ACK1 Tyrosine Kinase Interacts with Histone Demethylase KDM3A to Regulate the Mammary Tumor Oncogene HOXA1

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Abstract

Background: The molecular mechanism by which tyrosine kinases promote estrogen-independent ER-responsive gene expression is poorly understood.

Results: ACK1 drives the expression of oncogene HOXA1 by stimulating KDM3A histone demethylase activity by tyrosine phosphorylation.

Conclusion: Targeting ACK1 signaling by AIM-100 mitigates HOXA1 expression and inhibits breast cancer cell growth.

Significance: ACK1-KDM3A-HOXA1 signaling represents a novel target in overcoming tamoxifen resistance.

Hormone therapy with the selective estrogen-receptor modulator tamoxifen provides a temporary relief for patients with estrogen receptor α (ER)-positive breast cancers. However, a subset of patients exhibiting overexpression of the HER2 receptor tyrosine kinase displays intrinsic resistance to tamoxifen therapy. Therefore, elucidating the mechanisms promoting the estrogen (E2)-independent ER-regulated gene transcription in tamoxifen-resistant breast tumors is essential to identify new therapeutic avenues to overcome drug resistance and ameliorate poor prognosis. The non-receptor tyrosine kinase, ACK1 (also known as TNK2), has emerged as a major integrator of signaling from various receptor tyrosine kinases including HER2. We have uncovered that heregulin-mediated ACK1 activation promoted ER activity in the presence of tamoxifen, which was significantly down-regulated upon ACK1 knockdown or inhibition of ACK1 by small molecule inhibitors, AIM-100 or Dasatinib. We report that ACK1 phosphorylates the ER co-activator, KDM3A, a H3K9 demethylase, at an evolutionary conserved tyrosine 1114 site in a heregulin-dependent manner, even in the presence of tamoxifen. Consistent with this finding, ACK1 activation resulted in a significant decrease in the deposition of dimethyl H3K9 epigenetic marks. Conversely, inhibition of ACK1 by AIM-100 or Dasatinib restored dimethyl H3K9 methylation marks and caused transcriptional suppression of the ER-regulated gene HOXA1. Thus, by its ability to regulate the epigenetic activity of an ER co-activator KDM3A, ACK1 modulates HOXA1 expression in the absence of E2, conferring tamoxifen resistance. These data reveal a novel therapeutic option, suppression of ACK1 signaling by AIM-100 or Dasatinib, to mitigate HOXA1 up-regulation in breast cancer patients displaying tamoxifen resistance.

Breast cancer is the second leading cause of cancer-related mortality in women (1, 2). ER3 protein binds to its cognate ligand E2 with high affinity to form a functionally active complex. This ER-E2 complex differentially interacts with co-regulator proteins that modify histones within chromatin to modulate gene expression of the ER target genes (3). The preponderance of ER expression in breast cancer cells and the dependence of cells on estrogen for growth has led to the successful use of tamoxifen in the treatment of ER-positive breast cancer, which is able to diminish the breast cancer recurrence by almost 50% (4, 5). Although a majority of the breast tumors initially respond well to tamoxifen therapy, in about 15 months to 5 years, most women acquire tamoxifen resistance (6, 7). Despite intensive research efforts, the molecular mechanisms underlying tamoxifen resistance remain elusive. Various mechanisms such as mutations in ERα, the opposing transcriptional activity of ERβ, and amplification of the 6q25 genomic region encompassing the ER gene have been suggested to be involved in the acquisition of tamoxifen resistance (8–10). Because ER is functional in most tamoxifen-resistant breast cancers, paradoxically it indicates that hitherto unidentified ER-dependent but estrogen-independent molecular mechanisms may account for the tamoxifen resistance.

Although elevated ER protein levels via amplification of the ERα gene, observed in ~20% of breast cancers, may account for increased ER signaling, activation of receptor tyrosine kinase signaling such as EGFR and HER2 (neu/erbB2) by mutation or overexpression leading to ligand-independent ER hyperactivity may also be a causative factor in these breast cancers (11). For example, athymic mice injected with HER2-overexpressing MCF-7 cells developed resistance to tamoxifen rather quickly.
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(∼8 weeks) as compared with MCF-7 tumors treated with tamoxifen or placebo pellets for 6 months of therapy (12, 13). However, although Gefitinib, a selective EGFR kinase inhibitor, can only transiently reverse tamoxifen-resistant tumor growth, complete tumor regression is not observed (13). Similarly, Trastuzumab, a HER2 inhibitor, is also not completely effective to suppress cell survival signals (14). Based on these data, we rationalized that breast cancer cells may have adapted to the tamoxifen-rich environment by aberrantly activating downstream signaling pathways, i.e. non-receptor tyrosine kinases that bypass blockade of receptor tyrosine kinase inhibitors, neutralizing the effect of tamoxifen as an ER antagonist. Tamoxifen also acts as an agonist in experimentally engineered breast cancer cells with high levels of the HER2 growth factor receptor (13). Taken together, these data raise the possibility that HER2 cross-talk with ER transcriptional complex, either directly or via an intermediate tyrosine kinase, could enhance the agonist activity of tamoxifen toward ER. Thus, it could be an alternate pathway of acquisition of tamoxifen resistance in breast cancer. However, the tyrosine kinase(s) responsible for stimulating ER-regulated gene expression in the presence of tamoxifen is not known.

ACK1 is an ubiquitously expressed non-receptor tyrosine kinase that has been implicated in the processes of tumorigenesis, cancer cell survival, radiation resistance, and metastasis (15–19). ACK1 gene amplification is reported in several tumors including ovarian, cervical, and lung cancers (cBioPortal for Cancer Genomic, Memorial Sloan-Kettering Cancer Center) (20). Further, overexpression and activation are seen in multiple malignancies including breast cancer. Somatic autoactivating mutations and receptor tyrosine kinase (RTK) activation could also be utilized by cancer cells to achieve ACK1 overexpression (15–17, 19). Overexpression of ACK1 in a human breast cancer cell line followed by injection into immunocompromised mice induced tumor development (20). Furthermore, ACK1 expression was shown to correlate with breast cancer progression and inversely correlated with survival of patients (17). These studies validated ACK1 as a critical signaling intermediate of growth factor signaling and a prime target for anticancer drug development (15, 17, 18, 21, 22). AIM-100 and Dasatinib have emerged to be two major small molecule inhibitors that not only inhibit ACK1 kinase activity in vitro and in vivo, but also suppress the growth of cancer cells (19, 22–24). Unlike AIM-100, which exhibits high specificity toward ACK1, Dasatinib (SPRyCEL) is a multi-targeted tyrosine kinase inhibitor that inhibits several critical oncopgenic proteins, including BCR-ABL, SRC family kinases, c-KIT, PDGF receptor (PDGFR), and ACK1 (23–27). Taken together, these recent data indicate that ACK1 has a critical role in breast cancer survival. However, its role in drug resistance remains poorly understood.

