C/EBP β regulates sensitivity to bortezomib in prostate cancer cells by inducing *REDD1* and autophagosome-lysosome fusion

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Abbreviations: bZIP, basic region-leucine zipper; C/EBP β , CCAAT/enhancer binding protein β ; ChIP, chromatin immunoprecipitation; CQ, chloroquine; ER, endoplasmic reticulum; GFP, green fluorescent protein; KD, knockdown; NOD/SCID, non-obese diabetic/severe-combined immunodeficient; NSG, NOD/SCID;IL2R $\gamma^{-/-}$; PE, phosphatidylethanolamine; PCa, prostate cancer; qPCR, quantitative polymerase chain reaction; PB, PiggyBac; mRFP, monomeric red fluorescent protein; shRNA, short hairpin RNA; TSS, transcription start site.

ABSTRACT

The purpose of this study was to ascertain the mechanisms by which advanced prostate cancer cells resist bortezomib therapy. Several independent studies have shown that cells are protected from proteasome inhibition by increased autophagic activity. We investigated whether C/EBP β , a transcription factor involved in the control of autophagic gene expression, regulates resistance to proteasome inhibition. In PC3 cells over-expressing C/EBP β , turnover of autophagic substrates and expression of core autophagy genes were increased. Conversely, C/EBP β knockdown suppressed autophagosome-lysosome fusion. We also found that C/EBP β knockdown suppressed *REDD1* expression to delay early autophagy, an effect rescued by exogenous REDD1. Cells with suppressed C/EBP β levels showed delayed autophagy activation upon bortezomib treatment. Knockdown of C/EBP β sensitized PC3 cells to bortezomib, and blockade of autophagy by chloroquine did not further increase cell death in cells expressing shRNA targeting C/EBP β . Lastly, we observed decreased growth of PC3 cells and xenografts with C/EBP β knockdown and that such xenografts were sensitized to bortezomib treatment. Our results demonstrate that C/EBP β is a critical effector of autophagy via regulation of autolysosome formation and promotes resistance to proteasome inhibitor treatment by increasing autophagy.

Keywords: autophagy, C/EBP β , REDD1, prostate cancer, proteasome inhibitor

1. Introduction

Metastatic prostate cancer (PCa) is an incurable disease and a leading cause of cancer death in Europe and North America. When patients acquire resistance to hormonal therapy, second-line chemotherapy is administered, which can increase patient lifespan by several years. There has been interest in the use of bortezomib, a proteasome inhibitor, for treatment of PCa, but clinical studies showed only modest or no significant improvement in patient benefit when administered in combination with hormonal therapy or docetaxel [1-3]. However, a recent study evaluating microarray data from younger patients (<65 years of age) with localized prostate cancer and treated by radical prostatectomy revealed that gene sets representing proteasome subunits and protein catabolism are the strongest predictors of metastatic progression [4]. Thus, proteasome inhibition may still be of clinical value for certain subsets of patients with PCa.

Autophagy is a catabolic process which serves to turn over long-lived proteins and organelles by engulfing them in double-membrane bound vacuoles, autophagosomes. These structures eventually fuse with lysosomes to degrade their contents and recycle lipids, amino acids, and carbohydrates [5]. Autophagy is initiated by activation of a multi-protein type III PI3K complex, composed of Beclin 1, VPS34 and ATG14, which enriches a region of the endoplasmic reticulum (ER) in phosphatidylinositol-3-phosphate to serve as a platform for the recruitment of other proteins to generate an isolation membrane [6,7]. A group of evolutionary conserved proteins are responsible for elongation and closure of the isolation membrane to form a double-membrane autophagosome by proteolytic cleavage of LC3 and GABARAP family proteins, covalently linking them to phosphatidylethanolamine (PE) and incorporating them into the burgeoning autophagosome [8,9]. Autophagy is not only regulated at the initiating steps, as LC3-II can be delipidated by ATG4b to LC3-I to promote LC3 recycling [10]. Conversely, LC3-II delipidation

by ATG4b can be suppressed by REDD1, thereby promoting autophagy [11]. Proteins and organelles destined for degradation are trafficked to autophagosomes by the ubiquitin-binding proteins p62 or NBR1 [12-14].

Autophagy has been recognized as a central process that cancer cells utilize to adapt to stress brought about by hypoxia, anoikis, radiation, or chemotherapy. Recently, it has been suggested that autophagy plays a critical role in resistance of PCa cells to hormonal therapy or chemotherapy and that inhibition of autophagy synergizes with docetaxel or androgen deprivation [15,16]. Combined treatment of several different cancer cell lines with autophagy suppressive agents and bortezomib increases cell death and improves clinical response in patients with relapsed multiple myeloma [17-19]. Further, proteasome inhibitor-resistant PCa cells are thought to utilize autophagy to resist this class of drugs [20].

