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The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer

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Summary

Cellular levels of key regulatory proteins are controlled via ubiquitination and subsequent degradation. Deubiquitinating enzymes or isopeptidases can potentially prevent targeted destruction of protein substrates through deubiquitination prior to proteasomal degradation. However, only one deubiquitinating enzyme to date has been matched to a specific substrate in mammalian cells and shown to functionally modify it. Here we show that the isopeptidase USP2a (ubiquitin-specific protease-2a) interacts with and stabilizes fatty acid synthase (FAS), which is often overexpressed in biologically aggressive human tumors. Further, USP2a is androgen-regulated and overexpressed in prostate cancer, and its functional inactivation results in decreased FAS protein and enhanced apoptosis. Thus, the isopeptidase USP2a plays a critical role in prostate cancer cell survival through FAS stabilization and represents a therapeutic target in prostate cancer.

Introduction

Ubiquitination of targeted substrates is a reversible process. *Ub*iquitin processing *p*roteases (Ubps) are cysteine proteases also known as deubiquitinating enzymes or isopeptidases (Wilkinson, 2000). Despite the large number of Ubps identified, little is known about their physiological roles or their substrates. Ubps may "edit" the number of ubiquitin moieties in the polyubiquitin chain of poorly or erroneously ubiquitinated proteins or generate free ubiquitin from polyubiquitin chains released after proteasomal activity. More importantly, a preproteasomal action for isopeptidases has been hypothesized, resulting in the cleavage of the polyubiquitin tag from specific substrates, preventing and modulating their degradation. In fact, the deubiquitinating enzyme HAUSP (USP7) has been shown to stabilize p53 through deubiquitination even when the ubiquitin ligase mdm2 is in excess (Li et al., 2002). These findings confirm the existence and the importance of preproteasomal isopeptidases. In this report, we show that the isopeptidase USP2a is a preproteasomal, androgen-regulated isopeptidase and is a key regulator of prostate cancer cell survival through the stabilization of FAS.

Results and discussion

We intended to identify androgen-regulated deubiquitinating enzymes expressed in the prostate and functioning at the pre-

proteasomal level. In addition, we sought to determine their potential substrates. Using degenerate primers complementary to the conserved isopeptidase catalytic motifs, the CYS and HIS boxes, and RACE-PCR, several full-length cDNAs encoding isopeptidases were obtained from RNA preparations of both intact and androgen-deprived (castrated) rat prostate. The most abundantly expressed cDNA in the preparation from the prostate with an intact androgen environment was Ubp69. Ubp69 is a product of a gene which also gives rise to an alternatively spliced form, called Ubp45 (Park et al., 2002). These isopeptidases are also known as Ubp-t2 and Ubp-t1, respectively (Lin et al., 2000, 2001). We then utilized the LNCaP prostate cancer cell line to obtain its human homolog, USP2, and characterized the gene structure on chromosome 11q23.3 (Figure 1A). Since the official human nomenclature for the gene known to be coded by this locus is USP2 (GenBank # NM_004205), we renamed the human splice variants USP2a (GenBank # NP_004196) and USP2b (GenBank # NP_741994). We also identified putative androgen-regulatory elements in the immediate 5′ upstream region. The C terminus, shared by both USP2a and USP2b, contains the canonical isopeptidase CYS and HIS boxes (Figure 1A). An antibody generated against the C terminus recognizes both splice variants (Figure 1A, lane 1), while the USP2a-specific one recognizes only the 69 Kd band (Figure 1A, lane 2).

We next searched for proteins that interacted with USP2a.

S I G N I F I C A N C E

We report that the deubiquitinating enzyme USP2a functions at the preproteasomal level by preventing the degradation of fatty acid synthase (FAS). FAS overexpression occurs in the majority of epithelial tumors, including prostate cancer, and protects cancer cells from apoptosis. Because USP2a is overexpressed in prostate cancer and its inhibition results in apoptosis of transformed prostatic cells, this isopeptidase may be targeted to induce apoptosis in prostate cancer cells.
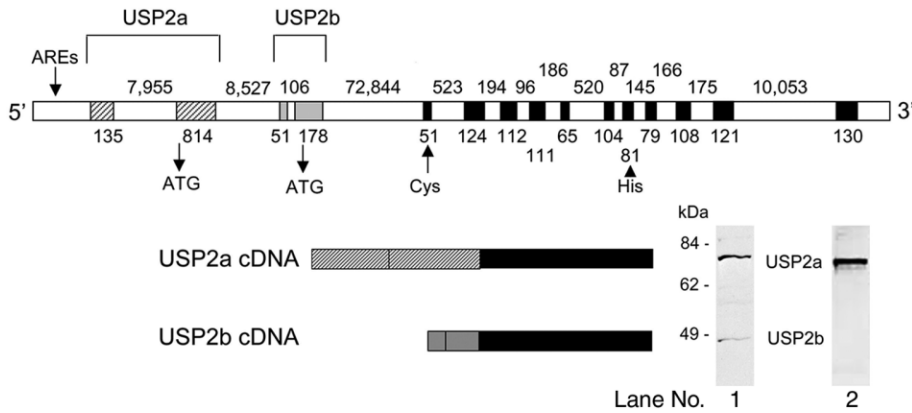

Figure 1. Chromosomal arrangement of the USP2 gene and its two splice variants

USP2a and b on chromosome 11q23.3. The homology between rat Ubp69 and human USP2a is 88% and between Ubp45 and USP2b is 90%. The numbers above and below the scheme represent the size in base pair of the introns and exons, respectively. An antibody raised against the USP2 region common to both splice variants (black boxes) recognizes both USP2a and b in an immunoblot analysis of LNCaP cell protein lysate. The anti-USP2a-specific antibody raised against the N terminus of USP2a (hatched boxes) recognizes only the 69 Kd form.

