NEOPLASIA
www.neoplasia.com

Volume 17 Number 6 June 2015 pp. 518–524 518

Deleterious *c-Cbl* Exon Skipping Contributes to Human Glioma[1,2]

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Abstract

c-Cbl, a RING-type ubiquitin E3 ligase, downregulates various receptor tyrosine kinases (e.g., epidermal growth factor receptor (EGFR)), leading to inhibition of cell proliferation. Moreover, patients with myeloid neoplasm frequently harbor *c-Cbl* mutations, implicating the role of c-Cbl as a tumor suppressor. Recently, we have shown that c-Cbl downregulates αPix-mediated cell migration and invasion, and the lack of c-Cbl in the rat C6 and human A172 glioma cells is responsible for their malignant behavior. Here, we showed that *c-Cbl* exon skipping occurs in the glioma cells and the brain tissues from glioblastoma patients lacking c-Cbl. This exon skipping resulted in generation of two types of c-Cbl isoforms: type I lacking exon-9 and type II lacking exon-9 and exon-10. However, the c-Cbl isoforms in the cells and tissues could not be detected as they were rapidly degraded by proteasome. Consequently, C6 and A172 cells showed sustained EGFR activation. However, no splice site mutation was found in the region from exon-7 to exon-11 of the *c-Cbl* gene in C6 cells and a glioblastoma tissue lacking c-Cbl. In addition, *c-Cbl* exon skipping could be induced when cells transfected with a *c-Cbl* mini-gene were grown to high density or under hypoxic stress. These results suggest that unknown alternations (e.g., mutation) of splicing machinery in C6 and A172 cells and the glioblastoma brain tissues are responsible for the deleterious exon skipping. Collectively, these findings indicate that the *c-Cbl* exon skipping contributes to human glioma and its malignant behavior.

Neoplasia (2015) 17, 518–524

Introduction

Glioblastoma multiforme (GBM) is the most invasive and aggressive human brain tumor. Disease-free survival of patients with GBM is poor even after surgical removal, radiotherapy, and chemotherapy because of malignant behavior of glioma cells [1,2]. Therefore, unlike for common types of solid cancer, current experimental therapies for GBM are mainly focused on inhibition of invasion [3–6]. Numerous proteins are involved in invasiveness of glioma cells. They include focal adhesion complex proteins, such as Pix, integrin, and paxillin, and receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) and c-Met [7]. Recently, we have shown that the expression of αPix is dramatically upregulated in the rat C6 and human A172 glioma cell lines and is critically involved in migration and invasion of the cells [8].

c-Cbl, a RING type E3 ubiquitin ligase, promotes the degradation of proteins associated with cell growth and migration, including EGFR, FAX, and paxillin [9–13]. Moreover, a wide variety of *c-Cbl* mutations have frequently been found in human myeloproliferative diseases, implicating the role of c-Cbl as a tumor suppressor. *c-Cbl* mutations include missense mutations, frame-shift mutations, insertions, deletion mutations, and primary transcript splicing mutations [14–20]. Of these, most of deletion mutations lead to elimination of a part or entire portion of exon-8 or exon-9

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[1]This work was supported by grants from the National Research Foundation of Korea (NRF-2005-084-C00025 and M10533010001-05N3301) and the Nobel Laureate Invitation Program of Seoul National University. M.W.S. and J.H.P. were the recipients of the BK21 fellowship. Conflict of interest: None of the authors of this work has any conflict of interest.
[2]This article refers to supplementary materials, which are designated by Figures S1 to S4 and are available online at www.neoplasia.com.
Received 2 February 2015; Revised 2 June 2015; Accepted 8 June 2015

1476-5586
http://dx.doi.org/10.1016/j.neo.2015.06.003

and thereby to inactivation of the c-Cbl ligase activity. We have recently shown that the expression of c-Cbl is dramatically downregulated in C6 and A172 cells, leading to marked accumulation of αPix. Surprisingly, however, the levels of c-Cbl mRNA in the glioma cells were found to be comparable to those in normal cells [8]. Therefore, we investigated whether c-Cbl in the glioma cells might be mutated and destabilized.

Here, we showed that deleterious *c-Cbl* exon skipping occurs in the brain tissues of GBM patients lacking c-Cbl as well as in C6 and A172 glioma cells. This exon skipping generated two types of c-Cbl isoforms: type I lacking exon-9 and type II lacking both exon-9 and exon-10. We further showed that both types of c-Cbl isoforms are inactivated and destabilized consistent with the fact that exon-9 encodes a part of RING finger domain essential for the function of c-Cbl as an ubiquitin E3 ligase. The lack of c-Cbl in C6 and A172 cells led to a sustained activation of epidermal growth factor (EGF) signaling for their increased cell growth and malignant behavior. However, no splice site mutation was found in the region from exon-7 to exon-11 of the *c-Cbl* gene in C6 cells and a GBM brain tissue lacking c-Cbl. Furthermore, *c-Cbl* exon skipping could be induced when cells transfected with a *c-Cbl* mini-gene were grown to high density or under hypoxic stress, suggesting that alteration in splicing machinery (e.g., mutation) is responsible for *c-Cbl* exon skipping. Taken together, our findings indicate that *c-Cbl* exon skipping contributes to human glioma and its malignant behavior.

