The CCAAT box-binding transcription factor NF-Y regulates basal expression of human proteasome genes

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ABSTRACT

Protein degradation by the proteasome plays an important role in all major cellular pathways. Aberrant proteasome activity is associated with numerous human diseases including cancer and neurological disorders, but the underlying mechanism is virtually unclear. At least part of the reason for this is due to lack of understanding of the regulation of human proteasome genes. In this study, we found that a large set of human proteasome genes carry the CCAAT box in their promoters. We further demonstrated that the basal expression of these CCAAT box-containing proteasome genes is regulated by the transcription factor NF-Y. Knockdown of NF-YA, an essential subunit of NF-Y, reduced proteasome gene expression and compromised the cellular proteasome activity. In addition, we showed that knockdown of NF-YA sensitized breast cancer cells to the proteasome inhibitor MG132. This study unveils a new role for NF-Y in the regulation of human proteasome genes and suggests that NF-Y may be a potential target for cancer therapy.

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1. Introduction

The 26S proteasome is the major cellular protease responsible for the degradation of regulatory and abnormal proteins [1]. Aberrant proteasome activity is often associated with human diseases including cancer and neurological disorders [2–7]. The 26S proteasome is comprised of a 20S core particle (CP) and one or two 19S regulatory particles (RPs). The 20S CP is a barrel-shaped structure consisting of a stack of four seven-subunit rings [8,9]. Both exterior rings contain one set of seven different α subunits; and both interior rings contain one set of seven different β subunits. The 20S CP performs three types of catalytic activities inside its chamber: chymotrypsin-like, trypsin-like, and caspase-like activities, which are provided by the $\beta 5$, $\beta 2$, and $\beta 1$ subunits, respectively. The 19S RP, which is attached to one or both ends of the 20S CP, is divided into two subcomplexes: the base and the lid [10,11]. The base is in contact with the 20S CP and consists of a ring of six different AAA + ATPases and three non-ATPase subunits, whereas the lid includes at least nine non-ATPase subunits. The connection between the lid and the base is stabilized by another non-ATPase subunit. The primary functions of the 19S RP include binding and unfolding substrates and facilitating their translocation into the 20S proteolytic chamber. Collectively, the 26S proteasome is formed by at least 33 different subunits, each encoded by a distinct gene.

How are the proteasome genes, in such a large number, regulated in the cell? Recent studies in the yeast *Saccharomyces cerevisiae* have provided important insight into the underlying mechanism [12]. The yeast proteasome genes are coordinately regulated by a transcription factor named Rpn4 [13,14]. An Rpn4 binding site, a 9-bp motif known as PACE (*P*roteasome-*A*ssociated *C*ontrol *E*lement), exists in the promoters of the proteasome genes. Deletion of the *RPN4* gene reduces the expression levels of proteasome genes. In fact, removal of the PACE motif from one of the proteasome genes leads to a substantial reduction in the abundance of assembled proteasome in the cell [15]. Interestingly, Rpn4 is an extremely short-lived protein ($t_{1/2} \leq 2$ min) and is degraded by the proteasome [14]. Stabilization of Rpn4 by inhibition of the proteasome activity results in upregulation of proteasome genes [16,17]. Thus, the proteasome homeostasis is controlled by a negative feedback circuit. On the one hand, Rpn4 induces the proteasome genes; on the other hand, Rpn4 is rapidly degraded by the proteasome. The Rpn4-proteasome feedback loop provides an efficient and sensitive means to gauge proteasome homeostasis. Disruption of the Rpn4-proteasome negative feedback circuit by inhibition of Rpn4 degradation or blockage of Rpn4-induced proteasome expression causes pleiotropic phenotypes, including a marked reduction in cell viability under stressed conditions [18,19].

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The proteasome genes in higher eukaryotes are also regulated by a feedback mechanism [12]. Knockdown of individual proteasome subunits by RNA interference (RNAi) results in upregulation of non-targeted subunits in *Drosophila* cells; and the feedback response is dependent on the 5′-untranslated regions (UTRs) of proteasome genes [20,21]. In addition, the suppression of proteasome activity by chemical inhibitors causes upregulation of proteasome genes in mammalian cells [7,22–25]. Several recent reports have suggested that nuclear factor erythroid-derived 2-related factor 1 (Nrf1) and factor 2 (Nrf2), which belong to the family of cap "n" collar-basic leucine zipper transcription factors, mediate feedback induction of proteasome genes in mammalian cells when the proteasome activity is compromised [22,26,27]. Nrf2 also plays an important role in upregulating proteasome genes in response to oxidative stress [28,29]. However, unlike Rpn4, which controls both basal expression and feedback induction of proteasome genes in yeast, the role of Nrf1 and Nrf2 in basal expression of mammalian proteasome genes is less clear. The basal expression levels of a number of proteasome subunits in $Nrf1^{-/-}$ and $Nrf2^{-/-}$ mouse embryonic fibroblasts (MEFs) and in the hepatic cells of $Nrf2^{-/-}$ mice are not significantly different from those in their wildtype counterparts [26,28]. These observations suggest that other transcription factors participate in the regulation of mammalian proteasome genes.

In this study, we set out to identify the transcription factor that controls basal expression of human proteasome genes. We found that a large set of human proteasome genes carries the CCAAT box in their promoters. We further demonstrated that the CCAAT box-binding transcription factor NF-Y binds these promoters. Knockdown of NF-YA, a subunit of NF-Y, reduced the expression levels of CCAAT box-containing proteasome genes, resulting in a lower cellular proteasome activity and sensitization of cells to proteasome inhibition. These results indicate that NF-Y plays a role in basal expression of human proteasome genes and suggest that NF-Y is a potential target for cancer therapy.

