# Hepatitis B Virus X Protein Induces IKKα Nuclear Translocation via Akt-dependent Phosphorylation to Promote The Motility of Hepatocarcinoma Cells

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## Abstract

Hepatitis B virus (HBV) X protein (HBx) has been implicated in HBV-associated carcinogenesis through activation of IκB kinase (IKK)/nuclear factor kappa B (NF-κB) signaling pathway. Besides activating NF-κB in the cytoplasm, IKKα was found in the nucleus to regulate gene expression epigenetically in response to various stimuli. However, it is unknown whether nuclear IKKα plays a role in HBx-associated tumor progression. Moreover, the molecular mechanism underlying IKKα nuclear transport also remains to be elucidated. Here, we disclosed HBx as a new inducer of IKKα nuclear transport in hepatoma cells. HBx induced IKKα nuclear transport in an Akt-dependent manner. HBx-activated Akt promoted IKKα nuclear translocation via phosphorylating its threonine-23 (Thr23). In addition, IKKα ubiquitination enhanced by HBx and Akt also contributed to the IKKα accumulation in the nucleus, indicating the involvement of ubiquitination in Akt-increased IKKα nuclear transport in response to HBx. Furthermore, inhibition of IKKα nuclear translocation by mutating of its nuclear localization signal and Thr23 diminished IKKα-dependent cell migration. Taken together, our findings shed light on the molecular mechanism of IKKα nuclear translocation and provide a potential role of nuclear IKKα in HBx-mediated hepatocellular carcinoma (HCC) progression.

## Introduction

Carcinogenesis of hepatocellular carcinoma (HCC) has been proven to be highly associated with a background of chronic and persistent infection of hepatitis B virus (HBV) (Marotta et al., 2004). The involvement of IκB kinase (IKK)/nuclear factor kappa B (NF-κB) pathway in HBV-induced hepatitis and HCC has been well-documented (Arsura and Cavin, 2005). NF-κB activity has been frequently found to be elevated in liver cancer and many other cancer types to mediate gene expressions related to cell proliferation and migration (Biswas et al., 2004; Nakshatri et al., 1997; Nakshatri and Goulet, 2002). The X protein of hepatitis B virus (HBx), associated with the tumorigenesis potential of HBV, is a potent activator of IKK and NF-κB (Chen and Li, 2010; Wang et al., 2004). NF-κB activation induced by HBx has been proven to promote malignant transformation and advantageous growth of HBV-infected liver cells (Wang et al., 2004). It has also been shown that kinase-dead mutant of IKKα can attenuate the HBx-mediated NF-κB activation (Ohata et al., 2003), suggesting the critical role of IKKα in HBx-mediated HCC progression.

While the classic IKK complex has been recognized as a key factor in the activation of NF-κB through phosphorylation and degradation of the inhibitory IκB protein, accumulating findings revealed that IKKα can be detected in the nucleus and plays important roles in regulating gene expressions in both NF-κB-dependent and -independent manners (Anest et al., 2003; Birbach et al., 2002; Yamamoto et al., 2003). In the nucleus, IKKα was found in a complex with NF-κB-inducing kinase (NIK) to phosphorylate p65 subunit at Ser536, and thereby enhances NF-κB activity through increasing p65 DNA binding activity (Jiang et al., 2003). In addition to targeting NF-κB directly in the nucleus, IKKα also regulates NF-κB-mediated gene expression via regulating epigenetic modifications. Nuclear IKKα, recruited to promoters by interacting with CREB-binding protein (CBP), contributes to the transcriptional regulation of NF-κB-targeted genes through phosphorylation of histone H3 at Ser10 (Anest et al., 2003; Yamamoto et al., 2003). This phosphorylation triggers subsequent acetylation of histone H3 at Lys14 by CBP, a crucial step in modulating chromatin accessibility at NF-κB-responsive promoters (Anest et al., 2003; Park and Christman, 2006; Yamamoto et al., 2003). Nuclear IKKα has also been reported to control prostate cancer metastasis by silencing maspin expression, a member of serpin family with tumor suppressive property, through up-regulation of histone H3 Ser10 phosphorylation (Luo et al., 2007). In addition to histone modifications, our previous study further demonstrated that nuclear IKKα phosphorylates CBP at Ser1382 and Ser1386 in response to TNF-α, CD40 and LTβR, which in turn increases both transcriptional and histone acetyltransferase (HAT) activities of CBP (Huang et al., 2007). Moreover, the IKKα-dependent phosphorylations also switch CBP binding preference from p53 to NF-κB and thereby promotes cell growth (Huang et al., 2007). Mayo and co-workers also established a role of nuclear IKKα, but not IKKβ, in de-repressing NF-κB-targeted genes through targeting the transcriptional repressor SMRT (Hoberg et al., 2006; Hoberg et al., 2004). Besides the role of nuclear IKKα in regulating NF-κB-dependent gene transcription

induced by TNF-α, IKKα also interacts with other factors to target on various NF-κB-independent promoters under the simulations with *Helicobacter pylori* (Hirata et al., 2006), estrogen(Tu et al., 2006), EGF(Xie et al., 2003), and cisplatin (Lara-Pezzi et al., 2002). These observations revealed that nuclear IKKα might function as a common and critical signal transducer in response to diverse stimuli. Our previous study showed that the nuclear IKKα-dependent CBP phosphorylations are significant higher in liver cancer cells than in normal liver cell lines (Huang et al., 2007), implying that IKKα in the nucleus may play a critical role in regulating tumor progression of HCC. However, it is unknown whether nuclear expression of IKKα is responsive to HBx and plays a role in HCC tumor progression. The molecular mechanism underlying the IKKα nuclear translocation also remains to be explored.

In the current study, we showed that HBx enhanced the nuclear transport of IKKα accompanied with its downstream functional activities, including histone and CBP phosphorylations and maspin down-regulation. We further explored that Akt mediates the HBx-induced nuclear translocation of IKKα through phosphorylation at Thr23 and ubiquitination of IKKα. Furthermore, inhibition of IKKα nuclear import by mutating its nuclear localization signal (NLS) and Thr23 diminished the migration and invasion of HCC cells. These findings provide the molecular insight into the mechanism of IKKα nuclear import and a potential role of nuclear IKKα in HBx-mediated HCC progression.

## Materials and Methods

*Plasmids, Antibodies, and Chemicals*- Mutations of indicated sites in Flag-IKKα were generated using a Quickchange site-directed mutagenesis kit (Strategene) according to the manufacturer's instructions and confirmed by DNA sequencing. We purchased antibodies against IKKα, phospho-IKKα Thr23, IKKβ, and maspin from Santa Cruz, and antibodies against Akt, phospho-Akt Ser473, phospho-histone H3 Ser10, and histone H3 from Cell Signaling Technology, and antibodies against myc-tag and HA-tag from Sigma-Aldrich. The validated siRNA for negative control and Akt1 were from Dharmacon (Lafayette, CO). Antibody to Ser1382 and Ser1386 phosphorylation sites of CBP was generated in collaboration with Cashmere Biotech (Taipei, Taiwan) as described previously (Huang et al., 2007).

*Cell Culture*- HEK-293, HepG2, HepG2x, Hep3B and Hep3Bx cell lines were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum.

*Transient Transfection*- Cells at 60% confluence were transfected with indicated plasmids or siRNA using Nanofectin (PAA, Cölbe, Germany) according to manufacturer's instruction. Briefly, plasmid DNA and nanofectin were mixed with 1:2 ratio in serum-free DMEM medium at room temperature for 30 minutes and then were added to Hep3B or HEK-293 cells for 6 h at 37°C followed by refreshment with complete DMEM medium. After 48 hrs, cells were subjected to cytoplasmic/nuclear protein

fractionation, total lysate preparation, immunofluorescence staining, or transwell migration and invasion assays.

*Cytoplasmic and nuclear protein fractionation*- Cells at 80-90% confluence were washed twice with 1X PBS, and then lysed in Nori buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP-40, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 μg/ml aprotinin), followed by incubation on ice for 10 min. Homogenates, obtained by stroking cell lysates for 40-50 times in a tightly fitting Dounce homogenizer, were centrifuged at 1,500g for 5 min. The supernatant was further centrifuged at 16,100g for 20 min to collect the subsequent supernatant as the cytoplasmic fraction. The former pellet was washed repeatedly for three times with Nori buffer without protease inhibitors followed by centrifugation at 1,500g for 5 min. Then the pellet was re-suspended in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl pH 8.0, 0.5% NP-40, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM PMSF, and 2 μg/ml aprotinin), followed by sonication and centrifugation at 16,100g for 20 min to collect the resulting supernatant as the nuclear fraction.

*Immunofluorescence staining and Confocal microscope analysis*- Cells transfected with indicated plasmids for 48 hr were washed three times with 1X PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.5% Triton X-100 for 15 min followed by blocking with 10% bovine serum albumin (BSA) for 1 hr. The fixed and permeabilized cells were incubated with mouse anti-Flag (1:500 dilution) and/or rabbit anti-myc antibody (1: 200 dilution) at room temperature for overnight followed by incubation with FITC- and/or Cy5-conjugated secondary antibodies (1:200 dilution) for 1hr, respectively. The fluorescence of FITC and Cy5 were visualized and captured by using Leica TCS SP2 confocal microscopy.

