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TITLE: The Role of Siah1-Induced Degradation of β-Catenin in Androgen Receptor Signaling

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## 14. ABSTRACT

The androgen receptor (AR) signaling-pathway plays crucial roles in the growth and progression of prostate cancer cells. Recent studies indicate that β-Catenin physically binds to AR and enhances its transcripational activity in a ligand dependent manner. p53 has also been implicated in AR signaling because of its ability to induce expression of Siah1, which binds and activates E3 ligase complexes which degrade β-Catenin. In this study, we demonstrated the biological significance and molecular mechanisms by which AR is regulated by the p53-induced Siah1 protein. Moreover, we identified the relevant proteins that are targeted for degradation by Siah1 besides β-Catenin. Thus, enhanced Siah function may suppress the ability of androgen to promote tumor cell growth. Understanding more about the functions of Siah-family proteins may therefore suggest novel strategies for chemoprevention and for improved treatment of prostate cancer.
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Introduction:
The androgen receptor (AR) signaling-pathway plays crucial roles in the growth and progression of prostate cancer cells. Recent studies indicate that β-Catenin physically binds to AR and enhances its transcriptional activity in a ligand dependent manner. Mutations that inactivate the p53 gene occur in one-third to half of all human prostate cancers and have been correlated with shorter patient survival. Loss of p53 is known to render tumor cells more resistant to a wide range of anticancer drugs and radiation [1, 2]. It would be highly desirable therefore to have a means of functionally restoring p53 activity in prostate cancers in which this gene has become inactivated. A strategy for accomplishing this is to identify the downstream effectors of p53's actions and to find ways of enhancing their activity in the absence of p53. The human Siah gene is localized on chromosome 16q12-13, a region reported to contain a candidate tumor suppressor gene in various types of cancer, including prostate cancer. Our previous results indicate that over-expression of the Siah1 protein can induce either growth arrest or apoptosis, depending on the cell line tested [3]. The findings suggest that p53-mediated induction of Siah1 expression could play an important role in the mechanisms by which this tumor suppressor inhibits cell proliferation and induces apoptosis. In this study, we designed experiments to test the hypothesis that Siah1 is an important mediator of p53's effects in prostate cancers. If the hypothesis proves to be correct, then the results derived from these studies will lay the groundwork for development of new strategies for restoring p53-like functions in prostate cancers, thus improving therapeutic outcomes for men with carcinoma of the prostate. Our preliminary data also suggest a model by which Siah-family proteins can regulate AR signaling through ubiquitation and degradation of β-Catenin. The central goal of this proposal is to test the validity of this hypothesize model, linking it ultimately to the regulation of tumor cell growth. However, at this point, essentially nothing is known about the relative importance of Siah1 for p53 responses compared to AR target genes. We therefore propose to address the following questions:

1. Is Siah1 a p53 primary response gene in the prostate?
2. What are the mechanisms by which Siah1 inhibits AR activity?
3. Does Siah1 control the sensitivity of human prostate cancer cell lines to anti-androgens in vitro and in xenograph mouse models?
4. What are the relevant proteins that are targeted for degradation by Siah1 besides β-Catenin?

The aims have not changed from the original proposal.
Body:
We have made excellent progress over the entire research period. A brief summary follows:

**Aim #1.** Examine the effects of p53 on expression of the Siah1 gene in prostate cells. The functional p53 binding site was identified in intron 1 of the Siah1 gene.

Using a computer algorithm, we identified nine potential p53-response elements (REs) in intron 1 of the Siah1 gene (Figure 1A). To explore the possibility that Siah1 is a p53-regulated gene, we cloned four fragments of intron 1 of the Siah1 gene into a luciferase reporter gene plasmid (named pGL3E-Siah1p53RE-1, 2, 3, and 4) and used transient transfection reporter gene assays to study the effects of p53 on its activity. PPC1, a p53-null prostate cancer cell line was transfected with each of the fragments with or without a plasmid containing wild-type p53. Among the four fragments, only p53RE-3 showed increased luciferase activity in response to p53 (Figure 1B). Since p53RE-3 still includes the potential p53 REs, we made three constructs including each of three p53 REs (named p53RE3-1, 3-2, and 3-3) and performed luciferase assays (Figure 1C). Among these fragments, only p53RE3-2 showed increased luciferase activity (Figure 1D). To examine this result, a vector was constructed with four point mutations in the potential p53 binding sequence (Figure 1E) and luciferase activity was assayed. As expected, the mutant did not respond to p53 (Figure 1F). We concluded that the functional p53 RE is located in the region p53RE3-2 of the Siah1 gene intron 1.
Figure 1. Transcriptional activation by p53 was identified in the intron 1 of the Siah1 gene. (A) Mapping of potential p53 responsive elements (REs) in intron 1 of the Siah1 gene. Potential p53 binding sites are shown by closed boxes. Lower arrows show the region of various inserts cloned into pGL3-enhancer (pGL3E) vector. The numbers show the name of pGL3E-Siah1p53RE vector. (B) Luciferase assay. PPC1 cells were transfected with the same amounts of the different pGL3E-Siah1 promoter vectors (pGL3E-Siah1p53RE-1, 2, 3, and 4), pCMV-p53wt, pCMV-p53mut, and pCMV-β-gal as a transfection efficiency control. pGL3E-Bax vector was used as a positive control. Luciferase activity was measured in cell lysates 24 hrs later and the data were normalized relative to β-galactosidase (mean ± SD; n=3). Lipofectamine™ 2000 (Invitrogen) was used for transfection. The same amounts of plasmid DNA was kept by the addition of empty expression vector. (C) Sequences of representative p53-inducible genes and three potential p53REs of the Siah1 gene inserted into pGL3E-Siah1p53RE3-1, 3-2, and 3-3 vector. The consensus p53 binding sequence (CBS) is shown above. (D) Luciferase assay using pGL3E-Siah1p53RE3-1, 3-2, and 3-3 promoter vectors. Luciferase activity was measured as described in (C). (E) The sequences of potential p53RE in the pGL3E-Siah1p53RE3-2 vector. Mutated points (pGL3E-Siah1p53RE3-2mut) are shown above. (F) Luciferase assay using pGL3E-Siah1p53RE3-2 and 3-2mut promoter vectors. Luciferase activity was measured as described in (C).

