

# The Metastasis Suppressor NME1 Regulates Expression of Genes Linked to Metastasis and Patient Outcome in Melanoma and Breast Carcinoma

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**Abstract.** *NME1* is a well-documented metastasis suppressor gene, with suppressor activity demonstrated across a wide spectrum of human cancers including melanoma and carcinomas of the breast, stomach and thyroid. A primary aim of the current study was to identify profiles of genes whose expression is regulated by *NME1* in cell lines of melanoma and thyroid carcinoma origin. Impact of *NME1* was determined by forcing its expression transiently in cell lines using a novel Ad5-based adenoviral vector (*Ad5-NME1*), followed 48 h later by analysis of RNA expression profiles using the U133A microarray chip. Robust *NME1* expression was achieved following infection with the *Ad5-NME1* adenovirus in the human metastasis-derived cell lines WM1158 (melanoma) and WRO82 (follicular thyroid carcinoma), resulting in wide-ranging effects on gene expression in both settings. A substantial proportion of the *NME1*-regulated genes identified in the analyses were of clear potential relevance to metastasis, such as matrix metalloproteinase-1 (*MMP1*), angiopoietin-2 (*ANGPT2*), *SERPINB9* and colony stimulating factor receptor-2B (*CSFR2B*). Nine genes were identified (false discovery rate

<0.1) that were regulated by *NME1* in both the WM1158 and WRO82 cell lines, each possessing one or more such metastasis-relevant activities as stress fiber formation and focal adhesion (*PPM1E*, *ZYX*, *PFN1*), chemotaxis (*CCR1*) epithelial-mesenchymal signaling (*WNT6*), differentiation and morphogenesis (*TBX4*, *ZFP36L2*), and G protein modulation (*GPR52* and *PFN1*). In addition, a number of the *NME1*-regulated genes were shown to be of prognostic value for distant disease-free survival and overall survival in melanoma and breast cancer. The combined expression of three *NME1*-regulated genes *CSFR2B*, *MSF4A1* and *SERPINB9* provided a strongly synergistic correlation with distant disease-free survival in the basal subtype of breast cancer ( $p < 3.5 \times 10^{-5}$ , hazard ratio = 0.33). Our study demonstrates that analysis of *NME1*-dependent gene expression is a powerful approach for identifying potential modulators of metastatic potential in multiple cancer types, which in turn may represent useful therapeutic targets. The study also highlights *NME1*-dependent genes as potential prognostic/diagnostic indices, which are profoundly lacking at present in melanoma.

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Metastasis accounts for most of the morbidity and mortality associated with cancer, yet the underlying mechanisms are poorly understood (1). A better understanding of those processes could provide new therapeutic approaches to block cancer dissemination, as well as targeting metastatic lesions that have already been established. Metastasis represents a complex cascade of events that requires a coordinated expression of gene networks to overcome barriers to their dissemination and growth in heterotopic environments. A growing number of metastasis-suppressor genes and their

encoded proteins are being identified that inhibit metastasis selectively, with minimal impact on growth of the primary tumor. Their metastasis-specific functions are providing critical insights into events driving the metastatic cascade in many cancers (2, 3). We recently verified metastasis suppressor activity of the NME1 and NME2 genes in melanoma for the first time *in vivo*, using a transgenic mouse model of UV-induced melanoma (4). Our site-directed mutagenesis analysis of the NME1 molecule has identified amino acid residues critical for its nucleoside diphosphate kinase and 3'-5' exonuclease activities (5) and metastasis suppressor function (6).

Considerable evidence suggests NME proteins regulate gene expression, although the underlying mechanisms involved have yet to be fully elucidated. Early studies demonstrated an affinity of NME1 and NME2 for single-stranded motifs in the CMYC (7) and PDGFA (5, 8) promoter regions, as well as their abilities to regulate transcription from those promoters. Chromatin immunoprecipitation analyses have further demonstrated physical associations of NME1 with non-B form DNA elements in the promoter regions of these and the TP53 genes (9-11). In addition, NME1 has been shown to regulate global gene expression profiles in the breast carcinoma cell line, MDA-MB-435, with the lysophosphatidic acid receptor EDG2 identified as a motility-driving target of NME1-mediated suppression (12). A primary intent of the current study was to analyze NME1-dependent gene expression profiles in the specific setting of melanoma, which had yet to be explored. In addition, we included thyroid carcinoma, for which a number of studies have indicated reduced NME1 expression in metastases and advanced forms of the disease (13-15). An important rationale for studying NME1-dependent gene expression in both melanoma and thyroid carcinoma cell lines was the potential for discovery of genes regulated across these diverse cancer types, which in turn could potentially enrich for genes with fundamental roles in metastasis suppressor function. This study has used a novel adenoviral vector for efficient forced expression of NME1, and microarray analysis of resulting gene expression profiles to reveal broad effects of NME1 on gene expression in metastatic cell lines of melanoma and thyroid carcinoma origins. A number of the NME1-dependent genes identified in these analyses are also shown to have prognostic value for survival in melanoma and breast cancer patients.

## Materials and Methods

**Cell lines and culture conditions.** The WM1158 (metastasis-derived) melanoma cell line was a gift of Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA, USA) and WRO82 cells (derived from a follicular thyroid carcinoma metastasis) were obtained from Dr. G. Juillard (University of California- Los Angeles, Los Angeles, CA, USA (16)). HEK-293 cells were obtained from the American Type

Culture Collection. WM1158 cells were cultured at 5% CO<sub>2</sub> in Tu2% media, composed of the following: MCDB:Leibovitz-15 medium (4:1, v/v; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM CaCl<sub>2</sub>, 2.5 µg/ml insulin and 2% fetal bovine serum (Life Technologies, Grand Island, NY, USA). The other cell lines were maintained at 10% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1 mM non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin.

**Plasmid and viral vectors.** A DNA fragment harboring full-length NME1 coding sequence and Kozak box with no 5'- or 3'- untranslated sequence was previously cloned into an expression plasmid (pCI- IRES-GFP) and designated pCI-H1(6). The key features of this plasmid are its CMV promoter, which is broadly expressed in most cell types, and an IRES (internal ribosome entry sequence) upstream of the marker gene GFP, which allows co-expression of NME1 and GFP from the same promoter. Using the restriction enzymes BglII which cuts just upstream of the CMV promoter, and ClaI which cuts downstream of the GFP, the entire expression cassette was transferred into the corresponding site of pAdLink as described (17) to create pAdLink-NME1. For construction of adenovirus *via* recombination, HEK-293 cells (2×10<sup>6</sup> cells) expressing the adenoviral *E1* gene were first seeded in 60-mm dishes. The cells were transfected 24 h later with 3.2 µg of *Cla*I-digested DNA isolated from wild type Ad5 (18), plus 4.8 µg of *Nhe*I-digested pAdLink-NME1. Following recombination, viral plaques expressing GFP were isolated and expanded in HEK-293 cells, and respective DNAs screened for the presence of NME1 by dot-blot analysis using a radiolabeled DNA probe generated by PCR from the NME1 cDNA sequence. Correct recombination of DNA fragments was verified by Southern blot using the same radiolabeled probe, followed by another round of plaque purification and Southern blotting. Potential contamination of the resulting virus, Ad5-NME1, by wild-type Ad5 was measured using PCR. A previously described Ad5-based vector also containing the IRES-GFP cassette plus a LacZ coding sequence (18, 19) was used as a negative control and is denoted herein as Ad5-clig. The Ad5-NME1 and Ad5-clig adenoviruses were purified by CsCl density gradient centrifugation (20) and eluted in 50 mM Hepes (pH 7.2), 0.9% NaCl, 0.5 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub> and 0.5% bovine serum albumin. Purified recombinant viruses were again screened for wild-type Ad5 adenovirus by PCR. Adenoviral titers were measured with the Adeno-X Rapid Titer Kit (Clontech; Mountain View, CA, USA) and were as follows: Ad5-clig, 4.6×10<sup>10</sup> i.f.u./ml; Ad5-NME1, 2.9×10<sup>10</sup> i.f.u./ml. Adenovirus stocks were stored in 5% glycerol in Dulbecco's phosphate buffered saline at -80°C.

**RNA isolation and microarray analysis.** WM1158 cells were seeded at a density of 1.5×10<sup>5</sup> per 100-mm plastic dish and grown for 3 days to 75-80% confluence in Tu2%. Total cellular RNA was harvested (RNeasy Extraction Kit, Qiagen, Valencia, CA, USA) from five independent dishes for each cell line and virus treatment group. Purified RNA samples were diluted to ~1 mg/ml with RNase-free deionized water and transferred to the University of Kentucky Microarray Core Facility for cDNA synthesis, hybridization and scanning using their established protocols (<http://www.research.uky.edu/microarray/AffymetrixGeneChip.html>). Genome-wide expression profiling was conducted using UI33A human genome arrays (Affymetrix, Santa Clara, CA, USA).

Gene expression values were calculated using Affymetrix® Expression Console™ Software version 1.1.2. Expression signals were subjected to background correction and scaled across all chips using quantile normalization. Resulting intensities were summarized and log<sub>2</sub> transformed using the Robust Multi-Array Average (RMA) algorithm (21). Primary data are available *via* the NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>).

**Kaplan-Meier analysis.** Gene expression profiles of 31 primary and 52 metastatic melanoma biopsies published by Xu *et al.* (22) were obtained from Gene Expression Omnibus (GEO) under series accession number GSE8401. The expression profiles were imported into Expression Console v1.1.2 software (Affymetrix) and RMA was used to normalize the expression data across the samples. SigmaPlot 12 (Systat Software, San Jose, CA, USA) was used to generate Kaplan-Meier survival curves and perform log-rank tests to assess statistical significance between “high” and “low” expression of individual genes in the metastatic melanoma samples. Samples were categorized as “high” or “low” expressers if the log<sub>2</sub> expression of gene was above or below, respectively, the median of all samples for that gene. Statistically significant differences in normalized log<sub>2</sub> expression of individual genes in GSE8401 and a second human melanoma GEOdataset by Kabbarah *et al.* (accession number GSE46517) (23) were analyzed by one-way ANOVA followed by pairwise multiple comparison testing (Holm-Sidak method, SigmaPlot 12). The GSE46517 dataset consisted of 9 nevi, 31 primary melanoma, and 73 metastatic melanoma biopsies.

Kaplan-Meier survival analyses in breast cancer were conducted using BreastMark, a newly-described breast cancer database and algorithm (24). The system contains integrated gene expression and survival data from 26 datasets on 12 distinct microarray platforms from a total of 4,738 patient samples, distributed across the four major breast cancer subtypes of luminal A, luminal B, Her2-positive and basal. Briefly, the BreastMark tool utilizes log-rank tests to measure differences in survival curves and Cox regression analyses to calculate hazard ratios *via* a web-based server (<http://glados.ucd.ie/BreastMark/>). The BreastMark tool conducts all calculations in the R statistical environment.

## Results

**Construction of an adenoviral vector for forced NME1 expression in metastatic cell lines of melanoma and thyroid carcinoma origin.** To identify genes dysregulated by loss of NME1 in metastatic cancers, gene expression profiling was conducted in the absence or presence of forced NME1 expression. The study was conducted in two human metastatic cell lines obtained from distinct cancers, melanoma and thyroid carcinoma, to identify NME1-responsive genes unique to the respective cancers, as well genes regulated across both. It was postulated that genes regulated by NME1 across these two diverse metastatic cancer types would be enriched for relevance to metastasis-suppressor function. The melanoma cell line, WM1158, was derived from a lymph node metastasis in a patient with a Stage II, superficial spreading melanoma lesion (<http://ccr.coriell.org/Sections/Collections/WISTAR/CellLine.s.aspx?PgId=572&coll=WC>). This metastatic line was

suitable for measuring effects of forced NME1 expression, as we showed previously that it exhibits markedly reduced NME1 and NME2 expression (6). WRO82 was originally obtained from a metastatic follicular thyroid carcinoma lesion, and expresses almost undetectable levels of NME1 and NME2.

Prior efforts at forced NME1 expression *via* stable transfection in metastatic melanoma cell lines have provided only modest (2- to 3-fold) increases at best in our laboratory, probably due to proteolytic degradation of NME1 in the lysosome, as we recently described (25). To maximize effects of NME1 expression on overall gene expression profiles in these studies, we investigated the utility of an adenoviral (Ad5-based) expression vector, constructed with an NME1 cDNA under control of the cytomegalovirus promoter/enhancer (Ad5-NME1) (Figure 1A). An internal ribosome entry sequence (IRES) and green fluorescent protein cDNA generates a bicistronic mRNA for co-translation of NME1 and GFP proteins. Infection with Ad5-NME1 provided robust expression of NME1 protein in both WM1158 and WRO82 cells, as indicated by immunoblot analysis (Figure 1B). GFP fluorescence was observed microscopically in >90% of infected cells in both lines, consistent with attainment of NME1 overexpression in a similar proportion of the cells (Figure 1C).

