The role of Cbl-mediated ubiquitination in the regulation of the Met receptor tyrosine kinase

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Abstract

The Met receptor tyrosine kinase (RTK) and its ligand, the hepatocyte growth factor/scatter factor (HGF/SF), which regulate epithelial cell remodelling, dispersal and invasion, are deregulated in many human cancers. In the last twenty years, mechanisms of RTK activation and signalling have been studied extensively. In contrast, very little is known about the mechanisms of down-regulation of RTKs. At the time I began my thesis work, the family of Cbl ubiquitin-protein ligases emerged as negative regulators for RTKs. In this thesis, I demonstrate that c-Cbl is an important negative regulator of the Met receptor. Upon HGF stimulation, c-Cbl promotes ubiquitination of the Met RTK. This requires the association of the c-Cbl tyrosine kinase binding (TKB) domain with a DpYR binding motif, including Y1003, present in the juxtamembrane of the Met receptor. A Met Y1003F receptor, which lacks the c-Cbl TKB binding site, is not ubiquitinated, transforms fibroblast and epithelial cells *in vitro* and is tumorigenic *in vivo*. I demonstrate that ubiquitination of the Met receptor is not required for its internalization from the plasma membrane, but is essential for its lysosomal degradation. In the presence of HGF, the Met Y1003F receptor is poorly degraded and remains phosphorylated, leading to sustained activation of the Ras-MAPK pathway. Moreover, fusion of a ubiquitin moiety to the carboxy-terminus of Met Y1003F is sufficient to decrease Met receptor stability, prevent sustained MEK1/2 activation and reduce cell transformation.

To examine the tumorigenicity of the Met Y1003F receptor in animals, I generated a murine model expressing Met Y1003F under the MMTV promoter, and observed that Y1003F substitution increases Met receptor tumorigenicity in mammary glands. This demonstrates for the first time that ubiquitination of a RTK is required for its biological functions *in vivo*. Furthermore, this highlights synergy between an activating mutation (M1250T) and a loss of down-regulation mutation (Y1003F) in the tumorigenicity of the Met receptor. Based on these results and on the observation that several RTKs escape the lysosomal degradative pathway in human tumours, I propose that loss of RTK down-regulation is a common mechanism for oncogenic activation of RTKs in human tumours.

Résumé

Le récepteur à activité tyrosine kinase (RTK) Met et son ligand, le facteur de croissance hépatocytaire HGF, qui jouent un rôle important dans le remodelage, la dispersion et l'invasion des cellules épithéliales, sont altérés dans plusieurs tumeurs. Au cours des vingt dernières années, les mécanismes sous-jacents à l'activation et à la signalisation des RTKs ont été amplement étudiés. Par contre, nous savons peu de choses à propos des mécanismes de désactivation des RTKs. Dans cette thèse, je démontre que la ligase de l'ubiquitine c-Cbl est une importante régulatrice négative du RTK Met. Ceci nécessite l'association du domaine TKB (tyrosine kinase binding) de c-Cbl au motif DpYR comprenant Y1003, qui est présent dans le domaine juxtamembranaire du RTK Met. Le RTK Met Y1003F, qui n'a pas de site de liaison pour le domaine TKB de c-Cbl, n'est pas ubiquitiné, transforme les cellules fibroblastes et épithéliales *in vitro* et est tumorigène *in vivo*. Je démontre que l'ubiquitination du RTK Met n'est pas nécessaire à son internalisation à partir de la membrane plasmique, mais est essentiel à sa dégradation dans le lysosome. En présence de HGF, le RTK Met Y1003F est peu dégradé et reste phosphorylé, menant à une activation soutenue de la voie Ras-MAPK. De plus, la fusion d'une protéine ubiquitine à la queue carboxy-terminale du RTK Met Y1003F suffit pour diminuer la stabilité du RTK Met, prévenir l'activation soutenue de la voie Ras-MAPK et diminuer la transformation cellulaire.

Pour examiner la tumorigénicité du RTK Met Y1003F chez les animaux, j'ai généré un modèle de souris exprimant Met Y1003F sous le contrôle du promoteur MMTV, et j'ai observé que la substitution Y1003F augmente la tumorigénicité de Met dans les glandes mammaires. Ceci démontre pour la première fois que l'ubiquitination d'un RTK est nécessaire à ses fonctions biologiques *in vivo*. Sur la base de ces résultats et de l'observation que plusieurs RTKs échappent à la voie de dégradation lysosomale dans les tumeurs chez l'humain, j'avance que la perte de régulation négative des RTKs constitue un mécanisme d'activation oncogénique des RTKs dans les tumeurs chez l'humain.

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First of all, I would like to thank my supervisor, Morag Park, for her mentorship, guidance, support, generosity and friendship. I have learned tremendously from her and she has been a real source of inspiration throughout my doctoral studies. I especially want to thank her for her critical appraisal and helpful comments.

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Preface

This thesis is a manuscript-based thesis. It contains 2 published manuscripts and 1 manuscript in press. The thesis is organized into six chapters:

1) a general introduction and literature review

2 to 4) manuscripts, each with their own preface, abstract, introduction, materials and methods, results, discussion and references

5) a general discussion of all the results with references

6) claims to original research

Reprint of a published minireview is included as appendix I at the end of the thesis

Publications arising from work of the thesis

First-author publications

1. Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. (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*, 995-1004.
2. Peschard, P., Ishiyama, N., Lin, T., Lipkowitz, S., and Park, M. (2004). A conserved DpYR motif in the juxtamembrane domain of the met receptor family forms an atypical c-Cbl/Cbl-b tyrosine kinase binding domain binding site required for suppression of oncogenic activation. J Biol Chem *279*, 29565-29571.
3. Abella, J.V.*, Peschard, P.*, Naujokas, M.A., Lin, T., Saucier, C, Urbé, S. and Park, M. (2005). Met/HGF receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. *Mol Cell Biol 25*, 9632-9645.

*These authors contributed equally to this work.

Contribution of Authors

1. T. Fournier observed that that the c-Cbl TKB domain binds to CSF-Met. L. Lamorte observed that the c-Cbl TKB domain does not bind to Tpr-Met. M Naujokas helped with the focus-forming assays.
2. N. Ishiyama did the structure modelling (Fig. 5). T. Lin generated the GST-Cbl-b TKB construct.
3. - I established the T47D cell lines (Fig. 1B).
   - I examined Met receptor ubiquitination (Fig. 1D).
   - I observed the delay in Met Y1003F degradation (Fig, 1E, F).
   - I performed the *in vitro* tumorigenesis assays (Fig. 2B, C).
   - I established the MDCK cell lines (Fig. 4).
   - I did the internalization assays using the trypsin-based assay and flow cytometry (Fig. 5A, B).

- I designed and made the Met-ubiquitin chimeric receptors used in Fig. 7, 8 and 9).
- I did the experiment demonstrating that Met Y1003F-ub is degraded (Fig. 8A).
- Finally, I did the focus-forming assays with Met Y1003F-ub (Fig. 9A,B).

J. Abella did the experiments on MAPK and Akt activation, performed the fluorescence microscopy and did the Hrs experiments (Fig. 1C, 2A, 3A,B,C, 4, 5C, 6, 7C, 8B,C).

T. Lin and M. Naujokas helped with establishing the T47D cell lines and the focus-forming assays.

C. Saucier helped with the *in vitro* tumorigenesis assays.

4. There are also unpublished data presented in Chapter 5 (General discussion). I designed and made the constructs and purified the DNA fragments used to generate the transgenic mice. I initiated the mouse colonies and setup the conditions for genotyping the mice. At that point, Stephanie Petkiewicz took over the project. She provided Table 2 and Figure 3 in Chapter 5.

Review

6. Peschard, P., and Park, M. (2003). Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. *Cancer Cell 3*, 519-523.

Other publications

7. Saucier, C., Khoury, H., Lai, K. M., Peschard, P., Dankort, D., Naujokas, M. A., Holash, J., Yancopoulos, G. D., Muller, W. J., Pawson, T., and Park, M. (2004). The Shc adaptor protein is critical for VEGF induction by Met/HGF and ErbB2 receptors and for early onset of tumor angiogenesis. Proc Natl Acad Sci U S A *101*, 2345-2350.

   - I helped with the in vivo tumorigenesis and matrigel plug assays.

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Chapter 1

Literature Review

1. Introduction

In a multicellular organism, development and homeostasis require cells to communicate with one another and to respond to the extracellular environment. Cells must know when to divide, differentiate, survive and migrate for the benefit of the whole organism. Deregulation of these processes can lead to the development of many human diseases including diabetes, neuropathies and cancers (1). Cell-surface receptors play an important role in detecting and transducing extracellular stimuli. In general, ligand binding to extracellular receptors will trigger intracellular signalling that will influence the fate of the cell. One major class of cell-surface receptors involved in cell signalling is the family of receptor tyrosine kinases (RTK).

2. Receptor tyrosine kinases

Receptors for most growth factors belong to the family of RTKs. They control a wide variety of cellular events in pluricellular organisms including cell proliferation, cell differentiation, cell migration and cell survival. They are single-pass transmembrane proteins that contain an extracellular ligand-binding domain and an intracellular domain that includes the kinase domain and several tyrosine residues that become phosphorylated upon receptor activation. Insight into the existence of RTKs was obtained in 1982 when Cohen et al. showed that a purified polypeptide bound to the epidermal growth factor (EGF) was associated with kinase activity (2), and Buhrow et al. demonstrated that it was able to bind ATP (3, 4). In 1984, Ullrich et al. cloned the first RTK, the EGF receptor (5, 6), and the following year, they cloned the cDNA for the insulin receptor (7). There are now 58 genes known to encode RTKs that are classified into 20 structural subfamilies (Fig. 1).

In normal cells, activation of RTKs is tightly regulated. Their inappropriate activation is associated with the development and progression of many human malignancies. Of the 58 genes encoding RTKs identified in the human genome, the deregulation of 30 has been associated with human tumours (8). In the past two decades, several mechanisms that lead to deregulation of RTKs in human tumours have been identified. These include receptor amplification, chromosomal translocation as well as point mutations (9). These changes can result in ligand-independent activation of the receptor, enhanced catalytic activity or altered

signalling (8, 9). In the past five years, work from this thesis and other laboratories has demonstrated that escape from normal down-regulation provides an additional mechanism that contributes to the oncogenic activation of RTKs. Hence, it is crucial to elucidate the mechanisms that ensure the proper down-regulation of RTK signalling in normal cells.

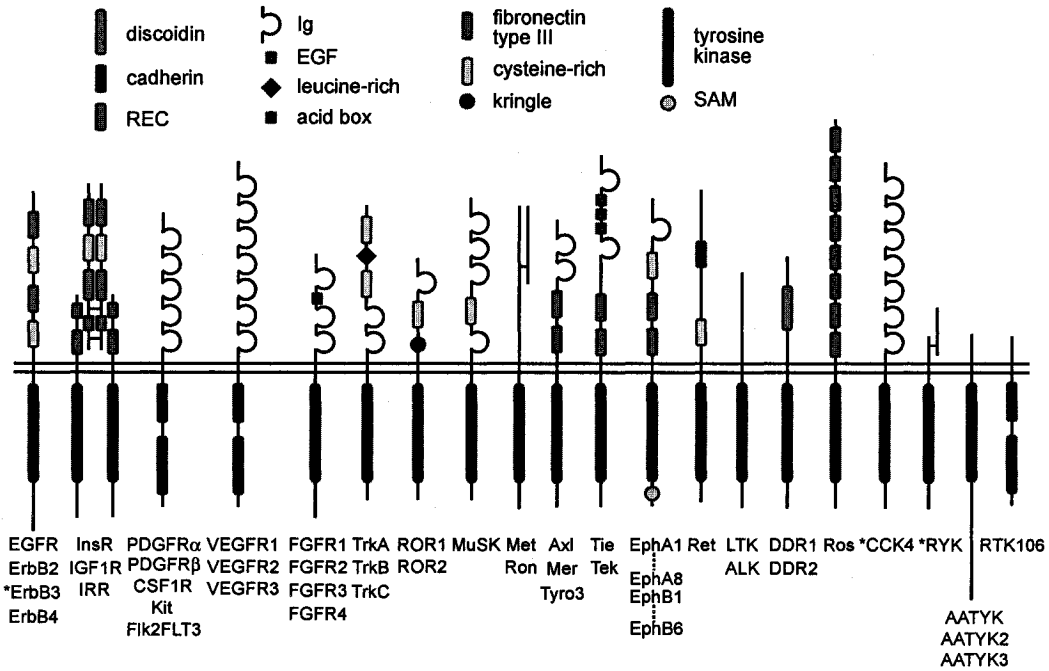


Figure 1. Human receptor tyrosine kinases

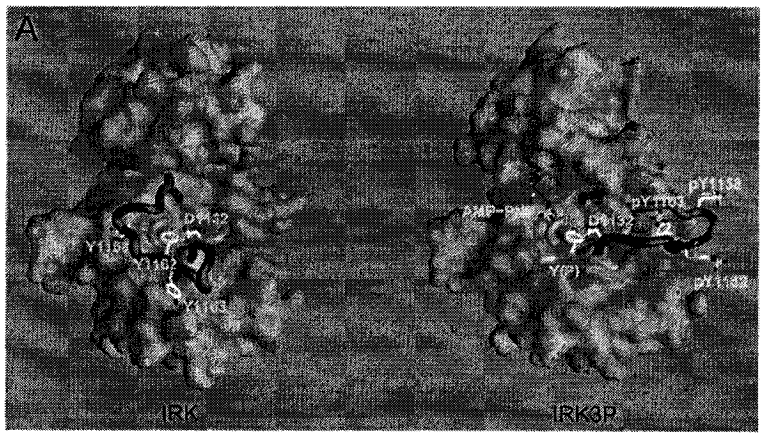
Human RTKs have been classified into 20 subfamilies based on their structure. Asterisks indicate receptors devoid of intrinsic kinase activity. Adapted from Blume-Jensen and Hunter. 2001. Nature 411: 355-65.

2.1 RTK activation

Members of the RTK family possess tyrosine kinase enzymatic activity that is conferred by a domain of 265 amino acids conserved between all members of the family of protein kinases. A seminal article by Hubbard *et al.* in 1994 described the structure of the kinase domain of the insulin receptor (IR) (10), which revealed that its structure is similar to serine and threonine kinases. The kinase domain is composed of two lobes separated by a linker region that varies in length. The amino-terminal lobe contains the ATP binding pocket in which an Mg^{2+} atom resides, whereas the carboxy-terminal lobe contains a conserved lysine residue involved in the transfer of the γ phosphate of ATP to tyrosine

residues (10-12). In the absence of ligand, RTKs are catalytically inactive. In an inactive state, a flexible loop, named the activation loop (A-loop), is located in the cleft between the amino and carboxy terminal lobes (Fig. 2) (10). For the insulin receptor, a tyrosine residue (Y1162) within the A-loop is hydrogen-bonded to the active site (D1132 in IR) and limits the access to protein substrates (10, 13). In general, binding of the ligand to the extracellular domain of the receptor promotes a conformational change and/or stabilizes receptor dimerization/oligomerization, leading to the phosphorylation of tyrosine residues within the A-loop. This induces a dramatic conformational change where the A-loop rotates away from the cleft, providing unrestricted access to the active site for ATP and substrate proteins (13). The phosphate group of a phosphotyrosine in the A-loop interacts with other amino acid residues of the A-loop, maintaining the A-loop in an open conformation (Fig. 2) (13). This allows the N-terminal lobe to swing towards the C-terminal lobe (10, 13). The receptor is now in an active state and this results in the phosphorylation of tyrosine residues located outside of the kinase domain. These then form binding sites for proteins that relay the biological signals to the inside of the cell.

The depth of the pocket containing the phosphotransfer site determines the specificity of the kinase towards tyrosine residues versus serine or threonine residues. This pocket is deeper in a tyrosine kinase and as a result, a serine or threonine side chain is too short for the hydroxyl group to reach the phosphotransfer site (10).



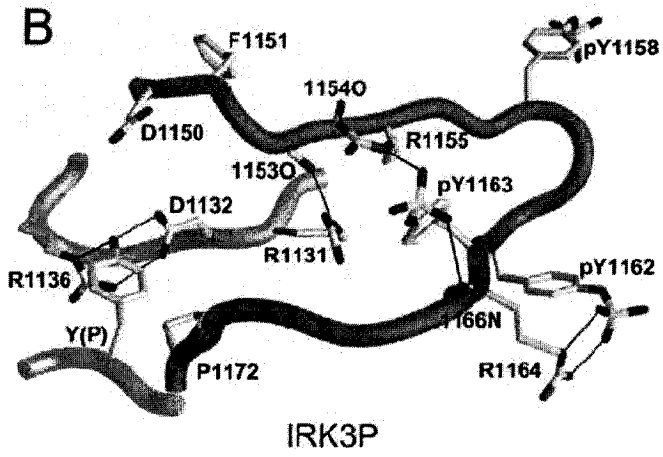


Figure 2. Structure of the kinase domain of the insulin receptor

A) Comparison of the A-loop conformations in IRK (insulin receptor kinase) and IRK3P (phosphorylated IRK). Whilst the A-loop is blocking the substrate and ATP binding sites IRK, it rotates away from the cleft, providing unrestricted access to the active site in IRK3P. **B)** In IRK3P, the phosphate group of a phosphotyrosine in the A-loop interacts with other amino acid residues of the A-loop, maintaining the A-loop in an open conformation. Reproduced from Hubbard. 1997. EMBO J **16**: 5572.

2.2 RTK signalling

Phosphorylation on tyrosine is a rare event in normal cells as it accounts for about 0.05% of total cellular phosphorylation (14). The first evidence for the role of tyrosine phosphorylation in cellular signalling came from studies on cellular transformation by v-Src published in 1980 (14, 15). Sefton and Hunter monitored a 6-10 fold increase in total cellular phosphotyrosine in cells transformed by the Rous sarcoma virus (14). They also observed that v-Src, the transforming protein of Rous sarcoma virus, is able to phosphorylate tyrosine residues, implying that tyrosine phosphorylation may have a role in cellular transformation (15). In the following three years, numerous publications demonstrated that several growth factors induce tyrosine-specific phosphorylation (16).

As mentioned above, phosphorylation of tyrosine residues in the cytoplasmic domain of RTKs forms sequence specific binding sites for proteins that contain SH2 and/or PTB phosphotyrosine-binding domains. Different classes of signalling proteins are recruited to activated RTKs. These include adaptor and docking proteins, kinases, phosphatases, phospholipases, transcription factors, guanine

nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) for small GTPases (8). Their recruitment to activated RTKs will allow their tyrosine phosphorylation, or promote conformational changes, which can modulate the catalytic activity and/or generate binding sites for other signalling molecules. The formation of signalling complexes following RTK activation will often require additional protein-protein and protein-lipid interactions that can occur through a wide variety of protein domains including the SH3, WW, PDZ, PH, and FYVE domains. The signalling complexes newly formed transmit signals from the cell surface to the nucleus to modulate gene expression, but also to various components of the cell such as the cytoskeleton, cell-matrix and cell-cell adhesion protein complexes.

2.3 Phosphotyrosine binding domains

The two major phosphotyrosine binding domains identified to date are the SH2 domain and the PTB domain. In addition to mediating reversible protein-protein interactions with phosphotyrosine proteins, phosphotyrosine binding domains also serve as an on-off switch in multiple proteins by forming an intramolecular interaction that keeps the protein in an inactive conformation (16, 17).

2.3.1 SH2 domain

Like the tyrosine kinase SH1 domain, the SH2 domain appeared in protozoa and Dictyostelium discoideum (18). The SH2 domain was first discovered by introducing dipeptide insertions in the v-Fps/Fes cytoplasmic tyrosine kinase to identify a potential domain regulating v-Fps kinase activity (19). Pawson's group identified a region of about 100 amino acids, conserved in Src and Abl, which is required for v-Fps transformation, leading to the name Src homology 2 (20). Disruption of the SH2 domain also modified both kinase activity and substrate recognition of v-Fps (16, 20). Four years later, in 1990, Pawson's group demonstrated that the SH2 domain of PLC-γ1, RasGAP and Src were able to bind tyrosine phosphorylated cytoplasmic proteins and activated RTKs (21, 22). Schlessinger's group also observed that the PLC-γ1 SH2 domain could prevent EGFR dephosphorylation (23). Hidesaburo Hanafusa and Bruce Mayer also contributed to this model by cloning v-Crk, a viral oncogene containing only an

SH2 and an SH3 domain (24). They observed that v-Crk is able to associate with phosphotyrosine-containing proteins in a phosphotyrosine-dependent manner (25, 26).

By sequence homology, over 50 mammalian proteins have been identified containing an SH2 domain. The amino acid residues located downstream of the phosphotyrosine residue confers the specificity to the interaction between SH2 domains and phosphotyrosine residues. For example, the SH2 domain of Src preferably binds to the pYEEI consensus sequence while the SH2 domain of p85 and Grb2 bind to the pYMxM and pYxN consensus sequences respectively (27-29). Structural studies on various SH2 domains provided an understanding of the specificity of SH2 domain interactions. SH2 domains form a compact module of about 100 amino acids. The structure of nine different SH2 domains has revealed a common fold. The amino and carboxy termini of SH2 domains are in close proximity in space, allowing their insertion in a polypeptide while leaving the ligand binding site exposed (16). All SH2 domains contain a central antiparallel β sheet that is flanked on either surface by a α-helix (17, 30). The phosphotyrosine residue fits into a positively charged pocket of the SH2 domain that contains an invariant arginine residue. The negative charge of the phosphate group interacts with the positively charged amino group of the arginine residue (31). Mutation of the invariant arginine residue abolishes the interaction of the SH2 domain with phosphotyrosine peptides. The two variable loops of the SH2 domain form a hydrophobic pocket that binds to the side chains of amino acids downstream of the phosphotyrosine residue. The amino acids forming the hydrophobic pocket dictate the specificity of an SH2 domain for a given consensus sequence. Waksman et al. described the interaction between the Src SH2 domain and the PQpYEEI peptide as "a complex that resembles a two-pronged plug engaging a two-holed socket" (31).

2.3.2 PTB domain

The PTB domain was first identified in Shc and SCK (IRS-1) proteins in 1994 (32). It is a 160 amino acid module that is structurally unrelated to the SH2 domain and that recognizes phosphotyrosine residues within an NPXpY consensus sequence (33-36). Surprisingly, the PTB domain fold is very similar to that of PH domains, which bind the headgroup region of phosphatidylinositol

phosphates. The PTB domain fold consists of a seven stranded β sandwich that is capped at one end by an α-helix (37-39). Two basic amino acid residues in PTB recognize the phosphotyrosine residue. Residues amino-terminal to the NPXpY motif in the insulin receptor forms an additional β strand in the β sheet of IRS-1 PTB (39). Whilst the phosphotyrosine peptides that bind to SH2 domains are disordered in solution and have an extended configuration once bound, the NPXpY peptides form stable turns in solution. This suggests that PTB domains recognize a preformed structure rather than a linear peptide (17). It is also worth mentioning that multiple PTB domains, such as the one in Dab and Numb, can bind to their ligand in a phosphotyrosine-independent manner (40).

3. Mechanisms of RTK oncogenic activation

As mentioned earlier, alterations in RTKs are frequently associated with human cancers. This occurs by multiple mechanisms, namely overexpression, the formation of an autocrine loop, point mutations and chromosomal translocation (Fig. 3). Several RTKs were first identified as the transforming agent incorporated into oncogenic retroviruses. This is the case for CSF-1R, identified as the cellular homologue of v-Fms, the product of the McDonough strain of feline sarcoma virus (41); the EGFR was identified as the cellular homologue of v-erbB protein encoded by the avian erythroblastosis virus (42); and c-Kit was identified as the cellular homologue of v-kit, the transforming protein of the Hardy-Zuckerman-4 strain of feline sarcoma viruses (43). However, RTK-derived retroviruses have not been identified in human cancers.

3.1 Autocrine loop

In general, different subsets of cells will express the RTK and its ligand (44, 45). In cancers, it is often observed that tumour cells express both the receptor and its ligand, resulting in continuous receptor activation. This can be observed *in vitro* where fibroblasts, expressing both the Met receptor and its ligand HGF, are tumorigenic in nude mice (46). There are several examples of the formation of autocrine loops in human tumours: some gliomas, astrocytomas, pancreatic and breast carcinomas express both PDGF and PDGFR while their normal counterparts do not (47-49). Inhibition of PDGFR with Gleevec (tyrosine kinase inhibitor) reduces growth and metastasis of human pancreatic carcinoma in nude

mice (49). Carcinomas expressing high levels of EGFR secrete one of its ligand, TGF-α (50).

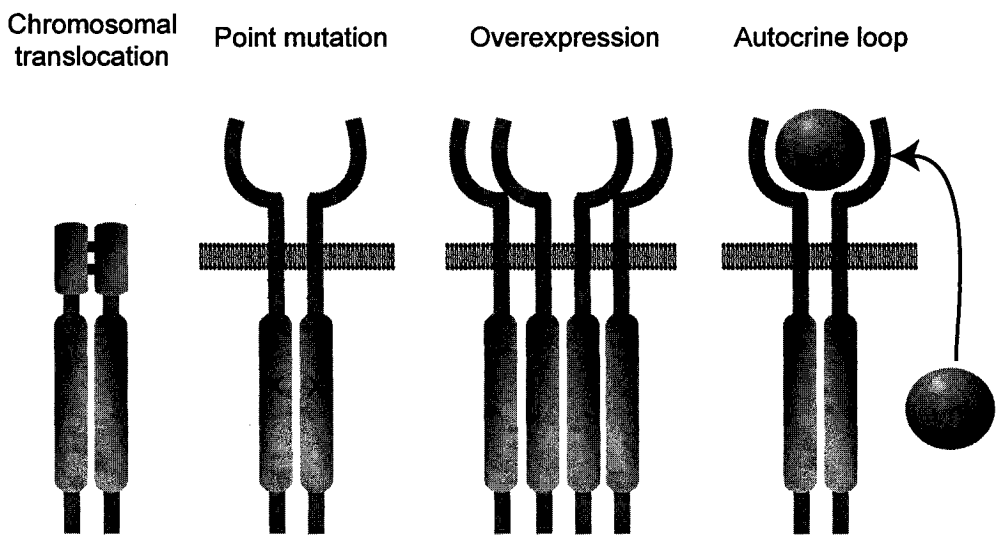


Figure 3. Mechanisms of oncogenic activation of RTKs in human tumours

3.2 Overexpression

Overexpression of RTKs increases the concentration of receptors at the cell surface. This may amplify the propagation of downstream signals in response to physiological levels of ligand. This may also lead to ligand-independent activation of RTKs by promoting their clustering. For instance, overexpression of RTKs in cell culture models often leads to their constitutive activation. Increase in protein level of RTKs can occur following genomic amplification or by other mechanisms that would enhance their transcription, translation and/or protein stability. Genomic amplification of RTKs has been reported in several human cancers (9). For example, ErbB2 is amplified in 10 to 30 percent of breast (51, 52), gastric (53), oesophageal (54) and ovarian (52) cancers while EGFR is amplified in 40 to 50 percent of glioblastomas (55), breast (56) and squamous cell carcinomas (57). PDGFRα is also amplified in glioblastomas (58). In the majority of cases, RTK overexpression is associated with poor prognosis.

3.3 Point mutation

There are multiple mechanisms through which point mutations can activate RTKs. The first point mutation identified in tumours was in ErbB2/Neu isolated from a rat neuroblastoma. A single point mutation, located in the transmembrane domain promotes receptor dimerization and kinase activation in the absence of ligand through dimerization of the transmembrane domain (59, 60). Point mutations that add or remove a cysteine residue from the extracellular domain of an RTK generate an uneven number of cysteine residues, which can promote the formation of a disulfide bond between receptors in close proximity, stabilizing their dimerization. For example, the loss of a single cysteine residue in the extracellular domain of the Ret receptor has been observed in the inherited multiple endocrine neoplasia (MEN) type 2A syndrome (61-63). Similarly, there is a gain of a cysteine residue within the extracellular or transmembrane domain of FGFR3 in multiple myeloma, bladder and cervical carcinomas, and short-limbed dwarfism (64, 65).

Most of the mutations identified are located in the kinase domain of RTKs. They have been observed in Ret in the MEN 2B syndrome (66, 67), in c-kit in mast cell tumours (68, 69), in the Met receptor in renal and hepatocellular carcinomas (70-72) and in FGFR3 in multiple myelomas (65, 73). Some mutations are thought to directly alter contacts between residues from the main body of the catalytic domain and from the activation loop in its inhibitory conformation, while other mutations located at hinge regions may facilitate the movement of sub-domains during kinase activation (74). In both cases, the mutations would reduce the threshold required to relieve auto-inhibition of kinase activity. Otherwise, mutations in the kinase domain may alter substrate specificity of the enzyme. A recent study demonstrated that a point mutation found in both Ret in MEN 2B and Met in renal papillary carcinomas specifically leads to the activation of STAT3 (75). This may explain how different mutations within Ret can lead to different diseases.

In addition, mutations in the juxtamembrane domain of RTKs have been identified in c-Kit (76, 77), Flt3 (78-80) and Met (81, 82). In recent years, several studies have revealed a role for the juxtamembrane domain in the autoinhibition of the VEGFR-1 (83), Eph (84, 85), Flt-3 (86) and c-Kit (87) RTKs. Structural studies demonstrated that a helix containing a juxtamembrane unphosphorylated

tyrosine residue adopts a conformation that distorts the small lobe of the kinase domain, preventing receptor activation. Upon receptor activation, phosphorylation of the juxtamembrane tyrosine promotes a conformational change that allows full activation of the receptor. Deletion of these tyrosine residues in c-Kit, Flt-3 and Eph receptors removes this inhibitory mechanism (85-87).

3.4 Chromosomal translocation

RTKs are frequently activated in human tumours following chromosomal translocation and to date, over 25 RTK-derived fusion proteins generated from 8 different RTKs have been identified (9). Radiation is a major cause of chromosomal instability leading to chromosome breaks and rearrangement. Many oncoproteins derived from the Trk and Ret RTKs have been identified in children exposed to the radioactive fallout following the Chernobyl reactor accident in 1986 (9). In general, the chromosomal translocation fuses a protein dimerization domain with the cytosolic kinase domain of an RTK, resulting in constitutive receptor dimerization and activation (88). The prototype for this class of RTK-derived oncoprotein, Tpr-Met, was generated following a chromosomal rearrangement that fuses Tpr, a sequence encoding a leucine zipper motif, in frame with the sequence encoding the cytoplasmic domain of Met (89). This rearrangement occurred following the treatment of a human osteogenic sarcoma (HOS) cell line with MNNG, a carcinogenic agent (90).

RTKs activated by chromosomal translocation include Met, FGFR1, FGFR3, PDGFRβ, Ret, Ros, TrkA and TrkC (9). In addition to Met, Tpr has also been found fused to TrkA, a neurotrophin receptor (91). Tel, a member of the ETS family of transcription factors, is another dimerization motif prone to be involved in activation of RTKs following chromosomal translocation since it has been fused to PDGFRβ (92) and TrkC (93-95). RTK-derived fusion oncoproteins are devoid of an amino-terminal signal peptide necessary for protein targeting to the membrane. Hence, with the exception of FIG-ROS that is targeted to the golgi (96), RTK-derived fusion oncoproteins are located within the cytoplasm. They may therefore activate a different group of signalling pathways compared to the membrane localized RTKs and, as this thesis will propose, their cytoplasmic localization may also impact on their down-regulation.

4. Mechanisms of RTK down-regulation

The biological consequences resulting from the activation of a RTK are determined by the duration, intensity and specificity of the signals activated downstream of the receptor. Regulation of signals occurs at multiple levels, including the RTK itself. Termination of RTK signalling has been correlated with receptor dephosphorylation, degradation or sequestration from the cytoplasm. PTPs could inactivate RTKs by completely dephosphorylating them or modulate their activity or signalling specificity by dephosphorylating specific tyrosine residues (97). However, at this time few studies have identified a role for PTPs in the dephosphorylation of RTKs under physiological conditions. The best evidence is provided by genetic studies (98-100). For example, mice null for a PTP (PTP-1B) display increase insulin sensitivity and prolonged insulin receptor phosphorylation following stimulation (98).

One of the predominant mechanisms for signal termination of RTKs may be mediated through the degradation of RTKs. Degradation of RTKs does more than interrupt their signalling. It is also a way to diminish the pool of RTKs present in the cell. Cellular proteins are degraded either by the proteasome or by the lysosome. Most nuclear and cytoplasmic proteins are degraded by the proteasome while most transmembrane proteins are thought to be degraded in the lysosome. The lysosomal degradation of membrane-associated proteins, including RTKs, first requires their internalisation in the endocytic pathway.

4.1 RTK internalization

Upon activation, RTKs are removed from the cell surface within minutes, rapidly undergoing clathrin-mediated endocytosis (Fig. 5). By facilitating fast and selective internalization of cell surface proteins such as transporters, channels and receptors, clathrin-mediated endocytosis plays a major role in regulating their cell-surface expression (101). There are two major types of clathrin-mediated endocytosis: constitutive endocytosis and ligand-induced endocytosis. Nutrient carrier proteins, such as the low-density lipoprotein (LDL) and transferrin (Tf) receptors are constitutively internalized irrespective of being occupied or not and recycle back to the plasma membrane after dissociation of the ligand (or iron in the case of the Tf-TfR complex). On the other hand, the internalization of GPCRs and RTKs is triggered by their ligand-induced activation and, for most receptors,

it results in their lysosomal degradation. In both cases, their recruitment into clathrin-coated vesicles (CCVs) preclude to their internalization.

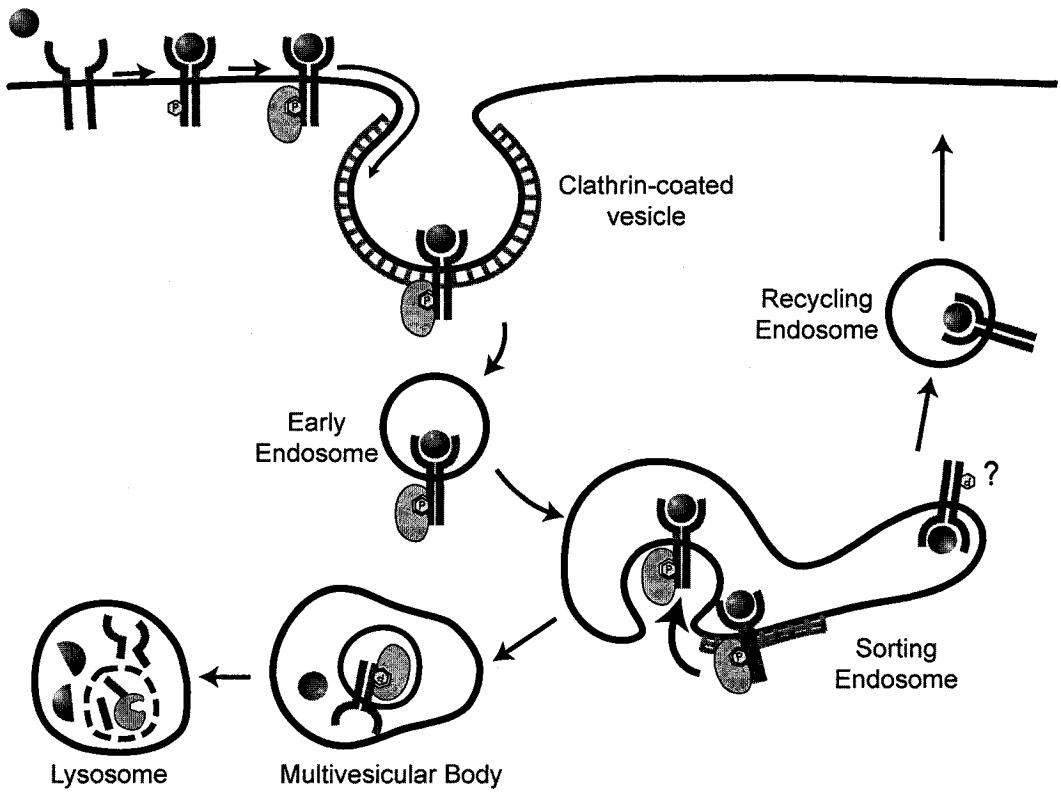


Figure 4. Mechanisms of RTK down-regulation

4.1.1 Clathrin-mediated endocytosis

The general structure of CCVs has been known for many years, although there is still active research to determine the structure of CCVs in more detail. The functional unit of CCVs is the triskelion, which is a hexamer containing three 180-kDa clathrin heavy chains each complexed with a 30-35 kDa light chain (102). The clathrin triskelia assemble into polyhedral lattices on the cytoplasmic surface of the plasma membrane. This process requires adaptor protein complexes that bind simultaneously to clathrin and phospholipids of the membrane. A second important function of adaptor proteins is to recruit the transmembrane proteins that are incorporated in the CCV. Once the clathrin coat is formed, the membrane deeply invaginates and fission of the clathrin-coated pit generates the CCV. Finally, the clathrin coat is disassembled and clathrin

triskelia can be recruited back at the plasma membrane for the formation of new CCVs.

Multiple proteins other than the adaptor proteins are involved in CCV formation at the plasma membrane. At early stages, AP-2, epsin, eps15, intersectin, synaptotagmin and CALM (clathrin assembly lymphoid myeloid leukemia) are involved in formation of the clathrin coat, whilst endophilin and amphiphysin are involved in promoting and/or sensing membrane curvature (103, 104). Fission of the clathrin-coated pit requires the cytoplasmic GTPase dynamin and the 5-phosphoinositide phosphatase synaptojanin-1 is involved in the disassembly of clathrin coat (105).

The adaptor protein complex-2 (AP-2) is the major clathrin adaptor protein located at the plasma membrane. It is composed of four related subunits called adaptins. There are two 100-kDa subunits (α and β2), one 50-kDa subunit (μ2) and one 17-kDa subunit (σ2). The 100-kDa subunits have similar structures composed of a large amino-terminal head domain, a proline-rich hinge, and a carboxy-terminal ear. The ear of the β2 subunit binds to the clathrin heavy chain, the ear of the α subunit interacts with multiple endocytic proteins such as Epsin, Dab2, Numb, Hip1, CALM and AP180 and the μ2 subunit recognizes endocytic motifs present in plasma membrane proteins (106). These interactions demonstrate the central role of AP-2 in coordinating the formation of the clathrin coat and the recruitment of the endocytic machinery and the transmembrane proteins to be internalized.

4.2 RTK trafficking and lysosomal degradation

After their internalization, RTKs travel through the endocytic pathway, a complex network of tubules and vesicles (Fig. 5). First, CCVs lose their clathrin coat to become early endosomes. From early endosomes, RTKs can recycle back to the plasma membrane or progress to sorting endosomes. At this stage, RTKs can undergo inward vesiculation, a process where small vesicles pinch off the limiting membrane into the lumen of sorting endosomes to generate multivesicular bodies (MVBs). This has three consequences on RTKs: 1) it prevents RTKs recycling back to the plasma membrane; 2) it terminates RTK signalling by sequestering their signalling-competent cytoplasmic domain; 3) it targets RTKs for lysosomal degradation. Several hypotheses have been

proposed to explain the transfer of the MVB content to lysosomes, although there is now strong evidence that MVBs fuse with lysosomes to form an hybrid organelle in which degradation occurs (107). These hybrid organelles have been observed *in vivo* and *in vitro* by electron microsocopy: they are less electron-dense than lysosomes and larger than MVBs and lysosomes (108-110). Lysosomes may subsequently bud from this hybrid compartment and be re-used as required.

4.3 Endosomal-lysosomal sorting signals

Sorting signals facilitate the internalization and lysosomal targeting of transmembrane proteins. They consist of short linear amino acid sequences located in the cytoplasmic domain of transmembrane proteins. There are two major classes of sorting motifs: the tyrosine-based and the dileucine-based motifs (Table 1). In addition to being involved in the internalization and lysosomal targeting of transmembrane proteins, sorting signals also facilitate protein trafficking between organelles (endosome-endosome, TGN-endosome) and protein sorting to the basolateral membrane in polarized epithelial cells. In general, the affinity of the recognition protein for the signal motifs is fairly low and therefore, these interactions are very difficult to study. In addition, it is becoming clear that multiple interactions take place between the sorting machinery and the transmembrane protein to be sorted, which adds to the difficulty of mapping these interactions. Consistent with this, the removal of a sorting signal from a transmembrane protein rarely abrogates sorting, but rather renders the process less efficient.

More recently, it has been established that ubiquitination of cytoplasmic lysine residues in transmembrane proteins also constitutes a sorting signal. This will be discussed in sections 5.4 and 5.5.

4.3.1 Tyrosine-based motifs

The first sorting motif was identified in the LDL receptor by the group of Brown and Goldstein (111). They identified a tyrosine-based NPXY motif, mutated in a patient with familial hypercholesterolemia, as being required for the rapid internalization of the LDL receptor (112, 113). The NPXY motif is also present in LDL receptor-related protein 1 (LRP1), megalin, the β subunits of

integrins and the β-amyloid precursor protein (β-APP). It is recognizes by clathrin, the μ2 subunit of AP-2 and the PTB domain of Dab2 (111).

Although identified later, the YXXΦ tyrosine-based motif, where Φ is a hydrophobic amino acid residue, is now known to be involved in the sorting of a much larger group of proteins. This group includes the transferrin receptor, TGN proteins such as furin and TGN38, lysosomal membrane proteins like LAMP-1 and LAMP-2, and mannose 6-phosphate intracellular sorting receptors (111). The surrounding and X amino acids also contribute to the strength and specificity of the YXXΦ motif. This motif is recognized by the μ subunits of AP-1, AP-2, AP-3 and AP-4.

Table 1. Sorting motifs

Motif	Examples of proteins	Recognition protein / domain	Function
NPXY	LDL receptor Integrin β-1 Insulin R EGFR	Clathrin, μ2, PTB of Dab2	Internalization
YXXΦ	Lamp-1 Transferrin R Furin CD-MRP	μ subunit of APs	Internalization Lysosomal targeting Basolateral targeting
(D/E)XXXL(L/I)	CD3-γ CD4 Glut4 TRP-1	μ and/or β subunits of APs	Internalization Lysosomal targeting Basolateral targeting
DXXLL	GGA 1 to 3 LRP10 CD-MRP	VHS domain of GGAs	TGN-to-endosome sorting
Acidic cluster	Furin Vamp4 Nef (HIV-1)	PACS-1	Endosome-to-TGN sorting
Ubiquitin	RTKs GPCRs ENaC	See table 2	Internalization Lysosomal targeting

Φ, hydrophobic amino acid residue
ENaC, epithelial sodium channel

4.3.2 Dileucine-based motifs

The second major class of sorting motifs are the dileucine-based motifs. They form a heterogeneous class of signals where again, the surrounding amino acids provide specificity to the motif. Whilst the DXXLL motif is recognized by the VHS domain of GGA1, GGA2 and GGA3 endocytic proteins, the EXXXLL motif is recognized by the adaptor protein complexes AP-1 and AP-3, which are located in the TGN and endosomes. The dileucine-based motifs are found in dozens of transmembrane proteins including Ig superfamily members CD3-γ chain and CD4, the glucose transporter GLUT4, mannose 6-phosphate receptors, LDL-receptor-related proteins LRP3 and LRP10 and the APP β-secretase (111).

4.4 Sorting motifs in EGFR

The only RTKs that have been carefully studied with regard to trafficking are the EGF and insulin receptors. As an example of the complexity of the process, I will summarize what we know about EGFR trafficking.

4.4.1 EGFR internalization motifs

The observation that a truncation at residue 973 prevents EGFR internalization (114) lead to the identification of two motifs, ^{973}FYRAL and ^{996}QQGFF, that are involved in ligand-induced EGFR internalization (115). The μ2 subunit of AP-2 was then shown to bind to the ^{973}FYRAL motif (116), while the function of ^{996}QQGFF remains unclear. Deletion of the ^{973}FYRAL motif delayed EGFR internalization only when the receptor was highly expressed (117), and mutation of the μ2 subunit of AP-2 attenuated transferrin receptor internalization, but not EGFR internalization (118). These experiments clearly demonstrate that there are multiple signals as well as sorting proteins involved in EGFR internalization.

Activation of the EGFR may also regulate its internalization by "activating" the endocytic machinery via tyrosine phosphorylation. EGFR activation leads to activation of Src, which can tyrosine phosphorylate clathrin and dynamin. Indeed, clathrin phosphorylation promotes its redistribution at the plasma membrane while dynamin phosphorylation promotes its assembly and increases its GTPase activity (119, 120). Mutation of the Src phosphorylation site on dynamin attenuates EGFR internalization (120).

The Grb2 adaptor protein may also play a role in EGFR internalization where blocking the recruitment of Grb2 to the EGFR inhibits receptor internalization (121). Furthermore, a recent study demonstrated that depletion of Grb2, using RNA interference, severely inhibits EGFR internalization (122). Similar results were obtained when the Grb2 binding sites in EGFR were mutated (122). The role of Grb2 in EGFR internalization may be to recruit dynamin (121), activate Rab5a via Ras and/or recruit Cbl ubiquitin ligases. The latter possibility will be discussed in section 7.2.

