Investigation of EPO-mediated rescue from p53-dependent apoptosis in DA3-EPOR cells

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Abstract

The usage of recombinant human erythropoietin in clinics to treat cancer-associated anemia has shown unfortunate unforeseen tumour response to cytokine treatment. Although other cytokines have previously been shown to have an effect on p53-dependent tumourigenesis and apoptosis *in vitro*, the effects of erythropoietin on cancer development have only recently been observed *in vivo*, of which a potential mechanism has yet to be elucidated. To determine the potential mechanism by which erythropoietin mediates the evasion of apoptosis in cells, we used the wild-type p53-expressing murine leukemic cell line DA3-EPOR and induced apoptosis via daunorubicin and doxorubicin treatment. Our findings suggest that EPO rescues cells from p53-dependent apoptosis and enhances proteasomal degradation of p53 with a concomitant decrease in miR-34a, miR-34b/c, and lincRNA-p21 expression. EPO was also observed to increase p53 recruitment to the p21 promoter followed by increased p21 expression, suggesting an orchestrated shift from p53-dependent apoptosis to cell cycle arrest.

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<u>**List of Abbreviations**</u>

ANOVA – analysis of variance

BEST – Breast Cancer Erythropoietin Trial

BIM – Bcl-2 interacting mediator of death

BTG2 – B-cell translocation gene 2

CDK – cyclin-dependent kinase

CHX – cycloheximide

DNR - daunorubicin

Dox – doxorubicin

DTT – diothiothreitol

ENHANCE – Erythropoietin in Head and Neck Cancer Trial

EPO – erythropoietin

EPOR – erythropoietin receptor

ESA – erythropoietin-stimulating agent

GENECAPP – Global ExoNuclease-based Enrichment of Chromatin-Associated Proteins for Proteomics

Grb2 – growth factor receptor-bound protein 2

HIF-2 – hypoxia-inducible factor 2

IgG – immunoglobulin G

IL – interleukin

JAK2 – Janus kinase 2

lincRNA – long intergenic non-coding RNA

MIF – macrophage inhibitor factor

miRNA – microRNA

p53RE – p53 response element

p53ts – p53 temperature-sensitive

PBS – phosphate buffer saline

pCMV – cytomegalovirus plasmid

PI3K - phosphatidylinositol-3 kinase

PIC – pre-initiation complex

PIDD – p53-induced protein with a death domain

PKB – protein kinase B

Pri-miRNA – primary microRNA

PTM – post-translational modification

PUMA – p53 upregulated modulator of apoptosis

qRT-PCR – quantitative reverse transcription polymerase chain reaction

qPCR – quantitative polymerase chain reaction

RBC – red blood cell

RPM – rotations per minute

RPMI – Roswell Park Memoriam Institute medium

rHEPO – recombinant human erythropoietin

SDS – sodium dodecyl sulphate

SEM – standard error of the mean

SH2 – src-homology 2 domain

shRNA – short hairpin RNA

STAT – signal transducer and activator of transcription

TUBE – Tandem Ubiquitin Binding Entity

1. Introduction

The evasion of apoptosis through the manipulation and circumvention of normal cellular processes is regarded as a hallmark of cancer (Hanahan and Weinberg 2000). The progression of tumourigenesis and acquisition of cancer hallmarks are typically associated with accumulation of mutations that have escaped DNA repair mechanisms. In some cases, tumours have also been found capable of utilizing factors present in their microenvironment, such as cytokines, to promote their own survival (Dranoff 2004). Erythropoietin (EPO), an erythropoiesis-stimulating cytokine, has recently been observed to promote the survival of tumours, which was discovered following EPO treatment of patients with cancer-associated anemia (Henke *et al.* 2003; Leyland-Jones 2003). We decided to take a p53 perspective in examining the mechanism by which EPO aids cancers in evading apoptosis. This is due to the well-established role of p53 in regulating apoptosis (Fridman and Lowe 2003) and the basis in which other cytokines have been found to affect p53-dependent apoptosis (Dranoff 2004). This introduction will focus on summarizing the role and interconnections between cancer-associated anemia, EPO, apoptosis, and p53.