**Profiling of NME1-regulated gene expression in WM1158 and WRO82 cells.** For RNA expression analysis, WM1158 and WRO82 cells were infected with the control adenovirus Ad5-clig, Ad-NME1, or were left uninfected, followed 48h later by harvesting of total cellular RNA (see Materials and Methods). RNA samples were analyzed using the U133A human microarray chip (Affymetrix), which analyzes expression for approximately 18,400 transcripts and variants. Genes were identified as NME1-regulated based on the following criteria. Signal intensities for the three experimental groups were compared pairwise using Welch two sample *t*-tests. Resulting *p*-values were adjusted for multiple comparisons using the correction method of Benjamini and Hochberg (26) and are listed as FDR *p*-values herein. Probe sets showing significant differences (unadjusted *p*<0.05) between the uninfected and Ad5-clig control groups were filtered out as non-specific, adenovirus infection-induced effects. NME1-induced expression changes were then identified as probe sets showing significant and consistent alterations as compared to both uninfected and Ad5-clig-infected cells at the adjusted *p*-values indicated.

Forced expression of NME1 in WM1158 cells resulted in up-regulation of 390 genes and down-regulation of 338 genes, as determined using a false discovery rate (FDR) of 0.1 (Figure 2). Ontology analysis using Ingenuity® pathway analysis software identified eight functional categories altered significantly by NME1 expression (Table I). The most

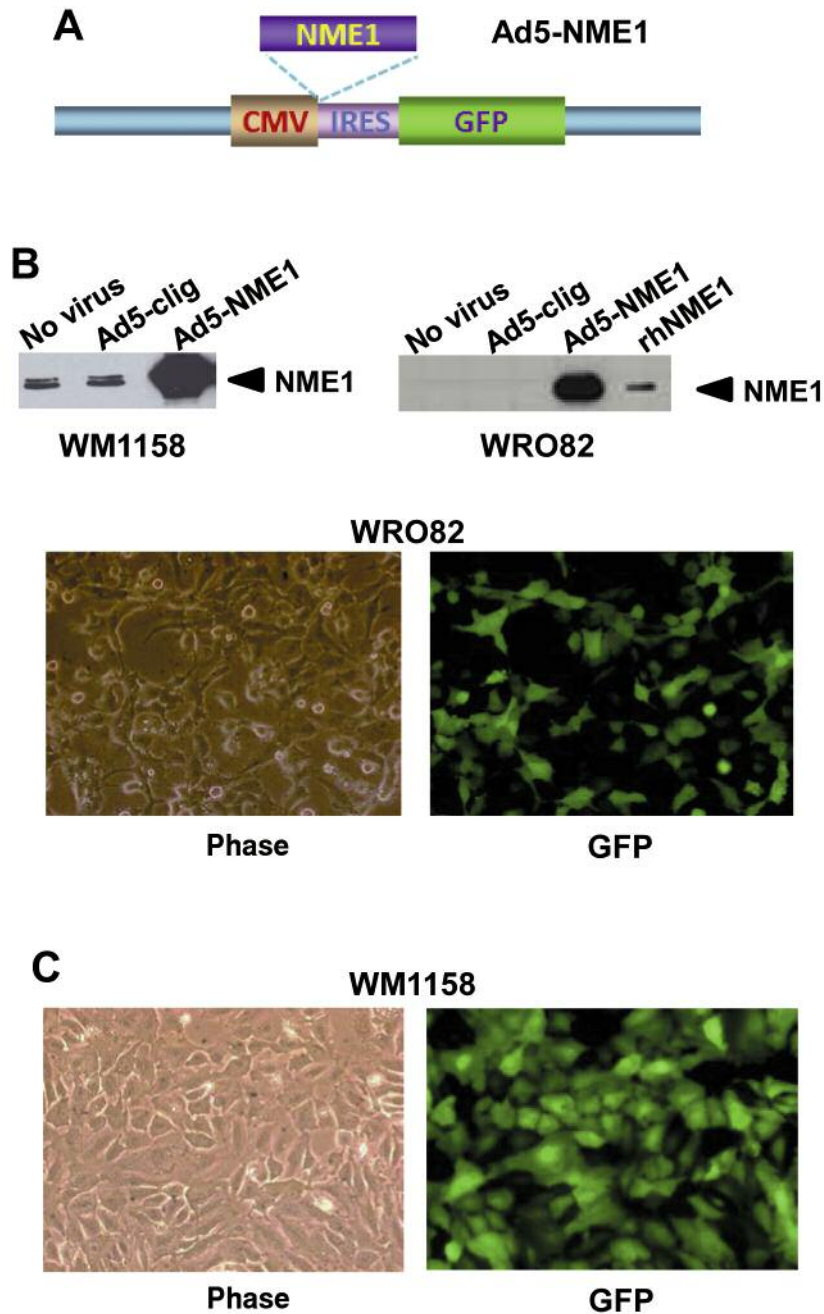


Figure 1. The adenoviral expression vector Ad5-NME1 provides strong NME1 expression in WM1158 and WRO82 cell lines. A: Salient features of Ad5-NME1 are shown: CMV, cytomegalovirus promoter/enhancer; NME1, NME1 cDNA; IRES, internal ribosome entry site/sequence; GFP, green fluorescence protein; LacZ,  $\beta$ -galactosidase. B: Immunoblot analysis of NME expression at 48h post-infection with Ad5-NME1. rhNME1, recombinant human NME1 (1  $\mu$ g) expressed in *E. coli* and purified as described (5). C: Representative phase and fluorescence microscopic images from WM1158 and WRO82 cells infected with pAd5-NME1.

pronounced effect was on the “cell death and survival” category which contained 276 genes related to apoptosis, cell death and viability functions. Other strongly represented categories included “small molecule biochemistry” (104 genes), “DNA replication, recombination and repair” (49

genes), “cell signaling” (49 genes), and “nucleic acid metabolism” (37 genes). NME1-mediated regulation of nucleotide metabolism genes is noteworthy in light of the nucleoside diphosphate kinase activity of NME1, whose activity would be expected to impact regulation of other genes

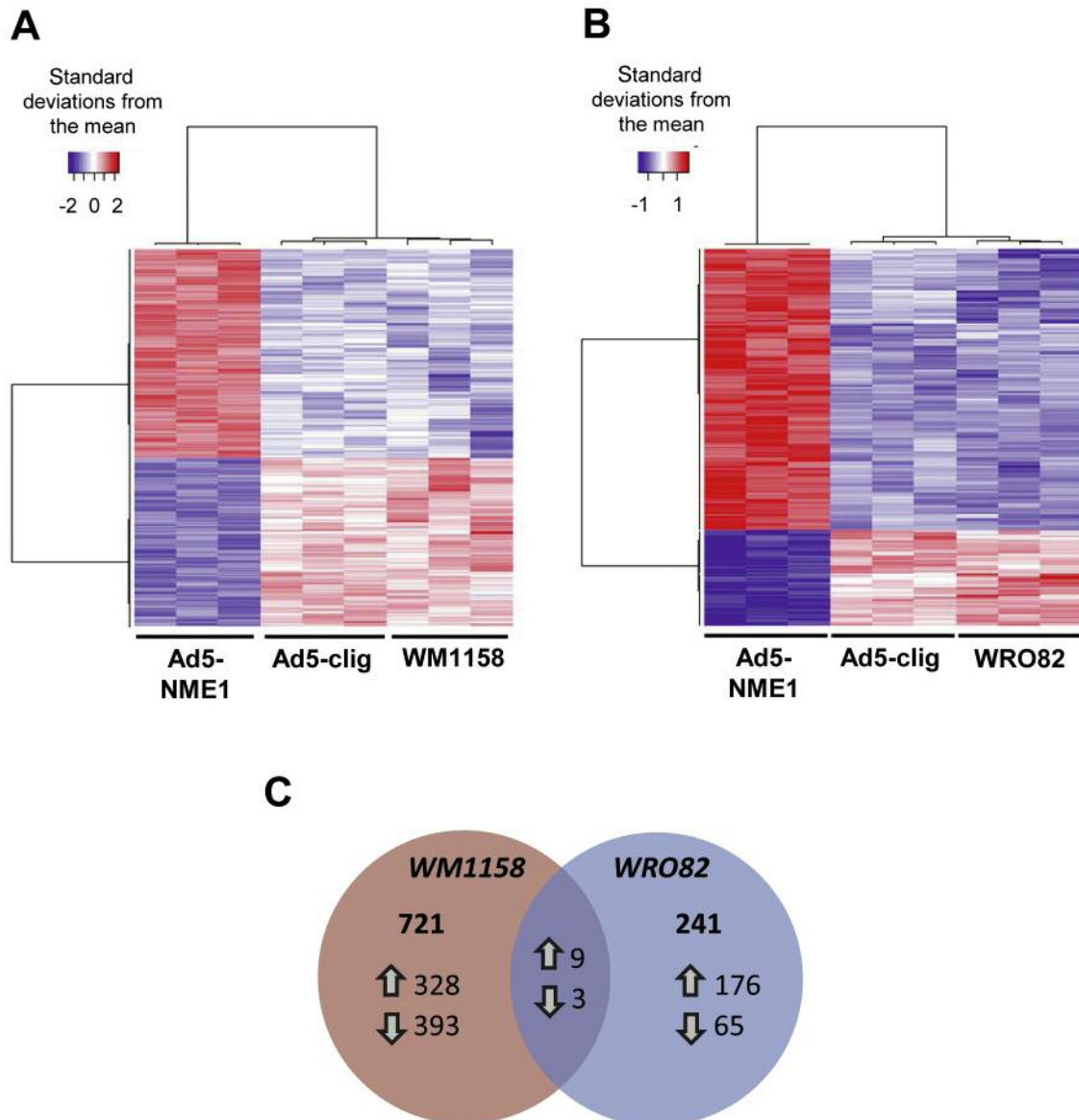


Figure 2. Supervised hierarchical clustering of genes differentially expressed in WM1158 and WRO82 cells in response to forced NME1 expression. Expression differences were identified by comparing cells with forced overexpression of NME1 using the Ad5-NME1 adenovirus to two control treatments (Ad5-clig control virus and untransduced) using *t*-test within the cell lines. A: Gene expression in WM1158 cells. B: Gene expression in WRO82 cells. False discovery rates were used for multiple test correction, with the most significant probesets (FDR  $p$ -value $<0.1$ ) displayed. The heatmaps depict relative expression levels for each significant probe set (rows) among each of triplicate samples (columns), based on the number of standard deviations from the mean. Lower relative expression levels are indicated in blue and higher levels in red. For WM1158, 787 probe sets corresponding to 733 genes were identified, while 267 probe sets (253 genes) were found in WRO82 cells. C: Venn diagram summary of NME1-regulated genes (FDR $\leq 0.1$ ) in WM1158 and WRO82 cells. The total number of NME1-regulated genes is given for each cell line, the number of up- and down-regulated genes (represented by up and down arrows) within them, and the intersection of twelve genes up-regulated in both.

in the category. Furthermore, a significant NME1-induced enrichment for members of the CDC42 signaling cascade (*ARPC5L*, *CDC42*, *CDC42EP2*, *FNBP1L*, *HLA-DOA*, *HLA-F*, *HLA-G*, *ITGB1*, *JUN*, *MYL7*, *MYL10*, *MYL12A* and *RASA1*) was identified (Fisher's exact test  $p < 0.014$ ). Using a more stringent FDR ( $\leq 0.05$ ), 13 down-regulated and 19 up-regulated

genes were identified (Table II). Of particular note in this list were strong inductions of such metastasis-relevant genes as matrix metalloproteinase 1 (*MMP1*), angiotensin 2 (*ANGPT2*), interleukins 8 and 11 (*IL8* and *IL11*), and induction of two genes associated with cellular apoptosis, *GADD45A* and programmed cell death 11 (*PDCD11*).

Table I. Enrichment of gene groups regulated by forced NME1 expression in metastatic WM1158 melanoma cells.

