Online Supporting Information for

"A Targeted Proteomic Approach for Heat Shock Proteins Reveals DNAJB4 as a Suppressor for Melanoma Metastasis"

Weili Miao, Lin Li and Yinsheng Wang*

Department of Chemistry, University of California, Riverside,

CA, 92521-0403, USA.

*Correspondence author: Yinsheng Wang. Telephone: (951)827-2700; E-mail:

yinsheng.wang@ucr.edu

Supplementary Materials and Methods

Western blot

Melanoma cells were cultured in a 6-well plate and the cells were lysed at 40-50% confluency following the above-described procedures. The concentrations of proteins in the resulting lysates were determined using Bradford Assay (Bio-Rad). The whole cell lysate for each sample (10 μg) was denatured by boiling in Laemmli loading buffer and subjected to SDS-PAGE separation. The proteins were subsequently transferred to a nitrocellulose membrane at 4°C overnight. The resulting membrane was blocked with PBS-T (PBS with 0.1% Tween 20) containing 5% milk (Bio-Rad) at 4°C for 6 h. The membrane was then incubated with primary antibody at 4°C overnight and subsequently with secondary antibody at room temperature for 1 h. After thorough washing with PBS-T buffer, the HRP signals were detected with Pierce ECL Western Blotting Substrate (Thermo).

Antibodies recognizing human DNAJB4 (Santa Cruz Biotechnology, sc-100711, 1:2000 dilution), DNAJC3 (Santa Cruz Biotechnology, sc-393559, 1:1000 dilution), and HSP40 (Santa Cruz Biotechnology, sc-398766, 1:1000 dilution) were employed as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG, IRDye® 680LT Goat anti-Mouse IgG (1:10000 dilution) were used as secondary antibodies. Membranes were also probed with anti-actin antibody (Cell Signaling #4967, 1:10000 dilution) to confirm equal protein loading.

Migration and invasion assay

Migration and invasion assay was performed using a Matrigel Transwell Chamber (Corning) with 8 μm pore polycarbonate filters. For migration assay, the harvested cells were suspended in 100 μl serum-free media at a final concentration of 3-8 × 10^5 cells/ml and were added to the upper

chambers of the transwell system. Medium containing 10% FBS was placed in the lower chamber. After incubation for the indicated periods of time, non-migrated cells and media on the upper chamber were removed by using cotton swab and the migrated cells on the bottom surface of the insert membrane were fixed by incubating with 75% methanol at room temperature for 15 min. The cells were then stained with 0.2% crystal violet in 10% ethanol for 15 min. After washing and drying, the insert membranes were imaged under a light microscope. For invasion assay, 200-400 μg/mL matrigel in serum-free media was coated on the upper-chamber of the filter and the matrigel-containing medium was removed after a 2-h incubation at 37°C. The cells were then dispersed, stained, and counted in a similar way as described for the migration assay. Since the same number of cells was suspended in each well, cell migration was determined based on the number of migrated cells and cell invasion was calculated by dividing the number of invaded cells with that of the migrated cells.

Gelatin zymography assay

Concentrated medium collected from cultured cells was loaded onto 10% SDS-PAGE gels containing 0.1% gelatin under non-reducing conditions. After electrophoresis, the gels were washed with 2.5% Triton X-100 (Sigma) to remove SDS and to renature the MMP-2 and MMP-9 proteins. The gels were subsequently incubated in a developing buffer with 1% Triton X-100 and 1 μM Zn^{2+} overnight to induce gelatin lysis by the renatured MMP-2 and MMP-9.[1] The relative amounts of MMP-2 and MMP-9 were then quantified based on their band intensities using ImageJ. Images were inverted using online image inversion tool (http://pinetools.com/invert-image-colors).

Real-time PCR

Melanoma cells were seeded in 6-well plates at 50% confluence level. Total RNA was extracted from cells using TRI Reagent (Sigma). Approximately 3 μg RNA was reverse transcribed by employing M-MLV reverse transcriptase (Promega) and an oligo$(dT)_{16}$ primer. After a 60-min incubation at 42°C, the reverse transcriptase was deactivated by heating at 85°C for 5 min. Quantitative real-time PCR was performed using iQ SYBR Green Supermix kit (Bio-Rad) on a Bio-Rad iCycler system (Bio-Rad), and the running conditions were at 95°C for 3 min and 45 cycles at 95°C for 15 sec, 55°C for 30 sec, and 72°C for 45 sec. The comparative cycle threshold (Ct) method (ΔΔCt) was used for the relative quantification of gene expression[2]. Primers are listed in Table S1. The mRNA level of each gene was normalized to that of the internal control (*GAPDH*).

Reference:

(1) Vandooren, J.; Geurts, N.; Martens, E.; Van den Steen, P. E.; Opdenakker, G. Nat. Methods 2013, 10, 211-220.

(2) Livak, K. J.; Schmittgen, T. D. Methods 2001, 25, 402-408.

Table S1. Sequences for RT-qPCR primers.

Gene Name	Forward Primer	Reverse Primer
MMP-2	5’-ATACAGGATCATTGGCTACACACCT-3’	5’-CCAAAGTTGATCATGATGTCTGCC-3’
MMP-9	5’-ACCAAGGATACAGTTTGTTCCTCGT-3’	5’-TAGAGGTGCCGGATGCCATTCACGT-3’
GAPDH	5’-CCATGGAGAAGGCTGGGG-3’	5’-CAAAGTTGTCATGGATGACC-3’

Table S2. Proteins and peptides included in the Skyline human heat shock protein library.

