

# StUbEx: Stable Tagged Ubiquitin Exchange System for the Global Investigation of Cellular Ubiquitination

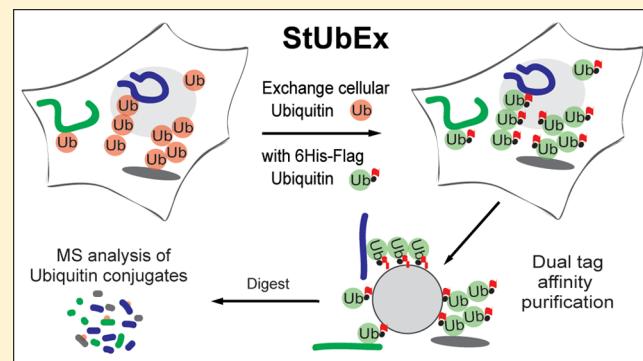
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 [Supporting Information](#)

**ABSTRACT:** Post-translational modification of proteins with the small polypeptide ubiquitin plays a pivotal role in many cellular processes, altering protein lifespan, location, and function and regulating protein–protein interactions. Ubiquitination exerts its diverse functions through complex mechanisms by formation of different polymeric chains and subsequent recognition of the ubiquitin signal by specific protein interaction domains. Despite some recent advances in the analytical tools for the analysis of ubiquitination by mass spectrometry, there is still a need for additional strategies suitable for investigation of cellular ubiquitination at the proteome level. Here, we present a stable tagged ubiquitin exchange (StUbEx) cellular system in which endogenous ubiquitin is replaced with an epitope-tagged version, thereby allowing specific and efficient affinity purification of ubiquitinated proteins for global analyses of protein ubiquitination. Importantly, the overall level of ubiquitin in the cell remains virtually unchanged, thus avoiding ubiquitination artifacts associated with overexpression. The efficiency and reproducibility of the method were assessed through unbiased analysis of epidermal growth factor (EGF) signaling by quantitative mass spectrometry, covering over 3400 potential ubiquitinated proteins. The StUbEx system is applicable to virtually any cell line and can be readily adapted to any of the ubiquitin-like post-translational modifications.

**KEYWORDS:** *Ubiquitin, SILAC, ubiquitin-like, proteomics, affinity purification, quantification*



## INTRODUCTION

A large number of post-translational modifications (PTMs) in eukaryotic cells orchestrate a variety of regulatory physiological mechanisms that are responsible for cell growth, survival, proliferation, and communication with the surroundings.<sup>1</sup> Progress in methodology and technology for the detection of PTMs has given rise to a catalogue of more than 200 different types of modifications.<sup>2</sup> Many of the PTMs are reversible and highly dynamic, implying their complex role in intracellular signaling processes including the formation of transient protein interaction networks via involvement of specific domains recognizing post-translationally modified proteins.<sup>3</sup>

One of the most complex and widespread PTMs in the cell is ubiquitination: a covalent attachment of ubiquitin, a small 8.5 kDa polypeptide, that is conjugated to proteins forming a isopeptide bond between its C-terminal glycine and the *ε*-amino group of lysine residues in the respective target protein. The process of ubiquitination consists of three consecutive steps of enzymatic reactions: activation of ubiquitin via ubiquitin-activating enzymes (E1), transfer of the ubiquitin moiety to ubiquitin-conjugating enzymes (E2), and conjugation of the ubiquitin molecule to a lysine residue of a target protein through the action of E3 ubiquitin ligases, which determine the

specificity of substrate recognition.<sup>4</sup> Counteracting the activity of ubiquitin ligases are the rapid actions of the deubiquitinating enzymes (DUBs) responsible for the removal of ubiquitin moieties from substrates, making ubiquitination a reversible and highly dynamic modification. Similar to other PTMs, ubiquitination can reside on a single site or simultaneously on several positions of the targeted protein, thereby forming mono- and multiubiquitination, respectively. However, the diversity of ubiquitin signaling is further determined by the ability of the ubiquitination machinery to build ubiquitin chains (polyubiquitination) via one of the seven internal lysine residues of ubiquitin itself or through its initial methionine.<sup>5</sup> The mode of protein modification (mono-, multi-, or polyubiquitination) as well as the type of conjugated ubiquitin chains creates a unique signal for ubiquitin recognition by specific ubiquitin-binding domains (UBDs) that determines the fate of ubiquitinated substrates.<sup>6</sup> For example, tagging of a protein with Lys-48 chains leads to its proteasomal degradation,<sup>7</sup> whereas mono- and polyubiquitination through Lys-63 chains have nonproteolytic functions, such as protein

**Received:** June 4, 2014

**Published:** August 5, 2014

trafficking, endocytosis, DNA damage repair, and autophagy.<sup>8–12</sup>

The role of quantitative mass spectrometry (MS)-based proteomics in elucidating functional consequences of PTMs is steadily growing.<sup>13,14</sup> High-resolution MS has become an inevitable tool for large-scale analyses of site-specific PTMs, expanding our knowledge of phosphorylation,<sup>15–20</sup> acetylation,<sup>21–23</sup> and N-glycosylation,<sup>24</sup> among other modifications. Significant progress in the analyses of protein ubiquitination by MS was made by the development of antibodies recognizing the diglycine (di-Gly) ubiquitin remnant that remains covalently attached to substrate lysine residues on ubiquitinated proteins following the trypsin digestion usually performed during MS sample preparation.<sup>25</sup> Recent studies utilizing this approach for enrichment of di-Gly-containing peptides have dramatically extended the list of identified ubiquitination sites.<sup>26–28</sup> Nonetheless, in addition to the generic challenges associated with peptide-enrichment strategies, such as sequence biases of the antibodies and a fraction of the proteolytic peptides residing outside the MS mass measurement range, di-Gly motif-directed analyses cannot distinguish between ubiquitination and at least two other ubiquitin-like modifiers (UbIs), namely, NEDD8 and ISG15, as they produce an identical di-Gly remnant.<sup>26,29</sup>

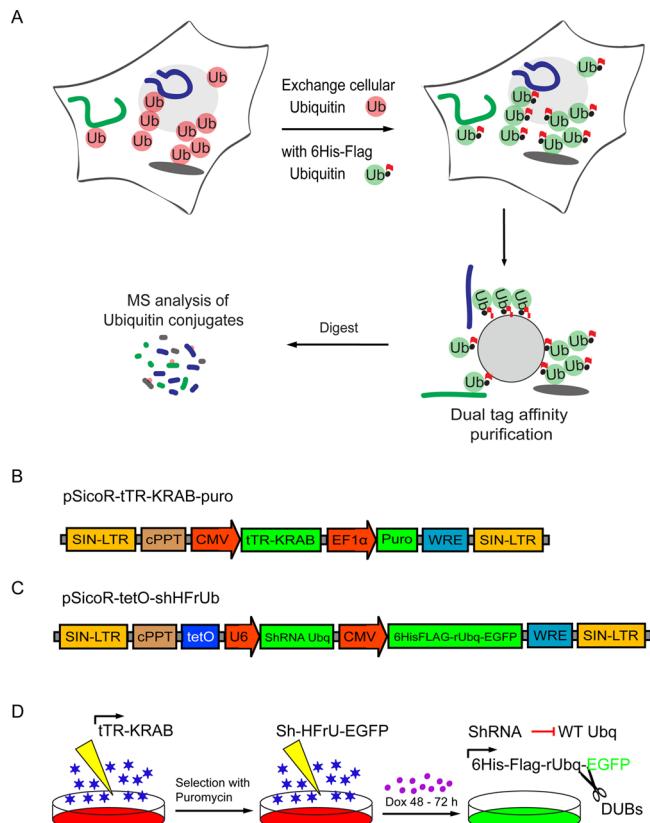
Another commonly applied approach for enriching ubiquitinated proteins for MS analysis on the global scale involves the ectopic expression of epitope-tagged ubiquitin.<sup>30–32</sup> An advantage of this method is the possibility of enriching ubiquitinated conjugates from cellular lysates using well-established purification tools and procedures targeting the corresponding tags. However, ectopic expression at low levels results in only modest levels of the overall ubiquitinated proteins being tagged, mainly due to strong competition with the endogenous ubiquitin, giving insufficient enrichment of ubiquitinated proteins.<sup>33,34</sup> On the other hand, overexpression of ubiquitin at higher levels leads to excessive nonphysiological ubiquitination of many cellular proteins.<sup>35</sup>

Considering these issues, we developed a generic cellular system for stable tagged ubiquitin exchange (StUbEx) in which about 80% of the endogenous ubiquitin is replaced by ectopically expressed tagged ubiquitin. In this way, the tagged ubiquitin is incorporated into the vast majority of the cellular ubiquitinome, while the overall pool of ubiquitin in the cell is maintained at physiological levels. We designed a dual tag, hexahistidine (6×His) in tandem with a Flag-epitope tag fused to the N-terminus of ubiquitin, to allow flexibility in the choice of purification conditions of ubiquitinated proteins under native or denaturing conditions as well as to provide the option for tandem affinity purification for maximal purity (Figure 1A). StUbEx utilizes lentiviral-driven shRNA silencing of endogenous ubiquitin and simultaneous expression of the 6×His-Flag-tagged version, resistant to the RNAi. The efficiency of StUbEx was assessed on three different cell lines, all displaying similar robust results. Finally, the utility of StUbEx for unbiased characterization of protein ubiquitination was demonstrated by global analyses of the EGF signaling network using SILAC-based quantitative proteomics.

