Review

E3 ubiquitin ligase-mediated regulation of bone formation and tumorigenesis

N Sévère¹,², F-X Dieudonné¹,² and PJ Marie*,¹,²

The ubiquitination–proteasome and degradation system is an essential process that regulates protein homeostasis. This system is involved in the regulation of cell proliferation, differentiation and survival, and dysregulations in this system lead to pathologies including cancers. The ubiquitination system is an enzymatic cascade that mediates the marking of target proteins by an ubiquitin label and thereby directs their degradation through the proteasome pathway. The ubiquitination of proteins occurs through a three-step process involving ubiquitin activation by the E1 enzyme, allowing for the transfer to a ubiquitin-conjugated enzyme E2 and to the targeted protein via ubiquitin-protein ligases (E3), the most abundant group of enzymes involved in ubiquitination. Significant advances have been made in our understanding of the role of E3 ubiquitin ligases in the control of bone turnover and tumorigenesis. These ligases are implicated in the regulation of bone cells through the degradation of receptor tyrosine kinases, signaling molecules and transcription factors. Initial studies showed that the E3 ubiquitin ligase c-Cbl, a multi-domain scaffold protein, regulates bone resorption by interacting with several molecules in osteoclasts. Further studies showed that c-Cbl controls the ubiquitination of signaling molecules in osteoblasts and in turn regulates osteoblast proliferation, differentiation and survival. Recent data indicate that c-Cbl expression is decreased in primary bone tumors, resulting in excessive receptor tyrosine kinase signaling. Consistently, c-Cbl ectopic expression reduces bone tumorigenesis by promoting tyrosine kinase receptor degradation. Here, we review the mechanisms of action of E3 ubiquitin ligases in the regulation of normal and pathologic bone formation, and we discuss how targeting the interactions of c-Cbl with some substrates may be a potential therapeutic strategy to promote osteogenesis and to reduce tumorigenesis.

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Facts

- E3 ubiquitin ligases are important proteins regulating bone turnover.
- The E3 ubiquitin ligases c-Cbl and Cbl-b interact with RTKs and other molecules to control bone cell proliferation, differentiation and survival.
- Inhibition of c-Cbl promotes osteoblast differentiation through decreased RTK degradation.
- c-Cbl expression is decreased in bone tumors and is a tumor suppressor in osteosarcoma.
- c-Cbl ectopic expression decreases RTK levels and reduces bone tumor growth and metastasis by inhibiting cell proliferation, migration and invasion.

Open Questions

- Identification of molecules interacting with c-Cbl that could be targeted to selectively promote osteogenesis in vivo.
- Determination of c-Cbl expression in relation to diagnosis and prognosis in solid tumors.
- Development of efficient and nontoxic tools targeting the interactions between c-Cbl and its specific substrates for a potential therapeutic use in bone disorders and cancers.

The ubiquitin-dependent proteolysis system (UPS) mediating protein degradation is an essential process involved in the

¹Laboratory of Osteoblast Biology and Pathology, INSERM U606, Paris, 75475, France and ²University Paris Diderot, Sorbonne Paris Cité, Paris, 75475, France

*Corresponding author: PJ Marie, INSERM U606, Hôpital Lariboisière, 2 rue Ambroise Paré, Paris Cedex 10, 75475 France. Tel: +33 1 49 95 63 89; Fax: +33 1 49 95 84 52; E-mail: pierre.marie@inserm.fr

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Abbreviations: RTK, receptor tyrosine kinase; UPS, ubiquitin-dependent proteolytic system; TKB, tyrosine kinase binding domain; UBA, ubiquitin-associated domain; MSC, mesenchymal stromal cells; Bzb, Bortezomb; BMP, bone morphogenetic protein; PTH, parathyroid hormone; IGFIR, insulin-like growth factor receptor; IRS-1, insulin receptor substrate-1; FGFR, fibroblast growth factor receptor; EGFR, epidermal growth factor receptor; PDGFR, platelet growth factor receptor; TGF-β1, transforming growth factor-β1; DUB, deubiquitinase; HECT, homologous to E6AP carboxyl terminus; GSK-3β, glycogen synthase kinase-3β; DKK1, Dickkopf1; Smurf1, Smad ubiquitination regulatory factor 1; TNF-α, tumor necrosis factor-α; Plekho1, casein-kinase 2 interacting protein-1; ATF4, activating transcription factor 4; PI3K, phosphatidylinositol-3-kinase; c-Fms, M-CSF receptor; RANK, receptor activator of NFκB.

