Additional Supporting Information (Figure Legends)

The significance of TNFAIP8 in prostate cancer response to radiation and docetaxel and disease recurrence

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Figure S1. (*a*) Schematic map of human TNFAIP8: genomic organization, transcript variants, and protein isoforms. TNFAIP8 isoforms a and b are differentially expressed in most cancer cells and tumor xenografts. Note: Three additional human *TNFAIP8* transcript variants are predicted (http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr5:118691596-118730294&db=hg19&ss=../trash/hgSs/hgSs_genome_65af_acbe70.pslx+../trash/hgSs/hgSs_genome_65af_acbe70.fa&hgsid=211126421). In addition, one TNFAIP8 protein isoform is predicted (190aa, 22 kDa; Uniprot: O95379-2); predicted coding exon 9 (N' terminus 2aa) and exon10 (remaining 188aa); predicted ORF (MDC-3.13-1): MAVATDVFNSKNLAVQAQKKILGKMVSKSIATTLIDDTSSEVLDELYRVTREYTQNKKE AEKIIKNLIKTVIKLAILYRNNQFNQDELALMEKFKKKVHQLAMTVVSFHQVDYTFDRN VLSRLLNECREMLHQIIQRHLTAKSHGRVNNVFDHFSDCEFLAALYNPFGNFKPHLQKL CDGINKMLDEENI. (*b*) Genomatix analysis of the *TNFAIP8* promoter region showing androgen response elements (ARE) and other key consensus sequences (http://www.genomatix.de/en/index.htm) . +1 denotes amino acid A of ATG, the initiation codon of TNFAIP8 isoform b (SCC-S2), derived from the first intron as shown in Fig. S1*a*. (-) denotes minus strand. TSS, predicted transcription start sites in the 5' UTR in Exon 1. TNFAIP8 expression is induced by TNF-α and NF-κB. *TNFAIP8* mRNA expression seems to be modulated by NKX3 family, PAX6, COUP-TF1, HOXC family, TGF-beta (GEO dataset GDS3710/210260_s_at/TNFAIP8/Homo sapiens), LIM1 (GEO dataset GDS1748/1416950_at TNFAIP8/Mus musculus) and HIF family (GEO data set GDS2761/GI_7657123-S/TNFAIP8/Homo sapiens). The Genomatix-based core and matrix similarities of the sequences indicated are as follows. Three GC Boxes, Core similarity, all 1, Matrix similarities, 0.914 (-152/-158), 0.953 (-217/-222), and 0.979 (-254/-261); KLF, Core similarity 1, Matrix similarity, 0.913; ARE (-408/-422), Core similarity 1, Matrix similarity, 0.863, ARE (-1248/-1263), manually predicted; NKX3 family, Core similarity, both 1, Matrix similarities, 0.974 (-549/-554)

and 0.962 (-1425/-1430); PAX6, Core similarity 1, Matrix similarity, 0.828; HOXC family, Core similarity, both 1, Matrix similarities, 0.968 (-590/-593) and 0.89 (-600/-603); NF-kappa B, Core similarity both 1, Matrix similarities, 0.974 (-766/-778) and 0.928 (-1894/-1905); LIM-1, Core similarity, both 1, Matrix similarities, 0.926 (-986/-993) and 0.83 (-1559/-1566); HIF family , Core similarity 1, Matrix similarity, 0.907; GATA1, Core similarity, both 1, Matrix similarities, 0.967 (-1783/-1787) and 0.984 (-2083/-2088).

Figure S2. Representative publicly available datasets (ONCOMINE and GEO) showing correlation of increased TNFAIP8 mRNA expression with hyperplasia, higher tumor grade and/or metastatic tumor tissues. (*a*) Adapted from https://www.oncomine.org. (*b*) Adapted from http://www.ncbi.nlm.nih.gov/geoprofiles?term=TNFAIP8.

