Protein phosphatase 2A Aα regulates Aβ protein expression and stability

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Protein phosphatase 2A (PP2A) represses many oncogenic signaling pathways and is an important tumor suppressor. PP2A comprises three distinct subunits and forms through a highly regulated biogenesis process, with the scaffolding A subunit existing as two highly related isoforms, Aα and Aβ. PP2A’s tumor-suppressive functions have been intensely studied, and PP2A inactivation has been shown to be a prerequisite for tumor formation. Interestingly, although partial loss of the Aα isoform is growth promoting, complete Aα loss has no transformative properties. Additionally, in cancer patients, Aα is found to be inactivated in a haplosufficient manner. Using both cellular and in vivo systems, colorectal and endometrial cancer cell lines, and biochemical and cellular assays, here we examined why the complete loss of Aα does not promote tumorigenesis. CRISPR/Cas9-mediated homozygous Aα deletion resulted in decreased colony formation and tumor growth across multiple cell lines. Protein expression analysis of PP2A family members revealed that the Aα deletion markedly up-regulates Aβ protein expression by increasing Aβ protein stability. Aβ knockdown in control and Aα knockout cell lines indicated that Aβ is necessary for cell survival in the Aα knockout cells. In the setting of Aα deficiency, co-immunoprecipitation analysis revealed increased binding of specific PP2A regulatory subunits to Aβ, and knockdown of these regulatory subunits restored colony-forming ability. Taken together, our results uncover a mechanism by which PP2A Aα regulates Aβ protein stability and activity and suggests why homozygous loss of Aα is rarely seen in cancer patients.

The serine/threonine protein phosphatase 2A (PP2A)2 is an important tumor suppressor protein which negatively regulates many oncogenic signaling pathways (1, 2). It is a heterotrimeric enzyme comprised of a scaffolding A subunit, catalytic C subunit, and one of several regulatory B subunits. The A subunit consists of two closely related isoforms, designated Aα and Aβ, which are 86% identical (3). The A subunit structure is made up of 15 tandem Huntingtin Elongation A subunit Tor (HEAT) repeats and the high sequence similarity between Aα and Aβ suggests that these two proteins have similar protein structures (3–5). However, the Aβ protein includes an N-terminal extension of 12 amino acids, which is not present in Aα (4) (Fig. S1). Both isoforms are targets of viral antigens that have been implicated in the initiation of cellular transformation. The Polyoma middle T viral antigen binds to both Aα and Aβ, whereas only Aα binds simian virus 40 (SV40) small T antigen, highlighting that there may be structural differences between the two isoforms (4, 6). Both Aα and Aβ have been identified to function as tumor suppressors, however the mechanisms of inactivation are unique to each isoform (6–8). Interestingly, whereas complete loss of Aβ results in transformation, Aα functions as a tumor suppressor in a haplosufficient manner. These data are reflective of what has been seen in large sequencing cohorts, including The Cancer Genome Atlas (TCGA). These studies have revealed that although the Aα isoform is altered in 35% of human cancers, homozygous deletions of Aα are exceedingly rare, occurring in only 0.3% of patient tumors (Fig. S2, A and B). In contrast, deletions of Aβ are more common, as the Aβ genePPP2R1B is located within the chromosomal region 11q23, a region commonly deleted in cancer (9–13).

To define the molecular basis for why homozygous Aα deletion appears to be unfavorable for cancer cell growth, we used a combination of biochemical and cellular assays to examine the functional ramifications of complete loss of the Aα subunit. CRISPR-Cas9 mediated homozygous deletion of the Aα subunit was growth suppressive across multiple cellular contexts. We examined the expression levels of various PP2A subunits in control and Aα-deficient cells and found that Aα loss lead to a robust increase in expression of the Aβ scaffold subunit as a result of increased Aβ protein stability. Knockdown of Aβ in the Aα knockout cells was lethal, suggesting that a minimum amount of PP2A activity is necessary for cell survival. Co-immunoprecipitation of Aβ protein in the presence and absence of Aα revealed that there was an increase in specific Aβ holoenzymes, including B56γ and PR130, upon Aα deletion. Modulating specific Aβ holoenzymes by knockdown of B56γ restored colony growth, indicating that B56γ-Aβ holoenzymes are at least partially responsible for the growth-suppressive effects of
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Aβ upon homozygous Aα loss. Together, these findings highlight why complete Aα loss is rarely seen in patients and reveals a unique mechanism through which Aα regulates Aβ protein levels and subsequent phosphatase activity.

