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# Thioredoxin stimulates MMP-9 expression, de-regulates the MMP-9/TIMP-1 equilibrium and promotes MMP-9 dependent invasion in human MDA-MB-231 breast cancer cells

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This paper is dedicated to the memory of Dr. Antonella Tacconelli

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ABSTRACT

**Increased expression of thioredoxin (Trx)-1 and matrix metalloproteinase (MMP)-9 associates with malignant breast cancer progression. Here, we describe a functional relationship between Trx-1 and MMP-9 in promoting MDA-MB-231 breast cancer cell invasive behaviour. Trx-1 overexpression stimulated MMP-9 expression, de-regulated the MMP-9/TIMP-1 equilibrium and augmented MMP-9 involvement in a more invasive phenotype. Trx-1 augmented MMP-9 transcription through NF-κB, AP-1 and SP1 elements; stimulated p50/p65 NF-κB activity and recruitment to the MMP-9 promoter; and facilitated MMP-9 promoter-accessibility to NF-κB by preventing HDAC recruitment and maintaining MMP-9 promoter histone acetylation. Our data provide a functional basis for Trx-1 and MMP-9 association in malignant breast cancer and identify Trx-1 and NF-κB as potentially druggable targets for reducing MMP-9 involvement in malignant behaviour.**


## 1. Introduction

Metastatic malignant breast cancer progression is a complex process involving metastasis-associated genes thioredoxin (Trx)-1 and matrix metalloproteinase (MMP)-9 [1–15].

Trx-1 is a 12 kDa multifunctional redox protein, which together with Trx reductase and NADPH, comprises a powerful protein cysteine disulphide oxi-reductase system [6]. Trx-1 redox activity resides within a $Cys^{32}$-Gly-Pro-$Cys^{35}$ catalytic active site, which undergoes reversible oxidation–reduction [1,4,16]. Trx-1 is over expressed in breast cancer and promotes aggressive behaviour in breast cancer models [1–6]; stimulates tumour cell proliferation whilst protecting against oxidative stress and apoptosis [16]; inactivates PTEN oncosuppressor [17]; promotes and deregulates angiogenesis [6,18]; promotes extracellular proteolysis by inhibiting TIMP function [19]; and regulates NIF, AP-1, NFκB and SP1 transcription factor activity [20–22]. Trx promotion of malignant breast cancer can be reversed by dominant negative (dn) C32S/C35S mutated Trx [3,4,16].

MMP-9 is involved in induction and maintenance of the malignant phenotype [7,12–15,23–25]; triggers tumour-associated angiogenesis [10,26], promotes tumour cell invasion [12,14,15,27,28], down-regulates tumour-associated immunological surveillance [29] and prepares organ parenchyma for metastatic tumour growth [30]. MMP-9 expression is regulated at transcriptional and post-transcriptional levels [31–33]. Basal and induced MMP-9 transcription depends upon AP-1, Ets, SP1, GTbox and NFκB elements within the MMP-9 promoter and cognate transcription

*Abbreviations:* Trx, thioredoxin; MMP-9, matrix metalloproteinase-9; TIMP, tissue inhibitor of metalloproteinase; HDAC, histone deacetylase; NF-κB, nuclear factor kappa binding; Mta, metastasis associated gene

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factors [27,28,32,34–36]; is regulated by promoter-associated histone acetylation and repressed by histone deacetylases (HDACs) recruited by transcriptional repressors, including metastasis associated gene-1 (Mta-1) [37]. Changes in mRNA stability regulate MMP-9 expression at the post transcriptional level [33].

In this study, we unveil a novel functional interaction between Trx-1 and MMP-9 in a MDA-MB-231 cell model of malignant p53 and ER-independent breast cancer. We report that Trx-1 stimulates MMP-9 expression, de-regulates the equilibrium between MMP-9 and its specific inhibitor TIMP-1 and increases MMP-9 involvement in a more invasive phenotype, we characterise a critical role for the transcription factor NF-κB in this effect and identify Trx-1 and NF-κB as druggable therapeutic targets to reduce MMP-9 involvement in breast cancer.

## 2. Materials and methods

### 2.1. Cells and reagents

MDA-MB-231 breast cancer cells and human MMP-9 and TIMP-1 antibodies, have been described previously [27,28]. Anti-catalytic MMP-9 antibody and recombinant human TIMP-1 were from Oncogene Science (Cambridge MA); p65, p50, HDAC-1, HDAC-2 and Mta-1 antibodies were Santa Cruz Biotechnology (Santa Cruz, CA); the acetylated Histone 3 antibody was from Upstate Biotechnology (Lake Placid, NY). The anti-Trx-1 antibody was from IMCO (Stockholm Sw). Dominant negative mutant I-κBα inhibitor of NF-κB in pmT2T vector was provided by Dr. U. Siebenlist (NIH, Bethesda, MD) [40]. Trx-1 pcDNA3.1 and C32S/C35S-mutated Trx-1 pcDNA3.1 (dn-Trx-1) vectors were obtained by sub-cloning previously characterised full length human Trx-1 and C32S/C35S mutated Trx-1 cDNAs [39] into mammalian expression vector pcDNA3.1 (Invitrogen). MDA-MB-231 cells stable transfected with pcDNA 3.1, using Lipofectamine™ as directed (Invitrogen), were isolated by 100 μg/ml Zeocin resistance.

### 2.2. Zymograms and Western blots

Gelatin zymograms and Western blots were performed as previously described [27,40].

### 2.3. Invasion assays

Invasion through reconstituted Matrigel (Collaborative Research, Bedford, MA) was performed as previously described [12], with inhibitors added directly to invasion medium at the concentrations stipulated.

