Ada3 Requirement for HAT Recruitment to Estrogen Receptors and Estrogen-Dependent Breast Cancer Cell Proliferation

Aleksandra Germaniuk-Kurowska,1,3 Alo Nag,1,3 Xiangshan Zhao,1,3 Manjari Dimri,2,3 Hamid Band,2,3,4 and Vimla Band1

Abstract

We have previously shown that evolutionarily conserved alteration/deficiency in activation (Ada) protein associates with and promotes estrogen receptor (ER)–mediated target gene expression. Here, we examined the role of endogenous Ada3 to recruit histone acetyl transferases (HAT) to an ER-responsive promoter and its role in estrogen-dependent cell proliferation and malignant phenotype. Using a combination of glycerol gradient cosedimentation and immunoprecipitation analyses, we show that Ada3, ER, and three distinct HATs [p300, (p300/CBP-associated factor) PCAF, and general control nonrepressed 5 (Gcn5)] are present in a complex. Using chromatin immunoprecipitation analysis, we show that short hairpin RNA (shRNA)–mediated knockdown of Ada3 in ER-positive breast cancer cells significantly reduced the ligand-dependent recruitment of p300, PCAF, and Gcn5 to the ER-responsive pS2 promoter. Finally, we use shRNA knockdown to show that Ada3 is critical for estrogen-dependent proliferation of ER-positive breast cancer cell lines in two-dimensional, as well as three-dimensional, culture. Knockdown of Ada3 in ER-positive MCF-7 cells induced reversion of the transformed phenotype in three-dimensional culture. Thus, our results show an important role of Ada3 in HAT recruitment to estrogen-responsive target gene promoters and for estrogen-dependent proliferation of breast cancer cells. [Cancer Res 2007;67(24):11789–97]

Introduction

Estrogens play pivotal functional roles in the development, differentiation, and oncogenesis in female reproductive organs, such as the mammary gland and uterus (1, 2). The effects of estrogens are mediated through estrogen receptor α (ERα) and ERβ, members of the nuclear hormone receptor (NR) family. These receptors serve as sequence-specific transcriptional regulators by binding to specific estrogen-responsive elements (ERE) within target gene promoters (3, 4). In the absence of bound ligands, NRs repress target gene transcription by associating with histone deacetylase–containing corepressor complexes. Ligand binding triggers the release of corepressors and subsequent association with coactivator complexes that are needed for crucial steps in gene transcription, including chromatin modification, remodeling, and recruitment of the RNA polymerase II holoenzyme (reviewed in refs. 4–6).

Two groups of NR coactivators have been widely studied: (a) the p300 and the highly related cAMP-responsive element binding protein–binding protein (CBP) and (b) the mammalian Mediator complexes (TRAP, DRIP, ARC, CRSP, SMCC, etc.; refs. 4–6). The p300 and CBP proteins are recruited to ligand-activated, DNA-bound NRs by the steroid receptor coactivator (SRC) family (SRC-1, SRC-2, and SRC-3) of bridging factors (4–6). The SRC proteins have receptor interaction domains containing LXXLL motifs that contact the ligand-binding domains (7, 8). The p300 and CBP proteins are histone acetyltransferases (HAT) that mediate the acetylation of nucleosomal histones, a covalent modification generally associated with the enhancement of transcription. Furthermore, the HAT-dependent acetylation of transcriptional activators themselves promotes their activity (9).

Recent studies have identified an evolutionarily conserved coactivator complex as a potential regulator of ER and other NRs. Initially identified through genetic studies in yeast, the core components of the alteration/deficiency in activation (Ada) complex include the adapter proteins Ada3 and Ada2 and general control nonrepressed 5 (Gcn5), a HAT (10). Mammalian Ada3 exists as a component of a yeast Ada-like complex that includes Ada2 and Gcn5, indicating evolutionary conservation of its function (11). Initial studies of mammalian retinoic X receptor (RXR) and GR expressed in the yeast indicated a requirement of yeast Ada complex, including the yAda3 gene product, for transcriptional activation (12). We and others have shown in mammalian cell systems that Ada3 indeed interacts with NRs, including RXR and ER (13, 14), as well as some non-NR transcriptional factors, such as p53 (15–17). Notably, overexpression of Ada3 enhanced ER-mediated target gene expression, whereas down-regulation of its expression had an opposite effect (14). These studies have raised the possibility that Ada3, as a component of ER coactivator complexes, may control ER-mediated biological outputs in mammalian cells. However, whether Ada3 at physiologically expressed levels indeed functions in this capacity is not known. This issue is particularly important because other multiple adaptor proteins, such as SRCs (see above), have been previously shown to control ER function in mammalian cells (1–6, 18). Furthermore, mammalian cells express Gcn5, as well as closely related HAT, called p300/CBP-associated factor (PCAF; ref. 19); the known interaction of PCAF with p300 might suggest that Ada3 may not play an important role in ER function. Alternatively, the ability of PCAF and apparently Gcn5 to interact with Ada3, as well as p300, might suggest that these proteins may function in concert and that Ada3 may play a crucial role in their...
retrieval to ligand-bound ER on target gene promoters and subsequent functional responses.