Histone methylation, a dynamic posttranslational modification of arginine and lysine residues, plays a critical role in both gene activation and gene repression events in euchromatic and heterochromatic regions (28). Methyl groups act as binding sites for a wide range of chromatin proteins, including the repressive heterochromatin protein 1 (HP1), which has been shown to bind methyl groups on histone H3 at lysine 9 or H3K9 (29). KDM3A (also known as JHDM2A or JmjD1a) is a histone demethylase that catalyzes the removal of mono- and dimethyl H3K9 in a Fe(II)- and α-ketoglutarate-dependent manner (30). KDM3A contains an 885LXLL889 sequence, which is a signature motif involved in nuclear hormone-receptor interaction (31). Indeed, KDM3A interacted with the androgen receptor (AR), resulting in KDM3A recruitment to AR target genes, e.g. PSA and NXX3.1, causing demethylation of dimethyl H3K9 at PSA and NXX3.1 enhancers (30). Thus, KDM3A is required for efficient demethylation of repressive dimethyl H3K9 at AR target genes promoting their transcriptional activation (30). Further, it was demonstrated that KDM3A is essential for spermatogenesis, as KDM3A-deficient mice exhibited post-meiotic chromatin condensation defects (32) and also obesity and hyperlipidemia (33).

Generally, ER-tamoxifen functions as an efficient suppressor of ER-E2-regulated genes by recruiting corepressor complexes that include distinctive sets of chromatin-modifying histone deacetylase (HDAC) complexes, HDAC3-NCoR or the HDAC1-NuRD (34). Conversely, ER-E2 complex recruits histone demethylases such as LSD1 and KDM3A to ER-regulated genes to activate gene transcription (30, 35). Further, whether histone demethylase activity is important for acquisition of tamoxifen resistance has not been explored. Unexpectedly, we observed that KDM3A but not LSD1 was Tyr-phosphorylated by ACK1 in tamoxifen-treated cells.4 Tyr-phosphorylated KDM3A promoted demethylation of dimethyl histone H3K9 at ACK1-ER-bound promoters to stimulate ER-regulated HOXA1 transcription. Our study therefore uncovers a novel ER coactivator, Tyr-phosphorylated KDM3A in potentiating ER-regulated gene HOXA1 transcription in the presence of tamoxifen. Thus, our data indicate that stimulating transcriptional activity of ER target genes by promoting epigenetic activity of KDM3A in the tamoxifen-rich environment could be one mechanism by which breast cancer cells could acquire tamoxifen resistance.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, Plasmids, and Inhibitors—T47D and MCF-7 cells were obtained from ATCC. ACK1 mAb (A11), α-tubulin (TU-O2), actin (I-19), Erα (Santa Cruz Biotechnology and GeneTrex), HRP-conjugated anti-pTyr (PY-20) (Santa Cruz Biotechnology), anti-pTyr-284 ACK1 (Millipore), HER2 Ab-2 (Clone 9G6.10) (Thermo Scientific), and EGFR (Epitomics) antibodies were purchased from the respective companies. Dimethyl H3K9 antibodies were obtained from Active Motif. Myc-tagged constitutively active ACK1 (caAck) and kinase-dead ACK1 (kdAck) have been described previously (36, 37). Heregulin, EGF, and 4-hydroxy-tamoxifen (4HT) were purchased from Sigma. ACK1-siRNAs were obtained from Dharmacon (D-003102-13 and D-003102-11). AIM-100 was made according to our published procedure (22).

Generation and Purification of Anti-pTyr-1114 KDM3A Antibody—Two KDM3A peptides coupled to immunogenic carrier proteins were synthesized. The phospho-peptide sequence is: Ac-TPEDRK[pY]GTTNLHLC-amide (where pY represents pTyr), whereas the non-phospho-peptide sequence is: Ac-CTPEDRKYGTTNLHL-amide. The antibodies were generated and purified according to standard procedures. The antibody was then purified on an anti-pTyr-1114 KDM3A column. The antibodies were used in Western blotting and immunoprecipitation experiments.

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custom-generated by 21st Century Biochemicals. In brief, two rabbits were immunized twice with phospho-peptide, and ELISA was performed to determine the relative titer of sera against phosphorylated and nonphosphorylated peptides. Two antigen affinity columns were used to purify the phospho-specific antibodies. The first column was the non-phospho-peptide affinity column. Antibodies recognizing the non-phospho-residues of the peptide bound to the column. The flow-through fraction was collected and then applied to the second column, the phospho-peptide column. Antibodies recognizing the phospho-residue bound to the column, which was eluted as phospho-specific antibodies.

Quantitative RT-PCR—qRT-PCR was performed as described earlier (38). In brief, for the construction of standard curves, serial dilutions of pooled sample RNA were used (50, 10, 2, 0.4, 0.08, and 0.016 ng) per reverse transcriptase reaction. One “no RNA” control and one “no Reverse Transcriptase” (No RT) control were included for the standard curve. Three reactions were performed for each sample: 10 ng, 0.8 ng, and a No RT (10 ng) control. Real-time quantitative PCR analyses were performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). All standards, the no template control (H2O), the No RNA control, the no Reverse Transcriptase control, and the no amplification control (Bluescript plasmid), were tested in six wells per gene (2 wells/plate and 3 plates/gene). PCR was carried out with SYBR Green PCR master mix (Applied Biosystems) using 2 μl of cDNA and the primers in a 20-μl final reaction mixture. After a 2-min incubation at 50 °C, AmpliTaq Gold was activated by a 10-min incubation at 95 °C followed by 40 PCR cycles consisting of 15 s of denaturation at 95 °C and hybridization of primers for 1 min at 55 °C. Dissociation curves were generated for each plate to verify the integrity of the primers. Data were analyzed using SDS software version 2.2.2 and exported into an Excel spreadsheet. The actin data were used for normalizing the gene values: i.e. ng of gene/ng of actin per well. The primer sequences for HOXA1 are: forward primer, 5’-CGGAACTGGAAGAGGTTC-3’, and reverse primer, 5’-TTCACCTGGCTCTGGTGG-3’. The primer sequences for actin are: forward primer, 5’-CGGAACTGGAAGAGGTTC-3’, and reverse primer, 5’-TTCACCTGGCTCTGGTGG-3’. The primer sequences for actin are: forward primer, 5’-CACCATTGGCAATGAGGGTTC-3’, reverse primer, 5’-AGGTCTTTGCGGATGTCCACGT-3’. HOXA1 primers were purchased from OriGene.

