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Regulation of ubiquitin-binding proteins by monoubiquitination

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Proteins containing ubiquitin-binding domains (UBDs) interact with ubiquitinated targets and regulate diverse biological processes, including endocytosis, signal transduction, transcription and DNA repair[1–3]. Many of the UBD-containing proteins are also themselves monoubiquitinated, but the functional role and the mechanisms that underlie this modification are less well understood. Here, we demonstrate that monoubiquitination of the endocytic proteins Sts1, Sts2, Eps15 and Hrs results in intramolecular interactions between ubiquitin and their UBDs, thereby preventing them from binding in *trans* to ubiquitinated targets. Permanent monoubiquitination of these proteins, mimicked by the fusion of ubiquitin to their carboxyl termini, impairs their ability to regulate trafficking of ubiquitinated receptors. Moreover, we mapped the *in vivo* monoubiquitination site in Sts2 and demonstrated that its mutation enhances the Sts2-mediated effects of epidermal-growth-factor-receptor downregulation. We propose that monoubiquitination of ubiquitin-binding proteins inhibits their capacity to bind to and control the functions of ubiquitinated targets *in vivo.*

The attachment of a single ubiquitin molecule (monoubiquitin) to a variety of cell-surface receptors is sufficient to drive their internalization and degradation[2,4–6]. Several endocytic adaptor proteins that control these processes — such as Eps15, epsins and Hrs — harbour one or more ubiquitin-binding domains (UBDs) that are able to recognize the ubiquitinated receptors and sort them along the endocytic pathway[2]. Interestingly, UBDs often mediate monoubiquitination of the proteins that contain them[2,3,7]. However, it is not yet understood whether and how monoubiquitination of ubiquitin-binding proteins may contribute to the regulation of their functions *in vivo*.

The suppressors of T-cell receptor signalling (Sts) 1 and 2 are ubiquitin-binding proteins that suppress signalling via T-cell receptors[8] and regulate endocytic sorting of receptor tyrosine kinases[9,10]. Sts1 and Sts2 are recruited to activated epidermal growth factor (EGF) and platelet-derived growth factor receptors via the ubiquitin ligase Cbl and bind to ubiquitinated receptor complexes through their amino-terminal ubiquitin-associated (UBA) domains. Both of these steps are required for the ability of Sts proteins to interfere with EGF receptor (EGFR) endocytosis and degradation, but the molecular mechanisms that underlie regulation of Sts in these processes remain elusive.

Monoubiquitination of Sts1 and Sts2, which is observed as a shift in their mobility on SDS–PAGE gels and which corresponds to the addition of a monoubiquitin to Sts1 and Sts2, is potently enhanced by overexpression of monoubiquitin in the cell (Fig. 1a). As several UBDs promote ubiquitination of their host proteins[3], we investigated whether ubiquitination of Sts1 and Sts2 was also dependent on the presence of functional UBA domains. Mutation of glycine (G) and phenylalanine (F) in the highly conserved MGF motif of the UBA of Sts1 (Sts1-GF/AA; Fig. 1b) abolished binding to monoubiquitin (Fig. 1b), as well as its monoubiquitination (Fig. 1a, b, lower panel). The same mutations did not completely block ubiquitin binding of Sts2 (Fig. 1b). However, mutation of Lys 40 in the UBA of Sts2 impaired its interaction with monoubiquitin (Fig. 1b) and monoubiquitination of Sts2 (Fig. 1, b). These results show that the UBA domains of Sts1 and Sts2 are crucial determinants of both their ubiquitin binding and monoubiquitination, and provide the first example of proteins that undergo monoubiquitination that is mediated by UBA domains.

To identify the lysine(s) in Sts proteins that had been monoubiquitinated *in vivo*, we purified human Sts2 by immunoaffinity columns and subjected the monoubiquitinated form to trypsin digestion and liquid chromatography-tandem mass spectrometry (LC-MS–MS) analysis (see Supplementary Information, Fig. S1). We identified Lys 202, which accounted for the majority of incorporated ubiquitin in the Sts2 molecule, in addition to three minor sites (Lys 15, Lys 309 and Lys 358) (Fig. 1c; and see Supplementary Information, Fig. S1). Mutation of Lys 202 to arginine ($Sts2^{K202R}$) efficiently impaired Sts2 monoubiquitination (Fig. 1c), supporting the notion that Lys 202 is the main monoubiquitination site of Sts2 *in vivo*.

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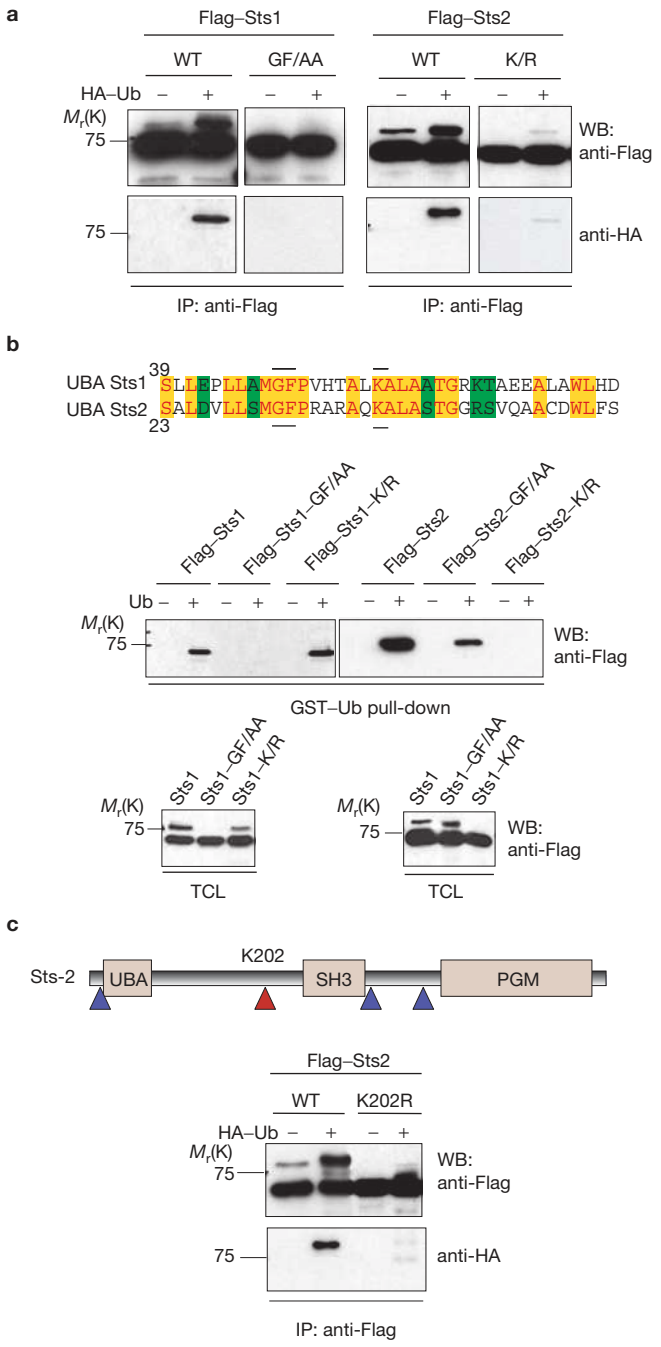

Figure 1 Functional UBA domains are required for monoubiquitination of Sts1 and 2 in cells. (**a**) Overexpression of ubiquitin (Ub) leads to UBA-dependent monoubiquitination of Sts1–2. Lysates of HEK293T cells co-expressing the indicated Flag–Sts1–2 constructs and HA–Ub or empty vector were subjected to immunoprecipitation (IP) using an α-Flag antibody, followed by western blotting (WB). (**b**) Alignment of the UBA domains of Sts1 and 2. Black bars indicate residues that have been mutated for Ub-binding and ubiquitination assays. Lysates of HEK293T cells overexpressing Flag–Sts1–2 wild-type (WT) or the indicated UBA mutants were subjected to GST–ubiquitin pull-down assays, followed by western blotting. TCL, total cell lysate. (**c**) Localization of minor (purple) and major (red, Lys 202) ubiquitination sites of Sts2 (upper panel). Lower panel: Lys 202 was mutated to arginine and the corresponding Flag–Sts2 construct was transfected in HEK293T cells, together with HA–ubiquitin or empty vector. The lysates were subjected to immunoprecipitation and western blotting.

