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Notch inhibition in Kaposi's sarcoma tumor cells leads to mitotic catastrophe through nuclear factor-κB signaling

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Abstract

Kaposi's sarcoma (KS) is the most common neoplasm in untreated AIDS patients and accounts for significant morbidity and mortality worldwide. We have recently reported that Notch signaling (which plays an important role in cell proliferation, apoptosis, and oncogenesis) is constitutively activated in KS tumor cells. Blockade of this activity using γ-secretase inhibitors resulted in apoptosis of SLK cells, a KS tumor cell line; however, this apoptosis was preceded by a prolonged G_2-M cell cycle arrest. This result led us to hypothesize that the cells were undergoing mitotic catastrophe, an abnormal mitosis that leads to eventual cell death. Here, we show that Notch inhibition in KS tumor cells using γ-secretase inhibitors or Notch-1 small interfering RNA resulted in G_2-M cell cycle arrest and mitotic catastrophe characterized by the presence of micronucleated cells and an increased mitotic index. Interestingly, Notch inhibition led to a sustained increase in nuclear cyclin B1, a novel observation suggesting that Notch signaling can modulate expression of this critical cell cycle protein. Further analysis showed the induction of cyclin B1 was due, at least in part, to increased nuclear factor-κB (NF-κB) activity, which was also required for the G_2-M growth arrest after Notch inhibition. Taken together, these studies suggest that Notch inhibition can initiate aberrant mitosis by inducing NF-κB activity that inappropriately increases cyclin B1 resulting in cell death via mitotic catastrophe. [Mol Cancer Ther 2007;6(7):1983–92]

Introduction

Notch proteins are evolutionarily conserved transmembrane receptors that serve as transcriptional activators and play a critical role in cell fate decisions. In mammalian cells, there are four Notch receptors (Notch-1, Notch-2, Notch-3, Notch-4) and five ligands [Jagged-1, Jagged-2, and Delta-like (Dll)-1, Dll-3, Dll-4]. The receptors are activated by ligand engagement, which triggers a two-step proteolytic cleavage by ADAM (a disintegrin and metalloprotease) and γ-secretase resulting in the release of the intracellular portion of the receptor, N^{IC} (1–3). N^{IC} translocates to the nucleus and binds CBF-1 (also termed RBP-Jκ), a transcription factor that is normally bound to DNA sequences in the presence of a corepressor complex containing silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT), CBF-1 interacting corepressor (CIR), and histone deacetylase-1 (HDAC-1) (4, 5). N^{IC} binding results in a release of the corepressor complex and recruits nuclear coactivators, such as Ski interacting protein (SKIP), mastermind-like–1 (MAML1), and histone acetyltransferases, resulting in transcription (6, 7).

Genes regulated by Notch include helix-loop-helix inhibitory transcription factors of the Hes (Hairy/Enhancer of Split) and the Hey (Hairy/Enhancer of Split related with YRPW) families (8, 9). Notch signaling can also influence nuclear factor-κB (NF-κB) activity. NF-κB refers to a family of transcription factors [p50, p65 (RelA), p52, RelB, c-Rel] that form homo- and heterodimers and regulate expression of various genes involved in proliferation and survival (10). NF-κB signaling is normally controlled by IκB proteins, which bind to and retain the NF-κB factors in the cytoplasm, thus preventing their translocation to the nucleus and binding to DNA. $N^{IC}1$ can function as a novel IκB protein by specifically interacting with the p50 subunit and blocking its activity (11, 12). Alternatively, Notch can increase NF-κB activity by directly interacting with NF-κB and promoting its nuclear retention (13, 14). It has been suggested that the ultimate positive or negative effect of Notch on NF-κB activity is dependent on factors such as dose, time, and cell type (13, 15).

Mitotic catastrophe is defined as abnormal mitosis that leads to eventual cell death. The cell death can occur either during mitosis or after mitosis; in the latter case, cells exit mitosis into an aberrant interphase, characterized by the formation of multiple micronuclei, which can be used as a surrogate measure of mitotic catastrophe. Ultimately, cells

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that have suffered mitotic catastrophe undergo apoptosis, nonapoptotic death, or senescence (16).

We have recently shown Kaposi's sarcoma (KS) tumor cells express constitutively active Notch receptors both *in vivo* and *in vitro*. We used γ-secretase inhibitors (pan-Notch inhibitors that block proteolytic cleavage of all Notch receptors preventing release of N^{IC}) to block Notch activation in SLK cells, a KS tumor cell line, which led to a G_2-M cell cycle arrest followed by apoptosis (17). This prolonged G_2-M arrest and subsequent death led us to hypothesize that Notch inhibition may induce mitotic catastrophe in KS tumor cells.

Materials and Methods

Cell Culture and Chemical Inhibitors

KS tumor cell lines (SLK, KS-IMM) were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin (18, 19). KS tumor cell line KS-Y1 was cultured in RPMI 1640 containing 1% sodium pyruvate, 10% FBS, 1% nonessential amino acids, 1% essential amino acids, 1% L-glutamine, 1% penicillin-streptomycin, and 1% Nu-Serum, and grown on gelatin-coated plates (20). It should be noted that these cell lines are not infected with the KS-associated herpesvirus (KSHV/HHV-8), which is consistently found in clinical tumors. The virus is rapidly lost from KS tumor cells in culture, and all known KS tumor cell lines are KSHV negative (21). Given the small number of cells available from fresh tumor isolates, it was not possible to perform these studies with authentic KS tumor cells infected with KSHV. Despite the lack of KSHV infection, these well-characterized cell lines possess the nonrandom chromosomal abnormalities associated with advanced KS lesions and have been routinely used in KS research for more than a decade (22).

Z-Leu-Leu-Nle-CHO (LLNle) and the NF-κB activation inhibitor (6-amino-4-(4-phenoxyphenylethylamino)quinazoline) were purchased from EMD Biosciences. LY-411,575 was resuspended in DMSO. When added to culture media, LY-411,575 precipitated, necessitating the use of high concentrations (500 μmol/L) to ensure adequate delivery of solubilized drug. Mixing LY-411,575 1:1 with Cremephor EL before use avoided much of the precipitation, and a 30 μmol/L concentration was used with similar results. Due to the short half-life of LY-411,575,[5] media was supplemented with the drug every 6 h.

