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PRECLINICAL STUDY

CIB1 depletion impairs cell survival and tumor growth in triple-negative breast cancer

Justin L. Black[1] · J. Chuck Harrell[2] · Tina M. Leisner[1] · Melissa J. Fellmeth[1] · Samuel D. George[3] · Dominik Reinhold[4,5] · Nicole M. Baker[3,7] · Corbin D. Jones[5,6] · Channing J. Der[3,7] · Charles M. Perou[3,8,9] · Leslie V. Parise[1,3]

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Abstract Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype with generally poor prognosis and no available targeted therapies, highlighting a critical unmet need to identify and characterize novel therapeutic targets. We previously demonstrated that CIB1 is necessary for cancer cell survival and proliferation via regulation of two oncogenic signaling pathways, RAF–MEK–ERK and PI3K–AKT. Because these pathways are often upregulated in TNBC, we hypothesized that CIB1 may play a broader role in TNBC cell survival and tumor growth. Methods utilized include inducible RNAi depletion of CIB1 in vitro and in vivo, immunoblotting, clonogenic assay, flow cytometry, RNA-sequencing, bioinformatics analysis, and Kaplan–Meier survival analysis. CIB1 depletion resulted in significant cell death in 8 of 11 TNBC cell lines tested. Analysis of components related to PI3K–AKT and RAF–MEK–ERK signaling revealed that elevated AKT activation status and low PTEN expression were key predictors of sensitivity to CIB1 depletion. Furthermore, CIB1 knockdown caused dramatic shrinkage of MDA-MB-468 xenograft tumors in vivo. RNA sequence analysis also showed that CIB1 depletion in TNBC cells activates gene programs associated with decreased proliferation and increased cell death. CIB1 expression levels per se did not predict TNBC susceptibility to CIB1 depletion, and CIB1 mRNA expression levels did not associate with TNBC patient survival. Our data are consistent with the emerging theory of non-oncogene addiction, where a large subset of TNBCs depend on CIB1 for cell survival and tumor growth, independent of CIB1 expression levels. Our data establish CIB1 as a novel therapeutic target for TNBC.

Keywords AKT · CIB1 · ERK · Non-oncogene addiction · PTEN · Triple-negative breast cancer

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✉ Leslie V. Parise
leslie_parise@med.unc.edu

1 Department of Biochemistry and Biophysics, University of North Carolina, 120 Mason Farm Rd Ste 3010, Chapel Hill, NC 27599, USA

2 Department of Pathology, Virginia Commonwealth University, Richmond, VA, USA

3 Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

4 Department of Mathematics and Computer Science, Clark University, Worcester, MA, USA

5 Carolina Center for Genomic Sciences, University of North Carolina, Chapel Hill, NC, USA

6 Department of Biology, University of North Carolina, Chapel Hill, NC, USA

7 Department of Pharmacology, University of North Carolina, Chapel Hill, NC, USA

8 Department of Genetics, University of North Carolina, Chapel Hill, NC, USA

9 Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC, USA

Introduction

Breast cancer is diagnosed in over 230,000 people each year in the United States [1]. Approximately 16 % of all new breast cancer diagnoses are triple-negative breast

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cancer (TNBC), a subtype of breast cancer that lacks expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) [2]. Many breast cancer therapies target one of these three receptors and are therefore ineffective for the treatment of TNBC.

In breast cancer, and other cancers, cell survival and cell proliferation are driven by oncogenic signaling pathways. A majority of TNBC cases are basal-like, and typically exhibit constitutively activated RAF–MEK–ERK and PI3K–AKT signaling pathways [2, 3]. Dual inhibition of both ERK and AKT signaling pathways has been identified as a promising approach to treat TNBC [3, 4]. However, preclinical and clinical studies have suggested that combined inhibition of both PI3 K and MEK may improve efficacy at the expense of increased toxicity [5–7]. New targeted therapies with enhanced efficacy and safety are necessary to improve patient outcomes [8, 9].

CIB1 is a small intracellular protein that regulates kinase activity and integrin biology [10–16], and has an emerging role in cancer cell survival and proliferation via regulation of oncogenic signaling pathways [10, 12, 14, 17, 18]. For example, CIB1 promotes AKT and ERK activation [10, 19], and may regulate these pathways via interaction with the serine/threonine kinase PAK1 [11, 20]. We recently showed that CIB1 depletion in two cancer cell lines (SK-N-SH neuroblastoma and MDA-MB-468 TNBC) disrupted both AKT and ERK signaling, resulting in the induction of a DNA damage response and a unique mechanism of non-apoptotic cell death [10].

Because of our initial observation that CIB1 is essential for MDA-MB-468 TNBC growth and survival in vitro, we hypothesized that CIB1 may have a broader role in TNBC and in tumor growth in vivo. Here we present evidence that CIB1 is necessary for proliferation and survival in TNBC cell lines with elevated AKT activation and/or low PTEN expression. We further demonstrate that CIB1 depletion results in dramatic TNBC tumor shrinkage in vivo. To gain further insight into the effects of CIB1 depletion, we present RNA sequence (RNAseq) analysis revealing that CIB1 depletion induces genetic programs that correlate with decreased proliferation, survival, and cell differentiation. We show that high CIB1 expression is not associated with susceptibility to CIB1 depletion or with TNBC patient prognosis. Taken together, these findings are consistent with the emerging concept of non-oncogene addiction, where a subset of TNBCs appear to be reliant on a non-oncogenic protein, CIB1, for cell survival and tumor growth. Our results further suggest that CIB1 may be a novel target for TNBC therapy.

