The Nuclear Import of Oncoprotein Hepatitis B X-interacting Protein Depends on Interacting with c-Fos and Phosphorylation of Both Proteins in Breast Cancer Cells

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Background: The oncoprotein HBXIP acts as a coactivator of transcription factor in cancer.

Results: The nuclear import of HBXIP depends on interacting with c-Fos and ATM-mediated phosphorylation of HBXIP and p-ERK1/2-mediated phosphorylation of c-Fos.

Conclusion: The nuclear import of HBXIP is required for collaboration with c-Fos.

Significance: We provide new insights into the mechanism that HBXIP imports into the nucleus in breast cancer cells.

Aberrant nuclear localization of oncopgenic transcription factors and coactivators always leads to the development of cancer. We have reported that the oncoprotein hepatitis B X-interacting protein (HBXIP) acts as a novel transcriptional coactivator to promote proliferation and migration of breast cancer cells. However, the mechanism of regulating the nuclear import of HBXIP remains unclear. In the present study, we found that HBXIP interacted with c-Fos through their leucine zipper domains in vitro and in vivo. Interestingly, the leucine zipper mutant of HBXIP (or c-Fos) was unavailable to bind to c-Fos (or HBXIP), resulting in the disappearance of nuclear localization of HBXIP. Moreover, we revealed that the nuclear import of HBXIP was required for phosphorylation of c-Fos at Thr232, Thr325, Thr331, and Ser374 by ERK1/2. In addition, the mutant of HBXIP at the Ser108 phosphorylation site failed to import into the nucleus. Strikingly, we found that the kinase ataxia telangiectasia mutated (ATM) phosphorylated HBXIP at Ser108. The knockdown of ATM by siRNA remarkably decreased the levels of ATM and blocked the nuclear import of HBXIP.

Consequently, we suggest that the nuclear import of the oncoprotein HBXIP requires interaction with c-Fos through their leucine zipper domains and phosphorylation of both proteins in breast cancer cells. Thus, our findings provide new insights into the mechanism of the nuclear import of HBXIP. Therapeutically, the block of the nuclear import of HBXIP is significant in breast cancer.

1 The abbreviations used are: HBXIP, hepatitis B X-interacting protein; NLS, nuclear localization signal; ATM, ataxia telangiectasia mutated; p-ERK, ERK1/2-mediated phosphorylation of c-Fos.

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site(s), and then influence protein subcellular localization (12). The nuclear import of proteins occurs through nuclear pore complexes and typically requires specific signals: the nuclear localization signal (NLS) (15, 16). Nuclear transport of proteins is always mediated by nuclear import receptors, which can recognize NLS (17). If proteins have no NLS, they should enter the nucleus together with other carrier proteins (18–21).

c-Fos is a proto-oncogene belonging to the immediate early gene family of transcription factors (22, 23), which has a leucine zipper binding domain and a transactivation domain at the C terminus. c-Fos is one member of the dimerizer activator protein 1 (AP-1) transcription factor family that regulates key cellular processes, including cell proliferation, death, survival differentiation, and oncogenic transformation (24, 25). It has been documented that c-Fos shuttles between the cytoplasm and the nucleus, which requires the operative MEK/ERK signal for phosphorylation of c-Fos to nuclear import (26). c-Fos may form heterodimers with c-Jun family members containing a basic leucine zipper, and travel into the nucleus using the NLS carrying those proteins. c-Fos can carry NLS-deleted c-Jun import into the nucleus through their leucine zippers (21).

In this study, we first provide a mechanistic explanation for nuclear import of the oncoprotein HBXIP in breast cancer cells. Our data uncover that nuclear import of HBXIP requires collaboration with c-Fos, and is dependent on interacting with c-Fos and phosphorylation of both HBXIP and c-Fos in breast cancer cells. The nuclear import of HBXIP contributes to its nuclear function. This finding provides new insights into the mechanism by which HBXIP imports into the nucleus in breast cancer cells.

EXPERIMENTAL PROCEDURES

Plasmids and siRNAs—pCMV-3V-c-Fos, pCMV-4A-c-Fos, and full-length HBXIP constructs were previously described (6, 27, 28). To identify the corresponding domain that activates c-Fos, full-length HBXIP was divided into 4 fragments, including amino acids 1–82, 83–173, 83–144, and 145–173. The leucine zipper mutant of HBXIP was constructed as described (29). The fragments and mutants of HBXIP were inserted into the pCMV-tag 2B plasmid. The full-length pCMV-c-Fos and pCMV-c-Jun plasmids were purchased from OriGene Technologies Co. The siRNAs directed against c-Fos 3’-UTR, ataxia telangiectasia mutated (ATM), and control siRNAs were purchased from Ribio Company (Guangzhou, China). The sequences of siRNA for HBXIP, c-Fos, ERK, and c-Jun were previously described (3, 30–32).