The androgen-dependent LNCaP cells were grown in the presence of 10% fetal bovine serum (FBS) or in androgen-deprived (charcoal stripped—cd) FBS for 48 hr. These two cell culture conditions were utilized because they reflected the intact and castrate rat prostate environments in vivo, respectively. LNCaP cells were metabolically labeled with ^{35}S-methionine. The protein lysates were then incubated with Ni-NTA resin coupled with the recombinant his-tagged USP2a, and one prominent band of approximately 250 kDa was detected (Figure 2A). This band was stronger in lysates prepared with cells grown in the presence of FBS than in charcoal-stripped, androgen-depleted conditions (cd-FBS), and absent in the uncoupled Ni-NTA. The 250 kDa band was analyzed by mass spectrometry and identified as being fatty acid synthase (FAS) (Figure 2B). The interaction between USP2a and FAS was then confirmed by coimmunoprecipitation performed in vitro (Figure 2C, lane 3) and reciprocal coimmunoprecipitation in vivo (Figure 2C, lanes 6 and 9). Finally, USP2a and FAS proteins colocalized in the cytoplasm of LNCaP cells (Figure 2D).

Next, we sought to determine whether USP2a is an androgen-regulated gene. LNCaP cells were grown in charcoal-stripped serum. Addition of dihydrotestosterone (DHT) to the medium induced, while the anti-androgen bicalutamide (Casodex) blocked, the expression of endogenous USP2a (Figure 3A,
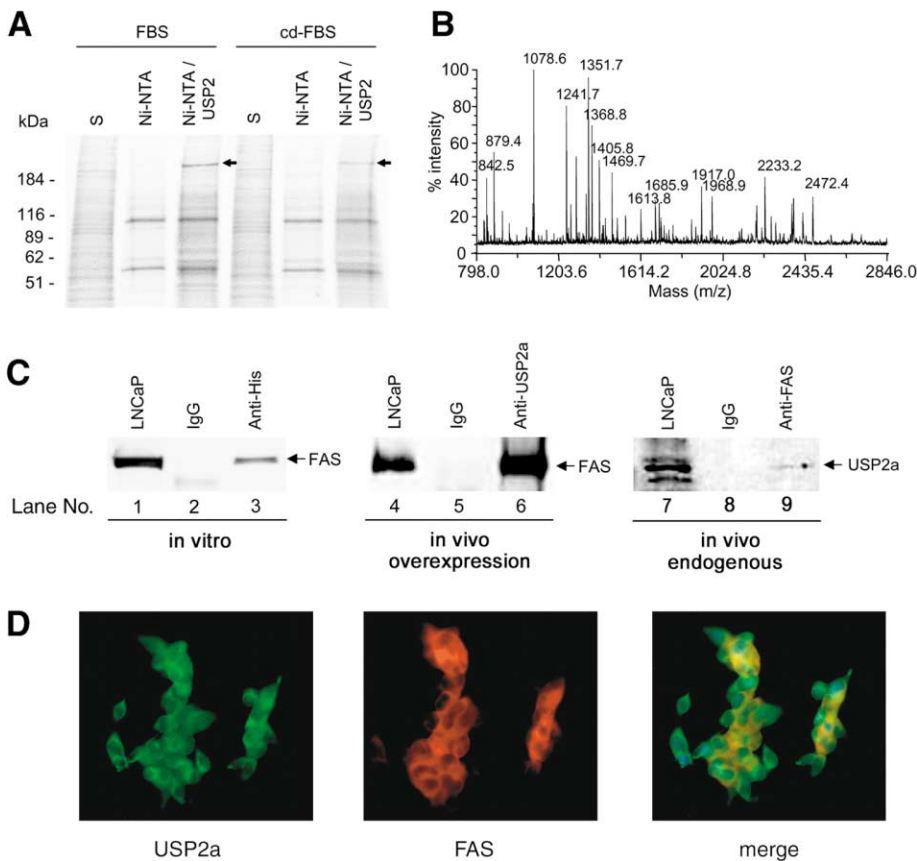

Figure 2. USP2a interacts with fatty acid synthase (FAS)

A: Gradient SDS-polyacrylamide gel of eluted proteins from the affinity chromatography: the arrows indicate the FAS band, which was stronger in LNCaP cells grown in the presence of FBS than with charcoal-stripped serum.
B: Mass spectrometry analysis of the band.
C: FAS is coimmunoprecipitated in vitro with the recombinant 6his-USP2a (left panel, lane 3), and in vivo after transient transfection and overexpression of USP2a (middle panel, lane 6). USP2a is identified by immunoblotting after immunoprecipitation of endogenous FAS (right panel, lane 9).
D: Immunofluorescence of USP2a (FITC—green) and FAS (rhodamine—red) demonstrating their colocalization in prostate cancer cells (right panel—nuclei counterstained with DAPI).
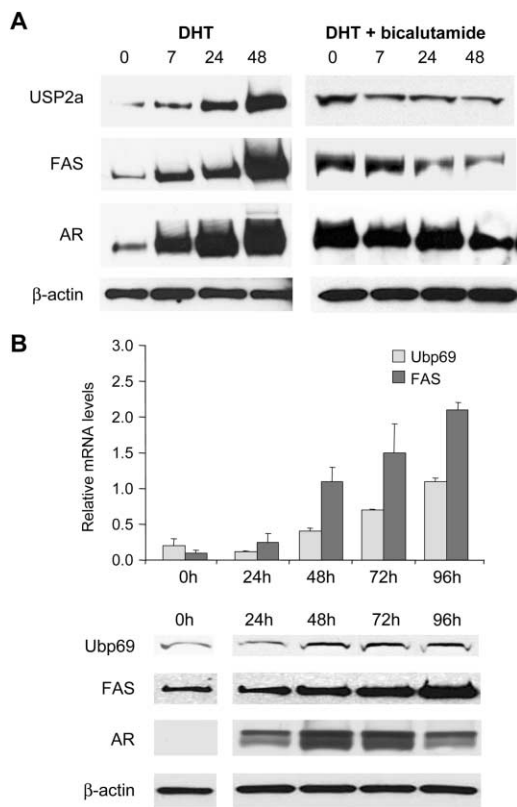
Figure 3. USP2a is regulated by androgens

A: Dihydrotestosterone (DHT) induces USP2a as well as FAS and AR protein expression in LNCaP cells grown in charcoal-stripped serum. Induction of all three proteins is blocked by the anti-androgen bicalutamide (Casodex) (right panel).

