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Supplemental Information

Extracellular Phosphorylation of TIMP-2 by Secreted c-Src Tyrosine Kinase Controls MMP-2 Activity

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Figure S1

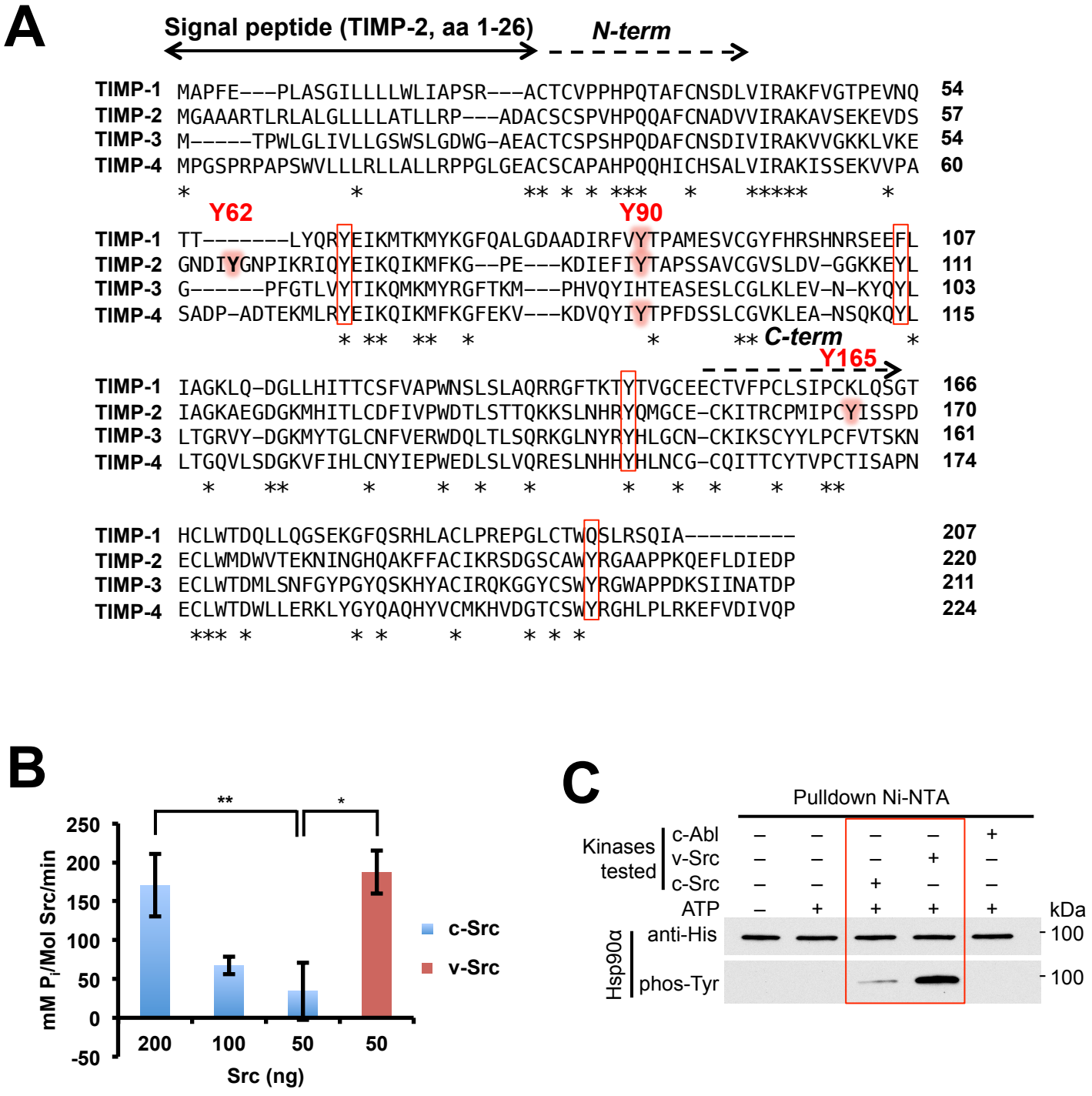
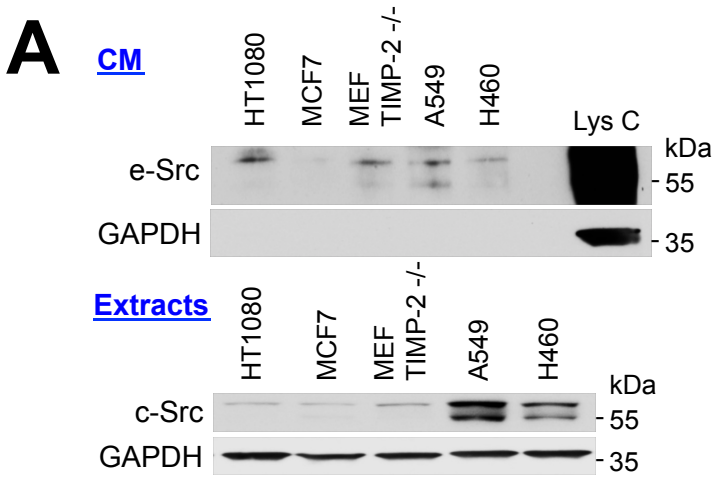

