

Hsp90 and PKM2 Drive the Expression of Aromatase in Li-Fraumeni Syndrome Breast Adipose Stromal Cells*

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Li-Fraumeni syndrome (LFS) patients harbor germ line mutations in the *TP53* gene and are at increased risk of hormone receptor-positive breast cancers. Recently, elevated levels of aromatase, the rate-limiting enzyme for estrogen biosynthesis, were found in the breast tissue of LFS patients. Although p53 down-regulates aromatase expression, the underlying mechanisms are incompletely understood. In the present study, we found that LFS stromal cells expressed higher levels of Hsp90 ATPase activity and aromatase compared with wild-type stromal cells. Inhibition of Hsp90 ATPase suppressed aromatase expression. Silencing Aha1 (activator of Hsp90 ATPase 1), a co-chaperone of Hsp90 required for its ATPase activity, led to both inhibition of Hsp90 ATPase activity and reduced aromatase expression. In comparison with wild-type stromal cells, increased levels of the Hsp90 client proteins, HIF-1 α , and PKM2 were found in LFS stromal cells. A complex comprised of HIF-1 α and PKM2 was recruited to the aromatase promoter II in LFS stromal cells. Silencing either HIF-1 α or PKM2 suppressed aromatase expression in LFS stromal cells. CP-31398, a p53 rescue compound, suppressed levels of Aha1, Hsp90 ATPase activity, levels of PKM2 and HIF-1 α , and aromatase expression in LFS stromal cells. Consistent with these *in vitro* findings, levels of Hsp90 ATPase activity, Aha1, HIF-1 α , PKM2, and aromatase were increased in the mammary glands of p53 null *versus* wild-type mice. PKM2 and HIF-1 α were shown to co-localize in the nucleus of stromal cells of LFS breast tissue. Taken together, our results show that the Aha1-Hsp90-PKM2/HIF-1 α axis mediates the induction of aromatase in LFS.

product of the *CYP19A1* gene, catalyzes the synthesis of estrogens from androgens (1). In postmenopausal women, the adipose tissue becomes the main site of estrogen biosynthesis, and particularly, the breast adipose tissue is considered an important source of estrogens that drive the growth of hormone-dependent breast cancers. Consequently, it is important to elucidate the mechanisms that regulate the transcription of the *CYP19A1* gene. The expression of aromatase is tightly regulated, with transcription being under the control of several distinct tissue-selective promoters (2–4). In normal breast adipose tissue, aromatase is expressed at low levels under the control of promoter I.4, whereas in obesity and cancer, the coordinated activation of the proximal promoters I.3 and promoter II (PII)³ causes a significant increase in aromatase expression (3–5). The proximal promoters I.3 and PII are located close to each other, activated by stimulation of the cAMP \rightarrow PKA \rightarrow cAMP response element-binding protein (CREB) pathway (6, 7), and aided by many other regulators including CREB-regulated transcription co-activator 2 (CRTC2), p300, and hypoxia-inducible factor-1 α (HIF-1 α) (8–11).

Several cytokines and tumor promoters, including prostaglandin E₂, tumor necrosis factor- α , and interleukin-1 β stimulate aromatase expression (4, 12). In addition, its expression is regulated by oncogenes such as HER-2/neu and tumor suppressor genes including BRCA1, LKB1, and p53 (9, 11, 13–18). Germ line mutations in the *TP53* gene, which encodes p53, lead to Li-Fraumeni Syndrome (LFS). Among women with LFS, the most common cancer is breast cancer, with the majority of breast cancers being hormone receptor-positive (19, 20). Aromatase expression has been shown to be increased in breast adipose stromal cells from LFS patients compared with non-LFS breast tissue (16). Recently, we showed that epithelial cells from LFS patients contained increased Hsp90 ATPase activity because of the increased expression of Aha1, a co-chaperone of Hsp90 (21, 22). Here, we extended these studies to breast adipose stromal cells and show that aromatase expression is increased in LFS *versus* wild-type stromal cells and that this increase is dependent on Hsp90 ATPase signaling involving

Estrogen is an important mediator in the development and progression of breast cancer. Cytochrome P450 aromatase, a

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³ The abbreviations used are: PII, promoter II; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; CREB, cAMP response element-binding protein; HIF-1 α , hypoxia-inducible factor-1 α ; Hsp, heat shock protein; LFS, Li-Fraumeni syndrome; PKM2, pyruvate kinase M2.

p53 Regulates Aromatase Expression via HIF-1 α /PKM2 Axis

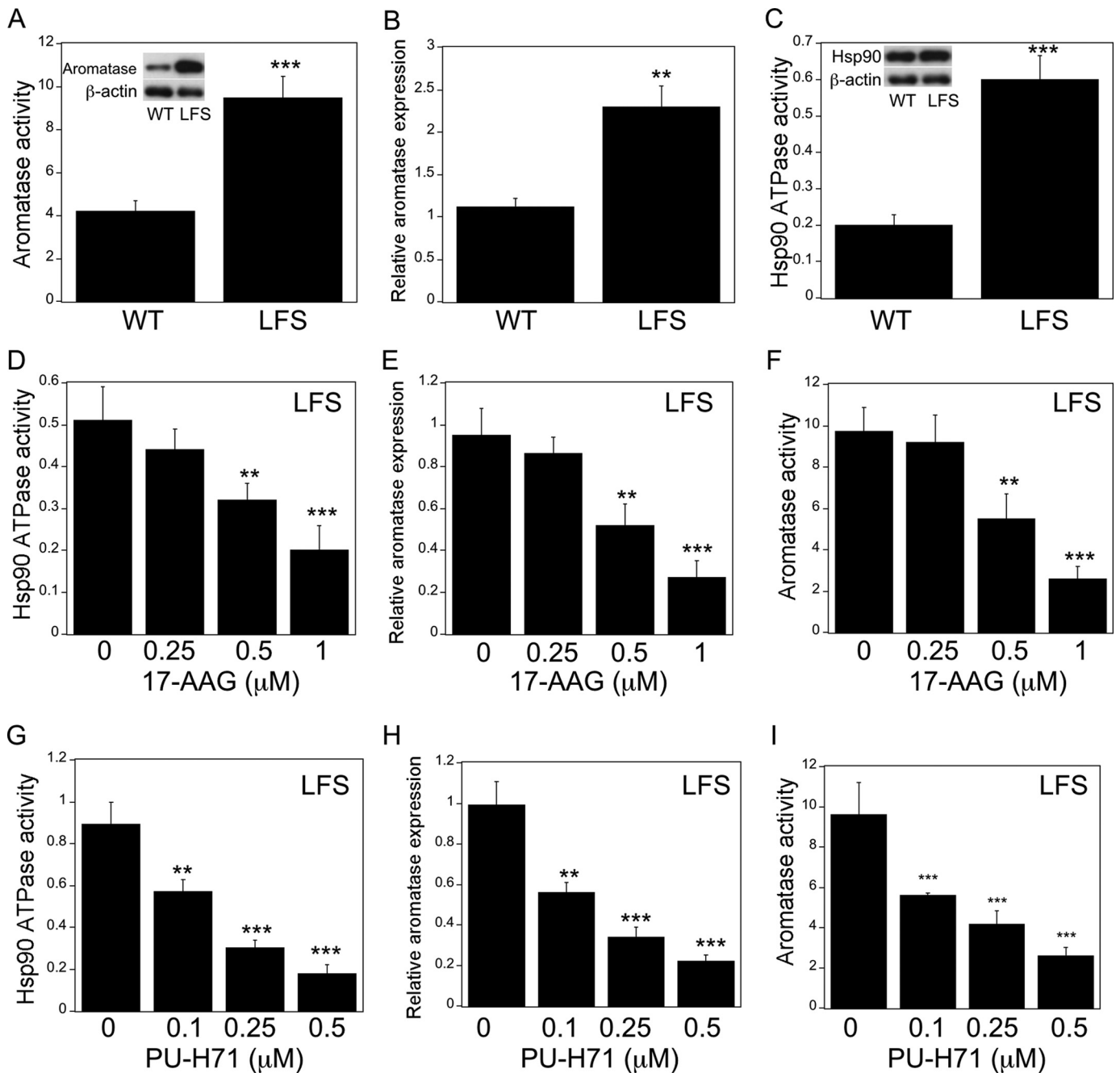


FIGURE 1. Hsp90 is important for the p53-mediated increase in aromatase levels in LFS stromal cells. A–C, WT and LFS stromal cells were used. In D–I, LFS cells were treated with the indicated concentrations of 17-AAG (D–F) or PU-H71 (G–I) for 24 h. In A, F, and I, aromatase activity was measured. In B, E, and H, aromatase mRNA levels were measured. In C, D, and G, Hsp90 ATPase activity was measured. A–I, means \pm S.D. (error bars) are shown, $n = 6$. **, $p < 0.01$; ***, $p < 0.001$ compared with wild-type stromal cells (A–C) and in D–I, compared with vehicle-treated cells.