In this study, we show that endogenously expressed Ada3 is present in a complex that includes ER and multiple HATs (p300, Gcn5, and PCAF) and that Ada3 is required for the recruitment of these HATs to an ER target gene promoter in the context of intact chromatin. We also show Ada3 is required for ER-mediated proliferation of breast cancer cells in two-dimensional, as well as three-dimensional, cultures, with a reversal of the transformed phenotype in three-dimensional cultures of MCF-7 cells upon Ada3 knockdown. These studies underscore a crucial role of Ada3 in orchestrating ER-mediated gene transcription, as well as downstream biological outputs in the context of breast cancer cells.

Materials and Methods

Cells and media. The ER-positive human breast cancer cell lines MCF-7 and ZR-75-1 were grown in α-MEM medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS). For some experiments, the ER-positive cell lines were cultured for 72 h in a phenol red–free medium (Hyclone) supplemented with 10% charcoal/dextran-treated FBS (Hyclone) to remove residual 17α-estradiol (E2).

Antibodies. Generation of anti-hAda3 rabbit polyclonal antiserum has been described previously (15). Antibodies against p53 (DO1), ERα, p300, PCAF, Gcn5, and β-actin were purchased from Santa Cruz Biotechnology, Inc. Anti-Flag–horseradish peroxidase (HRP) antibodies were from Sigma. β-Actin antibodies were from Abcam, Inc.

Generation of stable Ada3 short hairpin RNA knockdown cells. The hAda3-specific RNA interference sequences used in short hairpin RNA (shRNA) constructs are GCATCAGAAACGCCCTT (#1) and GGTGACAGCACCTTCTGCA (#2). The oligonucleotides were cloned in the pSUPER-Retro vector (OligoEngine). MCF-7 and ZR-75-1 cells were infected with hAda3 RNA interference retroviral supernatants as described previously (14, 17). Virally transduced cells were selected and maintained in 0.5 µg/mL puromycin for 72 h, and expression of endogenous hAda3 was assessed in the whole-cell lysate using anti-hAda3 polyclonal antibody followed by Western blotting.

Cell proliferation assay. Cells (2 × 10⁴) from MCF-7 or ZR-75-1, stably expressing scrambled shRNA or two independent shRNA against hAda3, were plated in six-well plates for growth analyses. Cells were seeded in regular α-MEM medium supplemented with 0.5 µg/mL of puromycin. After 24 h, medium was replaced with phenol red–free α-medium (plus 0.5 µg/mL puromycin) to remove residual E2 and, after that, treated with either vehicle or 100 pmol/L E2. The cells were trypsinized and counted at the indicated time points.

Three-dimensional culture on Matrigel. For the three-dimensional culture on Matrigel, the procedure was essentially as described previously (20, 21) with some modifications. Briefly, single-cell suspensions were prepared by trypsinization and 2.5 × 10⁵ cells in 0.4 mL of 2% Matrigel (phenol red–free Matrigel from BD Biosciences) in phenol red–free α-medium. For stable cells containing shRNAs, medium was supplemented with 0.5 µg/mL puromycin. Cells were plated in eight-well chamber slides (BD Biosciences) on top of a 40-µL polymerized layer of 100% Matrigel. The cultures were fed every 3 days. For some samples after E2 deprivation, 100 pmol/L E2 was included in the feeding medium through the end of experiment. Phase contrast images were documented periodically during the entire growth period.

