ATIP3, a novel prognostic marker of breast cancer patient survival, limits cancer cell migration and slows metastatic progression by regulating microtubule dynamics

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Abstract

Metastasis, a fatal complication of breast cancer, does not fully benefit from available therapies. In this study we investigated whether ATIP3, the major product of 8p22 MTUS1 gene, may be a novel biomarker and therapeutic target for metastatic breast tumors. We show that ATIP3 is a prognostic marker for overall survival among patients with breast cancer. Notably, among metastatic tumors, low ATIP3 levels associate with decreased survival of the patients. By using a well-defined experimental mouse model of cancer metastasis we show that ATIP3 expression delays the time-course of metastatic progression and limits the number and size of metastases in vivo. In functional studies, ATIP3 silencing increases breast cancer cell migration whereas ATIP3 expression significantly reduces cell motility and directionality. We report here that ATIP3 is a potent microtubule stabilizing protein whose depletion increases microtubule dynamics. Our data support the notion that by decreasing microtubule dynamics, ATIP3 controls the ability of microtubule tips to reach the cell cortex during migration, a mechanism that may account for reduced cancer cell motility and metastasis. Of interest, we identify a functional ATIP3 domain that associates with microtubules and recapitulates the effects of ATIP3 on microtubule dynamics, cell proliferation and migration. Our study is a major step toward the development of new personalized treatments against metastatic breast tumors that have lost ATIP3 expression.
Introduction

The occurrence of distant metastasis is a dreadful complication of breast cancer and a leading cause of death by malignancy in women worldwide. Metastasis is a multi-step process that involves cancer cell migration and invasion across the extracellular matrix to reach the blood flow, followed by extravasation and colonization of secondary organs (1). Among millions of invasive cancer cells that reach the blood circulation, only few will establish at distant sites and grow as metastases (2-5). Breast cancer metastases can remain latent for several years following primary tumor removal, and the identification of molecular markers that may predict the risk of metastasis occurrence and/or progression is of invaluable help for the follow-up of the patients and choice of therapeutic options (5, 6). Over the past decade, extensive studies have led to the classification of breast tumors into distinct molecular subtypes, allowing subsequent development of efficient targeted treatments for a majority of primary tumors (7-9). However, available therapies have limited effect on cancer metastasis and new genetic determinants contributing to essential steps of the metastatic process need to be characterized.

Microtubule (MT)-targeting drugs such as taxanes are used for standard first-line treatment of breast cancer metastasis and new MT-targeting agents, such as epothilones and eribulin, are under clinical evaluation (10). MTs are polarized and highly dynamic structures that rapidly switch between periods of polymerization (growth) and depolymerization (shrinkage) at the plus ends, a process termed dynamic instability (11-13). The extent and rate of MT growth, as well as transitions between growth and shrinkage, are parameters of dynamic instability that can be measured by tracking end-binding (EB) proteins at the MT plus ends (13-15). Dynamic instability is essential for the MT plus ends to explore the cytosol and ensure cytoskeleton reorganization during cell division and migration. Targeting the expression or activity of metastasis genes that regulate MT dynamics represents a promising option for cancer therapy.
ATIP3 is a MT-associated protein encoded by 8p22 candidate tumor suppressor gene MTUS1 (16-18). We have previously shown that ATIP3/MTUS1 levels are significantly down-regulated in 47.7% of invasive breast carcinomas and 62.4% of metastatic tumors (19). Restoring ATIP3 expression at normal levels in breast cancer cells significantly reduces cancer cell proliferation in vitro and tumor growth in vivo (19). However, effects of ATIP3 on breast cancer metastasis have not yet been evaluated.

In this study, we investigated whether ATIP3 may represent a new biomarker and therapeutic target for breast cancer metastasis. We present evidence that low ATIP3 levels correlate with decreased probability of survival among patients with breast cancer metastasis, and that ATIP3 expression into ATIP3-deficient cancer cells markedly impairs the establishment of metastatic foci in vivo. Loss of ATIP3 increases breast cancer cell migration and alters MT dynamics. We show that ATIP3 associates with MTs through a central basic domain that retains the functional properties of the full-length protein. Our study thus identifies ATIP3 as a new promising therapeutic target against metastatic breast tumors of poor prognosis.
Materials and methods

Breast tumor samples and gene arrays

Microarray data for a series of 150 infiltrating ductal primary breast carcinomas and 11 normal breast tissues from the Institut Curie (Paris) and clinical data for the patients were described elsewhere (19, 20). Gene expression profiles from an independent cohort of 162 invasive breast carcinomas were obtained from patients included in the prospective database of the Institut Gustave Roussy (IGR) (Villejuif, France) between 1984 and 1994. This study was approved by the institutional review boards of the IGR. Data have been submitted to the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-1389. MTUS1 gene expression in a meta-analysis of 2898 breast cancer patients with known clinical outcome was retrieved from Kaplan-Meier plotter database (21, 22).

