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Identification of Ubiquitin System Factors in Growth Hormone Receptor Transport

Johan A. Slotman, Peter van Kerkhof,
Gerco Hassink, Hendrik J. Kuiken and Ger J. Strous

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1. Introduction

Proteins can be covalently modified by ubiquitin. These modifications are the result of a coordinated enzymatic process and regulate many cellular networks and processes (Schwartz & Ciechanover, 2009, Weissman et al., 2011). First, ubiquitin is activated by an ubiquitin activating enzyme (E1), then the activated ubiquitin is transferred to the active cysteine of an ubiquitin conjugating enzyme (E2), which interacts with a substrate recognizing enzyme, the ubiquitin ligating enzyme (E3). This enzyme positions ubiquitin towards the substrate, after which ubiquitin is covalently linked via its C-terminal glycine residue to the ε-amino group of a lysine residue. The human genome contains two E1, 35 E2, and more than 1000 E3 enzymes. The E3s are subdivided in three types: Hect, Ring and U-box domain containing. Ubiquitin has seven internal lysine residues that can be modified with ubiquitin, resulting into chains of multiple ubiquitin moieties. Depending on the internal lysine that is modified, different types of ubiquitin chains can be synthesized. The most abundant ubiquitin chains are linked via lysine-48 (K48), lysine-63 (K63), or combinations, resulting in mixed chains (Goto et al., 2010). Additionally, it was recently discovered that linear ubiquitin chains are important in NFκB activation. These chains are formed by LUBAC ("linear ubiquitin chain-assembly complex"), by conjugating ubiquitin moieties head-to-tail (Iwai K & Tokunaga, 2009, Rahighi et al., 2009). Substrates can be modified by one ubiquitin moiety (mono-ubiquitylation), by one ubiquitin moiety on more than one lysine in the same substrate (multiple mono-ubiquitylation) or by chains of ubiquitin (poly-ubiquitylation). Analogous to phosphorylation, ubiquitin can be removed from substrates. A special class of proteases, the deubiquitylating enzymes (DUBs) of which there are approximately 100 genes in the human genome, can specifically remove ubiquitin moieties (reviewed in Soboleva & Baker, 2004). Modification by ubiquitin can have several outcomes for a substrate protein. Besides degradation by the proteasome, ubiquitin

modification can regulate a.o. DNA repair as well as sorting of transmembrane proteins, which is the topic of this chapter. Clathrin mediated endocytosis, a well characterized mode of endocytosis of membrane and cargo molecules, involves the recognition of cargo, assembly of the coat and the pinching off of the invagination (Marsh & McMahon, 1999). The cargo is acquired by adapter proteins such as AP-2 or by AP-2 binding proteins such as Epsin1 or Eps15, after which the clathrin lattice is formed and the membrane is curved by BAR domain-containing proteins (Kirchhausen, 1999). The curved domain is pinched off by dynamin and forms a cargo-containing coated vesicle. Subsequently, this vesicle is uncoated and fused with early endosomes (van der Bliek et al., 1993). From the early endosomes the cargo can either be transported toward the lysosome, ultimately resulting in degradation, or recycled back to the plasma membrane. These decisions are mediated by three endosomal sorting complexes, ESCRT-I, -II and -III (Jovic et al., 2010).

The involvement of ubiquitin in membrane traffic was first described in yeast, where ubiquitylation of both the α-factor receptor and carboxypeptidase S by the HECT E3 ligase Rsp5 is essential for endocytosis as well as for their correct trafficking into the multi vesicular bodies (MVB) (Hicke & Riezman, 1996). In mammalian cells, the role of ubiquitin was first studied for the growth hormone (GH) receptor and the sodium channel ENaC (Staub et al., 1996, Strous et al., 1996). ENaC is ubiquitylated by the HECT E3 Nedd4-2 and is subsequently recognized by the ubiquitin binding domain containing clathrin adapter Epsin1. After endocytosis, deubiquitylation of ENaC by the DUBs UHC-L3 and USP2 determines its fate at the multivesicular bodies. ENaC that remains ubiquitylated is recognized by the ESCRT complexes and degraded in the lysosome, whereas deubiquitylated ENaC is recycled back toward the plasma membrane (Butterworth & Johnson, 2008). A second DUB involved in ENaC down regulation is USP10, which can deubiquitylate sorting nexin 3, resulting in both decreased ENaC endocytosis and increased recycling. USP10 is also implied in endocytosis and recycling of the cystic fibrosis transmembrane conductance regulator CFTR (Bomberger et al., 2010, Boulkroun et al., 2008).

The role of ubiquitylation in endocytosis of receptor tyrosine kinases has been extensively studied for the tyrosine kinase receptor, the epidermal growth factor (EGF) receptor. The RING E3 ligase c-Cbl and the E2 enzyme Ube2D1-4 are involved in ubiquitylation of the EGF receptor. Its clathrin-dependent endocytosis is regulated via binding to the clathrin adapter AP-2, either directly or via the ubiquitin binding endocytosis adapter Eps15 or Epsin1. Ubiquitin plays also an important role in sorting of the EGF receptor from the endosomal system toward the lysosome. This receptor is actively sorted away from the recycling endosome by binding to Hrs, which in turn binds the flat clathrin coat on the endosome (Madshus & Stang, 2009). Hrs binds to the ESCRT-I component Tsg101 that delivers the EGF receptor to the ESCRT machinery. Incorporation of the EGF receptor into intraluminal vesicles depends on the DUBs AMSH and USP8 that are associated with the ESCRT-III machinery (Row et al., 2007). However, other studies have shown that USP8 can also act , earlier at the level of ESCRT-I, by preventing entry into multivesicular bodies promoting recycling. The interaction of USP8 to both ESCRT-I and ESCRT-III indicates a complex role for this DUB in sorting of endosomal cargo (Berlin et al., 2010).