Separation of native hAda3-associated complexes. MCF-7 cells were cultured for 72 h in phenol red–free α-medium (Hyclone) to remove all residual E2 and, after that, treated for 24 h with either vehicle or 100 pmol/L E2. Whole-cell extracts (~4 mg of protein) were dialyzed 3 h at 4°C against buffer containing 50 mmol/L HEPEs (pH 7.9), 50 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). The dialyzed material was centrifuged to remove all insoluble material and then layered onto 9 mL of a 10% to 30% linear glycerol gradient and centrifuged for 18 h at 41,000 rpm at 4°C using a TH641 rotor (Sorvall). Gradients were fractionated by collecting 360 µL aliquots. Fractions were trichloracetic acid precipitated and then analyzed by Western blotting using specific antibodies, as indicated in the figure. Additionally, high molecular weight markers (HMW; Sigma) were centrifuged on the separate gradients, and one of five from every fraction was separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. This allowed the calculation of the apparent molecular weights corresponding to particular fractions of the gradient.

Chromatin immuno precipitation analysis. The chromatin immuno-precipitation (ChIP) assay was performed as described previously (22). In brief, MCF-7 cells were cultured in phenol red–free medium (Hyclone) for 3 days followed by either mock treatment or 10 nmol/L E2 for 30 min. After that, cells were treated with 1% formaldehyde to cross-link the DNA-protein complexes. The cross-linking was quenched by adding glycin solution. Cells were washed with cold PBS. A small portion of the cross-linked, sheared chromatin solution was saved as input DNA (5%), and the remainder was used for immunoprecipitation with anti-ERα antibody (Ab-10, Neo Marker), anti-p300 (RW128, Upstate), anti-RNA polymerase II (8WG16, Abcam), anti-PCAF (E-8, Santa Cruz), anti-Gcn5 (H-75, Santa Cruz), and hAda3 (15). Immunoprecipitated DNA was deproteinized by phenol-chloroform extraction, precipitated by ethanol, and resuspended in TE buffer. PCR amplifications were performed with primers covering pS2 promoter (region from −353 to −30): forward primer 5'-GGCCACTCTCCTGCTTCGAGTC-3' and reverse primer 5'-GGCCAGGGCTGTTCTGTTAAAGAGGC-3'. Control PCR was performed using primers covering the region upstream to pS2 promoter (region from −679 to −501): forward primer 5'-GTGATCTCCGACTTAAACG-3' and reverse primer 5'-TGCGCCACTGGTCCTACCGTG-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as control (14).

Immunoblotting and immunoprecipitation. For immunoblotting, 80 µg of cell lysate was fractionated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antibodies. Enhanced chemiluminescence reagents were used for signal detection. For immunoprecipitation experiments, MCF-7 cells were maintained in a phenol red–free α-medium supplemented with 10% charcoal/dextran-treated FBS (Hyclone) for 72 h and then treated with either 100 pmol/L E2 or vehicle for 24 h.

Results

Ada3 cosediments with multiple HATs upon E2 treatment of breast cancer cell lines. Whereas yeast Ada3 functions as a component of the ADA complex with Gcn5 as the HAT (23), mammalian Ada3 was found to associate with p300 (16, 17) and was present in the same complex as PCAF in a cervical cancer cell line (24). Thus, Ada3 may form multiple independent coactivator complexes with various HATs or a larger complex containing multiple HATs. We used cosedimentation analysis on glycerol gradients to isolate intact Ada3-containing complexes from untreated and E2-treated breast cancer cell line MCF-7 to address this issue. Cells were lysed under mild conditions (50 mmol/L NaCl) to ensure that even potentially weak protein–protein interactions remained intact. Equal amounts of protein extracts were subjected to glycerol gradient centrifugation and Ada3-containing complexes were identified by Western immunoblotting of individual fractions. Internal standards were used to determine the relative molecular sizes of isolated complexes.

In estrogen-deprived MCF-7 cells, the majority of Ada3 was found in fractions 11 to 14 (Fig. L1–D). Upon E2 treatment, the Ada3 protein showed a shift toward higher molecular weight fractions (fractions 13–16), suggestive of association with additional proteins. As expected, a proportion of the ER protein, as well as the Ada3-binding partner p53, was also present in the same fractions as Ada3 (Fig. L1–D), confirming our previous studies (15, 17). Whereas p53 remained relatively unchanged in its location in the gradients, the ER peak shifted into higher molecular weight fractions (fractions 11–14 versus fractions 13–18).