Figure S3. Western blot analysis of the effects of inhibition of TNFAIP8 protein on key invasion and metastasis related signals in PC-3 cells. (*a*) and (*b*) PC-3 cells were treated with 100 nM of stealth TNFAIP8 siRNAs (Si2) or a scrambled stealth siRNA (ScrSi) (all from Invitrogen) as explained in Materials and Methods. The whole cell lysates were sequentially immunoblotted with indicated antibodies. The antibodies were used at the following dilutions: anti-TNFAIP8 antibody, 1:1000; anti-VEGF antibody, 1:100; anti-VEGFR2 antibody, 1:100; anti-MMP-9 antibody; 1:2000; anti-MT1-MMP antibody , 1:1000; anti-MMP-1 antibody, 1:1000; anti-MMP-2 antibody, 1:1000; and anti-GAPDH antibody. The signals were normalized against GAPDH signal in the corresponding lanes. C, untreated control, L, lipofectin-treated control. (*c*) Quantification of data shown above in panels (*a*) and (*b*) was performed using NIH ImageJ software (version 1.45). Expression of various molecules in TNFAIP8 siRNA treated PC-3 cells versus ScrSi control (100%): Expt 1, TNFAIP8, 51.11%, MMP-9 active isoform, 85.75%, VEGFR-2, 82.14%; Expt 2, TNFAIP8, 71.55%, MMP-1, 24.39%, MMP-2, 69.90%, MT1-MMP, 37.98%). Data shown are a representative of two repeat experiments.

Figure S4. Determination of TNFAIP8 knockdown efficiencies of TNFAIP8 antisense oligonucleotides *in vitro* and *in vivo*. (*a*) *In vitro* effects of TNFAIP8 antisense oligonucleotides. Two TNFAIP8 antisense oligonucleotides tested had identical 14-mer sequence and either single-base phosphorothioated at each end (AS5) or all bases phosphorothioated (AS1). In addition, two control mismatch oligonucleotides used had either single-base phosphorothioated at each end (MM) or all bases phosphorothioated (MM1). MDA-MB 435 cells were grown to approximately 70% confluency in T-25 flasks. On day 1, cells were rinsed twice with serum-

free medium and treated with 2 ml of serum-free medium containing 10.9 µl of 4 mg/ml oligo and 65 µl of lipofectin per T-25 flask for 6 hr (final AS5 or AS1 concentration, 5 µM). Control cells were treated with 2 ml of serum-free medium containing 8.0 µl of 4 mg/ml control oligo (MM or MM1) and 65 µl of lipofectin per T-25 flask (final MM or MM1 concentration, 5 µM). Additional control groups were either left untreated (UT) or treated with lipofectin alone (L). The cells were rinsed twice with complete medium containing serum and switched to 2 ml complete medium containing the oligo (as above) without lipofectin for 18 hr. On day 2, day 1 steps were repeated. On day 3, adherent and floating cells were collected, and the viable number of cells was determined by the trypan blue exclusion assay. Subsequently, the cells were lysed for Western blotting using anti-TNFAIP8 antibody (1:2000 dilution). The blot was reprobed with anti-GAPDH antibody (1:10,000 dilution). The signals were normalized against GAPDH signal in the corresponding lanes. Expression levels of TNFAIP8 in AS5 and AS1 treated cells relative to corresponding mismatch oligonucleotide MM and MM1, respectively, are shown. (*b*) Pilot experiment showing the *in vivo* efficiencies of systemically-delivered TNFAIP8 antisense oligonucleotides in PC-3 tumor bearing athymic mice. The sequences and chemistries of three TNFAIP8 antisense oligonucleotides tested (AS5, AS1 and AS2) are shown in panel (c). Liposome-entrapped AS5 (LE-AS5) formulation was prepared as described in Materials and Methods. Fully phosphorothioated antisense oligonucleotides, AS1 and AS2 were used as such for systemic deliveries. Six- to eight week old male athymic nude mice were inoculated in the left flank region with 5.0×10^6 PC-3 cells in 150 µl of PBS. When tumor sizes were in the range of 60-70 mm^3, animals were randomized into four treatment groups (n = 2) and treatment was initiated. The first day of treatment was designated as day 0. TNFAIP8 antisense oligonucleotide groups LE-AS5, AS1, and AS2 received 10 i.v. injections of 25.0 mg/kg/dose over 14 days (day 0, 1, 3, 4, 6, 7, 9, 10, 12, 13). Control groups were left untreated (UT). Tumor tissues were excised within 6-12 hr after the last treatment. TNFAIP8 protein expression was examined in whole cell extracts from tumor tissues by western blotting with anti-TNFAIP8 antibody, followed by reprobing the blot with anti-GAPDH antibody and quantification of the TNFAIP8 and GAPDH signals. The TNFAIP8 expression was normalized against GAPDH signal in the corresponding lane and TNFAIP8 levels relative to the UT control were plotted as mean ± SD (n=2). UT, 100%; AS1, 42.6 ± 4.1%; AS2, 90.2 ± 31.7%; LE-AS5, 63.6 ± 12%. (*c*) Table showing TNFAIP8 antisense oligonucleotides used in the above panels.

