Human epidermal growth factor receptor 4 (Her4) Suppresses p53 Protein via Targeting the MDMX-MDM2 Protein Complex

IMPLICATION OF A NOVEL MDMX SER-314 PHOSPHOSITE

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Deregulated receptor tyrosine kinase (RTK) signaling is frequently associated with tumorigenesis and therapy resistance, but its underlying mechanisms still need to be elucidated. In this study, we have shown that the RTK human epidermal growth factor receptor 4 (Her4, also known as Erbb4) can inhibit the tumor suppressor p53 by regulating MDMX-mouse double minute 2 homolog (MDM2) complex stability. Upon activation by either overexpression of a constitutively active vector or ligand binding (Neuregulin-1), Her4 was able to stabilize the MDMX-MDM2 complex, resulting in suppression of p53 transcriptional activity, as shown by p53-responsive element-driven luciferase assay and mRNA levels of p53 target genes. Using a phospho-proteomics approach, we functionally identified a novel Her4-induced posttranslational modification on MDMX at Ser-314, a putative phosphorylation site for the CDK4/6 kinase. Remarkably, inhibition of Ser-314 phosphorylation either with Ser-to-Ala substitution or with a specific inhibitor of CDK4/6 kinase blocked Her4-induced stabilization of MDMX-MDM2 and rescued p53 activity. Our study offers insights into the mechanisms of deregulated RTK-induced carcinogenesis and provides the basis for the use of inhibitors targeting RTK-mediated signals for p53 restoration.

p53 is either mutated or functionally inactive in most human cancers. Several tumor types that retain wild-type p53 (i.e. breast cancer) display higher protein levels of MDMX and/or MDM21 (1–8), the primary inhibitors of p53, representing alternative mechanisms of p53 inactivation. Typically, p53 protein levels are tightly controlled and modulated by its principle negative regulator, the MDMX-MDM2 complex. Given that MDMX and MDM2 are amplified in many tumors, modulation of their stability offers an attractive therapeutic strategy to restore p53 function and so improve tumor responsiveness to DNA-damaging therapy.

Although MDM2 is the main negative regulator of p53, there is now growing evidence supporting the notion that MDMX also plays a key role in modulating p53, mainly through its stabilization of MDM2 (9). In fact, the MDMX-MDM2 heterocomplex is the main form in which the two proteins are found in the cell (10, 11), and loss of this formation leads to p53 activation and embryonic lethality (9, 11–13).

Under non-stressed conditions, MDMX and MDM2 suppress p53 activity. However, under stress conditions, MDMX and MDM2 are posttranslationally modified and disabled for their inhibition of p53. As a result, p53 can then respond to the stress and repair any damage. MDM2 and MDMX are structural homologs, with the major distinction that MDM2 contains an E3 ligase domain, a nuclear localization signal domain, and a nuclear export signal domain (14). There are two major ways in which p53 activity can be suppressed by the complex. First, MDM2 and MDMX can cooperatively bind to the transcriptional domain of p53 and suppress its transcriptional activity. Second, MDM2 can act as an E3 ligase and induce p53 ubiquitination and subsequent proteasomal degradation (15).

Whether p53 inhibition by MDM2 and MDMX occurs through protein degradation or suppression of its transcriptional activity, one key aspect of this regulatory core is that p53 inhibition is more efficient when MDM2 and MDMX act as a heterocomplex, which is partly explained by the ability of MDMX to stabilize MDM2 and stimulate its intrinsic ligase function (11, 16).

Deregulation of receptor tyrosine kinase (RTK) signaling is a recurring feature in many tumors because it allows cancer cells to overrun tightly controlled cellular homeostatic processes, such as proliferation and apoptosis, leading to tumor development and therapy resistance. In particular, the EGF receptor family plays an important role in the initiation and maintenance of many tumors (17). Among its members, Her4 (Erbb4) is rarely mutated in human cancers and has been shown to display different expression patterns (18). These discrepancies among the reported data may be attributed to the diversity in Her4 signaling, stemming from mRNA splice variants (19) and variance in antigen site recognition by different antibodies that give different staining patterns (20). However, recent studies...
have generated a new interest to understand the mechanisms of action of Her4 in cancer. For instance, Her4 overexpression has been shown to stimulate breast cancer growth and transform mouse mammary cells into tumors both in vivo and in vitro (21–23). Also, the development of better Her4 antibodies with location specificity has prognostic significance in breast cancer (24–26). Unlike other EGF receptors, Her4 is processed into intracellular spliced variants. Full-length Her4 contains two sites that are cleaved upon ligand binding. The sequential cleavage process starts with a ligand-dependent proteolysis, then cleavage by the \( \beta / H9253 \)-secretase enzyme to produce a kinase-active cleaved product (23, 27).