Transfections and Plasmids

KS cell lines were transfected with ExGen 500 (Fermentas Life Sciences) using the manufacturer's instructions. The Hey-1 luciferase reporter construct and the dominant negative IκBα construct have been described (23, 24). The NF-κB-LUC vector was purchased from Clontech and contains a firefly luciferase gene driven by an NF-κB–responsive element. pSUPER plasmid (OligoEngine) was used to express small interfering RNA (siRNA) against Notch-1 (target sequence: 5′-AAGTGTCTGAGGCCAG-CAAGA-3′). Cells were analyzed at 24–96 h post-transfection. The LZRS retroviral expression vector encoding for constitutively active Notch-1 ($N^{IC}1$) has been described (25). The Phoenix packaging cell line was transfected with LZRS using standard $CaCl_2$ and 2× HBSS methodologies, and supernatants containing infectious retrovirus were harvested after 24–48 h (26). Target cells were transduced with 1 mL of viral supernatant containing 8 μg/mL polybrene, with centrifugation for 30 min. Fresh media was added to the wells, and the cells were analyzed after 48 h.

Immunofluorescent Staining

KS tumor cells were plated on sterile glass coverslips. The coverslips were washed in PBS, and the cells fixed in ice-cold methanol/acetone for 10 min. The coverslips were incubated with antibodies to cyclin B1 (Santa Cruz Biotechnology) diluted in 0.1% bovine serum albumin for 1 h, washed, and then incubated for an additional 1 h with species-specific Alexa Fluor secondary antibodies (Invitrogen). DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). To quantitate micronucleation, at least 1,000 cells in 10–20 random, high-powered fields were examined per experiment, and the number of micronucleated cells versus normal cells were determined. Cells were considered micronucleated if they had three or more nuclei.

Western Blot Analysis

Western blot analysis was done as previously described (17). Proteins were visualized using chemiluminescence, and even loading of proteins was confirmed by Ponceau S staining of the membranes and detection of actin on each blot.

Cell Cycle Analysis

DNA/propidium iodide (DNA/PI) staining was done using standard methodologies. Cells (1×10^6) were permeabilized with 100% ethanol in the presence of 15% FBS. Cells were washed and treated for 15 min at 37°C with 10 μg/mL RNase (Ambion). PI (5 μg/mL) was added, and the cells were incubated for 1 h at 4°C before analysis using a Coulter Epics MCL flow cytometer with 10,000 cells analyzed per gated determination.

Mitotic Index

Cells in G_2 and M phases were distinguished based on reactivity with mitotic protein monoclonal 2 (MPM2) antibodies (Millipore). Briefly, adherent and floating cells were fixed in 1% formaldehyde on ice for 15 min, washed, and exposed to 80% ice-cold ethanol. Cells were permeabilized with 0.25% Triton X-100 in PBS, washed, and incubated for 1 h with 2.5 μg/mL anti-MPM2 antibodies. Following a wash, the cells were incubated for 30 min with Alexa Fluor 488–labeled anti-mouse immunoglobulin G (Invitrogen) and treated with RNase (10 μg/mL) and 5 μg/mL PI. Cells were analyzed by two-color flow cytometry. Mitotic index was defined as the percentage of MPM-2–positive cells.

CDK1 Kinase Assay

CDK1 kinase activity was assessed using the CDK1/Cdc2 kinase assay kit (Upstate Biotechnology) following manufacturer's instructions. KS tumor cells were collected

[5] L. Miele, unpublished data.

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by scraping and incubated in lysis buffer for 20 min at 4°C. Debris was pelleted, and 500 μg protein was incubated with anti-CDK1 antibody (Santa Cruz Biotechnology) for 3 h at 4°C. About 50 μL of A/G agarose beads (Santa Cruz Biotechnology) were added, and the mixture was incubated for an additional 1 h. Immunoprecipitates were washed thrice in ice-cold PBS and resuspended in assay dilution buffer. Scintillation counting was done using a Packard Topcount microplate scintillation counter.

Results

Notch Inhibition Leads to a G_2-M Cell Cycle Arrest in KS Tumor Cells

We have recently shown that treatment of SLK cells with LLNle or LY-411,575 (two chemically distinct pharmacologic inhibitors of γ-secretase) significantly reduced constitutive Notch activation and led to a G_2-M cell cycle arrest followed by cell death (17). To confirm and extend these observations, DNA/PI staining was used to evaluate the cell cycle in three different KS cell lines (SLK, KS-IMM, and KS-Y1) treated with LLNle over an extended time course (1–96 h). The percentage of cells in the G_2-M phase increased in all three cell lines as early as 6 h after treatment and continued to increase from 18 to 72 h, after which the majority of cells shifted to a sub-G_0/G_1 DNA content, indicative of apoptosis (Fig. 1A). KS-Y1 cells underwent the same G_2-M arrest followed by apoptosis, but it occurred over an abbreviated time course (Fig. 1A).

To further confirm the role of Notch inhibition in the induction of G_2-M arrest, plasmid-based siRNA technology was used to knock down Notch-1 expression, thereby limiting its activation. Initial experiments showed that siRNA directed against Notch-1 (siNotch1) effectively reduced Notch-1 protein expression (both the full-length receptor and N^{IC}1) in SLK cells compared with cells transfected with a scrambled control siRNA (siControl; Fig. 1B). DNA/PI staining showed no significant alteration in the cell cycle profile of siControl expressing cells; however, siNotch1 led to a significant increase in cells in the G_2-M phase at 48, 72, and 96 h (Fig. 1C). Unlike the pharmacologic inhibitors, siNotch1 did not cause cell death at 96 h. This is may be due to incomplete blockade of Notch expression/activation with siNotch1, particularly at the early time points. Alternatively, this may be related to the blockade of only Notch-1 using the siRNAs, whereas the γ-secretase inhibitors are pan-Notch inhibitors and prevent activation of all four Notch receptors.

