Differential Regulation of c-Cbl and Cbl-b Ubiquitin Ligases Downstream of the Met Receptor Tyrosine Kinase

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December 2007

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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ISBN: 978-0-494-51264-7
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ISBN: 978-0-494-51264-7

Abstract

The Cbl family of E3 ubiquitin ligases are important negative regulators of multiple receptor and cytoplasmic tyrosine kinases, and participate in a wide variety of cellular processes. Uncoupling of Cbl-mediated negative regulation allows activated receptor tyrosine kinases such as the Met receptor to escape degradation, enhancing their oncogenic potential *in vitro* and *in vivo*. Despite the consequences of loss of Cbl-mediated negative regulation for human disease, little is known about the mechanisms regulating Cbl protein levels themselves.

In this thesis work, I demonstrate a differential regulation of c-Cbl and Cbl-b downstream of the Met receptor tyrosine kinase. Cbl-b protein levels decrease in response to Met kinase activity, whereas c-Cbl levels remain stable. Cbl-b is partially degraded in a proteasome-dependant manner. This requires Cbl-b ubiquitin ligase activity and a carboxy terminal domain region located between the RING and UBA domains. I conclude that the regulation of c-Cbl and Cbl-b differs downstream of Met, and propose that negative regulation of Cbl-b by a dysregulated Met receptor may contribute to tumourigenesis.

Résumé

La famille Cbl des ligases E3 d'ubiquitine constitue un méchanisme important de désactivation des récepteurs à activité tyrosine kinase (RTKs), et jouent un rôle important dans plusieurs processus cellulaires. La perte de la régulation négative exigée par Cbl permet au récepteurs activés d'échapper à la destruction, résultant en l'accroissance de leur potentiel oncogénique *in vitro* et *in vivo*. En dépit des consequences de la perte de Cbl comme un méchanisme de désactivation pour le cancer chez l'humain, nous savons peu de choses à propos des méchanismes de regulation et de désactivation de la famille Cbl elle-même.

Dans cette thèse, je compare l'effect du récepteur à activité tyrosine kinase Met actif sur la régulation des protéines c-Cbl et Cbl-b. Je démontre une régulation differentielle par Met, où le niveau d'éxpression de Cbl-b chute dramatiquement en réponse à Met, mais le niveau d'expression de c-Cbl reste constant. Cbl-b est dégradé par le protéasome, et c'est l'activité ligase d'ubiquitine et aussi la région entre les domaines RING et UBA de Cbl-b sont requises. D'abord, en conclusion, la régulation de c-Cbl et Cbl-b par Met n'est pas identique, et j'avance que la régulation négative de Cbl-b par un récepteur Met dérégulé pourrait contribuer au développement du cancer.

Acknowledgements

I would first like to thank my supervisor, Morag Park, for her guidance, support and friendship during my time in the lab. The insight she has given me into nearly all aspects of research has been of both great help and interest to me, and will continue to be well into the future.

I am also grateful to members of the lab whom I have had the enjoyment of working alongside. Their dedication, advice, and friendship have motivated me to do the best work I can, and they are a large part of why the lab is a great one to be a part of. I also thank Andrea Lai, Christine Parachoniak, Melanie Frigault, and Richard Vaillancourt for their help editing this thesis.

Special thanks also goes to Dr. Kurt Dejgaard, who taught me the theory and techniques of mass spectrometry, and sparked my interest in this subject.

I would also like to acknowledge the financial support of the Canadian Institute of Health Research Cancer Consortium.

I am also appreciative of my family for their continued support, and am especially grateful to my partner Marita for her unbroken patience, encouragement and understanding. This work would not have been a success without her.

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Literature Review

1. Introduction

The existence of multicellular organisms relies on the ability of all tissues, individual cells, biomolecular processes and subprocesses within each cell to respond to environmental stimuli in a coordinated fashion. Through millions of years of evolution, multicellular organisms have developed mechanisms of cellular signaling by which they coordinate a complex interwoven network of different tissue and cell types in order to control their own development, metabolism, growth, ability to respond to their environment, and maintain homeostasis. Only a tight regulation of these networks of cells and the networks of biological processes within individual cells through cellular signaling allows for continued existence of the organism. A complex process, cellular signaling involves the use of either direct cell-cell contacts, or a myriad of extracellular signaling molecules such as hormones, growth factors, proteins, peptides and smaller chemicals as a means of communicating from a signaling cell to produce a response in a target cell. Target cells receive ligand-encoded signals by means of extracellular and/or intracellular receptors which begin the process of cellular signal transduction through the initiation of signaling cascades within the cell. Numerous results can occur downstream of a signal or combination of signals via the specific proteins involved in its transduction, including immediate changes in cytoskeletal dynamics, cell survival, cell motility, proliferation and growth, as well as long-term genetic changes as a result of prolonged signaling.

2. Receptor tyrosine kinases and cellular signaling

Receptor tyrosine kinases (RTKs) represent a large family of receptors present at the cell surface and initiate many of the signaling responses of the cell to extracellular stimuli. 58 genes encoding RTKs have been identified, and are divided into 20 subfamilies based on their structure and function [1] (Fig.1). Despite differences between different subfamilies, all RTKs share an extracellular ligand-binding domain, a single-pass hydrophobic transmembrane domain, and an intracellular region containing a kinase domain. RTKs are generally monomers, with the exception of the insulin receptor and insulin-like growth factor receptor which exist constitutively as disulfide-linked dimers [2, 3]. Binding of a growth factor ligand to the extracellular domain of inactive receptor

monomers induces receptor dimerization or oligomerization which results in a conformational change in the cytoplasmic kinase domain, activating the intrinsic kinase activity of the receptor [4]. This results in autophosphorylation of the receptor on specific tyrosine residues in the intracellular region and recruitment of complexes of signaling proteins through adaptor proteins. The reversible binding of adaptor and

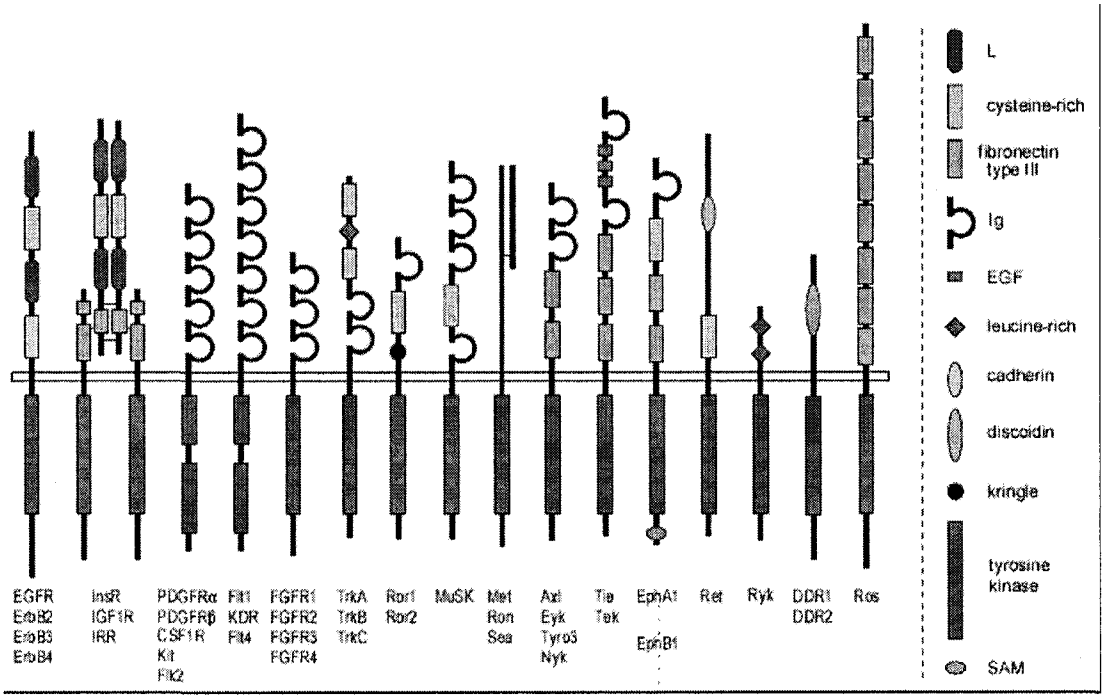

Figure 1. Domain organization of human receptor tyrosine kinase families. Adapted from Hubbard and Till. 2000. Annu. Rev. Biochem. **69**: 373-398.

scaffolding proteins to phosphorylated tyrosine (pTyr) residues occurs through Src-homology 2 (SH2) domains, protein domains specialized in binding specifically to phosphotyrosine-containing consensus sequences [5]. As signaling cascades often require the formation of complexes of signaling proteins, adaptor and scaffolding proteins contain combinations of different protein-protein interaction motifs, each recognizing and binding to other specific motifs and post-translational modifications in other proteins or lipids. Many protein-lipid and protein-protein interaction domains are involved in RTK signaling. These include SH2 domains, Src-homology 3 (SH3), protein tyrosine binding (PTB), pleckstrin homology (PH), Ubiquitin-associated (UBA), 14-3-3, and PDZ domains [5-10]. Furthermore, the signaling proteins recruited downstream of an

activated receptor may or may not possess an enzymatic activity of their own. For example, PI3K and SHP-2 can phosphorylate or dephosphorylate additional substrates, respectively, whereas Gab1, Grb2, and Crk have no enzymatic activity of their own and function only to recruit specific signaling molecules into large complexes in close proximity to each other. Adaptor and scaffolding proteins can participate in multiple different signaling processes, depending on the specific signals being received by the cell. As an end result, the extracellular message received by the cell is transduced to produce specific short- and long-term effects in the cell.

3. Met receptor tyrosine kinase signaling

The Met receptor was originally identified as Tpr-Met, an oncogene arising from a chromosomal rearrangement in cells treated with N-methyl-N'-nitrosoguanidine (MNNG) [11]. Subsequently, Met was identified as the physiological receptor for the

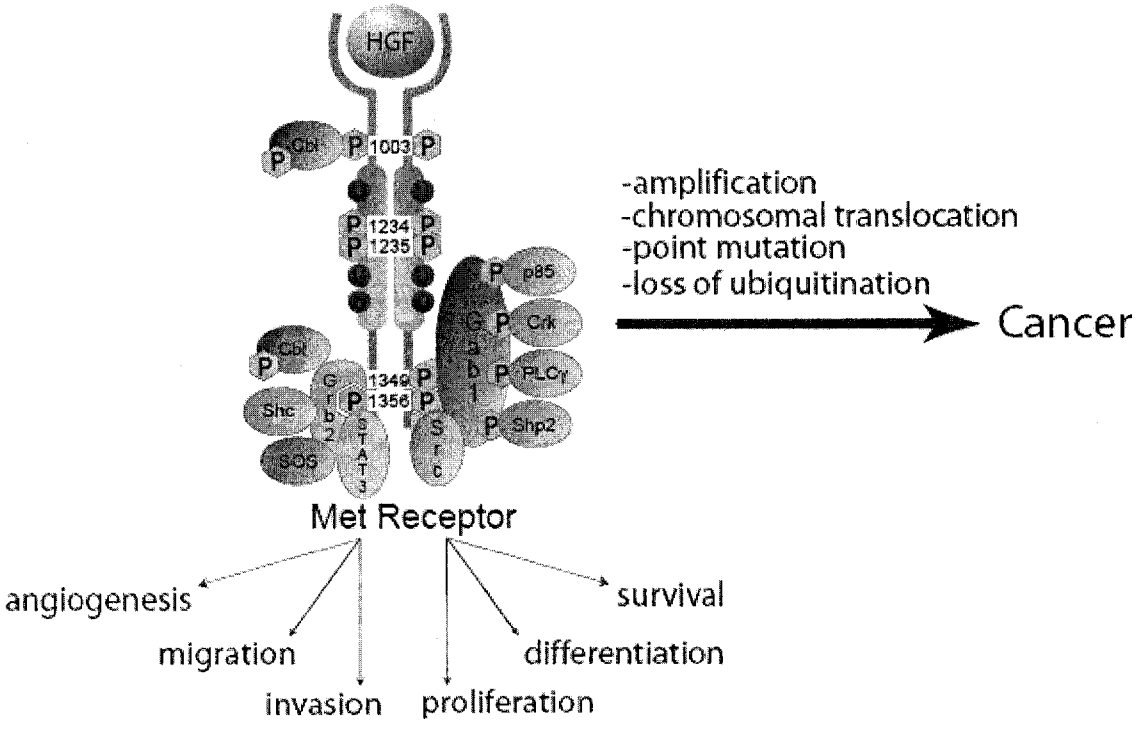

Figure 2. Proteins involved in the transduction of Met signaling and biological functions. Provided by Dr. Pascal Peschard.

hepatocyte growth factor/scatter factor (HGF/SF), a mitogen for rat hepatocytes and a scatter factor for sheets of epithelial cells [12-15]. Signaling from the Met RTK elicits a variety of cellular responses, making use of both the Ras/MAPK and PI3K/Akt pathways to induce cell proliferation, survival, migration, invasion, epithelial morphogenesis, angiogenesis, and when deregulated, tumourigenesis [16].

Following Met activation, tyrosines (Y) 1349 and 1356 in the Met C-terminus are phosphorylated, a process both sufficient and required for all biological functions of Met [17-19]. The Shc and Grb2 adaptor proteins are recruited to the second of these tyrosines (Y1356), linking Met to the Ras/MAPK pathway required for invasive growth. Shc also mediates Met-dependant angiogenic effects [20]. Additionally, Grb2 recruits the scaffold protein Gab1, providing Met with access to additional downstream signaling pathways through PI3K, PLC- γ, SHP-2 and Crk, all of which are recruited through Gab1. Gab1 is also recruited to Met directly through its Met binding motif (MBM) to Y1349. This represents a unique mode of recruitment that results in a sustained tyrosine phosphorylation of Gab1 and sustained activation of signaling pathways recruited through Gab1. For example, the PI3K/Akt pathway is required for Met-dependant cell survival and migration [21], and association of Gab1 with SHP-2 is necessary for sustained activation of Erk required for branching morphogenesis [22].

4. Regulation of cell signaling and the Cbl protein family

To maintain homeostasis, signals received by a cell must be kept in check by opposing negative regulatory signals controlling the duration and amplitude of the signal received. This can be achieved through a variety of mechanisms such as the transcription of genes of opposing function, the addition or removal of post-translational protein modifications from a protein, or the destruction of active signaling proteins. Disruption of this balance can lead to disease as cellular activities are no longer properly controlled, leading to physiological consequences [23]. An example of the importance of maintaining this balance is the interplay between RTKs and ubiquitin ligases. A major mechanism by which the activation and signaling of many RTKs is controlled is through the action of the Cbl family of ubiquitin ligases, as the destruction of many activated kinases subsequent to protein ubiquitination attenuates their signaling capabilities [24].

Cbl proteins have been found to be essential in an increasingly large number of cellular processes. As ubiquitin ligases, Cbl proteins fulfill an essential negative regulatory role downstream of multiple RTKs [25]. However, Cbl family members also possess many different protein-protein interaction domains making them multifaceted scaffolding proteins. This fact is reflected by the variety of different cellular processes in which Cbl proteins have been shown to play a role; Cbl has been shown to interact with over approximately 150 proteins [26]. The importance of the Cbl family in cell signaling is evident as perturbations of Cbl function have been implicated in both cancer and autoimmune disorders, such as type I diabetes [25, 27, 28].

5. Ubiquitin, the ubiquitination cascade, and ubiquitin ligases

Ubiquitination of cellular proteins is an absolutely essential method by which the cell eliminates old or improperly folded proteins, or controls the activity of other proteins by degradation or the alteration of localization and trafficking of an ubiquitinated protein. Proteins are marked by covalent conjugation of ubiquitin, a small 76-amino acid protein, to a lysine residue in the protein to be degraded. Ubiquitin is ubiquitously expressed in eukaryotes, being highly conserved from yeast to humans. The transfer of ubiquitin is achieved through a multi-enzyme cascade, involving three different families of enzymes: E1s (ubiquitin activating enzymes), E2s (ubiquitin conjugating enzymes) and E3s (ubiquitin-protein ligases). The E1 enzyme initially activates ubiquitin by the ATP-dependant formation of a thiol-ester bond with the carboxy-terminal glysine residue of ubiquitin. Subsequently, a transient thiol-ester bond is formed between ubiquitin and the E2 enzyme, transferring ubiquitin to the E2. Finally, substrate specificity for the ubiquitination reaction is mediated by the E3 enzyme which recruits the E2 to the substrate to be

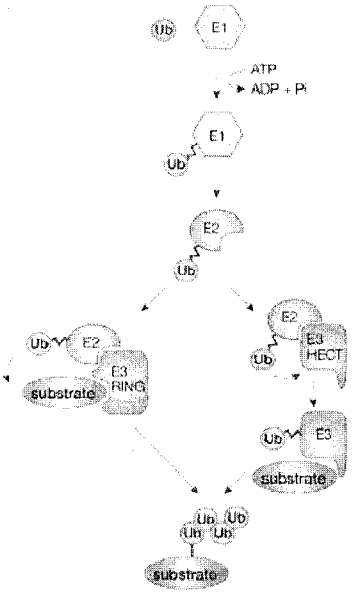

Figure 3. The ubiquitination cascade. Adapted from Woelk, T., et al. 2007. Cell division. **2**: 11-23.

ubiquitinated, and the E2 catalyzes the formation of an isopeptide bond between the activated carboxy terminus of ubiquitin and the epsilon amine group of a lysine residue in the target molecule [29]. No consensus motifs suggesting specificity for lysines to be ubiquitinated in the target protein have been elucidated to date. In addition to the E1, E2, and E3 enzymes, a class of U-box E4 enzymes which promote the extension of polyubiquitin chains already added by an E3 also has been characterized [30].

Approximately a thousand E3s have been identified in humans, ten times more than the number of E2s and 100 times the number of E1s [31]. Of the E3s, most, including the Cbl family, belong to the Really Interesting New Gene (RING) class of E3s and a lesser portion is of the Homologous to E6AP Carboxy Terminus (HECT) class. A number of differences define these two classes of E3. RING E3s function as scaffolding proteins, possessing no enzymatic activity of their own, but instead recruiting the E2 for direct ubiquitin transfer to a specific substrate [32]. RING finger domains bind two Zn^{2+} ions within a cysteine/histidine rich region and this ensures their correct folding and function as promoters of protein-protein and protein-DNA interaction. HECT domain E3s, on the other hand, are characterized by their ~350 a.a. HECT domain. The HECT domain has catalytic activity, containing a conserved cysteine residue which accepts transfer of an ubiquitin moiety by the E2 enzyme, and the HECT E3 then catalyzes the direct transfer of ubiquitin from itself to the substrate [33].

