Cyclophilin B Supports Myc and Mutant p53-Dependent Survival of Glioblastoma Multiforme Cells

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Abstract

Glioblastoma multiforme is an aggressive, treatment-refractory type of brain tumor for which effective therapeutic targets remain important to identify. Here, we report that cyclophilin B (CypB), a prolyl isomerase residing in the endoplasmic reticulum (ER), provides an essential survival signal in glioblastoma multiforme cells. Analysis of gene expression databases revealed that CypB is upregulated in many cases of malignant glioma. We found that suppression of CypB reduced cell proliferation and survival in human glioblastoma multiforme cells in vitro and in vivo. We also found that treatment with small molecule inhibitors of cyclophilins, including the approved drug cyclosporine, greatly reduced the viability of glioblastoma multiforme cells. Mechanistically, depletion or pharmacologic inhibition of CypB caused hyperactivation of the oncogenic RAS–mitogen-activated protein kinase pathway, induction of cellular senescence signals, and death resulting from loss of MYC, mutant p53, Chk1, and Janus-activated kinase/STAT3 signaling. Elevated reactive oxygen species, ER expansion, and abnormal unfolded protein responses in CypB-depleted glioblastoma multiforme cells indicated that CypB alleviates oxidative and ER stresses and coordinates stress adaptation responses. Enhanced cell survival and sustained expression of multiple oncogenic proteins downstream of CypB may thus contribute to the poor outcome of glioblastoma multiforme tumors. Our findings link chaperone-mediated protein folding in the ER to mechanisms underlying oncogenic transformation, and they make CypB an attractive and immediately targetable molecule for glioblastoma multiforme therapy. Cancer Res; 74(2); 1–13. ©2013 AACR.

Introduction

Malignant gliomas have an extremely poor prognosis (1, 2), and require improved understanding of the molecular mechanisms supporting tumor cell survival. Cyclophilins, intracellular receptors for cyclosporin A (CsA; ref. 3), have peptidyl-prolyl isomerase activity, which accelerates the folding of proteins (4). The first cyclophilin identified, cyclophilin A, mediates the immunosuppressive effect of CsA by binding to calcineurin (5, 6). Cyclophilin B (CypB) is a highly related cyclophilin in the endoplasmic reticulum (ER; ref. 7) and the nucleus (8). Previous research suggested that CypB participates in multiple functions, including hepatitis virus replication (9), immunosuppression (10), chemotaxis (11), and prolactin signaling (12). To determine its role in vivo, we generated CypB knockout mice (13), and found that, other than moderate osteoporosis at older ages, CypB is not required for viability, and is well tolerated.

Recent studies revealed several signaling pathways frequently activated in glioblastoma multiforme: the receptor tyrosine kinase (RTK) pathway, the retinoblastoma pathway, and the p53 pathway (1). Most glioblastoma multiforme tumors have mutations in all three to enhance cell proliferation and survival, while allowing the tumor cells to evade cell-cycle arrest, senescence, and death. Discovering ways to modulate these survival signals will improve therapeutic approaches for glioblastoma multiforme. Because they are “druggable,” cyclophilins are considered good therapeutic targets. Recently, reports have implicated CypB in Stat3 activation and in generation of reactive oxygen species (ROS) in other cancer cells (14, 15). Here, we identified CypB, for the first time, as a key regulator of several signals that fuel oncogenesis in glioblastoma multiforme, including mutant p53, c-Myc (MYC), and Chk1. These findings offer proof-of-principle that CypB inhibitors may be effective as novel therapeutics for glioblastoma multiforme.

Materials and Methods

Cell culture and reagents

Glioblastoma multiforme cell lines, primary human glioblastoma multiforme xenograft cells (Mayo Brain Tumor Core), and human astrocytes (ScienCell Research) were grown...
in Dulbecco’s Modified Eagle Medium, 10% FBS, and penicillin–streptomycin. Xenografts using U251 cells in nude mice were performed as described previously (16). Brain-tumor xenografts were obtained from the Mayo Brain Tumor Core Facility. Cell lines were obtained from the American Type Culture Collection (ATCC), and their identities were verified by the ATCC (except for U251, which is no longer tested by the ATCC). Reagents were purchased from Sigma [cyclosporine, chloroquine, 10058-F4, H2O2, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), AICAR, Tocris (thapsigargin, tunicamycin, and eeyarestatin), Enzo (etoposide, daunorubicin), or Calbiochem (WP1066, Stattic, MG132, and N1-(β-D-Ribofuranosyl)-5-aminoimidazole-4-carboxamide (ALLN)]. CsA dimer and compound 41 were from the Mayo Chemical Core.

Short hairpin RNA–mediated silencing
TRC1.5 and TRC2 lentiviral short hairpin RNA (shRNA) vectors (Sigma) were prepared using the manufacturer’s instructions, and transduced into glioblastoma multiforme cells for 14 hours, followed by puromycin selection 24 hours later. Specific sequences are listed in Supplementary Materials and Methods.