Materials and Methods

Plasmids and Antibodies

cDNAs for c-Cbl and its isoforms were inserted into pFlag-CMV2 or pcDNA-HisMax. A *c-Cbl* mini-gene construct was generated by inserting a fragment (6868 bp) of the *c-Cbl* gene in C6 cells (i.e., the region from exon-7 to exon-11 including introns) into pFlag-CMV2 and then by fusing green fluorescent protein (GFP) cDNA to the 3′-end of exon-11. All primers were purchased from Bioneer (Daejeon, Korea).

Antibodies against Flag (Sigma, St Louis, MO), Xpress (Invitrogen, Grand Island, NY), hemagglutinin (HA) (Roche, Pleasanton, CA), c-Cbl (Santa Cruz Biotechnology, Dallas, TX), extracellular signal-related kinase (ERK) (Cell Signaling Technology, Boston, MA), and phospho-ERK (pERK; Cell Signaling Technology) were used. Peroxidase-conjugated goat anti-rabbit and anti-mouse IgGs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). An anti-αPix antibody was generated as previously described [8].

Cell Culture and Hypoxia Induction

Cells were grown at 37°C under an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) supplemented with 1% (vol/vol) antibiotic-antimycotic solution (Welgene, Gyeongsan, Korea) and 10% (vol/vol) fetal bovine serum (Gibco, Grand Island, NY). Primary glial cells were prepared from rat brains of post-natal day 3. Dissociated glial cells were cultured in minimum essential medium containing 0.6% glucose, 1 mM pyruvate, 2 mM L-glutamine, 10% horse serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. HEK293T and COS7 cells were transfected with appropriate vectors by using JetPEI (Polyplus, Berkeley, CA), and C6 and A172 cells were done by electroporation (NEON; Invitrogen) according to the manufacturer's instructions.

For hypoxia experiments, C6 and A172 cells that had been transfected with a *c-Cbl* mini-gene were seeded at a density of ~5×10^5 cells per 100-mm culture dish. They were then exposed for various periods to an ambient O_2 concentration of 1%, 5% CO_2, and 94% N_2 at 37°C (using a controlled incubator with CO_2/O_2 monitoring and CO_2/N_2 gas sources).

Immunoprecipitation and Pull-Down Analysis

Cell lysates were prepared in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, and 1 × protease inhibitor cocktail (Roche). They were incubated with appropriate antibodies for 2 hours at 4°C and then with 30 μl of 50% slurry of protein A-Sepharose (Sigma) for the next 1 hour. The resins were collected by centrifugation, boiled in sodium dodecyl sulfate–sampling buffer, and subjected to immunoblot analysis. For pull-down analysis, cell lysates were prepared in Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 0.5% NP-40, and 1 × protease inhibitor cocktail. They were incubated with Ni^{2+}-NTA agarose bead (NTA; Qiagen, Valencia, CA) for 1 hour at 4°C. The resins were then collected by centrifugation, boiled in sodium dodecyl sulfate–sampling buffer, and subjected to immunoblot analysis.

Reverse Transcription–Polymerase Chain Reaction Analysis

The brain tissues of patients with GBM were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank at the University of Maryland (Baltimore, MD). The identification number for each tissue is given as follows: UMB# 5117 (normal), 1765 (patient 1: P1), 4538 (P2), 4557 (P3), 1786 (P4), 4517 (P5), 4736 (P6), 4754 (P7), and 5371 (P8). Total mRNAs from cell lines and brain tissues were isolated using TRIzol (Invitrogen). Reverse transcription–polymerase chain reaction (RT-PCR) was performed using SuperScript III (Invitrogen) by following the manufacturer's instructions. The resulting cDNAs were then used as templates for PCR amplification. To identify the skipped *c-Cbl* exons, the following primers were used: 5′-CTTTACCCGACTCTTTCAGCCCTGGTCCTC-3′ (forward) and 5′-TTGCTCCCCAGGTGGCAGTTTTGGCACAGG-3′ (reverse) for both human and rat c-Cbl. The sequences of primers used for semiquantitative PCR were 5′-GAACCTATTCAGGCATGG