Chromatin Immunoprecipitation (ChIP)—ChIP was performed using the Active Motif kit as per the manufacturer’s instructions. Cells pellets were lysed in RLB (receptor/cell lysis buffer) (22, 37) on ice for 10 min and sonicated for 25 s to shear DNA to an average length of 300–500 bp. The soluble chromatin was incubated overnight at 4 °C with anti-pTyr-1114 KDM3A, ACK1, and dimethyl H3K9 antibodies and 20 μl of protein-G magnetic beads. After incubation at 4 °C overnight, the complexes were washed, and ChIP DNA was eluted from the beads with elution buffer. ChIP DNA was subjected to proteinase-K treatment for 2 h. Genomic DNA (input) was prepared by treating aliquots of chromatin with proteinase-K. The ChIP DNA was quantitated by real-time PCR as described above, using SimpleChIP human HOXA1 intron 1 primers (Cell Signaling).

Luciferase Assay—MCF-7 cells were transfected with the 3×ERE (estrogen-responsive element)-vitellogenin-LUC reporter construct. The cells were serum-starved and treated with heregulin (10 nm, 1 h), 4HT (100 nm, 2 h), kinase inhibitor Dasatinib (100 nm, 16 h), or E2 (10 nm, 2 h). The cells were lysed, and luciferase activity was determined using the luciferase assay kit (Promega) and the LMax microplate luminometer (Molecular Devices, Sunnyvale, CA) as described earlier (22, 37).

Cell Proliferation and Drug Sensitivity Assays—MCF-7 and T47D cells were grown in serum-free media reconstituted with heregulin (10 ng/ml), E2 (10 nm), 4HT (100 nm), Dasatinib, or AIM-100 (10 μM) for 72 h, and cells were counted by trypan blue dye assay. The data were calculated based on the percentage of control (DMSO-treated) cells.

Microarray—MCF-7 cells were untreated or treated with AIM-100 (1 μM) for 4 h, and RNA was prepared. 100 ng of total RNA served as the mRNA source for microarray analysis. The poly(A) RNA was specifically converted to cDNA and then amplified and labeled with biotin using the Ambion MessageAmp premier RNA amplification kit (Life Technologies) following the manufacturer’s protocol initially described by Van Gelder et al. (39). Hybridization with the biotin-labeled RNA, staining, and scanning of the chips followed the prescribed procedure outlined in the Affymetrix technical manual and has been previously described (40). The oligonucleotide probe arrays used were the Human Genome U133 Plus 2.0 arrays. This array contains over 54,000 probe sets representing over 47,000 transcripts that were designed from GenBank, dbEST, and RefSeq sequences that were clustered based on builds 133 and 159 of the UniGene database. The clusters were further refined by analysis and comparison with a number of other publicly available databases, including the Washington University EST trace repository and the University of California, Santa Cruz GoldenPath human genome database. Each gene is represented by a series of oligonucleotides that are identical to the sequence in the gene as well as oligonucleotides that contain a homomeric (base transversion) mismatch at the central base position of the oligomer, which is used to measure cross-hybridization.

Data Analysis—Scanned output files were visually inspected for hybridization artifacts and then analyzed using Affymetrix GeneChip Operating Software (GCOS) using the MAS 5.0 algorithm. Signal intensity was scaled to an average intensity of 500 prior to comparison analysis. Using the default settings, the GCOS software identifies the increased and decreased genes between any two samples with a statistical algorithm that assesses the behavior of 11 different oligonucleotide probes designed to detect the same gene (41). Probe sets that yielded a change p value less than 0.002 were identified as changed (increased or decreased), and those that yielded a p value between 0.002 and 0.002667 were identified as marginally changed.
data, we reasoned that ACK1 could act as a downstream mediator of HER2/HER3 signaling in breast cancer cells. To assess this supposition, ER-positive MCF-7 breast cancer cells were treated with heregulin ligand; 40 min of heregulin treatment resulted in optimal ACK1 tyrosine phosphorylation (Fig. 1A). These data establish that heregulin-mediated HER2 activation