4.4.2 EGFR lysosomal sorting motifs

In the absence of ligand, EGFR internalizes slowly and recycles to the plasma membrane in about 5 minutes, whereas ligand-bound EGFR internalizes rapidly, but recycles at the much slower rate (123). Thus, there is endosomal retention of activated EGFR, which subsequently leads to its lysosomal degradation. There are three distinct signals in the cytoplasmic domain of EGFR that contribute to EGF-induced receptor degradation. The first signal, ^{679}LL, is a dileucine motif located in the juxtamembrane of EGFR. Mutagenesis of this signal affects EGFR degradation without affecting its internalization (124, 125). The second motif, ^{954}YLVI, is a tyrosine-based motif located at the distal border of tyrosine kinase domain. In a yeast two-hybrid screen, the protein sorting nexin 1 (SNX1) was found to bind ^{954}YLVI (126). SNX1 is a cytoplasmic protein that can bind to membranes via its PX phospholipid binding domain (127) and that is involved in lysosomal degradation of EGFR (126, 128, 129). SNX1 also binds recycling receptors such as the transferrin and LDL receptors (130, 131). The third element involved in EGFR degradation, discovered as I was starting my Ph.D. thesis, is tyrosine 1045 located toward the carboxy-terminus of the receptor (132). Tyrosine 1045 has been identified as the direct binding site for Cbl ubiquitin ligases and its substitution for a phenylalanine prevents EGFR ubiquitination and severely impairs EGR-induced receptor degradation (132). The function of ubiquitin as a sorting signal will be discussed in sections 5.4 and 5.5.

4.5 RTK proteasomal degradation

General proteasomal inhibitors, MG132 and lactacystin, inhibit ligand-induced degradation of the Met and EGF receptors (133-135), suggesting that the proteasome may be involved in RTK degradation. Further studies revealed that the proteasomal inhibitors impair the trafficking of Met, EGFR and the growth hormone receptor (GHR) to late endosomes. In the presence of proteasomal inhibitors, the Met receptor recycles to the cell surface (136) and electron microscopy studies showed that EGFR and GHR are not efficiently incorporated into luminal vesicles of MVBs (135, 137). Thus, the degradation of proteins by the proteasomal machinery may be required to sort RTKs for lysosomal degradation.

In search for inhibitors for the RTK ErbB2, a member of the EGFR family, it was observed that an irreversible tyrosine kinase inhibitor, in addition to inhibiting tyrosine kinase activity, promotes ErbB2 ubiquitination and degradation (138). It appeared that the degradative pathway stimulated by the tyrosine kinase inhibitor involves the heat shock protein Hsp90. Geldanamycin, an Hsp90 inhibitor, also promotes ErbB2 ubiquitination and proteasomal degradation, suggesting that Hsp90 is required for mature ErbB2 stability (139). Additional work has demonstrated that the ubiquitin ligase CHIP, a co-chaperone protein associated with Hsp70 and Hsp90, is responsible for geldanamycin-induced ErbB2 ubiquitination and proteasomal degradation (140-142). Whether this is common to other RTKs remains to be demonstrated.

5. Ubiquitination

Degradation of cellular regulatory proteins plays a critical role in controlling multiple physiological processes. Regulation of signalling pathways through the degradation of their key components has the advantage of being irreversible and *de novo* synthesis is required for re-activation of the pathway. In 2004, Aaron Ciechanover, Avram Hershko and Irwin Rose won the Nobel Prize in chemistry for discovering the ubiquitin-proteasome pathway as the major route for protein degradation. Substrates of this pathway comprise tumour suppressor proteins (p53), cell cycle proteins ($p27^{Kip1}$) and transcription factors (E2F-1, fos, jun, myc, NF-κB). More recently, receptor tyrosine kinases (RTKs), including the colony stimulating factor-1 receptor (CSF-1R) (143, 144), the EGFR (145-147), the Met

receptor (133), and the platelet-derived growth factor receptor (PDGFR) (148) have been identified as substrates for ubiquitination.

5.1 The ubiquitin pathway

Protein ubiquitination consists in the covalent addition of ubiquitin moieties to a lysine residue within the substrate protein (Fig. 6). Ubiquitin is a 76-amino-acid protein that is highly conserved from yeast to mammals. Several genes encode ubiquitin proteins fused in frame. Once translated, the ubiquitin moieties are cleaved by a class of cysteine proteases named ubiquitin carboxy-terminal hydrolases (UCH) (149).

Protein ubiquitination is mediated by an enzymatic cascade composed of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase (E3 (Fig. 6)). First, the E1 enzyme forms a thiol-ester bond with the carboxy-terminal glycine of ubiquitin by an ATP-dependent reaction. Next, the ubiquityl moiety is transferred to the E2 enzyme. The E3 enzyme provides the specificity to the process by recruiting the E2 to a finite set of substrates, thus promoting their ubiquitination. In humans, there is about ten E1s, hundred E2s and a thousand E3s (150). Until recently, it was thought that E3 ligases were the only component of the ubiquitination enzymatic cascade subjected to regulation, but evidence now indicates that E2s can also be regulated. Modification of the Hip2 E2 with the ubiquitin-like protein SUMO inhibits Hip2 by blocking its interaction with E1 (151).

There are two major classes of E3 ligases: Really Interesting New Gene (RING) and Homologous to E6AP Carboxy Terminus (HECT). RING E3s form by far the largest class of E3s. They have no intrinsic catalytic activity, but rather function as adaptor proteins that recruit E2s to substrates. RING E3s coordinate the direct transfer of the ubiquityl moieties from the E2 active site to the substrate. The metal-binding RING finger domain interacts with the E2 while other protein-protein interaction domains/motifs associate with the substrate to be ubiquitinated. There are two subclasses of RING E3s: the single-subunit, that contains Cbl and BRCA, and the multi-subunit, that includes SCF (Skp1, Cul1, F-box protein and Rbx1) and VCB (VHL, elongin C or B, Cul2 and Rbx1) complexes.

The ~350 amino acid HECT domain was first identified in E6AP. Upon binding to the E6 human papillomavirus protein, E6AP ubiquitinates host cell p53, resulting in p53 degradation and viral DNA replication (152). In the case of HECT E3s, the ubiquityl moieties are transferred from the E2 to the E3 before its conjugation to the substrate. A conserved cysteine residue in HECT E3s forms a thiol ester intermediate with ubiquitin.

A third class of E3s includes the relatively small family of U-box proteins. The U-box domain adopts a conformation similar to the RING domain structure and is able to interact directly with E2s (153, 154). However, two U-box E3s that have been studied, Ufd2 (155) and CHIP (156), display E4-like activity, meaning that they promote the polyubiquitination of a substrate that must be first ubiquitinated by another E3 ligase (157).

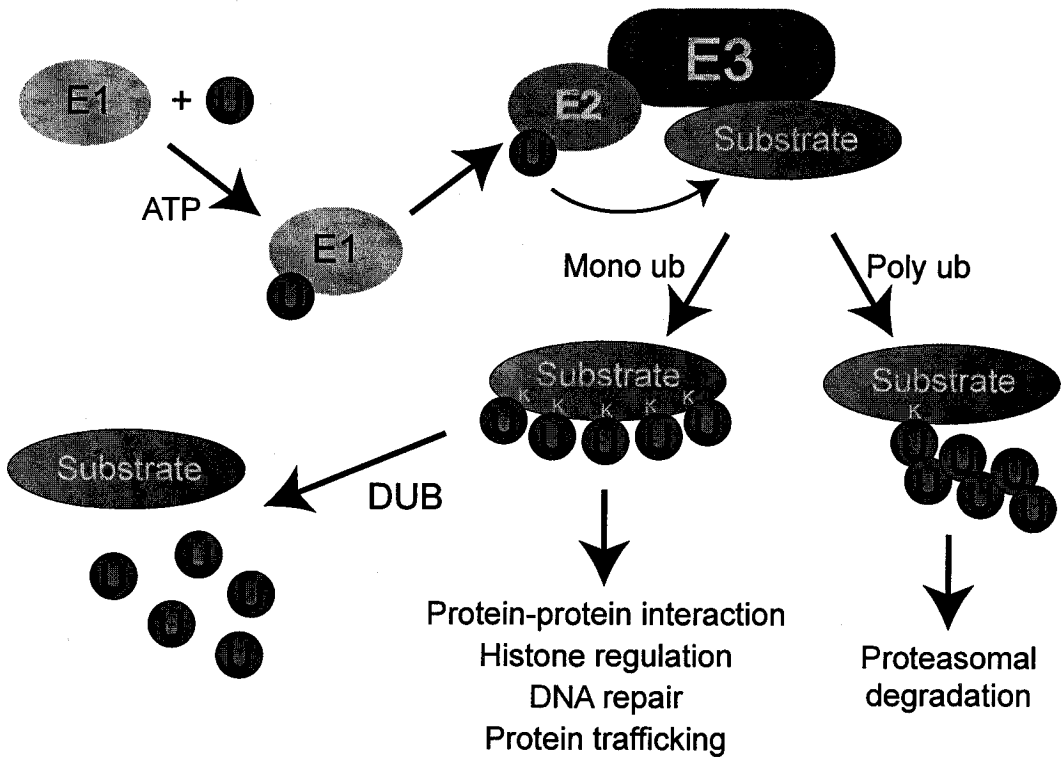


Figure 5. The ubiquitin pathway

Like phosphorylation, ubiquitination is a reversible process (Fig. 5). The deubiquitination reaction is catalyzed by deubiquitinating enzymes called DUBs (158). DUBs cleave ubiquitin-linked molecules after the terminal carbonyl of the last residue of ubiquitin (G76). There are a least five subclasses of DUBs, four of

them, including ubiquitin carboxy-terminal hydrolases (UCH), are cysteine proteases, while the fifth group are zinc-dependent metalloproteases. DUBs are involved in multiple processes such as processing of ubiquitin precursors, rescuing ubiquitinated proteins and recycling ubiquitin prior to the proteasomal degradation of ubiquitinated proteins (158).

5.2 Polyubiquitination

For most substrates, the carboxyl group of the carboxy-terminal glycine of ubiquitin is conjugated to the tertiary amino group (ε-NH_2) of lysine residues. In some cases, the first ubiquityl moiety is conjugated linearly to the α-NH_2 group of the amino-terminal residue (159). Following the covalent addition of a ubiquitin moiety to the substrate, additional ubiquitin moieties can be added to lysine residues within the linked ubiquitin to generate polyubiquitin chains. There are seven lysines present in ubiquitin (K6, K11, K27, K33, K48, K63) and chain formation on all lysines has been observed in yeast by mass spectrometry (160). The first polyubiquitin chain formation that has been identified is the K48-linked chain (161). The presence of a K48 polyubiquitin chain on many cytosolic and nuclear proteins targets them for degradation by the 26S proteasome and in yeast, K48-linked chains proved to be the principal proteasome delivery signal (162). It appears that a chain of at least four ubiquitin moieties is required for proteasomal targeting (163). The S5a subunit of the 26S proteasome, which contains more than 30 subunits, is able to bind to K48-linked chains (163) and overexpression of S5a specifically blocks the degradation of polyubiquitinated proteins (164).

K63-linked chains do not act as proteolytic signals. K63-linked chains were first identified as playing a role in DNA repair. Yeast cells defective in K63-linked chain formation are defective in DNA repair, but proteolysis is not affected (165). Whilst monoubiquitination of K164 in PCNA (proliferating cell nuclear antigen) results in an error-prone mode of bypass during replication, modification of K164 with a K63-linked chain results in an error-free mode of bypass (166-168). This exemplifies how different ubiquitin modifications on a same site can produce different outcomes. K63-linked chains are now known to be involved in three additional pathways: the inflammatory response, ribosomal protein synthesis and

protein trafficking. The other polyubiquitin chains remain poorly characterized (169).

5.3 Monoubiquitination

In the early 1980s, Goldknopf and Bush observed that histones 2A (H2A) and 2B (H2B) were monoubiquitinated in a reversible manner and that it may be "a mechanism for "moment-to-moment" control of the genome" (170). It took two decades to begin to understand the function of histone monoubiquitination (171). For example, the E2/E3 complex (Rad6/Bre1) ubiquitinates the carboxy-tail of H2B in yeast and this event is essential for mitotic and meiotic growth (172-174). This is also a prerequisite for H3 methylation, an important event in gene silencing (175-177). Mutations of the lysines that are monoubiquitinated in H2B abolish gene silencing at telomeric regions (177). Thus, monoubiquitination of histones is not a signal for proteasomal degradation, but rather modulates the function of histones as regulators of gene expression.

In addition to histone regulation, monoubiquitination plays a role in the activation of the NFκB pathway, which is involved in inflammatory and immune responses, as well as in DNA repair. Monoubiquitination is essential to the recruitment of the DNA repair machinery to the site of DNA damage. Fanconi anaemia (FA), an autosomal recessive cancer susceptibility syndrome, results from mutations in one of eight genes (FA genes) and is characterized by a defect in DNA repair. Five of the eight FA genes form a complex that is essential for the monoubiquitination of FANCD2, another FA gene. FANCD2 monoubiquitination occurs following DNA damage and leads to its recruitment to the sites of active DNA repair (178).

Finally, in yeast and possibly in mammalian cells, monoubiquitination is also involved in the trafficking of transmembrane proteins. Ubiquitin can regulate protein trafficking in two ways: 1) ubiquitin conjugated to a transmembrane protein can function as a sorting signal and 2) ubiquitination of the sorting machinery can regulate its activity (179). The role of ubiquitin in protein trafficking will be discussed more extensively in the following sections.

5.4 Ubiquitin as a signal for receptor internalisation

Whilst ubiquitination of nuclear and cytoplasmic proteins mainly induces their proteasomal degradation, ubiquitination of many cell-surface receptors correlates with their internalisation and lysosomal degradation. In 1986, amino-terminal protein sequencing of the PDGF and lymphocyte homing receptors revealed that they were covalently conjugated to ubiquitin (180, 181). In 1992, Mori et al. observed that PDGF induced PDGF receptor ubiquitination and that it required an intact kinase domain (182). At that time, it was hypothesized that ubiquitination may be involved in the regulation of plasma membrane proteins, but work performed later in yeast provided a function for the ubiquitination of plasma membrane proteins. In 1994, Kölling and Hollenberg reported that a ubiquitinated form of the multi-spanning membrane protein Ste6 accumulates at the cell surface when endocytosis is blocked and that Ste6 is stabilized in both ubiquitination and vacuolar defective yeast strains (183). These observations suggested that ubiquitination is important for the degradation of Ste6 in the vacuole, the equivalent of lysosomes in yeast.

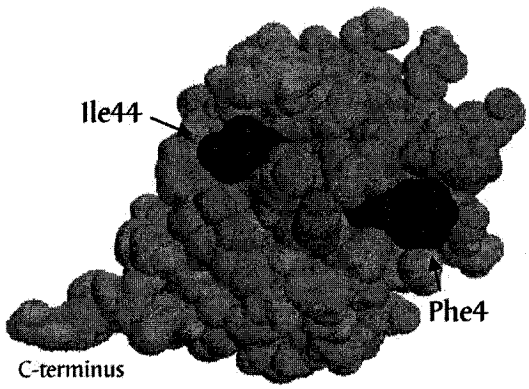


Figure 6. Ubiquitin and internalisation

Internalization information carried by ubiquitin is present in two surface patches. Residues essential for internalization are highlighted in magenta; surrounding residues that function in endocytosis but play a lesser role are shown in pink. Adapted from Shih et al. 2000. EMBO J **19**: 187.

Subsequently, studies of the α-factor G protein-coupled receptor Ste2p revealed that ubiquitination of the receptor itself is required for its internalisation (184). Mutation of a single lysine in a truncated form of the receptor prevented both its ubiquitination and internalisation. Importantly, monoubiquitination of the α-factor receptor is sufficient for its internalisation (185). This was demonstrated using ubiquitin mutants unable to form polyubiquitin chains as well as by fusing a single ubiquitin moiety in frame with a receptor devoid of cytoplasmic lysines (185). Hence, in yeast, ubiquitin itself and not a specific type of linkage to the

receptor can constitute an internalisation signal (185). Ubiquitin does not consist of a short linear peptide motif like previously identified sorting signals. Its sorting signal resides in its three-dimensional structure that contains two surface patches (Fig. 6). Mutation of either F4 or I44 in ubiquitin inhibits Ste2p internalisation (186). In contrast, proteasomal targeting of ubiquitinated proteins requires the I44 residue but not the F4 residue; both are essential for yeast cell viability (187). The patch containing I44 is involved in the recognition of ubiquitin by ubiquitin binding domains (discussed in section 5.6). However, the function of the patch containing F4 remains unknown.

In mammalian cells, RTKs were the first transmembrane proteins found to be ubiquitinated and it is only in the past few years that we have observed the ubiquitination of some G-protein coupled receptors and ion channels (188, 189). Haglund and co-workers recently demonstrated that monoubiquitin *could* promote the internalisation of a transmembrane protein in mammalian cells (190). They also provided evidence that EGFR and PDGFR are multi-monoubiquitinated rather then polyubiquitinated (190, 191). This was shown indirectly using two antibodies, one that recognizes only polyubiquitinated proteins and one that recognizes both monoubiquitinated and polyubiquitinated proteins. Only the latter detects ubiquitinated EGF and PDGF receptors (190). Similar results were obtained with the Met receptor (192). In addition, overexpression of a ubiquitin mutant unable to form chains did not impair EGFR and PDGFR ubiquitination (190, 191). However, it remains to be proven whether ubiquitination of a plasma membrane protein is required for its internalisation in mammalian cells.

5.5 Ubiquitin as a signal for receptor lysosomal degradation

In 1998, Levkowitz and colleagues provided the first clue that ubiquitination may target cell surface receptors for lysosomal degradation. They observed that whilst c-Cbl overexpression induces EGFR ubiquitination and lysosomal degradation, v-Cbl overexpression fails to induce EGFR ubiquitination and promotes receptor recycling (145). Neither c-Cbl nor v-Cbl overexpression affects EGF uptake, suggesting that Cbl acts after receptor internalisation (145). Ubiquitination of the G protein-coupled receptor CXCR4, an HIV co-receptor, is also important for its lysosomal degradation rather than for its internalisation

(193). Mutation of lysines located in the cytoplasmic domain of CXCR4 that are critical for receptor ubiquitination does not affect its internalisation, but blocks its lysosomal degradation (193).

Conclusive evidence that ubiquitination can target proteins for lysosomal degradation came from studies in yeast (179, 194). First, in 2001, it was observed that a cell surface protein (ste6p), normally sorted to the vacuolar lumen, is missorted to the limiting membrane of the vacuole in a yeast strain where ubiquitin is depleted. The phenotype is rescued by overexpressing ubiquitin (195). That same year, it was reported that resident proteins of the lumen of the vacuole are ubiquitinated in their cytoplasmic domain (196). Mutations that prevent ubiquitination of the vacuolar proteins block their entry into the vacuolar lumen and this is rescued by fusion of monoubiquitin to the vacuolar proteins (196, 197). Moreover, fusion of monoubiquitin to proteins that normally localize to the vacuolar limiting membrane missort them to the vacuolar lumen (198). In mammalian cells, fusion of ubiquitin to the transferrin receptor, which rapidly recycles to the cell surface, promotes its retention to the lumenal vesicles of MVBs (199). Together, these experiments demonstrate that ubiquitin can target proteins to vacuolar/lysosomal degradation by directing them to the lumen of vacuoles/MVBs.

In the past two years, studies on the EGFR revealed that its ubiquitination by Cbl is required for directing the receptor to internal vesicles of late endosomes and for subsequent lysosomal degradation of the EGFR (200-203). Moreover, the fusion of monoubiquitin to a severely truncated EGFR is sufficient, in the absence of other signals, to target this receptor to late endosomes, supporting a role for monoubiquitination in targeting RTKs for lysosomal degradation (190).

The group of Scott Emr made a substantial contribution to our understanding of the molecular mechanisms underlying the sequestration of ubiquitinated cargo into vacuoles/lysosomes (197, 204-206). They identified and characterized three ESCRT (endosomal sorting complex required for transport) complexes in yeast. Together, they are composed of 17 proteins that are required for cargo sorting to MVBs (194). Some of these proteins, like Vps23/Tsg101, contain ubiquitin binding domains (UBDs) (e.g. UBA, UEV or UIM) (see section 5.6). Ubiquitinated receptors are thought to be recognised by proteins of the endocytic pathway, including Hrs (HGF-regulated tyrosine kinase substrate), that possess UBDs

(179, 194, 207). The UIM-containing protein Hrs appears to be the first sorting protein recruited to endosomes and becomes enriched in regions of the endosome containing a specialised bilayered clathrin coat (199, 208, 209). Hrs is believed to be involved in the retention of ubiquitinated receptors within the bilayered clathrin coat and in the recruitment of ESCRT complexes (204, 210-214). Hrs becomes tyrosine phosphorylated upon activation of several RTKs (215). Hrs phosphorylation requires RTK internalisation (216) as well as an intact ubiquitin interacting motif (UIM) in Hrs (217), supporting a role for Hrs in retaining ubiquitinated RTKs at the limiting membrane of endosomes.

In summary, whilst it is still unclear whether ubiquitination of RTKs plays a role in their internalisation, the last five years have provided convincing evidence that ubiquitination of RTKs is essential for their lysosomal degradation.

5.6 Ubiquitin binding domains (UBDs)

The first UBD, the UBA (ubiquitin-associated) domain, was identified in 1996. Since 2001, eight distinct UBDs have been identified: CUE (coupling of ubiquitin conjugation to ER degradation protein), UIM (ubiquitin-interacting motif), UEV (ubiquitin- E2 variant), VHS (Vps27/Hrs/STAM), NZF (Np14 zinc finger), GAT (GGA and Tom1), GLUE (GRAM-like ubiquitin-binding in Eap45) and PAZ (polyubiquitin-associated zinc finger) (Table 2) (150). Their study in the past few years opens the way for the discovery of numerous mechanisms through which ubiquitination regulates biological processes.

Overall, UBDs have very different structures. However, where examined, they all recognize the hydrophobic patch containing Ile44 in ubiquitin (150). As mentioned in the previous section, UBDs are present in multiple proteins of the endocytic pathway and are involved in sorting ubiquitinated cargos. In addition, UBDs are involved in the recognition of ubiquitinated proteins by the 26S proteasome, which has several subunits that contain UBDs. Many UBDs are found in proteins of the ubiquitination and deubiquitination machinery where their functions remain to be identified, although they may be implicated in polyubiquitin chain formation (150). Finally, there are many more proteins involved in numerous cellular events that possess UBDs and therefore, one can anticipate that UBDs have functions yet unpredicted. In the three following sections, I

describe the UBA, CUE and UIM domains in more detail since they are the best characterized UBDs.

Table 2. Ubiquitin binding domains identified to date

Domain	Number of proteins in humans	Proteins	Length (aa number)	Structure	Interaction with ubiquitin
CUE	21	Rabex-5, Tollip	42-43	Three-helix bundle	Hydrophobic with the I44 patch
GAT	14	GGAs, Tom1, Tom1L1	135	Two parallel α-helices	?
GLUE	?	Eap45	~ 135	GRAM or PH	?
NZF	25	Vps36	~ 35	Zinc finger	Hydrophobic with the I44 patch
PAZ	16	HDAC6	~ 58	?	?
UBA	98	Cbl, Ede1, Rad23	~ 50	Three-helix bundle	Hydrophobic with the I44 patch
UEV	?	Tsg101	~ 145	~ UBC domain	Multiple
UIM	71	S5a, Hrs, Eps15, epsin, STAM	~ 20	A single α-helix	Hydrophobic with the I44 patch
VHS	28	STAM	150	?	?

Adapted from Hicke *et al.* 2005. Nat Rev Mol Cell Biol **6:**610-21 and Hicke and Dunn. 2003. Annu Rev Cell Dev Biol **19:**141-72.

5.6.1 UBA (Ubiquitin-associated domain)

The ubiquitin-associated domain (UBA) was the first UBD described. It was initially identified in proteins involved in the ubiquitin-proteasome pathway, including E2s, E3s and deubiquitinating enzymes (DUBs) (218). Several UBA domains have been shown to bind free mono- and poly-ubiquitin as well as

ubiquitinated proteins, predicting a role for UBA domains in protein-protein interaction and subcellular targeting (219, 220). Some UBA domains can bind to K29- and K48-linked ubiquitin chains, which target proteins for proteasomal degradation, but not to K63-linked ubiquitin chains, which don't have proteolytic functions (221). It has been proposed that UBA domains may play a role in delivering ubiquitinated proteins to the proteasome, in protecting ubiquitin chains from being disassembled by deubiquitinating enzymes or in preventing multi-ubiquitin chain assembly and consequently stabilizing the ubiquitinated proteins (222-224).

UBA is a small globular domain of 40 amino acids with limited sequence identity among the various UBA domains. NMR structure analysis of the two UBA domains present in the DNA repair protein Rad23 revealed that it is a compact 3 alpha-helix bundle (225). Two hydrophobic patches are located on opposite sides of the UBA domain and therefore, UBA may possibly bind two proteins simultaneously. In addition to ubiquitin, the carboxy-terminal UBA domain of Rad23 has been shown to bind four other proteins, namely Vpr, Png1, MPG and p300 (220). The Rad23 UBA(1) and UBA(2) domains utilize the same binding surface on ubiquitin. However, models for the Rad23 UBA(1)-ubiquitin and UBA(2)-ubiquitin complexes obtained by NMR chemical shift mapping studies, and by homology modelling with the solution structure of the CUE-ubiquitin complex, revealed that both UBA domains may have a different orientation on the surface of ubiquitin (226).

5.6.2 CUE (coupling of ubiquitin conjugation to ER degradation)

The CUE domain was first recognized in Cue1p, a yeast protein that recruits the E2 Ubc7p to an ER-associated complex (227). The capacity of the CUE domain to bind ubiquitin was revealed in yeast two-hybrid screens using monoubiquitin as bait (228, 229). The CUE domain is present in multiple proteins, including the endocytic proteins Tollip and Vps9, and in its mammalian homologue, Rabex-5. Vps9 and Rabex-5 are GEFs for Rab5 and promote the fusion of early endosomes to sorting endosomes (230). The 40-amino-acid CUE domain has an amino acid sequence and structure similar to the UBA domain. It consists of a 3-helix bundle that contains two ubiquitin binding surfaces. In an NMR study, Kang et al. suggest that in solution, the CUE domain of Cue2 binds

as a monomer to ubiquitin with an affinity of 155 μM (231). However, in an X-ray crystallography study, Prag et al. propose that the CUE domain of Vps9 forms a domain-swapped dimer that binds to a single ubiquitin moiety with high affinity (20 μM) (232). The hydrophobic surface patch on ubiquitin that contains the isoleucine residue 44 (I44) contributes to the interaction with the CUE domain (229). *In vitro*, the Vps9 CUE domain binds to K48-linked ubiquitin chains with higher affinity than to monoubiquitin (233), yet this interaction has not been examined at the structural level.

5.6.3 UIM (Ubiquitin-interaction motif)

UIM is a 20-amino acid sequence motif identified using iterative database searches with sequences from the S5a subunit of the 19S proteasome regulatory complex, that interact directly with polyubiquitin chains (234). It is also named LALAL motif because of the presence of a well-conserved central helical LALAL motif. It is present in multiple endocytic proteins including Epsin, Eps15, Hrs, STAM and Hse1, as well as in ubiquitin ligases, deubiquitinating enzymes and proteins with other cellular functions. As shown by NMR spectroscopy, the UIM domain forms an isolated short α-helix, which is uncommon in proteins. Conserved hydrophobic residues are located on one face of the helix. They form a contiguous hydrophobic patch that directly engages the complementary I44-containing hydrophobic patch on the ubiquitin surface (235-237). Hence, the same I44-containing hydrophobic patch on ubiquitin is implicated in UBA, CUE and UIM binding. Whether the interaction may interfere with ubiquitin chain elongation on K48 is not yet clear. Surprisingly, the second UIM of Vps27, the yeast homologue of Hrs, crystallizes as an anti-parallel four-helix bundle with the hydrophobic face of each helix packing into the middle of the bundle. Since UIM domain-containing proteins generally have multiple copies of the motif, it is possible that intramolecular oligomerization of UIMs occurs *in vivo* (237).

In addition to binding ubiquitin, many UIM and CUE domains promote the monoubiquitination of proteins which they are part of (229, 238-241). The mechanism is not yet well understood, but since the UIM and CUE domains do not possess ubiquitin ligase activity *per se*, they may recruit E2 or HECT E3 ubiquitin ligases.

6. The Met/HGF receptor

The Met receptor was first identified as a product of a human oncogene, *Tpr-Met*, which was generated following a chromosomal rearrangement induced by the treatment of a human osteogenic sarcoma (HOS) cell line with the carcinogen N-methyl-N'-nitronitrosoguanidine (MNNG) (90, 242, 243). The genomic rearrangement fuses two genetic loci, *Tpr (translocated promoter region)*, which encodes a dimerization leucine zipper motif, and *met,* which contributes the kinase domain and carboxy-terminus of the Met RTK (244, 245). The resulting 65 kDa cytoplasmic Tpr-Met oncoprotein forms a dimer and is therefore constitutively activated (89).

The Met proto-oncogene locus was mapped to human chromosome 7q31 (244) and its amino acid sequence revealed the features of a RTK: a 926-amino acid extracellular binding domain, a 23-amino acid transmembrane domain and a 435-amino acid intracellular domain containing the highly conserved kinase domain (Fig. 7) (90, 245). It was four years later that Met was identified as the receptor for the hepatocyte growth factor/scatter factor (HGF/SF) (246, 247). The phenotype of Met and HGF knockout mice are identical, demonstrating that during development, Met is likely to be the only receptor for HGF and HGF the only ligand for the Met receptor (248-250).

Met is the prototype receptor for a family of RTKs with sequence and structural homology. The Met family of RTKs contains two members in mammals, Met and Ron. The latter is the receptor for macrophage stimulating protein (MSP), which has the highest sequence similarity to HGF (45% identity) (251). A third member of the Met family, Sea, was identified in chicken as the cellular homologue of the v-Sea oncogene (252). Sea may be the avian orthologue of Ron, since chicken MSP is a ligand for the Sea receptor (253) and neither an avian orthologue of Ron nor a mammalian orthologue of Sea has been cloned. Intriguingly, three receptor genes of the Met family have been identified in the genome of the puffer fish *Fugu rubripes*, a model vertebrate with a compact genome (254).

6.1 HGF

HGF and SF were independently characterized as a potent mitogenic factor for rat hepatocytes (255) and as a motility factor for epithelial cells (256)

respectively. It was then demonstrated that HGF and SF are identical proteins encoded by a single gene (246, 257). HGF is produced by cells of mesenchymal origin (258, 259), platelets (255), macrophages (260), monocytes (261), endothelial cells (260), nonparenchymal liver cells (262), leukocytes (263), bone marrow stromal cells (264) and placental cells (265). Whilst many growth factors like insulin, and EGF are short polypeptides of about 6 kDa, active HGF is a 100 kDa growth factor that, surprisingly, shares homology with the proteinases of the plasminogen family (266). Plasminogen is a proenzyme circulating in the blood whose active form is responsible for the lysis of blood clots. HGF has multiple structural features of plasminogen proteases, including a pre-activation peptide loop, four kringle domains and a protease-like domain (267). However, the latter domain lacks enzymatic activity due to the absence of two critical amino acids.

HGF is synthesized as a 728-amino acid polypeptide (preproHGF) that is first cleaved intracellularly and secreted as an inactive precursor (proHGF). It is then cleaved by extracellular serine proteases to generate an active heterodimer (HGF), which consists of a 69 kDa α-chain and a 34 kDa β-chain linked by a disulfide bond (255, 265). There are several serine proteases, including urokinase plasminogen activator (uPA), that are able to cleave pro-HGF *in vitro*, but Naldini *et al.* demonstrated that an inhibitor of uPA blocks HGF activity in tissue culture assays (268). Like fibroblast growth factor (FGF), HGF binds to heparan sulfate and dermatan sulfate proteoglycans, proteins present at the cell surface and in the extracellular matrix (269). This prevents HGF to diffuse freely *in vivo* (45). The affinity of HGF for dermatan sulfate proteoglycans (19.7 nM) is 10 to 100-fold lower than for heparan sulfate proteoglycans (269), which is itself 10-fold lower than for the Met receptor (0,06 nM) (270).

6.2 Structural features of the Met receptor

Like HGF, the Met receptor is a disulphide-linked heterodimer. It is synthesized as a 150 kDa precursor polypeptide that becomes heavily glycosylated (271). Upon translocation to the cell surface, Met is cleaved extracellularly by a furin protease (272) to generate a disulphide-linked heterodimer composed of an extracellular α chain (307 amino acids - 45 kDa) and a transmembrane β chain (1083 amino acids - 145 kDa) that contains the intracellular kinase domain (Fig. 8) (271). The extracellular domain of the Met

receptor contains a Sema domain that is conserved in all semaphorins and plexins, a cysteine-rich PSI domain (found in plexins, semaphorins, and integrins), and four immunoglobulin-like repeats (also found in plexins) (Fig. 7). The Sema domain of Met, which is formed by the α chain and the first 212 residues of the β chain, is sufficient for HGF/SF binding.

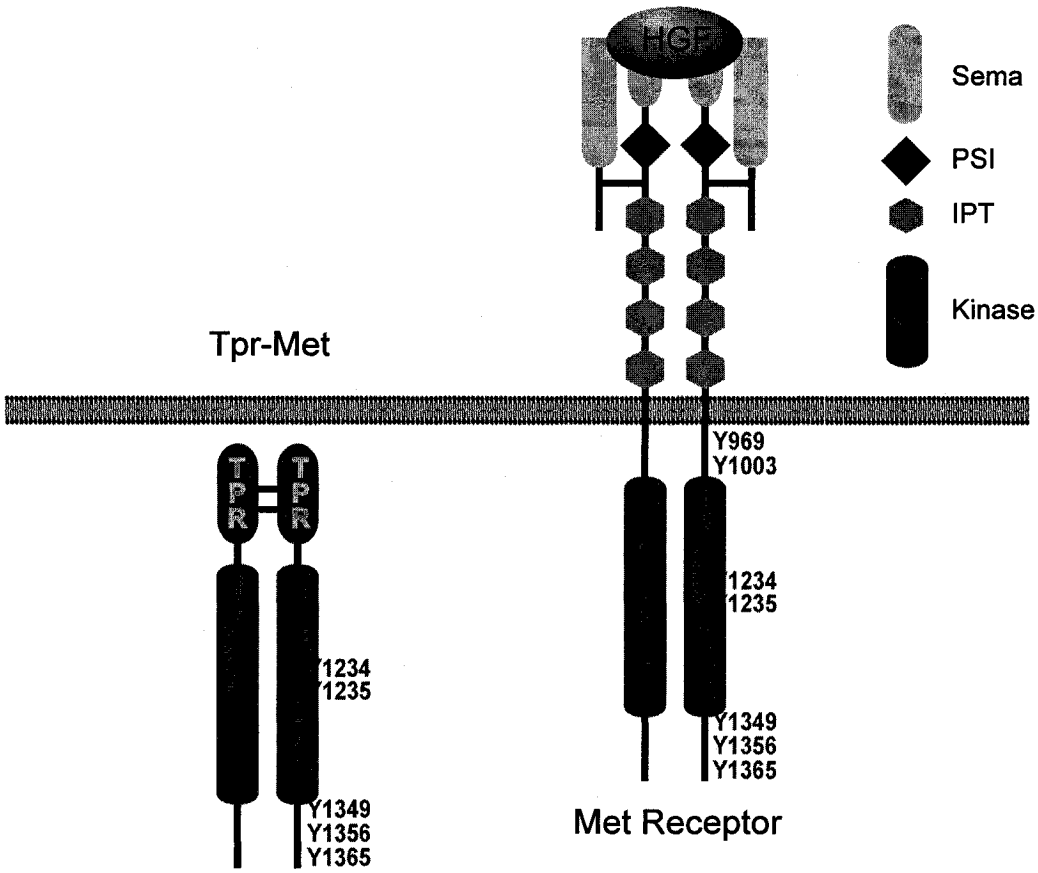


Figure 7. Structure of the Met receptor and Tpr-Met oncoprotein

The intracellular portion of Met is composed of 120-amino acid juxtamembrane region, a 265-amino acid kinase domain and a 50-amino acid carboxy-terminal region. The crystal structure of the Met receptor kinase domain revealed that it is most similar to the structure of the insulin and fibroblast growth factor receptor kinase domains with root-mean-square (rms) deviations of 1.2 A and 1.5 A respectively (273). Met contains 16 tyrosine residues in its intracellular portion, two of which (Y1234 & Y1235) are the conserved autophosphorylation sites within the kinase domain (274). The carboxy-terminal region contains three tyrosine residues (Y1349, Y1356 & Y1365), one of which is highly

phosphorylated (Y1356) (275) and represents the major docking site for signalling proteins (276-278). The two tyrosine residues located in the juxtamembrane region of Met (Y971 & Y1003) are absent in the Tpr-Met oncoprotein (Fig. 7).

6.3 HGF/Met biology

The Met receptor and its ligand HGF appeared late in evolution and are only found in vertebrates (254). In addition to being essential for mouse embryogenesis (248-250), Met and HGF are involved in multiple processes such as organ regeneration following kidney and liver damage and wound healing. In the adult, Met is predominantly expressed in epithelial cells of multiple organs, including intestine (279), kidney (279, 280), liver (279, 280), pancreas (281, 282), oesophagus (283), stomach (284), lung (280), limb bud (285) and tooth (286), as well as endothelial cells (287), neuronal cells (288), erythroid progenitors (289), melanocytes (290) and monocytes (261). Met regulates diverse cellular processes including cell proliferation, scattering, migration, invasion and survival (45).

6.3.1 Cell proliferation, migration, invasion and morphogenesis

HGF is also named scatter factor (SF) for its ability to induce cell scattering (256, 291, 292), a process that consists of the dissociation of epithelial sheets into individual cells that lose cell polarity and acquire a fibroblastic-like phenotype. It involves the breakdown of cell-cell junctions and reorganization of the cytoskeleton (293, 294). HGF also promotes epithelial cell migration/motility and invasion (295), which are important events during development, tissue repair and metastasis. Cell migration involves the formation of a leading edge, cytoskeleton remodelling and the constant formation and breakdown of focal adhesions (296). Invasion further requires the secretion of proteinases to degrade the extracellular matrix.

HGF is also a morphogenic factor: it promotes branching tubulogenesis of epithelial and endothelial cells when grown in a three-dimensional collagen matrix (297-299). This process has been mostly characterized using Madin Darby canine kidney (MDCK) epithelial cells (300). They form a hollow cyst of polarized cells in a three-dimensional collagen matrix where the apical membrane is facing

the lumen of the cyst. In the presence of HGF, there is a breakdown of cell-cell junctions and the cells start to proliferate and invade the collagen matrix (301). Within two weeks, the cells form branching tubules that are originating from the cyst. As for the cysts, the walls of the tubes consist of a sheet of polarized epithelial cells. The formation of the lumen requires apoptosis of the cells in the center of the tubules (302). This *in vitro* process mimics the formation of branching tubules observed in kidney, prostate and breast (301). Similarly, in three-dimensional collagen matrices, colon epithelial cells exposed to HGF form crypt-like structures with a brush border facing the lumen while lung epithelial cells form alveolar-like structures (299). These morphogenic programs are remarkably complex and alteration in the intensity, duration or specificity of Met signalling pathways may have deleterious consequences such as tumorigenesis.

6.3.2 Tissue repair

Nakamura et al. isolated HGF in serum from partially hepatectomized rats as a factor whose level increased 5-fold following surgery (303), and demonstrated that purified HGF acts as a potent mitogenic factor for rat hepatocytes (255). Other groups then observed that HGF serum levels are also elevated following injury of the kidney (304, 305), lung (260), stomach (306, 307) and intestine (308), and that HGF is a strong mitogen for the epithelial cells derived from these organs. Moreover, HGF prevents the onset or progress of hepatic fibrosis/cirrhosis (309), hepatic failure, acute and chronic renal failure and lung fibrosis, in addition to enhancing renal and lung regeneration (310). Administration of HGF to mice abrogated Fas-induced massive liver apoptosis (311) and mice lacking the *Met* gene in hepatocytes are hypersensitive to Fas-induced liver apoptosis (312). Collectively, these results suggest that HGF and Met are important following tissue injury to limit the extent of the damage and to promote tissue regeneration.

6.3.3 Angiogenesis

HGF is a potent angiogenic factor, inducing blood vessel formation *in vivo* (313, 314). This effect is mediated in part through direct action on endothelial cells, which express the Met receptor (287). HGF can stimulate the proliferation, migration, protease production and invasion of endothelial cells (287, 315-318)

and promotes their organization into capillary-like tubules (319). HGF also stimulates the proliferation of vascular smooth muscle cells and pericytes, two cell types that are involved in the formation of capillaries (320). In addition to its direct action on endothelial and vascular smooth muscle cells, HGF stimulates expression of other angiogenic factors such as VEGF and uPA by vascular smooth muscle cells (321), and inhibits the expression of anti-angiogenic factors like thrombospondin-1 (322, 323). Furthermore, different groups observed that HGF synergizes with uPA and VEGF in promoting vascular tubulogenesis in 3D endothelial cell cultures assays (324, 325).

Not only does HGF promote normal vascularization, it also plays a role in tumour angiogenesis (323, 326). A positive correlation between HGF expression and tumour angiogenesis has been observed in glioma and breast, lung and hepatocellular carcinomas (327-330).

6.3.4 Embryonic development

Both Met and HGF knockout mouse embryos are reduced in size and die at E14.5-16.5 (248-250). This is due to defects in placenta and liver development where a reduction in trophoblast cells and parenchymal cells respectively was observed (248-250). In addition to the development of epithelial organs, HGF and Met are essential for the migration of myogenic precursor cells in the limb bud, the diaphragm and the tip of tongue (250). Consequently, in a Met null mouse, skeletal muscles of the limb and diaphragm do not form (250).

6.3.5 Neuronal development

Both HGF and Met are expressed in the developing and adult brain (331-335). In addition to neurons, Met is expressed in non-neuronal cells of the nervous system, including microglia and Schwann cells (288, 336). Hence, whereas neurotrophins mainly act on neurons, HGF can act on both neuronal and non-neuronal cells (337). Initially, studies in chick embryos demonstrated that HGF plays a role in the early steps of neuronal induction (338-340). It was then found that HGF, which is secreted by the limb mesenchyme, is a guidance factor for developing spinal motor axons (341). It can also induce neurite outgrowth of sympathetic, thalamic, but not cortical neurons (342-344). HGF acts as well as a neurotrophic factor for spinal and cranial motor neurons (345-347), is

required for the differentiation, survival and growth of sympathetic neuroblasts (342, 348), and stimulates the migration of interneurons from the ventral to dorsal telencephalon (349). Therefore, Met and HGF are key players in the development of the nervous system by regulating the differentiation, outgrowth and/or survival of distinct neuronal populations (337).

6.4 Met, HGF and cancer

Chronic activation of the Met receptor is associated with several human and murine tumours (45, 350). The first evidence was provided by Berton Zbar's group who identified mutations in the Met receptor in hereditary and sporadic papillary renal carcinomas (70-72). They identified 9 germline and 6 somatic mutations, all located in the tyrosine kinase domain. Furthermore, they observed that thirteen percent (17/129) of the sporadic papillary renal carcinomas examined had a mutation in Met while they did not detect mutations in Met in 199 sporadic human solid tumours of various types (71). Mutations in the kinase domain of the Met receptor were also found in hepatocellular carcinomas (351). The mutations enhanced the kinase activity of the Met receptor towards an exogenous substrate when compared to wt Met, although the somatic mutations generally display greater kinase activity than the germline mutations (352). This could be explained by the possibility that a Met receptor mutant that displays high kinase activity may be embryonic lethal (352). Consistent with this, embryonic lethality was observed in mice homozygous for activated Met receptor mutants (353).

The mutations with the strongest transforming activity are located within the activation loop of the Met kinase domain and are thought to release a negative regulatory constraint imposed by the activation loop on Met catalytic activity (74). The mutations with intermediate transforming activity are located at the interface of the N- and C-terminal lobes of the kinase domain (74). Of considerable interest, several of the point mutations identified in Met were also found in other RTKs such as Ret and Kit (70), suggesting that the mechanisms of regulation and deregulation of kinase activity are conserved through RTKs. Importantly, the Met receptors containing an activating mutation in their kinase domain still require HGF-induced activation to transform epithelial and fibroblast cells, since either a deletion removing the HGF binding site in the Met receptor mutants or

the presence of HGF antagonists were sufficient to completely inhibit their transforming activity (354).

During the course of my thesis, mutations in the juxtamembrane domain of the Met receptor have been identified in gastric, breast and lung cancers (81, 82). As mentioned in section 3.3, several recent studies revealed a role for the juxtamembrane domain in the auto-inhibition of RTKs (355). It is tempting to speculate that the juxtamembrane domain of the Met receptor also acts as a negative regulatory domain and that the juxtamembrane mutations found in cancers may relieve this negative regulation; nevertheless, this remains to be demonstrated.

