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14. ABSTRACT

Aberrant DNA methylation of promoter region CpG islands is associated with gene silencing and serves as an alternative to mutations in the inactivation of tumor suppressor genes in human cancers. We identified a gene TMS1 (for Target of Methylation-mediated Silencing) that is subject to such epigenetic silencing in a significant proportion of human breast and other cancers. TMS1 encodes a bipartite intracellular signaling molecule with proposed roles in apoptosis and inflammation. However, the precise role of this protein in apoptosis has not been clearly defined, and the consequence of TMS1 silencing on the pathogenesis of breast cancer is unknown. In this study we identified two novel roles for TMS1 in apoptosis, activation of caspase-8 and subsequent apoptosis induced by TNFα, and apoptosis induced by detachment from the extracellular matrix. Importantly, loss of TMS1 expression severely inhibits apoptosis induced by these stimuli. Therefore, loss of TMS1 expression through epigenetic silencing may contribute to breast carcinogenesis by dampening the apoptotic response to TNFα, and allowing cells to bypass cell death induced by detachment from the extracellular matrix.

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A. Introduction

The behavior of cells, including growth properties, interactions with the surrounding microenvironment, and sensitivity to genetic and environmental insults, is dictated by the genes that are expressed in that cell. Alterations in gene expression, therefore, can lead to alterations in cellular behavior. Cancer can be thought of as a disease characterized by inherent genetic instability. This genetic instability allows for the accumulation of mutations that change the behavior of cancer cells and confer survival advantages. These mutations consist of gain of function mutations in oncogenes and loss of function mutations in tumor suppressor genes. There are various mechanisms that have been identified as causing such mutations. Oncogenes, for example, can acquire gain of function mutations through amplification of the gene sequence, point mutations that render the gene constitutively active, or translocation to the vicinity of a different, more active promoter [1-3]. Tumor suppressor genes can be inactivated by some of the same mechanisms, including point mutations and translocations, but contrary to oncogenes, these aberrations would render the gene inactive [3].

Recent evidence suggests that aberrant methylation of promoter-region CpG islands can serve as an alternative, epigenetic mechanism of tumor suppressor gene inactivation [4]. Methylation of DNA in the human genome occurs on the 5' position of cytosines in the dinucleotides 5'-CpG-3.' CpG sites are under-represented in the mammalian genome except in CpG islands, which are stretches of DNA with a high frequency of CpG sites [5]. These CpG islands are found at the promoters of approximately 50% of human genes and remain unmethylated in normal cells [5]. CpG islands can, however, acquire methylation during aging and carcinogenesis [4, 6]. This methylation is accompanied by a shift to a repressed chromatin conformation characterized by hypoacetylation of histones H3 and H4, as well as a shift in the local histone methylation pattern from one rich in H3 methylated at lysine 4 (H3-K4) to one hypermethylated at H3 lysine 9 (H3-K9) [7]. These modifications render the associated gene transcriptionally silent. Once established, this state is mitotically heritable and contributes to the stable silencing of the affected allele [4]. Importantly, genes silenced by methylation are structurally intact, thus the potential for re-expressing aberrantly-methylated tumor suppressor genes in patients could in theory be exploited to re-sensitize the patients to therapy. Indeed, identifying new therapeutic strategies aimed at reactivating aberrantly methylated and epigenetically silenced genes is the subject of much on-going research [7].

Our lab has identified a novel gene named TMS1 (for "target of methylation-mediated silencing," also known as ASC or PYCARD), which is commonly silenced by methylation in human cancer cells. We identified TMS1 in a screen for downstream targets of methylation-mediated gene silencing induced by overexpression of human DNA methyltransferase-1 [8]. TMS1 was also independently identified by Masumoto *et. al.* [9] as a protein that forms cytosolic "specks" during retinoic acid-induced and drug-induced apoptosis, and called ASC (for "apoptosis- associated speck-like protein containing a CARD"). Upon identifying TMS1, our lab examined the methylation pattern at the *TMS1* locus as well as the expression of TMS1 in breast epithelial and breast cancer cell lines. We found that TMS1 remained unmethylated and expressed in normal breast epithelial cell lines, but that many of the breast cancer cell lines examined had lost TMS1 expression, and this correlated with methylation of the promoter region CpG island of *TMS1* [8]. In addition, we have also shown that this gene is aberrantly methylated and silenced in approximately 40% of primary breast tumors and in some glioblastomas [8, 10]. Other investigators have reported that TMS1 is methylated and silenced in other cancers, including gastric, lung, colorectal, prostate and skin cancers [11-15]. The fact that TMS1 is

epigenetically silenced in a variety of tumor types suggests that it may be a novel tumor suppressor gene that plays a role in the pathogenesis of many different human cancers.

Through sequence homology searches, it was determined that the TMS1 gene encodes a 23 kilodalton (kDa) protein consisting of an N-terminal pyrin domain and a C-terminal caspase recruitment domain(CARD), two members of the death domain fold (DDF) superfamily [8, 16]. These motifs are often found in intracellular signaling molecules involved in the regulation of apoptosis and inflammation. That TMS1 protein contains these domains suggests that TMS1 may play a role in inflammatory and/or apoptotic signaling pathways. Consistent with a role in inflammation, several groups focusing on the role of TMS1 in immune cells have determined that TMS1 is an integral part of the "inflammasome," a multimeric complex responsible for the processing and secretion of the proinflammatory cytokines IL-1β and IL-18 in response to a variety of inflammatory "danger signals" [17-21]. Evidence from TMS1 knockout mice indicates that TMS1 is absolutely necessary for inflammasome function and serves as an activating adaptor for caspase-1. Thus, TMS1 plays an important role in caspase-1-dependent processing and proinflammatory cytokine secretion in immune cells.

In addition, TMS1 has been reported to play a role in apoptotic signaling. Previous work from our lab showed that TMS1 prevents colony-forming ability of breast cancer cells and induces apoptosis when overexpressed [8, 22]. TMS1-induced apoptosis could be blocked with a dominant-negative caspase-9, but not a dominant-negative caspase-8, which suggested it was part of intrinsic mitochondrial signaling [22]. Consistent with this idea, another group that independently identified TMS1 found that TMS1 localized to perinuclear aggregates ("specks") following treatment of HL-60 leukemia cells with retinoic acid or etoposide, and that reduction of TMS1 expression with antisense oligonucleotides conferred resistance of these cells to etoposide-induced apoptosis, which is a DNA damaging agent known to rely on caspase-9 and mitochondrial signaling [9].

It is our hypothesis that epigenetic silencing of TMS1 contributes to carcinogenesis by allowing breast cancer cells to bypass normal apoptotic cues and, as a consequence, may cause some cancers to be resistant to chemotherapy. There were three specific aims associated with this proposal. The first was to create a model of TMS1 loss-of-function in human breast cancer cells (Months 1-12). The second task of this proposal was to determine whether loss of TMS1 causes resistance of breast cancer cells to anticancer agents and proapoptotic stimuli, and to characterize the resulting apoptotic response using the cell lines created in Task 1 (Months 13-30). Task 3 was to determine the effects of TMS1 loss on anchorage-independent growth potential (Months 31-36). In this annual summary report, I will discuss the progress made on Tasks 1-3, as well as discuss other novel directions this project has taken, important findings, and reportable outcomes.

B. Body

The overall goal of this proposal was to model the loss of TMS1 seen in breast cancer and to determine the consequences of TMS1 silencing on breast cancer progression by specifically examining the cellular response to chemotherapy and other proapoptotic stimuli, as well as to examine anchorage-independent growth potential following TMS1 loss. The first task, which we proposed would take approximately twelve months, involved developing a model of TMS1 loss-of-function in human breast cancer cells. To create this system, we proposed to generate breast cancer cell lines stably knocked down for TMS1 using RNA interference (RNAi) technology. The specific goals within Task 1 were to a.) determine the optimal target site within TMS1 mRNA for blocking TMS1 expression with short interfering RNA (siRNA), b.) construct a TMS1 siRNA plasmid containing a U6 promoter driving the expression of a short hairpin RNA (shRNA) directed at the target site of TMS1 along with a selectable neomycin-resistance gene, c.) stably transfect MCF7 and MDA-MB468 breast cancer cell lines with the TMS1 shRNA plasmid or vector alone to create TMS1-null breast cancer cells and isolate neomycin (neo)-resistant clones, and d.) test individual clones for decreased TMS1 protein expression by western analysis. The majority of these goals were met, and this task was completed during the first reporting period (17 July 2003 – 16 July 2004), although through somewhat alternate methods than those originally proposed. An optimal RNAi targeting sequence was identified in transient transfection assays that resulted in a virtual 100% loss of TMS1 expression in MCF7 and MB468 cells. An shRNA plasmid directed at the same sequence was then generated in the pSilencer 1.0 vector (Ambion), and MCF7 cells were stably transfected with this plasmid or with vector alone. A large number of neo-resistant clones were isolated and tested for TMS1 expression by western blot analysis, and a significant majority of the isolated clones displayed some reduction in TMS1 protein, with several exhibiting no detectable TMS1 expression (data not shown). Unfortunately, the knockdown of TMS1 was transient, as all of the knockdown clones regained TMS1 expression after 10-20 passages in culture.

As an alternative to the siRNA approach, we proposed to create TMS1 knockdown cell lines using an antisense approach. A construct containing the TMS1 message in an antisense orientation driven by the cytomegalovirus (CMV) promoter was constructed and stably introduced into MCF7 cells. Neomycin resistant clones were isolated and tested for TMS1 expression by western blot analysis. Although a number of positive clones were identified, the knockdown of TMS1 expression was again transient in nature, with most cells regaining TMS1 expression levels after several passages. Taken together, these results may indicate that loss of TMS1 represents an unfavorable condition for breast cancer cells in culture. However, although stable knockdown of TMS1 was not possible, we have optimized the transfection of synthetic siRNA to TMS1 as an alternative approach for developing a model of TMS1 loss-of-function in human breast cancer cells, as reported in the first reporting period (17 July 2003 – 16 July 2004).

As discussed in the introduction, overexpression of TMS1 induces apoptosis, illustrating that TMS1 is a proapoptotic molecule. This suggests that epigenetic silencing of TMS1 may contribute to breast carcinogenesis by allowing cancer cells to bypass normal apoptotic cues. Therefore, the focus of Task 2 was to determine whether loss of TMS1 causes resistance of breast cancer cells to anticancer agents and other proapoptotic stimuli, and to characterize the resulting apoptotic response using the TMS1 loss-of-function model created in Task 1. In initial studies aimed at better understanding the function of TMS1 as an apoptotic signaling molecule, we utilized we utilized HEK 293 cells (which lack endogenous TMS1 expression) that had been engineered to express TMS1 from an ecdysone-inducible promoter (referred to as MTMS22)

[22]. Addition of the ecdysone analogue ponasterone A (ponA) to the cell culture media drives expression of TMS1 from the ecdysone-inducible promoter in a time- and dose-dependent manner. Using this system, we identified that TMS1 overexpression leads to activation of the initiator caspases-8 and -9 upstream of caspase-3 activation in human embryonic kidney 293 cells (HEK 293) (Appendix A, Figure 1). The impact of TMS1 expression on caspase-8 activation was not specific to MTMS22 cells, as overexpression of TMS1 also induced caspase-8 activation in MCF7 cells (Appendix A, Figure 2 and Appendix B, Figure 5c). In addition, TMS1-induced apoptosis could be blocked with caspase-8 inhibitors in MTMS22 cells, illustrating that TMS1-induced apoptosis relies upon caspase-8 activation (Appendix A, Figures 3 and 4 and Appendix B, Figure 5d).

The finding that TMS1 overexpression activates caspase-8, and that TMS1-induced apoptosis can be blocked with caspase-8 inhibitors, suggested that TMS1 might play a role in apoptosis and/or caspase-8 activation induced by natural upstream stimuli, such as the cytokines TRAIL and TNFα, which activate the extrinsic/death receptor apoptotic pathway. Using the TMS1 loss-of-function model created in Task 1, we determined that TMS1 was dispensable for caspase-8 activation induced by TRAIL or TNFα in MCF7 cells (Appendix A, Figure 5 and Appendix B, Figures 5a and 5b), despite the fact that overexpression of TMS1 activates caspase-8 in this cell type (Appendix A, Figure 2 and Appendix B, Figure 5c). Similarly, TMS1 was also dispensable for caspase-8 activation and apoptosis induced by TRAIL in MB468 cells (Appendix A, Figure 6). However, contrary to the findings in MCF7 cells, MB468 cells lacking TMS1 expression displayed reduced caspase-8 activation and apoptosis compared to control cells in response to TNFα (Appendix A, Figures 6 and 7). These data indicate that loss of TMS1 expression can cause breast cancer cells to be more resistant to apoptosis induced by death receptors such as TNFα. Therefore, the goals outlined in Task 2 of our approved statement of work have been met. These data have recently been published (Appendix B).

The focus of Task 3 was to determine the impact of TMS1 loss on anchorage-independent growth potential. In order to survive, epithelial cells require integrin-mediated adhesion to the extracellular matrix, which helps to dictate cellular properties such as shape, polarity and proliferation. Unligated integrins cause cells to undergo programmed cell death, which in this case is referred to as "anoikis," the Greek word for "homelessness" [23]. The reliance of epithelial cells on the extracellular matrix is termed "anchorage dependence." One of the hallmarks of cancer is the acquired ability to invade basement membranes and neighboring tissues, which requires a reversion from an anchorage dependent state to one of anchorage independence [24]. Overcoming anchorage dependence allows cancer cells to leave their primary site of growth, survive transit through the bloodstream, and colonize a foreign tissue. In addition, resistance to anoikis also underlies the local invasion of many epithelial tumors. Thus, understanding how cells avoid anoikis and gain anchorage independence is an important task in understanding, preventing and treating human cancer.

The majority of breast cancers are thought to arise in the epithelial cells that line the lobules and ducts of the breast. In particular, one of the earliest stages of breast cancer, termed ductal carcinoma *in situ* (DCIS), is characterized by ductal epithelial cells that have filled the luminal space. It is hypothesized that a failure of ductal epithelial cells to undergo anoikis contributes to filling of the lumen in DCIS. In normal breast tissue, TMS1 is selectively expressed in the ductal and lobular epithelium, but is absent from the underlying myoepithelium and stromal cells [16]. This localization, combined with the proposed roles of TMS1 as a proapoptotic, tumor-suppressor molecule, suggests that TMS1 may play a role in the apoptotic

response of epithelial cells to loss of substratum interactions. Consistent with this idea, when we examined the expression of TMS1 in DCIS lesions, we found that while the ductal epithelium retained TMS1 expression, several of the DCIS samples examined had reduced TMS1 expression in the majority of the epithelial cells that had filled in the breast duct (data not shown). Thus, loss of TMS1 expression may be contributing to the pathogenesis of early breast cancer development.

In our approved statement of work, we originally proposed to study anchorage-independent growth potential by using cells stably knocked-down for TMS1 expression and comparing these cells to control cells for their ability to form colonies in soft agar over the course of three weeks. However, as we have had to use a transient siRNA transfection method to knock down TMS1 expression, we cannot perform the soft agar experiments, as the knockdown of TMS1 expression will only last for one week using our transient transfection method. As an alternative, we have optimized a system whereby cell culture dishes are coated with poly(2-hydroxyethyl-methacrylate), which is a hydrogel used to prevent adhesion of cells to growth surfaces. Seeding cells onto poly-HEMA plates triggers anoikis, thus allowing us to study anchorage-independent growth in a short-term setting.

To examine the involvement of TMS1 in anoikis, we first studied the consequences of detachment in the nontransformed breast epithelial cell line MCF10A. TMS1 protein (Appendix A, Figure 8A) and mRNA (Appendix A, Figure 8B) were upregulated in a time-dependent manner following detachment. The proapoptotic BH3-only protein Bim is upregulated following detachment of MCF10A cells through both transcriptional and posttranslational means [25-27], and reduction of Bim expression confers resistance to anoikis [28]. We show in Appendix A, Figure 8A that upregulation of TMS1 protein paralleled that of Bim. These data illustrate that TMS1 expression is induced by detachment and further implicate TMS1 in anoikis. Despite their requirement for TNFα-induced upregulation of TMS1 (see below) [29], JNK and NF-κB signaling pathways are not required for induction of TMS1 following detachment (Appendix A, Figure 9). Further studies will be needed to elucidate the signaling pathways responsible for TMS1 induction during anoikis.

Consistent with our hypothesis that loss of TMS1 expression may be one mechanism by which breast epithelial cells avoid anoikis, we have found that knockdown of TMS1 causes MCF10A cells to be resistant to anoikis. In control cells, upregulation of Bim and TMS1 were evident by 8 and 24 hours in suspension, respectively. Activation of caspase-8 and cleavage of the downstream caspase target PARP occurred 24 hours after detachment in control cells (Appendix A, Figure 10). Strikingly, apoptosis was significantly delayed in TMS1 knockdown cells, as activation of caspase-8 and PARP cleavage were not observed until 48 hours (Appendix A, Figure 10). Upregulation of Bim protein was also significantly delayed in TMS1 knockdown cells (Appendix A, Figure 10). When the timecourse was extended to include a 12 hour timepoint, caspase-8 activation and PARP cleavage were observed as early as 12 hours in control cells, but not until 48 hours in cells lacking TMS1 expression (Appendix A, Figure 10B). Quantification of cell death after 24 hours in suspension showed that knockdown of TMS1 offered approximately 4-fold protection in cell death induced by detachment compared to lamin a/c knockdown cells (Appendix A, Figure 10C). In addition, we also found that knockdown of TMS1 reduces Bim mRNA expression in both resting and detached cells, and upregulation of Bim following detachment is delayed (Appendix A, Figure 11). Despite reports that cytochrome c is released during anoikis [28, 30], cleavage of caspase-9 was not observed in these experiments (data not shown). Taken together, these data illustrate that caspase-8 activation,

Bim upregulation, and PARP cleavage are all severely inhibited in cells lacking TMS1 expression, indicating that loss of TMS1 confers resistance to anoikis. Furthermore, these data suggest that TMS1 silencing may contribute to anchorage-independent growth of ductal epithelial cells in the early stages of breast cancer. Therefore, the overall goal of Task 3 has been met. These data are the subject of a manuscript in preparation and will be submitted for publication within the next month.

In addition to the specific aims outlined in the approved statement of work, this project also made progress in several other areas, as reported in the annual summaries for reporting periods 17 July 2003 - 16 July 2004 and 17 July 2004 - 16 July 2005. For example, during this project we made the novel discovery that the death receptor ligands TNFα and TRAIL are positive regulators of TMS1 expression in breast epithelial cells (Appendix A, Figures 12A-B and 13A-C, and Appendix B, Figures 1a-b and 2a-c). The upregulation of TMS1 by TNFα was not a consequence of transformation, as it occurred in the nontransformed breast epithelial cell line MCF10A (Appendix A, Figure 13C and Appendix B, Figure 2a-c). However, contrary to our findings in epithelial cells and previously published findings in immune cells [31, 32], TNFα and TRAIL had little impact on TMS1 expression in the fibroblast cell line IMR90 (Appendix A, Figures 12C and 13D, and Appendix B, Figures 1c and 2d). This was not due to the absence of TNF receptors or other cell-type dependent differences in components of the TNFα signaling pathway (see below), as treatment of IMR90 cells with TNFα induced an approximate 4-fold increase in the levels of IL-1β mRNA, a well-documented target of TNFα signaling, as measured by conventional RT-PCR (data not shown). These data confirm that the TNFα response is intact in IMR90 fibroblasts, and indicate that the upregulation of TMS1 by TNFα is cell-type dependent. In addition, despite a previous report that TMS1 is a direct transcriptional target of p53 [33], we found that DNA damaging agents that induce p53 stabilization and upregulation of p53 target genes have little impact on TMS1 expression in breast epithelial cells or fibroblasts (Appendix A, Figures 12 and 13, and Appendix B, Figures 1 and 2).
Using transient siRNA transfections, dominant-negative expression constructs and chemical inhibitors, we also determined that both JNK and NF-κB signaling activities are required for TNFα-induced upregulation of TMS1 (Appendix A, Figures 14 and 15, Appendix B, Figure 3a-f and Appendix C, Figure 1). Although the immediate promoter region of TMS1 does not respond to TNFα treatment as determined in luciferase reporter assays (Appendix A, Figure 16 and Appendix C, Figure 2), TNFα treatment does induce local changes the chromatin structure of the TMS1 locus (Appendix A, Figure 17). Furthermore, neither changes in the stability of TMS1 mRNA nor new protein synthesis are required for TNFα-induced upregulation of TMS1 (Appendix A, Figures 18 and 19 and Appendix B, Figure 3g-h). Taken together, these data suggest a direct effect of TNFα on TMS1 transcription. Importantly, TNFα treatment is not sufficient to restore TMS1 expression in cell lines that have methylated and silenced TMS1, such as MDA-MB231 cells (Appendix A, Figure 20). Comparison of DNase-I hypersensitivity between MCF7 cells and MB231 cells illustrated that MB231 cells completely lack CpG island-associated DNase-I hypersensitive sites at the TMS1 locus (Figure 21). Taken together, these data suggest that a pre-existing open chromatin conformation may be required for TNFα-induced upregulation of TMS1 and that treatment of cells with TNFα or TRAIL is not sufficient to overcome epigenetic silencing of TMS1. Thus, the inability to respond to cytokine signaling may be an important consequence of epigenetic silencing. These data have recently been published (Appendix B and Appendix C).