Western blotting
Cell lysates were prepared at indicated times by lysis with 1% NP-40, and fractionated on SDS-PAGE. Western blotting was conducted as described (13), using the indicated antibodies (sources are listed in Supplementary Materials and Methods).

Gene expression analysis
TRIzol-isolated total RNA was transcribed into cDNA using polyT primers and amplified to generate biotin-labeled cRNA. Gene expression microarray used the illumina HumanHT-12v4 BeadChip, visualized by staining with Streptavidin-Cy3 after hybridization. Expression values were calculated using illumina GenomeStudio Data Analysis Software (Gene Expression Omnibus (GEO) accession number GSE50756). Real-time PCR reactions used total RNA, reverse transcribed into cDNA, and were run in triplicate. mRNA levels were normalized to Actin or Gapdh, expressed as ΔΔCt values.

Cell viability, cell death, and colony formation assays
Cells seeded in triplicate were cultured for the indicated times, and then directly counted using trypan blue exclusion for proliferation assays. Cell viability tests used AlamarBlue (Invitrogen) according to the manufacturer’s instructions. Cell death was determined 3 to 5 days after lentiviral transduction by fluorescence-activated cell sorting (FACS) analysis of propidium iodide (PI)/Annexin V–Cy5 staining. For colony formation, cells suspended in complete medium containing 0.3% agar deposited onto solidified 0.6% agar in 6-well plates were quantified for colonies after 17 days.

Senescence-associated β-galactosidase assay
Cells plated in triplicate at 10^4 cells per well in 6-well plates were assessed for senescence-associated β-galactosidase (SA-β-gal) activity after 4 days (SA-β-gal Kit; Cell Signaling Technology) following the manufacturer’s instructions. Percentages were assessed by counting at least 300 cells for each.

Nuclear factor of activated T cells activation assay
Nuclear factor of activated T cells (NFAT) activation was quantified by FACS analysis of a CD8 reporter under the control of a trimeric NFAT-binding site in Jurkat cells stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin in the presence or absence of cyclophilin inhibitors.

Protein disulfide isomerase staining
Cells were cultured on glass cover slides, fixed with 2.5% paraformaldehyde in PBS and permeabilized using 0.2% Triton X-100 in PBS. Cells were blocked with PBS containing 5% goat serum and stained with anti-protein disulfide isomerase (PDI; Stressgen, ID3).

Statistical analyses
Two-tailed Student t tests were used to measure the significance of a difference deviation between two means. Data are shown as mean ± SD, and significant difference is represented by *P < 0.05 (*).

Results
CypB is overexpressed in brain tumors
Given the potential that CypB regulates Stat3-dependent cell proliferation (15), we asked whether CypB was expressed in neoplasms. Oncomine database (www.oncomine.org) inspection revealed that CypB was upregulated in 98 of 398 analyses of tumors (*P < 0.05). The highest upregulation was in brain tumors, with 14 studies comparing normal tissue with glioblastoma multiforme, astrocytomas, or oligodendrogliomas (Fig. 1A and Supplementary Fig. S1A). Rembrandt Repository (http://rembrandt.nci.nih.gov) analysis indicated highest CypB expression in glioblastoma multiforme tumors compared with all others, whereas lowest levels were in nontumor brain (Supplementary Fig. S1B). Measurement of CypB abundance by Western blot analysis in primary human glioblastoma multiforme cells revealed high amounts of CypB compared with primary human astrocytes (Fig. 1B and Supplementary Fig. S1C).

Genetic ablation of CypB reduces glioma cell survival and proliferation
We depleted CypB in human glioblastoma multiforme cells using lentiviral-delivered shRNA. Knockdown of CypB significantly decreased the viability of glioblastoma multiforme cells (Fig. 1C, D, and G). Increased death in CypB-depleted U251 cells was confirmed by increased sub-G1 fractions (Fig. 1E and F) and Annexin V/PI staining (Fig. 1H). CypB knockdown also reduced anchorage-independent colony growth in soft agar (Fig. 1I).