*Migration and invasion assays*- Transwell migration and invasion assays were carried out using transwell chambers (24-well insert; pore size, 8 μm; Costar Corp.). For migration assays, cells (5 x $10^4$ per well) were plated on the non-coated membrane of the upper chamber. For invasion assay, cells (2 x $10^5$ per well) were plated on the Matrigel (60 μg; BD Bioscience)-coated membrane of the upper chamber. After incubation for 48 hr, cells remained inside the upper chamber were removed with cotton swab. Cells migrated or invaded through the pores to the opposite side of the membrane were fixed with methanol and stained with crystal violet. The number of the migrated or invasive cells was counted under a light microscope. Student's t test was used to assess the statistical significance.

**Results**

*HBx induces the nuclear translocation and functions of IKKα.* To assess whether nuclear expression of IKKα is responsive to HBx overexpression, the nuclear level of IKKα in HBx-stable transfectant of

Hep3B (Hep3Bx) hepatocarcinoma cell line was examined by Western blot. As shown in Fig. 1A, the nuclear level of IKKα protein was enhanced in Hep3Bx cells as compared with the parental cells. The elevation of nuclear IKKα was also observed in HEK-293 cells transiently transfected with myc-HBx (Fig. 1B). To further confirm this finding, Hep3B cells were transfected with Flag-IKKα alone or co-transfected with Flag-IKKα and myc-HBx, and then were subjected to immunofluorescence staining. Consistently, the nuclear level of Flag-IKKα was significantly higher in the presence of myc-HBx (Fig. 1C). These results suggest HBx as an inducer of IKKα nuclear translocation. In addition to HBx in liver cancer cells, activation of EGFR by EGF (Supplementary Fig. 1A) and overexpression of HER2 (supplementary Fig. 1B), two receptor tyrosine kinase of HER family, also induced IKKα nuclear translocation in MCF-7 breast cancer cells, suggesting that the nuclear translocation of IKKα may be commonly occurred in response to a variety of oncogenic stresses.

Next, we further examined whether HBx is able to enhance the nuclear functions of IKKα, including Ser10 phosphorylation of histone H3, Ser1382/1386 phosphorylations of CBP, and down-regulation of maspin expression. In parallel to IKKα nuclear transport, histone H3 Ser10 phosphorylation in Hep3Bx cells was higher than that in the parental Hep3B cells (Fig. 2A). Transient transfection with HBx also dose-dependently increased histone H3 Ser10 phosphorylation in HEK-293 cells (Fig. 2B). This nuclear IKKα-dependent histone phosphorylation has been proposed to repress the gene expression of tumor suppressor maspin (Luo et al., 2007). Indeed, a lower protein level of maspin was found in Hep3Bx cells than in Hep3B cells (Fig. 2C). In addition, HBx also induced the nuclear IKKα-dependent phosphorylation of CBP at Ser1382/1386 in HepG2 cells (Fig. 2D). These results support that HBx induced IKKα nuclear translocation and its downstream signals in the nucleus.

*Akt-dependent Thr23 phosphorylation of IKKα promotes its nuclear translocation in response to HBx.* Next, we investigated the molecular mechanisms of IKKα nuclear translocation. The kinase activity of IKKα has been demonstrated to be required for its nuclear translocation. Most of NF-κB activation stimuli, such as TNF-α, induce the kinase activity of both IKKα and IKKβ subunits but only enhance the nuclear translocation of IKKα, but not IKKβ (Anest et al., 2003; Huang et al., 2007; Yamamoto et al., 2003), suggesting that IKKα nuclear import is regulated by a signal pathway specific for this subunit. Among the diverse receptor activation signals to IKK signalosome, Akt has been demonstrated to more specifically activate IKKα, but not IKKβ, via phosphorylating IKKα at Thr23 (Ozes et al., 1999). Intriguingly, those signals stimulating IKKα nuclear import, including HBx, EGF, HER2, and TNF-α, also commonly induce Akt activation. These clues prompted us to assess whether Akt promotes the nuclear translocation of IKKα through phosphorylating its Thr23 in response to HBx overexpression. In support to this notion, Akt phosphorylation at Ser473, an indicator for Akt activation, was significantly higher in Hep3Bx cells than that in Hep3B cells (Fig. 3A). The interaction between IKKα and Akt was also enhanced by overexpression of HBx (Fig. 3B). Furthermore, overexpression of myc-HBx

dose-dependently increased both Akt phosphorylation at Ser473 and IKKα phosphorylation at Thr23 (Fig. 3C). The HBx-induced IKKα phosphorylation at Thr23 was abolished by two pharmacological inhibitors of PI3K/Akt pathway in Hep3B cells (Fig. 3D). These data indicated that HBx activated Akt to phosphorylate IKKα at Thr23.

To further examine whether Akt regulates HBx-induced IKKα nuclear translocation, HEK-293 cells were co-transfected with Flag-IKKα and myc-HBx followed by treatment with PI3K/Akt inhibitors. As shown in Fig. 4A, inhibition of Akt activation by API2 and LY294002 reduced HBx-induced IKKα nuclear transportation. In addition to these pharmacological inhibitors, silence of Akt expression by siRNA also attenuated the nuclear accumulation of IKKα by HBx (Fig. 4B). Consistently, overexpression of constitutively active Akt dose-dependently increased the nuclear level of IKKα, but not IKKβ (Fig. 4C), supporting the crucial and specific role of Akt in regulating the nuclear import of IKKα. Next, we assessed the necessity of IKKα Thr23 phosphorylation by Akt in HBx-induced IKKα nuclear transport. By substituting IKKα Thr23 with Ala (IKKα T23A) to mimic the unphosphorylated status, the effect of this mutation on HBx-induced IKKα nuclear transportation was examined. As shown in Fig. 5A, the nuclear level of IKKα T23A mutant was not increased by overexpression of HBx. In contrast to the lower nuclear level of IKKα T23A mutant, the phosphorylation-mimicking mutant of IKKα (T23E) showed a higher nuclear expression as compared with wild-type IKKα (wtIKKα) in the absence of HBx (Fig. 5B). These results support that Akt promotes IKKα nuclear translocation via phosphorylating its Thr23 in response to HBx.

In addition to identifying Thr23 phosphorylation by Akt as a regulator for IKKα nuclear import, we further characterized the essential peptide signals for IKKα nucleo-cytoplasmic shuttling. The presence of a lysine-rich motif, Lys235, Lys236, and Lys237 (as illustrated in Fig. 5C upper panel) has been proposed as a putative nuclear localization signal (NLS) in IKKα (Sil et al., 2004). We further established the NLS-inactive IKKα mutant by substitutions of these three lysines with methionines (IKKα $NLS^{3KM}$) and examined its deficiency in nuclear transportation. Unexpectedly, the nuclear level of IKKα $NLS^{3KM}$ was not significantly reduced in comparison with wtIKKα. However, when we further mutated two additional residues at Lys233 and Lys240 (IKKα $NLS^{5KM}$), the nuclear expression of IKKα was dramatically attenuated (Fig. 5B). These results indicated that all five lysine residues may be required for the nuclear translocation of IKKα. In addition to nuclear import, IKKα nuclear export in a CRM-1-dependent fashion has also been observed (Birbach et al., 2002). However, the nuclear export signal (NES) required for IKKα nucleo-cytoplasmic shuttling remains unidentified. Sequence analysis revealed the presence of a putative leucine-rich NES within the C-terminal domain of IKKα (Fig. 5C, lower panel). Substitutions of leucine or isoleucine within this motif with alanines ($NES^{mut}$) enhanced the ability of IKKα to retain in the nucleus (Fig. 5D), indicating that the residues 714-724 in IKKα may be critical for its nuclear export.

*Akt mediates HBx-induced IKKα nuclear translocation via increasing ubiquitination of IKKα.* In addition to phosphorylation, ubiquitination is another critical posttranslational modification for protein trafficking to the nucleus (Geetha et al., 2005; Lohrum et al., 2001; Massoumi et al., 2006; Trotman et al., 2007). Although ubiquitinations of IKKβ (Carter et al., 2003) and IKKγ (Shambharkar et al., 2007; Tang et al., 2003) were identified and important for the activation of IKK complex in response to TNF-α, it is unknown whether IKKα is ubiquitinated in response to HBx overexpression. Interestingly, our data revealed that the increase in IKKα protein smear was detected in HBx-overexpressing cells (Fig. 6A), raising the possibility that HBx may also enhance IKKα ubiquitination. To test this hypothesis, Flag-tagged IKKα was overexpressed in HEK-293 cells and was immunoprecipitated and probed with anti-ubiquitin antibody. As shown in Fig. 6B, poly-ubiquitination of immunoprecipitated IKKα was detectable in HEK-293 cells and was significantly increased by overexpression of HBx. Interestingly, overexpression of Akt also enhanced the poly-ubiquitination of IKKα, which was further increased by ectopical expression of ubiquitin (Fig. 6C). Moreover, the Akt-increased nuclear expression of IKKα was further augmented by overexpression of ubiquitin (Fig. 6D), suggesting that ubiquitination may contribute to the Akt-dependent nuclear transportation of IKKα.

