THE ROLE AND REGULATION OF $p21^{WAF1/CIP1}$ IN MYELOPOIESIS

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Elevated levels of the molecular adaptor protein p21$^{waf1/cip1}$ (p21) and of the IL-3 receptor α chain are correlated with chemoresistance and poor prognosis in acute myeloid leukemia (AML). p21 is a core regulator of many biological functions including cell cycle control, apoptosis and differentiation. Our laboratory has demonstrated a decrease in p21 expression levels during cytokine-induced granulocytic differentiation, leading us to hypothesize that p21 antagonizes granulopoiesis. The proliferative cytokine IL-3 has been shown to prevent granulocytic differentiation of murine and human myeloid progenitor cells. We also hypothesized that IL-3 inhibition of differentiation is mediated in part by p21, and tested this in murine 32Dcl3 myeloblasts that are used to model granulopoiesis. Our findings demonstrated that p21 antagonized differentiation by promoting apoptosis of cells exposed to the differentiation inducer G-CSF. We also showed that p21 prevented premature expression of primary granule proteins and contributed to maintenance of the myeloblast phenotype. Furthermore, p21 knockdown accelerated morphologic differentiation of 32Dcl3 cells stimulated to differentiate with G-CSF. We then determined how IL-3 maintains p21 expression in myeloblast cells. We showed that IL-3 stabilized p21 mRNA in myeloblasts leading to high levels of p21 protein. This effect mapped to the 3' untranslated region (UTR) of the p21 transcript. p21 transcript stabilization by IL-3 was independent of PI3-kinase and ERK pathway signaling. *In vitro* binding assays provided evidence that distinct sets of RNA:protein interactions occur within the proximal 303 nucleotides of the p21 3' UTR and are regulated by IL-3 and G-CSF signaling. Association of a 60-65 kDa

protein with p21 riboprobes correlated with IL-3 mediated p21 mRNA stabilization, whereas binding by a 40-42 kDa protein was associated with destabilization of p21 transcripts in 32Dcl3 cells undergoing G-CSF-induced differentiation. These findings provide the first evidence for IL-3-mediated stabilization of mRNA transcripts in myeloid progenitor cells. The finding that p21 antagonized granulopoiesis is also novel. Because high levels of the IL-3 receptor and high p21 expression have separately been linked to poor outcomes in AML, IL-3 mediated p21 mRNA stabilization may contribute to differentiation blockade during AML pathogenesis.

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PREFACE

During my first explorations in the world of laboratory science a sage mentor who knew of my aspirations advised that I select a thesis advisor as if I were picking a spouse. He told me that the relationship between a student and a mentor is very intense, can be contentious at times and is filled with strong emotions, for better or worse. To Dr. Richard Steinman, my mentor, I am grateful that you welcomed me into your lab and put up with my often misplaced intensity, my contentiousness and the strong emotions emanating from my frequent misinterpretations of reality. To be sure, I am more grateful for the wisdom you shared, opening doors of scientific thought previously locked to me and creating in me a deep respect for the scientific method and those engaged in it. I am yet more grateful for the standards you held me to, for your endless patience and guidance, and for your unwavering support and belief in me. The things for which I am most grateful are your character and your friendship. To you, Richard, my boss, my mentor and friend, I am forever indebted, thank you.

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and most of all your friendship. To Dr.'s Pam Hershberger, Mick Jarzynka, Michelle Miranda, Lori Emert-Sedlak and Robert Redner, I thank you for sharing with me many enlightening scientific discussions, plentiful camaraderie and always provocative philosophical ruminations.

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1. Introduction

1.1. Overview

Acute myelogenous leukemia (AML) results from uncontrolled cell growth of immature myeloid progenitor cells and accounts for 34% of all leukemias and 30% of all hematological malignancies[1,2]. Despite advances in cancer therapeutics, the overall five-year survival of patients with AML remains at 14%. Elevated levels of the molecular adaptor protein $p21^{cip1/waf1}$ (p21) are correlated with chemoresistance and poor prognosis in AML[3]. p21 is a core regulator of many biological functions including cell cycle control, apoptosis and differentiation. Control of p21 expression and function is complex, being regulated by cellular stress, growth factors, and differentiation stimuli; however, how p21 is regulated in hematopoiesis is not well understood. Our laboratory has demonstrated that p21 undergoes dynamic changes in expression levels and subcellular compartmentalization during cytokine-induced myeloid differentiation, suggesting that p21 may play an important role in myeloid development. The mechanisms that control changes in p21 abundance and the resultant functional outcomes upon myelopoiesis have not been addressed and are the focus of this dissertation.

Studies of monocytic cell lines have provided evidence to support an anti-apoptotic role for p21 in myeloid cells. However, both the inhibition and promotion of apoptosis are functions that have been attributed to p21. During granulocytic differentiation of human CD34+ cells, p21 protein levels initially rise and then decline when blasts commit to terminal differentiation, a stage associated with decreased cell survival. These findings led us to hypothesize that p21 inhibits cell death during granulopoiesis, and that p21 downmodulation contributes to the

differentiation-associated decrease in survival as granulocytes mature. This hypothesis is explored in Chapter II using 32Dcl3 cells. 32Dcl3 cells are normal, diploid, myeloid progenitor cells of murine origin with high basal levels of p21 expression that depend upon IL-3 for survival, proliferation, and maintenance of an undifferentiated state[4,5]. These cells provide a good model to study the effects of p21 upon myelopoiesis because they undergo granulocytic differentiation in response to G-CSF and recapitulate the dynamic changes in p21 expression observed during granulocytic differentiation of CD34+ human hematopoietic progenitor cells. Data within this chapter demonstrates that artificial maintenance of p21 levels leads to an unexpected proapoptotic activity during the myeloblast to granulocyte transition, suggesting the downmodulation of p21 that occurs in differentiating 32Dcl3 cells is necessary for survival.

Experiments presented in Chapter III address why p21 levels decrease when IL-3 is replaced by G-CSF in the 32Dcl3 model system. Because post-translational modification of p21 protein can regulate its stability, it was hypothesized that IL-3 maintains p21 protein levels through inhibition of signal pathways linked with proteasomal decay of p21. Our data did not support this hypothesis. In contrast, our studies indicated that IL-3 acts posttranscriptionally at the level of p21 message. IL-3 stabilizes p21 transcripts through control of protein:RNA interactions within the p21 3' untranslated region. These findings provide the first evidence for IL-3-mediated stabilization of mRNA transcripts in myeloid progenitor cells.

Because IL-3 supports myeloid progenitor cell development *in vivo* and is an antagonist of terminal differentiation in 32Dcl3 cells, Chapter IV explores the hypothesis that IL-3 suppresses granulocytic differentiation, in part, through maintenance of high p21 levels. In support of an inhibitory role for p21 during differentiation, studies in primary keratinocyte precursors have demonstrated that p21 inhibits the expression of genes normally found in

terminally differentiated keratinocytes and prevents their morphologic maturation. Our investigations in differentiating myeloid cells revealed that siRNA-mediated knockdown of p21 accelerated the kinetics of G-CSF induced differentiation. This was associated with premature expression of primary granule proteins. The mechanism of this effect was at the posttranscriptional level. Since high levels of p21 have been described in subsets of AML, our finding of accelerated differentiation in p21 knockdown cells suggests that differentiation blockade by p21 may be one mechanism that contributes to AML pathogenesis.

Current literature regarding p21 structure and function is summarized in the Introduction in order to aid in the understanding of the experimental and interpretive framework used in our studies. All experiments were conducted in myeloid cells, so a brief review of myelopoiesis with an emphasis on granulocytic maturation is presented first. The extensive functional versatility of p21 warrants discussion of the role of p21 in cell cycle control and regulation of DNA synthesis, of how p21 contributes to apoptotic pathways, and the role of p21 in differentiation. Furthermore, it has become evident that a complex interplay of transcriptional, post-transcriptional and post-translational mechanisms regulate p21 expression. Background information on these mechanisms is therefore included. Lastly, a review of IL-3 biology as it pertains to myeloid development and AML pathogenesis will be discussed.

1.2. Myelopoiesis

Myelopoiesis is the process whereby myeloid cells, monocytes and granulocytes, develop from pluripotent hematopoietic stem cells (HSCs) in the bone marrow. Myeloid cells are critical mediators of antimicrobial and inflammatory responses. Their production results from a linear progression where HSCs become committed myeloid progenitors (CFU-GEMM), capable of

producing all myeloid blood elements (i.e., granulocytes, erythrocytes, macrophages and megakaryocytes), then form lineage-specific precursors restricted to production of monocytes and granulocytes (CFU-GM). Final maturation into granulocytes (i.e., neutrophils) or monocytes is influenced by specific cytokines and the sequential activation of lineage-specific transcription factors. Monocytic differentiation from CFU-GM precursors is dependent upon the cytokine M-CSF and PU.1/c-jun transcriptional activity[6-8]. Whereas IL-3 is important for the development of the common myeloid progenitor, (reviewed in section 1.6), the cytokine G-CSF is an important mediator of neutrophil maturation[9]. Indeed, mutations in the G-CSF receptor are responsible for a variety of clinical conditions characterized by insufficient neutrophil production[10]. Transition from myeloblasts, the first morphologically identifiable granulocytic precursor, to terminally differentiated neutrophils requires the activation of the transcription factor CCAAT/enhancer binding protein α, C/EBPα[6,11]. Expression of neutrophil granule proteins, enzymes responsible for neutrophil killing functions, also occurs during this transition and is mediated in part by the related transcription factor C/EBPε[12]. Mutations which impair C/EBPα transcriptional activity cause developmental arrest at the myeloblast stage and are responsible for the development of AML in some patient populations[13,14]. Failure to complete the myeloid differentiation program is a central etiologic factor in myeloproliferative syndromes and myeloid leukemia pathology.

1.2.1. Acute Myelogenous Leukemia

Acute myelogenous leukemia (AML) is a hematologic malignancy characterized by rapid proliferation of abnormal blast cells and impairment of normal blood cell production. AML constitutes the majority of all leukemias and is responsible for 1.2% of all cancer deaths in the United States[15]. Although AML can afflict both adults and children, it is predominantly a

disease of adulthood with a rising incidence in each decade after the age of 50. Therapies have improved overall 5-year survival rates to near 50% for all leukemias, however, five-year survival for patients with AML is under 20% and has not improved significantly over the past decade[2]. Combination chemotherapeutic regimens are used as first-line therapy to induce remission. Complications of this therapy include bone marrow suppression, which by itself is sometimes fatal, and the production of drug resistant leukemic blasts when therapy is unsuccessful.

Failure to complete the normal myeloid differentiation program at different stages is implicated as contributing to AML because of the heterogeneity of undifferentiated blasts found in subtypes of the disease. When this happens, accumulation of abnormal blasts in the bone marrow occurs, and serves to impair the synthesis of normal leukocytes, red cells, and platelets. The clinical manifestations of the disease stem mainly from this loss of production. Left untreated the course of the disease is rapid, leading to death within six to twelve months[16].

AML molecular pathogenesis is characterized by cytogenetic abnormalities as well as intragenic mutations. Morphologic subtypes of AML are often correlated with specific chromosomal translocations which create leukemogenic fusion proteins such as the AML-ETO fusion protein (t(8;21), M2 subtype)[17]. Activating mutations in receptor tyrosine kinases such as Flt3 or c-Kit, or activating mutations in the small GTPase Ras are also prevalent in AML[18-20]. Hematopoietic transcription factors such as C/EBPα and GATA-1 are subject to inactivating point mutations in many cases[13,21]. Often, the molecular defect underlying the cause of AML is not identified. Despite this, high levels of a growing number of genes are being correlated with disease severity and prognosis, including the IL-3 receptor α chain and p21, and therefore warrant further investigation[3,22].

1.3. p21 Structure

The human p21 gene is located on chromosome 6, maps to the p arm at 21.2 (6p21.2) and spans 8.6 kilobases. p21 has two characterized splice variants which both encode an identical protein of 164 amino acids (Genbank Accession # NM_078467, and NM_000389). These variants differ in length by 141 nucleotides in the 5' untranslated region, however, the functional significance of this difference remains to be identified. p21 function has undergone extensive study in murine systems. The homologous murine p21 gene spans 6.9 kilobases, is located on chromosome 17 at 15.23 cM (Genbank Accession # NM_007669) and produces a 159 amino acid protein. The nucleotide and protein sequences contain 76% and 79% similarity, respectively, with the human homologue.

The human p21 protein is an adapter molecule with no known catalytic function. It is grouped together by sequence homology with two other proteins, p27 and p57, into the Cip/Kip family of cyclin-dependent kinase inhibitors (CDKIs). Most p21 functions have been ascribed to either its N-terminal or C-terminal domains. The CDK (AA 49-71) and Cyclin (AA 21-26) binding domains of p21 reside in its N-terminal region and are conserved among the Cip/Kip family members. Sequence homology diverges at the C-terminus with that of p21 being promiscuous in its binding affinities. A secondary Cyclin-binding domain is found in the C-terminus (AA 153-159), which overlaps p21's nuclear localization sequence (AA 140-159) and its PCNA binding domain (AA-141-160). Structure-function and protein interaction mapping of p21 domains has been largely ascertained through mutational analysis. Although crystal structure data is available for small C-terminal polypeptides (<20 amino acids), atomic resolution of the full-length p21 protein has not been achieved.

Despite the lack of crystallographic data, elements of p21 tertiary structure have been inferred from analysis of crystal structures of the homologous kinase inhibitory domains of p27

in complex with Cyclin A and Cdk2[23]. p27 was shown to bind both Cyclin A and Cdk2 through its Cyclin and CDK binding domains respectively. Stabilization of these two domains occurs through formation of an α-helix by the intervening residues. Although sequence homology within the Cip/Kip family for this linker region is minimal, secondary structure prediction and circular dichroism analysis suggest an α-helical structure is present in all three proteins, and corresponds to amino acids 27-48 of p21[24,25]. Inhibition of the Cyclin A/Cdk2 complex was postulated to occur through two mechanisms. First, p27 binding was shown to alter the shape of the Cdk2 catalytic cleft; and second, a p27 helix caused a steric blockade of the ATP binding site. Understanding of p21 secondary and tertiary structure is further limited because p21 is intrinsically unstable in solution, failing to maintain a defined conformation[24]. This observation, coupled with spectroscopic examination of p27, prompted Lacy et. al. to hypothesize that the specificity of p21 interactions are governed by a folding-on-binding mechanism[25,26]. This hypothesis proposes that intrinsically disordered molecules such as p21 and p27 obtain a more ordered confirmation only after initial contact with a binding partner. It was further proposed that the specificity of interactions within multiple protein complexes is mediated by the first binding partner; and in the case of p27 or p21, initial binding to a particular Cyclin determines which conformation is adopted and therefore controls which other interactions will take place.

1.4. p21 Biological Functions

First identified in 1993 as a regulator of cell cycle progression, p21 is now known as a molecular adaptor protein with pleiotropic effects. p21 lacks an identified enzymatic activity and therefore functions through its association with an ever growing cohort of binding partners. Through these interactions, p21 exerts its influence on distinct cellular functions such as DNA replication, transcriptional control, apoptosis, differentiation and cell migration. p21 behavior is

complicated by observations that demonstrate p21 to have opposing functions that are tissue-type dependent or dependent upon its nuclear or cytoplasmic localization. For example, p21 can be both pro- and anti-apoptotic, can both promote and inhibit differentiation and transcription, and is associated with both active and inactive enzyme complexes (Fig. 1). The *in vivo* consequences of many p21 protein interactions remain to be identified, leaving open the possibility that p21 functions in other cellular processes.

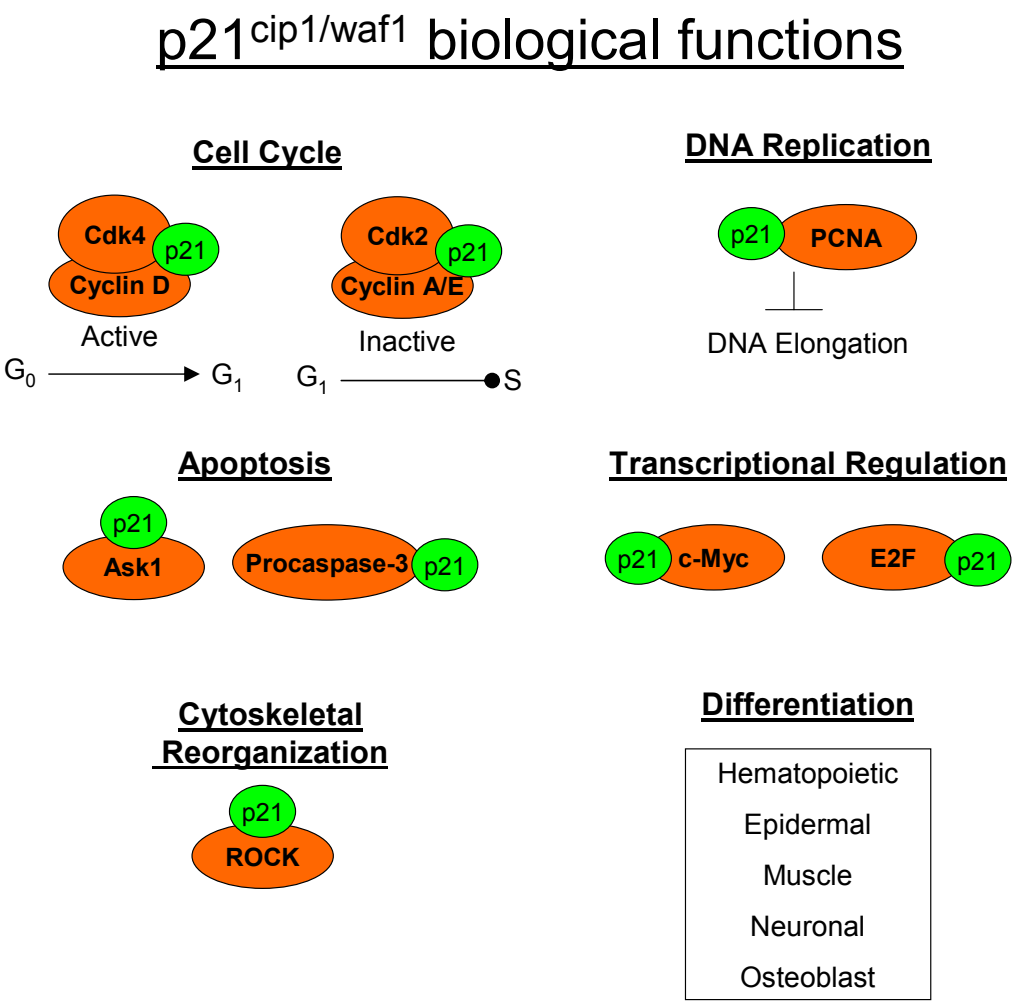

Figure 1. p21 Biological Functions.

p21 regulates multiple cellular processes. p21 both promotes and inhibits cell cycling through interactions with distinct Cyclin-Cdk complexes and can block DNA synthesis through inhibition of PCNA, a component of the DNA polymerase holoenzyme. p21 regulates the function of both enzymatic and non-enzymatic proteins to influence apoptotic and transcriptional processes, as well as cell motility. Although the mechanisms are ill-defined, p21 either promotes or inhibits differentiation in several tissue types. The diversity of specific intracellular protein interactions are highlighted.

1.4.1. Regulation of the Cell Cycle and DNA synthesis

Proliferating cells traverse through several phases before dividing. These phases are grouped in what is known as the cell division cycle, or cell cycle. The cell cycle is divided into two growth phases, G1 and G2, a DNA replication phase, known as the S phase, and mitosis. Cell cycle progression proceeds from G1 to the S phase, is followed by G2 and culminates with mitosis. Phase transitions are known as checkpoints and transition from one phase to another is dependent upon the activity of cyclin and cyclin-dependent kinase (CDK) complexes. Control of cyclin-CDK complexes is multifactorial, and includes negative regulation by cyclin-dependent kinase inhibitors (CDKI) which are comprised of two protein families, the INK4a (p15, p16, p18, p19) and the Cip/Kip (p21, p27, p57). INK4 proteins bind to Cdk4 and Cdk6 complexes, thereby liberating Cip/Kip proteins sequestered by these CDKs; this frees Cip/Kip proteins to block cycling by binding to nuclear Cdk2 complexes. Cip/Kip family proteins inhibit cyclin-CDK complexes through binding of both the cyclin and CDK proteins, and are not restricted to G1 regulation (for review, [27]). p21 was the first identified CDKI.

Early work on p21 described it as the downstream effector of p53-induced growth arrest (i.e. Wild-type p53 Activated Fragment or WAF1) [28]. An independent and simultaneous study identified p21 as a Cdk-interacting protein ($p21^{Cip1}$), causing arrest through direct interaction and inhibition of Cdk2 kinase activity [29]. Growth arrest following DNA damage and other cellular stressors occurred at the G1-to-S transition of the cell cycle[30], but it was later shown that p21 could also arrest cells in the G2 phase[31], and prevent G2 arrested cells from re-entering S phase following DNA damage[32]. In other early studies, Zhang et. al. identified p21 as a component of quaternary complexes in association with cyclins, CDKs and the proliferating cell nuclear

antigen (PCNA)[33]. PCNA is a subunit of DNA polymerase δ and is necessary for processivity of this enzyme. Through its interaction with PCNA, p21 was shown to be a potent inhibitor of DNA synthesis following DNA damage, doing so without inhibiting PCNA-dependent nucleotide excision repair [34,35].

p21 can bind to several different combinations of Cyclin and CDK complexes to control their activity. p21 is most efficient as an inhibitor of Cdk/Cyclin-A or Cdk/Cyclin-E complexes to cause cell-cycle arrest[36,37]. Conversely, p21 promotes assembly of Cdk/Cyclin-D complexes and remains associated with active complexes during G1 phase in proliferating cells [38-40]. A prevailing model suggests that the pool of available, or unbound, p21 is regulated through association with Cyclin/CDK complexes[27]. The model predicts that sequestration of free p21 into active Cdk/Cyclin-D complexes prevents its inactivation of Cdk2/Cyclin-E or -A complexes, enabling the unfettered progression of the cell cycle, and suggesting that p21 can serve as an assembly factor for Cyclin:Cdk complexes to drive proliferation[27].

S phase DNA replication is dependent upon expression of many genes that are controlled by the Rb (pRB, p107, p130) and E2F (E2F1 – E2F7) family of transcription factors. E2F proteins activate transcription of S phase genes and are targeted by negative regulators of the cell cycle. Hypophosphorylated Rb proteins act as transcriptional repressors and contribute to the maintenance of G1 cell cycle arrest. Rb repressor functions are executed through formation of a repressor complex with E2F proteins, as well as recruitment of histone deacetylases to E2F containing promoters[41,42]. Active Cdk4/6- and Cdk2-cyclin complexes phosphorylate Rb family proteins downstream of mitogenic signals. Hyperphosphorylation of Rb triggers the release and derepression of E2F proteins, allowing for E2F transactivation (for review, [43]. p21 regulation of this pathway is threefold. First, direct interaction and inhibition of Cdk2/Cyclin

complexes promotes the accumulation of hypophosphorylated Rb and repression of E2F[29]. Second, p21 can disrupt existing interactions between Cdk2 and p130-E2F complexes[44]. Last, accumulating evidence indicates that p21 can associate with promoter elements, bind E2F subunits directly and inhibit their transcriptional activity independent of Cdk2 modulation [45,46]. Although this analysis presents a simplification of the Rb-E2F transcriptional regulatory axis, it serves to illustrate that p21 control of the G1-S transition is accomplished through multiple mechanisms, acting as both an allosteric and competitive inhibitor, as well as a transcriptional repressor.

Induced expression of p21 was found to decrease the expression of genes associated with DNA synthesis and cell proliferation, and also increase the expression of genes associated with senescence[47]. Several studies have shown p21 to interact with and inhibit transcription factors which are key effectors of mitogenic signaling pathways such as Stat3, c-Myc and E2F as noted. Repression of Stat3 transcriptional activity was found to occur through direct interaction by p21 and did not require disruption of Stat3 DNA binding ability[48]. Although the p21 domain responsible for inhibition was not identified in this study, the C-terminal PCNA binding domain of p21 was found to act as a transcriptional repressor of c-Myc, preventing association of the c-Myc:Max heterodimer with E-box elements[49]. It is interesting to note that Kitaura et. al. also observed that titration of c-Myc was able to rescue DNA synthesis blocked by p21 repression of PCNA. This may represent a possible reciprocal regulatory circuit between p21 and c-Myc that is governed by the relative abundance of each molecule. It is likely that repression of mitogenic transcription factors and inhibition of DNA synthesis through PCNA repression are two complementary mechanisms used by p21 to exert S phase control. These mechanisms may also

support ongoing blockade of DNA synthesis and cell cycle progression that accompany p21 induced cellular senescence.

One early study utilizing a cDNA library expression screen determined that p21 expression was sufficient to block cell proliferation and induce a senescent phenotype[50]. Gene knockout studies revealed that the absence of p21 was sufficient to prevent senescence in fibroblasts[51], whereas in keratinocytes this phenotype depended upon ablation of both p21 and p14(ARF)[52]. Recent work has demonstrated that translational control of p21 by the RNA binding proteins CUGBP1 and Calreticulin contributes to the determination of cell fate in fibroblasts[53]. CUGBP1 was found to relieve translational repression by Calreticulin through competition for an element in the p21 5'UTR, causing an increase in p21 protein expression, growth arrest and a senescence phenotype. Furthermore, induction of senescence by p21 has been shown to correlate with radiation and chemoresistance[54]. Data support a role for p21 as an inducer of senescence, however, the mechanism of this function remains unclear. In addition to negative regulation of the cell cycle, data support an anti-apoptotic function of p21 during replicative senescence.

1.4.2. Regulation of Apoptosis and Stress Responses

p21 has been found to regulate apoptosis in response to many different stimuli including oxidative stress, chemotherapeutic agents, radiation, and death receptor activation. Both the inhibition and promotion of apoptosis by p21 has been described. The preponderance of literature defines p21 as an apoptotic inhibitor and the majority of the following discussion will address this function. Numerous studies of human cancers have linked high levels of p21 expression to chemoresistance and poor prognosis [3,55]. While only a few mechanisms have been

delineated, the effect of p21 on apoptosis is often dependent upon two factors, its abundance and its intracellular localization. The role of p21 in apoptosis is further characterized by its control of both p53-dependent and p53-independent apoptotic processes.

Evidence suggests that p21 protects cells from p53-induced apoptosis while mediating p53-dependent cell cycle arrest[56]. p53 tumor suppressor activity is essential for maintenance and repair of the genome, and promotes apoptosis or growth arrest following genomic insults. The mechanism of p53-induced apoptosis is not well understood, but results in part from transcriptional activation of genes controlling apoptosis that can activate mitochondrial and death receptor apoptosis pathways (for review[57]. p21 may serve to switch cells from the p53 death pathway to a growth arrest phase during which DNA repair can occur[58].

Studies utilizing melanoma and colorectal carcinoma cell lines demonstrated that high levels of p53 expression could trigger apoptotic responses[59,60]. Inactivation of p21 in colon carcinoma cell lines by homologous recombination was shown to increase the sensitivity of cells to p53-induced apoptosis[59]. Furthermore, murine embryonic fibroblasts (MEFs) derived from p21 knockout mice ($p21^{-/-}$) underwent high levels of apoptosis in response to overexpression of p53[60]. Ectopic expression of p21 in these MEFs, as well as in p53 sensitive melanoma cell lines, was found to protect against p53-induced apoptosis and enhance survival[60]. c-Myc has been reported to prevent activation of p21 by p53 following DNA damage, thereby increasing p53-directed cell death in damaged cells[61]. Together these findings suggest that p21 expression levels are an important determinant of the balance between growth arrest and apoptosis stimulated by p53 activation.

p21 can also oppose several p53-independent apoptotic pathways, such as those induced by cytokines or subclasses of chemotherapeutic agents that do not induce p53 expression. High

levels of p21 expression have been shown to inhibit TGF-β induced apoptosis in retinal endothelial cells[62]. In a model of monocytic differentiation, NF-κB mediated upregulation of p21 was shown to prevent apoptosis that was induced by autocrine production of TNF-α [63]. In glioma cells, downregulation of p21 using antisense oligonucleotides caused an increase in sensitivity to CD95-ligand induced apoptotic signals. In addition, STAT1-dependent p21 expression has been shown to be protective in the setting of IFN-γ induced apoptosis[64]. Gene knockout studies revealed that induction of p21 in bone marrow derived macrophages by IFN-γ treatment was required to protect against apoptotic stimuli such as growth factor withdrawal or treatment with lipopolysaccharide[65]. p21 has also been shown to oppose apoptosis induced by microtubule inhibitors (MTIs). p21 deficient cells were shown to experience more apoptosis following treatment with the MTIs Taxol and vincristine; and rescue of this phenotype was accomplished by ectopic expression of p21[66]. The HER2/neu oncogene is overexpressed in many breast cancers, and its expression is correlated with increased resistance to chemotherapy with Taxol[67]. It has been shown that HER2/neu-overexpressing cells induce resistance to Taxol through transcriptional activation of p21[68].

p21 may prevent apoptosis in part by antagonizing Cdk2. Cdk2 activity has been shown to be a necessary component of the effector phase of apoptosis[69-71]. These studies revealed that an increase in Cdk2/CyclinA nuclear localization and activity occurred as a result of caspase-3 activation. Partial blockade of apoptosis resulted from expression of either Cdk2 dominant negative mutants, pharmacologic inhibition of Cdk2, or, significantly, by expression of a caspase cleavage resistant mutant of p21.

Caspase 3 has been reported to undermine antiapoptotic functions of p21 by cleaving p21. Caspase-3 mediated cleavage of p21 has been shown to occur following γ-irradiation, treatment

with chemotherapy molecules, or oxidative stress[72-74]. In each circumstance, p21 cleavage was correlated with an increase in Cdk2 activation and with increased apoptosis. p21 cleavage occurs in the C-terminal region of the protein at D^{112}, an aspartic acid that is part of the caspase recognition motif ($DHVD^{112}L$)[70]. Cleavage separates the CDK inhibitory domain (AA 49-71) from the nuclear localization sequence (AA 140-159). Cytoplasmic localization of the residual N-terminal domain results as a consequence. Compartmental separation of p21 and Cdk2 was correlated with an increase in Cdk2 activity, as well as an increase in apoptosis[75].

