**Human Epidermal Growth Factor Receptor 2 (HER2) Impedes MLK3 Kinase Activity to Support Breast Cancer Cell Survival**

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Human epidermal growth factor receptor 2 (HER2) is amplified in ~15–20% of human breast cancer and is important for tumor etiology and therapeutic options of breast cancer. Upregulation of HER2 oncogene initiates cascades of events culminating to the stimulation of transforming PI3K/AKT signaling, which also plays a dominant role in supporting cell survival and efficacy of HER2-directed therapies. Although investigating the underlying mechanisms by which HER2 promotes cell survival, we noticed a profound reduction in the kinase activity of a pro-apoptotic mixed lineage kinase 3 (MLK3) in HER2-positive (HER2+) but not in HER2-negative (HER2−) breast cancer tissues, whereas both HER2+ and HER2− tumors expressed a comparable level of MLK3 protein. Furthermore, the kinase activity of MLK3 was inversely correlated with HER2+ tumor grades. Moreover, HER2-directed drugs such as trastuzumab and lapatinib as well as depletion of HER2 or HER3 stimulated MLK3 kinase activity in HER2+ breast cancer cell lines. In addition, the noted inhibitory effect of HER2 on MLK3 kinase activity was mediated via its phosphorylation on Ser674 by AKT and that pharmacological inhibitors of PI3K/AKT prevented trastuzumab- and lapatinib-induced stimulation of MLK3 activity. Consistent with the pro-apoptotic function of MLK3, stable knockdown of MLK3 in the HER2+ cell line blunted the pro-apoptotic effects of trastuzumab and lapatinib. These findings suggest that HER2 activation inhibits the pro-apoptotic function of MLK3, which plays a mechanistic role in mediating anti-tumor activities of HER2-directed therapies. In brief, MLK3 represents a newly recognized integral component of HER2 biology in HER2+ breast tumors.

Breast cancer is the second leading cause of cancer death in women worldwide (1). Overexpression of epidermal growth factor receptor 2 (ErbB2/HER2/Neu) and of endocrine receptors (i.e. ER3 and PR) has been implicated in the initiation, progression, and maintenance of breast cancer cells growth (2, 3) and serves as a prognostic marker for breast cancer treatment (3−5). Breast cancer is molecularly a heterogeneous disease, where 65–75% cases are ER/PR-positive and 15–25% cases are HER2-positive (6).

The amplification of HER2 and endocrine receptors trigger multiple downstream signaling pathways to drive breast cancer cell survival, proliferation, and metastasis (7). Therefore, there are agents, either in clinical use or under development, to target these deregulated pathways downstream of amplified receptors to block uncontrolled breast cancer cell growth (3). The basic premise of targeting HER2-amplified breast cancer is to block the aberrant HER2 signaling by using Food and Drug Administration-approved trastuzumab or pertuzumab, humanized monoclonal antibodies against HER2, or a small molecule tyrosine kinase inhibitor, lapatinib, that blocks HER2 signaling and thus promotes cell death (8).

The pro-apoptotic actions of anti-hormonal receptor therapies are fairly known; however, the pro-apoptotic pathways, mediated via anti-HER2 therapies, are not well understood. It is reported that central to anti-HER2 therapies, blocking of the PI3K-AKT pathway downstream of the receptor is essential because most of the survival signals are mediated in part via activation of PI3K-AKT pathway (9, 10). Here we report a new function of a pro-apoptotic kinase MLK3 in mediating the pro-apoptotic actions of HER2-directed therapies.

MLK3 is a member of a larger mixed lineage kinase (MLK) family, and the members are unique in the sense that their catalytic domains contain signature sequences of both serine/threonine and tyrosine kinases (11). Previous works by us and others

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Background: Amplification of HER2 suppresses downstream pro-apoptotic pathways for breast cancer cell survival.

Results: The pro-apoptotic MLK3 kinase activity is suppressed by HER2.

Conclusion: MLK3 mediates HER2-induced breast cancer cell viability.

Significance: Understanding the mechanisms of MLK3 suppression by HER2 provides a basis to target MLK3 in HER2 positive breast cancer.

