Supplemental Information

Figure S1

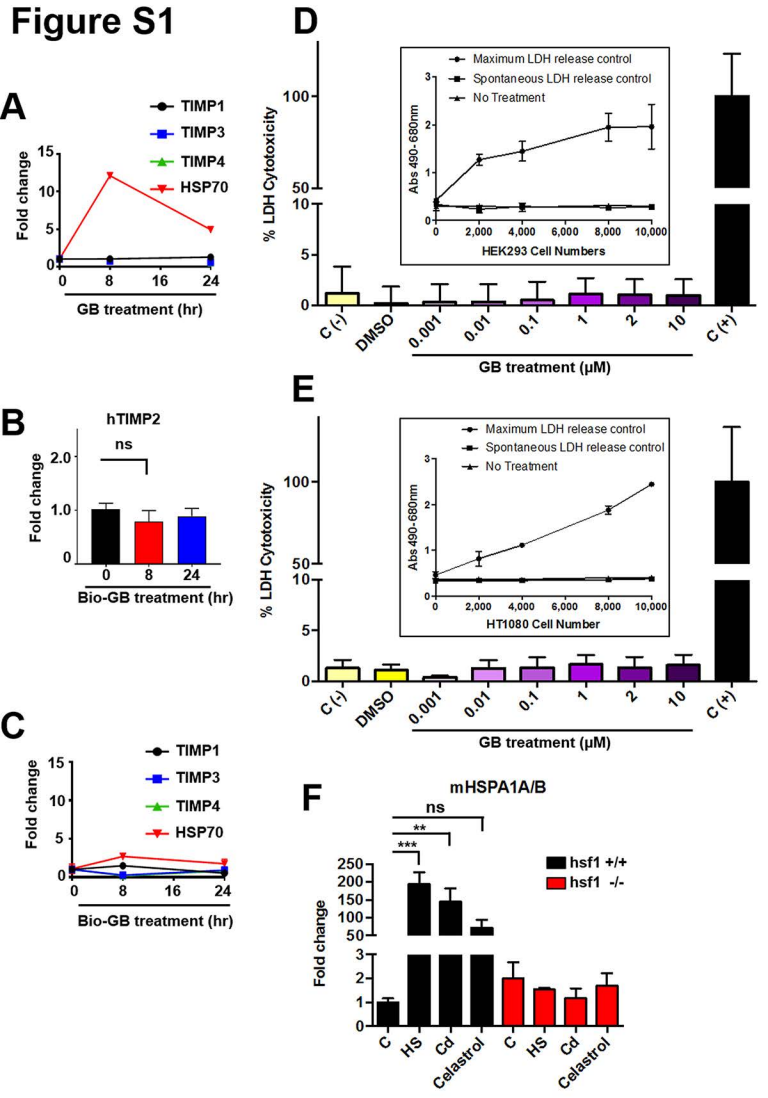

A
TIMP1
TIMP3
TIMP4
HSP70
Fold change
GB treatment (hr)
B
hTIMP2
ns
Fold change
Bio-GB treatment (hr)
C
TIMP1
TIMP3
TIMP4
HSP70
Fold change
Bio-GB treatment (hr)
D
Maximum LDH release control
Spontaneous LDH release control
No Treatment
Abs 490-680nm
HEK293 Cell Numbers
% LDH Cytotoxicity
C (-)
DMSO
GB treatment (μM)
C (+)
E
Maximum LDH release control
Spontaneous LDH release control
No Treatment
Abs 490-680nm
HT1080 Cell Number
% LDH Cytotoxicity
GB treatment (μM)
F
mHSPA1A/B
ns
hsf1 +/+
hsf1 -/-
Fold change
C
HS
Cd
Celastrol

Figure S1 - TIMPs mRNA expression upon stress. Related to Figure 1

(A) Following time course treatment with HSP90 inhibitor ganetespib (GB, 1μM), cDNA was prepared from HEK293 cells and qRT PCR was performed to determine transcript levels for the indicated human genes. *Hsp70* mRNA expression levels (positive control). Representative of at least n=2 independent experiments.