Given the potential role of Her4 in breast cancer development, we investigated the effect of Her4 on the MDMX-MDM2 complex and its influence on p53 activity in MCF-7 cells. Our findings suggest that Her4 influences the stability of the MDMX-MDM2 complex, leading to decreased p53 activity, through a process that is at least partly mediated by the novel phosphorylate Ser-314 on MDMX. The GPS kinase prediction software indicated CDK4/6 as a candidate kinase of the phosphosite. This prediction was supported by the abrogation of the Her4-mediated stabilization of MDMX and MDM2 when CDK4/6 was inhibited, leading to reactivation of p53. These findings suggest that Her4 induces phosphorylation of MDMX at Ser-314, likely through CDK4/6, resulting in MDMX-MDM2 stabilization and, hence, suppression of p53 activity.

Results

Her4 Overexpression Stabilizes the MDMX-MDM2 Complex and Suppresses p53—By screening an RTK library of constitutively active kinases in search of those capable of influencing the binding efficiency between MDMX and MDM2, Her4 was identified as a positive candidate. To validate this finding, various combinations of different expression vectors of MDMX, MDM2, and Her4 were cotransfected into HEK293T cells (Fig. 1A). Coexpression of MDMX and MDM2 resulted in increased...
levels of MDM2 compared with its level when expressed singularly, whereas their coexpression with Her4 resulted in higher levels of both MDMX and MDM2.

To substantiate the effect of Her4 on MDMX and MDM2, HEK293T cells transfected with MDMX, FLAG-MDM2, and either with or without Her4 were immunoprecipitated using FLAG-conjugated beads. It is important to point out that our FLAG-MDM2 is tagged at the N-terminal, given that C-terminal tagging of MDM2 affects heterodimer formation with MDMX. As shown in Fig. 1B, the presence of Her4 caused a greater pulldown of MDM2 along with MDMX. The same transfection result was obtained in MCF-7 cells by stimulating endogenous Her4 with NRG1 (Fig. 1C). This augmented MDMX-MDM2 complex suggests that the presence of Her4 would decrease p53 activity by increasing the levels of MDMX and MDM2. This was tested using a cell line harboring wild-type p53, U2OS, in which p53 activity was measured using a p53-responsive element-driven LacZ plasmid. Indeed, Her4 expression was associated with the inhibition of ionizing radiation (IR)-induced p53 activation (Fig. 1D). To complement such findings, p53 activity was measured in MCF-7 cells using a luciferase reporter containing 13 copies of the p53-binding consensus sequence (pg13). In line with the results obtained with U2OS cells, p53 transcriptional activity in MCF-7 was suppressed by Her4 in a concentration-dependent manner (Fig. 1E).

To assess the effect of Her4 on the localization of MDM2 and MDMX, U2OS cells were transfected with various combinations of MDMX, MDM2, and/or Her4 and evaluated by immunocytochemistry. As MDMX is normally found in the cytoplasm and MDM2 in the nucleus, their coexpression resulted in the relocalization of MDMX into the nucleus with MDM2 (Fig. 2A) and promoted the cytoplasmic concentration of the MDMX-MDM2 complex. We next set out to see the effect these results had on p53 localization. To visualize p53 in a system that can lead to its degradation by overexpression of the MDMX-MDM2 complex, a p53 construct with a deletion at amino acid position 92–112 was used because the region was identified previously as essential for MDM2-mediated p53 degradation. Although MDMX-MDM2 coexpression resulted in the nuclear export of p53, Her4 considerably concentrated p53 into the cytoplasm (Fig. 2B). Taken together, these results imply that Her4 increases the level of MDMX-MDM2, which inactivates p53 by promoting its nuclear export and degradation.

Her4 Ligand, NRG1, Stabilizes Endogenous MDMX and MDM2 and Decreases p53 Levels—To corroborate the results derived from experiments with Her4 overexpression, we tested the effect of endogenous Her4 stimulation. To this end, MCF-7 cells were treated with 50 ng/ml NRG1 to evaluate the effects on Her4, MDMX/2, and p53. As published previously (28), Her4 activity was determined indirectly by its cleavage and also by using an antibody that recognizes Her4 in its phosphoactive form (Fig. 3A). A similar stimulation experiment was performed using EGF, a ligand of EGFR. Results showed that EGF failed to induce the same effect produced by NRG1 (Fig. 3A).

