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c-Abl kinase regulates curcumin-induced cell death through activation of JNK

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Running Title: c-***Abl is required for curcumin-induced apoptosis***

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Abbreviations; IR, ionizing radiation, shRNA, short-inhibitor of RNA, NAC, N-acetyl cysteine, ROS, reactive oxygen species, PARP, poly (ADP-ribose) polymerase, CurC, Curcumin.

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Abstract

Curcumin, a natural phenolic compound found in turmeric (*curcuma longa*) exhibits anti-cancer properties, attributed to its anti-proliferative and apoptosis-inducing activity. The ubiquitously expressed non-receptor tyrosine kinase, c-Abl regulates stress responses induced by oxidative agents such as ionizing radiation and H_2O_2. Here we show that c-Abl is an important component of the cell death response activated by curcumin and that Abl mediates this response, in part, through activation of JNK. Consequently, inhibition of Abl by STI571 (Imanitib, Gleevac) treatment or downregulation of Abl expression through Abl specific ShRNA diminished cell death induction and JNK activation. Highlighting the inter-dependent nature of the Abl and JNK signaling in the curcumin-induced cell death response, a JNK inhibitor (SP600125) caused very little cell death inhibition in STI571-pretreated cells and in Abl ShRNA expressing cells. Moreover, treatment with Abl and JNK inhibitor alone or together caused similar levels of cell death inhibition. Although p53 induction in response to curcumin treatment is dependent on Abl, we found that Abl→p53 signaling is dispensable for curcumin-induced cell death. Taken together, the results demonstrate the differential roles played by Abl→p53 and Abl→JNK signaling events in modulating the cell death response to curcumin.

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Introduction

Curcumin (diferuloylmethane), the major yellow pigment extracted from turmeric (*Curcuma longa*) and commonly used as flavoring food additive exhibits potent antioxidant, anti-inflammatory and anticancer properties (Sharma et al, 2005). Curcumin's anti-cancer property is attributed mainly to its ability to activate cell death in a wide variety of tumor cells with low toxicity to normal cells (Chauhan, 2002). Thus, in cells derived from human prostate, lung, skin, large intestine, bone and leukemia, curcumin triggered a dose- and time-dependent caspase-mediated cell death response (Sharma et al., 2005). Similarly, immortalized NIH 3T3, mouse sarcoma S180, human colon cancer cell HT-29, human kidney cancer cell 293, and human hepatocellular carcinoma Hep G2 cells underwent apoptosis following treatment with curcumin (Jiang et al., 1996; Song et al., 2005). In many other normal and primary cells curcumin is either inactive or inhibits proliferation. For example, in primary untransformed mouse embryonic fibroblast C3H 10T1/2, rat embryonic fibroblast, and human foreskin fibroblasts, curcumin failed to initiate cell death although curcumin inhibited proliferation of both normal and transformed cells in a non-selective manner (Sharma et al., 2005; Gautam et al., 1998).

The mechanism behind curcumin's selective toxicity against tumor cells remains unclear but is thought to involve multiple downstream targets including NF-kB and NF-kB-regulated genes, COX-2, AKT, FOXO and GSK3 (Aggarwal et al., 2005; Goel et al., 2001; Hussain et al., 2006). Curcumin's apoptotic effect on

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colon cancer cell growth has been demonstrated to be due to stimulation of the trans-activating activity of peroxisome proliferator-activated receptor γ (PPAR-γ) (Chen and Xu, 2005). In primary effusion lymphoma (PEL) cells, curcumin suppressed JAK-1 and STAT-3 consequently triggering apoptosis (Uddin et al., 2005). Curcumin treatment also induced JNK-dependent sustained phosphorylation of c-jun and stimulation of AP-1 transcriptional activity, and treatment with the JNK-specific inhibitor SP600125 blocked c-jun phosphorylation and apoptosis (Collett and Campbell, 2004; Moussavi, et al 2006). It has been suggested that curcumin-mediated inhibition of NF-KB results in generation of reactive oxygen species (ROS) that ultimately triggers activation of JNK and apoptosis (Bhaumik et al., 1999). A recent study showed curcumin covalently binds and inhibits Thioredoxin reductase (TrxR) consequently leading to increased NADPH oxidase activity and production of reactive oxygen species (ROS) (Fang et al., 2005).

Curcumin-induced cell death is mediated through its effects on p53 although the requirement appears to be cell type-specific. For example, in human basal cell carcinoma curcumin induces a p53-dependent apoptotic response (Jee et al., 1998). In myeloid leukemic cells curcumin induced ubiquitin-independent degradation of WT p53 and inhibited p53-induced apoptosis *via* inhibition of NAD(P)H:quinone oxidoreductase 1 (NQO1) activity (Tsvetkov et al., 2005). Interestingly, in human melanoma cell lines curcumin induced apoptosis through a p53-independent but Fas receptor/caspase-8 dependent pathway

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(Bush et al., 2001). Similarly, in colorectal cancer cells curcumin induced a p53 independent cell death response (Jaiswal et al., 2002).

C-Abl is a Src-related non-receptor tyrosine kinase that is ubiquitously expressed and localized both in the nucleus and cytoplasm (Smith et al 2002). C-Abl contains a large C-terminal domain that is critical for its function as mice expressing a C-terminal truncated Abl displayed neonatal lethality similar to Abl-nullizygous mice (Tybulewicz et al., 1991; Schwartzberg et al., 1991). Several functional domains have been identified on to this C-terminus, including a motif that interacts with the C-terminal domain (CTD) of RNA polymerase II, a substrate of Abl, nuclear localization signal (NLS) and nuclear export signals (NES) (Smith et al., 2002). The catalytic function of Abl is normally tightly regulated but is up-regulated during S phase and following genotoxic stress (Baskaran et al., 1997; Kharbanda, et al., 1998). In response to IR treatment, the kinase activity is stimulated by ATM-mediated phosphorylation of Abl on S465 (Baskaran et al., 1997). Activated Abl, in turn, regulates the cellular responses to oxidative damage by triggering cytochrome *c* release and apoptosis (Kumar et al., 2004; Sun et al., 2000). Following genotoxic stress, c-Abl associates with and activates MEK kinase 1 (MEKK-1), an upstream effector of the SEK1→ SAPK pathway (Kharbanda et al., 2000). C-Abl stabilizes p53 through tyrosine phosphorylation of MDM-2 and potentiates p53 function through direct binding (Levav-Cohen et al., 2005).

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In this report we show that c-Abl tyrosine kinase function is important for the cell death response induced by curcumin. Further, we find that Abl mediates this response through activation of JNK. Although p53 induction is dependent on Abl function it is dispensable for curcumin-induced cell death. The results identify an Abl-to-JNK signaling event as a critical regulator of curcumin-induced apoptosis.

Materials and Methods

Materials: Curcumin was obtained from Sigma-Aldrich, USA, and dissolved in DMSO. STI571 (Gleevac) was a gift from Novartis. JNK inhibitor (SP600125) was purchased from Calbiochem, USA. MitoTracker Green FM and DCFH-DA were obtained from Molecular probes (Carlsbad, CA). Antibodies against phospho-JNK (Thr183/Tyr185), phospho-Abl (Y245) and cleaved PARP were obtained from Cell signaling (Danvers, MA). Antibodies against JNK, p53 (DO-1), c-Abl (K-12), phospho-c-jun (S63/S73) and tubulin were from Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). HRP-conjugated secondary antibodies were purchased from Novus Biologicals (Littleton, CO).

Cell lines: HeLa and HCT116 were obtained from American Tissue Type Collection (ATCC, Manassa, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 3% penicillin/streptomycin. HCT116 *p53*$^{+/+}$, and HCT116 *p53*$^{-/-}$, respectively, were generous gifts from Dr. Bert

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Vogelstein (Johns Hopkins University). The cells were cultured in McCoy's medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were grown at 37° C in a humidified 5% CO2 incubator. Wild type and c-*abl*$^{+/+}$ mouse fibroblasts (3T3 cells) were grown and maintained as described (Shangary et al., 2000).