Aha1, HIF-1 α , and PKM2. Consistent with these *in vitro* findings, levels of aromatase were increased in the mammary glands of p53 null *versus* wild-type mice. Taken together, this study provides new insights into the mechanism by which p53 regulates aromatase expression in stromal cells, which may be important for understanding the pathogenesis of estrogen-dependent breast cancer.

Results

Regulation of Aromatase by p53 Is Dependent on Hsp90—Initially, we compared levels of aromatase in stromal cells that were wild-type for p53 *versus* stromal cells from a LFS patient

that expressed mutant p53. As shown in Fig. 1 (A and B), levels of aromatase mRNA, protein, and activity were increased in the stromal cells derived from the LFS patient. Previously, we reported that mutant p53 led to increased Hsp90 ATPase activity in epithelial cells (21, 22). Consistent with this prior finding, Hsp90 ATPase activity was increased in LFS *versus* wild-type stromal cells (Fig. 1C). To determine whether the elevated level of Hsp90 ATPase activity was causally linked to the increased levels of aromatase in LFS stromal cells, two inhibitors of Hsp90 ATPase were used. Both 17-AAG and PU-H71 caused the dose-dependent suppression of Hsp90 ATPase activity and aroma-

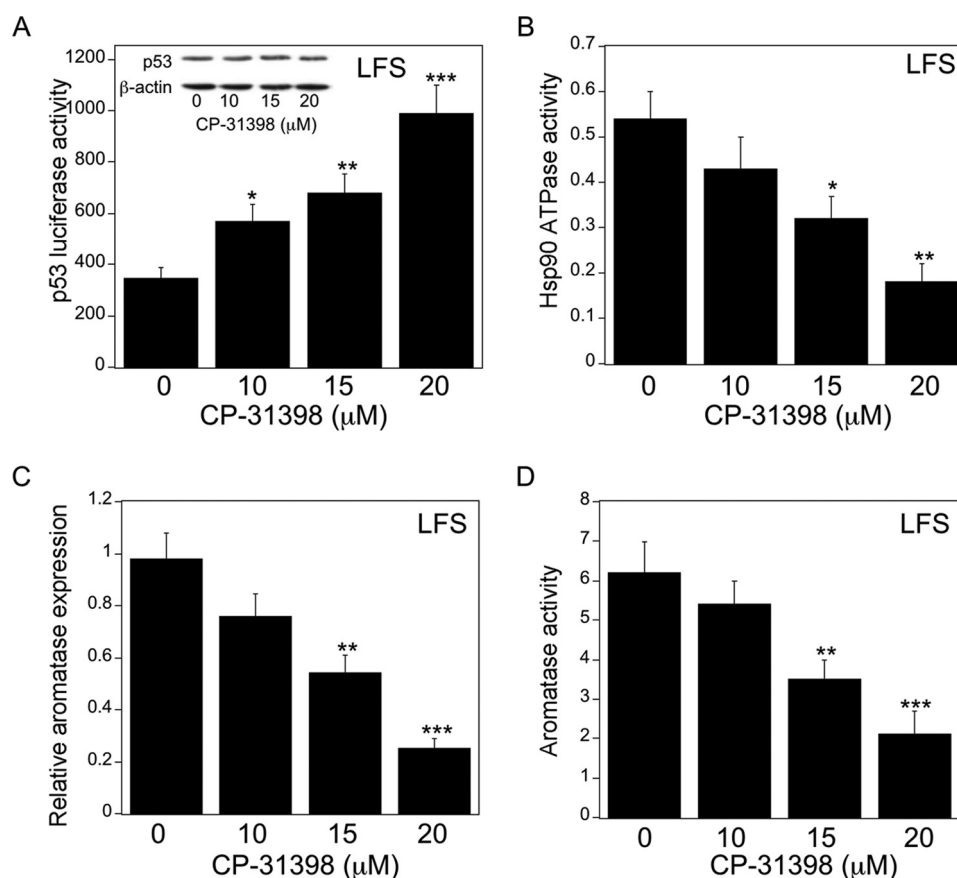


FIGURE 2. Reactivation of p53 leads to inhibition of aromatase activity in LFS stromal cells. In *A–D*, LFS stromal cells were transfected with 1.8 μ g of p53-luciferase construct and 0.2 μ g of *psv*- β -galactosidase construct. 24 h after transfection, the cells were treated with the indicated concentrations of CP-31398. 24 h later, the cells were harvested, and luciferase activity was measured. Luciferase activity was normalized to β -galactosidase activity. *Inset*, LFS cells were also treated with the indicated concentrations of CP-31398, lysates were subjected to Western blotting, and the blots were probed as indicated. In *B–D*, the cells were treated with the indicated concentrations of CP-31398 for 24 h. In *B*, Hsp90 ATPase activity was measured. In *C* and *D*, levels of aromatase mRNA and activity were measured in cell lysates. *A–D*, means \pm S.D. (error bars) are shown, $n = 6$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with vehicle-treated cells.

tase in LFS stromal cells (Fig. 1, *D–I*). To further interrogate the role of p53 in regulating aromatase levels, CP-31398, a p53 rescue compound was used (23). LFS stromal cells were transfected with a p53-luciferase construct. Treatment with CP-31398 caused the dose-dependent induction of p53-luciferase activity (Fig. 2*A*) without affecting p53 protein levels (Fig. 2*A*, *inset*). The same dose range of CP-31398 suppressed Hsp90 ATPase activity and down-regulated aromatase expression and activity (Fig. 2, *B–D*). Next, we determined the effects of silencing p53 on Hsp90 ATPase activity and aromatase expression in wild-type stromal cells. As shown in Fig. 3 (*A–C*), silencing of p53 led to increased Hsp90 ATPase activity and an associated increase in aromatase levels and activity. Silencing p53 with a different set of siRNAs also induced aromatase (data not shown). Similar inductive effects were observed in primary human adipose stromal cells (data not shown). Next we compared Hsp90 ATPase activity and aromatase levels in HCT116 cells that were wild-type or null for p53. Levels of Hsp90 ATPase activity and aromatase mRNA were increased in the HCT116 p53 null cell line (Fig. 3, *D* and *E*). To further investigate this relationship, we employed a p53 null cell line (EB-1) in which p53 is induced by exogenous ZnCl₂ (24, 25). Transient transfections were carried out to establish the concentration range of ZnCl₂ that stimulated p53-luciferase activity. Treat-

ment with 0–100 μ M ZnCl₂ led to a dose-dependent induction of p53-luciferase activity and p53 protein levels (Fig. 3*F*). The same concentration range of ZnCl₂ down-regulated Hsp90 ATPase activity and aromatase levels (Fig. 3, *G* and *H*). Previously, we demonstrated in epithelial cells that p53 suppresses Aha1, a co-chaperone of Hsp90, leading to inhibition of Hsp90 ATPase activity (21). Accordingly, we next determined whether this mechanism was operative in stromal cells and important for the regulation of aromatase expression. Levels of Aha1 were increased in LFS *versus* wild-type stromal cells (Fig. 4*A*). Silencing of p53 in a wild-type stromal cell line (Fig. 4*B*) or primary human adipose stromal cells (data not shown) induced Aha1 protein expression. Similar effects were found using a second set of siRNAs to p53 (data not shown). Similarly, levels of Aha1 were higher in HCT116 cells that were null for p53 (Fig. 4*B*). Treatment of LFS cells with CP-31398 caused the dose-dependent suppression of Aha1 levels (Fig. 4*C*). Consistent with the known function of Aha1, silencing of Aha1 down-regulated Hsp90 ATPase activity in LFS stromal cells (Fig. 4*D*). Importantly, silencing of Aha1 led to reduced aromatase expression and activity in these cells (Fig. 4, *E* and *F*).

p53 Regulates Hsp90 ATPase Activity Leading to the Stabilization of HIF-1 α and PKM2—HIF-1 α is a client protein of Hsp90 and a known regulator of aromatase expression (26–28).

