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14-3-3 fusion oncogenes in high-grade endometrial stromal sarcoma

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14-3-3 proteins are ubiquitously expressed regulators of various cellular functions, including proliferation, metabolism, and differentiation, and altered 14-3-3 expression is associated with development and progression of cancer. We report a transforming 14-3-3 oncoprotein, which we identified through conventional cytogenetics and whole-transcriptome sequencing analysis as a highly recurrent genetic mechanism in a clinically aggressive form of uterine sarcoma: high-grade endometrial stromal sarcoma (ESS). The 14-3-3 oncoprotein results from a t(10;17) genomic rearrangement, leading to fusion between 14-3-3ε (YWHAE) and either of two nearly identical FAM22 family members (FAM22A or FAM22B). Expression of YWHAE–FAM22 fusion oncogenes was demonstrated by immunoblot in a (10;17)-bearing frozen tumor and cell line samples. YWHAE–FAM22 fusion gene knockdowns were performed with shRNAs and siRNAs targeting various FAM22A exons in an t(10;17)-bearing ESS cell line (ES51): Fusion protein expression was inhibited, with corresponding reduction in cell growth and migration. YWHAE–FAM22 maintains a structurally and functionally intact 14-3-3ε (YWHAE) protein-binding domain, which is directed to the nucleus by a FAM22 nuclear localization sequence. In contrast to classic ESS, harboring JAZF1 genetic fusions, YWHAE–FAM22 ESS display high-grade histologic features, a distinct gene-expression profile, and a more aggressive clinical course. Fluorescence in situ hybridization analysis demonstrated absolute specificity of YWHAE–FAM22A/B genetic rearrangement for high-grade ESS, with no fusions detected in other uterine and nonuterine mesenchymal tumors (55 tumor types, n = 827). These discoveries reveal diagnostically and therapeutically relevant models for characterizing aberrant 14-3-3 oncogenic functions.

cytogenetic aberration | translocation | uterine neoplasm | NUT | leiomyosarcoma

The 14-3-3 protein family includes seven highly conserved dimorphic isofoms (β, γ, ε, η, σ, and r) that are expressed in all eukaryotic cells (1). Through interaction with phospho-serine or phospho-threonine motifs, 14-3-3 can regulate diverse cellular functions, including signal transduction, cytoskeletal configuration, metabolism, differentiation, survival, and transcription (2). 14-3-3 proteins are implicated in tumorigenesis (3, 4), as a tumor suppressor in the case of 14-3-3r (SFN), and as a putative oncoprotein in the case of 14-3-3ε (YWHAZ). 14-3-3ε expression is inhibited in premalignant and malignant cells (5), and loss of 14-3-3r results in polyploidy and failure to maintain G2/M cell-cycle arrest after DNA damage through cytoplasmic sequestration of CDC2/cyclin B1 (6, 7). 14-3-3ε expression is up-regulated in various cancers (8), and it induces epithelial-mesenchymal transition by activation of TGF-β1/Smad and inhibits apoptosis in anoikic cells, thereby potentiating tumor invasion and metastasis (9, 10). Although these observations demonstrate functional roles for altered expression of 14-3-3 in tumorigenesis, there have heretofore been no reported instances of genomically aberrant 14-3-3 oncogenes.

Endometrial stromal sarcoma (ESS) is a type of uterine sarcoma that, in its low-grade form, contains JAZF1 fusions with various polycistron complex proteins (SUZ12, PHF1, and EPC1) (11, 12). In contrast, some ESS are histologically high grade, and these tumors typically lack JAZF1 rearrangement. The genetic basis for high-grade ESS is undefined. In this study, we used a combination of conventional cytogenetics and next-generation sequencing to identify YWHAE–FAM22A/B genetic fusion as a frequent genetic event that is specific for high-grade ESS. We further demonstrated the transforming properties of the fusion protein and characterized the clinicopathologic significance of YWHAE–FAM22A/B genetic fusion. The discovery of this unique oncogenic mechanism has biologic, diagnostic, and therapeutic implications.