Notch Inhibition Leads to Micronucleation and Mitotic Arrest

Micronucleation, or the presence of multiple micronuclei with uncondensed chromatin, is a morphologic criteria used to define mitotic catastrophe (27, 28). To determine if Notch inhibition leads to micronucleation, SLK cells treated with LLNle or DMSO for 24 h were stained with DAPI to visualize the nuclei. Although SLK cells treated with DMSO showed sporadic micronucleation (average, 1.05 ± 0.33%), treatment with LLNle resulted in a significant

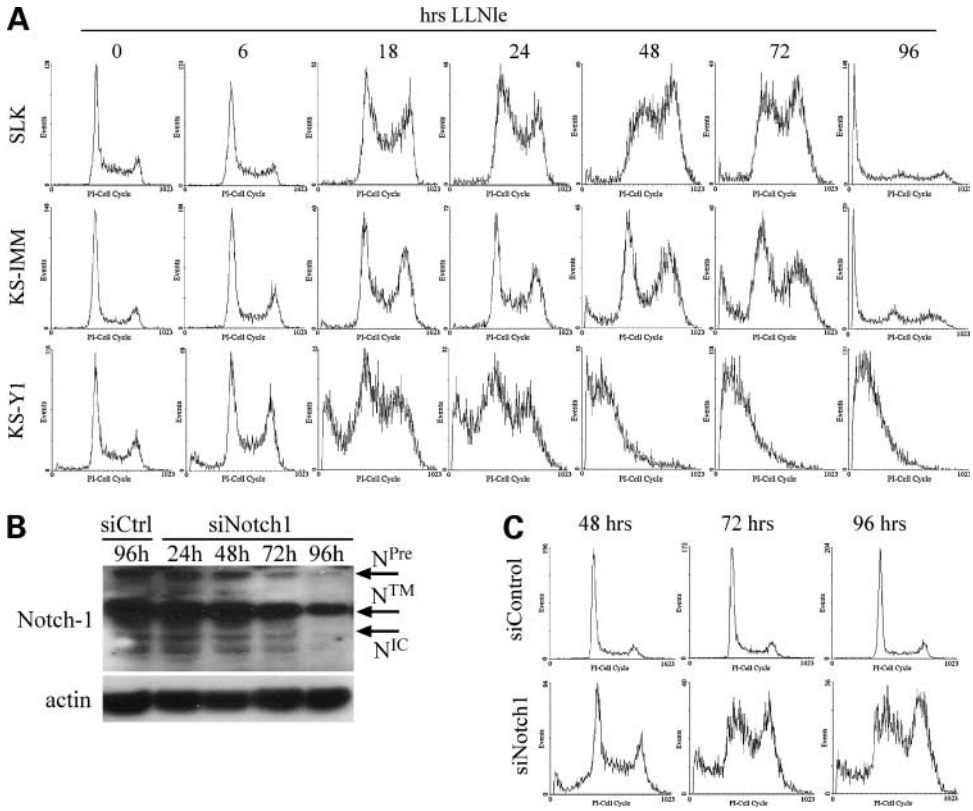

Figure 1. **A,** DNA/PI staining in three KS tumor cell lines (SLK, KS-IMM and KS-Y1) treated with LLNle showed an increase in the number of cells in the G_2-M phase of the cell cycle from 6 to 72 h, after which the majority of the cells were apoptotic (sub-G_0/G_1 DNA content). **B,** Western blot confirmed a decrease in the Notch-1 precursor protein (N^{Pre}), the transmembrane Notch-1 receptor (N^{TM}), and activated Notch (N^{IC}) expression in SLK cells treated with a Notch-1 siRNA (*siNotch1*). Notch-1 expression was decreased on average by 1.1-, 1.5-, 3.4- and 17-fold at 24, 48, 72 and 96 h, respectively, compared with cells transfected with control siRNA (*siCtrl*, 96-h time point is shown). Actin expression is provided as a protein loading control. **C,** DNA/PI staining of SLK cells transfected with siControl showed no significant change in the percentage of cells in the G_2-M phase (on average, 15.7 ± 0.7%, 16.45 ± 0.4%, and 16.7 ± 6.9% at 48, 72, and 96 h, respectively). In contrast, siNotch1-transfected SLK cells had significantly more cells in the G_2-M phase (on average, 32.7 ± 3.1%, 28.9 ± 6.2%, and 36 ± 4.38% at 48, 72, and 96 h, respectively). For each panel, representative data from three independent experiments are shown.

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increase in micronucleated cells (average, 34.8 ± 5.7%; Fig. 2A; $P < 0.01$). Similarly, LY-411,575 treatment increased micronucleation to 19.3 ± 2.1% (Fig. 2A; $P < 0.01$). To determine if the restoration of Notch-1 signaling could prevent micronuclei formation, SLK cells were transduced with a $N^{IC}1$-encoding retroviral vector and overexpression confirmed by Western blot (Fig. 2B, *inset*). We found a significant decrease in micronucleation in $N^{IC}1$ expressing SLK cells (average, 12.8 ± 1.8%) compared with cells transduced with the empty retroviral vector (average: 33.9 ± 6.2%; Fig. 2B; $P < 0.01$). Similar studies were done with KS-IMM and KS-Y1 cells, and micronucleated cells were readily detected in both cell types treated with LLNle compared with control-treated cells (data not shown). However, poor adherence of KS-IMM and KS-Y1 cells to the coverslips resulted in significant cell loss that prevented accurate quantitation. To confirm and extend these results, Notch-1 siRNA was used to knockdown Notch-1 expression, and micronucleation was evaluated. As anticipated, siNotch1 induced micronucleation in SLK cells (average, 22.0 ± 2.4%) compared with cells treated with siControl (average, 3.0 ± 1.1%; Fig. 2C; $P < 0.01$).

To confirm that Notch inhibition induces mitotic catastrophe, we measured the mitotic index in SLK cells by quantitating the accumulation of a mitotic marker, the MPM2 epitope. MPM2 antibodies recognize phosphoproteins that appear only during mitosis, allowing for the quantitation of cells arrested in the G_2 versus M phase of the cell cycle. The results showed that LLNle-treated SLK cells had a significant increase in mitotic index at time points corresponding to the G_2-M arrest (average at 24 h time point: LLNle, 38.6 ± 3.9%; DMSO, 2.0% ± 0.2% MPM2-positive cells; Fig. 2D; $P < 0.01$).