Conversely, in some cell types, cytoplasmic localization of the full length p21 protein exerts a protective effect against apoptosis. This effect is mediated through direct interactions with several molecules thought to be involved with the initiation and effector steps of apoptosis. Interaction between p21 and procaspase-3 was found to inhibit Fas-mediated apoptosis and block caspase activation in human hepatoma cells [76,77]. p21 prevented procaspase-3 proteolysis through blockade of the p3 serine protease recognition site. Furthermore, in a model of DR4 TRAIL receptor inducted apoptosis, p21 overexpression in breast, lung and colon cancer cell lines was shown to block cleavage of the initiator caspase, caspase 8[78]. Our laboratory has shown in the leukemic cell line K562 that induced expression of p21 promoted the stabilization of the apoptotic inhibitor protein c-IAP1 and correlated with an increase in chemoresistance[79]. These findings support the hypothesis that cytoplasmic p21 opposes the activity of central components of the apoptotic cascade and that the survival promoting effects are not dependent upon p21 nuclear localization in all cell types.

Signals initiated by cellular stressors such as TNF-α, radiation and oxidative stress are often transduced by cytoplasmic kinases resulting in growth arrest or engagement of the apoptotic machinery. Shim et. al. found that p21 expression was sufficient to inhibit the activation of

stress-activated protein kinase (SAPK or JNK), a terminal effector of stress pathway signaling whose activation can lead to apoptosis[80]. Subsequently, Asada et. al. determined that p21 mutants restricted to expression in the cytoplasm resulted in decreased SAPK activation and were able to inhibit apoptosis triggered by multiple stimuli[81]. Moreover, they found that p21 control of SAPK activity was a result of p21 binding and inhibition of the apoptotic-signal regulating kinase, ASK1, and that this regulation occurred within the cytoplasmic compartment. The molecular mechanism of p21 inhibition of these kinases remains unresolved, however, it is evident from these studies that p21 retains sufficient plasticity to interact with and inhibit multiple kinases, in addition to Cdk2, to enhance cell survival.

In contrast with the above reports, several studies have linked p21 to the promotion of apoptosis. These studies utilized either p21 overexpression or were conducted in systems that suppressed p21 expression. In overexpression studies, p21 was sufficient to promote apoptosis or promoted apoptosis in conjunction with death receptor activation[82,83], DNA damaging agents[84,85], ionizing radiation[86], or antioxidant treatments[87-89]. Exogenous p21 alone has been sufficient to induce apoptosis in cervical[90], esophageal[91], and breast cancer cell lines[92] and a peptide corresponding to the C-terminus of p21 has recently been shown to cause apoptosis in the U937 myelomonocytic cell line [93]. p21 suppression by gene deletion or antisense techniques attenuated apoptosis induced by death receptor activation in thymocytes[83], retinoids in hepatoma cell lines[94], and by expression of the DNA-repair protein RAD50 in colon carcinoma cells[89]. It has been speculated that p21 may exert its proapoptotic effects through interactions with the DNA repair machinery[56]. The proapoptotic effect induced by p21 C-terminal peptides was shown to require disruption of the outer mitochondrial membrane, but was independent of

caspase activation, and suggests a mechanism that can be separated from p21 activity in the nucleus[93].

In summary, numerous reports support a role for p21 in apoptosis regulation, although few details are known regarding how p21 regulates apoptosis. p21 antiapoptotic functions have been attributed to p21 compartmentalization either in the nucleus or cytoplasm. Direct binding and inhibition of apoptotic effectors, stabilization of negative regulators, and inhibition of stress pathway kinases are mechanisms mediated by cytoplasmic p21. As in the p53-dependent response to stress, where absolute levels of p21 expression are linked to cellular decisions to undergo either growth arrest or apoptosis, it is plausible that the ratio of cytoplasmic and nuclear p21 govern the apoptotic outcome. The antiapoptotic effects of p21 have been correlated with chemoresistance in numerous studies and implicate p21 as an important contributor to cancer cell survival. A proapoptotic function for p21 has also been characterized, but mechanisms remain speculative.

1.4.3. Control of Differentiation

Changes in p21 levels have been linked to differentiation in several cell types including muscle, neuronal, epidermal and hematopoietic cells. Differentiation and cell proliferation are coordinated in a precise fashion during development. Terminal differentiation is associated with growth arrest which is mediated by upregulation of one or several cell cycle regulatory proteins, particularly cyclin-dependent kinase inhibitor family members, including p21. This raised the speculation that p21 was responsible for exit of terminally differentiating cells from the cell cycle[95]. In differentiating myotubes, MyoD induction of p21 appeared to support this view[96]. However, high steady-state levels of CDKI's are found in both mitotic myeloid and erythroid

progenitors as well as in post-mitotic differentiated cells, suggesting that in these lineages CDKI's do not simply couple differentiation to growth arrest [95,97-100].

p21 has been shown to promote cellular differentiation in several tissues. p21 overexpression can promote differentiation of myelomonocytic, promyelocytic and erythroid leukemia cell lines[101-103]. In muscle, p21 expression can be directly activated by MyoD, a muscle-specific, differentiation-promoting transcription factor[104,105]. Furthermore, under conditions of differentiation suppression, exogenous expression of both p21 and $p16^{ink4a}$ in myoblasts was sufficient to induce muscle-specific genes and cell cycle withdrawal[106]. In a study of xenopus retinal progenitors, Ohnuma et. al. showed that p21 expression was sufficient to induce glial cell differenatiaion[107]. Analysis of oligodendrocytes from $p21^{-/-}$ mice demonstrated that differentiation required p21[108].

On the other hand, there is some support for an inhibitory role for p21 during differentiation. Our laboratory has shown that p21 expression is dramatically decreased in post-mitotic granulocytes in both normal cell lines and cord blood derived stem cells differentiated *in vitro*[100]. This supports the finding of high p21 levels in undifferentiated myeloblasts isolated from patients with chemoresistant AML[3]. Additionally, in primary keratinocyte precursors, p21 inhibits the expression of genes normally found in terminally differentiated cells and prevents morphologic maturation[109].

The differentiation effects of p21 have been shown to be independent of its cell cycle modulating ability in several of these systems. p21 can promote differentiation in promyelocytic leukemia cells without inhibiting cell-cycle progression[103]. In oligodendrocytes and retinal progenitors, neural differentiation stimulated by p21 was shown to be independent of its ability to regulate CDKs or cause growth arrest[107,108]. In keratinocyte progenitors, p21-induced

differentiation blockade was a function localized to the p21 C-terminal domain[109]. Deletion of the C-terminus abrogated the inhibitory effect, suggesting that inhibition was independent of CDK binding and control.

However, CDKI's are necessary to maintain cell cycle arrest in terminally differentiated cells. The timing of CDK inhibition is increasingly thought to be an important factor in proliferative and survival signaling during differentiation[98]. Using murine erythroleukemia cells in a model of erythropoiesis, Matushansky et. al. revealed that a specific order of CDK inhibition was required to commit proerythroblasts to differentiate[102,110]. Transition to the differentiated state required first the inhibition of Cdk2, followed by inhibition of Cdk6. They showed that either expression of p21 or the combined use of roscovitine, a Cdk2 inhibitor, followed by overexpression of p16, a Cdk4/6 inhibitor, could induce differentiation[110]. Reversing the order of inhibition did not result in differentiation, but caused cell death instead[110]. For p21 induced differentiation, they reasoned that p21 can directly inhibit Cdk2, but mediates indirect inhibition of Cdk6. p21 can promote dissociation of p16 from cytoplasmic Cdk4 complexes, thus making it available to inhibit Cdk6[110]. Growth arrest was correlated with late stage upregulation of p27, which is also seen in myeloid differentiation, and a late drop in Cdk4 activity[110]. It is conceivable that an analogous system of precise temporal inhibition of Cdk's is also controlled by p21 in epidermal, neural and muscle tissues.

The role of p21 in differentiation has also been characterized using mice carrying homozygous deletions of the p21 gene. Initial p21 knockout animals ($p21^{-/-}$) displayed no gross developmental abnormalities, suggesting that p21 is not a necessary component in differentiation pathways[30]. By contrast, p21 and p57 double null animals had severe defects in lung alveoli and skeletal muscle[111]. This implicates a functional redundancy of these proteins during

development, and illustrates the difficulty of interpreting findings from single gene knockout studies. However, closer scrutiny of cells derived from $p21^{-/-}$ single knockout animals initially revealed defects in both hematopoietic and epidermal stem cell development. In $p21^{-/-}$ hematopoietic stem cells, Cheng et. al. demonstrated that p21 inhibits stem cell cycling and helps to regulate stem cell population size by controlling the balance of self-renewal versus proliferation[112]. Absence of p21 in keratinocyte stem cells allows self-renewal to go unchecked and results in a similar expansion of the stem cell compartment [113]. These knockout studies link p21 to control of stem cell proliferation and normal differentiation in multiple tissues.

Data from knockout studies has also shown that p21 may have a tumor suppressor function. Long term study of p21 deficient mice (~2 years) revealed an increased susceptibility to hematopoietic, endothelial and epidermal tumors[114]. Ras-transformation of $p21^{-/-}$ primary keratinocytes also was shown to be sufficient to promote tumor formation in mice when compared to controls containing wild-type p21[115]. Subsequent animal studies demonstrated that $p21^{-/-}$ keratinocytes were more susceptible to carcinogenesis induced by DMBA and displayed a greater prevalence of higher grade undifferentiated skin tumors [113,116,117]. These studies implicate inactivation or suppression of p21 as a mechanism that contributes to malignancy in multiple tissues.

Knockout models have indicated two functions of p21 in differentiating muscle and neuronal cells. One function is to oppose differentiation-associated apoptosis. $p21^{-/-}$ myogenic progenitors were found to have defects in skeletal muscle regeneration following injury, with increased levels of apoptosis during differentiation [118]. In support of this function, forced expression of p21 alone was sufficient to inhibit differentiation-induced apoptosis in cell line models of myogenesis[119]. In neuroblastoma cells, antisense suppression of p21 revealed that p21

was antiapoptotic during differentiation[120]. Because p21 antiapoptotic effects have not been linked to differentiation in other tissues, these studies suggest that the role of p21 in cell survival during differentiation is tissue-specific and therefore not generalizable.

A second function of p21 derived from knockout studies is the promotion of cytoskeletal reorganization in neural cells. One early finding demonstrated that a delay in cerebellar myelination occurs in $p21^{-/-}$ mice[108]. Myelination requires the cytoplasm of glial cells (Schwann cells) to form processes that ensheath neural axons in response to axonal signals that have yet to be identified[121]. Cytoplasmic p21 has been found to regulate neurite remodeling during differentiation of retinal progenitors through association with and inhibition of Rho kinase (ROCK) [81,122]. Interestingly, cytoplasm restricted p21 mutants were found to stimulate axonal regeneration following spinal cord injury by promoting myelination of neuronal outgrowths, a process inhibited by active ROCK in this setting[123]. One can hypothesize from these findings that p21 is a node that integrates axonal myelination signals. In this capacity p21 may promote the formation of cytoplasmic outgrowths from axons that interact with and stimulate myelin production from surrounding myelin producing cells.

1.5. Regulation of p21

1.5.1. Gene Expression

Expression of the p21 gene is controlled through transcriptional activation and repression, and, to a much lesser extent, epigenetic silencing. Transcriptional activation of p21 was first described to be regulated by p53 in 1993[28]. In 1994, our laboratory determined that p21 undergoes p53-independent transactivation in the setting of myeloid differentiation[95]. Since then, many transcription factors that regulate p21 gene expression have been identified including

Sp1/Sp3, vitamin D receptor (VDR), retinoic acid receptor (RAR), AP2, Miz-1, STAT family proteins (STAT1/3/5), C/EBPα, C/EBPβ, E2F1/3 and c-Myc (for review, [124]).

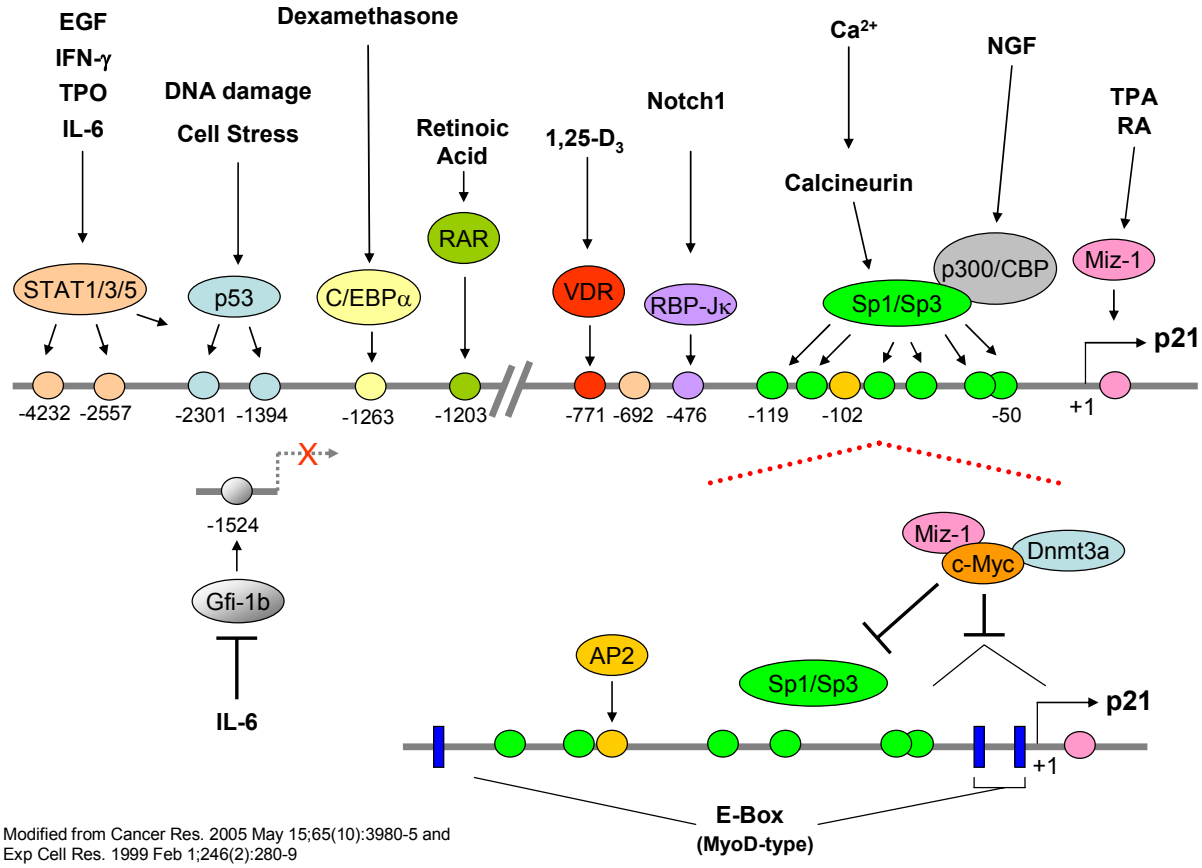

Figure 2. Transcriptional regulation of the p21 gene promoter.

An extensive array of response elements in the p21 promoter (filled ovals) are bound by transcriptional regulatory proteins downstream of an equally diverse set of extracellular stimuli resulting in p21 transcriptional activation. Transcriptional repression of p21 occurs through direct binding of the promoter (e.g., Gfi-1b) or through inhibition of transactivating factors.

There are two p53 binding elements in the human p21 promoter approximately 1.4 and 2.3 kilobases upstream of the transcriptional start site (1.9 and 2.8 Kb in mice) that are conserved in humans, rats and mice (Fig. 2). p53 binding to one of these elements is sufficient, but not required for p21 activation[125]. The promoter-proximal element is also bound by a repressor protein distinct from p53 in CD34+ progenitor cells; this protein disengages during myelopoiesis[100]. Additional evidence of p53-independence comes from p53 knockout mice ($p53^{-/-}$), where p21 expression is found in multiple embryonic and adult tissues[126]. The p53-dependent pathway of p21 activation is also subject to negative regulation by a variety of mechanisms. p53 can be bound and inactivated by phosphorylation[127], bound and targeted for destruction by viral proteins[128], or, alternatively, blocked from binding the p21 promoter by competition[129].

Although recent identification of a tumor suppressor function of p21 has been made in prospective studies of $p21^{-/-}$ mice[114], an extensive study of the p21 gene in human malignancies by single-strand conformation polymorphism analysis determined that intragenic mutations of p21 were rare[130]. However, there is evidence that p21 inactivation occurs as a result of p21 promoter hypermethylation within STAT1 and Sp1/Sp3 response elements [131,132]. Despite some conflicting reports, broader studies of p21 promoter methylation in both solid and humoral human malignancies indicate that p21 gene silencing secondary to hypermethylation is a rare event[133-136]. Epigentic silencing or coding region mutations do not seem to be implicated as mechanisms of p21 inactivation in human cancers. It remains to be determined if methylation of p21 DNA sequences contributes to p21 regulation during normal development or ongoing cellular activities in differentiated tissues.

Numerous differentiation signal pathways, activated independent of the p53 stress response, can also induce p21 transcription. In the myelomonocytic leukemia cell line U937, the

differentiation inducing agents phorbol ester (12-O-tetradecanoyl phorbol-13-acetate, TPA) and okadaic acid were shown to induce p21 transcription via GC-rich Sp1 binding sites in the p21 proximal promoter[137]. These agents also induce p21 transcription through activation of the AP2 transcription factor in K562 leukemia cells, suggesting that alternative pathways exist in leukemia subtypes that can activate p21 transcription [138]. Nerve growth factor (NGF) triggers neuronal differentiation in PC12 cells. In these cells, NGF stimulates the transcriptional coactivator p300/CBP, a histone acetylase, to associate with Sp1 and Sp3 at the p21 promoter, causing a cooperative induction of p21 transcription[139,140]. Although the p21 gene elements responsible for its function are unknown, MyoD, a muscle specific transcription factor that induces myogenesis, also activates p21 transcription in cooperation with p300/CBP[96,141].

In adipocytes and hepatocytes, expression of the transcription factor C/EBPα increases during differentiation and is highest in terminally differentiated, growth arrested cells [142,143]. A concomitant increase in CDKI expression, particularly p21, is also observed in these cell types[144,145]. C/EBPα can activate the p21 promoter and also stabilizes p21 protein expression through direct interaction[144,146]. Furthermore, cell cycle arrest stimulated by glucocorticoid receptor signaling in hepatoma cell lines was found to be dependent upon a consensus C/EBPα binding element in the p21 promoter[147].

Keratinocyte differentiation induced by calcium is mediated by calcineurin, a calcium dependent phosphatase, and is associated with upregulation of p21 transcription[148,149]. p21 promoter activation by calcineurin is dependent upon Sp1/Sp3 binding and may be synergized through association of NFAT1/2 transcription factors (nuclear factor of activated T cells). Activation of Notch signaling pathways also coordinate keratinocyte growth and differentiation.

Rangarajan et. al. identified RBP-Jκ as a protein that binds the endogenous p21 promoter and activates p21 expression in response to Notch1 signals[150].

p21 activation also occurs as a result of cytokine and lipid hormone induced differentiation in hematopoietic cells. 1,25-dihydroxyvitamin D3 can stimulate macrophage differentiation in U937 cells[151]. Liu et. al. determined that a vitamin D3 response element in the p21 promoter is bound by the activated form of the vitamin D3 receptor (VDR) to stimulate p21 transcription. Retinoic acid (RA) induced monocytic differentiation of U937 cells has also been correlated to p21 transcriptional activation. This effect occurs through binding of the retinoic acid receptor (RAR) to the RAR response element approximately 1.2 kilobases upstream of the p21 transcriptional start site[152]. An alternative mechanism of p21 transactivation has also been observed during monocytic differentiation. IL-6 induced monocytic differentiation and G1 arrest of M1 leukemia cells is associated with downregulation of Gfi-1B expression, a DNA-binding transcriptional repressor, and derepression of the p21 promoter[153]. Together, these studies demonstrate three independent mechanisms of p21 gene activation that occur in response to endogenous signaling molecules during monocytic differentiation, highlighting the importance of p21 to differentiation in this lineage. Thrombopoietin (TPO) induced megakaryocytic differentiation is also accompanied by the upregulation of p21 transcription[154]. Engagement of c-Mpl, the TPO receptor, triggers a signaling cascade mediated by JAK2 which results in activation of the STAT family of transcription factors[155]. STAT proteins undergo tyrosine phosphorylation, become dimerized, and translocate to the nucleus where they activate gene expression (for review, [156,157]). Both STAT3 and STAT5 have been observed to undergo phosphorylation in response to TPO in CMK cells, a megakaryoblastic leukemia cell line, however, only STAT5 was found to bind to the p21 promoter. Interestingly, p27 transcriptional

upregulation, but not that of p21, has been shown to occur following G-CSF mediated activation of STAT3 in 32Dcl3 myeloid cells[158].

Several other cytokine induced signals converge upon STAT proteins to facilitate activation of p21 such as those stimulated by EGF, IFN-γ and IL-6. Chin et. al. revealed that growth arrest stimulated by EGF or IFN-γ correlated with STAT1 activation and p21 transcriptional upregulation[159]. This effect was shown to depend upon three STAT1 binding sites in the p21 promoter. In MG63 osteosarcoma cells, STAT1 and STAT3 were found to bind and activate the p21 promoter following induction of differentiation by IL-6 and IL-6 soluble receptor[160]. More recent data shows that IL-6 induced p21 expression in a hepatoma cell line results from the simultaneous binding and cooperation of STAT3, the steroid receptor coactivator 1, NcoA/SRC1a, and p300/CBP on the p21 promoter[161]. A follow-up study noted that PI-3 kinase pathway signals mediated the association of p300/CBP with STAT3 and NcoA/Src1a on the p21 promoter following IL-6 stimulation in glioblastoma cell lines[162]. PI-3 kinase inactivation prevented p300/CBP recruitment to the p21 promoter without altering the binding of STAT3 and NcoA/Src1a. Inhibition of PI-3 kinase activity, by contrast, did not prevent the recruitment of all three factors to the c-Myc promoter, suggesting that constitutive activation of this pathway in transformed cells regulates growth arrest and proliferation through coordinated regulation of p21 and c-Myc transcription.

Direct protein interactions between p21 and c-Myc are responsible for reciprocal inhibition of each protein's function, however c-Myc also operates at an additional level as a repressor of p21 transcription [49](for review, [163]). Although the p21 promoter does not contain the canonical c-Myc binding element (CACGTG or CATGTG), it does contain three E-box elements that conform to the CANNTG motif which are sufficient for binding by c-Myc:Max dimers[164]. In a

study by Claassen et. al., c-Myc repression of p21 in fibroblasts acted through a region -62 bp and +16 bp of the p21 transcriptional start site, however direct binding of this sequence was not examined [165,166]. Another study demonstrated that expression and activation of c-Myc-estrogen receptor fusion proteins in p53 null fibroblasts could also suppress p21 transcription. *In vitro* and *in vivo* binding studies suggested that suppression was mediated by c-Myc interactions with both Sp1 and Sp3[165].

Seone et. al. demonstrated that c-Myc is recruited to the -62 bp to +16 bp p21 proximal promoter region by the DNA-binding protein Miz-1 (Myc-interacting zinc finger 1)[61]. Miz-1 has also been shown to be upregulated in multiple hematopoietic cell lines induced to undergo myeloid differentiation in response to TPA or retinoic acid[167]. Miz-1 expression was sufficient to activate the p21 core promoter during differentiation of these cell lines. The increase of Miz-1 protein expression from basal levels during differentiation was correlated with a decrease in c-Myc expression. Furthermore, c-Myc was shown to associate with the p21 promoter and repress its activation through direct interaction with Miz-1. The authors hypothesized that c-Myc and Miz-1 formed a repressor complex on the p21 promoter in undifferentiated cells, and that release of c-Myc from this complex was dependent upon differentiation signals. A recent study using chromatin immunoprecipitation analysis (ChIP) concluded that c-Myc targets the methyl transferase Dnmt3a to the p21 promoter to form a repressive ternary complex with Miz-1[168]. Moreover, p21 repression by c-Myc was found to require DNA methylation activity. These studies are consistent with the hypothesis that c-Myc specifically represses p21 transcription by multiple mechanisms to inhibit differentiation and promote cell proliferation.

1.5.2. mRNA Stability and Regulation

Regulation of messenger RNA turnover, like that of gene transcription, is a complex and important point of control that determines gene expression. The structural features of an mRNA molecule, the 5' and 3' untranslated regions (UTRs), protein coding region and poly A tail, can all serve as docking points for RNA binding proteins. Akin to transcription factors, these RNA-binding proteins are *trans*-acting factors that regulate splicing, polyadenylation, localization, translation or degradation of a particular transcript. Control of mRNA decay, once thought to occur in a non-specific fashion, is now known to be under tight regulation that is dependent upon evolutionarily conserved *cis*-acting sequences. Alterations in mRNA half-life in response to environmental signals permit a cell to effect a rapid change in transcript abundance and therefore its acute physiologic response. mRNA turnover is also an important regulatory element during differentiation and fetal development, as well as ongoing metabolic processes.

Failure to ensure proper mRNA decay has been linked to several human diseases. For example, short-lived transcripts encoding c-Myc, TNF-α, and many cell cycle regulatory proteins have altered stabilities due to 3' UTR mutations which correlate with disease phenotypes[169-172]. These pathologic states include cancer, cardiovascular disease, chronic inflammatory diseases, α-thalassemia and Alzheimer's disease and are attributable to two principle causes. First, the occurrence of sequence defects in 3'UTR *cis*-acting elements, and second, abnormal expression of the RNA-binding proteins that interact with these elements.

Much data points to the importance of AU-rich *cis*-acting elements (AREs) in the regulation of short-lived transcripts. Abundant in cytokine, transcription factor and cell cycle regulatory gene transcripts, AREs have been identified in ~1500 different genes to date[173,174]. Initial description of these motifs demonstrated that repeats of the AUUUA pentamer conferred a destabilizing effect upon the cytokine transcript GM-CSF[175]. It has since been determined that

triplets of the UUAUUUAUU nonamer are a more optimal destabilizing motif, however, this motif is not an absolute requirement since AU-rich elements that lack this sequence have been shown to be effective destabilizers[176].

The stability of a given ARE containing transcript depends upon two classes of *trans*-acting factors that work in opposition: ARE stabilizers and ARE destabilizers. The primary mRNA stabilizing proteins that have been studied are the Hu proteins: HuC, HuD (neuronal only) and HuR (ubiquitous) [177]. Hu protein overexpression is associated with poor outcomes in cancers [178-181]. Proliferation-associated transcripts that are stabilized by Hu proteins include cyclins A and B1 and c-fos [182,183]. Hu proteins may compete for binding with destabilizing proteins that recognize ARE sequences such as AUF1, KSRP and tristretraprolin (TTP) (e.g., [184]).

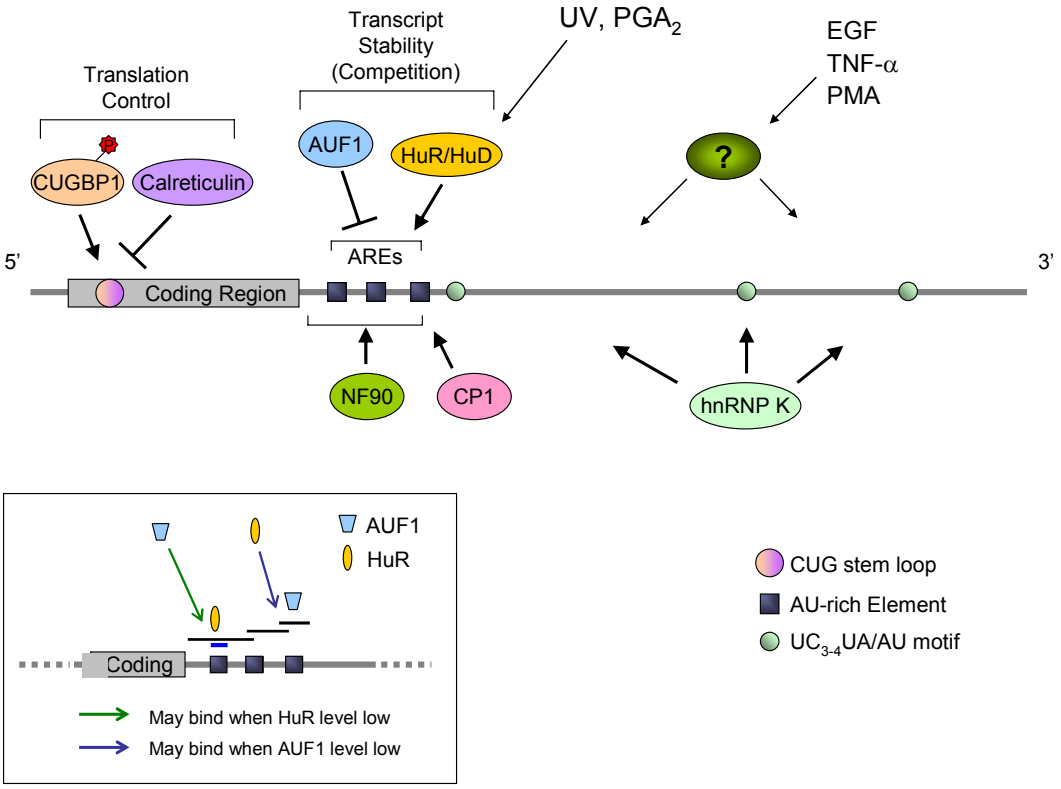

Figure 3. Human p21 mRNA: Regulatory elements and RNA-binding proteins.