Results

Knockout of PP2A Aα decreases colony formation

To eliminate PP2A Aα protein, we used CRISPR-Cas9 to create insertion and deletion mutations in exon 5, corresponding to HEAT 5 of the Aα protein (Fig. 1A; Fig. S3). The double nicking strategy using mutant Cas9n was utilized to limit off-target effects, and two separate knockout clones for each cell line (designated as KO.1 and KO.2) were used to verify the on-target specificity of our findings (14, 15). Colorectal cancer and endometrial cancer cell lines were chosen for these analyses because both heterozygous mutations as well as heterozygous loss of the Aα subunit are recurrent in these contexts, indicating that haploinsufficiency of Aα in these cancers may be a mechanism of PP2A inactivation (The Cancer Genome Atlas) (16, 17). Specifically, two colorectal cancer cell lines, SW620 and HCT-116, and two patient-derived endometrial cell lines, UT89 and UT150, were selected for the generation of

Figure 1. Knockout of PP2A Aα decreases colony formation. A, schematic of CRISPR sgRNA targeting design. The double nickase strategy utilizing Cas9n was used to increase specificity. The two guides target exon 5 of PPP2R1A. B, representative Western blotting of the PP2A A and C subunits in SW620 and HCT-116 (colorectal) control and two individual CRISPR clones; KO.1 and KO.2, cells. C, representative Western blotting of the PP2A A and C subunits in UT89 and UT150 (uterine) control and two individual CRISPR clones; KO.1 and KO.2, cells. D and E, quantification of the A and C subunits protein levels in SW620, HCT-116, UT89, and UT150 control and CRISPR KO cells. n = 3, error bars, mean ± S.D. (two-way ANOVA with Dunnett’s multiple comparisons test; p values: **** < 0.0001). F, representative images of colony formation assays in SW620, HCT-116, UT89, and UT150 control and CRISPR KO cells. n = 3. G, quantification of the colony formation assays. n = 3, error bars, mean ± S.D. (multiple t tests, control versus each KO; p values: * < 0.05, ** < 0.01).
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Aα knockouts. Knockout clones were first screened by DNA sequencing and subsequent Western blot analysis confirmed the knockout of Aα protein. Because of the high sequence homology between Aα and Aβ, an antibody targeting both isoforms was used for the detection of Aα. Knockout of Aα resulted in an almost complete loss of total A subunit protein expression across all cell lines analyzed, consistent with reports that the Aα isoform is the predominantly expressed isoform in most tissues (Fig. 1, B–E) (4). Consistent with previous literature, depletion of the Aα subunit also resulted in decreased levels of the PP2A C subunit expression (Fig. 1, B–E) (18, 19). To determine the effects of Aα subunit knockout on cell growth, clonogenic colony assays were performed. Interestingly, in these assays the loss of Aα subunit resulted in an approximate 20–30% decrease in colony formation across all cell lines and knockout clones analyzed (Fig. 1, F and G). Taken together, these findings suggest that although heterozygous Aα loss is tumor promoting, the complete loss of Aα is not growth promoting, but instead growth suppressive.

Loss of PP2A Aα results in altered protein expression of specific PP2A family members

To understand why knockout of the Aα subunit was growth suppressive, we investigated how the loss of Aα protein impacted other PP2A subunit family members. To do so, we performed Western blot analysis of regulatory subunits, including striatin, striatin 3, PR130, PR70, B55α, B56γ, and B56δ, as well as Aα’s closely related isoform Aβ (Fig. 2, A–D; Fig. S4). To analyze Aβ protein levels, an antibody directed toward the unique N-terminal region was used. Interestingly, the protein expression levels of only select regulatory subunit isoforms were affected by Aα knockout. Specifically, the expression of B55α and B56δ dramatically decreased upon Aα knockout, whereas other isoforms, including B56γ, PR130, and the striatin proteins were unaffected. Remarkably, although Aα and Aβ have been proposed to have distinct functions, the protein levels of Aβ increased ~5-fold across all Aα KO cell lines (Fig. 2, A–D). The increase in Aβ protein expression was not associated with changes in Aβ mRNA levels, as measured in SW620 and UT150 (Fig. S5), suggesting that the increase in Aβ expression occurred posttranscriptionally. Taken together, these results indicate that loss of the more abundantly expressed scaffold isoform, Aα, has widespread effects on PP2A subunit protein levels across all subunit families (scaffolding A, regulatory B, and catalytic C) and suggests that distinct PP2A subunits, in particular Aβ, may be directly dependent on the Aα scaffold for their stability.

Knockout of PP2A Aα decreases tumor growth and alters protein expression in vivo

To determine the effects of Aα depletion on tumor growth in vivo, we performed an SW620 xenograft model using control

![Figure 2. Knockout of PP2A Aα results in altered expression specific PP2A family members.](image-url)
and Aα KO.1 cells (Fig. 3). SW620 was selected for downstream analyses as it grows efficiently in vivo and is a well-characterized cell line, part of the NCI-60 panel. In this model, consistent with growth in vitro, the SW620 Aα KO.1 tumors grew significantly slower than the control tumors (Fig. 3A; Table S1). Subsequent analysis of PP2A subunit expression by Western blotting showed a trend consistent with what was observed in cell culture (Fig. 3B). Importantly, there was significantly less A subunit and C subunit protein in these tumors, confirming that the Aα subunit knockout was maintained (Fig. 3, B and C). Additionally, there was significant up-regulation of the Aβ subunit, consistent with data observed in cell culture models (Fig. 3, B). Finally, quantification of the regulatory subunits showed no significant changes in the striatin, PR70 family, or B56γ regulatory subunits consistent with in vitro analyses (Fig. 3, B and D). There was a trend of decreased B55α levels in the Aα knockout tumors, but this change did not reach statistical significance (Fig. 3, B and D). B56β levels could not be measured in vivo as the antibody did not detect adequate bands in any group (data not shown). In summary, these results showed that Aα knockout caused a reduction in tumor growth. Additionally, Aα knockout resulted in the up-regulation of Aβ and decrease of C subunit expression in vivo.

