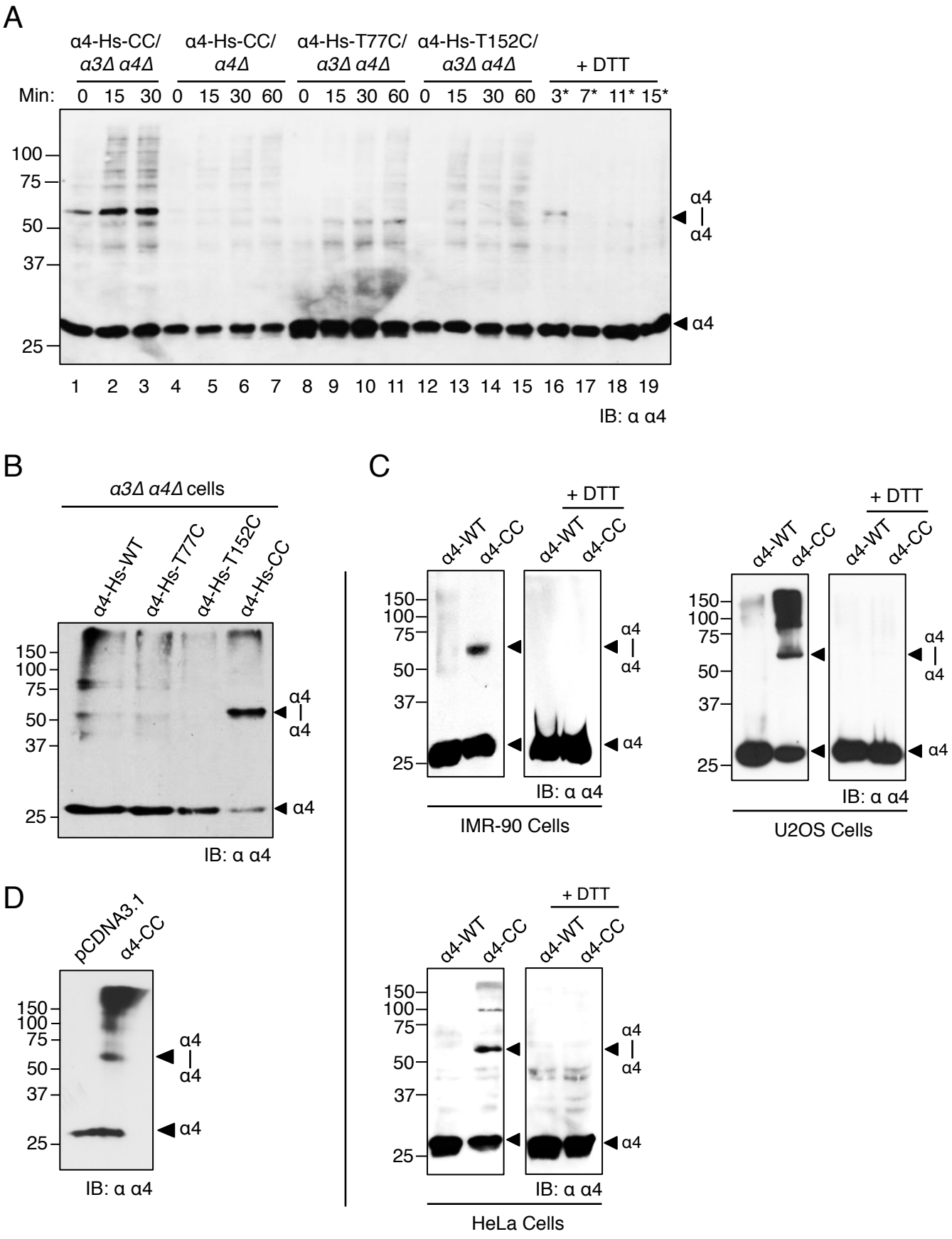
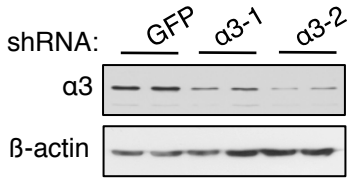
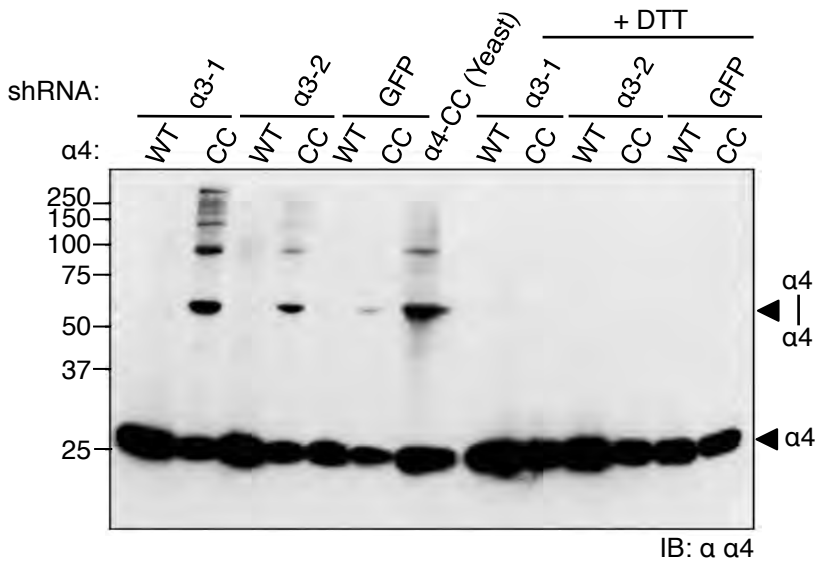

A
α4-Hs-CC/ α3Δ α4Δ
α4-Hs-CC/ α4Δ
α4-Hs-T77C/ α3Δ α4Δ
α4-Hs-T152C/ α3Δ α4Δ
+ DTT
Min: 0 15 30 0 15 30 60 0 15 30 60 0 15 30 60 3* 7* 11* 15*
100
75
50
37
25
α4 | α4
α4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
IB: α α4
B
α3Δ α4Δ cells
α4-Hs-WT
α4-Hs-T77C
α4-Hs-T152C
α4-Hs-CC
150
100
75
50
37
25
α4 | α4
α4
IB: α α4
C
+ DTT
α4-WT
α4-CC
α4-WT
α4-CC
150
100
75
50
37
25
α4 | α4
α4
IB: α α4
IMR-90 Cells
+ DTT
α4-WT
α4-CC
α4-WT
α4-CC
150
100
75
50
37
25
α4 | α4
α4
IB: α α4
U2OS Cells
D
pCDNA3.1
α4-CC
150
100
75
50
37
25
α4 | α4
α4
IB: α α4
+ DTT
α4-WT
α4-CC
α4-WT
α4-CC
150
100
75
50
37
25
α4 | α4
α4
IB: α α4
HeLa Cells

