Inhibition of Arginyltransferase 1 Induces Transcriptional Activity of Myocardin-related Transcription Factor A (MRTF-A) and Promotes Directional Migration*

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Myocardin-related transcription factor A (MRTF-A/MAL/MKL1/BSAC) regulates the expression of serum-response factor (SRF)-dependent target genes in response to the Rho-actin signaling pathway. Overexpression or activation of MRTF-A affects shape, migration, and invasion of cells and contributes to human malignancies, including cancer. In this study, we report that inhibition of arginyltransferase 1 (ATE1), an enzyme mediating post-transcriptional protein arginylation, is sufficient to increase MRTF-A activity in MCF-7 human breast carcinoma cells independently of external growth factor stimuli. In addition, silencing or inhibiting ATE1 disrupted E-cadherin-mediated cell-cell contacts, enhanced formation of actin-rich protrusions, and increased the number of focal adhesions, subsequently leading to elevated chemotactic migration. Although arginylated actin did not differentially affect MRTF-A, a rapid loss of E-cadherin and F-actin reorganization preceded MRTF-A activation upon ATE1 inhibition. Conversely, ectopic ATE1 expression was sufficient to render MRTF-A inactive, both in resting cells and in cells with exogenously activated RhoA-actin pathways. In this study, we provide a critical link between protein arginylation and MRTF-A activity and place ATE1 upstream of myocardin-related transcription factor.

Intracellular signaling pathways leading to the transcription of serum-response factor (SRF)2-dependent target genes play a pivotal role in regulating the cytoskeleton, cell migration, and invasion as a response to external physiological factors (1). Given that aberrant regulation of these pathways and effector proteins is associated with the occurrence of numerous human pathologies (2), it is of paramount importance to increase the understanding of the molecular mechanisms involved in their activation.

Myocardin-related transcription factor A (MRTF-A/MAL/MKL1/BSAC) belongs to the myocardin protein family of transcriptional co-activators controlling expression of subsets of SRF-dependent target genes (3). SRF regulates growth factor-induced expression of immediate-early- and muscle-specific genes as well as genes encoding regulators of actin dynamics via the Rho-actin pathway (1). Monomeric actin (G-actin) is known to sequester MRTF-A’s transcriptional activity of controlling SRF-dependent target gene expression by binding to its N-terminal RPEL motifs (3). This inhibitory complex formation may however be reversed in growth factor-stimulated cells, leading to nuclear accumulation and transcriptional activation of MRTF-A. The pivotal importance of adequate MRTF-A regulation in vivo manifests in patients expressing a constitutively active MRTF-A fusion protein (in conjunction with RBM15), leading ultimately to the development of acute megakaryoblastic leukemia in infants (4, 5).

Although few publications acknowledged a link between deregulated MRTF-A activity and carcinogenesis (6), its molecular role and mode of action in regulating cellular motility appear to depend on the cell type. Whereas silencing of MRTF-A expression in breast cancer cells reduced cellular motility in vitro as well as inhibited experimental metastasis in vivo (7, 8), partial reduction of MRTF-A in murine fibroblastic or epithelial cells induced cellular motility (8).

Here, we report that inhibition of arginyltransferase 1 (ATE1), an enzyme mediating protein arginylation, is sufficient to activate MRTF-A activity in resting MCF-7 cells in a manner that appears to be independent of direct N-terminal arginylation of β-actin. In resting cells, inhibition of ATE1 activity induced the formation of actin-rich protrusions and the induction of small focal adhesions, and ultimately it resulted in an increase in cellular motility. This study demonstrates an autonomous mechanism inducing MRTF-A-dependent cellular motility in a manner that is independent of external growth factor stimuli.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Treatments, and Reagents—The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC) and were routinely cultured in DMEM (Invitrogen) supple-
mented with 10% (v/v) fetal calf serum (FCS; Invitrogen) and 2 mM glutamine (Invitrogen). Cells were transfected with cDNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 16 h post-transfection, the medium was exchanged. If cells were serum-starved, the medium was exchanged to 0.5% FCS containing starvation medium 24 h prior to the experiment. FCS was added to a final concentration of 20%, where indicated, and cells were incubated for additional 7 h. Cells were treated with tannic acid for 24 h and with hemin (both purchased from Sigma) for 4 h at the indicated concentrations.

