Title
Ionizing radiation induces ATM-independent degradation of p21Cip1 in transformed cells

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Ionizing Radiation Induces ATM-independent Degradation of p21\textsuperscript{Cip1} in Transformed Cells

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in

Biomedical Sciences

by

Scott Stuart

Committee in charge:
Professor Jean J.Y. Wang, Chair
Professor Steven F. Dowdy
Professor Geoff Wahl
Professor Tony Wynshaw-Boris
Professor Huilin Zhou

2008
The Dissertation of Scott Andrew Stuart is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2008
Dedication

This dissertation is dedicated in memory of Evelyn F. Mahle and Natalie J. Stuart.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>A-T</td>
<td>Ataxia-Telangiectasia</td>
</tr>
<tr>
<td>Abl</td>
<td>Abelson murine leukemia viral oncogene homolog 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-Telangiectasia Mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine Triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM/Rad3 Related</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>Breakpoint Cluster Region-Abelson fusion protein</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>cdc</td>
<td>cell division cycle</td>
</tr>
<tr>
<td>cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>Chk1</td>
<td>Checkpoint kinase 1</td>
</tr>
<tr>
<td>Chk2</td>
<td>Checkpoint kinase 2</td>
</tr>
<tr>
<td>CHX</td>
<td>Cyclohexamide</td>
</tr>
<tr>
<td>Cip</td>
<td>Cdk-interacting protein</td>
</tr>
<tr>
<td>CKI</td>
<td>Cdk Inhibitor protein</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myelogenous Leukemia</td>
</tr>
<tr>
<td>CSN</td>
<td>COP9 signalosome</td>
</tr>
<tr>
<td>Cul</td>
<td>Cullin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>DCAF</td>
<td>DDB1-Cul4 associated factors</td>
</tr>
<tr>
<td>DDB1</td>
<td>DNA damage binding protein 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>E1</td>
<td>E1 Ubiquitin activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>E2 Ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>E3 Ubiquitin ligase</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte Macrophage Progenitor</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>HFF</td>
<td>Human Foreskin Fibroblast</td>
</tr>
<tr>
<td>INK4</td>
<td>Inhibitors of cdk4</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing Radiation</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal Ribosome Entry Site</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid Enhancer Factor</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
</tr>
<tr>
<td>MEP</td>
<td>Myeloid Erythroid Progenitor</td>
</tr>
<tr>
<td>mHPC</td>
<td>murine hematopoietic progenitor cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MSCV</td>
<td>Murine Stem Cell Virus</td>
</tr>
<tr>
<td>Nbs1</td>
<td>Nijmegen breakage syndrome 1</td>
</tr>
<tr>
<td>p210</td>
<td>BCR-ABL 210 kilodalton protein</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PIKK</td>
<td>PI3-like protein kinase</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1/Cul1/Rbx1/F-box complex</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>Skp2</td>
<td>S-phase kinase associated protein 2</td>
</tr>
<tr>
<td>Smc1</td>
<td>Structural maintenance of chromosomes protein 1</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TCF</td>
<td>Transcription Factor Gene</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet radiation</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>ZVAD-fmk</td>
<td>N-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethylketone</td>
</tr>
</tbody>
</table>
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our family, it all starts with him, and this would have been much more difficult without his support.

The text of Chapter 2, in part, has been submitted for publication to the *Journal of Biological Chemistry*. Scott A. Stuart and Jean Y.J. Wang. I was the primary investigator of this research as well as the primary author of the manuscript.

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VITA

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To obtain a postdoctoral position in a challenging biomedical sciences or biochemistry laboratory where I can conduct translational research that has the potential to better our understanding of human disease.

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Publications


Abstracts


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Responsibilities included designing and performing molecular biology experiments.
Abstract of the Dissertation

Ionizing Radiation Induces ATM-independent Degradation of $p21^{cip1}$ in Transformed Cells

by

Scott Stuart

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2008

Professor Jean J.Y. Wang, Chair

Eukaryotic organisms have developed a complex network of cell cycle control pathways in an attempt to ensure the faithful replication and division of the genome. One important component of this network is the cyclin-dependent kinase inhibitor $p21$ which is upregulated in normal cells in response to a number of cellular stresses, including ionizing radiation (IR). However, we have discovered that, in transformed cells, ionizing radiation leads to the degradation of $p21$. We show that despite being induced by IR, this process is independent of the most well characterized transducers of the DNA damage signal, the PI3-like family of protein kinases (PIKK). We demonstrate degradation of $p21$ after IR is ubiquitin-dependent and requires the Cul4-DDB1$^{Cdt2}$ E3 ligase. In addition, we show that mutations in $p21$ that prevent it from binding to PCNA also prevent its degradation after IR. As $p21$ degradation has been implicated in facilitating DNA repair, the specificity of this event for transformed cells
suggests that these cells may harbor an additional DNA damage repair pathway not present in normal cells.

In a separate project, we have worked to establish a mouse model of Chronic Myelogenous Leukemia (CML). CML is a chronic myeloproliferative disorder which inevitably progresses to a terminal blast crisis phase without treatment. In human patients, blast crisis is characterized by the presence of abnormal granulocyte macrophage progenitors (GMP) that have increased self-renewal capacity and elevated levels of nuclear β-catenin. We have found that expression of p210BCR-ABL in an established line of pluripotent E2A-knockout mouse bone marrow cells leads to expansion of a leukemia-initiating GMP-like population of cells. Upon transplantation into mice, these cells generate a CML-like disease that can be transplanted into secondary recipients. Analysis of the leukemic GMP demonstrated that these cells have higher levels of β-catenin activity, both in vitro and in vivo, than either the transformed non-GMP or the untransformed GMP. These data provide additional evidence that GMP-like cells with elevated levels of β-catenin activity may function as the leukemic stem cells in CML.
Chapter 1:
Introduction

With all that is now understood about the biology of cells, it’s hard to believe that as late as the mid-1800’s it had not been determined how new cells arise (1). Of course, it is now known that all cells arise from the replication and division of other cells, and that it is this process, known as the cell cycle, that allows all organisms to develop and reproduce. At its most basic level, the cell cycle involves the replication of genetic material by way of DNA synthesis (S phase), and the division of this genetic material into two daughter cells by way of mitosis (M phase). As the survival of all organisms depends on the fidelity of these two processes, it is not surprising that many organisms have developed an intricate regulatory network that monitors a cell’s readiness to enter and progress though each of these phases of the cell cycle. In addition to S-phase and M-phase, most eukaryotic cells also include gap phases (G1 prior to S-phase and G2 prior to M-phase) that allow the cell to evaluate the internal and external environment prior to committing to either S-phase or M-phase. Thus, the eukaryotic cell cycle is generally divided into four distinct phases: G1, S, G2, and M. Cyclin-dependent kinases (cdks) play integral roles during each of these phases (2,3).

Cyclin-Dependent Kinases

As their name implies, the activity of cyclin-dependent kinases requires binding to a member of the cyclin family of proteins. There are at least 12 cdks and 10
cyclin families in the human genome. Of these, 4 cdk's (cdk1, cdk2, cdk4, cdk6) and four classes of cyclins (cyclins A, B, D, and E) play major roles in governing transit through the cell cycle (4,5). And in each instance, the cyclin:cdk complexes have a positive effect on cell cycle progression, promoting the transition from one phase to the next. The D-type cyclins function in complex with cdk4 or cdk6 to promote progression through early G1, while cyclins A and E function in complex with cdk2 to initiate S-phase (6,7). Entry into M-phase then requires the activation of cyclinB:cdk1 complexes, which need to be destroyed prior to exit from mitosis (8). To ensure that these events only occur in the proper order and under the right conditions, there exist a number of mechanisms through which cdk's can be regulated.

The primary means of controlling cdk activity is through regulation of cyclin expression. While cdk levels remain fairly constant throughout the cell cycle, cyclins are expressed only periodically. In the absence of cyclins, cdk kinase activity is prevented by occlusion of the substrate binding site and improper orientation of the ATP \( \gamma \)-phosphate. Cyclin binding causes a conformational change that removes both of these restraints (3,9).

Cdns can also be regulated by phosphorylation, and although cyclin binding yields a kinase with basal activity, full activation of cdk's requires phosphorylation of a conserved threonine residue in a region known as the T-loop (10,11). Furthermore, phosphorylation and dephosphorylation of two residues in the N-terminal lobe of cdk's (T14,Y15) can function as a molecular switch controlling cdk activity, with the dephosphorylated form being the much more active kinase. Several kinases including
Wee1 and Myt1 are capable of phosphorylating these sites, while the Cdc25 family of phosphatases remove these phosphates and promote kinase activity (3). This mode of regulation is particularly evident in the activation of cyclinB:cdk1 complexes which are activated largely through the rapid dephosphorylation of these two N-terminal sites by Cdc25C (12).

A third way cyclin-cdk activity is controlled within the cell is through interaction with cdk inhibitory proteins (CKIs). There are two distinct families of CKIs in humans. Inhibitors of the INK family of CKIs (which includes p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}) specifically bind to and inhibit cdk4 and cdk6, resulting in the inhibition of cyclin D complexes. Overexpression of p16^{INK4a} results in G1 arrest, primarily by inhibiting the cdk4/6-dependent phosphorylation of the transcriptional repressor Rb (13). Unlike the INK inhibitor proteins, members of the Cip/Kip family of CKIs (which includes p21^{cip1}, p27^{kip1}, and p57^{kip2}) are capable of binding to each of the cyclin:cdk complexes in vivo (at least the aforementioned cdk complexes with cell cycle functions) and inhibiting each of these complexes in vitro (14,15). However, in vivo, the main target of these CKIs appear to be the cyclin A/E- cdk2 complexes, and upregulation or overexpression of these inhibitors arrests cells in late G1.

Cell Cycle Checkpoints

Cell cycle checkpoints are inhibitory signaling pathways that prevent cells from transitioning to one phase of the cell cycle before the previous phase has been
completed (16). Nearly all checkpoints inhibit cell cycle progression by targeting the cyclin-dependent kinases. In an unperturbed cell cycle, these checkpoint pathways ensure that DNA synthesis is completed prior to mitosis and that all chromosomes are attached to the mitotic spindle prior to chromosome segregation and cytokinesis (17,18). In addition, checkpoint pathways can be activated in response to cellular stresses to ensure that cells do not enter S-phase or M-phase with a damaged genome.

In eukaryotic cells, there exist three DNA damage checkpoints (G1, intra-S, and G2/M), and each of these functions primarily through inhibition of cdks (19). Another similarity shared by these checkpoints is that they each rely on members of the PI3-like family of protein kinases (PIKKs), which includes ATM, ATR, DNA-PK, and hSMG-1. Of these family members, ATM and ATR are most important for the checkpoint responses, with ATM responding primarily to damage generated by ionizing radiation (IR) and other agents that generate DNA double-strand breaks, while ATR primarily responds to damage generated by ultraviolet radiation (UV) and replication stress (20,21).

The IR-induced G1 checkpoint has been extensively studied and appears to be comprised of two separate pathways that lead to arrest with different kinetics. Both the rapid response and the prolonged response are dependent on the damage-induced activation of ATM (19). In the rapid response, which persists no more than a few hours after damage, activation of ATM leads to Chk2-dependent phosphorylation of the Cdc25A phosphatase, promoting its degradation and preventing cdk2 from being activated by dephosphorylation (22,23). In the prolonged response, ATM activation
leads to phosphorylation of p53 on a number of residues, which results in both increased stability and increased transcriptional activity of the protein (21,24-26). This leads to elevated expression of the cyclin-dependent kinase inhibitor p21\(^{cip1}\) (hereafter referred to as p21), which binds to and inhibits cyclin-cdk2 complexes (27,28).

Importantly, while p21 appears to be a critical mediator of the p53-induced G1 arrest, additional p53 target genes must also be involved as the G1 arrest observed in p21\(^{-/-}\) mouse embryonic fibroblasts (MEFs) in response to IR is intermediate that of WT and p53\(^{-/-}\) MEFs (29).

Irradiated cells also show an immediate, but transient, reduction in DNA synthesis, and this has been termed the intra-S phase checkpoint. Cells from patients with Ataxia Telangiectasia lack ATM activity and display almost no reduction in DNA synthesis immediately after IR, suggesting that the intra-S checkpoint is ATM-dependent (20). Similar to the G1 checkpoint, it is possible that the intra-S checkpoint is comprised of two pathways. The first pathway is likely to be the same ATM-Chk2-Cdc25A that functions in G1 cells. As this checkpoint has been shown to prevent the assembly of origins of replication (22), it seems reasonable to expect that it would function to inhibit origin firing both in late G1 and throughout S-phase. The second S-phase pathway appears to require ATM-dependent phosphorylation of the Nbs1 and Smc1 proteins (30,31), although how these events contribute to the intra-S checkpoint and whether they target cdks remains to be determined.

The G2/M checkpoint is similar to the other checkpoints in that it is ATM/ATR-dependent and targets a member of the Cdc25 phosphatases to prevent cdk
activation. In this case activation of ATM (or ATR) leads to the phosphorylation of Cdc25C, creating a binding site for 14-3-3 proteins leading to inactivation of the protein, at least in part due to cytoplasmic sequestration (12,19). Interestingly, the p21 cdk inhibitor has also been implicated in the G2/M checkpoint by a study showing that p21 and Cdc25C may compete for binding to cyclin-cdk-PCNA complexes (32). Thus, elevated levels of p21 may also function to inhibit the Cdc25C-dependent activation of Cdk1.