p53 Regulates Aromatase Expression via HIF-1 α /PKM2 Axis

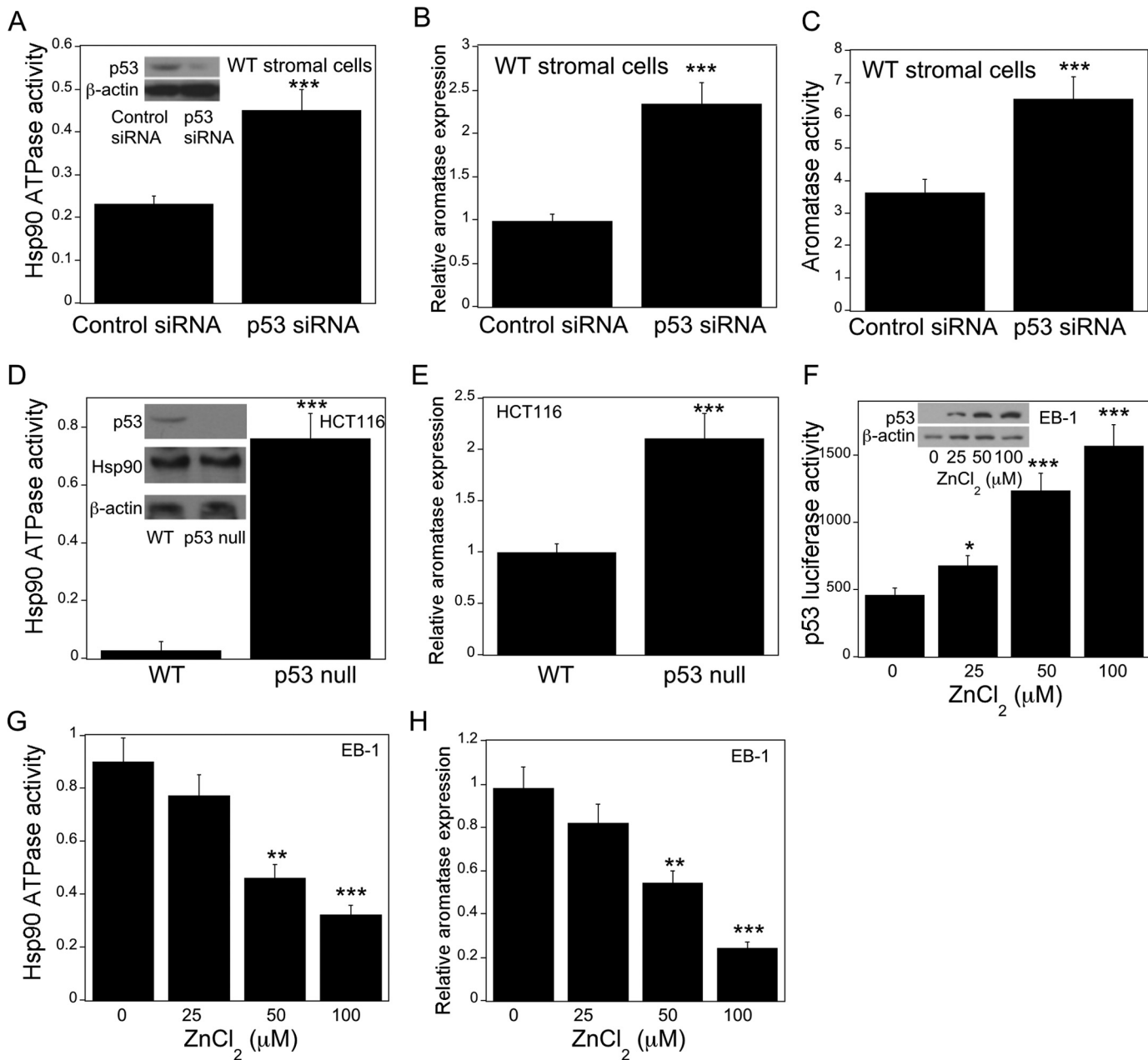


FIGURE 3. p53 regulates Hsp90 ATPase activity and aromatase expression. In A–C, wild-type stromal cells were transfected with 2 μ g of siRNA to GFP (control siRNA) or p53. 48 h after transfection, the cells were harvested, and levels of Hsp90 ATPase activity (A), aromatase mRNA (B), and aromatase activity (C) were measured. In D and E, HCT116 cells wild-type or null for p53 were used. The cells were harvested and Hsp90 ATPase activity (D) and aromatase expression (E) were measured. In F–H, EB-1 cells were used. In F, the cells were transfected with 1.8 μ g of p53-luciferase construct and 0.2 μ g of *psv*- β -galactosidase construct for 24 h. In F–H, EB-1 cells were treated with the indicated concentrations of ZnCl₂ for 24 h. Inset, EB-1 cells were treated with the indicated concentrations of ZnCl₂ for 24 h, and the lysates were subjected to Western blotting. In F, the cells were harvested, and luciferase activity was measured. Luciferase activity was normalized to β -galactosidase activity. In G and H, levels of Hsp90 ATPase activity and aromatase mRNA levels were measured, respectively. In A–H, means \pm S.D. (error bars) are shown, $n = 6$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with control siRNA-treated cells (A–C), wild-type cells (D and E), or vehicle-treated cells (F–H).

PKM2 is a co-activator of HIF-1 α -mediated gene expression (29–32). Hence, we investigated whether these two proteins could be important for mediating the effects of p53 on aromatase. Levels of HIF-1 α and PKM2 were increased in LFS stromal cells (Fig. 5A, left panel). Levels of PKM2 and HIF-1 α were also increased in p53 null versus wild-type HCT116 cells (Fig. 5A, right panel). Next, we investigated whether HIF-1 α and PKM2 were in a complex with Hsp90. In LFS compared with wild-type stromal cells, there was more HIF-1 α and PKM2 that co-immu-

noprecipitated with Hsp90 (Fig. 5B). Treatment of LFS stromal cells with CP-31398 led to a loss of HIF-1 α , PKM2, and Hsp90 complexes (Fig. 5C). In contrast, silencing of p53 in a wild-type stromal cell line increased levels of HIF-1 α and PKM2 and stimulated the interaction between HIF-1 α , PKM2, and Hsp90 (Fig. 5D). Increased levels of HIF-1 α and PKM2 were also found in primary human adipose stromal cells in which p53 was silenced (data not shown). To assess the role of Hsp90 ATPase activity in regulating levels of these client proteins, 17-AAG and

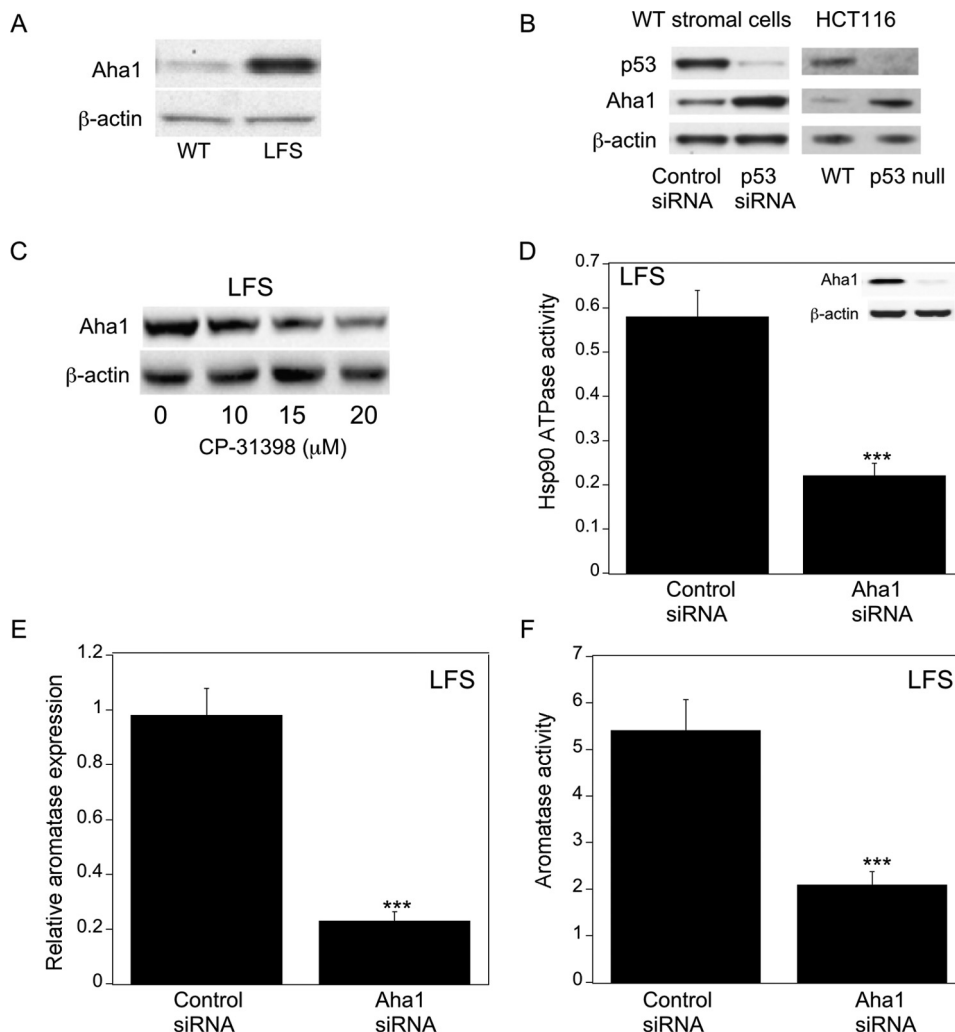


FIGURE 4. Aha1 is important for p53-mediated regulation of aromatase in LFS stromal cells. *A*, levels of Aha1 and β -actin in WT and LFS stromal cells were determined in cell lysates by immunoblotting. In *B* (left panel) and *D–F*, cells were transfected with 2 μ g of siRNA to GFP (control siRNA), p53 (*B*), or Aha1 (*D–F*). 48 h after transfection, the cells were harvested, and levels of Aha1 protein (*B*, left panel; and inset in *D*), Hsp90 ATPase activity (*D*), and aromatase mRNA (*E*), and aromatase activity (*F*) were measured. In *B* (right panel), lysates were prepared from p53 wild-type and p53 null HCT116 cells and subjected to Western blotting. In *C*, LFS cells were treated with the indicated concentrations of CP-31398 for 24 h. The cell lysates were prepared and subjected to Western blotting. The blots were probed as indicated. In *D–F*, means \pm S.D. (error bars) are shown, $n = 6$. ***, $p < 0.001$ compared with cells transfected with GFP siRNA.