2. Materials and methods

2.1. Cell culture and plasmids

The MCF-10A cell line was maintained in DMEM/F12 with 5% horse serum (Invitrogen, CA), 10 μg/ml of insulin (Sigma-Aldrich, MO), 20 ng/ml of epidermal growth factor (EGF) (Invitrogen, CA), 0.5 μg/ml of hydrocortisone (Sigma-Aldrich, MO) and 100 ng/ml of cholera toxin (Calbiochem, CA). HEK293 and MDA-MB-231 cells were grown in DMEM/F12 with 10% fetal bovine serum (Invitrogen, CA). The MCF-10A and MDA-MB-231 cell lines were obtained from the cell bank of Karmanos Cancer Institute. Cell culture was conducted at 37 °C in the presence of 5% CO2. Details of plasmid construction are available upon request. The human PSMA5 gene promoter from −3097 to +121 was amplified from the genomic DNA of MCF-10A cells using high fidelity DNA polymerase and inserted upstream of the firefly luciferase reporter gene in the pGL3-Basic vector. The promoters of PSMA2 (−400 to +46), PSMA3 (−391 to +40) and PSMB3 (−400 to +32) were also amplified from MCF-10A genomic DNA by PCR and subcloned into the pGL3-Basic vector. Truncation and point mutation mutants of the PSMA5 promoter were generated by a conventional PCR-mediated procedure. The coding region of NF-YA was amplified from cDNAs generated by a reverse transcription reaction with RNA from MCF-10A cells and subcloned into the pCDNA3.1(+) expression vector.

2.2. Mapping of transcription initiation sites using RLM-RACE

Total RNA was prepared from MCF-10A cells using Trizol reagent purchased from Invitrogen (Carlsbad, CA). RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) was performed using the kit obtained from Ambion (Austin, TX). Briefly, RNA was sequentially treated with calf intestine alkaline phosphatase and tobacco acid pyrophosphatase. After 5′-RACE adaptor ligation and reverse transcription, the 5′-end sequence of the PSMA5 transcript was amplified using nested PCR. The outer and inner primers were 5′-GCCCACTCATGGCACAACCT-3′ and 5′-TGTCTGGATCCCAATGGCTGT-3′, respectively. The PCR products were resolved by electrophoresis using an 8% polyacrylamide gel and the PCR bands were recovered and submitted to DNA sequencing to define the transcription initiation sites.

2.3. Electrophoresis mobility shift assay (EMSA)

Cells were washed once with ice-cold phosphate buffered saline (PBS) and scraped into Eppendorf tubes. After centrifugation at 1000 rpm for 2 min at 4 °C, the pellet was resuspended in 200 μl buffer A (25 mM HEPES, pH7.9, 5 mM KCl, 0.5 mM MgCl2, 0.5 mM DTT, 1× protease inhibitor cocktail). Cell membrane was disrupted by mixing with 200 μl buffer B (25 mM HEPES, pH7.9, 5 mM KCl, 0.5 mM MgCl2, 1% NP-40, 0.5 mM DTT, 1× protease inhibitor cocktail) at 4 °C for 20 min. Nuclei were pelleted by centrifugation at 2500 rpm for 2 min. The pellet was washed once with buffer A, resuspended in 200 μl buffer C (25 mM HEPES, pH7.9, 350 mM NaCl, 10% sucrose, 0.01% NP-40, 0.5 mM DTT, 1× protease inhibitor cocktail), and rotated at 4 °C for 1 h. After centrifugation at 13,200 rpm for 15 min, nuclear extracts were collected and quantified using the Bradford method and stored at −80 °C. EMSA was conducted at room temperature for 30 min in a 10 μl reaction containing 10 mM Tris–HCl, pH7.9, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 6 μg of nuclear extract, 1 μg of poly(dI-dC).poly(dI-dC), 50 fmol of [α-^{32}P]-dCTP labeled DNA probe with or without 2.5 pmol of unlabeled competitor DNA. For supershift assay, 1 μl anti-NF-YA antibody (Santa Cruz Biotechnology) was added into the reaction. The reaction was subjected to electrophoresis with 4% polyacrylamine gel containing 0.5× TBE (Tris/Borate/EDTA) buffer and 4% glycerol. Radioactive signals were detected using the PhorsphorImager system or autoradiography. The following double-stranded oligonucleotides were used in EMSA as probes and/or competitors: NFY, 5′-GACCGTACGTGATTGGTTAATCTCTT-3′; NFYm, 5′-AGACCGTAC-GAAATACGGGAATCTCTT-3′; C/EBP, 5′-TGCAGATTGCGCAATCTGCA-3′.

2.4. Luciferase reporter assay

Cells were seeded at a density of $3–5\times10^4$ cells/well in 24-well plates 24 h prior to transfection. Cell transfection was performed using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). Luciferase reporter analysis was carried out 48 h after transfection using the Dual-Luciferase Reporter Assay System from Promega. To measure the effect of knockdown of NF-YA on luciferase expression, 10 pmol of small interfering RNA (siRNA) specific to NF-YA or GFP (as a control) was cotransfected with 64 ng of reporter DNA and 16 ng of pRL-SV40, an internal control plasmid, into each well. siNF-YA and control siRNA were purchased from Santa Cruz Biotechnology. For gene overexpression assay, 520 ng of expression vector, 224 ng of reporter plasmid and 56 ng of pRL-SV40 were cotransfected into cells. pCDNA3.1(+) vector was used to adjust total DNA input.

2.5. Real-time reverse transcription-polymerase chain reaction

Total RNA was treated with DNase I to eliminate contaminated genomic DNA. Reverse-transcription was carried out with SuperScript II reverse transcriptase (Invitrogen), and quantitative real-time PCR (qRT-PCR) was performed using Fast SYBR Green Master Mix in the StepOne™ system following the manufacturer's instruction (Applied Biosystems). The following primer sets were used: PSMA5, 5′-

AGCAATTGGCTCTGCTTCAG-3′ and 5′-GCATTCAGCTTCTCCTCCAT-3′;
PSMC2, 5′-ATGTTGGTGGCTGTAAGG-3′ and 5′-CACGCATCAGTCC-
GATTA-3′; PSMD3, 5′-AGGCCUATCTCGCGCTCGTGTG-3′ and 5′-ACGC-
GUGATGTGGAAGGCAGCAT-3′; NF-YA, 5′-GAGTCTCGGCACCGTCAT-3′
and 5′-TGCTTCTTCATCGGCTTG-3′; NRF1, 5′-TTGGCGACAGGATATTGA-
3′ and 5′-CTGGAAATGTCTGCTGGA-3′; NRF2, 5′-AAACCAGTGGATCTGC-
CAC-3′ and 5′-GACCGGGAATATCAGGAACA-3′; GAPDH, 5′-CAGCCT-
CAAGATCATCAGCA-3′ and 5′-TGTGGTCATGAGTCCTTCCA-3′.

