Investigation of a role for the endosomal deubiquitylase USP8 in cancer cell survival pathways

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by:

Alice Emma Louise Howarth

December 2016

Investigation of a role for the endosomal deubiquitylase USP8 in cancer cell survival pathways

Alice Emma Louise Howarth

Ubiquitylation is a reversible post-translational modification regulated by a number of enzymes. E1 activating, E2 conjugating and E3 ligase enzymes facilitate the modification of proteins by adding the 8.5kDa protein, ubiquitin, to lysine residues. The presence of seven internal lysine residues within ubiquitin itself allows the construction of complex chains with different chain-linkages. This complexity means that ubiquitylation plays a role in a number of cellular events including protein degradation. This signal is reversed through the action of deubiquitylases (DUBs).

One of the roles for ubiquitylation is the targeting of proteins for lysosomal degradation and DUBs are important for this function. USP8 is an endocytic deubiquitylase involved in the regulation of a number of cell surface proteins. USP8 is important for endosomal sorting and the trafficking of cargo for degradation by the lysosome of a number of substrates. More recently USP8 has been linked to the regulation of cell viability of some cancer cells with a purported link to the PI3K-AKT cell survival pathway.

In this thesis I identify a role for USP8 in the survival of EGFR-mutant, gefitinib-resistant, non-small cell lung cancer cells. Depletion of USP8 induces apoptosis in these cells. I confirm a link with the PI3K-AKT signalling pathway evidenced by a synthetic lethal effect of inhibiting this pathway when combined with USP8 depletion.

In addition I identify a novel link between USP8 and the NRF2-KEAP1 reactive oxygen species (ROS) management pathway. USP8 depletion decreases protein levels of the NRF2 inhibitor, KEAP1. USP8 depletion sensitises NSCLC cells to induction of ROS.

I conclude that USP8 is a DUB involved in a number of pathways including cell survival.

Table of contents

List of figures

List of tables

Abbreviations

ABC-transporter	ATP-binding cassette transporter
ACTH	Adrenocorticotropic hormone
AMBA1	Activating molecule in Beclin related autophagy
AMSH	Associated molecule with the SH3 domain of STAM
AMSH-LP	AMSH like protein
AP	Adaptor protein
APC/C	Anaphase promoting complex/cyclosome
APF-1	ATP-dependent proteolysis 1
ARE	Antioxidant response element
ASK1	Apoptosis signalling related kinase 1
ATG	Autophagy related gene
ATG14L	Atg14 like protein
ATP	Adenosine triphosphate
BAD	BCL2-associated death promotor
BAK	BCL2 homologous antagonist/killer
BAR	Bin amphiphysin Rvs
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BIF-1	BAX-interacting factor 1
BTB	Bric-à-brac, tramtrack and broad complex

CALM	Clathrin assembly lymphoid myeloid leukaemia
CBP	CREB-binding protein
CCCP	Carbonyl cyanide m-chlorophenyl hydrosome
CCP	Clathrin coated pit
CCV	Clathrin coated vesicle
CHMP	Charged multivesicular body protein
CIN85	Cbl interacting protein of 85kDa
CME	Clathrin mediated endocytosis
CNC	Cap 'n' collar
CREB	cAMP response element binding
C-terminus	Carboxy terminus
Cul1	Cullin 1
Cul3	Cullin 3
Cvt	Cytoplasm to vacuole targeting
CXCR4	Chemokine receptor 4
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DDR	Discoidin domain receptor
DGR	Double glycine repeat
DiUb	Di-ubiquitin
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DUB	Deubiquitylase
E1	Ubiquitin activating enzyme

E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERAD	Endoplasmic reticulum associated degradation
ESCRT	Endosomal sorting complex required for trafficking
FOXO	Forkhead box
FRET	Förster resonance energy transfer
GEF	Guanine nucleuotide exchange factor
GGA	Golgi-localising, γ adaptin ear homology domain, ARF-binding proteins
GH	Growth hormone
GHR	Growth hormone receptor
GLUE	GRAM-like ubiquitin-binding in Eap45
GPCR	G-protein coupled receptor
GST	Glutathione s transferase
HD-PTP	His-domain-containing protein tyrosine phosphatase
ILV	Intralumenal vesicle
ITGA2	Integrin α 2
JAMM	JAB1/MPN/MOV34
KEAP1	Kelch-like ECH associated protein 1
KIR	KEAP1 interacting region
LAMP1	Lysosomal-associated membrane protein 1
LDL	Low-density lipoprotein

LIR	LC3 interacting region
M6PR	Mannose 6 phosphate receptor
MAPK	Mitogen-activated protein kinase
MCL1	Induced myeloid leukaemia cell differentiation protein
MDM2	Mouse double minute 2 homologue 2
MINDY	MIU-containing novel DUB
mTORC1	Mammalian TORC1
MVB	Multivesicular body
NAC	N-acetyl cysteine
NEAA	Non-essential amino acids
NEM	N-ethylaleimide
NGF	Nerve growth factor
NQO1	NAD(P)H:quinine oxidoreductase 1
NRF2	Nuclear factor (erythroid-derived 2)-like 2
NRG1	Neuregulin 1
NSCLC	Non-small cell lung cancer
N-terminus	Amino terminus
OTU	Ovarian tumour protease
OTULIN	OTU deubiquitylase with linear linkage specificity
PARP	Poly ADP-ribose polymerase
PAS	Pre-autophagosomal structure
PB1 domain	Phox and Bem1 domain
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor

PDK1	Phosphoinositide-dependent kinase 1
PE	Phosphatidylethanolamine
PGAM	Phosphoglycerate mutase
PH	Plekstrin homology
PI(3,5)P2	Phosphatidyl inositol 3, 5 bisphosphate
PI3K	Phosphoinositide 3 kinase
PI3*P*	Phosphatidyl inositol 3 phosphate
PINK1	PTEN induced putative kinase 1
PIP_2	Phosphatidyl inositol 4,5 bisphosphate
PIP_3	Phosphatidyl inositol 3,4,5 trisphosphate
PKC	Protein kinase C
POMC	Proopiomelanocortin
PTEN	Phosphatase and tensin homologue
PTP1B	Protein-tyrosine phosphatase 1B
PTPN23	Protein tyrosine phosphatase non-receptor type 23
RBR	RING between RING
RING	Really interesting new gene
RME	Receptor mediated endocytosis
RNAi	RNA interference
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RUBICON	Run domain Beclin1-interacting and cysteine-rich containing protein
SCF complex	Skp, Cullin, F box complex

SNARE	Soluble NSF attachment protein receptor
SOUBA	Solenoid of overlapping UBA
SQCC	Squamous cell carcinoma
SQSTM1	Sequestosome 1
STAM	Signal transducing adaptor protein
STAMBP	STAM binding protein
SUMO	Small ubiquitin-like modifiers
tBHP	tert-butylhydroperoxide
tBHQ	tert-butylhydroquinone
TBK1	TANK binding kinase
TEMED	Tetramethylethylenediamene
TGN	Trans-Golgi network
TKB	Tyrosine kinase-binding
TOM complex	Translocase at the outer membrane complex
TORC1	Target of rapamycin complex 1
TRAIL	TNF-related apoptosis-inducing ligand
UBA	Ubiquitin associated
Ub-AMC	Ubiquitin-amidomethylcoumarin
UBD	Ubiquitin binding domain
UBL	Ubiquitin like
UbVME	Ub-vinyl methyl ester
UbVS	Ubiquitin-vinyl sulfone
UCH	Ubiquitin C-terminal hydrolase

UIM	Ubiquitin interacting motif
USP	Ubiquitin specific protease
UVRAG	UV radiation resistance associated gene
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Vps	Vacuolar protein sorting
WIPI	WD-repeat protein interacting with phosphoinositides

Acknowledgement

Completing a PhD requires support and guidance in many forms and I have been remarkably fortunate to have both in abundance.

To my wonderful supervisors, Prof. Sylvie Urbé and Prof. Michael Clague – I give my thanks for both their academic and human support. They have taught me a lot about what it means to be a good scientist and I hope I can apply these lessons in my future career. I also give thanks to Prof. Ian Prior and Prof. Judy Coulson for giving their insights on things ranging from scientific projects to academic careers.

I am grateful for the inspiration, advice, discussions and friendship of my colleagues on the 5th floor past and present. Particularly I would like to thank Ewan MacDonald, Yvonne Tang and Claire Heride for being patient teachers. Also thanks to Joe Sacco for guidance on experiments. I thank, in no particular order; Doug, Lisa, Fiona, Leah, Emma, Dora, Aitor, Leila, Eri, Adam, Sofia, Elena, Andreas, Vicky, Amos, Viktor, Han, Rosanna, Jenny, Ali, Sarah, Jenna, Neil, Matthew, Zohra, Andrew, Vruti, Rachel, Jo, Amy and Arnaud. This list comprises a remarkable group of people who have contributed to a fun, welcoming and stimulating working environment.

To my family: my mother and father Kate and Mark are patient, supportive and encouraging. I am where I am because of their guidance. I also thank my brother Jonathan. I am grateful to Lynn and Alan for being my family away from home and for always asking after my cells.

To the friends who are the very dearest people in my heart. They have provided an immeasurable amount of support. To Marsh and Nicola for being incredible people whom I love, admire and value so very much. Your support has been invaluable. To Andrew, Emma, Laurie and Karin for being some of the most wonderful people I know and providing me with much love and laughter and being the best Eggs. To Mike, Katy, Lana, Tom, Celia, Kat,

Geraint, Ash and Chrissie for being ace and supportive and keeping me going.

And finally to my incredible fiancé, Warren. For providing me over a decade of love, happiness, laughter and support. For the things we've learned together, for his patience and for being my constant inspiration. Thank you for being my best friend and taking this journey with me.

Chapter one: Introduction

1.1. The discovery of ubiquitin-dependent protein degradation.

The concept of protein-turnover was first proposed in the late 1930s when studies using nitrogen isotope fed mice provided evidence for amino acid biosynthesis and recycling (Schoenheimer, Rittenberg et al. 1938). In the 1950s Christian De Duve and colleagues discovered a key degradative organelle involved in protein degradation, the lysosome, and characterised a number of pH sensitive lysosomal enzymes (see 1.2) (De Duve, Pressman et al. 1955, De Duve and Wattiaux 1966). For a long time, lysosomal degradation was thought to be the only pathway for protein degradation. However Poole et al. showed that blocking lysosomal degradation did not prevent the breakdown of the majority of cytosolic proteins (Poole, Ohkuma et al. 1977).

In 1977, Etlinger and Goldberg reported that degradation of a mutated form of haemoglobin in a cell-free reticulocyte lysate assay required ATP (Etlinger and Goldberg 1977). Proteolysis was optimal at a pH of around 7.8, which precluded the activity of acidic pH dependent lysosomal hydrolases.

Shortly after, Hershko, Ciechanover and Rose not only confirmed the reliance on ATP for proteolysis in the cell-free reticulocyte assay but also identified a small protein that was fundamental in this protein breakdown. Key to this discovery was the fact that they had separated the cell-free lysates into three fractions - I, IIA and IIB (Hershko, Ciechanover et al. 1979). Within fraction I they found a factor which they named ATP-dependent proteolysis factor 1 (APF-1). This was a small, 8.5kDa protein that was required to activate the ATP-dependent proteolytic activity of the combined IIA and IIB fractions. By the early 80s Ciechanover, Hershko and colleagues had shown that this small protein was conjugated to other proteins and that this modification required ATP (Ciechanover, Heller et al. 1980, Hershko, Ciechanover et al. 1980, Ciechanover 2005). Additionally, they reported that this protein could form chains (Hershko, Ciechanover et al. 1980). This work

eventually won Hershko, Ciechanover and Rose the 2004 Nobel Prize for Chemistry (Wilkinson 2005).

Prior to this, Goldstein and colleagues had identified a protein important for lymphocyte differentiation. They named this protein ubiquitin due to its ubiquitous expression profile (Goldstein, Scheid et al. 1975, Schlesinger and Goldstein 1975, Schlesinger, Goldstein et al. 1975). In the late 70s two articles suggested that ubiquitin was conjugated to histone H2A. The two proteins combined had been referred to as a nuclear protein, A2F, but analysis of the sequence and identification of an isopeptide bond suggested that A2F was a conjugate of these two proteins (Goldknopf and Busch 1977, Hunt and Dayhoff 1977).

In 1980, Wilkinson and colleagues suggested that APF-1 was, in fact, ubiquitin – a theory that was proven by comparison of the amino acid sequence of the two proteins (Ciechanover, Elias et al. 1980, Wilkinson, Urban et al. 1980, Wilkinson 2005).

1.1.1. Ubiquitylation: a reversible post-translational modification

Ubiquitiylation is a reversible post-translational modification in which the carboxy (C)-terminal glycine of ubiquitin is covalently linked to proteins predominantly at lysine residues. Ubiquitin contains itself 7 internal lysine residues allowing a daisy-chain of ubiquitin moieties to be added. This results in the formation of a series of chain linkages (Figure 1.1). Additionally, linear chains can be formed by the binding of a second ubiquitin moiety (distal ubiquitin) to the amino (N)-terminal methionine of the first (proximal ubiquitin). These chains can become more complex still by the formation of mixed and/or branched chain linkages (figure 1.1.B) (Ye and Rape 2009, Heride, Urbe et al. 2014).

The system can be likened to phosphorylation where phosphate groups are added to serine, threonine and tyrosine residues by kinases often marking enzymatic activation. Ubiquitylation is a much more complex modification than phosphorylation due to its chain linkage diversity. As kinases add

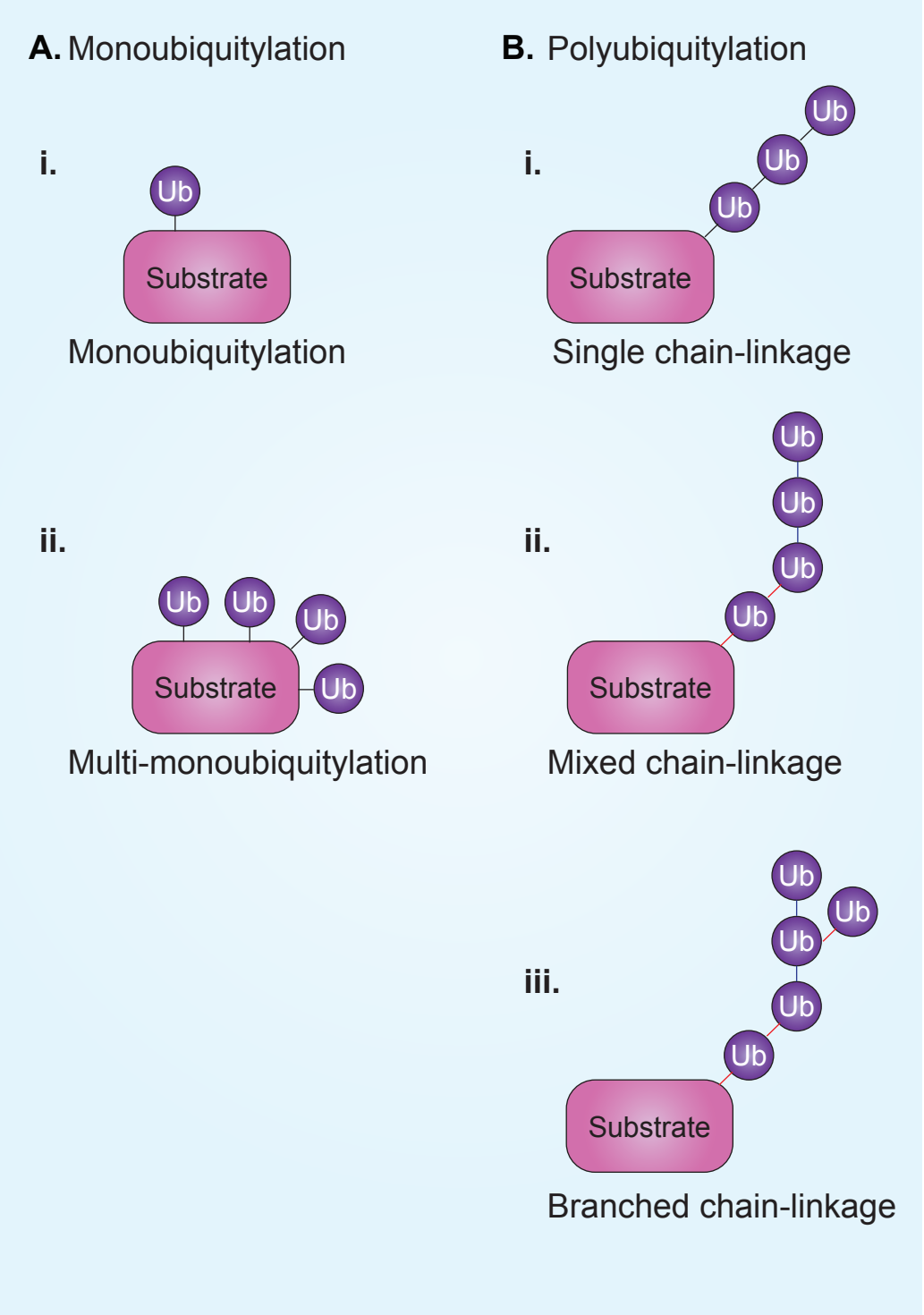


Figure 1.1. The variety of ubiquitylation types in cells. Ubiquitylation is the conjugation of a ubiquitin moiety to substrates. Substrates may be mono- or poly-ubiquitylated. (A.) Monoubiquitylation involves the linkage of a single ubiquitin via an isopeptide bond to the substrate. This can either be on a single lysine residue (i.) or on multiple lysines (ii.). (B.) Polyubiquitylation is the build up of ubiquitins into chains. Due to the variety of chain linkage types this can take the form of a chain made up of a single linkage type (i.), a single chain with multiple linkage types (ii.) or a branched chain with one ubiquitin residue acting as a bridge between two or more linkage types (iii.).

phosphate groups to substrates, so too are enzymes involved in the addition of ubiquitin to proteins. Again this is more complex in the case of ubiquitylation. Here, a sequence of enzymes, namely ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3), are the enzymes that add the ubiquitin to substrates (Clague, Heride et al. 2015).

1.1.2. Ubiquitin activation, conjugation, ligation

Three types of enzymes cooperate to attach ubiquitin on to substrates. The number of distinct proteins for each family of enzymes is hierarchical – there are 2 E1s, 40 E2s and over 600 E3s. However the actual copy number of proteins in each class tips the balance in favour of the E2s with an approximate E1:E2:E3 ratio of 1:3:2 in HeLa cells (Kulak, Pichler et al. 2014, Clague, Heride et al. 2015).

E1 conjugation enzymes rely on ATP to activate and bind ubiquitin. In vertebrates this group comprises UBA1 and UBA6 with the former outweighing the latter in abundance by 10:1 (Clague, Heride et al. 2015). E2s interact with both activated ubiquitin and the E1 enzyme to transfer the ubiquitin moiety to the active site cysteine of the E2. Different E2s play different roles; some determine chain specificity, whilst others are important for chain initiation or alternatively for conjugating ubiquitin to ubiquitin and therefore chain extension (Clague, Heride et al. 2015).

Substrate specificity is bestowed by the E3 ubiquitin ligases. This group of enzymes can be split into three main subgroups; the HECT E3s, RING E3s and RING between RING (RBR) E3s. They differ in how they facilitate the conjugation of ubiquitin to substrates. The HECT and RBR E3 ligases take the ubiquitin chain onto their own active-site cysteine from the E2 conjugating enzymes before passing it on to the substrate. RING E3s, however, lack this active-site cysteine so must bring E2 and substrate into close proximity allowing transfer directly from one to the other (figure 1.2) (Rotin and Kumar 2009, Heride, Urbe et al. 2014, Dove, Stieglitz et al. 2016). In addition to these families there are also complex multimeric ligases. An example of

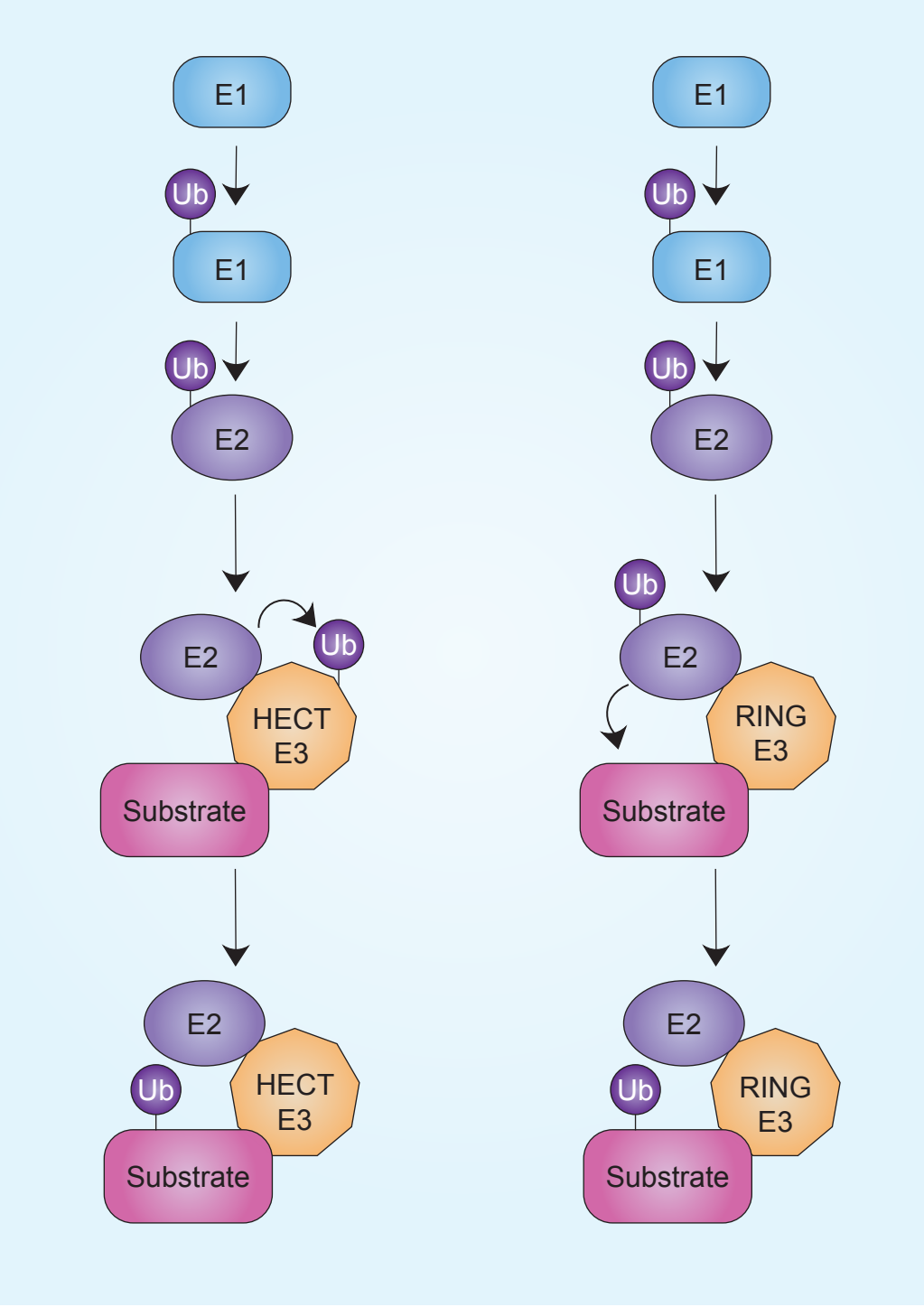


Figure 1.2. The process of ubiquitylation. In an ATP driven process, an E1 activating enzyme picks up ubiquitin which is transferred to an E2 conjugating enzyme. The E3 ligase then brings the E2 enzyme and substrate together. In the case of HECT and RBR E3 ligases (left) the ubiquitin moiety is taken and transferred to the substrate. In the case of RING E3 ligases (right) the E3 acts to bring the E2 enzyme bound to ubiquitin into close proximity of the substrate facilitating the transfer of ubiquitin without ubiquitin first being passed to the E3 ligase.

these comes from the large family of Cullin-RING E3-ligases which are composed of one of five Cullin scaffold proteins, a RING protein and a variety of other adaptor proteins. The first of these to be described, and perhaps the best characterised, is the Skp, Cullin, F-box (SCF) complex which is made up of the Skp1 adaptor, an F-box protein, Cullin1 (Cul1) and the RING ROC1/RBX1/HRT1 (Petroski and Deshaies 2005, Heride, Urbe et al. 2014). Another of these Cullin-RING E3s, the Cul3-RING complex will be described in greater detail in section 1.5. Similarly, a subunit of the anaphase promoting complex/cyclosome (APC/C) contains a Cullin homology domain and the complex itself acts as an E3 ligase in the regulation of the cell cycle (Petroski and Deshaies 2005, Bochis, Fetica et al. 2015).

1.1.3. Roles of ubiquitylation

As discussed above, ubiquitylation was first identified as a way of marking proteins for non-lysosomal ATP-dependent degradation. Now we know that ubiquitylation is the key signal targeting proteins for degradation by the 26s proteasome. The 26S proteasome was first partially purified by Rechsteiner and colleagues when they discovered an ATP-dependent protease for the degradation of ubiquitin-tagged lysozyme in rabbit reticulocyte lysates (Hough, Pratt et al. 1986, Hough and Rechsteiner 1986). This protease was 600kDa in mass. Later studies showed that, in fact, this multi-protein complex is comprised of a central catalytic 20S subunit between two 19S regulatory subunits as well as a number of associated accessory proteins including ubiquitin receptors (Hough, Pratt et al. 1987, Waxman, Fagan et al. 1987, Eytan, Ganoth et al. 1989, Driscoll and Goldberg 1990).

Primarily, K48-linked ubiquitylation is the major signal for this type of degradation (Chau, Tobias et al. 1989, Finley, Sadis et al. 1994, Grice and Nathan 2016). In particular longer chains (four or more) of K48-linked ubiquitin or multiple short K48-linked chains are optimal (Thrower, Hoffman et al. 2000, Lu, Lee et al. 2015). The proteasome recognises ubiquitylated proteins through the presence of three ubiquitin receptors (Chen, Randles et al. 2016, Shi, Chen et al. 2016). Two of these receptors, Rpn10 and Rpn13

show similar specificity for both K48- and K63-linked ubiquitin *in vitro*; however K63-linked chains are not as readily degraded by the proteasome in cells. The reasons for this are not yet fully clear (Jacobson, Zhang et al. 2009, Lu, Lee et al. 2015).

Mixed chain linkage types might also target proteins for proteasomal degradation. For example, whilst K11-linked chains do not in themselves act as a signal for proteasome-mediated proteolysis, heterotypic K11/K48 chains do (Grice, Lobb et al. 2015). Additionally, there is some evidence that on occasion multi-monoubiquitylation can serve as the signal for proteasomal degradation as is the case for cyclin B1 (see figure 1.1) (Lu, Lee et al. 2015).

In addition to proteasomal degradation, we now know that ubiquitylation, in particular via K63-linked poly-ubiquitin chains acts as a signal for lysosomal degradation. This will be discussed in detail in 1.2. Ubiquitylation might also act as a signal for the sequestration of misfolded proteins or damaged organelles into autophagosomes (see 1.2) (Stolz, Ernst et al. 2014).

Ubiquitylation is not just a target for different types of degradation. Due to its complexity it is involved in a host of signalling events including the cell cycle, DNA damage repair and modulation of transcription. Ubiquitylation can also play a role in protein localisation and protein interactions, which can subsequently regulate signalling (Komander and Rape 2012).

1.1.4. Deubiquitylases (DUBs)

As with phosphorylation, ubiquitylation can be reversed by an enzyme. In this case, deubiquitylases (DUBs) perform this function. There are around 100 DUBs encoded in the human genome of which 84 are proposed to be active (Komander, Clague et al. 2009, Clague, Barsukov et al. 2013). DUBs are split into five well-characterised families plus one newly identified family. The families are separated based on the structure of their catalytic domains. Five of the families are defined as cysteine proteases. These DUBs rely on the presence of a catalytic triad of conserved amino acids, which bear resemblance to other cysteine proteases such as papain. These five families

are the ubiquitin specific proteases (USPs), the ubiquitin C-terminal hydrolases (UCHs), the ovarian tumour proteases (OTU), the Josephins and the MINDY family. The sixth family are zinc metalloproteases JAB1/MPN/MOV34 (JAMMs) (Komander, Clague et al. 2009, Clague, Barsukov et al. 2013). The novel family of MINDY-DUBs comprises just four members, FAM63A, FAM63B, FAM188A and FAM188B (Abdul Rehman, Kristariyanto et al. 2016). These are cysteine proteases however they bear enough structural differences to the other cysteine protease families to be set within a unique group. These four novel DUBs have specificity for K48-linked poly-ubiquitin chains (Abdul Rehman, Kristariyanto et al. 2016). Rehman et al. showed that MINDY1 (FAM63A) has a preference for tetraubiquitin, a length specificity which may also apply to the other three members of the family (Abdul Rehman, Kristariyanto et al. 2016, Maurer and Wertz 2016).

DUBs have a variety of domains, many of which are important for their interaction with substrates and/or ubiquitin itself. These ubiquitin binding domains (UBDs) can be split further into subcategories including ubiquitin interacting motifs (UIMs) or ubiquitin associated domains (UBAs). Another common feature in DUBs are ubiquitin-like folds (UBL folds) which structurally look similar to ubiquitin but lack the di-Gly motif at the C-terminus and therefore cannot be cleaved by DUBs (Komander, Clague et al. 2009, Clague, Barsukov et al. 2013).

The variety within the DUBs and their UBDs allows for a range of substrate specificities and chain-linkage specificities. This was first shown for AMSH (also known as STAMBP), a JAMM-domain containing deubiquitylase with a key role in the internalisation of receptors which will be discussed in much greater detail in section 1.2. AMSH shows exquisite specificity for K63-linked ubiquitin chains in *in vitro* DUB activity assays (McCullough, Clague et al. 2004, McCullough, Row et al. 2006, Komander, Reyes-Turcu et al. 2009). A crystal structure of the highly related AMSH-like protein (AMSH-LP) in complex with K63-linked di-ubiquitin (diUb) has been solved (Sato, Yoshikawa et al. 2008). This structure shows how the enzyme binds to both the proximal and distal ubiquitin within the chain via the catalytic domain and two family specific insertions (Ins1 and Ins2). Understanding the molecular

structure of the interface between AMSH-LP and diUb provides a rationale for the linkage specificity of this DUB. The shared structure between AMSH and AMSH-LP suggests that the two share a common mechanism of action (Davies, Paul et al. 2011).

At least two other members of the JAMM family (BRCC36 and POH/PSMD14) also show K63-linkage specificity, suggesting that this may be a common feature of this family (Cooper, Cutcliffe et al. 2009).

In contrast, the USP family of DUBs show very little chain specificity (Faesen, Luna-Vargas et al. 2011). One exception is CYLD, which also shows a clear preference for K63- over K48-linked ubiquitin chains, but in contrast to AMSH also cleaves linear ubiquitin chains (Komander, Lord et al. 2008, Komander, Reyes-Turcu et al. 2009).

The DUB family with the most defined linkage specificity is the OTU family. Mevissen and colleagues purified the 16 OTU deubiquitylases which have a complete catalytic triad and performed *in vitro* DUB activity assays using all 8 types of diUb chains (Mevissen, Hospenthal et al. 2013). Cleavage of the isopeptide bond was assessed by separating diUb and monomeric ubiquitin by SDS-PAGE. Six of the DUBs they tested had a preference for a single chain linkage – these were Cezanne and Cezanne 2 which preferred K11-linked chains, OTUD4 and OTUB1 which preferred K48-linked ubiquitin, OTUD1 which cleaved K63-linked chains and OTULIN which cleaved linear chains. They also identified an additional four DUBs that had linkage specificity for two chains: OTUD3 cleaved K6- and K11- linked chains, A20 and VCPIP preferred K11- and K48-linked ubiquitin and phosphorylated OTUD5 cleaved K48- and K63-linked chains. Interestingly unphosphorylated OTUD5 was not active in their DUB assay (see below). Finally four OTU DUBs (OTUD2, OTUD6A, OTUB2 and TRABID) were able to cleave three or more chain linkages whereas the final two (ALG13 and OTUD6B) were inactive in their assay.

MALDI-TOF mass spectrometry offers an alternative approach to assess chain linkage specificity (Ritorto, Ewan et al. 2014). The authors expressed, purified and then incubated 42 DUBs with diubiquitin topoisomers

representing each chain-linkage type *in vitro*. They then used MALDI-TOF mass spectrometry to quantitate the output of monoubiquitin by each DUB with each linkage type to interpret their chain-linkage specificity. From there, they separated the DUBs they characterised into high specificity, moderate specificity and low specificity categories. Within the high specificity group was OTULIN, which cleaves linear M1-linked chains, OTUB1 which is specific for K48 and AMSH and AMSH-LP, which prefer K63-linked chains. The moderate specificity group included A20, which cleaves K48 but at higher concentrations will cleave K11, Cezanne, which is specific for K11 but can also cleave K48 and K63, and TRABID, which prefers K29 or K33 but will also cleave K63 at higher concentrations. They also identified a large number of DUBs that displayed very little chain linkage type specificity such as USP1, USP7, USP8 and USP15. In fact all of the USPs they used in their assay displayed low specificity, which is in line with other work showing that USPs tend to be more promiscuous than other DUB-families (Faesen, Luna-Vargas et al. 2011). Finally, Ritorto et al. found a number of DUBs with little to no activity including OTU1, ATXN3L and JOSD1.

DUBs themselves have three main roles. Firstly they are able to cleave the bonds between the linear chains of three or nine ubiquitins that make up two of the four gene products encoding ubiquitin. The other two genes encode a linear fusion between RPL40 and RPS27A (60s and 40s ribosomal subunits respectively) and ubiquitin and also require DUB activity to liberate one ubiquitin molecule. Thus DUBs generate a pool of free ubiquitin for the cell. Secondly, DUBs are important for terminating signalling events or rescuing proteins targeted for degradation. Once proteins are committed to the degradation pathway, DUBs also remove ubiquitin allowing it to be recycled by the cell. Finally DUBs are able to modulate signalling by trimming chains and allowing new branches to be built (figure 1.3) (Komander, Clague et al. 2009).

1.1.4.1. Regulation of DUBs

DUBs can be regulated in much the same way as many other enzymes. They can be regulated by post-translational modifications such as phosphorylation. For example, USP8 is phosphorylated at Ser680 triggering its interaction with and inhibition by 14-3-3 proteins. This phosphorylation is removed during the M-phase of the cell cycle when USP8 deubiquitylating activity is increased as cells divide (Mizuno, Kitamura et al. 2007). Conversely, evidence shows that phosphorylation of OTUD5 is sufficient and required for the enzymatic activity of this DUB. OTUD5 is phosphorylated at Ser177 which does not alter the structure of the apo (unbound) form (Huang, Ma et al. 2012). The crystal structure of OTUD5 in complex with ubiquitin highlights the importance of the phosphorylation at this residue. Once phosphorylated, OTUD5 is able to wrap around its substrate bringing the catalytic triad into an enzymatically competent configuration (Huang, Ma et al. 2012).

Another DUB, that requires a conformational change for activation is USP7. USP7 switches between the active and inactive state and the active state can be stabilised by interaction with GMP synthase, which helps bring the catalytic triad into the correct confirmation (Faesen, Dirac et al. 2011) .

In other cases, DUBs can be regulated by their localisation. USP19, for example, has a transmembrane domain inserting it into the endoplasmic reticulum membrane where it plays an important role in the endoplasmic reticulum associated degradation (ERAD) pathway and exit of newly synthesised and fully folded LRP6 from the ER (Hassink, Zhao et al. 2009, Perrody, Abrami et al. 2016).

Similarly, DUBs can be recruited to other complexes. For example the K63-ubiquitin specific JAMM deubiquitylase, AMSH, is brought to the early endosome due to its interaction with ESCRT-0 complex member STAM and multiple ESCRT-III components including CHMP3 and Clathrin (McCullough, Row et al. 2006). AMSH depletion enhances the degradation of the ESCRT-cargo and EGFR downregulation presumably by enhancing ubiquityation dependent sorting of EGFR into the multivesicular bodies (McCullough, Row et al. 2006). AMSH activity is enhanced by its direct interaction with STAM

which is reliant on the UIM domain of STAM (see section 1.2 for detail) (McCullough, Row et al. 2006). Another example of DUB interactions with other complexes can be seen in USP28. This DUB binds to Fbw7, a substrate adapter of the Skp1-Cul1 E3 ligase complex. This interaction is required for the recognition of USP28 - substrates which include c-Myc. In this case the DUB and the E3 ligase complex counteract one another fine-tuning c-Myc protein stability (Diefenbacher, Chakraborty et al. 2015).

1.1.4.2. Measuring DUB activity

There are a number of ways to assay the DUB activity of a protein. Some assays use fluorescence as an output, others use molecular weight shifts on a gel or rely on mass spectrometry (see above). One fluorescence-based technique monitors the cleavage of ubiquitin-amidomethylcoumarin (AMC) (Russell and Wilkinson 2005, Ovaa 2007). In this case, ubiquitin is fused to the fluorophore AMC and combined with a DUB *in vitro*. The fluorescence of this fluorogenic substrate increases when the DUB releases the AMC.

More recently, active-site directed probes have become useful tools for studying the activity of DUBs (Ovaa 2007, Ekkebus, Flierman et al. 2014). The first of these had a ubiquitin moiety with a vinyl sulfone (UbVS) group attached. This VS group irreversibly modifies UCH and USP deubiquitylases allowing a shift of approximately 8.5kDa (the size of ubiquitin) to be detectable by SDS-PAGE. Subsequently, different chemical groups have been used to increase the sensitivity of the probes. An example of these – the Ub-VME probe – is used and described in chapter four (McGouran, Gaertner et al. 2013).

Neither of the above two methods informs on linkage specificity. A simple assay for the chain-linkage specificity of DUBs is to combine diUb of different linkages and a DUB of interest *in vitro* and resolve the products on a gel, which can either be developed by silver stain or processed by western blot. DiUb runs differently on an SDS-PAGE gel and can be distinguished from monoubiquitin if the active DUB cleaves the bonds. Similarly, chain-length

specificity can be identified using increasing lengths of ubiquitin chains and monitoring the cleavage by SDS-PAGE.

A similar type of assay makes use of Förster Resonance Energy Transfer (FRET) whereby the DUB activity separates two fluorophores attached to isopeptide-linked ubiquitin moieties triggering a shift in the wavelength (Horton, Strachan et al. 2007). Similarly the Progenra CHOP-assays can be used whereby cleavage of the isopeptide bond releases a reporter enzyme usually from a single ubiquitin and allows generation of fluorescence from the enzyme acting upon its substrate.

Mass spectrometry has also been used to identify cleavages of di-ubiquitin as described above: Matthias Trost and colleagues recently generated diUb topoisomers of each chain linkage type using ^{15}N-ubiquitin, which could then be measured by MALDI-TOF mass spectrometry after *in vitro* incubation with a number of deubiquitylases (Ritorto, Ewan et al. 2014).

1.1.5. Ubiquitylation as a proteolysis signal

I described above that ubiquitylation was first identified for its role in proteasomal degradation. In the next section I will discuss the role of ubiquitylation in the regulation of both lysosomal and autophagosomal degradation.

1.2. Ubiquitin-dependent sorting in the endo-lysosomal pathway

1.2.1. The lysosome

Christian de Duve and colleagues first coined the term ‘lysosome’ in 1955 (De Duve, Pressman et al. 1955). It was named such due to the organelle’s proposed digestive function, as the word is Greek for digestive (lysis) body (soma). The publication in 1955 was preceded by a number of biochemical studies that first described the organelle (Berthet, Berthet et al. 1951, Berthet

and De Duve 1951, Appelmans, Wattiaux et al. 1955). These studies employed the use of a novel fractionation technique developed by Albert Claude that involved centrifugation to separate four fractions – a nuclear fraction, a large-granule fraction comprised mostly of mitochondria, a small-granule fraction referred to as microsomes and a final supernatant (Claude 1946, Claude 1946). This led to the discovery of an additional fraction, which could be identified by the presence of an enzyme called acid phosphatase (De Duve, Pressman et al. 1955). The activity profile of the enzyme varied based on the level of decay in the samples, suggesting that this enzyme was enclosed within a membrane and required release in order to interact with its substrate. The team worked out a five-fractionation protocol and identified a fraction that lay between those of the mitochondria and of the microsomes in density. By 1955 an additional four enzymes had been characterised in this fraction, all of which behaved in a similar way to acid phosphatase. These were β-glucoronidase, Cathepsin (now known as Cathepsin D), Ribonuclease and Deoxyribonuclease (de, Berthet et al. 1951, De Duve, Pressman et al. 1955). They also identified a fifth enzyme, which behaved differently and was later shown to belong to another novel cytoplasmic particle, the peroxisome (De Duve and Baudhuin 1966). Following this biochemical identification of the organelle, the group went on to visualise the lysosome by electron microscopy (Novikoff, Beaufay et al. 1956). The presence of these degradative enzymes within the lysosomes, gave us our first glimpse at a mechanism for protein turnover (Simpson 1953, Ciechanover 2005). We now know that the lysosome has two key degradative functions. Firstly to degrade proteins internalised by endocytosis and secondly to fuse with autophagic vacuoles to allow the turnover of cellular organelles and cytosolic proteins (Kornfeld and Mellman 1989).

The lysosome is comparable to the yeast vacuole, which serves as a key degradative organelle in yeast (Klionsky, Herman et al. 1990, de Marcos Lousa and Denecke 2016). Lysosomes and vacuoles differ in relative size and number: yeast cells contain one to five large 5μm vacuoles whereas mammalian cells contain 50-1000, small lysosomes between 0.1-1μm in size. However, like the lysosome, the yeast vacuole is an acidic compartment

containing pH-sensitive enzymes, which allow the breakdown of proteins delivered to this organelle (Klionsky, Herman et al. 1990).

1.2.2. Endocytosis

Endocytic processes can be categorised into macro-scale and micro-scale endocytosis. Macro-scale endocytosis includes phagocytosis and macropinocytosis, sometimes referred to as cell eating and drinking. In this type of endocytosis a cell can consume large particles or portions of extracellular liquid relative to the size of the cell itself. Micro-scale endocytosis is internalisation on a smaller scale of around 200nm (Kumari, Mg et al. 2010). This can be split further still into clathrin-mediated, caveolar and different types of clathrin- and dynamin-independent endocytosis. Here I will focus on clathrin-mediated endocytosis (CME), which is involved in delivering cell surface receptors to the lysosome for degradation.

1.2.3. Clathrin-mediated endocytosis

In 1964, Thomas Roth and Keith Porter published their study of the internalisation of yolk proteins by *Aedes aegypti* oocytes (Roth and Porter 1964). Building on the idea that the process of internalisation was similar to micropinocytosis, they took sequential electron micrographs and showed invagination of the cell membrane. The pits, and subsequently pinched-off vesicles, had a 20µm bristle-like coat, which Kanaseki and Kadota later showed to be comprised of a polygon lattice that appeared to surround the vesicle like a basket (Kanaseki and Kadota 1969). Prior to this, at the end of the 19th century, Metchnikoff had shown that cells internalised litmus paper from outside of the cell into acidic compartments and in the 1930s, W. H. Lewis imaged macrophages internalising extracellular fluid (Roth 2006). However, it was the detailed electron micrographs from Roth and Porter that gave us our first visualisation of the coat itself.

In the 1970s, the major protein component of this lattice-like coat was isolated and named Clathrin by Barbara Pearse (Pearse 1975, Pearse 1976).

1.2.4. The Clathrin coat

The now iconic structure of the Clathrin-basket has, over the years, become well characterised. Clathrin is comprised of three heavy chains each with an associated light chain (Crowther and Pearse 1981, Kirchhausen and Harrison 1981, Ungewickell and Branton 1981). These form a triskelion structure with the heavy chains providing three 'legs' each bent at the 'knee' and 'ankle' (Smith, Grigorieff et al. 1998, ter Haar, Musacchio et al. 1998, Musacchio, Smith et al. 1999). The light chains are believed to confer additional regulation in both yeast and mammalian cells (Nathke, Hill et al. 1990, Pley, Hill et al. 1995, Huang, Gullberg et al. 1997, Chu, Pishvaee et al. 1999). Pearse identified Clathrin as by far the most abundant protein of the Clathrin coat. However even in her first paper on this topic she identified the presence of other proteins which she suggested might "represent light impurities or minor proteins associated with vesicles" (Pearse 1975).

We now know that Clathrin itself cannot interact with the lipid bilayer. It is recruited to the membrane via its interaction with adaptor protein (AP) complexes. AP complexes were first identified as approximately 100kDa 'assembly polypeptides' (Zaremba and Keen 1983, Robinson 2015). When the fraction containing 100kDa proteins was combined with Clathrin itself, *in vitro* reassembly of disrupted Clathrin coated vesicles (CCVs) was enhanced (Keen, Willingham et al. 1979). AP complexes are hetero-tetrameric protein complexes comprised of an α-type (α, γ, δ, ε or ζ), β, μ and σ chain (Kirchhausen 2000). In mammalian cells there are five known AP complexes: AP-1, AP-2, AP-3, AP-4 and AP-5. The former three of these have been shown to be capable of binding Clathrin (Kirchhausen 2000, Park and Guo 2014). The most recent of these AP complexes to be identified, AP-5, has been shown to interact with the late endosome and plays a role in endosomal sorting but does not interact with Clathrin (Hirst, Barlow et al. 2011). Yeast

cells have three AP complexes of which only one has been shown to bind Clathrin (Huang, D'Hondt et al. 1999).

In addition to AP complexes, Clathrin can also bind to other adaptor proteins called Golgi-localising, γ-adaptin ear homology domain, ARF-binding proteins (GGA proteins) (Nakayama and Wakatsuki 2003). These are monomeric adaptors, conserved from yeast to humans that are found at the Golgi. GGAs, together with AP-1, interact with cargos such as the Mannose 6 Phosphate Receptor (M6PR) which binds to lysosomal hydrolases. GGAs therefore play a key role in transporting lysosomal hydrolases from the *trans*-Golgi network (TGN) to the endosome via CCVs (Braulke and Bonifacino 2009). Endosomal maturation and subsequent fusion with the lysosome allows the delivery of these enzymes to the lysosome. The M6PR dissociates from the hydrolases at the low pH of the endosome and is retrieved by retrograde trafficking facilitated by the retromer (Trousdale and Kim 2015).

AP complexes allow the recruitment of Clathrin to cargo, and cargo to Clathrin by acting as a bridge between the two (Keen, Willingham et al. 1979, Pearse and Bretscher 1981, Pearse and Robinson 1990, Brodsky, Hill et al. 1991, Kirchhausen, Bonifacino et al. 1997, Kirchhausen 2000). The AP complex required for Clathrin-mediated endocytosis is AP-2. AP-2 contains an α chain, a β2 chain, a μ2 chain and an σ2 chain (Robinson and Pearse 1986, Ahle, Mann et al. 1988, Hirst and Robinson 1998). The N-terminal region targets AP-2 to the plasma membrane whereas other AP complexes are targeted elsewhere and therefore play different roles in trafficking. For example AP-1 is targeted to the TGN via the small G protein Arf-1 and is important for Golgi to endosome trafficking alongside GGAs (Gaidarov, Chen et al. 1996, Gaidarov and Keen 1999, Zhu, Traub et al. 1999).

The flexibility of Clathrin itself allows curvature of the membrane. This is especially evident at the knee region of each heavy chain where the protein is not constrained by contacts with the light chain or adaptor complexes. This curvature is reliant on the structure of the combined Clathrin molecules of the coated pit (Musacchio, Smith et al. 1999). Clathrin assembles into a mixture of both hexagonal and pentagonal polygons and the diversity of these

shapes is fundamental to the curvature of the membrane in a similar way to the structure of a football (Kanaseki and Kadota 1969, Heuser 1980).

The exact method of Clathrin-coated pit (CCP) initiation is not yet fully understood. Historically it was believed that AP-2 recruitment to the plasma membrane was the first step, however in some cases CCPs form in the absence of AP-2 (Motley, Bright et al. 2003). A collection of other factors might be involved in the nucleation step, including cell surface phospholipids, the recruitment of other adaptor proteins such as Eps15 and the presence of cargo (Merrifield and Kaksonen 2014). The initiation process may also engage SNARE protein complexes, small Rab proteins and Epsin. The latter is involved in inducing membrane curvature and links Clathrin to cargo as well as binding ubiquitin as will be discussed below (Kirchhausen 2000).

1.2.5. Clathrin coated vesicle (CCV) propagation and scission

A number of proteins are involved in the growing CCV, including Bin Amphiphysin Rvs (BAR) domain-containing proteins and amphiphysin. These recognise and regulate membrane curvature. Curvature of the membrane is also dependent on the proportion and layout of pentagonal Clathrin polygons within the pentagonal/hexagonal complement. In addition, the brain protein AP-180 and its ubiquitous mammalian equivalent CALM are involved in regulating the size of CCVs (Kirchhausen 2000).

Amphiphysin recruits the membrane scission protein, dynamin, to the budding vesicle. The helix of this corkscrew-shaped protein pinches off the vesicle allowing its release into the cytosol. The Clathrin coat is rapidly removed once the vesicle is released into the cell (Kirchhausen 2000).

1.2.6. Receptor-mediated endocytosis

A particular subtype of Clathrin-mediated endocytosis is the internalisation of cell surface receptors (figure 1.3.). Anderson, Brown and Goldstein described ligand-induced receptor-mediated endocytosis of LDL and its receptor, which they first identified in 1974 (Brown and Goldstein 1974, Anderson, Brown et

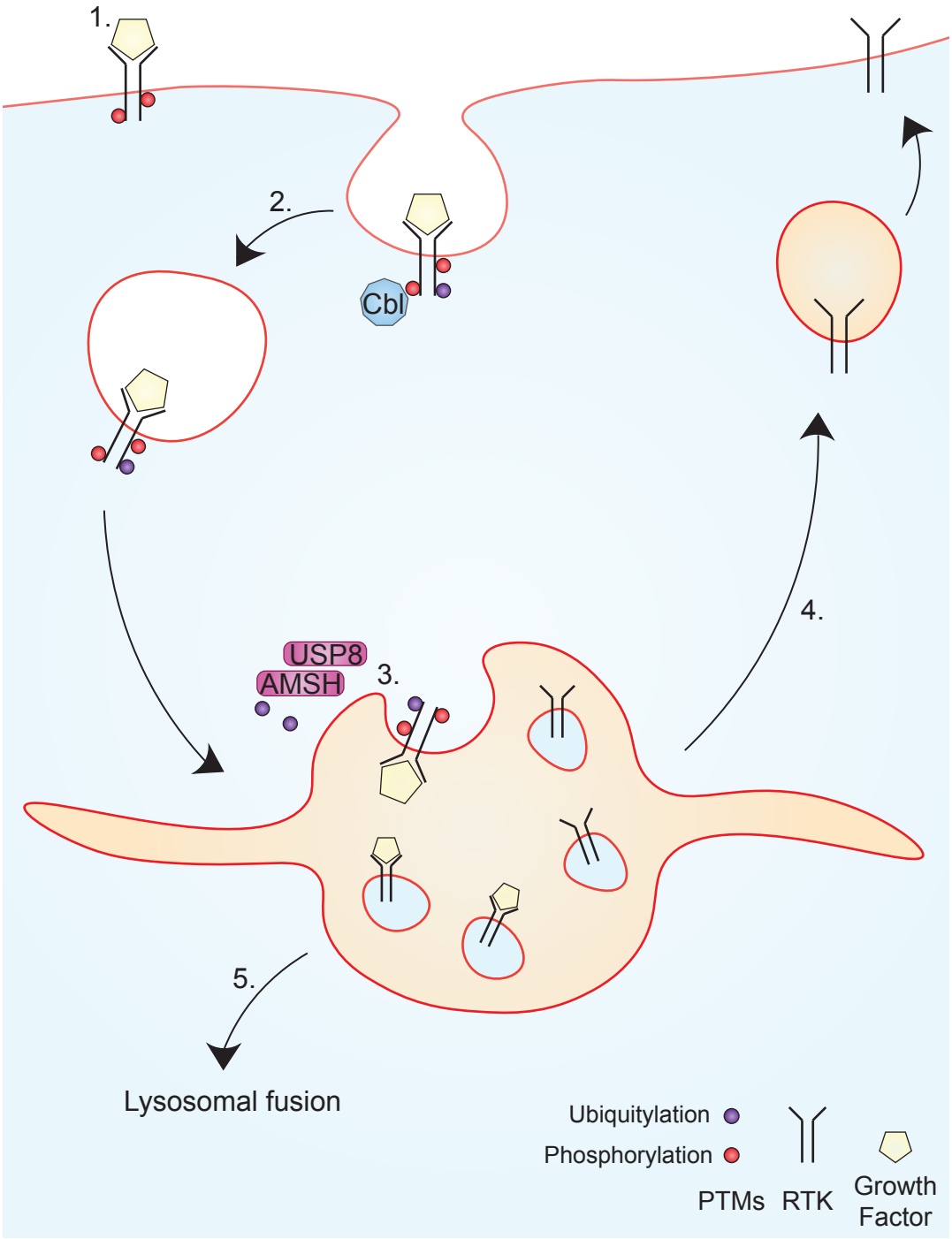


Figure 1.3. A simplified schematic of ligand-induced RTK internalisation and trafficking to the multivesicular body and recycling back to the cell surface. 1. Ligand binding triggers receptor dimerisation and activation by phosphorylation. 2. The activated receptor is ubiquitylated and internalised. 3. The receptor is trafficked to the early endosome where it is sorted into multivesicular bodies facilitated by ESCRT proteins and the endocytic DUBs, USP8 and AMSH (see figure 1.4.). 4. Receptors can be recycled to the plasma membrane or degraded upon endosome fusion wit the lysosome (5.).

al. 1977, Anderson, Goldstein et al. 1977, Goldstein, Anderson et al. 1979). Using human fibroblasts incubated for two hours at 4°C with Ferritin-labelled LDL, then fixed and imaged by electron microscopy, the authors showed that 60-70% of Ferritin labelling occurred at indented, coated regions, which comprised less than 2% of the cell surface area. When these cells were warmed up to 37°C it took only one minute for Ferritin to appear in coated endocytic vesicles. By ten minutes nearly all of the membrane-bound Ferritin-LDL had been internalised. These imaging studies allowed the visualisation of sequential internalisation steps and the uncoating of internalised vesicles. Tracking Ferritin-tagged LDL revealed its incorporation into structures that were morphologically similar to lysosomes, indicating a potential fusion of the endocytic carriers with lysosomes. Thus Anderson, Brown and Goldstein were fundamental in describing the process of receptor-mediated endocytosis. This was later confirmed by studies on insulin, EGF and α2-macroglobulin using similar experiments, whereby fluorescent derivatives of these ligands were imaged after initial binding at 4°C and subsequent internalisation at 37°C in Swiss 3T3 cells (Maxfield, Schlessinger et al. 1978). Over time, our understanding of receptor mediated endocytosis and some of the signals involved in its regulation have grown.

1.2.7. Ubiquitylation as a signal for receptor-mediated endocytosis

Ligand-activated receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR) become autophosphorylated and these phosphorylation sites recruit downstream signalling molecules (discussed in 1.4). In addition to the recruitment of signalling effectors, the phosphorylation sites can recruit E3 ligases such as c-Cbl (Joazeiro, Wing et al. 1999, Levkowitz, Waterman et al. 1999, Waterman, Levkowitz et al. 1999, Yokouchi, Kondo et al. 1999, Klapper, Waterman et al. 2000, Peschard, Fournier et al. 2001). Recruitment of Cbl can be direct via its tyrosine kinase-binding (TKB) domain, which interacts with Y1045 of EGFR, Y1003 of Met and Y1112 of ERBB2 (Levkowitz, Waterman et al. 1999, Klapper, Waterman et al. 2000, Peschard, Fournier et al. 2001). Cbl can also be indirectly recruited via adaptors such as Grb2 (which binds to Y1068 and Y1086 of

EGFR), APS (recruited to Y1021 of PDGFR) or a complex of Grb2 and FRS2 (in the case of FGFR) (Yokouchi, Wakioka et al. 1999, Waterman, Katz et al. 2002, Wong, Lamothe et al. 2002, Jiang, Huang et al. 2003).

Cbl then recruits an E2, UBCH7, via its RING domain facilitating the linkage of single ubiquitin molecules on to the receptor (Yokouchi, Kondo et al. 1999, Zheng, Wang et al. 2000). Cbl also interacts with the adaptor protein CIN85 which allows the coupling of Cbl to endophilins. This complex might induce the negative membrane curvature required for successful endocytosis (Soubeyran, Kowanetz et al. 2002). Other E3 ligases may also be involved in the endocytosis of cell surface receptors such as Nedd4 and the Cbl-like ligase Hakai which regulate the levels of insulin-like growth factor receptor and E-cadherin respectively (Vecchione, Marchese et al. 2003) (Fujita, Krause et al. 2002, Huang, Choi et al. 2015).

At the early stage of CCV formation, there are a number of proteins that are able to bind to ubiquitin. For example, tandem ubiquitin interacting motifs have been found in Epsin 1 and Epsin 2 as well as the yeast Epsin homologues Ent1p and Ent2p and human Eps15 (Hofmann and Falquet 2001, Klapisz, Sorokina et al. 2002, Shih, Katzmann et al. 2002). In yeast, the homologue of Eps15, Ede1p, contains a ubiquitin-associated (UBA) domain (Shih, Katzmann et al. 2002). Research in yeast has been fundamental in understanding this pathway. Initial studies in *Saccharomyces cerevisiae* showed that a GPCR (Ste2p) and two transporters, uracil permease and Ste6p, are mono- and/or poly-ubiquitylated (Kolling and Hollenberg 1994, Hicke and Riezman 1996, Galan and Haguenauer-Tsapis 1997). A ubiquitylated form of the ABC-transporter family member, Ste6p, accumulates at the plasma membrane in endocytic mutants of *S. cerevisiae* as evidenced by immunofluorescence and cell fractionation. In endocytic mutants, Ste6p is mostly localised to intracellular membranes with only a small portion found at the plasma membrane (Kolling and Hollenberg 1994). This redistribution of Ste6p gave an indication that this protein might be internalised into the endocytic pathway. Immunoprecipitation of Ste6p from cells expressing myc-tagged ubiquitin and western blotting with anti-myc antibodies confirmed its ubiquitylation. Additionally, Ste6p is stabilised in cells

defective in ubiquitin conjugation, and this is reversed by the re-expression of an E2 ligase, UBC5.