PU-H71 were used. Both of these inhibitors caused the dose-dependent suppression of HIF-1 α and PKM2 protein in LFS stromal cells (Fig. 5, *E* and *F*). By contrast, treatment with 17-AAG did not affect PKM2 mRNA levels (data not shown). Similar effects were observed when Aha1 was silenced (Fig. 5*G*). To evaluate whether p53 regulated this process, the effect of CP-31398 on levels of Aha1, HIF-1 α , and PKM2 was determined. Treatment of LFS stromal cells with this p53 rescue compound suppressed levels of each of these proteins (Fig. 5*H*). Similarly, ZnCl₂-mediated induction of p53 led to down-regulation of Aha1, HIF-1 α and PKM2 (Fig. 5*I*). Next, we explored the significance of HIF-1 α and PKM2 in regulating aromatase expression. Because levels of HIF-1 α and PKM2 (Fig. 5*A*), as well as aromatase (Fig. 1, *A* and *B*), were increased in LFS versus wild-type stromal cells, we investigated whether these differences were causally linked. Initially, we explored the possibility that HIF-1 α and PKM2 were in a complex. As shown in Fig. 6*A*, immunoprecipitating PKM2 led to co-precipitation of HIF-1 α and vice versa in LFS stromal cells.

Next, we used cell fractionation to determine whether HIF-1 α and PKM2 were detectable in the nucleus in LFS cells. As shown in Fig. 6*B*, both HIF-1 α and PKM2 were detectable in the nucleus of LFS cells with protein levels being higher in LFS compared with wild-type cells. Similar results were obtained when p53 was silenced in wild-type cells (data not shown). To determine whether HIF-1 α and PKM2 bound to the *CYP19A1* promoter, ChIP assays were performed. ChIP assays revealed increased binding of both HIF-1 α (Fig. 6*C*) and PKM2 (Fig. 6*D*) to the proximal promoter region of *CYP19A1*. Consistent with this binding being functionally important, silencing of either HIF-1 α (Fig. 6, *E–G*) or PKM2 (Fig. 6, *H–J*) in LFS stromal cells suppressed aromatase promoter activity, expression, and enzyme activity. Using a second set of siRNAs to PKM2 and HIF-1 α , similar results were obtained (data not shown).

Levels of Hsp90 ATPase Activity, Aha1, HIF-1 α , PKM2, and Aromatase Are Increased in p53 Null Mice—To assess the impact of p53 on the Hsp90-aromatase axis *in vivo*, we utilized

p53 Regulates Aromatase Expression via HIF-1 α /PKM2 Axis

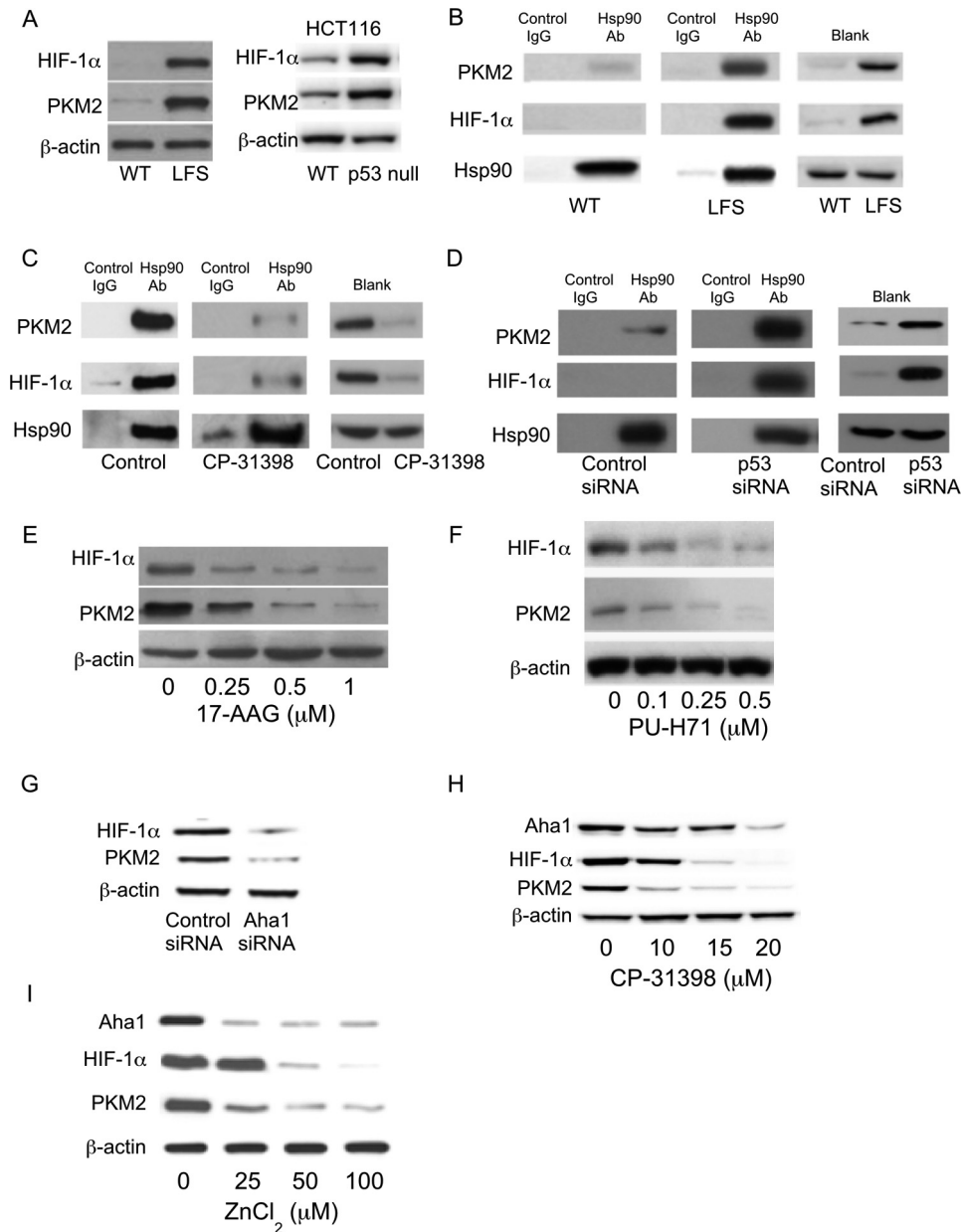


FIGURE 5. HIF-1 α and PKM2 are client proteins of Hsp90. *A*, cell lysates from wild-type and LFS stromal cells (*left panel*) and HCT116 cells (*right panel*) were subjected to Western blotting, and the blots were probed as indicated. In *B–D*, immunoprecipitation experiments were performed. In *B*, cell lysates were prepared from wild-type and LFS stromal cells; in *C*, LFS stromal cells were treated with 20 μ M CP-31398 for 3 h, and cell lysates were prepared; and in *D*, wild-type cells were transfected with 2 μ g of siRNA to GFP (control siRNA) or p53. 48 h after transfection, the cells were harvested. In *B–D*, Hsp90 was immunoprecipitated with control IgG or antibody to Hsp90. Immunoprecipitates were subjected to Western blotting, and the blots were probed as indicated. *Blank*, total cell lysates were subjected to Western blotting and probed as indicated. In *E* and *F*, LFS stromal cells were treated with the indicated concentrations of 17-AAG and PU-H71 for 24 h. In *G*, LFS stromal cells were transfected with 2 μ g of siRNA to GFP (control siRNA) or Aha1. 48 h after transfection, cells were harvested, and lysates were prepared. In *H*, LFS cells were treated with the indicated concentrations of CP-31398 for 24 h. The cells were harvested, and lysates were prepared. In *I*, EB-1 cells were treated with the indicated concentrations of ZnCl₂ for 24 h, and cell lysates were prepared. In *E–I*, the cell lysates were subjected to Western blotting, and the blots were probed with the indicated antibodies.

mammary gland tissue from wild-type and p53 null mice. p53 protein was not detected in the mammary glands of p53 null mice (Fig. 7A). Similar levels of Hsp90 were found in p53^{+/+} and p53^{-/-} mice (Fig. 7A). Compared with wild-type mice, p53 null mice exhibited increased levels of Aha1, Hsp90 ATPase activity, and HIF-1 α and PKM2 protein levels (Fig. 7, B and C). Moreover, levels of aromatase mRNA and activity were both elevated in mammary glands from p53 null *versus* wild-type mice (Fig. 7, D and E).