*Nuclear IKKα promotes HCC cell migration and invasion.* HCC has poor prognosis due to intra-hepatic metastasis, which is strongly associated with the expression level of HBx (Lara-Pezzi et al., 2002). Our data also showed that Hep3Bx cells possess higher invasion activity than Hep3B cells *in vitro* (Fig. 7A). Overexpression of IKKα also increased both migration and invasion of Hep3B cells (Fig. 7B). Treatment with IKK inhibitor reduced the HBx-mediated cell migration (Fig. 7C), indicating that IKKα mediated the migration in response to HBx. Moreover, inhibition of IKKα nuclear import either by substitution of Thr23 to Ala (T23A) or by mutation of NLS attenuated the IKKα-enhanced migration of Hep3B cells. These results suggest that nuclear IKKα may contribute to HBx-induced cell migration and invasion.

**Discussion**

Studies with knockout experiments have shown that IKKα is dispensable for IκB degradation although both IKKα and IKKβ are critical for NF-κB mediated gene induction. IKKα activity in concomitant with undetectable nuclear p65 and p52 expressions and bindings on NF-κB-targeted genes in colorectal cancer reflects the involvement of NF-κB-independent mechanisms by IKKα in tumorigenesis (Fernandez-Majada et al., 2007). Accumulating evidence revealed that IKKα is detectable in the nucleus and has its unique functions, whereas IKKβ were localized predominantly in the cytoplasm (Anest et al., 2003; Yamamoto et al., 2003), indicating a functional difference between these two related signaling kinases with respect of their intracellular distribution. The nuclear translocation of IKKα can respond to a variety of stimuli. However, the underlying molecular mechanisms regulating

IKKα nuclear import were unclear. In our previous study, the nuclear translocation of IKKα was found in IKKβ$^{-/-}$ and IKKγ$^{-/-}$ mouse embryonic fibroblast (MEF) cells upon TNF-α stimulation, suggesting that the nuclear transportation of IKKα is in an IKKβ- and IKKγ-independent manner (Huang et al., 2007). IKKα is able to translocate into the nucleus by a nuclear import mechanism that depends on its kinase activity (Birbach et al., 2002). Our current study further disclosed that Akt regulates IKKα nuclear trafficking in response to HBx overexpression.

Akt signaling plays a key role in several processes considered hallmarks of cancer through phosphorylating a variety of substrates implicated in tumor progression and drug resistance to anti-cancer therapies (Huang and Hung, 2009). In addition to regulating the enzymatic activity of most substrates, activated Akt also provokes its pleiotropic effects through changing the intracellular distribution of some targets. Notably, phosphorylation by Akt promotes nuclear export of forkhead family, transcription factors involved in cell cycle arrest and apoptosis, and thus inhibits their activity (Brunet et al., 1999; Tang et al., 1999; Wolfrum et al., 2003). Recently, Akt was also found to interact with and phosphorylate MST1 at Thr120, which leads to inhibitions of its kinase activity and nuclear translocation (Yuan et al., 2010). In contrast to promoting the nuclear exports of these substrates, phosphorylation by Akt also caused nuclear import of some target proteins. Expression of constitutively active Akt diminished cellular levels and transcriptional activity of p53 through enhancing nuclear entry of Mdm2 (Zhou et al., 2001). Akt also phosphorylates β-catenin at Ser552 to enhance its nuclear localization in intestinal stem cells (He et al., 2007). Our current findings further revealed that IKKα nuclear transportation is regulated by Akt phosphorylation at Thr23. These observations suggest that phosphorylation by Akt may function as a common signal to drive the intracellular trafficking. But other regulations involving interaction with nuclear importer or exporter may be required to decide the destination of the cargo proteins.

Our data further indicated that the Akt-regulated IKKα nuclear transportation involved IKKα ubiquitination. Ubiquitination has been found to be involved in protein nucleo-cytoplasmic shuttling (Geetha et al., 2005; Lohrum et al., 2001; Massoumi et al., 2006; Trotman et al., 2007). We found that ubiquitination enhanced the nuclear localization of IKKα. Moreover, expression of both HBx and constitutively active Akt can enhance IKKα ubiquitination. These results suggest that ubiquitination may contribute to the Akt-mediated IKKα nuclear translocation in response to HBx overexpression. Since Akt has been found to induce p53 ubiquitination via phosphorylating MDM2(Zhou et al., 2001), it cannot exclude the possibility that Akt may regulate IKKα ubiquitination indirectly via targeting its E3 ligase. However, it still awaits further investigations to identify the E3 ligase and the ubiquitination sites for IKKα. It will also be of great interest to know whether Thr23 phosphorylation by Akt affects the interaction of IKKα with its E3 ligase.

In this study, nuclear IKKα induced by HBx was found to promote cell migration and invasion of Hep3B liver cancer cells. This function may associate with down-regulation of tumor suppressor maspin.

Maspin, an anti-angiogenic serpin, inhibits tumor cell invasion and motility through the inhibition of urokinase-type plasminogen activator (Cher et al., 2003). Nuclear IKKα has been reported to promote tumor metastasis via suppressing the expression of maspin in prostate cancer cells (Luo et al., 2007). Our data discovered that overexpression of HBx reduced maspin expression in Hep3B cells. Expression of wtIKKα but not its NLS mutant also suppressed maspin expression in Hep3B cells (unpublished data), implying that nuclear IKKα may contribute to HBx-mediated cell migration and invasion via suppressing maspin expression. Although nuclear IKKα has been proposed to suppress maspin expression via phosphorylating histone H3 at Ser10, it is still unclear how this histone phosphorylation reduce*s maspin* promoter activity. HBx-induced tumor cell migration and invasion have also been reported to be associated with the elevation of several gene expressions including CD44 (Lara-Pezzi et al., 2001), monokine induced by the interferon-γ (Xia et al., 2009), matrix metalloproteinase 9 (MMP9) (Chung et al., 2004), MMP1 and cyclooxygenase-2 (COX-2) (Lara-Pezzi et al., 2002), and miR-143 (Zhang et al., 2009). It is also worthy to further study whether nuclear IKKα mediated the up-regulation of these genes.

In summary, we identified HBx as a new inducer of IKKα nuclear import and elucidated that both Thr23 phosphorylation and ubiquitination by Akt are obligate for HBx-induced IKKα nuclear translocation. Nuclear IKKα may thus contribute to HBx-mediated cell migration and invasion, leading to the malignance of HCC (as illustrated in Fig. 8).

**Reference**

Anest V, Hanson JL, Cogswell PC, Steinbrecher KA, Strahl BD, Baldwin AS. 2003. A nucleosomal function for I[kappa]B kinase-[alpha] in NF-[kappa]B-dependent gene expression. Nature 423(6940):659-663.

Arsura M, Cavin LG. 2005. Nuclear factor-kappaB and liver carcinogenesis Cancer Lett 229(2):157-169.

Birbach A, Gold P, Binder BR, Hofer E, de MR, Schmid JA. 2002. Signaling molecules of the NF-kappa B pathway shuttle constitutively between cytoplasm and nucleus. JBiolChem 277(13):10842-10851.

Biswas DK, Shi Q, Baily S, Strickland I, Ghosh S, Pardee AB, Iglehart JD. 2004. NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. ProcNatlAcadSciUSA 101(27):10137-10142.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg

ME. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96(6):857-868.

Carter RS, Pennington KN, Ungurait BJ, Arrate P, Ballard DW. 2003. Signal-induced ubiquitination of I kappaB Kinase-beta. J Biol Chem 278(49):48903-48906.

Chen B, Li W. 2010. [Current status of Akt in non-small cell lung cancer]. Zhongguo Fei Ai Za Zhi 13(11):1059-1063.

Cher ML, Biliran HR, Jr., Bhagat S, Meng Y, Che M, Lockett J, Abrams J, Fridman R, Zachareas M, Sheng S. 2003. Maspin expression inhibits osteolysis, tumor growth, and angiogenesis in a model of prostate cancer bone metastasis. Proc Natl Acad Sci U S A 100(13):7847-7852.

Chung TW, Lee YC, Kim CH. 2004. Hepatitis B viral HBx induces matrix metalloproteinase-9 gene expression through activation of ERK and PI-3K/AKT pathways: involvement of invasive potential 3. FASEB J 18(10):1123-1125.

Fernandez-Majada V, Aguilera C, Villanueva A, Vilardell F, Robert-Moreno A, Aytes A, Real FX, Capella G, Mayo MW, Espinosa L, Bigas A. 2007. Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer. ProcNatlAcadSciUSA 104(1):276-281.

Geetha T, Kenchappa RS, Wooten MW, Carter BD. 2005. TRAF6-mediated ubiquitination regulates nuclear translocation of NRIF, the p75 receptor interactor. EMBO J 24(22):3859-3868.

He XC, Yin T, Grindley JC, Tian Q, Sato T, Tao WA, Dirisina R, Porter-Westpfahl KS, Hembree M, Johnson T, Wiedemann LM, Barrett TA, Hood L, Wu H, Li L. 2007. PTEN-deficient intestinal stem cells initiate intestinal polyposis. Nat Genet 39(2):189-198.

Hirata Y, Maeda S, Ohmae T, Shibata W, Yanai A, Ogura K, Yoshida H, Kawabe T, Omata M. 2006. Helicobacter pylori induces IkappaB kinase alpha nuclear translocation and chemokine production in gastric epithelial cells. InfectImmun 74(3):1452-1461.

