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An oncogenic role for the multiple endocrine neoplasia type 1 gene in prostate cancer

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Abstract

Prostate cancer is the second leading cause of cancer related deaths in US men, largely because of metastasis, which is ultimately fatal. A better understanding of metastasis biology will lead to improved prognostication and therapeutics. We previously reported 11q13.1 gain was independently predictive of recurrence after radical prostatectomy. Multiple endocrine neoplasia I (*MEN1*) maps to this region of copy number gain in aggressive prostate tumors and was shown to be the only gene at this locus at increased expression in prostate cancer. Here, we demonstrate an oncogenic role for *MEN1* in prostate cancer using a variety of independent assays.

Keywords

MEN1; metastasis; oncogene

Introduction

The multiple endocrine neoplasia (MEN) tumor suppressor gene, *MEN1*, maps to 11q13.1 and encodes the menin protein that underlies the syndrome for which it is named, MEN 1.^{1–3} The *MEN1* gene is predicted to be capable of encoding a vast repertoire of splice variants and possibly proteins. It is well-established that mutations in *MEN1* cause MEN 1 in an autosomal dominant manner.⁴ Tumors in *MEN1* cases arise through a two-hit mechanism consistent with it being a tumor suppressor gene.⁵ Not surprisingly, *MEN1*'s role in biology and pathology has been studied predominantly in the parathyroid gland, pituitary gland and pancreas, however its function remains largely enigmatic. Several studies have shown a role for menin in transcriptional modulation through interaction with histone deacetylases and histone methyltransferases.^{6,7} Clinical manifestations of *MEN1* are the result of overproduction of hormones such as insulin, parathyroid hormone or growth hormone.^{8,9} With the exception of gastrinomas, most *MEN1*-associated tumors do not metastasize.

In a previous genome wide array comparative genomic hybridization (aCGH) study aimed at identification of DNA based biomarkers predictive of postoperative recurrence and metastasis,

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we identified the *MEN1* locus.¹⁰ Paradoxically, the narrow region centered on *MEN1* is gained and not lost in aggressive prostate tumors. Further, in an exhaustive gene expression study all other candidates at the locus were excluded.¹⁰ Moreover, expression of *MEN1* in a set of matched normal and prostate tumor pairs was higher in all tumors with 11q13.1 copy number gain, and in one case with normal *MEN1* copy number.¹⁰ The latter case suggests a mechanism independent of copy number gain for deregulation of *MEN1* gene expression in prostate tumors. It also provides compelling evidence the *MEN1* gene is the primary target of amplification. Elevated *MEN1* expression in prostate tumors has also been observed in studies using expression microarrays.¹¹ Taken together the evidence seems to suggest *MEN1* can act as an oncogene in prostate tumors. Here we present evidence demonstrating *MEN1*'s role in promoting growth and attenuating apoptosis further supporting *MEN1*'s role as an oncogene in cancer of the prostate.

Materials and methods

Fluorescent *in situ* hybridization to tissue microarray

The CTD-2220I9 BAC clone from Invitrogen (Carlsbad, CA, USA) containing *MEN1* was used to make a fluorescent *in situ* hybridization (FISH) probe. The clone was streaked on chloramphenicol LB plates. Single colonies were isolated for 3 ml starter cultures. DNA was isolated by following the manufacturer's protocol for the Plasmid Midi DNA kit (Qiagen, Valencia, CA, USA). The DNA was directly fluorescently labeled by following a modified random priming protocol. Briefly, two labeling reactions were set up that each contained 100 ng BAC DNA, 1 × Random Primers (Invitrogen BioPrime kit) and water such that the volume was 16 µl. The mixture was denatured at 99 °C for 15 min and then immediately put on ice. A total of 20 units of Klenow (Invitrogen BioPrime kit), 2 µl of FITC-dCTP (1 mM, PerkinElmer, Waltham, MA, USA) and 2 µl of a dNTP solution (2 mM dATP, dGTP and dTTP, and 0.5 mM dCTP) were added and then incubated O/N at 37 °C. The two labeling reactions were combined and ethanol precipitated in the presence of COT-1 (Human Hybloc; Applied Genetics, Melbourne, FL, USA). FISH studies were carried out in Dr Mark Rubin's Laboratory. For detailed FISH procedure see Perner *et al.*¹² FISH signals were analyzed using digital imaging microscopy. At least 100 cells were attempted to assess per case. The tissue microarray (TMA) consisted of 30 metastatic prostate tumors from various organ sites obtained at autopsy.¹³

Immunohistochemistry to TMA

Immunohistochemistry (IHC) was carried out with a polyclonal *MEN1* antibody (generously donated by Dr Francis Collins and Dr Lee Burns of the NIH) at a 1:1500 dilution. Antigen retrieval was performed by heating the slides for 7 min in 10 mM citric acid buffer. The TMA consisted of cores from 48 patients with prostate cancer that were at high risk of recurrence at the time of surgery according to the Kattan nomogram. Each case had three or more representative cores.

IHC calls were made independently by two pathologists (SP, JS). Pathologist 1 (SP) made a positive call if ≥5% of the cells showed *MEN1* staining. Pathologist 2 (JS) made a positive call if ≥5% of the cells showed staining with 2+ or 3+ staining. 2+ staining is observed with a ×10 and 3+ with a ×4 objective. The call was further defined, by both pathologists, as occurring in the nucleus or the cytoplasm.

Cell culture

Prostate cancer cell lines including PC3, DU145, LNCaP, MDAPCA2A, and a breast cancer cell line BT474 were obtained from American Type Culture Collection (Manassas, VA, USA) and grown according to manufacturer's protocols.