PKM2 Is Detectable in the Nucleus of LFS Stromal Cells and Is Positively Correlated with HIF-1 α and Aromatase—The subcellular localization of PKM2 was examined in adipose stromal cells from LFS patients and compared with normal breast tissue using immunofluorescence and confocal microscopy. In normal breast tissue and consistent with *in vitro* findings (Fig. 6B), PKM2 was observed in the cytoplasm (Fig. 8A), whereas immunoreactivity for PKM2 was detectable in the nucleus of LFS stromal cells (Figs. 6B and 8B).

p53 Regulates Aromatase Expression via HIF-1 α /PKM2 Axis

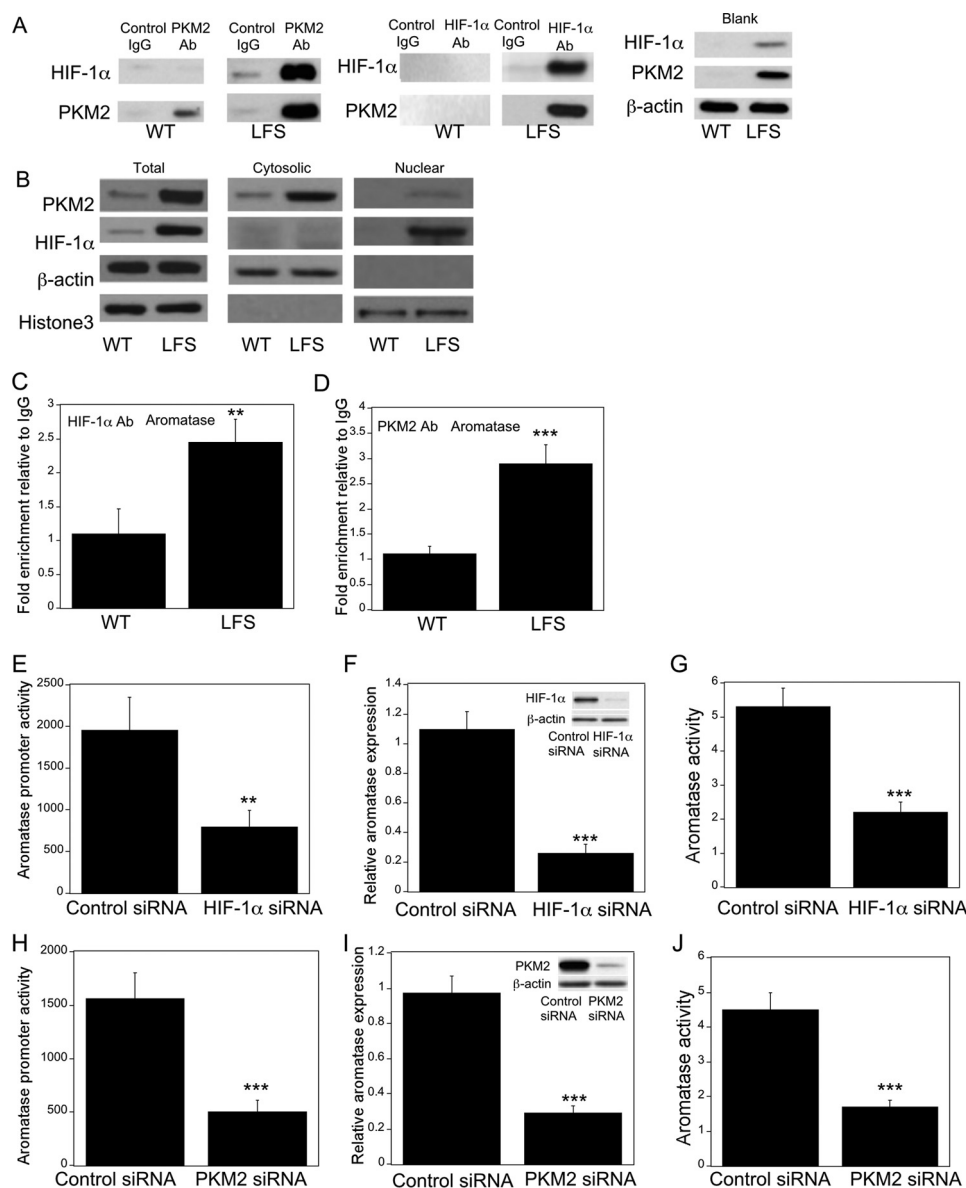


FIGURE 6. HIF-1 α and PKM2 are important for the p53-mediated regulation of aromatase. In *A*, cell lysates prepared from WT and LFS stromal cells were either subjected to Western blotting (*Blank*) or immunoprecipitated with control IgG, antibody to PKM2, or antibody to HIF-1 α and then subjected to Western blotting. In *B*, lysates were prepared from wild-type and LFS cells. Cytosolic and nuclear fractions were isolated, and 75 μ g of total protein and equal volumes of cytosolic and nuclear proteins were subjected to Western blotting, and the blots were probed as indicated. In *C* and *D*, ChIP assays were performed. Chromatin fragments were immunoprecipitated with antibodies against HIF-1 α (*C*) or PKM2 (*D*), and the aromatase promoter was amplified by real time PCR. DNA sequencing was carried out, and the PCR products were confirmed to be the aromatase promoter. This promoter was not detected when normal IgG was used or when antibody was omitted from the immunoprecipitation step. In *E* and *H*, LFS stromal cells were transfected with 0.9 μ g of aromatase promoter construct and 0.2 μ g of *psv* β -gal constructs. 24 h later, the cells also received either 0.9 μ g of control siRNA, HIF-1 α siRNA (*E*), or PKM2 siRNA (*H*). 48 h after transfection, the cells were harvested, and luciferase activity was measured. Luciferase activity was normalized to β -galactosidase activity. In *F*, *G*, *I*, and *J*, LFS stromal cells were transfected with 2 μ g of GFP siRNA (control), siRNA to HIF-1 α (*F* and *G*), or siRNA to PKM2 (*I* and *J*). In *F* and *I*, 48 h after transfection, the cell lysates were subjected to Western blotting (*insets*), and the blots were probed as indicated. In *F* and *I*, total RNA was isolated, and aromatase mRNA levels were measured. In *G* and *J*, aromatase activity was measured in cell lysates. In *C–J*, means \pm S.D. (*error bars*) are shown, $n = 6$. **, $p < 0.01$; ***, $p < 0.001$ compared with wild-type cells (*C* and *D*); cells that were transfected with GFP siRNA (*E–J*).

HIF-1 α and PKM2 also co-localized in the nucleus of LFS breast adipose stromal cells (Fig. 8C), and a significant positive correlation between HIF-1 α and PKM2 immunofluorescence intensity was found following quantification of cell-specific signals (Fig. 8D). Aromatase immunoreactivity was also detected in cells with nuclear PKM2 (Fig. 8E) and positively correlated with nuclear PKM2 (Fig. 8F) in LFS stromal cells. No staining was detected when primary antibodies were omitted (data not shown).