Knockout of PP2A Aα alters Aβ and C subunit protein stability

Based on the altered PP2A subunit expression upon Aα depletion, we characterized the effects of Aα expression on the stability of select PP2A subunits. To determine whether Aβ protein stability was altered in the absence of Aα protein, we treated control and knockout SW620 cells with cycloheximide and monitored Aβ protein expression by Western blotting over time (Fig. 4A). From these Western blot analyses, we graphed and calculated the Aβ protein half-life (Fig. 4, B and C). In control cells, the calculated half-life of Aβ was ~6 h. Conversely, in Aα knockout cells, the half-life of Aβ increased to ~23 h, suggesting that the increase in Aβ protein seen in Aα...
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A

SW620: Control
CHX (hrs) 0 6 12 24 36 48 60 72
Aβ
Vinculin

SW620: KO.1
CHX (hrs) 0 6 12 24 36 48 60 72
Aβ
Vinculin

B

SW620
Control
AaKO.1

0 1.0 0.5 0.0
Relative Level of Aβ

Time (hrs)
6 12 24 36 48 60 72

C

Cell Line Aβ Half-Life (Hours)
Control 6.2 ± 2.3
Aa KO.1 23.2 ± 4.0

D

SW620: Control
CHX (hrs) 0 0.5 1 3 6 12 24
Ca/β
Vinculin

SW620: KO.1
CHX (hrs) 0 0.5 1 3 6 12 24
Ca/β
Vinculin

E

SW620
Control
AaKO.1

0 1.0 0.5 0.0
Relative Level of Total C

Hours
6 12 18 24

F

Cell Line C Subunit Half-Life (Hours)
Control Not Calculated
Aa KO.1 2.2 ± 1.1

G

SW620: KO.1
Aβ
Ca/β
Vinculin

H

Aβ Levels (Relative to EGFP)

I

C Subunit Levels (Relative to EGFP)

J

SW620: Control
Control + - -

CHX (50 μg/mL) - + +

MG-132 (20 μM) - - +

Aβ
Vinculin

K

Relative Levels of Aβ (Normalized to Control)

L

SW620: AaKO.1
Control + - -

CHX (50 μg/mL) - + +

MG-132 (20 μM) - - +

Ca/β
Vinculin

M

Relative Levels of C Subunit (Normalized to Control)
KO cells was a result of increased protein stability (Fig. 4, A–C). We also examined whether C subunit stability was altered, based upon our observation of decreased C subunit protein expression in Aα knockout cells. Similarly, we treated control and knockout SW620 cells with cycloheximide and monitored C subunit protein levels by Western blotting over time (Fig. 4D). In control cells, the C subunit expression was only decreased 40% within 24 h, so a half-life could not be calculated (Fig. 4, E and F). Conversely, the calculated half-life of the C subunit in Aα knockout cells was ~2 h, suggesting that the stability of the C subunit may be partially dependent on the presence of the Aα subunit (Fig. 4, E and F). Additionally, B55α was also found to be less stable in the absence of Aα (Fig. S6). Re-expression of exogenous Aα protein restored Aβ and C subunit protein expression levels (Fig. 4, G–I). Finally, cotreatment of control SW620 cells with cycloheximide and MG-132, a proteasome inhibitor, rescued Aβ protein degradation, indicating that Aα regulation of Aβ protein stability is mediated through the proteasome (Fig. 4, J and K). Additionally, cotreatment of SW620 Aα knockout cells with cycloheximide and MG-132 rescued C subunit protein degradation, indicating that the Aα regulation of C subunit stability is also mediated through the proteasome (Fig. 4, L and M). Taken together, these data indicate that knockout of Aα alters the protein stability of both the Aβ and C subunit, suggesting that Aα may play a role in their regulation.

Aβ is essential for the survival of Aα knockout cells

The Aβ subunit of PP2A is an established tumor suppressor and complete knockout of Aβ leads to cellular transformation (6). To determine whether the baseline differences in growth observed in control and Aα knockout cells resulted from the up-regulation of Aβ and subsequent Aβ holoenzymes, we generated stable knockout cell lines with two different shRNAs targeting Aβ in the SW620 control and KO.1 cells (Fig. 5, A and B). Stable cell lines were generated using lentiviral transduction followed by selection in puromycin. Surprisingly, after selection, although all cell lines were resistant to puromycin, only the control cells had significant knockdown of Aβ, indicating that complete removal of all PP2A scaffolding subunit may not be tolerated in cells (Fig. 5, A and B). To determine whether the reduction of Aβ in Aα knockout cells impacted cell viability, we performed acute knockout of Aβ using two distinct sequence-specific siRNAs targeting Aβ in SW620 control and KO.1 cells (Fig. 5, C and D). Acute depletion of Aβ resulted in a significant decrease in Aβ protein expression in both the control and KO.1 cells (Fig. 5, C and D). However, there was a significant decrease in viability in only the Aα knockout cells upon knockdown of Aβ by siRNA as measured by MTT and Western blotting for cleaved PARP and cleaved caspase 3 (Fig. 5, E and F). Together, these data indicate that there is a minimum amount of scaffold required for cell survival and are suggestive that the up-regulation of Aβ, and the Aβ holoenzymes formed, allows for cell survival in the absence of the Aα subunit expression.