MEN1 knockdown with siRNAs

siRNAs targeting four different regions of the *MEN1* coding sequence were obtained from Qiagen. DU145 cells were transfected with siRNAs targeting *MEN1*, a scrambled siRNA (nonhomologous to any known human cDNA sequence) or transfection reagent alone in serum-free conditions, 24 h after seeding. Each of serum-free media (25 μ l) with 0.75 μ l of HiPerfect (Qiagen) and a 5 nM solution of siRNA were prepared separately, incubated for 5 min at room temperature (RT) and then combined. The liposome–siRNA complexes were then allowed to form, by further incubation for 15 min at RT. This 50 μ l transfection mixture was then added to existing media on the cells and left to incubate at 37 °C for at least 24 h before desired treatment. Volumes listed are for a 96-well format required for the ACEA experiments described below, and were adjusted accordingly for the six well assays in the fluorescence-activated cell sorting (FACS) and reverse transcription (RT)–PCR experiments.

ACEA

The ACEA (San Diego, CA, USA) RT-CES \times 16 system was used to monitor the cell growth index in *MEN1* knocked down cells. ACEA's technology measures the electronic impedance of sensor electrodes integrated on the bottom of microtiter plates. Cells are plated onto these special wells, and grown in a 37 °C incubator under 5% CO₂. The electrical impedance is then used to derive a cell index, which when continually monitored, gives a real time representation of the growth characteristics of the cells in the well.

Background impedance measurements were made in each well with media. A total of 1000 DU145 cells were then seeded, in a total of 150 μ l media, per well. At 24 h later, transfections were carried out in serum-free media. The cell index was then continuously monitored, during serum starvation as well as post-rescue with serum media, for the various experimental conditions. Serum starvation (48 h) was found to be the optimal time period to show arrest of cell growth in *MEN1* siRNA-transfected cells whereas maintaining viable cells. In addition, cell counts were confirmed manually (data not shown).

Fluorescence-activated cell sorting (Annexin V and propidium iodide)

Cells were seeded in duplicate six-well plates and transfected 24 h later in serum-free conditions. At 48 h post-serum starvation, cells were rescued with serum for a further 48 h before being collected by trypsinization. Cells grown in serum media, nontransfected serum starved cells, mock transfected (transfection agent only) serum starved cells, and scrambled siRNA-transfected serum starved cells were controls. Culture medium was discarded after cells were pelleted by centrifugation for 5 min. Cells were dissociated and suspended in 5 ml phosphate-buffered saline (PBS). A cell count was performed for every sample and equal number of cells (approximately 10⁶) were retained in 500 μ l PBS. The cells were then fixed in 5 ml of 70% ethanol for 30 min. Cells were spun down and ethanol removed. The cell pellet was suspended in 5 ml PBS and centrifuged again. Propidium iodide (PI)/Triton X-100 (1 ml; Sigma, St Louis, MO, USA; 1 mg/ml PI in 0.1% Triton X-100) staining solution with RNaseA was added to stain the cells. Flow cytometry was performed after 15 min of staining at RT. Forward scatter and side scatter (FSC/SSC), respectively, and fluorescence 2 (FL2) were measured by FACS (FACS Calibur System; Becton Dickinson, San Jose, CA, USA) and analytical software (Treestar, Ashland, OR, USA; FlowJo) was used to gate single cells based on light scatter and analyze the resulting cell cycle distribution patterns using peak deconvolution (Deen-Jett, Watson).

Similarly, cells were also analyzed for apoptosis using Annexin staining posttransfection. Cells were isolated by trypsinization, washed in PBS then suspended in 100 μ l of 1 \times Annexin-binding buffer before staining with Annexin V (Molecular Probes-Invitrogen), according to manufacturer's instructions. By FACS (Becton Dickinson FACS Calibur System), after FSC/

SSC gating was used to discriminate against debris and doublets, changes in live, dead and apoptotic subpopulation fractions were determined against negative treatment controls.

MTS

Cells were set up for transfection and treatment, as above, but in 96-well plates and in triplicates. At 48 h post-serum starvation, Promega's (Madison, WI, USA) CellTiter 96 Aqueous One Solution Cell Proliferation assay was used according to manufacturer's instructions to quantify NADH/NADPH levels in cells. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2,4-dimethylphenyl-2H-tetrazolium, inner salt (MTS) reduction was quantified by recording absorbance at 490 nm in a Wallac-PerkinElmer Victor2 1420 Multilabel counter.

Quantitative RT-PCR

RNA for the *MEN1* siRNA knocked down DU145 cells and corresponding controls were isolated using Trizol (Invitrogen) in accordance with the manufacturer's protocol and deoxyribonuclease (DNase) treated. cDNA was made using iScript First Strand Synthesis kit (BioRad, Hercules, CA, USA).

TaqMan assays to confirm *MEN1* knockdown in the siRNA experiments were carried out by the UCSF Genome Core. TaqMan to confirm ABI expression array results for DU145's *MEN1* siRNA experiments were carried out by Dr David Ginzinger (LinkageBio, San Francisco, CA, USA). All TaqMan assays were carried out according to ABI protocols. TaqMan primer-probe sets were purchased as Assays on Demand kits through ABI (Foster City, CA, USA). All gene expression results were calculated relative to the expression levels of the housekeeping gene *BGUS*.

ABI expression arrays

<http://bioconductor.org/packages/1.9/bioc/html/ABarray.html>). Probe signal intensities across microarrays were normalized using the quantile method.¹⁴ Features with signal/noise values ≥ 3 and quality flag values < 5000 were considered detected and were compared by *t*-test. The resulting gene lists with *P*-value < 0.05 were then classified using the PANTHER database (<http://www.pantherdb.org>).^{15,16}

Results

Fluorescence *in situ* hybridization to a tissue microarray

Fluorescence *in situ* hybridization was carried out to further investigate copy number gain of the *MEN1* locus in metastatic prostate cancer. FISH to a TMA containing cores from 30 metastatic prostate tumors was performed. The detailed results are reported in Supplementary Table S1. Two cases were excluded because of lack of tissue. Out of 28 readable cases, 26 had tissue from multiple metastatic sites. Defining gain of 11q13.1 as $\geq 10\%$ of cells having ≥ 3 signals in at least one core for a patient, 5 out of 28 (18%) cases were positive. However, if the threshold was defined as $\geq 5\%$ of cells having ≥ 3 signals in at least one patient core, 11 out of 28 cases (39%) were positive. Using the 5% threshold, 8 out of the 11 (73%) cases positive for 11q13.1 gain were positive in more than one metastatic site per patient. Figure 1a shows an example of increased copy number at the 11q13.1 locus in soft tissue and lung metastases from one patient. The TMA also included control cores of benign prostate tissue ($N = 2$), benign hyperplasia ($N = 2$) and prostatic intraepithelial neoplasia ($N = 1$) and normal breast tissue ($N = 1$), none of which were positive for *MEN1* by FISH.