Cell lines, plasmid constructs and transfections

Human breast cancer cell lines MDA-MB-468, MCF7 and stable clones were described previously (19). MDA-MB-231-Luc-D3H2LN breast cancer cells (designated D3H2LN) obtained from Caliper Life Science (Xenogen, MA, USA) were derived from an in vivo-selected metastatic subclone of MDA-MB-231 cells expressing luciferase and grown as described (23). HeLa cells were provided by Dr. Mounira Amor-Gueret (Institut Curie, Orsay). RPE-1 (h-TERT-immortalized, retinal pigment epithelial) cells were from Dr. Franck Perez (Institut Curie, Paris). MRC5-SV lung fibroblasts were grown in Akhmanova’s lab as described (24). All cells were used at passages 2 to 20 after thawing and grown as described by the provider. Cells were routinely authenticated by morphologic observation and tested for absence of mycoplasma contamination using MycoAlert Assay detection kit (Lonza, France). Plasmid constructs are described in Supplementary Methods. Transfections using ATIP3-
specific siRNAs (si#1 and si#2) were performed as described (19) and verified by immunoblotting using anti-MTUS1 polyclonal antibodies (ARP-44419, Aviva Systems, San Diego, CA, USA).

**Intracardiac experimental mouse model of metastasis**

Experimental metastasis was conducted as described (23, 25, 26) following intracardiac injection of stable ATIP3-negative (WT, GFP) or positive (Cl3, Cl6) D3H2LN cell clones. All injected cells showed similar viability as measured by AnnexinV apoptosis kit (Beckman Coulter, France). The experiment was carried out with the approval of the Département d'Expérimentation Animale, Institut d'Hématologie, Hopital St-Louis ethical committee, and was performed twice (9 mice per group).

**Clonogenicity, cell migration and adhesion assays.**

Analyses of colony formation, Boyden chambers chemotaxis, trans-endothelial migration, wound healing and cell adhesion were performed as described (23). Time-lapse videomicroscopy analyses of cell motility are described in Supplemental Methods. For cell polarity measurements, transiently transfected D3H2LN were allowed to migrate for 1h30 and analyzed using bright field microscopy. Polarized cells were identified based on nucleus position and cytoplasm extension at the leading edge.

**Immunostaining, fluorescence microscopy, analysis of MT dynamics**

Cells were plated on glass coverslips and transfected for 24 hrs (plasmids) or 72 hrs (siRNA), fixed in ice-cold methanol for 5 min and incubated for 1 hr at room temperature with anti-α-tubulin clone F2C (27), monoclonal anti-γ-tubulin (Sigma), anti-EB1 (clone 5; BD Bioscience) or anti-acetylated-tubulin (clone 6-11B-1; Sigma). Secondary antibodies and fluorescence images capture are described in Supplemental Methods.
Linescan analyses of α-tubulin and EB1 fluorescence intensity were done (ImageJ) on a 6 μm line along the length of MT tip. At least 10 MTs per cell in 4 separate cells were measured. EB1-comet maximal intensity was obtained by subtracting the intensity value of the EB1-dot (100 a.u.) to the maximal staining intensity.

Analyses of MT stability, regrowth, and MT dynamic instability are described in Supplemental Methods.

**Statistical analysis**

Statistical analyses were done using JMP-7 and GraphPad Prism softwares. OS curves were plotted according to the method of Kaplan-Meier and compared by the log-rank test. Data in bar graphs (mean +/- SD) were analyzed using two-tail unpaired t-test. Dot plot analyses were done using Mann-Whitney test. p<0.05 was considered statistically significant.
Results