Lastly, as discussed in the annual summary for reporting period 17 July 2004 - 16 July 2005, we also determined that TMS1 does not play a role in NF-κB activity. Beyond inducing apoptosis, proteins containing pyrin and CARD domains can also activate NF-κB and/or influence NF-κB activity induced by upstream stimuli (i.e. cytokines) [34]. TMS1 has been reported to positively regulate NF-κB activity through pyrin-pyrin domain interactions with pyrin or members of the PYPAF family, which are pyrin domain-containing adaptor proteins structurally related to the caspase-9 adaptor protein Apaf-1 [35-37]. In addition, other reports have found that TMS1 can negatively impact NF-κB activity induced by stimuli such as TNFα, Bcl-10 and Nod-1 [32]. As the majority of these studies utilized overexpression of TMS1 or forced interactions between TMS1 and other adaptor proteins, the physiologic role of TMS1 in NF-κB activation remained unclear. However, we have determined that TMS1 has little impact on NF-κB activity induced by death receptors, which are natural upstream inducers of NF-κB dependent transcription. We find here that levels of TMS1 neither negatively nor positively impact NF-κB activation induced by TRAIL, TNFα or Fas (Appendix A, Figures 22 and 23A-B, and Appendix B, Figure 4a-b). In addition, restoration of TMS1 expression in HEK 293 cells does not impact IκBα degradation in response to TNFα (Appendix A, Figure 24 and Appendix B, Figure 4c). These findings are consistent with initial studies performed in our lab suggesting that TMS1 overexpression alone is not sufficient to induce NF-κB activity [22]. Our findings here are also consistent with recent studies from TMS1 knockout mice, which showed that levels of IκBα degradation and NF-κB activation in response to LPS and TNFα are similar between macrophages from wild type and TMS1-/- mice [18].

C. Key Research Accomplishments

- Development of a model of TMS1 loss-of-function in human breast cancer cells
    - Identification of target sites within TMS1 mRNA amenable to blocking TMS1 expression with short interfering RNA
    - Optimization of transient siRNA transfection to knock down TMS1 expression
    - Generation of MCF7 cells stably expressing shRNA to TMS1
    - Generation of MCF7 cells stably expressing antisense to TMS1

- Functional characterization of TMS1 as an apoptotic signaling molecule
    - Determined that TMS1 overexpression induces activation of caspases-8, -9 and -3
    - Determined that TMS1-induced apoptosis relies upon caspase-8 activity
    - Determined that loss of TMS1 expression in MCF7 cells does not affect caspase-8 activation in response to TNFα or TRAIL
    - Determined that loss of TMS1 expression in MDA-MB468 cells does not impact caspase-8 activation or apoptosis induced by TRAIL
    - Determined that loss of TMS1 expression in MDA-MB468 cells inhibits both caspase-8 activation and apoptosis induced by TNFα plus cycloheximide

- Identified a novel role for TMS1 in detachment-induced apoptosis
    - Determined that TMS1 is upregulated following detachment
    - Determined that TMS1 upregulation following detachment is not due to JNK or NF-κB signaling pathways
    - Determined that loss of TMS1 expression in inhibits caspase-8 activation, Bim upregulation and apoptosis following detachment

- Characterized regulation of TMS1 by proapoptotic stimuli
    - Determined that TNFα is a positive regulator of TMS1 expression in transformed and non-transformed breast epithelial cells, but not normal human fibroblasts
    - Determined that the DNA-damaging agents etoposide and mitomycin c do not contribute to TMS1 expression, contrary to a previous report
    - Identified the RelA/p65 subunit of NF-κB as a mediator of TNFα-induced upregulation of TMS1 in MCF7 breast epithelial cells
    - Identified JNK as a mediator of TNFα-induced upregulation of TMS1 in MCF7 breast epithelial cells
    - Determined that neither secondary protein synthesis nor mRNA stability contribute to TNFα-induced upregulation of TMS1 in MCF7 breast epithelial cells
    - Identified local chromatin alterations at the TMS1 locus following TNFα treatment in MCF7 breast epithelial cells
    - Determined that in a breast epithelial cell line in which TMS1 is silenced (MDA-MB231), treatment with TNFα does not restore TMS1 expression
    - Determined that DNaseI hypersensitivity sites found in TMS1 expressing breast epithelial cells are lacking in TMS1 non-expressing breast epithelial cells

- Characterized the impact of TMS1 expression on death receptor-mediated NF-κB activity
    - Determined that levels of TMS1 expression do not impact NF-κB activity in response to death-receptor activation
    - Determined that IκBα degradation in response to TNFα is not impacted by levels of TMS1 expression

D. Reportable Outcomes

Abstracts:

- **Parsons, MJ**, McConnell, BB and Vertino, PM. "Role of TMS1/ASC in Death Receptor Mediated Apoptosis and Survival Pathways" 95th Annual AACR Meeting, Orlando, FL (March, 2004).
- **Parsons, MJ**, McConnell, BB and Vertino, PM. "Role of TMS1/ASC in Death Receptor Mediated Apoptosis and Survival Pathways" AACR Pathobiology of Cancer Workshop, Snowmass, CO (July, 2004).
- **Parsons, MJ** and Vertino, PM. "Regulation of TMS1/ASC by Death Receptor Signaling in Breast Epithelial Cells" AACR Special Conference Regulation of Cell Death in Oncogenesis, Waikoloa Village, HI (January 2005).
- **Parsons, MJ** and Vertino, PM. "Regulation of TMS1/ASC by Death Receptor Signaling in Breast Epithelial Cells" Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA (June 2005).
- **Parsons, MJ** and Vertino, PM. "Role of TMS1 in Detachment-Induced Apoptosis" 97th Annual AACR Meeting, Washington, DC (April, 2006).

Awards:

- 2005 AACR Brigid G. Leventhal Women in Cancer Research Scholar Award

Degrees Conferred:

- Ph.D., Program in Genetics and Molecular Biology, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, GA (May, 2006).

Presentations:

- Oral Presentation: Role of TMS1 Silencing in Breast Carcinogenesis (February, 2004). Graduate School Seminar. Emory University, Atlanta, GA.
- Poster Presentation: Role of TMS1/ASC in Death Receptor Mediated Apoptosis and Survival Pathways (March, 2004). 95th Annual AACR Meeting, Orlando, FL.
- Poster Presentation: Role of TMS1/ASC in Death Receptor Mediated Apoptosis and Survival Pathways (July, 2004). AACR Pathobiology of Cancer Workshop, Snowmass, CO.
- Oral Presentation: Role of TMS1 in Death Receptor Signaling (October 2004). Graduate School Seminar. Emory University, Atlanta, GA.
- Poster Presentation: Regulation of TMS1/ASC by Death Receptor Signaling in Breast Epithelial Cells (January 2005). AACR Special Conference: Regulation of Cell Death in Oncogenesis, Waikoloa Village, HI.
- Poster Presentation: Regulation of TMS1/ASC by Death Receptor Signaling in Breast Epithelial Cells (June 2005). Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA.
- Oral Presentation: Role of TMS1/ASC in Death Receptor Signaling and Breast Carcinogenesis (October, 2005). Graduate School Seminar. Emory University, Atlanta, GA.
- Oral Presentation: Functional Characterization of TMS1/ASC in Apoptotic Signaling (March, 2006). Oral Thesis Defense. Emory University, Atlanta, GA.

- Poster Presentation: Role of TMS1 in Detachment-Induced Apoptosis (April, 2006). 97th Annual AACR Meeting, Washington, DC (April, 2006).

Publications:

- **Parsons, MJ** and Vertino, PM (2006) Dual role of TMS1/ASC in death receptor signaling. Oncogene, May 22; [Epub ahead of print].
- **Parsons, MJ** and Vertino, PM (2006) Loss of TMS1/ASC inhibits anoikis in breast epithelial cells. In preparation.

E. Conclusions

Evasion of apoptosis is a hallmark of cancer that contributes to carcinogenesis by allowing cancer cells to persist under a variety of conditions that would otherwise trigger a cell death response. Circumventing apoptosis in human cancers is achieved through gain of function mutations in anti-apoptotic genes and loss of function mutations in proapoptotic genes. In this annual summary we have provided evidence that TMS1, which is subject to aberrant DNA methylation and epigenetic silencing in a high percentage of human breast and other cancers, is a positive regulator of apoptosis and caspase-8-dependent cell death processes. That TMS1 is a positive regulator of apoptosis suggests that epigenetic silencing of TMS1 may be one mechanism by which evasion of apoptosis is achieved in human cancers. Consistent with this, we have also shown in this dissertation that loss of TMS1 expression can confer resistance to apoptosis induced by both TNFα signaling and detachment from the extracellular matrix through a reduction in caspase-8 activation induced by these stimuli. Therefore, silencing of TMS1 in human breast and other tumors may contribute to evasion of apoptosis and resistance to caspase-8-dependent apoptotic stimuli.

That loss of TMS1 expression confers resistance to cell death induced by detachment (also referred to as "anoikis") is of particular importance for breast cancer biology, as avoiding anoikis contributes to the filling of the breast duct with aberrant epithelial cells in ductal carcinoma *in situ* (DCIS), which represents an early stage in breast cancer development. In this regard, epigenetic silencing of TMS1 could be one mechanism by which breast epithelial cells evade cell death induced by detachment from the extracellular matrix in early breast cancer development. Consistent with this, we have shown that TMS1 is expressed in the epithelial layer surrounding the breast duct, but that TMS1 expression is lost in the aberrant epithelial cells that fill the lumen of the breast duct in DCIS lesions. These data implicate TMS1 silencing in early breast cancer development, and raise the possibility that loss of TMS1 expression is one mechanism by which this early stage of breast cancer is established.

Currently, the molecular markers that predict the severity of breast cancer progression and the likelihood of developing invasive or metastatic disease are few in number. The lack of such prognostic factors is detrimental as it may lead to under-treatment of high risk women and unnecessary treatment of women who would likely not develop metastatic disease. This is especially problematic following diagnosis of DCIS, as there are few molecular markers that predict the potential of ductal carcinoma *in situ* to develop into invasive or metastatic disease. Loss of TMS1 expression and resistance to anoikis may contribute to the acquisition of anchorage-independence in breast epithelial cells, a necessary step in the progression to invasive and metastatic disease. Thus, assessment of TMS1 gene methylation or TMS1 expression status following the diagnosis of DCIS may have prognostic implications. That TMS1 expression could be used as a prognostic marker for aggressive disease is supported by recent studies in prostate cancer, which found that TMS1 methylation and silencing was significantly higher in adjacent normal prostate tissue of patients with biochemical recurrence of aggressive disease [11]. Taken together, these data suggest that TMS1 silencing may be a useful molecular marker for identifying patients likely to develop aggressive breast and other cancers.

Silencing of TMS1 in human cancers may also have therapeutic implications. Our work on death receptor signaling indicates that another important consequence of epigenetic silencing of TMS1 in breast and other cancers may be an attenuated response to cytokines such as TNFα and/or resistance to therapeutic regimens that rely on caspase-8-dependent apoptosis, such as TRAIL-related therapies. Our data suggests that the upregulation of TMS1 by death receptor

signaling in breast epithelial cells may function to amplify the apoptotic signal induced by TNFα and TRAIL by further promoting caspase-8 activation. In addition, TMS1 is required for apoptosis induced by TNFα in some breast cancer cells. Therefore, breast and other tumors in which TMS1 is silenced may be more resistant to death receptor-induced apoptosis or therapies that rely on caspase-8 activation.

Genes that are epigenetically silenced remain structurally intact, thus the potential exists for re-expressing epigenetically silenced genes such as TMS1. In fact, identifying new therapeutic strategies aimed at re-activating epigenetically silenced genes is the subject of much on-going research, which has led to the development of a number of agents that are currently in clinical trial [7]. Our lab has shown that treatment of breast cancer cells with de-methylating agents is sufficient to restore expression of TMS1 [8]. In addition, recent work by Collard *et. al.* [11] has shown that a combination of the DNA de-methylating agents Decitabine and Zebularine restored TMS1 expression in prostate cancer cells which have methylated and silenced TMS1. Thus, patients with tumors in which TMS1 has been silenced may benefit from treatment with de-methylating agents, which could theoretically restore sensitivity to existing chemotherapeutic agents that act through the activation of death receptor-mediated apoptosis, such as TRAIL.

In conclusion, the work presented in this final summary report suggests that epigenetic silencing of TMS1 may contribute to the evasion of apoptosis in breast cancer. Specifically, aberrant DNA methylation and epigenetic silencing of TMS1 may be important for early breast cancer progression by allowing cells to avoid anoikis in the establishment of ductal carcinoma *in situ*, as well as allow cancer cells to avoid apoptosis induced by therapeutic regimens that rely on the death receptor and caspase-8 apoptotic pathways. As genes that are silenced through epigenetic means are structurally intact, restoration of TMS1 expression in human breast and other cancers through treatment with de-methylating agents may restore sensitivity to apoptotic signaling. Taken together, our data suggests that TMS1 expression may be an important component of breast cancer progression.

My work over the last three years has led to the successful completion of a dissertation from the Emory University Graduate School of Arts and Sciences, two publications, a number of published abstracts, and multiple presentations at national meetings. Importantly, this work has also led to the creation of a loss-of-function model of TMS1 in breast epithelial cells. Using this model, we have gained a better understanding of TMS1 function in apoptotic signaling, as well as identified some important consequences of TMS1 silencing in breast cancer cells. Therefore, we feel that the goals of this proposal have been met, and that the role of TMS1 silencing in breast cancer has been thoroughly explored within the scope of our original proposal.

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Appendix A.

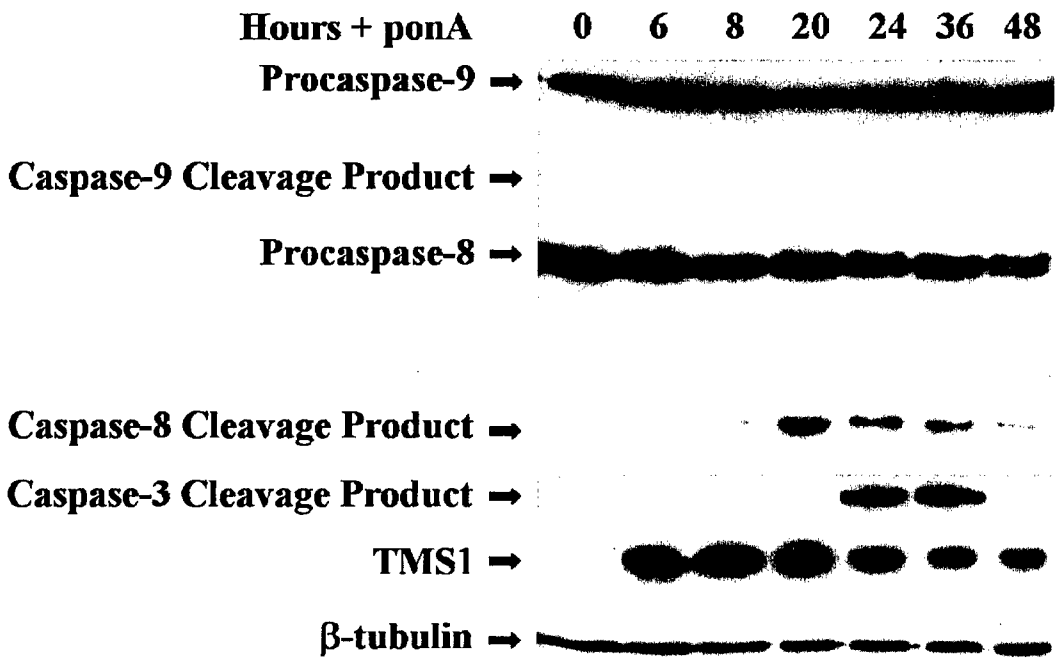


Figure 1. TMS1 overexpression induces activation of caspases-8, -9 and -3. MTMS22 cells were treated with 5 μM ponA for the indicated times, at which point protein lysates were collected and analyzed by western blotting with the indicated antibodies. Cleavage of caspases indicates that they have been activated; therefore, antibodies that recognize both the procaspase and subsequent cleavage products following activation were utilized in this experiment.

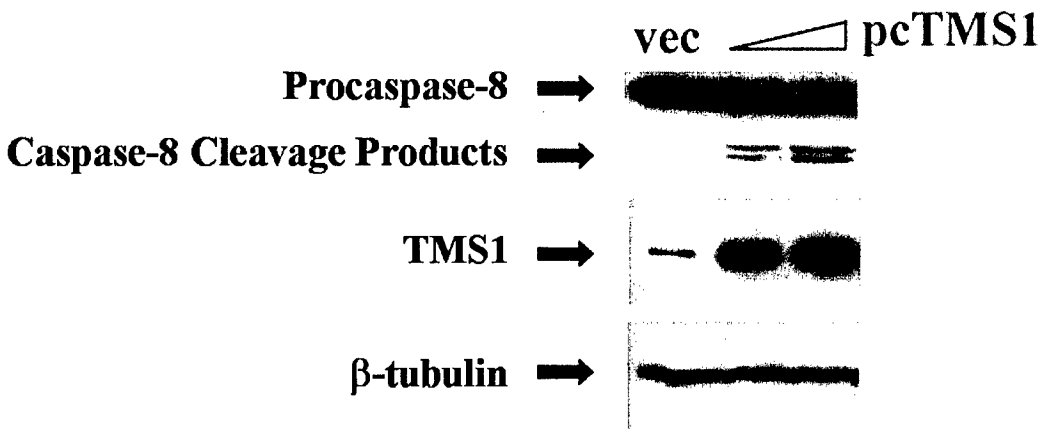


Figure 2. Overexpression of TMS1 induces caspase-8 cleavage in MCF7 cells. MCF7 cells were transfected with 1 μg of an empty vector (pcDNA3.1), or 1-2 μg of a TMS1 expression construct (pcDNA-TMS1). Cell lysates were collected after 24 hours and subjected to western blot analysis using the indicated antibodies.

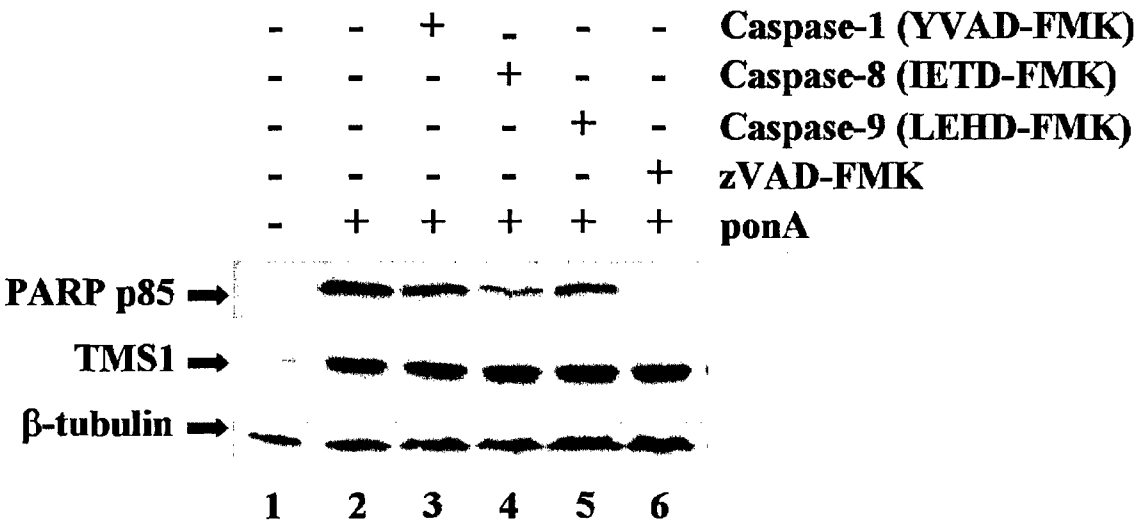


Figure 3. TMS1-induced apoptosis can be blocked with caspase inhibitors. MTMS22 cells were pretreated with 50 μM of the indicated caspase inhibitor for 1 hour, followed by addition of 5 μM ponA for 24 hours, at which time protein lysates were collected and subject to western blot analysis with the indicated antibodies.