Notch Inhibition Alters Expression, Localization, and Activity of G_2-M Checkpoint Proteins

Next, we examined the effect of Notch inhibition on the expression of several G_2-M checkpoint proteins. Altered expression of these proteins would further support

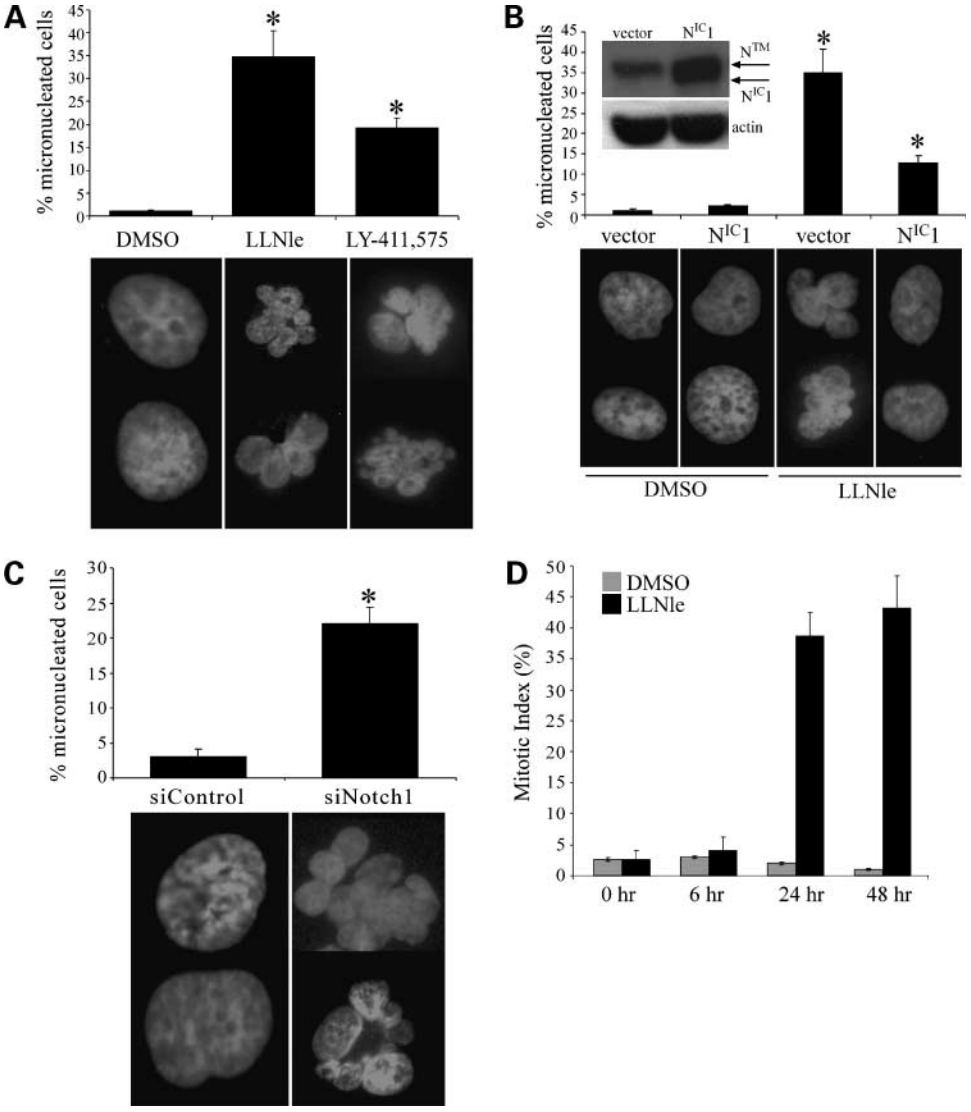

Figure 2. **A,** micronucleation was readily detected in SLK cells treated with LLNle or LY-411,575 after 24 h, whereas DMSO-treated cells showed predominantly round, intact nuclei (DMSO, 1.05 ± 0.33%; LLNle, 34.8 ± 5.7%; LY-411,575, 19.3 ± 2.1% micronucleated cells). Cells were stained with DAPI to visualize nuclei. **B,** micronucleation was significantly reduced in LLNle-treated SLK cells overexpressing constitutively active Notch-1 (SLK + vector + LLNle: 33.9 ± 6.2%, SLK + $N^{IC}1$ + LLNle: 12.8 ± 1.8% micronucleated cells). *Inset,* Western blot confirmed the overexpression of $N^{IC}1$ in SLK cells transduced with a retroviral vector encoding for $N^{IC}1$ compared with cells transduced with the empty vector. **C,** siRNA inhibition of Notch-1 in SLK cells resulted in an increase in the number of micronucleated cells at 72 h (siControl, 3 ± 1.1%; siNotch1, 22 ± 2.4% micronucleated cells). In **A – C**, quantitation of the results is presented as a bar graph (combined data from three to four independent experiments) above examples of nuclear morphology. *, $P < 0.01$. **D,** SLK cells treated with LLNle showed a gradual increase in MPM2 epitope expression, a marker of mitosis. In contrast, the percentage of DMSO-treated cells expressing MPM-2 was relatively constant over time. The results represent combined data from two independent experiments.

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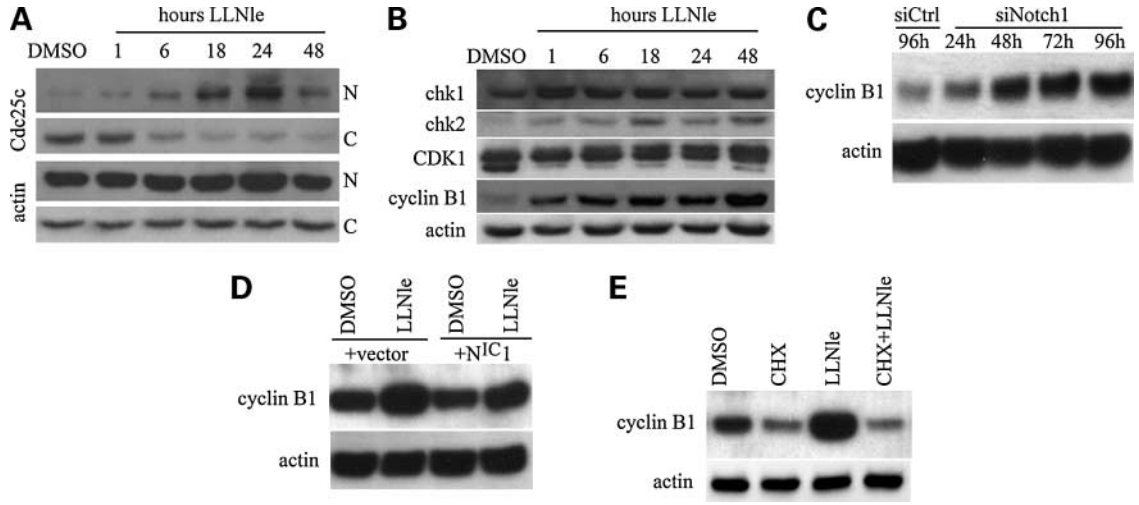