Met activation in human cancer often occurs through the formation of autocrine or paracrine loops. Whilst autocrine loops have been observed in sarcomas (356), gliomas (357), lung (358) and breast carcinomas (359-361), paracrine activation of Met is very common in carcinomas. The tumour cells express or overexpress Met while the surrounding stroma secretes HGF (45). Since Met activation induces cell migration and invasion, paracrine activation of Met promotes dissemination of the tumour cells. In addition to carcinomas, Met receptor overexpression has been reported in some sarcomas and hematopoietic malignancies (53). Met amplification has only been observed in gliomas (357) and in colon tumours and metastases (362). Whilst only 10% of the colon tumours examined had amplified Met, 89% (8/9) of the metastases did, suggesting that Met amplification may provide a selective advantage for the acquisition of metastatic potential (362).

Several transgenic mouse models have been generated to study the role of Met in tumorigenesis. Expression of the Tpr-Met oncoprotein under the metallothionein (MT) promoter induced the formation of mammary tumours in 42% (11/26) of female mice with an average onset of 13 months (363). Diffuse and thymic lymphomas, metastatic spindle-cell sarcoma, orbital osteosarcoma, squamous papilloma, and rectal spindle-cell sarcoma were also observed (363). Mice expressing HGF under the MT promoter developed a broad range of tumours from mesenchymal and epithelial origin (364). In a similar manner to Tpr-Met expressing mice, the most prevalent tumours were mammary tumours, which were present in 40% of the transgenic females. The MT-HGF transgenic mice also developed many skin tumours (364), liver tumours (hepatocellular

adenomas and carcinomas) (365), as well as renal tubular hyperplasia, polycystic disease, and glomerulosclerosis (366).

Mice expressing Met receptor mutants found in renal papillary carcinoma under the MT promoter were also generated (367). The female founders had severe breeding difficulties and developed mammary adenocarcinomas. Finally, mice with targeted mutations in the murine *met* locus were created (353). Interestingly, mice carrying different renal papillary carcinoma mutations developed unique tumour profiles; some lines harbouring specific mutants developed carcinomas, but not sarcomas and others the opposite (353). This could be explained by the fact that some mutations may alter substrate specificity of the receptor, leading to the activation of different signalling pathways (75). Altogether, these murine models provide convincing evidence that Met and HGF can promote the development and progression of tumours *in vivo*.

6.5 HGF/Met-dependent signalling pathways

Tyrosine 1356 is the major autophosphorylation site outside of the kinase domain in the Met receptor (275) and is essential for Met biological activity (Fig. 8) (277, 278). A unique feature of Y1356 is its ability to recruit multiple signalling proteins containing either a Src-homology-2 (SH2) or a phosphotyrosine binding (PTB) domain, given that Y1356 (NATpYVNV) is part of a PTB (NXXpY), a p85 SH2 (pYXXΦ) as well as a Grb2 (Growth factor receptor binding protein 2) SH2 (pYXNX) consensus binding site. The signalling proteins recruited to Y1356 include Grb2 (276, 368), Shc (368), Src (276), PLC-γ (276), SHP-2 (369), the p85 subunit of PI3'K (370), STAT3 (371), and SHIP (372). However, Grb2 remains the major protein recruited to Y1356 (277, 373-375). In addition to its central SH2 domain, Grb2 contains two SH3 domains that bind to proline-containing sequences. Grb2 recruits Son of Sevenless 1 (Sos1), a Ras GEF that activates the Ras-MAPK pathway (376-378), as well as Gab1 (Grb2-associated binding protein-1) (379), Cbl (380, 381), dynamin (382, 383) and Vav1 (384-386). Gab1 and Cbl are scaffolding adaptor proteins that are highly tyrosine phosphorylated upon Met activation (387-390).

Gab1, a 110 kDa docking protein containing a PH domain, is a major transducer of Met signalling (391, 392). Consistent with this, Gab1, Met and HGF knockout mouse embryos have very similar phenotypes (393). Gab1 is recruited

directly to Y1349 in Met and indirectly via Grb2 (394, 395). Gab1 binds multiple signalling proteins including SHP-2 (396), PLC-γ (397), the p85 subunit of PI3'K (398) and Crk adaptor proteins (389, 399-401). These events induce the activation of downstream effectors such as the Ras-MAPK pathway, Akt, Src, Jnk, Rho, Rac1, Cdc42 and PAK, and are necessary for Met biological activity (301, 402). Importantly, whereas MAPK activation is transient following EGF stimulation, it is sustained in response to HGF (403). The prolonged activation of MAPK downstream of HGF requires the recruitment of SHP-2 via Gab1 to the Met receptor and is essential for HGF-induced morphogenesis (396).

As mentioned previously, c-Cbl is highly tyrosine phosphorylated downstream of Tpr-Met and Met (387, 390, 399, 404). c-Cbl is recruited to the Met receptor in a phosphotyrosine-dependent manner via the Grb2 adaptor protein. Deletion of the Grb2 binding site in Met prevents c-Cbl tyrosine phosphorylation and abrogates most of Met biological activity, suggesting that c-Cbl may play an important role downstream of the Met receptor (373, 387). Moreover, since c-Cbl is an E3 ubiquitin ligase and Met is ubiquitinated upon activation (133), c-Cbl could be involved in Met receptor ubiquitination.

7. The Cbl family of proteins

The c-Cbl proto-oncogene was identified following the isolation of a viral oncogene v-Cbl, which was encoded by the pre-B cell lymphoma inducing retrovirus Cas NS-1 (405). v-Cbl was named for Casitas B-lineage lymphoma and encompasses only the first 355 amino acids of c-Cbl (405, 406). Cbl proteins are present in metazoans from nematodes to vertebrates (407). Whilst, there is one *cbl* gene in *C. elegans* (*sli-1*) and *Drosophila* (*d-cbl*), there are three mammalian *cbl* genes: *c-cbl*, *cbl-b* and *cbl-c* (also named *cbl-3*) (Fig. 8) (407). c-Cbl and Cbl-b proteins are ubiquitously expressed with highest expression levels in haematopoietic cells and testis (408, 409) and their subcellular localization is cytoplasmic (410). In contrast, Cbl-c mRNA is expressed mainly in organs rich in epithelium from the gastrointestinal tract, the respiratory, urinary and reproductive system as well as the epidermis and is expressed at low levels in hematopoietic tissues (411-413).

c-Cbl and Cbl-b deficient mice have no developmental abnormalities and are generally healthy (414-417). However, thymocytes and peripheral T-cells are

hypersensitive to TCR stimulation in c-Cbl and Cbl-b null mice respectively. Hence, whilst c-Cbl null mice displayed lymphoid hyperplasia, Cbl-b null mice are highly susceptible to auto-immune diseases (414-417). Importantly, the c-Cbl/Cbl-b double null mice are embryonic lethal, revealing that c-Cbl and Cbl-b have redundant key functions. Despite the lower levels of c-Cbl and Cbl-b proteins in epithelial tissues, the c-Cbl-deficient mice, in addition to having hematopoietic defects, develop mammary hyperplasia (415), signifying that c-Cbl is important for growth regulation of mammary epithelia. Cbl-c deficient mice are viable, healthy, fertile and exhibit no histological abnormalities (413). The epithelial tissues were normal and therefore, Cbl-c is not required for their development and function (413).

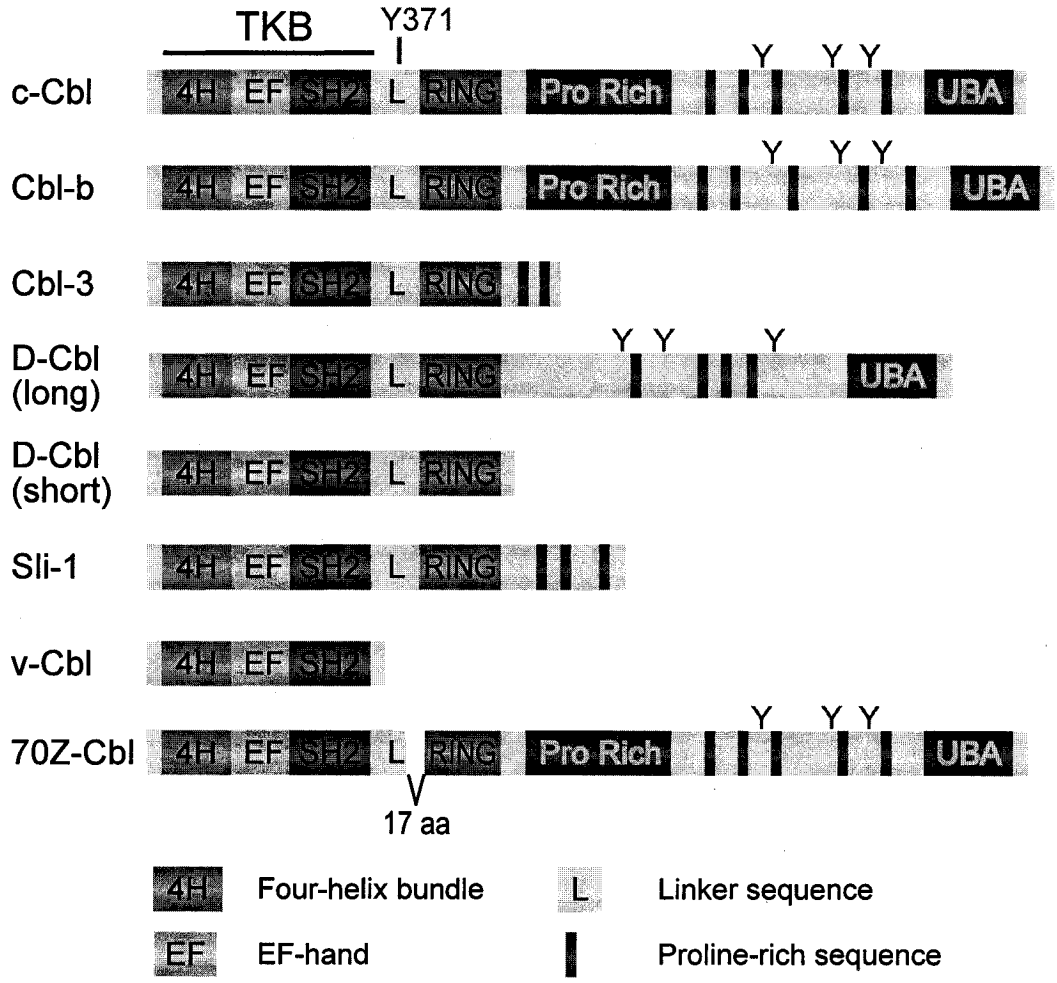


Figure 8. Cbl ubiquitin-protein ligases

Adapted from Thien and Langdon. 2001. Nat Rev Mol Cell Biol **2**:294-307.

7.1 Cbl structure

Cbl ubiquitin-protein ligases are modular proteins that contain, in their N-terminal half, a tyrosine kinase binding (TKB) domain and a RING finger domain that are conserved in all the members of the family. The C-terminal portion is much more variable in length and includes binding sites for SH2 and SH3 containing proteins (Fig. 8) (418, 419).

7.1.1 TKB domain

In 1995, Galisteo *et al.* observed that the first 486 amino acids of c-Cbl interact with the EGFR in a phosphotyrosine-dependent manner (420). Similarly, Lupher and co-workers reported that the first 357 amino acids of c-Cbl were able to bind directly to a phosphotyrosine residue in ZAP-70, a cytoplasmic protein tyrosine kinase (PTK) involved in T-cell signalling (421). These observations were intriguing since the N-terminal portion of c-Cbl containing the TKB domain has no sequence homology with known SH2 and PTB domains. A degenerate phosphopeptide library screen revealed the c-Cbl TKB domain has a preference for an aspartate or an asparagine residue at position pY-2 (D/NxpY) (422). These results suggested that the c-Cbl TKB domain behaved in a similar manner to a PTB domain, where the specific-determining interactions are located upstream of the phosphotyrosine residue (see Section 2.3.2).

Crystal structure studies of the c-Cbl TKB domain performed by Meng and co-workers revealed the true nature of the c-Cbl TKB domain (423). It is composed of a four-helix bundle (4H), an EF-hand calcium-binding domain, and a variant SH2 domain that, together, are able to bind to phosphotyrosine residues (Fig. 8) (423). The variant SH2 domain within the c-Cbl TKB domain is structurally similar to an SH2 domain, although there is only 11% identity at the amino acid level. The phosphotyrosine-binding pocket is well conserved, containing an invariant arginine residue that forms two hydrogen bonds with the phosphate group of the tyrosine residue (423). However, the SH2 domain in c-Cbl lacks the secondary beta-sheet and the loop that define the binding specificity of an SH2 domain with residues located downstream from the phosphotyrosine residue (see section 2.3.1) (30, 424).

Meng and co-workers also obtained the crystal structure of the c-Cbl TKB domain complexed to its binding site on ZAP-70 kinase. They observed that a

medium-sized hydrophobic residue (Φ) at position pY+4 and an acid residue (D/E) at position pY+3 constitute the primary specific-determining interactions (423). In addition, an aspartate residue at position pY-2 forms a hydrogen bond, in agreement with the results obtained with the degenerate phosphopeptide library screen (422, 423). Hence, the consensus sequence for the c-Cbl TKB domain was established to be D/NXpYXXD/EΦ. This sequence is found not only in the ZAP-70 kinase, but also in other c-Cbl TKB domain binding proteins including the Syk kinase, the EGFR and Sprouty adaptor proteins (132, 422, 425-427).

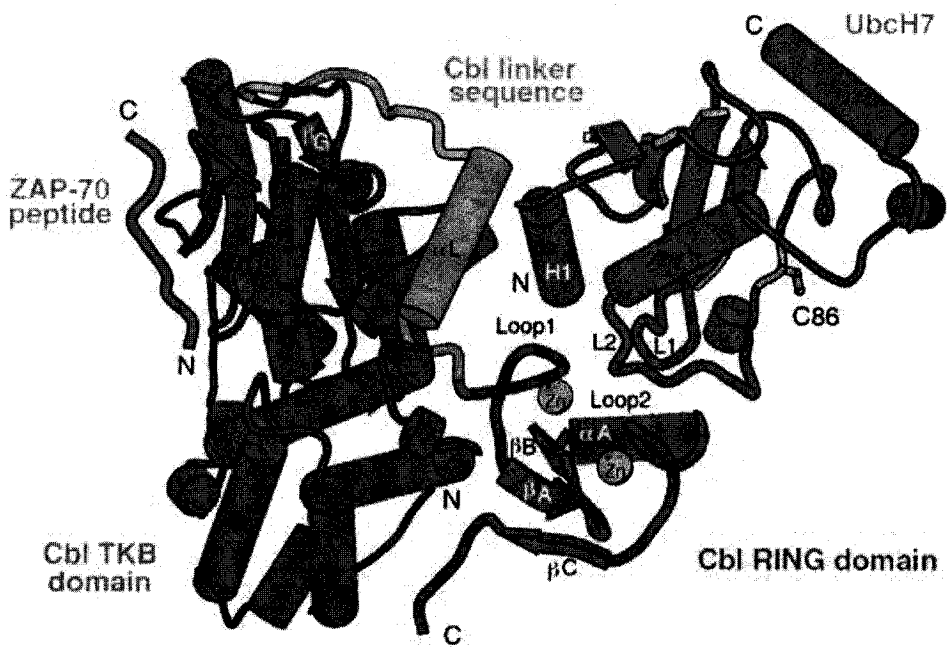


Figure 9. Structure of the c-Cbl-UbcH7 complex
Zheng *et al.* 2000. Cell **102**:533-539.

7.1.2 RING finger domain

RING finger domains are a subclass of Zinc finger domains that mediate only protein-protein interactions. The Cbl RING finger is a 55 amino acid compact domain that contains two zinc ions. The first one is coordinated by three cysteine and one histidine residue while the second one is coordinated by four cysteine residues (C3HC4 RING finger) (428). The RING finger domain, which is found in several hundred proteins, characterizes a family of E3 ubiquitin-protein ligases (429). Its function is to recruit the E2 ubiquitin conjugating enzyme (see section

5.1). At the beginning of my thesis, UbcH7 was identified as the E2 associated with the Cbl RING domain by yeast two-hybrid assays (146). A year later, Zheng and co-workers published the crystal structure of the c-Cbl TKB and RING domain in complex with UbcH7 and a ZAP-70 phosphopeptide (430). The RING domain is attached to the TKB domain by a 35 amino acid linker sequence that packs on the TKB domain, forming an ordered loop and an α-helix (Fig. 9) (430). The RING domain interacts with the 4H bundle through multiple hydrogen bonds and van der Waals contacts. UbcH7 binds primarily to a shallow groove in the c-Cbl RING domain, but also forms bonds with the α-helix in the linker sequence. The active cysteine residue of UbcH7 that binds ubiquitin is oriented away from the ZAP-70 phosphopeptide, both being separated by around 60 Å. Importantly, the structure of UbcH7 complexed with c-Cbl is very similar to the structure of isolated E2s, suggesting that UbcH7 does not undergo a conformational change upon binding to the c-Cbl RING domain (430).

Although it was proposed that Y371 of Cbl must be tyrosine phosphorylated for Cbl to become an active E3 ligase (132), the crystal structure revealed that Y371 forms a hydrogen bond with the TKB domain and is not readily accessible for phosphorylation. Hence, a Y371F substitution is more likely to abolish c-Cbl ubiquitin ligase activity by altering the structure of linker-TKB and linker-UbcH7 interfaces (430).

Overall, the TKB domain, the linker sequence, the RING finger domain and UbcH7 form a rigid structure, suggesting that Cbl does more than bring UbcH7 in the vicinity of the substrate. Cbl may actually orient UbcH7 toward specific lysine residues in the substrate (430).

7.1.3 UBA domain

Mammalian c-Cbl and Cbl-b proteins, as well as the long form of drosophila Cbl (D-Cbl), contain a ubiquitin-associated domain (UBA) at their carboxy-terminal end (Fig. 8). This domain is described in section 5.6.1. The c-Cbl and Cbl-b UBA domains are very similar at the amino acid sequence level (85% similarity) (431). However, whilst the UBA domain of Cbl-b binds ubiquitin and ubiquitinated proteins, the UBA domain of c-Cbl is unable to so (431).

The UBA domain of c-Cbl was previously thought to be a leucine zipper dimerization motif (432), which consists in a sequence of leucine/isoleucine

residues spaced every seventh amino acid residue along an α-helix. Thus, all the leucine residues of the motif are placed along one side of the α-helix. It was shown by yeast two-hybrid, far western and *in vitro* binding assays, that the putative leucine zipper motif can mediate c-Cbl homodimerization (432). Furthermore, the same motif could also mediate c-Cbl/Cbl-b heterodimerization (433). However, bioinformatics analyses lead to the discovery that the putatitive leucine zipper in c-Cbl and Cbl-b has the sequence pattern of a UBA domain (218). Since then, it has been observed that UBA domains found in other proteins are also able to dimerize (434).

The difference in the ability of the Cbl UBA domains to bind ubiquitin is intriguing at the structural level and may play a critical role in specifying their biological function. It also remains to be determined whether the Cbl-b UBA domain can dimerize and bind ubiquitin simultaneously.

7.2 Cbl proteins as negative regulators of RTKs

In 1995, genetic studies in *C. elegans* provided the first evidence that Cbl proteins act as negative regulators of RTKs (435). A loss-of-function mutation in the Cbl orthologue sli-1 rescued vulval induction mediated by a weakly active form of the EGFR orthologue Let-23, while the addition of an extra copy of sli-1 blocked vulval induction (435). Two years later, genetic studies in *Drosophila* revealed that D-Cbl is a negative regulator of EGFR during R7 photoreceptor development and dorsoventral patterning (436-438). However, the molecular mechanisms underlying the down-regulation of EGFR by Cbl was still obscure.

In 1998, Levkowitz and colleagues demonstrated that c-Cbl overexpression promotes the ubiquitination and degradation of EGFR, whereas overexpression of v-Cbl, which behaves as a dominant negative Cbl protein, induces the recycling of the EGFR to the cell-surface (145). Similar data were obtained for the PDGF and CSF-1 receptors (148, 439). Ligand-induced polyubiquitination and internalisation of the CSF-1R is delayed in macrophages from c-Cbl null mice, identifying c-Cbl as important for ubiquitination of the CSF-1 receptor (143). Several reports then demonstrated that c-Cbl possesses ubiquitin ligase activity *in vitro* (132, 146, 428). Mutagenesis studies demonstrated that both the TKB and RING finger domain of c-Cbl are required for the ubiquitination of RTKs (132, 146, 428, 439). The TKB domain interacts with specific phosphotyrosine residues

on RTKs, whereas the RING finger domain recruits the E2 ubiquitin-conjugating enzyme, UbcH7. The other members of the Cbl family, Cbl-b and Cbl-c, can also down-regulate RTKs. Both proteins are able to promote the ubiquitination and degradation of EGFR (147, 412, 440, 441), as well as inhibit EGFR signalling (412, 440, 442).

The aforementioned studies propose a role for Cbl in the down-regulation of RTKs, by affecting either their internalisation and/or lysosomal degradation. One must make a distinction between the role of Cbl versus the role of ubiquitination in RTK internalisation and lysosomal degradation, the latter being discussed in sections 5.4 and 5.5. For example, c-Cbl and Cbl-b can recruit the CIN85 adaptor protein that binds endophilin-1 and Dab2 to activated EGF and Met receptors (443-446). Endophilin-1 is thought to induce negative membrane curvature required for the invagination of the plasma membrane into pits (103), whereas Dab2 is an endocytic adaptor that binds clathrin and regulates its assembly *in vitro* (447). Therefore, by recruiting CIN85, Cbl could promote RTK internalisation in a ubiquitination-independent manner.

Additional lines of evidence support a role for c-Cbl in the internalisation of RTKs. The overexpression of c-Cbl enhances the rate of internalisation of EGFR (132, 444), whereas in c-Cbl null macrophages, CSF-1R has a slower rate of internalisation (143). However, Duan et al. observed that EGFR internalisation is not affected in c-Cbl/Cbl-b null mouse embryonic fibroblasts, although EGFR degradation is impaired (203). Altogether, the role of Cbl in RTK internalisation remains controversial and further studies including RTKs other than EGFR would help to elucidate this issue. The role of Cbl proteins in targeting RTKs for lysosomal degradation correlates with the ability of Cbl to induce RTK ubiquitination and is less controversial. The role of ubiquitination in RTK lysosomal degradation is discussed in section 5.5.

Finally, the stability of c-Cbl itself is regulated by its own ubiquitination (448). Tyrosine phosphorylation of c-Cbl by the c-Src protein tyrosine kinase promotes auto-ubiquitination of c-Cbl and its degradation in a proteasome-dependent manner (449, 450). The observation that Cbl proteins can also interact with ubiquitin-protein ligases containing HECT (homologous to E6-AP C-terminus) domains suggests a potential additional layer of regulation (440).

7.3 Cbl as scaffolding proteins

Multiple binding sites for SH2 and SH3 domain-containing proteins are located in the carboxy-terminal half of c-Cbl and Cbl-b (Fig. 8) (419, 451). These include proline-rich motifs that can mediate constitutive interactions with Grb2, Nck, CAP (Cbl-associated protein), PLCγ, CrkL, the p85 subunit of PI3'K and PTKs (Src, Syk, Fyn, Hck, Lck, Lyn and Btk) (419). Again, the constitutive association of Grb2 to Cbl plays an important role in the recruitment of Cbl to activated RTKs.

Multiple tyrosine residues in c-Cbl and Cbl-b are phosphorylated downstream of RTKs, cytokine receptors, integrins and antigen/immunoglobulin receptors (418). They form binding sites for several signalling proteins, including Crk proteins, p85, Vav and PTKs (Src, Abl, Fyn, Hck, Lck, Lyn, Blk and Fgr) (418, 419). Clearly, Cbl has the ability to recruit numerous signalling proteins in a constitutive and regulated manner and plays a positive role in signalling downstream of multiple PTKs and cell surface receptors.

For example, c-Cbl is involved in bone resorption downstream of Src, as c-Cbl specific antisense oligonucleotides inhibit bone resorption by osteoclast-like cells *in vitro* (452). Other studies demonstrated that the association of the p85 subunit of PI3'K to Y731 in c-Cbl is essential for granulocyte-CSF and interleukin-4 induced B-cell proliferation and survival (453, 454). Garcia-Guzman and colleagues observed that c-Cbl association with Crk is essential HGF-induced activation of Jnk but not Erk in HeLa cells (390). c-Cbl and Cbl-b are also involved in insulin-induced translocation of the glucose transporter GLUT4 to the cell surface (433). Insulin stimulates the tyrosine phosphorylation of Cbl following its recruitment to the insulin receptor via APS (adaptor containing PH and SH2 domain). Cbl then activates the small G protein TC10 (via Crk/C3G), which is required for GLUT4 translocation to the cell surface (455).

There are also numerous publications on the positive role that c-Cbl plays in actin cytoskeleton remodelling, cell adhesion and migration (418, 419). The overexpression of c-Cbl promotes cell adhesion and spreading of v-Abl transformed fibroblasts, and this is dependent on the ability of c-Cbl to bind p85 and CrkL (456). Integrin-dependent adhesion promotes the phosphorylation of c-Cbl by Src, which activates Rac by recruiting Crk and PI3'K to actin lamella (457-459). Truncated mutants of c-Cbl that cannot bind Crk inhibit the formation of

lamellipodia and membrane ruffles in NIH 3T3 cells (460). Integrin-mediated adhesion of macrophages is also dependent on c-Cbl, since treatment with antisense c-Cbl oligonucleotides, Src inhibitor or PI3'K inhibitor is sufficient to inhibit cell spreading on fibronectin (461).

Given that Cbl proteins are phosphorylated downstream of many cell surface receptors and that they bind to multiple signalling proteins, the biological functions of Cbl discovered to date probably represent only a fraction of Cbl functions.

7.4 Oncogenic activation of Cbl proteins

Several oncogenic forms of c-Cbl have been identified in mouse cell lines and tumours, but the mechanisms by which the oncogenic forms of Cbl transform cells remain poorly understood. As mentioned previously, c-Cbl was originally identified as v-Cbl, a viral oncogene that contains the first 355 amino acids of c-Cbl (405). This represents the TKB domain of c-Cbl without the α-helix of the linker sequence (406). v-Cbl is thought to transform cells by competing out endogenous Cbl proteins, preventing the down-regulation of RTKs and potentiating their downstream signals. This is supported by the fact that a v-Cbl protein, with a point mutation (G306E) that abrogates its ability to bind phosphotyrosine residues, is no longer oncogenic (462, 463). However, the addition of the α-helix of the linker sequence to v-Cbl abrogates its transforming activity without affecting its ability to bind phosphotyrosine residues (410).

Another oncogenic form of Cbl, 70Z-Cbl, has been identified in the 70Z/3 mouse pre-B cell lymphoma (406). 70Z-Cbl displays a 17-amino acid deletion (366-382) that removes most of the α-helix and the first cysteine of the RING finger domain (406, 410, 430). The oncogenicity of 70Z-Cbl, which has no ubiquitin ligase activity, could be explained in part by its ability to compete with endogenous Cbl. Expression of 70Z-Cbl in NIH 3T3 cells enhances EGFR phosphorylation and signalling (464). On the other hand, 70Z-Cbl induces epithelial-mesenchymal transition of MDCK cells without significantly elevating Met or EGFR phosphorylation (404). Furthermore, a careful study by Thien and Langdon revealed that mutations that abrogate the ubiquitin ligase activity of c-Cbl are not sufficient for its oncogenic activation (465). Instead, mutations that disrupt the α-helix of the linker sequence render c-Cbl oncogenic (410, 465). The

transforming activity of the α-helix mutants requires an intact C-terminal half, suggesting that it may require Cbl association with signalling proteins (418). More recently, another oncogenic variant of c-Cbl, identified in a macrophage cell line, has an 111-amino acid deletion that eliminates the α-helix of the linker sequence and the RING finger domain (466). Hence, all of the oncogenic forms of Cbl identified to date lack an intact α-helix in the linker sequence. As discussed in Section 7.1.2, the α-helix packs with the TKB domain and with UbcH7 (430). The results obtained in the transformation assays suggest that disruption of the α-helix does more than abrogating Cbl ubiquitin ligase activity. However, even the structural studies do not as yet provide an explanation for these observations.

There is little evidence to support a role for mutations in Cbl in human cancer. One study has reported that the *CBL* gene is fused to the *MLL* gene in an adult patient with *de novo* acute myeloid leukemia (467). The predicted protein product, MLL-Cbl, would contain the C-terminal half of Cbl. Since homodimerization of truncated MLL is sufficient to induce leukemia in mice (468), it is possible that the UBA domain of c-Cbl promotes MLL homodimerization and oncogenic activation (467). The reciprocal CBL-MLL fusion transcript was absent in the leukemic cells. The fact that no mutations in Cbl have been reported in human tumours does not mean that there is none, since large-scale studies have not yet been pursued.

Since their discovery, RTKs have been studied extensively with respect to their activation and signalling. In contrast, our understanding of their down-regulation is poor. The discovery in yeast that ubiquitination of plasma membrane receptors plays a role in their lysosomal degradation has provided a better understanding of the mechanisms underlying the down-regulation of RTKs. In this thesis, I have defined the mechanisms by which the Met RTK is ubiquitinated and what are the functional and biological consequences of uncoupling ubiquitination from the Met RTK.

Abbreviations

A-loop	Activation-loop
AP180	Assembly Protein 180
AP2	Adaptor Protein Complex-2 or (Assembly Polypeptide-2)
APP	Amyloid precursor protein
APS	Adaptor containing PH and SH2 Domain
CALM	Clathrin Assembly Lymphoid Myeloid Leukemia
Cbl	Casitas B-lineage Lymphoma
CCV	Clathrin Coated Vesicle
Cdc42	Cell Division Cycle 42
CHIP	Carboxyl Terminus of Hsc70-Interacting Protein
CIN85	85K Cbl-Interacting Protein
Crk	CT10 Regulator of Kinase
CSF	Colony Stimulating Factor
CUE	Coupling of Ubiquitin conjugation to ER degradation
CXCR4	Chemokine (C-X-C motif) Receptor 4
Dab2	Disabled-2
DUB	Deubiquitinating Enzyme
E1	Ubiquitin-Activating Enzyme
E2	Ubiquitin-Conjugating Enzyme
E3	Ubiquitin-Protein Ligase
E4	Polyubiquitin Protein Ligase
E6AP	E6 associated protein
EGF(R)	Epidermal Growth Factor (Receptor)
Eps15	Epidermal Growth Factor Receptor Substrate 15
Epsin	Eps15 Interactor
ESCRT	Endosomal Sorting Complex Required for Transport
FA	Fanconi Anemia
FANCD2	Fanconi Anemia Complementation group D2
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FIG-ROS	Fused In Glioblastoma
Flt3	FMS-Like Tyrosine kinase 3
Fms	Feline McDonough Strain
Fos	FBJ Murine Osteosarcoma
FYVE	Fab-1, YGL023, Vps27, and EEA1
Gab1	Grb2 Associated Binding Protein 1
GAP	GTPase activating Protein
GAT	GGA and Tom1
GEF	Guanine Nucleotide Exchange Factor
GHR	Growth Hormone Receptor
GLUE	GRAM-like Ubiquitin-binding in Eap45
GLUT4	Glucose Transporter-4
GPCR	G-Protein Coupled Receptor
Grb2	Growth Factor Receptor-Bound Protein 2
H2A/B	Histone 2A/B
HECT	Homologous to E6AP Carboxy Terminus
HGF	Hepatocyte Growth Factor
Hip1	Huntingtin interacting protein 1
HOS	Human Osteogenic Sarcoma

Hrs	Hepatocyte Growth Factor Regulated Tyrosine Kinase Substrate
Hse1	Yeast homologue of STAM
Hsp	Heat Shock Protein
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate-1
Jnk	Jun N-terminal Kinase
LAMP	Lysosome Associated Membrane Protein
LDL	Low Density Lipoprotein
LRP1	Low Density Lipoprotein-Related Protein 1
MAPK	Mitogen Activating Protein Kinase
MDCK	Madin Darby Canine Kidney
MEN	Multiple Endocrine Neoplasia
Met	Cloned in N-methyl-N'-nitronitrosoguanidine treated cells
MLL	Mixed Lineage Leukemia
MNNG	N-methyl-N'-nitronitrosoguanidine treated cells
MPG	3-Methyladenine-DNA Glycosylase Protein
MSP	Macrophage Stimulating Protein
MT	Metallothionein
MVB	Multi-Vesicular Bodies
Myc	Myelocytomatosis
NF-kB	Nuclear Factor Kappa B
NZF	Np14 Zinc Finger
p53	Tumour Suppressor Protein 53
p85	Regulatory Subunit of PI3'K
PAK	P21 Activated Kinase
PAZ	Polyubiquitin-associated Zinc Finger
PCNA	Proliferating Cell Nuclear Antigen
PDGF(R)	Platelet Derived Growth Factor (Receptor)
PH	Pleckstrin-homology
PI3'K	Phosphoinositide-3-Kinase
PLC-gamma	Phospholipase C
Png1	Peptide:N-Glycanase 1
PSI	Plexins,Semaphorins and Integrins
PTB	Protein Tyrosine Binding
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
PTP-1B	Protein Tyrosine Phosphatase-1B
PX	Phox Homology
Rab	Ras in the Brain
Rabex-5	Rab5 GTP Exchange Factor
Rho	Ras Homologous
RING	Really New Interesting Gene
rms	Root-Mean-Square
Ros	Recepteur d'Origine Nantais
RTK	Receptor Tyrosine Kinase
SCF	Skp1,Cul1,F-box Protein and Rbx1
Sea	Sarcoma, Erythroblastosis and anemia
SF	Scatter Factor
SH2	Src Homology 2
SH3	Src Homology 3
Shc	Src homology 2 domain containing

SHIP	SH2 Domain-containing Inositol Phosphatase
SHP-2	SH2 Domain-containing Protein-Tyrosine Phosphatase
SNX1	Sorting Nexin-1
Sos	Sons of Sevenless
STAM	Signal Transducing Adaptor Molecule
STAT3	Signal Transducer and Activator of Transcription 3
Syk	Spleen Tyrosine Kinase
TCR	T-Cell Receptor
Tf(R)	Transferrin (Receptor)
TGFalpha	Transforming Growth Factor alpha
TKB	Tyrosine Kinase Binding
Tollip	Toll-Interacting Protein
Tpr	Translocated Promoter Region
Trk	Tropomyosin-Related Kinase
Tsg101	Tumour Suppressor Gene 101
UBA	Ubiquitin-associated domain
UBD	Ubiquitin Binding Domain
UCH	Ubiquitin Carboxy-Terminal Hydrolases
UEV	Ubiquitin E2-Varient
Ufd2	Yeast Homologue of Ubiquitination Factor 2
UIM	Ubiquitin Interacting motif
uPA	Urokinase Plasminogen Activator
VCB	VHL, elongin C or BCul2 and Rbx1
VEGFR	Vascular Endothelial Growth Factor Receptor
VHS	Vps27p, Hrs and STAM
Vpr	Viral Protein
Vps23	Vacuolar Protein Sorting 23
WW	Domain that contains two highly conserved tryptophan residues
ZAP-70	Zeta-Associated Protein 70

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Chapter 2

Mutation of the c-Cbl TKB Domain Binding Site on the Met Receptor Tyrosine Kinase Converts it into a Transforming Protein

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Preface

The Met/HGF receptor tyrosine kinase (RTK) is involved in multiple cellular processes that include proliferation, migration, invasion, differentiation and survival. The Met receptor is deregulated in multiple human cancers by several mechanisms. For instance, Met is amplified and/or overexpressed in multiple carcinomas, some sarcomas and hematopoietic malignancies. Mutations in the juxtamembrane and kinase domain of Met have also been found in renal, hepatocellular, gastric, lung and breast carcinomas, establishing a new paradigm. In the absence of ligand, the Met receptor is catalytically inactive. Binding of HGF to Met induces receptor dimerization, tyrosine phosphorylation and activation of signalling pathways via the recruitment of multiple signalling proteins. Met receptor activation also induces its ubiquitination, internalisation and lysosomal degradation. However, the mechanisms underlying Met receptor internalisation and lysosomal down-regulation remain poorly understood. This chapter addresses the importance of appropriate down-regulation of the Met receptor for its biological activity.

One of the proteins recruited to an activated Met receptor is c-Cbl. c-Cbl was recently identified as a ubiquitin-protein ligase for the EGF, PDGF and CSF-1 receptors. Genetic evidence in Drosophila and C. elegans previously identified Cbl proteins as negative regulators of RTKs. In addition, overexpression of Cbl promotes EGFR internalisation and down-regulation. However, the biological consequence of preventing RTK ubiquitination had not been examined and therefore, the biological relevance of RTK ubiquitination remained to be shown. This chapter describes the mechanism of Cbl-mediated Met receptor ubiquitination and demonstrates that preventing ubiquitination of the Met receptor is sufficient to induce its oncogenic activation.

Abstract

The c-Cbl proto-oncogene is a negative regulator for several receptor tyrosine kinases (RTKs) through its ability to promote their polyubiquitination. Hence, uncoupling c-Cbl from RTKs may lead to their deregulation. In testing this, we show that c-Cbl promotes ubiquitination of the Met RTK. This requires the c-Cbl tyrosine kinase binding (TKB) domain and a juxtamembrane tyrosine residue on Met. This tyrosine provides a direct binding site for the c-Cbl TKB domain, and is absent in the rearranged oncogenic Tpr-Met variant. A Met receptor, where the juxtamembrane tyrosine is replaced by phenylalanine, is not ubiquitinated and has transforming activity in fibroblast and epithelial cells. We propose the uncoupling of c-Cbl from RTKs as a mechanism contributing to their oncogenic activation.

Abbreviations

4H	4 helix bundle
Cbl	Casitas B-lineage lymphoma
CSF-1	colony stimulating factor-1
EGFR	epidermal growth factor receptor
ErbB-2	erythroblastic leukemia viral oncogene homolog 2
FBS	fetal bovine serum
Grb2	Growth Factor Receptor-Bound Protein 2
GST	glutathione S-transferase
HGF	hepatocyte growth factor
MDCK	Madin-Darby Canine Kidney
PDGFR	platelet-derived growth factor receptor
PTB	phosphotyrosine binding
PTK	protein tyrosine kinase
RTK	receptor tyrosine kinase
SH2	Src-homology 2
SH3	Src-homology 3
Syk	Spleen tyrosine kinase
TKB	tyrosine kinase binding
WT	wild type
ZAP-70	70 kDa zeta-associated protein

Introduction

Tight control of receptor tyrosine kinase (RTK) activity is essential for normal cell growth and development. The deregulation of RTKs has been widely associated with human tumours and can occur via multiple mechanisms (1). Although the mechanisms of deregulation of RTKs vary, they generally result in a ligand-independent activation of the receptor. There are multiple mechanisms present within the cell that act to tightly regulate the activity of RTKs. Recent studies have implicated the proto-oncogene c-Cbl as a regulator of RTKs (2). From genetic studies in *Caenorhabditis elegans* and *Drosophila*, homologues of the c-Cbl protein are negative regulators of the epidermal growth factor receptor (EGFR) (3-5). In mammalian cells, the overexpression of c-Cbl enhances ligand-dependent down-regulation of the platelet-derived growth factor receptor (PDGFR) and the EGFR, by promoting their polyubiquitination and degradation and CSF-1 fails to stimulate CSF-1 receptor polyubiquitination in c-Cbl-/- cells (6-8).

In vitro assays have demonstrated that c-Cbl functions as an E3 ubiquitin-protein ligase (9-11). c-Cbl contains a RING finger domain, required for the recruitment of E2 ubiquitin-conjugating enzymes, and a tyrosine kinase binding (TKB) domain containing a four-helix bundle (4H), an EF-hand calcium binding domain and a variant SH2 domain that binds to phosphotyrosine residues (12, 13). Where tested, both the TKB and RING finger domains are essential for ligand-induced polyubiquitination of the receptor, implicating the c-Cbl TKB domain in targeting c-Cbl associated ubiquitin-conjugating enzymes to the receptor (14-17). In addition, c-Cbl contains a proline-rich region that associates with the N-terminal SH3 domain of Grb2, allowing the indirect recruitment of c-Cbl to RTKs via the Grb2 adaptor protein (18-20).

The partial or complete deletion of the c-Cbl RING finger domain and upstream linker domain generates transforming variants of c-Cbl. The v-Cbl oncogenic form of c-Cbl is thought to function as a dominant negative protein through competition with endogenous c-Cbl for association with RTKs (20). These studies suggest that c-Cbl may play an important role in the normal regulation of RTKs and consequently, that uncoupling c-Cbl from RTKs may lead to receptor deregulation. However, although implied, this hypothesis has not been tested.

The hepatocyte growth factor (HGF) receptor, Met, is an RTK that is primarily expressed in epithelial and endothelial cells. The chronic activation of the Met receptor is associated with the genesis and the progression of multiple types of human and murine tumours including carcinomas, melanomas and sarcomas (21). Stimulation of the Met receptor with its ligand, HGF, induces tyrosine phosphorylation of the receptor and stimulation with high levels of HGF leads to detectable Met receptor polyubiquitination and degradation (22-24). The ubiquitin-protein ligase responsible for Met receptor ubiquitination has yet to be identified.

The c-Cbl protein is highly phosphorylated following stimulation of the Met receptor as well as in cells expressing an oncogenic Met receptor variant, Tpr-Met (25-27), suggesting a potential role for c-Cbl in the regulation of Met signaling. In this paper, we show that c-Cbl overexpression promotes polyubiquitination of the Met receptor and this requires an intact c-Cbl TKB domain. Importantly, we have also identified a juxtamembrane tyrosine on the Met receptor required for recruitment of the c-Cbl TKB domain (Y1003) and that is absent in the rearranged oncogenic Met variant, Tpr-Met. Met receptor mutants containing a substitution of Y1003 with a phenylalanine residue are not ubiquitinated and are oncogenically active in both fibroblast and epithelial cells.

Results

Ubiquitination of the Met receptor is promoted by c-Cbl

To establish if c-Cbl induces ubiquitination of the Met receptor, we transiently transfected 293T HEK cells with constructs encoding the Met receptor, together with c-Cbl, a c-Cbl TKB domain mutant (G306E) that fails to bind phosphotyrosine residues, or 70Z-Cbl, that has an intact TKB domain, but contains a deletion in the linker and RING finger domains and lacks ubiquitin ligase activity. Immunoprecipitation of the Met receptor followed by an anti-ubiquitin immunoblot revealed that overexpression of the wild type c-Cbl protein promotes the ubiquitination of the Met receptor (Fig. 1). In contrast, overexpression of the c-Cbl G306E or 70Z-Cbl mutant proteins is unable to mediate Met receptor ubiquitination, although similar levels of c-Cbl proteins were expressed (Fig. 1). This is consistent with studies of the EGFR and PDGFR where intact c-Cbl TKB and RING finger domains are required for c-Cbl-dependent receptor ubiquitination.

Efficient c-Cbl tyrosine phosphorylation requires a functional Grb2 binding site in Met

To understand the structural requirements of the Met receptor for the recruitment of c-Cbl, we examined the phosphorylation status of endogenous c-Cbl in cells expressing various Met receptor mutants. For this, we have utilized chimeric CSF-Met receptor mutants that we have used extensively for biological and biochemical studies and that contain substitutions of residues important for Met receptor signaling and biological activities (Y1349F, Y1356F, N1358H) (28, 29). The endogenous c-Cbl protein is phosphorylated in 293T cells overexpressing the wild type Met receptor, the Y1349F Met mutant and the Tpr-Met oncoprotein, but not in cells expressing Met mutant receptors (Y1356F, N1358H and Y1349/1356F) (Fig. 2B) that fail to recruit the Grb2 adaptor protein (28). Moreover, the tyrosine phosphorylation of c-Cbl correlates with the ability of a fusion protein (GST-Cbl-C), containing several potential Grb2-binding sites (amino acids 358 to 906 of c-Cbl, Fig. 2A), to associate with the Met receptor or Tpr-Met oncoprotein (Fig. 2B), suggesting that the indirect recruitment of c-Cbl to Met or Tpr-Met via the Grb2 adaptor protein is sufficient for c-Cbl tyrosine phosphorylation.

The c-Cbl TKB domain associates with the Met receptor but not with the Tpr-Met oncoprotein

The mechanism of recruitment of the c-Cbl TKB domain to RTKs is poorly defined. Since a functional c-Cbl TKB domain is required for Met ubiquitination, we performed *in vitro* binding assays with the N-terminal portion of c-Cbl containing the TKB domain (GST-Cbl-N, Fig. 2A). In contrast to GST-Cbl-C, the GST-Cbl-N fusion protein associated with the wild type Met receptor and all Met receptor mutants, but failed to associate with the Tpr-Met oncoprotein (Fig. 2B). The Tpr-Met oncoprotein is derived from the Met receptor following a chromosomal translocation. Tpr-Met contains only a portion of the Met cytoplasmic domain (amino acids 1010-1390) fused to a protein dimerization motif (Tpr), leading to the exclusion from Tpr-Met of the extracellular, transmembrane and juxtamembrane regions of the Met receptor (30).