### 2.4. Nuclear extracts and EMSAs

Nuclear extracts were prepared, and regular and supershift EMSAs performed, as previously described [27]. For EMSAs, the following oligonucleotides were used:

- MMP-9 AP-1 (5′-CCTGACCCCTGAGTCAGCACTTGCCTGT-3′);
- MMP-9 NF-κB (5′-TGCCCCAGTGGAATTCCCCAGCCTTG-3′) and
- MMP-9 Sp1 (5′-GTGCCTTCCGCCCCCAGATGAA-3′).

All oligonucleotides were double stranded, complementary affinity strands are not indicated.

### 2.5. RT-PCR

Total RNAs (1 μg) were reverse transcribed using Moloney Murine Leukaemia virus reverse transcriptase kit (Life Technologies Inc., Paisley, UK), as directed. RT reactions were subjected to PCR amplification using primers sets for: Trx-1: 5′-GAATTCGCTTTGG ATCCATTTCCATCG-3′ and 5′-CAAGTTTAAATAGCCAATGGCTGGT-TA-3′; MMP-9 5′-TGGACGATGCCTGCAACGTG-3′ and 5′-GTCGTGC GTGTCCAAAGGCA-3′; TIMP-1 5′-AGCGCCCAGAGAGACACCAG-3′ and 5′-CCACTGCGGGCAGGATTCAG-3′ and GAPDH 5′-CGGAGT CAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGG TGAA-GAC-3′. RT-PCR products were resolved by 1.5% agarose gel electrophoresis.

### 2.6. Luciferase reporter gene and β-galactosidase assays plasmids

Luciferase reporter gene constructs -670MMP-9-Luc, -670AP1mu-Luc (mutation of the proximal -79 AP-1 site from TGAGTCA to gtcacCg), -670GTmu-Luc (mutation of the -54 GT box from GGGGTGGGG to GGGtTaacG), -670SP1mu-Luc (mutation of the -560 SP1 site from CCGCCCC to CgtCaaC) and -670NFκBmu-Luc (mutation of the -600 NFκB element from GGAATTCCCC to GGcAaccCgg) were prepared by sub-cloning previously characterised -670MMP-9; -670AP1mu; -670GTmu; -670SP1mu; -670NFκBmu and MMP-9 NF-kB sequences of -670MMP-9CAT; -670AP1mu-CAT; -670GTmuCAT, -670SP1muCAT, -670NFκBmu-CAT and Δ56NFkBCAT reporter gene constructs [27] into the PGL3 luciferase reporter plasmid (Promega Madison WI). Luciferase reporter genes were sequence verified and the mutations introduced into relevant binding sites verified as inhibiting specific binding by EMSA. Luciferase reporter gene and β-galactosidase assays were performed following 48 h transient transfection with luciferase and pRSVβgal reporter genes (Invitrogen), as previously described [41,42].

### 2.7. Chromatin immunoprecipitation

ChIp assays were performed as previously described [43]. Immunoprecipitated chromatin was subjected to PCR amplification using 100 ng/μl of each unlabeled oligonucleotide primer designed to amplify the region spanning -634 to -484 of the MMP-9 promoter (5′-ATTCAGCCTGCGGAAGACCAG-3′ and 5′-ACT-CCAGGCTCTGTCCTC TT-3, respectively) in a reaction containing 500 μM dNTPs, 67 mM Tris–HCl; 6.7 mM $MgCl_2$; 170 μg/ml BSA; 16.6 mM $(NH_4)_2SO_4$) and one unit of Taq Polymerase (35 cycles of 95 °C for 30 s; 56 °C for 30 s and 72 °C for 30 s). Products were separated by 10% non-denaturing acrylamide gel electrophoresis.

### 2.8. Statistical analysis

Differences between means were evaluated by Student's *t*-test, with *t*-value-associated probabilities of $\leqslant 0.05$ considered statistically significantly.

## 3. Results

### 3.1. Stable transfectants

Densitometric analysis of Western blots indicated that constitutive Trx protein expression in stable MDA-MB-231 pcDNA control transfectants (a and b) was moderately increased with respect to β-actin levels in both stable Trx-1 (a and b) and dnTrx-1 (a and b) transfectants (Fig. 1A). The presence of Trx-1 and dn-Trx1 expression vectors in stable transfectants was confirmed by PRC using Trx and pcDNA-specific primers (Fig. 1A, bottom panel).

### 3.2. Trx-1 stimulates MMP-9 expression

Differences in MMP-9 activity, MMP-9 and TIMP-1 expression were assessed by zymogram, Western blot and RT-PCR using equal

