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Gain-of-function *c-CBL* mutations associated with uniparental disomy of 11q in myeloid neoplasms

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Abstract

C-CBL (CBL) encodes a multifunctional protein engaged in the regulation of intracellular signaling pathways.[1,2] It was first identified as a cellular counterpart of the viral oncogene, *v-CBL*, that causes murine lymphoma.[3,4] Although no genetic evidence existed suggesting its role in human carcinogenesis, the recent discovery of c-*CBL* mutations in myeloid cancers has unveiled a unique oncogenic mechanism mediated by gain-of-function of a mutated tumor suppressor, closely associated with allelic conversion of 11q arms.[5-9] In this review, we summarize our current knowledge about *c-CBL* mutations and discuss the molecular mechanisms of their gain-of-function.

Keywords

c-CBL; 11qUPD; myeloproliferative neoplasms; gain-of-function; MDS/MPN; tyrosine kinases

Myeloproliferative Neoplasms and Related Disorders

Myeloproliferative neoplasms (MPNs) are a heterogeneous group of blood cancers, characterized by clonal hematopoiesis that causes excessive production of one or more components of mature blood cells with hypercellular bone marrow and extramedullary hematopoiesis.[10] Some patients also show abnormalities in cell morphology and

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differentiation with dysplastic bone marrow, and are classified into myelo-dysplastic/myeloproliferative neoplasms (MDS/MPN) in the World Health Organization (WHO) classification.[11] A genetic hallmark of MPN and MDS/MPN is frequent mutations of genes on signal transduction pathways, which have been causally linked to hypersensitivity of neoplastic progenitors to growth factors and cytokines.[10] A notable example is *JAK2 V617F* mutations found in most cases of polycythemia vera (PV), a form of MPNs that is characterized by overproduction of mature erythrocytes together with other blood components.[12-14]

JAK2 Mutations in MPNs

These mutants encode constitutive active kinases that transmit signals from erythropoietin receptor, and induce a hypersensitive proliferative response to erythropoietin.[12] Of particular interest about *JAK2* mutations in PV is the presence of one or more subclones showing acquired uniparental disomy (aUPD) involving the 9p arm that leads to homozygous *JAK2* mutations ($JAK2^{mut/mut}$) by allelic conversion (Fig. 1).[15] One of the initial discoveries of *JAK2* mutations relied on the detailed mapping of loss of heterozygosity (LOH) caused by aUPD in 9p.[13] The consequence of 9p-aUPD is loss of wild type *JAK2* and duplication of mutated *JAK2*, but the latter seems to be more important for the clonal selection of UPD clones, because mutated *JAK2* is duplicated without loss of wild-type allele in 9p trisomy in some cases.[16] Similarly gain-of-function mutations of *cMPL* are frequently found in primary myelofibrosis in close association with 1p-aUPD.[17] Thus, aUPD, or copy number neutral LOH, is associated not only with biallelic loss-of-function of classical tumor suppressor genes in the Knudson's paradigm,[18] but also with gain-of-function of proto-oncogenes. Moreover, genome-wide analysis of genetic imbalances in a variety of myeloid neoplasms revealed that aUPD is another genetic feature of MPNs, where 42% of chronic myelomonocytic leukemia (CMML) cases had one or more regions of aUPD and were grouped into several discrete clusters, which may or may not harbor mutations of known cancer related genes.[9] Among these one of the most prominent is the cluster that is defined by 11q-aUPD, from which mutated *c-CBL* proto-oncogene was identified.[9]

c-CBL Mutations in MDS/MPNs

Although *c-CBL* mutations have been reported in a variety of myeloid neoplasms including acute myeloid leukemia, myelodysplastic syndromes, as well as classical myeloproliferative disorders, the majority of *c-CBL*-mutated cases are MDS/MPN, including CMML (~15%), juvenile myelomonocytic leukemia (JMML) (~17%), and atypical chronic myeloid leukemia (~5%).[5-9,19,20] In most cases, *c-CBL* mutations are associated with 11q-aUPD involving *c-CBL* locus, which converts these mutations into a homozygous state. Loss of wild-type *c-CBL* is rarely caused by chromosomal deletion.[6,7,9] *c-CBL* mutations exclusively occur independent of *RAS* and *PTPN11* in CMML and JMML.[8,9] Notably, *c-CBL* mutations have a germline origin in some JMML cases.[8] Approximately half of the *c-C-BL* mutations in JMML cases involve Y371, while mutations are widely distributed within linker/RING finger domain in other neoplasms. *c-CBL* mutants strongly transform fibroblasts and enhance proliferation of hematopoietic progenitors in methylcellulose culture.[9] These genetic and functional observations indicate that mutant *c-CBL* may have some gain-of-

function, which promotes clonal evolution, especially of aUPD-positive clones carrying two copies of the mutations.