Inhibition of CypB kills glioblastoma multiforme cells
CypB inhibition by CsA also reduced glioblastoma multiforme cell viability and proliferation (Fig. 1J). Immuno-suppression by CsA is an obstacle to its use as a cancer
Figure 1. Knockdown or inhibition of CypB reduces glioblastoma cell survival. A, increased expression of CypB in biopsy samples (gray) from patients with glioblastoma multiforme (GBM; \( P = 7.9 \times 10^{-15} \)), astrocytoma (\( P = 1 \times 10^{-12} \)), oligodendroglioma (\( P = 4.6 \times 10^{-8} \)), and another astrocytoma study (\( P = 2.5 \times 10^{-5} \)), compared with normal brain tissue (dark gray; www.Oncomine.org). B, increased CypB protein expression in primary human glioblastoma multiforme cells. C, cell death induced by CypB knockdown (shCypB) in U251 or T98G cells compared with control lentivirus (shCON). Bar, 200 μm. D, AlamarBlue assay of cell viability in control U251 or two different CypB-knockdown U251 cultures (D-5 after knockdown). E and F, CypB silencing increases sub-G1 fraction and PI positivity of U251 cells (D-3 or D-5). G, growth rates measured by counting control or CypB-knockdown U251 cells. H, cell death due to CypB knockdown detected by Annexin-V/PI staining (D-4). I, decreased anchorage-independent tumor growth in CypB-depleted T98G cells analyzed in a soft agar growth assay. J, cell death induced in U251 cells treated for 48 hours with cyclosporine (CsA), cyclosporine dimer (CsA dimer), or compound 41 (41). K, cyclophilin inhibitors decreased the survival of U251 cells, as measured by AlamarBlue assay. Error bars, ± SD; *, \( P < 0.05 \).
therapeutic, so we synthesized a nonimmunosuppressive derivative of CsA (CsA dimer), which cannot inhibit calcineurin, yet retains cyclophilin-binding activity (17). CsA dimer had equal effectiveness in killing U251 cells (Fig. 1J), yet was not immunosuppressive, indicating that its cytotoxic effect results from cyclophilin inhibition, rather than calcineurin inhibition (Supplementary Fig. S1D). Also, compound 41, an unrelated small molecule shown previously to inhibit cyclophilin (18), killed glioblastoma cells (Fig. 1J and Supplementary Fig. S1E), yet it demonstrated little immunosuppression (Supplementary Fig. S1D). These inhibitors also killed two medulloblastoma cell lines (Fig. 1K; Supplementary Fig. S1F–S1H).

**CypB regulates Stat3-mediated glioblastoma multiforme survival by supporting Jak2 expression**

Stat3 nuclear localization in myeloma was reported to be dependent upon CypB (15). We tested whether CypB knockdown similarly altered Stat3 nuclear accumulation in glioblastoma multiforme cells. Unexpectedly, knockdown of CypB instead strongly decreased Stat3 phosphorylation upon oncostatin-M (OSM) stimulation (Fig. 2A), without affecting cell surface expression of its receptor (Fig. 2B). CypB-knockdown T98G cells had reduced amounts of the Stat3-specific kinase Jak2 (Fig. 2C and D), potentially explaining decreased Stat3 phosphorylation. Jak2 mRNA was not reduced (rather increased) by CypB silencing (Fig. 2E and F), suggesting that CypB regulates Jak2 expression posttranscriptionally. Consistent with this, Stat3 inhibitors (19) increased cell death upon CypB knockdown, but had little effect on control glioblastoma multiforme cells (Fig. 2G and H). Combined treatment with CsA dimer and Stat3 inhibitors also caused synergistic killing of tumor cells (Fig. 2I).

**CypB suppresses generation of ROS**

CypB-depleted glioblastoma multiforme cells showed higher ROS levels than control cells (Fig. 2J, K, and L), and developed higher increases in ROS following H2O2 exposure (Fig. 2M), indicating reduced ability to handle oxidative stress. More CypB-knockdown glioblastoma multiforme cells died following H2O2 treatment (Fig. 2N), which was reversed by the antioxidant, N-acetyl cysteine (Fig. 2N).

**CypB loss induces cellular senescence**

Microarray analysis of RNA from control or CypB-knockdown U251 cells revealed approximately 130 genes affected more than 2-fold by CypB depletion (Fig. 3A; Supplementary Tables S1 and S2). CypB-knockdown cells had a 3.4-fold reduction in the transcript for uncoupling protein-2 (UCP2), which suppresses ROS production during oxidative phosphorylation (20). These microarray results were similar to expression profiles of oncogenic Ras-induced senescent cells (Fig. 3D; ref. 21), including increased levels of members of the senescence-associated secretome, such as IL-11, IL-6, IL-8, PLAU, and SERPINE1. Several of these microarray results were validated by real-time PCR (Fig. 2B and O) and Western blot analysis (Fig. 3C).