ATIP3 is a prognostic marker of poor outcome in metastatic breast cancer

The prognostic value of ATIP3 as a marker for metastatic progression and overall survival (OS) was evaluated in three independent cohorts of breast cancer patients. Comparison of MTUS1 Affymetrix probeset intensities with clinicopathological data of the patients in a panel of 150 invasive breast carcinomas (Supplementary Table SI) showed that the overall probability of survival is strongly reduced in patients with tumors expressing low as compared to normal ATIP3 transcript levels (Fig.1A; Supplementary Fig.S1A). Relapse-free survival (RFS) of the patients was also significantly reduced in low ATIP3-expressing tumors (Supplementary Fig.S1B). Similar results were obtained by analyzing MTUS1 levels in an independent cohort of 162 breast cancer patients (Fig.1B, Supplementary Table SII) and in a meta-analysis of 2898 breast cancer patients (Fig.1C; Supplementary Fig.S1C, S1D). Of note, correlation between ATIP3 expression and OS of the patients was independent of the estrogen receptor (ER) status of the tumor (Fig.1D).

Tumors were then classified according to their metastatic properties and MTUS1 probeset intensities were compared with the probability of patient survival. As shown in Fig.1E, the percentage of patients with metastatic disease surviving after 5 years was markedly reduced when tumors expressed low ATIP3 (6.25%) compared to normal ATIP3 levels (31.6%) whereas in patients with non-metastatic tumors, 5-years survival (Fig.1E, Supplementary Fig.S1E) and OS rates (Fig.1F, 1H) were independent of the levels of ATIP3. Within patients with metastatic disease, OS rates (Fig.1F, 1H, Supplementary Fig.S1E) and survival time (Fig.1G, 1I) were also reduced when tumors expressed low levels of ATIP3. Thus, ATIP3 expression is an important indicator of clinical outcome for patients with metastatic breast tumors. Correlation between low ATIP3 levels and reduced survival rates among patients with advanced breast cancer suggests major effects of ATIP3 on metastatic progression.
ATIP3 limits breast cancer metastatic colonization in vivo

In vivo effects of ATIP3 on the metastatic potential of breast cancer cells were evaluated using a well-defined experimental mouse model of metastasis monitored by intravital bioluminescence imaging (23, 25, 26). Highly metastatic, ATIP3-negative, D3H2LN breast cancer cells were transfected with either GFP or GFP-ATIP3 and independent stable cell clones (Cl3, Cl6) expressing moderate levels of ATIP3 were selected (Fig.2A, left). All cell clones exhibited similar levels of luciferase activity (Fig.2A, right). Metastatic cancer cells were injected intracardiacally into the bloodstream of nude mice in order to recapitulate the late, rate-limiting, steps of the metastatic process, and examine metastatic dissemination to various organs while avoiding any effect of ATIP3 on primary tumor growth. Four groups of 18 mice were analyzed in two independent experiments. For each animal, the total number of metastatic foci and the number of photons/sec were quantified every two days for 24 days (Supplementary Table SIII). As shown in Fig.2B, the time-course of metastasis formation was markedly delayed in mice injected with ATIP3-positive as compared to ATIP3-negative cell clones. The number of cancer cells growing at secondary sites increased exponentially from day 17 after injection of ATIP3-positive clones, as compared to day 10 for mice injected with control cells (Fig.2B). As shown in Fig.2C, the number of mice developing metastasis was strongly diminished upon ATIP3 expression. Importantly, the number of detectable metastases per mouse was also significantly reduced at all times in the presence of ATIP3 (Fig.2D). At day 24, the number of mice invaded with large metastases reached 13/18 (72.2%) in the control group as compared to 2/18 (11.1%) following injection of ATIP3-positive cells (Fig.2E, 2F), indicating a prominent effect of ATIP3 on cancer cell growth and colonization at secondary sites. Accordingly, on day 24 the total number of photons/sec per mouse was 50- and 25-fold lower following injection with Cl3 and Cl6 clones, respectively, compared to WT (Supplementary Fig.S2A). For ethical reasons, mice had to be sacrificed at
day 24, therefore overall survival of the two groups of mice could not be quantified. Further ex-vivo and histological analysis of metastatic nodules (Supplementary Fig.S2B) confirmed that bioluminescent signals indeed correspond to metastases of human tumor cells having infiltrated mouse tissues. Metastases were mainly detected in the bones, the lungs and the brain, which are the most frequent sites of metastatic dissemination of human breast tumors. No preferential location of metastatic nodules in ATIP3-positive versus ATIP3-negative cell types could be observed. Altogether these results identify ATIP3 as a potent anti-metastatic molecule, and support a role for ATIP3 in metastatic growth and colonization in vivo.