A
Signal peptide (TIMP-2, aa 1-26)
N-term
TIMP-1 MAPFE---PLASGILLLLWLIAPSR---ACTCVPPHPQTAFCNSDLVIRAKFVGTPEVNQ 54
TIMP-2 MGAAARTLRLALGLLLLATLLRP---ADACSCSPVHPQQAFCNADVVIRAKAVSEKEVDS 57
TIMP-3 M-----TPWLGLIVLLGSWSLGDWG-AEACTCSPSHPQDAFCNSDIVIRAKVVGKKLVKE 54
TIMP-4 MPGSPRPAPSWVLLLRLLALLRPPGLGEACSCAPAHPQQHICHSALVIRAKISSEKVVPA 60
Y62
Y90
TIMP-1 TT-------LYQRYEIKMTKMYKGFQALGDAADIRFVYTPAMESVCGYFHRSHNRSEEFL 107
TIMP-2 GNDIYGNPIKRIQYEIKQIKMFKG--PE---KDIEFIYTAPSSAVCGVSLDV-GGKKEYL 111
TIMP-3 G------PFGTLVYTIKQMKMYRGFTKM---PHVQYIHTEASESLCGLKLEV-N-KYQYL 103
TIMP-4 SADP-ADTEKMLRYEIKQIKMFKGFEKV---KDVQYIYTPFDSSLCGVKLEA-NSQKQYL 115
C-term
Y165
TIMP-1 IAGKLQ-DGLLHITTCSFVAPWNSLSLAQRRGFTKTYTVGCEECTVFPCLSIPCKLQSGT 166
TIMP-2 IAGKAEGDGKMHITLCDFIVPWDTLSTTQKKSLNHRYQMGCE-CKITRCPMIPCYISSPD 170
TIMP-3 LTGRVY-DGKMYTGLCNFVERWDQLTLSQRKGLNYRYHLGCN-CKIKSCYYLPCFVTSKN 161
TIMP-4 LTGQVLSDGKVFIHLCNYIEPWEDLSLVQRESLNHHYHLNCG-CQITTCYTVPCTISAPN 174
TIMP-1 HCLWTDQLLQGSEKGFQSRHLACLPREPGLCTWQSLRSQIA--------- 207
TIMP-2 ECLWMDWVTEKNINGHQAKFFACIKRSDGSCAWYRGAAPPKQEFLDIEDP 220
TIMP-3 ECLWTDMLSNFGYPGYQSKHYACIRQKGGYCSWYRGWAPPDKSIINATDP 211
TIMP-4 ECLWTDWLLERKLYGYQAQHYVCMKHVDGTCSWYRGHLPLRKEFVDIVQP 224
B
mM Pi/Mol Src/min
250
200
150
100
50
0
-50
**
*
200
100
50
50
Src (ng)
c-Src
v-Src
C
Pulldown Ni-NTA
Kinases tested
c-Abl – – – – +
v-Src – – – + –
c-Src – – + – –
ATP – + + + +
kDa
Hsp90α
anti-His
100
phos-Tyr
100

Figure S1. [Alignment of human TIMPs protein family and *in vitro* kinase assay], Related to Figure 1

(**A**) Protein sequence alignment (ClustalW) of full length human TIMPs (TIMP-1 to -4). Asterisks (*) indicate conserved amino acid residues. Tyrosine residues (Y) subjected to phosphorylation are highlighted in red and tyrosine residues not targeted for phosphorylation are outlined in red.
(**B**) *In vitro* kinase activity of recombinant c-Src-GST and v-Src-GST tyrosine kinases as determined by measuring the amount of free inorganic phosphate in solution released for each active kinase in the presence of ATP. The experiment was carried out in duplicate. All the data represent mean ± SD. A Student's *t*-test was performed to assess statistical significance (*P<0.05 and **P<0.005).
(C) *In vitro* kinase assay to determine human Hsp90α phosphorylation by tested kinases. Hsp90α-His_6 pulldown and immunoblotting were performed to assess Hsp90α tyrosine phosphorylation using anti-His (for Hsp90α) and anti-pan-phos-Tyr antibodies.

Figure S2


A
CM
HT1080
MCF7
MEF
TIMP-2 -/-
A549
H460
Lys C
kDa
e-Src
55
GAPDH
35
Extracts
HT1080
MCF7
MEF
TIMP-2 -/-
A549
H460
kDa
c-Src
55
GAPDH
35

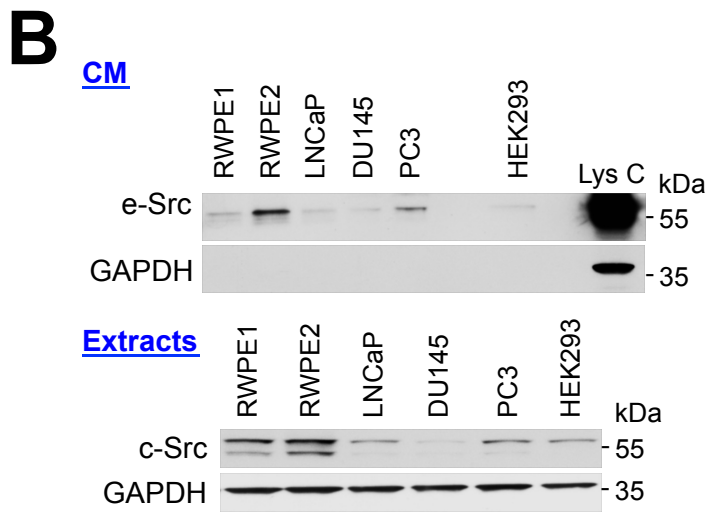

B
CM
RWPE1
RWPE2
LNCaP
DU145
PC3
HEK293
Lys C
kDa
e-Src
55
GAPDH
35
Extracts
RWPE1
RWPE2
LNCaP
DU145
PC3
HEK293
kDa
c-Src
55
GAPDH
35

Figure S2. [c-Src tyrosine kinase is present in cell conditioned media], Related to Figure 2

(**A)** Conditioned media (CM) and cell extracts were collected from human fibrosarcoma HT1080, human breast cancer MCF7, mouse embryonic fibroblasts MEF TIMP-2-/- ras/myc, human lung cancer A549 and H460;
B) and from immortalized (HEK293H and normal prostate epithelial RWPE1) or tumorigenic derivative RWPE2, human prostate tumor cell lines LNCaP, DU145 and PC3. GAPDH indicates equal cellular (extracts) protein content.