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have reported that MLK family members, including MLK3, activate c-Jun N-terminal kinase (JNK) (12). Furthermore, we also reported that AKT, a downstream target of PI3K, directly phosphorylates MLK3 on Ser674 residue, and this phosphorylation suppresses kinase activity and pro-apoptotic function of MLK3 (13).

In the present report we demonstrate that activation of HER2-mediated pathway inhibits MLK3 kinase activity and its pro-apoptotic function, contributing to an enhanced cell survival. Treatment of HER2+ breast cancer cell lines with HER2 inhibitors such as trastuzumab or lapatinib activates MLK3 kinase activity via inhibition of PI3K/AKT. The activation of MLK3 by trastuzumab or lapatinib was essential for their cytotoxic effects in HER2+ breast cancer cell lines. Moreover, the expression of constitutively active MLK3 resulted in suppression of HER2+ breast cancer cell viability. Interestingly, the expression of active-MLK3 (p-MLK3) was decreased in HER2+ human breast tumors and was further decreased in higher grade tumors. Taken together, our results demonstrate that inhibition of MLK3 by the HER2 pathway is one of the mechanisms for HER2-amplified breast cancer cells survival.

Experimental Procedures

Cell Culture and Treatments—Human ER−/PR−/HER2+ (SKBR3, HCC202, and HCC1954), ER−/PR−/HER2+ (MDA-MB-231, SUM159, and MDA-MB-468) breast cancer cell lines were purchased from ATCC, Manassas, VA. Cells were maintained in DMEM or RPMI1640 media containing 10% FBS, 2 mmol/liter glutamine and antibiotics (penicillin/streptomycin). Trastuzumab (10 μg/ml) (Genentech), lapatinib (1 μM), and erlotinib (100 nM) (Selleckchem) were treated for the indicated duration in cell culture media with 10% FBS. For PI3K/AKT inhibitors LY294002 (50 μM) (Calbiochem) and GDC-0941 (100 nM) (Selleckchem) treatment, cells were starved overnight in DMEM medium containing 2% FBS and pretreated for 2 h before trastuzumab treatment for 24 h. SKBR3 cells were treated with 100 ng/ml concentrations of human heregulin β-1 (Sigma) in DMEM medium with 10% FBS.

cDNA and siRNA Transfection—SKBR3 cells were transiently transfected either with FLAG-tagged MLK3 or FLAG-MLK3 (S674A) using Xtremegene-HP (Roche Applied Science). The endogenous Her1/2/3 were knocked down in SKBR3 cells using validated Her1/2/3 siRNAs (Accell SMARTpool) and the respective non-targeting control siRNAs, purchased from (Dharmacon/ThermoFisher Scientific Inc.) and transfected using Accell siRNAs delivery media (ThermoFisher Scientific Inc.) following the manufacturer’s instructions. To rule out any off-target effect of HER2 siRNA knockdown, second HER2 siRNA (Origene, Rockville, MD) was used along with negative control. Her1/2/3 silencing was verified by immunoblotting using specific antibodies.

Immunoblotting, Immunoprecipitation, and MLK3 Kinase Assay—Western blot analysis was performed following the protocols as reported earlier (13, 14) using primary antibodies against pMLK3 (Thr777/Ser281), EGF receptor, p-HER2 (Tyr1221/1222), HER2, HER3, pAKT (Ser473), AKT, ER-α (all from Cell Signaling Technology, Inc., Beverly, MA) and MLK3 (Abcam). The specific signals were finally detected using HRP-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagent. Endogenous MLK3 was immunoprecipitated by using an antibody raised against the C-terminal peptide of MLK3, developed in our laboratory. Ectopically expressed MLK3 was immunoprecipitated by using antibody against the FLAG-epitope-tag (Sigma). In vitro kinase assay was performed following our published protocol (12, 13). The incorporation of 32P into SEK1 kinase dead protein (SEK1 (K-R)) was quantified by phosphorimaging (STORM 820, GE Healthcare), and the kinase activity was presented as arbitrary phosphorimaging units.