c-CBL as a Tumor Suppressor Gene

c-CBL proto-oncogene is a cellular homologue of a viral oncogene, *v-CBL*, isolated from the Casitas-NS-lymphoma virus that induces murine lymphoma.[3,4] Together with other two homologues CBL-b and CBL-c, it comprises the CBL family of proteins. All c-CBL proteins have an N-terminal domain for binding to phosphorylated tyrosine kinases (TKB domain) connected through a linker sequence to the RING finger, but CBL-c lacks most of the C-terminal domains shared by c-CBL and CBL-b (Fig. 2A). While c-CBL has multivalent molecular functions in signal transduction and cytoskeletal regulation, the most intensively studied-function is its role in negative regulation of receptor tyrosine kinase (RTK) signalings, which depends on the E3 ubquitin ligase activity of this molecule.[1,21] After RTKs are phosphorylated on cytokine stimulation, c-CBL binds to the phosphorylated RTKs through the TKB domain, and mono-ubiquitinates these RTKs at multiple sites in concert with the E2 conjugating enzyme, which is followed by internalization and degradation/recycling of the phosphorylated RTKs.[21] Thus, c-CBL prevents excessive RTK signaling after cytokine/growth factor stimulation and potentially acts as a tumor suppressor. *c-CBL*$^{-/-}$ mice have an enlarged thymus, splenomegaly with extramedullary hematopoiesis.[22,23] In these mice, hematopoietic progenitor pools are expanded,[9,24] and their hematopoietic progenitors exhibit hypersensitive proliferative responses to cytokine stimulations. When introduced into *BCR/ABL* transgenic mice, a *c-CBL*$^{-/-}$ allele accelerates blastic crisis.[9] Moreover, *c-CBL*$^{-/-}$ mice developed invasive cancer spontaneously (in preparation), further supporting that *c-CBL* has tumor suppressor functions.

Gain of Function of CBL Mutants

How can we reconcile with the tumor suppressor functions of c-CBL on the one hand, and the oncogenic properties of c-CBL mutants on the other? A simple explanation would be an inhibition of tumor suppressor function of wild type c-CBL by mutant c-CBL. Most *c-CBL* mutations in MPNs occur within the linker/RING finger domains, through which c-CBL binds E2 conjugating enzymes, and thus are expected to compromise the E3 ligase activity of the molecule. In fact, when expressed in fibroblasts, tumor-derived linker and RING finger mutants show severely compromised E3 ubiquitin ligase activity.[9,20] Moreover, two linker mutants (Q367P and Y371S) have been shown to inhibit the activity of wild-type c-CBL protein, although they do not make direct contact with E2 enzymes but with the TKB domain.[25] As expected from the inhibitory action of these mutants with regard to E3 ubiquitin ligase activity, transduction of the latter mutants into NIH3T3 or hematopoietic cells lead to prolonged activation of tyrosine kinases after stimulation with a variety of cytokines and growth factors, including epidermal growth factor, stem cell factor (SCF), Interleukin 3 (IL3), thrombopoietin, and FLT3 ligand.[9,20] Given the diverse spectrum of kinase targets of CBL, the enhanced sensitivity of these cells to a variety of cytokines is well expected.