As suggested by these findings, although the majority of U251 cells died upon CypB knockdown, surviving cells showed microscopic changes characteristic of senescence, including large and flat morphology, binuclear cells, and massive vacuolization (Fig. 3E). Furthermore, CypB knockdown in U87 cells markedly increased SA-β-gal staining (Fig. 3H). Overexpression of oncogenic Ras (HRASV12) in glioblastoma multiforme cells evoked similar senescence-associated morphologic and molecular changes (Fig. 3F and G). Oncogenic Ras activates the mitogen-activated protein kinase/extracellular signal–regulated kinase (MAPK/ERK) cascades in senescent cells (21). CypB knockdown indeed increased Erk phosphorylation in U251 cells on days 3 and 4 following shRNA expression (Fig. 3I) and caused Ras activation (Fig. 3J). MEK inhibitor (U0126) treatment reduced this early hyperactivation of Erk and blocked cellular senescence in CypB-depleted glioblastoma multiforme cells (Supplementary Fig. S2). Ras hyperactivation causes suppression of its own downstream signals due to Ras-dependent induction of the negative feedback inhibitors Sprouty, RasGap120, and DUSP proteins (22, 23). Consistent with this, by day 5 after CypB depletion, phospho-Erk was reduced below the starting baseline (Fig. 3I). Furthermore, DUSP5 and Sprouty2 were among the top 35 upregulated genes in CypB-knockdown glioblastoma multiforme cells (Supplementary Table S1). CypB knockdown or inhibition also increased levels of the cell-cycle inhibitor p27/Kip1 (Fig. 3I, K, and L; ref. 24), and strongly reduced the p27-specific E3 ubiquitin ligase Skp2 (Fig. 3M). These results suggest that aberrant activation of the RAS–MAPK pathway occurs following CypB suppression, and induces cellular senescence in glioblastoma multiforme cells, possibly via increased p27.

**CypB regulates MYC stability**

We examined cells for known Ras targets previously implicated in cancer, such as MYC (25, 26). CypB depletion in glioblastoma multiforme cells reduced steady-state levels of MYC (Fig. 4A). Induction of MYC by OSM or AICAR (27) was also blocked by CypB knockdown (Fig. 4B and C). CypB regulation of MYC is posttranscriptional because its silencing did not reduce MYC mRNA (Fig. 4D).

Cyclophilin inhibitors also decreased MYC expression (Fig. 4E), which was reversed by the proteasome inhibitors MG132 and ALLN (Fig. 4F). Jak2 and phospho-Stat3 were similarly reduced in MYC-knockdown cells, suggesting that Stat3 activation is partially dependent upon MYC (Fig. 4G). Furthermore, MYC-knockdown cells demonstrated higher levels of ROS and decreased UCP2 mRNA (Fig. 4H and I), implicating MYC as a mediator of CypB-knockdown effects. MYC knockdown also blocked proliferation and survival of glioblastoma multiforme cells and induced morphologic changes similar to CypB knockdown (Fig. 4J and Supplementary Fig. S3D). The MYC inhibitor 10058-F4 (28) also reduced the survival and proliferation of glioblastoma multiforme cells, and reduced Jak2 expression and Stat3 phosphorylation (Fig. 4K and L). We conclude that CypB regulates MYC expression through enhancing its stability, an effect that is critical for glioblastoma multiforme cell viability.
Figure 2. CypB controls Jak2–Stat3 signals and ROS generation. A, control or CypB-knockdown U251 cells were treated with OSM for the indicated times and analyzed by Western blotting with the indicated antibodies (left). The results of phospho-Stat3 (pStat3) and total Stat3 Western blots were quantified using ImageJ. Relative intensity values of pStat3 to total Stat3 were plotted (right). B, normal cell surface expression of gp130 in CypB-depleted U251 cells, as measured by flow cytometry. C and D, CypB depletion reduced Jak2 protein abundance in T98G, U87, U138, and U373 glioblastoma multiforme cells. E and F, knockdown of CypB did not reduce Jak2 mRNA levels in U251 and T98G cells, measured by real-time PCR. G and H, decreased cell viability in CypB-depleted U251 cells in response to treatment with Stat3 inhibitors. Control or CypB-depleted U251 cells were treated with the indicated doses of Stat3 inhibitor, WP1066 (left), or Stattic (right) for 48 hours (G). U251 cells were treated with WP1066 (10 μmol/L) for 24 hours and cell viability was quantified by Annexin-V/PI staining (H). I, increased cell killing effects with the indicated compounds for 24 hours. Cell viability was observed by AlamarBlue assay. J–L, knockdown of CypB increased ROS generation in T98G (J), U251 (K), and U87 cells (L). Cellular ROS was measured using flow cytometry after staining with DCFH-DA (0.5 mmol/L for 30 minutes). M, CypB-depleted T98G cells had higher ROS production in response to H2O2 treatment. Cells were treated with H2O2 for 2 hours and cellular ROS was measured by DCFH-DA staining. N, CypB-depleted T98G cells showed increased sensitivity to oxidative stress-induced cell death. Cells were pretreated with 5 mmol/L of N-acetyl cysteine (NAC) or vehicle, and subsequently with H2O2 for 24 hours. Cellular viability was measured by PI stain exclusion. O, CypB silencing decreased UCP2 mRNA levels in U251 (left) or T98G (right) cells. Error bars, ± SD; *, P < 0.05.
CypB regulates p53 and Chk1 expression