Recently, several groups have shown that autophagy is regulated at the transcriptional level [21, 22]. C/EBP β , a basic region-leucine zipper (bZIP) transcription factor, was identified as a regulator of circadian autophagy in the liver by inducing a broad array of autophagy genes [23]. C/EBP β protein is translated from an intronless transcript from three, in-frame start codons as higher molecular weight, liver-enriched activating proteins (LAP and LAP*) or the truncated liver-enriched inhibitory protein (LIP). The dominant-inhibitory LIP isoform lacks all three N-terminal activation domain modules and can inhibit transcription by heterodimerizing with LAPs. Another group also showed that C/EBP β promotes differentiation of 3T3-L1 pre-adipocytes by activation of *ATG4b* gene expression and autophagy [24]. Intriguingly, treatment of Burkitt's lymphoma cells with bortezomib dramatically increases the expression of C/EBP β [25]. Other proteasome inhibitors, such as lactacystin or MG-132, also increase nuclear levels of C/EBP β and increase its DNA binding [26,27]. We therefore investigated the role of C/EBP β in PCa cell autophagy and

sensitivity to bortezomib. Our results suggest that autophagy is activated early after bortezomib treatment, that C/EBP β promotes autophagy in PCa cells via induction of autophagosome-lysosome fusion and via induction of *REDD1*, and that reducing C/EBP β expression increases prostate cancer cell sensitivity to bortezomib.

2. Materials and methods

2.1 Cell lines, reagents and mice

PC3 and LNCaP cells were maintained in RPMI media with 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT) supplemented with penicillin/streptomycin. Cells were grown in a humidified incubator maintained at 37°C with 5% CO₂. Cell lines transduced with short hairpin RNA (shRNA) or PiggyBac (PB) Tet-on vectors were grown in media supplemented with 10% tetracycline-screened FBS (Hyclone). For experiments involving inducible expression of shRNA or ectopic C/EBP β , 0.5 μ g/ml doxycycline was added to the media and replaced every 48 hrs. The pCMS-eGFP-RTP801 plasmid was purchased from Addgene (Cambridge, MA; plasmid #65057).

Non-obese diabetic/severe-combined immuno-deficient (NOD/SCID);IL2R γ ^{-/-} (NSG) mice were obtained from a breeding colony established in the Johns Hopkins University division of animal resources. Animals were subcutaneously engrafted with 2E06 PC3 cells in PBS in a 1:1 mixture with Matrigel. Animals were monitored daily after transplantation. When tumor volumes reached 100 mm³, the mice were placed on doxycycline-laced feed. When tumors reached a volume between 100 and 300 mm³, animals were randomly assigned to receive either DMSO vehicle or bortezomib, by intraperitoneal (IP) injection (1 mg/kg of body weight) on days 1, 4 and

8. Mice were sacrificed 11 days after engraftment. Tumor volumes were determined by caliper measurement using the ellipsoidal formula: length x width x height x 0.5236 [28].

All animal studies were conducted under protocols approved by the Johns Hopkins Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

2.2 Western Blotting

Protein samples from whole cell lysates or nuclear extracts were prepared and subjected to Western blotting as described [29]. Each experiment was repeated at least twice. Anti- β -actin (AC15) and anti-vinculin (V9131) antibodies were from Sigma-Aldrich (St. Louis, MO). Anti-C/EBP β (C-19), anti-C/EBP β (H-7), and anti-SQSTM1/p62 (H-290) antibodies were from Santa Cruz Biotechnologies (Dallas, TX). Anti-LC3B (2775), anti-ATG3 (3415), anti-ATG5 (8540), and anti-ATG-7 (8558) antibodies were from Cell Signaling Technologies (Danvers, MA). Anti-REDD1 antibody (10638-1-AP) was from Proteintech (Rosemont, IL).

2.3 Quantitative real-time PCR

RNA extraction, cDNA synthesis, and PCR reactions were performed as previously described [29]. Oligonucleotides were designed using PrimerBlast and custom ordered from Sigma-Aldrich. Their sequences are presented in Table 1.

2.4 Ectopic C/EBP β expression and shRNA

LNCaP cells stably transfected with the TET-on, transposon based PiggyBac vector PB-TRE, or PB-CEBPB expressing murine C/EBP β , were previously described [29]. PC3 cells were

transfected with these same vectors, and cells with stable integration were positively selected with puromycin (2 mg/ml). Lentiviral transduction of PC3 cells with pTRIPZ lentiviral vectors targeting *CEBPB* was also performed as previously described for LNCaP cells [29].

2.5 TALEN construction and *CEBPB* gene editing

TALEN DNA constructs targeting the human *CEBPB* open reading frame (ORF) were constructed as previously described [29]. *CEBPB* homology arm 1 (HA1) was amplified from 293T DNA with primers TGCtctagaCTGGTGGGAACAATGCCACC and ACTtgatcaTGGTGGCATTGTTCCCACCAG; restriction enzyme sites are in lower case. The resulting fragment was digested with *Xba*I and *Spe*I and ligated into the pSEPT donor plasmid [30]. *CEBPB* homology arm 2 (HA2) was amplified using GCatcgatGAACTTGTTCAAGCAGCTGCC and AGTcagctgAGGCTCCGGAATCTCTTCTC primers. The resulting fragment was digested with *Cla*I and *Sal*I and ligated into the pSEPT plasmid containing *CEBPB* HA1. PC3 cells were co-transfected with TALEN expression vectors targeting *CEBPB* and the pSEPT donor plasmid at a 1:1 ratio and after 48 hours were seeded into a 96-well plate at 1 cell/well with G418. Individual clones were screened for C/EBP β expression by Western blotting.