6. Mono- and multi-monoubiquitination

The outcome of protein ubiquitination can be complex, as ubiquitin can be transferred in multiple different forms. Monoubiquitination, where a single ubiquitin residue is transferred, can serve multiple purposes depending on the context. It can modulate protein function as in the case of histones, where monoubiqutination of H2B is a prerequisite for H3 methylation and ultimately, gene silencing [34, 35]. Additionally, monoubiquitination is becoming appreciated as a means to alter the subcellular localization of substrate proteins. The nuclear localization of tumour suppressor proteins p53, FOXO, and PTEN has been found to be regulated in part by monoubiquitination, and this is likely to affect their function [36]. Additionally, monoubiquitination plays a role in the endocytic trafficking of transmembrane proteins, such as RTKs. In fact, the

EGFR, PDGFR and Met receptors have been shown to be multi-monoubiquitinated [37-39], and this acts as a signal sorting them for degradation in the lysosome. In support of this, fusion of a single ubiquitin residue to a truncated EGFR was sufficient to target the EGFR to late endosomal compartments in the absence of other signals [37]. Moreover, a Met-Ub fusion protein was demonstrated to localize to endosomal compartments and have decreased half-life upon stimulation when compared to an ubiquitination-deficient mutant [40].

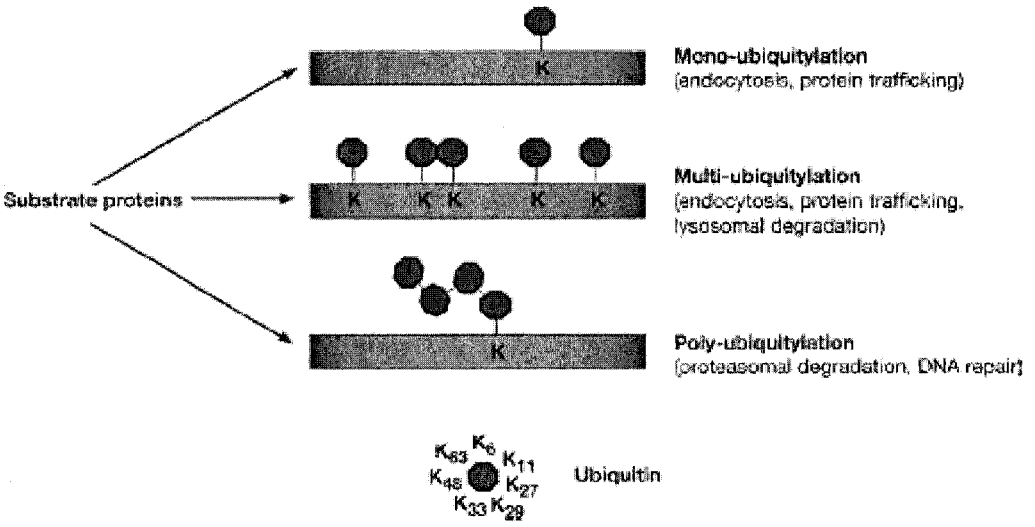

Figure 4. Different ubiquitin modifications. Adapted from Thien and Langdon. 2005. Biochem. J. **391**: 153-166.

7. Polyubiquitination

Polyubiquitination involves the addition of chains of ubiquitin to a target protein. Ubiquitin contains seven internal lysine residues (K6, K11, K27, K33, K48 and K63), to which additional ubiquitin moieties can be attached and all of which have been demonstrated, to varying extents, to participate in ubiquitin chain formation [41]. Polyubiquitination is capable of targeting proteins for degradation, however it does so by targeting K48-linked polyubiquitinated substrates to the 26s proteasome, rather than the lysosome. This is the major method of protein turnover used in both yeast and mammalian cells. Polyubiquitination can serve other functions besides protein degradation depending on the type of linkage involved in chain formation. K63-linked

polyubiquitin chains are generally not proteolytic signals, and are instead involved in the processes of DNA repair in yeast, as well as inflammatory responses, ribosomal protein synthesis and protein trafficking [42, 43].

8. Cbl family evolution and genomic organization

The first Cbl (Casistas B-lineage lymphoma) family protein was initially identified as a Cas NS-1 retrovirus-encoded oncoprotein responsible for the induction of pre- and pro-B lymphomas in infected mice [44]. Named v-Cbl, this 357 amino acid oncoprotein led to the identification of its full-length cellular counterpart, c-Cbl. Since then, Cbl family proteins have been identified in many species, such as the nematode *C. elegans*, fruit flies, fish, amphibians, birds, and mammals. Mammals possess three Cbl family members, c-Cbl, Cbl-b and Cbl-3, the genomic structure and evolution of which being outlined in detail in a study by Nau and Lipkowitz [45]. Located on chromosomes 11 and 3, respectively, both c-Cbl and Cbl-b genes retain identical intron-exon structures between mice and humans, whereas Cbl-3 (located on chromosome 19) only maintains this similarity up to exon 7, and diverges after. The c-Cbl gene encodes for a 906 amino acid, 120 kDa protein with no alternatively spliced forms identified in normal human tissue. On the contrary, the Cbl-b gene, encoding for a 982 amino acid wt protein, can be processed into several truncated splice variants although the significance of these variants has not been examined. The Cbl-3 gene is much smaller, having only 11 exons versus 16 and 19 for c-Cbl and Cbl-b, respectively, and encoding a protein of 474 amino acids. One alternatively spliced form of this protein has been described and has been shown to be defective in its ability to bind to the EGFR [46].With respect to tissue distribution, both c-Cbl and Cbl-b protein are ubiquitously expressed, with highest levels found in haematopoetic cells, as opposed to Cbl-3, which is only expressed in the epithelium, with highest levels in the gastrointestinal tract [46-50].

9. Cbl family protein domain structure and function

Key to the functional diversity of Cbl family proteins is the variety of functional domains found in these proteins (Fig 4). All Cbl proteins have a highly homologous N-terminal region that is evolutionarily conserved between invertebrate and vertebrate Cbl

homologues. This region contains the tyrosine kinase binding (TKB) domain, responsible for binding to phosphorylated tyrosines in substrate molecules, as well as the RING finger domain required for Cbl's E3 ligase activity. Connecting these two regions is a small, 17 amino acid linker region. Homology between Cbl family members is dramatically reduced in sequences C-terminal to the RING finger, which contain proline-rich regions as well as an UBA / leucine zipper domain. The shorter Cbl-3 protein contains fewer proline-rich regions and no UBA/LZ domain [45]. As the conservation of the N-terminal regions implies, mammalian Cbl family proteins have been found to have overlapping functions, however, variations in the C-terminal regions of these proteins allows for diversification of the roles these proteins play as adaptors in cell signaling

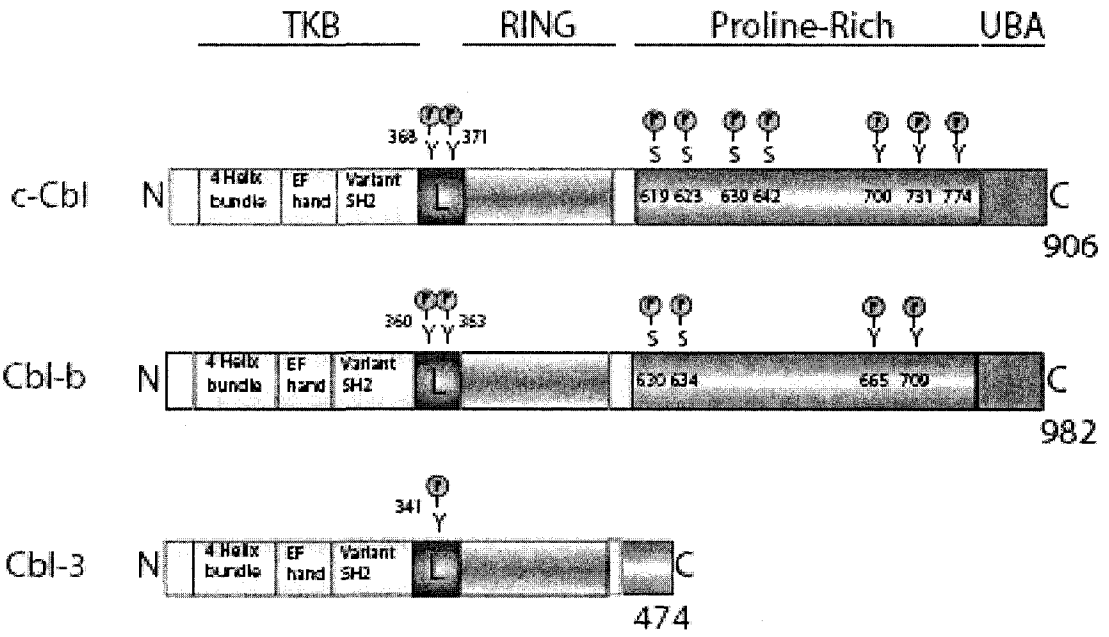

Figure 5. Domain organization of the mammalian Cbl protein family.

processes.

The principal function of the TKB domain is to bind to phosphorylated tyrosine residues on proteins destined for ubiquitination. As the TKB domain does not form strong interactions with associated proteins, it is unlikely to be the principal method by

which Cbl is recruited to target proteins and may determine instead the extent and type of ubiquitination that occurs [51]. Nevertheless, disengagement of the TKB domain from a target protein has been shown to be sufficient for cessation of target ubiquitination [52-54]. An initial structural and crystallographic study performed by Meng et al (1999) on the N-terminal region of c-Cbl in complex with a peptide corresponding to its binding site on the ZAP-70 tyrosine kinase revealed that the TKB region consisted of a four-helix bundle, a calcium-binding EF hand, and a variant SH2 domain. Specifically, the variant SH2 domain binds to phosphotyrosine in the same general orientation as does a traditional SH2 domain, however, the 4H bundle is positioned by the calcium-bound EF hand to complete the pTyr binding pocket. All three components of the TKB domain are required for pTyr binding [55]. The complexity of the TKB domain suggests a potential for plasticity in TKB-protein interactions, and numerous studies have since revealed additional consensus TKB binding motifs. For example, one consensus binding sequence, NXpY(S/T)XXP, is found in Src family kinases, ZAP-70, as well as the EGFR and some other RTKs, with residues C-terminal to the pTyr playing critical roles in the interaction. However, a consensus sequence RA(V/I)XNQpY(S/T) has since been identified in the APS family of adaptor proteins, where amino acids N-terminal to the pTyr are more important for the interaction [56]. Moreover, a DpYR sequence bearing no resemblance to other consensus binding sequences has been identified in members of the Met receptor family of RTKs, Met, Ron and the avian v-Sea [57]. Although much fewer in number, pTyr-independent interactions have also been described. The TKB domain of c-Cbl can bind tubulin and SLAP independent of tyrosine phosphorylation, as a critical mutation demonstrated to abrogate TKB-pTyr interactions (G306E) did not disturb these associations [58, 59]. Many further studies will be required to elucidate the subtleties of the TKB domain's role in modulating Cbl-protein interactions and Cbl-mediated ubiquitination.

Crucial for their E3 ubiquitin ligase activity, a second highly conserved domain in the N-terminal region of Cbl family proteins is the RING finger domain. The importance of the RING finger for Cbl-mediated ubiquitination was clear from an early stage, as Cbl proteins with mutations or deletions residing within the RING domain were unable to induce protein ubiquitination and had been identified in cancer as proto-oncogenes,

acting as dominant negatives of Cbl function as a negative regulator of activated kinases. With the development of in vitro ubiquitination assays, the RING domain of c-Cbl was found to be capable of independently associating with and activating the E2 ubiquitin-conjugating enzymes, and that this association was required for protein ubiquitination [60, 61]. Almost simultaneously, an additional study described the specific interaction between the c-Cbl RING domain and the human E2 UbcH7, demonstrating the requirement of this specific interaction for the ubiquitination of the EGFR [62]. Thus, Cbl RING mutants acted as dominant negatives due to their ability to maintain target protein binding through association with other adaptor proteins and/or the TKB domain while being defective as E3 ligases, thus competing for protein binding with wild-type Cbl proteins, reducing target ubiquitination and negative regulation.

Bridging the TKB and RING finger domains is a short, highly-conserved 17 amino acid linker helix. Crystallographic studies performed on the conserved c-Cbl N-terminal region in complex with ZAP-70 and UbcH7 revealed that the TKB, linker, and RING regions form multiple interactions between each other, folding into a compact structure of which the linker region was crucial from a structural standpoint. The linker region has two tyrosine residues, Y368 and Y371, which form central contacts in the structure [32]. The exact role of these tyrosines remains unknown but mutation of Y371 or deletion of either tyrosine eliminates c-Cbl E3 ubiquitin ligase activity, as do mutations of C381 in the RING domain. However, only deletion of Y371 converts c-Cbl into an oncogene; substitution to phenylalanine does not [63, 64]. Phosphorylation of Y371 removes negative regulation of E3 ligase activity by the TKB and linker regions, and structural studies predict Y371 phosphorylation to result in major conformational changes in the linker region. Furthermore, mutation to the phosphomimetic glutamate results in constitutively active E3 ligase activity, suggesting that phosphorylation of Y371 may be necessary for Cbl activation [65]. Thus, for a Cbl mutant to be transforming, loss of both E3 ligase activity as well as the structural contributions of tyrosines of the linker helix is required. As a cohesive unit, the TKB, RING and linker regions all collaborate in the proper binding and positioning of target proteins, making possible the transfer of ubiquitin from the E2 ubiquitin-conjugating enzyme.

Homology between the different Cbl family members tapers off in the C-terminal regions, where many of the adaptor-related associations between Cbl and an ever-growing list of binding partners take place. c-Cbl, Cbl-b, and Cbl-3 contain 15,17 and 5 polyproline rich regions, respectively, which serve as potential binding sites for SH3-domain containing proteins [51]. Although the c-Cbl and Cbl-b C-terminal regions bind some of the same proteins, much remains to be discovered concerning which proteins interact with polyprolines in one Cbl protein versus another in different circumstances. Importantly, both c-Cbl and Cbl-b make prominent associations with Grb2 and possess a Grb2 SH3 consensus sequence, PPVPPR, providing c-Cbl and Cbl-b with an indirect mode of recruitment to some RTKs [66-68]. CIN85, another significant binding partner, has been reported to bind to c-Cbl and Cbl-b, which both contain a PXXXPR CIN85-binding site [69]. Through CIN85, endophillins are in turn recruited to c-Cbl and Cbl-b, and these complexes have been shown to promote clustering of endocytic proteins at sites of activated EGFR [70], and are required for proper downregulation of the EGFR and Met [71, 72]. Having only five potential polyproline-SH3 interaction sites, Cbl-3 associated with a more restricted set of proteins through their SH3 domains than did Cbl-b in *in vitro* binding assays, and Cbl-3 notably did not bind Grb2 [46], one of the principal methods of recruitment of c-Cbl and Cbl-b to the EGFR and Met receptor.

The C-termini of c-Cbl and Cbl-b are also sites of serine and tyrosine phosphorylation. The major pTyr sites in c-Cbl, Y700, Y731, and Y774 form associations with the SH2 domains of Crk, PI3K, and Vav1, a GEF for the Rho family GTPases Rac and Cdc42. Cbl-b also possesses phosphorylated tyrosines corresponding to Y700 and Y774, however, it lacks an equivalent of Y731, highlighting a potential major difference in the function between two otherwise similar family members [51]. In accordance with this, the constitutively-activated oncoprotein BCR-Abl was found to induce association of c-Cbl and Cbl-b with different signaling complexes. c-Cbl was found to associate with activated PI3K and CrkL, whereas Cbl-b associated to a greater extent with Vav1 [73]. Serine phosphorylation also represents a potential divergence between c-Cbl and Cbl-b function, as following PMA stimulation c-Cbl was serine phosphorylated inducing association with 14-3-3 proteins tau and zeta, however, no 14-3-3 association with Cbl-b was detected [74, 75].

The final functional domain of Cbl proteins is the UBA (ubiquitin-associated) domain, found at the far C-terminus of c-Cbl and Cbl-b, but not Cbl-3. Originally thought to be leucine zipper dimerization motifs, UBA domains have been characterized as modules promoting the dimerization of a number of proteins, and indeed c-Cbl and Cbl-b can both homo- and heterodimerize in a UBA-dependant manner [76]. On the other hand, UBA domains can also bind to ubiquitin and ubiquitin-like proteins, however, among Cbl proteins this is only true for the Cbl-b UBA: the c-Cbl UBA cannot bind to ubiquitin [77].

Dimerization plays a large role in the function of Cbl, as c-Cbl mutants lacking the UBA domain are deficient in their tyrosine phosphorylation and recruitment to activated EGF, insulin, and Met receptors [76, 78, 79]. The discovery that c-Cbl required dimerization through an intact UBA domain to efficiently ubiquitinate and downregulate the Met receptor, showing the importance of the UBA for the biological function of c-Cbl. This stands in contrast to Cbl-b, which also requires an intact UBA for robust tyrosine phosphorylation and recruitment to the EGFR downstream of EGF, although deletion of the UBA does not affect its ability to ubiquitinate the EGFR [77]. Whether the differential ability of the two UBA domains to bind ubiquitin is involved in this difference is unclear, but as mentioned by Kovlov et al (2007), the capacity to heterodimerize implies that c-Cbl could acquire the ability to bind ubiquitin through association with Cbl-b, and both proteins may be able to expand the sets of proteins with which they can interact through heterodimerization [79]. Cbl-b is capable of associating with high-molecular weight complexes of ubiquitinated proteins through its UBA domain, and has been thought to be possibly recruited to the endocytic pathway via UBA-ubiquitin interactions since many endocytic proteins are monoubiquitinated and could serve as potential binding partners. Although this may be true, the Cbl-b UBA domain shows a preference for binding polyubiquitin chains, and the homodimerization of Cbl-b through the UBA domain is greatly increased by polyubiquitin chain binding rather than monoubiquitin [80]. While additional studies will be required to unravel the mechanisms by which the Cbl UBA domains affect their function, a nonsense c-Cbl mutant lacking the UBA domain has been discovered in a T-cell lymphoma cell line [81] and Cbl-b splicing isoforms lacking the UBA domain also exist [45, 48], providing

potential tools by which future studies may examine the contributions of the UBA domain to Cbl function.

