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ORIGINAL ARTICLE

Heat shock factor 1 represses estrogen-dependent transcription through association with MTA1

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Heat shock factor 1 (HSF1), the transcriptional activator of the heat shock genes, is increasingly implicated in cancer. We have shown that HSF1 binds to the corepressor metastasis-associated protein 1 (MTA1) *in vitro* and in human breast carcinoma samples. HSF1–MTA1 complex formation was strongly induced by the transforming ligand heregulin and complexes incorporated a number of additional proteins including histone deacetylases (HDAC1 and 2) and Mi2α, all components of the NuRD corepressor complex. These complexes were induced to assemble on the chromatin of MCF7 breast carcinoma cells and associated with the promoters of estrogen-responsive genes. Such HSF1 complexes participate in repression of estrogen-dependent transcription in breast carcinoma cells treated with heregulin and this effect was inhibited by MTA1 knockdown. Repression of estrogen-dependent transcription may contribute to the role of HSF1 in cancer.
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Keywords: heat shock factor; metastasis; associated protein

Introduction

Heat shock factor 1 (HSF1) is the transcriptional activator of the heat shock genes and is increasingly implicated in cancer (Calderwood *et al*., 2006). Many tumor types express high concentrations of HSP28, HSP70 and HSP90 that are correlated with increases in HSF1 level (Hoang *et al*., 2000; Ciocca and Calderwood, 2005; Khaleque *et al*., 2005; Tang *et al*., 2005). Elevated levels of HSP expression contribute directly to tumorigenesis through inhibition of programmed cell death in tumors (Jones *et al*., 2004; Ciocca and Calderwood, 2005), and thus afford protection of cancer cells from chemotherapeutics (Mosser and Morimoto, 2004; Ciocca and Calderwood, 2005; Calderwood *et al*., 2006). Our recent studies indicate a mechanism for elevated HSP levels in breast cancer involving heregulin, a potent transforming factor that increases HSF1 concentrations and induces HSP synthesis (Khaleque *et al*., 2005). We have also shown that HSF1 is a versatile repressor of transcription (Cahill *et al*., 1996; Chen *et al*., 1997; Xie *et al*., 2002, 2003). Our current experiments suggest a mechanism for gene repression by HSF1 as well as for its potential role in breast cancer (Calderwood *et al*., 2006).

We show here that HSF1 binds to the corepressor protein MTA1 and thus participates in repression of estrogen-regulated target promoters. MTA1 is a component of the NuRD complex that contains histone deacetylases (HDAC1 and 2), chromodomain proteins CHD3 and 4 and ATP-dependent chromatin-remodeling protein Mi2 (Xue *et al*., 1998; Mazumdar *et al*., 2001; Yao and Yang, 2003). The potential role of HSF1–MTA1 interaction in breast cancer is compelling as heregulin increases expression of both HSF1 and MTA1. We show that HSF1–MTA1–NuRD complexes form on the promoters of estrogen-induced genes after heregulin treatment and mediate repression of estrogen-inducible transcription.

Results

HSF1 associates with metastasis-associated protein 1

We screened for HSF1-associated regulatory proteins, by probing HeLa lysates with GST-HSF1, prior to mass spectrometric analysis (Figure 1a). Prominent among HSF1-binding proteins pulled down by GST-HSF1 was MTA1 (Figure 1a), an association confirmed by western analysis of proteins eluted from GST-HSF1 with anti-MTA1 antibodies (Figure 1b). We further examined this association by co-immunoprecipitation using cells overexpressing Myc-MTA1 and Flag-HSF1 to increase the probability of detecting additional coprecipitating proteins in the complexes (Figure 1c) (However, although not detected in unstimulated cells HSF1–MTA1 complexes can be readily detected by co-immunoprecipitation when HSF1 is activated, as shown later in Figure 2). In cells expressing Myc-MTA1, HSF1