To verify that the effect of NRG1 was specific to Her4, we performed a stimulation experiment with MCF-10A cells that lack Her4 expression (Fig. 3B, top panel). In these cells, NRG1 treatment had little effect on the levels of MDM2 (Fig. 3B, bottom panel), contrarily to what we observed in MCF-7 cells. To further verify the specificity of the NRG1 effect, we used a γ-secretase inhibitor to prevent Her4 activation by NRG1. In MCF-7 cells pretreated with the γ-secretase inhibitor, NRG1 did not stabilize the levels of MDMX and MDM2 (Fig. 3C), consistent with a requirement of Her4 activation for induction of the MDMX-MDM2 complex.

Kinetic studies on Her4 activation and MDMX/2 levels were performed with NRG1 on MCF-7 (Fig. 3, D–F). NRG1 was able to induce Her4 phosphorylation as early as 20 min post-stimulation (Fig. 3D) and gradually increase MDMX and MDM2 levels over the course of 48 h (Fig. 3, E and F). There was also a
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correlation between the intensity of Her4 activation and the increased levels of the MDMX-MDM2 complex. The fact that Her4 activation increases the protein abundance of exogenously expressed MDMX and MDM2 suggests a mechanism of posttranscriptional regulation. We validated this notion by measuring the level of MDMX transcripts in MCF-7 cells treated with NRG1 at different time points (6, 12, and 24 h). The result showed no statistically significant difference in MDMX mRNA levels after NRG1 treatment at any of the time points (Fig. 3G), consistent with sole regulation at the posttranscriptional level. We next explored the possibility of Her4-mediated modulation of protein turnover by determining the half-life of MDMX protein. MCF-7 cells were treated with NRG1 or solvent control for 24 h, washed, and cultured with fresh...
medium containing cycloheximide to inhibit protein synthesis. The cells were harvested at the indicated time for Western blotting analysis to monitor the disappearance of MDMX (Fig. 3H). We focused on the turnover of MDMX because our previous studies as well as those from others showed that the stability of the MDMX-MDM2 complex is primarily controlled by MDMX, which binds to and stabilizes MDM2 (32). The result of the anti-MDMX immunoblot revealed a considerably increased half-life of MDMX in NRG1-treated cells compared with the solvent controls. Collectively, the results indicate that Her4 activation was associated with stabilization of the MDMX-MDM2 complex.

**Her4 Stabilization of the MDMX-MDM2 Complex Causes a Reduction in p53 Protein Levels and Transcriptional Activity**—Having shown that Her4 can stabilize the MDMX-MDM2 complex, we wanted to evaluate whether this effect of Her4 would affect the p53 response to DNA-damaging agents. To this aim, MCF-7 cells were transfected with a Her4 constitutively active vector and irradiated 48 h later with a dose of 3 Gy. The results from the Western blotting analysis showed that MDMX and MDM2 levels were higher in Her4-transfected cells compared with the empty vector control prior to radiation treatment (Fig. 4A). Although, under DNA damaging conditions, Her4 induced slightly higher levels of the MDM complex, the 3-Gy treatment-induced p53 was considerably impeded in Her4-expressing cells relative to the control. The decrease of MDMX in IR-treated cells was caused by MDM2-mediated degradation, as shown previously by our group and others (29).

To extend this observation to endogenous Her4, MCF-7 cells werepretreated with NRG1 to induce sustained activation of this receptor. Cells were then treated with radiation or 5’ FU. NRG1 treatment increased the abundance of the MDMX-MDM2 complex and diminished p53 induction by irradiation (Fig. 4B). A similar effect on 5’FU-induced p53 activation was observed, where NRG1 pretreatment resulted in considerably weakened p53 induction (Fig. 4C). We validated Her4 ligand specificity by correlating a decrease in p53 levels with an increase in MDMX, MDM2, and Her4 phosphorylation after NRG1 treatment compared with EGF (Fig. 4D). Using RT-qPCR to quantify the transcript levels of MDM2 and p21, we detected significantly reduced p53 transcription activity in NRG1-treated cells, corroborating the compromised induction of p53 protein abundance (Fig. 4E). Altogether, these data demonstrate that Her4 activation is associated with considerably diminished p53 activation by 5’FU and radiation.