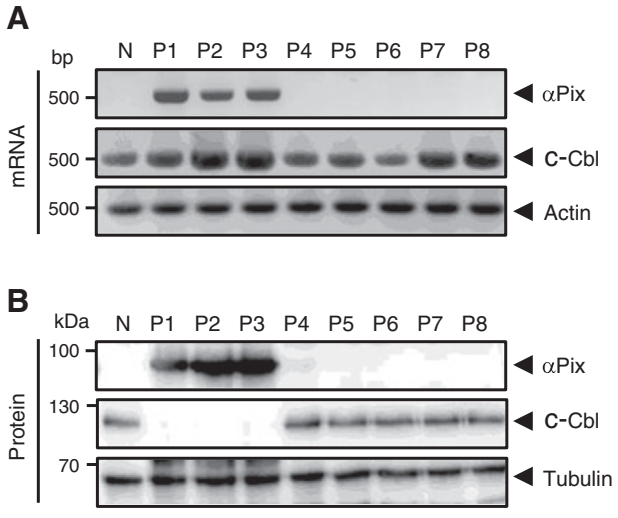

Figure 1. Expression of αPix and c-Cbl in the brain tissues of patients with GBM. (A) Total mRNAs were isolated from the brain tissues of normal (N) and GBM patients (P1-P8) by using TRIzol. They were then subjected to RT-PCR using primers specific to αPix and c-Cbl. (B) The same brain tissues were homogenized, and their soluble fractions were subjected to immunoblot with anti-αPix and anti–c-Cbl antibodies.

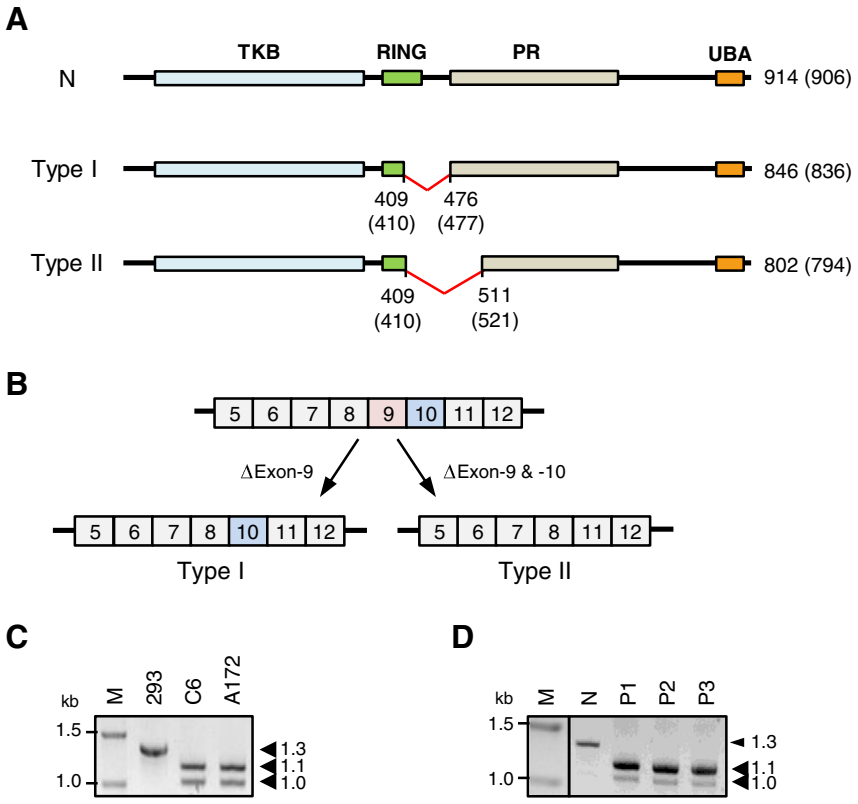

Figure 2. *c-Cbl* exon skipping in glioma cells and the brain tissues of GBM patients. (A) cDNAs for c-Cbl from normal rat cells (NCBI database, XP_576396), C6 and A172 glioma cells, and the brain tissues of P1 to P3 were subjected to sequence analysis. The primary sequences of normal c-Cbl (N) and its isoforms (types I and II) were shown with their functional domains. TKB, PR, and UBA indicate tyrosine kinase-binding domain, Pro-rich domain, and ubiquitin-associate domain, respectively. The numerals in parentheses indicate the position of amino acids in c-Cbl from normal human cells (NP_005179.2) and its isoforms from the glioma cells and the tissues of P1 to P3. Note that C6 and A172 cells were grown to confluence for preparation of *c-Cbl* cDNA from their total mRNAs. (B) Schematic diagram shows c-Cbl isoforms generated by two types of exon skipping: type I lacking exon-9 and type II lacking both exon-9 and exon-10. (C) Using the cDNAs as templates, PCR was performed with primers directed to a region between exon-4/exon-5 and exon-12. The length of PCR product from normal c-Cbl cDNA was about 1.3 kb. Skipping of exon-9 and both exon-9 and exon-10 led to the generation of PCR products with approximate sizes of 1.1 and 1.0 kb, respectively. The accession numbers for the sequences of cDNAs for c-Cbl isoforms are KJ944831 (type I) and KJ944832 (type II) from A172, KJ944833 (type I) and KJ944834 (type II) from C6, KJ944835 (type I) and KJ944836 (type II) from P1, KJ944837 (type I) and KJ944838 (type II) from P2, and KJ944839 (type I) and KJ944840 (type II) from P3.