10. Cbl proteins in the regulation of receptor tyrosine kinases

Binding of a receptor tyrosine kinase to its growth factor ligand induces dimerization and a conformational change in the cytoplasmic kinase domain, activating the intrinsic kinase activity of the receptor. This results in autophosphorylation of the receptor on specific tyrosine residues and recruitment of complexes of signaling proteins

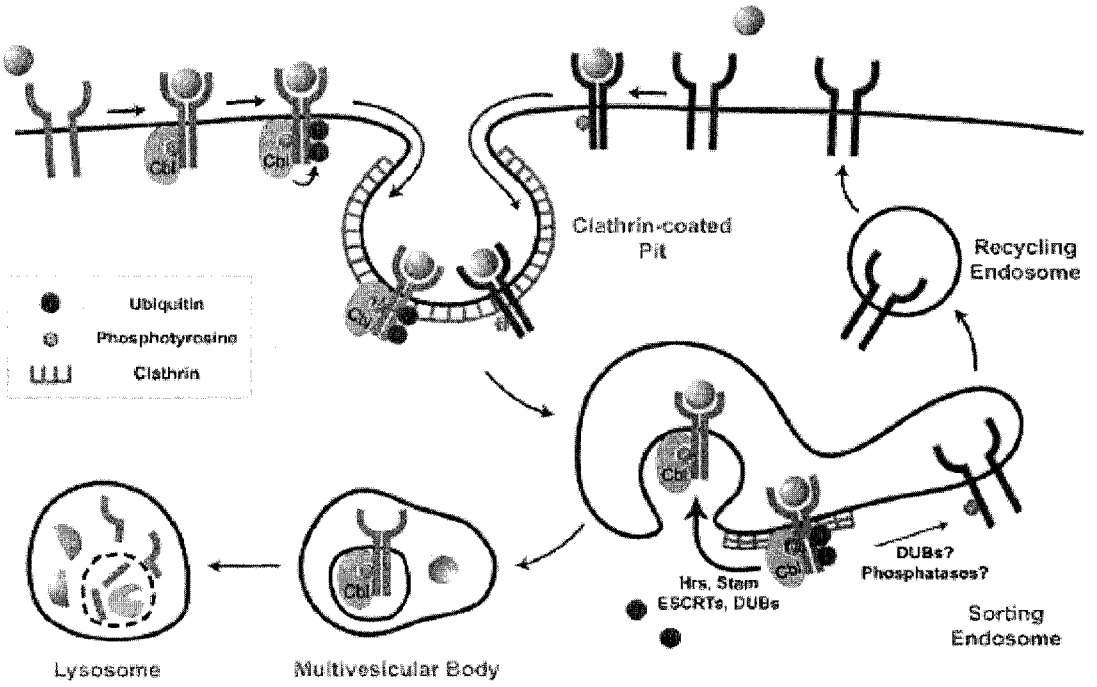

Figure 6. Schematic of the regulation of RTKs by Cbl proteins. Adapted from Peschard and Park. 2003. Cancer Cell. **3**: 519-23.

via SH2-pTyr interactions [1]. However, another result of receptor activation is the clustering of receptor dimers or oligomers in specialized membrane pits and their subsequent internalization, a process termed endocytosis. Endocytosis is a major mechanism of controlling the number of actively signaling receptors at the cell surface. Two major classes of endocytosis are thought to mediate receptor internalization: clathrin-dependant and clathrin-independent [82]. Under physiological circumstances, it

is believed that clathrin-dependent mechanisms are the major mode of RTK internalization [83]. Clathrin, a triskeleton protein, binds to the heavy chains of multiple other clathrin proteins with a precise geometry, forming a lattice meshwork encircling the pit, to form a clathrin-coated vesicle (CCV) [84]. The negative membrane curvature required for the formation of CCVs are the result of mechanical stresses put on the membrane by the clathrin lattice in combination with the recruitment of endocytic adaptor proteins linking clathrin to the membrane lipid bilayer. Next, clathrin-coated vesicles will pinch off from the membrane, and lose their clathrin coats, becoming early endosomal vesicles and progressing along the endocytic pathway [85]. At the level of the sorting endosome, activated receptors can be either sorted back to the plasma membrane where they continue to signal, or they are sorted for lysosomal degradation by the process of inward vesiculation, ceasing receptor signaling (Fig 6). Monoubiquitination of receptors is required for their inclusion in the inward vesiculation process, where proteins containing ubiquitin interacting motifs (UIMs) such as hepatocyte growth factor regulated substrate (Hrs) have been proposed to connect ubiquitinated receptors to the endosomal required for transport (ESCRT) complex. The ESCRT complex drives the formation of multivesicular bodies (MVBs), which are the result of inward vesiculation, which then mature into lysosomes and degrade their stored receptor cargo [86].

11. Regulation of Met by c-Cbl and Cbl-b

Cbl proteins are involved at multiple points along the endocytic pathway in the process of RTK degradation, and make use of both their E3 ubiquitin ligase and adaptor functions to regulate this process. The Met receptor is a target for ubiquitination by both c-Cbl and Cbl-b (but not Cbl-3) following activation [54, 57, 87, 88], and can serve as a paradigm for the role Cbl proteins play in the downregulation of many other RTKs, such as the EGFR, PDGFR, CSF-1R, and c-Kit, among others [89-92]. Post-HGF stimulation, Cbl is recruited to the Met receptor via two different modes of interaction. Cbl can associate with the Met receptor directly through binding of the TKB domain to phosphorylated Y1003 in the Met juxta-membrane region. In addition, Cbl can associate with Met indirectly through the binding of the Grb2 adaptor protein, where Grb2 binds a Cbl proline-arginine rich motif with its SH3 domain while binding to Met pY1356 by

way of its SH2 domain. This is highly similar to the EGFR, where c-Cbl and Cbl-b also bind using a dual mechanism of association; the TKB domain binds to pY1045, and Grb2 links Cbl proteins to the receptor indirectly [61, 66]. Both direct and indirect interactions are required for maximal ubiquitination of Met. A Met mutant (Y1003F) which cannot associate to the Cbl TKB domain is not ubiquitinated upon co-overexpression of the two proteins, and a Met mutant (N1358H), failing to associate with Grb2, shows reduced ubiquitination [54]. Both c-Cbl and Cbl-b have the capacity to ubiquitinate Met, as siRNA-mediated knockdown of both c-Cbl and Cbl-b was required to significantly reduce receptor ubiquitination upon HGF stimulation (unplublished data). Once a subject of controversy, the EGFR has now been demonstrated to be both multimono- and polyubiquitinated, and the same is likely to be true for Met [39, 93]. Monoubiquitination of Met is sufficient for Hrs phosphorylation [40], which in turn likely couples the receptor to components of ESCRT complex and lysosomal degradation. The role of receptor polyubiquitination, which is classically associated with proteasomal degradation, is less clear. However, degradation of the Met receptor has been demonstrated to require both the lysosome and proteasome, implying a role for polyubiquitination in receptor downregulation [39, 94].

Aside from ubiquitination, Cbl proteins may also contribute to receptor downregulation by affecting receptor internalization through their versatility as multivalent adaptor proteins. Following activation of the EGFR and Met, both c-Cbl and Cbl-b are bound by the SH3 domain of the adaptor protein CIN85 in their C-terminal regions, recruiting the CIN85/endophillin-1/Dab2 complex to activated receptors at the membrane. Endophillin-1 is thought to create the negative membrane curvature required for the formation of clathrin-coated pits [95], and Dab2 is an endocytic adaptor that has been shown to bind clathrin and regulate assembly of clathrin lattices *in vitro* [96]. Disruption of formation of these complexes inhibited the internalization and prolonged signaling of both the EGF and Met receptors, supporting an ubiquitin-independent role for c-Cbl and Cbl-b in receptor downregulation [71, 72].

12. Loss of Cbl-mediated negative regulation of RTKs in cancer

As mentioned previously, Cbl proteins were first identified as a viral oncogene, v-Cbl, although the mechanisms by which v-Cbl induced transformation were unknown. It was not until six years later when genetic studies revealed that the *C. elegans* Cbl homologue, Sli-1, opposed the function of Let-23, the EGFR homologue, during vulval induction [97, 98]. Since then, with the discovery of Cbl proteins acting as E3 ubiquitin ligases, the role of Cbl proteins in the downregulation of multiple different RTKs has been identified. The importance of this function gained appreciation because of two major revelations: 1) that previously identified oncogenic Cbl mutants all had disrupted E3 ligase activity, and 2) that many oncogenic RTKs harbour mutations that allow them to escape Cbl-mediated ubiquitination and degradation.

In the former case, the transforming ability of oncogenic Cbl proteins lies in their ability to act as dominant negative proteins, maintaining the capacity to bind to activated RTKs, while being deficient in their ability to ubiquitinate their targets [99]. In this way, oncogenic Cbl mutants compete for binding sites on activated RTKs with wt Cbl proteins, resulting in drastically reduced receptor degradation. In support of this, v-Cbl, which consists of only the c-Cbl TKB domain, has transforming activity. However, introduction of the G306E point mutation, which abrogates the ability of the TKB domain to bind to pTyr and hence activated RTKs, eliminates the transforming capabilities of v-Cbl [52, 100]. Other oncogenic Cbl mutants have been identified, including 70Z Cbl and p95Cbl [81, 101]. Found in the 70Z/3 pre-B cell lymphoma and the murine reticulum sarcoma cell line, respectively, both of these mutants as well as v-Cbl have disrupted or absent linker and RING finger domains, a feature common to oncogenic Cbl mutants identified to date.

The converse scenario, where mutations in activated receptors allow escape from Cbl-mediated degradation, is also a mechanism of tumourigenesis. Oncogenic mutants of Met, CSF-1R, EGFR, and c-Kit that have lost the ability to recruit Cbl in a TKB-dependant manner have been identified, and are illustrated in figure 6. In the case of the Met receptor, a Met mutant Y1003F is incapable of being bound by the Cbl TKB domain, and was shown to be transforming both *in vitro* in both fibroblast and epithelial cells, as well as *in vivo* in tumourigenesis assays [40, 54]. The oncogene Tpr-Met, the

result of a carcinogen-induced chromosomal translocation, also lacks Y1003. A recent report demonstrates that simple targeting of Tpr-Met to the plasma membrane is insufficient to induce its rapid degradation, and that re-insertion of the Y1003 Cbl binding site was a key requirement to target Tpr-Met for rapid turnover [88]. Moreover, a Met mutant lacking exon 14, which includes Y1003, has recently been discovered in human lung cancer. This mutant displays decreased Cbl binding, decreased receptor ubiquitination, and isolated tumour cells were dependent on the mutant receptor for proliferation [102].

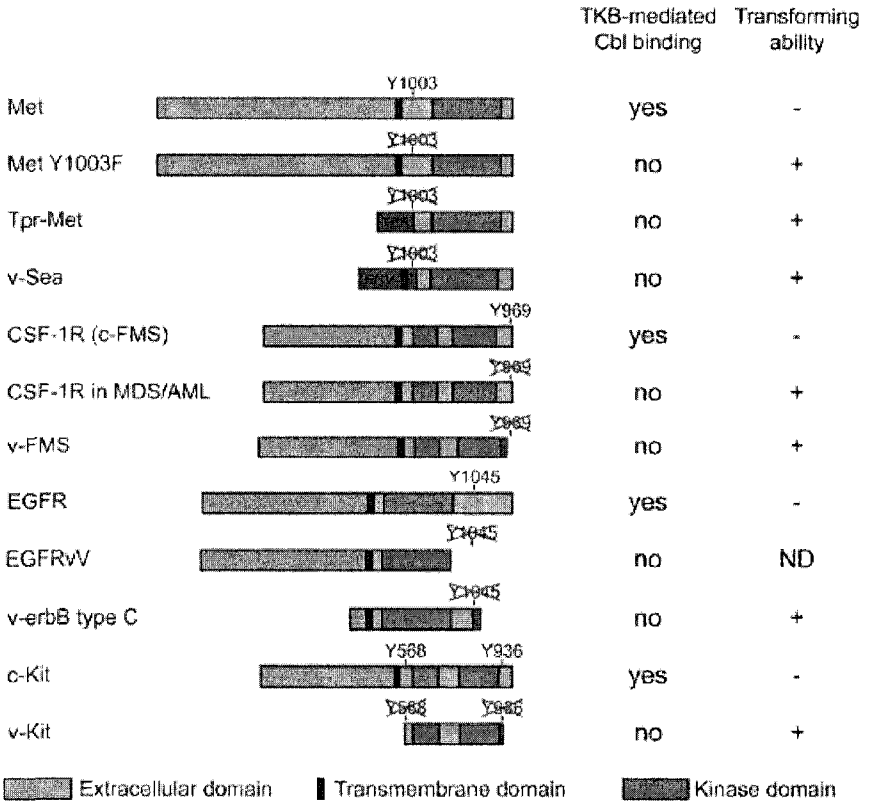

Figure 7. Oncogenic RTK mutants incapable of associating with the Cbl TKB domain. Adapted from Peschard and Park. 2003. Cancer Cell. **3**: 519-23.

In the EGFR and CSF-1R, mutations conferring similar characteristics to the receptor have been identified in cancer. In both cases, loss of the critical tyrosine residue mediating Cbl TKB-domain binding is lost [89, 103]. In the case of c-Kit, Cbl is recruited indirectly through the adaptor protein APS [90]. v-Kit, the oncogenic variant of

c-Kit, lacks Y568 and Y936, which are recruitment sites for APS [104, 105]. Altogether, the mutant receptors lacking Cbl binding sites identified to date emphasize the importance of Cbl-mediated negative regulation of RTKs both *in vitro* and in *in vivo* malignancy.

13. Regulation of Cbl proteins

Just as signaling processes must be attenuated by Cbl- mediated negative regulation, Cbl function must also be regulated to maintain cellular homeostasis. The regulation of Cbl can be grouped into three main categories: 1) Structural regulation, 2) Regulation by degradation, and 3) Regulation without degradation, as illustrated in Figure 8.

As mentioned in section 9, structural regulation of Cbl ubiquitin ligase activity is centered on conformational changes in the linker helix region and the impact they play on phosphorylation of Y371in the linker domain. *In vitro* studies demonstrate that the TKB domain is inhibitory to the E3 ubiquitin ligase function of Cbl, and that phosphorylation of Y371 is required for Cbl E3 ligase activity. Moreover, a Y371 mutation to glutamate functions as a phosphomimetic, constitutively activating Cbl [63, 65]. However, crystallographic studies indicate that Y371 is buried and unavailable for phosphorylation without a conformational change. These discrepancies are reconciled by a model in which interaction of Cbl with the target protein induces a conformational change, both relieving inhibition of the RING domain by the TKB domain, and exposing Y371 for phosphorylation. Hence, a proposed mechanism suggests that Cbl proteins would be regulated by their own structure, keeping the E3 activity turned off only until interaction with a protein to be targeted for ubiquitination [106].

Another important method by which Cbl proteins are regulated is by degradation. This can occur in multiple different ways, some of which are illustrated in Figure 8. Firstly, just as Cbl E3 ligases ubiquitinate and degrade other proteins, some E3 ligases of the HECT family of E3s have been shown to regulate Cbl in a similar manner. The HECT domain E3 ubiquitin ligases Nedd4 and Itch contain WW domains, which can interact with proline-rich domains present in all three mammalian Cbl family members. This interaction induces the ubiquitination and proteasomal degradation of Cbl proteins

dependant on the E3 ligase activity of the HECT ligase but not that of Cbl, as a Cbl-b mutant with abrogated E3 ligase activity (C373A) is still degraded by coexpression of Nedd4 or Itch [107]. Importantly, Nedd4 decreased the Cbl-b-mediated downregulation of the EGFR, allowing for prolonged receptor signaling. This is just one method by which Cbl proteins have been shown to be regulated downstream of the EGFR. In addition, c-Cbl and Cbl-b have been shown to undergo a degree of simultaneous degradation with some RTK substrates such as EGFR and c-Kit, and this degradation is dependant on both the proteasome and lysosome. This may act as a mechanism to control the activity of Cbl ubiquitin ligases as well as the EGFR-associated signaling molecules, since parallel EGFR-Cbl-b coordinated degradation appears to synchronize with the lysosomal degradation of many other proteins involved in an EGFR signaling

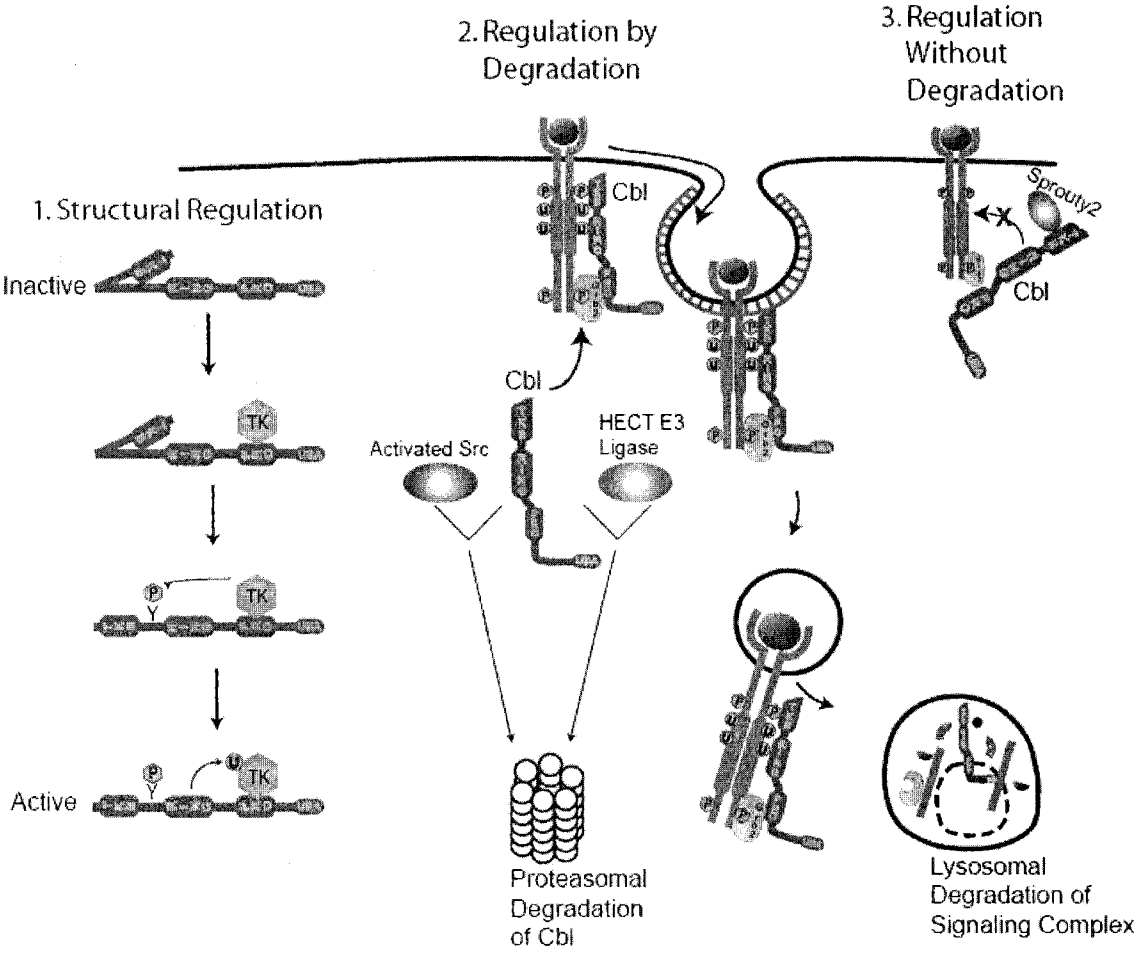

Figure 8. A schematic representation of major mechanisms of Cbl regulation.

complex [108]. Activated Src has also been shown to be capable of degrading c-Cbl. *In vitro* ubiquitination studies first demonstrated that Src activation could induce the tyrosine phosphorylation of c-Cbl and the subsequent ubiquitination of both proteins [109]. Subsequently, Yarden's group confirmed that it is the activation of Src downstream of the EGFR that in part mediates the loss of the pool of c-Cbl degraded by the proteasome downstream of EGFR activation [110]. In terms of biological consequences, activation of Src synergizes with the EGFR to contribute to cellular transformation [111, 112]. One reason for this synergy is likely to be due to the loss of Cbl proteins, as loss of Cbl would disrupt not only the proper negative regulation of both Src and the EGFR, but also other cytosolic TKs and RTKs known to be targeted for Cbl-mediated negative regulation. In addition to Src, other proteins such as the ubiquitin-interacting protein Sts-2, have been shown to induce the degradation of Cbl proteins downstream of the EGFR, although further studies are required to elucidate the physiological role of these proteins [113, 114]. Co-expression of Sts-2 with c-Cbl induces the ubiquitination and downregulation of c-Cbl, an effect that is increased by activation of the EGFR. However, Sts-2 is primarily expressed in haematopoetic cells, which do not express the EGFR, hence the mechanism and physiological relevance of a Sts-2/Cbl interaction remains questionable. Nevertheless, Sts-2 overexpression in Jurkat T-cells reverses the Cbl-mediated suppression of T-cell receptor (TCR) signaling, indicating a potential role for this interaction in T-cells [115]. Cbl-b is also subject to negative regulation in haematopoetic cells, where T-cell receptor (TCR) and CD28 costimulation targets Cbl-b for ubiquitination and degradation in the proteasome by an unknown mechanism [116].