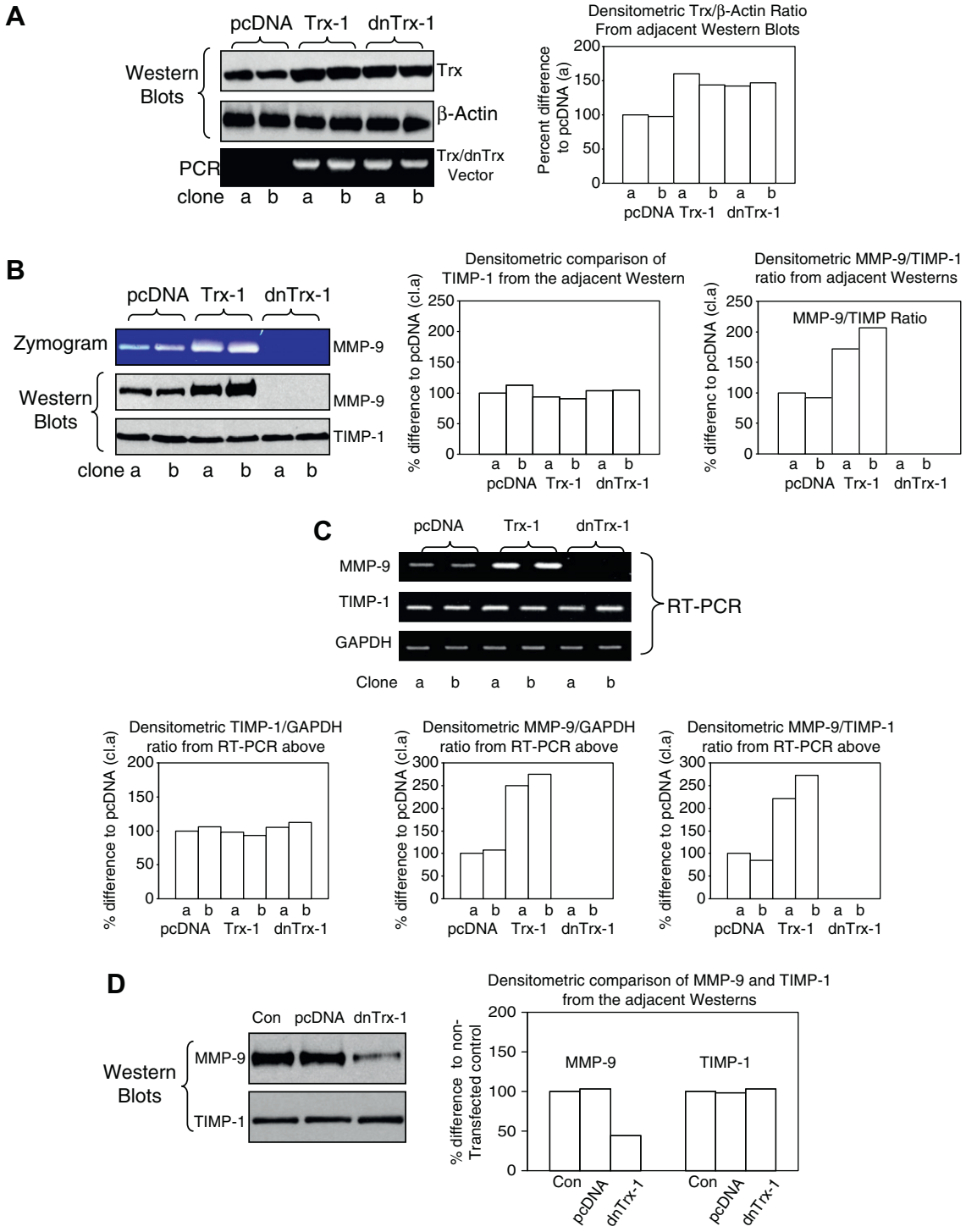


**Fig. 1.** (A) Western blots demonstrating Trx-1 and β-actin expression in stable pcDNA control, Trx-1 and dnTrx-1 transfectants, plus PCR presence of Trx/dnTrx-1 expression vector in Trx-1 and dnTrx-1 but not in pcDNA stable transfectants. The adjacent histogram shows the percent difference in Trx-1/β-actin ratio assessed by densitometry of the adjacent Western blots, using pcDNA (cl.a) (100%) as the baseline. (B) Gelatin zymogram (zym) and Western blots comparing MMP-9 activity and expression and TIMP-1 expression in equal numbers of pcDNA control, Trx-1 and dnTrx-1 transfectants. The adjacent histograms depict the percent differences in TIMP-1 levels and MMP-9/TIMP-1 ratios assessed by densitometry of the adjacent Westerns, using pcDNA (cl. A) as the baseline. (C) Agarose gels demonstrating RT-PCR levels of MMP-9, TIMP-1 and GAPDH mRNAs in control pcDNA, Trx-1 and dnTrx-1 transfectants. The histograms beneath show the percent densitometric differences in TIMP-1/GAPDH; MMP-9/GAPDH and MMP-9/TIMP-1 ratios with respect to pcDNA transfectant clone a (100%), in the gels above. (D) Western blots demonstrating MMP-9 and TIMP-1 protein levels in concentrated 48-h serum-free conditioned medium from untreated stable Trx-1 transfectants (con) and stable Trx-1 transfectants transiently transfected with either empty pcDNA expression vector (pcDNA) or dnTrx-1 expression vector (dn-Trx-1). The adjacent histogram shows the percent difference in MMP-9 and TIMP-1 expression assessed by densitometry of the adjacent Westerns, using untreated stable Trx-1 transfectant controls (100%) as the baseline.

numbers of pcDNA control, Trx-1 and dnTrx-1 transfectants. Densitometric analysis of zymograms, Western blots (Fig. 1B) and RT-PCR reactions (Fig. 1C) revealed that MMP-9 activity, mRNA and protein expression in pcDNA controls was more than doubled in Trx-1 stable transfectants relative to TIMP-1 expression. In contrast, MMP-9 but not TIMP-1 protein (Fig. 1B) and mRNA (Fig. 1C)

expression in dnTrx-1 transfectants (a and b) was abrogated, with MMP-9/TIMP-1 protein (Fig. 1B) and mRNA (Fig. 1C) ratios altered in favour of MMP-9 in Trx-1 transfectants and in favour of TIMP-1 in dn-Trx transfectants. Transient dn-Trx-1 transfection into stable Trx-1 transfectants reduced MMP-9 protein expression without affecting that of TIMP-1, compared to transient pmT2T-transfected counterparts (Fig. 1D).

