Mamm Genome (2010) 21:388–397
DOI 10.1007/s00335-010-9268-4

USP32 is an active, membrane-bound ubiquitin protease overexpressed in breast cancers

Shiva Akhavantabasi · Hesna B. Akman · Aysegul Sapmaz · Jennifer Keller · Elizabeth M. Petty · Ayse E. Erson

Received: 24 March 2010 / Accepted: 21 May 2010 / Published online: 13 June 2010

Abstract *USP32*, on chromosomal band 17q23.1-17q23.2, is a highly conserved but uncharacterized gene that gave rise during evolution to a well-known hominoid-specific proto-oncogene, *USP6*. We investigated the expression profile of *USP32* in human tissues and examined its functions to gain insight into this novel member of the well-conserved ubiquitination system. We detected ubiquitous *USP32* expression across tissues and confirmed the predicted deubiquitination function owing to the presence of conserved peptidase signature aspargine, cysteine, histidine, and aspartic acid domains of ubiquitin-specific proteases. A Golgi localization of GFP-fused *USP32* was detected by fluorescent protection assay and BODIPY-TR staining. In addition, stable silencing of *USP32* caused a significant decrease in the proliferation and migration rate of cells. Based on these and the fact that *USP32* maps to 17q23, which is commonly amplified in breast cancers, we analyzed *USP32* expression in breast cancer cells. We detected high expression of *USP32* in 50% (9 of 18) of breast cancer cell lines and 22% (9 of 41) of primary breast tumors compared to mammary epithelial cells. In summary, we report the preliminary characterization of this novel deubiquitinating enzyme on 17q23 and demonstrate its functional role in the ubiquitin system and its potential involvement in tumorigenesis.

S. Akhavantabasi, H. B. Akman, and A Sapmaz contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00335-010-9268-4) contains supplementary material, which is available to authorized users.

S. Akhavantabasi · H. B. Akman · A. Sapmaz · A. E. Erson (✉)
Department of Biological Sciences, Inonu Bulvarı, M.E.T.U, 06531 Ankara, Turkey
e-mail: erson@metu.edu.tr

J. Keller · E. M. Petty
Department of Internal Medicine and Human Genetics, University of Michigan, 5220 MSRB III, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0640, USA

Introduction

Ubiquitination alters stability, half-life, localization, activity, and conformation of proteins (Hussain et al. 2009). Therefore, ubiquitination and the removal of ubiquitin (deubiquitination) are likely to have important consequences in cells. The mammalian genome is thought to encode nearly 100 deubiquitinating enzymes (DUBs) of five related classes, one of which is the ubiquitin-specific proteases (USPs) (Nijman et al. 2005). One of the uncharacterized members of USPs is *USP32*, on 17q23, which harbors the peptidase signatures C-terminal aspargine, cysteine, histidine, and aspartic acid domains of USPs, suggesting that it plays a role in deubiquitination. This functional role, however, has not yet been confirmed.

USP32 is an ancient and highly conserved gene (Paulding et al. 2003) which, during evolution, gave rise to the hominoid-specific *USP6* proto-oncogene (aka *TRE2*) on 17p13. In fact, *USP6* evolved from the chimeric fusion of two genes, *USP32* and *TBC1D3*, indicated by phylogenetic analysis (Paulding et al. 2003). Therefore, *USP6* mRNA nucleotides 1–3193 have 89% sequence identity to *TBC1D3* (TBC1 domain family, member 3) that functions in Rab GTPase signaling (Wiemann et al. 2001) and nucleotides 3194–6063 have 97% sequence identity to *USP32*. Interestingly, *USP6* has also been indicated as a

translocation partner with strong promoters or as part of chimeric transcripts in cancer cells (Oliveira et al. 2004, 2006; Panagopoulos et al. 2008). In addition to *USP6*, other members of DUBs have also been indicated in cancer-related pathways, such as USP10 deubiquitinating p53 and reversing the Mdm2-induced p53 nuclear exportation and degradation (Yuan et al. 2010). An interesting role for USP33 has recently been described: a Robo1 receptor binding DUB and causing its relocation to the plasma membrane in response to Slit and taking part in the inhibition of breast cancer cell migration (Yuasa-Kawada et al. 2009).

In addition, the *USP32* gene maps to 17q23, which is amplified in breast cancers as well as other tumors (Haverty et al. 2008; Pärssinen et al. 2007; Sinclair et al. 2003). The structure of the breast cancer-associated amplicon is complex, with discontinuous regions of genomic amplification that cover several genes (Bärlund et al. 1997; Couch et al. 1999; Erson et al. 2001; Jonsson et al. 2005). Some members of the amplicon have already been extensively examined. Recently, a high-resolution study identified a discrete 17q23 region (Chr 17: 55,503–57,374 Kb) that is amplified in 20% of the HER2+ and 8% of the luminal breast tumors (Natrajan et al. 2009). This region harbors *CA4*, *C17orf64*, *APPBP2*, *PPM1D*, *BCAS3*, *BRIP1*, and *USP32*. In addition, an 81-gene copy-number signature to predict the metastatic capability of breast cancers identified *USP32* (among other 17q23 genes) as a gene that has an increased copy number in the estrogen receptor (ER)-positive tumors (Zhang et al. 2009). *USP32* was also reported as one of the upregulated transcripts in malignant breast epithelium compared to normal luminal epithelial cells (Grigoriadis et al. 2006). This suggests that upregulation of *USP32* in mammary epithelial cells may be important in the pathogenesis of breast cancer and/or serve as a useful biomarker in breast cancer cells.

Given the fact that *USP32* resides on an amplicon region important in breast cancers and has high sequence similarity to a known oncogene, we sought to better characterize *USP32* to begin to gain insight into its potential role in normal and neoplastic cells.

Materials and methods

Tissue cDNA samples and cell lines

Human normal multitissue cDNA panel (Clontech), human normal breast total RNA (50 μg) (Ambion), and human breast cancer I tissue PCR array (Origene) were used. BT20, CAL51, MDA-MB231, MCF7, BT474, DU4475, SK-BR3, and HeLa cell lines were obtained from ATCC (Manassas, VA, USA). EFM-19, JIMT-1, HCC1937, HCC1143, HDQ-P1, and CAL85-1 cell lines were obtained from DSMZ (Braunschweig, Germany) and grown under recommended conditions. SUM-225, SUM-159, SUM-149, SUM-185, and SUM-1315 cell lines and the human papilloma virus (HPV)-immortalized nontumorigenic mammary cell line HPV11-21 were developed at the University of Michigan (Ethier et al. 1993, 1996; Ignatoski and Ethier 1999).