(B) *TIMP2* transcriptional expression measured by real time RT-qPCR over housekeeping gene *GAPDH* from HEK293 cells treated with 1μM biotinylated ganetespib (Bio-GB). Error bars represent the SEM of n=2 independent experiments. (ns, not significant).

(C) Following time course treatment with HSP90 inhibitor biotinylated-GB (Bio-GB, 1μM), cDNA was prepared from HEK293 cells and qRT PCR was performed to determine transcript levels for the indicated human genes. Graphs are representative of at least n=2 independent experiments.

(D-E) Cell death of HEK293 or HT1080 human fibrosarcoma cells in response to 24 hours treatment with increasing concentrations of Ganetespib (GB), 1nM - 10μM or DMSO and untreated control, was measured using cell cytotoxicity assay. Optimum cell number was initially determined (insert, line graph) and then cell cytotoxicity was measured and plotted as a percentage of the Maximum LDH release control, C(+). Error bars represent SEM. Graph is representative of n=3 independent experiments.

(F) *hsf1*+/+ and *hsf1*-/- MEF were treated as in Figure 1E. The mRNA levels of HSPA1A/B (Hsp70) were analyzed as in Figure 1E.

Significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Figure S2

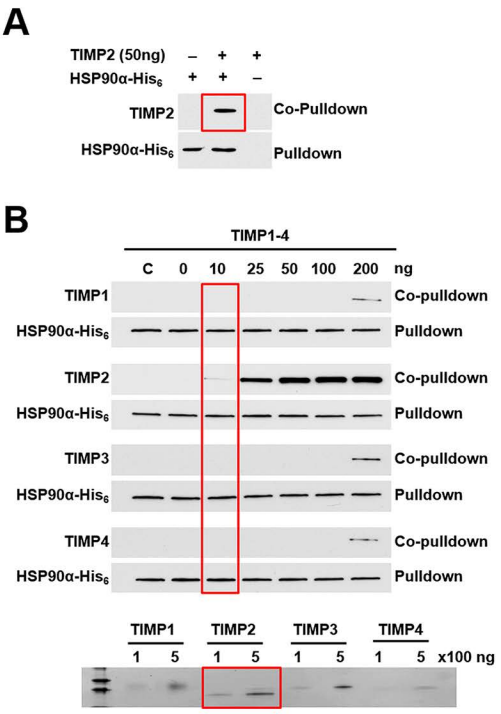
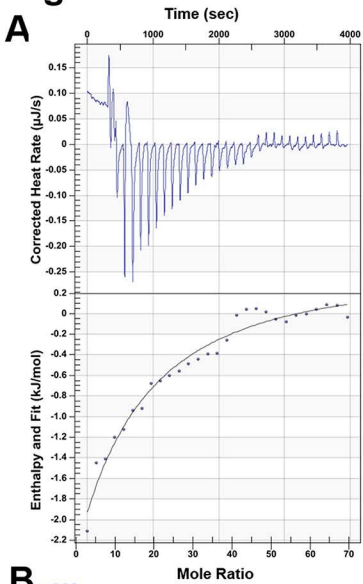


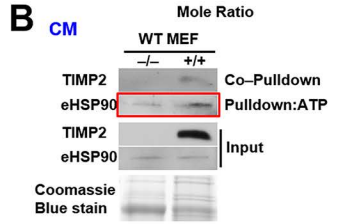
Figure S2 - TIMPs interaction with HSP90. Related to Figure 2

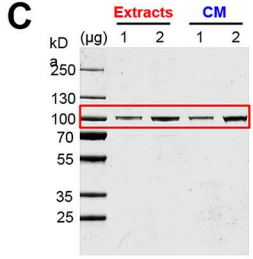
(A) Recombinant HSP90α-His_6 (or no protein control) was bound to Ni-NTA agarose followed by the addition of 50 ng recombinant human TIMP2. Pulldown and co-pulldown proteins were detected by immunoblotting.