**Her4 Increases MDMX-MDM2 and Suppresses p53 Activity**—To extend this observation to endogenous Her4, MCF-7 cells were treated with NRG1 for 6, 12, and 24 h, followed by Western blotting analysis of the cell lysates with the indicated antibodies. Expression of Her4 (top panel) C. MCF-7 cells werepretreated with or without γ-secretase inhibitor for 45 min, followed by 6 h of NRG1 treatment. Western blotting analysis was carried out with the indicated antibodies. D. Early time points. MCF-7 cells expressing endogenous Her4 were treated with 50 ng/ml of NRG1 for 20 and 40 min, and then extracted lysates were subjected to Western blotting analysis with the indicated antibodies. E, MCF-7 cells were treated with 50 ng/ml of NRG1 for 30 min, 1 h, and 12 h. The lysates were then subjected to Western blotting analysis with the indicated antibodies. F, late time points. MCF-7 cells were treated with 50 ng/ml of NRG1 for 6, 12, 36, and 48 h, followed by Western blotting analysis with the indicated antibodies. G, MCF-7 cells were treated with NRG1 for 6, 12, or 24 h. RNA samples were collected for RT-qPCR analysis of MDMX expression. Data are shown as the mean ± S.D. NS, not significant; H, MCF-7 cells were treated with NRG1 or solvent (control condition) for 24 h in DMEM/10% FBS, washed, and cultured with fresh medium containing cycloheximide (CHX, 10 μg/ml) to inhibit protein synthesis. The cells were harvested at the indicated time for Western blotting analysis to monitor the disappearance of MDMX. A–G, cells were treated with or without NRG1/EGF in DMEM/1% FBS.

**CDK4/6 Mediates Her4 Stabilization of MDMX-MDM2 and Its Suppression of p53 Activation by DNA Damage**—Figure 3. Her4 activation after NRG1 treatment increases MDMX levels, leading to the stabilization of MDM2. A, MCF-7 cells were treated with 50 ng/ml of NRG1 for Her4 activation or EGF for EGFR activation over a course of 3 h and then harvested for Western blotting analysis with the indicated antibodies. B, untreated MCF-7 and MCF-10A cells were compared for total Her4 and tubulin by Western blotting analysis (top panel). C, MCF-7 cells werepretreated with or without γ-secretase inhibitor for 45 min, followed by 6 h of NRG1 treatment. Western blotting analysis was carried out with the indicated antibodies. D, early time points. MCF-7 cells expressing endogenous Her4 were treated with 50 ng/ml of NRG1 for 20 and 40 min, and then extracted lysates were subjected to Western blotting analysis with the indicated antibodies. E, MCF-7 cells were treated with 50 ng/ml of NRG1 for 30 min, 1 h, and 12 h. The lysates were then subjected to Western blotting analysis with the indicated antibodies. F, late time points. MCF-7 cells were treated with 50 ng/ml of NRG1 for 6, 12, 36, and 48 h, followed by Western blotting analysis with the indicated antibodies. G, MCF-7 cells were treated with NRG1 for 6, 12, or 24 h. RNA samples were collected for RT-qPCR analysis of MDMX expression. Data are shown as the mean ± S.D. NS, not significant; H, MCF-7 cells were treated with NRG1 or solvent (control condition) for 24 h in DMEM/10% FBS, washed, and cultured with fresh medium containing cycloheximide (CHX, 10 μg/ml) to inhibit protein synthesis. The cells were harvested at the indicated time for Western blotting analysis to monitor the disappearance of MDMX. A–G, cells were treated with or without NRG1/EGF in DMEM/1% FBS.
Her4 signal (35). With the search criteria based on high stringency, the software identified two candidate kinases, CDK4/6 and p38 (Fig. 6A). To validate the prediction, we pretreated MCF-7 cells with inhibitors specific to each predicted kinase. CDK4/6 inhibitor IV impeded NRG1 stabilization of the MDMX-MDM2 complex (Fig. 6B), whereas the p38 inhibitor (doramapimod) did not appear to have a significant effect on MDMX levels despite inhibiting its target phosphorylation on p38 (Fig. 6C). A kinase assay was performed using CDK4 active or kinase dead along with wild-type MDMX or MDMX (S314A). The result confirmed that CDK4/6 directly phosphorylated MDMX (Fig. 6D).

Having shown that Her4 can induce the phosphorylation of Ser-314 on MDMX, likely through CDK4/6, we tested the role of this cyclin kinase along this signaling axis by pretreating MCF-7 cells with NRG1 for 24 h with or without the CDK4/6 inhibitor. Consistent with the negative effect of Her4 on the p53 response, NRG1 treatment hampered 3-Gy IR-induced p53 activation, as reflected by substantially reduced mRNA levels of MDM2 and p21. Remarkably, addition of the CDK4/6 inhibitor partly prevented the effect of NRG1 on p53 inhibition, leading to nearly complete restoration of IR-induced p53 transcriptional activity (Fig. 6E).

