UNIVERSITY OF CINCINNATI

Date: August 14, 2006

I, Wenjun Ni,

hereby submit this work as part of the requirements for the degree of:

Master of Science

in:

Molecular and Developmental Biology

It is entitled:

Involvement of Rac GTPase in p53-deficiency mediated lymphomagenesis

This work and its defense approved by:

Chair: Yi Zheng, PhD

Susanne Wells, PhD

Kenneth Campbell, PhD

Involvement of Rac GTPase in p53-deficiency mediated lymphomagenesis

A thesis submitted to the

Division of Research and Advanced Studies

of the University of Cincinnati

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in the Molecular and Developmental Biology Graduate Program

of the College of Medicine

August, 2006

By

Wenjun Ni, B.S.

Second Military Medical University, 2001

Committee Chair: Yi Zheng, PhD

ABSTRACT

My studies are based on the hypothesis that Rac GTPase signaling contributes to the p53 deficiency-mediated tumorigenesis through a functional connection of Rac with the p53-regulated cell growth and survival network. Combined methods of dominant negative Rac1 expression and pharmacologic treatment with a Rac-specific small molecule inhibitor, NSC23766, were applied. In human BL41 and mouse J3D cells in which p53 is defective, p53 functional expression caused a reduction of Rac1-GTP level. Expression of N17Rac1 mutant or treatment with NSC23766 resulted in decreased Rac1 activity accompanied by decreased proliferation of these cells. The suppression of lymphoma cell proliferation by Rac1 inhibition was associated with increased apoptosis and decreased S-phase transition, as well as reduced signaling activities of Rac-downstream effectors PAK1 and AKT. These results suggest Rac1 activity is inversely regulated by p53 in the lymphoma cells, and that targeting Rac-signaling pathway may be beneficial in suppressing lymphomagenesis associated with p53-pathway deficiency.

Acknowledgements

This work was supported by a grant from NIH. I thank my committee members, Yi Zheng, Susanne Wells, and Kenneth Campbell for their knowledge and support during this project. Finally, I appreciate my colleagues for their constant encouragement throughout the past years.

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CHAPTER 1-Introduction

1. Rac GTPases

The Rho GTPase family belongs to the Ras GTP-binding protein superfamily and consists of over twenty-two sequence and functionally related proteins, including three Rho isoforms A, B, and C; three Rac isoforms 1, 2, and 3; Cdc42, RhoD, Rnd1, Rnd2, RhoE/Rnd3, RhoG, TC10, and TCL; RhoH/TTF; Chp and Wrch-1; Rif, RhoBTB1, and 2; and Miro-1 and 2 [1]. These Rho GTPases cycle between an active GTP bound state and an inactive GDP-bound state in a tightly regulated manner controlled by several regulatory proteins: guanine exchange factors (GEFs); GTPase-activating proteins (GAPs); and guanine nucleotide dissociation inhibitors (GDIs) (Fig. 1). Many mitogenic signals, including those from growth factor receptors and integrins, can promote the exchange of GDP for GTP on Rho GTPases [2] and enable them to interact with an array of effector targets to elicit specific cellular effects [3]. These Rho family members are required for Ras transformation [3, 4-6], and their deregulation correlates with poor cancer prognosis in some cases [7]. This deregulation happens when the negative aspect of these multifunctional proteins arises in the context of scenarios that cause their constitutive activation including point mutations or overexpression and render them insensitive to regulatory signals. Under such circumstances, these GTPases trigger specific signals that can lead to uncontrolled cell growth, enhanced angiogenesis, inhibition of apoptosis, and genetic instability, all of which may contribute to tumor development [8].

The chemical compound NSC23766 was identified by a structure-based virtual screening of compounds that fit into a surface groove of Rac1 known to be critical for GEF specification.

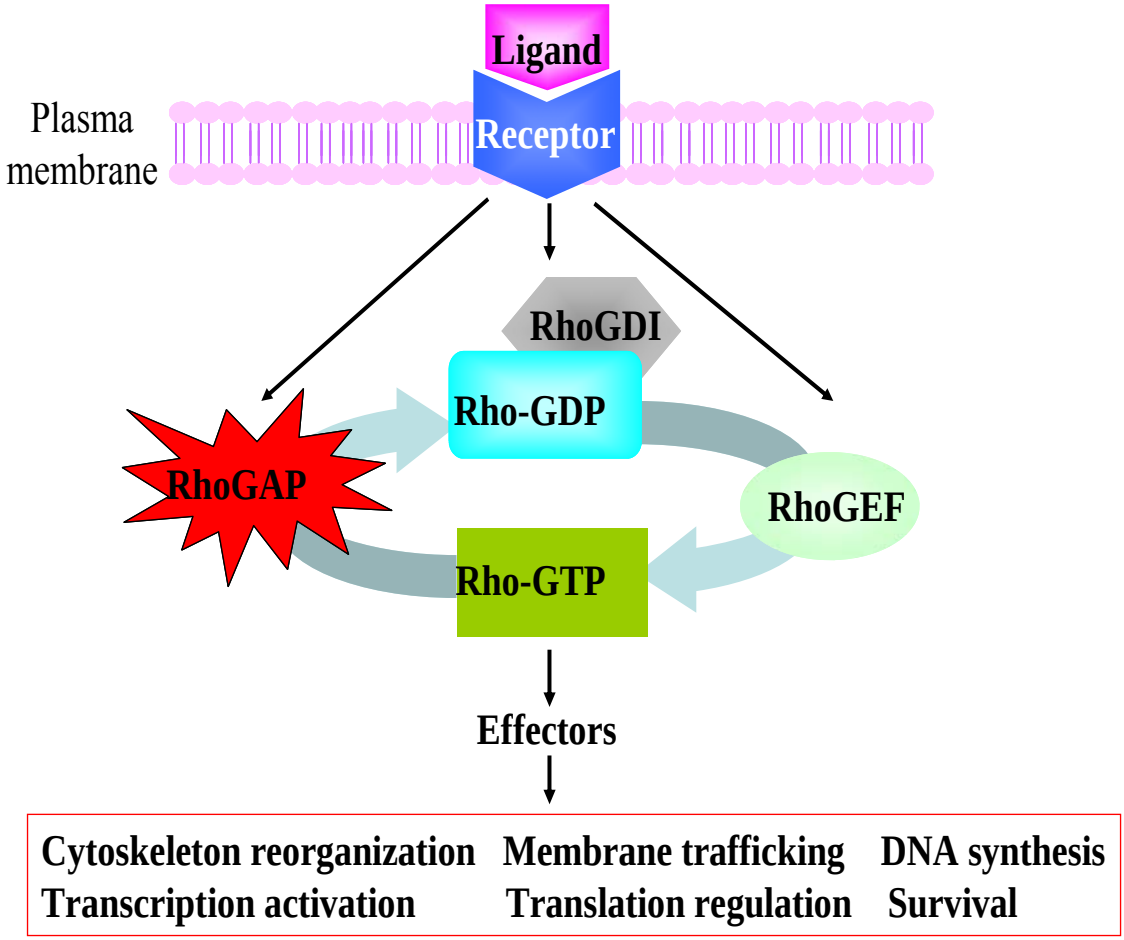


Figure 1. Rho GTPase signaling model. These Rho GTPases cycle between an active GTP bound state and an inactive GDP-bound state in a tightly regulated manner controlled by several regulatory proteins: guanine exchange factors (GEFs); GTPase-activating proteins (GAPs); and guanine nucleotide dissociation inhibitors (GDIs). Many mitogenic signals, including those from growth factor receptors and integrins, can promote the exchange of GDP for GTP on Rho GTPases and enable them to interact with an array of effector targets to elicit specific cellular effects. (*Moon SY, Zheng Y. 2003*)

In cells, it potently blocked serum or platelet-derived growth factor-induced Rac1 activation and lamellipodia formation without affecting the activity of endogenous Cdc42 or RhoA [9], providing a useful tool to pharmacologically target Rac GTPase function.