### 3.3. Trx-1 augments MMP-9 involvement in MDA-MB-231 invasion

Trx-1 MDA-MB-231 transfectants exhibited a significant 75 ± 2.9% (±S.E.) increase ($P \leqslant 0.05$, $n = 24$) (Fig. 2) and dnTrx-1 transfectants a significant 55 ± 3.1% decrease ($P \leqslant 0.05$, $n = 24$) in invasion, when compared to pcDNA transfectants (Fig. 2). Recombinant human TIMP-1 (10 μg/ml) and anti-catalytic anti-MMP-9 antibody (100 μg/ml) significantly inhibited pcDNA transfectant invasion by 35 ± 3.5% ($P \leqslant 0.05$, $n = 12$) and 35 ± 4.8% ($P \leqslant 0.05$, $n = 12$), respectively, and inhibited Trx-1 transfectant invasion by 51.5 ± 3.6% ($P \leqslant 0.05$, $n = 12$) and 45.8 ± 4.2% ($P \leqslant 0.05$, $n = 12$), respectively, when compared to untreated controls (Fig. 2). Recombinant human TIMP-1 (10 μg/ml) and anti-catalytic anti-MMP-9 antibody (100 μg/ml) did not reduce dnTrx-1 transfectant invasion (Fig. 2). Transient 48 h dn-Trx-1 transfection prior to invasion assay significantly inhibited pcDNA transfectant invasion by 33.8 ± 6.2% ($P < 0.05$ $n = 6$), inhibited Trx-1 transfectant invasion by 47.2 ± 5.8% ($P < 0.05$, $n = 6$), (Fig. 2) but did not reduce dn-Trx-1 transfectant invasion, when compared to pmT2T transiently-transfected controls (Fig. 2).

### 3.4. Trx-1 stimulates MMP-9 transcription

MMP-9 mRNA stability over 10 h, in the presence of actinomycin D (5 μg/ml) was not augmented in Trx-1 compared to pcDNA transfectants. MMP-9 but not TIMP-1 or GAPDH mRNA was lost by 10 h in both cell lines (Fig. 3A).

MMP-9 luciferase reporter gene assays revealed significant differences in transcription between pcDNA and Trx-1 MDA-MB-231 transfectants. Basal transcription in pcDNA transfectants (arbitrary value, 100%) was significantly augmented in Trx-1 transfectants by 65 ± 4.8% (±S.E.) ($P < 0.05$, $n = 12$) (Fig. 3B). GT box mutation (-670GTmu) did not reduce transcription in pcDNA or Trx-1 transfectants, Sp1 mutation (-670SPmu) significantly reduced transcription in pcDNA and Trx-1 transfectants by approximately 30% ($P < 0.05$, n6, for both pcDNA and Trx-1 transfectants); AP-1 mutation (-670AP-1mu) significantly reduced transcription in pcDNA and Trx-1 transfectants by approximately 60% ($P < 0.05$, n6, for both pcDNA and Trx-1 transfectants) and NF-κB mutation (-670 NF-κBmu) significantly reduced transcription by approximately 80% a in both pcDNA and Trx-1 transfectants ($P < 0.05$, $n = 6$, for both pcDNA and Trx-1 transfectants) (Fig. 3B). Transcription in dn-Trx transfectants was significantly attenuated by 46 ± 4.5% ($P < 0.05$, $n = 12$) compared to pcDNA controls and was significantly reduced further in AP-1 mutated (-670AP-1mu) and NF-κB mutated (-670NF-κBmu) but not -670GTmu or -670SP1mu constructs (Fig. 3B), indicating that abrogation of MMP-9 expression in dn-Trx transfectants is not accompanied by abrogation of transcription from the MMP-9 reporter gene.

In NF-κB luciferase reporter gene assays constitutive NF-κB activity in pcDNA transfectants was significantly augmented by 92 ± 7.4% ($P < 0.05$, $n = 12$) in Trx-1 transfectants and significantly reduced by 32 ± 4.3% ($P < 0.05$, $n = 12$) in dnTrx-1 transfectants (Fig. 3C). Transient transfection with the mutated dominant negative (dn) I-κBα inhibitor of NF-κB [40] significantly reduced MMP-9 luciferase reporter gene activity by 49.8 ± 10.3%, ($P < 0.05$, $n = 9$) in Trx-1 transfectants and by 35 ± 8.5% ($P < 0.05$, $n = 9$) in pcDNA transfectants, when compared to pmT2T vector transfected controls (Fig. 3D). Transient 48 h dn I-κBα transfection inhibited MMP-9 but not TIMP-1 expression by equivalent numbers of Trx-1 and pcDNA transfectants, assayed in 10 fold concentrated 48 h serum-free conditioned medium, by Western blot (Fig. 3E).

### 3.5. Trx-1 augments AP-1, SP-1 and NF-kB activity in EMSAs

AP-1, SP-1 and NF-κB site binding activity was assessed in nuclear extracts from pcDNA, Trx-1 and dnTrx-1 transfectants, by EMSA. Comparative densitometric analysis of the ratio between specific and non-specific binding complexes, using pcDNA transfectants as a baseline, revealed that Trx-1 MDA-MB-231 transfectants exhibited a 65% increase in AP-1, a 54% increase in SP-1 and a 90% increase in NF-κB site binding activity, whereas dn-Trx transfectants exhibited a 55% decrease in AP-1, a 32% decrease in SP-1 and a 22% decrease in NF-κB site binding activity (Fig. 4A). Binding specificity was confirmed by competition EMSA in which cold-specific but not cold non-specific oligonucleotides competed with labelled probe (data not displayed). The protein composition of NF-κB complexes determined by supershift EMSA confirmed the presence of p50 in NF-κB complexes from pcDNA, Trx-1 and dn-Trx-1 transfectants, and detected p65 exclusively in NF-κB complexes from Trx-1 transfectants (Fig. 4B). Densitometric analysis of Westerns revealed a 96% increase in p50 and 46% increase in p65, relative to Mta1, in nuclear extracts from Trx-1 transfectants, and a 40% decrease in p50 and 65% decrease in p65, relative to Mta1, in dn-Trx-1 transfectants, when compared to pcDNA transfectants (Fig. 4C).

### 3.6. Trx-1 regulates NF-κB recruitment to the endogenous MMP-9 promoter

ChIp assay detected p50 but not p65 at the endogenous MMP-9 promoter in pcDNA transfectants; p50 and p65 at the endogenous MMP-9 promoter in Trx-1 transfectants but did not detect either p50 or p65 at the endogenous MMP-9 promoter in dnTrx-1 transfectants (Fig. 5A).