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regulation of cell proliferation, differentiation and apoptosis. The ubiquitination process consists in adding ubiquitin chains on target proteins, which leads to mono- or polyubiquitination of the protein that is then degraded by the proteasome.\(^1\) The ubiquitination cascade requires the successive action of three enzymes. The first enzyme (E1) involved in the ubiquitination process is the ubiquitin-activating enzyme recruiting ubiquitin. The second enzyme (E2) is the ubiquitin-conjugating enzyme that transfers the ubiquitin to the targeted protein. The third enzyme (E3) is the ubiquitin ligase acting as a scaffold protein that interacts with the E2 enzyme and transfers ubiquitin to the target protein.\(^2,3\) This process is reversible through the action of deubiquitinases (DUBs) that remove ubiquitin chains linked on target proteins, which leads to mono- or polyubiquitination of the protein that is then degraded by the proteasome.\(^1\) More than 600 E3 ubiquitin ligases are expressed in the human genome, allowing for the specificity of the ubiquitination system.\(^5\) Based on their distinct function, E3 ubiquitin ligases have been classified as homologous to E6AP carboxyl terminus (HECT)-type and RING-finger like proteins. E3 ubiquitin ligases play a key role in cellular functions by determining the specificity for degradation of substrates, marking it for degradation by the proteasome or the lysosome.\(^6\) However, ubiquitination of a given protein does not necessarily lead to its degradation as ubiquitin may control other biological processes including DNA repair, endocytosis, autophagy, transcription, immunity and inflammation.\(^7\) Interestingly, E3 ligases are themselves regulated by ubiquitination and targeted for degradation, which makes the protein recognition system self-regulated.\(^6\)

During the recent years, it became apparent that E3 ubiquitin ligases regulate various cellular processes involved in the control of bone turnover. Initial studies showed that the Cbl family of E3 ubiquitin ligases regulates bone resorption by osteoclasts. Recent studies indicate that c-Cbl also plays a role in the control of osteogenesis and bone tumorigenesis. Here, we summarize the roles of the Cbl family of E3 ubiquitin ligases in the ubiquitination of specific proteins that control bone-forming cells and tumorigenesis, and we review the implications of this knowledge for controlling bone formation in normal and pathological conditions.

### The Ubiquitination and Degradation System and Bone Turnover

The skeleton is continuously renewed by bone remodeling that starts by the resorption of the bone matrix by osteoclasts,\(^9\) followed by the formation of a new bone matrix by osteoblasts.\(^10\) The early differentiation of osteoblasts is mainly controlled by the transcriptional factor Runx2, followed by the expression of osteoblast phenotypic genes and the synthesis of a bone matrix.\(^11,12\) At the end of the bone formation period, osteoblasts become flattened lining cells, are embedded into the bone matrix as osteocytes or die by apoptosis.\(^13\) All these stages are regulated by several transcription factors, hormones, growth factors and the extracellular matrix, most of them acting by regulating Runx2 gene expression or activity.\(^12,14,15\)