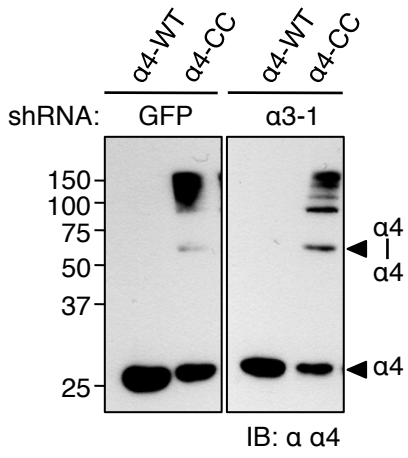
A

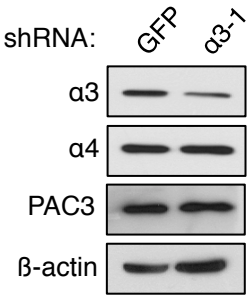

shRNA:
GFP
α3-1
α3-2
α3
ß-actin

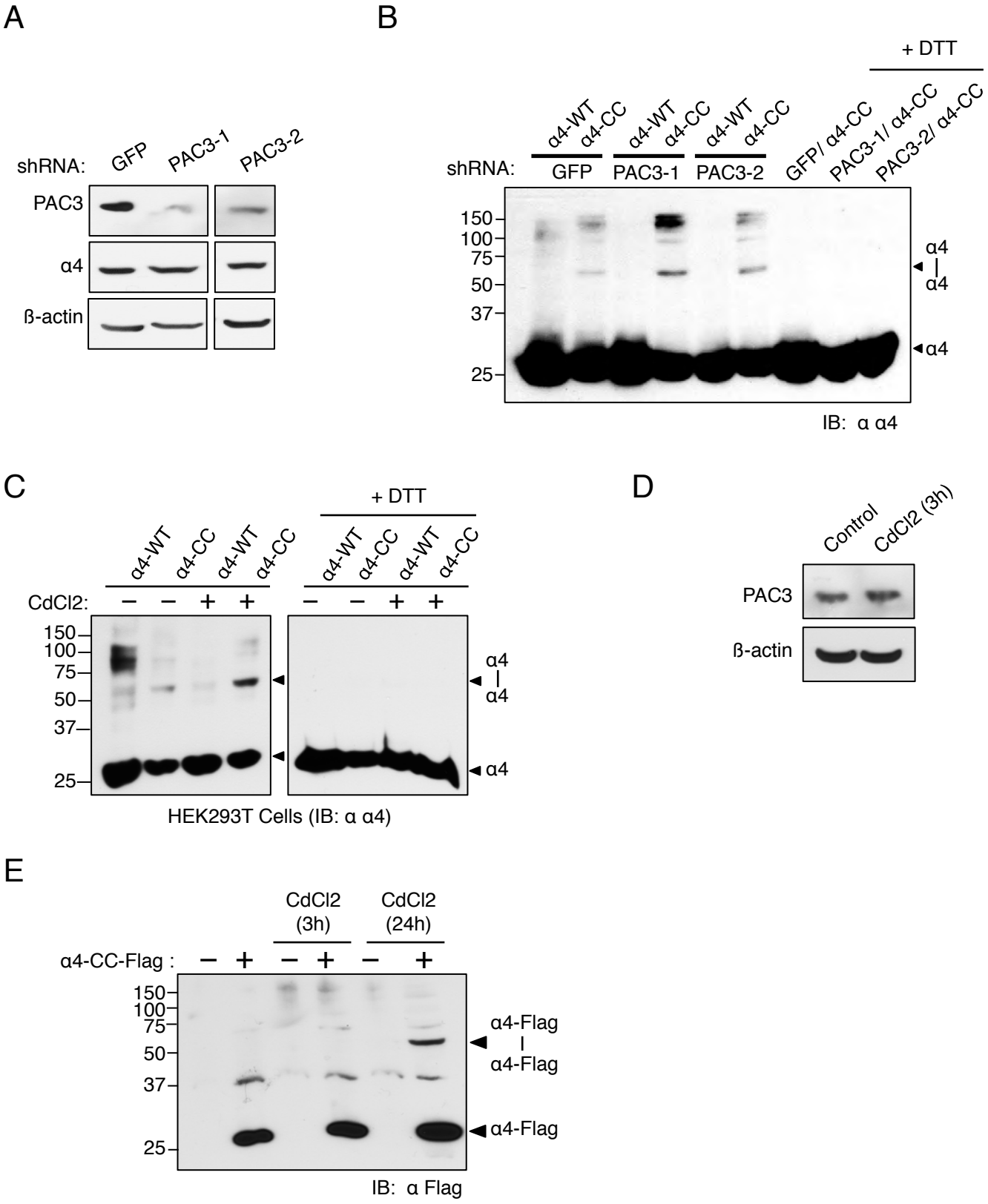
B


shRNA:
α3-1
α3-2
GFP
α4-CC (Yeast)
+ DTT
α4:
WT
CC
250
150
100
75
50
37
25
α4
α4
α4
IB: α α4

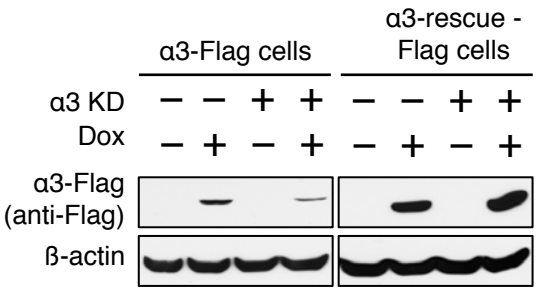
C


α4-WT
α4-CC
shRNA:
GFP
α3-1
150
100
75
50
37
25
α4
α4
α4
IB: α α4

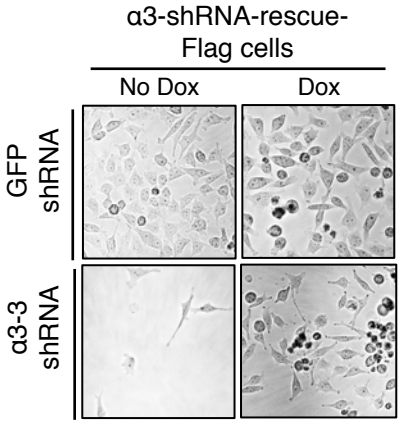

shRNA:
GFP
α3-1
α3
α4
PAC3
ß-actin


A
shRNA:
GFP
PAC3-1
PAC3-2
PAC3
α4
ß-actin
B
+ DTT
α4-WT
α4-CC
shRNA:
GFP
PAC3-1
PAC3-2
GFP/ α4-CC
PAC3-1/ α4-CC
PAC3-2/ α4-CC
150
100
75
50
37
25
α4
α4
α4
IB: α α4
C
+ DTT
α4-WT
α4-CC
CdCl2:
HEK293T Cells (IB: α α4)
D
Control
CdCl2 (3h)
PAC3
ß-actin
E
CdCl2 (3h)
CdCl2 (24h)
α4-CC-Flag :
α4-Flag
α4-Flag
α4-Flag
IB: α Flag