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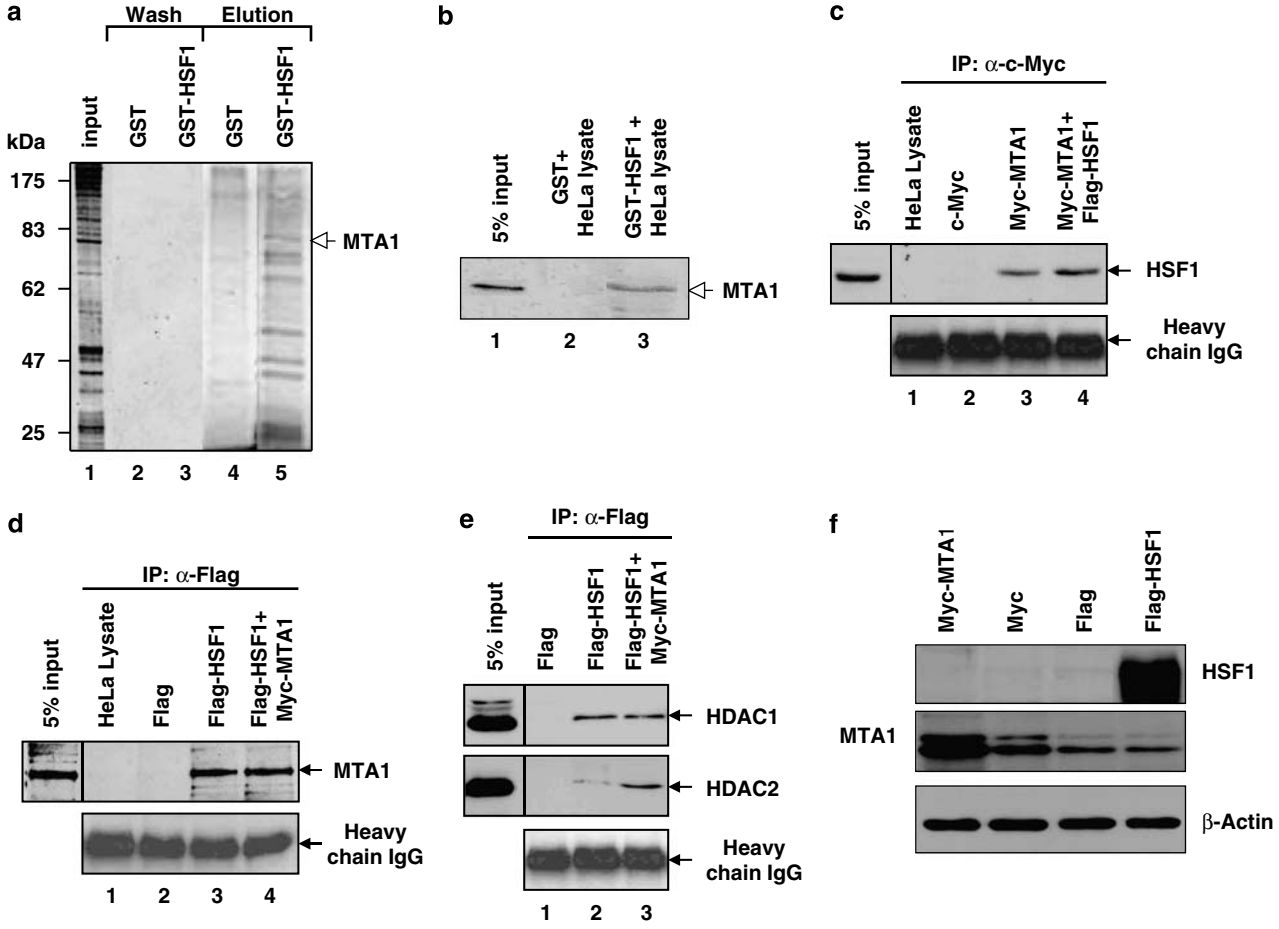


Figure 1 Heat shock factor 1 (HSF1) binds to metastasis-associated protein 1 (MTA1). (**a**) HeLa cell extracts were incubated with purified GST (lanes 2 and 4) or GST-HSF1 (lanes 3 and 5). After low salt wash, the interacting proteins were eluted with 150 mM NaCl. Then the wash (lanes 2 and 3) and elute (lanes 4 and 5) were analysed by SDS–PAGE silver staining. Arrow indicates MTA1. At least four peptides with sequence unique to MTA1, including (R)IEELNK(T), (K)SYPTKVR(L), (K)LLSSSETK(R) and (R)YQA-DITDLLK(E) were discovered in the digest of the band marked MTA1. (**b**) GST-HSF1-associated proteins were separated by SDS–PAGE and immunoblotted with anti-MTA1 antibody. (**c**) HeLa cells were either non-transfected (lane 1) transfected with control pBJ-Myc (lane 2), pBJ-*myc*/MTA1 (lane 3) or both pBJ-*myc*/MTA1 and pcDNA3.1-Flag-HSF1 (lane 4) for 24 h. Immunoprecipitation with anti-c-Myc antibodies preceded immunoblot with HSF1 or heavy chain immunoglobulin G (IgG) loading control. (**d**) HeLa cells were either non-transfected (lane 1), transfected with control plasmid (lane 2), pcDNA3.1-Flag-HSF1 (lane 3) or co-transfected with pcDNA3.1-Flag-HSF1 and pBJ-*myc*/MTA1 (lane 4) for 24 h. Immunoprecipitation with anti-Flag antibody preceded anti-MTA1 immunoblot. (**e**) Levels of Myc-MTA1 and Flag-HSF1 expression in transfectants. (**f**) Expression levels of MTA1 and HSF1 in transfectants.

was co-immunoprecipitated by anti-Myc antibodies and recovery was increased by overexpression (Figure 1c). We next examined HSF1 complexes using cells overexpressing Flag-HSF1 and observed coimmunoprecipitation of MTA1 with Flag-HSF1 but not the Flag control (Figure 1d). As MTA1 associates with the NuRD complex, we also examined NuRD components. Indeed HDAC1 and HDAC2 were detected in the Flag-HSF1 immunoprecipitates, especially in conditions of MTA1 overexpression and in the case of HDAC2 (Figure 1e). Relative levels of forced expression are shown in Figure 1f.