**Source, Cloning, and Mutagenesis of cDNA**—The p3DA-Luc and pRL-TK constructs have been described previously (3). The eGFP-tagged ATE1 (ATE1-eGFP) as well as the ubiquitin fusion actin constructs, both the nonarginylated (Ub-M-actin-eGFP) and the constitutively arginylated (Ub-R-actin-eGFP) plasmids, were kindly provided by A. Kashina (University of Pennsylvania, Philadelphia) (9, 10). To ensure efficient actin polymerization, the C-terminal eGFP tag was removed from the ubiquitin-actin fusion constructs, and the actin-ende
genous stop codon was re-inserted (Ub-M-actin and Ub-R-actin).

**siRNA Transfection**—A pool of four pre-designed siRNAs targeting different regions of the ATE1 transcript (siATE1) as well as non-targeting control siRNA (siCtrl) was purchased from Dhharmacon/Thermo Scientific (Lafayette, CO). Cells were seeded in a 6-well plate (3 × 10^5 cells/well) and allowed to adhere for 16 h. Targeted and non-targeted siRNA was trans
tected into cells at a final concentration of 25 nM, and cells were incubated for 24 h before the medium was exchanged. The total time of incubation with siRNA was 72 h.

**Luciferase Reporter Assay**—Luciferase reporter assays were performed using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI) as described previously (11). Briefly, cells were transfected with an MRTF-A/SRF-driven firefly luciferase reporter plasmid and the Renilla luciferase pRL-TK control plasmid. 18 h following transfection, medium was exchanged for serum-starving medium, where indicated, and cells were incubated for an additional 24 h. Subsequently, cells were lysed in 100 μl of 1 X Passive Lysis Buffer, and 20 μl of total cell lysate was assayed according to the manufacturer’s instructions. Data are shown as relative firefly luciferase expression normalized to Renilla luciferase for the indicated biological replicates. In parallel, the remaining cell lysate was immuno
tobloted to control for ectopic protein expression.

**Immunofluorescence**—Immunofluorescence was performed as described previously (12). A series of optical images was acquired using the Zeiss ApoTome microscope with a ×63 immersion objective. Quantification of foci adhesions was performed using the Volocity three-dimensional imaging and analysis software (Improvement, PerkinElmer Life Sciences).

**Real Time PCR**—RNA isolation (RNeasy mini kit, Qiagen, Hilden, Germany) and first-strand cDNA synthesis (Verso cDNA synthesis kit, Thermo Scientific, Epsom, UK) were performed according to the manufacturer’s instructions. In total, 1 μg of total RNA was reverse-transcribed using random hexamers. SYBR Green (Applied Biosystems, Darmstadt, Germany) gene expression profiling was performed on the LightCycler 480 system (Roche Applied Science). Fluorescent signal detection used ROX as an internal passive reference dye, and normal
ization of cellular expression level was normalized to human 18 S rRNA. Relative gene expression levels were calculated according to the 2^−ΔΔCT method.

**Immunoprecipitation and Protein Immunoblotting**—Immu
noprecipitation was performed as described previously (12). Briefly, cells were lysed in immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate). Cell lysates were incubated with anti-FLAG M2 magnetic beads (Sigma) for 90 min at 4 °C under constant rotation. Beads were washed four times for 10 min with immunoprecipita
tion buffer and resuspended in 2× Laemmli buffer. Denatured proteins were separated on 10% SDS-PAGE and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in NET-gelatin (blocking buffer) for 1 h at room temperature and probed with primary antibodies diluted in blocking buffer at 4 °C for 16 h at concentra
tions recommended by the manufacturers. Following washing steps (three times for 10 min), the membrane was incubated for 1 h with horseradish peroxidase-conjugated secondary anti
bodies (Jackson ImmunoResearch, Newmarket, UK) at room temperature. Immunoreactive bands were detected by en
hanced chemiluminescence (ECL detection kit, Millipore), and band intensities were quantified using the ImageJ image pro
cessing software from the National Institutes of Health (13).