2.6. Proteasome activity assay and immunoblotting analysis

Cells were washed three times with ice-cold PBS and scraped for
centrifugation. Cell pellets were resuspended in lysis buffer (50 mM
Tris-Cl, pH 7.5, 10 mM MgCl2, 100 mM KCl, 1% Triton X-100, 10% glyc-
erol, 1 mM DTT, 1× protease inhibitor cocktail) and mixed with rota-
tion at 4 °C for 2 h. Cell extracts were centrifuged at 13,200 rpm for
10 min at 4 °C. Protein concentration was determined using Bradford
Assay Kit (Bio-Rad, CA). To measure the proteasome activity, 20 μg of
cell extract was incubated with the Suc-Leu-Leu-Val-Tyr-AMC pep-
tide substrate (Boston BioChem, MA) at a final concentration of
40 μM. The reaction was set at 37 °C and lasted for 1 h. The released
fluorescence signals were recorded by VersaFluot Fluorometer (Bio-
Rad, CA) with an excitation filter of 355 nm and an emission filter of
460 nm. For immunoblotting analysis, cell lysate was denatured by
heating at 95 °C for 3 min and resolved by SDS-PAGE, followed by im-
munoblotting with an anti-NF-YA or anti-ubiquitin antibody (Santa
Cruz Biotechnology) and detection with the Odyssey Infrared Imaging
System (LI-COR Biosciences, NE). The blots were re-probed with an
anti-β-actin antibody (Sigma) to ensure comparable loading.

2.7. Cell viability assay

The MDA-MB-231 breast cancer cells were transfected with siNF-
YA or siGFP. Overnight after transfection, cells were seeded in 96-well
plates at a density of 4000–6000 cells per well and treated with 2 μM
MG132 or DMSO for 48 h. Cell viability was measured by the MTT
assay as previously described [45]. 3-(4, 5-dimethyl-2-thiazolyl)-2,
5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from
Sigma-Aldrich (St. Louis, MO), whereas MG132 was obtained from
BostonBiochem (Cambridge, MA). The values shown were the
means of three independent experiments.

2.8. DNA sequence analysis

The Dialign TF and MatInspector software from Genomatix [46]
was used for DNA sequence alignment and location of potential tran-
scription factor binding sites in the proteasome gene promoters.

3. Results

3.1. NF-Y binds human proteasome genes via the CCAAT box

To explore how the human proteasome genes are regulated, we
used the MatInspector and Dialign TF software to analyze the DNA se-
quences of the promoters of 14 20S proteasome genes (PSMA1-7 and
PSMB1-7) and 19 19S regulator genes (PSMC1-6, PSMD1-4 and
6–14). The analyzed sequences spanned ~400 bp upstream and
~100 bp downstream from the putative transcription initiation sites
annotated in databases. We found that a large set of human protea-
some genes, including 6 20S proteasome genes (PSMA2, PSMA3,
PSMA5, PSMB1, PSMB2 and PSMB3) and 6 19S regulator genes
(PSMC2, PSMC3, PSMC5, PSMD4, PSMD10 and PSMD14), contains
one or more CCAAT boxes in their promoters (Fig. 1). To examine if
NF-Y (nuclear factor Y) and C/EBP (CCAAT/Enhancer Binding Protein),
two known CCAAT box binding transcription factors [30], could bind
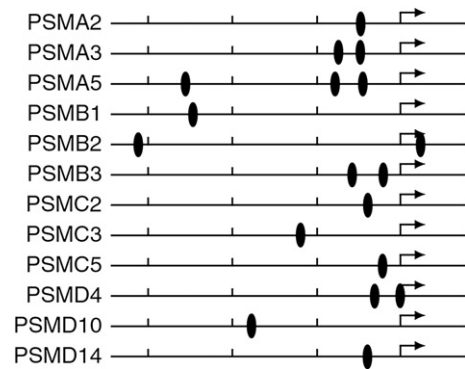the CCAAT box-containing proteasome genes, we used the

Fig. 1. Distribution of the CCAAT box in human proteasome genes. The promoter se-
quences from ~400 bp upstream and ~100 bp downstream from the annotated tran-
scription initiation sites of known human proteasome genes in databases were
analyzed by the MatInspector and Dialign TF software. The genes containing the
CCAAT box and the relative positions of the CCAAT boxes are presented.

electrophoresis mobility shift assay (EMSA). The PSMA5 promoter,
which was experimentally defined in this study (see below), was
first chosen for the EMSA. The PSMA5 promoter has three CCAAT
boxes ($-251/-247$, $-83/-79$, $-54/-50$) (Fig. 3B). The promoter
region from -126 to $+77$ was used as a probe. As shown in
Fig. 2A, the consensus NF-Y oligonucleotide (NFY) inhibited the bind-
ing of ^{32}P-labeled probe to nuclear proteins as efficiently as the unla-
beled (cold) probe (compare lanes 3, 4 to 2). In contrast, a mutant
NFY oligonucleotide (NFYm) with substitutions at the core CCAAT se-
quence showed no competition (lane 5). Another oligonucleotide
(A5-NFY-1) corresponding to the PSMA5 promoter sequence from
-64 to -38, including the proximal CCAAT box, also eliminated
the DNA–protein complex (lane 6). In addition, a DNA fragment
(A5-NFY-1 m) containing the middle CCAAT box of the PSMA5 pro-
moter displayed a substantial competition effect (lane 7). Moreover,
an antibody to NF-YA, a subunit of NF-Y, was able to form super-
shifted DNA–protein complexes (lane 9). Unlike NFY, the consensus
C/EBP oligonucleotide failed to compete with the ^{32}P-labeled probe
(lane 8). Together, these results demonstrate that NF-Y binds the
PSMA5 promoter via its CCAAT boxes.