**ATIP3 impairs breast cancer cell proliferation and migration**

Metastatic colonization involves cancer cell migration, invasion through the extracellular matrix and proliferation at the secondary site. As expected from our previous studies (19), cell proliferation was significantly reduced in ATIP3-positive clones Cl3 and Cl6 as compared to control (Supplementary Fig.S3A). In addition, Boyden chambers assays of chemotaxis and invasion revealed more than 90% reduction in the pro-migratory properties of Cl3 compared to GFP (Fig.3A). Similar effects were observed using stably transfected MDA-MB-231 cells (Supplementary Fig.S3B). Conversely, ATIP3-silencing in metastatic MDA-MB-468 breast cancer cells expressing endogenous ATIP3 induced a 2 to 2.5-fold increased chemotactic migration (Fig.3B), suggesting that cancer cells having lost ATIP3 may acquire a pro-migratory phenotype and may be more prone to develop distant metastasis.

The ability to migrate through a monolayer of endothelial cells (trans-endothelial migration) was significantly reduced (58±16%) in Cl3 compared to control (Fig.3C). Adhesion of clones Cl3 and Cl6 to endothelial cells was significantly elevated (3-fold and 2.8-fold, respectively) compared to WT (Fig.3D), suggesting that increased tumor-endothelial cell adhesion may account for reduced trans-endothelial migration. Cell adhesion to collagen I was also
increased in Cl3 (1.85-fold) and Cl6 (1.93-fold) compared to WT (Fig.3E). Altogether these data indicate that ATIP3 concomitantly increases cell adhesion and limits cell migration.

The consequences of ATIP3-silencing on cancer cell motility were analyzed in HeLa cells that express endogenous ATIP3 and are well suited for analyses of wound closure. As shown in Fig.3F, ATIP3-silencing in HeLa cells increased (1.84 to 2.6 fold) directional migration. Conversely, stable ATIP3 expression into D3H2LN (Cl3 and Cl6, Fig.3G) and MCF7 cells (Supplementary Fig.S3C) significantly reduced wound closure. Time-lapse microscopy (movies 1 and 2) and tracking of D3H2LN migrating cells further indicated that stable ATIP3 expression impairs both cancer cell velocity (0.34 μm/sec and 0.55 μm/sec for Cl3 and GFP clones, respectively) (Fig.3H) and directionality (Fig.3I). Similar results were obtained (Supplementary Fig.S3D, Fig.S3E) by analyzing cell tracking following transient transfection of GFP or GFP-ATIP3 into D3H2LN cells (movies 3 and 4). Of note, the number of GFP-ATIP3-positive cells reaching the wound edge was reduced compared to GFP-expressing cells. GFP-ATIP3 expressing cells were overtaken by non-transfected cells reaching the border of the wound (Supplementary Fig.S3F), further confirming the inhibitory effect of ATIP3 on cancer cell migration.

**ATIP3 alters microtubule dynamics**

We hypothesized that ATIP3, being closely associated with microtubules (19), may limit cell proliferation and migration by regulating MT dynamics. We first analyzed the consequences of ATIP3 depletion on the sensitivity of MTs to nocodazole, that prevents repolymerization of dynamic MTs. Stable MTs that are not affected by nocodazole treatment are typically stained by anti-acetylated tubulin. As shown in Fig.4A, ATIP3-silenced HeLa cells were highly sensitive to nocodazole. The number of cells retaining stable MTs was decreased by 51%±10 and 53%±14 following transfection of siRNA#1 and siRNA#2 compared to control. Conversely, stable transfection of GFP-ATIP3 into MCF7 cells significantly increased the
number of cells retaining stable, nocodazole-resistant, microtubules as assessed by anti-acetylated tubulin labelling (Fig.4B) and immunoblotting (Fig.4C). ATIP3 expression also significantly delayed MT regrowth following nocodazole washout (Fig.4D). At time 5 min, MT length around the centrosome was reduced by $57\pm20\%$ in GFP-ATIP3 compared to GFP-transfected clones, supporting the notion that ATIP3 may impair MT dynamics.