Cell Culture and Treatment—Breast cancer cell lines MCF-7 and SK-BR3 were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). The breast epithelial cell line MCF-10A was cultured in DMEM (Invitrogen) supplemented with 5% horse serum, 100 mg/ml of cholaer toxin, 20 ng/ml of epidermal growth factor, 10 μg/ml of insulin, 0.5 μg/ml of hydrocortisone, 50 units/ml of penicillin, and 50 mg/ml of streptomycin. Cells were collected and seeded in 6-, 24-, or 96-well plates for 24 h and then transected with siRNA or plasmid. All transfections were performed using Lipo-nectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Antibodies—Primary antibodies directed against c-Fos (sc-52), phosphorylated (Ser375)-c-Fos (sc-81485), GST (sc-138), ERK1/2 (sc-93), pERK1/2 (sc-7383), HBXIP (sc-134791), and HRP-coupled secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-Histone 3 (21237) was purchased from Signalway Antibody. Anti-S100A4 was purchased from Proteintech Group. Anti-FLAG and anti-β-actin were purchased from Sigma.

Immunofluorescence Staining and Confocal Microscopy—Cells were grown on acid-treated glass coverslips as described elsewhere (27). Treated cells were fixed with ice-cold 4% paraformaldehyde for 10 min, washed three times with phosphate-buffered saline (PBS), and permeabilized with 0.1% Triton X-100 in PBS for 20 min. After washing three times with 0.05% Tween 20 in PBS, samples were blocked in PBS containing 3% BSA for 1 h. Cells were incubated with primary antibodies for 2 h at room temperature. After being washed, the cells were incubated with fluorophore-conjugated secondary antibody (DAKO, Denmark) and DAPI. After washing, slides were mounted with glycerol and observed under an upright fluorescence microscope (Zeiss Axio Imager Z1) or a confocal microscopy (Leica TCS SP5). The cells in which HBXIP localized in the nucleus by observation of three randomly selected fields and 200 cells were counted.

Nucleocytoplasmic Fractionation and Western Blot Analysis—Nucleocytoplasmic fractionation was performed as described previously (33). In brief, cells were collected and resuspended in 1:5 diluted buffer A (50 mM Hepes, pH 7.4, 1 mM EDTA, 10 mM mannitol, 1 mM DTT, 2 μg/ml aprotinin, 2 μg/ml of leupeptin and 1 mM PMSF). After incubation on ice for 10 min, cells were centrifuged at 6,000 × g at 4 °C for 10 min; the cytoplasmic fraction was collected in the supernatant. The pellet was washed with buffer A and then resuspended in buffer B (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, and 1 mM PMSF). After centrifugation, the nuclear fraction was collected in the supernatant. The methods used for Western blots were described previously (34). In cell fractionation studies, the value under each lane in the Western blot images indicates the relative amounts of protein relative to the control group. The value was obtained by the intensity ratio between the target protein and β-actin band for cytoplasmic fractionation in each lane. The value was obtained by the intensity ratio between the target protein and histone H3 band for nuclear fractionation in each lane. Protein bands were quantified using Quantity One software (Bio-Rad).