To further investigate whether the p53 protein is actually able to bind this p53 RE, Electrophoretic Mobility-Shift Assay (EMSA) (Figure 2A) and Chromatin Immunoprecipitation (ChIP) assays (Figure 2B) were performed using MCF7 cells (Figure 2A). In both experiments, we observed actual p53 binding to the p53-RE-3-2. Thus, we concluded that p53 binds directly to the identified p53 RE of Siah1 in vitro and in vivo.

Figure 2. p53 binds directly to the p53RE of Siah-1 in vitro and in vivo. (A) Electrophoretic Mobility-Shift Assay was performed using a 27bp probe including the p53 binding site of Siah1 (Shown in Figure 2e) and recombinant p53 protein (30 ng, Active Motif) in the presence or absence of anti-p53 antibody (Pab421, Oncogene). Specific binding was determined by adding either unlabeled homologous probe DNA or mutant DNA (Shown in Figure 2e) at 50-fold molar excess. The positions of the free probe and shifted bands are indicated. (B) Chromatin Immunoprecipitation assay was performed as described previously (de Belle, I et al. 2000, BioTechniques). Chromatins from MCF7 cells with or without 24hr after UV-irradiation (10 J/m²) were immunoprecipitated with (IP p53) or without (IP ctrl) anti-p53 antibody (FL-393, Santa Cruz) overnight at 4°C and followed by incubation with protein A-Sepharose beads (Santa Cruz) for an additional 1hr. After the DNA fragments were purified, PCR amplification was performed using the Siah1 specific primers (5’-AGACATAGCTATTGCAGCCTTTAC-3’ and 5’-
TATTTTGAGGCTTCCAACCAAGC-3') designed to amplify a 280-bp fragment including p53 binding site. The same samples were used for PCR using Cyclophilin A primers (5’-CTCCTTTGAGCTGTTTGCA-3’ and 5’-CACCGACTGTTGCCATCC-3’) as a negative control. Total lysate was used as a positive control (input).

Next, to investigate whether Siah1 protein is actually expressed by p53 in cells, RNase protection assays and western blot analysis were performed using HEK293 cells. Results showed no difference of expression of Siah1 mRNA before and after when HEK293 cells were over-expressed with p53 (Figure 3A). However, a larger size mRNA band was highly expressed after these cells were over-expressed with p53 (especially 12 hours later). Expression of the long type of Siah1 protein, named Siah1L, was also confirmed by western blotting using Siah1-specific antibodies (Figure 3B). The protein sizes of endogenous Siah1 and Siah1L corresponded with transiently transfected Siah1 and Siah1L, respectively using expression vectors. The Siah1L expression was confirmed using the Siah1L specific short interfering RNA (siRNA) vector (pSuppress-Siah1L) (Figure 3C). The protein level of Siah1L was decreased by pSuppress-Siah1L, whereas that of Siah1 did not change. These results indicate that only Siah1L is upregulated in response to p53. Currently, we are analyzing other types of prostate cancer cell lines including LNCaP, PC3, ALVA31, Du145, JCA-1, Tsu-prl and the immortalized prostate epithelial cell line 267β1 cells to contrast the expression of Siah1 and β-Catenin proteins.

Figure 3. (A) RNase protection assay of Siah1 mRNA after p53 over-expression. HEK293 cells were transiently transfected with 10 µg of pCMV-p53wt. Total RNAs were extracted from cells at 0, 12, 24 and 48 hr after transfection and Siah1 RNA expression was measured using a probe containing 324 bp of Siah1 cDNA. RNase protection assay was performed as previously described [3]. (B) Left; MCF7 cells were treated with UV-irradiation (10 J/m²). Total proteins were extracted from cells at 0, 16, 24 hr after transfection. Right; PPC1 cells were transiently transfected with empty vector or pCMV-p53wt. Cells were lysed with RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS). The same amounts of cells lysates (60 µg per lane) were analyzed by immunoblotting, using antibodies specific for Siah1 (N-15, Santa Cruz), p53 (Pab421, Oncogene). The membrane was reprobed with goat anti-HSC70 antibody (K-19, Santa Cruz) as a control. (C) PPC1 cells were transiently transfected with pcDNA3-Siah1, pcDNA3-Siah1L, and pSuppress-Siah1L (siRNA-Siah1L) in various combinations, as indicated. Empty
pcDNA3 and pSuppress vectors were used as controls. After 24 hrs, the lysates (60 µg per lane) were analyzed by immunoblotting, using antibodies specific for Siah1. The membrane was reprobed with goat anti-HSC70 antibody as a control. The cDNAs encoding human Siah1 and Siah1L were generated by PCR as described previously [3]. pSuppress, an siRNA-expressing plasmid, was kindly provided by D. Billadeau (Mayo Clinic, Rochester, MN). The following Siah1L target sequence was inserted: 5'-CTCCTGCCTCCTTATGTAT-3'.

These results suggest that Siah1L is a direct transcriptional target of p53 and involved in p53-regulated pathway. This aim had been completed and a manuscript is being prepared.

**Aim #2. Determine the importance of p53-induced degradation of β-Catenin in Siah-mediated suppression of AR activity.**

Androgen plays an important role in progressing prostate cancers (CaP) through androgen receptor (AR)-mediated signaling. Recent studies indicate that β-Catenin binds directly to AR and enhances its activity [4-9]. On the other hand, p53 is reported to suppress AR activity indirectly [10], however, its functional role is still unclear. To determine whether the degradation of β-Catenin by Ebi is important for Siah-mediated suppression of AR activity, a dominat-negative Ebi mutant was constructed which fails to bind Skp1 but still binds to β-catenin. As expected, over-expression of the mutant Ebi abrogated the Siah1-induced suppression of AR activity, suggesting that Ebi is essential for the Siah1–induced suppression of AR activity (Figure 4A).

To further investigate whether Siah1L is necessary for p53-dependent regulation of AR activity, we used Siah1L-specific siRNA. AR activity was measured by luciferase assay using PPC1 cells transfected with pMMTV (AR responsive luciferase reporter plasmid). p53 over-expression resulted in a decrease of AR activity (Figure 4B). However, introduction of Siah1L siRNA failed to suppress AR activity by p53 (Figure 4B). These data suggest that Siah1L is important for p53-dependent β-catenin degradation pathway.