Hoberg JE, Popko AE, Ramsey CS, Mayo MW. 2006. IkappaB kinase alpha-mediated derepression of SMRT potentiates acetylation of RelA/p65 by p300. MolCell Biol 26(2):457-471.

Hoberg JE, Yeung F, Mayo MW. 2004. SMRT derepression by the IkappaB kinase alpha: a prerequisite to

NF-kappaB transcription and survival. MolCell 16(2):245-255.

Huang WC, Hung MC. 2009. Induction of Akt activity by chemotherapy confers acquired resistance. J Formos Med Assoc 108(3):180-194.

Huang WC, Ju TK, Hung MC, Chen CC. 2007. Phosphorylation of CBP by IKKalpha promotes cell growth by switching the binding preference of CBP from p53 to NF-kappaB. MolCell 26(1):75-87.

Jiang X, Takahashi N, Ando K, Otsuka T, Tetsuka T, Okamoto T. 2003. NF-kappa B p65 transactivation domain is involved in the NF-kappa B-inducing kinase pathway. BiochemBiophysResCommun 301(2):583-590.

Lara-Pezzi E, Gomez-Gaviro MV, Galvez BG, Mira E, Iniguez MA, Fresno M, Martinez AC, Arroyo AG, Lopez-Cabrera M. 2002. The hepatitis B virus X protein promotes tumor cell invasion by inducing membrane-type matrix metalloproteinase-1 and cyclooxygenase-2 expression. J Clin Invest 110(12):1831-1838.

Lara-Pezzi E, Serrador JM, Montoya MC, Zamora D, Yanez-Mo M, Carretero M, Furthmayr H, Sanchez-Madrid F, Lopez-Cabrera M. 2001. The hepatitis B virus X protein (HBx) induces a migratory phenotype in a CD44-dependent manner: possible role of HBx in invasion and metastasis. Hepatology 33(5):1270-1281.

Lohrum MA, Woods DB, Ludwig RL, Balint E, Vousden KH. 2001. C-terminal ubiquitination of p53 contributes to nuclear export. MolCell Biol 21(24):8521-8532.

Luo JL, Tan W, Ricono JM, Korchynskyi O, Zhang M, Gonias SL, Cheresh DA, Karin M. 2007. Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. Nature 446(7136):690-694.

Marotta F, Vangieri B, Cecere A, Gattoni A. 2004. The pathogenesis of hepatocellular carcinoma is multifactorial event. Novel immunological treatment in prospect. ClinTer 155(5):187-199.

Massoumi R, Chmielarska K, Hennecke K, Pfeifer A, Fassler R. 2006. Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. Cell 125(4):665-677.

Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ, Jr., Sledge GW, Jr. 1997. Constitutive activation of

NF-kappaB during progression of breast cancer to hormone-independent growth. MolCell Biol 17(7):3629-3639.

Nakshatri H, Goulet RJ, Jr. 2002. NF-kappaB and breast cancer. CurrProblCancer 26(5):282-309.

Ohata K, Ichikawa T, Nakao K, Shigeno M, Nishimura D, Ishikawa H, Hamasaki K, Eguchi K. 2003. Interferon alpha inhibits the nuclear factor kappa B activation triggered by X gene product of hepatitis B virus in human hepatoma cells FEBS Lett 553(3):304-308.

Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401(6748):82-85.

Park GY, Christman JW. 2006. Nuclear factor kappa B is a promising therapeutic target in inflammatory lung disease. CurrDrug Targets 7(6):661-668.

Shambharkar PB, Blonska M, Pappu BP, Li H, You Y, Sakurai H, Darnay BG, Hara H, Penninger J, Lin X. 2007. Phosphorylation and ubiquitination of the IkappaB kinase complex by two distinct signaling pathways. EMBO J 26(7):1794-1805.

Sil AK, Maeda S, Sano Y, Roop DR, Karin M. 2004. I[kappa]B kinase-[alpha] acts in the epidermis to control skeletal and craniofacial morphogenesis. Nature 428(6983):660-664.

Tang ED, Nunez G, Barr FG, Guan KL. 1999. Negative regulation of the forkhead transcription factor FKHR by Akt. J Biol Chem 274(24):16741-16746.

Tang ED, Wang CY, Xiong Y, Guan KL. 2003. A role for NF-kappaB essential modifier/IkappaB kinase-gamma (NEMO/IKKgamma) ubiquitination in the activation of the IkappaB kinase complex by tumor necrosis factor-alpha. J Biol Chem 278(39):37297-37305.

Trotman LC, Wang X, Alimonti A, Chen Z, Teruya-Feldstein J, Yang H, Pavletich NP, Carver BS, Cordon-Cardo C, Erdjument-Bromage H, Tempst P, Chi SG, Kim HJ, Misteli T, Jiang X, Pandolfi PP. 2007. Ubiquitination regulates PTEN nuclear import and tumor suppression. Cell 128(1):141-156.

Tu Z, Prajapati S, Park KJ, Kelly NJ, Yamamoto Y, Gaynor RB. 2006. IKK alpha regulates estrogen-induced cell cycle progression by modulating E2F1 expression. JBiolChem

281(10):6699-6706.

Wang T, Wang Y, Wu MC, Guan XY, Yin ZF. 2004. Activating mechanism of transcriptor NF-kappaB regulated by hepatitis B virus X protein in hepatocellular carcinoma. World JGastroenterol 10(3):356-360.

Wolfrum C, Besser D, Luca E, Stoffel M. 2003. Insulin regulates the activity of forkhead transcription factor Hnf-3beta/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization. Proc Natl Acad Sci U S A 100(20):11624-11629.

Xia LM, Huang WJ, Wu JG, Yang YB, Zhang Q, Zhou ZZ, Zhu HF, Lei P, Shen GX, Tian DA. 2009. HBx protein induces expression of MIG and increases migration of leukocytes through activation of NF-kappaB. Virology 385(2):335-342.

Xie X, Wacker HH, Huang S, Regitz E, Preuss KD, Romeike B, Parwaresch R, Tiemann M, Pfreundschuh M. 2003. Differential expression of cancer testis genes in histological subtypes of non-Hodgkin's lymphomas. Clin Cancer Res 9(1):167-173.

Yamamoto Y, Verma UN, Prajapati S, Kwak YT, Gaynor RB. 2003. Histone H3 phosphorylation by IKK-[alpha] is critical for cytokine-induced gene expression. Nature 423(6940):655-659.

Yuan Z, Kim D, Shu S, Wu J, Guo J, Xiao L, Kaneko S, Coppola D, Cheng JQ. 2010. Phosphoinositide 3-kinase/Akt inhibits MST1-mediated pro-apoptotic signaling through phosphorylation of threonine 120. J Biol Chem 285(6):3815-3824.

Zhang X, Liu S, Hu T, He Y, Sun S. 2009. Up-regulated microRNA-143 transcribed by nuclear factor kappa B enhances hepatocarcinoma metastasis by repressing fibronectin expression 2. Hepatology 50(2):490-499.

Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC. 2001. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol 3(11):973-982.

**Figure Legends**

Fig. 1. HBx induced IKKα nuclear translocation. *A.* Total, cytosolic, and nuclear extracts from Hep3B and Hep3Bx cell lines were prepared and subjected to Western blotting with anti-HBx, anti-IKKα, anti-lamin B, and anti-tubulin antibodies. *B.* HEK-293 cells transiently transfected with myc-HBx were subjected to nucleo-cytoplasmic fractionation and Western blotting analysis with indicated antibodies. *C.* Flag-IKKα was co-transfected with or without myc-HBx into Hep3B cells. The cellular localization of IKKα and HBx in Hep3B cells were examined by immunofluorescence confocal analysis with anti-Flag and anti-Myc antibodies.

Fig. 2. HBx enhanced the nuclear function of IKKα. *A and C.* Total lysates of Hep3B and Hep3Bx cells were prepared and subjected to Western blotting with indicated antibodies. *B.* Total lysates of HEK-293 cells transfected with increasing amount of myc-HBx were prepared and subjected to Western blotting with indicated antibodies. *D.* Total lysates of HepG2 and HepG2x cells were subjected to Western blotting with anti-phospho-Ser1382/1386 CBP or anti-CBP antibodies.

Fig. 3. HBx activates Akt to phosphorylate IKKα at Thr23. *A.* Total lysates of Hep3B and Hep3Bx cells were prepared and subjected to Western blotting with indicated antibodies. *B.* Total lysates of HEK-239 cells transfected with Flag-IKKα, HA-myr-Akt, and/or myc-HBx were subjected to immunoprecipitation with anti-IKKα antibody followed by Western blotting with anti-HA, anti-Flag, or anti-myc antibodies. *C.* Total lysates of HEK-293 cells transfected with increasing amount of myc-HBx were prepared and subjected to Western blotting with indicated antibodies. *D.* Hep3B cells transfected with Flag-IKKα and myc-HBx were treated with or without 20 μM API2 or 20 μM LY2943002 (LY) for 1hr. Then total lysates were prepared and subjected to Western blotting with indicated antibodies.

Fig. 4. Akt enhanced IKKα nuclear translocation in response to HBx overexpression. *A.* HEK-293 cells transfected with HBx and/or Flag-IKKα were treated with 20μM API2 or LY294002 for 1hr. Cytosolic and

nuclear protein was prepared and subjected to Western blotting with indicated antibodies. *B.* HEK-293 cells were co-transfected with myc-HBx, Flag-IKKα, and/or Akt siRNA for 48hr. Cytosolic and nuclear protein was prepared and subjected to Western blotting with indicated antibodies. *C.* HEK-293 cells were transfected with increasing amount of HA-myr-Akt for 48 hr and then subjected to nucleo-cytoplasmic fractionation. Protein expressions in the cytoplasm and nucleus were examined by Western blotting with indicated antibodies.