**Cell Motility Assay**—Cellular motility toward a serum gradi
cent was assessed using a transwell chamber assay. Millicell
hanging tissue culture inserts (Millipore; 8-μm pore size) were assembled in 24-well plates. The chemotactic migration of 1 × 10^5 cells seeded in 0.1% serum-containing medium (top cham
ber) was assessed toward a 10% serum gradient (lower cham
ber). Following a 6-h incubation at 37 °C, the chambers were disassembled, and the upper side of the membrane was wiped. Cells that have been migrated through the pores to the lower side of the membrane were fixed in 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature. Membranes with cells were washed subsequently in PBS, and filters were mounted onto slides using ProLong® Gold antifade reagent containing DAPI (Invitrogen). All cells within five different representative areas of each filter were counted. Five biological replicates were used per condition.

**Statistical Analysis**—Statistical analysis was performed using the GraphPad Prism® software (Version 5, GraphPad Software, Inc., San Diego) as well as the R package (R Core Team). Statis
tical significance was assessed using one-way analysis of vari
cance with a Student’s Newman-Keuls post hoc test, unless indi
cated otherwise. Direct comparisons between two samples were assessed using the Student’s t test. All numerical values shown represent means ±95% confidence intervals (CI) or ± S.E.

**RESULTS**

**Inhibition or Silencing of ATE1 Expression Induces MRTF-A Transcriptional Activity**—The ability of MRTF-A to control
SRF-dependent gene expression is impeded by its binding to
monomeric actin, a process that is known to be reversed by external growth factor stimulation (e.g. serum). β-Actin itself
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A. MCF-7 cells were serum-starved and treated with 15 μM tannic acid or 100 μM hemin. siCtrl or siATE1 was added, and cells were stimulated with FCS for 60 min. The localization of MRTF-A and F-actin was detected. Line scans of orthogonal sections along the indicated line demonstrate the distribution of MRTF-A (green line) and F-actin (red line). Green bars indicate the nuclear region in the line scan, and red arrows represent cell-cell borders. The localization of MRTF-A staining was determined by quantifying 100 cells each of four independent experiments. Shown is the mean ± S.E. C, cytoplasmic staining; C/N, cytoplasmic/nuclear staining; N, nuclear staining. B, MCF-7 cells were transfected with siCtrl or siATE1, respectively, and the efficacy of ATE1 knockdown was assessed by real time PCR (RNA level) and immunoblotting (protein level). Shown is the fold-change difference of siATE1 versus siCtrl of four (RNA level) or three (protein level) independent experiments ± 95% CI. C, MCF-7 cells were transfected with siCtrl or siATE1, respectively, and protein lysates were immunoblotted (IB) with an anti-ATE1, an anti-β-actin and an anti-tubulin antibody. D, cells were serum-starved and treated with the indicated concentrations of tannic acid or 100 μM hemin. siCtrl or siATE1 was added, and cells were stimulated with serum, tannic acid, or hemin. First, the localization of MRTF-A staining was determined by quantifying 100 cells each of four independent experiments. Shown is the mean ± S.E. C/N, cytoplasmic/nuclear staining; N, nuclear staining.