Figure S3

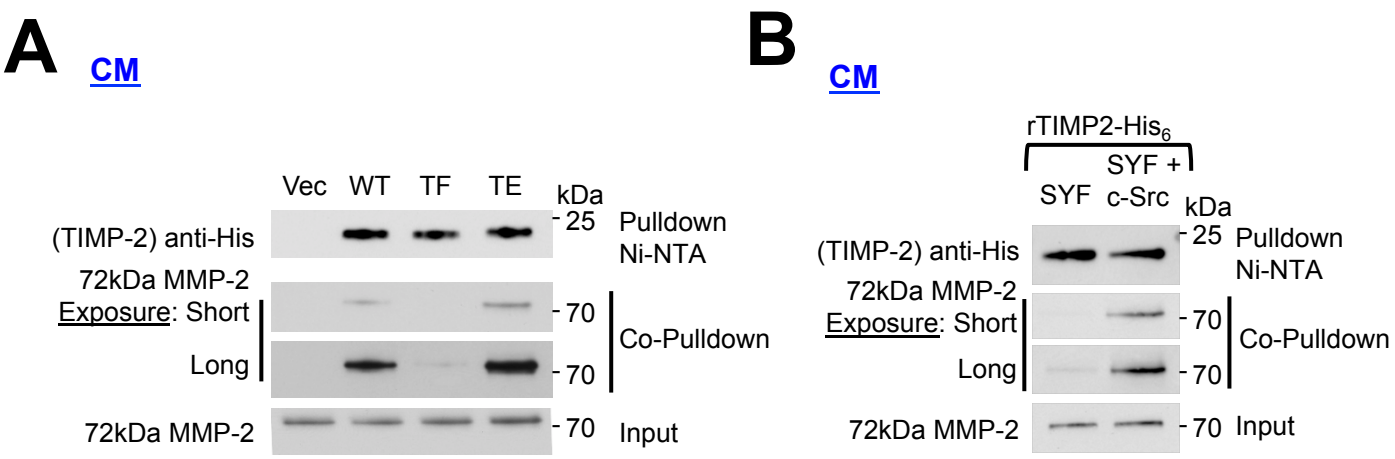
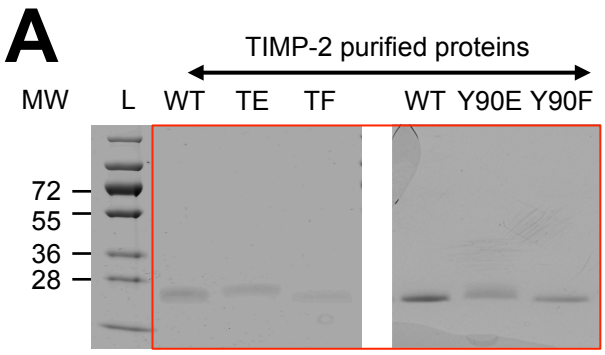

A
CM
Vec
WT
TF
TE
kDa
(TIMP-2) anti-His
25
Pulldown
Ni-NTA
72kDa MMP-2
Exposure: Short
70
Co-Pulldown
Long
70
72kDa MMP-2
70
Input
B
CM
rTIMP2-His6
SYF +
SYF
c-Src
kDa
(TIMP-2) anti-His
25
Pulldown
Ni-NTA
72kDa MMP-2
Exposure: Short
70
Co-Pulldown
Long
70
72kDa MMP-2
70
Input

Figure S3. [TIMP-2:MMP-2 interaction depends on TIMP-2 phosphorylation], Related to Figure 3

(**A**) HEK293H cells were transiently transfected with WT, TF (Y62F/Y90F/165F) and TE (Y62E/Y90E/Y165E) TIMP-2 mutants. CM were collected for pulldown and co-pulldown experiments and immunoblotted as indicated to assess interaction.
(B) SYF and SYF+c-Src cells were treated with 50ng/ml of exogenous recombinant TIMP-2-His_6 and CM were collected for immunobloting pulldown, co-pulldown experiments.