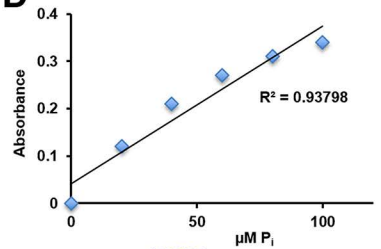
(B) Increasing amounts (ng) of recombinant proteins TIMP1, TIMP2, TIMP3 and TIMP4 were incubated with HSP90α-His_6-bound to Ni-NTA agarose beads. HSP90α was pulled down and interaction with each TIMP was determined by co-immunoprecipitation and immunoblotting. C, control HSP90α without TIMP. Coomassie brilliant blue was performed to confirm purity and equal loaded indicated protein levels (bottom, coomassie gel image).

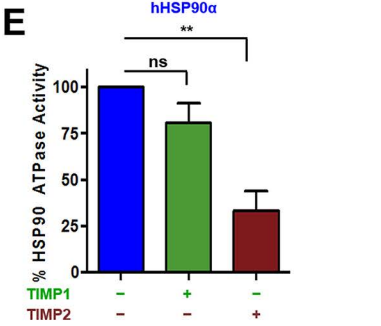
Figure S3

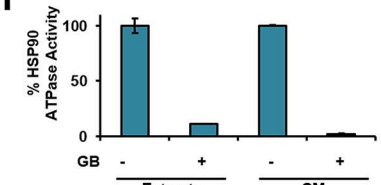

A
Time (sec)
Corrected Heat Rate (µJ/s)
Enthalpy and Fit (kJ/mol)
Mole Ratio


B
CM
WT MEF
–/–
+/+
TIMP2
eHSP90
Co–Pulldown
Pulldown:ATP
TIMP2
eHSP90
Input
Coomassie
Blue stain


C
Extracts
CM
kD
(µg)
1
2
1
2
250
130
100
70
55
35
25


D
Absorbance
R² = 0.93798
µM Pi


E
hHSP90α
**
ns
% HSP90 ATPase Activity
TIMP1
TIMP2


F
% HSP90 ATPase Activity
GB
Extracts
CM

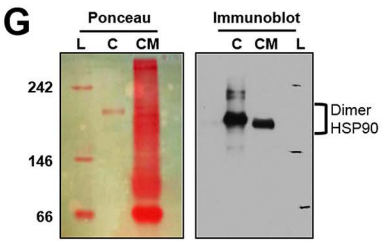

G
Ponceau
Immunoblot
L
C
CM
C
CM
L
242
146
66
Dimer HSP90

Figure S3 - TIMP2 effect on HSP90 function. Related to Figure 3

(A) ITC analysis of HSP90-His_6 binding to titrated ATP. Corrected heat rate (μJ/s) and Enthalpy/Fit (kJ/mol) are presented at the top and bottom respectively.

(B) ATP-beads were incubated with CM from wild type (+/+) and *TIMP2* knock-out (-/-) MEFs. eHSP90 and TIMP2 binding to ATP-beads were assessed by immunoblotting. Coomassie Blue stain (loading control for CM).

(C) HSP90α-FLAG was purified from HEK293 cell extracts and conditioned media (*CM*). Immunoprecipitated proteins were quantified and analyzed for purity by coomassie brilliant blue staining. Representative from at least n=2 independent purifications.

(D) Inorganic phosphate (P_i) standard curve with linear fit line was created from serial dilutions (0 to 100 μM). Graph is representative of n=3 independent experiments.

(E) ATPase activity of recombinant HSP90-His_6 was measured in the presence of 10ng (4.4 nM) of TIMP2 or TIMP1. Activity is shown as a % of HSP90-His_6 in the absence of TIMP. Error bars represent SEM of at least n=2 independent experiments, (ns, not significant).

(F) HEK293 cell extracts and CM-purified HSP90α ATPase activity. Measurements of P_i released per min from HSP90 ATP hydrolysis is shown both for cell extracts and CM and in the presence/absence of Ganetespib. Representative of n=3 independent experiments.