Table S3. Expression levels of heat shock proteins in WM-115/WM-266-4, IGR-39/IGR-37 and WM-793/1205Lu pairs of melanoma cells obtained from PRM method.

Figure Legend

Figure S1. Performance of the PRM method. (a) Correlation between iRT in library and measured RT for tryptic peptides from the lysates of WM-115 and WM-266-4 cells on a Q Exactive Plus mass spectrometer. (b) A Venn diagram showing the overlap between quantified heat shock proteins from LC-MS/MS analyses in the PRM or DDA mode. (c) Correlation between iRT in library and measured RT on a Q Exactive Plus mass spectrometer of IGR-39/IGR-37 paired melanoma cells. (d) A Venn diagram depicting the overlap between quantified heat shock proteins from forward (F) and reverse (R) SILAC labeling experiments for of IGR-39/IGR-37 paired melanoma cells. (e) Correlation between iRT in library and measured RT for tryptic peptides from heat shock proteins in WM-793/1205Lu paired melanoma cells on a Q Exactive Plus mass spectrometer. (f) A Venn diagram displaying the overlap between quantified heat shock proteins based on forward and reverse SILAC labeling experiments for WM-793/1205Lu paired melanoma cells.

Figure S2. Performances of the PRM method. (a) XIC traces for representative peptides from heat shock proteins. (b) Correlation between the ratios obtained from forward and reverse SILAC labeling experiments. (c) A heatmap showing the differences in expression of heat shock proteins in the 2 biological replicates in WM-115/WM-266-4 paired primary/metastatic melanoma cell lines (Table S3).

Figure S3. Differential expression of heat shock proteins in paired IGR-39/IGR-37 (a) and WM793/1205Lu (b) primary/metastatic melanoma cells. The data represent the mean of results obtained from one forward and one reverse SILAC labeling experiments. The ratios obtained from individual measurements are listed in Table S3.

Figure S4. Validation of quantification results obtained from PRM-based targeted proteomic method. (a) Validation of the differential expression of representative heat shock proteins by

Western blot analysis. (b) Quantitative comparison of ratios of heat shock proteins obtained from PRM and Western blot analysis (n =3).

Figure S5. Western blot results showing the siRNA-mediated knock-down of DNAJB4 in three lines of primary melanoma cells.

Figure S6. The migratory and invasive abilities of WM-115 (a) and WM-266-4 (b) upon siRNA-mediated down-regulation and ectopic overexpression of DNAJB4, respectively.

Figure S7. The migratory and invasive abilities of IGR-39 (a), IGR-37 (b) and 1205Lu (c) cells upon siRNA-mediated down-regulation and ectopic overexpression of DNAJB4.

Figure S8. Gelatin zymography assay showing the alterations in activities of secreted MMP-2 and MMP-9 in primary melanoma cells upon siRNA-mediated knockdown of DNAJB4 and metastatic melanoma cells upon ectopic expression of DNAJB4.

Figure S9. qRT-PCR showing the modulation in mRNA levels of *MMP2* and *MMP9* genes in primary melanoma cells upon siRNA-mediated knockdown of DNAJB4 or metastatic melanoma cells upon ectopic expression of DNAJB4.

Figure S1.

