Supplemental Information

Supplemental Data

Table S1. CRPC patient and tumor characteristics (Related to Figure 2). Site of CRPC, age, grade, and prior hormonal therapies are shown for each human tumor used in this study. DES is diethylstilbestrol and keto is ketoconazole.

Tumor	Site	Age	Gleason	Hormonal
UTSW1	Pelvic mass	52	5+4	leuprolide, bicalutamide, nilutamide
UTSW2	Lymph Node	66	4+3	orchiectomy, bicalutamide, nilutamide
UTSW3	Bronchial	57	3+4	leuprolide, bicalutamide
UTSW4	Pelvic	56	Unknown	orchiectomy, bicalutamide,
UTSW5	Lung	67	3+3	leuprolide
UTSW6	Lymph Node	68	3+4	leuprolide
UTSW7	Prostate/bladder	86	4+5	leuprolide, bicalutamide, nilutamide, keto
UTSW8	Liver	67	Unknown	Leuprolide, bicalutamide, nilutamide, keto, abiraterone
UTSW9	Prostate/bladder	64	3+3	leuprolide, bicalutamide
UTSW10	Prostate/bladder	87	5+5	orchiectomy, bicalutamide
UTSW11	Prostate/bladder	78	4+5	leuprolide, bicalutamide
UTSW12	Prostate/bladder	64	Unknown	leuprolide, bicalutamide
UTSW13	Pelvic	66	4+5	leuprolide, bicalutamide
UTSW14	Prostate/bladder	48	5+4	leuprolide, bicalutamide
UW1	Lymph Node	53	Unknown	orch, flutamide, keto, DES
UW2	Lymph Node	74	4+3	leuprolide, finasteride, flutamide, keto
UW3	Lymph Node	66	3+5	leuprolide, bicalutamide, DES
UW4	Lymph Node	43	4+5	leuprolide, bicalutamide, keto, DES
UW5	Lymph Node	80	4+3	bicalutamide
UW6	Lymph Node	54	5+4	Very limited notes
UW7	Lymph Node	62	7	leuprolide, bicalutamide
UW8	Lymph Node	77	9	leuprolide, bicalutamide, orchiectomy
UW9	Liver	63	n/a	leuprolide, bicalutamide
UW10	Retroperitoneal	57	4+5	nilutamide, bicalutamide, keto

UW11	Liver	78	4+5	leuprolide, orchiectomy, flutamide, finasteride
UW12	Lymph Node	74	4+3	leuprolide, bicalutamide, keto
UW13	Lymph Node	53	Unknown	leuprolide, bicalutamide
UW14	Lymph Node	70	4+4	leuprolide, flutamide, keto
UW15	Lymph Node	67	5+4	leuprolide, bicalutamide, keto, DES
UW16	Lymph Node	67	3+4	leuprolide, bicalutamide
UW17	Liver	58	4+5	bicalutamide, leuprolide, nilutamide, keto, DES
UW18	Pelvic Mass	79	Unknown	bicalutamide, goserelin, nilutamide
UW19	Lymph Node	73	3+4	leuprolide, DES
UW20	Lymph Node	75	5+5	leuprolide, bicalutamide, keto, DES
UW21	Lymph Node	66	4+4	bicalutamide, leuprolide, keto, DES
UW22	Lymph Node	64	3+3	Bicalutamide, keto
UW23	Lymph Node	62	3+3	Unknown
UW24	Lymph Node	62	4+3	bicalutamide, leuprolide, keto, DES
UW25	Lymph Node	60	5+4	leuprolide, bicalutamide, orchiectomy, DES
UW26	Lymph Node	54	4+5	bicalutamide, leuprolide, DES

Figure Legends

Figure S1 (Related to Figure 1). Increased metabolic flux from DHEA to AD associated with a point mutation is not attributable to transcriptional regulation and association of flux with the mutant occurs in other models. **A.** Expression of *HSD3B1* and *HSD3B2* transcripts

are comparable between LNCaP and LAPC4. Expression of *HSD3B1* (blue bars) and *HSD3B2* (green bars) isoenzymes by qPCR are shown in LAPC4 relative to LNCaP and normalized to *RPLP0* (above) and *GAPDH* (below). Error bars represent the SD from experiments performed in triplicate. **B.** Metabolic flux from [^{3}H]-DHEA (100 nM) to AD is robust in VCaP with 3βHSD1(367T) and limited with DU145, RWPE-1 and PzHPV7 with 3βHSD1(367N). Downstream flux to 5α-dione and DHT occurs in VCaP and is not detectable in DU145, RWPE-1 and PzHPV7. Steroids were quantitated at the designated time points by HPLC. Experiments were performed in triplicate, and error bars represent the SD.