2. The contribution of Rac GTPase-regulated signaling pathways to tumorgenesis

p21-activated kinases (Paks) are serine/threonine kinases that function as downstream nodes for Rac-regulated oncogenic signaling pathways. Except for their well-known regulators of cytoskeletal remodeling and cell motility, they have recently also been shown to promote cell proliferation, regulate apoptosis and accelerate mitotic abnormalities, which results in tumour formation and cell invasiveness [10]. Paks are regulated by many upstream signaling pathways, which include G-protein-coupled receptors. Signaling activates GEFs, which then stimulate GTP loading on Cdc42 or Rac1. The kinase activity of Paks is increased over the low level of basal activity after the binding of activated Cdc42 or Rac1 to the Pak PBD domain[11].

The Rho-LIM-kinase (LIMK) signaling pathway plays a critical role in stimulus-induced remodeling of cytoskeleton by linking signals from the Rho family GTPases to changes in cofilin activity. LIMK1 is reported to play an important role in cell motility [12], but the mechanism of induction of cell motility and the role of LIMK1 in tumor growth, angiogenesis and invasion are poorly understood.

In 1995, the Tsichlis laboratory discovered that the Akt/protein kinase B (PKB) serine/threonine protein kinase is a target of phosphoinositide 3-kinase [13]. Several laboratories have reported increased Akt activity, phosphorylation, and even protein expression in tumors of the breast, prostate, ovary, and pancreas [14]. These and numerous other studies have thus provided overwhelming evidence that efficient signaling through the PI3K/Akt signaling axis promotes growth and survival, and that any genetic perturbation of this pathway will increase the survival of cancer cells that would normally undergo apoptosis. In fibroblasts, Akt signaling enhances activation of various small GTPases, including Rac, and thus leads to remodeling of the actin cytoskeleton and enhanced cell motility [15].

Stimulation of a variety of tyrosine kinase receptors leads to a rapid elevation of the enzymatic activity of a family of closely related serine-threonine kinases, known as mitogen-activated protein kinase-MAP kinases. These kinases are able to convert extracellular stimuli to intracellular signals that control gene expression and, eventually, cell proliferation and differentiation [16-17]. MAP kinases have been classified into three subfamilies: ERKs, including ERK1 and ERK2 (also known as $p44^{mapk}$ and $p42^{mapk}$, respectively); SAPKs, also called c-jun N-terminus kinases (JNKs); and p38 kinase, the homologue of the *Saccharomyces cerevisiae HOG1* gene.

The extracellular signal-regulated kinase (ERK) signaling pathway is a major determinant in the control of diverse cellular processes such as proliferation, survival, differentiation and motility. This pathway is often up-regulated in human tumors and as such represents an attractive target for the development of anticancer drugs [18].

The c-Jun N-terminal kinases (JNKs) were originally identified by their ability to phosphorylate c-Jun in response to UV-irradiation, but now are recognized as critical regulators of various aspects of mammalian physiology, including: cell proliferation, cell survival, cell death, DNA repair and metabolism. Targeted disruption of the Jnk loci in mice studies revealed that the JNKs play important roles in numerous cellular processes, including: programmed cell death, T cell differentiation, negative regulation of insulin signaling, control of fat deposition, and epithelial sheet migration. Importantly, the JNKs have become prime targets for drug development in several important clinical areas, including: inflammation, diabetes, and cancer [19].

p38 is a MAPK that has been shown to induce a wide variety of biological effects in cell culture in response to a wide range of stimuli. These effects are dependent not only on the stimuli, but also on the cellular context. For example, p38 was shown to induce apoptosis in some cells, but prevent apoptosis in others. Similarly opposed effects had been observed with respect to cell cycle regulation. More recently, p38 function has been evaluated in vivo, and through these studies p38 has emerged as an important regulator of both embryonic development and cancer progression [20].

3. A possible functional relationship between Rac signaling and p53 tumor suppressor

p53 is a key transcription factor essential for the response to cellular stress from DNA damage, hypoxia, or oncogene activation, and when activated, can trigger cell cycle arrest, apoptosis or senescence [21]. Previous studies have not only implicated the p53 pathway in cell cycle control and/or apoptosis, but also have suggested a functional link between p53 and cellular reactive oxygen species (ROS) activity and genomic stability (Fig. 2).

With the appreciation of a central role of p53 in tumor suppression and the critical involvement of Rac GTPases in cell cycle progression, cell survival and ROS production, it is logical to envision a functional connection between the loss of p53 function and Rac-mediated signaling processes in tumorigenesis. Since upregulation of expression or activity, but rarely mutation, of Rac GTPases are associated with a wide spectrum of human tumors, it is particularly attractive hypothesis that Rac GTPases may serve as signal modifier or mediator for primary genetic "hits" such as p53 mutations to regulate tumor cell transformation. Using a genetic approach, we have previously shown that the $p19^{Arf}$-p53 pathway negatively modulates Rac1 activity and regulates cell actin cytoskeleton and proliferation mediated by

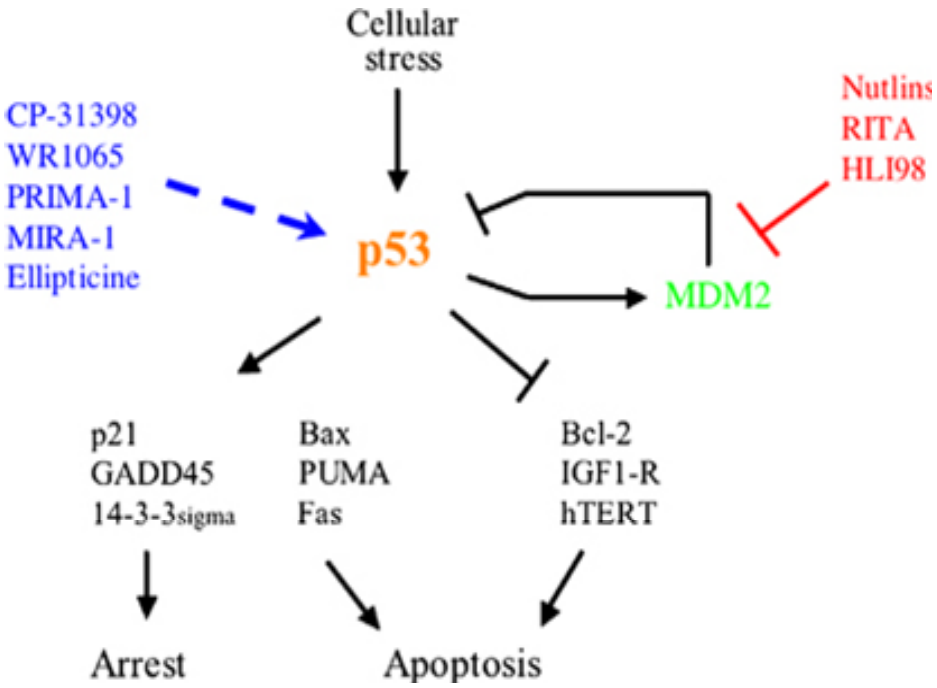


Figure 2. The p53 tumor suppressor pathway and novel small molecules that restore this pathway in human tumors. p53 regulates transcription of target genes including p21/CDKN1A, BAX, FAS, PUMA, BCL-2 and hTERT, resulting in a biological response. Ellipticine, CP-31398, WR1065, PRIMA-1, and MIRA-1 have been shown to reactivate mutant p53 or induce cell death preferentially in mutant TP53-carrying tumor cells. Nutlins, RITA, and HLI98 target wild-type p53 or MDM2 and inhibit MDM2-mediated p53 degradation, resulting in activation of wild-type p53 and a p53-dependent biological response. For both groups of compounds, the therapeutic aim is reactivation of p53-dependent apoptosis to rapidly eliminate tumor cells. (*Wiman KG. 2006*)

Rac1 in primary MEFs [22, 23]. Rac1 may contribute to the p53-mediated cell proliferation by regulating transcriptional and cell cycle machineries in this system. These observations suggest an intimate involvement of Rac signaling pathways in the p53 signaling network.