The effects of ATIP3 on MT dynamic instability parameters were further analyzed by measuring End-binding 1 (EB1) protein accumulation at the MT plus tips (13-15, 28, 29) in RPE-1 epithelial cells and lung fibroblasts (MRC5-SV) which have a sparse MT array and are well suited for distinguishing individual MT tips. As shown in Fig.5A, ATIP3 expression in RPE-1 cells led to a significant reduction in the number and size of EB1 comets that rather appeared as dots. Decreased accumulation of EB1 at MT plus ends was not associated with decreased EB1 expression (Supplementary Fig.S4A). In ATIP3-depleted HeLa cells, significantly more EB1 comets of increased length and intensity were detected compared to control cells (Fig.5B), suggesting that ATIP3 silencing increases MT dynamics. Time-lapse TIRF videomicroscopy analysis of EB1-GFP comets (movie 5) and subsequent MT-tips tracking indicated that MT growth episodes were significantly longer in ATIP3-silenced HeLa cells compared to control (Fig.5C). ATIP3-depletion increased MT growth rate and decreased the time spent in pause as well as the frequency of catastrophes (Fig.5C), accounting for increased MT dynamics. Conversely, videomicroscopy of EB3-GFP comets following expression of mCherry-ATIP3 in MRC5-SV cells (movie 6), and corresponding kymographs (Supplementary Fig.S4B), indicated that ATIP3 expression decreases MT dynamics and reduces the rate of MT growth.

MT stabilization and decreased growth rate at the cell periphery should be responsible for an inhibition of MT targeting and capture at the cell cortex (30). As shown in Fig.5D, in migrating D3H2LN cells MTs projected radially toward the cell periphery and MT plus ends were close to the cell edge (mean distance $1.43 \pm 0.7 \mu m$) whereas in the presence of ATIP3,
MTs were bended and more than 50% of MT tips did not reach the cell margin (mean distance 2.31 ±1.2 μm). Of note, reduced ability of MTs to reach the cell cortex in migrating ATIP3-positive cells was accompanied by a 34% decrease in cell polarity (Fig.5E). Taken together, these results suggest that ATIP3-dependent regulation of MT dynamics results in decreased ability of microtubules to reach the cell cortex, which contributes to reduced cell polarity and migration.

**MT-binding domain D2 recapitulates the functional effects of ATIP3**

The ATIP3 polypeptide was cleaved into three fragments designated D1, D2 and D3 (Fig.6A) which were fused to GFP and expressed in RPE-1 cells (Fig.6B). As shown in Fig.6C, the GFP-D1 fusion protein did not associate with MTs and was rather diffuse in the cytosol. Accordingly, GFP-D1 expression had no significant effect on the number, size or intensity of EB1 comets (Fig.6D). In contrast, GFP-D2 clearly colocalized with the microtubule cytoskeleton and centrosome in living cells (Fig.6C). As for GFP-ATIP3, GFP-D2 was entirely retrieved in the pellet fraction in MT co-sedimentation assays (Supplementary Fig.S5A). Of interest, upon expression of GFP-D2, accumulation of EB1 as comet-like structures at the MT plus ends was strongly impaired (Fig.6E), indicating that expression of the D2 domain is sufficient to stabilize microtubules. Expression of GFP-D3 (Fig.6C) led to the formation of large aggregates containing tubulin, probably due to oligomerization of coiled-coil motifs present in the C-terminal region of ATIP3 (31). Because of these aggregates, functional properties of GFP-D3 could not be evaluated further.

Altogether our results identify D2 as the ATIP3 domain able to associate with MTs and suppress their dynamics. Of importance, the D2 domain also retained the ability of ATIP3 to inhibit cell proliferation (91.6% inhibition for GFP-D2 and GFP-ATIP3 compared to GFP) (Fig.7A). In wound healing assays, cells expressing GFP-D2 showed reduced cell migration and directionality (Fig.7B). Cell tracking of transient transfectants (movie 7) indicated that
similar to GFP-ATIP3, GFP-D2 positive cells mostly remained at the back of the wound and were overtaken by untransfected cells (Fig.7C, Supplementary Fig.S5B). Thus, the MT-binding domain D2 is sufficient to recapitulate the functional features of ATIP3.
Discussion

We report here that ATIP3 is an important prognostic marker for survival of the patients with breast cancer, independently of the ER status of the tumor. Using three different patient cohorts, we show that among metastatic breast tumors, low ATIP3 levels correlate with reduced probability for overall survival of the patients, suggesting that ATIP3 may be an important indicator of metastatic progression. Examination of ATIP3 levels in breast tumors may contribute to identify a population of patients at high risk of fatal complication, who should be the subject of careful medical follow-up.

Using a bioluminescence-based experimental mouse model for cancer metastasis (23, 25, 26), we showed that restoring ATIP3 expression into highly metastatic ATIP3-deficient D3H2LN breast tumor cells significantly delays the time-course of metastasis and reduces the number of detectable metastases per mouse at all times examined. ATIP3 expression in cancer cells also strongly reduces the size of metastatic foci as well as the number of mice fully invaded with large metastases. These observations, together with above-mentioned results on human patients, suggest that ATIP3 may have a prominent effect on metastatic colonization.