Downloaded from cancerres.aacrjournals.org on April 20, 2017. © 2007 American Association for Cancer Research.
We also immunoblotted the fractionated proteins to determine the location of potential HAT partners of Ada3: p300, Gcn5, and PCAF. Each of these HATs showed partial cosedimentation with Ada3, which, with PCAF and Gcn5, was relatively modest before E2 treatment (Fig. 1A); however, the cosedimentation was more clearly observed upon E2 treatment (Fig. 1B), consistent with their incorporation into an Ada3-containing complex. The peak p300 fractions did not show a significant shift upon E2 treatment; however, we have frequently noticed increased intensity of p300 bands in E2 versus untreated cell fractions (Fig. 1). Cosedimentation of the three HAT proteins (p300, Gcn5, and PCAF), as well as the ER, with Ada3 and their shift into overlapping fraction upon E2 treatment supports the idea that Ada3 is present in a complex with these proteins.

**Figure 1.** Biochemical fractionation of native Ada3-containing complexes in breast cells. Whole-cell extracts from MCF-7, either vehicle-treated (A) or treated with 100 pmol/L of E2 for 24 h (B; ~4 mg of protein), as well as HMW (Sigma), were dialyzed 3 h at 4°C against buffer X (50 mmol/L HEPES (pH 7.9), 50 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mmol/L PMSF) and then layered on top of 9 mL of a 10% to 30% linear glycerol gradient and centrifuged O/N at 41,000 rpm at 4°C, using a TH641 rotor (Sorvall). Gradients were fractionated by collecting 360 μL aliquots from the top of the gradients. Fractions were TCA precipitated and then analyzed by Western blotting using specific antibodies, as indicated in the figure. One fifth from every fraction of the separated HMW markers was separated by 10% SDS-PAGE and stained with Coomasie Brilliant Blue (data not shown). This allowed the calculation of the apparent molecular weight corresponding to particular fractions of the gradient. The intensity of the bands in particular fractions of the gradients of cells, either vehicle-treated (C) or treated with 100 pmol/L of E2 for 24 h (D), was quantified by densitometry using Scion Image Software. The graphs show the intensity of the bands (Y axis) versus fraction number (X axis). The experiments were repeated at least thrice.
Ada3 associates with PCAF, Gcn5, and p300 in breast cancer cell lines. To further substantiate the association of Ada3 with various HATs, we performed coimmunoprecipitation analyses using lysates of untreated or E2-treated MCF-7 cells expressing a Flag-Ada3. Ectopically expressed Flag-Ada3 was used for these analyses to overcome the uncertainty about the efficiency of immunoprecipitation because of the comigration of endogenous Ada3 with the heavy chain of immunoprecipitation antibody (data not shown). Anti-Flag immunoprecipitation followed by anti-Flag-HRP immunoblotting showed efficient immunoprecipitation of Ada3 protein (Fig. 2). As expected, anti-ER immunoblotting showed a clear association between Ada3 and ER (Fig. 2). Notably, all three HATs, p300, PCAF, and Gcn5, were also coimmunoprecipitated with the Ada3 protein. These results complement the cosedimentation analyses, indicating that Ada3 associates with all three HATs in addition to the ER.

Ligand-dependent recruitment of Ada3 and HATs to an ER target gene promoter. Given the critical role of coactivator recruitment to transcriptional activators bound to target gene promoters (25) and our previous findings that Ada3 is recruited to native estrogen-responsive promoter pS2 (14), we performed ChIP analysis of untreated and E2-treated MCF-7 cell chromatin extracts to assess the corecruitment of Ada3 and HATs on pS2 promoter. As expected, both ER and Ada3 were found to be bound to pS2 promoter, and treatment with E2 led to a significant increase in the pS2 promoter occupancy by both ERα and Ada3 (Fig. 3). Parallel ChIP analysis of HATs showed that p300, Gcn5, and PCAF are recruited to the pS2 promoter upon E2 treatment (Fig. 3); concurrently, we also observed that RNA polymerase II was present in ChIPs, as anticipated (26). No significant signals were detected from either the preimmune serum or mouse or rabbit IgG (Fig. 3). Control PCR analysis of a region upstream of the ERE element in the pS2 promoter and GAPDH promoter also showed no detectable signals (Fig. 3). These results clearly indicated that all three HATs examined are recruited to the pS2 promoter in addition to Ada3 and ER and raised the possibility that Ada3 may play a role in the recruitment process (addressed below).