Growth factor and stress stimuli trigger RNA:protein interactions which regulate p21 expression levels. RNA-binding proteins interact with several conserved RNA-binding motifs residing in both the coding and 3' untranslated regions of the p21 mRNA. Competition for binding to these motifs regulates both p21 translation rates and p21 message stability. (Inset) Competition for access to AU-rich elements (ARE) between the mRNA stabilizing protein HuR and the mRNA destabilizer AUF1 has been hypothesized to represent a general phenomenon regulating mRNA stability[184,185].

p21 mRNA stability is regulated in response to various stimuli including UV radiation, phorbol ester, TNF-α, phenylephrine, EGF and Prostaglandin A_2[186-191]. Elements responsive to these stimuli are localized to the 3' UTR and include three AREs which contain the AUUUA pentanucleotide. Endogenous RNA binding proteins have been shown to bind these elements and include HuR, AUF1, NF90 and CP1[192-194]. Lethal defects in skeletal muscle formation in $NF90^{-/-}$ knockout mice were correlated with dramatic reductions in p21, MyoD and Myogenin[194]. Increased apoptosis and skeletal muscle abnormalities are also observed in p21/p57 double knockout mice[111]. These findings are consistent with the hypothesis that NF90 stabilization of p21 through binding its AREs is an important component of myogenesis. While CP1 binding was not attributable to a specific stimulus, binding of HuR to p21 ARE containing regions occurs in response to UV radiation, induction of myocyte and neuronal differentiation and Prostaglandin A_2 treatment[189,195,196]. Each of these stimuli caused a substantial increase in the cytoplasmic abundance of HuR, where it is thought to exert the majority of its mRNA stabilizing effect, and correlated with increased p21 mRNA binding and stabilization. However, HuR mRNA binding is not limited to the cytoplasm. In a study by Lal and colleagues in HeLa cells (cervical carcinoma), HuR and AUF1 binding to p21 mRNAs occurred in the nucleus. While both proteins could bind p21 transcripts in the nucleus, competitive binding of one or the other directed cytoplasmic p21 transcripts either to the polysome for translation or to the exosome for degradation [184]. Interestingly, independent suppression of HuR or AUF1 with siRNA demonstrated that each could bind the preferred site of the other; indicating that competition between HuR and AUF1 is a function of their relative expression levels. Further analysis revealed that upon UV irradiation, increased cytoplasmic HuR was found in association with polysome bound p21 mRNA in the absence of AUF1, suggesting that common targeting was a

phenomenon limited to the nuclear compartment. AUF1 has been found to interact with components of the exosome, a multisubunit machine responsible for 3'-5' mRNA degradation, and decay of AU-rich mRNAs has been associated with AUF1 ubiquitination and its proteasomal degradation[197,198]. Since cytosolic AUF1 copurified with exosomal components in ribosome free fractions, it was hypothesized that in the absence of UV stabilizing signals, AUF1 shuttles p21 mRNA to the exosome for decay, thus preventing p21 protein accumulation and growth arrest.

Competitive regulation of p21 translation also occurs and is mediated by RNA binding proteins that interact with ARE-independent elements. A GC-rich region in the p21 coding region can form a stem loop structure which contains a conserved CUG triplet. CUG binding protein 1 (CUGBP1), a protein implicated in the pathogenesis of the CUG triplet expansion disease myotonic dystrophy, was found to interact with this stem loop in a phosphorylation dependent manner CUGBP1 binding promoted p21 translation and a senescent phenotype in fibroblasts[53,199]. Calreticulin was demonstrated to also bind this GC-rich stem loop, functioning to repress p21 translation and promote proliferation. Competition between CUGBP1 and Calreticulin for binding the stem loop was shown to control the onset of senescence in this system.

Within the p21 3' UTR, there are several CU-rich regions that can be targeted by KH-domain containing proteins. The KH-domain, so named for the KH domain in hnRNP K, is an RNA-binding domain that has been conserved throughout evolution in both prokaryotes and eukaryotes[200]. Recent work utilizing a neuroblastoma model of neuronal differentiation showed that upregulation of p21 protein expression during differentiation was a function of translational control mediated by CU-rich elements in the p21 3'UTR[195]. Whereas overexpression of HuR was found to promote increases in p21 protein expression and neuronal differentiation,

exogenous hnRNP K inhibited both without alteration of mRNA stability. Optimal binding of hnRNP K occurs in sequence elements containing the $UC_{3-4}UA/AU$ motif[201]. Three $UC_{3-4}UA/AU$ motifs are present in the human p21 3'UTR, one of which is in close proximity to the AREs (Fig. 3). hnRNP K was found to bind the p21 3'UTR and could inhibit p21 translation of reporter genes containing this sequence. Because HuR and hnRNP K were found to associate within endogenous complexes and bind each other *in vitro*, the authors hypothesized that translational blockade in undifferentiated neuroblastoma cells was mediated by hnRNP K recruitment to the p21 3'UTR through interactions with HuR.

Regulation of p21 transcripts is not limited to interactions with RNA-binding proteins. Sequence specific targeting of microRNAs (miRNA) to mRNA transcripts has emerged as a mechanism that controls mRNA stability and translation. Regulation of cell growth, apoptosis and differentiation of neural and hematopoietic tissues are recently described functions of miRNA genes[202]. In addition, pluripotency in murine embryonic stem (ES) cells is associated with expression of specific miRNAs which become downregulated upon differentiation[203]. Mature 22-bp miRNAs are generated through Drosha endonuclease-mediated processing of a long polymerase II-transcribed pri-miRNA in the nucleus followed Dicer endonuclease-mediated cleavage of a ~80 bp pre-miRNA in the cytoplasm[204]. A recent study of *Drosophila melanogaster* germline stem cells (GSCs) carrying mutant forms of *dicer-1* revealed defects in germline cyst development and in G1 to S phase transitions due to elevated expression of the *Drosophila* CDKI Dap, a protein homologous to p21 and the cip/kip family proteins[205]. A partial phenotypic rescue of cyst production and restoration of Cyclin E levels in GSCs was accomplished by mutation of Dap genes to remove predicted miRNA binding sites in the 3'UTR. Because Dap expression was similarly reduced in GSCs containing either mutant dicer-1 or the

mutant Dap transgene, this suggests that the miRNA pathway plays a role in cell cycle control and Dap expression through a post-transcriptional regulatory mechanism in GSCs. It is conceivable that p21 regulation of mammalian stem cell cycling is also under the control of miRNAs acting on the p21 3'UTR.

In summary, post-transcriptional regulation of mRNA stability and translation is dependent upon both *cis*-acting elements and *trans*-acting factors. Regulated sequences in the p21 mRNA, mainly in the 3'UTR, include AU-rich elements that are targets for stabilization of short-lived transcripts, as well as CU-rich elements and CUG containing stem loops that control translation. These elements are bound by several distinct *trans*-acting factors whose binding, and effects, may be controlled through competition. Cytokines, cellular stressors and differentiation all can evoke changes in p21 message stability and p21 translation in both cell lines and animal models. Furthermore, there is provocative evidence from *Drosophila* studies which suggests that miRNAs may contribute to p21 translation control during GSC division through putative miRNA binding motifs in the 3'UTR. It has become clear that p21 expression is under complex control at the posttranscriptional level. This facilitates rapid coupling of p21 expression to environmental cues. A large network of regulatory proteins and perhaps small RNAs act both to control p21 mRNA stability and p21 protein turnover (see below).

1.5.3. p21 Protein Stability and Posttranslational Modification

1.5.3.1. Phosphorylation

p21 protein functions are governed by its stability, intracellular localization and the range of its binding partners. In broad terms, regulation of these characteristics is achieved within a cell very often through posttranslational modification. There are numerous functional groups that are

covalently attached to proteins to modify charge, conformation, and hydrophobicity including phosphates, sugars, lipids, methyl groups and small proteins such as ubiquitin. p21 has been described to undergo both phosphorylation and ubiquitination.

Phosphorylation of p21 by the serine/threonine kinase Akt/PKB has been shown to occur on two adjacent C-terminal residues, Thr145 and Ser146, which reside within an Akt phosphorylation motif [206-208]. Akt lies within the PI-3 kinase signaling pathway, is activated by phosphoinositides, a class of small phospholipids generated by upstream kinases, and regulates growth, survival and metabolism in many cell types. Scott et. al. utilized surface plasmon resonance and *in vitro* binding studies to show that p21:PCNA interactions were dependent upon the phosphorylation state of Thr145 and Ser146[206]. Phosphorylation at these sites prevented p21 binding to PCNA. Further examination of this phenomenon in endothelial cells showed that PCNA binding was disrupted when p21 was phosphorylated at Thr145 by Akt[209]. In this study, Thr145 phosphorylation limited the ability of p21 to inhibit DNA replication. In addition, data suggested that phosphorylation at this site led to decreased binding and repression of Cdk2, indicating that Thr145 phosphorylation serves to inactivate multiple mechanisms by which p21 inhibits cell proliferation. C-terminal phosphorylation of p21 at Ser146 was also found to be catalyzed by atypical protein kinase-ζ (PKCζ)[210]. Like Akt, PKCζ activity is thought to regulate growth, survival and differentiation, as well as regulate metabolic responses to insulin (for review, [211]. However, unlike Akt, PKCζ was found to only phosphorylate Ser146, and not Thr145, by *in vitro* kinase assay, doing so with a much higher affinity than Akt. In cell line experiments, overexpression of PKCζ resulted in a shortening of p21 protein half-life. Furthermore, it was found that an acute and transient decrease in p21 protein levels following insulin treatment of HCT116 cells (colon carcinoma) was dependent upon PKCζ activity. These

experiments suggest that Ser146 phosphorylation by PKCζ is a signal to increase p21 protein turnover *in vivo* and comprise a pathway through which acute changes in metabolic states are coordinated with regulation of cell cycle modulators. In glioblastoma cells, phosphorylation by Akt occurs at Thr145 and Ser146. Thr145 phosphorylation in this system confirmed an inhibition of p21:PCNA binding. While PKCζ-mediated phosphorylation at Ser146 caused a decrease in p21 half-life in colon carcinoma cell lines, Akt phosphorylation of this site, by contrast, was shown to *increase* the half-life of p21 protein and was associated with increased resistance to Taxol-induced apoptosis[208]. In transfection experiments using $p21^{-/-}$ MEFs, Akt activated by HER-2/neu overexpression was shown to phosphorylate p21 at Thr145 and result in p21 cytoplasmic relocalization. Using phospho-specific antibodies directed at Thr145 in HER-2/neu positive primary adenocarcinomas, phosphorylated forms of p21 were found to localize to the cytoplasm and were correlated with poor survival[55,212]. These studies demonstrate that survival signals integrated by Akt can regulate p21 phosphorylation state and abundance, in a cell type-specific manner, and thereby provide a regulatory mechanism of p21 anti-apoptotic function.

A countervailing mechanism could be mediated by glycogen synthase kinase-3β, a protein phosphorylated by Akt at Ser21/Ser9 to inhibit its activity[212]. GSK-3β regulates many intracellular metabolic processes including protein synthesis and breakdown (for review, [213]). *In vitro* phosphorylation of p21 by GSK-3β was shown to occur at Thr57 and overexpression of GSK-3β in human umbilical vein endothelial cells (HUVEC) was correlated with decreased expression of p21 protein. These observations create a possible scenario whereby opposing activities of Akt and GSK-3β control p21 protein levels to serve as one determinant of cell survival.

Recent data indicates that Thr57 phosphorylation, as well as phosphorylation at Ser130, may control p21 cell cycle effects. Dash et. al. determined that p21 undergoes a transient hyperphosphorylation at the G2/M phase of the cell cycle and that hyperphosphorylated p21 associated with CyclinB1-Cdc2 complexes[214]. Using a Thr57 phosphomimetic mutant of p21, p21 T57D, in $p21^{-/-}$ cells, they showed that cyclin B1 binding of this mutant was required for cyclin B1-associated kinase activity in G2/M. Their data suggests that Thr57 phosphorylation can specify p21:Cdk associations and promote kinase activation at the G2/M checkpoint. In HD3 colon carcinoma cells, p21 phosphorylation was shown to occur in response to TGF-β1 stimulation, resulting in an increase in p21 protein stability and growth arrest[215]. *In vitro* analysis determined that both JNK1 and p38α were p21 kinases. However, phosphopeptide mapping determined that only p38α was able to phosphorylate p21 both *in vitro* and *in vivo* following TGF-β1 stimulation, doing so at Ser130. Together, these findings link phosphorylation of p21 to protein stability as a transcription-independent phenomenon of cell cycle regulation. It should be noted that we have generated stable aspartate and alanine mutants of p21 phosphorylation targets (Thr145, Ser 146, and Thr57) but found that mutations did not alter p21 subcellular distribution in 32Dcl3 cells (data not shown).

1.5.3.2. Proteolysis and Ubiquitin

A major determinant of p21 protein stability is its rate of decay. Proteolysis of p21 is mediated by the proteasome, a multisubunit ATP-dependent protease. The proteasome is a polysubunit cylindrical structure with a 20S core complex containing protease activity and a 19S regulatory complex that detects and recruits ubiquitinated proteins and caps each end. The

covalent addition of ubiquitin (ubiquitination) to proteins is often a precursor step that targets proteins for proteasomal destruction. It has been well documented that p21 protein levels and half-life can be increased through the use of proteasomal inhibitors [216-218]. The ubiquitination of p21 occurs, but it is not the only mechanism that is correlated with p21 stability. The cellular setting and type of decay signals are also important parameters.

Protein ubiquitylation occurs over several steps and is mediated by at least three different enzymes. This pathway begins with free ubiquitin becoming activated by an E1 or ubiquitin activating enzyme. Next, ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2). In the final step, a ubiquitin protein ligase (E3) transfers ubiquitin from E2 to the substrate by catalyzing a covalent linkage between ubiquitin and the ε-amino group of a lysine residue on the substrate. Continuous ubiquitination results in the formation of polymeric ubiquitin chains on the substrate. Efficient proteasomal targeting requires a chain with a minimum of four ubiquitin subunits[219]. This is important because the addition of a single ubiquitin tag can have functional consequences independent of protein fate (for review, [220]). There are numerous E1, E2 and E3 class proteins, with substrate specificity most often defined by interactions with E3 ubiquitin ligases. One notable E3 ligase complex, the Skp1/Cullin/F box protein-related complex (SCF) controls the abundance of some cell cycle regulatory proteins, notably p27 and p57[221,222]. SCF-mediated ubiquitin conjugation of p21 occurs *in vitro* and may be responsible for S phase destabilization of p21[223].

Although ubiquitinated forms of p21 have been described, controversy remains over whether ubiquitination is necessary for basal p21 turnover by the proteasome. Sheaff et. al. used a lysine-free form of p21, which had its six lysine residues mutated to arginine, to determine if ubiquitination was a requirement for p21 proteasomal decay[218]. This mutant protein was shown

to retain its CDK-inhibitory properties, did not form ubiquitin conjugates, and had a short half-life, similar to that of wild-type p21. Moreover, its abundance underwent a significant increase in response to treatments with proteasomal inhibitors, indicating that p21 ubiquitination was not an absolute requirement for proteasomal turnover. Interestingly, mechanistic support for this finding arose in a study by Touitou et. al. where they demonstrated that purified 20S proteasome subunits could mediate ubiquitin-independent proteolysis of p21. They found that the last 31 amino acids of the p21 C-terminus were required for the effect and were responsible for direct binding to the C8 α-subunit (C8α) of the 20S proteasome [224]. A complementary study determined that introduction of the H-Ras oncogene into fibroblasts resulted in a Cyclin D1 dependent increase in p21 protein stability[225]. It was further demonstrated that Cyclin D1 could block interactions between p21 and C8α and that titration of Cyclin D1 could inhibit p21 proteolysis by purified 20S proteasomes. These studies suggest that a pathway for p21 degradation exists where the ubiquitin detection machinery in the 19S proteasomal subunits can be bypassed. In light of these findings however, a recent and provocative study suggests that p21 ubiquitination is required for proteasomal degradation[226]. Ubiquitin mutants that cannot form polyubiquitin chains were used to show that *in vivo* degradation of wild-type and lysine free p21 was dependent on a functional ubiquitin system. Moreover, lysine residues were found to be dispensable since *in vivo* ubiquitination was found to occur on the amino group of the N-terminal methionine in both wild-type and lysine-free p21 mutants and suggested that the sensitivity of previous ubiquitin analyses was insufficient. Nonetheless, further experimentation will be required to reconcile these conflicting observations.

The E3 ubiquitin ligase MDM2 is an oncoprotein that mediates p53 ubiquitination and targeting to the proteasome for destruction[227]. Like p21, MDM2 transcription is activated by

p53, however it operates in negative feedback to regulate p53 levels. Simultaneous and independent studies addressed the hypothesis that MDM2 inhibits p53-induced G1 arrest by also regulating p21 protein levels. These studies determined that MDM2 could interact with p21, reduce p21 half-life independent of ubiquitin transfer and could promote p21:C8α association *in vitro*[228,229]. MDM2 binding to p21 may be the physical bridge that links p21 with the 20S proteasomal machinery *in vivo*, and sets up a mechanism whereby MDM2 competition with p21 stabilizing proteins determines its expression level. In fact, recent work identified WISp39 as a novel p21 interacting protein that can prevent p21 proteasomal degradation. WISp39 was shown to bind the N-terminus of p21 and recruit Hsp90, via a tetratricopeptide (TPR) domain, into a ternary complex with p21. Absence of the TPR domain resulted in a loss of Hsp90 recruitment and loss of p21 stabilization. Moreover, p21 stabilization and growth arrest responses to ionizing radiation damage did not occur when WISp39 levels were reduced with siRNA or when Hsp90 was inhibited with Geldanamycin. This finding suggests that both p53 transcription and molecular chaperones are necessary to stabilize p21 following ionizing radiation and reinforces the hypothesis that competition between stabilizing and destabilizing interactions determines the net balance of p21 protein expression. This contrasts with a study by Bendjennat et. al., where it was found that low, and not high, doses of UV radiation caused a ubiquitin-dependent degradation of p21[230]. They showed that p21 ubiquitination was also dependent upon ATR activation and Skp2 ubiquitin ligase activity, and that lysine-free mutants of p21 did not get degraded following the UV stimulus. Because sustained levels of the lysine-free p21 mutant inhibited DNA repair, it may be that a ubiquitin-dependent pathway of p21 decay is selectively activated by the DNA repair machinery when DNA damage is not overwhelming, as is often the case in the setting of ionizing radiation.

1.6. IL-3 in Myelopoiesis and Acute Myeloid Leukemia

The growth factor Interleukin-3 (IL-3) is a pleiotropic cytokine protein that can promote the survival and proliferation of multipotent hematopoietic progenitors, as well as stimulate the development of multiple hematopoietic lineages[231-233]. IL-3 is mainly produced by activated T cells, whereas bone marrow stromal cell production has not been reported[234]. The human IL-3 receptor (IL-3R) is a heterodimer consisting of an α chain (IL-3Rα) and a common β chain (βc) which is the primary signal transducing subunit in the complex. The common β chain is shared with the GM-CSF and IL-5 receptors. Mice encode both a common β chain (βc) and an IL-3 specific β chain (β_{IL-3}). Homozygous deletion of the β_{IL-3} chain or IL-3 genes in mice results in no hematopoietic impairments[235,236]. This may be due to compensatory signals provided by GM-CSF and M-CSF, cytokines known to synergize with IL-3 to increase cell cycling and proliferation of bone marrow progenitors[237,238].

IL-3 promotes development of all myeloid lineages, but acts in opposition to development of lymphoid progenitors[239-241]. Most effective as a stimulator of granulocyte and monocyte colony formation (CFU-GM), IL-3 also contributes to eosinophil, mast cell, megakaryocyte and erythroid colony formation[238,242-244]. IL-3 is thought to be the main survival and differentiation factor for basophils[245,246]. In contrast to mast cells, circulating mature basophils express the IL-3R and can be activated to release histamine in response to IL-3[247].

Studies have demonstrated high expression levels of the IL-3 receptor in acute myeloid leukemias (AML), including leukemic stem cell populations[22,248]. IL-3 can activate DNA synthesis and proliferation in primary AML cells, as well as support self-renewal by promoting growth of AML-colony forming cells[249,250]. Primary AML cells with high IL-3Rα expression

have also been correlated with resistance to growth-factor withdrawal and increased cell cycling[251]. Moreover, patients were found to have higher levels of leukemic blasts at diagnosis, reduced rates of complete remission and decreased overall survival. These studies demonstrate the biological and clinical importance of IL-3 signaling pathways in myeloid development and myeloid malignancy.

Ligation of the IL-3 receptor results in the rapid activation of several signal transduction pathways. These include the Janus kinase (JAK)/STAT pathway, mitogen-activated protein kinase (MAPK/ERK) pathway and the PI-3 kinase pathway [252]. The Src-family of tyrosine kinases, notably c-Src, Hck, Fyn and Lyn, have also been reported to be activated by IL-3 in myeloid cells[253,254]. IL-3 regulates transcription through these pathways to stimulate the production of immediate-early genes such as c-Myc, c-Fos and c-Jun which are responsible for the survival, differentiation and proliferative effects of IL-3[252,255]. There is a sole report indicating that IL-3 contributes to gene stability at the post-transcriptional level. In mature human eosinophils, IL-3 was shown to stabilize the mRNA of its own receptor subunits, the IL-3Rα and βc chains[256]. Despite this observation, there has been no systemic study of posttranscriptional regulatory mechanisms controlled by IL-3.

2. Chapter II

A PROAPOPTOTIC FUNCTION OF p21 IN DIFFERENTIATING GRANULOCYTES

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2.1. Abstract

$p21^{waf1/cip1}$ (p21), best known for its ability to regulate the cell cycle, has been noted also to exert cell-cycle-independent effects on apoptosis and differentiation. Inhibition of apoptosis by p21 has been reported in hematopoietic models, particularly in monocytes exposed to apoptogenic agents. The effect of p21 on survival has not hitherto been analyzed during the myeloblast to granulocyte transition. Using 32Dcl3 murine myeloblasts, a cell line that proliferates in IL-3 and differentiates in G-CSF, we studied the effects of forced expression of p21 on cell survival. We hypothesized that exogenous p21 would suppress the modest levels of cell death associated with G-CSF mediated differentiation of 32Dcl3 cells. Contrary to expectations, we found that exogenous p21 enhanced apoptosis of cells removed from IL-3. p21 overexpression led to decreased cell growth, caspase 3 activation and annexin-V positivity. These effects occurred only in the presence of G-CSF. These findings suggest that p21 is proapoptotic in granulopoiesis, and that this effect is masked by IL-3-mediated survival signals. Our results also indicate there are distinct and opposing effects of p21 on monocytic and granulocytic survival. Aberrantly high levels of p21 may contribute to disease processes involving excessive apoptosis of granulocyte precursors.

2.2. Introduction

The multifunctional molecular adaptor protein $p21^{waf1/cip1}$ (cdkn1a, hereafter p21), best known for its ability to regulate the cell cycle, has been noted also to exert cell-cycle-independent effects on differentiation [81,97,103,122] and on apoptosis. Inhibition of apoptosis by p21 has been reported in nonhematopoietic and in hematopoietic models (reviewed in [56]). In the U937 monocytic cell model, p21 is upregulated concurrent with vitamin D-mediated differentiation. Ectopic p21 localized to the cytoplasm and prevented cell death by stress-signaling pathways and by autocrine TNF-α production [63,81]. We have previously demonstrated that p21 decreased chemotherapy-mediated apoptosis of K562 myeloid cells by preventing etoposide-induced degradation of the anti-apoptotic protein cIAP1 [79]. Other mechanisms proposed for anti-apoptotic action of p21 include procaspase-3 inhibition [76] and ASK1 inhibition [81]. A physiologic anti-apoptotic function of p21 in hematopoiesis is supported by the finding that exogenous p21 overexpression in CD34+ cells increases CFU-GEMM formation [257]. Moreover, high levels of endogenous p21 are linked to chemoresistance and poor prognosis in AML [3,257]. In aggregate, these reports indicate that p21 antagonizes apoptosis, particularly apoptosis triggered by p53 activation (for review, [56]).

During monocytic differentiation of the U937 cell line, p21 levels increase and p21 localizes to the cytoplasm where it is retained by the BRAP2 protein [81,258]. Cytoplasmic p21 inhibits apoptosis in U937 monocytes by binding to and inactivating ASK1 kinase, an intermediary in the JNK stress signaling pathway [81]. Normal human monocytes express exclusively cytoplasmic p21, supporting a physiologic function of p21 identical to that uncovered in cell line experiments [81].

In contrast to the p21 expression pattern in monocytes, we have demonstrated that p21 protein levels are high in normal human myeloblasts but decline when blasts commit to terminal differentiation despite sustained levels of p21 gene transcription [99]. Because neutrophils are shorter-lived than monocytes we speculated that the observed downmodulation of p21 during granulopoiesis may contribute to an increased sensitivity of differentiating granulocytes to apoptotic stimuli. A role for p21 in apoptosis during granulopoiesis has not hitherto been studied.

The murine 32Dcl3 myeloblast cell line was chosen to model p21 effects during granulopoiesis. This cell line proliferates in IL-3 and differentiates to neutrophils upon substitution of G-CSF for IL-3. As noted herein, 32Dcl3 differentiation recapitulates the expression pattern of p21 in normal granulocytic precursors with decreased p21 protein levels following initiation of differentiation. We expected that increasing p21 expression with exogenous p21 would inhibit apoptosis in these cells, as reported for monocytic U937 cells. Contrary to expectations, we found that exogenous p21 increased cell death during myelopoiesis. The increase in cell death was manifested in the presence of G-CSF but not while cells were proliferating in IL-3. These findings suggest that p21 is proapoptotic in granulopoiesis, and that this effect is masked by IL-3-mediated survival signals or by modification of p21 by IL-3. Downmodulation of p21 may be required to maximize the survival of granulocyte precursors after the myeloblast stage.

2.3. Materials and Methods

2.3.1. Cells and Culture

32Dcl3 cells (32D)[259] (kind gift from Alan Friedman, Johns Hopkins University) were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 I.U./ml and 100 ug/ml) and 2 mM L-Glutamine (Mediatech, Herndon, VA). Murine IL-3 (Peprotech, Rocky Hill, NJ) was used at 2 ng/ml during cell passaging. Serum and murine IL-3 are required for survival, proliferation and maintenance for the myeloblast phenotype of these cells in culture [4]. Unless otherwise noted, all experiments were carried out with cells maintained between $2x10^5$ and $1x10^6$ cells/ml, to minimize paracrine effects that may occur at density extremes, and incubated at 37°C with 5% CO_2. Phoenix A cells (ATCC, Manassas, VA), an amphotropic retroviral packaging cell line, were cultured in DMEM (Mediatech, Herndon, VA) with fetal bovine serum, penicillin-streptomycin and L-glutamine as noted above.

2.3.2. Differentiation

To induce differentiation, 32D cells were washed twice with 50 ml PBS to remove IL-3, resuspended in cytokine free media at $2x10^5$ cells/ml and incubated for 4 hours, followed by addition of G-CSF (Amgen, Thousand Oaks, CA) to a final concentration of 100 ng/ml. Cell viability and viable cell density was determined immediately prior to G-CSF addition by trypan dye exclusion assay using a Vi-Cell XR cell viability analyzer (Beckman-Coulter, Miami, FL) and was evaluated every 24 hours thereafter. Differentiation was confirmed by analysis of Wright-Giemsa stained cytospins at each time point assessed.

2.3.3. Statistics

Viability and cell growth characteristics were compared to negative controls with significance determined by a 2-tailed, type 2 student's t-test using 95% confidence intervals. All calculations were made using the Excel software package (Microsoft, Redmond, WA).

2.3.4. Plasmids

Retroviral constructs were derived from the MIG plasmid (a kind gift of Luk Van Parijs, Massachusetts Institute of Technology), which contains an IRES-GFP cassette downstream of its multiple cloning region. Using standard methods, the Gateway® vector conversion cassette (Invitrogen, Carlsbad, CA), reading frame B, was blunt-end cloned into the MIG Hpa I site and transformed into DB3.1 cells (Invitrogen) to create a Gateway® destination vector, MIGccd. Human p21 wild-type cDNA (accession# NM_078467) carrying a myc-his tag was PCR amplified and cloned into the pENTR-D-TOPO (Invitrogen) plasmid to create a Gateway® entry vector, pENTR-D hp21WT. The expression clone MIG hp21WT was synthesized by combining pENTR-D hp21WT and MIGccd in an LR clonase recombination reaction (Invitrogen) per manufacturer's instructions. The expression clone MIG CTL was synthesized by combining pENTR-D and MIGccd in an LR clonase recombination reaction. All plasmids were verified by sequencing analysis.

2.3.5. Retroviral Transduction

To generate retroviruses for infection of 32D cells (protocol modified from [260]), Phoenix A cells were transfected with retroviral MIG plasmids using the Polyfect Transfection Reagent (Qiagen, Valencia, CA). Phoenix cells were plated at a density of $2.5x10^6$ cells/dish in 60 mm

dishes and incubated for 16 hours. For each dish, 4 μg plasmid DNA was mixed with 40 μl Polyfect, incubated and applied to the cells per manufacturer's instructions for 60mm dishes. Transfection media was removed after 24 hours of incubation at 37°C and 4 ml fresh DMEM was added. Cells were incubated at 32°C for an additional 48 hours. Viral supernatants were harvested and passed through a 45 μM syringe filter to exclude producer cells. Viral supernatants were supplemented with 2 ng/ml murine IL-3 and 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). $2x10^5$ 32D cells were pelleted and resuspended in 4 ml of viral supernatant and aliquoted into one well of a 6-well plate. Using a microplate carrier, cells were centrifuged at 32°C for 90 minutes at 2500 RPM (H1000b rotor, Sorvall RT6000D centrifuge). Cells were incubated overnight at 32°C and placed in fresh viral supernatant with IL-3 and polybrene. Centrifugation was repeated and cells were cultured at 32°C for 24 hours. Following this incubation period, cells were pelleted, the viral supernatant was replaced with IL-3 supplemented RPMI growth media and the cells were incubated for an additional 48 hours at 37°C. Transduced cells were sorted on the basis of GFP expression using a Cytomation MoFlo (Cytomation, Fort Collins, CO). To derive clonal populations, one cell per well sorting parameters were used, isolating cells in a 96-well plate.

