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HTLV-1 Tax-Induced Rapid Senescence Is Driven by the Transcriptional Activity of NF-κB and Depends on Chronically Activated IKKα and p65/RelA

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The HTLV-1 oncoprotein Tax is a potent activator of classical and alternative NF-κB pathways and is thought to promote cell proliferation and transformation via NF-κB activation. We showed recently that hyperactivation of NF-κB by Tax triggers a cellular senescence response (H. Zhi et al., PLoS Pathog. 7:e1002025, 2011). Inhibition of NF-κB activation by expression of I-κBα superrepressor or by small hairpin RNA (shRNA)-mediated knockdown of p65/RelA rescues cells from Tax-induced rapid senescence (Tax-IRS). Here we demonstrate that Tax-IRS is driven by the transcriptional activity of NF-κB. Knockdown of IKKγ, the primary Tax target, by shRNAs abrogated Tax-mediated activation of both classical and alternative NF-κB pathways and rendered knockdown cells resistant to Tax-IRS. Consistent with a critical role of IKKα in the transcriptional activity of NF-κB, IKKα deficiency drastically decreased NF-κB *trans*-activation by Tax, although it only modestly reduced Tax-mediated I-κBα degradation and NF-κB nuclear localization. In contrast, although IKKβ knockdown attenuated Tax-induced NF-κB transcriptional activation, the residual NF-κB activation in IKKβ-deficient cells was sufficient to trigger Tax-IRS. Importantly, the phenotypes of NIK and TAK1 knockdown were similar to those of IKKα and IKKβ knockdown, respectively. Finally, double knockdown of RelB and p100 had a minor effect on senescence induction by Tax. These data suggest that Tax, through its interaction with IKKγ, helps recruit NIK and TAK1 for IKKα and IKKβ activation, respectively. In the presence of Tax, the delineation between the classical and alternative NF-κB pathways becomes obscured. The senescence checkpoint triggered by Tax is driven by the transcriptional activity of NF-κB, which depends on activated IKKα and p65/RelA.

Human T-lymphotropic virus type 1 (HTLV-1) is etiologically associated with adult T-cell leukemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Soon after HTLV-1 was discovered, the viral transactivator/oncoprotein Tax was identified and found to be essential for HTLV-1 to transform cells (24). However, the mechanism by which Tax promotes ATL development is not fully understood. As Tax is not expressed in most ATL cells (20, 30, 31), it is thought that Tax is required at the early stages of ATL development but is not needed for maintaining the transformed phenotype of ATL cells.

Tax is a potent activator of the IKK/NF-κB pathway by virtue of a direct interaction with the NF-κB essential modulator/I-κB kinase γ subunit (NEMO/IKKγ) (3, 15, 39). This interaction leads to persistent IKKα and IKKβ activation; I-κBα phosphorylation, polyubiquitination, and degradation; increased p100 expression and processing; and activation of both classical and alternative NF-κB pathways (3, 15, 37). While IKKγ is critical for IKKβ but not IKKα activation under physiological conditions (4, 5), Tax-mediated activation of IKKα and IKKβ both require IKKγ (29). Importantly, activation of IKKα and IKKβ depends on the phosphorylation of their respective activation loops by upstream kinases NF-κB induced kinase (NIK) and TGF-β activated kinase 1 (TAK1) (14, 25, 34, 36). Indeed, NIK and TAK1 have been implicated in Tax-mediated IKK activation previously (32, 35).

It has been amply demonstrated that the transforming activities of Tax resides in its ability to activate NF-κB (8, 12, 19, 39, 40). Paradoxically, despite the critical role of NF-κB in promoting cell proliferation and survival, Tax was also found to adversely affect cell cycle progression by prematurely activating the anaphase promoting complex/cyclosome (APC/C), which targets unscheduled proteasomal degradation of Skp2. The loss of Skp2 then stabilizes the cyclin-dependent kinase inhibitor (CDKI) $p27^{Kip1}$ (p27) (16). Furthermore, the mRNA level of another CDKI, $p21^{CIP1/WAF1}$ (p21), also increases significantly by Tax through promoter activation and mRNA stabilization (16, 42). The dramatic increase in both p21 and p27 proteins induces a type of cellular senescence known as Tax-induced rapid senescence (Tax-IRS) (16). We demonstrated recently that Tax-IRS is a cellular checkpoint response triggered by Tax-mediated NF-κB hyperactivation (43). Tax-IRS can be prevented by the expression of a degradation-resistant form of I-κBα or by small hairpin RNA (shRNA)-mediated knockdown of p65/RelA (43). The dramatic upregulation of p27 and p21 CDKIs that leads to senescence is therefore the downstream effect of hyperactivated NF-κB (43).

The mechanism by which chronically activated NF-κB induces senescence is not clear. Here we show that the nuclear localization of NF-κB alone is insufficient to induce senescence. Rather, the transcriptional activity of NF-κB stimulated by Tax is correlated with senescence induction. Of the cellular factors involved in IKK signaling, IKKγ, IKKα, and NIK are the most critical for Tax-

Received 19 January 2012 **Accepted** 20 June 2012
Published ahead of print 27 June 2012
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Supplemental material for this article may be found at http://jvi.asm.org/.

doi:10.1128/JVI.00158-12

mediated NF-κB *trans*-activation and cellular senescence. In cells deficient in IKKα, both I-κBα degradation and NF-κB nuclear transport as induced by Tax (and mediated by IKKβ) remained largely unaltered, yet the transcriptional activity of NF-κB was drastically curtailed, and as a result, Tax-IRS was blocked. In contrast, in cells knocked down for IKKβ and TAK1, Tax-induced NF-κB activation (via IKKα) was attenuated albeit sustained, and a senescence response was triggered. Furthermore, RelB and p100 double knockdown did not prevent Tax-IRS. Our results support the notion that by interacting with IKKγ/NEMO, Tax facilitates the recruitment of IKK kinases TAK1 and NIK to IKKα and IKKβ, respectively, to cause constitutive IKK activation. However, Tax-induced cellular senescence is triggered by persistent transcriptional activity that emanates mainly from hyperactivated IKKα and NF-κB (p65/50). Finally, cellular transformation by Tax requires the inactivation of this senescence checkpoint.

MATERIALS AND METHODS

Derivation of knockdown cell lines. IKKα, IKKβ, IKKγ, TAK1, and NIK genes were each targeted by short hairpin RNAs (shRNAs) expressed from a lentivirus vector containing the puromycin resistance gene. The sequences targeted for each gene are listed in Table S1 in the supplemental material). Pooled shRNA lentiviral vectors for each gene were generated as previously described (43) and used to transduce HeLa/18x21-EGFP (HeLa-G) or SupT1/18x21-EGFP (SupT1-G) (18) cells. The transduced cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO). Stable cell clones with knockdown of each of the relevant genes were subsequently isolated from 96-well plates after limiting dilution and validated by immunoblotting.

Derivation of NIK-DN cells. A dominant negative mutant NIK gene was obtained from Shao-Cong Sun and stably expressed in HeLa-G cells using a lentivirus vector containing the puromycin resistance gene. The transduced cells were grown as described above.