Recruitment of the c-Cbl TKB domain requires Y1003 in Met

The above results suggest that the c-Cbl TKB domain binds to a tyrosine residue that is present in the Met receptor, but not in the Tpr-Met oncoprotein. Two tyrosine residues present in the Met receptor juxtamembrane domain are not retained in the Tpr-Met oncoprotein (Y969 and Y1003) (31). Previous studies have demonstrated that the replacement of tyrosine 1003 by a phenylalanine residue leads to a partial gain-of-function of the Met receptor (32). To establish if this site is required for association with the c-Cbl TKB domain, we performed *in vitro* association and coimmunoprecipitation assays. When tyrosine 1003 has been replaced by a phenylalanine, a c-Cbl-N protein that contains the TKB domain is unable to interact with the Met receptor in *in vitro* association assays or following coimmunoprecipitation, (Fig. 2C and 2D). Furthermore, the ability of Cbl-N to coimmunoprecipitate with Met is dependent on a functional TKB domain, since a G306E Cbl-N mutant protein fails to coimmunoprecipitate with the Met receptor. In contrast, the presence of a Y1003F substitution in the Met receptor, or loss of 1003 in Tpr-Met, does not interfere with its ability to associate with the GST-Cbl-C fusion protein (Fig. 2B and 2C). These results demonstrate that Y1003 within the juxtamembrane of the Met receptor is critical for the recruitment of the c-Cbl TKB domain to the Met receptor, and indicate that two molecular

interactions take place between Met and c-Cbl. One interaction involves a Grb2-binding site in Met (Y1356), a C-terminal proline domain in c-Cbl, and Grb2 as an intermediate; the second interaction involves a juxtamembrane tyrosine (Y1003) in Met and the TKB domain in c-Cbl.

To establish if Y1003 represents a direct binding site for the c-Cbl TKB domain we tested the ability of phosphopeptides containing pY1003 to compete the interaction between the c-Cbl TKB domain (Fig. 2E). A peptide containing a tyrosine to phenylalanine substitution at Y1003 (Y1003F) in addition to phosphopeptide that efficiently competes Grb2 binding to Met Y1356 (pY1356) were used as controls. Whereas the pY1356 or Y1003F peptides failed to compete the ability of the c-Cbl TKB domain fusion protein to coimmunoprecipitate with wild type Met, the phosphopeptide corresponding to pY1003 showed efficient competition at 25uM (Fig. 2E). To confirm that the c-Cbl TKB domain interacts directly with Y1003 in Met as suggested from the peptide competition, a Far-Western assay was performed on wild type and Y1003F Met mutant proteins using a GST fusion protein containing the TKB domain of c-Cbl (GST-Cbl-N). Probing with anti-GST antibody revealed direct binding of the GST-Cbl-N fusion protein to wild type Met and no binding to the Y1003F Met mutant, although an additional GST-Cbl-N binding protein is observed, it runs with higher mobility than Met, and is at this moment unknown. Together, these data indicate that the c-Cbl TKB domain interacts directly with pY1003 in Met (Fig. 2F).

Ubiquitination of the Met receptor requires Y1003

c-Cbl dependent ubiquitination of the Met receptor requires an intact c-Cbl TKB domain (Fig. 1). Conversely, we tested whether the c-Cbl TKB binding site on Met (Y1003) is required for Met ubiquitination. When coexpressed with c-Cbl, the Y1003F Met receptor mutant and Tpr-Met oncogene fail to become ubiquitinated, demonstrating that the c-Cbl TKB binding site on Met is essential for Met receptor ubiquitination (Fig. 3). Met receptor mutants that are unable to associate with Grb2 (Met Y1356F, Y1349/1356F and N1358H) are ubiquitinated, although at reduced levels when compared with the wild type receptor (Fig. 3), indicating that the Grb2-dependent recruitment of c-Cbl is not essential for ubiquitination of Met. However, unlike the endogenous c-Cbl (Fig. 2B), when

overexpressed, c-Cbl is phosphorylated by Met receptors that fails to directly recruit Grb2.

A Y1003F substitution promotes oncogenic activation of the Met receptor

Ubiquitination of RTKs correlates with receptor downmodulation. Hence, we hypothesized that the uncoupling of the c-Cbl TKB domain from the Tpr-Met oncoprotein, and RTKs in general, may provide a mechanism through which receptor activity is no longer tightly regulated, leading to its oncogenic activation. To address this possibility, we first established whether the CSF-Met Y1003F receptor mutant can transform Rat1 fibroblasts. Whereas the wild type CSF-Met receptor failed to form foci in the absence of ligand, the CSF-Met Y1003F mutant formed foci of transformed Rat1 cells in the absence of ligand (Fig. 4A). As shown previously, when grown in the presence of ligand, cells transfected with the wild type CSF-Met receptor formed foci (33), although these were generally smaller than those induced by the Y1003F CSF-Met receptor mutant (Fig. 4A). Similarly the expression of a Y1003F Met receptor mutant induced morphological transformation of NIH3T3 cells in the absence of ligand whereas the wild type receptor failed to do so (Fig. 4B). To further determine if the uncoupling of c-Cbl from the Met receptor leads to its deregulation, we show that the expression of the Y1003F CSF-Met mutant, but not the wild type receptor, in MDCK epithelial cells, promotes epithelial mesenchymal transition and cell dispersal in the absence of ligand (Fig. 4C). Hence, the uncoupling of the c-Cbl TKB domain from the Y1003F Met receptor contributes to the oncogenic activation of the receptor in both fibroblast and epithelial cells.

The overexpression of c-Cbl enhances down-regulation of the PDGFR and EGFR, whereas EGFR mutants lacking a c-Cbl TKB binding domain show delayed receptor degradation. Moreover the overexpression of the c-Cbl TKB domain (Cbl-N) resulted in enhanced phosphorylation of the EGFR or PDGFR possibly through competition with endogenous c-Cbl for association with these RTKs (6-8). To understand the consequence of uncoupling c-Cbl from the Met receptor, we examined the stability of wild type and Y1003F CSF-Met receptor proteins in fibroblast cell lines. Similar steady state levels of wt and Y1003F Met proteins are expressed in fibroblast cell lines (Fig. 5C). However, following pulse-chase analysis in the presence of serum, the Y1003F mutant shows an extended

half-life (1.2h) when compared to the wild type Met receptor (0.7h) (Fig. 5A and 5B), suggesting that the uncoupling of c-Cbl prolongs the half-life of the receptor. To further determine if the uncoupling of c-Cbl from the Met receptor leads to its deregulation, we examined phosphotyrosine levels of wt and Y1003F Met receptors. Following serum starvation, both Met wild type and Met Y1003F proteins show similar basal levels of phosphotyrosine, whereas following stimulation, the Y1003F Met mutant consistently showed elevated and prolonged phosphotyrosine levels, up to 6h when compared to a wild type Met protein (Fig. 5C). Collectively these data support a role for c-Cbl in the downmodulation of the Met receptor.

Figure 1. c-Cbl promotes the ubiquitination of the Met receptor.
293T cells were transiently transfected with expression plasmids encoding CSF-Met, alone or with the indicated Cbl construct. Cell lysates (500 μg) were subjected to immunoprecipitation (IP) with Met antibodies followed by immunoblotting with anti-ubiquitin antibodies. The nitrocellulose membrane was stripped and reprobed with anti-Met. Whole cells lysates (25 μg) were subjected to immunoblotting with HA antibodies.

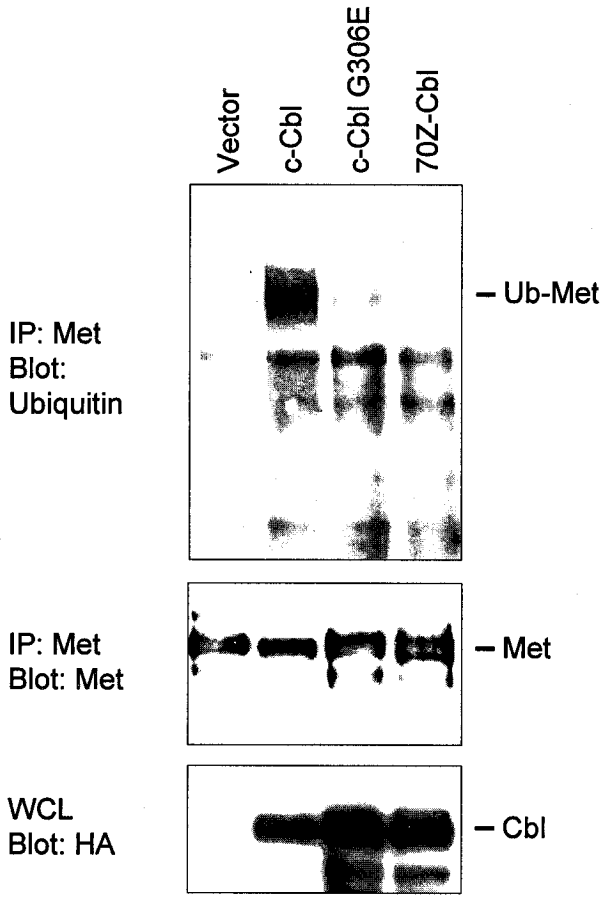
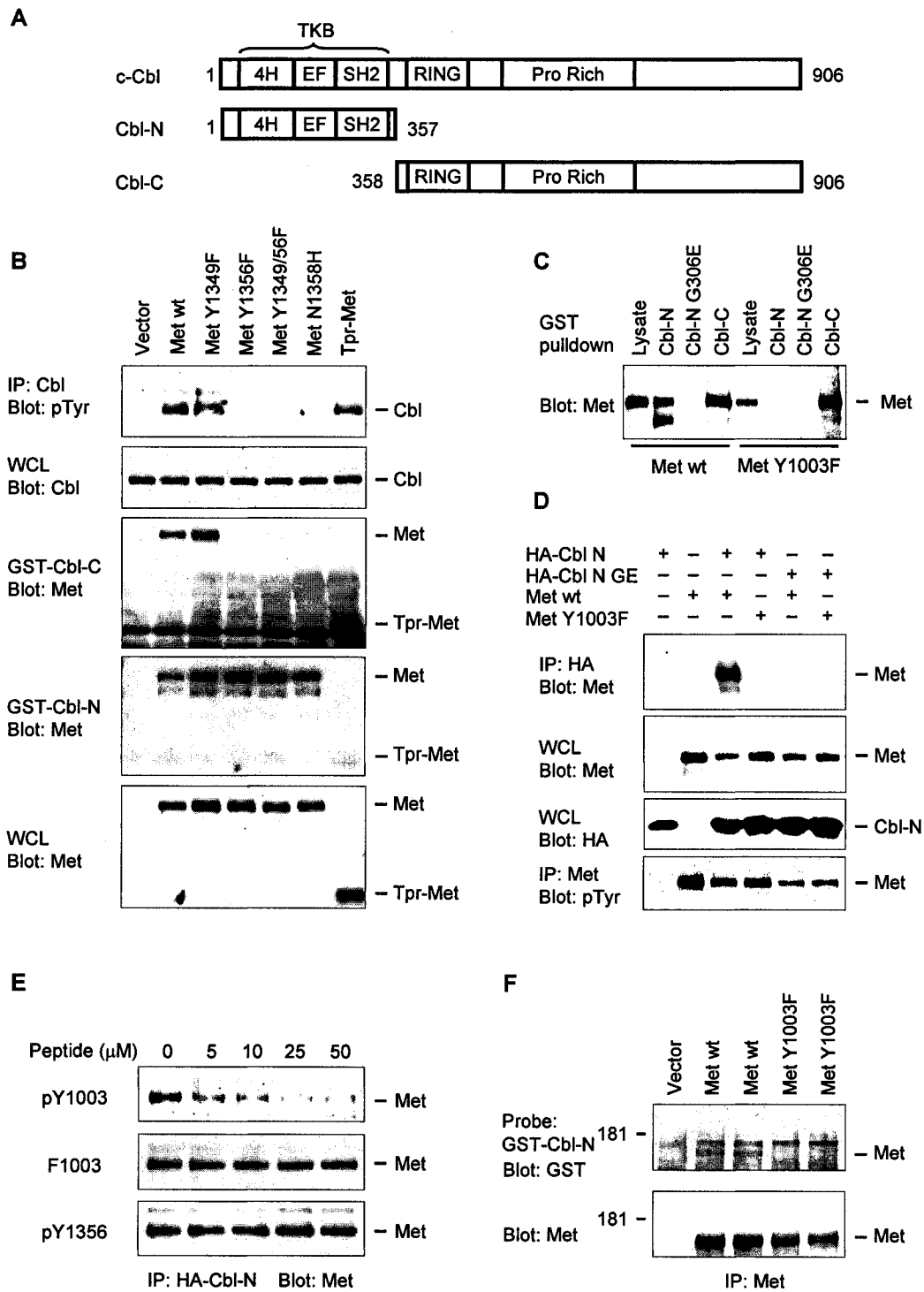


Figure 2. Identification of the Cbl TKB domain binding site in Met.
(A) Structure of the c-Cbl, Cbl-N and Cbl-C proteins. **(B)** 293T cells were transfected with expression plasmids encoding CSF-Met wild type, mutants or Tpr-Met. Proteins from serum-starved cells were immunoprecipitated with anti-c-Cbl and immunoblotted with anti-pTyr. In parallel, cell lysates were subjected to *in vitro* binding assays with GST-Cbl-N and GST-Cbl-C fusion proteins followed by immunoblotting with anti-Met. Whole cell lysates were subjected to immunoblotting with anti-Met. **(C)** Lysates (500 μg) of 293T cells transfected with Met wt or Y1003F, were subjected to *in vitro* binding assays as described in (B) and immunoblotted with anti-Met. **(D)** Proteins from 293T cells, transfected with indicated plasmids were immunoprecipitated with anti-HA and immunoblotted with anti-Met. The same lysates were immunoprecipitated with anti-Met and immunoblotted with anti-pTyr. Whole cell lysates were immunoblotted with Met and HA antibodies. **(E)** Competition of HA-Cbl-N from coimmunoprecipitation with wild type CSF-Met protein using peptides corresponding to the sequence surrounding Y1003 with either a phosphotyrosine residue (pY1003) or a phenylalanine residue (F1003) at position 1003 or using a phosphopeptide surrounding the tyrosine 1356 (pY1356). The immunoprecipitations were performed as described in (D). **(F)** CSF-Met was immunoprecipitated from transfected 293T cells transfected with either vector, CSF-Met wt or CSF-Met Y1003F. The proteins immunoprecipitated were resolved by SDS-PAGE and transferred on a nitrocellulose membrane. The membrane was immunoblotted with purified GST-Cbl-N proteins, then stripped and probed with Met antibody.

Figure 2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358
RING
Pro Rich
906
B
Vector
Met wt
Met Y1349F
Met Y1356F
Met Y1349/56F
Met N1358H
Tpr-Met
IP: Cbl
Blot: pTyr
Cbl
WCL
Blot: Cbl
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Met Y1003F
Met Y1003F
Probe:
GST-Cbl-N
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Met
Blot: Met
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Met
IP: Met

Figure 3. The Cbl TKB domain binding site in Met is required for c-Cbl induced ubiquitination of Met.

Lysates from 293T cells cotransfected with expression plasmids encoding c-Cbl, CSF-Met or Tpr-Met were subjected to immunoprecipitation with either anti-Met antibodies and immunoblotted with anti-ubiquitin or anti-HA-Cbl and immunoblotted with anti-pTyr. Whole cell lysates (25 μg) were immunoblotted with anti-Met and anti-HA.

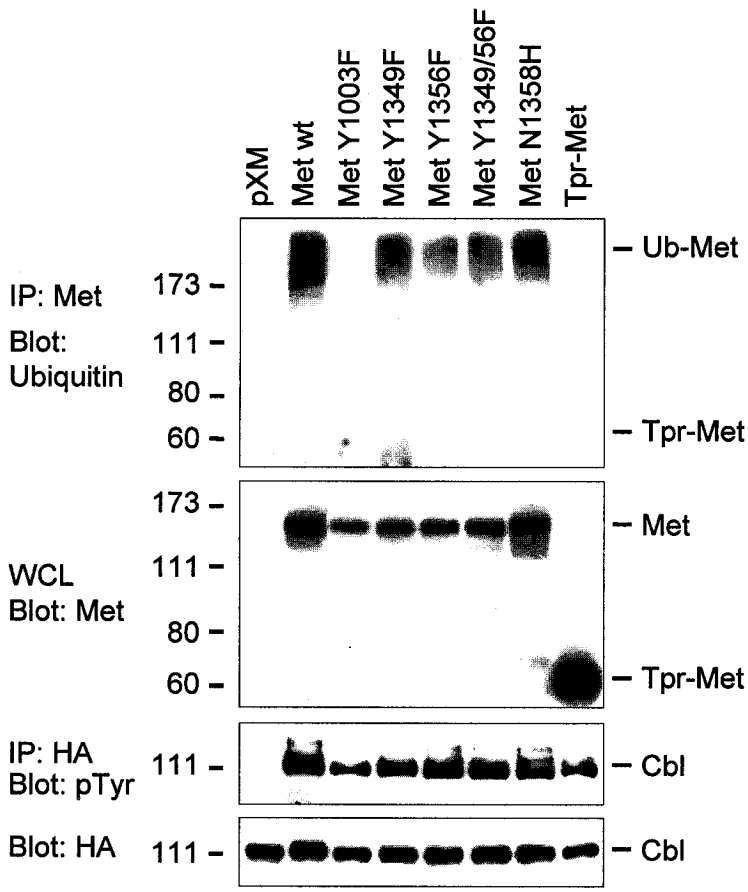
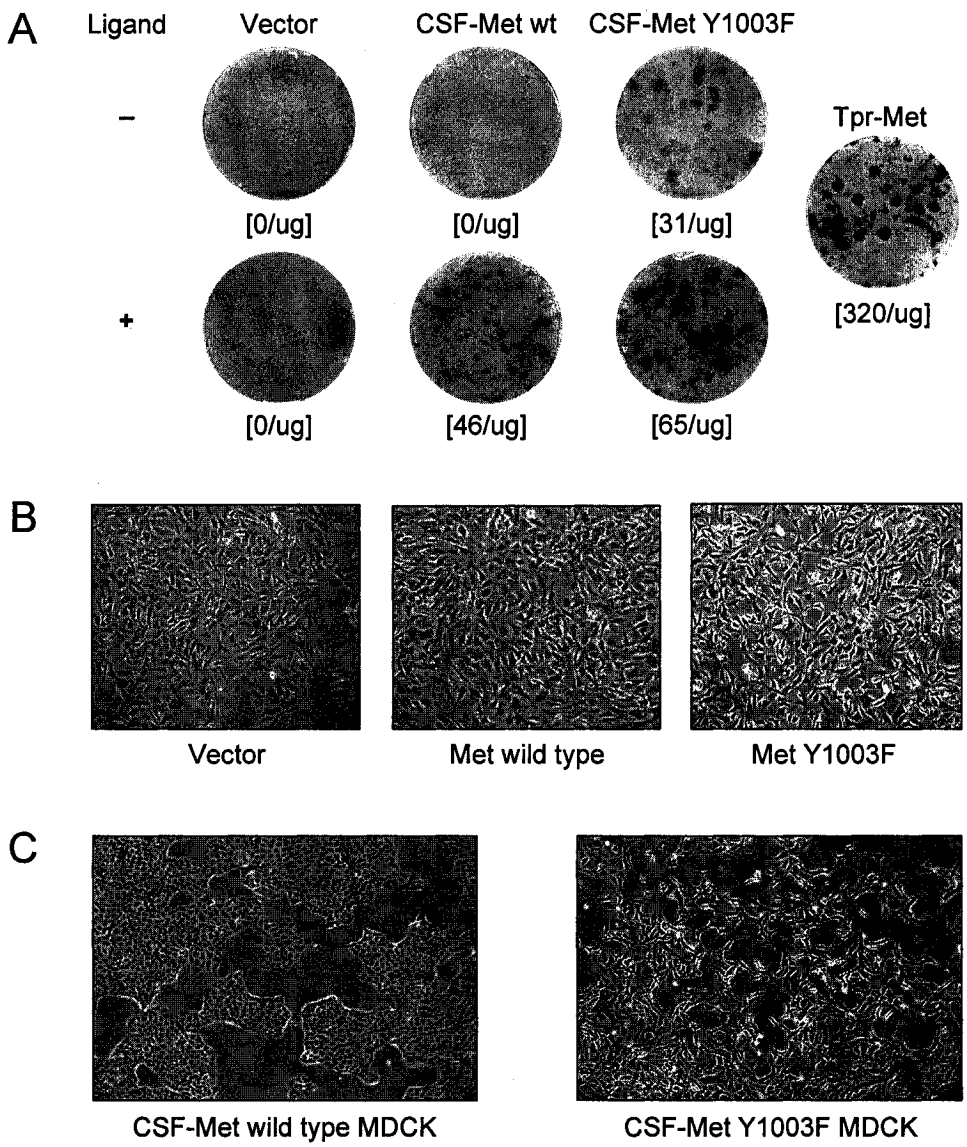


Figure 4. Tyrosine 1003 to phenylalanine substitution in Met promotes its oncogenic activation.

(A) Rat1 fibroblasts were transfected with either vector alone, CSF-Met wt or Y1003F (2 μg DNA/60 mm petri dish) or Tpr-Met (500 ηg DNA/60 mm petri dish) and grown in DMEM containing 10% FBS for two weeks in the presence or absence of ligand (10 ηg/ml CSF-1) as indicated. The numbers indicating the transforming frequency are indicative of 4 different experiments in which five different clones of CSF-Met Y1003F were tested. **(B)** NIH 3T3 mouse fibroblasts were infected with retroviruses encoding either Met wt or Met Y1003F. The cells were maintained for two weeks in 5% FCS under G418 selection. For each infection, 10 clones were picked, grown and pooled. Phase contrast images were taken three days after cells have been seeded. **(C)** Phase contrast images of stable lines of MDCK cells expressing either CSF-Met wt or Y1003F mutant.

Figure 4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Ligand
Vector
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[0/ug]
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[65/ug]
B
Vector
Met wild type
Met Y1003F
C
CSF-Met wild type MDCK
CSF-Met Y1003F MDCK

Figure 5. A Met receptor mutated at tyrosine 1003 is impaired in receptor down-regulation.

(A) Metabolic labelling of stable fibroblast cell lines expressing either wild type Met or the Y1003F mutant Met. Stable cell lines were pulse-labeled with 35S-methionine for 1hr. At indicated times, cell lysates were immunoprecipitated with anti Met antibodies, resolved by 8% SDS-PAGE and subjected to autoradiography. **(B)** Graphic representation of duplicate experiments. **(C)** Stable cell lines as in A were stimulated with ligand (CSF-1 500 ng/ml) for indicated times. Proteins were immunoprecipitated with anti-Met sera, separated by SDS-10% PAGE and transferred to membrane. Membranes were immunoblotted with phosphotyrosine antibody, stripped and reprobed with Met antibody.

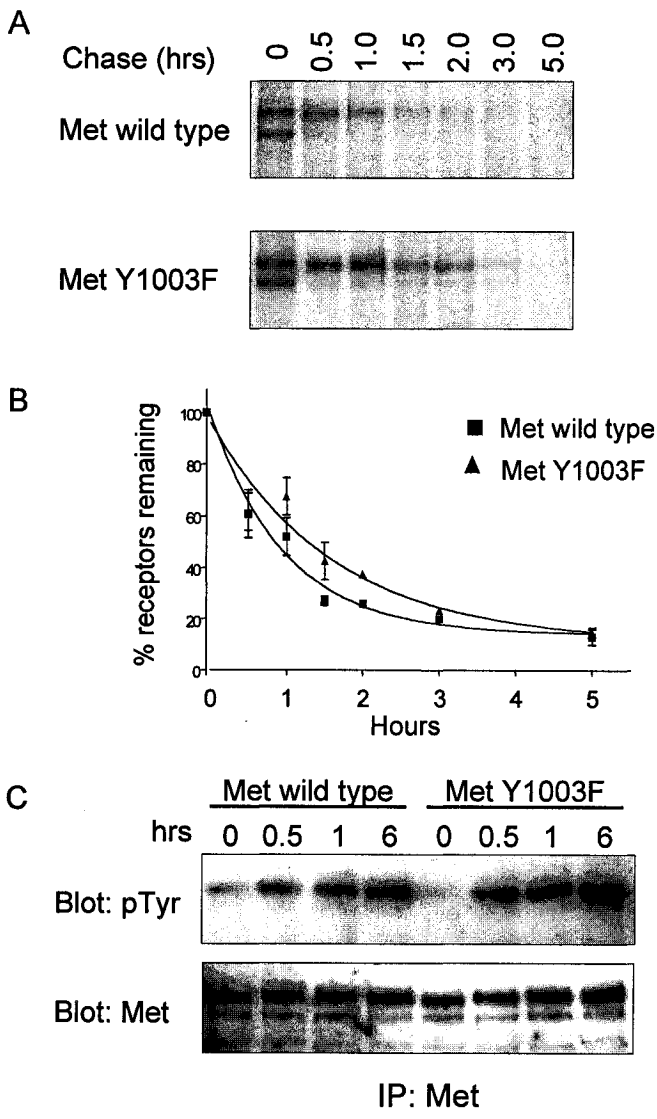
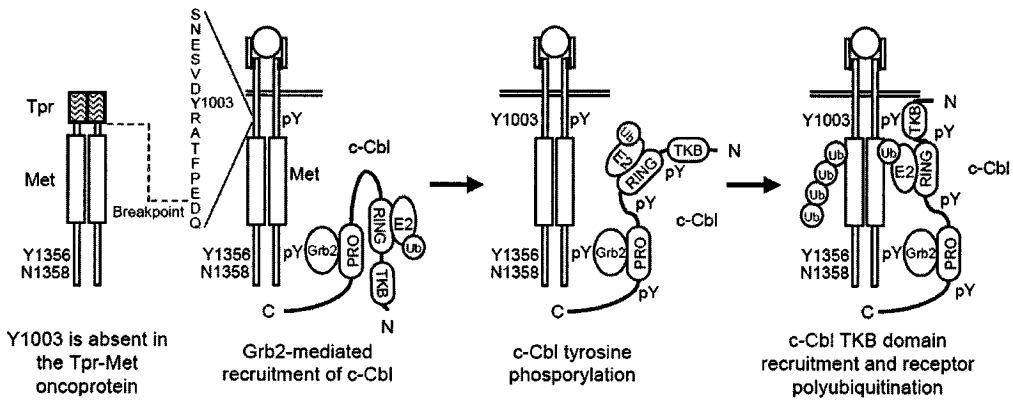


Figure 6. Model for the recruitment of c-Cbl to the Met receptor.



Discussion

The discovery that Cbl proteins function as ubiquitin-dependent protein ligases has supported their role as negative regulators of RTKs. Where examined, polyubiquitination of RTKs requires a functional c-Cbl TKB domain, which interacts with receptor targets, in addition to the RING finger domain that acts to recruit the ubiquitin-conjugating enzymes. Several oncogenic variants of c-Cbl lack ubiquitin ligase activity, but require a functional TKB domain for transforming activity (20, 34). From these results, it was reasoned that these oncogenic Cbl proteins compete with endogenous Cbl proteins, preventing the downmodulation of RTKs or other tyrosine kinases that recruit c-Cbl. However, since c-Cbl interacts with many cellular proteins and can elicit both negative and positive signals, the precise mechanism for cell transformation is unclear.

In this study, we have directly tested the possibility that uncoupling c-Cbl from a RTK would contribute to receptor deregulation and cell transformation. Since the deregulation of the Met receptor has been associated with its oncogenic activation (21), we analysed the consequence of uncoupling c-Cbl from the Met receptor. We have shown that c-Cbl overexpression promotes ubiquitination of the Met receptor (Fig. 1). This requires a functional TKB domain in c-Cbl and a juxtamembrane tyrosine (1003) in the Met receptor (Fig. 1 and 3). The substitution of Y1003 with phenylalanine abrogates the recruitment of the c-Cbl TKB domain to the Met receptor and receptor ubiquitination (Fig. 2 and 3), converting the receptor into a transforming protein in the absence of ligand (Fig. 4).

The c-Cbl protein is recruited to the Met receptor by two distinct mechanisms (Fig. 6). Tyrosine 1003 on the Met receptor is required for the recruitment of the c-Cbl TKB domain (Fig. 3), whereas a functional Grb2 binding site at Y1356 (YVNV) is required for the recruitment of the c-Cbl C-terminal proline-rich domain and for tyrosine phosphorylation of the endogenous c-Cbl protein (Fig. 2). Similarly, c-Cbl is recruited in a Grb2 and TKB dependent manner to distinct tyrosine residues on the EGFR (20). However, Grb2-dependent association of c-Cbl in the absence of Y1003 is not sufficient for ubiquitination of the Met receptor (Fig. 3), identifying Y1003 as a critical residue for efficient transfer of ubiquitin from the ubiquitin-conjugating enzyme to the Met substrate (Fig. 6). The finding that the segment between the c-Cbl TKB and RING domains must be modified by

tyrosine phosphorylation, suggests that a conformational change may be required to activate the E3 c-Cbl associated complex (10). Such a conformational change may also require the engagement of the c-Cbl TKB domain with a phosphotyrosine residue, consistent with the requirement for the presence of pY1003 for Met receptor ubiquitination.

The c-Cbl TKB binding site in the EGFR, ErbB-2, Syk and ZAP-70 proteins correlates with the (N/D)XpYXXXØ consensus sequence determined from a phosphopeptide library screen using the c-Cbl TKB domain, and from the crystal structure of the c-Cbl TKB domain (12, 35). The Met TKB binding site (Y1003) contains a hydrophobic amino acid residue at position pY+4 (VDpYRATF) (Fig. 6). Although Y1003 in Met does not conform to the consensus pYXXXP required for c-Cbl TKB binding observed in the EGFR, pY1003 in Met is bound directly by the c-Cbl TKB domain as shown by Far-Western analysis (Fig. 2F). Moreover a peptide containing pY1003 almost completely abolished the binding of the c-Cbl TKB domain with Met (Fig. 2E). This provides evidence that pY1003 in Met provides a direct binding site for the c-Cbl TKB domain.

Cbl family members share high homology within their TKB domain and studies of murine c-Cbl and Cbl-b knockout animals suggest that Cbl proteins are at least in part functionally redundant (2). The loss of a c-Cbl TKB binding site from a RTK would therefore be expected to provide a more direct measure of the regulation that Cbl family proteins impart on that receptor. In support of this, the uncoupling of the c-Cbl TKB domain from the Met receptor generates a receptor that promotes transformation of fibroblast cells and dispersal of epithelial cells in the absence of ligand (Fig. 4). These observations reflect the biological activity of the wild type Met receptor in the continuous presence of ligand (Fig. 4). These data are consistent with a previous study that identified Y1003 as a negative regulatory site for the Met receptor and provide a mechanism for this observation (32).

c-Cbl dependent polyubiquitination of RTKs can target them for degradation rather than recycling (7, 16). Hence, loss of c-Cbl recruitment may allow the accumulation of a pool of basally activated Met Y1003F receptors that would normally be modulated by c-Cbl. The Y1003F mutant Met protein is expressed at similar levels to the wild type protein and under serum starved conditions is phosphorylated at a similar basal level to that of the wild type receptor, indicating

that phosphorylated Y1003F mutant Met receptors do not accumulate to high levels (Fig. 5A and 5C). However, in the stable cell lines, the half-life of the Y1003F mutant is increased two fold over that of the wild type receptor (Fig. 5B) consistent with the increased stability of EGFR that lacks a c-Cbl TKB domain binding site (10). Hence, basally activated Y1003F mutant Met receptors may elicit a signal for a prolonged period when compared to wild type Met, and that this level of deregulation is sufficient for the weak transforming activity of this mutant observed in fibroblasts and epithelial cells. Moreover, following stimulation of stable cell lines, the Y1003F Met protein is phosphorylated at levels elevated to that of the wild type protein (Fig. 5C), supporting a role for c-Cbl in turnover of the Met receptor. In a similar manner to the Met Y1003F mutant, the overexpression of Cbl-N or 70Z-Cbl proteins in epithelial cells promotes epithelial cell dispersal (26). This requires a functional TKB domain and is consistent with the competition of Cbl-N or 70Z-Cbl with the endogenous c-Cbl protein, resulting in the deregulation of RTKs that regulate epithelial cell dispersal, such as the Met receptor. Together, these data implicate c-Cbl as an important negative regulator for the Met receptor. This has important consequences *in vivo*, since all epithelial cells express the Met receptor and most stromal tissues express its ligand, HGF.

Notably, the oncogenic variant of the Met receptor, Tpr-Met, lacks the juxtamembrane domain of Met including Y1003. Although we have shown that the oncogenic activation of Tpr-Met requires a leucine zipper within Tpr that promotes dimerization and robust activation of the Met kinase in the absence of ligand (30), from our present studies, the loss of the c-Cbl TKB binding domain, and lack of ubiquitination of Tpr-Met would be expected to contribute to its deregulation. This is supported from studies demonstrating that the addition of the Met juxtamembrane domain containing Y1003 to Tpr-Met decreased its transforming potential (36). This domain has been described to contain several negative regulatory sites, including a Protein Kinase C phosphorylation site (S985) (37) and a binding site for a protein tyrosine phosphatase (PTP-S) (Y1003) (38). However, the Y1003F substitution had no effect on binding of PTP-S (38) and it is therefore unlikely that uncoupling of PTP-S contributes to the oncogenic activation of the Met receptor that we observe. Similarly, the deletion of 25 amino acids that contains the c-Cbl TKB binding site in the EGFR (10) increases the transforming activity of an EGFR derived oncoprotein, v-erbB (39).

These observations suggest that loss of a Cbl TKB binding site may be a common mechanism that contributes to full oncogenic activation of RTKs. Tyrosine 1003 is conserved in the chicken c-Sea protein, which is a member of the Met RTK gene family. Notably, the predicted Cbl binding site is deleted in *env-sea*, the retrovirally transduced oncogenic variant of c-Sea (40, 41). Likewise, a putative Cbl binding site is deleted in v-fms, the retrovirally transduced oncogenic variant of the CSF-1 receptor. Moreover, several TrkC oncogenic variants generated in human tumours, following chromosomal rearrangements in acute myeloid leukemia and congenital fibrosarcoma, delete a putative c-Cbl TKB domain binding site (DTpYVQHI) (42, 43). Hence, the loss of negative control exerted through Cbl proteins, through chromosomal rearrangements or mutations that delete Cbl binding sites, may be an important contribution to the deregulation of Met and other RTKs observed in cancers. Moreover, the observation that c-Cbl can exert positive signals downstream from RTKs and integrins would have additional implications for a role for Cbl in tumour progression downstream from oncogenic RTKs that become uncoupled from Cbl-dependent polyubiquitination.

Experimental Procedures

Cell culture, DNA transfections and transformation assays

Human embryonic kidney 293T cells were transfected using the calcium phosphate method. Stable Madin-Darby canine kidney (MDCK) cell lines expressing wild type and Y1003F CSF-Met receptors were generated by retroviral infection as described previously (28, 29). Transformation assays in Rat1 fibroblasts were performed as described in (30). NIH 3T3 mouse fibroblast (ATCC) cells were cultured in DMEM containing 10% Fetal Calf Serum (FCS). Cells were infected with a retrovirus expressing either Met wt or Y1003F and maintained as described in (Jeffers et. al. 1997).

Antibodies and reagents

Antibodies raised in rabbit against a C-terminal peptide of human Met were used (44). HA antibody was purchased from BABCO (Richmond, CA), anti-ubiquitin (NCL-UBIQ) from Novocastra Laboratories Ltd (Newcastle, UK), ant-pTyr (4G10) from Upstate Biotechnology (Lake Placid, NY) and anti-c-Cbl and anti-GST (sc-138) from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Recombinant CSF-1 was provided by Genetics Institute (Boston, MA).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Tyrosine 1003 was converted to Phe using the 5'-GAATCTGTAGACTTCGAGCTACTTTTCCAG-3' primer and its complementary primer.

In vitro binding Assays

Bacteria expressing the amino-terminal portion of c-Cbl (amino acids 1-357) fused to GST (GST-Cbl-N), the mutant G306E Cbl-N (GST-Cbl-N G306E) as well as the carboxy-terminal portion of c-Cbl (amino acids 358-906) fused to GST (GST-Cbl-C) were provided by Dr. Hamid Band (45). Coupling of GST fusion proteins to Glutathione-Sepharose beads (Amersham Pharmacia Biotech (A.P.), Baie d'Urfe, Qc) was performed at room temperature for 1 hr. Complexes were

washed twice with TGH lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol) and then incubated with cell lysate overnight at 4°C, washed four times with TGH lysis buffer and resuspended in Laemmli sample buffer.

Immunoprecipitations, Western Blotting and Peptide competition

293T cells were serum-starved in 0.1% FBS overnight and harvested in TGH lysis buffer. Lysates were incubated with the indicated antibody overnight at 4°C with gentle rotation. Proteins collected on either protein A- or protein G-Sepharose were washed three times in TGH lysis buffer, resolved by SDS-PAGE and transferred to a nitrocellulose membrane as described (26). Proteins were visualized with an ECL detection kit (A.P.). For peptide competition assays, the following peptides were added to cell lysates at the indicated concentration 20 min prior to immunoprecipitation with the HA antibody: pY1003 (MVSNESVDpYRATFPEDQF), F1003 (MVSNESVDFRATFPEDQF) and pY1356 (IFSTFIGEHpYVHVNATpYVNVKCV). The peptides were synthesized at the Sheldon Biotechnology Center (Montreal, Qc).

Far-Western Blotting

For Far-Western analysis, the CSF-Met receptor was immunoprecipitated from 3 mg of 293T whole cell lysates, resolved on a 8% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked 1 hr in 20 mM Tris pH 7.5, 150 mM NaCl and 1 mM Na_3VO_4 containing 5% non-fat milk and 0,1% Tween-20, and then probed overnight at 4°C with 50 ug/ml of purified GST-Cbl-N proteins in blocking solution. After four washes of 5 min each, bound proteins were detected with anti-GST antibodies (Thien and Langdon 1997).

Pulse-chase Analysis

Rat1 cell lines derived from G418 selection and focus assays were seeded at $8x10^5$ cells/100 mm dishes in DMEM containing 10% FBS. The next day, cells were rinsed twice in labelling medium (DMEM without Methionine and Cystine (ICN Biomedicals Inc., Ohio)), then incubated 3 hrs in 2 ml labelling medium containing 2% FBS. The cells were pulsed 1 hr following the addition of 400 μCi of ^{35}S-labelled Methionine and Cystine (Easytag™, PerkinElmer, MA) per plate.

The cells were then washed once with DMEM and chased in DMEM containing 10% FBS and 3 mg/ml Methionine. The lysates were harvested at the indicated times in TGH lysis buffer, pre-cleared 1 hr with protein-A sepharose and incubated with the Met antibody for 2 hrs. The complexes were resolved by SDS-PAGE. The gels were first fixed for 30 min in 50% methanol, 10% acetic acid, then incubated in Amplify™ NAMP100 (A.P.) for 30 min and dried under vacuum at 80°C for 1 hr. The data were analysed with the ImageQuant (Molecular Dynamics) and Prism softwares.

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Chapter 3

A Conserved DpYR Motif in the juxtamembrane domain of the Met Receptor Family Forms an Atypical c-Cbl/Cbl-b TKB Domain Binding Site Required for Suppression of Oncogenic Activation

Preface

The Cbl tyrosine kinase binding (TKB) domain contains a variant SH2 domain and is able to bind to phosphotyrosine residues. The Cbl TKB domain interacts with phosphotyrosine residues located in protein tyrosine kinases (PTK) such as ZAP-70, Syk and Src, and in RTKs like EGFR and CSF-1 receptor. The crystal structure of the c-Cbl TKB domain complexed to its binding site on ZAP-70 kinase as well as a degenerate phosphopeptide library screen revealed that the c-Cbl TKB domain has a preference for the (D/N)XpYXX(D/E)Φ consensus sequence. This sequence is found not only in the ZAP-70 kinase, but also in other c-Cbl binding proteins including the Syk kinase, the EGFR and Sprouty adaptor proteins.

In chapter 2, I demonstrated that the Cbl TKB domain binds to the Y1003 residue located in the juxtamembrane domain of the Met receptor. I also established that substitution of Y1003 for phenylalanine in the Met receptor prevents Cbl TKB binding and ubiquitination of the Met receptor, and leads to oncogenic activation of the Met receptor. Hence, the interaction between the Cbl TKB domain and the Y1003 residue in Met is required for Cbl-mediated Met ubiquitination and down-regulation. Importantly, I also established by far western analysis that the c-Cbl TKB domain interacts directly with Y1003 in the Met receptor. Intriguingly, the amino acid sequence surrounding Y1003, VDpYRATF, does not correspond to the (D/N)XpYXX(D/E)Φ consensus sequence found in other Cbl TKB domain interacting proteins. Therefore, it is important to determine the molecular requirements for the interaction between the Cbl TKB domain and Y1003 in Met. I examined this by performing alanine-scanning mutagenesis of the sequence surrounding Y1003. The results are presented in this chapter.

Abstract

Activation and phosphorylation of Met, the receptor tyrosine kinase (RTK) for hepatocyte growth factor, initiates the recruitment of multiple signalling proteins, one of which is c-Cbl, a ubiquitin-protein ligase. c-Cbl promotes ubiquitination and enhances downmodulation of the Met receptor and other RTKs, targeting them for lysosomal sorting and subsequent degradation. Ubiquitination of Met by c-Cbl requires the direct interaction of the c-Cbl TKB domain with tyrosine 1003 in the Met juxtamembrane domain. Although a consensus for c-Cbl TKB domain binding has been established (D/N)XpYXX(D/E)Φ, this motif is not present in Met, suggesting that other c-Cbl TKB domain binding motifs may exist. By alanine-scanning mutagenesis, we have identified a DpYR motif, including Y1003, as being important for the direct recruitment of the c-Cbl TKB domain and for ubiquitination of the Met receptor. Substitution of Y1003 with phenylalanine, or substitution of either aspartate or arginine residues with alanine, impairs c-Cbl-recruitment and ubiquitination of Met, and results in the oncogenic activation of the Met receptor. We demonstrate that the TKB domain of Cbl-b, but not Cbl-3, binds to the Met receptor and requires an intact DpYR motif. Modeling studies suggest the presence of a salt bridge between the aspartate (D) and arginine (R) residues that would position pY1003 for binding to the c-Cbl TKB domain. The DpYR motif is conserved in other members of the Met RTK family, but is not present in previously identified c-Cbl binding proteins, identifying DpYR as a new binding motif for c-Cbl and Cbl-b.

Abbreviations

4H	4 helix bundle
Cbl	Casitas B-lineage lymphoma
CSF	colony stimulating factor
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
GST	glutathione S-transferase
HGF	hepatocyte growth factor
MDCK	Madin-Darby Canine Kidney
PTB	phosphotyrosine binding
PTK	protein tyrosine kinase
RTK	receptor tyrosine kinase
SH2	Src-homology 2
TKB	tyrosine kinase binding
VEGFR-1	vascular endothelial growth factor receptor-1
WT	wild type

Introduction

The Cbl family of proteins has been recently identified as ubiquitin-protein ligases that are involved in the ubiquitination and subsequent down-regulation of receptor tyrosine kinases (RTKs) (1, 2). There are three mammalian Cbl proteins: c-Cbl, Cbl-b and Cbl-3. The c-Cbl and Cbl-b genes are ubiquitously expressed, with the highest levels of expression in haematopoietic tissues (3, 4). In contrast, Cbl-3 mRNA is mainly expressed in organs rich in epithelium such as pancreas, liver, small intestine, colon and placenta, and is expressed at low levels in haematopoietic tissues (5, 6). Despite the lower levels of c-Cbl and Cbl-b proteins in epithelial tissues, the c-Cbl deficient mice, in addition to having haematopoietic defects, develop mammary hyperplasia (7), signifying that c-Cbl is important for growth regulation of mammary epithelia.

The conserved amino-terminal domain of Cbl proteins is composed of a tyrosine kinase binding (TKB) domain and a RING finger domain, which are both required for ubiquitin-protein ligase activity. The TKB domain mediates the recruitment of Cbl proteins to the tyrosine-phosphorylated substrate while the RING finger domain associates with the ubiquitin-conjugating enzyme (UbcH7) (8). The presence of several binding sites for SH2- and SH3-domain containing proteins within their carboxyl-terminal end confers to c-Cbl and Cbl-b the ability to function as adaptor proteins.

The Cbl TKB domain interacts with phosphotyrosine residues located in protein tyrosine kinases (PTK) such as ZAP-70, Syk and Src, and in RTKs like EGFR, Met and colony-stimulating factor-1 receptor (1, 9, 10). It is composed of a four-helix bundle (4H), an EF-hand calcium-binding domain, and a variant SH2 domain that, together, are able to bind to phosphotyrosine residues (11). The crystal structure of the c-Cbl TKB domain complexed to its binding site on ZAP-70 kinase reveals that a medium-sized hydrophobic residue (Φ) at position pY+4 and an acid residue (D/E) at position pY+3 would constitute the primary specific-determining interactions (11). In addition, an aspartate residue at position pY-2 forms a hydrogen bond. This is in accordance with a degenerate phosphopeptide library screen, using the c-Cbl TKB domain as a bait, which revealed a preference for an aspartate or an asparagine residue at position pY-2 (12). The D/NXpYXXD/EΦ sequence is found not only in the ZAP-70 kinase, but also in

other c-Cbl binding proteins including the Syk kinase, the EGFR and Sprouty adaptor proteins (12-16).