In the recent years, significant advances have been made in our understanding of the role of UPS in regulating bone cell functions. Initial studies using proteasome inhibitors demonstrated that the ubiquitination-degradation system is an important mechanism controlling bone turnover.\(^16,17\) Notably, inhibition of the proteasome leads to increased bone formation and to recovery of bone loss induced by ovariotomy in mice.\(^18\) Consistent with these findings, clinical studies showed that the proteasome inhibitor Bortezomib (Bzb) promotes osteoblastogenesis and inhibits bone resorption in patients with multiple myeloma.\(^19–21\) These effects are mediated by the inhibition of degradation of proteins controlling bone formation and resorption.\(^22\) One of the identified proteins is \(\beta\)-catenin that plays an essential role in chondrogenesis\(^23\) and bone cell function.\(^24\) Wnt binding to Wnt coreceptors LRPS and Frizzled leads to the inhibition of glycogen synthase kinase-3/\(\beta\) (GSK-3/\(\beta\)), leading to decreased \(\beta\)-catenin phosphorylation and prevention of its proteasomal degradation.\(^25\) In chondrocytes, Bzb increases \(\beta\)-catenin (a negative regulator of chondrogenesis) and thereby causes cell apoptosis and growth retardation in mice.\(^16,26\) In osteoblasts, \(\beta\)-catenin accumulation induced by proteasome inhibition leads to increased osteoblastic cell proliferation, differentiation and survival.\(^27\) In addition to \(\beta\)-catenin, the Bzb-induced bone formation is mediated by reduced degradation of Dikkopf1 (Dkk1), an extracellular Wnt/\(\beta\)-catenin antagonist.\(^20\) Another mechanism that is targeted by the proteasome is Hedgehog signaling. Specifically, proteasome inhibition decreases the degradation of the zinc-finger transcription factor GlI2 that mediates bone morphogenetic protein-2 (BMP2) expression in response to Hedgehog signaling, resulting in increased bone formation.\(^28,29\) Thus, several proteins can be targeted by proteasome inhibitors in osteoblasts, resulting in increased osteoblastogenesis and bone formation. In addition to its effect on osteoblasts, the inhibition of the proteasome has an important impact on bone resorption. Notably, proteasome inhibitors suppress osteoclastogenesis and decrease bone resorption mainly by acting on the NF-\(\kappa\)B signaling pathway, causing a reduction in the expression of receptor activator of NF-\(\kappa\)B ligand (RANKL), which is essential for osteoclastogenesis.\(^30–32\) Overall, these studies provided evidence that the UPS is highly implicated in the regulation of bone turnover by affecting the degradation of several proteins regulating bone cell metabolism.

### Role of E3 Ubiquitin Ligases in Bone-Forming Cells

E3 ubiquitin ligases play an important role in bone through their implication in directing proteins to proteasomal degradation. Smad ubiquitination regulatory factor 1 (Smurf1) is the first HECT domain ubiquitin ligase that was shown to regulate bone formation. Mechanistically, Smurf1 associates with the transcription factor Runx2 and mediates its degradation in ubiquitin- and proteasome-dependent manner, resulting in inhibition of osteoblast differentiation and bone formation.\(^33–35\) Consistently, Smurf1 expression induced by tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) results in Runx2 degradation.\(^36,37\) In contrast, inhibition of Smurf1 leads to increased Runx2 protein levels and increased osteoblast differentiation of mesenchymal cells, resulting in enhanced bone formation in vivo.\(^38\) The anabolic factor BMP2 stimulates Runx2 acetylation and thereby prevents Smurf1-induced Runx2 degradation.\(^39\) In addition to regulating Runx2 degradation, Smurf1 mediates the degradation of Smad1, a downstream
mediator of BMP receptors, and thereby inhibits BMP-induced osteoblast differentiation. Consistently, inhibition of Smurf1-mediated Smad1 degradation results in activation of osteoblast differentiation. It was recently shown that silencing of the gene encoding casein-kinase 2 interacting protein-1 (Plekh1), a cofactor for Smurf1, leads to enhanced osteoblast differentiation, bone formation and bone mass in osteopenic rats, which supports an important role of Smurf1 in osteogenesis. Smurf1 also mediates some effects of parathyroid hormone (PTH) in bone-forming cells. Continuous PTH treatment increases Smurf1 expression, leading to Runx2 degradation and attenuation of anti-apoptotic signaling in osteoblasts. In addition to the control of Runx2 degradation, Smurf1 negatively regulates osteoblast differentiation by increasing JunB and MEKK2 degradation through the ubiquitin–proteasome pathway. All these studies illustrate how the E3 ubiquitin ligases Smurf1 negatively regulate osteogenesis (Figure 1).