GAAGGAGATGATATTA-3′ (forward) and 5′-CTGCTGATGGTC TAAGTGGAGGTGCAGGTCGTAG-3′ (reverse) for αPix and 5′-ATGGCCGGCAACGTGAAGAAGAGCTCTGGGGCCG-3′ (forward) and 5′-TTCCTTTTAGTTCTGCCAGCATGTGGCT GAAGAT-3′ (reverse) for c-Cbl.

Thiazoyl Blue Trazolium Bromide Assay

C6 and A172 cells transfected with appropriate vectors were seeded at a density of 1.0×10^4 cells per well and incubated for increasing periods. Cell proliferation was then determined by incubation with thiazoyl blue trazolium bromide (MTT; AMERSCO, Solon, OH) for 1 hour. After incubation, the culture medium was replaced by DMSO to lyse the cells. Absorbance at 570 nm was then measured by using a Biokinetics plate reader (Bio-Tek Instruments, Winooski, VT).

DNA Sequencing

To determine the nucleotide sequence of genomic DNA (the region from exon-7 to exon-11 of the *c-Cbl* gene), total genomic DNAs were prepared from C6 cells and the brain tissue of patient P1 (see below). Successive PCRs were performed along the genomic DNA using appropriate primers to generate about 500 to 1500 bp, and the products were cloned into pGEM-T Easy (Promega, Madison, WI) vectors. The resulting vectors were then subjected to sequencing by using Sanger method.

Results

c-Cbl Exon Skipping in C6 and A172 Cells and Glioma Tissues

We have recently shown that c-Cbl ubiquitinates αPix for proteasomal degradation and that the rat C6 and human A172 glioma cells lack c-Cbl protein, although they normally express c-Cbl mRNA [8]. To determine whether the expression patterns of αPix and c-Cbl are also altered in the brain tissues of patients with GBM, we first compared the mRNA level of αPix in them (P1-P8). As was found in C6 and A172 cells [8], the expression of αPix mRNA was dramatically upregulated in the brain tissues from three patients with GBM (P1-P3), although not in five other GBM tissues or in a normal brain tissue (Figure 1*A*). Consistently, the expression of αPix protein could be seen only in P1 to P3 (Figure 1*B*). We then examined whether the mRNA and protein levels of c-Cbl might also be altered in the tissues tested. Remarkably, the c-Cbl protein could not be detected only in P1 to P3 (Figure 1*B*), despite the finding that c-Cbl