For the cytokine receptors interferon-α receptor chain 1 (IFNAR1), the prolactin, the erythropoietin and GH receptor, βTrCP, the substrate recognizing subunit of the E3 ligase complex skip-culling-F-box (SCF), is essential for endocytosis and degradation (Kumar et al., 2004, Li et al., 2004, Meyer et al., 2007, van Kerkhof et al., 2007). Clearly, cytokine receptors appear particularly well regulated by the ubiquitin system. To identify additional regulators of ubiquitylation events involved in trafficking of cytokine receptors we used our model, the GH receptor.

The GH receptor plays an important role in growth and metabolic pathways. In contrast to the EGF receptor, the GH receptor is constitutively endocytosed and degraded in lysosomes. Using a temperature-sensitive E1 mutant, we previously showed that endocytosis is clathrin-mediated and depends on an intact ubiquitin system (Strous et al., 1996). This activity is mediated via the ubiquitin-dependent endocytosis (UbE) motif, a 12 amino acid stretch in the cytosolic tail of the GH receptor (Govers et al., 1999). The ring E3 ligase SCF(βTrCP) binds to the UbE motif and this binding is required for proper endocytosis (van Kerkhof et al., 2007). Recently, it was shown that, in addition to endocytosis, βTrCP is involved in sorting the GH receptor from multivesicular bodies towards the lysosome (van Kerkhof et al., 2011). In the absence of βTrCP, transport of the GH-GH receptor complex is halted at the multivesicular bodies and routed back to the plasma membrane. Interestingly, ubiquitylation of the receptor itself is not required for proper sorting, but it does require binding of this E3 ligase. These findings suggest that the GH receptor travels from the cell surface to the lysosome using βTrCP as a cargo-specific adapter that ubiquitylates (an ancillary factor of) the machinery instead of the cargo itself. In this study we used small interfering ribonucleic acid (siRNA) in a cell-based assay to find additional regulators of ubiquitylation involved in the fate of GH receptors expressed at the cell surface. We screened a library of siRNAs targeting DUBs and other ubiquitylation factors for their involvement in GH receptor sorting towards the lysosomes.

2. Materials and methods

2.1. Antibodies, chemicals, and cells

The DUB siRNA library (Table 1) was obtained from Dharmacon, Thermo Scientific. Anti GH receptor (B) was previously described (van Kerkhof et al., 2000). Anti-actin was obtained from ICN. Lipofectamine2000 was obtained from InVitrogen. Butyrate was obtained from Sigma. NHis6-GH receptor expressing HepG2 cells ware grown in MEM (InVitrogen), supplemented with 10% FCS, 100 units/ml penicillin and 0.1 mg/ml streptavidin and geneticin. GH receptor U2OS cells were generated and propagated as described in van Kerkhof et al., 2011.

2.2. SiRNA transfection and screening

Mixtures of 0.07 µl lipofectamine2000 and siRNAs in a total volume of 10 µl Optimem with a final concentration of 48 nM siRNA per well (348 well plates) were incubated for 30 min at

room temperature. Four thousand Nhis6-GH receptor expressing HepG2 cells were added to the mixture and allowed to propagate. After 48 h, 10 mM butyrate was added to increase the expression of GH receptor. After 72 h, the cells were incubated at 37°C with 5 ng/μl Cy3-GH for 30 min, and fixed with 3% paraformaldehyde for 4 h at room temperature. After three 5-min washes with PBS, cells were incubated with 50 μl DAPI, 0.5 μg/ml for 10 min and washed with PBS. Automated image acquisition was performed by the BD Pathway 855 system (BD Bioscience). DAPI was used for focusing and 4 images per well were acquired using a 20x objective.

Gene Symbol	Accession Number	Gene Symbol	Accession Number	Gene Symbol	Accession Number	Gene Symbol	Accession Number
ATXN3L	XM045705	UBE1C	NM003968	UBL4	NM014235	USP33	NM015017
BAP1	NM004656	UBE1DC1	NM024818	UBL5	NM024292	USP34	XM291018
COPS5	NM006837	UBE1L	NM003335	UBR1	NM174916	USP35	XM290527
CXORF53	NM024332	UBE2A	NM003336	UBTD1	NM024954	USP36	NM025090
CYLD	NM015247	UBE2B	NM003337	UCHL1	NM004181	USP37	NM020935
DUB1A	XM377830	UBE2C	NM007019	UCHL3	NM006002	USP38	NM032557
DUB3	NM201402	UBE2D1	NM003338	UCHL5	NM015984	USP39	NM006590
FBXO7	NM012179	UBE2D2	NM003339	UEV3	NM018314	USP4	NM003363
FBXO8	NM012180	UBE2D3	NM003340	UFD1L	NM005659	USP40	NM018218
FLJ14981	NM032868	UBE2E1	NM003341	USP1	NM003368	USP41	XM036729
JOSD1	NM014876	UBE2E2	NM152653	USP10	NM005153	USP42	XM166526
LOC391622	NM212553	UBE2E3	NM006357	USP11	NM004651	USP43	XM371015
MJD	NM004993	UBE2G1	NM003342	USP12	NM182488	USP44	NM032147
MYSM1	XM055481	UBE2G2	NM003343	USP13	NM003940	USP45	XM371838
OTUB1	NM017670	UBE2H	NM003344	USP14	NM005151	USP46	NM022832
OTUB2	NM023112	UBE2I	NM003345	USP15	NM006313	USP47	NM017944
OTUD1	XM166659	UBE2J1	NM016021	USP16	NM006447	USP48	NM033236
OTUD4	NM017493	UBE2J2	NM058167	USP18	NM017414	USP5	NM003481
OTUD5	NM017602	UBE2L3	NM003347	USP19	XM496642	USP50	NM203494
OTUD6B	NM016023	UBE2L6	NM004223	USP2	NM004205	USP51	NM201286
OTUD7	NM130901	UBE2M	NM003969	USP20	NM006676	USP52	NM014871
PARP11	NM020367	UBE2N	NM003348	USP21	NM012475	USP53	XM052597
PRPF8	NM006445	UBE2NL	XM372257	USP22	XM042698	USP54	NM152586
PSMD14	NM005805	UBE2Q	NM017582	USP24	XM165973	USP6	NM004505
SBBI54	NM138334	UBE2R2	NM017811	USP25	NM013396	USP7	NM003470
SENP2	NM021627	UBE2S	NM014501	USP26	NM031907	USP8	NM005154
SHFM3P1	AF174606	UBE2V2	NM003350	USP28	NM020886	USP9X	NM004652
STAMBP	NM006463	UBE3A	NM000462	USP29	NM020903	USP9Y	NM004654
STAMBPL1	NM020799	UBE3B	NM130466	USP3	NM006537	VCPIP1	NM025054
TNFAIP3	NM006290	UBE4A	NM004788	USP30	NM032663	YOD1	NM018566
TRFP	NM004275	UBE4B	NM006048	USP31	NM020718	ZA20D1	NM020205
UBE1	NM003334	UBL3	NM007106	USP32	NM032582	ZRANB1	NM017580