B: mRNA levels of rat Ubp69 (homolog of the human USP2a) and of FAS assessed by quantitative PCR in the rat prostate at castration (time 0) and during regeneration (at the times shown) after testosterone replenishment (bar graph). Western blots of USP2a, FAS, AR, and β actin are shown below. mRNA levels of Ubp69 progressively increase after testosterone proprionate (TP) stimulation, while protein levels increase at 48 hr and then stabilize.

top panel). FAS mRNA is known to be induced by androgens in prostate cancer cells (Kuhajda, 2000; Swinnen et al., 2000). As expected, both FAS and the androgen receptor (AR) were induced by DHT and blocked by Casodex (Figure 3A, middle panels). In addition, rat Ubp69 mRNA expression was progressively induced by testosterone proprionate replenishment after castration in the regenerating prostate rat model (Waltregny et al., 2001) (Figure 3B). As expected, FAS mRNA and protein as well as AR protein were upregulated by testosterone in the same model (Figure 3B). Taken together, these results demonstrate that Ubp69/USP2a (hereon in referred to as USP2a) is regulated by androgens.

The association between USP2a and FAS prompted us to investigate posttranscriptional FAS regulation. It had been previously reported that the yeast homolog of FAS is regulated by ubiquitin-mediated degradation (Egner et al., 1993). Therefore, we next investigated whether the inhibition of the proteasome activity affects FAS turnover in LNCaP cells. Five hours following lactacystin or MG-132 treatment, the FAS protein accumulates

when compared to control cells (Figure 4A, lanes 3 and 4). Immunofluorescence with anti-FAS antibodies confirmed the accumulation of cytoplasmic FAS in LNCaP cells treated with proteasome inhibitors (Figure 4A). Importantly, ubiquitinated isoforms of FAS were identified by FAS immunoprecipitation followed by anti-ubiquitin immunoblot (Figure 4B). Finally, using pulse-chase experiments, proteasomal inhibition was able to significantly extend FAS protein half-life from 12.2 to 40.9 hr (Figure 4C). These results indicate that FAS is regulated by the ubiquitin-proteasome system in human cells.

Next, we confirmed that the catalytic core of USP2 (lacking the N terminus) has in vitro deubiquitinating activity as previously described (Lin et al., 2000, 2001) (Figure 5A, lane 4). In order to show that the ubiquitination state of FAS in vivo depends on USP2a, siRNAs designed to bind to the 5′ region specific to the USP2a isoform were utilized in the presence of proteasome inhibitors and FAS ubiquitination was determined. Indeed, FAS ubiquitination was highest in LNCaP cells treated with both anti-USP2a siRNA and MG-132 (Figure 5B, lane 3).

In order to assess the role of USP2a in vivo, we transiently transfected LNCaP cells with both wild-type and mutant USP2a (Δ276 Cys to Ala). Mutations in the cysteine box have traditionally been utilized to obtain catalytically inactive isopeptidase mutants which have been shown to often act as dominant negatives (DeSalle and Pagano, 2001; Li et al., 2002; Naviglio et al., 1998). Four hours after cycloheximide treatment, FAS protein levels decreased in LNCaP cells transfected with vector (Figure 5C, lane 3, upper panel) or, even more significantly, with mutant USP2a (Figure 5C, lane 9, upper panel), while wild-type USP2a protected FAS from degradation (Figure 5C, lane 6 compared to lanes 3 and 9, upper panel). Neither the wild-type nor the catalytic inactive mutant of the unrelated isopeptidase UNP (DeSalle et al., 2001) had an effect on FAS stability (data not shown). Thus, USP2a protects FAS from degradation in vivo.

To further understand the role USP2a plays in the regulation of FAS metabolism, we blocked USP2a expression in LNCaP cells with antisense oligonucleotides. Transfected cells were FACS-selected on the basis of cotransfection of CD19 in a 3:1 ratio. USP2a mRNA levels were reduced by approximately 90% in cells transfected with anti-USP2a oligonucleotides. As expected, protein levels were comparably suppressed (Figure 6A). Importantly, FAS protein levels were significantly reduced, despite unaltered FAS mRNA levels (Figure 6A). To investigate the biological consequences of FAS protein downregulation via USP2a inhibition, we assessed changes in cell cycle kinetics or induction of programmed cell death, since both had been reported as a result of direct inhibition of FAS. Apoptosis was obtained by treating prostate cancer cells with USP2a antisense oligonucleotides. As expected, a similar effect on apoptosis was observed after treatment of LNCaP cells with cerulenin, a well-known inhibitor of FAS (Figure 6A). In fact, RNA interference of FAS also results in apoptosis of LNCaP cells (Swinnen et al., 2003). A slightly higher apoptotic rate was obtained when non-sorted LNCaP cells were treated with siRNA specific for USP2a (Figure 6B) compared to antisense oligonucleotide treated cells. The mismatched control had no effect. The effect was also more protracted when compared to antisense oligonucleotides, since most cells treated with anti-USP2a siRNA (but not the scrambled control) were apoptotic at 120 hr following transfection (data not shown). No effect on the S phase fraction was observed with either gene silencing or cerulenin (not shown). These results
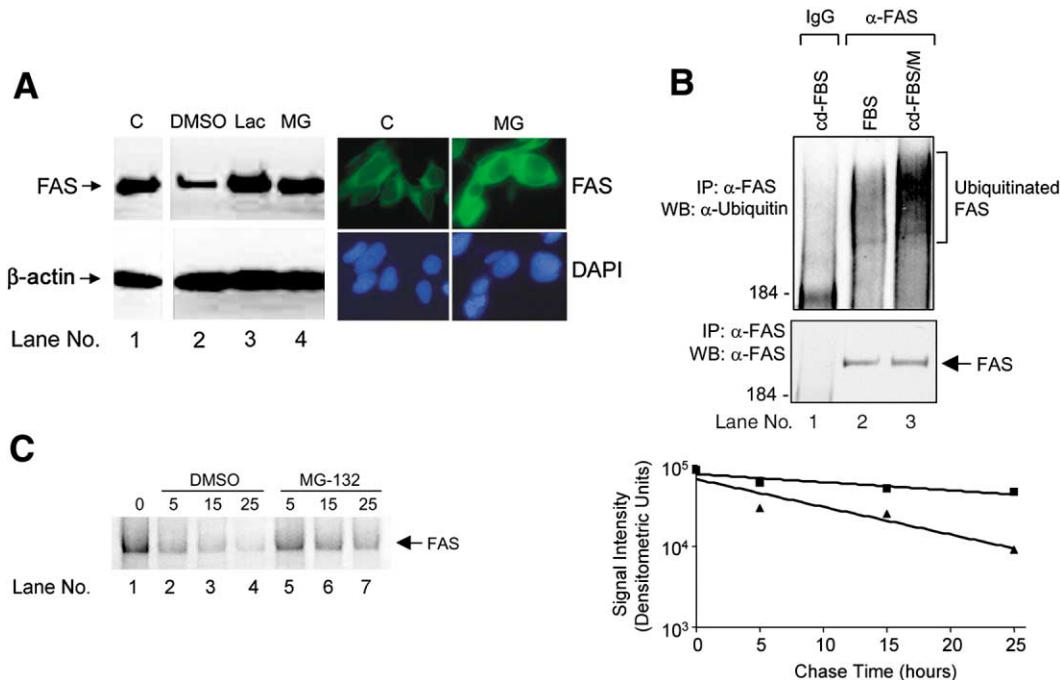
Figure 4. FAS is regulated by the ubiquitin-proteasome pathway