Monoubiquitination can have several functional consequences for the targeted protein, including changes in binding properties, subcellular localization and activity[1–3]. Using *in vitro* ubiquitin-binding assays, we found that monoubiquitinated Sts1 and Sts2 did not interact with exogenous monoubiquitin (Fig. 2a). We tested whether this phenomenon is also true for other endocytic adaptor proteins that are known to be monoubiquitinated, such as the ubiquitin interacting motif (UIM)-containing Eps15 and Hrs[7,11,12]. Indeed, monoubiquitinated forms of Eps15 (Fig. 2b) and Hrs (Fig. 2c) did not bind to glutathione *S*-transferase (GST)-fused monoubiquitin, whereas the same unmodified protein efficiently did so. To further validate these findings, we created permanently monoubiquitinated proteins by fusing a ubiquitin moiety to the carboxy-terminal part of Sts1, Sts2, Eps15 and Hrs (Fig. 2f). Sts1–ubiquitin, Sts2–ubiquitin, Eps15–ubiquitin and Hrs–ubiquitin chimerae maintained their ability to interact with non-ubiquitinated targets, including Cbl, epsin and STAM, respectively (see Supplementary Information, Fig. S2). However, when tested for binding to GST-fused ubiquitin, all ubiquitin chimerae were impaired in their ability to interact with exogenous ubiquitin (Fig. 2b–e). To exclude that this effect was due to misfolding imposed by the fusion of ubiquitin to these proteins, we introduced a mutation in the conserved hydrophobic patch of ubiquitin (Ile44) that abolishes its binding to known UBDs[3], including the UBA of Sts1 and Sts2 (Fig. 2d, e). Mutation of Ile44 to Ala in the ubiquitin chimerae completely restored the ability of Sts1, Sts2, Eps15 and Hrs to bind to GST–monoubiquitin (Fig. 2b–e). These data support the concept that monoubiquitination of both UIM- and UBA-containing proteins neutralizes their ubiquitin-binding capacities.

We hypothesized that the loss of ubiquitin binding of the monoubiquitinated proteins might be due to an intramolecular interaction between the UBD and the monoubiquitin on the same molecule, thereby preventing its binding to neighbouring ubiquitin targets. Given the fact that Eps15, Hrs and Sts proteins are able to dimerize or oligomerize, and are also found in multimeric protein complexes in cells[2,9], it is possible that their monoubiquitinated forms engage in intramolecular (within the single molecule), intermolecular (between different molecules of the homo-oligomeric complex) or transmolecular (between different proteins in heterologous complexes) interactions. We therefore investigated whether the intramolecular binding between monoubiquitin and the UBA of Sts1 and 2 is sufficient for its auto-inhibition. Dimerization-deficient Sts1–ΔPGM (phosphoglycerate mutase domain) and its ubiquitin chimera were expressed in *Escherichia coli*, which lacks the ubiquitin conjugation system as well as UBD-containing proteins. As shown in Fig. 3a, bacterially expressed Sts1–ΔPGM readily interacted with GST–ubiquitin, whereas Sts1–ΔPGM–ubiquitin was impaired in binding to exogenous ubiquitin. When the same constructs were subjected to chemical cross-linking under conditions in which wild-type Sts1 was completely cross-linked, there was no detectable dimerization of Sts1–ΔPGM–ubiquitin (Fig. 3b). This result confirms that there is no biochemical evidence for intermolecular interactions between the UBA of monomeric Sts1–ΔPGM and the attached ubiquitin to another Sts1–ΔPGM molecule. The same block in ubiquitin binding was also found in the context of the full molecule, and mutation of I44A in Sts1–ubiquitin was able to restore binding to ubiquitin (see Supplementary Information, Fig. S2e). Last, to directly confirm the conformational change resulting from intramolecular UBD–ubiquitin interactions in ubiquitinated Sts1–2–ΔPGM, we took advantage of fluorescence resonance energy

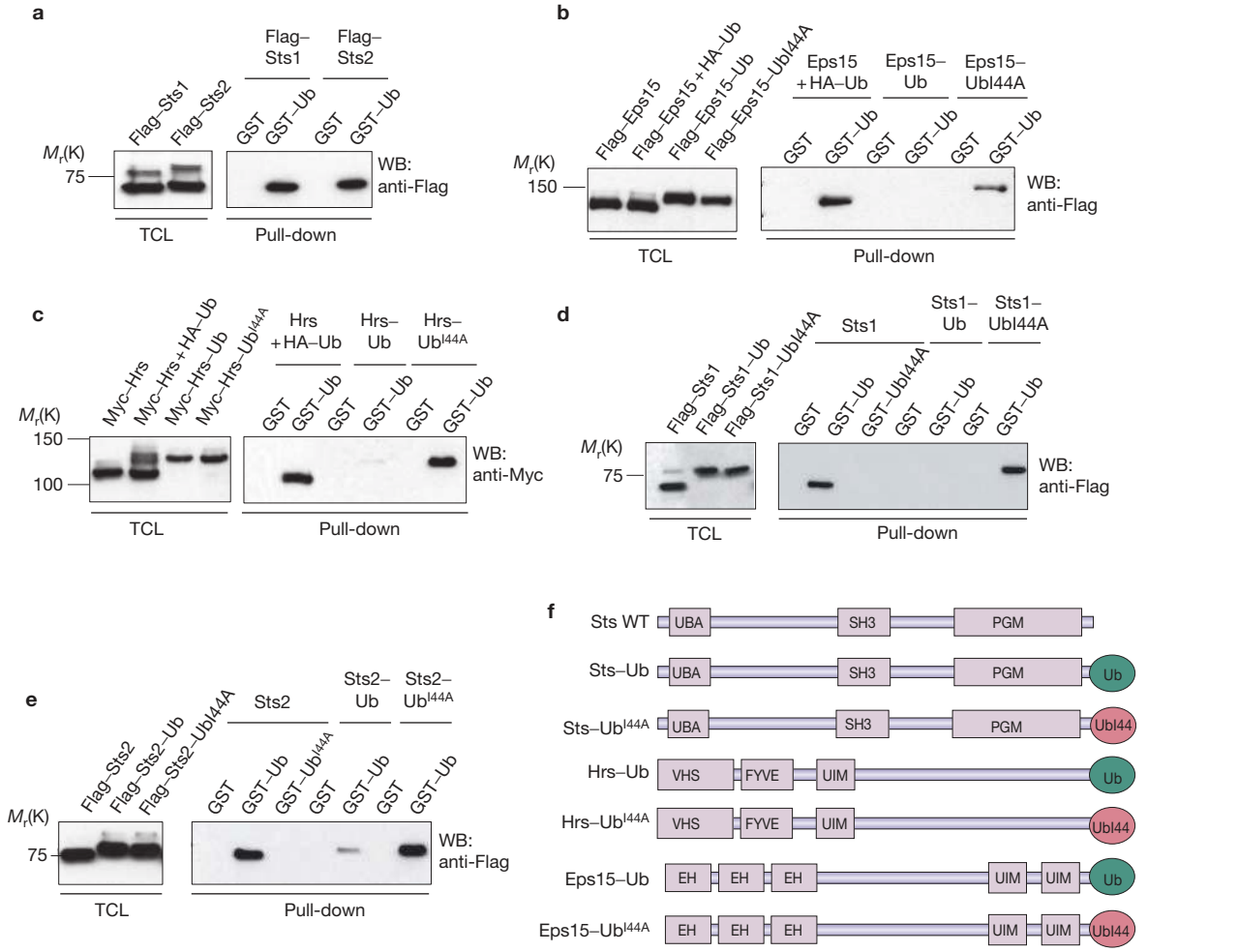

Figure 2 Monoubiquitination of Sts1–2, Hrs and Eps15 abolishes their binding to exogenous ubiquitin. (**a**) Lysates of HEK293T cells overexpressing either Flag–Sts1 or Flag–Sts2 and HA–ubiquitin were subjected to GST–ubiquitin pull-down assays, followed by western blotting (WB). (**b**) Neither natively monoubiquitinated Eps15 nor Eps15 with C-terminally fused monoubiquitin (a single ubiquitin molecule) bind to exogenous ubiquitin. Lysates of HEK293T cells overexpressing the indicated constructs were subjected to GST–ubiquitin pull-down assays, followed by western blotting. (**c**) Monoubiquitinated Hrs does not bind to GST–ubiquitin. Lysates of HEK293T cells overexpressing Myc–Hrs, together with HA–ubiquitin, Myc–Hrs–ubiquitin or Myc–Hrs–ubiquitin^{I44A}, were subjected to GST–ubiquitin pull-down assays, followed by western blotting. (**d**) Lysates of HEK293T cells overexpressing Flag–Sts1 wild type or the indicated chimerae were subjected to GST–ubiquitin pull-down experiments and analysed by western blotting. (**e**) Flag–Sts2 wild type or the indicated chimerae were subjected to GST–ubiquitin pull-down experiments and analysed by western blotting. (f) Schematic representation of ubiquitin chimerae that were used for the described experiments. EH, Eps15-homology domain; FYVE, PtdInsP_3 binding domain; SH3, Src-homology 3 domain; VHS, Vps27/Hrs/STAM domain.

transfer (FRET) technology. We attached the FRET donor cyan fluorescence protein (CFP) to the C terminus and the acceptor citrine to the amino terminus of Sts2–ΔPGM (Cit–Sts2ΔPGM–CFP, Fig. 3c). First, we confirmed that this construct retained the features of the untagged variant described above. For this purpose, we checked its ubiquitination as well as its ubiquitin-binding properties, and found that Cit–Sts2ΔPGM–CFP behaved normally in all assays (see Supplementary Information, Fig. S2f). When expressed in HEK293T cells, this construct led to a FRET signal that was significantly higher than when Cit–Sts2–ΔPGM and Sts2–ΔPGM–CFP were co-expressed (Fig. 3d), indicating that citrine and CFP are in close proximity. Importantly, mutation of the major ubiquitination site Lys 202 to arginine resulted in a decrease of the FRET signal to almost background level. The same decrease was observed in a ubiquitin-binding-deficient mutant in which Lys 40 was mutated to arginine (Cit–Sts2ΔPGM–UBA*–CFP; Fig. 3c, d). This indicates that, in these mutants, citrine and CFP are too distant from each other to enable energy transfer. These data demonstrate that intramolecular interactions between monoubiquitin and the UBA domain in Sts–ΔPGM occur and are sufficient to block the ubiquitin-binding ability of Sts1 and 2.