Co-immunoprecipitation (Co-IP) and GST Pull-down—Co-IP assays were performed as described previously (8). The c-Fos cDNAs were inserted into pGEX-4T-1 vector, and HBXIP cDNA was cloned into pET-28a vector. Proteins were expressed in Escherichia coli BL21 or BL21(DE3) and induced with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside at 16 °C for 16 h. The GST-c-Fos fusion proteins expressed in bacteria were purified by glutathione-Sepharose 4B (GE Healthcare) or nickel-nitriiotricatic acid-agarose beads (Qiagen). The beads were washed and purified HBXIP was added. The binding reac-
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HBXIP Is Able to Interact with c-Fos in Vivo and in Vitro—To identify the mechanism by which c-Fos mediates the nuclear import of HBXIP, we examined the relationships between HBXIP and c-Fos in the cells. Co-IP showed that c-Fos could be immunoprecipitated by HBXIP antibody. Reciprocally, HBXIP is present in the samples immunoprecipitated by c-Fos antibodies in MCF-7 and SK-BR3 cells (Fig. 2A). Then we analyzed the cellular distribution of HBXIP and c-Fos by immunofluorescence staining. Confocal images showed that HBXIP and c-Fos were co-localized in breast cancer cells with significant overlap (Fig. 2B). To determine that the interaction between HBXIP and c-Fos was direct or indirect, we performed GST pull-down experiments. Our data revealed that GST-c-Fos, but not GST alone, interacted with His-HBXIP (Fig. 2C). A gel filtration experiment also showed that HBXIP and c-Fos were found in the complexes of cytoplasm (Figs. 1C and 2D). To rule out the possibilities that interaction of HBXIP with c-Fos is involved in activation of AP-1, we examined the effect of the interaction of HBXIP with c-Fos on AP-1 target genes. The AP-1 element is found in the c-Fos, c-Jun, and S100A4 promoters.
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**FIGURE 1.** c-Fos is involved in the nuclear import of HBXIP. A, cellular localization of the HBXIP protein was examined by immunofluorescence staining in MCF-10A, SK-BR3, and MCF-7 cells, respectively. Immunofluorescence images of HBXIP (red) are shown. DAPI staining (blue) was included to visualize the nucleus. The scale bar represents 50 μm for immunofluorescence. B, nucleocytoplasmic HBXIP expression was determined by Western blot analysis in MCF-10A, SK-BR3, and MCF-7 cells, respectively. C and D, MCF-7 cells were transfected with FLAG-HBXIP, the FLAG-IP purified cytoplasmic fraction was subjected to gel filtration on a Superdex-200 column, and the expression levels of HBXIP in each subunit were detected by Western blot. E, a model of leucine zipper motif of HBXIP. The leucine zipper region is highlighted with yellow. F, nuclear localization of HBXIP in MCF-7 and SK-BR3 cells transfected with c-Fos siRNA. Immunofluorescence images of HBXIP (red) are shown. DAPI staining (blue) was included to visualize the nucleus. The appearance of HBXIP staining in the nucleus was scored and shown as the percentage of total cells. The scale bar represents 50 μm for immunofluorescence. G, nucleocytoplasmic HBXIP expression transfected c-Fos siRNA in MCF-7 and SK-BR3 cells was examined by Western blot analysis. The value under each lane in the Western blot images indicates the relative amounts of protein relative to control group. The value is obtained by the intensity ratio between the target protein and β-actin band for cytoplasmic fractionation in each lane. The value is obtained by the intensity ratio between the target protein and histone H3 band for nuclear fractionation in each lane. Protein bands were quantified using Quantity One software (Bio-Rad). H, the expression levels of c-Fos were examined by Western blotting after treatment with c-Fos siRNA in MCF-7 cells.

Fosl2 promoters (36, 37, 39). ChIP assays showed that HBXIP failed to bind to the promoters of c-Fos, c-Jun, and Fosl2 at AP1 sites in MCF-7 cells, respectively (data not shown), suggesting that the interaction of HBXIP with c-Fos is not involved in the activation of AP1. Thus, we conclude that HBXIP is able to interact with c-Fos in the cells, which may play an important role in the process of nuclear import of HBXIP, rather than activating AP1.
HBXIP Interacts with c-Fos through Their Leucine Zipper Domains to Import into the Nucleus—Next, we tried to identify the interaction domain of HBXIP binding to c-Fos. HBXIP was divided into 4 fragments (Fig. 3A). We found that the fragment of HBXIP (amino acids 83–144) including the leucine zipper was able to interact with c-Fos (Fig. 3A), suggesting that the leucine zipper of HBXIP may be responsible for interaction of HBXIP with c-Fos. To validate the hypothesis, we constructed the leucine zipper mutant of HBXIP, in which Leu^{130}, Leu^{137}, and Ile^{184} were mutated into valine (V) (termed pCMV-3V-HBXIP). We constructed the c-Fos leucine zipper mutant as well, in which Leu^{172}, Leu^{179}, and Leu^{186} were mutated into valine (V) (termed pCMV-3V-c-Fos). Co-IP assay showed that the c-Fos mutant failed to interact with HBXIP, and the HBXIP mutant also failed to interact with c-Fos in breast cancer cells (Fig. 3, B and C), suggesting that HBXIP interacts with c-Fos through their leucine zipper domains.

Next, we tested the effect of the c-Fos leucine zipper mutant on the nuclear import of HBXIP in breast cancer cells. We first removed the endogenous c-Fos using c-Fos 3’-UTR siRNA, then the leucine zipper mutant of c-Fos with FLAG tag (pCMV-3V-c-Fos) (Fig. 3D) was transfected into MCF-7 cells. Immunofluorescence staining and Western blot analysis showed that the exogenous c-Fos mutant failed to mediate the nuclear import of HBXIP. In control, the overexpression of wild type c-Fos worked well in the cells (Fig. 3E). Reciprocally, overexpression of the FLAG-HBXIP mutant also failed to import into the nucleus of MCF-7 cells by immunofluorescence staining and Western blot analysis (Fig. 3F). In addition, we showed that HBXIP was able to interact with c-Jun containing the leucine zipper as well. But, knockdown of c-Jun failed to affect the nuclear import of HBXIP in the cells (data not shown), suggesting that not all leucine zipper domains are required for the event. From these experiments, we conclude that the interaction of HBXIP with c-Fos through their leucine zipper domains is involved in nuclear import of HBXIP in breast cancer cells.