Essential steps of metastatic progression include the ability of cancer cells to reach a secondary organ and grow in the new micro-environmental context (3-5). This requires active cell migration and proliferation, two important biological processes that are significantly increased in breast cancer cells following ATIP3-silencing. By promoting dual effects on cancer cell proliferation and migration, ATIP3 likely regulates both early (tumorigenic) and late (metastatic) stages of cancer development. Beneficial actions of ATIP3 on a wide range of cancer-related processes, including invasion, trans-endothelial migration, cell migration and proliferation, may explain its potent anti-metastatic effects in pre-clinical studies.

Other studies have shown that the $MTUS1$ gene encoding ATIP3 is significantly down-regulated in various types of cancers including from the pancreas (32), ovary (33), head-and-neck (34, 35), colon (36) and bladder (37). Low $MTUS1$ levels were also correlated with
reduced overall survival of the patients with bladder cancer (37) and oral tongue squamous cell carcinomas (35), highlighting the potential importance of ATIP3 as a new prognostic marker in a variety of solid tumors.

At the molecular level, we show that ATIP3 is a microtubule-associated protein with potent MT-stabilizing effects. We propose that by stabilizing MTs, ATIP3 decreases their dynamics therefore leading to impaired ability of MT tips to reach the cell cortex during migration. MT dynamics at the cell cortex is essential for generating a polarized MT array, required for cell polarity and migration (30). Reduced MT dynamics may thus represent a major mechanism accounting for anti-migratory and anti-metastatic effects of ATIP3 in breast cancer. Accordingly, loss of ATIP3 leads to increased MT growth rate, less time spent in pause and decreased frequency of catastrophes. Alteration of MT dynamics parameters in ATIP3-depleted cells may explain uncontrolled cancer cell motility that is associated with increased metastasis and poor prognosis in ATIP3-negative breast cancer patients.

The association of ATIP3 with the MT lattice involves an internal basic region designated D2 whose expression is sufficient to recapitulate all effects of the full-length protein on MT stabilization, as well as cell proliferation, motility and directional migration. The MT-binding D2 region thus represents the functional domain of ATIP3. Further characterization of this domain and identification of intracellular interacting partners may help deciphering the molecular mechanisms by which ATIP3 limits breast cancer cell migration and hence, metastasis. Our study paves the way to the design of peptides or small molecules able to mimic the effects of ATIP3, which is a pre-requisite for the development of targeted therapy. These may be particularly beneficial to the subset of breast tumors that have lost ATIP3 expression and are prone to metastasize.
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References


Figure Legends

Figure 1. Low levels of ATIP3 predict poor outcome among metastatic tumors

(A) Overall survival (OS) curves for patients from the Institut Curie cohort, with tumors expressing normal (>0.5) or low (<0.3) ATIP3 levels, relative to the median value of MTUS1 probeset (212096_s_at) in normal tissues. (B) OS curves for patients from the IGR cohort, with tumors expressing normal or low ATIP3 levels (inferior or superior to the median value of MTUS1 probeset (A_23_P347169) intensities in the 162 tumors analyzed). (C) OS curves for patients with tumors expressing normal to high (grey) or low (black) MTUS1 (212096_s_at) using Kaplan-Meier plotter. The best performing threshold was used as a cut off. (D) OS curves from patients expressing normal or low ATIP3 as in (A), among ER negative (ER-, left) and positive (ER+, right) tumors. (E) Percentage of patients remaining alive after 5-years with non-metastatic (-) and metastatic (+) tumors expressing ATIP3 levels as in (A). (F) OS curves for patients with non metastatic (left) or metastatic (right) tumors expressing ATIP3 levels as in (A). (G) OS time (in months) for patients with metastatic tumors expressing ATIP3 levels as in (A). Median values are on the right. *p=0.0119. (H) OS curves as in (F) for patients from the IGR cohort. Non-metastatic (left) and metastatic (right) tumors were classified according to ATIP3 levels as in (B). (I) OS time (in months) for patients as in (G) with metastatic tumors classified as in (H). Number of patients is under brackets. *p=0.0172.