We went on to examine if NF-Y binds other CCAAT box-containing
proteasome genes. The promoters of PSMA2, PSMA3, and PSMB3
were analyzed by EMSA in this study, with PSMA5 as a positive con-
trol. Whereas the gel-shift patterns were somewhat different, the
NFY competitor eliminated at least one of the major DNA–protein
complexes formed by the PSMA2, PSMA3, and PSMB3 promoter se-
quences (Fig. 2B, marked by arrowheads). Moreover, the PSMA2,
PSMA3, and PSMB3 promoter sequences were able to inhibit the for-
mation of DNA–protein complex by the PSMA5 probe (Fig. 2C, com-
pare lanes 4–6 to 2). In contrast, the PSMD3 and PSMD9 promoters,
which lack the CCAAT box, could not compete with the PSMA5
probe (Fig. 2C, lanes 7, 8). Thus, NF-Y specifically binds to the protea-
some gene promoters carrying the CCAAT box.

3.2. The CCAAT box is important for the PSMA5 promoter activity

Our initial analysis of the relevance of the CCAAT box to regulation
of proteasome gene expression was focused on the PSMA5 promoter.
We began with the determination of its transcription initiation site(s)
using 5′-RLM-RACE, a method that specifically detects authentic
capped 5′ ends of mRNAs [31]. Total RNA was prepared from the
human mammary epithelial cell line MCF-10A [32]. Two bands were
obtained from the 5′ RACE PCR reaction (Fig. 3A). DNA sequencing
analysis of the recovered PCR bands located the transcription initia-
tion sites to 118 and 107 nucleotides upstream from the translation
start codon (Fig. 3B). The upstream transcription initiation site was
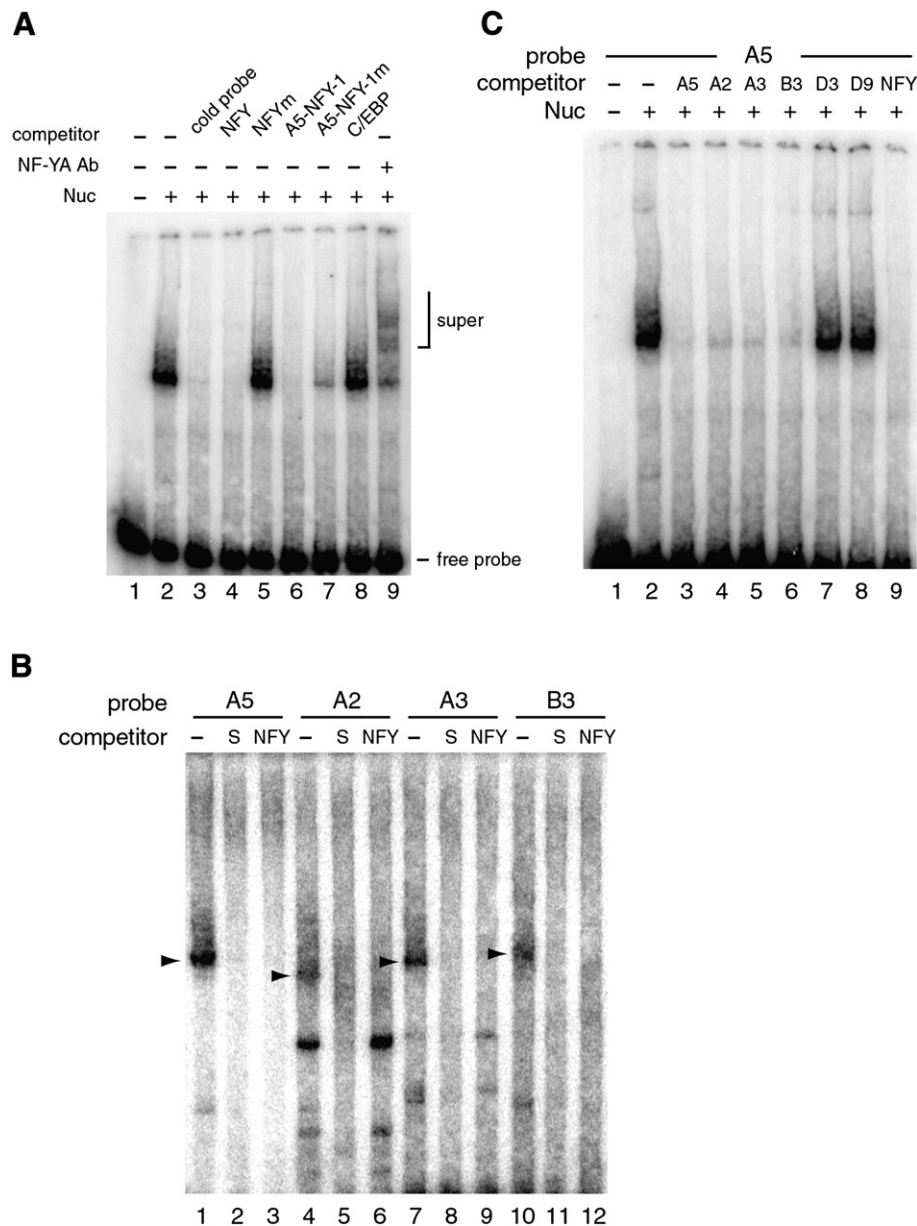numbered $+1$ to refer the promoter sequence analyzed in this