Gene ontology: disease and biological functions	Gene count in study	Enrichment <sup>1</sup>
Cell signaling	49	
Hydrolysis of GTP <sup>2</sup>	14	9.15×10 <sup>-6</sup>
Binding of GTP <sup>3</sup>	11	4.28×10 <sup>-4</sup>
Synthesis of nitric oxide <sup>4</sup>	27	8.47×10 <sup>-3</sup>
Rac protein signal transduction	4	1.45×10 <sup>-2</sup>
DNA replication, recombination and repair	49	
Hydrolysis of nucleotide <sup>3</sup>	18	7.26×10 <sup>-5</sup>
Cleavage of GDP <sup>3</sup>	3	1.78×10 <sup>-3</sup>
Cleavage of core promoter	2	3.36×10 <sup>-3</sup>
Catabolism of ATP <sup>3</sup>	11	1.06×10 <sup>-2</sup>
Hydrolysis of GDP <sup>5</sup>	2	1.86×10 <sup>-2</sup>
Dephosphorylation of DNA fragment	2	1.86×10 <sup>-2</sup>
Fragmentation of DNA <sup>6</sup>	18	1.95×10 <sup>-2</sup>
Nucleic acid metabolism	37	
Phosphorylation of nucleoside diphosphates <sup>4</sup>	2	3.36×10 <sup>-3</sup>
Catabolism of purine nucleotide <sup>4</sup>	12	1.01×10 <sup>-2</sup>
Small molecule biochemistry	104	
Accumulation of ganglioside GM2	4	6.49×10 <sup>-4</sup>
Accumulation of sphingomyelin	3	7.42×10 <sup>-4</sup>
Accumulation of asialo ganglioside GM2	3	1.78×10 <sup>-3</sup>
Accumulation of lysobisphosphatidic acid	3	1.78×10 <sup>-3</sup>
Clearance of cholesterol	3	1.78×10 <sup>-3</sup>
Degradation of platelet activating factor	2	3.36×10 <sup>-3</sup>
Dephosphorylation of L-amino acid	2	3.36×10 <sup>-3</sup>
Quantity of asialo ganglioside GM2	2	3.36×10 <sup>-3</sup>
Accumulation of ganglioside GM3	3	3.40×10 <sup>-3</sup>
Accumulation of glucosylceramide	3	3.40×10 <sup>-3</sup>
Accumulation of lactosylceramide	3	3.40×10 <sup>-3</sup>
Accumulation of cholesterol ester	5	4.82×10 <sup>-3</sup>
Quantity of 12-hydroxyeicosatetraenoic acid	4	7.02×10 <sup>-3</sup>
Metabolism of terpenoid	28	7.23×10 <sup>-3</sup>
Transport of sterol	7	7.44×10 <sup>-3</sup>
Secretion of steroid	13	7.54×10 <sup>-3</sup>
Homeostasis of sphingolipid	2	9.68×10 <sup>-3</sup>
Binding of heparin sulfate proteoglycan	2	9.68×10 <sup>-3</sup>
Degradation of proteoglycan	4	1.16×10 <sup>-2</sup>
Flux of sterol	11	1.36×10 <sup>-2</sup>
Transport of lactic acid	3	1.71×10 <sup>-2</sup>
Secretion of lipid	18	1.76×10 <sup>-2</sup>
Length of glycosaminoglycan	2	1.86×10 <sup>-2</sup>
Redistribution of glycosaminoglycan	2	1.86×10 <sup>-2</sup>
Accumulation of sterol	8	2.17×10 <sup>-2</sup>
Flux of lipid	12	2.23×10 <sup>-2</sup>
Efflux of sterol	10	2.86×10 <sup>-2</sup>
Accumulation of cholesterol	7	2.94×10 <sup>-2</sup>
Cell death and survival	276	
Apoptosis	216	9.92×10 <sup>-6</sup>
Necrosis	204	8.33×10 <sup>-5</sup>
Cell death	253	1.44×10 <sup>-4</sup>
Cell viability	105	1.36×10 <sup>-3</sup>
Cell survival	111	2.60×10 <sup>-3</sup>
Disintegration of secondary lens fiber cells	2	3.36×10 <sup>-3</sup>
Fragmentation of nucleus	6	1.24×10 <sup>-2</sup>
Loss of Purkinje cells	3	1.71×10 <sup>-2</sup>
Cytolysis of vascular endothelial cells	2	1.86×10 <sup>-2</sup>
Loss of germ cells	3	2.26×10 <sup>-2</sup>

<sup>1</sup>p-Value of enrichment based on Fisher's Exact Test using Ingenuity Pathway Analysis software; <sup>2</sup>Additional subcategory of "DNA Replication, Recombination and Repair" and "Nucleic Acid Metabolism"; <sup>3</sup>Additional subcategory of "Nucleic Acid Metabolism" and "Small Molecule Biochemistry"; <sup>4</sup>Additional subcategory of "Small Molecule Biochemistry"; <sup>5</sup>Additional subcategory of "Nucleic Acid Metabolism"; <sup>6</sup>Additional subcategory of "Cell Death and Survival".

Table II. *Genes regulated by forced NME1 expression in the metastatic melanoma cell line, WM1158 (FDR < 0.05).*

Gene symbol	Gene name	Gene ontology <sup>1</sup>	Fold-change <sup>2</sup>	p-Value
<i>CNPY2</i>	Canopy 2 homolog	Negative regulation of gene expression	-2.3	3.37×10 <sup>-5</sup>
<i>OSGEPL1</i>	O-sialoglycoprotein endopeptidase-like 1	tRNA processing	-1.8	1.62×10 <sup>-4</sup>
<i>PQBP1</i>	Polyglutamine binding protein 1	Regulation of transcription, RNA splicing	-1.7	2.13×10 <sup>-4</sup>
<i>TMEM189-UBE2V1</i>	read-through transcript	Regulation of transcription, DNA repair	-1.6	6.19×10 <sup>-5</sup>
<i>FKBP3</i>	FK506 binding protein	Protein folding	-1.6	1.27×10 <sup>-4</sup>
<i>POLR2G</i>	RNA polymerase II subunit G	Transcription, DNA-templated	-1.6	2.28×10 <sup>-4</sup>
<i>BRMS1</i>	Breast cancer metastasis suppressor 1	Negative regulation of transcription, DNA-templated	-1.6	2.26×10 <sup>-4</sup>
<i>PPP1CA</i>	Protein phosphatase 1, catalytic subunit, alpha isozyme	Glycogen metabolic process, TGF-beta signaling	-1.5	8.69×10 <sup>-5</sup>
<i>ATP5D</i>	ATP synthase, H <sup>+</sup> transporting, mitochondrial F1 complex, delta subunit	Oxidative phosphorylation	-1.5	1.40×10 <sup>-4</sup>
<i>PUM1</i>	Pumilio RNA-binding family member 1	Oxidative phosphorylation, post-Golgi vesicle transport	-1.4	7.15×10 <sup>-5</sup>
<i>NIT2</i>	Nitrilase family, member 2	Nitrogen compound metabolic process	-1.4	3.18×10 <sup>-4</sup>
<i>IRF1</i>	Interferon regulatory factor 1	Transcription from RNA polymerase II promoter, regulation of adaptive immune response	-1.3	3.72×10 <sup>-5</sup>
<i>ELL3/SERINC4</i>	ELL3/SERINC4 read-through transcript	DNA-templated transcription, elongation	-1.3	1.08×10 <sup>-4</sup>
<i>EFCAB2</i>	EF-hand calcium binding domain 2	- - -	1.2	2.13×10 <sup>-4</sup>
<i>HMX1</i>	H6 family homeobox 1	Transcription, DNA-templated	1.2	1.72×10 <sup>-4</sup>
<i>PPM1E</i>	Protein phosphatase, Mg <sup>2+</sup> /Mn <sup>2+</sup> dependent, 1E	Positive regulation of stress fiber assembly	1.3	1.29×10 <sup>-4</sup>
<i>MSR1</i>	Macrophage scavenger receptor 1	Receptor-mediated endocytosis	1.3	2.19×10 <sup>-4</sup>
<i>NOVA2</i>	Neuro-oncological ventral antigen 2	- - -	1.3	2.82×10 <sup>-4</sup>
<i>PDCD11</i>	Programmed cell death 11	rRNA, mRNA processing	1.4	9.00×10 <sup>-5</sup>
<i>WAC</i>	WW domain containing adaptor with coiled-coil	Transcription, DNA-templated	1.5	3.73×10 <sup>-5</sup>
<i>HSP90AA1</i>	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	Response to stress, unfolded protein	1.7	8.92×10 <sup>-5</sup>
<i>VAMP4</i>	Vesicle-associated membrane protein 4	Exocytosis, endocytosis	1.7	2.66×10 <sup>-5</sup>
<i>STC1</i>	Stanniocalcin 1	Cellular calcium ion homeostasis	2.0	3.30×10 <sup>-5</sup>
<i>GADD45A</i>	Growth arrest and DNA-damage-inducible, alpha	Positive regulation of apoptotic process	2.0	1.23×10 <sup>-4</sup>
<i>PP1F</i>	Peptidylprolyl isomerase F	Protein folding	2.1	1.32×10 <sup>-4</sup>
<i>AKAP12</i>	A kinase (PRKA) anchor protein 12	Positive regulation of protein kinase A signaling	2.4	2.23×10 <sup>-4</sup>
<i>PTH1H</i>	Parathyroid hormone-like hormone	Skeletal system development	3.6	2.62×10 <sup>-4</sup>
<i>IL11</i>	Interleukin 11	B cell, megakaryocyte differentiation	4.4	2.11×10 <sup>-4</sup>
<i>GEM</i>	GTP binding protein overexpressed in skeletal muscle	Small GTPase-mediated signal transduction	4.4	2.16×10 <sup>-5</sup>
<i>IL8</i>	Interleukin 8	Immune/inflammatory response, angiogenesis	5.5	3.13×10 <sup>-5</sup>
<i>ANGPT2</i>	Angiopoietin 2	Angiogenesis	6.1	9.62×10 <sup>-5</sup>
<i>MMP1</i>	Matrix metalloproteinase 1	Extracellular matrix organization	6.6	9.98×10 <sup>-5</sup>

<sup>1</sup>Biological process assignments based on AmiGO version 1.8; GO database release 2014-02-22; <sup>2</sup>Fold-change is the ratio of expression obtained with Ad5-NME1 adenovirus divided by expression obtained with control Ad5-clig adenovirus.

A substantial number of genes were also regulated by NME1 in WRO82 cells (FDR < 0.1), with 185 up-regulated and 68 down-regulated transcripts identified (Figure 2). NME1 expression also exerted an impact on multiple functional gene categories in WRO82 cells (Table III), with the strongest effects seen on “cell-mediated immune response” (5 genes), “cellular development” (32 genes), “cellular function and maintenance” (23 genes), hematological system development and function” (43 genes), and “hematopoiesis” (26 genes). At the more stringent level

(FDR < 0.05), a total of 6 down-regulated and 14 up-regulated genes were identified (Table IV). Effects of potential relevance to metastasis were the down-regulation of *BCL2L1* and *PALB2*, and the up-regulation of *CYP3A43* (3.0-fold), *SERPINB9* (2.9-fold), *KIF5A*, *PPM1E*, and *CSF2RB*. *KIF5A* and *PPM1E* were of particular interest for their roles in microtubule-based movement (27) and regulation of the nonreceptor tyrosine kinase PAK1 (28), respectively.

Having identified numerous NME1-regulated genes in two cell lines representing diverse forms of metastatic

Table III. *Enrichment of gene groups regulated by forced NME1 expression in metastatic WRO82 thyroid carcinoma cells.*