4. p53-defective lymphoma models

Burkitt lymphoma (BL) is an aggressive B-cell tumor that occurs in several clinical forms. Endemic BL (eBL) affects children and young adults in Africa and some other geographical areas and carries Epstein–Barr virus (EBV) in more than 95% of cases. In contrast, sporadic BL (sBL) among adolescents in Europe and North America are mostly EBV-negative. A third

type of BL is associated with HIV-infection in adults. BL has a high growth rate and a large fraction of cycling cells [24].

A majority of BL lines and at least 30% of BL biopsies carry p53 mutations [25-29]. Like in other tumor types, p53 mutations in BL cluster in the core domain and include so-called hot spot residues such as Arg-175, Arg-248, and Arg-273. Many of these mutations were shown to functionally affect p53 [30]. P53 mutation in BL shows no correlation with EBV status. In order to study the delineate function of Rac1 and p53, we used the EBV negative BL41 cell line with a temperature sensitive p53 mutant (Val135) that expresses p53 with a largely mutant conformation at 37°C and mostly wild-type conformation at 32°C. At 37°C, the p53-Val135 transfected cells behaved like the parental or neo transfected control cells. However, expression of exogenous wild-type p53 at 32 °C resulted in a rapid reduction of the number of viable cells while the parental and neo control cells remained unaffected [31].

5. Working hypothesis

The p53 tumor suppressor pathway constitutes a checkpoint in cell cycle progression, elicits apoptosis or senescence signals and maintains cellular ROS level and genomic integrity in normal cells in response to stimulation. Disruption of p53 or its regulatory genes leads to tumor formation in mice, and loss of function of genes in the p53 pathway is a common feature in human cancer. On the other hand, active Rac GTPase can transduce signals to the cell nucleus via ERK, JNK, cyclin D1 and/or Stat3 to promote cell transformation. In addition, Rac GTPase has been implicated as a key regulator of cell survival, cell cycle progression and ROS production. The observations that 1) defective p53 pathway leads to a significant increase in Rac activities in primary MEFs and 2) active Rac is required for the hyperproliferative phenotype of p53 defective MEFs suggest that Rac may serve as a useful

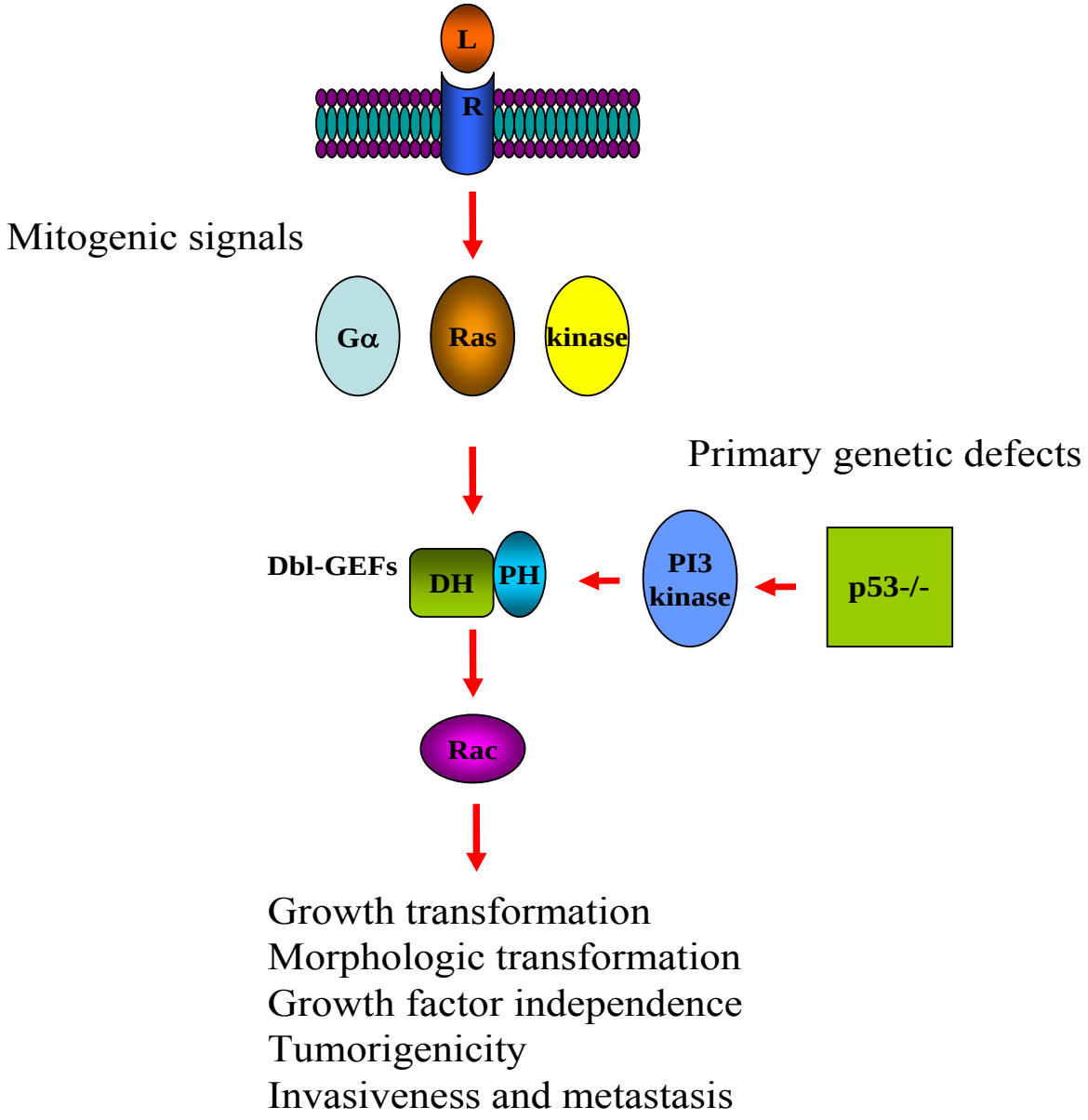


Figure 3. Hypothesis of the relationship between p53 defect and Rac function.

target in p53-defective tumor cells. Thus, the central hypothesis of my thesis work is that Rac activity provides essential signals as modifiers or mediators to the growth phenotype of p53 deficient lymphomas (Fig.3). In the following studies I have tested this hypothesis in B- and T-cell lymphoma models, and attempted to preliminarily define the molecular and cellular contributions by Rac to the proliferation of p53 defective lymphoma cells.

CHAPTER 2-Materials and Methods

DNA constructs. The Rac1 dominant negative mutant (Rac1N17) was generated by site-directed mutagenesis based on oligonucleotide-mediated PCR [32]. For retroviral expression, cDNAs encoding the dominant negative Rac1 were ligated into the *Bam*HI and *Eco*RI sites in frame with the nucleotides encoding a three-hemagglutinin (HA_3) tag at the 5' end of the retroviral vector MIEG3 that expresses enhanced green fluorescent protein bicistronically.