Results

CIB1 depletion induces cell death in a TNBC cell line panel

Recent reports have indicated that CIB1 promotes survival and proliferation in several cancer cell lines, including one TNBC cell line [10, 12, 17, 18]. We therefore screened a panel of eleven TNBC cell lines for their susceptibility to shRNA-mediated CIB1 depletion. We found that CIB1 depletion significantly increased cell death in eight of eleven cell lines tested (Fig. 1a). One cell line that showed only a moderate increase in cell death that was not statistically significant, HCC1143 (Fig. 1a, $P = 0.08$) did exhibit a significant decrease in proliferation rate (Supplementary Fig. S1A, $P < 0.003$). Ultimately, we observed some response in either cell viability, cell proliferation, or both, in nine out of eleven TNBC cell lines.

Pharmacological inhibition of both the ERK and AKT signaling pathways, but not either pathway alone, induces TNBC cell death [10, 21]. We previously showed that CIB1 depletion impaired both ERK and AKT activation, leading to significant cell death in MDA-MB-468 cells [10]. Therefore, we compared activated (phosphorylated) ERK (pERK) and AKT (pAKT) levels in CIB1-depleted versus control cells in the TNBC cell line panel (Fig. 1b). We first noted that CIB1 depletion resulted in decreased pERK and pAKT in most cell lines. Interestingly, we observed that CIB1 depletion increased cell death in all eight cell lines that have relatively high basal levels of pAKT. We observed elevated pERK in seven out of these eight cell lines, but also noted that pERK was elevated in two out of three cell lines that were insensitive to CIB1 depletion. Because the tumor suppressor PTEN is an upstream inhibitor of AKT activation and several of the cell lines from our TNBC panel have PTEN mutations (Supplementary Table 1), we also interrogated the PTEN status in each TNBC cell line. Interestingly, PTEN protein expression was absent or reduced in seven of eight cell lines that responded to CIB1 depletion (Fig. 1b), suggesting that PTEN status may be an additional predictor of responsiveness to CIB1 inhibition. These results suggest that pAKT and PTEN status, but not pERK, may be predictors of sensitivity to CIB1 depletion. To further explore differences between sensitive and insensitive cell lines, we examined gene expression microarray data [22] for each cell line in the panel. Using Significance Analysis of Microarrays, we identified two genes that were significantly (false discovery rate equal to zero) upregulated in cells that are insensitive to CIB1 depletion, NBEA (fold change +5.6) and FUT8 (fold change +4.9). As both of these genes are involved in cell differentiation, we