Results and Discussion

Conventional Cytogenetics and Whole-Transcriptome Sequencing Identifies YWHAE–FAM22A/B Fusion as a Frequent Recurrent Genetic Event in High-Grade ESS. To characterize the genetic basis of high-grade ESS, we performed prospective cytogenetic G-banding analyses, which identified a translocation, t(10;17)(q22;p13), as a recurrent and predominant aberration in 7 of 12 cases (Fig. L4 and Table S1). A spontaneously immortal cell line, ESS1, was established from one of these t(10;17)-bearing ESS. Fluorescence in situ hybridization (FISH) localized the ESS 17p13 translocation breakpoint to the YWHAE (14-3-3ε) gene (Fig. L1B). In contrast to the tumor cells, the adjacent normal myometrial tissues uniformly lacked YWHAE rearrangement by FISH, confirming the somatic nature of the rearrangement. One ESS had an unbalanced t(10;17), associated with deletion of the rearranged YWHAE 3’ end, thereby implicating the YWHAE 5’ end in a putative t(10;17)-associated fusion oncogene. FISH localizations mapped the 10q translocation breakpoint, in each t(10;17) ESS, to one of two regions (10q22.3


The authors declare no conflict of interest.

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Data deposition: The fusion gene sequences reported in this paper have been deposited in the GenBank database (accession nos. JN996980 and JN996989).

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and 10q23.2) separated by 7.8 megabases (Fig. S1): notably, these regions had genomic and organizational similarities, each containing two members of the FAM22 family. FISH mapping within these regions was hampered by the repetitive nature of the genomic sequences (Fig. S1). Because of the abundant expression of wild-type YWHAE, 3′ RACE analysis was unsuccessful.

To demonstrate a putative YWHAE fusion oncogene in these genomically repetitive 10q regions, we used whole-transcriptome sequencing as an unbiased method. Sequencing was performed against the t(10;17)-containing ESS1, and sequence reads were analyzed by using a custom-written deFuse algorithm designed to identify fusion transcripts in RNA sequencing datasets (13), including those involving members of highly homologous gene families. deFuse analysis identified in-frame YWHAE–FAM22A fusions of YWHAE exon 5 to FAM22A exon 2 (Fig. 1C and Table S2). FAM22A is located within the 10q23.2 breakpoint region, whereas the alternate breakpoint region, 10q22.3, contains FAM22B (encoding a protein with 99% amino acid identity to FAM22A) and FAM22E. RT-PCR with YWHAE forward primers and consensus reverse primers for FAM22A/B/E identified YWHAE–FAM22B fusion transcripts in each t(10;17) ESS that lacked YWHAE–FAM22A (Fig. 1D). Therefore, FAM22A and FAM22B are alternative YWHAE gene fusion partners (Fig. 1E). In all cases, the genetic rearrangements in transcribed YWHAE–FAM22 involved fusion of YWHAE exon 5 to FAM22A or FAM22B exon 2, creating a fusion coding sequence consistent with genomic breakpoints in YWHAE intron 5 and FAM22A/B intron 1. FAM22A and FAM22B have sequence homology with NUT, an oncogene fused to BRD4 and BRD3 bromodomain genes in NUT midline carcinoma (14, 15). The YWHAE–FAM22A fusion transcript is 2,970 bp in length, and the corresponding protein product contains 989 aa, with a predicted molecular mass of 108 kDa (Dataset S1 and GenBank accession nos. JN999698 and JN999699).
SHRNA1 targets exon 2, which is contained in the fusion YWHAE–FAM22A/B express in the breakpoint region. YWHAE–FAM22A/B oncoproteins were not detected in ESS or other sarcomas lacking t(10;17) nor were they detected in t(10;17) ESS by using antibodies to the YWHAE C-terminal region. Furthermore, endogenous ESS YWHAE–FAM22A/B fusion proteins comigrated with a FLAG-tagged YWHAE–FAM22A pcDNA3 construct expressed in HEK 293T cells (Fig. S2). These studies demonstrated equivalent YWHAE–FAM22A/B expression levels in t(10;17) ESS biopsy specimens compared with the ESS1 immortal cell line.