**Figure 4.** PPC1 cells were transiently transfected with pMMTV-Luc plasmid that contains an AR responsive element cloned upstream of a luciferase reporter gene together with pCMV-βGal as a transfection-efficiency control and the indicated plasmids encoding AR-encoding plasmid pSG5-AR(AR),
β-Catenin, p53, Siah1, Ebi (EbiΔF) or pSuppress-Siah1L (siRNA-Siah1L). Twenty-four hours after transfection, cells were stimulated with 1 nM R1881. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity at 48 h. Data were normalized using β-galactosidase, and results are expressed as –fold transactivation relative to cells transfected with the reporter gene alone (mean ± S.D.; n=3).

Next, we analyzed the effect of this pathway using various p53 wild-type (WT) and p53 mutated (MT) CaP cell lines. AR activity was measured by luciferase assay using PPC1 cells transfected with pMMTV (AR responsive luciferase reporter plasmid). Over-expression of β-Catenin enhanced AR activity in p53WT, but not in p53MT CaP cell lines (Figure 5). Thus, our findings suggest that Siah1 regulates AR-mediated signaling in CaP cell lines through p53-induced β-Catenin degradation and might be one of key proteins for CaP progression.

These results suggest that Siah1 is necessary for p53-dependent regulation of AR activity. This aim has been completed and a manuscript is being prepared.

**Figure 5. Dependency of p53 on AR activation by β-Catenin.**
The AR-encoding plasmid pSG5-AR(AR) was co-transfected into p53 wild-type prostate cancer cells (p53 WT) and p53 mutated prostate cancer cells (p53 MT) with pMMTV-luc reporter plasmid, pCMV-βGal and plasmid encoding β-Catenin and Siah1 as indicated. Twenty-four hours after transfection, cells were stimulated with 1 nM R1881. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity at 48 h. Data were normalized using β-galactosidase, and results are expressed as –fold transactivation relative to cells transfected with the reporter gene alone (mean ± S.D.; n=3).
**Aim #3.** Explore the effects of Siah1 on the control of sensitivity of human prostate cancer cell lines to anti-androgens in vitro and in xenograph mouse models.
We have attempted to establish stable cell lines that express TET-inducible Siah1. However, we could not obtain the cell line. We also had tried to use other expression systems, such as several metal-inducible systems, but none of them worked successfully. One major problem was a leaking expression of Siah1 in these the prostate cancer cell lines since Siah1 expression, even low amount, induced apoptosis in the cells. Therefore, we could not proceed to the experiment using xenograph mouse model.

**Aim #4.** What are the mechanisms by which Siah1 suppresses androgen receptor activity?

To identify potential targets of Siah1, yeast two-hybrid screens of cDNA libraries made from prostate cancer cell lines were performed using the human Siah1 protein as a bait. This effort has resulted in identification of a known Siah-binding protein AF4 [4, 5] and a novel protein that we call SIP2, for Siah1 Interacting Protein 2 (Figure 6).

Figure 6. Identification of SIP2 as a candidate Siah1-binding protein by two-hybrid cDNA library screening. Library screening by the yeast two-hybrid method was performed as described [3] using pGilda encoding human Siah1 as a bait, cDNA libraries derived from PPC1, and EGY48 strain yeast. Cells were grown in either YPD medium with 1% yeast extract, 2% polypeptone, and 2% glucose, or in Burkholder's minimal medium (BMM) fortified with appropriate amino-acids as described previously [3]. Transformations were performed by a LiCl method using 0.1 mg of pJG4-5-cDNA library DNA, and 5 mg of denatured salmon sperm carrier DNA. Clones that formed on Leu-deficient BMM plates containing 2% galactose / 1% raffinose were transferred to BMM plates containing leucine and 2% glucose, and filter assays were performed for β-galactosidase measurements. The specificity of two-hybrid interactions mediated by candidate cDNA clones was evaluating by mating with RFY206 cells, which contained one of 4 different indicator pGilda plasmids encoding the following LexA bait proteins: Ebi, Bax (1-171), Fas (191-335) or v-Ras.
**Siah1 binds a novel protein SIP2.** Among the other cDNA clones identified in the two-hybrid screen described above were 3 that encoded a novel protein we have termed SIP2, for Siah1 Interacting Protein 2.

SIP2 binds specifically to Siah1 in yeast two-hybrid assays. The minimal length cDNA clones that permitted positive interactions in yeast two-hybrid experiments encompassed the C-terminal 151 amino acids of SIP2. A search of the homology database shows that there is no homology against known proteins. Interestingly, at least 3 putative Siah-binding motifs [13] were identified in the C-terminal region of SIP2 protein (Figure 7). Site-directed mutagenesis of these Siah-binding motifs showed loss of binding of Siah1 to SIP2 by yeast 2-hybrid system (data not shown). Interaction of SIP2 with Siah1 has been confirmed by in vitro binding experiments and co-immunoprecipitation assays in which epitope tagged Siah1 and SIP2 proteins were co-expressed in 293T cells (Figure 8).

![Figure 7. Structure of SIP2 protein.](image)

![Figure 8. Analysis of Siah1 and SIP2 interactions.](image)

**Figure 8. Analysis of Siah1 and SIP2 interactions.** (A) Radiolabeled Siah1 protein was produced by in vitro translation (IVT) in reticulocyte lysates in the presence of $^{35}$S-L-methionine. $^{35}$S-Siah1 was incubated with 1 µg of GST, GST-CD40 cytosolic domain, or GST-SIP2 proteins immobilized on glutathione-Sepharose. After 1 hr, beads were washed extensively and analyzed by SDS-PAGE/autoradiography. As a control, 0.1 volume of input in vitro translated (IVT) $^{35}$S-Siah1 was loaded directly in the same gel. (B) 293T cells were transfected with plasmids producing HA-tagged Siah1 or nm23, myc-tagged SIP2, in various combinations. Controls (-) represent cells transfected with HA or myc-tag pcDNA3 lacking a cDNA insert. Lysates were either loaded directly in gels or subjected to immunoprecipitation using either
anti-HA antibody or control IgG. Immune-complexes were analyzed by SDS-PAGE/immunoblotting using anti-myc-tag antibody with ECL-based detection.