Immunoprecipitation and Immunoblotting: Mock (DMSO)- and curcumin-treated cells were washed with ice-cold PBS and lysed in cold 1X lysis buffer containing 10 mM Tris Hcl, pH 8, 240 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM PMSF, 1% Triton X-100, 1 mM sodium vanadate, and 1 μg/ml of leupeptin, pepstatin, aprotinin by incubation at 4 C for 20 mins. Lysates were cleared by centrifugation and ~240 μg of the supernatants were incubated overnight at 4^0 C with 1-3 μg of anti-p53, or anti-Abl antibodies (K-12). Protein A/B agarose (Santa Cruz Biotechnology) was subsequently added to each sample, and the incubation was continued for an additional 3 h at 4° C with gentle shaking. The immunoprecipitates were used for immunoblotting or for kinase assays. For immunoblotting, proteins were resolved by 4-12% gradient SDS-PAGE, transferred to PVDF membrane and probed with respective antibodies. When necessary the membrane was stripped by incubation at 40 C for 30 min. in a closed container containing 65 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol (BME) and 2% SDS and then re-probed with appropriate antibodies.

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Kinase Assay: Kinase reactions were performed as described (Shangary et al., 2000). Briefly, c-Abl immune-complexes were washed and resuspended in kinase buffer containing 25 mM HEPES, pH 7.5, 40 mM KCl, 0.5 mM EDTA, 5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Kinase reactions were carried out in a final reaction volume of 35 μl. One µg of the indicated purified recombinant fusion protein (GST-CTD) was added to the immune complex along with 5 μM cold ATP, 30 μCi of [γ-^{32}P]ATP (7,000 Ci/mmol, ICN). The kinase reaction was carried out at room temperature for 30 min and terminated by adding an equal volume of 3X SDS sample buffer followed by heat inactivation. The reaction products were resolved on 8% acrylamide gels and electrophoretically transferred onto Immobilon-P membrane. The membrane was subjected to autoradiography followed by immunoblotting with anti-Abl antibody (K-12, Santa Cruz). Quantitation was done using a Bio-Rad laser densitometer (GS-710 Calibrated imaging densitometer).

Preparation of Nuclear and Cytoplasmic extracts: Cells were gently homogenized in ice-cold hypotonic saline containing 10 mM HEPES, pH 7.9, 10 mM KCl and 0.1 mM EDTA. Nuclei were isolated by centrifugation at 15 K for 3 min. The supernatant was normalized for equal protein concentration and used for immunoblotting with phospho-specific (Y245) Abl antibody or for immunoprecipitation (IP) of Abl and in vitro kinase assay. Nuclei were lysed in 1X lysis buffer and clarified by centrifugation at 10,000 RPM for 10 min at 4° C

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and used for IP kinase reaction or immunoblotting. Lysates were adjusted for equal protein concentration by a Bio-Rad dye binding assay.

Microscopy: Cells were incubated with 240 nM MitoTracker Green FM (Molecular Probes) dye in pre-warmed medium for 30 min at 37° C for labeling. Cells were washed three times with HBBS containing 10 mM Hepes, 2 mM CaCl2, and 4 mg/ml BSA. Visualization and analysis was performed using a Nikon fluorescence microscope (TE S2000) equipped with CCD camera.

ShRNA: 5-GGATCAACACTGCTTCTGAT-3 (Abl) and 5–GAAGCAAGCGTGACAACAAT – 3 (JNK) was hybridized to their complementary oligonucleotides and cloned into pSIREN-RETRO-Q from Clontech into the BamH1 – EcoR1. An additional Xba1 site was inserted to confirm the presence of the insert. Following lipofectamine-mediated transfection into 293T cells, the supernatant containing the viral DNA was collected, filtered and used to infect HeLa cells. Cells were selected by incubation with Puromycin (1 μg/ml) for 4 days and downregulation of Abl or JNK expression was confirmed by immunoblotting.

Inhibitors: Cells were pretreated for 16 h with STI571 (1-5 μM) or 2 h with SP600125 (2 μM) and then exposed to 5 or 40 μM curcumin. Cells were collected at the indicated time-points and used for lysate preparation or flow cytometry.

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Flow cytometry: Mock (DMSO)- and Curcumin-treated cells were washed twice with 1X PBS and fixed in cold ethanol (70%). Samples were kept at room temperature for 30 min and stored at 4° C. Prior to flow cytometry, cells were incubated in PBS containing 1 mg/ml RNase A, 40 μg/ml propidium iodide (PI, Sigma) for 30 min in dark at 37° C (Beckman Coulter). In each sample more than 3 x 10^4 cells were counted and the cells with a lower DNA content than those of the G0/G1 phase were referred to as apoptotic cells and DNA histograms were analyzed using ModFit (Verity Software, Inc.) software.

ROS determination: ROS was measured by a dichlorofluorescein (DCF) assay described by Boissy, et al., 1989. Briefly, cells were grown to ~80% confluency and incubated with 2 μM DCFH-DA. After 1 h incubation with the dye, cells were washed free of Dye and resuspended in fresh media. Cells were treated with DMSO or curcumin (40 μM) for 2 h. NAC was added after the cells had been labeled for 1 h with DCHF-DA dye. The cells were then trypsinized, washed with PBS and fluorescence measured using flow cytometry. Cells were also treated with STI571 for 16h and then used for detection of ROS following curcumin treatment.

Statistical Analysis: All flow cytometry experiments were performed in triplicate. The paired Student's *t* test was used to determine the statistical significance. StatView software was used (Abacus Concepts, Berkeley, CA). A *P* value of less

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than 0.05 represented a statistically significant difference between the values of two group means.

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Results

Effect of STI571 on curcumin-induced cell death

The c-Abl kinase critically regulates cell death responses to oxidative stress induced by H_2O_2 and ionizing radiation (Kharbanda et al., 1998; Sun, Kumar et al., 2003). To determine Abl's involvement in curcumin-induced cell death we cultured HeLa cells in the presence of 5 μM of STI571, a concentration known to completely inhibit c-Abl kinase activity (Shangary et al., 2000 and Fig. 4*C*) and then assessed the cell death response to curcumin (40 μM) by examining hypodiploid DNA content by flow cytometry and by PARP cleavage. Consistent with a number of published reports, (Moussavi et al., 2006; Choudhuri et al., 2002; Radhakrishna et al., 2004), curcumin treatment resulted in marked accumulation of hypodiploid cells (Sub-G1=~31.7%) indicating robust cell death in the treated population (Fig. 1*A, upper panels*). However, cells pretreated with STI571 showed reduced cell death with ~11.7% Sub-G1 cells in response to curcumin treatment. Since STI571 alone caused very little cell death (~4.3% Sub-G1) the result demonstrate an inhibitory effect of STI571 on curcumin-induced cell death. A parallel study performed with a pharmacologically-relevant concentration of curcumin (5 μM) (Shoba et al., 1998; Lao et al., 2006) revealed noticeable cell death (Sub-G1=~18.6%) at 96 h post-treatment (*lower panels*). Again, in the presence of 1 μM of STI571 apoptosis was significantly reduced (~7.7% Sub-G1). Immunoblotting of lysates with anti-PARP antibody showed at each of the concentrations tested the presence of STI571 reduced PARP

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cleavage confirming the reduced cell death induction (Fig. 1*C*). Results obtained from three independent experiments showed that STI571 caused ~2.5- and 2.4-fold reductions in the apoptotic response induced by 40 μM and 5 μM of curcumin, respectively (Fig. 1*B*). Taken together, the results clearly demonstrate an inhibitory effect of STI571 on curcumin-induced apoptosis.

c-Abl$^{-/-}$ 3T3 cells are resistant to curcumin-induced cell death

As STI571 targets a number of kinases including PDGF, c-kit and Arg kinases (Krystal et al., 2004), we investigated the specific involvement of Abl in the curcumin-induced cell death by comparing the apoptotic induction in NIH 3T3 cells derived from wild type (WT) and Abl-nullizygous (*Abl$^{-/-}$*) mice. A previous report (Jiang et al.,1996) showed that curcumin treatment resulted in activation of an apoptotic response in immortalized NIH3T3 cells but not in primary culture of mouse embryonic fibroblasts C3H 10T1/2. In agreement with these results, a 24 h treatment of NIH 3T3 cells with 40 μM curcumin resulted in a ~35.7% Sub-G1 DNA containing population. However, under the same conditions *Abl$^{-/-}$* 3T3 cells showed ~17.2% Sub-G1 cells (Fig. 2*A*). Next, we exposed *Abl$^{+/+}$* and *Abl$^{-/-}$* 3T3 cells to varying concentrations of curcumin (40, 100 and 200 μM) and monitored cell death by flow cytometry. The results showed that at each of the concentrations tested, Abl-deficient mouse fibroblasts exhibited reduced apoptosis (as determined by % Sub-G1 events) compared to 3T3 cells expressing wild-type Abl (Fig. 2*B*). Similar results were obtained with 5 μM curcumin where noticeable differences in cell death induction (Sub-G1) between

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these two cell lines were observed beginning at 72 h after treatment with maximal difference observed at 96 h (~23.1% and ~10% in regular and $Abl^{-/-}$ 3T3 cells) (Fig. 2*C*).