Although these experimental data support a dominant negative mechanism of mutant *c-CBL*, a simple dominant negative model is defied by an experiment, in which mutant *c-CBL* was transduced into *c-CBL*$^{-/-}$ hematopoietic progenitors. Lin^{-}Sca1^{+}cKit^{+} (LSK) hematopoietic progenitors from *c-CBL*$^{-/-}$ mice showed enhanced survival or proliferative responses after stimulation with a variety of cytokines, including SCF, IL3, or thrombopoietin, as compared to those from *c-CBL*$^{+/+}$ mice. However, transduction of mutant *c-CBL* into *c-CBL*$^{-/-}$ progenitors dramatically augmented the responses to these cytokines and also to FLT3 ligand, while the effect of mutant *c-CBL*-transduction into *c-CBL*$^{+/+}$ progenitors was unremarkable even as compared to mock-transduced *CBL*$^{-/-}$ progenitors.[9] The augmented sensitivity to these cytokines in *c-CBL*$^{-/-}$ cells was nothing to do with the inhibition of c-CBL functions, and thus is considered to represent a true gain-of-function of the mutant c-CBL. The gain-of-function nature of *c-CBL* mutations is also predicted from the fact that in myeloid neoplasms, 11qLOH is caused by aUPD in most cases and rarely accompanies 11q deletion, although in this case the target gene has tumor suppressor functions. Interestingly, the effect of the gain-of-function effect largely disappears by introducing wild type *c-CBL* or in the presence of the wild-type *c-CBL* allele,[9] which might explain the observation that the wild type *c-CBL* allele was lost in most MDS/MPN cases with *c-CBL* mutations as a result of allelic conversion or aUPD.

Origin of the Gain-of-Function of Mutant CBL

The exact mechanism through which mutant c-CBL acquires oncogenic functions even in *c-CBL*$^{-/-}$ cells is still elusive. Because the gain-of-function of mutant c-CBL is largely neutralized by the presence of wild type c-CBL, one possibility is that it could be mediated by the inhibition of some ‘CBL-like’ activity still present in *c-CBL*$^{-/-}$ cells, most likely CBL-b. Both c-CBL and CBL-b are expressed in immature hematopoietic progenitors, and c-CBL mutant inhibits E3 ubiquitin ligase activity of both c-CBL proteins.[9,26] Although *c-CBL/CBL-b*-double knockout mice are embryonic lethal, conditional double knockout in T cells shows hypersensitive to anti-CD3 stimulations and prolonged TCR-signaling, as compared to *c-CBL* or *CBL-b* single null T cells.[27] This reminds us of the gain-of-function of mutated TP53, which explains the difference in the phenotypes between *TP53*$^{-/-}$ and *TP53*$^{mut/-}$ mice. *TP53*$^{-/-}$ mice develop tumors at a high frequency, but they are mostly sarcomas or lymphomas and development of carcinoma is very rare, whereas *TP53*$^{mut/-}$ mice also develop carcinoma in various organs. Thus, TP53 mutant has more than null functions, which are thought to be mediated by the inhibition of its homologues, TP63 and TP73, expressed in epithelial tissues (Fig. 3).[28,29] Like c-CBL, TP53 tumor suppressor gene was first identified as an oncogene through its mutated, oncogenic forms in cancer cells. On the other hand, the model of gain-of-function mediated through CBL-b inhibition fails to explain why *CBL-b* mutations are extremely rare in CMML. According to this model, essentially no difference would be expected between the mutations of *c-CBL* and *CBL-b*, as long as in either case, compromised E3 ubiquitin ligase activity would result. The linker-RING finger mutants of c-CBL would be expected also to be able to inhibit E3 ubiquitin ligase activity of the wild-type c-CBL.

Another, but not necessarily exclusive, explanation of the gain-of-function of mutant c-CBL would be related to positive roles of c-CBL as a signal transducer rather than an attenuator

(Figs. 3A and 4). c-CBL not only binds to a number of phosphorylated tyrosine kinases through its TKB domain, which is indispensable for the negative regulation of these kinases, but also interacts with more than 150 different proteins through a number of C-terminal domains and residues, and acts as a multi-domain adaptor protein, involved in signal transduction (Fig. 2B).[2] When recruited to phosphorylated tyrosine kinases, c-CBL is also phosphorylated at multiple tyrosine residues, and provides docking sites for the SH2 domains of Vav (pY700),[30] CrkL (pY700 and pY774)[31-34] and the p85 subunit of PI3 kinase (Y731).[35-37] c-CBL also binds to Grab2,[38-40] CAP,[41] and Src family tyrosine kinases[36] through the proline-rich domain. Several lines of evidence suggest that c-CBL positively transmits signals through these interactions. For example, c-CBL promotes cell survival and proliferation, depending on the PI3 kinase pathway,[42,43] and also enhances activation of MAP kinases after stimulation of Met tyrosine kinase.[44] c-CBL is also a key substrate/effector of Src kinase, which plays a central role in bone resorption and osteoclast migration.[45,46] It also is involved in cytoskeletal rearrangements through activation of Rac1, Cdc42, and R-Ras.[47,48] Normally, mediated by its E3 ligase activity, kinase-bound phosphorylated c-CBL rapidly undergoes degradation,[26] by which positive signaling should be terminated. Thus, once linker/RING finger mutations abolish the E3 ligase activity of c-CBL, the consequence would be prolonged signaling due not only to loss of negative regulation of tyrosine kinase, but also to enhanced positive regulatory functions, which should appear as gain-of-function (Fig. 4).