The region in Siah1 required for SIP2 binding was preliminarily mapped to the C-terminus of Siah1 (Figure 9), based on yeast two-hybrid assays using a panel of Siah-1 truncation mutants constructed in our previous work [13]. These two-hybrid assay results were confirmed by in vitro protein binding assays, using GST-SIP2 (659-810) fusion protein for binding to in vitro translated full-length Siah1, Siah1 (ΔRING), or Siah-1 (ΔC) proteins (not shown). Thus, the N-terminal RING domain (which binds UBCs) is not required for SIP2 binding. We therefore speculate that Siah can bridge UBCs to APC, using its N-terminal domain to bind UBC and its C-terminal domain to bind APC.

Figure 9. SIP2 Binds Carboxyl-Domain in Siah1. Yeast two-hybrid experiments were performed by transforming EGY48 (strain) cells. Wild-type Siah1 or various mutants as indicated were expressed from the plasmid pGilda and tested for interactions with SIP2, which were expressed from the pJG4-5 plasmid. Interactions were detected by trans-activation of LEU2 or β-galactosidase reporter genes. Growth on leucine-deficient medium at 30°C was examined 4 days later.

Siah1 regulates degradation of SIP2. We speculated that SIP2 might become a target of Siah1-induced protein degradation. To preliminarily explore this hypothesis, a myc-tagged SIP2 protein was expressed in PPC1 cells (a) alone, (b) with full-length Siah1, or (c) with a Siah1 (ΔRING) mutant that fails to bind UBCs. Co-expression of Siah1 with SIP2 resulted in a marked decrease in the levels of SIP2 protein (Figure 10). In contrast, the Siah1 (ΔRING) protein did not reduce SIP2 levels in cells.
Figure 10. Siah1 regulate SIP2 levels. PPC1 cells were transiently transfected with 0.2 mg pEGFP and plasmids encoding FLAG-Siah1 (0.2 µg), FLAG-Siah1AR (0.5 µg), or myc-SIP2 (0.5 µg) in various combinations, as indicated (total DNA amount normalized). After 24 h, cell lysates were prepared from duplicated dishes of transfectants, normalized for total protein content (40 µg per lane), and analyzed by SDS-PAGE/immunoblotting using antibodies specific for myc with ECL-based detection.

Explore whether alterations in the levels of Siah-family proteins occur during the pathogenesis of prostate cancer. To begin our studies of the regulation of SIP2 gene expression, we developed RNAase protection assays that allow for specific detection of each of the three known members of the Siah-family. We have first analyzed the level of expression of Siah-family genes in different tumor cell lines. The level of SIP2 expression was assayed by RNase protection assay in several prostate tumor lines. The results obtained demonstrated that the SIP2 gene was expressed in all prostate cancer cell lines. However, significant alterations in mRNA levels of SIP2 had not been observed in prostate cancer cell lines compared with other cancer cell lines or normal cell lines.

We have begun to raise mouse monoclonal antibodies specific for SIP2 using these reagents to assess the expression of SIP2 proteins in prostate cancer cell lines and prostate tumors by immunoblotting and immunohistochemistry.

Functional analysis of SIP2 in AR signaling pathway.
To explore the functional consequences of Siah1/SIP2 interactions, we examined the effect of the SIP2 proteins on AR activity. AR activity was measured by luciferase assay using LNCap cells transfected with plasmids encoding β-catenin, Siah1 or SIP2 in various combinations. However, induction of SIP2 did not affect on the β-catenin-activated AR activity. Furthermore, over-expression of the SIP2 had no influence on the Siah1-induced suppression of AR activity. Further analysis will be needed to determine the function of SIP2 using siRNA or gene targeting mouse in the future.

Key Research Accomplishment:
We have made excellent progress in the first year of funding towards accomplishing these goals. Two manuscripts are being prepared regarding Aim#1 and #2. However, we could not complete Aim#3 with technical problem. We also obtained a potential target of Siah1 termed SIP2. To explore the functional consequence of Siah1/SIP2 interaction, further analysis will be needed.
Reportable Outcomes:

Publication

Meeting Abstract


Conclusions:
We have determined that *Siah1* is a primary response gene for p53. We have also demonstrated that Siah1-specific siRNA showed down-regulation of AR activity by p53. These findings suggest that Siah1 is a critical regulator of AR signaling and there are new strategies for restoring tumor suppressive pathways lost in cancers that have suffered p53 inactivation.
References:
List of Personnel:
Shu-ichi Matsuzawa, Ph.D. Principal Investigator:
Dr. Matsuzawa has been served as the PI for the all project. Dr. Matsuzawa is responsible for the daily supervision of the project personnel and provided the overall scientific and administrative direction for the project. He is also responsible for progress reports and writing or editing of all research reports submitted for publication.

Toru Fukushima, M.D., Ph.D., Post-Doctoral Fellow (40% effort):
Dr. Fukushima is a post-doctoral fellow in the Matsuzawa laboratory who generated most of the data regarding the effects of Siah1 on androgen receptors. He led the investigations of the mechanism of Siah1 in androgen receptor signaling pathway.

John C. Reed, M.D., Ph.D. (Collaborator and Advisor):
Dr. Reed is a President/CEO at the Burnham Institute. Dr. Reed has provided Dr. Matsuzawa's group with reagents and advised for multiple aspects of the work on Siah effects on steroid hormone receptors. He served as a non-paid collaborator and advisor for the project.

Stan Krajewski, M.D., Ph.D. (Collaborator and Advisor):
Dr. Krajewski is a longstanding collaborator of Dr. Matsuzawa and expert pathologist. Dr. Krajewski served as a non-paid collaborator and advisor for the project.

Appendices:
1. Reprint
Critical Function for SIP, a Ubiquitin E3 Ligase Component of the β-Catenin Degradation Pathway, for Thymocyte Development and G1 Checkpoint

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Summary

β-catenin has been implicated in thymocyte development because of its function as a coactivator of Tcf/LEF-family transcription factors. Previously, we discovered a novel pathway for p53-induced β-catenin degradation through a ubiquitin E3 ligase complex involving Siah1, SIP (CacyBP), Skp1, and Ebi. To gain insights into the physiological relevance of this novel degradation pathway in vivo, we generated mutant mice lacking SIP. We demonstrate here that SIP−/− thymocytes have an impaired pre-TCR checkpoint with failure of TCRβ gene rearrangement and increased apoptosis, resulting in reduced cellularity of the thymus. Moreover, the degradation of β-catenin in response to DNA damage is significantly impaired in SIP−/− cells. SIP−/− embryonic fibroblasts show a growth-rate increase resulting from defects in G1 arrest. Thus, the β-catenin degradation pathway mediated by SIP defines an essential checkpoint for thymocyte development and cell-cycle progression.