Retrovirus Infection. Recombinant retroviruses were produced using the ecotropic Phoenix packaging cell system [33]. BL41 and J3D cells were infected with the retroviruses and harvested 48 hrs after infection. EGFP fluorescent protein-positive cells were isolated by fluorescence-activated cell sorting (FACS).

Immunoblotting. Whole-cell lysates were prepared by extraction of the BL41 or J3D cells by the lysis buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.2% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, 1 μg/mL of aprotinin, and 1 mM dithiothreitol for 30 min. Protein contents in the whole-cell lysates and nuclear lysates were normalized by the Bradford method. The lysates were separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Detection of phospho-PAK was by using phospho-PAK1 (Ser144)/PAK2 (Ser141) antibody (Cell Signaling). Detection of PAK was by PAK1 antibody (Cell Signaling). Detection of phospho-LIMK was by using phospho-LIMK1 (Thr508)/LIMK2 (Thr505) antibody (Cell Signaling). Detection of LIMK was by using LIMK1 antibody (Cell Signaling). Detection of

phospho-ERK was by phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling). Detection of ERK was by p44/42 MAP kinase antibody (Cell Signaling). Detection of phospho-JNK was by phospho-SAPK/JNK (Thr183/Tyr185) antibody (Cell Signaling). Detection of JNK was by SAPK/JNK antibody (Cell Signaling). Detection of phospho-p38 was by phospho-p38 MAP kinase (Thr180/Tyr182) antibody (Cell Signaling). Detection of p38 was by p38 MAP kinase antibody (Cell Signaling). Detection of phospho-Akt was by phospho-Akt (Tyr326) antibody (Cell Signaling). Detection of Akt was by Akt antibody (Cell Signaling).

Rho GTPase effector domain pull-down assays. Cells transiently expressing comparable total protein were lysed at 4 °C in a buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.2% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, 1 μg/mL of aprotinin, and 1 mM dithiothreitol. The cell lysates were then mixed with purified glutathionine-agrose immobilized fusion proteins (10 μg each) at 4 °C for 45min. After incubation, the beads were washed twice in the lysis buffer, and the bound proteins were separated on 12% SDS-PAGE, transferred on to the PVDF membrane, and immunoblotted with respective monoclonal antibodies [32].

Apoptosis and cell-cycle progression assays. To quantify the apoptotic cell population, 1 x 10^5 cells were stained with 7-AAD and APC-conjugated annexin-V per manufacturer's instructions (Pharmingen) and were analyzed by flow cytometry. For cell-cycle analysis, 1 x 10^5 cells were treated with RNase, labeled by propidium iodide (PI) and analyzed by flow cytometry.

Short term cell proliferation assay. Cells were plated in 24-well non-tissue culture dishes at day 2 $\times 10^3$ cells were plated per well. NSC23766 was added when plating the cells. Cells were collected and counted at day 2 and day 4.

CHAPTER 3-Results

p53 deficiency causes an increase of Rac1 activity in lymphoma cells. Previously, our lab found that endogenous Rac1 activity is subject to functional p53 regulation in primary MEF cells. In particular, introduction of a wild-type *p53* gene into *p53*$^{-/-}$ MEF cells suppressed Rac1-GTP levels [22]. To determine if such an inverse functional relationship between Rac1 GTPase and p53 occurs in tumor cells, we measured Rac1 activity in the p53 deficient BL41 Burkitt's lymphoma cells in the presence or absence of functional p53 expression. For this purpose, a temperature sensitive p53 mutant that is active at the permissive temperature of 32°C but inactive at the non-permissive temperature of 37°C was expressed in the BL41 cells, and the relative levels of Rac1-GTP species of the cells were estimated by the GST-effector domain pull-down assays under both the permissive and non-permissive conditions. As shown in Fig.4A, functional p53 expression effectively suppressed Rac1-GTP formation in the BL41 cells. To examine if this property may also apply to the T-cell lymphoma cells, we measured Rac1 activity in the *p53*$^{-/-}$ J3D T-cell lymphoma cells by a similar strategy. As shown in Fig.4B, at the permissive temperature, functional p53 expression led to a markedly decreased Rac1-GTP level in J3D cells. Together these results indicate that expression of functional p53 suppresses Rac1 activity and loss of p53 causes elevated Rac1 activity in both B- and T-lymphoma cells.

Dominant-negative Rac1 mutant expression or Rac1 inhibitor NSC23766 treatment suppresses Rac1 activity in lymphoma cells. NSC23766 was discovered by a rational approach to specifically target Rac1-GEF interaction [9]. It has been shown to have specific inhibitory effect on Rac1 activity in NIH 3T3 cells [9] and a number of other cell types

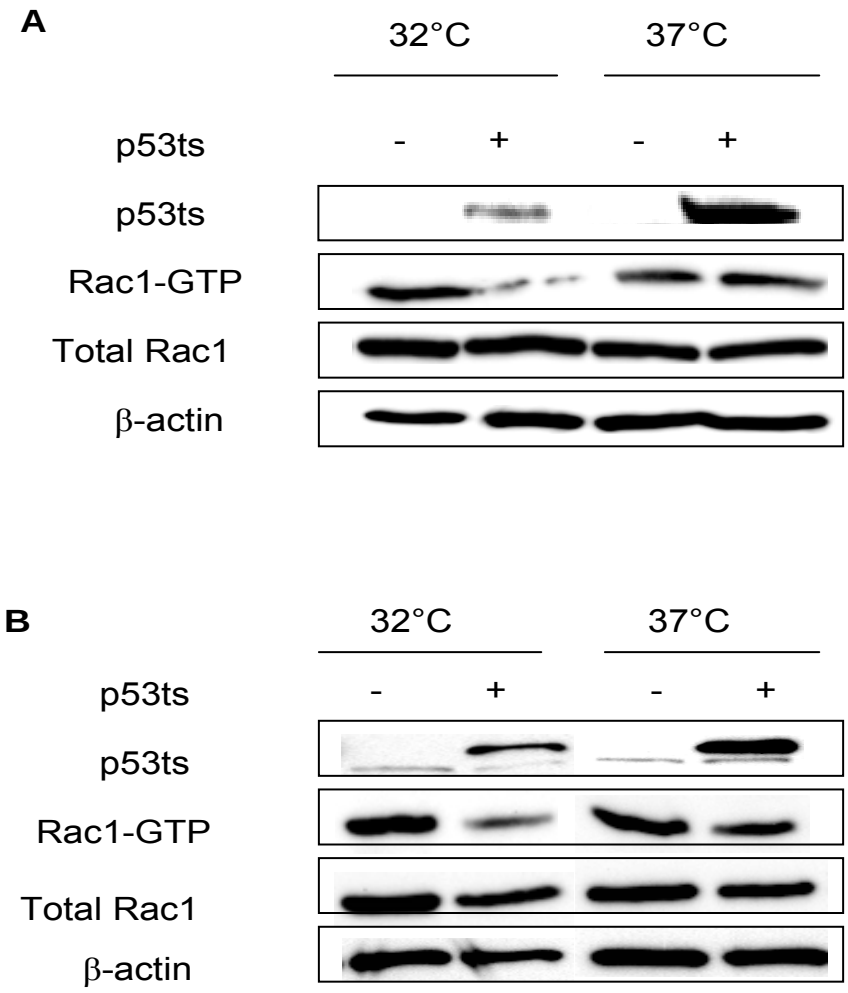


Figure 4. p53-/- tumor cells contained elevated Rac activity. BL41 and BL41-p53ts cells (A) and J3D and J3D-p53ts cells (B) were incubated in IMDM 10%FBS for 24 hours at 32° C or 37.5° C. Cell lysates were subjected to the GST-PAK1 pull-down assay and were processed for Western blot with anti-p53, anti-Rac1 or anti- β -actin antibodies. β -actin was used as internal control.

including hematopoietic stem/progenitors, leukemia and epithelial cells. In addition, the dominant-negative Rac1 mutant, Rac1N17, has been widely used to inhibit endogenous Rac1 activity and was shown to be effective in inhibiting Rac1 activity in *p53*$^{-/-}$ MEFs [10]. To test the efficacy of Rac1N17 or NSC23766 on Rac1 inhibition in BL41 and J3D lymphoma cells, we measured Rac1 activities in the p53 defective BL41 and J3D cells treated with two doses of NSC23766 (10μM and 20μM, respectively) or with Rac1N17 expression. As shown in Fig.