**FIGURE 1. Heregulin-mediated HER2 activation leads to ACK1 activation.** A, MCF-7 cells were treated with heregulin (10 ng/ml) for various time points, and equal amounts of protein lysates were subjected to immunoprecipitation with ACK1 (top panel) and HER-2 (second panel) antibodies followed by immunoblotting with pTyr antibodies. The lysates were also subjected to immunoblotting with KU antibodies to confirm equal loading of protein samples. p indicates phospho. B, activated ACK1 interacts with and Tyr-phosphorylates KDM3A, HEK293 cells were transfected with FLAG-tagged KDM3A and ACK1 constructs as described. 48 h after transfection, equal amounts of protein lysates were subjected to immunoprecipitation with FLAG beads followed by immunoblotting with anti-Myc Abs (top panel) or with anti-pTyr Abs to detect phosphorylated KDM3A (second panel). The remaining panels represent cell lysates subjected to immunoblotting with the indicated antibodies, Vec, empty vector; mock, mock-transfected. C, ACK1 activation regulates ER:ACK1 complex formation in the presence of 4HT. 293T cells were transfected with Myc-tagged ACK1 and FLAG-tagged ER and KDM3A constructs as shown. 36 h after transfection, the cells were treated with 4HT for 16 h. Cells were harvested, and equal amounts of protein lysates were immunoprecipitated with Myc beads followed by immunoblotting with anti-FLAG antibodies (top panel). The remaining panels represent cell lysates subjected to immunoblotting with the indicated antibodies. D, schematic representation of ACK1, KDM3A and their deletion constructs. SAM, sterile α motif; C, Cdc42/Rac interactive binding domain; CL, clathrin-interacting domain; ZF, zinc finger; L, LXxxL motif; JmjC, JmjC domain. E, HEK293 cells were transfected with FLAG-tagged KDM3A and Myc-tagged ACK1 deletion construct. 48 h after transfection, equal amounts of protein lysates were subjected to immunoprecipitation with FLAG beads followed by immunoblotting with anti-ACK1 monoclonal Abs (top panel) or with anti-pTyr Abs to detect phosphorylated KDM3A (second panel). The remaining panels represent cell lysates subjected to immunoblotting with the indicated antibodies. E, schematic representation of ACK1, KDM3A and their deletion constructs. SAM, sterile α motif; C, Cdc42/Rac interactive binding domain; CL, clathrin-interacting domain; ZF, zinc finger; L, LXxxL motif; JmjC, JmjC domain. F, HEK293 cells were transfected with FLAG-tagged aKDM3A or cKDM3A and ACK1 constructs. 48 h after transfection, equal amounts of protein lysates were subjected to immunoprecipitation with FLAG beads followed by immunoblotting with anti-pTyr Abs to detect 4HT phosphorylation (top panel) or anti-FLAG monoclonal Abs to detect expression of deletion constructs (second panel). G, activated ACK1 interacts with endogenous KDM3A. MCF-7 cells were treated with AIM-100 (5 μM, 16 h) followed by heregulin (10 nM, 40 min). Cell lysates were immunoprecipitated with ACK1 antibodies followed by blotting with anti-KDM3A Abs (top panel). The remaining panels represent cell lysates subjected to immunoblotting with the indicated antibodies (bottom panels).
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Endogenous ACK1-KDM3A interaction was also assessed in the presence of ACK1 inhibitor, AIM-100. ACK1 kinase inactivation decreased ACK1-KDM3A interaction (Fig. 1G). Taken together with earlier data, these experiments suggest that ACK1 upon activation interacts with the carboxyl-terminal KDM3A spanning LXXLL, a nuclear hormone-receptor interaction region and JmjC domains, subsequently phosphorylating KDM3A in the JmjC domain.

ACK1 Phosphorylates Endogenous KDM3A in the Presence of Tamoxifen—To test whether endogenous KDM3A is a substrate for ACK1 tyrosine kinase, MCF-7 cells were treated with heregulin (10 nm, 40 min). Cells were harvested and equal amounts of protein lysates were subjected to immunoprecipitation with KDM3A antibodies followed by immuno blotting with anti-pTyr Abs (top panel). The protein lysates were also subjected to immunoprecipitation with ACK1 (third panel) or HER2 antibodies (bottom panel) followed by immunoblotting with anti-pTyr antibodies. The remaining panels represent cell lysates subjected to immunoblotting with the indicated antibodies. D. Tyr phosphorylation of endogenous KDM3A is sensitive to ACK1 inhibitor, T47D cells were treated with heregulin (10 nm, 40 min), 4HT (100 nm, 40 min), and Dasatinib (0.4 μM, 16 h). Cells were harvested, and equal amounts of protein lysates were subjected to immunoprecipitation with KDM3A antibodies followed by immunoblotting with anti-pTyr Abs (top panel). The protein lysates were also subjected to immunoprecipitation with ACK1 (third panel) or HER2 antibodies (bottom panel) followed by immunoblotting with anti-pTyr antibodies. The remaining panels represent cell lysates subjected to immunoblotting with the indicated antibodies.

ACK1 Interacts with Carboxy-terminal KDM3A—To determine regions within ACK1 and KDM3A involved in the interaction that leads to KDM3A Tyr phosphorylation (Fig. 1B), co-immunoprecipitation studies were performed. FLAG-tagged KDM3A deletion constructs cKDM3A (881–1321 amino acids) and aKDM3A (1058–1321 amino acids) were generated (Fig. 1D). In addition, Myc-tagged ACK1 deletion constructs aACK1 and cACK1 that were generated earlier (17) were used in this study. Myc-tagged ACK1 and its deletion construct when co-expressed with full-length KDM3A both exhibited interaction with KDM3A, indicating that the amino terminus of ACK1 spanning SAM (sterile α motif) and kinase domain is likely to be involved in KDM3A recognition (Fig. 1E). FLAG-tagged KDM3A deletion constructs, aKDM3A and cKDM3A, were similarly co-expressed with caAck. cKDM3A construct exhibited robust phosphorylation; however, aKDM3A lacking LXXLL motif was not targeted for phosphorylation by caAck (Fig. 1F). Interestingly, both KDM3A deletion constructs, aKDM3A and cKDM3A, possess a Tyr-1114 phosphorylation site, suggesting that LXXLL motif and the adjoining region are critical for recognition of KDM3A by ACK1.
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**FIGURE 3.** ACK1 phosphorylates KDM3A at an evolutionarily conserved tyrosine 1114 site. A, alignment of KDM3A protein sequences from various organisms indicate that tyrosine residue at 1114 position is invariant from *Xenopus* to humans. B, the sequence homology between the JmjC domains of KDM3A (residues 1058–1281) and KDM3B (residues 1498–1721) is shown. C, crystal structure of JmjC domain of human histone 3 lysine-specific demethylase 3B (KDM3B) shown in ribbon form. The sequence DRRVGTTN is shown in green with valine-1554 highlighted in yellow. The manganese atom is shown in magenta, and co-factor mimic N-oxalylglycine is shown in green. In KDM3A, the critical tyrosine 1114 residue is located in a similar position to that occupied by valine-1554 in KDM3B. PyMOL was used to generate the picture of the protein. **D**, schematic representation of KDM3A and Y1114F point mutant proteins. ZF, Zink finger domain (662–687 amino acids); JmjC, JmjC domain (1058–1281 amino acids). **E**, 293T cells were transfected with FLAG-tagged KDM3A or KDM3A point mutant (Y1114F) and caAkk1 and harvested after 48 h. Equal amounts of protein lysates were immunoprecipitated with FLAG Abs. Immunoprecipitates were resolved by SDS-PAGE followed by blotting with anti-pTyr Abs (top panel). The lower panel represents cell lysates blotted with total KDM3A antibodies.

phorylate KDM3A. Cells treated with heregulin exhibited Tyr phosphorylation of endogenous KDM3A (Fig. 2A, top). The role of ACK1 in heregulin-mediated Tyr phosphorylation of KDM3A was further confirmed by transfecting the cells with ACK1 or control siRNA followed by heregulin treatment. Down-regulation of ACK1 resulted in decrease in KDM3A Tyr phosphorylation (Fig. 2B). Taken together with our earlier data (Fig. 2A), this suggests that ACK1 directly phosphorylates endogenous KDM3A.