\textbf{p21^{epI}}

p21 was initially identified more than 15 years ago as part of a quaternary complex that included cyclin D, cdk4, and PCNA (15). Additional studies showed that p21 was a potent inhibitor of many cyclin-cdk complexes (14,28), leading to the hypothesis that p21 may have an important role in cell cycle control. This hypothesis seemed to be substantiated by the identification of p21 as a transcriptional target of the p53 tumor suppressor and as a gene upregulated in senescent fibroblasts (27,33,34). Conclusive evidence that p21 was an important cell cycle regulator \textit{in vivo} came from aforementioned experiments showing that p21^{−/−} MEFs were partially defective in their G1 checkpoint response (29).

In addition to binding cyclin-dependent kinases, p21 was also shown to interact with proliferating cell nuclear antigen (PCNA), a processivity factor for DNA polymerase (35,36). This interaction was shown to require the C-terminal region of p21 and to inhibit DNA replication \textit{in vitro} (37,38). Based on these \textit{in vitro} data, it was
postulated that p21 upregulation following damage may serve as a means to target both the cell cycle machinery and the replication machinery to ensure that DNA synthesis does not occur. Whether this is actually the case \textit{in vivo} remains to be determined, as reports showing inhibition of PCNA-dependent processes by p21, both \textit{in vitro} or \textit{in vivo}, have all required a significant molar excess (on the order of 50:1) of p21 (37-39). In undamaged cells, p21 levels are relatively low, and even with upregulation of p21 after damage, it seems unlikely that p21 levels would ever reach even an equimolar ratio with an abundant protein like PCNA. This suggests that the cell cycle effects of p21 may be independent of its PCNA-binding function. Several studies showing that the PCNA-binding domain of p21 is dispensable for its growth inhibitory effect support this idea (37,38,40,41).

However, recent studies showing that UV-radiation leads to the degradation of p21 have complicated this issue (42-45). One of these studies showed that p21 degradation led to increases in chromatin-bound PCNA and increases in damage-induced DNA synthesis, suggesting that p21 degradation may be required for PCNA to facilitate DNA repair (42). In addition, another recent study has shown that PCNA-bound p21 plays an important role in controlling replication licensing, again suggesting that p21-dependent inhibition of PCNA may have important consequences \textit{in vivo} (46). Thus, whether the inhibition of PCNA is a critical function of p21 \textit{in vivo} is a question that continues to warrant future studies.

\textbf{Consequences of p21 Loss}
Based on the evidence that p21 was an important transcriptional target of the ATM-p53 pathway, and the fact that knockout of either ATM (47,48) or p53 (49) led to a significant increase in spontaneous tumorigenesis in mice, it was presumed that knockout of p21 would also lead to increased tumorigenesis. And while mice lacking p21 were initially reported to be developmentally normal and have no increased susceptibility to cancer, a later study demonstrated that these mice do in fact show an increased rate of spontaneous tumorigenesis, with an average age of onset of 16 months (29,50). However, this late age of onset suggests that the role of p21 in tumorigenesis is likely more complex than either ATM or p53. It is possible that other checkpoint mechanisms or other Cip/Kip family members compensate for p21 in its absence. Another possible explanation is that p21, which has been reported to play a role in the assembly of cyclin D complexes and in the inhibition of apoptosis (both of which could be oncogenic), has additional functions independent from its role as a cdk inhibitor that preclude it from functioning solely as a tumor suppressor (51,52).

**Regulation of p21 Levels**

Most short-lived proteins are degraded through the ubiquitin-proteasome system (53). In addition, the inducible degradation of many proteins, including several proteins involved in cell cycle control, are also mediated by ubiquitin-dependent degradation. Ubiquitin is a highly conserved protein of 76 amino acids that can be attached to the lysine side chains of target proteins through the sequential actions of three enzymes: E1, E2, and E3 (54). Although it is now understood that the attachment
of ubiquitin polymers can mediate a variety of intracellular processes depending on how many ubiquitin monomers are attached (and how these monomers are linked together), the most common fate of ubiquitinated proteins is degradation via the proteasome (55).

In its free state, p21 is relatively unstructured, likely acquiring a stable structure only when bound to cdks or other proteins (56). In vivo, most p21 is found in higher order complexes (57), and it is likely that the nature of these complexes influence the overall stability of the protein (58). In general, the half life of the protein is reported to be between 30-60 minutes (57,59,60). It is well established that degradation of p21 occurs through the proteasome as inhibition of the proteasome rapidly leads to p21 accumulation. In addition, inhibition of the proteasome also leads to the accumulation of several higher molecular weight forms of p21 demonstrating that p21 can be ubiquitinated in vivo (57,61,62). Whether this ubiquitination is absolutely required for p21 degradation remains to be determined. p21 can directly bind to the C8 subunit of the proteasome and several groups have reported ubiquitin-independent turnover of p21 suggesting that there may be multiple pathways leading to p21 degradation (43,62-64). Thus far, however, only the ubiquitin-dependent pathways have been well characterized in vivo.

It is the E3 enzyme that provides substrate specificity to the ubiquitination reaction, and there are two E3 ligases that have been implicated in the degradation of p21: SCF<sup>Skp2</sup> and Cul4-DDB1<sup>Cdt2</sup>. The SCF E3 ligases are quaternary complexes comprised of Skp1, Cul1, Rbx1, and an F-box adaptor protein, which actually binds
the substrate. In many cases, F-box proteins only bind phosphorylated forms of the substrate, providing a means to control the ubiquitination and degradation of substrates (65). The SCF-dependent degradation of p21 relies on the Skp2 F-box proteins and degradation by the SCF$^{\text{Skp2}}$ complex appears to be enhanced by phosphorylation of p21 at S130 (59,66).

The Cul4-DDB1 E3 ligases are quaternary complexes similar to the SCF E3 ligases, but use adaptor proteins known as DCAFs (DDB1-Cul4 associated factors) to bind substrates (65). The initial indication that p21 might be regulated by Cul4-DDB1 came from studies of a DDB1 conditional knockout mouse showing that deletion of DDB1 in the epidermis led to elevated p21 levels (67). More recently, two reports have demonstrated that the Cul4-DDB1$^{\text{Cdt2}}$ complex is required for degradation of p21 following UV damage (44,45), while another report has suggested that Cul4-DDB1$^{\text{Cdt2}}$ also plays a role in the degradation of p21 during S-phase (46).

Taken together, the above results indicate that there are likely several pathways that lead to the degradation of p21. With the potential impact of p21 loss on a number of critical cell cycle processes, it is important to determine how these pathways work together to control p21 levels.
References


Chapter 2:
Ionizing Radiation Induces ATM-independent Degradation of p21cip1 in Transformed Cells

Abstract

The cyclin-dependent kinase inhibitor p21cip1 plays an important role in the cellular response to DNA damage. In normal cells, genotoxic stress activates the ATM-p53 pathway which upregulates the expression of p21cip1 leading to cell cycle arrest. However, we have found that in many transformed cell lines, ionizing radiation leads to the proteasome-dependent degradation of p21cip1. This inducible degradation is ubiquitin dependent and occurs at IR doses as low as 2 Gray. This process is independent of the ATM pathway as it occurs in the presence of specific inhibitors of ATM and in immortalized A-T fibroblasts. Knockdown of Skp2, an F-box protein capable of regulating the normal turnover of p21cip1, does not prevent the IR-induced degradation of p21cip1. Instead, this process requires the Cul4-DDB1 E3 ligase as shRNA knockdown of DDB1 rescues p21cip1 degradation after IR. Mutating the PCNA binding site of p21cip1 also prevents its IR-induced degradation suggesting that the p21cip1-PCNA interaction is critical for this event. Downregulation of 21cip1 in transformed cells following irradiation may facilitate DNA repair and promote survival.
Introduction

It is important that eukaryotic cells respond appropriately to DNA damage to ensure that the integrity of the genome is maintained. One family of proteins that plays an important role in the coordination of this response is the PI3K-like family of protein kinases (PIKK), which includes ATM, ATR, and DNA-PK (1). Of these family members, ATM seems to be most important for the cellular response to ionizing radiation (IR) and other agents that generate DNA double-strand breaks. Following exposure to IR, ATM kinase activity increases within minutes and leads to the phosphorylation of a number of target proteins that regulate an array of cellular process including the activation of cell cycle checkpoints and the initiation of DNA repair (2). One of the most well characterized targets of ATM is the p53 tumor suppressor protein, which is essential for the G1 checkpoint after IR (3,4). ATM activation leads to phosphorylation of p53 at multiple sites resulting in both the increased stability and increased transcriptional activity of the protein (1). This ultimately leads to the increased expression of many p53 target genes, such as the cyclin-dependent kinase inhibitor p21cip1 (5).

p21cip1 was initially identified in cyclin D1 immunoprecipitates as a component of a quaternary protein complex that included cyclin D1, cdk2 or cdk4, and PCNA (6). Additional studies identified p21cip1 as a potent inhibitor of cyclin-dependent kinases (cdks), suggesting that it played an important role in cell cycle regulation (7-9). This was confirmed by several independent studies that identified p21cip1 as an important mediator of the G1 cell cycle arrest that occurs in response to a variety of cellular
stresses (9-11). In particular, it is now well established that p21<sup>cip1</sup> is critical for the p53-dependent G1 arrest that occurs following DNA damage (10,12-14).

In addition to binding cyclin-dependent kinases, p21<sup>cip1</sup> also directly binds the DNA polymerase processivity factor PCNA through its C-terminal region. This association has been shown to lead to inhibition of PCNA-dependent DNA replication <i>in vitro</i> (15-17). Thus, upregulation of p21<sup>cip1</sup> following DNA damage may potentially target both the cell cycle machinery and the DNA replication machinery. However, high levels of p21<sup>cip1</sup> may also inhibit PCNA-dependent repair following DNA damage. In fact, it has been shown that p21<sup>cip1</sup> is degraded following ultraviolet (UV) radiation and that this degradation is required to facilitate DNA repair (18). This UV-induced degradation of p21<sup>cip1</sup> was shown to require ATR, the PIKK family member known to be activated in response to UV (1). In addition, the F-box protein Skp2, which functions as an adaptor protein for the SCF E3 ligase, was also shown to be required, although several recent reports have suggested that the Cul4A-DDB1 complex may actually be the E3 ligase responsible for this event (19,20).

Here we report that in many transformed cell lines, ionizing radiation leads to the degradation of p21<sup>cip1</sup>. We show that this IR-inducible degradation is dependent on the Cul4A-DDB1 E3 ligase, but is independent of ATM and other PIKK family members.
Experimental Procedures

Reagents

Cyclohexamide, epoxomicin, wortmannin, N-Ethylmaleimide, and 1,10-phenanthroline were from Sigma. ZVAD and MG132 were from EMD biosciences. Complete protease inhibitor cocktail tablets were from Roche. KU-55933 was a gift from Graeme Smith (Kudos Pharmaceuticals). Phleomycin was a gift from Richard Kolodner (University of California, San Diego).

Plasmids

All HA-p21 plasmids were constructed using PCR to introduce the HA-tag and necessary restriction sites into p21, p21Δpcna, or p21K6R. All fragments were then cloned into the multiple cloning site of pcDNA3.1+ (Invitrogen). The original p21K6R plasmid was a gift from Jim Roberts (Fred Hutchinson Cancer Research Center).

Cell Culture

All cells were supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin-streptomycin (Mediatech) unless otherwise indicated. 293, COS-1, HeLa, HCT116 (3-6) cells were cultured in DMEM with high glucose and L-glutamine (Mediatech). A-T fibroblasts (Coriell Repository) were grown in DMEM and supplemented with 100µg/mL hygromycin B (Sigma). BJ normal human foreskin fibroblasts (between population doublings 30-45) were grown in Eagle’s minimal essential media with
Earle’s balanced salt solution (ATCC). Saos-2 cells were grown in McCoy’s 5a medium (Gibco) supplemented with 15% FBS and 1% penicillin-streptomycin.

Immunoblotting

Cells were lysed in RIPA buffer and protein concentration was determined using the Dc Protein Assay (BioRad). Equal amounts of protein were run on 5%, 12%, or 4-20% tris-glycine gels (Invitrogen). Proteins were transferred to PVDF membranes (Millipore) for 1hr at 100V or overnight at 40V using a cooling coil. Membranes were incubated in primary antibody for 2 hours at room temperature or overnight at 4 degrees and in secondary antibody for 1 hour at room temperature. Where LICOR analysis was used, PVDF-FL membranes (Millipore) were used.

Immunoprecipitation

Primary antibodies were conjugated to A/G UltraLink resin (Pierce) for at least 4 hours at 4° and 1.0μg conjugated antibody was incubated with 1.0mg whole cell lysate overnight at 4°. The resin was washed 3 times in RIPA buffer and resuspended in 25μL 3X sample buffer.

Antibodies

Antibodies from Santa Cruz were: p21cip1 (C-19 and H164), Chk1 (G4), Chk2 (A12), SV40 LT (Pab 101), and rabbit IgG. Antibodies from Cell Signaling were: p21 (DCS60 and 12D1), and Skp2 (4358). DDB1 was from BD Pharmingen (612488).
GFP was from Covance (B34). PCNA was from EMD biosciences (PC10). ATM pS1981 was from Rockland Immunochemicals. ATM 5C2 was a gift from Dr. Eva Lee (University of California, Irvine). Secondary antibodies were from Pierce and LICOR.

IR experiments
Cells were seeded at 75-90% confluence in 60mm dishes and media was changed at least every 48hrs. Cells were irradiated 2.5 or 4.5 days after seeding with a JL Shepherd Mark 1 $^{137}$Cs irradiator using a dose rate of 4 Gy/min.