Fig. 2. NF-Y binds human proteasome genes via the CCAAT box. (A) NF-Y but not C/EBP binds the PSMA5 promoter. The EMSA procedure was described in Materials and methods. A fragment of the PSMA5 promoter (-126 to $+77$) was used as a probe. Nuclear extracts (Nuc) were prepared from Hela cells. Fifty fold excess of competitor oligonucleotides or unlabeled (cold) probe was used for competition assays. Competitors used included the consensus NF-Y oligonucleotide (NFY), a mutant NFY oligonucleotide (NFYm), an oligonucleotide (A5-NFY-1) corresponding to the PSMA5 promoter sequence from -64 to -38, and a DNA fragment (A5-NFY-1m) containing the middle CCAAT box of the PSMA5 promoter. Supershifted bands formed by the anti-NF-YA antibody are marked (lane 9). (B) NF-Y binds various human proteasome genes carrying the CCAAT box. The probes used in EMSAs were derived from PSMA2 (-220 to -18), PSMA3 (-211 to -2), PSMA5 (-126 to $+77$), and PSMB3 (-177 to $+32$). The competitors included respective cold probes (S) and NFY. The bands eliminated by NFY are indicated by arrowheads. (C) The binding of NF-Y to the PSMA5 promoter is inhibited by other promoter fragments containing the CCAAT box. The ^{32}P-labelled PSMA5 probe was the same as in A. The competitors included cold probes of PSMA2, PSMA3, PSMA5 and PSMB3 as in B, and the promoter sequences of PSMD3 (-152 to $+48$) and PSMD9 (-132 to 65).

study. Note that there is no canonical TATA-box within a 468-bp region upstream of the translation start codon, suggesting that the PSMA5 gene may be driven by a TATA-less promoter.

We then delineated the proximal promoter region of the PSMA5 gene. A sequence from -3097 to $+121$ of the PSMA5 gene was amplified by PCR and ligated to the luciferase reporter gene in the pGL3-Basic vector. Based on this construct, we generated a series of 5′ end deletion mutants and measured their transcription activities in HEK293 cells using the luciferase assay (Fig. 3C). The transcription activity of the region from -311 to $+121$ was comparable to that from -3097 to $+121$. Further deletion to -84 significantly weakened the transcription activity. We noticed that a deletion from $+77$ to $+121$ appreciably reduced the expression level of the luciferase reporter.

This result indicates that the 5′ UTR is important for basal transcription of the PSMA5 promoter. Together, these results demonstrate that the region from -311 to $+121$ constitutes the proximal promoter of PSMA5. Interestingly, all three CCAAT boxes reside in this region.

To examine whether the CCAAT boxes are important for the activity of the PSMA5 promoter, we replaced individual CCAAT boxes or a combination of any two or all three boxes with irrelevant sequences, and measured the strength of the mutated promoters using luciferase assays (Fig. 3D). We found that mutation of the middle or the proximal CCAAT box substantially impaired the promoter activity. Moreover, simultaneous replacement of these two CCAAT boxes generated a marked additive effect. By contrast, mutation of the distal CCAAT
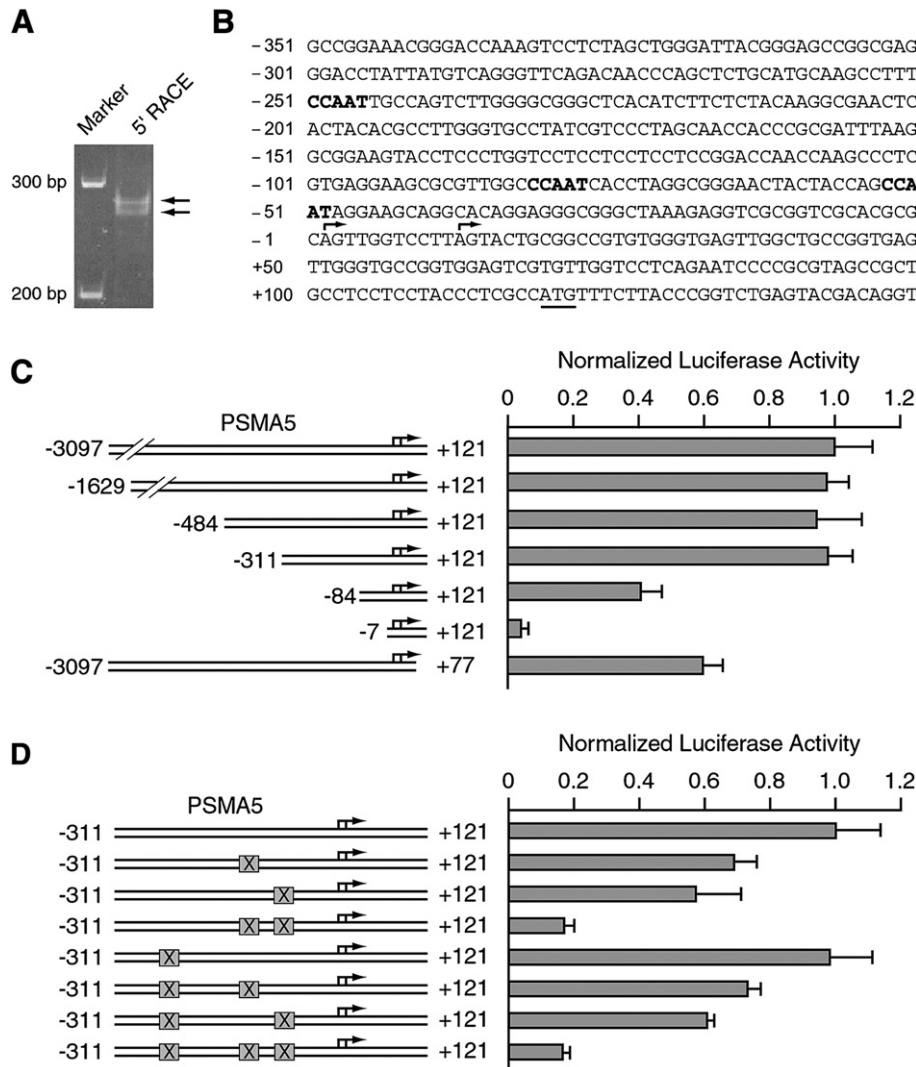
Fig. 3. The CCAAT box is critical for the PSMA5 promoter activity. (A) Determination of the transcription initiation sites of PSMA5 by 5′RLM-RACE. Two PCR products resulting from the PSMA5 transcripts are marked by arrows. (B) The nucleotide sequences flanking the transcription initiation sites of the PSMA5 promoter. The two transcription initiation sites are indicated by arrows, with the upstream one numbered +1 to refer the promoter sequence. The translational start codon is underlined. The CCAAT boxes are in bold. (C) The proximal PSMA5 promoter residues in the region from −311 to +121. A panel of firefly luciferase reporter constructs containing various PSMA5 promoter fragments were cotransfected with a *Renilla* luciferase expression vector into HEK293 cells. Firefly luciferase activity was normalized against *Renilla* luciferase activity, and the relative activity was set at 1.0 for the full-length PSMA5 promoter (−3097 to +121). The values shown are the means of at least three independent experiments. (D) Mutational analysis of the CCAAT boxes. Individual CCAAT boxes or a combination of two or three were mutated and their effects on the PSMA5 promoter activity were assessed by luciferase assays.

box did not affect the promoter activity. Thus, the proximal and the middle CCAAT boxes are critical for the PSMA5 promoter activity.