Introduction

β-catenin, an important regulator of cell-cell communication and embryonic development, associates with and regulates the function of Tcf/LEF-family transcription factors (Peifer and Polakis, 2000). Conditional degradation of β-catenin represents a central event in Wnt signaling pathways controlling cell fate and proliferation (Peifer and Polakis, 2000). Interaction of Wnt-ligands with frizzled-family receptors transduces signals that suppress GSK3β activity, via mechanisms requiring APC, Axin, and other proteins. Suppression of GSK3β reduces phosphorylation of β-catenin, thus eliminating binding of F box protein β-TrCP/Fbw1 and resulting in β-catenin accumulation due to reduced ubiquitin-mediated proteolysis (Hart et al., 1999; Kitagawa et al., 1999b; Latres et al., 1999; Winston et al., 1999). β-catenin accumulation facilitates its translocation to a nucleus where it collaborates with Tcf/LEF-family (high-mobility group) transcription factors to activate expression of target genes important for cell proliferation, such as c-myc (He et al., 1998) and cyclin D1 (Tetsu and McCormick, 1999). Mutations in β-catenin inappropriately activate various transcription factors, thereby promoting malignant transformation (Peifer and Polakis, 2000; Polakis, 2000).

Genotoxic stress triggers activation of checkpoints that delay cell-cycle progression (Abraham, 2001; Zhou and Elledge, 2000). Defective cell-cycle checkpoints lead to genomic instability and a predisposition to cancer. Cell-proliferation arrest is also required to allow T cell receptor (TCR) genes rearrangement in the thymus, which is a crucial step in T cell development (Borowskiet al., 2002; Hoffman et al., 1996). This checkpoint critically depends on stage-specific signals derived from the thymic microenvironment. In this regard, β-catenin has been implicated in T cell development, where it plays an essential role in differentiation of CD4+CD8− double-negative to CD4+CD8+ double-positive thymocytes (Ioannidis et al., 2001; Mulroy et al., 2003; Xu et al., 2003). Moreover, expression of active β-catenin in the thymus results in the generation of T cells lacking mature T cell antigen receptors (TCRs) and impairs thymocyte survival (Gounari et al., 2001).

Upon DNA damage, p53 is stabilized and activated via posttranslational mechanisms, leading to growth arrest or apoptosis (Hall and Lane, 1997; Hartwell and Kastan, 1994; Reed, 1996; Vogelstein et al., 2000). The p53 protein has been shown to transactivate a wide variety of target genes, including the cell-cycle inhibitor p21waf1 (El-Deiry et al., 1993; Harper et al., 1993). Upregulation of p21waf1 inhibits cyclin-dependent kinases, particularly those that function during the G1 phase of the cell cycle. However, waf1-deficient mice develop normally, and fibroblasts derived from p21−/−-deficient mouse embryos are only partially defective in their ability to undergo cell-cycle arrest in response to DNA damage (Brugarolas et al., 1995; Deng et al., 1995). These observations suggest the existence of an alternative p21waf1-independent pathway through which p53 can suppress cell proliferation.

Previously, we and others observed that β-catenin is downregulated by activated p53 (Liu et al., 2001; Matsuzawa and Reed, 2001; Sadot et al., 2001). Furthermore, we discovered that this β-catenin degradation pathway is mediated by a series of protein interactions involving Siah1, SIP (CacyBP), Skp1, APC, and Ebi, an F box protein that binds β-catenin independently of the phosphorylation sites recognized by β-TrCP/Fbw1 (Liu et al., 2001; Matsuzawa and Reed, 2001). Siah family proteins bind ubiquitin-conjugating enzymes and target proteins for proteasome-mediated degradation (Reed and Ely, 2002). We identified a novel Siah-interacting protein (SIP), an SGT1-related molecule that provides a physical link between Siah-family proteins and the SCF ubiquitin E3 ligase component Skp1 (Kitagawa et al., 1999a; Matsuzawa et al., 2003; Santelli et al., 2005). Expression of Siah1 is induced by p53 (Iwai et al., 2004; Matsuzawa et al., 1998), thereby linking genotoxic injury to destruction of β-catenin, thus reducing activity of β-catenin-binding Tcf/LEF transcription factors and contributing to cell-cycle arrest (Matsuzawa and Reed, 2001).

To gain insights into the function of SIP in p53-induced β-catenin degradation in vivo, we generated SIP knockout mice. First, we identified a specific stage of T cell development at which SIP is required for T cell
differentially, mimicking the previously reported effect of constitutive expression of β-catenin in thymocytes. Second, we show that embryonic fibroblasts derived from mice lacking SIP are deficient in their ability to arrest in G1 following DNA damage. These data underline the essential role of SIP as a component of the p53-regulated pathway that controls β-catenin levels in vivo and regulates the thymocyte development and G1 cell-cycle checkpoint.

Results

Generation of SIP-Deficient Mice

The murine ortholog of SIP was initially characterized as a calcyclin-binding protein (CacyBP) (Filipek and Kuznicki, 1998; Filipek and Wojda, 1996) and resides on chromosome 1H1. By searching the Omni Bank database (http://www.lexgen.com) of ES cell clones with retrovirus insertions, we identified a clone containing a retroviral integration in the first intron of the SIP locus. The closed rectangles denote exons 1 and 2 of SIP, and the open rectangles denote the retroviral LTRs.

Smaller Thymus and Spleen Size

in SIP-Deficient Mice

SIP−/− mice were fertile and grew normally during 18 months of observation. However, we noticed that SIP−/− mice have smaller thymus and spleen size than wild-type mice (Figure 2A). Indeed, 4-week-old SIP−/− mice have thymi and spleens that have ~50% the cellularity of organs from wild-type littermates (Figure 2B). This difference in cellularity was not as prominent in 8-month-old SIP−/− mice, which have ~85% of the cells in the thymus and spleen in comparison to wild-type littermates. This phenotype is similar to that of mice expressing stabilized β-catenin in the thymus (Gounari et al., 2001). Histological analysis of the thymus glands of SIP−/− mice showed slightly smaller medullas compared to SIP+/+ mice (Figure 2C). Also, in the spleen, the size of the T and B areas were reduced in SIP−/− mice (Figures 2D–2F), although the splenic structure
appeared normal (Figure 2B), thus suggesting a role for SIP in B and T lymphocytes homeostasis.