Ubiquitination Assay
To preserve endogenously ubiquitinated p21$^{cip1}$, we adopted a method developed for the detection of endogenously ubiquitinated IkB (Dr. G. Pineda, personal communication). Cells were immediately lysed in 500$\mu$L of 95° ubiquitin lysis buffer (20mM Tris pH7.5, 150mM NaCl, 1% SDS, 45$\mu$M N-Ethylmaleimide, 60$\mu$M 1,10-phenanthroline, 2X protease inhibitor cocktail) and boiled for 10 minutes. Lysates were sonicated continuously for 30 seconds and spun at 14,000rpm for 30 minutes. Clarified lysates were diluted in dilution buffer (lysis buffer without SDS) to a final volume of 5mL. Lysates were immunoprecipitated with 1.0ug p21$^{cip1}$ polyclonal antibody (Santa Cruz H-164) conjugated to 10uL protein A/G UltraLink resin (Pierce) overnight at 4°. The resin was then washed in 20mM Tris pH 7.5, 10% glycerol containing 150mM, 300mM, and 500mM NaCl (washed 2X with each buffer). The
resin was washed once more in wash buffer with 150mM salt prior to being run on a 4-20% tris-glycine gel and transferred to a PVDF membrane and incubated with a monoclonal primary antibody (Cell Signaling DCS60).

Lentiviral shRNA
All shRNAs were from Sigma. The shRNA sequences for DDB1 experiments were

CCGGCGACCGTAAGAAGGTGACTTTTCTCGAGAAAGTCACCTTCTTACGGTCGTTTTTG,
CCGGCGTGTACTCTATGGTGGAATTCTCGAGAATTCCACCATAGACGTTACACGTGTTTTTG,
CCGGCCTATCACAATGGTGACAAATCTCGAGATTTGTCACGATTGTGATAGGTTTTTG. The skp2 shRNA sequence was

CCGGGCCTAAGCTAAATCGAGAGAACTCGAGTTCTCTCGATTTAGCTTAGGCTTTTT.

For virus production the shRNA plasmids were cotransfected along with the pMDL, pRev, and pVSVG packaging plasmids into 293FT cells (Invitrogen) using the calcium phosphate method. Media was changed 12 hours after transfection and viral supernatants were collected 36 hours later. Viral supernatants were filtered and polybrene (Sigma) was added to 8μg/mL prior to infection of target cells. Target cells were infected for 48hrs and selected with 2.0μg/mL puromycin until control cells were dead and completely detached from dish.
siRNA
Skp2 sequences were CCUAUCAACUCAGUUAUAdTdT and CCUUAGACCUCACAGGUAAAdTdT (Ambion). The DDB1 sequence was ACUAGAUCCGAAUAAAAAdTdT (Qiagen). The Cdt2 sequence was GAAUUAUACUGCUUAUCGA (Dharmacon). siRNAs were transfected at 50nM with 10uL Lipofectamine 2000 (Invitrogen) 60 hours prior to irradiation.

QPCR
Total RNA was extracted from 293 cells with the RNeasy kit (Qiagen) and reverse transcribed into cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR was run on the 7900 HT Fast Real-time PCR System (Applied Biosystems) using the Power SYBR Green PCR master mix (Applied Biosystems). Primers were as follows: Actin CGAGAAGATGACCCAGATCATGTT (forward), CCTCGTAGATGGGCACAGTGT (reverse); p21\textsuperscript{cip1} GGCAGGCGCTGCATCCA (forward), AGTGGTGTCTCGGTGACAAAGTC (reverse); DDB1 TTTGTGGGCAGACAAGAGTT (forward), CATCCACGAAACCCCATCAGTT.

Flow cytometry
BrdU incorporation was determined using the BrdU Flow Kit (BD Pharmingen) according to the manufacturer’s suggestions.
Results

**p21cip1 is degraded following IR**

We have observed that irradiation of HEK293 cells leads to a transient reduction in p21cip1 protein levels between 30 and 90 minutes after IR (Fig 1A). Confluent HEK293 cells were used in these experiments because we have observed that these cells, which normally express p21cip1 at low levels, upregulate p21cip1 when they are cultured to confluence (Fig. S1), making it easier to evaluate the extent of p21cip1 degradation. As all other cell lines used in this study (many of which express p21cip1 at low levels presumably due to impaired p53 function) were also found to upregulate p21cip1 upon reaching confluence (data not shown), we conducted all subsequent experiments with confluent cultures of cells.

To determine if the IR-induced decrease in p21cip1 protein level was due to protein degradation, we pretreated HEK293 cells with cyclohexamide (CHX) and looked at the half-life of p21cip1 with or without IR (Fig 1B). We noticed that following a dose of 10 Gy IR, the half-life of p21cip1 decreased from more than an hour to less than 30 minutes. Importantly, we observed no decrease in the rate of p21cip1 transcription in these cells during this same time period, suggesting that the decrease in p21cip1 protein level is entirely due to a decrease in protein stability (Fig. S1).

To establish whether degradation of p21cip1 occurred over a broad range of IR doses, we irradiated HEK293 cells with doses ranging from 0.5-20 Gy. Although a dose of 0.5Gy had only a very modest effect, doses between 2 and 20 Gy all led to a
significant reduction in $p21^{cip1}$ half-life (Fig 1D, data not shown). In addition, we were also able to detect a decrease in $p21^{cip1}$ half-life following treatment of HEK293 cells with the DNA damaging agent phleomycin, suggesting that degradation of $p21^{cip1}$ may occur in response to other agents that induce DNA double-strand breaks (DSBs) (Fig 1E). Although $p21^{cip1}$ has been shown to be cleaved by caspase-3 in some cell lines following IR (21), both the IR-induced and phleomycin-induced degradation of $p21^{cip1}$ occurred in the presence of the broad specificity caspase inhibitor ZVAD-fmk (Fig 1E), demonstrating that the degradation we observe is not due to caspase cleavage of $p21^{cip1}$.

**$p21^{cip1}$ is Degraded in Transformed Cell Lines After IR**

We examined $p21^{cip1}$ levels in a number of cell lines following IR and observed that COS-1, Saos-2, and HeLa cells all rapidly degrade $p21^{cip1}$ after IR (Fig. 2A, data not shown). In contrast, HCT116 cells exhibited only a modest reduction in $p21^{cip1}$ after IR (Fig. 2A). Furthermore, irradiation of BJ human fibroblasts had no effect on $p21^{cip1}$ levels (Fig. 2B). Quantification of $p21$ levels showed that the half life of $p21$ in HEK293 cells went from 2 hours to less than 30 minutes after IR. Similar experiments with BJ fibroblasts confirmed that there is no change in the half life of $p21$ in these cells after IR (Fig. 2C).

We noticed that the cell lines that degrade $p21^{cip1}$ most efficiently after IR all lack functional p53 and Rb proteins, either due to impairment by viral proteins (HEK293, COS-1, HeLa) or by way of genetic losses (Saos-2). To test whether the
lack of p53 function was responsible for the different rates of p21cip1 degradation, we compared the half-life of p21cip1 in wild-type and p53-deficient HCT116 cells. Following irradiation, p53-deficient HCT116 cells exhibited only a modest decrease in p21cip1 stability, and this decrease was similar to that seen in the parental HCT116 cells (Fig. 2C). Thus, p53 loss alone does not permit p21cip1 degradation after IR. To determine if the combined loss of p53 and Rb function would cause a more robust degradation of p21cip1, we infected BJ fibroblasts with a control retrovirus or a retrovirus expressing the SV40 large T-antigen, which impairs both p53 and Rb (22). Cells expressing T-antigen exhibited no increase in the rate of p21cip1 degradation after IR relative to control cells (Fig. 2D), demonstrating that although the IR-induced degradation of p21cip1 appears to be most robust in cells that lack functional p53 and Rb, other unidentified factors must also influence whether p21cip1 is degraded after IR.

The IR-induced degradation of p21cip1 is independent of ATM

It is well established that the ATM protein plays an important role in orchestrating the cellular response to ionizing radiation (1). To determine if ATM is required for the IR-induced degradation of p21cip1, we treated HEK293 cells with KU55933, a small molecule inhibitor of the ATM kinase that does not target other PIKK family members (23). Pretreatment of cells with this inhibitor led to a significant reduction in the phosphorylation of multiple ATM target sites after IR, but failed to prevent the IR-induced degradation of p21cip1 (Fig. 3A), suggesting this event may occur independently of ATM. To further rule out a role for ATM in this response,
we compared the IR-induced degradation of p21\textsuperscript{cip1} in a pair of SV40 immortalized A-T fibroblasts, one of which has been reconstituted with human ATM. Following irradiation, the degradation of p21\textsuperscript{cip1} occurred with nearly identical kinetics in both cell lines, showing that ATM is not required for the IR-induced degradation of p21\textsuperscript{cip1} (Fig. 3B).

The possibility exists that in the absence of ATM, other PIKK family members, such as ATR, may stimulate the IR-induced degradation of p21\textsuperscript{cip1}. To address this possibility, we treated HEK293 cells with high concentrations (100\(\mu\)M) of the fungal metabolite wortmannin, a general inhibitor of the PI3K family of kinases. At these concentrations, wortmannin has been shown to inhibit ATM, ATR, and DNA-PK (24). As shown in Fig 3C, the IR-induced phosphorylation of ATM target sites was significantly reduced in the presence of 100\(\mu\)M wortmannin, while phosphorylation of an ATR target site on Chk1 was completely abolished. Despite the apparent inhibition of both ATM and ATR under these conditions, p21\textsuperscript{cip1} was still degraded in these cells after IR (Fig. 3C). This suggests that the IR-induced degradation of p21\textsuperscript{cip1} is likely independent of each of the PI3K-related kinases.

**Degradation of p21\textsuperscript{cip1} is dependent on the ubiquitin-proteasome system**

To assess the proteasome dependence of p21\textsuperscript{cip1} degradation after IR, we treated HEK293 cells with the proteasome inhibitor MG132 for 1 hour prior to IR. We found that pretreatment with this inhibitor completely prevented the IR-induced degradation p21\textsuperscript{cip1} (Fig. 4A). As MG132 inhibits proteases other than the proteasome
(25), we also treated cells with epoxomicin, a much more specific inhibitor of the proteasome (26). Pretreatment with this inhibitor also prevented degradation of p21\textsuperscript{cip1} (Fig. 4B), demonstrating that the IR-induced degradation of p21\textsuperscript{cip1} is proteasome-dependent.

To determine if p21\textsuperscript{cip1} is ubiquitinated after IR, we immunoprecipitated endogenous p21\textsuperscript{cip1} from MG132-treated HEK293 cells in the presence of N-ethylmaleimide and 1,10-phenanthroline, two inhibitors of deubiquitinating enzymes (27). As shown in Fig 4C, in the absence of MG132, significantly less p21\textsuperscript{cip1} was immunoprecipitated from irradiated cells, again showing that p21\textsuperscript{cip1} is degraded after IR (Fig 4C, lower panel). In cells pretreated with MG132, we observed several higher molecular weight forms of p21\textsuperscript{cip1}, consistent with reports that the normal turnover of p21\textsuperscript{cip1} can involve ubiquitination (28,29). Following irradiation of MG132 treated cells, we observed a time-dependent increase in the higher molecular weight forms of p21\textsuperscript{cip1} (Fig. 4C), suggesting that p21\textsuperscript{cip1} is ubiquitinated after IR. Importantly, the increase in these higher molecular weight forms of p21\textsuperscript{cip1} peaks around 30 minutes, which correlates well with the kinetics of p21\textsuperscript{cip1} degradation after IR (Fig. 1A).

To show that ubiquitination is required for p21\textsuperscript{cip1} degradation following IR, we transfected HEK293 cells with wild-type p21\textsuperscript{cip1} or a p21(K6R) mutant in which the six lysines of p21\textsuperscript{cip1} have all been mutated to arginines (28). These lysine mutations eliminate all potential ubiquitination sites within the protein (with the possible exception of the amine group at the N-terminus), and therefore should prevent any process that requires p21\textsuperscript{cip1} to be ubiquitinated. Because it has been reported that
N-terminal tags may affect the stability of p21cip1 (29,30), both the wild-type p21cip1 and p21(K6R) constructs were designed with HA-tags on the C-terminus (Fig. 4D). Following irradiation of cells expressing wild-type p21-HA, there was a significant reduction in both the endogenous and HA-tagged forms of p21cip1. In contrast, irradiation of cells expressing p21(K6R)-HA led only to a reduction in endogenous p21cip1 levels (Fig 4E), indicating that the p21(K6R)-HA mutant is protected from degradation, and suggesting that ubiquitination of p21cip1 is indeed necessary for its degradation after IR.

**Skp2 is dispensable for p21cip1 degradation after IR**

Skp2 is an F-box protein which functions as an adaptor for the Cul1-Skp1 E3 ligase, and which has previously been reported to be involved in both the normal and inducible turnover of p21cip1 (18,31-33). Since the IR-induced degradation of p21cip1 appeared to be ubiquitin-dependent, we asked if it was also Skp2-dependent. To investigate the role of Skp2 in this process, we infected HEK293 cells with a lentiviral shRNA against Skp2, or a non-target shRNA that targets no known or predicted human gene. Immunoblotting showed that Skp2 protein levels were depleted by more than 90% in cells infected with the Skp2 shRNA. In addition, these cells also had significantly elevated levels of p21cip1 suggesting that Skp2 function was in fact compromised by the knockdown (Fig. 5A). Despite this, irradiation of these cells still resulted in the degradation of p21cip1 (Fig. 5B). We obtained similar results using Skp2
siRNAs (Fig. 5C), suggesting that although Skp2 is involved in the normal turnover of p21cip1, it is dispensable for the IR-induced degradation of the protein.