A: FAS protein levels increase in Lactacystin (Lac) or MG132 (MG) treated (5 hr) LNCaP cells (lanes 3 and 4) compared to control (lane 2). Lane 1 shows endogenous levels of FAS. Cytoplasmic accumulation of FAS occurs in LNCaP cells after proteasome inhibition (MG, middle panel).

B: Immunoprecipitation with anti-FAS antibodies followed by Western blot with anti-Ub polyclonal antibodies show the smear-like positivity of the ubiquitinated isoforms of FAS in LNCaP cells. The reaction is strongest in protein extracts obtained from cells grown in charcoal-stripped serum (cd-FBS) (lane 3). The membrane was stripped and reblotted with anti-FAS antibodies (bottom panel).

C: LNCaP cells were pulse-labeled with ^{35}S-methionine, treated with 5 μM of the proteasome inhibitor MG-132 or DMSO, the protein extracts immunoprecipitated with antibodies against FAS, and autoradiography performed (left panel). The half-life of FAS shown in the semi-log plot (right panel) was extended from 12.2 hr to 40.9 hr in the cells treated with MG-132.

suggest that the USP2a enzyme acts by inhibiting FAS protein degradation by the proteasome, resulting in the protection of prostate cancer cells from apoptosis.

In order to prove this assumption, we generated LNCaP cells stably transfected with FAS, which we called FASCaP. This line expresses 2.3-fold levels of FAS protein and 8.7-fold levels of FAS mRNA (Figure 6C, lane 2) compared to the parent LNCaP cell line. When FASCaP cells were treated with siRNA against USP2a, FAS protein levels remained persistently higher than those in parent LNCaP cells, and no apoptosis was seen (Figure 6D). Finally, Figure 6E demonstrates a knockdown of USP2a protein (lanes 2 and 4) and mRNA (bar graphs) by the specific siRNA in both LNCaP and FASCaP lines. In contrast, FAS protein levels diminish only in LNCaP untransfected cells. These results strongly suggest that apoptosis resulting from USP2a inactivation is mediated by FAS.

To assess the relevance of the USP2a/FAS interaction in human prostate cancer, USP2a expression levels were first analyzed in human prostate cell lines. USP2a is expressed in the prostate cancer cell lines LNCaP, DU145, and PC3, but not in normal primary prostate epithelial (PrEC) cells (Figure 7A). We then assessed USP2a expression in human normal and tumor tissues by semiquantitative RT-PCR followed by densitometry (Figure 7B). Indeed, 46% of tumor samples showed increased expression of USP2a mRNA compared to the corresponding normal. Finally, utilizing the polyclonal antibody we generated

against the N terminus of USP2a, we found overexpression of USP2a protein in 51% of prostate tumors (Figure 7C). Thus, USP2a is overexpressed in a subset of prostate cancers both at the mRNA and protein level.

Despite the growing number of Ubp family members, their physiological functions remain mostly unknown. The high number and structural diversity of Ubps suggests that some of these enzymes have specific targets. The protein levels of specific substrates may be controlled by the removal of polyubiquitin chains, preventing and regulating their degradation. Here, we demonstrate that USP2a is an androgen-regulated isopeptidase and that downregulating USP2a in prostate cancer destabilizes FAS leading to programmed cell death, thus establishing a link between substrate regulation by a "preproteasomal" Ubp, a metabolic enzyme, and prostate cancer. USP2a thus appears to behave as an oncogene by stabilizing the antiapoptotic enzyme FAS.

FAS plays a central role in de novo lipogenesis. In addition, tumor cells synthesize 95% of fatty acids de novo, despite adequate nutritional supply (Ookhtens et al., 1984; Sabine et al., 1967; Weiss et al., 1986). FAS is overexpressed in prostate cancer, while overexpression has been associated with aggressive biologic behavior, suggesting a functional role for fatty acid synthesis in the growth or survival of cancer cells (Dhanasekaran et al., 2001; Kuhajda, 2000; Rossi et al., 2003; Singh et al., 2002a). Importantly, FAS is overexpressed in metastases arising
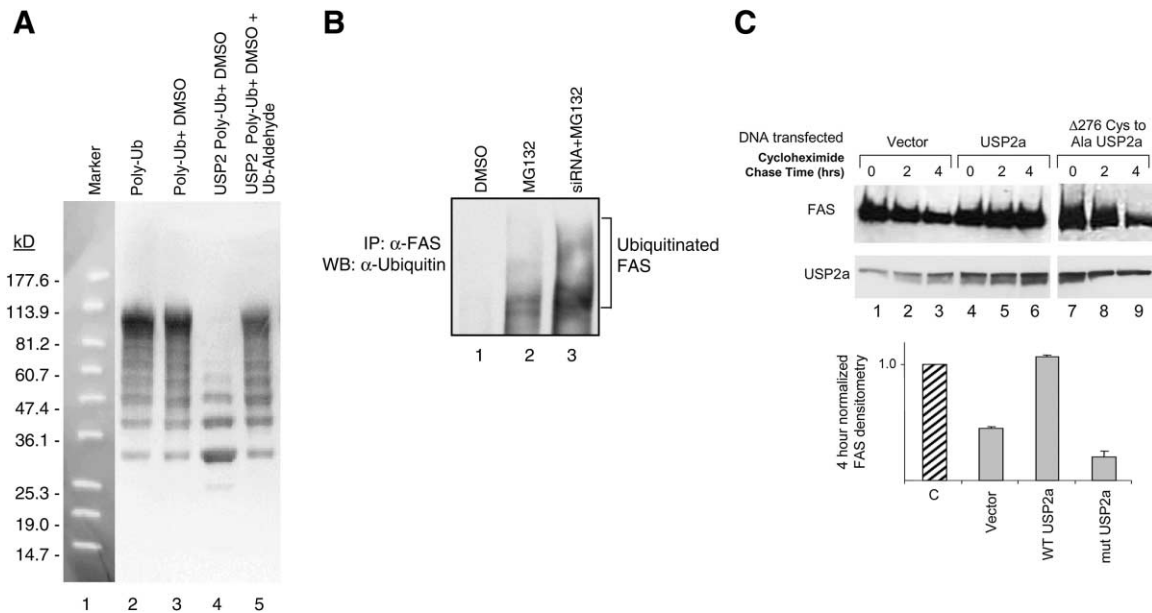
Figure 5. USP2a deubiquitinates FAS and protects it from proteasome-mediated degradation