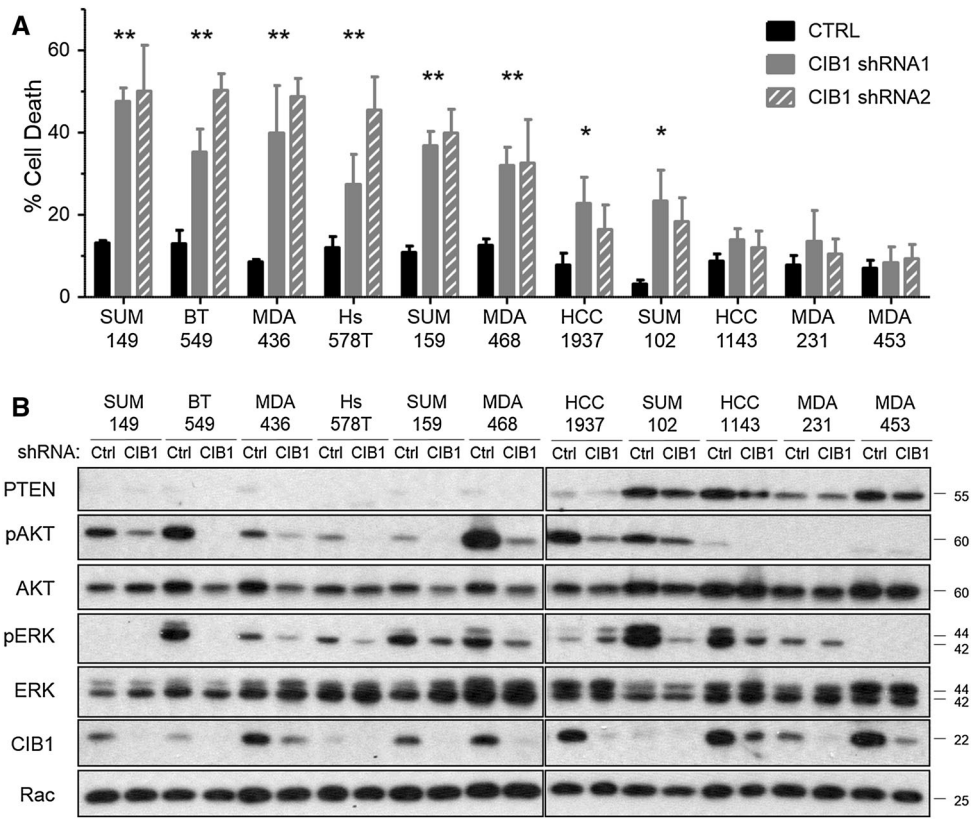


Fig. 1 CIB1 depletion induces cell death in a panel of TNBC cell lines**. a** A panel of 11 TNBC cell lines was transduced with either control (CTRL) or two separate CIB1 shRNA targeting sequences. Results are expressed as the mean percentage of dead cells (i.e., trypan blue positive cells) from both adherent and floating cell populations, data represent mean ± SEM from $n \geq 3$ experiments. P values were calculated using Student's t test. $^{**}P < 0.01$; $^{*}P < 0.05$. **b** Relative protein levels of PTEN, pAKT, AKT, pERK, ERK, CIB1, and Rac (additional loading control) in TNBC cell lines treated with CTRL or CIB1 shRNA as in (**a**). All membranes were processed under the same conditions. Blots are representative of three independent experiments

compared the average Differentiation Score [22, 23] of the sensitive and insensitive cell lines and found that cell lines that were not sensitive to CIB1 depletion trended toward a more differentiated state compared to the cell lines that were sensitive to CIB1 depletion (Supplementary Fig. S1B). Finally, we observed that CIB1 expression was variable in the TNBC cell line panel, and that there was no association between high CIB1 expression and sensitivity to CIB1 depletion. These results indicate that CIB1 inhibition may be a therapeutic approach to induce TNBC cell death regardless of CIB1 expression levels, particularly in cells with high basal levels of pAKT and/or low levels of PTEN.

To determine whether CIB1 depletion induces cell death in other breast cancer subtypes, we measured the effect of CIB1 depletion in three non-TNBC mammary cell lines: ZR-75-1 (Luminal A subtype); SKBR3 (HER2 overexpressing); and ME16C (non-cancerous mammary epithelial cell line). We observed a significant increase in cell death in CIB1-depleted ZR-75-1 cells (Supplemental Fig. S2). Consistent with our observations from the TNBC cell line panel, the ZR-75-1 cells are PTEN-null, whereas SKBR3 and ME16C are PTEN WT and do not exhibit increased cell death upon CIB1 depletion. These data suggest that, in addition to TNBC, CIB1 inhibition may be effective in additional PTEN-null breast cancers and other cancers.

CIB1 depletion from MDA-MB-468 TNBC cells decreases proliferation and increases cell death

Data presented here and elsewhere demonstrate that CIB1 depletion increased cell death in MDA-MB-468 (MDA-468) cells (Fig. 1) [10], but not in non-cancerous cells (Supplementary Fig. S2) [24, 25]. While these data suggest that CIB1 may be a promising target for TNBC therapy, we sought in vivo validation. We utilized a doxycycline-inducible shRNA system to regulate CIB1 expression in MDA-468 tumor xenografts. MDA-468 cells were engineered to express either CIB1 shRNA (MDA-468-CIB1shRNA) or control (scrambled) shRNA (MDA-468-SCRshRNA) in response to the antibiotic doxycycline (Dox). MDA-468-CIB1shRNA cells treated with doxycycline showed significant depletion of CIB1 by immunoblot (Supplementary Fig. S3C). Consistent with previous findings [10], CIB1 depletion decreased phosphorylation of ERK and AKT and increased phosphorylation of the DNA damage marker, γH2AX (Supplementary Fig. S3C).

Because treatment response in the 2D clonogenic survival assay in vitro typically agrees with tumor treatment response in vivo [26], we performed a 2D clonogenic assay to measure MDA-468-CIB1shRNA and MDA-468-SCRshRNA colony formation in 2D cell culture. CIB1 depletion in MDA-468 cells (MDA-468-CIB1shRNA + Dox) resulted in a complete

loss in the ability to form colonies (Fig. 2a). Importantly, doxycycline treatment of control cells (MDA-468-SCRshRNA + Dox) had no effect on colony formation ability. We next measured the effect of CIB1 depletion on MDA-468 cell proliferation and survival in culture. CIB1 depletion resulted in arrested proliferation and an ~12-fold increase in cell death (Supplementary Fig. S3A). To better quantify the cell death induced by CIB1 depletion, we performed flow cytometry to measure phosphatidylserine (PS) cell surface expression via Annexin V staining and cell permeability to 7-AAD. The majority of CIB1 depleted cells were in either early (Annexin V positive—22.6 %) or late (Annexin V positive and 7-AAD positive—37.3 %) stages of cell death (Supplementary Fig. S3B). Thus, in cell culture, conditional shRNA knockdown of CIB1 recapitulates the effects of CIB1 depletion using conventional shRNA knockdown.