(G) CM from HEK293 cells transiently transfected with HSP90α-His_6 was separated on a native PAGE alongside recombinant HSP90-His_6 control (C). Ponceau S staining of nitrocellulose membrane is shown as loading control with subsequent immunoblot of the native PAGE. L= ladder

Significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Figure S4

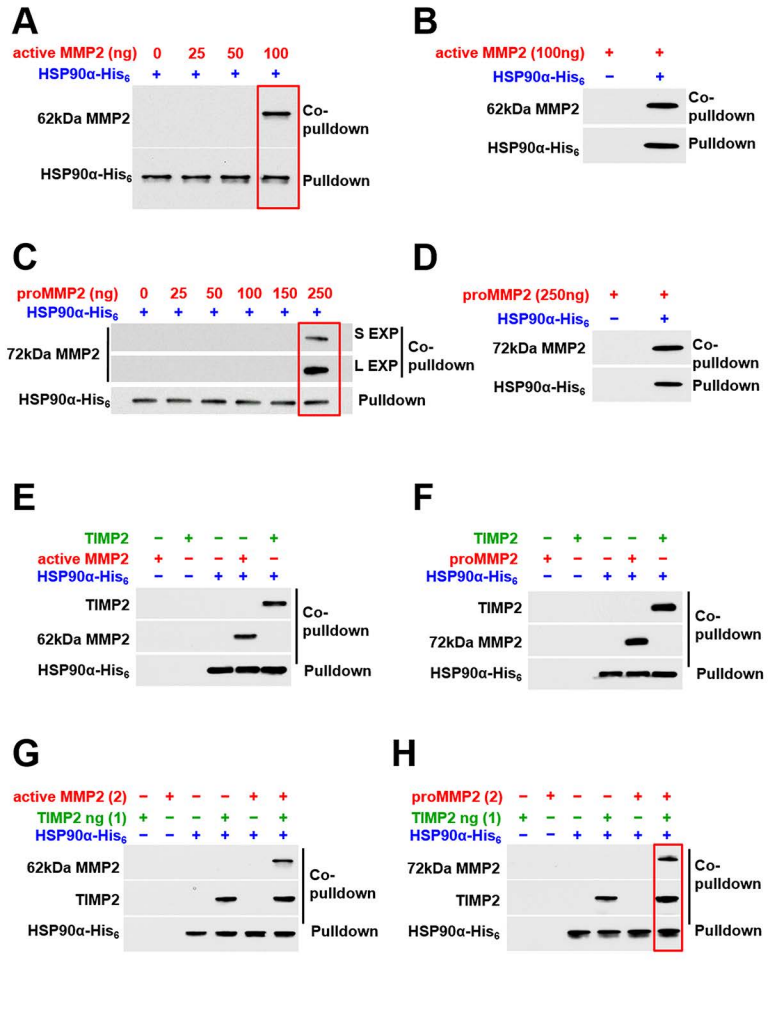

A
active MMP2 (ng) 0 25 50 100
HSP90α-His6 + + + +
62kDa MMP2
HSP90α-His6
Co-pulldown
Pulldown
B
active MMP2 (100ng) + +
HSP90α-His6 − +
62kDa MMP2
HSP90α-His6
Co-pulldown
Pulldown
C
proMMP2 (ng) 0 25 50 100 150 250
HSP90α-His6 + + + + + +
72kDa MMP2
HSP90α-His6
S EXP
L EXP
Co-pulldown
Pulldown
D
proMMP2 (250ng) + +
HSP90α-His6 − +
72kDa MMP2
HSP90α-His6
Co-pulldown
Pulldown
E
TIMP2 − + − − +
active MMP2 + − − + −
HSP90α-His6 − − + + +
TIMP2
62kDa MMP2
HSP90α-His6
Co-pulldown
Pulldown
F
TIMP2 − + − − +
proMMP2 + − − + −
HSP90α-His6 − − + + +
TIMP2
72kDa MMP2
HSP90α-His6
Co-pulldown
Pulldown
G
active MMP2 (2) − + − − + +
TIMP2 ng (1) + − − + − +
HSP90α-His6 − − + + + +
62kDa MMP2
TIMP2
HSP90α-His6
Co-pulldown
Pulldown
H
proMMP2 (2) − + − − + +
TIMP2 ng (1) + − − + − +
HSP90α-His6 − − + + + +
72kDa MMP2
TIMP2
HSP90α-His6
Co-pulldown
Pulldown

