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c-Cbl regulates αPix-mediated cell migration and invasion

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ABSTRACT

c-Cbl, a RING-type ubiquitin E3 ligase, down-regulates receptor tyrosine kinases, including EGF receptor, and inhibits cell proliferation. Moreover, c-Cbl mutations are frequently found in patients with myeloid neoplasm. Therefore, c-Cbl is known as a tumor suppressor. αPix is expressed only in highly proliferative and mobile cells, including immune cells, and up-regulated in certain invasive tumors, such as glioblastoma multiforme. Here, we showed that c-Cbl serves as an ubiquitin E3 ligase for proteasome-mediated degradation of αPix, but not βPix. Remarkably, the rat C6 and human A172 glioma cells were unable to express c-Cbl, which leads to a dramatic accumulation of αPix. Depletion of αPix by shRNA markedly reduced the ability of the glioma cells to migrate and invade, whereas complementation of shRNA-insensitive αPix promoted it. These results indicate that c-Cbl negatively regulates αPix-mediated cell migration and invasion and the lack of c-Cbl in the C6 and A172 glioma cells is responsible for their malignant behavior.

1. Introduction

Migration and invasion are malignant behaviors of cancer cells [1]. Glioblastoma multiforme (GBM) is the most invasive and aggressive human brain tumor. 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 (RTKs), including EGFR and c-Met [2].

The Pix proteins (also called COOL or Arhgef) are guanine nucleotide exchange factors (GEF) that activate the Rho family of small GTPases. Of these, αPix (COOL-2) activates Cdc42 and Rac1 by exchanging GDP to GTP [3–6]. Upon ligand binding to integrin, αPix functions in the activation of Pak kinase by forming a complex with GIT and Pak at the focal adhesion [7]. αPix is also involved in cell spreading, direction of migration, angiogenesis, and dendrite outgrowth of fetal neurons [6,8–12]. Consistently, αPix could be detected only in highly motile cells, including immune cells, microvascular cells, and growing fetal neurons, unlike βPix (COOL-1) that is ubiquitously expressed in all human tissues [10,12,13]. Moreover, the protein level of αPix is markedly up-regulated in AGS gastric cancer cells and GBM patient tissues [14,15]. These findings suggest that up-regulation of αPix in glioma cells might be responsible for their malignant behavior. However, underlying mechanism(s) for αPix up-regulation in the cancer cells and tissues remained unknown.

c-Cbl, a RING type E3 ubiquitin ligase, down-regulates proteins that are related with cell growth and migration, such as paxillin, FAK, and EGFR [16–20]. Moreover, numerous mutations in c-Cbl have been found frequently in myelo-proliferative diseases [21–25], implicating the role of c-Cbl as a tumor suppressor. Significantly, βPix interacts with c-Cbl, and prevents c-Cbl-mediated down-regulation of EGFR, thus allowing EGF-mediated signal transduction [26–30]. However, it remains unknown whether αPix interacts with c-Cbl.

Here, we showed that c-Cbl also interacts with αPix and ubiquitinates it, but not βPix, for degradation by proteasome. Remarkably, the rat C6 and human A172 glioma cells were unable to express c-Cbl, resulting in stabilization and accumulation αPix and in turn in promotion of cell migration and invasion. These results indicate that the malignant behavior of C6 and A172 glioma cells is mediated by the lack of c-Cbl.

2. Materials and methods

2.1. Plasmids and antibodies

cDNAs for αPix and c-Cbl were inserted into pFlag-CMV2 or pcDNA-HisMax. All primers were purchased from Bioneer (Daejeon, Korea). shRNA vectors for c-Cbl was purchased from Open Bio-

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systems (#RHS4529). shαPix was cloned into pSilencer 3.1 vector (Invitrogen) using 5′-GAGGCTGGTGGGAAGGCACATTAAA-3′, which is identical for αPix in all of human, mouse, and rat.

Antibodies against Flag (Sigma), Xpress (Invitrogen), HA (Roche), c-Cbl (Santa Cruz), and tubulin (Santa Cruz) were used. Peroxidase-conjugated goat anti-rabbit and anti-mouse IgGs were purchased from Jackson ImmunoResearch Laboratories. To generate anti-αPix antibody, we constructed a vector that can produce GST fusion of a αPix-specific region (amino acids 56–83). The fusion protein was expressed in *Escherichia coli*, purified by using GSH-Sepharose resin, and injected to rabbits.