A number of examples of Cbl interactions have now been found that negatively regulate the ability of Cbl proteins to downregulate RTKs without the need for Cbl degradation. One way involves competition with Cbl target proteins for TKB domain binding. The prototype for this regulatory mechanism is Sprouty2. Sprouty2 is weakly and constitutively bound to the Cbl RING finger, however, activation of the EGFR induces the phosphorylation of Sprouty2 on Y55 [117, 118]. In its phosphorylated form, Y55 forms a TKB domain binding site, and associates with the TKB domain of c-Cbl more favorably than with the RING finger domain. In occupying the TKB domain,

Sprouty2 prevents TKB binding to the EGFR, and as a result decreases the Cbl-mediated downregulation of the EGFR. The inhibition of Cbl by Sprouty2 is transient, as Sprouty2 is ubiquitinated by Cbl and degraded by the proteasome as a result of the interaction [118], although one may conjecture that in cells with upregulation of Sprouty2 expression, the inhibition would be less transient. Indeed, Sprouty2 has been found to be upregulated in a panel of human cancer cell lines [119] and hence may contribute to tumourigenesis by excessive inhibition of Cbl downregulation of cytoplasmic TKs and RTKs in general. In addition to Sprouty2, the GTPase Cdc42, the lipid phosphatase SHIP2, and the adaptor proteins Alix and Cortactin are also part of a growing list of proteins that regulate Cbl without inducing its degradation using a variety of mechanisms of competitive inhibition of Cbl-RTK interactions [106].

14. Differences in function and regulation of c-Cbl and Cbl-b

Most of what is known about mammalian Cbl protein function is derived from studies performed on c-Cbl. Cbl-b for many years was largely considered to have functions redundant with those of c-Cbl due to the high homology between the two proteins in their N-termini. c-Cbl and Cbl-b knockout mice are viable, whereas loss of both is embryonic lethal before day E10.5 [120], further implying redundancy in their functions. As a result, a large gap in our understanding still remains with respect to the differences in function of c-Cbl versus Cbl-b. Structurally, c-Cbl and Cbl-b begin to diverge in their C-termini. Both proteins have extensive proline-rich regions, with c-Cbl and Cbl-b having 15 and 17 potential SH3 domain binding sites, respectively. However, c-Cbl and Cbl-b only hold three of these 15 sites in common [99]. c-Cbl also contains two serine-rich 14-3-3 binding sites, however Cbl-b only has one, and as a result c-Cbl associates with 14-3-3 proteins whereas Cbl-b does not [75], although the consequences of this interaction are not clear. Additional differences in C-terminal tyrosine phosphorylation sites and the UBA domain mentioned previously in section 8 also contribute to differences in potential interactors of c-Cbl and Cbl-b.

Circumstances have been uncovered *in vivo* where differences exist both in the signaling complexes associated with c-Cbl and Cbl-b as well as their regulation. Some of the most apparent differences in their function are in T-lymphocytes. c-Cbl functions to

regulate proliferation of thymocytes by negative regulation of the TCR upon co-activation of CD4 [120], a fact reflected by the enhanced proliferation of thymocytes in c-Cbl $^{-/-}$ mice [121]. On the other hand, Cbl-b regulates the TCR in peripheral T-cells upon CD28 costimulation [122], setting the threshold required for T-cell activation, explaining how Cbl-b$^{-/-}$, but not c-Cbl$^{-/-}$ mice develop autoimmune disorders [123]. *In vitro*, the signaling complexes and regulation of c-Cbl and Cbl-b were found to be different in cells transformed by the oncogene BCR-Abl. Both Cbl proteins were found to associate with BCR-Abl, however, only c-Cbl associated with activated PI3K and CrkL. In contrast, Cbl-b associated with monoubiquitinated Vav. At the level of Cbl expression, c-Cbl protein levels were unaffected by transformation by BCR-Abl, but Cbl-b mRNA levels were decreased resulting in an associated loss of Cbl-b protein [73]. Unfortunately, not many direct comparisons have been performed with respect to examining regulation and function of c-Cbl versus Cbl-b outside of haematopoetic cells, despite the fact that c-Cbl and Cbl-b responses to stimulation of RTKs appear to differ. For example, c-Cbl tyrosine phosphorylation increases dramatically in response to EGF stimulation but not PDGF, despite Cbl proteins being negative regulators of both receptors. In contrast, Cbl-b tyrosine phosphorylation increases moderately downstream of both EGF and PDGF stimulation [124], but the reasons behind these differences remain unclear. There also remain cases, such as the effects of Sts-2, where only c-Cbl regulation has been examined, and not that of Cbl-b. The same is true in the case of the c-Cbl $^{-/-}$ and Cbl-b $^{-/-}$ mice, where c-Cbl $^{-/-}$ mice have increased ductal branching in mammary fat pads [121], but whether the Cbl-b $^{-/-}$ mice display this phenotype as well has not been reported. Further investigation into differences in regulation and the signaling molecules associating with c-Cbl versus Cbl-b in epithelial cells may reveal important findings considering the major role Cbl proteins play in the negative regulation of RTKs and their involvement in cancer.

15. Rationale

Much remains to be understood concerning the mechanisms by which members of the mammalian Cbl family of E3 ubiquitin ligases are regulated downstream of RTKs and cytoplasmic TKs. A common theme has emerged by which many oncogenic RTKs

escape Cbl-mediated negative regulation, and this enhances their oncogenicity. Loss of c-Cbl or Cbl-b-mediated negative regulation could also be achieved through excessive negative regulation of Cbl proteins themselves by activated RTKs. This would be hypothesized to affect signaling from multiple TKs simultaneously and could be one mechanism by which different RTKs synergize to promote oncogenesis. This thesis work aims to compare the regulation of c-Cbl and Cbl-b downstream of the Met receptor tyrosine kinase, as well as to elucidate the mechanism by which such regulation occurs (Chapter 2). Understanding how c-Cbl and Cbl-b are regulated downstream of Met may provide a model for the regulation of Cbl proteins downstream of other RTKs, and may provide an explanation for differences in the mechanisms by which Met and other RTKs contribute to oncogenesis.

Abbreviations

APS	Adaptor containing PH and SH2 domain
BCR-Abl	Breakpoint Cluster Region-Abl
Cbl	Casitas B-lineage Lymphoma
CCV	Clathrin-Coated Vessicle
CIN85	85K Cbl-Interacting Protein
Crk	CT10 Regulator of Kinase
CSF-1R	Colony-Stimulating Factor-1 Receptor
Dab2	Disabled-2
E1	Ubiquitin-Activating Enzyme
E2	Ubiquitin-Conjugating Enzyme
E3	Ubiquitin-Protein Ligase
E4	Polyubiquitin Protein Ligase
EGF(R)	Epidermal Growth Factor (Receptor)
ESCRT	Endosomal Sorting Complex Required for Transport
FGFR	Fibroblast Growth Factor Receptor
Fms	Feline McDonough Strain
FOXO	Forkhead box protein O
Gab1	Grb2 Associated Binding protein 1
Grb2	Growth factor Receptor-Bound protein 2
GEF	Guanine nucleotide Exchange Factor
H2A/B	Histone 2 A/B
H3	Histone 3
HECT	Homologous to E6AP Carboxy Terminus
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
Hrs	Hepatocyte growth factor Regulated tyrosine kinase Substrate
MAPK	Mitogen Activating Protein Kinase
MBM	Met Binding Motif
Met	MNNG-HOS transforming gene
MVB	Multi-Vesicular Body
p53	Tumour Suppressor Protein 53

PDGF(R)	Platelet-Derived Growth Factor (Receptor)
PH	Pleckstrin Homology
PI3K	Phosphatidyl Inositol-3-Kinase
PLC-γ	Phospholipase C gamma
PTB	Protein Tyrosine Binding
PTEN	Phosphatase and Tensin homolog
pTyr	phosphoTyrosine
RING	Really Interesting New Gene
RTK	Receptor Tyrosine Kinase
Sea	Sarcoma, Erythroblastosis and Anemia
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
SLAP	Src-Like Adaptor Protein
Sts	Suppressor of T-cell receptor Signalling
TCR	T-Cell Receptor
TK	Tyrosine Kinase
TKB	Tyrosine Kinase Binding
Tpr	Translocated Promoter Region
Ub	Ubiquitin
UBA	Ubiquitin-Associated domain
UIM	Ubiquitin Interacting Motif
WW	Domain that contains two highly conserved tryptophan residues
ZAP-70	Zeta-Associated Protein 70

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Chapter 2

Differential Regulation of the Ubiquitin Ligases c-Cbl and Cbl-b Downstream of the Met Receptor Tyrosine Kinase

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Abstract

The E3 ubiquitin ligases c-Cbl and Cbl-b are key negative regulators of many receptor tyrosine kinases (RTKs). Different mechanisms resulting in the loss of c-Cbl and Cbl-b mediated ubiquitination have been reported to impair downregulation of RTKs and have been identified in tumourigenesis. Despite this, little is known about the mechanisms by which c-Cbl and Cbl-b are themselves regulated, and it can be postulated that excessive negative regulation of these proteins could contribute to RTK-mediated tumourigenesis. Here we report a differential regulation of c-Cbl and Cbl-b downstream of the Met RTK, where Met selectively targets Cbl-b for degradation mediated in part by the proteasome. We demonstrate that loss of Cbl-b requires Met kinase activity, intact Cbl-b ubiquitin ligase activity, as well a domain between the Cbl-b RING and UBA domains, and proceeds via a mechanism distinct from that by which Cbl proteins induce Met degradation. Differential loss of Cbl-b appears specific to the Met receptor, as other active tyrosine kinases known to associate with Cbl proteins, such as the EGFR, Neu, and Src kinases, were incapable of promoting loss of Cbl-b. We also observe differential regulation of c-Cbl and Cbl-b in cancer cell lines known to overexpress a variety of tyrosine kinases, and postulate that losses of Cbl proteins by this and similar other mechanisms may represent additional means of abrogating Cbl-mediated negative regulation in cancer.

Introduction

Over the past several years, the Cbl family members c-Cbl and Cbl-b have emerged as versatile signaling proteins, playing important roles in a wide range of cellular signaling processes, principally downstream of tyrosine kinases [1, 2]. One aspect of their function is to serve as key scaffolding proteins, recruiting a variety of proteins to tyrosine kinases via interaction with the c-Cbl and Cbl-b C-terminal tails. The C-terminal halves of c-Cbl and Cbl-b contain multiple proline-rich motifs, phosphorylated tyrosine residues, and a UBA domain. In the case of Cbl-b, the UBA domain confers the ability to bind ubiquitin, as opposed to the c-Cbl UBA, which cannot [3, 4].

A well-characterized function of c-Cbl and Cbl-b is as E3 ubiquitin ligases, mediated by their highly-conserved N-terminal tyrosine-kinase binding (TKB) and RING finger domains [5]. The Cbl TKB domain consists of a four-helix bundle, EF-hand, and variant SH2 domain which together mediate direct binding of Cbl proteins to key phosphotyrosine residues on target proteins [6]. Ubiquitination is carried out through the recruitment of the E2 enzyme UbcH7 to the RING finger domain, which subsequently catalyzes ubiquitin transfer to the substrate, a process that requires both the TKB and RING domains [7-9]. Ubiquitination then generally marks the target protein for proteasomal or lysosomal degradation.

Receptor and non-receptor tyrosine kinases control many physiological cellular processes, such as growth, proliferation, and migration. These processes must be kept tightly regulated in order to ensure that cellular homeostasis is maintained and prevent malignancy, as deregulated RTK and TK signaling can lead to tumourigenesis [10, 11]. One mechanism by which this deregulation can occur is through the loss of cellular systems by which tyrosine kinase signaling is attenuated. Through their functions as E3 ligases and scaffold proteins, c-Cbl and Cbl-b have emerged as essential negative regulators, attenuating signaling of numerous receptor and non-receptor tyrosine kinases, including the EGFR, PDGFR, Ret, Syk, members of the Src family of kinases, and the Met family RTKs, Ron and Met [12-21]. Cbl function is critical for tight regulation of such signaling molecules. Cbl proteins possessing a mutant RING domain with defective E3 ligase activity act as dominant negative proteins and result in enhanced signaling by

both receptor and non-receptor tyrosine kinase Cbl targets, leading to transformation both *in vitro* and *in vivo*. Moreover, c-Cbl$^{-/-}$ mice develop mammary hyperplasias [22].

The reverse situation, whereby RTKs possess mutations disrupting their association with Cbl family proteins, has also been implicated in cancer (reviewed in [23]). c-Cbl and Cbl-b bind to the Met receptor both via a direct interaction of the TKB domain with phosphorylated tyrosine 1003 in the Met juxtamembrane region, and indirectly through interaction of Grb2 with a Cbl proline-rich region. Both of these modes of recruitment are required for optimal Met ubiquitination and downregulation, and a Y1003F Met receptor mutant lacking the ability to associate with the TKB domain directly has greater transforming ability *in vitro* [20], is more tumourigenic than wt Met *in vivo* [24], and a Met mutant lacking the exon in which this residue resides has been identified in human lung cancer [25].

Despite the importance of c-Cbl and Cbl-b as negative regulators of multiple tyrosine kinases involved in cellular transformation and oncogenesis, little is known about regulation of Cbl proteins themselves, differences in regulation between c-Cbl and Cbl-b, or the consequences of perturbations in the mechanisms regulating either Cbl activity and/or expression. In the case of the EGFR, following stimulation with EGF both c-Cbl and Cbl-b have been shown to undergo coordinated degradation with the EGFR in a lysosome and proteasome-dependant manner [26]. In the case of c-Cbl, Src activation also induces loss of c-Cbl in a proteasome-dependant manner, [27] although this has not yet been demonstrated for Cbl-b. Src-induced degradation of c-Cbl correlated with a 70-110% increase in EGFR levels, arguing in favour of Src-induced loss of c-Cbl playing a role in oncogenic synergy observed between the two tyrosine kinases [27]. Differential regulation of c-Cbl and Cbl-b has been demonstrated in only a few instances. In BCR-Abl transformed cells, c-Cbl levels remain unaffected, whereas expression of Cbl-b mRNA is decreased, resulting in a pro-migratory effect [28], again highlighting a potential role for Cbl-b downregulation in oncogenesis. In addition, CD28 stimulation in T-lymphocytes results in downregulation of Cbl-b through ubiquitination. Although it is not clear whether this occurs in an autocatalytic manner, the same does not occur for c-Cbl downstream of CD28 [29].

Here we demonstrate differential regulation of c-Cbl and Cbl-b downstream of the Met receptor tyrosine kinase, whereby activation of the Met receptor induces a preferential loss in Cbl-b, but not c-Cbl. Degradation of Cbl-b is mediated by the proteasome and appears to be Met-specific. Met kinase activity, as well as Cbl-b ubiquitin ligase activity and a region of the Cbl-b C-tail lying between the RING and UBA domains are required. We propose a mechanism by which Met-induced Cbl-b degradation may potentiate oncogenesis through multiple other RTKs as a result of the removal of this important negative regulator.

Materials and Methods

Cell Culture and Transfections.

All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and Gentamycin. Transfection of HEK293 cells was performed using Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturer's instructions. EGFR and NeuNT were provided by Dr. W. Muller, and Src Y527F was a gift from Dr. J. Brugge.

Antibodies and Reagents.

A polyclonal antibody was raised against the Met receptor (Rodrigues et al., 1991). HA monoclonal antibody was purchased from Covance (Berkeley, CA), EGFR Ab-17 was purchased from Lab Vision (Fremont, CA), ErbB2 Ab-3 from Calbiochem (San Diego, CA), α-tubulin (Boehringer Mannheim), actin AC-15 from Sigma (St. Louis, MO), pY20 and pY100 from Cell Signaling Technology (Boston, MA) and Anti- c-Cbl (sc-170), Cbl-b (H-121), ubiquitin (P4D1), and Src (B-12) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MG-132 was from Calbiochem (San Diego, CA).

Immunoprecipitations and Western blotting.