Immunohistochemistry to a tissue microarray

To investigate menin levels in prostate cancer, IHC was carried out with an *MEN1* antibody on a TMA comprised of cores from 48 patients with prostate cancer that were at high risk for recurrence at the time of surgery as defined by the Kattan nomogram.¹⁷ The result details are tabulated in Supplementary Table S2. Of these cases, 36 had at least two cores that were able to be evaluated by two pathologists. There was high concordance in their calls with only three disagreements calls. One was a benign case being classified as either staining positive or negative. The other two disagreements questioned whether the tissue was benign or PIN, but both called the staining positive. Five of the thirty-six (14%) tumor cases stained positive for *MEN1*. The staining was detected in the nuclei of the tumor cells (Figure 1b). There was benign tissue available for a subset of the cases. Five of the thirty-six cases (14%) stained positive for *MEN1* in the benign prostate glands. Interestingly, the staining in these cases was detected in the cytoplasm (Figure 1b).

MEN1 expression database query

A meta-analysis was conducted to evaluate *MEN1* expression in published and publicly available prostate cancer studies. The Oncomine database (<http://www.oncomine.org>) was queried for studies that identified *MEN1* expression in prostate tissue. Studies that contained cancer tissue and showed differential expression with a *P*-value ≤ 0.05 are shown in Figure 2. In general, the trend for *MEN1* expression in these studies was that *MEN1* expression increased in primary and metastatic tumors as compared to benign tissue.

MEN1 expression levels in cell lines

RNA was extracted from various prostate cell lines (PC3, DU145, LNCaP, MDAPCA2A) to quantify *MEN1* expression levels and to determine their suitability for *in vitro* knockdown experiments. BT474, a breast cancer cell line was included in the evaluation as well as lung cDNA as it is known to express low levels of *MEN1*. All of the cell lines expressed *MEN1*. Supplementary Figure S1 shows the TaqMan results for *MEN1* in the five cell lines and the control. MDAPCA2A and LNCaP are semi-adherent cell lines and were not pursued for phenotypic studies.

MEN1 siRNA knockdown experiments in DU145

The *in vitro* ability of *MEN1* to modulate cellular phenotypes such as growth was measured using RNAi. PC3, which is derived from a bone metastasis, did not respond to *MEN1* siRNA knockdown experiments. However, DU145, which is derived from a brain metastasis did show a knockdown phenotype and was therefore utilized in all subsequent experiments. Cell growth was monitored in real time with the ACEA Biosciences RT-CES System, a cell-based assay that indirectly monitors the cell growth index by measuring electrical impedance across sensor electrodes integrated on the bottom of microtiter plates. Four independent siRNAs targeting different regions of the *MEN1* transcript were evaluated. All appropriate controls were employed including scrambled siRNAs. Silencing of *MEN1* suppressed cell growth in DU145 cells approximately 30% relative to controls (Figure 3a). TaqMan was used to confirm attenuation of *MEN1* expression in the siRNA treated cells (Figure 3b). The siRNAs lowered the abundance of *MEN1* mRNA by about 60%.

The MTS assay, a proliferation assay that is directly proportional to the number of live cells in culture, was used as an independent measure of proliferation in siRNA treated DU145 cells. These results support that silencing of *MEN1* results in suppressed cell growth. Cell numbers were approximately 15% lower in *MEN1* siRNA treated cells when compared to controls 48 h posttransfection, in serum starved conditions (Figure 3c).

To investigate the mechanism by which silencing *MEN1* suppresses cellular proliferation, PI staining followed by FACS was used to quantify the distribution of cells in G1, S and G2/M phase in the different transfected cells (Figure 4a). *MEN1* suppression resulted in an increase in the number of cells in S and G2 relative to the controls. Annexin staining increased in cells where *MEN1* expression was silenced (Figure 4b). One can infer from these results that *MEN1* depletion can increase apoptosis and cell cycle arrest in DU145 cells treated with *MEN1* siRNA.

Expression arrays

To elucidate putative pathways perturbed by aberrant *MEN1* expression in prostate tumors, we undertook expression microarray studies with RNA from the siRNA experiments. RNA from DU145 cells with confirmed *MEN1* silencing (siRNA M4) was compared to control RNAs (every set in triplicate). Analysis was restricted to genes with expression changes with *P*-values <0.05. Supplementary Table S3 lists the biological function categories that were overrepresented and underrepresented for the *MEN1* siRNA expression studies. The expression of selected genes was then validated using TaqMan with *MEN1* as a control. Figure 5a shows the results for integrin- β 1 (*ITGB1*), caspase 8 (*CASP8*) and p53 effector related to PMP22 (*PERP*). Figure 5b shows the validation of these genes using quantitative RT-PCR.

Discussion

In previous work using aCGH, we determined approximately 25% of primary prostate tumors that progressed to metastasis had gain at the *MEN1* locus.¹⁰ Now using FISH on metastatic tumors we have detected gain at *MEN1* in 18–39% of metastatic tumors depending on the threshold used. The metastases used to construct the TMA come from a rapid autopsy program and for most autopsies multiple organ sites are represented on the TMA. This allowed us to investigate heterogeneity for *MEN1* gain across organ sites within individual cases. The majority of cases showed there was uniformity across organ sites. This may suggest a clonal origin for prostate cancer metastases and that *MEN1* is at least associated with aggressive prostate cancer. The possibility that *MEN1* may be associated with aggressive prostate cancer is supported by earlier work where gain at the *MEN1* locus was found to be an independent predictor of disease recurrence following radical prostatectomy.¹⁰ Thus, it is possible that in prostate cancer *MEN1* is a positive regulator of aggressive disease.