2.2. Cell culture and transfection

Cells were grown at 37 °C under atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 1% (v/v) antibiotic–antimycotic solution (Welgene) and 10% (v/v) fetal bovine serum (Gibco). HEK293T cell was transfected with appropriate vectors by using JetPEI (Polyplus), and C6 and A172 cells were done by electroporation (NEON, Invitrogen) according to the manufacturer's instructions.

2.3. Immunoprecipitation and pull-down analysis

Cell lysates were prepared in a lysis buffer consisting of 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 h at 4 °C and then with 30 μl of 50% slurry of protein A-Sepharose (Sigma) for the next 1 h. The resins were collected by centrifugation, boiled in SDS-sampling buffer, and subjected to immunoblot analysis.

2.4. Assay for ubiquitination

HA-ubiquitin and Flag- or HisMax-tagged αPix and c-Cbl were overexpressed in HEK293T cells. For detection of ubiquitinated αPix, cell lysates were subjected to immunoprecipitation with appropriate antibodies under denaturing conditions followed by immunoblot with anti-HA antibody.

2.5. Wound healing assay

C6 and A172 cells transfected with appropriate vectors were cultured in 5% CO_2 incubator at 37 °C until they became confluent. They were scratched with an autoclaved 200 μl pipette tip, washed twice with PBS to remove debris, incubated for 24 h, and photographed using a CCD camera-equipped microscope.

2.6. Cell invasion assay

Quantitative cell invasion assay was performed using 8-μm polycarbonate inserts (Merck Millipore) in 24-well cell culture plates as described by the manufacturer. The lower chamber was filled with DMEM containing 20% FBS and 1% antibiotic–antimycotic solution and the upper chamber was filled with serum-free media. Cells (5×10^4) were added to upper chamber, and incubated for 24 h at 37 °C. Non-invaded cells were removed from the upper surface of the membrane using cotton swab. The invaded cells were fixed and stained with Differential Quik Stain kit (Polysciences). The stained cells were photographed and the invasion was assessed by counting the number of stained cells in four randomly chosen fields per sample.

2.7. Semi-quantitative RT-PCR analysis

Total RNAs from cell lines were isolated using Trizol (Invitrogen). 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. The sequences of primers for PCR were: 5′-GAACCTATTCAGGCATGGGAAGGAGATGATATTA-3′ (forward) and 5′-CTGCTGATGGTCTAAGTGGAGGTGCAGGTCGTAG-3′ (reverse) for αPix, 5′-ATGGCCGGCAACGTGAAGAAGAGCTCTGGGGCCG-3′ (forward) and 5′- TTCCTTTTAGTTCTGCCAGCATGTGGCTGAAGAT-3′ (reverse) for c-Cbl, and 5′-ATGGATGATGATATCGCCGCGCTCGTCGTCGACA-3′ (forward) and 5′-CGTAGATGGGCACAGTGTGGGTGACCCCGTCACC-3′ (reverse) for β-actin.

3. Results

3.1. c-Cbl ubiquitinates and destabilizes αPix

c-Cbl is known to interact with βPix [27]. To determine whether c-Cbl also interacts with αPix, they were overexpressed in HEK293T cells. Immunoprecipitation analysis showed that c-Cbl binds not only to βPix but also to αPix (Fig. 1A). It has been shown that the Arg822 and Arg829 residues in the UBA domain of c-Cbl and the Trp43 and Trp44 residues in the SH domain of βPix are involved in their interaction [28]. To determine whether the corresponding Trp residues (Trp196 and Trp197) in αPix are also required for interaction with c-Cbl, the Trp residues in αPix and the Arg residues in c-Cbl were substituted with Lys and Ala, respectively. R822A/R829A could not interact with αPix and neither W196K/W197K could bind to c-Cbl (Supplementary Fig. S1), indicating that αPix and βPix share the same conserved sites for c-Cbl binding.

To determine whether c-Cbl is capable of ubiquitinating the Pix proteins, they were expressed in HEK293T cells with ubiquitin. Despite the finding that c-Cbl could interact with both αPix and βPix, it ubiquitinated αPix only (Fig. 1B). Furthermore, the expression of a c-Cbl-specific shRNA (shc-Cbl), but not a nonspecific shRNA (shNS), abrogated αPix ubiquitination (Fig. 1C). Knockdown of c-Cbl also led to a marked increase in the stability of αPix upon analysis by treatment with cycloheximide (Fig. 1D). These results indicate that c-Cbl serves as a αPix-specific ubiquitin E3 ligase.