Pre-TCR Checkpoint Is Impaired in SIP-Deficient Thymocytes

Thymocytes develop from a double-negative (DN) stage lacking expression of CD4 and CD8 to a double-positive stage (DP), and they then mature to single-positive (SP) T cells expressing either CD4 or CD8 and displaying surface αβ TCR expression (Figure 3A). The DN stage can be further divided into four successive developmental stages identified by the differential expression of CD44 and CD25 on the surface of DN thymocytes: DN1 (CD44+CD25+), DN2 (CD44+CD25−), DN3 (CD44−CD25+), and DN4 (CD44−CD25−). TCRβ gene rearrangements occur at the DN3 stage, resulting in the expression of a pre-TCR (receptor complexes of TCRβ, pTα, and CD3 chains lacking the TCR α-chain). The signals provided by the pre-TCR together with other signals provided by the thymic microenvironment, such as Wnt signaling, support the transition of DN cells to the DP stage. Thymocytes without pre-TCR signaling undergo cell-cycle arrest and apoptosis, defining the pre-TCR checkpoint (Wu and Strasser, 2001). Furthermore, p53 plays an important role in the pre-TCR checkpoint (Bogue et al., 1996; Guidos et al., 1996; Haks et al., 1999; Jiang et al., 1996).

To examine whether SIP-dependent β-catenin degradation is required for the pre-TCR checkpoint, we performed flow-cytometry analyses of thymocytes from 4-week-old wild-type and SIP−/− mice. Whereas both SIP−/− and wild-type mice have similar percentages of DP and CD4 and CD8 single-positive T cell populations (Figure 3B), the analysis of the DN population showed an accumulation of the DN3 population in SIP−/− mice (Figure 3B). Also, among the thymocytes that did manage to progress to the DN4 stage in SIP−/− mice, intracellular TCRβ expression was reduced, compared to wild-type mice (Figure 3C), similar to observations previously made for mice in which stabilized β-catenin was expressed in thymus (Gounari et al., 2001).

To further confirm that SIP−/− thymocytes are able to develop from DN3 to DN4 stage in the absence of TCRβ gene rearrangement, we measured catalytic activity of recombinase in SIP−/− thymocytes. Recombination-activating genes RAG1 and RAG2 are essential for the first step of V(D)J recombination in TCR gene rearrangement. Mice lacking either of these genes are unable to undergo V(D)J recombination, causing a complete block of thymocyte differentiation at the pre-TCR checkpoint (Mombaerts et al., 1992; Shinkai et al., 1992). To measure recombinase activity, we used a retroviral vector that contains the 12- and 23-recombination signal sequences (RSS), separated by a reverse EGFP cDNA relative to 5’LTR (Figure 3D). The retroviral vector integrates into the host genome, whereas the LTR induces bicistronic transcripts that allow for the simultaneous assessment of recombinase resulting in inversion of the GFP cassette and GFP expression (Liang et al., 2002). As shown in Figure 3E, recombinase activity...
was significantly reduced in *SIP*<sup>−/−</sup> DN4 cells compared with wild-type cells, but not in DN3 cells. Thus, the absence of SIP allows thymocytes to progress to the DN4 stage despite absence of recombinase activity, indicating that these SIP-deficient cells defy a checkpoint in thymic development, akin to observations from mice with constitutive expression of β-catenin in thymocytes (Gounari et al., 2001).

**Accumulation of β-catenin Protein and Increased Apoptosis in SIP-Deficient Thymocytes**

To confirm that SIP deficiency results in elevated β-catenin expression in the developing thymus, we isolated DN cells by cell sorting and compared β-catenin levels in wild-type and mutant mice. DN thymocytes were fractionated into Triton X-100-soluble and -insoluble fractions to distinguish cytosolic versus nuclear β-catenin (Sadot et al., 2000, Sadot et al., 2001). As shown in Figure 4A, the level of β-catenin in *SIP*<sup>−/−</sup> DN cells was remarkably increased in both the Triton X-100-soluble and -insoluble fractions compared to wild-type thymocytes. To analyze the β-catenin levels in thymocytes more quantitatively, we evaluated intracellular β-catenin immunostaining by flow cytometry and combined it with surface staining for CD4, CD8, CD25, and CD44 antigens (Figure 4B). Increased accumulation of β-catenin protein was observed in *SIP*<sup>−/−</sup> DN3 cells compared to wild-type DN3 cells, and to a lesser extent in DN4 thymocytes.

Altogether, these results suggest that SIP-deficient thymocytes reach the DN3 stage of thymic development, then either fail to complete differentiation or die. Because *SIP*<sup>−/−</sup> mice showed reduced thymic cellularity compared to wild-type mice, we hypothesized that immature *SIP*<sup>−/−</sup> thymocytes undergo apoptosis after disobeying the pre-TCR checkpoint. To examine this possibility, we analyzed the survival of thymocytes in culture by Annexin V staining. Apoptotic cells were increased among the DN4 (1.4-fold) and the DP (1.8-fold) populations in *SIP*<sup>−/−</sup> thymocytes (Figure 4C), suggesting that SIP deficiency increases apoptosis of developing thymocytes at these stages of development in a manner similar to what has been reported in mice with constitutive expression of β-catenin in thymocytes (Gounari et al., 2001). In contrast, the frequency of apoptotic cells...
was not increased within the DN3 population (data not shown). These data suggest that by disobeying the pre-TCR checkpoint, SIP−/− thymocytes inappropriately proceed from the DN3 to DN4 and DP stage, resulting in reduced survival.