Immunofluorescence and Immunohistochemistry—Immunofluorescence analyses were performed as described earlier (15). Briefly, after treatments, cells in chamber slides were fixed, permeabilized, and incubated with respective primary and Alexa Fluor-conjugated secondary antibodies. The nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) and photographed using a laser confocal microscope (Zeiss). For immune-histological analysis, a total of 46 cores from breast cancer array (Biomax) were analyzed for MLK3 and p-MLK3 expressions. The 46 cores analyzed were: 12, normal/non-malignant; 8, ER−/PR−/HER2−; 26, ER−/PR−/HER2+ cores represent: 4, grade I; 10, grade II; 12, grade III breast cancer tissues. The deparaffinized tissue array was blocked in goat normal serum and incubated overnight at 4°C with respective primary antibodies (pMLK3 and MLK3) followed by incubation with biotin-conjugated secondary antibodies for 1 h at room temperature. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and DAB (3, 3′-dianisobenzoindene) peroxidase substrate staining were used for visualization of pMLK3 and MLK3 as per the manufacturer’s protocol. The sections were counterstained with hematoxylin for nuclear staining and pictured under light microscope.

Stable MLK3 Knockdown Cell Line, Cell Viability, and Colony Formation Assay—The stable MLK3 knockdown in SKBR3 cells were created by utilizing validated MLK3-shRNA in lentiviral vector pLKO.1-Puro (Sigma). Lentivirus particles containing shRNAs were infected and selected with 0.6 μg/ml puromycin (Sigma). Knockdown of MLK3 expression was confirmed by PCR and Western blot analysis. Cell viability/proliferation was determined by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). For the assay, 2.5 × 103 cells were plated on 96-well plates and incubated overnight for cell attachment. Cells were treated for 48–72 h either with trastuzumab or PI3K inhibitors before analysis. For sequential treatment with PI3K inhibitors and trastuzumab, the PI3K inhibitors were pretreated for 4 h before trastuzumab treatment. Colony formation assay with SKBR3-WT and SKBR3 MLK3-knockdown stable cells were carried out by seeding (1000 cells per/well) into 6-well plates containing 10% FBS. Cells were treated with trastuzumab (30 μg/ml) and lapatinib (10 nM) every 2nd day up to 14 days in fresh DMEM containing 10% FBS and incubated at 37°C in a humidified, 5% CO2 atmosphere. The colonies were fixed in 4% paraformaldehyde and stained with crystal violet (0.05% w/v). The colonies were counted manually under a microscope in three different focusing zones and presented as the mean ± S.E. (% control).

Statistical Analysis—Data obtained from different sets of experiments are presented as the means ± S.E. Densitometry
analysis was performed using ImageJ 1.42 software (National Institutes of Health, Bethesda, MD). Two-way analysis of variance and Bonferroni’s multiple comparison test or unpaired Student’s t test were done using GraphPad Prism 5.0 (GraphPad Software Inc.). A p value < 0.05 was considered statistically significant.

Results

Down-regulation of Active MLK3 in HER2+ Breast Tumors and Cell Lines—To explore the potential role of MLK3 in HER2 biology and breast cancer pathogenesis, we examined the status of MLK3 and activated MLK3 (i.e. p-MLK3) in human breast tumors. There was no difference in MLK3 protein expression between HER2+ and HER2− tumors; however, the expression of p-MLK3 was significantly down-regulated in HER2+ tumors as compared with HER2− breast tumors (Fig. 1, A and B). Next, we examined the levels of MLK3 and p-MLK3 in different tumor grades of HER2+ breast tumors. Remarkably, the expression of MLK3 across all the grades of tumors was similar; however, the expression of p-MLK3 was progressively decreased and was least in Grade III tumors (Fig. 1C). To search for an experimental model system for mechanistic studies, we examined the expression of total and active MLK3 in HER2-positive and -negative cell lines. In agreement with the breast tumors, we found that the expression of p-MLK3 was significantly lower in HER2+ cell lines as compared with the levels in HER2− cell lines (Fig. 1E). Taken together, our results showed that MLK3 kinase activity is negatively regulated by HER2 pathway activation and, thus, might play a role in the progression of HER2+ breast cancer.