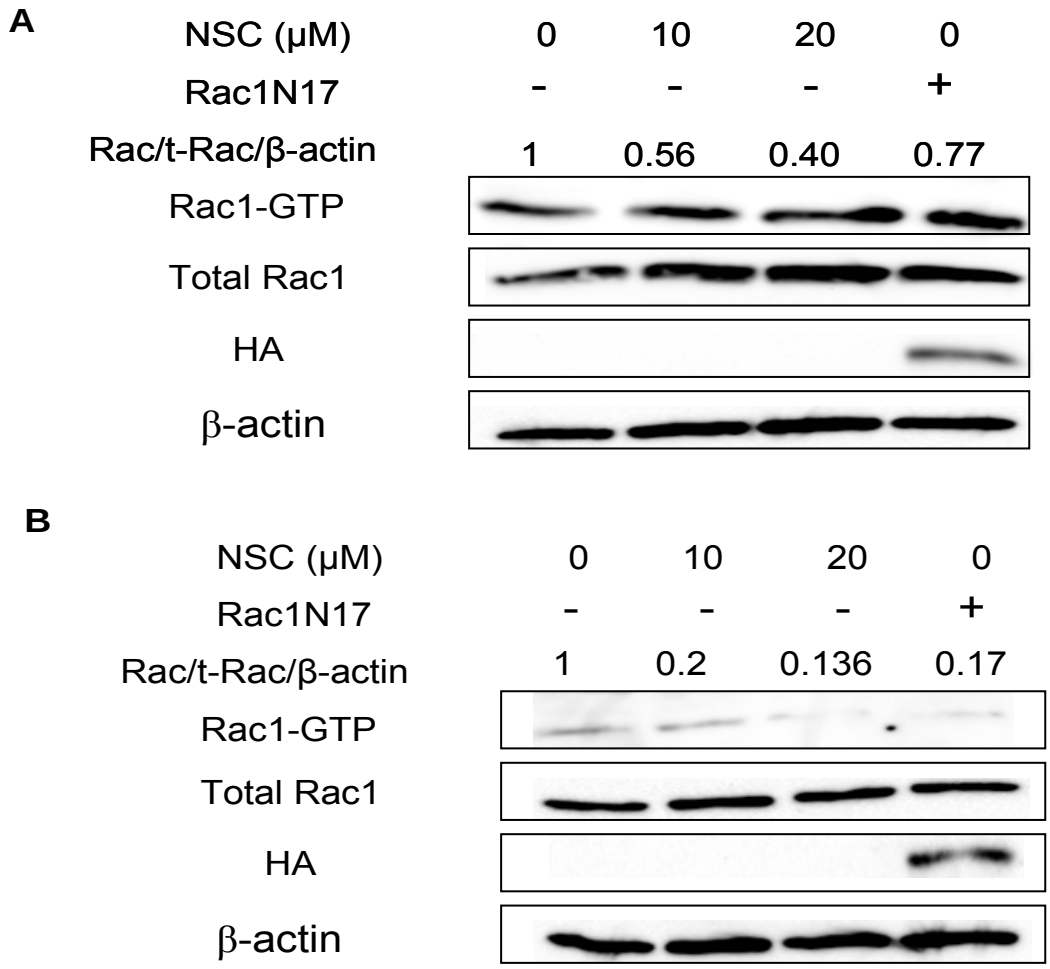
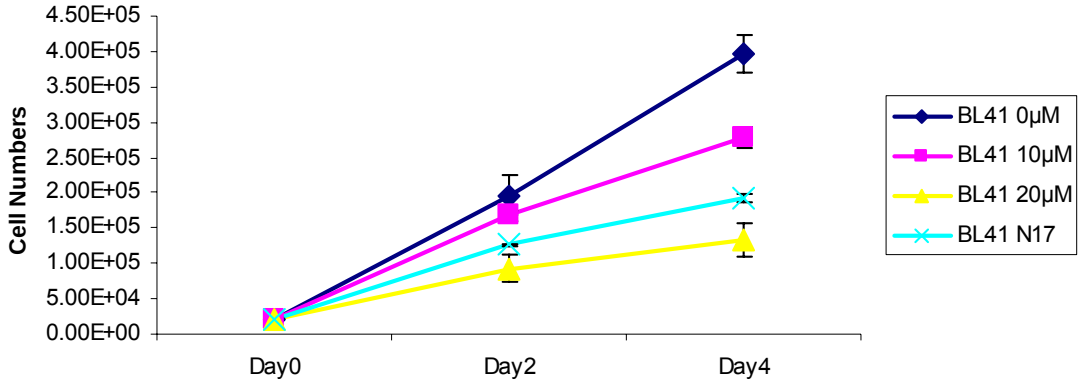


Figure 5. Rac1 inhibitor NSC23766 and dominant-negative Rac1 mutant (Rac1N17) can decrease Rac1 activity in BL41 and J3D cells. BL41 cells (A) and J3D cells (B) were incubated in the absence or presence of 10 μ M or 20 μ M of NSC23766 for 24 hours at 37°C. Cell lysates were subjected to the GST-PAK1 pull-down assay and were processed for Western blot with anti-Rac1, anti-HA, or anti- β -actin antibodies. β -actin was used as internal control.

5A, Rac1 activity in BL41 cells was inhibited by NSC23766 in a dose-dependent manner. In parallel, Rac1 activity was also reduced by Rac1N17 expression in BL41 cells. Similarly, as shown in Fig. 5B, Rac1 activity in J3D cells was potently inhibited by either Rac1N17 expression or NSC23766 treatment in a dose-dependent manner. The inhibitory effect on Rac1 activity in BL41 cells was relatively smaller when compared to that of the J3D cells. These results suggest that NSC23766 can be used in targeting Rac1 in the p53-defective BL41 or J3D lymphoma cells.

