at defined time points and mixed with equal volume of ACN/ddH₂O 1:1, 0.2% TFA, thereupon subjected to RP-UPLC analysis.

Analytical RP-UPLC

RP-UPLC analysis was conducted on a Vanquish Flex UHPLC System with a DAD detector, Split Sampler FT, Column Compartment H and binary pump F (Thermo Fisher Scientific, USA) using a Hypersil Gold Vanquish 1.9 µm, 150 x 2.1 mm RP-UPLC-column (Thermo Fisher Scientific, USA) with a flow rate of 0.5 mL/min. The following gradient was used: (A= H₂O + 0.1% FA, B = ACN + 0.1% FA) 5% B -1.0 - 0.0 min, 5% B 0.0 – 0.5 min, 5-95% B 0.5 - 5.5 min, 95% B 5.5 – 6.5 min, 95-5% B 6.5 – 7.0 min, 5% B 7.0 – 8.0 min. UV chromatograms were recorded at 220 nm.

Preparative HPLC

Preparative HPLC was conducted on a Dionex Ultimate 3000 HPLC System with a UltiMate 3000 AFC automated fraction collector (Thermo Fisher Scientific, USA) using a ReproSil-XR 120 C18, 5 µm, 250 x 6,5 mm column (Dr. Maisch, Germany) with a flow rate of 2 mL/min. The following gradient was used: (A= H₂O + 0.1% TFA, B = ACN + 0.1% TFA) 5% B -5.0 - 0.0 min, 5% B 0.0 – 1 min, 5-95% B 1 - 60 min, 95% B 60 – 64 min, 95-5% B 64 – 65 min, 5% B. UV chromatograms were recorded at 220 nm.

Chemoenzymatic labeling of GBP-tub and TscFv-tub with 3-azido-L-tyrosine

TTL catalyzed ligation of 3-azido-L-tyrosine (Watanabe Chemical Industries LTD) to Tub-tagged proteins was performed in 25 - 500 μ L reactions consisting of 50 μ M TscFv-tub or 100 μ M GBP-tub, 1/5 equivalent TTL and 1 mM 3-azido-L-tyrosine in TTL-reaction buffer (20 mM MES, 100 mM KCl, 10 mM $MgCl_2$, 2.5 mM ATP and 5 mM reduced glutathione) at 30°C for 3 h. TTL and excess 3-azido-L-tyrosine was removed by size exclusion chromatography on a Superdex 200 Increase 10/300 GL column (GE Healthcare) using PBS as a running buffer.

Chemoenzymatic labeling of GBP-tub with O-propargyl-L-tyrosine

TTL catalyzed ligation of O-propargyl-L-tyrosine to GBP-tub was performed in 25 - 500 μ L reactions consisting of 100 μ M GBP-tub, 20 μ M TTL and 10 mM O-propargyl-L-tyrosine in TTL-reaction buffer (20 mM MES, 100 mM KCl, 10 mM $MgCl_2$, 2.5 mM ATP and 5 mM reduced glutathione) at 30°C for 3 h followed by size exclusion chromatography on a Superdex 200 Increase 10/300 GL column (GE Healthcare). For optimization experiments the reactions were carried out in the same manner with O-propargyl-L-tyrosine concentration ranging from 1 mM to 20 mM and TTL concentrations of 5 μ M and 20 μ M. Reaction mixtures were desalted by buffer exchange to PBS with Zeba Spin desalting columns (7K MWCO, Thermo Scientific).

CuAAC for small molecule labeling of azido-GBP and alkynyl-GBP

CuAAC reactions were performed with 100 μ M azido-GBP or alkynyl-GBP and 1 mM biotin-PEG₄-alkyne (Sigma-Aldrich) or 1 mM 6-FAM-azide (6-Carboxyfluorescein azide, baseclick), respectively, 0.25 mM $CuSO_4$, 1.25 mM THPTA (Tris(benzyltriazolylmethyl)amine), 5 mM aminoguanidine, 5 mM sodium ascorbate in 1x PBS at room temperature. Reactions were quenched at different timepoints by buffer exchange to PBS. Proteins were separated by SDS-PAGE and stained with Coomassie stain. 6-FAM-conjugates were additionally visualized by detection of in-gel fluorescence on an Amersham Imager 600 system (GE Healthcare).