Figure S4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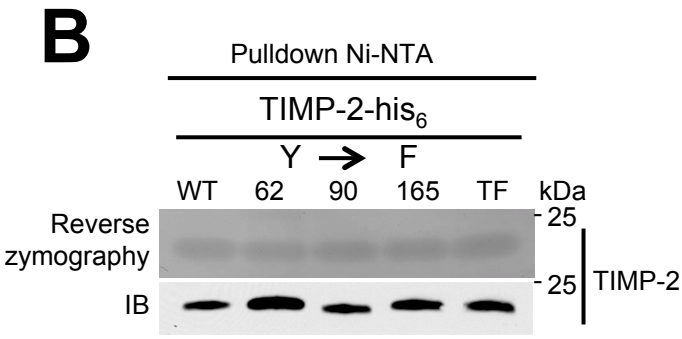
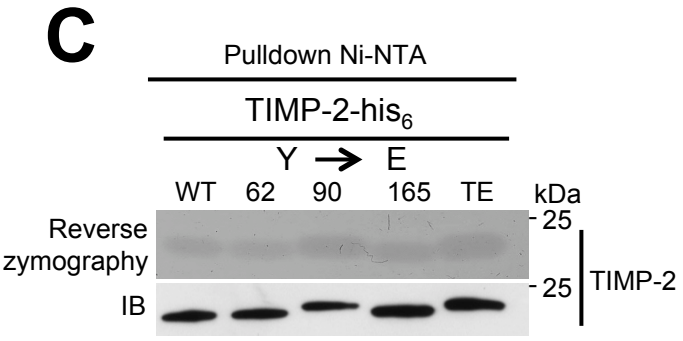
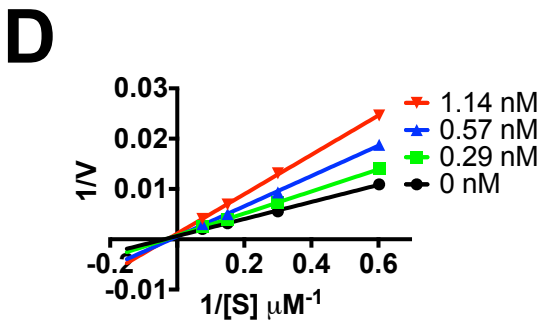
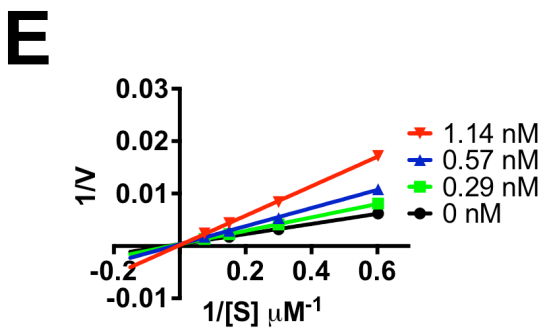
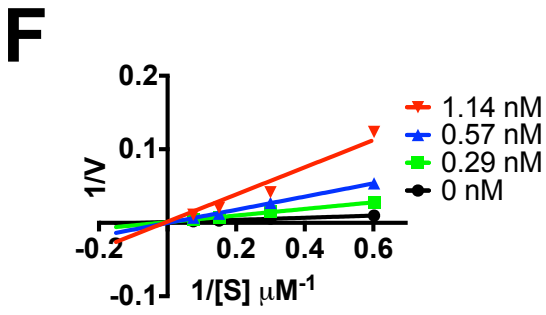
TIMP-2 purified proteins
MW
L
WT
TE
TF
WT
Y90E
Y90F
72
55
36
28


B
Pulldown Ni-NTA
TIMP-2-his6
Y → F
WT
62
90
165
TF
kDa
Reverse zymography
IB
25
25
TIMP-2


C
Pulldown Ni-NTA
TIMP-2-his6
Y → E
WT
62
90
165
TE
kDa
Reverse zymography
IB
25
25
TIMP-2


D
0.03
0.02
0.01
1/V
-0.2
-0.01
0.2
0.4
0.6
1/[S] μM-1
1.14 nM
0.57 nM
0.29 nM
0 nM


E
0.03
0.02
0.01
1/V
-0.2
-0.01
0.2
0.4
0.6
1/[S] μM-1
1.14 nM
0.57 nM
0.29 nM
0 nM


F
0.2
0.1
1/V
-0.2
-0.1
0.2
0.4
0.6
1/[S] μM-1
1.14 nM
0.57 nM
0.29 nM
0 nM

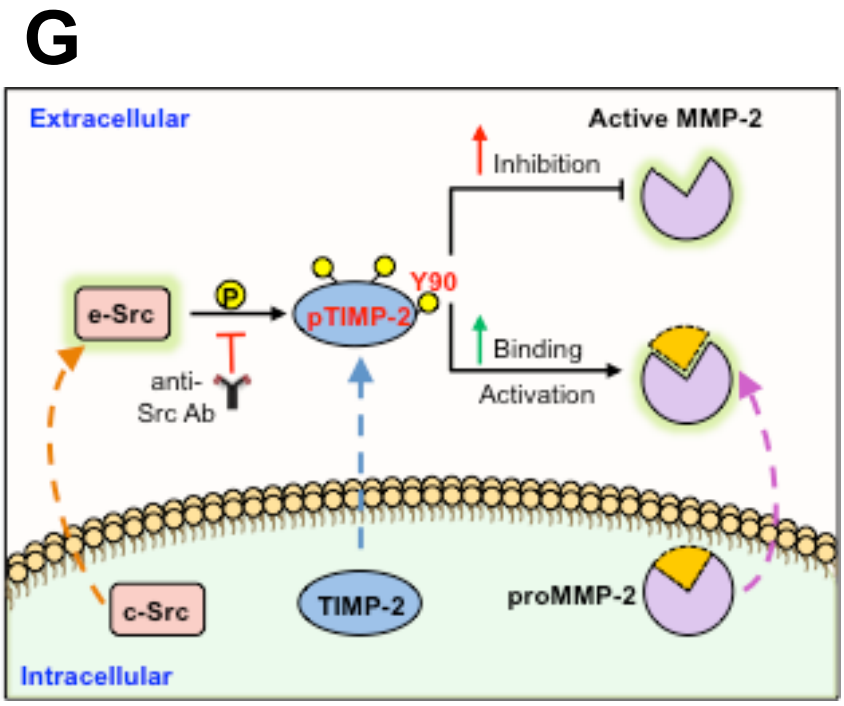

G
Extracellular
Active MMP-2
Inhibition
e-Src
P
pTIMP-2
Y90
anti-
Src Ab
Binding
Activation
c-Src
TIMP-2
proMMP-2
Intracellular