We have shown recently that the hepatocyte growth factor (HGF) receptor, Met, is also negatively regulated by c-Cbl (10). The Met receptor is primarily expressed in epithelial and endothelial cells and its activation leads to loss of cell-cell adhesion and enhances cell migration and proliferation (17). The chronic activation of the Met receptor is associated with the genesis and the progression of multiple types of tumors including carcinomas, melanomas and sarcomas (18). Stimulation of the Met receptor with its ligand, HGF, induces tyrosine phosphorylation and polyubiquitination and ultimately, degradation of the receptor (19-21). We have previously reported that the c-Cbl ubiquitin-protein ligase is recruited to the Met receptor by two distinct mechanisms. The carboxy-terminal region of c-Cbl can be recruited indirectly to Y1356 in the Met receptor via the Grb2 adaptor protein, while the TKB domain of c-Cbl associates directly to the juxtamembrane Y1003 in the Met receptor (10). The latter interaction is required for full ubiquitination of the Met receptor. A Met receptor lacking the c-Cbl TKB domain binding site (Y1003F) has a prolonged half-life and is oncogenic in cell culture, identifying c-Cbl and ubiquitination as important negative regulators for this receptor (10). Here, we have performed alanine-scanning mutagenesis of amino acids surrounding Y1003 and have identified a DpYR motif as being essential for the recruitment of the c-Cbl/Cbl-b TKB domain to the Met receptor as well as receptor ubiquitination and down-regulation. Based on the c-Cbl•ZAP-70 complex crystal structure, we also propose a structural mechanism for the association of the Cbl TKB domain to the DpYR motif in the Met receptor.

Results

Mapping of the c-Cbl TKB domain binding site in Met

By far-western analysis and peptide competition experiments, we have shown that the c-Cbl TKB domain binds directly to the phosphorylated Y1003 in the Met receptor (10). The sequence surrounding Y1003 differs from the identified c-Cbl TKB domain binding site in the ZAP-70 and Syk kinases and EGFR (Fig. 1*A*). To establish the molecular requirements for the association of the c-Cbl TKB domain to Y1003 in the Met receptor, we performed an alanine-scanning mutagenesis of the amino acid residues surrounding Y1003 (N998 to P1008) (Fig. 1*B*). The mutant Met receptors were expressed to similar levels and, with the exception of Met Y1003F, were phosphorylated on Y1003 to similar levels as the wt Met receptor as detected using anti-phosphotyrosine 1003 sera. In addition, mutant receptors were all comparably phosphorylated on the conserved twin tyrosine residues (Y1234-1235) located in the activation loop (Fig. 1*C*). These tyrosines are required for full activation of the Met kinase (22). This indicates that activation of the Met kinase and subsequent tyrosine phosphorylation of Y1003 are not detectably altered in the alanine substitution mutants.

A DpYR motif is required for recruitment of the c-Cbl TKB domain

To define the c-Cbl TKB domain-binding site, the ability of each mutant to coimmunoprecipitate with an HA-tagged c-Cbl TKB domain protein was assessed following transient co-expression. As shown previously (10), the substitution of tyrosine 1003 in the Met receptor with a non-phosphorylatable phenylalanine residue abrogates coimmunoprecipitation of Met with the c-Cbl TKB domain protein (Fig. 2*A*). In addition, the substitution of both D1002 and R1004 with alanine residues significantly reduces the ability of the c-Cbl TKB domain protein to coimmunoprecipitate with these mutant Met receptors (Fig. 2*A*), even if Y1003 is present and phosphorylated to similar levels as the wt Met receptor (Fig. 1*C*). All other Met alanine substitution mutants tested coimmunoprecipitate with the c-Cbl TKB domain protein to levels similar to the wt Met receptor (Fig 2*A*). This indicates that D1002 and R1004 are independently required for coimmunoprecipitation with the c-Cbl TKB domain.

To establish if there is a requirement for residues D and R for the direct binding of the c-Cbl TKB domain, we performed a Far-Western analysis using a

GST-c-Cbl TKB domain fusion protein. Probing with GST-c-Cbl TKB conjugated to glutathione-HRP revealed direct binding of the GST-c-Cbl TKB fusion protein to wt Met receptor and to Met alanine scanning mutants, with the exception of the D1002A, Y1003F and R1004A Met receptor mutants (Fig. 2*B*). This indicates that, in addition to Y1003, the D and R residues are also independently required for the direct association of the Met receptor with the c-Cbl TKB domain and together, these data demonstrate that the DpYR residues of the Met receptor form an alternative-binding motif for the c-Cbl TKB domain.

The DpYR motif is required for the recruitment of the Cbl-b TKB domain

The TKB domain is well conserved throughout the Cbl protein family (2). Therefore, we examined the ability of the Cbl-b and Cbl-3 TKB domains to interact with the Met receptor. We performed *in vitro* binding assays using GST-TKB fusion proteins. When compared with the GST-c-Cbl TKB fusion protein, the GST-Cbl-b TKB fusion protein bound to similar levels to the Met receptor (Fig. 3*A*). Moreover, as observed for the c-Cbl TKB domain, the substitution of the aspartate or the arginine residue impairs the association of the Cbl-b TKB domain with the Met receptor (Fig. 3*C*). We did not detect association of the GST-Cbl-3 TKB domain to the Met receptor under the same conditions (Fig. 3*A*) even though similar levels of GST-fusion proteins were used (Fig. 3B)

An intact DpYR motif is required for Met receptor ubiquitination and suppression of transformation

We have previously demonstrated that the uncoupling of the c-Cbl TKB domain from the Met receptor by the Y1003F mutation strongly impairs ubiquitination of the receptor and converts the receptor into a transforming protein (10). Therefore, we examined whether the D1002A and R1004A mutations, which uncouple the c-Cbl TKB domain from the Met receptor, affect receptor ubiquitination and transforming activity. When co-expressed with c-Cbl, the D1002A and R1004A receptor mutants, like the Met Y1003F receptor mutant, are ubiquitinated to levels significantly less than the wt Met receptor and other alanine-scanning mutant receptors (Fig. 4*A*). The biological activity of the alanine-scanning mutant Met receptors was tested by examining their ability to induce foci of transformed cells on confluent monolayers of Rat-1 fibroblasts.

Consistent with their inability to recruit the c-Cbl TKB domain and their reduced ubiquitination, both the D1002A and R1004A mutants are able to transform Rat-1 fibroblast cells (Fig. 4*B* and 4*C*), although the transforming activity of the D1002 receptor mutant was consistently less than that of the Y1003F or R1004A receptor mutants. In three independent experiments, the wt Met receptor and other alanine-scanning mutant receptors failed to induce foci (Fig. 4*C*). This demonstrates that, in addition to Y1003, the D1002 and R1004 residues are essential to suppress the transforming activity of the Met receptor.

To further examine the role of the DpYR motif in Met receptor regulation, we established Rat-1 fibroblast cell lines expressing the wt Met receptor as well as V1001A, D1002A, Y1003F and R1004A Met receptor mutants. Multiple (2-5) stable cell lines were isolated that express each mutant. The steady-state protein levels of the D1002A, Y1003F and R1004A Met receptor mutants were consistently higher than that of wt Met and Met V1001A receptors and we were unable to generate cell lines with equal protein levels (Fig. 5*A*). Moreover, baseline and ligand-induced phosphorylation of each mutant receptor reflects their protein levels and is elevated in D1002A, Y1003F and R1004A receptor mutants (Fig. 5*A*). Ligand stimulation provokes robust ubiquitination of Met wt and Met V1001A whereas ligand-induced ubiquitination of Met D1002A, Y1003F and R1004A receptors was low or not detectable (Fig. 5*A* and 5*B*). The low levels of ubiquitination in response to stimulation correlate with the higher transforming activity of the Y1003F and R1004A Met receptor mutants when compared to the Met D1002A receptor mutant (Fig. 4 and 5).

A molecular model predicts that D1002 and R1004 form a salt bridge required for projection of pY1003 into the c-Cbl TKB domain binding pocket

To determine how the substitution of either D1002 or R1004 with alanine in the DpYR motif could affect the binding of the Met receptor with c-Cbl, a model of the Met peptide S1000-P1008 bound to the TKB domain of c-Cbl was constructed based on the structure of the c-Cbl•ZAP-70 peptide complex (11) (Fig. 6). The modeled structure of the Met peptide S1000-P1008 predicts that D1002 and R1004 in the DpYR motif of the Met receptor form a salt bridge. This would stabilize the peptide in the conformation most favourable to expose pY1003 towards the phosphotyrosine-binding pocket of the c-Cbl TKB domain.

Furthermore, the main chain carbonyl and amino groups of D1002 and R1004 form hydrogen bonds with the TKB domain of c-Cbl to provide additional support for binding pY1003 to the phosphotyrosine-binding pocket. The specific association of the Met receptor to the TKB domain of c-Cbl could be reinforced by the binding of F1007, positioned at pY+4, to the hydrophobic pocket (Fig. 6).

Figure 1. The c-Cbl TKB binding site in Met is distinct from the identified c-Cbl TKB domain binding site in the ZAP-70, Syk and EGFR tyrosine kinases.
(A) Alignment of the c-Cbl TKB direct binding site identified in human EGFR, ZAP-70, Syk, Sprouty1 and Met proteins. **(B)** Schematic representation of the Met receptor. The c-Cbl TKB direct binding site (Y1003) is located in the juxtamembrane domain of the Met receptor. Amino acids from N998 to P1008 residues have been substituted individually for alanine residues. **(C)** 293T cells were transiently transfected with plasmids expressing CSF-Met wt or mutants. Cell lysates were subjected to immunoprecipitation (IP) with Met antibodies followed by immunoblotting with phosphorylation site-specific antibodies raised against the juxtamembrane Y1003 residue and against the kinase domain Y1234 and Y1235 residues. Nitrocellulose membranes were stripped and reprobed with Met antibodies.

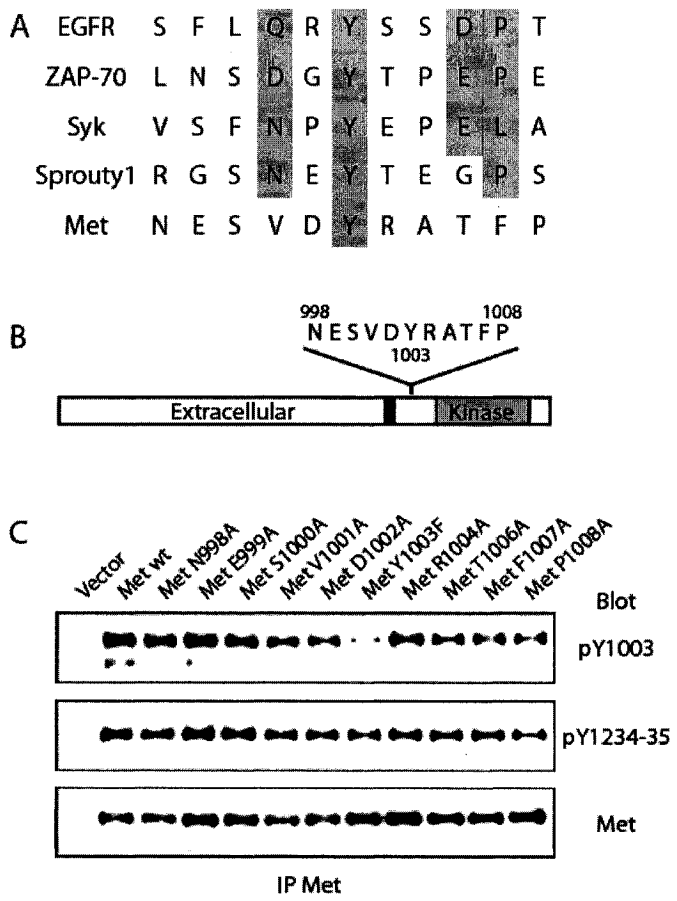


Figure 2. D1002, Y1003 and R1004 residues are required for the direct binding of the c-Cbl TKB domain to the Met receptor.

(A) Protein lysates from 293T cells transiently co-transfected with plasmids expressing HA-Cbl-TKB and CSF-Met wt or mutants were subjected to immunoprecipitation with HA antibodies. Membranes were immunoblotted with Met antibodies, stripped and reprobed with HA antibodies. Whole-cell lysates were immunoblotted with Met antibodies. **(B)** CSF-Met receptors were immunoprecipitated from 293T cells transfected with CSF-Met wt and mutants, resolved by SDS-PAGE and transferred to a nitrocellulose membrane that was then immunoblotted with purified GST-Cbl-TKB proteins conjugated to HRP-glutathione. The membrane was stripped and reprobed with Met antibodies.

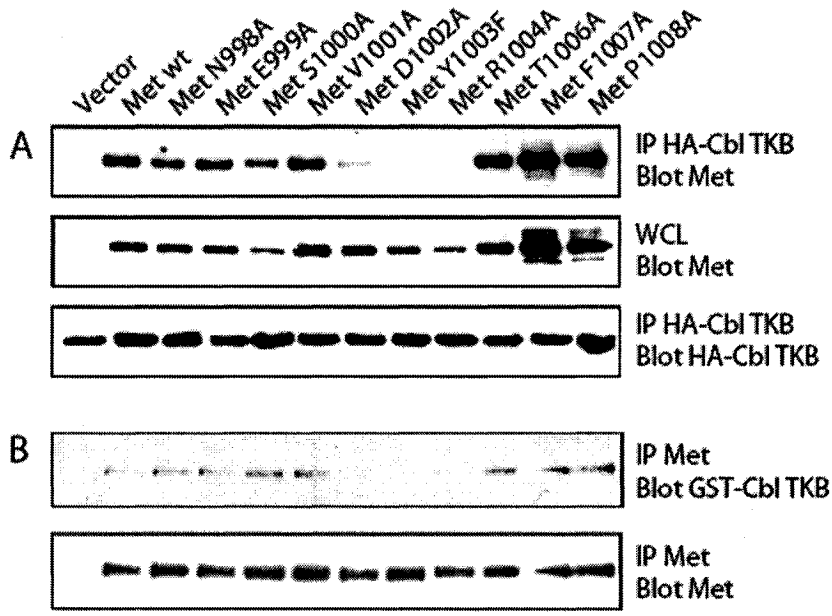


Figure 3. An intact DpYR motif in the Met receptor is required for its association with the Cbl-b TKB domain.

(A) Protein lysates from 293T cells transiently transfected with vector alone or plasmids expressing CSF-Met wt or Y1003F were subjected to *in vitro* binding assays with either GST, GST-c-Cbl-TKB, GST-Cbl-b-TKB or GST-Cbl-3-TKB. The membrane was immunoblotted with Met antibodies. **(B)** The amount of GST-Cbl-TKB fusion proteins used for the *in vitro* binding assays was determined by coomassie staining. **(C)** 293T cells were transiently transfected with plasmids expressing CSF-Met wt or mutants. Cell lysates were subjected to *in vitro* binding assays with GST-c-Cbl-TKB and GST-Cbl-b-TKB followed by immunoblotting with Met antibodies. Whole cell lysates were resolved separately and immunoblotted with Met antibodies.

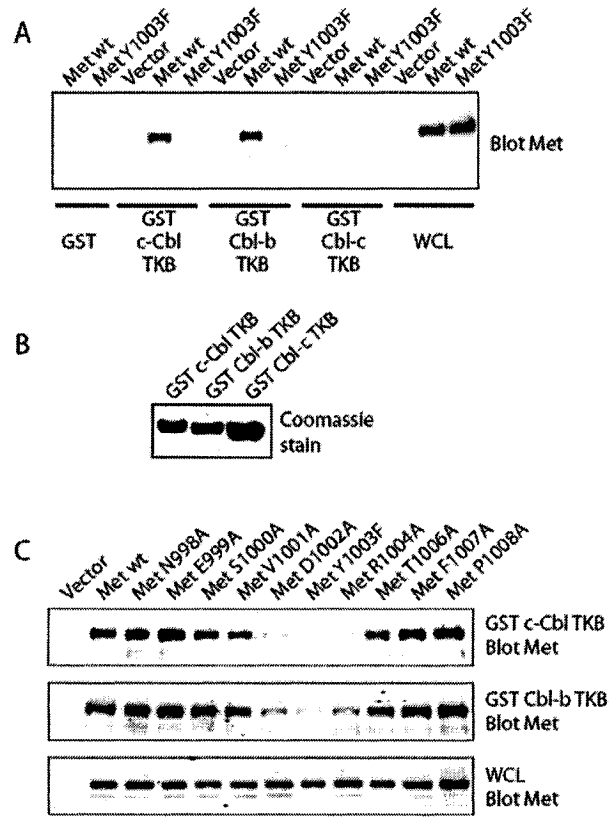


Figure 4. Mutation of the DpYR motif prevents Cbl-mediated Met receptor ubiquitination and promotes receptor oncogenic activation.

(A) Lysates from 293T cells co-transfected with HA-ubiquitin, c-Cbl and CSF-Met wt or mutants were subjected to immunoprecipitation with Met antibodies and immunoblotted with HA antibodies. The membrane was then stripped and reprobed with Met antibodies. Whole-cell lysates were immunoblotted with c-Cbl antibodies. **(B)** Rat-1 fibroblast cells were transfected with either CSF-Met wt or mutants (2 µg DNA/60 mm petri dish) and grown in DMEM containing 5% FBS in the absence of ligand until the appearance of foci (~2 weeks). **(C)** The graph represents the relative number of foci per dish from 4 different experiments.

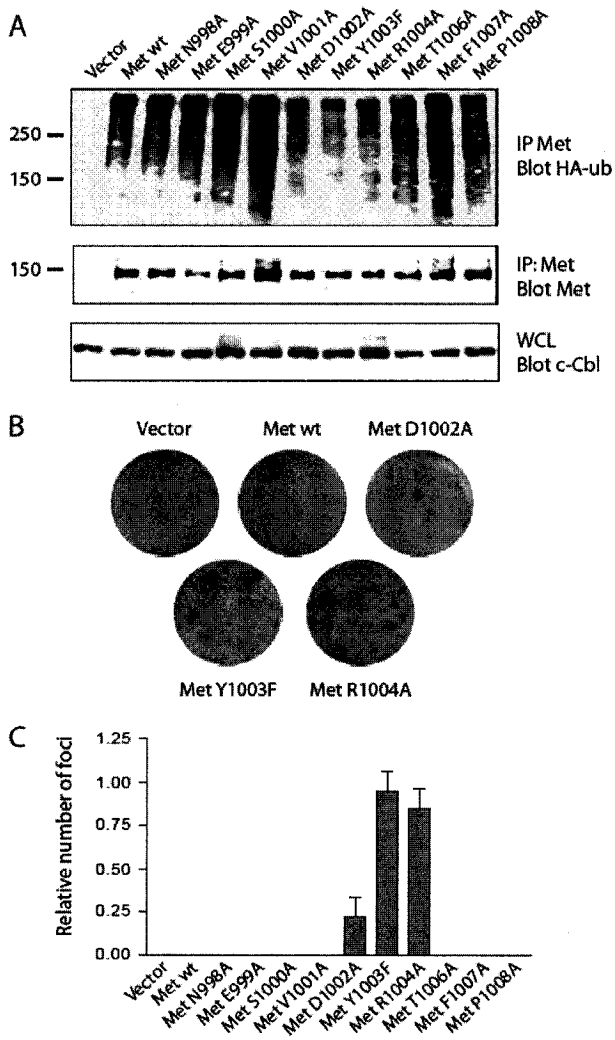


Figure 5. Mutation of the DpYR motif abrogates ligand-induced ubiquitination of the Met receptor in stable cell lines.

(A) Rat-1 fibroblast cell lines expressing either Met wt or Met mutants receptors were stimulated for 5 min with CSF-1 and lysed immediately. The CSF-Met receptor proteins were immunoprecipitated, resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was first immunoblotted with P4D1 ubiquitin antibodies, stripped and reprobed with Met antibodies, then stripped again and reprobed with 4G10 phosphotyrosine antibodies. **(B)** Quantification of Met ubiquitination; the anti-ubiquitin blot and the anti-Met blot in 5*A* were quantified using the ImageJ 1.31v software from NIH, USA. The values from the anti-ubiquitin blot were normalized for the Met protein levels in the absence of stimulation.

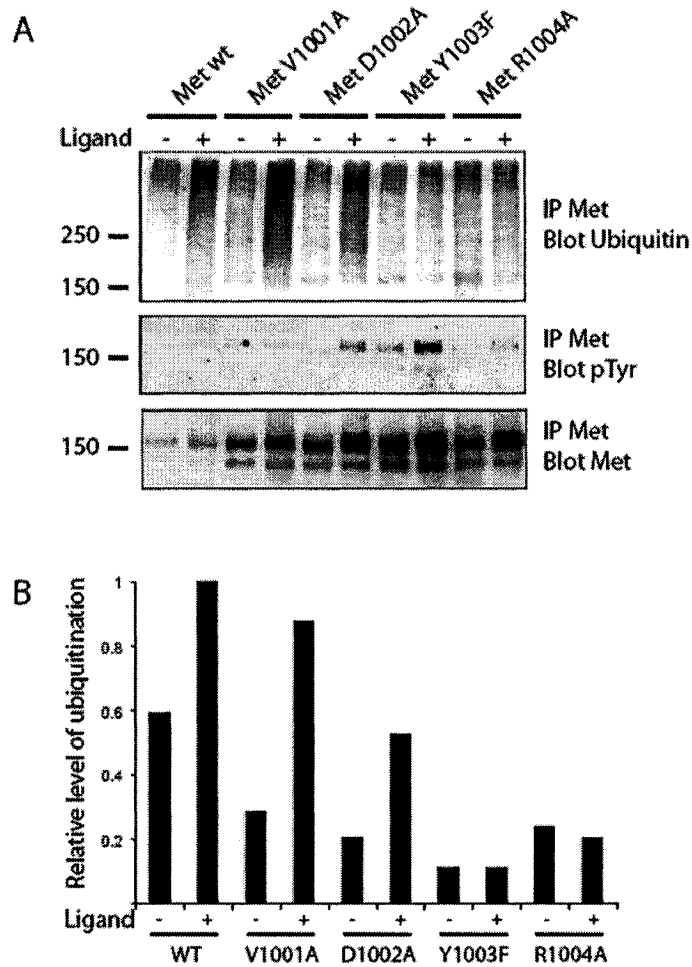


Figure 6. Role of salt bridge formation between D1002 and R1004 in the DpYR motif for the binding of the Met receptor to the TKB domain of c-Cbl.

The Met peptide S1000-P1008 (green) containing the DpYR motif was modeled based on the structure of the ZAP-70 pY292 peptide co-crystallized with c-Cbl (PDB code: 2CBL) (11). The side chains of both D1002 and R1004 extend out along the surface of the TKB domain of c-Cbl in the opposite direction as the side chain of pY1003 and can form a salt bridge (magenta dashed line). The electrostatic potential (positive in blue and negative in red) of the molecular surface of the TKB domain of c-Cbl was calculated with the program GRASP (23). It indicates that neither D1002 nor R1004 is associated with a highly charged surface of c-Cbl. In contrast, pY1003 binds to the positively charged pY-binding pocket, which is composed of three domains: SH2 (light yellow), EF-hand (light purple) and 4H (light blue). The specific binding between the Met receptor and c-Cbl is further supported by the binding of F1007 to the hydrophobic pocket. The figure was created using MOLSCRIPT, POVSCRIPT+ and Raster3D (24-26).

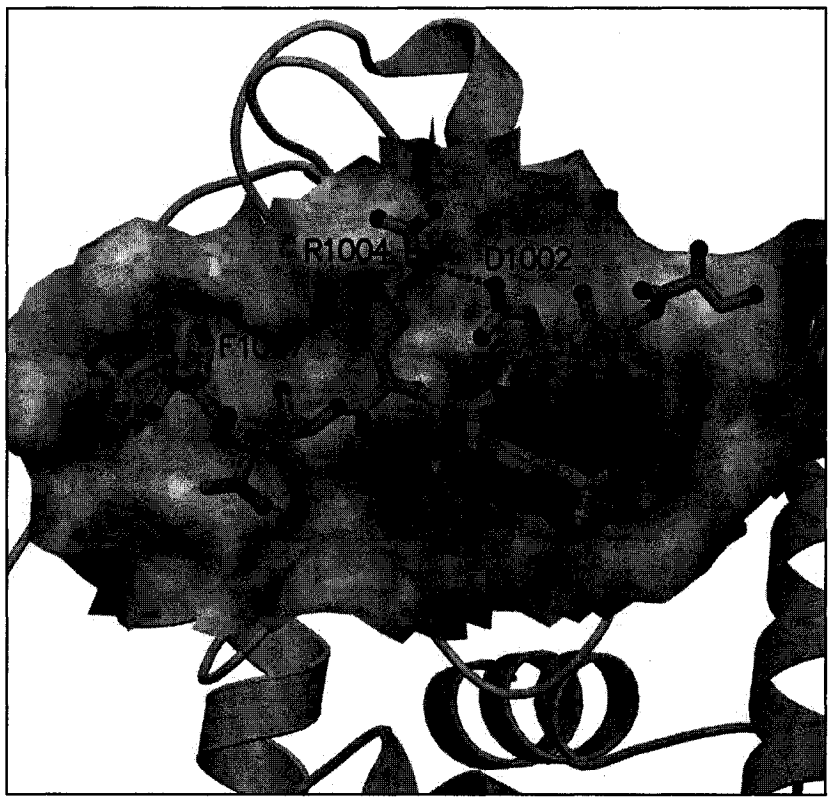
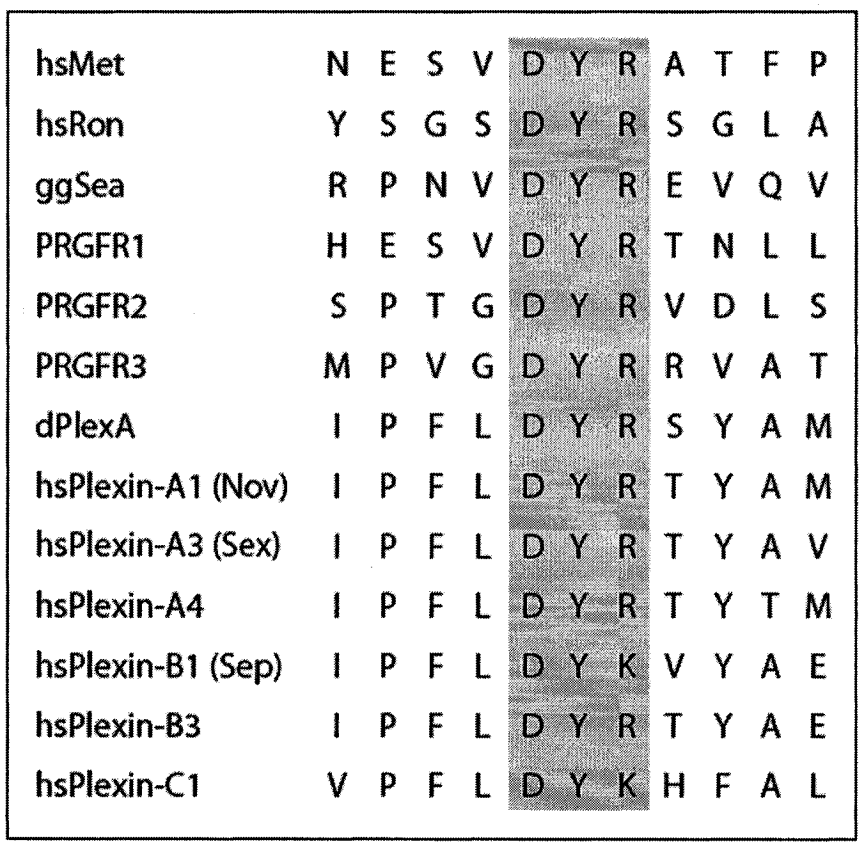


Figure 7. The DYR motif is conserved in the Met receptor family as well as in the Plexin receptor family.

Alignment of the DYR motif conserved in all the members of the Met receptor family and in members of the Plexin receptor family. Plexins are the receptors for semaphorins. In all cases, the DYR motif is located in the juxtamembrane region of the receptor (hsMet, human Met; hsRon, human Ron; ggSea, chicken Sea; PRGFR, Puffer fish plasminogen-related growth factor receptor; dPlexA, drosophila PlexA; hsPlexin, human plexin).



Discussion

The Cbl family of proteins has been reported to interact through their TKB domains with a variety of proteins. The TKB domain of c-Cbl can associate directly with the juxtamembrane tyrosine 1003 on the Met RTK and this interaction is required for Met receptor ubiquitination and downmodulation (10). The crystal structure of the c-Cbl TKB domain, complexed to its binding site on the ZAP-70 kinase, has revealed a consensus binding site, D/NXpYXXD/EΦ, that is found in three other c-Cbl TKB domain binding proteins: the Syk kinase, EGFR and Sprouty (12-16) (Fig 1). However, the c-Cbl TKB domain binding site in the Met receptor does not conform to this consensus sequence, suggesting that other c-Cbl TKB domain binding motifs may exist.

Mutagenesis studies of the Cbl TKB binding site on the Met receptor have revealed a Cbl TKB binding core motif, DpYR (Fig. 2). This motif binds c-Cbl as well as the Cbl family member Cbl-b, but not Cbl-3 (Fig. 3). The decreased association of c-Cbl and Cbl-b TKB domain with the D1002A and R1004A Met receptor mutants does not reflect decreased phosphorylation of Y1003, or of other tyrosine residues required for full catalytic activity of the receptor (Y1234-Y1235), as demonstrated by using phosphorylation site-specific Met antibodies (Fig. 1*C*). This identifies D and R as important for Cbl TKB domain interaction (Fig. 2 and 3). The 4H, EF-hand and SH2-like domains of c-Cbl and Cbl-b are highly conserved. However, only the SH2-like domain is conserved in Cbl-3, the 4H and EF-hand domains being notably different. This suggests a role for the 4H and EF-hand domains in c-Cbl and Cbl-b binding to the Met receptor, which is in agreement with the c-Cbl•ZAP-70 crystal structure (11).

The c-Cbl TKB domain is structurally similar to an SH2 domain, although there is only 11% identity at the amino acid level. The phosphotyrosine-binding pocket is well conserved, containing an invariant arginine residue that forms two hydrogen bonds with the phosphate group (11). However, the SH2 domain in c-Cbl lacks the secondary beta-sheet and the loop that define the binding specificity of an SH2 domain with residues located downstream from the phosphotyrosine residue (27, 28).

The crystal structure of a peptide from ZAP-70 indicates that residues positioned at pY-2 and pY+3 directly interact with c-Cbl to assist the specific binding of pY292 in ZAP-70 to the phosphotyrosine-binding pocket of c-Cbl (11).

The modeled structure of the Met peptide predicts that it utilizes a slightly different mechanism than ZAP-70 to promote the specific binding of pY1003 to the TKB domain of c-Cbl (Fig. 6). The Met receptor is predicted to form a salt bridge between D1002 and R1004 in the DpYR motif to stabilize the peptide conformation most favourable to expose pY1003 of Met towards the phosphotyrosine-binding pocket of c-Cbl (Fig. 6). Therefore, the substitution of either D1002 or R1004 in the DpYR motif would result in loss of the salt bridge, which would be expected to alter the projected orientation of pY1003 towards the phosphotyrosine-binding pocket of c-Cbl, decreasing the binding affinity of the TKB domain of c-Cbl to the Met receptor.

The crystal structure of the c-Cbl•ZAP-70 complex also revealed that a medium-sized hydrophobic residue (Φ) at position pY+4, proline in the case of ZAP-70, binds to a hydrophobic pocket (11). In the case of the Met receptor, the phenylalanine residue at position pY+4 also lies in that hydrophobic pocket. However, the F1007A substitution does not seem to affect the association of the c-Cbl/Cbl-b TKB domain with Y1003 in the Met receptor (Fig. 2), even though alanine is less capable of forming hydrophobic interactions than phenylalanine.

The specific substitution of Y1003 in Met with a non-phosphorylatable phenylalanine residue (F) uncouples the recruitment of the c-Cbl TKB domain, significantly diminishes ubiquitination of the receptor and leads to enhanced receptor stability and oncogenic activation (10). An intact DpYR motif, necessary for c-Cbl and Cbl-b TKB domain binding, is also required for efficient ubiquitination of the Met receptor following stimulation (Fig. 4*A* and 5*A*). Moreover, the substitution of the aspartate or arginine residues with alanine is sufficient to endow the Met receptor with transforming activity (Fig. 4*B* and 4*C*), where the transforming activity of these Met receptor mutants corresponds to their degree of ubiquitination. Whereas the Y1003F and R1004A Met receptor mutants show no detectable increase in ubiquitination following ligand stimulation and transform with similar efficiency, the D1002A Met receptor mutant is ubiquitinated at low levels and shows a decreased efficiency of transformation when compared to the Y1003F and R1004A Met receptor mutants. The lower levels of ubiquitination of the DpYR Met mutant receptors in stable Rat-1 fibroblast cell lines correlate with their elevated steady-state protein levels and elevated baseline phosphotyrosine levels when compared to wt Met and Met

V1001A mutant receptors (Fig. 5). Consistent with these observations, MDCK epithelial cells expressing D1002A and R1004A Trk-Met hybrid receptor mutants acquire a fibroblastoid phenotype in the absence of HGF stimulation (29). These observations further support a role for the D1002 and R1004 residues in Cbl-mediated down-regulation of the Met receptor.

Juxtamembrane tyrosine residues play a role in the autoinhibition of the VEGFR-1 (30), Eph (31, 32), and c-Kit (33) RTKs. Structural studies demonstrated that a helix containing the juxtamembrane unphosphorylated tyrosine residue adopts a conformation that distorts the small lobe of the kinase domain, preventing receptor activation. Upon receptor activation, phosphorylation of the juxtamembrane tyrosine promotes a conformational change that allows full activation of the receptor. The substitution of this residue with a non-phosphorylatable phenylalanine residue prevents activation of the Eph receptor (31, 32), whereas deletion of these tyrosine residues in c-Kit and Eph receptors removes this inhibitory mechanism (32, 33). In the case of the Met receptor, the substitution of Y1003 in the juxtamembrane domain with a phenylalanine residue does not prevent the activation and tyrosine phosphorylation of the Met receptor (Fig 1*C*). This suggests a distinct mechanism for the negative regulation of Met by the juxtamembrane Y1003 residue, consistent with the requirement for Y1003 and surrounding amino acids D1002 and R1004 for binding the c-Cbl TKB domain and c-Cbl-dependent ubiquitination and down-regulation of the Met receptor (Fig. 2 and 3) (10).

The DpYR motif is conserved within Met family members Met, Ron and Sea, as well as in Met orthologues in Puffer fish (Fig. 7), suggesting a conserved function for this motif in Cbl recruitment and negative regulation of the Met receptor family. In support of this, in a similar manner to the Met receptor, this tyrosine in the Ron RTK (Y1017) is required for the recruitment of the c-Cbl TKB domain and is essential for ubiquitination and degradation of Ron (34). Moreover, a DpYR motif is conserved in plexins, which are receptors for semaphorins that promote cell repulsion (35). Although mammalian plexins were identified through their homology with the extracellular domain of the Met receptor, homology with the cytosolic domain of the Met receptor family has not yet been reported (36). The presence of a conserved DpYR motif in plexins raises the possibility that this

motif may represent an unsuspected Cbl recruitment site in these receptors that modulates their stability.

There is no precedent for the association of a given phosphotyrosine-binding domain, either PTB or SH2, to unrelated consensus binding sites. This work demonstrates the versatility of the Cbl TKB domain to bind to different consensus sequences, which constitutes a unique feature, and highlights the need to identify and characterize other Cbl TKB domain binding sites.

Experimental Procedures

Antibodies and reagents

Antibodies raised in rabbit against a C-terminal peptide of human Met were used (37). Met pY1003 and pY1234-1235 phosphorylation site-specific antibodies were purchased from Biosource (Nivelles, Belgium), HA antibody from BABCO (Richmond, CA), anti-pTyr (4G10) from Upstate Biotechnology (Lake Placid, NY) and anti-c-Cbl (sc-170) and anti-ubiquitin (P4D1) from Santa Cruz Biotechnology Inc (Santa Cruz, CA). The HA-ubiquitin expression plasmid is described in (38).

Cell culture, DNA transfections and transformation assays

Human embryonic kidney 293T cells were transfected using the calcium phosphate method. For transformation assays in Rat-1 fibroblasts, $4X10^5$ cells were seeded in 60 mm plates in DMEM containing 10% FBS and transfected the next day with 2 ug DNA using the calcium phosphate method. After 12 hours, cells were washed twice with PBS and maintained in DMEM containing 10% FBS for two days. The cells were then maintained in DMEM containing 5% FBS, the medium being changed every 3-4 days, until the appearance of foci. For the generation of Rat-1 stable cell lines, foci were obtained in the presence of 25 ng/ml CSF-1, picked and expended in DMEM containing 10% FBS.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions using CSF-Met (39) in pMX-139 as a template. Asn 998 was converted to Ala using the 5'-GAAATGGTTTCAGCTGAATCTGTAGAC-3' primer; Glu 999 to Ala using the 5'-GGTTTCAAATGCATCTGTAGACTACCG-3' primer; Ser1000 to Ala using the 5'-GTTTCAAATGAAGCAGTCGACTACCGAGCTAC-3' primer; Val 1001 to Ala using the 5'-GTTTCAAATGAATCTGCAGACTACCGAGC-3' primer; Asp 1002 to Ala using the 5'-GTTTCAAATGAATCTGTCGCCTACCGAGCTAC-3' primer; Arg 1004 to Ala using the 5'-GAATCTGTAGACTACGCGGCTACTTTTCCAG-3' primer; Thr 1006 to Ala using the 5'-CTGTAGACTACCGAGCAGCTTTTCCAGAAGATCAG-3' primer; Phe 1007 to Ala using the 5'-

CTACCGAGCTACTGCGCCAGAAGATCAG-3' primer and Pro 1008 to Ala using the 5'-CTACCGAGCTACTTTTGCAGAAGATCAGTTTC-3' primer.

In vitro binding Assays

The amino-terminal portion of c-Cbl and Cbl-3 fused to GST were provided by Dr. Hamid Band (40) and Dr. Vincent Ollendorff (41) respectively. Coupling of GST fusion proteins to Glutathione-Sepharose beads (Amersham Pharmacia Biotech (A.P.), Baie d'Urfe, Qc) was performed at 4°C for 1 hr. Complexes were washed three times with TGH lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium vanadate, and then incubated with cell lysate for 2 hrs at 4°C, washed four times with TGH lysis buffer and resuspended in Laemmli sample buffer.

Immunoprecipitations and Western Blotting

293T cells were serum-starved in 0.1% FBS overnight and harvested in TGH lysis buffer. Lysates were incubated with the indicated antibody overnight at 4°C with gentle rotation. Proteins collected on either protein A- or protein G-Sepharose were washed three times in TGH lysis buffer, resolved by SDS-PAGE and transferred to a nitrocellulose membrane as described (42). Proteins were visualized with an ECL detection kit (A.P.). To detect CSF-Met receptor ubiquitination in Rat-1 cell lines, cells were stimulated for 5 min with 500 ug/ml CSF-1 and immediately lyzed in RIPA buffer (0.05 % SDS, 50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium desoxycholate) containing 25 mM N-ethylmaleimide (Sigma), 1 mM PMSF, 10 ug/ml aprotinin, 10 ug/ml leupeptin and 1 mM sodium vanadate.

Far-Western Blotting

For far-western analysis, the CSF-met receptor was immunoprecipitated from 1 mg of 293T whole cell lysates, resolved on an 8% SDS-page gel and transferred to a nitrocellulose membrane. The membrane was blocked overnight in 20 mM Tris ph 7.5, 150 mM NaCl and 1 mM Na_3Vo_4 containing 10% non-fat milk and 0,1% tween-20, and then probed 1 hr at room temperature with 40 ug/ml of purified GST-Cbl-N proteins previously coupled to 1 ug HRP-glutathione (Sigma

Aldrich, g-6400) (30 min, room temperature) in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 0,1% Tween-20). After four washes of 5 min each with TBST, bound proteins were detected with an ECL detection kit (A.P.) (43).

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Chapter 4

Met/HGF receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation

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Preface

In chapter 2 and 3, I demonstrated that a point mutation in the juxtamembrane of the Met receptor (Y1003F) prevents Cbl-mediated ubiquitination of the Met receptor and is sufficient for oncogenic activation of the Met receptor in epithelial and fibroblast cells in vitro. In this chapter, I observed that loss of ubiquitination of the Met receptor also leads to tumorigenesis in vivo.

Numerous genetic studies in yeast revealed that ubiquitination of cell-surface proteins plays a role in their internalisation and degradation in the vacuole. Whether this is the case for RTKs in mammalian cells remained to be shown. During the course of my Ph.D., several publications addressed the role of ubiquitination of the epidermal growth factor receptor (EGFR) in its internalisation and lysosomal degradation. Whilst evidence for the requirement for the ubiquitination of RTKs for their lysosomal degradation has accumulated, the role of ubiquitination for RTK internalisation remains controversial. In addition, the consequence of preventing ubiquitination of EGFR or other RTKs on the activation of downstream signalling pathways has not been examined carefully. Hence, in this chapter, I have addressed how loss of ubiquitination of the Met receptor affects Met down-regulation and signalling, leading to its oncogenic activation. To do so, I generated epithelial cell populations stably expressing a wt Met and a Met Y1003F receptor mutant and examined Met receptor ubiquitination, degradation, internalisation, trafficking and signalling.

Abstract

The Met receptor tyrosine kinase (RTK) regulates epithelial remodelling, dispersal and invasion and is deregulated in many human cancers. It is now accepted that impaired down-regulation, as well as sustained activation, of RTKs could contribute to their deregulation. Down-regulation of the Met receptor involves ligand-induced internalisation, ubiquitination by Cbl ubiquitin ligases, and lysosomal degradation. Here we report that a ubiquitination-deficient Met receptor mutant (Y1003F) is tumorigenic *in vivo.* The Met Y1003F mutant is internalised, and undergoes endosomal trafficking with kinetics similar to the wt Met receptor, yet is inefficiently targeted for degradation. This results in sustained activation of Met Y1003F and downstream signals involving the Ras-MAPK pathway, cell transformation and tumorigenesis. Although Met Y1003F undergoes endosomal trafficking and localizes with the cargo sorting protein Hrs, it is unable to induce phosphorylation of Hrs. Fusion of monoubiquitin to Met Y1003F is sufficient to decrease Met receptor stability and prevent sustained MEK1/2 activation. In addition, this rescues Hrs tyrosine phosphorylation and decreases transformation in a focus forming assay. These results demonstrate that Cbl-dependent ubiquitination is dispensable for Met internalisation, but is critical to target the Met receptor to components of the lysosomal sorting machinery, and to suppress its inherent transforming activity.

Abbreviations

Cbl	Casitas B-lineage lymphoma
CSF-1	colony stimulating factor-1
EEA1	early endosome antigen 1
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ESCRT	endosomal sorting complex required for transport
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
Gab-1	Grb2 associated binder-1
GFP	green fluorescent protein
GST	glutathione S-transferase
HEK	human embryonic kidney
HGF	hepatocyte growth factor
Hrs	HGF-regulated tyrosine kinase substrate
MAPK	mitogen-activated protein kinase
MDCK	Madin-Darby Canine Kidney
MVB	multivesicular bodies
PDGFR	platelet-derived growth factor receptor
PFA	paraformaldehyde
PI3K	phosphatidylinositol-3-kinase
RTK	receptor tyrosine kinase
SH2	Src-homology 2
TKB	tyrosine kinase binding
TCL	total cell lysate
UIM	ubiquitin interacting motif
wt	wild type

Introduction

Growth factor RTKs are involved in a variety of cellular processes including proliferation, differentiation, migration and survival. RTK activation is tightly controlled through several levels of regulation to achieve an appropriate biological response. The inappropriate activation of RTKs is associated with the development and progression of many human malignancies. Of the 58 transmembrane RTKs identified to date, deregulation of 30 RTKs has been associated with human tumours (1). RTK deregulation can occur through receptor amplification, point mutations and chromosomal translocations, leading to elevated and ligand–independent RTK activation. More recently, the uncoupling of the Met/Hepatocyte Growth Factor RTK from ligand-induced ubiquitination and down-regulation revealed another mechanism leading to deregulation and oncogenic activation of RTKs (2, 3).

Met, the receptor for the hepatocyte growth factor/scatter factor (HGF/SF), is predominantly expressed in epithelia *in vivo*, and in epithelial cell lines *in vitro*. Its ligand, HGF/SF, is expressed by mesenchymal cells and promotes dissociation of epithelial cells in culture, increasing their motility and invasiveness, and acts as a mitogen for primary epithelia (4). Many of these are processes that are normally regulated by Met and HGF during development, and reflect the ability of HGF to promote cell survival, cell migration and to activate the intrinsic invasive morphogenic programs of different epithelial cells in three-dimensional matrix cultures (4). Deregulated Met signalling is associated with tumour progression and metastasis in a variety of tumours (5). Activating mutations in Met were first identified in both hereditary and sporadic papillary renal carcinomas (6). Many missense mutations in Met have now been reported in a variety of human cancers (7, 8).