Other E3 ubiquitin ligases were shown to play important roles in osteogenesis. The transcription factor Runx2 is controlled by the E3 ubiquitin ligase CHIP that promotes Runx2 degradation in preosteoblasts, resulting in inhibition of osteoblast differentiation. Another E3 ubiquitin ligase, Wwp1, interacts with Runx2 and the zinc-finger adapter protein Schnurri-3, resulting in increased Runx2 polyubiquitination and proteasome degradation. Consistently, Schnurri-3 deficiency in mice increases osteoblast gene expression and osteoblast activity, and Wwp1 deletion in mice leads to increased Runx2 and bone mass. Wwp1 also promotes the ubiquitination and degradation of JunB, an AP-1 transcription factor that positively regulates osteoblast differentiation. The role of Wwp1 in osteogenesis was demonstrated in Wwp1 knockout mice that do not exhibit the TNF-α-induced JunB ubiquitination and subsequent inhibition of osteoblast differentiation observed in wild-type mice. On the other hand, intermittent PTH treatment was found to increase activating transcription factor 4 (ATF4) protein level as a result of decreased proteasomal degradation by the E3 ubiquitin ligase β-TrCP1, resulting in increased osteoblastogenesis. Finally, overexpression of the E3 ligase complex SCF that regulates the ubiquitination and degradation of p57 (KIP2) induced by transforming growth factor-β1 (TGF-β1) inhibits osteogenic differentiation, whereas the expression of a dominant-negative form of SCF promotes osteogenic differentiation. Overall, these studies emphasize the important roles of these specific E3 ubiquitin ligases and UPS in the control of bone-forming cells and bone formation (Figure 1).

**The E3 Ubiquitin Ligase Cbl Protein Family**

The Cbl proteins are RING domain-type E3 ubiquitin ligases that are important regulators of signal transduction and cell functions. The first member of Cbl family, v-Cbl, is a truncated form of the wild-type form c-Cbl that was isolated from a retrovirus Cbs NS-1 that induces lymphoma in mice. Since then, three isoforms of c-Cbl have been described in mammals (c-Cbl, Cbl-b and Cbl-3), two in drosophila (d-Cblα, d-Cblβ), and one in Caenorhabditis elegans (Sli-1). Cbl proteins are scaffold proteins with multiple interaction domains (Figure 2a). Two domains, the tyrosine kinase binding domain (TKB) and the RING (really interesting new gene) domain, are highly conserved. The TKB domain is essential for the interaction of Cbl proteins with phosphorylated tyrosine-containing peptides. The RING domain controls the ubiquitin ligase activity of Cbl proteins by binding to the E2 ubiquitin-conjugating enzymes. Sprouty interacts with the RING domain of Cbl proteins and thereby sequesters Cbl away from activated RTKs. The linker domain bearing two important tyrosines (Tyrl and Tyrl) is an important link between the TKB and the RING domains. Notably, Tyrl phosphorylation activates Cbl by inducing conformational changes that eliminate autoinhibition. The C-terminal part is much less conserved among Cbl proteins. The proline-rich domain interacts with SH3 domain proteins of Src and Grb2. The ubiquitin-associated domain (UBA) of Cbl proteins interact noncovalently with (mono) ubiquitin or preferentially with polyubiquitinated chains. The most abundant Cbl proteins in bone, Cbl and Cbl-b, share sequence similarity in the N-terminal half, including the TKB domain that binds phosphorylated tyrosine residues, the linker domain and the RING domain that binds the E2 ubiquitin-conjugating enzymes. However, the two Cbl proteins exhibit structural differences in the C-terminal parts such as the presence of Y731 in c-Cbl that acts as a docking site for the Src homology 2 (SH2) domain of the p85 subunit of phosphorylated phosphatidylinositol-3’ kinase (PI3K), and sequence differences in the UBA domains that differ in their ability to bind polyclubiquitinated chains and ubiquitylated proteins. Because of these multiple domains, Cbl proteins can interact with a large number of proteins. Most importantly, Cbl proteins act as negative regulators of growth factor receptors and signal transduction.
nonreceptor tyrosine kinases that play essential roles in normal and pathological bone cell functions.