Gene Ontology: disease and biological functions	Gene count in study	Enrichment <sup>1</sup>
Cell-mediated immune response	5	
Differentiation of memory T lymphocytes <sup>2</sup>	5	1.65×10 <sup>-4</sup>
Granularity of natural killer cells	1	1.91×10 <sup>-2</sup>
Cellular development	32	
Differentiation of lymphatic system cells	12	3.08×10 <sup>-4</sup>
Clonal expansion of cytotoxic T cells <sup>3</sup>	2	1.07×10 <sup>-3</sup>
Differentiation of tumor cell lines	17	1.97×10 <sup>-3</sup>
Proliferation of bone marrow cells	8	4.09×10 <sup>-3</sup>
Tumorigenesis of plasmacytoma	2	5.16×10 <sup>-3</sup>
Proliferation of bronchial epithelial cells	2	7.13×10 <sup>-3</sup>
Immortalization of myeloid progenitor cells	2	7.13×10 <sup>-3</sup>
Differentiation of bone marrow cells <sup>4</sup>	9	7.57×10 <sup>-3</sup>
Proliferation of T lymphocytes <sup>3</sup>	5	8.90×10 <sup>-3</sup>
Branching of epithelial tissue	2	1.19×10 <sup>-2</sup>
Maturation of blood cells	11	1.21×10 <sup>-2</sup>
Differentiation of erythroid progenitor cells <sup>3</sup>	5	1.41×10 <sup>-2</sup>
Proliferation of macrophages <sup>3</sup>	4	1.81×10 <sup>-2</sup>
Proliferation of B lymphocytes <sup>3</sup>	11	1.89×10 <sup>-2</sup>
Maturation of embryonic stem cells	1	1.91×10 <sup>-2</sup>
Proliferation of mesonephric cells	1	1.91×10 <sup>-2</sup>
Immortalization of bone marrow cells	1	1.91×10 <sup>-2</sup>
Branching morphogenesis of ovarian cancer cell lines	1	1.91×10 <sup>-2</sup>
Ciliogenesis of kidney cancer cell lines <sup>5</sup>	1	1.91×10 <sup>-2</sup>
Expansion of endothelial cell lines	1	1.91×10 <sup>-2</sup>
Expansion of megakaryoblasts <sup>6</sup>	1	1.91×10 <sup>-2</sup>
Expansion of nervous tissue cell lines	1	1.91×10 <sup>-2</sup>
Expansion of promegakaryocytes	1	1.91×10 <sup>-2</sup>
Formation of Lewy neuritis <sup>5</sup>	1	1.91×10 <sup>-2</sup>
Formation of large-cell follicular lymphoma	1	1.91×10 <sup>-2</sup>
Arrest growth of stomach cancer cell lines	1	1.91×10 <sup>-2</sup>
Metastasis of sarcoma cell lines	1	1.91×10 <sup>-2</sup>
Morphogenesis of B lymphocytes <sup>6</sup>	1	1.91×10 <sup>-2</sup>
Arrest in sprouting of neurites <sup>5</sup>	1	1.91×10 <sup>-2</sup>
Cellular function and maintenance	23	
Function of lymphatic system cells	4	4.69×10 <sup>-3</sup>
Hyperpolarization of mitochondria	2	5.16×10 <sup>-3</sup>
Maintenance of T lymphocytes	3	1.03×10 <sup>-2</sup>
Release of granules	3	1.15×10 <sup>-2</sup>
Function of red blood cells	2	1.47×10 <sup>-2</sup>
Respiration of mitochondria	3	1.57×10 <sup>-2</sup>
Endoplasmic reticulum stress response	1	1.91×10 <sup>-2</sup>
Formation of pre-aggregosomes	1	1.91×10 <sup>-2</sup>
Formation of ribbon synapse	1	1.91×10 <sup>-2</sup>
Localization of G-actin	1	1.91×10 <sup>-2</sup>
Localization of melanoblasts	1	1.91×10 <sup>-2</sup>
Mineralization of bone cancer cell lines	1	1.91×10 <sup>-2</sup>
Polymerization of actin cytoskeleton	1	1.91×10 <sup>-2</sup>
Hematological System Development and Function	43	
Activation of T lymphocytes	4	3.18×10 <sup>-4</sup>
Cell viability of hematopoietic progenitor cells	6	2.82×10 <sup>-3</sup>
Abnormal morphology of T lymphocytes	7	3.50×10 <sup>-3</sup>
Differentiation of monocytes	6	7.38×10 <sup>-3</sup>
Maturation of hematopoietic progenitor cells <sup>4</sup>	5	8.90×10 <sup>-3</sup>
Maturation of T lymphocytes	5	1.07×10 <sup>-2</sup>
Inhibition of T lymphocytes	4	1.12×10 <sup>-2</sup>
Abnormal morphology of megakaryocytes	2	1.19×10 <sup>-2</sup>
Differentiation of antigen presenting cells <sup>4</sup>	8	1.46×10 <sup>-2</sup>
Quantity of blood cells	32	1.47×10 <sup>-2</sup>
Recruitment of mononuclear leukocytes	6	1.51×10 <sup>-2</sup>
Lymphocyte migration	13	1.53×10 <sup>-2</sup>

Table III. *Continued*



Table III. *Continued*

Gene Ontology: disease and biological functions	Gene count in study	Enrichment <sup>1</sup>
Priming of cytotoxic T cells	2	1.78×10 <sup>-2</sup>
Survival of pro-B lymphocytes	2	1.78×10 <sup>-2</sup>
Hematocrit	6	1.86×10 <sup>-2</sup>
Attachment of alveolar macrophages	1	1.91×10 <sup>-2</sup>
Mitogenesis of pro-B lymphocytes	1	1.91×10 <sup>-2</sup>
Recovery of plasma cells	1	1.91×10 <sup>-2</sup>
Hematopoiesis	26	
Abnormal morphology of hematopoietic progenitor cells	8	2.42×10 <sup>-3</sup>
Maturation of natural killer cells	4	3.04×10 <sup>-3</sup>
Quantity of red blood cells	11	3.17×10 <sup>-3</sup>
Maturation of dendritic cells	7	6.70×10 <sup>-3</sup>
Chemotaxis of mesenchymal stem cells	1	1.91×10 <sup>-2</sup>
Generation of erythroid cells	1	1.91×10 <sup>-2</sup>

<sup>1</sup>*p*-Value of enrichment based on Fisher's Exact Test using Ingenuity Pathway Analysis software; <sup>2</sup>Additional subcategory of "Cellular Function and Maintenance", "Hematological System Development and Function", and "Hematopoiesis"; <sup>3</sup>Additional subcategory of "Hematological System Development and Function"; <sup>4</sup>Additional subcategory of "Hematopoiesis"; <sup>5</sup>Additional subcategory of "Cellular Function and Maintenance"; <sup>6</sup>Additional subcategory of "Hematological System Development and Function" and "Hematopoiesis".

Table IV. *Genes regulated by forced NME1 expression in the metastatic thyroid carcinoma cell line, WRO82 (FDR < 0.05).*

Gene symbol	Gene name	Gene ontology <sup>1</sup>	Fold-change <sup>2</sup>	<i>p</i> -Value
<i>BCL2L1</i>	BCL2-like 1	Negative regulation of apoptotic process	-3.2	5.51×10 <sup>-5</sup>
<i>PALB2</i>	Partner and localizer of BRCA2	Double-strand break repair <i>via</i> homologous recombination	-2.8	4.34×10 <sup>-5</sup>
<i>TEAD4</i>	TEA domain family member 4	Cell fate specification, DNA-templated transcription	-2.0	8.27×10 <sup>-5</sup>
<i>PSMB5</i>	Proteasome (prosome, macropain) subunit, beta type, 5	Mitotic cell cycle, processing /presentation of peptide antigen via MHC class I	-2.0	4.71×10 <sup>-5</sup>
<i>BLMH</i>	Bleomycin hydrolase	Response to drug	-1.7	6.52×10 <sup>-5</sup>
<i>C20orf4</i>	AAR2 splicing factor homolog	- - -	-1.4	6.85×10 <sup>-5</sup>
<i>MGC4859</i>	LOC79150		1.2	1.13×10 <sup>-5</sup>
<i>DIRAS2</i>	DIRAS family, GTP-binding RAS-like 2	Small GTPase-mediated signal transduction	1.3	1.71×10 <sup>-5</sup>
<i>PNLIP</i>	Pancreatic lipase	Lipid digestion	1.5	8.37×10 <sup>-5</sup>
<i>SPARCL1</i>	SPARC-like 1 (hevin)	Signal transduction	1.6	1.70×10 <sup>-5</sup>
<i>SLC4A4</i>	Solute carrier family 4	Ion transport	1.7	6.21×10 <sup>-5</sup>
<i>GOLGA6A</i>	Golgin A6 family, member A	- - -	1.9	7.78×10 <sup>-5</sup>
<i>NFKB2</i>	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	Transcription, DNA-templated; toll-like receptor signaling pathway	2.0	1.52×10 <sup>-5</sup>
<i>MS4A1</i>	Membrane-spanning 4-domains, subfamily A, member 1	Humoral immune response	2.0	9.59×10 <sup>-5</sup>
<i>CSF2RB</i>	Colony stimulating factor 2 receptor, beta	Signal transduction	2.1	1.78×10 <sup>-5</sup>
<i>SLCO1A2</i>	Solute carrier organic anion transporter family, member 1A2	Organic anion transport	2.1	2.08×10 <sup>-5</sup>
<i>PPM1E</i>	Protein phosphatase, Mg <sup>2+</sup> /Mn <sup>2+</sup> dependent, 1E	Negative regulation of protein kinase; stress fiber induction	2.1	4.39×10 <sup>-5</sup>
<i>KIF5A</i>	Kinesin family member 5A	Microtubule-based movement	2.3	9.00×10 <sup>-5</sup>
<i>SERPINB9</i>	Serpin peptidase inhibitor, clade B	Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	2.9	2.26×10 <sup>-5</sup>
<i>CYP3A43</i>	Cytochrome P450, family 3, subfamily A, polypeptide 43	Xenobiotic metabolic process	3.0	5.82×10 <sup>-5</sup>

<sup>1</sup>Biological process assignments based on AmiGO version 1.8; GO database release 2014-02-22; <sup>2</sup>Fold-change is the ratio of expression obtained with Ad5-NME1 adenovirus divided by expression obtained with control Ad5-clig adenovirus.

Table V. Genes regulated similarly by forced NME1 expression in both WM1158 and WRO82 cells (FDR<0.1).

Gene symbol	Gene name	Gene ontology <sup>1</sup>	WM1158		WRO82	
			Fold-change <sup>2</sup>	p-Value	Fold-change	p-Value
<i>CCRI</i>	Chemokine (C-C motif) receptor 1	Chemotaxis	1.7	4.34×10 <sup>-5</sup>	2.0	7.05×10 <sup>-4</sup>
<i>WNT6</i>	Wingless-type MMTV integration site family, member 6	Epithelial-mesenchymal cell signaling, Wnt signaling pathway	1.5	1.13×10 <sup>-5</sup>	2.7	6.54×10 <sup>-4</sup>
<i>TBX4</i>	T-box 4	Epithelial morphogenesis, transcription	1.5	6.52×10 <sup>-5</sup>	1.9	1.65×10 <sup>-3</sup>
<i>PPM1E</i>	Protein phosphatase, Mg <sup>2+</sup> /Mn <sup>2+</sup> dependent, 1E	Negative regulation of protein kinase; stress fiber induction	1.3	5.51×10 <sup>-5</sup>	2.1	4.39×10 <sup>-5</sup>
<i>GPR52</i>	G protein-coupled receptor 52	G protein-coupled receptor activity	1.2	6.85×10 <sup>-5</sup>	2.0	1.02×10 <sup>-3</sup>
<i>KCNA3</i>	Potassium voltage-gated channel, shaker-related subfamily, member 3	Potassium ion transport; synaptic transmission	1.2	4.43×10 <sup>-3</sup>	1.4	1.67×10 <sup>-3</sup>
<i>FMO5</i>	Flavin containing monooxygenase 5	NADP binding, flavin adenine dinucleotide binding; N,N-dimethylaniline monooxygenase activity	1.5	1.10×10 <sup>-3</sup>	1.6	3.91×10 <sup>-4</sup>
<i>GTDC1</i>	Glycosyltransferase-like domain containing 1	Glycosyltransferase activity	1.2	3.76×10 <sup>-3</sup>	2.3	1.69×10 <sup>-3</sup>
<i>SERPINB3</i>	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	Regulation of proteolysis, serine-type endopeptidase inhibitor activity; response to virus	1.2	4.40×10 <sup>-3</sup>	1.8	1.52×10 <sup>-3</sup>
<i>ZYX</i>	Zyxin	Focal adhesion, adherens junction; stress fiber	-1.4	8.37×10 <sup>-5</sup>	-3.2	5.81×10 <sup>-4</sup>
<i>ZFP36L2</i>	ZFP36 ring finger protein-like 2	Stem cell differentiation; transcription factor activity	-1.4	1.71×10 <sup>-5</sup>	-2.9	1.23×10 <sup>-3</sup>
<i>PFN1</i>	Profilin 1	Actin skeleton organization; Rho GTPase binding	-1.3	1.70×10 <sup>-5</sup>	-1.8	1.00×10 <sup>-3</sup>

<sup>1</sup>Biological process assignments based on AmiGO version 1.8; GO database release 2014-02-22; <sup>2</sup>Fold-change is the ratio of expression obtained with NME1-expressing adenovirus Ad5-NME1 divided by expression obtained with control Ad5-clig adenovirus.

Table VI. Summary of genes regulated by NME1 in both WM1158 and WRO82 cells that predict survival similarly in melanoma and at least one subtype of breast cancer. Blue checks represent genes associated with longer patient survival, and those in red are negatively associated with survival.

Gene	Progression/Distant Disease Free Survival					Overall Survival				
	Melanoma	Breast Cancer				Melanoma	Breast Cancer			
		LumA	LumB	HER2	Basal		LumA	LumB	HER2	Basal
<i>CSF2RB</i>	✓	✓			✓	✓	✓	✓	✓	
<i>MS4A1</i>	✓		✓		✓		✓		✓	
<i>PPIF</i>	✓		✓		✓		✓		✓	
<i>SERPINB9</i>	✓			✓	✓				✓	
<i>ZFP36L2</i>	✓	✓	✓			✓	✓		✓	
<i>BRMS1</i>		✓			✓	✓			✓	
<i>TEAD4</i>					✓		✓		✓	

cancer, an effort was also made to identify genes that were similarly regulated across both lines. Using an FDR<0.1, a total of nine such genes were identified, six of which were up-regulated and three down-regulated (Table V). Interestingly, three (*PPM1E*, *ZYX*, *PFN1*) of those genes are participants in the highly metastasis-relevant processes of stress fiber formation and focal adhesion. On the whole, each of the eight genes possessed metastasis-relevant activities, such as chemotaxis (*CCRI*), epithelial-

mesenchymal signaling (*WNT6*), differentiation and morphogenesis (*TBX4*, *ZFP36L2*), and G protein modulation (*GPR52* and *PFN1*). Taken together, the genes coregulated by NME1 across metastatic cell lines from these distinct cancers appear to be enriched for functions of probable relevance to metastatic potential.