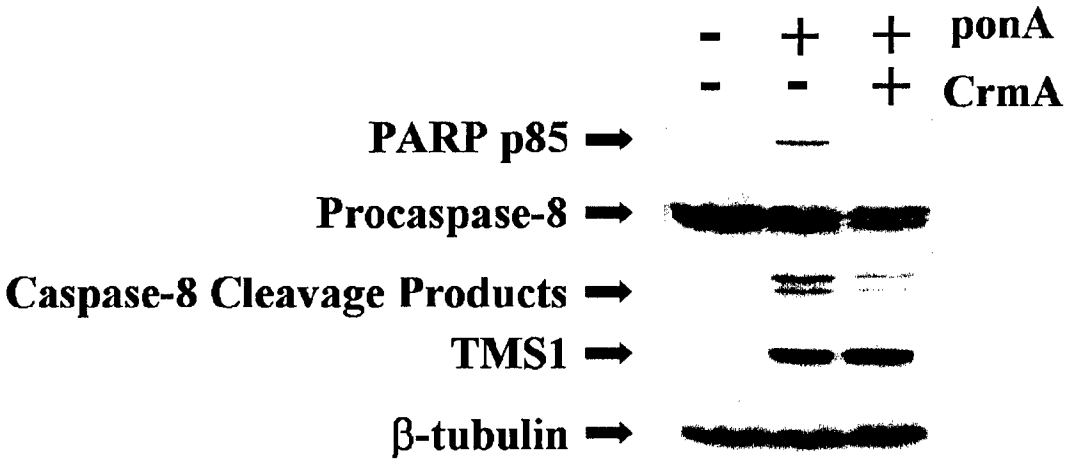


Figure 4. TMS1-induced caspase-8 activation and PARP cleavage is blocked by coexpression of CrmA. MTMS22 cells were transfected in 10 cm dishes with 6 μg empty vector (pcDNA3.1) or 6 μg of a CrmA expression construct (pcDNA-CrmA). After 48 hours, cells were left untreated or treated with 5 μM ponA. The following day, total cellular protein was isolated and subjected to western blot analysis using antibodies to TMS1, caspase-8, PARP p85, and β-tubulin.

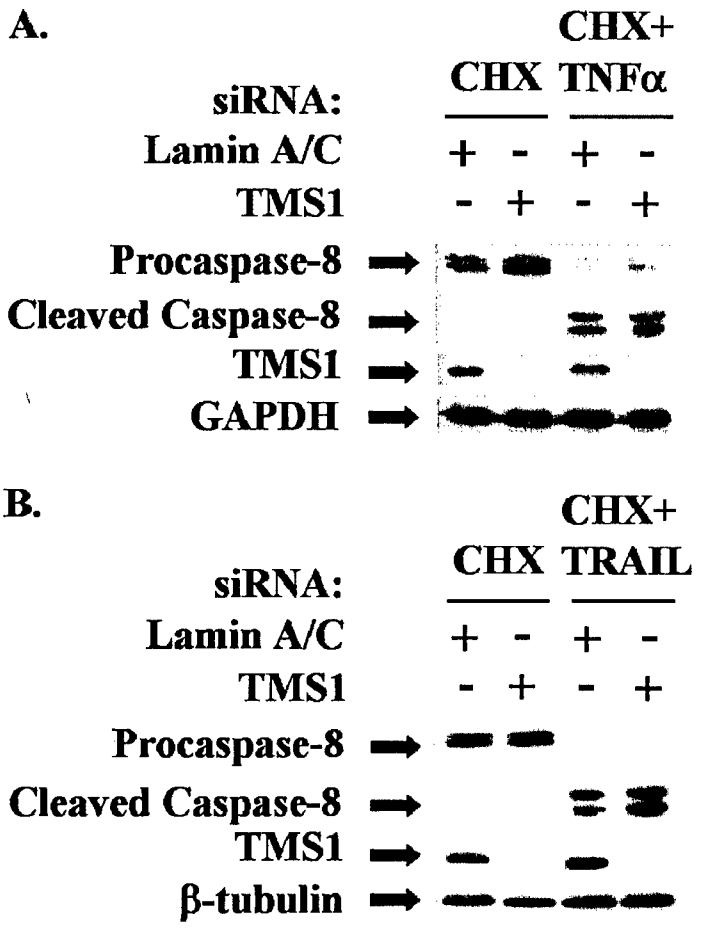


Figure 5. Impact of TMS1 downregulation on caspase-8 activation induced by TNFα or TRAIL in MCF7 cells. MCF7 cells were transfected with 200 nM siRNA targeted to TMS1 or lamin a/c. After 48 hours, cells were pretreated with 2 μg/ml cycloheximide for 30 minutes, followed by treatment with 30 ng/ml TNFα (*A*) or 100 ng/ml TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody (*B*) for 4 hours. Protein lysates were subjected to western blot analysis for caspase-8, TMS1 and either GAPDH or β-tubulin as indicated.

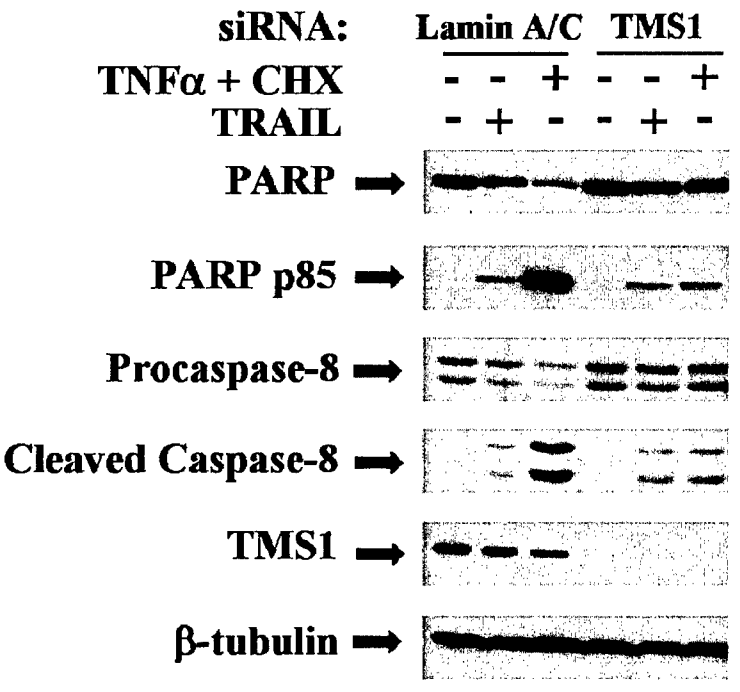


Figure 6. Loss of TMS1 expression in MB468 cells causes a reduction in caspase-8 activation and PARP cleavage induced by TNFα. MB468 cells were transfected with 200 nM siRNA targeted to TMS1 or lamin a/c. After 72 hours, cells were treated with 50 ng/ml TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody or pretreated with 2 μg/ml cycloheximide for 1 hour, followed by treatment with 100 ng/ml TNFα for 5 hours. Protein lysates were subjected to western blot analysis or caspase-8, PARP, PARP p85, TMS1 and β-tubulin as indicated.

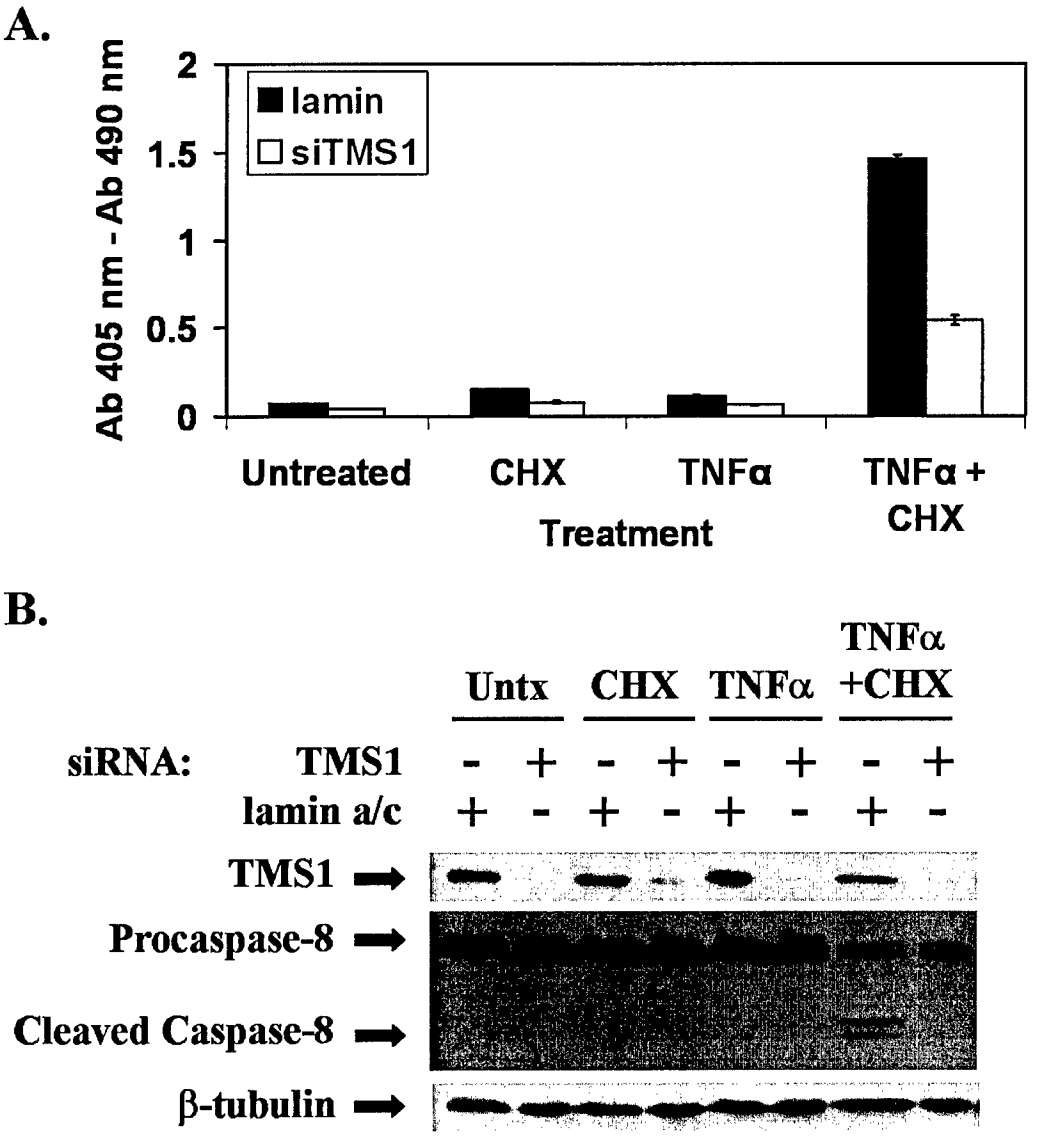


Figure 7. TNFα-induced apoptosis and caspase-8 activation are significantly reduced in MB468 cells lacking TMS1 expression. Cells were transfected with 200 nM siRNA against lamin a/c or TMS1. After 72 hours, cells were treated with 2 μg/ml cycloheximide, 50 ng/ml TNFα, or pretreated with 2 μg/ml cycloheximide for 1 hour followed by treatment with 50 ng/ml TNFα. *A*, Cells were harvested and analyzed for apoptosis using a cell death ELISA as described in Material and Methods. Bars represent the mean of duplicate determinations. *B*, Protein was isolated and subjected to western blot analysis using the indicated antibodies.

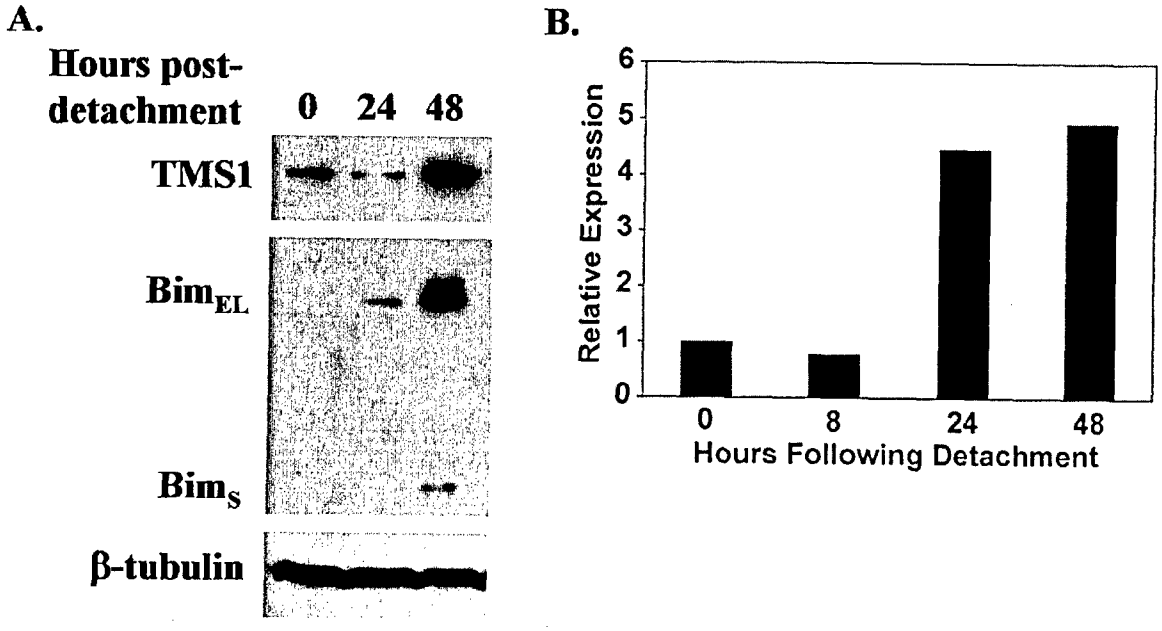


Figure 8. TMS1 protein and mRNA are upregulated during anoikis. MCF10A cells were plated on 10 cm dishes coated with poly-HEMA, and protein (*A*) and mRNA (*B*) were isolated at the indicated timepoints. *A*, Protein lysates were subjected to western analysis using antibodies against TMS1, Bim, and β-tubulin. *B*, Total cellular RNA was isolated at the indicated timepoints. TMS1 expression levels were quantified by reverse transcription and real-time PCR analysis. Data represent the levels of TMS1 mRNA normalized to that of an 18s rRNA internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%.

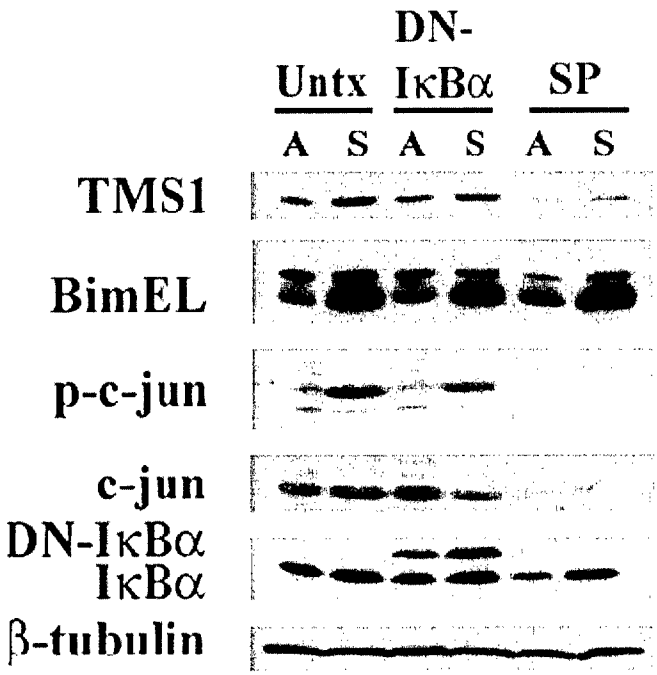
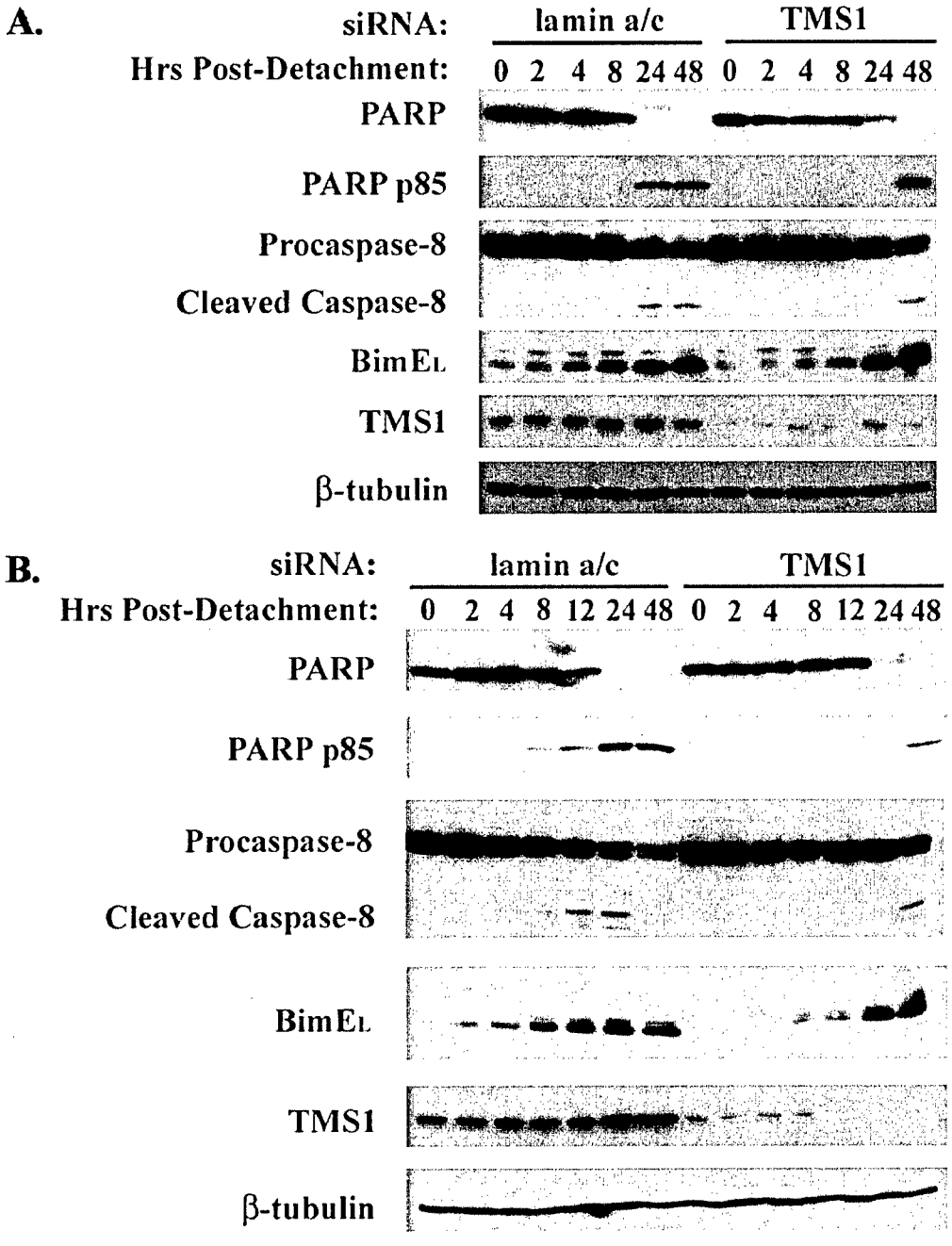


Figure 9. Effects of NF-κB and JNK inhibition on TMS1 upregulation following detachment. For NF-κB inhibition, MCF10A cells were infected with 20 m.o.i. of a dominant-negative IκBα adenoviral construct the night before plating onto poly-HEMA plates. For JNK inhibition, MCF10A cells were pre-treated with 20 μM SP600125 (SP) for 30 minutes. Cells were left attached (A) or plated on poly-HEMA coated 10 cm dishes (S) for 24 hours, at which time cells were harvested for protein. Protein lysates were subjected to western blot analysis with the indicated antibodies.


A.
siRNA:
lamin a/c
TMS1
Hrs Post-Detachment:
0 2 4 8 24 48 0 2 4 8 24 48
PARP
PARP p85
Procaspase-8
Cleaved Caspase-8
BimEL
TMS1
β-tubulin
B.
siRNA:
lamin a/c
TMS1
Hrs Post-Detachment:
0 2 4 8 12 24 48 0 2 4 8 12 24 48
PARP
PARP p85
Procaspase-8
Cleaved Caspase-8
BimEL
TMS1
β-tubulin
C.
Ab 405 nm - Ab 490 nm
1
0.8
0.6
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0.2
0
lamin
TMS1
Attached
Detached
siRNA: TMS1 - +
Lamin A/C + -
TMS1
β-tubulin

Figure 10. Effects of TMS1 knockdown on the apoptotic response to detachment. *A* and *B*, MCF10A cells were transfected with 200 nM siRNA against lamin a/c or TMS1. After 36 hours, cells were plated on poly-HEMA coated dishes, and protein was isolated at various timepoints and subjected to western analysis with the indicated antibodies. *C*, MCF10A cells were transfected as in panels *A* and *B*. After 36 hours, cells were plated on poly-HEMA coated dishes. The following day, the cells were harvested and subjected to a cell death detection ELISA as described in Materials and Methods. At least 5.0×10^4 cells were used per sample. The remaining cells from each sample were harvested for protein and subjected to western blot analysis for TMS1 and β-tubulin as a loading control (*right panel*).