### 3.7. HDACs at the MMP-9 promoter mediate MMP-9 repression

ChIp assays also detected acetylated histone 3 but not HDAC-1, HDAC-2 or Mta1 at the endogenous MMP-9 promoter in pcDNA and Trx-1 transfectants, and detected HDAC-1, HDAC-2 and

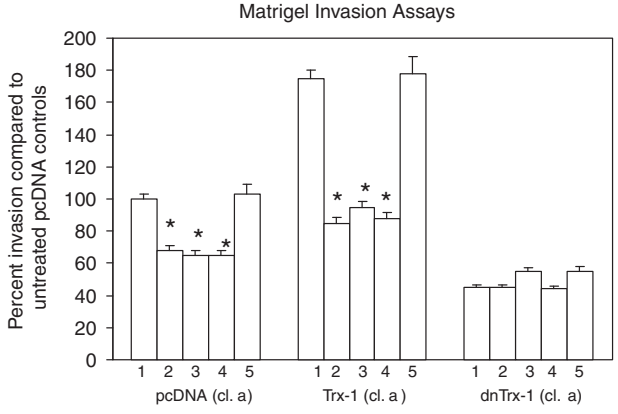


**Fig. 2.** Histogram depicting Matrigel invasion by stable pcDNA, Trx-1 and dn-Trx-1 transfectants in invasion medium (1) alone; (2) plus 1 μg/ml of exogenous recombinant human TIMP-1; (3) plus 100 μg/ml of anti-catalytic anti-MMP-9 antibody; (4) following transient transfection with dnTrx-1 vector, and (5) following transient transfection with 10 μg pmT2T vector. Results are displayed as percent change in mean invasion (±S.E.), compared to invasion of pcDNA controls in the presence of 100 μg/ml of pre-immune IgG (100%).