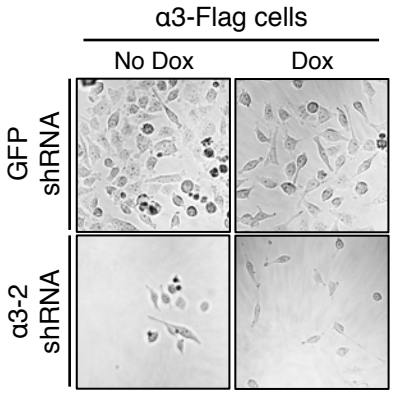
A


α3-Flag cells
α3-rescue -
Flag cells
α3 KD
Dox
α3-Flag
(anti-Flag)
ß-actin

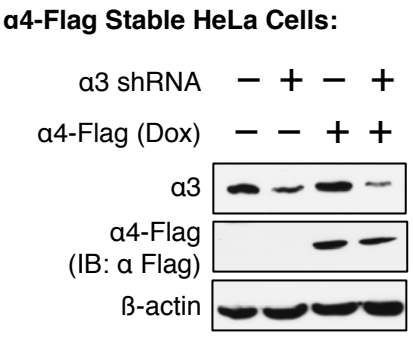
B


α3-shRNA-rescue-
Flag cells
No Dox
Dox
GFP
shRNA
α3-3
shRNA

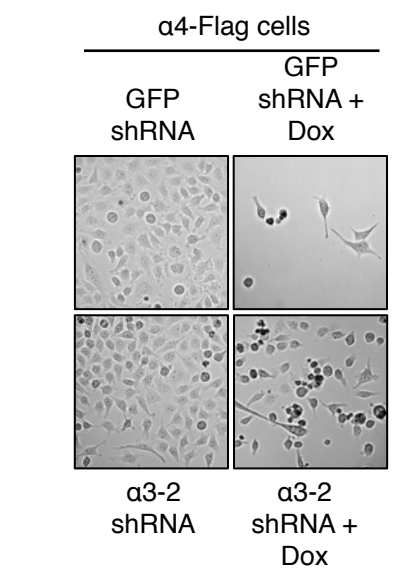
C


α3-Flag cells
No Dox
Dox
GFP
shRNA
α3-2
shRNA

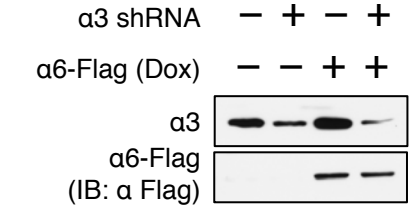
D


α4-Flag Stable HeLa Cells:
α3 shRNA
α4-Flag (Dox)
α3
α4-Flag
(IB: α Flag)
ß-actin

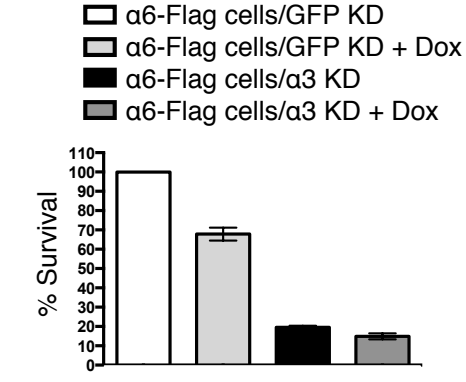
E


α4-Flag cells
GFP
shRNA
GFP
shRNA +
Dox
α3-2
shRNA
α3-2
shRNA +
Dox

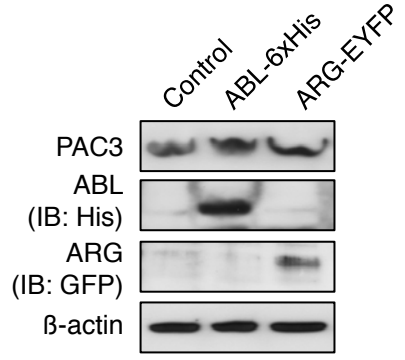
F


α6-Flag Stable HeLa Cells:
α3 shRNA
α6-Flag (Dox)
α3
α6-Flag
(IB: α Flag)