HSF1 association with MTA1 and HDACs in breast cancer cells

We next asked if MTA1 and HSF1 interact in breast cancer. We investigated breast carcinoma cells because MTA1 is induced by heregulin in such cells and mediates repression of estrogen-dependent genes, an effect linked to metastasis (Mazumdar *et al*., 2001). Exposure to heregulin-induced MTA1 in HeLa cells, MCF7 cells (estrogen receptor (ER^+) breast cancer) and MDA-MB-231 (ER^-) (Figure 2a). (Results for *hsf1* +/+ MEF cells are also included here as we later show some experiments comparing *hsf1* knockout with wild-type MEF). Our previous studies indicated that heregulin also induces HSF1 through binding of heregulin to its receptor c-*erb*B3 and subsequent activation of c-*erb*B2 (Khaleque *et al*., 2005). Heregulin induction of MTA1 and HSF1 both evidently involve similar upstream signals, as indicated by c-*erb*B2 tyrosine kinase inhibitors and c-*erb*B3 blocking antibodies (Figure 2b). In addition, MTA1 induction by heregulin is *hsf1* dependent, and in *hsf1*−/− cells MTA1 fails to increase MTA1

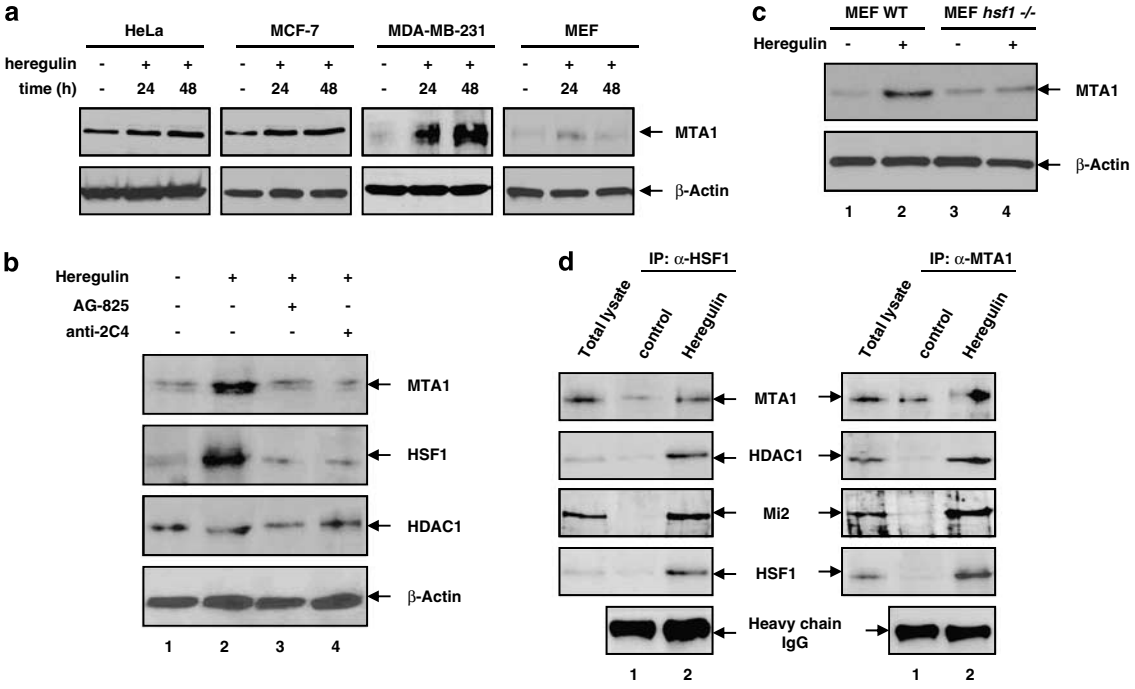


Figure 2 Heregulin induces metastasis-associated protein 1 (MTA1) expression in a heat shock factor 1 (HSF1)-dependent manner. (**a**) HeLa, MCF7, MDA-MB-231 and MEF cells were treated with 1 nM HRGβ1 for 24 h or 48 h, prior to immunoblot assay for MTA1 and β-actin. (**b**) MCF7 cells were treated with (lanes 2–4) or without (lane 1) 1 nM HRGβ1. Selected HRGβ1-treated cultures were exposed to 50 μM AG-825 (lane 3) for 2 h or 10 μg ml^{-1} anti-2C4 antibody (lane 4) for 1 h prior to the HRGβ1 treatment. Cells were then lysed and probed for MTA1, HSF1, histone deacetylases 1 (HDAC1) and β-actin. (**c**) MEF WT or MEF *hsf1*−/− cells were treated with 1 nM HRGβ1 for 24 h and cell lysates immunoblotted for MTA1, HSF1 and β-actin. (**d**) Heregulin-treated and control cell lysates were immunoprecipitated with anti-HSF1 (left panels) or anti-MTA1 (right panels) prior to immunoblot with anti-MTA1, anti-HDAC1, anti-Mi2 or anti-HSF1 antibodies or heavy chain immunoglobulin G (IgG). Experiments were reproducibly repeated twice.

above basal levels (Figure 2c). We next investigated whether HSF1 and MTA1 are found in protein complexes and could be co-immunoprecipitated in MCF7 cells (Figure 2d). Immunoprecipitation analysis with anti-HSF1 antibodies resulted in minimal recovery of the proteins in control cells but efficient co-precipitation of HSF1 with MTA1 after heregulin (Figures 2a and b). In addition, heregulin induced the co-association with HSF1 of NuRD complex components HDAC1 and Mi2α as well as MTA1 (Figure 2d). Complementary results were obtained by MTA1 immunoprecipitation, indicating co-precipitation of HSF1, HDAC1 and Mi2α with MTA1 (Figure 2d).