Just two years later Hicke and Riezman published their work on the yeast GPCR Ste2p (Hicke and Riezman 1996). Ste2p is mono-ubiquitylated and internalised in response to ligand binding and subsequently trafficked to the vacuole for degradation. Double ubiquitin E2 enzyme mutants (*ubc1ubc4* and *ubc4ubc5*) internalise the ligand-bound receptor 5- and 15-times slower than wild-type, and a K337R mutant Ste2p is no longer ubiquitylated or internalised. Meanwhile Galan and Haguenauer-Tsapsis showed that uracil permease is ubiquitylated in yeast and that this is ablated by mutation of the ubiquitin ligase Npi1p (Galan and Haguenauer-Tsapis 1997). Npi1p mutant cells also have an increase in plasma membrane localised uracil permease. Likewise, ubiquitin-conjugated uracil permease accumulates at the cell surface of cells in which internalisation is blocked due to a temperature sensitive actin (*act1*) mutation (Galan, Moreau et al. 1996). The authors went on to show that in fact, K63-linked ubiquitylation is important in enhancing endocytosis of this protein and that endocytosis is impaired in cells lacking the deubiquitylase Doa4p (Galan and Haguenauer-Tsapis 1997).

Prior to these findings in yeast, receptors had been shown to be ubiquitylated in mammalian cells. In 1992, Mori et al. showed that PDGF binding to the PDGFβ receptor induces its polyubiquitylation (Mori, Heldin et al. 1992). Mutation of K63 to an alanine in the receptor prevented its ubiquitylation and ligand-induced degradation. Meanwhile in 1994, two groups showed that the c-Kit receptor was subjected to ligand-induced polyubiquitylation, which enhanced its internalisation and turnover, and reduced its cell surface levels (Miyazawa, Toyama et al. 1994, Yee, Hsiau et al. 1994). Thus the link between ubiquitylation and receptor degradation was evident, however that this process required endocytosis in mammalian cells was not shown until studies by Strous et al. in 1996 made use of Chinese hamster ovary (CHO) cells with a temperature sensitive defect in ubiquitin conjugation (CHO-ts20) (Strous, van Kerkhof et al. 1996). The authors transfected these cells as well as the wild-type (CHO-E36) cells with growth hormone receptor (GHR) and stimulated them with the growth hormone (GH) ligand. Using this method

they showed that GHR ubiquitylation is ligand dependent, ligand induces internalisation and that internalisation is ubiquitin dependent. At 42°C the CHO-ts20 cells are unable to undergo ubiquitin conjugation. Cells treated with GH at this temperature failed to internalise the fluorescently labelled receptor. Endo-lysosomal degradation inhibitors NH4Cl and bafilomycin A1 blocked the ligand-induced downregulation of GHR at the permissive temperature. We now know that ubiquitylation acts as an internalisation signal for a number of receptors in mammalian cells. In addition ubiquitylation accelerates the slow, ligand-independent, constitutive internalisation of RTKs such as EGFR family members, MET, and PDGFR (Levkowitz, Waterman et al. 1998, Miyake, Lupher et al. 1998, Marmor and Yarden 2004).

In 1998 a further link between ubiquitylation and receptor-mediated endocytosis was reported. Levkowitz et al. showed that the E3 ligase c-Cbl increases the rate of EGFR degradation and that it does so by interacting with the receptor itself (see above). Upon stimulation, EGFR was shown to be ubiquitylated by Cbl and is subsequentally degraded in the lysosome (Levkowitz, Waterman et al. 1998). Conversely, overexpression of inactive oncogenic viral Cbl (v-Cbl) interfered with the ability of c-Cbl to sort receptors, which were subsequently recycled instead of degraded – pointing to a role for ubiquitylation in the targeting of receptors to the lysosome.

Experiments in yeast linked the deubiquitylase Doa4 to sorting of Ste6p-GFP to the vacuolar lumen. Mutant Doa4 led to the entrapment of Ste6p-GFP at the vacuolar membrane, indicating that the protein was successfully internalised but unable to cross into the vacuole (Losko, Kopp et al. 2001). This led to the proposition that reversible ubiquitylation is required for sorting into multivesicular bodies.

Mono-ubiquitylation on one or several lysines is sufficient for internalisation of a number of RTKs. For example EGFR- or PDGFR-ubiquitin chimeric proteins were internalised although the ubiquitin in these chimeras was mutated such that it could not be conjugated to additional ubiquitin moieties (Haglund, Sigismund et al. 2003, Mosesson, Shtiegman et al. 2003). These receptors as well as the MET receptor were shown to crossreact with

antibodies that recognise mono-ubiquitylated proteins, but not with antibodies specific for polyubiquitin (Carter, Urbe et al. 2004, Marmor and Yarden 2004). These observations are in line with some of the earlier findings in yeast outlined above, in which Ste2p was mono-ubiquitylated, subsequently internalised and degraded (Hicke and Riezman 1996).

There is evidence, however, that also in mammalian cells, short K63-linked chains might be more efficient signals for endocytosis at least in some cases. One such example of this comes from mass spectrometry analysis of ubiquitylated EGFR (Huang, Zeng et al. 2013). Huang and colleagues found that the majority of ubiquitin associated with EGFR was found in the form of poly-ubiquitin chains, mostly linked through K63 moieties. Additionally, EGFR expressed as a fusion protein with the K63 specific deubiquitylase AMSH reduced polyubiquitylation, increased mono-ubiquitylation and delayed degradation of EGFR. However these mono-ubiquitylated receptors still underwent the initial internalisation step suggesting that K63-linked chains may be important for a downstream sorting pathway (Huang, Zeng et al. 2013). Incidentally, Cbl may remain associated with activated EGFR beyond the initial internalisation of the receptor as it is seen to colocalise with EGFR in endosomes (Levkowitz, Waterman et al. 1998).

Studies in yeast already hinted at a role for K63-linked chains. Firstly, Galan et al. showed that uracil permease requires K63-linked chains for its degradation as described above (Galan and Haguenauer-Tsapis 1997). Additionally, the *S. cerevisiae* HECT E3 ligase, RSP5, which ubiquitylates a number of cell surface receptors including Ste2p and Gap1 permease, produces K63-linked ubiquitin chains (Kim and Huibregtse 2009). Finally, Lauwers et al. showed that K63-linked ubiquitylation is required for MVB sorting of Gap1 permease in yeast, although monoubiquitylation was sufficient for the protein's initial internalisation (Lauwers, Jacob et al. 2009).

1.2.8. The ESCRT machinery

Initial studies in yeast identified 46 vacuolar protein sorting (*vps*) mutants that were involved in the transport of cargo to the vacuole, the yeast equivalent to

the mammalian lysosome (Bryant and Stevens 1998). Thirteen of these mutants produced an exaggerated, aberrant 'class E' compartment, which is equivalent to an aberrant endosome. Later work, pioneered by the Emr lab, characterised three ESCRT complexes – ESCRT I, II and III – which in combination with the already-characterised Vps4 complex and the upstream-acting ESCRT-0 complex comprise the 5-step pathway required for the biogenesis of multivesicular bodies (figure 1.4.) (Katzmann, Babst et al. 2001, Babst, Katzmann et al. 2002, Babst, Katzmann et al. 2002, Bilodeau, Urbanowski et al. 2002, Katzmann, Stefan et al. 2003). Equivalent ESCRT complexes were characterised in mammalian cells (table 1.1.) (Williams and Urbe 2007, Raiborg and Stenmark 2009, Hurley 2010).

The initial step in MVB biogenesis is the recognition of ubiquitylated proteins by ESCRT-0. The mammalian ESCRT-0 complex is made up of HRS and STAM1/2 (Vps27 and Hse1 respectively in yeast). These two proteins constitutively interact with one another via anti-parallel coiled-coil and GAT domains which bring the ubiquitin binding regions of the two proteins into close proximity (Prag, Watson et al. 2007, Ren, Kloer et al. 2009). HRS possesses a FYVE domain which interacts with phosphatidylinositol-3-phosphate (PI3*P*), a lipid that is enriched on early endosomes (Burd and Emr 1998, Gaullier, Simonsen et al. 1998, Kutateladze, Ogburn et al. 1999, Misra and Hurley 1999, Urbe, Mills et al. 2000). This interaction between HRS and PI3*P* recruits the ESCRT-0 complex as a whole to the endosome (Raiborg, Bache et al. 2001, Raiborg, Bremnes et al. 2001). The ESCRT-0 complex is involved in the initial sorting of cargo. It recognises ubiquitylated cargo via the VHS domains of both HRS and STAM, alongside the multiple UIMs possessed by the two proteins (Mizuno, Kawahata et al. 2003, Hong, Ahn et al. 2009, Ren, Kloer et al. 2009, Ren and Hurley 2010). Both mammalian HRS and yeast Vps27 are capable of binding three ubiquitin moieties. In addition to the ubiquitin interacting N-terminal VHS domain HRS carries a double-sided UIM and Vps27 has two tandem UIMs (Urbe, Sachse et al. 2003, Hirano, Kawasaki et al. 2006). STAM contains both the N-terminal VHS domain and a traditional UIM (Mizuno, Kawahata et al. 2003). Therefore, in total, ESCRT-0 can interact with up to five ubiquitin moieties or

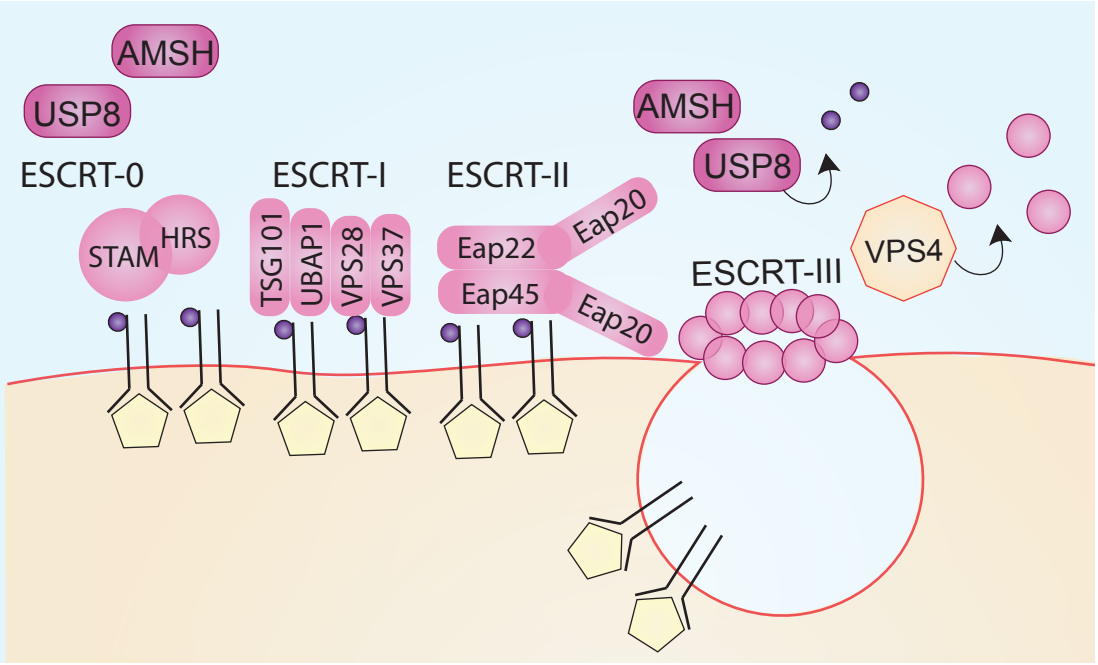


Figure 1.4. MVB biogenesis at the early endosome. The MVB biogenesis pathway is composed of four ESCRT complexes - ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III. Ubiquitylated receptors are first recognised by the ESCRT-0 complex. The ESCRT complexes act together to sort cargo into multivesicular bodies at the early endosome. Finally the VPS4 complex dissociates the ESCRT machinery. USP8 and AMSH are recruited to both the ESCRT-0 and ESCRT-III complex and are involved in cargo deubiquitylation.

	ESCRT components	
	Yeast	**Mammalian**
ESCRT-0	Vps27	HRS
	Hse1	STAM
ESCRT-I	Vps23	TSG101
	Vps28	VPS28
	Vps37	VPS37
	UBAP1	Mvb12
ESCRT-II	Vps36	Eap45
	Vps22	Eap22
	Vps25	Eap20
ESCRT-III	Vps20	CHMP6
	Snf7	CHMP4
	Vps24	CHMP3
	Vps2	CHMP2
ESCRT-III accessory	Did2	CHMP1
	Vps60	CHMP5
	Ist1	IST1
	Bro1	ALIX
	Bro1	HD-PTP
VPS4-AAA	Vps4	VPS4
	Vta1	VTA1

Table 1.1. Componenets of the ESCRT complexes in yeast and mammals.

multiple ubiquitin moieties on a poly-ubiquitylated protein. In mammalian cells, HRS and STAM also interact with Clathrin via the Clathrin-binding domain present in HRS (Raiborg, Bache et al. 2001). The early endosome is associated, through HRS, with a flattened Clathrin coat comprised of both the Clathrin heavy and light chains but lacking adaptor protein (AP) complexes (Clague 2002, Sachse, Urbe et al. 2002, Hurley 2010). Invagination of the membrane into intralumenal vesicles (ILVs) of the endosome during MVB biogenesis was seen adjacently rather than directly below Clathrin coated regions (Sachse, Urbe et al. 2002). It has been proposed that this coat might favour membrane remodelling to occur consistently in the correct direction – internalising vesicles into the MVB rather than vesicles budding outwards (Clague 2002, Sachse, Urbe et al. 2002).

In addition to interaction with PI3*P,* Clathrin and ubiquitylated receptors, ESCRT-0 is able to recruit the next complex in the sequence, ESCRT-I. This is in part via interactions between P(S/T)XP motifs within Vps27/HRS and the ubiquitin E2 variant (UEV) domain within the ESCRT-I complex member Vps23/TSG101. Finally, ESCRT-0 also recruits a pair of endocytic DUBs, which will be described in greater detail below (Clague and Urbe 2006).

ESCRT-I is comprised of TSG101 (Vps23 in yeast), Vps28, Vps37 and Mvb12/UBAP1. As described above, the complex is recruited by the direct interaction of TSG101 with HRS. Together with ESCRT-II, this complex is believed to be involved in initiation and stabilisation of vesicle budding. Additionally, ESCRT-I encodes multiple UBDs, which allow it to interact with and contribute to the sorting of ubiquitylated cargo. One such UBD is the Ubiquitin E2 Variant (UEV) domain within the N-terminus of yeast Vps23 and human TSG101 (Williams and Urbe 2007, Hurley 2010). The mammalian proteins that fulfil the function of yeast Mvb12 (although without sequence conservation) are MVB12A, MVB12B and UBAP1. UBAP1 is endosome-specific, regulates ubiquitin-dependent EGFR sorting and is not required for the other known functions of the ESCRT machinery – cytokinesis or viral packaging (Stefani, Zhang et al. 2011, Agromayor, Soler et al. 2012, Wunderley, Brownhill et al. 2014). UBAP1 contains a solenoid of overlapping

UBA (SOUBA) domain comprised of three rigid UBA regions (Agromayor, Soler et al. 2012).

ESCRT-II is made up of Vps36/Eap45, Vps22/Eap22 and two Vps25/Eap20 molecules. Vps36/Eap45 and each Vps22/Eap22 interact with one another and one of the two Vps25/Eap20 proteins giving the complex a 'Y' shape. Vps36/Eap45 also interacts with Vsp28 of ESCRT-I, PI3*P* of the endosome and the ubiquitin moieties of sorted cargo via a GLUE domain which carries a plekstrin homology (PH) lipid binding region at its core (Gill, Teo et al. 2007, Williams and Urbe 2007, Hurley 2010).

Whilst the ESCRT machinery may pass off cargo step-wise from one complex to the next, it seems more plausible that these complexes work in concert with one another. As mentioned, some of the complexes have overlapping functions – such as ESCRT-I and ESCRT-II – and many share interaction partners.

The complicated role of the ESCRT-III complex is not fully elucidated yet. It is comprised of a number of proteins: Vps20/CHMP6, Snf7/CHMP4, Vps24/CHMP3, Vps2/CHMP2 and the accessory components Did2/CHMP1, Vps60/CHMP5 and Ist1 (Williams and Urbe 2007, Hurley 2010, Schmidt and Teis 2012, Christ, Raiborg et al. 2016). Each of the proteins are brought to the membrane and activated from their monomeric auto-inhibition by conformational changes in a step-wise fashion. They form two sub-complexes made up of Vps2/CHMP2-Vps24/CHMP3 and Vps20/CHMP6-Snf7/CHMP4 (Christ, Raiborg et al. 2016). The complete role of ESCRT-III is not fully understood but it is known to play a key part in the membrane remodelling required for the completion of budding and scission of internal vesicles into the lumen of the nascent MVB. A key component of this might be the filaments comprised predominantly of CHMP4 (and to a lesser extent CHMP2 and CHMP3) which recruit another ESCRT associated protein ALIX (Bro1 in yeast) (Saksena, Wahlman et al. 2009, Christ, Raiborg et al. 2016). It has been proposed that these curved filaments might be involved in the membrane remodelling and typically associate with membrane neck regions (Christ, Raiborg et al. 2016). They also confer additional links between

ESCRT-I and ESCRT-III since ALIX interacts with the ESCRT-I complex member TSG101 via a P(S/T)XP motif (Raiborg, Bache et al. 2001, Bilodeau, Urbanowski et al. 2002, Katzmann, Stefan et al. 2003). Other subunits of the ESCRT-I and ESCRT-II complexes can help bring ESCRT-III components to the endosomal membrane. For example Eap20 of ESCRT-II and Vps28 of ESCRT-I can interact with CHMP6 (Yorikawa, Shibata et al. 2005). Additionally both CHMP6 (through myristoylation) and CHMP4 (through an amphipathic helix) can interact with the endosomal membrane (Yorikawa, Shibata et al. 2005, Buchkovich, Henne et al. 2013).

Another key ESCRT-III binding protein is HD-PTP (Doyotte, Mironov et al. 2008). This protein switches between binding to ESCRT-0 complex member STAM and binding to CHMP4 (Christ, Raiborg et al. 2016). Its role as an ESCRT-III interactor is not fully understood; however the switch between each ESCRT complex is regulated by the recruitment of the deubiquitylase USP8 (also known as UBPY) to STAM (Ali, Zhang et al. 2013). Its interaction with ESCRT-0 is known to modulate cargo sorting into ILVs. Additionally, HD-PTP interacts with the ESCRT-I complex member UBAP1 (Stefani, Zhang et al. 2011).

The Vps4 complex is made up of Vps4 and a co-factor, Vta1. Vps4 belongs to the AAA-ATPase family of which the membrane fusion protein NSF is also a member. NSF is involved in synaptic vesicle fusion, a pathway modulated by SNARE complexes. Vps4 is recruited to ESCRT-III both via its microtubule interacting and transport (MIT) domain and the two MIT domains within its co-factor Vta1. In particular, Vta1 binds very well to VPS60/CHMP5 which is a late acting, ESCRT-III associated protein (Hurley 2010, Christ, Raiborg et al. 2016). The recruitment of the Vps4 complex promotes disassembly of ESCRT-III in an ATP-dependent manner thus promoting the dissociation and recycling of upstream ESCRT complexes.

1.2.9. Endocytic DUBs

The mammalian ESCRT-III components also recruit the endocytic DUBs AMSH and USP8, while yeast Doa4 may be recruited to both the proteasome

and endosomes (Papa, Amerik et al. 1999, Amerik, Nowak et al. 2000, Clague and Urbe 2006, McCullough, Row et al. 2006). At this stage, it is believed that deubiquitylation of the cargo is required to a) release the earlier ESCRT complexes that bind ubiquitin moieties and b) allow recycling of ubiquitin whilst permitting cargo targeted for lysosomal degradation to be fully ensconced within MVBs (Clague and Urbe 2006). The cargo is then committed for degradation as the maturing endosome fuses with the lysosome (Futter, Pearse et al. 1996).

The yeast deubiquitylase Doa4 plays a role in proteasomal degradation through its interaction with the 26S proteasome (Papa, Amerik et al. 1999). It is also recruited to the endosome via direct interactions with Snf7 (of the ESCRT-III complex) and the ESCRT associated protein Bro1 (ALIX in mammalian cells) both of which are described above (Amerik, Nowak et al. 2000, Bowers, Lottridge et al. 2004, Luhtala and Odorizzi 2004). The role of Doa4 in yeast appears to be related to the recycling of ubiquitin since Doa4-deleted strains have reduced ubiquitin levels in the stationary phase (Swaminathan, Amerik et al. 1999, Amerik, Nowak et al. 2000, Losko, Kopp et al. 2001). This can be partially recovered by the blockade of the MVB pathway by preventing MVB fusion with the vacuole (Amerik, Nowak et al. 2000). These Doa4Δ cells also had proteolytic defects and less efficient protein sorting to the vacuole, which could be rescued by the addition of free ubiquitin again lending weight to the argument that Doa4 is important in the recycling of ubiquitin at the late stage of the MVB sorting pathway (Swaminathan, Amerik et al. 1999, Losko, Kopp et al. 2001). However deubiquitylation of cargo is not essential for MVB sorting in yeast as Doa4-deleted cells still had cargo present on the vacuolar membrane (Reggiori and Pelham 2001). In addition to this role in recycling ubiquitin, Doa4 has been linked to internalisation of surface proteins in yeast. One study showed that Doa4Δ cells are no longer able to degrade the Gap1 permease in response to ammonia treatment and instead the protein remains active on the cell surface. Nikko and André suggested that this was due to a failure of the cells to sort and target Gap1 for degradation. They proposed that Gap1 was still endocytosed but recycled back to the plasma membrane since additional

deletion of a gene important in recycling results in partially internalised GAP1 (Nikko and Andre 2007).

There are two endocytic DUBs recruited to the ESCRT machinery during cargo sorting and MVB biogenesis in mammalian cells. AMSH is a JAMM metalloprotease with specificity for K63-linked ubiquitin chains, whereas USP8 is a USP with relatively little chain linkage specificity (see section 1.1) (McCullough, Clague et al. 2004, Mizuno, Iura et al. 2005, McCullough, Row et al. 2006). Both USP8 and AMSH are able to interact with the ESCRT-0 member STAM through their single (AMSH) or three (USP8) SH3 domains which bind to a non-canonical SH3 binding motif PX(V/I)(D/N)RXXKP within STAM (Tanaka, Kaneko et al. 1999, Kato, Miyazawa et al. 2000, Kaneko, Kumasaka et al. 2003). Both DUBs also interact with several ESCRT-III components via their MIT domains (McCullough, Row et al. 2006, Row, Liu et al. 2007). In particular AMSH is able to interact with CHMPs1, 2, 3 and 4, whereas USP8 interacts with CHMP1, CHMP2 and CHMP4 (Agromayor and Martin-Serrano 2006, Tsang, Connell et al. 2006, Ma, Boucrot et al. 2007, Row, Liu et al. 2007).

AMSH is also localised to endosomes via its binding to Clathrin (McCullough, Row et al. 2006, Nakamura, Tanaka et al. 2006). The activity of AMSH is positively regulated by its direct interaction with STAM (McCullough, Row et al. 2006). This relies on the ability of STAM to bind ubiquitin as mutation of the UIM prevents its regulation of AMSH activity. This might be due to the additional binding of ubiquitylated cargo facilitating cleavage of K63-linked ubiquitin as the chain is held in a favourable orientation (Clague and Urbe 2006, McCullough, Row et al. 2006, Davies, Paul et al. 2013).

Expression of a catalytically-inactive mutant form of AMSH leads to an accumulation of endosomal ubiquitin in HeLa cells and its siRNA-mediated depletion leads to the acceleration of EGFR degradation (McCullough, Clague et al. 2004, Bowers, Piper et al. 2006). In addition, a recent siRNA screen for DUBs involved in the regulation of EGFR levels confirmed that AMSH depletion accelerated EGFR degradation (Savio, Wollscheid et al. 2016). Based on these findings it has been proposed that AMSH

deubiquitylation of cargo early in the MVB pathway might rescue cargo from lysosomal degradation and contribute to its recycling back to the plasma membrane (McCullough, Clague et al. 2004, Clague and Urbe 2006, Liu, Urbe et al. 2012). In principle, USP8 may also contribute to this function. As a K63-linkage specific DUB, AMSH may be unable to remove the last ubiquitin from polyubiquitylated cargo, but USP8 has less specificity and therefore might work cooperatively with AMSH. Further support for ubiquitin as a lysosomal sorting signal at the endosome comes from the observation that the transferrin receptor, which normally recycles constitutively, can be forced down the lysosomal degradation pathway through tagging or fusing ubiquitin onto its C-terminus (Raiborg, Bache et al. 2002). Finally, as mentioned above, fusion of AMSH onto EGFR leads to delayed degradation of the receptor despite its successful internalisation into early endosomes (Huang, Zeng et al. 2013).

Conversely, some studies report that AMSH depletion reduces the degradation of EGFR and other receptors such as CXCR4 (Ma, Boucrot et al. 2007, Sierra, Wright et al. 2010). In particular, the G-protein coupled receptor CXCR4 is internalised when AMSH is depleted but the overall levels of the receptor were reported to increase (Sierra, Wright et al. 2010).

1.2.10. USP8

Ubiquitin specific protease 8 (USP8), also called UBPY, was first identified in 1998 as a growth regulated deubiquitylase with similarities to the yeast DUB Doa4 (Naviglio, Mattecucci et al. 1998). Naviglio and colleagues showed that this protein had DUB activity using ubiquitylated proteins extracted from rabbit reticulocyte lysate. Having first treated the lysate with NEM to block endogenous cysteine DUB activity the authors treated with GST-USP8 with or without additional NEM and separated the lysates by SDS-PAGE. Western blot analysis using an anti-ubiquitin antibody showed that USP8 was able to remove high molecular weight bands which corresponded to ubiquitylated forms of proteins (Naviglio, Mattecucci et al. 1998).

USP8 was first described from its cDNA sequence, which predicted a 1118 amino acid protein (Naviglio, Mattecucci et al. 1998). Today, data from Ensembl describes three protein-coding transcripts accounting for two known isoforms. The first isoform, annotated by NCBI as isoform a is coded for by transcripts USP8_001 and USP8_003, CCCDS10137. This protein has a UniProt reference of 40818-1. Isoform a is the full length, 1118 amino acid protein with a predicted molecular weight (mW) of 128kDa. Isoform b is coded for by transcript USP8_002, CCCDS561632. The UniProt reference for isoform b is 40818-2. This protein misses amino acids 35-111 corresponding to exons 2 and 3 in addition to amino acids 601-629 coded for by exon 11. This protein is 1012 amino acid in size with a predicted mW of 115kDa. The second isoform was identified following an effort to better characterise human cDNA from tissue, primary cell cultures and cell lines (Ota, Suzuki et al. 2004). To date, no isoform specific functions have been reported for either of these however isoform b lacks a key domain involved in the recruitment of the protein to ESCRT machinery, the MIT domain an interaction fundamental to the function of USP8 in particular contributing to its role in regulating the stability of STAM (see below for detail). This might hint at an alternative localisation of the smaller protein, which retains the full catalytic domain and could allude to an additional, ESCRT machinery independent function of USP8. In addition to these two reported isoforms there are three isoforms predicted by computational analysis according to NCBI. These are USP8_X1, USP8_X2 and USP8_X3. The first corresponds in protein sequence to the full-length protein whereas isoforms X2 and X3 are predicted to comprise 1089 and 928 amino acids respectively. Predicted isoform X2 would differ to the full length protein only in its loss of amino acids 601 to 629 corresponding to exon 11 whilst retaining exons 2 and 3 in contrast to isoform a.

By far the best characterised role for USP8 is its interaction with ESCRT machinery proteins and regulation of receptor degradation by the lysosome. Only two years after USP8 was first described in the literature, Kato et al. identified an interaction between USP8 and an HRS binding protein Hbp, the mouse orthologue of STAM2 (Kato, Miyazawa et al. 2000). Then, in the mid-

2000s a spate of papers linked USP8 to the downregulation of EGFR, Met and ERBB3 as well as to the regulation of global ubiquitylated protein levels and endosomal morphology. First, Mizuno et al. reported in 2005 that overexpression of USP8 led to a decrease in EGF-stimulated ubiquitylation of EGFR and a delay in its degradation, whereas RNAi depletion of the DUB led to an increase in EGF-stimulated ubiquitylation and accelerated degradation of EGFR (Mizuno, Iura et al. 2005). Conversely, in 2006, Row et al. showed that RNAi depletion of USP8 led to the increase in global ubiquitylated proteins and delayed ligand-induced EGFR degradation. In the same year, Mizuno et al. also showed that lysosomal trafficking of endocytosed EGF was blocked in USP8-depleted cells, a finding apparently incongruent with their initial finding that USP8-depletion accelerated the degradation of EGFR (Mizuno, Kobayashi et al. 2006). Row and colleagues showed that over-expressed wild-type USP8 was recruited to early endosomes upon EGF stimulation, whereas a pool of the catalytically-inactive mutant was permanently associated with endosomes (Row, Prior et al. 2006). The same study also reported an accumulation of enlarged aberrant endosomes and an increase in the number and size of MVBs, which clustered in the perinuclear area. STAM, and to a lesser extent HRS, protein levels were severely reduced, and residual HRS was associated with these perinuclear structures while its cytoplasmic pool was depleted. Importantly, STAM stability was shown to depend not only on the presence of USP8 but also the DUB's recruitment to ESCRT-III components via its MIT domain: the expression of an siRNA resistant form of USP8 could partially rescue HRS and STAM levels, and this was dependent on USP8 catalytic activity and its MIT domain (Row, Liu et al. 2007). This suggests that deubiquitylation of STAM by USP8 might rescue the protein from proteasomal degradation. In agreement with this, ubiquitylated STAM could be stabilised by proteasome inhibitors in USP8 depleted cells. In the absence of USP8, AMSH would be unable to compensate due to its specificity for K63 chains leaving the proteasomal targeting K48-linked ubiquitin untouched. Although USP8 is able to interact with ESCRT-0 via binding to STAM, this is not required for its recruitment to the endosome. Just as for AMSH, the group showed that USP8 activity is regulated by STAM (Row, Liu et al. 2007).

USP8 knockout in mice is embryonically lethal and conditional knockout of USP8 in adult mice eventually leads to severe liver failure (identified by apoptotic hepatocytes) and death (Niendorf, Oksche et al. 2007). Niendorf and colleagues showed that in USP8 knockout mice there is a reduction in the overall protein levels of EGFR. In hepatocytes, where a small amount of EGFR does remain, the protein is found within the cell rather than at the cell surface which might point towards enhanced internalisation and lysosomal degradation of the protein. The authors also reported a reduction in ERBB3 and c-MET levels. The knockout mice show a reduction in HRS and STAM levels and immortalised mouse embryonic fibroblasts derived from these mice present with enlarged aberrant endosomes and an accumulation of MVBs, much like that seen in siRNA depleted HeLa cells described above (Niendorf, Oksche et al. 2007).

As mentioned above, USP8 interacts with a number of components of the ESCRT machinery. Through its SH3 domains, it is able to interact with the ESCRT-0 component, STAM, an interaction that also increases its DUB activity (Row, Prior et al. 2006, Row, Liu et al. 2007). Through its MIT domain, USP8 is able to interact with several ESCRT-III associated CHMPs (Row, Liu et al. 2007). Additionally, Ali et al. showed that USP8 interacts with HD-PTP, an ESCRT accessory protein which cycles between ESCRT-0 and ESCRT-III (Ali, Zhang et al. 2013). Their work suggested a complicated interaction between HD-PTP, CHMP4, STAM and USP8 in which USP8 competed with HD-PTP for STAM binding. Based on their findings, the authors proposed a mechanism whereby USP8 is recruited to HD-PTP first via CHMP4 which both proteins interact with. From here USP8 would displace HD-PTP from ESCRT-0, allowing it to cycle towards ESCRT-III. Meanwhile, USP8 deubiquitylation of STAM-associated cargo would allow the passage of that cargo through the ESCRT pathway. In fact, the authors showed that HD-PTP did recruit USP8 to ubiquitylated EGFR, and depletion of HD-PTP phenocopied depletion of USP8 (Ali, Zhang et al. 2013).

As well as the impact on EGFR degradation, Row et al. found that HGF-induced Met degradation was also delayed upon USP8 depletion (Row, Prior et al. 2006). In agreement with this, Alwan and van Leeuwen reported that

expression of a dominant negative, catalytically inactive form of USP8 resulted in delayed EGFR degradation (Alwan and van Leeuwen 2007). Interestingly, this study also showed that USP8 is phosphorylated in response to EGFR activation and that this might be due to Src kinase activity (Alwan and van Leeuwen 2007). Prior studies have shown that HRS is phosphorylated downstream of EGFR, and that this phosphorylation can be prevented by a Src-family kinase inhibitor (PP1) (Bache, Raiborg et al. 2002, Row, Clague et al. 2005).

USP8 also indirectly regulates ERBB3 receptor levels, albeit by an alternative mechanism (Wu, Yen et al. 2004, Cao, Wu et al. 2007). The degradation of ERBB3 follows a different pathway to EGFR, PDGFR and MET, which are ubiquitylated by the E3 ligase c-Cbl and targeted to the lysosome (Levkowitz, Klapper et al. 1996). Instead, the ubiquitin E3 ligase Nrdp1 regulates basal levels of ERBB3. Nrdp1 is in turn stabilised by USP8 in a ligand dependent manner, which the authors propose relies on USP8 phosphorylation by AKT (Wu, Yen et al. 2004, Cao, Wu et al. 2007). Neuregulin (NRG1) stimulation caused the accumulation of Nrdp1 in cells expressing catalytically-active but not catalytically-inactive USP8. Conversely, USP8 depletion correlated with reduced ERBB3 ubiquitylation and degradation (Cao, Wu et al. 2007).

At this stage, it became clear that USP8 plays an important role in the regulation of a number of RTKs. This has since been extended to other membrane proteins including GPCRs and even some ion channels. For example the GPCR, Chemokine Receptor 4 (CXCR4) is stabilised upon USP8 depletion (Berlin, Higginbotham et al. 2010). USP8 also regulates the ubiquitylation status of the E3 ligase for activated CXCR4, Itch (also known as AIP4) (Bhandari, Trejo et al. 2007, Panner, Crane et al. 2010). Balut et al. reported that USP8 depletion also led to accumulation of the potassium channel KCa3.1 in a ubiquitylated form and inhibits its degradation (Balut, Gao et al. 2010, Balut, Loch et al. 2011).

USP8 has become well established as a regulator of cell surface protein stability however it is possible that some of the observed effects on trafficking are due to general pleiotropic effects on the MVB pathway, since USP8

depletion also severely reduces ESCRT-0 levels and has major effects on the morphology of endosomes. The current list of proposed USP8 substrates is summarised in table 1.2.

As mentioned above, ESCRT complexes are also involved in the regulation of cytokinesis. It is then of interest that USP8, and in fact, AMSH have been observed at the mid-body of cells undergoing cytokinesis (Mukai, Mizuno et al. 2008). The two DUBs localise in overlapping but distinct regions of the mid-body. The ESCRT-0 component and USP8 binding protein HRS is localised to this region but with a different pattern. ESCRT-III complexes have a similar double-banded staining pattern at this region as USP8 suggesting their potential relevance in recruiting the DUB (Morita, Sandrin et al. 2007). Furthermore, cells depleted of USP8 or AMSH had impaired cytokinesis as detected by an increase in multi-nucleated cells.

1.2.12. Other DUBs associated with endocytic trafficking

While AMSH and USP8 are well established as DUBs important for trafficking and downregulation of cell surface receptors, there are a number of other DUBs that may play a role in this process. A study from Polo and colleagues sought to identify all of the DUBs involved in the ligand-induced degradation of EGFR (Savio, Wollscheid et al. 2016). They used an siRNA library to transiently deplete each active DUB in HeLa cells that they then stimulated with EGF. Using immunoblotting and an ELISA-based assay they measured EGFR degradation and categorised each knockdown into one of three groups – no change, delayed EGFR degradation or accelerated EGFR degradation. As described above they found AMSH or USP8 depletion to accelerate and delay EGFR degradation respectively. They also confirmed a study implicating USP2a in the downregulation of EGFR (Liu, Zanata et al. 2013). In total they identified 15 DUBs that affect EGFR degradation kinetics. In particular the group focused on the depletion of USP9X. Depletion of this DUB led to a severe delay in ligand-induced EGFR degradation and accumulation of the ubiquitylated receptor on endosomes (Savio, Wollscheid et al. 2016). In addition, Savio et al. showed that ubiquitylation of the

Protein	RNAi/OE	Cell line	Publications
BACE1	RNAi	H4 neuroglioma	Yeates and Tesco 2016
BRUCE	OE		Sippel et al. 2009
CXCR4	OE/RNAi	HEK293, HeLa	Berlin et al 2010
EGFR	OE/RNAi	COS7, HeLa	Mizuno et al. 2005
EGFR	RNAi	HeLa	Row et al. 2006
EGFR	RNAi	HeLa	Mizuno et al. 2006
EGFR	OE	HEK293	Alwan and van Leeuwen 2007
ErbB2	OE	HEK293	Meijer et al 2010
$FLIP_L$	RNAi	293FT, HeLa	Jeong et al. 2016
$FLIP_S$	OE/RNAi	Glioblastoma xenograft cells	Panner et al. 2010
Frizzled	OE	HeLa	Mukai et al 2010
KCa3.1	OE/RNAi	HEK293	Balut et al 2011
Nrdp1	OE	COS7, 293T	Wu X et al. 2004
Nrdp1	OE/RNAi	293T, MCF7	Cao et al. 2007
PAR2	OE	HEK-PAR2	Hasdemir et al
Smoothened	OE/RNAi	Drosophila S2	Xia et al. 2012, Li et al. 2012
STAM	RNAi	HeLa	Row et al. 2006
VEGFR	RNAi	HUVEC	Smith et al. 2016
α-synuclein	OE/RNAi	HEK293T, SH-SY5Y	Alexopoulou et al. 2016
TrkA	RNAi	PC12	Ceriani et al. 2015
ENaC	OE	HEK293	Zhou et al. 2013
LDLR	OE/RNAi	HeLa, HEK293	Scotti et al. 2013, Sorrentino et al. 2013

Table 1.2. Proposed USP8 substrates compiled from data in the literature. OE: data from USP8 overexpression. RNAi: data from USP8 depletion by RNA interference.

endocytic adaptor protein Eps15 was increased upon USP9X depletion and that Eps15 was a substrate of this DUB. They postulated that the regulation of EGFR degradation by USP9X might hinge on this adaptor since depletion of the two combined did not have an additive effect on receptor internalisation (Savio, Wollscheid et al. 2016).

Another DUB siRNA library screen identified the Otubain Cezanne/OTUD7B as a DUB involved in the regulation of EGFR. Pareja et al. showed that simultaneous depletion of Cezanne 1 and Cezanne 2 led to enhanced degradation of EGFR. Overexpression of Cezanne 1 led to a reduction in EGFR ubiquitylation and subsequent stabilisation of the receptor. Cezanne 1 binds to and deubiquitylates EGFR and receptor downregulation was reliant on Cezanne 1 catalytic activity in HeLa cells (Pareja, Ferraro et al. 2012).

1.3. Autophagy

Autophagy is a mechanism by which the cell can degrade organelles and other cytosolic material in response to starvation or damage. It relies on ultimate degradation by the lysosome and makes use of some of the machinery outlined above. During autophagy, membrane is acquired from other intracellular organelles to form the phagophore or isolation membrane. As this membrane grows it begins to surround and sequester material, including proteins and organelles, which eventually become engulfed by a double membrane which closes to become the autophagosome. The autophagosome subsequently fuses with the lysosome, allowing hydrolases from this digestive organelle to break-down the contents, including the inner membrane of the autophagosome. The amino acids acquired from this digestion are then released back into the cytosol.

As with our understanding of the MVB biogenesis pathway, much of what we know about the process of autophagy began with genetic research in *S. cerevisiae.* To date, 37 autophagy-related genes (ATG) have been identified, and many of these are conserved beyond yeast in multicellular eukaryotes (Ohsumi 2014, Stanley, Ragusa et al. 2014). The initial work came from a number of groups who defined autophagy mutants. Firstly, the Ohsumi lab

identified a yeast mutant that failed to accumulate autophagic bodies when starved of nitrogen. This autophagy defective mutant, apg1, was able to grow and survive normally in full media conditions, but under starvation conditions the cells failed to degrade proteins and eventually died. The group used this viability defect to screen for other Apg mutants which they confirmed using light microscopy to verify their inability to accumulate autophagic bodies. They identified another 99 autophagy defective mutants which could be split into fifteen complementation groups (Tsukada and Ohsumi 1993). This screen formed the basis of the work for which Ohsumi was awared a Nobel Prize in Physiology or Medicine in 2016. Meanwhile, Thumm and colleagues studied *S. cerevisiae* that were unable to produce vacuolar autophagic bodies under starvation conditions and identified six so-called Aut mutants (Thumm, Egner et al. 1994). The Klionsky lab studied the cytosol to vacuole targeting (Cvt) pathway which shares the same membrane dynamics as autophagy. Following α-aminopeptidase I from the cytosol to the vacuole, they identified Cvt mutants which were unable to transport this enzyme into the vacuole, and which were later shown to be largely the same as the Apg mutants described above (Harding, Morano et al. 1995, Baba, Osumi et al. 1997). Later these mutants, combined with others from groups working on specific types of selective autophagy, were unified into one gene nomenclature system, re-naming all the genes associated with autophagy as *ATG* genes.

1.3.1. Atg complexes

The protein (Atg) products of *ATG* genes are assembled into six complexes that are involved in the biogenesis of the autophagosome (Ohsumi 2014, Stanley, Ragusa et al. 2014). Upon induction of autophagy, these complexes are recruited to the pre-autophagosomal structure (PAS) (figure 1.5.) (Suzuki, Kirisako et al. 2001, Suzuki and Ohsumi 2010). In yeast the presence of the PAS is constitutive as it is involved in Cvt (Suzuki and Ohsumi 2010, Stanley, Ragusa et al. 2014). However mammalian cells lack this constitutive Cvt pathway and the ATG complexes are recruited to the Omegasome, a PI3*P*-rich subdomain of the endoplasmic reticulum (ER) (Mizushima, Yoshimori et

al. 2011). To date we do not fully understand all of the roles each complex plays in the biogenesis of the autophagosome, however we do know that the recruitment of these complexes of Atg proteins is hierarchical (Stanley, Ragusa et al. 2014) .

1.3.2. The Atg1 kinase complex

The first Atg protein described was the protein kinase Atg1. Yeast Atg1 is able to bind to Atg13, which under normal conditions is phosphorylated by TORC1 kinase. Under starvation conditions, Atg13 is dephosphorylated, increasing its affinity for Atg1 (Kamada, Funakoshi et al. 2000). In addition, the binding of Atg13 to Atg1 enhances the kinase activity of Atg1. The Atg1-Atg13 complex is then able to interact with a stable trimer of Atg17-Atg29-Atg31, forming a pentameric complex (Kabeya, Noda et al. 2009, Ragusa, Stanley et al. 2012). In yeast, Atg17-Atg29-Atg31 is associated with the PAS regardless of nutritional status (Suzuki, Kubota et al. 2007, Kabeya, Noda et al. 2009).

In mammalian cells the equivalent complex is formed by ULK1 and ULK2, which have homology to Atg1, alongside the mammalian ATG13, ATG101, and a scaffold protein FIP200 which is orthologous to ATG17 (Hara, Takamura et al. 2008, Ganley, Lam du et al. 2009, Mercer, Kaliappan et al. 2009, Cheong, Lindsten et al. 2011, McAlpine, Williamson et al. 2013). Mammalian TORC1 (mTORC1) forms part of the complex under normal conditions but dissociates during starvation resulting in dephosphorylation of ULK1, ULK2 and ATG13, ultimately releasing ULK1 from the inhibitory effect of phosphorylation (Hosokawa, Hara et al. 2009, Wirth, Joachim et al. 2013).

1.3.4. Atg9

ATG9 is a transmembrane protein expressed in both yeast and mammals. In yeast, Atg9 is found within the membranes of small vesicles that contribute the membrane required for the phagophore (Noda, Kim et al. 2000, Yamamoto, Kakuta et al. 2012). In mammalian cells, ATG9 is found in the trans-Golgi network and late endosomes, however, upon starvation parts of the ATG9-positive membranes from these organelles are donated to the

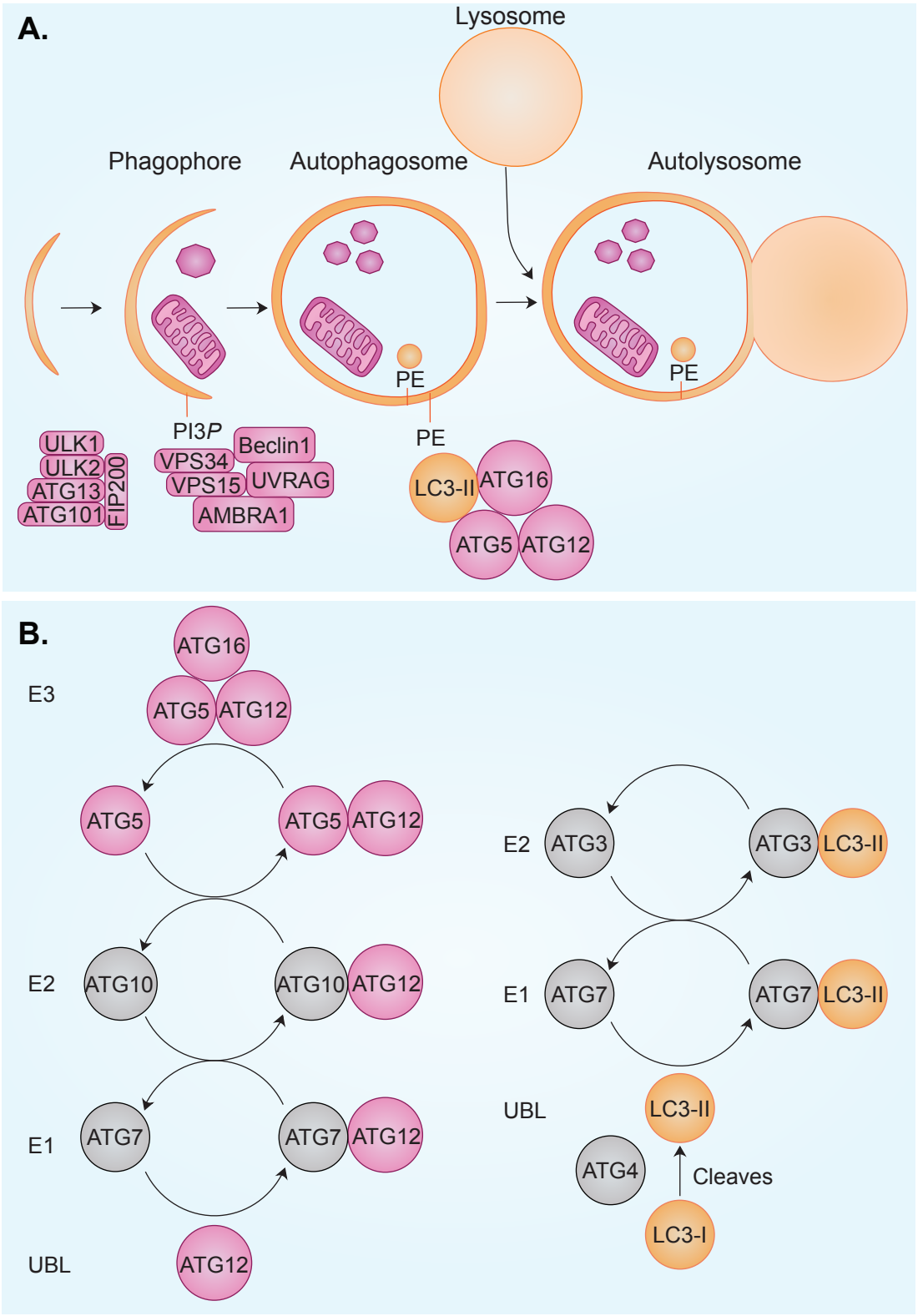


Figure 1.5. Mammalian autophagy. (adapted from Helgason, Karvela et al. 2011) A. Shows the hierarchy of ATG proteins at each stage of autophagy. B. Two ubiquitin-like conjugation systems are involved in autophagy. In the first (left), ubiquitin-like ATG12 is conjugated to ATG5 facilitated by ATG16. This complex can act as an E3 for lipidation of phosphatidylethanolamine (PE) with LC3-II. LC3-I is processed to LC3-II by ATG4 cleavage which is activated by the E1 ATG7 and transferred to the E2 ATG3 (right). LC3-II is conjugated to PE facilitated by the ATG5-ATG12-ATG16 complex.

phagophore (Tooze 2010, Yang and Klionsky 2010). This serves as a nucleation site for membrane biogenesis (Ohsumi 2014). As these small vesicles accumulate, their membranes fuse assisted by soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptors (SNAREs) (Stanley, Ragusa et al. 2014).

1.3.5. PI3K complex

The phosphoinositide 3 kinases (PI3Ks) are a family of lipid kinases that, in yeast, comprise only one member – Vps34. This kinase forms a component of two complexes; complex II is important for vacuolar protein sorting, whereas complex I is required for autophagy (Ohsumi 2014). Complex I comprises Vps34, Vps15, Vps30/Atg6, Atg14 and the recently described Atg38 (Ohashi, Soler et al. 2016). Vps34 generates phosphatidylinositol 3 phosphate (PI3*P*) which resides on the inner membrane of the autophagosome and is believed to recruit other proteins required for autophagosome formation, such as Atg18 (described below).

In mammalian cells VPS34 is a class III PI3K (the classes are described in greater detail in section 1.3) and the complex as described above is composed of VPS34, Beclin1 (the mammalian orthologue of Atg6), VPS15 and the human ATG38 – NRBF2 (Yang and Klionsky 2010). In addition Beclin1 interacts with a number of regulatory proteins such as UV radiation resistance associated genes (UVRAG), activating molecule in Beclin1 regulated autophagy (AMBRA1) and autophagy related protein 14-like protein (Atg14L) (Fimia, Stoykova et al. 2007, Itakura, Kishi et al. 2008, Yang and Klionsky 2010). Other mammalian regulatory components of this complex include the positive regulator BAX-interacting factor 1 (BIF-1) and its inhibitory partner run domain Beclin1-interacting and cysteine-rich containing protein (RUBICON) which are brought to the complex via Beclin1's interaction with UVRAG (Takahashi, Coppola et al. 2007, Zhong, Wang et al. 2009). Additionally, the anti-apoptotic protein B-cell lymphoma 2 (BCL-2) interacts directly with the BH3 domain of Beclin1 and acts in an inhibitory way on the complex (Abrahamsen, Stenmark et al. 2012).

1.3.6. Atg18

Atg18 forms a complex with Atg2 and binds both the Vps34 generated PI3*P* and phosphatidylinositol 3,5 bisphosphate (PI(3,5)P2) (Ohsumi 2014). PI(3,5)P2 is generated from PI3*P* by the PI3*P* 5-kinase Fab1 (PIKfyve in mammalian cells) (Jin, Lang et al. 2016). Whilst PI(3,5)P2 functions mostly in the regulation of vacuolar size it is also involved at some level in autophagy since mutation in PI(3,5)P2 results in a defect in the degradation of autophagosomes that are directed to the vacuole in yeast (Jin, Lang et al. 2016). Atg18 appears to have roles in autophagy, vacuolar size and possibly endocytosis. Although its function still requires full elucidation, we do know that its regulation of autophagosome formation is PI3*P* dependent (Stromhaug, Reggiori et al. 2004, Krick, Henke et al. 2008). In humans the WIPI proteins share sequence homology with Atg18. WIPI1 and WIPI2 have both been shown to be recruited to the autophagosomal membrane through a preferred interaction with PI3*P* (Proikas-Cezanne, Ruckerbauer et al. 2007, Polson, de Lartigue et al. 2010, Dooley, Razi et al. 2014, Proikas-Cezanne, Takacs et al. 2015).

1.3.7. Atg12 conjugation

Atg12 is a ubiquitin-like protein which is activated by the E1 enzyme Atg7. Following transfer to the E2 enzyme Atg10, Atg12 is conjugated by isopeptide bond to K149 of Atg5 (Mizushima, Noda et al. 1998). Atg12-Atg5 dimerisation is facilitated by Atg16 homodimers (Mizushima, Noda et al. 1999, Kuma, Mizushima et al. 2002). The Atg12-Atg5 conjugate can function as an E3-like enzyme to enhance Atg8 lipidation (figure 1.5.B.) (Hanada, Noda et al. 2007).

1.3.8. Atg8 lipidation

Like Atg12, Atg8 is a ubiquitin-like protein. During starvation conditions the expression of Atg8 is increased and it is partially localised to the

autophagosome and the membranes of autophagic bodies (Kirisako, Baba et al. 1999). Atg8 is synthesised as a precursor, processed by the cysteine protease Atg4 and activated by Atg7 (Ichimura, Kirisako et al. 2000, Kirisako, Ichimura et al. 2000). Through the E2 enzyme Atg3 and with facilitation from the Atg12-Atg5 conjugate, Atg8 can be passed onto the head group of phosphatidylethanolamine (PE) (Ichimura, Kirisako et al. 2000). The amount of Atg8 on the growing isolation membrane regulates the final size of the autophagosome (Xie, Nair et al. 2008).

In mammalian cells the homologue for Atg8 is LC3 which is converted to LC3-I by ATG4 cleavage and subsequent lipidation to LC3-II at both faces of the phagophore (figure 1.5.B.). Its removal from the outer membrane precedes fusion of the autophagosome with the lysosome (Yang and Klionsky 2010).

1.3.9. Selective autophagy and ubiquitylation

Selective autophagy is reliant on different autophagy receptors which are brought to autophagosomes through their interaction with Atg8/LC3 (Stolz, Ernst et al. 2014). Many of these receptors contain LC3-interacting regions (LIR) with a consensus sequence W/F/YxxL/I/V (Pankiv, Clausen et al. 2007, Khaminets, Behl et al. 2016).

Whilst the role for ubiquitylation in autophagy is mostly understood through ubiquitin-dependent cargo recognition in selective autophagy (as will be exemplified below), there is some evidence that ubiquitylation is also involved, to some degree, in the regulation of core autophagy components. For example, free p85b subunits of the Vps34 PI3K complex are targeted for degradation by the Skp, Cullin, F-box-containing complex (SCP) E3 ligase which prevents formation of the PI3K complex (Kuchay, Duan et al. 2013). SUMOylation is the addition of small ubiquitin-like modifiers (SUMOs) to proteins. Vps34 can be SUMOylated by the E3 SUMO ligase KRAB-associated protein 1 (KAP1) which enhances its interaction with Beclin1, Atg14L and UVRAG complexes (Yang, Fiskus et al. 2013).

A recent study has linked the DUB USP8 to the regulation of autophagy in *Drosophila melanogaster* (Jacomin, Bescond et al. 2015). The authors coexpressed dsRNA targeting 25 DUBs of USP and UCH families, together with the autophagy marker GFP-LC3B, in the larval fat body (Jacomin, Bescond et al. 2015). Under normal conditions GFP-LC3B is cytosolic, but upon induction of autophagy it is recruited to the membranes of autophagosomes. Using larvae with low basal levels of autophagy, the authors identified four DUBs out of 25 for which depletion increased the accumulation of LC3B positive dots in a minimum of 50% of cells. The phenotype for two of these DUBs, USP8 and USP12, could be further confirmed using an alternative RNAi system.

For their follow up experiments the authors made use of transgenic Drosophila lines expressing GFP and mCherry double tagged Atg8a, the Drosophila paralogue of LC3B. GFP is quenched in the acidic environment of the autolysosome resulting in a switch from green and red autophagosomes to uniquely red autolysosomes. Using this method they showed that USP8 depletion increased basal autophagy as the number of GFP-mCherry positive punctate structures increased. They also suggested that there was a block in autophagic flux since there was no accumulation of autolysosomes despite the activation of autophagy. Similar results were obtained with expression of catalytically inactive USP8. Electron-microscopy based analysis of these USP8-depleted cells showed double membrane bound autophagosomes with undigested content and significantly smaller lysosomes. Likewise GFP-LAMP1 staining was redistributed from large perinuclear structures to smaller vesicles that were distributed throughout the cytosol. The authors suggested that these might correspond to vesicles transporting enzymes from the biosynthetic secretory pathway (i.e. the trans-Golgi network) to the endo-lysosomal pathway.

Interestingly, USP8 has been implicated in this pathway once before. A key protein in lysosomal enzyme delivery from the biosynthetic pathway is the Mannose-6 phosphate receptor (M6PR) which is both involved in delivering newly synthesised hydrolases from the TGN to the lysosomes and also scavenging secreted enzymes from the plasma membrane. In 2014,

MacDonald et al. reported that USP8 depletion leads to M6PR accumulation in endosomes due to a failure to recycle back to the TGN (MacDonald, Urbe et al. 2014). The receptor was thus unable to pick up new lysosomal enzymes from the TGN which prevented the delivery of these degradative enzymes to the lysosome. As a result the hydrolase, Cathepsin D, was found to be constitutively secreted rather than delivered to the lysosome.