Our experimental results were additionally evaluated by comparing the thermodynamic properties of intramolecular versus transmolecular ubiquitin binding. The presented biophysical estimates and mathematical equations indicate that monoubiquitinated Sts1 and Sts2 in solution will exclusively bind intramolecularly to their own ubiquitin and not to exogenous ubiquitin (see Supplementary Information). However, if these proteins are localized on scaffolds or platforms (for example, on endosomes), they can engage in transmolecular interactions as the equilibrium between intramolecular versus transmolecular binding depends on the geometrical arrangement of the complex and on the number of ubiquitins that are attached to the target protein (see Supplementary Information). Notably, there will be a dynamic exchange between the intramolecular and transmolecular bound state of UBD-containing

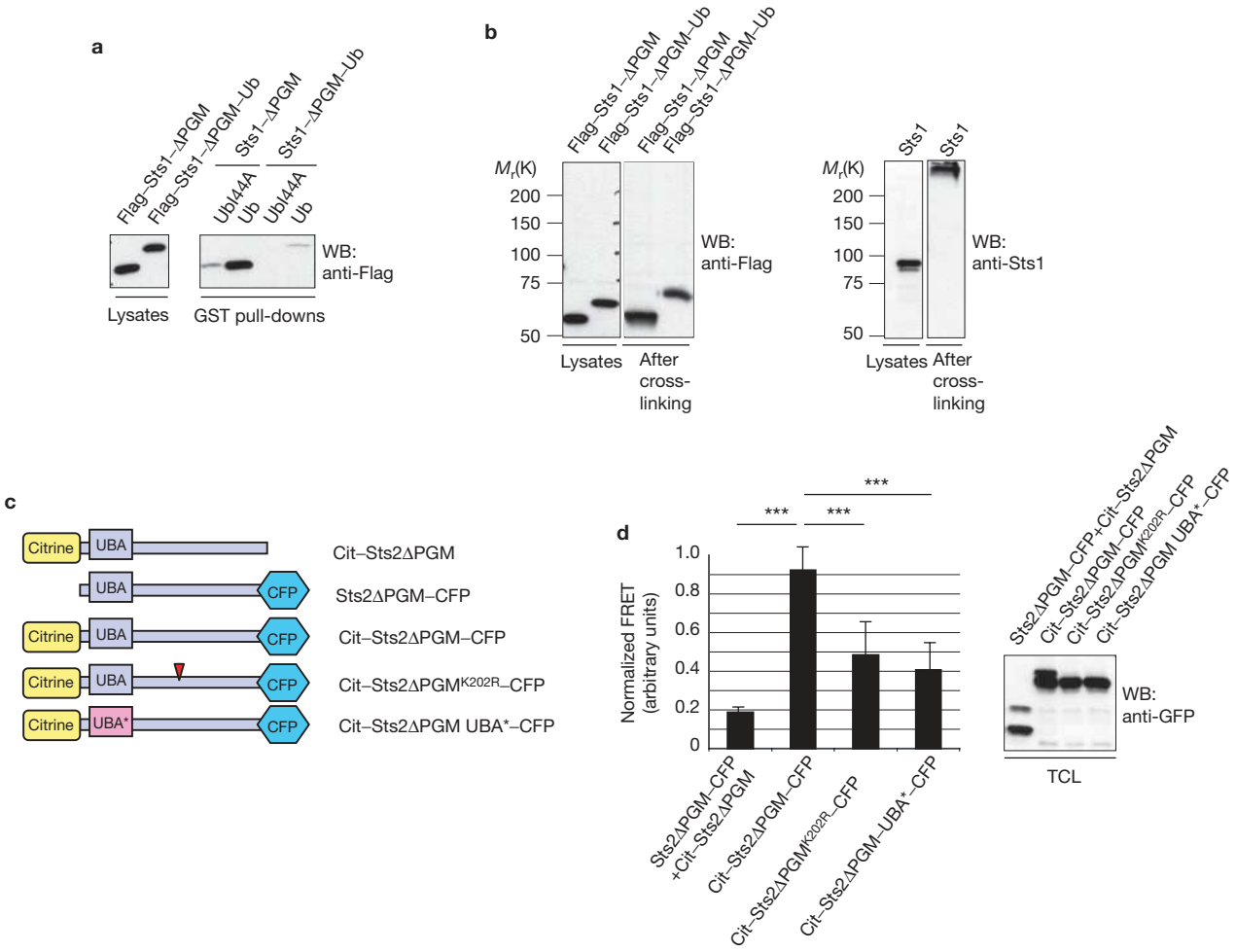

Figure 3 Attachment of ubiquitin to bacterially expressed Sts1 leads to intramolecular UBD–ubiquitin interactions that impairs binding to exogenous ubiquitin. (**a**) Bacterial lysates containing dimerization-deficient Sts1–ΔPGM or Sts1–ΔPGM–ubiquitin were subjected to GST pull-downs and then analysed by western blotting (WB). (**b**) Attachment of ubiquitin does not induce dimerization of Sts–ΔPGM. Bacterial lysates containing Flag–Sts1–ΔPGM or Flag–Sts1–ΔPGM–ubiquitin (left panel) and full-length Sts1 (right panel) were subjected to chemical cross-linking using bis(sulphosuccinimidyl) subarate (BSS) and then analysed by western blotting. K, relative molecular mass in thousands. (**c**) Schematic representation of the constructs used for fluorescence resonance energy transfer (FRET) experiments. CFP, cyan fluorescence protein. (**d**) Left panel: Cell lysates expressing the indicated constructs were analysed using a Victor^3 multilabel reader (Perkin Elmer). *** = P value < 0.001 in a one-tailed Student's t-test with unequal variance. The FRET signal is shown as FRET–CFP in arbitrary units, normalized within each experimental replicate so that the maximum signal equals 1. The error bars represent the mean ± 1SD. Right panel: The same lysates were subjected to immunoblotting.

proteins that allows a flexible adaptation to changes in the local environment and might also explain the observations that Sts–ubiquitin chimerae are monoubiquitinated to a certain extent (data not shown).

Having created the Sts2–ubiquitin chimera, which mimicks permanent monoubiquitination and the Sts2 mutant (Sts2^{K202R}) that is not monoubiquitinated, we were able to analyse the functional importance of monoubiquitination of Sts proteins in cells. Sts1 and Sts2 have been shown to inhibit EGFR degradation by binding to the ubiquitin ligase Cbl and interacting with ubiquitinated receptor complexes via their UBA domains[9]. To reliably detect differences in the ability of Sts2, Sts2–ubiquitin and Sts2^{K202R} mutant to interfere with EGFR degradation even in the presence of endogenous Sts1, we overexpressed them along with EGFR. We made use of the green fluorescent protein (GFP)-tagged EGFR, the degradation kinetics of which are the same as those of wild-type and endogenous receptors (data not shown). Expression of Sts2 in HEK293T cells caused stabilization and accumulation of EGFR–GFP following ligand stimulation (Fig. 4a). By contrast, overexpression of Sts2–ubiquitin, but not of the Sts–ubiquitin^{I44A}, chimerae in cells caused significantly decreased EGFR levels (Fig. 4a). Equivalent data were obtained for Sts1 and Sts1–ubiquitin chimerae on EGFRs at steady state (data not shown) and after EGF stimulation (see Supplementary Information, Fig. S3a). More importantly, Sts2^{K202R}, which cannot be monoubiquitinated in cells, stabilized EGFRs more significantly than Sts2 wild type following EGF stimulation (Fig. 4a), indicating that monoubiquitination of Sts2 inhibits its capacity to block ligand-induced degradation of EGFRs. Sts2 therefore represents the first UBD-containing protein, which is monoubiquitinated at a defined lysine residue and the mutation of which is functionally significant *in vivo*.