Figure S4. [TIMP-2 WT and mutant protein function and effect on MMP-2 activity], Related to Figure 4

(**A**) Purified WT and mutant proteins (TE, TF, Y90E and Y90F) were analyzed for purity in coomassie stained gels.
(**B)** Purified TIMP-2-His_6 (WT and non-phosphorylatable F mutants;
(C) or phosphomimetic E mutants, were tested for MMP-2 inhibitory activity by reverse zymography. Equal amounts of protein were analyzed by western blot (IB).
(**D-F**) Lineweaver-Burke plots for the inhibition of active MMP-2 by purified TIMP-2-His_6 WT (D), Y90F (E) and Y90E (F).
(G) A Model for the extracellular phosphorylation of TIMP-2 and effect on MMP-2 activity. Related to discussion.

Table S1. [Information of antibodies used], Related to Figures 1-4

Antibody	Company	Cat Number	Dilutions used
pan-phosphotyrosine (4G10)	Millipore	#05-1050	1:1,000
6x-his	Thermo Fisher Scientific	#MA1-21315	1:2,000 - 1:20,000
TIMP-2 (D18B7)	Cell Signaling	#5738	1:1,000 - 1:5,000
TIMP-2 (T2-101)	Abcam	#ab3161	1:1,000
MMP-2 (D8N9Y)	Cell Signaling	#13132	1:1,000 - 1:2,000
MMP-2	Millipore	#MAB3308	1:1,000 - 1:4,000
GAPDH	Enzo Life Sciences	#ADI-CSA-335-E	1:10,000 - 1:20,000
c-Src (36D10)	Cell Signaling	#2109	1:1,000
c-Src (L4A1)	Cell Signaling	#2110	1:1,000
c-Src (32G6) mAb1	Cell Signaling	#2123	1:1,000
c-Src (327537)	R&D Systems	MAB3389	1:1000
Mouse IgG_{2A} Isotype Control	R&D Systems	MAB003	1:1000
goat anti-mouse IgG-HRP	Santa Cruz Biotechnology	#sc-2005	1:4,000
goat anti-rabbit IgG-HRP	Santa Cruz Biotechnology	#sc-2004	1:4,000

Table S2. [Generated constructs], Related to Figure 1 and 3

Mammalian expression vectors	Generated constructs	Other names used	Number of mutated tyrosines introduced
pcDNA3.3 TOPO	TIMP-2-6xHis-wt	WT	No mutation
	TIMP-2-6xHis-Y62F	Y62F	One
	TIMP-2-6xHis-Y71F	Y71F	One
	TIMP-2-6xHis-Y90F	Y90F	One
	TIMP-2-6xHis-Y110F	Y110F	One
	TIMP-2-6xHis-Y148F	Y148F	One
	TIMP-2-6xHis-Y165F	Y165F	One
	TIMP-2-6xHis-Y204F	Y204F	One
	TIMP-2-6xHIS Y62E	Y62E	One
	TIMP-2-6xHIS Y90E	Y90E	One
	TIMP-2-6xHIS Y165E	Y165E	One
	TIMP-2-6xHIS Y62F/Y90F	Y62F/Y90F	Two
	TIMP-2-6xHIS Y62F/Y165F	Y62F/Y165F	Two
	TIMP-2-6xHIS Y90F/Y165F	Y90F/Y165F	Two
	TIMP-2-6xHIS Y90E/Y165F	Y90E/165F	Two
	TIMP-2-6xHis-Y62F/Y90F/Y165F	Triple F (TF)	Three
	TIMP-2-6xHis-Y62E/Y90E/Y165E	Triple E (TE)	Three
	TIMP-2-6xHis-Y62F/Y90E/Y165F	Y62F/Y90E/Y165F	Three
	TIMP-2-6xHis-Y62E/Y90E/Y165F	Y62E/Y90E/Y165F	Three

Table S4. [PCR mutagenesis primer sequences], Related to Figure 3

TIMP-2 mutant	Primer sequences
TIMP-2-Y62E	Forward: 5'-GAC TCT GGA AAC GAC ATT **GAG** GGC AAC CCT ATC AAG AGG
	Reverse: 5'-CCT CTT GAT AGG GTT GCC **CTC** AAT GTC GTT TCC AGA GTC
TIMP-2-Y90E	Forward: 5'-AAG GAT ATA GAG TTT ATC **GAG** ACG GCC CCC TCC TCG GCA
	Reverse: 5'-TGC CGA GGA GGG GGC CGT **CTC** GAT AAA CTC TAT ATC CTT

Table S5. [Buffer composition], Related to Figure 4

NP40 Lysis buffer	TBST	Protein loading buffer	TIMP-2 Reconstitution buffer
0.1% or 1% NP40 (IGEPAL)	2.42g Trizma	1.25 mL Tris pH 6.8 1M	50 mM Tris pH 7.4
1 mM MgCl2	8g NaCl	1mL Glycerol	10 mM CaCl2
100 mM NaCl	1.3 mL HCl	1mL 20% SDS	150 mM NaCl
20 mM Tris pH 7.4	1 mL Tween	215µl Bromophenol blue	0.05% Brij-35 pH 7.5
20 nM sodium molybdate	1 L dH2O	500µl Beta-mercaptoethanol	
1 phos stop tablet		6mL dH20	
1 protease inhibitor tablet			