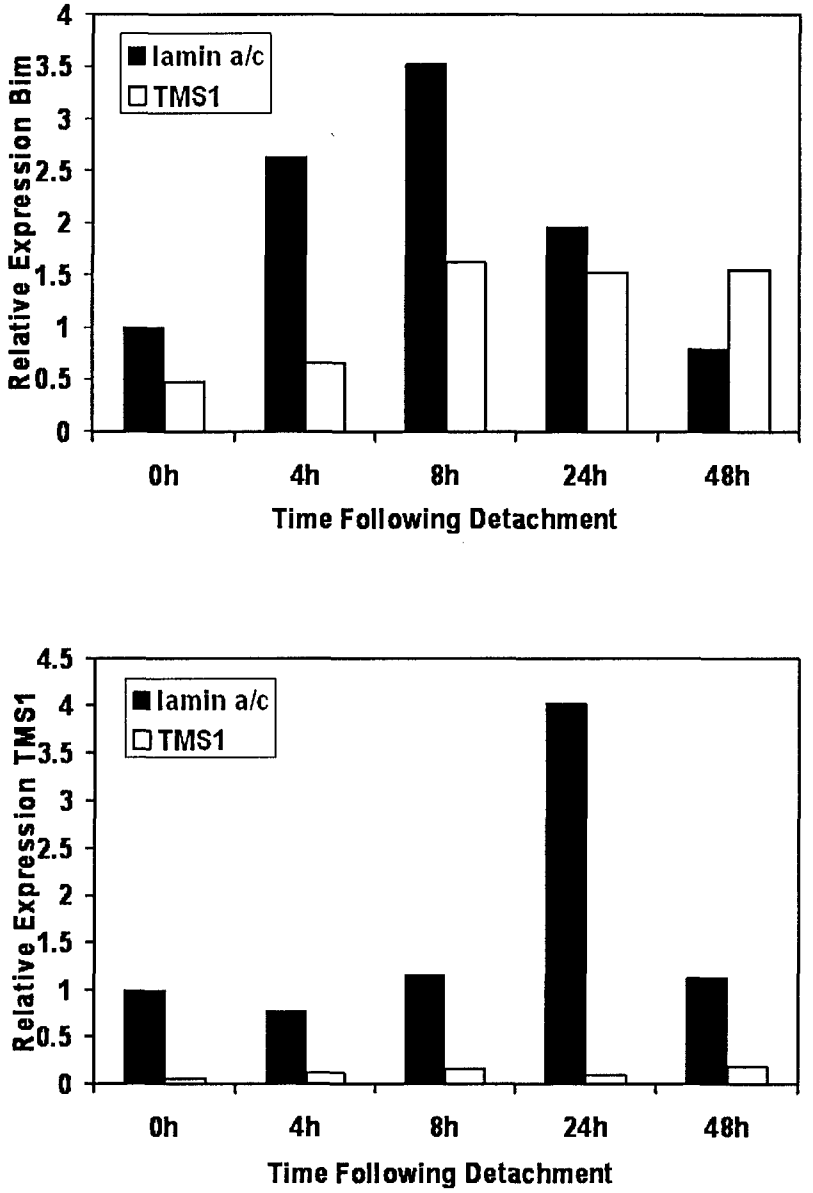

Figure 11. Loss of TMS1 reduces Bim mRNA levels and delays upregulation of Bim during anoikis. MCF10A cells were transfected with 200 nM siRNA against TMS1 or lamin a/c as described in Materials and Methods. After 36 hours, cells were plated onto poly-HEMA coated plates and total cellular RNA was isolated at the indicated timepoints. Bim (*top panel*) and TMS1 (*bottom panel*) expression levels were quantified by reverse transcription and real-time PCR analysis. Data represent the levels of mRNA normalized to that of an 18s rRNA internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%.

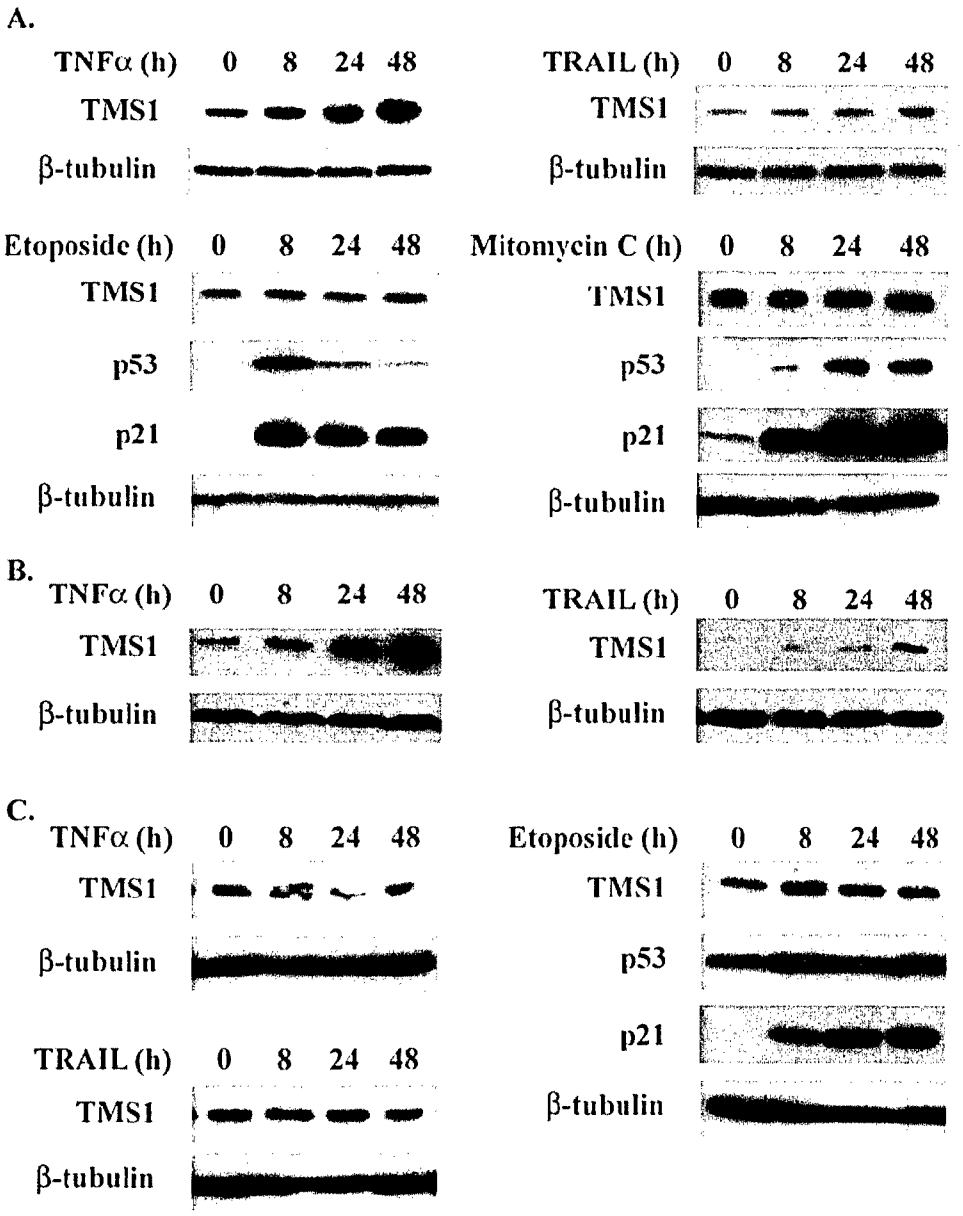

Figure 12. TMS1 is upregulated in response to TNFα and TRAIL, but not DNA damaging agents. *A*, MCF7 cells were treated with 30 ng/ml TNFα, 100 ng/ml TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody, 50 μM etoposide, or 0.25 mg/ml mitomycin C and cell lysates were collected at the indicated time points. Total cellular proteins were subjected to western blot analysis using antibodies to TMS1, p53 and p21 as indicated. β-tubulin levels were similarly analyzed and served as a loading control. *B*, MDA-MB468 cells were treated over 48 hours with 30 ng/ml TNFα or 100 ng/ml TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody and analyzed for TMS1 and β-tubulin protein levels by western blot analysis as in panel *A*. *C*, IMR90 cells were treated over 48 hours with 30 ng/ml TNFα, 100 ng/ml TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody or 50 μM etoposide and analyzed for TMS1, p53, p21 and β-tubulin protein levels by western blot analysis as in panel *A*.

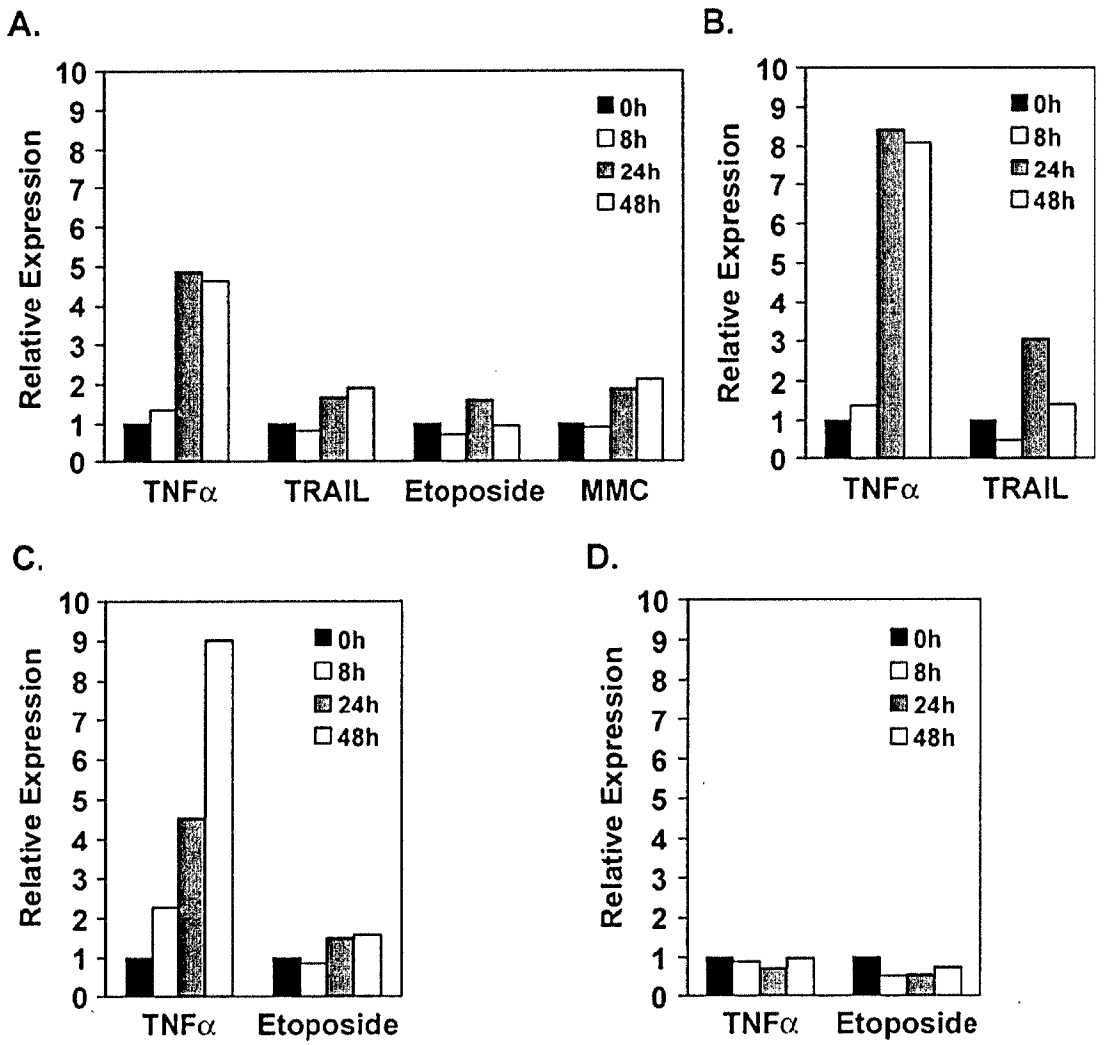

Figure 13. Effect of death receptor ligands and DNA damaging agents on the expression of TMS1 mRNA. *A*, MCF7 cells were treated with 30 ng/ml TNFα, 100 ng/ml TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody, 50 μM etoposide or 0.25 mg/ml mitomycin c (MMC), and total cellular RNA was isolated at the indicated timepoints. TMS1 expression levels were quantified by reverse transcription and real-time PCR analysis. Data represent the levels of TMS1 mRNA normalized to that of an 18s rRNA internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative experiment which was repeated at least twice with similar results. *B*, MDA-MB468 cells were treated with 30 ng/ml TNFα or 100 ng/ml TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody over 48 hours and analyzed for TMS1 expression as in panel *A*. *C* and *D*, MCF10A breast epithelial cells (*C*) and IMR90 fibroblasts (*D*) were treated with 30 ng/ml TNFα or 50 μM etoposide and analyzed for TMS1 expression as in panel *A*. TMS1 expression levels at time zero differed between the cell lines such that IMR90 cells and MCF10A cells express approximately 10-20 fold less TMS1 than MCF7 and MDA-MB468 cells.

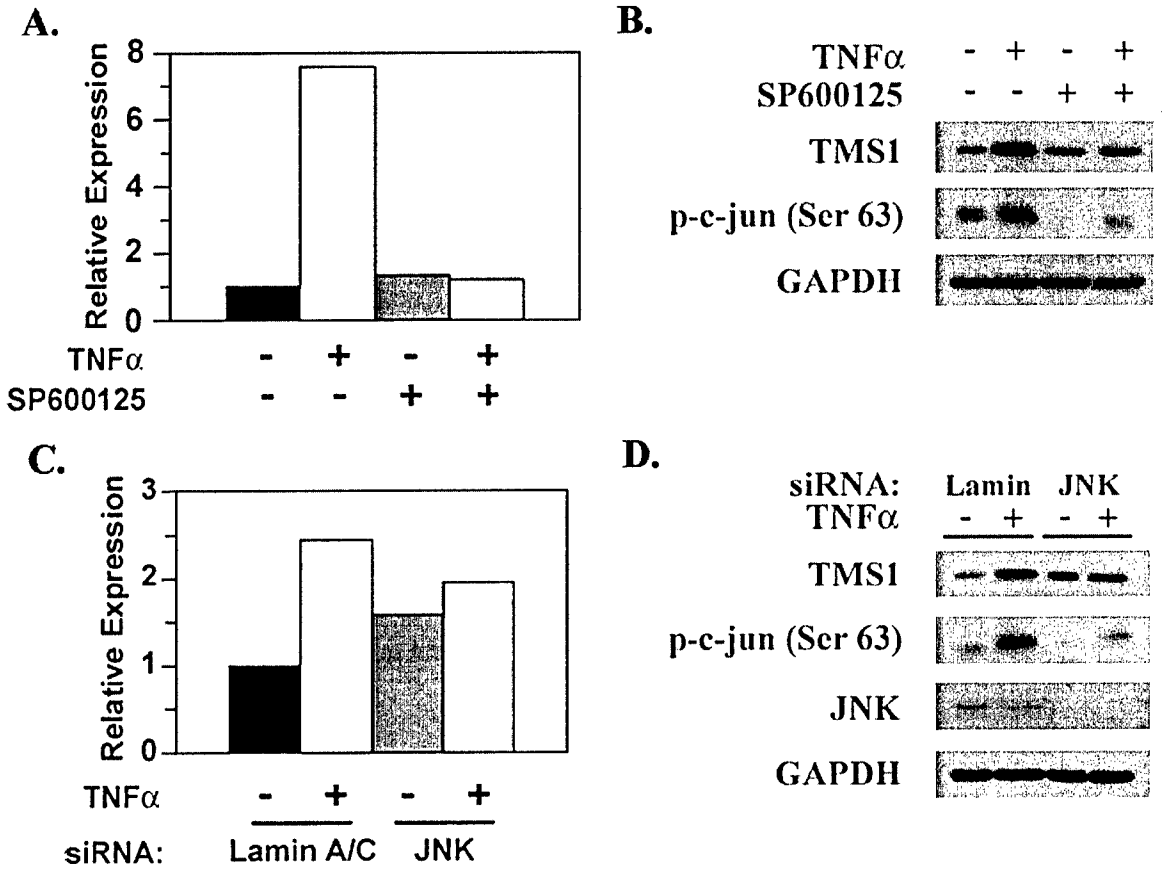

Figure 14. JNK signaling is required for TNFα-induced upregulation of TMS1. *A and B*, MCF7 cells were either left untreated or were pretreated with 20 μM SP600125 for 30 minutes. TNFα was then added to a final concentration of 30 ng/ml for 48 hours. *A*, TMS1 mRNA expression was determined by real time RT-PCR as described in Figure 2. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative of two independent experiments with similar results. *B*, MCF7 cells were treated as described in *A*. Protein lysates were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to TMS1, phospho-c-jun (Ser 63), and β-tubulin as indicated. *C and D*, MCF7 cells were transfected with 200 nM siRNA targeted to JNK or lamin a/c. After 24 hours, cells were left untreated or treated with 30 ng/ml TNFα for an additional 48 hours. *C*, TMS1 mRNA expression was determined by real time RT-PCR. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative of two independent experiments. *D*, Protein lysates from parallel cultures were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to TMS1, phospho-c-jun (Ser 63), JNK and GAPDH.

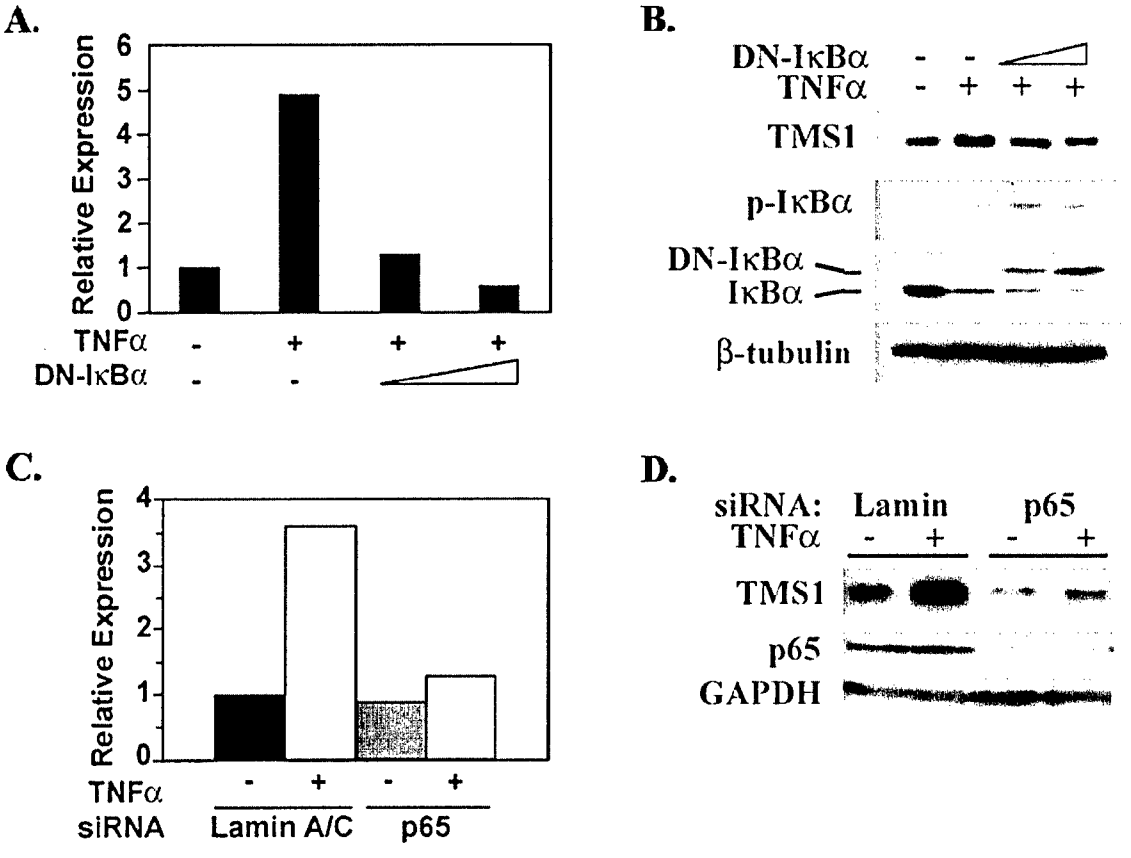

Figure 15. NF-κB signaling is required for TNFα-induced upregulation of TMS1. *A and B,* MCF7 cells (1.0×10^6) were infected with 0, 20 or 100 m.o.i. of an adenoviral construct expressing dominant-negative IκBα (Ad-mIκBα). After 18 hours, cells were left untreated or treated with 30 ng/ml TNFα and incubated for an additional 48 hours. *A*, TMS1 mRNA expression was determined by real time RT-PCR as described in Figure 2. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative of two independent experiments with similar results. *B*, Protein lysates from parallel cultures were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to TMS1, IκBα, phospho-IκBα, and β-tubulin. *C and D*, MCF7 cells were transfected with 200 nM siRNA targeted to p65/RelA or lamin a/c. After 24 hours, cells were left untreated or treated with 30 ng/ml TNFα for an additional 24 hours. *C*, TMS1 mRNA expression was determined by real time RT-PCR. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative of three independent experiments. *D*, Protein lysates from parallel cultures were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to TMS1, p65/RelA and GAPDH.