Knockdown of B56γ restores colony formation of Aα knockout cells

Homozygous deletion of Aα reduced tumor growth and was associated with up-regulation of Aβ, suggesting that increased activity of Aβ containing holoenzymes may be tumor suppressing in this context. To determine the tumor-suppressive Aβ containing holoenzymes, we performed co-immunoprecipitation experiments to identify which B subunits were interacting with Aβ. Using an Aβ-specific antibody, we immunoprecipitated Aβ and its binding partners in the presence and absence of Aα using control and Aα KO SW620 cells and measured the interactions of select PP2A subunits by immunoblotting (Fig. 6, A and B). To quantify interactions, the amount of each protein was first normalized to the amount of Aβ to control for the expression differences in Aβ between the two cell lines. After normalization, the resulting values were graphed relative to the SW620 controls to determine whether binding ratios were altered upon knockout of Aα (Fig. 6B). Through this analysis, it was determined that specific regulatory subunits, including PR130, B56δ, and B56γ, were more highly bound to Aβ in the absence of Aα, although the increase in PR130 binding did not reach statistical significance. Interestingly, the loss of regulatory subunits PR130 and B56γ have previously been implicated in cellular transformation (8, 20), leading us to hypothesize that the increased formation of holoenzymes containing these regulatory subunits may contribute to the growth-suppressive phenotype seen upon loss of Aα. Conversely, knockout of B56δ has been shown to be unfavorable to transformation and resulted in a further reduction of colony growth (8). To determine whether the colony growth phenotype depends on specific regulatory subunits, we generated stable knockout lines using specific shRNAs, focusing on the regulatory subunits previously implicated in cellular transformation, PR130 and B56γ, which also displayed increased binding to Aβ in the absence of Aα, and one regulatory subunit B55α, which displayed unaltered binding to Aβ upon Aα loss. A nontargeting shRNA con-
struct was used as a control (Fig. 6, C and D). Stable knockdown of B55α and B56γ was efficiently achieved in both control and Aα KO SW620 cells. However, stable knockdown of PR130 was only obtained in the control SW620 cells, paralleling the results seen with stable knockdown of Aβ, raising the possibility that PR130/Aβ holoenzymes are essential for cell viability in the
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A

| SW620 | IP: IgG | IP: Aβ |
|-------|--------|--------|
|       | C KO.1 | C KO.1 |
| Aβ    |        |        |
| C Subunit |      |        |
| PR130 |        |        |
| B55α  |        |        |
| B56δ  |        |        |
| B56γ  |        |        |
| Striatin |      |        |

B

Binding of Aβ Interacting Proteins (Relative to Control)

C

SW620

| shControl | shPR130 | shB55α | shB56γ |
|-----------|---------|--------|--------|
| Control   | Aα KO.1 |
| Aα/β      |         |        |
| Aβ        |         |        |
| PR130     |         |        |
| B55α      |         |        |
| B56δ      |         |        |
| B56γ      |         |        |
| Vinculin  |         |        |

D

Relative Protein Level (Normalized to Vinculin)

E

F

Colony Number (Relative to shControl)
absence of Aα. To determine the effects of the regulatory subunit knockdown on cell growth, clonogenic colony formation assays were performed (Fig. 6, E and F). Interestingly, the loss of each regulatory subunit resulted in similar growth changes in both the control and Aα KO cells; regulatory subunit knockdown resulted in either decreased (shB55α) or increased (shB56γ) colony growth. However, only the knockdown of B56γ in the Aα KO cells restored colony growth to baseline, suggesting that the B56γ/Aβ holoenzymes are critical for the growth-suppressive effects seen upon Aα loss (Fig. 6F). Taken together, these data show that there is an increase in the formation of specific Aβ-containing holoenzymes in the absence of Aα, and the increase in Aβ-B56γ holoenzymes is at least partially responsible for the decreased colony growth seen upon loss of Aα.

Discussion

The goal of this study was to elucidate the functional ramifications and potential clinical relevance of the complete loss of the PP2A Aα subunit. Here, we demonstrate that complete loss of Aα suppressed cell and tumor growth and describes a compensatory mechanism by which PP2A Aα regulates Aβ protein stability and activity, which may suggest why homozygous loss of Aα is rarely seen in patients.

Previous literature suggests that the Aα and Aβ scaffolding subunits have distinct functions within a cell (4, 6, 7). Specifically, in cell-based transformation assays, the exogenous overexpression of the Aα isofrom was unable to compensate for the loss of Aβ on transformation (6). However, our results indicate that there are some overlapping functions between the two scaffold subunits. In a transformed, tumorigenic cell, loss of Aα is tolerated because of a compensatory increase in Aβ protein. Subsequent removal of the Aβ caused a decrease in cell viability, indicating that the complete loss of PP2A scaffolding subunits is not tolerated. Thus, the overlapping functions of Aα and Aβ are likely essential functions of PP2A necessary for cellular survival, whereas the distinct actions of the two scaffolds may represent more specialized activities. The interplay between the two subunits is more complicated on a physiological level, as evidenced by Aα knockout mouse models. In mice, complete loss of Aα is embryonically lethal and total body inducible knockout of Aα in adult mice is also lethal, suggesting that Aβ is not able to completely compensate for Aα during development or in normal physiology (21, 22).