A: Recombinant GST-Polyubiquitin was incubated in presence or absence of Ubiquitin-aldehyde with recombinant core USP2 (lacking the N terminus) for 30 min at 37°C. The FK2 antibody (Affiniti Research Products, Exeter, UK) was used to detect polyubiquitin in the Western blot. The catalytic core of USP2 degraded polyubiquitin chains (lane 3), while ubiquitin aldehyde almost completely inhibited core-USP2-mediated polyubiquitin degradation activity (lane 4).

B: Incremental ubiquitination of FAS is seen starting with untreated LNCaP cells (lane 1), in LNCaP cells treated with MG-132 (lane 2), and in LNCaP cells treated with anti-USP2a siRNA as well as proteasome inhibitor (lane 3).

C: FAS protein levels in LNCaP cells treated with cyclohexamide and transfected with empty vector (lanes 1–3), wild-type USP2a (lanes 4–6), and catalytic inactive mutant (Δ276 Cys to Ala) USP2a (lanes 7–9). Membranes were stripped and reblotted with anti-USP2a antibodies (lower panel). Quantitation of the 4 hr time points normalized to the 0 hr control show that wild-type, but not mutant USP2a, prevents degradation of FAS.

in the TRAMP mouse model of prostate cancer (Pflug et al., 2003) and, together with its transcriptional regulator steroid regulatory element binding protein-1 (SREBP1), in human prostate cancer metastatic to bone (Rossi et al., 2003).

Taken together, direct inactivation of FAS (Swinnen et al., 2003) and the results we obtained suggest that FAS overexpression confers selective advantage to prostate cancer cells by inhibiting apoptosis. While the mechanism by which FAS overexpression protects cancer cells from apoptosis is still not known, several hypotheses have been put forth. First, it has recently been shown that FAS plays a major role in the synthesis of cellular membrane phospholipids (Swinnen et al., 2003). Alterations in membrane lipid composition may, in turn, have profound effects on relevant signal transduction pathways, such as the phosphatidylinositol 3′-kinase pathway (Van de Sande et al., 2002). Alternatively, the cytotoxicity which occurs as a result of FAS downregulation may be attributed to direct activation of apoptotic pathways. In fact, FAS inhibition results in the release of cytochrome c and caspase activation (Heiligtag et al., 2002). Finally, FAS overexpression may restore redox balance in limiting oxygen conditions. Specifically, excessive lactate produced by anaerobic glycolysis limits the respiratory chain and results in decreased oxidizing power. Interestingly, both lower organisms tolerant to hypoxia and prostate cancer cells favor synthesis and chain elongation of fatty acids because they supply oxidizing power for key oxidative steps (Hochachka et al., 2002). Thus, overexpression and enhanced activity of FAS would result in a significant improvement in redox balance de-

spite hypoxic conditions, possibly preventing mitochondrial initiation of the apoptotic cascade.

Not only do FAS inhibitors, such as C75, the mycotoxin cerulenin, and interference of FAS message result in apoptosis of prostate cancer cells in culture (Kuhajda et al., 2000; Swinnen et al., 2003), but they also result in decrease tumor size in animal models of prostate cancer that overexpress the enzyme (Pflug et al., 2003; Pizer et al., 2001), underscoring the relevance of FAS inhibition in vivo. Targeting FAS directly in cancer, however, may result in undesirable side effects such as anorexia and significant weight loss (Clegg et al., 2002), and may even have teratogenic consequences (Chirala et al., 2003). Since we show here that FAS protein levels are modulated via deubiquitination by USP2a (Figure 8), our data suggest that FAS downregulation with the resultant induction of programmed cell death could be achieved by targeting USP2a in prostate cancer.

In conclusion, the results of the present study indicate that USP2a is a preproteasomal, androgen-regulated isopeptidase and is a key regulator of prostate cancer cell survival via the stabilization of FAS protein. USP2a may represent a potential therapeutic target in prostate cancer.