3.3. NF-Y controls the expression of CCAAT box-containing proteasome genes

NF-Y is a heteromeric complex composed of 3 subunits, NF-YA, NF-YB and NF-YC, which all are required for binding the CCAAT box [30]. To address the role of NF-Y in basal expression of human proteasome genes, we measured the effect of NF-YA knockdown on transcription of CCAAT box-containing proteasome genes. siRNA oligos for NF-YA (siNF-YA) or GFP (as a control) were transfected into HEK293 cells. Immunoblotting analysis showed that the expression level of NF-YA was substantially reduced by siNF-YA (Fig. 4A). Using quantitative real time-PCR (qRT-PCR), we found that the mRNA levels of PSMA5 and PSMC2 were at least 50% lower in the cells transfected with siNF-YA than control cells transfected with siGFP (Fig. 4B). In line with the qRT-PCR data, knockdown of NF-YA reduced the

expression levels of the luciferase reporter driven by the promoters of PSMA2, PSMA3, PSMA5 and PSMB3, respectively (Fig. 4C). It is of interest to note that knockdown of NF-YA did not affect the mRNA level of the PSMD3 proteasome gene whose promoter lacks the CCAAT box (Fig. 4B). Thus, NF-Y is required for normal level expression of CCAAT box-containing proteasome genes.

We then examined if overexpression of NF-YA could enhance transcription of CCAAT box-containing proteasome genes. An NF-YA overexpression vector or a void vector (control) was cotransfected into HEK293 cells with a luciferase reporter expressed from the promoters of PSMA2, PSMA3, PSMA5 or PSMB3. Luciferase assays showed that the transcription levels of these promoters were indeed increased by overexpression of NF-YA (Fig. 5A). The effect of NF-YA overexpression was also revealed by qRT-PCR (Fig. 5B). The mRNA levels of PSMA5 and PSMC2 were elevated by overexpression of NF-YA. In contrast, the PSMD3 mRNA level was not altered. Together, the knockdown and overexpression experiments demonstrate that NF-Y controls the transcription of CCAAT box-containing human proteasome genes.
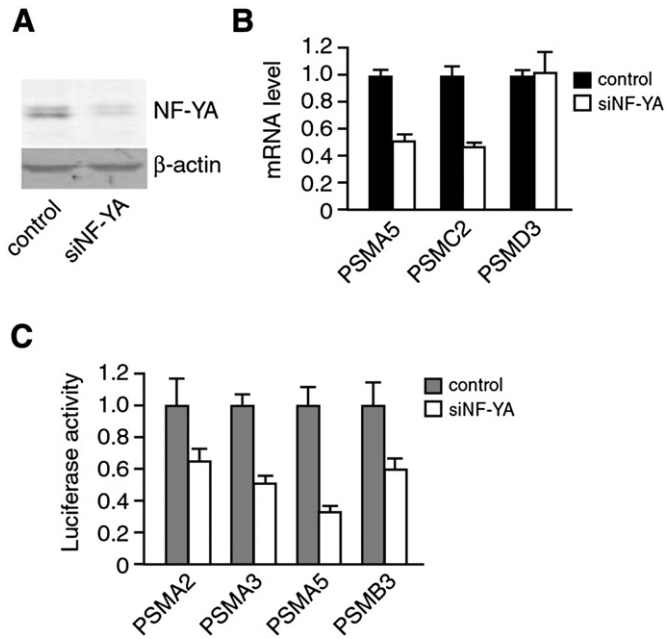
A

NF-YA

β-actin

control   siNF-YA

B

C

Fig. 4. Knockdown of NF-YA reduces the basal expression of CCAAT box-containing human proteasome genes. (A) Immunoblotting analysis shows knockdown of NF-YA by siNF-YA. HEK293 cells were transfected with siRNA oligos for NF-YA (siNF-YA) or GFP (siGFP). The latter served as a control. Forty-eight hours after transfection, cell extracts were prepared and examined by immunoblotting with an anti-NF-YA antibody (upper panel). Comparable loading was confirmed by re-probing the blot with an anti-β-actin antibody (lower panel). (B) Knockdown of NF-YA reduces the mRNA levels of PSMA5 and PSMC2 but has no effect on PSMD3. siNF-YA and siGFP were transfected into HEK293 cells, respectively. Total RNA was prepared 48 h after transfection and subjected to qRT-PCR analysis. The mRNA levels from siGFP transfectants were set at 1.0. (C) Knockdown of NF-YA impairs the transcription activity of CCAAT box-containing proteasome gene promoters. siNF-YA or siGFP was cotransfected into HEK293 cells with luciferase reporters expressed from the promoters of PSMA2 (-400 to $+46$), PSMA3 (-391 to $+40$), PSMA5 (-311 to 121), or PSMB3 (-400 to $+32$). The effect of NF-YA knockdown on transcription of the promoters was measured by luciferase assays. The relative luciferase activity with control siRNA was set at 1.0.
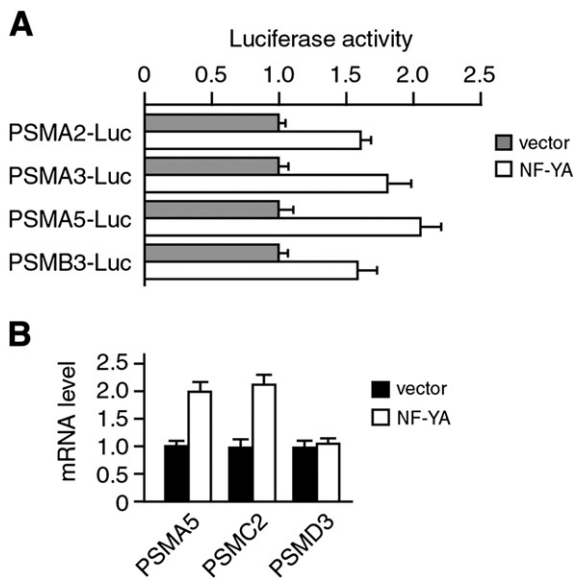
3.4. Knockdown of NF-YA reduces cellular proteasome activity and sensitizes breast cancer cells to proteasome inhibitor MG132