Figure S4 – *In vitro* HSP90 interaction with TIMP2 and MMP2. Related to Figure 4

(A-B) Recombinant HSP90α-His_6 (or no protein control) was bound to Ni-NTA agarose followed by the addition of 0-100 ng recombinant 62kDa MMP2.

(C-D) Recombinant HSP90α-His_6 (or no protein control) was bound to Ni-NTA agarose followed by the addition of 0-250 ng recombinant 72kDa proMMP2.

(E-F) HSP90α-His_6 recombinant protein (or no protein control) was bound to Ni-NTA resin, 62kDa active MMP2 or 72kDa pro-MMP2 or TIMP2 were subsequently added.

(G-H) Ni-NTA resin was incubated with HSP90α-His_6 (or no protein control), TIMP2 (*1*), and 62kDa active or 72kDa pro-MMP2 (*2*) were added sequentially.

Pulldown and co-pulldown proteins were detected by immunoblotting with indicated antibodies.

Figure S5

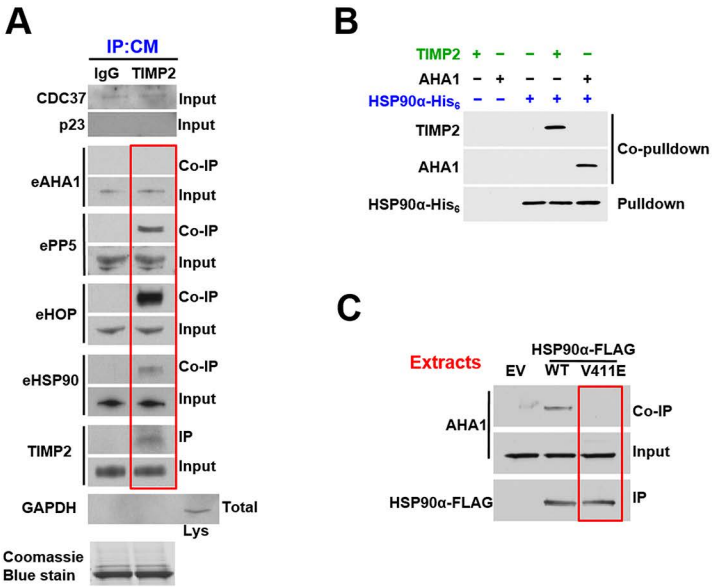


Figure S5 – TIMP2 interaction with secreted co-chaperones. Related to Figure 5

(A) Endogenous TIMP2 was immunoprecipitated from HEK293 cell-conditioned media (CM) using anti-TIMP2 antibody or IgG control. Presence of other co-chaperones in the CM (input) was determined by immunoblotting using antibodies against each protein. Co-immunoprecipitation was performed to determine interaction of TIMP2 with secreted co-chaperones. GAPDH was used as a control for intracellular protein present only in cell extracts (ext). Coomassie Blue stain (loading control for CM).

(B) Ni-NTA resin was left empty or bound to HSP90α-His_6. AHA1 and TIMP2 were subsequently added to the resin. Following pulldown, HSP90α-His_6, AHA1, and TIMP2 levels were determined by immunoblot.

(C) HSP90α-FLAG mutant V411E (AHA1 non-binding) and WT HSP90α-FLAG were transiently transfected into HEK293-T cells. Following serum starvation, cell extracts bound to FLAG affinity agarose and immunoprecipitated for HSP90 (IP) and AHA1 (Co-IP).