Table 1. DUB siRNA library. ²Underlined genes are ubiquitin-related proteins without DUB activity

2.3. Western blotting

GH receptor expressing U2OS cells were transfected with the siRNAs to silence the indicated genes. Cells were lysed in 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF in PBS for 20 min and centrifuged at 13,000 x g for 5 min at 4°C. The supernatant was boiled in Laemmli sample buffer, and the proteins were separated on SDS-PAGE and transferred to Immobilon-FL polyvinylidenedifluoride membrane (Millipore). Blots were immunostained with primary antibodies followed by Alexa Fluor 680 or IRDye 800 conjugated anti-mouse or anti-rabbit antibodies. An Odyssey system was used for detection (Li-Cor Biosciences, Lincoln NE).

3. Results

3.1. High throughput screening

GH receptor transport from the plasma membrane toward the lysosome is a complex process that requires, in addition to general endocytosis factors, specific regulatory proteins. As the ubiquitin system was implied in this process (Govers et al., 1997), we set up an assay to identify novel modifiers of regulatory ubiquitilation involved in GH uptake.

Since the liver is an important target of GH, we used the human hepatocellular carcinoma cell line HepG2 for this assay. These epithelial cells are non-tumorigenic and synthesize a variety of liver-specific proteins, such as the asialoglycoprotein receptor and several plasma proteins (albumin, transferrin and the acute phase proteins fibrinogen, β2-macroglobulin, α1-antitrypsin, and plasminogen). Although HepG2 cells are GH sensitive, they have insufficient GH receptors to probe the effect of gene silencing. Therefore, we constructed a HepG2 cell line that expresses 100-200,000 rabbit GH receptors per cell. To identify genes involved in GH receptor endocytosis and degradation, we transfected the GH receptor expressing HepG2 cells with siRNAs as indicated. After 72 h, the cells were allowed to take up GH conjugated with Cy3 (Cy3-GH) for 30 min at 37°C, after which they were immediately fixed and assessed for Cy3-GH uptake (Fig. 1).

We screened a commercial siRNA library targeting 84 DUBs and 44 other ubiquitylation-related enzymes (Table 1) for regulators of GH uptake and receptor sorting (Dharmacon). This arrayed library consists of siRNA pools constituted of 4 unique siRNA sequences, targeting a specific gene (Dharmacon). The screen was performed in a 384 well plate format and an automated spinning-disc confocal microscope was used for unbiased image acquisition, 4 images per well, of Cy3-GH and DAPI signal. The experiment itself was performed in duplicate yielding eight data points per gene. Hits were called when at least 3 out of 4 wells showed a similar phenotype. The assessment of hits was done manually in a double blind fashion by two researchers, independently. The hits were ordered in three categories: Category I, Cy3-GH enrichment on the plasma membrane, category II, Cy3-GH enrichment on both the plasma membrane and intracellular, and category III, intracellular enrichment only. Clathrin heavy chain and Tsg101 depletion, involved in clathrin-mediated endocytosis and in sorting at multivesicular bodies, respectively, were used as controls. Fig.

1 (first row) shows that clathrin depletion resulted in accumulation of Cy3-GH at the plasma membrane, whereas Tsg101 depletion resulted in Cy3-GH accumulation in both internal vesicles and on the plasma membrane. These data show that our screen setup has sufficient sensitivity to detect accumulation of GH receptor. Using the same method we identified 13 genes: 7 in category I, 3 in category II and 3 in category III (Table 2). The E1 enzyme (UBE1) was identified as a category I hit, in concordance with its previously described role in GH receptor endocytosis (Strous et al., 1997)
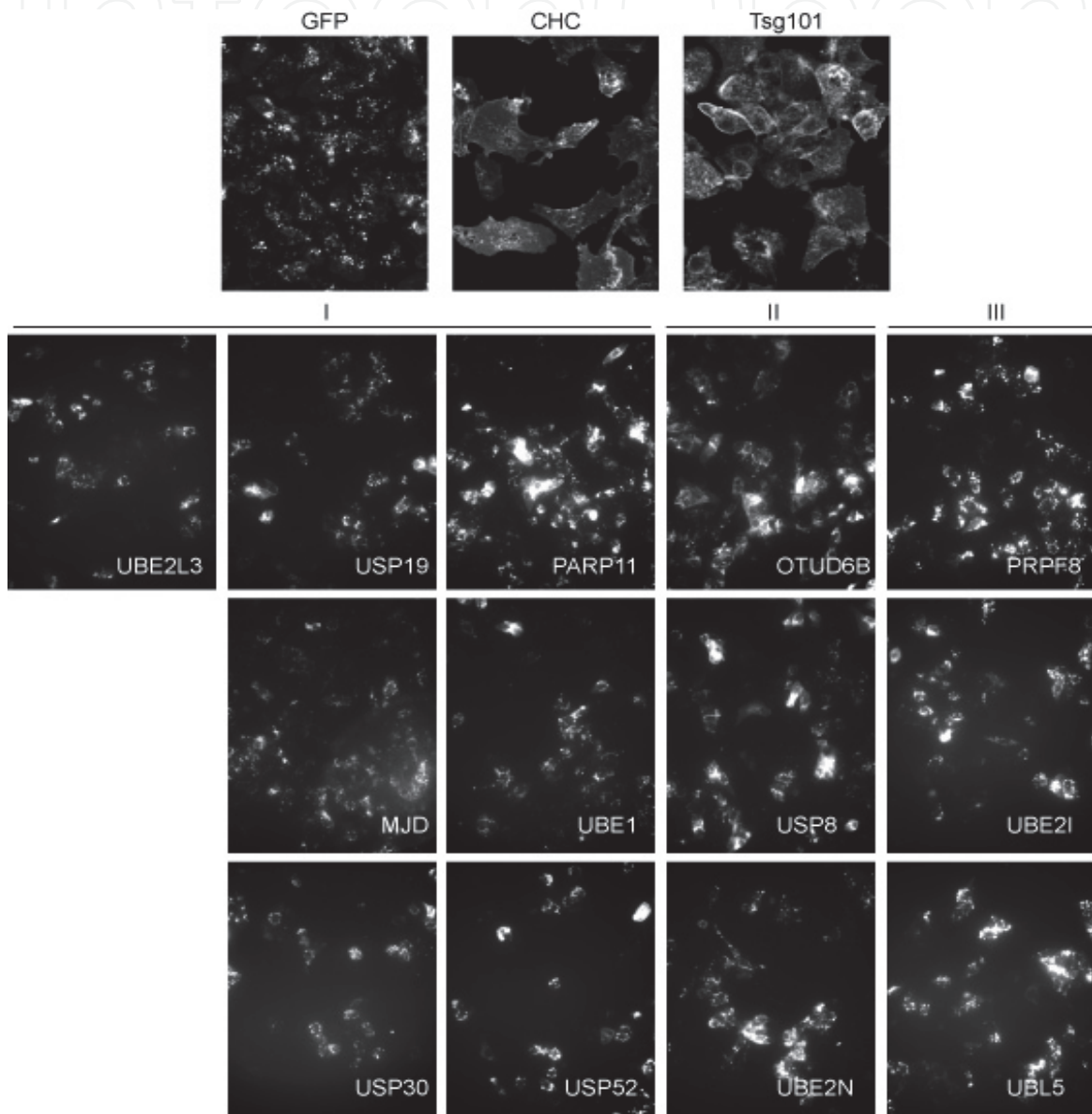