Discussion

The majority of LFS patients carry germ line mutations in *TP53*, the gene that encodes p53, with affected individuals having an increased risk of hormone receptor-positive breast cancer (16, 19, 20). This suggests an important causal role for p53 inactivation in breast carcinogenesis. Recently, p53 was found to down-regulate aromatase expression (16). Consistent with this finding, aromatase expression was increased in breast adi-

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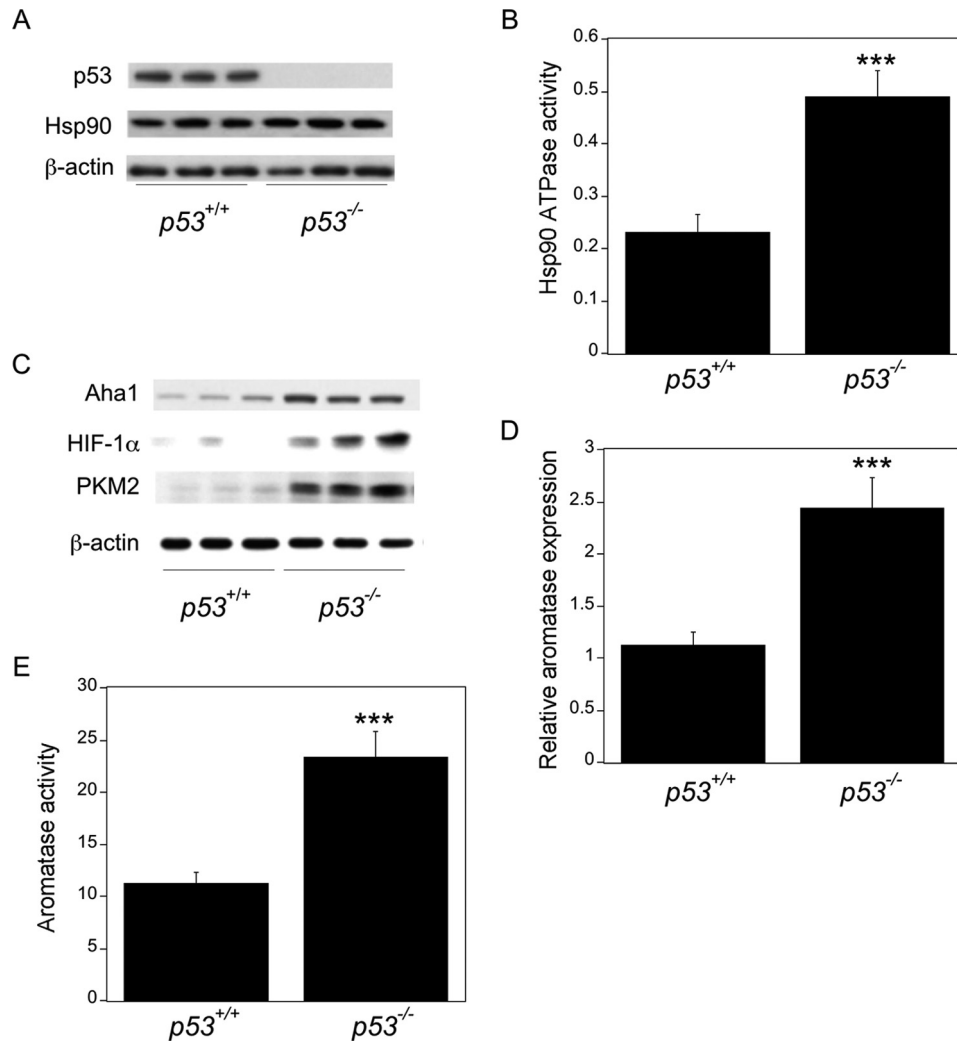


FIGURE 7. Levels of aromatase are increased in mammary glands of p53 null mice. Mammary gland tissue from female p53 wild-type and p53 null mice was used. *A*, lysates were subjected to Western blotting and probed as indicated. *B*, tissue lysates were used to measure Hsp90 ATPase activity. *C*, lysates were subjected to Western blotting and probed as indicated. *D*, total RNA was prepared, and poly(A) RNA was isolated. Relative expression of aromatase was quantified by real time PCR. The values were normalized to levels of β -actin. *E*, tissue lysates were used to measure aromatase activity. In *B*, *D*, and *E*, means \pm S.D. (error bars) are shown, $n = 6$. ***, $p < 0.001$ compared with p53^{+/+} mice.

pose stromal cells from LFS compared with non-LFS patients. However, the complex molecular mechanisms underlying the effect of p53 on aromatase expression are poorly understood. In the present study, we have not only confirmed previous results demonstrating that p53 inhibits aromatase expression (16, 17) but also have identified a novel mechanism by which this occurs in LFS stromal cells. Importantly, we show that wild-type p53 suppresses Aha1 levels leading to inhibition of Hsp90 ATPase activity. This results in reduced amounts of the Hsp90 client proteins HIF-1 α and PKM2 and suppression of aromatase levels (Fig. 9).

Our data demonstrate that Hsp90 plays a significant role in stimulating aromatase expression in LFS-derived stromal cells. Previously, we showed that loss of p53 led to the induction of Aha1, which was critical for increased Hsp90 ATPase activity in colon cancer cells (21, 22). In the current study, we demonstrate that silencing of Aha1 leads to the suppression of Hsp90 ATPase activity, as well as aromatase expression in LFS-derived stromal cells. Taken together, these results show for the first time that p53 regulates aromatase expression in an Hsp90-de-

pendent fashion. Additionally, we show that Hsp90 ATPase activity is increased in LFS-derived stromal cells, suggesting the potential use of Hsp90 ATPase inhibitors for the prevention or treatment of breast cancer in LFS patients. Previously, we reported that HDAC6 functions as an Hsp90 deacetylase and that inhibition of HDAC6 leads to Hsp90 hyperacetylation, its dissociation from p23, a co-chaperone, and a loss of chaperone activity (33). Inhibition of HDAC6 has been reported to down-regulate aromatase expression (34), suggesting a possible role for reduced Hsp90 ATPase in mediating this effect. Based on the current results, future studies are warranted to test this possibility.

HIF-1 α , a client protein of Hsp90 (26), has been shown to stimulate aromatase in human breast adipose stromal cells (10). Here, we show that increased Hsp90 ATPase activity found in LFS-derived stromal cells leads to enhanced HIF-1 α levels, the increased binding of HIF-1 α to the proximal promoter of *CYP19A1* (PII promoter), and a consequent increase in aromatase expression. This is consistent with previous findings demonstrating that HIF-1 α binds to aromatase PII and is required

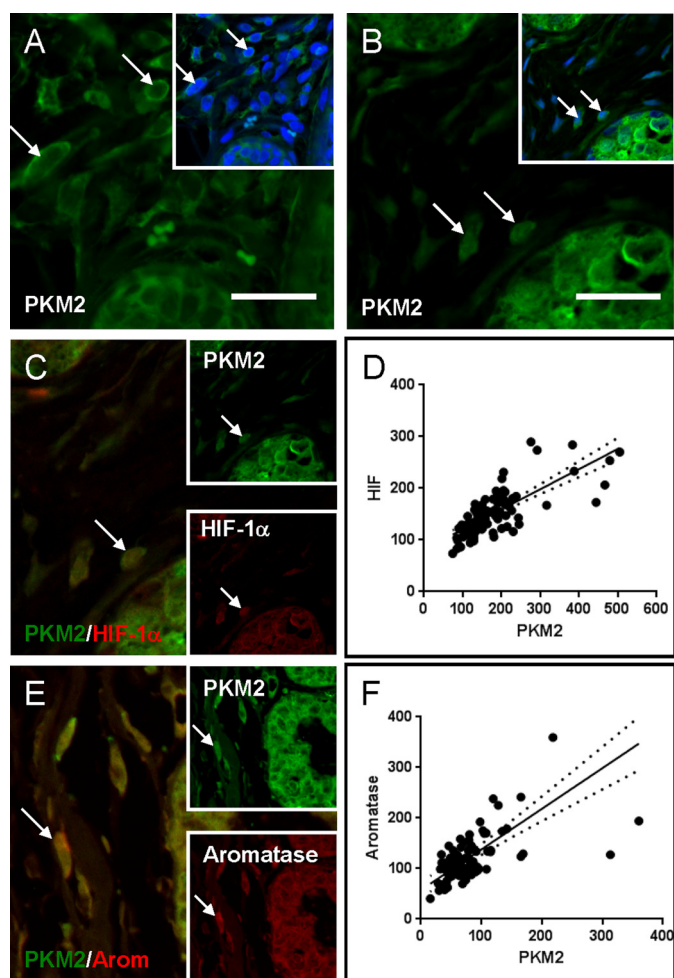


FIGURE 8. PKM2 is detectable in the nucleus of LFS stromal cells and is positively correlated with HIF-1 α and aromatase. PKM2, HIF-1 α , and aromatase immunoreactivity and localization were examined in adipose stromal cells from normal breast tissue and breast tissue from LFS patients using immunofluorescence and confocal microscopy. *A*, PKM2 (green) is detectable in the cytoplasm of stromal cells from normal breast tissue (white arrows). In LFS breast adipose stromal cells, PKM2 (*B*, *C*, and *E*; green) and HIF-1 α (*C*; red) are detectable in the cytoplasm and nucleus. Co-localization of PKM2 and HIF-1 α is visible in the nucleus of LFS stromal cells (*C*; white arrows), and the relative fluorescence intensity for HIF-1 α is positively correlated with nuclear PKM2 (*D*). *E*, PKM2 (green) and aromatase (red) also co-localize in adipose stromal cells from LFS (arrows). *F*, aromatase immunofluorescence staining is positively correlated with nuclear PKM2 in these cells. *Insets* in *A* and *B* are two-channel images where Hoescht nuclear staining is in blue and PKM2 in green. Images presented (LFS6) are representative of samples examined ($n = 7/\text{group}$). Scale bars in *A* and *B* represent 25 μm . Arom, aromatase.

for the PGE₂-mediated induction of aromatase (10), whereas PGE₂ also suppresses p53 in breast adipose stromal cells (16). We also confirm that HIF-1 α is a client protein of Hsp90 in LFS stromal cells as silencing Aha1 or treatment with Hsp90 inhibitors suppressed HIF-1 α protein levels. Our data support growing evidence that HIF-1 α has roles that go beyond those of mediating responses to hypoxia. The direct causal relationship between the loss of p53 and increased expression of HIF-1 α was further supported by evidence that silencing p53 leads to an increase in HIF-1 α expression in wild-type stromal cells. Moreover, rescuing p53 in LFS-derived stromal cells led to inhibition of HIF-1 α levels.