SiRNA-mediated inhibition of Abl expression suppressed curcumin-induced cell death

To demonstrate the specific involvement of Abl in curcumin-induced cell death in human cells we tested HeLa cells with stably knocked-down c-Abl protein level. After confirming efficient knock down of Abl expression by immunoblotting of the lysates with anti-Abl (K-12) antibody (Fig. 3*A*, *top panel [0 h]*), these cells along with control ShLuc cells were exposed to curcumin (40 μM) and cell death was assessed by immunoblotting the lysate with anti-caspase-3 antibody. At 24 and 48 h post-treatment, high levels of cleaved caspase-3 was observed in ShLuc cells, indicative of robust cell death induction. In ShAbl cells very little caspase-3 cleavage was observed. Additionally, curcumin-treated ShAbl cells showed decreased PARP cleavage. When cells were stained with mitochondria-specific Mitotracker Green FM (Molecular Probes) dye we observed that, compared to mock- treatment, ShLuc cells showed significantly reduced staining, presumably due to mitochondrial membrane collapse following curcumin treatment (Fig. 3*B*). Curcumin-treated ShAbl cells showed intact labeling in the vast majority of cells, consistent with attenuated cell death induction. To determine the requirement of Abl on cell death activated by pharmacologically relevant doses of curcumin, ShLuc and ShAbl cells were exposed to 5 μM

curcumin and cell death was monitored at different time points by flow cytometry. Similar to the results obtained in 3T3 cells, differences in cell death induction was observed at 72 h and 96 h after treatment (Fig. 3*C*). At 96 h cell death (Sub-G1) was ~23.7% in ShLuc and ~11.1% in ShAbl cells. Together, the results clearly demonstrate that the function of Abl kinase is important for cell death induction by curcumin.

Curcumin activates c-Abl kinase through generation of ROS

Cells expressing kinase-defective Abl display defective apoptotic response to genotoxins, indicating that the kinase function of Abl is essential for the response (Kharbanda et al., 1998). Further, in response to treatment with IR and H_2O_2, the catalytic activity of c-Abl is upregulated several fold (Baskaran et al., 1997; kharbanda et al 1998; Sun, et al., 2000). Thus, we examined if curcumin stimulated Abl kinase activity. Activation of Abl was measured by immunoprecipitation (IP) of Abl followed by *in vitro* phosphorylation of GST-CTD (RNAP II-C-terminal domain) in the presence of ^{32}P-γATP (Baskaran et al., 1997). A time-course analysis using whole lysate preparations indicated that a 4 h treatment with 40 μM curcumin resulted in optimal activation of Abl (*data not shown*). Using this time point, we next assessed whether curcumin activated nuclear and/or cytoplasmic Abl. As shown in Fig. 4A, Abl immunoprecipitated from nuclear extracts prepared from curcumin exposed cells revealed ~3- fold increase in activity as measured by the ^{32}P-GST-CTD/Abl ratio (Fig. 4*A*, compare lane 1 to 2). No detectable Abl activation was observed in cytoplasmic extracts

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(compare lane 3 to 4). Activation of nuclear Abl by curcumin was confirmed by immunoblotting the nuclear extract with phosphospecific Abl (Y245) antibody which showed increased reactivity to the antibody (Fig. 4*B* and *C*). As expected, treatment with STI571 blocked Abl activation/phosphorylation by curcumin. To gain insight into the mechanism of Abl activation we measured pAbl in the presence of N-acetyl cysteine (NAC). NAC alone caused no changes in Abl (Y245) phosphorylation. However, Curcumin+NAC treated cells showed attenuated Y245 phosphorylation of Abl (Fig. 4*B* and *C*). Corroborating well with this result, curcumin treatment resulted in elevated ROS and addition of NAC suppressed curcumin-induced ROS formation as measured by DCF fluorescence using flow cytometry (Fig. 4*D, compare upper and middle panels*). STI571 had no effect on curcumin-generated ROS (*bottom panel*). Taken together, the data strongly suggests an involvement of ROS in c-Abl activation by curcumin.

c-Abl is required for p53 induction and JNK activation by Curcumin

Recent work demonstrated a role for JNK in curcumin-induced apoptosis (Moussavi et al., 2006; Collett and Campbell, 2004). Also, in certain cell types, curcumin activated p53-dependent apoptosis involving activation of caspase-8 and caspase-3 (Bush et al., 2001). Given that Abl is linked to p53 induction and JNK activation triggered by genotoxic stress (Kharbanda et al., 1998), we examined the Abl dependency of these signaling events during curcumin treatment. ShLuc and ShAbl cells were exposed to curcumin (40 μM) and p53 induction and JNK activation were assessed by western blotting against

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phospho- and non-phospho-specific antibodies. As shown in Fig. 5*A*, p53 upregulation by curcumin was observed in ShLuc cells at 4, 8 and 12 h. ShAbl cells showed very little increase in p53. Anti-tubulin immunoblotting showed comparable levels of protein in both the cell populations indicating the Abl-dependent nature of p53 induction. Similar results were obtained in HeLa cells pretreated with STI571 where the Abl kinase inhibitor blocked curcumin-induced p53 stabilization (Fig. 5*B*). Determination of fold-increase in p53 levels (at 12 h) by densitometry showed ~2-3 fold decreased p53 induction in Abl inhibited cells in response to curcumin treatment (Fig. 5*C*). When the samples were analyzed for JNK activation by immunoblotting with phospho-specific JNK (Thr183/Tyr185) antibody, we observed a biphasic activation of JNK1 and 2 that peaked at 4 h and 12 h in curcumin treated ShLuc cells (Fig. 5A, *middle panel*)**.** Interestingly, ShAbl cells displayed dually phosphorylated JNK at 4 h but not 12 h after treatment (Fig. 5A). Immunoblotting of the lysate with phospho-Jun (S63/S73) antibody further confirmed the deficient activation of JNK at 12 h in Abl-knocked down cells (Fig. 5A). A similar result was observed in STI571+curcumin treated cells wherein intact JNK activation and Jun phosphorylation was observed only at the early (4 h) time point but not at 12 h after treatment (Fig. 5B). These studies indicate an upstream role for Abl in JNK activation in response to curcumin treatment especially evident at the 12 h time point.

Since HeLa cells express E6 and curcumin-induced p53 induction has been argued to be due to E6 inhibition (Bech-Otschir et al., 2001), we examined the Abl dependence of signaling events in HCT116 cells treated with curcumin

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(Fig. 5D). HCT116 cells pretreated with 1 μM STI571 were exposed to 5 μM curcumin and p53 induction and JNK activation were assessed by immunoblotting. The results showing attenuated p53 induction and JNK activation (Fig. 5*D*) demonstrated that these signaling events are indeed Abl-mediated.

Abl→p53 signaling is dispensable for Curcumin-induced cell death

Next, we determined the p53 requirement for curcumin-induced apoptotic signaling through c-Abl and/or JNK. For this purpose we used HCT116, a poorly differentiated human colorectal adenocarcinoma cell line and isogenic HCT116 $p53^{-/-}$ cells derived by targeted disruption of the p53 alleles. Studies by Jaiswal et al., (2002) established that curcumin induced a p53-independent cell death in these cell types. At the curcumin concentration employed (20 μM) these authors observed cell cycle arrest at G2/M (67%) and a Sub-G1 population (11%) in p53-positive cells. Thus, to better examine the dispensable nature of p53 in curcumin-induced cell death we used 40 μM curcumin. Curcumin treatment (40 μM) resulted in similar levels of cell death in HCT116 and HCT116/$p53^{-/-}$ (28-34% sub-G1) with essentially no G2/M blockade (Fig. 6A). We next evaluated the effect of Abl inhibition on cell death induction in both cell types. Results showed that, irrespective of p53 status, STI571 exerted a similar ~2 fold reduction in curcumin-induced cell death (Fig. 6*B*). Immunoblotting of the lysate with phospho-JNK antibody (T183/Y185) and phospho-c-jun (S63/73) antibody showed comparable levels of JNK2 (54 kDa)

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activation in both cell populations although JNK1 (46 kDa) activation was greater in wild-type compared to p53-nullizygous cells (Fig. 6*C*). The dispensable nature of p53 in curcumin-induced cell death and JNK activation reinforce the notion that Abl→p53 signaling is not a requirement in the cell death response triggered by curcumin.