3.2. αPix is up-regulated in C6 and A172 glioma cell lines

It has been reported that both the mRNA and protein levels of αPix are up-regulated in the brain tissues of GBM patients [14,31]. To determine whether αPix expression is also up-regulated in glioma cell lines, we compared the mRNA level of αPix in various cell lines. The expression of αPix mRNA was dramatically up-regulated in two glioma cell lines, rat C6 and human A172, but not in other human glioma cells or HEK293T cells (Fig. 2A). Consistently, the expression of αPix protein could be seen only in C6 and A172 cells. We then examined whether the mRNA and protein levels of c-Cbl might also be altered in the cell lines tested. Strikingly, c-Cbl protein could not be detected only in C6 and A172 cells, despite the finding that c-Cbl mRNA was expressed to a similar extent in all cell lines tested. These results suggest that the elevation of αPix protein level in C6 and A172 cells is due to the lack of c-Cbl protein.

We next examined whether ectopic expression of c-Cbl can destabilize endogenous αPix in the rat C6 cells. αPix ubiquitination was markedly promoted by the expression of c-Cbl, but not by that of its catalytically inactive mutant (C381A), of which the active site Cys381 was replaced by Ala (Fig. 2B). Furthermore, the expression of c-Cbl, but not C381A, dramatically reduced