Based on the data from these two papers it seems possible that depletion of USP8 affects autophagy by dysregulating lysosome biogenesis thus preventing autophagosome fusion with the lysosome. This would prevent the generation of, and degradation by the autolysosome. In addition, components of the autophagy machinery described above have been linked to components of the ESCRT machinery. For example, Atg12 has recently been shown to form a conjugate with Atg3 which is then able to interact with the ESCRT-III associated protein ALIX. It is proposed that this interaction might play a role in late endosome distribution and basal autophagy but not starvation-induced autophagy (Murrow, Malhotra et al. 2015).

More recently a study has implicated another DUB in autophagy. Xu et al. showed that AKT phosphorylates and activates the proteasome associated DUB USP14. USP14 activation promotes the removal of K63-linked ubiquitin chains from Beclin1 whilst its depletion leads to an increase in autophagy (Xu, Shan et al. 2016).

1.3.10. Mitophagy

The best example for the role of ubiquitin in selective autophagy is its role in mitophagy. Two E3 ligases, Parkin and Glycoprotein 78, have been implicated in this mechanism which serves to degrade damaged mitochondria (Geisler, Holmstrom et al. 2010, Fu, St-Pierre et al. 2013, Khaminets, Behl et al. 2016). Most famously, the RING between RING (RBR) ligase Parkin and its activating kinase PINK1 play a key role in this pathway. Loss of function of Parkin and PINK1 in the substantia nigra have been associated with Parkinson's disease (Durcan and Fon 2015). Under normal conditions the PTEN induced putative kinase 1 (PINK1) is constitutively

imported into the mitochondrial inner membrane where it is cleaved by presenilin-associated rhomboid-like protein (PARL) and then subsequently degraded by the proteasome in the cytosol (Jin, Lazarou et al. 2010, Bingol and Sheng 2016). When mitochondria become damaged, PINK1 is stabilised at the mitochondrial outer membrane due to impairment of the translocase of the outer membrane (TOM) complex which can be blocked by a change in membrane potential (Hamacher-Brady and Brady 2016). At the outer mitochondrial membrane, PINK1 phosphorylates Mitofusin 2 (Mfn2) at Ser442 and Thr111 and other mitochondrial outer membrane proteins and accumulates at the TOM complex (Bingol and Sheng 2016). This recruits Parkin to the outer membrane (Chen and Dorn 2013). PINK1 then phosphorylates the ubiquitin-like (UBL) domain of Parkin at Ser65 as well as ubiquitin itself at the topologically equivalent residue (Shiba-Fukushima, Imai et al. 2012, Kane, Lazarou et al. 2014, Koyano, Okatsu et al. 2014). This triggers a conformational change in the E3 ligase causing its activation. Activated Parkin ubiquitylates a number of outer mitochondrial membrane proteins including both Mitofusin 1 and Mitofusin 2 (Sarraf, Raman et al. 2013). Following Parkin activation, there is an increase in K48-, K63-, K6- and K11- linked ubiquitylation on mitochondria. Some of the mitochondrial outer membrane proteins are degraded by the proteasome, which is essential for mitophagy (Chan, Salazar et al. 2011, Ordureau, Sarraf et al. 2014, Bingol and Sheng 2016). In addition, autophagy receptors are recruited by ubiquitylation which then bridge the target mitochondria to the autophagy machinery as described above (Hamacher-Brady and Brady 2016). These receptors include the ubiquitin-binding domain containing proteins p62/SQSTM1, NBR1 and Optineurin (Hamacher-Brady and Brady 2016). In addition, the Beclin1-interacting protein AMBRA1 also interacts with Parkin at the outer mitochondrial membrane, providing another link between the autophagy machinery and damaged mitochondria (Fimia, Stoykova et al. 2007).

Recently, USP8 has been linked to the regulation of mitophagy through its purported DUB activity against Parkin. Using an unbiased siRNA screen, Durcan et al. depleted DUBs in U2OS cells stably expressing GFP-Parkin

and looked for defects in the recruitment of Parkin to depolarised mitochondria in cells treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Durcan, Tang et al. 2014). USP8 depletion delayed the recruitment of GFP-Parkin and increased the levels of steady-state Parkin. Overexpression of USP8 reduced Parkin steady-state levels and rescued the defect in Parkin recruitment caused by USP8 depletion. In addition, USP8 depletion delayed successful autophagy since the cells retained mitochondrial protein TOM20 for longer than cells treated with non-targeting siRNA. An *in vitro* DUB activity assay confirmed activity of USP8 against K6-linked auto-ubiquitylation of Parkin. Other DUBs have been linked to mitophagy. Depletion of both USP15 and USP30 promotes mitophagy however neither of these USPs deubiquitylate Parkin directly. Instead they are thought to act downstream of this E3 ligase (Bingol, Tea et al. 2014, Cornelissen, Haddad et al. 2014, Durcan, Tang et al. 2014, Liang, Martinez et al. 2015) .

1.3.11. p62/Sequestosome 1

The scaffold protein p62/Sequestosome 1 (p62/SQSTM1) is an autophagy receptor that possesses a ubiquitin associated (UBA) domain, a LC3-interacting region (LIR) and a PB1 domain permitting its self-oligomerisation. In addition, p62 contains a KEAP1-interacting region (see 1.5. for detail) (Katsuragi, Ichimura et al. 2015). There is disagreement in the literature as to whether p62 is essential for mitophagy, however it is known to promote aggregation of damaged mitochondria and link the autophagy machinery to these aggregates (Hamacher-Brady and Brady 2016). Optineurin, another ubiquitin adapter, is recruited to ubiquitylated proteins at the mitochondria by its LIR and brings TANK-binding kinase (TBK1) into proximity of p62. This results in the phosphorylation of p62 within its UBA domain at Ser403 which increases its binding affinity for ubiquitin chains, acting as a positive feedback mechanism (Matsumoto, Wada et al. 2011, Richter, Sliter et al. 2016).

On top of the involvement of p62 in the regulation of mitophagy, it has also been implicated both in the autophagosomal degradation of protein

aggregates (aggrephagy) and bacteria (xenophagy), again due to its ability to interact with ubiquitin (Katsuragi, Ichimura et al. 2015, Hamacher-Brady and Brady 2016). For example, misfolding of Tau is associated with the formation of p62 positive protein aggregates and neurodegeneration (Kuusisto, Salminen et al. 2001). K63-linked ubiquitylation of mutant Tau has been shown to correlate with its degradation by autophagy (Tan, Wong et al. 2008).

Interestingly, studies investigating the degradation of p62-positive protein aggregates by autophagy have also highlighted a potential role for the ESCRT machinery in this process. Depletion of the ESCRT-0 component HRS, was shown to result in the accumulation of both ubiquitylated proteins and p62 (Oshima, Hasegawa et al. 2016). The accumulation of p62 is typically a marker of a block in autophagy since the protein in itself is targeted for degradation by this pathway. Since we already know that DUBs interact so closely with the ESCRT-machinery, this might hint at another link between ubiquitylation and autophagy. Indeed the endocytic DUB, USP8 has been linked to the clearance of α-synuclein aggregates in Lewy body disease. Lewy body accumulation is a feature of Parkinson's disease. A recent study showed strong staining for K63-linked ubiquitin in α-synuclein positive Lewy bodies in neurons with pathological inclusions (Alexopoulou, Lang et al. 2016). This K63-ubiquitin immunostaining was reduced in the substantia nigra whereas USP8 levels were increased in the same tissue.

The authors showed that USP8 interacts with α-synuclein and its overexpression stabilised total α-synuclein levels while reducing the K63-linked pool.

Conversely USP8 depletion enhanced lysosomal degradation of α-synuclein. Hence the authors propose that USP8 is required for K63-linked deubiquitylation of this protein, rescuing it from degradation. Importantly, USP8 depletion protected dopaminergic neurons in Drosophila from α-synuclein induced locomotor deficits and cell loss suggesting that USP8 might be an interesting therapeutic target in Parkinson's disease.

1.4. Signal transduction pathways

1.4.1. Signalling

While individual cells have all the genes they need for their own survival, as part of a complicated multi-cellular organism they are reliant on external signals from other cells or changes in the environment around them such as availability of nutrients. This is why the signalling receptors on the cell surface are fundamentally important in order for cells to respond to these changes via activation of signalling pathways that allow for a change in gene expression. Unicellular organisms such as yeast also possess cell surface receptors as they must also engage with their external environment in a similar way.

There are a number of different types of receptor on the surface of our cells. Broadly speaking these can be split into three groups; ion channel-linked receptors, enzyme-linked receptors and G-protein-coupled receptors.

Here I will focus on a subset of the enzyme-linked receptors, receptor tyrosine kinases (RTKs).

1.4.2. Receptor tyrosine kinases

The human genome comprises genes for 90 tyrosine kinases. Of these, 58 are transmembrane receptors. These 58 are classed into 20 families including the growth factor receptor families, such as epidermal growth factor receptor (EGFR), PDGFR, MET and the vascular endothelial growth factor receptor (VEGFR); the collagen receptor family known as the discoidin domain receptors (DDRs); and the ephrin receptors which bind a membrane bound ligand, ephrin (Robinson, Wu et al. 2000).

Receptor tyrosine kinases share the same basic structural layout. The extracellular domain contains a ligand-binding region whilst the intracellular domain comprises the tyrosine kinase domain and additional regulatory

features; a single transmembrane region links the two (Lemmon and Schlessinger 2010). Upon ligand binding, the majority of RTKs homo- or hetero-dimerise prior to their transactivation. This leads to the transphosphorylation of the activation loop within one receptor monomer by its partner and vice versa thus creating an active receptor dimer (Stamos, Sliwkowski et al. 2002). Activation of the receptor dimer allows autophosphorylation of a number of other tyrosine residues. In the case of EGFR these are Y992, Y1045, Y1068, Y1086, Y1148 and Y1173 (Downward, Parker et al. 1984, Hsuan, Totty et al. 1989, Margolis, Lax et al. 1989, Walton, Chen et al. 1990, Levkowitz, Waterman et al. 1999). Phosphorylation of tyrosine residues within the intracellular domain allows for the recruitment of a number of downstream effectors triggering the signalling output of that particular receptor. This is exemplified below for the epidermal growth factor receptor (EGFR) family.

1.4.3. Epidermal growth factor receptor family

In the 1950s and 1960s, Rita Levi-Montalcini and Stanley Cohen published their study on the identification of two growth factors, ultimately winning a Nobel Prize for their discoveries (Weltman 1987). Rita Levi-Montalcini had initially identified a tumour-derived agent which altered the development of the sympathetic nervous system when tumours from mice were implanted into chicken embryos (Levi-Montalcini 1952). Working together with Stanley Cohen, they purified this factor which was later dubbed ‘nerve growth factor’ (NGF). The epidermal growth factor (EGF) was also identified by Cohen injecting salivary gland extracts into new-born mice, an intervention that triggered accelerated growth of the mice (Cohen and Levi-Montalcini 1957, Levi-Montalcini and Cohen 1960, Cohen 1962).

Soon after their work was published, the first RTK, EGFR, was discovered (O'Keefe, Hollenberg et al. 1974, Carpenter, Lembach et al. 1975). Carpenter and colleagues showed that ^{125}I-labelled EGF specifically binds to human fibroblasts and that the growth factor is subsequently degraded. This was a process that could reach saturation point dependent on time and temperature

(Carpenter, Lembach et al. 1975). Later *in vitro* work showed that EGF binds to membrane proteins and triggers phosphorylation of endogenous proteins in A-431 tumour cells (Carpenter, King et al. 1978). The authors proposed that the mechanism for increased phosphorylation of endogenous proteins indicates that the receptor might be a protein kinase. The full amino acid sequence was reported in 1984 (Ullrich, Coussens et al. 1984).

Subsequently, three additional members of the EGFR family have been described, ERBB2, ERBB3 and ERBB4 with EGFR (ERBB1) completing the set (figure 1.6.A.) (Stern D.F. 1986, Bargmann and Weinberg 1988, Plowman, Whitney et al. 1990, Plowman, Culouscou et al. 1993)

1.4.3.1. EGFR family activation

A number of ligands associate with the different EGFR family members. EGFR itself is the best characterised member within this family and to date seven ligands are known for this receptor (Singh, Carpenter et al. 2016). As yet, no ligand has been found for ERBB2. This orphan receptor does, however, activate signalling pathways through its dimersation and cooperative activation with other EGFR family members (Citri, Skaria et al. 2003). Due to its conformation, ERBB2 is the preferred dimerisation partner of all the other EGFR family members (Tzahar, Waterman et al. 1996, Graus-Porta, Beerli et al. 1997, Citri, Skaria et al. 2003). In particular, ERBB2 heterodimerises very well with ERBB3, and this pairing is especially beneficial since ERBB3 does not have an active kinase domain but does bind ligands, the neuregulins (Tzahar, Waterman et al. 1996, Citri, Skaria et al. 2003). ERBB4 also binds to and is activated by neuregulins. This receptor is subsequently cleaved and the intracellular domain makes its way into the cell to activate its downstream effectors (Schlessinger 2002, Arasada and Carpenter 2005, Lemmon 2009).

Whilst there is some evidence for pre-existing dimers of these receptors on the cell surface, the conformation of these receptors prevents both ligand binding and dimerisation in the basal state. The receptors possess a tether within their extracellular region that obstructs the binding of ligands and the

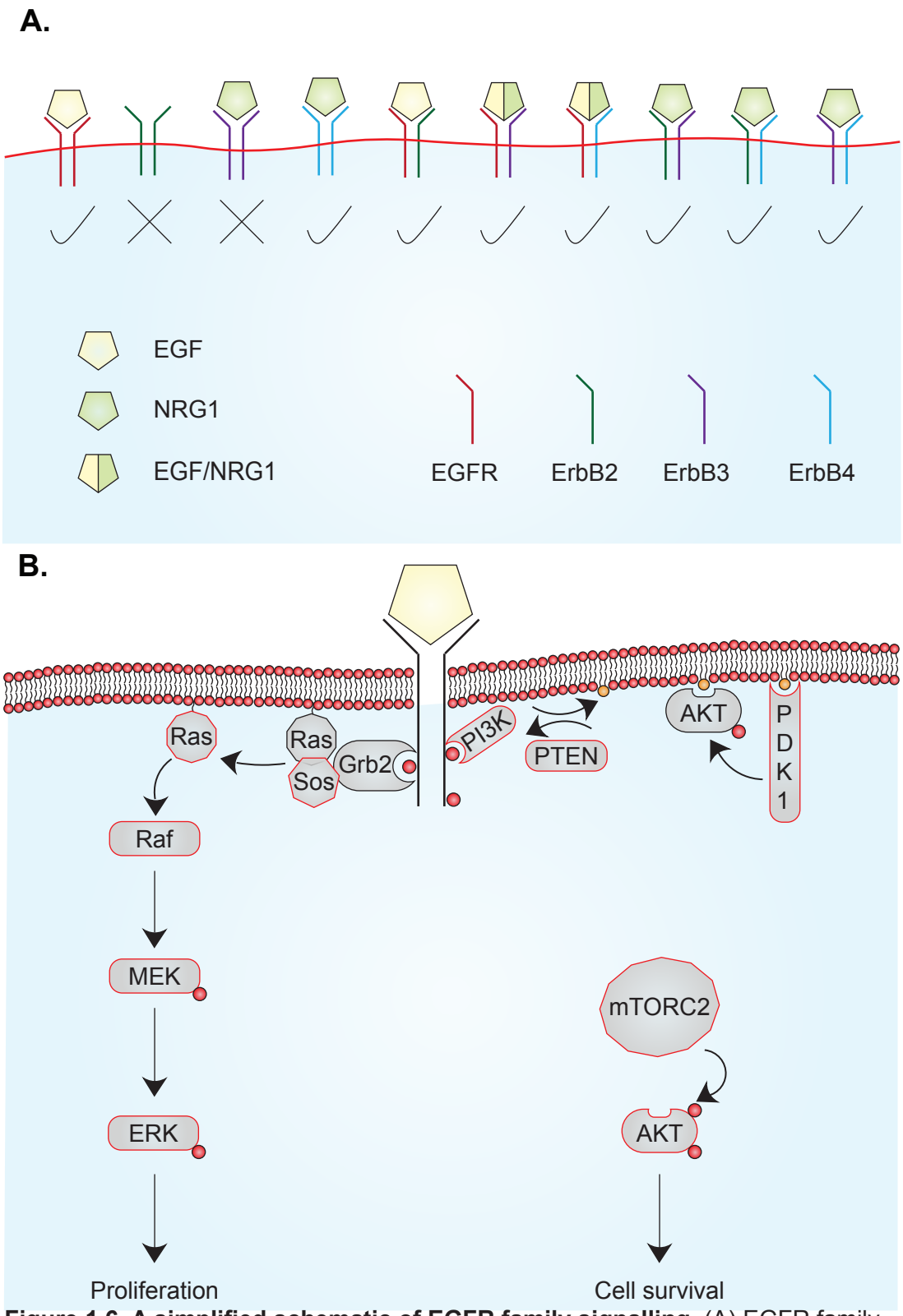


Figure 1.6. A simplified schematic of EGFR family signalling. (A) EGFR family potential dimers , ligands and whether or not they can signal (indicated by a tick or cross). (B) A simplified outline of the MAPK and PI3K signalling pathways. The MAPK pathway is activated by the recruitment of adapter proteins to phosphorylated RTKs triggering activation of downstream effectors. PI3K recruitment and active RTKs allows lipidphosphorylation and recruitment of AKT and one of its two activating kinases PDK1. Following mTORC2 phosphorylation active AKT can act on downstream effectors promoting cell survival.

dimerisation domain (Holbro and Hynes 2004, Hynes and Lane 2005, Lemmon and Schlessinger 2010). EGF-like ligands are bivalent but instead of bridging two receptors as is the case for other RTKs, the ligand binds in two places within the same monomer. This breaks the tether and allows dimerisation and subsequent activation of the receptor. Different subpopulations of the EGFR family have different ligand binding affinities, both high and low. Those dimeric forms described above may account for the high-affinity subpopulation (Schlessinger 2002, Lemmon 2009).

Ligand induced hetero- and/or homo-dimerisation of EGFR family members triggers their activation and autophosphorylation (figure 1.6.). These receptors transactivate by phosphorylation of tyrosines within the C-terminus. This phosphorylation recruits SH2 domain containing proteins to the cell surface allowing the activation of downstream pathways (figure 1.6.B.).

1.4.3.2. MEK/ERK signalling

One of the proteins, activated EGFR recruits to the cell surface, is the adaptor protein GRB2. This protein contains both SH2 and SH3 domains. The SH3 domain allows GRB2 to recruit another protein, Son of sevenless (SOS). SOS is a guanine nucleotide exchange factor (GEF) for the small GTPase superfamily, RAS. Activated GTP-bound RAS can in turn activate the MAP kinase kinase kinase (MAPKKK) protein, RAF that subsequently activates downstream kinase MEK (figure 1.6.B) (Katz, Amit et al. 2007). MEK phosphorylates and activates the MAPK ERK, which in its activated form can phosphorylate cytosolic and cytoskeletal proteins or other kinases. Alternatively it translocates to the nucleus where it can activate transcription factors such as FOS and JUN (Murphy and Blenis 2006, Katz, Amit et al. 2007). Activation of this pathway stimulates cell proliferation through regulation of cell cycle proteins and migration through transcriptional regulation or phosphorylation of proteases that alter focal adhesions and other cytoskeletal components (Murphy and Blenis 2006, Katz, Amit et al. 2007).

1.4.3.3. PI3K pathway signalling

The other main canonical pathway activated by EGFR family members is the phosphatidyl inositide 3-kinase (PI3K)-AKT signalling pathway. In a similar way to the MEK/ERK signalling pathway, it all begins with the recruitment of a protein. In this case, PI3K is recruited to phosphorylated tyrosines within the C-terminus of the receptor. There are several classes of PI3K. In the case of RTK activation, it is the heterodimeric class I kinases that are recruited and activated (Vanhaesebroeck, Stephens et al. 2012). The Class I family of PI3Ks comprises four kinases within two subgroups. The class IA subgroup is made up of 3 enzymes each containing the p110α, p110β or p110δ catalytic subunit paired with the p85 regulatory subunit. Class IB is made up of the p110γ catalytic subunit paired with either p101 or p84/p87 regulatory subunits (Vanhaesebroeck, Stephens et al. 2012). Class IB PI3Ks are activated by G-protein-coupled receptors (Andrews, Stephens et al. 2007). Activated RTKs recruit the p85 subunit of class IA PI3K via its SH2 domain. This is something that the ERBB2/ERBB3 heterodimer does particularly well due to the presence of a large number of p85 recruiting domains on the intracellular region of ERBB3. As described, ERBB3 has ligand binding potential but no kinase activity and typically heterodimerises with the orphan receptor ERBB2 for its activation (Citri, Skaria et al. 2003).

Once recruited to the activated receptor, PI3K can be phosphorylated and activated. Then it phosphorylates phosphatidyl inositol 4,5 bisphosphate (PIP_2) to phosphatidyl inositol 3,4,5 trisphosphate (PIP_3), a process which can be reversed by the phosphatase PTEN. This acts as a docking site for PH domain containing proteins (figure 1.6.B.). In this way PIP_3 recruits PDK1 and AKT and triggers a conformational change in AKT, which allows PDK1 to phosphorylate Thr308 within its activation loop. AKT activation requires the phosphorylation by a second kinase, mTORC2 at Ser473 within the hydrophobic pocket of the kinase.

Activated AKT has a wide variety of outputs as it is able to phosphorylate a large number of substrates, including pro-apoptotic BAD and FOXO proteins. Perhaps most importantly, AKT has pro-survival functions which will be discussed below.

1.4.3.4. AKT and cell survival

AKT plays a very complex role in cell survival. It both promotes normal cell cycle progression and inhibits pro-apoptotic pathways in a direct and indirect way. One of the best-characterised pathways that AKT regulates is that of the forkhead family of transcription factors. This superfamily consists of FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX) and FOXO6 which contain two (FOXO6) or three (FOXO1, FOXO3 and FOXO4) AKT phosphorylation sites within their structure (Tzivion, Dobson et al. 2011). These transcription factors stimulate the transcription of a number of genes that promote apoptosis and cell cycle arrest such as p130, p27 and TRAIL. Activated AKT phosphorylates FOXO proteins precluding them from the nucleus. Once trapped in the cytosol, FOXOs are bound and sequestered by 14-3-3 proteins ultimately leading to their proteasomal degradation.

Additionally, AKT can regulate the cell cycle directly by regulating p21 and p27 (Shin, Yakes et al. 2002, Viglietto, Motti et al. 2002). It also regulates the levels of p53 through phosphorylation of its E3 ligase, MDM2 promoting the degradation of p53 (Zhou, Liao et al. 2001, Ogawara, Kishishita et al. 2002). AKT also activates IKKα leading to the phosphorylation and proteasomal degradation of IκB (Bai, Ueno et al. 2009). This releases NFκB from its inhibition allowing its translocation to the nucleus where it activates transcription of pro-survival genes. Finally, AKT phosphorylates the pro-apoptotic BH3-only protein BAD resulting in its dissociation from the BAD/BCL-X_L complex (del Peso, Gonzalez-Garcia et al. 1997). This prevents BAD from activating the pro-apoptotic Bcl2-family members BAX and BAK, which are normally held in check by anti-apoptotic members of the family including BCLXL, BCL2 and MCL1. Activation of BAX and BAK leads to the permeabilisation of the outer mitochondrial membrane, release of Cytochrome C and activation of apoptotic caspases (Elmore 2007).

1.5. The ROS signalling pathway

In this introduction I have described the role for ubiquitylation in lysosomal degradation of RTKs and depicted the link between one of those RTK signalling pathways and cell survival. Thus ubiquitylation can play a role in cell survival in an indirect way through modulating the signalling output of this pathway.

Another way in which ubiquitylation contributes to the regulation of cell survival and response to stress is in the management of reactive oxygen species (ROS). Endogenous ROS are generated by the mitochondria during normal cellular metabolism. Under oxidative phosphorylation the generation of ATP causes the production of ROS such as superoxide and hydrogen peroxide. This can lead to the production of free radicals, which can damage the cell, cause DNA damage, and ultimately requires the cell to undergo apoptosis. It follows that ROS levels must be carefully regulated. There is evidence that ROS may also have beneficial effects for the cell. In particular, ROS are involved in propagation of cell growth and survival pathways such as the MAPK and PI3K pathways. In the case of the PI3K pathway, there is evidence that ROS can modify the phosphatase for PIP_3, PTEN, thus inhibiting it and allowing sustained PI3K pathway activation (Ray, Huang et al. 2012).

1.5.1. Identification of NRF2

NRF2 was isolated from a screen for DNA-binding proteins that recognise the NFE2 motif present in the β-globin locus (Moi, Chan et al. 1994). Whereas NFE2 is an erythroid-specific transcription factor involved in erythropoiesis and platelet development, NRF2 is expressed ubiquitously. Like NFE2, NRF2 belongs to a subset of the basic region leucine zipper (bZip) transcription factors; the cap ‘n’ collar (CNC) family (Moi, Chan et al. 1994). NRF2 was later implicated in the regulation of drug mediated enzymes (DMEs) such as glutathione S-transferase (GST) and NAD(P)H:quinine oxidoreductase 1 (NQO1) that are upregulated in response to antioxidants and electrophiles (Venugopal and Jaiswal 1996, Itoh, Chiba et al. 1997). These genes had in

common an antioxidant response element (ARE), which had been identified in the early 1990s as a region that was important in expression of the Glutathione S-transferase Ya subunit induced by tert-butylhydroquinone (tBHQ) (Rushmore and Pickett 1990). This ARE showed homology to the NFE2 motif described above. An investigation into the binding of CNC-bZip transcription factors subsequently linked NRF2 to genes under ARE control and showed an increase in the expression of genes bearing the ARE motif with NRF2 overexpression (Venugopal and Jaiswal 1996).

Meanwhile, work on NRF2 knockout mice showed that whilst these mice survived and grew normally, they were prone to pathologies with oxidative elements such as lupus-like nephritis (Yoh, Itoh et al. 2001, Itoh, Mimura et al. 2010). Itoh et al. reported that the induction of ARE regulated genes, GST and NQO1, was reduced in the livers and intestines of NRF2 knockout mice (Itoh, Chiba et al. 1997). The same study also identified a binding partner for NRF2 in small Maf proteins.

1.5.2. NRF2 binding and transcriptional activation

The basic region of NRF2 facilitates its interaction with DNA whereas its bZip domain is important for its heterodimerisation with small musculo-aponeurotic fibrosarcoma proteins (Mafs). Mafs are capable of binding to ARE motifs but do not possess a transactivation domain required to facilitate transcriptional activation (Nguyen, Huang et al. 2000, Nioi, McMahon et al. 2003, Hayes, McMahon et al. 2010). NRF2 has six Nrf2-ECH homology domains two of which (Neh4 and Neh5) allow its binding to cAMP response element-binding protein (CREB)-binding protein (CBP) (Katoh, Itoh et al. 2001). CBP has histone acetyl-transferase activity and may act as a co-activator for NRF2 by allowing the DNA into a more ‘open’ configuration and giving accessibility for the recruitment of the transcription machinery (Hayes, McMahon et al. 2010).

NRF2 itself is involved in the transcriptional regulation of over 100 genes which orchestrate a number of different pathways (Sporn and Liby 2012). As well as modulating the levels of NQO1 and GSTs described above, NRF2 can regulate the expression of antioxidant proteins such as glutathione

peroxidase and drug-metabolising enzymes (Jaramillo and Zhang 2013). NRF2 also regulates the synthesis and metabolism of glutathione which serves as a ROS neutralising agent in its reduced state. This can be through control of enzymes such as glutamate-cysteine ligase catalytic subunit which is rate limiting in the glutathione synthesis process (Jaramillo and Zhang 2013).

Since NRF2 plays such a key role in the stress response it makes sense that it would be involved in the cancer response. In fact, NRF2 can both act as a tumour suppressor and an oncogene (Sporn and Liby 2012, Jaramillo and Zhang 2013). NRF2 knockout mice are more prone to tumour formation triggered by exposure to carcinogens and this is correlated with a reduction in the expression of ARE controlled genes (Ramos-Gomez, Kwak et al. 2001, Sporn and Liby 2012, Jaramillo and Zhang 2013). However other studies show that NRF2 is elevated in a number of cancers and prognosis with elevated NRF2 is poor (Jaramillo and Zhang 2013).

1.5.3. Identification of KEAP1

The Neh4 and Neh5 domains of NRF2 each confer transcriptional activation activity to the protein whereas Neh2 is, in fact, inhibitory (Itoh, Wakabayashi et al. 1999). In HD3 erythroblasts, the expression of wild-type NRF2 leads to only mild gene activation however mutation of the Neh2 domain enhances the ability of NRF2 to activate transcription. Additionally the mild transcriptional activation upon wild-type NRF2 expression in HD3 cells was enhanced by increasing amounts of co-expressed isolated Neh2 domain suggesting that an inhibitory protein might interact with this domain in a competitive way. Using this domain as bait in a yeast two-hybrid screen, the authors identified a novel protein, which due to sequence homology with an actin binding protein in Drosophila, was named Kelch-like ECH associated protein 1 (KEAP1).

Soon after the identification of KEAP1 in HD3 cells, Dhakshinamoorthy and Jaiswal cloned a cytosolic inhibitor of NRF2 from rat livers which they named Inhibitor of Nrf2 (INrf2). They characterised this protein as a 624 amino acid,

cysteine-rich polypeptide of approximately 70kDa that contains a bric-à-brac, tramtrack and broad complex (BTB) domain and a KELCH domain which Itoh et al. had also described in KEAP1 (Itoh, Wakabayashi et al. 1999, Dhakshinamoorthy and Jaiswal 2001). We now know the two proteins to be the same.

KEAP1 has two protein binding domains. The Kelch repeat or double glycine repeat (DGR) domain allows for its interaction with NRF2's Neh2 domain (Li, Zhang et al. 2004, Lo, Li et al. 2006). The bric-à-brac, tramtrack and broad complex (BTB) domain recruits the scaffold protein and E3 ligase, Cullin3 (Cul3) (Cullinan, Gordan et al. 2004, Kobayashi, Kang et al. 2004, Furukawa and Xiong 2005). Thus KEAP1 is a substrate adapter for the Cullin3-RING E3 ligases. The BTB domain also permits KEAP1 dimerisation, which is important for its interaction with NRF2 (Zipper and Mulcahy 2002).

1.5.4. NRF2 binding to KEAP1

There are two independent binding sites for KEAP1 in the Neh2 domain of NRF2 (figure 1.7.). The low-affinity DLG motif and the high-affinity ETGE motif bind one each of the two β-propeller protein-docking sites presented by the KEAP1 dimer (see figure 1.4) (McMahon, Thomas et al. 2006, Tong, Kobayashi et al. 2006). This creates what is described as a hinge and latch mechanism wherein newly synthesised NRF2 is picked up by dimeric KEAP1 at the high-affinity ETGE motif thus promoting DLG motif binding (figure 1.7.). Through its binding to NRF2, KEAP1 performs two roles. Firstly, it retains NRF2 in the cytosol, preventing its nuclear import. Immunofluorescence experiments showed that expression of NRF2 and KEAP1 together retained the transcription factor in the cytosol whereas NRF2 expressed alone was nuclear (Itoh, Wakabayashi et al. 1999). In the early 2000s several studies showed that the stability of NRF2 is regulated by proteasomal degradation and that this is dependent on KEAP1 (Itoh, Wakabayashi et al. 2003, McMahon, Itoh et al. 2003, Nguyen, Sherratt et al. 2003, Stewart, Killeen et al. 2003, Zhang and Hannink 2003).

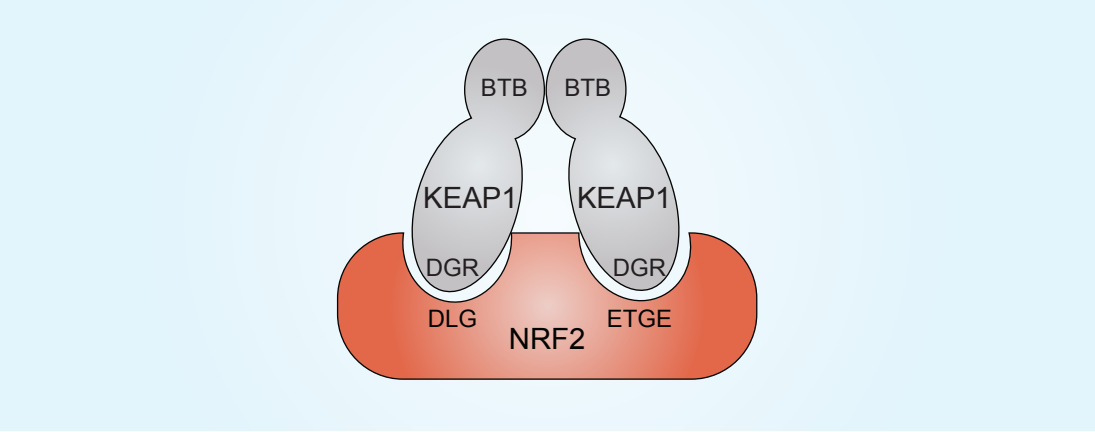


Figure 1.7. A simplified schematic of NRF2-KEAP1 binding. The two binding regions in NRF2 are the high affinity ETGE motif and the low affinity DLG which each bind to one of the two DGRs in the KEAP1 homodimer.

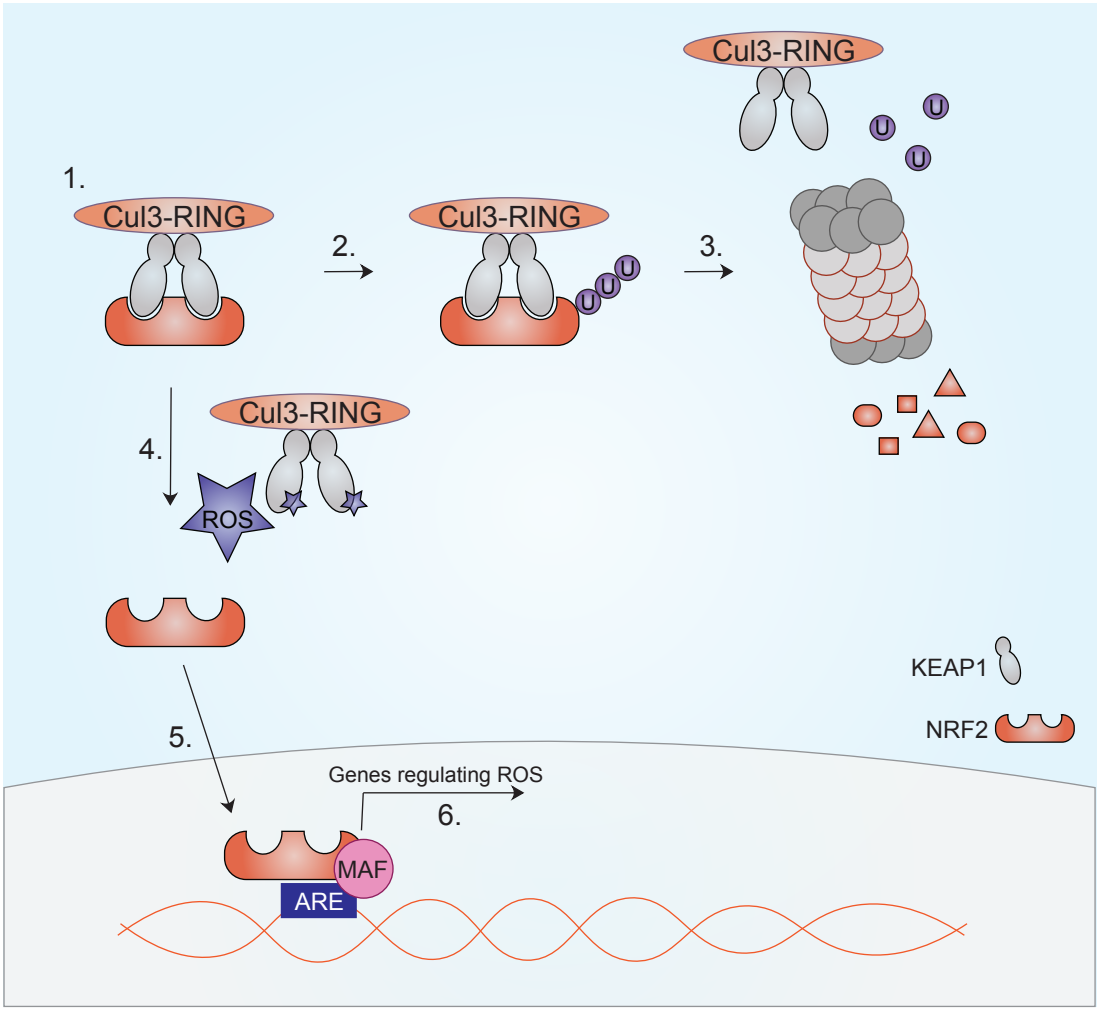


Figure 1.8. A simplified schematic of NRF2 regulation by KEAP1. The KEAP1 homodimer binds to NRF2 and recruits the Cul3-RING complex (1). This promotes NRF2 ubiquitylation (2) and degradation by the proteasome (3). ROS modifies reactive cysteines in KEAP1 and block its binding with NRF2 (4). Released NRF2 translocates to the nucleus where it heterodimerises with small MAFs and binds ARE promoters (5). NRF2 activates genes involved in ROS regulation (6).

1.5.4. Ubiquitin-mediated degradation of NRF2

Coincidentally, work in *C. elegans* had identified a link between the E3 ligase scaffold protein Cullin3 (Cul3) and the BTB domain of MEL-26 (Furukawa, He et al. 2003, Pintard, Willis et al. 2003, Xu, Wei et al. 2003). At the time, the role of Cullin3 had not fully been understood, however it was proposed that it might form a similar complex to the SCF E3 ligase complex comprised of Cullin1 (Cul1), Skp1, an F box protein and the E3 ligase RBX1/ROC1/HRT1 (Deshaies 1999). The finding in *C. elegans* lent weight to this argument since not only did Cul3 interact with the BTB domain containing protein MEL-26, it also promoted the ubiquitylation and subsequent degradation of the meiotic protein, MEI-1.

Mass spectrometry to identify Cul3 binding proteins first reported two proteins that both had BTB domains (KIAA1309 and KIAA1354). This was later extended to a total of four proteins with the addition of BTBD1 and MEL-26 which were identified by yeast two-hybrid (Furukawa, He et al. 2003). In addition the authors expressed the BTB domains from a select number of proteins showing that, at least *in vitro*, Cul3 bound to 13 distinct BTB domain. Meanwhile, Xu et al. identified 11 proteins that bound to a Cul3 bait in a yeast two-hybrid screen all of which contained BTB domains which they confirmed by biochemistry (Xu, Wei et al. 2003).

This, plus a number of other studies have allowed us to understand the formation of the Cul3-BTB E3 ligase complex (reviewed in (Pintard, Willems et al. 2004). In the case of NRF2 regulation, KEAP1 recruits NRF2 to the Cul3-RING box protein 1 (Rbx1) complex (figure 1.8.). NRF2 is polyubiquitylated at several lysine residues within the Neh2 domain and between the two KEAP1 binding motifs (Cullinan, Gordan et al. 2004, Kobayashi, Kang et al. 2004, Furukawa and Xiong 2005). This targets NRF2 to the proteasome for degradation and keeps its half-life to less than 20 minutes (figure 1.8.) (Kobayashi, Kang et al. 2004). The level of reactive oxygen species in the cell regulates this process: the conformation of KEAP1 can be altered by modification at several reactive cysteine residues which

weakens its association with NRF2. This in turn, reduces NRF2 ubiquitylation and allows its translocation to the nucleus (figure 1.8.) (Kobayashi, Kang et al. 2004, Hayes, McMahon et al. 2010). In particular cysteine residues in the intervening region of KEAP1 between the BTB and DGR domains are modified by ROS and this may block the association with NRF2 (Dinkova-Kostova, Holtzclaw et al. 2002, Tong, Kobayashi et al. 2006).

1.5.5. Disruption of the KEAP1-NRF2 interaction

Other proteins can disrupt the binding of the Neh2 domain to KEAP1 and thereby regulate NRF2 activity. This occurs when proteins competitively bind to either NRF2 or KEAP1. For example, the cyclin-dependent kinase p21 knockout mouse also has reduced NRF2 levels (Chen, Sun et al. 2009). This p53-regulated protein, p21, can interact with the DLG motif of NRF2 via a basic tripeptide KRR region. By stabilising NRF2, increased p21 levels might therefore increase transcription of anti-apoptotic genes that are under ARE regulation (Chen, Sun et al. 2009). Moreover, p53 itself can be activated by oxidative stress thus potentially feeding in to this pathway (Han, Muller et al. 2008).

There are a number of important proteins that can interact with KEAP1. Phosphoglycerate mutase family member PGAM5 is a mitochondrial phosphatase, which interacts with KEAP1 via an ESGE motif that looks similar to the EGTE motif of NRF2 (Lo and Hannink 2006). Similarly p62/Sequestosome 1 (p62/SQSTM1) possesses an STGE motif, which facilitates its interaction with KEAP1 (Komatsu, Kurokawa et al. 2010). The potential role of KEAP1 interaction with these two proteins is discussed below however at the very least they might release NRF2 from its inhibition by KEAP1 allowing its translocation to the nucleus. Evidence for this comes from Atg7 knockout mice. Atg7 negatively regulates p62 and in the Atg7 knockout mice the levels of NRF2 activity are increased (Komatsu, Kurokawa et al. 2010). In itself, p62 is an NRF2 target gene and this might play a role in a positive feedback of this pathway (Jain, Lamark et al. 2010).

1.5.6. KEAP1 and autophagy

As described above, macroautophagy is a pathway controlling the degradation of long-lived proteins, damaged organelles and proteins that tend to form aggregates.

P62/SQSTM1 is able to act as a specific autophagy receptor through its interaction with both ubiquitylated proteins and LC3-II of the growing phagophore. In this way it triggers selective autophagy by bringing cargo to the autophagy machinery.

Under treatment with the oxidative stressor sodium arsenite, p62 forms aggregates. Following its aggregation, phosphorylation of p62 at Ser351 by mTORC1 increases the binding affinity of p62 for KEAP1 (Ichimura, Waguri et al. 2013). Over time Ichimura and colleagues saw a reduction in KEAP1 protein levels suggesting that the protein was first sequestered and then degraded by selective autophagy. This led to the persistent activation of NRF2. The authors identified this when they expressed a phospho-mimetic p62 mutant in human hepatocellular carcinoma cells and saw increased tumourigenesis in mouse xenografts. This effect was ablated by NRF2 depletion (Ichimura, Waguri et al. 2013).

In addition Taguchi et al. showed that KEAP1 accumulates in the livers of autophagy deficient mice (either p62 or Atg7 deficient) again suggesting that KEAP1 protein levels might be regulated by autophagy (Taguchi, Fujikawa et al. 2012).

1.5.7. KEAP1 and cell death

As already mentioned above, KEAP1 has been proposed to also bind to PGAM5 (Lo and Hannink 2006). PGAM5 is an outer mitochondrial membrane protein with phosphatase activity that binds the anti-apoptotic BCL-2 family member BCL-$_{XL}$ (Stepkowski and Kruszewski 2011). In addition to this, PGAM5 regulates the activity of apoptosis signal-regulated kinase 1 (ASK1) through its dephosphorylation of this protein (Takeda, Komuro et al. 2009). In

this way, PGAM5 is important in preventing apoptosis triggered by sustained activation of the JNK and p38 pathways, which activate ASK1 (Takeda, Komuro et al. 2009, Stepkowski and Kruszewski 2011).

Interestingly, it has been shown that KEAP1-deficient mouse embryonic fibroblasts (MEFs) are resistant to apoptosis induced by the ROS-inducer paraquat (Stepkowski and Kruszewski 2011). This was shown to be due to a failure to activate ASK1 in KEAP1-deficient MEFs (Niso-Santano, Gonzalez-Polo et al. 2010). A subsequent study has shown that PGAM5 stability is in fact regulated by its interaction with KEAP1 (Xu, Fang et al. 2013). KEAP1 can be recruited to mitochondrial membranes by PGAM5. It has been proposed that KEAP1 in turn can recuit the Cul3-Rbx1 complex and promote the polyubiquitylation and subsequent proteasomal degradation of PGAM5 (Lo and Hannink 2006).

Chapter two: Materials and methods

2.1. Mammalian cell culture

2.1.1. Cell culture inhibitors, reagents and antibodies

Unless otherwise stated, cell culture reagents, plasticware and chemicals were from Gibco (Invitrogen, UK), Corning Inc (New York, USA) and Sigma-Aldrich (Pool, UK) respectively. Inhibitors were obtained from the companies indicated in table 2.1. Inhibitors were diluted in dimethyl sulphoxide (DMSO) (Sigma-Aldrich) and stored at -20°C in aliquots. Prior to use the inhibitors were thawed and diluted in warm media at the concentrations indicated in table 2.1. Recombinant growth factors neuregulin (NRG1) and epidermal growth factor (EGF) were obtained from Peprotech (London, UK). Supplements for the growth of MCF10A cells were from Sigma-Aldrich. Antibodies for western blotting and immunofluorescence were purchased from the companies detailed in tables 2.2, 2.3, 2.4, 2.5 and 2.6 and used at the concentrations indicated. Tert-butyl hydroperoxide was purchased from Sigma Aldrich.

Inhibitor	Source	Catalogue number	Targets (IC50)	Concentration used
PI3K Pathway inhibitors				
Wortmannin	Sigma	W1628-1MG	Non-specific PI3K inhibitor (2-4nM)	100mM
LY294002	Calbiochem	440202	Non-specific PI3K inhibitor (1.4nM)	20μM
PI-103	Calbiochem	528100	p110α (4nM), p110β (8nM), p110δ (>500nM), DNA-PK (2nM), mTORC1 (20nM), mTORC2 (80nM)	100nM
GDC-0491	Axon	1377	p110α (3nM), p110β (33nM), p110δ (3nM), p110γ (75nM) DNA-PK (1230nM), mTOR (580nM)	1μM
MK2206	Selleck	S1078	AKT1 (8nM), AKT2 (12nM), AKT3 (65nM)	1μM
DUB inhibitors				
P22077	Merck Millipore	662142-25MG	USP7 (8.01μM), USP47 (8.74μM)	50μM
HBX-41108	R&D Systems	4285	USP7 (424nM)	1μM
HBX90,397	Hybrigenics		USP8 (0.559μM), USP7/USP5/UCHL1 (>100μM), UCHL2 (10μM)	1μM/2.5μM
HBX90,659	Hybrigenics		USP8 (0.850μM), USP7/USP5/UCHL1/UCHL3 (>100μM)	1μM/2.5μM

Table 2.1. Inhibitors used.

2.1.2. Cell culture

HeLa, MCF7, U2OS and HEK293T cells were cultured in 5% CO_2 at 37°C. Culture medium was Dulbecco's modified Eagle's medium (DMEM) +GlutaMAX supplemented with 10% fetal bovine serum (FBS) and 0.1mM minimum essential medium (MEM) non-essential amino acids (NEAA). The split ratio was 1:5-1:7, 1:3-1:5, 1:4-1:5 and 1:9-1:12 every 2-3 days for HeLa, MCF7, U2OS and HEK293T cells respectively. NCI-H1650 and NCI-H1975 cells (ATCC, LGC Standards Ltd, Middlesex, UK) were cultured in RPMI-1640 media plus L-Glutamine supplemented with 10% FBS. These cells were split 1:2-1:4 every 2-3 days. MCF10A parental and isogenic cell lines (Horizon Discovery, Cambridge, UK) were cultured in DMEM/F12 GlutaMAX supplemented with 5% horse serum (HS), EGF (20ng/ml), hydrocortisone (500ng/ml), insulin (0.01mg/ml) and cholera toxin (100ng/ml). The MCF10A cells were split at a ratio of 1:5-1:8 every 2-3 days. FKHRL1-U2OS cells (Thermo Scientific, Bioimage products, Lafayette, USA) stably expressing FKHRL1 (FOXO3) fused to the N-terminus of enhanced green fluorescent protein (EGFP) under a CMV promotor were cultured in DMEM +GlutaMAX supplemented with 0.5mg/ml Geneticin (G418). These cells were split at a ratio of 1:3-1:4 every 2-3 days. For maintenance, cells were dissociated from flasks or dishes with 0.05% trypsin with EDTA.

2.1.3. SiRNA transfection

SiRNA transfection used the siRNA oligomers in table 2.4. All siRNA was purchased from either Qiagen (Crawley, UK) or GE Dharmacon (Lafeyette, USA) as indicated. Transfection of cells either followed a forward, fast-forward or reverse protocol using Lipofectamine® RNAiMAX transfection reagent (Thermo Fisher Scientific, Massachusetts, USA). For the forward protocol, cells were seeded in 6 well plates, 6cm or 10cm dishes and transfected the following day following the RNAiMAX protocol. For 6 well plates siRNA to a final concentration of 10 or 40nM was combined with opti-MEM in a final volume of 180µl. Separately; 2µl RNAiMAX was combined with 18µl opti-MEM. Both mixtures were incubated at room temperature for 5-7min before they were mixed, incubated together for a further 20min and

added to the cells in a final volume of 1ml. This was scaled-up according to plate size.

For the fast-forward protocol, the cells were seeded 4-8 hours prior to transfection following the above protocol later that day. For the reverse protocol, cells were seeded directly on to a transfection reagent mixture. SiRNA was diluted in 200µl opti-MEM to a final concentration of 10 or 40nM. 2µl RNAiMAX was added to the mixture and the mixture incubated in the 6 well plate for 10-20 minutes at room temperature. Cells were seeded directly on to this mixture making a final volume of 1ml and a final siRNA concentration of 10 or 40nM.

2.1.4. DNA transfection

For transient DNA transfections, 1µg DNA was diluted in 150µl opti-MEM and mixed. 3µl of GeneJuice (Merck-Millipore, Nottingham, UK) was added to this mixture, mixed and incubated for 20 minutes at room temperature. This was added, dropwise, to cells that had been seeded up to 24 hours earlier. Cells were incubated with the transfection mixture for 24 to 72 hours.

2.1.5. Crystal violet staining

For staining of cells with crystal violet, cells in a 6 well plate were washed once with room temperature PBS and fixed with ice cold methanol for 10 minutes at -20°C. The cells were washed once more with PBS and stained with 1mg/ml crystal violet in methanol (Sigma-Aldrich, Poole, UK) for 30 minutes at room temperature. The cells were washed 4-5 times with ddH_2O and left to air-dry overnight before scanning on an Epson scanner.

Entrez gene ID	NCBI gene symbol	Name	Company	Type	Catalogue number	Sequence
9101	USP8	HS_USP8_1 (QI1)	QIAGEN	Flextube siRNA	S1000073014	CAGGGTCAATTCAATCTACA
9101	USP8	HS_USP8_2 (QI2)	QIAGEN	Flextube siRNA	S1000073024	AAGGCTCGTATTCATGCAGAA
9101	USP8	HS_USP8_3 (QI3)	QIAGEN	Flextube siRNA	S1000073031	CAGGTTCAGGCAAGCCATTTA
9101	USP8	HS_USP8_5 (QI4)	QIAGEN	Flextube siRNA	S103103604	GAGGATACAGACGATACCGAA
9101	USP8	Oligo 1 (Dh1)	Dharmacon	siGENOME	D-005203-02	TGAAATACGTGACTGTTTATT
9101	USP8	Oligo 2 (Dh2)	Dharmacon	siGENOME	D-005203-03	GGACAGGACAGTATAGATATT
54764	ZRANB1	TRABID/ ZRANB1 (TBD)	Dharmacon	ON TARGET plus	LQ-009270-00	GACCAAGGTGAATCTTC, GAAGTACGCTTGCTGAATC, CAACAAGCAGCAAAGTGTA, AGACCTAGTGGAACAATTA
23032	USP33	Oligo 6 (USP33)	Dharmacon	ON TARGET plus	J006081-06	GGGCATGTCTGGAGAATAGTT
N/A	N/A	Non-targeting 1 (NT1)	Dharmacon	ON TARGET plus	D-001810-01	TGGTTTACATGTGTTTTCTGA
N/A	N/A	Non-targeting 3 (NT3)	Dharmacon	ON TARGET plus	D-001810-03	TGGTTTACATGTCGACTAA
N/A	N/A	AllStars Negative Control (SiC)	QIAGEN	AllStars negative control	1027280	Not provided

Table 2. 2. SiRNA oligos used for RNA interference

2.1.6. Scepter cell count

Where indicated, cell counts were taken using a Scepter 2.0 (Merck-Millipore, Wisconsin, USA). For this cells were dissociated with trypsin and resuspended in full media as usual. 100µl of singularised cells were added to 900µl PBS and a cell count taken using 60µm tips (Merck-Millipore). To count only live cells the Scepter was set to count cells with a minimum of 12 and maximum of 30µm diameter.

2.2. Microscopy

2.2.1. Immunofluorescence staining

For immunofluorescence, cells were grown on glass coverslips. At the end of the experiment the coverslips were washed twice with PBS at room temperature and fixed with 4% paraformaldehyde in PBS for 15 minutes. Cells were washed twice more with PBS and the fixation quenched with 50mM ammonium chloride in PBS for 10 minutes followed by another two PBS washes. The cells were permeabilised with 0.2% Triton-X-100 in PBS for 4 minutes and blocked with 10% goat serum or 3% BSA depending on the antibody (table 2.5) for 30 minutes. The primary antibody was incubated with the coverslips for 20 minutes or 1 hour (table 2.5) and the coverslips washed three times with PBS. The secondary antibody was incubated with the coverslips for 20 minutes (table 2.6) and the coverslips washed three times with PBS, rinsed once with ddH_2O and mounted on slides with Mowiol plus 4',6-diamidino-2-phenylindole (DAPI) stain (Molecular Probes, Thermo Fisher Scientific, Massachusetts, USA). Where indicated DRAQ7 (0.3μM) and annexin V-AF350 (5μl per 1ml) (Molecular Probes and Thermo Fisher Scientific respectively) were used to visualize dead cells and apoptotic cells, respectively.

Target protein	Species	Source (catalogue number)	Blocking buffer	Incubation buffer	Dilution (time)
EEA-1	mouse	BD Transduction (610456)	10% goat serum	5% goat serum	1:500 (20m)
EEA-1	rabbit	Home-made (243)	10% goat serum	5% goat serum	1:2000 (20m)
EGFR	mouse	CRUK (R1)	10% goat serum	5% goat serum	1:1000 (20m)
USP8	rabbit	Sigma-Aldrich (HPA004869)	10% goat serum	5% goat serum	1:1000 (20m)

Table 2.3. Primary antibodies used for immunofluorescence.

Antibody	Catalogue number	Incubation buffer	Dilution (RT, 20m)
Donkey anti-rabbit AF488	A21206	5% goat serum	1:1000
Donkey anti-mouse AF488	A21202	5% goat serum	1:1000
Donkey anti-rabbit AF594	A21207	5% goat serum	1:500
Donkey anti-mouse AF594	A21203	5% goat serum	1:500

Table 2.4. Secondary antibodies used for immunofluorescence

2.2.3. Time-lapse microscopy

For time-lapse microscopy, cells in 6 well plates were kept in the presence of 5% CO_2 at 37°C for the duration of the experiment. Cells were imaged using a fully motorised NikonTi-E eclipse microscope (CFI Super Plan Fluor 20x/0.45 NA) at intervals as indicated per experiment. Movies were processed using NIS-Elements software (Nikon, Amsterdam, Netherlands) and FijiIsJustImageJ 1.48 or ImageJ 1.47v (NIH).

2.3. Protein biochemistry

2.3.1. Cell lysis

Cells were lysed as indicated with either RIPA (10mM Tris-HCl pH 7.5, 150mM NaCl, 1% w/v Triton X-100, 0.1% SDS, 1% sodium deoxycholate) or NP40 (0.5% NP40, 25mM Tris-HCl pH 7.5, 100mM NaCl, 50mM NaF) lysis buffer. In both cases the lysis buffers were supplemented with PhosSTOP phosphatase inhibitor cocktail tablets (Roche, Basel, Switzerland) and mammalian protease inhibitors (Sigma Aldrich, Poole, UK). Cells were rinsed twice with ice cold PBS on ice then incubated with 150µl (per 6 well plate well), 250µl (6cm dish) or 500µl (10cm dish) lysis buffer for 10minutes on ice. The lysate was centrifugated at 14,000rpm for 5 minutes at 4°C and the supernatant collected for protein assay.

2.3.2. Protein assay

Protein concentration was assessed using the Bio-Rad bicinchroninic acid (BCA) assay kit (#23225 Pierce, UK) according to the manufacturer's instructions using IgG as a standard.

2.3.4. SDS polyacrylamide electrophoresis (SDS-PAGE)

Concentration adjusted protein lysates were combined with a 1x final concentration sample buffer (3% w/v SDS, 62.5mM Tris-HCl pH 6.8. 10% glycerol, 3.2% β-mercaptoethanol stained with bromophenol blue), boiled for 5 minutes at 98°C and separated by SDS-PAGE. Samples were either loaded onto 4-12% precast Bis-Tris gradient gels (NuPAGE, Invitrogen, Paisley, UK) or 8% mini-gels poured using a Bio-Rad mini-protean® 3 system (BioRad, California, USA) (resolving gel (2 gels): 5.3ml protogel, 5.2ml protogel resolving, 9.2ml water, 15µl tetramethylethylenediamene (TEMED) (VWR, Lutterworth, UK), 150µl 10% APS (Sigma-Aldrich, Poole, UK); stacking gel (2 gels): 1.3ml protogel, 2.5ml protogel stacking, 6.1ml water, 50µl TEMED, 10µl 10% APS). Separation typically took place either at a constant voltage of 200V for 60min (NuPAGE) or 90V for 15min followed by 180V for 45min (BioRad). Samples were run alongside Broad Range marker (New England BioLabs, Herts, UK) and Rainbow Marker (GE Healthcare, Amersham, UK).