The IR-induced degradation of p21cip1 requires DDB1Cdt2 and PCNA binding

One complex with an established role in the IR-induced degradation of proteins is the Cul4-DDB1Cdt2 E3 ligase, which has been shown to degrade the replication licensing factor Cdt1 after both UV and IR (34-36). Interestingly, DDB1Cdt2 was also recently implicated in both the normal and UV-inducible turnover of p21cip1 (19,20,37). To address the role of DDB1 in the IR-induced degradation of p21cip1, we infected HEK293 cells with a non-target shRNA or three separate shRNAs against DDB1. Similar to what was seen with knockdown of Skp2, knockdown of DDB1 led to an upregulation of p21cip1 in untreated cells (Fig. 5D), suggesting that DDB1 contributes to the normal turnover of p21cip1. However, in contrast to what was seen with Skp2, knockdown of DDB1 also prevented the IR induced degradation of p21cip1 (Fig. 5E and S2). Similar results were obtained with siRNA (Fig. 5F). We also used siRNA to knockdown Cdt2, which functions as an adaptor protein for DDB1, is required for the degradation of Cdt1 and the UV-induced degradation of p21cip1 (19,20,35,36). Knockdown of Cdt2 also lead to modest increases in the steady-state state levels of p21cip1, and more importantly, completely prevented degradation of p21cip1 after IR (Fig. 6A). Taken together, these results demonstrate that while both Skp2 and DDB1Cdt2 are capable of regulating the normal turnover of p21cip1, only DDB1Cdt2 is required for its IR-induced degradation (Fig 6D).
As previously mentioned, the Cul4-DDB1 E3 ligase is required for the DNA-damage induced degradation of Cdt1 (38). Like p21\(^{cip1}\), Cdt1 binds PCNA, and it has been shown that this interaction is required for degradation of Cdt1 following UV and IR (35,39). To determine if PCNA binding is required for the DDB1-dependent degradation of p21\(^{cip1}\) following IR, we transfected cells with p21-HA or p21\(^{Δpcna}\)-HA, which harbors three point mutations in the C-terminus and has been shown to be defective in PCNA-binding (Fig 6B). Irradiation of cells transfected with wild-type p21-HA led to degradation of both the endogenous and HA-tagged p21\(^{cip1}\). In contrast, irradiation of cells transfected with p21\(^{Δpcna}\)-HA led only to degradation of endogenous p21\(^{cip1}\) (Fig. 6C), suggesting that PCNA binding is likely critical for the IR-induced degradation of p21\(^{cip1}\).

Increased BrdU Incorporation in COS-1 cells

While characterizing the pathway leading to the IR-induced degradation of p21\(^{cip1}\) is informative, the ultimate goal is to determine the biological consequences of this degradation in transformed cells. It has been reported that the UV-induced degradation of p21\(^{cip1}\) is required to free PCNA to participate in repair (18). To attempt to determine if there are increases in DNA repair in cells that degrade p21\(^{cip1}\) after IR, we examined bromodeoxyuridine (BrdU) incorporation in irradiated COS-1 cells. BrdU is a nucleic acid analog that cells incorporate into DNA and it is therefore useful as an indicator of both DNA synthesis and DNA repair. To compare the BrdU incorporation in irradiated or unirradiated COS-1 cells, we pulsed these cells with
BrdU for the 5 hour window (2-7 hours post-IR) during which p21 levels had been determined to be lowest in these cells (Fig. 7A). Using this strategy, we were able to determine that the percentage of positive cells that incorporated BrdU was consistently higher in irradiated COS-1 cells when compared to unirradiated control cells (Fig. 7B). Thus, p21cip1 degradation coincides with increased BrdU incorporation in COS-1 cells which may indicate that p21cip1 needs to be degraded in these cells to allow them to efficiently repair DNA.

Overexpression of p21cip1 in COS-1 cells appears to saturate the degradation machinery leading to p21cip1 expression even in irradiated cells (data not shown). Therefore, overexpressing p21cip1 in these cells should rescue any event that relies on the IR-induced degradation of p21cip1. To see if p21cip1 degradation was directly responsible for the increased BrdU incorporation in irradiated COS-1 cells, we transfected these cells with p21cip1 and compared the BrdU incorporation in the transfected and non-transfected populations. In these experiments, irradiation of cells overexpressing p21cip1 no longer led to an increase significant in BrdU incorporation (Fig 7C), suggesting that p21cip1 may be directly influencing DNA repair.
Discussion

We have observed that in many transformed cell lines, ionizing radiation leads to the degradation of the cyclin-dependent kinase inhibitor p21cip1. In addition, phleomycin, a DNA damaging agent that produces both single and double strand breaks (40), also leads to p21cip1 degradation. Based on these results, it seems probable that many agents that produce DNA strand breaks may trigger p21cip1 degradation in these cells. However, as we have been unable to detect degradation of p21cip1 following treatment with either doxorubicin or etoposide (data not shown), two other DNA-damaging agents capable of generating strand breaks, it is likely that the type of damage and/or the kinetics with which the damage occurs, influence whether p21cip1 is degraded following damage.

Degradation of p21cip1 following ionizing radiation occurs in both a proteasome and ubiquitin-dependent manner, but is surprisingly independent of ATM as it occurs in immortalized A-T fibroblasts. It is unlikely that this ATM independence is due to compensation by other ATM family members as degradation also occurs in the presence of wortmannin, which inhibits ATM, ATR, and DNA-PK. In an attempt to determine if other serine/threonine kinases play a role in the IR-induced degradation of p21cip1, we treated cells with the general kinase inhibitor staurosporine, but have observed no effect on p21cip1 degradation (data not shown). In addition, we have used two-dimensional gel electrophoresis to search for IR-induced modifications of p21cip1, but have been unable to detect any changes in the isoelectric point or mobility shift of
p21cip1 (data not shown). Taken together, these results suggest that p21cip1 may not need to be covalently modified prior to its ubiquitination and degradation after IR.

We show here that the IR-induced degradation of p21cip1 requires DDB1 and is dependent on the p21cip1-PCNA interaction. The best characterized target of the Cul4-DDB1 E3 ligase is the replication licensing factor Cdt1, which is degraded by Cul4-DDB1 both during S-phase and following DNA damage induced by UV or IR. (34-36,38). Similar to p21cip1, Cdt1 binds PCNA and this interaction is necessary for both its S-phase and DNA damage-induced degradation (35,39,41). Interestingly, several studies published during preparation of this manuscript have also shown that Cul4-DDB1 is responsible for the degradation of p21cip1 both during S-phase and following UV damage (19,20,42). In each of these reports, the degradation of p21cip1 was also shown to be dependent on its interaction with PCNA. Thus, PCNA seems to serve as a critical intermediary for the two of the most well characterized DDB1 substrates.

Interestingly, PCNA has been shown to be recruited to chromatin within minutes of IR, and this recruitment has been shown to be both transient and ATM-independent (43,44). As we have shown that the IR-induced degradation of p21cip1 is also transient and ATM-independent, it is possible that PCNA-mediated recruitment of p21cip1 to chromatin is the rate-limiting step in p21cip1 degradation after IR. Since DDB1 has been shown to degrade a number of chromatin-bound proteins (45), this model would also help explain why the IR-induced degradation of p21cip1 is dependent on Cul4-DDB1 while the normal turnover of the protein can be regulated by both Cul4-DDB1 and SCF^{Skp2}. 
A unique feature of the IR-induced degradation of p21cip1 is that while it occurs in most of the cell lines we have tested, it is significantly more robust in a subset of transformed cell lines that lack functional p53 and Rb proteins. We have observed that loss of p53 alone does not increase the rate at which p21cip1 was degraded after IR. Introduction of the SV40 large T-antigen, which impairs both the p53 and Rb proteins, also failed to accelerate p21cip1 degradation after IR. Thus, the increased rate of degradation we observe in cells lacking functional p53 and Rb may be a unique feature of these cells or it may be a result of a more complex transformation process. In either case, as p21cip1 degradation after damage has been reported to facilitate DNA repair (18), it is possible that the IR-induced degradation of p21cip1 in these transformed cells functions to enhance DNA repair and therefore promote survival.

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Figure 1  Ionizing radiation induces degradation of p21cip1.  A) HEK293 cells were irradiated with 10Gy IR and collected at the indicated times.  A western blot was performed on equal amounts of whole cell lysate.  NS is a non-specific band of the p21cip1 antibody (C-19) used as a loading control.  B) Western blot of HEK293 cells pretreated with 25μg/mL cyclohexamide (CHX) prior to treatment with no IR or 10Gy IR.  C) Western blot of HEK293 cells pretreated with 25μg/mL CHX for 1 hour prior to irradiation with 0, 0.5, 2, or 10Gy IR.  D) Western blot of HEK293 cells pretreated with 50μM ZVAD-fmk, 25μg/mL CHX, and 100μg/mL pleomycin (or vehicle) for 1hr.
Figure 2  IR-induced degradation of p21cip1 occurs in transformed cells. A, B) The indicated cells were pretreated with 25μg/mL cyclohexamide (CHX) and left untreated or irradiated with 10Gy IR. All immunoblots were run using equal amounts of whole cell lysate and are representative of multiple experiments. C) Same as in A and B, except LICOR secondary antibodies were used and LICOR imaging software was used to quantify p21 levels. p21 levels were normalized to tubulin. Graphs are from three independent experiments with representative experiments shown below. D) Western blot of HCT116 or HCT116 p53-/- cells pretreated with 25μg/mL CHX for 1 hour prior to 10Gy IR. E) Western blot of BJ human foreskin fibroblasts infected with a control retrovirus or a retrovirus expressing SV40 T-antigen. Infected cells were selected with 2.0μg/mL puromycin until control cells were dead and detached from the dish. Selected cells were cultured to confluence and pretreated with 25μg/mL CHX for 1 hour prior to 10Gy IR. Vertical lines indicate gel lanes that were spliced together.
Figure 3 IR-induced degradation of p21cip1 is independent of ATM. A) Western blot of whole cell lysates from HEK293 cells pretreated for 1 hour with DMSO or 10μM KU55933 prior to 10Gy IR. Phospho-specific antibodies to ATM or the ATM target Chk2 were used to verify the efficacy of the inhibitor. B) Western blot of whole cell lysates from immortalized A-T fibroblasts or reconstituted A-T fibroblasts pretreated with 25μg/mL cyclohexamide for 1 hour prior to 10Gy IR. C) Western blot of whole cell lysate from HEK293 cells pretreated with 25μg/mL CHX and DMSO or 100μM wortmannin for 1 hour prior to 10Gy IR. Phospho-specific antibodies to ATM or ATR target sites were used to verify the efficacy of the inhibitor.
Figure 4  p21cip1 is degraded by the ubiquitin-proteasome system following IR.  A) Western blot of whole cell lysates from HEK293 cells pretreated with 25μg/mL CHX and DMSO or 25μM MG132 for 1hr prior to 10 Gy IR. B) Western blot of whole cell lysates from HEK293 cells pretreated with 25μg/mL CHX and DMSO or 5μM epoxomicin for 1hr prior to 10 Gy IR. C) HEK293 cells were pretreated with DMSO or 25μM MG132 for 30 min prior to IR. Cells were collected at the indicated timepoints as described in the materials and methods. p21cip1 was immunoprecipitated from 1.0mg whole cell lysate using a polyclonal antibody to p21cip1 and a western blot was performed using a p21cip1 monoclonal antibody. D) Schematic of the p21-HA and p21(KGR)-HA constructs. E) HEK293 cells were transfected with 5.0μg total DNA including 50ng GFP and 100ng p21-HA or p21(KGR)-HA. Cells were pretreated with 25μg/mL CHX for 1 hour prior to irradiation with 10 Gy IR. Cells were collected 1 hour after IR and equal amounts of whole cell lysate were analyzed by Western blot.
Figure 5. DDB1, but not Skp2, is required for the IR-induced degradation of p21cip1. A) HEK293 cells were infected with a nontarget lentiviral shRNA (NT) or an shRNA against Skp2. Infected cells were selected with 2.0 μg/mL puromycin. Knockdown of Skp2 was assessed by western blot using dilutions of the NT sample. B) NT or Skp2 knockdown cells were pretreated with 25 μg/mL CHX for 1 hour prior to irradiation with 10 Gy IR. Cells were collected at the indicated timepoints and analyzed by western blot. C) HEK293 cells were transfected with a nontarget siRNA or two separate siRNAs against Skp2. 60 hours post-transfection, cells were irradiated with 10 Gy. Cells were collected 1 hour after IR and whole cell lysates were analyzed by western blot. D) HEK293 cells were infected with a nontarget lentiviral shRNA or an shRNA against DDB1 and assessed for DDB1 knockdown and p21 cip1 upregulation as in A. E) NT or DDB1 knockdown cells were pretreated with 25 μg/mL CHX for 1 hour prior to irradiation with 10 Gy IR. Cells were collected at the indicated timepoints and analyzed by western blot. F) HEK293 cells were transfected with a nontarget siRNA or an siRNAs against DDB1. 60 hours post-transfection, cells were pretreated with 25 μg/mL CHX for 1 hour prior to irradiation with 10 Gy. Cells were collected 1 hour after IR and whole cell lysates were analyzed by western blot. Vertical lines indicate gel lanes that were spliced together.
Figure 6 The IR-induced degradation of p21cip1 requires Cdt2 and the p21-PCNA interaction. A) HEK293 cells were transfected were with a nontarget siRNA or an siRNA against Cdt2. 60 hours post-transfection, cells were irradiated with 10Gy. Cells were collected 1 hour after IR and whole cell lysates were analyzed by western blot. B) HEK293 cells were transfected with 5.0μg DNA including 500ng p21-HA or p21Δpcna-HA and 100ng GFP. 60 hrs post-transfection, p21cip1 was immunoprecipitated from 1.0mg of whole cell lysate and immunoblots were performed with antibodies to p21cip1 or PCNA. C) HEK293 cells were transfected with 5.0μg DNA including 100ng p21-HA or p21Δpcna-HA and 50ng GFP. 60 hrs after transfection, cells were treated with 25μg/ml CHX for 1 hour prior to irradiation with 10Gy IR. Samples were collected 1 hour after IR. Equal amounts of whole cell lysate were analyzed by western blot for expression of the indicated proteins. D) Model depicting degradation of p21cip1. Both Skp2 and DDB1 can regulate the normal turnover of p21cip1, but only DDB1 is responsible for the IR inducible turnover.
Figure 7 Ionizing radiation causes increased BrdU incorporation in COS-1 cells. A) Western blot of whole cell lysates from COS-1 cells irradiated with 10 Gy and collected at the indicated timepoints. B) COS-1 cells were irradiated with 10 Gy, 2 hours post-IR, BrdU was added to the culture medium at a concentration of 10μM. Cells were collected 5 hours later and fixed for BrdU analysis by FACS. C) COS-1 cells were transfected with 5.0 μg vector or FLAG-p21 and 100ng GFP. 48 hours later cells were irradiated with 10 Gy, 4 hours post-IR cells were pulsed with 10μM BrdU for 1 hour before being fixed for FACS analysis. BrdU incorporation was determined in the transfected (GFP+) and untransfected (GFP-) populations by using separate gates for these two populations.
Figure 8  Decrease in p21cip1 levels following IR is independent of transcription.  A) 5.0x10⁶ HEK293 cells were seeded in 60mm dishes and cultured for the indicated times.  Equal amounts of whole cell lysate were then analyzed by western blot for p21cip1 expression.  B) HEK293 cells or BJ-HFF's were irradiated with 10Gy IR and total RNA was collected at the indicated timepoints and reversed transcribed to cDNA.  QPCR was performed on cDNA using primers specific for p21cip1 or actin.  Graph in the upper panel shows the level of p21cip1 mRNA induction normalized to actin mRNA.  Lower panel shows a western blot from the same experiment.
Figure 9  Multiple shRNAs against DDB1 prevent the IR-induced degradation of p21cip1.
A) HEK293 cells were infected with shRNA #2 against DDB1 and selected with puromycin. Selected cells were seeded and cultured to confluence. Cells were then pretreated with 25µg/mL CHX for 1 hour prior to 10Gy IR. Whole cell lysates were analyzed by western blot for expression of p21cip1 and DDB1. B) Same as in A except using shRNA #3 against DDB1.
References