Abl→JNK signaling critically regulates cell death response to curcumin

To evaluate the requirement of Abl→JNK signaling events in mediating the apoptotic response to curcumin we assessed the combined effect of Abl and JNK inhibitors on curcumin-induced cell death and p53 induction. Consistent with a recent report (Collett and Campbell, 2004;Moussavi et al**.,** 2006), JNK inhibitor SP600125 abrogated cell death as demonstrated by ~14.1% apoptosis (Sub-G1) in the presence of inhibitor and 37.7% apoptosis in its absence (Fig 7*A*). As expected, treatment with Abl inhibitor (STI571) reduced the cell death to ~12%. Interestingly, co-treatment with both inhibitors caused only a modest further inhibition of cell death induced by curcumin (~9.6% apoptosis). Corroborating well with these results, comparably reduced PARP cleavage was observed in the presence of Abl or JNK inhibitors or both (Fig. 7*B*). The lack of significant additive interaction between these two inhibitors demonstrates the linear nature of these two signaling events in activating the cell death response to curcumin. Immunoblotting of the lysate with phospho-jun antibody confirmed inhibition of JNK by both the SP600125 and STI571. As

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expected, NAC treatment blocked cell death, p53 induction and JNK activation induced by curcumin. Inhibition of JNK, however, had no effect on p53 induction.

To further evaluate the Abl→JNK signaling involvement in curcumin-induced cell death, we next examined the effect of STI571 in cells suppressed for JNK expression by ShRNA. After confirming the efficient knock-down of JNK expression in ShJNK RNA cells by immunoblotting (Fig. 8*B*), these cells along with control luciferase knock down cells were exposed to curcumin in the presence or absence of STI571 and cell death induction was assessed. As control, cells were exposed to STI571 alone. Consistent with a requirement for JNK function, flow cytometric analysis of the mock- and curcumin-treated cells showed that ShLuc but not ShJNK cells displayed significant cell death induction. Further, treatment with STI571 decreased cell death in ShLuc cells more than 2-fold (CurC-33.1% Sub-G1 versus CurC+STI571-13.2% Sub-G1). STI571 treatment had very little effect on cell death in ShJNK cell populations (CurC-15.8% Sub-G1 versus CurC+STI571-14.2% Sub-G1) (Fig. 8*A*). Treatment with STI571 alone had the same effect as DMSO (mock) controls. Immunoblotting of the lysate showed that, when compared to controls, ShJNK cells diminished PARP cleavage in response to curcumin treatment (Fig. 8*B*). Also, STI571 treatment has very little effect on PARP cleavage. Together, these results demonstrate the linearity and the indispensability of Abl→JNK signaling events in regulating the cell death response to curcumin.

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Discussion

In this report we show that c-Abl tyrosine kinase is an important component in the cell death response activated by curcumin. Consistent with this view, inhibition of Abl kinase by STI571 treatment or suppression of Abl expression by short hairpin RNA specific for Abl attenuated the apoptotic response induced by curcumin. Additionally, we have observed that compared to wild-type, cells derived from *Abl*-nullizygous mice displayed decreased sensitivity to curcumin toxicity. These findings establish Abl's involvement in curcumin-induced apoptosis. The activation of Abl kinase activity by curcumin further supports the participation of Abl-mediated signaling event in cell death activation by curcumin.

Despite documented anti-cancer properties, the mechanism by which curcumin triggers cell death is not known. Available evidence, however, suggests a role for p53 and JNK1/2 in triggering the apoptotic response. Previous studies (Bush et al., 2001; Choudhuri et al., 2005) document a cell-type specific role for p53; however, in colorectal cancer cells curcumin triggered a JNK-dependent cell death response since inhibition of JNK1/2 by SP600125 treatment blocked cell death (Collett and Campbell, 2004; Moussavi et al., 2006). Inhibition of related MAP kinases such as p38 and Erk1 had no effect on cell death induced by curcumin (Collett and Campbell, 2004) indicating that JNK, in particular, is targeted for activation during induction of this cell death pathway. The results of our study show that although both p53 stabilization and JNK activation triggered

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by curcumin is dependent on Abl function, JNK but not p53 is critically required for the cell death response elicited by Abl. Accordingly, inhibition of Abl by STI571 which suppressed JNK activation (at 12 h) reduced cell death induction by curcumin. Moreover, the Abl inhibitor, STI571, failed to significantly impact cell death in JNK knock-down cells and JNK- inhibitor treated cells. These findings indicate interdependency between Abl and JNK in eliciting the cell death response to curcumin. Inhibition of JNK by SP600125 and ShRNA specific for JNK blocked cell death but had no effect on Abl activation by curcumin (*data not shown*). Thus, we conclude that Abl→JNK represents a linear signaling event important for the cell death response elicited by curcumin with Abl playing a more upstream role. Abl does not appear to activate JNK directly since an association between these molecules was not detected (*data not shown*). It is possible that JNK activation may occur via an indirect mechanism such as through activation of MEKK1, a known target of Abl in the genotoxin-induced stress response pathway (Kharbanda, et al., 2000). Interestingly, a recent study showed that c-Abl targets IkBα for phosphorylation and induces it to accumulate in the nucleus consequently inhibiting NF-kB transcription activity and increasing sensitivity to apoptosis (Kawai et al., 2002). Since curcumin treatment results in inactivation of NF-kB and this is linked to ROS generation and JNK activation, future studies will determine whether NF-kB inhibition is mediated by curcumin-activated Abl.

The function of p53 was found to be dispensable for curcumin-induced cell death in HeLa and HCT116 cells. Both HCT116 and HCT116/p53$^{-/-}$ cells

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displayed comparable levels of cell death. Irrespective of the status of p53, STI571 exerted a ~2 fold reduction in curcumin-induced cell death in both HCT116 and HCT116/p53$^{-/-}$ cells. In addition, inhibition of JNK which blocked cell death in HeLa cells had very little effect on p53 induction. Thus, Abl→p53 represents a parallel signaling event that is dispensable for curcumin-induced cell death.

A simplistic correlation of JNK activity with curcumin-induced cell killing is complicated by the observation that curcumin-induced JNK1 activity is increased to a greater extent in HCT116 wild-type compared to p53 null cells yet there is similar curcumin-induced cell killing in these cell lines (Fig. 6). However, JNK2 activity is similar in p53 wild-type and null cells (Fig. 6C). In addition, in HeLa cells knock down of JNK2 suggests that this isoform may be more central to cell killing (Fig. 8b). Parsing out the role(s) of JNK1 and JNK2 in curcumin-induced cell killing will be the subject of future investigations.

Curcumin activates the apoptotic pathway via a ROS-associated mechanism that converges on JNK activation (Moussavi et al., 2006; Bhaumik et al., 1999). Consequently, treatment with a ROS scavenger, NAC, blocked both JNK activation and cell death induced by curcumin (Moussavi et al., 2006). Our observation that NAC blocked curcumin-induced formation of ROS and also blocked Abl activation suggests the involvement of ROS in catalytically upregulating Abl function. The precise mechanism of Abl activation is not known but the activation of nuclear Abl suggest probable involvement of DNA damage induced by curcumin-generated ROS. Reactive oxygen species can induce DNA

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strand breaks and oxidative base and nucleotide modifications such as formation of 8-oxo-deoxyguanine (Storz, 2005). Consistent with this notion, lysates prepared from curcumin treated cells displayed p53 phosphorylation on S15 (*data not shown*), a site that is targeted by ATM kinase following IR exposure. Such observations raise the possibility that ATM may be involved in curcumin induced activation of Abl. A recent study showed that curcumin can irreversibly inhibit *Thioredoxin reductase* activity by forming covalent adducts and converting it into an NADPH oxidase resulting in ROS production (Fang et al., 2005). It would be interesting to determine if activation of Abl is impaired in cells deficient in Thioredoxin reductase, given the involvement of ROS. Since curcumin binds and inactivates a number of key cellular targets, it is possible that inactivation of Abl regulatory proteins such Abi-1 and Abi-2, implicated in Abl activation, may contribute to Abl activation by curcumin (Smith et al., 2002). However, the present study demonstrates that i) Abl activation is dependent on formation of ROS; 2) treatment with NAC suppressed formation of ROS and also blocked Abl and JNK activation and cell death. These results support activation of a ROS→Abl→JNK signaling pathway by curcumin that regulates the cell death response.