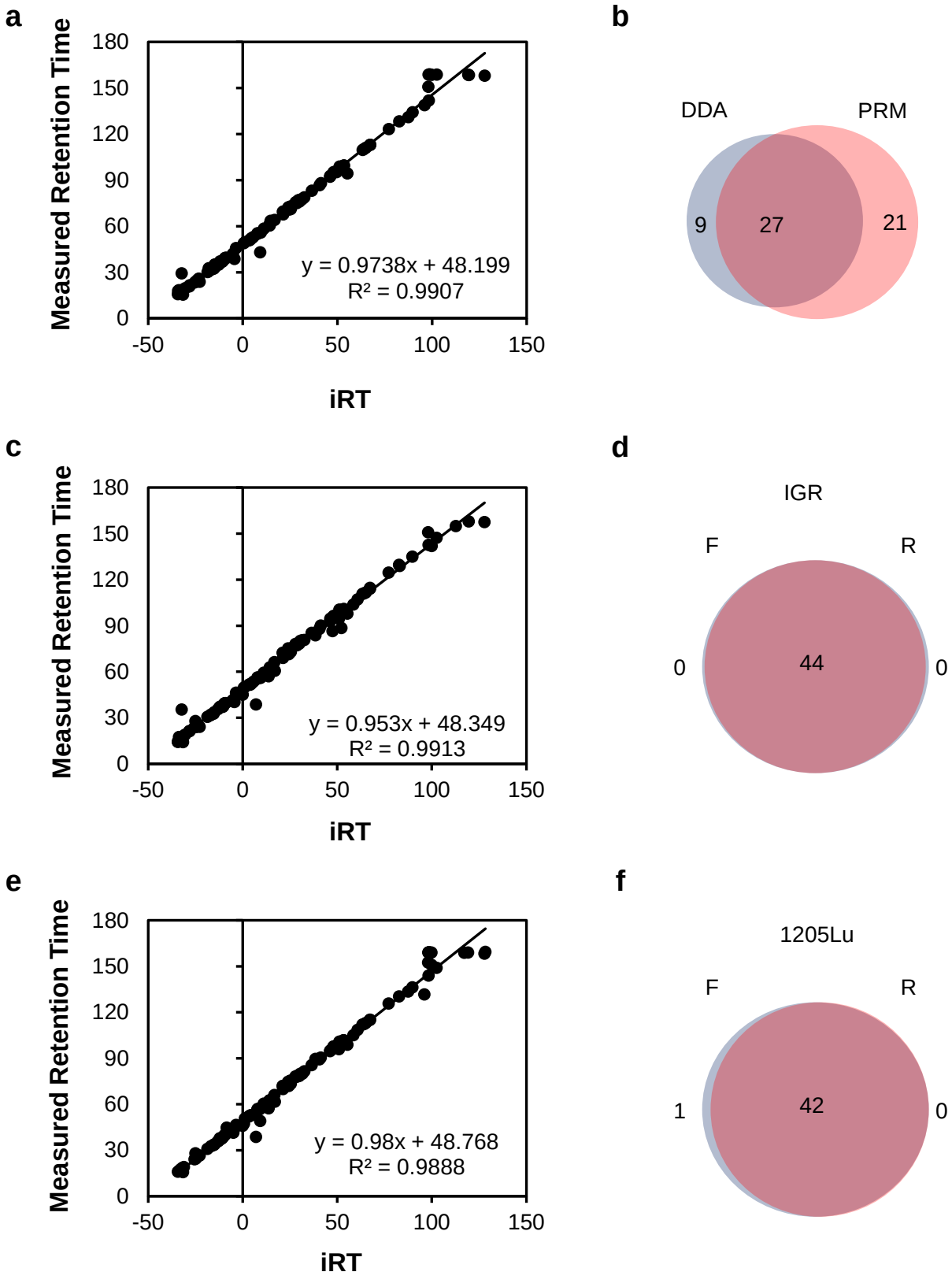

a
Measured Retention Time
180
150
120
90
60
30
0
-50
0
50
100
150
iRT
y = 0.9738x + 48.199
R² = 0.9907
b
DDA
PRM
9
27
21
c
Measured Retention Time
iRT
y = 0.953x + 48.349
R² = 0.9913
d
IGR
F
R
0
44
0
e
Measured Retention Time
iRT
y = 0.98x + 48.768
R² = 0.9888
f
1205Lu
F
R
1
42
0

Figure S2.