has been shown to undergo a series of post-translational modifications, which ultimately affect its monomer–polymer equilibrium (14). Among those, the recently documented arginylation, a process mediated by arginyltransferase 1 (ATE1) (9), still remains poorly understood on the molecular level. Although arginylated proteins are routinely marked for proteasomal degradation via the N-end rule pathway (15), arginylated proteins remain stable in fibroblasts and exhibits altered dynamics (9). Moreover, arginylation of actin could affect its binding to the RPEL domain of MRTF-A, which is rich in positively charged residues. To investigate this possibility, we sought to inhibit ATE1’s activity either by silencing ATE1 expression by siRNA (siATE1) or by treating cells with tannic acid or hemin, compounds known to reduce ATE1 activity as well as to induce its degradation (16–18). Therefore, MCF-7 cells were transfected with nontargeting siRNA control (siCtrl) or siATE1 or were treated with serum, tannic acid, or hemin. First, the localization of MRTF-A was assessed. As shown in Fig. 1A, MRTF-A’s localization was diffuse in the control cells (−FCS) as well as in cells transfected with siCtrl. Serum stimulation (+FCS) of cells induced the translocation of MRTF-A to the nucleus, paralleling observations obtained so far in HeLa cells (19) and routinely in murine fibroblasts (3, 20).

Surprisingly, either silencing of ATE1 expression by siRNA transfection or inhibition of ATE1 using tannic acid or hemin led to an increased nuclear accumulation of MRTF-A (Fig. 1A).
These observations were subsequently quantified, showing an increase in nuclear localization in ATE1 inhibited/silenced cells, both in a line scan and an MRTF-A compartmentalization assessment (Fig. 1A, right panel, line scan; MRTF-A is represented by the green lines). Noteworthy, we routinely observed an increase in F-actin in ATE1-inhibited cells, especially at cell-cell contacts, as quantified in the line scan of micrographs (Fig. 1A, red arrows).

The efficiency of ATE1 knockdown achieved by employing a pool of four different siRNAs targeted to different regions of the ATE1 transcript was confirmed both on the mRNA level using real time PCR (Fig. 1B; mRNA change-fold expression, 0.22 siATE1 versus siCtrl; 95% CI, 0.1403–0.3108) and on the protein level by immunoblotting (Fig. 1, B and C; protein expression, 0.13 siATE1 versus siCtrl; 95% CI, −0.0618–0.3302). The comparable signal intensity of β-actin in the immunoblot further confirms the stability of β-actin in control cells compared with siATE1 (Fig. 1C). The ATE1-inhibitory capacity of tannic acid and hemin was assessed by immunoblotting treated samples with an anti-RGS4 antibody, a known target for ATE1-dependent proteasomal degradation (21). Hence, an accumulation of RGS4, as observed in Fig. 1D, corresponds to a decreased ATE1 activity.

Although nuclear translocation, as shown in Fig. 1A, is necessary for MRTF-A to exhibit its function, the sole quantification of MRTF-A translocation is not sufficient to confirm its transcriptional activity. Therefore, we performed a luciferase reporter gene assay measuring MRTF-A/SRF-dependent transcription levels (3). Transfection of MCF-7 cells with this MRTF-A/SRF-dependent luciferase reporter revealed a significant up-regulation of MRTF-A activity in serum-stimulated cells (Fig. 2A; p = 0.0352). However, treatment of cells with tannic acid or hemin showed no statistically significant difference in MRTF-A activity in stimulated cells (Fig. 2A; control + FCS versus tannic acid + FCS, p = 0.0764; control + FCS versus hemin + FCS, p = 0.4187). Interestingly however, a strong MRTF-A induction was observed in resting cells treated with the inhibitors, which were shown to be within the 95% confidence limits of FCS treatment (Fig. 2A; control − FCS versus tannic acid − FCS, p = 0.0208; control − FCS versus hemin − FCS, p = 0.0112; 95% CI: control + FCS, 4.0–36.96; tannic acid − FCS, 2.171–6.119; hemin − FCS, 4.065–12.68). This induction of MRTF-A activity exhibited a dose dependence, and concentrations of 50 μM led to comparable MRTF-A activities as observed upon serum stimulation (Fig. 2B; the 95% CI of serum-stimulation is indicated, see dotted lines). Treatment of resting cells with siATE1 showed a similar induction of MRTF-A activity, paralleling results observed upon ATE1 inhibition (Fig. 2C; p = 0.0277). Furthermore, addition of FCS to cells lacking ATE1 expression does not add an additional means of increasing MRTF-A activity compared with siATE1-transfected serum-starved cells (p = 0.9794), indicating that indeed silencing of ATE1 expression mimics the effect observed upon serum stimulation. Given that MRTF-A activity is directly controlled by its binding to monomeric actin, we tested whether inhibition of ATE1 activity affected the actin-MRTF-A complex. To assess this, MCF-7 cells were co-transfected with HA-tagged MRTF-A (MRTF-A-HA) and FLAG-tagged actin (FLAG-actin), and cell lysates were immunoprecipitated with an anti-FLAG antibody (M2, Sigma). Immunoprecipitates and input controls were immunoblotted with an anti-HA and an anti-FLAG antibody. As shown in Fig. 2D, inhibition of ATE1 indeed reduced the actin-MRTF-A complex, as expected from the reporter gene assays and the immunofluorescence data. These observations were confirmed by quantifying three independent immunoprecipitations (Fig. 2E). The reduction of actin-MRTF-A complexes could further be confirmed by siATE1-treated cells co-transfected with HA-tagged MRTF-A (MRTF-A-HA) and FLAG-tagged actin (FLAG-actin). Similar to the observations obtained upon ATE1 inhibition, silencing of ATE1 expression decreased the amount of co-immunoprecipitated actin-MRTF-A complexes (Fig. 2F).