2.6 Chromatin immunoprecipitation (ChIP)

1E06 PC3 cells were used in each ChIP reaction, using rabbit-anti C/EBP β antiserum or normal rabbit IgG (Santa Cruz Biotechnology) as previously described [31]. DNA fragments corresponding to the promoter of *REDD1* were detected by qPCR using the primers presented in Table 2.

2.7 Cell viability and proliferation assays

To assess cell proliferation, 1E05 PC3 cells were seeded in growth media containing 0.5 µg/ml doxycycline. On days 2 and 4, cells were trypsinized and stained with Trypan blue dye, and viable cells were enumerated using a hemocytometer. Experiments were repeated three times. For clonogenic assays, PC3 cells were trypsinized and seeded into 6-well plates at 200 cells per well. 12 days after plating, cells were fixed and stained with 4% formaldehyde and 0.05% crystal violet. After washing with tap water, colonies were manually counted under a bright field microscope. Clusters of cells containing more than 50 cells were counted as a colony.

2.8 Autophagosome-lysosome fusion assay

PC3 cells were plated on 60 mm dishes to obtain 80% confluence the next day. Cells were transfected with tandem mRFP-GFP fluorescent-tagged LC3 (ptfLC3) (Addgene #21074) [32]. On day two post-transfection, cells were trypsinized and plated on sterile glass bottom dishes (Mattek, Ashland, MA) at a 40-50% confluency. Cells were allowed to adhere to glass and spread for 24 hrs. Cells were then imaged using a live cell Zeiss LSM780-FCS Single-point, laser scanning confocal microscope at the JHU Core Imaging facility. Images were processed using ImageJ software. Cells with predominantly yellow RFP/GFP (autophagosome) or red RFP (autolysosome) punctae were counted and analyzed as described [32].

2.9 Q74-EGFP degradation assay

PC3 cells seeded in 24-well plates were transfected with 500 ng of Q74-EGFP (Addgene #40262) [33] or pMax-GFP (Lonza, Basel, Switzerland). Forty-eight hours after transfection, fluorescent micrographs of GFP-positive cells were taken using a Nikon Ti-E inverted microscope

at 400X magnification from at least five non-overlapping regions of interest. Cells were manually counted using ImageJ software and the percentage of GFP-positive cells displaying punctuate staining was determined.

2.10 Statistical analysis

Statistical comparison of two groups was conducted using the Student's t-test. Comparisons of multiple groups was performed using analysis of variance followed by multiple comparisons with the Student's t-test and the Holm-Bonferonni correction. P-values are ordered from highest to lowest and α is adjusted to $\alpha/(m+1-k)$ for $p_{(k)}$, where m = number of comparisons and corresponds to the highest p-value. k corresponds to the p-value rank. α was set to 0.05 for comparisons. For analysis of tumor growth, linear regression analysis was used to determine statistical significance in growth rates between groups of mice using Graph Pad Prism statistical software.

3. Results

3.1 *C/EBP β LAP:LIP isoform ratio regulates autophagy in prostate cancer cells*

To understand the role of C/EBP β in prostate cancer cell autophagy, we first ectopically expressed murine C/EBP β , which shares 98.8% similarity to the human C/EBP β bZIP domain and 80-100% similarity to the human trans-activation and regulatory domains, in the PC3 PCa cell lines using the PB-TRE Tet-on PiggyBac inducible expression vector we had used previously to obtain LNCaP cells harboring doxycycline-inducible C/EBP β [29]. Induction of PiggyBac-CEBPB (PB-CEBPB) in PC3 cells led to a decline in the total levels of LC3-I and LC3-II and p62 compared to cells expressing the empty PB-TRE vector (Fig. 1A, left). Decreases in p62 levels typically indicate increased autophagic degradation, while changes in LC3-II can imply blockade

or activation of autophagosome formation [34]. We further evaluated other proteins involved in autophagosome nucleation and elongation and found increases in ATG3, ATG5, ATG7 and Beclin-1, suggesting that autophagy is increased upon induction of C/EBP β (Fig. 1A, right).

In contrast with these results in PC3 cells, LNCaP cells showed increased total levels of LC3-I and LC3-II and no change in p62 in response to C/EBP β induction (Fig. 1B). Of note, ectopic C/EBP β is predominantly expressed as the shorter, dominant inhibitory LIP isoform in LNCaP cells as is endogenous C/EBP β . Thus induction of CEBPB in LNCaP cells may reduce C/EBP β trans-activation activity to impair autophagy.

As C/EBP β was shown to directly regulate autophagic genes at the transcriptional level [23,24,35], we also evaluated the expression of several such genes by quantitative real-time PCR (qPCR). We found a modest increase in *CTSL*, *GABARAP*, *ATG7*, *BNIP3*, *BNIP3L* and *ULK1* mRNAs in PC3 cells and that nearly all of these genes were suppressed upon ectopic C/EBP β expression in LNCaP cells (Fig. 1C).