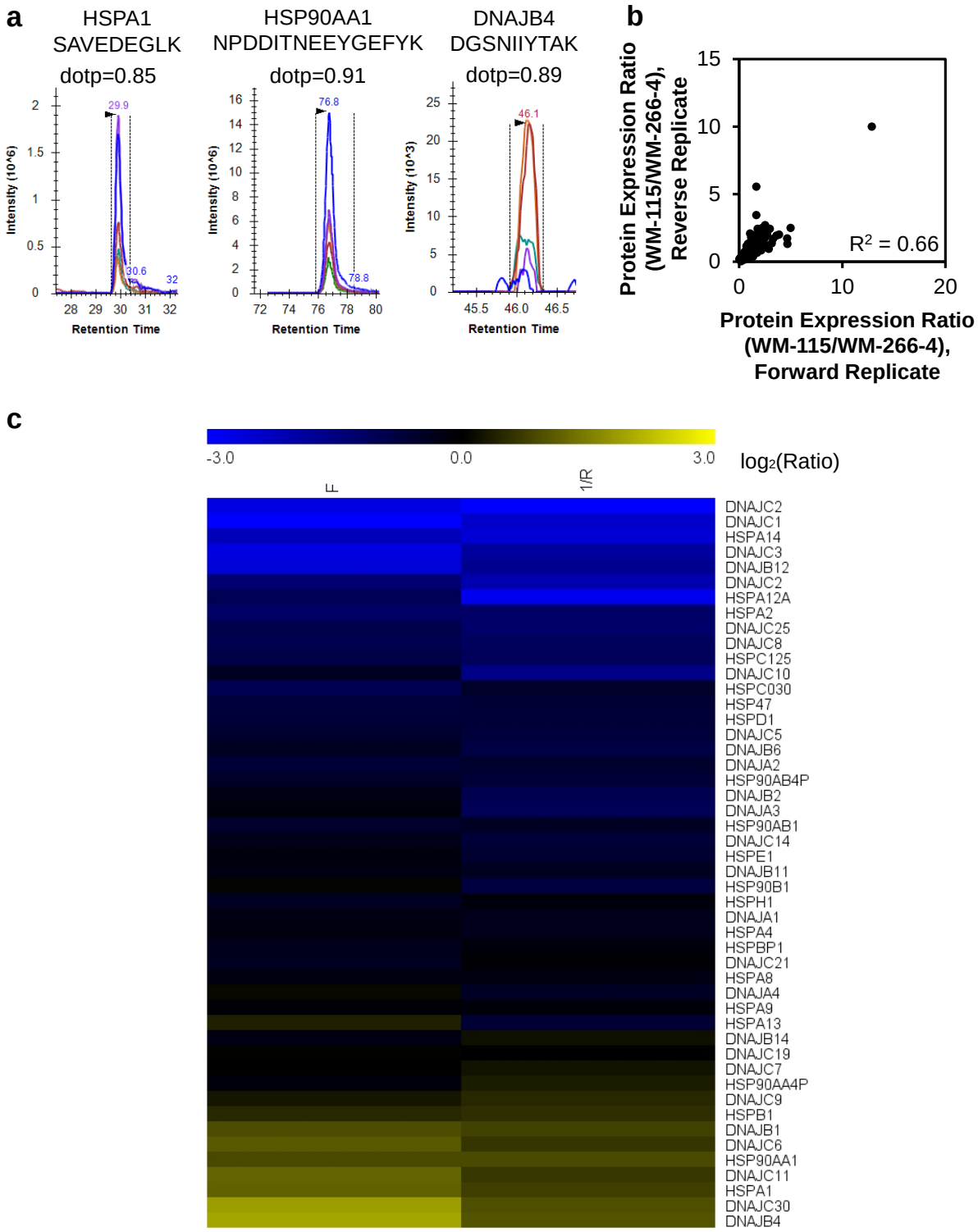

a
HSPA1
SAVEDEGLK
dotp=0.85
HSP90AA1
NPDDITNEEYGEFYK
dotp=0.91
DNAJB4
DGSNIIYTAK
dotp=0.89
Intensity (10^6)
Intensity (10^3)
Retention Time
29.9
30.6
32
76.8
78.8
46.1
b
Protein Expression Ratio (WM-115/WM-266-4), Reverse Replicate
Protein Expression Ratio (WM-115/WM-266-4), Forward Replicate
R² = 0.66
c
-3.0
0.0
3.0
log2(Ratio)
F
1/R
DNAJC2
DNAJC1
HSPA14
DNAJC3
DNAJB12
DNAJC2
HSPA12A
HSPA2
DNAJC25
DNAJC8
HSPC125
DNAJC10
HSPC030
HSP47
HSPD1
DNAJC5
DNAJB6
DNAJA2
HSP90AB4P
DNAJB2
DNAJA3
HSP90AB1
DNAJC14
HSPE1
DNAJB11
HSP90B1
HSPH1
DNAJA1
HSPA4
HSPBP1
DNAJC21
HSPA8
DNAJA4
HSPA9
HSPA13
DNAJB14
DNAJC19
DNAJC7
HSP90AA4P
DNAJC9
HSPB1
DNAJB1
DNAJC6
HSP90AA1
DNAJC11
HSPA1
DNAJC30
DNAJB4

Figure S3.