To date, there is very little known about what mechanisms regulate Aα and Aβ expression. Transcription factors responsible for controlling the expression of Aα and Aβ have been described for both isoforms (23, 24) and may help explain their differential expression levels and tissue distribution. Our results suggest that these proteins are also regulated posttranslationally. Here we show that genetic loss of Aα led to a compensatory up-regulation of Aβ through increased Aβ protein stability. When Aα is present, Aβ undergoes rapid degradation via the proteasome and loss of Aα greatly increased the half-life of the Aβ protein. Further exploration into the ubiquitination sites of both isoforms and the identification of the E3 ligases targeting these proteins will be critical in understanding the posttranslational mechanisms regulating the expression of the PP2A scaffolding subunits. Additionally, studies on proteasomal regulation of the scaffolding subunits may give further insight into tissue-specific expression differences that exist between the two scaffolds. Ubiquitination is a mechanism of regulation for other PP2A subunits, as proteasomal degradation contributes to the brain-specific expression of the B′β regulatory subunit (25).

Furthermore, we show that CRISPR/Cas9 mediated-knockout of Aα resulted in altered expression of not only the closely related Aβ isoform, but of other PP2A subunits, including the C subunit and B55α. The loss of C subunit expression upon loss of the Aα scaffold has been well-documented (18, 19). This finding has potential implications beyond PP2A, as similar mechanisms likely occur in other multimeric proteins, including other protein phosphatases, such as PP4 and PP6. Therefore, when altering protein expression with CRISPR/Cas9 careful characterization of not only the target protein but other subunit family members or interactors may be important in the interpretation of results obtained when using these methodologies. Interestingly, this may not apply to all regulatory subunits as depletion of B56α was shown to not affect expression of other subunits (26). Additionally, the decrease in C subunit and B55α subunit half-life upon Aα loss may indicate that these proteins rely on Aα for stability and could provide insight into how PP2A holoenzymes are stabilized.

Finally, we probed the Aβ interactome in the presence and absence of Aα to determine whether there were alterations in regulatory B subunit binding to Aβ in this context. Indeed, we determined that specific regulatory subunits had increased Aβ complex formation in the absence of Aα, which may indicate that there are affinity differences between PP2A subunits and the two A isoforms. Further, we examined the dependence of the growth-suppressive effect of Aβ up-regulation on the expression of these regulatory subunits and determined that the removal of B56γ restored colony growth in Aα knockout cells, suggesting that Aβ-B56γ holoenzymes display tumor-suppressive activity. Further exploration into the PP2A substrates responsible for the growth-suppressive effect of Aβ-B56γ holoenzymes will be critical for understanding the signaling pathways regulating Aβ-B56γ-dependent cell growth.

Figure 6. Knockdown of specific Aβ holoenzymes restores colony formation ability and tumor growth in Aα KO cells. A, representative immunoblot of lysate from co-immunoprecipitation of endogenous Aβ from SW620 control or Aα KO.1 cells. B, quantification of binding of PP2A subunits, normalized to Aβ. n = 3; error bars, mean ± S.D. C, representative immunoblot of SW620 control or Aα KO.1 stable cell lines expressing shRNAs directed at select B subunits. n = 3. D, quantification of PP2A B subunit levels in C, normalized to vinculin and graphed relative to shControl. n = 3, error bars, mean ± S.D. (two-way ANOVA with Dunnett’s multiple comparisons test, compared with shControl; p values: *** < 0.001, **** < 0.0001, all other values not significant). E, representative image from clonogenic assays of SW620 control or Aα KO.1 expressing the designated shRNA constructs. n = 3 (two technical replicates per biological replicate). F, quantification of the clonogenic assays in E. Colonies were counted using ImageJ and relative colony number was graphed relative to the SW620 control shControl cell line. n = 3; error bars, mean ± S.D. (two-way ANOVA with Dunnett’s multiple comparisons test, compared with control shControl; p values: * < 0.05, ** < 0.01, all other values not significant).
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In conclusion, our results demonstrate a novel mechanism by which PP2A Aα regulates Aβ protein and activity. The knockout of Aα and subsequent increase in Aβ results in decreased cell and tumor growth and the selective removal of B56γ in these cells ameliorates the growth-suppressive effects caused by Aα loss. Taken together, these data suggest that homozygous loss of Aα is rarely seen in patients because of an increase in tumor-suppressive Aβ holoenzyme activity.

**Experimental procedures**

**Cell lines and reagents**

SW620 and HCT-116 (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (Corning Mediatech, Inc., Manassas, VA) or McCoy’s 5A (Corning Mediatech) respectively. H293T (American Type Culture Collection), UT89, and UT150, obtained from Dr. Analisa DiFeo, were grown in DMEM (Corning Mediatech). All media were supplemented with 10% fetal bovine serum (VWR International, Avantor Performance Materials, Center Valley, PA) and 50 units/ml of penicillin-streptomycin solution (GE Healthcare). Avantor performance materials, Center Valley, PA) and 50 mM, and stored at 80 °C.