If increased *MEN1* copy number is causally related to aggressive disease, then an increase in *MEN1* gene expression would be expected in the corresponding tumor. Indeed, such an increase in expression in primary tumors has been reported.¹¹ Further, a meta-analysis of public *MEN1* gene expression data in prostate cancer reveals a trend toward increased *MEN1* expression in prostate tumors and metastases compared to normal tissue in several different studies.^{18–22} The collective evidence suggests that *MEN1* can act as an oncogene. If the *MEN1* gene is acting as an oncogene then it should confer a phenotype consistent with that function. To test this, *MEN1* transcripts were silenced using siRNA and cell proliferation monitored in real time using the ACEA RT-CES system. In multiple independent experiments, silencing of *MEN1* in a prostate cancer cell line was accompanied by decreased cell proliferation and increased apoptosis. According to the manufacturer, curve shapes generated by the ACEA RT-CES system are indicative of specific cellular responses. These curves suggest a cytostatic response, which also was confirmed by manual cell counting. Furthermore, these results, a decrease in cell proliferation and an increase in apoptosis, were reproduced using the MTS assay and FACS analysis. These experiments provide the first evidence that *MEN1* can act as an oncogene *in vitro* in the proper cellular context.

The siRNA experiments demonstrated that down-regulation of *MEN1* enhances apoptosis. Therefore, we conducted expression microarray studies to identify downstream effectors of

menin. The expression array studies identified genes co-regulated with *MEN1*. Two genes, *PERP* and *CASP8*, can induce apoptosis and were observed to have increased expression when *MEN1* is suppressed. Therefore, when *MEN1* is over expressed in prostate tumors these positive effectors may be down-regulated, attenuating apoptosis. The *CASP8* finding is in contradiction to what has been reported when *MEN1* acts as a tumor suppressor. In that context, menin suppresses tumorigenesis partly by upregulating *CASP8* expression.²³ Similarly, *ITGB1*'s expression increases when *MEN1* expression is decreased. Therefore, when *MEN1* dosage is increased, *ITGB1*'s expression is decreased. Integrins are involved in cellular adhesion and proliferation and their deregulation can facilitate metastasis. This suggests a link for *MEN1* in promoting prostate cancer metastasis.

Menin shows no homology to any known protein. Other than two nuclear localization signals,^{24,25} menin has no identifiable functional motifs. IHC studies by Guru *et al.* of interphase somatic cells showed that the menin protein is located in the nucleus.²⁴ In an effort to elucidate the role of *MEN1* in prostate cancer, we performed IHC studies. In benign prostate glands adjacent to tumor cells, IHC staining revealed menin localized to the cytoplasm although nuclear localization was observed in tumor cells. The gene's two functional nuclear localization signals are consistent with our observation that the menin protein can localize to either the cytoplasm or nucleus. Interestingly, both normal adjacent prostate tissue and tumor can also stain negatively for menin.