To determine whether ACK1 phosphorylates endogenous KDM3A in the presence of tamoxifen, MCF-7 cells were treated with heregulin, 4HT, and tyrosine kinase inhibitor, Dasatinib. Heregulin treatment of MCF-7 cells resulted in Tyr phosphorylation of endogenous KDM3A (Fig. 2C, top panel, lane 2). The 4HT treatment did not affect Tyr phosphorylation of KDM3A (Fig. 2C, top panel, compare lanes 2 and 4). However, treatment with Dasatinib resulted in a significant decrease in KDM3A Tyr phosphorylation (Fig. 2C, top panel, lanes 5 and 6). Similarly, when another ER-positive breast cancer cell line T47D was treated with heregulin or heregulin+4HT, ACK1-dependent KDM3A Tyr phosphorylation was observed (Fig. 2D). Thus, collectively these data indicate that heregulin-mediated KDM3A Tyr phosphorylation is unaffected by 4HT treatment but is critically dependent upon ACK1 kinase activation. This observation opens up a novel possibility that ACK1 modulates ER coactivator KDM3A by Tyr phosphorylation and may have a role in conferring tamoxifen resistance.

Identification of a Novel Tyr Phosphorylation Site in KDM3A—Aberrant activation of oncogenic tyrosine kinases initiates a phosphorylation cascade in cells to deliver signals that ulti-
mately translate into hormone-independent cell growth, suppression of apoptosis, pro-angiogenesis, invasion, and metastasis (37, 44–47). Estrogen-independent ER transcriptional activation may occur due to the recruitment of specific tyrosine kinases at ER target genes that modulate ER co-activator activity in response to specific signaling cues. Because there was increased ACK1-ER complex formation in an E2-deficient but tamoxifen-rich environment (Fig. 1C), we reasoned that the tyrosine 1114 residue of ACK1 could phosphorylate KDM3A on tyrosine residue. To evaluate Tyr phosphorylation status of KDM3A in ER target gene activation, we employed an artificial neural network-based method for predicting potential Tyr phosphorylation sites in KDM3A (48). Artificial neural networks have the ability to classify complex and nonlinear biological sequence patterns and recognize the correlation between positions. Therefore, the artificial neural network algorithms have been widely used in biological sequence analysis. The artificial neural network method can computationally identify catalytic kinase-specific phosphorylation sites and may predict phosphorylation sites in independent sequences with the sensitivity in the range from 69 to 96%. This method yielded a putative Tyr phosphorylation site, Tyr-1114, in the JmjC domain (Score 0.982). Sequence analysis revealed that Tyr-1114 is located in a highly conserved region of the JmjC domain (Fig. 3A).

The crystal structure of the JmjC domain of KDM3A has not been reported. However, the crystal structure of the JmjC domain of its homolog KDM3B has been deposited in the Protein Data Bank (PDB code 4C8D). The sequence homology between the JmjC domains of KDM3A (residues 1058–1281) and KDM3B (residues 1498–1721) is 91% (83% of residues are identical and 8% are equivalent) (Fig. 3B). We note that in KDM3A, the Tyr-1114 is embedded in the sequence DRRVGTTN. The equivalent sequence in KDM3B is DRRVGTNN, having the tyrosine substituted by valine. In the crystal structure of KDM3B (shown in Fig. 3C), the DRRVGTTN sequence is highlighted in green, and the valine residue (Val-1554) is highlighted in yellow. The valine residue is located in the substrate peptide binding groove, and is accessible and close to the catalytic site (with the manganese ion and the α-ketoglutarate mimic N-oxalylglycine shown). We therefore reasoned that the tyrosine 1114 residue in KDM3A would also be accessible and close to the catalytic and substrate binding site, and its phosphorylation might therefore be expected to significantly alter the activity of the enzyme.

Site-directed mutagenesis was performed to mutate the predicted tyrosine to phenylalanine in KDM3A to generate the mutant, Y1114F (Fig. 3D). The FLAG-tagged KDM3A point

FIGURE 4. Characterization of the anti-pTyr-1114 KDM3A antibodies. A, HEK293 cells were treated with EGF ligand (1 nM) for various time intervals. Cells were harvested, and equal amounts of protein lysates were subjected to immunoblotting with pTyr-1114 KDM3A antibodies (pKDM3A) at 1:1500 dilution (top panel). The bottom panel represents cell lysates subjected to immunoblotting with the antibodies as indicated. pEGFR, phospho-EGFR. B, MCF-7 cells were treated with heregulin (Hrg) ligand (10 nM) for 45 min. Cells were harvested, and equal amounts of protein lysates were subjected to immunoblotting with pTyr-1114 KDM3A antibodies as described above. C, MCF-7 cells were electroporated (Amaxa) with ACK1 or control siRNA, and 48 h after transfection, cells were treated with heregulin, and lysates were subjected to immunoblotting with pTyr-1114 KDM3A antibodies (pKDM3A(Tyr-1114)) as described above. D, MCF-7 cells were electroporated (Amaxa) with KDM3A or control siRNA, and 48 h after transfection, cells were treated with heregulin, and lysates were subjected to immunoblotting with pTyr-1114 KDM3A antibodies as described above. E, MCF-7 cells were treated with ACK1 inhibitor AIM-100 or Dasatinib followed by heregulin ligand treatment. Cell lysates were subjected to immunoblotting with pTyr-1114 KDM3A antibodies as described above.
mutant (Y114F) was co-expressed with caAck in HEK293 cells. Cells were immunoprecipitated with FLAG-beads followed by immunoblotting with pTyr antibodies. Loss of KDM3A Tyr phosphorylation was seen in the Y1114F mutant, suggesting it to be a major Tyr phosphorylation site (Fig. 3E).