**Antibodies and immunoblot analysis**

Antibodies used in this study can be found in Table S2. Proteins from whole cells were lysed in RIPA buffer (Thermo Fisher Scientific). All lysis buffers were supplemented with protease and phosphatase inhibitors (Roche). Protein concentrations of cell extracts were determined by Pierce BCA Protein Assay kit (Thermo Fisher Scientific) and equal quantities of protein isolated from clones with deletions in exon 5 were analyzed by immunoblotting. Primary antibodies were detected with goat anti-mouse (Abcam, Cambridge, MA) or donkey anti-rabbit (GE Healthcare) conjugated to horseradish peroxidase using the Bio-Rad ChemiDoc XRS chemiluminescence imager. Densitometry quantification was performed within the Bio-Rad Image Lab software.

**Generation of CRISPR/Cas9-mediated knockout cell lines: sgRNA design and cloning**

The MIT CRISPR tool was used to design the pairs of sgRNAs against the gene coding for PP2A Aα (PP2P2R1A). A ~200-bp sequence within exon 5 of PPP2R1A was submitted to the CRISPR design tool for sgRNA design. The top and bottom strands of the sgRNA were purchased from Integrated DNA Technologies and cloned into PX461-pSpCas9n(BB)-2A-GFP (Addgene plasmid 48410), following the previously described protocol (15). The guide RNA sequences used to cleave PPP2R1A were as follows: Guide 1a, GCTGCGGCAGGGCCGCAACATG; Guide 1b, CAAGCTGGGGGAGTTCTGCGCAGGG (Fig. S3).

**Transfection and isolation of knockout clones**

Plasmids were transfected into the target cell lines using Lipofectamine 3000 (Thermo Fisher Scientific). 72 h post transfection, GFP-positive cells were sorted 1 cell/well into 96-well plates using FACS and incubated for ~3 weeks. At this time, each 96-well plate was split into two 48-well plates (one master plate and one replica plate).

**Genotyping and validation of isolated clones**

The replica plate was used to isolate genomic DNA using QuickExtract DNA reagent. Genomic DNA was amplified using PCR primers spanning exon 5 (forward: TACTTCCGGAACCTGTGCTC; reverse: CCAGGAAGCAAAA-CTCA-CCT) and sent for Sanger sequencing to identify deletions. Protein isolated from clones with deletions in exon 5 were analyzed for the presence of knockouts by immunoblotting.

**Clonogenic assays**

100 (SW620), 250 (UT89 and UT150), or 1000 (HCT-116) cells were plated in 6-well plates and grown for 10 days, changing the media every 3–4 days. Colonies were fixed in a solution of 10% acetic acid and 10% methanol, stained with crystal violet (1% crystal violet in methanol), and counted using ImageJ.

**Xenograft tumor formation**

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University. Animal use and care was in strict compliance with institutional guidelines and all experiments conformed to the relevant regulatory standards by Case Western Reserve University. 5 × 10⁶ SW620 cells or AαKO.1 SW620 cells were injected subcutaneously in 50% Matrigel into the right flanks of 6- to 8-week-old female Balb/c nu/nu mice. Tumor volume was assessed by caliper measurement every other day. Tumor tissue was both formalin-fixed and snap frozen in liquid nitrogen for immunoblot analysis.

**qPCR analysis**

RNA was isolated from cells (Roche, 11828665001) and cDNA was prepared (Roche, 5893151001). mRNA levels were determined using SYBR Green (Roche, 04887352001) and primers directed at control or target genes and measured on the Roche LightCycler 480. Actin was used as a reference gene (forward: CCCACACTGTGCCCATCTAC; reverse: GCACCTCA-CTTAATGGTACGC). Three independent primer sets specific to PPP2R2A (Aα) or PPP2R1B (Aβ) were used for this study.

Primer sequences are as follows: PPP2R2A, Set 1F: AAGGCGAGTGGAGTCCCTACG, Set 1R: AGGTTCGCGGAAGTACTGTGCG, Set 2F: GGGGGATTTGGCAAGGTG, Set 2R: TCTGGAACCTGTGCACTTCTT, Set 3F: ATGTCCCAGATGGAGTCCTTACG, Set 3R: TCGGGCCACTCATCTCCTCACG; PPP2R1B, Set 1F: GTGGTTGGTGCGACGTCTTCTG, Set 1R: CAGCTGGGTGTTGAATCTCTT, Set 2F: TTGTTGGTGCCAGCTTCTC, Set 2R: TTGAGTATATCCGAGTCTG, Set 3F: TTTCTAGAGTTTGTAGTG, Set 3R: GCCTGGCCATCATTTGAGTAT.
**Constructs and shRNAs**

Gateway V5-tagged lentiviral expression vector pLX304-PP2A-α was obtained by DNASU Plasmid Repository (HSCD00444402) deposited by The ORFeome Collaboration (27). pLX304-PP2A-α was sequence verified to be WT. pLX304-EGFP was created by Gateway cloning EGFP into pLX304 vector. After cloning all constructs were sequence verified by Sanger sequencing. shRNAs targeting αβ were obtained from Sigma’s Mission shRNAs (shβ.1: TRCN0000010710; shβ.2: TRCN0000010711). shRNAs targeting the regulatory B subunits (PR130, B55α, and B56γ) were a gift from Dr. Alejandro Gutierrez.