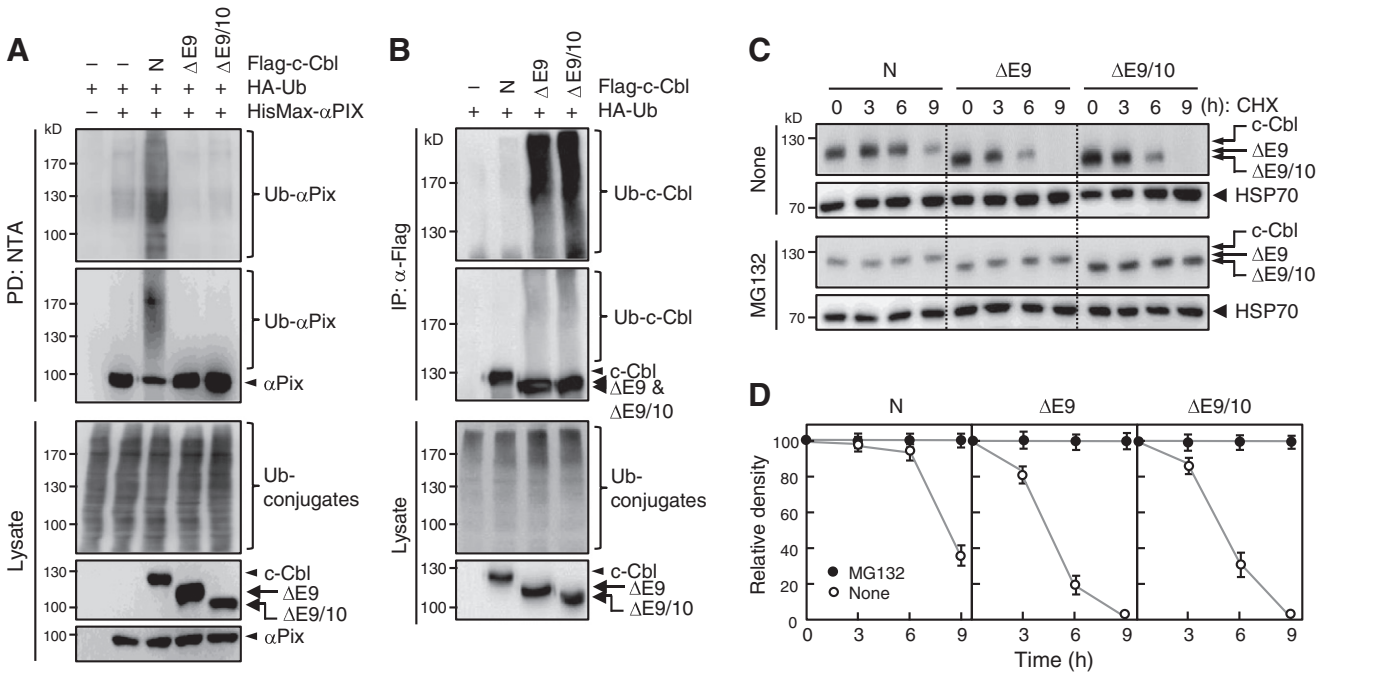

Figure 3. Exon skipping leads to inactivation and destabilization of c-Cbl. (A) Flag-tagged c-Cbl (N) and its isoforms (ΔE9 and ΔE9/10) were expressed in HEK293T cells with HisMax-αPix and HA-ubiquitin. After incubation with 20 μM MG132 for 8 hours, cell lysates were subjected to pull-down with NTA resins followed by immunoblot with anti-HA and anti-Xpress antibodies. (B) Flag-tagged c-Cbl and its isoforms were expressed in C6 cells with HA-ubiquitin. They were then treated as in A. (C) C6 cells expressing Flag-tagged c-Cbl and its isoforms were incubated with 200 μg/ml cycloheximide in the presence and absence of 20 μM MG132. Cell lysates were then subjected to immunoblot with anti-Flag antibody. (D) The c-Cbl bands in C were scanned using a densitometer, and their densities were quantified by using "ImageJ" software. The density seen at each "0" time point was expressed as 100% and the others as its relative values. Data are the means ± S.D. ($n = 3$).

mRNA was expressed to a similar extent in all tissues tested (Figure 1*A*). These results indicate that the elevation of αPix protein level in P1 to P3 is due to the lack of c-Cbl protein, which serves as an αPix-specific ubiquitin E3 ligase [8]. These findings also raised a possibility that c-Cbl in the brain tissues of P1 to P3 as well as in C6 and A172 cells might be mutated and destabilized, resulting in αPix accumulation.

To test this possibility, total mRNAs were prepared from the brain tissues and the glioma cells and subjected to RT-PCR followed by cDNA sequencing. Comparison with the known sequences of c-Cbl cDNA (from the National Center for Biotechnology Information (NCBI) database) revealed that cDNAs from C6 and A72 cells and the tissues from P1 to P3 encode two types of c-Cbl isoforms (Figure 2*A*): type I lacking the amino acid sequence corresponding to the exact length of exon-9 and type II lacking that of both exon-9 and exon-10 (Figure 2*B*). To show more clearly the exon skipping, PCR was performed using primers directed to the sequences in the region between exon-4/exon-5 and exon-12. Two types of *c-Cbl* exon skipping could evidently be seen in the cDNAs from C6 and A172 glioma cells (Figure 2*C*) and the tissues of P1 to P3 (Figure 2*D*). These results demonstrate that *c-Cbl* exon skipping occurs in both C6 and A172 glioma cells and the brain tissues of P1 to P3. Henceforth, the c-Cbl isoforms generated by skipping of exon-9 and both exon-9 and exon-10 were referred to as ΔE9 and ΔE9/10, respectively.

c-Cbl Isoforms (ΔE9 and ΔE9/10) Are Inactivated and Destabilized

Skipping of exon-9 leads to elimination of approximately one-half of the RING domain (see Figure 2*A*), which is required for the catalytic function of c-Cbl as an ubiquitin E3 ligase. Thus, it is likely that both ΔE9 and ΔE9/10 are unable to ubiquitinate αPix. To confirm this, the cDNAs for ΔE9 and ΔE9/10 were cloned into pFlag-CMV vector. Flag-tagged ΔE9 and ΔE9/10 were then overexpressed in HEK293T cells with αPix and ubiquitin. NTA pull-down analysis showed that c-Cbl, but not ΔE9 and ΔE9/10, could ubiquitinate αPix (Figure 3*A*), indicating that both types of exon skipping abrogate the ligase function of c-Cbl.

However, neither ΔE9 nor ΔE9/10 could be detected in C6 and A172 cells and the brain tissues of P1 to P3, suggesting that the c-Cbl isoforms are unstable, possibly due to the lack of the amino acid sequence corresponding to exon-9 or both exon-9 and exon-10. To test this possibility, experiments were performed as in Figure 3*A* but using C6 cells and without αPix expression. Unlike normal c-Cbl, both ΔE9 and ΔE9/10 were heavily ubiquitinated (Figure 3*B*). Similar results were obtained when ΔE9 and ΔE9/10 were expressed in A172 cells (Figure S1). To determine whether ΔE9 and ΔE9/10 are indeed degraded more rapidly than normal c-Cbl and their degradation is mediated by proteasome, C6 cells expressing c-Cbl and its isoforms were incubated with cycloheximide in the presence and absence of MG132, a proteasome inhibitor. The stability of both ΔE9 and ΔE9/10 was much lower than that of normal c-Cbl, and their degradation could be abrogated by treatment with MG132 (Figure 3, *C* and *D*). These results indicate that the lack of c-Cbl proteins in C6 and A172 cells and the brain tissues of P1 to P3 is due to *c-Cbl* exon skipping, which results in rapid proteasomal degradation of both ΔE9 and ΔE9/10.

c-Cbl Exon Skipping Upregulates EGF Signal

c-Cbl is known to play an important role in switching off EGF signal by downregulating EGFR [17]. Therefore, we examined whether *c-Cbl* exon skipping leads to a sustained activation of EGFR.