Experimental procedures

Degenerate PCR and RACE RT-PCR
One microgram of total RNA from the rat prostate was reverse-transcribed and used to PCR-amplify the CYS and HIS boxes with the following primer set: Cys-box primer: 5′ AAC CTG GG(C,G) AAC AC(C,T) TGC T(G,T)C ATG AAC 3′; His-box primer: 5′ GTT CTT C(C,T,A)C (A,G)TA (G,T)G(T,C) (A,G)GT
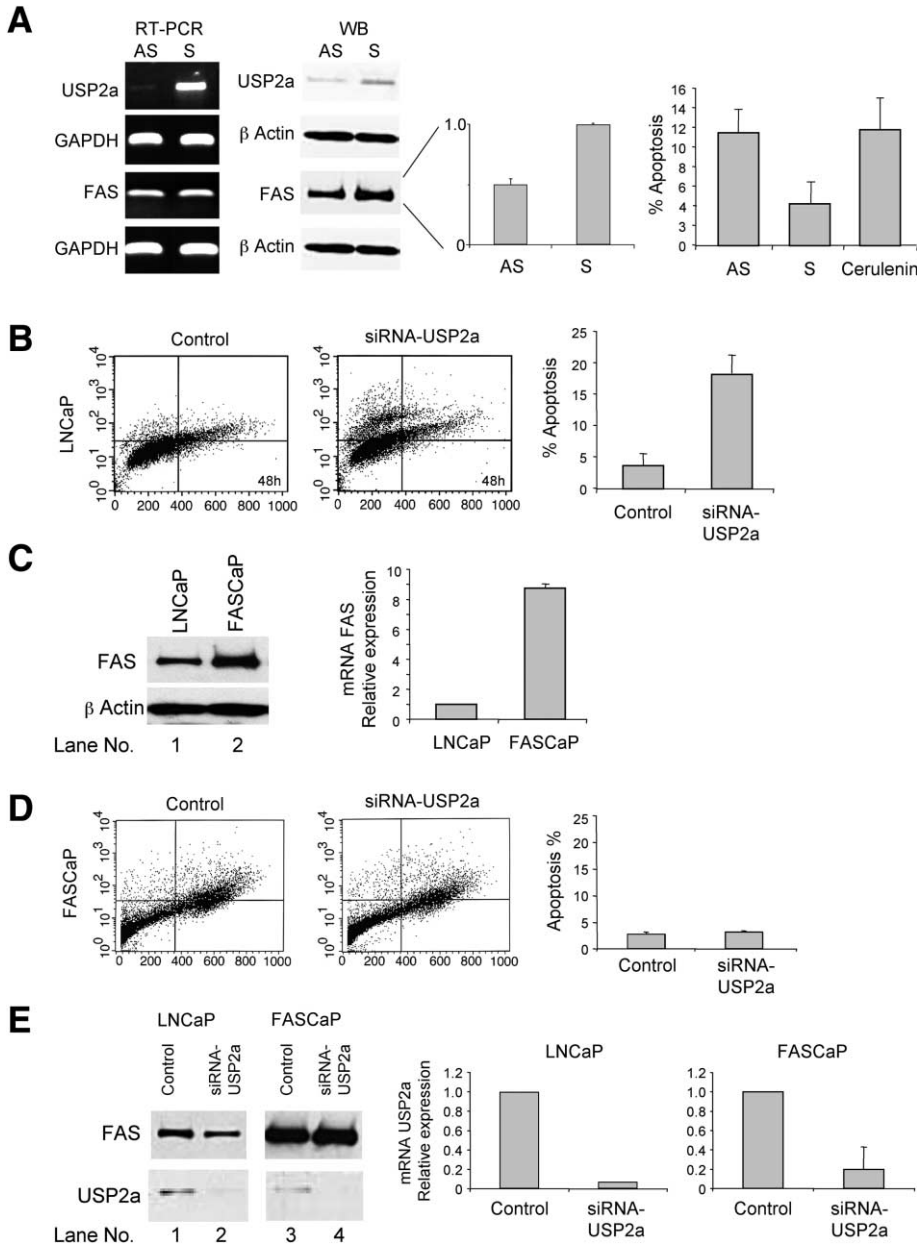
Figure 6. Antisense oligonucleotides and siRNA against USP2a induce apoptosis in prostate cancer cells

Apoptosis is rescued by FAS overexpression.
A: Antisense experiment in LNCaP cells. mRNA (RT-PCR) and protein (WB) levels of USP2a and FAS from CD19-sorted LNCaP cells transfected with antisense (AS) or sense (S) oligonucleotides. USP2a mRNA levels were reduced by approximately 90%, and FAS mRNA levels were not affected. Western blot reactions show a 33% decrease in USP2a and a 50% decrease in FAS levels (average of three experiments shown in histogram) in the cells transfected with AS oligonucleotides for USP2a. AS oligonucleotides to USP2a, as well as the FAS inhibitor cerulenin, induce apoptosis.
B: siRNA experiment in LNCaP cells. siRNA against USP2a, but not the mismatched siRNA, induces apoptosis in LNCaP cells. Quantitation of apoptosis in unselected LNCaP cells was measured by CaspACE/FACS analysis following anti USP2a siRNA or nonsilencing siRNA in four independent experiments. SSC (x axis) refers to cell granularity and FL (y axis) represents fluorescence of cells.
C: Quantitation of FAS protein (2.3×) and mRNA (8.7×) by qPCR in LNCaP cells stably transfected with FAS (FASCaP cells).
D: siRNA experiment in FASCaP cells. FAS overexpression rescues apoptosis in LNCaP cells: apoptosis is not induced by siRNA against USP2a in FASCaP cells.
E: Levels of USP2a mRNA and protein decrease in both lines (lanes 2 and 4), while FAS protein levels diminish only in LNCaP untransfected cells (lane 2, upper blot), but not in FASCaP cells (lane 4, upper blot).

GTA GTG GCC 3′. The SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) was utilized to obtain the full-length cDNAs.

Semiquantitative and real-time RT-PCR
One microgram of RNA from castrate or testosterone-replenished rat prostates, as well as normal and neoplastic human prostate and LNCaP cell lines, were reverse-transcribed with random primers and amplified with FAS, Ubp69, and USP2a-specific primers. The sequences for rat Ubp69 and FAS primers are 5′ CAG ACC CGT GGC AAT GAA A 3′ (Ubp69 forward), 5′ GCT GTT CGA TTT CTT CTG GC 3′ (Ubp69 reverse), 5′ CCT CAG TCC TGT TAT CAC CCG A 3′ (FAS forward), and 5′ GCT GAA TAC GAC CAC GCA CTA 3′ (FAS reverse). The sequences for the human USP2a and FAS primers are 5′ ATG CTT GTG CCC GGT TCG AC 3′ (USP2a forward), 5′ CTA CAT TCG GGA GGG CGG GCT 3′ (USP2a reverse), 5′ CAGCCATGGAGGAGGTGGT GATT 3′ (FAS forward), 5′ GGGACATGCCTAACACCTACGTCTA 3′ (FAS reverse). For semiquantitation on the human samples, the PCR products were visualized on 1.3% agarose gels stained with ethidium bromide and analyzed using the Kodak 440CF imager. The expression of USP2a cDNAs

was normalized by GAPDH. Real-time RT-PCR on the rat prostates and LNCaP cells was performed using an ABI-PRISM 7700 Sequence Detector and the SYBR Green PCR Core Reagent kit (PE Applied Biosystems, Foster City, CA), as previously described (Waltregny et al., 2001). All samples were run in triplicate, and the relative amounts of Ubp69, USP2a, FAS, rat, and human GAPDH mRNAs calculated by comparison with standard curves.