Figure S2 (Related to Figure 2). *HSD3B1* LOH in human CRPC tissues. **A.** LOH of the *HSD3B1*(1245C) allele encoding 3βHSD1(367T) does not occur in CRPC. Shown are the remaining cases with germline heterozygosity not shown in Figure 2B. These 8 cases maintain both alleles in genomic DNA extracted from CRPC tumors. **B.** *HSD3B1* mRNA expression in UW tumors with LOH does not explain elevated 3βHSD1 protein expression. Expression is normalized to *RPLP0* and error bars represent the SD from experiments performed in triplicate.

Figure S3 (Related to Figure 4). Protein half-life of 3βHSD1(367T) and 3βHSD1(367N). **A.** HA-tagged 3βHSD1(367T) (T-HA) has a prolonged half-life compared to wild-type 3βHSD1(367N) (N-HA) protein in the DU145 prostate cancer cell line. Cells were transiently transfected with empty vector alone (vector), constructs encoding for wild-type (N-HA) and mutant (T-HA) protein and treated with CHX, protein was collected at

the designated time points, Western blot was performed and signal was quantitated and normalized to time zero and β-actin. The calculated half-lives of 3βHSD1(367N) and 3βHSD1(367T) proteins are 2.7 and > 100 hours, respectively. **B.** Stable expression in LAPC4 demonstrates a longer half-life of HA-tagged 3βHSD1(367T) (T-HA) compared to 3βHSD1(367N) (N-HA) protein. Expression was induced after lentiviral infection with a doxycycline-inducible expression construct or vehicle (v). Cells were treated with CHX, protein was collected at the time points indicated, Western blot was performed, and signal was quantitated and normalized to time zero and β-actin. The calculated half-lives of wild-type and mutant proteins are 3.7 and > 100 hours, respectively. **C.** Proteosome inhibition with MG132 (10 uM; 8 hours) results in no increase in polyubiquitinated 3βHSD1(367T) protein in LNCaP as evidenced by immunoprecipitation with an anti-ubiquitin antibody.

Extended Experimental Procedures

Cell lines

LNCaP and DU145 were purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 medium with 10% fetal bovine serum. VCaP was purchased from ATCC and maintained in DMEM containing 10% fetal bovine serum. LAPC4 was generously provided by Dr. Charles Sawyers (Memorial Sloan Kettering Cancer, New York, NY) and was maintained in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum. RWPE-1 was obtained from ATCC and cultured in Keratinocyte Serum Free Medium (K-SFM) (Invitrogen, Carlsbad, CA). PzHPV7 was generously provided by Dr. JT Hsieh (UT Southwestern) and maintained in PrEGM (Lonza, Allendale, NJ). All cells except for VCaP were incubated in a 5% CO_2 humidified incubator. VCaP cells were grown in a 10% CO_2 humidified incubator.

Steroid metabolism

Cells (300,000-400,000 cells per well) were plated in 12-well plates coated with poly-L-ornithine. Twelve hours after seeding, medium was replaced with 1 mL serum-free medium containing [^{3}H]-labeled DHEA (100nM, 300,000-600,000 cpm) purchased from PerkinElmer (Waltham, MA). Cells were incubated at 37°C and aliquots of medium (0.25-0.3mL) were collected for up to 48 hours. To hydrolyze the β-D-glucuronic acid group from steroids, 1000 units of β-glucuronidase (*H. pomatia*; Sigma-Aldrich, St. Louis, MO) were added to each aliquot and incubated at 65°C for 4 hours. The deconjugated steroids were extracted with 1 mL 1:1 ethyl acetate:isooctane, and the reagents were evaporated under nitrogen stream. The dried samples were dissolved in 50% methanol and injected on a Breeze 1525 system equipped with model 717 plus autoinjector (Waters Corp., Milford, MA). The steroid metabolites were separated on a