3.2 *C/EBP β promotes autophagosome-lysosome fusion in PC3 cells*

Bafilomycin A1, a vacuolar-ATPase inhibitor, increases lysosomal pH and alters Ca²⁺ gradients of the lysosome, subsequently inhibiting fusion with autophagosomes, blocking autophagic flux and leading to accumulation of LC3-I and LC3-II [36,37]. We treated cells for four hours with bafilomycin A1 and then evaluated lysates by Western blot analysis. Although the total levels of LC3-I and LC3-II were increased in PC3 cells expressing shCEBPB, the ultimate level of LC3-II achieved in response to bafilomycin A1 was not different from the control cells transduced with shNTV (Fig. 2A, top). In addition, ectopic expression of C/EBP β did not enhance autophagic flux in bafilomycin A1-treated cells, as again the peak levels of LC3-II, 4.1 and 3.9, were nearly

identical (Fig. 2A, bottom). These data suggest that C/EBP β does not play a dominant role in autophagosome formation, as otherwise CEBPB shRNA might have been predicted to reduce and ectopic C/EBP β to increase the change in LC3-II in the presence of bafilomycin A1.

We next tested whether C/EBP β regulates turnover of autophagy substrates. We transiently expressed mutant huntingtin-EGFP (Q74-EGFP) fusion protein in PC3 cells together with either shNTV or shCEBPB [33]. Expression of Q74-EGFP results in protein aggregation which can be observed as punctuate GFP-positive staining. Forty-eight hours after transfection, we quantified the number of GFP-positive cells displaying aggregates and found that there were significantly more cells with GFP aggregates in PC3 cells expressing shRNA targeting CEBPB (Fig. 2B, left and center). These data indicate that turnover of autophagosomes is augmented by C/EBP β . In addition, there were significantly more non-viable, Trypan blue dye positive cells in cultures lacking C/EBP β (Fig. 2B, right).

The first step in autophagosome turnover is fusion with lysosomes. We therefore next evaluated autophagosome-lysosome fusion in PC3 cells using plasmid ptfLC3, expressing a mRFP-GFP-LC3 fusion protein [32]. As GFP fluorescence is quenched by the acidic environment of the lysosome, yellow punctae visible under fluorescence microscopy, representing signal from both RFP and GFP, indicates autophagosomes, whereas red punctae represent autolysosomes. Doxycycline induction of shRNA from pTRIPZ also induces RFP and so is not amenable to this assay. We therefore utilized TALEN targeting of genomic *CEBPB* as an alternative means of knockdown (KD). We utilized homologous recombination to insert a neomycin resistance cassette and generate a deletion in the *CEBPB* locus with a pSEPT donor plasmid containing homology arms of CEBPB flanking the TALEN cut site (Supplementary Figure S1) [30]. We generated a neomycin-resistant subclone which expressed decreased levels of C/EBP β relative to the control

subclone (Fig. 2C). When TALEN KD cells were transfected with the mRFP-GFP-LC3 reporter, we found that there was a remarkable, 4-fold increase in the percentage of cells with RFP/GFP-positive autophagosomes relative to control cells and a similar decrease in the percent of cells with RFP-positive autolysosomes, implying that fusion with lysosomes is impaired (Fig. 2D). Collectively, these data demonstrate that C/EBP β is critical for basal autophagy in PC3 cells by regulating autophagosome-lysosome fusion.

3.3 C/EBP β LAP promotes autophagy in PC3 cells by augmenting *REDD1* expression

Although C/EBP β is reported to broadly regulate the expression of autophagy genes in the liver [23], we did not observe differential expression of all genes analyzed in PC3 cells over-expressing C/EBP β . Recently, it was shown that *REDD1* could promote autophagy by suppressing ATG4b-mediated LC3-II delipidation to LC3-I, an effect most critical during periods of metabolic stress [11]. This function of *REDD1* prevents recycling of LC3-II and leads to greater turnover of autophagosomes. *REDD1* can also inhibit mTORC1, which suppresses autophagy by phosphorylating ULK1 and ATG13, upstream autophagy regulators [38]. C/EBP β was previously shown to cooperate with ATF4 to promote *REDD1* transcription by binding to a non-consensus site centered 1,004 bp upstream from the *REDD1* transcription start site (TSS), but only in cells under oxidative stress [39,40]. We evaluated *REDD1* gene expression changes in PCa cells expressing shRNA targeting *CEBPB* by qPCR. In contrast to cells over-expressing C/EBP β , we found that none of the genes that we had analyzed in Figure 1 were affected by C/EBP β KD in LNCaP or PC3 cells (Fig. 3A). However, in PC3 cells with *CEBPB* deficiency, we found significant 2-fold down-regulation of *REDD1* transcript levels (Fig 3A). We also found that LNCaP cells showed increased *REDD1* upon shCEBPB induction, likely due to decreased

expression of the dominant-inhibitory LIP isoform (Fig. 3A). Notably, *REDD1* mRNA showed stronger up-regulation upon C/EBP β over-expression in PC3 cells than other autophagy genes analyzed (Fig. 3B). Conversely, over-expression of C/EBP β in LNCaP cells suppressed *REDD1* transcript levels (Fig. 3B).