*NME1-regulated genes are strong predictors of survival in melanoma. As a metastasis suppressor in cell culture and in*

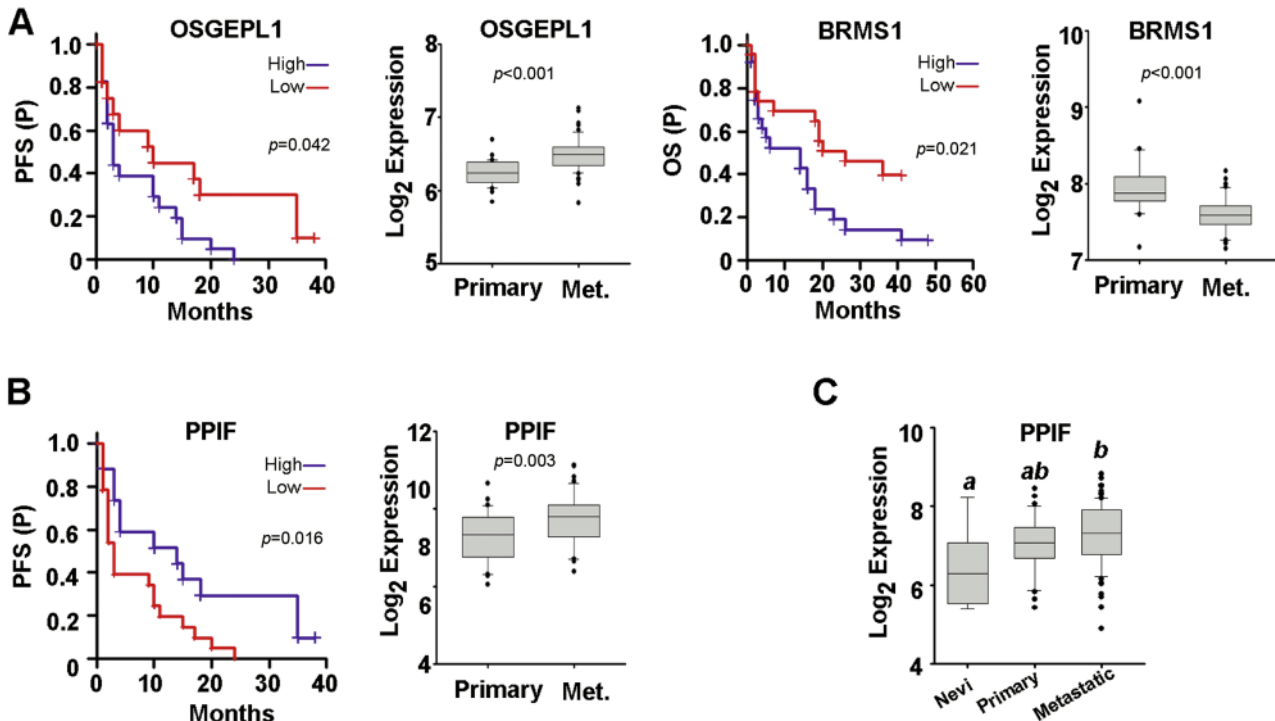


Figure 3. Genes regulated by NME1 in WM1158 cells are predictive of survival and RNA expression in metastatic melanoma patients. A: Shown are Kaplan-Meier plots for NME1-regulated genes that displayed significant effects on both patient survival (left panels: progression-free, PFS; overall, OS) and differences in RNA expression in primary versus metastatic melanoma biopsies (right panels). Both analyses employed the GEO dataset GSE8401 (22). B: Kaplan-Meier survival plots for the PPIF gene in metastatic melanoma samples (left) and RNA expression in primary metastatic versus primary melanoma biopsies from GSE8401 (right). C: Expression of the PPIF RNA in the human melanoma progression GEO dataset GSE46517. Groups not sharing a common superscript are statistically significantly different ( $p \leq 0.05$  by ANOVA, Holm-Sidak post-hoc testing).

*in vivo* settings, NME1 may regulate the expression of genes that determine metastatic potential and overall survival in human cancer patients. To determine whether the NME1-regulated genes identified in the microarray analyses were of predictive value, we conducted correlational analysis between expression of these genes and indices of patient metastasis and survival in existing RNA expression databases from melanoma patients. Expression of NME1 RNA in human melanoma samples was not well-correlated with many of the NME1-regulated genes identified in the WM1158 and WRO82 cell lines. This was expected, as NME1 down-regulation is exerted at the level of protein expression *via* lysosomal degradation (25) rather than RNA expression in the majority of cancer cell lines we have analyzed to date. Nevertheless, it was considered of potential value to analyze the predictive power of the NME1-regulated genes, with the premise that many are likely under control of NME1 protein expression in melanoma tumors. Analyses could not be conducted in thyroid carcinoma patients due to a lack of robust, publicly available RNA expression datasets.

Of the top twenty genes regulated by NME1 in the melanoma cell line WM1158, three were found to have a significant predictive value in either progression-free survival (PFS) or overall survival (OS) of metastatic melanoma patients and also display significantly different RNA expression in primary *versus* metastatic melanoma samples in the GEO dataset, GSE8401 (Figure 3). Consistent with being repressed by the metastasis suppressor NME1 (Table II), expression of *OSGEPL1* and *BRMS1* were both associated with shorter PFS and OS, respectively (Figure 3A). Expression of the NME1-induced gene, *PPIF*, was correlated with longer progression-free survival of metastatic melanoma patients (Figure 3B, upper left). Interestingly, *PPIF* expression was significantly higher in metastatic samples *versus* primary melanomas or benign nevi in both the GSE8401 dataset (Figure 3B) and another (GSE46517) (Figure 3C), suggesting complexity in its regulation by NME1 as well as potential survival value in metastatic melanoma.

Interestingly, a number of genes regulated by NME1 in the metastatic thyroid carcinoma cell line WRO82 also exhibited significant predictive value for survival in metastatic

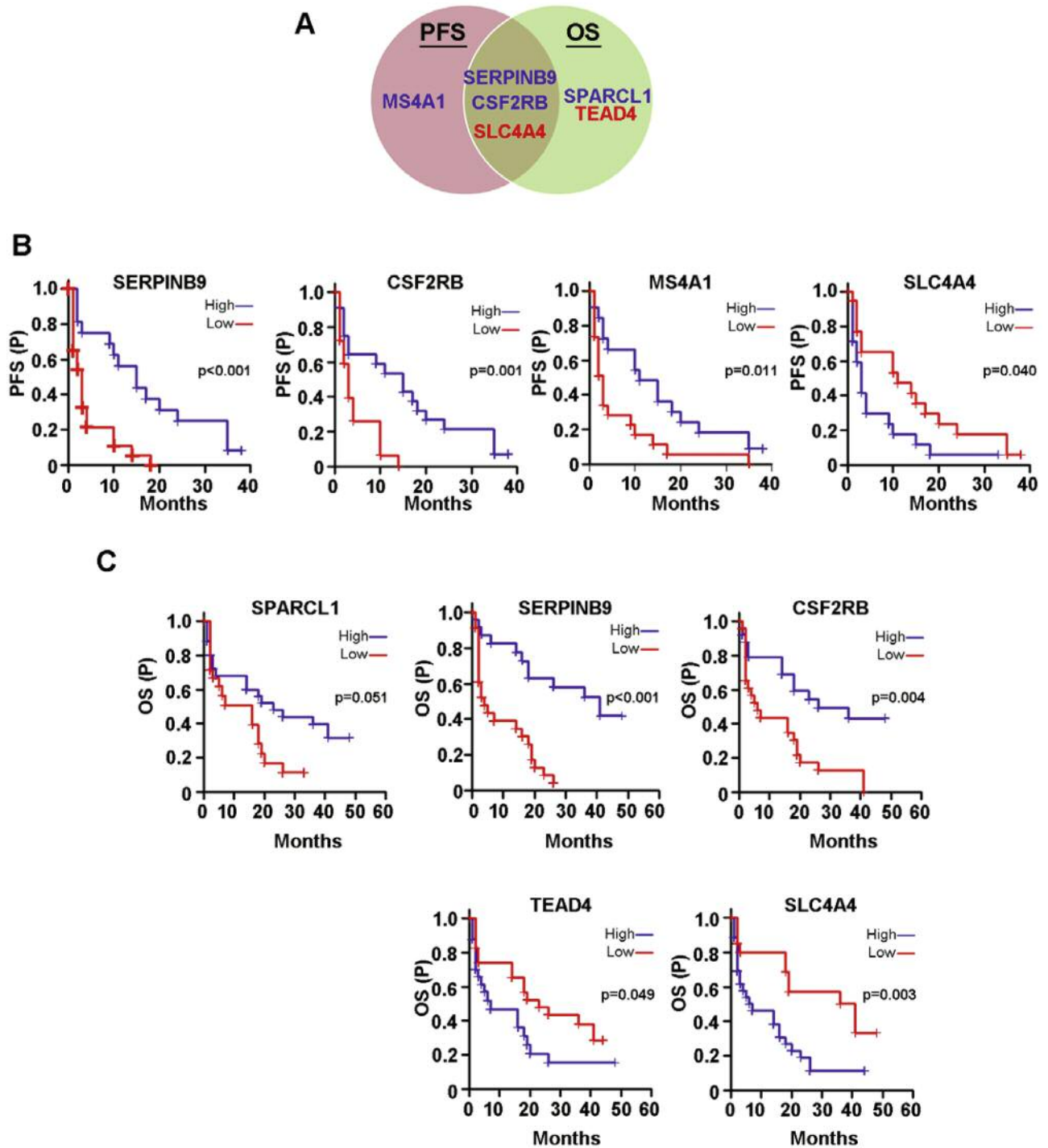


Figure 4. *Continued.*

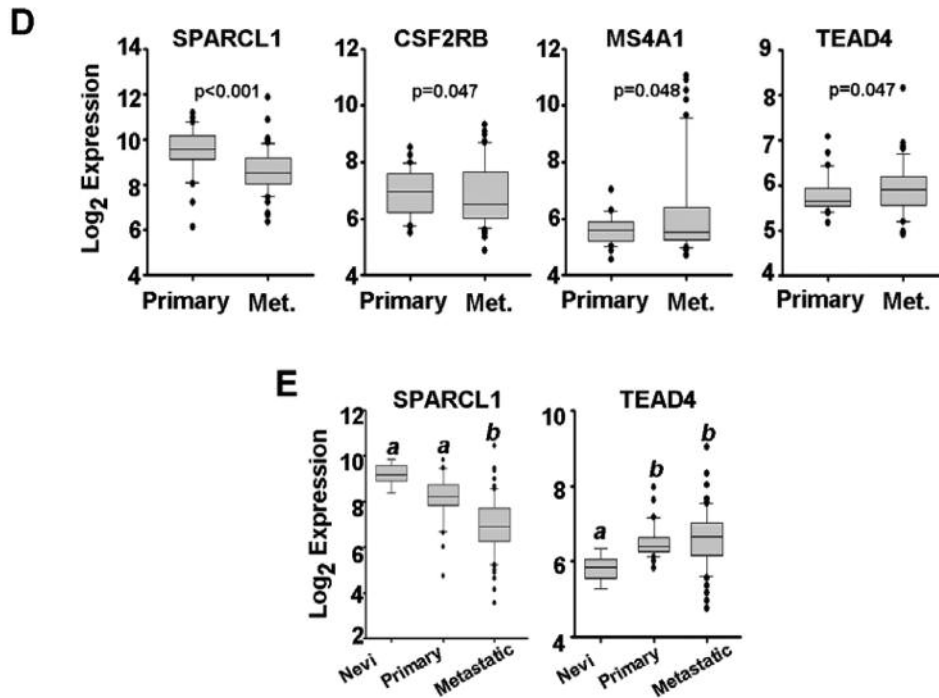


Figure 4. Summary of Kaplan-Meier survival plots in metastatic melanoma for genes regulated by NME1 in WR082 cells. A: Venn diagram summarizing NME1-regulated genes predictive of progression-free survival (PFS) and/or overall survival (OS) in melanoma patients. B: NME1-regulated genes that were predictive of PFS and C: OS in metastatic melanoma patients from the GSE8401 GEO dataset. D: Genes with significantly different RNA expression in melanoma dataset GSE8401. E: Genes with significantly different RNA expression in melanoma dataset GSE46517. Groups not sharing a common superscript are statistically significantly different ( $p \leq 0.05$  by ANOVA, Holm-Sidak post-hoc testing).

melanomas (Figure 4A-C). Three genes, *SERPINB9*, *CSF2RB* and *SLC4A4*, were positively correlated with both PFS (Figure 4B) and OS (Figure 4C). In order to identify NME1-regulated genes with the most potential for clinical application, we filtered for genes that displayed both 1) significant predictive value for melanoma survival and 2) differential expression in metastatic melanoma relative to primary lesions or benign nevi in two independent melanoma datasets. Two genes, *SPARCL1* and *TEAD4*, met those selection criteria. Consistent with metastasis suppressor function and its induction by NME1, *SPARCL1* was a strong predictor of increased OS and its expression was significantly decreased in more advanced melanoma samples (Figures 4D and 4E). Conversely, *TEAD4*, which was repressed by NME1 (Table IV), was inversely associated with OS and also significantly increased in metastatic melanomas compared to primary melanomas in dataset GSE8401 (Figure 4D) and to benign nevi in dataset GSE46517 (Figure 4E).