**Generation and Validation of Anti-pTyr-1114 KDM3A Antibodies**—To determine whether phosphorylation at Tyr-1114 is of physiological relevance, we have raised specific antibodies to the phosphorylated Tyr-1114 site in KDM3A (anti-pTyr-1114 KDM3A antibodies). Both ACK1 and KDM3A are ubiquitously expressed proteins, detectable in a variety of cell lines and tissues. Hence to detect endogenous anti-pTyr-1114 KDM3A antibodies, HEK293 and MCF-7 cells were either untreated or treated with heregulin (3 h), 100 nM Dasatinib (16 h), or 100 nm 4HT (3 h before and during heregulin treatment), and luciferase activity was measured. The experiment was performed three times, and a representative data are shown. *p < 0.05. B, ACK1 modulates ER transcriptional activity in tamoxifen-treated breast cancer cells. MCF-7 cells were transfected with 0.2 μM control or ACK1 siRNA. On the next day, cells were transfected with 25 ng of 3×ERE-vitellogenin-LUC reporter in serum-free media. Cells were either untreated or treated with heregulin (3 h), 100 nm Dasatinib (16 h), or 100 nm 4HT (3 h before and during heregulin treatment), and luciferase activity was measured. The experiment was performed three times, and a representative data are shown. *p < 0.05. C, ACK1 inhibitors suppress breast cancer cell proliferation. MCF-7 cells were treated with various agents (AIM-100, 10 μM; Dasatinib, 10 μM; heregulin, 10 nM; 4HT, 200 nM; E2, 10 nM) for 72 h, and cells were counted by trypan blue viability assay. Data shown are a percentage of the cell number as compared with DMSO-treated sample (control). *p < 0.05. D, ACK1 inhibitors suppress T47D breast cancer cell proliferation. T47D cells were treated with various agents as described above for 48 h, and cells were counted by trypan blue viability assay. Data shown are a percentage of the cell number as compared with DMSO-treated sample (control). *p < 0.05.

**ACK1 Inhibitors Suppress KDM3A Tyr Phosphorylation**—To further explore the functional role of ACK1 kinase in KDM3A Tyr-1114 phosphorylation, MCF-7 cells were treated with heregulin ligand or/and ACK1 inhibitor Dasatinib and AIM-100. Although both Dasatinib and AIM-100 are potent ACK1 inhibitors, AIM-100 is highly specific for ACK1 (18, 22). Not surprisingly, both the ACK1 inhibitors suppressed KDM3A Tyr-1114 phosphorylation (Fig. 4E), suggesting that ACK1 kinase activity is critical for KDM3A Tyr-1114 phosphorylation in breast cancer cells.
ACK1 Is Required for ER-dependent Gene Expression in Tamoxifen-rich Environment—To examine the precise role of ACK1 in endogenous estrogen-independent ER transcriptional function, ER reporter activity was monitored. MCF-7 cells were transfected with the 3×ERE-vitellogenin-LUC reporter construct. The cells were then treated with E2, hergulin, 4HT, and Dasatinib, and luciferase activity was determined. Luciferase activity of MCF-7 cells treated with hergulin was treated as 100%, and the changes with respect to these level are shown (Fig. 5A). E2 treatment of MCF-7 cells resulted in significant increase in luciferase activity that was suppressed upon 4HT treatment. Interestingly, hergulin treatment also led to increased luciferase activity, and this estrogen-independent ER activity was unaffected by 4HT treatment. However, when cells were treated with Dasatinib, a significant decrease in the luciferase activity was observed (Fig. 5A).

To assess the effect of knockdown of endogenous ACK1 on endogenous estrogen-independent ER reporter activity, MCF-7 cells were transfected with control and ACK1-siRNAs and with the 3×ERE-vitellogenin-LUC reporter construct. The cells were treated with hergulin, 4HT, and Dasatinib, and luciferase activity was determined. Luciferase activity of MCF-7 cells transfected with control siRNA and treated with hergulin was treated as 100%, and the changes with respect to these levels are shown (Fig. 5B). Hergulin-treated samples exhibited high ER transcriptional activity, which was unaffected by 4HT treatment; however, ACK1 knockdown significantly reduced hergulin-mediated ER transcriptional activation. Further, upon Dasatinib treatment, ER transcriptional activity was significantly reduced (Fig. 5B).

To determine the effect of ACK1 inhibitor on growth of breast cancer cells in a tamoxifen-rich environment, a cell proliferation assay was performed. As expected, significant cell proliferation was observed in E2-treated samples, whereas tamoxifen treatment exhibited modest increase in cell number, as compared with control (untreated) samples (Fig. 5C). In contrast, hergulin treatment did not exhibit substantial increase in cell number; however, both ACK1 inhibitors, AIM-100 and Dasatinib, severely sensitized cell growth. Interestingly, the effectiveness of ACK1 inhibitors seems to have been enhanced in a tamoxifen-rich environment. Similar data were obtained in T47D cells (Fig. 5D). Taken together, this suggests that ACK1 is critical for E2-independent ER transcriptional activation and cell proliferation in a tamoxifen-rich environment. Thus, activation of ACK1 could be one of the mechanisms of acquisition of tamoxifen resistance by breast cancer cells.