As we observed that *REDD1* expression correlated with full-length C/EBP β levels in the absence of oxidative stress, we analyzed the human *REDD1* promoter to identify additional binding sites for C/EBP β . We found a site matching the C/EBP β consensus at -603 bp and a near-consensus site at -99 bp (Supplementary Figure S2). To test whether C/EBP β binds to these regions, we subjected nuclear extracts from PC3 cells to chromatin immunoprecipitation (ChIP). Using two different primer pairs, each flanking one of the putative C/EBP β binding sites, we observed between 6 and 8-fold greater levels of amplified product in extracts precipitated with anti-C/EBP β antiserum relative to normal rabbit IgG (Fig. 3C), indicating that endogenous C/EBP β binds to these regions of the *REDD1* promoter.

C/EBP β regulation of *REDD1* suggests that C/EBP β is not only critical for autophagosome-lysosome fusion, but also might play a role in autophagosome maturation. Residual *REDD1* present upon shCEBPB-mediated *CEBPB* KD must be sufficient to prevent a complete block at this step in autophagy. Knockdown of *REDD1* was problematic, as all siRNAs tested also reduced C/EBP β (not shown), and of note *REDD1*(-/-) MEFs show greatly reduced autophagosome formation [11]. Therefore, to further test whether C/EBP β drives early autophagy via *REDD1*, we transiently over-expressed *REDD1* in PC3 cells. We found that expression of *REDD1* from the pCMS-EGFP-*REDD1* vector [41] increased LC3-II in PC3 cells harboring shNTV, as predicted from its role in early autophagy, but mildly reduced LC3-II in shCEBPB cells, compared to

controls expressing the pMax-GFP plasmid (Fig. 3D). These results suggest that C/EBP β promotes autophagy in part by regulating *REDD1* expression.

3.4 *C/EBP β promotes autophagy following bortezomib treatment*

Previous reports suggest that proteasome inhibitors increase expression and activity of C/EBP β [25, 26]. As proteasome inhibitors also activate autophagy [42,43], we tested whether C/EBP β is required for autophagy following bortezomib treatment. Gene expression analysis of several autophagy genes revealed that *ULK1* and *p62* were markedly increased 16 hours after bortezomib treatment of PC3 cells (Fig. 4A). *ATG5* and *ATG4B* were unchanged and *ATG7* and *REDD1* were mildly upregulated by bortezomib. The changes in *p62* and *ULK1* were not mediated by C/EBP β because shCEBPB cells showed the same increase in gene expression upon bortezomib treatment (Fig. 4B). Treatment of LNCaP or PC3 cells with bortezomib for 24 hours markedly increased C/EBP β (LAP), LC3-II, and p62 protein expression (Fig. 4C). To further evaluate the effects of bortezomib on PCa cell autophagy, we analyzed LC3 and p62 by time course analysis. We found that REDD1 increased from 4-8 hours following 25 nM bortezomib treatment, but then decreased between 8 and 16 hours (Fig. 4D). LC3-II and p62 protein levels showed the strongest increase 16 hours after treatment. Lastly, we tested whether C/EBP β was critical for autophagy following bortezomib exposure. We treated PC3 cells expressing shCEBPB with bortezomib and evaluated autophagy markers by time course analysis. C/EBP β deficiency decreased REDD1 expression and prevented increases in LC3-II, though shCEBPB cells had higher basal levels of LC3-II (Fig. 4E). These data indicate that bortezomib promotes autophagy and that C/EBP β is critical for activating autophagy early after bortezomib treatment.

3.5 Reduced C/EBP β slows PC3 tumor growth and increases sensitivity to bortezomib

Autophagy positively regulates “cellular fitness” in a variety of cell types [44]. We therefore evaluated the effect of C/EBP β on PC3 proliferation. We quantified cell growth over a four day time course and found that inhibition of C/EBP β by shRNA targeting or TALEN reduced cell accumulation by more than 2-fold (Fig. 5A). We also evaluated clonogenic growth and found that cells deficient in C/EBP β due to TALEN targeting or shRNA expression showed a more than two-fold average decrease in colony number (Fig. 5B). We treated shNTV and shCEBPB PC3 cells with 25 or 50 nM of bortezomib for 24 hours and evaluated viability by Trypan blue dye exclusion. Suppression of C/EBP β increased cell death relative to shNTV cells by 2-fold with either dose of bortezomib (Fig. 5C, left). Phase contrast images of cells treated with 50 nM bortezomib and shNTV or shCEBPB are also shown (Fig. 5C, right). To evaluate whether autophagy contributed to increased cell death in shCEBPB cells we treated cells with chloroquine (CQ) in combination with bortezomib. Chloroquine is a weak base that accumulates in the acidic compartments of late endosomes and lysosomes, alkalinizes these structures, and subsequently inhibits autophagosome-lysosome fusion [45,46]. We found that there was no difference in shNTV versus shCEBPB cell death in cultures treated with the combination of CQ and bortezomib, indicating that increased death in shCEBPB cultures was due to a block in autophagy (Fig. 5D). We next evaluated PC3 tumor growth and sensitivity to bortezomib *in vivo*. NSG mice subcutaneously engrafted with shNTV or shCEBPB PC3 cells were treated with either vehicle or bortezomib (1 mg/kg) on days 1, 4 and 8 (Fig. 5E). Consistent with our *in vitro* findings, we observed a decreased rate of growth of shCEBPB PC3 tumors ($p=0.026$). Further, we observed no difference in the rate of growth in control tumors receiving either vehicle or bortezomib. In contrast, bortezomib treatment and CEBPB KD strikingly synergized to further decrease tumor growth ($p=0.017$). These results