CIB1 is required for MDA-MB-468 xenograft tumor growth

To test whether CIB1 was necessary for TNBC tumor growth and survival in vivo, we used a xenograft model and injected MDA-468-CIB1shRNA and MDA-468-SCRshRNA cells subcutaneously into the flanks of immunocompromised mice. Once tumors reached a volume of approximately 100 mm^3, mice were randomized into groups receiving sucrose, or sucrose plus doxycycline, and tumor volume was monitored for 5 weeks. We observed a rapid arrest of tumor growth followed by a drastic decrease in tumor volume in CIB1-depleted tumors (Fig. 2b). In contrast, control tumors continued to grow steadily throughout the treatment period. After 5 weeks, CIB1-depleted tumors were not visible compared to control tumors, which were visibly bulging from the flanks of the mice. Upon completion of the study, tumors were resected and weighed. The average mass of CIB1-depleted tumors was significantly smaller than control tumors (Fig. 2c).

To better understand how CIB1 depletion affects TNBC tumors, resected xenograft tumors were fixed, stained, and analyzed by microscopy. Histological analysis revealed that CIB1-depleted tumors had relatively few remaining cells and were composed mostly of non-cellular tissue (pink), whereas control tumors were composed of densely packed cells (blue) (Fig. 2d). Because CIB1 is essential for

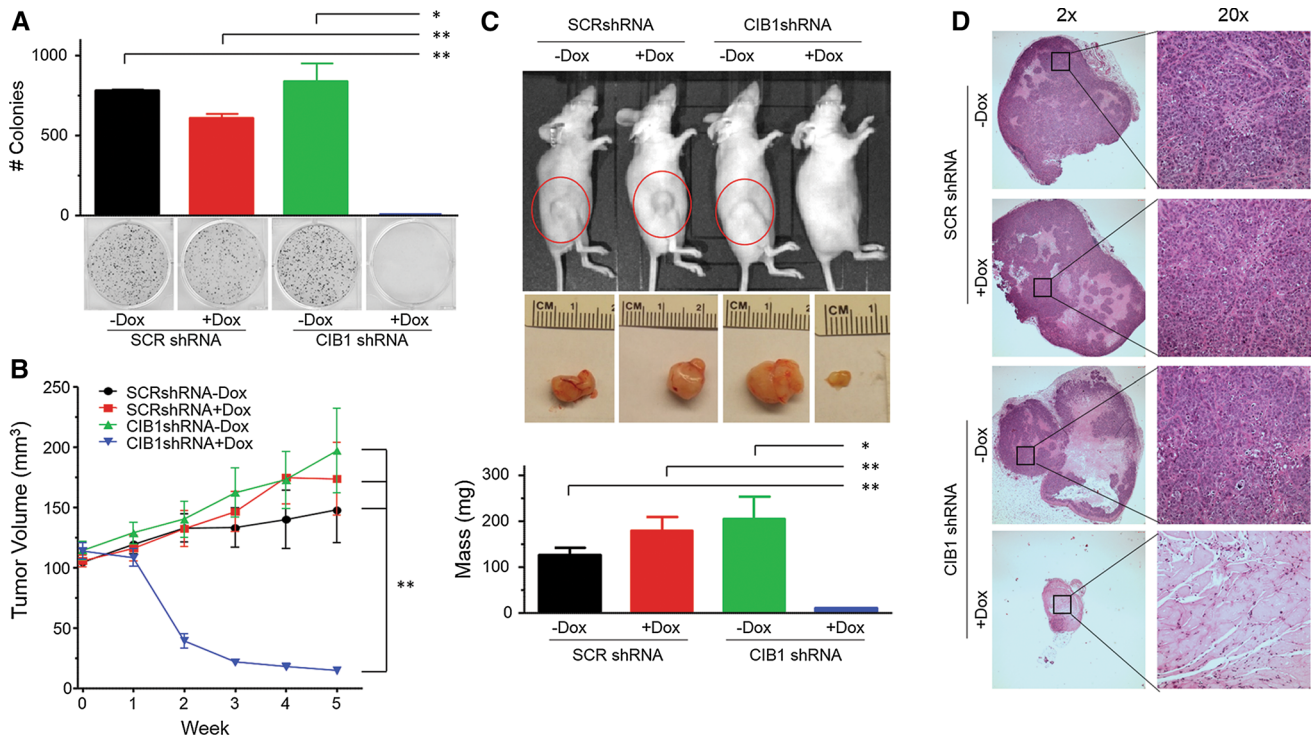


Fig. 2 CIB1 depletion shrinks TNBC tumors in vivo. MDA-468 cells were engineered to stably express doxycycline (Dox)-inducible CIB1 shRNA (MDA-468-CIB1shRNA) or scrambled shRNA (MDA-468-SCRshRNA). **a** CIB1 depletion in MDA-468 cells results in complete loss of cell proliferation and colony formation in a 2D clonogenic assay. Data represent mean ± SEM, $n = 3$. $^{**}P < 0.005$, $^{*}P < 0.01$. **b** MDA-468 xenograft studies. Graph represents average tumor volume ± SEM. $N = 8$ mice per treatment group. P values were calculated by Student's t test for the average final tumor volume $^{**}P < 0.005$. **c** Representative images show tumors bulging from the flanks of control mice, but not CIB1shRNA + Dox mice (*upper panel*). After 5 weeks, mice were sacrificed and resected tumors were imaged (*middle panel*) and weighed (*lower panel*). Data represent average mass ± SEM ($n = 8$) $^{**}P < 0.005$, $^{*}P < 0.01$. **d** Representative images of H&E stained tumor sections show that CIB1-depleted tumors are less dense than control tumors. Pink (eosin)—non-cellular tissue. Blue (hematoxylin)—cell nuclei