Figure S6

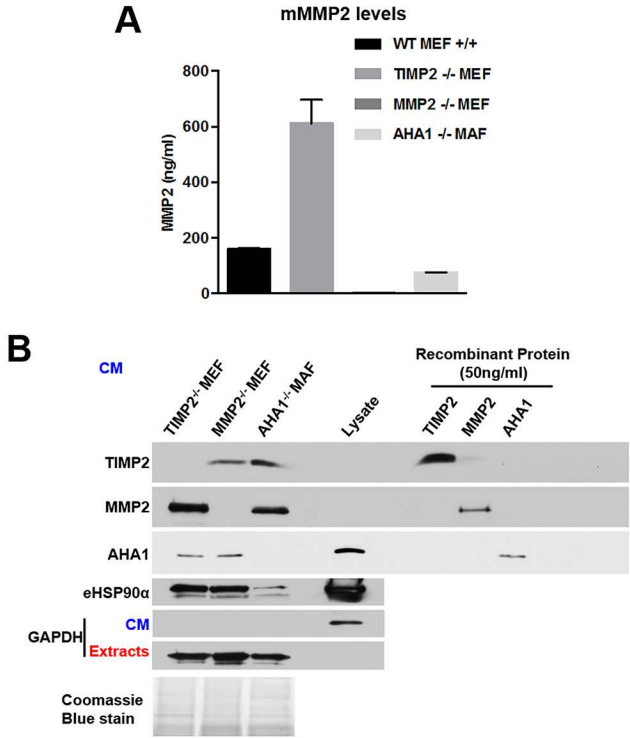


Figure S6 – Extracellular protein levels. Related to Figure 6

(A) Enzyme-linked immunosorbent assay (ELISA) was performed to determine secreted levels of MMP2 in mouse fibroblast CM. Error bars represent the SEM of at least n=2 independent experiments.

(B) Following normalization to cell extracts, TIMP2-/- MEF, and MMP2-/- MEF, AHA1-/- MAF CM were analyzed by immunoblot. Protein expression was determined using indicated antibodies in cell extracts and CM. MEF WT extract control (total protein 10 μg) or recombinant proteins (50 ng/ml) were added for comparison. Coomassie Blue stain (loading control for CM).