HSF1 and MTA1 cooperate in gene repression

Previous studies have shown that MTA1 promotes breast cancer metastasis by repressing estrogen-dependent transcription (Mazumdar *et al*., 2001). We have therefore investigated whether HSF1 and MTA1 might cooperate in repressing estrogen responsive element (ERE)-regulated genes. To investigate the role of MTA1 we prepared RNAi constructs, each of which inhibit MTA1 expression (Figure 3a). Most subsequent experiments involved the construct used in lane 3 (MTA1-RNAi(2)) although confirmatory results were obtained with the vector used for lane 2. Controls using a scrambled sequence rather than the RNAi sequences used for lanes 2, 3 showed no depletion of MTA1 (data not shown). We next examined whether HSF1 overexpression inhibits estrogen-dependent transcription, using an ERE-*Luc* promoter–reporter system. Both HSF1 and MTA1 directly repressed estradiol-mediated luciferase accumulation (Figure 3b). These effects of HSF1 were strikingly reversed by RNAi-mediated MTA1 knockdown, using MTA1-RNAi(2) consistent with MTA1 acting as an HSF1 corepressor (Figure 3b). In addition, heregulin exposure led to repression of estrogen-induced transcription and repression was relieved by MTA1 knockdown (Figure 3b). Cells transfected with a control vector expressing a scrambled sequence did not antagonize the effects of heregulin. Selective inhibitors of heregulin signaling, including c-*erb*B3 blocking antibody 2C4 and c-*erb*B2 kinase inhibitor AG-825 likewise antagonized the effects of heregulin on ERE (Figure 3c). Relative levels of MTA1 and HSF1 in cells treated with estradiol with or without MTA1 overexpression, HSF1 overexpression or heregulin are shown (Figure 3d). Heregulin increases expression of both MTA1 and HSF1 (Figure 3d). In addition, as previous studies show that the *c-fos* promoter is a target for HSF1, we used c-*fos* as a positive control (Xie *et al*., 2003). Reporter construct pGL-*fos* was activated by Ras overexpression and such activity was repressed by both HSF1 and MTA1 (Figure 3e). MTA1 therefore plays a versatile role in co-repression of gene promoters by HSF1.