Figure 1. Representative images of observed phenotypes in the screen. Cells were transfected with smartpool siRNA, containing 4 individual siRNAs directed against a specific target. After 72 h, cells were incubated with Cy3-GH at 37°C for 30 min and fixed. Four images of the samples were automatically acquired, and analysed by eye. All the targets that were considered hits are shown. The image is a representative of 2 experiments. The images are grouped according to three pheno-typical categories: I: Cy3-GH enrichment on the plasma membrane, II: Cy3-GH enrichment on both the plasma membrane and intracellular, and III: intracellular enrichment only.

Gene	Protein	Function	Cat
USP19	Ubiquitin carboxyl-terminal hydrolase 19	DUB functioning in ER folding	I
MJD	Ataxin-3	DUB functions in IGF-1 signalling and longevity in *C. Elegans*	I
USP30	Ubiquitin specific protease 30	DUB located at outer mitochondrial membrane, function unknown	I
OTUD6B	OTU domain containing 6B	Member of the otubain DUB family, function unknown	II
PARP11	poly (ADP-ribose) polymerase family, member 11	Poly (ADP-ribose) polymerase family member, mRNA processing	I
PRPF8	pre-mRNA processing factor 8 (PRPF8 homolog, yeast)	mRNA processing factor functions in the spliceosome	III
UBE1	Ubiquitin activating enzyme E1	Human ubiquitin-activating enzyme E1	I
USP52	Ubiquitin specific peptidase 52	DUB, function unknown	I
USP8	ubiquitin specific peptidase 8	DUB, involved in EGF receptor sorting at multivesicular bodies	II
UBE2I	ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	Human homolog of UBC9, activating enzyme for ubiquitin like protein SUMO	III
UBE2L3	ubiquitin-conjugating enzyme E2L 3	Ubiquitin conjugating enzyme, cell cycle, can function together with Triad1	I
UBE2N	ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	Ubiquitin conjugating enzyme able to catalyse K-63 linked ubiquitin chains	II
UBL5	ubiquitin-like protein 5 (BEACON homolog, human)	Ubiquitin like protein, differentially expressed between lean and obese	III

Table 2. Screening results: Genes with a phenotype

3.2. Validation of hits

To validate candidate genes, we tested 8 siRNA pools with the strongest phenotypes in the first screen (USP19, Ubc9, Usp52, Usp8, Ubc13, Ubl5, PRPF8, and OTUD6B) in a second screen using a different cell type. For this purpose, we transfected osteosarcoma U2OS cells with rabbit GH receptor and selected a clone that expressed a limited number of exogenous GH receptors (Fig. 2A). In our experience, U2OS cells have a limited capacity to endocytose cargo via the clathrin-mediated pathway, a feature that renders the endocytosis assay particularly sensitive. In addition to the siRNA pools we transfected the individual siRNAs that constituted the pools (data not shown). In all cases at least 2 out of 4 siRNAs showed a similar phenotype as the pool, decreasing the likelihood of off-target effects. These 8 hits were examined in more detail.