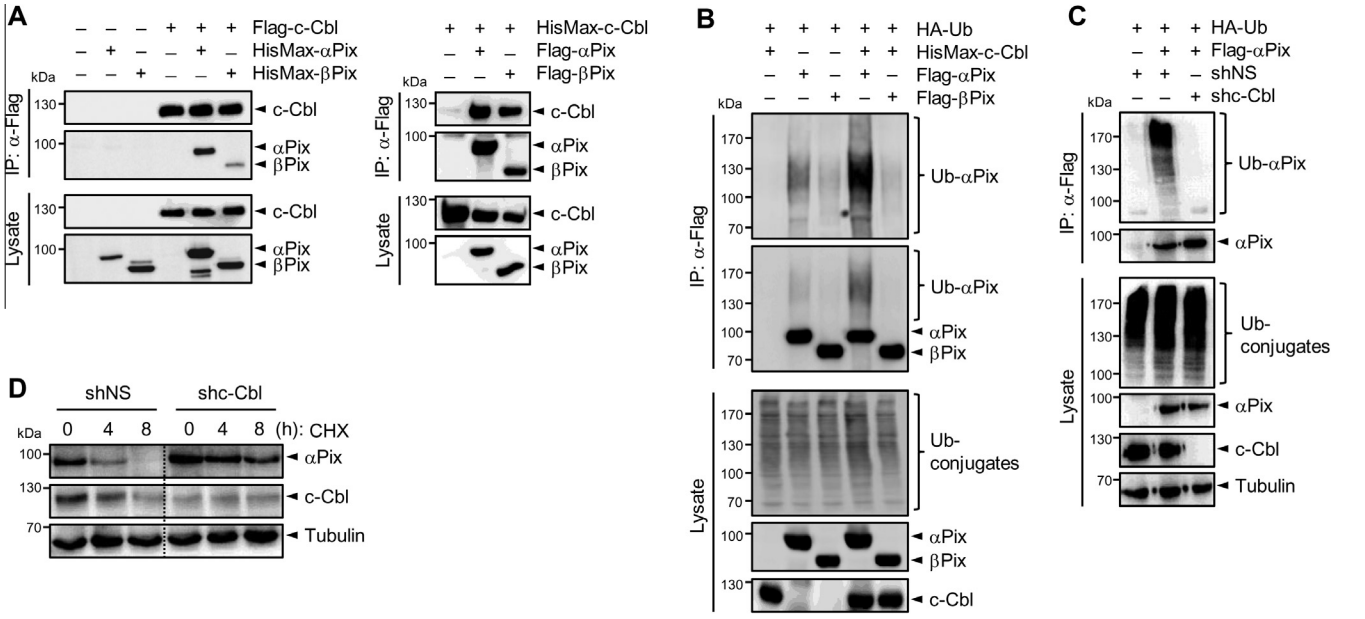

Fig. 1. c-Cbl ubiquitinates and destabilizes αPix. (A) Flag-c-Cbl was expressed in HEK293T cells with HisMax-tagged Pix proteins. Cell lysates were subjected to immunoprecipitation (IP) with anti-Flag antibody followed by immunoblot with anti-Flag and anti-Xpress antibodies (left panel). Reciprocal immunoprecipitation was performed by using HisMax-c-Cbl and Flag-tagged Pix proteins (right panel). (B) Flag-tagged Pix proteins were expressed in cells with HisMax-c-Cbl and HA-ubiquitin (HA-Ub). Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot with anti-HA and anti-Flag antibodies. (C) shc-Cbl or shNS was expressed in cells with Flag-αPix and HA-ubiquitin. Immunoprecipitation analysis was performed as in B. (D) shc-Cbl or shNS was expressed in cells with Flag-αPix. Cells were then subjected to incubation with 200 μg/ml of cycloheximide (CHX) followed by immunoblot with anti-Flag and anti-c-Cbl antibody.

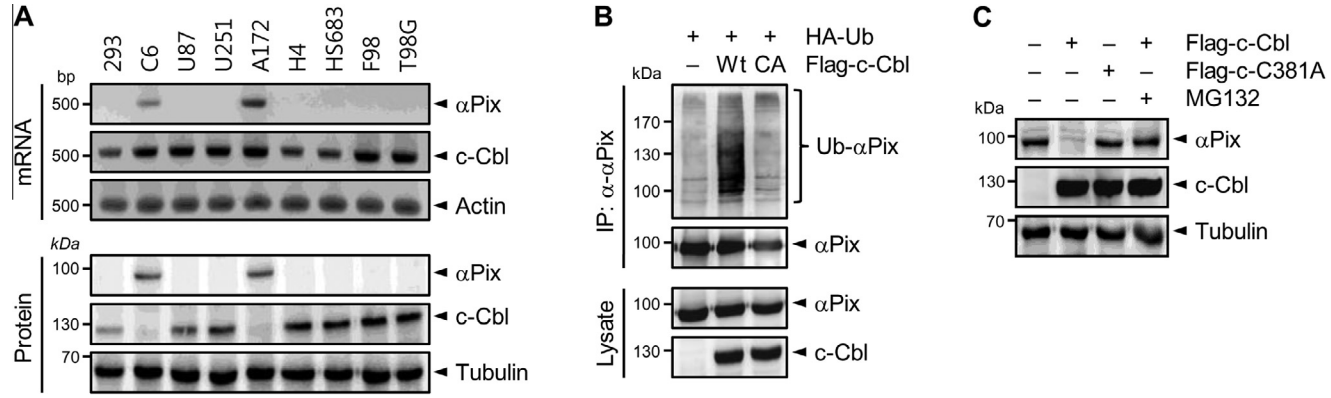

Fig. 2. Expression of αPix and c-Cbl in glioma cell lines. (A) The mRNA levels of αPix and c-Cbl in glioma cell lines were determined by semi-quantitative RT-PCR. Their protein levels in the cells were also determined by immunoblot with anti-αPix and anti-c-Cbl antibodies. 293 Indicates HEK293T cells. C6 and F98 are rat glioma cells and the others are human glioma cells. (B) Flag-tagged c-Cbl (Wt) and its C381A mutant (CA) were expressed in C6 cells with HA-ubiquitin. After incubation with 20 μM MG132 for 8 h, cells were subjected to immunoprecipitation with anti-αPix antibody followed by immunoblot with anti-HA antibody. (C) Flag-tagged c-Cbl and or its C381A mutant was expressed in C6 cells with or without 20 μM MG132. Cell lysates were then subjected to immunoblot analysis.

the level of αPix and this decrease could be prevented by treatment with MG132, a proteasome inhibitor (Fig. 2C), indicating that the ubiquitin–proteasome system is responsible for αPix degradation. Similar results were obtained with A172 cells (Supplementary Fig. S2). These results indicate that αPix up-regulation in C6 and A172 cells is due to the lack of its ubiquitin E3 ligase, c-Cbl.