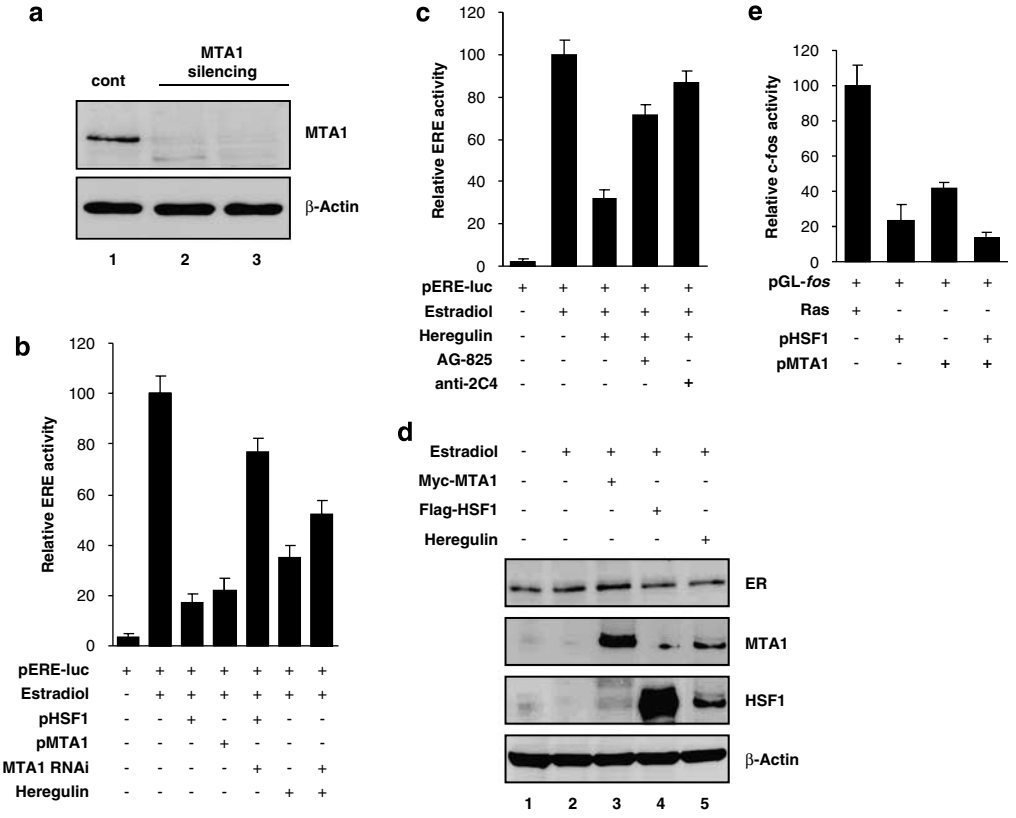


Figure 3 Heat shock factor 1 (HSF1) and metastasis-associated protein 1 (MTA1) cooperate in gene repression. (**a**) MCF7 cells were non-transfected (lane 1) or co-transfected (lanes 2 and 3) with MTA1 RNAi expression plasmid and pEGFP-C1. After 72 h cells were sorted, lysates were prepared from GFP-positive cells and analysed by immunoblot for MTA1 and β-actin. (**b**) MCF7 cells were transiently transfected in triplicate with pERE-Luc (1.6 μg) alone or with pHSF1 (0.8 μg), pMTA1 (0.8 μg) or MTA1 RNAi (0.8 μg). In each culture total amount of plasmid was adjusted to 4 μg with control plasmid. Then cells were treated with 17β-estradiol (1 ng ml^{-1}) for 6 h except lane 1. Two sets of samples were also treated with 1 nM HRGβ1 for 24 h. Cells were then lysed for luciferase assay. Mean relative luciferase activity, corrected for transfection efficiency, was expressed ± s.d. (**c**) MCF7 cells were transiently transfected in triplicate with pERE-Luc (1.6 μg) plus control plasmids to make the total plasmid amount 4 μg. Then cells were treated with 17β-estradiol alone or with HRGβ1. Prior to HRGβ1 treatment some cells were treated with 50 μM AG-825 or 10 μg ml^{-1} anti-2C4 antibody as indicated. Cells were then lysed and luciferase activity examined in the extracts. (**d**) MCF7 cells were transfected with pBJ-*myc*/MTA1 (lane 3) or pcDNA3.1-Flag-HSF1 (lane 4) and treated with 17β-estradiol (lanes 2–5) and HRGβ1 (lane 5) prior to immunoblot for estrogen receptor (ER), MTA1, HSF1 and β-actin. (**e**) MCF7 cells were co-transfected with Ras expression plasmid, pGL-*fos* and pCMV-βGal transfection efficiency control alone (lane 1) with pFlag-HSF1 (lane 2), pMyc-MTA1 (lane 3) or pFlag-HSF1 and pMyc-MTA1 (lane 4). Incubations were carried out in triplicate and luciferase assayed as above. Experiments were repeated (reproducibly) three times.

MTA1 and HSF1 localize to ERE- and HSE-containing promoters

We next examined whether these effects of HSF1 on reporter activation are reflected in the binding to gene promoters. Chromatin immunoprecipitation (ChIP) analysis was carried out on three genes, including heat shock element (HSE)-containing HSP70.1 (positive control), and ERE-containing presenilin 2 (*pS2*) and c-*Myc* (Figure 4a). Exposure of MCF7 cells to estradiol causes the binding of ERα to the ERE regions of *pS2* and c-*Myc*, an effect which was not affected by overexpression of HSF1 and MTA1 (Figure 4b). We also investigated the association of HSF1 and MTA1 with *HSP70.1*, *pS2* and c-*Myc* promoters in cells sequentially exposed to estradiol and heregulin. Heregulin increased the association of both HSF1 and MTA1 with the *HSP70.1* promoter and the ERE-regulated promoters of the *pS2* and c-*Myc* genes (Figure 4c). As an additional control, we carried out ChIP using anti-histone H3 antibodies under the conditions used here (Figure 4d). The experiments indicate that none of the conditions markedly modulate histone H3 association with the c-*Myc* promoter indicating reproducible performance of the ChIP assay and absence of global effects of the conditions on chromatin structure. We also examined the ability of heregulin and forced expression of HSF1 or MTA1 to modulate the levels of c-Myc protein (Figure 4e). C-Myc levels were increased by exposure to estradiol and this increase was blocked by heregulin, and overexpression of HSF1 or MTA1 (Figure 4e).

HSF1 and MTA1 are co-localized in breast carcinoma

As our experiments suggest a link between HSF1 and MTA1 in breast cancer, we examined potential co-expression of HSF1 and MTA1 using immunohistochemical analysis of serial paraffin sections from advanced human breast carcinomata (Figure 5A). Since