2.3.5. Western blotting

Separated proteins were transferred on to 0.45µm Protran nitrocellulose membrane (Geneflow, Lichfield, UK). The transfer was performed using the GenieBlotter system (Idea Scientific, Minneapolis, USA) at a constant current of 08-09A for one hour. Transferred proteins were visualised with Ponceau S (Sigma-Aldrich, Poole, UK) staining which was subsequently removed with PBS. The membrane was blocked with 0.5% Marvel TBS-T (20mM Tris, 137mM NaCl, pH 7.6, 0.1% Tween-20) or 0.5% Marvel PBS-T (1x PBS, 0.1% Tween-20) for one hour at room temperature or overnight at 4°C. Membranes were incubated with primary antibody as indicated below. Prior to the addition

of the secondary antibody (see table 2.3) membranes were washed three times with TBS-T or PBS-T for 15 minutes in total. The secondary antibody was added to the membrane for one hour and the membrane washed again two times with TBS-T/PBS-T and one time with TBS/PBS. Protein bands were visualised using the LI-COR Odyssey Classic scanner (LI-COR, Nebraska, USA).

Antibody	Catalogue number	Incubation buffer	Dilution (RT, 1h)
IRDye 800CW donkey anti-rabbit	926-32213	5% milk TBS-T/PBS-T	1:15,000
IRDye 800CW donkey anti-mouse	926-32212	5% milk TBS-T/PBS-T	1:15,000
IRDye 800CW donkey anti-goat	926-32214	5% milk TBS-T/PBS-T	1:15,000
IRDye 680LT donkey anti-rabbit	926-68023	5% milk TBS-T/PBS-T	1:15,000
IRDye 680LT donkey anti-mouse	926-68020	5% milk TBS-T/PBS-T	1:15,000
IRDye 680 donkey anti-goat	926-32224	5% milk TBS-T/PBS-T	1:15,000

Table 2.5. Secondary antibodies used for western blotting

2.4. Quantitative real-time polymersase chain reaction (qRT-PCR)

2.4.1. Reverse Transcription

mRNA was prepared from cell lysates harvested using the Qiagen RNeasy Kit following the manufacturer's guidelines. The RNA concentration was determined using a NanoDrop Spectrophotometer ND1000 at 260nm. 1µg RNA was diluted in sterile water to a final volume of 10µl. Poly(A) tails of the mRNA were primed by incubation of the solution with 1µl oligo dT primers at 70°C for 5 minutes. 8µl reaction buffer (4µl 5x reverse transcription buffer, 2µl PCR nucleotide mix, 0.5µl RNasin, 1.5µl nuclease free deionised water) was combined with the mRNA and oligo dT primer mix. The mixture was incubated for 5 minutes at 37°C before the addition of 1µl MuLV reverse transcriptase. The reaction was carried out at 42°C for 1 hour then 70°C for

10 minutes. The final reaction mixture was made up to 100µl with sterile water.

2.4.2. qRT-PCR

Reaction buffer (per single reaction: 5µl 2x SYBR Mastermix, 1.66µl water, 0.17µl 20µM forward primer, 0.17µl 20µM reverse primer) was prepared using iTaq^{TM} Universal SYBR® Green Supermix (Bio-Rad) according to the manufacturer's protocol. KEAP1 forward primer (5'-CAG ATT GGC TGT GTG GAG TT-3') and reverse primer (5'-GCT-GTT-CGC-AGT-CGT-ACT-TG-3') were gifts from Prof. Ian Copple (Universit of Liverpool). cDNA from 2.4.1. was diluted 3-fold before 3µl was added to the reaction buffer. qRT-PCR was performed using the CFX Connect^{TM} Real-Time PCR detection system (Bio-Rad). Denaturation took place at 95°C for 3 minutes. A two-step amplification protocol consisted of incubation at 95°C for 10 seconds followed by 60°C for 30 seconds for 40 cycles. The melt curve was analysed after 40 cycles. The Ct values for test genes were normalised to actin and relative expression represented as $2^{-[\Delta\Delta Ct]}$.

Target protein	Species	Source (catalogue number)	Blocking buffer	Incubation buffer	Dilution (temperature, time)
Actin	mouse	Abcam (ab6276)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:10,000 (RT, 1h)
AKT	rabbit	Cell Signaling (#9272)	5% Milk TBS-T/PBS-T	5% BSA TBS-T/PBS-T	1:1000 (4°C, o/n)
p-AKT	rabbit	Cell Signaling (#4060)	5% Milk TBS-T/PBS-T	5% BSA TBS-T/PBS-T	1:2000 (4°C, o/n)
EGFR	goat	Santa Cruz (SC03-1005)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:200 (4°C, o/n)
ErbB3	rabbit	Cell Signaling (#12708)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:1000 (4°C, o/n)
HRS	goat	Everest Biotech (EB07211)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:2000 (4°C, o/n)
HRS	rabbit	Home-made (864) (Sasche, Urbé et al. 2002)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:1000 (4°C, o/n)
HRS	rabbit	Bethyl (A300-989A)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:1000 (4°C, o/n)
MET	mouse	Cell Signaling (#3127)	5% Milk TBS-T/PBS-T	5% BSA TBS-T/PBS-T	1:1000 (4°C, o/n)
PARP p85	mouse	Cell Signaling (#9546)	5% Milk TBS-T/PBS-T	5% BSA TBS-T/PBS-T	1:2000 (4°C, o/n)
USP33	mouse	Sigma-Aldrich (WHO002032M1)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:1000 (4°C, o/n)
USP8	rabbit	Sigma-Aldrich (HPA004869)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:1000 (4°C, o/n)
USP8	rabbit	Bethyl (A300-929A)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:2000 (4°C, o/n)
USP8	sheep	R&D Systems (AF7735)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:500 (RT, 2h)
STAM2	rabbit	Home-made (198) (Row, Clague et al. 2005)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:1000 (RT, 1h)
ERK	rabbit	Cell Signaling (#4695)	5% Milk TBS-T/PBS-T	5% BSA TBS-T/PBS-T	1:1000 (4°C, o/n)
p-ERK	rabbit	Cell Signaling (#4370)	5% Milk TBS-T/PBS-T	5% BSA TBS-T/PBS-T	1:2000 (4°C, o/n)
p-MEK	rabbit	Cell Signaling (#9154)	5% Milk TBS-T/PBS-T	5% BSA TBS-T/PBS-T	1:1000 (4°C, o/n)
p-MEK1/2	rabbit	Cell Signaling (#9121)	5% Milk TBS-T/PBS-T	5% BSA TBS-T/PBS-T	1:1000 (4°C, o/n)
NRF2	rabbit	Abcam (ab62352)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:500 (4°C, o/n)
KEAP1	mouse	Abcam (ab119403)	5% Milk TBS-T/PBS-T	5% Milk TBS-T/PBS-T	1:400 (4°C, o/n)

Table 2.6. Primary antibodies used for western blotting

2.5. FACs analysis

The media from adherent cells was collected and cells were dissociated from dishes with 0.05% trypsin EDTA. All media and trypsin was retained. The collected cells, media and trypsin were were centrifugated at 2000rpm for 3 minutes. The cell pellet was resuspended in 1ml Annexin V buffer (Ca2+/Mg2+ rich) with Annexin-V-FITC (1:20,000 dilution) and incubated for 5-8 minutes. 5µl propidium iodide (50µg/ml) was added and incubated for 2 minutes. The FITC and PI staining was measured using an Attune® NxT Acoustic Focusing Cytometer. All reagents were gifts from Prof. Gerald Cohen and Dr Shankar Varadarajan (University of Liverpool).

Chapter three: The role of the PI3K pathway in regulating USP8

USP8 is well characterised as an important DUB that regulates the trafficking of growth factor receptors to the lysosome (Clague and Urbe 2006). Thus by regulating the levels of these receptors this DUB also influences the subsequent outputs of these important signalling molecules. Endocytic trafficking of growth factor receptors does not only promote their downregulation, there is also evidence that internalised receptors might signal en route through the trafficking system (Villasenor, Kalaidzidis et al. 2016).

In addition to this indirect effect on signalling, there is increasing evidence that USP8 may also interact with downstream effectors of these growth factor receptors adding an extra layer of regulation.

Interestingly, a number of studies report direct or indirect interactions between the PtdIns-3 kinase (PI3K) -AKT signalling pathway and USP8 with a particular focus on a purported interaction between USP8 and p-AKT (Cao, Wu et al. 2007, Cai, Crotty et al. 2010, Panner, Crane et al. 2010). In particular, these studies show that changes in p-AKT levels correlate with changes in USP8 levels. It has been suggested that USP8 is, in fact, a direct substrate of activated AKT and that USP8 phosphorylation (either by AKT, or other kinases) regulates the stability, subcellular localisation and activity of this DUB (Ballif, Cao et al. 2006, Cao, Wu et al. 2007, Mizuno, Kitamura et al. 2007, Cai, Crotty et al. 2010, Panner, Crane et al. 2010, Meijer, Kerperien et al. 2013). However, currently the data linking USP8 to the PI3K-AKT signalling pathway are contradictory. In 2007 Cao and colleagues reported that stimulation of the PI3K pathway using NRG1 correlated with an increase in USP8 stability (Cao, Wu et al. 2007). This was complemented by data using PI3K pathway inhibition, which showed a decrease in AKT was associated with a decrease in USP8 (Cai, Crotty et al. 2010). However a study in transformed mouse astrocytes and human glioblastoma xenografts indicated an inverse correlation between AKT activation and USP8 levels

(Panner, Crane et al. 2010). In this chapter, my aim was to investigate this discrepancy in the literature and to understand the role, if any, for the PI3K pathway in regulating USP8.

In order to further investigate this proposed interaction between USP8 and the PI3K pathway I assembled a toolkit of reagents and cell lines to use in this chapter. First I used EGF and neuregulin to activate EGFR and ERBB3 in two cell lines. HeLa cells had been used in a previous investigation in our lab assessing the role of USP8 in EGFR degradation and these MCF7 cells, together with HeLa cells had been employed in some of the studies mentioned above (Row, Prior et al. 2006, Cao, Wu et al. 2007). Neuregulin is a particularly potent activator of the PI3K pathway by activating the ERBB3-ERBB2 heterodimer. ERBB2 is an orphan receptor but can heterodimerise with ERBB3 which has a particular propensity for recruiting PI3K due to a large number of PI3K recognition sites within its intracellular domain (Citri, Skaria et al. 2003).

In order to examine the role of PI3K specifically I made use of a panel of isogenic MCF10A cell lines from Horizon Discovery, comparing parental cells to their PI3K mutant counterparts. Additionally, my toolkit comprised inhibitors of the PI3K pathway (LY294002, Wortmannin, PI-103 and GDC-0491) as well as the AKT inhibitor MK-2206.

3.1. Objective

The objective of this chapter was to investigate whether the PI3K pathway exerts an influence on USP8 levels, distribution or activity.

3.2. Results

3.2.1. USP8 levels do not change in response to RTK activation

Following on from previous reports suggesting that activation of AKT leads to an increase in the levels of USP8, I investigated the activation response of both HeLa and MCF7 cells to EGF or NRG1 stimulation, both of which activate AKT. I first stimulated serum-starved HeLa cells with EGF and observed a characteristic peak in p-Ser473-AKT levels following 5 minutes of stimulation, which drops down to basal levels by 180 minutes (figure 3.1.A). A similar AKT activation profile is evident in NRG1-stimulated cells (figure 3.1.A). Despite the clear activation of AKT, the levels of USP8 remain unchanged with either growth factor throughout the time-course.

In MCF7 cells the AKT activation profile in response to EGF looks very similar to that observed in the HeLa cells (figure 3.1.B). Interestingly, with NRG1 stimulation the AKT activation is more sustained in these cells, peaking at 5 minutes as for EGF but retaining activation for the remainder of the 6 hour time-course. Importantly, neither the EGF nor the sustained NRG1 activation of this pathway triggers a change in USP8 levels (figure 3.1.B).

3.2.2. USP8 levels do not change with constitutive PI3K activation with or without EGFR activation

I next looked for a different way to activate AKT that would be independent of growth factor receptor activation. Mutation of histidine 1047 to an arginine renders the PI3K enzyme constitutively active (Chakrabarty, Rexer et al. 2010). I used isogenic MCF10A cell lines in which this H1047R mutation has been introduced in one allele of the endogenous gene and compared their p-Ser473-AKT and USP8 levels with those of parental MCF10A cells. The standard growth medium for MCF10A cells contains a large number of supplements including cholera toxin, hydrocortisone, insulin, and importantly EGF. Parental cells show decreased p-Ser473-AKT levels when EGF is

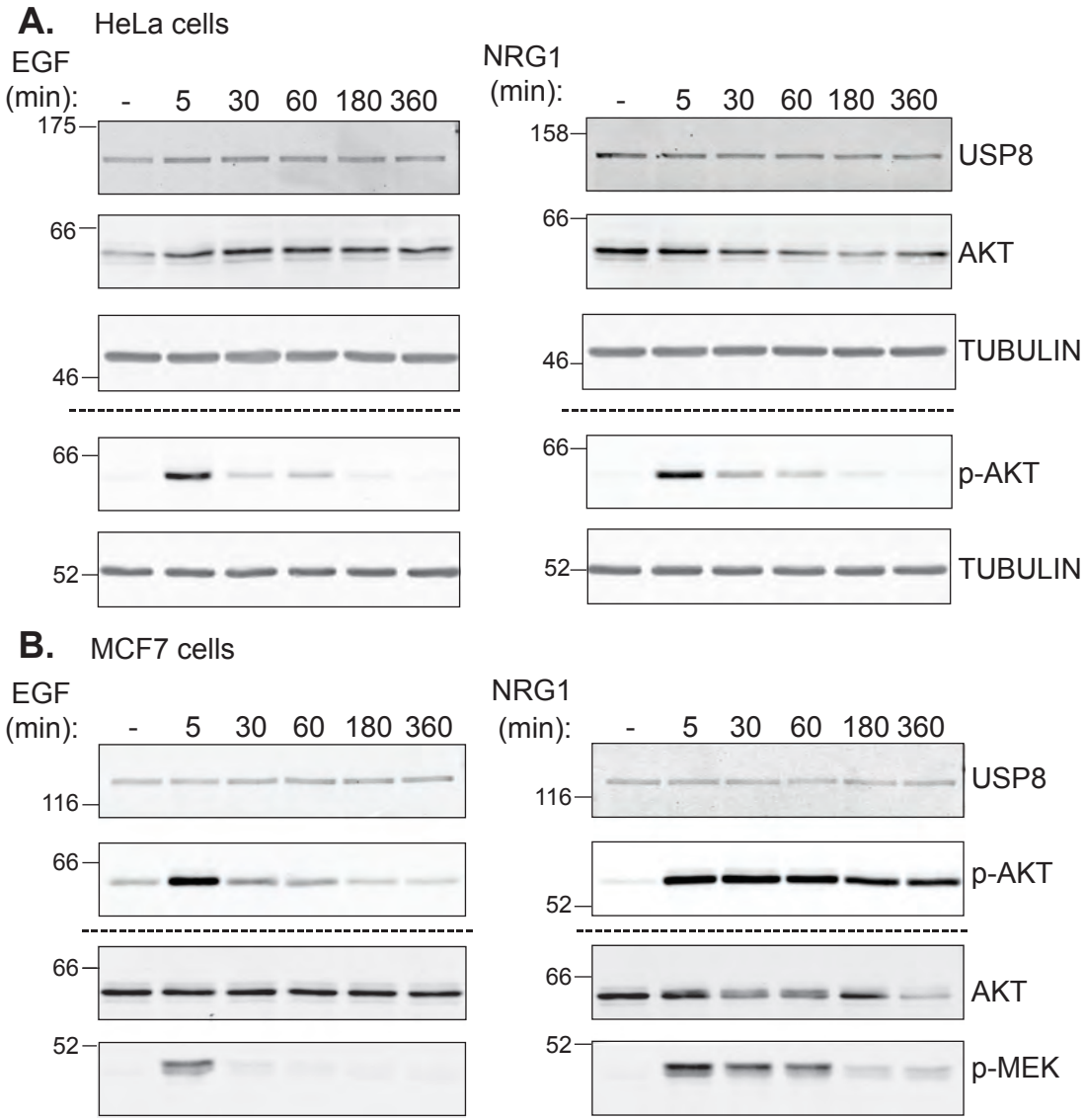


Figure 3.1. USP8 levels do not change with RTK activation. (A) HeLa cells were starved for 18h before stimulation with 20ng/ml EGF or 50nM NRG1 over a 6h time course followed by RIPA lysis at the indicated time points (n=1). Lysates were separated by SDS-PAGE and western blots probed with the indicated antibodies. (B) MCF7 cells were starved for 18h then stimulated with EGF or NRG1 for up to 6h (n=1). RIPA lysates were separated by SDS-PAGE and western blots probedwith the indicated antibodies. Dotted lines separate individual western blots.

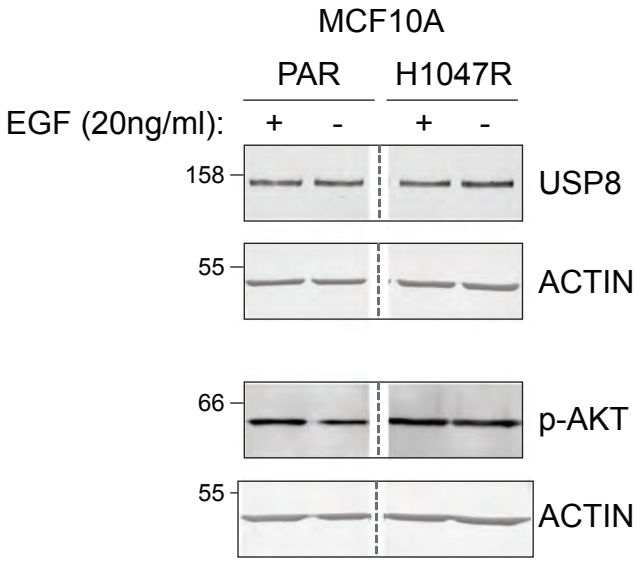


Figure 3.2. USP8 levels do not change in response to constitutive PI3K activation with or without RTK activation. Parental (PAR) or PI3K mutant (H1047R) isogenic MCF10A cells were grown to confluence in media with or without EGF. Cells were lysed in RIPA buffer, samples separated by SDS-PAGE and probed with the indicated antibodies. PAR and H1047R samples were run on the same gels (n=1).

withdrawn from their culture medium for 6 hours. In contrast to the parental cells, PI3K mutant cells retain high levels of p-Ser473-AKT independently of the presence of EGF in the medium. There is no change in USP8 level when comparing the two cell lines to one another, nor is there a change in USP8 level when comparing plus or minus EGF conditions within each cell line.

3.2.3. USP8 levels are not affected by inhibitors of the PI3K pathway

Inhibition of the PI3K pathway or AKT directly should likewise be informative on the potential regulation of USP8 by this pathway. Acute inhibition (30 minutes) of the PI3K pathway with LY294002, PI-103 or Wortmannin should result in reduced AKT activitation. I used these inhibitors plus an allosteric inhibitor of AKT (figure 3.3.A). The LY294002 inhibitor is a reversible, ATP competitive inhibitor of a number of kinases (McNamara and Degterev 2011). As well as PI3K family members, at doses of over 10μM, it also inhibits mTOR, one of the two activating kinases of AKT, DNA-PK and other, unrelated proteins including a number of channels. Following 24 hour treatment the LY294002 clearly decreased p-Ser473-AKT levels in HeLa cells, whilst Wortmannin only had a very small effect. The AKT inhibitor MK-2206 most strikingly abolishes AKT activation. However USP8 levels were not affected by either PI3K or AKT inhibition (figure 3.3.B).

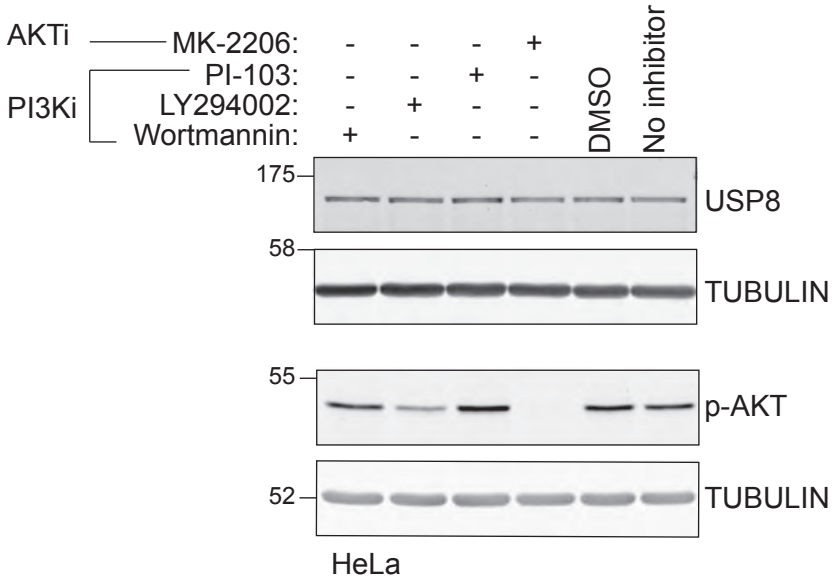
I next conducted a time-course experiment in both HeLa (left) and MCF7 (right) cells (figure 3.3.C). The cells were treated for up to 24 hours with LY294002 the only PI3K inhibitor that markedly affected AKT levels in panel B. Strong inhibition of AKT activity is obvious up to 8 hours in HeLa cells and 24 hours in MCF7 cells. In HeLa cells, basal p-Ser473-AKT levels appear unaffected in the 24 hour treatment sample although this is unlikely due to instability of the drug given the successful p-Ser473-AKT reduction in MCF7 cells.

In neither HeLa cells nor MCF7 cells does prolonged inhibition of PI3K decrease the levels of USP8.

A.

Inhibitor	Main targets (reported IC50)
PI-103	p110α (4nM), p110β (8nM), p110δ (>500nM), DNA-PK (2nM), mTORC1 (20nM), mTORC2 (80nM)
LY294002	Non-specific PI3K (1.4μM)
Wortmannin	Non-specific PI3K (2-4nM)
GDC-0491	p110α (3nM), p110β (33nM), p110δ (3nM), p110γ (75nM), DNA-PK (1230nM), mTOR (580nM)
MK-2206	AKT1, AKT2, AKT3 (8, 12 and 65nM respectively)

B.



C.

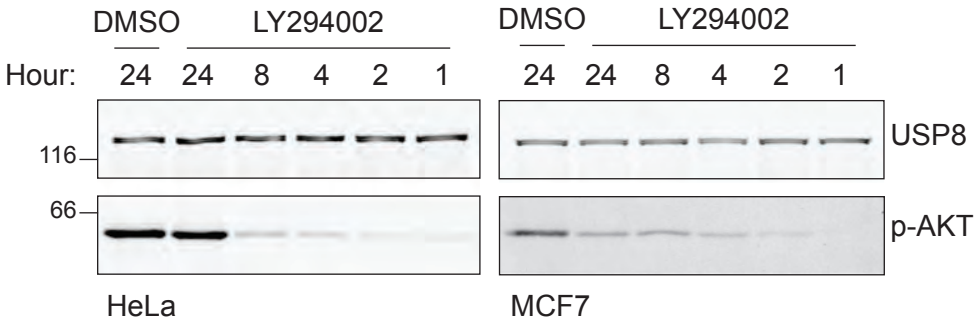


Figure 3.3. USP8 levels do not change with PI3K pathway inhibition. (A) PI3K pathway inhibitors used. (B) HeLa cells were treated with the PI3K inhibitors PI-103 (100nM), LY294002 (20μM) or Wortmannin (100nM) or the AKT inhibitor MK-2206 (1μM) or DMSO for 24h prior to RIPA lysis. Lysates were separated by SDS-PAGE and western blots probed for the indicated proteins (n=1). Tublin acts as a loading control for each separate western blot. (C) HeLa or MCF7 cells were treated with LY294002 for up to 24h. Lysates were separated by SDS-PAGE, transfered to nitrocellulose membrane and probed for USP8 and p-AKT (n=1).

3.2.4. USP8 distribution does not change upon PI3K pathway inhibition

My data suggest that USP8 levels do not change upon PI3K pathway manipulation. However, it is possible that the distribution of this DUB is affected by the PI3K pathway. Following activation, EGFR is internalised to endosomes where it co-localises with USP8. Co-localisation of overexpressed GFP-USP8 with EGFR can be seen in HeLa cells stimulated for 10 minutes with EGF (figure 3.4). Pre-treating cells for 10 minutes prior to addition of EGF (total treatment with inhibitors: 20 minutes) with PI3K or AKT inhibitors, LY294002 or MK-2206 respectively, did not alter this distribution and USP8 remained co-localised with EGFR at punctate structures within the stimulated cells.

3.2.5. The availability of the USP8 catalytic site is not affected by EGF stimulation

In order to assess activity changes in USP8 with EGF stimulation I used a DUB-activity or suicide inhibitor probe, ubiquitin-VME (Ub-VME) which binds irreversibly to the active site in DUBs that have a catalytic cysteine (McGouran, Gaertner et al. 2013). MCF7 cells were pre-treated with EGF for 10 minutes, then lysed and extracted proteins were incubated with increasing concentrations of the probe for 15 minutes at 37°C (figure 3.5). Binding of the probe to the active site of the DUB results in an approximate 8kDa shift in the apparent molecular weight of that DUB (figure 3.5.A). Probing samples separated by SDS-PAGE with a USP8 antibody thus allowed me to compare any changes in the ability of USP8 to react with this probe with and without EGF stimulation. Incubation with 10 and 20ng/ml Ub-VME results in an upshift of approximately 20 and 55% respectively of the USP8 band (figure 3.5.B) Pre-treatment of cells with EGF does not appear to alter the proportion of USP8 that is able to interact with the probe. Thus I was unable to detect any changes in USP8 activity upon EGF stimulation.

The ubiquitin-VME probe is conjugated to a small HA tag which allows for identification of global changes in reactive proteins by western blot analysis.

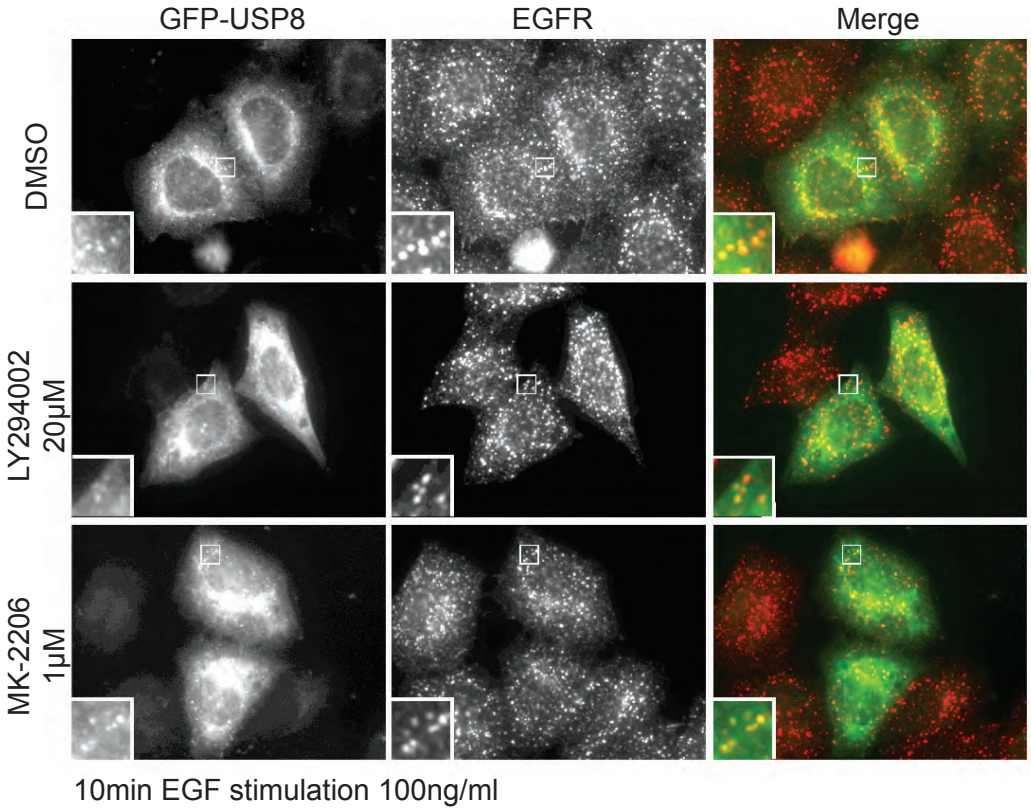


Figure 3.4. USP8 punctate co-localisation does not change with PI3K pathway inhibition. HeLa cells grown on coverslips were transfected with GFP-USP8 and starved for 18h, 24h after transfection. Cells were pre-treated with the PI3K inhibitor LY294002 (20μM), the AKT inhibitor MK-2206 (1μM) or DMSO for 30min prior to stimulation with EGF (100ng/ml) for 10min. Cells were fixed with 4% PFA in PBS, stained with EGFR antibody and AlexaFluor 594 coupled secondary antibody and imaged on a Nikon Eclipse TiE microscope (n=1).

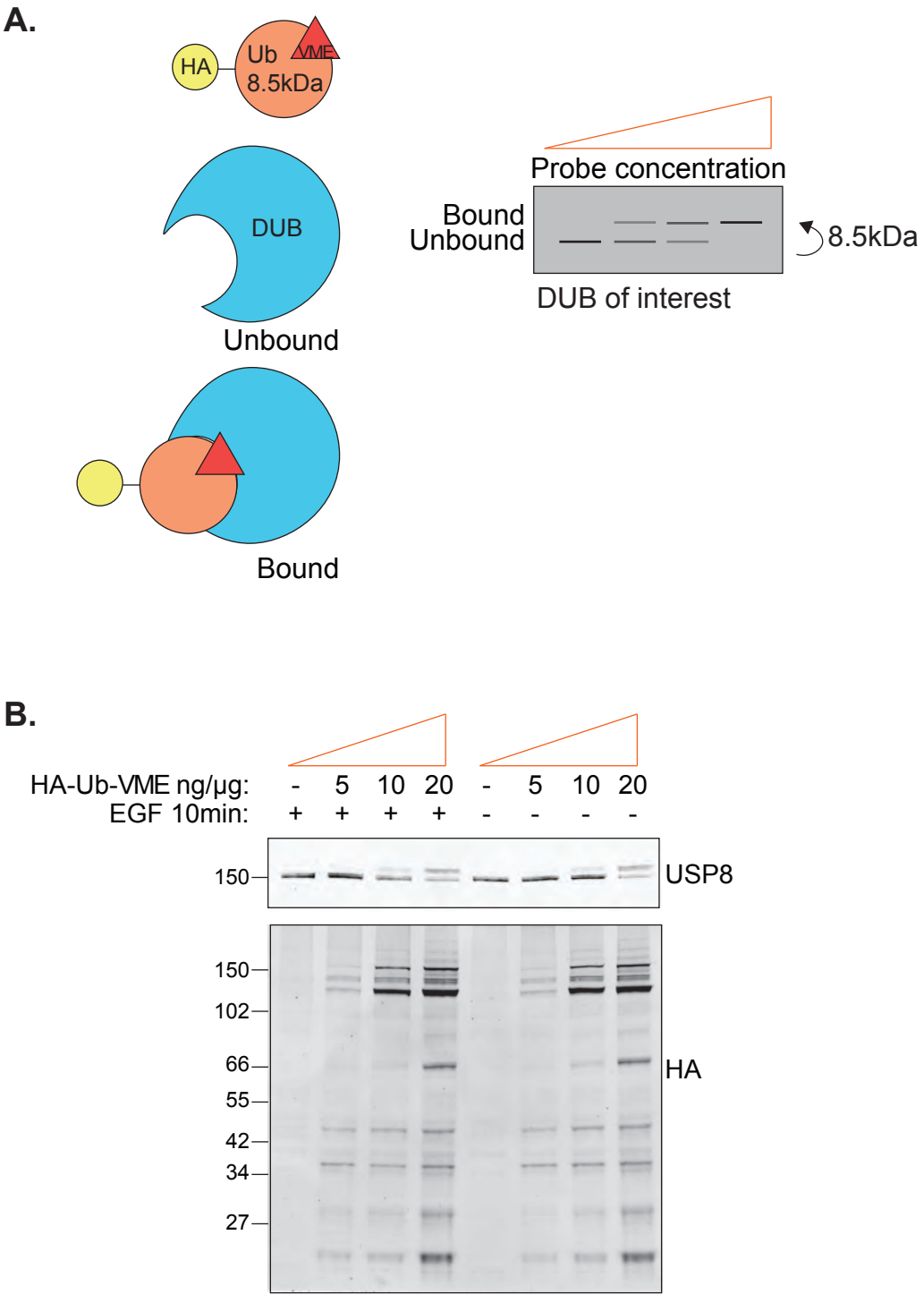


Figure 3.5. The availability of the USP8 active site does not change with EGF stimulation. (A) Ubiquitin active site probe consists of a vinyl methyl ester (VME) warhead and HA tag. The irreversible reaction of Ubiquitin-VME with a DUB results in a covalently linked complex that is detectable by an 8.5kDa upshift in a western blot. (B) MCF7 cells were stimulated with EGF at a concentration of 20ng/ml for 10min prior to their lysis and incubation at 37°C for 15min. Samples were separated by SDS-PAGE and western blots probed with HA or USP8 antibodies (n=1).

This would not include all DUBs, only those with a reactive cysteine as described above. In addition to deubiquitylases the Ub-VME probe also binds to a number of ubiquitin conjugating enzymes as E1, E2 and some E3 proteins also possess reactive cysteines and ubiquitin binding domains. Based on the banding pattern seen in the HA blot, I did not detect any major changes in global Ub-VME probe binding upon 10 minutes of EGF stimulation (figure 3.5.B).

3.2.6. Synthetic lethality of PI3K inhibition and USP8 depletion

Joe Sacco, a previous student in our lab, showed that USP8-depleted U2OS cells expressing GFP-tagged FOXO3 (referred to hereafter as FKHRL1-U2OS cells) die when they are treated with the PI3K inhibitor PI-103 (Sacco 2012). My data did not support an effect on USP8 levels, distribution or activity by the PI3K pathway, however Joe's finding alludes to some functional interplay between USP8 and this pathway.

In order to revisit this finding, I first depleted FKHRL1-U2OS cells of USP8 and treated the cells with the more specific PI3K inhibitor, GDC-0491, for 6 hours. Two out of the three USP8 siRNA oligos showed an increase in cell death as identified by morphological changes in the cells when combined with PI3K inhibition (figure 3.6).

Examining this further, I used PARP cleavage (PARP p85) as a marker for apoptosis by western blotting. In addition to the FKHRL1-U2OS cells I also analysed wild-type U2OS cells. FKHRL1, also called FOXO3, is a pro-apoptotic transcription factor that regulates expression of proteins involved in apoptosis and the cell cycle including p130, p21 and TRAIL. Under basal conditions FOXO3 shuttles between the cytosol and the nucleus however PI3K pathway inhibition promotes the translocation of this transcription factor to the nucleus. Here it can trigger the expression of genes important in apoptosis. The overexpression of this pro-apoptotic protein in the FKHRL1-U2OS cells might sensitise them to cell death. To account for this I analysed the wild-type U2OS cells.

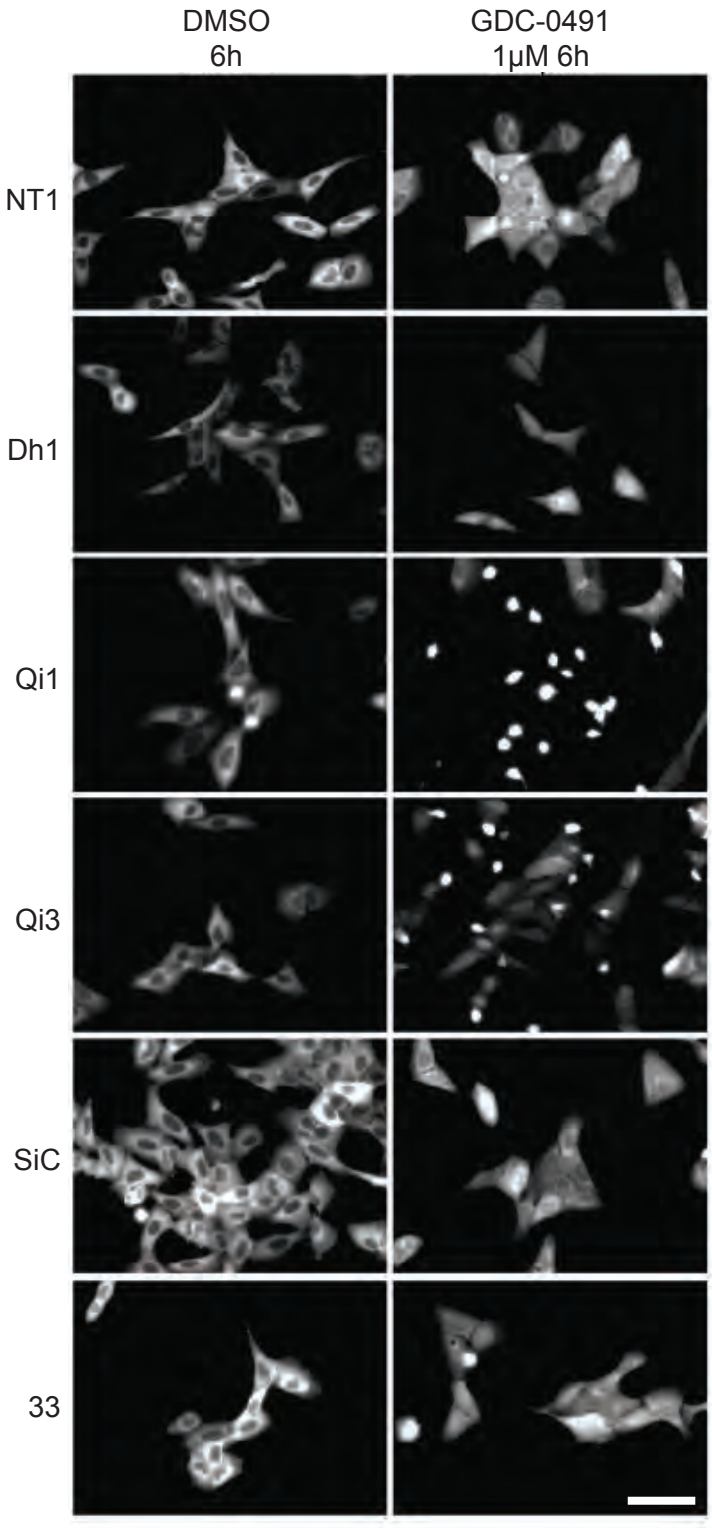


Figure 3.6. USP8 depleted cells die when subjected to PI3K inhibition. U2OS cells stably expressing GFP-FOXO3 cells were transfected with USP8 siRNA (Dh1, Qi1 or Qi3), non-targeting controls (NT1 or SiC) or an siRNA targeting the unrelated DUB USP33 (33). The cells were seeded onto coverslips. 48 hours post-transfection the cells were treated with 1μM GDC-0491 or DMSO for 6h, fixed with PFA and imaged with brightfield using a Nikon TiEclipse microscope (20x objective) (n=1). Scale bar is 400μm in width.

Both FKHRL1-U2OS and U2OS cells exhibited increased cell death when depleted of USP8 for 48 hours followed by 6-8 hour treatment with 1μM GDC-0491 compared to DMSO (figure 3.7.A and 3.7.B respectively).

Data from Cai et al suggest that the non-small cell lung cancer cell line NCI-H1650 (H1650) may be sensitive to changes in USP8 levels (Cai, Crotty et al. 2010). In their study considering the role of DGKδ in regulating USP8 stability (described in more detail below), the authors found that a reduction of USP8 levels as a result of DGKδ depletion caused a decrease in cell viability of this cell line. Therefore I also examined H1650 cells and another NSCLC cell line NCI-H1975 (H1975). Both H1650 and H1975 cell lines showed increased PARP p85 upon 48 hour USP8 depletion combined with 6-8 hour PI3K pathway inhibition by GDC-0491 treatment at a concentration of 1μM (figure 3.7.B). In the H1975, and indeed in both the FKHRL1-U2OS and U2OS cells, this effect was most evident with the Qi2 and Qi3 siRNA oligos whereas the H1650 cells only exhibited increased PARP p85 upon treatment with Qi3 siUSP8. The Dh1 oligo had little to no effect and was not used with the FKHRL1-U2OS cells in figure 3.7.A.

3.3. Summary of results

- USP8 levels are stable in HeLa and MCF7 with an activated or inhibited PI3K pathway
- Constitutive activation of PI3K in MCF10A isogenic cells does not affect USP8 protein stability
- Overexpressed wild-type USP8 is not redistributed upon inhibition of the PI3K pathway
- The availability of the USP8 active site is not altered by activation of the PI3K pathway through EGF stimulation
- USP8-depleted U2OS, FKHRL1-U2OS and H1975 cells undergo apoptotic cell death with PI3K pathway inhibition

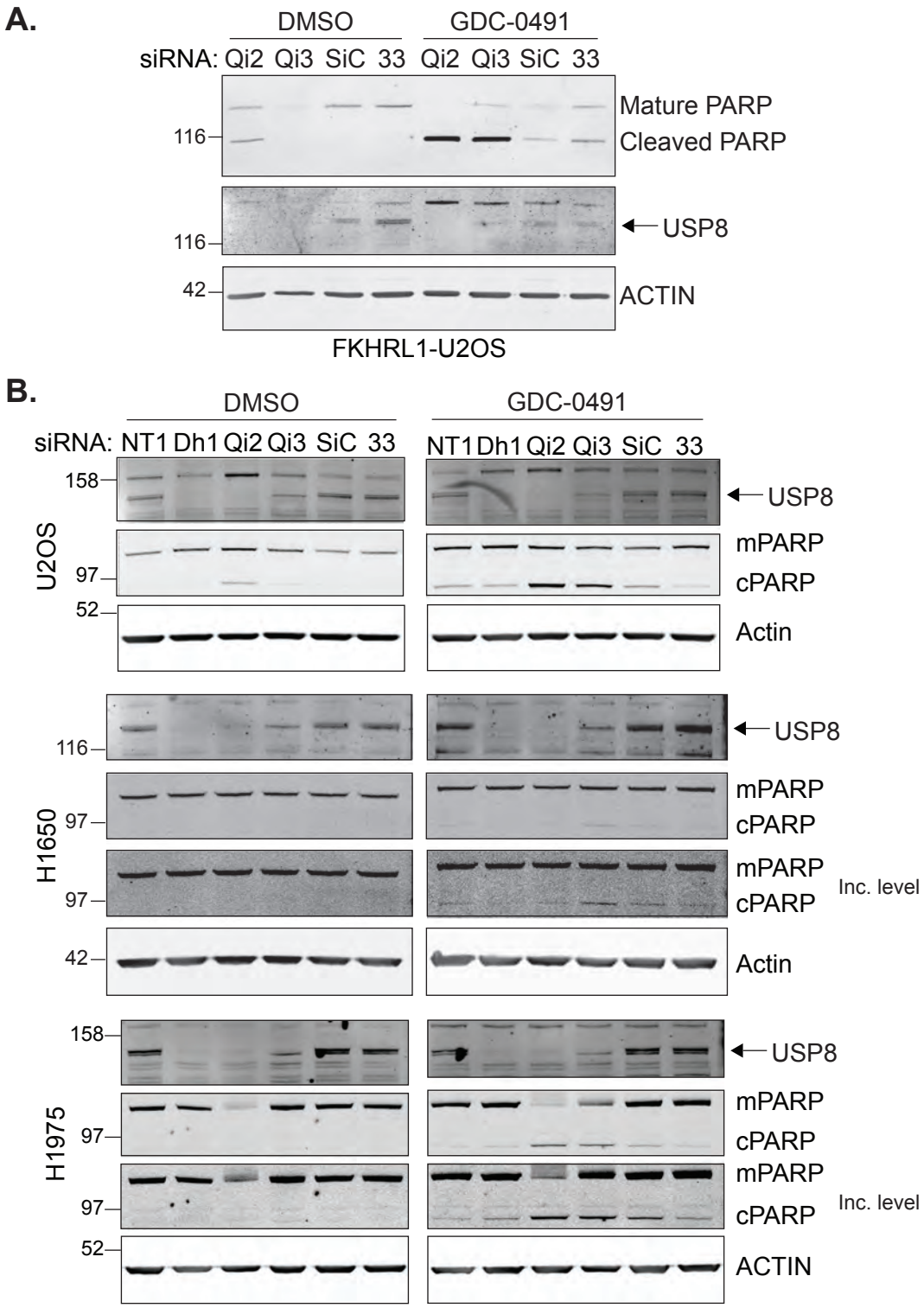


Figure 3.7. USP8 depleted cells die by apoptosis when treated with the PI3K pathway inhibitor GDC-0491. (A) FKHRL1-U2OS cells were depleted of USP8 for 48h prior to treatment with 1μM GDC-0491 or DMSO for 6-8h. NP40 lysates were separated by SDS-PAGE and western blots probed with the antibodies indicated (n=1). Actin is representative for each of the above blots. (B) U2OS, H1650 or H1975 cells were depleted of USP8 for 48h prior to treatment with 1μM GDC-0491 or DMSO for 6-8h. NP40 lysates were separated by SDS-PAGE and western blots probed as indicated (n=1). The same images were relevelled at a higher intensity (inc. level) where indicated. mPARP: mature PARP, cPARP: cleaved PARP. Actin is representative for each of the above blots.

3.4. Discussion

The aim of this chapter was to investigate the relationship between USP8 and the PI3K-AKT signalling pathway. A number of studies described below report changes in USP8 levels linked to AKT activation (Cao, Wu et al. 2007, Cai, Crotty et al. 2010, Panner, Crane et al. 2010). One study focused on the role of DGKδ in regulating p-AKT levels and the subsequent effect on USP8 (Cai, Crotty et al. 2010). DGKδ metabolises diacylglycerol (DAG), a second messenger generated by phospholipase C enzymes, which can be activated by EGFR activation (Crotty, Nakano et al. 2013). DAG recruits and activates PKC, which in turn increases activated AKT levels.

The authors found that DGKδ deficiency in HeLa cells led to increased ubiquitylation of EGFR, which in turn led to decreased steady state levels and increased ligand-induced EGFR degradation. This was not due to changes in levels or activity of the EGFR E3 ligase Cbl. Nor was it due to changes in the interaction between Cbl and EGFR. Instead the authors reported that USP8 levels were reduced both by DGKδ depletion and PI3K pathway inhibition using the LY294002 inhibitor. This correlated with reduced p-Ser473-AKT levels and reduced threonine phosphorylated USP8 levels.

Conversely, Panner et al. found an inverse correlation between AKT activation and USP8 levels in transformed mouse astrocytes and human glioblastoma xenografts (Panner, Crane et al. 2010). They reported that knock-out of PTEN, which led to increased activated AKT levels, results in a decrease in USP8 levels. They also showed that AKT inhibition increased USP8 levels.

Whilst both papers described above considered USP8 regulation in the context of EGFR signalling, Cao et al. looked at its regulation upon activation of other EGFR family receptors which do not rely on Cbl ubiquitylation for their ligand-induced degradation (Cao, Wu et al. 2007). Using MCF7 cells the authors report that ERBB3 activation by NRG1 leads to increased levels of activated AKT, which results in phosphorylation of USP8 and its subsequent stabilisation.

Here we thus have contradictory studies, reporting either a positive or a negative correlation between AKT activation and USP8 levels respectively. These papers used a variety of ways to activate or inhibit the PI3K pathway or, indeed, inhibit AKT directly. In my experiments I found no evidence for changes in USP8 levels in response to EGF or NRG1 stimulation, nor upon PI3K nor AKT inhibition in HeLa or MCF7 cells, nor upon stable expression of constitutively active PI3K in MCF10A cells. I did not observe any obvious changes in USP8 distribution or co-localisation with EGFR, nor did I find any evidence for changes in USP8 activity, based on its ability to bind DUB-activity probes.

However, the ubiquitin-VME probe assay may not be suitable to pick up subtle changes in DUB activity. The probe relies on the availability of the active site, which in many but not all DUBs is readily accessible. One exception is USP7, which requires a ubiquitin binding mediated conformational change for its activation, which can be further stabilised by interaction with GMP-synthase. The conformational change brings the catalytic triad of USP7 into an organisation that allows its enzymatic activity (Faesen, Dirac et al. 2011).

The crystal structure of an apo-form of USP8 has been solved which shows that this DUB has the correctly aligned organisation of the catalytic triad however there is a subdomain which seems to block access to this active site (Avvakumov, Walker et al. 2006). Thus in principle, it would be possible that phosphorylation for example by AKT could in principle assist the binding of ubiquitin to USP8. A ubiquitin bound form of the DUB is yet to be crystallised.

However not all DUBs require conformational changes for their activity and therefore bind the probe with or without additional modifications (McGouran, Gaertner et al. 2013). As such this probe may be better able to detect DUB abundance rather than activity.

Other assays that may be more informative are direct *in vitro* activity measurements using, for example, ubiquitin-AMC. Here a ubiquitin peptide is conjugated to a fluorophore which only fluoresces once the ubiquitin moiety has been cleaved off. Similarly mass spectrometry quantitation of conversion

of di-ubiquitin to mono-ubiquitin can be used to monitor DUB activity (Ritorto, Ewan et al. 2014).

There is only one paper, which suggests USP8 is phosphorylated by AKT. Cao and colleagues found phosphorylation of mouse USP8 at Thr907, which fits within the AKT recognition sequence RXRXXS/T (although this is not the case for the equivalent residue in human USP8). This phosphorylation was seen upon NRG1 stimulation when both proteins were overexpressed in HEK293Ts. The levels of pThr on immunoprecipitated USP8 were increased in cells depleted of DGKδ (which leads to increased AKT activation) (Cao, Wu et al. 2007).

In addition to the studies described above, a yeast two-hybrid screen from Hybrigenics suggests a direct interaction between USP8 and AKT (Daviet and Colland 2008). In addition to this, the same Cao et al. study described above showed co-immunoprecipitation of overexpressed USP8 and AKT in HEK293T cells. I therefore undertook preliminary co-immunoprecipitation experiments using overexpressed GFP-USP8 and myc-AKT, which did not provide evidence for an interaction between USP8 and AKT.

There are known ubiquitylation sites within the AKT protein. For example the E3 ligase TRAF-6 is believed to ubiquitylate AKT within its PH domain which is important for its subsequent activation and potentially its recruitment to the cell surface (Yang, Wang et al. 2009). Ubiquitylation of AKT by TRAF6 has been shown to be via K63-linked chains and its deubiquitylation has been assigned to the linear- and K63-chain specific CYLD (Lim, Jono et al. 2012). It is nevertheless conceivable that USP8 also contributes to AKT regulation rather than AKT modulating USP8 levels as proposed by the studies described above.

In contrast to the negative results concerning effects of PI3K and AKT modulation on USP8 protein levels, I did find evidence for synthetic lethality combining PI3K pathway inhibition and USP8 depletion. Additionally, Cai et al. found that the NSCLC cell line H1650 has reduced cell viability when both USP8 and p-AKT levels are reduced by DGKδ depletion. Elsewhere in the literature there is evidence that USP8 depletion sensitises some cancer cell

lines to cell death (Byun, Lee et al. 2013). This will be discussed further in both chapters four and five.

Chapter four: The role of USP8 in non-small cell lung cancer (NSCLC) cell viability

In chapter three I discussed my work on the potential role for USP8 in EGFR family signalling through the PI3K pathway in cervical HeLa and breast MCF7 cancer cells. In the meantime, a new study reported a role for USP8 depletion or inhibition in controlling cell viability in gefitinib resistant EGFR mutant non-small cell lung cancer (NSCLC) cell lines (Byun, Lee et al. 2013).

The cells under study were the NSCLC cell lines NCI-H1650 and NCI-H1975 (hereafter referred to as H1650 and H1975 respectively) and they were compared to normal human bronchial cells and human lung fibroblasts. The authors monitored cell viability of USP8 depleted cells and cells treated with one of two EGFR inhibitors gefitinib or erlotinib using Giemsa staining. As expected, gefitinib resistant cells did not respond to the EGFR inhibitors, however depletion of USP8 for 72 hours markedly reduced cell viability. In contrast, the two normal lung cell lines showed reduced cell viability in response to EGFR inhibition but were insensitive to USP8 depletion.

Comparison of the RTK phosphorylation profile of the USP8 depleted cells by RTK array identified a decrease in EGFR, ERBB2, ERBB3 and MET phosphorylation upon USP8 depletion. The authors found that this was due to a reduction in basal receptor levels as assessed by western blotting. Byun et al. further extended their work to the alleged USP8 inhibitor 9-Ethyloxyimino-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile and found that H1650, H1975 and a number of both gefitinib resistant and sensitive NSCLC cell lines showed reduced viability in the presence of this inhibitor in contrast to normal human lung cells. The proposed USP8 inhibitor also caused a reduction in the levels of EGFR, ErbB2, ErbB3 and c-MET as well as phosphorylated forms of downstream effectors STAT3, ERK and AKT, which was not evident in cells treated with gefitinib.

Since I had previously been working on a link between USP8 and the PI3K pathway and had observed a synthetic effect on cell viability, I wanted to investigate the above findings by Byun et al. further.

4.1 Objective

In this chapter I set out to assess whether USP8 depletion and inhibition reduces cell viability by affecting RTK protein levels.

4.2 Results

4.2.1. siRNA-mediated depletion reveals two isoforms of USP8 expressed in lung cancer cell lines

First I set out to characterise our USP8 siRNA oligos in the two EGFR mutant, gefitinib resistant NSCLC lines described by Byun et al (Byun, Lee et al. 2013). These cell lines carry a point mutation or deletion respectively in EGFR that renders the kinase constitutively active (figure 4.1.A). In the case of the H1975 cells, a second mutation in the kinase domain of the receptor (T790M) renders the cells gefitinib resistant. In the H1650 cells a secondary, unknown mechanism is responsible for their resistance to gefitinib. The USP8 siRNA oligos I used are shown in figure 4.1.B and their recognition sequences are mapped to the domain structures of USP8 isoforms in figure 4.1.C.

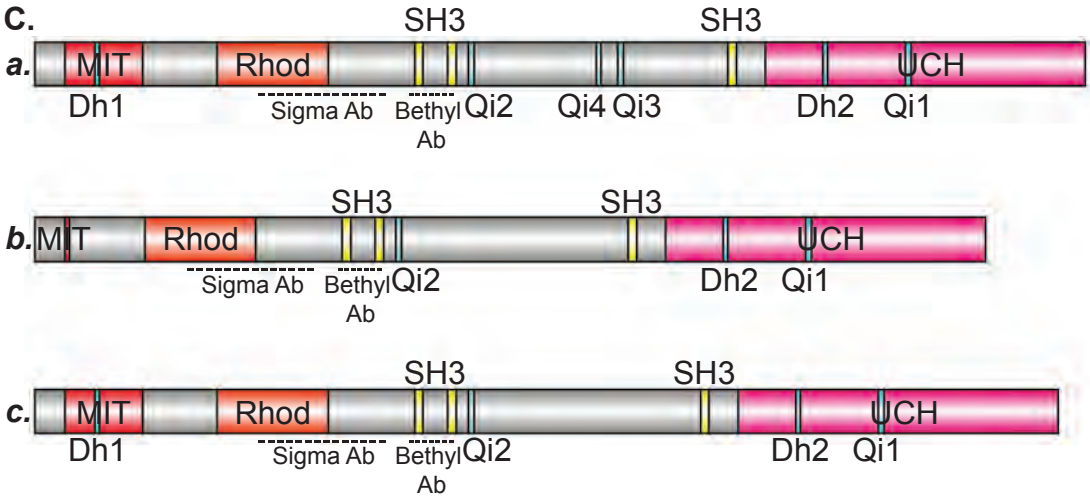
USP8 depletion for 48 hours in both cell lines clearly showed that all oligos reduce USP8 protein levels efficiently (figure 4.1.D). However it became clear that USP8 separated by SDS-PAGE as a doublet (close up – figure 4.1.E). I found that some of the siRNA oligos depleted both bands of the USP8 doublet. However the Qi3 and Qi4 siRNA mapped to a region unique to only the larger (figure 4.1.Ca) of the two known isoforms and consequently only depleted one band of the USP8 doublet. Interestingly the Dh1 oligo depletes both USP8 bands yet since it maps to the MIT domain which is missing from

A.