1.1 Erythropoietin

1.1.1 Cancer-associated anemia and EPO

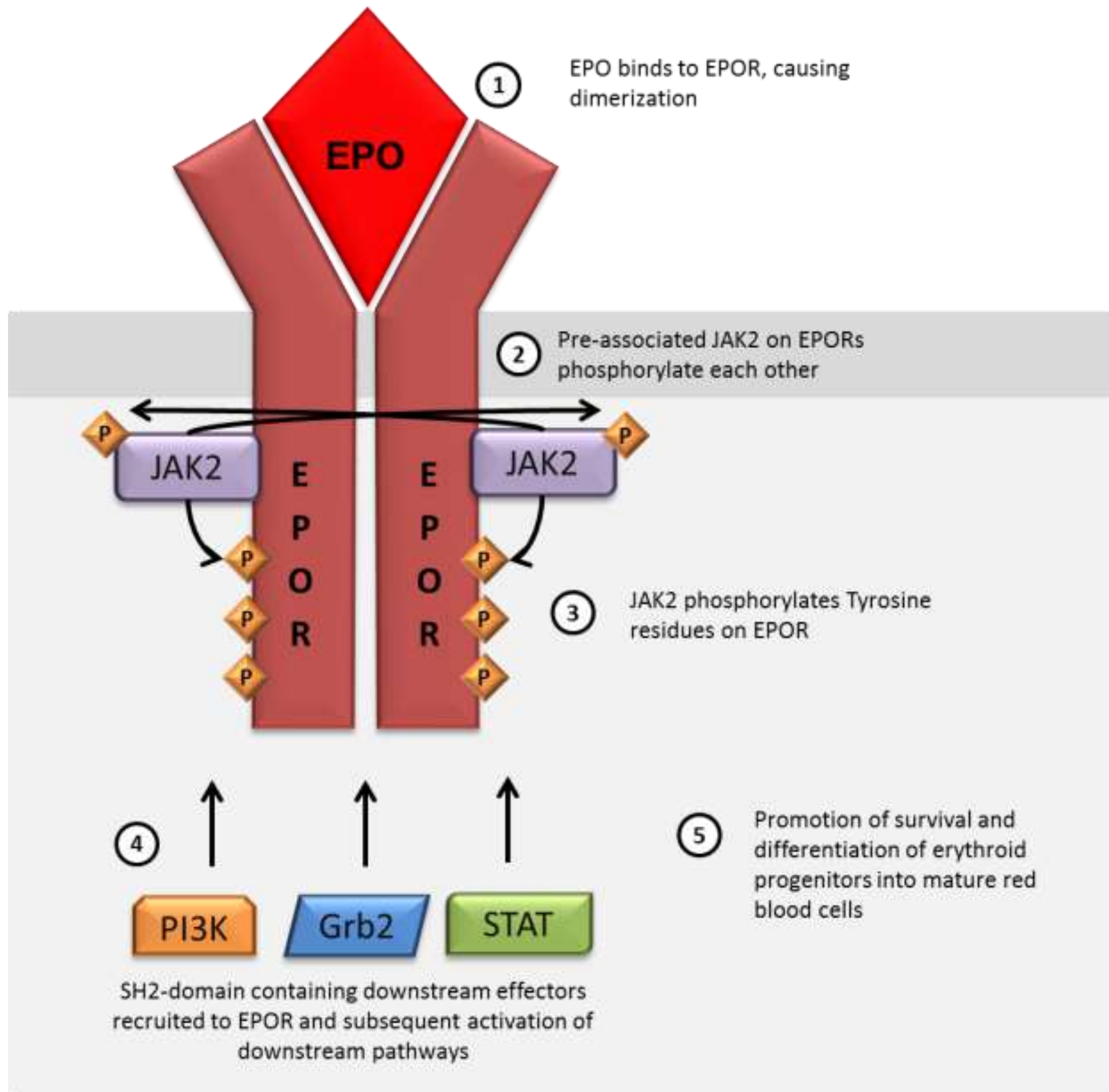
Anemia is a condition denoted as a decrease in red blood cell (RBC) volume and can be associated with the manifestation of numerous different types of illnesses, including but not limited to renal failure, dysfunctional erythropoiesis in the bone marrow, iron deficiency, sickle cell disease, and cancer (Steinberg 1989). In cancers, the origin of anemia tends to be difficult to diagnose as it may arise from both the cancer itself as well as the chosen cancer therapies. Cancer-associated anemia can result from impairment in RBC production caused by chemotherapy, hematopoietic cancers, blood loss-inducing cancers, advanced metastatic cancers, and/or vascular tumours among many other factors (Steinberg 1989). Although the causes of cancer-associated anemia may vary it is generally agreed upon that anemia in patients may greatly influence their quality of life and prognosis. In a comprehensive review of the MEDLINE database, Caro *et al*. found that in the 60 articles surveyed, there was a 20-43% reduction in median survival for cancer patients diagnosed as anemic compared with non-anemic patients across a large variety of cancer types (2001). As such, treatment of anemia in cancer patients is imperative as it has been shown to increase overall survival and quality of life (Steinberg 1989). To this end, anemic cancer patients are typically treated with recombinant human erythropoietin (rhEPO) or erythropoietin-stimulating agents (ESAs). These treatments utilize the EPO pathway to bolster normal erythropoiesis in the body and reduce the need for blood transfusions (Szenajch *et al*. 2010).