Figure 3. **A,** Western blot of nuclear (*N*) and cytoplasmic (*C*) extracts from LLNle-treated SLK cells showed a gradual increase in Cdc25c in the nucleus with a corresponding decrease in the cytoplasm. In contrast, Cdc25c levels were relatively constant in control, DMSO-treated cells (48-h time point is shown). **B,** Western blot of total cellular protein from LLNle-treated SLK cells revealed no significant change in Chk1 levels over time and only a modest increase in Chk2 expression. LLNle treatment rapidly induced the expression of a higher molecular weight form of CDK1 (representing the phosphorylated or activated form of the protein), and there was a significant increase in cyclin B1 expression over time. Elevated expression of both CDK1 and cyclin B1 were sustained throughout the 48-h experiment. **C,** cyclin B1 protein expression was also increased in SLK cells treated with Notch-1 siRNA compared with control siRNA (96-h time point is shown). **D,** cyclin B1 protein expression was increased in SLK cells transduced with an empty retroviral vector before treatment with LLNle for 24 h. The cyclin B1 increase was partially attenuated (22% decrease) in LLNle-treated cells overexpressing $N^{IC}1$. **E,** treatment of SLK cells with cycloheximide (*CHX*) blocked new protein synthesis resulting in a decrease in cyclin B1 expression. Similarly, pretreatment of SLK cells with cycloheximide before LLNle treatment prevented the increase in cyclin B1 induced by LLNle alone. This figure shows representative data from two to three independent experiments.

aberrant entry of these cells into mitosis and initiation of mitotic catastrophe (29). Cdc25C is a phosphatase whose nuclear import early in mitosis is required for activation of cyclin-dependent kinase 1 (CDK1; ref. 30). We found that Cdc25C was primarily localized to the cytoplasm of control-treated SLK cells, but translocated to the nucleus after LLNle treatment (Fig. 3A). Increased nuclear levels were detectable as early as 1 h posttreatment and continued to increase for 24 h. Chk1 and Chk2 are kinases upstream of Cdc25C; Chk1 is thought to recognize UV-induced DNA damage sensed by ATM and Rad-3-related (ATR) kinase, and Chk2 recognizes double-strand breaks sensed by ataxia telangiectasia mutated (ATM) kinase (31, 32). Expression of Chk2 increased following LLNle treatment and remained elevated throughout the experiment (range, 1.8-fold at 1 h to 4.3-fold at 48 h; Fig. 3B). Chk1 expression was not significantly increased (range, 1.5- to 1.8-fold, Fig. 3B). LLNle treatment also resulted in the accumulation of the phosphorylated (or active) form of CDK1, the binding partner for cyclin B1, as detected by a shift in the molecular weight of the protein on Western blot (Fig. 3B).

Because increased levels of cyclin B1 have been implicated in mitotic catastrophe, the effect of Notch inhibition on cyclin B1 expression was investigated. Western blot showed a rapid and sustained increase in the total amount of cyclin B1 in LLNle-treated SLK cells (Fig. 3B). Similar results were seen in SLK cells treated with LY-411,575 and those treated with Notch-1 siRNAs (data not shown and Fig. 3C). To confirm that the elevation in cyclin B1 expression was due to Notch inhibition, we overexpressed $N^{IC}1$ in SLK cells before LLNle treatment. As anticipated, LLNle treatment led to an increase in cyclin B1 expression in vector-transduced SLK cells that was partially attenuated in cells overexpressing $N^{IC}1$ (25% decrease on average; Fig. 3D). Next, we wanted to determine if the increase in cyclin B1 following LLNle treatment was dependent on new protein synthesis. Pretreatment of SLK cells with cycloheximide before Notch inhibition with LLNle prevented the increase in cyclin B1, indicating that protein synthesis was required (Fig. 3E).

The localization of cyclin B1 plays a role in its activation, and increases in nuclear cyclin B1 have been implicated in the induction of mitotic catastrophe (33, 34). Immunofluorescent staining of LLNle-treated SLK cells revealed localization of cyclin B1 to the nucleus, as determined by colocalization with DAPI-stained DNA (Fig. 4A). To verify these findings, nuclear protein extracts from LLNle-treated SLK cells were analyzed and showed a rapid accumulation of cyclin B1 that was sustained throughout the experiment (Fig. 4B).

The Western blot data demonstrating an increase in the phosphorylated form of CDK1 in LLNle-treated SLK cells (Fig. 3B) suggest that CDK1 is functionally active. Therefore, we assessed CDK1 activity by measuring CDK1-mediated phosphorylation of histone H1 in SLK cells treated with LLNle or DMSO (Fig. 5A). As compared with control-treated cells, LLNle treatment led to an increase

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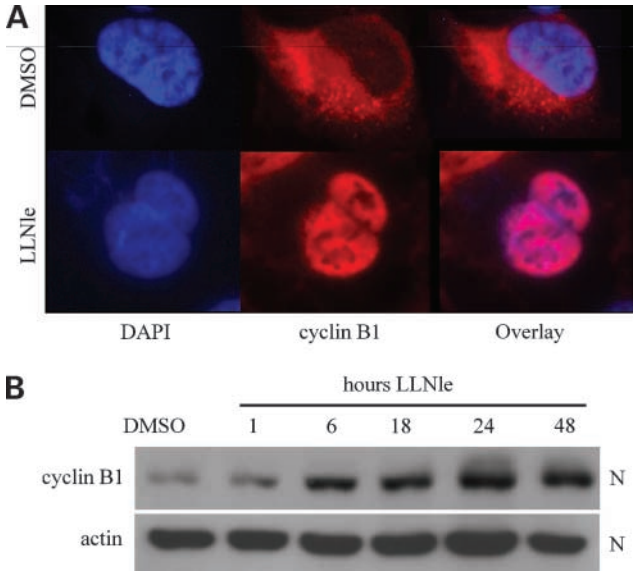