In contrast to the CBL-b inhibition model, the uni-laterality of c-CBL mutations could be more easily explained, because c-CBL and CBL-b have distinct biological functions, as clearly shown by the phenotypes of *c-CBL*$^{-/-}$ and *CBL-b*$^{-/-}$ mice.[22,23,49,50] For example, CBL-b lacks one of the major phosphorylated tyrosines, Y731, that provides a docking site for the p85 subunit of PI3 kinase. Although the exact molecular basis for the distinct functions between both CBL proteins remains to be elucidated, c-CBL-specific positive regulatory function in immature hematopoietic progenitors may be important for the pathogenesis of myeloid neoplasms.

Conclusion

Allelic conversion leading to aUPD is an important genetic mechanism of clonal evolution in the pathogenesis of MPN, and associated not only with loss-of-function of tumor suppressor genes, but also with gain-of-function mutations of proto-oncogenes. Homozygous *c-CBL* mutations that characterize a subset of MDS/MPD carrying 11q-aUPD, represent a unique example of gain-of-function mutations of tumor suppressor/proto-oncogene. These linker/RING finger mutations convert c-CBL, which otherwise act as a tumor suppressor, to a gain-of-function oncogenic protein. Although its exact molecular mechanism is still unknown, the gain-of-function of oncogenic c-CBL mutants seems to be related to disintegration of negative and positive regulatory machineries of normal c-CBL protein. Detailed analysis of the oncogenic mechanisms of c-CBL mutants is warranted, which should shed light on a novel aspect of physiological function of c-CBL. Considering their expression and functions in a broad spectrum of tissues, *CBL* family genes may be mutated in other human cancers.