TRANSPARENT METHODS

Cell culture, primers, plasmids, transfection and antibodies

Cell lines were purchased from ATCC and cultured at low passages. No authentication method was used. Experiments were performed in cell cultures maintained up to one month before they were renewed. Cell lines had been tested for mycoplasma contamination at the early stages of the experiments. All cultures were analyzed when cells reached up to 70% confluency. HEK293H, SYF, SYF+c-Src, HT1080, MCF7, MEF TIMP-2 $^{-/-}$ ras/myc, A549 were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen). RWPE1 and RWPE2 were cultured in Keratinocyte Serum Free Medium (K-SFM) supplemented with 2% FBS (Invitrogen). LNCaP, DU145, PC3 and H460 were cultured in RPMI supplemented with 10% FBS. MEF TIMP-2 $^{-/-}$ ras/myc was obtained from Dr Soloway (Wang et al., 2000). Wild type TIMP-2 and mutants contained a Six-Histidine (His_6) tag at their carboxyl-terminus. HEK293H cells (293H) were cultured overnight. The next day they were transfected using *Trans*iT-2020 Reagent (Mirus, #MIR5405) with 2µg plasmid DNA by manufacturer's protocol. For conditioned media (CM) collection, culture media were replaced with serum free media for an additional 24 hours. Media collected for analysis (see below). Gene synthesis, site-directed mutagenesis and sequence verification were performed by Genewiz and/or using in-house PCR: PfuUltra HotStart (Agilent Technologies) and primers (Eurofins MWG Operon). See Table S2. [Generated constructs]. Mutagenesis primers used in this study are listed in Table S4. [PCR mutagenesis primer sequences]. The following antibodies were used in this study: anti-pan-phosphotyrosine (4G10) (Millipore, #05-1050), anti-6x-His (ThermoFisher Scientific, #MA1-21315), anti-TIMP-2 (Cell Signaling, #5738 and Abcam, ab3161), anti-MMP-2 antibodies (Cell Signaling, #13132 and Millipore, #MAB3308), anti-MMP-9 (Cell Signaling, #3852), anti-GAPDH mAb (Enzo Life Sciences, #ADI-CSA-335-E), anti-c-Src (Cell Signaling, #2109, #2110, #2123), goat anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc., #sc-2005) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., #sc-2004).

Anti-c-Src antibodies for blocking experiments: rabbit anti-Src mAb1 (32G6, biotinylated) (Cell Signaling, #8077), rabbit (DA1E mAb IgG XP™ Isotype control, biotinylated) (Cell Signaling, #4096). Antibody dilution for each assay is provided in Table S1. [Information of antibodies used]. Buffer composition is shown in Table S5. [Buffer composition]. TIMP-2 sequences are provided in Table S3. [Sequences of TIMP-2 constructs]**.**

Immunoblotting

Conditioned media were obtained 24 hours after serum starvation and treatments. Cell media were centrifuged at 1000 rpm for 5 minutes to remove any floating cells without lysing them, and supernatant from this step was used in experiments. Cell extracts were obtained by washing confluent cells with ice-cold PBS, lysing with 0.1% NP40 lysis buffer containing protease and phosphatase inhibitors (Roche) (see Table S5. [Buffer composition]), and centrifuging at 14000 rpm in a microcentrifuge at 4°C for 10 min. Protein concentrations of the resulting supernatants were determined using Bradford assay (Bio-Rad). Equal amounts of protein in 5X protein loading buffer were boiled for 5 min, loaded on 4-20% polyacrylamide gradient gels (Bio-Rad), and electrophoresis was performed in denaturing conditions. After transfer to nitrocellulose membranes (Bio-

Rad), samples were blocked in TBST (TBS + 0.1% Tweenx20) with 5% non-fat dry milk and incubated with primary antibodies at room temperature for 1-2 hours or at 4°C overnight (Table S1. [Information of antibodies used]). Blots were incubated with the appropriate HRP-conjugated secondary antibodies for 1 hour at room temperature. Bands were visualized by incubating with ECL 2 substrate (Thermo Scientific), followed by different exposures to CLASSIC X-Ray film (Research Products International Corp).

Pulldown Ni-NTA and Immunoprecipitation

Following transient transfections, equal amounts of isolated cell extract proteins were incubated with HisPur Ni-NTA Resin (ThermoScientific) for 2 hours at 4°C. Immunopellets were washed 4 times with fresh lysis buffer (20mM HEPES pH 7.0, 100mM NaCl, 1mM $MgCl_2$, 0.1% NP40, protease inhibitor cocktail (Roche) and PhosSTOP (Roche). Proteins bound to Ni-NTA agarose were washed with 50 mM imidazole in lysis buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, protease inhibitor cocktail and PhosSTOP) and eluted with either 300 mM imidazole in lysis buffer or with 5x Laemmli buffer.