Immunoblotting. A standard protocol was used for immunoblotting. Briefly, cells were harvested and lysed. The protein concentration for each of the whole-cell lysates was then quantified with a Bio-Rad protein assay. A total of 10 to 20 μg of proteins was loaded per sample. The HTLV-1 Tax hybridoma monoclonal antibody 4C5 was generated in this lab. IKKα, IKKβ, IKKγ, TAK1, and NIK antibodies were purchased from Cell Signaling (Denver, MA). I-κBα, p65/RelA, p52/NF-κB2, RelB, p21, p27, β-actin, hemagglutinin (HA), HDAC1, and GAPDH antibodies were purchased from Santa Cruz (Santa Cruz, CA). MG132 was purchased from Sigma-Aldrich (St. Louis, MO).

Adenovirus vectors. The recombinant adenovirus vectors Ad-Tax and Ad-tTA were propagated and their titers were determined as previously published (16).

Luciferase reporter assay. The NF-κB (E-selectin) and HTLV-1 LTR luciferase reporter plasmids, together with pRL-TK, an internal control plasmid that contains the HSV thymidine kinase (TK) promoter-driven *Renilla* luciferase reporter cassette, were used in the dual-luciferase reporter assay system (Promega, Madison, WI) following the manufacturer's protocol.

Cell counting. Ten thousand cells were seeded in each well of a 6-well plate, transduced with Ad-Tax at a multiplicity of infection (MOI) of 1, and harvested at 2 and 5 days posttransduction. Trypan blue dye was used to label dead cells, and the total viable enhanced green fluorescent protein-positive ($EGFP^+$) cells were counted using a Cellometer Vision automated fluorescence-based cell counter (Nexcelom Biosciences, Lawrence, MA).

Immunofluorescence. Wild-type HeLa-G and various knockdown cell lines were plated on cover slides and transduced with Ad-Tax at an MOI of 1 for 48 h. They were then fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were immunostained overnight with the indicated primary antibodies followed by Alexa Fluor 568 secondary antibodies (Invitrogen, Carlsbad, CA). The slides were then mounted in a mounting medium containing DAPI (4′,6′-diamidino-2-phenylindole) (Vectashield; Vector Lab Inc., Burlingame, CA) and kept at 4°C in the dark. Images were captured using an Olympus IX81 fluorescence microscope or a Pascal confocal microscope.

Subcellular fractionation. Cells were transduced with Ad-Tax at an MOI of 1 for 48 h and harvested for subcellular fractionation according to the manufacturer's protocol (Thermo Scientific, Waltham, MA).

RESULTS

IKKα and IKKγ knockdown abrogate Tax-induced rapid senescence. To identify components in the IKK/NF-κB signaling pathway that are responsible for Tax-mediated NF-κB activation and Tax-IRS, we generated stable IKKα, IKKβ, and IKKγ/NEMO (referred to as IKKγ here) knockdown (KD) cell clones in a Tax-responsive HeLa (HeLa-G) reporter cell line that was previously described (18, 41). After transduction with the respective shRNA-expressing lentiviral vectors, individual cell clones were isolated, expanded, and validated by immunoblotting (Fig. 1A). Knowing that the IKK complex plays a critical role in regulating NF-κB activation, we used E-selectin luciferase reporter assay to determine the ability of the knockdown cell lines to support NF-κB transactivation by Tax. While Tax was able to induce a robust NF-κB luciferase reporter activity in wild-type HeLa-G cells, the IKKα-, IKKβ- and IKKγ-KD cell lines each displayed a significant reduction in NF-κB reporter activity. Specifically, IKKα- and IKKγ-KD cells had only minimal reporter activity (~5% of wild-type), whereas IKKβ-KD cells retained approximately 30% of the NF-κB reporter activity (20-fold *trans*-activation over the basal level) of wild-type HeLa-G cells (Fig. 1B, left). In contrast, robust LTR-Luc reporter transactivation was readily detected in each of the three knockdown clones (Fig. 1B, right). It should be pointed out that throughout our experiments when NF-κB activity was blocked, there was a general reduction in transcriptional activity of most promoters. Further, Tax expression is driven by the cytomegalovirus (CMV) immediate early enhancer/promoter, which is inducible by NF-κB. Therefore, the levels of Tax expression were mostly reduced in the various KD cells. These factors, we think, contribute to the decrease in LTR *trans*-activation in the KD cell lines. These results, nevertheless, confirmed the importance of IKKγ in Tax-mediated IKK/NF-κB activation and indicated that IKKα plays a much more important role than IKKβ in Tax-induced NF-κB *trans*-activation. This supports earlier publications demonstrating the essential role of IKKα in tumor necrosis factor α (TNF-α)- and Tax-mediated NF-κB activation (1, 21, 38).

To assess the contribution of each IKK subunit to Tax-IRS, the knockdown cell clones and the parental wild-type HeLa-G cells were sparsely plated and infected with Ad-Tax (an adenovirus vector for Tax) at a multiplicity of infection (MOI) of 1. The Ad-Tax-transduced cells were then incubated for 5 days. HeLa-G cells contain a reporter cassette in which the EGFP gene is transcriptionally regulated by 18 copies of the 21-bp-repeat Tax-responsive enhancer element. In the presence of Tax, EGFP is abundantly induced, rendering Tax-expressing cells easily identified by fluorescence microscopy (41). As previously described (43), for wild-type HeLa-G cells, the majority of the EGFP-positive (Tax-positive) cells were arrested as single cells, whereas EGFP-negative (Tax-negative) cells continued to proliferate (Fig. 1C, upper left). In contrast, HeLa-G cells expressing the I-κBα superrepressor, ΔN-IκBα, continued to proliferate in the presence of Tax (Fig. 1C,