Figure 4. **A,** cyclin B1 (*red*) was localized to the cytoplasm in control-treated SLK cells, but colocalized to the nucleus with DNA (*DAPI*; *blue*) in cells treated with LLNle. Representative examples of fluorescently stained cells are shown. **B,** LLNle-treated SLK cells showed a gradual increase in nuclear (*N*) cyclin B1 compared with DMSO-treated cells (48-h time point is shown). Both show representative data from three independent experiments.

in CDK1 kinase activity ($P < 0.05$). To indirectly confirm CDK1 activity, disruption of nuclear lamin B, an event downstream of CDK1 activation, was assessed using fluorescent microscopy (35–37). In control-treated SLK cells, lamin B was localized to the nucleus in a majority of the cells with only a small percentage showing cytoplasmic localization (average, 1.6 ± 0.4%; Fig. 5B and C). However, LLNle-treated SLK cells revealed a significant increase in the number of cells with cytoplasmic lamin B (average, 15.8 ± 6%; Fig. 5B and C; $P < 0.01$).

Similar studies that were also done with the KS-IMM and KS-Y1 cells to confirm the results were consistent among KS tumor cell lines and not specific for SLK cells. As expected, there were no major differences in protein expression patterns between the cell lines with respect to Cdc25c, chk1, chk2, CDK1, and cyclin B1 with the exception that KS-IMM constitutively expressed higher levels of total CDK1 than SLK or KS-Y1 cells (data not shown). CDK1 activity was also increased in KS-IMM and KS-Y1 cells treated with LLNle (data not shown) along with a significant increase in cytoplasmic lamin B expression (KS-IMM + DMSO, 4.2 ± 0.8%; KS-IMM + LLNle, 25.4 ± 4.4%; and KS-Y1 + DMSO, 1.75 ± 0.4%; KS-Y1 + LLNle, 33.8 ± 2.4%; $P < 0.01$).

Notch-1 Regulates Expression of Cyclin B1 through NF-κB

To determine the mechanism through which Notch-1 may regulate cyclin B1 expression, the promoter of cyclin B1 was analyzed for binding sites of proteins known to be regulated by Notch signaling. A putative binding site (GGAGTTACCT) with 85% homology to the consensus NF-κB p65 binding site was found at position −1162 and led us to hypothesize that Notch-1 regulation of NF-κB may in turn increase cyclin B1 expression. To test this hypothesis, SLK cells were transfected with a NF-κB–responsive

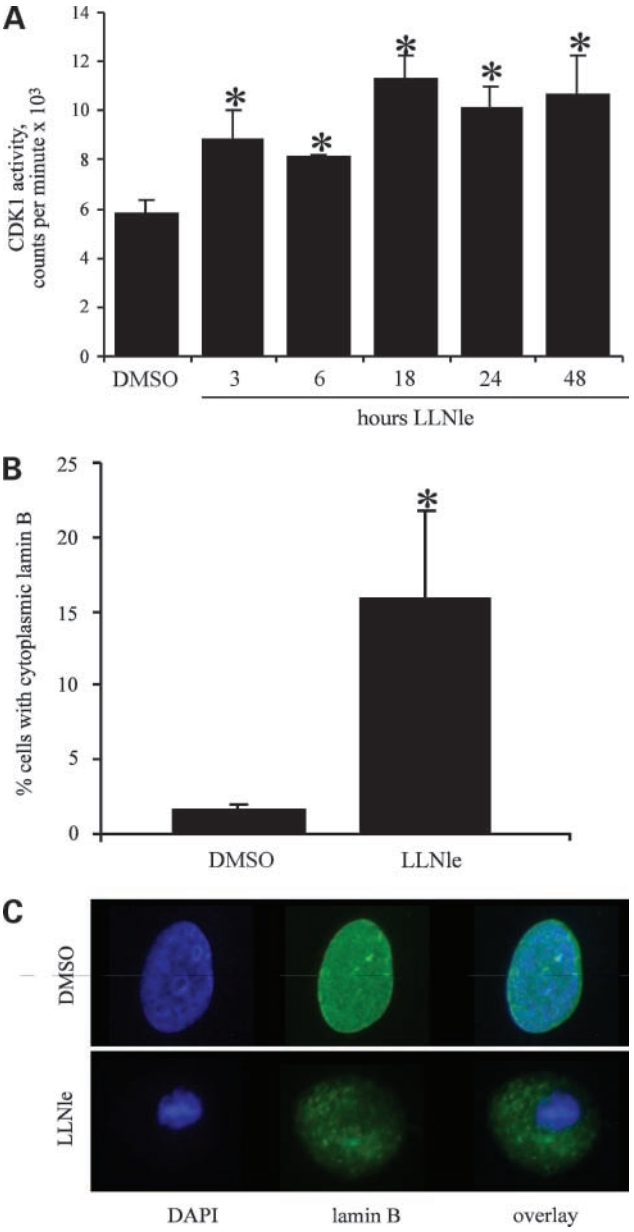

Figure 5. **A,** CDK1 activity was significantly higher in LLNle-treated SLK cells compared with DMSO treated cells (48-h time point is shown). Data are averaged counts per minute ±SD from two independent experiments. *, $P < 0.05$. **B,** lamin B (*green*) colocalized to the nucleus with DNA (*DAPI*; *blue*) in DMSO-treated SLK cells, with only a small percentage demonstrating cytoplasmic localization (1.6 ± 0.4%). In contrast, LLNle treatment for 24 h resulted in a significant increase in cells with cytoplasmic lamin B (15.75 ± 6%) Quantitation of the results is presented as a bar graph (combined data from three independent experiments) above examples of fluorescent staining (**C**). *, $P < 0.01$.