Inhibition or Silencing of ATE1 Leads to Formation of Actin-rich Cell Protrusions—Given the increase in F-actin observed in cells treated with ATE1 inhibitors or siATE1 (Fig. 1A), we sought to assess the ability of cells to form lamellipodium-like protrusions in the absence of active ATE1. Therefore, MCF-7 cells were serum-starved in 0.5% FCS-containing medium and stimulated with tannic acid for 24 h and with hemin or serum for 4 h. Cells transfected with siRNA were cultivated for 72 h and subsequently fixed, and the actin cytoskeleton was visualized by immunostaining with phallolidin. As expected, cells stimulated with serum to a final concentration of 20% predominately formed stress fibers, whereas a significant induction of actin-rich membrane protrusions was revealed in cells treated with the ATE1-blocking inhibitors or siATE1 (Fig. 3A). These observations were subsequently quantified (Fig. 3B), showing a significant increase in these lamellipodium-like structures in siATE1-transfected (p < 0.0001 versus siCtrl, unpaired t test), in hemin-stimulated (p < 0.0001 versus −FCS control, unpaired t test), and in tannic acid-treated cells (p < 0.0001 versus −FCS control, unpaired t test). Along with the formation of stress fibers in serum-stimulated cells, the appearance of actin-rich protrusions was also increased (p = 0.001 versus −FCS control, Fig. 3B). However, this increase was significantly lower than those observed upon ATE1 inhibition.

Silencing of ATE1 Expression Leads to an Increase in Focal Adhesion Formation in Resting Cells—Considering that MRTF-A function has been linked to a contractile and migratory phenotype in breast cancer cells (7) and we have shown that reduction of ATE1 activity in MCF-7 cells led to an increase in actin-rich cell protrusions, we tested whether this phenotype is linked to an increase in the number of focal adhesions. Therefore, the localization of paxillin and F-actin was assessed in serum-starved cells (−FCS control) and in cells treated with serum (+FCS) as well as in siCtrl- or siATE1-transfected cells (Fig. 4A). Although only a limited number of small focal adhesions in −FCS and siCtrl cells were visualized (Fig. 4B), stimulation of cells with serum led to the formation of mature focal adhesions (mean size, 10.56 μm; 95% CI, 8.841–12.27; Fig. 4, B and C). In contrast, silencing of ATE1 expression by siRNA led to an increase in small-sized focal adhesions (mean size, 4.058 μm; 95% CI, 3.654–4.461; Fig. 4, B and C). The focal adhesions observed in siATE1-transfected cells were found to be significantly more than counted in siCtrl cells (p = 0.001; unpaired Student’s t test, Fig. 4B) as well as significantly
smaller than those observed under serum-stimulated conditions ($p = 0.0442$ in the category $<2 \mu m$; $p = 0.0003$ in the category $>12 \mu m$; Fig. 4C).