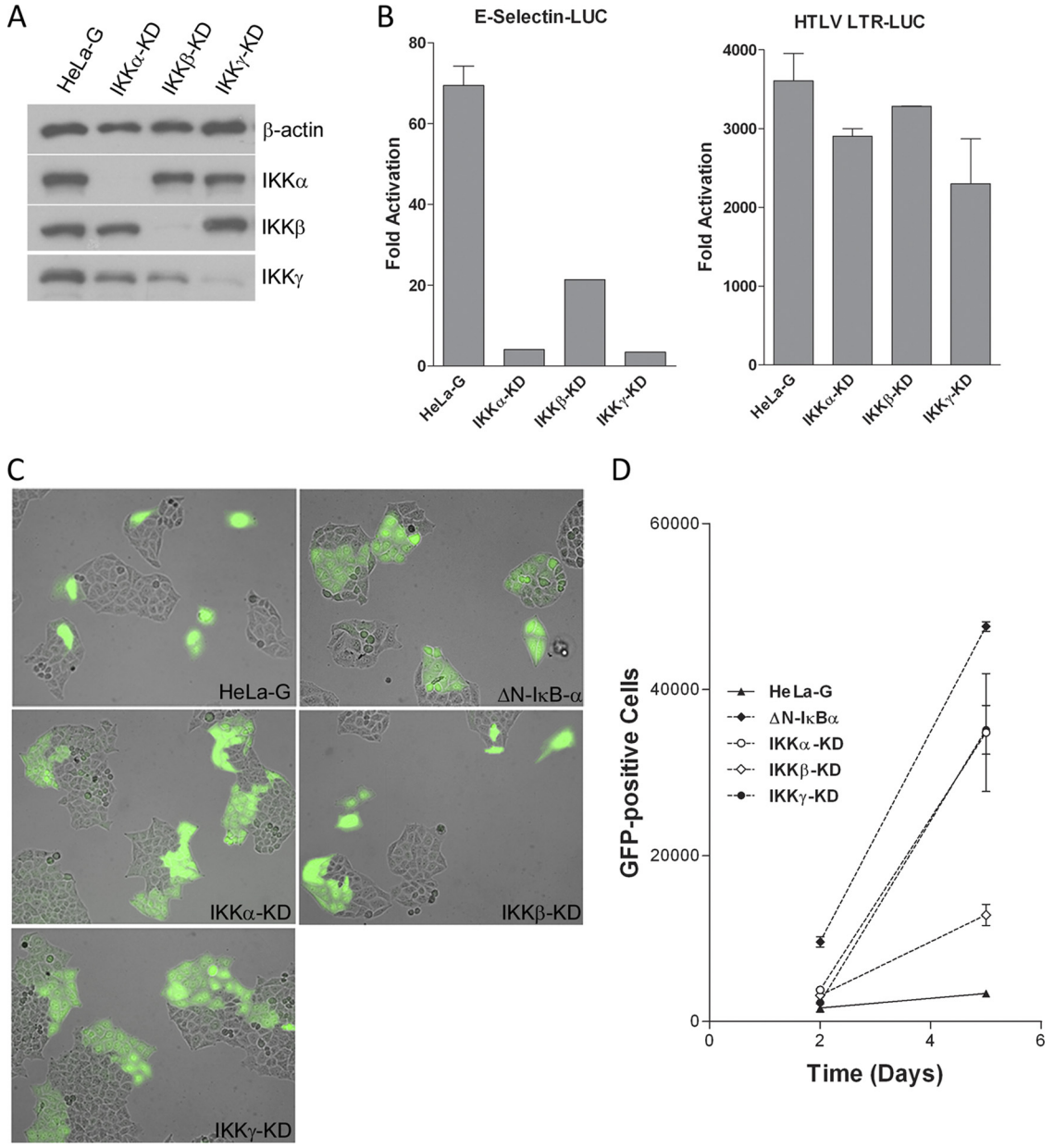

FIG 1 IKKα and IKKγ are essential for Tax-mediated NF-κB activation as well as Tax-IRS. (A) Isolation and characterization of IKKα-, IKKβ-, and IKKγ-KD cell lines. IKKα-, IKKβ-, and IKKγ-KD clones were established in a HeLa-G reporter cell line by individually knocking down the α, β, and γ subunits of the IKK complex using their respective shRNA-expressing lentiviral vectors as described in Materials and Methods. The cells were cloned via limiting dilutions, expanded, and validated by immunoblotting. (B) Tax-mediated NF-κB activation in IKKα-, IKKβ-, and IKKγ-KD cells. IKKα-, IKKβ-, and IKKγ-KD cells were cotransfected with E-selectin-Luc or LTR-Luc and pRL-TK with or without Tax. Luciferase assays were performed at 48 h posttransfection. Activation was calculated as the ratio of reporter activities in cells with Tax over those in cells without Tax. (C) Tax-induced senescence requires IKKα and IKKγ. The three IKK knockdown cell clones, ΔN-IκBα cells, and the wild-type HeLa-G cells were plated at low density and transduced with Ad-Tax at an MOI of 1. After 5 days, cells were examined and photographed using an Olympus IX81 inverted fluorescence microscope equipped with a charge-coupled device (CCD) camera as previously reported (41). (D) Deficiency in IKKα or IKKγ confers resistance to Tax-IRS. The growth curve of Ad-Tax-transduced cells of each KD cell line was determined by counting the total number of $EGFP^+$ cells at 2 and 5 days after transduction using an automated fluorescence-based cell counter as described in Materials and Methods. The data were then plotted on a line graph using Prism software.

upper right). Similarly, EGFP-positive IKKα-KD and IKKγ-KD cells also continued to grow and divide alongside EGFP-negative cells, indicating that Tax-IRS was largely abrogated with the loss of IKKα or IKKγ (Fig. 1C, left middle and bottom). Interestingly, despite a 70% reduction in NF-κB activation by Tax (Fig. 1B), senescence induction in IKKβ-KD cells was not significantly altered, as demonstrated by the presence of mostly nonproliferating EGFP-positive cells mixed with a small population of proliferating counterparts (Fig. 1C, lower right). Growth curves of Ad-Tax-transduced HeLa-G, ΔN-IκBα, and IKK KD (EGFP-positive) cells determined by quantifying the number of EGFP-positive cells at 2 and 5 days post-Ad-Tax transduction also reflect the importance

of IKKα and IKKγ, and to a much lesser extent IKKβ, in senescence induction (Fig. 1D).

In aggregate, these results support the notion that IKKα, rather than IKKβ, is the primary driver of the senescence response. In IKKβ knockdown cells, NF-κB activation by Tax, while curtailed, remains significant (~30% of parental that of HeLa-G cells) and capable of senescence induction. Thus, it appears that once a threshold of NF-κB activity is reached (in IKKβ knockdown cells), the senescence response is triggered. As IKKα, but not IKKβ, is crucial for the transcriptional activity of NF-κB, these results support the notion that the transcriptional activity of NF-κB is the primary mediator of cellular senescence response.

In an attempt to further validate our data, a Tax-responsive SupT1/18x21 EGFP (SupT1-G) reporter cell line (18) was used to generate IKKα, IKKβ, and IKKγ-KD cell clones, as shown by the immunoblot in Fig. 1A. An E-selectin luciferase reporter assay was then performed to confirm the role of the IKK subunits in supporting Tax-mediated NF-κB activation. In contrast to HeLa-G cells, SupT1-G IKKα, IKKβ, and IKKγ-KD cells exhibit attenuated but sufficient Tax-mediated NF-κB transactivation (data not shown). As a result, these KD cells continued to undergo Tax-IRS (data not shown). The discrepancy is most likely because basal NF-κB activity is critical for the proliferation and survival of T cells, and therefore, substantial NF-κB activity remained in the knockdown cell lines to drive senescence induction. This outcome is reminiscent of an unsuccessful attempt to express a degradation-resistant superrepressor form of I-κBα (ΔNIκBα) in SupT1 cells (43).