ROS are regulated downstream of several transcription factors, including NRF2 and p53 (29). Although NRF2 levels were normal in CypB-depleted glioblastoma multiforme cells (data not shown), p53 expression was remarkably reduced (Fig. 5A, B, and D). Immortalized CypB−/− fibroblast cells also

Figure 3. CypB suppresses cellular senescence. A, representative gene expression changes in CypB-depleted U251 cells. B, increased IL-11 (left) or decreased glial fibrillary acidic protein (GFAP; right) mRNA levels in CypB-depleted U251 cells were confirmed by real-time PCR. C, increased SERPINE1 and decreased SERPINA3 expression was confirmed by Western blot analysis in CypB-depleted U251 cells. D, cross-match analysis between microarray results from CypB silencing and those from oncogenic H-Ras–activated senescent cells. E, morphologic changes in CypB-depleted U251 cells. CypB-knockdown cells had cytoplasmic vacuolization and a large flat appearance. Bar, 200 μm. F and G, morphologic features (F) and molecular changes (G) of oncogenic H-Ras–expressed glioblastoma multiforme cells. U251 cells were transduced with a lentivirus expressing oncogenic H-Ras (HRASV12) or control lentivirus (vector), and cell morphology was examined by microscopy (F). Ras expression, subsequent Erk activation, and expression changes in SERPINE1 and SERPINA3 were measured by Western blot analysis (G). Bar, 200 μm. H, increased positive staining of the SA-β-gal marker in CypB-silenced U87 cells. Bar, 200 μm. I, increased Erk activation and p27 accumulation in CypB-depleted U251 cells. Cells were transduced with shCypB (48 or 49) or control lentivirus (C), and analyzed at days 3, 4, and 5. J, increased Ras activation in CypB-depleted U251 cells. Active form of Ras (RAS-GTP) was pulled down with Raf-1 RBD (Millipore) and detected by Western blot analysis. K and L, increased expression of p27 in CypB-depleted T98G (K) or U87 cells (L). M, CsA treatment (20 μmol/L) reduced Skp2 expression in U251 cells. Error bars, ± SD; *P < 0.05.
Figure 4. CypB controls MYC stability. A, reduced MYC protein in U251 cells transduced with shCypB (48 or 49). B, MYC induction by OSM was reduced by CypB knockdown in U251 cells. C, control or CypB-depleted U251 cells were treated with AICAR or bafilomycin A1 (Baf-A1) for 12 hours. MYC levels were measured by Western blotting. D, CypB silencing did not reduce MYC mRNA levels, as measured by real-time PCR. E, pharmacologic inhibition of CypB reduced the levels of MYC, Jak2, and phospho-Stat3. T98G cells were treated with cyclophilin inhibitors (CsA, CsA dimer, or 41) for 24 hours. F, treatment with proteasome inhibitors (MG132 and ALLN for 4 hours) rescued MYC expression in CypB-silenced glioblastoma multiforme cells; chloroquine had no effect. G, MYC depletion reduced levels of Jak2 and phospho-Stat3 in U251 cells. H and I, increased levels of ROS and decreased UCP2 transcription in MYC-depleted T98G cells. ROS levels were monitored by DCF-DA staining (H) and UCP2 mRNA levels were measured by real-time PCR (I). J, shRNA-mediated MYC depletion reduced U251 cell survival. U251 cells were transduced with shMYC or control lentivirus and observed by phase microscopy. Bar, 200 μm. K and L, pharmacologic inhibition of MYC reduced JAK2 expression and Stat3 phosphorylation. U251 (K) or T98G cells (L) were treated with the MYC inhibitor, 10058-F4, for 24 hours. Error bars, ± SD; *, P < 0.05.
had decreased p53 (Fig. 5C). CsA, CsA dimer, and compound 41 also reduced p53 expression in glioblastoma multiforme cells (Fig. 5E).

CypB silencing decreased the amount of p53 transcript by approximately 5-fold (Fig. 5F). Mutant p53 expression in U251 cells was dependent upon endogenous MYC, because knockdown of MYC suppressed p53 expression (Fig. 5G and H). Mutant p53 was also suppressed by the knockdown of Stat3 (Fig. 5G and I).

In U87 and MCF-7 cells (with wild-type p53), CypB knockdown suppressed induction of p53 and its transcriptional target p21 by etoposide or daunorubicin (Fig. 5J; Supplementary Fig. S3A and S3B). Silencing of endogenous mutant p53 in glioblastoma multiforme cells reduced survival, repressed UCP2 expression, and induced ROS (Fig. 5K and L; Supplementary Fig. S3C).