The Phosphorylation of c-Fos Is Involved in the Nuclear Import of HBXIP—It has been reported that phosphorylation of c-Fos plays an important role in its nuclear localization (26). Thus, we are interested in the phosphorylation role of c-Fos in the nuclear import of HBXIP process. We validated that phosphorylated c-Fos was able to interact with HBXIP in the total lysate and cytoplasmic fraction of MCF-7 cells (Fig. 4A). Usually, phosphorylation of c-Fos is activated by phosphorylated ERK1/2 (p-ERK1/2) (40). Interestingly, we observed that HBXIP failed to transport into the nucleus when MCF-7 cells were treated with PD98059 (an inhibitor of ERK) or ERK1/2 siRNA by immunofluorescence staining and Western blot analysis (Fig. 4, B and C). The RNA interference efficiency of ERK and effect of PD98059 on phosphorylated c-Fos were validated by Western blot analysis (Fig. 4, D and E). It suggests that phosphorylation of c-Fos by ERK1/2 may be involved in the nuclear import of HBXIP.

To confirm the ERK1/2 role in phosphorylation of c-Fos in the event of the nuclear import of HBXIP, we constructed the ERK phosphorylated site mutants vector of c-Fos, containing four amino acid phosphorylation sites at Thr^{232}, Thr^{325}, Thr^{331}, and Ser^{374} (termed pCMV-4A-c-Fos) (27), and single mutants (termed T232A, T325A, T331A, and S374A, respectively). To rule out the effect of endogenous c-Fos on the nuclear import of HBXIP, the endogenous c-Fos was silenced by siRNA in MCF-7 cells. Then, immunofluorescence staining displayed that the protein levels of HBXIP in the nucleus were slightly reduced relative to the wild type c-Fos control. For pCMV-4A-c-Fos, immunofluorescence staining and immunoblotting showed that the protein levels of HBXIP in the nucleus were remarkably decreased (Fig. 5, A and B). Moreover, we validated that the total c-Fos protein levels had no change, but the levels of phosphorylated c-Fos was decreased in the cells (Fig. 5C), suggesting that our system is working well. Co-IP assay showed that HBXIP was still able to interact with all of the above mutants (Fig. 5D). The four mutated sites did not exist in the leucine zipper (amino acids 160–196) of c-Fos, which is the binding domain to HBXIP. It suggests that phosphorylation of c-Fos at Thr^{232}, Thr^{325}, Thr^{331}, and Ser^{374} is responsible for the nuclear import of HBXIP. In addition, according to our previous report that HBXIP was able to activate ERK1/2 (41), we hypothesized...
that HBXIP might increase the phosphorylation levels of c-Fos through activating ERK1/2. As expected, our data confirmed the phosphorylation of c-Fos, which could be activated by HBXIP through p-ERK1/2 (Fig. 5, E and F). Taken together, we conclude that phosphorylation of c-Fos is involved in the nuclear import of HBXIP through interaction of c-Fos with HBXIP.

The Phosphorylation of HBXIP at Ser<sup>108</sup> by ATM Is Involved in Nuclear Import of HBXIP—According to the observation that the phosphorylation of c-Fos is involved in the nuclear import of HBXIP, we are interested in whether phosphorylation of HBXIP is involved in the event as well. It has been reported that there are three potential phosphorylation sites in HBXIP, such as Thr<sup>94</sup>, Ser<sup>108</sup>, and Thr<sup>128</sup>, using structure analysis (29). In this study, we observed that HBXIP was phosphorylated in MCF-7 cells by Western blot analysis using antibodies against the phosphorylated threonine or serine. To validate whether HBXIP could be phosphorylated in the cells, we constructed three mutants of HBXIP, which replaced the above potential phosphorylation sites with alanine. Our data showed that the phosphorylation levels of the three mutants of HBXIP were remarkably reduced relative to those of the wild type HBXIP in MCF-7 cells by Western blot analysis using the antibodies against phosphorylated threonine or serine (Fig. 6A).