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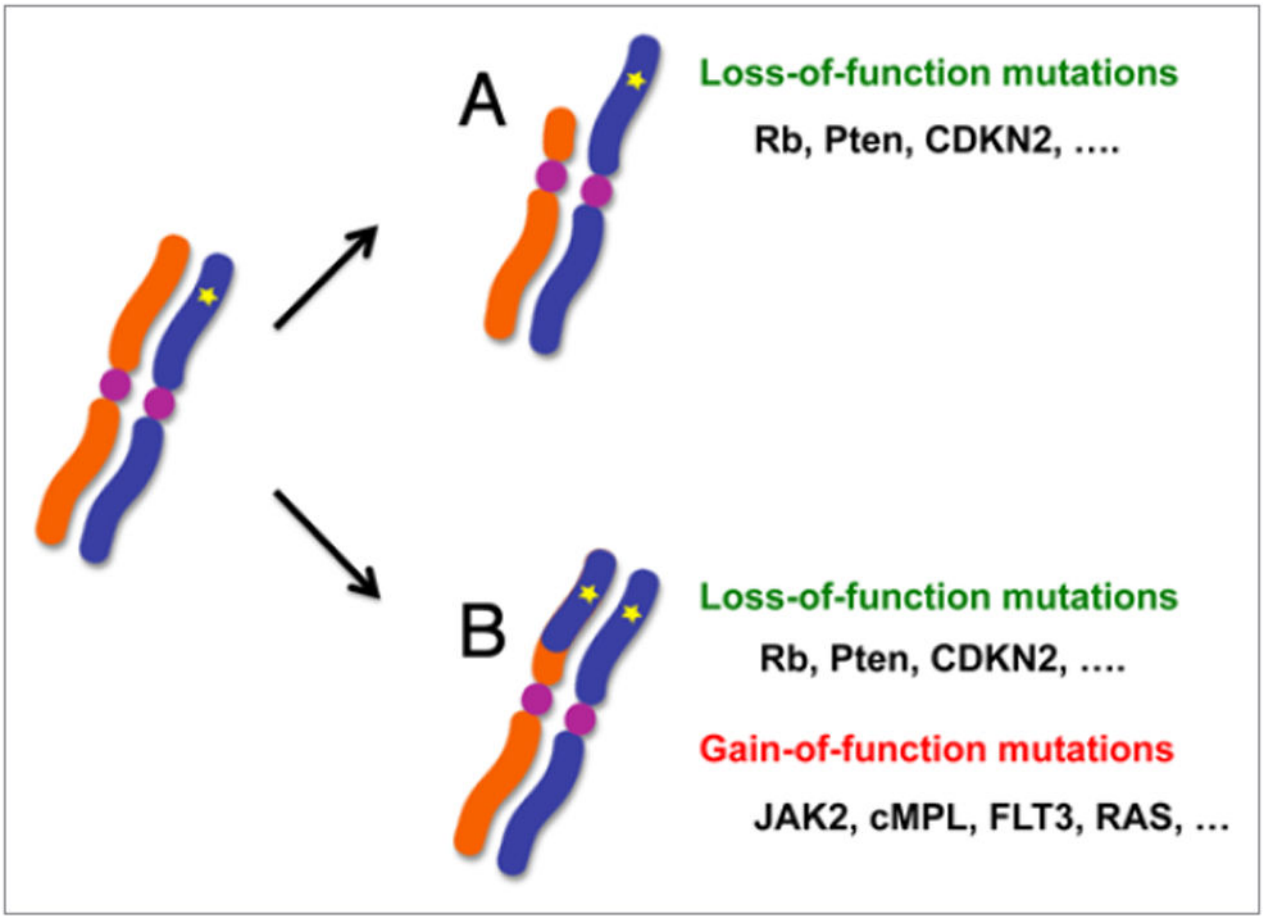

Figure 1.

In cancer cells, LOH is frequently associated with a mutated tumor suppressor locus, in which a normal copy of the tumor suppressor is lost by simple allelic deletion (A), or replaced by the mutated copy through allelic conversion that leads to copy number neutral LOH or aUPD (B). In either case, the common consequence is biallelic loss-of-function of the tumor suppressor. In addition, LOH caused by aUPD is also implicated in the common mechanism of homozygous mutations of proto-oncogenes. A number of gain-of-function oncogenic mutations found in aUPD regions have been shown to exist in a homozygous state, including mutations of *JAK2* (9pUPD), *MPL* (1pUPD), *NRAS* (1pUPD), *KRAS* (12pUPD), *BRAF* and *FLT3* (13qUPD). The clonal outgrowth of aUPD-positive clones indicates that two copies of mutations confer a growth advantage to aUPD positive cells through their gain-of-function.