The text of Chapter 2, in part, has been submitted for publication to the

*Journal of Biological Chemistry.* Scott A. Stuart and Jean Y.J. Wang. I was the

primary investigator of this research as well as the primary author of the manuscript.
Chapter 3:  
Transformation of E2A-Deficient Pluripotent Progenitors by BCR-ABL  
Generates Leukemic Stem Cells in the GMP Compartment

Abstract

During blast crisis of chronic myelogenous leukemia (CML), abnormal granulocyte macrophage progenitors (GMP) with nuclear β-catenin acquire self-renewal potential and may function as leukemic stem cells (Jamieson, et al. *N Engl J Med*, 2004). To develop a mouse model for CML-initiating GMP, we expressed p210BCR-ABL in an established line of E2A-knockout mouse bone marrow cells that retain pluripotency in *ex vivo* culture. Expression of BCR-ABL in these cells reproducibly stimulated myeloid expansion in culture and generated leukemia-initiating cells specifically in the GMP compartment. The leukemogenic GMP displayed higher levels of β-catenin activity than either the non-transformed GMP or the transformed nonGMP, both in culture and in transplanted mouse bone marrow. While E2A-deficiency may have contributed to the formation of leukemogenic GMP, restoration of E2A-function did not reverse BCR-ABL-induced transformation. These results provide further evidence that BCR-ABL-transformed GMP with abnormal β-catenin activity can function as leukemic stem cells.
Introduction

Chronic myelogenous leukemia (CML) is caused by the expression of a constitutively active BCR-ABL tyrosine kinase from the abnormal Philadelphia chromosome, which is present in the hematopoietic stem cells and progenitor cells of CML patients (1, 2). The clinical success of the ABL kinase inhibitor imatinib (Gleevec™) in treating chronic phase CML serves as a model for molecular targeted therapy of cancer (3-6). However, CML patients in accelerated phase or blast crisis are refractory to imatinib due to the rapid emergence of drug-resistant BCR-ABL mutant clones (7, 8). In CML blast crisis patients, granulocyte macrophage progenitors (GMP) with an aberrant potential for self-renewal were detected (9). This finding suggests that BCR-ABL-transformed GMPs may function as the leukemic stem cells during blast crisis (9).

Retroviral transduction of human BCR-ABL into 5-fluorouracil-activated primary mouse bone marrow cells has been shown to generate a CML-like disease (2), with the leukemia-initiating cells found in the hematopoietic stem cell compartment (10). By contrast, transduction of BCR-ABL into primary GMP isolated from the mouse bone marrow did not lead to the formation of leukemic stem cells (11). To maximize the potential of transforming the myeloid precursors, and to achieve \textit{ex vivo} propagation of leukemic stem cells, we expressed BCR-ABL in an established long-term culture of murine hematopoietic progenitor cells (mHPC) derived from the bone marrow of \textit{E2A}-knockout mice, which are defective in B-cell development (12). Previous studies have shown that these cultured mHPC can repopulate the myeloid, erythroid and T/NK compartments when
they are co-transplanted with normal bone marrow cells into lethally irradiated congenic mice (12). Following retroviral transduction of mHPC with p210BCR-ABL, we observed an immediate expansion of myeloid cells, including GMP, MEP (megakaryocyte erythroid progenitors) and the myeloid lineage-positive (CD11b+) cells in culture. Using this experimental system, we were able to identify, isolate and propagate the cells that cause myeloid leukemia upon transplantation into congenic mice. Similar to what has been reported for blast crisis patients, these leukemia-initiating cells were found to reside in the GMP compartment and display elevated levels of β-catenin activity.
Experimental Procedures

Cell Culture and Retroviral Infection.

Pluripotent murine hematopoietic progenitor cells (mHPC) derived from E2A-deficient mouse (C57BL/6; CD45.2) bone marrow were co-cultured with the S17 stromal cells plus stem cell factor (SCF), Flt-3-ligand (FL) and interleukin-7 (IL-7) (R&D Systems, Minneapolis, MN) as previously described (12). The MSCV-IRES-GFP, the MSCV-p210-IRES-GFP and the MSCV-p210KD-IRES-GFP plasmids were kindly provided by Dr. Ruibao Ren (Brandeis University, Waltham, MA) (13). The pCSretTAC-E2A-ER-IRES-hCD25 vector was previously described (24). Retrovirus production was performed using the Phoenix™ Retroviral Packaging System (Orbigen, San Diego, CA) and FuGENE 6 (Roche Applied Science, Indianapolis, IN) for DNA transfection. Viral supernatants (500 µl/well, 24-well plates) with polybrene (Sigma-Aldrich, Saint Louis, MI; final concentration; 2 µg/ml) were added to the mHPC (at 10^6/100µl/well), the plates were centrifuged at 2,500 rpm for 1.5 hours at 30 C°. After centrifugation, the viral supernatants were removed and complete media (2 ml/well) was added. After another 6-hour incubation, infected cells were transferred onto 6-well plates coated with S17 stromal cells for expansion using the established mHPC culture conditions (12).

Mouse Transplantation and Analysis for Leukemic Phenotype.

Recipient C57BL/6-Tyr<sup>ε-2J</sup>/J mice (CD45.1, 8 weeks old) were irradiated (1,000 rads) between 6 to 8 hours prior to transplantation. GFP<sup>+</sup> cells (1x10^6) from the mHPC/GFP
or mHPC/p210 cultures in 100 µl of IMDM media with 1% fetal bovine serum were mixed with 2x10⁵ normal bone marrow cells from C57BL/6J-Tyr<sup>c-21</sup>/J mice (CD45.1, 8 weeks old) in 100 µl of media and injected into the tail veins of recipient mice (12, 14). Mice were monitored daily for cachexia, lethargy and ruff coats and the distressed animals were euthanized (25). Peripheral blood collection and blood examinations were performed by the UCSD Murine Hematology and Coagulation Core Facility, and histopathological analyses were performed by the UCSD Histology & Immunohistochemistry Core Facility.

**Statistical Analysis.**

Kaplan-Meier analysis and statistical analysis were performed using PRISM 4 (2003) from GraphPad (San Diego, CA) as previously described (25). Data were expressed as mean ± s.e.m. determined from the indicated number. Values of $P < 0.05$ was considered statistically significant.

**Flow-Cytometric Analysis and Cell Sorting.**

Flow-cytometric analysis and sorting were performed with FACS Calibur and FACS Aria (Becton Dickinson, San Jose, CA), using established methods (9, 16, 26). Biotin-conjugated antibodies against seven lineage markers (CD3, CD5, CD8, CD11b, Gr-1, B220 and Ter119), Sca-1 and IL-7R, as well as APC-anti-CD11b, PE-anti-B220, APC-anti-cKit, PE-anti-FcγR, PerCP-Cy5.5-anti-CD34 and PE-anti-Sca-1 antibodies were from eBioscience (San Diego, CA). PE-Cy7-anti-streptavidin and APC-anti-
human CD25 antibodies were from CALTAG Laboratories (Carlsbad, CA). Staining with propidium iodide (PI) to measure PI-uptake (intact cells) and DNA contents were performed with standard procedures as previously described (27).

**Immunoblotting.**

Anti-Abl antibody (8E9) and anti-E2A antibody were from BD Biosciences. Anti-phospho-c-Abl (Tyr245) and anti-β-catenin were from Cell Signaling Technology (Beverly, MA). Anti-Tublin antibody and anti-GFP antibody were from Sigma-Aldrich. To measure protein concentrations of cell lysates, Bio-Rad Protein Assay Kit was used. (Bio-Rad Laboratories, Hercules, CA). Immunoblotting were performed with standard protocols as previously described (28, 29), and developed with enhanced chemiluminescence (Amersham, Little Chalfont, UK).

**Restoration of E2A-Function.**

The E2A-ER-IRES-hCD25-infected mHPC/p210 cells were selected by hCD25-expression and PI-negativity using FACS Aria. The levels of E2A-ER protein were determined by immunoblotting with anti-E2A antibody. The activity of E2A-ER was induced by treating cells with 4-hydroxytamoxifen (4-OHT) (Sigma-Aldrich) at 1 µM for 24-48 hours as previously described (19).

**Real-Time Quantitative PCR.**
Total RNA was prepared using the RNeasy kit (Qiagen, Valencia, CA), and cDNA prepared with Superscript II reverse transcriptase (Invitrogen) or the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR using the Power SYBR Green PCR master mix (Applied Biosystems) was conducted using the 7900 HT Fast Real-time PCR System (Applied Biosystems). The primers used were: Id2 (forward) CGGAAGGAAAAACTAAGGATG and (reverse) TGTAGAAAGGGCCTGAAAG; Hes1 (forward) TTGGCTGAAAAGTTACTGTGG and (reverse) ACTATTCCAGGACCAAGGAG; Luciferase (forward) TGCACATATCGAGGTGGACATC and (reverse) GCCAACCGAACGGACATTT; Actin (forward) CGAGAAGATGACCCAGATCATGTT and (reverse) CCTCGTAGATGGGCCACAGTGT.

**β-Catenin Reporter.**

The Topflash sequence was amplified from the Super8XTopflash plasmid by polymerase chain reaction and cloned into a self-inactivating (SIN) lentiviral vector. The pgk-puro sequence was amplified from pMSCV-puro (Clontech, Mountainview, CA) and inserted downstream of the Topflash sequence. The lenti-Topflash plasmid and lentiviral packaging plasmids were then transfected into 293FT cells (Invitrogen) using calcium phosphate. Media was changed 18 hours after transfection and virus from twelve 15-cm dishes was harvested 48 hours later and concentrated into a final volume of 400 μL. mHPC/GFP and mHPC/p210 were then infected with 50 μL viral
supernatant containing 8 µg/mL polybrene. After 48 hours, cells were replated in 10-cm dishes and infected cells were selected by addition of 0.5 mg/mL puromycin (Sigma-Aldrich) to the media. For in vivo-experiments, mice were injected with mHPC/GFP-Top or mHPC/p210-Top as described above. Three weeks after injection, mice were euthanized and GFP-positive cells were sorted into GMP and nonGMP populations and luciferase expression was determined by real-time quantitative PCR.
Results

Transformation of cultured hematopoietic progenitor cells by BCR-ABL

We infected the mHPC, cultured on top of the S17 stromal cells (12), with an ecotropic retrovirus expressing either GFP alone or p210BCR-ABL-IRES-GFP (13). The GFP and p210 proteins were detected in GFP+ populations isolated by fluorescence-activated cell sorting (FACS) 3 days after infection (Fig. 1A and 1B). The levels of p210 protein and tyrosine phosphorylation (Y245) in mHPC/p210 cells were comparable to those in the human blast crisis CML cell line K562 (Fig. 1B). We then expanded the GFP+ cells in culture and transplanted 1x10^6 mHPC/GFP or mHPC/p210, along with 2x10^5 freshly isolated normal bone marrow cells, into lethally irradiated congenic mice (12, 14). Mice were sacrificed weekly after transplantation, and the fraction of GFP+ cells in the bone marrow (BM), peripheral blood (PB) and spleen was determined. Beginning at 6 weeks post-transplantation, a dramatic expansion of GFP+ cells in the BM, PB and spleen of mice transplanted with mHPC/p210 was observed (Supporting Fig. 6A). At week 7, the mHPC/p210-transplanted mice displayed an increase in the number of white blood cells (WBC) in the periphery (Fig. 1C). By week 8, the mHPC/p210-transplanted mice displayed phenotypes of bone marrow hyper-cellularity (Fig. 1D and Supporting Fig. 6C), splenomegaly (Fig. 1E, and Supporting Fig. 6B, C), and infiltration of leukemic cells into the liver (Supporting Fig. 6C). Of the 20 mice transplanted with mHPC/p210, 18 had to be sacrificed at week 8, whereas none of the mHPC/GFP-transplanted mice (n=6) developed leukemia (Fig. 1F).
Transplantation of total BM cells from the leukemic mice into secondary recipients propagated the leukemic phenotypes. The disease latency was reduced in secondary and tertiary recipients as BM hyper-cellularity and splenomegaly were observed 4 weeks after transplantation with either 1x10^6 or 1x10^5 BM cells from primary leukemic mice (Fig. 1F and data not shown). In control experiments, bone marrow cells from mHPC/GFP-transplanted primary recipients did not cause leukemia in secondary recipients (Fig. 1F). We performed Southern-blotting experiments using splenic DNA from the leukemic mice to examine the retroviral insertion sites (Supporting Fig. 7). In primary and secondary leukemic mice derived from one batch of mHPC/p210, we observed two EcoRI fragments suggesting an oligoclonal, transplantable disease (Supporting Fig. 7B, left panels). In a separate set of secondary leukemic mice, derived from mHPC/p210 generated in an independent retroviral transduction experiment, we observed one predominant EcoRI band, suggesting a monoclonal disease (Supporting Fig. 7B, right panel). These results show that expression of p210^{BCR-ABL} in mHPC reproducibly generates transplantable leukemic cells that can be monoclonal or oligoclonal in origin.