Meanwhile, we validated the data by another method, a radioactive labeling in vitro kinase assay. HBXIP was shown in the system by Western blotting (Fig. 6B), suggesting that Thr<sup>24</sup>, Ser<sup>108</sup>, and Thr<sup>128</sup> are three phosphorylation sites of HBXIP. Co-IP assay indicated that the above mutants of HBXIP were still able to interact with c-Fos (Fig. 6C), due to the fact that the phosphorylation sites of HBXIP do not exist in the leucine zipper domain. Surprisingly, we found that the Ser<sup>108</sup>A mutant of HBXIP failed to enter the nucleus by immunofluorescence staining and Western blot, whereas the Thr<sup>94</sup>A and Thr<sup>128</sup>A
mutants of HBXIP worked well (Fig. 6D). In addition, we constructed a phosphorylation mutant HBXIP Ser108D analog, which replaced Ser108 with aspartate (Asp) and found that it was mainly localized in the nucleus by immunofluorescence staining (Fig. 6E). Interestingly, we observed that HBXIP Ser108D and phosphorylated c-Fos could be co-localized in MCF-7 cells with a significant overlap by confocal assays (Fig. 6F). Moreover, the co-IP assay indicated that HBXIP Ser108D was able to interact with phosphorylated c-Fos in the cytoplasm of MCF-7 cells (Fig. 6G), suggesting that phosphorylated HBXIP at Ser108 is able to interact with phosphorylated c-Fos in the cytoplasm of cells. Thus, it strongly suggests that the Ser108 phosphorylation of HBXIP is necessary for the nuclear localization of HBXIP.

Next, we tried to determine the kinases for HBXIP phosphorylation. Because the nuclear import of HBXIP could be influenced by p-ERK1/2, we are interested in whether ERK1/2 is responsible for HBXIP phosphorylation. Our data showed that the phosphorylation levels of serine and threonine of HBXIP were not affected by ERK1/2 when the cells were treated with ERK1/2 siRNA (Fig. 7A). In addition, HBXIP could not interact with ERK1/2 (Fig. 7B), suggesting that ERK1/2 is not responsible for HBXIP phosphorylation.

It has been reported that the kinase ATM has phosphorylation consensus sites in its substrates: Ser-Gln and Thr-Gln motifs. HBXIP appears to have the motif Ser108-Gln109 (29) and is able to be identified by antibodies to phospho-Ser-Gln by protein IP (42). Accordingly, we silenced ATM by siRNA in MCF-7 cells (Fig. 7C), and found that the serine phosphorylation of HBXIP, but not threonine, was strongly reduced by Western blot analysis using antibodies against the phosphorylated threonine or serine (Fig. 7A). Radioactive labeling of the in vitro kinase assay also showed that HBXIP phosphorylation was strongly reduced by ATM siRNA, meanwhile, HBXIP was shown in the system by Western blotting (Fig. 7D). Furthermore, the co-IP assay validated that HBXIP was able to interact with ATM in the cells (Fig. 7E). We observed that HBXIP almost stayed in the cytoplasm by immunofluorescence staining and Western blot analysis when ATM was knocked down by siRNA (Fig. 7F and G), suggesting that ATM is responsible for HBXIP Ser108 phosphorylation. Thus, we conclude that ATM is responsible for HBXIP phosphorylation at Ser108 through interacting with HBXIP, contributing to the nuclear import of HBXIP.

The Relative Factors Involving HBXIP Nuclear Import Are Responsible for the Nuclear Function of HBXIP—We have reported that HBXIP acts as a transcription coactivator of factors, such as STAT4, SP1, E2F1, and TFIID to transactivate S100A4, LMO4, Skp2, and Lin28B in the promotion of growth and migration of breast cancer cells (8–11). To validate the role of the relative factors involving the nuclear import of HBXIP in the nuclear function of HBXIP, we examined the effect of these factors on the nuclear function of HBXIP in breast cancer cells. ChIP assays showed that HBXIP could bind to the promoters of S100A4, LMO4, Skp2, and Lin28B in MCF-7 cells, respectively. But, the HBXIP with leucine zipper mutants (pCMV-3VH-
BXIP) or with a phosphorylation site of ATM at Ser\textsuperscript{108} (pCMV-S108A) failed to work (Fig. 8A). When the endogenous c-Fos was silenced by siRNA in MCF-7 cells, HBXIP could not bind to the above promoters by ChIP assays. Interestingly, the add-back of wild type, but not c-Fos mutants, restored the binding (Fig. 8B). Next, to validate the mechanism by which c-Fos, p-ERK1/2, ATM, and the phosphorylation sites of c-Fos or HBXIP are responsible for the target genes of HBXIP, we examined the effect of these factors on luciferase activities of \( S100A4 \) mediated by HBXIP as a co-activator of transcription factors. Our data revealed that inhibition of the above factors remarkably decreased the luciferase reporter activities of \( S100A4 \) (Fig. 8C). Meanwhile, Western blot analysis showed the same results (Fig. 8D). In addition, we found that the mutant of HBXIP S108A failed to activate ERK1/2 (data not shown), suggesting that phosphorylation of HBXIP at S108A is associated with the phosphorylation of c-Fos activated by ERK1/2. Thus, we conclude that the relative factors involving the nuclear import of HBXIP, such as c-Fos, p-ERK1/2, ATM, and phosphorylation sites of c-Fos (or HBXIP), are responsible for the nuclear function of HBXIP.