Expression analysis

RNA isolation and DNase treatment were performed as described (Erson et al. 2001). cDNA samples were synthesized with 1 μg DNase-treated RNA using RevertAid First Strand cDNA Kit (Fermentas). Various primer sets were used for *USP32* and *USP6* (full length = 210 and 213) RT-PCR and *USP32* mutation analysis primer sequences are available upon request. For real-time RT-PCR (qRT-PCR) SYBR® Green Master (Roche) was used. *USP32* (196 bp) F: 5′-CTGCAAGCAGGACACA ACTGGTTT-3′, R: 5′-TCACGTAACTGAGGCTGCTTC CAA-3′ and *B2M* (270 bp) F: 5′-CCAGCAGAGAATG GAAAGTC-3′, R: 5′-CCTCATGATGCTGCTTACA-3′ primers were used. *USP32* expression levels were calculated using the ΔΔCt method as previously described (Livak and Schmittgen 2001). The fold change for *USP32* was normalized against the housekeeping control (*B2M*) and compared to normal breast tissue. For the already normalized primary breast cancer tissue PCR array, the ΔCt method was used (Livak and Schmittgen 2001).

In vivo deubiquitination assay

Ubiquitin-met-β-galactosidase (Ub-β-gal) in a pA-CYC184-based plasmid (a gift from Dr. M. Hochstrasser) and pGEX-4T-*USP32* constructs were cotransformed into DH5α cells to assess deubiquitination activity as previously described (Papa and Hochstrasser 1993). DH5α cells were grown in 100 μg/ml ampicillin and 10 μg/ml chloramphenicol containing 2XYTA medium until A_{600} reached 0.6-0.8. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 0.2 mM for 4 h to induce *USP32* expression from the pGEX-4T construct. Cells were then lysed in phosphate-buffered saline (PBS) containing 100 μg/ml lysozyme, protease inhibitor (Roche Complete Mini Protease Inhibitor Cocktail Tablet in 10 ml 1 × PBS buffer), and 10 U/ml DNase I, followed by multiple freeze/thaw cycles. The following antibodies were used for immunoblotting: monoclonal mouse anti-β-galactosidase antibody (1:1000; Cell Signaling) and goat anti-mouse IgG-HRP (1:2000; Santa Cruz Technologies).

pGEX-Ubp3 (a gift from Dr. M. Hochstrasser) was used as positive control for DUB activity (Baker et al. 1992).

Fluorescence protection assay and subcellular localization

The full-length *USP32* coding sequence (4.8 Kb, NM_032582) was PCR amplified with *Xho*I and *Apa*1 restriction enzyme site containing primers F: 5′-CCGCTCGA GATGGGTG CCAAGGAGTCAC-3′ and R: 5′-GGGCCC GCTGTAACACACAGTACTTTTTGTAATCAG-3′ and cloned into pEGFPN1 (Clontech). After sequence confirmation, HeLa cells were transfected with 2 μg *USP32-GFP* construct (*USP32* in pEGFPN1) and 2 μg control pEGFPN1 in 6-well plates, using Fugene 6 (Roche) according to the manufacturer's instructions. Twenty to 24 h post transfection, cells were washed three times for 1 min in KHM buffer (110 mM CH_3COOK, 20 mM HEPES, and 2 mM $MgCl_2$) at room temperature and were incubated with 20 μM digitonin for permeabilization (Lorenz et al. 2006). Pre and post-permeabilization images (20×) were captured and recorded using Zeiss LSM 510 (Central Lab, METU). To stain Golgi, BODIPY-TR (Molecular Probes) was used. Transfected cells were incubated with 5 μM BODIPY-TR in PBS/HEPES for 30 min at 4°C followed by washing steps. Cells were again incubated for 30 min at 37°C in fresh medium. After a final wash with the KHM buffer, cells were analyzed under the microscope (100×).

Stable *USP32* silencing via shRNA

Synthetic sense (S) and anti-sense (AS) oligos (IDT) corresponding to *USP32* cDNA nucleotide positions 1886-1904 were annealed and cloned into the pSUPERretroneoGFP (Oligo Engine) (S: 5′-GATCCCCCCAGT AAAGGCTACATCATTTCAAGAGAATGATGTAGCC TTT ACTGGTTTTTA-3′ and AS: 5′-AGCTTAAAAAC CAGTAAA GGCTACATCATTCTCTT GAAATGATGT AGCCTTTACTGGGGG-3′) to form the shRNA. Similarly, control oligos with no homologies to human genome were annealed and cloned into pSUPERretroneoGFP: (S: 5′-GATCCCCGTACGTTACGCGTAACGTATTCAAGA GATACGTTACGCGTAACGTACTTTTTA-3′, AS: 5′-A GCTTAAAAAGTACGTTACGCGTAACGTATCTCTTG AATACGTTACGC GTAACGTACGGG-3′). Anti-*USP32*, control shRNA, and mock transfections were done with Fugene-HD (Roche). Positive mono- and polyclones in both HeLa and MCF7 cells were selected by 750 μg/ml G418 treatment. Silencing of *USP32* was confirmed by qRT-PCR as described above, and statistical analysis of data was done by one-way ANOVA followed by Tukey's multiple-comparison test ($p < 0.05$).

Proliferation rate determination

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Roche) according to the manufacturer's instructions. A total of 5×10^3 cells were plated in 96-well plates in complete DMEM to be assayed at 24, 48, 72, and 96 h. The absorbance was measured using a Bio-Rad microplate reader at 570 nm. Data obtained for each cell group at 48, 72, and 96 h was normalized to the corresponding group's OD values at 24 h to eliminate initial cell counting and/or plating differences. Experiments were done in triplicate. Statistical analysis of the data was done by one-way ANOVA followed by Tukey's multiple-comparison test ($p < 0.05$).

Transwell migration assay

Transwell migration assay was performed as previously described (Cimen et al. 2009). HeLa cells (3×10^4) or MCF7 cells (10×10^4) were plated in the upper chamber of the transwell plates (8 μm pore size; Greiner Bio-one) in starvation medium (1% FBS). The lower side of the chamber was filled with 10% FBS containing medium. Cells were allowed to migrate for 24 h; nonmigrated cells were removed by scrubbing with sterile cotton swabs. The chambers were fixed in 100% methanol for 10 min, stained with Giemsa solution for 2 min, and washed twice in distilled water (Cimen et al. 2009). The migration of cells was quantified under a light microscope with 4× objective by counting five random fields per membrane. Statistical analysis of data was done by one-way ANOVA followed by Tukey's multiple-comparison test ($p < 0.05$).