Taken together, our results show that Her4 displays its oncogenic behavior at least in part by phosphorylating MDMX on Ser-314 in a CDK4/6-dependent manner. This, in turn, leads to increased stability of MDMX and the MDMX-MDM2 heterocomplex, resulting in p53 inactivation as outlined in Fig. 7.

**Discussion**

Given its growth inhibitory role, p53 is either mutated or functionally inactive in most tumors. It is therefore important to understand the mechanisms behind p53 loss to gain insights in both tumorigenesis and therapy resistance.

Deregulated RTK signaling is common in breast cancer, where p53 mutation frequency is relatively low, implicating
mechanisms other than gene mutation in p53 inactivation. In line with this notion, we uncovered a novel role of Her4 in stabilization of the MDMX-MDM2 heterocomplex leading to p53 inactivation. Interestingly, Her4 has been gaining recognition for its role in breast cancer, and it has been shown to mediate resistance to treatments against Her2-positive breast cancers (25). Activating oncogenic Her4 mutations also exist in advanced esophageal or head and neck squamous cell carcinoma (37). It is therefore fitting to extend our understanding of the Her4 pathway in wild-type p53 breast cancer cells to delineate the signaling circuitry for MDM heterocomplex formation.

Her4 Increases MDMX-MDM2 and Suppresses p53 Activity

A

\begin{tabular}{|c|c|c|}
\hline
 & FNSPSKR Ser314 & TISAPVVRPK Ser367 \\
\hline
MDMX/2 & 30 & 13 \\
MDMX/2+ Her4 & 85 & 17 \\
\hline
\end{tabular}

B

\begin{itemize}
\item Empty-vector
\item MDMX
\item MDMXmS314A
\item MDM2
\item Her4
\end{itemize}

C

\begin{itemize}
\item + + + MDMX
\item + + MDMXmS314A
\item + + Her4
\end{itemize}

D

\begin{itemize}
\item Empty-vector
\item 0.5ug Her4-FLAG
\item 1ug Her4-FLAG
\end{itemize}

E

\begin{itemize}
\item Empty-vector
\item MDMX
\item MDMXmS314A
\item Her4
\end{itemize}
Her4 Increases MDMX-MDM2 and Suppresses p53 Activity

Our studies showed that overexpression of a constitutively active Her4 leads to increased levels of both MDMX and MDM2. A similar effect was observed by stimulating Her4 in MCF-7 cells with its ligand, NRG1. This effect of Her4 seemed to be mediated by a mechanism of posttranscriptional regulation, as there was little change at the transcript level. Consistent with this notion, we observed an increased half-life of the MDMX protein in response to Her4 activation. Through binding with MDM2, the increased MDMX level led to elevated abundance of the MDMX-MDM2 heterocomplex. The functional significance of Her4-induced stabilization of the MDMX-MDM2 complex was highlighted by the reduced protein levels and activity of p53 and markedly impaired p53 activation in response to DNA damage. Our data imply that Her4 contributes to tumorigenesis and therapy resistance by interfering in the p53 pathway.

The role of Her4 in breast cancer development has been considered ambiguous, partly because of a lack of mechanistic knowledge about its mode of action (21, 31–35). In this context, our study sheds light on the mechanism of action of Her4 by showing that its active form is required to stabilize the MDMX-MDM2 complex, as we demonstrated using a γ-secretase inhibitor to prevent Her4 activation in response to NRG1 stimulation. This is consistent with previous studies where the γ-secretase inhibitor suppressed NRG1-dependent tumor growth (27, 36). In fact, Her4 intracellular spliced variants were shown to be prominent in several cancer samples (21, 24, 37). This may be a possible explanation for the immunohistochemistry studies showing that the prevalence of intact Her4-positive expression in the increased survival rate of breast cancer patients (38).