Ada3 is required for optimal association of HATs and ERα to the pS2 promoter in a native chromatin context. As Ada3 serves as an adapter within the ADA complexes, we surmised that it might play a role in Gcn5 recruitment to ER target promoters; as the mechanisms of PCAF and p300 recruitment to ER target promoter remain to be determined, we also asked if Ada3 plays any role in their recruitment. For this purpose, we generated MCF-7 cells stably expressing either a scrambled shRNA or two independent shRNAs against Ada3. Anti-Ada3 immunoblotting of cell lysates showed the specific knockdown of Ada3 in Ada3 shRNA but not in control shRNA-expressing cells (Fig. 4). Extracts of untreated or E2-treated cells were then subjected to ChIP analyses to assess recruitment of various proteins to pS2 promoter. MCF-7 cells expressing the scrambled shRNA control showed the anticipated ligand-promoted recruitment of Ada3, ERα, p300, PCAF, Gcn5, and RNA polymerase II to the pS2 promoter with no signals in negative control lanes (preimmune serum, mouse or rabbit IgG, or PCR of upstream region in pS2 promoter or GAPDH promoter; Fig. 5). Notably, ChIPs of cells expressing shRNAs against hAda3 showed a substantial decrease in the recruitment of ER and all three HATs to the pS2 promoter compared with cells expressing the control shRNA (Fig. 5). These results suggest that endogenous Ada3 plays an essential role in the recruitment of all three HATs examined to an ER target promoter. Interestingly, Ada3 knockdown also resulted in reduced ChIP with anti-ER antibodies, indicating that Ada3 also contributes to the stability of ER association with a target promoter in native chromatin context.

Ada3 is required for ER-dependent cell proliferation. In view of the critical role of endogenous Ada3 in the recruitment of HATs (and apparently to the stability of ER occupancy) to an ER target promoter, as revealed by ChIP analyses of Ada3 knockdown cells, we hypothesized that Ada3 will be required for downstream functional consequences of ER-dependent transcriptional activation. Therefore, we compared the level of E2-induced cell proliferation in control versus Ada3 shRNA-expressing MCF-7 cells, as well as a similar pair of another ER-positive breast cancer cell line, ZR-75-1. Western blot analysis of cell lysates showed the specific knockdown of Ada3 protein in Ada3 shRNA-expressing cells compared with scrambled shRNA-expressing cells, with more substantial knockdown with Ada3 shRNA #2 versus #1 (Fig. 4). Assessment of cell proliferation in response to E2 over time showed the expected E2-induced proliferation of control shRNA-expressing MCF-7 and ZR-75-1 cells scrambled shRNA, whereas no significant proliferation was observed in the absence of E2 (Fig. 4). In contrast, cells with Ada3 knockdown showed a substantial reduction in E2-induced cell proliferation at all times; the inhibition was more marked (~50% reduction at day 7) in cells expressing shRNA #2, in which a higher degree of knockdown was observed compared with those expressing shRNA #1 (~25% reduction at day 7; Fig. 4). These results underscore the important role of Ada3 in estrogen-dependent cell proliferation.

Essential role of Ada3 in estrogen-dependent malignant phenotype of breast cancer cells in three-dimensional Matrigel culture. Compared with the conventional two-dimensional tissue culture system, the Matrigel-supported three-dimensional culture system allows analysis of normal and cancerous breast epithelial...
cell proliferation that more closely mimics that observed in vivo (27). We therefore assessed the role of endogenous Ada3 in the E2-dependent proliferation of MCF-7 cells in three-dimensional culture. For this purpose, MCF-7 cells expressing scrambled or Ada3 shRNA were cultured for 4 days in phenol red–free Matrigel, using previously established methods (28), followed by feeding with medium containing E2 (100 pmol/L) for a period of 2 weeks. In the absence of exogenous E2, neither the control nor the Ada3 knockdown cells displayed any proliferation (data not shown). However, when E2 was included in the growth medium, the control shRNA-expressing cells formed large irregular colonies typical of breast cancer cell growth on Matrigel (Fig. 6A; refs. 20, 21, 27). In contrast, MCF-7 cell lines expressing two independent shRNAs developed markedly smaller structures (Fig. 6A). The extent of reduction in colony size was quantified by enumerating small (0.004–0.01 cm²), medium (0.01–0.3 cm²), or large (0.3 cm² or more) colonies. Whereas parental and control shRNA-expressing MCF-7 cells form predominantly large and medium-sized colonies, those with Ada3 shRNA form primarily small colonies (Fig. 6B). Notably, the colonies formed by Ada3 knockdown MCF-7 cells were more regular and acinus-like, similar to those reported by the Bissell laboratory for other treatments that induce a reversal of the malignant phenotype of breast cancer cells on Matrigel (Fig. 6A; ref. 29). These results provide evidence that Ada3 protein is required for E2-dependent proliferation under three-dimensional growth conditions that closely mimic in vivo cell proliferation.