Results

USP32 as a ubiquitously expressed and membrane-bound DUB

Because *USP6* is an oncogene with high sequence similarity to *USP32* and is suspected to have alternatively spliced variants (Papa and Hochstrasser 1993) that may resemble the 3′ coding sequence of *USP32*, we first determined *USP6* and *USP32* expression patterns to examine the possibility of *USP6* function overlapping with *USP32*. Therefore, we designed primers to amplify different-sized PCR products for *USP32* and *USP6* transcripts in a multitissue cDNA panel to distinguish their expression patterns. Earlier, *USP6* was reported to be expressed only in testis (Paulding et al. 2003), but we also demonstrated *USP6* transcript in ovary, whereas *USP32* was ubiquitously expressed in the multitissue cDNA panel (Fig. 1a).

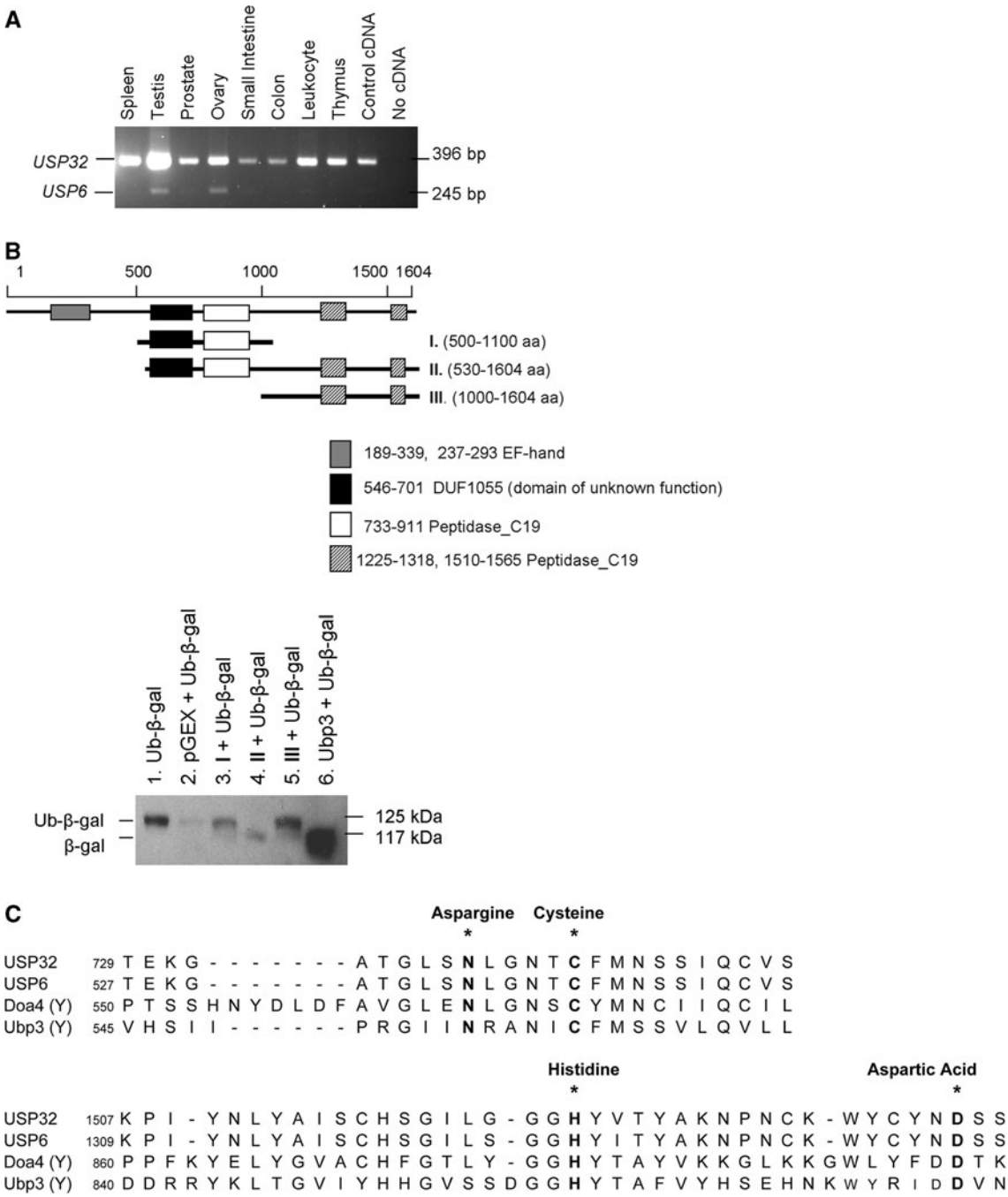


Fig. 1 *USP32* expression and deubiquitination function. **a** *USP32* (396 bp) and *USP6* (245 bp) RT-PCR with a multitissue panel of spleen, testis, prostate, ovary, small intestine, colon, leukocyte, and thymus cDNAs. **b** Domain structure of partial *USP32* constructs (I, II, III) and in vivo deubiquitination assay. Plasmid combinations (pGEX-*USP32* and Ub-β-gal) were cotransformed into in DH5α cells. 1. Ub-β-gal plasmid only. 2. Empty pGEX + Ub-β-gal. 3. pGEX-*USP32*-**I** +Ub-β-gal. 4. pGEX-*USP32*-**II** + Ub-β-gal. 5. pGEX-*USP32*-**III** + Ub-β-gal. 6. pGEX-Ubp3 + Ub-β-gal [Ubp3 was used as a positive control for deubiquitination activity (Baker et al. 1992)]. Ub-β-gal is 125 KDa, β-gal is 117 KDa. **c** Conserved residues required for deubiquitination activity in *USP32* and other DUBs [e.g., USP6, Doa4 (Y: yeast), Ubp3 (Y: yeast)] are shown. Bold letters show the conserved amino acids. * indicates active aspargine (738th amino acid of *USP32*), cysteine (743rd amino acid of *USP32*), histidine (1526th amino acid of *USP32*), and aspartic acid (1543rd amino acid of *USP32*) residues found in DUBs as indicated by ENTREZ

USP32 (1604 aa) is predicted to be a ubiquitin protease due to the C-terminal aspargine, cysteine, histidine, and aspartic acid residues in the peptidase domains (Fig. 1b, c) conserved in DUBs. To confirm the predicted function of USP32, an in vivo deubiquitination assay (Papa and Hochstrasser 1993) was performed.