Figure 2. ATIP3 expression slows metastatic progression in vivo

(A) Characterization of stably transfected D3H2LN cell clones. Left: Immunoblots of non-transfected (WT) and GFP-ATIP3-expressing D3H2LN clones (Cl3, Cl6) using anti-GFP antibodies, reprobed with anti-α-tubulin (α-tub) antibodies. Right: Measurement of luciferase activity per cell (n=3). (B) Number of photons/sec per mouse (n=9) at days 6 to 24 following
tumor cell inoculation. **C**) Left: Representative pictures of bioluminescence (5 out of 9 mice) at day 17 following intra-cardiac injection. Scale is on the right. **Right:** Number of mice with at least one detectable metastasis at day 17. **D**) Total number of metastatic sites per mouse at indicated days after inoculation of control (Ctrl: WT and GFP, n=18) and ATIP3-positive (ATIP3: Cl3 and Cl6, n=18) cells. **p<0.01, ***p<0.001. **E**) Number of mice with large metastases at different times after inoculation as in (D). **F**) Representative pictures (day 24) as in (C).

**Figure 3. ATIP3 reduces breast cancer cell migration**

**A**) Boyden chamber migration of stable D3H2LN cell clones across filters coated (coll) or not (no coll) with collagen I. Results (percent) are mean±SEM (n=3). **Right:** Representative picture of cells migrating to the bottom of the well. **B**) Boyden chamber assay using ATIP3-positive (WT and siCtrl) and ATIP3-negative (si#1 and si#2) MDA-MB-468 cells. Results are shown as in (A). **Upper:** Immunoblots of MDA-MB-468 cells after siRNA silencing (anti-MTUS1, reprobed with anti-α-tubulin (α-tub) antibodies). **Right:** Representative pictures of the lower face of the filter. **C**) Trans-endothelial migration, mean±SEM (n=3). **D and E**) Cancer cell adhesion (mean±SEM, n=3) to endothelial cells (D) and collagen (E). **F**) Migration of ATIP3-positive (siCtrl) and ATIP3-silenced (si#1, si#2) HeLa cells (n=3). **Left:** Representative pictures of wound at times T0 and T22. **Right:** Quantification (percent) of wound closure at T22. Results are mean±SEM (n=2). Upper: Immunoblots of siRNA-transfected HeLa cells as in (B). **G**) Directional migration of stably transfected D3H2LN clones (n=4). **Left:** Representative pictures of wound at times T0 and T7. **Right:** Quantification of wound closure at T4 and T7. Results are mean±SD (n=3). **H and I**) Cell tracking of D3H2LN stable clones during wound closure. **H**) Cell velocity scattered dot plot. **I**) Diagrams of migration trajectories (12 hrs). Number of cells is under brackets.
Directionality coefficient (Dir) is inside the graph. (A, B, F, G) Magnification x100. *p<0.05, **p<0.001, ***p<0.0001.

**Figure 4. ATIP3 reduces nocodazole sensitivity and microtubule outgrowth**

(A) Immunostaining (anti-α-tubulin (α-tub) and anti-acetylated tubulin (Ac-tub) antibodies) of ATIP3 positive (siCtrl) and negative (si#1) HeLa cells incubated without (0) or with 1μM nocodazole (Nz). **Right:** Immunoblots of HeLa cells after siRNA silencing (anti-MTUS1, reprobed with anti-tubulin antibodies). **Lower:** Quantification (%) of cells retaining stable MTs, mean±SEM (n=3). (B) Immunostaining of stable MCF7 clones incubated with or without 10μM nocodazole, as in (A). **Right:** Quantification as in (A), mean±SEM (n=3). (C) Immunoblot analysis of acetylated-tubulin (Ac-tub) and ezrin content in stably transfected MCF7 clones, either non-treated (-) or treated with DMSO (D) or increasing concentrations of nocodazole. **Right:** Quantification of the ratio between Ac-tub and ezrin intensity. (D) MT regrowth in transiently transfected RPE-1 cells (n=4). Shown is α-tubulin staining at indicated times after nocodazole (10μM) washout. **Right:** Quantification of MT density at 4 μm around the centrosome, mean±SD (n= 4 to 10 cells). *p<0.05. **p<0.001. Scale bar: 10 μm.