YWHAE–FAM22A oncogenic roles were evaluated in t(10;17) ESS1 cells by using shRNAs and siRNAs targeting FAM22A. FAM22A shRNA1 targets exon 2, which is contained in the fusion transcript. A control sequence, FAM224 shRNA2, targets exon 1, which is not in the fusion transcript, and is expected to inhibit wild-type FAM22A/B/D/E. The nonfusion transcript is minimal to absent in virtually all adult tissues and cancers (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene). The nonfusion transcript is minimal to absent in virtually all adult tissues and cancers (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene).

plaque density compared with 293T cells expressing a YWHAE–FAM22A construct. In contrast to the predominantly cytoplasmic staining (and nuclear localization controls, whereas GAPDH is a cytosolic protein). FOXO3A and PARP are nuclear localization controls. Five second-gen of YWHAE–FAM22A expression is nuclear in 293T cells, indicating that wild-type YWHAE–FAM22A expression is nuclear in 293T cells, indicating that wild-type YWHAE–FAM22A is predominantly nuclear (18), while YWHAE–FAM22A/B was predicted to be predominantly nuclear (18–20). YWHAE–FAM22A/B nuclear localization was confirmed in ESS1 (Fig. 3A) in 293T cells expressing a YWHAE–FAM22A/B construct (Fig. 3B).

YWHAE–FAM22 Maintains 14-3-3 Binding Properties and Shows Aberrant Nuclear Localization. Structurally, the YWHAE–FAM22A/B oncoproteins contain an intact YWHAE protein-interaction domain (16), and loss of the YWHAE C-terminal end (encoded by YWHAE exon 6) and fusion to FAM22A/B are not predicted to functionally impair this rigid YWHAE protein-interaction domain or its ability to dimerize (Fig. 2D). Further analysis of FAM22A/B protein sequences revealed a bipartite nuclear localization sequence (Arg-805 to Arg-822) encoded by exons 7 of FAM22A and FAM22B. In contrast to native YWHAE protein, which is predominantly cytoplasmic (17), YWHAE–FAM22A/B was predicted to be predominantly nuclear (18–20). YWHAE–FAM22A/B nuclear localization was confirmed in ESS1 (Fig. 3A) and in 293T cells expressing a YWHAE–FAM22A construct (Fig. 3B).

Fig. 2. Oncogenic roles of YWHAE–FAM22A fusion oncoprotein and structural considerations. (A) 3T3 cells transfected with YWHAE–FAM22A pcDNA3 at three different plating densities compared with 3T3 cells transfected with YWHAE pcDNA3. Error bars indicate SEs. (B and C) 3T3 cells transfected (Lipofectamine) with YWHAE–FAM22A pcDNA3 migrated more rapidly than 3T3 cells transfected with YWHAE pcDNA3, as shown by assays for quantitative cell migration (B) and wound healing (C). Error bars indicate SEs. (D) Structural modeling of YWHAE–FAM22A (including the protein sequences encoded by exons 1 to 5 of YWHAE and FAM22A exon 2) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene). The nonfusion transcript is minimal to absent in virtually all adult tissues and cancers (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene). The nonfusion transcript is minimal to absent in virtually all adult tissues and cancers (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene).

Fig. 3. Oncogenic fusion to FAM22 enables aberrant nuclear localization of YWHAE. (A) Endogenous YWHAE–FAM22A is predominantly nuclear, whereas native YWHAE is predominantly cytoplasmic. FOXO3A and poly(ADP-ribose) polymerase (PARP) are nuclear localization controls, whereas GAPDH is a cytoplasmic protein. (B) Induced YWHAE–FAM22A expression is nuclear in 293T cells, as shown by FLAG immunoprecipitation (Upper) and YWHAE immunohistochemistry (Lower) after transient expression of FLAG-tagged YWHAE–FAM22A pcDNA3 construct. In contrast to the predominantly cytoplasmic staining (and absent nuclear staining) seen in nontransfected 293T cells (representing wild-type YWHAE), YWHAE immunostaining in YWHAE–FAM22A-expressing 293T cells showed the presence of nuclear staining, indicating nuclear localization of the fusion protein.
mRNA sequencing demonstrated a distinctive expression profile in YWHAE–FAM22A/B ESS compared with JAZF1-rearranged ESS and uterine leiomyosarcoma (Fig. 4B). Genes involved in the regulation of cell proliferation (CCND1 and CEBPA) and tissue invasion (MMP15, FSCN1, and TIMP1) were up-regulated in YWHAE–FAM22A/B ESS compared with JAZF1-rearranged ESS (Table S4). Clinically, patients with YWHAE–FAM22A/B ESS presented with higher-stage disease and experienced more frequent disease recurrence compared with patients with JAZF1-rearranged ESS (Fig. 4C and D). FISH analysis demonstrated absolute diagnostic specificity of YWHAE–FAM22A/B rearrangement for high-grade ESS (Table 1). In addition, YWHAE–FAM22A/B rearrangement and JAZF1 rearrangement were mutually exclusive, and YWHAE–FAM22A/B rearrangement was not found in low-grade ESS (n = 38) or in various uterine and nonuterine mesenchymal tumors (55 tumor types, n = 827) (Table 1). These findings show that YWHAE–FAM22A/B rearrangement defines a group of uterine sarcomas that is genetically, histologically, and clinically distinct from classic JAZF1-rearranged ESS. This evidence prompts reconsideration of the current classification of endometrial sarcomas. In the present study, we refer to this genetically unique subgroup as YWHAE–FAM22A/B ESS. An alternative classification consideration would be “14-3-3 ESS,” which has the advantage of brevity while reflecting the expected biological contributions of YWHAE dysregulation. A biologic classification seems preferable to “high-grade ESS,” which misleadingly suggest a biologic continuum with the genetically distinct JAZF1 low-grade ESS.