USP32 cDNA encoding the C-terminal peptidase domains was cloned as three overlapping inserts into pGEX vectors as GST fusion peptides. These constructs (I, II, and III) and Ub-β-gal plasmids were cotransformed into DH5α cells for ectopic expression. Deubiquitination assay tested the protease ability of the peptides expressed from pGEX based on ubiquitin removal from the Ub-β-gal. DH5α cells cotransformed with both pGEX-*USP32* and pACYC184-Ub-β-gal constructs were lysed and USP32 peptide expression was confirmed by Western blotting (GST antibody, data not shown). A β-gal antibody showed the cleavage of Ub-β-gal into β-gal by both *USP32* (construct II, has all three active peptidase domains) (lane 4) and the positive control Ubp3 (lane 6) (Baker et al. 1992), showing the deubiquitination activity of USP32 (Fig. 1b). Construct I (lane 3), which harbored only the first peptidase region (amino acids 733-911), and construct III (lane 5), which harbored only the second and the third peptidase regions (amino acids 1225-1318 and 1510-1565), had minimal enzymatic activity as faint β-gal bands could be visualized for darker exposures of the film (darker exposures not shown).

Next, we examined the cellular localization of USP32. Fluorescence protection assay relies on the time course loss of the fluorescence signal of any soluble protein after the permeabilization of the plasma membrane (Lorenz et al. 2006). We checked for but did not observe any photobleaching of transfected cells during the assay period (Fig. 2a). Transfected cells were then treated with digitonin to permeabilize the plasma membrane. The signal of *USP32*-GFP (pEGFP-*USP32*, third row) did not fade out, even 270 s after digitonin treatment as it did in GFP-only-expressing cells (pEGFP only, second row) in 90 s. This initial observation suggested a membrane- and/or organelle-bound localization of *USP32*, as also predicted by ENTREZ Gene (Maglott et al. 2007). The GFP signal for vector-transfected cells was undetectable after 145 s (Fig. 2a).

To further confirm these observations, we examined the subcellular localization of the full-length and partial USP32-GFP fusion peptides in HeLa cells. Full-length USP32 (USP32-GFP) seemed to localize to Golgi (stained with BODIPY-TR). Other N terminus constructs had similar localization, whereas construct 3 (USP32-**3**) always had a very clear cytoplasmic diffusion pattern (Fig. 2b, c). We have observed that localization of USP32-**1** and USP32-**2** was consistent with USP32-GFP, but cytoplasmic signals were also detected in some cells for these partial constructs, almost comparable to GFP alone (Fig. 2b).

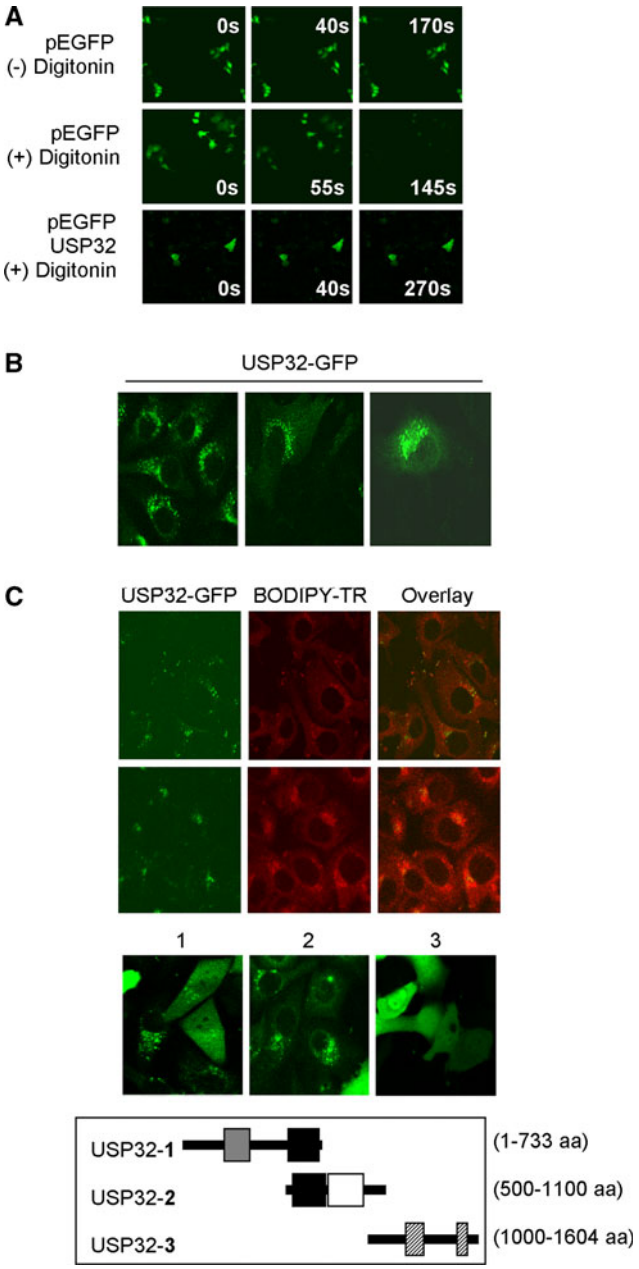


Fig. 2 Fluorescence protection assay and subcellular localization of *USP32* in HeLa cells. **a** First row: Empty pEGFP vector-transfected cells without digitonin treatment were imaged for 0, 40, and 170 s to demonstrate lack of photobleaching. Second row: pEGFP-transfected cells were treated with 20 μM digitonin and were imaged for 0, 55, and 145 s until the GFP signal faded. Third row: pEGFP-*USP32*-transfected cells were treated with 20 μM digitonin and cells were imaged for as long as 270 s (20 ×). **b** Three images of full-length *USP32* (*USP32*-GFP) localization (100×). **c** Full-length *USP32* (*USP32*-GFP) (first column), BODIPY-TR dye staining of Golgi (second column), and overlay of the two images (third column). Below are cells transfected with *USP32*-**1**-GFP (1), GFP-*USP32*-**2** (2), and GFP-*USP32*-**3** (3) together with the structures of the constructs (100×)