HER2 Negatively Regulates MLK3 Kinase Activity—Next, we sought to determine whether MLK3 kinase activity might be
negatively regulated by HER2 signaling. Because there is no known ligand of HER2, we therefore took an reverse approach to block the HER2-initiated signaling by using trastuzumab and lapatinib, Food and Drug Administration-approved drugs for HER2+/HER2- breast cancer patients. HER2+/HER2- cell lines SKBR3 and HCC1954 were treated either with 10 μg/ml trastuzumab or with 1 μM lapatinib for different period of times as indicated in Fig. 2. The endogenous MLK3 kinase activity was determined using the physiological substrate of MLK3 SEK1 (K-R) protein. There was a robust activation of the endogenous MLK3 kinase activity by both trastuzumab and lapatinib in a time-dependent manner (Fig. 2). The kinase activity of MLK3 started to increase by 6 h of trastuzumab treatment and peaked at 24 h (Fig. 2, A and C). However, the lapatinib treatment rapidly activated MLK3 kinase activity and peaked after 2 h (Fig. 2, B and D). To confirm that MLK3 activation is specifically mediated via HER2, cell line, MDA-MB-231 was treated with 1 μM lapatinib at different time points, and MLK3 kinase activity was determined. There was no activation of MLK3 kinase activity by lapatinib in the HER2- cell line (data not shown), suggesting that the effect of lapatinib on MLK3 activation is mediated via HER2. Furthermore, to rule out any role of the HER1/EGF receptor in MLK3 inhibition, the HER1 inhibitor, erlotinib treatment did not alter the activity of MLK3 (data not shown). Collectively, these results clearly showed that inhibition of HER2 pathway activates MLK3 kinase activity and, therefore, activation of HER2 pathway might inhibit MLK3 activity.

Inhibition of MLK3 Kinase Activity Is Mediated via HER2-HER3 Pathways—There is no known ligand for HER2; however, HER2 is reported to be activated either via homodimerization or heterodimerization with other HER family members (2). The homo- or heterodimerization of HER2 eventually initiates downstream signaling in cancer cells for their survival, proliferation, and invasion (2). Therefore, to further corroborate that the activation of HER2 pathway down-regulates MLK3 kinase activity and also to determine the nature of the HER family member(s) that dimerizes with HER2 in the observed negative regulation of MLK3, we knocked down HER1, HER2, and HER3 individually in SKBR3 cells and measured the MLK3 kinase activity. Our results clearly showed that knockdown of HER1 does not alter MLK3 kinase activity (Fig. 3B); however, knock-
down of HER2 alone was sufficient to activate MLK3 kinase activity to a similar extent, like trastuzumab and lapatinib treatments (Fig. 3A). To rule out any off-target effects of HER2 siRNA, we also used a second siRNA against HER2; here again, the HER2 knockdown activated MLK3 kinase activity (data not shown). Similarly, knockdown of HER3 also activated MLK3 kinase activity (Fig. 3B), suggesting that HER3 is probably the dimerization partner of HER2 in MLK3 kinase activity inhibition.

To further verify the role of HER3 as a dimerization partner and in MLK3 inhibition, we used heregulin, a ligand of HER3 to activate HER2/HER3-mediated downstream signaling. Treatment of SKBR3 cells with heregulin alone did not alter the basal activity of MLK3; however pretreatment of breast cancer cells with heregulin almost completely blocked trastuzumab- and lapatinib-induced MLK3 kinase activation (Fig. 3C). The immunofluorescence analysis of SKBR3 cells also confirmed that inhibitors of HER2 activate MLK3 kinase activity, whereas heregulin alone had no effect on MLK3 kinase activation (data not shown). These results suggested that HER2/HER3 or HER2/HER2 dimers mediate downstream signals leading to suppression of the MLK3 kinase activity.