To investigate whether a similar negative regulation by monoubiquitination can be found in other components of the endocytic sorting machinery, we tested the role of monoubiquitination of Hrs in Hela cells. Hrs has been previously implicated in the sorting of ubiquitinated transmembrane receptors into clathrin-coated microdomains of the early endosome[13–15]. Because overexpression of Hrs inhibits

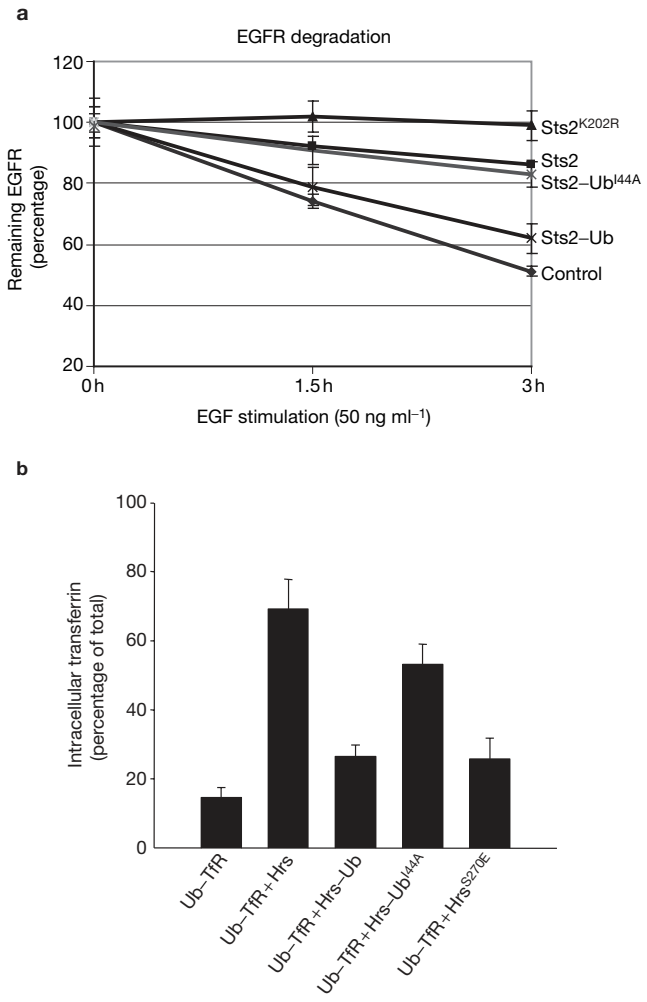

Figure 4 Monoubiquitination of Sts1–2 and Hrs affects their ability to regulate cargo sorting. (**a**) Sts2^{K202R} shows enhanced stabilization of EGFR compared with wild-type Sts2. HEK293T cells co-expressing EGFR–GFP and either of the indicated Flag–Sts2 constructs were stimulated with EGF and analysed by flow cytometry. The data represent the mean ±SEM of three experiments. (**b**) HeLa cells were co-transfected with ubiquitin–TfR, Myc-tagged Hrs, Hrs–ubiquitin or Hrs^{S270E} and Alexa^{568}-transferrin (Tf) was internalized for 15 min. After a 2-h chase period in the presence of cycloheximide and leupeptin, the cells were processed for confocal microscopy. Cell-associated Alexa^{568}-transferrin was quantified as described in Methods. Error bars denote ± SEM. Ub–TfR: $n = 10$; Ub–TfR + Hrs: $n = 40$; Ub–TfR + Hrs–Ub: $n = 40$; Ub–TfR + Hrs^{S270E}: $n = 25$.

recycling of an endocytosed ubiquitin-transferrin receptor (TfR) chimera (ubiquitin–TfR) through a mechanism that requires ubiquitin binding[4], we investigated whether monoubiquitination of Hrs would promote recycling of ubiquitin–TfR. For this purpose, HeLa cells were co-transfected with ubiquitin–TfR and the indicated Hrs constructs, and the intracellular accumulation of endocytosed transferrin following a 2-h chase period was measured. Overexpression of wild-type Hrs or the Hrs–ubiquitin^{I44A} chimera led to a strong cellular retention of transferrin, whereas Hrs–ubiquitin, which mimics a mutant with a non-functional UIM, Hrs-S270E, was unable to retain transferrin in endosomes (Fig. 4b; see Supplementary Information, Fig. S4c). The ability of Hrs to bind and recruit clathrin is thought to be important for its function as an endosomal sorting receptor[16], and we considered the possibility that ubiquitination could affect clathrin recruitment to endosomes. However, overexpression of both Hrs and the Hrs–ubiquitin chimera equally recruited clathrin to early endosomes, whereas Hrs(1–706), which lacks the clathrin-binding C terminus, did not cause any clathrin recruitment (see Supplementary Information, Fig. S3b). Taken together, monoubiquitination of Hrs does not significantly affect its ability to recruit clathrin to early endosomes, but leads to functional inactivation of the UIM domain, thereby affecting trafficking of ubiquitin–TfR.

Recent studies have shed new light on the possible functions of Eps15, Eps15R and epsin in regulating the endocytic route taken by ubiquitinated cargoes in cells[17,18]. It was proposed that the ubiquitin-binding competent Eps15, Eps15R and epsin may engage in *trans* interactions with ubiquitinated cargoes, thereby promoting clathrin-independent endocytosis following stimulation with high doses of EGF[18]. Moreover, it was shown that the UIM of Eps15 is required for its membrane recruitment and co-localization with ligand-bound EGFRs[19,20]. To test whether monoubiquitination of Eps15 regulates its co-localization with activated EGFR, we overexpressed Eps15, Eps15–ubiquitin and Eps15–ubiquitin^{I44A} chimerae in Hela cells that had been treated with high doses of EGF. In these assays, Eps15 and Eps15–ubiquitin^{I44A} chimerae showed significant co-localization with EGFR-positive vesicles, whereas Eps15–ubiquitin chimerae were diffusely expressed in the cytoplasm and were not associated with endocytosed EGFRs (Fig. 5a; see Supplementary Information, Fig. S4). These data indicate that monoubiquitination of Eps15 inhibits its association and co-localization with EGFR-containing endocytic vesicles.

Our results demonstrate that monoubiquitination of UBD-containing proteins triggers intramolecular interactions with the UBDs, thereby preventing them from binding in *trans* to ubiquitinated targets. This is a common phenomenon for several UBDs, including UBA (Fig. 2) and UIM (Fig. 2), as well as the novel UBM and UBZ domains[21]. Changes in their ubiquitination status seem to induce a conformational switch from a ubiquitin-binding state of these proteins to an intramolecular monoubiquitin-inhibited state (Fig. 5b). This could explain how UBD proteins that constitute the endocytic sorting machinery can dynamically exchange their ubiquitinated cargoes along the endosomal compartments. Although the main outcome of monoubiquitination of UBD proteins is inhibition of their ubiquitin-binding capacity, broader functional consequences can also be conceived, including changes in enzymatic activity[1,3], binding properties[17,22] or intracellular localization (Fig. 5a). Biophysical calculations reveal an important difference in the behaviour of proteins in solution and of proteins that are anchored on scaffolds. Freely diffusible monoubiquitinated UBD-containing proteins will invariably engage in intramolecular UBD–ubiquitin interactions due to the high local concentration of ubiquitin being attached to the same molecule. However, a significant pool of proteins is embedded into multimeric complexes *in vivo*, which constrains the mobility of the protein components. In such conditions, the reaction equilibrium shifts towards transmolecular interactions (Fig. 5b). At the same time, the attached monoubiquitin becomes accessible and is either cleaved off or, alternatively, can be available as an additional binding surface, thereby positively promoting the assembly of ubiquitin-linked protein networks. Taken together, ubiquitin plays a dual role in endocytic pathways: it acts as a sorting tag on trafficking cargoes and as a regulatory signal on UBD-containing proteins.

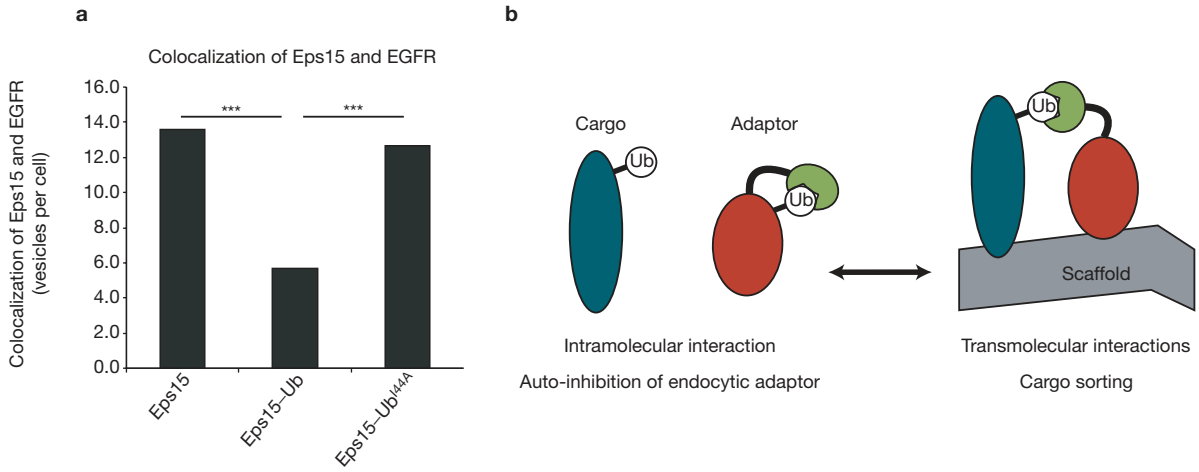

Figure 5 Monoubiquitination of Eps15 impairs localization of Eps15 to EGFR-positive endosomes. (**a**) Eps15 and Eps15–ubiquitin^{I44A} strongly colocalized with endoctyosed endogenous EGFR in Hela cells that have been stimulated with 100 ng ml^{-1} EGF for 5 min, whereas Eps15-ubiquitin was mostly cytoplasmic. Colcalization was quantified by counting endosomal vesicles that were double-positive vesicles for endogenous EGFR and the indicated constructs. Statistical analysis was performed using the two-sided Wilcoxon test: Eps15 WT > Eps15–ubiquitin (***, $P < 0.0001$); Eps15–ubiquitin < Eps15–ubiquitin^{I44A} (***, $P < 0.0001$); Eps15 WT = Eps15–ubiquitin^{I44A} ($P = 0.34$). Eps15: $n = 21$; Eps15–ubiquitin: $n = 21$; Eps15–ubiquitin^{I44A}: $n = 21$. (**b**) Proposed mechanism of monoubiquitin-mediated regulation of endocytic adaptor proteins: In solution, monoubiquitinated UBD-containing proteins adopt a closed, auto-inhibited conformation due to intramolecular UBD–ubiquitin interactions. This pool of proteins will be inactive with respect to transmolecular binding to ubiquitinated targets; for example, cargo sorting. At the same time, a significant pool of the adaptor protein is captured on scaffolds or platforms (for example, on endosomes or complexes on EGFRs). Depending on the protein, different scenarios will take place: Monoubiquitination precludes localization of the adaptor on the scaffold (as is the case for Eps15). Therefore, the adaptor must be de-ubiquitinated to actively participate in cargo sorting. Alternatively, the monoubiquitinated adaptor can be recruited to the scaffold but transmolecular UBD interactions are dependent on the geometrical arrangement of the domains. Multiple monoubiquitination of cargo can, additionally, shift the equilibrium from intra- to transmolecular ubiquitin binding.