ACK1 Inhibition Suppresses HOXA1 Transcription—Earlier we demonstrated that AIM-100, a highly selective ACK1 inhibitor, causes G1 arrest, leading to eventual apoptosis in treated cancer cells (18, 21). These data also revealed that those cancer cells that are “addicted” to ACK1 signaling are highly sensitive to ACK1 inhibitors. We thus hypothesized that the selective sensitivity of breast cancer cells to ACK1 inhibition may relate to impaired function of a potent oncogene. Toward the goal of identifying the potential oncogene targeted specifically by ACK1-KDM3A-HOXA1 signaling, we profiled the AIM-100-sensitive breast cancer cell line, MCF-7. Cells were untreated or treated with 1 μM AIM-100 overnight followed by hergulin stimulation for 3 h, and total RNA was isolated. cDNA was

TABLE 1

| Gene symbol | Description | Hrg signal | Hrg + AIM signal | -Fold change |
|-------------|-------------|------------|-----------------|--------------|
| UGT2B10     | UDP glucuronosyltransferase 2 family polypeptide | 103.8 | 9.5 | -10.93 |
| HOXA4       | Homeobox A1 | 60.3 | 5.8 | -10.6 |
| EPHAX7      | EPH receptor A7 | 70.9 | 7.3 | -9.71 |
| SPINK7      | Serine peptidase inhibitor, Kazal type 7 | 108.5 | 11.4 | -9.52 |
| BMP4        | Bone morphogenetic protein 4 | 151.2 | 16.4 | -9.22 |
| TXNIP       | Thioredoxin interacting protein | 1049 | 119 | -8.82 |
| ANKRD34A    | Ankyrin repeat domain 34A | 57.1 | 7 | -8.16 |
| NAP1L5      | Nucleosome assembly protein 1-like 5 | 59.7 | 7.6 | -7.86 |
| FDP5S5      | Farnesyl diphosphate synthase pseudogene 5 | 147.9 | 19.2 | -7.70 |
| ID2         | Inhibitor of DNA binding 2 | 1058 | 157.5 | -6.72 |
| UHRF1       | Ubiquitin-like with PHD and ring finger domains 1 | 1011.3 | 153.6 | -6.58 |
| ZNF821      | Zinc finger protein 821 | 65.4 | 10.1 | -6.48 |
| ANXA8L2     | Annexin A8-like 2 | 122.8 | 20.6 | -5.96 |
| UBE2N       | Ubiquitin-conjugating enzyme E2N | 513.3 | 88.4 | -5.78 |
| CCNE2       | Cyclin E2 | 899.7 | 159.7 | -5.63 |
| C12orf60    | Chromosome 12 open reading frame 60 | 53.1 | 10 | -5.31 |
| KR76C       | Keratin 6C | 828.1 | 157.1 | -5.27 |
| RR2M        | Ribonucleotide reductase M2 | 2639 | 505.5 | -5.22 |
| MGP         | Matrix Gla protein | 190 | 177.4 | -5.19 |
| PKP1        | Plakophilin 1 (skin fragility syndrome) | 182.3 | 35.9 | -5.08 |
| KHL24       | Kelch-like 24 | 26.1 | 133.6 | 5.12 |
| DCBLD2      | Discoidin, CUB, and LCCL domain-containing 2 | 13.6 | 72.7 | 5.35 |
| RUNX3       | RUN and FYVE domain-containing 3 | 10.3 | 60.7 | 5.89 |
| FAM1129A    | Family with sequence similarity 129, member A | 34.6 | 205.2 | 5.93 |
| LTB1P       | Latent transforming growth factor beta binding | 15.2 | 96.5 | 6.35 |
| LOC284570   | Hypothetical protein LOC284570 | 18.1 | 122.9 | 6.79 |
| FBXO9       | F-box protein 9 | 10.2 | 71.7 | 6.79 |
| FAM1129A    | Family with sequence similarity 129, member A | 94.5 | 693.9 | 7.34 |
| SQSTM1      | Sequestosome 1 | 26.5 | 211.6 | 7.98 |
| ANKRD20A1   | Ankyrin repeat domain 20 | 14.6 | 119.8 | 8.21 |
| CYP1A1      | Cytochrome P450, family 1, polypeptide 1 | 21.2 | 255.2 | 12.04 |
| PS4G        | Pregnancy-specific β-1-glycoprotein 4 | 4.7 | 74 | 15.74 |
| CREG2       | Cellular repressor of E1A-stimulated genes 2 | 3.2 | 51.6 | 16.13 |
| OTUD4       | OTU domain-containing 4 | 3.1 | 52.4 | 16.90 |
ACK1·KDM3A·HOXA1 Signaling in Estrogen Independence

generated followed by hybridization to Affymetrix human promoter 1.0R arrays following the Affymetrix procedure. Differentially regulated genes that exhibit at least 5-fold change are shown in Table 1. These genes are significantly modulated on the basis of the false discovery rate <0.05 for signal-to-noise in the comparison of heregulin versus heregulin + AIM-100-treated sample. The second most down-regulated gene was observed to be HOXA1.

Homeobox A1 (HOXA1) gene, a potent oncogene, has attracted significant interest in breast cancer. Forced expression of HOXA1 was sufficient to cause oncogenic transformation of immortalized human mammary epithelial cells with an ability to form aggressive in vivo tumors (49). To validate the microarray results demonstrating the requirement of ACK1 in HOXA1 transcription, we evaluated the effect of ACK1 inhibitors on HOXA1 gene expression in breast cancer cells by real-time reverse transcriptase PCR (qRT-PCR). HOXA1 expression was significantly increased upon heregulin treatment, but it was significantly down-regulated in AIM-100- and Dasatinib-treated MCF-7 breast cancer cells (Fig. 6A). These data suggest that ACK1 signaling is an E2-independent regulator of HOXA1 transcription.

ACK1 Inhibitors Displace ER and KDM3A from the HOXA1 Promoter Region to Suppress HOXA1 Expression—To determine whether ACK1 kinase activity is needed for the recruitment of ER and pTyr-1114 KDM3A complex at the HOXA1 locus, we performed ChIP with ER and dimethyl H3K9 antibodies followed by real-time PCR (quantitative PCR). ChIP analysis revealed the presence of ER at the HOXA1 intron 1 in a heregulin-dependent manner (Fig. 6B). In contrast, treatment with AIM-100 resulted in the displacement of ER from HOXA1 intron 1, providing a mechanistic explanation for the observed AIM-100-dependent decrease in HOXA1 transcription (Fig. 6B).

KDM3A demethylates mono- and dimethyl H3K9 in vitro and in vivo (30). To determine whether ACK1 modulates the KDM3A demethylase activity at the HOXA1 promoter to activate HOXA1 gene transcription, ChIP was performed using dimethyl H3K9 antibodies followed by real-time PCR (quantitative PCR). We observed that dimethyl H3K9 marks were partly erased at the HOXA1 intron 1 in heregulin-stimulated condition (Fig. 6C). However, the deposition of transcriptionally suppressive dimethyl H3K9 epigenetic marks was significantly increased at the HOXA1 intron 1 upon AIM-100 or Dasatinib treatment (Fig. 6C), suggesting that ACK1-mediated modulation of ER co-repressor activity is critical to promote HOXA1 gene transcription.