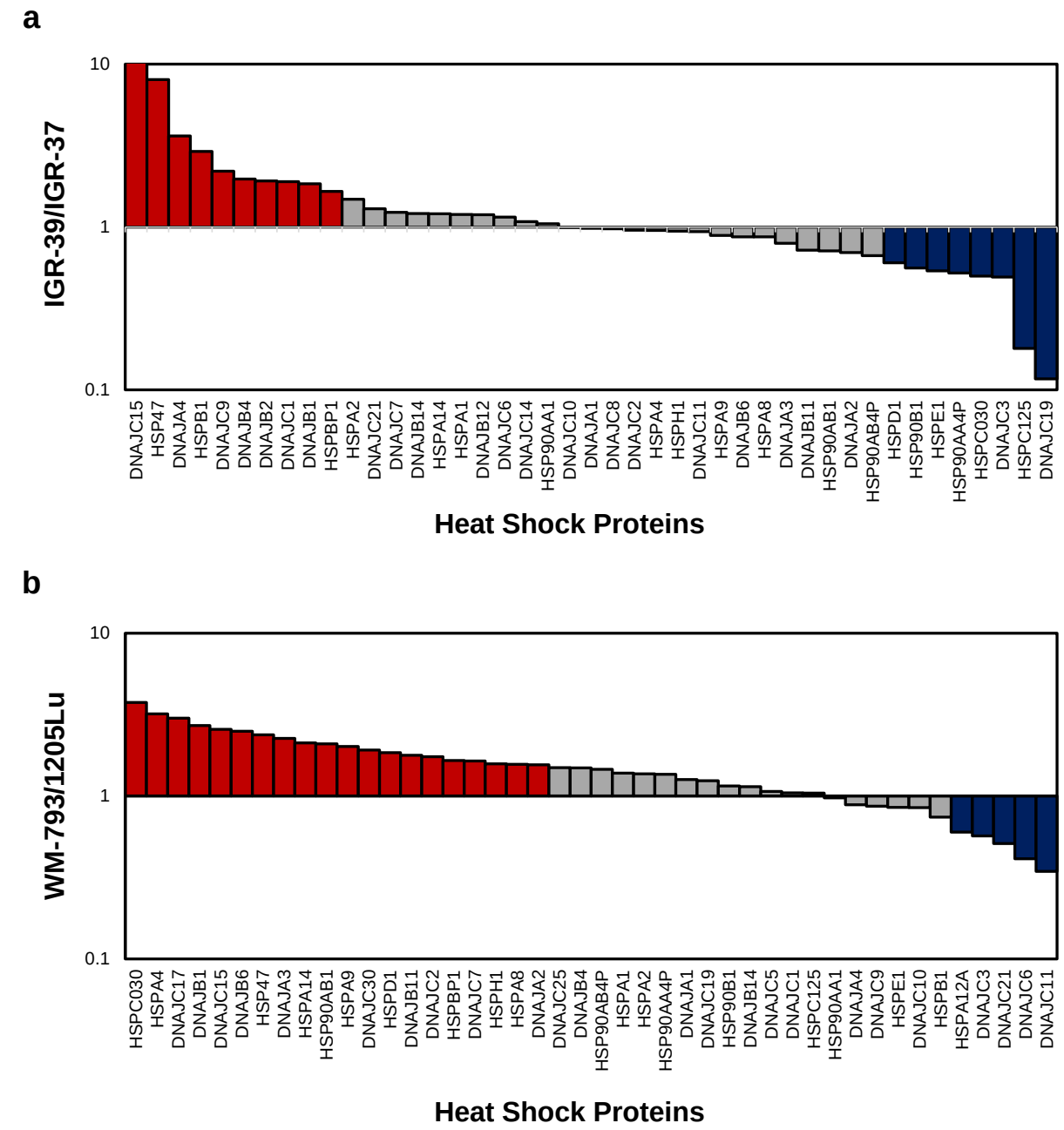

a
IGR-39/IGR-37
10
1
0.1
DNAJC15 HSP47 DNAJA4 HSPB1 DNAJC9 DNAJB4 DNAJB2 DNAJC1 DNAJB1 HSPBP1 HSPA2 DNAJC21 DNAJC7 DNAJB14 HSPA14 HSPA1 DNAJB12 DNAJC6 DNAJC14 HSP90AA1 DNAJC10 DNAJA1 DNAJC8 DNAJC2 HSPA4 HSPH1 DNAJC11 HSPA9 DNAJB6 HSPA8 DNAJA3 DNAJB11 HSP90AB1 DNAJA2 HSP90AB4P HSPD1 HSP90B1 HSPE1 HSP90AA4P HSPC030 DNAJC3 HSPC125 DNAJC19
Heat Shock Proteins
b
WM-793/1205Lu
10
1
0.1
HSPC030 HSPA4 DNAJC17 DNAJB1 DNAJC15 DNAJB6 HSP47 DNAJA3 HSPA14 HSP90AB1 HSPA9 DNAJC30 HSPD1 DNAJB11 DNAJC2 HSPBP1 DNAJC7 HSPH1 HSPA8 DNAJA2 DNAJC25 DNAJB4 HSP90AB4P HSPA1 HSPA2 HSP90AA4P DNAJA1 DNAJC19 HSP90B1 DNAJB14 DNAJC5 DNAJC1 HSPC125 HSP90AA1 DNAJA4 DNAJC9 HSPE1 DNAJC10 HSPB1 HSPA12A DNAJC3 DNAJC21 DNAJC6 DNAJC11
Heat Shock Proteins

Figure S4.

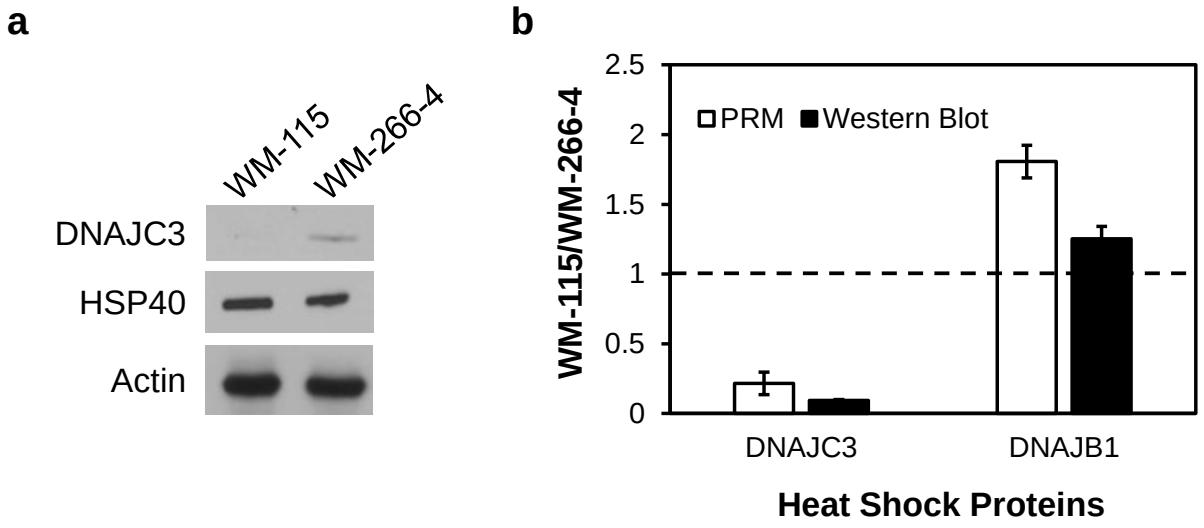

a
WM-115
WM-266-4
DNAJC3
HSP40
Actin
b
WM-115/WM-266-4
2.5
2
1.5
1
0.5
0
PRM
Western Blot
DNAJC3
DNAJB1
Heat Shock Proteins

Figure S5.