There are many observations of *MEN1* that imply molecular and biological complexity. First is not only can there be multiple foci per affected organ in MEN, but also that many different endocrine organs can be affected simultaneously. Second, several alternative splicing patterns exist for *MEN1*, suggesting the potential for multiple protein isoforms. Whereas most *MEN1* tumors are benign, a subset are malignant. Interestingly, *MEN1* patients who develop gastrinomas possess metastases at diagnosis, with 15% progressing to an aggressive malignancy.^{26–28} Even the traditional role of *MEN1* as a tumor suppressor has been shown to be complex. *MEN1* can act as a transcriptional activator, to maintain the expression of growth regulatory genes, and a transcriptional corepressor, negatively regulating growth promoting genes.^{7,29,30} Although *MEN1* has historically been thought to act as a tumor suppressor, menin is considered an oncogene in mixed-lineage leukemia (MLL).³¹ Yokoyama *et al.* elegantly demonstrated that the physical interaction between the oncogenic MLL fusion protein and menin was required for the initiation of MLL-mediated leukemogenesis. Therefore, *MEN1* can serve as an oncogenic cofactor in MLL. Finally, Karnik *et al.* also demonstrated that *MEN1* is regulated by prolactin and progesterone raising the possibility that defects in signaling pathways regulated by steroid hormones might be linked to phenotypes associated with *MEN1* inactivation.³² Conversely, it is conceivable that increased expression of *MEN1* might play a role in hormone driven cancers, such as prostate cancer, as suggested here. Further studies will need to be performed to further elucidate the mechanism for *MEN1*'s oncogenic potential as well as clarify the role of co-regulatory genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Chandrasekharappa SC, Teh BT. Functional studies of the *MEN1* gene. *J Intern Med* 2003;253:606–615. [PubMed: 12755956]
2. Pannett AA, Thakker RV. Multiple endocrine neoplasia type 1. *Endocr Relat Cancer* 1999;6:449–473. [PubMed: 10730900]
3. Guru SC, Manickam P, Crabtree JS, Olufemi SE, Agarwal SK, Debelenko LV. Identification and characterization of the multiple endocrine neoplasia type 1 (*MEN1*) gene. *J Intern Med* 1998;243:433–439. [PubMed: 9681840]
4. Lemos MC, Thakker RV. Multiple endocrine neoplasia type 1 (*MEN1*): analysis of 1336 mutations reported in the first decade following identification of the gene. *Hum Mutat* 2008;29:22–32. [PubMed: 17879353]
5. Larsson C, Skogseid B, Oberg K, Nakamura Y, Nordenskjold M. Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma. *Nature* 1988;332:85–87. [PubMed: 2894610]
6. Hughes CM, Rozenblatt-Rosen O, Milne TA, Copeland TD, Levine SS, Lee JC, et al. Menin associates with a trithorax family histone methyltransferase complex and with the *hoxc8* locus. *Mol Cell* 2004;13:587–597. [PubMed: 14992727]
7. Yokoyama A, Wang Z, Wysocka J, Sanyal M, Aufiero DJ, Kitabayashi I, et al. Leukemia proto-oncoprotein MLL forms a SET1-like histone methyltransferase complex with menin to regulate *Hox* gene expression. *Mol Cell Biol* 2004;24:5639–5649. [PubMed: 15199122]
8. Schussheim DH, Skarulis MC, Agarwal SK, Simonds WF, Burns AL, Spiegel AM, et al. Multiple endocrine neoplasia type 1: new clinical and basic findings. *Trends Endocrinol Metab* 2001;12:173–178. [PubMed: 11295574]
9. Trump D, Farren B, Wooding C, Pang JT, Besser GM, Buchanan KD, et al. Clinical studies of multiple endocrine neoplasia type 1 (*MEN1*). *QJM* 1996;89:653–669. [PubMed: 8917740]
10. Paris PL, Andaya A, Fridlyand J, Jain AN, Weinberg V, Kowbel D, et al. Whole genome scanning identifies genotypes associated with recurrence and metastasis in prostate tumors. *Hum Mol Genet* 2004;13:1303–1313. [PubMed: 15138198]
11. Lapointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K, et al. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc Natl Acad Sci USA* 2004;101:811–816. [PubMed: 14711987]
12. Perner S, Wagner PL, Demichelis F, Mehra R, Lafargue CJ, Moss BJ, et al. EML4–ALK fusion lung cancer: a rare acquired event. *Neoplasia* 2008;10:298–302. [PubMed: 18320074]
13. Rubin MA, Putzi M, Mucci N, Smith DC, Wojno K, Korenchuk S, et al. Rapid ('warm') autopsy study for procurement of metastatic prostate cancer. *Clin Cancer Res* 2000;6:1038–1045. [PubMed: 10741732]
14. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;19:185–193. [PubMed: 12538238]
15. Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al. PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res* 2003;13:2129–2141. [PubMed: 12952881]
16. Thomas PD, Kejariwal A, Guo N, Mi H, Campbell MJ, Muruganujan A, et al. Applications for protein sequence-function evolution data: mRNA/protein expression analysis and coding SNP scoring tools. *Nucleic Acids Res* 2006;34:W645–W650. [PubMed: 16912992]
17. Kattan MW, Wheeler TM, Scardino PT. Postoperative nomogram for disease recurrence after radical prostatectomy for prostate cancer. *J Clin Oncol* 1999;17:1499–1507. [PubMed: 10334537]
18. Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, et al. Delineation of prognostic biomarkers in prostate cancer. *Nature* 2001;412:822–826. [PubMed: 11518967]
19. Dhanasekaran SM, Dash A, Yu J, Maine IP, Laxman B, Tomlins SA, et al. Molecular profiling of human prostate tissues: insights into gene expression patterns of prostate development during puberty. *FASEB J* 2005;19:243–245. [PubMed: 15548588]
20. Tomlins SA, Mehra R, Rhodes DR, Cao X, Wang L, Dhanasekaran SM, et al. Integrative molecular concept modeling of prostate cancer progression. *Nat Genet* 2007;39:41–51. [PubMed: 17173048]

21. Vanaja DK, Cheville JC, Iturria SJ, Young CY. Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. *Cancer Res* 2003;63:3877–3882. [PubMed: 12873976]
22. Varambally S, Yu J, Laxman B, Rhodes DR, Mehra R, Tomlins SA, et al. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. *Cancer Cell* 2005;8:393–406. [PubMed: 16286247]
23. Schnepf RW, Mao H, Sykes SM, Zong WX, Silva A, La P, et al. Menin induces apoptosis in murine embryonic fibroblasts. *J Biol Chem* 2004;279:10685–10691. [PubMed: 14688275]
24. Guru SC, Goldsmith PK, Burns AL, Marx SJ, Spiegel AM, Collins FS, et al. Menin, the product of the *MEN1* gene, is a nuclear protein. *Proc Natl Acad Sci USA* 1998;95:1630–1634. [PubMed: 9465067]
25. La P, Desmond A, Hou Z, Silva AC, Schnepf RW, Hua X. Tumor suppressor menin: the essential role of nuclear localization signal domains in coordinating gene expression. *Oncogene* 2006;25:3537–3546. [PubMed: 16449969]
26. Jensen RT. Management of the Zollinger–Ellison syndrome in patients with multiple endocrine neoplasia type 1. *J Intern Med* 1998;243:477–488. [PubMed: 9681846]
27. Norton JA, Fraker DL, Alexander HR, Venzon DJ, Doppman JL, Serrano J, et al. Surgery to cure the Zollinger–Ellison syndrome. *N Engl J Med* 1999;341:635–644. [PubMed: 10460814]
28. Weber HC, Venzon DJ, Lin JT, Fishbein VA, Orbuch M, Strader DB, et al. Determinants of metastatic rate and survival in patients with Zollinger–Ellison syndrome: a prospective long-term study. *Gastroenterology* 1995;108:1637–1649. [PubMed: 7768367]
29. Milne TA, Hughes CM, Lloyd R, Yang Z, Rozenblatt-Rosen O, Dou Y, et al. Menin and MLL cooperatively regulate expression of cyclin-dependent kinase inhibitors. *Proc Natl Acad Sci USA* 2005;102:749–754. [PubMed: 15640349]
30. Agarwal SK, Guru SC, Heppner C, Erdos MR, Collins RM, Park SY, et al. Menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription. *Cell* 1999;96:143–152. [PubMed: 9989505]
31. Yokoyama A, Somervaille TC, Smith KS, Rozenblatt-Rosen O, Meyerson M, Cleary ML. The menin tumor suppressor protein is an essential oncogenic cofactor for MLL-associated leukemogenesis. *Cell* 2005;123:207–218. [PubMed: 16239140]
32. Karnik SK, Chen H, McLean GW, Heit JJ, Gu X, Zhang AY, et al. Menin controls growth of pancreatic beta-cells in pregnant mice and promotes gestational diabetes mellitus. *Science* 2007;318:806–809. [PubMed: 17975067]
33. Luo J, Duggan DJ, Chen Y, Sauvageot J, Ewing CM, Bittner ML, et al. Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling. *Cancer Res* 2001;61:4683–4688. [PubMed: 11406537]
34. Snijders AM, Nowak N, Segreaves R, Blackwood S, Brown N, Conroy J, et al. Assembly of microarrays for genome-wide measurement of DNA copy number. *Nat Genet* 2001;29:263–264. [PubMed: 11687795]