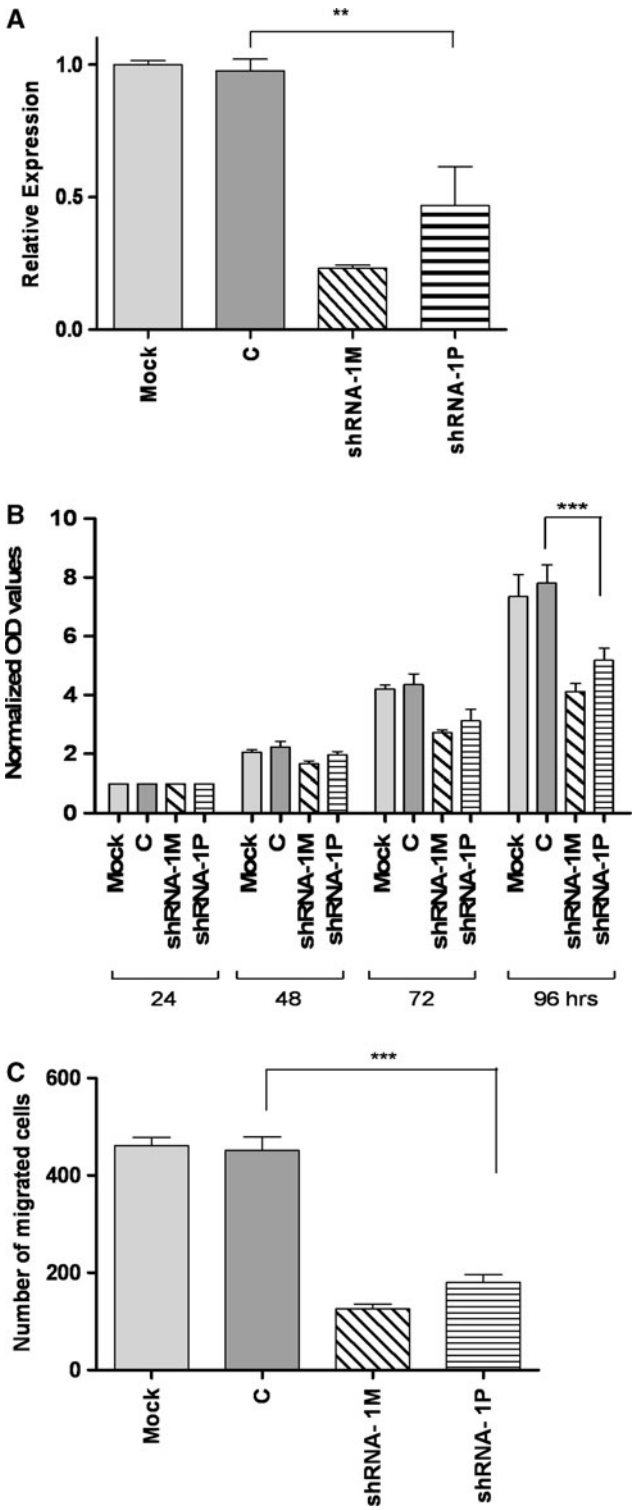


Fig. 3 Stable *USP32* silencing and its effect in HeLa cells. **a** Relative ▶ expression of *USP32* in mock (empty vector), control shRNA (C), and anti-*USP32* shRNA-transfected cells was determined by qRT-PCR (calculated by the ΔΔCt method). shRNA-1 M: *USP32*-silenced monoclonal cells; shRNA-1P: *USP32*-silenced polyclonal cells (same shRNA used for both). The baseline for the mock-transfected HeLa cells was set to 1. ** indicates significant difference between C and shRNA-1P cells, $p < 0.05$ (Tukey's multiple-comparison test). **b** 5000 mock, control shRNA (C), and anti-*USP32* shRNA-transfected cells [monoclonal (shRNA-1 M) and polyclonal (shRNA-1P)] were plated and assayed for proliferation at 24, 48, 72, and 96 h by MTT. Data obtained for each cell group at 48, 72, and 96 h were normalized to the corresponding cell group's OD values at 24 h to eliminate cell counting and plating differences. *** indicates significant difference between C and shRNA-1P cells, $p < 0.05$ (Tukey's multiple-comparison test). **c** Transwell migration assay was done in the presence of serum as a chemoattractant. *USP32*-silenced shRNA-1 M and shRNA-1P cells migrated through the 8-μm pores of the transwell chamber in significantly less numbers (*** $p < 0.05$) compared to the control and mock-transfected cells

USP32 silencing and its effect on proliferation rate

Anti-*USP32* and control shRNA vectors were stably transfected into HeLa cells. Silencing of *USP32*, detected with qRT-PCR (Fig. 3a), resulted in a more than 30% reduction in the proliferation rate of transfected cells (both poly- and monoclonal) at 96 h post plating detected by MTT (Fig. 3b) and growth curve assays (Supplementary Fig. 1). Moreover, *USP32*-silenced HeLa cells had lower migration abilities compared to control cells as indicated by the transwell migration assay (Fig. 3c). However, no evident apoptosis or significant difference in the cell cycle profiles of *USP32*-silenced and control cells was observed when stained with propidium iodide for flow cytometry analysis (data not shown).

USP32 overexpression in breast cancer cells

Based on *USP32*'s effect on the proliferation and migration rate and the fact that it maps to 17q23, which is commonly amplified in breast cancers, we expanded our *USP32* transcript analysis to breast cancer cell lines and primary tumors compared to normal breast tissue and immortalized nontumorigenic mammary cells (HPV11-21) by qRT-PCR. *USP32* was found to be overexpressed more than twofold in 9 of 18 breast cancer cell lines compared to normal breast tissue and HPV11-21 (Fig. 4a) and in 22% of (9 of 41) primary breast tumors compared to the mean of 7 normal breast tissue samples, indicated as C1-7 (Fig. 4b). As a representative cell line for *USP32* overexpression, all 34 exons of *USP32* in MCF7 cells were PCR amplified and sequenced to determine if the wild type or mutated transcript was being produced. No mutations were detected in the coding sequence or intron-exon junctions, a result consistent with the COSMIC (Catalogue of Somatic Mutations in Cancer) database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) which reports the lack of mutations in *USP32* in the examined samples (Forbes et al. 2008). Moreover, no *USP6* or its known alternatively spliced variants [known as Clones 210 and 213 (Nakamura et al. 1992)] were detected in the normal breast tissue and in two of the representative breast cancer

cell lines with high and normal *USP32* expression (MCF7 and MDA-MB-231, respectively) (Supplementary Fig. 2 and data not shown). Therefore, we eliminated the possibility of the presence of the *USP6* transcript that may have overlapping expression profiles and/or functions due to sequence similarity with *USP32* in normal breast and the examined breast cancer cells.

Since *USP32* silencing caused a decrease in the proliferation and migration properties of HeLa cells, we also stably transfected MCF7 cells with anti-*USP32* and control shRNA vectors (Fig. 5a) to examine if the same effect would be observed in breast cancer cells. A reduction in the proliferation rate of MCF7 cells was detected by MTT analysis (Fig. 5b) at the end of 96 h. Moreover, a more than 30% reduction in the migration ability of *USP32*-silenced MCF7 cells was observed in the transwell migration assay (Fig. 5c).