Role of Cbl E3 Ubiquitin Ligases in Bone Resorption

Deletion of both c-Cbl and Cbl-b genes induces early embryonic lethality, indicating that the two proteins play essential roles during development.65 Both c-Cbl and Cbl-b are expressed by osteoclasts66 in association with podosomes67 that are required for normal osteoclast maturation and resorption activity. c-Cbl regulates the actin cytoskeleton and stabilizes microtubules68 that are important for osteoclast activity. The extinction of Cbl-b increases osteoclast activity and induces bone loss in mice,69 whereas deletion of c-Cbl reduces osteoclast migration and bone resorption during development.66 The opposite effects of these two Cbl proteins on osteoclast differentiation, function and survival are likely related to their interactions with distinct proteins in osteoclasts.69–71 Specifically, the structural differences could account for the different bone phenotypes of c-Cbl- and Cbl-b-deficient mice include phosphorylation of Tyr731, a binding site for PI3K, in Cbl but not in Cbl-b,63 and the different ubiquitin-binding properties of the UBA domains of c-Cbl and Cbl-b.61

Some mechanisms involved in the effects of Cbl and Cbl-b in osteoclasts have been identified.71 The extinction of c-Cbl expression decreases osteoclast motility, adhesion and resorbing activity by modulating the degradation of Src kinase.62,67,72,73 In contrast, Cbl-b overexpression decreases osteoclast activity by regulating NF-κB, ERK and p38 signaling pathways.69 Also, c-Cbl promotes osteoclast survival by ubiquitylating Bim, a pro-apoptotic BH3-only Bcl-2 family member.74 Osteoclast survival is also controlled by the interaction of c-Cbl with PI3K. A mutant form of c-Cbl abrogating the interaction between c-Cbl and PI3K leads to decreased osteoclast activity and bone resorption, resulting in increased bone volume in mice.75 In addition to these functions, Cbl proteinsmodulate the assembly of signaling proteins downstream of the M-CSF receptor (c-Fms) and RANK that are required for osteoclastogenesis.79 Thus, both c-Cbl and Cbl-b control osteoclast activity through distinct ubiquitylation-dependent mechanisms, and some functions of one Cbl protein cannot be compensated by the other homolog.69 These studies underscore the multifunctional roles of Cbl proteins in the control of essential molecules regulating osteoclastogenesis and bone resorption.

Role of Cbl E3 Ubiquitin Ligases in Bone-Forming Cells

Recent studies have emphasized the role of Cbl proteins in osteoblastogenesis and bone formation. The analysis of c-Cbl / − and Cbl-b / − mice did not reveal detectable changes in bone formation,66 possibly because of redundancy of the two isoforms in osteoblast precursor cells and in mature osteoblasts. However, studies carried out in normal and pathological conditions revealed that Cbl proteins regulate the degradation of proteins that control osteoblasts (Figure 2b). After activation of receptor tyrosine kinases (RTKs), c-Cbl forms complexes with PI3K through interactions between SH2 and SH3 domains and the proline-rich region of c-Cbl.53 The first demonstration that c-Cbl controls osteoblastic cell proliferation came from our finding that the expression of a mutant G306ECbl that abrogates the interaction between c-Cbl and RTKs increases human mesenchymal stromal cell (MSC) proliferation.76 In bone, PI3K controls osteoblasts by interacting with various molecules.77 Specific inhibition of the PI3K/c-Cbl interaction using a mutant Y737F c-Cbl increased osteoblastic cell proliferation, osteoblast number and bone volume in vivo, showing that abrogation of the c-Cbl-PI3K interaction leads to increased bone formation.78 A role for c-Cbl in osteoblastic cell proliferation is also supported by our analysis of the Saethre–Chotzen syndrome, a human disease characterized by premature fusion of the cranial sutures induced by Twist1 haploinsufficiency.79 In this disease, c-Cbl...
expression is decreased in osteoblasts, resulting in increased PI3K/Akt signaling and increased osteoblastic cell proliferation that contributes to the disease. These results indicate that c-Cbl downregulates osteoblastic cell proliferation by interacting with targeted proteins such as PI3K (Figure 2b).

In addition to negatively regulating cell proliferation, Cbl proteins regulate osteoblast differentiation through ubiquitination and degradation of RTKs and other molecules. After binding of their ligands, RTKs are downregulated, resulting in their ubiquitination and degradation by the proteasome or lysosomes. This mechanism is an essential mechanism that negatively controls activated signaling pathways. The recruitment of c-Cbl to RTKs allows polyubiquitination of in vitro osteogenesis (Figure 3).