**Virus production and infection**

Lentiviruses were packaged in 293T cells using XtremeGENE transfection reagent (Sigma, 12260). Supernatant media containing virus were collected at 24–48 h, filtered using 0.45 μm syringe filters, and supplemented with 4 μg/ml polybrene (Santa Cruz, sc-134220). Cells were transduced for 24 h and cultured for 48 h before being selected with 16 μg/ml of blasticidin (SW620 and UT150) (InvivoGen, ant-bl-5b).

**siRNAs**

siRNAs targeting αβ were obtained from Sigma (siαβ.1: SASHLs01_000040968; siαβ.2: SASHLs01_000040970). Control siRNA was obtained from Sigma (cat no. SIC001). siRNA was transfected at 10 nM concentration using Sigma’s Mission siRNA transfection reagent (S1452) using the manufacturer’s protocol. Knockdown was measured by immunoblot at 72 h post transfection.

**Co-immunoprecipitation analysis**

Cell lines were plated to 70% confluency in 150-mm plates. After 24 h, cells were harvested and co-immunoprecipitation was performed per Dynabeads Co-Immunoprecipitation Kit protocol (Thermo Fisher, 14321D). Aβ antibody (Novus) was coupled at a concentration of 7 μg/mg of Dynabeads. Fresh conjugated beads were prepared for each biological replicate, three biological replicates were performed for each experiment.

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**References**

1. Sangodkar, J., Farrington, C. C., McClinch, K., Galsky, M. D., Kastrinsky, D. B., and Narla, G. (2016) All roads lead to PP2A: Exploiting the therapeutic potential of this phosphatase. *FEBS J* 283, 1004–1024 CrossRefMedline

2. Ruvolo, P. P. (2016) The broken “off” switch in cancer signaling: PP2A as a regulator of tumorigenesis, drug resistance, and immune surveillance. *BBA Clin.* 6, 87–99 CrossRefMedline

3. Hemmings, B. A., Adams-Pearson, C., Maurer, F., Müller, P., Goris, J., Merlevede, W., Hofsteenge, J., and Stone, S. R. (1990) α- and β-forms of the 65-kDa subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure. *Biochemistry* 29, 3166–3173 CrossRefMedline

4. Zhou, J., Pham, H. T., Ruediger, R., and Walter, G. (2003) Characterization of the α and αβ subunit isoforms of protein phosphatase 2A: Differences in expression, subunit interaction, and evolution. *Biochem. J.* 369, 387–398 CrossRefMedline

5. Grimaldi, A., Tam, X., Weiser, B., Karplus, M., and Kleckner, N. (2010) PR65, the HEAT-repeat scaffold of phosphatase PP2A, is an elastic connector that links force and catalysis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2467–2472 CrossRefMedline

6. Sablin, A. A., Chen, W., Arroyo, J. D., Corral, L., Hector, M., Bulmer, S. E., DeCaprio, J. A., and Hahn, W. C. (2007) The tumor suppressor PP2A αβ regulates the RalA GTPase. *Cell* 129, 969–982 CrossRefMedline

7. Sablin, A. A., and Hahn, W. C. (2007) The role of PP2A α subunits in tumor suppression. *Cell Adh. Migr.* 1, 140–141 CrossRefMedline

8. Sablin, A. A., Hector, M., Colpaert, N., and Hahn, W. C. (2010) Identification of PP2A complexes and pathways involved in cell transformation. *Cancer Res.* 70, 10474–10484 CrossRefMedline

9. Döhner, H., Stilgenbauer, S., James, M. R., Benner, A., Weilguni, T., Bentz, M., Fischer, K., Hunstein, W., and Lichter, P. (1997) 11q deletions identify a new subset of B-cell chronic lymphocytic leukemia characterized by extensive nodal involvement and inferior prognosis. *Blood* 89, 2516–2522 CrossRefMedline

10. Keldysh, P. L., Dragani, T. A., Fleischman, E. W., Konstantinova, L. N., Perevoschikov, A. G., Pierotti, M. A., Della Porta, G., and Kopnin, B. P. (1993) 11q deletions in human colorectal carcinomas: Cytogenetics and restriction fragment length polymorphism analysis. *Genes Chromosomes Cancer* 6, 45–50 CrossRefMedline

11. Petzmann, S., Ullmann, R., Halbwedl, I., and Popper, H. H. (2004) Analysis of chromosome-11 aberrations in pulmonary and gastrointestinal carcinoids: An array comparative genomic hybridization-based study. *Virchows Arch.* 445, 151–159 CrossRefMedline

12. Petzmann, S., Ullmann, R., Klemen, H., Renner, H., and Popper, H. H. (2001) Loss of heterozygosity on chromosome arm 11q in lung carcinoids. *Hum. Pathol.* 32, 333–338 CrossRefMedline