suggest that C/EBP β is critical for prostate tumor growth and that decreasing C/EBP β increases sensitivity to bortezomib *in vivo*.

4. Discussion

Although clinical trials investigating bortezomib in PCa have produced lackluster results, a recent study suggests that the proteasome may still be a valid target in a subset of prostate cancer patients [4]. In patients younger than 65 with localized cancer, proteasome and protein catabolism genes were shown to be strong predictors of metastatic progression after radical prostatectomy. Understanding the response of PCa cells to proteasome inhibition is therefore of potential clinical utility.

The results of our study suggest that C/EBP β regulates fusion of lysosomes and autophagosomes in PCa cells. The effects of C/EBP β on autophagy were dependent upon the expression ratio of the C/EBP β translational isoforms, with higher relative levels of LIP:LAP expression suppressing autophagy in LNCaP cells and vice versa in PC3 cells. The observed effect of LIP down-regulating autophagic gene expression is consistent with its role as a transcriptional repressor owing to its lack of N-terminal trans-activation domains. Our data are consistent with previous reports that show a link between C/EBP β and autophagy. C/EBP β was reported to control circadian autophagy in mouse liver by broadly activating autophagy gene transcription [23]. Guo and colleagues showed that C/EBP β activates autophagy by directly regulating *ATG4B* in 3T3-L1 pre-adipocytes to promote differentiation to mature adipocytes [24]. However, the specific defect in autophagosome-lysosome fusion that we have revealed in PCa cells is a distinct mechanism from that reported in these earlier studies (Fig. 6). Analogous to what we have found however, C/EBP β (-/-) macrophages exhibit defects in bactericidal activity and fusion of phagosomes with lysosomes

[47]. Overall, our findings and those of others show that the effect of C/EBP β on autophagy is cell type and context specific.

Our results also demonstrate that C/EBP β further promotes autophagy through direct induction of *REDD1* gene expression (Fig. 6). Previous reports had shown that C/EBP β cooperates with ATF4 by binding to adjacent half-sites at -1,004 bp in the *REDD1* promoter in cells challenged by oxidative stress [39,40]. Our ChIP data and over-expression/knockdown studies suggest that C/EBP β can also bind to a consensus site centered at -603 bp and a near consensus site at -99 bp in the *REDD1* proximal promoter in the absence of oxidative stress to induce *REDD1* expression. REDD1 indirectly promotes autophagosome turnover by inhibiting LC3-II delipidation and thereby preventing LC3 recycling [11]. The latter function is consistent with our findings that show decreased LC3-II and p62 levels and increases in core autophagy regulators in PC3 cells over-expressing C/EBP β . Because REDD1 is not known to directly mediate or control autophagosome-lysosome fusion, it is likely that there are additional genes regulated by C/EBP β whose protein products promote this function independent of REDD1. And as noted, shCEBPB expression in PC3 cells only reduced REDD1 2-fold, likely allowing autophagosome formation, albeit at a slower rate. However, we were able to partly rescue the defect in autophagy in shCEBPB cells by ectopic expression of REDD1. In particular, LC3-II was not increased by shCEBPB in the presence of exogenous REDD1, suggesting increased flux through autophagy. The latter effect suggests that in the presence of shCEBPB, where autophagosome-lysosome fusion is suppressed, ectopic expression of REDD1 may have positive feedback on the autophagy pathway or additional activities beyond its ability to induce LC3-II.

During periods of metabolic stress such as exercise, starvation, or hypoxia, REDD1 is critical for autophagosome maturation by inducing LC3-II and regulating proper autophagosome size [11].

REDD1 transcript levels were not increased 16 hrs after 25 nM bortezomib treatment despite elevation of C/EBP β LAP, indicating that bortezomib interferes with C/EBP β induction of *REDD1* transcription, and protein levels were increased more than two-fold at this time point suggesting stabilization of REDD1 protein. Consistent with the idea that bortezomib affects REDD1 protein levels independent of C/EBP β , knockdown of *CEBPB* did not prevent up-regulation of REDD1 protein by bortezomib, though basal levels were reduced. We also found that LC3-II proteins were up-regulated within 4 hours of bortezomib treatment. However, in cells expressing shCEBPB, LC3-II levels showed only modest changes relative to control cells. These findings suggests that regulation of basal *REDD1* transcript and protein levels by C/EBP β is crucial for LC3B processing early after proteasome inhibitor treatment.