Our early work has shown that downregulation of a single proteasome gene is sufficient to reduce the proteasome activity in yeast cells [15]. We reasoned that knockdown of NF-YA could lead to a similar effect in human cells even though not all human proteasome genes are regulated by NF-Y. To test this hypothesis, we transfected siNF-YA into the MDA-MB-231 breast cancer cells using siGFP as a control. Cell extracts were prepared from the transfectants and their proteasome activities were compared by two assays. In the first assay, the cell extracts were incubated with the fluorogenic peptide substrate Suc-LLVY-AMC. Degradation of Suc-LLVY-AMC by the proteasome was measured by the release of the fluorescent AMC group. We found that the proteasome activity was lower in the siNF-YA transfectants than the control cells (Fig. 6A). In the second assay, the cell extracts were resolved on SDS-PAGE and subjected to immunoblotting analysis with an anti-ubiquitin antibody. Clearly, more ubiquitylated proteins were accumulated in the siNF-YA transfectants than the control cells (Fig. 6B). Together, these results indicate that knockdown of NF-YA causes a reduction in cellular proteasome activity.

The effect of knockdown of NF-YA on cellular proteasome activity prompted us to examine if it could sensitize cancer cells to proteasome inhibitors. As a proof-of-concept, we applied the proteasome inhibitor MG132 to treat MDA-MB-231 cells transfected with siNF-YA or siGFP. The cell viability was measured by the MTT assay. As shown in Fig. 6C, knockdown of NF-YA indeed enhanced the cytotoxic effect of MG132.

4. Discussion

It is well documented that the expression of proteasome genes in yeast is coordinately controlled by Rpn4 via the PACE motif in their promoters [12]. The proteasome genes in mammalian cells are also regulated in a concerted manner, but current knowledge of the cis-
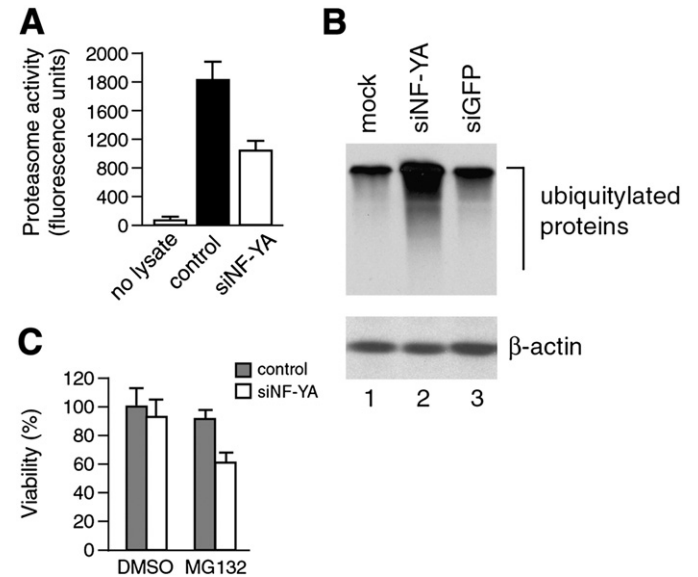
A

B

C

Fig. 5. Overexpression of NF-YA increases the expression of CCAAT box-containing proteasome genes. (A) Overexpression of NF-YA enhances the transcription of PSMA2, PSMA3, PSMA5 and PSMB3 promoters. An NF-YA overexpression vector or a control plasmid was cotransfected into HEK293 cells with luciferase reporters expressed from the promoters of PSMA2, PSMA3, PSMA5 and PSMB3, respectively. The effect of NF-YA overexpression was assessed by luciferase assays. (B) Overexpression of NF-YA increases the expression of PSMA5 and PSMC2 but not PSMD3. The mRNA levels of PSMA5, PSMC2 and PSMD3 in the cells overexpressing NF-YA or a control vector were measured by qRT-PCR.

Fig. 6. Knockdown of NF-YA impairs cellular proteasome activity and sensitizes cells to proteasome inhibition. (A) Extracts prepared from cells transfected with siNF-YA or siGFP were incubated with the fluorogenic peptide substrate Suc-LLVY-AMC. The proteasome activity reflected on the degradation of Suc-LLVY-AMC was measured by recording the release of the fluorescent AMC group. (B) Cellular extracts as in A were resolved on SDS-PAGE and subjected to immunoblotting analysis with an anti-ubiquitin antibody. Accumulated ubiquitylated proteins are marked. (C) Sensitization of MDA-MB-231 breast cancer cells to proteasome inhibitor MG132 by knockdown of NF-YA. MDA-MB-231 cells were transfected with siNF-YA or siGFP, followed by treatment with 2 μM MG132 or DMSO. Cell viability was measured by the MTT assay.

acting regulatory elements and transcription factors that are involved remains limited. In this study, we demonstrate that the NF-Y transcription factor controls basal expression of a large set of human proteasome genes through binding the CCAAT box in their promoters. Knockdown of NF-YA leads to a reduction in cellular proteasome activity due to downregulation of CCAAT box-containing proteasome genes. Phylogenetic analysis of the PSMA5 promoter shows that the CCAAT box is conserved both in sequence and in position (Fig. 7), suggesting that NF-Y contributes to basal expression of proteasome genes in other mammals. This is consistent with NF-Y being a well conserved and ubiquitous transcription factor. Previous studies have shown that NF-Y supports the transcription of a variety of genes, including some key players in controlling cell proliferation and differentiation such as E2F1, cdc25B, cdc25C, cyclin A, cyclin B1, FGF, and ODF [33–39]. Our study, for the first time, reveals a role for NF-Y in basal expression of proteasome genes. It is of interest to note that NF-YA, the limiting subunit of NF-Y, is subject to ubiquitin-dependent proteasomal degradation [47]. This suggests that NF-Y may also participate in feedback regulation of human proteasome genes.