In a cDNA microarray experiment, *USP32* was reported among a group of genes that were responsive to estrogen (E2) and antiestrogen treatments in ER-positive ZR-75.1 breast cancer cells (Scafoglio et al. 2006). To determine experimentally if any canonical and/or noncanonical E2 response elements exist in the *USP32* promoter, we cloned a 2.7-kb immediate upstream promoter region of *USP32* into a dual luciferase reporter system but did not detect any E2 responsiveness in these regions (Supplementary methods and Supplementary Fig. 3) in MCF7 (ER +) cells. Although *in silico* prediction programs suggested the presence of estrogen response elements within this 2.7-kb region, these sites were not conserved between mice and human *USP32* upstream sequences (data not shown).

Discussion

It has long been known that DUBs have a role in the pathogenesis of many diseases, including cancer. They are known to take part in protein degradation, receptor endocytosis, and possibly other processes. The role of DUB enzymes in the Golgi apparatus is also just beginning to be

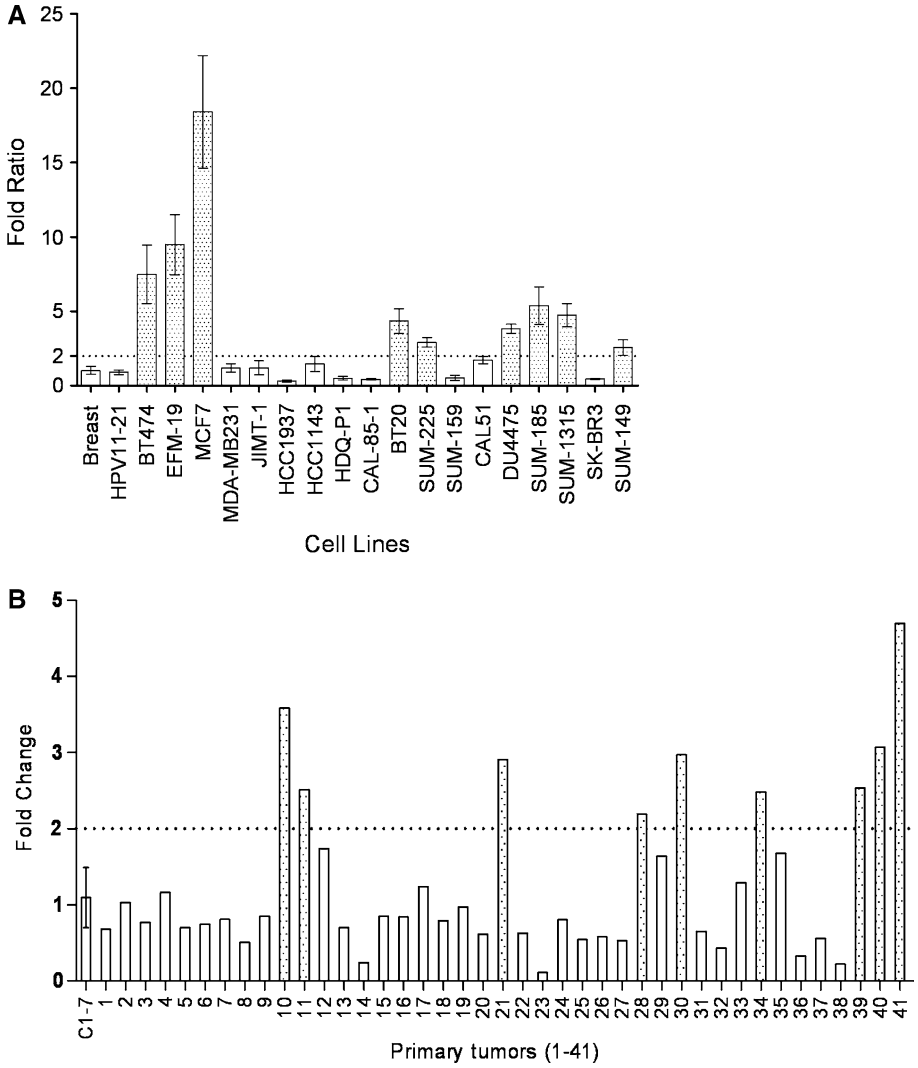
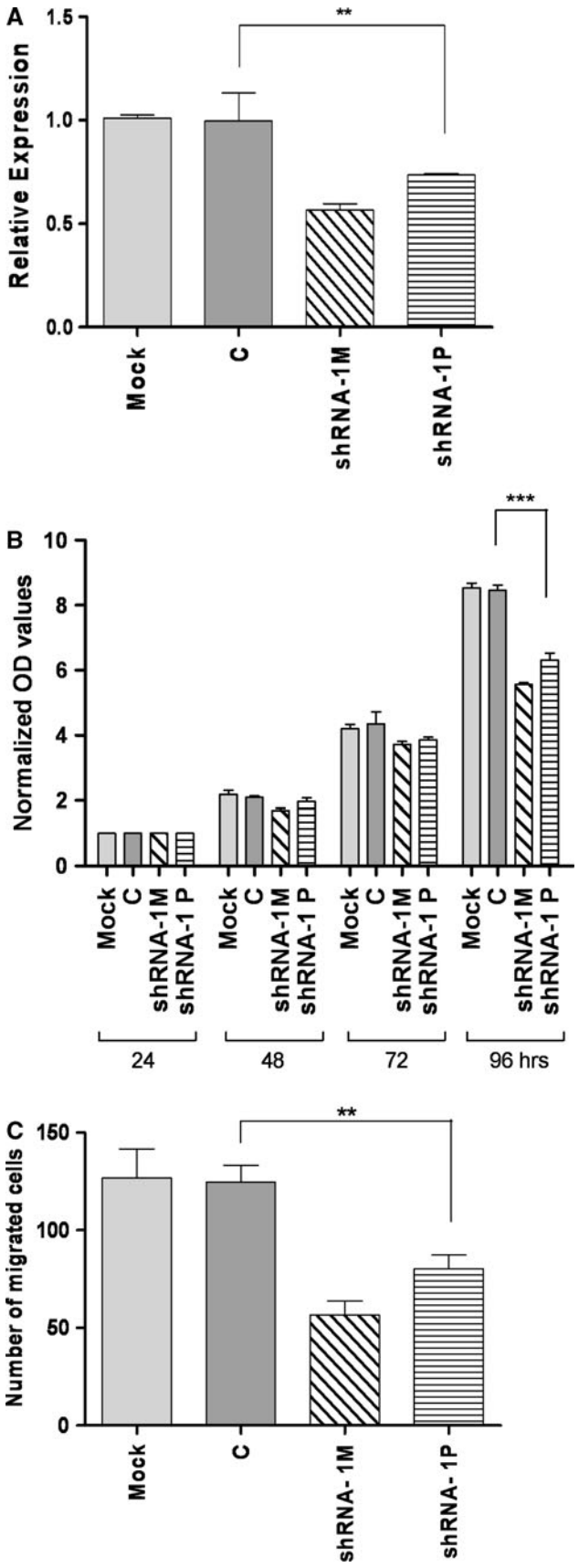