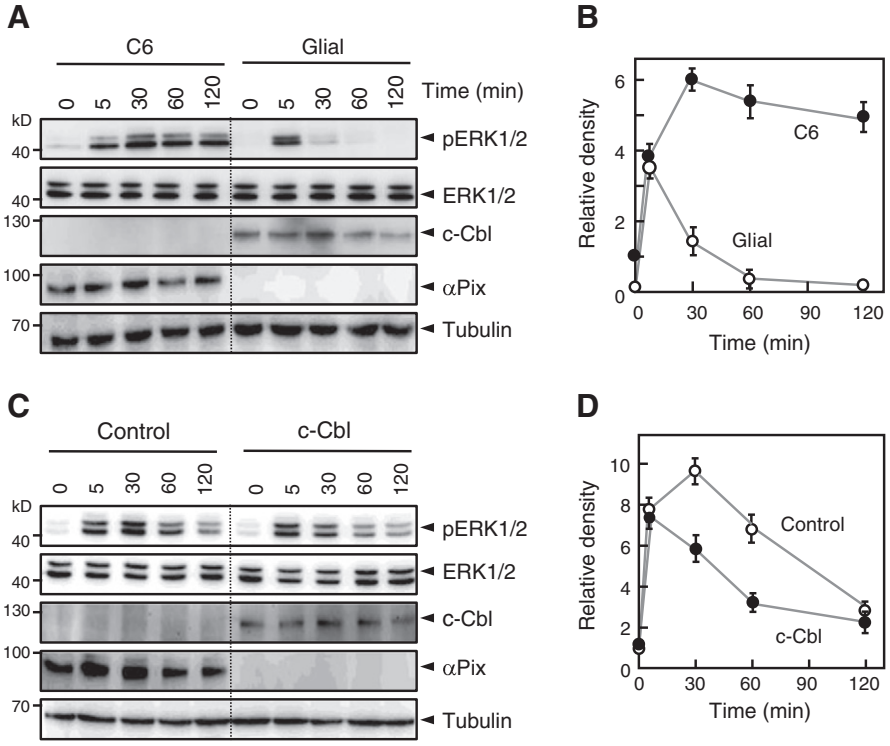

Figure 4. *c-Cbl* exon skipping upregulates EGFR activation. (A) C6 and glial cells treated with 0.5 ng/ml EGF were incubated for various periods and then subjected to immunoblot with anti-pERK1/2 and anti-ERK1/2 antibodies. (B) The pERK bands in A were scanned and quantified. The density seen at each "0" time point was expressed as 100% and the others as its relative values. (C) Experiments were performed as in A, except the use of C6 cells transfected with an empty vector (Control) or a vector expressing Flag–c-Cbl. (D) The pERK bands in C were scanned and quantified. Data of B and D are the means ± S.D. ($n = 3$).

EGF treatment resulted in persistent phosphorylation of ERK even after 2 hours in C6 cells, unlike glial cells, in which EGFR activation transiently occurred as early as 5 minutes after the treatment (Figure 4, *A* and *B*). Furthermore, ERK phosphorylation was significantly reduced when C6 cells were complemented with normal c-Cbl (Figure 4, *C* and *D*). However, overexpression of ΔE9 and ΔE9/10 in COS7 cells led to an increase in ERK phosphorylation (Figure S2), suggesting that the overexpressed ΔE9 and ΔE9/10 could upregulate EGF signaling by acting dominant negatively to endogenous c-Cbl. In addition, MTT assay revealed that the proliferation of C6 cells could significantly be attenuated by the expression of normal c-Cbl but not by that of its catalytically inactive mutant (C381A), of which the active site Cys381 is replaced by Ala (Figure S3). These results suggest that the exon skipping in C6 cells blocks c-Cbl–mediated down-regulation of EGF signal and, in turn, promote EGF-mediated cell proliferation and tumorigenesis.

Alteration of Trans-Element(s) Is Responsible for c-Cbl Exon Skipping

Exon skipping is commonly generated by mutations in splice sites (*cis*-elements) or in splicing machinery (*trans*-elements). To determine whether any mutation in *cis*- or *trans*-element is responsible for *c-Cbl* exon skipping, we carried out sequence analysis of the region from exon-7 to exon-11 of the *c-Cbl* gene in C6 cells and the brain tissue of P1. The accession numbers for the genomic DNA sequences from C6 cells and P1 tissues are KP406160 and KP203813, respectively. However, no splice site mutation was found in the region, suggesting that *c-Cbl* exon skipping is mediated by certain unknown alteration (e.g., mutation) of *trans*-elements.