## EXPERIMENTAL PROCEDURES

### RNAi and DNA Constructs

The lentiviral vectors pSicoR (Addgene Plasmid 11579),<sup>36</sup> pLVCT-tTR-KRAB (Addgene Plasmid 11643),<sup>37</sup> and plasmids



**Figure 1.** Strategy and engineering of StUbEx cell lines for doxycycline-inducible replacement of endogenous ubiquitin with 6×His-Flag-Ubiquitin. (A) Overview of the StUbEx strategy and general workflow. (B) Schematic of the lentiviral construct pSicoR-tTR-KRAB-puro, which is used for inserting the tetracycline repressor-KRAB domain fusion protein into the cells' genome. The puromycin resistance gene is used as a selection marker. (C) The lentiviral construct pSicoR-tetO-shHFrUb designed for drug-inducible silencing of endogenous ubiquitin and simultaneous expression of the tagged ubiquitin resistant to the RNAi (see also Supporting Information Figure 1). (D) Flow chart of the two-step lentiviral-based platform for the generation of StUbEx cells.

of the third generation packaging system for producing viral particles, pMD2.G (Addgene Plasmid 12259), pMDLg/pRRE (Addgene Plasmid 12251), and pRSV-Rev (Addgene Plasmid 12253),<sup>38</sup> were obtained from Addgene in accordance with material transfer agreements (MTAs). The lentiviral vector pCDF1-MCS2-EF1-Puro was purchased from System Biosciences Co.

RNAi sequences targeting UBC and UBB genes of ubiquitin were designed using available web resources ([www.dharmacon.com](http://www.dharmacon.com)) according to recommendations for siRNA design.<sup>39,40</sup> shRNA constructs were designed under guidelines from ref 36 and available web resources. Briefly, forward and reverse DNA oligomers containing shRNA coding sequences and hairpin linkers were designed in the following format: 5'-T(N19)-TTCAAGAGA(rN19)TTTTTC and 5'-TCGAGAAAAAA-(N19)TCTCTTGAA(rN19)A, where N19 is the sense of the target sequence and rN19 is the antisense. The oligonucleotides were annealed as described<sup>40</sup> and directly cloned into the pSicoR vector followed by DNA sequencing for verification (pSicoR-shUb, Supporting Information Figure 1A). For screening purposes, we used the following sequences for RNAi: sh85, AAGATCCAAGATAAGGAAG (targeting the

UBC gene); sh137, CAGGCAAGCAGCTGGAAGA (targeting the UBB gene); sh1, ATGCAGATCTCGTGAAGA, sh170, CTGACTACAACATCCAGAA; sh53, AGCCCAGTGACAC-CATCGA (the last three targeting the UBC and UBB genes of ubiquitin).

The shRNA-resistant ubiquitin sequence containing an N-terminal fusion with the 6×His-Flag epitope (HFrUb) was created by direct annealing of long forward and reverse synthetic oligonucleotides encoding the human ubiquitin sequence where every codon was mutated but still encoding the same amino acid (Supporting Information Figure 1C). The frequency of each codon type was also taken into consideration. The product of the annealing reaction was cloned into the pSicoR vector in frame with the EGFP (enhanced green fluorescent protein) sequence at the C-terminus of ubiquitin. The sequences of several clones were verified by DNA sequencing, and an error-free clone was chosen for the subcloning of the tagged ubiquitin sequence into the pSicoR vector containing the shRNA candidates (pSicoR-shUbHFrUb) (Supporting Information Figure 1B).

The construct pSicoR-tTR-KRAB-puro (Figure 1B) was created by cloning of the sequence encoding tTR-KRAB (from the vector pLVCT-tTR-KRAB) into pSicoR downstream of the CMV promoter. The DNA cassette EF1-Puro (from the vector pCDF1-MCS2-EF1-Puro), encoding the EF1 $\alpha$  promoter and the puromycin resistance gene, was subcloned into the same pSicoR lentiviral vector between tTR-KRAB and the WRE element.

The construct pSicoR-tetO-shHFrUb (Figure 1C) was made with conventional cloning methods by inserting the tetracycline response element (TRE) from the pLVCT-tTR-KRAB vector into the pSicoR-shUbHFrUb construct upstream of the U6 Pol III promoter. Design of primers for gene amplification, oligonucleotides for shRNA and 6×His-Flag-rUbiquitin, and selection of restriction enzymes for cloning was done using the licensed software CLC workbench (version 4.1.2).

#### Generation of Stable Cell Lines

Lentiviral particles were produced as described<sup>36</sup> with modifications. In brief, 10  $\mu$ g of lentiviral vector and 5  $\mu$ g of each packaging plasmid were co-transfected in HEK-293T cells using the transfection reagent METAFECTENE (Biontex Laboratories) according to manufacturer's instructions. Supernatant was harvested 48 and 72 h after infection, and viral particles were concentrated by ultracentrifugation at 115 000 rcf for 2 h. The viral stocks were diluted in cell culture media and used for infection of HeLa, U2OS, and Skov3 cells to generate stable cell lines expressing the described transgenic DNA constructs (Figure 1B,C). Cells stably expressing the pSicoR-tTR-KRAB-Puro construct (Figure 1B) were grown in the presence of 2  $\mu$ g/mL puromycin for 5 days and used for infection with viral particles containing the inducible version of the rescue ubiquitin transgene pSicoR-tetO-shHFrUb (Figure 1C). Two rounds of infection were performed in order to create stable cell lines uniformly expressing the transgenic ubiquitin (StUbEx ubiquitin), which was monitored by GFP expression in live cells and western blotting analysis.

#### Cell Culture and Affinity Purification

HeLa, U2OS, Skov3, and HEK-293T cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 2 mM glutamine (Lonza), 50 U/mL penicillin and 50 U/mL streptomycin (Lonza). For MS-based proteomic experiments, StUbEx HeLa cells were grown in DMEM deficient in

lysine and arginine supplemented with 10% dialyzed FBS from Invitrogen (Carlsbad, CA), antibiotics, and glutamine. Cells were differentially SILAC labeled<sup>41</sup> with L-arginine (Arg0) and L-lysine (Lys0); L-arginine-<sup>13</sup>C<sub>6</sub> (Arg6) and L-lysine-<sup>2</sup>H<sub>4</sub> (Lys4). Eight 15 cm dishes were used for each SILAC condition. Cells were treated with 1  $\mu$ g/mL doxycycline 60 h prior to harvesting, serum starved for 16 h, and either stimulated with 150 ng/mL EGF for 6 min or left untreated before lysis under strong denaturing conditions (binding buffer: 6 M Guanidinium-HCl, 50 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 500 mM NaCl, 15 mM imidazole, 2 mM  $\beta$ -mercaptoethanol). The first step of affinity purification was performed as described<sup>42</sup> with modifications: lysates were collected, mixed together, sonicated to reduce sample viscosity, and centrifuged at 11000 rcf for 30 min at room temperature. To purify ubiquitin conjugates, 100  $\mu$ L of nickel sepharose beads (GE Healthcare) was mixed with the sample and incubated for 1 h at room temperature followed by washes with a 50× bead volume of the binding buffer and washing buffers 1–4 (WB1: 8 M Urea, 50 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 20 mM imidazole, and 2 mM  $\beta$ -mercaptoethanol; WB2: same as WB1 but with 0.2% Triton X-100; WB3: same as WB2 but at pH 6.3; WB4: 8 M Urea, 25 mM Tris, pH 8.0, 150 mM NaCl). The ubiquitin conjugates were eluted with an elution buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, and 300 mM imidazole. The sample was diluted five times with the ice-cold Triton X-100 buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton X-100 plus protease inhibitors cocktail (Roche)) and incubated with Flag-agarose beads for 3 h at 4 °C. After washing the Flag beads three times with Triton X-100 buffer and three times with 150 mM NaCl, the ubiquitin conjugates were eluted with 0.1% TFA solution and neutralized using 1 M Tris, pH 8.0.