These activities are mediated by the SH2 domain, which binds specific proline-containing motifs, and the SH3 domain, which binds phosphotyrosine, and the kinase domain. The binding of proteins to SH2 and SH3 can allow a switch from an inactive to an active state, leading to Src auto-phosphorylation and activation. The binding of c-Cbl to Src leads to Src inactivation by overactive FGFR2. Src-like kinases are substrates of activated Cbl in response to RTK activation, leading to the phosphorylation of a tyrosine residue in Cbl that phosphorylates some components of these complexes.

Another mechanism by which c-Cbl may control osteoblast formation in pathological conditions. Apert syndrome is a human disease characterized by premature fusion of cranial sutures (craniosenosis) induced by constitutive activation of FGFR2. This effect results in increased osteoblast differentiation and cranial osteogenesis. Among the mechanisms involved in this effect, we found that activated FGFR2 enhances EGFR and PDGFR-α mRNA expression via activation of PKC-α-dependent AP-1 transcriptional activity. The increased EGFR protein expression in mutant osteoblasts also results from a posttranscriptional mechanism involving increased Sprouty2-c-Cbl interaction due to FGFR2 constitutive activation, which leads to c-Cbl sequestration and reduced EGFR ubiquitination. These studies identified molecular crosstalks between c-Cbl, activated FGFR2 and EGFR and PDGFR-α expression that functionally contribute to the regulation of osteoblast functions (Figure 3).

In addition to controlling RTK degradation, c-Cbl regulates the ubiquitination and degradation of proteins acting downstream of RTK signaling such as Src nonreceptor tyrosine kinases. Src may act as an adapter protein that links other signaling proteins within complexes, or as a tyrosine kinase that phosphorylates some components of these complexes.

The interaction of c-Cbl with RTKs may also play an important role in the control of osteogenesis in pathological conditions. The E3 ubiquitin ligase c-Cbl mediates Lyn and Fyn ubiquitination and degradation by the proteasome ubiquitin system (UPS), resulting in increased osteoblast differentiation. c-Cbl induces ubiquitination and degradation of PI3K and α5 integrin, resulting in cell apoptosis. Abrogating the interaction between c-Cbl and FGFR2 and PDGFR-α by a c-Cbl mutant (G306E) promotes mesenchymal cell proliferation and osteoblast differentiation mediated by increased ERK1/2 and PI3K signaling. c-Cbl regulates the ubiquitination and degradation of the transcription factor STAT5 in mesenchymal osteoprogenitor cells and thereby promotes bone-forming cell differentiation. The isoform Cbl-b mediates ubiquitination and degradation of IGFR1 receptors and downregulates osteoblastogenesis (see the text for details).

**Figure 3** Signaling pathways and mechanisms involved in c-Cbl-mediated control of bone forming cells. The E3 ubiquitin ligase c-Cbl mediates Lyn and Fyn ubiquitination and degradation by the proteasome ubiquitin system (UPS), resulting in increased osteoblast differentiation. c-Cbl induces ubiquitination and degradation of PI3K and α5 integrin, resulting in cell apoptosis. Abrogating the interaction between c-Cbl and FGFR2 and PDGFR-α by a c-Cbl mutant (G306E) promotes mesenchymal cell proliferation and osteoblast differentiation mediated by increased ERK1/2 and PI3K signaling. c-Cbl regulates the ubiquitination and degradation of the transcription factor STAT5 in mesenchymal osteoprogenitor cells and thereby promotes bone-forming cell differentiation. The isoform Cbl-b mediates ubiquitination and degradation of IGFR1 receptors and downregulates osteoblastogenesis (see the text for details).
activation triggers proteasome degradation of the α5 integrin subunit, resulting in decreased integrin expression, reduced osteoblast attachment and increased apoptosis.\textsuperscript{104} PI3K and c-Cbl are known to interact with growth factors in the cell membrane microdomains called lipid rafts that serve as platforms where signaling molecules are recruited to membrane-associated kinases, allowing functional interactions.\textsuperscript{105,106} We showed that FGFR2 activation in osteoblasts leads to c-Cbl recruitment associated with PI3K/Akt in lipid rafts. This effect results in PI3K ubiquitination and degradation, attenuation of PI3K/Akt signaling and increased osteoblast apoptosis \textit{in vitro} and \textit{in vivo}.\textsuperscript{107,108} Conversely, a mutant G306ECbl that abrogates c-Cbl–RTK interaction was shown to protect human MSCs from apoptosis.\textsuperscript{76} Overall, these findings support a role of c-Cbl in the control of osteoblast survival through modulation of PI3K/Akt and integrin degradation (Figure 3).