Proteasome inhibition increases cytosolic and endoplasmic reticulum (ER) protein content and promotes amino acid deprivation [42,48]. These events activate the amino acid sensitive protein kinase GCN2 and the unfolded protein response in the ER, leading to phosphorylation of eIF2 α and suppression of protein translation [49]. Autophagy is thought to promote cell survival in cells treated with proteasome inhibitors by restoring amino acid homeostasis and suppressing ER stress [42]. We found that PCa cells treated with bortezomib show a dynamic regulation of autophagy that was dependent upon C/EBP β expression. Suppression of autolysosome catabolism has been reported in ovarian and breast cancer cell lines treated with bortezomib, owing to a decrease in cathepsins D and B [50,51]. The conclusions of these studies are consistent with our findings which showed strong up-regulation of *p62* and *ULK1* but suppression of *Cathepsin L* (not shown) in PC3 cells treated with bortezomib.

Finally, our findings also show decreased cell and tumor growth rates in PC3 cells lacking C/EBP β coincident with a decline in basal autophagy. This decline in autophagy could explain the

poor growth rates of cells lacking C/EBP β as autophagy maintains cellular fitness by turnover of damaged organelles and long-lived proteins [44]. We also found that suppression of C/EBP β increased PC3 cell and tumor susceptibility to bortezomib. Further, CQ did not additively increase PC3 cell death following bortezomib treatment, indicating that bortezomib-induced cell death was related to inhibition of autophagy. These results are in accordance with previous studies wherein combined autophagy and proteasome blockade additively increase tumor cell killing [52-54]. In particular, our results implicate C/EBP β as a key mediator of autophagy in PCa and validate the utility of targeting C/EBP β in combination with bortezomib or other proteasome inhibitors as a novel approach to PCa therapy.

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6. Author's contributions

DJB, SKK, IPP, and ADF conceived and designed the research. TB and JZ generated shCEBPB and shNTV constructs and cell lines. JM conducted autophagosome-lysosome fusion analysis. DJB conducted the balance of the experiments. DJB and ADF wrote the manuscript. All authors read and approved the final manuscript.

7. References

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Fig. 1. C/EBP β isoforms LAP and LIP differentially regulate autophagy in PCa cells. (A) PC3 cells stably transduced with PB-TRE or PB-CEBPB were treated for 72 hrs with 0.5 μ g/ml doxycycline and cell lysates were analyzed by Western blotting for the indicated proteins. Numbers below the blots indicate the relative band density normalized to β -actin. (B) LNCaP cells stably transduced with PB-TRE or PB-CEBPB were analyzed similarly for C/EBP β , p62, LC3, and β -actin. (C) PC3 or LNCaP cells transduced with PB-TRE or PB-CEBPB were cultured similarly followed by qPCR analysis for the indicated genes. The dashed line represents gene expression levels in control cell lines expressing PB-TRE. Bar graphs represent the average of three experiments, error bars represent standard error of the mean (SEM). (* p <0.05).

Fig. 2. C/EBP β promotes autophagosome-lysosome fusion in PC3 cells. (A) Western blot analyses in PC3 cells expressing NTV or CEBPB shRNAs (top panels) or ectopically expressing mouse C/EBP β from a PiggyBac vector (PB-CEBPB) or the PB-TRE control vector (bottom panels). In both sets of experiments, PC3 cells were treated for 72 hrs with 0.5 μ g/ml doxycycline and then treated with either vehicle (DMSO) or 100 nM bafilomycin A1 for 4 hrs. Western blotting for C/EBP β , LC3, and β -actin was then conducted. (B) Quantification of EGFP-Q74 aggregates in GFP⁺, transfected PC3 cells expressing shNTV or shCEBPB (left). Sample fluorescence micrograph (center); white arrows indicate cells with aggregates. Cell viability was quantified by Trypan blue dye exclusion in cultures transfected with control pMax-GFP or EGFP-Q74 (right). (C) Western blot analysis of cell lysates from PC3 control and TALEN KD subclones. (D) Confocal micrographs of PC3 TALEN control and CEBPB KD cells transfected with the tandem fluorescent-tagged mRFP-GFP-LC3 (tfLC3) construct (top panels). Quantification of percent of cells with LC3 present in autophagosomes or autolysosomes is shown below (bottom). In the

red/green overlay images, yellow foci represent autophagosomes and red foci autolysosomes. Bar graphs throughout represent the average of three experiments, error bars represent SEM. (* $p < 0.05$).

Fig. 3. C/EBP β promotes autophagy in PC3 cells by regulation of *REDD1* expression. (A) qPCR analysis of autophagic genes in PC3 and LNCaP cells expressing shRNA targeting *CEBPB*. The dashed line represents gene expression levels in control cell lines expressing shNTV. (B) *REDD1* gene expression in cells expressing C/EBP β from the PB-CEBPB vector or harboring the control PB-TRE vector after 72 hr culture with doxycycline. (C) qPCR analysis, using two primer pairs, of genomic DNA extracted from PC3 cell chromatin following immunoprecipitation with anti-C/EBP β antibody or IgG control. Fold-increase in C/EBP β binding versus IgG is shown. (D) PC3 cells expressing shNTV or shCEBPB were transiently transfected with pMax-GFP or pCMS-EGFP-REDD1, and 48 hours later whole cell lysates were subject to Western blot analysis for the indicated proteins. Data presented is representative of three independent experiments. Bar graphs represent the average of three experiments, error bars represent SEM. (* $p < 0.05$).