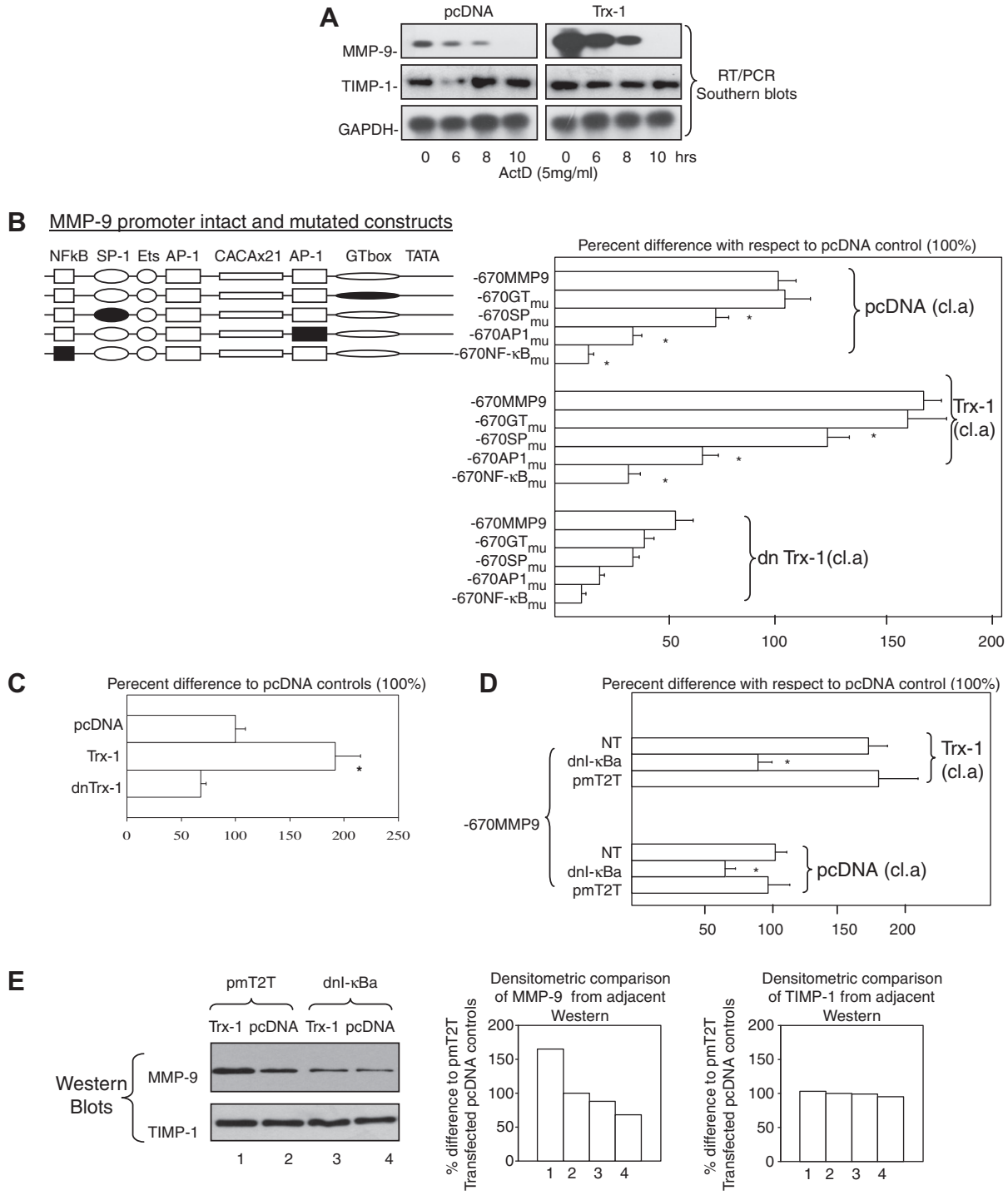


**Fig. 3.** (A) RT-PCR/Southern blots comparing the effects of actinomycin D (5 μg/ml) on MMP-9, TIMP-1 and GAPDH mRNA levels in stable pcDNA control and Trx-1 transfectants over 10 h. (B) Diagram outlining the MMP-9 promoter intact and mutated constructs plus histogram comparing percent differences in luciferase reporter gene activity from intact; GTbox mutated (-670MMP-9GT$_{mu}$); AP1 mutated (-670MMP-9AP-1$_{mu}$), SP1 mutated (-670MMP-9SP1$_{mu}$) and NFκB mutated (-670MMP-9NFκB$_{mu}$) reporter gene constructs in stable pcDNA, Trx-1 and dnTrx-1 transfectants. (C) Histogram demonstrating differences in NF-κB-luciferase reporter gene activity in pcDNA, Trx-1 and dnTrx-1 transfectants, displayed as mean percent difference in activity (±S.E.), compared to pcDNA controls (arbitrary value, 100%), in three independent experiments performed in duplicate. (D) Histogram comparing percent differences in luciferase reporter gene activity from -670MMP-9Luc reporter gene following 48 h transient transfection of Trx-1 and pcDNA stable transfectants with either nothing (NT), dn-I-κBα or pmT2T control vector. Results are displayed as the mean percentage difference to luciferase activity (±S.E.) from the intact promoter in non-treated pcDNA transfectant controls (arbitrary value, 100%), in three independent experiments performed in duplicate (*significant difference). (E) Western blots demonstrating differences in MMP-9 relative to TIMP-1 expression in 48 h serum-free conditioned medium from equal numbers of stable Trx-1 and pcDNA transfectants transiently transfected with either pmT2T control or dn-I-κBα vector. The adjacent histogram shows the percent difference in MMP-9 and TIMP-1 levels assessed by densitometry of the adjacent Westerns, using transient pmT2T-transfected pcDNA control (100%) as the baseline.

Mta-1 but not acetylated histone 3 at the MMP-9 promoter in dn-Trx-1 transfectants (Fig. 5A). Densitometric analysis of Western blots revealed similar levels of Mta1 in nuclear extracts from pcDNA, Trx-1 and dn-Trx-1 transfectants (Fig. 4C). The HDAC inhibitor TSA (100 ng/ml for 48 h) restored MMP-9 expression to dn-Trx-1 transfectants, without effecting the expression of