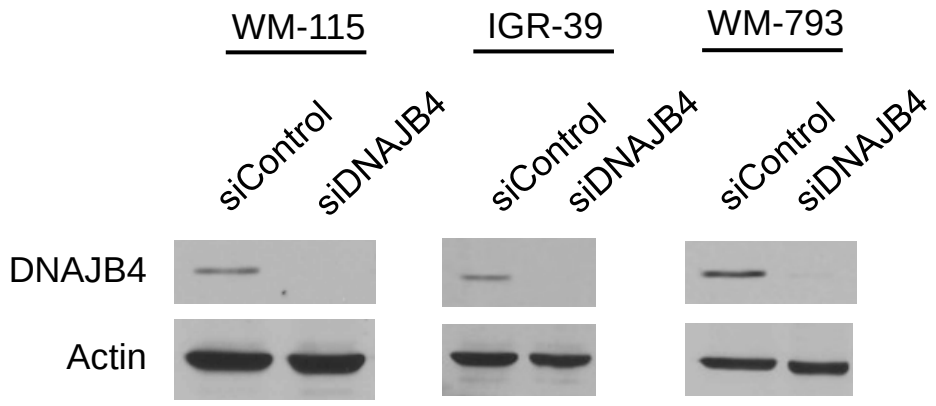

WM-115
IGR-39
WM-793
siControl
siDNAJB4
siControl
siDNAJB4
siControl
siDNAJB4
DNAJB4
Actin

Figure S6.

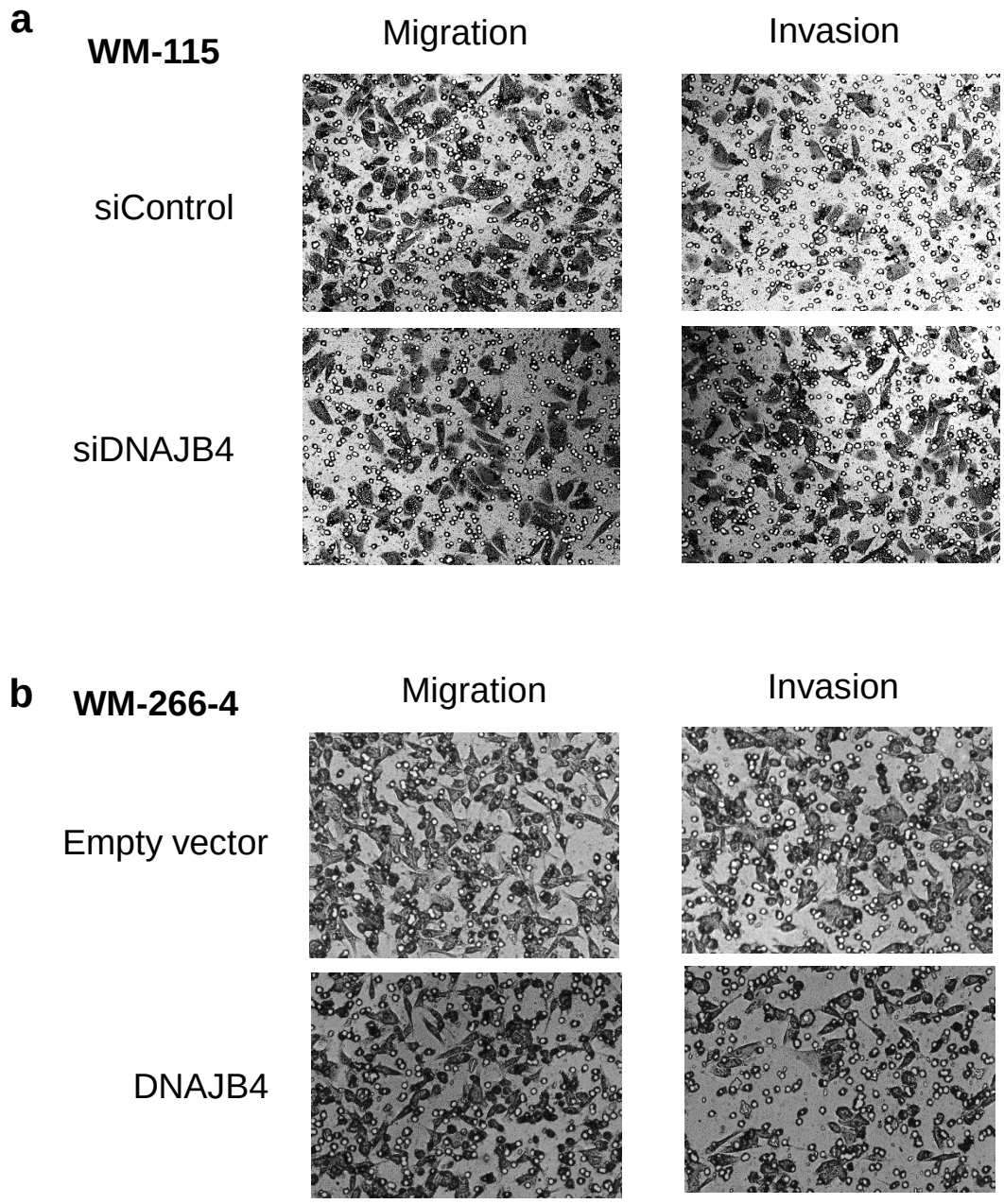

a
WM-115
Migration
Invasion
siControl
siDNAJB4
b
WM-266-4
Migration
Invasion
Empty vector
DNAJB4

Figure S7.