13. Walch, A. K., Zitzelsberger, H. F., Aubele, M. M., Matts, A. E., Bauchinger, M., Candisis, S., Prätzer, H. W., Werner, M., and Höfler, H. (1998) Typical and atypical carcinoid tumors of the lung are characterized by 11q deletions as detected by comparative genomic hybridization. *Am. J. Pathol.* 153, 1089–1098 CrossRefMedline

14. Ran, F. A., Hsu, P. D., Lin, C. Y., Gootenberg, J. S., Konermann, S., Trevino, A. E., Scott, D. A., Inoue, A., Matoba, S., Zhang, Y., and Zhang, F. (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154, 1380–1389 CrossRefMedline

15. Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. *Nature Protoc.* 8, 2281–2308 CrossRefMedline

16. Haesen, D., Abbasi Asbagh, L., Derua, R., Hubert, A., Schrauwen, S., Merlevede, W., Hofsteenge, J., and Stone, S. R. (1990) Loss of heterozygosity on chromosome arm 11q in lung carcinoids. *Human Pathol.* 21, 173–178 CrossRefMedline

17. Shih, I.-M., Panuganti, P. K., Kuo, K. T., Mao, T. L., Kuhn, E., Jones, S., Velculescu, V. E., Kurman, R. J., and Wang, T. L. (2011) Somatic mutations in ovarian and uterine carcinomas. *Am. J. Pathol.* 178, 1442–1447 CrossRefMedline

18. Xiao, G., Chan, L. N., Klemm, L., Braas, D., Chen, Z., Geng, H., Zhang, Q. C., Aghajanianrehaf, A., Cosgun, K. N., Sadas, T., Lee, J., Mirzapoiazza, T., Salgia, R., Ernst, T., Hochohaus, A., et al. (2018) B-cell-specific diversion of glucose carbon utilization reveals a unique vulnerability in B cell malignancies. *Cell* 173, 470–484.e14 CrossRefMedline

19. Strack, S., Cribs, J. T., and Gomez, L. (2004) Critical role for protein phosphatase 2A heterotrimers in mammalian cell survival. *J. Biol. Chem.* 279, 47732–47739 CrossRefMedline
PP2A Aα regulates Aβ protein expression and stability

20. Chen, W., Possemato, R., Campbell, K. T., Plattner, C. A., Pallas, D. C., and Hahn, W. C. (2004) Identification of specific PP2A complexes involved in human cell transformation. Cancer Cell 5, 127–136 CrossRef Medline

21. Ruediger, R., Ruiz, J., and Walter, G. (2011) Human cancer-associated mutations in the Aα subunit of protein phosphatase 2A increase lung cancer incidence in Aα knock-in and knockout mice. Mol. Cell. Biol. 31, 3832–3844 CrossRef Medline

22. Lange, L., Marks, M., Liu, J., Wittler, L., Bauer, H., Piehl, S., Bläss, G., Timmermann, B., and Herrmann, B. G. (2017) Patterning and gastrulation defects caused by the tα8 lethal are due to loss of Ppp2r1a. Biol. Open 6, 752–764 CrossRef Medline

23. Liu, J., Ji, W., Sun, S., Zhang, L., Chen, H. G., Mao, Y., Liu, L., Zhang, X., Gong, L., Deng, M., Chen, L., Han, W. J., Chen, P. C., Hu, W. F., Hu, X., et al. (2012) The PP2A-Aβ gene is regulated by multiple transcriptional factors including Ets-1, SP1/SP3, and RXRα/β. Curr. Mol. Med. 12, 982–994 CrossRef Medline

24. Chen, H. G., Han, W. J., Deng, M., Qin, J., Yuan, D., Liu, J. P., Xiao, L., Gong, L., Liang, S., Zhang, J., Liu, Y., and Li, D. W. (2009) Transcriptional regulation of PP2A-Aα is mediated by multiple factors including AP-2α, CREB, ETS-1, and SP-1. PloS One 4, e7019 CrossRef Medline

25. Oberg, E. A., Nifoussi, S. K., Gingras, A. C., and Strack, S. (2012) Selective proteasomal degradation of the Bβ subunit of protein phosphatase 2A by the E3 ubiquitin ligase adaptor Kelch-like 15. J. Biol. Chem. 287, 43378–43389 CrossRef Medline

26. Little, S. C., Curran, J., Makara, M. A., Kline, C. F., Ho, H. T., Xu, Z., Wu, X., Polina, I., Musa, H., Meadows, A. M., Carnes, C. A., Biesiadecki, B. J., Davis, J. P., Weisleder, N., Györke, S., Wehrens, X. H., Hund, T. J., and Mohler, P. J. (2015) Protein phosphatase 2A regulatory subunit B56α limits phosphatase activity in the heart. Sci. Signal. 8, ra72 CrossRef Medline

27. Wiemann, S., Hu, P. C. Y., Hunter, P., Harbers, M., Amiet, A., Bethel, G., Busse, M., Carninci, P., Dunham, I., Hao, T., Harper, J. W., Hayashizaki, Y., Heil, O., Hennig, S., Hotz-Wagenblatt, A., et al. (2016) The ORFeome Collaboration: A genome-scale human ORF-clone resource. Nat. Meth. 13, 191–192 CrossRef Medline