2.3.6. Western Blotting

Protein samples were prepared using two methods. For comparison of relative transgene expression in clonal lines, 32Dcl3 extracts were prepared in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% Na-Deoxycholate, 1.0% NP-40, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 0.5 mM DTT, 1.0 mM Vanadate, 1:250 Protease Inhibitor Cocktail (Sigma-Aldrich, Cat: P-8340) and quantified using a BCA protein assay (Pierce, Rockford, IL). To minimize

upregulated protease activity in cells stimulated with G-CSF, either $1.5x10^5$ or $3.0x10^5$ cells were harvested, washed once in ice-cold PBS, lysed directly in 15 µl 2X sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol, 2% 2-mercaptoethanol, 0.03 mM Bromphenol Blue), and boiled for 5 minutes. Lysates containing equal amounts of total protein were run on 12% SDS-PAGE gels. Alternatively, an equivalent number of whole cells, lysed directly in 2x sample buffer, were analyzed by SDS-PAGE. Proteins were electrotransferred onto Protran membranes (Schleicher and Schuell, Keene, NH), which were incubated with Ponceau S (Sigma-Aldrich) to confirm transfer efficiency. Membranes were then incubated with blocking buffer (5% w/v nonfat dry milk, 1x TBS, 0.1% Tween-20) for 1 hour. Antibodies were diluted in blocking buffer and included goat polyclonal p21(C-19)-HRP (1:1000, Cat: SC-397HRP, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal β-Actin (AC15) (1:20,000, Cat: A5441 Sigma-Aldrich), mouse monoclonal p27-HRP (1:2500, Cat: K25025, BD Transduction Laboratories, San Diego, CA), mouse monoclonal α-Tubulin (DM1A) (1:2500, Cat: T9026, Sigma-Aldrich), mouse monoclonal Lamin B1 (1:1000, Cat: 33-2000, Zymed, San Francisco, CA), and rabbit polyclonal cleaved Caspase-3 (1:1000, Cat: 9661, Cell Signaling Technology, Beverly, MA). Where appropriate, secondary HRP-conjugated antibodies were diluted in blocking buffer and included goat anti-mouse IgG (H + L)-HRP (Cat: 170-6516, Bio-Rad, Hercules, CA) and goat anti-rabbit IgG (H + L)-HRP (Cat: 170-6515, Bio-Rad). Signals were developed using the Western Lightning chemiluminescence reagent (PerkinElmer, Boston, MA). Scanned images of exposed film underwent signal density analysis using Image J 1.31v software (http://rsb.info.nih.gov/ij).

2.3.7. Northern Blotting

RNA was prepared from $1x10^7$ 32D cells propagating in IL-3, cytokine starved for 4 hours, or stimulated with G-CSF for each time point analyzed using RNeasy total RNA purification kits (Qiagen, Valencia, CA). Blots were performed as described previously [95]. In brief, radiolabeled probes were prepared at $1x10^6$ CPM/ml hybridization buffer, incubated with membranes overnight, washed and subjected to autoradiography at -80°C for 1-5 days. The murine p21 probe contained nucleotides 100-578 (Genbank Accession # NM_007669) which were amplified by PCR using the following primers: forward – CACCATGTCCAATCCTGGTGATG, reverse – TCAGGGTTTTCTCTTGCAGAAG. The GAPDH probe was human in origin and contained nucleotides 100-941 (Genbank Accession #NM_002046) which were amplified by PCR using the following primers: forward – AACGGATTTGGTCGTATTGGG, reverse – TCGCTGTTGAAGTCAGAGGAGAC. Scanned images of exposed film underwent signal density analysis using Image J 1.31v software (http://rsb.info.nih.gov/ij). Multiple exposures were obtained to optimize linearity of signal.

2.3.8. Cell Cycle Analysis

Cell cycle distribution and DNA content were determined by measurement of propidium iodide uptake in ethanol-fixed cells using a Coulter EPICS XL flow cytometer and Expo32 ADC software (Beckman Coulter, Miami, FL). Treated 32D cells were washed once, resuspended in 0.5 ml PBS, transferred to ice-cold 70% ethanol to a final density of $1x10^6$ cells/ml and stored at -20°C overnight. For each sample, one million fixed cells were washed once in 5 ml PBS, resuspended in 1 ml propidium iodide staining solution (20 μg/ml propidium iodide, 200 μg/ml

RNase A, 0.1% Triton X-100 in PBS), incubated in the dark at 37°C for 15 minutes, and analyzed.

2.3.9. Annexin-V/Sytox Blue Staining

Apoptosis was assayed using the Annexin V-PE Apoptosis Detection Kit I (Cat: 559763, BD Pharmingen, San Diego, CA) with modification. 7-Amino-Actinomycin-D (7-AAD) was substituted with Sytox Blue nucleic acid stain (Cat: S-11348, Molecular Probes, Eugene, OR). $1x10^5$ cells resuspended in 100 µl 1x binding buffer per manufacturer's instructions were treated with 5 µl Annexin-V PE solution and Sytox Blue was titrated to 1 µM. Following a 15 minute, room temperature incubation in the dark, 400 µl of 1x binding buffer was added and the cells were analyzed using a CyAn LX flow cytometer (DakoCytomation, Fort Collins, CO) and the Summit software package (DakoCytomation).

2.4. Results

2.4.1. p21 downregulation by G-CSF in 32D cells

To determine if p21 protein levels were modulated in 32D cells during differentiation, p21 temporal expression patterns were examined in cells stimulated to differentiate with G-CSF. G-CSF induces differentiation of 32D cells along the granulocytic pathway which was confirmed by the appearance of segmented neutrophils in wright-giemsa stained cytospins (data not shown). Western blot analysis (Fig. 4A) revealed that a four hour cytokine starvation (Ø) results in a 20%

increase in p21 protein levels which dropped immediately following the G-CSF stimulus, decreasing by 50% by 3 days and thereafter. p21 mRNA levels (Fig. 4B), experienced a similar acute reduction in expression, with a trough at 3 days, closely paralleling p21 protein levels during this period (Fig. 4C). However, we observed that p21 mRNA levels increased after 3 days, without a corresponding increase in p21 protein. This parallels findings reported elsewhere for differentiating CD34+ cells [99,100].

Because p21 protein has been reported to inhibit apoptosis when localized to the cytoplasm during monocytic differentiation, we examined the compartmental distribution of p21 during granulocyte differentiation [81]. In 32D cells maintained in the undifferentiated state with IL-3, we observed an equal cytoplasmic and nuclear distribution of p21 (Fig. 4D). Transition to G-CSF revealed an almost complete shift to the cytoplasm, which was maintained through terminal differentiation. We also observed an upregulation and predominant cytoplasmic localization of p27, a cip/kip family member closely related to p21.

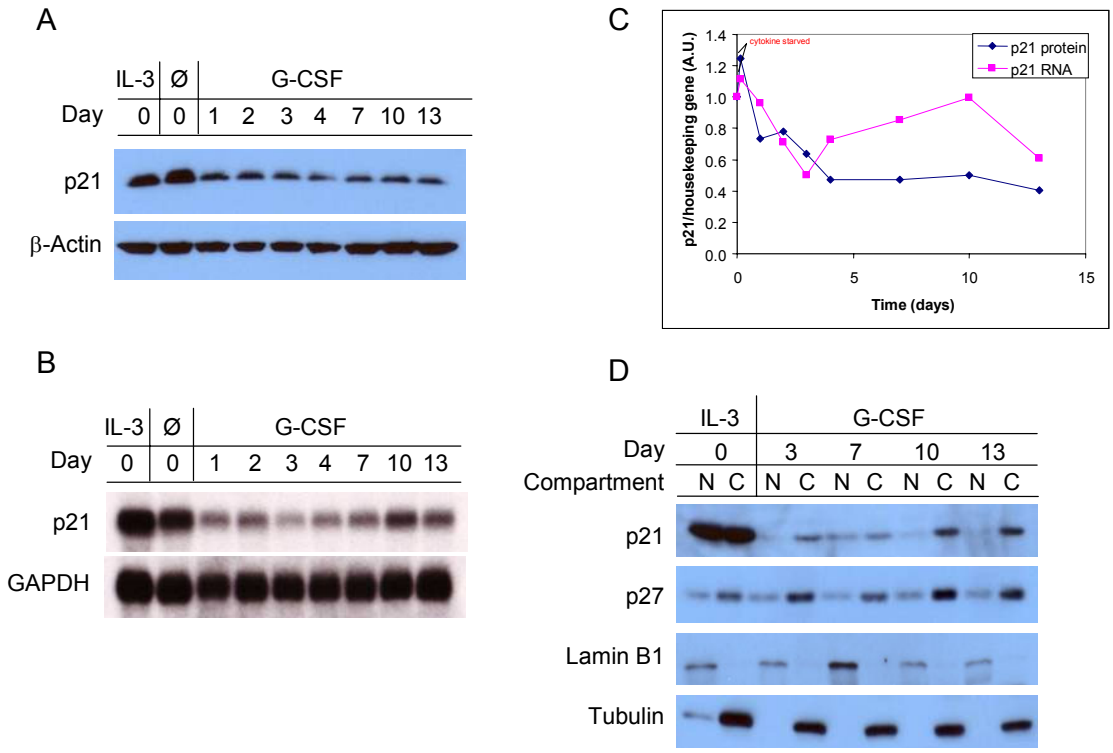


Figure 4. p21 expression decreases in G-CSF treated 32D cells.

a) p21 protein levels decrease in differentiating 32D cells. Cells propagating in IL-3 were cytokine starved, (Ø), and stimulated to differentiate with G-CSF. Each lane contains $3x10^5$ viable cells harvested daily, prepared directly in laemmli buffer, which were subjected to SDS-PAGE. p21 levels were determined by immunoblot with β-actin provided as a loading control. b) p21 message levels change during differentiation. Total RNA prepared at each time point was probed by northern blot for p21 mRNA. GAPDH signal is provided as a loading control. c) p21 protein and p21 message are regulated independently during differentiation. Quantification of signal density for p21 protein (1a) and mRNA (1b) was normalized against β-actin and GAPDH, respectively, and plotted. d) p21 shifts to cytoplasm during differentiation. Nuclear and cytoplasmic extracts were prepared at each time point and subjected to SDS-PAGE and

immunoblot analysis in sequence for p21, p27, with Lamin B1 and Tubulin provided as compartmentalization controls.

2.4.2. p21 suppresses 32D cell growth in IL-3 and G-CSF

To determine if p21 regulates survival in 32D cells during differentiation, we first engineered 32D cells to overexpress a myc-his tagged human p21 transgene by retroviral transduction with MIG hp21WT or a control virus, MIG CTL. We evaluated clonal cell lines by western blot and isolated two clones, clones 6 and 9, expressing comparable levels of the p21 transgene (Fig. 5A). First described as the downstream effector of p53-induced growth arrest [28,29], p21 can inhibit the cell cycle through binding and inactivation of Cyclin E or Cyclin A/Cdk2 complexes. It is thought that this occurs when p21 levels rise to sufficient levels to overcome sequestration by other binding partners ([27] for review), most notably Cdk4/CyclinD complexes. To determine if overexpression of p21 inhibited cell growth, we evaluated the expansion of cells propagating in IL-3 (Fig. 6B) or those stimulated with G-CSF (Fig. 6C). Whereas cell growth in IL-3 was modestly attenuated, cells treated with G-CSF underwent dramatic growth suppression compared to controls. Consistent with a reduced growth rate, cell cycle analysis (Fig. 7D) revealed that p21 overexpression was correlated with a higher percentage of cells residing in the G1 phase during IL-3 stimulated expansion. G-CSF treatment for 24 hours led not only to reduced S and G2/M phase populations, but a substantial increase in Sub-G1 populations in both p21 overexpressing clones compared to the control. This result was consistent with increased apoptosis of p21 overexpressing clones in G-CSF.

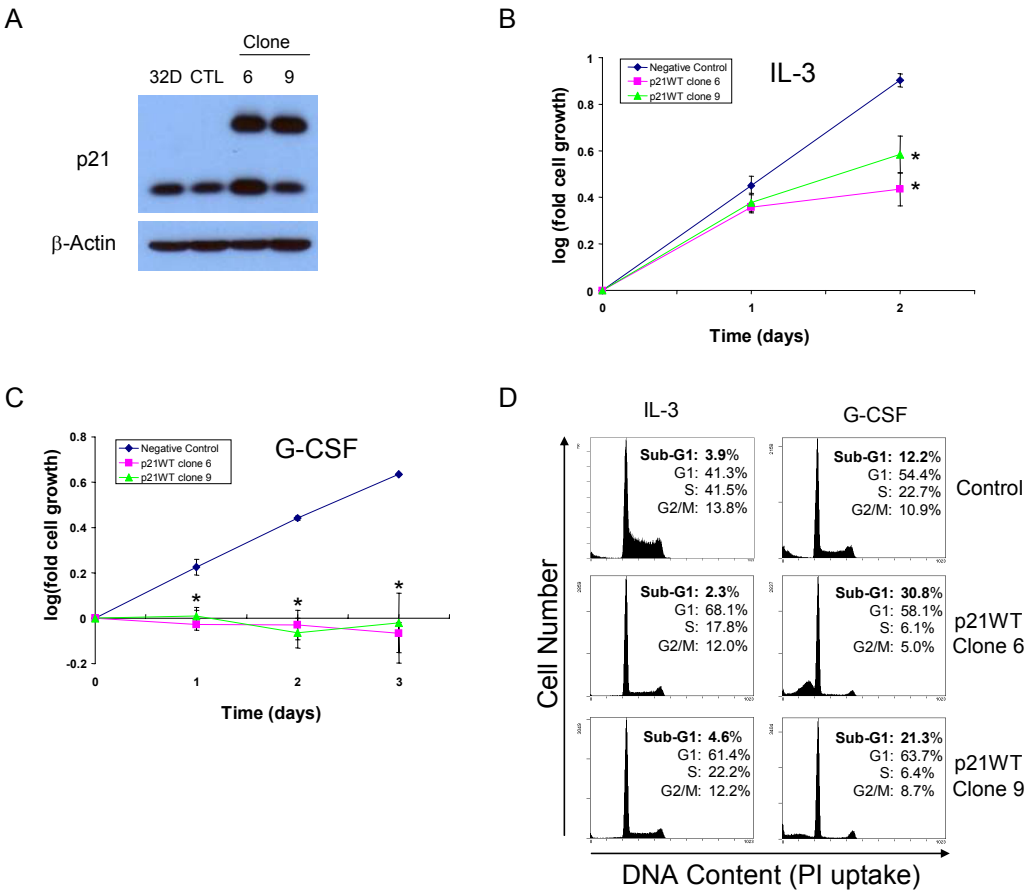


Figure 5. Exogenous p21 inhibits 32D cell growth in IL-3 and G-CSF.

a) 32D cells were transduced with either a control virus (CTL) or virus carrying myc-his tagged, wild-type human p21 (clones 6 and 9). Protein lysates were prepared from generated clones and were assessed for equivalent transgene expression and compared to control (CTL) and parental (32D) cells by immunoblot analysis using antibodies against p21. β-actin signal is provided as a loading control. b and c) Exogenous p21 inhibits proliferation in IL-3 and G-CSF. Cell numbers were determined by trypan blue dye assay and are presented as viable cells/ml cell suspension (*$p<0.05$; bars denote standard error of the mean). d) Cell cycle distribution of cells

propagating in IL-3 or treated with G-CSF for 24 hours. DNA content was assessed on the basis of propidium iodide uptake.

2.4.3. Exogenous p21 promotes apoptosis upon IL-3 withdrawal and during 32D cell differentiation with G-CSF.

Between 10-20% of 32D cells switched from IL-3 to G-CSF undergo apoptosis within the first 24 hours. Since p21 inhibits apoptosis during monocytic differentiation [81], we examined whether sustained expression with exogenous p21 could mitigate the observed differentiation-induced apoptosis in a granulocytic model. p21 increased apoptosis 2-fold after 2 days of G-CSF stimulation in clone 6 (35.3% +/-2.4% vs. 15.2% +/-3.6%, $p<0.05$), and increased apoptosis in clone 9 by 2-fold after 3 days (36.3% +/- 6.0% vs. 14.1% +/- 1.8%, $p<0.05$) (Fig. 3A). To determine if the decrease in viability was correlated with caspase activation, we assayed total cellular protein for the 19 kDa cleavage product of Caspase-3 (Fig. 6B). In both clones 6 and 9, caspase activation was markedly higher following G-CSF stimulation compared with control cells. Activation was characterized by a signal peak after one day. This was diminished after 48 hours, presumably because of decreased p21 expression at day 2 (Fig. 6C). To evaluate early events in apoptosis, we assessed Annexin V reactivity of cells treated with G-CSF. At 24 hours, clone 6 exhibited a 4-fold increase in Annexin V binding when compared with the control population (24.51% +/- 4.48% vs. 6.06% +/- 0.62%, $p<0.02$) (Fig. 7).

In order to determine whether G-CSF signals cooperated with p21 in promoting cell death or whether IL-3 deprivation enabled the p21 proapoptotic effect, we analyzed cell death at high and low IL-3 concentrations. Cells were cultured in IL-3 at concentrations ranging from 1 ng/ml to 1 pg/ml and cell viability at 48 hours was determined (Fig. 6C). At doses of 10 pg/ml both clones exhibited a ~1.5 fold decrease in viability relative to control (clone 6: 64.5% +/- 6.4%,

$p<0.03$, clone 9: 57.8% +/- 3.0%, $p<0.01$, vs. control: 40.7% +/- 0.5%), with clone 6 showing a 2-fold decrease in viability at 100 pg/ml (35.0% +/- 1.2% vs. 17.6% +/- 1.7%, $p<0.01$). At 1 pg/ml of IL-3, both control and p21 overexpressing clones experienced ~70% viability loss. This indicates that accelerated cell death associated with p21 did not require G-CSF signaling, but rather loss of IL-3 signaling.

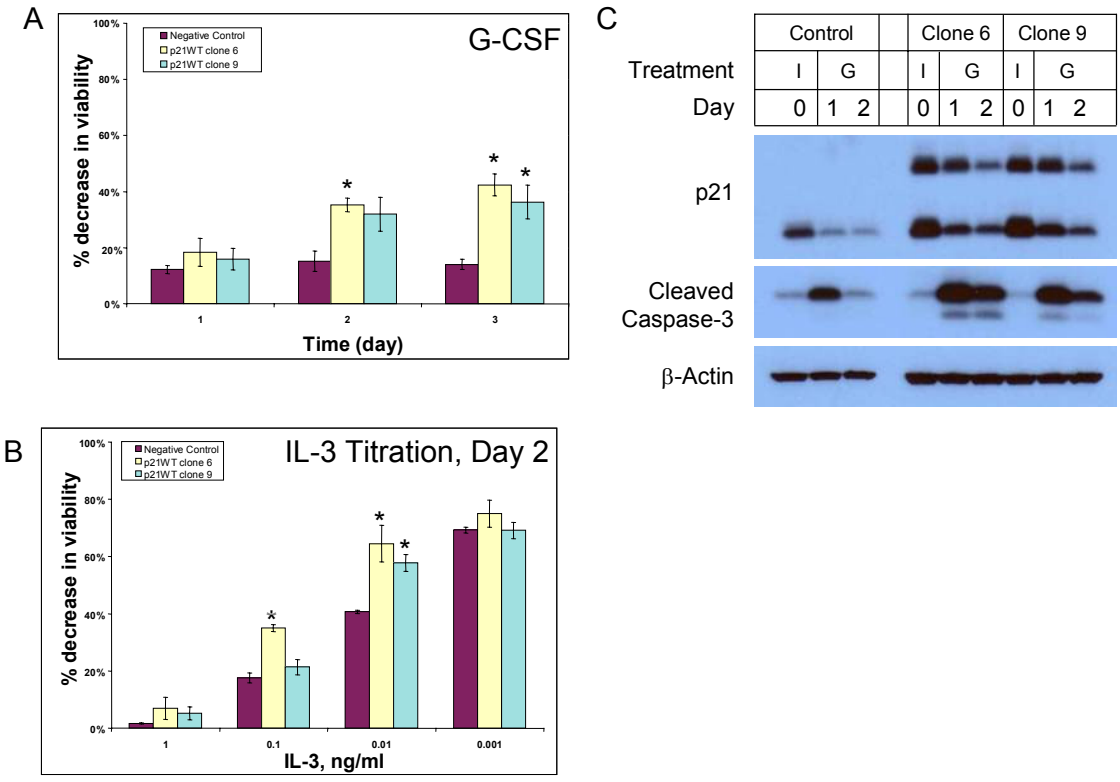
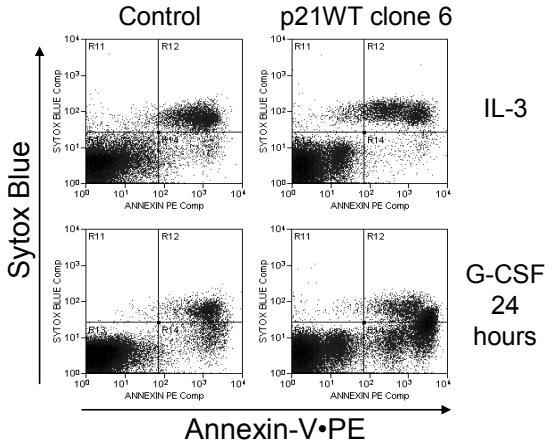


Figure 6. Exogenous p21 is proapoptotic during 32D cell differentiation.

a) 32D cells induced to differentiate with 100 ng/ml G-CSF were assessed for viability daily by trypan blue dye assay. Data reported as change in viability from initial G-CSF stimulus at day zero. b) Exogenous p21 sensitizes 32D cells to IL-3 withdrawal. IL-3 was titrated from 1 ng/ml to 1 pg/ml and cells were assessed for viability by trypan blue dye assay. Data presented for day 2, reported as change in viability from day zero ((a and b) *$p<0.05$; bars denote standard error of the mean). c) Exogenous p21 promotes activation of Caspase-3 during differentiation. $1.5x10^5$ cells from a) were harvested, prepared directly in laemmli buffer and subjected to SDS-PAGE and immunoblot analysis. Nitrocellulose membranes were probed in sequence with antibodies against cleaved Caspase-3, p21 and β-Actin. (I = IL-3, G = G-CSF).



	Control G-CSF	p21WT clone 6 G-CSF	t-test (2-tailed, type 2) p-value
Negative	85.52 +/- 0.96%	63.30 +/- 4.30%	**0.007**
Annexin-V	6.06 +/- 0.62%	24.51 +/- 4.48%	**0.015**
Sytox/Annexin	7.76 +/- 0.17%	11.55 +/- 0.38%	**0.001**
Sytox Blue	0.66 +/- 0.24%	0.63 +/- 0.04%	0.897

Figure 7. Exogenous p21 increases apoptosis in G-CSF stimulated cells measured by Annexin-V labeling. Negative control cells (left column) and a representative p21 overexpressing clone (clone 6, right column) were analyzed by Annexin V-PE/Sytox Blue staining after a 24 hour stimulation with IL-3 (top row) or G-CSF (bottom row). Table reports percent positive for G-CSF treated cells.

2.5. Discussion

We tested the hypothesis that exogenous overexpression of p21 would suppress differentiation-associated apoptosis in 32D cells. We demonstrate, in contrast, that overexpression of p21 promoted apoptosis within the first 48 hours of G-CSF stimulation (Figs. 5D and 6A). Apoptosis was manifested by increases in Caspase-3 cleavage and Annexin V reactivity (Figs. 6 and 7). p21-mediated apoptosis occurred in two independent clones as well as in polyclonal culture (2-fold increase in apoptosis compared with control polyclonal culture, data not shown), indicating that it was not related to clonal selection. These findings are significant because they suggest that p21 function in cell survival is distinct and opposite between granulocytes and monocytes.

p21 has been previously described as an antiapoptotic protein in monocytic lineages, with upregulation occurring immediately following differentiation stimuli [81,101]. In monocytes, antiapoptotic effects depend on cytoplasmic localization of p21, where p21 binds to and inhibits ASK1 kinase[81,261]. In contrast, proapoptotic effects of p21 occur despite its presence in the cytoplasm (Fig. 4D) and presumed accessibility to ASK1 in 32Dcl3 cells.

p21 overexpression sensitizes 32D cells to cell death only upon IL-3 withdrawal. This finding is distinct from other reports linking p21 overexpression to apoptosis. In those systems exogenous p21 is sufficient to promote apoptosis or promotes apoptosis in conjunction with death receptor activation[82,83], DNA damaging agents[84,85], ionizing radiation[86], or antioxidant treatments[87-89]. Exogenous p21 alone has been sufficient to induce apoptosis in cervical[90], esophageal[91], and breast cancer cell lines[92] and a peptide corresponding to the C-terminus of p21

has recently been shown to cause apoptosis in the U937 cell line [93]. In our system p21 overexpression was insufficient to cause apoptosis in proliferating cells. IL-3 withdrawal or cytokine substitution with G-CSF was required to trigger apoptosis. IL-3 withdrawal has been described to upregulate the secreted lipocalin 24p3, causing an autocrine induction of apoptosis which is insensitive to repletion of IL-3[262]. Whereas exogenous lipocalin 24p3 is sufficient to trigger apoptosis in hematopoietic cells propagating in IL-3, 32Dcl3 cells supported by G-CSF are insensitive to lipocalin 24p3-mediated cell death[263]. Because p21 promoted apoptosis in the setting of IL-3 withdrawal or in the presence of G-CSF, its mechanism of action is unlikely to involve lipocalin 24p3.

Why would IL-3, and not G-CSF, suppress p21 augmented apoptosis? Both IL-3 withdrawal and substitution of G-CSF for IL-3 unmask proapoptotic effects of p21. This could reflect either IL-3-specific modification of p21 to minimize its apoptotic potential or differences between IL-3 and G-CSF in the strengths or repertoire of survival signals they transmit. Khanna et. al. have noted differences in p21 migration on 2-D gels in IC.DP premast cell derivatives cultured in the presence and absence of IL-3. Phosphorylation of p21 on Thr-145 by AKT has been published [209,264], however both IL-3 and G-CSF have been reported to activate AKT in 32D cells [265,266]. Future work will characterize post-translational modification of p21 in 32D cells in IL-3 and G-CSF in order to map and validate p21 residues which are modified in association with apoptosis induction. Differences in survival signaling between IL-3 and G-CSF [158,262,267,268] also may account for differential suppression of p21-induced apoptosis.

p21-induced apoptosis during differentiation has also been reported in muscle cells deficient in the Rb protein [269]. In this myotube differentiation model, Peschiaroli et. al. demonstrated that initiation of differentiation by MyoD is required for apoptosis to occur and

that p21 knockout was protective. The association of p21-mediated apoptosis with a differentiation trigger observed in muscle parallels our observations in the 32Dcl3 system.

Promotion of apoptosis by p21 may be related to dysregulated Cdk function. In the myogenesis system utilized by Peschiaroli, p21 expression and apoptosis coincided with nuclear redistribution of Cdk's. In an erythroblast differentiation model, Matushansky et. al. demonstrated that a specific order of Cdk inhibition was required to commit proerythroblasts to differentiate[102,110]. Apoptosis occurred when this sequence of Cdk-inhibition was reversed. Based on these observations in muscle and erythroid differentiation systems, it is conceivable that an analogous system of precise temporal inhibition of Cdk's and/or intracellular localization is required for both differentiation and survival in myeloid cells.

In 32D cells, p21 levels decrease when cells are switched from IL-3 to G-CSF, whereas the levels of p27 increase (Fig. 4). This parallels findings in muscle, where a successive spike in expression of distinct cyclin dependent kinase inhibitors (e.g., p21 peaking before p18) occurs upon transition to a more differentiated state and parallels sequential induction of p21 and p27 which we have noted in a K562 myeloid model expressing inducible p21[97,270,271]. p27 appears to play a primary role in terminal differentiation arrest in 32D cells [158]. Our results suggest that p21 downmodulation enhances cell survival during the final stages of differentiation in which p27 levels increase.

In conclusion, we have detected a proapoptotic function of p21 in 32D cells which is unmasked by cytokine withdrawal of IL-3 or substitution of G-CSF for IL-3. Our finding expands the number of examples in blood cells [83] and other cell systems in which p21 is proapoptotic [56]. Accumulated evidence tends to support a model wherein p21 is antiapoptotic

when coupled to p53 signals and is proapoptotic with other death signals. Our results indicated that p21 promotion of cell death can be cytokine specific in the absence of other death signals.

Promotion of apoptosis by p21 at the myeloblast to myelocyte developmental transition may be a mechanism utilized to put a brake on myelopoiesis in response to acute changes in the cytokine environment. It is conceivable that aberrant modulation of p21 could contribute to disease processes characterized by excessive apoptosis during the myeloblast to myelocyte transition such as that occurring in a subset of myelodysplastic syndromes.

2.6. Acknowledgements

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3. Chapter III

IL-3 STABILIZATION OF p21 TRANSCRIPTS IN MYELOID CELLS IS ASSOCIATED WITH RNA-BINDING PROTEIN INTERACTIONS IN THE p21 3' UNTRANSLATED REGION

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3.1. Introduction

Cellular differentiation is a complex process mediated by growth and developmental factors that requires precise control of gene expression. Posttranscriptional regulation of mRNA is an important mechanism that modulates both rapid and long-acting responses to differentiation cues. Recent work has demonstrated that neuronal and myogenic differentiation depends upon RNA-binding proteins that regulate mRNA turnover. For example, induction of neurite outgrowth and regulation of cell fate in neural progenitor cells is dependent on the expression and RNA-binding activity of neuronal Hu proteins[272,273]. In addition, during myogenesis, the RNA-binding proteins (RNA-BPs) HuR and NF90 mediate mRNA stabilization of the critical myogenic developmental factors myogenin, MyoD and the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ (p21) through interactions with AU-rich elements in their 3' untranslated regions[194,196]. Such transcript stabilization contributes to the steady rise in p21 expression during muscle and neuronal differentiation. The binding of stabilizing or destabilizing RNA-BP's to common sequence elements in 3' UTR's facilitates coordinated expression of gene products beyond that achieved by transcription alone. This control of multigene stability has been compared to "eukaryotic operons" and is critical in highly regulated processes such as differentiation[274]. A result in muscle cells is an increase in transcription factor levels concurrent with a steady increase in p21 during differentiation.

p21 does not steadily increase during granulocytic differentiation[95,99,100]. Our data indicates that p21 is posttranscriptionally regulated in this setting also. Posttranscriptional regulation of many genes, including c-Myc, neutrophil elastase and multiple cytokines, has also

been demonstrated in leukemic cell lines induced to undergo myeloid differentiation[275-278]. However, neither the mechanisms that control mRNA stabilization during myelopoiesis nor the role of RNA-binding proteins in myelopoiesis are well understood.