A



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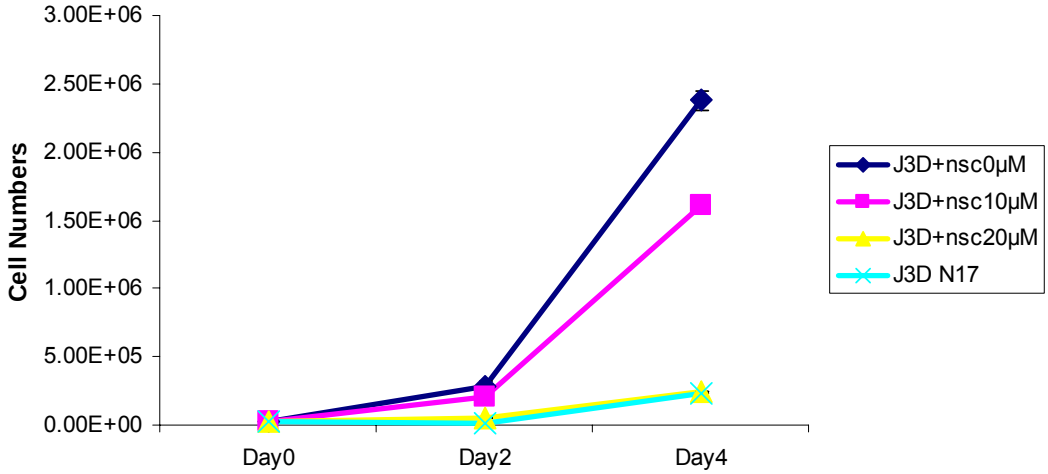

Figure 6. Rac1 targeting by NSC23766 or dominant-negative Rac1 inhibits the tumor cell proliferation. BL41 and BL41-N17 cells (A) or J3D and J3D-N17cells (B) were incubated in the absence or presence of 10 μ M or 20 μ M of NSC23766 for indicated times at 37° C.

Rac1 targeting by NSC23766 or dominant-negative Rac1 mutant inhibits the p53-deficient lymphoma cell proliferation. Since active Rac1 GTPase appears to contribute to the promotion of hyperproliferation in p53-/- MEFs [10], we next asked if the proliferation phenotype of lymphoma cells can be inhibited in the p53 deficient BL41 or J3D cells when Rac1 is targeted by NSC23766 or Rac1N17. As shown in Figs. 6A and 6B, treatment of either cell types by NSC23766 or Rac1N17 were able to inhibit cell growth. Furthermore, the

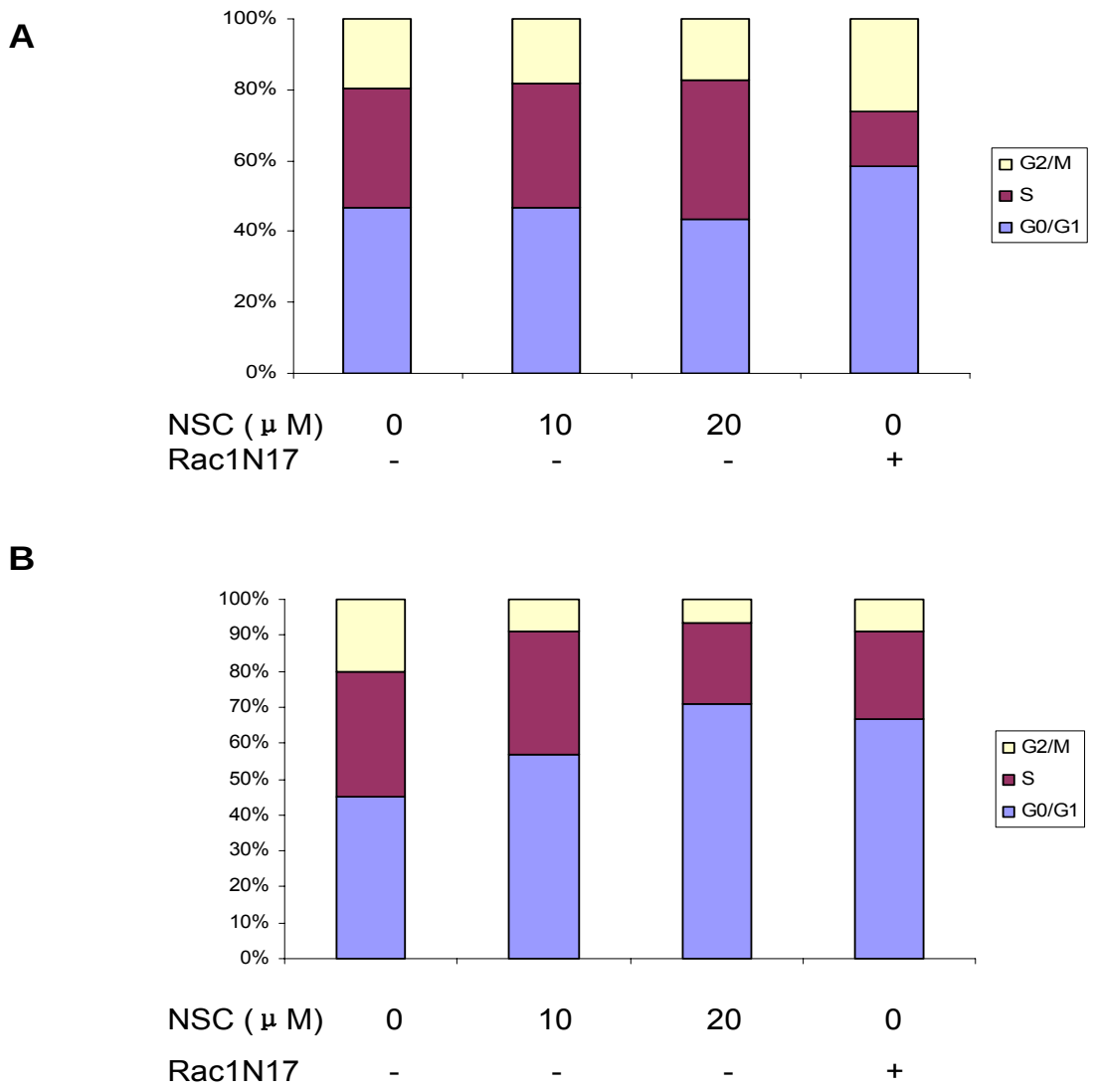

Figure 7. Rac1 signaling modulates cell cycle progression of p53 deficient BL41 and J3D cells. BL41 and BL41-N17 cells (A) and J3D and J3D-N17 cells (B) were incubated in the absence or presence of 10 μ M or 20 μ M of NSC23766 for 48 hours at 37° C and analyzed for propidium iodide–stained DNA content by FACS-Canto machine. Values indicate the percentage of cells with hypodiploid DNA content.

inhibition of the cell growth by NSC23766 was dose-dependent. Thus, Rac1-targeting appears to be a promising strategy to inhibit p53-deficient lymphoma cell proliferation.

Rac1 targeting modulates cell cycle progression and apoptotic response of p53 deficient lymphoma cells. To begin to address the role of Rac1 in p53-mediated cell proliferation, we