Cell line	Protein	Mutation
H1975	EGFR	L858R
	EGFR	T790M
	p16	E69*
	p53	R274H
	PI3K p110a	G118D
H1650	EGFR	Δ746-750
	PI3K p110a	C1524R
	p53	Intronic
	p53	Intronic

B.

siRNA	Sequence
Qi1	CAGGGTCAATTCAATCTACA
Qi2	AAGGCTCGTATTCATGCAGAA
Qi3	CAGGTTCAGGCAAGCCATTTA
Qi4	GAGGATACAGACGATACCGAA
Dh1	TGAAATACGTGACTGTTTATT
Dh2	GGACAGGACAGTATAGATATT



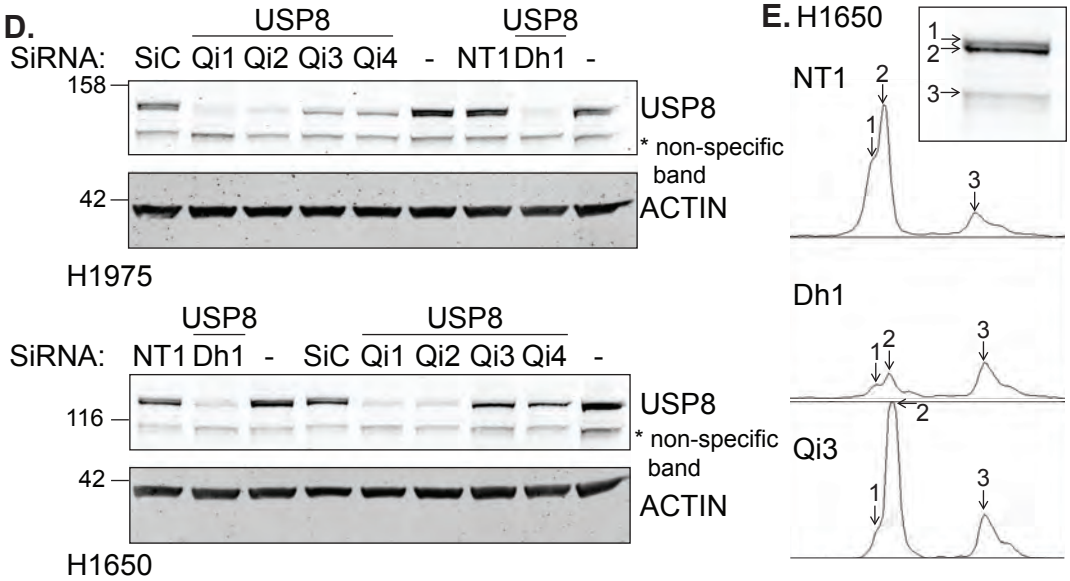


Figure 4.1. SiRNA-mediated depletion reveals two isoforms of USP8 expressed in lung cancer cell lines. (A) Mutational status of each cell line. (B) USP8 siRNA oligo sequences (Dh2 is only used in chapter 5). (C) USP8 isoforms (a and b) and proposed isoform (c) with siRNA target regions indicated in blue and antibody recognition sites indicated with dotted line. (D) 48h USP8 depletion in NSCLC cell lines H1975 and H1650 using USP8 targeting siRNA (n=1). (E) Line graph illustrating the USP8 banding pattern. Peaks corresponding to isoform a, proposed isoform c and a non-specific band are indicated (1, 2 and 3 respectively).

isoform b (figure 4.1.Cb) one might expect only one band to be affected. For this reason I propose a third isoform that is missing the section recognised by the Qi3 and Qi4 oligos but retains the MIT domain (figure 4.1.Cc).

4.2.2. The effect of USP8 depletion on RTK expression levels in H1975 and H1650 lung cancer cells

Once I had confirmed the effectiveness of the siRNA oligos I looked at the basal growth factor receptor expression levels in these two cell lines. The H1650 cells express slightly more EGFR and clearly more ERBB2 but considerably less ERBB3 and MET than the H1975 cells (figure 4.2.A). Given that USP8 is a long-lived protein with a reported half-life of over 80 hours I used a 120 hour knockdown protocol with reseeding between the two siRNA treatments (Schwanhausser, Busse et al. 2011).

I used three different negative control siRNAs: non-targeting SiC and NT1 as well as an siRNA targeting another unrelated DUB, TRABID (TBD). As previously observed in a number of cell lines, USP8 depletion resulted in a loss of HRS protein in both H1650 and H1975 cells (figure 4.2.B) (Row, Prior et al. 2006). The ERBB3 levels in H1650 cells vary across conditions but the changes are not consistently associated with USP8 depletion. In fact I repeated this experiment five times with siRNA depletion time courses ranging from 72 to 120 hours and saw no consistent changes in ErbB3 levels upon USP8 depletion. The same is true for EGFR and MET protein levels, which are not consistently reduced in either cell line. One thing to note is that the Qi2 oligo dramatically reduced EGFR protein levels in both cell lines however I noticed that this oligo is highly toxic to the cells and thus this is most likely an off target effect. Although in this particular experiment it appears that EGFR levels are also more mildly reduced by the other USP8-targeting siRNAs, this effect was not reproducible over the course of five experiments.

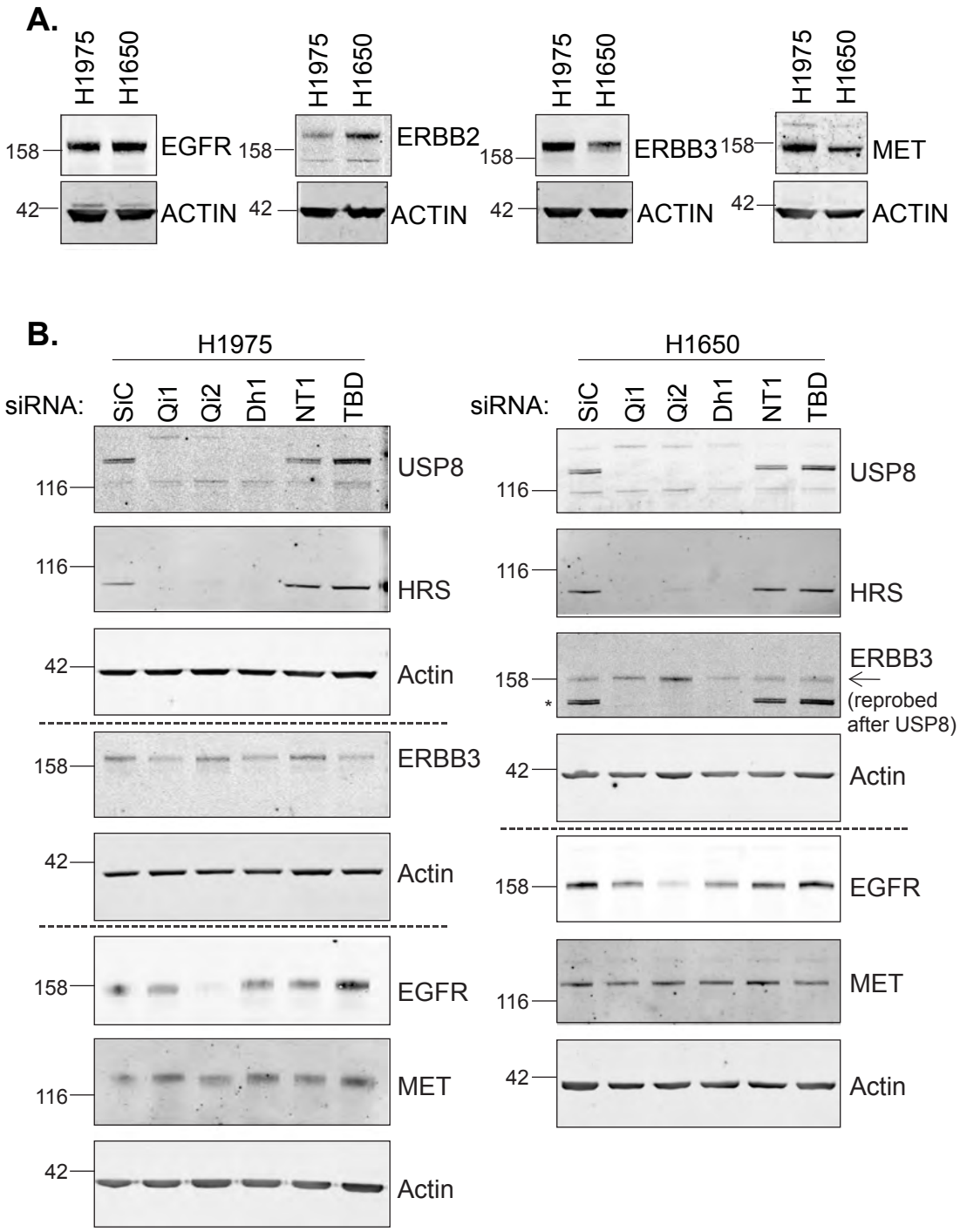


Figure 4.2. Effect of USP8 depletion on RTK expression levels in H1975 and H1650 lung cancer cells. (A) Western blot showing RTK levels in NP40 lysates dervied from H1975 and H1650 cell lines. (B) H1975 and H1650 cells were treated with siRNAs targeting USP8 (Qi1, Qi2, Dh1), Trabid (TBD) or non-targeting siRNAs (SiC and NT1) for 120h. Blots are representative of five individual experiments with depletion durations ranging from 72-150h (n=5). Cells were lysed in NP40 buffer and samples analysed by western blotting as indicated.The dotted lines separate western blots from distinct gels each with corresponding actin as a loading control.

4.2.3. USP8 inhibition does not consistently affect RTK or HRS levels in H1975 or H1650 cells

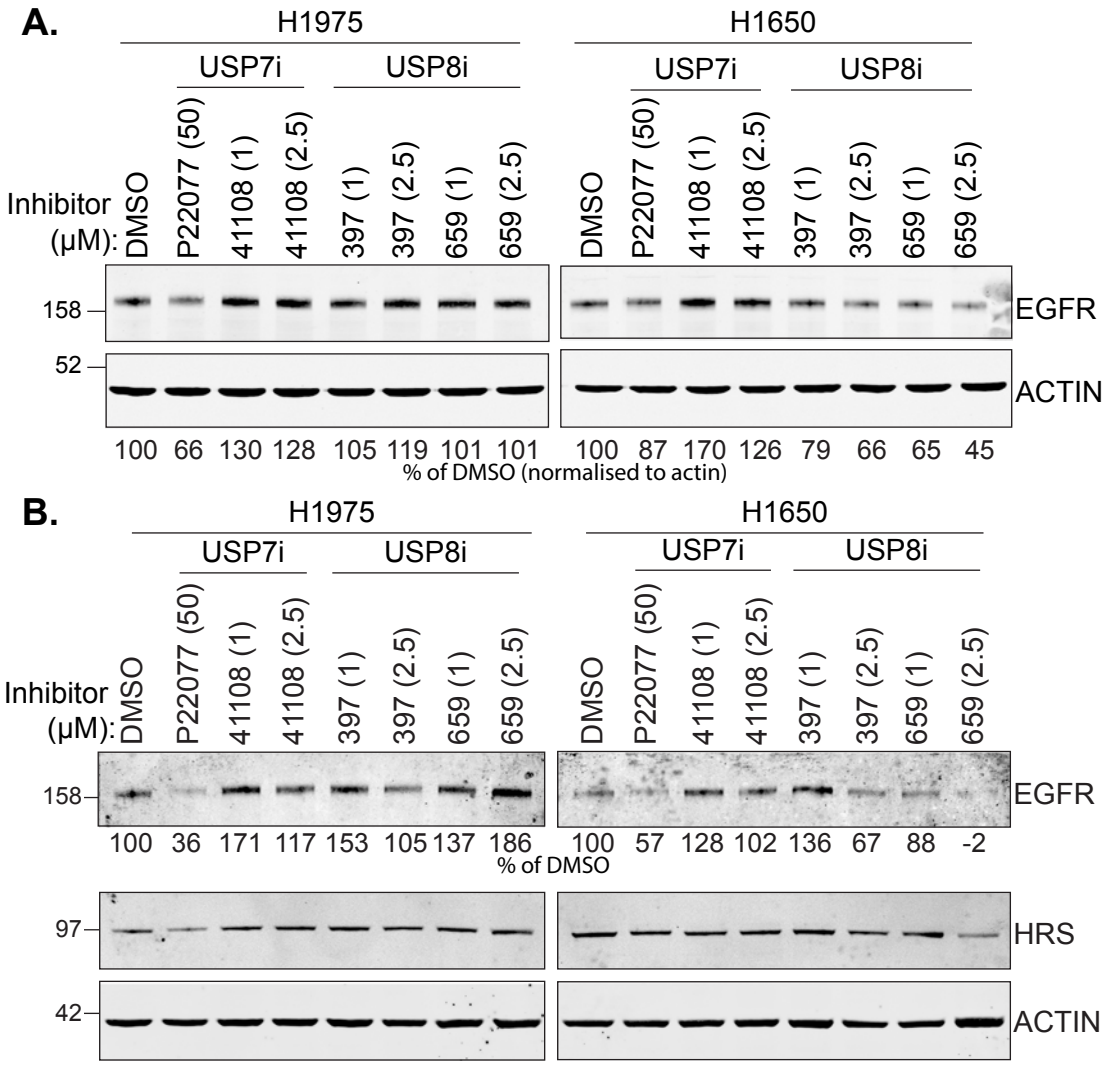
As an alternate approach, I used a small selection of previously reported USP8 inhibitors in both H1650 and H1975 cells. I treated the two cell lines for 18 hours with the inhibitors at the indicated concentrations (figure 4.3.A and figure 4.3.B represent two independent experiments).

In H1975 cells EGFR expression levels were not markedly affected by the USP8 inhibitors in either experiment. In the H1650 cells, however, the two USP8 inhibitors in both experiments consistently reduced EGFR levels. The 41108 inhibitor is marketed as a USP7 inhibitor however unpublished data from Forma Therapeutics suggested that this may also inhibit USP8 to some degree (figure 4.3.C). In both H1650 and H1975 cells this inhibitor increases the levels of EGFR. Neither did USP8 inhibition decrease HRS levels in the H1975 cells (figure 4.3.B). In the second experiment I also assessed the effect of these putative USP8 inhibitors on HRS. Neither inhibitor reproduced the effect of USP8 depletion as HRS protein levels were unaffected in both cell lines. The variation across all three putative USP8 inhibitors and the two individual experiments make this data hard to interpret and therefore inconclusive.

In contrast, the validated USP7 inhibitor, P22077, consistently reduced EGFR protein levels in both cell lines.

4.2.4. USP8 depletion decreases cell viability as assessed by crystal violet staining and cell counting

As described above, Byun and colleagues reported that USP8 depletion reduced viability of H1650 and H1975 cells. Whilst the authors saw a striking effect by 48 hours of depletion, I only observed a mild reduction in cell viability after 72-96 hours. For this reason I first decided to assess cell viability in a semi-quantitative manner using crystal violet staining after long-term USP8 depletion. Cells were treated for 120 hours with two hits of USP8



C.

Name in figure	Name	From	IC50 (μM) Main target
397	HBX 90,397 (22c)	Hybrigenics	USP8 0.559
659	HBX 90,659 (22d)	Hybrigenics	USP8 0.850
41108	HBX 41108	Tocris	USP7 0.424
P22077	P22077	Merck Millipore	USP7 8.01, USP47 8.74

Name in figure	IC50 (μM) Other targets	Unpublished (Forma)
397	USP7 >100, USP5 >100, UCHL1 >100 UCHL3 10	
659	USP7 >100 USP5 >100 UCHL1 >100 UCHL3 >100	
41108	p53 DUB by USP7 0.8	USP2 4.189, USP7 0.8956, USP8 0.06030
P22077	USP47 8.74	

Figure 4.3. USP8 inhibition does not consistently affect RTK or HRS levels in H1975 or H1650 cells across two independent experiments. (A and B) H1975 and H1650 cells were treated with USP8 inihibitors (397 and 659 at 1μM or 2.5μM), USP7 inhibitors (P2207 at 50μM and 41108 at 1μM or 2.5μM) or DMSO. Lysates were separated by SDS-PAGE and probed for the indicated proteins. Quantitation of EGFR levels is expressed as a percentage of the control below the relevant blot (n=2). (C) The inhibitors used and their IC50s for a range of targets.

targeting siRNA, fixed with methanol and stained with crystal violet followed by several washes with PBS (figure 4.4.A). The plates were then imaged using an Epson Perfection 3200 PhotoScanner (figure 4.4.B). Here, a slight reduction in cell viability is detectable when comparing the USP8 depleted H1975 cells to those treated with NT1 non-targeting control siRNA. This is particularly clear with the Qi2 oligo. This staining also highlights a mild effect of the SiC non-targeting control oligo compared to the NT1 siRNA. The effect on cell viability is dramatic in the H1650 cells with a clear reduction in cell viability across all three USP8 siRNA oligos compared to all three control siRNA oligos. In the same experiment, I also reseeded USP8-depleted cells 48 hours after the first siRNA treatment into three separate six well plates at an equal density. I then used an automatic cell counter to count the cells at 72 hours, 120 hours and 150 hours after the initial transfection (figure 4.4.C). In the H1975 cells a reduction in cell number is apparent with all three USP8 targeting siRNAs when compared to NT1. In particular the Qi2 treated cells decline in number from the time of reseeding for the remainder of the experiment whereas the Qi1 and Dh1 treated cells begin to grow after reseeding before the effect on viability becomes apparent at 120 hours. The Dh1 treated cells exhibit the least growth inhibition of the three siRNA oligos.

The effect is even more striking in the H1650 cells (figure 4.4.C). Here, the three USP8 siRNA treated wells exhibit either no change in cell number over the course of the experiment (Dh1, Q1) or a clear decline in cell number (Qi2). An effect on viability with the SiC control siRNA oligo is apparent.

Figure 4.4.D shows an exact repeat of this experiment with the two cell lines. Comparing the three USP8 depleted samples to the NT1 control treated samples it is clear in both cell lines that viability is consistently reduced. Again, the Dh1 treated H1975 cells grow well at earlier time points before reaching a plateau at 120 hours. As seen above, the Qi2 siRNA reduces cell numbers drastically across the duration of the experiment indicating the potential for off target toxicity effects. In this case there is a clear effect on viability with the SiC control siRNA oligo in both cell lines confirming the notion that this control oligo exerts some toxicity on the cells.

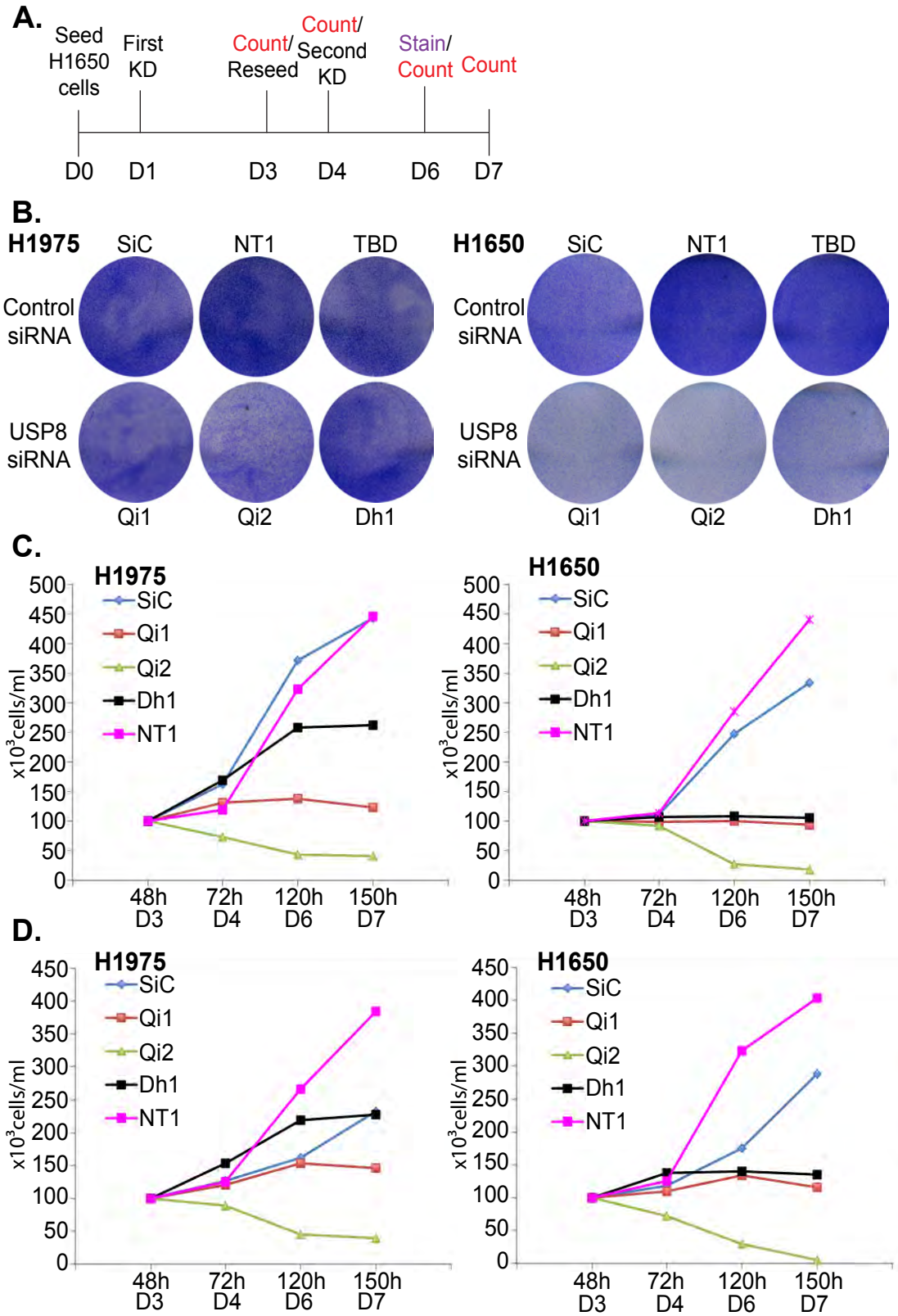


Figure 4.4. USP8 depletion decreases cell viability as assessed by crystal violet staining and cell counting. (A) Experimental procedure in which cells were either counted or stained on the indicated days (D1-7). (B) H1650 or H1975 cells were fixed with methanol and stained with crystal violet on day 6. (C) H1650 or H1975 cells were counted at indicated intervals after siRNA depletion. (D) H1650 or H1975 cells were counted at indicated intervals following siRNA depletion. (C) and (D) represent two individual experiments (n=2)

4.2.5. USP8 depletion in H1650 cells results in increased annexin V staining indicative of increased apoptosis

The cell viability effect was most striking in the H1650 cells and therefore it made sense to focus on this cell line for follow up studies. In order to investigate whether this effect is caused by apoptotic cell death I used annexin V staining in live H1650 cells. I incubated cells depleted of USP8 for 120 hours with annexin V-FITC for 20 minutes prior to imaging. In these experiments I replaced the non-targeting SiC oligo with an alternative control siRNA (NT3). In figure 4.5 it is clear that compared to the two non-targeting control siRNA (NT1, NT3) treated cells, the USP8 siRNA treated cells accumulate considerably more annexin V-FITC staining. This suggests that the effect USP8 depletion has on viability is at least in part due to an increase in apoptosis.

4.3. Summary of results

- Differential immunoblot banding patterns with a variety of USP8 siRNA oligos alludes to the existence of an as yet unreported USP8 isoform
- There is no consistent effect of USP8 depletion on basal EGFR levels
- Long-term USP8 depletion reduces cell viability in both H1650 and H1975 cells – an increase in apoptosis is reported in the H1650 cells

4.4. Discussion

The aim of this chapter was to explore and extend the findings by Byun and colleages describing the effect of USP8 depletion on cell viability and basal RTK levels in non-small lung cancer cells.

The initial, unexpected finding was one alluding to the existence of a third, as yet unreported, isoform of USP8. Data from Ensembl describes three protein-coding transcripts USP8_001, USP8_002 and USP8_003. Transcripts 001 and 003 both encode the full length, 1118 amino acid, predicted 128kDa

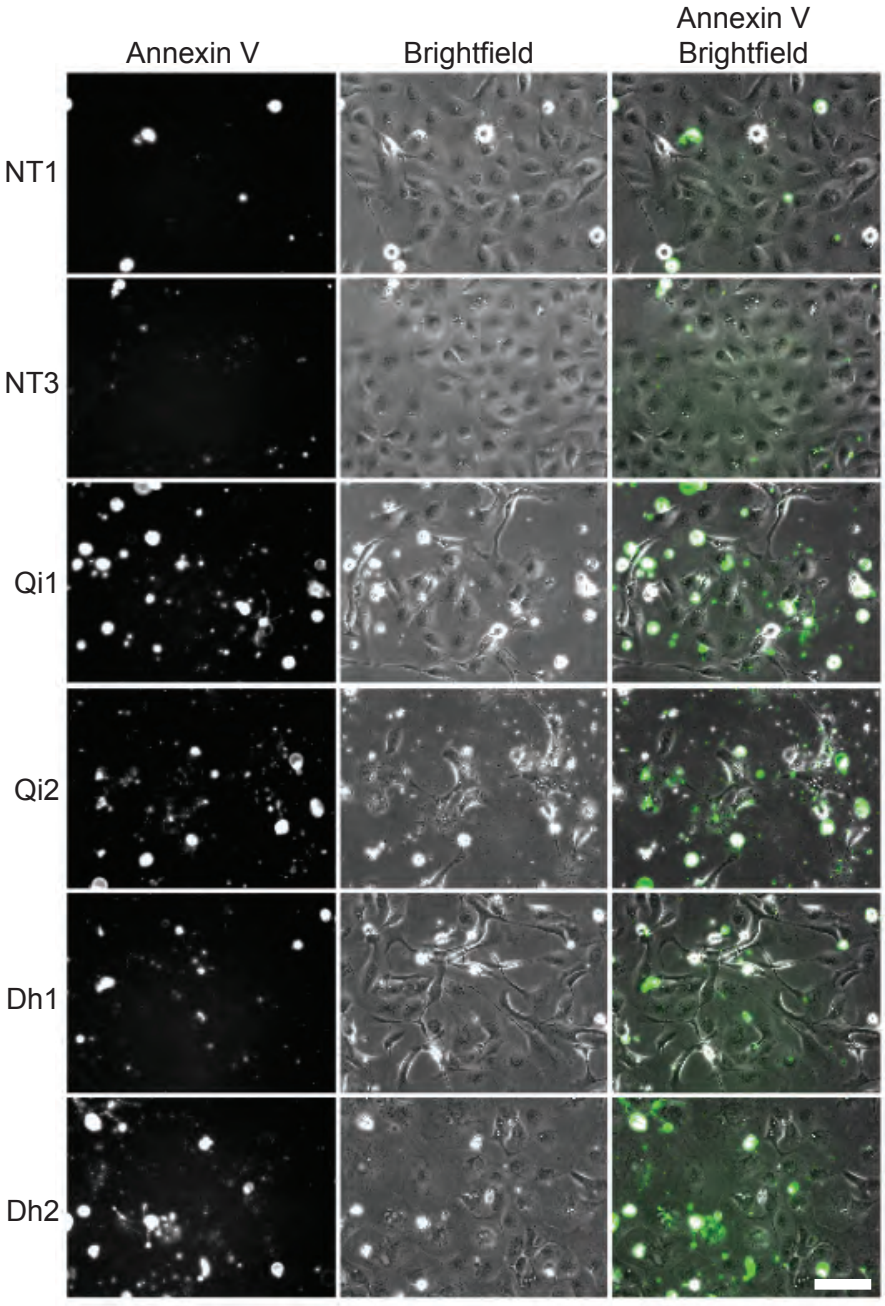


Figure 4.5. USP8 depletion in H1650 cells results in increased annexin V staining indicative of increased apoptosis. Cells were treated with USP8 targeting (Qi1, Qi2, Dh1 or Dh2) or non-targeting (NT1 or NT3) siRNA for 120h using a two-hit protocol with reseeding at 48h following the first transfection. The cells were then incubated with FITC conjugated annexin V (green) for 30min and imaged with a Nikon TiEclipse microscope (n=1). The scale bar is 400μm wide.

protein CCCDS10137 (Uniprot reference: 40818-1). Transcript 002, however, encodes a shorter 1012 amino acid, predicted 115kDa protein CCCDS61632 (Uniprot reference: 40818-2). The second isoform misses the amino acids 35 to 111 corresponding to exons 2 and 3 which encodes most of the MIT domain (see figure 5.1.C) and amino acids 601 to 629 corresponding to exon 11. When I mapped the sequence of our available siRNA oligos I found that the oligos Qi3, Qi4 and Dh1 would only target one of the two isoforms however the banding patterns seen by western blotting showed that the Dh1 oligo depleted both bands of the observed doublet. This led me to propose a third USP8 isoform that retains exons 2 and 3, thus the MIT domain, but loses amino acids 601 to 629. This would have a predicted molecular weight of 124kDa. The presence of this isoform might simply be brought about by the loss of one exon, exon 11, by alternative splicing. I would need to use PCR primers specific for the proposed isoform to confirm this hypothesis by RT-PCR. If the second band is this proposed third isoform and not the known second isoform of USP8 it would indicate that either the antibody I used does not recognise this second isoform (which it should since the epitope is not within the MIT domain region – see figure 5.1.C) or that these cells do not express detectable levels of this second isoform.

The most important finding I made was to confirm that USP8 depletion does indeed reduce cell viability in H1975 cells and most strikingly in the H1650 cells. These are both gefitinib resistant EGFR mutant NSCLC cell lines however they do carry very different mutations in EGFR and in other proteins. In particular H1975 cells have an L858R and T790M mutation in EGFR and a G118D mutation in PI3K p110a whilst H1650 cells have a Δ746-750 mutation in EGFR and a C1524R mutation in PI3K p110a (figure 4.1.A). In fact according to the Catalogue of Somatic Mutations in Cancer (COSMIC) the H1650 cells carry 227 known mutations and the H1975 cells carry 612.

A key finding here is that in the H1650 cells the reduction in cell viability with USP8 depletion can at least partially be explained by an increase in apoptosis. This is supported in the literature in a number of ways. Firstly, Byun et al. and Jeong et al. show that both H1650 cells and gefitinib resistant HCC827GR cells exhibit increased apoptosis upon USP8 depletion or

inhibition respectively (Byun, Lee et al. 2013, Jeong, Lee et al. 2016). USP8 has been linked to mediation of death receptor induced apoptosis through its regulation of $FLIP_L$ levels (Jeong, Lee et al. 2016). Here USP8 depletion prevents protection of $FLIP_L$ from ubiquitin-dependent proteasomal degradation and renders cells more susceptible to activation of the apoptotic pathway whilst USP8 overexpression enhances the stability of $FLIP_L$. Thus USP8 was proposed to regulate the stability of this long form of FLIP but not the short form, $FLIP_S$. Both the short and long forms of FLIP play a role in death receptor mediated, extrinsic, apoptosis. Typically, death receptors such as TRAIL recruit initiator caspases 8 and 10 and adaptors allowing the formation of the death inducing signalling complex (DISC) (Yang, Wilson et al. 2010, Safa 2013). Caspases 8 and 10 can activate downstream caspases and also feed into the intrinsic, mitochondrial apoptotic pathway through cleavage of the pro-apoptotic BCL2 family member, Bid. Truncated Bid triggers release of Cytochrome C from the mitochondria thus activating the intrinsic pathway (Safa 2013).

Long and short forms of FLIP act against apoptosis through their binding with death receptors and inhibition of caspase 8 and 10 activation (Peter 2004, Safa 2013). Caspase 8 activation relies on the homodimerisation of pro-caspase 8 and subsequent cleavage of the pro form (Lavrik and Krammer 2012). $FLIP_L$ is structurally similar to and the same length as caspase 8 but is catalytically inactive and has been shown to inhibit caspase 8 activation (Peter 2004, Safa 2013). However $FLIP_L$ has also been shown to play a pro-apoptotic role through caspase 8 activation via its heterodimerisation with pro-caspase 8 in some circumstances. In particular this might be dependent on the levels of $FLIP_s$ (Safa 2013).

Importantly, Jeong and colleagues found that overexpression of $FLIP_L$ could rescue the effect of USP8 depletion on sensitivity to TRAIL. This suggests that maintenance of specific $FLIP_L$ levels, or perhaps the ratio of $FLIP_L$ to $FLIP_s$, is required to suppress apoptosis. Whilst Jeong et al. found a role for USP8 in regulating $FLIP_L$ stability in a number of cancer cells routinely used in the lab, Panner et al. looked at glioblastoma multiforme primary cells and found a role for USP8 in the regulation of $FLIP_s$ stability (Panner, Crane et al.

2010). This, the authors reported, was due to regulation of USP8 by AKT which lead to the deubiquitylation of the $FLIP_s$ E3 ligase AIP4 and subsequent degradation of $FLIP_s$. This could, in principle, make cells more sensitive to death receptor mediated apoptosis although the authors did not directly show this with their data. Importantly, in both studies USP8 was proposed to increase the ratio of $FLIP_L$ to $FLIP_s$.

It is also possible that USP8 depletion leads to a defect in cell proliferation. Immortalised MEFs from inducible USP8-knockout mice showed growth arrest once USP8 depletion was induced (Niendorf, Oksche et al. 2007). This was evidenced in both a reduction in cell number and a reduction in bromodeoxyuridine incorporation into replicating DNA which indicates cell proliferation. USP8 activity has also been linked to this DNA synthesis phase (S-phase) of the cell cycle. USP8 phosphoryation was reported to inhibit DUB activity through 14-3-3 protein binding to p-S680-USP8. Dephosphorylation and dissociation from 14-3-3 in the S-phase enhanced USP8 activity (Mizuno, Kitamura et al. 2007). Naviglio et al. reported that USP8 inhibition through microinjection of fibroblasts with an anti-sense plasmid blocked cells from entering S-phase (Naviglio, Mattecucci et al. 1998). Conversely, recent studies have linked USP8 expression to the levels of a member of the inhibitor of apoptosis protein (IAP) family, BRUCE (Sippel, Rajala et al. 2009, Ge, Che et al. 2015, Ge, Che et al. 2015). In neural progenitor cells, treatment with a synthetic growth hormone dexamethasone led to an increase in USP8 levels and subsequent destabilisation of BRUCE through the stabilisation of E3 ligase Nrdp1 by USP8 (Sippel, Rajala et al. 2009). This promoted the proliferation of these neural progenitor cells. Other studies link USP8 and BRUCE via their joint recruitment to double strand breaks (Ge, Che et al. 2015, Ge, Che et al. 2015).

The effect of USP8 depletion on H1650 cell viability reported in this chapter is evident only after a prolonged knockdown protocol. However, in chapter three I described a synthetic lethal effect between PI3K pathway inhibition and USP8 depletion in FKHRL1-U2OS, U2OS and H1975 cells. Of course if there is a link between USP8 and the PI3K pathway as my data in chapter 3 suggested then it may make sense that disruption of USP8 could lead to

increased cell death since the AKT-PI3K pathway is a known regulator of apoptosis. However my data in chapter three showed that, at least in FKHRL1-U2OS, U2OS, H1975 and to some degree H1650 cells, there is a requirement for simultaneous depletion of USP8 and inhibition of the PI3K pathway to stimulate this cell death.

Synthetic lethality is an effect in which combining two or more non-lethal mutations results in cell death. This terminology is also used to describe synthetic effects observed due to simultaneous loss of function induced by RNAi or drug inhibition. This may be of particular therapeutic benefit as cancer cells with acquired mutations in particular pathways might subsequently be sensitised to drugs inhibiting other pathways. As such, synthetic lethality screens are now used in order to identify potential therapeutic targets in cancer cells. One example of success in this area comes from the use of PARP inhibitors in BRCA mutant breast cancers. In this case the homozygous loss of BRCA sensitises cells to PARP inhibition (Farmer, McCabe et al. 2005).

To fully explain the reason for USP8 depletion induced cell death requires further research, which I begin to explore in chapter five.

Importantly, I was unable to confirm any significant and specific changes in growth factor receptor levels upon USP8 depletion alone. USP8 is a deubiquitylase that regulates the levels of a number of growth factor receptors. This is best characterised for EGFR and specifically observed following acute EGF stimulation. USP8 depletion in HeLa cells severely delays the downregulation of EGFR and MET in response to their respective ligands (Row, Prior et al. 2006, Row, Liu et al. 2007). USP8 overexpression stabilises the levels of the E3 ligase Nrdp1 which itself regulates ERBB3 levels (Wu, Yen et al. 2004, Cao, Wu et al. 2007). It would be of interest to perform a time course stimulation of the H1975 and H1650 cell lines with EGF, NRG1 or HGF and see how the receptor levels respond with and without USP8 depletion. There is evidence from USP8 knockout mice that points to a reduction in global basal EGFR levels with removal of this DUB. This may suggest that complete ablation of the protein is required to see this

effect on basal growth factor levels as RNAi may not remove the protein entirely. With the advent of CRISPR technology, future work may elucidate this further as complete knockout cell lines can be produced.

One might also study this further using USP8 inhibitors however current commercially available USP8 inhibitors are not truly specific for USP8. For example, the USP8 inhibitors I used above also inhibit USP7, USP5, UCHL1 and UCHL3 (Colombo, Vallese et al. 2010, Ritorto, Ewan et al. 2014). In particular, many USP inhibitors target the catalytic cysteine of DUBs and are thus unable to discriminate between different members of the family. Matthias Trost and colleagues analysed DUB activity using a MALDI-TOF based mass spectrometry technique. They considered 11 DUB inhibitors and found that none of the compounds, including one for USP8 and the USP7/USP8 41108 inhibitor used in this chapter, were specific for only a single DUB and many inhibited the majority of DUBs in their panel (Ritorto, Ewan et al. 2014).

The findings in this chapter led me to conclude that there may be another reason for the effect of USP8 depletion on cell viability. Conversation with the biopharmaceutical company, Amgen suggested a new avenue to follow up in this vein, which I discuss in chapter five.

Chapter five: The role of USP8 in the regulation of ROS management and cell death

In March 2015, Olivier P. Nolan-Stevaux PhD, a Senior Scientist in the Department of Oncology at the biopharmaceutical company Amgen Inc., contacted us to discuss preliminary data concerning cell viability effects they observed upon USP8 depletion. Their findings, described below, spoke to my own findings described in chapter four.

Initially Amgen screened 53 cell lines by RNA interference with three genomic siRNA libraries looking for effects on cell viability. They identified three cell lines that showed sensitivity to both USP8 and HRS depletion; breast cancer cells HCC1954 and HCC70 and primitive neuro-ectodermal tumour cells PFSK1. HRS sensitivity is of interest here since it is a core component of the ESCRT-0 complex that USP8 interacts with as part of its function in endocytic trafficking. Importantly, depletion of USP8 has been shown to reduce HRS levels in a number of cell lines thus sensitivity to both USP8 and HRS increases the confidence in these observations (Row, Prior et al. 2006) (see also chapter four, figure 4.2). Amgen further validated this sensitivity to USP8 depletion in the two breast cancer cell lines HCC1954 and HCC70. They went on to show that both lines were also sensitive to depletion of TSG101 and PTPN23, two further ESCRT components of the multi-vesicular body (MVB) sorting pathway. This finding further supports a requirement for endocytic trafficking for viability of these cells.

Comparison of the RNA expression profiles of the three identified cell lines initially led Amgen to identify the squamous cell carcinoma (SQCC) marker Serpin B3 as a potential common denominator even though these cell lines did not belong to the SQCC type. These three cell lines expressed more of this marker than any of the other 50 cell lines tested in the initial screen. Amgen followed this finding up by analysing other cell lines of the SQCC type and identified a further two cell lines with USP8, TSG101 and PTPN23

sensitivity. However, these were Serpin B3 negative lung SQCC cell lines CALU1 and EBC1. At this stage it became clear that Serpin B3 expression is not a universal marker for USP8 sensitivity.

In a further attempt to identify any common mutations between USP8 dependent cell lines, Amgen performed an unbiased association study across the entire pool of cell lines they had previously studied. They found that two of the USP8-dependent cell lines (CALU1 and EBC1) had a mutation in the NFE2L2 gene and two cell lines (PFSK1 and HCC70) had a mutation in the NFE2L3 gene. One USP8-independent cell line (LK2) also had a mutation in NFE2L2.

As discussed in the introduction, the protein product of NFE2L2 is NRF2. NRF2 is a protein involved in the ability of the cell to respond to reactive oxygen species (ROS) and mismanagement of this system can lead to a decrease in cell viability. With this in mind it was a logical step to further investigate a link between USP8 sensitivity and NRF2.

5.1. Objective

In this chapter, my main objective was to establish if there is a link between USP8 and the NRF2/KEAP1 pathway as suggested by the data from Amgen described above. I also intended to explore a functional role of the NRF2/KEAP1 pathway in the reduced cell viability observed in USP8 depleted cells.

5.2. Results

5.2.1. Testing the specificity of different NRF2 and KEAP1 antibodies by western blotting and by RNA interference

Whilst reviewing the literature I came across a common problem with western blot analysis of NRF2. Whilst the predicted molecular weight of this protein is approximately 70kDa, studies in the literature consistently show it running at

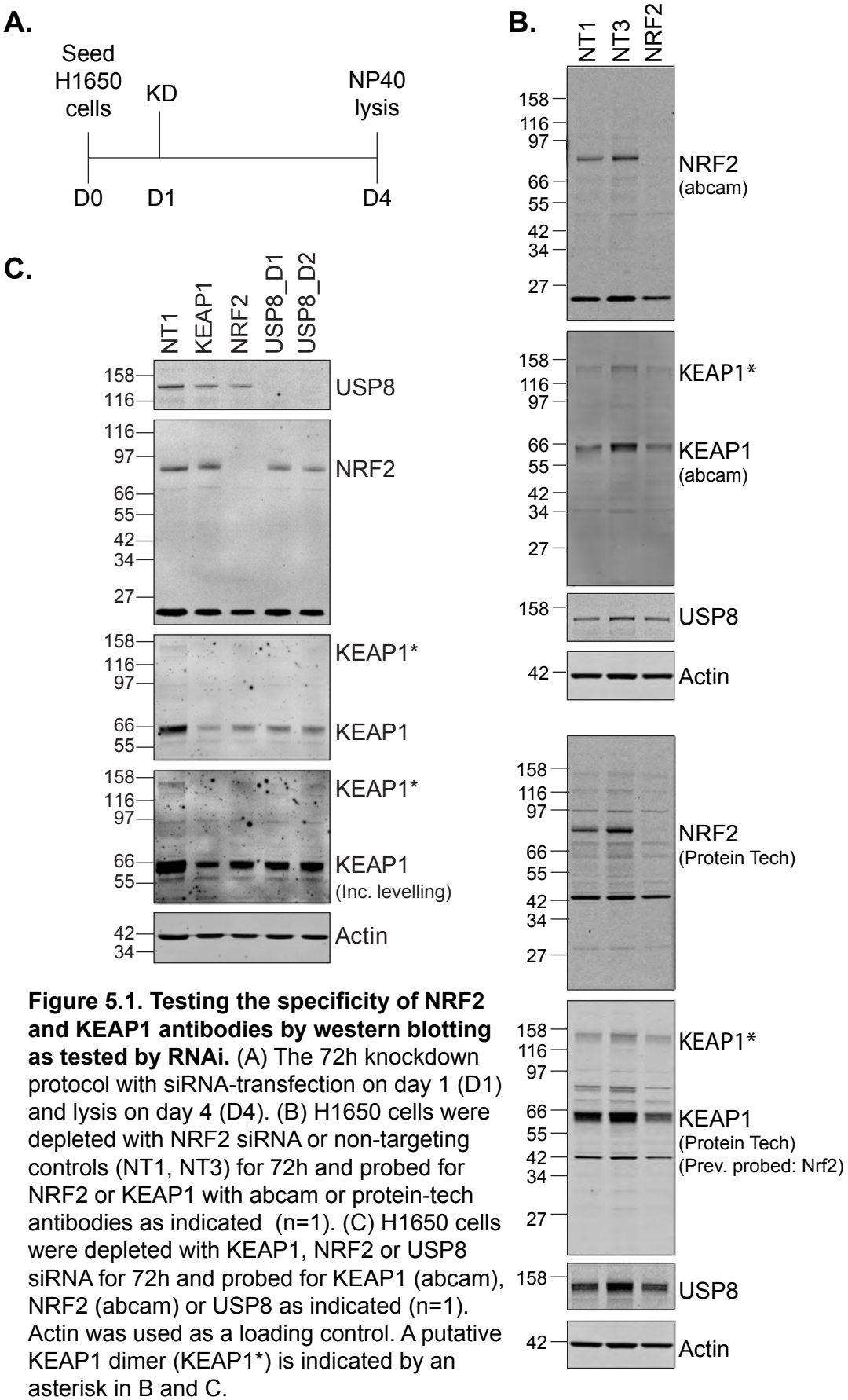


Figure 5.1. Testing the specificity of NRF2 and KEAP1 antibodies by western blotting as tested by RNAi. (A) The 72h knockdown protocol with siRNA-transfection on day 1 (D1) and lysis on day 4 (D4). (B) H1650 cells were depleted with NRF2 siRNA or non-targeting controls (NT1, NT3) for 72h and probed for NRF2 or KEAP1 with abcam or protein-tech antibodies as indicated (n=1). (C) H1650 cells were depleted with KEAP1, NRF2 or USP8 siRNA for 72h and probed for KEAP1 (abcam), NRF2 (abcam) or USP8 as indicated (n=1). Actin was used as a loading control. A putative KEAP1 dimer (KEAP1*) is indicated by an asterisk in B and C.

around 100kDa on western blots. In order to verify the behaviour of NRF2 and its inhibitor, KEAP1, on my gels I decided to first test two independent antibodies against each protein. Furthermore I used RNA interference in H1650 cells against each protein to identify specific and non-specific bands. Figure 5.1.A introduces the RNA interference protocol. H1650 cells were treated with siRNA against NRF2, KEAP1, USP8 or non-targeting controls (NT1 or NT3) the day after seeding. The cells were lysed in NP40 buffer 72 hours after the transfection.

H1650 cells express wild-type NRF2, however their sensitivity to USP8 depletion makes them an interesting point of study in the first instance. In figure 5.1.B it is clear that NRF2 depletion removed a band running between the 66 and 97 kDa marker that was recognised by both NRF2 antibodies. Although other studies find this band running a little higher, this finding suggests that in 4-12% NuPAGE gradient gels with MOPS-based running buffer, the protein runs at its predicted molecular weight. NRF2 depletion also decreased KEAP1 levels as detected with both KEAP1 antibodies (figure 5.1.B). The predicted molecular weight of KEAP1 is also 70kDa but on these gels it runs just under the 66kDa marker. In this panel I also identified a second KEAP1 band just under the 158kDa marker that followed the same pattern as the major KEAP1 band. This leads me to suggest that this may correspond to a dimeric form of KEAP1.

In order to test the KEAP1 antibodies, I depleted KEAP1 using a pool of four siRNA oligos (Figure 5.1.C.) This led to a marked reduction of both afore-mentioned bands confirming that these are specific for KEAP1. Concurrently, I treated cells with siRNA oligos targeting NRF2 and reconfirmed the reduction in KEAP1 levels with NRF2 knockdown. KEAP1 is a known transcriptional target of NRF2 acting as a negative feedback regulator of the transcription factor thus it makes sense that depletion of NRF2 reduces KEAP1 levels (Chorley, Campbell et al. 2012). Finally, I also treated cells with two individual USP8-targeting siRNA oligos. This revealed a clear decrease in KEAP1 levels in cells depleted of USP8.

5.2.2. USP8 knockdown results in a decrease in KEAP1 protein levels in H1650 cells

In order to further investigate the reduction in KEAP1 protein levels upon USP8 depletion I subjected the same NSCLC H1650 cells to a longer RNA interference protocol (figure 5.2.A). Cells were treated twice over 120 hours with siRNA using a two hit protocol and were reseeded between the hits on day three. Using three independent USP8 oligos – Qi1, Dh1 and Dh2 – I observed a reduction in KEAP1 levels with two different KEAP1 antibodies that was apparent for both the major KEAP1 band and the higher molecular weight band (figure 5.2.B).

Whilst this reduction in KEAP1 levels could suggest KEAP1 may be stabilised by USP8, it could, in principle, also indicate a redistribution of the protein. In order to ensure that I had solubilised all proteins, I compared my standard NP40 lysis protocol to RIPA lysis in figure 5.2.C. Lysis with RIPA buffer, which contains SDS, sodium deoxycholate and triton X-100 allows for extraction of nuclear as well as cytosolic proteins. The presence of ionic detergent in RIPA buffer allows better solubilisation of proteins and the disruption of non-covalent bonds. NP40 buffer, however, relies on the gentler, non-ionic detergent Nonidet P-40 which is non-denaturing. I found a similar reduction in KEAP1 levels upon USP8 depletion using the RIPA lysis protocol. Interestingly, the RIPA lysis also highlighted an increase in NRF2 levels. This may indicate an increase in the nuclear pool of NRF2 as a result of USP8 depletion and subsequent KEAP1 reduction. Figure 5.2.D shows a quantitation of several 120 hour USP8 depletion experiments all using the NP40 lysis buffer protocol confirming the reduction in KEAP1 protein levels. Error bars indicating standard deviation are shown where the number of experiments exceeded three.

5.2.3. USP8 depletion in EBC1 cells reduces KEAP1 protein levels

As discussed above, Amgen found that EBC1 cells are sensitive to USP8 depletion. These cells have a heterozygous D77V mutation in NRF2, which prevents KEAP1 from coupling to the protein. I first performed a 96 hour

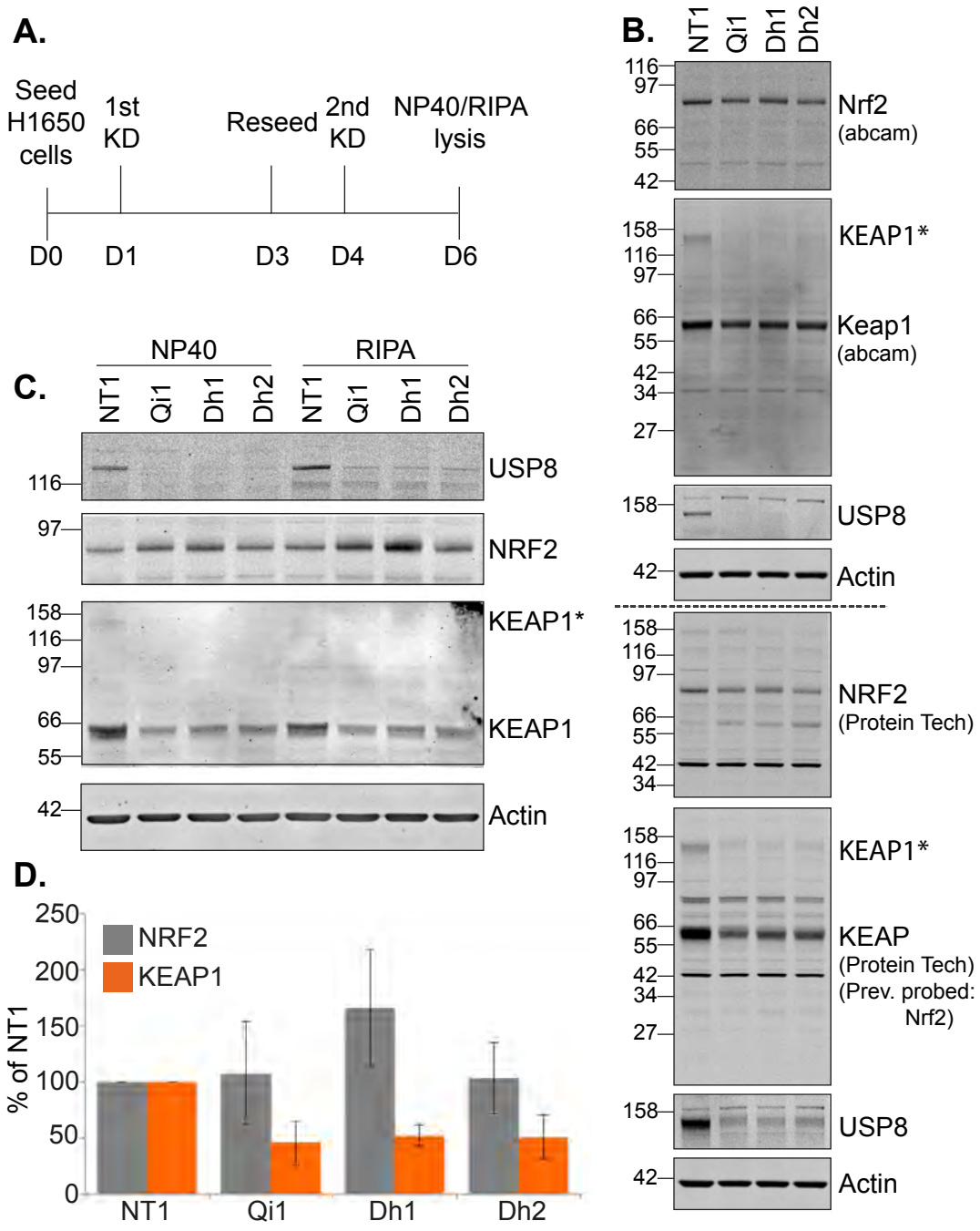


Figure 5.2. USP8 depletion decreases KEAP1 protein levels in H1650 cells. (A) The 120h protocol entails knockdown (KD) on days 1 (D1) and 4 (D4), re-seeding on day 3 (D3) and lysis on day 6 (D6). (B) H1650 cells depleted with USP8 siRNA for 120h were lysed with NP40 lysis buffer (n=4). Proteins were separated by SDS-PAGE then blotted with the indicated abcam/protein tech antibodies. Actin was used as a loading control for each of the blots divided by the dotted line. (C) H1650 cells depleted with USP8 siRNA for 120h were lysed with NP40 (n=4) or RIPA buffer (n=1), separated by SDS-PAGE and blotted with the indicated antibodies. (D) Quantitation of KEAP1 and NRF2 protein levels from several independent experiments using the protocol shown in A. Relative protein levels are expressed as a percentage of those seen in cells targeted with control siRNA (NT1). Error bars show standard deviation for Dh1 (n=4) and Dh2 (n=3), range for Qi2 (n=2), NT1 set at 100% (n=4).

knockdown in the EBC1 cells (figure 5.3.A). I found that KEAP1 levels were indeed reduced upon USP8 depletion. Since this was the first time I had depleted USP8 in this cell line I also analysed the levels of HRS (figure 5.3.A). HRS levels were also reduced in USP8-depleted EBC1 cells just as I previously observed in H1650 and H1975 cells (chapter 4 figure 4.2). USP8 is a long-lived protein with an 80 hour half-life and a 120h knockdown protocol tends to result in a better knockdown (see chapter four, figure 4.2; (Schwanhausser, Busse et al. 2011)). Therefore I performed the longer, two hit protocol as described in figure 5.3.2. This gave a very good USP8 depletion in these cells and further confirmed the reduction in KEAP1 in cells lysed with NP40 buffer (figure 5.3.B).

In this experiment I also depleted the cells of HRS since Amgen had found that these cells are sensitive to knockdown of HRS as well. Here I found a similar reduction in KEAP1 levels as with the USP8 depletion. I further validated the specificity of the antibody in these cells by KEAP1 siRNA.

In addition to the KEAP1 reduction in figure 5.3.B, I also identified a mild increase in NRF2 levels upon USP8 and HRS depletion that is only evident in this longer knockdown experiment and mirrors my observation in RIPA extracted H1650 lysates (see figure 5.2).

5.2.4. HRS depletion and its effect on KEAP1 protein levels in H1650 and EBC1 cells

Following up on the finding that HRS depletion caused a decrease in KEAP1 protein levels, I depleted it in both H1650 (figure 5.4.A) and EBC1 (figure 5.4.B) cells using the same 120h knockdown protocol as described above (figure 5.4.C). It is evident that HRS depletion reduced KEAP1 levels to a similar level as USP8 depletion in both cell lines. The high molecular weight, putative dimeric band for KEAP1 is clearly evident, albeit only in H1650 cells. This band is decreased to the same degree in both USP8 and HRS depleted cells (figure 5.4.A). In both cell lines I found an increase in NRF2 levels upon USP8 and HRS depletion although this is most striking in the EBC1 cells. Note that all these lysates were prepared using NP40 lysis buffer.

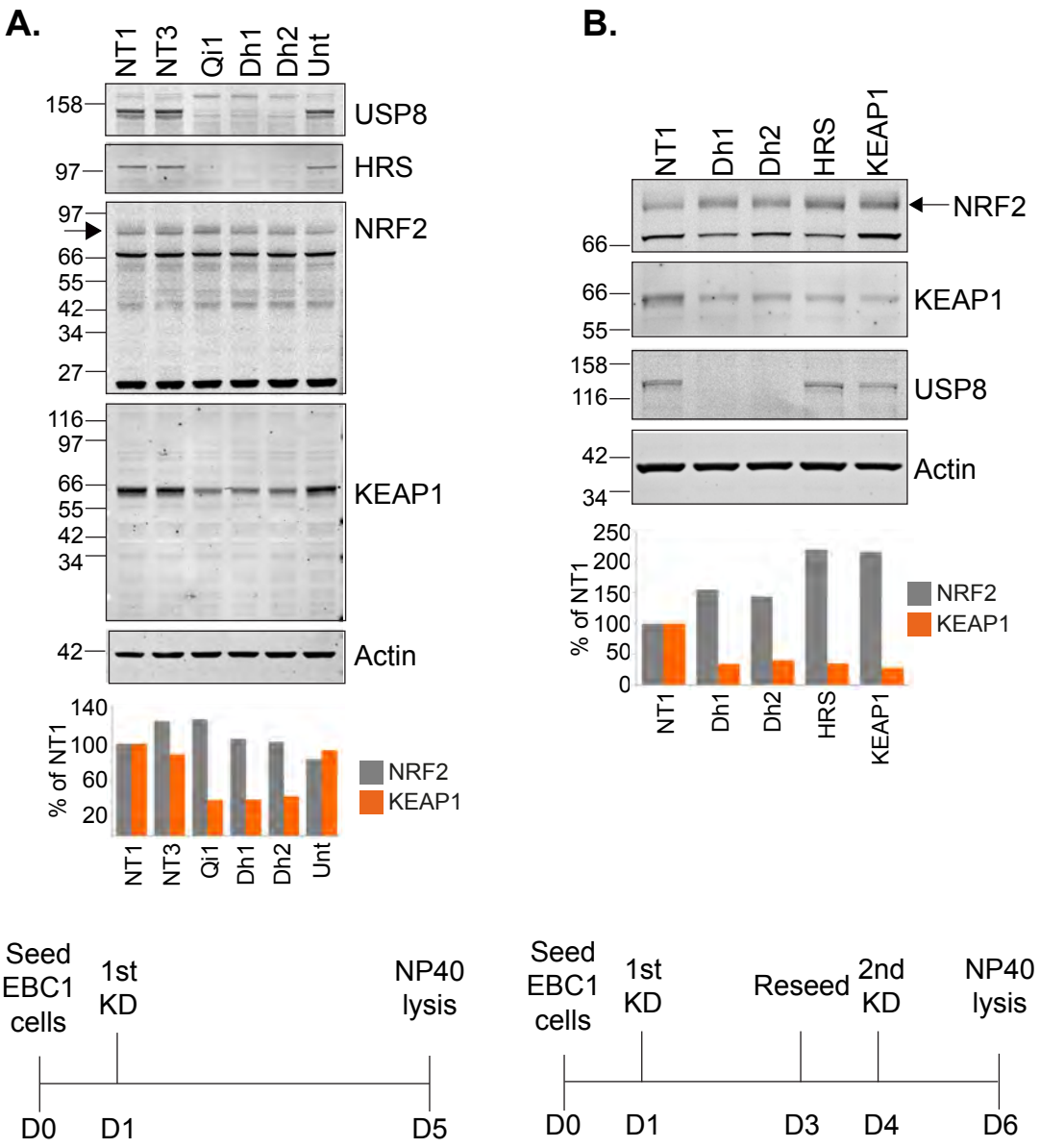


Figure 5.3. Deacreased KEAP1 protein levels in USP8-depleted EBC1 cells. (A) EBC1 cells treated with USP8 siRNA (Qi1, Dh1, Dh2) for 96h were lysed with NP40 lysis buffer, separated by SDS-PAGE and blotted with the indicated antibodies (n=1). Actin represents loading for the above immuno-blots. Bar chart shows corresponding quantitation of relative NRF2 and KEAP1 protein levels. Schematic shows protocol with knockdown on day 1 (D1) and lysis on day 5 (D5). (B) EBC1 cells treated with USP8 siRNA (Qi1, Dh1, Dh2) for 120h were lysed with NP40 buffer, separated by SDS-PAGE and blotted with the indicated antibodies (n=1). Actin represents loading for the above immuno-blots. Bar chart shows corresponding quantitation of relative NRF2 and KEAP1 protein levels. Schematic shows protocol with knockdowns on day 1 (D1) and day 4 (D4), reseeding on day 3 (D3) and lysis on day 6 (D6).