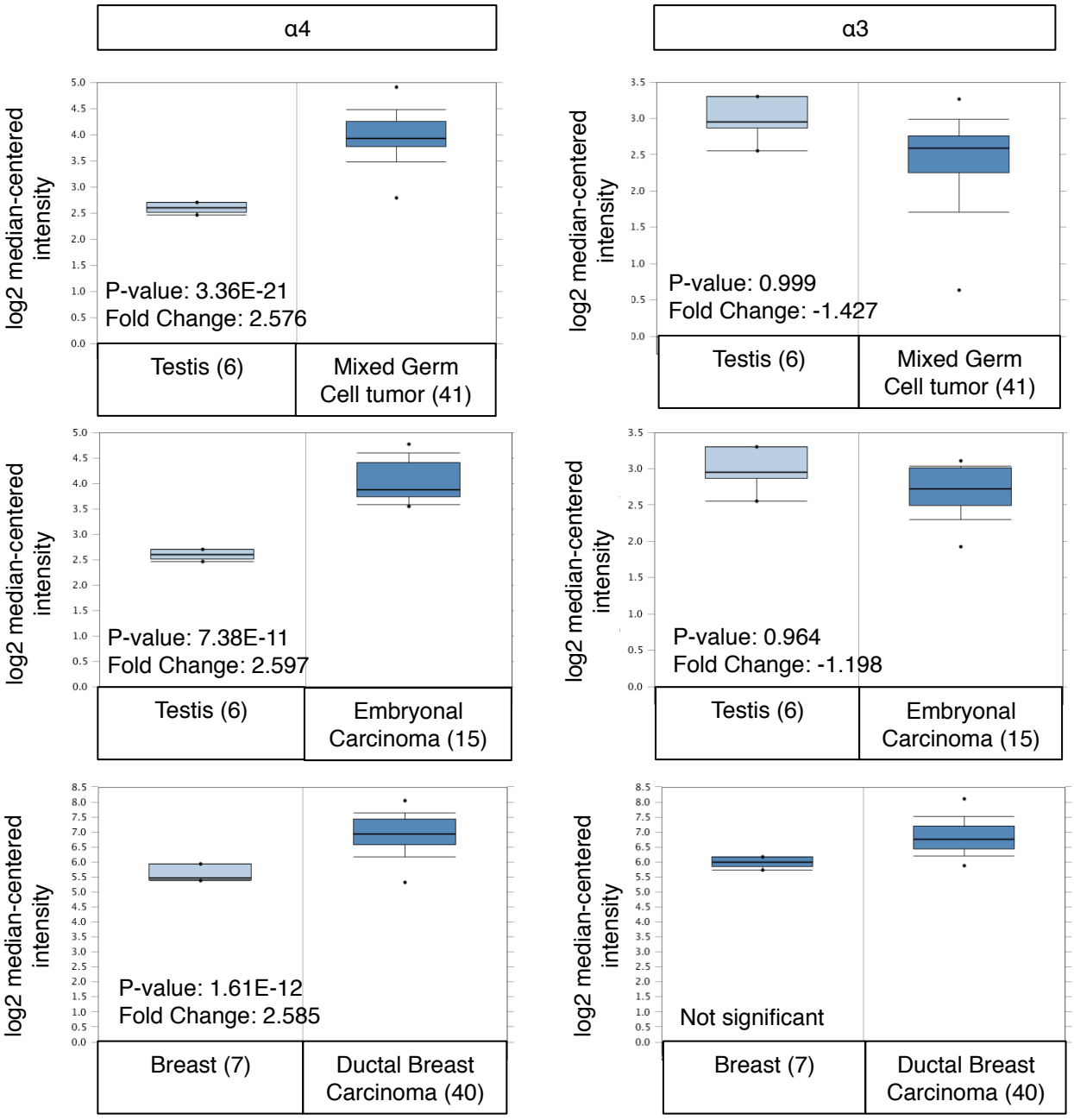
G


α6-Flag cells/GFP KD
α6-Flag cells/GFP KD + Dox
α6-Flag cells/α3 KD
α6-Flag cells/α3 KD + Dox
% Survival
110
100
90
80
70
60
50
40
30
20
10
0