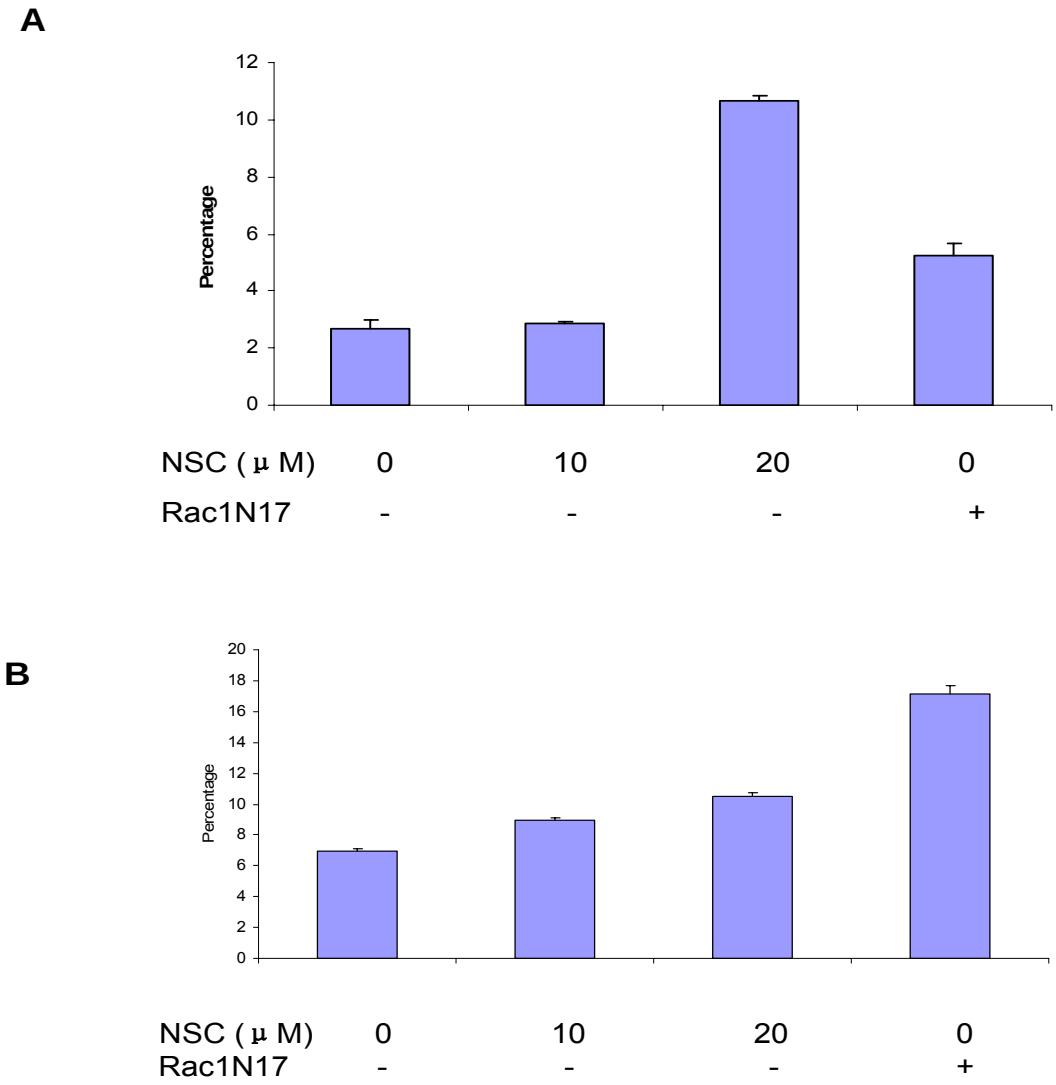

Figure 8. Rac1 signaling modulates apoptotic response of p53 deficient BL41 and J3D cells. BL41 and BL41-N17 cells (A) and J3D and J3D-N17 cells (B) were incubated in the absence or presence of 10 μM or 20 μM of NSC23766 for 48 hours at 37° C and analyzed by PE-conjugated annexin V staining followed by FACS analysis on a FACS-Canto machine.

carried out a set of experiments comparing the cell cycle and apoptotic properties of p53-defective BL41 B-cell and J3D T-cell lymphomas by applying different dosage of NSC23766 or expressing the dominant-negative Rac1 mutant. As shown in Figs.7A and 7B, when the cell cycle progression profiles of BL41 and J3D cells with or without treatment by NSC23766 or Rac1N17 were analyzed by PI staining and FACS, the dominant-negative Rac1 mutant was found to be able to extend the G_1 phase and suppress the G_2/M phase of BL41 and J3D cells,

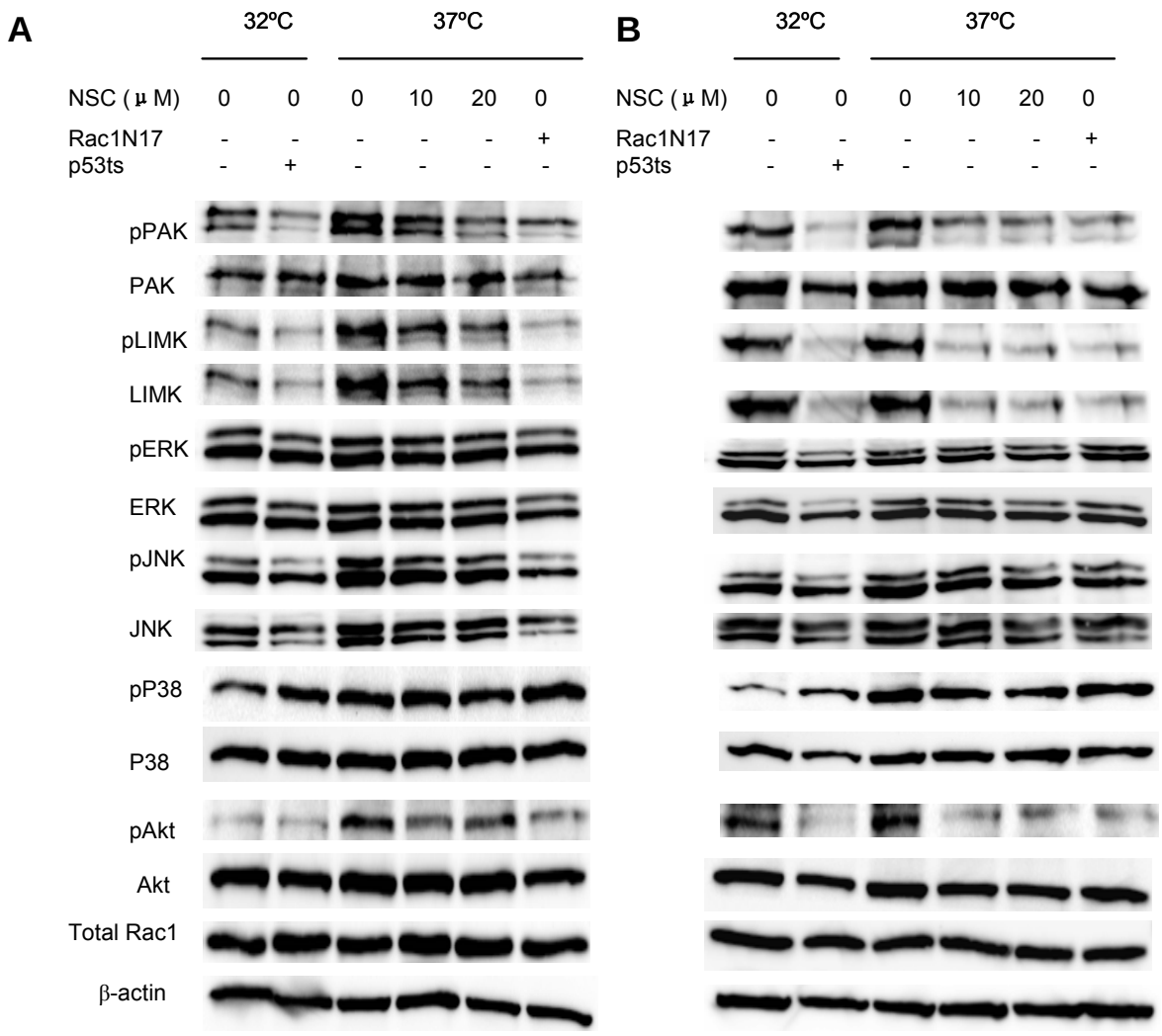

Figure 9. Inhibition of Rac1 affects downstream PAK, LIMK and Akt pathways. BL41, BL41-p53ts, and BL41-N17 cells (A) or J3D, J3D-p53ts cells and J3D-N17 cells (B) were incubated in the absence or presence of 10μM or 20μM of NSC23766 for 24 hours at 32 or 37°C. Cell lysates were processed for Western blot with antibodies as described in Metarials and Methods. Total Rac1 and β-actin were probed as internal controls.

while NSC23766 was also effective in a dose-dependent manner in J3D cells. However, no significant effect on cell cycle regulation in BL41 cells was observed, even when the concentration of NSC23766 was at 20μM. On the other hand, treatment of the cells with NSC23766 or Rac1N17 led to an increase in the rate of apoptosis compared with that of BL41 or J3D cells (Figs. 8A and 8B). Expression of exogenous p53ts mutant in these cells at the permissive temperature (32°C) caused increased apoptosis and decreased cell cycle S-phase

cell population compared with the p53 deficient parental cells (data not shown). These results indicate that Rac1 may contribute to p53-regulated cell proliferation by modulating both cell cycle progression and apoptosis regulation and Rac1-targeting may suppress the hyperproliferation of p53-defective tumor cells by stimulating a p53-independent apoptotic signal.