METHODS

Reagents, cells, plasmids and antibodies. We generated a polyclonal antibody that recognizes the C-terminal peptide of Sts1: CPTGGFNWRETLLQE. Antibodies against extracellular-regulated kinase (ERK)-2 (C14) and ubiquitin (P4D1) were purchased from Santa Cruz (Heidelberg, Germany), mouse anti-HA (12CA5) antibodies were obtained from Roche (Mannheim, Germany) and anti-FLAG (M2 and M5) antibodies were obtained from Sigma (Taufkirchen, Germany). Anti-FLAG M2 antibodies were used for immunoprecipitation and M5 for western blotting. Anti-Cbl (RF) and anti-EGFR (RK2) antibodies were described previously. Affinity-purified rabbit antibodies against recombinant Hrs have been described previously[16]. Mouse monoclonal antibodies against the human transferrin receptor (B3–25) were obtained from Boehringer Mannheim (Mannheim, Germany). Cy2- and Cy5-labelled secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA). Alexa^{568}-transferrin was obtained from Molecular Probes (Eugene, OR).

EGF was purchased from Peprotech (London, UK). For overexpression experiments, cells were transfected using Lipofectamine Reagent (Invitrogen) according to the manufacturer's instructions. Thirty-six hours after transfection, cells were either lysed or starved for an additional 12 h and then subjected to stimulation with 50 ng ml^{-1} EGF for the indicated times, and then lysed.

Constructs for expressing GFP–EGFR, EGFR, HA–c-Cbl and HA-tagged ubiquitin have been described previously. The EGFR–ubiquitin, pcDNA3–Flag–epsin-1 and the pcDNA3–Flag–Eps15 chimera constructs were kindly provided by P.P. Di Fiore (FIRC, Italy). The pcDNA3–Myc–Hrs and pcDNA3–Myc–Hrs–UIM^{S270E} constructs have been described recently[4]. HA-tagged hStam2 was kindly provided by S. Urbe (University of Liverpool, UK). The constructs for mammalian expression of Sts1 were all generated by polymerase chain reaction (PCR) using pcDNA3–FLAG (Invitrogen) and have been described recently[9]. The Sts1–ubiquitin and Sts2–ubiquitin chimerae were generated by subcloning the cDNAs for ubiquitin wild-type or I44A mutant, and then amplification by PCR in frame with the 3′ terminus of Sts1–2 or their deletions that had previously been subcloned into pcDNA3–FLAG. The stop codon in the sequence of Sts1–Sts2 was removed by mutagenesis to allow expression of the corresponding chimeric proteins. The same procedure was applied to generate Hrs–ubiquitin and Eps15–ubiquitin chimerae. For bacterial expression of Sts1, Sts1–ubiquitin, Flag–Sts1ΔPGM and Flag–Sts1ΔPGM–ubiquitin were cloned into the *Sal*I and *Not*I sites of the pET24–SUMO vector and were expressed in BL21 cells according to the manufacturer's instructions (Lifesensor, Malvern, PA). GST Cbl–CT, containing the proline-rich sequences of Cbl (amino acids 450–860 of human c-Cbl), was expressed in BL21 cells and purified as described previously[9]. The constructs used in the FRET experiments were generated by subcloning citrine into the *Nhe*I and *Hind*III sites of pcDNA3–CFP and subsequent insertion of Sts2, Sts2^{K202R}, Sts2ΔPGM and Sts2ΔPGM K202R into the *Kpn*I and *BamH*I sites of the same vector. pcDNA3–CFP and pcDNA3–citrine were kindly provided by P. Bastiaens (EMBL, Germany).

HEK293T, CHO, Hela and CCL-185 cell lines were purchased from the American Type Culture Collection and grown according to the manufacturer's instructions.

Biochemical assays. For ubiquitin-binding assays, HEK293T cells were transfected with the indicated Flag-tagged Sts1–2 constructs, lysed for 10 min on ice in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton-X-100, 25 mM NaF, 10 μM $ZnCl_2$, pH 7.5) containing protease inhibitors (aprotinin, leupeptin and PMSF). Cell lysates were collected, centrifuged for 15 min (13,000*g*) to remove the insoluble fraction and incubated with GST–ubiquitin or GST coupled to Glutathione sepharose 4B (Amersham Biosciences, Frieburg, Germany) for 4 h at +4 °C. After incubation, the sepharose matrix was washed three times with lysis buffer. Bound proteins were analysed by immunoblotting using α-Flag antibodies.

Chemical cross-linking was performed by incubating the cell lysates with 2 mg ml^{-1} BS^3 (Pierce, Bonn, Germany) for 30 min at room temperature. The reaction was stopped by adding Laemmli buffer. Cross-linked proteins were then analysed by SDS–PAGE and immunoblotting.

Mass spectrometry. Protein bands containing monoubiquitinated Sts1 or Sts2 were excised from the gel and subjected to in-gel reduction, alkylation, trypsin digestion and subsequent sample desalting, as described previously[23]. The peptide mixtures were then analysed by nanoscale LC-MS–MS using Agilent 1100 nano-flow system connected to a 7-Tesla Finnigan linear quadrupole ion trap-Fourier

transform (LTQ-FT) mass spectrometer (Thermo Electron, Bremen, Germany), which was equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark), essentially as described previously[24]. Protein identification was performed with the Mascot software package (Matrix Science, London, UK).

FRET experiments. FRET measurements were performed as recently described[25]. Briefly, HEK293T cells were transfected with either Cit–Sts2ΔPGM + Cit–Sts2ΔPGM–CFP, Cit–Sts2ΔPGM–CFP, Cit–Sts2ΔPGM^{K204}–CFP or Cit–Sts2ΔPGM–UBA*–CFP. Following 24–30 h of transfection, cells were lysed and the lysates were analysed using a Wallac Victor^3 1420 multilabel counter (Perkin Elmer, Wiesbaden, Germany), using the following filters: CFP: 430 nm/8 nm (excitation), 486 nm/10 nm (emission); citrine: 510 nm/10 nm (excitation), 535 nm/25 nm (emission). The FRET signal is shown as FRET–CFP in arbitrary units, normalized within each experimental replicate so that the maximum signal equals 1.

Measurement of transferrin recycling. Transfected HeLa cells were incubated with Alexa^{568}-transferrin (50 μg ml^{-1}) for 15 min at 37 °C in DMEM supplemented with 10% fetal calf serum (FCS). The cells were either fixed directly or chased for 2 h in DMEM with 10% FCS, containing 10 μg ml^{-1} cycloheximide (Sigma), 0.3 mM leupeptin (Peptide Institute, Inc., Osaka, Japan) and 5 mM nitrilotriacetic acid (Sigma). The cells were then processed for confocal microscopy. Coverslips were examined using a Zeiss LSM 510 META confocal microscope equipped with a Plan-Apochromat 63/1.4 oil immersion objective. For quantification of cell-associated Alexa^{568}-transferrin, confocal images of single cells that expressed ubiquitin-TfR and Hrs constructs were recorded at fixed intensity settings below pixel value saturation and analysed by post-image processing. All pixel values above background level (defined as mean values obtained in untransfected cells) were integrated for each cell using the histogram function in the Zeiss LSM Software, version 3.2. To adjust for different receptor expression levels, the transferrin signal was correlated to the level of overexpressed ubiquitin–TfR in each cell by dividing the measured intensity of transferrin by the intensity of the receptor. Intracellular transferrin in each cell after 2 h of chase was represented as the percentage of total cell-associated transferrin after 15 min uptake (defined as the mean intensity from 20 cells).

Measurement of Eps15–EGFR colocalization. Hela cells were transfected with 2 μg DNA using MATra transfection reagent (IBA, Göttingen, Germany) and seeded onto coverslips 12 h post-transfection. After serum starvation for 15 h, the cells were stimulated for 5 min with 100 ng ml^{-1} EGF. Cells were fixed with 4% PFA, permeabilized with digitonin and stained for EGF receptor with a monoclonal mouse antibody (MAb 108; 10 μg ml^{-1}) and for Flag–Eps15 using a polyclonal anti-Flag antibody (1:300; Sigma). Secondary antibodies conjugated with fluorochromes (anti-rabbit-FITC and anti-mouse-Cy3; Jackson Immunoresearch) were used to visualize the primary antibodies. Images were prepared using a Zeiss 510 Meta confocal microscope.