maintaining double-strand break repair in TNBC cells [10, 27], we asked whether CIB1-depleted TNBC tumors exhibited increased TUNEL staining, which detects dead or dying cells by labeling DNA double-strand breaks. Images of TUNEL-stained sections revealed that more of the remaining CIB1-depleted cells were TUNEL-positive compared to control tumors (Supplementary Fig. S3E). Finally, a portion of each tumor was lysed for analysis by immunoblotting. Consistent with CIB1 depletion in vitro, CIB1-depleted tumors had lower CIB1 expression, and decreased pERK and pAKT levels compared to control tumors (Supplementary Fig. S3D). This initial examination of the role of CIB1 in tumor growth in vivo suggests that CIB1 inhibition may be an effective therapeutic strategy for the treatment of TNBC tumors.

PAK1 activation partially rescues cells from CIB1 depletion

CIB1 binds and activates PAK1 [11], and we previously hypothesized that the role of CIB1 in promoting AKT and ERK activation was mediated by PAK1 [10]. To test whether PAK1 activation could rescue cells from CIB1 depletion-induced cell death, we overexpressed constitutively active PAK1 (caPAK1) in MDA-468 cells, then knocked down CIB1 and measured cell death. We observed that expression of caPAK1 resulted in a partial rescue of cell death in response to CIB1 depletion (Supplementary Fig. S4). These data suggest that CIB1-PAK1 binding is not exclusively responsible for CIB1-dependent cell survival, and that additional factors may contribute to CIB1 signaling to promote survival and proliferation.

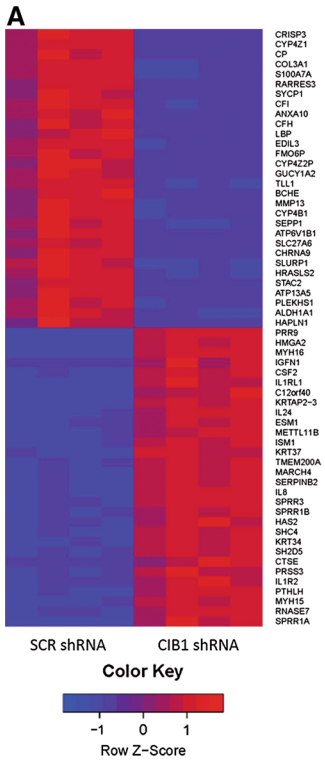
CIB1 depletion induces genetic programs that reduce proliferation and survival

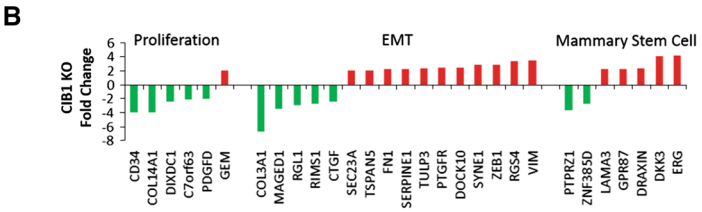
Because CIB1 depletion induces cell death by a unique, non-apoptotic mechanism that is only partially understood [10], we measured global changes in gene expression by RNAseq analysis to gain additional mechanistic insight into the effects of CIB1 depletion. Total mRNA was isolated from *viable* control and CIB1-depleted MDA-468 cells <96 h after shRNA induction, since extended CIB1 depletion induces nearly complete MDA-468 cell death (Supplementary Fig. S3A). RNAseq analysis identified 812 genes that showed significant differential expression after CIB1 depletion (Fig. 3a; Supplementary Table 2). Because sensitivity to CIB1 depletion in the TNBC cell line panel was associated with cellular differentiation, as measured with the Differentiation Score (see Supplementary Fig. S1B), we asked whether CIB1 depletion-induced changes in gene expression were associated with genes involved in cell differentiation. We compared the CIB1 depletion-induced differentially expressed genes (CIB1 KD gene signature) to 10,508 known gene signatures (from public databases, such as GSEA and also from manual curation). Interestingly, several gene signatures that had strong Pearson correlation values with the CIB1 KD gene signature were prominent in genetic programs that mediate differentiation and cancer stem cell function (Supplementary Table 3). For example, we observed an increase in 5 out of 7 genes from a mammary stem cell gene signature [28] and an increase in 11 out of 16 genes from an epithelial to mesenchymal transition (EMT) gene signature [29] (Fig. 3b). We also observed a decrease in 5 out of 6 genes from a breast cancer proliferation gene signature [30]. These results support previous observations that CIB1 depletion correlates with decreased cell proliferation, and indicate that CIB1 depletion also activates genetic programs consistent with mammary stem cells and EMT. Interestingly, we observed nearly complete cell death in MDA-468 cells after extended CIB1 depletion (Supplementary Fig. S3A), suggesting that CIB1-depleted cells do not become stem cells, but rather acquire some stem-like characteristics as they are dying. As we described previously, CIB1 depletion in MDA-468 cells results in cell death by a unique non-apoptotic mechanism [10]. It is possible that the observed differential gene expression is a downstream cellular response to overcome the negative effects of CIB1 depletion, rather than a direct effect of loss of CIB1. Further experiments are required to follow-up on this interesting observation.