Transfected cells were lysed 48 hours post-transfection with RIPA lysis buffer (50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1 mmol/L PMSF, 1 mmol/L NaV, 50 mmol/L NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin). The specified antibodies were incubated with equal amounts of whole cell lysate for 1 hour at 4°C with rotation, and then isolated by incubation with Protein A Sepharose beads (Amersham Biosciences) and washed three times with RIPA lysis buffer. Protein samples were then subjected to SDS-PAGE, transferred to nitrocellulose membrane (Amersham Biosciences), and blocked with 3% BSA in TBST (10mmol/L Tris pH 8.0, 150 mmol/L NaCl, 2.5 mmol EDTA, 0.1% Tween-20) for 1 hour. Immunoreactive bands were visualized using the Enhanced Chemiluminescence kit (Amersham Biosciences), except for when blotting for ubiquitin, where the SuperSignal West Femto Maximum Sensitivity Substrate kit was used (Pierce).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer's instructions to create the Cbl-b. An AgeI cut site was created and Pro 505, Pro 508, and Arg 510 were mutated to Ala using the 5'-CATCTAAGCCTGGCACCGGTGGCTCCTGCCCTGGATCTAATTC-3' primer and its complementary primer.

Quantitative real-time PCR.

Total cellular RNA was extracted from HEK293 cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. 5 μg of RNA was reverse transcribed and amplified as previously described [24]. 5-aminolevulinic acid synthase (ALAS1) was used as a control to normalize mRNA levels. Primer sequences were as follows: ALAS1, sense, 5'- CTGCAAAGATCTGACCCCTC –3', and antisense, 5'– CCTCATCCACGAAGGTGATT –3'. Both c-Cbl and Cbl-b used the same sense primer directed to the HA tag: 5'- GCCTACCCTTATGATGTGCC -3'. c-Cbl, antisense, 5'-CCAGCACTTCTCCACCATCT -3', and Cbl-b, antisense, 5'-CAAGTCTTCTCCACGGTCCT -3'. The mean cycle (Ct) value for each transcript was normalized by dividing it by the mean Ct value for the ALAS1 transcript for that sample. Measurements were taken four times for each set of transfected cells, and three sets of transfected cells were measured. The level of c-Cbl or Cbl-b RNA in the presence of Met is expressed as the mean fold difference in expression relative to the level of c-Cbl or Cbl-b alone +/- standard error.

Results

c-Cbl and Cbl-b protein levels are differentially regulated in breast cancer cell lines.

The ubiquitin ligases c-Cbl and Cbl-b have been increasingly characterized as key negative regulators of receptor and non-receptor tyrosine kinases, including Src family kinases, CSF-1R, EGFR, and Met [23, 30]. Impaired negative regulation generally occurs through loss of Cbl-mediated ubiquitination, leading to the prolonged activation, half-life, and downstream signaling of target kinases, thereby increasing their oncogenic potential. Cbl proteins themselves are regulated by a variety of mechanisms [31], although much remains to be understood with regards to the extent which these regulatory mechanisms occur for c-Cbl and Cbl-b, respectively, and the contribution they may have for oncogenesis. Therefore, we hypothesized that differences or aberrations in c-Cbl and Cbl-b regulation may contribute to the simultaneous deregulation of multiple tyrosine kinases and their role in cancer progression. To evaluate this possibility, a panel of breast cancer cell lines were lysed and protein levels of endogenous c-Cbl and Cbl-b were quantified (Figure 1). Compared to lysates from HeLa cells, which consistently express high levels of both Cbl family members, we observed significant differences in protein levels of both c-Cbl and Cbl-b between cell lines. Importantly, changes in protein levels of c-Cbl were not necessarily paralleled by similar changes in Cbl-b (Figure 1, compare MDA 231 to MDA 468 for c-Cbl vs. the same lysates for Cbl-b), showing that c-Cbl and Cbl-b are differentially regulated in these breast cancer cell lines.

Met Expression Induces a Preferential Loss of Cbl-b.

These results raised the possibility that low protein levels of one or both Cbl proteins may contribute to the progression of the parental tumours. In the case of the EGFR, ligand-induced receptor activation has been demonstrated to result in recruitment of both c-Cbl and Cbl-b, ubiquitination of the receptor, and a subsequent coordinated degradation of EGFR, c-Cbl, and Cbl-b via the lysosome [12, 26, 32]. Additionally, regulation of c-Cbl protein levels has been shown to occur downstream of an activated EGFR through an EGF-induced association of c-Cbl with activated Src, leading to the phosphorylation and ubiquitination of both proteins and ending in their proteasome-

mediated degradation [27, 33]. Considering this evidence, and that we have previously shown Met to be an RTK targeted for c-Cbl and Cbl-b -mediated ubiquitination and degradation [20], this raised the question as to whether Met activation would affect c-Cbl or Cbl-b protein levels. As shown in Figure 2A, transient co-expression of increasing amounts of Met expression vector with either c-Cbl or Cbl-b in HEK293 cells induced a dramatic loss of Cbl-b protein levels, whereas c-Cbl levels remained unaltered. It is also important to note that high levels of Met expression induce its ligand-independent activation and tyrosine phosphorylation, as indicated by immunoblotting using antibodies to phosphotyrosine (Figure 2A). The same effect was observed in the COS-7 cell line (data not shown). Met receptor levels decreased in the presence of both c-Cbl and Cbl-b (Figure 2A), consistent with the role of Cbl proteins as negative regulators of the Met receptor. The preferential loss of Cbl-b when compared with c-Cbl was also observed following co-expression of increasing amounts of Tpr-Met, a cytosolic, constitutively activated form of the Met receptor (Figure 2B). Notably, unlike Met, levels of Tpr-Met remained stable when co-expressed with either Cbl protein, due to the fact that it lacks the Cbl TKB domain binding site, Y1003, escaping Cbl-mediated ubiquitination [20, 34, 35]. To determine whether preferential loss of Cbl-b could be observed with endogenous proteins, the level of endogenous Cbl-b in cell lines known to express differing amounts of Met was examined. The Okajima and MKN-45 cancer cell lines, which are known to possess an amplified Met locus, resulting in high expression levels and ligand-independent activation of the receptor [36, 37], also express lower amounts of endogenous Cbl-b than do cell lines with lower Met expression (Figure 2C). Therefore, high levels of activated endogenous Met may promote a preferential loss of Cbl-b.

Preferential loss of Cbl-b protein is Met-specific.

Both c-Cbl and Cbl-b are recruited to other RTKs, such as EGFR and Neu/ErbB2 tyrosine kinases [12, 32, 38], as well as the cytosolic Src family of tyrosine kinases [39-41]. To establish whether preferential loss of Cbl-b represented a general effect induced by overexpression of tyrosine kinases known to associate with Cbl proteins, increasing amounts of EGFR, as well as constitutively activated forms of Neu (NeuNT) and Src (Src Y527F) were co-expressed with either c-Cbl or Cbl-b and Cbl protein levels were

examined. Increasing expression of either the EGFR or Src Y527F resulted in a loss of both c-Cbl and Cbl-b (Figure 3 A,B), in agreement with previous reports [26, 27, 33]. Conversely, no loss of either c-Cbl or Cbl-b protein occurred as a result of expressing NeuNT (Figure 3C), consistent with the observation that Cbl proteins have been demonstrated to associate only very weakly with this RTK, compared to the EGFR [38].

Met-induced downregulation of Cbl-b requires Met kinase activity.

All signaling cascades induced by Met are dependant on dimerization of the receptor, which leads to autoactivation of the kinase domain and phosphorylation of key tyrosine residues serving as recruitment sites for signaling molecules [42]. As Cbl proteins have been demonstrated to bind both directly and indirectly to the Met receptor and both of these modes of association require phosphorylation of key tyrosine residues [20], we used a kinase dead Met mutant (K1110A) illustrated in Figure 4A, to examine whether Met kinase activity was required for Met-induced downregulation of Cbl-b. As expected, coexpression of a kinase dead Met mutant with Cbl-b resulted in abrogated downregulation of Cbl-b levels when compared to wt Met (Figure 4B). Still, it is apparent that some Cbl-b loss does occur, possibly the result of high protein levels occuring from overexpression of both constructs.

Intact Cbl-b ubiquitin ligase activity and C-tail region are required for Met-induced Cbl-b downregulation.

To elucidate the domains in Cbl-b required for loss we performed structure-function studies. c-Cbl possesses the ability to self-ubiquitinate, leading to its proteasomal degradation [27], implicating the RING finger as a potentially important domain. Moreover, Cbl-b possesses an UBA domain capable of binding to ubiquitin, whereas the c-Cbl UBA domain cannot [3, 4]. The ability to bind to ubiquitin confers upon some proteins the ability to associate with ubiquitinated cargo present in the endosomal pathway of internalization destined for degradation in the lysosome [43, 44]. To test the contributions of each of these domains and others to Met-induced Cbl-b downregulation, Cbl-b constructs with mutations specifically abrogating the respective functions of these domains, illustrated in Figure 5A, were expressed in the absence or

presence of Met. A Cbl-b TKB domain mutant protein (G298E) that lacks the ability to bind to phosphotyrosine [45], was lost in the presence of Met (Figure 5B). Similarly, deletion of the Cbl-b UBA domain in its entirety (Cbl-b ΔUBA), or a point mutation specifically disabling the UBA domain from binding to ubiquitin (Cbl-b A937E) [46] were ineffective at stabilizing Cbl-b in the presence of Met. Notably, specific abrogation of Cbl-b ubiquitin ligase activity (C373A) resulted in not only stabilization of the receptor to levels far exceeding those when Met is expressed alone, but also in stabilization of Cbl-b protein levels (Figure 5B). This indicates that the ubiquitin ligase activity of Cbl-b is not only essential for Cbl-b – mediated negative regulation of Met, but is also a key requirement for the mechanism by which Met induces a differential negative regulation of Cbl proteins. Finally, a Cbl-b mutant lacking the entire C-tail but with an intact RING domain is stable in the presence of Met (Figure 5C), implying a role for protein-protein interactions unique to the Cbl-b C-tail. Therefore, we conclude that both the ubiquitin ligase activity and C-tail region of Cbl-b are necessary to induce Cbl-b downregulation.

Cbl-b associates with ubiquitinated proteins downstream of Met.

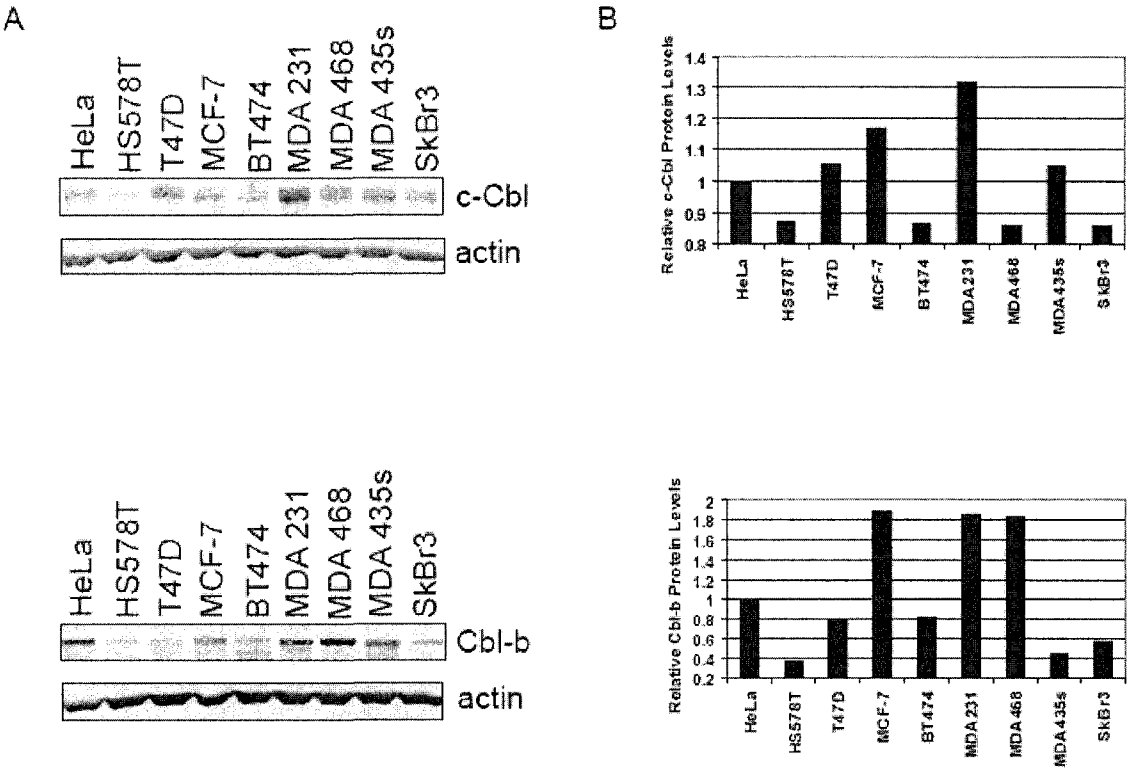
Ubiquitination through K48-linked polyubiquitin chains targets proteins to the 26s proteasome for degradation. Ubiquitination of Cbl proteins has previously been characterized as a mechanism used to control Cbl protein levels. During the Src-induced degradation of c-Cbl downstream of the EGFR, c-Cbl becomes ubiquitinated although an ubiquitin ligase deficient mutant of c-Cbl is still susceptible to degradation, suggesting that other ubiquitin ligases may be responsible [33]. Moreover, the HECT E3 ubiquitin ligases Nedd4 and Itch are capable of ubiquitinating and downregulating c-Cbl and Cbl-b, also independent of Cbl ligase activity [47]. Having shown that the ubiquitin ligase activity of Cbl-b is necessary for Cbl-b downregulation, we hypothesized that Cbl-b may autoubiquitinate in response to Met, resulting in its own degradation. To test this, c-Cbl and Cbl-b were immunoprecipitated from lysates used in Figure 2A, and blotted for ubiquitin. Both proteins were found to associate with ubiquitinated proteins in a Met-dependant fashion, however, loss of this association paralleled loss of Cbl-b levels, and not c-Cbl levels (Figure 6).

Cbl-b loss is mediated in part by proteasomal degradation.

Cbl-mediated ubiquitination has been demonstrated to result in varying effects on the stability of a target protein. In the case of cytosolic proteins, polyubiquitination of many proteins known to associate with c-Cbl and Cbl-b, such as Sprouty2, Fyn, and Lyn results in their proteasomal degradation [15, 48, 49]. However, in T-cells, Cbl-b-mediated ubiquitination of the p85 subunit of PI3K does not affect its protein levels, but rather results in its inactivation [50]. Given that the loss of Cbl-b requires Cbl-b ubiquitin ligase activity and that Cbl-b is a cytosolic protein, the effects of proteasomal inhibition on levels of Cbl-b in the presence or absence of Met were examined. MG132, an inhibitor of the proteasome, showed a modest rescue of Cbl-b protein levels in the presence of Met, suggesting that the proteasome is responsible for some of the Met-induced degradation of Cbl-b (Figure 7A). Given that we did not see complete rescue of Cbl-b, levels of exogenous Cbl-b RNA were measured and compared to those of c-Cbl in the presence or absence of Met using real-time PCR. No loss of Cbl-b at the RNA level was detected, and on the contrary, Met induced an increase of both c-Cbl and Cbl-b RNA compared to cells transfected with only c-Cbl or Cbl-b (Figure 7B), despite the same preferential degradation of Cbl-b occurring at the protein level (Figure 7C). Proteasomal degradation is thus responsible for part of the Cbl-b protein loss downstream of Met.

Figure 1. c-Cbl and Cbl-b are differentially regulated in breast cancer cell lines. (A) Cell lines indicated were lysed and 50μg of whole cell lysate were subjected to SDS-PAGE, and immunoblotted for c-Cbl, Cbl-b, and actin. (B) c-Cbl and Cbl-b protein levels were quantified using the Odyssey system and normalized using actin protein levels.

Figure 1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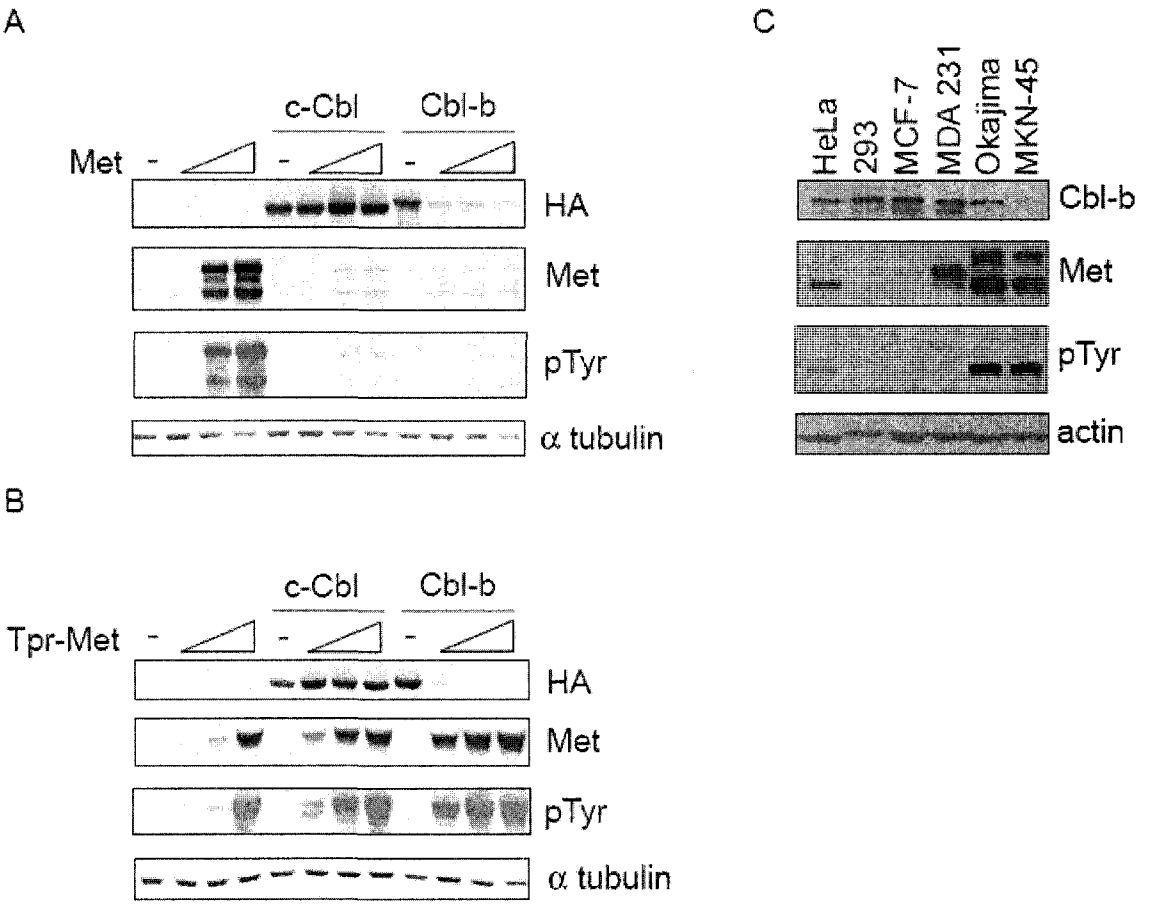
T47D
MCF-7
BT474
MDA 231
MDA 468
MDA 435s
SkBr3
c-Cbl
actin
Cbl-b
actin
B
Relative c-Cbl Protein Levels
Relative Cbl-b Protein Levels

Figure 2. Met Expression Induces a Preferential Loss of Cbl-b.