#### Sample Preparation for MS Analyses

The eluted proteins were subjected to in-solution proteolytic digestion essentially as described<sup>16</sup> with modifications: eluates were diluted to 25 mM Tris, pH 8.0, urea was added to attain a final concentration of 8 M, and the mixture was subjected to reduction with 1 mM DTT (at room temperature for 40 min) and alkylation with 5.5 mM chloroacetamide for 30 min at room temperature.<sup>43</sup> Lysyl endopeptidase (Wako Chemicals) was added (1:100 w/w) for 4 h followed by dilution of the urea buffer with distilled water to achieve a urea concentration below 2 M. Trypsin (sequencing grade, Promega, Madison, WI) was added (1:100 w/w) to the sample for an overnight incubation at room temperature. Tryptic peptide mixtures were acidified with TFA to a final concentration 0.3% and concentrated using the C<sub>18</sub> Sep-Pak cartridge (Waters, Milford, MA) according to the manufacturer's instructions. After peptide elution, the organic solvent (80% acetonitrile) was evaporated, and the sample was divided into two parts for two technical replicates. The peptides were subjected to pH gradient fractionation (pISep, Cryobiophysica) as described.<sup>44</sup> In brief, the peptide mixture was diluted two times with buffer A (pISep concentrate A (Cryobiophysica), 25% acetonitrile, 1% acetic acid, pH 2.8) and loaded to a small column containing a mixture of strong-cation and weak-cation exchange material (PolySULFOETHYL A and PolyCAT A, PolyLC INC). Peptides were eluted by a stepwise increase of the pH of an elution buffer from pH 3.5 to 10.0 that was achieved by mixing buffers A and B in different volumes. Buffer B was composed of pISep concentrate B (Cryobiophysica), 25% acetonitrile, 0.5% ammonium hydro-

chloride, pH 10.8. The flow through and 11 elution fractions were collected for each technical replicate. Each fraction was dried in a speedvac and adjusted to a final concentration of 1.7% ACN/0.33% TFA; peptides were then concentrated and desalting using C<sub>18</sub> STAGE tips.<sup>45</sup>

For the first technical replicate, the peptide mixtures were analyzed using an Agilent 1100 nanoflow system (Agilent Technologies) connected online to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray ion source (Proxeon Biosystems). For chromatographic separation, peptides were injected into a fused silica column packed in-house with 3 μm C<sub>18</sub> beads (Reprosil, Dr. Maisch), applying a 120 min gradient from 8 to 64% acetonitrile in 0.5% acetic acid at a flow rate of 250 nL/min. We set up the instrument methods for the LTQ-Orbitrap Velos in the data-dependent mode, and the seven most intense ions after full scan survey (MS spectra from *m/z* 300–2000) were subjected to MS/MS fragmentation using the HCD activation technique with the following settings: resolution MS *r* = 30 000, MS/MS *r* = 7500, normalized collision energy of 40%, with 0.1 ms activation time, and isolation window 2.5 Th. The second technical replicate was performed on the same instrumentation with slightly different mass spectrometric parameters: 15 most intense peaks were chosen for MS/MS fragmentation with CID activation, FTMS resolution was 60 000, normalized collision energy of 35%, activation *q* = 0.25, and activation time of 10 ms. Samples used for estimation of total ubiquitin pool in StUbEx cells (see below) were acquired on an LTQ-Orbitrap XL mass spectrometer with the following parameters: 10 most intense peaks were chosen for MS/MS fragmentation with CID activation, resolution MS was 60 000, normalized collision energy of 35%, activation *q* = 0.25, and activation time of 30 ms. For all MS runs, ions selected for fragmentation were dynamically excluded for 45 s, and a lock mass ion *m/z* 445.120024 was used for internal mass calibration.<sup>46</sup>

## Data Analyses

Processing of the 46 raw files from the two biological experiments, with two technical replicates each, was done with MaxQuant software (version 1.0.13.13) essentially as described.<sup>47</sup> In brief, peak lists were generated by the Quant console of MaxQuant program with the following parameters used for searching: double SILAC with labels Arg6/Lys4; maximum two missed trypsin cleavages; six most intense peaks per 100 Da interval used for MS/MS peak lists; mass tolerance was 7 ppm on precursors and 0.5 Da (CID), 0.02 Da (HCD) for fragment ions. A fixed modification was carbamidomethyl (C), and variable modifications were oxidation (M), N-term protein acetylation, and ubiquitination of lysines (GlyGly and Lys4 + GlyGly).

The MaxQuant-generated peak lists were searched by Mascot v.2.3 (<http://www.matrixscience.com>) against the human International Protein Index database v.3.69 (174 784 entries, including the reversed decoy sequences). The acquired Mascot DAT files together with the raw files were processed and quantified by the Identify console of MaxQuant with the following parameters: peptide, protein, and site of modifications false discovery rate (FDR) was below 1%, as assessed by the number of hits in the reverse database; minimum peptides length was six; and a minimum of one unique peptide for protein identification was required. All unmodified and modified peptides, except ubiquitination (GlyGly), were used

for protein quantification based on both razor and unique peptides requiring a minimum ratio count of two. Only the proteins identified by at least two peptides in each of the independent replicas and quantified in at least one replica were accepted. Common contaminants were removed from the identification list as well.<sup>48</sup> The significance score value calculated by MaxQuant was used to reveal regulated candidates using a significance B value less than 0.01 as a threshold for assigning candidates as being differentially regulated.

GO terms enrichment analysis was performed on a web-based application GORilla,<sup>49</sup> using all identified proteins as a background set. Pearson correlation analyses was assessed in GProX software,<sup>50</sup> and the KEGG pathways analysis was done using the web-based tool KEGG Mapper v1.6.<sup>51</sup>

## Immunoprecipitation and Western Blotting

For the comparative WB analysis of HeLa, U2OS, and Skov3 cells, the cells were lysed with buffer containing 1% SDS, and the lysates were briefly sonicated to reduce viscosity, centrifuged, and mixed with 6× sample buffer for SDS-PAGE analysis. HeLa, U2OS, and Skov3 StUbEx cells were grown and treated in the same way except for incubation with doxycycline (2 μg/mL) for 60 h or other amounts of time, as indicated. Around 50 μg of each lysate was loaded on Novex 4–12% Bis-TRIS gradient gels (Invitrogen, Carlsbad, CA) using MES running buffer followed by protein transfer to nitrocellulose membrane, blocking with 2% BSA, and incubation with primary and HRP-conjugated secondary antibodies.

For IP of EGFR and Eps15, we used two 15 cm dishes of WT and StUbEx HeLa cells per condition that were grown to 90% confluence. Cells were incubated with doxycycline (2 μg/mL) for 60 h, serum-starved for 16 h, left untreated or treated with EGF for 6 min, and lysed in ice-cold lysis buffer (1% v/v NP-40, 150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA, 10 mM chloroacetamide, and protease inhibitors (complete tablets; Roche)). After centrifugation of lysates, they were supplemented with SDS to 1% and incubated for 30 min to destroy co-purifying protein complexes.<sup>52</sup> The lysates were diluted 10 times with lysis buffer and subjected to IP using the indicated antibodies bound to protein A beads (GE Healthcare) for 5 h at 4 °C followed by five washes of beads with the lysis buffer. For IP of Shc1 and ASAP1, we used WT HeLa cells following the protocol described above.

For the purification tests, cellular lysates from WT and StUbEx cells (four 15 cm dishes each) were subjected in parallel to the TAP purification procedure described above. For the loading of input and flow-through control lanes, 6 M guanidinium-HCl was exchanged with 8 M urea using 3 kDa cutoff membrane spin tubes (Vivaspin 500, Sartorius Stedim Biotec) according to the manufacturer's instructions.

## Real-Time PCR

Real-time PCRs were done essentially as described.<sup>53</sup> Cells were lysed with TRIzol (Invitrogen), and total RNA was isolated according to the manufacturer's instructions. Prior to reverse transcription using the MMLV reverse transcriptase (Invitrogen), genomic DNA was degraded by incubating 1 μg RNA in first strand buffer with RQ1 DNase (Promega) for 30 min at 37 °C in a 20 μL reaction. Following denaturation of the DNase at 75 °C for 5 min, dNTPs (0.8 mM), random hexamers (0.02 μg/μL), and MMLV (8 units/μL) were added, giving a total reaction volume of 25 μL. Reactions were incubated at room temperature for 10 min and then at 37 °C

for 1 h. The MMLV reverse transcriptase was inactivated at 70 °C for 15 min. Finally, cDNA was diluted with 100 μL of water.