1.1.2 Erythropoiesis and EPO's signalling pathway

EPO treatment utilizes the normal erythropoiesis pathway in the body to stimulate RBC production and offset cancer-associated anemia. Normally erythropoiesis is stimulated in the body under hypoxic or anemic conditions, where a decrease in blood volume or blood oxygen levels upregulates the expression of EPO's primary transcription factor hypoxia-inducible factor 2 (HIF-2). HIF-2 then subsequently stimulates the production of EPO by the kidney. In the fetus however, EPO is produced from the liver and is later produced primarily in the kidney as an adult, although it can still be produced minutely in the adult liver (Kapitsinou *et al.* 2010).

EPO is released into the bloodstream where it travels until it reaches cells expressing an EPO-receptor (EPOR). EPORs are normally found on the cell surface of erythroid progenitors in the bone marrow and are lost upon differentiation into mature RBCs. To a lesser extent, EPORs have also been found expressed in the brain, heart, and kidney tissues, and are believed to have a tissue protective effect (Chateauvieux *et al*. 2011; Jelkmann *et al*. 2010). Binding of EPO to an EPOR on erythroid progenitors in the bone marrow causes dimerization of the EPOR molecules which then triggers a conformational change in the receptors' extracellular domain. EPOR does not have intrinsic kinase activity of its own and downstream signalling from EPOR depends on the activation of pre-associated Janus kinase 2 (JAK2). The conformational change in EPOR activates pre-associated JAK2 via an auto-phosphorylation event. Activated JAK2 then phosphorylates tyrosine domains on the cytoplasmic region of EPOR (Witthuhn *et al*. 1993). This phosphorylation event recruits various Src homology-2 domain-containing (SH2) downstream effectors to the activated EPOR homodimer. A few of the effectors recruited include members of the signal transducer and activator of transcription (STAT) family, phosphatidylinositol-3 kinase (PI3K) and subsequently protein kinase B (PKB), and the adaptor

protein growth factor receptor-bound protein 2 (Grb2) which activates the RAS/RAF pathway (Bouscary *et al.* 2003; Constantinescu *et al.* Haq *et al.* 2002; Socolovsky *et al.* 2001). Activation of these downstream effectors leads to upregulation of various regulated genes, such as B-cell lymphoma-extra-large (Bcl-xL), and downregulation of other genes including caspase-3 (Gregory *et al.* 1999; Zermati *et al.* 2001). As result, the EPOR pathway promotes the survival and differentiation of erythroid progenitors into mature RBCs (Ghezzi and Brines 2004; Iwatsuki *et al*. 1997) (Figure 1). The usage of rhEPO and ESAs has since become an essential component to the treatment regimen of cancer patients suffering from anemia.