In an effort to identify the signaling events that linked Her4 activation to the stabilization of MDMX-MDM2, we performed an LC/MS-MS analysis of the MDMX-MDM2 complex and identified the novel phosphosite Ser-314 on MDMX. The significance of the site was evident upon its mutation because Her4-induced stabilization of the complex was almost completely lost. Among the only two candidate kinases predicted by the model, p38 and CDK4/6, only CDK4/6 was shown to mediate the effect of Her4 on the stability of the MDMX-MDM2 complex (Fig. 6B), leading to suppression of p53 activity (Fig. 6E). Interestingly, the CDK4/6 pathway is functional in many cancers that retain wild-type p53 and have elevated levels of MDM proteins, such as breast cancer, prostate cancer, and colon cancer (39–41). Specifically, recent studies showed that inhibition of the CDK4/6 pathway can be effective against breast cancer (39, 42). In fact, the CDK4/6 kinase appeared to be the converging point of various cross-talking oncogenic pathways because inhibition of one pathway upstream of CDK often could not effectively block CDK activity because of signals from other pathways (42). This may also be the mechanism by which Her4 acquires resistance to the Her2 inhibitor trastuzumab in Her2-positive breast cancer (25). In this context, our results suggest that the stabilization of the MDMX-MDM2 complex is the converging point of oncogenic signals (i.e. Her4 activation) through CDK4/6.

As our key observation, we present Her4 phosphorylation of the novel Ser-314 site on MDMX through mediation of CDK4. However, we should consider that signaling pathways are rarely linear. This partially explains the lack of full suppression in the case of our MDMX(S314A) expression vector and CDK4 in vitro kinase assay. The partial inhibition of total serine phosphorylation on MDMX S314A implies that CDK4/6 might phosphorylate other sites (Fig. 6D). Another indication of the non-linearity of the signaling pathway is the fact that, as shown in Fig. 6E, the CDK4/6 inhibitor failed to fully abrogate the NRG1-induced suppression of p53. A reason for this could be provided by our phosphodata (Fig. 5A). Apart from the prominent Ser-314 site, the second most phosphorylated site influenced by Her4 was Ser-367, which has been shown previously to be phosphorylated by Akt, leading to MDMX stabilization (30). Given that NRG1/Her4 is capable of activating the Akt pathway (43) and that we observed an increase in the Akt-targeted Ser-367 phosphosite on MDMX, we speculate that the lack of a full rescue by the CDK4/6 inhibitor may be due to the effect of the phosphosite Ser-367.

We present here, to the best of our knowledge, the first report of Her4 mediating the stabilization of MDMX via phosphorylation of a novel MDMX Ser-314 site, likely through CDK4/6. Interestingly, the zinc finger domain of MDMX does not bind with any region of MDM2, but it has been shown to play a role in kinase signaling (44). In particular, it was shown to be essential for interaction with CK1ε and, hence, to be necessary for stimulating MDMX-p53 binding. Based on our results and considering the role of the MDMX zinc finger domain in transducing kinase signaling, we propose a model in which Her4 activates CDK4/6, leading to MDMX phosphorylation on Ser-314. This phosphorylation event causes a conformational change in MDMX that increases its stability. MDMX, in turn, binds to and stabilizes MDM2, resulting in stabilization of the MDMX-MDM2 complex and enhanced suppression of p53 activity (Fig. 7).

FIGURE 5. Her4 stabilizes MDMX through the phosphorylation of Ser-314, leading to MDM2 stabilization and p53 degradation. A, i, peptide count of the phosphosites Ser-314 and Ser-367 of MDMX in FLAG-MDMX and MDM2 versus FLAG-MDMX-, MDM2-, and Her4-transfected HEK293T cells (left panel, # designates the phosphorylation site to the left amino acid). Right panel, representative MS-MS spectrum sample of the phosphorylation event on MDMX Ser-314. ii, MDMX phosphorylation map localizing various sites, including the reported site Ser-367 (red) and the novel phosphosite Ser-314 (yellow). B, HEK293T cells were transfected with various combinations of expression vectors for wild-type MDMX, mutant MDMX S314A, MDM2, and Her4 and then harvested 48 h later for Western blotting analysis using the indicated antibodies. I.E., low exposure; H.E., high exposure. C, HEK293T cells were transfected for 24 h with either wild-type MDMX or mutant MDMX S314A with or without overexpression of Her4. Cell lysates were incubated with GST-MDM2 and then pulled down by GST beads. To compare binding affinity, the amount of MDMX protein was adjusted so comparable abundances of MDMX protein in Her4-transfected and non-transfected cells were used to incubate with GST-MDM2. Western blotting analysis was carried out with the indicated antibodies. D, MCF-7 cells were transfected with or without 0.5 or 1 μg Her4 and then harvested after 24 h for Western blotting analysis with the indicated antibodies. E, U2OS cells were transfected with various combinations of expression vectors for wild-type MDMX, mutant MDMX S314A, and Her4 and then harvested after 24 h for Western blotting analysis using the indicated antibodies.
Experimental Procedures