Fig. 4 *USP32* overexpression in breast cancer cells. **a** qRT-PCR amplification of the *USP32* transcript in 18 breast cancer cell lines compared to normal breast tissue and HPV11-21 (calculated by the ΔΔCt method). Dotted bars indicate cell lines with *USP32* expression more than twofold compared to controls (normal breast tissue and HPV11-21). **b** qRT-PCR amplification of the *USP32* transcript in 41 primary breast tumors compared to 7 normal breast (C1-7) (calculated by the ΔCt method). Dotted bars indicate primary tumors, with *USP32* expression more than twofold compared to C1-7



◀ **Fig. 5** Stable *USP32* silencing and its effect in MCF7 cells. **a** Relative expression of *USP32* in mock (empty vector), control shRNA (C), and anti-*USP32* shRNA-transfected cells was determined by qRT-PCR (calculated by the ΔΔCt method). shRNA-1 M: *USP32*-silenced monoclonal cells; shRNA-1P: *USP32*-silenced polyclonal cells (same shRNA used for both). The baseline for the mock-transfected MCF7 cells was set to 1. ** indicates significant difference between C and shRNA-1P MCF7 cells, $p < 0.05$ (Tukey's multiple-comparison test). **b** 5000 mock, control shRNA (C), and anti-*USP32* shRNA-transfected cells (shRNA-1 M and shRNA-1P) were plated and assayed for proliferation at 24, 48, 72, and 96 h by MTT. Data obtained for each cell group at 48, 72, and 96 h were normalized to the corresponding cell group's OD values at 24 h to eliminate cell counting and plating differences. *** indicates significant difference between C and shRNA-1P cells, $p < 0.05$ (Tukey's multiple-comparison test). **c** Transwell migration assay showed significantly (** $p < 0.05$) less migration of *USP32*-silenced cells through the 8-μm pores of the transwell chamber compared to controls

understood as more evidence points to regulatory roles of ubiquitin in the sorting of proteins at the trans-Golgi network (Piper and Luzio 2007) and Golgi membrane dynamics (Meyer 2005; Wang et al. 2004). Ubp3p, for example, together with Bre5p has roles in the regulation of COPI and COPII complexes in endoplasmic reticulum to Golgi trafficking (Cohen et al. 2003a, b) and autophagy (Kraft et al. 2008).

In this study we confirmed the DUB activity of *USP32*. To get initial evidence on what this DUB might be doing in cells, we performed a fluorescence protection assay to determine if *USP32* is a soluble or membrane/organelle-bound protein. Contrary to soluble GFP alone, the GFP-fused *USP32* signal did not leave the cell when the membranes were permeabilized with digitonin, whose activity is limited to only the cholesterol-rich plasma membranes (Lorenz et al. 2006). Subcellular localization studies also confirmed these results and suggested a possible Golgi colocalization. Localization of the partial proteins (*USP32*-**1** and *USP32*-**2**) was consistent with the full-length *USP32*, but we also noticed cytoplasmic signals in some transfected cells for N-terminal partial constructs, almost comparable to GFP alone. This may be explained by the presence of a Golgi localization or retention signal around the C terminus of *USP32*-**1** and the N terminus of *USP32*-**2**, lowering the efficiency of localization of these proteins. It is also possible that localization pattern inconsistencies for these two partial peptides are due to a different cell cycle status of the transfected cells or abnormal folding of partial peptides. The roles of *USP32* in connection to Golgi, its potential partners, and the functions of the N-terminus EF-hand domains remain to be investigated.

We also showed *USP32* overexpression in breast cancer cell lines and primary breast tumors. No mutations were detected in MCF7 cells as a representative cell line for *USP32* overexpression, indicating that it is the wild-type transcript that is overexpressed. The fact that no mutations

have been identified in *USP32* in cancer cells to date (Forbes et al. 2008) suggests that the increased expression observed in cancer cells is most likely associated with amplification or other epigenetic mechanisms.

No *USP6* or its variants (excluding as of yet undetected isoforms) were found in normal breast or the examined breast cancer cell lines. It was crucial to know the *USP6* expression status in breast cells because of the high sequence similarity between *USP6* and *USP32*.

Although E2-induced expression of *USP32* has been reported, we failed to detect any E2 responsive regions in the 2.7-kb upstream region, but we cannot exclude any other indirect effects of E2 or further upstream regions harboring E2 response elements. Such effects will need to be investigated for E2 responsiveness of *USP32*.

In short, *USP32* is a novel DUB that maps to 17q23 which harbors an amplicon in breast cancer cells (Erson et al. 2001; Sinclair et al. 2003). Since the amplicon structure is complex and gene rich, a functional understanding of genes in this region and how they may contribute to breast tumorigenesis is crucial. Together with its effect on proliferation and migration as well as its overexpression in breast cancer cells, further investigation of USP32 function in Golgi will be interesting. Specific roles of *USP32* (by itself or with other 17q23 genes) in breast carcinogenesis will be important to investigate next.

Acknowledgments This project was funded by the Scientific and Technological Research Council of Turkey (TUBITAK) (104S241, 108S408) and METU internal funds. A. Sapmaz is funded by OYP (YYU, Van). Contributions by J. Keller and E. Petty were supported by NIH-NCI RO1 CA72877-01A1. We thank Drs. C. Yakicier and U. Tazebay for sharing resources, T. Acun for technical help, Drs. C. Ozen, P. Ballar, and C. Son for guidance with microscopy, Dr. M. Gürsel for flow cytometry, Dr. M. Hochstrasser for constructs/protocols, and Dr. V. Seyrantepe for pEGFP vectors.