Chk1 contributes to the DNA damage response and is induced by MYC (30, 31). CypB knockdown or inhibition reduced Chk1 expression (Fig. 5A, M, and N), as did knockdown of MYC or Stat3 by siRNA decreased mutant p53 expression in T98G or U251 cells. H and I, shRNA-mediated MYC (H) or Stat3 (I) silencing reduced mutant p53 expression in U251 cells. J, CypB silencing in U87 cells ablated (wild-type) p53 induction after treatment with DNA damaging drug, etoposide. K, increased ROS in p53-depleted U251 cells. L, p53 silencing reduced UCP2 mRNA levels in U251 (left) and T98G cells (right). M, reduced Chk1 expression in CypB-depleted glioblastoma multiforme cells. Cells were transduced with two different shCypB (48 and 49) or shCON lentiviruses. N, treatment with cyclophilin inhibitors (CsA, CsA dimer, or 41) reduced Chk1 expression in U251 cells. O, MYC silencing decreased Chk1 expression in U251 cells. P, MYC depletion did not decrease Chk1 mRNA levels. Q, reduced cell viability in response to 24-hour treatment with daunorubicin in CypB-depleted U251 cells. Error bars, ± SD; * , P < 0.05.
or inhibition of CypB in glioblastoma multiforme cells causes multiple deleterious effects due to loss of MYC, mutant p53, Chk1, and Janus-activated kinase (JAK)/STAT3 signaling.

**CypB loss causes ER stress and an abnormal unfolded protein response**

Because CypB chaperones some ER proteins (13), we asked whether CypB knockdown might impact cells through the unfolded protein response (UPR; ref. 32). ER tracker (Molecular Probes; 250 nmol/L for 30 minutes) staining showed that CypB-depleted cells have increased ER content (Fig. 6A and B). CypB silencing also induced a dot-like PDI staining pattern (Fig. 6C), similar to thapsigargin treatment (Fig. 6C; ref. 33), HRASV12 expression in glioblastoma multiforme cells also increased ER content (Fig. 6D) and induced similar PDI dots (Fig. 6E).

The ER stress sensor pancreatic endoplasmic reticulum kinase (PERK; ref. 32) was also increased in CypB-depleted cells (Fig. 6F), HRASV12-expressing cells (Fig. 6H and I), and by treatment with tunicamycin or thapsigargin (Fig. 6J). Induction of C/EBP homologous protein (CHOP), but not BiP, by thapsigargin or eeyarestatin was impaired following CypB knockdown (Fig. 6G).

CypB-knockdown cells were more easily killed by thapsigargin (Fig. 6K). Tunicamycin also increased susceptibility of glioblastoma multiforme cells to CsA dimer cytotoxicity (Fig. 6L). Calculation of the combination index and isobologram analysis indicated a true synergistic interaction of CsA dimer with thapsigargin or eeyarestatin was impaired following CypB knockdown (Fig. 6G).

**CypB targeting in primary human glioblastoma multiforme cells**

Knockdown or inhibition of CypB decreased the viability of primary human glioblastoma multiforme cells from freshly isolated human glioblastoma multiforme xenografts passaged in nude mice (Fig. 7A and D–G), and reduced Stat3 and Erk activation in response to OSM (Fig. 7B). Cyclophilin inhibitors reduced Chk1, mutant p53, and Skp2 expression (Fig. 7C). Inhibition of Chk1 killed both primary (22RG and 79RG) as well as temozolomide (TMZ)-resistant recurrent glioblastoma multiforme cells (22TMZ and 79TMZ; Fig. 7H and I).

Rembrandt database inspection revealed that overall survival was better in patients with glioma with lower CypB expression in their tumors (Fig. 7J), in part due to highest levels in glioblastoma multiformes, which have the worst prognosis. Among astrocytoma cases, higher CypB expression was associated with shorter overall survival (Supplementary Fig. S6). Also, patients whose tumors had deletions of PPIB gene encoding CypB did better than those with PPIB amplification \( P = 0.0492; \) Supplementary Fig. S7. To determine the effect of CypB inhibition on tumor cell growth in vivo, we implanted control- or CypB-depleted U251 cells into nude mice and measured the rates of tumor formation. Ablation of CypB strongly suppressed tumor formation in this xenograft model (Fig. 7K).

**Discussion**

Gene expression studies revealed that CypB is highly upregulated in malignancies, suggesting a widespread role in folding of ER proteins to reduce ER stress, a known problem for cancers (34, 35). Previous studies found that CypB may support the survival of transformed cells through suppression of ROS or by enhancing the nuclear localization of Stat3. However, the mechanisms underlying these effects were unclear. We report here that ablation of CypB expression in glioblastoma multiforme cells suppresses several canonical oncogenic signaling pathways, which cause the dramatic induction of cellular senescence and loss of tumor cell survival (Supplementary Fig. S8).

A key feature of CypB is its ability to sustain the expression of MYC (Fig. 4), which is essential for many cancer cells (26). We found that MYC induces Jak2 expression and, subsequently, STAT3 activation (Fig. 4G). MYC knockdown increased ROS in glioblastoma multiforme cells, thus explaining several of the CypB-dependent phenotypes (Fig. 4H). ROS generation downstream of CypB loss was likely due to reduced UCP2 (Fig. 2J–O). Although MYC was not known previously to regulate UCP2, we found that knockdown of MYC decreased UCP2 mRNA by 80% (Fig. 4I).