Discussion

Estrogens play crucial roles in the development and function of various female reproductive tissues and are critically important in human cancer (1, 2). For example, nearly 70% to 80% breast cancers are ER-positive and antiestrogens, and aromatase inhibitors are now used as a first line of treatment for such patients (1, 2, 30). Similar to NRs in general, the ability of estrogens to serve as activating switches to turn on ER-responsive genes and the ensuing control of cellular functions requires coregulatory proteins; corepressors keep ER targets repressed, whereas coactivators promote target gene expression (5–8). The latter is mediated through the recruitment of HATs, which allow chromatin remodeling required for optimal transcriptional activation (5–8, 25). Thus far, the best characterized ER coactivators are members of the SRC family, which have been linked to the recruitment of HAT proteins p300 and PCAF (5–8, 31–34). We have previously identified Ada3, an evolutionarily conserved component of ADA3 coactivator complex, as a coactivator of ER-mediated target gene expression.
Here, we show that Ada3 associates with multiple HATs (p300, PCAF, and GCN5) and that endogenous Ada3 in human ER-positive breast cancer cell lines is essential for the recruitment to ER target gene promoters, suggesting its role as a key organizer of HAT recruitment and ER-dependent transcription. We further show that Ada3 is essential for ER-dependent functional responses, as shown by a marked inhibition of estrogen-dependent proliferation of human breast cancer cell lines with Ada3 knockdown and reversal of the malignant behavior of such cells when grown in three-dimensional culture on Matrigel. Thus, our results suggest a critical role of Ada3 as a regulator of ER function in the context of human ER-positive breast cancer cell proliferation and malignant phenotype.

It is noteworthy that estrogen-regulated gene transcription, proliferation, and oncogenesis in mammary cell systems require members of the SRC family (31–34). For example, estrogen-dependent proliferation of MCF-7 cells was reduced upon SRC-3 depletion (35). Similarly, depletion of SRC-1 or SRC-2 inhibit the...
expression of estrogen-responsive genes and estrogen-dependent cell proliferation (32, 33). Furthermore, SRC-3 deletion suppressed MMTV-v-Ha-ras transgene-induced mammary tumorigenesis in mice (34). Complementing these studies development and function of mammary gland, as well as other female reproductive tissues, is affected in SRC-1
/C0
/C0
/C0
, SRC-2
/C0
/C0
/C0
, or Src-3
/C0
/C0
/C0
 mice (32–35). Thus, the requirement for endogenous Ada3 for HAT recruitment to ER target gene promoters and, in ER-dependent functional responses, functions ascribed to SRC proteins was therefore surprising. Several potential models can explain why both Ada3 and SRC proteins may be required to promote ER-dependent gene expression and functional responses. One possibility is that these proteins play similar but redundant roles. Second, it is possible that a multiple coactivator protein play temporally distinct roles, accounting for their obligatory, rather than redundant, roles. In this regard, p300 together with SRC-3 was found to be rapidly and transiently (15–30 min after E2 addition) associated with ER-responsive CATD promoter, whereas PCAF was found associated much later (45 min; ref. 36). Finally, it is possible that Ada3 and SRC proteins are components of a supramolecular coactivator complex and loss of either protein induces loss of functional complex. Using glycerol gradient cosedimentation and immunoprecipitation analyses, we showed