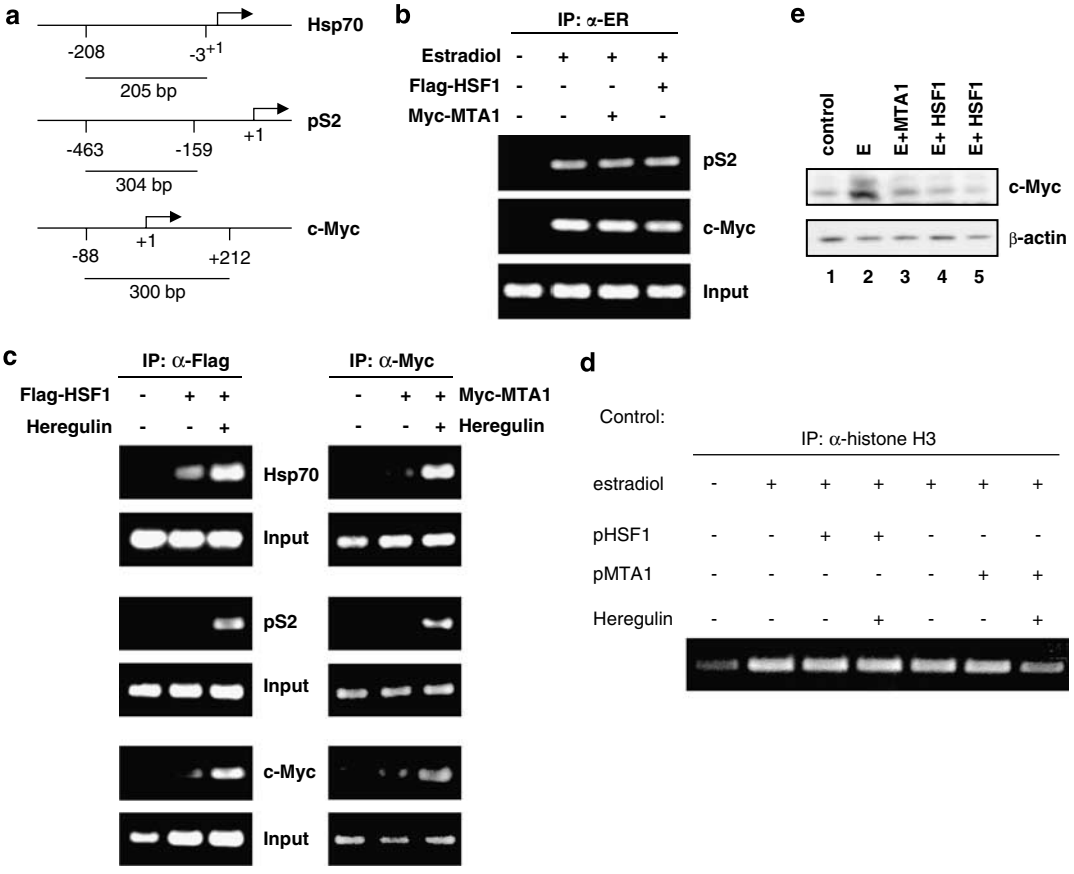


Figure 4 Heregulin promotes association of metastasis-associated protein 1 (MTA1) and heat shock factor 1 (HSF1) with HSP70.1 and ERE-responsive promoters. (**a**) Promoter elements amplified by PCR from HSP70.1 (205 bp), *pS2* (304 bp) and *c-Myc* (300 bp). (**b**) MCF7 cells were treated with or without 17β-estradiol for 6 h prior to transfection of pFlag-HSF1 and pMyc-MTA1 for 24 h. Chromatin lysates were immunoprecipitated with anti-estrogen receptor (ER) antibody. Bands show PCR analysis of *pS2* (304 bp) and c-*Myc* (300 bp) promoter fragments associated with ER. Lower panel shows PCR analysis of the DNA input. (**c**) MCF7 cells were transfected with or without pFlag-HSF1 and pMyc-MTA1 for 24 h and then treated with 1 nM HRGβ1. Cross-linked lysates were immunoprecipitated with anti-Flag (left panels) or anti-Myc (right panels) antibodies and samples prepared for PCR. PCR products shown from *HSP70.1* (205 bp), *pS2* (304 bp) *and c-Myc* (300 bp) associated with HSF1 (left panels) and MTA1 (right panels). DNA input was also analysed by PCR (Input). (**d**) Extracts from (**c**) were probed by ChIP assay using immunoprecipitation with anti-histone H3 antibody as a control. (**e**) Relative levels of c-Myc in control cells, or cells stimulated with estradiol without (E) or with MTA1 expression, HSF1 expression or pre-exposure to heregulin (lane 5). Experiments were carried out three times with similar results.

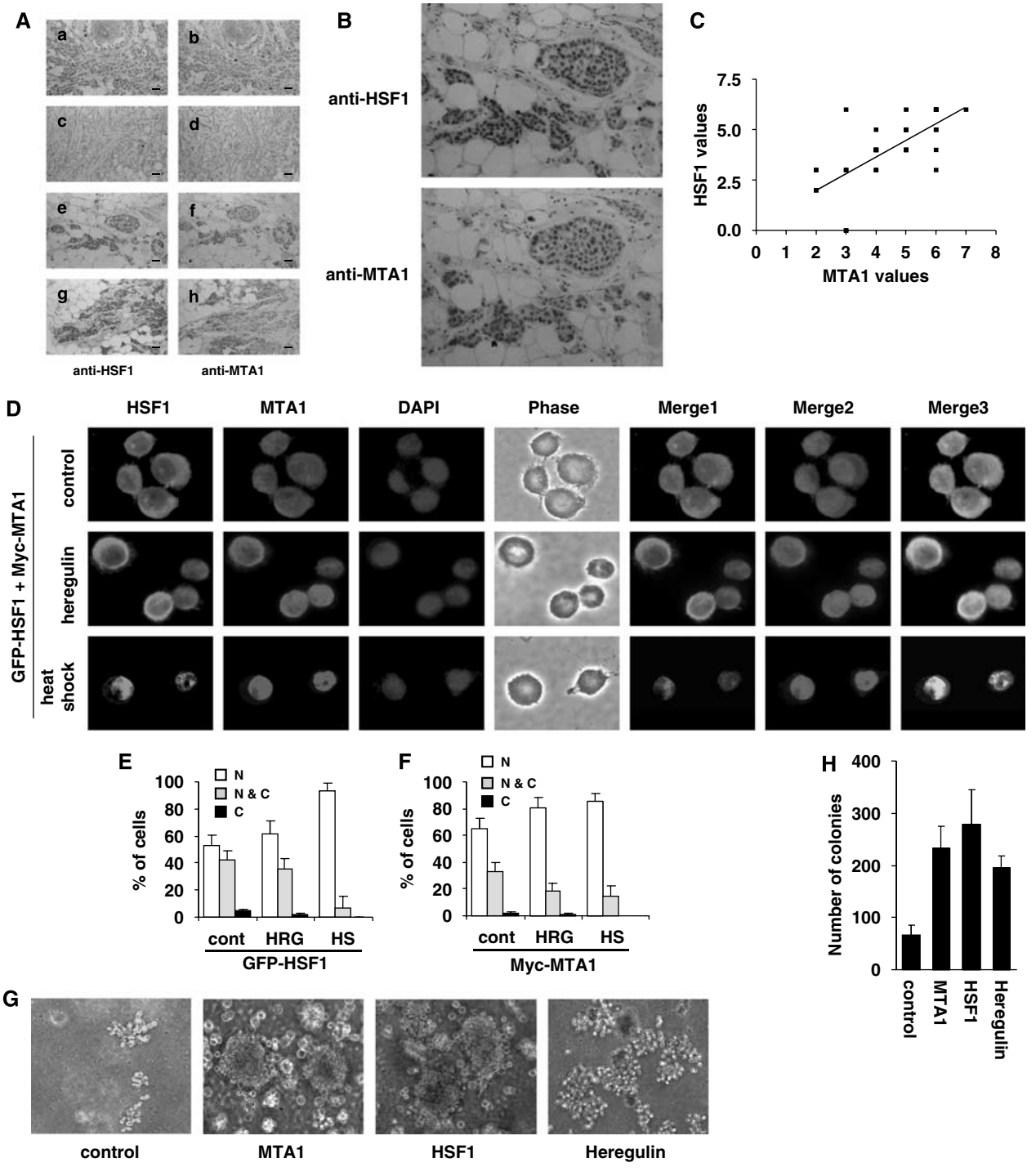
MTA1 is implicated in metastasis, the samples that we tested were from patients who developed distant metastases. Relative HSF1 and MTA1 expression in large tumor fields can be seen in four tumor samples at low power in Figure 5A and one series of serial sections is shown at higher power in Figure 5B. The higher power photomicrograph shows the co-localization of both proteins in nuclei in the *in situ* component of the breast carcinoma as well as in the invasive carcinoma (Figure 5B). The analysis indicates a close correlation