(A) Increasing amounts of Met or (B) Tpr-Met were transiently transfected into HEK293 cells with or without HA-c-Cbl or HA-Cbl-b. 48 hrs post-transfection, cells were lysed, and equal amounts of protein extracts were subjected to SDS-PAGE, and immunoblotted with Met, HA, pTyr, and α-tubulin antibodies. (C) Cell lines indicated were lysed, subjected to SDS-PAGE, and immunoblotted for Met, Cbl-b, pTyr, and actin.

Figure 2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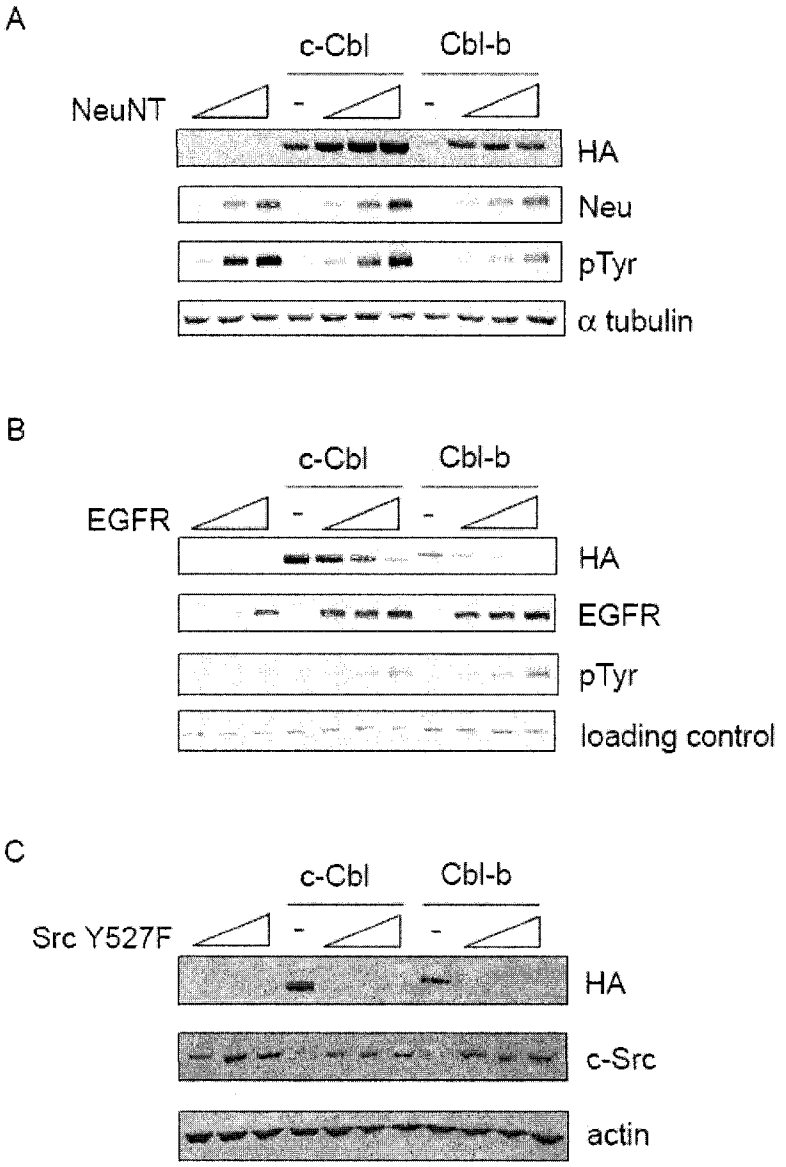
c-Cbl
Cbl-b
Met
-
-
-
HA
Met
pTyr
α tubulin
B
c-Cbl
Cbl-b
Tpr-Met
-
-
-
HA
Met
pTyr
α tubulin
C
HeLa
293
MCF-7
MDA 231
Okajima
MKN-45
Cbl-b
Met
pTyr
actin

Figure 3. Preferential loss of Cbl-b protein is Met-specific.

(A) Increasing amounts of NeuNT, (B) EGFR, or (C) Src Y527F were transiently transfected into HEK293 cells with or without HA-c-Cbl or HA-Cbl-b. 48 hrs post-transfection, cells were lysed, and equal amounts of protein extracts were subjected to SDS-PAGE, and immunoblotted with Neu, EGFR, c-Src, HA, pTyr, actin, and α-tubulin antibodies.

Figure 3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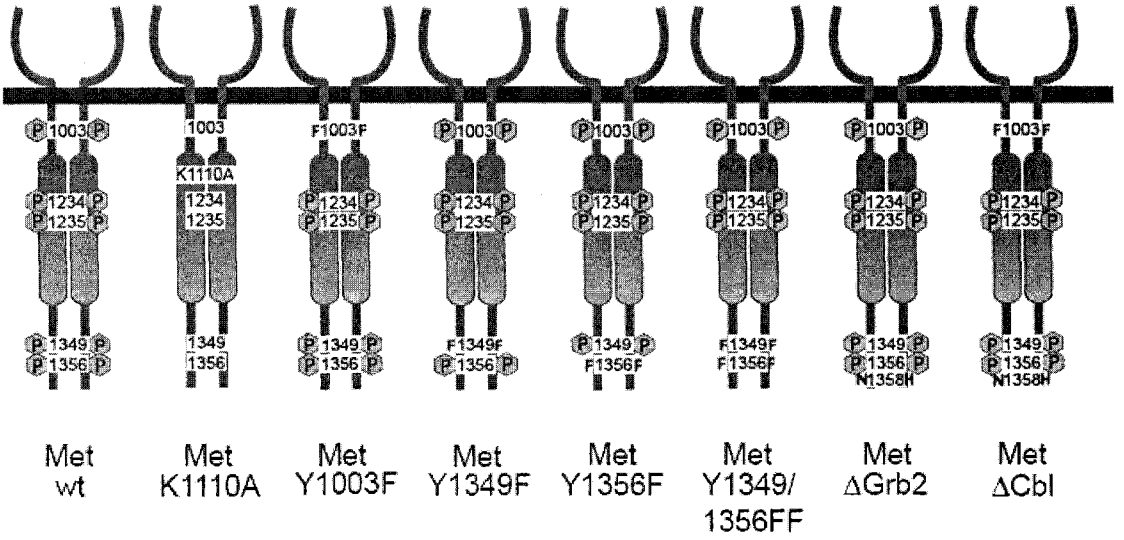
c-Cbl
Cbl-b
NeuNT
HA
Neu
pTyr
α tubulin
B
c-Cbl
Cbl-b
EGFR
HA
EGFR
pTyr
loading control
C
c-Cbl
Cbl-b
Src Y527F
HA
c-Src
actin

Figure 4. Met-induced downregulation of Cbl-b requires Met kinase activity.
(A) A schematic representation of the panel of Met mutants. (B) A panel of Met mutants was transfected into HEK293 cells alone or with HA-Cbl-b or (C) HA-c-Cbl. Cells were lysed 48-hours post-transfection, and equal amounts of protein extracts were subjected to SDS-PAGE and immunoblotted with Met, HA, and actin antibodies.

Figure 4

A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P 1234 P
P 1235 P
P 1349 P
P 1356 P
1003
K1110A
1234
1235
1349
1356
F1003F
F1349F
F1356F
N1358H
Met wt
Met K1110A
Met Y1003F
Met Y1349F
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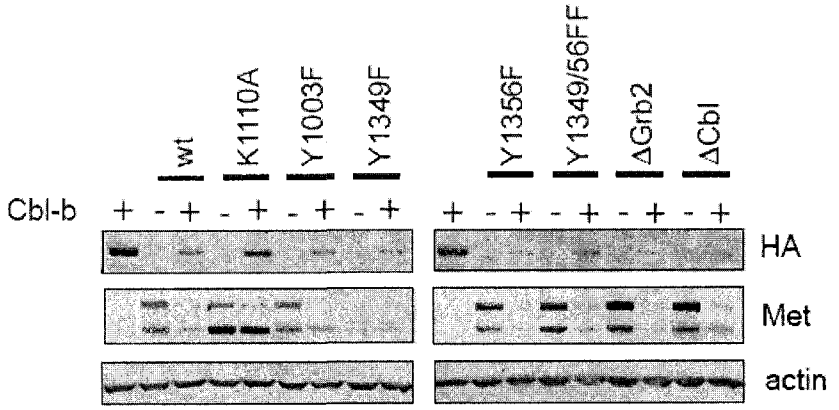
Met ΔGrb2
Met ΔCbl

B


wt
K1110A
Y1003F
Y1349F
Y1356F
Y1349/56FF
ΔGrb2
ΔCbl
Cbl-b
+ - + - + - + - + + - + - + - + - +
HA
Met
actin

C

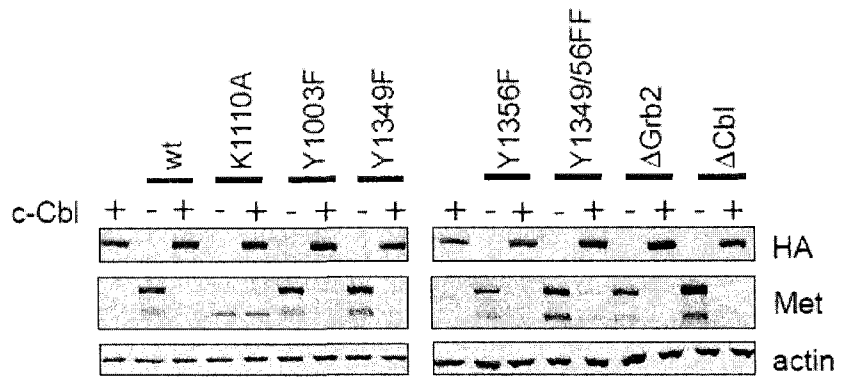
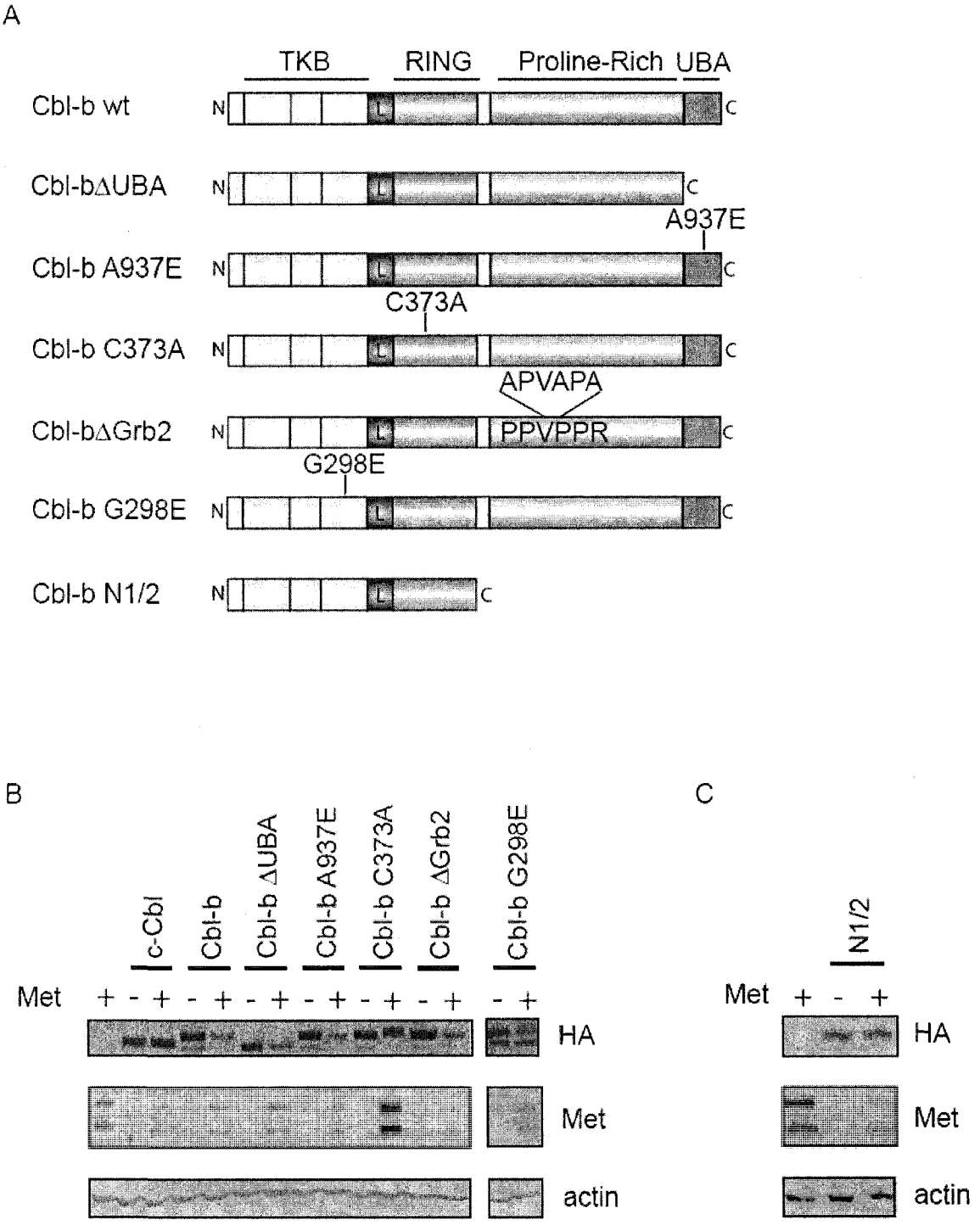

wt
K1110A
Y1003F
Y1349F
Y1356F
Y1349/56FF
ΔGrb2
ΔCbl
c-Cbl
+ - + - + - + - + + - + - + - + - +
HA
Met
actin

Figure 5. Intact Cbl-b ubiquitin ligase activity and C-tail region are required for Met-induced Cbl-b downregulation.

(A) A schematic representation of the panel of Cbl-b mutants used. (B,C) Indicated Cbl constructs were transfected with or without Met in HEK293 cells. Preparation of lysates and immunoblotting was performed as in Figure 4.

Figure 5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TKB
RING
Proline-Rich
UBA
Cbl-b wt
Cbl-bΔUBA
A937E
Cbl-b A937E
C373A
Cbl-b C373A
APVAPA
Cbl-bΔGrb2
PPVPPR
G298E
Cbl-b G298E
Cbl-b N1/2
N
C
L
B
c-Cbl
Cbl-b
Cbl-b ΔUBA
Cbl-b A937E
Cbl-b C373A
Cbl-b ΔGrb2
Cbl-b G298E
Met
HA
Met
actin
C
N1/2
Met
HA
Met
actin

Figure 6. Loss of ubiquitinated proteins associated with Cbl-b downstream of Met parallels loss of Cbl-b.

The same lysates used in Figure 2A were subjected to immunoprecipitation with anti-HA antibodies. Immunoprecipitated HA-tagged proteins and corresponding whole cell lysates were separated using SDS-PAGE, and immunoblotted with HA and actin antibodies. Immunoprecipitated HA-tagged proteins were further immunoblotted for ubiquitin.

Figure 6

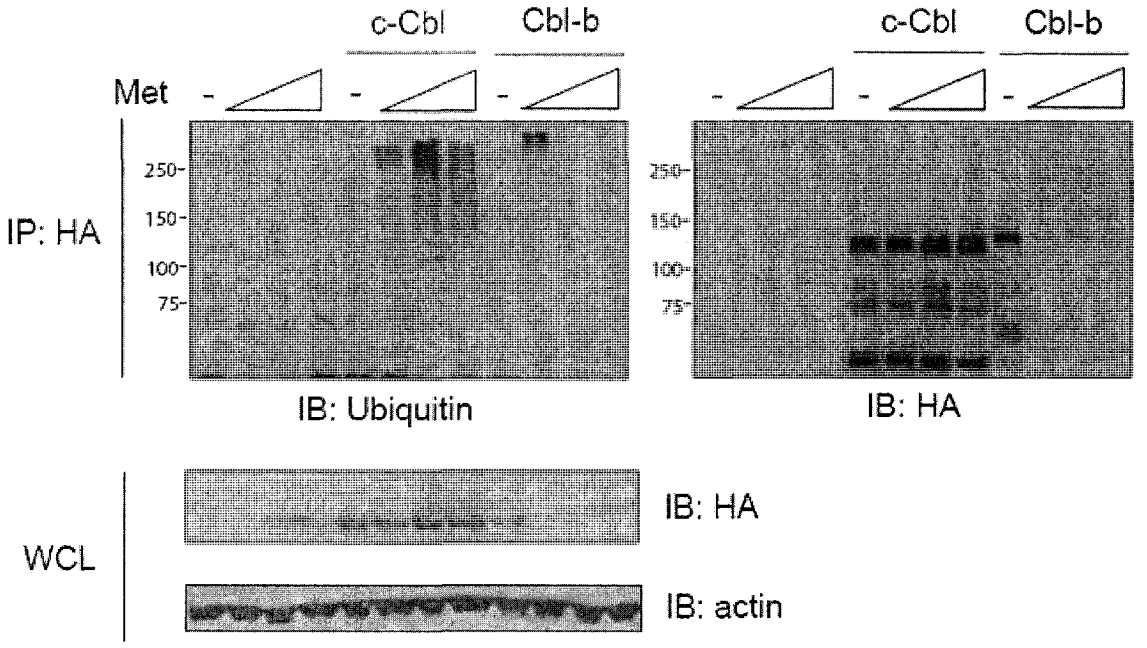
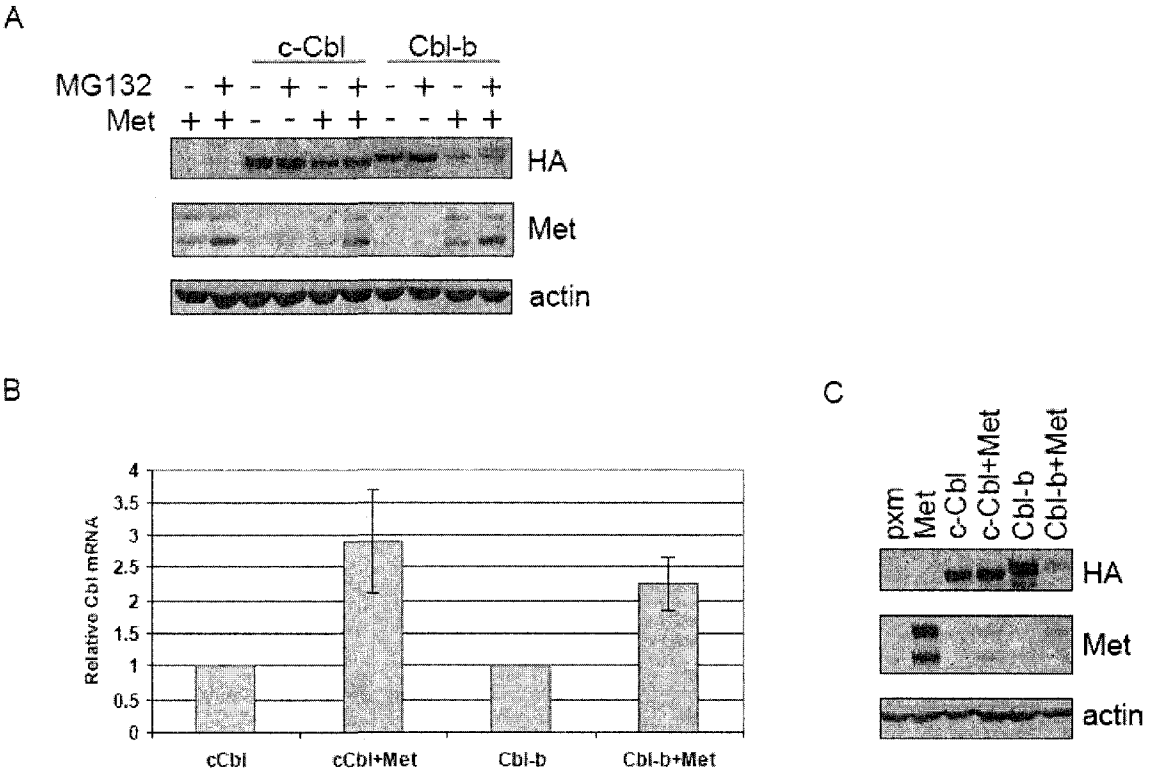

c-Cbl
Cbl-b
c-Cbl
Cbl-b
Met
- - - - - -
IP: HA
250-
150-
100-
75-
250-
150-
100-
75-
IB: Ubiquitin
IB: HA
WCL
IB: HA
IB: actin