Fig. 5. Regulation of IKKα nuclear transportation by Thr23 phosphorylation, nuclear localization signal (NLS) and nuclear export signal (NES). *A.* HEK-293 cells co-transfected with HBx and Flag-IKKα WT or its T23A mutant were subjected to nucleo-cytoplasmic fractionation followed by Western blotting with indicated antibodies. *B.* HEK-293 cells transfected with Flag-IKKα WT, T23A, T23E, $NLS^{3KM}$, or $NLS^{5KM}$ mutants were subjected to nucleo-cytoplasmic fractionation followed by Western blotting with indicated antibodies. *C.* Illustration of putative NES and NLS sites within IKKα and their mutant sequences. *D.* HEK-293 cells transfected with Flag-IKKα WT or NES mutant were subjected to nucleo-cytoplasmic fractionation followed by Western blotting with indicated antibodies.

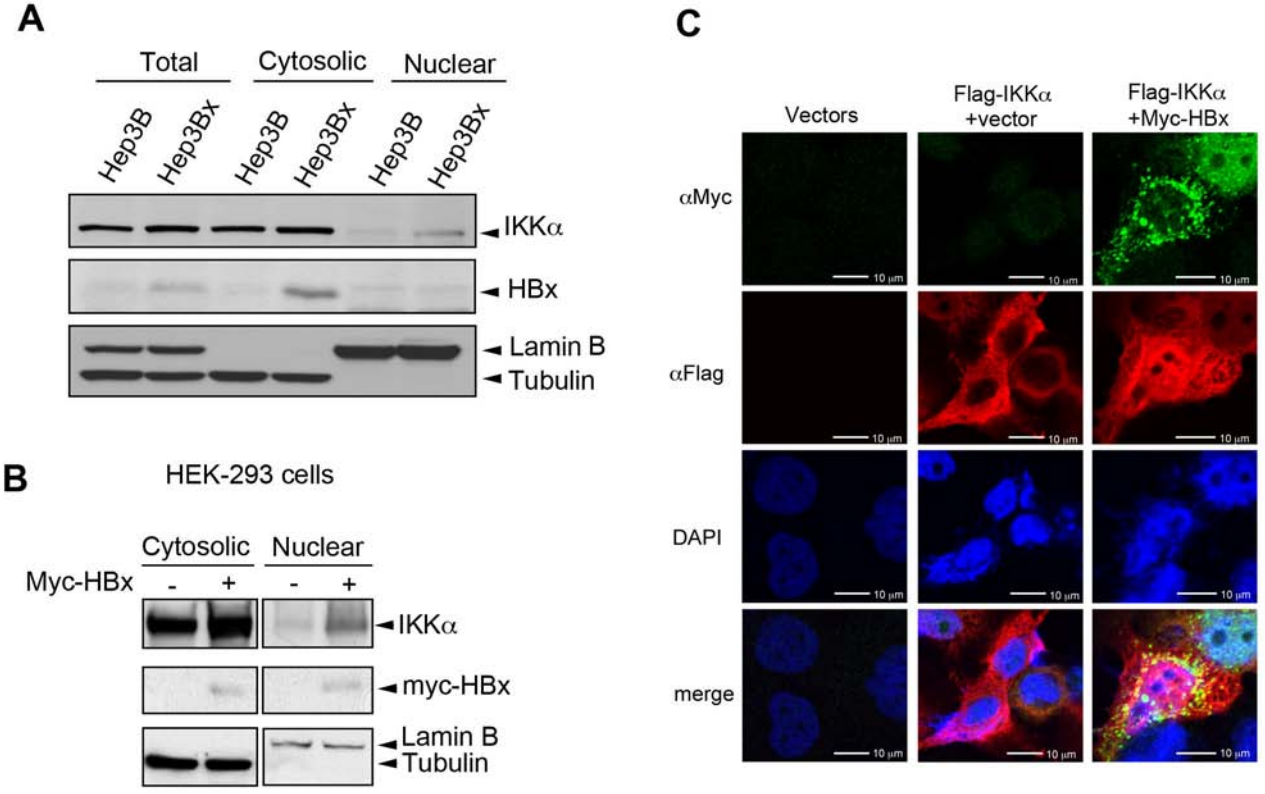
Fig. 6. The involvement of ubiquitination in IKKα nuclear translocation. *A.* Total lysates of Hep3B cells co-transfected with Flag-IKKα WT and/or HBx were subjected to Western blotting with indicated antibodies. *B.* HEK-293 cells transfected with indicated plasmids were subjected to immunoprecipitation with anti-IKKα antibody followed by Western blotting with anti-ubiquitin or anti-IKKα antibodies. *C.* HEK-293 cells transfected with indicated plasmids were subjected to immunoprecipitation with anti-Flag antibody followed by Western blotting with anti-ubiquitin or anti-Flag antibodies. *D.* HEK-293 cells transfected with indicated plasmids were subjected to nucleo-cytoplasmic fractionation followed by Western blotting with anti-Flag, anti-lamin B, or anti-tubulin antibodies.

Fig. 7. Nuclear IKKα contributes to HBx-enhanced migration and invasion ability of Hep3B cells. *A.* The

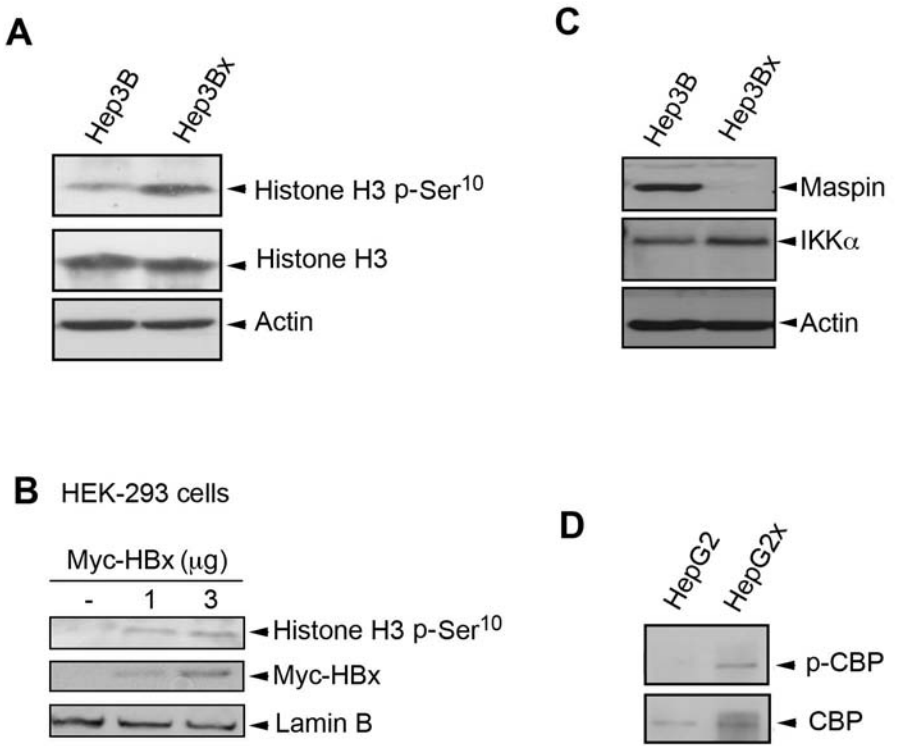
invasion of Hep3B and Hep3Bx cells were examined by transwell invasion assay for 48hr. The represented pictures of invaded cells visualized by using an optic microscope with 40X magnification were shown in the middle panel. *B.* Hep3B cells were transfected with or without Flag-IKKα and then subjected to transwell migration and invasion assays for 48hr. *C.* Hep3B and Hep3Bx cells were treated with or without 10 μM IKK inhibitor-II and subjected to transwell migration assays for 24hr. *D.* Hep3B cells were transfected with or without Flag-IKKα WT or its indicated mutants for 24 hr, and then subjected to transwell migration assays for 48hr. The quantitative data in *A-D* are represented as mean ± SD of three independent experiments. *, $P< 0.05$; **, $p<0.01$; ***, $p<0.001$.

Fig. 8. The proposed model for the molecular mechanism underlying HBx-induced IKKα nuclear translocation. Akt phosphorylates IKKα at Thr23 and increases IKKα poly-ubiquitination to enhance its nuclear translocation in response to HBx overexpression. Nuclear IKKα may regulate gene expressions through phosphorylation of histone H3 at Ser10 to promote HBx-mediated migration and invasion of HCC.