3.2.1. Usp19

Usp19 depletion showed an overall increase in Cy3-GH signal as well as some cell surface labelling (Fig 2A). Usp19 is an ER-localized membrane protein (Hassink et al., 2009), which was originally identified as a DUB induced in atrophying muscle (Combaret et al., 2005). It stabilizes ER localized proteins like CFTR and TCRα (Hassink et al., 2009) as well as cytosolic proteins like Hif1α, Siah1 and 2, c-IAP1 and 2, and the cyclin-dependent kinase inhibitor regulating ubiquitin ligases KPC1 and 2 (Altun et al., 2012, Lu et al., 2009, Mei et al., 2011). Interestingly, the catalytic activity of USP19 does not seem to be required for the stabilization of its substrates, but for stabilization of itself, facilitated via self-association (Altun et al., Mei et al., 2011). It also modulates transcription of major myofibrillar proteins (Sundaram et al., 2009), probably in a similar way as the earlier mentioned proteins. Furthermore, Usp19 is upregulated in smoke-induced muscle atrophy conditions in mice (Liu et al., 2011).
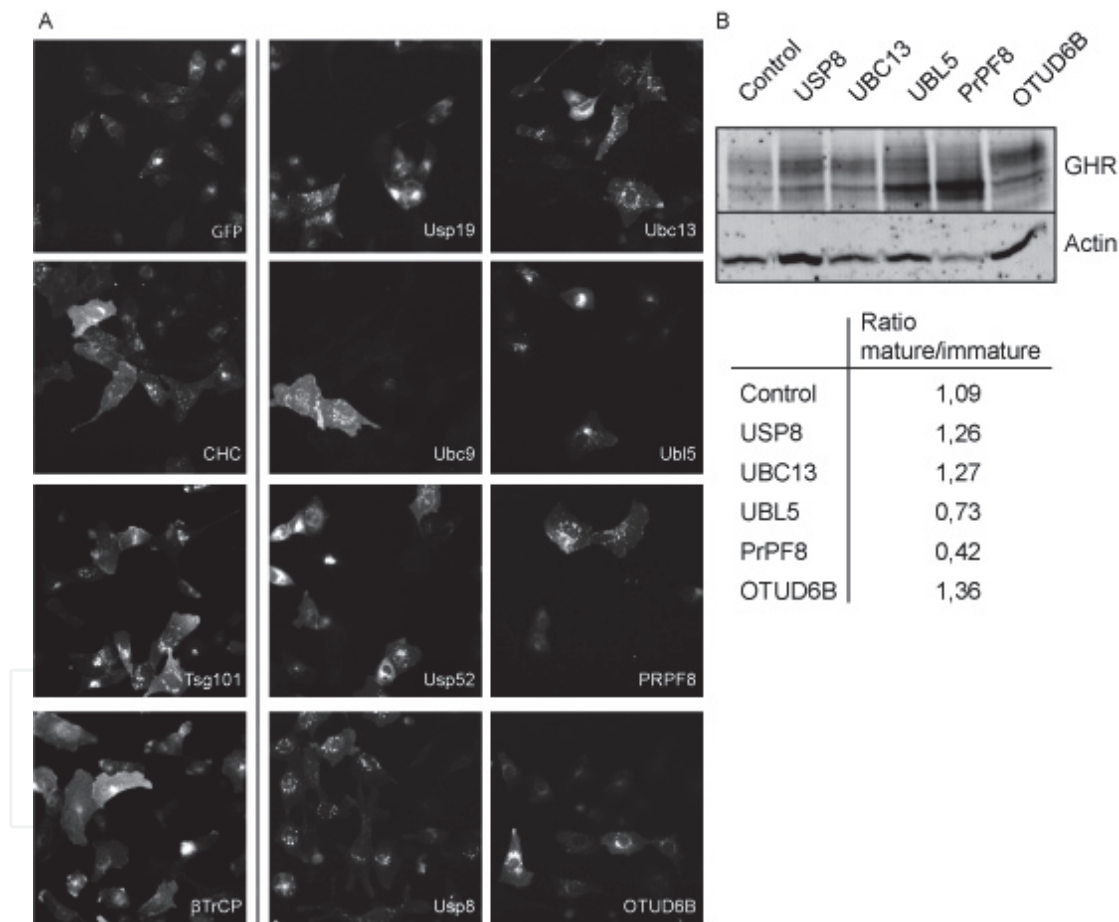

	Ratio mature/immature
Control	1,09
USP8	1,26
UBC13	1,27
UBL5	0,73
PrPF8	0,42
OTUD6B	1,36

Figure 2. Validation of hits obtained by the initial screen. GH receptor expressing U2OS cells were transfected with siRNAs as indicated. A) After 72 h, the cells were allowed to take up Cy3-GH for 30 min at 37°C and immediately fixed. Cy3-GH uptake was assessed by automated confocal microscopy; images are representative of 4 experiments. B) After 72 h, cells were lysed and proteins were separated by SDS-PAGE followed by Western blot analysis for GH receptor (GHR) using actin as a loading control. The upper GHR band represents the mature GHR, while the lower band is the immature (ER) form. The ratio mature/immature for the GHR species was taken as a measure for the turnover of the GHR related to the steady state situation in control cells.

Since the predominant splice variant is a type IV membrane protein, USP19 might act as an ER resident protein with no direct role in GH receptor regulation at the endosomal level. Further characterization was beyond the scope of this study. Alternative roles in the cytosol will undoubtedly become clear in the future.

3.2.2. Usp8

Usp8, also known as UBPY, is a deubiquitylating enzyme that functions in protein sorting at multivesicular bodies. Depletion of Usp8 showed a perinuclear accumulation of Cy3-GH punctae (Fig. 2A), reminiscent of the phenotype resulting from Tsg101 depletion. To characterize the effects of gene silencing on the GH receptor degradation further we performed Western blot analysis for the GH receptor. This approach reveals additional details related to the role of USP8 in GH receptor trafficking. At steady state, Western blot analyses of the GH receptor show an approximately 1:1 ratio of the amount of 'immature' (high mannose oligosaccharides-containing) receptor, mainly located in the rough ER, and the amount of the 'mature', complex-glycosylated GH receptor, mainly present at the plasma membrane. Previously, we determined the half-life of both GH receptor species to be 50 min at 37°C. The half-life of the high-mannose GH receptor is defined as the time it takes for the GH receptor to exit the ER and arrive at the Golgi complex to receive the 'complex' sugar moieties, while the half-life of the 'mature' GH receptor is defined as the time required for its transport from plasma membrane to the lysosomes. As Usp8 depletion caused a relative increase in mature over immature GH receptor levels, the results suggest that Usp8 promotes GH receptor degradation.