Quantitative PCR was performed in 20 μL reactions containing SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), 2 μL of diluted cDNA, and 300 nM each primer. Reaction mixtures were preheated at 95 °C for 2 min followed by 40 cycles of melting at 95 °C for 15 s, annealing at 62 °C for 30 s, and elongation at 72 °C for 45 s. Expression of the general transcription initiation factor IIB (TFIIB) was used for normalization. Primer sequences are as follows: RPS27A: forward, gtaaggccaagatccaggataagg; reverse, accttataatattcaggacaggcgc. UBA52: forward, attgaggctcttcggccag; reverse, cttcttctggcggcagg. UBB: forward, tcttcagtcatggcattcgac; reverse, cgaaaccttattaacatttgaacagg. UBC: forward, tcataactcgcccttagaaccc; reverse, ttgtcaagtgacgatcacagcg. RecUBI: forward, ggaatcccctgatcaacaacg; reverse, ggagcctgagcagcagatgg. TFIIB: forward, gcgttaccagccgttgg; reverse, cacatcaaaccggcgtacc.

#### SILAC-Labeling Experiment to Estimate the Total Ubiquitin Pool in the StUbEx Cells

The StUbEx and WT HeLa cells were SILAC-labeled using L-arginine (Arg0) and L-lysine (Lys0); L-arginine-<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>4</sub> (Arg10) and L-lysine-<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>2</sub> (Lys8) (Arg0/Lys0 and Arg10/Lys8, respectively) as described.<sup>52</sup> The StUbEx cells were induced with the doxycycline for 24, 48, and 72 h or left untreated. The WT cells and StUbEx cells from each time point were lysed in 1% SDS, 25 mM Tris, pH 8.0, buffer and mixed in 1:1 protein ratio to resolve 25 μg of each mix on the Bis-TRIS gel (Invitrogen) using MES running buffer. After colloidal blue staining (Invitrogen), gel slices in MW range ~60 to 98 kDa, containing ubiquitin conjugated to proteins (mono- and polyubiquitinated), as well as slices containing free ubiquitin species (MW 6 to 14 kDa), were cut off and subjected to an in-gel digestion procedure as described.<sup>54</sup> Peptides mixtures were run on the LTQ-Orbitrap Velos and LTQ-Orbitrap XL mass spectrometers as described earlier, and raw data were processed using the MaxQuant platform (see above). Two biological replicates were performed, and the ratios of a minimum of five unique peptides from ubiquitin from each experiment were used to compare the levels of the total ubiquitin pool for each time point of doxycycline-treated StUbEx cells.

#### Microscopy

To obtain the live cell images of doxycycline-induced cells, StUbEx HeLa cells were grown on 6 cm dishes and induced with Dox for the indicated time points as described above. Phase contrast images were obtained for visualization of cells together with images acquired in the green channel for fluorescent detection. All images were acquired in live cells using a Zeiss CellObserver with a CoolSNAP HQ2 cooled CCD camera (Photometrics) with an Plan-Apochromat 20× 0.8 n.a. objective.

#### Antibodies

For IP and WB, the following antibodies were used: rabbit anti-EGFR, rabbit anti-Eps15, mouse anti-ubiquitin (clone P4D1) (all from Santa Cruz Biotechnology, Inc.), mouse polyubiquitin K63-linkage-specific (clone HWA4C4, Enzo Life Sciences), mouse anti-Flag (Sigma-Aldrich), mouse anti-ASAP1 (Abnova), and rabbit anti-Shc1 (BD Biosciences). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Amersham Pharmacia Biotech.

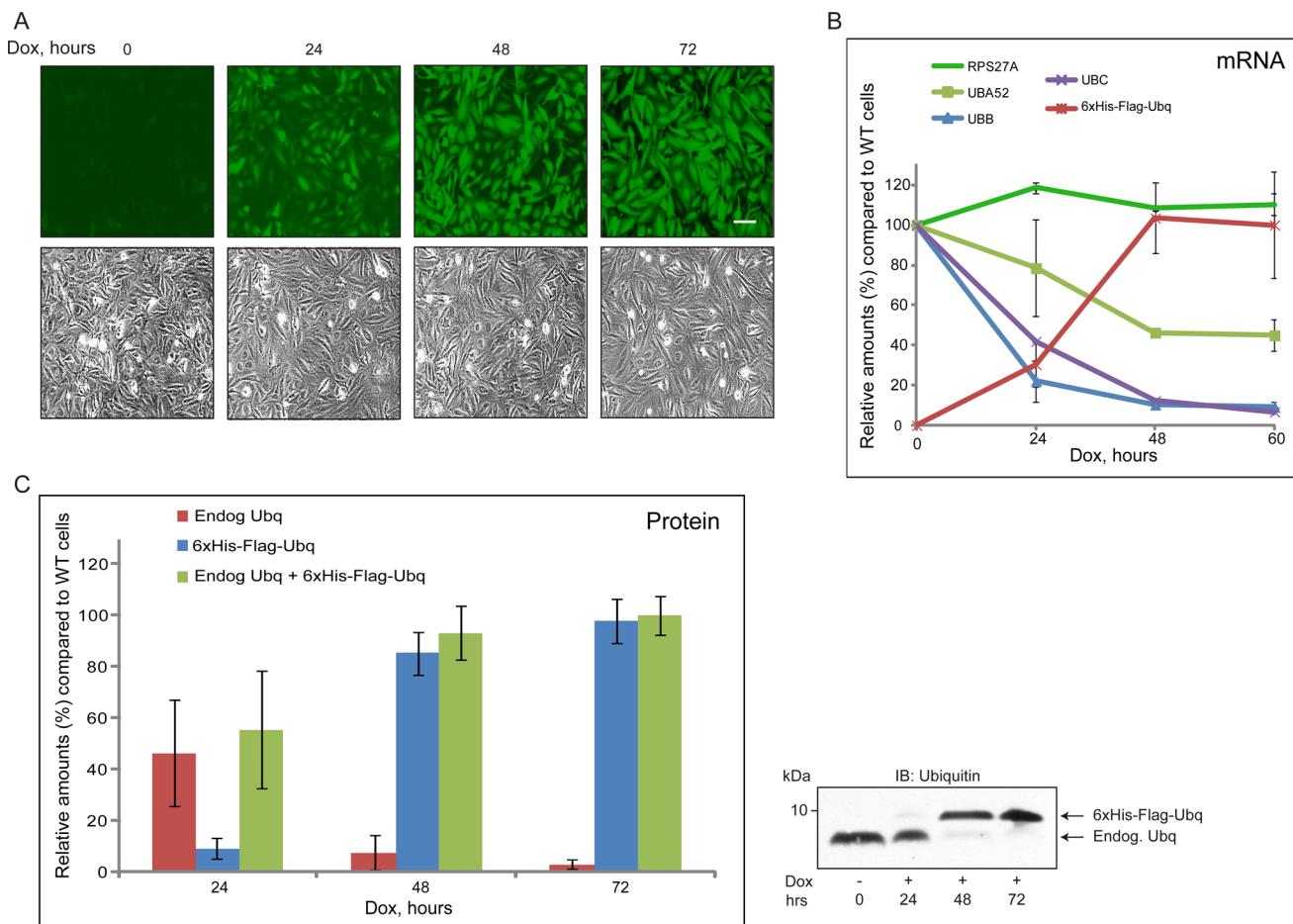
## RESULTS

### Engineering of StUbEx for Replacing Endogenous Ubiquitin with a 6×His-Flag-Tagged Version

To overcome the issues associated with ectopic expression of ubiquitin, we aimed to develop an RNAi-based system to knockdown endogenous ubiquitin and, at the same time, to reintroduce to the cell similar amounts of epitope-tagged ubiquitin. A similar ubiquitin replacement strategy has already been applied successfully to study Lys-63 chain specific polyubiquitination events in human cells.<sup>55</sup> In that manner, we intended to reach a significantly higher incorporation of the tagged version into ubiquitinated proteomes while maintaining the ubiquitin pool in the cell at physiological levels. We utilized a lentiviral delivery system for generation of stable cell lines, providing a drug-inducible shRNA silencing of endogenous ubiquitin with simultaneous replacement by tagged-ubiquitin resistant to the shRNA. However, there are four genes encoding ubiquitin in humans, producing, altogether, 14 copies of ubiquitin. The UBC and UBB genes encode polyubiquitin precursors composed of 9 and 3, respectively, head-to-tail ubiquitin repeats. Two other genes, namely, UBA52 and RPS27A, generate single copies of ubiquitin fused to the ribosomal proteins L40 and S27a, respectively.