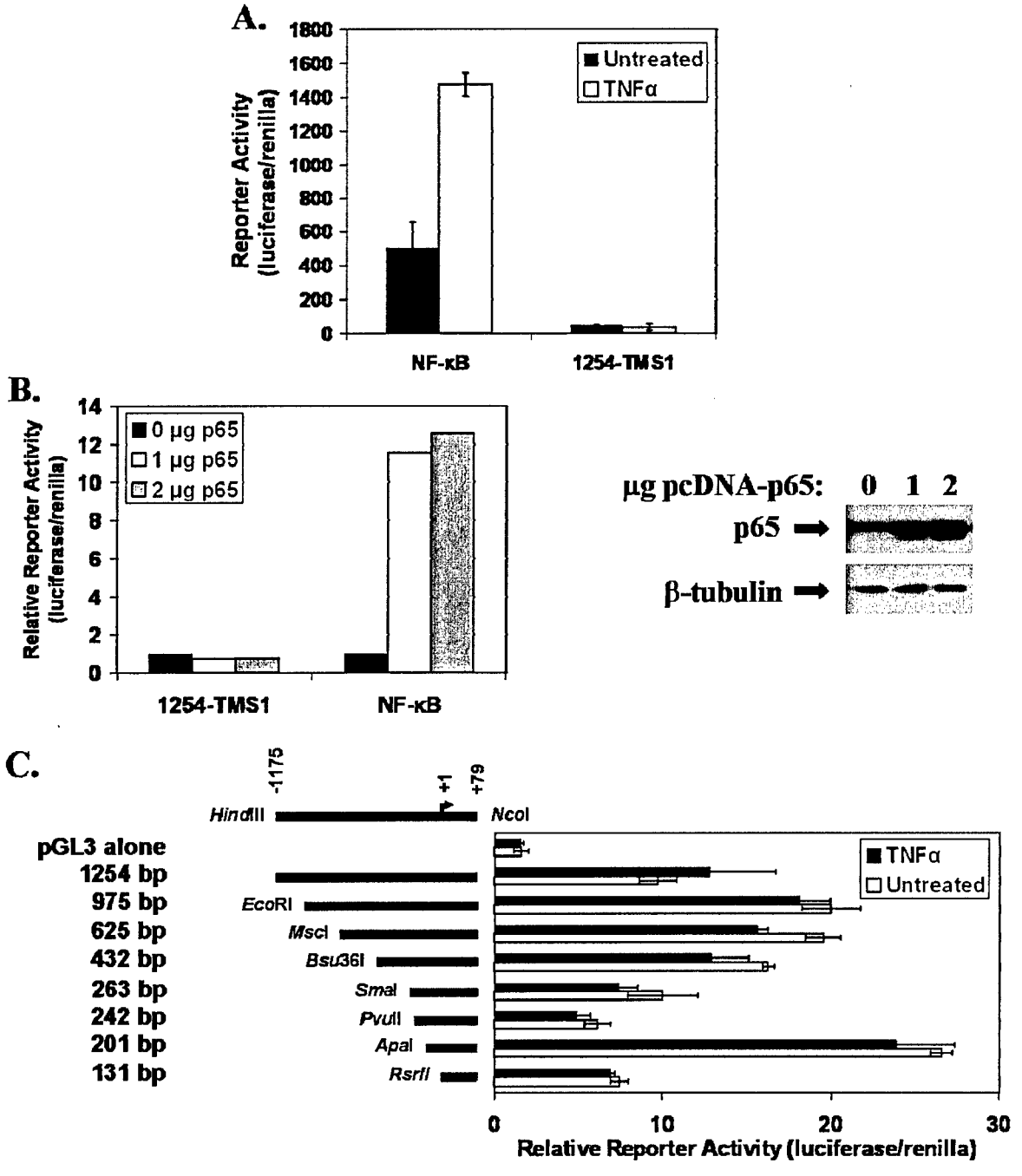

Figure 16. Effect of TNFα and p65/RelA on the TMS1 promoter. *A*, MCF7 cells were transfected with 200 ng of an NF-κB-responsive luciferase reporter plasmid (pNF-κB-Luc, Stratagene) or a TMS1 promoter luciferase reporter plasmid (pTMS1-1254-Luc) using the FuGene reagent. Ten ng of a renilla luciferase plasmid (pRL-TK) was included as a control for transfection efficiency. After 18 hours, cells were left untreated or treated with 30 ng/ml TNFα for 48 hours, at which time luciferase activity was determined. Data represent the mean +/- standard deviation of triplicate determinations after correction for transfection efficiency. *B*, *Right panel*, MCF7 cells were transfected with 500 ng of an NF-κB-responsive luciferase

reporter plasmid (pNF-κB-Luc, Stratagene) or a TMS1 promoter luciferase reporter plasmid (pTMS1-1254-Luc), along with the indicated amounts of a p65 expression construct (pcDNA-p65) using Lipofectamine. Fifty ng of a renilla luciferase plasmid (pRL-TK) was included as a control for transfection efficiency. Total amount of DNA was kept constant at 2.5 μg using pcDNA3.1 (Invitrogen). *Left panel*, Luciferase activity was determined after 48 hours. Data represent the average fold increase in reporter activity of four independent determinations after correction for transfection efficiency. Determinations did not differ by more than 10% of the mean. *Right panel*, Parallel cultures were subjected to western analysis using antibodies against p65/RelA and β-tubulin as a loading control. *C*, MTMS22 cells were transfected with 200 ng of the indicated plasmids using FuGene. Ten ng of a renilla luciferase plasmid (pRL-TK) was included as a control for transfection efficiency. Cells were treated as in *A*.

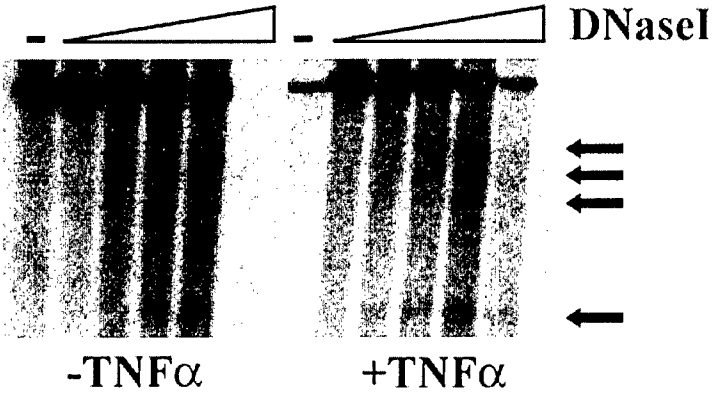

Figure 17. DNaseI hypersensitive site analysis of MCF7 cells treated with TNFα. MCF7 cells were left untreated or treated with 30 ng/ml TNFα for 24 hours, at which point nuclei were isolated, digested with DNaseI and analyzed by Southern blot analysis as described in Materials and Methods. Arrows indicate bands which are more distinct in TNFα-treated cells.

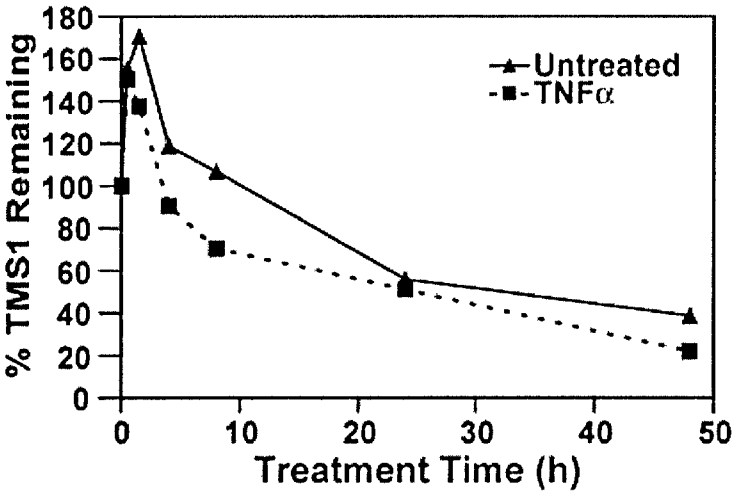

Figure 18. Effect of TNFα on TMS1 mRNA stability. MCF7 cells were pretreated for 30 minutes with 1 μg/ml actinomycin D, followed by the addition of 0 or 30 ng/ml TNFα. Total RNA was isolated at the indicated times and analyzed for TMS1 mRNA levels by real-time RT-PCR as described in Figure 2. TMS1 levels are expressed relative to the value at time zero after normalization to the levels of an 18S rRNA internal control. Data represent the mean of duplicate PCR determinations which varied by less than 10%.

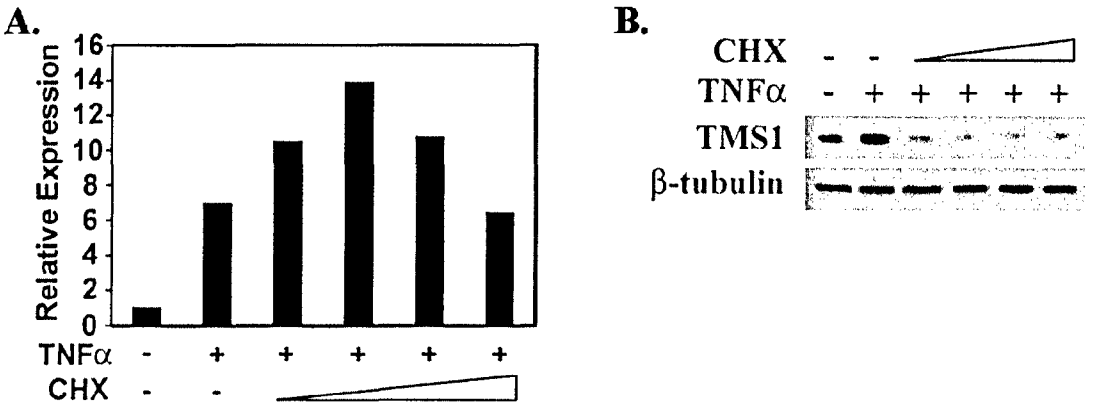

Figure 19. Secondary protein synthesis is not required for TNFα-induced upregulation of TMS1 mRNA. *A and B*, MCF7 cells were left untreated or were pretreated with 0.5, 1, 2 or 4 μg/ml cycloheximide for one hour, followed by treatment with 30 ng/ml TNFα for 24 hours. TMS1 mRNA and protein expression were analyzed by real-time RT-PCR (*A*) and western blot analysis (*B*) as described in Figure 2. Shown is a representative of three independent experiments.

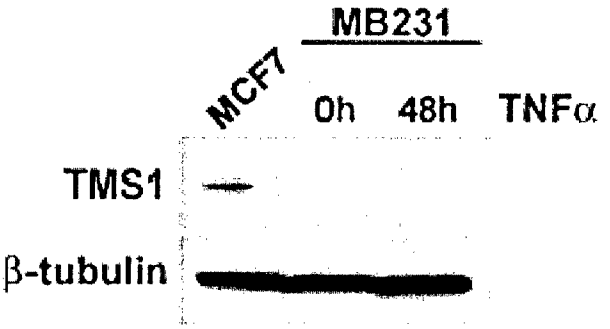

Figure 20. TNFα treatment is not sufficient to restore TMS1 expression in MB231 cells. MB231 cells were treated with 30 ng/ml TNFα for 48 hours. Protein lysates were immunoblotted for TMS1 or β-tubulin as a loading control. Protein lysates from MCF7 cells served as a positive control for TMS1 expression.

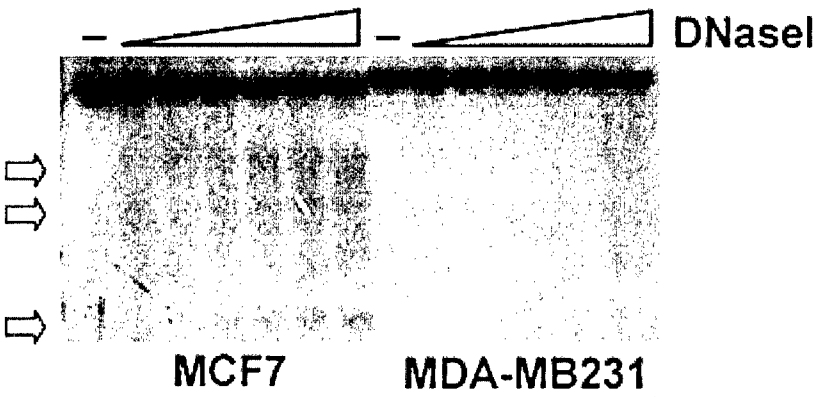

Figure 21. DNaseI hypersensitive site analysis of MCF7 and MDA-MB231 breast epithelial cells. Nuclei were isolated from MCF7 and MDA-MB231 breast epithelial cells and digested with increasing amounts of DNaseI. DNA was isolated and subjected to Southern blot analysis with a radiolabelled probe anchored to the 3' end of the TMS1 locus. Arrows indicate DNaseI hypersensitive sites.

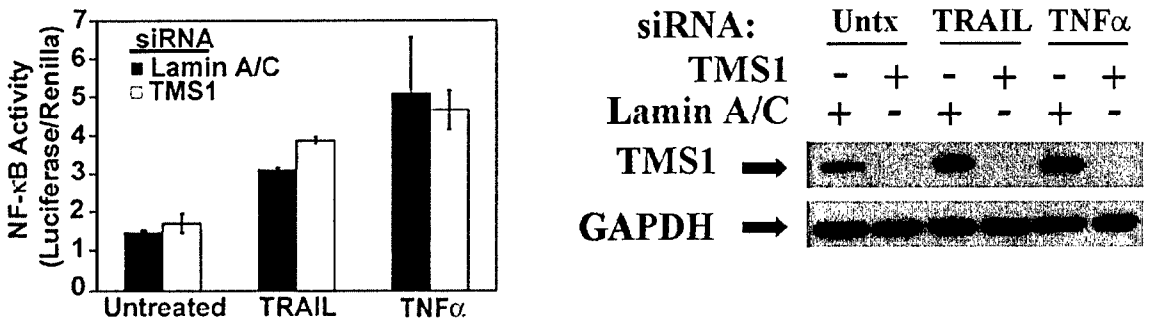

Figure 22. TMS1 is dispensable for death receptor-induced activation of NF-κB in MCF7 cells. MCF7 cells were transfected with 200 nM siRNA targeted to TMS1 or lamin a/c for 48 hours, followed by transfection with 1 μg of the NF-κB-responsive luciferase reporter plasmid (pNF-κB-Luc) and 50 ng of a renilla luciferase control plasmid (pRL-TK) as a control for transfection efficiency. After 24 hours, cells were left untreated or treated with 100 ng/ml TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody or 30 ng/ml TNFα for an additional 8 hours at which time luciferase activity was determined (*left panel*). Data represent the mean +/- standard deviation of duplicate determinations after correction for transfection efficiency. *Right panel*, Total cellular protein collected from parallel cultures was analyzed for TMS1 and GAPDH by western blot analysis.

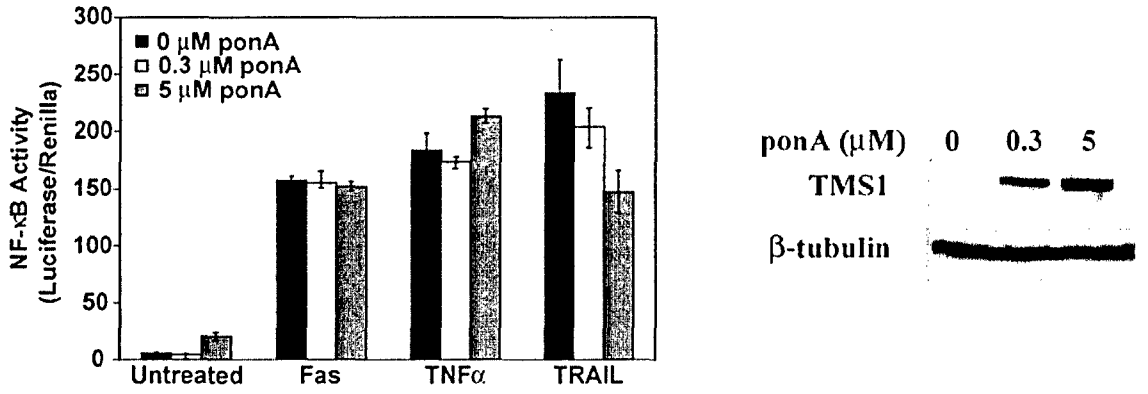

Figure 23. TMS1 levels do not affect NF-κB activity induced by death receptors in MTMS22 cells. MTMS22 cells were transfected with 200 ng of pNF-κB-Luc and 10 ng pRL-TK. After 24 hours, cells were left untreated or treated with 0.3 μM or 5 μM ponasterone A to induce TMS1 expression. The following day, cells were treated with 1 μg/ml Fas, 20 ng/ml TNFα or 20 ng/ml TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody for 8 hours, at which time luciferase activity was determined (*left panel*). Data represent the mean +/- standard deviation of triplicate determinations after correction for transfection efficiency. Total cellular protein collected from parallel cultures was analyzed for TMS1 and β-tubulin by western blot analysis (*right panel*).

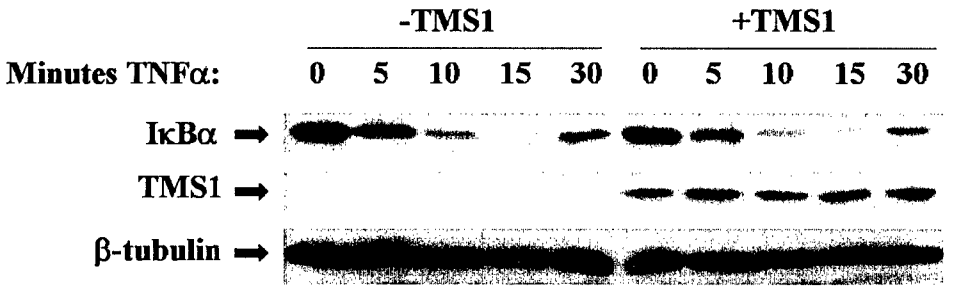

Figure 24. Levels of TMS1 do not affect the kinetics or degradation of IκBα in response to TNFα treatment. MTMS22 cells were left untreated or treated with 0.3 μM ponasterone A. The following day, cells were treated with 20 ng/ml TNFα, and total cellular protein was isolated at the indicated timepoints. Protein lysates were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to IκBα, TMS1, and β-tubulin.

Appendix B.

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ORIGINAL ARTICLE

Dual role of TMS1/ASC in death receptor signaling

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Aberrant DNA methylation of promoter region CpG islands is associated with gene silencing and serves as an alternative to mutations in the inactivation of tumor suppressor genes in human cancers. We identified a gene *TMS1* (for *T*arget of *M*ethylation-mediated *S*ilencing) that is subject to such epigenetic silencing in a significant proportion of human breast and other cancers. Also known as ASC and PYCARD, *TMS1* encodes a bipartite intracellular signaling molecule with proposed roles in apoptosis and inflammation. However, the precise role of this protein in the pathogenesis of breast and other cancers has not been clearly defined. In this study, we examined the role of TMS1/ASC in death receptor signaling. We found that TMS1/ASC is upregulated in response to treatment with TNF-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor-α (TNFα) in breast epithelial cells, but not in human fibroblasts. This upregulation was not dependent on the synthesis of a TNFα-regulated intermediate or alterations in mRNA stability, suggesting a direct effect on TMS1/ASC transcription. Induction of TMS1/ASC by TNFα was blocked by co-expression of a dominant negative IκBα, small interfering RNA-mediated knockdown of RelA/p65, or concurrent treatment with SP600125, indicating a requirement for the nuclear factor-κB (NF-κB) and jun kinase signaling pathways. Although previous work has suggested that TMS1/ASC may be directly regulated by p53, we found that whereas treatment of breast epithelial cells or normal diploid fibroblasts with DNA damaging agents resulted in the stabilization of endogenous p53 and a concomitant increase in p21, it had little impact on the expression of TMS1/ASC mRNA or protein. We further show that whereas TMS1/ASC is not required for TNFα or TRAIL-induced activation of NF-κB or caspase-8, it can promote caspase-8 activation independently of death receptor–ligand interactions. Taken together, these data suggest that upregulation of TMS1/ASC by TNFα and subsequent activation of caspase-8 could function to amplify the apoptotic signal induced by death receptors in some cell types, including breast epithelial cells.
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Introduction

Acquired genetic alterations that lead to defects in apoptosis are common in human cancers and are thought to promote tumorigenesis by allowing cells to survive under conditions that would otherwise trigger a cell death response. Unlike normal cells, cancer cells survive despite DNA damage, in the absence of required growth factors and under the hypoxic conditions encountered when a tumor outgrows its blood supply. To metastasize, tumor cells must survive transit through the blood stream and colonize a foreign tissue, although normal epithelial cells die when deprived of substratum interactions. Importantly, the propensity of tumor cells to undergo apoptosis is a critical determinant of their sensitivity to many chemotherapeutic agents. Therefore, defects in apoptotic signaling contribute to tumor initiation and progression, and can lead to drug resistance and treatment failure.