Figure S5. (*a*) Specificity and sensitivity of the anti-TNFAIP8 antibody by Western blotting of whole cell extracts from human tumor cell lines, and human tumor xenografts grown in athymic mice. Immunoblotting was performed as described in Materials and Methods and legend to Fig. 4. (*b*) Validation of the anti-TNFAIP8 antibody by Western blotting of whole cell extracts from matched human tumor and benign clinical specimens from breast cancer patients. Breast tumor (T) and matched benign/normal tissues (N). Immunoblotting of the clinical specimens was performed as described earlier.[6] (*c*) Validation of the anti-TNFAIP8 antibody by immunoprecipitation and immunoblotting. Immunoprecipitations of whole cell extracts were performed using anti-TNFAIP8 antibody (lane 1), anti-Raf-1 antibody (lane 2), and normal rabbit IgG (lane 3), followed by immunoblotting with anti-TNFAIP8 antibody as described in Materials and Methods and legend to Fig. 4.

Figure S6. Immunohistochemical evaluation of TNFAIP8 in prostate tumor tissue micro-array and standard histological sections of clinical specimens from prostate cancer patients. (*a*) Immunohistochemical (IHC) evaluation of TNFAIP8 in a prostate tumor tissue microarray (TMA). High grade prostatic adenocarcinomas showing intense cytoplasmic (top left panel) and intense nuclear TNFAIP8 expression (top right panel) in two different cores on the *same* prostate cancer TMA. Summary of the IHC findings in prostate tumor TMA (bottom panel). As shown both cytoplasmic and nuclear TNFAIP8 was noted in 1/26 low grade PACs and 5/38 intermediate to high grade PACs. (*b*) Summary of the IHC findings showing differences in TNFAIP8 immunostaining in PAC versus adjacent benign tissue in formalin-fixed, paraffin-embedded sections. LG, low grade (Gleason score ≤ 6); HG, intermediate/high grade (Gleason score ≥ 7); *1, TNFAIP8 in PAC > adjacent benign; -1, TNFAIP8 in PAC < adjacent benign TNFAIP8. (*c*) Summary of the IHC findings showing differences in nuclear TNFAIP8 immunostaining in PAC versus adjacent benign tissue in formalin-fixed, paraffin-embedded sections from recurrent and non-recurrent cases. *0, no change in nuclear TNFAIP8 in PAC vs adjacent benign; 1, nuclear TNFAIP8 in PAC > adjacent benign.

Figure S7. Subcellular localization of TNFAIP8 protein prostate cancer cells. The hormone-dependent (LNCaP) and hormone-independent prostate cancer cells (PC-3 and DU-145) were fractionated into nuclear and cytosolic fractions and analyzed by Western blotting using 4-12% NuPAGE (approx 30μg protein/ lane). The blots were sequentially probed with anti-TNFAIP8 (1: 1000 dilution), anti-Lamin B1 (1:1000 dilution) or anti-PARP (1:200 dilution), and anti-α-

Tubulin antibodies (1:1000 dilution) and signals were quantified using NIH ImageJ software (version 1.45). WCE, Whole cell extract; ND, not determined.

Figure S8. Connectivity map of TNFAIP8 interacting proteins identified by the Ingenuity Pathway Analysis. Top three functions associated with this map are Cell-to-Cell signaling and interaction, tissue development, and cellular movement. Green, high expression or binding in TNFAIP8 immune-complex from ScrSiRNA treated PC-3 cells versus TNFAIP8 siRNA. Gray, no change, Empty, not on our antibody microarray.

Figure S9. TNFAIP8-centric interactive pathways (modified from STRING database (http://string-db.org/newstring_cgi/show_input_page.pl?UserId=CTc7ww7hklju&sessionId=SzChjSHd3U2J) Arrows indicate molecules identified/validated in this study.