I-κBα degradation and p65/RelA nuclear localization are necessary but not sufficient for senescence induction. We next examined the biochemical correlates of NF-κB activation and cellular senescence mediated by Tax in IKK-KD cells. In agreement with the established paradigm, expression of Tax caused drastic I-κBα degradation in a HeLa-G reporter cell line (Fig. 2, lanes 1 versus 2). As expected, immunofluorescence data indicated that p65/RelA was abundantly localized to the nucleus upon Tax expression (see the correlation between EGFP/Tax expression and nuclear p65/RelA for HeLa-G cells [Fig. 3A]). IKKγ deficiency substantially stabilized I-κBα (Fig. 2, lanes 7 versus 8) and prevented nuclear localization of most of the p65/RelA induced by Tax, as indicated by immunofluorescence (Fig. 3A). Unlike IKKγ knockout cells, IKKγ expression was not completely obliterated by shRNAs. As a result, some of the IKKγ-KD cells appeared to show low but detectable levels of Tax-induced nuclear localization of p65/RelA due to residual levels of IKKγ protein. Nevertheless, subcellular fractionation clearly indicated that p65 nuclear localization in Tax-expressing IKKγ-KD cells is significantly less than that of IKKα-KD and IKKβ-KD cells (Fig. 3B, lanes 2 and 6 versus lane 10). Furthermore, the deficiency of IKKγ markedly reduced both steady-state and Tax-driven p100 expression (both dependent on p50/p65 NF-κB) and processing (dependent on IKKα) (Fig. 2, lanes 7 versus 8). The p21 and p27 upregulation by Tax was also abrogated by IKKγ knockdown (Fig. 2, lanes 7 versus 8).

Interestingly, significant I-κBα degradation continued to occur in cells deficient in either IKKα or IKKβ after transduction by Tax (Fig. 2, lanes 3 and 5 versus lanes 4 and 6). This correlates with p65/RelA nuclear localization in both IKKα KD and IKKβ KD cells, as indicated by p65/RelA immunofluorescence (Fig. 3), and subcellular fractionation (Fig. 3B, lanes 2 and 6 versus lanes 4 and 8). Tax-mediated upregulation of p100 expression and processing, surrogate indicators of alternative NF-κB pathway activation, were significantly reduced by IKKα KD (Fig. 2, lanes 3 versus 4) but only moderately affected by IKKβ KD (Fig. 2, lanes 5 versus 6), again consistent with the critical role of IKKα, but not IKKβ, in both classical and alternative NF-κB activation by Tax.

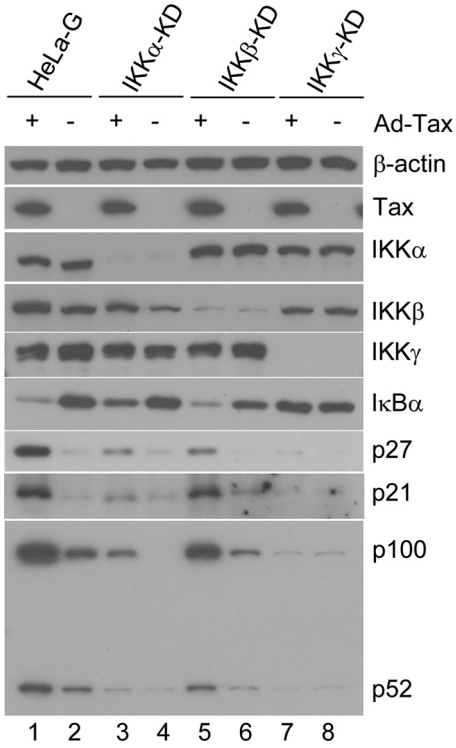

FIG 2 Distinct impairments of I-κBα degradation, p100 processing, and p21 and p27 upregulation in IKKα-, IKK-β-, and IKKγ-deficient cells. The IKKα, IKK-β, and IKKγ knockdown cell clones (lanes 3 to 8) and the control wild-type HeLa-G cells (lanes 1 and 2) were transduced with Ad-Tax or Ad-tTa (a control adenoviral vector for the *tet trans*-activator) at an MOI of 10 for 48 h. The cells were then harvested and immunoblotted for the indicated proteins.

In agreement with the cell proliferation assays in Fig. 1C, upregulation of the cyclin-dependent kinase inhibitors and markers of senescence p21 and p27 was observed in IKKβ-KD cells transduced with Ad-Tax (Fig. 2, lanes 5 versus 6). In contrast, no significant increases in p21 and p27 levels were seen in similarly treated IKKα-KD (where IKKβ remained active) or IKKγ-KD cells (Fig. 2, lanes 3 and 7). As I-κBα degradation and nuclear localization of p65/RelA induced by Tax continued to occur in IKKα-KD cells (Fig. 3A; Fig. 3B, lanes 2 versus 4), these data indicate that the nuclear transport of NF-κB alone is insufficient for senescence induction.

The critical role of IKKα in Tax-IRS is particularly noteworthy. Previous studies have indicated that I-κBα degradation and p65/RelA nuclear localization are necessary but not sufficient for TNF-α or Tax-induced NF-κB transcriptional activation. Rather, active IKKα is essential for the transcriptional activity of NF-κB (21). The loss of the NF-κB transcriptional activity in IKKα-KD cells (Fig. 1B) correlated with the loss of Tax-IRS (Fig. 1C and D) and strongly suggests that the abrogation of the transcriptional activity emanating from NF-κB effectively prevented senescence induction. Together with our earlier publication showing that

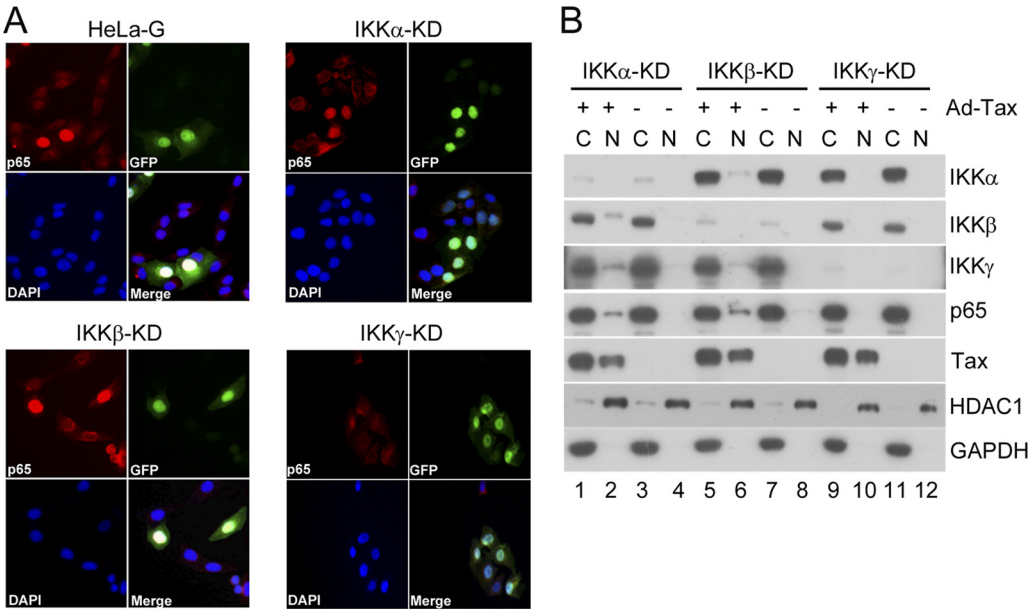

FIG 3 Nuclear p65/RelA and activated IKKα are both needed for Tax-induced NF-κB *trans*-activation and for triggering senescence response. (A) HeLa-G, IKKα-KD, IKKβ-KD, and IKKγ-KD cell clones were plated on chamber slides and transduced with Ad-Tax at an MOI of 1 for 48 h. The cells were then fixed, permeabilized, and immunostained for p65/RelA (red). Nuclei were stained with DAPI (blue). EGPF fluorescence indicates Tax expression. (B) The indicated cell clones were transduced with either Ad-tTa or Ad-Tax at an MOI of 10 for 48 h. Cells were harvested and fractionated into cytoplasmic and nuclear fractions and immunoblotted for the indicated proteins. GAPDH and HDAC1 were used as indicators for cytoplasmic and nuclear fractions, respectively.

p65/RelA knockdown can prevent Tax-IRS (43), these results support the notion that nuclear p65/RelA and activated IKKα act in concert to promote NF-κB transcription, which, when persistently activated by Tax, triggers a senescence response.