Chk1 loss downstream of CypB depletion (Fig. 5A and M) or inhibition (Fig. 5N) may also be mediated via MYC, because MYC knockdown similarly extinguished its expression (Fig. 5O). Chk1 has been proposed to be a potential target for therapy for MYC-driven lymphomas (36). Glioblastoma multiforme cells lacking CypB were significantly more sensitive to death caused by the DNA damage drug daunorubicin (Fig. 5Q).

Most importantly, knockdown of MYC recapitulated the dramatic killing of U251 cells that we observed following CypB knockdown (Fig. 4J). CypB knockdown affected MYC post-transcriptionally (Fig. 4D), and MYC protein was significantly rescued by proteasome inhibition (Fig. 4F). Clinically useful inhibitors of MYC have been difficult to develop, so our finding that the druggable protein CypB supplies a critical level of support for MYC in glioblastoma multiforme cells provides an attractive approach for targeting it therapeutically.

Mutant p53 drives malignancy, as shown here (Supplementary Fig. S3C) and elsewhere (37), and we found that its expression depends upon CypB. Although wild-type p53 and its target p21 also required CypB for induction after DNA damage, we note that U87 and primary glioblastoma multiforme cells that have wild-type p53 were effectively killed by CypB knockdown.

Gene expression pattern changes in CypB-knockdown cells were most consistent with a Ras-induced senescence signature (Fig. 3D; ref. 21), as further demonstrated by activated Ras expression in glioblastoma multiforme cells. Although knockdown of CypB caused a transient increase in Ras activation, not all of its effects could be induced by exogenous expression of activated Ras. In particular, destabilization of MYC protein was not seen in Ras-overexpressing cells, and we attribute this to an alternative effect of CypB loss.

CypB knockdown induced dilation of the ER and PDI aggregation, indicators of ER stress and altered redox status (33, 38). Although CypB suppression did not evoke BiP and CHOP induction, it did upregulate PERK. Alteration of UPR sensors in CypB-knockdown cells may underlie the defective UPR response to ER stress, as evidenced by reduced CHOP in...
Figure 6. Suppression of CypB induces abnormal ER stress. A, increased ER content in CypB-depleted U251 cells. ER content was measured using ER-tracker staining. Bar, 40 μmol/L. B, quantification of ER-tracker signal by flow cytometry in U251 (left) or U87 cells (right). C, increased PDI staining in ER stress-induced (thapsigargin-treated) or CypB-depleted U251 cells. Bar, 40 μmol/L. D and E, increased staining for ER tracker (D) or PDI (E) in HRASV12-transduced U251 cells. Bar, 40 μmol/L. F, increased expression of PERK in CypB-depleted U251 cells. G, ER stress-mediated CHOP induction was decreased in CypB-depleted glioblastoma multiforme cells. Control or CypB-depleted cells were treated with thapsigargin (4, 8, and 20 hours) or eeyarestatin I (ESI; 8 hours). Induction of ER chaperones, PERK, BiP, or CHOP, was measured by Western blotting. H, increased ROS levels in HRASV12-transduced U251 cells (HRASV12). I, activation of oncogenic Ras induced molecular changes similar to CypB silencing, including increased PERK expression and reduced levels of mutant p53, Skp2, and phospho-Stat3. J, ER stress suppressed Chk1 and mutant p53 expression and Stat3 activation. Cells were pretreated with 17-AAG, thapsigargin (Tg), tunicamycin (Tm), or MG132 (MG) and stimulated with OSM. K, reduced cell viability in response to ER stress in CypB-depleted cells. Control or CypB-depleted U251 cells (D-1) were treated with Tg for 48 hours. Cell viability was measured by AlamarBlue assay. L, combined treatments with CsA dimer and tunicamycin increased tumor cell killing effects in T98G cells, as determined by AlamarBlue assay. Bar, 200 μm. Error bars, ± SD; *, P < 0.05.
Figure 7. Targeting of CypB in primary human glioblastoma multiforme (GBM) cells. A, GBM22 cells were transduced with shCypB or control lentivirus. Bar, 40 μm. B, control or CypB-knockdown GBM43 cells (D-5) were treated with OSM for the indicated times, harvested, and analyzed by Western blotting with the phospho-Stat3 or phospho-Erk antibody. C, pharmacologic inhibition of CypB reduced levels of Chk1, mutant p53, and Skp2. GBM43 cells were treated with cyclophilin inhibitors (CsA, CsA dimer, or 41) for 24 hours. D–F, treatment with cyclophilin inhibitors decreased the survival of primary human glioblastoma multiforme cells. Cells were treated with indicated doses of CsA, CsA dimer, or 41 for 72 hours. Cell survival was measured using AlamarBlue assay in GBM43 cells (D), GBM6 cells (E), and GBM14 cells (F). G, glioblastoma multiforme cells were treated with vehicle or compound 41. Neurosphere formation was observed by microscopy. Bar, 200 μm. H and I, treatment with cyclophilin inhibitors decreased the viability and survival of both TMZ sensitive (22RG and 79RG) and resistant (22TMZ and 79TMZ) matched sets of primary human glioblastoma multiforme cells. Cells were treated with indicated doses of compound 41 for 72 hours. J, Kaplan–Meier curves showing that overall survival of patients with brain tumors having higher PPIB gene expression (≥2.0; 167 cases) was significantly lower than those with a gene expression of 2.0 or below (176 cases). Data were obtained from the Rembrandt database of the National Cancer Institute. K, shRNA-mediated CypB depletion inhibited in vivo tumor cell growth. U251 cells expressing shRNAs targeting CypB or control shRNAs were implanted into the flanks of nude mice. The resulting tumor sizes were visualized and tumor volumes quantified. Genetic ablation of CypB significantly reduced in vivo tumor formation. Error bars, ±SD; *, P < 0.05.
response to ER stress. Moreover, CypB knockdown rendered cells more vulnerable to ER stress-related death (Fig. 6K–L). CypB has a crucial role in ER protein quality control through the removal from the ER of ERAD-LS substrates (39), consistent with our finding of a dramatic defect in eeyarestatin-induced CHOP. Although our results suggest that CypB regulates ER stress and UPR signaling, further studies will be required to define the precise molecular mechanism of regulation of the UPR by CypB, which may involve its known interactions with other ER chaperones, including BiP, Grp94, calnexin, and calreticulin (14).