E-cadherin-mediated Cell-Cell Contacts Are Disrupted in Cells Lacking ATE1 Activity—We have shown that cells lacking ATE1 expression exhibit an increased number of focal adhesions and actin-rich protrusions, indicating a potentially increased motile phenotype. To test whether ATE1 knockdown or inhibition indeed leads to a decrease in cell-cell adhesion, the presence of intact E-cadherin-mediated contacts was assessed. Cells were cultivated under serum-starving conditions, and ATE1 activity was abolished by either siRNA transfection or
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This study provides evidence for a link between the inhibition of ATE1 activity and an increase in MRTF-A activity in breast carcinoma cells. This study was supported by grants from the National Institutes of Health.

FIGURE 2. MRTF-A-dependent transcription in resting MCF-7 cells is increased in cells lacking ATE1 activity. A, MCF-7 cells were transfected with the firefly and the Renilla luciferase plasmids and were treated with 30 μM tannic acid (Tannic A.) or hemin (100 μM). Data represent four independent experiments (tannic acid, three independent experiments) with mean ± S.E. a.u., arbitrary units. B, cells were transfected as before (A) and were treated with increasing concentrations of tannic acid or hemin. Cells were serum-starved in 0.5% FCS-containing medium for 24 h prior to the assay. Data represent four independent experiments with means ± 95% CI. The 95% CI for ± FCS control is indicated (dotted lines), a.u., arbitrary units. C, cells were transfected as before (A), and siRNA was added. Cells were either serum-starved (white bars) or stimulated for 7 h with FCS (black bars). Data represent three independent experiments with mean ± S.E. a.u., arbitrary units. D-F, MCF-7 cells were transfected with FLAG-actin and MRTF-A-HA. Cells were treated with siRNA for 72 h, for 2 h with FCS, for 24 h with indicated concentrations of tannic acid, or for 4 h with hemin, and cell extracts were immunoprecipitated (IP) with an anti-FLAG antibody. Immunoprecipitates and input controls were immunoblotted (IB) with an anti-HA and an anti-FLAG antibody. The semiquantitative analyses of band intensities of three independent immunoprecipitations (D) ± S.E. are shown on the right side (E).

This study demonstrates that ATE1 activity is sufficient to restrain MRTF-A activity, and that ATE1 inhibition by siRNA leads to an increase in MRTF-A activity. The increase in MRTF-A activity is correlated with an increase in focal adhesions and actin-rich protrusions in ATE1-silenced cells. This study provides evidence for a link between ATE1 activity and MRTF-A activity in breast carcinoma cells.
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A

|          | Cell-cell contacts | Phalloidin | Leading edge |
|----------|--------------------|------------|--------------|
| -FCS     | ![Image]           | ![Image]   | ![Image]     |
| +FCS     | ![Image]           | ![Image]   | ![Image]     |
| siCtrl   | ![Image]           | ![Image]   | ![Image]     |
| siATE1   | ![Image]           | ![Image]   | ![Image]     |
| Tannic Acid | ![Image]   | ![Image]   | ![Image]     |
| Hemin   | ![Image]           | ![Image]   | ![Image]     |