Activation of the Met receptor through HGF binding promotes tyrosine phosphorylation of its intracellular domain and the recruitment of signalling protein complexes required for the activation of downstream signalling pathways and the generation of a biological response (9-13). In addition to the recruitment of positive effectors, acute HGF stimulation of the Met receptor leads to receptor internalisation into clathrin-coated vesicles and its down-regulation by as yet incompletely understood mechanisms (14).

Following ligand stimulation, internalised receptors are subject to two distinct fates: either to recycle back to the plasma membrane or to be degraded via the lysosomal pathway (15). The rapid removal of growth factor receptors from the cell surface and their subsequent targeting to lysosomal degradative compartments provides a mechanism of down-regulation that is important to prevent the sustained activation of downstream signalling pathways, which could potentially lead to cellular transformation. Following clathrin-mediated internalisation, ligand-activated RTKs, including the Met receptor, are eventually delivered to sorting endosomes (14, 16). At this stage, RTKs can be sorted into intralumenal vesicles of multivesicular bodies (MVBs) that subsequently fuse with lysosomes (17, 18). This event terminates RTK signalling by sequestering their signalling-competent intracellular domain and prevents RTKs from recycling to the cell surface.

In the last few years, genetic and biochemical studies have begun to elucidate the molecular mechanisms through which plasma membrane proteins are recognised for degradation in the lysosome. Studies in yeast have shown that monoubiquitination of several cell surface proteins is required for their internalisation and trafficking to the vacuole for degradation (17, 19, 20). Although multiple signals are used in the regulation of the internalisation of plasma membrane proteins in mammalian cells (21), several studies now support a role for monoubiquitination in the trafficking and targeting of the EGFR for lysosome degradation (22-26).

Monoubiquitinated receptors are thought to be recognised by multiple proteins of the endocytic pathway that contain ubiquitin interacting domains (UBA, UIM, UEV, CUE) (17, 18, 27). One of these, Hrs (HGF-regulated tyrosine kinase substrate), appears to be the first protein recruited to endosomes and becomes enriched in regions of the endosome containing a specialised bilayered clathrin coat (28-30). Hrs is believed to be involved in the retention of ubiquitinated receptors within the bilayered clathrin coat and in the recruitment of ESCRT (endosomal sorting complex required for transport) complexes (31-35). Hrs becomes tyrosine phosphorylated upon activation of several RTKs (36). Hrs phosphorylation requires RTK internalisation (37) as well as an intact ubiquitin interacting motif (UIM) in Hrs (38), supporting a role for Hrs in retaining ubiquitinated RTKs at the limiting membrane of endosomes.

The recruitment of the Cbl family of ubiquitin-protein ligases to RTKs is important for ligand-induced degradation of several RTKs, among them the EGFR, the platelet-derived growth factor receptor (PDGFR), the colony-stimulating factor-1 receptor (CSF-1R) and the Met/HGF receptor (2, 22, 39-41). Cbl is thought to mediate multi-monoubiquitination of RTKs rather than polyubiquitination, through the demonstration that antibodies specific for polyubiquitination fail to recognise ubiquitinated forms of the EGF, PDGF or Met receptors (23, 24, 42). When overexpressed, Cbl can positively regulate endocytosis of the EGF and Met receptors, possibly through its ability to act as an adaptor for endophilin, an enzyme that may be involved in membrane curvature (22, 43, 44). Ubiquitination of the EGFR by Cbl is required for directing the receptor to internal vesicles of late endosomes and for subsequent lysosomal degradation of the EGFR (25, 26, 45, 46). Moreover, the fusion of monoubiquitin to a truncated EGFR is sufficient, in the absence of other signals, to target this receptor for constitutive internalisation, and trafficking to late endosomes, supporting a role for monoubiquitination as a signal in mammalian cells for the trafficking of the EGFR (23).

We have previously demonstrated that the specific uncoupling of the Met receptor from Cbl-dependent ubiquitination promotes cell transformation by this receptor (2, 41). Moreover, several mechanisms have been identified that reduce Cbl-mediated ubiquitination of RTKs through enhanced Cbl degradation or sequestration, leading to cell transformation (47-50). In addition, mutations in RTKs or Cbl proteins that impair Cbl-mediated ubiquitination of RTKs have been observed in multiple tumours (3). Together, this provides support that the uncoupling of RTKs from ubiquitination may play an important role in tumorigenesis (3, 16, 51, 52). However, the molecular consequences of diminished RTK ubiquitination, at the level of receptor trafficking and signalling, as well as the biological outcomes, remain poorly understood.

Here, we provide a mechanistic understanding for oncogenic activation of the Met RTK through uncoupling from ubiquitination. We demonstrate that a ubiquitination-deficient Met receptor mutant (Met Y1003F) shows increased stability and signalling of downstream pathways, including the Ras-MAPK pathway, and oncogenic activation *in vivo*. We show that the Met Y1003F receptor mutant is internalised and can reach Hrs containing endosomes in a

manner similar to the wt Met receptor, yet is unable to induce tyrosine phosphorylation of Hrs. The fusion of monoubiquitin to the Met Y1003F receptor rescues Hrs phosphorylation, decreases the stability of the Met Y1003F mutant, and suppresses Met receptor signalling and transforming activity.

Results

Loss of Cbl-mediated ubiquitination results in sustained tyrosine phosphorylation and enhanced stability of the Met RTK

To study the *in vivo* relevance of loss of ubiquitination of the Met receptor, we established populations of T47D epithelial breast cancer cells expressing either the wt Met receptor or a Met receptor mutant (Y1003F) (Fig. 1B). The Y1003F mutant does not associate with the Cbl TKB domain (Fig. 1A) and is only weakly ubiquitinated in transient assays (2, 41). Unlike the majority of epithelial cells, T47D cells do not express detectable levels of endogenous Met receptor (53), providing a unique epithelial cell model in which to analyse the consequence of loss of Cbl ubiquitination. Stable cell populations were established that express wt Met and Met Y1003F mutant receptors (Fig. 1B). The level of expression of the Met Y1003F mutant receptor in stable cell populations is systematically higher than that of the wt Met receptor even though Met RNA levels are similar (Fig. 1B and 1C). Following stimulation with 3 nM HGF, the wt Met receptor is robustly ubiquitinated whereas the Met Y1003F receptor mutant shows only low levels of ubiquitination (Fig. 1D). Consistent with a role for ligand-induced ubiquitination in Met degradation, after 2 hours of stimulation, the steady-state levels of the wt Met receptor are decreased by 75%, whereas the levels of the Met Y1003F receptor mutant are decreased by only 20% (Fig. 1E and 1F). In addition to enhanced stability, tyrosine phosphorylation of the Met Y1003F receptor mutant is sustained up to 2 hours following HGF stimulation, whereas tyrosine phosphorylation of the wt Met receptor is decreased within 30 minutes (Fig. 1E), indicating that the Met Y1003F receptor, which is poorly ubiquitinated, induces prolonged signalling of downstream pathways.

The Met Y1003F receptor mutant is transforming *in vitro* and *in vivo*

The enhanced stability of the Met Y1003F mutant is reflected by its enhanced biological activity. T47D cell lines stably expressing the Met Y1003F receptor, but not the wt Met receptor, display loss of epithelial organisation, a hallmark of epithelial cell transformation that is enhanced by HGF stimulation (Fig. 2A). Furthermore, NIH 3T3 cells expressing a Met Y1003F receptor, when subcutaneously injected into nude mice, formed solid tumours (0.10 cm^3) with short latencies (20 days), which reached a volume of 0.70 cm^3 within 35 days. In

contrast, control NIH 3T3 cells, or cells expressing a wt Met receptor, formed only a small mass of 0.10 cm^3 by 35 days post-injection (Fig. 2B and 2C). Although the cell populations injected into nude mice expressed similar amounts of Met proteins, the tumours that developed contained higher levels of Met Y1003F than wt Met proteins (Fig. 2C). We conclude that the specific uncoupling of the Met receptor from Cbl-mediated ubiquitination is sufficient to increase both the *in vitro* and *in vivo* tumorigenicity of the Met receptor.

The Met Y1003F receptor preferentially promotes sustained activation of the Ras-MAPK pathway

Several studies have indicated that recruitment of Cbl is required for the ubiquitination and ligand-induced down-regulation of the Met and EGF receptors (2, 22, 41, 54). However, reports on Met or EGF receptor mutants, uncoupled from Cbl-mediated ubiquitination, have failed to address the consequence of loss of ubiquitination on receptor signalling. To understand at the mechanistic level why the Met Y1003F receptor mutant is tumorigenic and promotes enhanced epithelial scattering, we examined its cellular signalling and trafficking in T47D cell populations stably expressing the Met wt or Y1003F receptor. Signalling downstream from the Met receptor is mediated through the recruitment of the adaptor proteins Grb2 and Shc, which couple the Met receptor to the Ras signalling pathway, and the scaffold protein Gab1. Gab1 couples the Met receptor to the MAPK pathway as well as to phosphatidylinositol-3-kinase (PI3K), phospholipase Cγ, Crk and the tyrosine phosphatase SHP2 (12, 55-57). Consistent with the sustained phosphorylation of the Met Y1003F receptor, tyrosine phosphorylation of downstream signals, such as Gab1 and c-Cbl, are prolonged in cells expressing the Met Y1003F receptor (Fig. 3A). Furthermore, Ras activation, as measured by an *in vitro* Ras binding assay, is transient (1 hr) downstream from the wt Met receptor, while it is sustained downstream of the Met Y1003F receptor (4 hrs, Fig. 3B). Activation of signalling pathways downstream from Ras, MEK1/2 and Erk1/2, as detected using phosphorylation site-specific antibodies, showed a similar trend (Fig. 3B). HGF stimulation induces a robust but relatively transient activation of MEK1/2 (1 hr) and Erk1/2 (decreased by 60 min) in T47D cells expressing a wt Met receptor, whereas activation of these proteins is sustained for 4 hrs in cells expressing the Met

Y1003F receptor mutant (Fig. 3B). Indirect immunofluorescence of activated MEK1/2 in T47D cell populations, using phospho-specific antibodies, revealed a sustained and perinuclear signal in Met Y1003F expressing cells when compared to a transient and perinuclear signal in cells expressing the wt receptor (Fig. 3C), demonstrating that the subcellular localization of the pMEK1/2 signal is not detectably different following sustained activation. Alternatively, in these cells, PI3K-dependent activation of Akt/PKB, as detected using a phospho-Ser473 antibody, is similar downstream from both the wt and Y1003F receptors (Fig. 3A), revealing a selective enhancement of the Ras signalling pathway.

The requirement of the Ras-MAPK and PI3K dependent signals for biological activity of the Met Y1003F receptor were examined in T47D and MDCK epithelial cells. These cells undergo an epithelial-mesenchymal like transition (EMT) following stable expression of the Met Y1003F receptor (Fig. 2A and 4) (2). Treatment of fibroblast-like MDCK and T47D cells expressing Met Y1003F, with a pharmacological inhibitor of MEK1/2 (U0126), induces a reversal in cell morphology and cells begin to form tight colonies 24 hours post-treatment, in the presence or absence of ligand (Fig. 4 and data not shown). In contrast, minimal changes in the fibroblast-like morphology of the Met Y1003F expressing cells were observed after treatment with an inhibitor of PI3K (LY294002) (Fig. 4 and data not shown). This demonstrates a specific requirement for the Ras-MAPK pathway in the maintenance of the epithelial scattered phenotype induced by the Met Y1003F receptor mutant.

Met wt and Y1003F receptors internalise with similar kinetics

The enhanced stability of the Met Y1003F receptor may indicate that this receptor is retained on the cell surface. Although several reports support the ability of ubiquitination to promote the internalisation of cell-surface receptors in yeast (17), whether ubiquitination of RTKs is directly involved in their internalisation has only been examined for EGFR and remains controversial (25, 26, 45, 46, 54, 58). To address the requirement for Met receptor ubiquitination for its internalisation, we compared internalisation of the Met wt and Y1003F receptors in T47D cells, using trypsin and flow cytometry-based assays. Following HGF stimulation, the amount of internalised, trypsin-protected, receptors increases at a similar rate for Met wt and Y1003F (Fig. 5A). In addition,

using flow cytometry, we observed that the Met wt and Y1003F receptors are internalised at similar rates following stimulation (Fig. 5B). In agreement with this, by immunofluorescence microscopy, both Met wt and Y1003F mutant receptors internalise and co-localize with the early endosome marker EEA1, 5 minutes post-HGF treatment, and this co-localization is enhanced at 15 and 30 minutes (Fig. 5C). These results demonstrate that there is no significant difference between the rate of internalisation and subcellular localization of wt Met and Met Y1003F receptors.

Met RTK ubiquitination is required for Hrs phosphorylation but not for trafficking to Hrs-positive endosomes

The enhanced stability of the Met Y1003F receptor could also indicate that the endosomal sorting machinery does not recognize the poorly ubiquitinated Met Y1003F receptor, a process thought to be required for efficient targeting of the receptor to the sorting endosome. Hrs, one member of the endosomal sorting machinery, was first identified as a tyrosine phosphorylated protein downstream from the Met receptor (36, 59). Therefore, as a readout for Met receptor recruitment by endosomal sorting proteins, we examined whether the Met Y1003F receptor mutant can phosphorylate Hrs. Following stimulation of T47D cells with HGF, the endogenous Hrs protein is tyrosine phosphorylated upon activation of a wt Met receptor, but is not detectably phosphorylated in cells expressing the Met Y1003F receptor (Fig. 6A). To determine if the inability of the Met Y1003F mutant receptor to induce Hrs phosphorylation reflects its inability to reach an Hrs positive endosome, we examined the subcellular localization of both Met wt and Y1003F receptors with either endogenous or transiently transfected Hrs. As observed by indirect immunofluorescence, both the Met wt and Y1003F receptors traffic to Hrs-positive endosomes (Fig. 6B and data not shown). Moreover, this occurs with similar kinetics, where both the Met wt and Y1003F receptors begin to localize with either endogenous or GFP-tagged Hrs as early as 5 minutes post-HGF stimulation (data not shown) and co-localization increases up to 30 minutes post-HGF stimulation (Fig. 6B and data not shown). At the latter time point (30 min), both receptors also begin to localize to GFP-Rab7 positive endosomes (a marker for MVBs/ late endosomes) (Fig. 6C) (60, 61). Hence, although the ubiquitination-deficient Met Y1003F receptor mutant

reaches Hrs and Rab7-positive endosomes, it does not induce Hrs tyrosine phosphorylation. This demonstrates that Cbl-mediated ubiquitination of the Met receptor is not essential for its targeting to Hrs and Rab7 positive endosomes, but is required to induce Hrs tyrosine phosphorylation.

Monoubiquitination of Met Y1003F decreases its stability and rescues Hrs phosphorylation

The addition of monoubiquitin to the transferrin receptor results in its partial exclusion from the recycling compartment and its retention within late endosomes (29). Similarly, the fusion of ubiquitin to the carboxy-terminus of a severely truncated EGFR was sufficient for this receptor to traffic to late endosomes (23). However, the ability of these proteins to interact with components of the cargo sorting pathway and their stability was not examined. To establish whether the addition of monoubiquitin to the Met Y1003F receptor can rescue its targeting to the lysosomal degradative pathway and if this enhances its ability to be recruited to the endosomal sorting complex containing Hrs, we generated Met-ubiquitin receptor chimeras. We fused a single ubiquitin moiety that cannot form ubiquitin chains, where the 7 lysine residues in ubiquitin are substituted for arginine residues (20), to the carboxy-terminus of the full-length wt and Y1003F Met receptors (Fig. 7A). Following transient overexpression in HEK 293 cells where the Met receptor is activated in a ligand-independent manner, both proteins are detected as a discrete band with an antibody raised against ubiquitin (Fig. 7B). Moreover, both proteins associate with known receptor binding proteins to similar levels. Both the Grb2 adaptor protein and the Met binding domain (MBD) of Gab1 associate with the chimeric receptors in *in vitro* binding assays, demonstrating that the chimeric receptors are properly folded, tyrosine phosphorylated and can interact with signalling proteins (Fig. 7B). In stable T47D cell populations, the chimeric receptors are expressed at the cell surface, as determined by flow cytometry (data not shown) and by immunofluorescence microscopy (Fig. 7C). Upon HGF stimulation, both chimeric receptors internalise and co-localize with EEA1 positive endosomes (Fig. 7C). When examined for stability, the Met Y1003F receptor mutant is stable for up to 6 hours after stimulation with HGF, whereas the Met Y1003F-ub chimeric receptor is degraded after 2 hours of stimulation (Fig. 8A), in a similar manner to the wt Met protein (Fig. 1E).

Consistent with this, the prolonged activation of the Ras-MAPK signalling pathway observed downstream of the Met Y1003F receptor (4 hrs, Fig. 8B) is reversed by the addition of monoubiquitin to this receptor. MEK1/2 activation downstream from Met Y1003F-ub is transient (60 min, Fig. 8B), as it is downstream of the wt Met receptor (Fig. 3B). These data indicate that the fusion of monoubiquitin to the Met receptor is sufficient to reverse the prolonged stability and signalling of the Met Y1003F receptor mutant. This suggests that monoubiquitination is sufficient to engage the Met Y1003F receptor mutant with components of the cargo-sorting pathway. To test this, we examined its ability to induce phosphorylation of Hrs in HEK293 transient transfection assays, where Met is constitutively activated due to over-expression. We observed that while the Met Y1003F receptor is severely reduced in its ability to induce robust tyrosine phosphorylation of Hrs when compared with a wt Met receptor, the Met Y1003F-ub chimeric receptor induces robust Hrs phosphorylation (Fig. 8C). Hence, this demonstrates that monoubiquitination of the Met Y1003F receptor is sufficient to target the receptor for degradation and for it to phosphorylate Hrs, a component of the cargo sorting machinery.

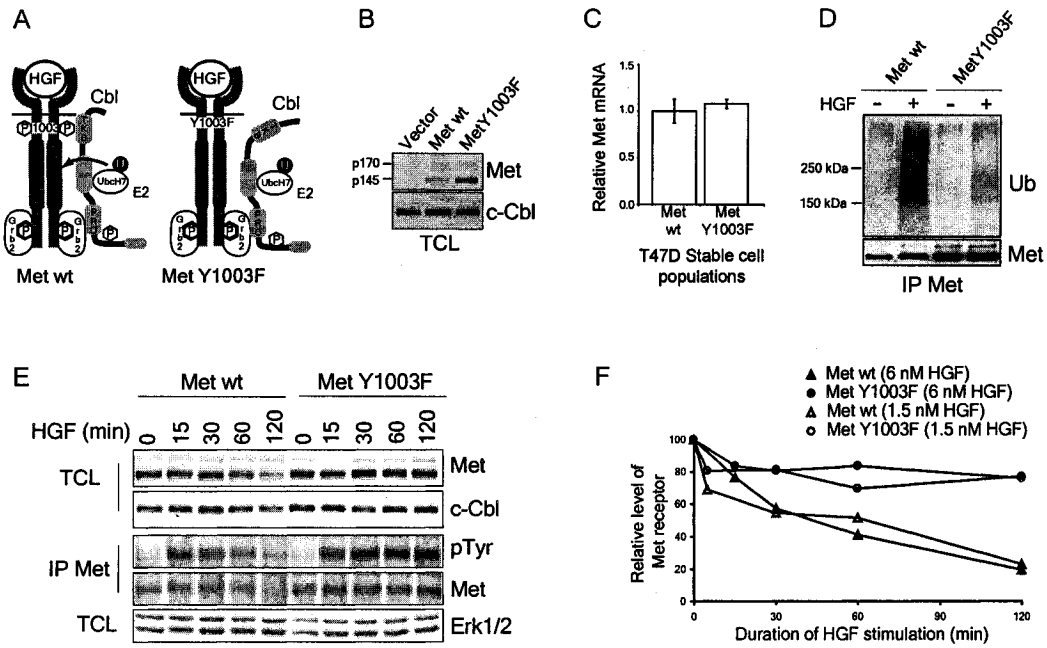
Monoubiquitination of the Met RTK suppresses its transforming activity

Since uncoupling the Met receptor from Cbl-dependent ubiquitination results in enhanced stability, signalling and cell transformation, we examined the ability of the Met-ubiquitin chimeric receptors to transform Rat-1 fibroblasts using a focus-forming assay. The addition of a monoubiquitin moiety to the Met Y1003F receptor mutant decreases its transforming activity by 60% (Fig. 9). Moreover, the addition of monoubiquitin to a wt Met receptor reduces its transforming activity in the presence of ligand by 90% (Fig. 9). Thus, monoubiquitination of the Met RTK is sufficient to suppress the oncogenic activity of both the Met wt and Y1003F receptors. Altogether, fusion of monoubiquitin to the Met receptor rescues the phenotypes associated with the Y1003F mutation, namely increased Met stability, prolonged MEK activation, loss of Hrs phosphorylation and Met oncogenic activation (Fig. 8 and 9). Hence, this demonstrates that these phenotypes result from the loss of ubiquitination of the Met Y1003F receptor and are unlikely caused by another mechanism.

Figure 1. The Met Y1003F receptor mutant is poorly ubiquitinated and its degradation is delayed upon HGF stimulation.

(A) Schematic representation of Cbl recruitment to the Met receptor. The Y1003F substitution abrogates binding of the Cbl TKB domain to the Met receptor. The Met Y1003F receptor mutant still recruits and tyrosine phosphorylates Cbl via the Grb2 adaptor. **(B)** Expression levels of the Met wt and Y1003F receptors in retrovirally infected stable T47D cell populations. After selection with neomycin for 2 weeks, cells expressing Met at their cell surface were sorted using FACS. Total cell lysates (TCL) were blotted with Met (144) and c-Cbl antibodies. The p170 band represents the uncleaved Met precursor and the p145 band represents the processed α chain of the receptor. **(C)** Met RNA expression levels from T47D cell populations were determined using quantitative real-time PCR. The graph represents the mean $\pm$ SD of triplicate samples. **(D)** T47D cells were stimulated with 3 nM HGF for 2 min and lysed immediately in boiling 2% SDS. Lysates were boiled for 10 min, diluted to 0.4% SDS, 2% Triton and then Met receptor proteins were immunoprecipitated with Ab 144 and blotted with ubiquitin antibodies, stripped and reblotted with Met antibodies. **(E)** T47D cells were stimulated with either 6 nM HGF (top two panels) or 1.5 nM HGF (bottom three panels) for the indicated amount of time. Lysates were treated as indicated. **(F)** Met protein levels were quantified using the ImageJ 1.63 software and were corrected using Cbl (6 nM HGF) and Erk2 (1.5 nM HGF) protein levels.

Figure 1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HGF
Cbl
Y1003
P
Ub
UbcH7
E2
Grb2
Met wt
Met Y1003F
B
Vector
Met wt
MetY1003F
p170
p145
Met
c-Cbl
TCL
C
Relative Met mRNA
1.5
1.0
0.5
0.0
Met wt
Met Y1003F
T47D Stable cell populations
D
Met wt
MetY1003F
HGF
250 kDa
150 kDa
Ub
Met
IP Met
E
Met wt
Met Y1003F
HGF (min)
0
15
30
60
120
TCL
Met
c-Cbl
IP Met
pTyr
Met
TCL
Erk1/2
F
Met wt (6 nM HGF)
Met Y1003F (6 nM HGF)
Met wt (1.5 nM HGF)
Met Y1003F (1.5 nM HGF)
Relative level of Met receptor
100
80
60
40
20
0
0
30
60
90
120
Duration of HGF stimulation (min)

Figure 2. NIH 3T3 fibroblast cells expressing the ubiquitination-deficient Met Y1003F receptor are tumorigenic.

(A) Met Y1003F expressing cells have an altered morphology. Phase contrast pictures of T47D cells unstimulated or stimulated for 24 hours with 1.5 nM HGF. **(B)** NIH 3T3 cell populations expressing either wt Met or Met Y1003F were injected subcutaneously in nude mice. The results represent the mean tumour volume obtained from 8 measurements and are representative of two independent experiments. **(C)** Pictures of representative tumours as well as Met protein expression in the NIH 3T3 cell populations injected and in excised tumours. The tumours were lysed in TGH buffer and total cell lysates (TCL) were blotted with Met (DL-21) antibodies. Then, the membrane was stained with coomassie brillant blue.

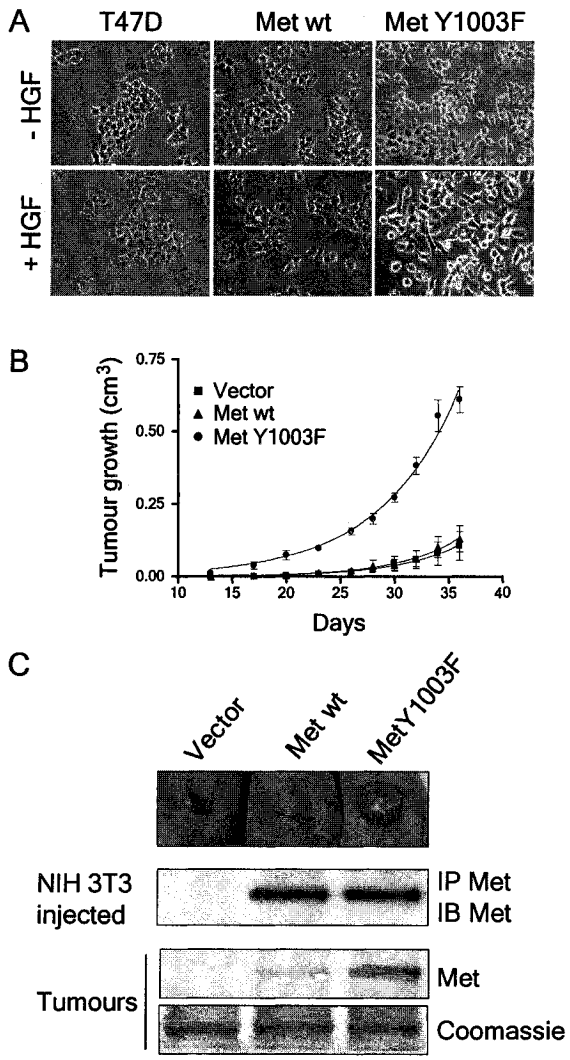
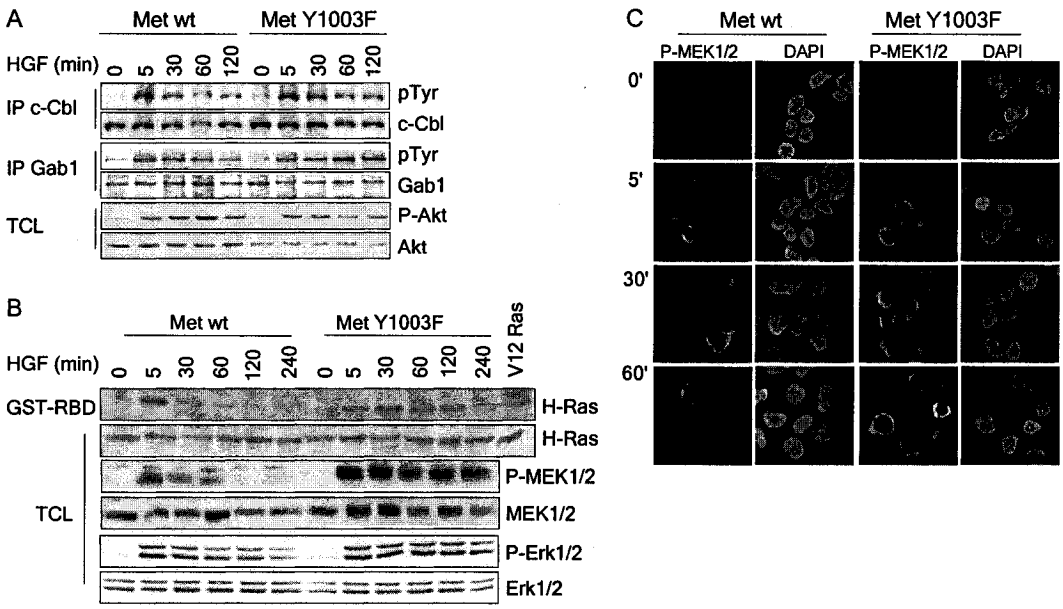


Figure 3. Activation of the Ras-MAPK but not the PI3K pathway is sustained downstream from Met Y1003F.

(A) T47D cells were stimulated with 1.5 nM HGF for the indicated time points and lysed in TGH buffer. In the upper two panels, c-Cbl proteins were immunoprecipitated and immunoblotted with phosphotyrosine antibodies. Membranes were stripped and blotted with Cbl antibodies. In the middle two panels, Gab1 proteins were immunoprecipitated and blotted with both anti pTyr, 4G10 and Gab1 antibodies using the Odyssey Infrared Imaging System (LI-COR). In the bottom two panels, lysates were blotted with either phospho-Ser473 or total Akt antibodies as indicated using the Odyssey system. **(B)** T47D cells were stimulated with 1.5 nM HGF and lysed as above. Upper two panels; Ras activation was determined using an in vitro binding assay with the GST-RBD fusion protein, with HEK293T cells expressing V12 H-Ras as a positive control. Panels were blotted with an H-Ras antibody using the Odyssey System. In the bottom four panels, cell lysates were blotted with phospho-Ser217/221 MEK1/2, total MEK1/2, phospho-Thr202/Tyr204 Erk1/2 and total Erk1/2 antibodies as indicated using the Odyssey system. **(C)** T47D cells were plated on cover slips, serum starved for 16 hours and stimulated with 1.5 nM HGF at 37°C for the indicated time points. Cover slips were fixed in 3% paraformaldehyde (PFA), and stained with phospho-Ser217/221 MEK1/2 (first pannel). Cell nuclei were visualized using DAPI (second panel). Confocal images were taken with a 100x objective and 2x zoom. Bar represents 5 mm.

Figure 3


A
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Met Y1003F
HGF (min)
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pTyr
c-Cbl
IP Gab1
pTyr
Gab1
TCL
P-Akt
Akt
B
Met wt
Met Y1003F
HGF (min)
0 5 30 60 120 240 0 5 30 60 120 240 V12 Ras
GST-RBD
H-Ras
H-Ras
P-MEK1/2
TCL
MEK1/2
P-Erk1/2
Erk1/2
C
Met wt
Met Y1003F
P-MEK1/2
DAPI
P-MEK1/2
DAPI
0'
5'
30'
60'

Figure 4. The Ras-MAPK is required for Met Y1003F increased biological activity.

MDCK cells stably expressing either wt CSF-Met or CSF-Met Y1003F, where indicated, were treated with CSF (2.7 nM), UO126 (20 mM) and LY294002 (25 mM) for 24 hours. After 24 hours, phase-contrast pictures of live cells were taken with a Zeiss Axiovision 135 microscope under a 10x objective. Bar represents 100 mm.

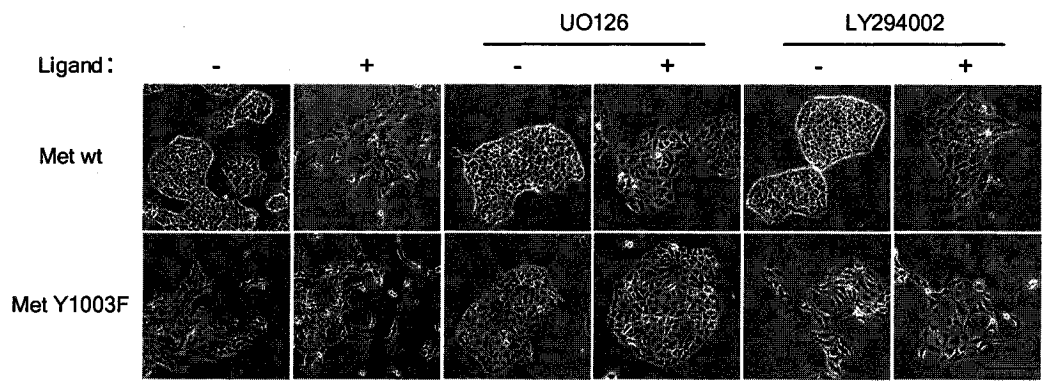


Figure 5. The ubiquitination-deficient Met receptor mutant is internalised.
(A) T47D cells were stimulated with 3 nM HGF at 37°C and then transferred on ice. Cells were acidified for 10 min and then treated with trypsin for 30 min. Trypsin was inhibited before cells were lysed. Lysates were blotted with Met (DL-21) and c-Cbl antibodies. **(B)** T47D cells were stimulated with 3nM HGF at 37°C and then transferred on ice. Cells were acidified for 10 min and then labelled with Met AF276 antibody as described under methods. The mean fluorescence intensity per cell was measured by flow cytometry and the percentage of Met receptors remaining at the cell surface over time is plotted. The graph represents the mean ±SD of three independent experiments. **(C)** Both Met wt and Met Y1003F internalise and traffic to early endosomes. T47D cells were plated on cover slips, and 16 hours later stimulated with 1.5 nM HGF at 37°C for the indicated time points. Cover slips were fixed in 3% PFA, and stained with Met AF276 (red) and EEA1 (green) antibodies. Confocal images were taken with a 100x objective and 2x zoom. Yellow staining represents co-localization between Met and EEA1. Bar represents 5 mm.

Figure 5

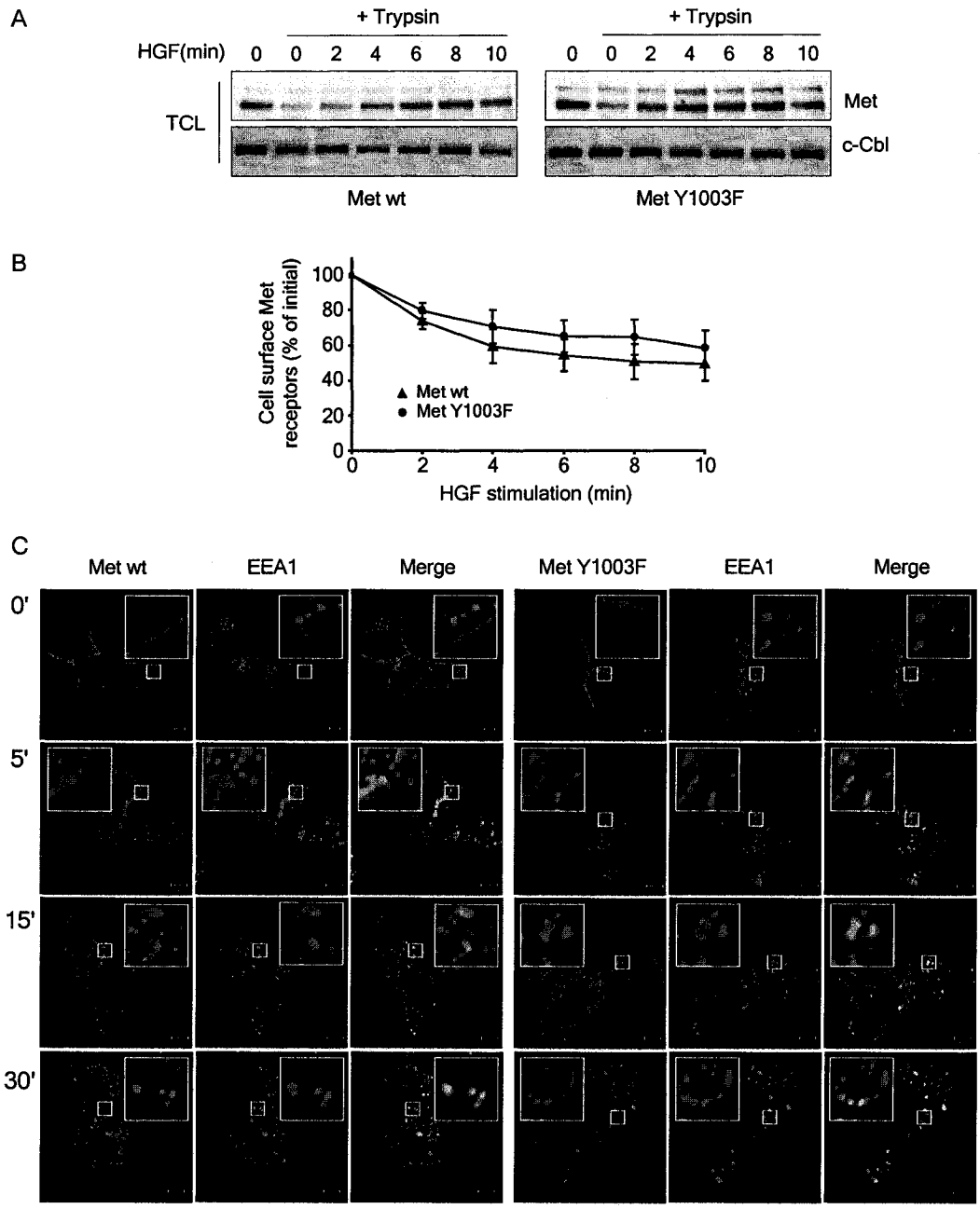

A
+ Trypsin
+ Trypsin
HGF(min) 0 0 2 4 6 8 10
0 0 2 4 6 8 10
TCL
Met
c-Cbl
Met wt
Met Y1003F
B
Cell surface Met receptors (% of initial)
100
80
60
40
20
0
Met wt
Met Y1003F
0 2 4 6 8 10
HGF stimulation (min)
C
Met wt
EEA1
Merge
Met Y1003F
EEA1
Merge
0'
5'
15'
30'

Figure 6. A ubiquitination-deficient Met receptor mutant is unable to induce Hrs tyrosine phosphorylation.

(A) T47D cells were serum starved for 16 hours and stimulated where indicated with 1.5 nM HGF for 8 minutes. Cells were then lysed in TGH buffer and Hrs and Met proteins were immunoprecipitated and first blotted with phosphotyrosine antibodies, stripped and reblotted with Hrs and Met antibodies as indicated. **(B and C)** wt Met and Met Y1003F both co-localize with endogenous Hrs and transfected GFP-Rab7. T47D cells, non-transfected (B) and transfected (C) were plated on cover slips and 16 hours later stimulated with 1.5 nM HGF at 37°C for the indicated time points. Cover slips were fixed with 3% PFA and stained with (B and C) Met AF276 (red) and (B) Hrs (green) antibodies. Yellow staining represents co-localization between Met and Hrs or Met and GFP-Rab7. Confocal images were taken with a 100x objective and 2x zoom. Bar represents 5mm.

Figure 6

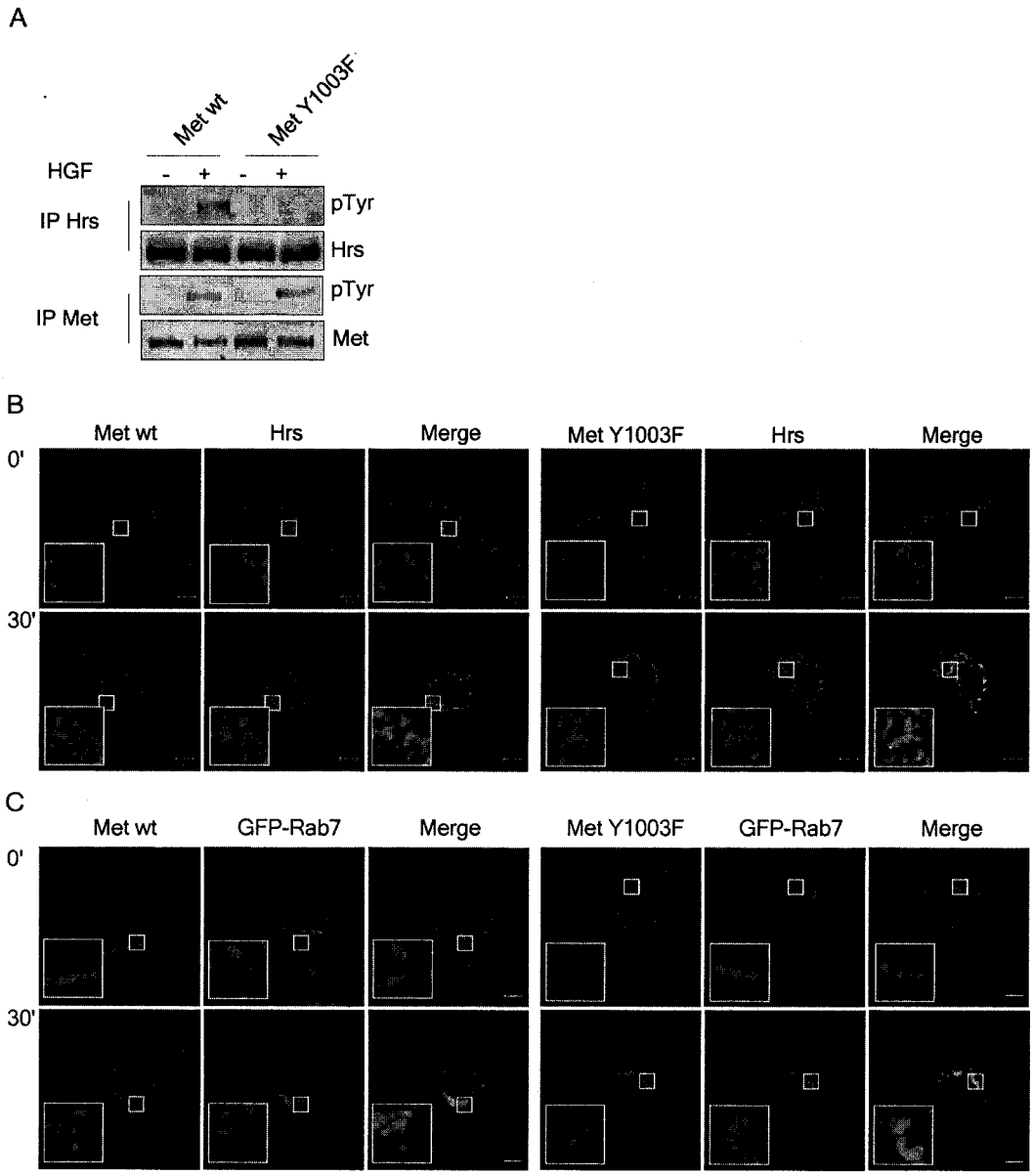
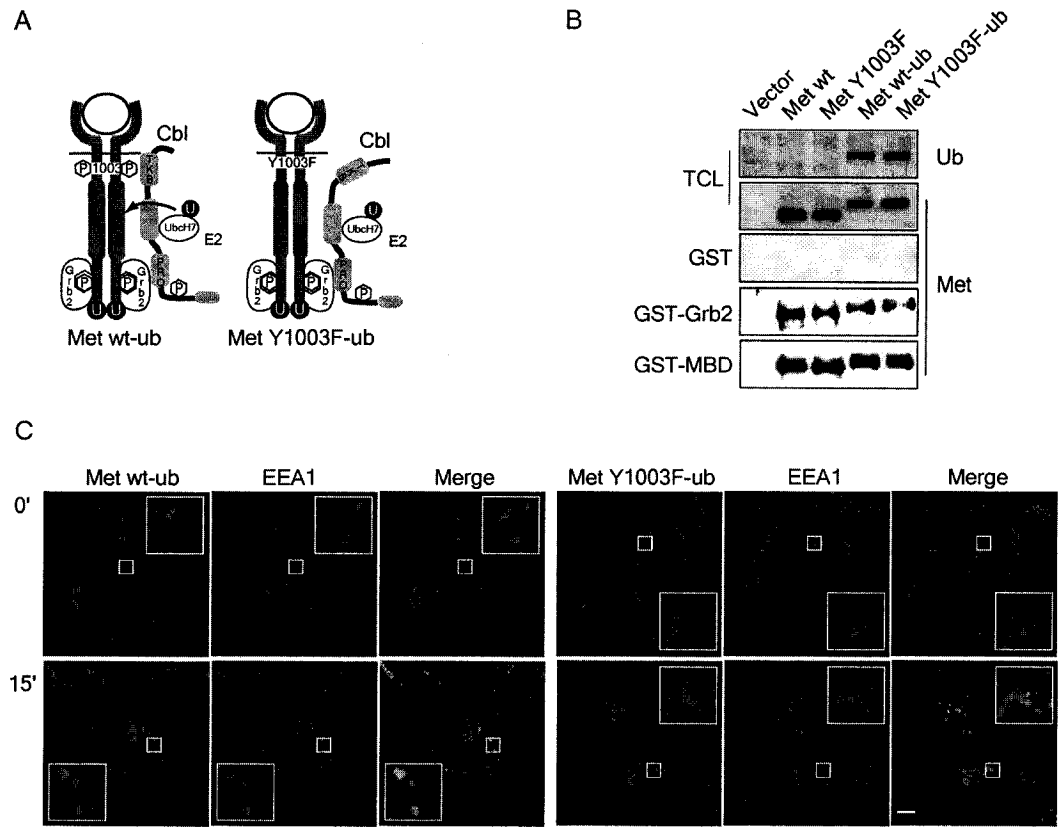

A
Met wt
Met Y1003F
HGF
- + - +
IP Hrs
pTyr
Hrs
IP Met
pTyr
Met
B
Met wt
Hrs
Merge
Met Y1003F
Hrs
Merge
0'
30'
C
Met wt
GFP-Rab7
Merge
Met Y1003F
GFP-Rab7
Merge
0'
30'

Figure 7. Fusion of monoubiquitin to the carboxy-terminus of the Met receptor does not alter its maturation to the cell surface, recruitment of signalling proteins and its HGF-induced internalisation.