Cell Culture and Transfection—HEK293T, U2OS, MCF-7, and MCF-10A cells (American Type Culture Collection) were seeded in tissue culture flasks and cultured until confluence in, respectively, DMEM, McCoy medium, or DMEM/F-12 supplemented with 10% FBS (HyClone) and antibiotics at 37 °C in a humidified atmosphere of 5% CO2/95% air. Cells were pretreated with DMEM 1% FBS for 4 h and then stimulated with 50 ng/ml NRG1 for 6 h. D, a kinase assay was carried out using immunopurified FLAG-tagged wild-type MDMX or mutant MDMX S314A incubated with cell lysates expressing constitutively active CDK4 or kinase-dead (K.D.) CDK4 in the presence of ATP. The products were analyzed by Western blotting analysis using anti-phosphoserine antibody (top panel) and anti-MDMX antibody (bottom panel). E, MCF-7 cells were treated for 24 h with or without 5 μM CDK4/6 inhibitor IV, followed by 6-h treatment with 50 ng/ml NRG1. Cells were then irradiated with 3 Gy, and RNA samples were collected 3 h after irradiation for RT-qPCR analysis of MDM2 and p21 mRNA expression. Data are shown as the mean ± S.D. *p < 0.05; t-test comparing treatments versus control; n = 3. Cells were treated with or without NRG1 in DMEM/1% FBS.

Preparation of Whole Cell Extracts and Western Blotting Analysis—Between 50 and 100 μg of cellular protein was extracted into radioimmune precipitation assay buffer or SDS-
PAGE loading buffer from control and treated cells. The samples were incubated at 95 °C for 10 min and resolved by SDS-PAGE through 10% gels (16 × 20 cm, final concentration of acrylamide) under reducing conditions and transferred onto nitrocellulose membranes (Schleicher & Schüll). Following blocking with 5% milk TBS-Tween (TBS-T), the membranes were incubated overnight at 4 °C with the primary antibody in TBS-T. The second anti-rabbit antibody-HRP conjugate (Cell Signaling Technology, Danvers, MA; 1:2000 dilution) was subsequently incubated with membranes for 1 h at room temperature, washed extensively for 30 min with TBS-T, and given a final rinsing with TBS-T at room temperature. Membranes were probed with the following antibodies as indicated: anti-MDMX (BL1258, Bethyl Laboratories), anti-MDM2 (Santa Cruz Biotechnology), anti-HER4 (Cell Signaling Technology), anti-phospho-HER4 (Millipore), anti-FLAG M2 (Sigma), anti-p53 (Cell Signaling Technology), anti-phospho-tyrosine (9411, Cell Signaling Technology), and anti-β-actin (AC-15, Sigma).

Proteins were visualized with an enhanced chemiluminescence detection system (PerkinElmer Life Sciences).

**Immunoprecipitation Analysis**—Cells were lysed on a rocking platform for 1 h with cold 1× lysis buffer (20 mM Tris (pH 7.6), 150 mM NaCl, 1 mM Na₂ EDTA, 1% Nonidet P-40, and Roche protease and phosphatase inhibitor) at 4 °C. The cell lysate was then sonicated at 15-watt output for 5 s and centrifuged for 10 min at 13,500 × g at 4 °C. Protein concentration was determined using the Pierce BCA protein assay (Thermo Scientific). Between 500 μg to 1 mg of protein was incubated with anti-FLAG M5 beads (Sigma) for 4 h at 4 °C. After centrifugation, the supernatant was removed, and the beads were washed five times with 500 μl of wash buffer (50 mM Tris buffer, 100 mM NaCl, 0.5% Nonidet P-40, and 1 mM DTT). The beads were then boiled in 1× SDS solution for 10 min in a water bath, followed by centrifugation, resolution by SDS-PAGE, and silver staining before MS-MS analysis. For MS-MS analysis, gel bands were excised, washed in acetonitrile, and sent for digestion and
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tandem mass spectrometry (Taplin Mass Spectrometry Facility, Boston, MA).

Methods for Phosphorylation Analysis by LC/MS-MS—Excised gel bands were cut into ~1-mm³ pieces, reduced with 1 mm DTT for 30 min at 60 °C, and then alkylated with 5 mm iodoacetamide for 15 min in the dark at room temperature. Gel pieces were then subjected to a modified in-gel trypsin digestion procedure (27). Gel pieces were washed and dehydrated with acetonitrile for 10 min, followed by removal of acetonitrile. Gel pieces were then completely dried in a SpeedVac, rehydrated with 50 mm ammonium bicarbonate solution containing 12.5 ng/µl modified sequencing-grade trypsin (Promega) at 4 °C, and then incubated at room temperature overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The extracts were then dried in a SpeedVac for ~1 h and stored at 4 °C until analysis.