**BCR-ABL-transformed leukemia-initiating cells reside in the GMP compartment**

We analyzed ex vivo-cultures of mHPC/GFP and mHPC/p210 using established cell surface markers for the murine hematopoietic system (15, 16). Previous studies have found that the mHPC express low levels of multiple lineage
markers at the level of mRNA and a predominant expression of the B-lineage marker B220 at the cell surface (12). Similarly, we found that cells in the mHPC/GFP culture was mostly positive for B220 (Fig. 2A). By contrast, the majority (~90%) of the mHPC/p210 culture was positive for CD11b, a myeloid lineage marker (Fig. 2A). The shift from B220⁺ to CD11b⁺ in the mHPC/p210 cultures occurred within 7 days of retroviral infection (Fig. 2A). Microarray-based gene profiling results also indicated an upregulation of myeloid lineage markers in the mHPC/p210 cultures (Supporting Fig. 8A and B). In addition, the mHPC/p210 cells readily acquired cytokine and stromal independent growth, consistent with transformation by BCR-ABL (Supporting Fig. 8C). Interestingly, however, transplantation of mHPC/p210 grown in the absence of cytokines and S17 stromal cells did not induce leukemia in congenic mice (data not shown), suggesting propagation and/or maintenance of cells with leukemogenic potential is dependent on cytokine and stromal support. This result also indicates that cytokine- and stromal-independent proliferation may be necessary but is not sufficient to confer leukemogenic potential.

Expression of p210BCR-ABL also affected the lineage-negative progenitors (i.e., CD3⁻, CD5⁻, CD8⁻, CD11b⁻, Gr-1⁻, B220⁻ and Ter119⁻) in the mHPC cultures. The GFP⁺-GMP fraction (Lin⁻, IL-7R⁻, Sca-1⁻, c-Kit⁺, CD34⁺, FcγR⁺) in the mHPC/p210 culture was 0.3%, which was 10 fold higher than the GFP⁺-GMP fraction in the mHPC/GFP culture (Fig. 2B). The GFP⁺-MEP fraction (Lin⁻, IL-7R⁻, Sca-1⁻, c-Kit⁺, CD34⁻, FcγR⁻) was 0.3% and 8% in the mHPC/GFP and mHPC/p210 cultures,
respectively (Fig. 2B). We isolated the GFP\(^+\)-GMP population by step-wise sorting of Lin\(^-\)/Sca-1\(^-\)/IL-7R\(^-\), GFP\(^+\)/c-Kit\(^+\) and Fc\(\gamma R^+\)/CD34\(^+\) (Supporting Fig. 9B) and transplanted them along with normal bone marrow cells into lethally irradiated congenic mice (Fig. 2C). We found that injection of as few as 50 such cells from the mHPC/p210 culture induced leukemia in three out of six mice (Fig. 2C and Supporting Fig. 9A). We also transplanted cells from the GMP-depleted fraction (GFP\(^+\)-nonGMP) for comparison. While transplantation of 5,000 GFP\(^+\)-GMP induced leukemia in five out of five mice, only one of seven mice transplanted with GFP\(^+\)-nonGMP developed leukemia (Fig. 2C and Supporting Fig. 9A).

We next sorted the GFP\(^+\)-GMP population from the leukemic mouse bone marrow to determine its leukemia-initiating potential (Fig. 2D and Supporting Fig. 9B). The GMP and MEP fractions in bone marrow of non-transplanted mice were similar to those previously reported for C57BL/6 mice (Fig. 2D) (16, 17). Eight weeks after transplantation with mHPC/p210, the GFP\(^+\)-GMP fraction in leukemic bone marrow was 10-15 fold higher than that in mice receiving mHPC/GFP (Fig. 2D). By contrast, there was not a significant difference in the GFP\(^+\)-MEP fractions of mHPC/p210 and mHPC/GFP transplanted mice (Fig. 2D). Thus, the expansion of the p210-transformed GMP population occurred both ex vivo and in vivo, while the expansion of the MEP population only occurred ex vivo.
The GFP⁺-GMP and GFP⁺-MEP sorted from leukemic mice were then injected along with normal bone marrow helper cells into lethally irradiated secondary recipients (Fig. 2E and Supporting Fig. 9C). Again, as few as 50 cells from the GFP⁺-GMP population isolated from the leukemic bone marrow were sufficient to transfer the disease to secondary recipients (Fig. 2E and Supporting Fig. 9C). In contrast, the sorted GFP⁺-MEP did not induce leukemia, nor did the GFP⁺-GMP isolated from the bone marrow of mHPC/GFP-transplanted mice (Supporting Fig. 9C).

To ascertain that the GFP⁺-GMP from mHPC/p210 cultures were functional myeloid progenitors, we plated the isolated cells in culture, with or without the S17 stromal cells, and analyzed the formation of CD11b⁺ (a myeloid lineage marker) cells with time (Supporting Fig. 10). Upon replating, the GFP⁺-GMP expanded to form CD11b⁺ cells and the conversion to these differentiated myeloid cells was slower in the presence of S17 stromal cells (Supporting Fig. 10), consistent with the idea that stromal support is required for the maintenance of the leukemic stem cell population. Taken together, the above results suggest that the GFP⁺-GMP isolated by lineage markers were functional precursors as they generated myeloid lineage cells in culture and myeloid leukemia in mice.

**BCR-ABL kinase activity is required to initiate the transformation of mHPC**

The results of numerous previous studies have supported the conclusion that BCR-ABL tyrosine kinase activity is essential for its transforming function and the
clinical success of imatinib has validated that conclusion (1-6). We expressed a kinase-defective p210 (p210KD) (18) in mHPC and found that this mutant did not cause an expansion of GFP\(^+\) or CD11b\(^+\) cells (Fig. 2A). Expression of p210KD also failed to cause the expansion of myeloid progenitors (GMP and MEP) (Fig. 2B). Furthermore, transplantation of mHPC/p210KD into congenic mice failed to induce leukemia or the expansion of GMP in the bone marrow (Fig. 1D, E and Fig. 2D). Thus, BCR-ABL tyrosine kinase activity is required for the transformation of mHPC.

**Restoration of E2A-function does not suppress the transforming function of p210**

As the mHPC culture was derived from the bone marrow of E2A-knockout mice (12), we sought to determine whether restoration of E2A function could reverse the transformation of GMP. Therefore, we stably infected mHPC/p210 cultures with a retrovirus expressing an E2A-ER fusion protein and the cell surface marker CD25 (Fig. 3A). We selected CD25/GFP-double positive cells, verified the expression of the E2A-ER protein (Fig. 3B), and demonstrated 4-hydroxytamoxifen (4-OHT)-dependent activation of E2A-target genes: Id2 and Hes-1 (Fig. 3C) (19). We then examined whether E2A activation by 4-OHT could affect the lineage distribution within the mHPC/p210 culture (Fig. 3D). We found that stable expression and activation of E2A-ER did not alter the fraction of CD11b\(^+\) cells, which remained at the \(~90\)% level (Fig. 3D). Similarly, expression of E2A-ER and treatment with 4-OHT did not affect the fraction of GMP, which remained at the 0.3% level (Fig. 3D). While E2A restoration did not affect the lineage distribution within this transformed mHPC/p210...
culture, we cannot rule out the possibility that *E2A*-deficiency and the *ex vivo*-culturing conditions might have created a permissive condition for BCR-ABL to transform GMP into leukemic stem cells.

**Higher levels of β-catenin activity in BCR-ABL-transformed GMP**

It was reported that the self-renewal potential of GMP from blast crisis CML patients was a result of the aberrant activation of β-catenin (9). To measure β-catenin activity, we infected mHPC/GFP and mHPC/p210 cultures with a lentiviral reporter in which 7 TCF/LEF sites lie upstream of the luciferase gene (Fig. 4A,B). The resultant cells, mHPC/GFP-Top or mHPC/p210-Top, were cultured *ex vivo*, sorted into GMP and nonGMP, and the β-catenin activity in the four populations assessed by quantification of the levels of luciferase mRNA. Interestingly, the GMP fraction of mHPC/p210-Top cells exhibited a significant increase in β-catenin activity relative to the other three populations (Fig 4C). To determine if this increase in β-catenin activity was maintained *in vivo*, we injected mHPC/GFP-Top or mHPC/p210-Top cells into mice. As was observed in earlier experiments, only mice injected with mHPC/p210-Top developed leukemia (data not shown). To determine if the GMP fraction of these mice also contained elevated levels of active β-catenin, we sorted GMP and nonGMP populations from bone marrow at 3 weeks after transplantation and measured the luciferase mRNA in each population. We observed that while the GFP-Top/GMP and p210-Top/nonGMP had slightly elevated levels of luciferase expression relative to the GFP-Top/nonGMP population, the p210-Top/GMP cells had
a nearly 20-fold increase in luciferase expression (Fig. 4D). Thus, cells in the p210-transformed GMP compartment exhibited an increase in β-catenin activity that was further enhanced by the *in vivo* bone marrow microenvironment. Taken together, these results suggest that leukemia induced by mHPC/p210 is propagated by GMP cells with abnormal β-catenin activity.
**Discussion**

By expressing BCR-ABL in an *ex vivo* culture of *E2A*-knockout pluripotent progenitor cells (mHPC), we have generated BCR-ABL-transformed GMP that exhibit higher β-catenin activity and acquire the ability to initiate leukemia in mice. Our finding is consistent with the report that BCR-ABL-positive GMP isolated from CML blast crisis patients acquire self-renewal potential through activation of the β-catenin pathway (9). A recent study using β-catenin-deficient mice has also confirmed the importance of this self-renewal pathway in BCR-ABL-dependent myeloid leukemogenesis (20). It has been shown that BCR-ABL cannot convert primary GMP isolated from the mouse bone marrow into leukemic stem cells (11). We show here that BCR-ABL can transform *ex vivo* cultured, *E2A*-deficient, mHPC into leukemia initiating cells, which are found in the GMP compartment. Together, these results suggest that BCR-ABL-induced formation of leukemogenic GMP may require other genetic and/or epigenetic alterations that have occurred in the *E2A*-deficient mHPC during *ex vivo* culture. We have examined the levels of β-catenin protein in the GMP and nonGMP fractions isolated from the bone marrow of wild type and *E2A*-knockout mice, as well as from *ex vivo* cultures of mHPC/p210 (Supporting Fig. 11). We did not observe any significant differences in the levels of total β-catenin between the wild type and *E2A*-deficient GMP, thus ruling out abnormal β-catenin levels as the permissive factor for transformation of *E2A*-deficient GMP by BCR-ABL. We did observe a two-folder higher β-catenin level in *ex vivo*-cultured p210/GMP (Supporting Fig 11), consistent with the increase in reporter activity (Fig. 4C). Because the restoration of *E2A*
function did not suppress the propagation of GMP in mHPC/p210 cultures, it appears that E2A itself is unable to reverse the transforming activity of BCR-ABL. The factors that collaborate with BCR-ABL to convert GMP into leukemic stem cells remain to be identified.

A unique feature of the experimental system developed here is the *ex vivo* propagation of BCR-ABL-transformed GMP and nonGMP in the same cultures (Fig. 5). At steady state, the mHPC/p210 cultures contain a very low level of GMP (~0.3%) and a high level of CD11b+ myeloid cells (~90%). The CD11b+ cells are likely to be derived from the GMP as demonstrated by the replating experiments (Supporting Figure 10). This mixed culture provides an experimental system to compare the pathobiological properties of BCR-ABL-transformed leukemic stem cells (GMP) and BCR-ABL-transformed leukemic cells (nonGMP). It has been shown that BCR-ABL kinase can directly activate β-catenin (21, 22). Those studies observed a 2 to 3 fold activation of the Topflash reporter in transient co-transfection experiments employing established cell lines. Using a stably integrated Topflash in the mHPC/p210 experimental system, we also detected a two-fold increase in the nonGMP population of mHPC/p210 relative to mHPC/GFP. However, we observed a further increase in the reporter activity in the GMP population of mHPC/p210. This increase was even more dramatic with the GMP population isolated from the bone marrow of mHPC/210-transplanted mice. These results suggest that the activation of β-catenin by BCR-ABL is modulated by the cell context (GMP versus nonGMP) and possibly
also the microenvironment (S17 stromal cells versus bone marrow). Gene expression profiling experiments have suggested that deregulation of the β-catenin pathway is a hallmark of CML disease progression (23). If the activation of β-catenin by BCR-ABL is more efficient in the leukemic stem cells, the overall activity of this pathway detected by gene profiling experiments may represent the relative levels of leukemic stem cells in blast crisis CML patient samples. The mHPC/p210 experimental system developed in this study may be useful in testing the efficacy of anti-β-catenin therapies in the treatment of blast crisis CML.