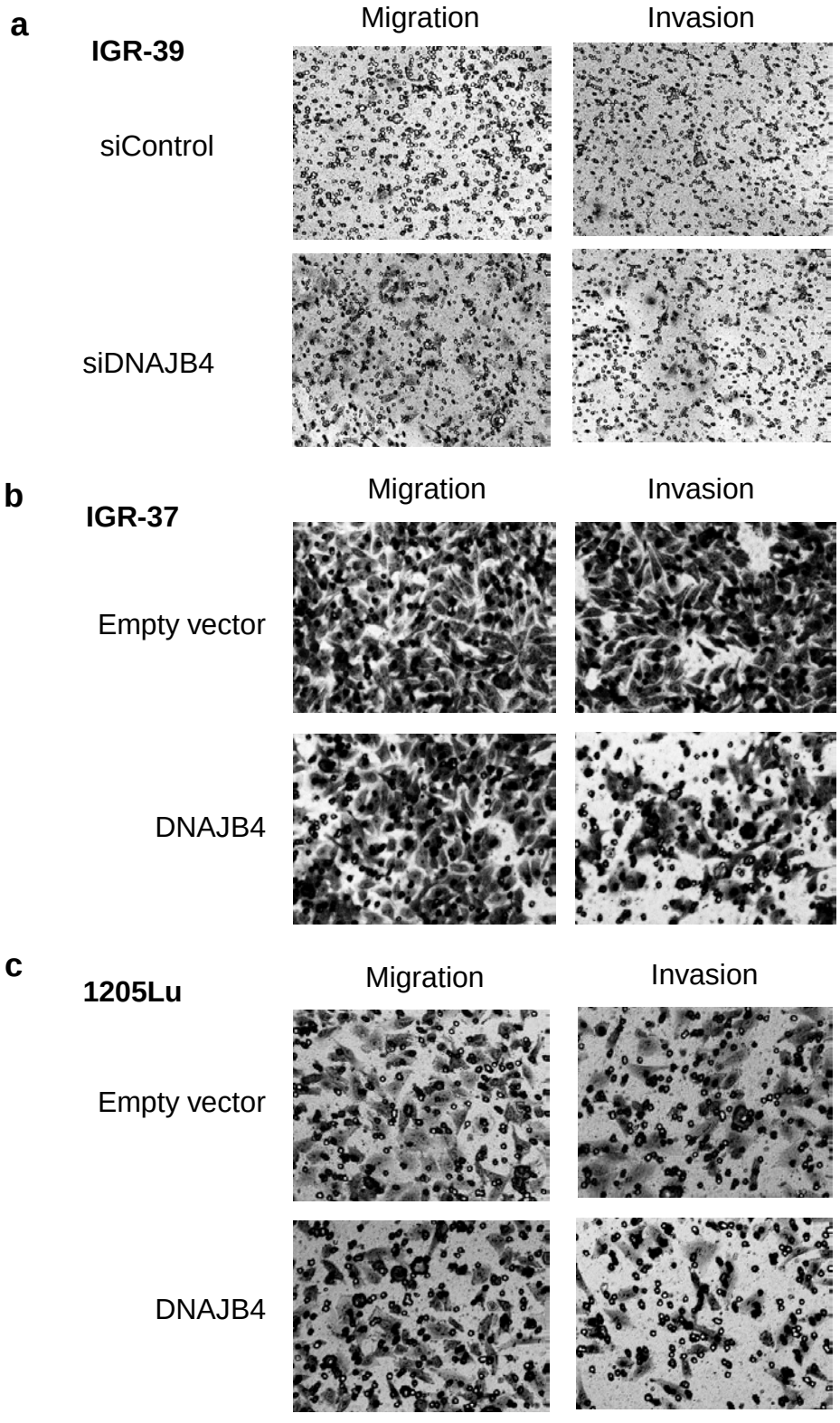

a
IGR-39
Migration
Invasion
siControl
siDNAJB4
b
IGR-37
Migration
Invasion
Empty vector
DNAJB4
c
1205Lu
Migration
Invasion
Empty vector
DNAJB4

Figure S8.