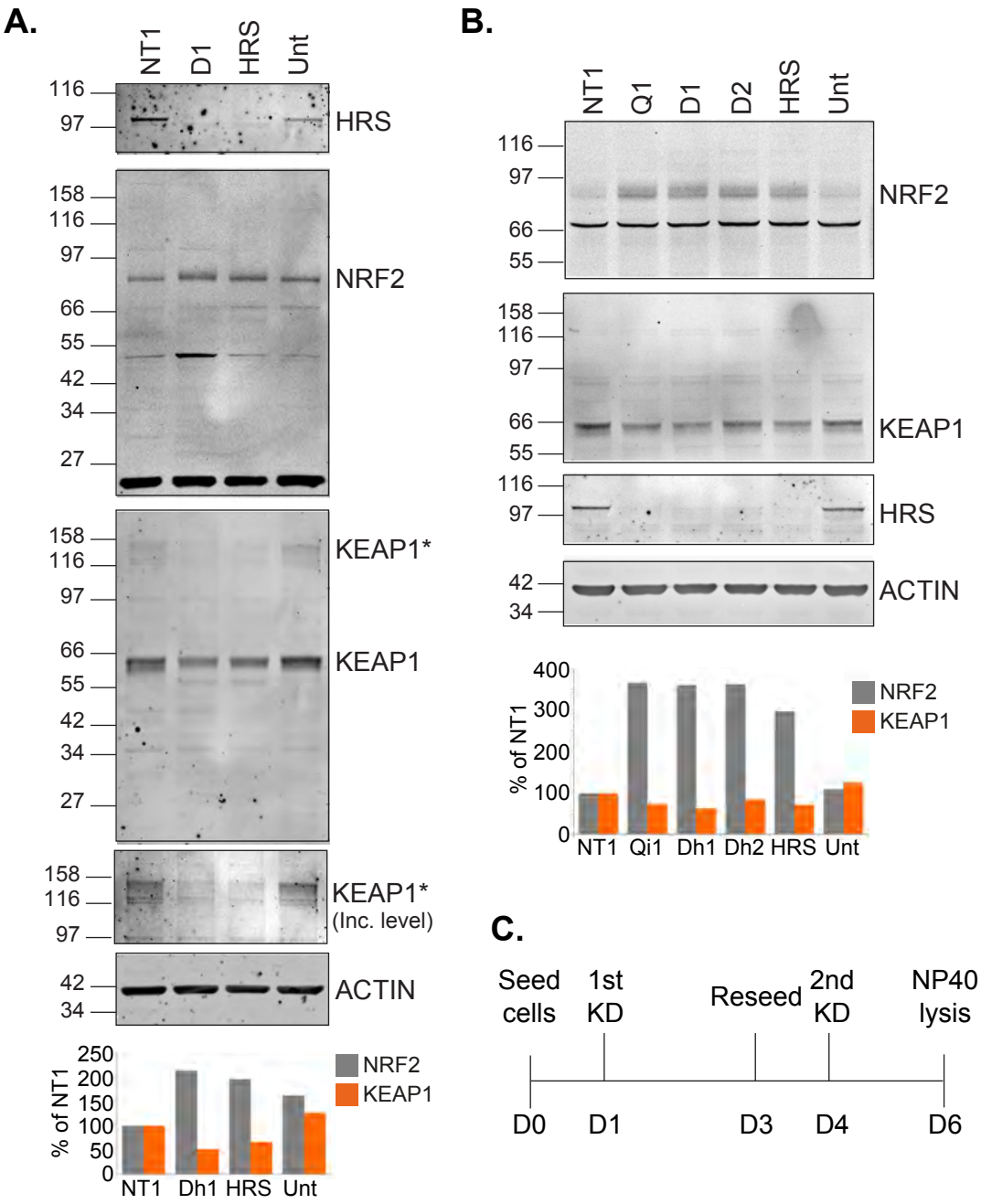


Figure 5.4. HRS depletion and its effect on KEAP1 and NRF2 protein levels in H1650 and EBC1 cells. (A and B) H1650 cells (A) or EBC1 cells (B) were treated with USP8 or HRS siRNA for 120h, lysed with NP40 buffer and separated by SDS-PAGE. Blots were probed with the indicated antibodies and protein levels quantitated as indicated (n=1 for each cell line). Actin represents loading for each of the above immuno-blots (C) The 120h protocol involves a knockdown on days 1 (D1) and 4 (D4), reseeding on day 3 (D3) and lysis on day 6 (D6). KEAP* indicates the putative KEAP1 dimer.

5.2.5. KEAP1 mRNA levels do not change upon USP8 depletion

The levels of a particular protein in a cell are dependent on the balance between the rate of protein synthesis and the rate of protein degradation. Changes in KEAP1 protein levels following USP8 depletion could indicate changes in protein stability, especially since USP8 is a DUB, and thus may rescue proteins from degradation. However, the rate of protein synthesis could alternatively be affected. In order to test this, I used quantitative qRT-PCR to analyse the levels of KEAP1 mRNA. Figure 5.5.A shows that KEAP1 mRNA levels are clearly decreased by KEAP1- but not USP8- or HRS-targeting siRNA in EBC1 cells. If anything there is a trend towards an increase in KEAP1 mRNA upon USP8 and HRS depletion. Likewise in H1650 cells, USP8 and HRS depletion do not affect KEAP1 mRNA (figure 5.5.B). In contrast NRF2 siRNA does have a mild effect, in keeping with the above mentioned transcriptional regulation of KEAP1 by NRF1.

5.2.6. USP8 depleted H1650 cell viability is not rescued by treatment with the ROS scavenger N-acetyl cysteine

In addition to understanding the mechanism by which KEAP1 may be decreased upon USP8 depletion, I also wanted to find out whether the effect of USP8 depletion on cell viability might be linked to changes in NRF2 or KEAP1. Cells with a deregulated ROS management pathway may be less able to handle this stress and therefore undergo cell death more readily.

This leads to the following question: do cells depleted of USP8 have an impaired ability to handle basal ROS levels and could this contribute to the effects on cell viability? If this were the case, one would expect that ROS scavengers such as N-acetyl cysteine (NAC) may rescue the reduced cell viability triggered by USP8 depletion.

I tested this theory by treating USP8 depleted cells with NAC (figure 5.6.A). The literature reports that NAC may be toxic to cells over long time periods. For this reason I added the NAC for only the last 36 hours of the 120 hour

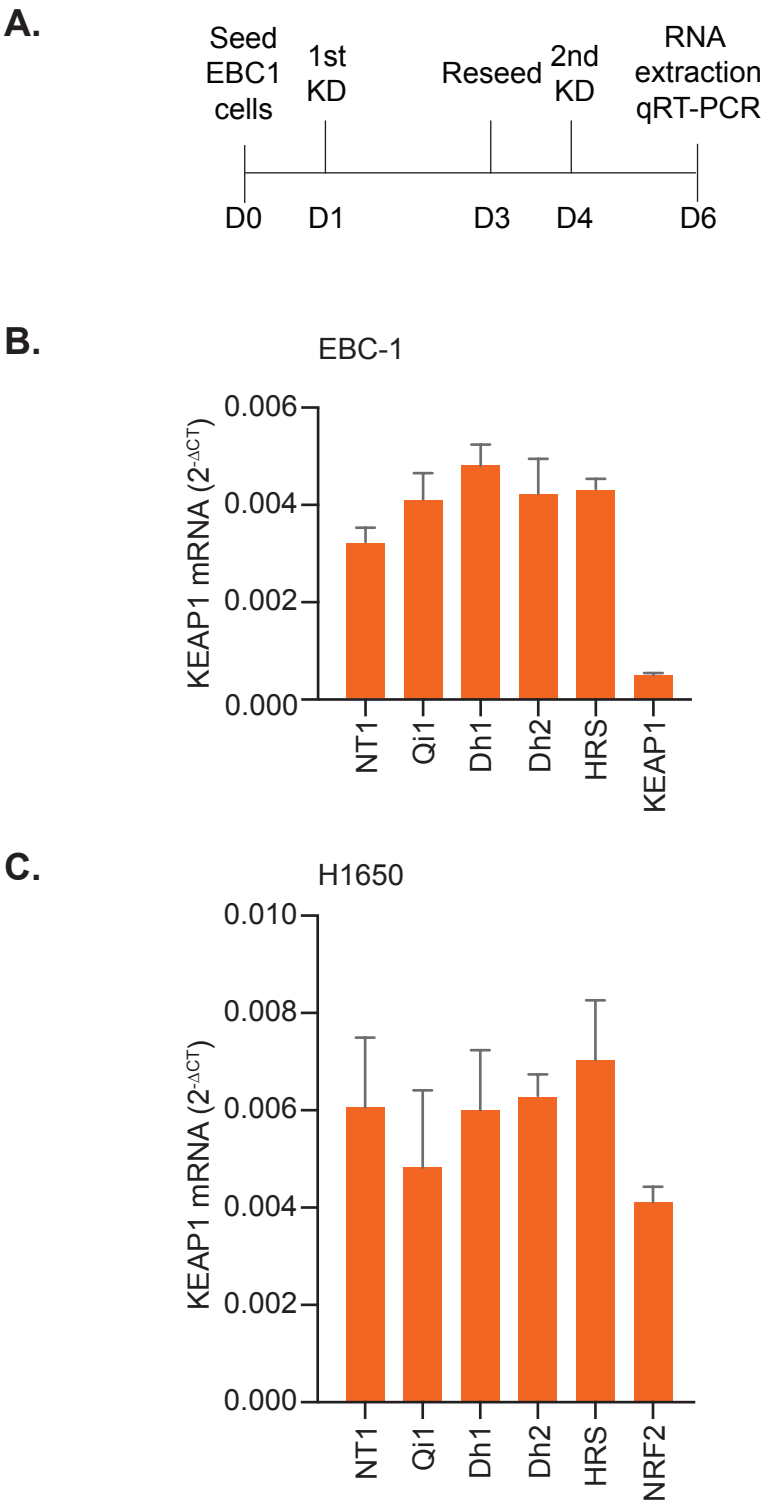


Figure 5.5. KEAP1 mRNA levels do not change with USP8 depletion. (A) Experimental procedure with RNAi KD on day 1 (D1) and day 4 (D4) and re-seeding on day 3 (D3) (B) EBC-1 cells were depleted of USP8, HRS or KEAP1 for 120h. RNA extraction and reverse transcription preceded qPCR with KEAP1 specific primers (n=1). (C) H1650 cells were depleted of USP8, HRS or NRF2 for 120h. RNA extraction and reverse transcription preceded qPCR with KEAP1 specific primers (n=1).

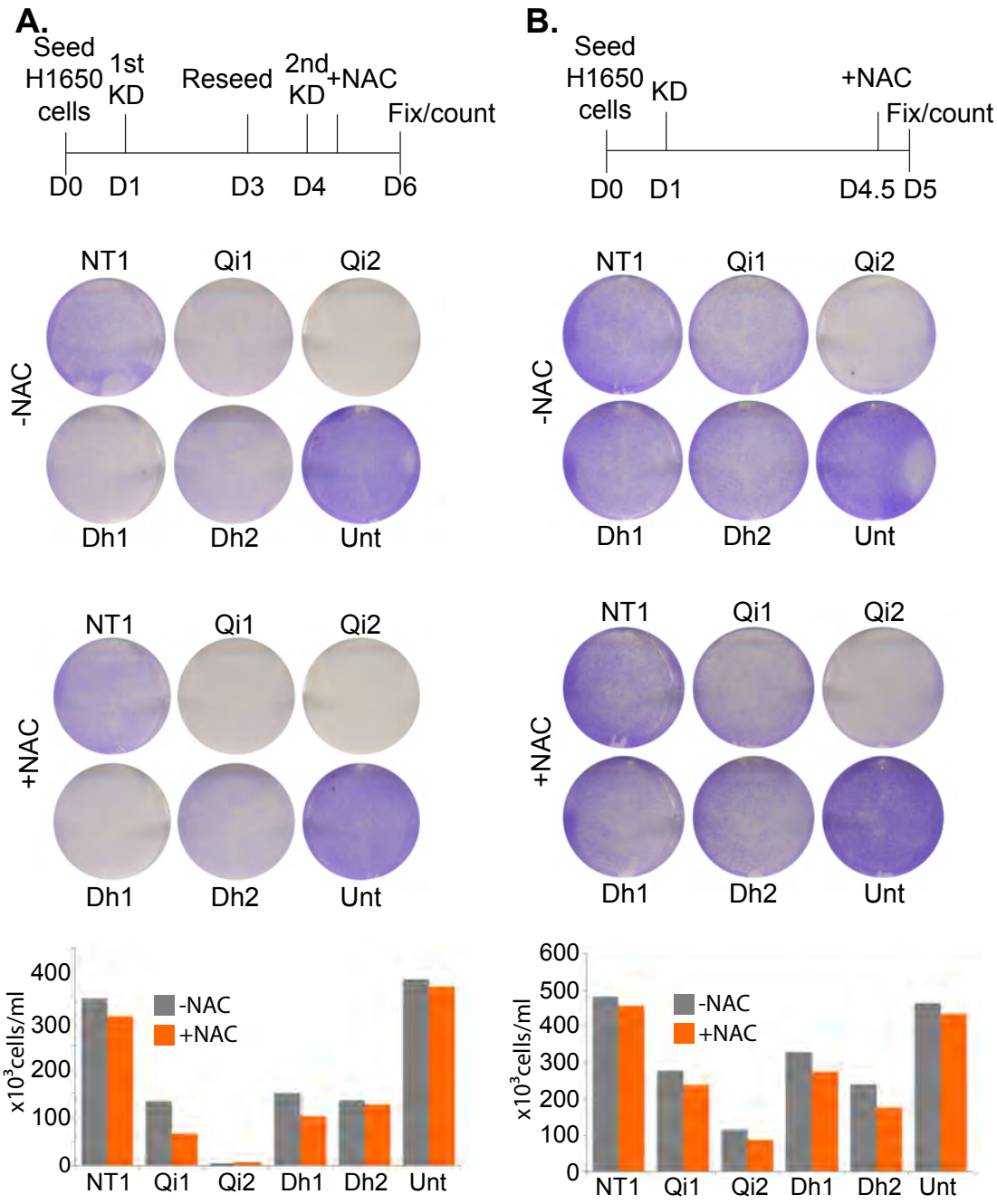


Figure 5.6. USP8 depleted H1650 cell viability is not rescued by treatment with the ROS scavenger N-acetyl cysteine. USP8 depleted cells were treated with the ROS scavenger N-acetyl cysteine (NAC) at a concentration of 10mM according to the protocol time-line indicated. Cells were fixed and stained with crystal violet to identify and changes in cell viability. Cells of two independent experiments were counted following 120h or 96h depletion (A and B respectively).

RNAi protocol. The cells were reseeded the day before this treatment and either fixed with methanol prior to crystal violet staining or counted with an automatic cell counter after 120 hours of RNAi.

In USP8 depleted H1650 cells that were not treated with NAC the reduction in cell viability is striking with both crystal violet staining and cell counting. It is also clear from the NT1 treated control cells that there is little, if any, additional cell death with NAC treatment. This allays the fears about toxicity. However, the addition of NAC to USP8 depleted cells does not rescue the cell number as assessed either by crystal violet staining or cell counting.

In this experiment the cells were depleted of USP8 for a long time and the cells were subjected to considerable stress with reseeding in between the two siRNA treatments. In figure 5.5.B I mitigated this by ending the experiment 24 hours earlier, 96 hours after knockdown, avoiding the need for reseeding and a second siRNA hit. In this case, the effect of USP8 depletion on cell viability is less striking with crystal violet staining, although still evident with cell counting. Also with this protocol I was unable to detect any rescue of cell viability in NAC-treated cells.

5.2.7. H1650 cells are sensitised to ROS induction as assessed by live cell imaging

I next asked the question whether USP8 depleted cells are more sensitive to ROS. To answer this, I used the ROS inducer tert-butyl hydroperoxide (tBHP). When designing this experiment I applied a shorter RNAi protocol. A 72 hour protocol gives sufficient USP8 depletion, as evidenced in figure 5.1.C. However, from my own observations in handling the H1650 cells – the effects on cell viability become most evident at 96 hours. By selecting this shorter protocol I could preclude cell death from USP8 depletion itself and focus on any sensitisation to cell death provoked by the ROS inducer. In figure 5.7 the control cells were loaded with FITC-annexin V after 72 hours of USP8 depletion and, though only a single time point is presented here, the cells were imaged every hour by live-cell imaging. Here any cell death due to USP8 depletion is negligible. Conversely with a 6 hour tBHP treatment there

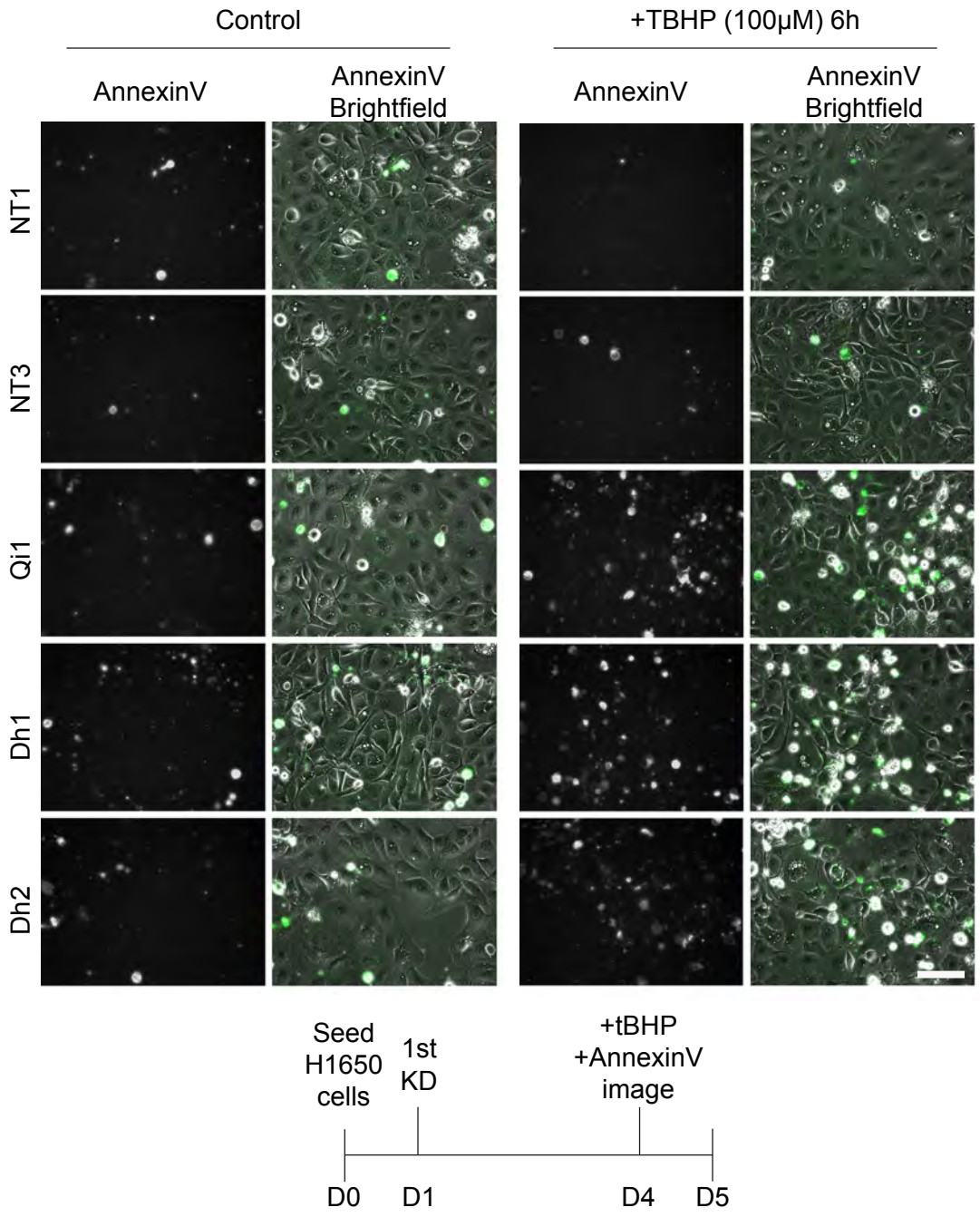


Figure 5.7. USP8 depleted H1650 cells are sensitised to ROS induction. H1650 cells were treated with siUSP8 or control oligos (NT1, NT3) for 72h prior to addition of 100μM tert-butyle hydroperoxide (tBHP) or H2O vehicle (control). The cells were pre-loaded with FITC-annexin V for 30min. Cells were imaged using a Nikon TiEclipse (20x objective) hourly and the 6h time-point is presented here (n=1). The scale bar is 400μm in width.

is a prominent increase in annexin V staining in siUSP8, but not siNT1 treated cells. This indicates that H1650 cells depleted of USP8 are more sensitive to ROS than control cells.

5.2.8. USP8 depleted cells are sensitised to ROS induction as assessed by FACS analysis

Investigating this sensitisation to ROS in a more quantitative fashion, I used FACS analysis with propidium iodide (PI) and FITC-annexin V staining following USP8 depletion and subsequent tBHP treatment. H1650 cells depleted for 72 hours of USP8 were treated with tBHP or vehicle control (H_2O) for 6 or 24 hours (as shown in protocol 5.8.A and figure 5.8.B.) and 10,000 events were measured for each condition as illustrated in the gated plots for the Qi1 oligo. The proportion of total cells stained either with PI or annexin V is presented. Here, despite some slight variation across the NT1 controls for each of the three conditions there is a clear and striking effect following 24 hours of tBHP treatment. The fold increase in cell death with tBHP in combination with USP8 depletion ranges from approximately 3-fold (with the Dh1 siRNA) to approximately 7-fold (with the Dh2 siRNA). I repeated the same experiment focusing on only the 24 hour time point (figure 5.8.C). Here I also included an HRS depleted and a KEAP1 depleted sample. It is important to note the differences in scale between figure 5.8.B and 5.8.C. Whilst the largest percentage of cell death in panel B was a little over 70%, in panel C it was just below 20%. Part of this may be due to variations in seeding density as higher cell confluency appears to confer some level of protection to these cells. Despite this, there is still an evident increase in cell death when combining tBHP and both the Qi1 and Dh2 siRNA with a maximum fold increase of over 7-fold. As with figure 5.8.B the least striking effect is seen when combining tBHP with the Dh1 oligo. Whilst there is a slight increase in cell death combining tBHP with HRS depletion, the proportion is too small to draw any definitive conclusions from this single experiment. It may be difficult to see increases in cell death as HRS depletion is generally very inefficient unless the knockdown is conducted for longer than 96 hours. Note that the half-life of HRS is 41 hours (Schwanhausser,

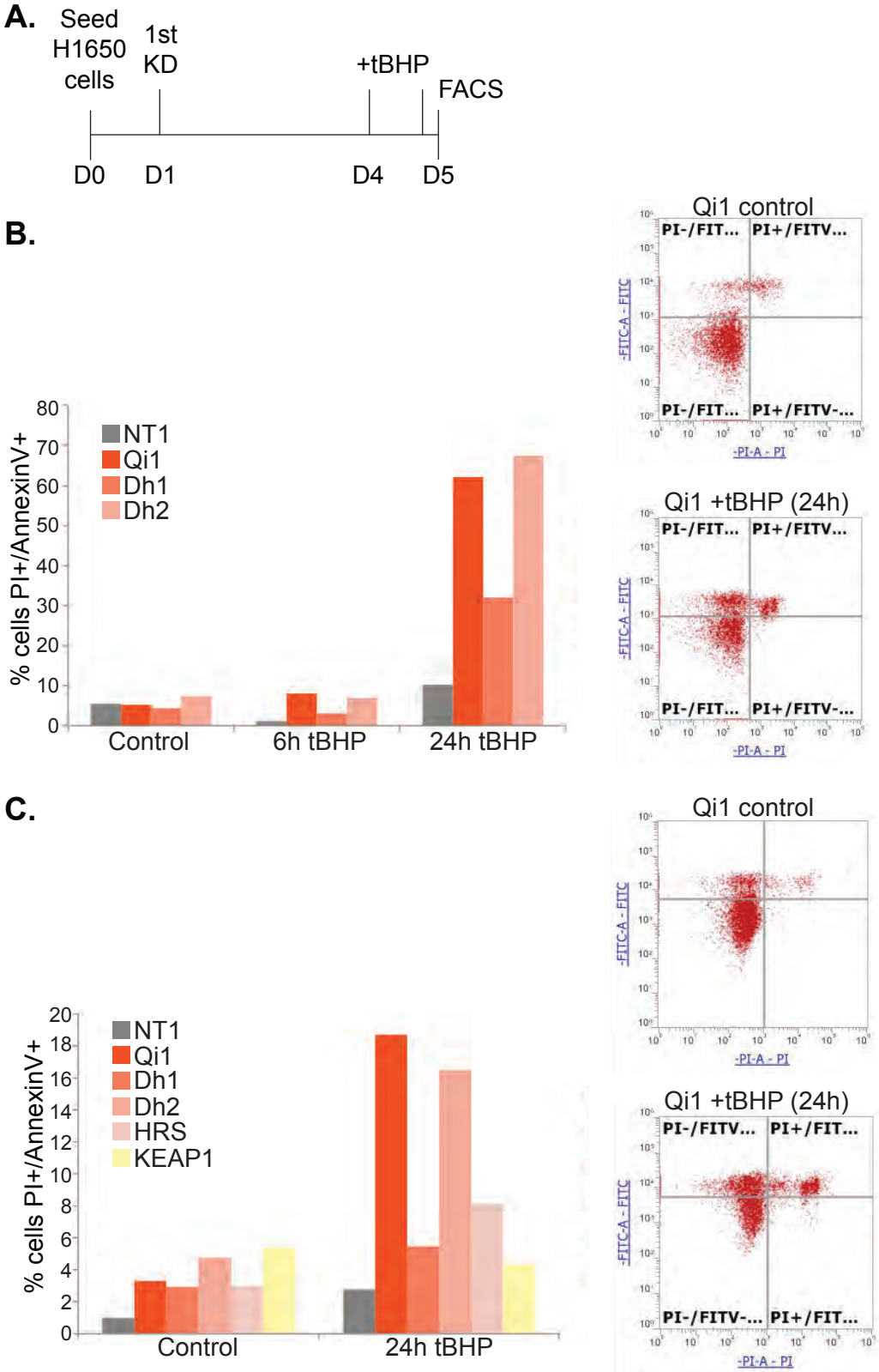


Figure 5.8. USP8 depleted H1650 cells are sensitised to ROS induction. (A) Experimental procedure with RNAi KD on day 1 (D1) and tBHP treatment on day 4 (D4). (B) USP8 depleted cells were treated with tBHP for the indicated times. Cells were trypsinised, stained with PI and FITC-annexin V prior to FACS analysis. (C) USP8 depleted cells were treated with tBHP for 24h, trypsinised, stained with PI and FITC-annexin V then analysed by FACS. (A) and (B) constitute two independent experiments (n=2).

Busse et al. 2011). Disappointingly, there is no evidence of increased cell death upon KEAP1-depletion indicating that the effect of USP8-depletion on cell viability is likely not due to the reduction of KEAP1 (figure 5.8.B).

5.2.9. USP8 depleted EBC1 cells are not sensitised to ROS induction as assessed by live-cell imaging

Next I carried out the same tBHP live-cell imaging experiment as in figure 5.7 this time focusing on EBC1 cells (figure 5.9). As described above I depleted the cells of USP8 or HRS or KEAP1 for 72 hours before the addition of the ROS inducer. The cells were incubated with FITC-annexin V and imaged once per hour to monitor apoptosis. One time point is presented in figure 5.9. It is clear that there is no increase in cell death upon short-term treatment with siRNAs targeting USP8, HRS or KEAP1 and that viability is not differentially affected by the addition of tBHP for 6 hours. This suggests EBC1 cells are not sensitised to ROS induced cell death by short-term depletion of USP8, HRS or KEAP1.

5.2.10. USP8 depleted EBC1 cells are not sensitised to ROS induction as assessed by FACS analysis

To confirm this in a more quantitative manner I employed FACS analysis with PI and annexin V staining in the EBC1 cells. The cells were treated with tBHP for 24 hours before staining with the two dyes and the proportion of total cells stained with either marker of cell death presented in figure 5.10. Whilst there is a low level of cell death in the cells depleted of USP8, HRS or KEAP1 compared to the control targeted cells, this is not increased with tBHP treatment. This confirms that EBC1 cells are not sensitised to ROS induction by depletion of these proteins. The gated plots in figure 5.10.C show the increase of events detected in the upper right quadrant (PI/FITC stained) with USP8 depletion when compared to NT1 in both control and tBHP treated cells.

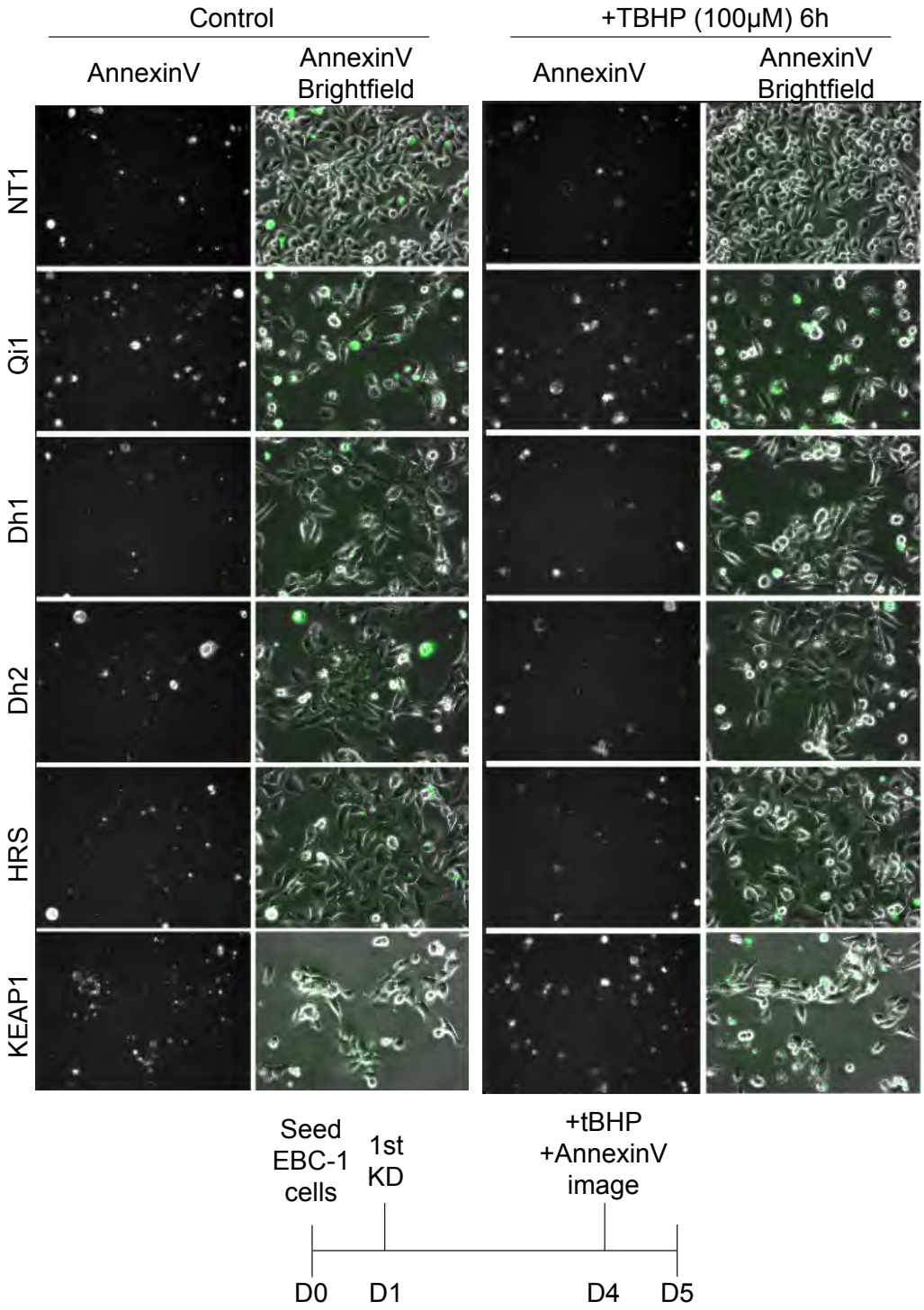


Figure 5.9. USP8 depleted EBC1 cells are not sensitised to ROS induction. EBC1 cells were treated with USP8, control (NT1), HRS or KEAP1 siRNA oligos for 72h prior to additionof 100μM tert-butyle hydroperoxide (tBHP) or control (n=1). The cells were pre-loaded with FITC-annexin V for 30min. Cells were imaged using a Nikon TiEclipse hourly and the 6h time-point presented here.

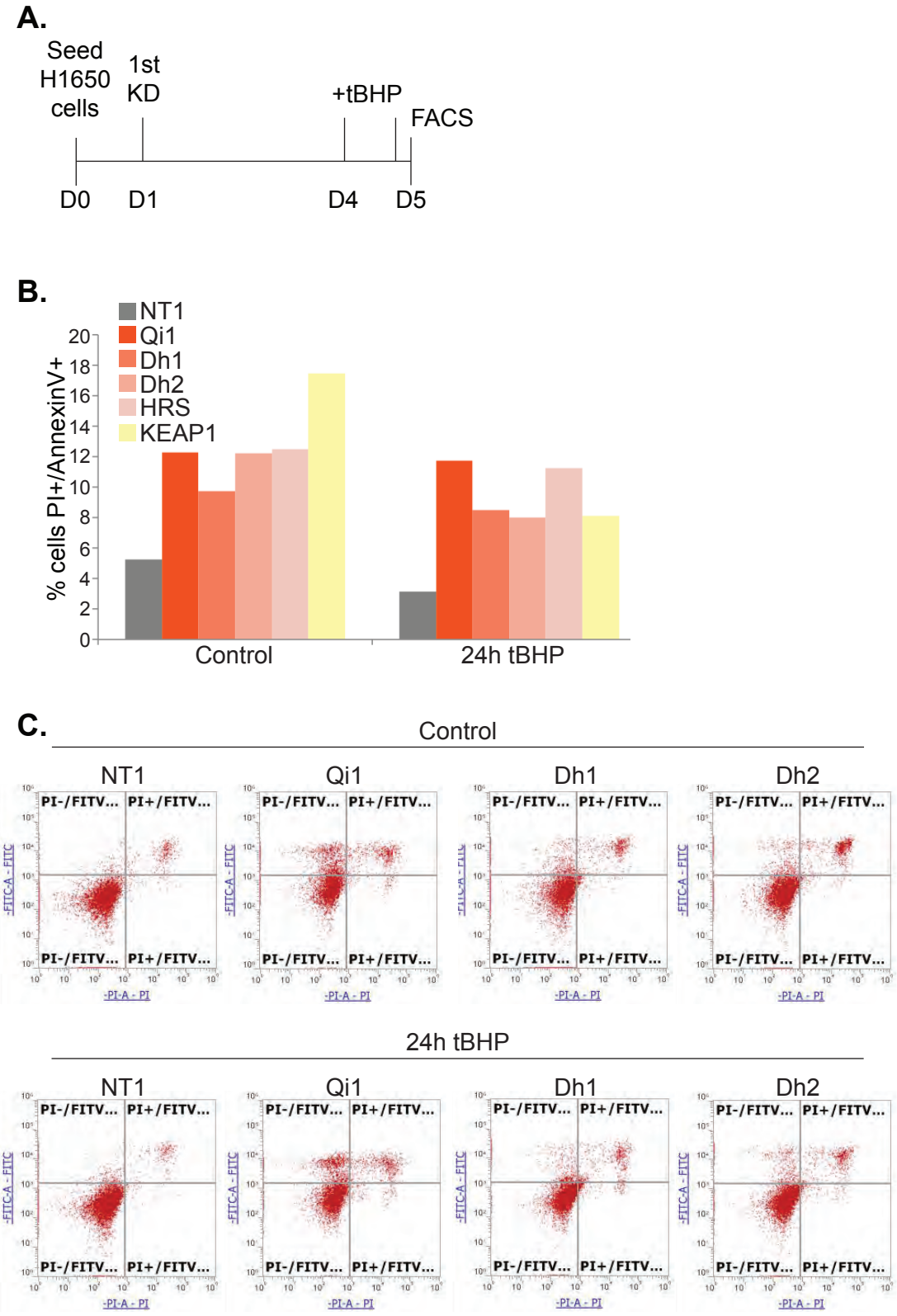


Figure 5.10. USP8-depleted EBC1 cells are not sensitised to ROS induction. (A) Experimental procedure with RNAi KD on day 1 (D1) and tBHP treatment on day 4 (D4). (B) USP8, HRS or KEAP1 depleted cells were treated with tBHP for 24h. Cells were trypsinised, stained with PI and FITC-annexin V prior to FACS analysis (n=1). (C) The gated plots for each of the siNT1 or siUSP8 samples.

5.3. Summary of results

- Depletion of USP8 or HRS reduces KEAP1 protein levels in two cell lines with reported sensitivity to USP8 depletion from two different cancer backgrounds (NSCLC and SQCC)
- Reduction in KEAP1 protein levels was not due to a decrease in KEAP1 mRNA levels
- The cell viability defect caused by USP8 depletion in H1650 cells (described in chapter four) was not rescued by treatment with a ROS scavenger
- USP8-depleted H1650, but not EBC1, cells were sensitised to ROS induction

5.4. Discussion

In this chapter the chief aim was to investigate a suggested link between USP8 and HRS depletion and the NRF2/KEAP1 pathway. Data from Amgen provided evidence that some cell lines with sensitivity to USP8 depletion carried mutations in NRF2 preventing its interaction with and regulation by its inhibitor, KEAP1. This led us to investigate the ability of USP8-depleted cells to manage ROS levels within the cell and the levels of both NRF2 and KEAP1.

5.4.1. USP8 and KEAP1

I showed above that USP8 depletion has apparent and consistent effects on KEAP1 protein levels within both the NSCLC H1650 and the SQCC EBC1 cells. I have shown that this is not due to a change in gene expression as the mRNA levels remain unchanged thus indicating either a change in protein stability or a change in translation of the protein. One question remaining is how USP8-depletion brings about this reduction in KEAP1 levels. USP8 is a DUB that regulates the levels of a number of proteins including STAM and several membrane spanning receptors such as EGFR and Smoothened

(Row, Prior et al. 2006, Alwan and van Leeuwen 2007, Xia, Jia et al. 2012). It is thus conceivable that changes in KEAP1 levels in response to USP8 depletion are due to changes in the protein's stability. A simple way to assess this would be to perform a cycloheximide chase experiment under USP8 depletion conditions to identify changes in protein turnover. Additionally, lysosomal and proteasomal inhibitors could be employed to see whether these can rescue KEAP1 protein levels.

Whilst KEAP1 is cytosolic, its levels can be regulated by autophagy. KEAP1 has been shown to interact with the selective autophagy receptor, p62. Selective autophagy of protein aggregates is known as aggrephagy (Svenning and Johansen 2013). p62 is recruited to ubiquitylated protein aggregates via its ability to interact with ubiquitin. It then recruits the autophagic machinery through its LC3 interacting region (LIR) allowing protein aggregates to be enveloped by the autophagosome (Katsuragi, Ichimura et al. 2015). The autophagosome ultimately fuses with the lysosome forming the autolysosome where its contents are degraded. KEAP1 has been linked to this pathway through an interaction between the DGR of KEAP1 and the STGE motif within the KEAP1 interacting region (KIR) of p62 (Komatsu, Kurokawa et al. 2010). This interaction is facilitated by phosphorylation of aggregated p62 by mTORC1 and subsequently allows release and activation of NRF2 (Komatsu, Kurokawa et al. 2010, Ichimura, Waguri et al. 2013). Preliminary experiments undertaken by Amgen show that USP8-depleted U2OS cells stably expressing GFP-p62, accumulate p62 positive punctae or aggregates upon USP8 depletion. Thus it is possible that USP8 depletion enhances the formation of p62-positive aggregates that may sequester KEAP1 into autophagosomes.

In line with this, a recent study reported that USP8 depletion by dsRNA in Drosophila increased basal autophagy identified by an accumulation of GFP-LC3B positive dots (Jacomin, Bescond et al. 2015). However the authors went on to show that USP8 depletion caused a block in autophagic flux since the accumulation of autophagosomes was not accompanied by an increase in the number of autolysosomes. In addition, large perinuclear lysosomes were lost in USP8-depleted cells whilst smaller vesicles were retained

(Jacomin, Bescond et al. 2015). The authors suggested that these vesicles may correspond to vesicles transporting lysosomal enzymes from the biosynthetic pathway. In fact, USP8 depletion in HeLa cells had previously been shown to prevent recycling of the mannose 6 phosphate receptor (M6PR) back from endosomes to the TGN (MacDonald, Urbe et al. 2014). As a result newly synthesized lysosomal enzymes (Cathepsin D) failed to be delivered to the lysosome and instead were found to be secreted in the medium. Over a long period this may result in the accumulation of non-functional lysosomes that are depleted of lysosomal enzymes.

A block in autophagic flux could also in principle reflect the destabilisation of ESCRT-0 components in USP8-depleted cells. The ESCRT machinery has also been linked to aggrephagy. In particular, depletion of the ESCRT-0 component HRS increases p62 levels and ubiquitylated proteins in mice (Oshima, Hasegawa et al. 2016). The same study showed a block in autophagic flux in HRS-depleted PC12 cells alongside accumulation of p62. Given that USP8 is known to regulate HRS and STAM protein levels, one might expect USP8 depletion to inhibit aggrephagy and thus prevent degradation of protein aggregates (Row, Prior et al. 2006).

In principle it is possible that USP8 depletion causes the sequestration of KEAP1, through its interaction with p62, in autophagosomes which are not subsequently degraded due to an additional block in autophagic flux. If KEAP1 is sequestered in autophagosomes which are unable to fuse with the lysosome one might see a reduction in cytosolic KEAP1 as it is sequestered within membrane bound structures. However it seems unlikely that the reduction in KEAP1 I observed in USP8-depleted cells is due to its sequestration and failed degradation as I saw a similar reduction in KEAP1 with RIPA lysis. RIPA lysis buffer contains SDS detergent and should extract proteins sequestered within such membrane structures. This was shown in a recent proteomics study which sought to isolate autophagosomes and identify proteins within (Dengjel, Hoyer-Hansen et al. 2012). The authors used SDS lysis to solubilise autophagosomal proteins prior to processing the samples for mass spectrometry and were able to identify key

autophagosomal proteins such as LC3 and p62 (Dengjel, Hoyer-Hansen et al. 2012).

Importantly, USP8 itself has been proposed to reduce the clearance of α-synuclein positive protein aggregates (Lewy bodies) through its removal of K63-linked ubiquitin chains from α-synuclein (Alexopoulou, Lang et al. 2016). Overexpression of wild-type USP8 was shown to reduce ubiquitylation of endogenous α-synuclein whilst USP8-depletion promoted its lysosomal degradation (Alexopoulou, Lang et al. 2016). It is unclear at the moment whether the autophagy machinery is involved in this process.

A number of experiments might be useful to assess the potential role of USP8 and the association of KEAP1 with the autophagic pathway. Firstly it will be important to understand if USP8 depletion causes any changes in the levels and/or distribution of key autophagy components such as LC3-II and p62. This can be assessed by western blotting and immunofluorescence. Secondly, if p62 is important in recruiting KEAP1 to autophagosomes then one might expect depletion of p62 would rescue the reduction in KEAP1 levels with USP8 depletion. Additionally – as mentioned above – lysosomal inhibitors could provide information on this pathway since the lysosomal inhibitor bafilomycin blocks autophagy. If this inhibition rescues the KEAP1 levels we can have some level of confidence that the pathways are linked.

In addition to understanding how KEAP1 is degraded - it would be of interest to determine if KEAP1 is a direct substrate of USP8. Co-immunoprecipitation would tell us whether USP8 and KEAP1 interact directly. We can also assess the ubiquitylation status of KEAP1 upon USP8 depletion or co-expression with a catalytically inactive USP8-mutant.

5.4.2. USP8 and ROS

The other key finding in this chapter is that H1650 cells, but not EBC1 cells, are sensitised to ROS induction with USP8 depletion. This could, in part, be due to the different NRF2 status in the two cell lines. As mentioned above, EBC1 cells have a mutation in one of the two KEAP1 binding domains, the

ETGE motif. This motif is absolutely required for the NRF2-KEAP1 interaction and therefore mutation in this motif uncouples NRF2 from its inhibitor, KEAP1. H1650 cells however have wild-type NRF2, which should be under the control of KEAP1. A loss of KEAP1 in USP8-depleted cells should therefore result in the stabilisation of NRF2. Surprisingly I observed an increase in NRF2 protein levels not just in H1650 but even most strikingly in EBC1 cells (see figure 5.4). It would be interesting to see whether this also results in the increased transcription of NRF2-responsive genes. This could be assessed by qRT-PCR using primers for transcriptional targets of NRF2 such as NQO1, or using an NRF2-responsive Luciferase reporter. Importantly, it is unlikely that the loss of KEAP1 protein levels is directly responsible for the sensitisation of USP8-depleted cells to ROS as a) KEAP1 levels are decreased equally in both sensitised (H1650) and non-sensitised (EBC1) cell lines, and b) direct KEAP1 depletion by siRNA in H1650 cells does not sensitise cells to ROS.

It is possible that USP8 depletion increases the basal ROS levels in the cells, which make any additional increase in ROS more problematic for the cells. In order to test this I attempted to use a probe for ROS using live cell imaging. The probe I used was CM-H_2DCFDA which upon oxidation generates a fluorescent adduct which can be measured by flow cytometry or fluorescent microscopy. Whilst this attempt was unsuccessful, researchers at Amgen did work with a similar probe and did not see a detectable increase in ROS in USP8-depleted EBC1, HCC70 or LK2 cells. However they did not assess ROS levels in H1650 cells, thus this negative result needs confirming.

Although the USP8-depleted cells do not have increased basal levels of ROS, it is still possible that the cells are less well equipped to deal with ROS and that this might explain the synthetic lethal effect of combining ROS induction with USP8 depletion. Investigating changes in transcription of downstream effectors of NRF2, such as NQO1, by qRT-PCR would tell us if the pathway is responding as expected. Interestingly, a recent study has provided further evidence that this pathway is linked to autophagy by suggesting that p62 might be under transcriptional control of NRF2 (Jain, Lamark et al. 2010). If cells without NRF2 have reduced autophagic gene

expression then we could consider that the reverse may be true: NRF2 released from its inhibition by depleted KEAP1 levels could lead to increased autophagy. Investigating whether cells depleted of USP8, and therefore KEAP1, show increased expression of autophagy related genes would be an interesting question to answer.

KEAP1 has also been linked to regulation of cell death. KEAP1 is reported to interact with PGAM5, an outer mitochondrial protein which binds to the anti-apoptotic BCL2 family member BCL-XL (Stepkowski and Kruszewski 2011). PGAM5 is a phosphatase which can dephosphorylate apoptosis signalling kinase 1 (ASK1) and therefore prevent apoptosis. If KEAP1 is degraded by autophagy, one might suppose that PGAM5 is recruited to autophagosomes through this interaction with KEAP1. This might sensitise cells to stress such as ROS induction. However, KEAP1-deficient MEFs are resistant to cell death by ROS induction due to a failure to activate ASK1 (Stepkowski and Kruszewski 2011). This lends weight to the argument that the synthetic cell death induced by ROS in USP8-depleted cells is not caused by a loss in KEAP1. It would thus also be of interest to monitor the levels of apoptosis related proteins upon USP8 depletion in relation to the cell death effects identified.

Overall this chapter has answered some interesting and novel questions about a potential link between the NRF2/KEAP1 pathway and USP8 and led to several avenues worthy of further investigation. It also offers some hint for ways to explore the finding in the previous chapter that USP8 depletion causes cell death.

Chapter six: final discussion

In this thesis I report that USP8-depletion reduces cell viability of a NSCLC cell line (chapter 4) and that combination of USP8-depletion with ROS induction has an additive effect in that cell line (chapter 5). Meanwhile, a USP8-depleted SQCC cell line is not sensitised to ROS induced cell death (chapter 5). USP8-depletion has an additional synthetic lethal effect when combined with PI3K pathway inhibition in U2OS cells stably expressing FOXO3, parental U2OS cells and H1975 cells (chapter 3).

6.1. USP8 and cell viability

My findings in this thesis point to multiple roles for USP8 in cell viability. There are two key findings: 1) USP8 plays an unidentified role in cell viability and 2) USP8-depletion sensitises some cells to additional stresses.

USP8-depletion reduces cell viability of two NSCLC cell lines reported here. Cell numbers were reduced in H1975 and H1650 cells upon 120 hour USP8 depletion and FITC-annexin V staining was increased in the H1650 cells (figures 4.4 and 4.5). Whilst I do not show data here pertaining to the cell viability effect of USP8-depletion on EBC1 cells, Amgen found these cells and a number of others (HCC70, HCC1954 – see chapter 5) to be sensitive to both USP8- and HRS- depletion. In the course of my experiments, I also observed a reduction in cell viability upon USP8-depletion in EBC1 cells.

This is in line with evidence, discussed throughout this thesis, that USP8 depletion reduces cell viability in NSCLC cell lines (Panner, Crane et al. 2010, Byun, Lee et al. 2013). In addition, USP8 depletion reduces the viability of three multiple myeloma cell lines (KMS11, 8226 and JJN3) (Tiedemann, Zhu et al. 2012). USP8 was found by two of three analyses in a genome-wide pooled, shRNA screen of gene essentiality to be essential for proliferation in NSCLC cells (Cheung, Cowley et al. 2011). The genetic knockout of USP8 in mice is embryonically lethal (Niendorf, Oksche et al. 2007).

In particular, I noticed the cell viability effect was most evident following long USP8-depletion protocols. In figure 5.7, H1650 cells treated with USP8 siRNA for approximately 78 hours do not undergo apoptosis, whereas cells treated for 120 hour with USP8 siRNAs do (figure 4.5). RNAi experiments are typically performed on shorter time scales which may conceal the effect of USP8- depletion on cell viability. In order to assess the extent of the effect of USP8-depletion on cell viability, it would therefore be of interest to treat a panel of cancer and non-cancer cells with USP8 siRNA over long, 120 hour experiments. This reliance on longer knockdown experiments may also allude to a requirement for a complete USP8 knockdown in order to elicit this effect. The embryonic lethality of USP8 knockout in mice lends weight to this argument. Another way to test this would be the generation of CRISPR knockout cell lines which would allow for complete removal of USP8.

The other effect USP8-depletion has on cell viability is the sensitisation of U2OS cells, U2OS stably expressing GFP-FOXO3 and H1975 cells to PI3K pathway inhibition. This pathway is frequently mutated in cancer leading to its hyperactivation, with the gene for the p110α subunit (PIK3CA) one of the most commonly mutated genes in breast cancer (Kan, Jaiswal et al. 2010). Inhibitors of the pathway such as class I PI3K inhibitor GDC-0491 and the p110α inhibitor GDC-0032 are under clinical evaluation (Thorpe, Yuzugullu et al. 2015). In addition I observed a USP8-depletion dependent sensitisation to cell death by ROS induction, which I will discuss below.

6.2. USP8 and ROS

My results suggest that the sensitisation of USP8-depleted cells to ROS is not universal: Only H1650 but not EBC1 cells showed enhanced apoptotic cell death in response to treatment with tBHP. One way in which H1650 and EBC1 cells differ is their mutational status of NRF2. H1650 cells have wild-type NRF2, whilst the EBC1 cells carry a heterozygous mutation which disrupts the binding of NRF2 to its inhibitor, KEAP1.

In chapter five I showed that USP8 depletion reduces the protein levels of KEAP1. This would mean H1650 cells depleted of USP8 might already be

sensitised to ROS induction as a reduction in KEAP1 levels releases some NRF2 from its inhibition. Further disruption of the NRF2-KEAP1 interaction by ROS induction might then enhance transcription of downstream NRF2 effectors. Whether this is truly the case would be an interesting question, which could be answered using luciferase assays or qRT-PCR to assess the basal mRNA levels of downstream NRF2 targets such as NQO1. Conversely, half of the NRF2 in EBC1 cells is already uncoupled from KEAP1 through the heterozygous D77V mutation. USP8 depletion reduces KEAP1 levels which might allow the release of the wild-type NRF2 from its inhibition but ROS induction does not have an additive effect. Why EBC1 cells are not sensitised remains to be investigated. It is also possible that the mechanism for this sensitisation does not hinge on the decrease in KEAP1 as KEAP1-depletion does not sensitise H1650 cells to ROS induction.

Both H1650 and EBC1 cells are NSCLC cell lines however H1650s are derived from epithelial adenocarcinoma whereas EBC1 are derived from squamous cell carcinoma. H1650 cells have an activating mutation in EGFR (Δ746-750). According to the catalogue of somatic mutations in cancer (COSMIC), EBC1 cells do not carry an EGFR mutation. EBC1 cells, however, do carry a MET amplification (Lutterbach, Zeng et al. 2007).

Studies have shown that a phosphatase involved in the regulation of both EGFR and MET, PTP1B, can be inactivated through ROS oxidation of its active site cysteine (Lee, Kwon et al. 1998, Tonks 2005, Heppner and van der Vliet 2016). In addition, PTP1B depletion delays ligand-induced degradation of EGFR and MET by preventing their internalisation (Sangwan, Abella et al. 2011). One difference between these two cell lines is their potential sensitivity to ligand-activation. In the case of EBC1 cells MET is amplified but not constitutively activated thus should be sensitive to activation by its ligand, HGF. Conversely the mutation in EGFR the H1650s carry, leads to constitutive activation of the receptor thus these cells should not be sensitive to activation by EGF. MET and EGFR signalling pathways bear some similiarities to one another in that both activate downstream MAPK and PI3K signalling pathways (Organ and Tsao 2011). Based on the studies in PTP1B, one would expect that both cell lines have a reduction in PTP1B

activation with ROS induction and therefore delayed ligand-induced degradation of MET and EGFR. USP8 is linked to the ligand-induced downregulation of both of these receptors; therefore one future avenue for investigation might be whether ligand treatment alters the sensitisation to ROS. This is of particular interest as each cell line should have different ligand responses as described above (Row, Prior et al. 2006).

6.3. USP8 substrates

USP8 is a DUB with many reported substrates (see chapter one). The reduction in KEAP1 levels following USP8 depletion might point to KEAP1 as a novel substrate. It would be of interest to see if the two proteins interact directly and to assess the ubiquitylation status of KEAP1 both upon USP8 depletion and overexpression of wild-type versus catalytically inactive USP8. Many USP8 substrates are degraded by the lysosome. However, KEAP1 can be sequestered in autophagosomes and degraded by autophagy (Taguchi, Fujikawa et al. 2012). USP8 has also been linked to regulation of this pathway and has been linked to the degradation of aggregated proteins such as α-synuclein (Jacomin, Bescond et al. 2015, Alexopoulou, Lang et al. 2016). It would therefore be interesting to see if blocking autophagy rescues the effect of USP8 depletion on KEAP1 levels.