SIP-Deficient Cells Show Impaired β-Catenin Degradation and Defective G1 Cell-Cycle Checkpoint After γ Irradiation and UV Irradiation

p53 plays an important role in the G1 cell-cycle checkpoint induced by UV damage (Kastan et al., 1992; Wahl and Carr, 2001). MEFs provide an ideal cell model system for studying p53-mediated G1 arrest (Attardi et al., 2004). For example, wild-type MEFs treated with γ irradiation undergo p53-dependent G1 arrest, whereas p53−/− MEFs fail to arrest (Kastan et al., 1992). For exploring the role of SIP in the G1 checkpoint induced by DNA damage, early-passage MEFs from SIP+/+ and SIP−/− embryos were subjected to γ irradiation (20 Gy), and the percentage of cells entering S phase was determined 24 hr later (Figure 5A). Prior to γ irradiation, SIP−/− MEFs entered S phase more rapidly than wild-type MEFs upon serum addition, but less than p53−/− MEFs. After 20 Gy γ irradiation, S phase entry by wild-type MEFs was markedly suppressed. In contrast, p53−/− MEFs underwent S phase progression regardless of γ irradiation. SIP−/− cells displayed a phenotype intermediate between wild-type and p53−/− MEFs (Figure 5A). This phenotype of SIP−/− cells is quite similar to MEFs from p21−/− mice (Deng et al., 1995). These results suggest that SIP is partially required for the G1-phase checkpoint induced by γ irradiation.

Next, we examined levels of endogenous β-catenin protein by immunoblotting. As expected, γ irradiation triggered a decline in β-catenin levels in SIP+/+ MEFs. In contrast, SIP−/− and p53−/− MEFs failed to downregulate β-catenin in response to γ irradiation (Figure 5B).

In addition to the G1 checkpoint, p53 is also known to control the mitotic spindle checkpoint (Cross et al., 1995). To examine whether SIP also has a role in mitotic spindle-checkpoint regulation, we compared the induction of polyploidy by nocodazole in wild-type, SIP+/+, and p53−/− MEFs. Nocodazole-treated SIP+/+ MEFs arrested with 4N DNA content (Figure 5C). In contrast, p53−/− MEFs failed to arrest, proceeding through cell cycle to become polyploid. Cells lacking SIP showed an intact mitotic checkpoint, thus indicating that SIP is not required for the mitotic spindle checkpoint.
Having observed that SIP is required for β-catenin degradation and G1 arrest following γ irradiation, we next examined the effects of SIP deficiency in response to UV irradiation, which we have described induces Siah1 expression and β-catenin degradation (Iwai et al., 2004; Matsuzawa et al., 1998). For these experiments, early-passage MEFs were prepared from SIP+/+ and SIP−/− embryos and subjected to UV irradiation, and then the number of viable cells was counted at various times thereafter. Low-dose UV irradiation (10–20 J/m²) induced proliferation arrest of wild-type (WT) MEFs without inducing substantial apoptosis, whereas SIP−/− MEFs continued to proliferate at nearly normal rates (data not shown). UV irradiation triggered a decline in endogenous β-catenin protein levels in MEFs from SIP+/+ (Figure 6A) and SIP+/− mice (data not shown). In contrast, SIP−/− MEFs failed to downregulate β-catenin (Figure 6A). Similarly, β-catenin levels were not reduced in UV-irradiated p53−/− MEF cells, consistent with the hypothesis that p53 and SIP operate in the same β-catenin degradation pathway. A similar result was obtained with splenocytes from SIP−/− mice (Figure 6B). Interestingly, the basal levels of endogenous β-catenin protein were higher in SIP+/− lymphocytes than in SIP+/+ and SIP−/− cells, further supporting a role for SIP in regulating endogenous β-catenin expression.

**SIP-Deficient MEFs Show Enhanced Proliferative Properties**

Using early-passage MEFs from SIP+/+, SIP−/−, and SIP−/− embryos, we compared the proliferative properties of cells with different levels of SIP. At low passage (e.g., passage 3), growth rates of MEFs from SIP−/− and SIP−/− MEFs were higher than MEFs from SIP+/+ and SIP+/− embryos. All MEFs showed contact inhibition, but the monolayers formed by SIP−/− MEFs were more crowded than those formed by SIP+/+ and SIP+/−. In addition, the saturation densities of SIP−/− MEFs were significantly higher than those of wild-type and SIP−/− MEFs (Figure 7A). At later passage (e.g., passage 7), proliferation of SIP+/+ and SIP+/− MEFs was significantly reduced, whereas the proliferation of SIP−/− MEFs remained robust (Figure 7A).

Activation of Tcf/LEF-family transcription factors by β-catenin is known to induce expression of cyclin D1, c-myc, and other genes important for cell proliferation (He et al., 1998; Tetsu and McCormick, 1999). To further examine the role of SIP in cell-cycle progression, we
analyzed the expression of β-catenin target genes by using serum-starved wild-type, SIP<sup>−/−</sup>, and p53<sup>−/−</sup> MEFs. After serum starvation, cells were cultured in complete media and harvested 3 hr and 9 hr later, respectively, and the levels of induction of Cyclin D1 and c-Myc were compared. As shown in Figure 7B, SIP<sup>−/−</sup> MEFs showed faster and higher induction of Cyclin D1 and c-Myc protein expression than wild-type MEFs, similar to what is observed in p53<sup>−/−</sup> MEFs. These data are consistent with previous reports describing a role for β-catenin as a transcriptional regulator of cyclin D1 and c-myc genes (He et al., 1998; Tetsu and McCormick, 1999).

Discussion

In this study, we showed that SIP-deficient embryonic fibroblasts have a faster growth rate and express cyclin D1 and c-myc at increased levels in comparison to wild-type MEFs. We also show that these cells are partially...
deficient in their ability to arrest in G1 following DNA damage. SIP-deficiency also inhibits β-catenin downregulation induced by p53-activating stimuli. p21\textsuperscript{wt-1} is a well-known G1 cell-cycle inhibitor induced by p53. However, p21\textsuperscript{−/−} MEFs show only a partial defect in the G1 cell-cycle checkpoint in response to DNA damage, suggesting the existence of other p53-dependent G1 checkpoint pathways. Taken together with previous results, these observations demonstrate that SIP is required for this alternative p53-dependent G1 checkpoint. Although a G1-checkpoint failure may lead to cancer, it is interesting to note that neither p21\textsuperscript{−/−} nor SIP\textsuperscript{−/−} mice develop spontaneous tumors, suggesting that combined deficiency of p21 and SIP may be necessary for tumorigenesis.