NF-Y is not the sole transcription factor responsible for basal expression of human proteasome genes. Some of the human proteasome genes do not contain the CCAAT box. Indeed, the expression level of PSMD3 was not affected by knockdown or overexpression of NF-Y (Figs. 4B, 5B), demonstrating that proteasome genes lacking the CCAAT box are regulated by other transcription factors. We noticed that nuclear proteins besides NF-Y were recruited to the promoters of PSMA2 and PSMA3 in EMSA (Fig. 2B), implying that even CCAAT box-containing proteasome genes may be regulated by other transcription factors in addition to NF-Y. A recent report suggested that heat shock factor 2 (HSF2) may play a role in regulating basal transcription of mouse proteasome genes [40]. This assumption was based on the observation that the expression levels of a large number of proteasome genes are lower in $Hsf2^{-/-}$ mouse embryonic fibroblasts (MEFs) than in their wildtype counterparts. It is currently unclear, though, whether HSF2 regulates proteasome genes directly by binding their promoters or via an indirect manner. Nrf1 and Nrf2 are two other transcription factors that may be involved in basal expression of human proteasome genes. Lee et al. recently showed

that the expression levels of a subset of proteasome genes are downregulated in the neuron cells of $Nrf1^{-/-}$ knockout mice [41]. The involvement of Nrf2 in basal expression of human proteasome genes was hinted by the correlation of increased proteasome gene expression with overexpression of Nrf2 in human colon cancer cells [42]. We also found that knockdown of Nrf1 or Nrf2 reduced basal expression of several proteasome genes in the MCF-7 breast cancer cell line (data not shown). In line with the observations in mammalian cells, silencing of Cnc-C, the Drosophila counterpart of Nrf1/Nrf2, caused a decline in proteasome gene expression in Drosophila cells [43]. Nrf1 and Nrf2 appear to regulate only a subset of proteasome genes. It was shown that the basal expression of some proteasome genes in $Nrf1^{-/-}$ and $Nrf2^{-/-}$ MEFs and in the hepatic cells of $Nrf2^{-/-}$ mice is not significantly different from that in their wildtype counterparts [26,28]. It is likely that different transcription factors control different sets of proteasome genes. This explains why multiple transcription factors participate in the regulation of proteasome genes in mammalian cells. It will be of interest to categorize all the human proteasome genes into different groups based on the relevant transcription factors.

It is worthy of note that downregulation of NF-YA sensitizes MDA-MB-231 breast cancer cells to proteasome inhibitor MG132. This is in line with our early observation that depletion of Rpn4 displayed synthetic lethality with mutation of proteasome genes in yeast cells [16]. Similar to the effect of NF-YA knockdown, silencing of individual proteasome genes by RNAi enhances the cytotoxicity of proteasome inhibitors in cancer cells [44]. In fact, we have demonstrated in yeast cells that downregulation of a single proteasome gene is sufficient to reduce cell viability when the proteasome activity is crippled by stressed conditions [15]. The current study suggests that NF-Y may be a potential target for cancer therapy, especially in combined therapy with proteasome inhibitors.

5. Conclusion

In summary, our data show that NF-Y regulates a large set of human proteasome genes via binding their CCAAT boxes. Knockdown of NF-Y reduces the expression of these proteasome genes and
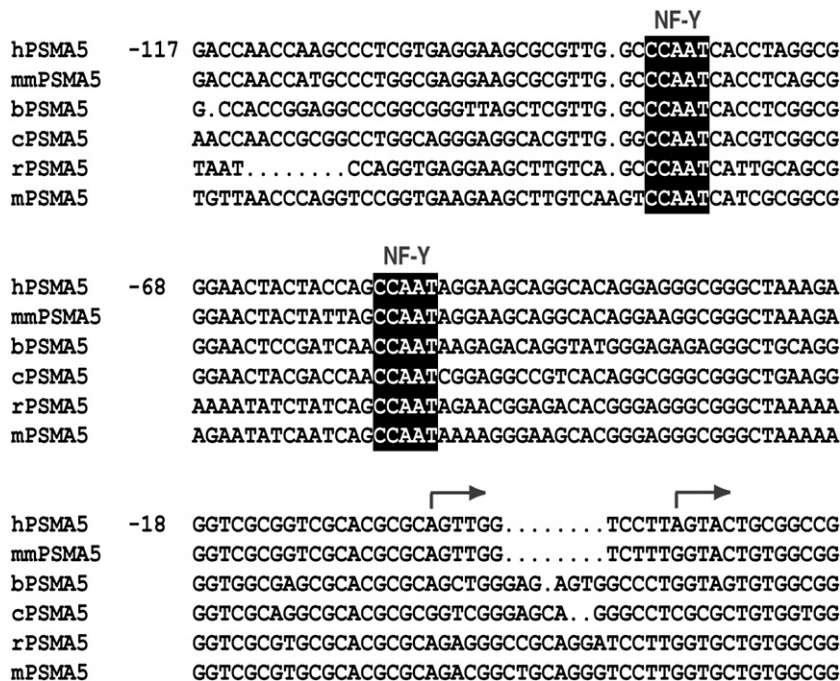

Fig. 7. Phylogenetic analysis of the PSMA5 promoter. The sequences of the PSMA5 promoters from human (h), *Macaca mulatta* (mm), *Bos taurus* (b), *Canis familiaris* (c), rat (r), and mouse (m) were aligned using Dialign TF software. The CCAAT boxes are highlighted. The arrows mark the transcription initiation sites of human PSMA5.

compromises the cellular proteasome activity. In addition, knockdown of NF-Y sensitizes breast cancer cells to the proteasome inhibitor MG132. It will be of great interest to investigate if silencing of NF-Y may also synergize the cytotoxic effect of proteasome inhibitors in other types of cancer cells.

Acknowledgements

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