For knocking down endogenous ubiquitin, we initially selected five sequences targeting either one or both polyubiquitin encoding genes, UBC and UBB. We decided to avoid manipulation of UBA52 and RPS27A, as these two genes do not significantly contribute to the general pool of ubiquitin in the cell<sup>56</sup> and to minimize possible interference with the ribosomal system. Each shRNA candidate was cloned into the pSicoR lentiviral vector<sup>36</sup> with or without rescue of endogenous ubiquitin by simultaneous expression of 6×His-Flag-Ubiquitin (Supporting Information Figure 1A,B). The highest efficiency of silencing was achieved by a shRNA targeting both polyubiquitin genes corresponding to the sequence at positions 53 to 71 of the UBB and UBC coding regions (Supporting Information Figure 1C) and was hence used for all subsequent experiments. The cDNA for ubiquitin resistant to the shRNAs was generated by mutating at least one base pair in every codon of the original ubiquitin coding sequence but maintaining the amino acid sequence (Supporting Information Figure 1C). In addition, EGFP sequence was fused in-frame to the C-terminal part of the tagged ubiquitin, which was used for monitoring the efficiency of infection. In the cell, the EGFP is cleaved off from the fusion protein cotranslationally by ubiquitin C-terminal hydrolase enzymes.<sup>57,58</sup>

Our next step was to generate drug-inducible StUbEx cell lines to allow precise timing of the silencing/replacement of the cellular ubiquitin and to avoid possible long-term instability of RNAi.<sup>59</sup> We designed a two-step lentiviral-based system where we first introduced into cells a plasmid that encodes a fusion protein between Kruppel-associated box (KRAB) domain and Tet repressor protein tetR (tTR-KRAB).<sup>37</sup> As a selective marker, a puromycin resistance gene was cloned into the same DNA construct as well (Figure 1B). In the second step, cells selected with puromycin and expressing the tTR-KRAB protein were transduced with another lentivector bearing both the shRNA against the two polyubiquitin genes and the cassette for expressing the 6×His-Flag-Ubiquitin resistant to the shRNA (Figure 1C). This vector was further modified by inserting a multiple tetracycline operator (tetO) sequence between cPPT and the U6 promoter (Figure 1C). In absence of doxycycline,



**Figure 2.** 6×His-Flag-Ubiquitin efficiently replaces endogenous ubiquitin in StUbEx cells while maintaining normal levels of total ubiquitin in the cells. (A) Fluorescent microscopy images at different time points after doxycycline treatment of StUbEx cells. GFP expression serves as a fluorescent marker for monitoring the expression of the 6×His-Flag-Ubiquitin. Scale bar = 50 μm. The corresponding phase-contrast images are displayed below each panel. (B) Q-PCR analyses of the mRNA levels in StUbEx cells of the four human transcripts encoding ubiquitin, analyzed by qPCR, relative to that in WT cells. The mRNA level for the 6×His-Flag-Ubiquitin (red line) is shown relative to its level at 60 h of doxycycline treatment. (C) Protein levels of endogenous ubiquitin (red bars), 6×His-Flag-Ubiquitin (blue bars), and total cellular ubiquitin (green lines) measured by densitometry of the WB images of StUbEx cellular lysates (representative image on the right). Also, see Supporting Information Figure 2.

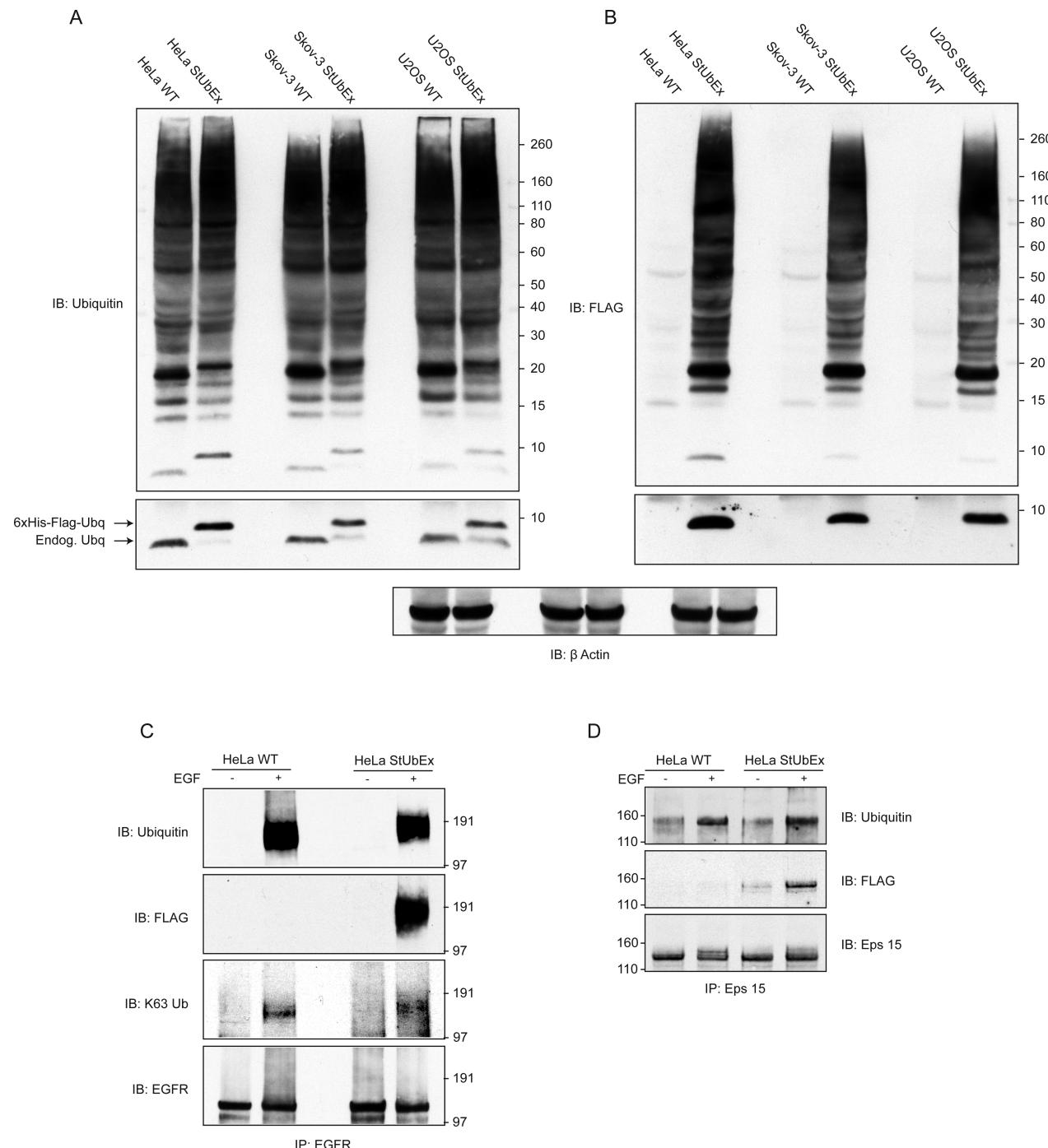
the tTR-KRAB protein binds to the tetO sequence and blocks both the U6 and the CMV promoters (Supporting Information Figure 1D). Addition of doxycycline to cell culture media results in the drug competing off the tTR-KRAB protein from the tetO sequence and releasing the KRAB-mediated silencing of the U6 and the CMV promoters. Thereby, induction of the StUbEx cells with doxycycline leads to a steady replacement of endogenous ubiquitin by its RNAi-resistant 6×His-Flag-tagged version (Figure 1D and Supporting Information Figure 1D).

#### StUbEx Provide Efficient Replacement of Endogenous Ubiquitin with the Dual-Tagged Version and Maintain the Ubiquitin Pool in the Cell at Physiological Levels

In order to determine the optimal timing of ubiquitin replacement, we subjected StUbEx cells to a time course treatment with doxycycline. Because the 6×His-Flag-Ubiquitin in StUbEx cells is translated with EGFP C-terminal extension (the EGFP being subsequently cleaved off by DUBs), it was possible to follow its expression in the cells by monitoring the levels of the fluorescent marker during the course of doxycycline incubation. As seen in Figure 2A, EGFP expression was readily detectable in most cells 1 day after doxycycline treatment, and strong expression was uniformly seen in all cells following 48 h of treatment. To assess the exact rates of

ubiquitin exchange at the mRNA and protein levels, StUbEx cells were harvested at different time points after doxycycline treatment and quantitatively analyzed by qPCR and immunoblotting, respectively. Changes in the mRNA levels of all four genes encoding endogenous ubiquitin were measured as well as the mRNA for the recombinant 6×His-Flag-Ubiquitin (Figure 2B). Concomitant with strong induction of the mRNA for the recombinant ubiquitin was a marked decrease in the mRNA levels of both polyubiquitin transcripts. Namely, the UBB and UBC mRNAs displayed approximately 90% reduced abundance following 48 h of doxycycline treatment. The expression of UBA52 transcript was also decreased to a smaller degree, whereas RPS27A mRNA remained unaffected (Figure 2B).