The Relative Factors Involving Nuclear Import of HBXIP Promotes Proliferation and Migration of Breast Cancer Cells—MTT assays and colony formation showed that HBXIP was able to promote the proliferation of MCF-7 cells, whereas treatments as described above abolished the enhancement (Fig. 9, A and B). In addition, wound healing assays revealed that HBXIP enhanced the migration capability of MCF-7 cells, whereas inhibition of the nuclear import of HBXIP abolished enhancement in the cells (Fig. 9C). These data strongly suggest that the relative factors involving the nuclear import of HBXIP, such as c-Fos, p-ERK1/2, ATM, and the phosphorylation sites of c-Fos (or HBXIP), enhances the proliferation and migration of breast cancer cells.

DISCUSSION

Aberrant nuclear localization of the oncogenic transcription factor and coactivator always leads to tumor development (13). It has been reported that HBXIP is a component of Ragulator, which is a guanine nucleotide exchange factor for the Rag GTPases that signal amino acid levels to mTORC1 (5). It suggests that HBXIP plays an important cytoplasmic function in normal cells. However, our recent reports posit that HBXIP acts as a novel coactivator of transcription factors in the nucleus to promote proliferation and migration in breast cancer cells. HBXIP was mainly localized in the cytoplasm in the majority of breast cancer cells in clinical breast cancer tissues by immunohistochemistry staining, however, there were also some in the...
nucleus, but few were equivalent in the cytoplasm and the nucleus (8–11). Interestingly, we observed that HBXIP was mainly localized in the cytoplasm of the normal epithelial breast cell line MCF-10A. However, the proliferative active cells, such as MCF-7 and SK-BR3 cell lines, sustained high levels of HBXIP in the nucleus. Therefore, nuclear accumulation of HBXIP should be tightly regulated to promote the expression of cancer-related proteins in cancer cells. Thus, we are interested in the mechanism of nuclear import of HBXIP in breast cancer cells.

Basically, proteins smaller than ~40 kDa can diffuse freely between the nucleus and cytoplasm (13). But, some small proteins in a cell, such as MCF-7 and SK-BR3 cell lines, sustained high levels of HBXIP in the nucleus. Therefore, nuclear accumulation of HBXIP should be tightly regulated to promote the expression of cancer-related proteins in cancer cells. Thus, we are interested in the mechanism of nuclear import of HBXIP in breast cancer cells.

It has been reported that c-Fos is a nuclear proto-oncogene belonging to the AP-1 family of transcription factors. It has been reported that c-Fos can carry the NLS-deleted c-Jun import into the nucleus through their leucine zippers (21). Because HBXIP has a leucine zipper domain (29), we supposed that c-Fos might be involved in nuclear import of HBXIP through its leucine zipper. As expected, c-Fos was able to interact with HBXIP through its leucine zipper in vitro and in vivo.