3.3. c-Cbl blocks αPix-mediated cell migration and invasion

αPix is known to promote cell migration [31], a critical process required for malignant behavior of cancer cells, such as glioma and AGS gastric cancer cells. To determine whether the up-regulated αPix in C6 cells (see Fig. 2A) could indeed promote the cells' ability to migrate, wound-healing assay was performed. Expression of a αPix-specific shRNA (shαPix) markedly reduced the ability of C6 cells in migration as compared to that of shNS (Fig. 3A–C). Moreover, overexpression of c-Cbl, but not its inactive variant (C381A), reduced the cell migration concurrent with a decrease in αPix level (Fig. 3D–F), indicating that αPix is at least in part responsible for C6 cell migration.

We next examined whether αPix-mediated cell migration promotes the ability of C6 cells in invasion. Matri-gel analysis revealed that the invasive activity of C6 cells was markedly reduced by shαPix expression, but could be restored by co-expression of shRNA-insensitive αPix (Fig. 4A–C). Moreover, overexpression of c-Cbl, but not the C381A mutant, strongly inhibited the invasive activity. Similar results were obtained when A172 cells were used for cell migration and invasion assays (Supplementary Fig. S3). Collectively, these results strongly suggest that the malignant behavior of the glioma cells is mediated by up-regulated expression of αPix due to the lack of c-Cbl.

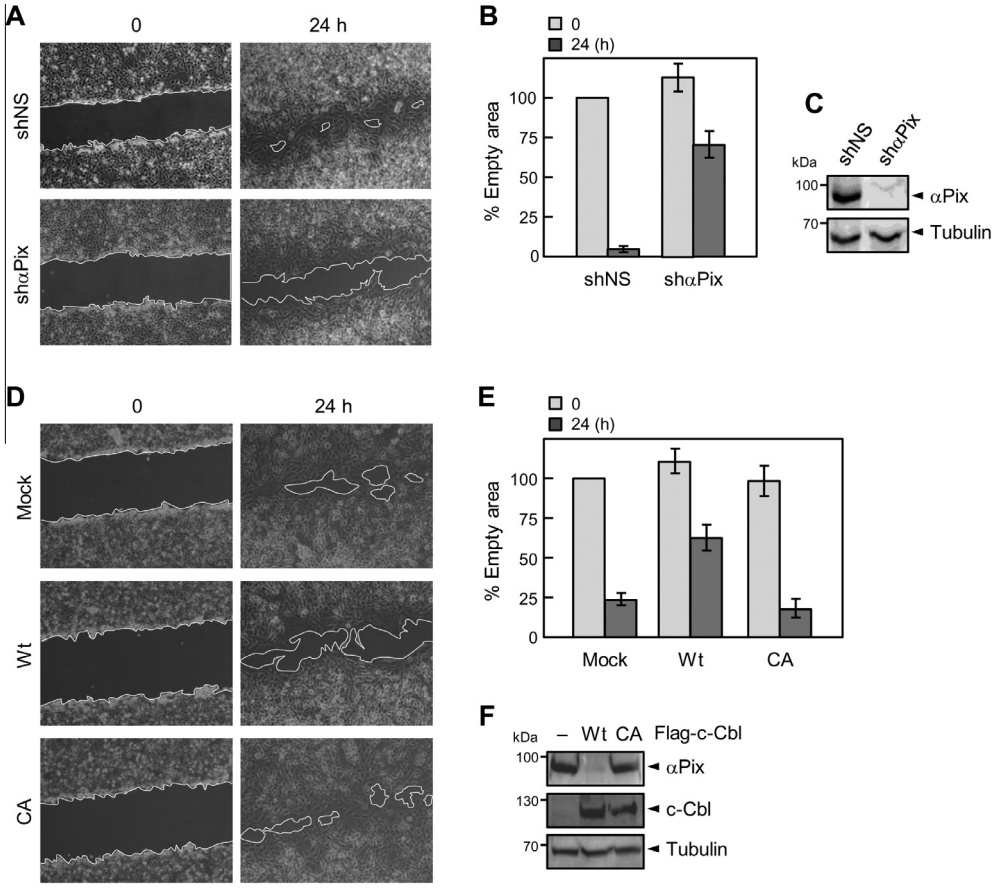

Fig. 3. c-Cbl blocks αPix-mediated migration of C6 cells. (A) Cells expressing shNS or shαPix were subjected to wound healing assay. (B) Empty areas in A were quantified by using "Image J" software. The area seen at '0' time with cells transfected with shNS was expressed as 100% and the others were as its relative values. (C) Cells used in A were subjected to immunoblot analysis to verify αPix knockdown. (D) Cells expressing c-Cbl (Wt) or its C381A mutant (CA) were subjected to wound healing assay. Mock indicates the cells transfected with an empty vector. (E) Empty areas were quantified as in B, but by setting the empty area of mock cells at '0' time as 100%. (F) Cells used in D were subjected to immunoblot analysis to verify the expression of αPix and c-Cbl proteins. Data are the mean ± s.d (n = 3).