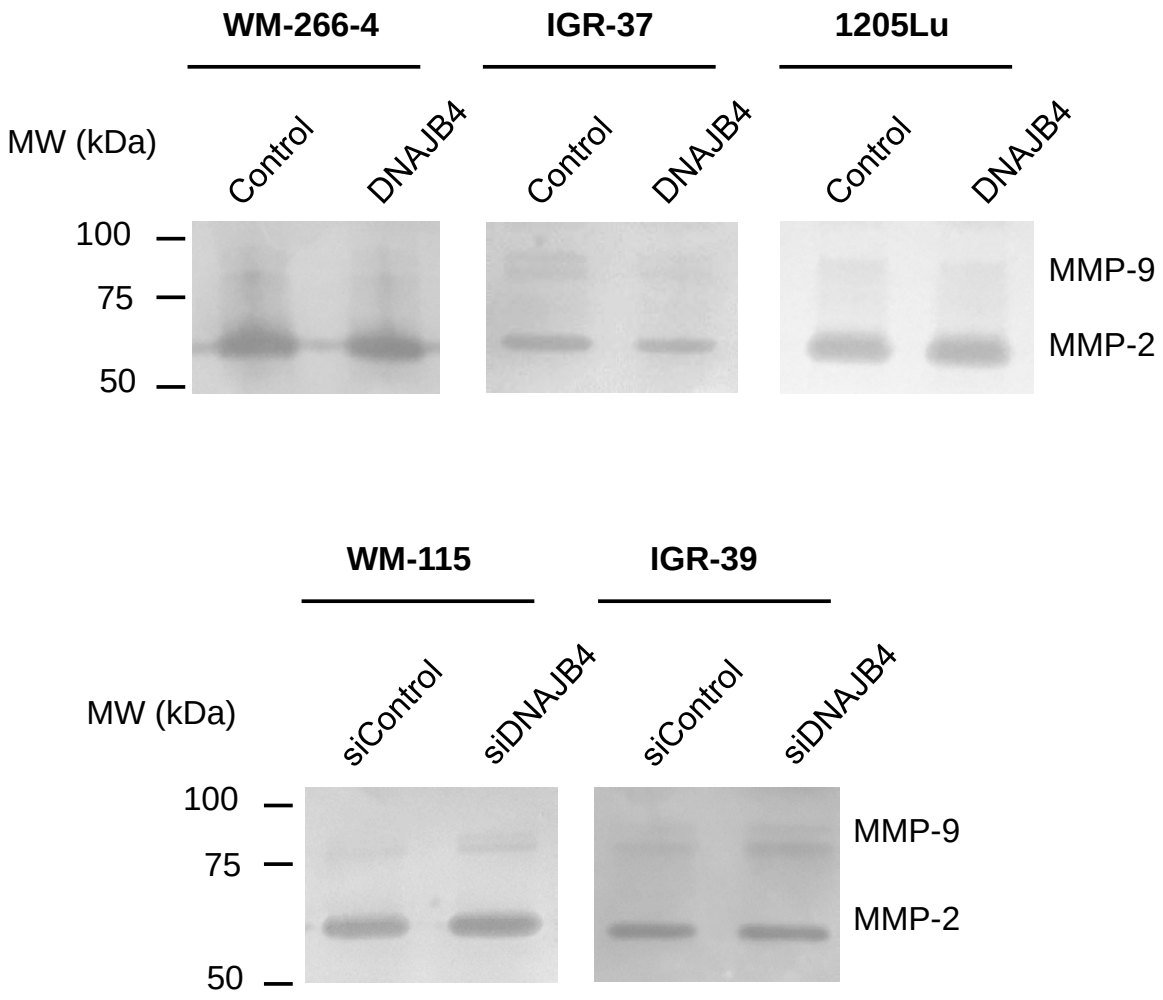
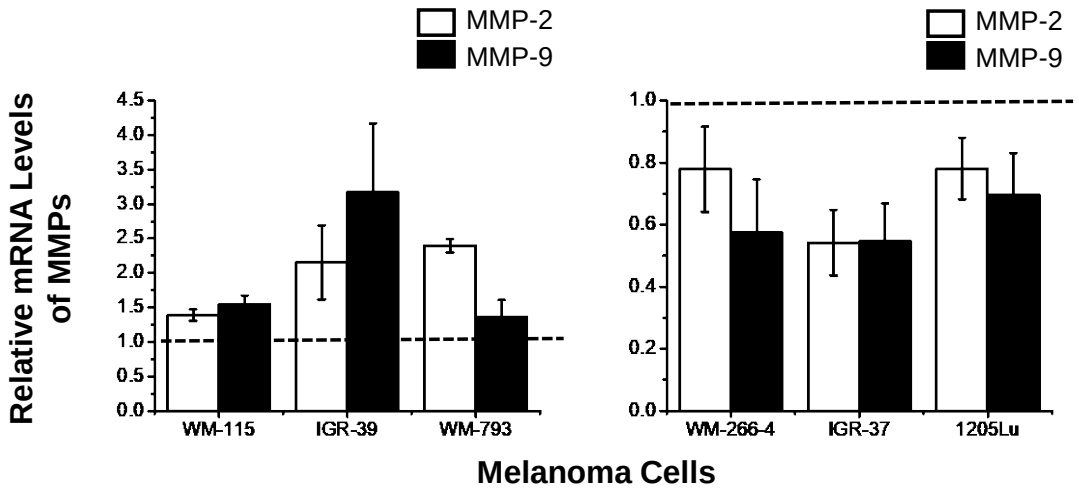

WM-266-4
IGR-37
1205Lu
MW (kDa)
Control
DNAJB4
Control
DNAJB4
Control
DNAJB4
100
75
50
MMP-9
MMP-2
WM-115
IGR-39
MW (kDa)
siControl
siDNAJB4
siControl
siDNAJB4
100
75
50
MMP-9
MMP-2

Figure S9.


MMP-2
MMP-9
MMP-2
MMP-9
Relative mRNA Levels of MMPs
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.0
0.8
0.6
0.4
0.2
0.0
WM-115
IGR-39
WM-793
WM-266-4
IGR-37
1205Lu
Melanoma Cells