One way to investigate cell surface USP8 substrates in an unbiased way is to combine mass spectrometry with cell surface biotinylation. In preliminary experiments which are not reported within the context of this thesis, I treated SILAC labelled MCF7 cells with USP8 siRNA oligos (Dh1 or Dh2) or non-targeting (NT1) siRNA. In order to identify proteomic changes on the cell surface I labelled cell surface proteins with membrane impermeable biotin. I lysed the cells in NP40 buffer then mixed the lysates 1:1:1 and used a streptavidin pulldown to isolate biotin labelled proteins. I then used LC/MS/MS to quantitate the protein levels across light (NT1), medium (Dh1) and heavy (Dh2) labelled cells. In this first preliminary experiment approximately 40% of the proteins identified were surface proteins. Selecting only proteins that were enriched or de-enriched beyond a set threshold I

found two proteins that were of interest for follow up. In both Dh1 and Dh2 treated cells the collagen RTK receptor discoidin domain receptor 1 (DDR1) was enriched. Dh1 treated cells had almost five times as much DDR1 and Dh2 treated cells had twice as much of this receptor compared to the NT1 treated cells. Meanwhile a subunit of the integrin α2/β1 collagen receptor, integrin α2 (ITGA2) was de-enriched compared to control treated cells by one and one and a half times in Dh1 and Dh2 treated cells, respectively. I confirmed these changes biochemically using western blotting in MCF7 cells. Here we have two collagen receptors changing in opposing directions with USP8 depletion which may hint at a compensatory mechanism. Interestingly, both proteins are involved in the migration of cells. In particular, DDR1 has been shown to play a role in the formation of linear invadosomes whereas ITGA2 is enriched in focal adhesions (Juin, Di Martino et al. 2014). In fact, collagen mediated epithelial to mesenchymal transition (EMT) has been linked to cooperative α2/β1 and DDR1 signalling (Shintani, Fukumoto et al. 2008). One might postulate that an increase in DDR1 and a decrease in ITGA2 encourages migration of USP8 depleted cells although this would require much further investigation. One way to assess this would be to use live-cell imaging of cells as they adhere to collagen and see if USP8 depletion alters this adhesion. Live-cell imaging would also tell us if USP8 depletion alters the motility of cells on a collagen substrate.

6.4. USP8 and disease

Recent studies have linked USP8 to Cushing's disease (CD). Cushing's disease is caused by pituitary corticotroph adenomas which secrete elevated levels of adrenocorticotropic hormone (ACTH).

The first of these studies used exome sequencing to identify protein-altering mutations in corticotroph adenomas (Reincke, Sbiera et al. 2015). Four of ten tumours derived from patients with CD had a somatic missense mutation in USP8. All of these mutations were between amino acids 713 and 720 corresponding to the region conferring 14-3-3 binding. Previous studies have shown that USP8 binding to 14-3-3 inhibits its activity and that

phosphorylation of S680 in the M phase ablates this binding (Mizuno, Kitamura et al. 2007). Reincke et al. reported that the USP8-mutants, which they identified in an additional six of seventeen corticotroph adenoma tumours, showed reduced or no 14-3-3 protein binding. In addition they identified a 90kDa and a 40kDa USP8 band by western blotting, which they proposed might correspond to proteolytical cleavage products. The 40kDa, C-terminal region is cleaved between amino acids 714 and 715 and has high DUB activity as it contains the USP domain (see figure 4.3 for USP8 domain structure). This short USP8 fragment does not comprise the MIT domain and only retains one of the three SH3 domains required for localisation to the ESCRT machinery. COS7 cells transfected with the active point mutants lacking 14-3-3 binding or the 40kDa truncated USP8 showed reduced ubiquitylated EGFR and increased recycling of this receptor to the plasma membrane following EGF-stimulated internalisation.

Subsequent studies have reiterated that USP8 gain-of-function mutations are associated with CD. In the same year, Ma et al. reported three types of USP8 mutation in 8 out of 12 ACTH-secreting pituitary adenomas identified by whole-exome sequencing (Ma, Song et al. 2015). Following up with targeted sequencing they found 17 USP8 variants across an additional 67 of 103 ACTH-secreting pituitary adenomas. Again, USP8 mutation disrupted 14-3-3 interaction and increased EGFR protein abundance. Importantly, USP8 depletion or EGFR inhibition with the small molecule inhibitor, gefitinib, reduced ACTH secretion in primary cells derived from patient tumours. In fact the presence of USP8 mutations is also correlated with a positive response to the CD treatment pasireotide (Hayashi, Inoshita et al. 2016).

Studies in mice showed that the EGFR pathway is essential for the synthesis of the ACTH precursor proopiomelanocortin (POMC) (Fukuoka, Cooper et al. 2011). Gefitinib treatment inhibits hyper-secretion of ACTH, inhibits proliferation and enhances apoptosis of corticotroph tumour cells in mouse models. Since USP8 is a major regulator of EGFR trafficking, its hyperactivation might promote ACTH secretion by promoting the recycling of EGFR to the plasma membrane and thereby increasing EGFR levels at the cell surface.

A role for USP8 activity in gefitinib resistant non-small cell lung cancer cells (NSCLC) has also been suggested (Byun, Lee et al. 2013). USP8 depletion reduced the viability of H1650 and H1975 cells. The authors reported a reduction in basal growth factor receptor levels such as EGFR, ERBB3 and MET. This was a finding I was unable to reproduce in the same cells in chapter four. However I did see an effect on cell viability following long-term (120 hours) depletion of USP8 in these cells. Whilst the reason for the decrease in the survival of these cells is unclear, it is possible that these cells, which have constitutively active EGFR, are particularly reliant on the EGFR pathway through oncogene addiction (Sharma and Settleman 2007). USP8 depletion delays EGFR degradation however the receptor is still internalised and is stalled at enlarged, aberrant endosomes in its ubiquitylated state (Row, Prior et al. 2006). Although there is some evidence that receptors can still signal from endosomes, typically endocytosis attenuates signalling (Sorkin and von Zastrow 2009). Thus cells with an activating EGFR mutation (and possible oncogene addiction) might be sensitised to cell death by entrapment of the active EGFR within the lumen of the endosome or multi-vesicular body.

6.5. USP8 as a drug target

In chapter four I showed that USP8 depletion reduced cell viability of H1975 and in particular, H1650 cells. Byun and colleagues also reported this effect which they were able to extend to an USP8 inhibitor (Byun, Lee et al. 2013). This was shown to be due to an increase in apoptosis (also see figure 4.5) (Jeong 2015). The literature suggests that this is specific to gefitinib resistant NSCLC. This makes USP8 a promising target in gefitinib resistant NSCLC. My results confirm that USP8 depletion in H1650 results in reduced cell viability. Future work might be useful to establish the effect of USP8 depletion on cell viability in a range of cancer types to investigate this further. In particular we should like to compare the effect of USP8 depletion in MCF10A parental cell lines with MCF10A expressing constitutively active EGFR. As mentioned above, USP8 depletion also reduced cell viability in multiple myeloma cells (Tiedemann, Zhu et al. 2012).

USP8 is also of general interest as a therapeutic target in cancer due to its role in the regulation of receptors often associated with tumourigenesis. Amongst the proposed substrates of USP8 are EGFR and MET. EGFR family members have been linked to lung cancer, breast cancer and glioblastoma, whilst MET receptor is linked to gastric and oesophageal carcinomas, medulloblastomas and liver metastases from colon cancer (Organ and Tsao 2011, Lemmon, Schlessinger et al. 2014).

USP8 depletion delays the EGF-induced degradation of EGFR; therefore at face value USP8 inhibition might not hold promise in EGFR positive cancers. However, the effect of USP8 inhibition on constitutively active EGFR has not yet been studied. Furthermore, inhibiting the catalytic activity of USP8 may not be equivalent to removing the whole protein by siRNA. In addition there are studies that suggest USP8 depletion enhances EGFR degradation – this may depend on the level and duration of USP8 depletion since conditional USP8 knockout mice also have reduced basal EGFR levels (Niendorf, Oksche et al. 2007). Mizuno et al. noted that they only saw endosomal aberration in 20-30% of USP8-depleted cells and proposed that cells without a complete USP8 depletion might degrade EGFR more rapidly (Mizuno, Kobayashi et al. 2006). The use of USP8 inhibition in cancer treatment has yet to be established, in part due to the lack of commercially available, specific USP8 inhibitors. As discussed in chapter five, the currently available USP8 inhibitors also inhibit a variety of other DUBs (Ritorto, Ewan et al. 2014).

Another way USP8 might be of interest as a therapeutic target in cancer is in combinatorial therapies. I described above the synthetic lethal effect of combining USP8 depletion and PI3K pathway inhibition and that the PI3K pathway is frequently mutated in cancer. In fact, the PI3K pathway has been proposed as a target for combinatorial therapies before. This is of particular use when cancer cells have acquired resistance to inhibitors at other levels (either up or downstream) of the pathway (Thorpe, Yuzugullu et al. 2015).

6.6. Concluding remarks

USP8 is a promiscuous DUB which is involved in the regulation of a whole range of pathways, substrates and ubiquitin chain-linkages. It has been linked to the regulation of endocytosis of cell surface proteins such as EGFR and MET, stability of cytosolic proteins such as Nrdp1 and STAM, delivery of lysosomal enzymes such as Cathepsin D, autophagic flux and selective autophagy (Row, Prior et al. 2006, Durcan, Tang et al. 2014, MacDonald, Urbe et al. 2014, Jacomin, Bescond et al. 2015). Here, I report a novel role for USP8 in regulating KEAP1 protein levels and reducing the sensitivity of cells to ROS. Whether the mechanism by which USP8 promotes the turnover of KEAP1 is linked to the endocytic pathway remains to be investigated.

Importantly, I show that USP8 is important in cancer cell viability. Preliminary, unpublished data hint at an additional role for USP8 in migration and outlines a technique for unbiased investigation of novel USP8 substrates. USP8 depletion has effects on endosomal morphology, global ubiquitin levels and the accumulation of ubiquitylated proteins at the endosome (Row, Prior et al. 2006). All of these suggest that USP8 depletion has pleiotropic effects on a number of pathways.

This DUB plays a role in the regulation of a number of proteins upregulated in cancer making USP8 an interesting potential drug target. However the pleiotropic effects described above, and that there is a potential variability in response depending on the degree and duration of USP8-depletion make its role as a drug target, complicated.

This thesis provides many new avenues for investigation to establish the role of USP8 in novel pathways such as ROS management and cell viability.

Bibliography

Abdul Rehman, S. A., Y. A. Kristariyanto, S. Y. Choi, P. J. Nkosi, S. Weidlich, K. Labib, K. Hofmann and Y. Kulathu (2016). "MINDY-1 Is a Member of an Evolutionarily Conserved and Structurally Distinct New Family of Deubiquitinating Enzymes." Mol Cell **63**(1): 146-155.

Abrahamsen, H., H. Stenmark and H. W. Platta (2012). "Ubiquitination and phosphorylation of Beclin 1 and its binding partners: Tuning class III phosphatidylinositol 3-kinase activity and tumor suppression." FEBS Lett **586**(11): 1584-1591.

Agromayor, M. and J. Martin-Serrano (2006). "Interaction of AMSH with ESCRT-III and deubiquitination of endosomal cargo." J Biol Chem **281**(32): 23083-23091.

Agromayor, M., N. Soler, A. Caballe, T. Kueck, S. M. Freund, M. D. Allen, M. Bycroft, O. Perisic, Y. Ye, B. McDonald, H. Scheel, K. Hofmann, S. J. Neil, J. Martin-Serrano and R. L. Williams (2012). "The UBAP1 subunit of ESCRT-I interacts with ubiquitin via a SOUBA domain." Structure **20**(3): 414-428.

Ahle, S., A. Mann, U. Eichelsbacher and E. Ungewickell (1988). "Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane." EMBO J **7**(4): 919-929.

Alexopoulou, Z., J. Lang, R. M. Perrett, M. Elschami, M. E. Hurry, H. T. Kim, D. Mazaraki, A. Szabo, B. M. Kessler, A. L. Goldberg, O. Ansorge, T. A. Fulga and G. K. Tofaris (2016). "Deubiquitinase Usp8 regulates alpha-synuclein clearance and modifies its toxicity in Lewy body disease." Proc Natl Acad Sci U S A **113**(32): E4688-4697.

Ali, N., L. Zhang, S. Taylor, A. Mironov, S. Urbe and P. Woodman (2013). "Recruitment of UBPY and ESCRT exchange drive HD-PTP-dependent sorting of EGFR to the MVB." Curr Biol **23**(6): 453-461.

Alwan, H. A. and J. E. van Leeuwen (2007). "UBPY-mediated epidermal growth factor receptor (EGFR) de-ubiquitination promotes EGFR degradation." J Biol Chem **282**(3): 1658-1669.

Amerik, A. Y., J. Nowak, S. Swaminathan and M. Hochstrasser (2000). "The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways." Mol Biol Cell **11**(10): 3365-3380.

Anderson, R. G., M. S. Brown and J. L. Goldstein (1977). "Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts." Cell **10**(3): 351-364.

Anderson, R. G., J. L. Goldstein and M. S. Brown (1977). "A mutation that impairs the ability of lipoprotein receptors to localise in coated pits on the cell surface of human fibroblasts." Nature **270**(5639): 695-699.

Andrews, S., L. R. Stephens and P. T. Hawkins (2007). "PI3K class IB pathway." Sci STKE **2007**(407): cm2.

Appelmans, F., R. Wattiaux and C. De Duve (1955). "Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver." Biochem J **59**(3): 438-445.

Arasada, R. R. and G. Carpenter (2005). "Secretase-dependent tyrosine phosphorylation of Mdm2 by the ErbB-4 intracellular domain fragment." J Biol Chem **280**(35): 30783-30787.

Avvakumov, G. V., J. R. Walker, S. Xue, P. J. Finerty, Jr., F. Mackenzie, E. M. Newman and S. Dhe-Paganon (2006). "Amino-terminal dimerization, NRDP1-rhodanese interaction, and inhibited catalytic domain conformation of the ubiquitin-specific protease 8 (USP8)." J Biol Chem **281**(49): 38061-38070.

Baba, M., M. Osumi, S. V. Scott, D. J. Klionsky and Y. Ohsumi (1997). "Two distinct pathways for targeting proteins from the cytoplasm to the vacuole/lysosome." J Cell Biol **139**(7): 1687-1695.

Babst, M., D. J. Katzmann, E. J. Estepa-Sabal, T. Meerloo and S. D. Emr (2002). "Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting." Dev Cell **3**(2): 271-282.
Babst, M., D. J. Katzmann, W. B. Snyder, B. Wendland and S. D. Emr (2002). "Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body." Dev Cell **3**(2): 283-289.
Bache, K. G., C. Raiborg, A. Mehlum, I. H. Madshus and H. Stenmark (2002). "Phosphorylation of Hrs downstream of the epidermal growth factor receptor." Eur J Biochem **269**(16): 3881-3887.
Bai, D., L. Ueno and P. K. Vogt (2009). "Akt-mediated regulation of NFkappaB and the essentialness of NFkappaB for the oncogenicity of PI3K and Akt." Int J Cancer **125**(12): 2863-2870.
Ballif, B. A., Z. Cao, D. Schwartz, K. L. Carraway, 3rd and S. P. Gygi (2006). "Identification of 14-3-3epsilon substrates from embryonic murine brain." J Proteome Res **5**(9): 2372-2379.
Balut, C. M., Y. Gao, S. A. Murray, P. H. Thibodeau and D. C. Devor (2010). "ESCRT-dependent targeting of plasma membrane localized KCa3.1 to the lysosomes." Am J Physiol Cell Physiol **299**(5): C1015-1027.
Balut, C. M., C. M. Loch and D. C. Devor (2011). "Role of ubiquitylation and USP8-dependent deubiquitylation in the endocytosis and lysosomal targeting of plasma membrane KCa3.1." FASEB J **25**(11): 3938-3948.
Bargmann, C. I. and R. A. Weinberg (1988). "Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene." Proc Natl Acad Sci U S A **85**(15): 5394-5398.
Berlin, I., K. M. Higginbotham, R. S. Dise, M. I. Sierra and P. D. Nash (2010). "The deubiquitinating enzyme USP8 promotes trafficking and degradation of the chemokine receptor 4 at the sorting endosome." J Biol Chem **285**(48): 37895-37908.
Berthet, J., L. Berthet, F. Appelmans and C. De Duve (1951). "Tissue fractionation studies. II. The nature of the linkage between acid phosphatase and mitochondria in rat-liver tissue." Biochem J **50**(2): 182-189.
Berthet, J. and C. De Duve (1951). "Tissue fractionation studies. I. The existence of a mitochondria-linked, enzymically inactive form of acid phosphatase in rat-liver tissue." Biochem J **50**(2): 174-181.
Bhandari, D., J. Trejo, J. L. Benovic and A. Marchese (2007). "Arrestin-2 interacts with the ubiquitin-protein isopeptide ligase atrophin-interacting protein 4 and mediates endosomal sorting of the chemokine receptor CXCR4." J Biol Chem **282**(51): 36971-36979.
Bilodeau, P. S., J. L. Urbanowski, S. C. Winistorfer and R. C. Piper (2002). "The Vps27p Hse1p complex binds ubiquitin and mediates endosomal protein sorting." Nat Cell Biol **4**(7): 534-539.
Bingol, B. and M. Sheng (2016). "Mechanisms of mitophagy: PINK1, Parkin, USP30 and beyond." Free Radic Biol Med.
Bingol, B., J. S. Tea, L. Phu, M. Reichelt, C. E. Bakalarski, Q. Song, O. Foreman, D. S. Kirkpatrick and M. Sheng (2014). "The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy." Nature **510**(7505): 370-375.
Bochis, O. V., B. Fetica, C. Vlad, P. Achimas-Cadariu and A. Irimie (2015). "The Importance of Ubiquitin E3 Ligases, SCF and APC/C, in Human Cancers." Clujul Med **88**(1): 9-14.
Bowers, K., J. Lottridge, S. B. Helliwell, L. M. Goldthwaite, J. P. Luzio and T. H. Stevens (2004). "Protein-protein interactions of ESCRT complexes in the yeast Saccharomyces cerevisiae." Traffic **5**(3): 194-210.
Bowers, K., S. C. Piper, M. A. Edeling, S. R. Gray, D. J. Owen, P. J. Lehner and J. P. Luzio (2006). "Degradation of endocytosed epidermal growth factor and virally ubiquitinated major histocompatibility complex class I is independent of mammalian ESCRTII." J Biol Chem **281**(8): 5094-5105.

Braulke, T. and J. S. Bonifacino (2009). "Sorting of lysosomal proteins." Biochim Biophys Acta **1793**(4): 605-614.
Brodsky, F. M., B. L. Hill, S. L. Acton, I. Nathke, D. H. Wong, S. Ponnambalam and P. Parham (1991). "Clathrin light chains: arrays of protein motifs that regulate coated-vesicle dynamics." Trends Biochem Sci **16**(6): 208-213.
Brown, M. S. and J. L. Goldstein (1974). "Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity." Proc Natl Acad Sci U S A **71**(3): 788-792.
Bryant, N. J. and T. H. Stevens (1998). "Vacuole biogenesis in Saccharomyces cerevisiae: protein transport pathways to the yeast vacuole." Microbiol Mol Biol Rev **62**(1): 230-247.
Buchkovich, N. J., W. M. Henne, S. Tang and S. D. Emr (2013). "Essential N-terminal insertion motif anchors the ESCRT-III filament during MVB vesicle formation." Dev Cell **27**(2): 201-214.
Burd, C. G. and S. D. Emr (1998). "Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains." Mol Cell **2**(1): 157-162.
Byun, S., S. Y. Lee, J. Lee, C. H. Jeong, L. Farrand, S. Lim, K. Reddy, J. Y. Kim, M. H. Lee, H. J. Lee, A. M. Bode, K. Won Lee and Z. Dong (2013). "USP8 is a novel target for overcoming gefitinib resistance in lung cancer." Clin Cancer Res **19**(14): 3894-3904.
Cai, J., T. M. Crotty, E. Reichert, K. L. Carraway, 3rd, D. M. Stafforini and M. K. Topham (2010). "Diacylglycerol kinase delta and protein kinase C(alpha) modulate epidermal growth factor receptor abundance and degradation through ubiquitin-specific protease 8." J Biol Chem **285**(10): 6952-6959.
Cao, Z., X. Wu, L. Yen, C. Sweeney and K. L. Carraway, 3rd (2007). "Neuregulin-induced ErbB3 downregulation is mediated by a protein stability cascade involving the E3 ubiquitin ligase Nrdp1." Mol Cell Biol **27**(6): 2180-2188.
Carpenter, G., L. King, Jr. and S. Cohen (1978). "Epidermal growth factor stimulates phosphorylation in membrane preparations in vitro." Nature **276**(5686): 409-410.
Carpenter, G., K. J. Lembach, M. M. Morrison and S. Cohen (1975). "Characterization of the binding of 125-I-labeled epidermal growth factor to human fibroblasts." J Biol Chem **250**(11): 4297-4304.
Carter, S., S. Urbe and M. J. Clague (2004). "The met receptor degradation pathway: requirement for Lys48-linked polyubiquitin independent of proteasome activity." J Biol Chem **279**(51): 52835-52839.
Chakrabarty, A., B. N. Rexer, S. E. Wang, R. S. Cook, J. A. Engelman and C. L. Arteaga (2010). "H1047R phosphatidylinositol 3-kinase mutant enhances HER2-mediated transformation by heregulin production and activation of HER3." Oncogene **29**(37): 5193-5203.
Chan, N. C., A. M. Salazar, A. H. Pham, M. J. Sweredoski, N. J. Kolawa, R. L. Graham, S. Hess and D. C. Chan (2011). "Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy." Hum Mol Genet **20**(9): 1726-1737.
Chau, V., J. W. Tobias, A. Bachmair, D. Marriott, D. J. Ecker, D. K. Gonda and A. Varshavsky (1989). "A Multiubiquitin Chain is Confined to Specific Lysine in a Targeted Short-Lived Protein." Science **243**(4898): 8.
Chen, W., Z. Sun, X. J. Wang, T. Jiang, Z. Huang, D. Fang and D. D. Zhang (2009). "Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response." Mol Cell **34**(6): 663-673.
Chen, X., L. Randles, K. Shi, S. G. Tarasov, H. Aihara and K. J. Walters (2016). "Structures of Rpn1 T1:Rad23 and hRpn13:hPLIC2 Reveal Distinct Binding Mechanisms between Substrate Receptors and Shuttle Factors of the Proteasome." Structure **24**(8): 1257-1270.
Chen, Y. and G. W. Dorn, 2nd (2013). "PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria." Science **340**(6131): 471-475.

Cheong, H., T. Lindsten, J. Wu, C. Lu and C. B. Thompson (2011). "Ammonia-induced autophagy is independent of ULK1/ULK2 kinases." Proc Natl Acad Sci U S A **108**(27): 11121-11126.
Cheung, H. W., G. S. Cowley, B. A. Weir, J. S. Boehm, S. Rusin, J. A. Scott, A. East, L. D. Ali, P. H. Lizotte, T. C. Wong, G. Jiang, J. Hsiao, C. H. Mermel, G. Getz, J. Barretina, S. Gopal, P. Tamayo, J. Gould, A. Tsherniak, N. Stransky, B. Luo, Y. Ren, R. Drapkin, S. N. Bhatia, J. P. Mesirov, L. A. Garraway, M. Meyerson, E. S. Lander, D. E. Root and W. C. Hahn (2011). "Systematic investigation of genetic vulnerabilities across cancer cell lines reveals lineage-specific dependencies in ovarian cancer." Proc Natl Acad Sci U S A **108**(30): 12372-12377.
Chorley, B. N., M. R. Campbell, X. Wang, M. Karaca, D. Sambandan, F. Bangura, P. Xue, J. Pi, S. R. Kleeberger and D. A. Bell (2012). "Identification of novel NRF2-regulated genes by ChIP-Seq: influence on retinoid X receptor alpha." Nucleic Acids Res **40**(15): 7416-7429.
Christ, L., C. Raiborg, E. M. Wenzel, C. Campsteijn and H. Stenmark (2016). "Cellular Functions and Molecular Mechanisms of the ESCRT Membrane-Scission Machinery." Trends Biochem Sci.
Chu, D. S., B. Pishvaee and G. S. Payne (1999). "A modulatory role for clathrin light chain phosphorylation in Golgi membrane protein localization during vegetative growth and during the mating response of Saccharomyces cerevisiae." Mol Biol Cell **10**(3): 713-726.
Ciechanover, A. (2005). "Proteolysis: from the lysosome to ubiquitin and the proteasome." Nat Rev Mol Cell Biol **6**(1): 79-87.
Ciechanover, A., S. Elias, H. Heller, S. Ferber and A. Hershko (1980). "Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes." J Biol Chem **255**(16): 7525-7528.
Ciechanover, A., H. Heller, S. Elias, A. L. Haas and A. Hershko (1980). "ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation." Proc Natl Acad Sci U S A **77**(3): 1365-1368.
Citri, A., K. B. Skaria and Y. Yarden (2003). "The deaf and the dumb: the biology of ErbB-2 and ErbB-3." Exp Cell Res **284**(1): 54-65.
Clague, M. J. (2002). "Membrane transport: a coat for ubiquitin." Curr Biol **12**(15): R529-531.
Clague, M. J., I. Barsukov, J. M. Coulson, H. Liu, D. J. Rigden and S. Urbe (2013). "Deubiquitylases from genes to organism." Physiol Rev **93**(3): 1289-1315.
Clague, M. J., C. Heride and S. Urbe (2015). "The demographics of the ubiquitin system." Trends Cell Biol **25**(7): 417-426.
Clague, M. J. and S. Urbe (2006). "Endocytosis: the DUB version." Trends Cell Biol **16**(11): 551-559.
Claude, A. (1946). "Fractionation of mammalian liver cells by differential centrifugation; experimental procedures and results." J Exp Med **84**: 61-89.
Claude, A. (1946). "Fractionation of mammalian liver cells by differential centrifugation; problems, methods, and preparation of extract." J Exp Med **84**: 51-59.
Cohen, S. (1962). "Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal." J Biol Chem **237**: 1555-1562.
Cohen, S. and R. Levi-Montalcini (1957). "Purification and properties of a nerve growth-promoting factor isolated from mouse sarcoma 180." Cancer Res **17**(1): 15-20.
Colombo, M., S. Vallese, I. Peretto, X. Jacq, J. C. Rain, F. Colland and P. Guedat (2010). "Synthesis and biological evaluation of 9-oxo-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile analogues as potential inhibitors of deubiquitinating enzymes." ChemMedChem **5**(4): 552-558.
Cooper, E. M., C. Cutcliffe, T. Z. Kristiansen, A. Pandey, C. M. Pickart and R. E. Cohen (2009). "K63-specific deubiquitination by two JAMM/MPN+ complexes: BRISC-associated Brcc36 and proteasomal Poh1." EMBO J **28**(6): 621-631.

Cornelissen, T., D. Haddad, F. Wauters, C. Van Humbeeck, W. Mandemakers, B. Koentjoro, C. Sue, K. Gevaert, B. De Strooper, P. Verstreken and W. Vandenberghe (2014). "The deubiquitinase USP15 antagonizes Parkin-mediated mitochondrial ubiquitination and mitophagy." Hum Mol Genet **23**(19): 5227-5242.
Crotty, T. M., T. Nakano, D. M. Stafforini and M. K. Topham (2013). "Diacylglycerol kinase delta modulates Akt phosphorylation through pleckstrin homology domain leucine-rich repeat protein phosphatase 2 (PHLPP2)." J Biol Chem **288**(3): 1439-1447.
Crowther, R. A. and B. M. Pearse (1981). "Assembly and packing of clathrin into coats." J Cell Biol **91**(3 Pt 1): 790-797.
Cullinan, S. B., J. D. Gordan, J. Jin, J. W. Harper and J. A. Diehl (2004). "The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase." Mol Cell Biol **24**(19): 8477-8486.
Davies, C. W., L. N. Paul and C. Das (2013). "Mechanism of recruitment and activation of the endosome-associated deubiquitinase AMSH." Biochemistry **52**(44): 7818-7829.
Davies, C. W., L. N. Paul, M. I. Kim and C. Das (2011). "Structural and thermodynamic comparison of the catalytic domain of AMSH and AMSH-LP: nearly identical fold but different stability." J Mol Biol **413**(2): 416-429.
Daviet, L. and F. Colland (2008). "Targeting ubiquitin specific proteases for drug discovery." Biochimie **90**(2): 270-283.
de, D. C., J. Berthet, L. Berthet and F. Appelmans (1951). "Permeability of mitochondria." Nature **167**(4245): 389-390.
De Duve, C. and P. Baudhuin (1966). "Peroxisomes (microbodies and related particles)." Physiol Rev **46**(2): 323-357.
De Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans (1955). "Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue." Biochem J **60**(4): 604-617.
De Duve, C. and R. Wattiaux (1966). "Functions of lysosomes." Annu Rev Physiol **28**: 435-492.
de Marcos Lousa, C. and J. Denecke (2016). "Lysosomal and vacuolar sorting: not so different after all!" Biochem Soc Trans **44**(3): 891-897.
del Peso, L., M. Gonzalez-Garcia, C. Page, R. Herrera and G. Nunez (1997). "Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt." Science **278**(5338): 687-689.
Dengjel, J., M. Hoyer-Hansen, M. O. Nielsen, T. Eisenberg, L. M. Harder, S. Schandorff, T. Farkas, T. Kirkegaard, A. C. Becker, S. Schroeder, K. Vanselow, E. Lundberg, M. M. Nielsen, A. R. Kristensen, V. Akimov, J. Bunkenborg, F. Madeo, M. Jaattela and J. S. Andersen (2012). "Identification of autophagosome-associated proteins and regulators by quantitative proteomic analysis and genetic screens." Mol Cell Proteomics **11**(3): M111 014035.
Deshaies, R. J. (1999). "SCF and Cullin/Ring H2-based ubiquitin ligases." Annu Rev Cell Dev Biol **15**: 435-467.
Dhakshinamoorthy, S. and A. K. Jaiswal (2001). "Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene." Oncogene **20**(29): 3906-3917.
Diefenbacher, M. E., A. Chakraborty, S. M. Blake, R. Mitter, N. Popov, M. Eilers and A. Behrens (2015). "Usp28 counteracts Fbw7 in intestinal homeostasis and cancer." Cancer Res **75**(7): 1181-1186.
Dinkova-Kostova, A. T., W. D. Holtzclaw, R. N. Cole, K. Itoh, N. Wakabayashi, Y. Katoh, M. Yamamoto and P. Talalay (2002). "Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants." Proc Natl Acad Sci U S A **99**(18): 11908-11913.

Dooley, H. C., M. Razi, H. E. Polson, S. E. Girardin, M. I. Wilson and S. A. Tooze (2014). "WIPI2 links LC3 conjugation with PI3P, autophagosome formation, and pathogen clearance by recruiting Atg12-5-16L1." Mol Cell **55**(2): 238-252.
Dove, K. K., B. Stieglitz, E. D. Duncan, K. Rittinger and R. E. Klevit (2016). "Molecular insights into RBR E3 ligase ubiquitin transfer mechanisms." EMBO Rep **17**(8): 1221-1235.
Downward, J., P. Parker and M. D. Waterfield (1984). "Autophosphorylation sites on the epidermal growth factor receptor." Nature **311**(5985): 483-485.
Doyotte, A., A. Mironov, E. McKenzie and P. Woodman (2008). "The Bro1-related protein HD-PTP/PTPN23 is required for endosomal cargo sorting and multivesicular body morphogenesis." Proc Natl Acad Sci U S A **105**(17): 6308-6313.
Driscoll, J. and A. L. Goldberg (1990). "The proteasome (multicatalytic protease) is a component of the 1500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins." J Biol Chem **265**(9): 4789-4792.
Durcan, T. M. and E. A. Fon (2015). "The three 'P's of mitophagy: PARKIN, PINK1, and post-translational modifications." Genes Dev **29**(10): 989-999.
Durcan, T. M., M. Y. Tang, J. R. Perusse, E. A. Dashti, M. A. Aguileta, G. L. McLelland, P. Gros, T. A. Shaler, D. Faubert, B. Coulombe and E. A. Fon (2014). "USP8 regulates mitophagy by removing K6-linked ubiquitin conjugates from parkin." EMBO J **33**(21): 2473-2491.
Ekkebus, R., D. Flierman, P. P. Geurink and H. Ovaa (2014). "Catching a DUB in the act: novel ubiquitin-based active site directed probes." Curr Opin Chem Biol **23**: 63-70.
Elmore, S. (2007). "Apoptosis: a review of programmed cell death." Toxicol Pathol **35**(4): 495-516.
Etlinger, J. D. and A. L. Goldberg (1977). "A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes." Proc Natl Acad Sci U S A **74**(1): 54-58.
Eytan, E., D. Ganoth, T. Armon and A. Hershko (1989). "ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin." Proc Natl Acad Sci U S A **86**(20): 7751-7755.
Faesen, A. C., A. M. Dirac, A. Shanmugham, H. Ovaa, A. Perrakis and T. K. Sixma (2011). "Mechanism of USP7/HAUSP activation by its C-terminal ubiquitin-like domain and allosteric regulation by GMP-synthetase." Mol Cell **44**(1): 147-159.
Faesen, A. C., M. P. Luna-Vargas, P. P. Geurink, M. Clerici, R. Merkx, W. J. van Dijk, D. S. Hameed, F. El Oualid, H. Ovaa and T. K. Sixma (2011). "The differential modulation of USP activity by internal regulatory domains, interactors and eight ubiquitin chain types." Chem Biol **18**(12): 1550-1561.
Farmer, H., N. McCabe, C. J. Lord, A. N. Tutt, D. A. Johnson, T. B. Richardson, M. Santarosa, K. J. Dillon, I. Hickson, C. Knights, N. M. Martin, S. P. Jackson, G. C. Smith and A. Ashworth (2005). "Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy." Nature **434**(7035): 917-921.
Fimia, G. M., A. Stoykova, A. Romagnoli, L. Giunta, S. Di Bartolomeo, R. Nardacci, M. Corazzari, C. Fuoco, A. Ucar, P. Schwartz, P. Gruss, M. Piacentini, K. Chowdhury and F. Cecconi (2007). "Ambra1 regulates autophagy and development of the nervous system." Nature **447**(7148): 1121-1125.
Finley, D., S. Sadis, B. P. Monia, P. Boucher, D. J. Ecker, S. T. Crooke and V. Chau (1994). "Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant." Mol Cell Biol **14**(8): 5501-5509.
Fu, M., P. St-Pierre, J. Shankar, P. T. Wang, B. Joshi and I. R. Nabi (2013). "Regulation of mitophagy by the Gp78 E3 ubiquitin ligase." Mol Biol Cell **24**(8): 1153-1162.
Fujita, Y., G. Krause, M. Scheffner, D. Zechner, H. E. Leddy, J. Behrens, T. Sommer and W. Birchmeier (2002). "Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex." Nat Cell Biol **4**(3): 222-231.

Fukuoka, H., O. Cooper, A. Ben-Shlomo, A. Mamelak, S. G. Ren, D. Bruyette and S. Melmed (2011). "EGFR as a therapeutic target for human, canine, and mouse ACTH-secreting pituitary adenomas." J Clin Invest **121**(12): 4712-4721.
Furukawa, M., Y. J. He, C. Borchers and Y. Xiong (2003). "Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases." Nat Cell Biol **5**(11): 1001-1007.
Furukawa, M. and Y. Xiong (2005). "BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase." Mol Cell Biol **25**(1): 162-171.
Futter, C. E., A. Pearse, L. J. Hewlett and C. R. Hopkins (1996). "Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes." J Cell Biol **132**(6): 1011-1023.
Gaidarov, I., Q. Chen, J. R. Falck, K. K. Reddy and J. H. Keen (1996). "A functional phosphatidylinositol 3,4,5-trisphosphate/phosphoinositide binding domain in the clathrin adaptor AP-2 alpha subunit. Implications for the endocytic pathway." J Biol Chem **271**(34): 20922-20929.
Gaidarov, I. and J. H. Keen (1999). "Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits." J Cell Biol **146**(4): 755-764.
Galan, J. M. and R. Haguenauer-Tsapis (1997). "Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein." EMBO J **16**(19): 5847-5854.
Galan, J. M., V. Moreau, B. Andre, C. Volland and R. Haguenauer-Tsapis (1996). "Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease." J Biol Chem **271**(18): 10946-10952.
Ganley, I. G., H. Lam du, J. Wang, X. Ding, S. Chen and X. Jiang (2009). "ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy." J Biol Chem **284**(18): 12297-12305.
Gaullier, J. M., A. Simonsen, A. D'Arrigo, B. Bremnes, H. Stenmark and R. Aasland (1998). "FYVE fingers bind PtdIns(3)P." Nature **394**(6692): 432-433.
Ge, C., L. Che and C. Du (2015). "The UBC Domain Is Required for BRUCE to Promote BRIT1/MCPH1 Function in DSB Signaling and Repair Post Formation of BRUCE-USP8-BRIT1 Complex." PLoS One **10**(12): e0144957.
Ge, C., L. Che, J. Ren, R. K. Pandita, J. Lu, K. Li, T. K. Pandita and C. Du (2015). "BRUCE regulates DNA double-strand break response by promoting USP8 deubiquitination of BRIT1." Proc Natl Acad Sci U S A **112**(11): E1210-1219.
Geisler, S., K. M. Holmstrom, D. Skujat, F. C. Fiesel, O. C. Rothfuss, P. J. Kahle and W. Springer (2010). "PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1." Nat Cell Biol **12**(2): 119-131.
Gill, D. J., H. Teo, J. Sun, O. Perisic, D. B. Veprintsev, S. D. Emr and R. L. Williams (2007). "Structural insight into the ESCRT-I/-II link and its role in MVB trafficking." EMBO J **26**(2): 600-612.
Goldknopf, I. L. and H. Busch (1977). "Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24." Proc Natl Acad Sci U S A **74**(3): 864-868.
Goldstein, G., M. Scheid, U. Hammerling, D. H. Schlesinger, H. D. Niall and E. A. Boyse (1975). "Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells." Proc Natl Acad Sci U S A **72**(1): 11-15.
Goldstein, J. L., R. G. Anderson and M. S. Brown (1979). "Coated pits, coated vesicles, and receptor-mediated endocytosis." Nature **279**(5715): 679-685.
Graus-Porta, D., R. R. Beerli, J. M. Daly and N. E. Hynes (1997). "ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling." EMBO J **16**(7): 1647-1655.
Grice, G. L., I. T. Lobb, M. P. Weekes, S. P. Gygi, R. Antrobus and J. A. Nathan (2015). "The Proteasome Distinguishes between Heterotypic and Homotypic Lysine-11-Linked Polyubiquitin Chains." Cell Rep **12**(4): 545-553.

Grice, G. L. and J. A. Nathan (2016). "The recognition of ubiquitinated proteins by the proteasome." Cell Mol Life Sci **73**(18): 3497-3506.
Haglund, K., S. Sigismund, S. Polo, I. Szymkiewicz, P. P. Di Fiore and I. Dikic (2003). "Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation." Nat Cell Biol **5**(5): 461-466.
Hamacher-Brady, A. and N. R. Brady (2016). "Mitophagy programs: mechanisms and physiological implications of mitochondrial targeting by autophagy." Cell Mol Life Sci **73**(4): 775-795.
Han, E. S., F. L. Muller, V. I. Perez, W. Qi, H. Liang, L. Xi, C. Fu, E. Doyle, M. Hickey, J. Cornell, C. J. Epstein, L. J. Roberts, H. Van Remmen and A. Richardson (2008). "The in vivo gene expression signature of oxidative stress." Physiol Genomics **34**(1): 112-126.
Hanada, T., N. N. Noda, Y. Satomi, Y. Ichimura, Y. Fujioka, T. Takao, F. Inagaki and Y. Ohsumi (2007). "The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy." J Biol Chem **282**(52): 37298-37302.
Hara, T., A. Takamura, C. Kishi, S. Iemura, T. Natsume, J. L. Guan and N. Mizushima (2008). "FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells." J Cell Biol **181**(3): 497-510.
Harding, T. M., K. A. Morano, S. V. Scott and D. J. Klionsky (1995). "Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway." J Cell Biol **131**(3): 591-602.
Hassink, G. C., B. Zhao, R. Sompallae, M. Altun, S. Gastaldello, N. V. Zinin, M. G. Masucci and K. Lindsten (2009). "The ER-resident ubiquitin-specific protease 19 participates in the UPR and rescues ERAD substrates." EMBO Rep **10**(7): 755-761.
Hayashi, K., N. Inoshita, K. Kawaguchi, A. Ibrahim Ardisasmita, H. Suzuki, N. Fukuhara, M. Okada, H. Nishioka, Y. Takeuchi, M. Komada, A. Takeshita and S. Yamada (2016). "The USP8 mutational status may predict drug susceptibility in corticotroph adenomas of Cushing's disease." Eur J Endocrinol **174**(2): 213-226.
Hayes, J. D., M. McMahon, S. Chowdhry and A. T. Dinkova-Kostova (2010). "Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway." Antioxid Redox Signal **13**(11): 1713-1748.
Hasdemir, B., J. E. Murphy, G. S. Cottrell and N. W. Bunnett (2009). "Endosomal deubiquitinating enzymes control ubiquitination and down-regulation of protease-activated receptor 2." J Biol Chem **284**(41): 28453-28466.
Heppner, D. E. and A. van der Vliet (2016). "Redox-dependent regulation of epidermal growth factor receptor signaling." Redox Biol **8**: 24-27.
Heride, C., S. Urbe and M. J. Clague (2014). "Ubiquitin code assembly and disassembly." Curr Biol **24**(6): R215-220.
Hershko, A., A. Ciechanover, H. Heller, A. L. Haas and I. A. Rose (1980). "Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis." Proc Natl Acad Sci U S A **77**(4): 1783-1786.
Hershko, A., A. Ciechanover and I. A. Rose (1979). "Resolution of the ATP-dependent proteolytic system from reticulocytes: a component that interacts with ATP." Proc Natl Acad Sci U S A **76**(7): 3107-3110.
Heuser, J. (1980). "Three-dimensional visualization of coated vesicle formation in fibroblasts." J Cell Biol **84**(3): 560-583.
Hicke, L. and H. Riezman (1996). "Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis." Cell **84**(2): 277-287.
Hirano, S., M. Kawasaki, H. Ura, R. Kato, C. Raiborg, H. Stenmark and S. Wakatsuki (2006). "Double-sided ubiquitin binding of Hrs-UIM in endosomal protein sorting." Nat Struct Mol Biol **13**(3): 272-277.
Hirst, J., L. D. Barlow, G. C. Francisco, D. A. Sahlender, M. N. Seaman, J. B. Dacks and M. S. Robinson (2011). "The fifth adaptor protein complex." PLoS Biol **9**(10): e1001170.

Hirst, J. and M. S. Robinson (1998). "Clathrin and adaptors." Biochim Biophys Acta **1404**(1-2): 173-193.
Hofmann, K. and L. Falquet (2001). "A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems." Trends Biochem Sci **26**(6): 347-350.
Holbro, T. and N. E. Hynes (2004). "ErbB receptors: directing key signaling networks throughout life." Annu Rev Pharmacol Toxicol **44**: 195-217.
Hong, Y. H., H. C. Ahn, J. Lim, H. M. Kim, H. Y. Ji, S. Lee, J. H. Kim, E. Y. Park, H. K. Song and B. J. Lee (2009). "Identification of a novel ubiquitin binding site of STAM1 VHS domain by NMR spectroscopy." FEBS Lett **583**(2): 287-292.
Horton, R. A., E. A. Strachan, K. W. Vogel and S. M. Riddle (2007). "A substrate for deubiquitinating enzymes based on time-resolved fluorescence resonance energy transfer between terbium and yellow fluorescent protein." Anal Biochem **360**(1): 138-143.
Hosokawa, N., T. Hara, T. Kaizuka, C. Kishi, A. Takamura, Y. Miura, S. Iemura, T. Natsume, K. Takehana, N. Yamada, J. L. Guan, N. Oshiro and N. Mizushima (2009). "Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy." Mol Biol Cell **20**(7): 1981-1991.
Hough, R., G. Pratt and M. Rechsteiner (1986). "Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates." J Biol Chem **261**(5): 2400-2408.
Hough, R., G. Pratt and M. Rechsteiner (1987). "Purification of two high molecular weight proteases from rabbit reticulocyte lysate." J Biol Chem **262**(17): 8303-8313.
Hough, R. and M. Rechsteiner (1986). "Ubiquitin-lysozyme conjugates. Purification and susceptibility to proteolysis." J Biol Chem **261**(5): 2391-2399.
Hsuan, J. J., N. Totty and M. D. Waterfield (1989). "Identification of a novel autophosphorylation site (P4) on the epidermal growth factor receptor." Biochem J **262**(2): 659-663.
Huang, F., X. Zeng, W. Kim, M. Balasubramani, A. Fortian, S. P. Gygi, N. A. Yates and A. Sorkin (2013). "Lysine 63-linked polyubiquitination is required for EGF receptor degradation." Proc Natl Acad Sci U S A **110**(39): 15722-15727.
Huang, K. M., K. D'Hondt, H. Riezman and S. K. Lemmon (1999). "Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast." EMBO J **18**(14): 3897-3908.
Huang, K. M., L. Gullberg, K. K. Nelson, C. J. Stefan, K. Blumer and S. K. Lemmon (1997). "Novel functions of clathrin light chains: clathrin heavy chain trimerization is defective in light chain-deficient yeast." J Cell Sci **110 (Pt 7)**: 899-910.
Huang, O. W., X. Ma, J. Yin, J. Flinders, T. Maurer, N. Kayagaki, Q. Phung, I. Bosanac, D. Arnott, V. M. Dixit, S. G. Hymowitz, M. A. Starovasnik and A. G. Cochran (2012). "Phosphorylation-dependent activity of the deubiquitinase DUBA." Nat Struct Mol Biol **19**(2): 171-175.
Huang, Z., B. K. Choi, K. Mujoo, X. Fan, M. Fa, S. Mukherjee, N. Owiti, N. Zhang and Z. An (2015). "The E3 ubiquitin ligase NEDD4 negatively regulates HER3/ErbB3 level and signaling." Oncogene **34**(9): 1105-1115.
Hunt, L. T. and M. O. Dayhoff (1977). "Amino-terminal sequence identity of ubiquitin and the nonhistone component of nuclear protein A24." Biochem Biophys Res Commun **74**(2): 650-655.
Hurley, J. H. (2010). "The ESCRT complexes." Crit Rev Biochem Mol Biol **45**(6): 463-487.
Hynes, N. E. and H. A. Lane (2005). "ERBB receptors and cancer: the complexity of targeted inhibitors." Nat Rev Cancer **5**(5): 341-354.
Ichimura, Y., T. Kirisako, T. Takao, Y. Satomi, Y. Shimonishi, N. Ishihara, N. Mizushima, I. Tanida, E. Kominami, M. Ohsumi, T. Noda and Y. Ohsumi (2000). "A ubiquitin-like system mediates protein lipidation." Nature **408**(6811): 488-492.

Ichimura, Y., S. Waguri, Y. S. Sou, S. Kageyama, J. Hasegawa, R. Ishimura, T. Saito, Y. Yang, T. Kouno, T. Fukutomi, T. Hoshii, A. Hirao, K. Takagi, T. Mizushima, H. Motohashi, M. S. Lee, T. Yoshimori, K. Tanaka, M. Yamamoto and M. Komatsu (2013). "Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy." Mol Cell **51**(5): 618-631.
Itakura, E., C. Kishi, K. Inoue and N. Mizushima (2008). "Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG." Mol Biol Cell **19**(12): 5360-5372.
Itoh, K., T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamoto and Y. Nabeshima (1997). "An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements." Biochem Biophys Res Commun **236**(2): 313-322.
Itoh, K., J. Mimura and M. Yamamoto (2010). "Discovery of the negative regulator of Nrf2, Keap1: a historical overview." Antioxid Redox Signal **13**(11): 1665-1678.
Itoh, K., N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J. D. Engel and M. Yamamoto (1999). "Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain." Genes Dev **13**(1): 76-86.
Itoh, K., N. Wakabayashi, Y. Katoh, T. Ishii, T. O'Connor and M. Yamamoto (2003). "Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles." Genes Cells **8**(4): 379-391.
Jacobson, A. D., N. Y. Zhang, P. Xu, K. J. Han, S. Noone, J. Peng and C. W. Liu (2009). "The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26 s proteasome." J Biol Chem **284**(51): 35485-35494.
Jacomin, A. C., A. Bescond, E. Soleilhac, B. Gallet, G. Schoehn, M. O. Fauvarque and E. Taillebourg (2015). "The Deubiquitinating Enzyme UBPY Is Required for Lysosomal Biogenesis and Productive Autophagy in Drosophila." PLoS One **10**(11): e0143078.
Jain, A., T. Lamark, E. Sjottem, K. B. Larsen, J. A. Awuh, A. Overvatn, M. McMahon, J. D. Hayes and T. Johansen (2010). "p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription." J Biol Chem **285**(29): 22576-22591.
Jaramillo, M. C. and D. D. Zhang (2013). "The emerging role of the Nrf2-Keap1 signaling pathway in cancer." Genes Dev **27**(20): 2179-2191.
Jeong, C. H. (2015). "Inhibition of Ubiquitin-specific Peptidase 8 Suppresses Growth of Gefitinib-resistant Non-small Cell Lung Cancer Cells by Inducing Apoptosis." J Cancer Prev **20**(1): 57-63.
Jeong, M., E. W. Lee, D. Seong, J. Seo, J. H. Kim, S. Grootjans, S. Y. Kim, P. Vandenabeele and J. Song (2016). "USP8 suppresses death receptor-mediated apoptosis by enhancing FLIPL stability." Oncogene.
Jiang, X., F. Huang, A. Marusyk and A. Sorkin (2003). "Grb2 regulates internalization of EGF receptors through clathrin-coated pits." Mol Biol Cell **14**(3): 858-870.
Jin, N., M. J. Lang and L. S. Weisman (2016). "Phosphatidylinositol 3,5-bisphosphate: regulation of cellular events in space and time." Biochem Soc Trans **44**(1): 177-184.
Jin, S. M., M. Lazarou, C. Wang, L. A. Kane, D. P. Narendra and R. J. Youle (2010). "Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL." J Cell Biol **191**(5): 933-942.
Joazeiro, C. A., S. S. Wing, H. Huang, J. D. Leverson, T. Hunter and Y. C. Liu (1999). "The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase." Science **286**(5438): 309-312.
Juin, A., J. Di Martino, B. Leitinger, E. Henriet, A. S. Gary, L. Paysan, J. Bomo, G. Baffet, C. Gauthier-Rouviere, J. Rosenbaum, V. Moreau and F. Saltel (2014). "Discoidin domain receptor 1 controls linear invadosome formation via a Cdc42-Tuba pathway." J Cell Biol **207**(4): 517-533.

Kabeya, Y., N. N. Noda, Y. Fujioka, K. Suzuki, F. Inagaki and Y. Ohsumi (2009). "Characterization of the Atg17-Atg29-Atg31 complex specifically required for starvation-induced autophagy in Saccharomyces cerevisiae." Biochem Biophys Res Commun **389**(4): 612-615.
Kamada, Y., T. Funakoshi, T. Shintani, K. Nagano, M. Ohsumi and Y. Ohsumi (2000). "Tor-mediated induction of autophagy via an Apg1 protein kinase complex." J Cell Biol **150**(6): 1507-1513.
Kan, Z., B. S. Jaiswal, J. Stinson, V. Janakiraman, D. Bhatt, H. M. Stern, P. Yue, P. M. Haverty, R. Bourgon, J. Zheng, M. Moorhead, S. Chaudhuri, L. P. Tomsho, B. A. Peters, K. Pujara, S. Cordes, D. P. Davis, V. E. Carlton, W. Yuan, L. Li, W. Wang, C. Eigenbrot, J. S. Kaminker, D. A. Eberhard, P. Waring, S. C. Schuster, Z. Modrusan, Z. Zhang, D. Stokoe, F. J. de Sauvage, M. Faham and S. Seshagiri (2010). "Diverse somatic mutation patterns and pathway alterations in human cancers." Nature **466**(7308): 869-873.
Kanaseki, T. and K. Kadota (1969). "The "vesicle in a basket". A morphological study of the coated vesicle isolated from the nerve endings of the guinea pig brain, with special reference to the mechanism of membrane movements." J Cell Biol **42**(1): 202-220.
Kane, L. A., M. Lazarou, A. I. Fogel, Y. Li, K. Yamano, S. A. Sarraf, S. Banerjee and R. J. Youle (2014). "PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity." J Cell Biol **205**(2): 143-153.
Kaneko, T., T. Kumasaka, T. Ganbe, T. Sato, K. Miyazawa, N. Kitamura and N. Tanaka (2003). "Structural insight into modest binding of a non-PXXP ligand to the signal transducing adaptor molecule-2 Src homology 3 domain." J Biol Chem **278**(48): 48162-48168.
Kato, M., K. Miyazawa and N. Kitamura (2000). "A deubiquitinating enzyme UBPY interacts with the Src homology 3 domain of Hrs-binding protein via a novel binding motif PX(V/I)(D/N)RXXKP." J Biol Chem **275**(48): 37481-37487.
Katoh, Y., K. Itoh, E. Yoshida, M. Miyagishi, A. Fukamizu and M. Yamamoto (2001). "Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription." Genes Cells **6**(10): 857-868.
Katsuragi, Y., Y. Ichimura and M. Komatsu (2015). "p62/SQSTM1 functions as a signaling hub and an autophagy adaptor." FEBS J **282**(24): 4672-4678.
Katz, M., I. Amit and Y. Yarden (2007). "Regulation of MAPKs by growth factors and receptor tyrosine kinases." Biochim Biophys Acta **1773**(8): 1161-1176.
Katzmann, D. J., M. Babst and S. D. Emr (2001). "Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I." Cell **106**(2): 145-155.
Katzmann, D. J., C. J. Stefan, M. Babst and S. D. Emr (2003). "Vps27 recruits ESCRT machinery to endosomes during MVB sorting." J Cell Biol **162**(3): 413-423.
Keen, J. H., M. C. Willingham and I. H. Pastan (1979). "Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets." Cell **16**(2): 303-312.
Khaminets, A., C. Behl and I. Dikic (2016). "Ubiquitin-Dependent And Independent Signals In Selective Autophagy." Trends Cell Biol **26**(1): 6-16.
Kim, H. C. and J. M. Huibregtse (2009). "Polyubiquitination by HECT E3s and the determinants of chain type specificity." Mol Cell Biol **29**(12): 3307-3318.
Kirchhausen, T. (2000). "Clathrin." Annu Rev Biochem **69**: 699-727.
Kirchhausen, T., J. S. Bonifacino and H. Riezman (1997). "Linking cargo to vesicle formation: receptor tail interactions with coat proteins." Curr Opin Cell Biol **9**(4): 488-495.
Kirchhausen, T. and S. C. Harrison (1981). "Protein organization in clathrin trimers." Cell **23**(3): 755-761.
Kirisako, T., M. Baba, N. Ishihara, K. Miyazawa, M. Ohsumi, T. Yoshimori, T. Noda and Y. Ohsumi (1999). "Formation process of autophagosome is traced with Apg8/Aut7p in yeast." J Cell Biol **147**(2): 435-446.

Kirisako, T., Y. Ichimura, H. Okada, Y. Kabeya, N. Mizushima, T. Yoshimori, M. Ohsumi, T. Takao, T. Noda and Y. Ohsumi (2000). "The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway." J Cell Biol **151**(2): 263-276.
Klapisz, E., I. Sorokina, S. Lemeer, M. Pijnenburg, A. J. Verkleij and P. M. van Bergen en Henegouwen (2002). "A ubiquitin-interacting motif (UIM) is essential for Eps15 and Eps15R ubiquitination." J Biol Chem **277**(34): 30746-30753.
Klapper, L. N., H. Waterman, M. Sela and Y. Yarden (2000). "Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2." Cancer Res **60**(13): 3384-3388.
Klionsky, D. J., P. K. Herman and S. D. Emr (1990). "The fungal vacuole: composition, function, and biogenesis." Microbiol Rev **54**(3): 266-292.
Kobayashi, A., M. I. Kang, H. Okawa, M. Ohtsuji, Y. Zenke, T. Chiba, K. Igarashi and M. Yamamoto (2004). "Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2." Mol Cell Biol **24**(16): 7130-7139.
Kolling, R. and C. P. Hollenberg (1994). "The ABC-transporter Ste6 accumulates in the plasma membrane in a ubiquitinated form in endocytosis mutants." EMBO J **13**(14): 3261-3271.
Komander, D., M. J. Clague and S. Urbe (2009). "Breaking the chains: structure and function of the deubiquitinases." Nat Rev Mol Cell Biol **10**(8): 550-563.
Komander, D., C. J. Lord, H. Scheel, S. Swift, K. Hofmann, A. Ashworth and D. Barford (2008). "The structure of the CYLD USP domain explains its specificity for Lys63-linked polyubiquitin and reveals a B box module." Mol Cell **29**(4): 451-464.
Komander, D. and M. Rape (2012). "The ubiquitin code." Annu Rev Biochem **81**: 203-229.
Komander, D., F. Reyes-Turcu, J. D. Licchesi, P. Odenwaelder, K. D. Wilkinson and D. Barford (2009). "Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains." EMBO Rep **10**(5): 466-473.
Komatsu, M., H. Kurokawa, S. Waguri, K. Taguchi, A. Kobayashi, Y. Ichimura, Y. S. Sou, I. Ueno, A. Sakamoto, K. I. Tong, M. Kim, Y. Nishito, S. Iemura, T. Natsume, T. Ueno, E. Kominami, H. Motohashi, K. Tanaka and M. Yamamoto (2010). "The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1." Nat Cell Biol **12**(3): 213-223.
Kornfeld, S. and I. Mellman (1989). "The biogenesis of lysosomes." Annu Rev Cell Biol **5**: 483-525.
Koyano, F., K. Okatsu, H. Kosako, Y. Tamura, E. Go, M. Kimura, Y. Kimura, H. Tsuchiya, H. Yoshihara, T. Hirokawa, T. Endo, E. A. Fon, J. F. Trempe, Y. Saeki, K. Tanaka and N. Matsuda (2014). "Ubiquitin is phosphorylated by PINK1 to activate parkin." Nature **510**(7503): 162-166.
Krick, R., S. Henke, J. Tolstrup and M. Thumm (2008). "Dissecting the localization and function of Atg18, Atg21 and Ygr223c." Autophagy **4**(7): 896-910.
Kuchay, S., S. Duan, E. Schenkein, A. Peschiaroli, A. Saraf, L. Florens, M. P. Washburn and M. Pagano (2013). "FBXL2- and PTPL1-mediated degradation of p110-free p85beta regulatory subunit controls the PI(3)K signalling cascade." Nat Cell Biol **15**(5): 472-480.
Kulak, N. A., G. Pichler, I. Paron, N. Nagaraj and M. Mann (2014). "Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells." Nat Methods **11**(3): 319-324.
Kuma, A., N. Mizushima, N. Ishihara and Y. Ohsumi (2002). "Formation of the approximately 350-kDa Apg12-Apg5.Apg16 multimeric complex, mediated by Apg16 oligomerization, is essential for autophagy in yeast." J Biol Chem **277**(21): 18619-18625.
Kumari, S., S. Mg and S. Mayor (2010). "Endocytosis unplugged: multiple ways to enter the cell." Cell Res **20**(3): 256-275.