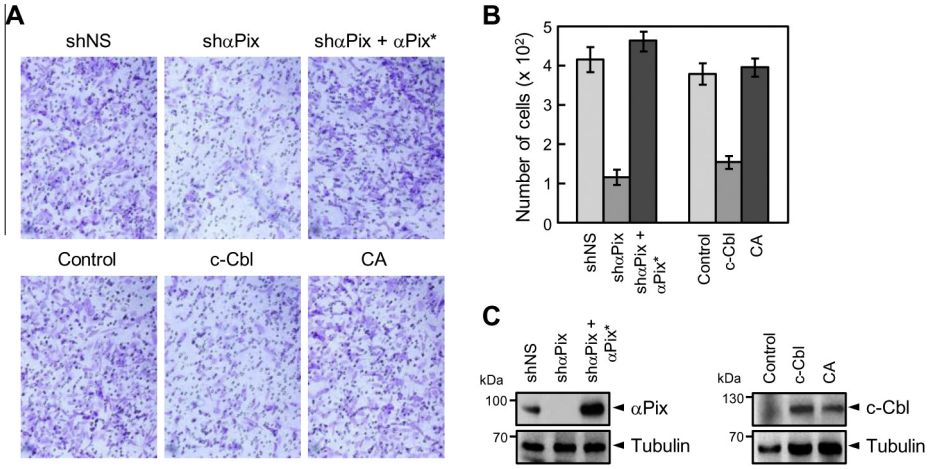

Fig. 4. c-Cbl blocks αPix-mediated invasion of C6 cells. (A) Cells expressing shNS or shαPix (upper panel) and Flag-tagged c-Cbl or its C381A mutant (CA) (lower panel) were subjected to cell invasion assay. αPix* indicates shRNA-insensitive αPix. (B) From the data of A, the number of cells in four independently chosen fields were counted and averaged. Data are the mean ± s.d. (n = 3). (C) Cells used in A were subjected to immunoblot analysis to verify the expression of proteins.

4. Discussion

In the present study, we demonstrated that c-Cbl serves as an ubiquitin E3 ligase for degradation of αPix. Intriguingly, the C6 and A172 glioma cells were found to be unable to express c-Cbl, which leads to a marked accumulation of αPix. The accumulated αPix promoted cell migration and invasion, which would provoke the malignant behavior of the cancer cells. As c-Cbl is known to down-regulate EGFR [32], the lack of c-Cbl in C6 and A172 cells would lead to sustained activation of the receptor

tyrosine kinase, which would in turn promote cell proliferation and tumorigenesis. Collectively, these findings demonstrate that c-Cbl plays a crucial role in down-regulation of αPix and the lack of c-Cbl in C6 and A172 cells is responsible for their malignant behavior.

Of note, however, is the finding that αPix mRNA was not expressed in the glioma cells tested in this study except C6 and A172, whereas both c-Cbl mRNA and c-Cbl protein were normally expressed. Thus, it is unlikely that the absence of αPix protein in those cells is due to c-Cbl-mediated destabilization. Interestingly, tissue transglutaminase (tTG) has been shown to up-regulate EGF signaling by preventing c-Cbl-mediated EGFR ubiquitination, and tTG is up-regulated in several glioma cells including U87 and T98G [33]. However, U87, U251, F98, and T98G cells lacking αPix are known to be highly invasive, although H4 and HS683 cells are derived from low-grade gliomas [34,35]. Thus, αPix-independent mechanism(s) appears to also operate in the malignant glioma cell lines that lack of αPix.

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 (mostly leading to elimination of a part or entire portion of exon-8), and primary transcript splicing mutations [21–25,36,37]. In addition, it has recently been shown that c-Cbl mutations also contribute to the pathogenesis of solid tumors [17]. Thus, it appears likely that the rat C6 and human A172 glioma cells harbor mutation(s) in the *c-Cbl* gene or the splicing machinery for its primary transcripts.

Conflict of interest

None of the authors of this work has any conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.129.

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