Subcellular localization of the IKK complex in Tax-expressing cells. Cytokine-mediated NF-κB activation often results in rapid nuclear accumulation of the NF-κB subunits. Several groups have independently demonstrated that the nuclear transport of NF-κB is necessary but not sufficient for NF-κB transcriptional activation. In the absence of IKKα, the activation of NF-κB (p50/p65)-directed gene transcription is completely abrogated (1, 38). This defect occurs in spite of I-κBα degradation and nuclear translocation of NF-κB as mediated by IKKβ. Importantly, when activated, IKKα was found to transport to the nucleus, where it associates with nuclear p65 and phosphorylates histone H3 adjacent to NF-κB-binding sites at Ser-10 to facilitate histone acetylation and transcriptional activation (1, 38). To confirm the nuclear localization of IKKα in the presence of Tax, subcellular fractionation of HeLa-G cells transduced with either Ad-tTa or Ad-Tax was carried out (Fig. 4A). Indeed, upon Tax expression, IKKα accumulated in the nucleus compared to the control (Fig. 4A). As might be expected, both Tax and p65/RelA were also present at appreciable levels in the nucleus. Interestingly, nuclear IKKβ and IKKγ were also readily detected (Fig. 4A). This is most likely facilitated by their association with Tax. Laser scanning confocal microscopy further revealed that nuclear IKKα exists in the form of nuclear speckles (Fig. 4B, lower left). Intriguingly, we also found that a significant fraction of IKKα localizes to the perinuclear region of Tax-expressing cells (Fig. 4B). This is consistent with a previous report detecting IKK localization to the Golgi apparatus (11). However, the biological significance of perinuclear IKKα is not clearly understood.

Tax-IRS is not executed via the alternative NF-κB pathway. As IKKα plays a crucial role in activating the alternative NF-κB pathway, we examined the latter's involvement in senescence induction. To this end, RelB and p100 expression were knocked

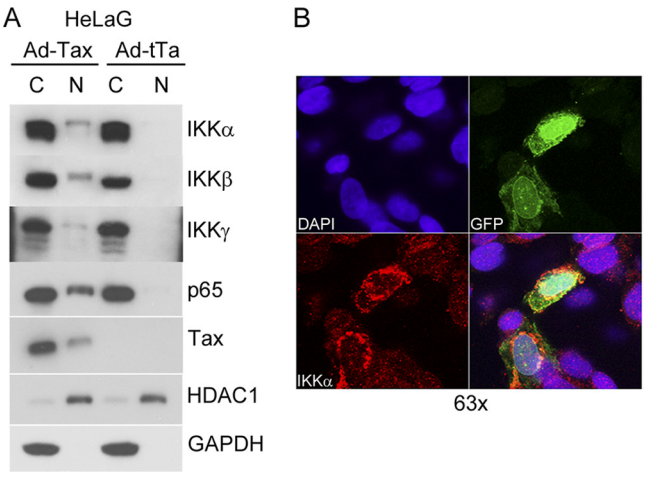

FIG 4 Tax induces nuclear localization of IKK complex. (A) HeLa-G cells were transduced with either Ad-tTa or Ad-Tax at an MOI of 10 for 48 h. Cells were then harvested and fractionated into nuclear and cytoplasmic fractions and immunoblotted for the indicated proteins. HDAC1 and GAPDH were used as markers for nuclear and cytoplasmic compartments, respectively. (B) HeLa-G cells were plated on chamber slides and transduced with Ad-Tax at an MOI of 1 for 48 h. The cells were then fixed, permeabilized, and immunostained for IKKα (red). Nuclei were stained with DAPI (blue). EGPF fluorescence indicates Tax expression. The immunostaining was examined and photographed using the 63× objective of a Pascal confocal microscope equipped with a CCD camera.

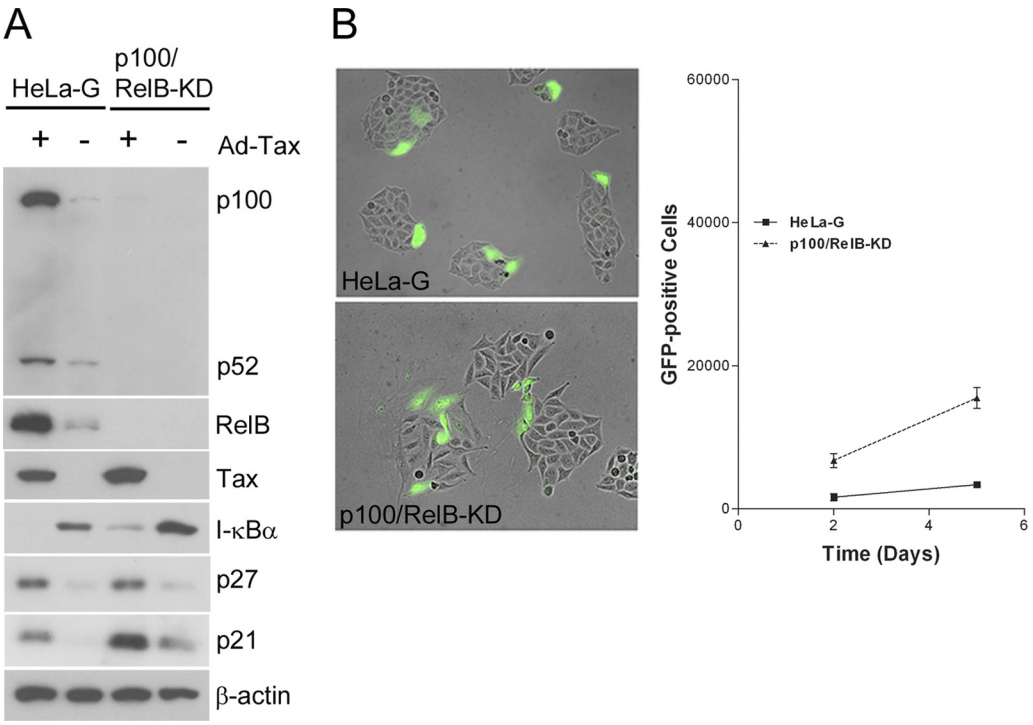

FIG 5 The alternative NF-κB pathway is not required for Tax-induced cellular senescence. The RelB and p100 double KD cells were derived by methods similar to Fig. 1 except that a pool of high-titer shRNA vectors targeting both RelB and p100 was used. A clone that displayed the most significant knockdown of both RelB and p100 was then used for the study. (A) Cells of parental HeLa-G and RelB/p100-KD clone were transduced with Ad-Tax (+) or Ad-tTa (−) at an MOI of 10 for 48 h. The cells were then harvested and immunoblotted for the indicated proteins. (B) (Left) HeLa-G cells (top) and the RelB/p100-KD clone (bottom) were transduced with Ad-Tax as for Fig. 1C and photographed at day 5. (Right) The growth curve of Ad-Tax-transduced RelB/p100-KD cells was determined as in Fig. 1D.