FIGURE 6. The phosphorylation of HBXIP at Ser^{108} is required for its nuclear import. A, the phosphorylation site mutant and wild type HBXIP with a FLAG tag were purified by anti-FLAG antibodies coupled to protein G-Sepharose beads. The anti-serine and anti-threonine antibodies were used for Western blot analysis. B, MCF-7 cells transfected with wild type and mutants of HBXIP, and immunoprecipitation with antibody to FLAG tag was conducted in the presence of HBXIP. The immune complexes were mixed with [γ-^{32}P]ATP, and in vitro phosphorylation was performed. Autoradiography (Autorad) was visualized by phosphorimaging, and levels of FLAG-HBXIP were detected with immunoblots. C, the phosphorylation site mutant of HBXIP was transfected into MCF-7 cells, and interaction of the phosphorylation site mutant of HBXIP with c-Fos was determined by co-IP experiments. D, immunofluorescence images of FLAG-HBXIP (green) were shown. DAPI staining (blue) was included to visualize the nucleus. The appearance of HBXIP staining in the nucleus was scored and shown as the percentage of total cells. The scale bar represents 50 μm for immunofluorescence. Nucleocytoplasmic HBXIP expression transfected wild type HBXIP or phosphorylation site mutants in MCF-7 cells were examined by Western blot analysis. E, nucleocytoplasmic HBXIP expression was examined by immunofluorescence staining in MCF-7 cells transfected with pCMV-S108D (or control pCMV-HBXIP). Images of FLAG-HBXIP (green) are shown. DAPI staining (blue) was included to visualize the nucleus. The appearance of HBXIP staining in the nucleus was scored and shown as the percentage of total cells. The scale bar represents 50 μm. F, pCMV-S108D and phosphorylated c-Fos are co-localized in MCF-7 cells. The expression of phosphorylated c-Fos (red) and FLAG-pCMV-S108D (green) was detected in MCF-7 cells by confocal microscopy. DAPI (blue) shows the nucleus. The scale bar represents 50 μm for immunofluorescence. G, the interaction of pCMV-S108D with endogenous phosphorylated c-Fos in the cytoplasm was examined by co-IP. The immunoprecipitates (IP) were analyzed by Western blotting. IB, immunoblot.
Our finding provides evidence that c-Fos contributes to the nuclear import of HBXIP lacking NLS through binding to the leucine zipper of HBXIP. However, we showed that c-Jun was not involved in the nuclear import of HBXIP in the cells. In addition, HBXIP could not bind to the promoters of c-Fos, c-Jun, and Fosl2 at AP-1 sites in MCF-7 cells. These data suggest that the interaction of HBXIP with c-Fos only involves the nuclear import of HBXIP, but not for HBXIP activating AP-1.

Phosphorylation is one of the most important modifications of proteins. The phosphorylated function motif was found to be required for both passive translocation of the protein to the nucleus and facilitated import via its binding to the nuclear importer (13). The phosphorylation of c-Fos is important to its nuclear localization (26). Interestingly, our findings showed that phosphorylation of c-Fos was also associated with the nuclear import of HBXIP. MAP kinase ERK1/2 is widely involved in eukaryotic signal transduction (47). Four amino acids of c-Fos at Thr232, Thr325, Thr331, and Ser374 could be phosphorylated by p-ERK1/2 (27). We previously demonstrated that HBXIP could strongly activate ERK1/2 (42). As expected, we found that HBXIP could increase the phosphorylation levels of c-Fos through activating ERK1/2, which is a benefit for the nuclear import of HBXIP.

Growing evidence reveals that ATM kinase, one of the critical DNA damage response elements, is hyperactive in late stage clinical breast tumor tissues of patients with lymph node metastasis. ATM phosphorylates Snail, an epithelial mesenchymal transition marker, suggesting that the ATM-Snail pathway promotes tumor metastasis (48). We have demonstrated that HBXIP promotes migration of breast cancer cells (6, 8). Structural analysis showed that HBXIP appears to have the Ser108–Gln109 motif, which is a phosphorylation site specifically recognized by ATM (29). According to the report that there are three potential phosphorylation sites, such as Thr94, Ser108, and Thr128, in HBXIP by bioinformatics analysis (29), we were interested in whether phosphorylation of HBXIP itself is

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**FIGURE 7.** The phosphorylation of HBXIP at Ser108 by ATM is required for its nuclear import. A, the FLAG-tagged HBXIP in MCF-7 cells transfected with ERK siRNA or ATM siRNA were purified by anti-FLAG antibodies coupled to protein G-Sepharose beads. The anti-serine and anti-threonine antibodies were used for Western blot analysis. B, co-IP experiments were carried out with MCF-7 cell extracts. The immunoprecipitates (IP) were analyzed by Western blotting. C, the expression levels of ATM were examined by Western blotting in MCF-7 cells treated with ATM siRNA. D, MCF-7 cells transfected with FLAG-HBXIP and ATM siRNA, and immunoprecipitation with antibody to the FLAG tag was conducted in the presence of HBXIP. The immune complexes were mixed with [γ-32P]ATP, and in vitro phosphorylation was performed. Autorad was visualized by phosphorimaging, and levels of FLAG-HBXIP were detected with immunobots. E, co-IP experiments were carried out with MCF-7 cell extracts. The immunoprecipitates were analyzed by Western blotting. F and G, subcellular localization of the HBXIP protein in MCF-7 cells transfected with ATM siRNA was examined by immunofluorescence staining and Western blot analysis, respectively. Immunofluorescence images of HBXIP (red) are shown. DAPI staining (blue) was included to visualize the nucleus. The appearance of HBXIP staining in the nucleus was scored and shown as the percentage of total cells. The scale bar represents 50 μm for immunofluorescence. Nucleocytoplasmic HBXIP expression was examined by Western blot analysis in MCF-7 cells transfected with ATM siRNA, IB, immunoblot.
required for its nuclear import. In this study, we validated that the three phosphorylation sites of HBXIP could be phosphorylated in our system. Strikingly, we found that Ser108, but not Thr94 and Thr128 of HBXIP were required for the nuclear import of HBXIP. Next, we tried to identify the mechanism of HBXIP phosphorylation. ATM substrate analysis showed that HBXIP was involved in the extensive protein networks through protein IP using phospho-Ser-Gln antibody (42). Interestingly, we revealed that Ser108, but not Thr94 and Thr128 of HBXIP, could be phosphorylated by ATM. Our data suggest that ATM is involved in the phosphorylation of HBXIP in the nuclear import of HBXIP process. However, the mechanism