**Figure 5. ATIP3 regulates MT dynamics**

(A) Immunostaining (anti-EB1, anti-mCherry antibodies) of RPE-1 cells transiently transfected with mCherry-ATIP3 (Ch-ATIP3). Insets: EB1 comet-like structures in ATIP3-negative (1) and positive (2) cells. Distribution of EB1 (black), α-tubulin (dashed) and ATIP3 (grey) at the MT tip (linescans) and quantification of comets intensity (scattered dot plot). Number of comets analyzed is under brackets. Shown is one experiment out of 5. (B) EB1 localization in siRNA-silenced HeLa cells. Insets: EB1 comet-like structures in ATIP3-positive (1) and ATIP3-negative (2) cells. Distribution of EB1 (black) and α-tubulin (dashed)
at the MT tip (linescans). Quantification of comets intensity as in (A). Shown is one experiment out of 3. (C) Time-lapse images of siRNA-silenced HeLa cells expressing EB1-GFP. Arrowhead indicates the position of EB1 comet over time (in seconds). Parameters of MT dynamics (EB1-GFP comets) in siRNA-transfected HeLa cells (n=100 comets) are shown in scattered dot plot and histograms. (D) Immunostaining (anti-α-tubulin (α-tub) and anti-GFP) of transfected D3H2LN in migration. Arrows indicate the direction of migration. Cell margin (black line) is visualized by bright field microscopy. Insets show MT array at the cell border of ATIP3-negative (1, 2) and GFP-ATIP3-positive (3, 4) cells. **Right:** Immunoblots (anti-GFP, anti-tubulin) of transfected D3H2LN cells. **Lower left:** Quantification of MTs reaching given distance from the cell cortex in GFP- and GFP-ATIP3- expressing cells. **Lower right:** Mean distance between MTs and cell cortex. Number of MTs analyzed is under brackets. (E). Quantification (percent) of polarized D3H2LN cells during migration. Number of cells is under brackets. *p<0.05, **p<0.001, ***p<0.0001. (A, B, C, D) Scale bar: 10 μm.

**Figure 6. The D2 region of ATIP3 decorates and stabilizes MTs**

(A) Scheme of ATIP3 regions D1, D2 and D3. Amino acid numbering is according to accession number NP_001001924. (B) Immunoblots (anti-GFP, anti-tubulin) of RPE-1 cells transfected (24 hr) with GFP-D1, GFP-D2 and GFP-D3. (C) Immunostaining (anti-GFP, anti-tubulin) of RPE-1 cells transiently transfected as in (A). (D) Anti-EB1 immunostaining of GFP-D1-transfected RPE-1 cells. Insets show EB1 comets in GFP-D1-negative (1) and GFP-D1-positive (2) cells. Distribution of EB1 (blue), α-tubulin (pink) and GFP-D1 (green) at the MT tip (linescans) and quantification of comets intensity (scattered dot plot). Number of comets is under brackets. (E) GFP-D2-transfected RPE-1 cells stained with anti-EB1 antibodies and analyzed as in (D). ***p<0.0001. (B, C, D) Scale bar represents 10 μm.
Figure 7. MT-binding domain D2 is the functional domain of ATIP3

(A). Colony formation of GFP-, GFP-ATIP3-, GFP-D2- transfected MCF7 cells and quantification (mean±SD, n=4). Shown is one representative experiment out of 3. (B) Migration trajectories (17 hrs) covered by GFP- (n=30), GFP-ATIP3- (n=17), and GFP-D2- (n=24) expressing D3H2LN cells. Directionality coefficient (Dir) is inside the graph. (C) Aligned dot plots show Euclidean distance covered by untransfected (-) and transfected cells (+) cells as indicated. *p<0.05, **p<0.001, ***p<0.0001.
Figure 1 from Molina et al.:

A. Patients with ER tumors
B. Patients with ER+ tumors
C. ATIP3 level
D. Patients without metastasis
E. Overall survival
F. Patients with metastasis

Percent OS over time for different groups, with statistical significance indicated (P-values) for each comparison.
Figure 2: Metastasis Model

A. GFP-ATIP3 expression in WT, CI3, and CI6 cells.

B. Photons/sec per cell (x10^6) for WT, GFP, CI3, and CI6 cells at different days (10, 17, 24).

C. Number of mice with metastasis at day 17 for WT, GFP, CI3, and CI6.

D. Number of metastases per mouse for Ctrl and ATIP3 at different days (10, 17, 20, 24).

E. Number of mice with metastatic burden (>10^8 photons/sec) for Ctrl and ATIP3 at different days (10, 17, 20, 22, 24).

F. Imaging of WT, GFP, CI3, and CI6 mice at different days.

Molina et al, Figure 2
Molina et al, Figure 3
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Molina et al, Figure 6
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