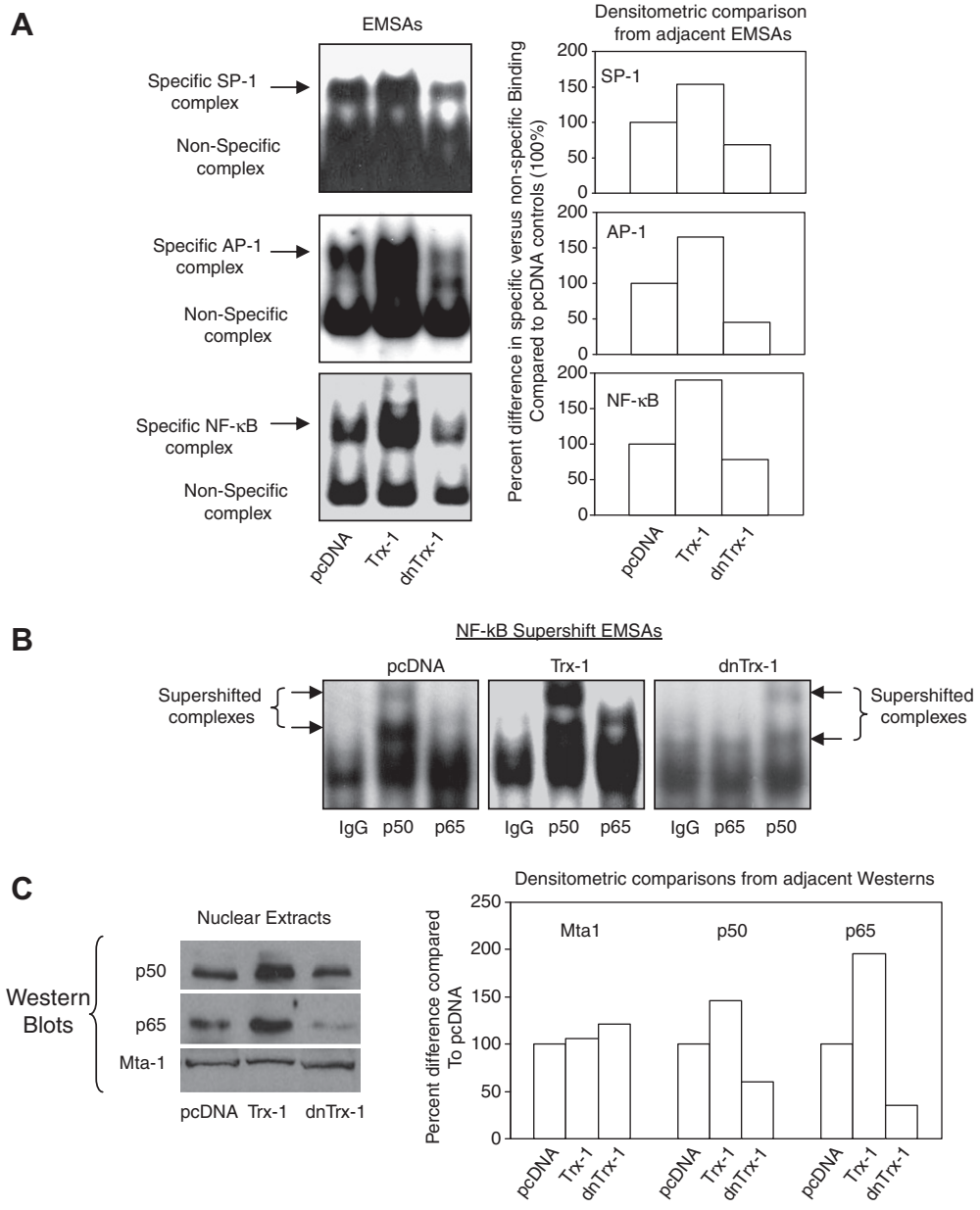


**Fig. 4.** (A) EMSAs demonstrating specific AP-1, SP-1 and NF-kB site binding activity and non-specific binding activity (NS) in nuclear extracts from pcDNA, Trx-1 and dn-Trx-1 transfectants. The adjacent histograms show the percent difference in specific AP-1, SP-1 or NF-κB versus non-specific binding activity, assessed by densitometry of adjacent EMSAs, using pcDNA controls (100%), as the baseline. (B) Supershift EMSAs demonstrating supershifting (arrows) of p50 and p65-containing NF-κB binding complexes in nuclear extracts from pcDNA, Trx-1 and dn-Trx-1 transfectants. (C) Western blots demonstrating p50, p65 and Mta1 levels in nuclear extracts from pcDNA, Trx-1 and dn-Trx1 transfectants. The adjacent histogram shows differences in Mta1, p50 and p65 levels, assessed by densitometry of adjacent Westerns, using pcDNA controls (100%) as the baseline.

TIMP-1 and GAPDH. TSA did not increase MMP-9 expression in either pcDNA or Trx-1 transfectants (Fig. 5B).

## 4. Discussion

We report a novel functional relationship between breast cancer metastasis-associated genes Trx-1 and MMP-9 in a MDA-MB-231 cell model of malignant p53 and ER-independent breast cancer. This relationship is characterised by Trx-1 stimulation of MMP-9 expression; de-regulation of the equilibrium between MMP-9 and its tissue specific inhibitor TIMP-1; and stimulation of MMP-9 involvement in a more invasive phenotype. We show that Trx-1 stimulates MMP-9 expression at the transcriptional level through AP-1, SP-1 and NF-κB sites within the MMP-9 promoter, modifies NF-κB DNA binding activity and recruitment to the endogenous MMP-9 promoter, increasing NF-κB-mediated MMP-9 transcription. Finally, we show that these Trx-1-mediated effects are reversed by dominant negative inhibition of both Trx-1 and NF-κB.

Our observations are the first to report a functional relationship between Trx-1 and MMP-9 in breast cancer cells and provide a potential explanation for the association between the overexpression of Trx-1 and MMP-9 in malignant breast cancer [1–15]. Increased Trx-1 expression in breast tumours, modelled in this study, is likely to reflect not only tumour-associated oncogenic activity but also oxidative-stress within the tumour microenvironment, which stimulates Trx-1 redox system expression in order to buffer oxidative stress-associated toxicity [43]. The net effect of this, combined

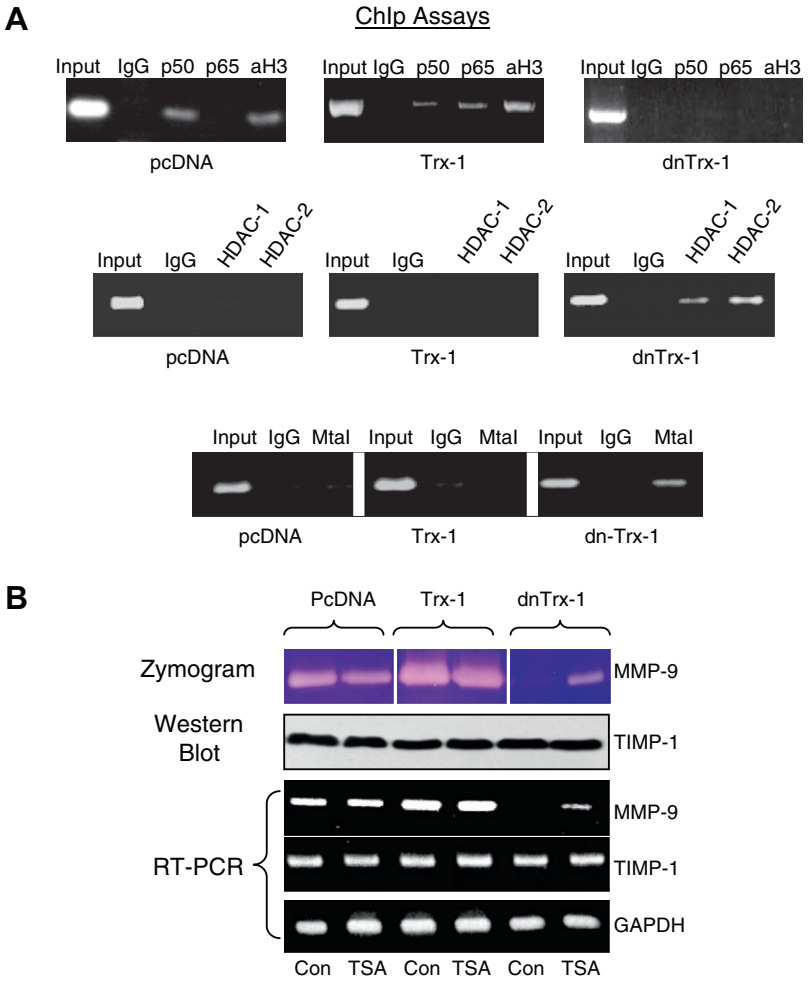


**Fig. 5.** (A). Agarose gels demonstrating endogenous distal MMP-9 promoter NF-κB binding site PCR products from anti-p50, p65, acetylated histone 3, HDAC 1, HDAC 2 and Mta-1 ChIp assays using chromatin from untreated pcDNA; Trx-1 and dn-Trx-1 transfectants. Input extracts and pre-immune IgG immunoprecipitates are also displayed. (B) Gelatin Zymograms (Zy), TIMP-1 Western blot (WB) and RT-PCR reactions demonstrating TSA (100 ng/ml for 48 h) restoration of MMP-9 relative to TIMP-1 expression in dn-Trx compared to pcDNA and Trx-1 transfectants.

with Trx-1 stimulation of MMP-9-dependent invasion, provides cues for survival within and escape from the stressful tumor microenvironment, both of which characterise malignant progression.