Luna 150 x 3 mm, 3.0 μM C_{18} reverse-phase column (Phenomenex, Torrance, CA) with methanol/water gradients at 25°C. The column effluent was mixed with Liquiscint scintillation cocktail (National Diagnostics, Atlanta, GA) and analyzed by a β-RAM model 3 in-line radioactivity detector (LabLogic, Brandon, FL). All metabolism studies were performed in triplicate and repeated in independent experiments.

For steroid metabolism analysis of LAPC4 with transient enzyme expression, pCMV5-HSD3B1 was kindly provided by J. Ian Mason (Lorence et al., 1990), sequenced and confirmed as encoding for 3βHSD1(367T). The plasmid encoding wild type 3βHSD1(367N) was derived by using Quick Change Site directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) with primer set (Forward: 5'-GGACCGGCACAAGGAGAACCTGAAGTCCAAGACTCAG-3' and Reverse: 5'-CTGAGTCTTGGACTTCAGGTTCTCCTTGTGCCGGTCC-3'). Plasmid DNA (20 ng) was transfected into 300,000 cells per well using Lipofectamine together with PLUS reagent (Life Technology, Grand Island, NY). Twenty-four hours after transfection, cells were treated with 25 μM CHX for 12 hours and then steroids were analyzed as described above.

For steroid metabolism studies with stable expression, HSD3B1, wild type (367N) or mutant (367T) was PCR amplified with primer set (Forward: 5'-TCCGCGGCCGCGGAGTGATTCCTGCTA-3' and Reverse: 5'-AAGACGCGTGAGCTCTAGTAGTCAAAA-3') and sub-cloned into the pLVX-Tight-Puro vector (Clontech, Mountain View, CA) by Not1 and Mlu1 restriction sites. Lentiviral particles were packaged in 293T cells by co-transfecting 10 μg of each pLVX-Tight-Puro vector, pMD2.G, and psPAX2 vector. After lentiviral infection and 2μg/ml puromycin selection for 2 weeks, 300,000 cells per well were used for analysis of metabolic flux.

Human tissues

Matching CRPC and normal tissues (UW1-UW26) were obtained from the University of Washington rapid autopsy program with IRB approval number 39053. At UT Southwestern, matching tumor and normal tissues (UTSW1-UTSW14) were obtained using IRB approved protocols STU-032011-187 and STU-062010-212. All sequencing studies were independently repeated.

DNA isolation and *HSD3B1* sequence analysis

Genomic DNA was prepared from cell lines and clinical samples (metastatic CRPC tumor and matched peripheral blood or normal tissue) using DNeasy Blood and Tissue Kit (QIAGEN, Germantown, MD). PCR products of the promoter region, all exons, exon-intron junctions and the 3'-UTR were sequenced to identify mutations in *HSD3B1*. The primers and annealing temperature were described previously (Chang et al., 2002). To sequence the 3' flanking region of *HSD3B1*, primer set (Forward: 5'-ATGTGGAGGGAGGTGTGAGT-3' and Reverse: 5'-ACGGAGATGGGTCTCTTCCA-3') were used with an annealing temperature of 62°C. Genotyping PCR reaction (50 μl) consisted of 30-100 ng genomic DNA, 1 x PCR buffer with 0.2 mM dNTP, 0.2 μM of each primer, and 0.5 μl Phusion High-Fidelity DNA Polymerase (New England BioLabs Inc, Ipswich, MA). DNA sequencing and polymorphism analysis was carried out at the McDermott Center, UT Southwestern.