down. Our published results have indicated that deficiency in p100 did not affect senescence induction by Tax, while deficiency in RelB moderately attenuated senescence induction by Tax by decreasing the extent of p21 upregulation (43). Likewise, expression of Tax in a HeLa-G-derived cell line doubly knocked down for p100 and RelB (Fig. 5A) induced upregulation of p27 and, to a lesser extent, increase in p21 (Fig. 5A). For reasons that are unclear at present, the basal p21 level was higher in cells deficient in both RelB and p100. As expected, Tax-IRS is only moderately attenuated by the loss of p100 and RelB, as revealed by fluorescence microscopy and the growth property of EGFP-positive p100/RelB-KD cells (Fig. 5B), supporting the notion that nuclear NF-κB (p65/RelA) and activated IKKα are primarily responsible for triggering a senescence response, with a minor contribution from the alternative NF-κB pathway.

TAK1 and NIK are required for potent NF-κB activation by Tax, but only deficiency in NIK effectively prevents Tax-IRS. In order to identify additional components in the NF-κB pathways that are involved in Tax-mediated NF-κB transactivation, NIK and TAK1 were individually knocked down using their respective lentiviral shRNA vectors. These two kinases have been widely reported to act upstream of IKKα and IKKβ subunits, respectively (reviewed in references 9, 10, and 33). NIK- and TAK1-KD cell clones were isolated, expanded, and validated by immunoblotting (Fig. 6A, compare lanes 1 and 2 for TAK1). Because NIK undergoes constitutive proteasomal degradation as a cellular safeguard to prevent basal activation of the alternative NF-κB pathway, it could be detected only after cells were treated with a proteasome inhibitor MG132. With MG132 treatment, the shRNA-mediated knockdown of NIK was confirmed (Fig. 6A, lanes 3 to 6 versus 7 to 10).

E-Selectin luciferase reporter assay was then carried out to determine the role of TAK1 and NIK in supporting Tax-mediated NF-κB activation. As indicated by the E-selectin luciferase reporter activities, NF-κB activation by Tax is attenuated (by approximately 50%) in TAK1-KD cells but greatly reduced (by approximately 90%) in NIK-KD cells (Fig. 6B). In TAK1-KD cells, Tax expression (as determined by EGFP positivity) caused the majority of cells to become arrested as single cells, as indicated by fluorescence microscopy (Fig. 6C, upper left). In contrast, NIK deficiency prevented Tax-IRS (Fig. 6C, lower left). The growth curves of Ad-Tax-transduced EGFP-positive cells in the respective KD background also confirm the critical role of NIK in senescence induction (Fig. 6C, right). Finally, consistent with the senescence or proliferation phenotypes seen in Fig. 6C, Tax greatly induced p21 and p27 levels in TAK1-KD cells (Fig. 6D, lane 5 versus lanes 4 and 6) but not in NIK-KD cells (Fig. 6D, lane 11 versus lanes 10 and 12). These results support the notion that IKK activation by Tax requires the recruitment and participation of the IKK kinases TAK1 and NIK. In agreement with the results showing that activated IKKα and nuclear NF-κB trigger the senescence response, the deficiency in NIK, the upstream activating kinase of IKKα, has a phenotype similar to that of the deficiency in IKKα. Likewise, the loss of TAK1 is phenotypically similar to the loss of IKKβ. The role of TAK1 in Tax-induced NF-κB activation has been controversial (7, 35). The present results are in agreement with those of Wu and