FIGURE 8. The relative factors involving nuclear import of HBXIP are responsible for the nuclear function of HBXIP. A and B, the ChIP assays were performed in MCF-7 cells, using HBXIP or FLAG antibodies. The DNAs harvested in the ChIP assay were amplified by RT-PCR, using the primers in the S100A4, LMO4, SKP2, and lin28B promoters. C, the promoter activity of S100A4 in MCF-7 cells transfected with pCMV-HBXIP, HBXIP mutants, or c-Fos mutants was examined by luciferase reporter gene assays, respectively. The same response was observed in MCF-7 cells transfected with ATM or c-Fos siRNAs (0.04 – 0.08 nmol). Luciferase activities were measured 24 h after transfection. Bars, mean ± S.E. (n = 3). Student’s t test, * < 0.05; ** < 0.01; *** < 0.001). D, MCF-7 cells were transfected with pCMV-HBXIP, HBXIP mutants, or c-Fos mutants, respectively. Then, the expression levels of S100A4 were also determined by Western blotting.
of whether ATM activation in the event is associated with a DNA damage response in breast cancer cells needs to be elucidated further.

We have reported that HBXIP acts as a coactivator of transcription factors, such as STAT4, SP1, E2F1, and TFIID to transactivate S100A4, LMO4, Skp2, and Lin28B in promotion of growth and migration of breast cancer cells (8–11). To validate the mechanism of HBXIP, we examined the effect of these factors involving the nuclear import of HBXIP on the nuclear function of HBXIP. ChIP and luciferase reporter gene assays demonstrated that the nuclear function of HBXIP depended on interaction of HBXIP with c-Fos and phosphorylation of both HBXIP and c-Fos. Furthermore, MTT, colony formation, and wound healing assays strongly supported that the nuclear import of HBXIP was crucial for its function in promoting proliferation and migration of breast cancer cells.

In Fig. 10, we summarize the mechanism of he oncoprotein nuclear import of HBXIP in a model. The interaction of HBXIP with c-Fos is unavailable to result in the nuclear import of HBXIP when both HBXIP and c-Fos are not phosphorylated in the cells. Notably, phosphorylation of c-Fos at Thr232, Thr325, Thr331, and Ser374 by p-ERK1/2 and HBXIP at Ser108 by ATM is necessary for the nuclear import of HBXIP, based on the interaction of HBXIP with c-Fos in the cells. The nuclear import of HBXIP is crucial for its function in promoting cell proliferation and migration. Potentially, the regulatory factors of nuclear
FIGURE 10. A model of the nuclear import of HBXIP in breast cancer cells. The nuclear import of HBXIP is precisely regulated in breast cancer cells. The interaction of HBXIP with c-Fos is unavailable to result in the nuclear import of HBXIP when both HBXIP and c-Fos are not phosphorylated in the cells. Notably, phosphorylation of c-Fos at Thr122, Thr125, Thr130, and Ser118 by p-ERK1/2 and HBXIP at Ser108 by ATM is necessary for the nuclear import of HBXIP, based on the interaction of HBXIP with c-Fos in the cells.

FIGURE 10. A model of the nuclear import of HBXIP in breast cancer cells. The nuclear import of HBXIP is precisely regulated in breast cancer cells. The interaction of HBXIP with c-Fos is unavailable to result in the nuclear import of HBXIP when both HBXIP and c-Fos are not phosphorylated in the cells. Notably, phosphorylation of c-Fos at Thr122, Thr125, Thr130, and Ser118 by p-ERK1/2 and HBXIP at Ser108 by ATM is necessary for the nuclear import of HBXIP, based on the interaction of HBXIP with c-Fos in the cells.

import of HBXIP may be served as therapeutic targets for breast cancer.

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