Kutateladze, T. G., K. D. Ogburn, W. T. Watson, T. de Beer, S. D. Emr, C. G. Burd and M. Overduin (1999). "Phosphatidylinositol 3-phosphate recognition by the FYVE domain." Mol Cell **3**(6): 805-811.
Kuusisto, E., A. Salminen and I. Alafuzoff (2001). "Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies." Neuroreport **12**(10): 2085-2090.
Lauwers, E., C. Jacob and B. Andre (2009). "K63-linked ubiquitin chains as a specific signal for protein sorting into the multivesicular body pathway." J Cell Biol **185**(3): 493-502.
Lavrik, I. N. and P. H. Krammer (2012). "Regulation of CD95/Fas signaling at the DISC." Cell Death Differ **19**(1): 36-41.
Lee, S. R., K. S. Kwon, S. R. Kim and S. G. Rhee (1998). "Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor." J Biol Chem **273**(25): 15366-15372.
Lemmon, M. A. (2009). "Ligand-induced ErbB receptor dimerization." Exp Cell Res **315**(4): 638-648.
Lemmon, M. A. and J. Schlessinger (2010). "Cell signaling by receptor tyrosine kinases." Cell **141**(7): 1117-1134.
Lemmon, M. A., J. Schlessinger and K. M. Ferguson (2014). "The EGFR family: not so prototypical receptor tyrosine kinases." Cold Spring Harb Perspect Biol **6**(4): a020768.
Levi-Montalcini, R. (1952). "Effects of mouse tumor transplantation on the nervous system." Ann N Y Acad Sci **55**(2): 330-344.
Levi-Montalcini, R. and S. Cohen (1960). "Effects of the extract of the mouse submaxillary salivary glands on the sympathetic system of mammals." Ann N Y Acad Sci **85**: 324-341.
Levkowitz, G., L. N. Klapper, E. Tzahar, A. Freywald, M. Sela and Y. Yarden (1996). "Coupling of the c-Cbl protooncogene product to ErbB-1/EGF-receptor but not to other ErbB proteins." Oncogene **12**(5): 1117-1125.
Levkowitz, G., H. Waterman, S. A. Ettenberg, M. Katz, A. Y. Tsygankov, I. Alroy, S. Lavi, K. Iwai, Y. Reiss, A. Ciechanover, S. Lipkowitz and Y. Yarden (1999). "Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1." Mol Cell **4**(6): 1029-1040.
Levkowitz, G., H. Waterman, E. Zamir, Z. Kam, S. Oved, W. Y. Langdon, L. Beguinot, B. Geiger and Y. Yarden (1998). "c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor." Genes Dev **12**(23): 3663-3674.
Li, S., Y. Chen, Q. Shi, T. Yue, B. Wang and J. Jiang (2012). "Hedgehog-regulated ubiquitination controls smoothened trafficking and cell surface expression in Drosophila." PLoS Biol **10**(1): e1001239.
Li, X., D. Zhang, M. Hannink and L. J. Beamer (2004). "Crystal structure of the Kelch domain of human Keap1." J Biol Chem **279**(52): 54750-54758.
Liang, J. R., A. Martinez, J. D. Lane, U. Mayor, M. J. Clague and S. Urbe (2015). "USP30 deubiquitylates mitochondrial Parkin substrates and restricts apoptotic cell death." EMBO Rep **16**(5): 618-627.
Lim, J. H., H. Jono, K. Komatsu, C. H. Woo, J. Lee, M. Miyata, T. Matsuno, X. Xu, Y. Huang, W. Zhang, S. H. Park, Y. I. Kim, Y. D. Choi, H. Shen, K. S. Heo, H. Xu, P. Bourne, T. Koga, H. Xu, C. Yan, B. Wang, L. F. Chen, X. H. Feng and J. D. Li (2012). "CYLD negatively regulates transforming growth factor-beta-signalling via deubiquitinating Akt." Nat Commun **3**: 771.
Liu, H., S. Urbe and M. J. Clague (2012). "Selective protein degradation in cell signalling." Semin Cell Dev Biol **23**(5): 509-514.
Liu, Z., S. M. Zanata, J. Kim, M. A. Peterson, D. Di Vizio, L. R. Chirieac, S. Pyne, M. Agostini, M. R. Freeman and M. Loda (2013). "The ubiquitin-specific protease USP2a prevents endocytosis-mediated EGFR degradation." Oncogene **32**(13): 1660-1669.

Lo, S. C. and M. Hannink (2006). "PGAM5, a Bcl-XL-interacting protein, is a novel substrate for the redox-regulated Keap1-dependent ubiquitin ligase complex." J Biol Chem **281**(49): 37893-37903.
Lo, S. C., X. Li, M. T. Henzl, L. J. Beamer and M. Hannink (2006). "Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling." EMBO J **25**(15): 3605-3617.
Losko, S., F. Kopp, A. Kranz and R. Kolling (2001). "Uptake of the ATP-binding cassette (ABC) transporter Ste6 into the yeast vacuole is blocked in the doa4 Mutant." Mol Biol Cell **12**(4): 1047-1059.
Lu, Y., B. H. Lee, R. W. King, D. Finley and M. W. Kirschner (2015). "Substrate degradation by the proteasome: a single-molecule kinetic analysis." Science **348**(6231): 1250834.
Luhtala, N. and G. Odorizzi (2004). "Bro1 coordinates deubiquitination in the multivesicular body pathway by recruiting Doa4 to endosomes." J Cell Biol **166**(5): 717-729.
Lutterbach, B., Q. Zeng, L. J. Davis, H. Hatch, G. Hang, N. E. Kohl, J. B. Gibbs and B. S. Pan (2007). "Lung cancer cell lines harboring MET gene amplification are dependent on Met for growth and survival." Cancer Res **67**(5): 2081-2088.
Ma, Y. M., E. Boucrot, J. Villen, B. Affar el, S. P. Gygi, H. G. Gottlinger and T. Kirchhausen (2007). "Targeting of AMSH to endosomes is required for epidermal growth factor receptor degradation." J Biol Chem **282**(13): 9805-9812.
Ma, Z. Y., Z. J. Song, J. H. Chen, Y. F. Wang, S. Q. Li, L. F. Zhou, Y. Mao, Y. M. Li, R. G. Hu, Z. Y. Zhang, H. Y. Ye, M. Shen, X. F. Shou, Z. Q. Li, H. Peng, Q. Z. Wang, D. Z. Zhou, X. L. Qin, J. Ji, J. Zheng, H. Chen, Y. Wang, D. Y. Geng, W. J. Tang, C. W. Fu, Z. F. Shi, Y. C. Zhang, Z. Ye, W. Q. He, Q. L. Zhang, Q. S. Tang, R. Xie, J. W. Shen, Z. J. Wen, J. Zhou, T. Wang, S. Huang, H. J. Qiu, N. D. Qiao, Y. Zhang, L. Pan, W. M. Bao, Y. C. Liu, C. X. Huang, Y. Y. Shi and Y. Zhao (2015). "Recurrent gain-of-function USP8 mutations in Cushing's disease." Cell Res **25**(3): 306-317.
MacDonald, E., S. Urbe and M. J. Clague (2014). "USP8 controls the trafficking and sorting of lysosomal enzymes." Traffic **15**(8): 879-888.
Margolis, B. L., I. Lax, R. Kris, M. Dombalagian, A. M. Honegger, R. Howk, D. Givol, A. Ullrich and J. Schlessinger (1989). "All autophosphorylation sites of epidermal growth factor (EGF) receptor and HER2/neu are located in their carboxyl-terminal tails. Identification of a novel site in EGF receptor." J Biol Chem **264**(18): 10667-10671.
Marmor, M. D. and Y. Yarden (2004). "Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases." Oncogene **23**(11): 2057-2070.
Matsumoto, G., K. Wada, M. Okuno, M. Kurosawa and N. Nukina (2011). "Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins." Mol Cell **44**(2): 279-289.
Maurer, T. and I. E. Wertz (2016). "Length Matters: MINDY Is a New Deubiquitinase Family that Preferentially Cleaves Long Polyubiquitin Chains." Mol Cell **63**(1): 4-6.
Maxfield, F. R., J. Schlessinger, Y. Shechter, I. Pastan and M. C. Willingham (1978). "Collection of insulin, EGF and alpha2-macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization." Cell **14**(4): 805-810.
McAlpine, F., L. E. Williamson, S. A. Tooze and E. Y. Chan (2013). "Regulation of nutrient-sensitive autophagy by uncoordinated 51-like kinases 1 and 2." Autophagy **9**(3): 361-373.
McCullough, J., M. J. Clague and S. Urbe (2004). "AMSH is an endosome-associated ubiquitin isopeptidase." J Cell Biol **166**(4): 487-492.
McCullough, J., P. E. Row, O. Lorenzo, M. Doherty, R. Beynon, M. J. Clague and S. Urbe (2006). "Activation of the endosome-associated ubiquitin isopeptidase AMSH by STAM, a component of the multivesicular body-sorting machinery." Curr Biol **16**(2): 160-165.
McGouran, J. F., S. R. Gaertner, M. Altun, H. B. Kramer and B. M. Kessler (2013). "Deubiquitinating enzyme specificity for ubiquitin chain topology profiled by di-ubiquitin activity probes." Chem Biol **20**(12): 1447-1455.

McMahon, M., K. Itoh, M. Yamamoto and J. D. Hayes (2003). "Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression." J Biol Chem **278**(24): 21592-21600.
McMahon, M., N. Thomas, K. Itoh, M. Yamamoto and J. D. Hayes (2006). "Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex." J Biol Chem **281**(34): 24756-24768.
McNamara, C. R. and A. Degterev (2011). "Small-molecule inhibitors of the PI3K signaling network." Future Med Chem **3**(5): 549-565.
Meijer, I. M., J. Kerperien, A. M. Sotoca, E. J. van Zoelen and J. E. van Leeuwen (2013). "The Usp8 deubiquitination enzyme is post-translationally modified by tyrosine and serine phosphorylation." Cell Signal **25**(4): 919-930.
Mercer, C. A., A. Kaliappan and P. B. Dennis (2009). "A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy." Autophagy **5**(5): 649-662.
Merrifield, C. J. and M. Kaksonen (2014). "Endocytic accessory factors and regulation of clathrin-mediated endocytosis." Cold Spring Harb Perspect Biol **6**(11): a016733.
Mevissen, T. E., M. K. Hospenthal, P. P. Geurink, P. R. Elliott, M. Akutsu, N. Arnaudo, R. Ekkebus, Y. Kulathu, T. Wauer, F. El Oualid, S. M. Freund, H. Ovaa and D. Komander (2013). "OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis." Cell **154**(1): 169-184.
Misra, S. and J. H. Hurley (1999). "Crystal structure of a phosphatidylinositol 3-phosphate-specific membrane-targeting motif, the FYVE domain of Vps27p." Cell **97**(5): 657-666.
Miyake, S., M. L. Lupher, Jr., B. Druker and H. Band (1998). "The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha." Proc Natl Acad Sci U S A **95**(14): 7927-7932.
Miyazawa, K., K. Toyama, A. Gotoh, P. C. Hendrie, C. Mantel and H. E. Broxmeyer (1994). "Ligand-dependent polyubiquitination of c-kit gene product: a possible mechanism of receptor down modulation in M07e cells." Blood **83**(1): 137-145.
Mizuno, E., T. Iura, A. Mukai, T. Yoshimori, N. Kitamura and M. Komada (2005). "Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated deubiquitination at endosomes." Mol Biol Cell **16**(11): 5163-5174.
Mizuno, E., K. Kawahata, M. Kato, N. Kitamura and M. Komada (2003). "STAM proteins bind ubiquitinated proteins on the early endosome via the VHS domain and ubiquitin-interacting motif." Mol Biol Cell **14**(9): 3675-3689.
Mizuno, E., N. Kitamura and M. Komada (2007). "14-3-3-dependent inhibition of the deubiquitinating activity of UBPY and its cancellation in the M phase." Exp Cell Res **313**(16): 3624-3634.
Mizuno, E., K. Kobayashi, A. Yamamoto, N. Kitamura and M. Komada (2006). "A deubiquitinating enzyme UBPY regulates the level of protein ubiquitination on endosomes." Traffic **7**(8): 1017-1031.
Mizushima, N., T. Noda and Y. Ohsumi (1999). "Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway." EMBO J **18**(14): 3888-3896.
Mizushima, N., T. Noda, T. Yoshimori, Y. Tanaka, T. Ishii, M. D. George, D. J. Klionsky, M. Ohsumi and Y. Ohsumi (1998). "A protein conjugation system essential for autophagy." Nature **395**(6700): 395-398.
Mizushima, N., T. Yoshimori and Y. Ohsumi (2011). "The role of Atg proteins in autophagosome formation." Annu Rev Cell Dev Biol **27**: 107-132.
Moi, P., K. Chan, I. Asunis, A. Cao and Y. W. Kan (1994). "Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem

NF-E2/AP1 repeat of the beta-globin locus control region." Proc Natl Acad Sci U S A **91**(21): 9926-9930.
Mori, S., C. H. Heldin and L. Claesson-Welsh (1992). "Ligand-induced polyubiquitination of the platelet-derived growth factor beta-receptor." J Biol Chem **267**(9): 6429-6434.
Morita, E., V. Sandrin, H. Y. Chung, S. G. Morham, S. P. Gygi, C. K. Rodesch and W. I. Sundquist (2007). "Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis." EMBO J **26**(19): 4215-4227.
Mosesson, Y., K. Shtiegman, M. Katz, Y. Zwang, G. Vereb, J. Szollosi and Y. Yarden (2003). "Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation." J Biol Chem **278**(24): 21323-21326.
Motley, A., N. A. Bright, M. N. Seaman and M. S. Robinson (2003). "Clathrin-mediated endocytosis in AP-2-depleted cells." J Cell Biol **162**(5): 909-918.
Mukai, A., E. Mizuno, K. Kobayashi, M. Matsumoto, K. I. Nakayama, N. Kitamura and M. Komada (2008). "Dynamic regulation of ubiquitylation and deubiquitylation at the central spindle during cytokinesis." J Cell Sci **121**(Pt 8): 1325-1333.
Mukai, A., M. Yamamoto-Hino, W. Awano, W. Watanabe, M. Komada and S. Goto (2010). "Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt." EMBO J **29**(13): 2114-2125.
Murphy, L. O. and J. Blenis (2006). "MAPK signal specificity: the right place at the right time." Trends Biochem Sci **31**(5): 268-275.
Murrow, L., R. Malhotra and J. Debnath (2015). "ATG12-ATG3 interacts with Alix to promote basal autophagic flux and late endosome function." Nat Cell Biol **17**(3): 300-310.
Musacchio, A., C. J. Smith, A. M. Roseman, S. C. Harrison, T. Kirchhausen and B. M. Pearse (1999). "Functional organization of clathrin in coats: combining electron cryomicroscopy and X-ray crystallography." Mol Cell **3**(6): 761-770.
Nakamura, M., N. Tanaka, N. Kitamura and M. Komada (2006). "Clathrin anchors deubiquitinating enzymes, AMSH and AMSH-like protein, on early endosomes." Genes Cells **11**(6): 593-606.
Nakayama, K. and S. Wakatsuki (2003). "The structure and function of GGAs, the traffic controllers at the TGN sorting crossroads." Cell Struct Funct **28**(5): 431-442.
Nathke, I., B. L. Hill, P. Parham and F. M. Brodsky (1990). "The calcium-binding site of clathrin light chains." J Biol Chem **265**(30): 18621-18627.
Naviglio, S., C. Mattecucci, B. Matoskova, T. Nagase, N. Nomura, P. P. Di Fiore and G. F. Draetta (1998). "UBPY: a growth-regulated human ubiquitin isopeptidase." EMBO J **17**(12): 3241-3250.
Nguyen, T., H. C. Huang and C. B. Pickett (2000). "Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK." J Biol Chem **275**(20): 15466-15473.
Nguyen, T., P. J. Sherratt, H. C. Huang, C. S. Yang and C. B. Pickett (2003). "Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome." J Biol Chem **278**(7): 4536-4541.
Niendorf, S., A. Oksche, A. Kisser, J. Lohler, M. Prinz, H. Schorle, S. Feller, M. Lewitzky, I. Horak and K. P. Knobeloch (2007). "Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytic trafficking in vivo." Mol Cell Biol **27**(13): 5029-5039.
Nikko, E. and B. Andre (2007). "Evidence for a direct role of the Doa4 deubiquitinating enzyme in protein sorting into the MVB pathway." Traffic **8**(5): 566-581.
Nioi, P., M. McMahon, K. Itoh, M. Yamamoto and J. D. Hayes (2003). "Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence." Biochem J **374**(Pt 2): 337-348.

Niso-Santano, M., R. A. Gonzalez-Polo, J. M. Bravo-San Pedro, R. Gomez-Sanchez, I. Lastres-Becker, M. A. Ortiz-Ortiz, G. Soler, J. M. Moran, A. Cuadrado, J. M. Fuentes and N. Centro de Investigacion Biomedica en Red sobre Enfermedades (2010). "Activation of apoptosis signal-regulating kinase 1 is a key factor in paraquat-induced cell death: modulation by the Nrf2/Trx axis." Free Radic Biol Med **48**(10): 1370-1381.
Noda, T., J. Kim, W. P. Huang, M. Baba, C. Tokunaga, Y. Ohsumi and D. J. Klionsky (2000). "Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways." J Cell Biol **148**(3): 465-480.
Novikoff, A. B., H. Beaufay and C. De Duve (1956). "Electron microscopy of lysosomerich fractions from rat liver." J Biophys Biochem Cytol **2**(4 Suppl): 179-184.
O'Keefe, E., M. D. Hollenberg and P. Cuatrecasas (1974). "Epidermal growth factor. Characteristics of specific binding in membranes from liver, placenta, and other target tissues." Arch Biochem Biophys **164**(2): 518-526.
Ogawara, Y., S. Kishishita, T. Obata, Y. Isazawa, T. Suzuki, K. Tanaka, N. Masuyama and Y. Gotoh (2002). "Akt enhances Mdm2-mediated ubiquitination and degradation of p53." J Biol Chem **277**(24): 21843-21850.
Ohashi, Y., N. Soler, M. Garcia Ortegon, L. Zhang, M. L. Kirsten, O. Perisic, G. R. Masson, J. E. Burke, A. J. Jakobi, A. A. Apostolakis, C. M. Johnson, M. Ohashi, N. T. Ktistakis, C. Sachse and R. L. Williams (2016). "Characterization of Atg38 and NRBF2, a fifth subunit of the autophagic Vps34/PIK3C3 complex." Autophagy **12**(11): 2129-2144.
Ohsumi, Y. (2014). "Historical landmarks of autophagy research." Cell Res **24**(1): 9-23.
Ordureau, A., S. A. Sarraf, D. M. Duda, J. M. Heo, M. P. Jedrychowski, V. O. Sviderskiy, J. L. Olszewski, J. T. Koerber, T. Xie, S. A. Beausoleil, J. A. Wells, S. P. Gygi, B. A. Schulman and J. W. Harper (2014). "Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis." Mol Cell **56**(3): 360-375.
Organ, S. L. and M. S. Tsao (2011). "An overview of the c-MET signaling pathway." Ther Adv Med Oncol **3**(1 Suppl): S7-S19.
Oshima, R., T. Hasegawa, K. Tamai, N. Sugeno, S. Yoshida, J. Kobayashi, A. Kikuchi, T. Baba, A. Futatsugi, I. Sato, K. Satoh, A. Takeda, M. Aoki and N. Tanaka (2016). "ESCRT-0 dysfunction compromises autophagic degradation of protein aggregates and facilitates ER stress-mediated neurodegeneration via apoptotic and necroptotic pathways." Sci Rep **6**: 24997.
Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R., Wakamatsu, A., Hayashi, K., Sato, H., Nagai, K., Kimura, K., Makita, H., Sekine, M., Obayashi, M., Nishi, T., Shibahara, T., Tanaka, T., Ishii, S., Yamamoto, J., Saito, K., Kawai, Y., Isono, Y., Nakamura, Y., Nagahari, K., Murakami, K., Yasuda, T., Iwayanagi, T., Wagatsuma, M., Shiratori, A., Sudo, H., Hosoiri, T., Kaku, Y., Kodaira, H., Kondo, H., Sugawara, M., Takahashi, M., Kanda, K., Yokoi, T., Furuya, T., Kikkawa, E., Omura, Y., Abe, K., Kamihara, K., Katsuta, N., Sato, K., Tanikawa, M., Yamazaki, M., Ninomiya, K., Ishibashi, T., Yamashita, H., Murakawa, K., Fujimori, K., Tanai, H., Kimata, M., Watanabe, M., Hiraoka, S., Chiba, Y., Ishida, S., Ono, Y., Takiguchi, S., Watanabe, S., Yosida, M., Hotuta, T., Kusano, J., Kanehori, K., Takahashi-Fujii, A., Hara, H., Tanase, T., Nomura, Y., Togiya, S., Komai, F., Hara, R., Takeuchi, K., Arita, M., Imose, N., Musashino, K., Yuuki, H., Oshima, A., Sasaki, N., Aotsuka, S., Yoshikawa, Y., Matsunawa, H., Ichihara, T., Shiohata, N., Sano, S., Moriya, S., Momiyama, H., Satoh, N., Takami, S., Terashima, Y., Suzuki, O., Nakagawa, S., Senoh, A., Mizoguchi, H., Goto, Y., Shimizu, F., Wakebe, H., Hishigaki, H., Watanabe, T., Sugiyama, A., Takemoto, M., Kawakami, B., Yamazaki, M., Watanabe, K., Kumagai, A., Itakura, S., Fukuzumi, Y., Fujimori, Y., Komiyama, M., Tashiro, H., Tanigami, A., Fujiwara, T., Ono, T., Yamada, K., Fujii, Y., Ozaki, K., Hirao, M., Ohmori, Y., Kawabata, A., Hikiji, T., Kobatake, N., Inagaki, H., Ikema, Y., Okamoto, S., Okitani, R., Kawakami, T., Noguchi, S., Itoh, T., Shigeta, K., Senba, T., Matsumura, K., Nakajima, Y., Mizuno, T., Morinaga, M., Sasaki, M., Togashi, T., Oyama, M., Hata, H., Watanabe, M., Komatsu, T., Mizushima-Sugano, J., Satoh,

T., Shirai, Y., Takahashi, Y., Nakagawa, K., Okumura, K., Nagase, T., Nomura, N., Kikuchi, H., Masuho, Y., Yamashita, R., Nakai, K., Yada, T., Nakamura, Y., Ohara, O., Isogai, T., Sugano, S. (2004) "Complete sequencing and characterization of 21,243 full-length human cDNAs" Nat Genet. **36**(1):40-5.

Ovaa, H. (2007). "Active-site directed probes to report enzymatic action in the ubiquitin proteasome system." Nat Rev Cancer **7**(8): 613-620.

Pankiv, S., T. H. Clausen, T. Lamark, A. Brech, J. A. Bruun, H. Outzen, A. Overvatn, G. Bjorkoy and T. Johansen (2007). "p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy." J Biol Chem **282**(33): 24131-24145.

Panner, A., C. A. Crane, C. Weng, A. Feletti, S. Fang, A. T. Parsa and R. O. Pieper (2010). "Ubiquitin-specific protease 8 links the PTEN-Akt-AIP4 pathway to the control of FLIPS stability and TRAIL sensitivity in glioblastoma multiforme." Cancer Res **70**(12): 5046-5053.

Papa, F. R., A. Y. Amerik and M. Hochstrasser (1999). "Interaction of the Doa4 deubiquitinating enzyme with the yeast 26S proteasome." Mol Biol Cell **10**(3): 741-756.

Pareja, F., D. A. Ferraro, C. Rubin, H. Cohen-Dvashi, F. Zhang, S. Aulmann, N. Ben-Chetrit, G. Pines, R. Navon, N. Crosetto, W. Kostler, S. Carvalho, S. Lavi, F. Schmitt, I. Dikic, Z. Yakhini, P. Sinn, G. B. Mills and Y. Yarden (2012). "Deubiquitination of EGFR by Cezanne-1 contributes to cancer progression." Oncogene **31**(43): 4599-4608.

Park, S. Y. and X. Guo (2014). "Adaptor protein complexes and intracellular transport." Biosci Rep **34**(4).

Pearse, B. M. (1975). "Coated vesicles from pig brain: purification and biochemical characterization." J Mol Biol **97**(1): 93-98.

Pearse, B. M. (1976). "Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles." Proc Natl Acad Sci U S A **73**(4): 1255-1259.

Pearse, B. M. and M. S. Bretscher (1981). "Membrane recycling by coated vesicles." Annu Rev Biochem **50**: 85-101.

Pearse, B. M. and M. S. Robinson (1990). "Clathrin, adaptors, and sorting." Annu Rev Cell Biol **6**: 151-171.

Perrody, E., L. Abrami, M. Feldman, B. Kunz, S. Urbe and F. G. van der Goot (2016). "Ubiquitin-dependent folding of the Wnt signaling coreceptor LRP6." Elife **5**.

Peschard, P., T. M. Fournier, L. Lamorte, M. A. Naujokas, H. Band, W. Y. Langdon and M. Park (2001). "Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein." Mol Cell **8**(5): 995-1004.

Peter, M. E. (2004). "The flip side of FLIP." Biochem J **382**(Pt 2): e1-3.

Petroski, M. D. and R. J. Deshaies (2005). "Function and regulation of cullin-RING ubiquitin ligases." Nat Rev Mol Cell Biol **6**(1): 9-20.

Pintard, L., A. Willems and M. Peter (2004). "Cullin-based ubiquitin ligases: Cul3-BTB complexes join the family." EMBO J **23**(8): 1681-1687.

Pintard, L., J. H. Willis, A. Willems, J. L. Johnson, M. Srayko, T. Kurz, S. Glaser, P. E. Mains, M. Tyers, B. Bowerman and M. Peter (2003). "The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase." Nature **425**(6955): 311-316.

Pley, U. M., B. L. Hill, C. Alibert, F. M. Brodsky and P. Parham (1995). "The interaction of calmodulin with clathrin-coated vesicles, triskelions, and light chains. Localization of a binding site." J Biol Chem **270**(5): 2395-2402.

Plowman, G. D., J. M. Culouscou, G. S. Whitney, J. M. Green, G. W. Carlton, L. Foy, M. G. Neubauer and M. Shoyab (1993). "Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family." Proc Natl Acad Sci U S A **90**(5): 1746-1750.

Plowman, G. D., G. S. Whitney, M. G. Neubauer, J. M. Green, V. L. McDonald, G. J. Todaro and M. Shoyab (1990). "Molecular cloning and expression of an additional epidermal growth factor receptor-related gene." Proc Natl Acad Sci U S A **87**(13): 4905-4909.

Polson, H. E., J. de Lartigue, D. J. Rigden, M. Reedijk, S. Urbe, M. J. Clague and S. A. Tooze (2010). "Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation." Autophagy **6**(4): 506-522.
Poole, B., S. Ohkuma and M. J. Warburton (1977). "The accumulation of weakly basic substances in lysosomes and the inhibition of intracellular protein degradation." Acta Biol Med Ger **36**(11-12): 1777-1788.
Prag, G., H. Watson, Y. C. Kim, B. M. Beach, R. Ghirlando, G. Hummer, J. S. Bonifacino and J. H. Hurley (2007). "The Vps27/Hse1 complex is a GAT domain-based scaffold for ubiquitin-dependent sorting." Dev Cell **12**(6): 973-986.
Proikas-Cezanne, T., S. Ruckerbauer, Y. D. Stierhof, C. Berg and A. Nordheim (2007). "Human WIPI-1 puncta-formation: a novel assay to assess mammalian autophagy." FEBS Lett **581**(18): 3396-3404.
Proikas-Cezanne, T., Z. Takacs, P. Donnes and O. Kohlbacher (2015). "WIPI proteins: essential PtdIns3P effectors at the nascent autophagosome." J Cell Sci **128**(2): 207-217.
Ragusa, M. J., R. E. Stanley and J. H. Hurley (2012). "Architecture of the Atg17 complex as a scaffold for autophagosome biogenesis." Cell **151**(7): 1501-1512.
Raiborg, C., K. G. Bache, D. J. Gillooly, I. H. Madshus, E. Stang and H. Stenmark (2002). "Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes." Nat Cell Biol **4**(5): 394-398.
Raiborg, C., K. G. Bache, A. Mehlum, E. Stang and H. Stenmark (2001). "Hrs recruits clathrin to early endosomes." EMBO J **20**(17): 5008-5021.
Raiborg, C., B. Bremnes, A. Mehlum, D. J. Gillooly, A. D'Arrigo, E. Stang and H. Stenmark (2001). "FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes." J Cell Sci **114**(Pt 12): 2255-2263.
Raiborg, C. and H. Stenmark (2009). "The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins." Nature **458**(7237): 445-452.
Ramos-Gomez, M., M. K. Kwak, P. M. Dolan, K. Itoh, M. Yamamoto, P. Talalay and T. W. Kensler (2001). "Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice." Proc Natl Acad Sci U S A **98**(6): 3410-3415.
Ray, P. D., B. W. Huang and Y. Tsuji (2012). "Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling." Cell Signal **24**(5): 981-990.
Reggiori, F. and H. R. Pelham (2001). "Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting." EMBO J **20**(18): 5176-5186.
Reincke, M., S. Sbiera, A. Hayakawa, M. Theodoropoulou, A. Osswald, F. Beuschlein, T. Meitinger, E. Mizuno-Yamasaki, K. Kawaguchi, Y. Saeki, K. Tanaka, T. Wieland, E. Graf, W. Saeger, C. L. Ronchi, B. Allolio, M. Buchfelder, T. M. Strom, M. Fassnacht and M. Komada (2015). "Mutations in the deubiquitinase gene USP8 cause Cushing's disease." Nat Genet **47**(1): 31-38.
Ren, X. and J. H. Hurley (2010). "VHS domains of ESCRT-0 cooperate in high-avidity binding to polyubiquitinated cargo." EMBO J **29**(6): 1045-1054.
Ren, X., D. P. Kloer, Y. C. Kim, R. Ghirlando, L. F. Saidi, G. Hummer and J. H. Hurley (2009). "Hybrid structural model of the complete human ESCRT-0 complex." Structure **17**(3): 406-416.
Richter, B., D. A. Sliter, L. Herhaus, A. Stolz, C. Wang, P. Beli, G. Zaffagnini, P. Wild, S. Martens, S. A. Wagner, R. J. Youle and I. Dikic (2016). "Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria." Proc Natl Acad Sci U S A **113**(15): 4039-4044.
Ritorto, M. S., R. Ewan, A. B. Perez-Oliva, A. Knebel, S. J. Buhrlage, M. Wightman, S. M. Kelly, N. T. Wood, S. Virdee, N. S. Gray, N. A. Morrice, D. R. Alessi and M. Trost (2014). "Screening of DUB activity and specificity by MALDI-TOF mass spectrometry." Nat Commun **5**: 4763.

Robinson, D. R., Y. M. Wu and S. F. Lin (2000). "The protein tyrosine kinase family of the human genome." Oncogene **19**(49): 5548-5557.
Robinson, M. S. (2015). "Forty Years of Clathrin-coated Vesicles." Traffic **16**(12): 1210-1238.
Robinson, M. S. and B. M. Pearse (1986). "Immunofluorescent localization of 100K coated vesicle proteins." J Cell Biol **102**(1): 48-54.
Roth, M. G. (2006). "Clathrin-mediated endocytosis before fluorescent proteins." Nature Reviews Molecular Cell Biology **7**: 6.
Roth, T. F. and K. R. Porter (1964). "Yolk Protein Uptake in the Oocyte of the Mosquito Aedes Aegypti. L." J Cell Biol **20**: 313-332.
Rotin, D. and S. Kumar (2009). "Physiological functions of the HECT family of ubiquitin ligases." Nat Rev Mol Cell Biol **10**(6): 398-409.
Row, P. E., M. J. Clague and S. Urbe (2005). "Growth factors induce differential phosphorylation profiles of the Hrs-STAM complex: a common node in signalling networks with signal-specific properties." Biochem J **389**(Pt 3): 629-636.
Row, P. E., H. Liu, S. Hayes, R. Welchman, P. Charalabous, K. Hofmann, M. J. Clague, C. M. Sanderson and S. Urbe (2007). "The MIT domain of UBPY constitutes a CHMP binding and endosomal localization signal required for efficient epidermal growth factor receptor degradation." J Biol Chem **282**(42): 30929-30937.
Row, P. E., I. A. Prior, J. McCullough, M. J. Clague and S. Urbe (2006). "The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation." J Biol Chem **281**(18): 12618-12624.
Rushmore, T. H. and C. B. Pickett (1990). "Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants." J Biol Chem **265**(24): 14648-14653.
Russell, N. S. and K. D. Wilkinson (2005). "Deubiquitinating enzyme purification, assay inhibitors, and characterization." Methods Mol Biol **301**: 207-219.
Sacco, J. (2012). "Investigating roles for the deubiquitylating enzymes in the PtdIns3-K/PKB pathway in cancer." University of Liverpool.
Sachse, M., S. Urbe, V. Oorschot, G. J. Strous and J. Klumperman (2002). "Bilayered clathrin coats on endosomal vacuoles are involved in protein sorting toward lysosomes." Mol Biol Cell **13**(4): 1313-1328.
Safa, A. R. (2013). "Roles of c-FLIP in Apoptosis, Necroptosis, and Autophagy." J Carcinog Mutagen **Suppl 6**.
Saksena, S., J. Wahlman, D. Teis, A. E. Johnson and S. D. Emr (2009). "Functional reconstitution of ESCRT-III assembly and disassembly." Cell **136**(1): 97-109.
Sangwan, V., J. Abella, A. Lai, N. Bertos, M. Stuible, M. L. Tremblay and M. Park (2011). "Protein-tyrosine phosphatase 1B modulates early endosome fusion and trafficking of Met and epidermal growth factor receptors." J Biol Chem **286**(52): 45000-45013.
Sarraf, S. A., M. Raman, V. Guarani-Pereira, M. E. Sowa, E. L. Huttlin, S. P. Gygi and J. W. Harper (2013). "Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization." Nature **496**(7445): 372-376.
Sato, Y., A. Yoshikawa, A. Yamagata, H. Mimura, M. Yamashita, K. Ookata, O. Nureki, K. Iwai, M. Komada and S. Fukai (2008). "Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains." Nature **455**(7211): 358-362.
Savio, M. G., N. Wollscheid, E. Cavallaro, V. Algisi, P. P. Di Fiore, S. Sigismund, E. Maspero and S. Polo (2016). "USP9X Controls EGFR Fate by Deubiquitinating the Endocytic Adaptor Eps15." Curr Biol **26**(2): 173-183.
Schlesinger, D. H. and G. Goldstein (1975). "Molecular conservation of 74 amino acid sequence of ubiquitin between cattle and man." Nature **255**(5507): 423-424.

Schlesinger, D. H., G. Goldstein and H. D. Niall (1975). "The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells." Biochemistry **14**(10): 2214-2218.
Schlessinger, J. (2002). "Ligand-induced, receptor-mediated dimerization and activation of EGF receptor." Cell **110**(6): 669-672.
Schmidt, O. and D. Teis (2012). "The ESCRT machinery." Curr Biol **22**(4): R116-120.
Schoenheimer, R., D. Rittenberg, G. L. Foster, A. S. Keston and S. Ratner (1938). "The Application of the Nitrogen Isotope N15 for the Study of Protein Metabolism." Science **88**(2295): 599-600.
Schwanhausser, B., D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen and M. Selbach (2011). "Global quantification of mammalian gene expression control." Nature **473**(7347): 337-342.
Scotti, E., M. Calamai, C. N. Goulbourne, L. Zhang, C. Hong, R. R. Lin, J. Choi, P. F. Pilch, L. G. Fong, P. Zou, A. Y. Ting, F. S. Pavone, S. G. Young and P. Tontonoz (2013). "IDOL stimulates clathrin-independent endocytosis and multivesicular body-mediated lysosomal degradation of the low-density lipoprotein receptor." Mol Cell Biol **33**(8): 1503-1514.
Sharma, S. V. and J. Settleman (2007). "Oncogene addiction: setting the stage for molecularly targeted cancer therapy." Genes Dev **21**(24): 3214-3231.
Shi, Y., X. Chen, S. Elsasser, B. B. Stocks, G. Tian, B. H. Lee, Y. Shi, N. Zhang, S. A. de Poot, F. Tuebing, S. Sun, J. Vannoy, S. G. Tarasov, J. R. Engen, D. Finley and K. J. Walters (2016). "Rpn1 provides adjacent receptor sites for substrate binding and deubiquitination by the proteasome." Science **351**(6275).
Shiba-Fukushima, K., Y. Imai, S. Yoshida, Y. Ishihama, T. Kanao, S. Sato and N. Hattori (2012). "PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy." Sci Rep **2**: 1002.
Shih, S. C., D. J. Katzmann, J. D. Schnell, M. Sutanto, S. D. Emr and L. Hicke (2002). "Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis." Nat Cell Biol **4**(5): 389-393.
Shin, I., F. M. Yakes, F. Rojo, N. Y. Shin, A. V. Bakin, J. Baselga and C. L. Arteaga (2002). "PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization." Nat Med **8**(10): 1145-1152.
Shintani, Y., Y. Fukumoto, N. Chaika, R. Svoboda, M. J. Wheelock and K. R. Johnson (2008). "Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1." J Cell Biol **180**(6): 1277-1289.
Sierra, M. I., M. H. Wright and P. D. Nash (2010). "AMSH interacts with ESCRT-0 to regulate the stability and trafficking of CXCR4." J Biol Chem **285**(18): 13990-14004.
Simpson, M. V. (1953). "The release of labeled amino acids from the proteins of rat liver slices." J Biol Chem **201**(1): 143-154.
Singh, B., G. Carpenter and R. J. Coffey (2016). "EGF receptor ligands: recent advances." F1000Res **5**.
Sippel, M., R. Rajala, L. Korhonen, B. Bornhauser, A. L. Sokka, M. Naito and D. Lindholm (2009). "Dexamethasone regulates expression of BRUCE/Apollon and the proliferation of neural progenitor cells." FEBS Lett **583**(13): 2213-2217.
Smith, C. J., N. Grigorieff and B. M. Pearse (1998). "Clathrin coats at 21 A resolution: a cellular assembly designed to recycle multiple membrane receptors." EMBO J **17**(17): 4943-4953.
Smith, G. A., G. W. Fearnley, I. Abdul-Zani, S. B. Wheatcroft, D. C. Tomlinson, M. A. Harrison and S. Ponnambalam (2016). "VEGFR2 Trafficking, Signaling and Proteolysis is Regulated by the Ubiquitin Isopeptidase USP8." Traffic **17**(1): 53-65.
Sorkin, A. and M. von Zastrow (2009). "Endocytosis and signalling: intertwining molecular networks." Nat Rev Mol Cell Biol **10**(9): 609-622.

Sorrentino, V., J. K. Nelson, E. Maspero, A. R. Marques, L. Scheer, S. Polo and N. Zelcer (2013). "The LXR-IDOL axis defines a clathrin-, caveolae-, and dynamin-independent endocytic route for LDLR internalization and lysosomal degradation." J Lipid Res **54**(8): 2174-2184.
Soubeyran, P., K. Kowanetz, I. Szymkiewicz, W. Y. Langdon and I. Dikic (2002). "Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors." Nature **416**(6877): 183-187.
Sporn, M. B. and K. T. Liby (2012). "NRF2 and cancer: the good, the bad and the importance of context." Nat Rev Cancer **12**(8): 564-571.
Stamos, J., M. X. Sliwkowski and C. Eigenbrot (2002). "Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor." J Biol Chem **277**(48): 46265-46272.
Stanley, R. E., M. J. Ragusa and J. H. Hurley (2014). "The beginning of the end: how scaffolds nucleate autophagosome biogenesis." Trends Cell Biol **24**(1): 73-81.
Stefani, F., L. Zhang, S. Taylor, J. Donovan, S. Rollinson, A. Doyotte, K. Brownhill, J. Bennion, S. Pickering-Brown and P. Woodman (2011). "UBAP1 is a component of an endosome-specific ESCRT-I complex that is essential for MVB sorting." Curr Biol **21**(14): 1245-1250.
Stepkowski, T. M. and M. K. Kruszewski (2011). "Molecular cross-talk between the NRF2/KEAP1 signaling pathway, autophagy, and apoptosis." Free Radic Biol Med **50**(9): 1186-1195.
Stern D.F., H. P. A. a. W. R. A. (1986). "p185, a Product of the neu Proto-Oncogene, Is a Receptorlike Protein Associated with Tyrosine Kinase Activity." Molecular and Cellular Biology **6**(5): 12.
Stewart, D., E. Killeen, R. Naquin, S. Alam and J. Alam (2003). "Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium." J Biol Chem **278**(4): 2396-2402.
Stolz, A., A. Ernst and I. Dikic (2014). "Cargo recognition and trafficking in selective autophagy." Nat Cell Biol **16**(6): 495-501.
Stromhaug, P. E., F. Reggiori, J. Guan, C. W. Wang and D. J. Klionsky (2004). "Atg21 is a phosphoinositide binding protein required for efficient lipidation and localization of Atg8 during uptake of aminopeptidase I by selective autophagy." Mol Biol Cell **15**(8): 3553-3566.
Strous, G. J., P. van Kerkhof, R. Govers, A. Ciechanover and A. L. Schwartz (1996). "The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor." EMBO J **15**(15): 3806-3812.
Suzuki, K., T. Kirisako, Y. Kamada, N. Mizushima, T. Noda and Y. Ohsumi (2001). "The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation." EMBO J **20**(21): 5971-5981.
Suzuki, K., Y. Kubota, T. Sekito and Y. Ohsumi (2007). "Hierarchy of Atg proteins in pre-autophagosomal structure organization." Genes Cells **12**(2): 209-218.
Suzuki, K. and Y. Ohsumi (2010). "Current knowledge of the pre-autophagosomal structure (PAS)." FEBS Lett **584**(7): 1280-1286.
Svenning, S. and T. Johansen (2013). "Selective autophagy." Essays Biochem **55**: 79-92.
Swaminathan, S., A. Y. Amerik and M. Hochstrasser (1999). "The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast." Mol Biol Cell **10**(8): 2583-2594.
Taguchi, K., N. Fujikawa, M. Komatsu, T. Ishii, M. Unno, T. Akaike, H. Motohashi and M. Yamamoto (2012). "Keap1 degradation by autophagy for the maintenance of redox homeostasis." Proc Natl Acad Sci U S A **109**(34): 13561-13566.
Takahashi, Y., D. Coppola, N. Matsushita, H. D. Cualing, M. Sun, Y. Sato, C. Liang, J. U. Jung, J. Q. Cheng, J. J. Mule, W. J. Pledger and H. G. Wang (2007). "Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis." Nat Cell Biol **9**(10): 1142-1151.

Takeda, K., Y. Komuro, T. Hayakawa, H. Oguchi, Y. Ishida, S. Murakami, T. Noguchi, H. Kinoshita, Y. Sekine, S. Iemura, T. Natsume and H. Ichijo (2009). "Mitochondrial phosphoglycerate mutase 5 uses alternate catalytic activity as a protein serine/threonine phosphatase to activate ASK1." Proc Natl Acad Sci U S A **106**(30): 12301-12305.
Tan, J. M., E. S. Wong, D. S. Kirkpatrick, O. Pletnikova, H. S. Ko, S. P. Tay, M. W. Ho, J. Troncoso, S. P. Gygi, M. K. Lee, V. L. Dawson, T. M. Dawson and K. L. Lim (2008). "Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases." Hum Mol Genet **17**(3): 431-439.
Tanaka, N., K. Kaneko, H. Asao, H. Kasai, Y. Endo, T. Fujita, T. Takeshita and K. Sugamura (1999). "Possible involvement of a novel STAM-associated molecule "AMSH" in intracellular signal transduction mediated by cytokines." J Biol Chem **274**(27): 19129-19135.
ter Haar, E., A. Musacchio, S. C. Harrison and T. Kirchhausen (1998). "Atomic structure of clathrin: a beta propeller terminal domain joins an alpha zigzag linker." Cell **95**(4): 563-573.
Thorpe, L. M., H. Yuzugullu and J. J. Zhao (2015). "PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting." Nat Rev Cancer **15**(1): 7-24.
Thrower, J. S., L. Hoffman, M. Rechsteiner and C. M. Pickart (2000). "Recognition of the polyubiquitin proteolytic signal." EMBO J **19**(1): 94-102.
Thumm, M., R. Egner, B. Koch, M. Schlumpberger, M. Straub, M. Veenhuis and D. H. Wolf (1994). "Isolation of autophagocytosis mutants of Saccharomyces cerevisiae." FEBS Lett **349**(2): 275-280.
Tiedemann, R. E., Y. X. Zhu, J. Schmidt, C. X. Shi, C. Sereduk, H. Yin, S. Mousses and A. K. Stewart (2012). "Identification of molecular vulnerabilities in human multiple myeloma cells by RNA interference lethality screening of the druggable genome." Cancer Res **72**(3): 757-768.
Tong, K. I., A. Kobayashi, F. Katsuoka and M. Yamamoto (2006). "Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism." Biol Chem **387**(10-11): 1311-1320.
Tonks, N. K. (2005). "Redox redux: revisiting PTPs and the control of cell signaling." Cell **121**(5): 667-670.
Tooze, S. A. (2010). "The role of membrane proteins in mammalian autophagy." Semin Cell Dev Biol **21**(7): 677-682.
Trousdale, C. and K. Kim (2015). "Retromer: Structure, function, and roles in mammalian disease." Eur J Cell Biol **94**(11): 513-521.
Tsang, H. T., J. W. Connell, S. E. Brown, A. Thompson, E. Reid and C. M. Sanderson (2006). "A systematic analysis of human CHMP protein interactions: additional MIT domain-containing proteins bind to multiple components of the human ESCRT III complex." Genomics **88**(3): 333-346.
Tsukada, M. and Y. Ohsumi (1993). "Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae." FEBS Lett **333**(1-2): 169-174.
Tzahar, E., H. Waterman, X. Chen, G. Levkowitz, D. Karunagaran, S. Lavi, B. J. Ratzkin and Y. Yarden (1996). "A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor." Mol Cell Biol **16**(10): 5276-5287.
Tzivion, G., M. Dobson and G. Ramakrishnan (2011). "FoxO transcription factors; Regulation by AKT and 14-3-3 proteins." Biochim Biophys Acta **1813**(11): 1938-1945.
Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger and et al. (1984). "Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells." Nature **309**(5967): 418-425.
Ungewickell, E. and D. Branton (1981). "Assembly units of clathrin coats." Nature **289**(5796): 420-422.

Urbe, S., I. G. Mills, H. Stenmark, N. Kitamura and M. J. Clague (2000). "Endosomal localization and receptor dynamics determine tyrosine phosphorylation of hepatocyte growth factor-regulated tyrosine kinase substrate." Mol Cell Biol **20**(20): 7685-7692.
Urbe, S., M. Sachse, P. E. Row, C. Preisinger, F. A. Barr, G. Strous, J. Klumperman and M. J. Clague (2003). "The UIM domain of Hrs couples receptor sorting to vesicle formation." J Cell Sci **116**(Pt 20): 4169-4179.
Vanhaesebroeck, B., L. Stephens and P. Hawkins (2012). "PI3K signalling: the path to discovery and understanding." Nat Rev Mol Cell Biol **13**(3): 195-203.
Vecchione, A., A. Marchese, P. Henry, D. Rotin and A. Morrione (2003). "The Grb10/Nedd4 complex regulates ligand-induced ubiquitination and stability of the insulin-like growth factor I receptor." Mol Cell Biol **23**(9): 3363-3372.
Venugopal, R. and A. K. Jaiswal (1996). "Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene." Proc Natl Acad Sci U S A **93**(25): 14960-14965.
Viglietto, G., M. L. Motti, P. Bruni, R. M. Melillo, A. D'Alessio, D. Califano, F. Vinci, G. Chiappetta, P. Tsichlis, A. Bellacosa, A. Fusco and M. Santoro (2002). "Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer." Nat Med **8**(10): 1136-1144.
Villasenor, R., Y. Kalaidzidis and M. Zerial (2016). "Signal processing by the endosomal system." Curr Opin Cell Biol **39**: 53-60.
Walton, G. M., W. S. Chen, M. G. Rosenfeld and G. N. Gill (1990). "Analysis of deletions of the carboxyl terminus of the epidermal growth factor receptor reveals self-phosphorylation at tyrosine 992 and enhanced in vivo tyrosine phosphorylation of cell substrates." J Biol Chem **265**(3): 1750-1754.
Waterman, H., M. Katz, C. Rubin, K. Shtiegman, S. Lavi, A. Elson, T. Jovin and Y. Yarden (2002). "A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling." EMBO J **21**(3): 303-313.
Waterman, H., G. Levkowitz, I. Alroy and Y. Yarden (1999). "The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor." J Biol Chem **274**(32): 22151-22154.
Waxman, L., J. M. Fagan and A. L. Goldberg (1987). "Demonstration of two distinct high molecular weight proteases in rabbit reticulocytes, one of which degrades ubiquitin conjugates." J Biol Chem **262**(6): 2451-2457.
Weltman, J. K. (1987). "The 1986 Nobel Prize for Physiology or Medicine awarded for discovery of growth factors: Rita Levi-Montalcini, M.D., and Stanley Cohen, Ph.D." N Engl Reg Allergy Proc **8**(1): 47-48.
Wilkinson, K. D. (2005). "The discovery of ubiquitin-dependent proteolysis." Proc Natl Acad Sci U S A **102**(43): 15280-15282.
Wilkinson, K. D., M. K. Urban and A. L. Haas (1980). "Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes." J Biol Chem **255**(16): 7529-7532.
Williams, R. L. and S. Urbe (2007). "The emerging shape of the ESCRT machinery." Nat Rev Mol Cell Biol **8**(5): 355-368.
Wirth, M., J. Joachim and S. A. Tooze (2013). "Autophagosome formation--the role of ULK1 and Beclin1-PI3KC3 complexes in setting the stage." Semin Cancer Biol **23**(5): 301-309.
Wong, A., B. Lamothe, A. Lee, J. Schlessinger and I. Lax (2002). "FRS2 alpha attenuates FGF receptor signaling by Grb2-mediated recruitment of the ubiquitin ligase Cbl." Proc Natl Acad Sci U S A **99**(10): 6684-6689.
Wu, X., L. Yen, L. Irwin, C. Sweeney and K. L. Carraway, 3rd (2004). "Stabilization of the E3 ubiquitin ligase Nrdp1 by the deubiquitinating enzyme USP8." Mol Cell Biol **24**(17): 7748-7757.

Wunderley, L., K. Brownhill, F. Stefani, L. Tabernero and P. Woodman (2014). "The molecular basis for selective assembly of the UBAP1-containing endosome-specific ESCRT-I complex." J Cell Sci **127**(Pt 3): 663-672.
Xia, R., H. Jia, J. Fan, Y. Liu and J. Jia (2012). "USP8 Promotes Smoothened Signaling by Preventing Its Ubiquitination and Changing Its Subcellular Localization." PLoS Biol **10**(1): e1001238.
Xie, Z., U. Nair and D. J. Klionsky (2008). "Atg8 controls phagophore expansion during autophagosome formation." Mol Biol Cell **19**(8): 3290-3298.
Xu, D., B. Shan, H. Sun, J. Xiao, K. Zhu, X. Xie, X. Li, W. Liang, X. Lu, L. Qian and J. Yuan (2016). "USP14 regulates autophagy by suppressing K63 ubiquitination of Beclin 1." Genes Dev **30**(15): 1718-1730.
Xu, L., Y. Wei, J. Reboul, P. Vaglio, T. H. Shin, M. Vidal, S. J. Elledge and J. W. Harper (2003). "BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3." Nature **425**(6955): 316-321.
Xu, Y., F. Fang, S. Miriyala, P. A. Crooks, T. D. Oberley, L. Chaiswing, T. Noel, A. K. Holley, Y. Zhao, K. K. Kiningham, D. K. Clair and W. H. Clair (2013). "KEAP1 is a redox sensitive target that arbitrates the opposing radiosensitive effects of parthenolide in normal and cancer cells." Cancer Res **73**(14): 4406-4417.
Yamamoto, H., S. Kakuta, T. M. Watanabe, A. Kitamura, T. Sekito, C. Kondo-Kakuta, R. Ichikawa, M. Kinjo and Y. Ohsumi (2012). "Atg9 vesicles are an important membrane source during early steps of autophagosome formation." J Cell Biol **198**(2): 219-233.
Yang, A., N. S. Wilson and A. Ashkenazi (2010). "Proapoptotic DR4 and DR5 signaling in cancer cells: toward clinical translation." Curr Opin Cell Biol **22**(6): 837-844.
Yang, W. L., J. Wang, C. H. Chan, S. W. Lee, A. D. Campos, B. Lamothe, L. Hur, B. C. Grabiner, X. Lin, B. G. Darnay and H. K. Lin (2009). "The E3 ligase TRAF6 regulates Akt ubiquitination and activation." Science **325**(5944): 1134-1138.
Yang, Y., W. Fiskus, B. Yong, P. Atadja, Y. Takahashi, T. K. Pandita, H. G. Wang and K. N. Bhalla (2013). "Acetylated hsp70 and KAP1-mediated Vps34 SUMOylation is required for autophagosome creation in autophagy." Proc Natl Acad Sci U S A **110**(17): 6841-6846.
Yang, Z. and D. J. Klionsky (2010). "Mammalian autophagy: core molecular machinery and signaling regulation." Curr Opin Cell Biol **22**(2): 124-131.
Ye, Y. and M. Rape (2009). "Building ubiquitin chains: E2 enzymes at work." Nat Rev Mol Cell Biol **10**(11): 755-764.
Yeates, E. F. and G. Tesco (2016). "The Endosome-associated Deubiquitinating Enzyme USP8 Regulates BACE1 Enzyme Ubiquitination and Degradation." J Biol Chem **291**(30): 15753-15766.
Yee, N. S., C. W. Hsiau, H. Serve, K. Vosseller and P. Besmer (1994). "Mechanism of down-regulation of c-kit receptor. Roles of receptor tyrosine kinase, phosphatidylinositol 3'-kinase, and protein kinase C." J Biol Chem **269**(50): 31991-31998.
Yoh, K., K. Itoh, A. Enomoto, A. Hirayama, N. Yamaguchi, M. Kobayashi, N. Morito, A. Koyama, M. Yamamoto and S. Takahashi (2001). "Nrf2-deficient female mice develop lupus-like autoimmune nephritis." Kidney Int **60**(4): 1343-1353.
Yokouchi, M., T. Kondo, A. Houghton, M. Bartkiewicz, W. C. Horne, H. Zhang, A. Yoshimura and R. Baron (1999). "Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7." J Biol Chem **274**(44): 31707-31712.
Yokouchi, M., T. Wakioka, H. Sakamoto, H. Yasukawa, S. Ohtsuka, A. Sasaki, M. Ohtsubo, M. Valius, A. Inoue, S. Komiya and A. Yoshimura (1999). "APS, an adaptor protein containing PH and SH2 domains, is associated with the PDGF receptor and c-Cbl and inhibits PDGF-induced mitogenesis." Oncogene **18**(3): 759-767.
Yorikawa, C., H. Shibata, S. Waguri, K. Hatta, M. Horii, K. Katoh, T. Kobayashi, Y. Uchiyama and M. Maki (2005). "Human CHMP6, a myristoylated ESCRT-III protein, interacts directly

with an ESCRT-II component EAP20 and regulates endosomal cargo sorting." Biochem J **387**(Pt 1): 17-26.
Zaremba, S. and J. H. Keen (1983). "Assembly polypeptides from coated vesicles mediate reassembly of unique clathrin coats." J Cell Biol **97**(5 Pt 1): 1339-1347.
Zhang, D. D. and M. Hannink (2003). "Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress." Mol Cell Biol **23**(22): 8137-8151.
Zheng, N., P. Wang, P. D. Jeffrey and N. P. Pavletich (2000). "Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases." Cell **102**(4): 533-539.
Zhong, Y., Q. J. Wang, X. Li, Y. Yan, J. M. Backer, B. T. Chait, N. Heintz and Z. Yue (2009). "Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex." Nat Cell Biol **11**(4): 468-476.
Zhou, B. P., Y. Liao, W. Xia, Y. Zou, B. Spohn and M. C. Hung (2001). "HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation." Nat Cell Biol **3**(11): 973-982.
Zhou, R., V. R. Tomkovicz, P. L. Butler, L. A. Ochoa, Z. J. Peterson and P. M. Snyder (2013). "Ubiquitin-specific peptidase 8 (USP8) regulates endosomal trafficking of the epithelial Na+ channel." J Biol Chem **288**(8): 5389-5397.

Zhu, Y., L. M. Traub and S. Kornfeld (1999). "High-affinity binding of the AP-1 adaptor complex to trans-golgi network membranes devoid of mannose 6-phosphate receptors." Mol Biol Cell **10**(3): 537-549.

Zipper, L. M. and R. T. Mulcahy (2002). "The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm." J Biol Chem **277**(39): 36544-36552.