CuAAC for GBP-GBP homodimer formation

CuAAC reactions for GBP homodimer formation were performed with 20 μ M azido-GBP, 20 μ M alkynyl-GBP, 0.25 mM $CuSO_4$, 1.25 mM THPTA, 5 mM aminoguanidine, 5 mM sodium ascorbate in either 100 mM MES pH 5.5 or 1x PBS/10 % (v/v) DMSO at 4 °C and 30 °C. Reactions were quenched at different timepoints by addition of 125 mM EDTA and buffer exchange to PBS. Proteins were separated by SDS-PAGE and stained with Coomassie stain. Conversion efficacy was estimated by densitometric analysis of coomassie stained SDS-PAGE gels. For preparative isolation of GBP-GBP dimers CuAAC reactions were quenched with 125 mM EDTA (Ethylenediaminetetraacetic acid) after 3 h and subjected to size exclusion chromatography as described above. To optimize the CuAAC reaction various buffer components, pH values and additives were tested. The common set up of these reactions was 15 μ M azido-GBP, 15 μ M alkynyl-GBP, 0.25 mM $CuSO_4$, 1.25 mM THPTA, 5 mM aminoguanidine, 5 mM sodium ascorbate, 100 mM buffer component X, pH X at 25 °C for 2 h. The effect of DMSO was assessed in 1x PBS, of NaCl in 20 mM Tris pH 7.5 and of SDS in 100 mM MES pH 5.5. CuAAC reactions were quenched with 125 mM EDTA and buffer exchanged to PBS with Zeba Spin desalting columns (7K MWCO, Thermo Scientific). Samples were analysed by SDS-PAGE as described above.

Conjugation efficiencies were assessed from scanned Coomassie stained SDS-PAGE gels. For this, the Gel Analyzer plugin of the Fiji software was used to quantify band intensities. For small molecule conjugation efficiency was calculated using the following equation: $\text{Intensity conjugate} / (\text{intensity conjugate} + \text{intensity unconjugated protein})$. For protein-protein conjugation the following equation was used: $\text{Intensity conjugate} \times (M_w(\text{protein A})/M_w(\text{protein A+B})) / (\text{intensity protein A} + \text{intensity conjugate} \times (M_w(\text{protein A})/M_w(\text{protein A+B})))$, where protein B was used in excess to protein A.

CuAAC for GBP-TscFv heterodimer formation

CuAAC reactions for TscFv-GBP heterodimer formation were performed with 15 μM azido-TscFv, 60 μM alkynyl-GBP, 0.25 mM CuSO_4 , 1.25 mM THPTA (Tris(benzyltriazolylmethyl)amine), 5 mM aminoguanidine, 5 mM sodium ascorbate in 100 mM MES pH 5.5 at 4 $^\circ\text{C}$ and 30 $^\circ\text{C}$. Reactions were quenched at different timepoints by addition of 125 mM EDTA and buffer exchange to PBS. Proteins were separated by SDS-PAGE and stained with Coomassie stain. Conversion efficacy was estimated by densitometric analysis of coomassie stained SDS-PAGE gels. GBP-TscFv samples were dialyzed two times against 2 L 20 mM Tris, 50 mM EDTA, pH 8.0 over night at 4 $^\circ\text{C}$ to remove copper ions.

eGFP binding assay and analytic size-exclusion chromatography

9 μM purified GBP-GBP was mixed with 0, 1 or 4 equivalents of eGFP in PBS and incubated for 10 min at room temperature. Complex formation was assessed by size exclusion chromatography on an Aekta Pure system equipped with a Superdex 200 Increase 10/300 GL column. Absorption at 280 nm and 488 nm was monitored to detect proteins and eGFP containing complexes.

Her2 binding assay and fluorescence microscopy

SKBR3 and MDAMB468 cells were seeded on sterile coverslips and incubated overnight at 37 $^\circ\text{C}$, 5 % CO_2 for cell attachment. Cells were washed three times with 1x PBS prior to fixation for 10 min in 1x PBS/4% PFA (formaldehyde). Fixation was stopped by addition of an equal volume 1x PBST (PBS + 0.05 % Tween20) followed by two more washes with PBST. TscFv-GBP heterodimer was added and incubated for 1 h at room temperature (RT). Unbound TscFv-GBP was removed by three washes with PBS. eGFP was added and incubated for 30 min at RT followed by three washes with PBS. Coverslips were mounted on glass slides and images were acquired on a Leica SP5 confocal microscopy system equipped with a 63x1.40 oil immersion objective. Laserlines 405 nm and 488 nm were used in combination with standard DAPI and GFP filter settings. Image processing was carried out with ImageJ 1.5.1h software extended by the Fiji processing package.

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