Cellular senescence is emerging as a fundamental mechanism of tumor suppression (40). Here, we showed that senescence is induced by disruption of the stress response machinery by targeting CypB. ER stress chaperones, including CypB, are apparently induced during glioblastoma multiforme development, and we suspect that the increased buffering capacity of the ER serves to counterbalance cellular stress in the transformed cells. CypB silencing leads to suppression of several inhibitors of senescence, including MYC and Skp2, as well as the hyperactivation of the oncogenic RAS–MAPK cascade. The fact that CypB silencing drives cellular senescence in the absence of p53 or p16 suggests that additional tumor-suppressive mechanisms may be involved. Could targeting of other ER chaperones induce similar pro-senescent effects? Several secreted proteins, including interleukins and PAI1, can contribute to senescence in different cell types (41). Because CypB silencing induces upregulation of these secreted proteins, evaluation of their impact on the senescence response to inhibition of CypB will be important to study in the future.

An additional impact of CypB silencing in glioblastoma multiforme was development of increased ROS and oxidative stress sensitivity. ROS are byproducts of protein oxidation in the ER. Because perturbation of ER stress chaperones induces oxidative stress, persistent ROS elevation, particularly in the context of a compromised ER stress response, may initiate a vicious cycle, leading to ER collapse and cell death (42). In addition to oxidative stress from the increased demand of protein folding in the ER, altered metabolism in rapidly proliferating cancer cells produces abnormally high levels of mitochondrial ROS. Cells typically relieve these effects of ROS by increasing production of cellular antioxidants, including reduced glutathione, thioredoxin, and NADPH. These antioxidant programs are activated by multiple intracellular signaling pathways, including MYC and mutant p53 (29). CypB likely constrains the mitochondrial redox state by controlling the transcription of UCP2, which is at least partially due to the maintained expression of MYC and mutant p53. We conclude that CypB regulates mitochondrial redox homeostasis in glioblastoma multiforme cells.

Our proof-of-principle study suggests that pharmacologic inhibition of CypB induces several tumor suppressive responses, including cell death and senescence in glioblastoma multiforme cells. Although CypB is ubiquitously expressed, because inhibition of CypB lacks serious systemic toxicity (13), it is attractive as a novel molecular target for therapy for glioblastoma multiforme. The elucidation of molecular mechanisms downstream of CypB, including modulation of p27, mutant p53, MYC, Chk1, ROS, and Stat3, further suggest combinatorial approaches that will have synergistic effects on tumor cell killing, as we found when testing Stat3 inhibitors with CypB-knockdown cells. However, all cyclophilin inhibitors tested in this study inhibit multiple isoforms of cyclophilin encoded by different genes. It should be considered that other closely related cyclophilins serve different functions in different cellular compartments and, more importantly, have distinct roles in cell proliferation, apoptosis, and necrosis (15, 43). We anticipate that the development of compounds that more specifically inhibit CypB will yield better treatment of multiple cancer types, including brain tumors such as glioblastoma multiforme.

Disclosure of Potential Conflicts of Interest

J.N. Sarkaria received commercial research grants from Merck, Basilea, Sanofi, and Genentech, and is a consultant/ advisory board member of Biomarin. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.W. Choi

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