The list of genes coregulated by NME1 across both the metastatic WM1158 and WRO82 cell were next tested for their potential association with survival in melanoma patients. Of the nine genes found to be regulated by NME1, two genes, *CCR1* and *ZFP36L2*, displayed significant

predictive value in patient survival times. Expression of each gene was associated with prolonged PFS (Figure 5A), while *CCR1* was also predictive of improved OS (Figure 5B). This commonality is intriguing as *CCR1* was induced by NME1 while *ZFP36L2* was repressed by NME1 (Table V). In addition, *CCR1* was significantly increased in more advanced lesions while *ZFP36L2* expression was decreased in advanced melanomas (Figure 5C). By combining all of the individual lists of NME1-regulated genes, we have identified seven genes with potential diagnostic value in terms of predicting melanoma patient survival as well as differential expression in more advanced samples.

*A number of genes regulated by NME1 in melanoma and thyroid carcinoma cell lines are predictors of survival in breast cancer.* In light of the well-documented metastasis suppressor function of NME1 in human breast cancer (45), we also examined the utility of NME1-regulated genes identified in WM1158 and WRO82 cells as prognostic indicators of breast cancer survival. Analyses were conducted using the breast cancer database and algorithm, BreastMark (24), which contains integrated gene expression and survival data from 26 datasets obtained with 12 distinct microarray

platforms on a total of 4,738 patient samples, distributed across the four major breast cancer subtypes of luminal A, luminal B, Her2-positive and basal (<http://glados.ucd.ie/BreastMark/>). A number of the NME1-regulated genes identified in WM1158 melanoma cells provided statistically significant predictive value in the luminal A subtype (Figure 6A). All expressions were inversely correlated with survival, including *MMP1* (distant disease-free survival, or DDFS,  $p < 0.048$ ), *FKBP1* (DDFS;  $p < 0.026$ ), *HSP90AA1* (DDFS, 0.013; overall survival or OS,  $p < 0.006$ ), and *BRMS1* (DDFS,  $p < 0.021$ ; OS,  $p < 0.018$ ). Moreover, the combination of *HSP90AA1*, *MMP1* and *FKBP1* expression exhibited a highly cooperative association with poor DDFS ( $p < 0.004$ , hazard ratio or HR of 1.9) and OS ( $p < 0.006$ , HR of 1.9). In the setting of the luminal B subtype of breast cancer, expression of six NME1-regulated genes identified in WM1158 cells was associated with patient survival (Figure 6B), with two showing a correlation with improved DDFS (*PPIF* and *PDCD11*) and four inversely correlated with OS (*PQBP1*, *CNPY2*, *ANGPT2* and *IL8*). Expression of the genes *AKAP12* (positive) as well as the previously cited genes *ANGPT2* and *MMP1* (both inverse) were associated with OS in the Her2 subtype (Figure 6C), while expression of *FKBP3* (inverse), *PPIF* and *POLR2G* (both positive) were correlated with DDFS in the basal subtype (Figure 6D).

A number of the genes identified as NME1-regulated in the metastatic thyroid carcinoma cell line WRO82 also had significant predictive value for survival across the four breast cancer subtypes. Expression of two genes, *PSMB5* and *PPM1E*, was inversely correlated with both DDFS and OS in the luminal A subtype (Figure 7A). In addition, expression of the colony-stimulating factor receptor isoform *CSFR2B* was positively correlated with DDFS ( $p < 0.04$ ) in luminal A patients. Moreover, the combination of *PPM1E*, *PSMB5* and *CSFR2B* exhibited significant synergy in predicting OS for this subtype ( $p < 0.002$ , HR=2.23). In luminal B patients (Figure 7B), expression of *MS4A1* exhibited a strong inverse correlation with both DDFS ( $p < 0.0009$ ) and OS ( $p < 0.0004$ ). As also seen in luminal A patients, expression of *PSMB5* ( $p < 0.026$ , positive correlation) and *CSFR2B* ( $p < 0.036$ , inverse correlation) had predictive value for OS in the luminal B subtype. Expression of another gene, *TEAD4*, was also positively correlated with OS ( $p < 0.013$ ). In Her2-positive patients (Figure 7C), *NFKB2* expression was correlated strongly with DDFS ( $p < 0.0009$ ) and approached significance for OS ( $p < 0.07$ ), and *CSFR2B* was again correlated with DDFS ( $p < 0.036$ ). In the basal subtype (Figure 7D), expression of *SERPINB9* was a strong predictor for both DDFS ( $p < 0.005$ ) and OS ( $p < 0.016$ ), as again were *CSFR2B* ( $p < 0.0003$  and 0.00007 for DDFS and OS, respectively) and *MSF4A1* ( $p < 0.0002$ , for DDFS only). In the strongest example of cooperativity observed in the overall survival analyses, expression levels of *CSFR2B*,

*MSF4A1* and *SERPINB9* together were highly predictive of DDFS in the basal subtype ( $p < 3.5 \times 10^{-5}$ , HR=0.33).

A total of seven genes identified in WM1158 and WRO82 cells as NME1-regulated were shown to have predictive value in melanoma and at least one subtype of breast cancer (Table VI). As might be expected, each gene had the same positive or negative effect on patient survival across the different cancers (*i.e.* genes associated with longer/shorter survival in melanoma were also associated with longer/shorter survival in one or more breast cancer subtypes). The most robust of these genes was *CSF2RB*, expression of which was associated with longer PFS in melanoma and the breast cancer subtypes LumA and basal, as well as longer OS in melanoma and LumB, Her2 and basal breast cancers. The other NME1-regulated genes exhibiting associations with survival to varying degrees across these cancers consisted of *MS4A1*, *PPIF*, *SERPINB9*, *ZFP36L2*, *BRMS1* and *TEAD4*. Taken together, these analyses of melanoma and breast cancer datasets demonstrate that NME1-regulated genes identified in the two metastatic cell lines of melanoma (WM1158) and thyroid carcinoma (WRO82) origin were enriched in predictive value for survival in melanoma and breast cancer patients, possibly due to their impact on the metastatic phenotype.

## Discussion

While considerable evidence suggests that the metastasis suppressor function of NME1 is mediated directly *via* inhibitory physical interactions with motility-driving proteins, other studies indicate it regulates expression of motility and metastasis-relevant genes as well (12, 29). This study was conducted to address a current gap in knowledge regarding the impact of NME1 on global profiles of gene expression in two cancers, melanoma and thyroid carcinoma. To this end, a novel adenovirus expression vector was constructed that successfully provided robust and efficient expression of NME1 in both melanoma (WM1158) and thyroid carcinoma (WRO82) cell lines. Forced NME1 expression resulted in broad impacts on gene expression profiles in both cell lines, with ontology analysis revealing enrichment of a number of gene groups (Tables I and III). One gene group of interest identified in the WM1158 melanoma cell line was DNA replication, recombination and repair (49 genes), which may well relate to our recent observations that NME1 contributes to the repair of UV-induced DNA damage in yeast and mammalian cells (4, 30). Other gene groups identified in the ontology analysis, such as cell death and survival, cellular development/ differentiation, and cell signaling strongly suggest NME1 regulates expression of genes capable of impacting metastatic potential in both cancers. Of interest was the significant regulation of *CDC42* and a number of its downstream effectors in WM1158 melanoma cells. While NME1 has been shown to induce motility suppression in

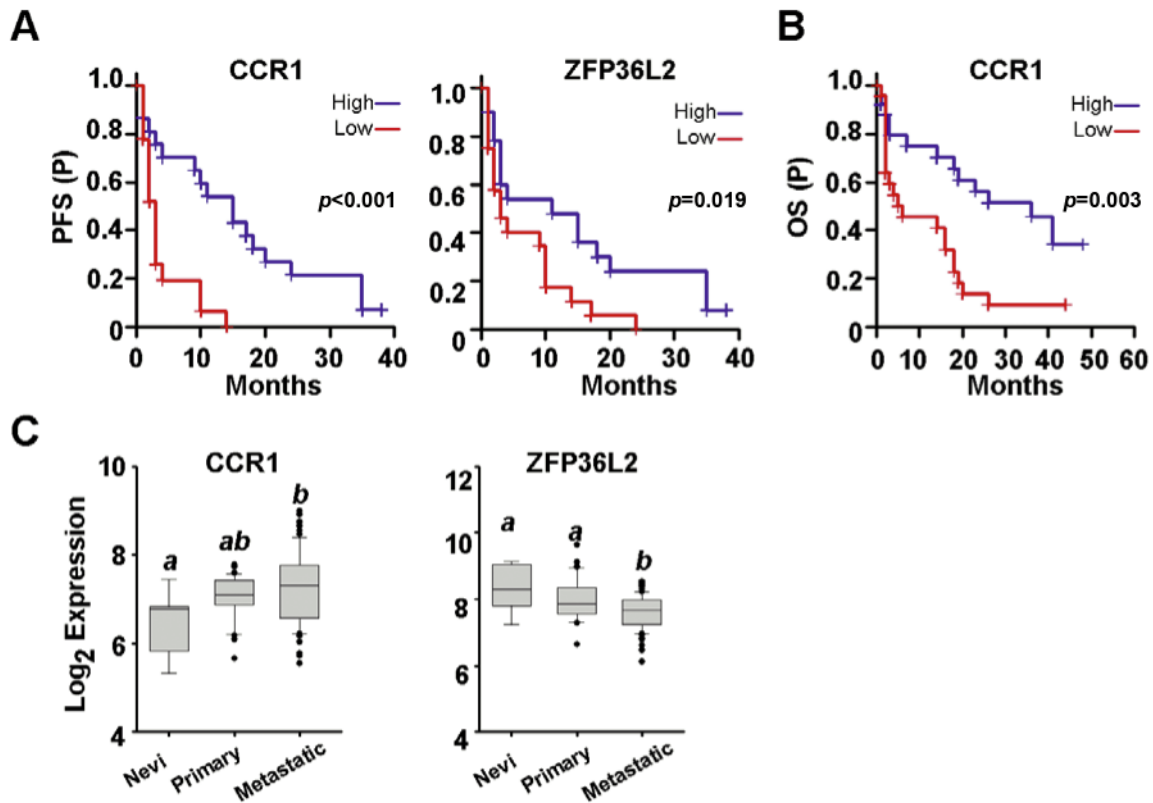


Figure 5. Genes regulated by NME1 in both WM1158 and WR082 cells are predictive of survival in metastatic melanoma. Shown are NME1-regulated genes that displayed significant effects on A: progression-free survival (PFS) or B: overall survival (OS) of metastatic melanoma patients from the GSE8401 dataset. C: RNA expression of CCR1 and ZFP36L2 in the human melanoma progression GEOdataset GSE46517. Groups not sharing a common superscript are statistically significantly different ( $p \leq 0.05$  by ANOVA, Holm-Sidak post-hoc testing).

MDA-MB-435 cells through a physical interaction with CDC42 to modulate downstream signaling (30), our data suggest an alternative pathway of regulating expression of CDC42 pathway genes in the context of melanoma.

Interestingly, the majority of NME1-down-regulated genes identified in WM1158 cells with known interactions with cancer (4/5) had the expected anti-motility/metastatic functions. For example, the NME1-down-regulated gene *CNPY2* potentiates FGF-induced cell motility in glioma and neurite outgrowth in neuroblastoma cells (31). In fact, we and others observe an association of neurite-like outgrowth with more aggressive phenotypes in melanoma and melanoma cell lines (32). Additional examples were *PPP1CA*, a carcinoma oncogene (33) and risk factor for recurrence of bladder carcinoma (34), and *ELL3/SERINC4* which has been documented to induce the cancer stem cell properties of breast carcinoma cells. While our observation of NME1-down-regulation of the well-characterized melanoma metastasis suppressor BRMS1 (35) is at first glance counter-intuitive, additional complexity is indicated by recent observations that

its subcellular localization may be as important as expression level (36). In contrast, a majority of NME1-up-regulated genes (5/6), such as *MMP1* (37), *ANGPT2* (38), *IL8* (39) and *IL8* (40), and *HSP90AA1* (11, 41) were associated with functions that could be metastasis-driving. One potential factor may have been the robust NME1 overexpression achieved in our studies with the Ad5clig-based adenoviral vector, which was much higher than achieved with plasmid-based stable transfection in our laboratory. While we have observed the expected low NME expression pattern in many metastatic cell lines of melanoma and other cancer origins, we have also observed marked NME overexpression in other melanoma cell lines and patient samples, suggesting that strong NME overexpression may have deleterious impacts on gene expression and cancer phenotypes as well. Alternatively, the roles played by these genes in metastasis are not fully understood, and theoretically could be suppressive in certain cellular and physiological conditions.