PKM2 is a key mediator of the Warburg effect, the phenomenon by which proliferating cells alter their mode of energy

production from oxidative phosphorylation to aerobic glycolysis, and its expression is increased in cancer cells (35, 36). Although the metabolic function of PKM2 is well established, our findings support a role for this protein as a regulator of gene expression via direct interactions with HIF-1 α . Here, we show that the tumor suppressor p53 is a regulator of PKM2 expression, providing a novel mechanism for the regulation of PKM2 in LFS-derived stromal cells. First, we show that PKM2 levels are higher in LFS compared with wild-type stromal cells. Moreover, silencing p53 in wild-type cells enhanced the expression of PKM2, whereas reactivation of p53 led to a decrease in PKM2 levels in LFS cells. In LFS patients, PKM2 was present in both the cytosol and nucleus of adipose stromal cells. In contrast, PKM2 was barely detectable in the nuclei of adipose stromal cells from women without LFS. The observation that PKM2 can be found in the nucleus of LFS adipose stromal cells is consistent with recent findings demonstrating increased nuclear localization of PKM2 in breast cancer patients (37). Next, we show that p53 regulates PKM2 expression via Hsp90 and that PKM2 is a client protein of Hsp90. To our knowledge, this is the first study to report that PKM2 is a client protein of Hsp90. Consistent with this, silencing Aha1 or treatment with Hsp90 inhibitors down-regulated PKM2 levels. Immunoprecipitating Hsp90 co-precipitated PKM2, suggesting a complex between Hsp90 and PKM2. The increased expression of PKM2 is causal to the increased expression of aromatase observed in LFS *versus* normal adipose stromal cells. Recently, PKM2 has been shown to have a role in the nucleus as a transcriptional regulator. In particular, it can co-activate HIF-1 α and modulate expression of HIF-1 α target genes (29–32). In LFS cells, we show that PKM2 and HIF-1 α form a complex that is localized in the nucleus. Previous findings had demonstrated that p53, via binding to the aromatase promoter, suppressed basal levels of aromatase (16). Our data suggest that p53 regulates aromatase expression in a HIF-1 α and PKM2-dependent manner (Fig. 9).

Consistent with our *in vitro* results, increased Hsp90 ATPase activity, HIF-1 α , PKM2, and aromatase expression were observed in mammary tissues of p53 null compared with p53 wild-type mice. These results are consistent with previous findings demonstrating that aromatase is elevated in p53-inactivated mammary epithelial cells (17). In this previous study, the increase in aromatase was attributed to effects on a known aromatase stimulator, CREB. Interestingly, HIF-1 α has previously been shown to act cooperatively with CREB to stimulate aromatase expression (27), suggesting that a larger complex containing HIF-1 α , PKM2, and CREB may be required for maximal induction of aromatase via this pathway.

LFS, despite being a rare disorder, also provides us with potential new insights into the biology of sporadic breast cancers, of which more than 30% have mutations in p53 (38). Based on the current results, we posit that processes that chronically suppress levels of p53 or stimulate Hsp90 ATPase activity will induce aromatase, thereby increasing the risk of hormone receptor-positive breast cancer.

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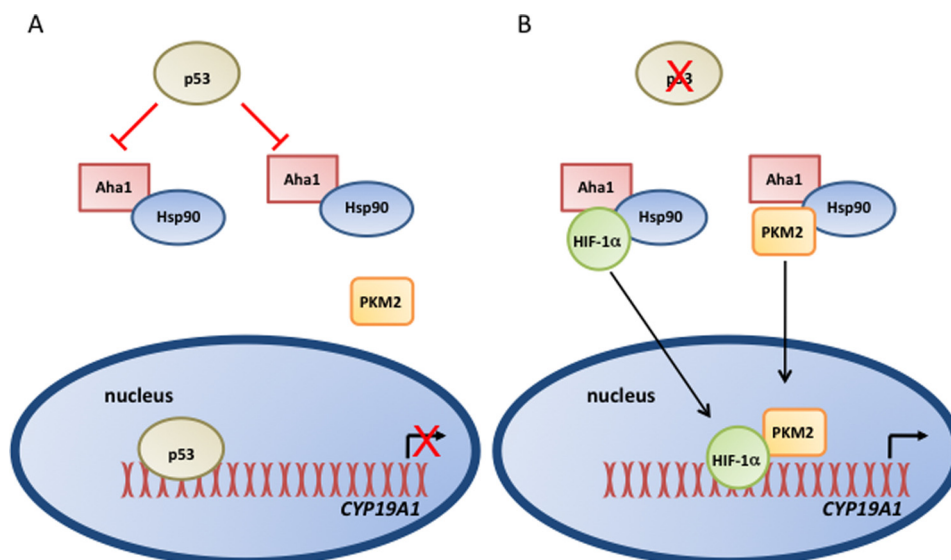


FIGURE 9. **Signaling pathway by which p53 regulates aromatase in LFS stromal cells.** A, in stromal cells with wild-type p53, Aha1 is suppressed leading to the decreased ATPase activity of Hsp90 and the decreased stabilization of HIF-1 α and PKM2. B, mutation or loss of p53 leads to an increase in Aha1 expression, which leads in turn to enhanced Hsp90 ATPase activity. This results in stabilization of HIF-1 α and PKM2, their increased binding to the *CYP19A1* promoter, and the up-regulation of aromatase expression.

Experimental Procedures

Materials—Glucose-6-phosphate, pepstatin, leupeptin, glucose-6-phosphate dehydrogenase, zinc chloride, DMSO, L-lactate dehydrogenase, G418, phosphoenolpyruvate, pyruvate kinase, NADH, antibodies to β -actin, and primers for PKM2 and β -actin were purchased from Sigma. CP-31398 was provided by the National Cancer Institute Chemopreventive Agent Repository. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) was obtained from Cayman Chemicals. PU-H71 was purchased from Tocris Bioscience. Monoclonal aromatase antibody 677 was obtained from the Baylor College of Medicine (39). Antibodies to PKM2 (Western blotting 1:1000; 4053, and immunofluorescence) and HIF-1 α (1:1000; D2U3T) were from Cell Signaling Technology, whereas the HIF-1 α antibody used for immunofluorescence was purchased from BD Transduction Laboratories. Antibodies to Hsp90 (1:500; SC-101494) and p53 (1:1000; SC6243) were from Santa Cruz. Antibody to Aha1 was purchased from Abcam (1:1000; EPR13888). NE-PER nuclear and cytoplasmic extraction kit, control siRNA and siRNAs to Aha1, HIF-1 α , PKM2, and p53 were from Thermo Scientific. A second set of siRNAs to GFP, p53, PKM2, and HIF-1 α was purchased from Qiagen. EpiTect ChIPone-day kits were purchased from SA Bioscience. Western blotting detection reagents were from PerkinElmer Life Sciences. p53-luciferase plasmid was from Panomics. Reagents for the luciferase assay and pSV β gal were from Promega. Aromatase promoter (*CYP19A1*) was kindly provided by Dr. S. Chen (City of Hope, Duarte, CA). 1β -[3 H]Androstenedione was from PerkinElmer Life Sciences.

Cell Culture—Human mammary stromal cells HMS32-hTERT (wild-type p53) and IUSM-LFS-HMS were provided by Dr. Brittney-Shea Herbert (Indiana University School of Medicine) and grown as previously described (40, 41). The IUSM-LFS-HMS cells express a heterozygous *TP53* 12141delG germ line frameshift mutation (41). EB-1, a human colon carcinoma

cell line, was kindly provided by Dr. Arnold J. Levine (Princeton University) (24, 25). EB-1 cells were maintained in RPMI medium with 10% FBS and supplemented with 0.5 g/liter G418. HCT116 cells (p53 wild-type and p53 null) were obtained from Dr. Bert Vogelstein (42). These cells were grown in McCoy's 5A medium supplemented with 10% FBS. Human preadipocytes (adipose stromal cells) derived from subcutaneous fat and preadipocyte growth medium were purchased from Cell Applications, Inc. Cellular cytotoxicity was assessed by measurements of cell number, lactate dehydrogenase release, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. No evidence of cell toxicity was detected in any of the experiments described below (data not shown).