Several recent reports have shown that control of p21 expression in response to cellular stress, growth factors, and differentiation stimuli occurs through posttranscriptional mRNA stabilization; however, how p21 is regulated during myeloid development remains unclear[186-191,193]. High basal levels of p21 expression can be found in the IL-3 dependent, myeloid progenitor cell line 32Dcl3(Figure 4 and [279]). p21 expression patterns in 32Dcl3 cells undergoing granulocytic differentiation in response to the cytokine G-CSF mirror those observed during granulocytic differentiation of CD34+ human hematopoietic progenitor cells(Figure 4 and [100]). Therefore, 32Dcl3 cells provide an excellent model system to study p21 mRNA stability during myelopoiesis.

IL-3 is responsible for maintenance of the undifferentiated state in 32Dcl3 cells and can promote the survival and proliferation of myeloid progenitors *in vivo*[231-233]. IL-3 is often secreted by leukemic myeloblasts[280,281] and can activate DNA synthesis and proliferation in primary AML cells, as well as support self-renewal by promoting growth of AML-colony forming cells[249,250]. Although IL-3 can increase mRNA stability of its own receptor subunits in mature eosinophils[256], investigations of posttranscriptional mechanisms elicited by IL-3 during differentiation, or in the setting of leukemic transformation, are limited.

Conserved *cis*-acting sequences within an mRNA often serve as docking points for RNA-binding proteins that regulate mRNA turnover. p21 mRNA contains three AU-rich elements (AREs) in its proximal 3' UTR that are specifically bound by stabilizing proteins including HuR and NF90, or destabilizing proteins such as AUF1[194]. For example, stabilization of p21 mRNA

by Prostaglandin A_2 has been shown to depend upon ERK-induced HuR binding to p21 AREs[189]. One mechanism recently reported suggests that steady-state levels of p21 mRNA result from competition between HuR and AUF1 for ARE binding[184]. Competition for access to ARE sites between HuR and other destabilizing RNA-binding proteins such as TTP and KSRP has also been described and has been hypothesized to represent a general phenomenon regulating mRNA stability[184,185]. In light of these studies, we hypothesized that competition between RNA-binding proteins may be a cytokine regulated phenomenon that controls p21 mRNA levels in 32Dcl3 cells during differentiation.

We report here that IL-3 maintains high levels of p21 expression in 32Dcl3 cells through stabilization of p21 mRNA requiring a functional 3'UTR region. The decrease in p21 mRNA stability noted during G-CSF-induced differentiation was rescued by IL-3 and independent of PI3-kinase and ERK pathway signaling. *In vitro* binding assays provide evidence that distinct sets of RNA:protein interactions occur within the p21 3' UTR and are regulated by IL-3 and G-CSF signaling. These findings provide the first evidence for IL-3-mediated stabilization of mRNA transcripts in myeloid progenitor cells and suggest that IL-3 mediated mRNA stabilization may be an important mechanism that contributes to differentiation blockade.

3.2. Materials and Methods

3.2.1. Cells and Culture

RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 I.U./ml and 100 ug/ml) and 2 mM L-Glutamine (Mediatech, Herndon, VA) was used to culture 32Dcl3 cells[259] (kind gift from Alan Friedman, Johns Hopkins University). Cells were maintained between $2x10^5$ and $1x10^6$ cells/ml with Murine IL-3 (Peprotech, Rocky Hill, NJ)

used at 1.5 ng/ml during cell passaging. All incubations were conducted at 37°C with 5% CO_2. p21 transduced 32D cells, 32D hp21WT clone 6, generated as described (see 2.3.1, ([279]), were cultured in IL-3 supplemented RPMI as above. Cytokine independent v-Abl transduced 32D cells generated as described [282] were cultured in RPMI with fetal bovine serum, penicillin-streptomycin and L-glutamine as noted above.

3.2.2. Differentiation

32Dcl3 cells were washed twice with 50 ml PBS to remove IL-3, resuspended in cytokine free media to $2x10^5$ cells/mL and treated with 100 ng/mL G-CSF (Amgen, Thousand Oaks, CA) to trigger differentiation. Partial media changes were executed every two days to maintain cell densities below $1x10^6$ cells/mL and to replenish G-CSF. Granulocytic differentiation was confirmed by analysis of Wright-Giemsa stained cytospins at each time point assessed (data not shown).

3.2.3. Western Blotting

For differentiation analysis, whole cell protein extracts were prepared by direct lysis in sample buffer to minimize upregulated protease activity. For each time point analyzed, $3.0x10^5$ viable cells, determined by trypan blue dye assay using a Vi-Cell XR cell viability analyzer (Beckman-Coulter, Miami, FL), were harvested, washed once in ice-cold PBS, lysed directly in 15 µl 2X sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol, 2% 2-mercaptoethanol, 0.03 mM Bromphenol Blue), and boiled for 5 minutes. p21 protein half-life determinations were performed using extracts prepared in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% Na-Deoxycholate, 1.0% NP-40, 1 mM EDTA, 1 mM PMSF,

1 mM NaF, 0.5 mM DTT, 1.0 mM Vanadate, 1:250 Protease Inhibitor Cocktail (Sigma-Aldrich)), quantified using a BCA protein assay (Pierce, Rockford, IL), and boiled as noted in 2X sample buffer. 32D v-Abl cells at $1x10^6$ cells/mL were treated with either 2 μM STI571 (kind gift from Tom Smithgall, University of Pittsburgh) or DMSO as a vehicle control. Cells were harvested at 0, 2 and 8 hour time points, washed in ice-cold PBS and protein extracts were prepared in modified RIPA buffer containing 50 nM Calyculin A (Cell Signaling Technology, Beverly, MA).

Lysates containing equal amounts of total protein, or an equivalent number of whole cells lysed directly in 2x sample buffer, were separated using 12% SDS-PAGE. Proteins were electrotransferred onto Protran membranes (Schleicher and Schuell, Keene, NH), which were incubated with Ponceau S (Sigma-Aldrich) to confirm transfer efficiency. Membranes were then incubated with blocking buffer (5% w/v nonfat dry milk, 1x TBS, 0.1% Tween-20) for 1 hour. Primary antibodies were diluted in blocking buffer and included mouse monoclonal β-Actin (AC15) (1:20,000, Cat: A5441 Sigma-Aldrich), rabbit polyclonal IgG Myeloperoxidase (1:3500, Cat: 91541, Assay Designs, Ann Arbor, MI), goat polyclonal IgG TTP (1:200, Cat: SC-8458, Santa Cruz), and mouse monoclonal IgG_1 DRBP76 (NF-90) (1:250, Cat: 612154, BD Transduction Laboratories, San Diego, CA). Additional primary antibodies including goat polyclonal p21(C-19)-HRP (1:1000, Cat: SC-397HRP), goat polyclonal IgG Proteinase-3 (1:200, Cat: SC-19748), mouse monoclonal IgG_1 HuR (3A2) (1:500, Cat: SC-5261), rabbit polyclonal IgG hnRNP A/B (1:200, Cat: SC-15385), and rabbit polyclonal IgG hnRNP K (H-300) (1:200, Cat: SC-25373) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes probed with mouse monoclonal $IgG_{2bκ}$ anti-phosphotyrosine (4G10 clone, 1:500, Cat: 05-321, Upstate Biotechnology, Lake Placid, NY) antibodies were blocked and probed using BSA buffer

(5% w/v BSA, 1x TBS, 0.1% Tween-20). All rabbit polyclonal IgGs including Akt (1:1000, Cat: 9272), phospho-Akt (Thr308) (1:1000, Cat: 9275), p44/42 MAP kinase (1:1000, Cat: 9102), and phospho-p44/p42 MAP kinase (Thr202/Tyr204) (1:1000, Cat: 9101) obtained from Cell Signaling Technology were utilized per manufacturer's instructions. Where appropriate, secondary HRP-conjugated antibodies were diluted in blocking buffer and included goat anti-mouse IgG (H + L)-HRP (Cat: 170-6516, Bio-Rad, Hercules, CA), goat anti-rabbit IgG (H + L)-HRP (Cat: 170-6515, Bio-Rad) and donkey anti-goat IgG HRP (Cat: SC-2033, Santa Cruz). Signals were developed using the Western Lightning chemiluminescence reagent (PerkinElmer, Boston, MA). Scanned images of exposed film underwent signal density analysis using Image J 1.31v software (http://rsb.info.nih.gov/ij).

3.2.4. Half-life determination

3.2.4.1. Protein

Log phase 32Dcl3 cells cultured in IL-3 were washed with 50 mL PBS and resuspended to $1x10^6$ cells/mL in cytokine free media. Cycloheximide (Sigma-Aldrich) was titrated to 10 μg/mL, cell suspensions were divided into equal volumes and IL-3 was titrated to either 1 ng/mL or 10 pg/mL. Cells were further treated with either 12.5 μg/mL MG132 (Sigma-Aldrich) or DMSO as a vehicle control and were harvested at 0, 1, 2, 3, 4 and 6 hour time points. Harvested cells were washed once in ice cold PBS, snap-frozen in a dry ice/ethanol bath, and lysates prepared with modified RIPA buffer. For half-life assessments made in G-CSF, cells were washed twice with 50 mL PBS and resuspended to $1x10^6$ cells/mL in cytokine free media, G-CSF was titrated to 100 ng/mL and cells were incubated for 18 hours to initiate the granulocytic

differentiation program. Cells stimulated with G-CSF for 18 hours were treated with cycloheximide or DMSO and harvested as above for the indicated time points.

3.2.4.2. RNA

32Dcl3 cells or 32D hp21WT cl6 cells were washed and resuspended as noted above. Actinomycin-D (Sigma-Aldrich) was titrated to 10 μg/mL and the culture was divided into two equal parts. IL-3 was titrated to either 1 ng/mL or 10 pg/mL and RNA was prepared from $1x10^7$ cells at 0, 2, 4, and 6 hour time points using RNeasy total RNA purification kits (Qiagen, Valencia, CA). For half-life assessments made in G-CSF, cells were prepared as noted above and incubated for 18 hours. Actinomycin-D was titrated to 10 μg/mL and cell cultures were divided into two equal parts. Cells received either 1 ng/mL IL-3 or an equal volume of water as a control. RNA was prepared from cells harvested at 0, 2, 4 and 6 hour time points. Where indicated, cells were treated with 50 μM LY294002 (Cell Signaling Technology) or an equal volume of DMSO as a vehicle control and incubated for 30 minutes prior to addition of Actinomycin-D. Similarly, following G-CSF stimulation, cells were treated with 20 μM U0126 (Cell Signaling Technology) or DMSO for 2 hours prior to addition of Actinomycin-D. 32D v-Abl cells at $1x10^6$ cells/mL were treated with either 2 μM STI571 or DMSO as a vehicle control and incubated for 2 hours. Actinomycin-D was titrated to 10 μg/mL and cells were harvested at 0, 1, 2, 4 and 6 hour time points. Northern blotting for all experiments was performed as described in section 2.3.7. Signal density analysis of scanned images was performed using Image J 1.31v software (http://rsb.info.nih.gov/ij). Multiple exposures were obtained to optimize linearity of signal.

3.2.5. Nuclear/Cytoplasmic fractionation

Cells treated with IL-3 and/or G-CSF as described above were harvested for preparation of nuclear and cytoplasmic protein extracts. Pelleted cells were washed with 1 mL of ice-cold PBS, treated with cytoplasmic extraction buffer (10 mM HEPES pH 7.9, 14 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 0.2% NP-40, 10 mM NaF, 1.0 mM DTT, 1.0 mM Vanadate, 50 nM Calyculin A, 1.0 mM Pefabloc, 2% CLAP cocktail – (20 μg/ml each chymostatin, leupeptin, antipapain, and pepstatin A)), mixed by pipetting 10-15 times and incubated on ice for 20 minutes. Intact nuclei were verified by microscopic examination in the presence of trypan blue dye. Nuclei were pelleted at 3000 RPM for 3 minutes at 4 C° (Heraeus 7500 3325 rotor) and cytoplasmic supernatants were transferred to pre-chilled tubes and snap-frozen in a dry ice/ethanol bath. Nuclei were washed once with cytoplasmic extraction buffer and pelleted as above. Pellets were resuspended by pipetting in nuclear extraction buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 20% Glycerol, 1.0 mM DTT, 1.0 mM Vanadate, 50 nM Calyculin A, 2% CLAP cocktail, 1.0 mM Pefabloc) and subjected to three cycles of freezing and thawing on dry ice and wet ice, respectively. Insoluble material was pelleted at 13,000 RPM for 30 minutes and supernatants were transferred to pre-chilled tubes and snap-frozen.

3.2.6. Riboprobes

T7 recognition-sequence containing PCR products of the murine p21 3' untranslated region (Accession: NM_007669) were amplified from phRL-p21 3'utr-FL (kind gift from Hirotaka Okano, Keio University[195]). Gel purified amplicons were used as templates to synthesize [α-^{32}P]-UTP (Perkin Elmer, Boston, MA) labeled riboprobes *in vitro* using the Maxi-Script kit with T7 polymerase (Cat: 1312, Ambion, Houston, TX). Biotinylated RNA probes synthesized *in*

vitro were end-labeled by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) using [γ-^{32}P]-ATP. The biotinylated probe sequence, h/m 37, was: TGTGTGTCTTAATTATTATTTGTGTTTTAATTTAAA. Riboprobes were separated from unincorporated nucleotides using NucAway Spin Columns (Cat: 10070, Ambion) per the manufacturer's instructions. Riboprobes without radioactive label were synthesized as above using 0.5 mM UTP.

3.2.7. Northwestern Blotting

Nitrocellulose membranes containing transferred proteins, as described above, were incubated in northwestern buffer [194] (10 mM Tris-HCl pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 0.1% gelatin, 0.1% Tween-20, 50 µg/ml salmon sperm DNA) for 16 hours at room temperature. A ^{32}P labeled T7-RNA probe ($5x10^6$ CPM/mL) containing nucleotides 580-1905 of the murine p21 3'UTR (Accession: NM_007669) was hybridized to the membrane in northwestern buffer with 50 µg/mL yeast tRNA for 2 hours at room temperature. The membrane was washed in northwestern buffer twice for 5 minutes at room temperature and once for 2 hours at 45° C. Dried membranes were subjected to autoradiography.

3.2.8. UV Crosslinking Assay

Cytoplasmic extracts derived from cytokine treated cells were used in UV Crosslinking assays performed as described with modification[283]. 30 µg of protein extract was equilibrated in RNA binding buffer on ice for 20 minutes, T7-riboprobes ($1x10^5$ CPM/reaction) were added, and the reactions incubated for an additional 25 minutes on ice. Reaction mixtures maintained on ice were exposed to UV light (254-nm) at a distance of 3 cm from the source for 16 minutes in a UV

Stratalinker™ 1800 (Stratagene, La Jolla, CA). RNase T1 (5 U/reaction, Ambion) and Heparin sulfate (30 μg/reaction, Sigma-Aldrich) were added and reactions were incubated at room temperature for 10 minutes. RNase A (Sigma-Aldrich) was titrated to 30 μg/ml and mixtures were incubated for 10 minutes at 37° C. An equal volume of 2X sample loading buffer was added and samples were boiled for 5 minutes. Samples were separated by 8% SDS-PAGE. Dried gels were subjected to autoradiography.

3.2.9. Statistics

Protein and RNA half-life determinations were compared for all conditions tested with significance assessed by a 2-tailed, type 2 student's t-test using 95% confidence intervals. All calculations were made using the Excel software package (Microsoft, Redmond, WA).

3.3. Results

3.3.1. p21 protein stability is independent of IL-3 or G-CSF stimulation in 32Dcl3 cells.

The myeloid progenitor cell line 32Dcl3 is a good model system for studying cytokine controlled mechanisms that regulate p21 gene expression during myeloid differentiation. 32Dcl3 cells are dependent upon IL-3 for propagation, survival and maintenance of an undifferentiated, myeloblast state. Withdrawal of IL-3 and treatment with G-CSF stimulates them to differentiate along the granulocytic pathway and leads to the formation of terminally differentiated neutrophils. To validate the competence of this cell line to initiate granulocytic differentiation and establish p21 protein expression kinetics during this period, we treated 32Dcl3 cells with G-CSF for 3 days. Primary granule protein expression is the molecular hallmark of early

granulocytic differentiation[284]. Western blot analysis revealed that two primary granule proteins, myeloperoxidase and proteinase-3, are strongly upregulated within 48 hours following G-CSF stimulation (Fig 8A). We next examined the effect of IL-3 and G-CSF on endogenous p21 protein levels in 32Dcl3 cells. Whereas basal levels of p21 protein were high in cells proliferating in IL-3, a rapid decrease is observed following stimulation with G-CSF, decreasing by 70% within 48 hours (Fig 8A). This pattern of p21 protein expression mirrors the changes in p21 expression noted by our laboratory during cytokine-induced neutrophil differentiation of human CD34+ umbilical cord stem cells[99,100].

p21 protein half-life has been reported to be controlled by several stimuli including insulin, TGF-β, and ionizing radiation, but has not been studied in myeloid cells during differentiation[210,215,230]. To determine if the observed decrease in p21 protein levels occurred due to changes in protein stability, we assessed whether changes in p21 protein half-life were a function of IL-3 concentration or occurred when G-CSF was substituted for IL-3. 32Dcl3 cells were treated with high or low levels of IL-3, or treated with G-CSF, and subjected to a cycloheximide chase analysis(Fig 8B, C). Under all three conditions, there was no significant difference in p21 protein levels at each time point evaluated with protein half-life ~3.5 hours ($t_{1/2}$ 3.6 hrs vs. 3.1 hrs vs. 3.4 hrs (high IL-3 vs. low IL-3 vs. G-CSF), ($p>0.05$, $n = 3$)). These findings suggest that p21 protein stability in 32Dcl3 cells is not a function of IL-3 concentration or a destabilizing effect of G-CSF. p21 protein levels were enhanced by coincubation with the proteasome inhibitor MG132 either in high or low IL-3 (Fig 8B). This suggests that p21 protein is actively metabolized by the proteasome independently of the presence of IL-3.

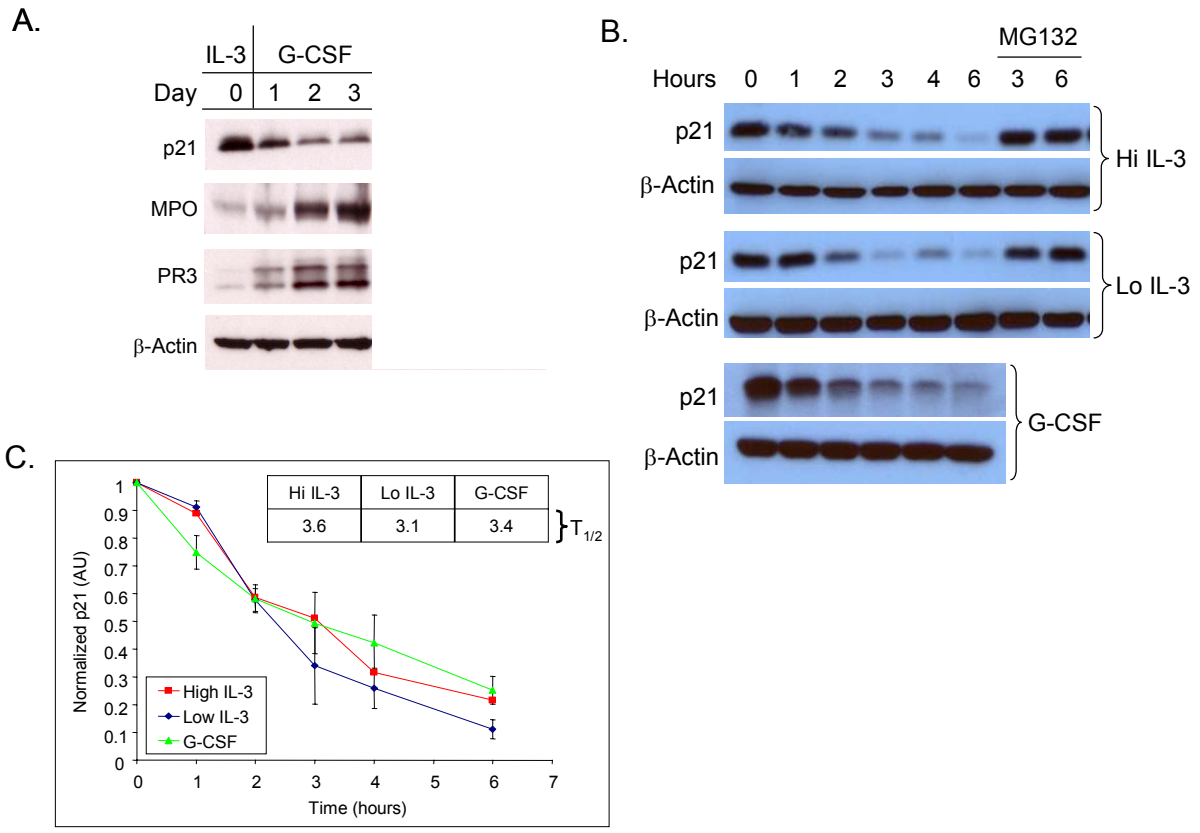

Figure 8. p21 protein stability is constant during differentiation.

a) p21 protein levels decrease during differentiation of 32Dcl3 cells. Cells propagating in 1 ng/mL IL-3 were washed and stimulated to differentiate with 100 ng/mL G-CSF. Each lane contains $3x10^5$ viable cells that were harvested daily and boiled directly in laemmli buffer. Samples were subjected to SDS-PAGE and immunoblot analysis with p21, myeloperoxidase (MPO) and proteinase-3 (PR3) antibodies. β-actin is provided as a loading control. b) p21 cycloheximide chase analysis in 32Dcl3 cells. 32Dcl3 cells were washed and treated with high or low doses of IL-3 (1 ng/mL vs. 10 pg/mL), with or without the proteasomal inhibitor MG132

(15 μg/mL), and 10 μg/mL cycloheximide. Total protein was extracted at 1, 2, 3, 4 and 6 hours after cycloheximide treatment and analyzed by immunoblotting. Washed 32Dcl3 cells were incubated with G-CSF (100 ng/mL) for 18 hours and then treated with 10 μg/mL cycloheximide. Whole cell extracts were prepared in modified RIPA buffer and analyzed by SDS-PAGE and immunoblot with p21 and β-actin antibodies. c) Results in b) were subjected to signal density analysis using NIH Image 1.31v. p21 values were normalized against β-actin and plotted. Error bars denote standard error of the mean (n=3).

3.3.2. IL-3 stabilizes p21 mRNA

Because p21 levels can be regulated either by changes in mRNA stability or changes in translation or transcription, we next examined the effect of IL-3 and G-CSF induced differentiation upon endogenous p21 mRNA expression levels in 32Dcl3 cells. Withdrawal of IL-3 and transition of 32Dcl3 cells to G-CSF was accompanied by a 65% decrease in p21 RNA expression within 24 hours that remained depressed up to 72 hours (Fig. 9A). To determine if this effect occurred at the posttranscriptional level and was simply a function of IL-3 withdrawal, we assessed p21 mRNA half-life by actinomycin-D chase analysis using high and low concentrations of IL-3 (Fig. 9B). Because 32Dcl3 cells are dependent upon IL-3 for survival, we utilized low doses of IL-3, rather than the absence of IL-3, to prevent the activation of apoptotic cascades during the experimental time period. By using low levels of IL-3 that were insufficient for ongoing expansion of cells, we maintained cell viability above (90%) throughout the duration of the experiment. Densitometric analysis revealed a significant difference in p21 mRNA levels at all time points tested with a ~3.5-fold difference in half-life between high and low IL-3 concentrations (3.7 vs. 0.95 hours, $p<0.01$) (Fig 9D). This data supports the hypothesis that IL-3 signals are required for stabilization of p21 mRNA in 32Dcl3 cells. We next sought to determine if IL-3 signals could stabilize p21 mRNA in the presence of differentiation signaling by G-CSF. We first treated 32Dcl3 cells with G-CSF for 18 hours to initiate the granulocytic differentiation program. Following actinomycin-D treatment, we observed a rapid decrease in p21 mRNA levels in these cells, and noted the p21 mRNA half-life was similar to that observed for cells exposed to low levels of IL-3 (1.0 vs. 0.95 hours, G-CSF vs. Low IL-3) (Fig 9B & C). Addition of IL-3 to these differentiating cells significantly increased the half-life to 3.3 hours (1.0 vs. 3.3

hours, G-CSF vs. G-CSF + IL-3, $p<0.03$) (Fig 9E). Together these finding support stabilization of p21 message by IL-3 rather than destabilization by G-CSF.

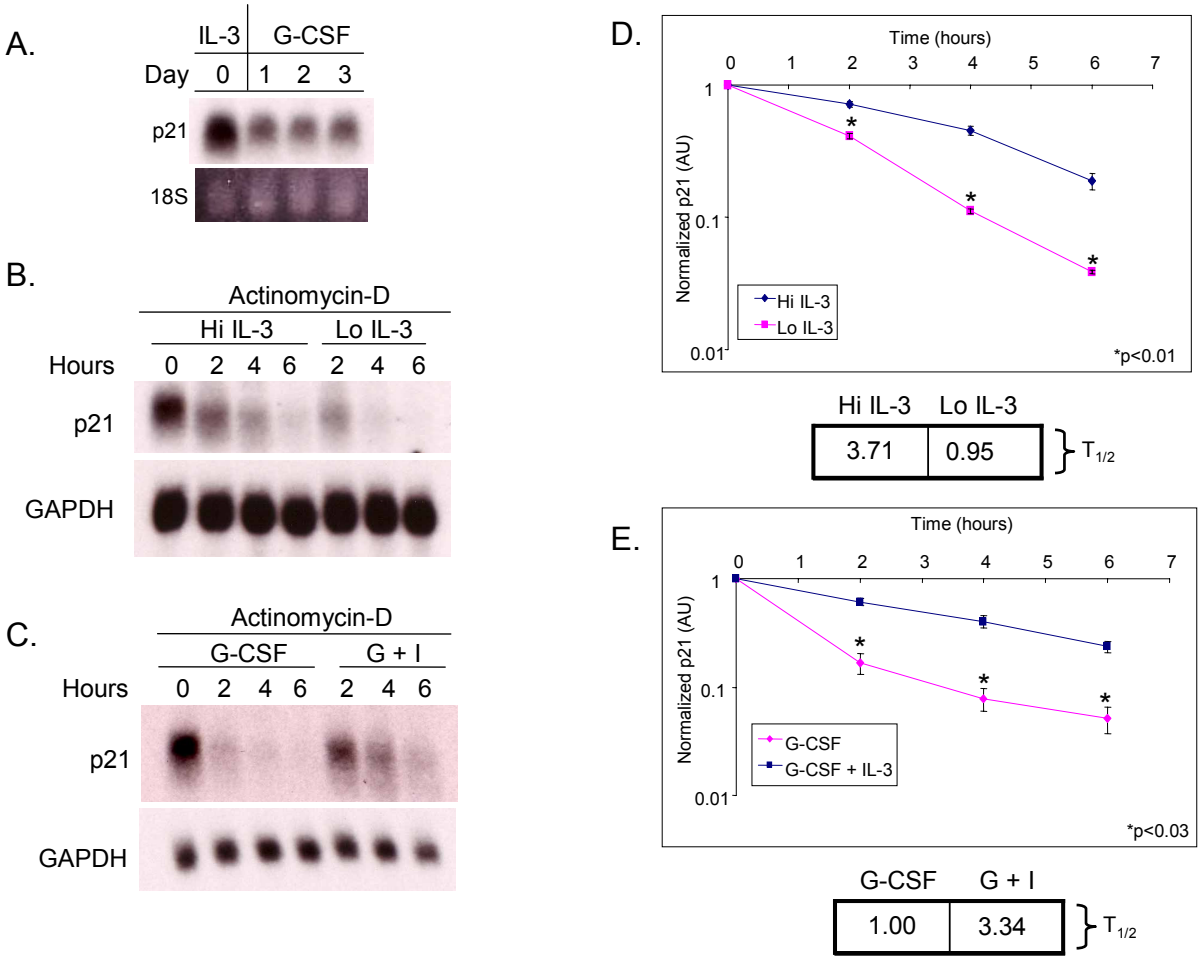

Figure 9. IL-3 stabilizes p21 mRNA.

A) Northern blot analysis of total RNA from 32Dcl3 cells propagating in IL-3 (1 ng/mL) or following treatment with G-CSF (100 ng/mL). RNA harvested daily was probed with ^{32}P-labeled murine p21 cDNA. Ethidium bromide stained 18S RNA is provided as a loading control. B) p21 stability is a function of IL-3 concentration. 32Dcl3 cells were treated with 10 µg/mL actinomycin-D and either 1 ng/mL (Hi IL-3) or 10 pg/mL (Lo IL-3) murine IL-3. Total RNA was extracted at 0, 2, 4 and 6 hours following actinomycin-D treatment and assessed by northern blot with ^{32}P-labelled p21 and GAPDH probes. C) IL-3 stabilizes p21 mRNA in the presence of G-CSF. 32Dcl3 cells were incubated with 100 ng/mL G-CSF for 18 hours, treated with 10

μg/mL actinomycin-D and either water (0.01%) as a vehicle control (G-CSF) or 1 ng/mL murine IL-3. Total RNA was harvested and analyzed as in B). D & E) Quantification of autoradiograms depicted in B) and C) were performed by NIH Image 1.31v. p21 signals normalized against GAPDH are plotted (*, $p<0.05$) with p21 mRNA half-life (hours) tabulated. Error bars denote standard error of the mean (n = 3).