Mechanism of Rac1 targeting in the lymphoma cells may involve downstream PAK, LIMK and Akt pathways. To begin to unveil the molecular pathways affected by targeting Rac1 in the p53-deficient lymphoma cells, we next examined a few key effectors that are thought to be regulated by Rac1 related to cell growth control. As shown in Figs. 9A and B, treatment of the BL41 or J3D cells with the Rac1 inhibitor, NSC23766, or Rac1N17 mutant, led to an inhibition of phospho-PAK, phoshpo-LIMK, and phospho-Akt levels that correlate with their respective activities, and the inhibition by NSC23766 occurred in a dose-dependent manner. However, the activities of several other potential effectors of the MAP kinase module, i.e. ERK, JNK, and p38, were not affected when the Rac1 activity was inhibited by NSC23766 or Rac1N17. These results suggest that our Rac1-targeting strategy may primarily work through the Rac1-PAK1-AKT signaling pathway, but not through MAP kinases, to affect the lymphoma cell survival and/or proliferation properties.

CHAPTER 4-Discussion

Summary of the results. In the present study, we demonstrate that intracellular Rac1 activity is inversely regulated by functional p53 in both B- and T-cell lymphoma cells and that Rac1 contributes to p53 deficiency-induced hyperproliferation of the cells. The contribution of Rac1 to the growth phenotype of p53 deficient tumor cells may come in part through PAK, LIMK, and/or Akt pathways, but independently from the ERK, p38 or JNK MAP kinase module (Fig. 9). Significantly, we show that Rac1 is involved in p53-regulated cell proliferation by modulating both the cell cycle and a p53-independent apoptotic signal. Although more detailed mechanisms of the functional connection between Rac1 GTPase and the p53 tumor suppressor pathway remain to be explored, these results help establish a novel relationship of Rac GTPase with the p53 pathway, defects of which occur in many cases of human cancer [34, 35]. The findings, therefore, may have important implications for strategies that target Rac1 GTPase in anticancer therapy.

The functional relationship between Rac1 and p53 pathways. Previous studies have shown that endogenous Rho GTPase activities including that of Rac1 were significantly elevated in *p53-/-* MEF cells [10]. In the present study, we further determined the relationship between Rac1 activity and functional p53 in the Burkitt's B-lymphoma BL41 cells and T-lymphoma J3D cells that are defective in p53 by using a temperature-sensitive p53 mutant (p53ts) expression approach. The results obtained from these lymphoma cells provide evidence that Rac1 activity is inversely regulated by p53 in p53-deficient tumor cells. By expressing the dominant-negative Rac1, Rac1N17, in the tumor cells, we have shown that Rac1 activity is required for the proliferative phenotype, to an extent that is similar to reconstitution of

functional p53. These observations suggest that Rac1 targeting by pharmacological methods, such as utilization of the Rac1 inhibitor, NSC23766, could be a useful approach in reversing aspects of tumor cell transformation resulting from p53-pathway deficiency.

Possible mechanism of Rac1 contribution to p53 deficiency-induced lymphomagenesis. Cell cycle checkpoints are regulatory mechanisms that ensure the proper timing of cell cycle events by enforcing the dependency of late events on the completion of early events [36]. p53-induced G1 cell cycle arrest is, to a large extent, mediated by transactivation of the cyclin/cyclin-dependent kinase inhibitor $p21^{WAF1}$ [37]. Rac1, a member of the Rho family GTPases, has been implicated in the regulation of a wide range of biological processes including G1 cell cycle progression [38, 39]. Treatment of p53 deficient cells with dominant negative Rac1 mutant seems to induce a cell cycle G1 phase accumulation in both cell lines and Rac1 inhibitor seems to induce J3D cell cycle G1 phase accumulation(Fig. 7), suggesting that Rac1 may work in regulating G1/S phase transition in the p53 defective setting. In addition, the observed increase in apoptosis of the cells when Rac1 activity was inhibited suggests that Rac1 may also be involved in a survival pathway independent from p53 (Fig. 8). These results raise the possibility that the elevated Rac1 activity in p53 defective lymphocytes may act cooperatively with p53-deficiency to promote cell cycle progression and survival in B-cell and T-cell transformation.

Although Rac-targeting with dominant negative mutant or NSC23766 showed efficacy toward both BL41 and J3D cells, it appears that J3D T-lymphoma cells are more susceptible to Rac inhibition, particularly in suppressing cell cycle progression. One reason might be that Rac GTPase is not as critical to the B-lymphma cell proliferation as to the T-lymphomas. Consistent with this possibility, NSC23766 was found less effective on pro-B cell proliferation by my colleagues (unpublished data).

Rac GTPase as a potential target in p53-deficient tumor cells. Introduction of wild-type p53 or p53-regulated signaling components is one way to reverse the growth phenotypes of various types of tumor cells that carry endogenous p53 mutant [21]. The effective inhibition of BL41 and J3D cell growth by exogenous expression of wild-type p53 are consistent with this scenario [31, 40]. Typically such an approach requires gene-therapy efforts that may bring on potential complications. Our observations that Rac1 inhibition, particularly by using the small molecule inhibitor NSC23766, results in effective suppression of growth of p53-deficient lymphoma cells present an alternative possibility for p53 deletion associated cancer therapy. The concept of targeting Rac1 and Rac1-mediated signaling that can act independently from p53-regulated cell cycle machinery and survival regulatory circuit is in line with recent appreciations that interference with modifier or mediator signals can be beneficial to suppressing tumor cell growth and development. In this sense, our data suggest that the small molecule inhibitor approach to target Rac1 or Rac1-mediated signals may be useful to down-regulate the necessary modifier or mediator signals that cooperate with p53-deficiency in lymphomagenesis. We believe that this approach merits a more detailed and systematic analysis and testing in a broader spectrum of tumor cells and in animal models that would provide more conclusive information on the validity of the Rac-targeting method.

Future Perspectives. Our results suggest a functional dependence of p53-deficient tumor cell growth on the Rac1 regulated pathway. With the exciting finding that Rac1 activity in the p53-defective lymphoma cells can be inhibited in a dose-dependent manner by using a small molecule inhibitor resulting in a suppression of the tumor cell growth, we propose that such a strategy has the potential to be further developed into future anti-cancer therapeutics after medicinal chemistry improvement of the lead Rac-targeting compound (i.e. NSC23766)

followed by more stringent in vitro and in vivo examination of toxicity and efficacy. The molecular and cellular mechanisms of the Rac-targeting strategy requires further analysis, ideally combining the examinations of various human tumor cell lines with primary tumor specimens. In addition, development of a genetically manipulable animal model, e.g. by bone marrow transplantation of p53 deficient hematopietic stem cells or xenograft human lymphoma cells, will be particularly important in establishing the usefulness of the Rac targeting strategy and in testing future generation of Rac-specific inhibitors in vivo.

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