HER2/HER3 Inhibits MLK3 Kinase Activity and Cell Death via the PI3K/AKT Pathway—It is widely believed that upon HER2/HER3 heterodimerization, the intrinsic tyrosine kinase activity of HER2 is activated and the intracellular kinase domain of HER2 trans-phosphorylates HER3 and creates a binding site for PI3K (16). The recruitment of PI3K to the HER3 intracellular domain activates downstream signaling, including activation of AKT for cell survival (17). Earlier we reported that AKT directly phosphorylates MLK3 on Ser674 residue, and this phosphorylation was necessary to suppress MLK3 kinase activity by estrogen (18) and IGF-1 (13). In these studies we also found that MLK3 inhibition by IGF-1 provides signals for cancer cell survival (13). To understand a potential role of PI3K/AKT pathway in HER2/HER3-mediated inhibition of MLK3 kinase activity, we pretreated SKBR3 cells with PI3K inhibitors, LY-294002 (19) and GDC-0941 (20) before treatment with trastuzumab. Pretreatment of SKBR3 cells with PI3K inhibitors completely blocked trastuzumab-induced activation of MLK3 kinase activity (Fig. 4A) probably via direct phosphorylation of MLK3 on Ser674 site. To corroborate any direct role of AKT in HER2/HER3-mediated inhibition of the MLK3 kinase activity, we overexpressed constitutively active AKT (i.e. Myr-AKT) in the SKBR3 cells and determined MLK3 kinase activity upon treatment with trastuzumab or lapatinib. Interestingly, overexpression of active-AKT completely blocked trastuzumab- and lapatinib-induced MLK3 kinase activation, suggesting that perhaps AKT activation downstream of HER2-HER3 inhibits the MLK3 kinase activity (Fig. 4B). To independently confirm a direct role of AKT in MLK3 kinase activity inhibition by the HER2/HER3 pathway, we transfected an MLK3 mutant lacking an AKT phosphorylation site (i.e. MLK3 S674A) (13) in SKBR3 cells. The MLK3 S674A mutant was not activated by trastuzumab, whereas MLK3-WT showed a robust activation (Fig. 4C) as expected from the earlier results. These results confirm that direct phosphorylation of MLK3 by AKT, downstream of the HER2/HER3 pathways, is necessary to suppress the MLK3 kinase activity in HER2+ breast cancer cells.

Because trastuzumab causes cell death in HER2+ cells and we have reported that MLK3 activation is required to induce cell death in breast cancer cells (18), we next determined the effect of PI3K/AKT inhibition on MLK3-mediated cell death by HER2 inhibitor trastuzumab. The overexpression of MLK3, which leads to constitutive activation of MLK3 kinase activ-
ity, or trastuzumab treatment alone, triggered significant cell death in the SKBR3 cells, which was further potentiated by PI3K/AKT inhibitors, LY-294002 and GDC-0941 (Fig. 4D). These results suggested that the activation of PI3K/AKT pathway downstream of HER2/HER3 inhibits MLK3 kinase activity as well as cytotoxic effects of trastuzumab in HER2+ breast cancer cells.

MLK3 Is Necessary for Cell Death by HER2-directed Therapies—To determine a physiological role of MLK3 in HER2-directed therapies, we generated HER2+ SKBR3 stable clones, where MLK3 was stably knocked down. The stable MLK3 knockdown clones showed a complete absence of MLK3 protein (Fig. 5A). Next, we determined the viability of these cells as a function of the trastuzumab dose. The parental SKBR3 cells were sensitive to trastuzumab-induced cell death in a dose-dependent manner; however, the MLK3 knockdown cells showed complete resistance to trastuzumab-induced cell death, even at a much higher dose (100 μg/ml; Fig. 5B). We also determined the colony formation ability of these stable cell lines, and again the parental cell line showed a significantly low number of colonies compared with MLK3 knockdown cells in the presence of trastuzumab or lapatinib (Fig. 5C). Specially, the lapatinib almost completely abolished the SKBR3 parental cell colonies, whereas large numbers of MLK3 knockdown colonies were resistant to cell death by lapatinib (Fig. 5, C and D). Taken together, these results supported the finding that MLK3 plays an important role in promoting the survival of HER2+ breast cancer cells and
that suppression of MLK3 kinase activity by HER2 contributes to enhanced breast cancer cell survival.