DISCUSSION

Our studies have uncovered the ACK1·KDM3A·HOXA1 regulatory signaling axis that operates in breast cancer cells. The functional outcome of this signaling pathway is primarily achieved by assembly of the ACK1·ER·pTyr·KDM3A complex at the HOXA1 intron 1, promoting its expression even in the absence of E2. Further, in cells exposed to ACK1 inhibitors, not only is ACK1 phosphorylation suppressed, but it also prevented KDM3A Tyr-1114 phosphorylation, blocking ER co-repressor activity at the HOXA1 intron 1. This caused robust down-regulation of HOXA1 expression in breast cancer cells (Fig. 7).

Overall, these data uncover a novel epigenetic mechanism of acquisition of tamoxifen resistance orchestrated by a non-receptor tyrosine kinase (Fig. 7).

Over the last decade, a number of histone demethylases have been identified followed by their biochemical characterization; however, the precise functional significance of the majority of these remains unknown, especially in cancer cells. In this study, we have uncovered an important role for the histone demethylase KDM3A in breast cancer. Histone demethylases erase H3K4 and H3K9 epigenetic marks generated by histone methyl transferases, upon estrogen stimulation to activate ER-regu-
lated genes, e.g. pS2 and GREB1, in MCF7 cells (35). Surprisingly, we noticed that upon binding to the ACK1 tyrosine kinase, ER recruits the chromatin-modifying transcriptional coactivator KDM3A, which covalently modifies histone proteins within the \( \text{HOXA1} \) promoter to regulate its expression in the estrogen-deficient and tamoxifen-rich environment. Thus, estrogen-bound ER and ACK1-bound ER could target distinct genes and utilize different histone demethylases to regulate gene expression. This would explain why tamoxifen-resistant breast cancer cells are insensitive to tamoxifen but undergo cell death when treated with ACK1 kinase inhibitors.

Tamoxifen resistance is a major clinical concern in patients with breast cancer, and a better understanding of this phenomenon could help HER2-ER-positive patients significantly. We observed that heregulin-activated ACK1 targeted the Tyr-1114 phosphorylation site in ER coactivator KDM3A, in the absence of estrogen. Indeed, our data indicate that ACK1 facilitates transcription of ER target genes such as \( \text{HOXA1} \) in an estrogen-deficient environment. These data uncover a novel molecular mechanism of acquisition of tamoxifen resistance in HER2-overexpressing breast tumors.

The roles of Homeobox (HOX) genes have been well documented in embryogenesis and organogenesis; however, evidences suggesting their roles in various malignancies are steadily accumulating (50, 51). Recently, a computational biology approach that involved evaluation of gene expression changes in the context of the entire gene regulatory network led to identification of the \( \text{HOXA1} \) as a critical mediator of mammary tumor progression (52). The importance of \( \text{HOXA1} \) in breast cancer progression has further acquired considerable significance when it was demonstrated that silencing this gene in cultured mouse or human mammary tumor spheroids using siRNA resulted in suppression of mammary epithelial cell proliferation, increased estrogen and progesterone receptor expression, and reduction in tumor incidence by 75% (52). \( \text{KDM3A} \) expression has recently been shown to be significantly elevated in human bladder carcinomas, and \( \text{KDM3A} \) knockdown resulted in the suppression of cell proliferation (51). Interestingly, it was observed that \( \text{KDM3A} \) activates transcription of the \( \text{HOXA1} \) gene through demethylation of histone H3 at lysine 9 epigenetic marks, and its down regulation arrested cancer cells in G1 phase. These data establish an important role of \( \text{KDM3A} \); however, targeted inhibition of its expression in cancer cells remained to be a difficult task. Our data demonstrate that ACK1 inhibitors AIM-100 and Dasatinib are effective suppressors of \( \text{KDM3A} \) phosphorylation and estrogen-independent ER transcriptional activation of the \( \text{HOXA1} \) oncogene.

With multiple mechanisms promoting ACK1 activation in multiple cancers including breast cancer, ACK1 inhibitors have emerged as a potential therapeutic option for the treatment of hormone deprivation therapy-resistant tumors (16). Moreover, the availability of pTyr-1114 KDM3A antibodies will allow screening of breast cancer patients positive for ACK1 signaling and likely to acquire tamoxifen resistance. Further, determination of \( \text{HOXA1} \) mRNA expression levels by qRT-PCR in breast tumor biopsies could be used as an additional step in this “companion diagnostic test” for ACK1 inhibitor therapy. This “personalized medicine” approach could be highly beneficial for tamoxifen-resistant breast cancer patients, unveiling ACK1 inhibitor treatments such as Dasatinib treatment, which is
already a Food and Drug Administration (FDA)-approved drug, as an adjuvant therapeutic option.

Not only has ACK1 kinase been shown to be an effector of a plethora of kinases, HER2, EGFR, PDGF receptor, and MERTK (Mer tyrosine kinase), but ACK1 gene amplification and somatic autoactivating mutations have also emerged to be other mechanisms of ACK1 activation in a wide variety of cancers (15, 16, 19). ACK1 gene amplification (3.4%) and somatic autoactivating mutations (0.1%) are relatively rare events in breast cancers. So, how does ACK1 accomplish increasing HOXA1 expression in an estrogen-deficient environment? Recently, it was reported that induction of ER signaling by estrogen induces seven in absentia homolog, SIAH2 ubiquitin ligase, which can catalyze the polyubiquitination and proteasomal degradation of ACK1 (53). Accordingly, in the absence of estrogen, breast cancer cells continued to express ACK1, highlighting the key role ACK1 may play in initiating a distinct transcription program in the absence of estrogen in breast cancer patients.

Overall, the identification of the ACK1 resembled the KDM3A-HOXA1 signaling axis reveals a new mechanism likely to be employed by breast cancer cells to acquire tamoxifen resistance, and for the KDM3A-Tyr-1114 phosphorylation or HOXA1 overexpression by specific inhibitors could be indicative of disease remission in chemotherapy trials.

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Note Added in Proof—Table 1 was inadvertently omitted from the manuscript version that was published in Papers in Press.

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