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3.3.3. Stabilization of p21 mRNA by IL-3 is independent of the PI3-kinase and ERK pathways

The hematopoietic cytokines M-CSF, GM-CSF and IL-3 have been reported to stimulate p21 expression in macrophages through the PI3-kinase pathway[285]. It has previously been reported that activation of this pathway by IL-3 is rapid and responsible for survival signaling in 32Dcl3 cells[265]. We wanted to determine if this pathway was activated by IL-3 in the setting of ongoing G-CSF signaling in our system. Phosphorylation of Akt, a downstream target of PI3-kinase, was evident within 15 minutes of IL-3 stimulation (Fig. 10A), indicating that IL-3 was sufficient to rapidly activate the PI-3 kinase pathway. Next, we sought to determine if PI3-kinase activity was required for the IL-3 mediated increase in p21 mRNA stability. To facilitate this analysis, we employed the PI3-kinase inhibitor LY294002 and found that it could effectively reduce IL-3 stimulated Akt phosphorylation at a dose of 50 μM (Fig. 10A, lanes 7-9). Importantly, a 30 minute treatment with this compound alone did not alter steady-state levels of p21 mRNA (Fig. 10B, lanes 10 vs. 11). p21 mRNA half-life was unchanged in cells treated with G-CSF and either LY294002 or a vehicle control (Fig 10B, lanes 1-5 vs. 11-15). Upon IL-3 stimulation, a 2.5 fold increase in half-life was observed in both LY294002 and vehicle treated cells (Fig 10B, lanes 6-9 vs. 16-19), indicating that stabilization of p21 mRNA by IL-3 signal pathways does not require PI3-kinase activity in 32Dcl3 cells.

ERK activity has been shown to regulate mRNA stability of several genes including, TGF-β, Cox-2 and the p21 relative $p27^{kip1}$ [286-288]. A recent report noted that Prostagladin-A_2 induced stabilization of p21 mRNA in the H1299 lung carcinoma cell line required ERK activation[189]. ERK inhibition blocked stabilization by preventing HuR binding to the p21

3'UTR. Since the ERK pathway is activated downstream of IL-3 receptor ligation[289,290], we next sought to determine if this pathway was mediating p21 mRNA stabilization by IL-3 during differentiation. Western blot analysis was used to first verify that IL-3 could activate the ERK pathway in G-CSF treated 32Dcl3 cells. Activation of ERK requires phosphorylation upon specific threonine and tyrosine residues which can be detected by phospho-specific antibodies. We noted a sustained ERK phosphorylation that was detectable within 15 minutes of IL-3 treatment (Fig. 11A). Optimal blockade of ERK activation was executed using 20 μM U0126, an inhibitor of the activating ERK kinase, MEK (Fig 11A). p21 mRNA half-life was unchanged in G-CSF treated cells stimulated with IL-3 in the presence and absence of U0126 ($t_{1/2}$ 3.0 vs. 3.2 hours, DMSO vs. U0126) (Fig 11B & C), suggesting that ERK pathway activation is not required for IL-3 mediated stabilization of p21 mRNA.

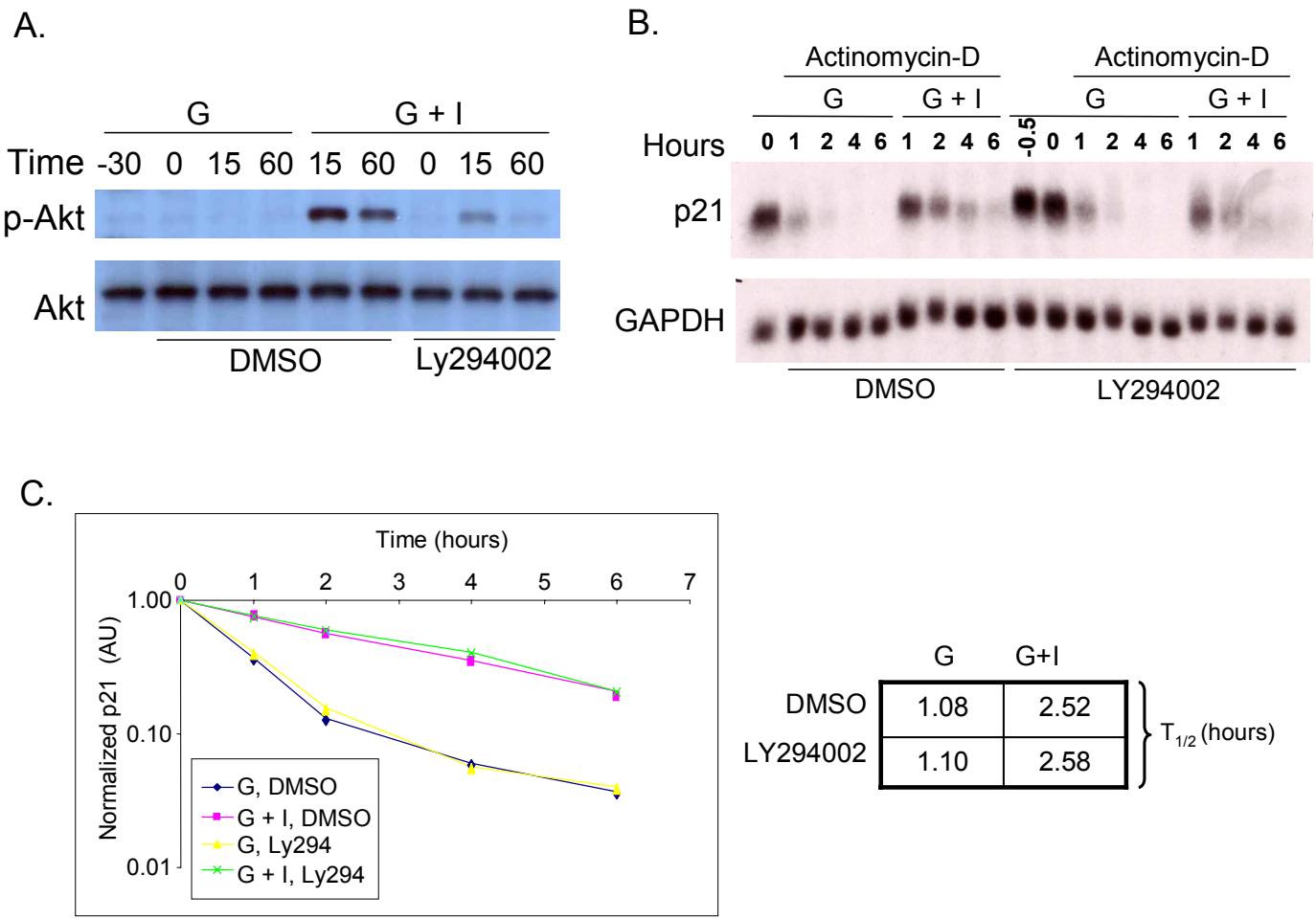


Figure 10. IL-3 stabilization of p21 mRNA is independent of PI3-kinase activity

A) IL-3 activates the PI3-kinase pathway. 32Dcl3 cells were incubated with G-CSF for 18 hours, treated for 30 minutes with 50 μM LY294002 or 0.01% DMSO as a vehicle control and then stimulated with 1 ng/mL IL-3. Western blot analysis was conducted on whole cell protein extracts (15 μg/lane) prepared from cells harvested at the indicated time points (minutes) to detect phosphorylated Akt (p-Akt). Equal loading was verified by detection of total Akt levels.

B) Northern blot analysis of p21 mRNA expression. 32Dcl3 cells incubated with G-CSF for 18 hours were pretreated with DMSO or LY294002 as in A). Actinomycin D was titrated to 10 μg/mL and cells were treated with either 1 ng/mL murine IL-3 (G + I) or water as a vehicle control (G). Total RNA was harvested at the indicated time points and probed with ^{32}P-labeled murine p21 and GAPDH cDNAs. C) Densitometric analysis of autoradiograms depicted in B) with p21 signals normalized to GAPDH density. p21 mRNA half-life is tabulated (hours).

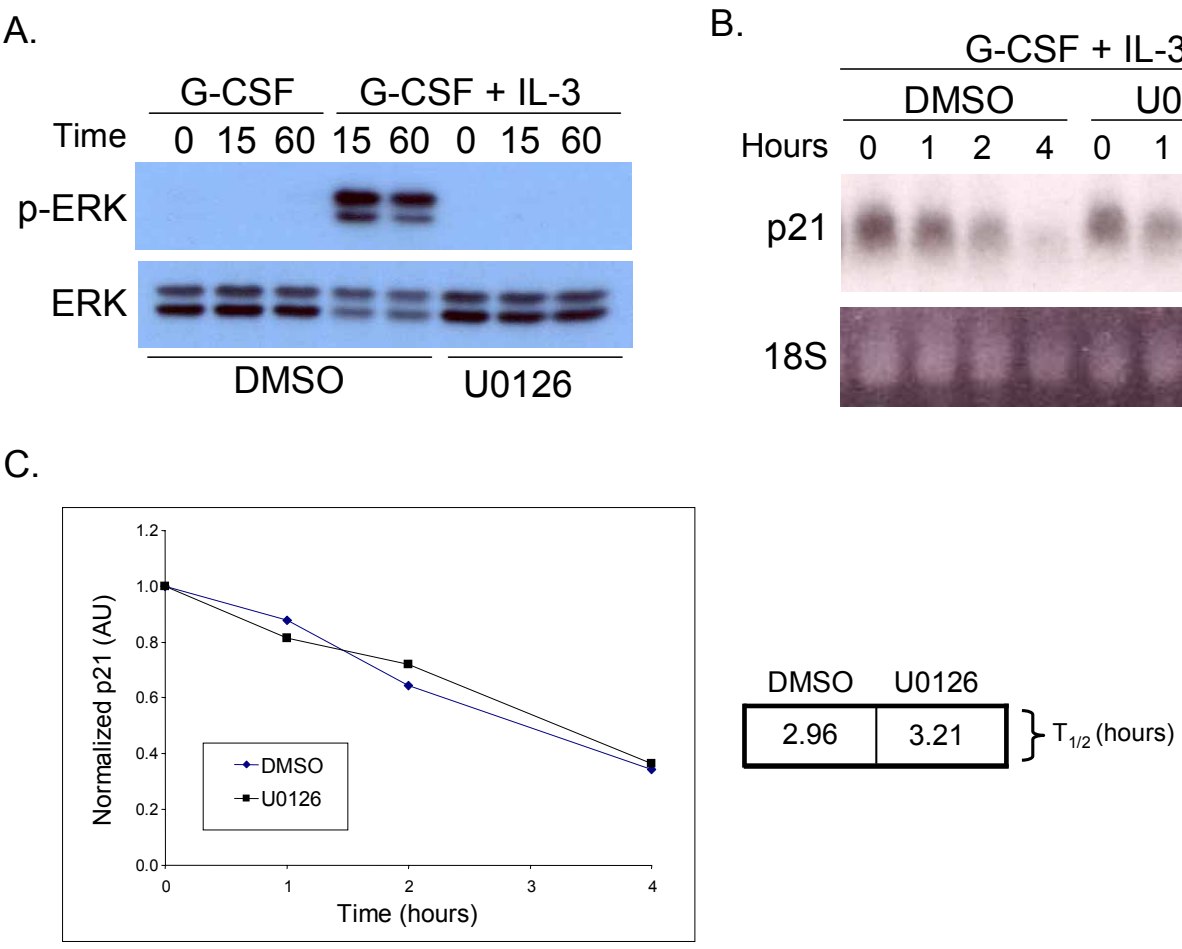


Figure 11. IL-3 stabilization of p21 mRNA is independent of MEK activity

A) IL-3 activates the ERK pathway. 32Dcl3 cells were incubated with G-CSF for 18 hours, treated for 2 hours with 20 μM U0126 or 0.01% DMSO as a vehicle control and then stimulated with 1 ng/mL IL-3. Western blot analysis was conducted on whole cell protein extracts (15 μg/lane) prepared from cells harvested at the indicated time points (minutes) to detect phosphorylated ERK (p-ERK). Equal loading was verified by detection of total ERK levels. B) Northern blot analysis of p21 mRNA expression. 32Dcl3 cells incubated with G-CSF for 18

hours were pretreated with DMSO or U0126 as in A). Actinomycin D was titrated to 10 μg/mL and cells were treated with 1 ng/mL murine IL-3. Total RNA was harvested at 0, 1, 2 and 4 hours and subjected to northern blot analysis with a ^{32}P-labeled murine p21 cDNA probe. Ethidium bromide stained 18S RNA is provided to demonstrate equal loading. C) Densitometric analysis of panels in B) with p21 signals normalized to 18S RNA density are plotted. p21 mRNA half-life is tabulated (hours).

3.3.4. IL-3 stabilization of p21 mRNA requires the 5' or 3' untranslated regions

Cis-acting elements within the p21 3'UTR are required for p21 mRNA stabilization in several experimental systems[189,191,193,196]. It is plausible that *cis*-elements within the p21 mRNA untranslated regions are necessary for IL-3 mediated stabilization in 32Dcl3 cells. To test this hypothesis, we utilized 32D cells expressing a p21 transgene lacking either the 5' or 3' UTR (see Fig. 5A) [279]. Using the endogenous p21 transcript as an internal control, we examined p21 mRNA half-life in response to high or low IL-3 levels. We found that half-life of p21 transcripts devoid of UTR sequences was independent of IL-3 concentration (6.1 vs. 4.5 hours, high vs. low IL-3) (Fig 12 A & B). Endogenous p21 transcripts recapitulated the ~3.5 fold difference in half-life demonstrated in parental 32Dcl3 cells (3.2 vs. 0.9 hours, high vs. low IL-3). This data suggests that regulatory elements reside in either the 5' or 3' UTR of p21 and are required for stabilization of p21 mRNA by IL-3.

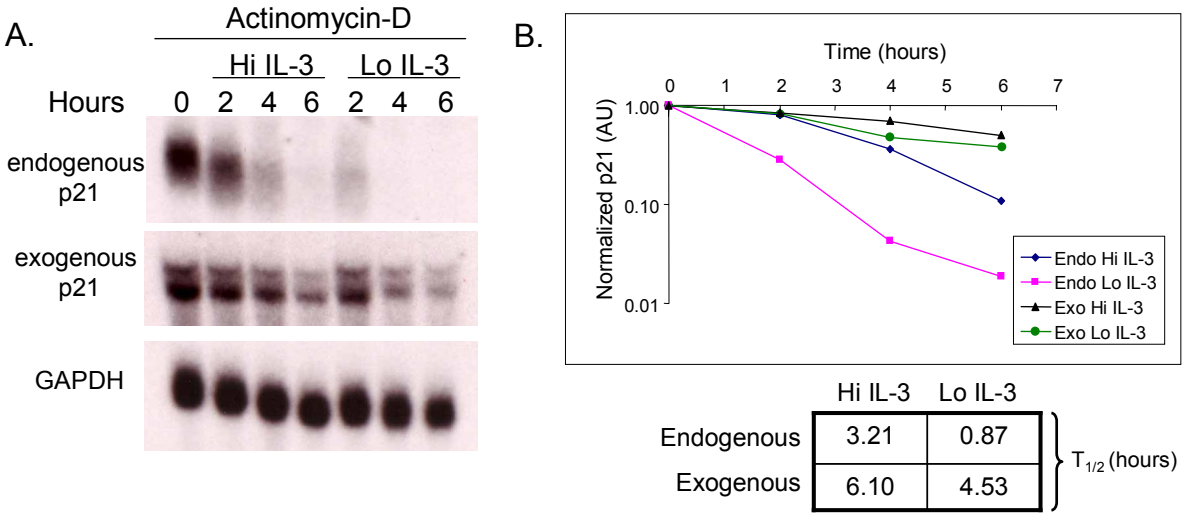


Figure 12. p21 mRNA stabilization by IL-3 requires the 3'UTR.

A) Northern blot analysis of p21 mRNA expression. 32D hp21WT cl6 cells were washed, treated with 10 μg/mL Actinomycin-D, and stimulated with 1 ng/mL IL-3 (Hi IL-3) or 10 pg/mL IL-3 (Lo IL-3). Total RNA was harvested at 0, 2, 4 and 6 hours and subjected to northern blot analysis with ^{32}P-labeled p21 and GAPDH probes. C) Densitometric analysis of autoradiograms in A) with p21 signals normalized to GAPDH density is plotted. p21 mRNA half-life is tabulated (hours).

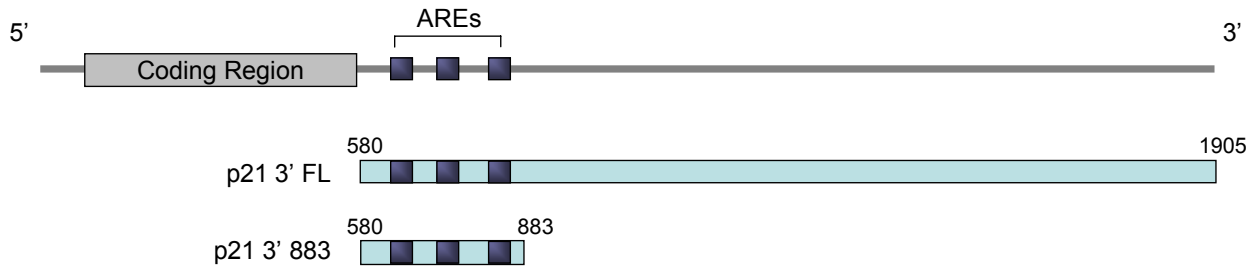


Figure 13. Schematic representation of the murine p21 mRNA and RNA probes.

Schematic representation of the murine p21 mRNA (nt 1-1909, Genbank Accession # NM_007669) with coding region denoted in gray and conserved AU-rich elements (AREs) represented by blue squares. *In vitro* transcribed RNA probes used in this study are depicted.

3.3.5. IL-3 stimulated p21 mRNA binding proteins localize to the cytoplasm

To determine whether RNA binding proteins in 32Dcl3 cells are capable of binding the p21 3'UTR under various cytokine conditions we utilized northwestern blotting and UV crosslinking assays. Two RNA probes were used in these studies: p21 3' FL, which contained the full length murine 3'UTR sequence (nt 580-1905), and p21 3' 883, which contained the proximal 303 nucleotides, including the three AU-rich elements that are conserved between human, mouse and rat species. Northwestern blot analysis with the p21 3' FL probe labeled a prominent 90 kDa interacting protein in whole cell extracts from 32Dcl3 treated with either IL-3 or G-CSF (Fig 14A). Because the fate of p21 mRNA can be determined by interactions with cytoplasmic RNA-binding proteins such as HuR and AUF1[184], we determined the subcellular distribution of this 90 kDa RNA binding protein and whether its compartmentalization changed

as a function of IL-3 or G-CSF exposure. Although an RNA-binding activity was not observed in cytoplasmic extracts, a similar 90 kDa protein was labeled by the p21 3'FL probe in nuclear extracts (Fig. 14B). This signal did not vary with changes in IL-3 dose or in response to G-CSF stimulation. To evaluate the possibility that the absence of binding in cytoplasmic extracts was due to insufficient protein loading (15 μg/lane), we probed membranes containing 80 μg of cytoplasmic extract per lane (loading used in Fig 14A). No detectable labeling of proteins was observed under these conditions (data not shown). These results demonstrate an RNA-binding activity in 32Dcl3 cells and identify a 90 kDa nuclear protein that is capable of binding the p21 3'UTR.

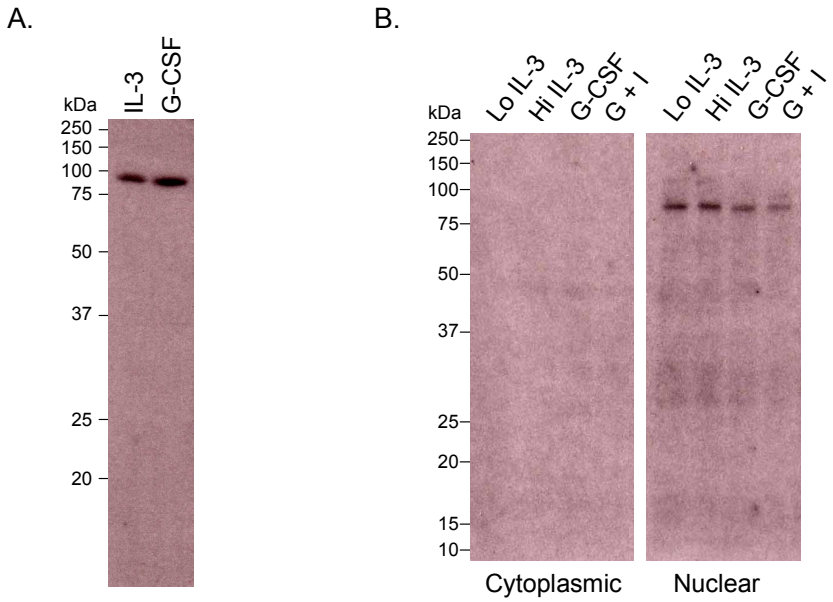
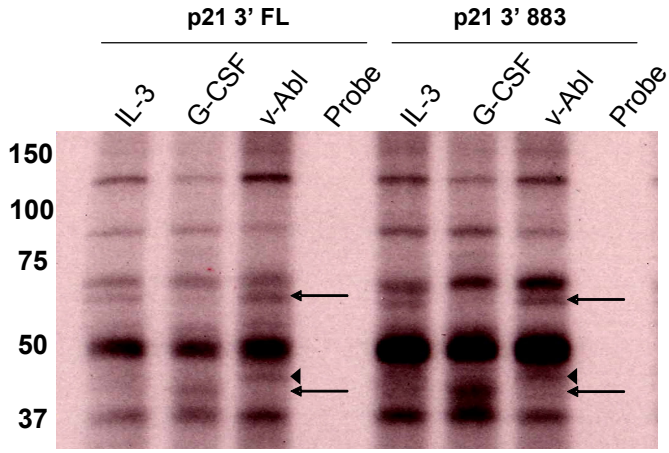


Figure 14. RNA-binding proteins in 32Dcl3 cells interact with the p21 3'UTR.

A) Northwestern blot analysis of whole cell protein extracts (80 μg/lane) from 32Dcl3 cells treated with either 1 ng/mL IL-3 or 100 ng/mL G-CSF for 18 hours. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with *in vitro* transcribed ^{32}P-labeled p21 3'FL riboprobes and signals determined by autoradiography. B) Northwestern blot of nuclear and cytoplasmic extracts (15 μg/lane) derived from 32Dcl3 cells treated with cytokines as follows: 10 pg/mL IL-3 (Lo IL-3) or 1 ng/mL IL-3 (Hi IL-3) for 6 hours, 100 ng/mL G-CSF for 24 hours (G-CSF), and 100 ng/mL G-CSF for 18 hours with 1 ng/mL IL-3 for an additional 6 hours (G + I). Membranes were prepared and probed as noted in A).

To characterize other potential cytokine responsive p21 mRNA binding proteins, UV crosslinking (UVXL) assays were utilized. UVXL assays provide high sensitivity resolution of RNA-protein interactions, including those that may be transient in nature or of low affinity, such as those found in the cytoplasm[283]. Both the p21 3'FL and p21 3'883 riboprobes were used to identify ARE binding proteins whose binding activity may be modulated during differentiation. Cytoplasmic extracts derived from cells treated with IL-3 or G-CSF for 18 hours were examined. Several proteins were found to interact with the p21 3'FL and p21 3'883 probes (Fig. 15A). Although binding to both riboprobes by the majority of proteins was independent of cytokine stimulation, RNA-protein complex formation was differentially regulated by IL-3 and G-CSF for two proteins (Fig 15A, arrows). An RNA-protein complex of ~60-65 kDa apparent mass was noted in IL-3 extracts, but absent in G-CSF extracts. Conversely, an RNA-protein complex of ~40-42 kDa apparent mass was found in G-CSF extracts, but was absent in IL-3 extracts. This pattern was observed for both riboprobes, suggesting that these RNA-protein complexes are localizing to the proximal 303 nucleotides of the p21 3'UTR. Analysis of several known RNA-binding proteins, including those previously reported to bind p21 mRNA, by western blot revealed an exclusively nuclear localization for NF90, hnRNP K, HuR, TTP, and hnRNP A/B with no detectable changes in subcellular localization in response to G-CSF or IL-3 stimulation (Fig 16). However, AUF1 was found to localize in both the nucleus and cytoplasm. Multiple AUF1 isoforms were detected, with a small percentage of total AUF1 found in cytoplasmic extracts, present at an apparent mass of ~40-42 kDa(Fig. 16).

A.



B.

RNA BP's	kDa
HuR	36
hnRNP K	66
TTP	33.6
hnRNP A/B	30.8
NF90*	90, 110
AUF1*	37, 40, 42, 45
CUGBP1	55
Calreticulin	47.9
CP1	37.5

Figure 15. Cytoplasmic p21 mRNA-binding proteins are differentially regulated by IL-3 and G-CSF.

A) UV crosslinking analysis of p21 3'UTR binding proteins. Extracts from IL-3 or G-CSF treated 32Dcl3 cells, or from unstimulated 32Dcl3 v-Abl, were incubated with either ^{32}P-labeled p21 3'FL or p21 3'883 riboprobes, crosslinked by UV exposure and separated by 8% SDS-PAGE. Dried gels were analyzed by autoradiography. Labeled riboprobe RNA in the absence of 32Dcl3 extracts was included as a control (Probe). Migration of prestained molecular mass markers is indicated (kDa). Arrows denote proteins with binding changes between IL-3 and G-CSF. Arrowhead denotes a protein unique to v-Abl transduced cells. B) Known RNA-binding proteins with their respective molecular masses are tabulated (*masses of known isoforms indicated).

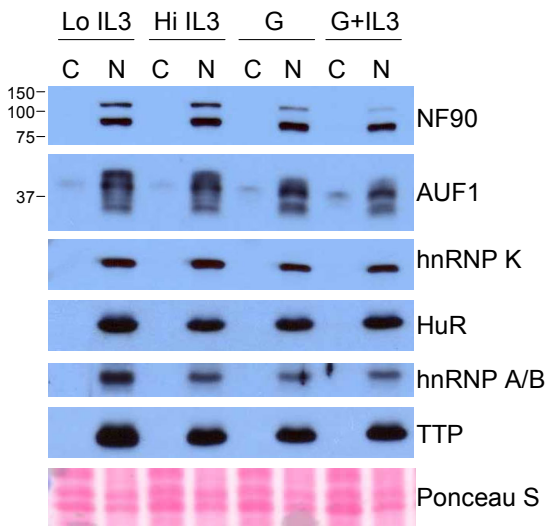


Figure 16. Compartmental distribution of RNA-binding proteins is unchanged during differentiation. Cytoplasmic (C) and nuclear (N) extracts (15 μg/lane) were separated by SDS-PAGE and immunoblotted in sequence with antibodies directed against NF90, AUF1, hnRNP K, HuR, hnRNP A/B and TTP. Ponceau S staining of nitrocellulose membranes is shown as a loading control.

3.3.6. p21 mRNA half-life is independent of v-Abl kinase activity

High levels of p21 expression were noted in the 32Dcl3 v-Abl cell line (Fig 17A). These cells were transduced with the v-Abl oncogene, do not require cytokines for survival, and are resistant to differentiation signals provided by G-CSF[95]. We utilized these cells to determine if RNA-protein complexes associated with IL-3 stabilized p21 mRNA were recapitulated in cytokine independent cells that have been transformed by a nonreceptor tyrosine kinase associated with leukemia. Comparison of p21 RNA-protein complexes from 32Dcl3 v-Abl cell extracts with those from IL-3 treated cells demonstrated a similar set of interactions with both the p21 3'UTR and p21 3'883 riboprobes. One RNA-protein complex of ~42-48 apparent mass was unique to 32Dcl3 v-Abl extracts, and also absent in G-CSF treated cells (Fig 15A, arrowhead). These findings suggest that IL-3 independent p21 RNA-binding activity is present in 32Dcl3 v-Abl cells and includes a distinct RNA-protein complex.

Since p21 mRNA-protein complexes were present in 32Dcl3 v-Abl cells, we sought to determine if v-Abl kinase activity was required for stabilization of the p21 transcript. To accomplish this we employed the tyrosine kinase inhibitor STI-571, a compound with high specificity for the Abl kinase domain. Effective kinase inhibitory concentrations for this compound have been reported to range from 1-4 μM *in vitro* and *in vivo*[291]. We found that a 2 μM dose of STI-571 decreased total cellular tyrosine phosphorylation within 2 hours and did not impact cell viability (Fig. 17A and data not shown). While p21 protein expression was reduced within 2 hours of treatment with STI-571 (Fig. 17A), this compound did not alter p21 transcript half-life as determined by actinomycin chase analysis (Fig. 17 B & C). Evaluation of p21 protein stability in 32Dcl3 v-Abl cells demonstrated that p21 protein half-life was independent of

v-Abl kinase activity (data not shown). Taken together, these results suggest that v-Abl kinase activity may be promoting p21 expression at the level of transcription or translation. We therefore determined whether the decrease in p21 protein seen in STI-571 cells still occurred in the presence of the transcriptional inhibitor Actinomycin D. p21 protein levels were decreased by STI-571 whether transcription was blocked or not (data not shown). In aggregate, these results suggest that v-Abl may be enhancing p21 translation, as Bcr-Abl has been reported to do for the C-Myc and MDM2 genes[292].