Figure 6. Ada3 regulates estrogen-dependent proliferation of breast cancer cells in three-dimensional cultures and induces reversion phenotype. A, MCF-7 cells stably expressing either scrambled shRNA or two independent shRNAs against hAda3 were grown in phenol red-free Matrigel in three-dimensional tissue culture system in the presence or absence of 100 pmol/L E2. After 16 days of growth incubation, the images were taken under phase microscopy with either 4× or 15× objective. B, at least 200 acinar structures from different fields were counted and plotted as a percentage of total counted. Colony size was measured using the NIH Image J 1.34 program and was defined by size as small (0.004–0.01 cm²), medium (0.01–0.3 cm²), or large (0.3 cm² or more).
that Ada3 is present in the same complex as ER, as well as three HATs (p300, PCAF, and GCN5), consistent with the model that Ada3 is a critical component of a large complex (composed of Ada3, Ada2, GCN5, p300, and PCAF), which plays an essential role in transcriptional activation by ER and potentially other transcriptional activators. Future studies using combinatorial knockdown in cells and compound mouse knockouts should help test these models.

Ada3 was initially identified as a core component of the yeast ADA coactivator complex that includes an adaptor protein Ada2, which in turn associates with a HAT protein GCN5 (37). Ada3 interacts directly with the activation domains of transcriptional complexes, thus serving as a key component in the recruitment of Ada complex and its associated HAT activity to transcriptional activators bound to specific promoters. Given our current observations that Ada3 facilitates the recruitment of multiple HATs, including GCN5, to ER target promoters, and recent studies indicating that Ada3 (and other Ada components) assemble into multiple complexes that can vary significantly from the trimeric yeast ADA complex, Ada3 may play multiple functional roles in the context of different transcriptional activators. Adding to this complexity, mammalian cells seem to contain a GCN5 complex that does not include Ada2 (38). Similarly, two distinct Ada2 proteins exist in higher eukaryotic cells, and studies in *Drosophila* show that both Ada2 proteins are essential for development, indicative of distinct functions (39, 40). In general, however, Ada3 has been found as an invariant component of many ADA-related complexes in mammals and other higher eukaryotic cells, suggesting a pivotal role of Ada3 in coactivator functions of these complexes. In addition, Ada3 may serve as a binding partner of the human papilloma virus E6 oncoprotein (15), suggesting the possibility that viral oncoproteins may alter the function of Ada3 during oncogenesis. Thus, detailed analysis of Ada3 and mechanisms of its function are likely to elucidate basic regulation of gene expression, as well as its aberrations in cancer.

In conclusion, we provide evidence that Ada3 associates with and is required for the recruitment of multiple HAT proteins (p300, GCN5, and PCAF) to ER target gene promoters and that Ada3 is required for ER-mediated cell proliferation. Future studies using total and mammary gland targeted knockout mice should further delineate the important role of Ada3 versus other known ER coactivators, such as SRC1 to SRC3 in ER function, either as redundant regulators or as part of a single complex of temporally coordinated coactivator complexes. Elucidation of the role of coactivators in ER function should provide insights relevant to normal reproductive tissue development and function, as well as oncogenesis.

**Acknowledgments**

Received 7/18/2007; revised 9/27/2007; accepted 10/12/2007.

**Grant support** NIH grants CA94143, CA96844, CA81076 (V. Band), CA87986, CA76118, CA99900, and CA99163 (H. Band), National Cancer Institute (NCI) Center of Cancer Nanotechnology Excellence Grant NCI U54 CA119341-01 (H. Band and V. Band), NCI Breast Cancer Specialized Programs of Research Excellence and AVON Breast Cancer Fund at Robert H. Lurie Cancer Center, Northwestern University, Duckworth Family Chair for Breast Cancer Research (V. Band), and Jean Ruggles- Robinson Chair for Cancer Research (H. Band).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Michael A. Sassaack for help in this project and Drs. Qingshen Gao and Gobheidhan Dimri and the members of Band laboratories for helpful suggestions and discussion.

**References**


Correction: Ada3 Requirement for ER-Dependent Cell Proliferation

In the article on Ada3 requirement for ER-dependent cell proliferation in the December 15, 2007 issue of Cancer Research (1), the figure legends on page 11794 are switched; the legend for Fig. 4 is next to Fig. 5, and the legend for Fig. 5 is next to Fig. 4.

Ada3 Requirement for HAT Recruitment to Estrogen Receptors and Estrogen-Dependent Breast Cancer Cell Proliferation

Aleksandra Germaniuk-Kurowska, Alo Nag, Xiangshan Zhao, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/24/11789

Cited articles

This article cites 40 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/24/11789.full.html#ref-list-1

Citing articles

This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/67/24/11789.full.html#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.