Usp8 can bind to ESCRT-III components and its depletion leads to enlarged endosomes and diminished incorporation of EGF receptors into the intraluminal vesicles (ILV) of the multivesicular bodies (Bowers et al., 2006). In addition, it has been reported that Usp8 can interact with the ESCRT-0 component STAM via its SH3 domain and that its depletion results in accelerated degradation of EGF receptor in the lysosome, strongly suggesting that Usp8-dependent deubiquitylation of EGF receptor prevents EGF receptor from being recognized by the ESCRT machinery, a recognition depending on ESCRT-0 component Hrs (Berlin et al., 2010, Rao et al., 2011). Furthermore, the ability of Usp8 to bind both ESCRT-I and ESCRT-III suggests a dual function for Usp8 in receptor sorting. Indeed, at the plasma membrane Usp8 can inhibit degradation of receptors by removing the ubiquitin tag that directs them to the multivesicular bodies, while at the multivesicular bodies Usp8 can promote degradation of receptors by allowing incorporation of receptors into the ILVs (Wright et al., 2011). We demonstrate here that depletion of Usp8 increases the level of intracellular GH receptor indicative of a defect in sorting towards the lysosome, most likely at the level of ESCRT-III. The latter is supported by previous data showing that the transport of GH receptor from multivesicular bodies to the lysosome does not depend on ESCRT-0 components. Apparently, the GH receptor enters the ESCRT complexes at the level of ESCRT-I (van Kerkhof et al., 2011).

3.2.3. Ubl5

Ubl5 depletion leads to an increased accumulation of Cy3-GH in endosomes. The Western blot analyses showed a complex pattern wherein both the immature GH receptor band and the mature GH receptor were increased compared to control cells. Ubl5 is a small ubiquitin like modifier that, in contrast to other ubiquitin like molecules, does not have a C-terminal glycine residue. A remarkable finding implicated a mutation in non-coding regions of Ubl5 in metabolic syndrome-related phenotypes (Bozaoglu et al., 2006). In a recent study, Mishra and co-workers show that its yeast homologue Hub1 alters the splice specificity for certain non-canonical 5' splice sites by non-covalent attachment to elements of the spliceosomes (Mishra et al., 2011). Combining the above renders it plausible that expression levels of Ubl5, altered by mutations in the non-coding region, attenuate the expression of proteins ultimately affecting metabolism. Since the GH receptor is tightly involved in regulation of metabolic pathways, the involvement of Ubl5 in GH receptor trafficking toward the lysosome is interesting. Either the GH receptor itself or GH receptor-regulating factors might be affected by Ubl5. Preliminary data from ^{125}I-GH uptake experiments suggest that depletion of Ubl5 does not alter the internalization and degradation kinetics of the receptor. Furthermore, a direct interaction between the GH receptor and Ubl5 could not be identified (data not shown). Since steady state levels of GH receptor as measured with Western blot analyses did increase as a result of Ubl5 silencing (Fig. 2B), the data suggest that Ubl5 either influences transcription or synthesis of the GH receptor. To fully understand the role of Ubl5 more investigation is required.

3.2.4. PrPF8

Pre-mRNA-processing-splicing factor 8 (PrPF8) is a large nuclear protein that functions in the U2, U12 and trans-spliceosome, and has an active role in processing of pre-mRNA (reviewed in Grainger & Beggs, 2005). PrPF8 knockdown showed an intracellular accumulation of Cy3-GH. In addition, the Western blot analysis showed an increase in the immature form of GH receptor and a complex band pattern of slower migrating bands. This might indicate that multiple pathways are affected. Because of PrpF8's role in the spliceosome, it is likely that depletion of this protein can influence correct splicing of many factors including the GH receptor and proteins of the endocytosis machinery. We did not yet elucidate whether PrPF8 is directly or indirectly involved in GH receptor endocytosis. For example, previously, we have shown that alternative splicing of βTrCP isoforms may affect their localization, which may have consequences for GH receptor endocytosis (Putters et al., 2011b). Because the GH receptor mRNA was artificially transcribed from a plasmid driven by a CMV promoter (pcDNA3) it is possible that this might indirectly lead to alterations in sorting of GH receptor at the plasma membrane or at endosomes by exceeding the sorting capacity of these systems. Alternatively, absence of PrPF8 may affect the balance of folding factors in the ER, resulting in ER overload of GH receptors that cannot leave the ER, e.g. due to defective dimerization (van den Eijnden et al., 2006).

3.2.5. OTUD6B

Ovarian tumour (OTU) domain containing 6B (OTUD6B) is a member of the OTU-domain Ubal-binding protein (otubain) domain containing protein family that constitutes a specific subset of deubiquitylating enzymes. The function of OTUD6B is not known. Substrates of two other OTU domain containing proteins, OTUB1 and OTUB2, are GRAIL (Soares et al., 2004), oestrogen receptor (Stanisic et al., 2009), p97 (Ernst et al., 2009), RhoA (Edelmann et al.), Traf3 and Traf6 (Li et al., 2010). They have been implicated in non-canonical DNA-damage response (Nakada et al., 2010). Furthermore, OTUB1 shows substrate specificity towards K48 linked ubiquitin chains (Wang et al., 2009). We have identified OTUD6B as a novel factor in GH receptor endocytosis. Its depletion leads to a phenotype where mature GH receptor accumulates, mainly in the endosomes but also at the plasma membrane. Fig. 2B clearly shows a decreased degradation with little effect on its biosynthesis. Analogous to Tsg101 depletion, this might point to a role in multivesicular body function. Further investigations are necessary to determine whether OTUB6D acts specific on the GH receptor or has a general role in endosomal sorting.

3.2.6. Ubc13 and associated E3s

The presence of UBE2N, encoding for the E2 enzyme Ubc13, in our hit list was striking. Previously, after our finding that ubiquitylation is required for GH receptor endocytosis and degradation, we identified SCF(βTrCP) as an E3 that specifically binds to the UbE motif of the GH receptor and conjugates K48 poly-ubiquitin chains to the GH receptor (Putters et al., 2011a, van Kerkhof et al., 2007). With the identification of Ubc13 it became plausible that also K63-linked ubiquitin chains might be involved in GH receptor endocytosis, as Ubc13 together with the pseudo E2s UEV1A and MMS2 can synthesize K63 linked ubiquitin chains (Deng et al., 2000).