Figure 7. Cbl-b loss is mediated in part by proteasomal degradation.
(A) HEK293 cells were transiently transfected with the indicated constructs. Twenty-four hours post-transfection, cells were treated with 20μM MG-132 or an equivalent volume of DMSO for 6 hours. Lysates were prepared and immunoblotting was performed as described in Figure 4. (B) RNA levels of Cbl constructs from transfected HEK293 cells were determined using quantitative real-time PCR. The graph represents the mean fold change of Cbl RNA levels in the presence of Met normalized to levels of Cbl RNA when transfected alone +/- standard error. (C) Cells transfected in parallel as those in (B) were used to visualize corresponding protein levels. Lysates were prepared as described in Figure 4 and protein levels from one representative experiment are shown.

Figure 7


A
c-Cbl
Cbl-b
MG132 - + - + - + - + - +
Met + + - - + + - - + +
HA
Met
actin
B
Relative Cbl mRNA
4
3.5
3
2.5
2
1.5
1
0.5
0
cCbl
cCbl+Met
Cbl-b
Cbl-b+Met
C
pxm
Met
c-Cbl
c-Cbl+Met
Cbl-b
Cbl-b+Met
HA
Met
actin

Discussion

Members of the Cbl family of E3 ubiquitin ligases act as important negative regulators of numerous RTKs. RTKs that become uncoupled from Cbl-mediated ubiquitination through point mutations or chromosomal translocations have longer half-lives, display prolonged downstream signaling, and increase the transforming capacities of the receptor [23, 35]. Similarly, loss of Cbl function due to Cbl mutants acting as a dominant negative protein results in cell transformation and both of these methods of loss of Cbl function have been identified as causes of human malignancies [25, 51-53]. This work demonstrates a specific loss of Cbl-b through differential regulation of Cbl proteins downstream of a receptor tyrosine kinase. We provide evidence that regulation of c-Cbl and Cbl-b differs between breast cancer cell lines, and show that, compared to other tyrosine kinases, the Met RTK is uniquely capable of contributing to differential regulation of Cbl proteins through the selective downregulation of Cbl-b. Moreover, we demonstrate that the loss of Cbl-b relies on one or more protein-protein interactions mapped to the Cbl-b C-tail region between its RING and UBA domains, resulting in part in proteasomal degradation of Cbl-b.

As previous work from our laboratory has shown, both c-Cbl and Cbl-b are capable of inducing downregulation of the Met receptor. We show that an actively signaling Met receptor is capable of inducing downregulation of both transfected and endogenous Cbl-b (Figure 2A,C) regardless of membrane localization (Figure 2B). This downregulation of Cbl-b is similar to data published concerning the overall negative effects of the EGFR on Cbl-b stability. However, unlike the EGFR, Met specifically targets Cbl-b and not c-Cbl, whereas the EGFR is capable of targeting both.

The EGFR, at high levels of expression, becomes tyrosine phosphorylated and only then induces degradation of both c-Cbl and Cbl-b, consistent with studies demonstrating loss of c-Cbl and Cbl-b levels upon EGFR stimulation (Figure 3A). On the other hand, a constitutively activated form of Neu does not induce significant loss of either c-Cbl or Cbl-b (Figure 3B), also in agreement with previous studies [38]. Notably, expression of activated Src induced loss of both c-Cbl and Cbl-b (Fig 3C), consistent with previous observations for c-Cbl [27]. Thus, as far as we have examined, in a system

accurately replicating known effects of activated tyrosine kinases on c-Cbl and Cbl-b, only Met is capable of specific downregulation of Cbl-b.

c-Cbl is recruited to Met directly through binding of the TKB domain to Met pY1003 and indirectly through Grb2, and both interactions are required for maximal ubiquitination of Met. The Cbl-b TKB domain is also capable of binding Met pY1003, and Cbl-b shares a Grb2 binding motif in its C-tail with c-Cbl. In Figure 4B, we performed structure-function studies using mutants of the Met receptor to determine which regions of Met play a role in Cbl-b downregulation. The Met mutant lacking kinase activity was impaired in its ability to downregulate Cbl-b. Although the slight amount of Cbl-b still lost in the presence of this mutant may be due to high levels of expression of both proteins, the robust loss of Cbl-b downstream of a receptor that lacks Y1003 and is incapable of recruiting Grb2 (MetΔCbl) is suggestive that other important modes of Cbl-b recruitment to Met may exist. c-Cbl, in contrast, remained stable downstream of all receptor mutants tested (Figure 4C).

We also provide additional evidence for a unique Cbl-b/Met interaction through structure-function studies of Cbl-b. Both the ubiquitin ligase activity of Cbl-b (Figure 5B) and the C-tail region lying between the RING and UBA domains (Figure 5C) are necessary for Cbl-b degradation. Neither the phosphotyrosine binding region of the TKB domain or the Grb2 binding sequence, methods by which Cbl proteins associate with Met, were required for Cbl-b degradation. c-Cbl also has a RING domain with E3 ubiquitin ligase activity that is highly homologous to that of Cbl-b, suggesting that the cause of differential Cbl-b instability lies in its C-tail. The C-tails of c-Cbl and Cbl-b have 15 and 17 potential SH3 interaction sites, respectively, but share only three of them, one of which being the Grb2 binding site [1]. Additionally, Cbl-b lacks the phosphotyrosine site known to recruit the p85 regulatory subunit of PI3K through its SH2 domain, but instead recruits this protein through the p85 SH3 domain [50]. Any one of these differences may be the site responsible for the Met-dependant selective downregulation of Cbl-b. To our knowledge, the only precedent for selective Cbl-b protein degradation is downstream of CD28 stimulation in T-cells, but the mechanism by which this occurs is not understood [29].

Autoubiquitination of Cbl proteins as a means of their regulation has not been extensively studied. Autoubiquitination has been demonstrated to occur for c-Cbl downstream of the EGFR and PDGFR upon interaction with Sts-2 [54, 55] or activated Src [33], but no studies have examined Cbl-b. Although we could not confirm direct ubiquitination of Cbl-b, in the presence of Met immunoprecipitation of Cbl-b and Western blotting for ubiquitin revealed a smear indicative of one or more polyubiquitinated or multimonoubiquitinated proteins, whose disappearance paralleled that of Cbl-b (Figure 6). Moreover, the treatment of cells with the proteasomal inhibitor MG-132 resulted in a partial rescue of Cbl-b levels, suggesting proteasomal degradation as the mechanism through which Cbl-b is degraded (Figure 7A). The rescue of Cbl-b levels would likely be further enhanced through the use of lactacystin, a more specific inhibitor of the proteasome with fewer cytotoxic effects. In some instances, ubiquitinated Cbl proteins are thought to be degraded in the lysosome with their downregulated RTK substrates. However, the molecular weight of ubiquitinated species immunoprecipitating with Cbl-b is too high to represent multimonoubiquitinated Cbl-b. More likely, the ubiquitinated species are polyubiquitinated, and thus destined for proteasomal degradation. However, the contribution of the lysosome in Cbl-b degradation remains to be addressed.

In summary, we present a selective downregulation of the ubiquitin ligase Cbl-b downstream of a receptor tyrosine kinase. Met kinase activity, as well as Cbl-b ubiquitin ligase activity and C-tail combine to induce proteasomal degradation of Cbl-b. Given the importance of Cbl proteins as negative regulators of active tyrosine kinases for transformation and that c-Cbl and Cbl-b levels are differentially altered between various cancer cell lines (Figure 1), this study suggests that the excessive loss of Cbl proteins by this, and perhaps other mechanisms not yet elucidated may contribute to the development or progression of these cancers.

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Chapter 3

General Discussion

1. Introduction

Cbl proteins are well documented to play numerous roles in the cell as multivalent scaffold proteins and E3 ubiquitin ligases. They are now regarded as the principal family of E3 ubiquitin ligases responsible for downregulation of RTKs, and the majority of research in this field has focused on the mechanisms by which Cbl proteins act as such. Work done in this area has revealed that loss of Cbl function has the potential to contribute to the progression of cancers driven by RTKs that are now deregulated as a result of Cbl loss [1]. This said, surprisingly little research has focused on how the cell regulates Cbl proteins themselves, and whether excessive negative regulation contributes to tumourigenesis. Even less is known about differences that may exist in the function or regulation of different Cbl family members, partially because the high degree of sequence homology between the N-termini of Cbl proteins suggested that they may function similarly as E3 ubiquitin ligases. Nevertheless, thorough investigation of mechanisms of Cbl function on prominent Cbl targets such as the EGFR have produced evidence indicative that Cbl proteins are indeed regulated downstream of some tyrosine kinases, and that this may contribute to oncogenic synergy between tyrosine kinases. This thesis work aimed to investigate specifically the effects of the Met RTK on regulation of c-Cbl and Cbl-b, and the mechanisms by which any regulation occurred.

2. The Met RTK is capable of downregulating Cbl-b but not c-Cbl, and is unique in this respect compared to Neu, EGFR, and Src

Previous work from our lab has shown the Met RTK to associate with the c-Cbl ubiquitin ligase and upon activation, become ubiquitinated and be targeted for lysosomal degradation as a result of this interaction [2-4]. Cbl-b was shown to associate with Met through its TKB domain, and is also capable of Met ubiquitination as siRNA-mediated depletion of both ligases is required to significantly reduce Met ubiquitination (unpublished observations). The EGFR, another RTK targeted by c-Cbl and Cbl-b for ubiquitination, has been shown to reduce the level of both c-Cbl and Cbl-b upon activation [5]. Thus, we aimed to evaluate the role of Met on regulation of c-Cbl versus Cbl-b. Transient co-transfections of increasing amounts of Met with constant amounts of either c-Cbl or Cbl-b induced a preferential loss of Cbl-b that paralleled the increasing

amounts of transfected Met. The effect of Met was drastic, as even low levels of Met lead to a large decrease in Cbl-b levels compared to levels of Cbl-b without transfected Met. Ubiquitination of Met occurs as early as two minutes after stimulation (unpublished observations), suggesting that it occurs at the plasma membrane. To test whether membrane localization of Met was required for Cbl-b downregulation, co-transfections of c-Cbl or Cbl-b with Tpr-Met, a constitutively activated cytoplasmic form of Met, were performed and demonstrate that membrane localization is not required. This result was unexpected, as Tpr-Met does not possess the Cbl TKB domain binding site, Y1003, and has been shown to escape Cbl-mediated ubiquitination [2, 6]. This lead us to hypothesize that selective loss of Cbl-b might be a general property of tyrosine kinases in our experimental system. However, similar titrations of increasing EGFR, NeuNT, and activated Src not only failed to select Cbl-b over c-Cbl for downregulation, but reproduced effects on c-Cbl and Cbl-b levels already shown in previously published studies [5, 7, 8], supporting our data. Although the EGFR construct used, upon overexpression, does not become highly activated in a ligand independent manner as do Met and the other mutant constructs, probing for phosphotyrosine revealed that the EGFR does undergo a degree of activation when high levels are transfected, and in turn is able to decrease levels of the Cbl constructs. However, EGFR was unable to selectively downmodulate Cbl-b levels as does the Met RTK.

3. Met kinase activity, as well as Cbl-b ubiquitin ligase activity and C-tail are required for Met-induced negative regulation of Cbl-b

We next sought to discover which domains in Met and Cbl-b were required for the observed preferential loss of Cbl-b. c-Cbl is recruited to Met directly through its TKB domain, and also indirectly through association with Grb2. Cbl-b also binds Met directly via the TKB domain, and shares an identical Grb2 binding site with c-Cbl. We predicted that, of the mutants examined, a Met mutant lacking these two binding sites (MetΔCbl) or a kinase-dead Met mutant (Met K1110A) would be unable to downregulate Cbl-b. Indeed Met K1110A was severely impaired in its ability to downregulate Cbl-b, proving that Met kinase activity is required; however, MetΔCbl retained this ability. The adaptor protein Shc, which also binds Met, is capable of recruiting Grb2, providing an alternative

mechanism for Cbl recruitment to Met. However, creation of an additional mutation abrogating Shc binding to the MetΔCbl construct was also able to induce Cbl-b downregulation (data not shown), implying either the existence of a novel interaction or that association of Met and Cbl-b is not required and Cbl-b downregulation is a downstream effect of Met signaling.

To gain further insight on the mechanism, we evaluated the role of domains and known protein binding sites in Cbl-b itself. Mutations of the TKB domain and Grb-2 binding site were used (Cbl-b G298E and Cbl-bΔGrb2), as they are ways by which Cbl-b associates with the receptor. Downstream of the EGFR, c-Cbl E3 ubiquitin ligase activity was shown to be required for its autoubiquitination [7], and thus we also tested a ligase-dead Cbl-b construct (C373A). We also hypothesized that regions with documented differences between c-Cbl and Cbl-b would likely be involved in the differential regulation of the two. Possible sources of differences in c-Cbl and Cbl-b regulation lie in the Cbl-b C-tail region, which contains different proline-rich regions than does the c-Cbl C-tail, and the UBA domain, which binds ubiquitin in the case of Cbl-b, but not c-Cbl. Thus, mutants specifically unable to bind ubiquitin or lacking the UBA domain altogether (Cbl-bΔUBA, Cbl-b A937E) as well as a Cbl-b lacking all sequences C-terminal to the RING domain (Cbl-b N1/2) were also evaluated. Upon co-transfection, only Cbl-b C373A and Cbl-b N1/2 were stable in the presence of Met, demonstrating that E3 ubiquitin ligase activity and the C-tail region that lies between the RING and UBA domains are both necessary for Cbl-b loss, but are insufficient to do so without the other (Ch.2, Fig. 5 B,C). Therefore, we hypothesized that a specific protein-protein interaction between an unidentified protein and the Cbl-b C-tail is induced by Met, and that Cbl-b ubiquitination, either of itself or another protein, results in its downregulation. It will be of interest to perform further structure/function analysis on the Cbl-b C-tail region, as mutation of tyrosine residues known to be phosphorylated as well as other proline-rich regions such as the CIN85 binding site will reveal the precise location of the interaction and perhaps lead to the identification of the unknown interacting protein.

4. Proteasomal degradation contributes to the downregulation of Cbl-b

Polyubiquitination of proteins is now accepted as being responsible for targeting them for degradation in the proteasome, whereas mono- and multimonoubiquitination targets proteins for lysosomal degradation [9]. Having observed the requirement for ubiquitin ligase activity (Ch.2, Fig 5B), and having detected no monoubiquitination of Cbl-b (Ch.2, Fig 6), we hypothesized that Cbl-b was being targeted for proteasomal degradation. Treating cells with MG-132, an inhibitor of the proteasome, resulted in a slight rescue of Cbl-b levels, showing that the proteasome is responsible for at least some of the Cbl-b degradation (Ch.2 Fig 7A). Rescue of Met levels was used as an internal positive control, as Met degradation has been shown to require both the proteasome and lysosome [10]. It may have been possible to observe greater Cbl-b rescue using an inhibitor more specific for the proteasome, such as lactacystin. Previous studies have shown that Cbl-b can be selectively downregulated at the mRNA level by the oncogenic kinase BCR-Abl [11]. This, in combination with the modest level of Cbl-b rescue by MG-132 lead us to hypothesize that loss of Cbl-b was mostly due to loss of Cbl-b mRNA. However, reverse transcriptase real-time PCR analysis using primers selective for transfected Cbl-b revealed no drop in levels of Cbl-b mRNA in the presence of Met (Ch.2 Fig 7B). The lysosome may also be responsible for Cbl-b degradation, however, this is unlikely considering immunoprecipitated Cbl-b associates with a smear of ubiquitinated protein that is of too high a molecular weight to be multimonoubiquitinated Cbl-b. If Cbl-b is among these ubiquitinated proteins, the high molecular weight suggests polyubiquitination, and hence the observed proteasomal degradation (Ch.2 Fig. 7A). However, whether Cbl-b is itself ubiquitinated still remains unclear as we were unable to observe conclusive Cbl-b ubiquitination. Further work aimed at proving that Cbl-b is itself ubiquitinated will be necessary. Also necessary will be the use of inhibitors of the lysosome to answer whether any degradation of Cbl-b occurs via this mechanism.