Recent studies have suggested that the duration of sustained JNK activation is a critical determinant of a cell's fate in response to stress signals (Li et al., 2005; Tang et al., 2001). Activation of JNK in response to stress signals occurs in a biphasic manner; an early time point activation that is associated with

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cell survival and another late time point activation that plays a critical role in cell death. Sustained activation may be required to induce a change in gene expression necessary for cell death induction. For example, sustained activation of JNK may disrupt negative modulation of JNK activation exerted by NFκB responsive genes such as XIAP (Tang et al., 2001). In support of this notion, Li et al, (2005) showed that in cisplatin-resistant ovarian carcinoma cells initial activation of JNK was intact but these cells failed to display prolonged JNK activation, correlating well with reduced cell death. Further, transfection of wt JNK restored prolonged JNK activation and sensitized cells to CDDP-induced cell death (Li et al., 2005). Similarly, Campell et al., (2004) observed that curcumin treatment resulted in biphasic JNK activation. Results of our study also showed biphasic JNK activation. However, while the function of Abl is dispensable for JNK activation at early time points, $Abl^{-/-}$ cells displayed diminished levels of activated JNK at later time-points. The attenuated cell death response observed in curcumin-treated Abl-deficient cells is consistent with deficient JNK activation at later time points that is a critical determinant of the cell death response. Further investigation is required to elucidate the critical nature of biphasic JNK activation in cell death response elicited by curcumin.

In sum, results of our study demonstrate that curcumin-generated ROS stimulates the catalytic function of c-Abl kinase. Activated Abl, in turn stimulates two parallel independent pathways; Abl$\rightarrow$p53 and Abl$\rightarrow$JNK signaling. In the cell culture models that we have tested, p53 is found to be dispensable for cell death induction whereas inhibition of Abl blocked JNK activation and cell death thus

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identifying Abl→JNK signaling as an important event in cell death induction by curcumin.

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Footnotes

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Figure Legends

Fig. 1. STI571 inhibits curcumin-induced cell death

A. HeLa cells were either mock (DMSO)-treated or exposed to curcumin (40 or 5 μM) and cell death induction at 24 and 96 h, respectively was assessed by flow cytometry following staining with propidium iodide. Cells were also pre-treated with STI571 (5 or 1 μM) for 16 h and then mock-treated or exposed to curcumin. **B.** The percentage of cell death (%Sub-G1) was determined by flow cytometry and the results of three independent experiments performed in triplicates with *S.D* is given. * denotes statistically significant reduction in the apoptotic cell population after treatment with curcumin+STI571 compared to curcumin treatment alone ($p < 0.01$). **C.** Lysates were normalized for equal protein concentrations and subjected to immunoblotting with anti-PARP and anti-tubulin antibody.

Fig. 2. C-Abl is required for apoptotic induction by curcumin.

A. Regular NIH3T3 and *Abl*$^{-/-}$ 3T3 cells were mock (DMSO)-treated or incubated with 40 μM Curcumin for 24 h and then stained with propidium iodide (PI) and subjected to flow cytometric analysis. **B.** Concentration-dependent activation of cell death in NIH and *Abl*$^{-/-}$ 3T3 cells, assessed by counting the number of Sub-G1 events 24 h after treatment. Shown are the mean +/- SD of three independent experiments. **C.** NIH3T3 and *Abl*$^{-/-}$ 3T3 cells were incubated with 5 μM curcumin for 0, 24, 48, 72 and 96 h and % Sub-G1 cells were assessed by

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flow cytometry and plotted against time (h). The experiment was repeated three times and the mean with *S.D* is given.

Fig. 3. ShRNA-mediated suppression of Abl renders cells resistant to cell death induction by curcumin.

A. HeLa cells stably expressing Luciferase or Abl ShRNA were exposed to 40 μM curcumin and collected at 0, 24 and 48 h. Lysates were normalized for equal protein concentration and then subjected to immunoblotting with anti-Abl (K-12) and anti-caspase-3 antibody. The membrane was reprobed with PARP antibody. Anti-tubulin immunoblotting shows equal protein loading. **B.** Mock (DMSO)- and curcumin (40 μM)-treated cells were fixed (at 24 h) and stained with MitoTracker Green FM (Molecular Probes) dye and visualized with a 40X objective mounted on a Nikon fluorescence microscope. Representative images are shown. **C.** ShlLuc and ShAbl cells were either mock (DMSO)- treated or exposed to (5 μM) of curcumin and %Sub-G1 cells were assessed at varying time points by flow cytometry and plotted against time (h). The experiment was repeated three times and the mean with *S.D* is given. Mock-treated cells showed <3% sub-G1 populations at 96 h (*data not shown*).

Fig. 4. Activation of c-Abl kinase by curcumin.

A. HeLa cells were either mock (DMSO)-treated or exposed to curcumin (40 μM) and 4 h later nuclear and cytoplasmic extracts were prepared as described in

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Methods. Extracts were normalized and c-Abl was immunoprecipitated using anti-Abl antibody (K-12). The immune-complex was washed and used to phosphorylate GST-CTD in the presence of ^{32}P-γATP. The reaction products were analyzed by SDS-PAGE and the proteins were transferred to Immobilon-P. The membrane was subjected to autoradiography (*top panel*) and then subjected to immunoblotting with anti-Abl antibody (*bottom panel*). Fold induction was determined by densitometric scanning of the ^{32}P-GST-CTD and Abl bands and determining their ratio. The ratio obtained with mock treatment was set to 1. **B.** Nuclear extracts prepared from mock-treated cells or cells exposed to curcumin in the presence or absence of NAC (10 mM) or STI571 (5 μM) were normalized for equal protein concentration and subjected to immunoblotting with Y-245 phospho-specific Abl antibody (top panel) or Abl antibody (bottom panel). **C.** Fold increase in Abl activity was determined by quantification of p-Abl and Abl bands by densitometric scanning and determination of their ratio. Shown are the mean +/- SD of three independent experiments. **D.** ROS production in the presence of CurC, CurC+NAC or CurC+STI571. Cells were labeled by incubation with 2 μM DCFH-DA and ROS generated by curcumin in the presence of DMSO (Mock), NAC and STI571 was assessed at 4 h by flow cytometry.

Fig. 5. p53 induction and JNK activation by curcumin are dependent on c-Abl function.

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A. ShLuc and ShAbl cells were exposed to Curcumin (40 μM) and collected at 0, 4, 8 and 12 h. Lysates were formed and then subjected to immunoblotting with anti-p53 (DO1), anti-tubulin, phospho-specific JNK (Y183/T185) and c-Jun (S63/S73) antibody. **B.** Effect of STI571 on curcumin-induced p53 stabilization and JNK activation. HeLa cells were pretreated with STI571 for 16 h and then exposed to 40 μM curcumin. p53 induction and JNK phosphorylation was assessed at 0, 4 and 12 h after treatment by immunoblotting the lysate with anti-p53, phospho-JNK and phospho-Jun antibody **C.** Fold increase in p53 levels at 12 h was assessed by quantification of p53 and tubulin bands and determining the ratio. The ratio obtained at 0 time point was set to 1. Shown are the mean +/- SD of three independent experiments. **D.** HCT116 were pretreated with 1 μM of STI571 and then exposed to 5 μM of Curcumin and p53 induction and JNK phosphorylation was assessed by immunoblotting the lysates prepared at 24 h after treatment.

Fig. 6. p53 is dispensable for curcumin-induced cell death.

A. HCT116 and HCT116/p53$^{-/-}$ cells were either mock (DMSO)- treated or exposed to 40 μM curcumin and the number of Sub-G1 events at 24 h were assessed by flow cytometry. **B.** Cells were pretreated with STI571 (5 μM) for 16 h and then mock-treated or exposed to curcumin (40 μM). Cell death was assessed at 24 h by flow cytometry. Shown are the mean +/- SD of three independent experiments performed in triplicates. * denotes statistically

significant reduction in the apoptotic cell population in curcumin+STI571 treated cells compared to curcumin treatment alone ($p < 0.05$). **C.** Cells were either Mock-treated or exposed to CurC (40 μM) for 24 h. Lysates were analyzed for JNK activation by immunoblotting with phospho-specific JNK and phospho-jun antibody.