Figure S7

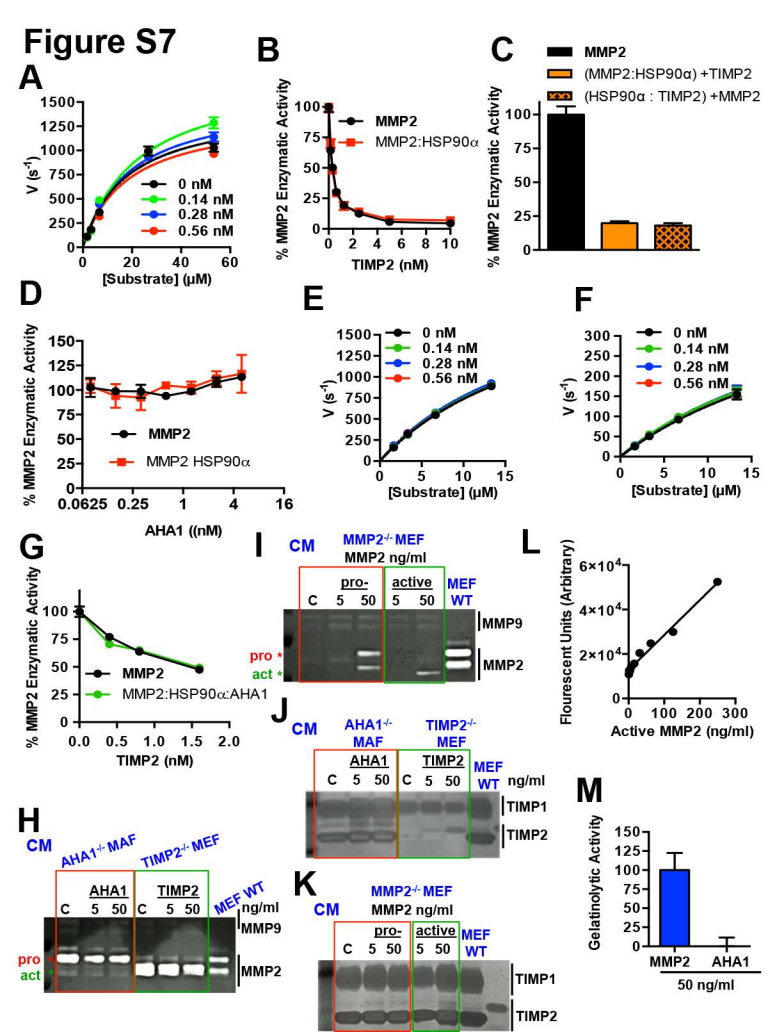

A
V (s-1)
[Substrate] (μM)
0 nM
0.14 nM
0.28 nM
0.56 nM
B
% MMP2 Enzymatic Activity
TIMP2 (nM)
MMP2
MMP2:HSP90α
C
MMP2
(MMP2:HSP90α) +TIMP2
(HSP90α : TIMP2) +MMP2
% MMP2 Enzymatic Activity
D
% MMP2 Enzymatic Activity
MMP2
MMP2 HSP90α
AHA1 ((nM)
E
V (s-1)
[Substrate] (μM)
0 nM
0.14 nM
0.28 nM
0.56 nM
F
V (s-1)
[Substrate] (μM)
0 nM
0.14 nM
0.28 nM
0.56 nM
G
% MMP2 Enzymatic Activity
MMP2
MMP2:HSP90α:AHA1
TIMP2 (nM)
I
CM
MMP2-/- MEF
MMP2 ng/ml
pro-
active
MEF WT
C 5 50 5 50
MMP9
MMP2
pro *
act *
L
Flourescent Units (Arbitrary)
Active MMP2 (ng/ml)
J
CM
AHA1-/- MAF
TIMP2-/- MEF
AHA1
TIMP2
MEF WT
ng/ml
C 5 50 C 5 50
TIMP1
TIMP2
M
Gelatinolytic Activity
MMP2
AHA1
50 ng/ml
H
CM
AHA1-/- MAF
TIMP2-/- MEF
AHA1
TIMP2
MEF WT
ng/ml
C 5 50 C 5 50
MMP9
MMP2
pro *
act *
K
CM
MMP2-/- MEF
MMP2 ng/ml
pro-
active
MEF WT
C 5 50 5 50
TIMP1
TIMP2

Figure S7 – HSP90, TIMP2 and AHA1 impact on MMP2 enzymatic activity. Related to Figure 7

(A) MMP2 was pre-incubated with increasing concentrations of recombinant HSP90α and the rate of an MMP2 substrate hydrolysis was determined. Varying concentrations of a fluorescent MMP2 peptide substrate were used and values showing the rate of hydrolysis were fit to the Michaelis-Menten model. Graph is representative from n=3 independent experiments.

(B) Varying concentrations of recombinant TIMP2 (0-10 nM) were added to MMP2 alone or to the MMP2:HSP90α complex. The rate of MMP2 substrate hydrolysis is shown as percentage compared to untreated control. Graph is representative from n=3 independent experiments.

(C) Active MMP2 was incubated with HSP90 with and without TIMP2 pre-incubation for 1 hour. The rate of MMP2 substrate hydrolysis is shown as percentage compared to control MMP2 alone. Error bars represent the SEM. Graph is representative from n=2 independent experiments.

(D) MMP2 was pre-incubated with and without HSP90α prior to addition of different amounts of human recombinant AHA1 (0-10 nM). % MMP2 activity was determined. Error bars represent SEM of n=2 independent experiments.