References

Baker RT, Tobias JW, Varshavsky A (1992) Ubiquitin-specific proteases of *Saccharomyces cerevisiae*. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. J Biol Chem 267:23364–23375

Bärlund M, Tirkkonen M, Forozan F, Tanner M, Kallioniemi O et al (1997) Increased copy number at 17q22–q24 by CGH in breast cancer is due to high-level amplification of two separate regions. Genes Chromosom Cancer 20:372–376

Cimen I, Tunçay S, Banerjee S (2009) 15-Lipoxygenase-1 expression suppresses the invasive properties of colorectal carcinoma cell lines HCT-116 and HT-29. Cancer Sci 100:2283–2291

Cohen M, Stutz F, Belgareh N, Haguenauer-Tsapis R, Dargemont C (2003a) Ubp3 requires a cofactor, Bre5, to specifically de-ubiquitinate the COPII protein, Sec23. Nat Cell Biol 5:661–667

Cohen M, Stutz F, Dargemont C (2003b) Deubiquitination, a new player in Golgi to endoplasmic reticulum retrograde transport. J Biol Chem 278:51989–51992

Couch F, Wang X, Wu G, Qian J, Jenkins R et al (1999) Localization of PS6K to chromosomal region 17q23 and determination of its amplification in breast cancer. Cancer Res 59:1408–1411

Erson A, Niell B, DeMers S, Rouillard J, Hanash S et al (2001) Overexpressed genes/ESTs and characterization of distinct amplicons on 17q23 in breast cancer cells. Neoplasia 3:521–526

Ethier SP, Mahacek ML, Gullick WJ, Frank TS, Weber BL (1993) Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. Cancer Res 53:627–635

Ethier SP, Kokeny KE, Ridings JW, Dilts CA (1996) erbB family receptor expression and growth regulation in a newly isolated human breast cancer cell line. Cancer Res 56:899–907

Forbes SA, Bhamra G, Bamford S, Dawson E, Kok C et al (2008) The catalogue of somatic mutations in cancer (COSMIC). Curr Protoc Hum Genet Chapter 10:10.11

Grigoriadis A, Mackay A, Reis-Filho JS, Steele D, Iseli C et al (2006) Establishment of the epithelial-specific transcriptome of normal and malignant human breast cells based on MPSS and array expression data. Breast Cancer Res 8:R56

Haverty P, Fridlyand J, Li L, Getz G, Beroukhim R et al (2008) High-resolution genomic and expression analyses of copy number alterations in breast tumors. Genes Chromosom Cancer 47:530–542

Hussain S, Zhang Y, Galardy PJ (2009) DUBs and cancer: the role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors. Cell Cycle 8:1688–1697

Ignatoski KM, Ethier SP (1999) Constitutive activation of pp125fak in newly isolated human breast cancer cell lines. Breast Cancer Res Treat 54:173–182

Jonsson G, Naylor TL, Vallon-Christersson J, Staaf J, Huang J et al (2005) Distinct genomic profiles in hereditary breast tumors identified by array-based comparative genomic hybridization. Cancer Res 65:7612–7621

Kraft C, Deplazes A, Sohrmann M, Peter M (2008) Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. Nat Cell Biol 10:602–610

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25:402–408

Lorenz H, Hailey D, Wunder C, Lippincott-Schwartz J (2006) The fluorescence protease protection (FPP) assay to determine protein localization and membrane topology. Nat Protoc 1:276–279

Maglott D, Ostell J, Pruitt KD, Tatusova T (2007) Entrez Gene: gene-centered information at NCBI. Nucleic Acids Res 35:D26–D31

Meyer HH (2005) Golgi reassembly after mitosis: the AAA family meets the ubiquitin family. Biochim Biophys Acta 1744:108–119

Nakamura T, Hillova J, Mariage-Samson R, Onno M, Huebner K et al (1992) A novel transcriptional unit of the tre oncogene widely expressed in human cancer cells. Oncogene 7:733–741

Natrajan R, Lambros MB, Rodriguez-Pinilla SM, Moreno-Bueno G, Tan DS et al (2009) Tiling path genomic profiling of grade 3 invasive ductal breast cancers. Clin Cancer Res 15:2711–2722

Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM et al (2005) A genomic and functional inventory of deubiquitinating enzymes. Cell 123:773–786

Oliveira AM, Hsi BL, Weremowicz S, Rosenberg AE, Dal Cin P et al (2004) USP6 (Tre2) fusion oncogenes in aneurysmal bone cyst. Cancer Res 64:1920–1923

Oliveira AM, Chou MM, Perez-Atayde AR, Rosenberg AE (2006) Aneurysmal bone cyst: a neoplasm driven by upregulation of the USP6 oncogene. J Clin Oncol 24:e1; author reply e2

Panagopoulos I, Mertens F, Lofvenberg R, Mandahl N (2008) Fusion of the *COL1A1* and *USP6* genes in a benign bone tumor. Cancer Genet Cytogenet 180:70–73

Papa F, Hochstrasser M (1993) The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene. Nature 366:313–319

Pärssinen J, Kuukasjärvi T, Karhu R, Kallioniemi A (2007) High-level amplification at 17q23 leads to coordinated overexpression of multiple adjacent genes in breast cancer. Br J Cancer 96:1258–1264

Paulding CA, Ruvolo M, Haber DA (2003) The Tre2 (USP6) oncogene is a hominoid-specific gene. Proc Natl Acad Sci USA 100:2507–2511

Piper RC, Luzio JP (2007) Ubiquitin-dependent sorting of integral membrane proteins for degradation in lysosomes. Curr Opin Cell Biol 19:459–465

Scafoglio C, Ambrosino C, Cicatiello L, Altucci L, Ardovino M et al (2006) Comparative gene expression profiling reveals partially overlapping but distinct genomic actions of different antiestrogens in human breast cancer cells. J Cell Biochem 98:1163–1184

Sinclair C, Rowley M, Naderi A, Couch F (2003) The 17q23 amplicon and breast cancer. Breast Cancer Res Treat 78:313–322

Wang Y, Satoh A, Warren G, Meyer HH (2004) VCIP135 acts as a deubiquitinating enzyme during p97–p47-mediated reassembly of mitotic Golgi fragments. J Cell Biol 164:973–978

Wiemann S, Weil B, Wellenreuther R, Gassenhuber J, Glassl S et al (2001) Toward a catalog of human genes and proteins: sequencing and analysis of 500 novel complete protein coding human cDNAs. Genome Res 11:422–435

Yuan J, Luo K, Zhang L, Cheville JC, Lou Z (2010) *USP10* regulates p53 localization and stability by deubiquitinating p53. Cell 140:384–396

Yuasa-Kawada J, Kinoshita-Kawada M, Rao Y, Wu JY (2009) Deubiquitinating enzyme USP33/VDU1 is required for Slit signaling in inhibiting breast cancer cell migration. Proc Natl Acad Sci USA 106:14530–14535

Zhang Y, Martens JW, Yu JX, Jiang J, Sieuwerts AM et al (2009) Copy number alterations that predict metastatic capability of human breast cancer. Cancer Res 69:3795–3801