EGFR downregulation assays using flow cytometry. HEK293T cells were transfected with EGFR–GFP and either Sts1, Sts1–ubiquitin, Sts1–ubiquitin^{I44A} or empty vector (control) in 10 cm cell culture dishes. After 24 h, cells were split into 12-well dishes and starved overnight in serum-free medium. The following day, cycloheximide (20 μg ml^{-1}) was added to the cells 2 h before they were left unstimulated or were incubated with EGF (50 ng ml^{-1}) for 30 or 60 min at 37 °C. After stimulation, cells were harvested and analysed using the Epics XL flow cytometer (Beckman-Coulter, Krefeld, Germany). For each sample, 10,000 GFP-positive cells were analysed to determine the amount of remaining EGFR. Mean fluorescence intensity of each sample was calculated using Expo 32 ADC software. Equal expression of the transfected proteins was checked by immunoblotting.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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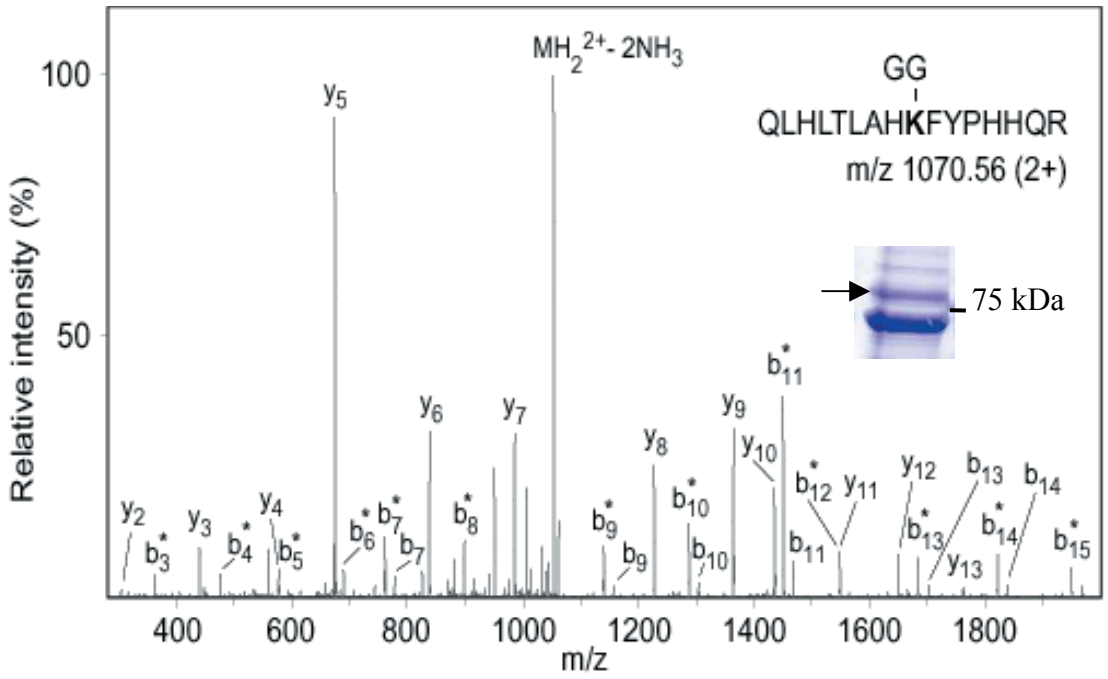


Figure S1 Identification of ubiquitylated lysines in Sts2 by mass spectrometric analysis. The digestion of ubiquitylated proteins with trypsin results in two glycine residues remnant from the C-terminus of ubiquitin attached to the targeted lysine. This leads to a mass shift of 114 Da and a trypsin missed cleavage at the modified lysine. The figure shows the fragmentation pattern of the doubly-charged tryptic peptide derived from Sts2 that contain the ubiquitylated K202. The y series of ions (C-terminus containing fragments) that are produced due to fragmentation are labeled, as well as those from the b ion series (N-terminus containing fragments). The * symbol indicates neutral loss of ammonia for the corresponding fragments.

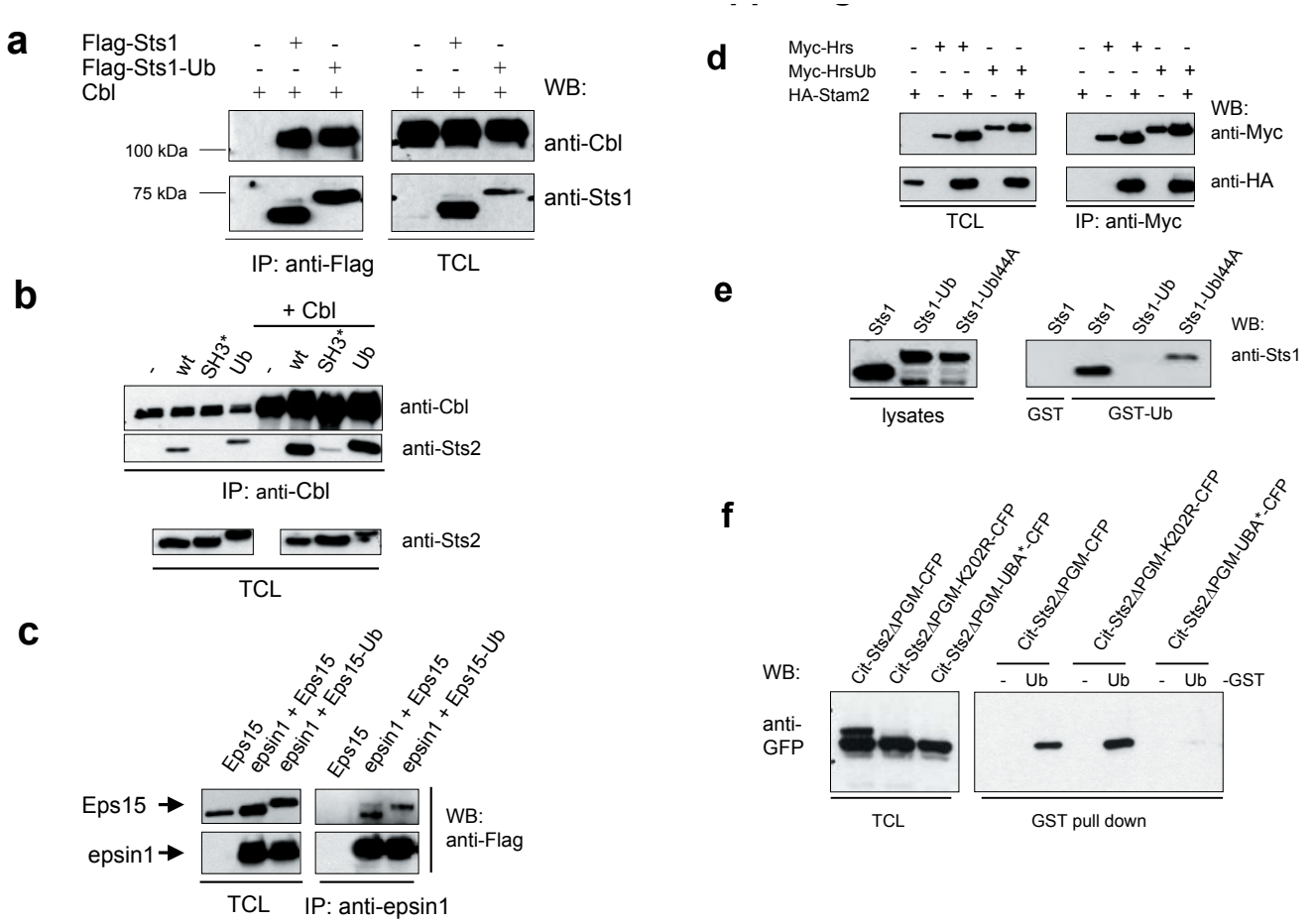
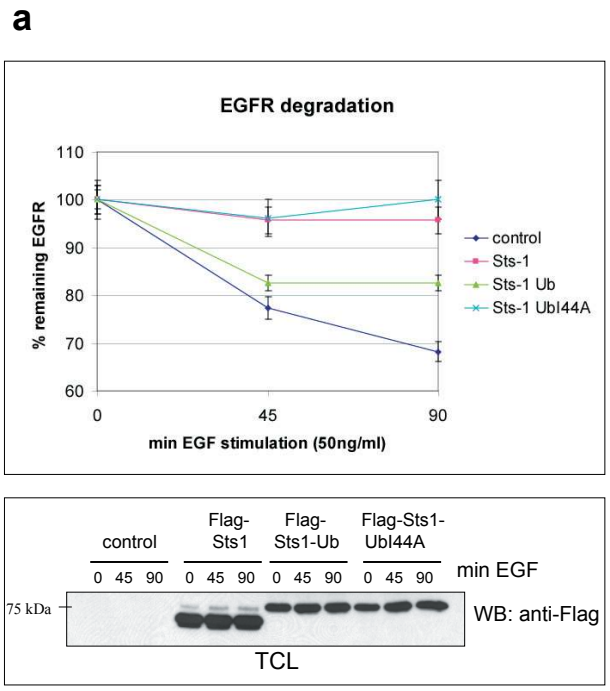