On the day of analysis, the samples were reconstituted in 5–10 µl of HPLC solvent A (2.5% acetonitrile and 0.1% formic acid). A nanoscale reverse-phase HPLC capillary column was created by packing 2.6-µm C18 spherical silica beads into a fused silica capillary (100 µm inner diameter × ~25 cm length) with a flame-drawn tip (28). After equilibrating the column, each sample was loaded via a Famos autosampler (LC Packings, San Francisco, CA) onto the column. A gradient was formed, and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile and 0.1% formic acid).

As each peptide was eluted, they were subjected to electrospray ionization, and then they entered an LTQ Orbitrap Velos Pro ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Eluting peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein or translated nucleotide databases with the acquired fragmentation pattern with the software program SEQUEST (ThermoFinnigan, San Jose, CA) (29). The modification of 79.9663 mass units to serine, threonine, and tyrosine was included in the database searches to determine phosphopeptides. Phosphorylation assignments were determined by the Ascore algorithm.

Preparation of Plasmids and Proteins—pcDNA3-MDMX (S314A) mutants were generated using the QuiChange II XL site-directed mutagenesis kit (Agilent Technologies) by two-step site-directed PCR mutagenesis utilizing the following primer: Ser-314 to Ala, 5'-TGTCGCTCCTGTCAACCTGTTCGGT and 3'-CTCCGTCCTAATGGTTTCTCG. Full-length Her4, MDMX, full-length MDM2, full-length FLAG-MDMX, and full-length FLAG-MDM2 were cloned into pcDNA4 (Invitrogen). GFP-p53Δ92–112 has been described previously by our group (45).

Immunofluorescence—Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton-X-100/phosphate-buffered saline. Coverslips were then incubated with primary antibody diluted in 1% BSA/phosphate-buffered saline overnight. After washing, coverslips were incubated in secondary antibody diluted in 1% BSA/phosphate-buffered saline for 1 h. Coverslips were washed, stained with DAPI, mounted, and analyzed by fluorescence microscopy.

Quantitative Real-time PCR—Total RNA was isolated using TRIzol. The extracted RNA was resuspended in 50 µl of RNase-free water. The quantity and purity of each RNA sample were determined by spectrophotometry (Nanodrop, Fisher Scientific). cDNA was synthesized from 2 µg of total RNA using the high-capacity cDNA reverse transcription kit (Bio-Rad). RT-qPCR was carried out using the Fast SYBR Green PCR kit according to the instructions of the manufacturer (TaKaRa). Amplifications were performed in an ABI PRISM 7500 sequence detection system (Applied Biosystems). The relative expression of the target genes was normalized to the expression of the housekeeping gene 18S using the Excel-based software qGENE (30). The value of each genotype was identified by three samples, and each sample was repeated three times independently.

Kinase Assay—HEK293T cells were transiently transfected with CDK4-expressing plasmid. Another set of HEK293T cells was transfected with FLAG-MDMX plasmid or MDMX (S314A) mutant plasmid. 48 h post-transfection, MDMX and MDMX(S314A) were immunoprecipitated by FLAG IP and then incubated with cell lysates expressing CDK4 in kinase assay buffer in the presence of 10 µM ATP. After 30-min incubation at 30 °C, the reaction was terminated by the addition of SDS loading dye and boiled. The products were analyzed by Western blotting analysis using anti-phosphoserine antibody.

GST-Protein Binding Assay—The glutathione S-transferase (16) construct of MDM2 has been generated previously in our laboratory. GST-MDM2 transfected cells were harvested and lysed in 0.5% Nonidet P-40 lysis buffer for 1 h at 4 °C. Cell lysates were incubated with 10 µl of GST beads containing MDM2 for 3 h at 4 °C. Beads were then washed in lysis buffer and 0.1% Nonidet P-40, and protein complexes were liberated by boiling the beads in SDS-PAGE sample buffer for 5 min.

Statistical Analysis—Each experiment was repeated three times independently. All data are expressed as mean ± S.D. Statistical analysis was determined using one-way analysis of variance or unpaired Student’s t test. p < 0.05 was considered statistically significant.

Author Contributions—Z. M. Y., C. G., and J. B. L. conceived and designed the study. C. G. performed the experiments. A. d. P. and M. E. K., and Z. M. Y. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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