Gene	Protein	Function	Reference
ARIH2*	Triad1	Cell cycle, cell growth	(Marteijn et al., 2009)
CHFR*	CHFR	Cell cycle	(Bothos et al., 2003, Loring et al., 2008)
PARK2*	Parkin	Neuroprotection, cell death	(Lo Bianco et al., 2004)
RNF8*	RNF8	DNA repair	(Plans et al., 2006)
Rad5	Rad5	DNA repair	(Torres-Ramos et al., 2002)
Rad18	PCNA	DNA repair	(Ulrich & Jentsch, 2000)
STUB1*	CHIP	Heat shock system	(Zhang et al., 2005)
TRAF2	Traf2	TNF signalling	(Habelhah et al., 2004)
TRAF6	Traf6	TNF signalling	(Wooff et al., 2004)

Table 3. E3 ligases known to interact with Ubc13

Furthermore, these K63-linked ubiquitin chains have been implied in endocytosis in yeast (Lauwers et al., 2009) and in mammalian cells (Kamsteeg et al., 2006). To investigate whether K63-linked ubiquitylation is indeed involved in GH receptor endocytosis, we selected nine E3 ubiquitin ligases that are known to interact with Ubc13 (listed in Table 3) (Bothos et al., 2003, Deng et al., 2000, Loring et al., 2008, Marteijn et al., 2009, Plans et al., 2006, Sun et al., 2004, Torres-Ramos et al., 2002, Zhang et al., 2005). As gene silencing of Traf2 and Traf6 induced cell death in previous experiments (data not shown), and rad5 and rad18 are mainly involved in DNA repair, we excluded them from further investigation and performed gene silencing experiments for the 5 remaining E3 ligases using validated siRNAs (Table 3, asterisk).

We transfected GH receptor-expressing U2OS cells with these siRNAs and propagated them for 3 days prior to performing the assay as described above. Cells, treated with siRNAs for ARIH2 and STUB1, encoding Triad1 and CHIP, respectively, showed a strong phenotype (Fig. 3). Not only was the label intensity much higher, silencing of both genes also induced accumulation of Cy3-GH signal on the plasma membrane. Recently, we published details about the role of the ubiquitin ligase CHIP in GH receptor endocytosis involving a direct interaction with specific amino acid sequences in the cytosolic tail of the receptor. The same study shows that CHIP acts in collaboration with UBC13, implicating K63-specific ubiquitylation in the GH receptor endocytosis (Slotman et al., 2012). This is in accordance with Fig. 2.
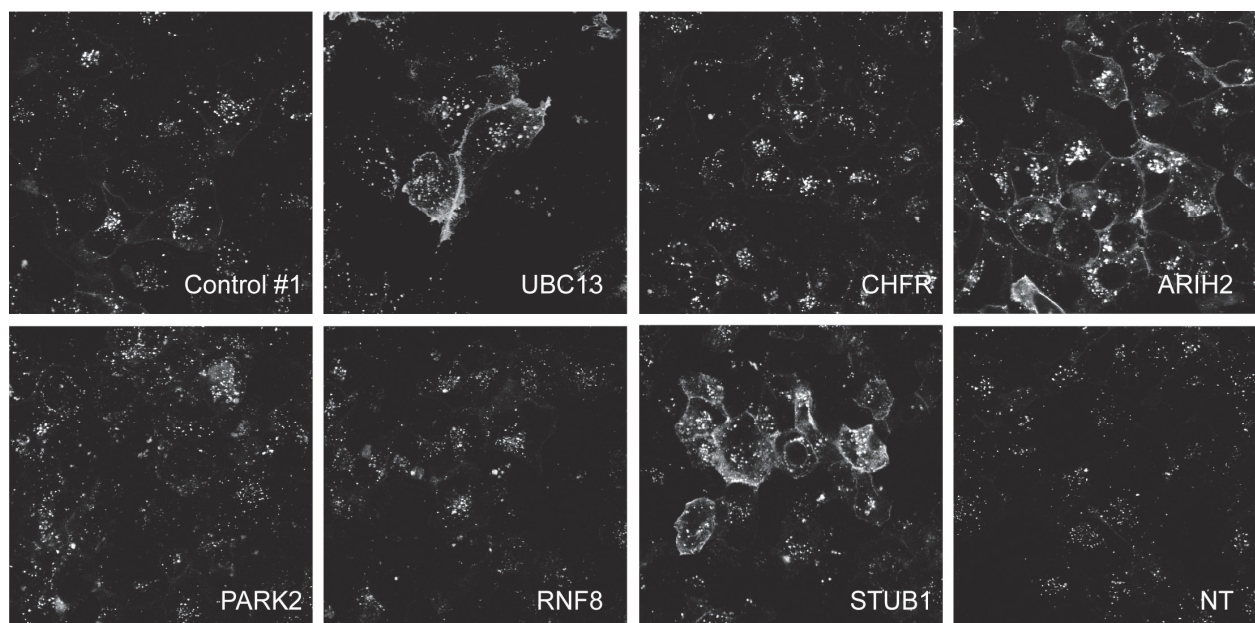

Figure 3. Mini-screen for E3s involved in Ubc13 mediated ubiquitylation. GH receptor-expressing U2OS cells were transfected with validated siRNAs targeting 5 E3 ligases that can function together with the E2 Ubc13. After 72 h, the cells were allowed to take up Cy3-GH at 37°C for 30 min after which they were fixed. Cy3-GH uptake was assessed by confocal microscopy. Shown cells are representative for all cells in the samples of 2 independent experiments.

Triad1-depleted cells showed larger internal vesicles with an altered pattern as compared to wild type. Triad1 is an E3 ligase that has 2 RING domains and, consequently, has the ability to bind two ubiquitin conjugases. Previously, Marteijn and co-workers identified both Ubc13 and UbcH7 as E2s involved in Triad1 action (Marteijn et al., 2009). As seen in Fig. 1 and Table 2, UBCH7 (UBE2L3) also tested positive in our initial screen, strengthening the involvement of Triad1 in the sorting of GH receptors in endosomes (Fig. 1, Table 2)

In conclusion, we identified three novel genes in GH-induced endocytosis via the GH receptor, two of which (UBC13 and STUB1) demonstrated identical phenotypes, while silencing of ARIH2 resulted in a different pattern. All three enzymes are involved in K63-linked ubiquitin chain formation. Further studies are being performed to determine whether ARIH2 acts specifically on the GH receptor or whether it has a broader function in endocytosis and/or lysosomal degradation (Hassink et al., 2012).