Figure 5 Heat shock factor 1 (HSF1) and metastasis-associated protein 1 (MTA1) co-localization. (**A**) Immunohistochemical detection of HSF1 and MTA1 in breast carcinoma sections **a**, **b**; **e**, **f**; **g**, **h**; and the absence of both proteins in normal breast tissue (**c** and **d**), bar = 80 μm. (**B**) HSF1 and MTA1 co-localization in a human breast cancer biopsy samples taken at higher magnification (same tissue sample as in (**a**); **e** and **f**). In upper panel are the HSF1 immunostained cells while in lower panel, MTA1 immunostained cells. (**C**) Correlation between HSF1 and MTA1 values in breast cancer biopsy samples from patients with invasive carcinomas ($n = 37$). The statistical analysis revealed a significant correlation in the expression of both molecules ($P < 0.001$, 95% CI 0.4577–0.8232, Pearson's r 0.6808, R^2 0.4635). (**D**) MCF7 cells transfected with pHM6-green fluorescent protein (GFP)-HSF1 and pBJ-*myc*/MTA1 for 48 h were either non-treated (cont), given 1 nM heregulin β1 (HRGβ1) for 24 h (HRG) or heat shocked (HS) prior to assay. GFP-HSF1 was visualized by green fluorescence, MTA1 by anti-Myc/Texas red immunofluorescence and nuclei by 4′-6-diamidino-2-phenylindole (DAPI) autofluorescence. Merge 1: co-localization of green fluorescence and DAPI; Merge 2: red fluorescence (MTA1) and DAPI, Merge 3: green (HSF1) and red (MTA1) (× 400 magnification). (**E** and **F**) Quantitation of data in (**D**); subcellular localization of GFP-HSF1 and MTA1 in MCF7 cells scored for nuclear (N), nuclear and cytoplasmic (N & C) or predominantly cytoplasmic (C) staining. Means ± s.d. are from three replicates. (**G**) Cells were transfected with pMyc-MTA1 or pFlag-HSF1 for 24 h or given 1 nM HRGβ1 for 24 h. Then 20 000 cells were plated on 0.6% agar in triplicate. Colonies were assayed after 2 weeks and × 400 phase contrast images captured. (**H**) Colonies of > 10 cells were scored blind from three plates each and expressed as mean ± s.d.

between HSF1 and MTA1 expression in the breast carcinoma cells (Figure 5C).

We also investigated the relative intracellular locations of HSF1 and MTA1 in MCF7 breast carcinoma cells *in vitro* by fluorescence microscopy (Figures 5D–F). Under resting conditions, green fluorescent protein (GFP)-HSF1 was evenly distributed between nucleus and cytoplasm and its distribution overlapped to a considerable degree with MTA1, although cytoplasmic HSF1 appeared to be slightly more abundant (Figure 5D). Exposure to heregulin increased nuclear localization of both proteins, while exposure to heat shock led to quantitative nuclear localization of each protein (Figures 5D–F).

Finally, we examined potential effects of HSF1 and MTA1 on MCF7 cell growth *in vitro* under anchorage-dependent or -independent conditions. Heregulin exposure and overexpression of HSF1 and MTA1 each strongly promote anchorage-independent cell growth. Each treatment increased the growth rates of cells on



soft agar (Figures 5G and H), while having minimal effects on growth under anchorage-dependent conditions (not shown).