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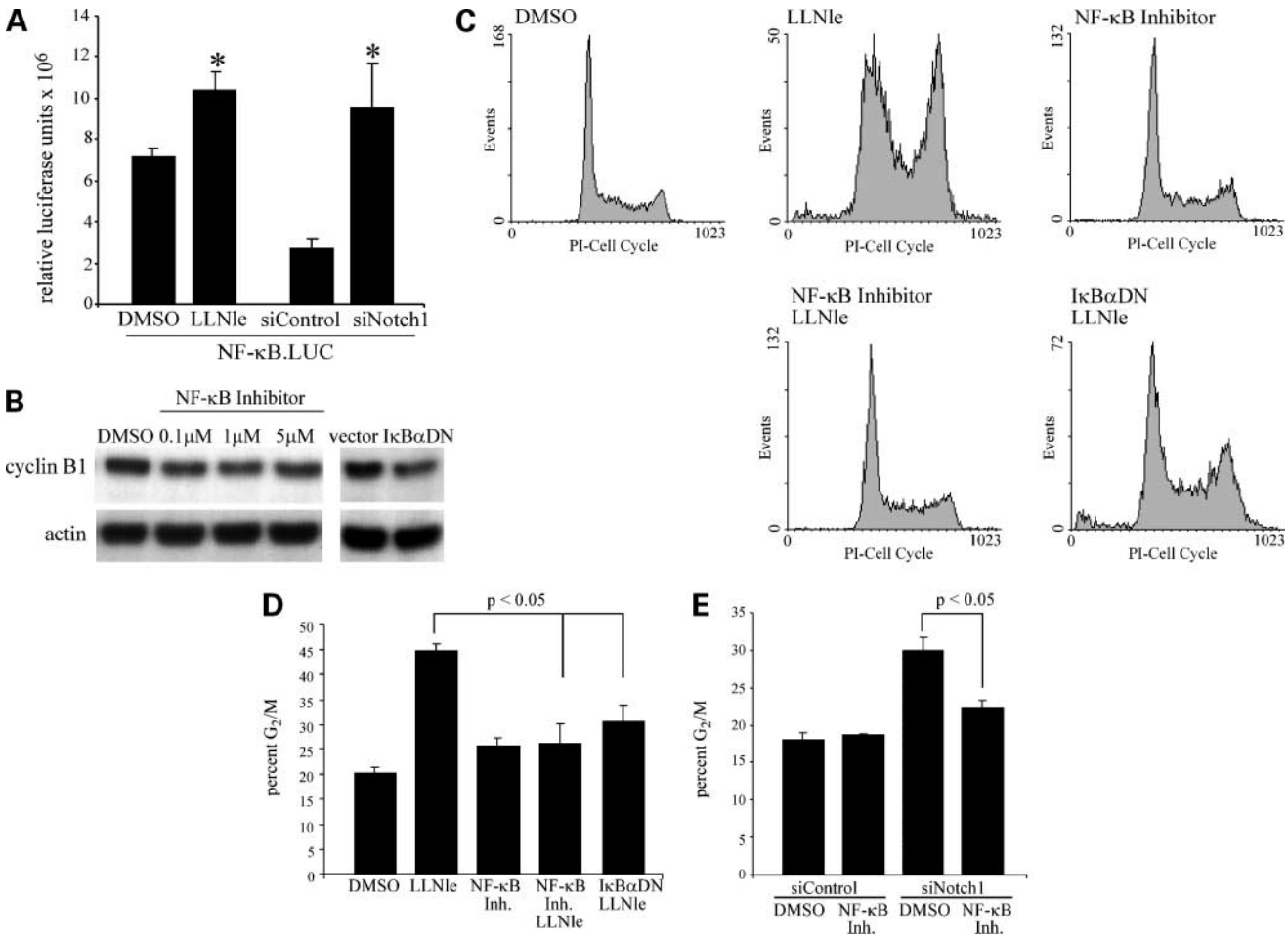

Figure 6. **A,** there was a significant increase in NF-κB–dependent luciferase reporter activity in SLK cells after LLNle treatment compared with DMSO. Similarly, inhibition of Notch activity with siNotch1 induced significant luciferase activity compared with siControl in these cells. *, $P < 0.01$. **B,** inhibition of NF-κB signaling with 6-amino-4-(4-phenoxyphenylethylamino)quinazoline resulted in a reduction in cyclin B1 protein expression (average decrease, 27.5 ± 2.0%, 37.0 ± 1.4%, and 30.0 ± 2.3% at 0.1, 1, and 5 μmol/L, respectively). Similarly, cyclin B1 was decreased, on average, by 55 ± 2.9% in SLK cells expressing a dominant negative IκBα (IκBαDN) compared with control-treated cells. **C,** DNA/PI staining revealed inhibition of NF-κB signaling with either the pharmacologic inhibitor, or the IκBαDN construct significantly reduced the number of SLK cells in G_2-M arrest following LLNle-treatment. Data from the 24-h time point is shown. **D,** graphical presentation of data in **C**. Note the significant reduction in SLK cells in G_2-M when the cells were pretreated with an NF-κB inhibitor before LLNle. **E,** pretreatment of SLK cells with an NF-κB inhibitor before blocking Notch-1 signaling with an siRNA results in attenuation of the G_2-M arrest. No effect was seen in cells treated with the NF-κB inhibitor before control siRNA treatment.

luciferase reporter and then treated with LLNle or DMSO for 24 h. A significant increase in luciferase activity was detected in LLNle-treated cells (Fig. 6A; $P < 0.01$). Similar experiments were done in SLK cells transfected with the NF-κB luciferase reporter and the Notch-1 siRNA. Again, there was a significant increase in NF-κB–mediated luciferase activity in cells where Notch activity was blocked (Fig. 6A; $P < 0.01$).

To determine if cyclin B1 expression could be regulated by NF-κB activity, SLK cells were treated with 6-amino-4-(4-phenoxyphenylethylamino)quinazoline, a cell-permeable inhibitor of NF-κB transcriptional activation, or transduced with a previously characterized retroviral vector encoding for a dominant negative form of IκBα (IκBαDN; ref. 24). Initial studies showed that both inhibitors significantly blocked NF-κB–mediated luciferase activity under our conditions (data not shown). The results showed a reproducible decrease in cyclin B1 expression with both the pharmacologic inhibitor (average, 37.0 ± 1.4% reduction for 1 μmol/L concentration) and the IκBαDN retrovirus (average, 55.0 ± 2.9% decrease) compared with control-treated cells (Fig. 6B).