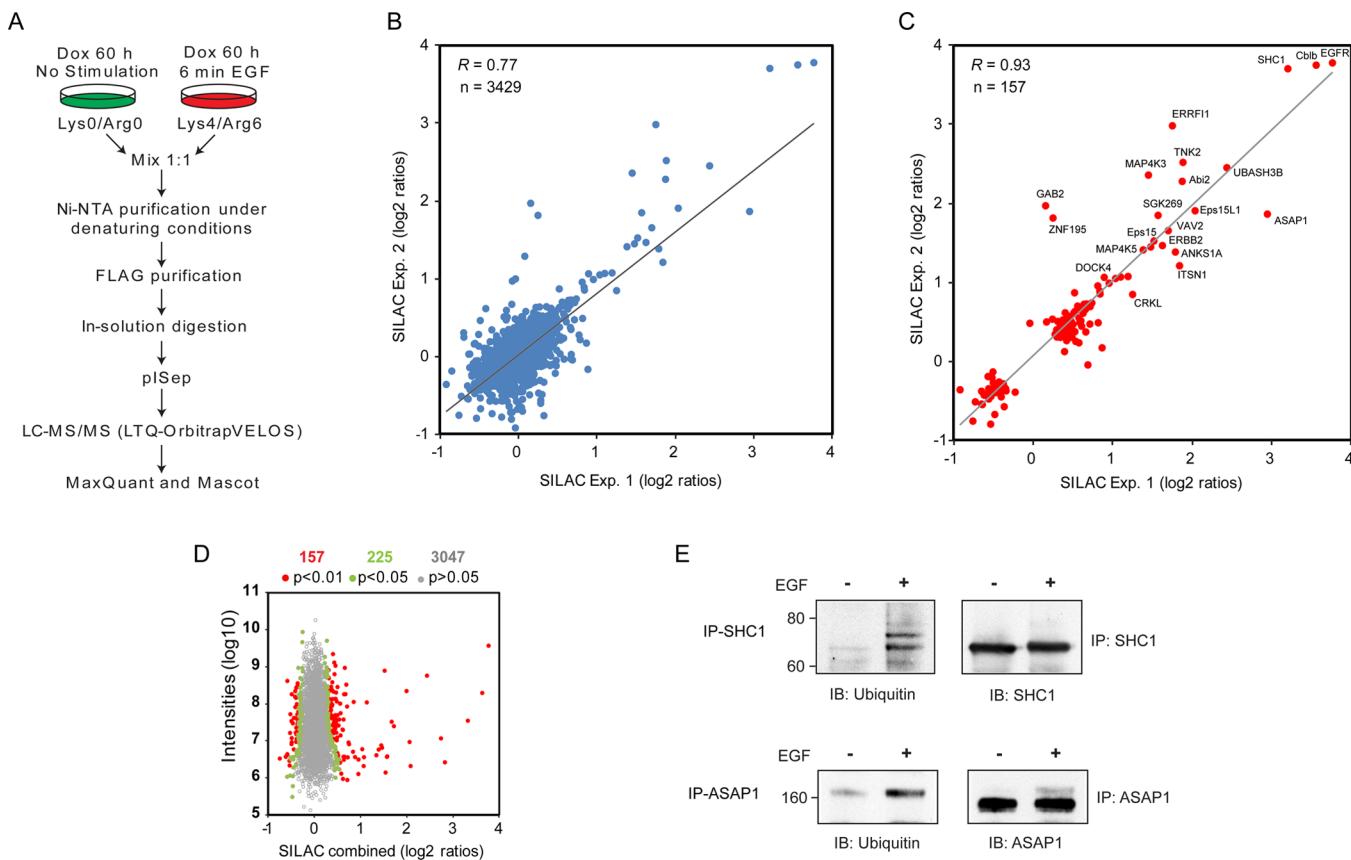
To follow the process of ubiquitin exchange at the protein level, StUbEx cells were also subjected to quantitative immunoblotting analyses during the doxycycline treatment. Due to the addition of 6×His-Flag sequences, the recombinant ubiquitin migrated slightly slower on a gradient SDS-PAGE compared to that of endogenous ubiquitin. This shift in migration allowed simultaneous visualization of both protein species by western blotting using anti-ubiquitin antibodies (Figure 2C). Consistent with the results observed for the mRNA levels, doxycycline treatment in the range of 48 to 72 h



**Figure 3.** 6×His-Flag-Ubiquitin in StUbEx cells is utilized for protein ubiquitination similar to ubiquitination in WT cells. (A, B) Similar overall ubiquitination patterns in StUbEx and WT cells. Immunoblots with the indicated antibodies on whole cell lysates from WT and corresponding StUbEx cell lines. The panels below each image show the lower part of the blots containing the free unconjugated ubiquitin, enhanced by a longer exposure time. Immunoblotting against β-actin (bottom panel, middle) served as a loading control. (C, D) Similar ubiquitination patterns of EGFR and Eps15 in WT and StUbEx HeLa cells in response to EGF stimulation. EGFR and Eps15 were immunoprecipitated from the specified EGF-treated or nontreated WT and StUbEx cells followed by western blot analyses with the indicated antibodies. K63Ub refers to an antibody specifically recognizing polyubiquitin chains linked through the Lys-63 residue of ubiquitin.

resulted in efficient replacement of the endogenous ubiquitin protein with the 6×His-Flag-Ubiquitin in StUbEx cells (Figure 2C). Importantly, the densitometric measurements of western blotting images from four independent experiments further revealed that the total pool of free ubiquitin in the cells was maintained at normal physiological levels (Figure 2C and Supporting Information Figure 2A,B).

Taking into account the semiquantitative nature of the WB densitometry, we performed SILAC-based experiments to better compare the total ubiquitin pool between StUbEx and wild-type (WT) cells in terms of the free ubiquitin fraction and the fraction of ubiquitin conjugated into proteins. For that purpose, WT HeLa cells were labeled with heavy lysine and arginine, whereas StUbEx HeLa cells were labeled with the light



**Figure 4.** Quantitative proteomics analyses of EGF-induced ubiquitination in StUbEx HeLa cells. (A) Schematic of the SILAC-based experimental workflow. (B, C) Reproducibility assessment of the measured SILAC ratios between the two biological replicas (B) for all identified proteins and (C) only the proteins with significant changes in response to EGF ( $p < 0.01$ ).  $R$  is Pearson's correlation coefficient. (D) Overall fold changes in protein ubiquitination status after 6 min EGF stimulation as a function of protein intensity in the MS. Protein ratios are color-coded according to their statistical significance, as indicated above the plot. (E) EGF-induced ubiquitination of endogenous Shc1 and ASAP1 in WT HeLa cells.

versions of arginine and lysine; both cell lines were subsequently induced with doxycycline for different time periods (Supporting Information Figure 2C). Cell lysates from the WT and StUbEx cells were then mixed in equal amounts, digested with trypsin, and quantitatively analyzed by LC–MS/MS. Quantitative MS data based on two independent replicates showed that the pool of ubiquitin in StUbEx is indeed maintained at normal physiological levels, fluctuating within 20% from the levels in the parental WT cells (Supporting Information Figure 2D).

#### StUbEx Is Applicable to Diverse Cell Systems and Preserves Normal Protein Ubiquitination Characteristics

To test whether the StUbEx system is applicable to diverse cell cultures, we created stable cell lines using HeLa, U2OS, and Skov3 cells, and in all three cases, endogenous ubiquitin was efficiently exchanged with the 6×His-Flag-Ubiquitin (Figure 3A,B). At the same time, WT cell lines and corresponding StUbEx lines showed similar levels of the overall protein ubiquitination, as visualized by western blotting with an antibody recognizing free, mono-, and polyubiquitin (Figure 3A). Further immunoblotting using a Flag specific antibody revealed that the 6×His-Flag-Ubiquitin is successfully utilized by the cellular ubiquitination machinery, as it is indeed widely conjugated into the proteomes of all three StUbEx cell lines (Figure 3B).

These results, in concordance with the quantitative data from the SILAC-based measurements described above (Supporting

Information Figure 2C,D), indicated similar conjugation efficiencies of ubiquitin into targeted proteins in the WT and StUbEx cells. However, we also needed to assess the ability of the StUbEx system to reconstitute normal cellular responses, in terms of protein ubiquitination, in response to physiological stimuli. For that purpose, we compared the ubiquitination status of well-characterized target proteins in StUbEx HeLa cells and the corresponding WT cells upon stimulation with epidermal growth factor (EGF).