(A) Schematic representation of Met wt-ubiquitin and Met Y1003F-ubiquitin chimeric receptors. Met receptor chimeras were generated with a single ubiquitin moiety fused to the carboxy-terminal end of the full-length wt and Y1003F Met receptors. The seven lysine residues within the ubiquitin moiety are substituted for arginine residues to prevent polyubiquitination and the carboxy-terminal glycine residue is substituted for a valine residue to prevent conjugation to free amino groups. **(B)** The chimeric receptors are properly expressed and tyrosine phosphorylated. HEK293 cells were transiently transfected with the indicated CSF-Met constructs. In the top two panels, lysates were blotted with Met (144) and ubiquitin antibodies. In the bottom three panels, lysates were incubated with GST alone, GST-Grb2 or GST-MBD (Met Binding Domain of Gab1). In vitro binding assays and TCL were immunoblotted for Met. **(C)** T47D cells were plated on cover slips and 16 hours later, stimulated with 1.5 nM HGF at 37°C for 15 min. Cover slips were fixed in 3% PFA, and stained with Met AF276 (red) and EEA1 (green) antibodies. Confocal images were taken with a 100x objective and 2x zoom. Yellow staining represents co-localization between Met and EEA1. Bar represents 5 mm.

Figure 7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Cbl
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E2
UbcH7
Met wt-ub
Cbl
Y1003F
E2
UbcH7
Met Y1003F-ub
B
Vector
Met wt
Met Y1003F
Met wt-ub
Met Y1003F-ub
TCL
Ub
GST
GST-Grb2
GST-MBD
Met
C
Met wt-ub
EEA1
Merge
Met Y1003F-ub
EEA1
Merge
0'
15'

Figure 8. Monoubiquitination of the Met Y1003F receptor leads to its degradation and restores HGF-induced Hrs tyrosine phosphorylation.

(A) T47D cells treated with 100 μg/ml cycloheximide were stimulated with 3 nM HGF for the indicated amount of time. TCL (30 mg) were immunoblotted for Met (DL-21) and Erk. **(B)** T47D cells were stimulated with 1.5 nM HGF for the indicated time points and lysed in TGH buffer. Cell lysates were blotted with phospho-Ser217/221 MEK1/2 and total MEK1/2. **(C)** HEK293 cells were co-transfected with GFP-Hrs and vector or the indicated CSF-Met constructs. GFP-Hrs and Met proteins were immunoprecipitated and blotted with phosphotyrosine antibodies then stripped and reblotted for GFP and Met respectively.

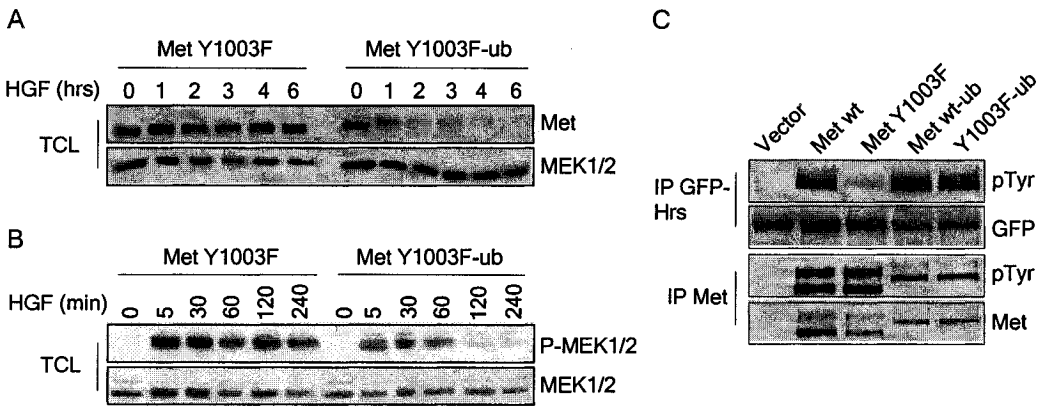
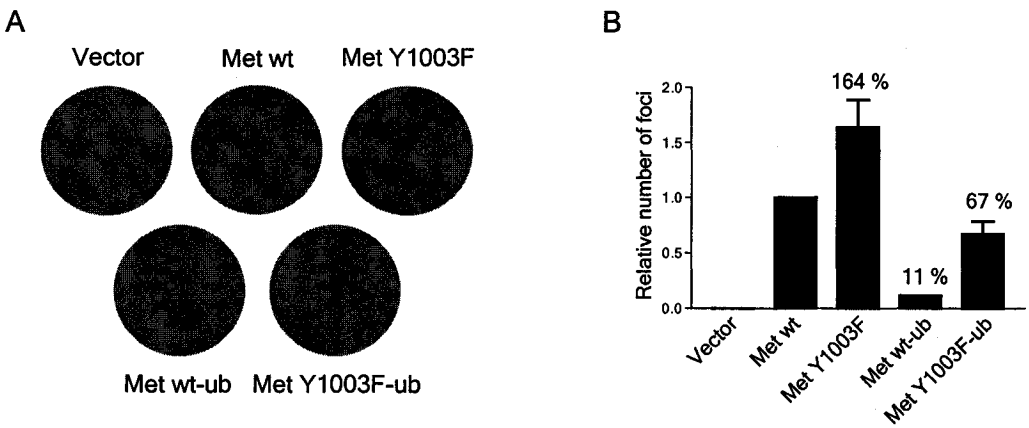


Figure 9. Monoubiquitination of the Met Y1003F receptor mutant is sufficient to reverse cell transformation.

Rat-1 fibroblast cells were infected with retroviruses encoding the various CSF-Met constructs and grown for 3 weeks in the presence of 0.3 nM of CSF-1. **(A)** Representative pictures of cell monolayers. **(B)** The graph represents the relative number of foci per dish corrected for the plaque-forming unit of each retrovirus, from 2 independent experiments.



Discussion

In this study, we provide a mechanistic understanding for the oncogenic activation of the Met RTK through its uncoupling from ubiquitination. We demonstrate that the specific uncoupling of the Met RTK from Cbl-mediated ubiquitination, (Met Y1003F), promotes oncogenic activation of Met *in vivo* and *in vitro* (Fig. 2). Moreover, we show that cell transformation by the Met Y1003F receptor mutant, or a ligand-activated wt Met receptor, can be reversed through the fusion of monoubiquitin to these receptors (Fig. 9), demonstrating the importance of ubiquitination as a mechanism to suppress the inherent oncogenic activity of deregulated RTKs.

When uncoupled from Cbl-mediated ubiquitination, the Met receptor, as shown for the EGFR (26, 62), has a prolonged half-life (Fig. 1D and E), which leads to elevated Met protein levels in the stable cell populations and in the tumours (Fig. 1B and 2C). This supports a previous study where ubiquitination of the Met receptor was shown to correlate with ligand-induced degradation (63). Importantly, the enhanced stability of Met is reversed in stable cell lines following the fusion of monoubiquitin to the carboxy-terminus of the Met Y1003F mutant receptor (Fig. 8A). This provides direct evidence that monoubiquitin is sufficient to target the Met receptor to a degradative pathway. This is in agreement with the observation that monoubiquitination of EGFR is sufficient to target this receptor for degradation (24).

Although proteasomal inhibitors inhibit Met receptor degradation and appears to promote its recycling to the cell surface (59), it remained unknown whether ubiquitination of the Met receptor itself is important for its internalisation and trafficking. We demonstrate here that the inability of the Met Y1003F receptor mutant to become ubiquitinated by Cbl does not alter its ability to internalise or enter the endocytic pathway in response to ligand. Using several approaches, we show that both the Met wt and Y1003F receptors are internalised into the endocytic pathway and can traffic to endosomes that contain the cargo sorting protein, Hrs (Fig. 5 and 6B) and late endosomes containing Rab 7 (Fig. 6C). This indicates that signals other than ubiquitination can promote ligand-induced Met receptor internalisation. These may involve Grb2 (62), the adaptor role of Cbl (43, 44), as well as Cbl-mediated ubiquitination of proteins other than Met (58), since Cbl is still recruited to the Met Y1003F receptor indirectly via Grb2 (2).

Monoubiquitination of a severely truncated or full-length EGFR was shown to be sufficient for constitutive internalisation when overexpressed in transient assays (23, 24). We observed that in stable cell populations, the constitutively monoubiquitinated Met wt-ub and Y1003F-ub chimeric receptors are mainly localized at the cell surface and do not co-localize with EEA1 in the absence of stimulation (Fig. 7C). Hence, monoubiquitination of the Met RTK is not sufficient for its internalisation, which remains ligand-dependent. This may reflect differences between transient overexpression versus stable cell lines, where receptor activation is dependent on ligand. Alternatively, a monoubiquitin moiety on a truncated EGFR lacking its entire cytosolic domain may be recognised in a different manner than a monoubiquitin moiety fused to a full-length Met RTK. This highlights the need to fully understand the interplay between monoubiquitin and other signals involved in internalisation, which may be specific for each receptor.

The ubiquitination of lysine residues in Met could control the interaction of Met with endosomal sorting proteins that contain ubiquitin interacting domains, such as Hrs. Although Hrs is phosphorylated following stimulation of cells with HGF or EGF (36), a requirement for the direct ubiquitination of RTKs for Hrs phosphorylation had not been tested. Our data demonstrates that the ability of Met to promote phosphorylation of Hrs is dependent on ubiquitination of the Met receptor. When compared to wt Met, the Met Y1003F receptor mutant fails to robustly phosphorylate Hrs (Fig. 6A). Importantly, this can be rescued through the fusion of monoubiquitin to the carboxy-terminus of the Met Y1003F receptor (Fig. 8C). Hence, our data reveal that both the stability of Met and the phosphorylation of Hrs are dependent on the direct ubiquitination of the Met receptor. Interestingly, recent data of Row *et al.* have established that the Hrs UIM domain is required for Hrs phosphorylation downstream from the Met receptor (64). Together, this provides support for a model where the interaction of a ubiquitinated Met RTK with the sorting machinery, involving Hrs, is important for the targeting of the Met receptor for efficient lysosomal degradation. Consistent with this, the specific depletion of Hrs with siRNA enhances the stability of the Met receptor (59).

In addition to enhanced stability, the Met Y1003F receptor also has prolonged tyrosine phosphorylation following ligand stimulation. This results in sustained activation of the Ras-MAPK pathway, whereas no significant difference was

observed in the activation of Akt, a downstream target of PI3K (Fig. 3). In agreement with this, the transforming and scatter activity of the Met Y1003F receptor are dependent on the activation of MEK1/2 but not PI3K (Fig. 4). Whereas activation of Akt is thought to occur at the plasma membrane, the MAPK pathway can be activated from several subcellular compartments, including endosomes (15). Our results are consistent with the interpretation that the enhanced signalling of the Met Y1003F receptor, in particular to the Ras-MAPK pathway, reflects a receptor that is not efficiently targeted to the sorting machinery through its inability to couple to Hrs, and that instead remains in a signalling-competent endosomal compartment.

A decrease in ubiquitination of both the Met (2, 3, 50) and EGF RTKs (47-49), mediated through multiple mechanisms, has been associated with their oncogenic activation in cell-based models. Moreover, the observation that a Met receptor mutant identified in human lung cancer is missing exon 14, which contains the Cbl binding site (65), and that several proteins involved in RTK internalisation and lysosomal degradation are altered in human tumours and transformed cells (52, 66), strongly supports the notion that loss of RTK down-regulation is a common mechanism for oncogenic activation of RTKs in human tumours. Our studies now raise the need to understand how ubiquitination and altered trafficking of other RTKs impacts on their signalling and oncogenic activity.

Experimantal Procedures

Reagents and antibodies

Antibody 144 was raised against a carboxy-terminal peptide of the human Met protein (67). HA antibody was purchased from BABCO (Richmond, CA). pTyr (4G10), Met DO-24 and DL-21 and Gab1 antibodies are from Upstate Biotechnology (Lake Placid, NY). Anti-c-Cbl (SC-170), anti-ubiquitin (P4D1), are from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Met antibody AF276 is from R&D Systems (Minneapolis, MN). GFP antibody and Alexa 488-conjugated secondary antibodies are from Molecular Probes (Eugene, OR). Total and phospho-specific Erk1/2 (pThr202/pTyr204), MEK1/2 (pSer217/221) and Akt1 (pSer473) are from Cell Signaling Technology (Mississauga, ON). H-Ras and EEA1 antibodies were purchased from BD Biosciences (Mississauga, ON). HA monoclonal antibody was purchased from Covance (Berkeley CA). Hrs antibodies used for immunoprecipitation were previously described (59) and the Hrs antibody used for immunofluorescence was a kind gift of Dr. Stenmark. Odyssey Blocking Buffer, IRDye800 anti-rabbit and Cy5.5 anti-mouse secondary antibodies for use with the Odyssey Infrared Imaging System (Li-COR Biosciences Lincoln, NE) was purchased from Rockland (Gilbertsville, PA). U0126 inhibitor was purchased from Promega (Madison, WI), LY294002 was purchased from BIOMOL Research Labs (Plymouth meeting, PA). HGF was a kind gift from Dr. George Vande Woude (Van Andel Research Institute, Grand Rapids, MI) and recombinant CSF-1 was provided by the Genetics Institute (Boston, MA).

Quantitative real-time PCR

Total RNA was extracted from T47D cells using TRIzol reagent (Invitrogen Life Technologies) following the manufacturer's protocol. 5 μg of RNA was reverse transcribed with oligo dT (Invitrogen Life Technologies), and the cDNA was amplified using LightCycler-FastStart Reaction Mix SYBR Green I (Roche Molecular Biochemicals) and Rotor-Gene 3000 lightcycler (Corbett Research, Sydney, Australia). GAPDH was used as a control to normalize mRNA levels. Primer sequences were as follows: GAPDH, sense, 5'-ACCACAGTCCATGCCATCAC-3', and antisense, 5'-

TCCACCACCCTGTTGCTGTA-3'; Met, sense, 5'-GTTTGTCCACAGAGACTTGGCTG-3', and antisense, 5'-AGTTCAGAAAAGGATGGGCG-3'. The mean threshold cycle (Ct) value for each transcript was normalized by dividing it by the mean Ct value for the GAPDH transcript for that sample. Normalized transcript levels were expressed relative to sample obtained from T47D expressing wt Met.

***In vivo* tumorigenesis assays**

For tumorigenesis assays, NIH 3T3 fibroblast cells were infected with retroviruses expressing either nothing, wt Met or Met Y1003F and selected with 10 μg/ml G418 for two weeks. The obtained cell populations were injected subcutaneously ($5x10^5$ cells per 100 μl) into 4- to 5-week-old female nude mice (CD1 *nu/nu*, Charles River Breeding Laboratories). Tumours were measured periodically and mice were sacrificed prior to the tumours reaching 1 cm^3 or undergoing ulceration.

Cell culture, transfections and focus formation assays

T47D breast epithelial cells, Madin-Darby canine kidney (MDCK) cells and HEK 293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). MDCK cell lines expressing wt CSF-Met and CSF-Met Y1003F were established as described previously (2). T47D cell lines expressing wt Met and Met Y1003F were generated by retroviral infection. Following infection, cells were selected for three weeks in 4 μg/ml G418. Selected cell populations were then FACS sorted to select cells expressing Met at their cell surface using a MoFlo flow cytometer (Dako Cytomation, Fort Collins, CO). Cells were labelled with anti-Met (1:50) (R&D Systems) for 1 hour and with Alexa 488 donkey anti-goat (1:250) for 30 min in FACS buffer (DMEM containing 1% heat-inactivated FBS, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and then incubated for 5 min in FACS buffer containing 5 mM EDTA. $1x10^5$ to $2x10^5$ sorted cells were collected and plated in DMEM containing 10% FBS. Transient transfections in T47Ds were performed via electroporation. Cells were trypsinized, resuspended in PBS and electroporated at 0.30 kV and 0.975 μFD. Transient transfections in HEK 293 cells were performed using Lipofectamine plus reagent according to the manufacturer's instructions

(Invitrogen Life Technologies). The focus formation assays were performed as described previously (9). For T47D phase contrast pictures, $8x10^5$ cells were seeded in 60 mm dishes and 16 hours later were treated where indicated with 1.5 nM HGF for 24 hours in the presence of serum. For MDCK scatter assay, $1x10^4$ cells/well were seeded in 12 well plates and 18 hours later were treated where indicated with CSF (50 ng/ml), U0126 (20 μM), LY294002 (25 μM) in the presence of serum for 24 hours. Phase contrast images were then taken with a Zeiss Axiovision 135 microscope with a 25x objective (Carl Ziess Canada Ltd, Toronto, ON). Image analysis was carried out using Northern Eclipse version 6.0 (Empix Imaging, Mississauga, ON).

Immunoprecipitation and Western Blotting

Following stimulation with HGF, T47D cells were harvested in TGH lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium vanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin). HEK 293 transfections were harvested 22 hours post-transfection with RIPA buffer (0.05% SDS, 50mM Tris pH8.0, 150nM NaCl,1% Nonidet P-40, 0.05% sodium deoxycholate) or modified RIPA buffer (0.1% SDS, 25mM Tris pH 8.2, 50mM NaCl, % 0.5% Nonidet P-40, 0.5% sodium deoxycholate) supplemented with 1 mM PMSF, 1 mM sodium vanadate, 1mM sodium fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Lysates were incubated with the indicated antibody for 2 hours at 4°C with gentle rotation. Proteins collected on either protein A- or G-Sepharose were washed three times in their respective lysis buffers, resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in 3% BSA in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 0.1% Tween-20) for 1 hour, incubated with primary and secondary antibodies in TBST for 2 hours and 1 hour respectively. After four washes with TBST, bound proteins were visualized with an ECL detection kit (Amersham Biosciences). Analyses with the Odyssey infrared imaging system were performed according to the manufacturer's instructions. To detect Met receptor ubiquitination, cells from a 10 cm dish were lysed in 200 μl boiling buffer (2% SDS, 1 mM EDTA). Lysates were boiled for 10 min and diluted to 1 ml with a buffer containing 2.5% Triton, 12.5 mM Tris pH 7.5, 187.5 mM NaCl and proteasomal inhibitors (38).

Internalisation assays using trypsin and flow cytometry

Cells were seeded in 60-mm dishes and the following day, stimulated with 3 nM HGF. Stimulations were terminated by placing cells on ice and rinsing them with ice-cold DMEM. On ice, cells were acidified for 10 min with cold DMEM at pH 4.0 containing 1% BSA. For the trypsin assays, cells were rinsed once with ice-cold PBS and then incubated with 2 ml of 1 mg/ml trypsin in PBS (pH 7.4) for 30 min on ice. The reaction was stopped by adding 2 ml of 5 mg/ml soybean trypsin inhibitor in ice-cold PBS. Cells were collected, centrifuged for 5 min at 200 g at 4°C, washed once with 1 ml of 5 mg/ml soybean trypsin inhibitor in PBS (ice-cold) and lysed in TGH lysis buffer. For flow cytometry analysis, cells were washed once in ice-cold FACS buffer (PBS containing 1% heat-inactivated FBS, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). On ice, cells were then incubated with Met antibody (AF206) in ice-cold FACS buffer for 45 min, washed 3 times with FACS buffer, incubated with donkey anti-goat Alexa 488 in FACS buffer for 20 min, washed 3 times and incubated 5 min in 0.5 ml FACS buffer containing 5mM EDTA. The cells were scraped and analysed with the FACScan flow cytometer (BD Biosciences).

Confocal Immunofluorescence microscopy

Cells were seeded at $2x10^5$ on glass cover slips (Bellco Glass Inc. Vineland, NJ) in 24 well plates (Nalge NUNC, Rochester, NY) and 16 hours later were serum starved for 2 hours prior to HGF treatment (1.5nM) where indicated. Cover slips were washed once with PBS then fixed with 3% paraformaldehyde (PFA, Fisher Scientific) in PBS for 20 minutes. Cover slips were then washed four times in PBS and residual PFA was removed with three 5-minute washes with 100 mM glycine in PBS. Cells were permeabilised with 0.3% Triton X-100/ PBS and blocked for 30 minutes with blocking buffer (5% BSA, 0.2%Triton X-100, 0.05%Tween-20, PBS). Cover slips were incubated with primary and secondary antibodies diluted in blocking buffer for 1 hour and 40 minutes respectively at room temperature. Cells stained for Hrs were permeabilised with 0.05% saponin and fixed with 3% PFA as described previously (68). Cover slips were mounted with immu-mount (Thermo-Shandon, Pittsburgh, PA). Confocal images were taken using a Zeiss 510 Meta laser scanning confocal microscope (Carl Ziess,

Canada Ltd, Toronto, ON) with 100x objective and 2x zoom. Image analysis was carried out using the LSM 5 image browser (Empix Imaging, Mississauga, ON).

Generation of the Met-ubiquitin chimera receptors and other plasmids

We first inserted a XmaI site just before the stop codon in the human Met receptor cDNA using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the 5'-CCTCCTTCTGGGAGACATCAGCCCGGGGCTAGTACTATGTCAAAGC-3' primer and its reverse complement. Next we amplified by PCR a yeast ubiquitin cDNA that had the seven lysine residues mutated to arginine residues (generous gift of Dr. Linda Hicke) using the forward primer 5'-CGATTCCCGGGGTATGCAGATCTTCGTC-3' (XmaI site underlined) and reverse primer 5'-GAACTGCGGCCGCTAAACACCTCTTAGTCTTAAGACAAG-3' (NotI site and V76 underlined). The PCR product was fused in frame with Met cDNA using XmaI and NotI. The Hrs-HA construct has been described previously (38, 69). The GST-Grb2 and GST-MBD constructs have been described previously (13). GFP-Rab7 was a generous gift from Robert Lodge.

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Chapter 5

General discussion

1. The Met Y1003F receptor: a tool to examine the biological role of ubiquitination of the Met receptor

During the first year of my thesis work, several articles were published demonstrating that Cbl proteins possess ubiquitin ligase activity (1-3). Different research groups were examining Cbl-mediated ubiquitination and down-regulation of the EGF and PDGF receptors at the biochemical level. c-Cbl is highly tyrosine phosphorylated following stimulation of the Met receptor and in cells expressing the oncogenic Met receptor variant Tpr-Met (4-6), indicating that c-Cbl may be an important regulator of Met signalling. I decided to study Cbl-mediated regulation of the Met receptor and examine the biological significance of it. I first examined whether overexpression of c-Cbl promotes Met receptor ubiquitination. Indeed, expression of wt c-Cbl, but not of TKB or RING finger mutants of c-Cbl, induces Met receptor ubiquitination (Chapt. 2, Fig. 1). As for the EGFR, the TKB and RING finger domain of c-Cbl are required for c-Cbl-dependent receptor ubiquitination. We had observed that the c-Cbl TKB domain binds to the Met receptor, but not to the Tpr-Met oncoprotein (Chapt. 2, Fig. 2B), implying that the binding site for the c-Cbl TKB domain has been lost following chromosomal rearrangement. Two tyrosine residues (Y969 and Y1003) in the juxtamembrane domain of Met are absent in Tpr-Met and previous work had demonstrated that the Y1003F substitution in Met leads to a partial gain-of-function of the Met receptor (7). Thus, I tested whether Y1003 is the c-Cbl TKB binding site. The Y1003F substitution, but not the Y1349F or Y1356F substitution, prevents c-Cbl TKB domain association with the Met receptor (Chapt. 2, Fig. 2). To further prove that Y1003 is the c-Cbl TKB binding site in the Met receptor, I reintroduced Y1003 and its surrounding sequence in Tpr-Met and demonstrated that this Tpr-Met variant is now able to bind to the c-Cbl TKB domain (data not shown). Since the TKB domain of c-Cbl is required for c-Cbl-mediated Met receptor ubiquitination, I examined whether the c-Cbl TKB domain binding site in Met, Y1003, is required for c-Cbl-mediated ubiquitination. Consistent with a requirement for Y1003 for c-Cbl TKB domain binding, the Met Y1003F receptor is poorly ubiquitinated by c-Cbl (Chapt. 2, Fig. 3).

In epithelial cell populations stably expressing the Met Y1003F receptor, the Met Y1003F is weakly ubiquitinated following stimulation with HGF whereas the wt Met receptor is robustly ubiquitinated (Chapt. 4, Fig. 1D). This implies that Cbl

proteins are the major ubiquitin ligases for the Met receptor, unless other ubiquitin ligases promote Met receptor ubiquitination in a pY1003-dependent manner. The Met Y1003F receptor mutant remains associated with c-Cbl via the Grb2 adaptor protein and is able to induce tyrosine phosphorylation of c-Cbl to a similar level as the wt Met receptor (Chapt. 2, Fig. 2B,D). Thus, the signalling and adaptor functions of c-Cbl are not detectably altered by the inability of the c-Cbl TKB domain to engage with Y1003F (Fig. 1).

This supports similar studies with the EGFR where phosphorylation of one tyrosine (1045) is required for the recruitment of the c-Cbl TKB domain and another tyrosine is required for the indirect recruitment of c-Cbl through Grb2. However, unlike the EGFR, where the TKB domain of all three Cbl family members (c-Cbl, Cbl-b and Cbl-c) interacts with tyrosine 1045, only the c-Cbl and Cbl-b TKB domains interact with Met (Chapt. 3, Fig. 3).

Several studies have demonstrated that Cbl-b can induce the ubiquitination of EGFR to a similar level as c-Cbl (8). Therefore, it is likely that Cbl-b can induce ubiquitination of the Met receptor, although this has not yet been tested. Hence, the Met Y1003F receptor represents a unique tool to study the role of ubiquitination of the Met receptor at the biochemical and biological level.

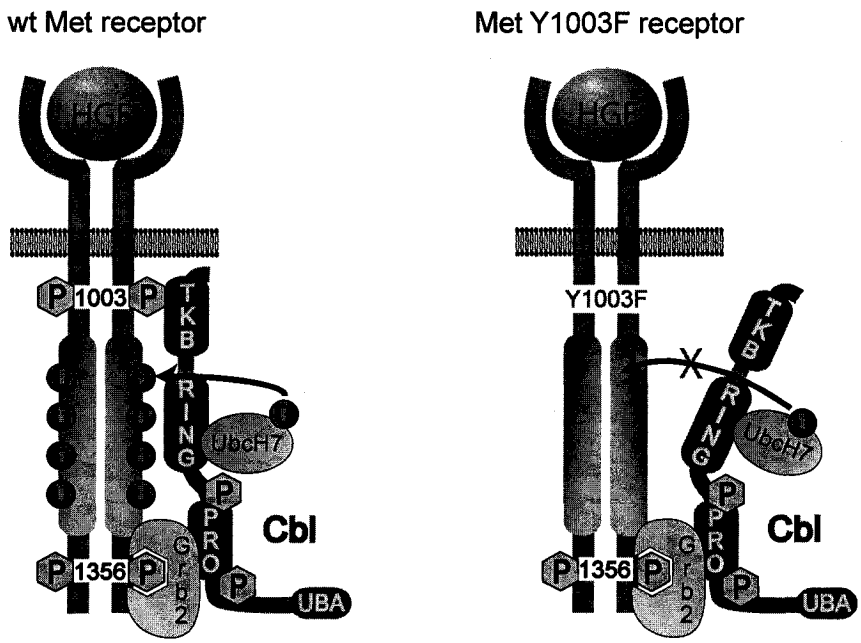

Figure 1. Dual interaction between c-Cbl and the Met receptor.

2. The uncoupling of ubiquitination from the Met receptor leads to its oncogenic activation both *in vitro* and *in vivo*

When I started my thesis, several studies provided data supporting a role for ubiquitination in the down-regulation of RTKs (9-11). The corollary of this observation is that blocking the ubiquitination of a RTK should enhance its biological activity. I tested this hypothesis by examining the biological activity of the Met Y1003F receptor in different biological assays. In focus forming assays in rodent fibroblasts, whilst the wt Met receptor is unable to transform Rat1 fibroblasts in the absence of ligand, the Met Y1003F shows transforming activity (Chapt. 2, Fig. 4A - Chapt. 3, Fig. 4B,C), which may reflect low levels of ligand produced by fibroblasts in culture. Consistent with this, the Met receptor is tyrosine phosphorylated in Rat1 cells in the absence of exogenous ligand.

Similarly, the expression of the Met Y1003F receptor in populations of NIH 3T3 fibroblasts cells induces morphological transformation in the absence of exogenous ligand, whereas the wt Met receptor fails to do so (Chapt. 2, Fig. 4B). Moreover, NIH 3T3 cells stably expressing the Met Y1003F receptor form tumours at a much faster rate than NIH 3T3 cells stably expressing the wt Met receptor when injected subcutaneously into nude mice (Chapt. 4, Fig. 2B,C). Hence, the specific uncoupling of ubiquitination from the Met receptor leads to Met receptor oncogenic activation both in *in vitro* and *in vivo* tumorigenesis assays. In epithelial cells, where the Met receptor is normally expressed, the Met Y1003F receptor induces an epithelial-mesenchymal like transition in the absence of ligand (Chapt. 2, Fig. 4C - Chapt. 4, Fig. 2A). This is a hallmark of an activated Met receptor. Why this receptor elicits a biological activity in the absence of ligand is not fully understood, but may reflect an enhanced stability of the protein and elevated basal kinase activity. It has been reported that cell adhesion elicits ligand-independent activation of the Met receptor and this may also explain Met Y1003F receptor biological activity in the absence of ligand (12, 13). Although we do see elevated levels of the Met Y1003F receptor in stable cell lines when compared to the wt Met receptor, this is rarely more than two fold.

To understand why the Met Y1003F receptor is transforming, we examined its cellular signalling. In response to ligand, we observed that Ras-MAPK pathway activation is sustained downstream of Met Y1003F, while it is transient downstream of wt Met (Chap. 4, Fig. 3). Furthermore, a pharmacological inhibitor

of MEK1/2 (UO126) induced a reversal in the epithelial-mesenchymal-like transition mediated by the Met Y1003F receptor. These results support numerous studies implicating the activation of the Ras-MAPK pathway in cellular transformation (14).

To formally prove that Met Y1003F is transforming because it is poorly ubiquitinated, a ubiquitin moiety devoid of lysine residues was fused to the C-terminus of wt Met and Met Y1003F (Chapt. 4, Fig. 7). The presence of a single ubiquitin moiety at the C-terminus of the Met Y1003F receptor is sufficient to revert its transforming activity in a focus-forming assay (Chapt. 4, Fig. 8). Consistent with this, the activation of the Ras-MAPK pathway is transient downstream of the Met Y1003F-ubiquitin chimeric receptor (Chapt. 4, Fig. 8B)

Altogether, these results demonstrate that the ubiquitination of the Met RTK is critical to regulate its normal biological functions.

3. Dual interaction between c-Cbl and the Met receptor

In Chapter 2, I demonstrate that c-Cbl is recruited to the Met receptor by two mechanisms: indirectly via the Grb2 adaptor protein binding to Y1356 and directly via the TKB domain binding to Y1003 (Fig. 1). c-Cbl is recruited in a similar manner to EGFR (15). The N-terminal SH3 domain of Grb2 is constitutively bound to a proline-rich domain in c-Cbl (16, 17). Upon Met receptor activation, the Grb2-Cbl complex is recruited to Y1356 in Met and c-Cbl becomes tyrosine phosphorylated. Grb2-mediated recruitment of c-Cbl seems to be more predominant than the TKB-mediated recruitment. Deletion of the Grb2 binding site in Met, but not of the TKB binding site, abrogates the recruitment and tyrosine phosphorylation of c-Cbl (Chapt. 2, Fig. 2B,D) (4, 5), whereas the direct association of the TKB domain with Y1003 is required for Met receptor ubiquitination. The stoichiometry of the Cbl-Grb2-Met interaction has not been examined and it remains to be determined whether the same c-Cbl molecule can bind to Y1003 and to Y1356 via Grb2. The reason why the c-Cbl TKB domain/RTK interaction is required for RTK ubiquitination is also obscure. The crystal structure of c-Cbl complexed with UbcH7 revealed that the TKB domain, linker sequence and RING finger domain form a compact structure with UbcH7 (18). This implies that the c-Cbl TKB and RING finger domain may orient UbcH7 in a precise way with respect to the substrate to ubiquitinate (18). Possibly, Grb2-

mediated recruitment of c-Cbl does not orient UbcH7 properly with respect to the substrate. This can be overcome by vastly overexpressing Grb2 and Cbl, which can then promote ubiquitination of an EGFR mutant lacking the Cbl TKB binding site (15). Insights on the structure of the C-terminal half of c-Cbl, where the Grb2 binding site is located, would help elucidate this question.

4. Identification of a new binding motif for the TKB domain of c-Cbl and Cbl-b

Studies on the c-Cbl TKB domain opened new avenues for our understanding of phosphotyrosine-binding domains. The discovery that the c-Cbl TKB domain contains the fold of an SH2 domain generated a lot of interest given that it is divergent at the amino acid level (19). This suggests that there may be more SH2 domains in nature than the number estimated by sequence analysis (20). In addition, from the work presented in Chapter 3 and a recent publication by Hubbard's group (21), it appears that the c-Cbl TKB domain is able to bind to multiple consensus sequences, a first for a phosphotyrosine-binding domain.

The c-Cbl TKB domain was known to bind to the (D/N)XpY(S/T)X(D/E)Φ consensus sequence present in Zap-70, EGFR, Syk, Src, Fyn and Sprouty-1/2. However, the c-Cbl TKB domain binding site in Met (Y1003) does not match this consensus sequence (Chapt. 3, Fig. 1A). For this reason, I performed alanine-scanning mutagenesis of the sequence surrounding Y1003 and identified the DpYR motif as being required for c-Cbl TKB association (Chapt. 3, Fig. 2,3). This motif is conserved throughout the Met receptor family as well as in plexins, which are receptors for semaphorins (Chapt. 3, Fig. 7). Consistent with this, the DpYR motif found in Ron is required for the recruitment of the c-Cbl TKB domain and for c-Cbl-mediated ubiquitination and degradation of Ron (22).

In collaboration with Noboru Ishiyama and Albert Berghuis at McGill University, we attempted to crystallize the c-Cbl TKB domain in complex with the Met pY1003 phosphopeptide. In the crystals analyzed, the structure of the c-Cbl TKB domain was virtually identical to the one published (19). However, phosphotyrosine was the only detectable residue in the pY1003 phosphopeptide. This implies that the phosphopeptide backbone is poorly ordered, which would reflect the biochemical results where none of the amino acids surrounding the DpYR core motif were required for the c-Cbl TKB domain/Y1003 association.

Noboru Ishiyama modeled the pY1003 phosphopeptide on the structure of the c-Cbl/ZAP-70 complex and predicted the presence of a salt bridge between the side chains of D1002 and R1004 amino acid residues (Chapt. 3, Fig. 6). Mutation of either of these two residues would disrupt the salt bridge that could be required to orient pY1003 in the phosphotyrosine binding pocket.

Recently, Hubbard's group identified a new consensus sequence for the c-Cbl TKB domain present in APS, SH2-B and Lnk: RA(V/I)XNQpY(S/T) (21). This consensus sequence was defined based on x-ray crystallography, site-directed mutagenesis and calorimetric studies. The interaction between the c-Cbl TKB domain and the APS phosphopeptide is mediated essentially by the phosphotyrosine and amino acid residues N-terminal to the phosphotyrosine, which form several bonds with the four-helix (4H) bundle (21). This is in contrast with the c-Cbl TKB domain/Zap-70 phosphopeptide interaction that is mediated primarily by the phosphotyrosine and amino acid residues C-terminal to the phosphotyrosine (Table 1) (19).

Table 1. Consensus sequence of different phosphotyrosine binding domains

Domain	Consensus sequence	
Src SH2	**pY** X X I	
p85 SH2	**pY** X X M	
Grb2 SH2	**pY** X N X	
Shc PTB	N P X **pY**	
c-Cbl TKB	D/N X **pY** (S/T) X (D/E) Φ	(EGFR, Zap-70, etc)
	D **pY** R	(Met, Ron, plexins)
	R A (V/I) X N Q **pY** (S/T)	(APS, SH2-B, Lnk)

Other well-studied phosphotyrosine binding domains, such as the Src SH2, p85 SH2, Grb2 SH2 and Shc PTB are known to bind one consensus sequence (Table 1). The c-Cbl TKB domain is unique in its ability to bind more than one consensus sequence. This correlates with the presence of the 4H bundle and EF-hand calcium-binding domain, which render the c-Cbl TKB domain more complex than classic SH2 and PTB domains. We can speculate that the c-Cbl

TKB domain evolved in this way to enable c-Cbl to interact with an increasing number of proteins.

I observed that the TKB domain of c-Cbl and Cbl-b, but not of Cbl-c, is able to bind to the Met receptor (Chapt. 3, Fig. 3). This is in contrast with the ability of the Cbl-c TKB domain to bind to the EGFR (23). Unless the Cbl-c TKB domain binds to a phosphotyrosine other than Y1045 in EGFR (c-Cbl TKB binding site), this means that whilst the (D/N)XpY(S/T)X(D/E)Φ consensus sequence found in EGFR binds the TKB domain of c-Cbl, Cbl-b and Cbl-c, the DpYR core motif binds only the c-Cbl and Cbl-b TKB domain. Since Cbl-c and Met are both expressed in most epithelial cells, the absence of Cbl-c binding site in Met may be important for Met biological activity.

5. Ubiquitination of the Met receptor is not required for internalization

In Chapter 4, we examined the ability of the Met Y1003F receptor to internalize upon HGF stimulation. Using biochemical, flow cytometry and confocal immunofluorescence techniques, we observed that Met wt and Y1003F receptors internalize with similar kinetics (Chapt. 4, Fig. 5). This is in agreement with five studies that demonstrated that the ubiquitination-deficient EGFR mutant (Y1045F) internalizes with similar kinetics to that of the wt EGFR. Depending on the cell type used for the assays, internalization kinetics were either virtually identical or the EGFR Y1045F receptor was internalized at 70 to 80% of the rate of wt EGFR (2, 15, 24-26). Moreover, overexpression of v-Cbl, which prevents EGFR ubiquitination, did not affect EGFR internalization (10). Overall, we can conclude that the ubiquitination of the Met and EGF receptor is not required for their internalization. Consistent with this, ubiquitination of the G protein-coupled β2-adrenergic receptor is required for its degradation, but not for its internalization (27). This is in contrast with what is observed in yeast, where several cell-surface proteins must be ubiquitinated to be internalized (28). How can we explain this discrepancy between yeast and mammals?

Clathrin-mediated internalization is essential for the uptake of many cell-surface proteins in both mammalian cells and yeast (29, 30). In mammalian cells, the adaptor protein complexes (APs) as well as the AP180/CALM proteins play a pivotal role in clathrin coat assembly and are required for clathrin-mediated internalization (30). However, in yeast, this is not the case. Simultaneous

disruption of genes encoding for AP subunits and AP180 did not alter clathrin-mediated internalization, implying that other factors may stimulate clathrin coat assembly (31, 32). It is possible that ubiquitin may be involved in clathrin coat assembly in yeast and that mammalian cells evolved in such a way that APs and AP180/CALM are now fulfilling this role. This could in part explain why ubiquitination is less important for the internalization of mammalian cell surface proteins. Nevertheless, it seems that mammalian cells have retained the ability to recognize ubiquitin as an internalization motif, since the fusion of a monoubiquitin moiety to the extracellular and transmembrane domain of EGFR is sufficient to induce the internalization of the truncated receptor (33). We observed that fusion of monoubiquitin to the full-length Met receptor does not promote its constitutive internalization, as the Met-ubiquitin chimeric receptor is mainly localized at the cell-surface in the absence of HGF (Chapt. 4, Fig. 7C), indicating that signals other than ubiquitination are required for Met receptor internalization. There are several putative sorting signals present within the cytoplasmic portion of Met and structure-function studies will be necessary to identify the ones involved in Met receptor internalization.

6. Ubiquitination of the Met receptor is required for lysosomal degradation

When I began my thesis, the first evidence that ubiquitination may target the EGF receptor for lysosomal degradation was published (10). Overexpression of c-Cbl, Cbl-b or Cbl-c induces EGFR ubiquitination and degradation (2, 8, 10, 34) and an EGFR mutant lacking the Cbl TKB binding site (Y1045F) is refractive to EGF-induced EGFR degradation (2, 26). Similarly, I observed that ligand-induced degradation of the Met Y1003F receptor is greatly delayed when compared to the degradation of wt Met receptor (Chapt. 4, Fig. 1E,F).

Initially, the stability of the CSF-Met wt and Y1003F chimeric receptors were compared in Rat1 fibroblast cells in the absence of ligand. I established by pulse chase analysis that the half-life of the CSF-Met Y1003F receptor (1.2 hours) is prolonged when compared to the CSF-Met wt receptor (0.7 hours) (Chapt. 2, Fig. 5A,B). In contrast, the half-life of the Met receptor in epithelial cells in the absence of ligand is approximately 5 hours (data not shown) (35). Discrepancies in the half-life of CSF-Met and Met receptors may be due to a difference in the basal kinase activity of the receptors and/or the cell type used. The difference in

the stability of the Met wt and Y1003F receptors is more pronounced in the presence of ligand (Chapt. 4, Fig. 1E,F). This is consistent with the concept that HGF stimulation induces the ubiquitination of the Met receptor and that the Met Y1003F receptor escapes ubiquitination-mediated degradation.

Lysosomal degradation of cell surface receptors requires their internalization and trafficking to sorting endosomes where they must undergo inward vesiculation. The latter prevents receptor recycling to the cell surface and terminates signalling by sequestering the cytoplasmic domain of the receptor. A fraction of the limiting membrane of sorting endosomes is covered by a flat bilayered clathrin coat where EGFR and Hrs are enriched (36, 37). Hrs is an endosomal protein that possesses a ubiquitin-interacting motif (UIM), a phosphatidylinositol 3-phosphate binding domain (FYVE) as well as a clathrin binding domain (clathrin box). Studies in yeast and mammalian cells suggest that Hrs may be involved in the retention of ubiquitinated receptors within the bilayered clathrin coat, leading to receptor inward vesiculation (38).

As discussed in the previous section, Met wt and Y1003F receptors internalize at similar rates. In addition, we have established that both receptors traffic to early endosomes (EEA1), sorting endosomes (Hrs) and late endosomes (Rab7) (Chapt. 4, Fig. 5,6). However, only the wt Met receptor is able to induce tyrosine phosphorylation of Hrs, implying that the Met Y1003F receptor is not retained within the bilayered clathrin coat by Hrs (Chapt. 4, Fig. 5A). Hence, we predict that the Met Y1003F receptor remains at the limiting membrane of sorting endosomes, late endosomes and ultimately, lysosomes and that Met Y1003F may keep signalling from some of these compartments. This would be consistent with the prolonged activation of the Ras-MAPK pathway observed downstream of the Met Y1003F receptor. The fate of receptors at the limiting membrane of lysosomes is also unclear. The extracellular domain of the receptor is in the lumen of the lysosome, but is not necessarily degraded since it is highly glycosylated. Electron microscopy studies of the Met wt and Y1003F receptors, and signalling partners like Grb2, would be required to learn more about Met receptor signalling from endosomes.

Fusion of ubiquitin to the C-terminus of Met Y1003F receptor rescues ligand-induced Met receptor degradation and its ability to induce the tyrosine phosphorylation of Hrs (Chapt. 4, Fig. 8A,C). This demonstrates that a single

ubiquitin moiety appended to the receptor is sufficient for its retention within the bilayered coat and its lysosomal degradation. In addition, the position of the appended ubiquitin moiety does not seem to be critical for Met receptor sorting.

7. The Y1003F mutation increases the tumorigenicity of the Met RTK in mammary glands

Most of the studies on the ubiquitination of RTKs have been performed *in vitro* or transient transfection assays where Cbl and/or RTKs are overexpressed and cells are stimulated with non-physiological amounts of ligand. Therefore, one major concern is the physiological relevance of the ubiquitination of RTKs. The fact that Cbl proteins are responsible for the ubiquitination of many RTKs, including EGFR, PDGFR α and β, CSF-1R, Met and Ron, and that c-Cbl/Cbl-b double null mice are embryonic lethal, may indicate that Cbl-mediated ubiquitination of RTKs plays a critical role *in vivo*. However, functions of Cbl other than RTK ubiquitination, for example in the regulation of T-cell signalling in thymocytes and peripheral T-cells, may be responsible for the embryonic lethality of the c-Cbl/Cbl-b double null mice. To examine the implications of loss of ubiquitination of the Met receptor *in vivo*, I generated transgenic mice expressing the Met Y1003F receptor.