Trx-1 exhibits both redox dependent and redox independent biological activity [16,44]. Here, we report that Trx-1 stimulation of MMP-9 expression and invasion is redox active site-dependent, since both were inhibited by the dominant negative C32S/C35S-mutated Trx-1 redox active site inhibitor [3,39]. This active site cysteine to serine mutant acts as a dominant negative inhibitor of Trx-1 by competing for Trx substrates including Trx reductase, impairing their capacity to reduce oxidised Trx-1 [3,39].

MMP-9 involvement in malignant behaviour depends upon alteration in equilibrium with its tissue specific inhibitor, TIMP-1 [7]. MMP-9 involvement in Trx-1 stimulated invasion was confirmed using exogenous TIMP-1 and an anti-catalytic anti-MMP-9 antibody, both of which inhibited Trx-1 transfectant but not non-MMP-9 expressing dnTrx-1 transfectant invasion. MMP-9 involvement in invasion was associated with increased MMP-9 but not TIMP-1 expression, producing an altered MMP-9/TIMP-1 equilibrium in favour of MMP-9. This adds to our previous report that extracellular Trx-1 de-regulates the MMP-9/TIMP-1 equilibrium and stimulates human neuroblastoma cells invasion by direct TIMP inhibition [19], and indicates that Trx-1 can de-regulate the MMP-9/TIMP-1 equilibrium to stimulate invasion at both the transcriptional (this study) and post translational [19] level. We do not exclude, however, that Trx-1 may also influence other aspects of the invasion process, since it is chemotactic and alters cellular adhesive interactions with extracellular matrices [18,45].

Trx-1 stimulation of MMP-9 expression did not result from increased mRNA stability but rather increased transcription. This involved combined increased transcription through AP-1 (-79), SP1 (-560) and NF-κB(-600) elements, confirming reports that these element function in MMP-9 transcription [27,28,32,34–36] and that Trx-1 regulates AP-1, Sp1 and NF-κB transcription factor activity [20–22]. Although Trx-1 increased both AP-1 and NF-κB binding activity, we chose to study NF-κB in more detail as AP-1 involvement in MMP-9 transcription is NF-κB dependent [34]. Focussing on NF-κB, we observed that Trx-1 overexpression altered NF-κB DNA binding activity, promoted formation of NF-κB complexes containing p50 and p65 and their recruitment to the endogenous MMP-9 promoter, resulting in increased NF-κB-mediated MMP-9 transcription. NF-κB involvement in MMP-9 transcription was confirmed by dominant negative mutated I-κBα inhibition of MMP-9 transcription [38]. Therefore, Trx-1 redox activity promotes p50/p65 NF-κB heterodimer formation, function and recruitment to the endogenous MMP-9 promoter, increasing MMP-9 transcription. Furthermore, dn-I-κBα also inhibited the invasion of both control and Trx-1 but not dnTrx-1 transfectants, confirming NF-κB involvement in invasion.

In contrast to control and Trx-1 transfectants, stable dn-Trx-1 MDA-MB-231 transfectants exhibited reduced invasion insensitive to MMP-9 inhibitors and did not express MMP-9, helping to explain why transient dn-Trx transfection did not further reduce invasion of this cell line. However, somewhat paradoxically, dn-Trx-1 transfectants exhibited constitutive MMP-9 and NF-κB transcriptional activity in reporter gene assay, constitutive NF-κB site binding activity in EMSAs and the presence of p50 and p65 in nuclear extracts. Stable dn-Trx-1 transfectants did not, however, exhibit p50 or p65 recruitment to the endogenous MMP-9 promoter, suggesting that inhibition of Trx-1 activity impedes NF-κB access to the MMP-9 promoter, providing a potential explanation for the abrogation of MMP-9 expression observed and highlighting the limit of reporter gene assays that fail to take into account chromatin structure. This possibility was supported by ChIp assay, which detected HDACs, involved in chromatin remodelling, in the absence of acetylated histone 3 and NF-κB at the endogenous MMP-9 promoter in dn-Trx-1 transfectants, but detected acetylated histone 3 and NF-κB but not HDACs at the MMP-9 promoter in control and Trx-1 transfectants. A potential role for HDACs in MMP-9 transcriptional repression was confirmed using the HDAC inhibitor Trichostatin A [46], which restored MMP-9 expression to dn-Trx-1 transfectants, adding to a report that HDACs mediate MMP-9 transcriptional repression [37] and identifying Trx-1 as a potential regulator of this process. These data suggest that increased Trx-1 activity stimulates NF-κB activity but blocks HDAC recruitment to the MMP-9 promoter facilitating MMP-9 expression, whereas reduced Trx-1 activity inhibits MMP-9 expression by promoting HDAC recruitment to the endogenous MMP-9 promoter, impeding NF-κB access as a consequence of histone de-acetylation. Although we did not assess the direct effects of Trx-1 and dnTrx-1 upon HDAC expression, our data indicate that within the context of the MMP-9 promoter Trx-1 regulates NF-κB and HDAC recruitment oppositely, with NF-κB recruitment a consequence of HDAC absence and vice versa. Although, the mechanism through which Trx-1 regulates HDAC involvement in MMP-9 expression remain to be fully elucidated, the HDAC recruiter and MMP-9 transcriptional repressor Mta-1 [37] was also detected at the MMP-9 promoter in dn-Trx-1 but not pcDNA nor Trx-1 transfectants, suggesting that Mta-1 may mediate HDAC recruitment and MMP-9 transcriptional repression in dnTrx transfectant. This interesting possibility is under current investigation.

In conclusion, our data provide a functional transcriptional basis for the association between MMP-9 and Trx-1 overexpression in breast cancer, characterise NF-κB as a critical determinant of this effect and identify both Trx-1 and NF-κB as a pivotal potential druggable therapeutic targets for reducing MMP-9 involvement in malignant breast cancer.

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