To validate this finding further, we generated a *c-Cbl* mini-gene by inserting the genomic DNA fragment (from C6 cells) into pFlag-CMV followed by fusion of GFP cDNA to the 3′-end of exon-11 (Figure 5*A*). Various glioma cell lines were then transfected with the mini-gene, grown to a high density (2.5×10^7 cells per 100-mm dish), and subjected to RT-PCR by using primers specific to exon-7 and GFP. Exon skipping of *c-Cbl* mini-gene could be observed in C6 and A172 cells, but not in HEK293T, HeLa, or other glioma cell lines, including U87 and H4 (Figure 5*B*), indicating that *c-Cbl* exon skipping is due to unknown alteration of *trans*-elements in C6 and A172 cells, by which the function of splicing machinery might be constitutively activated or temporally induced under specific environmental conditions.

To test this possibility, C6 and A172 cells that had been transfected with *c-Cbl*-mini gene were grown to high cell density or exposed to hypoxic stress, both of which are typical environment of cancer cells. *c-Cbl* exon skipping, which could not be observed at a density of 5×10^5 cells per 100-mm dish, was markedly increased upon increasing the cell density, i.e., at above 5×10^6 cells per dish (Figure 5*C*). Exposure to hypoxic stress also induced *c-Cbl* exon skipping even at a low cell density, i.e., at 5×10^5 cells per dish (Figure 5*D*). Unlike in C6 and A172 cells, however, no *c-Cbl* exon skipping could be observed in HeLa and H4 cells under both high cell density and hypoxic conditions. To determine whether exon skipping indeed occurs in the primary transcripts of c-Cbl in C6 and A172 cells under

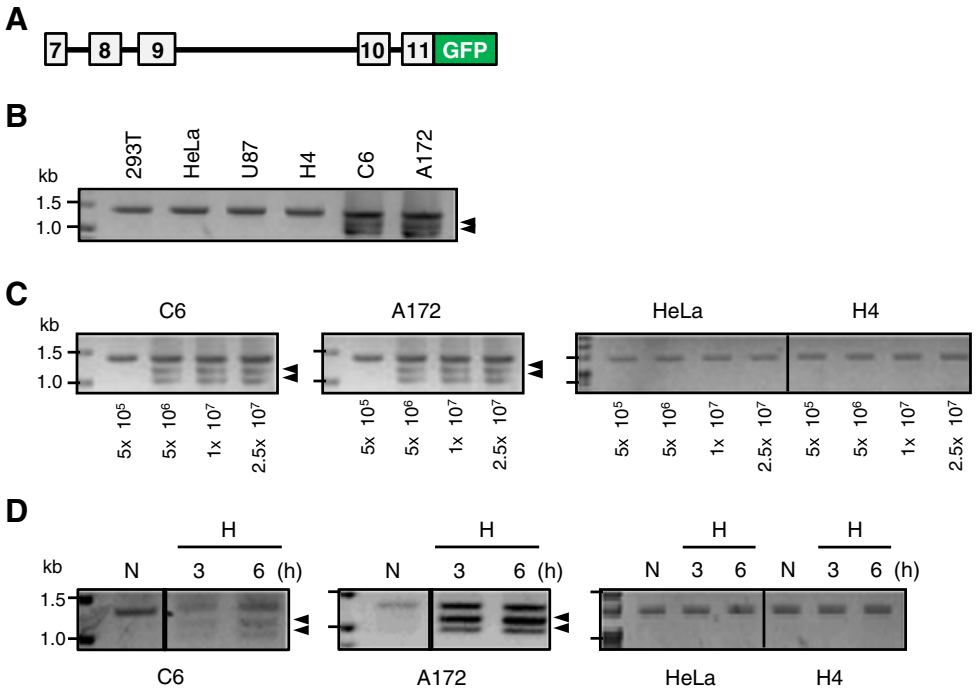

Figure 5. Alteration of *trans*-elements is responsible for *c-Cbl* exon skipping. (A) Schematic diagram of a *c-Cbl* mini-gene construct. The numerals indicate the exon number, and the thick black lines show introns. (B) The mini-gene was transfected into the indicated cell lines. After incubating the cells for 48 hours to reach a density of 2.5×10^7 cells per 100-mm dish, they were harvested with TRIzol reagent. Total RNAs were then subjected to RT-PCR by using the primers specific to exon-7 (forward) and GFP (reverse). (C) C6, A172, HeLa, and H4 cells transfected with the mini-gene were seeded in 100-mm dishes at the indicated cell densities. After culturing for 24 hours, RT-PCR was performed as in B. (D) C6, A172 HeLa, and H4 cells transfected with the mini-gene were seeded at a density of 5×10^5 cells per 100-mm dish. After culturing for 24 hours, cells were incubated under hypoxic conditions followed by RT-PCR as in B. N and H indicate normoxia and hypoxia, respectively.

high cell density conditions, total mRNAs were prepared from the glioma cells and subjected to RT-PCR. *c-Cbl* exon skipping, which could not be seen at a density of 5×10^5 cells, was dramatically increased upon increasing the density of C6 and A172 cells but not in HeLa and H4 cells (Figure S4). These results suggest that cell-to-cell contact and hypoxia somehow influence certain *trans*-elements to trigger *c-Cbl* exon skipping in C6 and A172 cells. These results also suggest that the glioma cells may overcome contact inhibition and hypoxia by *c-Cbl* exon skipping for their survival and malignant behavior.