Pulldowns were also performed from CM concentrated ~10X using Amicon Ultra 10K centrifugal filters (Millipore) according to the manufacturer's protocol. HiPur Ni-NTA Resin was used to pulldown His_6-tagged TIMP-2 from concentrated CM. Briefly, HisPur Ni-NTA Resin was washed 3 times by vortexing resin with 0.1% NP40 lysis buffer and pipetting off supernatant. Concentrated CM was combined with washed HisPur Ni-NTA Resin, and placed on rotator at 4°C for 1 hour. Wash step was repeated 4 times with 1% NP40 buffer + 150 mM NaCl + 50 mM imidazole to reduce non-specific binding of proteins to resin. 5X protein loading buffer was added to resin and boiled for 5 minutes. Samples were created from supernatant after removing resin by centrifugation at 15000 rpm for 30 seconds. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Precipitated and co-precipitated proteins were detected by immunoblotting with indicated antibodies (Table S1. [Information of antibodies used]).

Immunoprecipitation (IP) of endogenous TIMP-2 was performed by incubating CM with TIMP-2 antibody (T2-101) (or IgG control), followed by protein G agarose for 2 hours at 4°C. Immunoprecipitates were washed four times with fresh lysis buffer (20mM HEPES pH 7.0, 10mM NaCl, 1mM $MgCl_2$, 0.1% NP40, plus protease and phoSTOP inhibitors) and eluted with 5 x Laemmli buffer. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting. Protein inputs were detected using anti-HIs or anti-TIMP-2 specific antibodies. Co-immunoprecipitated proteins were also incubated with specific antibodies (Table S1. [Information of antibodies used]).

***In vitro* kinase assay**

Purified recombinant human TIMP-2-His_6 protein was incubated with 50 µl of Ni-NTA agarose (Qiagen) for 2hr. The Ni-NTA agarose beads were washed with 30mM imidazole and then incubated with 50ng of baculovirus- expressed and purified active cSrc-GST, vSrc-GST and c-Abl (SignalChem) for *in vitro* kinase assay in the presence or absence of ATP (Sigma). When necessary and after extra washes, recombinant human proMMP-2 was added. The assay was carried out in 50mM Tris-HCl (pH7.5),

10mM $MgCl_2$ and 0.2mM ATP, at 28˚C for 15 min. The reaction was quenched by addition of an equal volume of 5xprotein loading buffer and immunoblotting was performed in denaturing conditions (as described above).

Quantification of Src kinase activity

Activity of recombinant human c-Src and v-Src was measured as described the PiPer Phosphate Assay Kit instructions for use (Life Technologies). Standard curve with linear fit line was created from 0 to 100 mM P_i final concentration reactions. 50ng of v-Src and 50, 100, and 200ng of c-Src were run in duplicate, incubated at 37°C for 1 hour with 1mM ATP as substrate. ATP turnover was calculated as mmol Pi per mol Src per minute and relative ATPase activity was calculated from those values, with the value of v-Src representing 100% activity.

Brefeldin A Treatment

HEK293H cells were seeded overnight. Cells were transiently transfected, as described above, with empty vector control or wild-type TIMP-2-His_6 DNA. After 18-20 hrs, brefeldin A (BFA) (eBioscience Inc. San Diego, CA; REF: 00-4506-51, LOT: E00021-1633) was added to the serum free media (1:1000 dilution) for a period of 12 hours, to inhibit the ER-Golgi secretion pathway. DMSO was used as a control in the non-treated cells. Both cell extracts and CM were collected for analysis (see Immunoblotting and pulldown experiments). Pulldowns were performed to assess TIMP-2 tyrosine phosphorylation.

TIMP-2 and anti-c-Src antibody treatments

For experiments related to exogenous TIMP-2 treatment and determination of extra- or intracellular phosphorylation, H1080 cells were seeded overnight, followed by serum starvation for 24 hours. Recombinant TIMP-2-His_6 (250ng/ml) was exogenously added for 2, 8 and 16 hours. Both lysates and CM were collected for immunoblot analyses. Pulldowns Ni-NTA were performed as described above to detect tyrosine phosphorylation.

For the anti-Src blocking experiments in extra- intracellular phosphorylation, serum starved HT1080 cells were treated with anti-Src mAbs or isotype IgG controls for 1 hour followed by addition of TIMP-2 at 250ng/ml for 8 hours.

For proMMP-2 activation and gelatin zymography experiments, HT1080 cells were seeded in a 96-well plate (20,000 cells per well in 100µl) for 24 hours. Media were replaced with serum free media for 24 hours followed by treatments with HEK293H-derived purified wild type TIMP-2-His_6 protein (40ng/ml) for another 24 hours. Conditioned media was collected for gelatin zymography to detect gelatinase activity. This experiment was performed three times using WT TIMP-2 from the same preparation and twice using TIMP-2 protein from different preparations. Media collected and analyzed as described (see Gelatin zymography).

Detection of c-Src in conditioned media (CM) of normal and cancer cell lines

Cell lines were cultured and serum starved for 24 hours. Cell extracts and CM were collected and processed as described above. Non-concentrated protein samples from CM were equalized to the cellular protein levels prior to immunoblotting (see above).