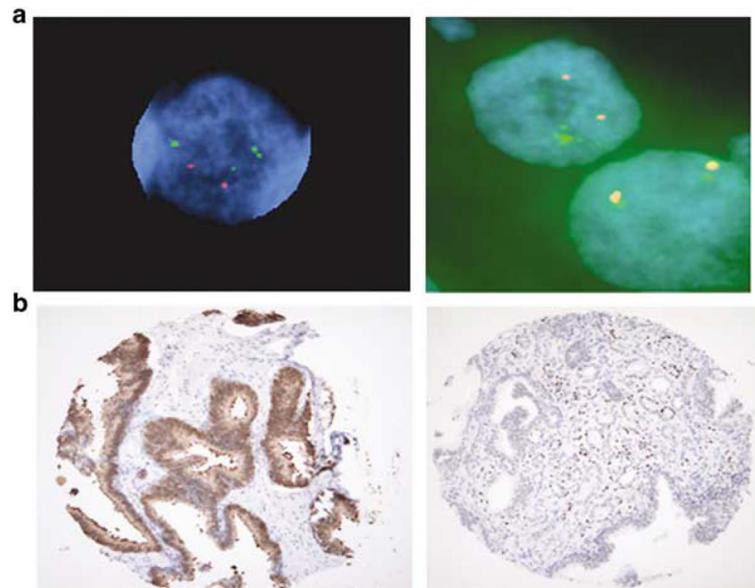


Figure 1. Fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC) examples. **(a)** FISH validation of 11q13.1 copy number gain in soft tissue (left) and lung (right) metastases from the same patient. Green signals represent the probe spanning the multiple endocrine neoplasia I (*MEN1*) locus, red signals represent the reference probe. **(b)** Representative IHC staining demonstrating *MEN1* expression in the cytoplasm of benign prostate tissue (left) and nuclear *MEN1* expression in a tumor sample (right) from different patients.

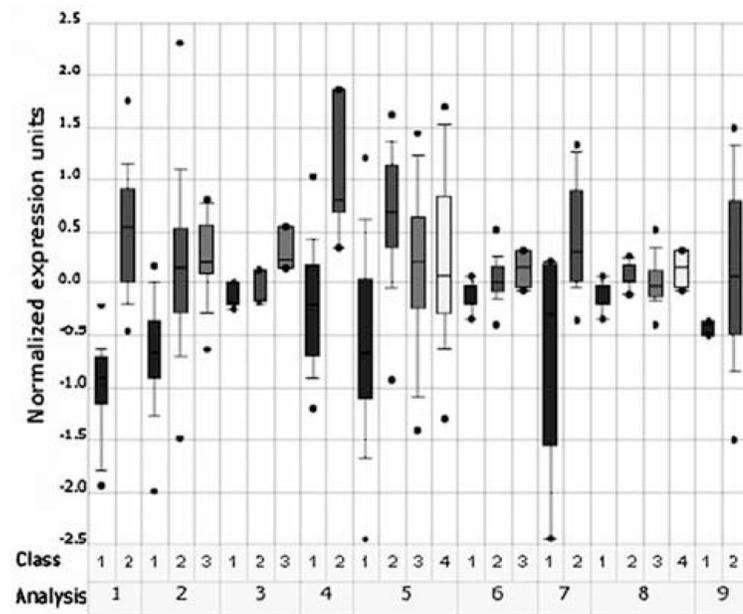
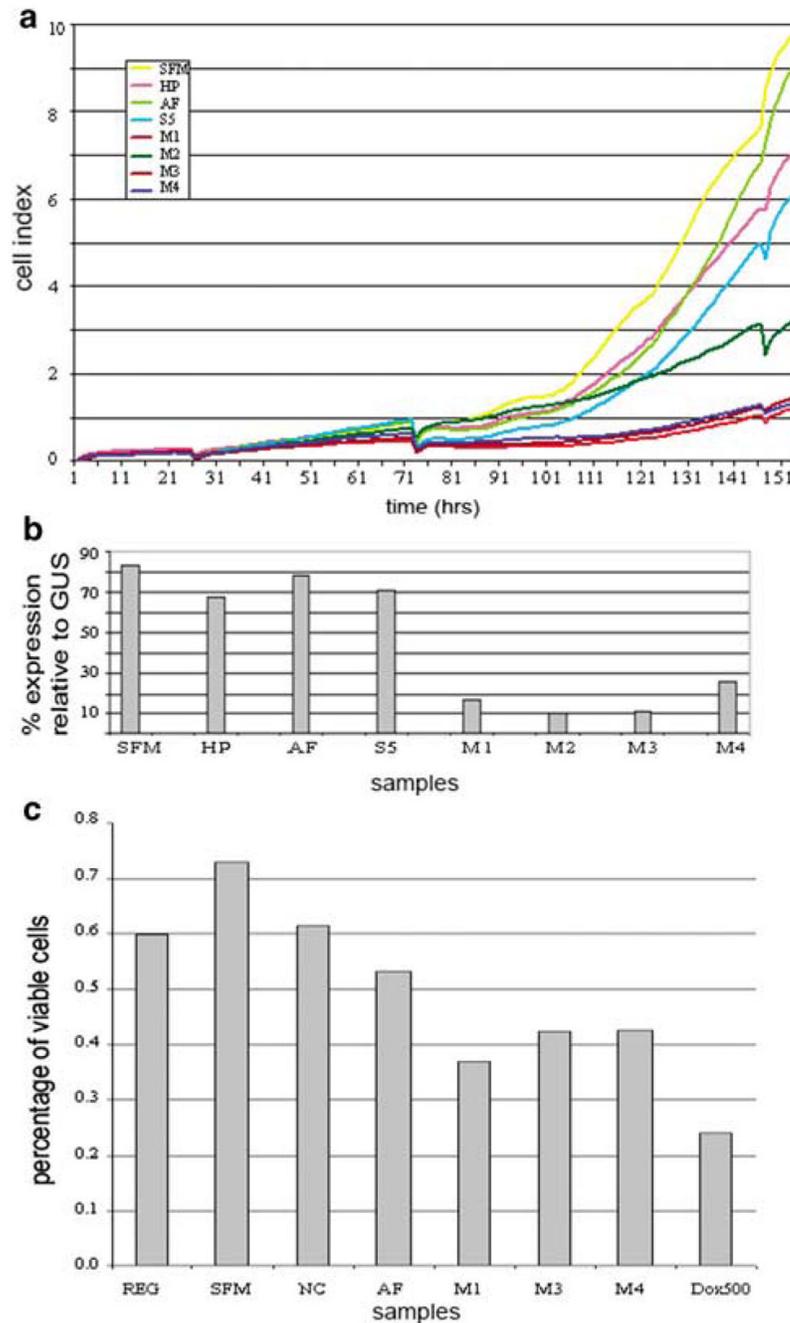