Because CIB1 depletion induces MDA-468 cell death, we next examined the RNAseq data for differential expression of genes involved in cell survival and cell death. We identified 99 differentially expressed genes that were positively associated with increased cell death (several of these genes are listed in Fig. 3c, d). Interestingly, CIB1 depletion resulted in decreased expression of several known cancer drug targets, suggesting that inhibiting CIB1 could broadly inhibit multiple targets simultaneously (Fig. 3d). For example, CIB1 depletion led to decreased expression of two isoforms of glutathione-*S*-transferase, an enzyme that protects cells from oxidative stress and is implicated in chemotherapy drug resistance, indicating that CIB1 interference may sensitize TNBC cells to chemotherapy or other stress-inducing targeted therapies [31]. We propose that examination of CIB1-dependent differentially expressed genes could lead to identification of additional novel drug targets or potential combination therapies.

CIB1 mRNA expression does not correlate with TNBC prognosis

Recent reports have suggested that CIB1 expression may have prognostic implications in breast cancer [32]. Since CIB1 protein levels did not appear to correlate with





C

Gene Symbol	Gene Name	Log2 Fold Change
IL24	Interleukin-24	+5.6
DKK3	Dickkopf-3	+4.1
PDCD1 LG2	Programmed Cell Death 1 Ligand 2	+3.5
NLRP3	NOD-like receptor, pyrin domain-containing 3	+3.0
FYN	FYN	+2.5
IL18	Interleukin-18	+2.4
ABL2	ABL2	+2.2
DUSP6	Dual-specificity phosphatase 6	+2.1

D

Gene Symbol	Gene Name	Log2 Fold Change	Inhibitor
TF	Tissue Factor	-4.3	Ixolaris
CLU	Clusterin	-3.5	OGX-011
EDNRB	Endothelin receptor, type B	-3.5	BQ788
BDNF	Brain Derived Neurotrophic Factor	-3.5	
KIT	KIT	-2.5	PKC412
GSTP1	Glutathione S-transferase P1	-2.4	Quercetin
NRAS	NRAS	-2.4	
GSTA1	Glutathione S-transferase alpha 1	-2.1	Curcumin
CCR2	Chemokine CC motif Receptor 2	-2.1	
HDAC6	Histone Deacetylase 6	-1.8	Tubacin
JAK1	Janus Kinase 1	-1.6	Ruxolitinib

Fig. 3 CIB1 depletion results in differential expression of 812 genes. **a** Heat map of top 60 genes differentially expressed upon CIB1 depletion (*red* upregulated; *blue* downregulated). **b** Overlap of 812 differentially expressed genes with three known breast cancer gene signatures [28–30]. Five of six genes from a proliferation signature decreased, eleven of sixteen genes from an EMT signature increased, and five of seven genes from a mammary stem cell signature increased. **c**, **d** Selected upregulated (**c**) and downregulated (**d**) genes predicted to increase cell death. Several gene products have known inhibitors that have been tested for efficacy in cancer

susceptibility to CIB1 depletion in the TNBC cell line panel examined in Fig. 1, we predicted that CIB1 mRNA expression might not be prognostic of survival in TNBC patients. We therefore tested the association of CIB1 and disease progression in an 855 human tumor database [33]. Kaplan–Meier survival analyses found no significant association ($P < 0.05$) of patient relapse-free survival and CIB1 mRNA level within estrogen receptor-negative tumors or triple-negative tumors (Supplementary Fig. S5). These results were confirmed in three other independent datasets [23, 34, 35] and indicate that CIB1 expression levels alone are not a reliable indicator of prognosis in TNBC.

Discussion

TNBC is a breast cancer subtype with generally poor prognosis and no available targeted treatment options [9]. Two oncogenic pathways, RAF–MEK–ERK and PI3K–AKT, are aberrantly active in the majority of TNBC [3]. Because CIB1 promotes both of these signaling pathways [10], we hypothesized that CIB1 might be essential to TNBC cell survival. The data presented here provide evidence that CIB1 depletion impairs cell survival in a majority of TNBC cell lines and shrinks TNBC xenograft tumors, suggesting that CIB1 may have a broad role in TNBC survival and tumor growth. Furthermore, dependence on CIB1 expression is associated with active AKT and/or low PTEN expression. PTEN mutation or deletion is significantly associated with incidence of basal-like breast cancer in mice and humans [36, 37]. These data suggest that CIB1 inhibition may be an effective therapeutic option for TNBC patients with PTEN-deficient tumors.