Discussion

In the present study, we demonstrated that two types of *c-Cbl* exon skipping occur in C6 and A172 glioma cells and the brain tissues of several GBM patients: type I lacking exon-9 and type II lacking both exon-9 and exon-10. These exon skipping abrogated the ubiquitin E3 ligase function of c-Cbl, resulting in stabilization of αPix. The accumulated αPix promoted cell migration and invasion [8], both of which are typical malignant behavior of cancer cells, such as GBM. Thus, it appears that *c-Cbl* exon skipping critically contribute to human glioma and its malignant behavior.

c-Cbl isoforms generated by exon skipping were found to be much more unstable than its normal form. However, it is unlikely that the catalytic activity of c-Cbl is responsible for ubiquitination and destabilization of itself, because both isoforms lack most part of the RING domain. It has been reported that c-Cbl can be ubiquitinated by the HECT-type ubiquitin E3 ligases, such as Itch or Nedd4, for proteasomal degradation [21]. Thus, it appears possible that Itch or Nedd4 may preferentially ubiquitinate the c-Cbl isoforms generated by exon skipping over the normal c-Cbl ligase.

Human myeloid neoplasms have been shown to be associated with a variety of *c-Cbl* mutations, including missense mutations, frame-shift mutations, insertions, deletion mutations, and primary transcript splicing mutations. Recently, it has been shown that *c-Cbl* mutations also contribute to the pathogenesis of solid tumors [10]. Somatic mutations were found in eight of 119 patients with non–small cell lung tumors, although only one was inside the linker and RING-finger regions unlike the mutations that contribute to myeloid neoplasm that are mostly within the regions. Thus, GBM appears to represent the second example of c-Cbl–associated solid tumor, although *c-Cbl* exon skipping rather than somatic mutations in the *c-Cbl* gene itself contributes to GBM. In this respect, it would be of interest to see if disruption of c-Cbl function may also contribute to the pathogenesis of other solid tumors.

Of note was the finding that overexpression of c-Cbl isoforms generated by exon skipping are capable of upregulating EGF signaling, suggesting that the overexpressed isoforms could act dominant negatively to endogenous c-Cbl. Cbl-b, a close homolog of c-Cbl, is thought to also negatively regulate tyrosine kinase signaling, primarily through its ubiquitin E3 ligase activity [14,22,23]. Significantly, it has been shown that overexpression of c-Cbl mutants lacking the E3 ligase activity in *c-Cbl*$^{-/-}$ LKS cells markedly increases their sensitivity to a broad spectrum of cytokines, including SCF, TPO, and FLT3 ligand, as compared to that in *c-Cbl*$^{+/+}$ cells, suggesting that the gain-of-function of the c-Cbl mutants may be

mediated by their ability to inhibit Cbl-b [24]. Thus, it appears also possible that the reduction of EGF signaling by c-Cbl isoforms might be due to inhibition of Cbl-b.

Interestingly, both the type I and type II *c-Cbl* exon skipping eliminated the exact length of exon-9 and both exon-9 and exon-10, respectively. Previously, it has been reported that genomic splice site mutations (*cis*-element mutations) cause *c-Cbl* exon skipping [19,20]. However, no mutation was found in *c-Cbl* genomic DNA sequence (at least in the region from exon-7 to exon-11 including introns) from C6 cells and the brain tissue of P1, suggesting that certain unknown mutation(s) in *trans*-elements are responsible for *c-Cbl* exon skipping. Moreover, *c-Cbl* exon skipping could be observed when cells were grown to high density or under hypoxic conditions. These results suggest that the environmental factors induce the activation of *trans*-elements to catalyze *c-Cbl* exon skipping. However, how the environmental factors make the *trans*-elements exert its splicing function is not at all known. Neither is known about the nature of *trans*-elements involved in *c-Cbl* exon skipping and what kind of mutation(s) is present in the *trans*-elements. Nevertheless, this study is the first report showing that certain mutation(s) in *trans*-elements could acquire a gain-of-function in splicing under certain environmental conditions, such as high cell density and hypoxic stress. In addition, *c-Cbl* exon skipping is clearly different from common alternative splicing, because only one transcript of c-Cbl has so far been reported in the transcriptome database unlike alternative splicing, which generates two or more transcripts from one gene. In this respect, *c-Cbl* exon skipping is a new type of splicing, which would require a new term for future use. For this, we suggest "deleterious exon skipping."

Appendix A. Supplementary Materials

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2015.06.003.

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