Affinity chromatography and mass spectrometry
His-tagged USP2a coupled to Ni-NTA beads (Qiagen, Valencia, CA) was incubated for 1 hr at 4°C with 300 μg of a ^{35}S-labeled LNCaP cell lysate in a buffer containing 50 mM NaH$_2$PO$_4$ (pH 8.0), 150 mM NaCl, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin (Roche Molecular Diagnostics, Indianapolis, IN), 2 mM NEM (Sigma, St. Louis, MO), and 100 μM MG-132 (Oncogene Research Product, La Jolla, CA). Following extensive washing with the same buffer, the Ni-NTA pellets were resuspended and boiled for 5 min in Laemmli buffer, and the eluted proteins resolved in 4%–20% gradient SDS-polyacrylamide gels and exposed to X-Omat AR films (Eastman Kodak Co., Rochester, NY). To identify the USP2a interactors,
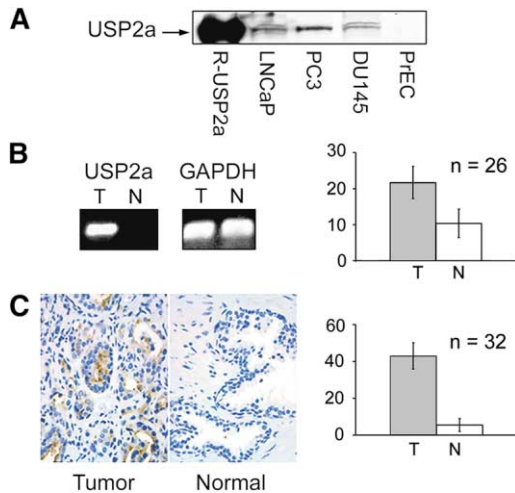
Figure 7. USP2a is overexpressed in prostate carcinoma

A: Western blot analysis using the USP2a-specific antibody shows that USP2a is expressed in prostate cancer cell lines LNCaP, DU145, and PC-3, but not in primary prostate (PrEC) cells.

B: RT-PCR for USP2a demonstrating tumor overexpression of the isopeptidase. Densitometry shows increased expression of USP2a in tumor samples compared to adjacent normal tissue.

C: Immunohistochemistry using the USP2a-specific antibody shows abundant expression of USP2a in tumor tissue and no expression in the corresponding normal tissue. Mean values of USP2a gene expression in normal and tumor samples is shown on the right.

this experiment was scaled up, and the stained bands cut out from the gel and analyzed by mass spectrometry (Maldi-TOF/Voyager DE/STR – Molecular Biology Core Facility, Dana-Farber Cancer Institute, Boston, MA). Among the bands that exhibited good signals and significant matches using the Protein Prospector Database (USCF), two were identified as being FAS (66% and 57% peptide match).

Coimmunoprecipitation
For in vitro coimmunoprecipitation, His-tagged USP2a protein was added to LNCaP protein lysates and FAS was detected in the His immunoprecipitate
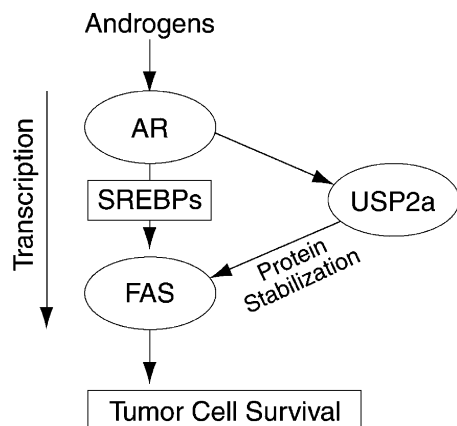
Figure 8. Hypothetical model of FAS regulation by USP2a

FAS is transcriptionally regulated via steroid regulatory element binding proteins (SREBPs), which, in turn, are regulated by the ligand-activated androgen receptor (AR). USP2a, also androgen-regulated, stabilizes FAS and contributes to tumor cell survival.

(Figure 2C, lane 3). For endogenous in vivo coimmunoprecipitation experiments, USP2a was overexpressed in LNCaP cells, and FAS immunoblotting was performed on anti-USP2a immunoprecipitate (Figure 2C, lane 6). Anti-USP2a immunoblot was performed on anti-FAS immunoprecipitate in untransfected cells (Figure 2C, lane 9).

Proteasome inhibitor treatment
LNCaP cells were incubated in RPMI 1640 medium containing the proteasome inhibitors Lactacystin (final concentration of 20 μM) (Oncogene Research Product, La Jolla, CA) or MG132 (50 μM) (Oncogene Research Product, La Jolla, CA) for 5 hr before being harvested for protein extraction (with or without prior FAS immunoprecipitation) or fixation for immunofluorescence.

Pulse chase
LNCaP cells were washed with methionine- and cysteine-free RPMI (Invitrogen, Carlsbad, CA) and pulse-labeled with 25 μCi/ml of ^{35}S-methionine (Perkin Elmer Life Sciences, Boston, MA) for one hour. Cells were then incubated for 5, 15, and 25 hr in RPMI with 10 μM of MG-132 or the equivalent amount of DMSO, harvested with cold PBS, and used to prepare protein lysates. Fifty micrograms of each lysate were immunoprecipitated for 12 hr at 4°C with 8 μg of anti-FAS antibodies (Transduction Laboratories, Lexington, KY) and incubated with protein G-Sepharose for 1 hr at 4°C. Eluted proteins were resolved in 6% SDS-polyacrylamide gels and exposed to X-ray films for 6–12 days at −80°C.