Because CIB1 is essential for TNBC survival and tumor growth, we asked whether CIB1 expression is prognostic of TNBC patient survival. Recently CIB1 expression was reported to be relatively higher in hepatocellular carcinoma tumor center compared to non-tumorous liver tissues from 100 patient samples [17], as well as in breast cancer tissue compared to matched non-cancerous breast tissue from nine patient samples [32]. We found no association between CIB1 mRNA expression and patient relapse-free survival in both TNBC and ER-negative breast cancer. In contrast to previous reports, our study used gene expression data from thousands of breast cancer patients across four established datasets [23, 33–35]. While the data presented here suggest that CIB1 expression is not prognostic in TNBC, it is possible that CIB1 does have prognostic

implications in other types of cancer. Our results indicate that CIB1 expression is not predictive of TNBC patient prognosis, and further suggest that CIB1 overexpression does not promote tumorigenesis per se.

CIB1 appears to have a critical role in promoting AKT activation and cell survival in cells reliant on the AKT oncogenic pathway. However, CIB1 itself has never been described as an oncogene. Although we find that CIB1 depletion is lethal to TNBC cells with high pAKT/low PTEN activity (Fig. 4), CIB1 depletion is tolerated in non-cancerous cells (Supplementary Fig. S2 and [24]) and in TNBC cells that do not rely on AKT signaling (Fig. 1a). Furthermore, CIB1 knockout mice have no developmental defects [25], suggesting that CIB1 could be a potentially safe therapeutic target. The properties of CIB1 observed here are consistent with non-oncogene addiction, a phenomenon in which cancer cells require, or become 'addicted' to a non-mutated, non-overexpressed gene/protein that is nonetheless essential to maintain oncogenic signaling pathways [38, 39]. For example, ATM-deficient tumor cells display non-oncogene addiction to the enzyme DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and DNA-PKcs has been identified as a potential drug target in ATM-defective malignancies [40]. Based on this example, our data suggest that PTEN-defective TNBC tumors may display non-oncogene addiction to CIB1 and implicate CIB1 as a novel drug target in TNBC.

In summary, CIB1 inhibition induces TNBC cell death in cell culture and tumor regression in vivo. These results warrant further investigation of CIB1 in non-oncogene addiction and as a candidate for TNBC therapy.

Methods

Cell lines and cell culture

Cell lines and cell culture conditions are listed in Supplementary Table 4.

Mice and xenografts

MDA-468-CIB1shRNA and MDA-468-SCRshRNA (5×10^6 cells) in PBS were mixed 1:1 with Cultrex Basement Membrane Extract Type III (Trevigen, Gaithersburg, MD) and injected subcutaneously into the flanks of 6-week-old female Nu/Nu mice (Charles River Laboratories, Wilmington, MA). Mice were enrolled at a tumor size of ~ 100 mm^3 in the following treatment arms: 1 % Sucrose (Sigma, St. Louis, MO), 1 % sucrose + 2 mg/mL doxycycline (Sigma, St. Louis, MO); administered via drinking water 3×/week. Tumors were measured twice per week with calipers (tumor volume = length × width × width/2). Mice were euthanized