Gene silencing associated with aberrant methylation of CpG island-containing gene promoters serves as an alternative, epigenetic mechanism contributing to loss of gene function in human cancers (reviewed in Jones and Baylin, 2002; Laird, 2005). CpG islands are CpG dense regions that flank the 5′ end of more than half the genes in the human genome (Bird, 1986; Lander *et al.*, 2001). Although normally maintained in an unmethylated state, CpG islands can become aberrantly methylated in cancer. This methylation is accompanied by a shift to a repressed chromatin conformation, which renders the associated gene transcriptionally silent. Once established, this state is mitotically heritable and contributes to the stable silencing of tumor suppressor and other genes. Several proapoptotic genes succumb to epigenetic silencing in human tumors, including *CASP8*, the TNF-related apoptosis-inducing ligand (TRAIL) receptors *TNFRSF10A* and *TNFRSF10B*, the caspase-9 adaptor *APAF1* and the death-associated protein kinase, *DAPK* (Kissel *et al.*, 1997; Teitz *et al.*, 2000; Soengas *et al.*, 2001; van Noesel *et al.*, 2002; Furukawa *et al.*, 2005; Horak *et al.*, 2005), suggesting that acquired epigenetic alterations also contribute to apoptotic resistance during tumor progression.

In a screen for targets of methylation-mediated silencing, we identified a gene *TMS1* (for *T*arget of *M*ethylation-mediated *S*ilencing), that is methylated and silenced in a significant proportion of human breast cancers (Conway *et al.*, 2000). TMS1 was also independently identified by Masumoto *et al.* (1999) as a protein that forms cytoplasmic 'specks' during retinoic acid induced differentiation or drug-induced apoptosis in HL60 cells, and called ASC. Subsequent studies by our lab and others have implicated the epigenetic silencing of *TMS1* in a number of other tumor types, including melanomas, glioblastomas, non-small cell lung cancers, gastric and colorectal cancers (Moriai *et al.*, 2002; Guan *et al.*, 2003; Virmani *et al.*, 2003; Yokoyama *et al.*, 2003; Stone *et al.*, 2004). Ectopic expression of TMS1 suppresses the growth of breast cancer cells, consistent with a role in tumor suppression (Conway *et al.*, 2000).

At present, the precise function of TMS1 and the consequences of its silencing during carcinogenesis are not known. *TMS1* encodes a bipartite adaptor protein containing an N-terminal pyrin domain (PYD) and a C-terminal caspase recruitment domain (CARD), two members of the death domain fold superfamily of protein–protein interaction domains that are found in proteins that mediate apoptosis and inflammation. There is evidence supporting a role for TMS1 in both of these processes. Evidence from overexpression studies indicates that TMS1 can drive apoptosis in a Bax and caspase-9-dependent manner (McConnell and Vertino, 2000; Ohtsuka *et al.*, 2004), and antisense-mediated knockdown of TMS1 protects cells from apoptosis induced by cytotoxic agents (Masumoto *et al.*, 1999). TMS1 has also been implicated in the extrinsic apoptotic pathway, as forced oligomerization or co-expression with CARD12/Ipaf stimulates caspase-8-dependent apoptosis (Masumoto *et al.*, 2003). TMS1 is also reported to be an integral component of the 'inflammasome,' a multiprotein complex that regulates the maturation of proinflammatory cytokines in cells of myeloid lineage (Martinon *et al.*, 2002). Indeed, recent evidence from knockout mice indicates that TMS1 is required for the secretion of interleukin-1β (IL-1β) in response to bacterial pathogens in macrophages (Mariathasan *et al.*, 2004; Yamamoto *et al.*, 2004). Whether it plays a similar, or perhaps additional, role in epithelial cells is not known.

Currently, the natural upstream stimulus for TMS1-dependent apoptosis is not well defined, and its regulation and function in epithelial cells has not been extensively studied. In this study, we examined the role of TMS1 in the cellular response to initiators of the extrinsic (death receptor) and intrinsic (mitochondrial) cell death pathways in breast epithelial cell lines and normal diploid fibroblasts. We find that TMS1 expression is induced in breast epithelial cells in response to the death receptor ligands TRAIL and tumor necrosis factor-α (TNFα). The induction by TNFα was specific to epithelial cells and required both the nuclear factor-κB (NF-κB) and jun kinase (JNK) signaling pathways. In contrast, we found little impact of DNA damaging agents on TMS1 expression in this study. Furthermore, we show that TMS1 is not required for NF-κB or caspase-8 activation induced by death receptor ligands in MCF7 cells, but promotes cleavage and activation of caspase-8 independently of death receptor–ligand interactions. These data indicate that TMS1 is both regulated by and promotes death receptor signaling in breast epithelial cells. Epigenetic silencing of TMS1 may therefore contribute to carcinogenesis by dampening the cellular response to death receptor ligands.

Results

TMS1 is normally highly expressed in immune cells, particularly in neutrophils and cells of the macrophage/monocyte lineage. Previous studies have shown that TMS1 is induced in these cell types in response to pro-inflammatory stimuli including IL-1β, LPS and TNFα (Shiohara *et al.*, 2002; Stehlik *et al.*, 2003), although the exact mechanism of this upregulation has not been addressed. TMS1 is also expressed in many epithelial cell types (Masumoto *et al.*, 2001). We have previously shown that in normal breast tissue, TMS1 is selectively expressed in the ductal and lobular epithelium, but is absent from the underlying myoepithelium and stromal cells (McConnell and Vertino, 2004). At present little is known about the factors that normally regulate TMS1 expression, and the function of the protein in cells of non-immune origin has not been extensively studied.

To gain insight into the regulation and function of TMS1, we characterized the response of TMS1 to death receptor ligands and genotoxic agents in normal and neoplastic breast epithelial cells. TMS1 protein levels were determined by Western blot analysis (Figure 1) and mRNA levels were determined by quantitative real-time reverse transcriptase PCR (RT-PCR) (Figure 2). Treatment of the breast cancer cell line MCF7 with TNFα resulted in a time-dependent increase in TMS1 protein levels (Figure 1a) and a 5-fold induction in TMS1 mRNA after 24 h (Figure 2a). TRAIL treatment also resulted in an upregulation of TMS1 protein and message, although to a lesser extent (2-fold) and with delayed kinetics relative to TNFα (Figures 1a and 2a). In contrast, treatment with mitomycin C resulted in a modest upregulation of TMS1 at later timepoints (Figures 1a and 2a), while etoposide treatment had no effect on TMS1 expression in MCF7 cells at either the protein or mRNA level (Figures 1a and 2a). That treatment with etoposide and mitomycin C elicited a normal p53-dependent DNA damage response in this cell line was confirmed by an observed stabilization of endogenous p53 and a concomitant upregulation of the p53 target gene p21 (Figure 1a).

A second TMS1 positive breast cancer cell line, MDA MB468, was also tested with similar results (Figures 1b and 2b). Treatment with TNFα induced a robust upregulation of TMS1 protein and message, whereas the effects of TRAIL were more modest (Figures 1b and 2b). MDA MB468 cells are p53 compromised and thus would not be expected to undergo p53-dependent

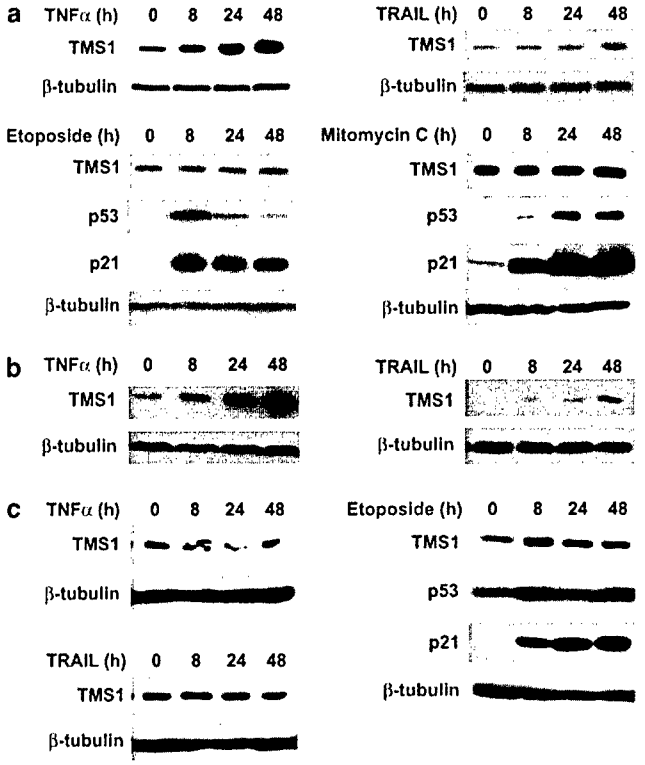

Figure 1 TMS1 is upregulated in response to TNFα and TRAIL, but not DNA damaging agents. (**a**) MCF7 cells were treated with 30 ng/ml TNFα, 100 ng/ml TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody, 50 μM etoposide, or 0.25 mg/ml mitomycin C and cell lysates were collected at the indicated time points. Total cellular proteins were subjected to Western blot analysis using antibodies to TMS1, p53 and p21 as indicated. β-tubulin served as a loading control. (**b**) MDA MB468 cells were treated over 48 h with 30 ng/ml TNFα or 100 ng/ml TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody and analysed for TMS1 and β-tubulin protein levels by Western blot analysis. (**c**) IMR90 cells were treated over 48 h with 30 ng/ml TNFα, 100 ng/ml TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody or 50 μM etoposide and analysed for TMS1, p53, p21 and β-tubulin protein levels by Western blot analysis.

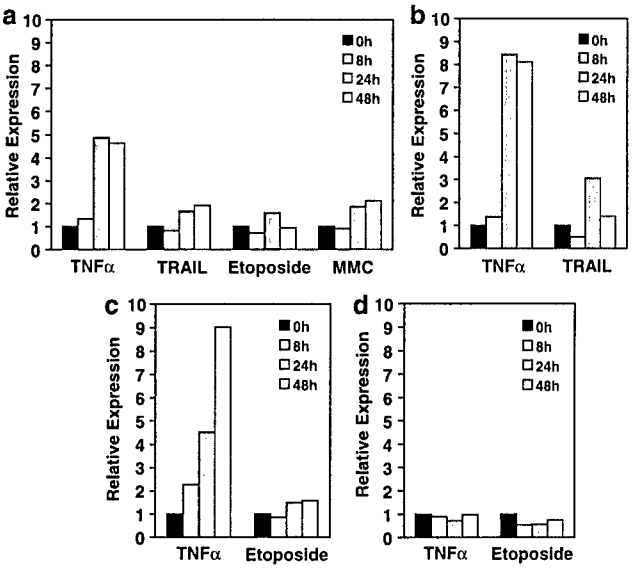

Figure 2 Effect of death receptor ligands and DNA damaging agents on the expression of TMS1 mRNA. (**a**) MCF7 cells were treated with 30 ng/ml TNFα, 100 ng/ml TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody, 50 μM etoposide or 0.25 mg/ml mitomycin C, and total cellular RNA was isolated at the indicated timepoints. TMS1 expression levels were quantified by reverse transcription and real-time PCR analysis. Data represent the levels of TMS1 mRNA normalized to that of an 18 s rRNA internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations, which in general varied by < 10%. Shown is a representative experiment, which was repeated at least twice with similar results. (**b**) MDA MB468 cells were treated with 30 ng/ml TNFα or 100 ng/ml TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody over 48 h and analysed for TMS1 expression as in (**a**). (**c** and **d**) MCF10A breast epithelial cells (**c**) and IMR90 fibroblasts (**d**) were treated with 30 ng/ml TNFα or 50 μM etoposide and analysed for TMS1 expression as in panel **a**. TMS1 expression levels at time zero differed between the cell lines such that IMR90 cells and MCF10A cells express approximately 10- to 20-fold less TMS1 than MCF7 and MDA MB468 cells.

transcriptional response to DNA damage. Accordingly, the DNA damaging agents etoposide and mitomycin C had no effect on the expression of TMS1 in this cell line (data not shown).

To determine if the effects of these agents on TMS1 expression were particular to epithelial cells, we also examined the impact of death receptors and DNA damaging agents on TMS1 expression in IMR90 normal human diploid fibroblasts. Unlike breast epithelial cells, TRAIL and TNFα had little effect on the expression of TMS1 protein (Figure 1c) or mRNA (Figure 2d) in IMR90 fibroblasts. That TNFα failed to affect TMS1 levels could be due to the absence of TNF receptors or other cell type-dependent differences in components of the TNFα signaling pathway (see below). To test this, we also examined the expression of IL-1β, a well-documented target of TNFα stimulation, in these cells. Treatment of IMR90 cells with TNFα induced an approximately 4-fold increase in the levels of IL-1β mRNA, as measured by conventional RT-PCR (data not shown), confirming that the TNFα response is intact in IMR90 fibroblasts.

These data indicate that the upregulation of TMS1 by TNFα is cell type-dependent. Although etoposide treatment induced the stabilization of endogenous p53 and upregulation of p21 as expected for these p53 wild-type cells, it had a relatively minor effect on TMS1 protein levels (1.5- to 1.8-fold increase, Figure 1c) and no effect on TMS1 mRNA levels (Figure 2d).

We also examined the effect of TNFα treatment on TMS1 expression in the immortalized, non-transformed breast epithelial cell line MCF10A. TNFα treatment resulted in a ninefold upregulation of TMS1 message in this cell line (Figure 2c), thus the upregulation of TMS1 by TNFα in the breast cancer cell lines does not appear to be a consequence of transformation. Consistent with the findings in MCF7 cells and IMR90 cells, etoposide treatment had no effect on TMS1 mRNA expression in MCF10A cells (Figure 2c). Taken together, these data indicate that TMS1 is upregulated by TNFα in breast epithelial cells, but not human fibroblasts, whereas agents that induce DNA damage and elicit an endogenous p53 response have little effect on TMS1 expression in either cell type.

We next investigated the mechanism of TNFα-induced upregulation of TMS1. The interaction of TNFα with its membrane receptors TNFRI and II initiates downstream signaling through the jun kinase (JNK) pathway and the IκB kinase (IKK) pathway, culminating in activation of the transcription factors activator protein 1 (AP-1) and NF-κB, respectively (Natoli *et al.*, 1997; Devin *et al.*, 2000; Wajant *et al.*, 2003). To determine whether activation of JNK plays a role in TNFα-induced upregulation of TMS1, MCF7 cells were treated with TNFα in the presence of the JNK inhibitor SP600125 (Bennett *et al.*, 2001). Inhibition of JNK signaling blocked the TNFα-induced upregulation of TMS1 mRNA and protein in a dose-dependent manner (Figure 3a and b). To control for the efficiency of JNK inhibition by SP600125, parallel samples were also analysed for phosphorylated c-jun (Ser 63) protein levels (Figure 3b). At concentrations of SP600125 that were sufficient to block TNFα-induced phosphorylation of c-jun, upregulation of TMS1 was also blocked (Figure 3b).

To further investigate the role of the JNK pathway, we also examined the response of TMS1 to TNFα treatment in the presence of small interfering RNA (siRNA) directed against JNK1 and 2 (Li *et al.*, 2004). We found that knockdown of JNK1/2 resulted in a slight upregulation (1.5-fold) in the basal levels of TMS1 mRNA and protein. However, TNFα-induced upregulation of TMS1 was blocked in the presence of JNK1/2 siRNA, as was the phosphorylation of c-jun, similar to the results observed with the chemical inhibitor (Supplementary Figure 1). Taken together, these results indicate that TNFα-induced TMS1 upregulation is mediated at least in part by JNK signaling.

To determine if TNFα-induced activation of NF-κB contributes to TMS1 upregulation, MCF7 cells were treated with TNFα in the absence or presence of an adenoviral vector expressing a dominant-negative form of inhibitor of κB (IκBα) (Ad-mIκBα, S32A, S36A) (Mayo *et al.*, 2003). The IκBα super-repressor contains alanines instead of serines at positions 32 and 36, and as a consequence cannot be phosphorylated by the IKK complex or degraded by the 26S proteasome (Brockman *et al.*, 1995; Mayo *et al.*, 2003). Expression of the dominant-negative IκBα thus sequesters NF-κB in the cytoplasm, thereby preventing NF-κB dependent transcription (Brockman *et al.*, 1995; Mayo *et al.*, 2003). The expression of IκBα super-repressor blocked TNFα-induced upregulation of TMS1 mRNA and protein in a dose-dependent manner (Figure 3c and d). As shown in Figure 3d, TNFα induced the phosphorylation and subsequent degradation of endogenous IκBα, confirming that the NF-κB pathway was activated. As expected, the stabilized dominant-negative IκBα mutant was unaffected by TNFα-induced activation of the NF-κB pathway (Figure 3d).

Canonical NF-κB acts as a heterodimer comprised of the p65/RelA and p50 subunits (Baeuerle and

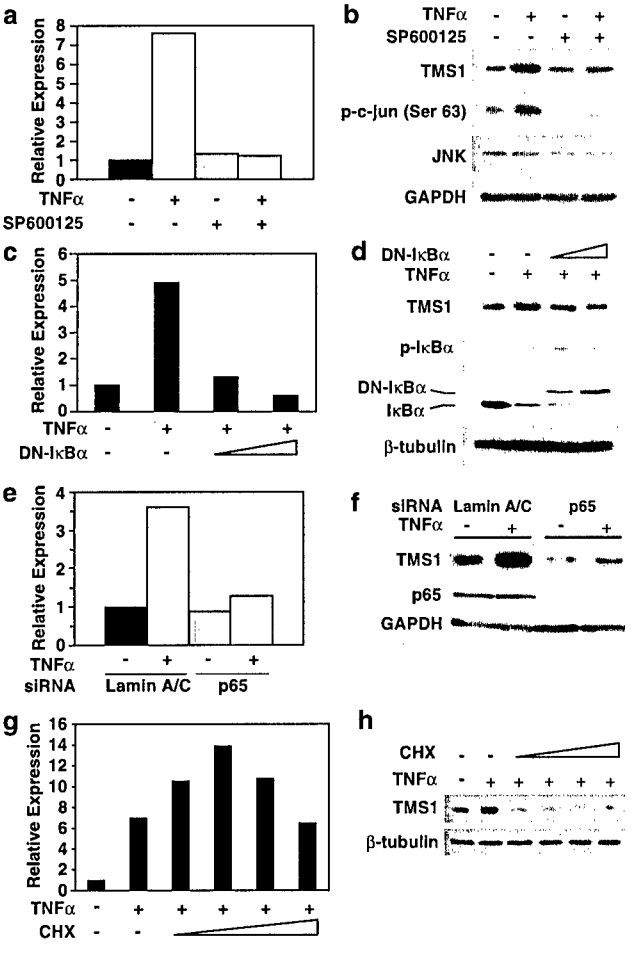

Figure 3 JNK and NF-κB signaling are required for the induction of TMS1 by TNFα. (**a** and **b**) MCF7 cells were either left untreated or were pretreated with 20 μM SP600125 for 30 min. TNFα was then added to a final concentration of 30 ng/ml for 48 h. (**a**) TMS1 mRNA expression was determined by real time RT–PCR as described in Figure 2. Data represent the mean of duplicate PCR determinations, which in general varied by < 10%. Shown is a representative of two independent experiments with similar results. (**b**) MCF7 cells were treated as described in (**a**). Protein lysates were fractionated by SDS–PAGE and subjected to Western blot analysis using antibodies to TMS1, phospho-c-jun, and β-tubulin as indicated. (**c** and **d**) MCF7 cells were infected with 0, 20 or 100 m.o.i. of an adenoviral construct expressing dominant-negative IκBα (Ad-mIκBα). After 18 h, cells were left untreated or treated with 30 ng/ml TNFα and incubated for an additional 48 h. (**c**) TMS1 mRNA expression was determined by real time RT–PCR. Data represent the mean of duplicate PCR determinations, which in general varied by < 10%. Shown is a representative of two independent experiments with similar results. (**d**) Protein lysates from parallel cultures were fractionated by SDS–PAGE and subjected to Western blot analysis using antibodies to TMS1, IκBα, phospho-IκBα, and β-tubulin. (**e** and **f**) MCF7 cells were transfected with 200 nM siRNA targeted to p65/RelA or Lamin A/C. After 24 h, cells were left untreated or treated with 30 ng/ml TNFα for an additional 24 h. (**e**) TMS1 mRNA expression was determined by real time RT–PCR. Data represent the mean of duplicate PCR determinations, which in general varied by less than 10%. Shown is a representative of three independent experiments. (**f**) Protein lysates from parallel cultures were fractionated by SDS–PAGE and subjected to western blot analysis using antibodies to TMS1, p65/RelA and GAPDH. (**g** and **h**) MCF7 cells were left untreated or were pretreated with 0.5, 1, 2 or 4 μg/ml cycloheximide for 1 h, followed by treatment with 30 ng/ml TNFα for 24 h. TMS1 mRNA and protein expression were analysed by real-time RT–PCR (**g**) and Western blot analysis (**h**). Shown is a representative of three independent experiments.