For *HSD3B1* transcript analysis, total RNA was harvested and mRNA was reverse transcribed to cDNA by iScript cDNA synthesis kit (Bio-Rad). Cloning primer set (Forward: 5'-ACTGAATTCCAGGCCAATTTACACCTATCG-3'; Reverse: 5'-ACTCTCGAGTCAAACTATGTGAAGGAATGGA-3') were used to PCR amplify the 3' region

of *HSD3B1* exon 4 and subcloned into the pCMX vector. Colonies with inserts were picked for sequencing.

Gene knockdown by lentiviral vector or RNA interference

Lentiviral vector construction, viral packaging and infection were performed as previously described (Chang et al., 2011). Briefly, three miR30-styled shRNA sequences (#1: 5'-TGCTGTTGACAGTGAGCGACCTCATACAGAAAGTGACAAGTAGTGAAGCCACAGATGTACTTGTCACTTTCTGTATGAGGCTGCCTACTGCCTCGGA-3'; #2: 5'-TGCTGTTGACAGTGAGCGAAGAGGAAAGACCATGTGGTTTTAGTGAAGCCACAGATGTAAAACCACATGGTCTTTCCTCTGTGCCTACTGCCTCGGA-3') against *HSD3B1* were PCR amplified and cloned into the pGIPZ vector (Open Biosystems, Huntsville, AL) and confirmed by sequencing. Virus packaging was carried out in 293T cells by co-transfecting 10 μg each of pGIPZ, pMD2.G, and psPAX2 vector. To increase transduction efficiency, 30 mL supernatant containing lentiviral particles were collected, filtered with a 0.45 μm nitrocellulose membrane and concentrated by ultracentrifugation at 19,000 rpm for 2 hours and 20 minutes at room temperature without brake. Pellets containing viral particles were re-suspended with 3 mL RPMI 1640 with 10% FBS and 1 mL was used to infect LNCaP cells supplemented with polybrene (6 μg/mL). After 24 hours, the infected cells were selected with 2 μg/mL puromycin for 2 weeks before evaluation for knockdown efficiency.

For RNA interference, 25nM siGENOME Human siRNA (Thermo Fisher Scientific, Waltham, MA) was transfected into LAPC4 ($1x10^6$ cells per well in 6 well plate coated with poly-L-ornithine) by Lipofectamine RNAiMax (Life Technology, Grand Island, NY). Cell lysate was

collected at 48 hours after transfection and knockdown efficiency was determined by Western blot with rabbit anti-AMFR and rabbit anti-SKP2.

Mouse xenograft studies

Mouse xenograft studies and abiraterone acetate treatment were done as described in detail previously (Li et al., 2012). Briefly, male NOD/SCID mice 6 to 8 weeks of age were obtained from the UT Southwestern Animal Resources Center, underwent surgical orchiectomy and a DHEA pellet (5 mg 90-day sustained-release) implantation and 2 days later underwent subcutaneous injection with 7 x 10^6 LAPC4 cells. Tumors reaching 300 mm^3 volume were treated with intraperitoneal abiraterone acetate or vehicle (n = 8 mice per treatment) once daily for 5 days per week for 4 weeks. Tumors were fresh frozen upon mouse sacrifice. For the studies of mutant 3βHSD1 knockdown, LNCaP cells (7 x 10^6) stably expressing shHSD3B1 #1, #2 and shCTRL were subcutaneously injected with matrigel into eugonadal NOD/SCID mice (n=15 per group). Mice with tumors reaching 100 mm^3 volume underwent surgical castration and DHEA pellet implantation. Time from castration to tumor volume $\geq$ 600 mm^3 was assessed. For the comparison between wild type and mutant 3βHSD1, LAPC4 cells (7 x 10^6) stably expressing pLVX-Tight-Puro-HSD3B1 (367N) or pLVX-Tight-Puro-HSD3B1 (367T) were subcutaneously injected into surgically orchiectomized NOD/SCID mice supplemented with a DHEA (5 mg 90-day sustained-release) pellet. Tumor diameters were measured by digital calipers two or three times per week.