Fig. 7. Effect of JNK and Abl inhibition on curcumin-induced p53 induction and apoptosis.

A. HeLa cells were pretreated with STI571 (5 μM for 16 h), SP600125 (0.1 μM for 2 h), STI571+SP600125 and NAC (10 mM for 2h) and then exposed to curcumin (40 μM) and apoptotic induction (%Sub-G1) was assessed at 24 h by flow cytometry. Control treatments include treatment with DMSO (mock), STI571 alone, SP600125 alone and NAC alone. The result obtained from three independent experiments with S.D is given. * denotes statistically significant reduction in the apoptotic cell population in comparison to curcumin treatment alone ($p < 0.05$). **B.** Lysates prepared after 24 h were immunoblotted against anti-PARP antibody. Lysates formed at 12 h were used for immunoblotting with phospho-jun and anti-p53 antibody and anti-tubulin antibody.

Fig. 8. Effect of Abl inhibition on curcumin-induced apoptosis in ShJNK cells.

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A. ShLuc and ShJNK cells were pretreated with STI571 (5 μM) for 16 h and then mock (DMSO)-treated or exposed to curcumin (40 μM). Cell death induction was assessed at 24 h by determination of Sub-G1 events by flow cytometry. **B.** Lysates were analyzed for cleavage of PARP by immunoblotting with anti-PARP antibody. Lysates formed at 12 h were normalized and immunoblotted with phospho-JNK, JNK and tubulin antibody.