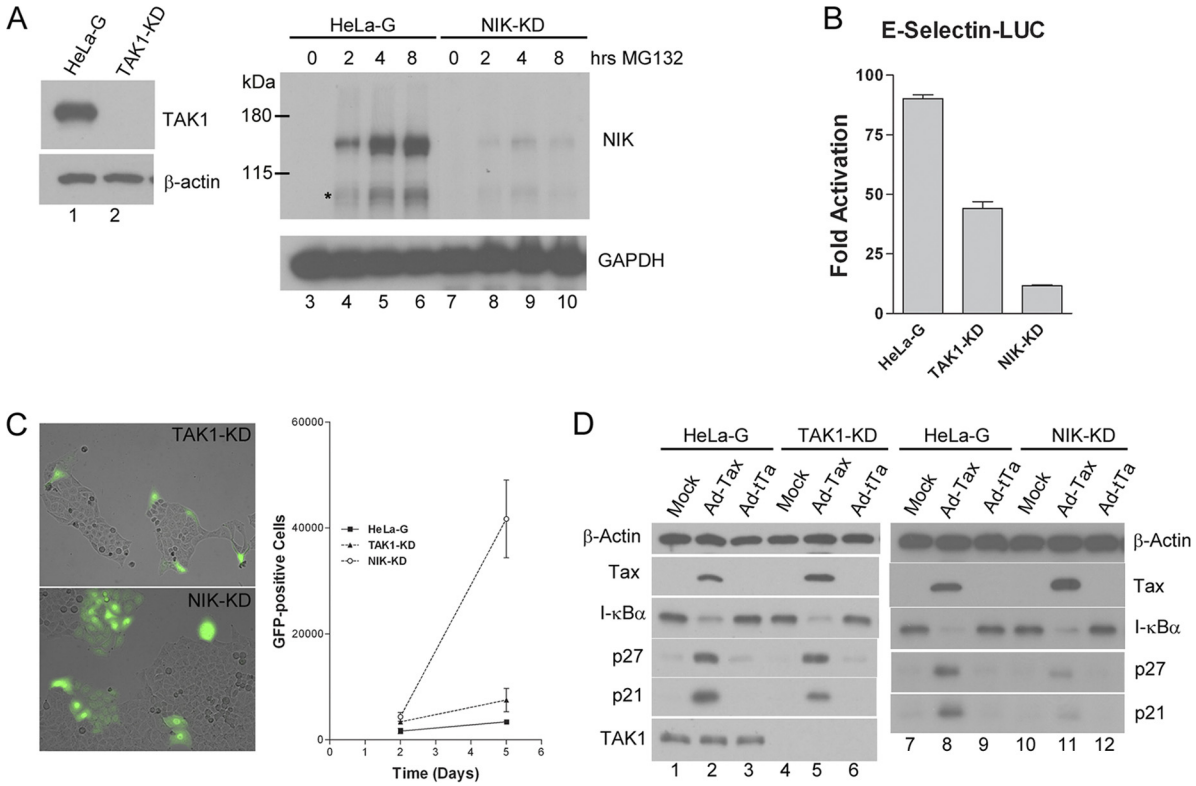

FIG 6 Phenotypes of NIK and TAK1 knockdown parallel those of IKKα and IKKβ knockdown, respectively. (A) Derivation and characterization of TAK1-KD and NIK-KD cell lines. TAK1-KD and NIK-KD clones were derived as in Fig. 1. To verify NIK knockdown, wild-type HeLa-G and NIK-KD cells were treated with 50 μM proteasome inhibitor MG132 for 0, 2, 4, and 8 h to inhibit the constitutive proteasomal degradation of NIK. Cells were subsequently harvested and immunoblotted for NIK. (The asterisk indicates a degradation product of NIK protein.) (B) Deficiency in TAK1 compromised but deficiency in NIK abrogated Tax-mediated NF-κB activation. TAK1-KD and NIK-KD cells were cotransfected with E-selectin-Luc and pRL-TK with or without Tax. Luciferase reporter assay was performed at 48 h posttransfection. Activation was calculated as in Fig. 1B. (C) NIK-KD but not TAK1-KD prevented Tax-IRS. TAK1-KD (top left) and NIK-KD (bottom) cells were plated and transduced with Ad-Tax as for Fig. 1C. Growth curves were produced as described in Fig. 1D. (D) I-κBα, p21, and p27 levels in untreated (mock), Ad-tTa, and Ad-Tax-transduced HeLa-G, TAK1-KD, and NIK-KD cells. Cells were left untreated or were transduced with Ad-Tax or Ad-tTa at an MOI of 10 for 48 h. The cells were then harvested and immunoblotted for the indicated proteins.

Sun (35) in showing the importance of TAK1 and IKKβ in achieving optimal Tax-mediated IKK activation.

Finally, to confirm the critical role of NIK in senescence induction, a dominant negative NIK mutant (NIK-DN) was stably expressed in HeLa-G cells by using a lentivirus vector (Fig. 7A). In agreement with results from NIK-KD cells, expression of NIK-DN blocked Tax-mediated NF-κB activation as well as moderated Tax-induced senescence (Fig. 7B and C).

DISCUSSION

HTLV-1 Tax is a potent activator of IKK and NF-κB. Chronic IKK/NF-κB activation by Tax has been causally linked to cellular transformation and leukemia development (8, 12, 19, 39, 40). Paradoxically, expression of Tax leads rapidly to upregulation of the CDK inhibitors p21 and p27 and to cellular senescence (16, 18, 42, 43). Recently, we demonstrated that Tax-induced rapid senescence (Tax-IRS) constitutes a host checkpoint response to the potentially oncogenic hyperactivation of NF-κB by Tax; and p65/RelA is responsible for triggering this response (43). In this study, we examined the role of the IKK subunits IKKα, IKKβ, and IKKγ and the IKK kinases NIK and TAK1 in senescence induction. We found that when IKKγ, IKKα, or NIK was knocked down, Tax-induced NF-κB transcriptional activity became drastically reduced and Tax-IRS was essentially abrogated. In contrast, although IKKβ or TAK1 deficiency curtailed NF-κB activation by Tax, the residual transcriptional activity of NF-κB as driven by IKKα remained significant and was sufficient to trigger senescence. These results support the notion that the chronic transcriptional activity of NF-κB mediated by Tax drives senescence induction.

Our data are also consistent with the notion that IKK activation by Tax requires the participation of the IKK kinases NIK and TAK1 (for IKKα and IKKβ, respectively). Specifically, IKKβ and TAK1 potentiate the magnitude of NF-κB activation by Tax by activating IKK and targeting I-κBα for degradation. However, while this step is necessary, it is insufficient to trigger the level of NF-κB transcriptional activity that is required for senescence induction. For this reason, when activated in IKKα-deficient condition, IKKβ causes I-κBα degradation, which then permits nuclear transport of NF-κB, but little NF-κB-driven transcription was stimulated in the absence of IKKα; thus, nuclear NF-κB failed to trigger a senescence response. In contrast, NIK and IKKα are essential for Tax-mediated NF-κB transcriptional activity, and their chronic activation elicits a robust senescence response. However, RelB and p100 double knockdown did not prevent Tax-IRS, indicating that the alternative pathway is not critical for Tax-IRS.

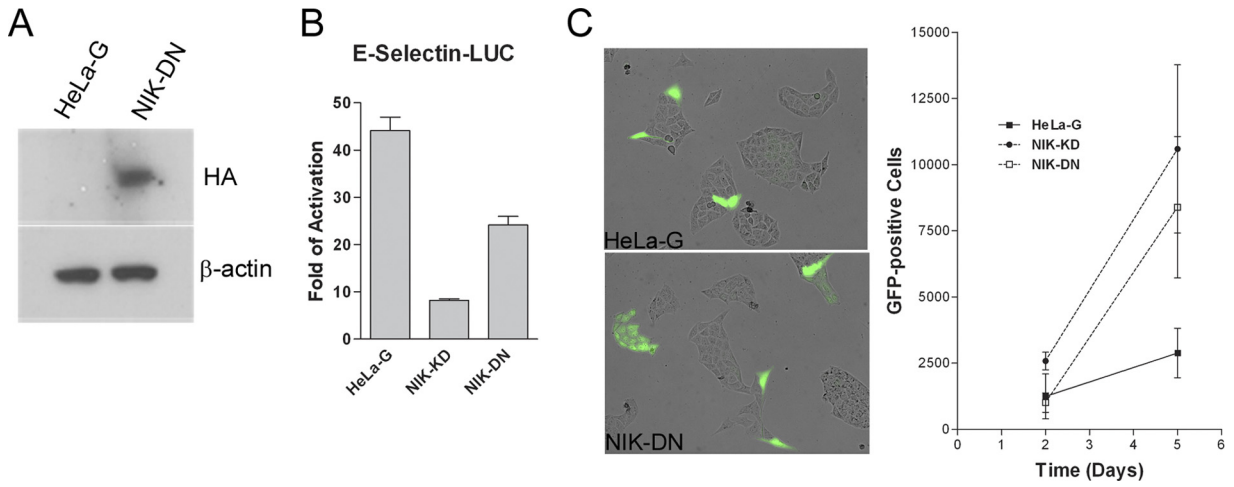

FIG 7 Phenotypes of cells expressing dominant negative NIK mutant (NIK-DN) parallel those of NIK-KD cells. (A) Derivation and characterization of NIK-DN cells. An HA-tagged dominant negative NIK mutant is stably expressed in wild-type HeLa-G cells via lentiviral vector and puromycin selection. Cells were subsequently harvested and immunoblotted for HA. (B) Expression of dominant negative NIK mutant compromised Tax-mediated NF-κB activation. HeLa-G, NIK-DN, and NIK-KD cells were cotransfected with E-selectin-Luc and pRL-TK with or without Tax. Luciferase reporter assay was performed at 48 h posttransfection. Activation was calculated as for Fig. 1B. (C) NIK-DN prevented Tax-IRS. Wild-type HeLa-G (left, top) and NIK-DN (bottom) cells were plated and transduced with Ad-Tax as in Fig. 1C. Growth curves were calculated as described in Fig. 1D. The growth curve of NIK-KD cells was included as a positive control.