Discussion

The receptor-tyrosine kinase HER2 serves as a prognostic marker and is targeted with agents that primarily relegate hyperactive downstream signaling pathways in breast cancer cells to promote cell death (8). Multiple lines of evidence suggest that the common endocrine therapies in breast cancer are associated with hyperactivation of RTK signaling pathways as well as therapeutic resistance and cell survival. In addition to HER2 amplification in breast cancer, IGF-1 receptor is also amplified in the majority (90–95%) of breast cancers (21). These observations suggest that the HER2 and IGF-1 receptor-mediated pathways might be mechanistically linked in conferring breast cancer cell survival, therapies resistance, and recurrence. Therefore, investigating the nature of a nodal molecule could resolve the concurrent compensatory roles of these receptors in breast cancer cell survival and can provide a viable future target for HER2-directed therapies.

Here we report a common downstream target that is reported to be down-modulated by estrogen ER (18) as well as by IGF1-IGF-1 receptor (13) through a shared mechanism. In addition, we also reported recently that MLK3 kinase activity was down-regulated by IGF-1 and estrogen via activation of PI3K/AKT pathway (11). Specifically, the AKT activation, either by IGF-1 or estrogen directly, phosphorylates MLK3 on the Ser674 site (13), and this phosphorylation suppresses the MLK3 kinase activity and prevents cancer cell death (13). Because HER2/HER3 dimerization also promotes cell survival, in part via PI3K/AKT activation (9, 10), we therefore explored the role of HER2 in MLK3 regulation and its effect on the cell survival. Our results suggest that HER2 inhibits MLK3 kinase activity and its pro-apoptotic function in breast cancer cells.

The initial clue that activation of the HER2 pathway might down-regulate MLK3 kinase activity, stemmed from our original observation that in HER2+ breast tumors, the expression of activated MLK3 (p-MLK3) was significantly lower compared with HER2− breast tumors, whereas there was no change in the levels of total MLK3 (Fig. 1A). These results suggest the decrease in p-MLK3 signal in HER2+ tumors is conceivably due to post-translational modification of MLK3 (Fig. 1A). We modulated HER2 signaling using trastuzumab and lapatinib, two Food and Drug Administration-approved HER2 inhibitors...
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in HER2+ breast cancer patients. We found that the treatment of HER2+, SKBR3, and HCC1954 cell lines with trastuzumab and lapatinib activated the kinase activity of MLK3 (Fig. 2). In contrast, the lapatinib treatment failed to activate MLK3 kinase activity in the HER2−, MDA-MB-231 breast cancer cell line. Furthermore HER2 amplification inhibited MLK3 kinase activity as knockdown of HER2 or HER3 activated MLK3 kinase activity (Fig. 3, A and B). These results also suggest that HER3 is the preferred partner of HER2 in SKBR3 cells because HER1 knockdown had no effect on MLK3 activation (Fig. 3B).

The anti-tumor actions of trastuzumab and lapatinib are mediated in part on their ability to down-regulate PI3K/AKT signaling (9, 10, 22). We examined whether the PI3K/AKT pathway directly impacts on the pro-apoptotic function of MLK3 in a HER2+ cell line. The fact that overexpression of MLK3 itself caused significant cell death in the HER2+ cell line (Fig. 4D), which was further synergized with PI3K/AKT inhibitors, suggests that indeed the PI3K/AKT pathway mediates pro-survival signals in HER2+ breast cancer cells, in part, via inhibition of MLK3 activity (this study). Our results suggest that MLK3 activity is necessary for HER2-directed therapies-induced cell death (Fig. 5).

In summary, our results suggest that the HER2 pathway down-regulates MLK3 kinase activity via activation of PI3K/AKT pathway. More specifically, the inhibition of MLK3 kinase activity by the HER2 pathway was mediated via direct phosphorylation of a common serine residue (i.e. Ser674) by AKT. We propose that MLK3 may be a nodal point by which breast cancer cells survive via activation of PI3K/AKT pathway by HER2 and IGF-1 receptor and perhaps other cell surface receptor-initiated signaling (Fig. 6). Therefore, targeting MLK3 by using its activator could block the escape pathways that promote breast cancer cell survival, therapy resistance, and metastasis.

**Author Contributions**—A. R., S. D., C. O., B. R., and G. T. conceived and designed the work. A. R., S. D., and C. O. helped to develop the methodologies. S. D., G. S., R. S. N., and N. V. helped in acquisition and interpretation of data. A. R., S. D., C. O., B. R., and G. T. analyzed the data. A. R. and S. D. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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