A

Fig. 1

Fig. 1

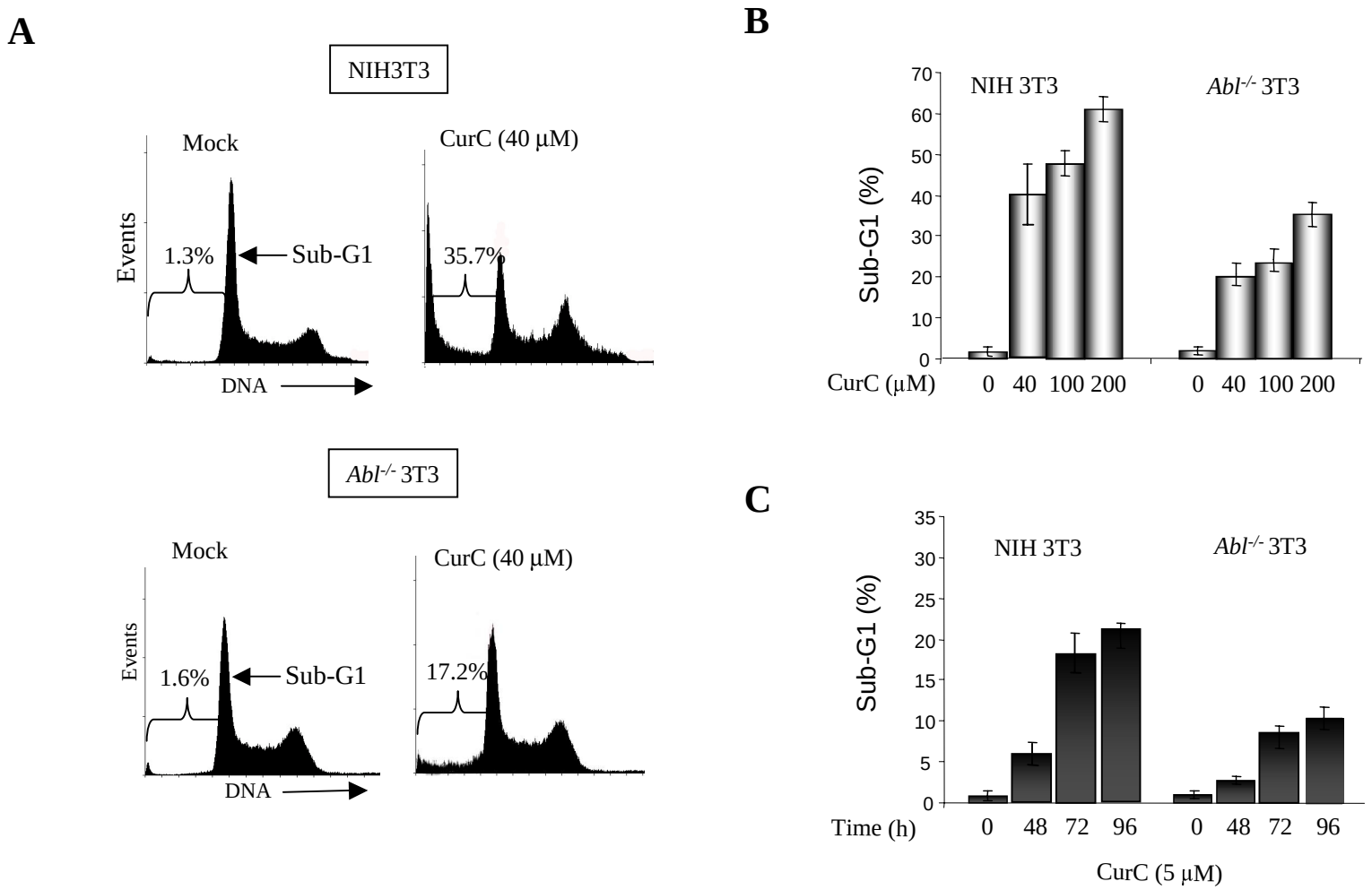


Fig. 2

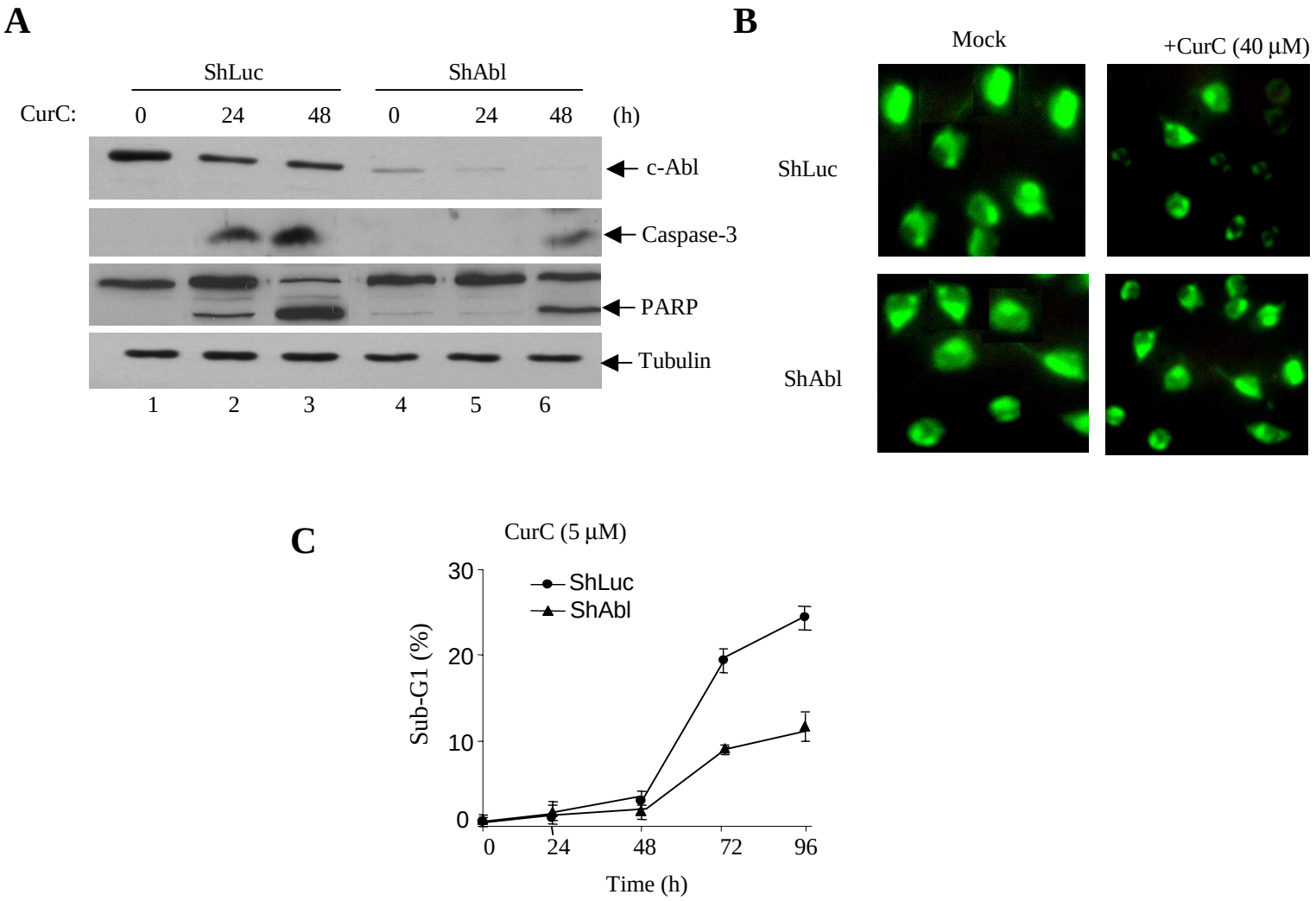
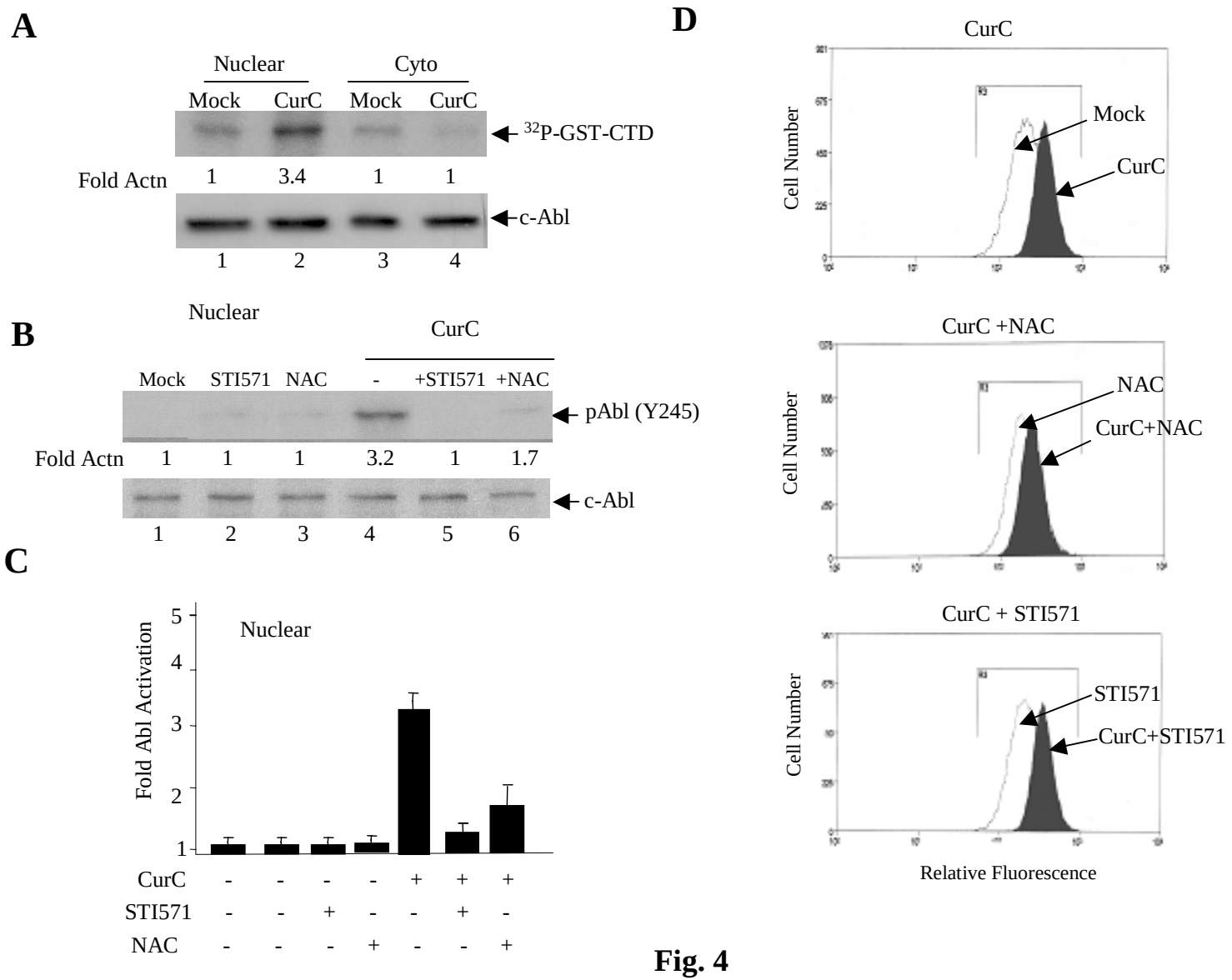


Fig. 3



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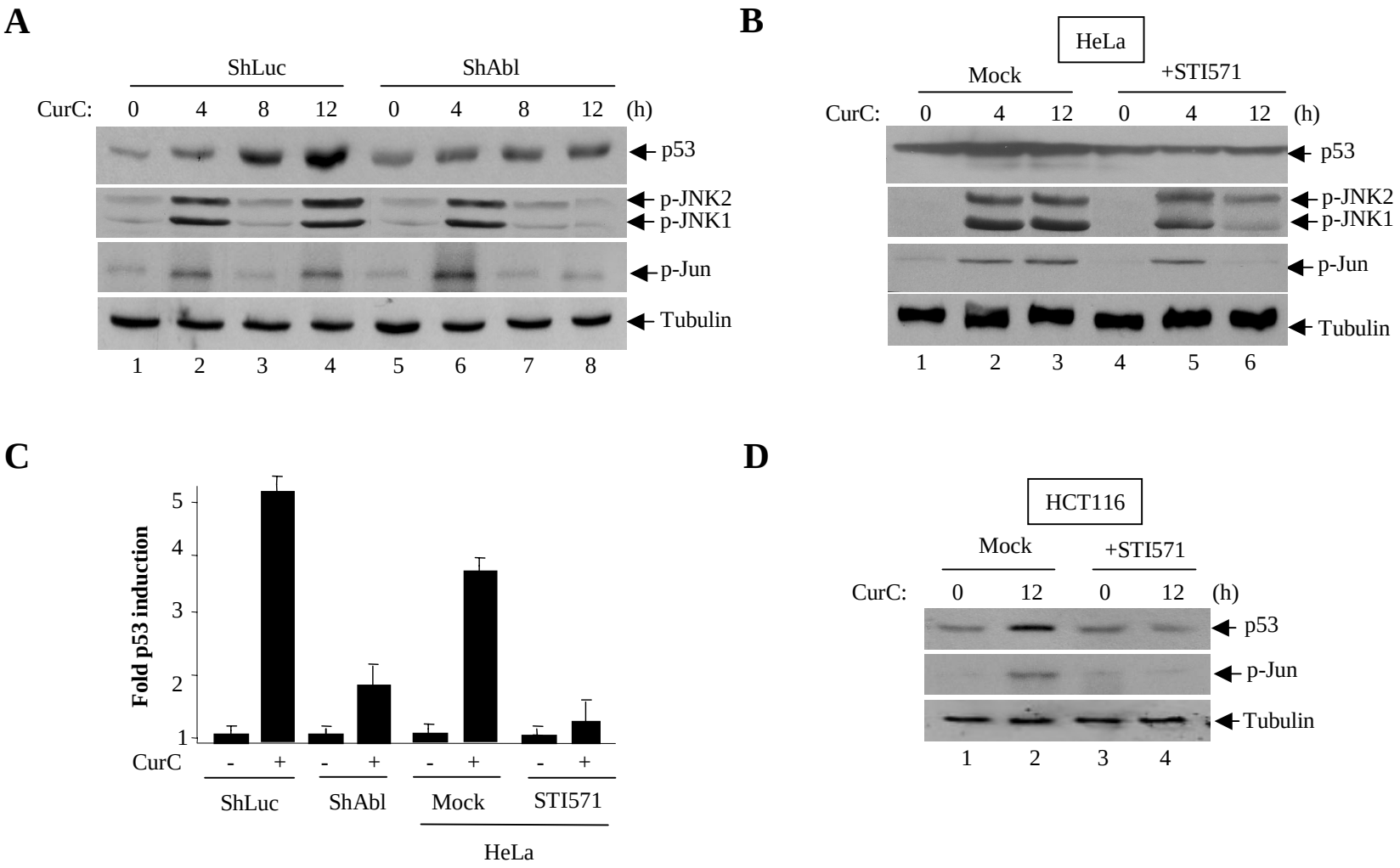