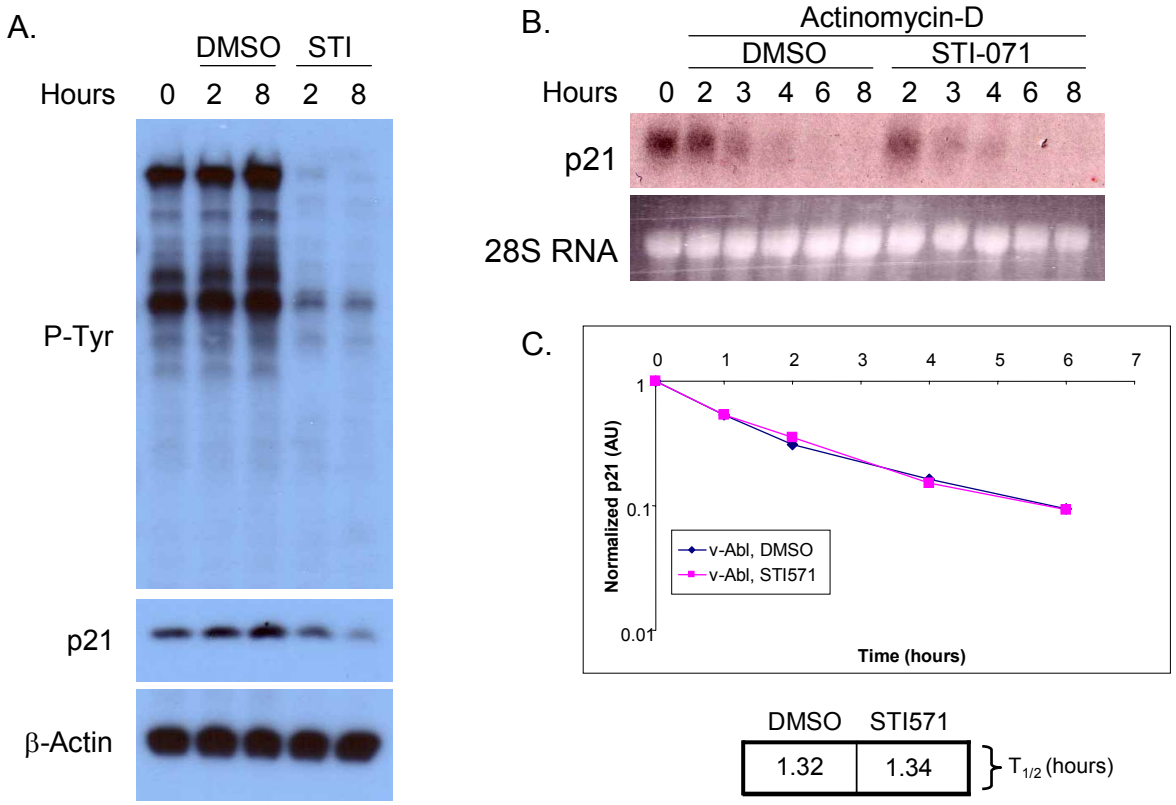


Figure 17. p21 mRNA half-life does not depend upon v-Abl kinase activity.

A) p21 protein expression decreases following v-Abl inhibition. Western blot analysis of 32Dcl3 v-Abl cells treated with 2 μM STI-571 or 0.02 % DMSO as a vehicle control. Whole cell extracts were prepared from cells harvested at 0, 2, and 8 hours after treatment, separated by SDS-PAGE (15 μg/lane) and transferred to nitrocellulose membranes. Membranes were probed in sequence with antibodies directed against phospho-tyrosine, p21 and β-actin. B) Northern blot analysis of p21 mRNA expression. 32Dcl3 v-Abl cells (t = 0) treated with 2 μM STI-571 or 0.02 % DMSO for two hours (t = 2) as in A) were exposed to 10 mg/mL Actinomycin-D. Total RNA was extracted from cell at the indicated time points and subjected to northern blot analysis

with a ^{32}P-labeled murine p21 cDNA probe. Ethidium bromide stained 28S RNA is provided to demonstrate equal loading. C) Densitometric analysis of panels in B) with p21 signals normalized to 28S RNA density are plotted. p21 mRNA half-life is tabulated (hours).

3.4. Discussion

IL-3 is an important regulator of myeloid progenitor cell development. In several organ systems, differentiation and development require changes in gene expression that are regulated at the posttranscriptional level by RNA binding proteins. However, the role of posttranscriptional regulation in myeloid development, and particularly posttranscriptional effects mediated by cytokines such as IL-3, are poorly understood. We used the IL-3 dependent myeloid progenitor cell line 32Dcl3 to test the hypothesis that IL-3 controls p21 expression during differentiation through posttranscriptional stabilization of its mRNA. Here we have demonstrated that IL-3 has no effect on p21 protein stabilization, but rather operates to stabilize p21 mRNA in 32Dcl3 cells (Figures 8 and 9). This effect required p21 3'UTR sequences. The decrease in levels of p21 as 32Dcl3 cells differentiated was shown to result from the faster turnover of p21 message in G-CSF than in IL-3. Our data also indicates that p21 mRNA stability downstream of IL-3 receptor activation is independent of ERK and PI3-kinase pathway activation (Figures 10 and 11). Several proteins have previously been reported to interact with the p21 3'UTR. Using 32Dcl3 protein extracts, we established that p21 RNA:protein complexes occur *in vitro,* identifying a 90 kDa nuclear protein that binds the p21 3'UTR independently of IL-3 or G-CSF signaling. In UV-crosslinking assays we demonstrate that distinct cytoplasmic p21 RNA:protein interactions occur within the proximal ARE region of the p21 3'UTR in response to either IL-3 or G-CSF. Association of a ~60-65 kDa protein with the p21 ARE region correlated with IL-3 mediated p21 mRNA stabilization, whereas binding by a ~40-42 kDa protein correlated with destabilization of p21 transcripts in 32Dcl3 cells undergoing G-CSF-induced differentiation. These findings are

significant because they provide the first evidence for IL-3-mediated stabilization of mRNA transcripts in myeloid progenitor cells.

Our data maps the IL-3 responsive domain of the p21 message to untranslated regions of the p21 transcript. When only the coding region of p21 was transfected into 32Dcl3 cells, the stability of that transcript did not vary with IL-3 concentration upon Northern blot analysis (Fig 12). This was consistent with data from heterologous reporter assays that demonstrated that the p21 3'UTR sequences were required to increase reporter expression in response to stimuli that stabilize p21 transcripts[191-193]. These effects were localized to nucleotides between 570-830 in the human 3'UTR [192,193]; a region containing three AREs which is homologous to the murine 580-883 sequence used in our UV-crosslinking assays. For each cytokine tested in 32Dcl3 cells, UV crosslinking data showed that patterns of RNA:protein complexes were conserved between the full length 3' untranslated region of p21 3'FL and truncated transcripts consisting of the 303 proximal base pairs of the p21 3' UTR (nucleotides 580-883, i.e., the p21 3' 883 riboprobe) (Fig 15). This indicates that RNA:protein complex formation resulting from the use of the p21 3'883 riboprobe was not an artifact of abnormal secondary structure arising from truncation of the full length 3'UTR, underscoring the utility of this riboprobe. The change in RNA:protein complex formation noted in our UV crosslinking assay suggests a binary model where on- and off-binding to the proximal p21 3'UTR region by two specific RNA-binding proteins follows an IL-3 to G-CSF switch.

IL-3 has previously been shown to increase the mRNA stability of its receptor subunits, the IL-3 receptor α chain and the β common subunit, in mature human eosinophils[256]. High levels of the IL-3 receptor α chain are found in subsets of AML and correlate with poor prognosis[22,248]. Since IL-3 is often secreted by myeloid leukemia cells[280,281], it is possible that high levels of the

IL-3 receptor α chain are maintained in an autocrine fashion by IL-3 in leukemic myeloblasts. Although the β common subunit lacks AREs in its mRNA sequence, the human IL-3 receptor α chain contains one AUUUA motif in its 5'UTR. The study by Wang et al. did not explore a mechanism for stabilization of IL-3 receptor mRNA by IL-3[256].

Our data suggests that IL-3 accomplishes mRNA stabilization by targeting specific RNA-binding proteins to the p21 3'UTR. A similar mechanism may be responsible for stabilization of IL-3 receptor α chain mRNA in normal and leukemic cells. Since IL-3 is necessary to prevent differentiation of 32Dcl3 cells, it may be that IL-3 directs stabilizing RNA-binding proteins to a set of transcripts (including p21) that function to inhibit differentiation in IL-3 responsive myeloid progenitor cells or in IL-3 responsive leukemic myeloblasts.

Posttranscriptional stabilization of p21 has been studied in animal and cell line models of muscle differentiation. p21 is an important regulator of survival and differentiation in skeletal muscle, with peak levels of p21 protein found in terminally differentiated cells[96,105,111,118]. Increases in p21 expression during differentiation of C2C12 myogenic progenitor cells are associated with a 6-fold increase in p21 mRNA stability[196]. In contrast to muscle, p21 expression decreases as 32Dcl3 cells undergo granulocytic differentiation (Fig 8A). This decrease in p21 protein levels is associated with a 3.5-fold decrease in p21 mRNA stability (Fig 9A & B). In myeloid cells, as in muscle, p21 protein expression appears to be a function of changes in mRNA stability, although the direction of change is opposite in the two cell types.

Our data suggests that HuR is not a cytokine responsive p21 mRNA stabilizing protein. Exposure of 32Dcl3 cells to G-CSF, or the readdition of IL-3, had no effect on either HuR abundance or intracellular distribution in 32Dcl3 cells (Fig 16). HuR was exclusively localized to the nucleus and undetectable in the cytoplasm, suggesting that compartmental redistribution of

HuR may not be regulated by differentiation signals in 32Dcl3 cells. This is important because cytoplasmic localization of HuR is associated with its ability to stabilize mRNAs[293,294]. HuR has been shown to promote p21 mRNA stability in muscle through binding of AREs in the p21 3'UTR[196]. Analysis of cytoplasmic extracts from differentiating myoblasts by UV crosslinking identified HuR as a 37 kDa protein whose p21 ARE-binding activity was differentiation-induced[196]. This effect was correlated with cytoplasmic accumulation of HuR that was triggered by the myogenic differentiation stimulus.

HuR independent stabilization of p21 mRNA has also been described and occurs in response to EGF or phenylephrine. Although HuR binding to the p21 ARE region was demonstrated in MDA-468 breast cancer cells, it did not mediate EGF-induced p21 mRNA stabilization in this system, suggesting that additional RNA-binding proteins can regulate p21 stability even in the presence of HuR binding[193]. In addition, the α_1 adrenergic agonist phenylephrine promoted p21 mRNA stability independent of HuR in the hepatoma cell line HepG2[191]. Interestingly, phenylephrine induced the binding of two proteins of 24 and 52 kDa apparent mass to a 78 nucleotide riboprobe encompassing the conserved p21 AREs (bp 648-726). In both studies, the proteins responsible for p21 RNA stabilization were not identified.

Our data indicates that binding of a 60-65 kDa protein, or prevention of the binding of a 40-42 kDa protein, to the p21 3'UTR is a cytokine regulated phenomenon associated with p21 mRNA stability. UV-crosslinking assays demonstrated a cytoplasmic 60-65 kDa protein only from 32Dcl3 cells in IL-3 bound to both p21 3'FL and p21 3'883 riboprobes (Fig. 15) In light of this, we examined the expression profile of hnRNP K, a 66 kDa protein that has been demonstrated to bind the p21 3'UTR and inhibit p21 translation. We found that hnRNP K was detectable solely in the nuclear compartment in 32Dcl3 cells, with expression levels independent

of the type of cytokine stimulus(Fig. 16). Other p21 RNA-binding proteins with masses in the 60-65 kDa range have not been described. Due to the high sensitivity of UV crosslinking assays, hnRNP K cannot be ruled out as a candidate for our 60-65 kDa protein based solely upon subcellular localization analysis. Since hnRNP K has been shown to inhibit p21 translation it is unlikely that hnRNP K:p21 mRNA interactions would lead to increased p21 protein expression levels. Therefore, we speculate that the observed 60-65 kDa protein represents a novel IL-3 regulated p21 3'UTR interacting protein that may act to stabilize p21 transcripts.

Destabilization of p21 mRNA was correlated with binding of the 40-42 kDa protein to the p21 3'UTR. Data from UV-crosslinking assays indicates that a cytoplasmic 40-42 kDa protein is activated to bind the p21 3'UTR when 32Dcl3 cells are switched out of IL-3 and induced to differentiate with G-CSF (Fig 15). This coincides with the disappearance of the 60-65 kDa interacting protein. AUF1, an RNA-binding protein that promotes mRNA decay, has been demonstrated to bind and destabilize p21 mRNA in competition with HuR[184]. AUF1 expression is characterized by four splice variants, p37, p40, p42 and p45, all of which are capable of binding mRNA through their RNA recognition motifs (RRMs)[295,296]. Most abundant in the nucleus, steady-state levels of these isoforms can also be found in the cytoplasm depending upon the cell type examined[296]. Western blot analysis demonstrated multiple AUF1 isoforms present in 32Dcl3 nuclear extracts in cells propagating in IL-3 or undergoing differentiation in G-CSF(Fig. 16). AUF1 was also present in the cytoplasm, but with a restricted isoform distribution. The apparent mass of the cytoplasmic signal is consistent with the presence of the 40 and/or 42 kDa isoforms. In THP-1 monocytic leukemia cells, induction of monocytic differentiation with TPA triggers dephosphorylation of the AUF1 p40 isoform, although the signal pathway mediating this effect was not established. Dephosphorylation precipitated

changes in its RNA binding affinity and correlated with stabilization of ARE-containing transcripts in these cells. Interestingly, TPA did not induce a shift in the compartmental distribution of any AUF1 isoforms; the p37 and p40 isoforms were expressed in the cytoplasm, and the p42 and p45 isoforms expressed in the nucleus. This is important because it indicates that posttranslational modification of AUF1 regulates its RNA binding activity and the half-life of its targets without stimulating shifts in localization during differentiation of myeloid cells. Thus, it is reasonable to speculate that posttranslational tuning of AUF1 binding affinity is sufficient to regulate p21 mRNA decay in response to cytokine signaling during granulocytic differentiation. Ongoing investigations seek to determine if the 40-42 kDa p21 mRNA binding protein is AUF1.

Deletion of the RNA-binding protein NF90 causes perinatal lethality in mice due to skeletal muscle defects that lead to respiratory failure[194]. NF90, and its splice variant NF110 (90 and 110 kDa, respectively), are predominantly nuclear proteins that function in RNA processing, mRNA export and localization, and initiation of translation[297]. Shi et al. demonstrated that p21 mRNA and protein expression were dramatically reduced, along with the myogenic regulators MyoD and Myogenin, in skeletal muscle from $NF90^{-/-}$ animals[194]. Specific NF90 binding to nucleotides 511-883 in the p21 3'UTR was demonstrated by northwestern blot analysis using murine tissue extracts and recombinant NF90. Our northwestern blotting data demonstrates a 90 kDa nuclear protein in 32Dcl3 cells that is capable of binding the p21 3'UTR (Fig 14). Given the importance of NF90 to myogenic differentiation and its ability to bind p21 3'UTR sequences, it is conceivable that the 90 kDa protein we have detected is NF90. We have confirmed that NF90, and its isoform NF110, are expressed in 32Dcl3 cells (Fig 16). Both isoforms are localized to the nucleus and do not experience intracellular shifts to the cytoplasm during G-CSF induced

differentiation. NF90 may stabilize p21 primary transcripts in the nucleus and/or facilitate their export to the cytoplasm during myogenesis. It is possible that NF90 binding is a required step for maintenance of primary p21 transcripts in the nucleus of 32Dcl3 cells.

Because discreet signal transduction pathways have been linked with IL3 signaling, we have undertaken a preliminary characterization of which IL3 signaling pathway could be involved in the stabilization of p21 message. IL-3 has been reported to activate the ERK and PI3-kinase pathways[265,290]. Moreover, The ERK and PI3-kinase signaling pathways have been implicated in the induction and maintenance of p21 expression in hematopoietic cells[285,298]. Our data indicates that IL-3 can induce ERK and Akt phosphorylation, markers of ERK and PI3-kinase pathway activation respectively, when administered following exposure of 32Dcl3 cells to G-CSF (Fig 10A and 11A). However, neither of these pathways is required for IL-3-mediated stabilization of p21 mRNA in our model system (Fig 10C and 11C). This contrasts with p21 mRNA stabilization induced by PGA_2 in H1299 lung carcinoma cells, where stabilization required ERK-induced HuR binding to the p21 3'UTR. Since PI3-kinase pathway activation by IL-3 was required for increased p21 expression in macrophages, it is possible that IL-3 promotes p21 expression via distinct mechanisms in mature and immature myeloid cells in response to PI3-kinase activity[285]. IL-3 receptor ligation can also activate JAK2 and c-Src family tyrosine kinases, as well as the stress pathway kinases $p38^{MAPK}$ and JNK[252,253]. Given these findings, further examination of IL-3 activated tyrosine or stress kinase signal transduction pathways, and their role in p21 mRNA stabilization in 32Dcl3 cells, is warranted.

We have previously observed that p21 expression is elevated in 32Dcl3 cells transformed with the v-Abl oncogene[95]. Since these cells do not depend upon IL-3 for survival, they

provided a good model to determine if v-Abl activity was simply replacing IL-3 signal pathways to stabilize p21 mRNA. Our data did not support this mechanism. Inhibition of v-Abl kinase activity with STI-571 revealed a decrease in p21 protein levels, but demonstrated no change in p21 mRNA stability. p21 protein stability was also unaffected by v-Abl inhibition (data not shown) suggesting that v-Abl activity may act at the level of transcription or translation. Recent work has demonstrated that BCR-ABL promotes leukemic transformation in myeloid cells through translational inhibition of C/EBPα and translational activation of MDM2[299,300]. These effects are mediated by BCR-ABL-dependent upregulation of several RNA binding proteins which specifically bind C/EBPα and MDM2 mRNA transcripts. We have demonstrated that a distinct 46-48 kDa protein in 32Dcl3 v-Abl cells interacts with the p21 3'FL and p21 3'883 riboprobes. Translational upregulation of p21 by specific RNA-binding proteins may contribute to differentiation blockade and/or to v-Abl induced leukemogenesis. Further analysis of p21 expression in 32Dcl3 v-Abl cells will focus on mechanisms that regulate translation and identification of p21 RNA-binding proteins that may impact this process.

In conclusion, the findings presented here suggest that high levels of p21 protein in proliferating 32Dcl3 myeloid progenitor cells results from IL-3-mediated stabilization of p21 mRNA. This work provides the first evidence for IL-3-mediated stabilization of mRNA transcripts in myeloid progenitor cells and may represent a mechanism that contributes to leukemic phenotypes in IL-3 secreting myeloblasts or those overexpressing the IL-3 receptor α chain. Future investigations will focus on characterization of p21 RNA-binding proteins and the signal pathways which regulate their binding activity.

4. Chapter IV

p21 REGULATES MPO TRANSLATION

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4.1. Introduction

Neutrophil differentiation is a 7 to 10 day process that is often divided into stages based upon cell morphology and the expression of distinct cytoplasmic granules. Granule constituents are proteins that are responsible for the inflammatory and microbicidal effects initiated by neutrophils as part of the innate immune response. As neutrophil differentiation proceeds, granule proteins accumulate first in primary azurophilic granules, then secondary and tertiary, or "specific," granules. These granules comprise vesicles that sequester granule proteins to prevent their premature activation and are identified based upon their affinity for specific dyes. The protein complement within a particular granule is thought to be regulated through synchronized expression of genes at the level of transcription rather than by a specific protein sorting mechanism that varies during differentiation[301,302]. Although the specific transcription factors that activate granule protein gene expression have been well characterized[6,301], the mechanisms that regulate the timing of gene expression during differentiation are not well understood.

Changes in p21 levels have been linked to differentiation in several cell types including epidermal, bone forming and hematopoietic cells. In support of an inhibitory role for p21 during differentiation, our laboratory has shown that p21 expression is highest in myeloblasts and then dramatically decreased in post-mitotic granulocytes in both normal cell lines and cord blood derived stem cells differentiated *in vitro*[99,100]. This supports the finding of high p21 levels in undifferentiated myeloblasts isolated from patients with chemoresistant AML[3]. There had been one report, in primary keratinocytes, of differentiation blockade mediated by p21. In keratinocytes exposed to differentiation inducers, ectopic p21 inhibited the expression of genes normally found in terminally differentiated cells and prevented morphologic maturation[109].

More recent work has indicated that p21 delays osteoblast differentiation kinetics[303]. The effect of p21 upon expression of genes associated with granulocytic differentiation has been unexplored.

IL-3 is required for maintenance of an undifferentiated state in 32Dcl3 cells. In Chapter 3, we demonstrated that IL-3 can stabilize p21 mRNA, contributing to the high levels of p21 protein expression found in these myeloid progenitor cells. Since p21 expression decreases with the onset of differentiation (Fig. 4 and Fig. 8), we hypothesized that p21 is a downstream effector of IL-3-mediated differentiation blockade in 32Dcl3 cells. To address this hypothesis we utilized short interfering RNAs (siRNAs) to specifically downmodulate p21 in 32Dcl3 cells. We demonstrate here that loss of p21 is sufficient to induce the premature expression of primary granule proteins in myeloblasts and accelerates G-CSF induced granulocytic differentiation.

4.2. Materials and Methods

4.2.1. Cells and Culture

– see section 2.3.1

4.2.2. Differentiation

– see section 2.3.2

4.2.3. Plasmids

Retroviral constructs were based on the MIG plasmid (a kind gift of Luk Van Parijs, Massachusetts Institute of Technology), which contains an IRES-GFP cassette downstream of its multiple cloning region. Using standard methods, the Gateway® vector conversion cassette (Invitrogen, Carlsbad, CA), reading frame B, was blunt-end cloned into the MIG Hpa I site and

transformed into DB3.1 cells (Invitrogen) to create a Gateway® destination vector, MIGccd. Human p21 wild-type cDNA (accession# NM_078467) carrying a myc-his tag was PCR amplified and cloned into the pENTR-D-TOPO (Invitrogen) plasmid to create a Gateway® entry vector, pENTR-D hp21WT. The expression clone MIG hp21WT was synthesized by combining pENTR-D hp21WT and MIGccd in an LR clonase recombination reaction (Invitrogen) per manufacturer's instructions.

The pSilencer 2.1-U6 Hygro expression vector (Ambion, Austin, TX) was utilized to deliver hairpin siRNA targeting murine p21. Complementary oligonucleotides based upon nucleotides 140-162 (Genbank Accession # NM_007669) of the murine p21 transcript (Primer A - GATCCGCAAAGTGTGCCGTTGTCTCTTCCTGTCAAGACAACGGCACACTTTGCTTTTTCGGCCGGAA; Primer B – AGCTTTCCGGCCGAAAAAGCAAAGTGTGCCGTTGTCTTGACAGGAAGAGACAACGGCACACTTTGCG) were annealed and cloned into the BamHI and HindIII sites of pSilencer 2.1-U6 Hygro to generate pSilencer mp21si. pSilencer 2.1-U6 Hygro was used to deliver negative control siRNA (agcttttccaaaaaactaccgttgttataggtgtctcttgaacacctataacaacggtagtggatccc). Retroviral siRNA expression vectors were synthesized by cloning siRNA cassettes from the above pSilencer plasmids, which consisted of the U6 promoter and hairpin sequences, by blunt-end cloning of PCR amplicons (forward primer – caccgaggagaagcatgaattcc, reverse primer – cgttgtaaaacgacggccag) into the HpaI site of MIG to generate MIG mp21si and MIG CTLsi. All plasmids were verified by sequencing analysis.

4.2.4. siRNA Transfection and Stable cell lines

32Dcl3 cells were transfected with pSilencer siRNA plasmids (Ambion, Austin, TX) by electroporation ($1.5x10^7$ cells in Opti-Mem I (Invitrogen, Carlsbad, CA), 10 μg plasmid DNA, 960 uF, 280 V, 0.4 cm gap), allowed to recover for 48 hours and selected with 1 mg/ml hygromycin (Calbiochem, La Jolla, CA) for 10 days in IL-3 supplemented RPMI as noted in section 2.3.1. Survivors were cloned by limiting dilution and passaged thereafter with 500 ug/ml hygromycin.

4.2.5. Retroviral Transduction

– see section 2.3.5

4.2.6. Western Blotting

–see section 3.3.3

4.2.7. Northern Blotting

– see section 2.3.7

4.3. Results

We have previously demonstrated that 32Dcl3 cells are a good model system for studying the initiation of granulocytic differentiation (Fig 8A). To determine if p21 expression patterns were inversely correlated with those of differentiation-specific genes, we established the temporal expression patterns of p21 and the primary granule proteins myeloperoxidase (MPO) and proteinase-3 (PR-3) in 32Dcl3 treated with G-CSF for 13 days. Western blot analysis demonstrated that p21 levels decrease by >75% within 48 hours of the switch from IL-3 to G-CSF and remained low in terminally differentiated cells at day 13 (Fig 18). In contrast, both MPO and PR-3 expression is induced to high levels within 48 hours of G-CSF stimulation.

These proteins are synthesized as proenzymes which become processed into smaller forms during differentiation. The high molecular weight form of PR-3 is maximal at 48 hours and diminishes thereafter, indicating that molecular processes associated with proper neutrophil maturation are intact in our experimental system. Morphologic differentiation was confirmed by the presence of segmented neutrophils in Diff-Quik stained cytospins (data not shown).

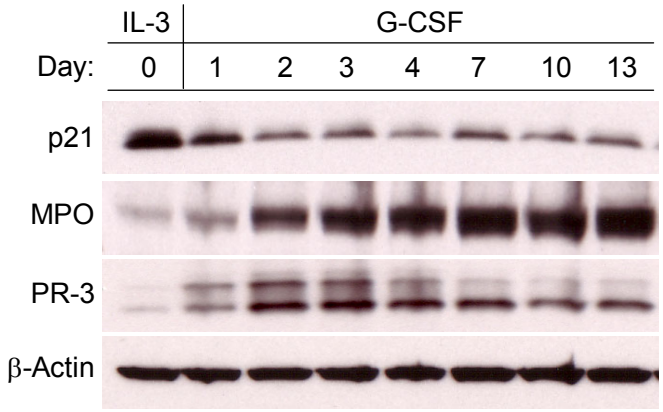

Figure 18. Primary granule protein expression is inversely correlated with that of p21 in 32Dcl3 cells.
32Dcl3 cells propagating in 1 ng/mL IL-3 were washed and stimulated to differentiate with 100 ng/mL G-CSF. Each lane contains $3x10^5$ viable cells that were harvested daily and boiled directly in laemmli buffer. Samples were subjected to SDS-PAGE and immunoblot analysis with p21, myeloperoxidase (MPO) and proteinase-3 (PR3) antibodies. β-actin is provided as a loading control.

We next sought to address the effect of reducing physiological levels of p21 on 32Dcl3 cell differentiation. To knock down p21 expression, we generated stable sublines of 32Dcl3 cells expressing U6 promoter driven hairpin siRNA targeting murine p21 and sublines expressing negative control siRNA. To facilitate targeting of dsRNA to the cytoplasm, where RNA interference primarily occurs, the *human mir-23* microRNA loop motif (CTTCCTGTCA) was incorporated into the hairpin sequence. p21 protein knockdown was >95% in both polyclonal and clonal cell lines compared to control cells (Fig. 19). Northern blot analysis of polyclonal cells demonstrated a comparable decrease in p21 mRNA expression (Fig. 20B, IL-3 lanes).

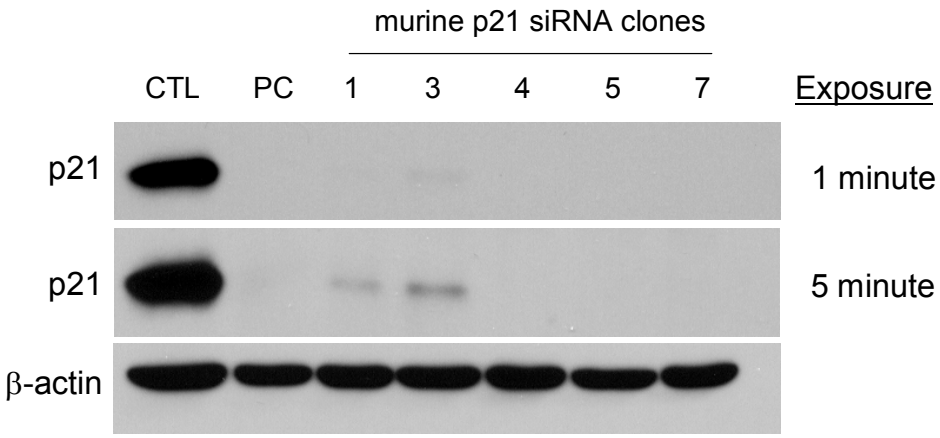

Figure 19. siRNA mediated knockdown of p21 in 32Dcl3 cells.

A) Western blot analysis of 32Dcl3 stable transfectants harboring control siRNA (CTL) or murine p21 siRNA. Polyclonal (PC) and clonal murine p21 siRNA cell lines (#) are indicated. Whole cell protein extracts were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted in sequence with antibodies against p21 and β-actin (loading control).

To determine if p21 knockdown altered the expression of differentiation specific genes, polyclonal cells expressing control or p21 siRNA were treated with G-CSF for 3 days and MPO expression was examined. As in the parental 32Dcl3 cells, MPO protein expression in control siRNA cells increased dramatically after 48 hours, correlating with a steep decrease in p21 expression (Fig 20A). In cells expressing p21 siRNA, MPO protein expression was high in cells propagating in IL-3 and accelerated in response to the G-CSF stimulus. By comparison MPO mRNA expression was low both in control and p21 siRNA expressing cells, rising only after the initiation of differentiation (Fig 20B). Because premature upregulation of MPO protein is not accompanied by an increase in mRNA, this indicates that the high level of MPO protein in p21 siRNA cells does not result from transcriptional upregulation or from increased stability of mRNA. The p21 siRNA is augmenting MPO protein formation or slowing MPO protein decay, suggesting that p21 inhibits MPO translation or MPO protein stability in 32Dcl3 cells.