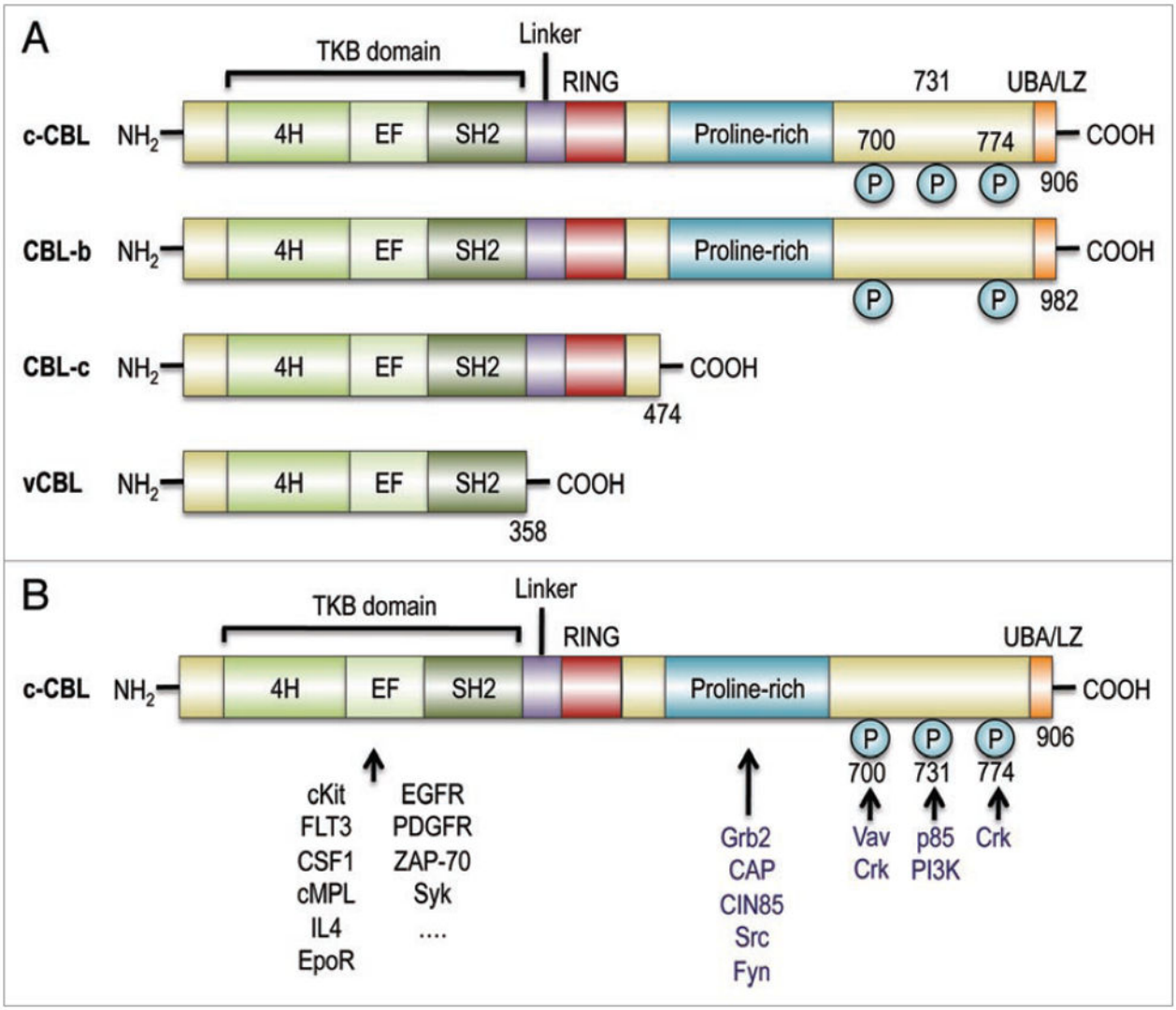

Figure 2.

(A) Structure of CBL family proteins. CBL family proteins in mammals have highly conserved domains, where an N-terminal TKB domain, consisting of a four-helix bundle (4H), a Ca^{2+}-binding EF (EF), and a src-homology (SH2) domains, is connected to a RING finger domain via a linker. c-CBL and CBL-b, but not CBL-c, have a proline-rich and other C-terminal components that end with a ubiquitin-associated and leucine zipper (UBA/LZ) domain. Their viral form, v-CBL, is truncated just after its SH2 domain. (B) CBL family proteins interact with a number of signal transducing molecules. Through their TKB domain, CBL family proteins target phosphrylated tyrosine kinases, including growth factor receptors and cytokine receptors, as well as, non-receptor tyrosine kinases. Ubiquitin conjugating enzymes have contact with CBL proteins via the linker/RING finger domain, which is central to the E3 ubiquitin ligase activity. The proline-rich domain provides a binding site for SH3 domains of Grab2, CAP and Src-family kinases. The C-terminal portion contains three tyrosine residues, Y700, Y731 and Y774, which are the major

phosphorylated tyrosines, and which bind to the p85 subunit of PI3 kinase (Y731), Vav (Y700) and Crk proteins (Y700 and Y774).