Taken together, these data suggest that Tax recruits both NIK and TAK1 for the activation of IKKα and IKKβ, respectively; however, only activated IKKα, which is crucial for hyperactivating the transcriptional activity of NF-κB, can trigger cellular senescence. Our results and those of many others suggest that in the presence of Tax, the line separating the canonical and the noncanonical pathways becomes blurred, allowing Tax-expressing cells to indiscriminately activate all IKK complexes and downstream NF-κB transcriptional activity, which then triggers a senescence response.

Earlier studies showed that IKKα plays a crucial role in the nucleosomal and transcriptional activity of NF-κB (1, 38). Nuclear transport of NF-κB alone is insufficient for transactivation when IKKα is absent (1, 38). Activated IKKα was found to transport to the nucleus, assemble to the NF-κB promoter region in association with nuclear p65/RelA and CBP/p300, and phosphorylate histone H3 adjacent to NF-κB-binding sites at Ser-10 to facilitate additional H3 acetylation and transcriptional activation (1, 38). In addition to its role in chromatin modification, IKKα has also been shown to be crucial for Tax-mediated NF-κB activation by phosphorylating the COOH-terminal Ser-529 and Ser-536 residues of p65/RelA, with Ser-536 phosphorylation playing a crucial role in NF-κB-driven transcription (21). Consistent with these observations, the results herein indicate that although IKKα and IKKβ can individually cause I-κBα degradation and nuclear transport of NF-κB, only activated IKKα together with p65/RelA plays a crucial role in Tax-dependent NF-κB activation, which triggers a senescence response. Immunofluorescence and subcellular fractionation confirmed that IKKα is localized in the nucleus in Tax-expressing HeLa-G cells, consistent with its role in chromatin modification. It remains to be determined whether chromatin modification, the act of constant mRNA transcription from multiple NF-κB-regulated promoters, or the induction of specific NF-κB-regulated genes is responsible for transmitting a danger signal for senescence induction.

We have also observed a fraction of IKKα in Tax-expressing cells to localize to the perinuclear region. This is in agreement with earlier reports by others showing that Tax relocalizes IKK to the Golgi (11). Surprisingly, unlike in TNF-α-treated HeLa-G cells (1, 38), appreciable amounts of both IKKβ and IKKγ were found to localize to the nucleus in Tax-expressing cells. As Tax shuttles between nuclear and cytoplasmic compartments, IKKβ and IKKγ may translocate to the nucleus by associating with Tax. The unusual Tax-associated localizations of IKKβ, IKKγ, and especially IKKα likely potentiate the activation of IKK and the transcriptional activity of NF-κB.

The mechanism by which Tax activates IKK remains to be elucidated. Our results and previously published data (32, 35) suggest that Tax most likely facilitates the recruitment of IKK kinases, namely, NIK and TAK1, to IKKα and IKKβ, respectively (Fig. 8). Over the past few years, the theme of Lys-63 (K63) polyubiquitin serving as a platform for IKK activation has emerged (reviewed in references 9, 10, and 33). Upon TNF-α or IL-1 stimulation, E3 ubiquitin ligases such as TRAF6, TRAF2/5, and cIAP1/2 and signaling molecules such as IRAK1 and RIP1 become recruited to activated receptors, where extensive K63 polyubiquitination takes place on TRAFs, RIP1, IRAK1, and other molecules (9, 10, 33). Via the K63 polyubiquitin-binding domains in IKKγ and TAB2 (a subunit of TAK1), IKK and TAK1 assemble on K63 polyubiquitin; thereupon, TAK1 phosphorylates and activates IKK (IKKβ). As our data indicate that the activation of IKKα and IKKβ by Tax requires the participation of NIK and TAK1, it would appear that Tax may usurp the K63 polyubiquitination process to achieve IKK activation (Fig. 8). In this vein, it is interesting that Tax is extensively modified posttranslationally by phosphorylation, ubiquitination, and sumoylation (6, 17, 23). K63 polyubiquitination of IKKγ and monoubiquitination of IKKβ are highly induced by Tax (2). Recent data have also suggested that UBC13/UBE2N, an E2-conjugating enzyme involved in IKK activation, can associate with both Tax and IKKγ and is critical for their K63 polyubiquitination and Tax-mediated IKK activation (27), a conclusion further sup-

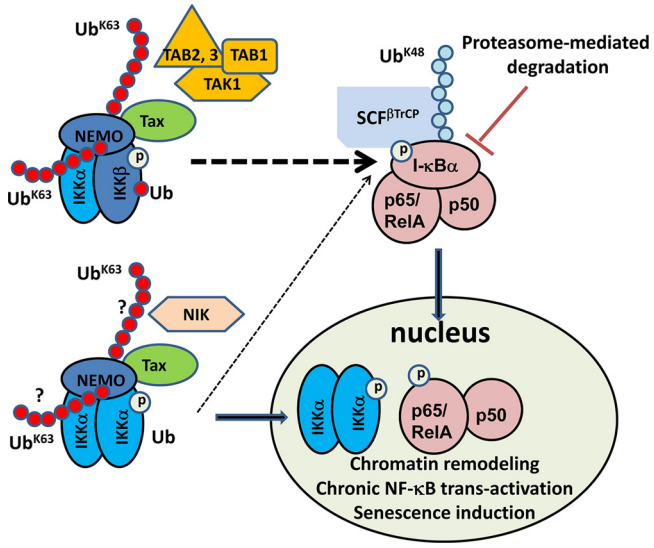

FIG 8 Schematic representation of the possible mechanisms by which Tax interacts and chronically activates the NF-κB pathway. Tax interacts with NEMO and recruits TAK1 and NIK to IKK complexes, which initiate the activation of predominantly the classical pathway and, to a lesser extent, the alternative NF-κB pathways. The Tax-mediated recruitment of TAK1 to IKK likely involves K63 polyubiquitination, as suggested by recent results (13, 27, 28). Whether NIK recruitment requires K63 polyubiquitin is not clear. Nuclear localization of p65/RelA and IKKα as mediated by Tax induces chromatin remodeling, chronic NF-κB transcriptional activation, and, subsequently, cellular senescence.

ported by a cell-free IKK activation system for Tax elegantly established by Shibata et al. (28). Finally, a binding partner of Tax, Tax1BP1, has been shown to constitute one subunit of a protein complex containing A20, a K63 deubiquitinase involved in down-regulating IKK/NF-κB signaling (22, 26). How Tax interacts with signaling molecules, ubiquitin E2 and E3 enzymes, and the A20-containing deubiquitinase that is involved in IKK regulation would be a topic of significant interest.

ACKNOWLEDGMENTS

We thank the Uniformed Services University Biomedical Instrumentation Center (BIC) for providing laser scanning confocal microscopy services.

This work was supported by grants from the National Institutes of Health (R01CA140963 and R01 CA115884) and the USU intramural research program (RO73NV) to C.-Z.G.

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