Acknowledgements

We gratefully acknowledge Dr. Ruibao Ren and Dr. William G. Bornmann for providing us with materials and David Ditto for assistance with blood examination. Super8X Topflash was a kind gift from Dr. Randall Moon. We thank R. Levenzon, Y. Nomura and K. Klingenberg for their technical and secretarial assistance and members of the Wang lab for their critical comments throughout the course of this work. This work was supported by NIH grant CA043054 to J.Y.J.W.
Figure 1 Transformation of murine pluripotent hematopoietic progenitor cells (mHPC) by BCR-ABL.

(A) Retroviral-mediated expression of GFP. Three days after infection with the MSCV-IRES-GFP or the MSCV-p210-IRES-GFP retrovirus, GFP-expression was determined by FACS, with mock-infected mHPC as negative control.

(B) Expression of BCR-ABL and GFP. Lysates (50 µg protein) of the indicated cells were probed with anti-pTyr245-Abl, anti-Abl (8E9) and anti-GFP antibodies by immunoblotting. Levels of the endogenous ABL protein were used as loading control.

(C) Peripheral blood analyses. PB samples from the indicated mice were collected at 7 weeks post transplantation. The white blood cell (WBC) counts, the levels of hemoglobin (Hb) and the platelet (PLT) counts are shown as mean ± s.e.m. from the indicated number (n) of mice. A representative Wright-Giemsa staining (original magnification, × 1,000) of PB from an mHPC/p210-transplanted mouse is shown.

(D) Expansion of GFP+ cells in mHPC/p210-transplanted mice. C57BL/6J-Tyr<sup>c-2</sup>/J mice (CD45.1, 8 weeks old) were transplanted as described in Experimental Procedures. Percentages of GFP+ cells in the bone marrows from mice sacrificed at 8 weeks post transplantation are shown. The data shown are mean ± s.e.m. from the indicated number (n) of mice. BM: bone marrow.

(E) Splenomegaly in mHPC/p210-leukemic mice. The spleen weights are shown as mean ± s.e.m. determined from the indicated number (n) of mice.

(F) Summary of transplantation experiments. Leukemia was scored by two criteria: splenomegaly (> 400 mg) and dominant GFP-positive cells (> 70 %) in bone marrows by FACS. NBM: normal bone marrow.
**Figure 2** BCR-ABL-transformed leukemia-initiating cells occur in the GMP population.

(A) Expansion of CD11b<sup>+</sup> cells in mHPC/p210 cultures. The expression of GFP and two lineage markers: B220 and CD11b was determined by FACS at 7 days post infection with MSCV-IRES-GFP (Vector), MSCV-p210-IRES-GFP (p210) and MSCV-p210KD-IRES-GFP.

(B) Analyses of myeloid progenitors in *ex vivo*-cultures. Infected cultures within 2 passages (7 days) after GFP-sorting were used for the analyses of GMP (Lin<sup>−</sup>, IL-7R<sup>−</sup>, Sca-1<sup>−</sup>, c-Kit<sup>+</sup>, CD34<sup>+</sup>, Fcγ R<sup>+</sup>) and MEP (Lin<sup>−</sup>, IL-7R<sup>−</sup>, Sca-1<sup>−</sup>, c-Kit<sup>+</sup>, CD34<sup>−</sup>, Fcγ R<sup>−</sup>) as percentages of total GFP<sup>+</sup> cells. The data shown are mean ± s.e.m. from three independently derived mHPC/GFP and mHPC/p210 cultures.

(C) Kaplan-Meier survival curves. The indicated numbers of GFP<sup>+</sup>-GMP and GFP<sup>+</sup>-nonGMP sorted from the mHPC/p210 cultures were mixed with normal bone marrow cells (2x10<sup>5</sup>) and injected into lethally irradiated recipient mice. The disease free survival of the indicated number of mice was monitored for 90 days.

(D) Analyses of progenitors in bone marrow cells. Bone marrow samples from mice transplanted with mHPC/GFP or mHPC/p210 were collected at 8 weeks post transplantation. Bone marrow samples from untreated healthy mice were also collected to detect distribution of normal myeloid progenitors in the bone marrow cells. Percentages show each population of total GFP-positive cells. The data are shown as mean ± s.e.m. of the indicated number (n) of mice.

(E) Kaplan-Meier survival curves. The indicated number (n) of GFP<sup>+</sup>-GMP from mHPC/p210-leukemic primary mice (8 weeks post transplantation) were mixed with
normal bone marrow cells (2x10^5) and injected into lethally irradiated recipient mice. The disease free survival of the indicated number of mice was monitored for 80 days.
Figure 3 Restoration of E2A-function does not eliminate p210-transformed GMP.


(B) Expression of E2A-ER. The indicated cells were treated with 4-OHT (1 µM, 24 hours) on S17 stromal cells plus cytokines, and total lysates (50 µg protein) were immunoblotted with anti-E2A and anti-tubulin antibodies.

(C) Activation of E2A-regulated genes after 4-hydroxytamoxifen (4-OHT)-treatment. E2A-ER-expressing mHPC/p210 cells were treated with 4-OHT (1 µM, 24 hours) and the levels of Id2, Hes-1 and GAPDH RNA were determined by quantitative real-time PCR as described in Methods. The ΔCT values were standardized by actin and the standardized mean values without 4-OHT-treatment were set as 1. The data shown are mean ± s.e.m. from three independent experiments.

(D) Analysis of GFP⁺-CD11b⁺ and GFP⁺-GMP cells. The mHPC/p210 and mHPC/p210/E2A-ER cells were cultured under the indicated conditions with or without 4-OHT (1 µM) for 24 hours. The percentages of CD11b⁺ and GMP cells among the total GFP⁺ cells were determined by FACS. The data shown are mean ± s.e.m. from three independent experiments.
Figure 3

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Figure 4  BCR-ABL-transformed GMP-like cells displayed higher levels of β-catenin activity than the nonGMP cells.

(A) Schematic of lenti-Topflash. Seven TCF/LEF binding sites upstream of a minimal promoter (P_{TA}) drive expression of firefly luciferase. The pgk-puro sequence is inserted downstream of the luciferase gene and allows infected cells to be selected with puromycin.

(B) Infection of mHPC cells with lenti-Topflash. Total RNA was collected from control or puromycin-selected cells following infection with lenti-Topflash and reverse transcribed into cDNA. Infection was monitored by polymerase chain reaction using primers specific for the luciferase gene.

(C) β-Catenin activity from ex vivo-cultured mHPC/p210-Top. Three batches of ex vivo-cultured mHPC/GFP-Top or mHPC/p210-Top cells were sorted into GFP⁺-GMP and GFP⁺-nonGMP fractions. cDNA was made from total RNA and transcript levels of the luciferase reporter were determined by quantitative RT-PCR. The ΔCT values were standardized by those of actin and the standardized mean values of GFP⁺-nonGMP from the mHPC/GFP cultures were set as 1. The data shown are mean ± s.e.m. from three biological repeats and triplicate PCR reactions per sample.

(D) β-Catenin activity from mouse bone marrow. 1x10⁶ mHPC/GFP-Top or mHPC/p210-Top cells were injected into each of 3 mice along with 2x10⁵ freshly isolated normal bone marrow cells. Three weeks after injection, bone marrow cells were collected and sorted into GFP⁺-GMP and GFP⁺-nonGMP fractions, and quantitative RT-PCR was carried out and analyses were done as described in Fig. 4C. The standardized mean values of GFP⁺-nonGMP from mHPC/GFP-Top-transplanted
mice were set as 1. The data shown are mean ± s.e.m. from three independently transplanted mice with triplicate PCR reactions per sample.
Figure 5  Summary of mHPC transformation by p210\textsuperscript{BCR-ABL}. Expansion of GMP (minority) and CD11b\textsuperscript{+} cells (majority) in mHPC cultures requires p210\textsuperscript{BCR-ABL} kinase activity. Once established, the CD11b\textsuperscript{+} and GMP cells co-exist in the same cultures. The leukemic stem cells reside in the GMP population with high β-catenin activity. The differentiated myeloid leukemic cells with low β-catenin activity develop cytokine and stromal independence but do not cause leukemia in mice.
**Figure 6** Generation of leukemic mice.

(A) Expansion of GFP$^+$ cells in mHPC/p210-transplanted mice. C57BL/6J-Tyr$^{c-21}$/J mice (CD45.1, 8 weeks old) were transplanted as described in Methods. Representative FACS profiles for GFP$^+$ cells in the indicated tissues from mice sacrificed at 6 weeks post transplantation are shown. BM: bone marrow, PB: peripheral blood.

(B) Representative spleens from the indicated mice at 8 weeks post transplantation.

(C) Histopathology. Representative tissue sections stained with hematoxylin and eosin (H&E) (original magnification, $\times$ 400) from the indicated mice collected at 8 weeks post transplantation.
Figure 6
Figure 7 Southern blotting of proviral insertions in leukemic splenocytes.

(A) The MSCV-BCR-ABL-IRES-GFP construct, the relevant restriction enzyme sites and the probe used for Southern blotting of total genomic DNA.

(B) Genomic DNA was isolated from the spleens of leukemic mice using the Promega Wizard genomic DNA purification kit (Promega, Madison, WI). 30 μg of genomic DNA was digested with EcoRI (E) or HindIII (H) as indicated, transferred onto Bio-Rad zeta membrane by alkaline capillary method, then hybridized with 32P-labeled 2.2 kb probe as indicated. Left panels: splenic DNA from two primary and two secondary recipient mice transplanted with a freshly infected mHPC/p210. Right panel: splenic DNA from two secondary recipient mice receiving bone marrow from primary leukemic mice transplanted with a different batch of freshly infected mHPC/p210.
Figure 7
Figure 8 Properties of the mHPC/p210 cultures.

(A) Scatter plot of microarray data. The fluorescence intensities from two biological repeats were normalized using the R package (DNAMR, http://www.rci.rutgers.edu/~cabrera/DNAMR) and fitted to the linear model using the LIMMA package (30). The significantly up- and down-regulated genes in mHPC/p210 are labeled with red and green, respectively ($p < 0.001$).

(B) Representative alterations in lineage-associated genes.

Gene Expression Profiling of mHPC/p210. Two million viable mHPC and mHPC/p210 were separated from S17 stromal cells by sorting for GFP$^+$, PI-exclusion and CD45$^+$ cells. Total RNA was isolated from the sorted cells using RNeasy kit (Qiagen, Valencia, CA). The quality of the RNA was assessed by the Agilent Bioanalyzer, the Nanodrop Bioanalyzer and by electrophoresis on 0.6% agarose gel. RNA samples were submitted to the UCSD BioMedical Genomics Microarray Core Facility (BIOGEM) for labeling (Amino Allyl MessageAmp™ II with Cy™5) and hybridization to Codelink Mouse 20K slides (Amersham, Piscataway, NJ) (19).

(C) Cytokine- and stromal-independent growth. Freshly infected and GFP-sorted mHPC/GFP and mHPC/p210 as well as mock-infected mHPC were each plated under the indicated culture conditions in the absence of S17 stromal cells. At the indicated time after stromal removal, viable cells were counted by Trypan blue exclusion and cell cycle profiles determined by DNA histogram. The values in the histograms refer to the percentage of cells with sub-G1 DNA content.
Figure 8A
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Figure 8B
Figure 8C
Figure 9  BCR-ABL-transformed leukemia-initiating cells occur in the GMP population.

(A) Summary of transplantation experiments shown in Fig. 2C. The spleen weights are shown as mean ± s.e.m. of the indicated number (n) of leukemic mice. NBM: normal bone marrow.

(B) Expansion of GFP^+ GMP in the bone marrow of leukemic mice. FACS analyses of bone marrow samples obtained from mice transplanted with mHPC/GFP or mHPC/p210 were collected at 8 weeks post transplantation as in Fig. 2D. The percentage of GMP cells among GFP^+ bone marrow cells is shown. The data are mean ± s.e.m. from the indicated number (n) of mice.

(C) Summary of mouse transplantation. GMP and MEP were sorted from leukemic bone marrow by the analyses shown in (B) and injected into secondary recipients as in Fig. 2E. The data of spleen weights and WBC counts are shown as mean ± s.e.m.. Leukemia was scored by two criteria as shown in Fig. 1F. NBM: normal bone marrow.
### A

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### B

![Flow cytometry plots](image_url)

### C

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**Figure 9**
**Figure 10** GFP⁺-GMP generated myeloid progenies.

*Ex vivo*-cultured mHPC/p210 within 2 passages after GFP-sorting (Fig. 1A) were used for the sorting of GFP⁺-GMP. The sorted cells were replated with cytokines in the presence or absence of the S17 stromal cells. FACS analyses of GFP and CD11b were performed on the indicated dates after replating. Representative quadrant dot plots for CD11b⁺ and GFP⁺ cells are shown. Similar results were obtained in three independent experiments.
Figure 10
Figure 11  Levels of β-catenin protein in GMP and nonGMP fractions.