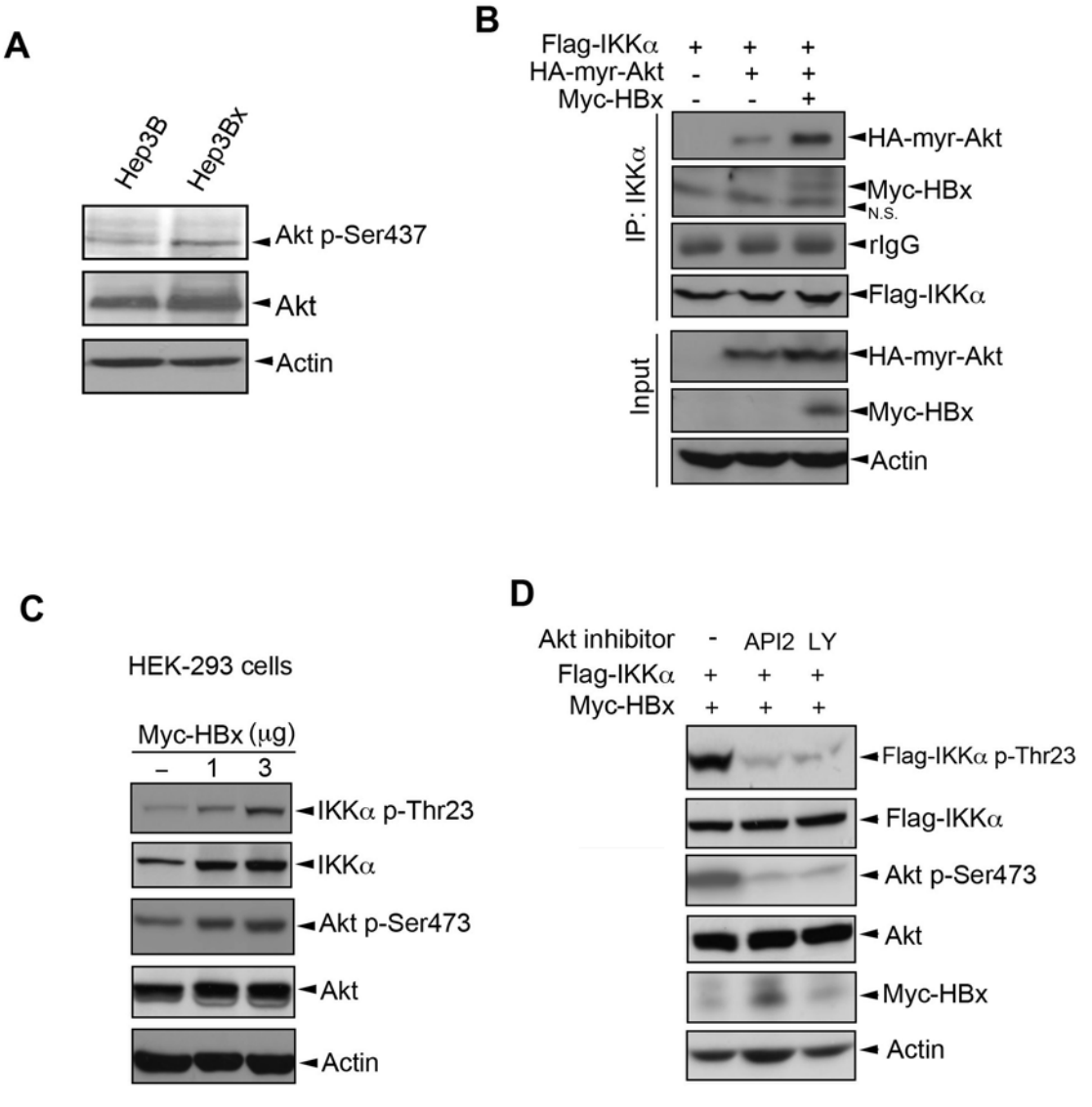
# Figure 1


A
Total
Cytosolic
Nuclear
Hep3B
Hep3Bx
Hep3B
Hep3Bx
Hep3B
Hep3Bx
IKKα
HBx
Lamin B
Tubulin
B
HEK-293 cells
Cytosolic
Nuclear
Myc-HBx
-
+
-
+
IKKα
myc-HBx
Lamin B
Tubulin
C
Vectors
Flag-IKKα +vector
Flag-IKKα +Myc-HBx
αMyc
αFlag
DAPI
merge
10 μm

# Figure 2


A
Hep3B
Hep3Bx
Histone H3 p-Ser10
Histone H3
Actin
B
HEK-293 cells
Myc-HBx (μg)
-
1
3
Histone H3 p-Ser10
Myc-HBx
Lamin B
C
Hep3B
Hep3Bx
Maspin
IKKα
Actin
D
HepG2
HepG2x
p-CBP
CBP

# Figure 3


A
Hep3B
Hep3Bx
Akt p-Ser437
Akt
Actin
B
Flag-IKKα + + +
HA-myr-Akt - + +
Myc-HBx - - +
IP: IKKα
HA-myr-Akt
Myc-HBx
N.S.
rIgG
Flag-IKKα
Input
HA-myr-Akt
Myc-HBx
Actin
C
HEK-293 cells
Myc-HBx (μg)
– 1 3
IKKα p-Thr23
IKKα
Akt p-Ser473
Akt
Actin
D
Akt inhibitor - API2 LY
Flag-IKKα + + +
Myc-HBx + + +
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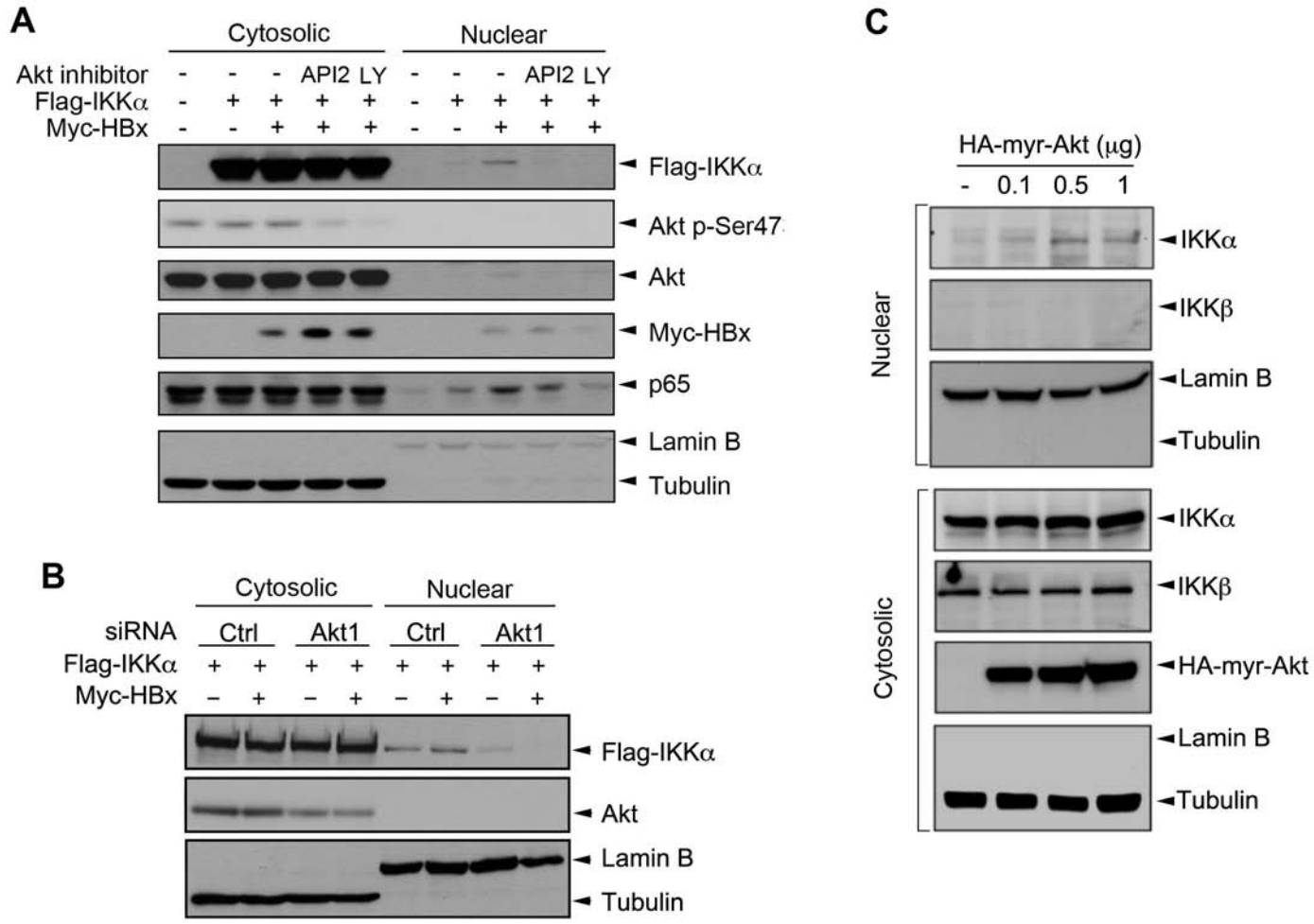
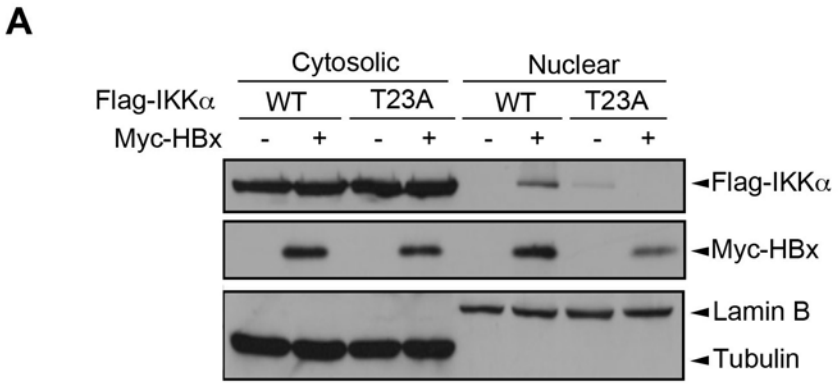
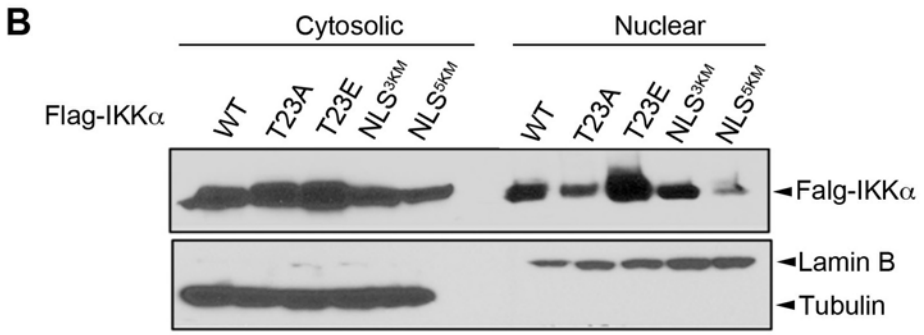
Flag-IKKα
Akt p-Ser473
Akt
Myc-HBx
Actin