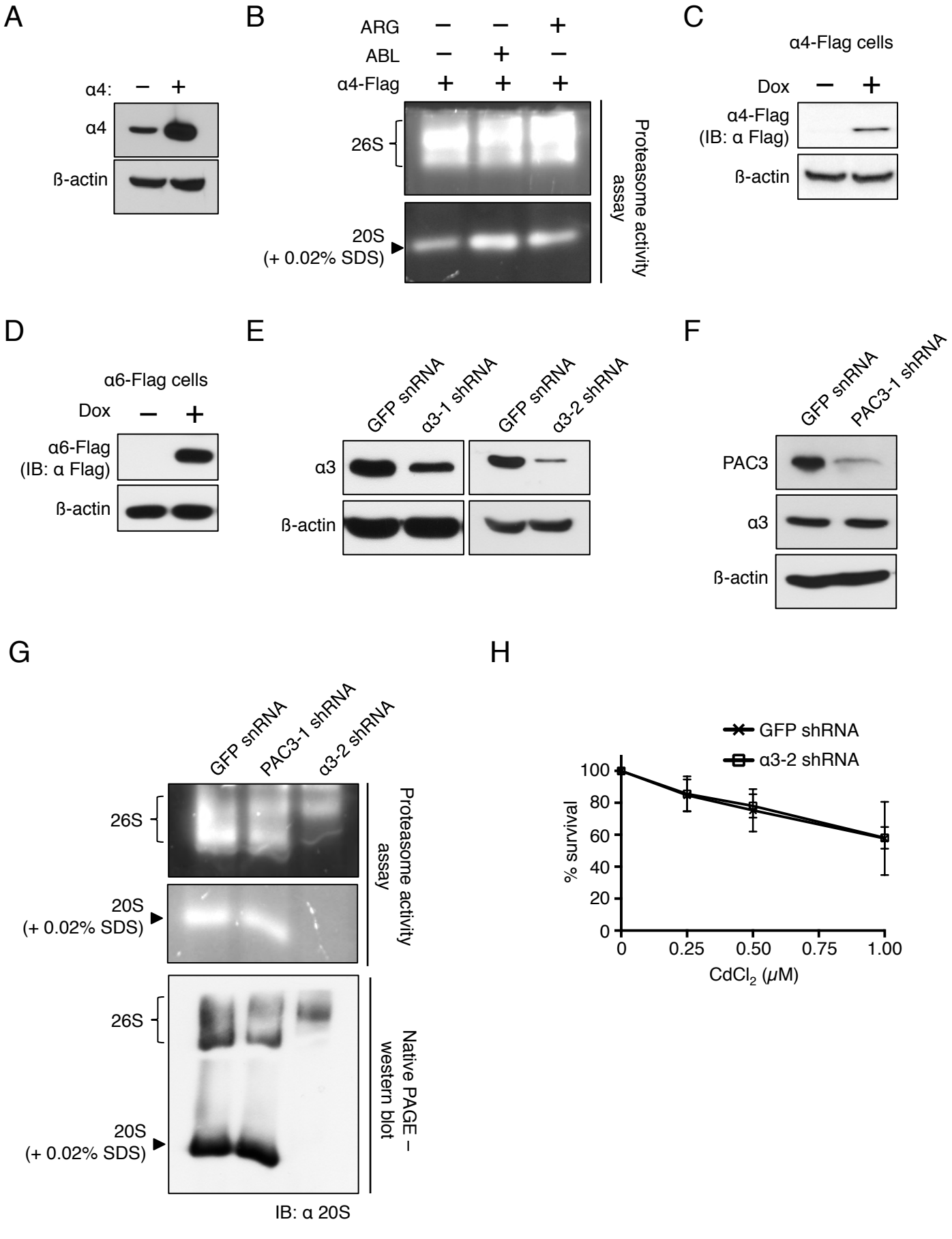
H

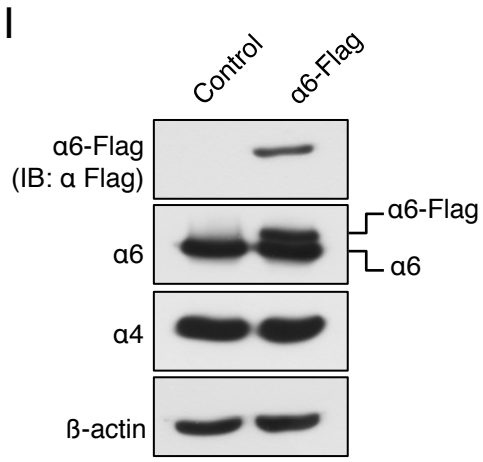

Control
ABL-6xHis
ARG-EYFP
PAC3
ABL
(IB: His)
ARG
(IB: GFP)
ß-actin

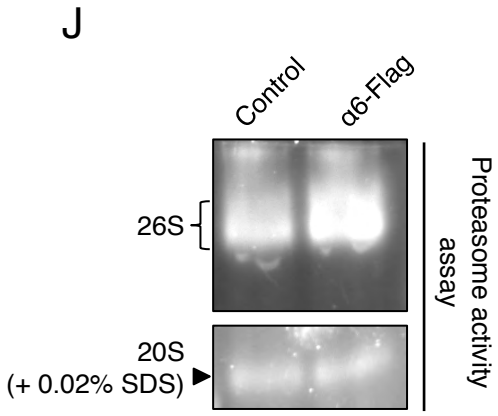
I

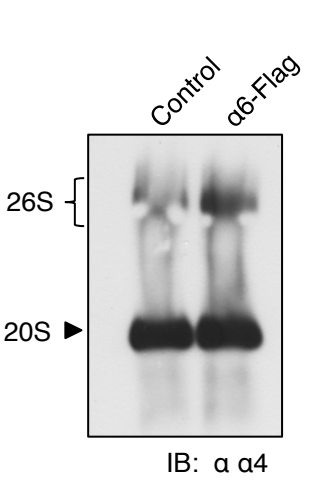


(Data retrieved from Oncomine. Korkola et al., 2006; Richardson et al., 2006.)


A
α4: – +
α4
ß-actin
B
ARG – – +
ABL – + –
α4-Flag + + +
26S
20S
(+ 0.02% SDS)
Proteasome activity assay
C
α4-Flag cells
Dox – +
α4-Flag
(IB: α Flag)
ß-actin
D
α6-Flag cells
Dox – +
α6-Flag
(IB: α Flag)
ß-actin
E
GFP snRNA
α3-1 shRNA
GFP snRNA
α3-2 shRNA
α3
ß-actin
F
GFP snRNA
PAC3-1 shRNA
PAC3
α3
ß-actin
G
GFP snRNA
PAC3-1 shRNA
α3-2 shRNA
26S
20S
(+ 0.02% SDS)
Proteasome activity assay
26S
20S
(+ 0.02% SDS)
Native PAGE – western blot
IB: α 20S
H
GFP shRNA
α3-2 shRNA
% survival
100
80
60
40
20
0
0
0.25
0.50
0.75
1.00
CdCl2 (μM)


I
Control
α6-Flag
α6-Flag
(IB: α Flag)
α6-Flag
α6
α6
α4
ß-actin


J
Control
α6-Flag
26S
20S
(+ 0.02% SDS)
Proteasome activity
assay


K
Control
α6-Flag
26S
20S
IB: α α4

SUPPLEMENTAL INFORMATION

Legends for Supplemental Figures

Figure S1 (related to Fig 1). Alternative α4-α4 proteasome assembly detected by α4-CC crosslinking in yeast and mammalian cells.

(A) Disulfide crosslinking between two α4-Hs (human α4) subunits requires both engineered cysteines in α4. Time of treatment with the oxidant $CuCl_2$ is indicated above the blot. Overexpression of α4-Hs-CC, but not α4-Hs-T77C or α4-Hs-T152C single cysteine mutants, in *α3*Δ *α4*Δ yeast resulted in disulfide bond formation. Sensitivity of the ~60 KDa 'α4- α4' band to DTT was consistent with disulfide bond formation between the α4 subunits. 3*, 7*, 11* and 15* are the same samples used in lane 3, 7, 11 and 15, respectively, but treated with DTT before loading on the gel.

(B) Formation of disulfide bond-linked α4-α4 dimers in gel-isolated functional 26S proteasomes from *α3*Δ *α4*Δ yeast cells also requires both engineered cysteine residues in α4.

(C) The ability to form α4-α4 proteasomes is conserved across both transformed and primary human cell lines. Overexpression of α4-CC in HeLa, U2OS and IMR-90 primary lung fibroblast cells resulted in assembly of α4-α4 proteasomes as indicated by the presence of a DTT-sensitive α4- α4.

(D) Crosslinking of neighboring human α4 subunits can be detected in functional human 26S proteasomes isolated from HEK293T cells expressing ectopic α4-CC. No α4-α4 dimers are seen in proteasomes isolated from cells transiently transfected with the vector pcDNA3.1.

Figure S2 (related to Fig 2). α3 regulates assembly of alternative α4-α4 proteasomes in mammalian cells.

(A) Different α3 shRNAs achieve different α3 knockdown efficiency.

(B) Knockdown of α3 using shRNAs targeting different regions of α3 results in increased in α4-α4 proteasome assembly in HeLa cells.

(C) shRNA-mediated knockdown of α3 in U2OS cells (bottom panel) results in increased α4-α4 proteasome assembly (top panel).

Figure S3 (related to Fig 3). Proteasome assembly chaperone PAC3 regulates assembly of alternative α4-α4 proteasomes in mammalian cells.

(A) Different PAC3 shRNAs achieve different PAC3 knockdown efficiency.

(B) Knockdown of PAC3 using shRNAs targeting different regions of PAC3 increases α4-α4 proteasome assembly in HeLa cells.

(C) $CdCl_2$ treatment (10 μM) increases α4-α4 proteasome levels in HEK293T cells transiently transfected with either α4-WT or α4-CC.

(D) Short-term treatment (3 h) $CdCl_2$ treatment has no effect on PAC3 levels in HeLa cells.

(E) Long-term $CdCl_2$ treatment (24 h), but not short-term treatment (3 h), results in increased α4-α4 proteasome assembly in α4-CC-Flag stable HeLa cells.

Figure S4 (related to Fig 4). Increased α4 levels are observed in cancers and can rescue α3 knockdown induced loss of cell viability.