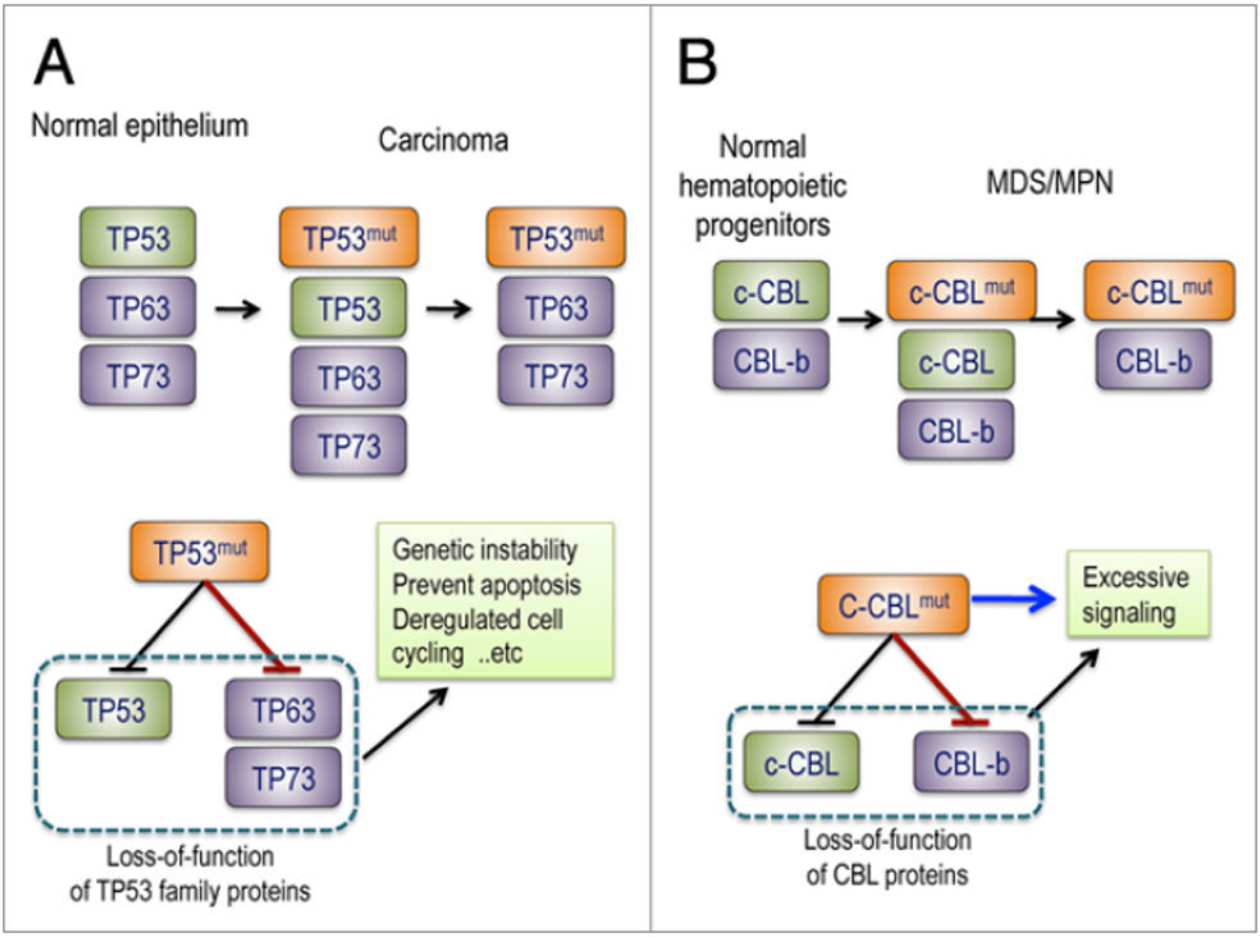

Figure 3.

Possible mechanisms of gain-of-function of mutated TP53 and c-CBL. The gain-of-function of TP53 mutants is associated with their potential to induce carcinoma in mice as well as in human, which is considered to be mediated by inhibition of TP63 and TP73. TP53-deficient mice frequently develop sarcomas and lymphomas but only rarely carcinomas, which are thought to be suppressed by TP53 homologues, TP63 and TP73, in epithelial tissues, in the face of loss of TP53. Mutant TP53 inhibits tumor suppressor functions of TP63 and TP73, and compromises TP53-like activity. Similarly, the gain-of-function of CBL mutants found in MDS/MPN may be explained by the inhibition of CBL-b (red arrow), which would result in more profound defects in negative regulation of tyrosine kinase signaling compared to simple loss of c-CBL. On the other hand, c-CBL is thought to have positive regulatory functions that are not directly related to the E3 ubiquitin ligase activity and could be the source of the gain-of-function of c-CBL mutants (blue arrow).