Baltimore, 1996; Baldwin, 1996). As a further test for the requirement of NF-κB, we examined the impact of siRNA-mediated knockdown of p65/RelA on TNFα-induced upregulation of TMS1. Knockdown of p65/RelA also blocked TNFα-induced upregulation of TMS1 mRNA and protein (Figure 3e and f), suggesting that NF-κB containing the p65/RelA subunit mediates TNFα-induced upregulation of TMS1. Together with the above results, these data indicate that both JNK and NF-κB signaling contribute to the TNFα-induced upregulation of TMS1.

NK-κB and JNK activity are known to stimulate the rapid expression of a number of proinflammatory and antiapoptotic genes, some of which could themselves be contributing to the TNFα-induced upregulation of TMS1. The time-frame of TMS1 induction following TNFα stimulation, which was not observed until 24 h after treatment (Figures 1 and 2), is also suggestive of the involvement of a TNFα-regulated intermediate. To address this possibility, MCF7 cells were treated with TNFα in the presence of cycloheximide to block new protein synthesis. At concentrations sufficient to block new protein synthesis (as indicated by a block in TMS1 protein induction by TNFα), cycloheximide had no effect on TNFα-induced upregulation of TMS1 mRNA (Figure 3g and 3h). We also examined the effects of TNFα on the stability of the TMS1 message. MCF7 cells were pretreated with 1 μg/ml Actinomycin D for 1 h followed by the addition of 30 ng/ml TNFα and TMS1 message levels were determined by real time RT–PCR. The half-life of the TMS1 message was approximately 24 h in control cells and was not significantly altered by treatment with TNFα (data not shown). These data indicate that neither the synthesis of a TNFα-regulated intermediate nor alterations in message stability contribute to the TNFα-induced upregulation of TMS1 message.

The above data are most consistent with a direct affect of TNFα on TMS1 transcription. To address this possibility, reporter studies were performed. A luciferase reporter construct containing 1254 bp upstream of the TMS1 translation start site (Levine *et al.*, 2003) was transfected into MCF7 cells, and the response to TNFα treatment or co-expression of p65/RelA was determined. A reporter construct containing five copies of a consensus NF-κB response element driving luciferase was used as a positive control. Although TNFα induced a 3-fold increase in luciferase expression from the consensus NF-κB reporter, it had no effect on TMS1 promoter-driven reporter activity (Supplementary Figure 2). Similarly, although co-expression of p65/RelA induced a 12-fold increase in luciferase activity from the NF-κB reporter, there was no effect on the TMS1 promoter construct (Supplementary Figure 2). To rule out the possibility that a *cis*-acting sequence contained within the TMS1 promoter was masking the effect of TNFα on TMS1 transcription, we also examined the response of a series of TMS1 promoter deletion constructs to TNFα. Again, TNFα had little impact on the activity of the TMS1 promoter (data not shown). Taken together, these data indicate that the effect of TNFα on TMS1 expression may require sequences distal to the promoter.

We next sought to test whether the upregulation of TMS1 might play a role in the downstream cellular events induced by TNFα. In macrophages, TMS1 is an integral part of the inflammasome, a multimeric complex responsible for the activation of proinflammatory caspases (i.e. caspases-1 and -5) and subsequent processing and secretion of cytokines (Mariathasan *et al.*, 2004). Whether or not this complex forms in response to physiologic stimuli in epithelial cells, or if it forms in response to TNFα, has not been addressed. We therefore examined the impact of TNFα-induced upregulation of TMS1 on caspase-1 activation. MCF7, MDA MB468 and MCF10A cells were treated with TNFα over the course of 48 h as described in Figure 1, followed by Western blot analysis using an antibody recognizing procaspase-1 and its cleavage products. We found that despite upregulation of TMS1, TNFα treatment had no impact on the basal levels of procaspase-1 and did not induce significant cleavage of caspase-1 in MCF10A or MDA MB468 cells (data not shown). Interestingly, procaspase-1 was undetectable in MCF7 cells. Thus, the TNFα-induced upregulation of TMS1 does not appear to drive caspase-1 activation in breast epithelial cells.

As discussed above, TNFα signaling stimulates the activities of the NF-κB and JNK signaling pathways. This is achieved through binding of TNFα to the TNFα receptor (TNFR), which in turn recruits TRADD and RIP or TRAF2 to induce activation of the IKK complex (Natoli *et al.*, 1997; Kelliher *et al.*, 1998) and JNK (Yeh *et al.*, 1997) respectively. Previous reports have suggested that TMS1 can either promote or inhibit NF-κB signaling depending on cell type, the coexpression of specific adaptor proteins and/or TMS1 expression levels (Grenier *et al.*, 2002; Manji *et al.*, 2002; Stehlik *et al.*, 2002; Wang *et al.*, 2002). The vast majority of these studies have utilized ectopic expression of TMS1. To examine the involvement of endogenous TMS1 in NF-κB signaling, we determined the impact of TMS1 knockdown on TNFα or TRAIL-induced activation of NF-κB in MCF7 cells. Knockdown of TMS1 with siRNA had no effect on activation of NF-κB in response to TRAIL or TNFα (Figure 4a). These data indicate that TMS1 is not required for TNFα- or TRAIL-induced activation of NF-κB in breast epithelial cells.

To further test the involvement of TMS1 in NF-κB signaling, we utilized HEK 293 cells (which lack endogenous TMS1 expression) that have been engineered to express TMS1 from an ecdysone-inducible promoter (referred to as MTMS22) (McConnell and Vertino, 2000). Cellular levels of TMS1 can be titrated in this system by varying the concentration of the inducing agent, ponasterone A (ponA). In pilot experiments, we determined conditions that induce near-physiologic levels (0.3 μM ponA) or supraphysiologic levels (5 μM ponA) of TMS1 by comparison to the endogenous protein levels in MCF7 cells (data not shown). At supraphysiologic levels, TMS1 alone induced a modest (5-fold) increase in NF-κB activity, which was significantly less (approximately 30- to

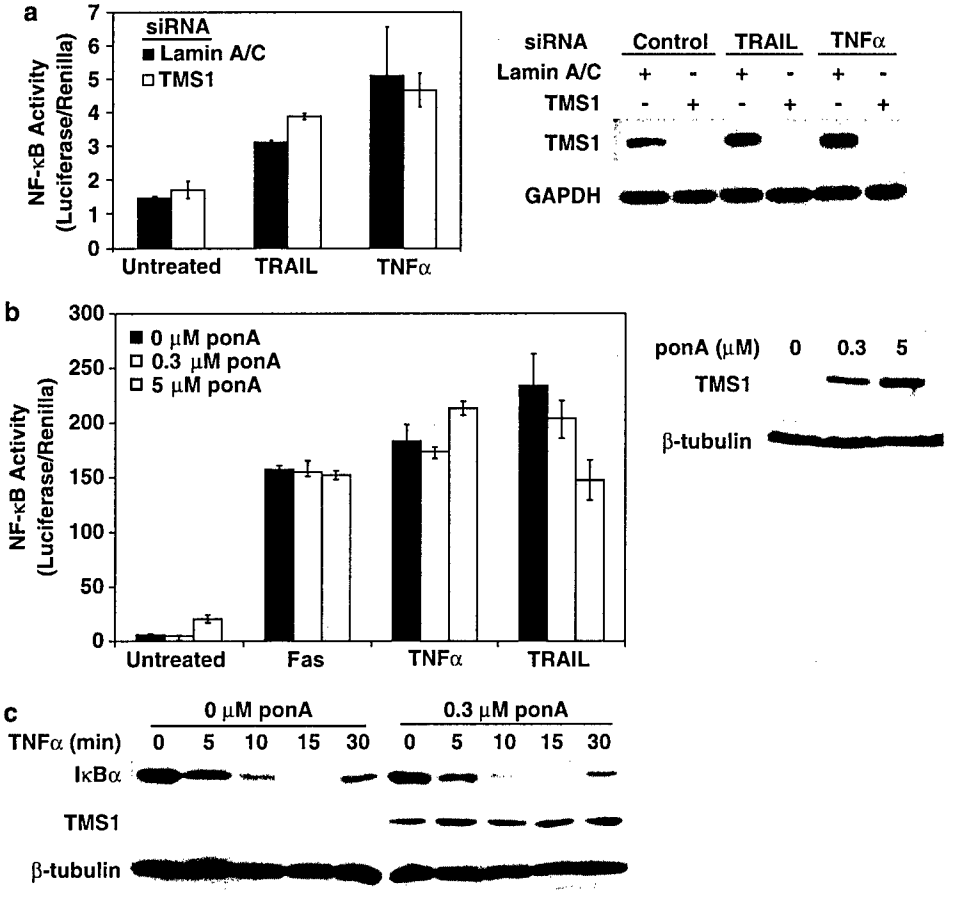

Figure 4 TMS1 is dispensable for death receptor-induced activation of NF-κB. (**a**) MCF7 cells were transfected with 200 nM siRNA targeted to TMS1 or Lamin A/C for 48 h, followed by transfection with 1 μg of the NF-κB-responsive luciferase reporter plasmid, pNF-κB-Luc and 50 ng of a renilla luciferase plasmid (pRL-TK) as a control for transfection efficiency. After 24 h, cells were left untreated or treated with 100 ng/ml TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody or 30 ng/ml TNFα for an additional 8 h, at which time luciferase activity was determined (*left panel*). Data represent the mean ± s.d. of duplicate determinations after correction for transfection efficiency. Total cellular protein collected from parallel cultures was analysed for TMS1 and GAPDH by Western blot analysis (*right panel*). (**b**) MTMS22 cells were transfected with 200 ng of pNF-κB-Luc or 10 ng pRL-TK. After 24 h, cells were left untreated or treated with 0.3 or 5 μM ponA to induce TMS1 expression. The following day, cells were treated with 1 μg/ml Fas, 20 ng/ml TNFα or 20 ng/ml TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody for 8 h, at which time luciferase activity was determined (*left panel*). Data represent the mean ± s.d. of duplicate determinations after correction for transfection efficiency. Total cellular protein collected from parallel cultures was analysed for TMS1 and β-tubulin by Western blot analysis (*right panel*). (**c**) MTMS22 cells were left untreated or treated with 0.3 μM ponA. The following day, cells were treated with 20 ng/ml TNFα, and total cellular protein was isolated at the indicated timepoints. Protein lysates were fractionated by SDS–PAGE and subjected to Western blot analysis using antibodies to IκBα, TMS1, and β-tubulin.

40-fold) than that induced by physiologic stimuli (see below). Using this system, we examined the impact of TMS1 expression on NF-κB activity induced by Fas, TNFα or TRAIL. Treatment of MTMS22 cells with Fas, TNFα or TRAIL alone resulted in a robust (150- to 200-fold) induction of NF-κB activity (Figure 4b). However, even at supraphysiologic levels, TMS1 expression had no effect on TNFα- or Fas-induced NF-κB reporter activity (Figure 4b). Similarly, neither the degree nor the kinetics of IκBα degradation were altered by TMS1 expression in MTMS22 cells (Figure 4c). At high TMS1 expression levels, there was an approximate 25% inhibition of TRAIL-induced NF-κB activity (Figure 4b). Taken together these data indicate that TMS1 does not significantly induce NF-κB activity on its own, nor does it influence NF-κB signaling induced by TNFα or Fas.

In addition to stimulating NF-κB and JNK signaling, TNFα and TRAIL can induce apoptosis. In this scenario, TNF-bound TNFRI (or TRAIL to the TRAIL receptors DR4 or DR5) recruits the adaptor protein FADD via homophilic death domain interactions to form the death-inducing signaling complex (DISC) (Chinnaiyan *et al.*, 1996; Hsu *et al.*, 1996; Ashkenazi and Dixit, 1998). The subsequent recruitment of procaspase-8 to the DISC allows for its cleavage and activation resulting in the activation of downstream effector caspases (i.e. caspase-3 and -7) and ultimately apoptosis. To test the involvement of TMS1 in death receptor-induced apoptosis, MCF7 cells were treated with TRAIL or TNFα in the presence of siRNA against TMS1. In the presence of cycloheximide, TNFα and TRAIL induced cleavage of caspase-8 (Figure 5a and b), which was not impacted by siRNA-mediated knock-

down of TMS1. These data indicate that TMS1 is dispensable for caspase-8 activation induced by death receptor ligands in breast epithelial cells.

To further examine the impact of TMS1 on caspase-8-dependent apoptosis, we determined the effect of TMS1 overexpression in MCF7 cells. Western analysis illustrated that TMS1 overexpression induced cleavage of caspase-8, and the caspase-8 target c-FLIP (Figure 5c). We also examined the role of TMS1 in caspase-8-dependent apoptosis in MTMS22 cells. Treatment of MTMS22 cells with 5 μM ponA to induce TMS1 expression resulted in the cleavage of caspase-8 and subsequent cleavage of the death substrate PARP, an indication of the downstream activation of effector caspases (e.g. caspase-3) and apoptosis. TMS1-induced cleavage of caspase-8 and PARP was blocked by co-expression of CrmA, a viral protein that specifically inhibits caspase-8 (Tewari and Dixit, 1995; Srinivasula *et al.*, 1996), illustrating that TMS1-induced apoptosis is dependent on caspase-8 activity. Taken together, the above data indicate that TMS1 is not required for death receptor-induced activation of caspase-8, but is sufficient to induce cleavage of caspase-8 and subsequent apoptosis, independent of a death receptor stimulus. Thus, the upregulation of TMS1 following death receptor signaling may function as a feed-forward loop that amplifies the apoptotic stimulus induced by TNFα or TRAIL.

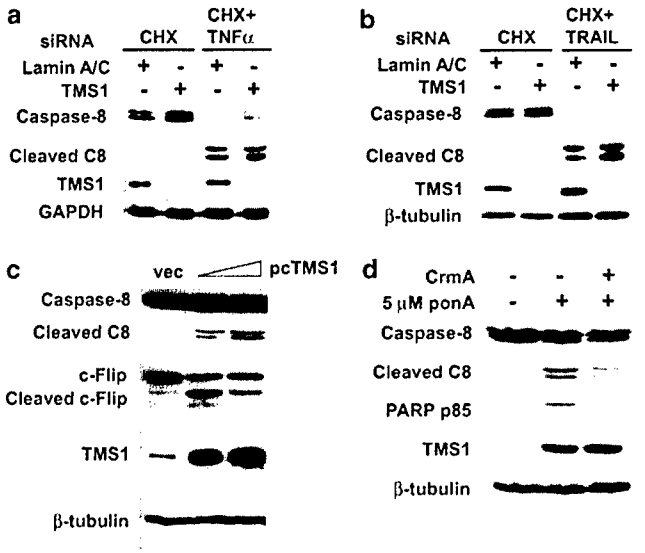

Figure 5 Effect of TMS1 on the extrinsic cell death pathway. (**a** and **b**) Impact of TMS1 downregulation on caspase-8 activation induced by TNFα or TRAIL. MCF7 cells were transfected with 200 nM siRNA targeted to TMS1 or Lamin A/C. After 48 h, cells were pretreated with 2 μg/ml cycloheximide for 30 min, followed by treatment with 30 ng/ml TNFα (**a**) or 100 ng/ml TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody (**b**) for 4 h. Protein lysates were subjected to Western blot analysis for caspase-8, TMS1 and either GAPDH or β-tubulin as indicated. (**c**) Overexpression of TMS1 induces caspase-8 cleavage in MCF7 cells. MCF7 cells were transfected with 1 μg empty vector (pcDNA3.1), or 1–2 μg TMS1 expression construct (pcDNA-TMS1). Cell lysates were collected after 24 h and subjected to Western blot analysis using antibodies to TMS1, capsase-8, and β-tubulin. (**d**) TMS1-induced capsase-8 activation and PARP cleavage is blocked by CrmA. MTMS22 cells were transfected with 6 μg empty vector (pcDNA3.1) or 6 μg of a CrmA expression construct (pcDNA-CrmA). After 48 h, cells were left untreated or treated with 5 μM ponA. The following day, total cellular protein was isolated and subjected to Western blot analysis using antibodies to TMS1, caspase-8, PARP p85, and β-tubulin.

Discussion

TMS1 is a novel tumor suppressor gene that is subject to frequent epigenetic silencing in several different tumor types, including breast, gliomas, melanomas and non-small cell lung cancers, however the mechanism by which *TMS1* silencing promotes carcinogenesis is not well understood (Conway *et al.*, 2000; Moriai *et al.*, 2002; Guan *et al.*, 2003; Virmani *et al.*, 2003; Yokoyama *et al.*, 2003; Stone *et al.*, 2004). Here, we examined the role of TMS1 in the cellular response of breast epithelial cells and human fibroblasts to death receptor activation and DNA damaging agents. We find that, in both transformed and non-transformed breast epithelial cells, TMS1 is upregulated in response to TNFα. This response was cell type-dependent and did not occur in normal fibroblasts. In contrast, DNA damaging agents that elicit a p53-dependent apoptotic response have a relatively minor impact on TMS1 expression in either cell type. In testing whether the upregulation of TMS1 might play a role in the downstream events induced by TNFα, we found that TMS1 was not necessary for TNFα-induced activation of caspase-8 or NF-κB activation, but could drive caspase-8 cleavage and subsequent apoptosis independently of a death receptor stimulus. These data suggest that TMS1 is both regulated by, and is an effector of, TNFα-induced apoptosis.

Previous studies indicate that TMS1 is upregulated in response to cytokines such as IL-1β, IFN-γ, TNFα and LPS in immune cells (Shiohara *et al.*, 2002; Stehlik *et al.*, 2003), however, the mechanism of this regulation was not determined. We found here that TMS1 is also regulated by TNFα in breast epithelial cells. Upon binding to its surface receptors TNFRI or TNFRII, TNFα induces receptor trimerization and recruitment of death domain-containing adaptor protein TRADD to the receptor's cytoplasmic death domains. The subsequent recruitment of RIP and TRAF2 leads to activation of downstream kinases and signaling through the IKK/NF-κB and JNK/AP-1 pathways (Yeh *et al.*, 1997; Kelliher *et al.*, 1998; Lin *et al.*, 2000). We found that the upregulation of TMS1 by TNFα was dependent upon the activities of both the NF-κB and JNK signaling pathways, and in particular required the p65/RelA subunit of NF-κB. The finding that TNFα-induced upregulation of TMS1 message and protein did not involve the synthesis of an intermediate or alterations in the stability of the TMS1 message suggests that there is a direct effect of TNFα on TMS1 transcription. Indeed, there are several putative AP-1 and NF-κB sites both upstream and downstream of the TMS1 transcription start site that could contribute to TNFα-induced upregulation of TMS1. In subsequent studies aimed at

defining the *cis* elements that mediate transcriptional regulation by TNFα, we found that there is no effect of TNFα treatment or p65 co-expression on transcription from a TMS1 reporter construct containing >1000 bp of upstream promoter sequence in several cell types (Supplementary Figure 2 and data not shown). One potential explanation for these findings is that TNFα-induced upregulation of TMS1 requires *cis* elements distal to the promoter. Alternatively, the regulation of TMS1 by TNFα may require a specific chromatin environment not represented in the transiently transfected reporter construct. Consistent with this idea, we have observed that TNFα-treatment induces local alterations in chromatin structure at the TMS1 locus by DNaseI hypersensitive site analysis (data not shown). Further studies will be needed to elucidate the exact mechanism by which TNFα influences TMS1 expression in breast epithelial cells.

NF-κB signaling is important for proper immune function and is often dysregulated in cancer. One proposed role for TMS1 is in the regulation of NF-κB. Previous studies addressing the impact of TMS1 on NF-κB activity are largely conflicting and have shown that TMS1 can promote or inhibit NF-κB activity depending on the stimulus and the co-expression of other adaptor proteins such as the PYPAFs or members of the pyrin family. For example, co-expression of TMS1 with the adaptor proteins PYPAF1/cryopyrin, PYPAF5 or PYPAF7 stimulates NF-κB activity (Grenier *et al.*, 2002; Manji *et al.*, 2002; Wang *et al.*, 2002), whereas other reports indicate that overexpression of either a full-length TMS1 or the pyrin domain of TMS1 inhibits NF-κB activity induced by TNFα, Bcl-10 or Nod-1 (Stehlik *et al.*, 2002). Most of these studies utilize overexpression of TMS1 or forced interactions between TMS1 and other adaptor proteins, which may not be physiologically relevant. To this end, a recent study by Mariathasan *et al.* (2004) showed that TMS1 is dispensable for IκBα degradation, ERK1/2 activation and NF-κB activation in response to LPS and TNFα in macrophages from *TMS1*$^{-/-}$ mice. In addition, cryopyrin does not induce NF-κB activity alone or in the presence of physiologic levels of TMS1 in HEK 293 cells (Yu *et al.*, 2005). Similarly, we find here that neither complete knockdown of endogenous TMS1 in breast epithelial cells nor restoration of TMS1 expression in HEK 293 cells has any impact on NF-κB activity or IκBα degradation in response to TNFα, TRAIL or Fas. Thus, it seems that TMS1 is dispensable for NF-κB signaling. Whether it functions in a feedback mechanism to inhibit NF-κB, as suggested by some studies (Stehlik *et al.*, 2002), remains to be determined. However, in our hands even high-level expression of TMS1 in MTMS22 cells had no effect on TNFα- or Fas-induced activation of NF-κB.