Discussion

We have shown evidence for a gene corepressor in HSF1 mediated repression, MTA1 that may contribute to the role of HSF1 in cancer. HSF1 binds MTA1, a metastasis-associated protein, and a number of its associated proteins in the NuRD, including HDAC1, 2 and Mi2 (Bowen *et al*., 2004). Our experiments also suggest that the HSF1–MTA1 interaction may play a role in a subset of breast cancers. HSF1 binds avidly to MTA1 in a range of breast cell lines and the proteins are coassociated in human breast tissue, leading to ERE repression (Figures 2–4 and 5A). Our experiments indicate that HSF1 participates in breast cancer at the intersection of two essential but competing signal transduction pathways, the estrogen–ER pathway and the c-*erb*B2 pathway. Although the expression of ER can promote breast carcinoma growth, ER may play an antagonistic role in invasion and metastasis (Bewick *et al*., 1999; Kumar *et al*., 2003; Neubauer *et al*., 2003). The influence of ER expression can however be overridden by c-*erb*B2 overexpression. Our studies show that stimulation of the c-*erb*B2 pathway by heregulin promotes HSF1 association with MTA1 leading to repression of ER-dependent promoters (Figures 2 and 4). HSF1 may also participate in the progression of gastrointestinal cancers by repressing the promoter of the XAF1 gene. XAF1 is a suppressor of XIAP, a caspase 3 inhibitory protein and loss of XAF1 thus leads to protection from caspase-dependent apoptosis (Wang *et al*., 2006). Elevation of HSF1 during malignant transformation may thus be implicated in tumorigenesis through multiple mechanisms, including expression of antiapoptotic proteins HSP70 and XIAP and repression of ERE-regulated genes including pro-apoptotic c-*Myc* (Wang *et al*., 2004b; Khaleque *et al*., 2005).

Materials and methods

Materials and constructs

Heregulin β1 (HRGβ1) was from Neo Markers (Fremont, CA, USA), Tyrphostin AG-825 from Calbiochem (La Jolla, CA, USA) and 17β-Estradiol (E2) from Sigma (St Louis, MO, USA). Anti-2C4 antibody was from Genentech (San Francisco, CA, USA).

pBJ-*myc*/MTA1 and pBJ-Myc were described (Toh *et al*., 2000). The *c* pGL-*fos*, pGL-*HSP70B*, pcDNA3.1-Flag-HSF1 pHM6-GFP-HSF1 and pEGFP-C1 plasmids were described previously (Wang *et al*., 2003, 2004a) (20). Control pHM6 was from Roche (Nutley, NJ, USA), Ha-Ras expression plasmid pH06T1 from Dr MC Ostrowski and pERE-Luc construct from Michael M Wang (University of Michigan, USA). pGST-HSF1 is described in (22) and pGEX-2T from Amersham Biosciences (Piscataway, NJ, USA).

To prepare an MTA1 RNAi, we used pSuper vector (OligoEngine, Seattle, WA, USA)-based silencing and selected three 19-mer-gene sequences. The optimal sequence predicted was 1115–1133, accession number U-35113 (5′-GCCAAA TCCGAACCAAATC-3′). Total length of primer was 64 mer including the MTA1 sequence. HeLa cells were transfected with pSuper-MTA1 and pEGFP-C1, sorted and lysates were prepared from GFP-positive cells after 72 h.

Anti-HSF1, anti-HSP70, anti-human ERα (SRA-1010) were from Stressgen (Vancouver, CA, USA), anti-β-actin and anti-Flag from Sigma, anti-MTA1 from Bethyl (Montgomery, TX, USA), polyclonal anti-HDAC1 and anti-HDAC2 from Affinity BioReagents (Golden, CO, USA), polyclonal anti-Mi2 (H-242) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-c-Myc, BD Science (Mountain View, CA, USA).

Cell culture

HeLa, MCF7, MEF and MEF *hsf1*−/− cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics. MDA-MB-231 human breast cancer cell from ATCC were grown in Leibovitz's L-15 with 10% FBS without CO_2. For estrogen experiments, cells were grown in phenol red-free DMEM.

Mass spectrometry

HeLa proteins adsorbed to GST (control) or GST-HSF1 were eluted by GSH, and proteins analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining. Bands were digested in-gel with trypsin and eluted peptides analysed by matrix-assisted laser desorption/ionization time of flight-mass spectroscopy by Voyager DE-PRO (Applied Bio-systems, Foster City, CA, USA) and proteins identified by mass fingerprinting using database.

Chromatin immunoprecipitation

Cells were cross linked with 1% formaldehyde for 10 min and ChIP performed as in the Upstate Biotechnology (Lake Placid, NY, USA) protocol. A total of 25–30 cycles of PCR were carried out with 5 μl of eluted DNA and primers: *pS2* (forward 5′-CCTTCCCTTCCCCCTGCAAGGT-3′ and reverse 5′-TATCAAAGGTGTTTCCTAGACATG-3′) amplify the region including the ER-responsive element from position −463 to −159; *c-Myc* (forward 5′-CATAAGCGCCCCTCCCGG GTTCC-3′ and reverse 5′-GCTGGAATTACTACAGC GAGTTAG-3′) amplify the region from −88 to +212 and *HSP70.1* (forward 5′-CCCAGAAGACTCTGGAGAGT-3′ and reverse 5′-ATCCGGACCGCTTGCCCC-3′) amplify region from −208 to −3. Amplified PCR products were analysed by agarose gel/ethidium bromide.

Immunohistochemistry

Human breast cancer biopsy samples ($n=37$) were retrieved from our tumor bank (Gago *et al*., 2006). Antigen retrieval with microwave oven was used to unmask MTA1 (30 min in citrate buffer, pH 6.0). Immunostaining was graded as: 0 = no staining; 1 = weak staining; 2 = moderate staining and 3 = strong staining and the proportion of cells staining positive: 0% = 0; 1–9% = 1; 10–33% = 2; 34–66% = 3 and >66% = 4. Both scores were combined for the final score. Linear regression analysis was performed to determine significance.

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