**Figure 4**


A
Cytosolic
Nuclear
Akt inhibitor - - - API2 LY - - - API2 LY
Flag-IKKα - + + + + - + + + +
Myc-HBx - - + + + - - + + +
Flag-IKKα
Akt p-Ser47
Akt
Myc-HBx
p65
Lamin B
Tubulin
B
Cytosolic
Nuclear
siRNA Ctrl Akt1 Ctrl Akt1
Flag-IKKα + + + + + + + +
Myc-HBx – + – + – + – +
Flag-IKKα
Akt
Lamin B
Tubulin
C
HA-myr-Akt (μg)
- 0.1 0.5 1
Nuclear
IKKα
IKKβ
Lamin B
Tubulin
Cytosolic
IKKα
IKKβ
HA-myr-Akt
Lamin B
Tubulin

# Figure 5

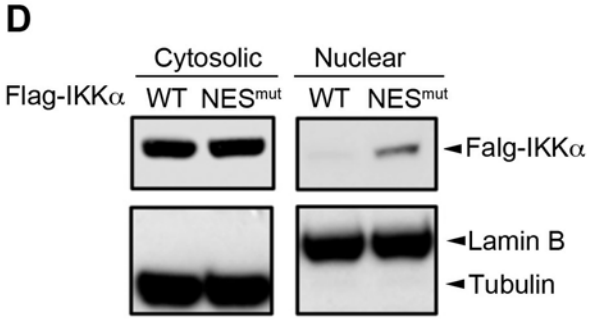



C

| Lysine-rich NLS motif | KKK |
|---|---|
| IKKα WT | 233-KIKKKDPK-240 |
| IKKα NLS$^{3KM}$ | KIMMMDPK |
| IKKα NLS$^{5KM}$ | MIMMMDPM |

| Leucine-rich NES motif | LX$_{2-3}$LX$_{2-3}$LXLI/V |
|---|---|
| IKKα WT | 714-LNCLGHLSTII-724 |
| IKKα NES$^{mut}$ | ANCAGHASTAA |



# Figure 6

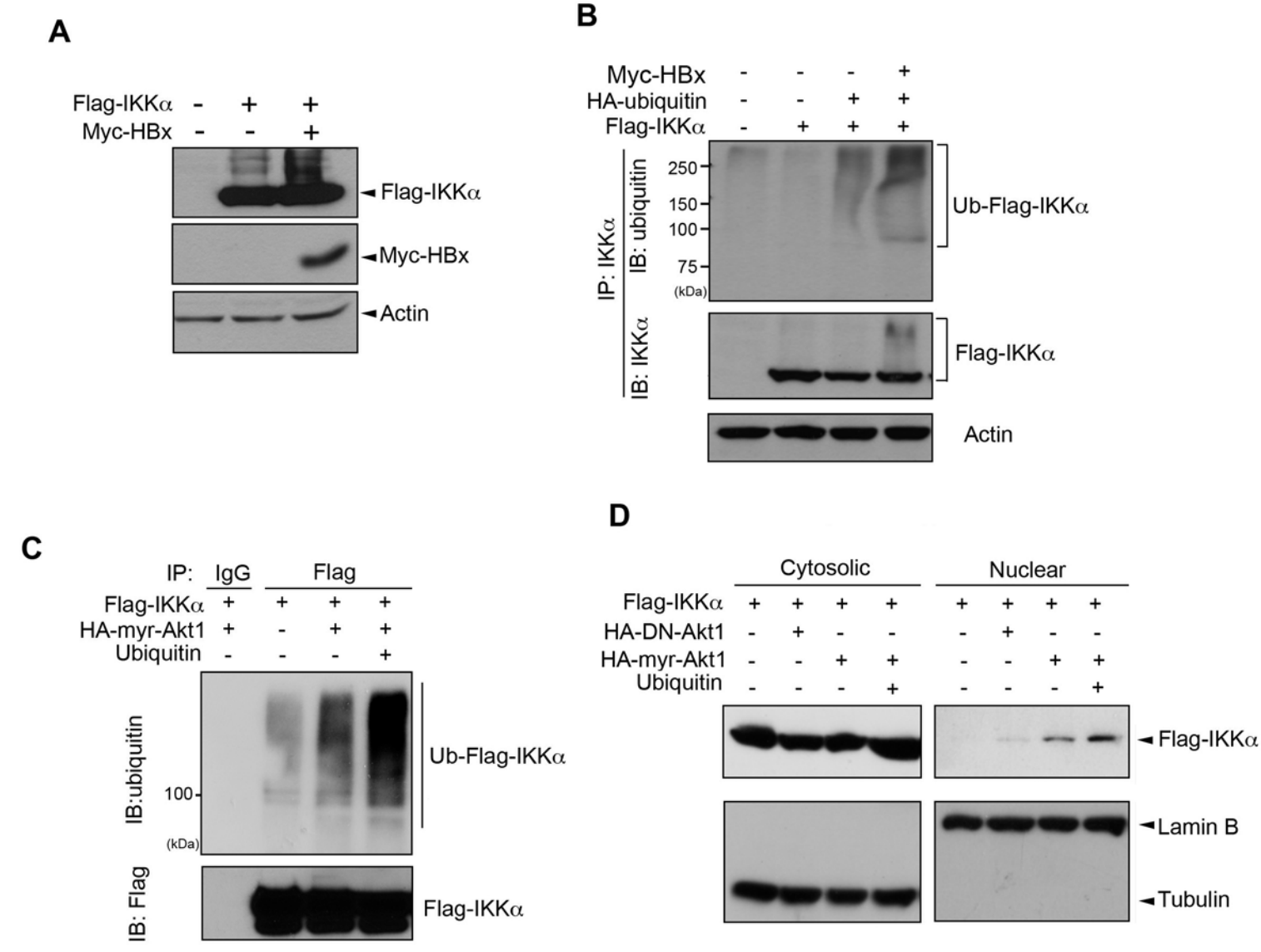
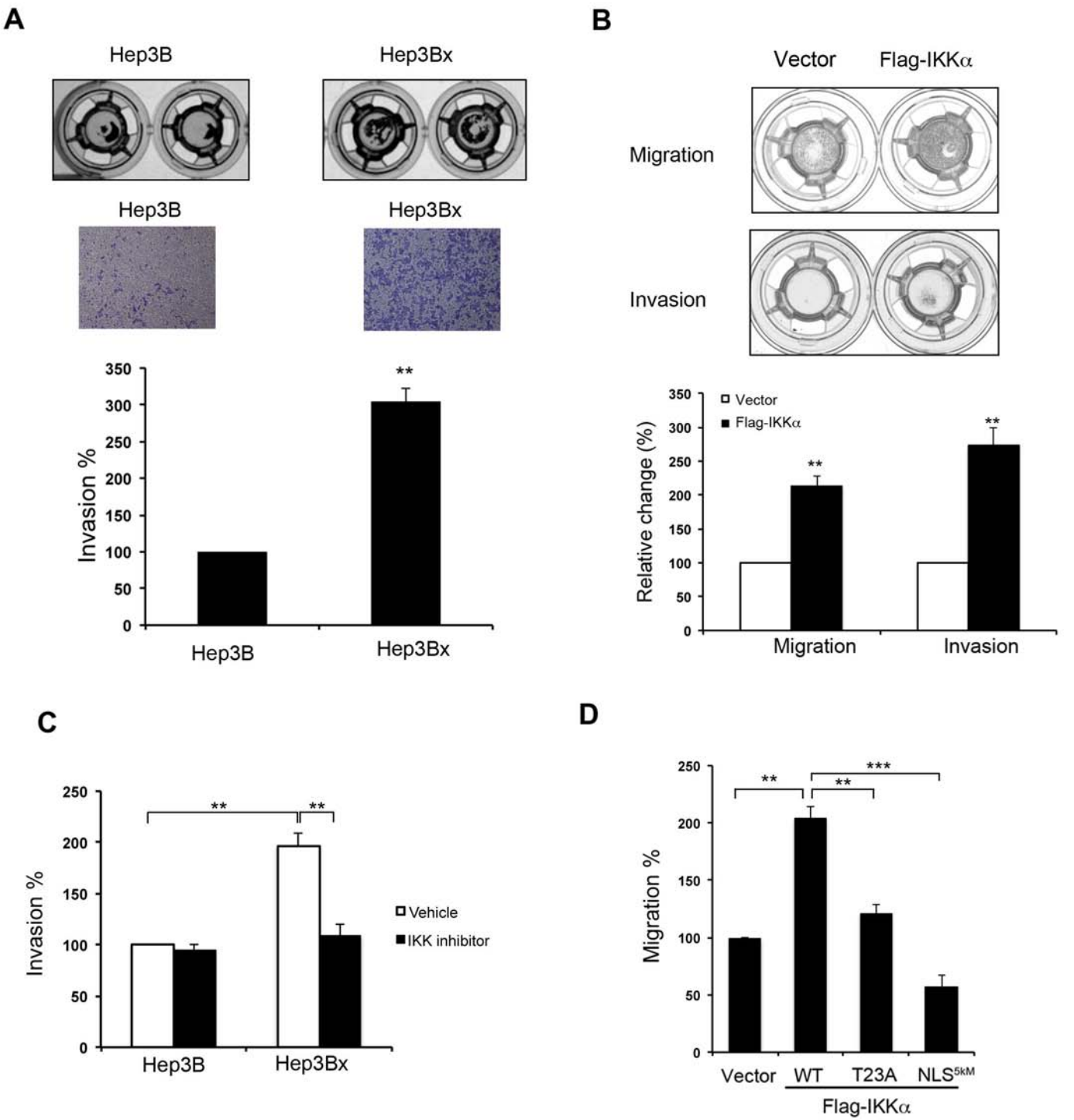