B

![Bar chart]
FIGURE 3. Formation of actin-rich protrusions is increased in resting cells lacking ATE1 activity. A, MCF-7 cells were treated with 30 μM tannic acid and 150 μM hemin. siRNA was added, and FCS was added to a final concentration of 20% for 60 min. The localization of F-actin was detected. Middle panels show magnified portions of each image (paxillin staining), as indicated (rectangles). Arrows point toward the formation of actin-rich protrusions, and open arrowheads indicate stress fibers. B, number of cells without actin-rich protrusions, with actin-rich protrusions, or ambiguous morphology was quantified by counting 100 cells of four independent experiments. ns, not significant. Data are shown as mean ± 95% CI with p < 0.0001 (**).
breast cancer cells. It is well established that MRTF-A activity is tightly regulated by its binding to monomeric actin, a complex that is disrupted by growth factor-mediated stimulation of the RhoA pathway. Here, we show an autonomous way of MRTF-A activation that is independent of external growth factor stimuli by serum but depends on the absence of post-translational protein arginylation. Karakozova and co-workers (9) have previously demonstrated that lack of ATE1 expression leads to a reduction of actin polymer levels, a phenotype that is associated with impaired cellular motility, reduced lamella formation, and defective cell adhesion in murine fibroblastic cells. In contrast, in this study we present data showing that ATE1 silencing or inhibition led to a decrease in cell-cell adhesion and an increase in focal adhesions and lamellipodia formation, mimicking the phenotype observed in cells expressing active MRTF-A. These differences observed might be attributed to the different cell types, although previous studies used murine fibroblastic cell lines or primary mouse fibroblasts, we employed human breast

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**FIGURE 5.** E-cadherin-mediated cell-cell adhesion is reduced upon ATE1 inhibition or depletion. A, MCF-7 cells were treated with 30 μM tannic acid or 150 μM hemin. siRNA was transfected, and FCS was added to a final concentration of 20% for 60 min. The localization of MRTF-A and E-cadherin was detected. *Inset panels* show magnified portions of each image (E-cadherin) as indicated (rectangles). B, in parallel, samples of A were lysed and immunoblotted (IB) with an anti-E-cadherin and an anti-tubulin antibody. The semiquantitative analyses of band intensities of three independent immunoblots are shown on the right side. Data are shown as mean ± S.E. ns, not significant.
carcinoma cell lines as a model. This notion is supported by recent evidence, showing that ablation of ATE1 expression in platelets in vivo did not lead to an impairment in actin polymerization but rather induced the contractile capacity of these cells during clot retraction (22). Also, MRTF-A activity has been shown to play different roles depending on the cell type, the tissue environment, and the signaling pathways in which it is involved (6). For example, a role for MRTF-A has emerged in tumorigenesis, i.e. silencing of MRTF-A expression decreased tumor cell motility and metastatic colonization of tumor xenografts of human breast carcinoma and mouse melanoma cells (7). However, MRTF-A overexpression has been associated with an anti-proliferative, anti-migratory, and pro-apoptotic phenotype in mouse fibroblastic cells (8, 11, 23).

By having established that reduction of ATE1 activity is sufficient to activate MRTF-A in breast cancer cells, the underlying molecular mechanism still remains elusive. β-Actin itself is a target for arginylation and has been shown to remain stable in vivo (9), raising the possibility that the actin-MRTF-A complex is affected due to the positively charged Arg residues. However, we were not able to observe an effect of constitutively arginylated actin on MRTF-A activity, excluding a direct role of

FIGURE 6. ATE1-dependent cytoskeletal rearrangements occur prior to MRTF-A activation. A, MCF-7 cells were treated with 150 μM hemin for increasing time intervals, as indicated. Cells were subsequently immunoblotted (IB) with an anti-E-cadherin, an anti-MRTF-A, and an anti-tubulin antibody. The fold change differences in protein expression compared with t = 0 min are indicated below the respective blots. B and C, MCF-7 cells were serum-starved, and 150 μM hemin was added for the indicated time intervals. F-actin and MRTF-A localization was visualized with phalloidin or an anti-MRTF-A antibody, and the percentage of cells exhibiting actin-rich protrusion (black bars) or MRTF-A nuclear localization (red line), respectively, was counted for 200 cells in two independent experiments (C; ± S.E.) D, MCF-7 cells were transfected with the firefly and the Renilla luciferase plasmids. Cells were serum-starved and treated with 150 μM hemin for indicated time points. Data represent three independent experiments with mean ± S.E. *, p < 0.05; **, p < 0.01.
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N-terminal β-actin arginylation. It is important to stress the point that we employed an actin construct harboring only an N-terminal arginine that is not accounting for potential internal arginylation sites as postulated by Terman et al. (14). Another link between ATE1 and MRTF-A is the arginylation-mediated stabilization of cell-cell adhesions. Zhang et al. (24) have demonstrated that ATE1 not only regulates calpain II-mediated cleavage of talin resulting in a 70-kDa N-terminal fragment but also mediates the arginylation of this fragment. This process was shown to be critical for the homeostasis and maintenance of E-cadherin-mediated adherens junctions.