In this study, we identified an oncogenic mechanism for 14-3-3 proteins in the form of a transforming YWHAE–FAM22A/B fusion oncoprotein. The translocation-mediated YWHAE–FAM22A/B fusions define a previously unrecognized group of uterine sarcoma, which is clinically more aggressive and histologically higher grade than JAZF1-rearranged ESS. YWHAE–FAM22A/B oncogenic fusion results in nuclear accumulation of the functionally intact YWHAE protein-interaction domain. Known cytoplasmic YWHAE protein–protein interactions are thereby likely redirected to the nuclear compartment. Disruption of YWHAE interaction in the nuclear compartment therefore would appear to be a rational therapeutic approach. This unique genetic fusion provides a compelling opportunity to characterize 14-3-3 functions in cancer development and progression.

**Methods**

**Study Samples.** The study samples include frozen and formalin-fixed paraffin-embedded tissues retrieved from tumor banks and pathology archives at Brigham and Women’s Hospital, Catholic University of Leuven, Vancouver General Hospital, and Stanford University Medical Center with the approval of the respective institutional research boards. Cell lines, including ESS1, ESS-JAZF1, gastrointestinal stromal tumor (GIST430), and leiomyosarcoma (LMS03), were developed at Brigham and Women’s Hospital.

![Figure 4](https://example.com/figure4.png)

**Fig. 4.** YWHAE–FAM22 ESS is associated with distinctive histology, gene-expression profiles, and clinical behavior. (A) YWHAE–FAM22 ESS, in contrast to JAZF1-SUZ12 ESS, has high-grade histology, with larger and more irregular nuclei and increased mitotic activity. (B) 3′ sequencing gene-expression profiling with unsupervised hierarchical clustering demonstrates distinct gene-expression signatures between YWHAE–FAM22 ESS (YWHAE ESS), JAZF1-rearranged ESS (JAZF1 ESS), and uterine leiomyosarcoma (LMS). (C) Patients with YWHAE–FAM22 ESS present with higher International Federation of Gynecology and Obstetrics (FIGO) stage disease compared with patients with JAZF1-rearranged ESS. (D) YWHAE–FAM22 ESS (average follow-up period of 3.5 y) more frequently recurs compared with JAZF1-rearranged ESS (average follow-up period of 10 y). NED, no evidence of disease; AWD, alive with disease; DOD, died of disease.
Cytogenetic Analysis and FISH. Cytogenetic analysis was performed on Giemsa-banded metaphase spreads per standard protocol (21). FISH analyses were performed on 4-μm tissue sections that were prebaked for 2 h at 60 °C. The sections were deparaffinized in xylene three times for 1 min each and dehydrated twice in 100% ethanol for 2 min. The slides were immersed in Tris-EDTA (100 mM Tris base and 50 mM EDTA (pH 7.0)) for 45 min at 95–99 °C and rinsed in 1× PBS for 5 min. Proteinolytic digestion of the sections was performed using Digest-ALL 3 (Invitrogen) at 37 °C for 20 min, twice. The sections were then sequentially dehydrated in alcohol (70%, 85%, 95%, and 100%) for 2 min each and air-dried. The YWHAE break-apart probe was composed of two sets of overlapping BAC clones (Children’s Hospital Oakland Research Institute), telomeric (RP11-143L7 and RP11-22G12, biotin-labeled) and centromeric (RP11-100F18 and RP11-60C18, digoxigenin-labeled), detected with streptavidin Alexa Fluor 594 conjugate (Invitrogen) and FITC anti-digoxigenin (Roche Diagnostics). The 10q22.3 (FAM22A/B) region breakpoint flanking probes were RP11-1005L9 (biotin-labeled) and RP11-210E13 (digoxigenin-labeled), and the 10q22.3 (FAM22B) region breakpoint flanking probes were RP11-715A21 (biotin-labeled) and RP11-668E21 (digoxigenin-labeled). One hundred nuclei per case were evaluated. Paired signals were defined as an orange and green signal less than two signal diameters apart or a single yellow (overlapping) signal, whereas unpaired signals were those separated by greater than or equal to two signal diameters. Only cases with clearly visible probe signals observed in at least 100 nuclei were considered interpretable. A case was considered to be positive for rearrangement if unpaired signals were seen in >20% of nuclei.