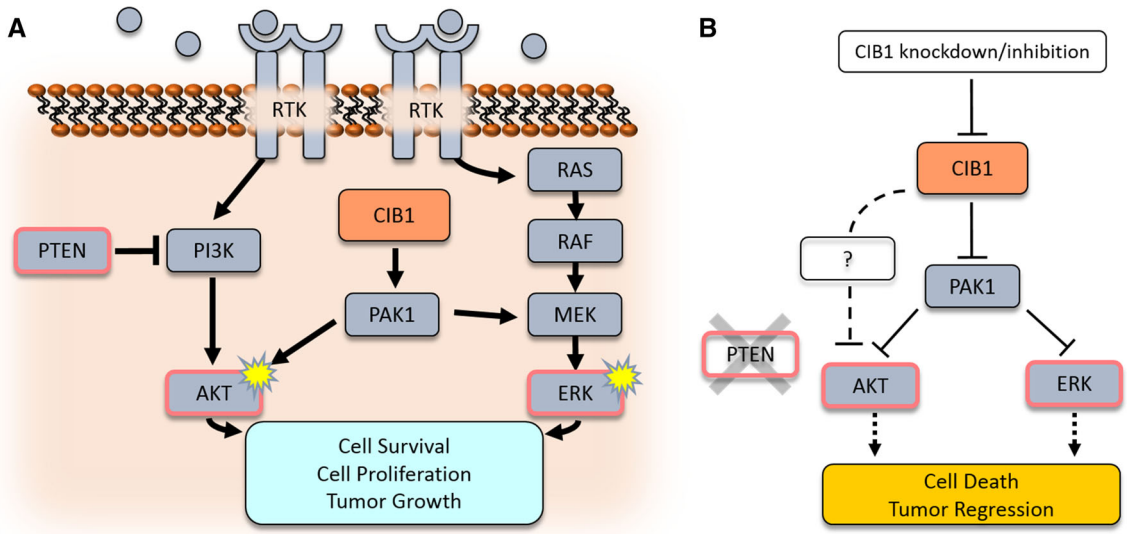


Fig. 4 Proposed mechanism of CIB1 regulation of TNBC cell survival and potential role of CIB1 in non-oncogene addiction. **a** CIB1 promotes TNBC cell survival, proliferation, and tumor growth via AKT and ERK signaling pathways. **b** CIB1 depletion results in loss of AKT and ERK. This effect is mediated in part by PAK1, but also likely involves additional, undetermined factors (*dotted line*). CIB1 depletion is most effective in PTEN-deficient cells and/or cells with elevated AKT activation. Because PTEN also acts as an upstream regulator of PI3K/AKT signaling, inactivating mutations or deletions of PTEN commonly result in hyper-activation of this pathway. Thus, TNBC cells with low or absent PTEN show increased sensitivity to CIB1 depletion. Together with the observations that CIB1 depletion/loss has minimal effect on non-cancerous cells [24] or TNBC cells with wild-type PTEN, our findings suggest a role for CIB1 in the concept of non-oncogene addiction

after 5 weeks of treatment and tumors were resected for further analysis.

RNAseq analysis

MDA-468_SCRshRNA and MDA-468_CIB1shRNA cells were treated with doxycycline for <96 h. After removing dead cells, RNA was isolated from viable cells (RNeasy kit, Qiagen, Venlo, Netherlands) and cDNA generated (QuantiTect Reverse Transcription kit, Qiagen). cDNA was sequenced at the UNC High Throughput Sequencing Facility on an Illumina HiSeq2000 (Illumina, San Diego, CA). Differential gene expression analysis was performed using DESeq 2 [41], and differentially expressed genes were selected based on Log2 fold change $\geq \pm$ 2 and Benjamini–Hochberg adjusted P value <0.05. Differentially expressed genes were analyzed using Ingenuity Pathway Analysis (Qiagen). The median-centered gene expression dataset and methods from Prat et al. [22] were used for Significance Analysis of Microarrays on the CIB1 KD sensitive versus insensitive cell lines, and for the identification of cell line Differentiation Scores; both of these analyses were performed with R version 3.1. To identify other gene signatures with similar profiles in human breast tumors [33], 10,508 gene signatures were retrieved from the GSEA database and via manual curation, each signature score was identified for each tumor by taking the average value of all signature genes within the median-centered gene set, then Pearson Correlation Values were obtained in Excel contrasting the CIB1 KD signature with all signatures.

Colony formation assay

MDA-468-control and -CIB1shRNA cells were treated ±1 μg/ml Dox for 48 h prior to plating at a density of 2000 cells/well. Cells were allowed to grow 9 days in the absence or presence of Dox, with media changes every 4 days. Cells were stained with crystal violet (0.05 % w/v in 4 % formaldehyde) (Sigma, St. Louis, MO) and colonies counted using ImageJ software.

RNA interference

Cells were transduced with either control shRNA (ACCG CTCTTCACACAGATCCTCTTCAAGAGAGAGGACTGT TGTGAAGAGCTTTTTC), CIB1 shRNA 1 (ACCGTGCC CTTCGAGCAGATTCTTCAAGAGAGAATCTGCTCGAA GGGCACTTTTTC), or CIB1 shRNA 2, (CAGCCTTAGC TTTGAGGACTTCTCGAGAAGTCCTCAAAGCTAAGGC TG). For inducible RNAi experiments, MDA-468 cells were transduced with either inducible control shRNA (GCTAC ACTATCGAGCAATTTTGGA TCCAAAATTGCTCGATAGTGTAGC) or inducible CIB1 shRNA (GGCTTAGTGCGTCTGAGATTTGGATCCAAA TCTCAGACGCACTAAGCC) using the pLV-H1-TetO-Puro lentiviral plasmid (Biosettia, San Diego, CA). Lentiviral particles were prepared as described previously [10].

Immunoblotting

Cell and tumor lysates were prepared using CHAPS lysis buffer (20 mM HEPES, 150 mM NaCl, 5 % v/v glycerol, 10 mM CHAPS, 0.1 mM $CaCl_2$, 0.05 mM $MgCl_2$, 20 mM NaF, 10 mM β-glycerophosphate, 0.1 mM Sodium Pervanadate, 1.25 mg/mL *N*-ethylmalemide, and Protease Inhibitor Cocktail III (BioVision). Protein concentration of tumor lysates was determined using BCA Assay (Thermo Scientific), equal amounts of total protein were separated by SDS-PAGE, transferred to PVDF, and incubated with indicated primary antibodies overnight at 4 °C, and visualization was performed using ECL2 (Pierce). The following antibodies were used: CIB1 chicken polyclonal antibody was produced as described previously [11]; antibodies against $pAKT^{473}$ (9271), pERK (9101), total AKT (4691), and γH2Ax (9718) were obtained from Cell Signaling Technology (Danvers, MA); ERK (sc-94), PTEN (sc-9145) and PAK1 (sc-882) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX); Rac monoclonal antibody was purchased from EMD Millipore (Billerica, MA).

Statistical analysis

P values were calculated using Student's t test.

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Conflict of interest JL Black, TM Leisner, and LV Parise are co-founders of Reveris Therapeutics, LLC. CM Perou is an equity stock holder, consultant, and member of the board of directors of BioClassifier LLC and GeneCentric Diagnostics. The other authors declare no potential conflicts of interest.

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