(E-F) MMP2 (without or with preincubation with HSP90α-His_6) was incubated with different amounts of recombinant AHA1. MMP2 activity was determined at varying concentrations of a specific fluorescent MMP2 peptide substrate. Graph is representative from n=3 independent experiments.

(G) MMP2 was pre-incubated alone or with recombinant HSP90α and AHA1, at a stoichiometry of 1:1:1. MMP2 activity was measured in the absence and presence of varying concentrations of TIMP2. The rate of substrate hydrolysis is shown as percentage compared to control MMP2 alone or MMP2:HSP90α:AHA1. Graph is representative of n=3 independent experiments.

(H-I) $AHA1^{-/-}$ MAF or $TIMP2^{-/-}$ MEF or $MMP2^{-/-}$ MEF cells were left untreated (C) or treated with increasing amounts (0-50ng/ml) of AHA1 or TIMP2 or MMP2, respectively. Gelatin zymography was performed and net MMP gelatinase activity (MMP2 and MMP9) was detected.

(J-K) $AHA1^{-/-}$ MAF or $TIMP2^{-/-}$ MEF or $MMP2^{-/-}$ MEF were left untreated (*C*) or treated with increasing amounts (0-50 ng/ml) of AHA1 or TIMP2 or MMP2. Reverse zymography was performed and TIMP2 inhibitory activity on MMP2-mediated gelatin degradation was detected.

(L) Standard curve of gelatinolytic activity of 62kDa active MMP2 (0-300 ng/ml) was generated in DQ gelatin degradation assay. End point fluorescence was measured. Graph is representative of n=3 independent experiments.

(M) 62kDa active MMP2 or AHA1 were incubated with DQ fluorescent gelatin at 50 ng/ml. Gelatinolytic activity was measured as an end point fluorescence. Error bars are SEM of n=3 independent experiments.

Table S1 – Primer sequences used in qRT-PCR. Related to STAR Methods.

Primer Name	Sequence
Human TIMP1 qRT-PCR Fwd	ACAGACGGCCTTCTGCAATTC
Human TIMP1 qRT-PCR Rev	CCTTTATACATCTTGGTCATCTTGATCTC
Human TIMP2 qRT-PCR Fwd	ACAGGCGTTTTGCAATGCA
Human TIMP2 qRT-PCR Rev	GGGTTGCCATAAATGTCGTTTC
Human TIMP3 qRT-PCR Fwd	TGCTCTCTGTCTCTTTTTTCAGCTT
Human TIMP3 qRT-PCR Rev	CTACAGTGTGTTGTCTGCTGCTTTT
Human TIMP4 qRT-PCR Fwd	CACCTGCCTCTCAGGAAGGA
Human TIMP4 qRT-PCR Rev	GGCTTGATCTTCAGGACTCTTGA
Human HSP70 qRT-PCR Fwd	AGGCCAACAAGATCACCATC
Human HSP70 qRT-PCR Rev	TCGTCCTCCGCTTTGTACTT
Human GAPDH qRT-PCR Fwd	GGAAGGTGAAGGTCGGAGTCA
Human GAPDH qRT-PCR Rev	GCAACAATATCCACTTTACCAGAGTTAA
Mouse TIMP2 qRT-PCR Fwd	CACGCTTAGCATCACCCAGA
Mouse TIMP2 qRT-PCR Rev	GTCAGCCTTCTTACGGGTCC
Mouse HSPA1A/B qRT-PCR Fwd	AGGTGCTGGACAAGTGCCAG
Mouse HSPA1A/B qRT-PCR Rev	AACTCCTCCTTGTCGGCCA
Mouse HSPA1A/B qRT-PCR Probe	FAM-CATCTCCTGGCTGGACTCCAACACG-BHQ
Mouse 18sRNA qRT-PCR Fwd	GCAATTATTCCCCATGAACG
Mouse 18sRNA qRT-PCR Rev	GGGACTTAATCAACGCAAGC
Mouse 18sRNA qRT-PCR Probe	FAM-TTCCCAGTAAGTGCG GGTC-BHQ