RT-PCR and Sequencing. RNAs from frozen tumor and cell line samples were extracted with a miRvana miRNA Isolation Kit (Ambion) according to the manufacturer’s protocol. Reverse transcription was subsequently performed with an Script CDNA Synthesis Kit to generate CDNA with 1 μg of RNA sample. Forward primers specific for YWHAE (exon 1A: 5'-AGAGGCTGAGAGATCTGGAGACA CTA-3'; exon 1B: 5'-TATGGATGATCGAGAGGATCTGGTG-3', and exon 5: 5'-GAGAAC TGGATACGC TGAGT GAAGAA-3') and a reverse primer specific for FAM22A/B (exon 2: 5'-CTCATAAGACACT CCTGG GGTACAGG-3') were used. PCR was performed with PCR SuperMix (11306; Invitrogen) according to the manufacturer’s protocol with the following cycling conditions: 1 cycle at 94 °C for 2 min followed by 30 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min, 68 °C for 2 min, and a final extension of 68 °C for 5 min. PCR products were evaluated on a 1% agarose gel alongside 1 Kb Plus DNA Ladder (Invitrogen) visualized with ethidium bromide staining. The PCR amplicon bands were excised from the gel, purified with a Qiagen Gel Purification Kit, and sequenced with BigDye Terminator v3.0 Ready Reaction Cycle Sequencing (Applied Biosystems) on an ABI PRISM 310.

Fusion Construct and Cloning. YWHAE–FAM22A–FLAG fusion CDNA containing BamHI (YWHAE end) and EcoRI (FLAG end) restriction sites was synthesized (GenScript) based on the sequences of the fusion transcript present in ES1 and cloned in pUC57 vector. The fusion gene sequence was validated by sequencing. It was further subcloned in pCDNA3(+) by EcoRI and BamHI (GenScript). The construct integrity was verified by sequencing. The fusion construct was expressed in 293T cells by a Lipofectamine-based transfection method according to the manufacturer’s instructions (Invitrogen Life Technologies).

Cell Lysate Preparation. Whole-cell lysates were prepared in lysis buffer [1% Nonidet P-40, 50 mM Tris HCl (pH 8.0), 100 mM sodium chloride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 5 mM EDTA, and 2 mM sodium orthovanadate] containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Nuclear and cytoplasmic fraction lysates were prepared by using a Qproteome Cell Compartment Kit (Qiagen) according to the manufacturer’s protocol. Protein concentrations were determined by using the Bio-Rad Protein Assay.

Western Blotting and Immunoprecipitation Studies. Electrophoresis and Western blotting were performed as described previously (25). In short, 30 μg of protein was loaded on a 4–12% Bis-Tris gel (NuPAGE; Invitrogen) and blotted onto a nylon membrane. Immunoprecipitations were performed by incubating 1 mg of precleared cell lysate with anti-FLAG (mouse monoclonal, F1804; Sigma) for 2 h at 4 °C, followed by addition of 20 μL of protein A Sepharose (Zymed Laboratories) for overnight incubation at 4 °C. The immunoprecipitates were
then washed three times with lysis buffer and one time with 750 μL of 10 mM Tris (pH 7.4) buffer for 10 min each at 4 °C, before being resuspended in SDS/ PAGE loading buffer containing 7.5% β-mercaptoethanol, heated at 95 °C for 5 min, resolved on 4–12% SDS/polyacrylamide gradient gels (NuPAGE, Invi- trogen), and transferred to nylon membranes. Adequate protein transfer was demonstrated by staining the membranes with Ponceau S (Sigma Chemical).