5. Cbl-b-mediated ubiquitination and degradation of Met proceeds via a different mechanism than the Met-mediated selective degradation of Cbl-b

As mentioned above, Cbl-mediated multimonoubiquitination of RTKs targets them for lysosomal degradation. Cbl proteins are recruited similarly to both the EGFR

and Met via direct and indirect binding, and in the case of the EGFR, Cbl proteins directly interacting with the EGFR are degraded with the EGFR in the lysosome [5]. Interestingly, unlike Cbl-EGFR interactions, the interactions by which Cbl-b ubiquitinates Met are not required for the mechanism by which Met in turn downregulates Cbl-b. The simplest and clearest evidence illustrating this point is that both c-Cbl and Cbl-b have been shown to bind to and ubiquitinate Met, but only Cbl-b is degraded in turn. The co-transfection of Cbl-b with Tpr-Met, which escapes Cbl-mediated ubiquitination, also illustrates this point: only Tpr-Met remains stable upon co-transfection with Cbl-b, but both Met and Tpr-Met are capable of selectively downregulating Cbl-b. Moreover, the MetΔCbl construct which also escapes Cbl-mediated ubiquitination and should not associate with Cbl-b is likewise capable of Cbl-b downregulation. Collectively, these data suggest that the mechanism by which Cbl-b ubiquitinates Met and the mechanism by which Met induces Cbl-b degradation are separate entities.

6. Summary and proposed mechanism

From work presented in this thesis, it is clear that the Met receptor, when activated, induces degradation of Cbl-b. It is also clear that this requires ubiquitin ligase activity of Cbl-b and the Cbl-b C-tail region. That this does not occur for c-Cbl implies that Met induces a different, unknown interaction in the Cbl-b C-tail, and that the specific site at which this interaction occurs is not present in c-Cbl. This interaction, in combination with Cbl-b-mediated ubiquitination of itself and/or other proteins results in proteasomal degradation of Cbl-b. A model for how the Met-induced degradation of Cbl-b occurs is illustrated in Figure 1. Some important questions remain unanswered. Although this mechanism may be at work in cancers overexpressing the Met receptor and we see differential regulation of c-Cbl and Cbl-b levels in breast cancer cell lines, whether Met-induced loss of Cbl-b is contributing to the development or progression of these cancers remains unknown. Studies using siRNA to selectively knock-down Cbl-b in cancer cell lines may help address the role of Cbl-b loss in the context of cancers with or without Met overexpression. We speculate that drastic losses of Cbl-b may contribute to the simultaneous elevated signaling of multiple RTKs known to be targeted for

degradation by Cbl-b, but this remains to be shown. How much c-Cbl is able to compensate for loss of Cbl-b in the context of downregulation of RTKs is also a key issue to be considered. If found to play a role in cancer, this will further our understanding of the mechanisms by which Met contributes to oncogenesis and further guide clinicians in the development of therapeutics.

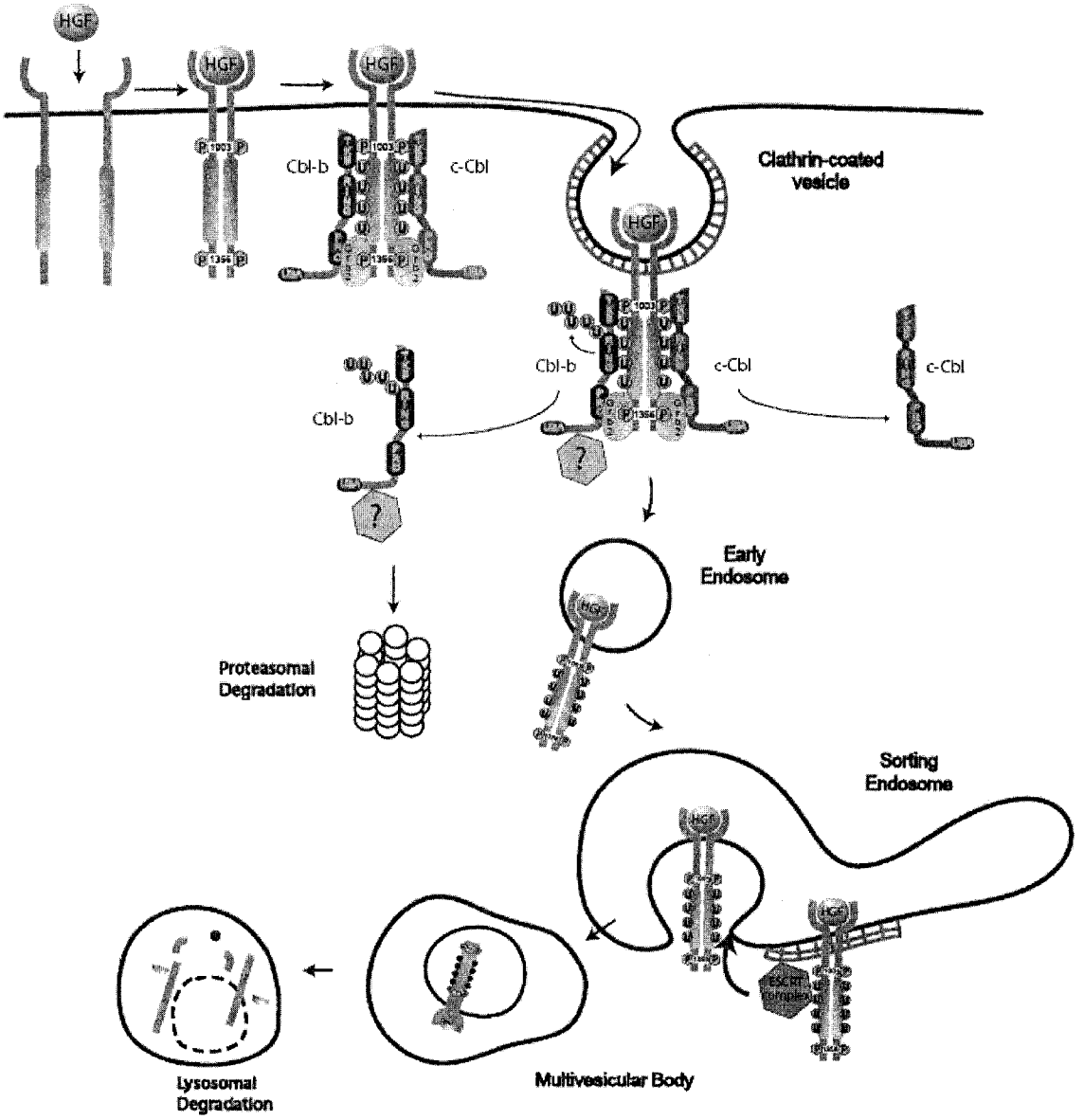

Figure 1. Schematic of proposed model.

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Chapter 4

Future Perspectives: the Identification of Novel Roles and Regulation of Cbl Family Proteins Downstream of the Met Receptor Tyrosine Kinase

1. Introduction

As discussed in Chapter 1.13, the function of Cbl proteins can be regulated by a variety of different mechanisms, not all of which result in degradation of Cbl. Novel, less obvious methods used by the cell to accomplish this can be uncovered by identifying the proteins that interact with Cbl, and at the same time, the elucidation of novel Cbl interacting proteins can also result in the identification of novel functions of Cbl. As mentioned in previous chapters of this thesis, c-Cbl, Cbl-b and Cbl-3 are E3 ubiquitin ligases and multivalent scaffold proteins, and over recent years have been documented as important negative regulators of tyrosine kinases. One Cbl domain required for this process is the TKB domain, which is thought to provide specificity in the targets to be ubiquitinated. The TKB domain is also part of the Cbl N-terminal region which is highly conserved between Cbl proteins over evolution [1]. One such example of a protein which interacts with the TKB domain and regulates Cbl function is Sprouty2, which has been demonstrated to bind this domain in order to competitively inhibit c-Cbl from binding the EGFR [2]. Other proteins known to sequester Cbl from activated EGFR may behave similarly.

Cbl proteins have also been documented as having a variety of roles in other cellular processes besides regulating tyrosine kinases, such as in the activation of the MAPK pathway, regulation of small GTPases and the actin cytoskeleton, and effects on the microtubular network [3]. Involvement in these other processes stems from the scaffolding functions of Cbl, but some reports have now implicated the TKB domain in these processes as well, such as in binding to the APS adaptor protein [4].

Major gaps still remain in our understanding of the functions of the TKB domain. That the TKB domain would have additional roles other than binding phosphotyrosine residues on activated tyrosine kinases is not surprising. Initial molecular modeling of the TKB domain in complex with a tyrosine-phosphorylated peptide from ZAP-70 suggested that the four-helix bundle, EF hand and variant SH2 of the TKB domain all contributed to phosphotyrosine binding, making it a much more complex interaction than classical SH2-pTyr interactions [5]. The authors of these studies suggested that the TKB domain would be capable of binding multiple different peptide sequences, a result confirmed by the fact that three different TKB domain consensus binding sequences have been

identified [6]. Given that c-Cbl and Cbl-b become tyrosine phosphorylated downstream of Met, the complexity of the TKB domain, and the emerging variety of proteins that it is capable of interacting with, we hypothesized that the TKB domain is involved in multiple other cellular processes downstream of Met, and the TKB domain may also be bound by novel proteins as a means of regulating Cbl function. The identification of these alternate functions of Cbl proteins downstream of Met through the discovery of novel TKB binding proteins will not only expand our understanding of Cbl's functions and regulation, but also may shed light on additional ways Met signaling contributes to tumourigenesis through Cbl proteins.

2. Aim and methodology

In related thesis work, I aimed to identify other processes in which Cbl is playing a role or by which Cbl is regulated downstream of Met through the identification of novel tyrosine-phosphorylated c-Cbl TKB domain binding proteins. Two major experimental components were necessary to accomplish this task. The first was the isolation of tyrosine-phosphorylated proteins bound by the c-Cbl TKB domain in a Met-dependant manner using in vitro binding assays and subsequent analysis of bound proteins by mass spectrometry. The second component involves the use of classical molecular biology techniques to confirm the identity and determine the biological function of the isolated TKB-bound phosphoproteins.

The experimental design used to accomplish the first component is illustrated in Figure 1. We have generated GST-fusion proteins containing the c-Cbl, Cbl-b and Cbl-3 TKB domains. Focusing on c-Cbl, in vitro binding assays were established using glutathione sepharose beads for purification of the GST-c-Cbl TKB domain fusion protein from bacterial lysates. Whole cell lysates prepared from human embryonic kidney (293) cells that were either untransfected or transfected with the constitutively activated form of Met, Tpr-Met, were used in the in vitro binding assays to supply a set of potential TKB binding proteins that are tyrosine phosphorylated downstream of Met. Tpr-Met was also used because it lacks the Cbl TKB binding site, Y1003, and hence would provide a Met signal without competing for TKB-binding with endogenous proteins when overexpressed. As negative controls, GST protein alone controlled for

proteins binding to the glutathione sepharose beads and GST-cCbl-TKB G306E, which represents a TKB domain mutant incapable of binding phosphotyrosine, was used to control for proteins binding to the TKB domain in a non-phosphotyrosine-dependant fashion. After the in vitro binding assay, the GST fusion proteins and bound proteins were eluted, separated on an SDS-PAGE gel, Coomassie stained, and digested into peptides with trypsin. TiO2 columns have been previously demonstrated to isolate phosphorylated peptides [7], and were used to enrich for phosphopeptides from the sample for subsequent analysis by mass spectrometry. A small fraction of each sample was run in parallel on a separate gel for Western blot analysis, where anti-phosphotyrosine antibodies were used to visualize the specific set of phosphoproteins bound by the TKB domain and confirm that none were bound by GST alone or the G306E mutant. The evaluation of the biological functions of identified novel TKB domain binding proteins has yet to be performed.

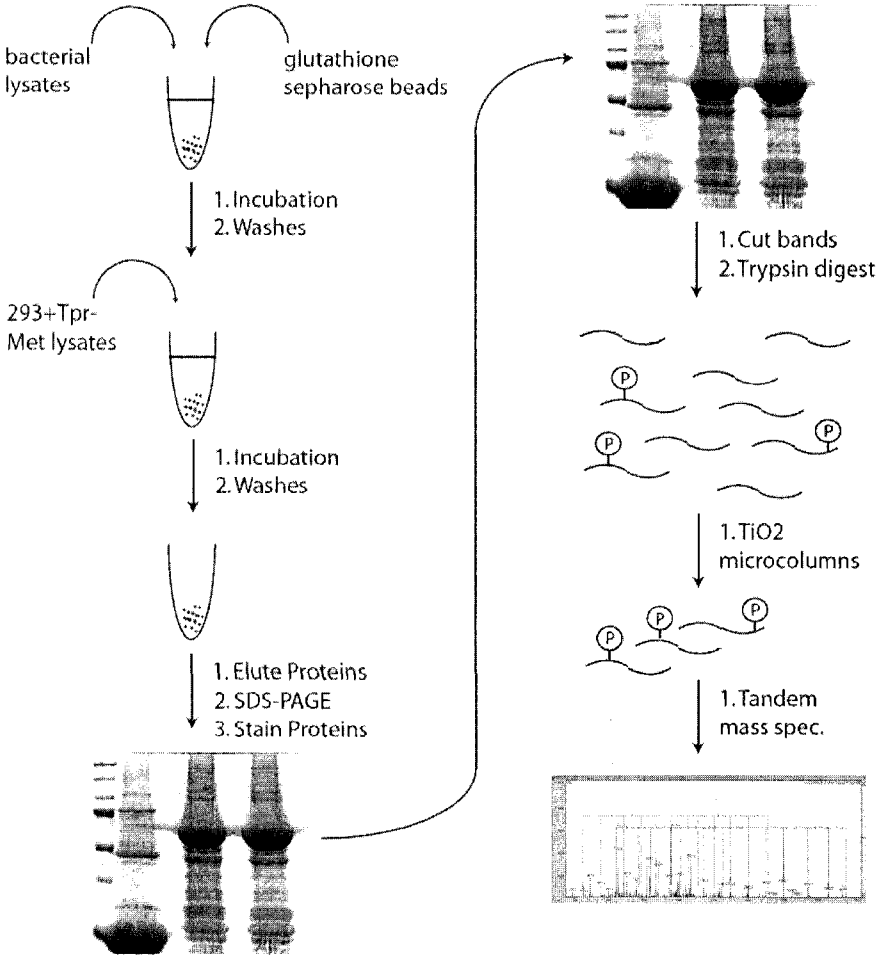

Figure 1. Schematic of experimental methodology

3. Preliminary results and future perspectives

Analysis of isolated phosphopeptides by mass spectrometry yielded hundreds of TKB-associated proteins; however, multiple caveats in the experiment needed to be addressed. Although TiO2 columns were used to isolate phosphorylated peptides, they enrich for serine and threonine phosphorylated peptides as well as those which are tyrosine phosphorylated. As tyrosine phosphorylation represents only about 0.05% of total cellular phosphorylation [8], the Ser/Thr-phosphorylated to Tyr-phosphorylated peptide ratio may have caused some Tyr-phosphorylated peptides to be missed in our experiment. Identification of a peptide of TKB-bound protein that is Ser/Thr phosphorylated does not negate the possibility that the protein was also tyrosine phosphorylated in another location on the protein, making the process of choosing which identified "hits" to validate a difficult one. In the same vein, this implies that proteins bound to the TKB domain that are heavily phosphorylated will be preferentially enriched by the TiO2 columns and detected by the mass spectrometer whereas potentially important TKB-binding proteins that have, for example, only one phosphotyrosine residue may be missed. To minimize the impact of these caveats, the experiment was repeated three times and three separate mass spectrometry analyses were performed in order to identify a broader range of TKB-interacting proteins and to pinpoint which proteins were detected in multiple analyses.

The TKB-interacting proteins identified by mass spectrometry represented numerous different pathways of cell signaling involved in a wide variety of cellular processes and functions. As mentioned in chapter 1, both structural studies and far-Western binding experiments have combined to identify three different consensus TKB domain-binding sequences. Thus, it is expected that some protein "hits" identified by the mass spectrometer would contain one or more sequences corresponding to one of the three known consensus sequences. Encouragingly, many protein "hits" did contain these consensus binding sites; some were present in the peptides identified directly by the mass spectrometer, and others were found to be present in proteins identified by the mass spectrometer only upon further visual inspection of the protein's complete amino acid sequence. In the latter case, the TKB-binding consensus sequence might have evaded direct detection for a number of reasons. First, the corresponding peptide may have

simply failed to bind the TiO_2 column or be detected in the semi-complex mixture by the mass spectrometer due to competition from other peptides. Second, the protein hit identified may not interact directly with the TKB domain at all, instead interacting indirectly through another protein. Lastly, the absence of known TKB domain-binding motifs does not rule out the presence of novel TKB-binding sequences not yet identified in the known literature. Interestingly, only two of the three known consensus sequences were identified; not a single protein hit was found to contain the consensus sequence identified in the APS family of adaptors, whereas there were many of both the DpYR and NXpY(S/T)XXP consensus sequences. This may be due to the fact that the APS family of adaptors only comprises three known proteins [6], however, the DpYR sequence has similarly only been identified as useful for binding Cbl in the case of the Met family of RTKs, comprising only two proteins in mammals, and the plexin family [9].

Multiple proteins already established as TKB domain binders were identified, validating our experimental design. In addition, many putative novel TKB binding proteins were identified. Whether these represent direct binders, or proteins recruited indirectly by virtue of the fact they are present in a complex with direct binders of the TKB domain remains uncertain and necessitates validation of every mass spectrometry "hit" using in vitro binding assays followed by western blot analysis for the protein in question and far western assays to determine whether the association is direct or indirect.

Another caveat of this approach lies in the fact that it does not address the role played by compartmentalization of proteins in the cell. c-Cbl is primarily localized in the cytosol of the cell, but lysis of the 293 cells solubilizes a number of different subcellular compartments. Thus, proteins normally incapable of association with the Cbl TKB domain *in vivo* due to localization in subcellular compartments kept separate from access to c-Cbl may become potential TKB domain binders in the context of *in vitro* binding assays. This will necessitate coimmunoprecipitation experiments as well as studies of colocalization using fluorescence microscopy to address whether binding occurs *in vivo* as well.

The mass spectrometry analysis will not only open new doors in the understanding of c-Cbl function downstream of Met, but can also be elaborated on. This technique can be used in future experiments to perform the same analysis on the Cbl-b

and Cbl-3 TKB domains, providing further insight into differences in the function and regulation of different Cbl family members downstream of Met. Furthermore, the same experiment can be performed in cancer cell lines dependent on Met for their survival to discover the role the Cbl family plays in response to Met specifically in the context of cancer. Although more work is needed to optimize the reproducibility and minimize the impact of the caveats of such experiments, further proteomic analyses can open new doors in our understanding of Met/Cbl signaling dynamics and function.

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