Figure S2 Monoubiquitylation of Sts1/2, Hrs and Eps15 does not affect interactions with Cbl, Stam2 or Epsin, respectively. (a) 293T lysates co-expressing Cbl and either of the indicated constructs were subjected to co-immunoprecipitation using a Flag(M2)-antibody followed by immunoblotting. (b) 293T lysates co-expressing Cbl and either Sts2 wild type, Sts2SH3* (a mutant unable to bind to Cbl) or Sts2Ub were subjected to co-immunoprecipitation using a Cbl-specific antibody followed by immunoblotting. (c) 293T lysates co-expressing the indicated Eps15 constructs and epsin1 were subjected to co-immunoprecipitation using epsin-specific antibodies and analyzed by immunoblotting using anti-Flag antibodies. (d) 293T lysates overexpressing the indicated Hrs constructs with or without HA-Stam2 were subjected to co-immunoprecipitation using an α-Myc antibody. (a) Bacterially expressed Sts1-Ub is blocked in Ub-binding. Bacterial lysates containing Sts1, Sts1-Ub or Sts1-UbI44A were subjected to GST pulldowns using GST-Ub (upper right panel) and GST-Cbl-CT (lower right panel). Precipitated proteins were then analyzed by immunoblotting. (f) Characterization of dimerization-deficient Sts-ΔPGM constructs used for FRET. Attachment of Citrine (Cit) to the N-terminus and CFP to the C-terminus of Sts2-ΔPGM constructs does not affect their ubiquitylation or ubiquitin binding properties. Cell lysates from 293T cells expressing the indicated constructs were subjected to GST-Ub pull downs and analyzed by western blotting.



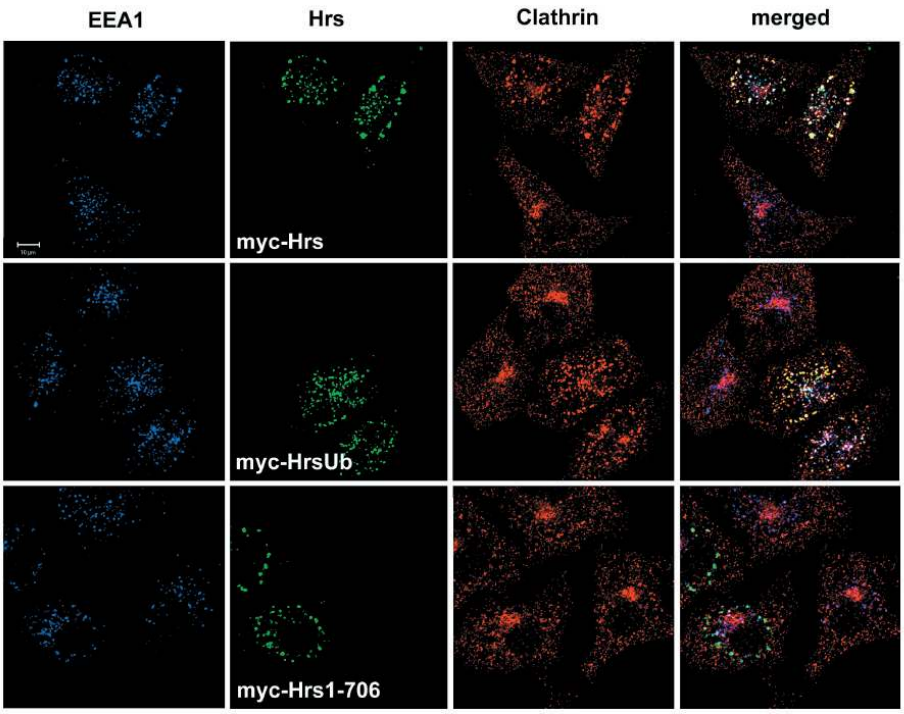


Figure S3 Attachment of Ub to Sts1 inhibits its ability to block EGFR degradation upon EGF stimulation and monoubiquitylation of Hrs does not affect its ability to recruit clathrin to EEA1-positive early endosomes. (a) 293T cells co-expressing EGFR-GFP, Cbl and either of the indicated Flag-Sts1 constructs were starved overnight and then stimulated with EGF for 0, 45 and 90 min. EGFR levels were analyzed by flow cytometry. Equal expression levels of the Sts1 constructs were checked by immunoblotting (lower panel). The data represent the mean (standard error of the mean (S.E.M.) of three experiments. (b) Monoubiquitination of Hrs does not affect its ability to recruit clathrin to EEA1-positive early endosomes. Hela cells were transfected with myc-Hrs, myc-HrsUb or myc-Hrs1-706 (a mutant unable to bind to Clathrin) and immunostaining for endogenous EEA1 (blue), endogenous Clathrin (red) and Hrs (green) was performed.

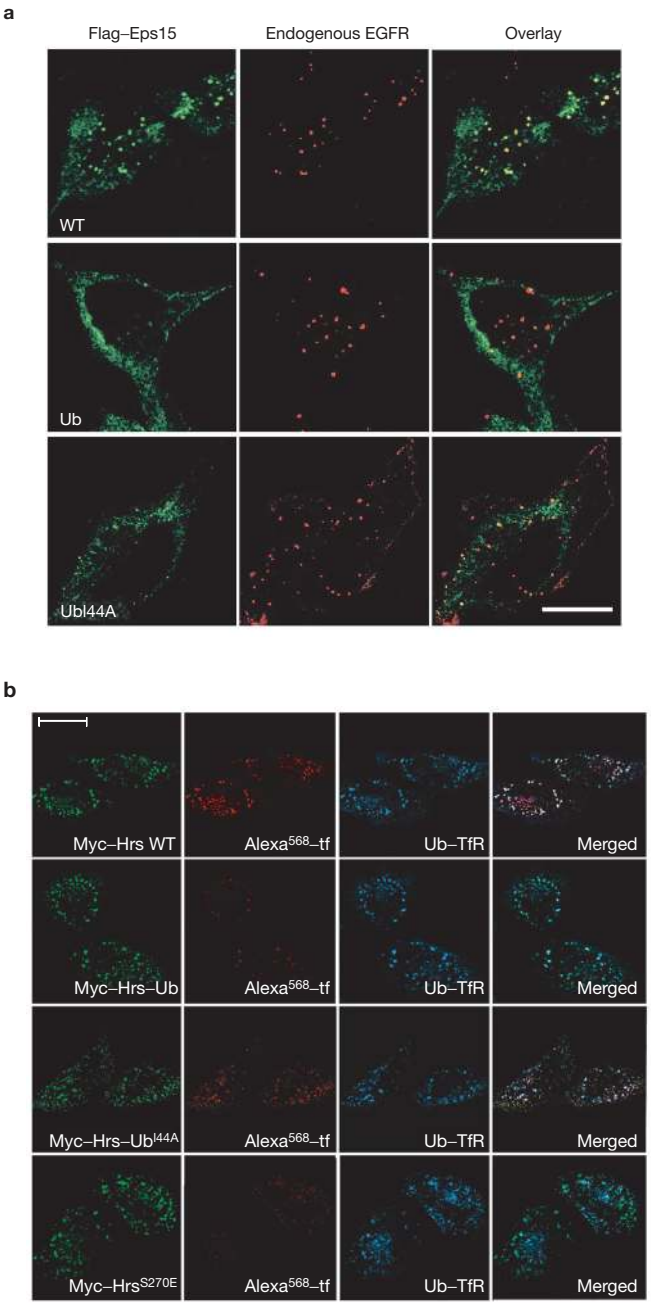


Figure S4 Monoubiquitination of Hrs and Eps15 affects their ability to control ubiquitinated proteins. (a) HeLa cells were co-transfected with Ub-TfR and myc-tagged Hrs, Hrs-Ub or Hrs^{S270E} and Alexa^{568}-transferrin was internalized for 15 min. After a 2hr chase in the presence of cycloheximide and leupeptin, the cells were processed for confocal microscopy. (b) Monoubiquitination of Eps15 impairs localization of Eps15 to EGFR positive endosomes. Hela cells were transfected with Flag-Eps15, Flag-Eps15-ubiquitin or Flag-Eps15-ubiquitin^{I44A}, starved for 15 h and stimulated with 100ng/ml EGF for 5 min. Immunostaining for endogenous EGF receptor and Eps15 was performed. Eps15 and Eps15-ubiquitin^{I44A} colocalized with endocytosed EGFR in vesicular structures, whereas Eps15-ubiquitin was mostly cytoplasmic.

Equilibrium thermodynamics of intra- and transmolecular ubiquitin binding

Consider the following possible reactions of ubiquitylated endocytic adaptor proteins:

$$\text{intramolecular}: \quad \mathrm{UA_{open}} \leftrightarrows \mathrm{UA_{closed}} \tag{1}$$

$$\text{transmolecular}: \quad \mathrm{UA_{open}} + \mathrm{UC} \leftrightarrows \mathrm{UAC}, \tag{2}$$

where $\mathrm{UA_{open}}$ is the ubiquitylated form of an endocytic adaptor protein in the open conformation and $\mathrm{UA_{closed}}$ its closed form where the ubiquitin moiety is bound intramolecularly to the ubiquitin binding domain (UBD). UC is an ubiquitylated cargo (or free ubiquitin) and UAC the transmolecular complex of adaptor and cargo via the UBD of the former (the same reasoning would apply in the case of an intermolecular interaction in a homooligomeric complex). The corresponding equilibrium constants are:

$$K_1 = \frac{[\mathrm{UA_{closed}}]}{[\mathrm{UA_{open}}]} \tag{3}$$

$$K_2 = \frac{[\mathrm{UAC}]}{[\mathrm{UA_{open}}][\mathrm{UC}]} \tag{4}$$

If the intramolecular and transmolecular reactions occur in parallel in free solution in the cytosol, rearranging and combining equations 3 and 4 yields:

$$\frac{[\mathrm{UAC}]}{[\mathrm{UA_{closed}}]} = \frac{K_2}{K_1}[\mathrm{UC}] \tag{5}$$

This means that the ratio of cargo-binding endocytic adaptor (UAC) and intramolecularly inactivated adaptor ($\mathrm{UA_{closed}}$) is independent of the concentration of adaptor protein itself and only dependent on the concentration of ubiquitylated cargo, [UC], and the ratio of the two equilibrium constants, K_2/K_1.