Protein purification of TIMP-2-His_6 WT and mutants from HEK293H conditioned media

To purify TIMP-2-His_6 mutants, we used 50 times concentrated CM from 293H cells in which the TIMP-2-His_6 WT and mutants had been transiently expressed. Precipitation pulldown protocol was followed as described before until the second wash step. After washing resin 4 times with 1% NP40 buffer + 500mM NaCl, TIMP-2 reconstitution buffer containing 0.5 M imidazole was added to resin and placed on rotator at 4^{o}C for 1 hour (Table S5. [Buffer composition]). Samples were centrifuged at 3000 rpm for 30 seconds to release the protein and supernatant was loaded into Amicon Ultra 10K centrifugal filters (EMD Millipore). Resin was washed once with TIMP-2 reconstitution buffer (without imidazole) and this wash was also processed through Amicon Ultra 10K centrifugal filters. Purified TIMP-2-His_6 proteins were concentrated down to ~50 µl. For a second round of purification, this fraction was combined with 1% NP40 buffer + 500 mM NaCl and placed on rotator at 4^{o}C for 15 min. Using Amicon Ultra 50K centrifugal filters (Millipore), samples were concentrated down to ~100 µl. Flow-through was kept for analysis. Concentrated sample above filter was then washed with 1% NP40 buffer + 500 mM NaCl and concentrated down to ~100 µl again. This wash step was repeated 3 times. Combined flow-through was saved and placed in Amicon Ultra 10K centrifugal filters to concentrate the purified TIMP-2-His_6 samples and remove salt from buffer. Samples were concentrated down to ~30 µl, and flow-through from this step was discarded. Buffer exchange was done by placing TIMP-2 reconstitution buffer above filter and concentrating again to yield a final volume of ~30 µl. The purity of the isolated proteins was examined by Coomassie staining of SDS-PAGE gels using GelCode Blue Safe Protein Stain (Thermo Scientific). TIMP-2 concentrations were determined using the human TIMP-2 Quantikine ELISA kit (R&D).

Gelatin Zymography

Gelatinase activity in CM was detected by gelatin zymography (Kleiner and Stetler-Stevenson, 1994). HT1080 cell cultures in 24-hour serum-free media were treated with different concentrations of purified TIMP-2 (wild type or mutants) for another 18 hours. Equal amounts of CM from HT1080 cells with and without treatments were subjected to electrophoresis using 8% acrylamide gels containing 0.1% gelatin. The gels were incubated for 30 min at room temperature in zymogram renaturing buffer (Novex, Invitrogen), 30 min at room temperature in zymogram developing buffer (Novex, Invitrogen), and then transferred to fresh zymogram developing buffer for overnight incubation at 37°C. Gels were then stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and briefly destained in 10% acetic acid, 40% methanol and distilled water. They were imaged using an Epson Perfection V700 scanner. Gelatinase activity was detected as transparent bands on a dark background. Recombinant human proMMP-2 was run alongside CM as a control to confirm the identity of MMP-2 in the samples. At least two independent proMMP-2 activation experiments were performed using proteins prepared from different purifications for treatment.

Reverse Zymography

Reverse gelatin zymography was performed to test WT and mutant TIMP-2 proteins inhibitory function towards MMP-2. Equal amounts (1ng) of purified TIMP-2-His_6 (WT and mutants) were run in 15% acrylamide gels containing 0.225% gelatin (Sigma) and 50 ng/ml recombinant proMMP-2. The gels were incubated for 2 hours at room temperature in zymogram renaturing buffer, 30 min at room temperature in zymogram developing buffer, and then transferred to fresh zymogram developing buffer for overnight incubation at 37°C. Gels were stained and imaged as described in gelatin zymography. TIMP-2 inhibitory activity was detected as dark positive staining bands over a clear background. Recombinant human TIMP-2 (Abcam) was run alongside purified TIMP-2-His_6 mutants as a positive control.

Active MMP-2 enzyme kinetic assays

Different concentrations of an MMP-2 substrate fluorescence peptide Dabcyl-GPLGMRGK(FAM)-NH_2 (1.66-13.32 μM) were titrated against active MMP-2 (62kDa MMP-2) in the presence of different concentrations of TIMP-2 WT or mutants Y90E and Y90F (0-1.14 nM) proteins. 62kDa MMP-2 was diluted in 50mM Tris pH 7.4, 2mM $CaCl_2$, 150mM NaCl, 5μM $ZnSO_4$ and 0.01% Brij-35. 50μL of each sample was then added to the 96-well assay plate and incubated for 15 minutes at room temperature. Fluorescent peptide was subsequently added to the plate at a volume of 50μL. Fluorometric analysis was performed at excitation of 485 and emission of 530nm. Fluorescence measurements were taken for 1 hour, at 5 minutes intervals. The initial velocities of the reaction were determined for each measurement. The rate of reaction was then plotted in relation to the amount of substrate and concentration of inhibitor. Following this, Michaelis-Menten and Lineweaver Burk Plots were produced through GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Km apparent values were determined for each of the inhibitor concentrations and using a rearrangement of the classic competitive inhibition formula, -1/Km(app) = -1/(Km (1 + [I]/KI)), K_i was produced for each individual TIMP-2 protein. A simple Student's *t*-test was used to calculate significance levels between the replicates of the experiments. The significance of the sample was marked with a *, P<0.05 = *, P<0.01 = **, P<0.001 = ***. Error bars correspond to s.e.m± s.d. from two to three technical replicates. Two experimental replicates using protein purifications from different preparations were performed. Measurements were taken using SpectraMaxi3 (Molecular Devices).

Statistical analysis

No statistical methods were used to predetermine sample size. *P* values for MMP-2 enzymatic activity assay were calculated using unpaired two-tailed Student's *t*-tests with Welch's correction for unequal SDs when two groups were compared (GraphPad Prism 6). *P* values with asterisk indicate significance. In the experiments, *n* represents the number of technical replicates. The overall presented experiments in the manuscript are representative of minimum two, generally three biological replicates. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.

Supplemental References

Kleiner, D.E., and Stetler-Stevenson, W.G. (1994). Quantitative zymography: detection of picogram quantities of gelatinases. Anal Biochem *218*, 325-329.