We and others have previously shown that the dissociation of cell-cell adhesions is indeed sufficient to induce MRTF-A activity in a variety of different cell types (25–29), playing a crucial role during the epithelial to mesenchymal transition. It may be hypothesized that reduced ATE1 activity renders talin unprocessed and enables it to assemble in focal adhesions rather than forming core elements of adherens junctions. Considering that talin is 2-fold reduced in MRTF-depleted MDA-MB-231 cells (7) and that talin represses the expression of E-cadherin in Drosophila (30), it will be interesting to clarify the role of talin in ATE1-mediated MRTF regulation.

Interestingly, we observed that ATE1 expression per se is sufficient to reduce MRTF-A activity, both in resting and in serum-stimulated conditions, suggesting a feedback loop feeding into the ability of actin to inhibit MRTF-A activity. Considering that ATE1 activity is required for the maturation and maintenance of adherens junctions, we hypothesize that mature cell-cell adhesions are sufficient to block MRTF-A activity, independent of serum stimulation. This notion is supported by our previous work showing that overexpression of E-cadherin in AGS cells (normally lacking E-cadherin expression) is indeed suppressing MRTF-A transcriptional activity (26). Moreover, highly invasive MDA-MB-231 breast cancer cells, which lack E-cadherin expression, exhibit a constitutively nuclear localization of MRTF-A. Despite similar expression and activity of ATE1, MRTF-dependent transcription in MDA-MB-231 is not further induced by serum (data not shown). This suggests that the ATE1 involvement is overruled by the absence of E-cadherin in tumor cells.

Intriguingly, ATE1 activity, as measured by RGS4 expression, appears to be reduced in breast carcinoma samples, whereas a decrease in E-cadherin gene expression is associated with a poor prognosis in patients (data not shown). Whether these observations indicate a functional connection between ATE1, E-cadherin, MRTF activity, and tumor progression remains to be investigated.

In conclusion, we have provided evidence that in ATE1-lacking cells, the activity of MRTF-A is induced in a manner that is independent of external growth factor stimuli by serum, thus inducing cellular motility. This work unravels an unex-

![FIGURE 7. ATE1 activity is sufficient to sequester MRTF-A activity independent of actin arginylation. A, MCF-7 cells were transfected with the firefly and the Renilla luciferase plasmids and were co-transfected with ATE1-eGFP. Cells were serum-starved (white circles) and stimulated for 7 h with FCS (black circles), as indicated. Data represent four independent experiments with mean ± S.E. ns, not significant. B, MCF-7 cells were transfected with the firefly and the Renilla luciferase plasmids and were co-transfected with MRTF-A-HA, Ub-M-actin or Ub-R-actin, as indicated. Cells were serum-starved for 24 h. Data represent three independent replicates with mean ± S.E. ns, not significant.](image)

![FIGURE 8. Silencing of ATE1 expression increases cellular motility. Cells were either transfected with siRNA or transfected with pEF-MRTF-A-HA-ΔN or respective empty plasmid control (pEF-HA control). Wild-type control (WT) remained untreated. Cell migration was quantified using a modified Boyden chamber assay. Data represent the mean number of cells per field of view (five different fields of view) of five independent biological replicates (pEF-HA control plasmid and pEF-MRTF-A-HA-ΔN transfected cells were assayed in duplicate) ± S.E. Representative field of view is shown below the plot.](image)
expected function of ATE1 that paves the way for future analysis in tumor cell migration, invasion, and metastasis.

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