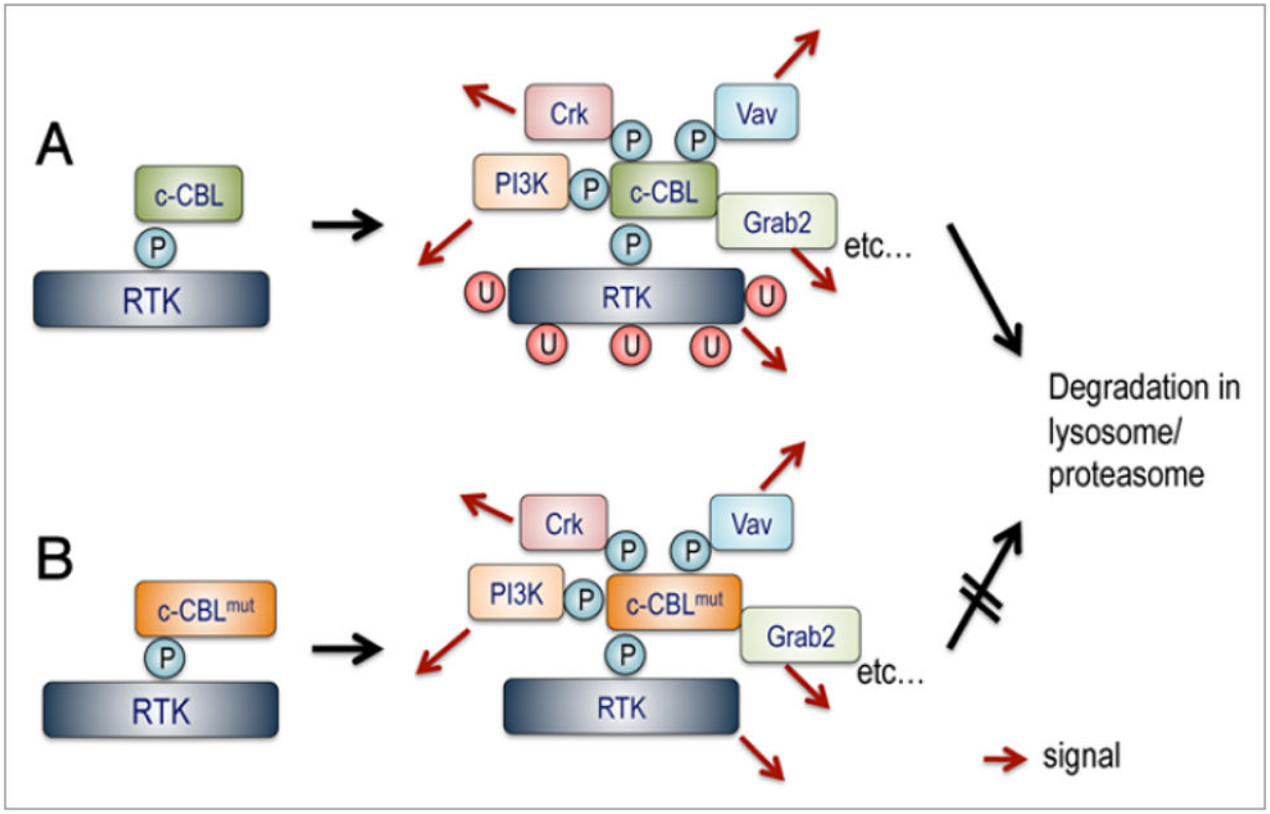

Figure 4.

Positive regulation of signal transduction by c-CBL. (A) Having E3 ubiquitin ligase activity for negative regulation of signaling, c-CBL also works as an adaptor protein for multiple signal transduction molecules. When bound to phosphorylated tyrosine kinases, c-CBL is rapidly phosphorylated at multiple tyrosine residues, which in turn provide binding sites for a number of signal transduction molecules. Several lines of evidence suggest that binding to these molecules plays important roles in positive regulation of signal transduction (red arrows). Normally, phosphorylated c-CBL undergoes degradation, which is mediated by its E3 ubiquitin ligase activity. Thus, degradation of mutated c-CBL could be retarded, leading to prolonged transmission of positive signals (B).