To determine if NF-κB activation is involved in the induction of the G_2-M cell cycle arrest seen following Notch inhibition, SLK cells were either transduced with the IκBαDN retrovirus or pretreated for 1 h with 6-amino-4-(4-phenoxyphenylethylamino)quinazoline before addition of LLNle. The results showed that NF-κB inhibition prevents the G_2-M cell cycle arrest induced by Notch inhibition (percentage G_2-M: LLNle: 44.8 ± 1.5%, NF-κB inhibitor and LLNle; 25.7 ± 1.7%, IκBαDN and LLNle: 30.7 ± 3%; Fig. 6C and D, $P < 0.05$). The results were further

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confirmed using Notch-1 siRNA in the presence and absence of the NF-κB inhibitor. Again, there was a significant reduction in the number of cells arrested in G_2-M when NF-κB activity was blocked in addition to Notch signaling (percentage G_2-M: DMSO and siNotch1, 28.85 ± 1.9%; NF-κB inhibitor and siNotch1, 22 ± 1.3%; Fig. 6E; $P < 0.05$).

Discussion

Notch signaling has been implicated in the pathogenesis of various human malignancies, including breast, lung, hematologic, and skin cancers, and it has been suggested that inhibition of this signaling may be of therapeutic value in these tumors (38, 39). Because most tumor cells express multiple Notch receptors, inhibition of γ-secretase (which blocks cleavage and activation of all Notch receptors) has been seen as a clinically viable option (40). Recently, we showed that Notch signaling is constitutively active in KS tumor cells (17). These studies showed that Notch inhibition in actively growing KS cell tumors in nude mice resulted in tumor regression and/or growth arrest, which further supports the potential use of Notch inhibitors as therapeutic agents in cancer.

Interestingly, we noted that Notch inhibition resulted in a prolonged G_2-M cell cycle arrest before induction of apoptosis. This led us to hypothesize that Notch inhibition in KS tumor cells results in mitotic catastrophe, which eventually resolves in cell death via apoptosis. Mitotic catastrophe has been described as a type of cell death resulting from aberrant mitosis, ending in the formation of cells with multiple nuclei and decondensed chromatin (27, 28). Altered expression of several G_2-M checkpoint regulators has been observed in mitotic catastrophe, including cyclin B1 and CDK1 (33, 41). For example, inappropriate increases in nuclear cyclin B1 have been found in mitotic catastrophe in colorectal adenocarcinoma, nasopharyngeal carcinoma, and colon cancer (33, 34). It has been suggested that elevated levels of cyclin B1 or premature activation and nuclear entry of CDK1/cyclin B1 may be sufficient to induce mitosis before the completion of DNA replication, thus causing cell death during mitosis (42–44). Furthermore, increases in CDK1 activity were shown in mitotic catastrophe caused by low dose doxorubicin treatment in hepatoma cell lines (45).

Here, we show that Notch inhibition in KS tumor cells by pharmacologic or genetic approaches results in mitotic catastrophe as characterized by micronucleation and an increased mitotic index. The sustained increase in cyclin B1 and increased CDK1 activity in these cells is consistent with other studies and may contribute mechanistically to mitotic catastrophe. To our knowledge, this is the first report indicating Notch signaling can up-regulate cyclin B1 expression. The Notch pathway has, however, been previously linked to other components of the cell cycle. In *Drosophila*, removal of Notch in follicle cells, or the Notch-ligand Delta in the germ line, leads to a failure of the mitotic/endocycle transition (46, 47). In the follicle cells, down-regulation of the mitotic activator string/Cdc25 and up-regulation of fzr at the mitotic/endocyctic transition are Notch dependent, as is the expression of the homeodomain protein Cut, although the effects of Notch signaling in the follicle cells has been suggested to affect the G_1-S and M-G_1 cell cycle transitions as well (48–50). In mammals, Notch-1 has been shown to influence S phase entry by inducing transcription of the S-phase kinase-associated protein 2 (SKP2), and the F-box subunit of the ubiquitin-ligase complex SCF^{SKP2}, thus enhancing degradation of cyclin-dependent kinase inhibitors ($p27^{Kip1}$ and $p21^{Cip1/WAF1}$) and promoting entry into the S phase (51). Overexpression of $N^{IC}1$ in hepatocellular carcinoma inhibits cell proliferation and leads to a decrease in cyclins A and D1, and a G_1-G_0 cell cycle arrest, whereas in RKE and 293T cells, it leads to an increase in cyclin D1 transcription and leads to transformation, highlighting the differential outcomes of Notch signaling depending on cell type (52, 53).

Our results also indicate that Notch signaling regulates cyclin B1 expression, at least in part, through altered NF-κB activity. Previous studies have shown that Notch can either increase or inhibit NF-κB activity depending on factors such as dose, timing, and cellular context (11–13). However, studies have not previously linked Notch signaling or NF-κB activation to mitotic catastrophe. In fact, a recent study by Eom et al. (37) reported that mitotic catastrophe induced by low-dose doxorubicin in hepatoma cell lines was not associated with the activation of NF-κB signaling. The observed differences are likely related to the variation in the experimental conditions and the cell types analyzed. Ongoing studies will further dissect the link between Notch and NF-κB signaling with mitotic catastrophe, including the identification of the involved NF-κB subunits and to determine if Notch alters IKK or IκB expression or function.

In our previous report, histologic analysis was shown and revealed numerous tumor cells with enlarged nuclei in LLNle-treated tumors that were not found in control-treated tumors (17). These enlarged nuclei are consistent with the results presented here as the tumor cells would have replicated their DNA, but not completed mitosis. Studies are currently in progress to further extend the *in vitro* findings of this report to our *in vivo* model system, including examination of cyclin B1 expression and NF-κB activity in treated tumors, as well as the examination of additional tumor models to determine if Notch inhibition induces mitotic catastrophe in other systems.

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Notch inhibition in Kaposi's sarcoma tumor cells leads to mitotic catastrophe through nuclear factor- κB signaling

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