Besides c-Cbl, there is also evidence that Cbl-b is involved in the control of osteoblast function. \textit{In vitro}, Cbl-b was shown to enhance Runx2 protein stability and osteoblast-related genes, indicating that Cbl-b may control osteoblast differentiation at the posttranscriptional level.\textsuperscript{109} \textit{In vivo}, bone loss induced by denervation in mice is associated with increased Cbl-b expression and decreased insulin receptor substrate-1 (IRS-1), PI3K and Akt levels in osteoblastic cells, a phenotype that is prevented in Cbl-b-deficient mice. One mechanism by which Cbl-b may downregulate bone formation is through modulation of IGF signaling because Cbl-b overexpression induces IRS-1 ubiquitination and degradation, resulting in impaired mitogenic response to IGF1.\textsuperscript{110} Overall, these studies indicate that Cbl proteins control osteoblast proliferation, differentiation and survival through the ubiquitination and degradation of RTKs and other c-Cbl-targeted proteins (Figure 3).

**Cbl-Mediated Ubiquitination: A Potential Target for Therapeutic Intervention**

Because the ubiquitination/degradation system controls many cellular processes, targeting proteins involved in this mechanism may provide therapeutic clues in pathological conditions. As discussed above, proteasome inhibitors were shown to increase bone formation while reducing bone resorption.\textsuperscript{18,19,22} A more specific approach for promoting bone formation may be to directly target E3 ubiquitin ligases that control osteoblastogenesis. In support of this concept, we recently showed that inhibition of c-Cbl activity using shRNA in murine and human MSCs promoted osteoblast differentiation and survival through activation of RTK signaling\textsuperscript{76} and STAT5-Runx2 transcriptional activity (F-X Dieudonné \textit{et al.}, submitted) (Figure 4). This finding may have functional implications as specific inhibitors of the interactions between c-Cbl and RTK have been recently identified. Notably, a pentapeptide was found to bind the c-Cbl TKD domain with high affinity.\textsuperscript{111} Targeting the interaction between Cbl and target proteins using this or other molecules may be an attractive strategy to activate osteoblastogenesis.

Several dysfunctions of the UPS lead to a large number of pathologies such as cancer. This may be because of an increased ubiquitination and degradation of tumor-suppressor proteins or a decrease in ubiquitination of oncopgenic proteins.\textsuperscript{112} Therefore, targeting the ubiquitin–proteasome system may represent a valid therapeutic strategy in cancers.\textsuperscript{113–115} In support of this concept, proteasome inhibitors were efficient at reducing tumorigenesis,\textsuperscript{114,116} specifically in myeloid disorders.\textsuperscript{17,32} Proteasome inhibition was also shown to reduce breast cancer tumor growth.\textsuperscript{117} However, some cancers are resistant to proteasome inhibitors and more selective strategies are needed to circumvent this limitation.\textsuperscript{118} One emerging aspect in tumorigenesis is the implication of E3 ubiquitin ligases\textsuperscript{119,120} that control cancer cell adhesion, migration and metastasis.\textsuperscript{121} A potential target is c-Cbl that is implicated in different cancers. Genetic mutations localized in the RING or the linker domain of c-Cbl are known to cause myelodysplasia and acute myeloid leukemia in children.\textsuperscript{122} Additionally, several oncopgenic RTKs have lost their Cbl-binding sites and thereby escape downregulation following ligand stimulation, leading to human tumors.\textsuperscript{123} Although Cbl activity is inhibited in lung cancer,\textsuperscript{124} it is increased in breast cancer,\textsuperscript{125} indicating that Cbl can act as an oncogene or a tumor-suppressor protein depending on its interaction with target proteins. In lung cancer, c-Cbl overexpression inhibits tumor metastasis and tumor growth \textit{in vivo}, indicating that c-Cbl can be targeted to reduce tumorigenesis.\textsuperscript{126}