The following primary antibodies were used for staining: antibodies raised against N-terminal (amino acids 1–70) YYHAE (rabbit polyclonal, HP008445; Sigma) and against C-terminal (amino acids 239–255) YYHAE (rabbit polyclonal, BML-SA475R; Enzo Life Sciences), anti-FLAG (mouse monoclonal, F1804; Sigma), anti-FOXO3A (rabbit polyclonal, 9467; Cell Signaling), anti-β-ADP- ribose polymerase (PARP, mouse monoclonal, 33–3100; Zymed), and anti- GADPH (mouse monoclonal, G8795; Sigma). Detection was by ECL (Amersham Pharmacia Biotechnology) with a Fuji LAS1000 Plus chemiluminescence im- aging system.

Preparation of Lentiviral FAM22A shRNA Constructs and Lentiviral Infections. FAM22A shRNAs were from Broad Institute RNAi Consortium: FAM22A shRNA1 (NM_001099938.1–311921c1), 5′-TCTCCTGTCGCTCAGTTTGTG-3′; and FAM22A shRNA2 (NM_001099338.1–59821c1), 5′-TATGGTCGGACAGGCTTTA-3′. Lentiviral preparations were produced by cotransfecting empty vector pLKO.1 puro with FAM22A shRNA and helper virus packaging plasmids pCMVΔR8.91 and pMD2.G (a gift from Dr. Ruth锡) into 293T cells. Transfections were carried out containing with Lipofectamine and PLUS reagent. Lentiviruses were harvested at 24, 36, 48, and 60 h posttransfection. Virions were frozen at −80 °C in aliquots at ap- propriate amounts for infection. ESS1 cells were seeded in 6-well plates. Infections were carried out in the presence of 8 μg/mL polybrene. After transduction, ESS1 were selected with 2 μg/mL puromycin for 15 d, then lyed for Western blot analysis. Cell culture images were obtained by using a Spot RT Slider Camera and Spot software (Version 4.6 for Windows) and a Nikon Eclipse TE2000-S inverted microscope.

In Vitro Wound-Healing Assays. Cell-wounding studies were carried out via standard methods (26). A blast was created in confluent cell cultures, using the tip of a P-100 Pipetman, at 8 h after shRNA transduction with puromycin se- lection. The plates were photographed at 0, 72, and 96 h with Spot software (Version 4.6 for Windows) and a Nikon Eclipse TE2000-S inverted microscope.

3′ End Sequencing Gene-Expression Analysis. We prepared 3 sequence libraries as previously described (27). Total RNA was purified from formalin-fixed par- affin-embedded sections after deparaffinization with a xylene incubation, eth- anol wash, and protease/DNase digestion (RecoverAll Total Nucleic Acid Isolation Kit; Ambion) per the manufacturer’s protocol. Isolation of the mRNA 3′ ends was achieved by oligo(dT) selection on 20 μg of total RNA with the Oli- gotex miniRNA Mini Kit (Qiagen). Insufficiently fragmented RNA was heat- sheared to ~100–200 bp. The poly(A)-selected RNA was then subjected to first- and second-strand cDNA synthesis and illumina library synthesis. To obtain 36-base single-end sequence reads, 3′-end sequencing for expression quantifi- cation (3SEQ) libraries were sequenced with illumina GA llx machines. Reads were mapped first to the transcriptome (refMrna, downloaded from the UCSC genome browser, genome.ucsc.edu) by using SOAP2, allowing at most two mismatches (28). Unmapped and nonuniquely mapping reads were then mapped against the human genome (hg19), also using SOAP2, and reads mapping to RefSeq exons (same strand) were determined. Total sequence reads for each gene symbol from the transcriptome mapping and genome mapping were summed to create the gene-expression profile matrix. The data were then normalized by expressing the number of reads as transcripts per million reads (TPM) and filtered to select genes with a value of ≥2 TPM in at least two samples and an absolute difference of ≥2 TPM across the series. From these genes, those with an SD ≥ 200 as determined by Cluster 3 software were log-transformed, centered by gene using Cluster 3 software, subjected to unsupervised hierar- chical clustering by centroid linkage, and visualized with Java TreeView. Sig- nificance analysis of microarrays (SAM; http://www-stat.stanford.edu/~tibs/ SAM) was used to identify genes expressed differentially between the tumor groups.

siRNA Study, Cell Viability Assay, and Quantitative Cell Migration Assay. The detailed methods are available in Supplemental Methods.

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