Three genes regulated by NME1 in WRO82 cells possess activities in cancer consistent with being effectors of

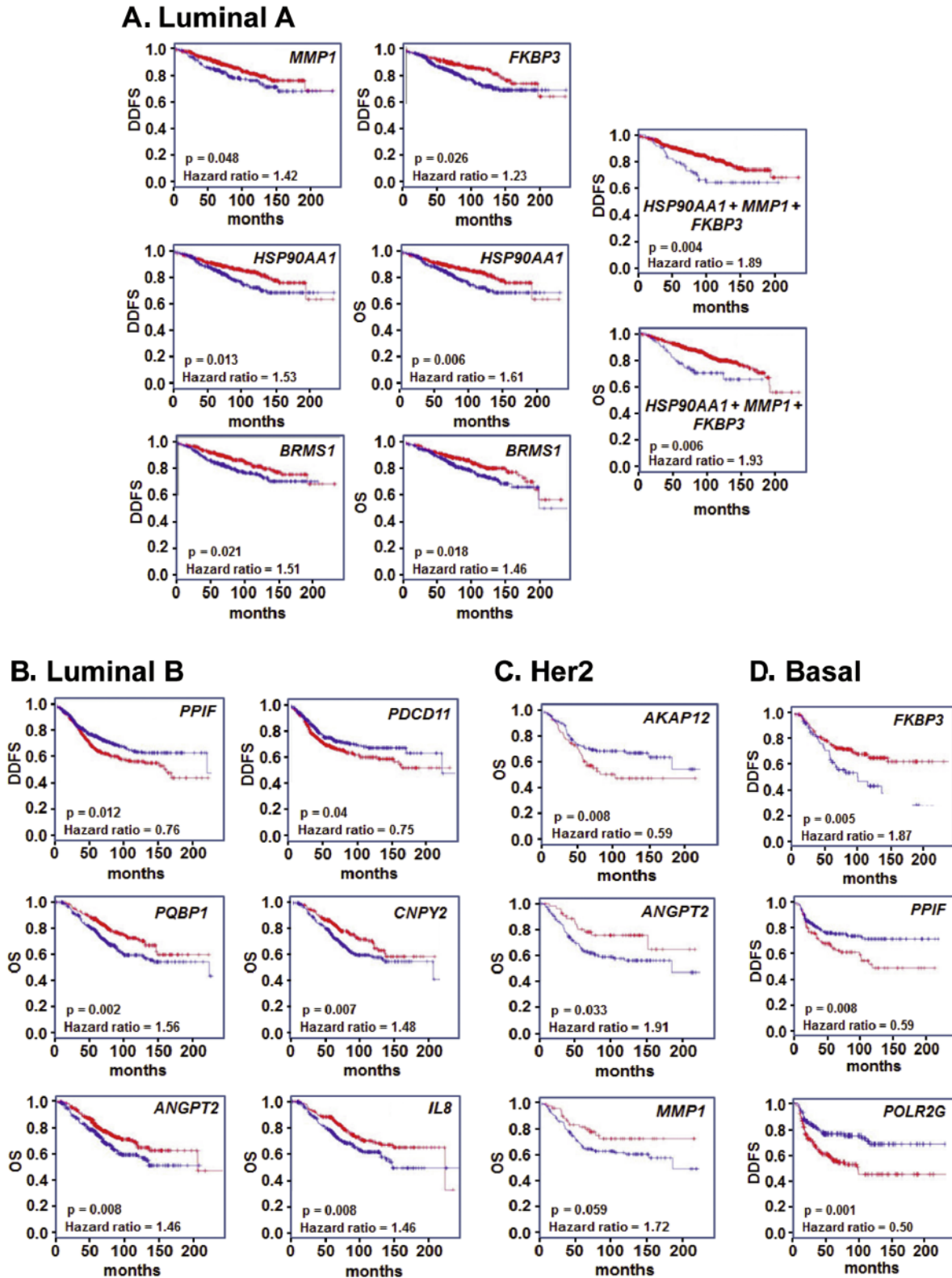


Figure 6. Genes regulated by NME1 in WM1158 cells are predictive of survival across multiple breast cancer subtypes, as identified using the BreastMark RNA expression database and algorithm (24). Kaplan-Meier plots shown in red represent survival data for patients in whom expression of the queried gene is significantly below the median, while those in blue represent expression above the median. DDFS, distant disease-free survival; OS, overall survival.



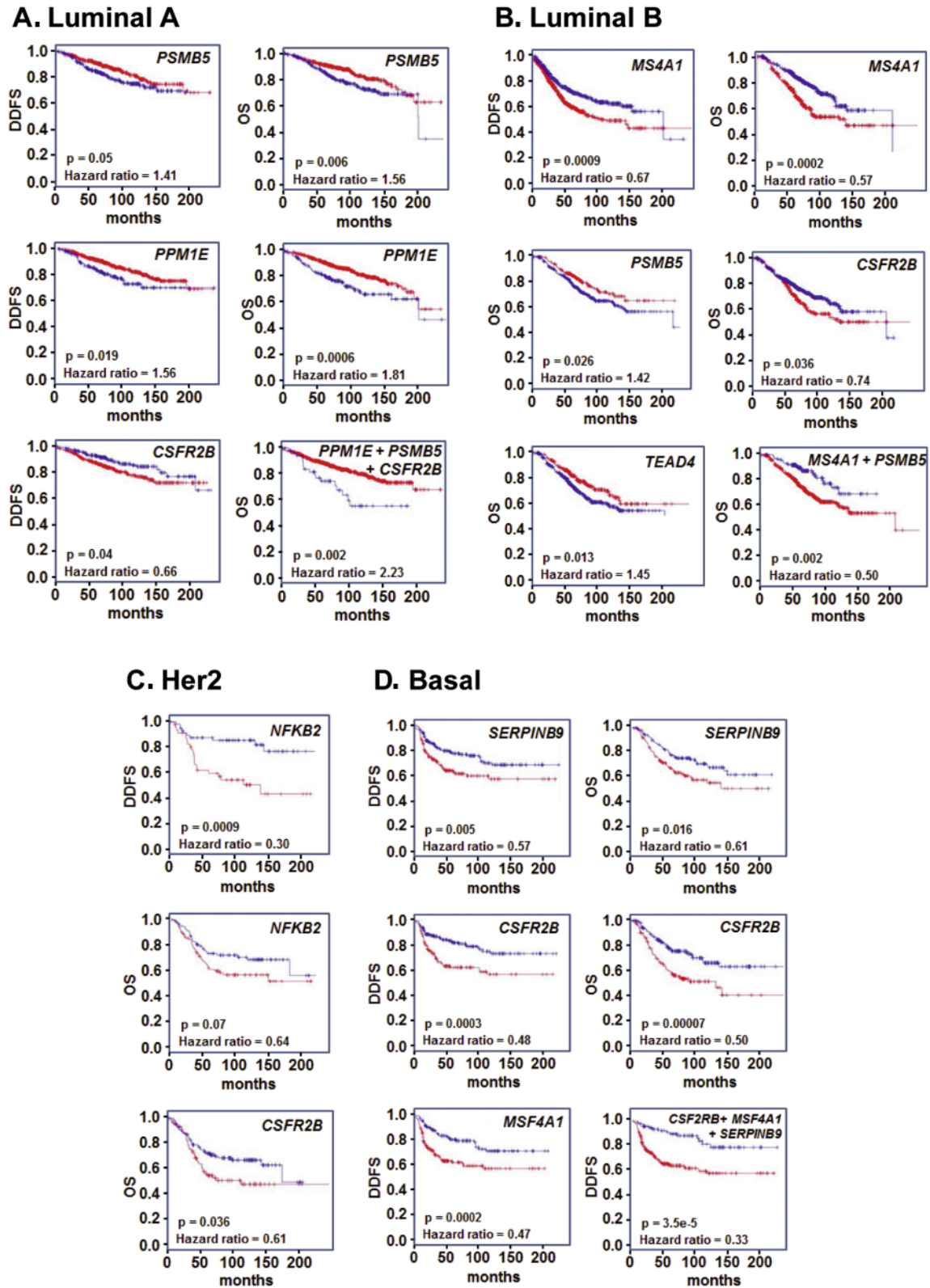


Figure 7. Genes regulated by NME1 in WRO82 cells are predictive of survival across multiple breast cancer subtypes, as identified using the BreastMark RNA expression database and algorithm (24). Kaplan-Meier plots shown in red represent survival data for patients in whom expression of the queried gene is significantly below the median, while those in blue represent expression above the median. DDFS, distant disease-free survival; OS, overall survival.

suppressor activity. In fact, the NME1-up-regulated gene *SPARCL1* has been reported to exhibit *bona fide* metastasis suppressor activity in carcinomas of the prostate (42), breast (43) and colon (44). Another NME1-up-regulated gene *PPM1E*, plays a key role in PAK1-driven breakdown of cytoskeletal stress fibers (28) and is found within a susceptibility locus for testicular germ cell tumor (45), the latter suggesting a suppressor function. Conversely, the NME1-down-regulated gene *TEAD4* is reported to be a tumor driver and mediator of chemoresistance in hepatocellular carcinoma (46), and is associated with poor prognosis in ovarian and gastric carcinoma (47).

A substantial number of the genes identified as NME1-regulated in WM1158 and WRO82 cells were of predictive value for survival in melanoma and the major subtypes of breast cancer, another NME1-dependent cancer. Moreover, many of those possessed significant concordance between the directionality of regulation by NME1, their canonical functions in cancer, and their impact on survival. Perhaps the strongest concordance was observed with the NME1-up-regulated metastasis suppressor *SPARCL1*, whose expression was positively correlated with survival in both melanoma datasets (GSE8401 and GSE46517), as well as with overall survival (OS) in LumA (also disease-free survival, DFS), LumB and Her2 subtypes of breast cancer. As predicted, low expression for many NME1-down-regulated genes in WM1158 cells (*i.e.* *CNPY2*, *OSGEP1*, *PQBPI*, *FKBP3*, *POLR2G*, *BRMS1*, *ATP5D* and *ELL3SERINC4*) was associated with better survival in melanoma and/or breast cancer. For example, low *CNPY2* expression was associated with improved DFS and OS in LumB breast cancers, while low expression of *FKBP3* was associated with better survival across all four breast cancer subtypes (LumA, DDFS; LumB, DFS; Her2, DFS and OS; basal, DDFS and OS). Interestingly, expression of the NME1-down-regulated gene *BRMS1* was reduced in metastatic melanoma, consistent with its metastasis suppressor function in melanoma but, surprisingly, was inversely correlated with OS in melanoma, as well as survival in the LumA (DFS, DDFS and OS) and OS in LumB subtypes of breast cancer. Thus, the down-regulatory effect of NME1 on *BRMS1* expression, while superficially discordant with prediction, was actually concordant with the association between *BRMS1* expression and poorer survival in melanoma and breast cancer patients. As the metastasis suppressor function and expression profiles associated with *BRMS1* have been established convincingly (35), our data suggest further complexity, possibly due to heterogeneity within these cancer populations.

A recent paper has described NME1 as a factor whose function is in “binding up the bad guys” in cancer (48). Our study as well as previous analyses of gene expression in breast cancer cell lines, indicates NME1 also regulates expression of RNAs that encode metastasis drivers and

suppressors in melanoma. Clearly much remains to be done to further elucidate the mechanisms underlying this regulation, which may reside not only in transcriptional regulation but all other events in the regulatory cascade including RNA splicing, export and stability. The NME1-regulated genes uncovered in this analysis highlights potential candidates for diagnosis and prognosis in melanoma and other NME1-dependent cancers. Moreover, they represent candidate effectors of its metastasis suppressor function that could be exploited as targets in therapy of such cancers.