Cell proliferation studies

LNCaP was seeded in triplicate at 100,000 cells/well in 12-well plates coated with poly-L-ornithine and grown in the presence of 20nM DHEA or vehicle control for up to 7 days. Relative cell numbers were determined by staining nucleic acid with Hoechst (Kan et al., 2007). Briefly, cells were washed with PBS and frozen with 250 µL Milli-Q water. To stain the nucleic acid, plates were thawed completely and 500µL Hoechst staining buffer (Hoechst 10 µg/mL in 1mM EDTA, 2M NaCl and 10mM Tris, pH=7.5) was added to each well. After shaking plates gently in a dark at room temperature for 2 hours, fluorescence in each well was determined by excitation at 360 nm and measuring emission at 460 nm with a plate reader. DNA quantities were estimated by comparison to the standard curve.

Gene expression

To accurately quantitate each gene transcript, qPCR was performed as previously described (Chang et al., 2011). Briefly, the iTaq SYBR Green Supermix with ROX kit (Bio-Rad, Hercules, CA) was applied for the thermocycling reaction in an ABI-7500 Real-Time PCR machine (Applied Biosystems, Foster City, CA). Total RNA (1 µg) collected by RNeasy kit (QIAGEN) was used in a RT reaction with the iScript cDNA synthesis kit (Bio-Rad). The qPCR analysis was carried out in triplicate with the following primer sets: *HSD3B1* (Forward: 5'-CCATGTGGTTTGCTGTTACCAA-3'; Reverse: 5'-TCAAAACGACCCTCAAGTTAAAAGA -3'), *PSA* (Forward: 5'-GCATGGGATGGGGATGAAGTAAG-3'; Reverse: 5'-CATCAAATCTGAGGGTTGTCTGGA -3'), *TMPRSS2* (Forward: 5'-CCATTTGCAGGATCTGTCTG-3'; Reverse: 5'-GGATGTGTCTTGGGGAGCAA-3'), the housekeeping gene *large ribosomal protein P0* (*RPLP0*) (Forward: 5'-

CGAGGGCACCTGGAAAAC-3'; Reverse: 5'-CACATTCCCCCGGATATGA-3') and *GAPDH* (Forward: 5'-AGAAGGCTGGGGCTCATTTG-3'; Reverse: 5'-AGGGGCCATCCACAGTCTTC-3'). Each mRNA transcript was quantitated by normalizing the sample values to *RPLP0* or *GAPDH* and to non-silencing control cells (for knockdown) or to vehicle treated cells (for steroid treated cells). All gene expression studies were repeated in independent experiments.

Enzyme kinetics

Recombinant human 3βHSD1 was expressed in *S cerevisciae* strain W303B using yeast vector V10-3βHSD1 for wild-type and 367T proteins, and microsomes were prepared as described(Li et al., 2012). Incubations containing [^{3}H]-DHEA (0.5-40 μM, 100,000 cpm) and 25 μg microsomal protein in 0.25 ml of 50 mM potassium phosphate (pH 7.4) were pre-incubated at 37°C for 1 min before initiating the reaction with NAD^+ (0.1 mM). After 20 min at 37°C, the steroids were extracted with 1 ml dichloromethane, concentrated, and resolved on an Agilent 1260 HPLC equipped with a Kinetex 2.1 x 100 mm, 2.6 μm C_{18} reverse-phase column (Phenomenex, Torrance, CA) using methanol-water gradients at 0.4 ml/min. The column effluent was analyzed with a β-RAM4 (LabLogic) in-line scintillation counter and Bio-SafeII cocktail (Research Products International). Means of triplicate determinations were plotted as v versus [S], and kinetic constants K_m and V_{max} were obtained by fitting the data to the Michaelis-Menten equation using Origin version 7.5.

Protein half-life determination

For transient expression, LAPC4 or DU145 cells ($1x10^6$ cells per well) were seeded on poly-L-ornithine coated 6-well plate for 24 hours. Constructs encoding wild type 3βHSD1(367N) or 3βHSD1(367T) protein (pCMX-HSD3B1-HA plasmids; 150 ng per well) were transfected using Lipofectamine, together with PLUS reagent (Life Technology). After 24 hours, cells were treated with 25 μM CHX (Sigma-Aldrich, St. Louis, MO) in serum-free IMDM containing 100 nM DHEA for up to 8 hours. Cell lysates were harvested using RIPA buffer with Protease Inhibitor Cocktail (Roche).