Ubiquitin conjugation plays an important role in receptor-tyrosine kinase (RTK) signaling, creating a growth factor-dependent signal for receptor downregulation by endocytosis and lysosomal degradation.<sup>52,60</sup> In response to its ligand, the EGF receptor itself undergoes extensive ubiquitination on multiple lysine residues, with part of it forming Lys-63 chains.<sup>52,61,62</sup> The ubiquitination status of EGFR in WT and StUbEx cells upon ligand stimulation is shown in Figure 3C, visualized by antibodies specifically recognizing Lys-63 ubiquitin chains, total ubiquitin, or the Flag epitope. The overall pattern appeared very similar in both cell lines, with a small increase in the molecular weight of ubiquitinated EGFR in StUbEx cells due to the slightly heavier 6×His-Flag-Ubiquitin that is being utilized for ubiquitination instead of the endogenous ubiquitin. The anti-Flag immunoblotting demonstrated that the tagged-ubiquitin is indeed used for the ubiquitination of EGFR upon ligand stimulation. It is also worth emphasizing that EGFR ubiquitination was not detected

in the unstimulated StUbEx cells, particularly because it has been previously observed that ectopically (over)expressed ubiquitin in mammalian cells can lead to excessive, ligand-independent protein ubiquitination, especially on growth factor receptors and endocytic adaptors.<sup>61,63</sup> Finally, the degree of Lys63-type ubiquitin chains on EGFR appeared to be very similar between the WT and StUbEx cells (Figure 3C).

Several endocytic adaptor proteins downstream of the receptors, like Eps15, become ubiquitinated in response to growth factor stimulation as well; however, this occurs mainly in the form of monoubiquitination.<sup>64,65</sup> Accordingly, we observed a characteristic monoubiquitination pattern on Eps15 in StUbEx cells, virtually identical to the Eps15 ubiquitination in the WT cells (Figure 3D).

#### Sequential Affinity Purification of (6×His-Flag-Tagged) Ubiquitinated Proteins from StUbEx Cells

Tandem affinity purification (TAP) tags are, in general, designed to provide clean and efficient isolation of the tagged protein from bacterial or eukaryotic cell lysates.<sup>30,33,34,66,67</sup> In StUbEx, we utilized hexahistidine and Flag tags because of their small sizes, efficiency of purification protocols, and flexibility in terms of purification conditions, which is provided by the combination of these two tags. The 6×His tag allows direct purification of ubiquitinated proteins under denaturing conditions, and the Flag tag directed purification alone permits usage of milder lysis buffers for studies focusing on ubiquitination-dependent protein complexes, whereas the highest purity is achieved by dual purification.

We thoroughly optimized both the 6×His and the Flag-directed purification procedures using StUbEx HeLa cells. In parallel, WT HeLa cells were subjected to the same purification workflow to assess nonspecific binding of proteins to the affinity matrixes. The efficiency of enrichment and purity of ubiquitinated proteins were monitored by immunoblotting and SDS-PAGE gel staining. As a first step in our dual-purification procedure, nickel-based affinity matrix was used to pulldown 6×His-Flag-ubiquitinated proteins from StUbEx cells. The enrichment was performed under stringent denaturing conditions to eliminate protein–protein interactions and thereby to minimize co-purification of nonubiquitinated proteins. This resulted in efficient depletion of ubiquitinated proteins from StUbEx cell lysates and a corresponding strong enrichment of those proteins in the eluate from the nickel-coated matrix (Supporting Information Figure 3). However, some contaminant proteins, possibly proteins containing multiple histidine stretches, were easily detected in the eluate originating from WT HeLa cells despite their being no visible depletion of ubiquitinated proteins from the WT cellular lysate. To remove the undesired contaminants, a second round of purification was performed using anti-Flag antibodies. As seen in Supporting Information Figure 3, the contaminants were efficiently diminished in the dual-purified WT eluate, whereas ubiquitinated proteins were still retained in the corresponding StUbEx sample.

#### StUbEx System for Unbiased MS-Based Quantitative Proteomics Studies

To formally assess the suitability of the StUbEx system for unbiased investigations of ubiquitination-related processes in mammalian cells using MS-based proteomics, we set up a proof-of-principle experiment focused on EGFR signaling. For that purpose, two populations of StUbEx HeLa cells were differentially SILAC-labeled, treated with doxycycline for 60 h,

and either stimulated with EGF for 6 min or left untreated for control. Ubiquitinated proteins were then purified from the combined cellular lysates using the dual-purification procedure described above, proteolytically digested, and subjected to nanoLC–MS/MS analysis on an LTQ-Orbitrap Velos instrument (Figure 4A).

Two independent biological experiments were performed, yielding a total of 4785 proteins, of which 3988 proteins (83.3%) were identified in both experiments (see Experimental Procedures). To increase the confidence in protein quantitation, we included an additional criterion that each protein should be identified with at least two peptides in each of the replicate experiments. This resulted in a highly confident data set of 3429 protein hits (Supporting Information Table 1) with a very high level of reproducibility of the measured SILAC ratios between the two biological replicas (Figure 4B). This data set was then used for all of the subsequent bioinformatics analyses.

In addition, 277 unique ubiquitination sites on 189 proteins were identified (Supporting Information Table 2), including a novel site on human EGFR at position 1061 (Supporting Information Figure 4), despite the fact that we did not perform specific enrichment for di-Gly-modified peptides. The characteristic ubiquitin-linkage peptides for all seven branched polyubiquitin chains were also identified (Supporting Information Table 2), further indicating the utility of the 6×His-Flag-Ubiquitin for protein conjugation and polyubiquitin chain formation in StUbEx cells.

In the set of 3429 proteins, 157 displayed a significant change in their ubiquitination status upon 6 min EGF treatment ( $p < 0.01$ ), of which 115 proteins showed increased and 42 showed decreased ubiquitination (Figure 4C,D and Supporting Information Table 1). GO term enrichment analyses revealed that the EGFR signaling pathway was vastly overrepresented in the group of regulated proteins, with the top eight most significantly enriched terms all related to ErbB signaling (Supporting Information Figure 5A). Indeed, most of the known proteins involved in early stages of EGFR signal transduction were found to be ubiquitination-regulated in response to the 6 min EGF treatment, including Grb2, PI3K, Vav2, Cbl, Sts1/UBASH3B, Epsin, Eps15, Stam2, Ymer/CCDC50, and the EGF receptors<sup>64,68–72</sup> (Supporting Information Figure 5B and Supporting Information Table 1). There were, however, several key regulatory proteins in the EGFR signaling pathways, like Shc1 and ASAP1,<sup>73,74</sup> that have not been previously shown to undergo ligand-dependent ubiquitination. To assess this finding and in order to exclude any possible artifacts from the ectopically expressed tagged-ubiquitin in StUbEx cells, we used WT HeLa cells and examined the ubiquitination status of the endogenous ASAP1 and Shc1 upon EGF stimulation. As seen in Figure 4E, both proteins displayed ligand-dependent ubiquitination, thereby further consolidating the utility of the StUbEx system for studying physiologically relevant ubiquitination events.

#### ■ DISCUSSION

Here, we present and characterize StUbEx, an RNAi-based cell system for exchanging endogenous ubiquitin with an epitope-tagged-ubiquitin, engineered to study ubiquitination processes in mammalian cells using mass spectrometry. One of the first reports providing a successful strategy for investigation of global ubiquitination by MS described the usage of a yeast strain in which the endogenous ubiquitin genes were removed

and replaced with 6×His-ubiquitin.<sup>31</sup> Because it is very technically challenging to replace all ubiquitin genes in mammalian cells, overexpression of tagged ubiquitin has instead become a widely used approach in the repertoire of strategies for enriching ubiquitinated proteins for MS-based analysis.<sup>31,34,57,67,75</sup> This, however, requires keeping the expression of the tagged ubiquitin at relatively low levels<sup>33,34</sup> in order to avoid unwanted artifacts associated with excess ubiquitin in the cell. Several reports have shown that a surplus of ectopically expressed ubiquitin in mammalian cells can lead to excessive nonspecific ubiquitination of cellular targets.<sup>35,61,63</sup> During our screening and optimization experiments for StUbEx, we also observed that expression of tagged ubiquitin alone resulted in strong overubiquitination of several cellular proteins such as EGFR and endocytic adaptor proteins (data not shown). At the same time, a low level of expression of tagged ubiquitin demands a significantly large amount of input material for the enrichment of ubiquitinated substrates, which, consequently, also increases the nonspecific background during the purification using any type of affinity matrix.