Fig. 5

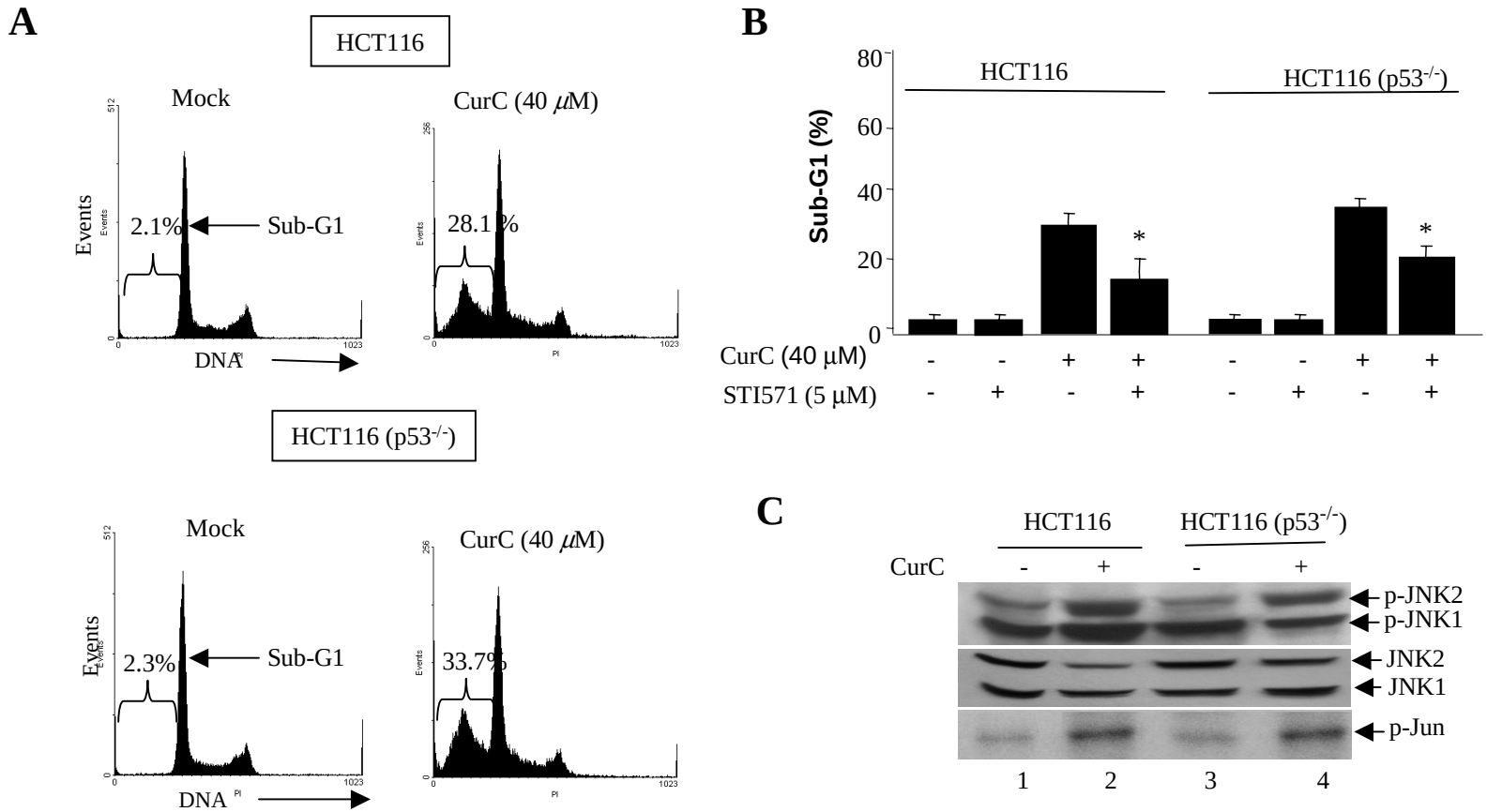
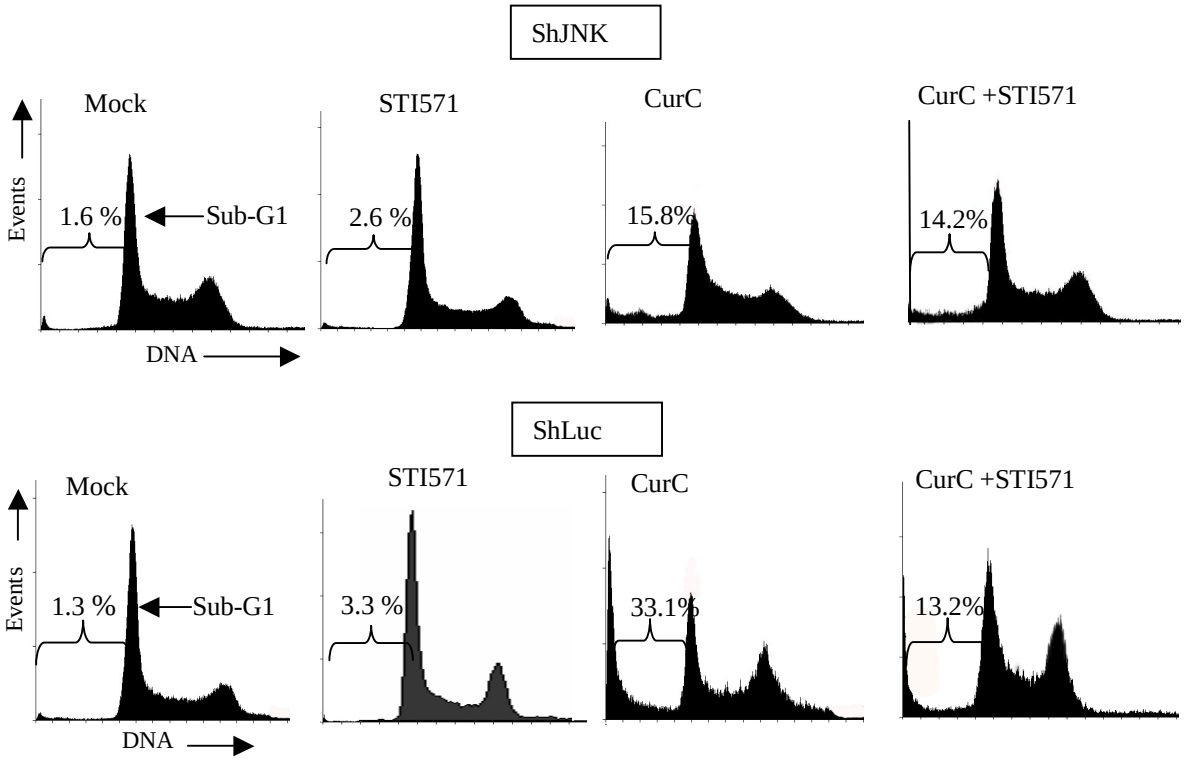


Fig. 6

Fig. 7

A



B

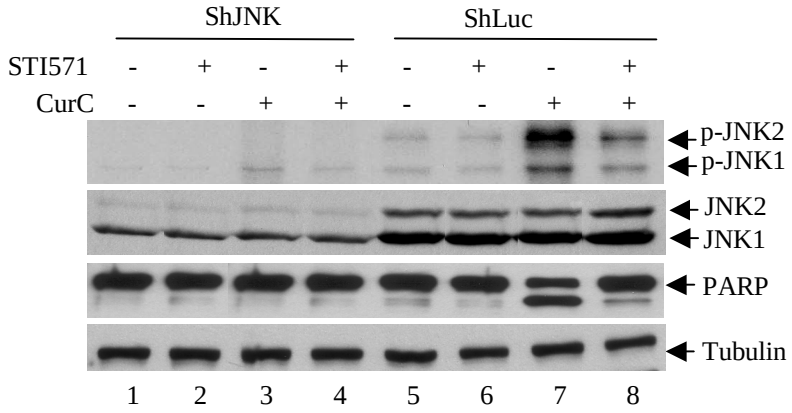


Fig. 8