Fig. 4. C/EBP β promotes autophagy driven by bortezomib challenge. (A) qPCR analysis for indicated mRNAs from PC3 cells that had been treated with bortezomib or vehicle for 16 hrs. (B) qPCR analysis of autophagy genes in PC3 cells expressing shNTV or shCEBPB and that had been treated for 16 hrs with 25 nM bortezomib or vehicle. (C) LNCaP or PC3 parental cells were treated for 24 hrs with the indicated concentrations of bortezomib and cell lysates were then subjected to Western blot analysis. (D) PC3 cells were treated with 25 nM bortezomib for the indicated times and cell lysates were then analyzed by Western blot analysis for indicated proteins.

(E) PC3 cells expressing NTV or CEBPB shRNAs were treated with 50 nM bortezomib for the indicated times and cell lysates were again analyzed by Western blot analysis. Numbers below blots indicate relative density after normalizing to β -actin. Bar graphs represent the average of three experiments, error bars represent SEM. (* $p < 0.05$).

Fig. 5. C/EBP β deficiency suppresses PC3 growth and improves *in vivo* efficacy of bortezomib.

(A) Growth of indicated PC3 lines after 3 and 5 days of culture (n=3). (B) Quantification of clonogenic growth of the indicated PC3 lines 12 days after seeding in 6-well plates at 200 cells/well. Crystal violet staining of representative wells (top) and mean colony numbers (bottom) are shown. (C) PC3 cells were incubated for 72 hrs in 0.5 μ g/ml doxycycline prior to treatment with the indicated concentrations of bortezomib for 24 hrs. Quantification of cell viability by Trypan blue dye exclusion is shown (left). Representative phase contrast images showing shNTV and shCEBPB PC3 cells that had been treated with 50 nM bortezomib for 24 hrs (right). (D) Quantification of cell viability by Trypan blue dye exclusion in PC3 cultures that had been treated with the indicated drugs for 24 hrs. Bort - 25 nM bortezomib; CQ - 50 μ M chloroquine. (E) Quantification of PC3 tumor growth in NSG mice treated with vehicle or bortezomib (1 mg/kg) on days 1, 4 and 8. (n = 7). Image to the right of the figure shows representative tumors from the indicated groups. Statistically significant differences were determined by linear regression analysis. Alpha adjusted by Holm-Bonferroni correction. Bar graphs represent the average of three experiments, error bars represent SEM. (* $p < 0.05$; ** $p < 0.025$; *** $p < 0.0167$).

Fig. 6. Model for C/EBP β driven autophagy in PCa cells. C/EBP β promotes autophagy in PCa cells by promoting autophagosome-lysosome fusion and by driving *REDD1* gene expression.

REDD1 suppresses LC3-II delipidation to LC3-I to increase autophagosome formation. Bortezomib may promote autophagy by increasing REDD1 protein levels. In addition, autophagosome-lysosome fusion is directly driven by C/EBP β through an as of yet undescribed mechanism (indicated by black box).

Table 1
PCR primers.

Gene	Forward (5' - 3')	Reverse (5' - 3')
<i>ACTB</i>	GACCTGGCTGGCCGGGACCT	GGCCATCTCTTGCTCGAAGT
<i>ATG4B</i>	ATGAGCATCGCGGAGCTTG	CTAGGGACAGGTTTCAGGACG
<i>ATG5</i>	GAAGCTGTTTCGTCCTGTGG	GATGTTCACCTCAGCCACTGC
<i>ATG7</i>	ACAGCTTGTTCTTCCAAAGTTCT	TCTCAGATGGTCTCATCATCGC
<i>CTSL</i>	CCTCAGCATGAGGAACACGA	AAAAGGTGCTGGTGGAGGTT
<i>BNIP3</i>	CCTCAGCATGAGGAACACGA	AAAAGGTGCTGGTGGAGGTT
<i>BNIP3L</i>	TGGAGCCATGAAGAAAGGGG	ACTTCACAGGTCACACGCAT
<i>GABARAP</i>	GGCTCCCAAAGCTCGGATAG	CTGGTACAGCTGACCCATTGT
<i>REDD1</i>	CGAACTCCCACCCCAGATCG	AACGACACCCCATCCAGGTA
<i>p62</i>	GATTCGCCGCTTCAGCTTCTG	GTCACTGGAAAAGGCAACCAA
<i>ULK1</i>	CGCCATCTCCTCAAGTTGGA	TGCAAGTCAGACAGGTTGGG

Table 2
ChIP primers.

Region	Forward (5' - 3')	Reverse (5' - 3')
REDD1 [-198 : -17]	GGGTTCGACTGCTGCGAGCTTTC	AGCAGCCTATAAGGACTAGCG
REDD1 [-720 : -534]	CTCTCCTTGGGAGCAGTTGG	TGCAAAGGCTCGGGATATGG