(A). Silent mutations in α3 were engineered to make Dox-inducible stable HeLa cells that express the α3-3-shRNA-resistant version of α3 (**right panel**) as measured by immunoblot analysis. Dox-inducible stable cells expressing α3 WT were used as control (**left panel**).

(B) Ectopic expression of shRNA-resistant α3 rescues α3-3 shRNA-mediated loss of cell viability. The cells were treated with α3-3 shRNA for 4 days. Cells were treated with Dox to induce ectopic α3 expression simultaneously with shRNA treatment.

(C) Stable cells expressing α3-WT are still susceptible to cell death induced by α3 knockdown. The cells were treated with α3-3 shRNA for 4 days. Cells were treated with Dox to induce ectopic α3 expression simultaneously with shRNA treatment.

(D) Immunoblot showing knockdown of α3 (α3-2 shRNA) in α4-Flag stable cells. Addition of Dox induces the expression of α4-Flag.

(E) Ectopic expression of α4 protects cells from cell death induced by α3-2 shRNA. The cells were treated with α3-2 shRNA for 8 days. Cells were treated with Dox to induce ectopic α4 expression simultaneously with shRNA treatment.

(F) Immunoblot showing knockdown of α3 (α3-2 shRNA) in α6-Flag stable cells. Addition of Dox induces α6-Flag expression in these cells.

(G) Ectopic expression of α6 does not rescue cell death induced by α3 shRNA. The cells were treated with α3-2 shRNA for 8 days. Cells were treated with Dox to induce ectopic α6 expression simultaneously with shRNA treatment. Data from four experiments were quantified; error bars represent standard deviation.

(H) Transient overexpression of ABL-6xHis and ARG-EYFP in HEK293T cells has no effect on endogenous PAC3 levels.

(I) Data retrieved from Oncomine reveal *α4 (PSMA7)* mRNA is upregulated in multiple cancers (Korkola et al., 2006; Richardson et al., 2006). The level of *α3* (*PSMA4)* mRNA did not change (or was reduced) in these tumors.

Figure S5 (related to Fig 5). Effect of ABL/ARG overexpression, α3 knockdown and PAC3 knockdown on proteasome activity and levels.

(A) Immunoblot showing increased α4 levels in transiently transfected HEK293T cells.

(B) Concomitant transient transfection of ABL or ARG with α4 in HEK293T cells results in increased 26S and 20S proteasome activity. Cell lysates were resolved by native PAGE, and proteasome activity was measured by a substrate-overlay assay using the flurogenic peptide substrate Suc-LLVY-AMC.

(C) Expression of α4-Flag in the α4-Flag Flp-In stable HeLa cells is induced by addition of Dox and evaluated by immunoblotting using anti-Flag antibody.

(D) α6-Flag expression in α6-Flag stable HeLa cells was induced by adding Dox and evaluated by immunoblotting using anti-Flag antibody.

(**E**) Immunoblot analysis of HeLa cell lysate treated with shRNA constructs targeting α3 reveals significantly higher knockdown of α3 by α3-2 shRNA (right panel) compared to α3-1 shRNA (left panel). α3-1 shRNA knockdown cells were used for viability assay in Fig. 5H. α3-2 shRNA knockdown cells were used in Figs. S5G and S5H.

(F) Immunoblot analysis confirming PAC3 knockdown by PAC3-1 shRNA in cells used for viability assays in Figs. 5I and in Fig. S5G.

(G) shRNA-mediated knockdown of PAC3 and α3 in HeLa cells results in decreased 26S and 20S proteasome activity (top panel). Cell lysates were resolved by native PAGE, and proteasome activity was measured by a substrate-overlay assay using the flurogenic peptide substrate Suc-LLVY-AMC. Native PAGE immunoblot analysis of the same gel (bottom panel) reveals slightly lower 26S proteasome level in PAC3 and α3 knockdown cells compared to control (GFP shRNA) cells. α3 knockdown results in complete loss of free 20S proteasomes in cells.

(H) α3 knockdown in HeLa cells using α3-2 shRNA does not enhance the ability of HeLa cells to resist cadmium ($CdCl_2$) toxicity. shRNA-mediated knockdown of α3 in HeLa cells was carried out for 8 days. Cells were treated with $CdCl_2$ for 24 hours prior to measuring cell viability using the CellTiter-Glo® luminescent cell viability assay. N=3, and error bars represent standard deviations.

(I) Immunoblot showing induced overexpression of α6-Flag in α6-Flag Flp-In stable HeLa cells by the addition of Dox.

(J) Overexpression of α6-Flag in HeLa cells (see Fig. S5I) results in increased 26S proteasome activity (top panel). Cell lysates were resolved by native PAGE, and proteasome activity was measured by a substrate-overlay assay using the flurogenic peptide substrate Suc-LLVY-AMC.

(K) Native PAGE-western blot analysis of α6-Flag Flp-In HeLa cell lysate reveals increase in 26S proteasome levels upon ectopic α6-Flag expression (same cell lysates as in Fig. S5I and S5J).

TABLE S1: Yeast Strains used in this study.

Strain	Genotype	Source
MHY1601	MATα *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 pre6Δ::HIS3*	(Velichutina et al., 2004)
MHY1603	MATα *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 pre6Δ::HIS3 pre9Δ::HIS3*	(Velichutina et al., 2004)

TABLE S2: shRNA constructs used for α3 and PAC3 knockdown.

Name	Target Sequence	Target
GFP	GCAAGCTGACCCTGAAGTTCAT3 (Gift from Christian Schlieker, Yale Univ.)	GFP
α3-1	CCGGGTACTAAATAAGACCATGGATCTCGAGATCCATGGTCTTATTTAGTAC TTTTT (Clone ID:NM_002789.x-722s1c1)	α3
α3-2	CCGGGCAACACTAACAAGAGAGAATCTCGAGATTCTCTCTTGTTAGTGTTGC TTTTT (Clone ID:NM_002789.x-776s1c1)	α3
α3-3	CCGGGATGCTAATGTTCTGACTAATCTCGAGATTAGTCAGAACATTAGCATC TTTTT (Clone ID:NM_002789.x-380s1c1)	α3
PAC3-1	CCGGGTCTTTGCAAAGAACCTGGTACTCGAGTACCAGGTTCTTTGCAAAGA CTTTTTTG (Clone ID:NM_032302.1-462s1c1)	PAC3
PAC3-2	CCGGGCGTTTGTGTCTCAAGAAGCTCTCGAGAGCTTCTTGAGACACAAACG CTTTTTTG (Clone ID:NM_032302.1-483s1c1)	PAC3
PAC3-3	CCGGGTTTGGGAAGATGGGCACCCTCTCGAGAGGGTGCCCATCTTCCCAAA CTTTTTTG (Clone ID:NM_032302.1-347s1c1)	PAC3

Table S3: Plasmids used in this study.