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### References

- Smith SC and Theodorescu D: Learning therapeutic lessons from metastasis suppressor proteins. *Nat Rev Cancer* 9: 253-264, 2009.
- Hurst DR and Welch DR: Metastasis suppressor genes at the interface between the environment and tumor cell growth. *Int Rev Cell Mol Biol* 286: 107-180, 2011.
- Stegg PS and Theodorescu D: Metastasis: a therapeutic target for cancer. *Nat Clin Pract Oncol* 5: 206-219, 2008.
- Jarrett SG, Novak M, Dabernat S, Daniel JY, Mellon I, Zhang Q, Harris N, Ciesielski MJ, Fenstermaker RA, Kovacic D, Slominski A and Kaetzel DM: Metastasis suppressor NM23-H1 promotes repair of UV-induced DNA damage and suppresses UV-induced melanomagenesis. *Cancer Res* 72: 133-143, 2012.
- Ma D, McCorkle JR and Kaetzel DM: The metastasis suppressor NM23-H1 possesses 3'-5' exonuclease activity. *J Biol Chem* 279: 18073-18084, 2004.
- Zhang Q, McCorkle JR, Novak M, Yang M and Kaetzel DM: Metastasis suppressor function of NM23-H1 requires its 3'-5' exonuclease activity. *Int J Cancer* 128: 40-50, 2011.
- Postel EH, Berberich SJ, Flint SJ and Ferrone CA: Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. *Science* 261: 478-480, 1993.
- Ma D, Nutt CL, Shanehsaz P, Peng X, Louis DN and Kaetzel DM: Autocrine platelet-derived growth factor-dependent gene expression in glioblastoma cells is mediated largely by activation of the transcription factor sterol regulatory element binding protein and is associated with altered genotype and patient survival in human brain tumors. *Cancer Res* 65: 5523-5534, 2005.
- Cervoni L, Egistelli L, Eufemi M, d'Abusco AS, Altieri F, Lascu I, Turano C and Giartosio A: DNA sequences acting as binding sites for NM23/NDPK proteins in melanoma M14 cells. *J Cell Biochem* 98: 421-428, 2006.
- Cervoni L, Pietrangeli P, Chichiarelli S, Altieri F, Egistelli L, Turano C, Lascu I and Giartosio A: *In vivo* cross-linking of nm23/nucleoside diphosphate kinase to the PDGF-A gene promoter. *Mol Biol Rep* 30: 33-40, 2003.

- 11 Chang W, Ma L, Lin L, Gu L, Liu X, Cai H, Yu Y, Tan X, Zhai Y, Xu X, Zhang M, Wu L, Zhang H, Hou J, Wang H and Cao G: Identification of novel hub genes associated with liver metastasis of gastric cancer. *Int J Cancer* *125*: 2844-2853, 2009.
- 12 Horak CE, Lee JH, Elkahoulou AG, Boissan M, Dumont S, Maga TK, Arnaud-Dabernat S, Palmieri D, Stetler-Stevenson WG, Lacombe ML, Meltzer PS and Steeg PS: Nm23-H1 suppresses tumor cell motility by down-regulating the lysophosphatidic acid receptor EDG2. *Cancer Res* *67*: 7238-7246, 2007.
- 13 Arai T, Yamashita T, Urano T, Masunaga A, Itoyama S, Itoh K, Shiku H and Sugawara I: Preferential reduction of nm23-H1 gene product in metastatic tissues from papillary and follicular carcinomas of the thyroid. *Mod Pathol* *8*: 252-256, 1995.
- 14 Liu Y, Jiang C and Tan Y: Pathological study on the expression of cell adhesion molecules and metastasis suppressor gene in thyroid follicular carcinoma and papillary carcinoma. *Zhonghua Bing Li Xue Za Zhi* *31*: 322-326, 2002.
- 15 Wei Q and Fan S: Expression of CD44V6 and nm23-H1 in thyroid papillary adenocarcinoma and lymph node metastasis. *Hunan Yi Ke Da Xue Xue Bao* *23*: 5-7, 30, 1998.
- 16 McLaughlin PJ, Zagon IS, Park SS, Conway A, Donahue RN and Goldenberg D: Growth inhibition of thyroid follicular cell-derived cancers by the opioid growth factor (OGF) – opioid growth factor receptor (OGFr) axis. *BMC Cancer* *9*: 369, 2009.
- 17 Mishra A, Ormerod AK, Cibull ML, Spear BT, Kraner SD and Kaetzel DM: PDGF-A promoter and enhancer elements provide efficient and selective antineoplastic gene therapy in multiple cancer types. *Cancer Gene Ther* *16*: 298-309, 2009.
- 18 Gonzalez M, Ruggiero FP, Chang Q, Shi YJ, Rich MM, Kraner S and Balice-Gordon RJ: Disruption of Trkb-mediated signaling induces disassembly of postsynaptic receptor clusters at neuromuscular junctions. *Neuron* *24*: 567-583, 1999.
- 19 Norris CM, Kadish I, Blalock EM, Chen KC, Thibault V, Porter NM, Landfield PW and Kraner SD: Calcineurin triggers reactive/inflammatory processes in astrocytes and is up-regulated in aging and Alzheimer's models. *J Neurosci* *25*: 4649-4658, 2005.
- 20 Kanegae Y, Makimura M, Saito I: A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn J Med Sci Biol* *47*: 157-166, 1994.
- 21 Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* *4*: 249-264, 2003.
- 22 Xu L, Shen SS, Hoshida Y, Subramanian A, Ross K, Brunet JP, Wagner SN, Ramaswamy S, Mesirov JP and Hynes RO: Gene expression changes in an animal melanoma model correlate with aggressiveness of human melanoma metastases. *Mol Cancer Res* *6*: 760-769, 2008.
- 23 Kabbarah O, Nogueira C, Feng B, Nazarian RM, Bosenberg M, Wu M, Scott KL, Kwong LN, Xiao Y, Cordon-Cardo C, Granter SR, Ramaswamy S, Golub T, Duncan LM, Wagner SN, Brennan C and Chin L: Integrative genome comparison of primary and metastatic melanomas. *PLoS One* *5*: e10770, 2010.
- 24 Madden SF, Clarke C, Gaule P, Aherne ST, O'Donovan N, Clynes M, Crown J and Gallagher WM: BreastMark: An integrated approach to mining publicly available transcriptomic datasets relating to breast cancer outcome. *Breast Cancer Res* *15*: R52, 2013.
- 25 Fiore LS, Ganguly SS, Sledziona J, Cibull ML, Wang C, Richards DL, Neltner JM, Beach C, McCorkle JR, Kaetzel DM and Plattner R: c-Abl and Arg induce cathepsin-mediated lysosomal degradation of the NM23-H1 metastasis suppressor in invasive cancer. *Oncogene*, in press.
- 26 Benjamini Y and Hochberg Y: Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B-Methodological* *57*: 289-300, 1995.
- 27 De S, Cipriano R, Jackson MW and Stark GR: Overexpression of kinesins mediates docetaxel resistance in breast cancer cells. *Cancer Res* *69*: 8035-8042, 2009.
- 28 Koh CG, Tan EJ, Manser E and Lim L: The p21-activated kinase PAK is negatively regulated by POPX1 and POPX2, a pair of serine/threonine phosphatases of the PP2C family. *Curr Biol* *12*: 317-321, 2002.
- 29 Horak CE, Mendoza A, Vega-Valle E, Albaugh M, Graff-Cherry C, McDermott WG, Hua E, Merino MJ, Steinberg SM, Khanna C and Steeg PS: Nm23-H1 suppresses metastasis by inhibiting expression of the lysophosphatidic acid receptor EDG2. *Cancer Res* *67*: 11751-11759, 2007.
- 30 Yang M, Jarrett SG, Craven R and Kaetzel DM: YNK1, the yeast homolog of human metastasis suppressor NM23, is required for repair of UV radiation- and etoposide-induced DNA damage. *Mutat Res* *660*: 74-78, 2009.
- 31 Bornhauser BC, Olsson PA and Lindholm D: MSAP is a novel MIR-interacting protein that enhances neurite outgrowth and increases myosin regulatory light chain. *J Biol Chem* *278*: 35412-35420, 2003.
- 32 Voura EB, Ramjeesingh RA, Montgomery AM and Siu CH: Involvement of integrin alpha(v)beta(3) and cell adhesion molecule L1 in transendothelial migration of melanoma cells. *Mol Biol Cell* *12*: 2699-2710, 2001.
- 33 Nohata N, Hanazawa T, Kikkawa N, Sakurai D, Fujimura L, Chiyomaru T, Kawakami K, Yoshino H, Enokida H, Nakagawa M, Katayama A, Harabuchi Y, Okamoto Y and Seki N: Tumour suppressive microRNA-874 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. *Br J Cancer* *105*: 833-841, 2011.
- 34 Brems-Eskildsen AS, Zieger K, Toldbod H, Holcomb C, Higuchi R, Mansilla F, Munksgaard PP, Borre M, Orntoft TF and Dyrskjot L: Prediction and diagnosis of bladder cancer recurrence based on urinary content of hTERT, SENP1, PPP1CA, and MCM5 transcripts. *BMC Cancer* *10*: 646, 2010.
- 35 Shevde LA, Samant RS, Goldberg SF, Sikaneta T, Alessandrini A, Donahue HJ, Mauger DT, Welch DR: Suppression of human melanoma metastasis by the metastasis suppressor gene, BRMS1. *Exp Cell Res* *273*: 229-239, 2002.
- 36 Riker AI and Samant RS: Location, location, location: The BRMS1 protein and melanoma progression. *BMC Med* *10*: 19, 2012.
- 37 Liu H, Kato Y, Erzinger SA, Kiriakova GM, Qian Y, Palmieri D, Steeg PS and Price JE: The role of MMP-1 in breast cancer growth and metastasis to the brain in a xenograft model. *BMC Cancer* *12*: 583, 2012.
- 38 Huang Y, Song N, Ding Y, Yuan S, Li X, Cai H, Shi H and Luo Y: Pulmonary vascular destabilization in the premetastatic phase facilitates lung metastasis. *Cancer Res* *69*: 7529-7537, 2009.
- 39 Melnikova VO and Bar-Eli M: Bioimmunotherapy for melanoma using fully human antibodies targeting MCAM/MUC18 and IL-8. *Pigment Cell Res* *19*: 395-405, 2006.

- 40 Luis-Ravelo D, Anton I, Zanduetta C, Valencia K, Ormazabal C, Martinez-Canarias S, Guruceaga E, Perurena N, Vicent S, De Las Rivas J and Lecanda F: A gene signature of bone metastatic colonization sensitizes for tumor-induced osteolysis and predicts survival in lung cancer. *Oncogene* 2013.
- 41 Wang J, Cui S, Zhang X, Wu Y and Tang H: High expression of heat shock protein 90 is associated with tumor aggressiveness and poor prognosis in patients with advanced gastric cancer. *PLoS One* 8: e62876, 2013.
- 42 Hurley PJ, Marchionni L, Simons BW, Ross AE, Peskoe SB, Miller RM, Erho N, Vergara IA, Ghadessi M, Huang Z, Gurel B, Park BH, Davicioni E, Jenkins RB, Platz EA, Berman DM and Schaeffer EM: Secreted protein, acidic and rich in cysteine-like 1 (SPARCL1) is down-regulated in aggressive prostate cancers and is prognostic for poor clinical outcome. *Proc Natl Acad Sci USA* 109: 14977-14982, 2012.
- 43 Cao F, Wang K, Zhu R, Hu YW, Fang WZ and Ding HZ: Clinicopathological significance of reduced SPARCL1 expression in human breast cancer. *Asian Pac J Cancer Prev* 14: 195-200, 2013.
- 44 Hu H, Zhang H, Ge W, Liu X, Loera S, Chu P, Chen H, Peng J, Zhou L, Yu S, Yuan Y, Zhang S, Lai L, Yen Y and Zheng S: Secreted protein acidic and rich in cysteines-like 1 suppresses aggressiveness and predicts better survival in colorectal cancers. *Clin Cancer Res* 18: 5438-5448, 2012.
- 45 Chung CC, Kanetsky PA, Wang Z, Hildebrandt MA, Koster R, Skotheim RI, Kratz CP, Turnbull C, Cortessis VK, Bakken AC, Bishop DT, Cook MB, Erickson RL, Fossa SD, Jacobs KB, Korde LA, Kraggerud SM, Lothe RA, Loud JT, Rahman N, Skinner EC, Thomas DC, Wu X, Yeager M, Schumacher FR, Greene MH, Schwartz SM, McGlynn KA, Chanock SJ and Nathanson KL: Meta-analysis identifies four new loci associated with testicular germ cell tumor. *Nat Genet* 45: 680-685, 2013.
- 46 Mao B, Hu F, Cheng J, Wang P, Xu M, Yuan F, Meng S, Wang Y, Yuan Z and Bi W: SIRT1 regulates YAP2-mediated cell proliferation and chemoresistance in hepatocellular carcinoma. *Oncogene* 33: 1468-1474, 2014.
- 47 Xia Y, Chang T, Wang Y, Liu Y, Li W, Li M and Fan HY: YAP promotes ovarian cancer cell tumorigenesis and is indicative of a poor prognosis for ovarian cancer patients. *PLoS One* 9: e91770, 2014.
- 48 Marino N, Marshall JC and Steeg PS: Protein-protein interactions: a mechanism regulating the anti-metastatic properties of Nm23-H1. *Naunyn Schmiedebergs Arch Pharmacol* 384: 351-362, 2011.

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