Furthermore, we found that SIP\textsuperscript{−/−} mice have smaller thymus glands and spleens than wild-type mice as a result of reduced lymphocyte cellularity. Recently, β-catenin has been implicated in T cell development. Indeed, deletion of β-catenin, Tcf-1, or adenomatous polyposis coli (APC) in the thymus impairs the transition from DN3 to DN4 stage (Gounari et al., 2005; Ioannidis et al., 2001; Xu et al., 2003). In contrast, conditional stabilization of active β-catenin in the thymus results in escape from the pre-TCR checkpoint and promotes thymocyte transition from the DN3 to DN4 and then to the DP stage in the absence of TCRβ selection (Gounari et al., 2001). However, while promoting differentiation, expression of active β-catenin in thymocytes results in reduced cellularity of the thymus, apparently as a result of apoptosis induction (Gounari et al., 2001). SIP deficiency produces a remarkably similar phenotype as enforced expression of β-catenin protein in DN cells, further underlining the essential role of β-catenin in thymocyte development. Indeed, DN3 SIP\textsuperscript{−/−} thymocytes appeared to transit to the DN4 stage in the absence of pre-TCR signaling, giving that SIP\textsuperscript{−/−} DN4 cells contained abnormally low levels of TCRβ. Also, like β-catenin-overexpressing mice, the thymus glands of SIP\textsuperscript{−/−} mice have reduced cellularity, and thymocytes undergo increased apoptosis in culture.

The phenotype of SIP\textsuperscript{−/−} mice is relatively modest compared with mice in which stabilized β-catenin was expressed or APC gene was disrupted in thymocytes (Gounari et al., 2001, Gounari et al., 2005). As shown in Figures 4A and 4B, levels of β-catenin in SIP\textsuperscript{−/−} DN thymocytes were 3–4 times higher than in wild-type mice. However, β-catenin-overexpressing mice and APC\textsuperscript{−/−} mice show a huge accumulation of β-catenin in thymocytes (Gounari et al., 2001, Gounari et al., 2005), suggesting that the severity of the phenotype is determined by the magnitude of β-catenin accumulation. For Siah1a, we found that the integration site for the retrovirus was located in exon 2 of mouse SIP gene, which was amplified by PCR methods. Genomic DNA was isolated from MEFs of wild-type and SIP-deficient littermate mice. A total of 10 μg of DNA was digested with XbaI and size fractionated in agarose-gel electrophoresis, followed by transfer to Hybond N+ nylon membranes. Membranes were incubated with random primed \(^{32P}\)-labeled mouse SIP cDNA probe in 10% dextran-sulfate, 1% SDS, and 1 M NaCl solution at 68ºC for overnight. Membranes were washed with 2x SSC, 0.1% SDS at room temperature, followed by 1 x to 0.1 x SSC at increased temperature. Autoradiography was performed overnight at −80ºC with Kodak AR film.

Experimental Procedures

Generation of SIP-Deficient Mice and Genotyping

Searching the Omni Bank database (http://www.lexgen.com) for ES cell clones with retrovirus insertions in the SIP gene, we found a clone in which the integration site for the retrovirus was located in the first intron of mouse SIP gene. The position of retrovirus insertion was determined by Southern blotting with probes derived from exon 2 of mouse SIP gene, which was amplified by PCR methods. Genomic DNA was isolated from MEFs of wild-type and SIP-deficient littermate mice. A total of 10 μg of DNA was digested with XbaI and size fractionated in agarose-gel electrophoresis, followed by transfer to Hybond N+ nylon membranes. Membranes were incubated with random primed \(^{32P}\)-labeled mouse SIP cDNA probe in 10% dextran-sulfate, 1% SDS, and 1 M NaCl solution at 68ºC for overnight. Membranes were washed with 2x SSC, 0.1% SDS at room temperature, followed by 1 x to 0.1 x SSC at increased temperature. Autoradiography was performed overnight at −80ºC with Kodak AR film.
Apoptosis Analysis
DN4 stages. The cells were then fixed for 10 min in PBS with 2% Thymocytes were labeled for cell-surface markers to define DN3 and Intracellular-Thymocyte Staining ware (Tree Star).

The following monoclonal antibodies (mAbs) for flow cytometry were obtained from BD Biosciences Pharmingen: anti-CD4, anti-CD8, anti-CD45, anti-CD4, anti-TCR(α/β)/H57-597, anti-TCR-γ/δ(GL3), anti-CDX, and anti-B220. These mAbs were directly coupled to fluo-

rescein isothiocyanate (FITC), phycoerythrin, cyanine-dye-coupled anti-DX5, and anti-B220. These mAbs were directly coupled to fluo-

tropicin chlorophyll protein (PerCP-Cy5.5), or allophycocyanin.

Thymocytes were incubated with 2.4G2 (anti-CD16/32) to reduce the background. Cells were flow sorted with a FACSCanto (BD Bio-
siences), or with 65

thymocytes with FITC-conjugated anti-BrdU mAb (BD Biosciences). The sections were visualized by SDS-PAGE (12% gels) and transferred to nitrocellulose mem-

branes. Proteins were detected with anti-β-catenin monoclonal antibody (14, BD Biosciences), and blots were reprowed with anti-

tubulin (TU-01, Zymed Laboratories) and PARP (C2-10, BD Biosciences).

Retroviral-Infection GFP Reporter Assay
The retroviral 12/23 rEGFP vector was kindly provided by Dr. Cortez (Mt. Sinai School of Medicine). The vector contains a reverse EGFP reporter (rEGFP) between 12 and 23 RSS. For retroviral infection, 293T cells at 60%–70% confluence were transfected with 5 μg PMD.G (encodes vesicular stomatitits G Protein), 8 μg pMD.OGP (en-
codes gag-pol), and 10 μg retroviral 12/23 rEGFP vector for 48 hr. Thymocytes from Sip+/− and Sip−/− mice were infected with retro-

viral supernatant containing 50 μM β-mercaptoethanol and 4 μg/ml polybrene (Sigma) at 1 × 10^6 cells/ml in 24-well plates. For increas-
ing infection efficiency, plates were centrifuged with a swing-bucket rotor at 1400 × g, 30°C for 60 min. After 48 hr infection at 37°C/6% CO2, cells were harvested and analyzed by flow cytometry.

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References
Bender, T.P., Kremer, C.S., Kraus, M., Buch, T., and Rajewsky, K. (2004). Critical functions for c-Myb at three checkpoints during thy-

tion and rescue of V(D)J rearrangement in scid mouse thymo-


fective in G1 checkpoint control. Cell 82, 675–684.