For stable expression of 3βHSD in LAPC4, cells expressing wild type 3βHSD1(367N) or 3βHSD1(367T) HA-tag fused to *HSD3B1* were constructed by pLVX-Tight-Puro vector system (Clontech, Mountain View, CA). One million cells were seeded on each well of poly-L-ornithine coated 6-well plates in IMDM containing 10% Tet System Approved FBS (Clontech). Expression of 3βHSD was induced by 2 ng/mL doxycycline for 24 hours. CHX treatment and lysate collection were performed as described above. Proteins were analyzed by SDS-PAGE and Western blot. Films were scanned and quantitated by ImageJ (http://rsbweb.nih.gov/ij/). Protein half-life calculation was done as previously described(Bloom et al., 2003) . Briefly, 3βHSD1 signal was normalized to β-actin and time zero. The $t_{1/2}$ was calculated by an equation derived from the logarithmic trend line. All experiments were independently repeated.

Western blot analysis and immunoprecipitation

For Western blot analysis, whole cell protein extract was harvested using RIPA buffer (Sigma-

Aldrich) with protease inhibitor cocktail (Roche). Protein concentration was determined by BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL), and 20 μg protein was resolved by 8.5% SDS-PAGE. The protein was transferred to a PVDF membrane and was detected by mouse anti-3βHSD1 (Sigma), rabbit anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-β-actin (Sigma) antibodies. To determine the role of the ERAD pathway in 3βHSD1 stability, cells were treated with Eeyarestatin (Sigma) for 6 hours before harvesting cell lysate.

To purify endogenous ubiquitin modified 3βHSD1, total cell lysate from ten million LAPC4 cells treated with 10 μM MG132 for 6 hours (EMD Millipore, Billerica, MA) was collected with IP lysis buffer (20 mM HEPES pH 7.9, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 10 mM glycerol phosphate, 10 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM NaF, 1 mM Na_3VO_4, 0.1% Nonidet P-40), supplemented with protease inhibitor cocktail and 20mM N-ethylmaleimide (Sigma). Pre-cleared lysate was incubated with mouse anti-ubiquitin antibody (Santa Cruz) at 4°C for 3 hours and followed by adding 40 μl of Protein AG UltraLink Resin (Thermo Scientific) for another hour for pull down. After extensive washing with lysis buffer, the purified proteins were eluted with 25 μl 2X SDS sample buffer and analyzed by Western blot.

For co-immunoprecipitation of 3βHSD1 and AMFR, 4 dishes of 293T cells (at 60% confluence) were transfected with 5μg of wild type 3βHSD1(367N) or 3βHSD1(367T) pCMX-HSD3B1-HA by polyethylenimine (Polysciences, Warrington, PA) for 36 hours. Immunoprecipitation assay was performed as described above. All immunoprecipitation studies were repeated with independent experiments.

***In vivo* ubiquitination assay**

For purification of 6xHis-ubiquitin conjugated proteins, experiments were conducted as previously described, with minor modifications (Rodriguez et al., 1999; Xirodimas et al., 2004). Briefly, HEK293T cells were transfected with pcDNA3-6xHis-ubiquitin together with wild-type 3βHSD1(367N) or 3βHSD1(367T) pCMX-HSD3B1-HA for 36 hours. Transfected cells were harvested by scraping in ice-cold PBS. Twenty percent of the cell suspension was pelleted and lysed with RIPA lysis buffer. Heterologously-expressed proteins were analyzed by Western blot. The remaining cells were pelleted and lysed with 4 mL lysis buffer (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 8.0, 5 mM imidazole, and 10 mM β-mercaptoethanol). Proteins covalently conjugated by 6xHis-ubiquitin were pulled down by adding 40 µl Ni-NTA-agarose (QIAGEN Inc, Valencia, CA), incubated at room temperature for 2 hours and successively washed with the following buffers: (1) 6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 8.0, 5 mM imidazole plus 10 mM β-mercaptoethanol; (2) 8 M Urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 8.0, 10 mM imidazole, 10 mM β-mercaptoethanol plus 0.1% Triton X-100; (3) 8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 6.3, 10 mM β-mercaptoethanol (buffer A), 20 mM imidazole plus 0.2% Triton X-100, twice; (4) buffer A with 10 mM imidazole plus 0.1% Triton X-100; (5) buffer A with 10 mM imidazole plus 0.05% Triton X-100. After the last wash, the proteins were eluted with 25 µl 2X SDS sample buffer containing 200 mM imidazole and 10 µL of elute was then analyzed by SDS-PAGE and Western blot. To determine the ubiquitin conjugation sites, lysine residues were replaced with arginine by using Quick Change Site directed Mutagenesis kit (Agilent Technologies) with primer set (K70R Forward: 5'-GAT GAG CCA TTC CTG AGG AGA GCC