Generation of the stable FASCaP line
To generate FASCaP, the LNCaP cell line stably expressing FAS, the 7536 base pair human fatty acid synthase (FAS) cDNA was cloned into the pWZL-blasticidin-resistant retrovirus vector. 293T packaging cells were cotransfected with retroviral vector pWZL-FAS and packaging plasmid pCL. Tissue culture supernatants containing retrovirus derived from transfected 293T cells were used to infect LNCaP cells in the presence of 5 μg/ml polybrene (Sigma, St. Louis, MO). Successfully infected LNCaP cells were selected in the presence of 1.5 μg/ml blasticidin. Expression of transfected sequences was confirmed by Western blotting and qRT-PCR.

In vivo androgen responsiveness
LNCaP cells were grown in phenol red-free RPMI medium 1640 supplemented with 10% charcoal-dextran-stripped fetal bovine serum (cd-FBS) for 48 hr and treated with 10 nM DHT in the presence or absence of 10 μM of bicalutamide (Casodex). The cells were washed, harvested at 0, 7, 24, and 48 hr into 2 ml ice-cold phosphate-buffered saline, pelleted by centrifugation, and protein extracts immunoblotted with antibodies against USP2a, FAS, AR, and β actin.

Rat prostate castration-regeneration model
Twelve-week-old male Noble rats were purchased from Charles River Laboratories (Boston, MA). Fourteen days after surgical castration, rats were injected with 6.6 mg/kg Testosterone Propionate (TP) (Sigma Chemical Co., St. Louis, MO) in tocopherol-stripped corn oil (ICN Biomedicals, Inc., Aurora, OH) i.m., once a day, for 4 days. At 24, 48, 72, and 96 hr after the beginning of TP injections, animals (seven rats per time point) were sacrificed by CO_2 asphyxiation. The ventral prostate was then harvested, and immediately flash frozen in liquid nitrogen for subsequent RNA and protein isolation.

Deubiquitination assay
The cDNA encoding the recombinant GST-Polyubiquitin (accession number AB003730, GI:2627128 [Nenoi et al., 1998]; Invitrogen, Basel, Switzerland) was subcloned into pGEX4T1 (Amersham Biosciences, Otelfingen, Switzerland), expressed in the *E. coli* strain BL21(DE3) (Invitrogen) and purified by affinity chromatography on a GSTrap FF column according to the manufacturer's recommendations (Amersham). 3.75 μg of recombinant GST-Polyubiquitin was incubated in presence or absence of 150 ng Ubiquitin-aldehyde (Boston Biochem Inc., Cambridge, MA) with 150 ng of recombinant core USP2 (lacking the N terminus) for 30 min at 37°C.

Transfection with anti-sense oligonucleotides and siRNA
Anti-sense (AS) oligonucleotides (Oligos Etc., Wilsonville, OR) were designed to complement the 5′ end of the USP2a coding region (GAG AGC TGG GAC ATC CTT CA). A scrambled (S) oligonucleotide (AAG ATC AGA GAC CTC

CGT CT) was used as control. The CD19 plasmid was cotransfected with the antisense or sense oligonucleotides (3:1, w/w) in order to select by FACS only the transfected cells. LNCaP cells were transfected for 36–46 hr with 2 mg/ml of Lipofectin reagent (Invitrogen, Carlsbad, CA), 300 nM of AS or S oligonucleotides, and 0.19 nM of CD19 plasmid in OPTIMEM (Invitrogen, Carlsbad, CA). For protein and cell cycle analysis, cells were harvested after an additional 40 hr. The pellets were incubated with 1.5 μl anti-CD19 FITC conjugated (Caltag Laboratories, Burlingame, CA) at 4°C for 2 hr, washed twice with cold PBS, stained with propidium iodide, and analyzed by flow cytometry.

For the siRNA (Qiagen-Xeragon, Germantown, MD) experiment, $2.5 \times 10e5$ cells per 6 cm dish were seeded in RPMI 1640 medium containing 10% FBS. 24 hr later, cells were transfected either with 80 nM anti-USP2 siRNA, r(UGCUUGUGCCCGGUUCGAC)d(TT), or 80 nM nonsilencing control siRNA, r(UUCUUCGAACGUGUCACGU)d(TT), using Oligofectamin (Invitrogen, Carlsbad, CA).

Apoptosis measurement
Forty-eight hours post siRNA transfection, cells were labeled by adding 10 μM of the CaspACE FITC-VAD-FMK apoptosis in situ marker (Promega, Madison, WI), a cell-permeable derivative of the pan-caspase inhibitor Z-VAD-FMK binding to activated Caspase. After 20 min incubation in the dark, cells were trypsinized, washed with medium and PBS, and fluorescence was analyzed on a PARTEC PAS flow cytometer at 530 nm (excitation at 488 nm). Percentage of apoptotic cells was calculated by the FlowMax software.

Transient transfection and cycloheximide chase experiments
QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) was used to construct the catalytic site inactive mutant USP2a (Cys276Ala). The wild-type, mutant USP2a as well as wild-type and mutant Unp (DeSalle et al., 2001) genes were inserted in pcDNA3.1 expression vectors and transiently transfected into LNCaP cells with Lipofectamin Plus reagent (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, Cycloheximide (25 μg/ml) was added to the medium. At indicated times, the cells were washed, harvested into 2 ml ice-cold phosphate-buffered saline, pelleted by centrifugation, and protein extracts immunoblotted with the indicated antibodies.

Immunohistochemistry and immunofluorescence
Immunohistochemistry was performed with the USP2a rabbit polyclonal antibody we generated, on 5 μm tissue microarray sections of formalin-fixed, paraffin-embedded prostate adenocarcinoma and its adjacent normal tissue from a database we previously characterized (Singh et al., 2002b). The study was carried out in an automated immunostainer, the Optimax Plus (BioGenex, San Ramon, CA). Antigen-antibody reactions were revealed with standardized development times, using the streptavidin method with diaminobenzidine (DAB) as substrate. Five representative cores per patient were scored for expression of cytoplasmic USP2a as previously described (Rossi et al., 2003). Immunofluorescence was performed as described (Waltregny et al., 2001).

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Accession numbers

The GenBank accession numbers for the human splice variants USP2a and USP2b are NP_004196 and NP_741994, respectively.