Bone marrow collected from normal C57BL/6J-Tyr<sup>e-2J/J</sup> and E2A-deficient mice (8 weeks old) were sorted into GMP and nonGMP fractions, and lysates from 5x10<sup>5</sup> cells of each fraction was immunoblotted with the indicated antibodies. GMP and nonGMP fractions from <i>ex vivo</i>-cultured mHPC/p210 were similarly sorted and lysates from 5x10<sup>5</sup> cells of each fraction examined by immunoblotting for β-catenin.
References


The text of Chapter 3 is a reprint of the material as it appears in *Proceedings of the National Academy of Sciences*, 2008, Vol. 105, No. 46, Yosuke Minami, Scott A. Stuart, Tomokatsu Ikawa, Yong Jiang, Asoka Banno, Irina C. Hunton, Dennis J Young, Tomoki Naoe, Cornelis Murre, Catriona H. Jamieson, and Jean Y.J. Wang. I was the secondary author of this paper. My contributions included designing and performing the experiments analyzing β-catenin levels in mHPC cells. I also designed and constructed all the lentiviral vectors used in this study and contributed to writing the manuscript.
Chapter 4: Discussion

In this study, we have characterized many of the molecular components of a novel pathway leading to the degradation of the cyclin dependent kinase inhibitor p21p21 after IR. Future studies will be needed to address the biological significance of this degradation and to determine whether proteolysis of p21 contributes to the survival of transformed cells after IR.

Chromatin recruitment and the ATM-independence of p21 degradation

One of the unique features of the IR-induced degradation of p21 is that it is independent of ATM and related PIKK family members. An explanation for this observation can be derived from the fact that this degradation requires both the p21-PCNA interaction and the Cul4-DDB1Cdt2 E3 ligase, both of which can be recruited to chromatin independently of ATM. It has been shown that PCNA is rapidly recruited to chromatin following treatment with ionizing radiation or bleomycin (a structurally related form of phleomycin), with maximal levels of chromatin associated PCNA being observed within 5 minutes of damage (1). This recruitment to chromatin occurs with similar kinetics in both normal and A-T fibroblasts, demonstrating that PCNA is recruited to chromatin after damage independently of ATM (1). As p21 is known to bind PCNA (2), this also provides a mechanism for p21 to be recruited to chromatin independently of ATM (Fig. 1).
Once recruited to chromatin, p21 likely becomes a potential target of the Cul4-DDB1\textsuperscript{Cdt2} as the known targets of this protein are degraded on chromatin (3). While relatively little is known about how Cul4-DDB1\textsuperscript{Cdt2} is activated, it is known that this activation can occur independently of PIKKs as this complex has been shown to degrade the replication licensing factor Cdt1 in an ATM/ATR-independent manner after ionizing radiation (4). Taken together, the above data are suggestive of a model whereby IR leads to a PCNA-dependent recruitment of p21 to chromatin.

Simultaneously, Cul4-DDB1\textsuperscript{Cdt2} is recruited to chromatin and activated through a poorly defined mechanism. Once activated, the Cul4-DDB1\textsuperscript{Cdt2} complex mediates the degradation of chromatin-bound p21 (Fig. 1).

A number of assumptions made in the construction of this model need to be addressed. First and foremost, as this model is predicated on p21 being recruited to chromatin after IR, it needs to be demonstrated that this actually happens. Simple cell fractionation experiments can be conducted to determine if this occurs. Furthermore, the model would suggest that only chromatin-bound p21 would be ubiquitinated and degraded. Cell fractionation experiments could also confirm this hypothesis, provided they were done in the presence of MG132 and inhibitors of deubiquitinating enzymes. Finally, resolving how the Cul4-DDB1\textsuperscript{Cdt2} complex finds p21 would help clarify the model. It has been shown that Cdt2 and p21 coimmunoprecipitate when overexpressed in HEK293 cells. However, whether this interaction is direct remains to be determined (5). In vitro binding assays using bacterially expressed Cdt2 and p21 would be informative in this regard. Failure of these two proteins to interact in vitro would
suggest that modification of the proteins or the presence of another factor (factor “X” in Fig. 1) regulates the p21/Cul4-DDB1\textsuperscript{Cdt2}. interaction and thus p21 degradation.

**Importance of p53 and Rb Impairment**

To date, we have only observed the IR-induced degradation of p21 in transformed cells that lack functional Rb and p53 pathways. Interestingly, although no connection has been suggested between p53-status and the IR-induced degradation of Cdt1, this event has also only been shown in transformed cells lacking p53 (HEK293, HeLa, and H1299) (4,6). It would certainly be worthwhile to test whether the IR-induced degradation of Cdt1 occurs in fibroblasts that fail to degrade p21 after IR. Should these cells also fail to degrade Cdt1, it would suggest that the IR-induced degradation of multiple Cul4-DDB1\textsuperscript{Cdt2} targets may only occur in transformed cells deficient for p53.

Furthermore it would allow us to make a definitive conclusion about the nature of cells that degrade p21 and Cdt1 after IR. Impairment of Rb would be entirely ruled out as a contributing factor as H1299 cells, which degrade Cdt1 after IR (6), are Rb-proficient. Our experiments with p53\textsuperscript{−/−} HCT116 cells have ruled out an exclusive role of p53. Therefore, the conclusion would be that cells that rapidly degrade these proteins after IR require multiple “hits”, one of which is likely impairment of p53.

**Subunit Rearrangement in Virally Transformed Cells**
It has been reported that T-antigen and other viral oncoproteins can affect the intracellular distribution of p21. Shortly after p21 was first observed in complexes with cyclins, cdks, and PCNA, it was reported that these complexes undergo subunit rearrangement in cells transformed by viral oncoproteins (7). In these experiments, antibodies to various cyclins and cdks were used to immunoprecipitate cdk complexes from $^{35}$S-labeled lysates of normal cells or cells transformed with the SV40 T-antigen. While p21 and PCNA were readily detectable in the cdk complexes of normal cells, they were absent from the same complexes in transformed cells (Fig 2A, path a). In the absence of p21 and PCNA, two new proteins, p16 and p19 (now known to be cdk inhibitors of the INK family), became associated with cdk complexes (7).

Similar results were obtained with HEK293 cells and HeLa cells, which are transformed by unrelated viral oncoproteins, suggesting this was a common feature of virally transformed cells. Li-Fraumeni cells, which contain no viral oncoproteins, but are transformed in part through loss of p53 also yielded similar results (7). Based on the fact that p21 and PCNA were no longer detectable in cdk immunoprecipitates from all these cells, the authors proposed that p21 and PCNA dissociate from cdk complexes in transformed cells that lack p53 (Fig 2A, path a).

The specificity of this subunit rearrangement for transformed cell lines lacking functional p53 is almost identical to what we see for the IR-induced degradation of p21, and may help to explain why p21 is only degraded in these cells. As mentioned above, the mechanism through which Cul4-DDB1Cd2 recognizes p21 is unresolved. It is possible this interaction does not occur when p21 is bound to cdks. Therefore, in
normal cells, where most p21 is cdk-bound, p21 would not be degraded after IR (Fig 2B). However, in transformed cells, where there is a large pool of p21 unable to bind cdks, p21 would be rapidly degraded by Cul4-DDB1\textsuperscript{Cdt2} (Fig 2B). Importantly, the recruitment of p21 to chromatin is unlikely to be different in these cells as we believe this is a PCNA-dependent process and PCNA is recruited to chromatin after IR in normal cells(1).

**Subunit Rearrangement and p53-dependent transcription of p21**

It is important to note that the study describing the subunit rearrangement of cdk complexes was published prior to the discovery that p21 was a transcriptional target of p53, and therefore the effect of viral oncoproteins on p53-dependent p21 expression was not considered. With the knowledge that p21 levels are often low in p53 impaired cells, it seems quite possible that viral oncoproteins do not cause p21 to dissociate from cdk complexes, but simply lead to significantly reduced p21 expression which results in much less p21 being detected in cdk immunoprecipitates (Fig 2A, path b). Since PCNA is appears to be recruited to most cdk complexes by binding to p21 rather than by directly binding cyclins or cdks (8), this explanation would also still explain the absence of PCNA from cdk complexes.

Therefore, in pursuing a connection between subunit rearrangement and the IR induced degradation of p21, it will be important to more carefully examine the cdk bound fraction of p21 in normal versus virally transformed cells. When the initial studies on subunit rearrangement were done, there were no antibodies available to
p21, and thus immunoprecipitations of p21 could not be done. Determining if subunit rearrangement does occur should be as simple as doing these immunoprecipitations. If subunit rearrangement does occur, the percentage of immunoprecipitated p21 that is cdk-bound should be significantly lower in T-antigen transformed cells. Should p21 immunoprecipitations show that the percentage of cdk-bound p21 is the same in normal and T-antigen transformed cells, it would suggested that the reported dissociation of p21 from these complexes was simply an artifact of reduced p21 expression caused by the impairment of p53.

**Biological Impact of IR-induced p21 Degradation**

The biological consequences of p21 degradation in transformed cells following IR are currently not known. The most well studied functions of p21 are its inhibition of cdkS and PCNA (2). Degradation of p21 in these contexts would lead to increased cdk activity or an increased ability of PCNA to participate in DNA synthesis. It is unlikely that transformed cells would degrade p21 to liberate cdkS, as these cells already impair Rb (9). In contrast, it is reasonable to expect that transformed cells, which are genetically unstable and are constantly having to cope with DNA damage (10), may have developed a means to degrade p21 in order to free PCNA to participate in DNA repair. Such an increase in repair may promote survival in these cells after damage.

The best assay to use to look for a survival advantage in irradiated cells is the clonogenic survival assay, in which a known number of irradiated cells are seeded at
low densities and cultured for up to two weeks to determine the number of cells that survive IR and form colonies. We have attempted to set up such an experiment by infecting HEK293 cells with a p21 lentivirus or a lentivirus expressing a non-degradable p21 (which expresses an N-terminal tag that seems to block degradation). However, these experiments have been complicated by the fact that overexpression of p21 in these cells cause them to acquire a senescent-like phenotype and ultimately leads to apoptosis. As, this experiment is the best way to definitely prove (or rule out) a role for p21 degradation in the survival of transformed cells after IR, future efforts should focus on designing a clonogenic survival assay in which p21 levels can be expressed at tolerable levels. One potential strategy is to transiently express a non-degradable p21 such that it is present at roughly physiological levels at the time of irradiation. Transgene expression would quickly decline over the course of the 10-14 day assay allowing cells to recover and proliferate in the absence of p21 overexpression.

**Insights from Cdt1 degradation**

The work discussed above demonstrates that ionizing radiation induces the degradation of p21 in a number of transformed cells lines that lack functional p53 and Rb. In addition, it shows that this degradation is dependent on the Cul4-DDB1$_{Cdt2}$ E3 ligase and that the p21-PCNA interaction is critical for this event. Interestingly, this degradation does not occur in normal human fibroblasts.
Shortly after we observed the IR-induced degradation of p21, it was reported that UV radiation can also cause degradation of p21 (11). This has since been confirmed by several groups and was shown to occur in a Cul4-DDB1\textsuperscript{Cdt2} and PCNA-dependent manner (12-14). In addition, this pathway has also been shown to be required for degradation of p21 in S-phase (5). In contrast to the IR-induced degradation of p21, both the degradation after UV and the degradation in S-phase have been observed in a number of cell lines, many of which maintain functional p53 and Rb pathways (11).

The Cul4-DDB1\textsuperscript{Cdt2}-dependent degradation of p21 is almost indistinguishable from the degradation of the Cul4-DDB1\textsuperscript{Cdt2}-dependent replication licensing factor Cdt1. Cdt1 is degraded by this complex in response to UV and IR (4,6,15-17), and to date, the only published experiments reporting degradation of Cdt1 after IR were conducted in transformed cell lines lacking functional p53 (4,6). In addition Cdt1 is also degraded by Cul4-DDB1\textsuperscript{Cdt2} in S-phase (18,19).

As mentioned above, it will be quite interesting to look at Cdt1 degradation in our fibroblasts to determine if, similar to p21, Cdt1 fails to be degraded in these cells after IR. The fact that the IR-induced pathway appears to be unique to transformed cells suggests that transformed cells may acquire a pathway to repair DNA double-strand breaks that normal cells do not have. This may be naturally selected for in transformed cells which are constantly coping with high levels of genetic instability.
Concluding Remarks

In the case of Cdt1, it has been shown *in vivo* that degradation by Cul4-DDB1\(^{Cdt2}\) prevents the S-phase entry of damaged cells (4). In contrast, there is not yet conclusive evidence showing that degradation of p21 has biological consequences *in vivo*. The current hypothesis is that p21 degradation is required to allow PCNA to participate in DNA repair, but this has not yet been convincingly demonstrated. As mentioned above, the only way to prove that there is a biological impetus for p21 degradation after DNA damage is to ask whether there is decreased survival in irradiated cells expressing a non-degradable, full length p21 at near physiological levels. Future studies in our lab should be able to answer just that question.
Figure 1 Model for p21 degradation by Cul4-DDB1<sup>core</sup>. Ionizing radiation leads to recruitment of PCNA-bound p21 to chromatin. The Cul4-DDB1<sup>core</sup> complex is also recruited to chromatin and activated by a poorly defined mechanism. The recognition of p21 by Cul4-DDB1<sup>core</sup> may be direct, potentially through Cdt2 (8), or may require an as of yet unidentified protein ("X").
Figure 2 Transformed cells lacking p53 may preferentially degrade p21 after IR. A) Model depicting subunit rearrangement in cells transformed by viral oncoproteins (or in other transformed p53-null cells). In path a, p21 is unable to bind cyclin-cdk complexes in these cells, leading to a pool of p21 free of cdkks. In path b, p21 is able to bind cdkks, but must compete with p16 and p19, leading to a pool of p21 that is not associated with cyclin-cdk complexes. p21 levels are decreased in both pathways due to a lack of p53-dependent expression. B,C) p21 free of cyclin-cdk complexes may be more accessible to the Cul4-DDB1<sup>apo</sup> than cdk-bound p21, and therefore more rapidly degraded.
References