TGC CAG GAC-3'; K70R Reverse: 5'-GTC CTG GCA GGC TCT CCT CAG GAA TGG CTC ATC-3'; K352R Forward: 5'-GAG GAA GCC AAG CAG AGA ACG GTG GAG TGG GTT-3'; K352R Reverse: 5'-AAC CCA CTC CAC CGT TCT CTG CTT GGC TTC CTC-3'). Ubiquitination studies were repeated with independent experiments.

Mass Spectrometry

Materials

1M triethylammonium bicarbonate (TEAB) solution, dl-dithiothreitol (DTT), iodoacetamide, and proteomics sequencing grade trypsin were purchased from Sigma. LC/MS grade acetonitrile and LC/MS grade trifluoroacetic acid (TFA) were purchased from Fisher Scientific.

In-gel digestion

Protein samples were separated by SDS-PAGE and stained with SimplyBlue SafeStain (Invitrogen) following the standard procedure. Briefly, the gel was rinsed with ultrapure water 3 times for 5 minutes and stained with 20 mL SimplyBlue SafeStain for 1 hour at room temperature. After washing twice with 100 mL ultrapure water for 1 hour, each gel lane was then cut into three pieces such that each would contain roughly equal amounts of proteins. Each excised gel band was then further chopped down into 1mm cubes. In-gel digestion was performed following the protocol below. Coomassie blue stain was removed by a 30 min incubation at 37°C in 50mM triethylammonium bicarbonate (TEAB)/acetonitrile (1:1, v/v). Gel pieces were dehydrated with acetonitrile at room temperature, followed by reduction/alkylation using DTT and iodoacetamide. Gel pieces were then dehydrated with acetonitrile and rehydrated

with trypsin solution (400ng/µg in 50mM acetic acid). Trypsin digestion was carried out at 37°C overnight. Peptides were extracted after 30min incubation at 37°C with extraction buffer to a final concentration of 50% acetonitrile and 3.3% TFA. All steps were carried out on a thermomixer shaker (Eppendorf, NJ) unless stated otherwise. Extracts were dried in vacuum centrifuge. Salts were removed using Oasis HLB µElution plate (Waters, MA) before LC-MS/MS analysis.

LC-MS/MS analysis

One-dimensional liquid chromatography was performed on an Ultimate 3000 nano HPLC system (Dionex), equipped with a 75 µm i.d. x 50 cm Thermo Scientific Easy-Spray column packed with 2µm resin. Separation of peptides was carried out at 350nl/min by a 200 min linear gradient of 1% to 25% acetonitrile in 0.1% formic acid. Column temperature was raised and maintained at 60°C using an Easy-Spray source (Thermo Electron). Mass spectrometric analyses were performed on a QExactive instrument (Thermo Electron) using a data-dependent top 20 method, with the full-MS scans acquired at 70K resolution (at m/z 200) and MS/MS scans acquired at 17.5K resolution (at m/z 200). Under-fill ratio was set at 0.1%, with a 3 m/z isolation window and fixed first mass of 100 m/z for the MS/MS acquisitions. The charge exclusion was applied to exclude the unassigned and singly charged species, and dynamic exclusion was used with a duration of 15 sec. SILAC MS data were analyzed using MaxQuant (version 1.3.0.5) (Cox and Mann, 2008) with default parameters, except that GlyGly(K) was specified as a variable modification.

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