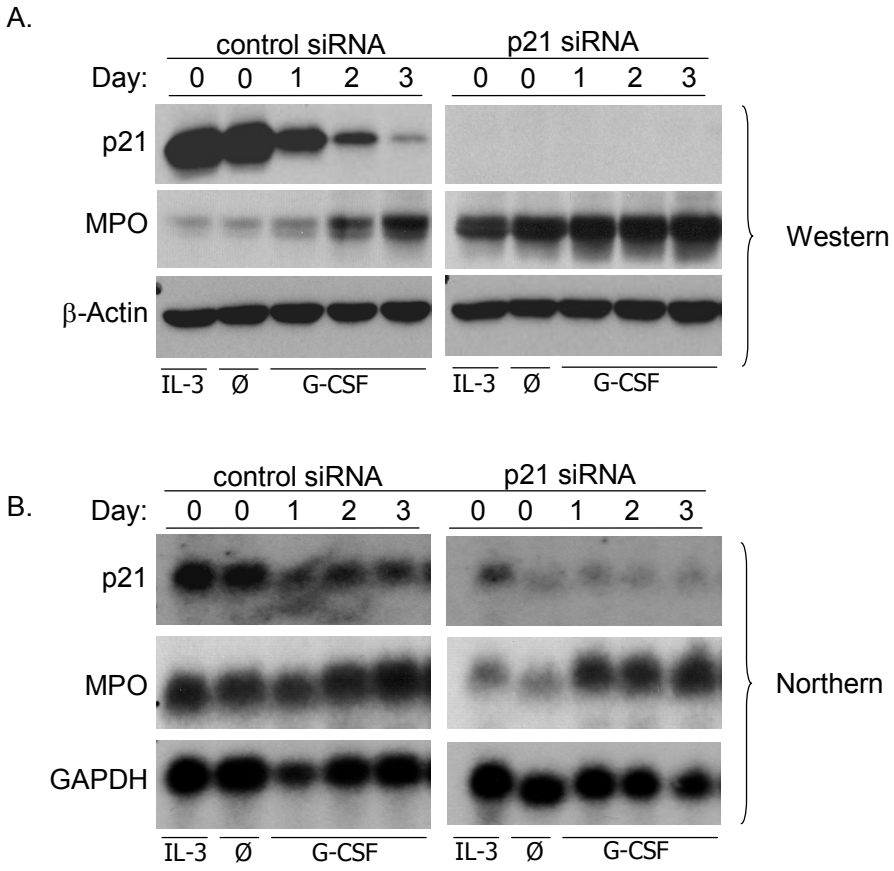

Figure 20. Loss of p21 triggers premature myeloperoxidase expression

A) Polyclonal 32D cells expressing control siRNA or murine p21 siRNA propagating in 1 ng/mL IL-3 underwent a four hour cytokine starvation (Ø) and were stimulated to differentiate with 100 ng/mL G-CSF. Whole cell protein extracts were prepared from cells harvested daily, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted in sequence with p21, myeloperoxidase (MPO) and β-Actin antibodies (loading control). b) Northern blot analysis of total RNA harvested from cells in A) at the indicated time points were subjected to northern blot analysis with ^{32}P-labeled p21, MPO and GAPDH (loading control) cDNA probes.

To validate these findings we utilized an independent retroviral siRNA delivery system. 32Dcl3 cells were transduced with retroviruses carrying control siRNA or p21 siRNA and sorted on the basis of GFP expression. Polyclonal and clonal sublines were established and analyzed for p21 protein expression by western blotting (Fig. 21A). p21 expression was reduced in excess of 80% in two clonal cell lines expressing p21 siRNA compared to control siRNA or parental cell lines. Whereas MPO protein expression is minimal in controls, expression is markedly upregulated in p21 siRNA cell lines in the absence of a differentiation stimulus. This data recapitulates the findings derived with plasmid delivered siRNA and reinforces the observation that p21 downmodulation is sufficient to increase primary granule protein expression in 32Dcl3 cells.

Because this data indicated an inhibitory effect of p21 on expression of differentiation-associated granule proteins, we hypothesized that p21 might slow myeloid differentiation. We therefore examined cell morphology to determine if p21 knockdown altered the kinetics of 32Dcl3 morphological differentiation. Because 32Dcl3 cells take 10-14 days to differentiate into neutrophils, we examined myeloblasts exposed to control or to p21 siRNA after just 4 days of G-CSF treatment. Following exposure to G-CSF at 30 ng/ml for 4 days, segmented neutrophils are apparent in 32Dcl3 sublines expressing p21 siRNA, but absent in controls, suggesting that knockdown of endogenous p21 accelerates granulocytic differentiation (Fig. 21B).

We next sought to determine if premature MPO upregulation in p21 siRNA expressing cells could be suppressed by introduction of a siRNA insensitive p21 transgene. 32Dcl3 cells carrying the p21 siRNA that inhibited endogenous murine p21 were transduced with a control retrovirus or retrovirus expressing a human p21 cDNA that was resistant to the murine p21 siRNA (data not shown). Polyclonal and clonal "rescue" sublines were established and

examined for human p21 transgene expression by western blotting (Fig 21C). Two rescue sublines, 1 and 4, exhibited levels of human p21 transgene expression comparable to those found in parental 32Dcl3 cells. MPO and PR-3 protein expression was suppressed in these clones when compared to the siRNA cells transduced with control virus. This confirmed that the increased MPO in p21-knockdown clones could be rescued by exogenous human p21 and that modulation of MPO levels is a direct effect of p21 and not a nonspecific effect of siRNA.

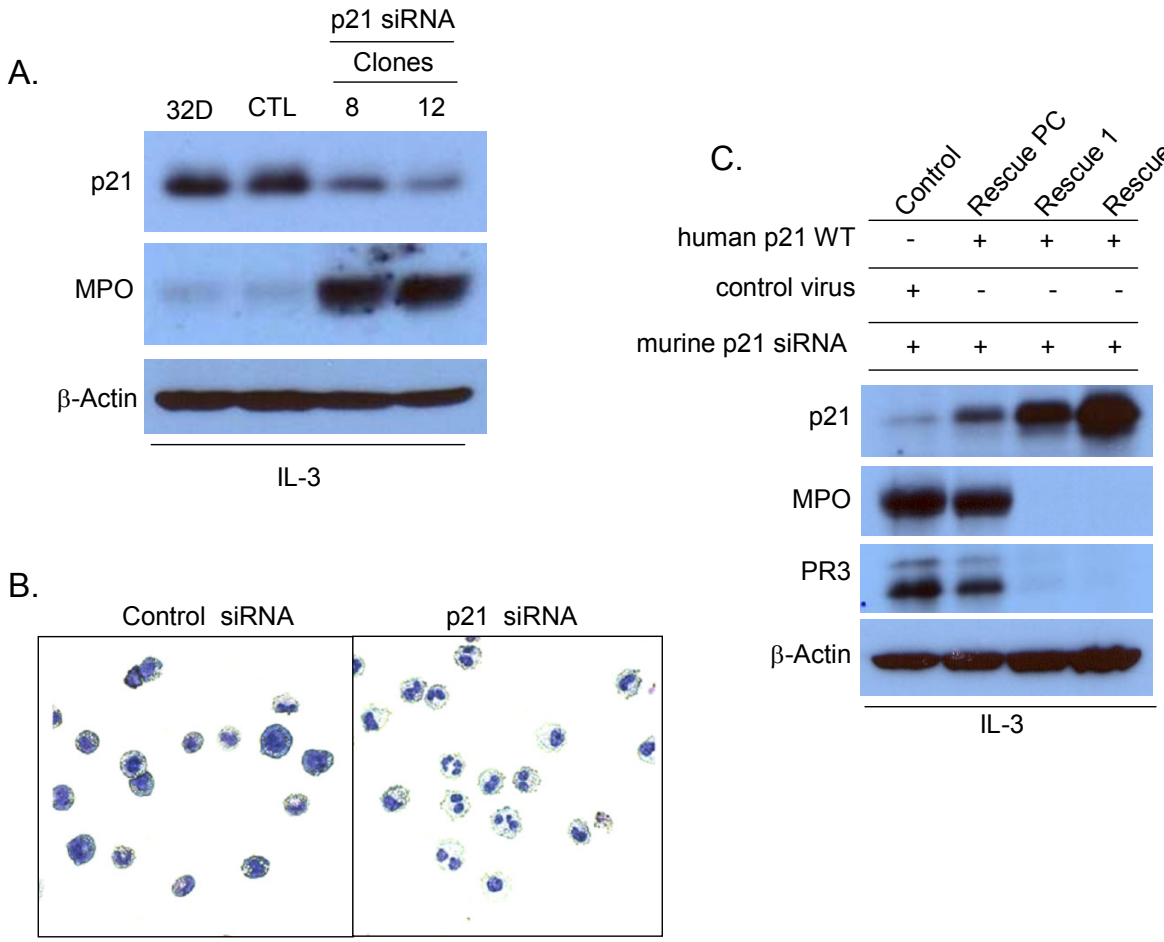

Figure 21. Rescue of p21 siRNA with exogenous human p21 suppresses premature granule protein expression.

A) Retroviral siRNA reduces endogenous p21 protein in 32Dcl3 cells. Parental 32Dcl3 cells (32D) were transduced with retroviruses containing control siRNA (CTL) or p21 siRNA (two representative clones depicted). Whole cell protein extracts were immunoblotted in sequence with antibodies against p21, myeloperoxidase (MPO) and β-actin (loading control). B) Loss of p21 accelerates granulocytic differentiation. 32Dcl3 cells from A) were washed and treated with 30 ng/mL G-CSF for 4 days. Cytospins of harvested cells were prepared with Diff-Quik stain. C) Rescue of p21 restores myeloperoxidase suppression. 32Dcl3 cells expressing stable plasmid

[96]p21 siRNA were transduced with either a control virus or one carrying siRNA resistant human p21 and maintained in 1 ng/mL IL-3. Polyclonal (PC) and two clonal cell lines were derived by cell sorting based on GFP expression. Protein extracts were immunoblotted in sequence with antibodies targeting p21, myeloperoxidase (MPO), proteinase-3 (PR-3) and β-actin.

4.4. Discussion

Defining the molecular mechanisms that prevent myeloid progenitor cells from maturing is important because defects in maturation contribute to the development of myeloproliferative and myelodysplastic diseases, including AML. IL-3 is an important developmental factor for myeloid progenitor cells and is required to maintain the undifferentiated state in 32Dcl3 cells. The mechanisms employed by IL-3 to block differentiation are not well understood. Since IL-3 maintains high levels of p21 in 32Dcl3 cells, we tested the hypothesis that p21 is an inhibitor of myeloid differentiation. Here we have demonstrated that siRNA knockdown of p21 is correlated with premature expression of the primary granule proteins MPO and PR-3, proteins not abundant in cells maintained as myeloblasts by IL-3. Rescue with human p21 in these cells suppressed this early expression. These findings indicate that p21 may contribute to maintenance of the myeloblast phenotype and may regulate the tightly controlled expression of primary granule proteins. p21 knockdown was also found to accelerate morphologic granulocytic differentiation in 32Dcl3 cells stimulated with G-CSF, indicating that p21 may antagonize differentiation at an early regulatory point and not simply cause primary granule protein suppression.

Our data demonstrates that knockdown of p21 expression is sufficient to cause an increase in MPO and PR-3 expression in 32Dcl3 cells. Moreover, the concurrent effect of p21 on two primary granule proteins suggests that this is a coordinated pathway. In support of this, rescue of the siRNA phenotype with exogenous human p21 was evident for both MPO and PR-3. However, it is possible that the demonstrated upregulation of MPO and PR-3 was an artifact of clonal selection. This is unlikely because premature expression occurred in both polyclonal and clonal sublines, and was observed using two independent platforms of p21 siRNA delivery. At

this point we do not know if p21 effects on granule protein expression arise from direct or indirect p21 binding to RNA transcripts or as a downstream manifestation of other p21 functions.

MPO protein upregulation was not accompanied by an increase in mRNA, implying that p21 regulation of MPO occurs at a posttranscriptional level in 32Dcl3 cells. Our data suggests that p21 inhibits MPO translation and/or promotes MPO decay. Although MPO mRNA synthesis is transient, occurring primarily at the promyelocytic stage, MPO protein is long-lived and abundant in terminally differentiated neutrophils[301]. This occurs because, once synthesized, MPO is stored in granules until they are exocytosed and therefore protected from degradation[304]. On this basis, we speculate that it is more likely p21 is regulating MPO translation. Analysis of MPO half-life in our system would help to clarify whether MPO degradation is a function of p21 levels.

Translational repression has been identified as an important mechanism mediating differentiation blockade in leukemic cells[305]. Activation of the RNA-binding proteins hnRNP E2 and calreticulin by leukemic fusion proteins has been demonstrated to suppress translation of the critical myeloid developmental factor C/EBPα in primary AML cells[300,306]. Since IL-3 can induce calreticulin expression in myeloid cells[307], it is possible that calreticulin is activated as a translational repressor by IL-3 in a fashion similar to its recruitment by leukemogenic signaling pathways. Interestingly, calreticulin acts as a molecular chaperone during MPO biosynthesis in the endoplasmic reticulum, facilitating the insertion of a critical heme group into the MPO proenzyme[308]. Although interactions with p21 have not been reported, it is reasonable to speculate that p21 acts downstream of IL-3 to engage calreticulin as a translational repressor of primary granule proteins such as MPO.

Our data demonstrates that downmodulation of p21 with siRNA accelerates differentiation in 32Dcl3 cells. This data is consistent with findings of accelerated osteoblast differentiation in cells derived from $p21^{-/-}$ mice, and p21 inhibition of keratinocyte differentiation[109,303]. Moreover, our data suggests that the high levels of p21 found in subsets of AML patients may contribute to pathogenesis in part by antagonizing differentiation[3]. However, peripheral white blood cell counts in $p21^{-/-}$ mice are normal, suggesting that the absence of p21 does not result in accelerated neutrophil differentiation in an animal model. This may be due to compensation by other cip/kip family proteins, which has been demonstrated to occur in muscle through analysis of p21/p57 double knockout animals[3]. Since siRNA strategies induce a somatic loss of gene expression, interpretation of data is not confounded by compensatory mechanisms that may occur in the germline or at the tissue level. Future investigations using siRNA-mediated knockdown of p21 in human myeloid progenitor cells or conditional p21 knockouts in animal models will be needed to more fully determine the role of p21 in normal myeloid differentiation.

In conclusion, our investigation in differentiating myeloid cells revealed that siRNA-mediated knockdown of p21 accelerated the kinetics of G-CSF induced differentiation. This effect was associated with premature expression of primary granule proteins due to mechanisms operating at the posttranscriptional level. Since high levels of p21 have been described in subsets of AML, our finding of accelerated differentiation in p21 knockdown cells suggests that differentiation blockade by p21 may be one mechanism that contributes to AML pathogenesis.

5. Chapter V

5.1. Discussion and Future Directions

Prior work has delineated changes in the level of expression of the multi-functional adaptor protein p21 during myeloid differentiation. However, the functional role of p21 in differentiating myeloid cells (particularly during granulopoiesis) has not hitherto been examined, nor have mechanisms of its regulation been fully elucidated.

Studies in chapter 2 demonstrated that p21 was an important regulator of survival during granulocytic differentiation. Evidence published elsewhere supported an anti-apoptotic role for p21 in myeloid cells undergoing monocytic differentiation. Therefore, the hypothesis that p21 inhibits apoptosis during granulocytic differentiation was explored. Contrary to expectations, exogenous expression of p21 had a proapoptotic effect in 32Dcl3 cells during the myeloblast to granulocyte transition. This finding was consistent with our overall hypothesis that p21 is an inhibitor of differentiation insofar as p21 downmodulation was required for cells to survive to maturity. A proapoptotic role for p21 during the myeloblast to myelocyte transition is also significant because it suggests that p21 function in cell survival is distinct and opposite between granulocytes and monocytes. Our finding groups granulocytes with several other cell types in which p21 has been noted to promote cell death rather than survival [56]. Of note, cells cultured in high levels of IL-3 were resistant to p21-promoted apoptosis that was manifested when cells were cultured in low IL-3 concentrations or in G-CSF. The ability of cells cultured in high levels of IL-3 to resist an apoptotic function of p21 could have several causes. One potential mechanism is that post-translational modifications of p21 stimulated by IL-3 were operating to minimize its apoptotic potential. Alternatively, differences in IL-3 and G-CSF survival signaling

may account for the relative sensitivity to p21 proapoptotic effects. Our results indicate that p21 promotion of cell death can be a cytokine specific phenomenon that occurs in the absence of other death signals. This may be a mechanism utilized to put a brake on myelopoiesis in response to acute changes in the cytokine environment.

Several methodological limitations should be noted. This study was conducted using a murine cell line model of myeloid development. Findings generated in cell line model systems do not always recapitulate *in vivo* physiology and may be phenomena that are cell line-, or species-specific. Although the 32Dcl3 system has yielded many insights about myeloid development, validation of p21 proapoptotic effects in other cell lines, particularly of human origin, and in primary human cells is required. Secondly, the process of generating polyclonal and clonal sublines overexpressing p21 may have resulted in adaptations that both accommodated the high levels of p21 and distorted the response to differentiation stimuli. This potential problem could be addressed though synthesis of a control cell line overexpressing cdk-inhibitors related to p21 such as p27. Such a control would help to discriminate between possible cell cycle effects that may be promoting apoptosis and proapoptotic effects specific to p21. We believe that p27 is not expected to recapitulate p21 antagonism of cell survival because p27 levels rise with 32Dcl3 differentiation in contrast to the observed fall in p21 expression (Fig 4D)[158]. Lastly, our series of experiments is subject to the usual concerns related to overexpression studies such as nonphysiologic sequestration of p21 binding partners, abnormal subcellular localization, or other effects that may aberrantly stress the 32Dcl3 cells when removed from IL-3 survival signals.

Candidate mechanisms through which p21 could promote apoptosis have yet to be delineated. While our data demonstrated that p21 induced apoptosis was characterized by

activation of the effector caspase caspase-3, this does not pin down a mechanism since multiple pathways associated with intrinsic and extrinsic apoptotic triggers can lead to caspase-3 activation. Future experimentation should establish which of these pathways is recruited by p21. Presumably such experiments will highlight a mechanism that differs between IL3 and G-CSF, since these cytokines are divergent in their sensitivity to p21-induced cell death. Among the many questions raised by our findings are: 1) Is there a survival advantage for cells in G-CSF if p21 levels are dropped (e.g., by RNAi) below the basal level that remains after IL-3 withdrawal? 2) Is p21 protein modified by IL-3 such that its compartmentalization or binding partners change in a way to minimize p21's proapoptotic potential? 3) Would p21 mutants that lack cdk2-binding activity remain capable of promoting apoptosis? 4) Does IL-3 alter the expression or localization of p21 binding partners with a resultant change in p21 function? Experimental approaches to dissect these possibilities include 2-D gel electrophoresis to screen for p21 posttranslational changes, followed by mass spectroscopy and peptide sequencing. Correlation of findings from 32Dcl3 cells, or human myeloid progenitors, with p21 posttranslational modifications that occur in p21 expressing leukemic cells would serve to identify residues with modifications that are specifically altered in leukemia.

Chapter 3 addressed how the cytokines IL-3 and G-CSF regulated p21 expression in myeloid progenitor cells. We found that p21 expression was controlled at the level of mRNA stability rather than protein stability. These findings are significant because they provide the first evidence for IL-3-mediated stabilization of mRNA transcripts in myeloid progenitor cells. Heretofore, gene expression regulated by IL-3 has been primarily attributed to changes in transcriptional activity [252]. Post-transcriptional regulation of mRNA transcripts in response to changes in cytokine signaling has been relatively unexplored in myelopoiesis. Our data indicate

that changes in cytokine signaling can produce changes in RNA:protein interactions. This implies that cytokine-activated RNA-binding factors can coordinate gene expression at the post-transcriptional level in myeloid progenitor cells. Such a mechanism would facilitate rapid developmental responses to changes in the cytokine milieu within the marrow cavity, such as those occurring during emergency granulopoiesis following infection. Cytokine-coordinated changes in mRNA stability also may be an important mechanism in leukemogenesis.

Given the likely biological impact of cytokines acting on posttranscriptional targets, it is important to dissect the signaling mechanism that underlies this action. The fact that IL-3 altered p21 stability even in the presence of actinomycin D indicates that no new gene transcription was necessary to alter the p21 transcript half-life. The rapidity of the IL-3 effect is most compatible with a model in which IL-3 alters the phosphorylation status of a target RNA binding protein (yet to be confirmed) and enables it to bind (or alternatively to be released from) the p21 transcript. Such a model makes it worthwhile to explore IL-3 signaling pathways in order to determine which phosphorylation cascade could be responsible for this phenomenon. The PI3-kinase pathway was of particular interest because a recent report had indicated that IL-3 increased p21 levels in macrophages through a PI3-kinase-dependent mechanism[285]. However, our data ruled out involvement of this pathway. Similarly, the ERK pathway has been shown to promote high levels of p21 expression in AML cells and to stabilize p21 transcripts in response to PGA_2 treatment in colon carcinoma cell lines[189,298]. Although activated by IL-3 in 32Dcl3 cells, the ERK pathway did not mediate p21 stabilization. Several other signaling molecules can be activated by IL-3 including the $p38^{MAPK}$ and JNK stress kinases, JAK2 and members of the c-Src family of tyrosine kinases[252,253]. Given these findings, further examination of IL-3 activated tyrosine or stress kinase signal transduction pathways, and their role in p21 mRNA stabilization

in 32Dcl3 cells, is warranted. It is also possible that multiple pathways can independently stabilize p21 mRNA downstream of IL-3 receptor ligation. Such redundant stabilizing signals could be investigated by the simultaneous application of signal pathway inhibitors and may be informative should experiments targeting inhibition of isolated pathways be indeterminate.

Interpretation of data in chapter 3 should be tempered by limitations of the methods and approach used. As in chapter 2, all experiments were conducted using a cell line model and are subject to the same caveats previously noted. Similar experiments should be conducted in human cell lines, or primary human stem cells differentiated *in vitro*, to validate our findings. In addition, demonstration of RNA:protein interactions was dependent upon the use of *in vitro* binding assays. The main caution with *in vitro* studies is that they have the inherent limitation of being conducted in the absence of intact cells, tissues or whole animals, where findings may not truly represent physiologic reality. They remain, however, a suitable starting point to narrow the focus of subsequent *in vivo* studies. An additional concern is that these assays employed *in vitro* transcribed riboprobes. While riboprobe titration can be tightly controlled, riboprobe misfolding in experimental solutions may create artificial secondary structures, thereby confounding the interpretation of results.

The nucleotide sequences that are responsible for IL-3-mediated stabilization of p21 mRNA have not been determined. We have demonstrated that RNA-binding proteins from 32Dcl3 cells can interact with the p21 3'UTR in a cytokine-regulated manner. Because we have not ruled out the importance of sequences downstream of the proximal 303 nucleotides, future studies should attempt to fine map IL-3 responsive elements using UV-crosslinking and RNA electrophoretic mobility shift (REMSA) assays. Short sequences that are found to be regulated by IL-3 can be incorporated into heterologous reporter constructs and analyzed *in vivo* to verify IL-3

responsiveness. Plasmids containing luciferase reporter genes fused to p21 3'UTR sequences have been described[195] and have been made available to our laboratory. In addition, IL-3 responsive elements could be utilized as baits to affinity purify cytokine responsive RNA-binding proteins from differentiating cells. Purified proteins would be identified by mass spectrometry with p21 RNA binding activity validated *in vitro* by REMSA supershift assays and *in vivo* utilizing IP-RT-PCR assays (i.e., a RNA "ChIP" assay). REMSA supershift assays will also be used to determine if putative p21 binding proteins identified on the basis of molecular mass in UV-crosslinking assays, such as AUF1, are present in RNA-binding complexes.

Chapter 4 investigated the hypothesis that p21 is an effector of differentiation blockade downstream of IL-3 signals in myeloid progenitors. Work from our laboratory, and published work of others, has previously demonstrated that IL-3 inhibits G-CSF-induced granulocytic differentiation in 32Dcl3 cells[282,309]. In Chapter 3, we demonstrated that IL-3 maintains high levels of p21 expression, in part, through stabilization of its mRNA. Results from experiments utilizing siRNA mediated suppression of p21 indicate that p21 may contribute to maintenance of the myeloblast phenotype and may regulate the tightly controlled expression of primary granule proteins. The observed concurrent effect of p21 on two primary granule proteins suggests that this is a coordinated pathway. Together, our findings support a model where p21 functions at the posttranscriptional level to inhibit translation of primary granule proteins. p21 has not been reported to regulate protein translation; therefore, validation of such a model would define a novel function for p21. Our data also indicated that p21 knockdown caused an acceleration of granulocytic differentiation in 32Dcl3 cells, suggesting that p21 may antagonize differentiation at an early regulatory point in addition to causing primary granule protein suppression. Given the high expression levels of p21 reported in AML subsets, the physiologic role of p21 as an

inhibitor of normal myelopoiesis may be a mechanism that is co-opted by leukemogenic signals to promote differentiation blockade.

Validation of the inhibitory effect p21 exerts upon myeloid differentiation and the potential mechanism through which p21 is operating requires further clarification. Due to limitations of cell line experimental systems, future investigations using siRNA-mediated knockdown of p21 in primary human myeloid progenitor cells, or conditional p21 knockouts in animal models will be needed to more fully determine the role of p21 in normal myeloid differentiation. Mechanistic studies would include determining whether suppression of primary granule proteins by p21 occurs due to translational inhibition or as a result of increased protein degradation. To complement this approach, siRNA rescue experiments using p21 mutants would provide a functional mapping of p21 domains, and/or phosphorylation sites, required for primary granule protein suppression. If posttranslational modification of p21 is implicated, a broader dissection of IL-3 activated signal pathways would then be pursued.

5.2. Conclusion

Previous work from our laboratory has characterized dynamic changes in p21 protein expression and subcellular compartmentalization during myelopoiesis, suggesting that p21 plays an important role in myeloid development. The studies described in this thesis utilized the 32Dcl3 myeloblast cell line model to explore the hypothesis that p21 is an inhibitor of granulocytic differentiation. A model of our findings is presented in Figure 22. In support of our hypothesis, the data herein demonstrates that 1) p21 antagonizes survival signals provided by G-CSF in differentiating cells, 2) p21 inhibits the expression of genes associated with early myeloid differentiation, and 3) that loss of p21 accelerates granulocytic differentiation. We also

demonstrate that p21 mRNA stability is dependent upon IL-3 signaling events, providing the first evidence for IL-3-mediated stabilization of mRNA transcripts in myeloid progenitor cells (Fig. 23). Because high levels of p21 have been described in subsets of AML, our findings suggest that differentiation blockade by p21 is one mechanism that contributes to AML pathogenesis.

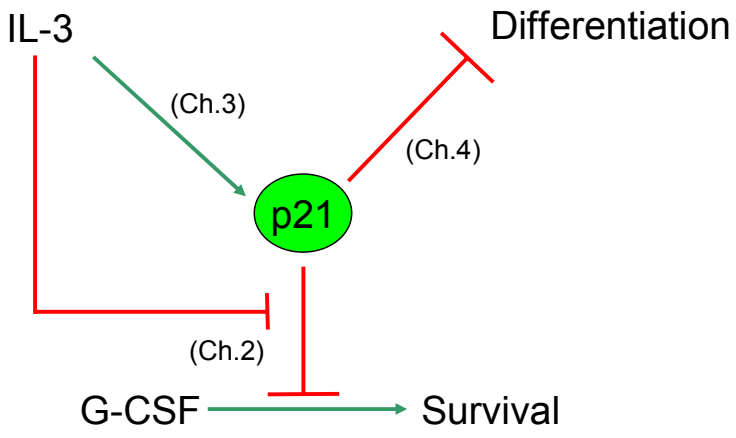

Figure 22. Role of p21 in myelopoiesis.

p21 antagonizes survival and primary granule protein expression during early myeloid differentiation. IL-3 inactivates the proapoptotic effects of p21 and maintains high levels of p21 in undifferentiated cells.

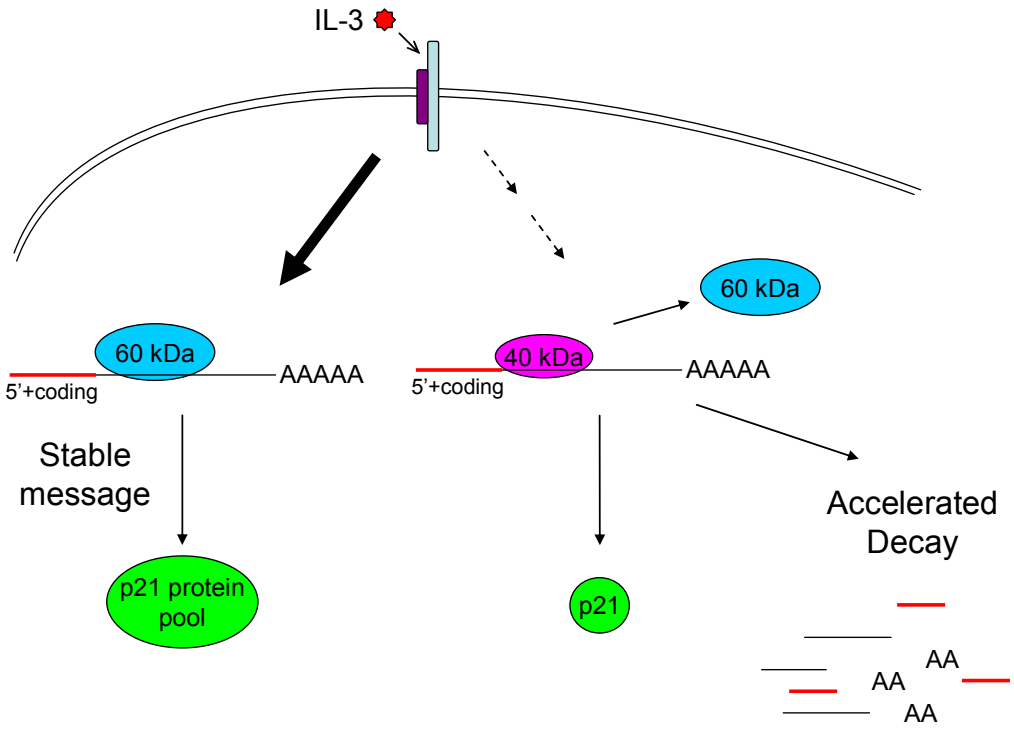

Figure 23. Proposed model of p21 mRNA stabilization by IL-3.

IL-3 activates signal pathways in 32Dcl3 cells (bold arrow) which promote stabilizing RNA:protein interactions in the p21 3'UTR leading to robust p21 protein expression. Loss of IL-3 signaling (dashed arrows) causes destabilizing RNA:protein interactions, accelerates p21 mRNA decay and results in decreased p21 protein expression.

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