Name	Expression system	Source
pRS424-α4-Sc	Yeast	This study
p424-GPD-α4-Hs	Yeast	This study
p424-GPD-α4-T77C	Yeast	This study
p424-GPD-α4-T152C	Yeast	This study
p424-GPD-α4-T77C T152C	Yeast	This study
pcDNA3.1-α4	Mammalian Cells	This study
pcDNA3.1-α4-T77C	Mammalian Cells	This study
pcDNA3.1-α4-T152C	Mammalian Cells	This study
pcDNA3.1-α4-T77C T152C	Mammalian Cells	This study
pcDNA3.1-α3	Mammalian Cells	This study
pcDNA3.1-PAC3	Mammalian Cells	This study
pcDNA3.1-ABL-6xHis	Mammalian Cells	Gift from Anthony J. Koleske, Yale Univ.
pcDNA3.1-ARG-EYFP	Mammalian Cells	Gift from Anthony J. Koleske, Yale Univ.
pBABE-Puro-HA-BRCA1	Mammalian Cells	Gift from Narendra Wajapeyee, Yale Univ.
pcDNA5-FRT/TO-α4	Mammalian Cells	This study
pcDNA5-FRT/TO-α4- T77C:T152C	Mammalian Cells	This study
pcDNA5-FRT/TO-α3	Mammalian Cells	This study
pcDNA5-FRT/TO-α3-shRNA rescue	Mammalian Cells	This study
pcDNA5-FRT/TO-α6	Mammalian Cells	This study

Table S4. Antibodies used in this study

Antibody	Manufacturer	Clone/Catalog No.	Dilution
Anti-α3	Enzo lifesciences	MCP257	1:1000
Anti-α4	Enzo lifesciences	MCP234 MCP72	1:2000 1:1000
Anti-α6	Enzo lifesciences	MCP20	1:1000
Anti-PAC3	Enzo lifesciences	EX-7	1:1000
Anti-ß-actin	Abcam Sigma	ab8226 A5441	1:5000 1:10000
Anti-GFP	Rockland	600-401-215	1:1000
Anti-Flag	Sigma	M2, monoclonal	1:5000
Anti-20S	Enzo lifesciences	MCP231	1:500

Table S5. Primers used for quantitative RT-PCR analysis.

Target	Primer	Product size
PAC3	Fwd: 5′ GAAGACGGAGGTGGTGTGC 3′ Rev: 5′ GCACAGGCTTGCTGACCTC 3′	151 bp
GAPDH	Fwd: 5′ GAAGGTGAAGGTCGGAGT 3′ Rev: 5′ GAAGATGGTGATGGGATTTC 3′	226 bp

Supplemental Experimental Procedures

Disulfide crosslinking assay to detect α4-α4 proteasomes in yeast

Yeast cells grown to mid-log phase was harvested, washed with 1 ml ice-cold sterile water and resuspended in 100 µL zymolyase buffer (1.2 M sorbitol, 50 mM Tris-HCl pH 8.0, 0.5 mM MgCl2) containing 30 mM DTT. After 15 minutes incubation at room temperature, the contents were spun down and the pellet was resuspended in 100 µL zymolyase buffer containing 4 µL of 15 mg/ml zymolyase 100T (zymolyase-100T Athrobacter Luteus, MP) and incubated at 30^{o}C for 30 minutes with agitation. The contents were spun down and pellet was washed once with 500 µL zymolyase buffer. After centrifugation, the supernatant was discarded and the pellet was resuspended in 100 µL ice-cold lysis buffer (50 mM HEPES pH7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100). Lysis was achieved by vortexing three times for 30 sec with 1 minute on ice in between. The contents were centrifuged at 20000xg for 15 min at 4^{o}C and the supernatant was transferred to a fresh eppendorf tube. Protein concentration was determined by Bradford assay (Bio-Rad Protein Assay Kit). 60 µg of total protein in 100 µL of lysis buffer was divided into five equal parts. 2 µL of 10x stop buffer (10 mM sodium iodoacetate and 50 mM N-ethylmaleimide) was added to one part. Disulfide cross-linking of the remaining extract was induced using 0.2 mM $CuCl_2$ at room temperature. 2 µL of 10X stop solution was added to each part at regular intervals and incubated on ice. 15 µl of non-reducing sample buffer was added to the 20 µL aliquots. The samples were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibody against α4.

Disulfide crosslinking assay to detect α4-α4 proteasomes in human cells

To perform disulfide crosslinking assays with human cell lysates, tissue culture cells were harvested and lysed by incubation in lysis buffer (50 mM Tris-HCl pH 7.5, 75 mM NaCl, 0.5% NP-40, 5 mM $MgCl_2$) on ice for 1-1.5 h. After incubation, the cell lysate was spun down and the protein concentration of the supernatant was determined by Bradford assay (Bio-Rad Protein Assay Kit). 60 – 100 µg of protein was diluted in 20 µL lysis buffer. For the reduced sample control, 2 µl of 1 M dithiothreitol (DTT) was added to a 20 µl aliquot and left at room temperature for 10 min. 15 µl of non-reducing sample buffer was added to the 20 µL aliquot and the samples were boiled for 3 minutes at 95 °C. Samples were resolved on a 10% SDS-PAGE gel and proteins were electrotransferred to a PVDF membrane. Immunoblotting was performed using either anti- α4 or anti-Flag antibodies as indicated.

Lentivirus production in HEK293T cells

Lentiviruses used for shRNA knockdown were produced in low-passage packaging HEK293T cells. HEK293T cells were grown to 70% confluence in a 10 cm plate and transfected with 1.6 µg hairpin pLKO.1-puro-based constructs (MISSION® shRNA -Sigma-Aldrich), 1.4 µg second generation packaging plasmid pCMV-dR8.91 (Sigma-Aldrich) and 160 ng envelope plasmid VSV-G/pMD2.D (Sigma-Aldrich) using the TransIT-LT1 transfection reagent (Mirius Bio). The specific pLKO.1-puro-based constructs used for shRNA knockdown of α3 and PAC3 are listed in Table S2. The transfection mix was added drop-wise on cells covered in 6 ml of high glucose DMEM containing no antibiotics. At 18 h post-transfection, the media was replaced with 10 ml of high serum

growth media (DMEM, 30% FBS, 1X penicillin/streptomycin). The medium was harvested 24 h later and replaced with fresh 10 ml of high serum growth medium, which was harvested after additional 24 h incubation at 37 ˚C. The harvested media containing lentiviral particles were pooled, sterile filtered and stored at -80^{o}C.