Figure 2. Results of Oncomine database query for multiple endocrine neoplasia I expression in prostate cancer (differential expression $P < 0.05$). The shades of gray within an analysis are to help distinguish each tissue type represented. The corresponding table legend describes each analysis.

Analysis number	Class 1	Class 2	Class 3	Class 4	P-value	References
1	Normal adjacent prostate ($N = 2$), normal adult prostate ($N = 7$), normal pubertal prostate ($N = 3$)	Prostate cancer ($N = 25$)			$8.9E-9$	19
2	Normal prostate ($N = 6$), benign prostatic hyperplasia ($N = 16$)	Primary prostate cancer ($N = 59$)	Metastatic prostate cancer ($N = 20$)		$4.1E-5$	18
3	Benign prostate ($N = 6$)	Prostate carcinoma ($N = 7$)	Hormone refractory metastatic prostate carcinoma ($N = 6$)		$1E-4$	22
4	Prostate cancer ($N = 25$)	Metastatic prostate cancer ($N = 6$)			0.002	19

Analysis number	Class 1	Class 2	Class 3	Class 4	P-value	References
5	Atrophic epithelium ($N = 5$), benign prostate ($N = 22$)	Prostatic intraepithelial neoplasia ($N = 13$)	Prostate carcinoma ($N = 30$)	Metastatic prostate cancer ($N = 19$)	0.006	20
6	Normal prostate ($N = 8$)	Prostate adenocarcinoma ($N = 27$)	Metastatic prostate cancer ($N = 5$)		0.009	21
7	Benign prostatic hyperplasia ($N = 9$)	Prostate carcinoma ($N = 16$)			0.01	33
8	Benign ($N = 8$)	Gleason score 6 ($N = 12$)	Gleason score 9 ($N = 15$)	Metastatic ($N = 5$)	0.051	21
9	Naive ($N = 3$)	Refractory ($N = 16$)			0.058	20

**Figure 3.**

Phenotypic effects of multiple endocrine neoplasia I (*MEN1*) siRNA silencing. **(a)** *In vitro* cell proliferation assay using an ACEA instrument showing decreased cell proliferation of DU145 cells transfected with four different *MEN1* siRNAs (M1-4). Transfections were carried out in serum starved conditions; cells were rescued and maintained in serum media subsequently for 48 h. Controls include nontransfected (SFM), transfection reagent alone (HP) and scrambled siRNAs (AF, S5). **(b)** shows corresponding TaqMan data for the same samples confirming knockdown of *MEN1* expression in cells with the siRNA, 72 h posttransfection. **(c)** DU145 cells were transfected in serum-free conditions and then rescued with serum media, 48 h later. Cell viability was quantified using the MTS assay, after 72 h of rescue. Samples include DU145

cells transfected with three different *MEN1* siRNAs (M1, M3 and M4). Controls include nontransfected (SFM), transfection reagent alone (NC) and scramble siRNA (AF). Doxorubicin treated DU145 cells were included as an additional control (Dox500).

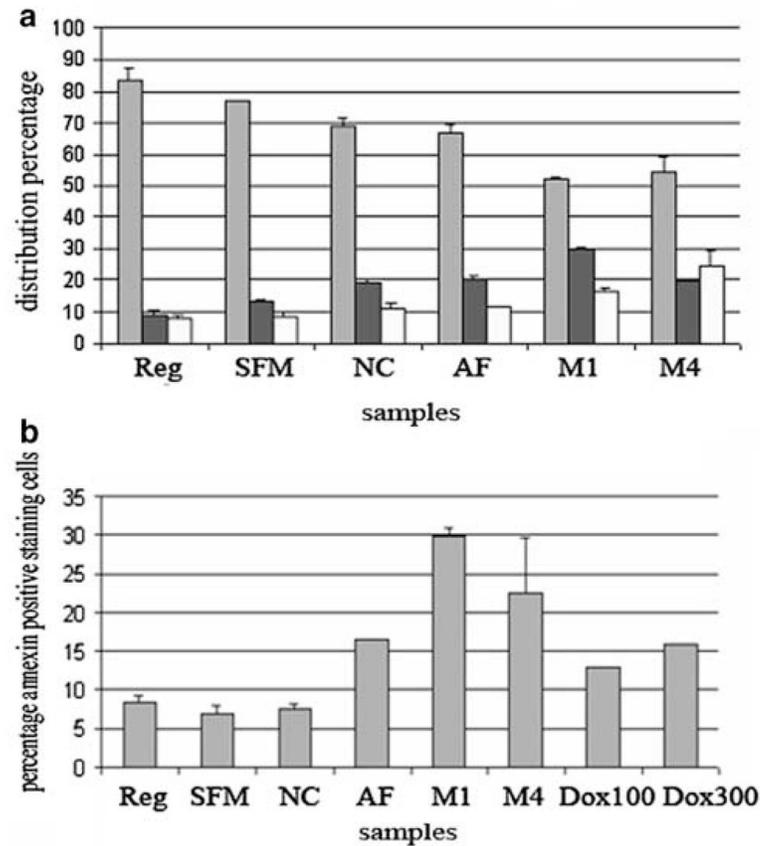


Figure 4.