Recent data indicate that c-Cbl may play a role is osteosarcoma, a primary bone tumor occurring in children and young adults. Various loss-of-function mutations in p53 and/or pRB tumor-suppressor genes have been found in osteosarcoma\textsuperscript{127} and both tumor suppressors have been linked to the expression and/or activity of Runx2.\textsuperscript{128} Dysregulations of Runx2 cause abnormal cell growth and differentiation.\textsuperscript{129–132} Therefore, targeting Runx2 may lead to
inhibition of tumor propagation. Indeed, the inhibition of the proteasome in osteosarcoma cells can increase Runx2, reduce cell growth and increase cell apoptosis, resulting in bone tumor regression in vivo. On the other hand, RTK expression was found to be increased in some osteosarcoma, and pharmacological inhibitors or specific RTK blocking antibodies in combination with chemotherapy could reduce bone tumor formation and metastasis. Consistently, we recently showed that c-Cbl protein expression is decreased in face of increased EGFR and PDGFR-α expression in human osteosarcoma tumors compared with normal bone. This finding has therapeutic implications as ectopic c-Cbl expression in osteosarcoma cells could reduce the expression of EGFR and PDGFR-α, resulting in decreased cell proliferation and survival, and reduced incidence of lung metastasis in mice. Thus, promoting RTK ubiquitination and degradation by targeting c-Cbl may prove to be efficient at reducing tumor growth and metastasis in cancers in which RTK expression is excessive (Figure 4). This strategy may also be applied to Ewing sarcoma that is associated with increased IGF receptor activity.

Another potential strategy to increase RTK degradation in cancers is to inhibit the interaction between c-Cbl and its negative regulators. For example, inhibiting the interaction between c-Cbl and Sprouty 2, which sequesters c-Cbl away from activated RTKs, may lead to increased c-Cbl available for interacting with RTKs (Figure 4). An alternative indirect strategy to downregulate the aberrant signaling in cancers would be to target deubiquitinases that are negative regulators of the ubiquitin cascade. These enzymes break ubiquitin chains that are linked to the targeted proteins, thereby avoiding the degradation of the targeted proteins.

DUB dysregulations have been associated with human cancers, where they may act as oncogenes or tumor suppressors depending on the substrate, suggesting that DUBs may be targeted in cancer therapeutics. This proposal is supported by the finding that downregulation of the deubiquitinating enzyme USP9X, which interacts with c-Cbl and the cancer-related ErbB2/HER2 receptor, results in increased ErbB2 degradation in cancer cells. A recent study showed that the deubiquitinase USP1, which acts by maintaining MSCs in a proliferative stage and inhibiting their osteogenic differentiation, is overexpressed and promotes the stability of the oncogenic protein ID2 in osteosarcoma cells. Interestingly, USP1 knockdown causes osteoblast differentiation in these cells. It is thus possible that inhibitors of the activity of specific DUBs may enhance the degradation of specific substrates such as RTKs and thereby reduce cell tumorigenicity and bone tumor development (Figure 4).

Conclusion

The ubiquitination system is an important mechanism regulating protein degradation. This system is highly involved in the regulation of cell proliferation, differentiation and survival, and dysregulations in this system often lead to pathologies such as cancers. Recent studies have underscored the role of E3 ubiquitin ligases in the control of bone cell functions and tumorigenesis. Some advances have been made in the understanding of the role of these ligases in the regulation of signaling pathways and transcription factors that control osteoblast differentiation and survival. Key proteins that interact with the E3 ubiquitin ligase c-Cbl and are regulated by c-Cbl-mediated degradation have been identified. Targeting the interactions between the identified proteins and c-Cbl proved to be efficient in modulating c-Cbl functions with subsequent changes in cell proliferation, differentiation and survival in normal osteoprogenitor cells and in bone tumor cells. This knowledge may pave the way for developing therapeutic strategies targeting c-Cbl interaction with specific substrates with the goal of promoting osteoblast function in diseases characterized by defective bone formation, or for correcting the abnormal osteoblast differentiation and reducing tumorigenesis in bone cancers.

Conflict of Interest

The authors declare no conflict of interest.

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