With StUbEx, we also utilized the advantages of having epitope-tagged ubiquitin for enrichment of ubiquitinated substrates, namely, 6×His-Flag-Ubiquitin, thereby benefiting from the existing tools for detection and enrichment of such tagged proteins. Instead of overexpression, however, in StUbEx, the tagged version replaces the majority of the endogenous ubiquitin, which is beneficial in two ways: (i) the proportion of the 6×His-Flag-Ubiquitin is much higher compared to the level of endogenous ubiquitin (in the range of 4:1), resulting in a larger fraction of the cellular ubiquitinome containing tagged ubiquitin that it is then subjected to enrichment. (ii) The total level of ubiquitin in the cell is maintained at normal levels, thereby avoiding overexpression-related artifacts. A lentiviral delivery system was utilized to generate the stable cell lines that allowed tedious clonal selection procedures to be avoided due to the high transduction efficiency of the lentiviral particles. Furthermore, the use of a single lentiviral vector carrying both the shRNA sequence targeting endogenous ubiquitin and the RNAi-resistant 6×His-Flag-Ubiquitin sequence ensured simultaneous gene silencing and transgene expression in the same cell.

We demonstrated that the StUbEx system is applicable to various cell lines, that the cells maintain normal protein ubiquitination characteristics, and that the system is suitable for unbiased ubiquitination studies. In a proof-of-principle experiment, we utilized StUbEx in combination with SILAC and mass spectrometry to quantitatively analyze the ubiquitination changes in HeLa cells in response to EGF stimulation. More than 3400 potentially ubiquitinated proteins were identified from this screen, of which 157 displayed a significant change in their ubiquitination status as a result of the treatment. Many of those, e.g., Shc1, INPPL1, Vav2, Ack1, Stam2, Odin/ANKS1A, Ymer, MAP4K3, ERRFI1, ITSN1, ASAP1, have well been associated with various aspects of EGFR signaling.<sup>70,71,74,76–81</sup> In addition, for several other proteins in this group, like EGFR, Cbl-b, Sts1, Eps15, and Epsin1, ligand-induced ubiquitination has already been shown to serve as an internalization or trafficking signal.<sup>65,82–84</sup> We also identified a large number of known participants of the EGFR signaling network that did not undergo significant changes in their ubiquitination status at the studied 6 min time point (Supporting Information Figure 5B and Supporting Information Table 1). Considering the highly dynamic nature of ubiquitination and the fact that only one

time point was analyzed, it would not be surprising if the majority of the proteins involved in the initial stages of EGFR signal transduction are subjected to regulatory ubiquitination but at different temporal dimensions.

Although a possible interplay between ubiquitin and some Ubis have been reported earlier,<sup>42,85</sup> a few leads ascended from our unbiased ubiquitination screen that may provide further basis to consolidate and broaden this intriguing notion. The pool of ubiquitinated substrates contained several Ubis, like SUMO1, SUMO2, NEDD8, and ATG12 (Supporting Information Table 1), which might be due to simultaneous conjugation of ubiquitin and these Ubis on the same proteins. However, we also identified direct ubiquitination sites on SUMO1, SUMO2, and NEDD8 (Supporting Information Table 2), which is indicative of the formation of mixed chains of ubiquitin and the corresponding Ubis. In addition, E1 activating enzymes for SUMO (namely, UBA2 and SAE1), NEDD8 (UBA3), and ATG12 (ATG7); several E3 ligases for SUMO (NSMCE2, TOPORS, RANBP2, CBX4, and PIAS1), and ATG12 (ATG5); an E2 conjugating enzyme for ATG12 (ATG3) and a SUMO-specific protease (PIAS1) were all found in the list as well. The presence of such large number of proteins involved in the SUMO/NEDD8/ATG12 conjugation processes tempts us to speculate that ubiquitination may also serve as a major signal directly regulating the processing-conjugation machinery for these Ubis.

In conclusion, we developed and characterized an RNAi-based system, namely, StUbEx, in which endogenous ubiquitin is stably replaced by an epitope-tagged ubiquitin in cultured mammalian cells. Combined with MS-based quantitative proteomics, StUbEx is an efficient and powerful system for unbiased studies of protein ubiquitination and ubiquitination-driven cellular processes.

## ASSOCIATED CONTENT

### Supporting Information

Figure 1: Schematic representation of the different lentiviral constructs. (A, B) Constructs used for the screening and optimization of the StUbEx system for (A) ubiquitin silencing alone and (B) with simultaneous expression of the RNAi-resistant 6×His-Flag-Ubiquitin. LTR, long terminal repeat; SIN, self-inactivating; U6, mouse U6 promoter; CMV, cytomegalovirus promoter; cPPT, central polypurine Tract; WRE, Woodchuck regulatory element. (C) Alignment of the DNA sequences of the human UBB gene (one of the monomers) with the transgenic RNAi-resistant 6×His-Flag-Ubiquitin. The target sequence for the shRNA used for generation of the stable StUbEx cell lines is indicated with a yellow bar. (D) The final doxycycline-controllable lentiviral system used for generation of the stable StUbEx cell lines. The tetO sequence was inserted into the pSicoR-shUbHFrUb vector as indicated. tTR-KRAB binding to the tetO sequence results in simultaneous repression of both U6 and CMV promoters. Addition of doxycycline induces the simultaneous production of the shRNA and the 6×His-Flag-Ubiquitin by competing away tTR-KRAB from the tetO sequence. Figure 2: 6×His-Flag-Ubiquitin efficiently replaces endogenous ubiquitin in the ubiquitinated proteome of StUbEx cells while maintaining normal levels of total ubiquitin in the cells. (A) Western blot images on total cellular lysates from StUbEx HeLa cells treated with doxycycline for indicated times, probed with anti-ubiquitin (left) and reprobed with anti-Flag antibodies. (B)

WB images derived from four independent experiments (same experimental setup as in panel A). Only the low-molecular-weight regions containing the free ubiquitin are shown. (C) MS-based quantitative comparison of the total levels of ubiquitin in WT and StUbEx HeLa cells. Lysates from differentially SILAC-encoded WT and StUbEx cells were mixed and separated on SDS-PAGE. Two regions of the gel, containing either the free ubiquitin or protein–ubiquitin conjugates, were analyzed separately. (D) The corresponding quantitation for conjugated, unconjugated, and total ubiquitin pool from two independent experiments is shown in Figure 3: Dual purification of 6×His-Flag-Ubiquitinated proteome form StUbEx cells. Equal amounts of StUbEx and WT HeLa cell lysates were used for the optimization of the double-affinity purification. (Left) Western blotting using antiubiquitin antibodies. (Right) Colloidal blue staining of the SDS-PAGE. Input, lysates before purification; Flowthr, flow-through, lysates after indicated purification step; Elution, eluates from indicated purification matrix. Figure 4: Identification of EGFR ubiquitination on the lysine residue at position 1061. The MS/MS spectrum (HCD fragmentation) of the EGFR peptide containing ubiquitinated lysine-1061. The di-Glycine remnant from ubiquitin on the lysine residue following trypsin digestion is indicated by GG- in the peptide sequence. Figure 5: GO terms and network analysis of the proteins with significant changes in ubiquitination status upon 6 min EGF stimulation. (A) Significantly overrepresented GO biological function categories for the proteins with regulated ubiquitination in comparison to all identified proteins. Only terms with significance  $p < 0.0001$  are presented. (B) KEGG map of the ErbB signaling pathway. Color coding: red, proteins with significant changes  $p < 0.01$ ; pink, proteins with  $p$  values between 0.01 and 0.05; proteins with not significant changes in response to 6 min EGF stimulation; white, not identified. Table 1: Proteins identified in the SILAC-based ubiquitination screen. Table 2: Ubiquitinated peptides identified in the SILAC-based screen. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We would like to thank Jakob Bunkenborg and Lasse Falkenby for helpful discussions and technical assistance. Drs. Tyler Jacks, Patrick Aebscher, and Didier Trono are acknowledged for kindly depositing lentiviral plasmids to the Addgene repository. This work was supported in part by grants from the Danish Council for Independent Research | Natural Sciences (FNU), the Novo Nordisk Foundation, and The Lundbeck Foundation.

## ABBREVIATIONS

DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; DUB, deubiquitinating enzyme; EGF, epidermal growth factor; EGFR, EGF receptor; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; GO, Gene Ontology; GProX, Graphical Proteomics Data Explorer; IP, immunoprecipitation; IPI, International Protein Index; KEGG, Kyoto Encyclopedia of Genes and Genomes; KRAB, Kruppel-associated box; LC, liquid chromatography; Lys, lysine; MS, mass spectrometry; PTM, post-translational modification; RNAi, RNA interference; shRNA, short hairpin RNA; SILAC, stable isotope labeling by amino acids in cell culture; StUbEx, stable tagged ubiquitin exchange; TRE, tetracycline response element; UBD, ubiquitin-binding domain; UbIs, ubiquitin-like proteins; WB, western blotting; WT, wild type

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