7.1 Design of the transgenes

Several groups have generated mouse models expressing HGF or oncogenic forms of the Met receptor (39-43). Transgenic mice ubiquitously expressing an oncogenic Met receptor under the metallothionein promoter typically developed mammary tumours and some had severe breeding difficulties (39, 43). For these reasons, we decided to use the mouse mammary tumour virus (MMTV) promoter/enhancer. This promoter is active in mammary epithelium starting at puberty and its activity is elevated during pregnancy. Since human Met is not efficiently activated by mouse HGF (44), a chimeric receptor was generated consisting of the extracellular domain of the murine Met receptor fused to the transmembrane and intracellular domain of human Met (Fig. 2). Importantly, this allowed us to easily distinguish the chimeric Met receptor from the endogenous Met receptor by using an antibody that specifically recognizes the carboxy-terminus of human Met, but not of mouse Met. The murine-human Met Y1003F

chimeric receptor and the human Met Y1003F receptor display similar transforming activity in focus-forming assays (Stephanie Petkiewicz, unpublished data), establishing that the murine-human Met chimeras are functional receptors. Several mutations located in the kinase domain of Met, which enhance Met kinase activity, were identified in papillary renal and hepatocellular carcinomas (e.g. M1250T) (45-49). A Met receptor lacking exon 14, which contains Y1003, has recently been identified in lung carcinomas (50). Moreover, there is cumulating evidence that other events observed in human tumours may prevent the ubiquitination of RTKs, including the Met receptor. For example, overexpression of Src, Sprouty2 or Cdc42 sequesters Cbl proteins and impairs RTK ubiquitination (51-55). To examine the possibility of synergy between events leading to increase tyrosine kinase activity and loss of down-regulation of the Met receptor, I created a chimeric mouse/human Met receptor carrying two point mutations, Y1003F and M1250T. The latter is located in the kinase domain of Met and has been identified in papillary renal carcinomas (45, 46). MMTV-Met mice were derived on a uniform FVB/N genetic background that is known to be permissive for mammary tumorigenesis (56). For each of the following transgene: wt Met, Met Y1003F, Met M1250T and Met Y1003F/M1250T, we obtained about nine founder mice and kept three independent lines in which the transgene was passed to offspring with the expected frequency.

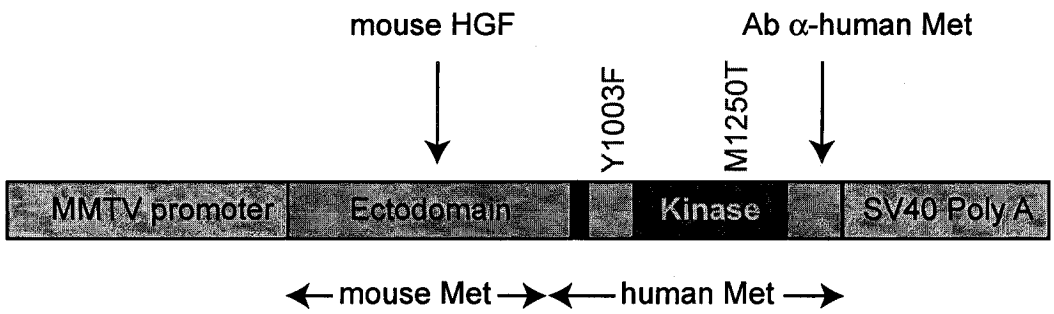

Figure 2. Design of the transgenes

7.2 Synergistic effect of the Y1003F and M1250T mutations on the tumorigenicity of the Met receptor

To date, one of the transgenic mice carrying the chimeric mouse/human wt Met allele developed a mammary tumour whereas two and three mice, respectively carrying a Met Y1003F and Met M1250T allele, developed mammary tumours (Table 1). Although it is too early to draw conclusions, both the Y1003F and M1250T mutations may be sufficient to increase to a detectable level the tumorigenicity of the Met receptor in mammary glands. Moreover, there is clearly synergy between the Y1003F and M1250T mutation in increasing the tumorigenicity of Met, as 24% (10/42) of the mice carrying a Met Y1003F/M1250T allele developed mammary tumours (Table 1). In addition, the average latency of tumour appearance is shorter in the transgenic mice carrying a Met Y1003F/M1250T allele (Table 1). Formalin-fixed paraffin-embedded tissue sections were prepared for each tumour and examined following hematoxylin-and-eosin (H&E) staining. All tumours retain a glandular phenotype, most frequently papillary adenocarcinomas, but display varying nuclear grades (Fig. 3A-D). Some Y1003F/M1250T tumour-bearing mice developed metastatic nodules in the lung. Protein lysates prepared from mammary tumours and normal adjacent tissue revealed high levels of the mouse/human chimeric Met receptor in the tumours, as detected with antisera specific to the carboxy-terminal region of human Met (Fig. 3E). This observation implies that there is a natural selection for cells that express the Met Y1003F/M1250T receptor. The phenotype we observed in the Met Y1003F/M1250T transgenic mice is reminiscent of the mammary hyperplasia observed in c-Cbl null mice, suggesting that enhanced RTK activity may be responsible for mammary hyperplasia in c-Cbl null mice. Hence, the Y1003F substitution in the Met receptor undoubtedly enhances its biological activity *in vivo. These results constitute the first evidence that ubiquitination of an RTK is required for its biological functions. Moreover, this is the first time that synergy between an activating mutation (M1250T) and a loss of down-regulation mutation (Y1003F) is observed in RTK-mediated tumorigenesis and we predict that this is likely to occur in human cancers.*

Overall, the Met transgenic mice we generated will constitute an important animal model to study the role of the Met receptor in mammary tumorigenesis and metastasis.

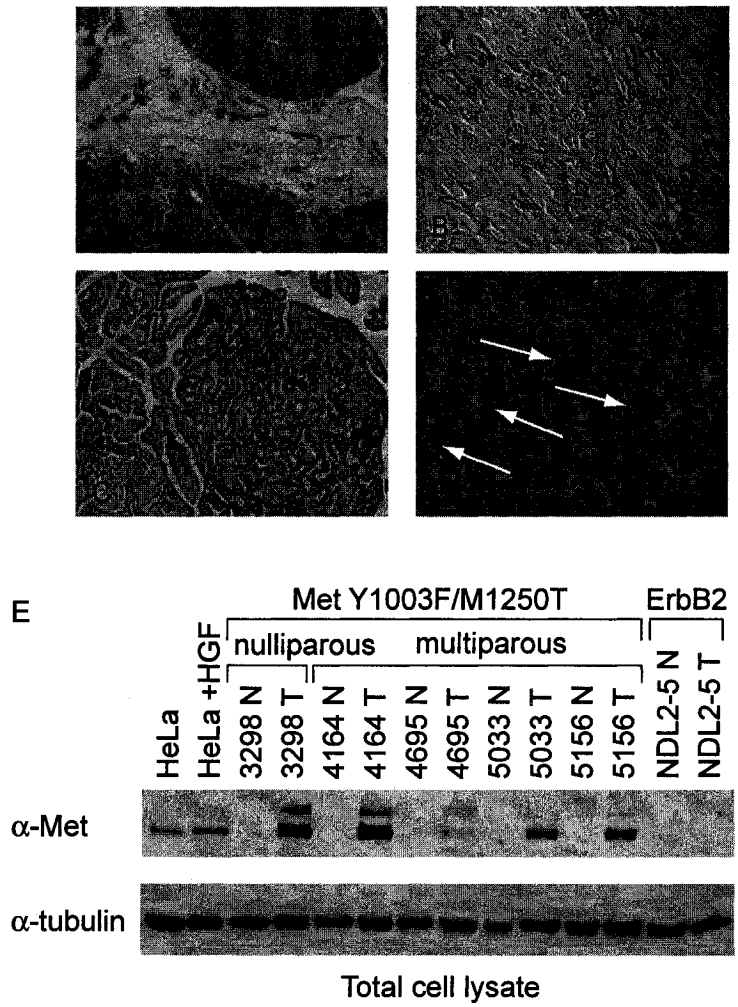

Figure 3. Mice expressing the Met Y1003F/M1250T receptor developed mammary adenocarcinomas.

A-D) Morphologies of the mammary tumours in mice expressing the Met Y1003F/M1250T receptor. **A)** Solid nodular ErbB2-like adenocarcinoma (10X). **B)** Well-differentiated invasive tubular adenocarcinoma invading muscle tissue (20X). **C)** Well-differentiated tubulo-papillary adenocarcinoma (20X). **D)** High nuclear grade showing polymorphic nuclei and several mitotic figures including one abnormal figure (arrows) (40X).
E) The chimeric mouse/human Met Y1003F/M1250T is overexpressed in mammary adenocarcinomas. Total cell lysate was immunoblotted with antibodies raised against the intracellular domain of human Met, stripped and reblotted with α-tubulin antibodies. N, normal adjacent tissue; T, tumour tissue.

Figure by Stephanie Petkiewicz

Table 2. Mammary tumour development in MMTV-Met Transgenic Mice

	Nulliparous			Multiparous			Overall
Strain	mice with tumours/total mice	average latency (days)	mice with multiple tumours	mice with tumours/total mice	average latency (days)	mice with multiple tumours	frequency of tumours
FVB	0/15	N/A	0	0/10	N/A	0	0
WT	0/25	N/A	0	1/13 (8%)	474	0	1/38 (3%)
Y1003F	0/23	N/A	0	2/14 (14%)	670	0	2/37 (5%)
M1250T	0/12	N/A	0	3/11 (27%)	547	1/3 (33%)	3/23 (13%)
Y1003F M1250T	2/19 (11%)	441	1/2 (50%)	8/23 (35%)	378	6/8 (75%)	10/42 (24%)

Data provided by Stephanie Petkiewicz

8. Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases

Recently, it has become evident that several oncogenic forms of RTKs have lost the ability to recruit Cbl in a TKB-mediated manner (Met/HGFR, CSF-1R, c-Kit/SCFR, EGFR). These receptors normally recruit Cbl following ligand stimulation, and for several of them, Cbl has been shown to promote receptor ubiquitination and enhanced degradation. I have summarized the molecular alterations that uncouple oncogenic RTKs from Cbl-mediated ubiquitination (Appendix 1) (57).

8.1 Met/HGF receptor

An oncogenic form of the Met receptor, Tpr-Met, was generated following a carcinogen-induced chromosomal rearrangement that fused a protein dimerization domain (Tpr) to the kinase domain of the Met receptor. This results in the deletion of the juxtamembrane tyrosine-binding site for c-Cbl (Y1003). The Tpr-Met RTK oncoprotein is constitutively activated, but fails to bind c-Cbl and is not ubiquitinated (Fig. 4) (58). This suggests that loss of Cbl recruitment and ubiquitination contribute to the oncogenic deregulation of Tpr-Met. Another member of the Met family of RTKs, c-Sea, was originally isolated as the cellular homologue of the avian erythroblastosis retroviral oncoprotein v-Sea. Like Tpr-Met, the c-Cbl TKB binding site located in the juxtamembrane domain of c-Sea is

deleted in the v-Sea oncogenic receptor (Fig. 4). More recently, a Met receptor splicing variant that is missing exon 14, which contains Y1003, has been identified in lung carcinomas by two independent research groups (50). As predicted, one of the groups observed that the MetΔexon14 receptor is refractive to Cbl-mediated ubiquitination (personal communication).

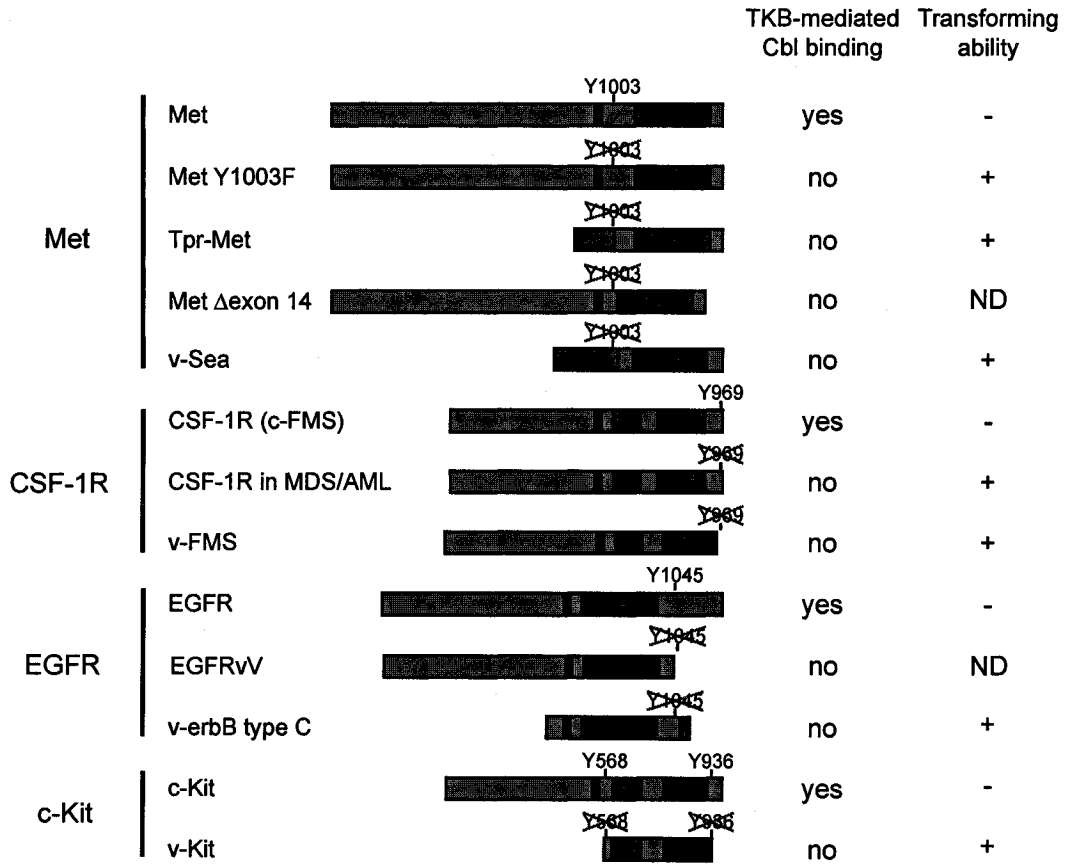

Figure 4. Loss of TKB-mediated Cbl binding in several RTK-derived oncoproteins

The binding of the c-Cbl TKB domain to the EGF, CSF-1 and Met receptors is required for receptor ubiquitination and degradation. Loss of TKB-mediated Cbl binding to RTKs correlates with a gain of transforming activity, suggesting that it constitutes an important event in RTK oncogenic deregulation.

8.2 Colony stimulating factor-1 receptor

In a similar manner, the CSF-1R was first identified as a viral oncogene, v-Fms, the product of the McDonough strain of feline sarcoma virus. One of the several structural differences between c-Fms/CSF-1R and v-Fms is the replacement of the CSF-1R carboxy-terminal 50 amino acids that contain the direct binding site for the c-Cbl TKB domain (tyrosine 969) by 14 unrelated amino acids in v-Fms (Fig. 4) (59, 60). The deletion of the c-Cbl direct binding site in v-Fms renders v-Fms refractory to c-Cbl-dependent ubiquitination and the addition of the c-Cbl TKB domain binding site to v-Fms decreases its transforming activity (59). Consistent with these observations, mutation of the C-terminal tyrosine 969, the direct binding site for c-Cbl, enhanced the transforming ability of CSF-1R in fibroblasts (61). Mutations of the Cbl binding site are frequently observed in CSF-1R in human myelodysplasia (8 out of 67 cases) and acute myeloblastic leukemia (8 out of 48 cases), further implicating loss of c-Cbl binding in oncogenic deregulation of CSF-1R in human cancer (62).

8.3 Stem cell factor receptor, c-Kit

The recruitment of c-Cbl to the stem cell factor (SCF) receptor/c-Kit and to PDGFRβ occurs indirectly via the APS (adaptor containing PH and SH2 domains) protein. This association leads to PDGFR downregulation (63). Notably, the two APS binding sites in c-Kit, tyrosine 568 located in the juxtamembrane domain and tyrosine 936 located in the carboxy-terminal region of the receptor, (64) are absent in v-Kit, the transforming protein of the Hardy-Zuckerman-4 strain of feline sarcoma virus (Fig. 4). Moreover, the deletion of APS binding sites in c-Kit greatly enhanced its transforming ability (65) although some of this may be attributed to the loss of negative regulation through juxtamembrane tyrosine 568 and enhanced catalytic activity of the receptor (66).

8.4 Epidermal growth factor receptor

Upon activation of EGFR (ErbB1), c-Cbl proteins are rapidly recruited and remain associated with the receptor as it progresses through the endocytic pathway (67). Tyrosine 1045 constitutes the direct binding site for the c-Cbl TKB domain and is required for c-Cbl-mediated ubiquitination and degradation of EGFR (2). Interestingly, the v-erbB protein encoded by the avian erythroblastosis

virus AEV-C has, among other alterations, an internal deletion of 21 amino acids that comprises the c-Cbl TKB domain binding site (Fig. 4) (68). Moreover, in a similar manner to both Met and CSF-1 receptors, an EGFR mutant lacking only the direct Cbl-binding site elicits stronger mitogenic signals than the wt receptor (15).

Up to 40% of glioblastomas express oncogenic mutants of EGFR. The most common genetic alteration consists of a deletion of exons 2-7 located in the extracellular domain of EGFR (67% of all EGFR genetic alterations) (69). The resulting protein (EGFRvIII) is constitutively activated, but has low levels of tyrosine phosphorylation. EGFRvIII receptors have a reduced ability to recruit Cbl proteins and CIN85, and are neither ubiquitinated nor internalized (70), suggesting that basal receptor activation is sufficient to confer tumorigenicity, but not sufficient to trigger Cbl-mediated downregulation. Another genetic alteration of EGFR identified in human glioblastoma generates a truncated receptor (EGFRvV) that has an intact kinase domain, but that is missing the c-Cbl TKB direct binding site (Y1045) and the internalization signals (Fig. 4) (69).

Another member of the EGF receptor family, HER2/ErbB2, is overexpressed in many human tumours such as breast, ovary, prostate and brain tumors. Overexpression of HER2 shifts the formation of EGFR homodimers towards the formation of EGFR/HER2 heterodimers. While ligand stimulated EGFR homodimers undergo rapid ubiquitination, internalization and degradation, EGFR/HER2 heterodimers recruit c-Cbl to a reduced extent, are slowly internalized and recycle rapidly to the cell surface (71, 72). The delay in EGFR/HER2 heterodimer degradation potentiates EGFR-dependent cell proliferation, migration and anti-apoptotic signals. Hence, the overexpression of HER2 constitutes a mechanism through which EGFR escapes Cbl-mediated downregulation. Indeed, overexpression or amplification of Cbl-binding RTKs, as frequently observed in human cancers, could act as a mechanism to sequester Cbl proteins, enhancing the stability of other RTKs that are Cbl substrates. In addition, the deregulation of Src, which occurs in human colon, breast, lung and brain cancers, leads to the stabilization of the EGFR by promoting c-Cbl ubiquitination and degradation (52).

Besides point mutations and overexpression, RTKs are frequently activated in human tumours following chromosomal translocation. In general, this fuses a

protein dimerization domain with the cytosolic kinase domain of the receptor, resulting in constitutive receptor dimerization and activation (73). Over 25 RTK-derived fusion proteins have been identified in human tumours. In each case, the N-terminal signal peptide, necessary for protein targeting to the membrane, is deleted in the rearranged kinase and, with the exception of FIG-ROS that is targeted to the golgi (74), where studied, these proteins are cytosolic (73). Localization to the cytosol would preclude their entry in the endocytic pathway and hence, their lysosomal targeting and degradation. However, it remains to be determined whether these oncoproteins are ubiquitinated and targeted for degradation by the proteasomal pathway.

9. Summary and perspectives

In the past few years, the discovery that Cbl proteins are ubiquitin-protein ligases and that ubiquitination regulates receptor sorting to lysosomes has greatly improved our understanding of the molecular mechanisms responsible for the down-regulation of RTKs. In this thesis, I have demonstrated that loss of ubiquitination increases the tumorigenicity of the Met receptor *in vivo*. Section 8 of this discussion brings to light evidence that many RTK-derived oncoproteins avoid down-regulation by loss of Cbl-binding sites, inefficient Cbl recruitment, Cbl degradation, or through the formation of fusion proteins that escape lysosomal degradation. These constitute some mechanisms among several that promote the stabilization of RTKs. Overexpression of a RTK, which is often observed in human tumours, may also be sufficient to deplete Cbl proteins and thus decrease the efficiency of downregulation of other RTKs that are not overexpressed.

Overall, an understanding of the mechanisms through which RTKs are down-regulated will allow new therapeutic approaches to target these RTKs for degradation in cancer. In this respect, Trastuzumab, a drug used in combination with chemotherapy for the treatment of metastatic breast cancer, is a weak agonist of HER2. Trastuzumab is thought to force receptor dimerization and potentiate recruitment of Cbl and receptor downregulation (75). Similar drugs designed to force other RTKs towards a degradation pathway would likely be important in the design of therapeutic strategies for cancer.

Abbreviations

4H	Four-helix bundle
AP180	Assembly Protein 180
APS	Adaptor containing PH and SH2 Domain
CALM	Clathrin Assembly Lymphoid Myeloid Leukemia
CIN85	85K Cbl-Interacting Protein
CSF	Colony Stimulating Factor
EEA1	Early Endosome Autoantigen-1
EGF(R)	Epidermal Growth Factor (Receptor)
FIG	Fused In Glioblastoma
FVB/N	Friend Leukemia B Virus strain
FYVE	Fab-1, YGL023, Vps27, and EEA1
Grb2	Growth Factor Receptor-Bound Protein 2
H&E	Hematoxylin and Eosin
HER2	Heregulin Receptor 2
HGF	Hepatocyte Growth Factor
Hrs	Hepatocyte Growth Factor Regulated Tyrosine Kinase Substrate
Lnk	Lymphocyte specific adapter protein
MAPK	Mitogen Activating Protein Kinase
MEK1/2	MAP Kinase Kinase 1/2
MMTV	Mouse Mammary Tumour Virus
p85	Regulatory Subunit of PI3'K
PDGF	Platelet Derived Growth Factor (Receptor)
PH	Pleckstrin-homology
Pro	Proline Rich Domain
PTB	Protein Tyrosine Binding
Rab7	Ras in the Brain7
RING	Really New Interesting Gene
Ron	Recepteur d'Origine Nantais
RTK	Receptor Tyrosine Kinase
SCF	Stem Cell Factor
Sea	Sarcoma, Erythroblastosis and anemia
SH2	Src Homology 2
SH3	Src Homology 3
Shc	Src homology 2 domain containing
Syk	Spleen Tyrosine Kinase
T-Cell	Thymocyte
TKB	Tyrosine Kinase Binding
Tpr	Translocated Promoter Region
UBA	Ubiquitin-Associated domain
UIM	Ubiquitin Interacting motif
ZAP-70	Zeta-Associated Protein 70

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Chapter 6

Original contributions to research

Original contributions to research

1. Demonstrated that c-Cbl promotes Met receptor ubiquitination and that this is dependent on the direct association of the c-Cbl TKB domain with the Y1003 residue in the juxtamembrane of the Met receptor.
2. Demonstrated that ubiquitination of the Met receptor is critical to suppress its inherent transforming activity. The ubiquitination-deficient Met Y1003F receptor transforms different epithelial and fibroblast cell lines and is tumorigenic *in vivo*. The ability of the Met Y1003F receptor mutant to transform fibroblast cells is reversed by the fusion of monoubiquitin to its carboxy-terminus.
3. Demonstrated that the oncogenic form of Met, Tpr-Met, lacks the c-Cbl/Cbl-b TKB binding site and is not ubiquitinated by c-Cbl. c-Cbl overexpression promotes the ubiquitination of the Met receptor, but not of the Tpr-Met oncoprotein.
4. Demonstrated that ubiquitination of the Met receptor is required for its lysosomal degradation. In the presence of HGF, the ubiquitination-deficient Met Y1003F receptor is not degraded efficiently and this is reversed by the fusion of monoubiquitin to the carboxy-terminus of the Met Y1003F receptor mutant.
5. Demonstrated that ubiquitination of the Met receptor is not required for its internalisation. The ubiquitination-deficient Met Y1003F receptor is internalised upon HGF stimulation.
6. Identified a new binding motif (DpYR) for the c-Cbl and Cbl-b TKB domain that is conserved in the juxtamembrane domain of all the members of Met receptor tyrosine kinase family. Identified for the first time that the Cbl phosphotyrosine binding domain can associate with more than one consensus sequence.
7. Demonstrated that the DpYR motif in Met is required for the ubiquitination of the Met receptor and for suppression of Met receptor oncogenic activation. Demonstrated that substitution of any residue in the DpYR motif blocks Met

receptor ubiquitination and increases the transforming activity of the Met receptor in a focus-forming assay.

8. Generated a mouse model that will be useful to study the role of the Met receptor in mammary tumorigenesis and metastasis. The transgenic mice expressing Met Y1003F/M1250T under the MMTV promoter constitute the first mouse model overexpressing a full-length Met receptor that systematically develops mammary tumours.

9. Demonstrated for the first time that ubiquitination of a receptor tyrosine kinase is required for its normal biological functions *in vivo*. The Y1003F substitution in the Met receptor increases Met receptor tumorigenicity in mouse mammary glands.

10. Demonstrated that there is synergy between an activating mutation (M1250T) and a loss of down-regulation mutation (Y1003F) in the tumorigenicity of the Met receptor.

11. Proposed in a minireview that escape from Cbl-mediated down-regulation contributes to the oncogenic deregulation of many receptor tyrosine kinases.

Escape from Cbl-mediated downregulation: A recurrent theme for oncogenic deregulation of receptor tyrosine kinases

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Deregulation of growth factor receptor tyrosine kinases (RTKs) is linked to a large number of malignancies. This occurs through a variety of mechanisms that result in enhanced activity of the receptor. Considerable evidence now supports the idea that loss of negative regulation plays an important role in receptor deregulation. RTKs are removed from the cell surface via endocytosis and many are subsequently degraded in the lysosome. Lysosomal targeting has recently been linked with receptor ubiquitination. We review here molecular alterations that uncouple RTKs from ubiquitination and implicate loss of ubiquitination as a process that plays a significant role in the pathogenesis of cancer.

Degradation of cellular regulatory proteins following their ubiquitination plays a critical role in controlling multiple physiological processes. Substrates of this pathway include tumor suppressor proteins (p53), cell cycle proteins ($p27^{Kip1}$), and transcription factors (E2F-1, fos, jun, myc, NF-κB). More recently, receptor tyrosine kinases (RTKs), including the colony-stimulating factor-1 receptor (CSF-1R), the epidermal growth factor receptor (EGFR), the hepatocyte growth factor receptor (HGFR/Met), and the platelet-derived growth factor receptor (PDGFR), have been identified as substrates for ubiquitination.

RTKs are single pass transmembrane proteins that control a wide variety of cellular events in pluricellular organisms including cell proliferation, cell differentiation, cell migration, and cell survival. In normal cells, RTK activation is tightly regulated. Their inappropriate activation is associated with the development and progression of many human malignancies. Of the 58 genes known to encode RTKs, the deregulation of 30 has been associated with human tumors (Blume-Jensen and Hunter, 2001). In the past two decades, several mechanisms that deregulate RTKs, such as receptor amplification, chromosomal translocation, and point mutations, have been identified. These changes result in ligand-independent activation or enhanced catalytic activity of RTKs (Blume-Jensen and Hunter, 2001; Lamorte and Park, 2001). However, in addition to these positive mechanisms, there is growing evidence that escape from negative regulatory mechanisms is an important event in RTK deregulation. In this review, we will examine how different RTK-derived oncoproteins escape downregulation.

In the absence of ligand, most RTKs are catalytically inactive. Binding of the ligand promotes receptor dimerization/oligomerization and induces a conformational change that triggers receptor kinase activity. Tyrosine residues on the receptor then become phosphorylated, forming binding sites for proteins that relay the biological signals. RTK activation promotes their internalization via clathrin-coated pits. The downregulation of tyrosine kinase activity and hence signaling can be modulated reversibly through the action of tyrosine phosphatases as well as irreversibly through their lysosomal degradation. Recent publications have established that ubiquitination plays a major role in RTK downregulation by targeting receptors to the lysosome (Shtiegman and Yarden, 2003).

Protein ubiquitination is mediated by an enzymatic cascade composed of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). A single 76 amino acid ubiquitin moiety is covalently conjugated to a lysine residue within the substrate protein. Additional ubiquitin residues can be added to lysine residues within the linked ubiquitin to generate polyubiquitin chains. The presence of a polyubiquitin chain on many cytosolic and nuclear proteins targets them for degradation by the 26S proteasome. In contrast, ubiquitination of many cell surface receptors correlates with their internalization and lysosomal degradation. Monoubiquitination of EGFR is sufficient for its internalization, and some evidence supports that EGFR and PDGFR are multimonoubiquitinated rather then being polyubiquitinated (Haglund et al., 2003; Mosesson et al., 2003).

Monoubiquitin moieties can constitute binding sites for proteins that contain ubiquitin binding domains such as ubiquitin interaction motif (UIM), ubiquitin-associated (UBA), and ubiquitin-conjugating-like (UBC-like) domains. The UIM-containing proteins, HRS and STAM, are thought to recruit ubiquitinated RTKs to ESCRT complexes that retain receptors in specialized microdomains of sorting endosomes characterized by a bilayered clathrin coat, from which receptors internalize into the endosomal lumen (Figure 1) (Clague, 2002). Sorting endosomes mature into multivesicular bodies (MVBs) where receptors remained trapped within internal vesicles. Fusion of MVBs with lysosomes leads to the degradation of internal vesicles and their content. RTKs that are not ubiquitinated are not sorted in the bilayered clathrin coat, can recycle back to the cell surface, and escape lysosomal degradation (Figure 1) (Katzmann et al., 2002).

The Cbl family of ubiquitin-protein ligases (c-Cbl, Cbl-b, and Cbl-3) plays a major role in the ligand-dependent ubiquitination of many RTKs (Thien and Langdon, 2001). Several receptors including EGFR, PDGFR, CSF-1R, and Met (HGFR) are ubiquitinated following recruitment of c-Cbl. Cbl ubiquitin-protein ligases are modular proteins that contain a conserved N-terminal tyrosine kinase binding (TKB) domain and a RING finger domain in addition to other protein interaction motifs (Thien

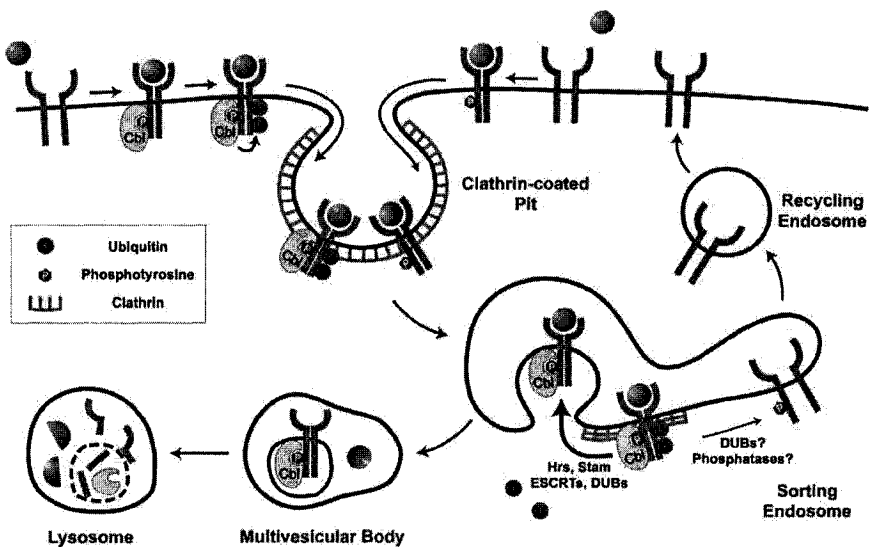

Figure 1. RTK ubiquitination and downregulation

Subsequent to the activation of several RTKs, c-Cbl is recruited to the receptor and induces receptor ubiquitination. Following internalization, ubiquitinated RTKs (green) are enriched in an endosomal microdomain characterized by a bilayered clathrin coat. Receptors are subsequently internalized in inner vesicles. The process of receptor enrichment and subsequent internalization involves multiple proteins that contain ubiquitin-interacting motifs (Hrs, Stam, and proteins of the ESCRT complexes) as well as deubiquitinating enzymes (DUBs) that remove ubiquitin moieties from the receptors. Fusion of multivesicular bodies with lysosomes leads to the degradation of inner vesicles and their content by lysosomal proteases. RTKs that are not ubiquitinated (red) are not sequestered in the bilayered clathrin coat of the sorting endosomes and can be recycled to the cell surface where they can be reactivated. Such receptors may be inactivated through dephosphorylation.

and Langdon, 2001). Where tested, the TKB domain interacts with specific phosphotyrosine residues on RTKs. The RING finger domain recruits the E2 ubiquitin-conjugating enzyme, UbcH7. Both domains are required for the transfer of ubiquitin residues to RTKs. Moreover, the stability of c-Cbl is itself regulated by ubiquitination. Tyrosine phosphorylation of c-Cbl by the c-Src protein tyrosine kinase promotes auto-ubiquitination of c-Cbl and its degradation in a proteasome-dependent manner (Yokouchi et al., 2001). The observation that Cbl proteins can also interact with ubiquitin-protein ligases containing HECT (*h*omologous to *E*6-AP *C* terminus) domains suggests a potential additional layer of regulation (Courbard et al., 2002).

In addition to the targeting of RTKs for lysosomal degradation following ubiquitination, several lines of evidence support a role for c-Cbl in the internalization of RTKs. The overexpression of c-Cbl enhances the rate of internalization of EGFR (Levkowitz et al., 1999; Soubeyran et al., 2002), whereas in c-Cbl null macrophages, CSF-1R has a slower rate of internalization (Lee et al., 1999). It has been proposed that c-Cbl promotes the internalization of EGFR and the Met receptor through its ability to recruit CIN85 and endophilins (Petrelli et al., 2002; Soubeyran et al., 2002). During RTK internalization, endophilins are thought to induce negative membrane curvature required for the invagination of the plasma membrane into pits.

Consistent with its role in the downmodulation of RTKs, mutant c-Cbl proteins that lack ubiquitin ligase activity have been identified in mouse tumors and as retrovirally transduced transforming proteins (Thien and Langdon, 2001). These proteins retain the ability to bind to phosphotyrosine residues on RTKs, yet fail to ubiquitinate the receptor. Hence, they are thought to compete with the binding of wt Cbl proteins to RTKs and to transform cells through their ability to potentiate RTK signals.

Recently, it has become evident that several oncogenic RTKs have lost the ability to recruit Cbl in a TKB-mediated manner (Met/HGFR, CSF-1R, c-Kit/SCFR, EGFR). These receptors normally recruit Cbl following ligand stimulation, and for several of them, Cbl has been shown to promote receptor ubiquitination and enhanced degradation.

Hepatocyte growth factor receptor

The c-Cbl TKB domain binds to a juxtamembrane tyrosine (1003) residue on the Met receptor, and this interaction is essential for ubiquitination and degradation of the Met receptor (Peschard et al., 2001). An oncogenic form of the Met receptor, Tpr-Met, was generated following a carcinogen-induced chromosomal rearrangement that fused a protein dimerization domain (Tpr) to the kinase domain of the Met receptor. This results in the deletion of the juxtamembrane tyrosine binding site for c-Cbl (Y1003). The Tpr-Met RTK oncoprotein is constitutively activated, but fails to bind c-Cbl and is not ubiquitinated (Figure 2) (Peschard et al., 2001). This suggested that loss of Cbl recruitment and ubiquitination contribute to the oncogenic deregulation of Tpr-Met. Notably, a Met receptor mutant that lacks only the c-Cbl TKB domain binding site (Y1003F) has a prolonged half-life and is oncogenic in cell culture and tumorigenesis assays, identifying c-Cbl and ubiquitination as important negative regulators for this receptor (Peschard et al., 2001). Another member of the Met family of RTKs, c-Sea, was originally isolated as the cellular homolog of the avian erythroblastosis retroviral oncoprotein v-Sea. Like Tpr-Met, the c-Cbl TKB binding site located in the juxtamembrane domain of c-Sea is deleted in the v-Sea oncogenic receptor (Figure 2).

Colony-stimulating factor-1 receptor

In a similar manner, the CSF-1R was first identified as a viral oncogene, v-Fms, the product of the McDonough strain of feline sarcoma virus. One of the several structural differences between c-Fms/CSF-1R and v-Fms is the replacement of the CSF-1R carboxy-terminal 50 amino acids that contain the direct binding site for the c-Cbl TKB domain (tyrosine 969) by 14 unrelated amino acids in v-Fms (Figure 2) (Mancini et al., 2002; Wilhelmsen et al., 2002). The deletion of the c-Cbl direct binding site in v-Fms renders v-Fms refractory to c-Cbl-dependent ubiquitination, and the addition of the c-Cbl TKB domain binding site to v-Fms decreases its transforming activity (Mancini et al., 2002). Consistent with these observations, mutation of the C-terminal tyrosine 969, the direct binding site for c-Cbl, enhanced the transforming ability of CSF-1R in fibroblasts (Roussel et al., 1988). Mutations of the Cbl binding site are frequently observed

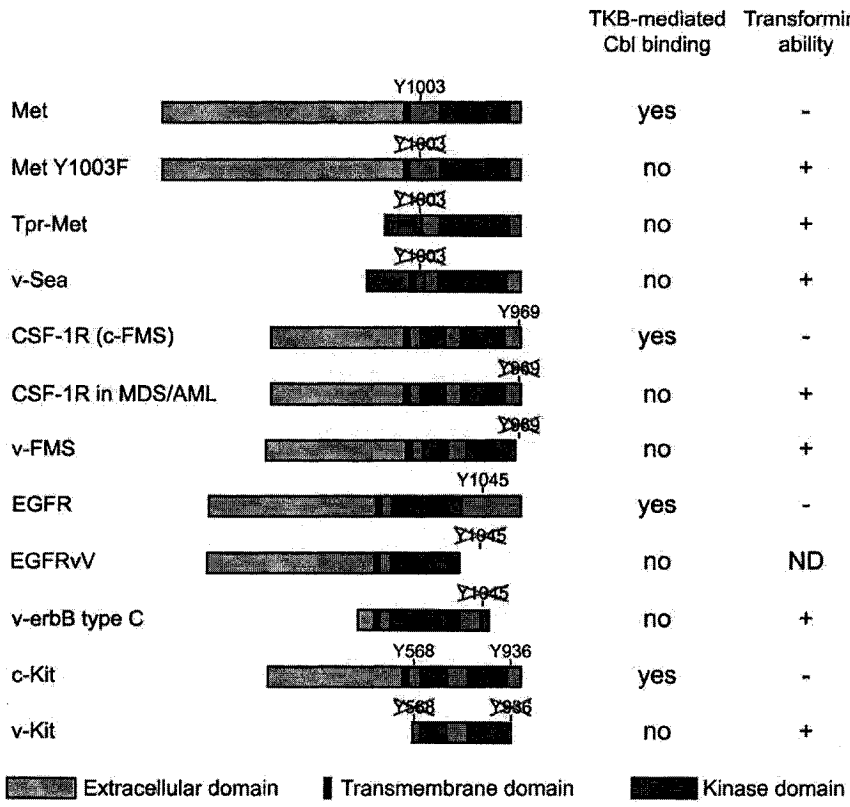

Figure 2. Loss of TKB-mediated Cbl binding in several RTK-derived oncoproteins

The binding of the c-Cbl TKB domain to the EGF, CSF-1, and Met receptors is required for receptor ubiquitination and degradation. Loss of TKB-mediated Cbl binding to RTKs correlates with a gain of transforming ability, suggesting that it constitutes an important event in RTK oncogenic deregulation.

in CSF-1R in human myelodysplasia (8 out of 67 cases) and acute myeloblastic leukemia (8 out of 48 cases), further implicating loss of c-Cbl binding in oncogenic deregulation of CSF-1R in human cancer (Ridge et al., 1990).

Stem cell factor receptor, c-Kit

The recruitment of c-Cbl to the stem cell factor (SCF) receptor/c-Kit and to PDGFRβ occurs indirectly via the APS (*a*daptor containing *P*H and *S*H2 domains) protein. This association leads to PDGFR downregulation (Yokouchi et al., 1999). Notably, the two APS binding sites in c-Kit, tyrosine 568 located in the juxtamembrane domain and tyrosine 936 located in the carboxy-terminal region of the receptor (Wollberg et al., 2003), are absent in v-Kit, the transforming protein of the Hardy-Zuckerman-4 strain of feline sarcoma virus (Figure 2). Moreover, the deletion of APS binding sites in c-Kit greatly enhanced its transforming ability (Herbst et al., 1995), although some of this may be attributed to the loss of negative regulation through juxtamembrane tyrosine 568 and enhanced catalytic activity of the receptor (Chan et al., 2003).

Epidermal growth factor receptor

Upon activation of EGFR (ErbB1), c-Cbl proteins are rapidly recruited and remain associated with the receptor as it progresses through the endocytic pathway (de Melker et al., 2001). Tyrosine 1045 constitutes the direct binding site for the c-Cbl TKB domain and is required for c-Cbl-mediated ubiquitination and degradation of EGFR (Levkowitz et al., 1999). Interestingly, the *v-erbB* protein encoded by the avian erythroblastosis virus AEV-C has, among other alterations, an internal deletion of 21 amino acids that comprises the c-Cbl TKB domain binding site (Figure 2) (Choi et al., 1986). Moreover, in a similar manner to both HGF and CSF-1 receptors, an EGFR mutant lacking only the direct Cbl binding site elicits stronger mitogenic signals than the wt receptor (Waterman et al., 2002).

Up to 40% of glioblastomas express oncogenic mutants of EGFR. The most common genetic alteration consists of a deletion of exons 2–7 located in the extracellular domain of EGFR (67% of all EGFR genetic alterations) (Frederick et al., 2000). The resulting protein (EGFRvIII) is constitutively activated, but has low levels of tyrosine phosphorylation. EGFRvIII receptors have a reduced ability to recruit Cbl proteins and CIN85, and are neither ubiquitinated nor internalized (Schmidt et al., 2003), suggesting that basal receptor activation is sufficient to confer tumorigenicity, but not sufficient to trigger Cbl-mediated downregulation. Another genetic alteration of EGFR identified in human glioblastoma generates a truncated receptor (EGFRvV) that has an intact kinase domain, but that is missing the c-Cbl TKB direct binding site (Y1045) and the internalization signals (Figure 2) (Frederick et al., 2000).

Another member of the EGF receptor family, HER2/ErbB2, is overexpressed in many human tumors such as breast, ovary, prostate, and brain tumors. Overexpression of HER2 shifts the formation of EGFR homodimers toward the formation of EGFR/HER2 heterodimers. While ligand-stimulated EGFR homodimers undergo rapid ubiquitination, internalization, and degradation, EGFR/HER2 heterodimers recruit c-Cbl to a reduced extent, are slowly internalized, and recycle rapidly to the cell surface (Lenferink et al., 1998; Muthuswamy et al., 1999). The delay in EGFR/HER2 heterodimer degradation potentiates EGFR-dependent cell proliferation, cell migration, and antiapoptotic signals. Hence, the overexpression of HER2 constitutes a mechanism through which EGFR escapes Cbl-mediated downregulation. Indeed, overexpression or amplification of Cbl binding RTKs, as frequently observed in human cancers, could act as a mechanism to sequester Cbl proteins, enhancing the stability of other RTKs that are Cbl substrates. In addition, the deregulation of Src, which occurs in human colon, breast, lung, and brain cancers, leads to the stabilization of the EGFR by promoting c-Cbl ubiquitination and degradation (Bao et al., 2003).

Besides point mutations and overexpression, RTKs are frequently activated in human tumors following chromosomal translocation. In general, this fuses a protein dimerization domain with the cytosolic kinase domain of the receptor, resulting in constitutive receptor dimerization and activation (Lamorte and Park, 2001). Over 25 RTK-derived fusion proteins have

been identified in human tumors. In each case, the N-terminal signal peptide, necessary for protein targeting to the membrane, is deleted in the rearranged kinase and, with the exception of FIG-ROS that is targeted to the golgi (Charest et al., 2003), where studied, these proteins are cytosolic (Lamorte and Park, 2001). Localization to the cytosol would preclude their entry in the endocytic pathway and hence, their lysosomal targeting and degradation. However, it remains to be determined whether these oncoproteins are ubiquitinated and targeted for degradation by the proteasomal pathway.

Conclusion

In the past few years, the discovery that Cbl proteins are ubiquitin-protein ligases and that ubiquitination regulates receptor sorting to lysosomes has greatly improved our understanding of the molecular mechanisms that downregulate RTKs. We bring to light evidence that many RTK-derived oncoproteins avoid downregulation by loss of Cbl binding sites, inefficient Cbl recruitment, Cbl degradation, or through the formation of fusion proteins that escape lysosomal degradation. These constitute some mechanisms among several that promote the stabilization of RTKs. Overall, an understanding of the mechanisms through which RTKs are downmodulated will allow new therapeutic approaches to target these RTKs for degradation in cancer. In this respect, Trastuzumab, a drug used in combination with chemotherapy for the treatment of metastatic breast cancer, is a weak agonist of HER2. Trastuzumab is thought to force receptor dimerization and potentiate recruitment of Cbl and receptor downregulation (Klapper et al., 2000). Similar drugs designed to force other RTKs toward a degradation pathway would likely be beneficial in the design of therapeutic strategies for cancer.

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