Although TNFα-induced signaling through the NF-κB and JNK pathways is generally thought to promote survival and proliferation through the upregulation of anti-apoptotic and pro-inflammatory target genes, there is also evidence that the NF-κB and JNK pathways promote apoptosis under certain circumstances. For example, JNK knockout mice are resistant to UV-induced apoptosis (Tournier *et al.*, 2000). Likewise, c-jun−/− cells are resistant to apoptosis and instead undergo cell cycle arrest in response to UV damage (Shaulian *et al.*, 2000). Fas ligand is upregulated by NF-κB and AP-1 following T-cell activation, and the subsequent Fas-mediated cell death is dependent upon intact NF-κB signaling (Kasibhatla *et al.*, 1998, 1999). Indeed, recent profiling studies examining the spectrum of TNFα-induced, NF-κB-regulated genes include several with pro-apoptotic functions (Zhou *et al.*, 2003; Tian *et al.*, 2005). TMS1 may be another example of an NF-κB/JNK-regulated factor that functions in a proapoptotic capacity. Previous work from our lab and others has shown that TMS1 induces apoptosis when overexpressed (Masumoto *et al.*, 1999; McConnell and Vertino, 2000; Richards *et al.*, 2001; Ohtsuka *et al.*, 2004). This apoptosis can be blocked by dominant negative mutants of caspase-9 and is dependent on Bax, implicating the mitochondrial pathway (McConnell and Vertino, 2000; Ohtsuka *et al.*, 2004). In contrast, apoptosis induced by co-expression of TMS1 with the adapter Ipaf/CARD12 or by forced oligomerization of TMS1 was shown to be dependent on caspase-8 (Masumoto *et al.*, 2003). We show here that TMS1 can induce the activation of caspase-8 and apoptosis, both of which are blocked by CrmA. Thus, the mechanism by which TMS1 induces apoptosis may be stimulus and/or context-dependent. Nevertheless, the implications of our studies are that NF-κB- and JNK-mediated upregulation of TMS1 serves to promote apoptosis.

The finding that TMS1 is upregulated by TNFα and can induce apoptosis prompted us to examine the role of TMS1 in death receptor-mediated apoptosis. Our siRNA experiments indicate that TMS1 is not required for activation of caspase-8 in response to TNFα or TRAIL, yet TMS1 overexpression promotes caspase-8 cleavage and subsequent apoptosis independent of a death receptor stimulus in both HEK 293 and MCF7 epithelial cells. One possibility is that the upregulation of TMS1 by TNFα, while not required for initial activation of caspase-8, serves to amplify the apoptotic signal induced by death receptor ligation. Current models indicate that death receptors elicit an apoptotic response through ligand-induced recruitment of the adaptor protein FADD and procaspase-8 to the receptors' cytoplasmic surface to form the DISC (Ashkenazi and Dixit, 1998; Sharp *et al.*, 2005), allowing for activation of the procaspase through induced proximity (Salvesen and Dixit, 1999; Shi, 2004). However, there is mounting evidence that caspase-8 can also be activated independently of DISC formation. For example, neither FADD nor caspase-8 are recruited to the membrane-associated TNFRI signaling complex in response to TNFα, yet both factors are required for TNFα-induced apoptosis (Harper *et al.*, 2003). Recent studies suggest that, unlike that of TRAIL and Fas, apoptosis induced by TNFα involves the sequential activation of two independent complexes; one at the cell membrane consisting of the TNFRI, the adaptor

TRADD, and the NF-κB signaling adaptors TRAF2 and RIP1, and a second cytoplasmic complex containing FADD and caspase-8 that forms after receptor internalization and/or dissociation of the ligand from the receptor (Harper *et al.*, 2003; Micheau and Tschopp, 2003; Muppidi *et al.*, 2004). Thus, the possibility exists that TMS1 drives caspase-8 cleavage by engaging a cytoplasmic pool of caspase-8. Indeed, there is evidence that TMS1 interacts directly with caspase-8, and co-localizes with caspase-8 in the cytoplasm in co-transfection experiments (Masumoto *et al.*, 2003; Hasegawa *et al.*, 2005). Consistent with this idea, Masumoto *et al.* (2003) showed that TMS1-induced apoptosis cannot be blocked by a dominant-negative FADD mutant consisting only of the death domains. Such mutants might be expected to interfere with recruitment of FADD and caspase-8 to the receptor, but perhaps not activities within the cytoplasmic complex.

Recently, it was suggested that TMS1 is a direct downstream target of the p53-mediated response to genotoxic stress in human fibroblasts and breast epithelial cells (Ohtsuka *et al.*, 2004). These authors showed that TMS1 is strongly induced by adenoviral-driven or inducible overexpression of p53, and to a lesser extent by treatment with mitomycin C or ionizing radiation, the latter of which was dependent on p53. Here we find that relative to cytokines like TNFα, such agents have a relatively minor impact on TMS1 expression. In particular, quantitative real-time RT–PCR showed no more than a 2-fold induction of TMS1 message in response to etoposide or mitomycin C treatment (Figure 2). Similar results were obtained in all three wild-type p53 cell types tested (MCF7, MCF10A and IMR90) and with agents known to induce different forms of DNA damage, including etoposide, mitomycin C and UV irradiation (data not shown). In contrast, the upregulation of TMS1 by TNFα appeared to be p53-independent and occurred in both p53 wild-type (MCF10A, MCF7) and p53 mutant (MDA MB468) epithelial cells. Moreover, reporter constructs containing an intact TMS1 promoter, including a putative p53-binding site, failed to respond to etoposide when transfected into MCF7 or IMR90 cells (data not shown). Thus, whereas p53 may have some impact on expression of TMS1, it may be indirect. Alternatively, the regulation of TMS1 by genotoxins may involve transcriptional crosstalk between p53 and NF-κB (Webster and Perkins, 1999) as has been described for other pro-apoptotic NF-κB target genes, such as DR5 (Shetty *et al.*, 2005).

In summary, we have shown here that TMS1 is upregulated by TNFα in breast epithelial cells in an NF-κB and JNK-dependent manner. While TMS1 is not required for the activation of caspase-8 following death receptor stimulation, overexpression of TMS1 can promote caspase-8 activation. Our data are consistent with a model in which the upregulation of TMS1 by death receptor signaling in breast epithelial cells may function to amplify the apoptotic signal induced by TNFα. Therefore, one important consequence of epigenetic silencing of *TMS1* in breast and other cancers may be an attenuated response to cytokines such as TNFα and/or resistance to therapeutic regimens that rely on caspase 8-dependent apoptosis, such as TRAIL-related therapies. Indeed, TNFα and TRAIL have no impact on TMS1 expression in breast cancer cell lines where the endogenous gene is methylated, such as MDA MB231 breast cancer cells (Levine *et al.*, 2003, and unpublished observations). Thus, reactivation of TMS1 through inhibition of DNA methylation may provide an opportunity to restore sensitivity to death receptor and caspase 8-dependent signaling and apoptosis.

Materials and methods

Cell lines, drug treatments and reagents

MCF7 and MDA MB468 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM (4.5 g/l glucose) plus 10% fetal calf serum and 2 mM glutamine. MCF10A cells were obtained from the Karmanos Cancer Institute (Detroit, MI, USA) and maintained in DMEM/F12 plus 5% FCS, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 2 mM glutamine. IMR90 normal human diploid fibroblasts were obtained from the National Institute on Aging Cell Repository and maintained in EMEM with 10% FCS and 2 mM glutamine. The generation of HEK 293 cells expressing an ecdysone-inducible myc-tagged TMS1 (MTMS22) have been described previously (McConnell and Vertino, 2000) and were maintained in DMEM (4.5 g/l glucose) plus 10% FCS, 2 mM glutamine, 600 μg/ml G418 and 400 μg/ml zeocin (Invitrogen, Carlsbad, CA, USA). Etoposide, mitomycin C and cycloheximide were purchased from Sigma (St Louis, MO, USA). His-tagged recombinant human TRAIL, 6 × polyhistidine crosslinking antibody and recombinant human TNFα were purchased from R&D Systems (Minneapolis, MN, USA). The JNK inhibitor SP600125 was purchased from Biomol (Plymouth Meeting, PA, USA). The dominant-negative IκBα adenovirus (Ad-mIκBα) was a gift from Dr Leland Chung (Emory University). PonA was purchased from Invitrogen. Activating anti-Fas antibody was purchased from Upstate Biotechnology.

Immunoblotting and antibodies

Cells were pelleted, washed three times in 1 × PBS, and lysed with RIPA buffer containing 1 × protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche, Indianapolis, IN, USA), 1 mM sodium orthovanadate, and 10 mM sodium fluoride (Sigma, St Louis, MO, USA). Total protein (100 μg) was separated on a 15% SDS–PAGE gel, transferred to PVDF or nitrocellulose (BioRad, Hercules, CA, USA), and probed with the indicated primary antibody. Immunocomplexes were detected by incubation with horseradish peroxidase-conjugated secondary antibody and chemiluminescence detection (Pierce, Rockford, IL, USA). The antibodies used were: anti-ASC (MBL), β-tubulin (Sigma), p53 (AB-6, Oncogene), p21 (Ab-1, Oncogene), GAPDH (Abcam), phospho-c-jun (Ser 63, Cell Signaling Technologies, Danvers, MA, USA), JNK (Cell Signaling Technologies), (phospho-IκBα (Ser 32/36, Cell Signaling Technologies), IκBα (Cell Signaling Technologies), NF-κB p65 (Santa Cruz, Santa Cruz, CA, USA), caspase-8 (1C12, Cell Signaling Technologies), caspase-1 (Biomol), c-FLIP (NF6, Alexis Biochemicals, San Diego, CA, USA) and poly (ADP-ribose) polymerase (PARP) p85 fragment (Promega, Madison, WI, USA).

Reverse transcription and real-time PCR
Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA was treated with amplification-grade DNase I (Invitrogen) and reverse-transcribed (RT) using random hexamer priming and MMLV-reverse transcriptase (Invitrogen). A 50 × dilution (1 μl) of the RT reaction was amplified in duplicate using iQ SYBR Green Supermix (BioRad) and the MyIQ real-time detection system. Reaction conditions included a hot start (3 min, 95°C), followed by 50 cycles of (95°C, 10 s; 55°C, 60 s). Melt curve analysis was performed to ensure a single product species. Parallel reactions were performed using primers to 18S rRNA as an internal control. Relative starting quantities were calculated by comparison to a common standard curve generated with a dilution series of MCF10A cell cDNA that was included in each run. Primers for real-time PCR analysis were for TMS1, 5′-TCC AGC AGC CAC TCA ACG-3′ and 5′-GCA CTT TAT AGA CCA GCA-3′; and for 18S, 5′-GAG GGA GCC TGA GAA ACG G-3′ and 5′-GTC GGG AGT GGG TAA TTT GC-3′.

siRNA experiments
MCF7 cells (2.0×10^5) were seeded in six-well plates and transfected the next day with 200 nM of the indicated siRNA using Oligofectamine (Invitrogen). TMS1 and p65 siRNA duplexes were purchased from Dharmacon (Lafeyette, CO, USA). The sequence of the TMS1 siRNA was: 5′-CGA GGG UCA CAA ACG UUG A dTdT-3′ (sense), and the sequence of the p65 siRNA was: 5′-GCC CUA UCC CUU UAC GUC A dTdT-3′ (sense). The JNK siRNA was purchased from Sigma (St Louis, MO, USA) and targets a common sequence in both Jnk1 and Jnk2 mRNA (Li *et al.*, 2004). The sequence of the JNK1/2 siRNA was: 5′-AAA GAA UGU CCU ACC UUC U dTdT-3′ (sense). Dharmacon Lamin A/C siControl was used to control for any non-specific off-target effects of siRNA transfection.

NF-κB reporter assays
For MCF7 cells, 2.0×10^5 cells were seeded in six-well plates and transfected the following day with 200 nM siRNA targeting TMS1 or Lamin A/C as described above. After 48 h, cells were transfected with 1 μg of an NF-κB responsive luciferase reporter construct (pNF-κB-Luc, Stratagene) and 50 ng of a renilla luciferase reporter (pRL-TK, Promega) as a control for transfection efficiency using 3 μl FuGene6 (Roche) per well. After 24 h, the transfection medium was replaced with fresh medium or medium containing 30 ng/ml TNFα or 100 ng/ml 6 × his-tagged recombinant human TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody. After an additional 8 h, cells were lysed and firefly luciferase and renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's directions. For MTMS22 cells, 8.0×10^4 cells were seeded in 24-well plates and transfected the next day with 200 ng of the NF-κB luciferase reporter construct and 10 ng of pRL-TK using 0.6 μl of FuGene6 (Roche) per well. The following day, TMS1 expression was induced by the addition of 0.3 or 5 μM ponA to the medium. After 24 h, the transfection medium was replaced with fresh medium or medium containing 1 μg/ml anti-Fas activating antibody, 20 ng/ml TNFα, or 20 ng/ml 6 × his-tagged recombinant human TRAIL plus 1 μg/ml 6 × polyhistidine crosslinking antibody. After 8 h, cells were lysed and firefly luciferase and renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega).

Plasmid transfections
MCF7 cells, (4.0×10^5) were seeded in six-well plates and transfected the following day with 1 μg pcDNA3.1 + or 1–2 μg pcTMS1 (McConnell and Vertino, 2000) using 6 μl Lipofectamine (Invitrogen). Protein lysates were collected 24 h following transfection. For MTMS22 cells, 4.0×10^6 cells were seeded in 10 cm dishes and transfected the following day with 6 μg pcDNA3.1 + or pcDNA-CrmA (gift from Dr Margaret Offermann, Emory University) using 36 μl Lipofectamine. TMS1 expression was induced the following day by the addition of 5 μM ponA, and protein lysates were collected after 24 h.

TMS1 promoter studies
A genomic *Hind*III–*Nco*I fragment containing 1254 bp upstream of the TMS1 translation start codon was cloned into the pGL3 luciferase reporter plasmid (Stratagene) to generate pTMS1–1254-Luc, as previously described (Levine *et al.*, 2003). MCF7 or MTMS22 cells (8.0×10^4) were seeded in 24-well plates and transfected the next day with the indicated plasmids using 0.6 μl of FuGene6 (Roche) per well. A Renilla luciferase reporter (10 ng of pRL-TK, Promega) was included to control for transfection efficiency. After 24 h, medium was replaced with fresh media with or without 30 ng/ml TNFα. After an additional 48 h, cells were lysed and firefly luciferase and renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega). For p65/RelA co-transfection experiments, 2.0×10^5 MCF7 cells were seeded in six-well plates and transfected the following day with 500 ng of pTMS1–1254-Luc plus 0–2 μg of a human p65/RelA expression construct (pcDNA-p65, a gift from Dr Jeremy Boss, Emory University) using Lipofectamine. Renilla luciferase plasmid (pRL-TK, 50 ng) was included as a control. The total amount of DNA was kept constant at 2.5 μg using pcDNA3.1 (Invitrogen). After 48 h, luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega).

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

Appendix C.

Supplemental Data:

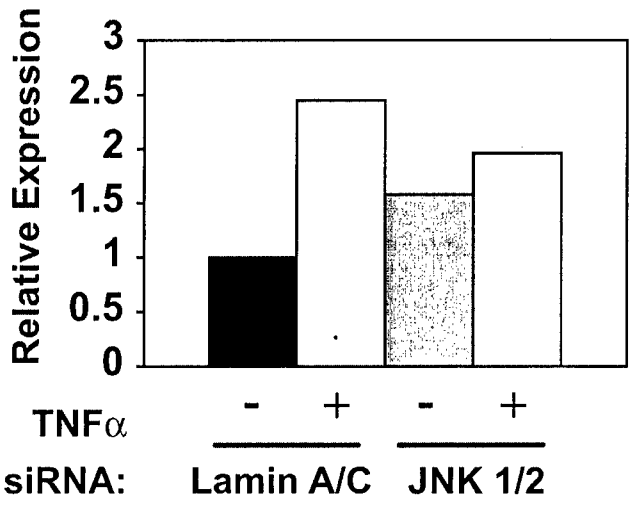
Legends

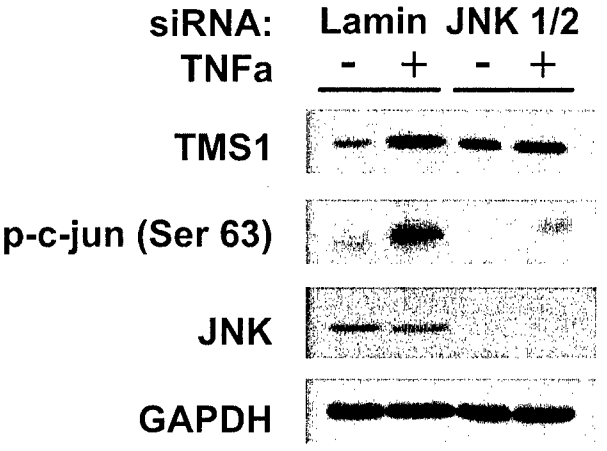
Supplemental Figure 1. JNK signaling is required for TNFα-induced upregulation of TMS1. MCF7 cells were transfected with 200 nM siRNA targeted to JNK1/2 or lamin A/C. After 24h, cells were left untreated or treated with 30 ng/ml TNFα for an additional 48h. *Left Panel,* TMS1 mRNA expression was determined by real time RT-PCR. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative of two independent experiments. *Right Panel*, Protein lysates from parallel cultures were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to TMS1, phospho-c-jun, JNK and GAPDH as indicated.

Supplemental Figure 2. Effect of TNFα and p65/RelA on the TMS1 promoter. *A*, MCF7 cells were transfected with 200 ng of an NF-κB-responsive luciferase reporter plasmid (pNF-κB-Luc, Stratagene) or a TMS1 promoter luciferase reporter plasmid (pTMS1-1254-Luc) using the FuGene reagent. Ten ng of a renilla luciferase plasmid (pRL-TK) was included as a control for transfection efficiency. After 18h, cells were left untreated or treated with 30 ng/ml TNFα for 48h, at which time luciferase activity was determined. Data represent the mean +/- standard deviation of triplicate determinations after correction for transfection efficiency. *B*, MCF7 cells were transfected with 500 ng of an NF-κB-responsive luciferase reporter plasmid (pNF-κB-Luc, Stratagene) or a TMS1 promoter luciferase reporter plasmid (pTMS1-1254-Luc), along with the indicated amounts of a p65 expression construct (pcDNA-p65) using Lipofectamine. Fifty ng of a renilla luciferase plasmid (pRL-TK) was included as a control for transfection efficiency. Total amount of DNA per transfection was kept constant at 2.5 μg using pcDNA3.1 (Invitrogen).

Left Panel, Luciferase activity was determined after 48h. Data represent the average fold increase in reporter activity of four independent determinations after correction for transfection efficiency. Determinations did not differ by more than 10% of the mean. *Right Panel*, Parallel cultures were subjected to western analysis using antibodies against p65/RelA and β-tubulin as a loading control.

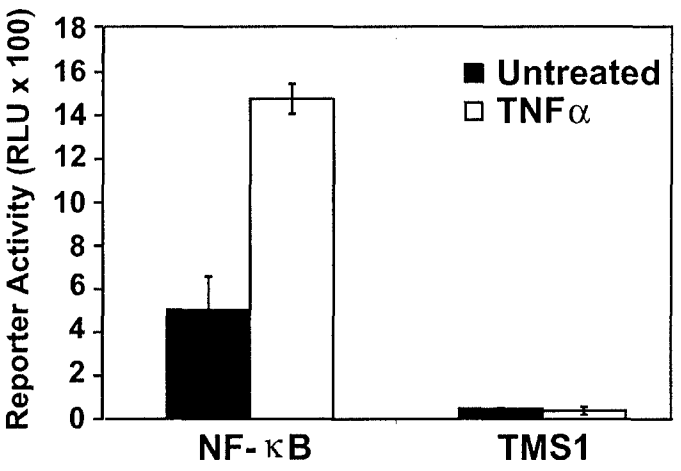
Supplemental Figure 1

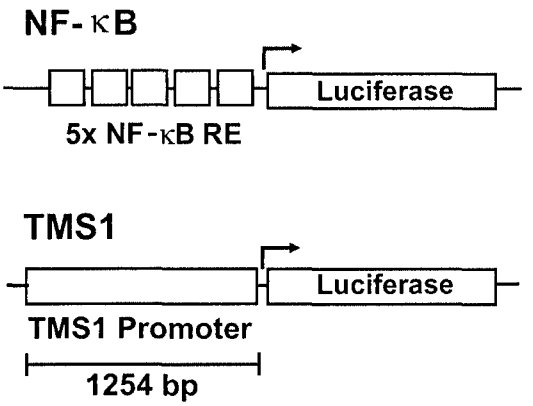




Supplemental Figure 2

A.





B.