A
Flag-IKKα - + +
Myc-HBx - - +
Flag-IKKα
Myc-HBx
Actin
B
Myc-HBx - - - +
HA-ubiquitin - - + +
Flag-IKKα - + + +
IP: IKKα
IB: ubiquitin
250
150
100
75
(kDa)
Ub-Flag-IKKα
IB: IKKα
Flag-IKKα
Actin
C
IP: IgG Flag
Flag-IKKα + + + +
HA-myr-Akt1 + - + +
Ubiquitin - - - +
IB:ubiquitin
100
(kDa)
Ub-Flag-IKKα
IB: Flag
Flag-IKKα
D
Cytosolic
Nuclear
Flag-IKKα + + + + + + + +
HA-DN-Akt1 - + - - - + - -
HA-myr-Akt1 - - + + - - + +
Ubiquitin - - - + - - - +
Flag-IKKα
Lamin B
Tubulin

**Figure 7**


A
Hep3B
Hep3Bx
Hep3B
Hep3Bx
Invasion %
350
300
250
200
150
100
50
0
**
Hep3B
Hep3Bx
B
Vector
Flag-IKKα
Migration
Invasion
Relative change (%)
Vector
Flag-IKKα
**
**
Migration
Invasion
C
Invasion %
250
200
150
100
50
0
**
**
Vehicle
IKK inhibitor
Hep3B
Hep3Bx
D
Migration %
250
200
150
100
50
0
**
**
***
Vector
WT
T23A
NLS5kM
Flag-IKKα

# Figure 8

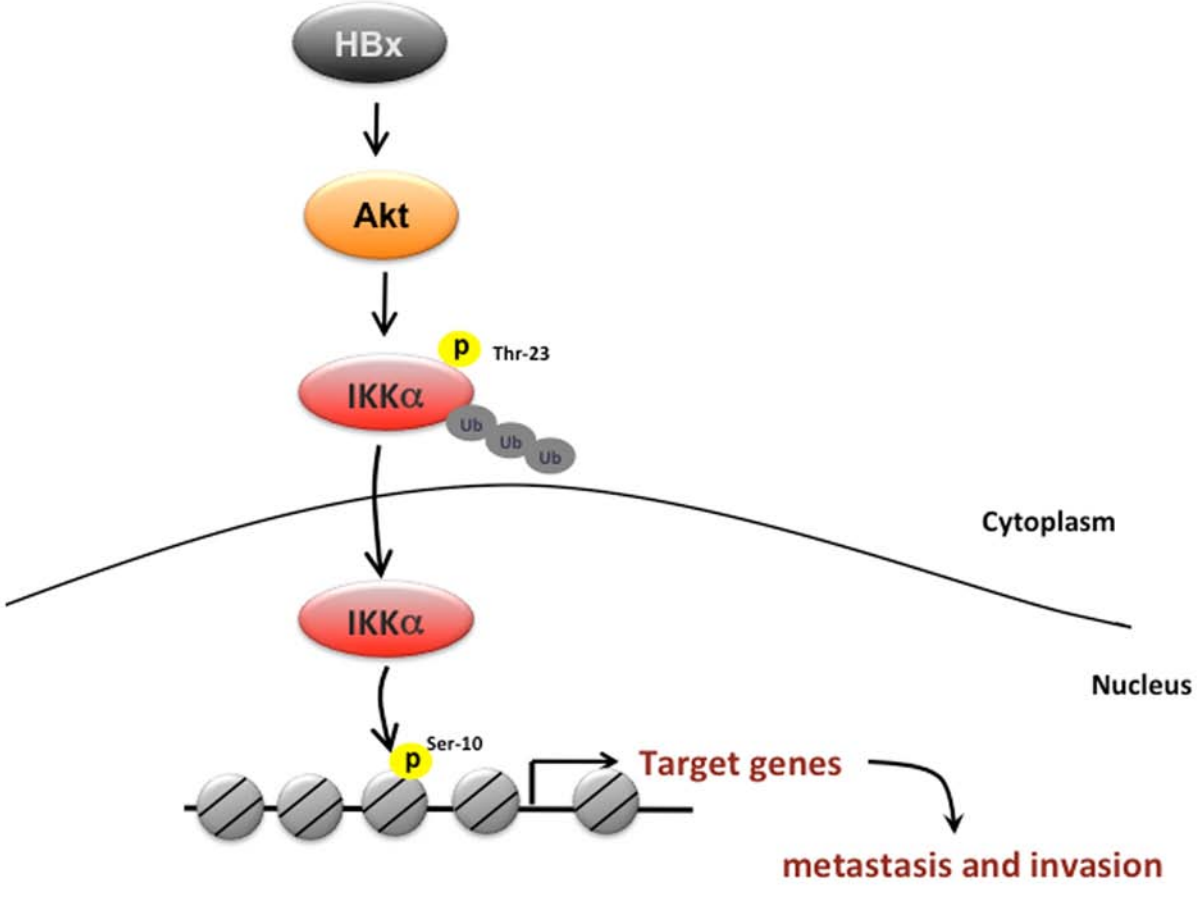
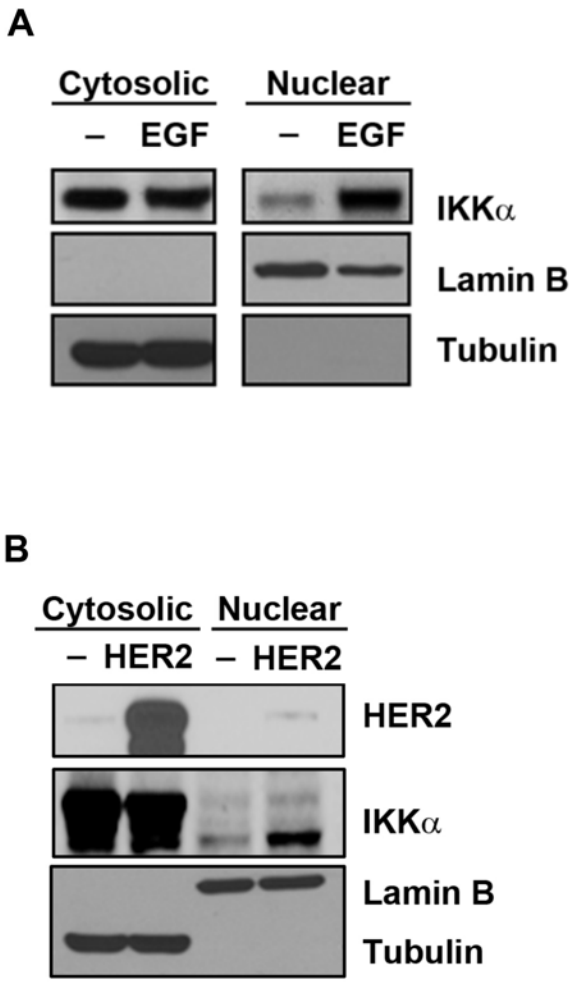

HBx
Akt
p
Thr-23
IKKα
Ub
Ub
Ub
Cytoplasm
IKKα
Nucleus
p
Ser-10
Target genes
metastasis and invasion

**Figure S1**


A
Cytosolic
Nuclear
– EGF
– EGF
IKKα
Lamin B
Tubulin
B
Cytosolic
Nuclear
– HER2
– HER2
HER2
IKKα
Lamin B
Tubulin