4. Discussion

We performed a siRNA screen in which we assayed 84 DUBs and 44 other enzymes of the ubiquitin and related systems. In the first screen we identified 13 genes that showed an altered uptake or degradation pattern of Cy3-GH. As expected, the UBE1 gene, encoding the E1 enzyme, one of two ubiquitin activating enzymes in mammalian cells, was among the 13 hits. It is known that by disrupting the enzymatic activity of the E1 enzyme GH receptor endocytosis is blocked (Strous et al., 1996). The observation that E1 silencing results in a similar phenotype shows that the screen setup is valid and able to pick up factors of the ubiquitin system that are involved in (GH receptor) endocytosis. Furthermore, the large number of hits in this screen clearly shows that GH receptor trafficking by ubiquitin is a complex and well regulated process.

Among the proteins that did not appear in our screen as a hit, but would be expected to show a phenotype, is the activating enzyme (E1) of the ubiquitin like modifier ISG15. ISG15ylation of Ubc13 disrupts its function (Zou et al., 2005) and depletion of this E1 would lead to less ISG15ylation of Ubc13 that might result in an increased endocytosis of GH receptor. Unfortunately, our approach lacks the sensitivity to register accelerated endocytosis. An assay that would pick up such an effect must be suited to measure endocytosis in a quantitative way rather than qualitatively. Another factor that we expected to find was UBE2M, which functions as the E2 for neddylation of cullins, the modification by the ubiquitin like protein Nedd8. Cullin neddylation activates SCF(βTrCP), which is essential for GH receptor ubiquitylation and degradation (van Kerkhof et al., 2007). Recently, the ubiquitin conjugase UBE2F was identified as a Nedd8 conjugating enzyme (Huang et al., 2009). Therefore, UBE2M and UBE2F might have a redundant function, possibly explaining the absence of UBE2M among our hits. Of note, UBE2F was not targeted by our siRNA library and was therefore not tested in our assay.

The screen identified two proteins involved in ubiquitin-like mechanisms, UBE2I, also known as UBC9, the E2 for SUMOylation, and UBL5, an ubiquitin like protein. This is the first time that these two pathways are implicated in GH receptor trafficking. Many similarities exist between the ubiquitin-based and ubiquitin-like mechanisms with many examples of interplay. In GH receptor trafficking SUMOylation might block ubiquitylation and *vice versa* (Denuc & Marfany, 2010). The action of UBL5 is more mysterious, since it cannot be conjugated to substrates due to a lack of a C-terminal glycine residue. Hypothetically, ubiquitin binding domains could be blocked by the structurally very similar UBL5 and in this a way UBL5 may regulate ubiquitylation events (McNally et al., 2003). A correlation between obesity and UBL5 mRNA levels was found in P. *obesus* (Walder et al., 2002) but this finding is still debated in humans (Bozaoglu et al., 2006, McNally et al., 2003). Since GH receptor signalling is highly involved in growth and obesity (Erman et al., 2010, Gao et al., 2010), altering the sorting of GH receptor could be involved. Hence, GH receptor sorting defects, UBL5, and growth and metabolic diseases may be linked.

We identified two novel ubiquitin ligases that are involved in GH receptor trafficking, CHIP and Triad1; thus, together with SCF(βTrCP), at least three E3s have a regulatory role in GH receptor degradation. This indicates that both K48 and K63-linked ubiquitin chain formation are involved. In addition, we identified six DUBs involved in GH receptor endocytosis and homeostasis: USP19, MJD, USP30, OTUD6B, UPS52 and USP8. These DUBs might antagonize the action of the ligases mentioned above, providing a high level regulation. Together, these proteins are part of a complex system that regulates ubiquitylation of many substrates. In part by spatial en temporal regulation, ubiquitylation and deubiquitylation events modulate the sorting of GH receptor, and thereby respond quickly to different forms of stress. Additionally, the number of distinct E2, E3, and DUB enzymes involved in ubiquitylation provides specificity in regulating different classes of receptors in disparate ways within the same endocytic system.

These findings underscore the complexity of the ubiquitylation system and endocytosis process, and, at the same time, offer interesting opportunities to discover drugs that specifically target the GH receptor. As this receptor is implicated both in anabolism, in longevity and insulin sensitivity (Bartke, 2012), drugs that can either up- or down regulate its activity are highly relevant.

5. Conclusion

The ubiquitin proteasome system plays an essential role in trafficking of the growth hormone receptor from the plasma membrane to the lysosomes, where degradation takes place. When ubiquitylation is disabled, the GH receptor accumulates at the plasma membrane and its degradation is inhibited. As endosomal sorting is a highly regulated process that depends on a variety of ubiquitylation events, we set up a cell-based, high content siRNA screen targeting 128 genes of the ubiquitylation system. In this study we report the identification of 13 modifiers of regulatory ubiquitylation events that are involved in trafficking and degradation of the GH receptor. As the GH receptor is a key regulator of

metabolism, this study highlights the complexity of pathways that underlie its regulation. These findings may guide the development of specific drugs that either up- or down-regulate GH-based signal transduction. As GH signalling is implicated in longevity, insulin-sensitivity, and cancer, studies focussed at factors that regulate ubiquitylation and GH receptor levels are highly relevant.

Author details

Johan A. Slotman, Peter van Kerkhof, Gerco Hassink and Ger J. Strous
Department of Cell Biology, University Medical Center Utrecht and
Institute of Biomembranes, Utrecht, The Netherlands

Hendrik J. Kuiken
Netherlands Cancer Institute, Division of Molecular Carcinogenesis, Amsterdam, The Netherlands

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