Fluorescence-activated cell sorter (FACS) analysis. **(a)** Cell cycle distribution of multiple endocrine neoplasia I (*MEN1*) siRNA treated DU145 cells. Transfected cells were analyzed by FACS to ascertain the effects of *MEN1* knockdown on the cell cycle. DU145 cells were transfected and starved in serum-free media for 48 h before rescue with serum –media. 48 h later, cells were fixed in ethanol and stained with PI. Samples include DU145 cells transfected with 2 different *MEN1* siRNAs (M1 and M4). Controls include DU145 cells (REG), nontransfected (SFM), transfection reagent alone (NC) and scrambled siRNA (AF). Light gray bars represent cells in G1 phase, dark gray bars represent S phase, and G2 phase cells are represented by white bars. Each sample was run in duplicate and the average is shown. **(b)** Transfected cells were analyzed by FACS to study the effects of *MEN1* silencing on the induction of apoptosis. DU145 cells were transfected and starved in serum-free media for 48 h before rescue with serum media. After 48 h, cells were stained with Annexin to track phosphatidyl serine flipping in cell membranes that is a hallmark of early apoptosis. Samples include DU145 cells transfected with two different *MEN1* siRNAs (M1 and M4). Controls include DU145 cells (Reg), nontransfected (SFM), transfection reagent alone (NC) and scramble siRNA (AF). Two different concentrations of doxorubicin treated DU145 cells were included as additional controls. Each sample was run in duplicate and the average is shown.

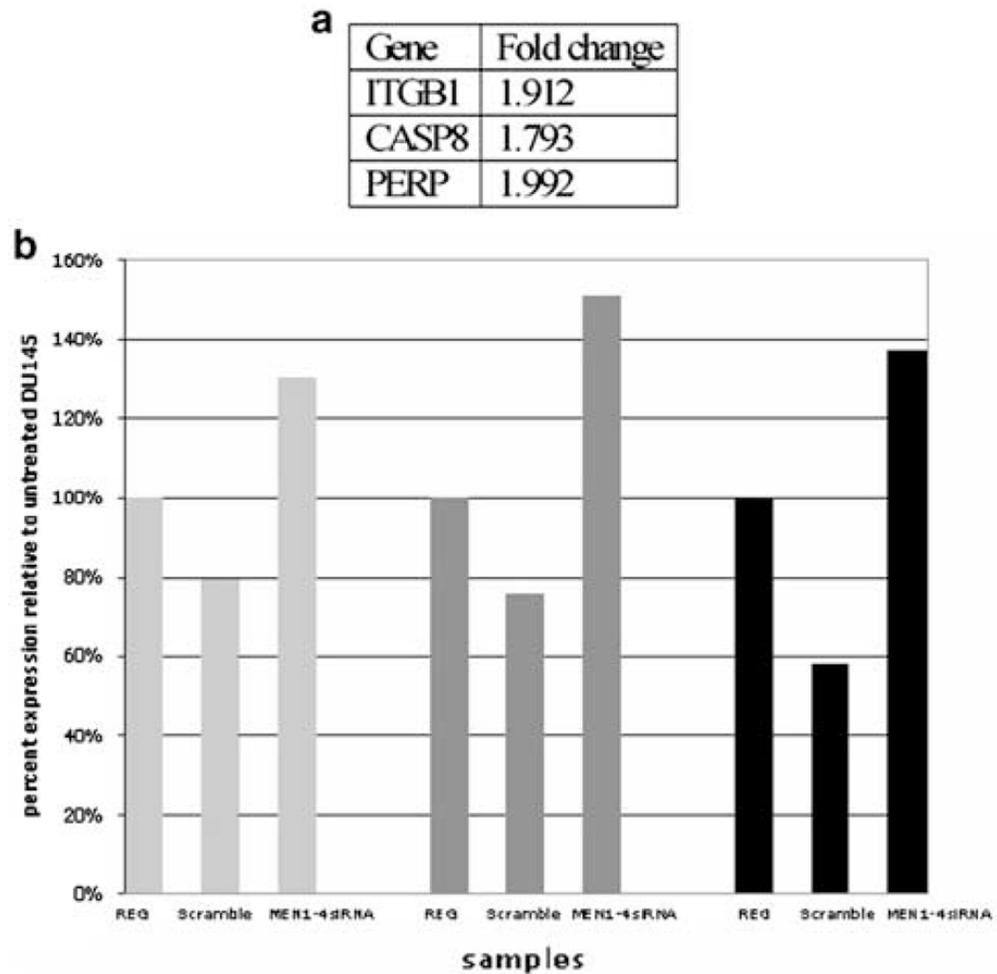


Figure 5. (a) DU145 expression array results of selected genes. The gene and the corresponding expression array fold change are listed. Fold change is calculated as the ratio of the expression in multiple endocrine neoplasia I (*MEN1*) knocked down DU145 cells compared to the controls (no siRNA and scramble combined). (b) TaqMan validation of DU145 expression array results. Every gene's expression was measured relative to that of the house keeping gene *BGUS*. TaqMan results are shown for *ITGB1* (light gray), *CASP8* (dark gray) and *PERP*.³⁴ Each result is plotted as the percent expression relative to untreated DU145. Reg represents DU145 cells without siRNA, scramble are DU145 cells that were treated with siRNAs that share no homology to known cDNAs, *MEN1* siRNAs are the DU145 cells treated with siRNA. Each bar represents two biological replicates that were each run in triplicate before averaging the results.