Lentiviral mediated gene knockdown in mammalian cells

For lentiviral infection, 1 ml of virus-containing medium was added to 70% confluent cells in a 6-well plate covered with 1 ml of DMEM media containing 10% FBS and 16 µg/ml polybrene (Millipore). 24 h post-infection, the lentivirus-infected cells were selected by replacing the medium with DMEM medium (+10% FBS) containing 5 µg/ml puromycin. Successful knockdown of the target protein was confirmed by immunoblotting.

Generation of Dox-inducible isogenic Flp-In HeLa stable cells lines

The Flp-In™ System (Invitrogen) was used for stable integration and generation of isogenic HeLa cell lines expressing α4-Flag, α4-CC-Flag, α6-Flag and α3-Flag; the integrated construct in the last of these cell lines was designed to be resistant to α3-2 and α3-3 shRNAs. HeLa cells containing an integrated FLP Recombination Target (FRT) site for Flp recombinase-mediated integration of the target gene was obtained from Dr. Christian Schlieker (Yale University). *α4*, *α4-CC*, *α6* and shRNA-resistant *α3* were cloned with a sequence encoding a C-terminal Flag tag into the pcDNA™5/FRT/TO vector (Invitrogen). HeLa cells containing a stably integrated FRT site were co-transfected with pcDNA™5/FRT/TO vector containing the gene of interest and pOG44 vector as per the manufacturer's instructions. 48 h post-transfection, the cells were trypsinized and plated at a dilution of 1:6 into a new 10 cm tissue culture dish. The cells were selected using hygromycin (50 µg/ml) for 10-14 d until distinct colonies appeared. Successful stable integration of pcDNA™5/FRT/TO vector containing the desired insert at the FRT site ensures survival of these cells under hygromycin selection to form isogenic colonies. These cells were pooled and tested for the induction of the transgene upon Dox (2 µg/ml) addition.

Native PAGE to resolve 26S and 20S proteasomes

26S and 20S proteasomes in whole cell lysates were resolved by native PAGE. Yeast cell extracts were prepared as described previously (Hochstrasser and Funakoshi, 2012). Mid to late log-phase cells (OD_{600} = 1.5–2.0) grown in YPD were washed with ice-cold water and frozen in liquid nitrogen. The frozen cells were ground liquid nitrogen with mortar and pestle, and the resulting cell powder was thawed in 26S Buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 10% glycerol, 1 mM ATP). To analyze mammalian proteasomes, cells were lysed in buffer containing 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2 mM ATP, 0.5 mM EDTA pH 8, 0.5% NP-40. Proteasome complexes were resolved using a 4% resolving gel (90 mM Tris-Borate pH 8.3, 2.5% sucrose, 4% acrylamide, 0.1% bis-acrylamide, 5 mM $MgCl_2$, 1 mM ATP) and a 3% stacking gel (50 mM Tris-Cl pH 6.8, 2.5% sucrose, 3% acrylamide, 0.6% bis-acrylamide, 5 mM $MgCl_2$, 1 mM ATP). Prior to gel loading, 5x loading buffer (50 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 10% glycerol, 5 mM ATP, 1.5 µg/ml xylene cyanol) was added to the samples. Native gels were run at 95 V for 3 hours at 4 °C.

Disulfide crosslinking in native gels

Disulfide crosslinking in native gel was performed as described previously (Kusmierczyk et al., 2008). Briefly, whole cell lysates containing 26S proteasomes were resolved by native PAGE and active doubly capped 26S proteasomes was identified by a fluorogenic substrate overlay assay; the bands were excised, and subunits were resolved by non-reducing SDS PAGE followed by immunoblot analysis. The human 26S proteasome was excised immediately after incubation with the overlay buffer and transferred to the top of a 10% SDS-polyacrylamide gel. Yeast 26S proteasomes were crosslinked in-gel. Gels were first incubated with minus-$CuCl_2$ buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 10 % glycerol) for 5 min, and then 0.2 mM $CuCl_2$ was added to the buffer and incubated for 30 min at room temperature. The gel was washed in 55 ml of Gel Wash Buffer with 1X Stop Solution (187.5 mM Tris-HCl pH 6.8, 10 mM sodium iodoacetate, 50 mM NEM) for 5 min. The 26S proteasomes were visualized under UV light, cut out and transferred to a 10% SDS-polyacrylamide gel. Packing solution (125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.7% agarose, bromphenol blue) was used to fill the empty space around the native gel piece atop the SDS-polyacrylamide gel.

Quantitative PCR Analysis

Total RNA from mammalian cells was extracted using the RNeasy Kit (Qiagen). DNAase I treatment was performed to eliminate contaminating DNA. DNAase was inactivated using the DNAase inactivating reagent. Purified total RNA was reverse-transcribed using the Superscript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed in triplicates as per the instructions in the iScript qRT-PCR kit (Bio-Rad). Fold difference in gene expression was determined after normalizing against GAPDH mRNA. Primers used for qRT-PCR are listed in Table S5.

Supplemental References:

Hochstrasser, M., and Funakoshi, M. (2012). Disulfide engineering to map subunit interactions in the proteasome and other macromolecular complexes. Methods in molecular biology *832*, 349-362.
Korkola, J.E., Houldsworth, J., Chadalavada, R.S., Olshen, A.B., Dobrzynski, D., Reuter, V.E., Bosl, G.J., and Chaganti, R.S. (2006). Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13.31, is associated with in vivo differentiation of human male germ cell tumors. Cancer research *66*, 820-827.
Kusmierczyk, A.R., Kunjappu, M.J., Funakoshi, M., and Hochstrasser, M. (2008). A multimeric assembly factor controls the formation of alternative 20S proteasomes. Nature structural & molecular biology *15*, 237-244.
Richardson, A.L., Wang, Z.C., De Nicolo, A., Lu, X., Brown, M., Miron, A., Liao, X., Iglehart, J.D., Livingston, D.M., and Ganesan, S. (2006). X chromosomal abnormalities in basal-like human breast cancer. Cancer cell *9*, 121-132.
Velichutina, I., Connerly, P.L., Arendt, C.S., Li, X., and Hochstrasser, M. (2004). Plasticity in eucaryotic 20S proteasome ring assembly revealed by a subunit deletion in yeast. The EMBO journal *23*, 500-510.