Co-immunoprecipitation—This assay was performed using a catch and release reversible immunoprecipitation system from Upstate Biotechnology. Cell lysate or tissue lysate (500–1000 μ g) protein was used for immunoprecipitation at room temperature. The immunoprecipitates were then subjected to Western blot analysis.

Western Blotting—Cells and tissues were lysed by suspending in lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml trypsin inhibitor, and 10 μ g/ml leupeptin) followed by sonication. Cell and tissue protein extracts were clarified following centrifugation to remove particulate material. The protein concentration was determined according to Lowry *et al.* (43). Mouse mammary gland tissue lysates were either subjected to Western blotting for Hsp90 and β -actin or immunoprecipitated with antisera to p53, PKM2, Aha1, and HIF-1 α followed by Western blotting. Lysates were subjected to SDS-PAGE under reducing conditions on 10% polyacrylamide gels. Separated proteins were transferred onto nitrocellulose sheets and incubated with the indicated antisera followed by a secondary antibody to

TABLE 1
Li-Fraumeni Syndrome sample characteristics

ID	Age ^a	TP53 mutation	Receptor status	Correlation with nuclear PKM2 (r)	
				HIF-1 α	Aromatase
1	62	g.14069 C>T (R248W)	ER+, PR-, HER2-	0.7969 ^b	0.6525 ^b
2	40	dup exons 2_6	ER+, PR+, HER2-	0.5715 ^b	0.8178 ^b
3	45	c.215C>G (P72R) ^c	ER+, PR+, HER2-	0.8331 ^b	0.7175 ^b
4	49	c.215C>G (P72R)	ER+, PR-, HER2-	0.7244 ^b	0.4079 ^b
5	33	c.524 G>A (R175H)	ER+, N/A, HER2-	0.7187 ^b	0.7312 ^b
6	43	c.393_395 Δ CAA (N131 Δ)	ER+, PR+, HER2-	0.7537 ^b	0.6623 ^b
7	27	c.584 T>C, c.215 C>G	ER-, PR-, HER2+	0.9259 ^b	0.6092 ^b

^a Age (in years) at time tissue was obtained.^b $p < 0.0001$.^c BRCA2 mutation carrier.

horseradish peroxidase conjugated IgG. The blots were then probed with the ECL Western blot detection system.

Quantitative Real Time PCR—Total RNA was isolated from cells using the RNeasy mini kit (Qiagen). For mammary gland tissue analyses, poly(A) RNA was prepared with an Oligotex mRNA mini kit (Qiagen). Poly(A) or total RNA was reversed transcribed using murine leukemia virus reverse transcriptase and oligo(dT)₁₆ primer. The resulting cDNA was then used for amplification. The primers for murine and human aromatase have been described previously (5, 9, 44, 45). The primers for PKM2 were 5'-GTCTGGAGAAACAGCCAAGG-3' (forward) and 5'-CGGAGTTCCTCGAATAGCTG-3' (reverse) (46). β -Actin was used as an endogenous normalization control; primers were purchased from Qiagen. Real time PCR was done using 2 \times SYBR green PCR master mix on a 7500 real time PCR system (Applied Biosystems). Using the ddC_T (relative quantification) analysis protocol, relative fold induction was determined.

Transient Transfections—The cells were grown to 60–70% confluence in 6-well dishes. The cells were then transfected using Lipofectamine 2000 (Invitrogen) for 24 h. Following transfection, the medium was replaced with serum-free medium for another 24 h. Luciferase and β -galactosidase enzyme activities were measured in cellular extracts. Luciferase activity in cell lysates was normalized to β -galactosidase enzymatic activity.

RNA Interference—The cells were transfected with 2 μ g of siRNA oligonucleotides using DharmaFECT 4 transfection reagent according to the manufacturer's instructions.

ChIP Assay—ChIP assay was performed using the EpiTect ChIPone-day kit from SA Bioscience. Approximately, 4 \times 10⁶ cells were cross-linked in a 1% formaldehyde solution at 37 $^{\circ}$ C for 10 min. Cross-linked cells were then lysed and sonicated to generate 200–1000-bp DNA fragments. Cell lysates were subjected to centrifugation, and the cleared supernatant was incubated with 4 μ g of the PKM2 or HIF-1 α antibodies at 4 $^{\circ}$ C overnight. Immune complexes were precipitated, washed, and eluted as described in the manufacturer's protocol. DNA-protein cross-links were reversed by heating at 65 $^{\circ}$ C for 4 h, and the DNA fragments were purified and used as a template for PCR amplification. Quantitative real time PCR was carried out. For ChIP analysis, the *CYP19A1* oligonucleotide sequences for PCR primers were 5'-AAC CTG ATG AAG TCA CAA-3' (forward) and 5'-TCA GAC ATT TAG GCA AGA CT-3' (reverse). This primer set covers the *CYP19A1* promoter I.3/II segment from nucleotide -302 to -38. PCR was performed at 94 $^{\circ}$ C for

30 s, 62 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 45 s for 35 cycles, and real time PCR was performed at 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 60 s for 40 cycles. The PCR product generated from the ChIP template was sequenced, and the identity of the *CYP19A1* promoter was confirmed.

Aromatase Activity—Aromatase activity assay was based on measurement of tritiated water released from 1 β -[³H]androstenedione (5, 9, 18, 44, 45). The reaction was also done in the presence of letrozole, a specific aromatase inhibitor, as well as a specificity control and without NADPH as a background control. Aromatase activity was normalized to protein concentration.

Hsp90 ATPase Activity—The ATPase assay was based on a regenerating coupled enzyme assay and was performed as described earlier (21, 22, 47). Hsp90 ATPase activity is expressed as pmol/min/mg protein.

Animal Model—p53 knock-out mice carrying the Trp53^{tm1Tyj} allele were maintained on the 129S6 inbred strain background. Genotyping was performed by PCR analysis of DNA extracted from tail tip biopsies (48). Tissues were harvested from female p53^{-/-} and p53^{+/+} littermate control mice at 6–10 weeks of age. Mammary gland tissue was isolated and rinsed once with PBS and then snap frozen for subsequent molecular analysis. All animal use was performed in accordance with federal and institutional guidelines, under a protocol approved by the Cornell University Institutional Animal Care and Use Committee.

Immunofluorescence and Confocal Microscopy—Breast tissue from LFS patients was obtained from kConFab (Melbourne, Australia) and compared with normal breast tissue from reduction mammoplasty from women of similar ages. The studies have been approved by Monash Health Human Research Ethics Committee B and the Peter MacCallum Cancer Centre. LFS patient details are included in Table 1. Formalin-fixed paraffin-embedded sections from normal breast tissue and LFS patients were dewaxed with xylene and rehydrated in descending grades of ethanol. Immunofluorescence was performed after antigen retrieval in 100 $^{\circ}$ C water bath with 10 mM Tris, 1 mM EDTA buffer for 30 min. Sections were then washed in PBS and blocked with 0.5% BSA/PBS for 30 min. Primary antibodies (1:200 rabbit mAb PKM2 4053P from Cell Signaling Technology and 1:100 mouse mAb HIF-1 α , 610958 from BD Transduction Laboratories and 1:500 mouse mAb Aromatase #677 (Dean Edwards, Baylor College of Medicine, Houston, TX)) were diluted in PBS and incubated overnight at 4 $^{\circ}$ C followed by the application of 1:750 of anti-mouse Alexa Fluor 546, 1:750 anti-rabbit Alexa Fluor 488, and 1:2000 Hoechst 33342 (Invitrogen) for 2 h. The sections were then

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washed and mounted with ProLong[®] Gold antifade mountant (P36934 from Thermo Fisher Scientific). Imaging was done using the Nikon inverted confocal microscope.

Statistics—Comparisons between groups were made by Student's *t* test. A difference between groups of *p* < 0.05 was considered significant. For correlation studies, the Pearson correlation coefficient *r* was calculated from *n* = 100 cells using GraphPad Prism 6.

Author Contributions—K. S. designed, performed, and analyzed experiments using cells and mouse tissue and helped draft the manuscript. K. A. B. designed and interpreted the confocal microscopy experiments and assisted in drafting the manuscript. H. Z. carried out the confocal microscopy experiments, assisted in data interpretation and manuscript preparation. G. B. maintained the p53^{+/+} and p53^{-/-} mice, prepared mammary tissue and provided constructive suggestions on the manuscript. R. S. W. helped with the design of the mouse experiments shown in Fig. 7 and assisted with editing the manuscript. B.-S. H. generated cell lines that were used in the study and assisted with manuscript preparation. A. J. D. assisted in the design of the experiments, data interpretation, and manuscript preparation.

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