The ratio of the two equilibrium constants can be estimated based on a few simple geometric and thermodynamic assumptions. If we replace the equilibrium constants by the underlying kinetic rate constants of the forward and backward reaction we obtain

$$\frac{K_2}{K_1} = \frac{k_{2forward}}{k_{2reverse}} \frac{k_{1reverse}}{k_{1forward}} \tag{6}$$

If intramolecular and transmolecular complex share the same binding strength, then $k_{1reverse}$ and $k_{2reverse}$ will be equal and cancel out. For the sake of the argument, we can split the rate constant $k_{2forward}$ into two virtual constants: $k_{2forward} = K_{hit} \times k_{react}$, where K_{hit} is associated with the initial approach of the two reacting molecules and k_{react} is the rate constant of their actual transformation into a bound complex. We can now choose the two virtual constants in such a way that $k_{react} = k_{1forward}$, in which case our equation becomes $K_2/K_1 = K_{hit}$. Assuming that diffusion is rapid compared to complex formation K_{hit} will then be the equilibrium constant associated with the formation of a "pre-reaction complex", PRE, which behaves in exactly the same way as if the two interacting domains were part of the same molecule. The original rate equation

$$\frac{\mathrm{d[UAC]}}{\mathrm{d}t} = k_{2forward}\ [\mathrm{UA_{open}}][\mathrm{UC}] \tag{7}$$

then becomes

$$\frac{\mathrm{d[UAC]}}{\mathrm{d}t} = k_{react}\ [\mathrm{PRE}]. \tag{8}$$

The domains in the intramolecular reaction are at most a distance d_{max} apart, defining a "reactive volume" $V = \frac{4}{3}\pi(\frac{d_{max}}{2})^3$ in which the intramolecular reaction has to take place. We assume that any ubiquitylated cargo, UC, that enters a volume V in the vicinity of a ubiquitin binding domain, $\mathrm{UA_{open}}$, will behave as if the consequent reaction is intramolecular. This is a conservative assumption that almost certainly will lead to an over-estimate of the rate of the transmolecular complex formation. The concentration of "pre-reaction complex" in a homogeneous dilute solution at any given time is then given by

$$[PRE] = [\mathrm{UA_{open}}]\ [\mathrm{UC}]\ V\ N_A, \tag{9}$$

where N_A is Avogadro's number. Here, [UC] V N_A is the number of UC molecules within the "reactive volume" V of a single $\mathrm{UA_{open}}$ molecule, which at low concentrations of UC is a good approximation for the fraction of $\mathrm{UA_{open}}$ molecules that have at least one UC molecule in their vicinity V. From 7, 8 and 9 we then obtain

$$\frac{K_2}{K_1} = K_{hit} = V\ N_A, \tag{10}$$

and consequently

$$\frac{[\mathrm{UAC}]}{[\mathrm{UA_{closed}}]} = V\ N_A[\mathrm{UC}]. \tag{11}$$

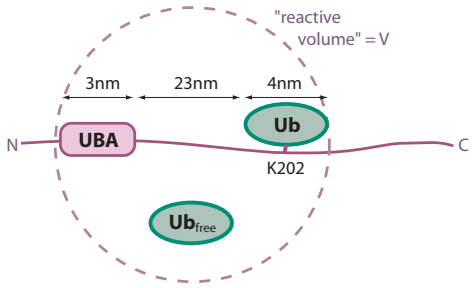


Figure 1: Estimate of the geometrical dimensions of the intramolecular ubiquitin binding reaction in STS-1. The approximate size of ubiquitin and of the ubiquitin binding domain is based on X-ray structures, the maximal length of the intervening sequence is estimated as the length of a fully extended alpha-helix. Combining these estimates leads to a rough (and conservative) estimate for the "reactive volume" V described in the text.

Based on X-ray data we know that the diameter of the UBD is about 3nm and that of ubiquitin about 4nm. In the intramolecular reaction the two domains are separated by a 150 amino acid linker between UBD and K202 of STS-2, which in an extended all-alpha helix conformation would have a length of 23nm. Thus, d_{max} in our case can be estimated as $\leq$ 30nm, so that $VN_A = \frac{4}{3}\pi\ 15^3\ \mathrm{nm}^3\ 6 * 10^{23}\mathrm{mol}^{-1} = 8482\mathrm{M}^{-1}$.

This means that when the concentration of ubiquitylated cargo is [UC] = 1nM = 10^{-9}M, only one in one hundred thousand adaptors will be able to bind cargo, while the remainder are inactivated intramolecularly. Even when the concentration of cargo is higher, e.g. in the high μM range in the case of free ubiquitin, the intermolecular complex will still be only a fraction of the intramolecular interaction.

These biophysical estimates indicate that ubiquitylated endocytic adaptor proteins in free solution will almost exclusively bind intramolecularly to their

own ubiquitin. They would thus be inactive with respect to cargo binding. However, equation 5 shows several ways in which the equilibrium may be shifted towards the transmolecular interaction:

- **Effect of multiple ubiquitylation.** If the cargo protein is ubiquitylated at N sites, the effect will at most be an increase by a factor of N of the active concentration of cargo. In free solution this will not be able to shift the equilibrium considerably towards cargo binding.

- **Changes in the K_2/K_1 ratio.** If the affinity for the *in cis* reaction where much lower than the affinity for the transmolecular reaction, the equilibrium would shift towards cargo binding. However, to achieve even an equal distribution of the adaptor protein between cargo-bound and closed conformation at nanomolar concentrations of cargo, the affinity difference would have to be by a factor of about 10^5. This would only be conceivable if the protein changes its conformation so drastically that the internal ubiquitin can no longer reach the UBA.

- **Changes in the active concentration** [UC]**.** While the cargo protein will never be present at millimolar concentration in a cell, local concentrations may be much higher than the average. As for the the case of changes in the K_2/K_1 ratio, to have relevant consequences the change in concentration would need to be by a factor of many orders of magnitude. Simple recruitment of endocytic adaptors to certain cellular compartments is unlikely to be sufficient for this magnitude of change. However, there is an alternative and related mechanism that could cause the necessary shift: If both endocytic adaptor and cargo are tightly attached to a common scaffold complex, both reactions could be considered as intramolecular. Equations 1 and 2 would then become

$$\mathrm{UA}_{\mathrm{open}} :: \mathrm{UC} \leftrightarrows \mathrm{UA}_{\mathrm{closed}} :: \mathrm{UC} \tag{12}$$

$$\mathrm{UA}_{\mathrm{open}} :: \mathrm{UC} \leftrightarrows \mathrm{UAC} ::, \tag{13}$$

where :: denotes the scaffold complex. Assuming equal affinities for both reactions, the ratio of closed and cargo-bound form will then be

$$\frac{[\mathrm{UAC}::]}{[\mathrm{UA_{closed}}::\mathrm{UC}]} = 1, \tag{14}$$

i.e., the UBD will be distributed equally between the two binding modes. In this case, the details of the geometrical arrangement of the protein domains could even shift the equilibrium towards the transmolecular complex. In addition, N-fold ubiquitylation of cargo within such a scaffolded arrangement could lead to a noticable increase in the ratio by a factor of up to N.

If the binding of ubiquitylated endocytic adaptor proteins to UBDs would always be transmolecular, equations 1 and 2 would become

$$\text{binding to inhibitor } \textit{in trans}: \quad \mathrm{UA_{open}} + \mathrm{Inhibitor} \leftrightarrows \mathrm{UA_{inhibited}} \tag{15}$$

$$\text{binding to cargo } \textit{in trans}: \quad \mathrm{UA_{open}} + \mathrm{UC} \leftrightarrows \mathrm{UAC}, \tag{16}$$

and the ratio of the corresponding inhibited and cargo-bound complexes would be

$$\frac{[\mathrm{UAC}]}{[\mathrm{UA_{inhibited}}]} = \frac{K_2^*}{K_1^*}\,\frac{[\mathrm{UC}]}{[\mathrm{Inhibitor}]} \approx \frac{[\mathrm{UC}]}{[\mathrm{Inhibitor}]}. \tag{17}$$

In this case, the two equilibrium constants (K_2^* and K_1^*) will be approximately equal, because they both represent an transmolecular binding between a ubiquitin moiety and a UBD. Thus the ratio between the two complexes is approximately equal to the ratio of the cargo and inhibitor. Thus, adding an excess of cargo, e.g. in the form of free ubiquitin, will lead to a competitive shift of the binding equilibrium towards the cargo-bound form.