<u>Figure 1: EPOR signalling pathway in erythroid progenitor cells</u>. 1) Erythropoietin binds to its EPO-receptor causing dimerization. 2) Dimerization of EPOR bring pre-associated JAK2 into closer proximity with each other, leading to auto-phosphorylation of JAK2. 3) JAK2 then phosphorylates tyrosine residues on the cytoplasmic domain of EPOR. 4) SH2-domain containing downstream effectors, such as PI3K, Grb2, and the STAT family, are recruited to EPORs' phosphorylated tyrosines. 5) Downstream signalling of the PI3K, Grb2/RAS/RAF, and STAT pathways results in promotion of survival and differentiation of erythroid progenitors into mature red blood cells.

1.1.3 EPO and cancer

In 2003 the first documented cases of an *in vivo* tumour response to EPO treatment were observed, resulting in significant changes to how EPO was to be prescribed to anemic cancer patients in future. In The Breast Cancer Erythropoietin Trial (BEST), a higher mortality rate was found in patient groups treated with chemotherapy and epoietin, a rhEPO, compared with groups treated only with chemotherapy (Leyland-Jones 2003). Similarly, patients pre-treated with epoietin in the Erythropoietin in Head and Neck Cancer trial (ENHANCE) along with radiation therapy to prevent cancer-associated anemia fared worse than patients who were not pre-treated (Henke *et al.* 2003). There have also been a number of reports linking usage of ESAs in conjunction with radiation therapy being especially detrimental to patients' overall survival (Machtay *et al*. 2007; Shenouda *et al*. 2015). EPOR expression in cancers has since been linked to aggressive tumour behaviour, risk of thromboembolism, increased angiogenesis in cancers, and drug resistance, raising concerns about EPO's continued usage in cancer-associated anemia treatment (Farrell and Lee 2004; Lin *et al.* 2012; Zhang *et al.* 2012).

However there is still a lack of consensus on whether EPOR expression is present and functional in many cancers, with some studies reporting cancers expressing a non-functional EPOR or having no response to EPO treatment despite functional EPOR expression (Radwan *et al.* 2016; Senger *et al.* 2016; Bennet *et al.* 2016). There have also been a number of clinical trials that have found no difference in overall survival between cancer patients treated with ESAs compared with control groups (Aapro *et al.* 2008; Crawford *et al.* 2007; Grote *et al*. 2005). In response to the reports indicating a potential detrimental effect of ESA and rhEPO treatment of cancer-associated anemia in patients, many professional organizations have issued revised guidelines to clinicians on how to evaluate when cancer-associated anemia can be treated with

EPO. These guidelines emphasize a full risk-assessment of the necessity of palliative EPO treatment for only chemotherapy-treated cancer patients in addition to continued evaluation of patient haemoglobin levels in case of potential tumour response (Rizzo *et al*. 2010).

Despite the conflicting results on the role of EPO in cancer treatment, it remains clear that EPOR's role in cancer development and progression is still uncertain and more research is required to further elucidate how some cancers may be utilizing the EPOR pathway. Studies have since mainly focused on the following: new methods to detect EPOR expression and function, observing for EPO-induced phenotypic changes in conjunction with drug treatments and delineating a possible mechanism if a change is observed, clinical studies observing for effects of EPO treatment, and determining relative expression and function of EPOR in cancers (Fuge *et al.* 2015; Jin *et al.* 2015; Maxwell *et al.* 2015; Park *et al.* 2014).

1.2 P53

1.2.1 p53 and cancer

P53 is a tumour suppressor whose importance in guarding against tumourigenesis is undisputed. There are three main lines of evidence that support p53's classification as a tumour suppressor gene: frequent loss of p53 function through mutation in cancers, germline p53 mutations present in families with Li-Fraumeni Syndrome, and susceptibility of p53-null mice to tumour development. Somatic p53 mutations occur in a large majority of cancers, with prevalence of over 40% in 8 out of 29 cancer types covered by the datasets from the IARC TP53 Database and TCGA/ICGC Database as of 2016 (Bouaoun *et al.* 2016). In cancers with a low rate of p53 mutation, wild-type p53 (WTp53) expression can be circumvented via increased degradation (Kubbutat *et al.* 1997). Inherited germline p53 mutations, characterized as Li-Fraumeni Syndrome, predispose their carriers to a higher risk of cancer development (Li and Fraumeni 1969). Homozygous p53 null mice are highly susceptible to tumour development, with tumours developing on average within 45 weeks with 100% penetrance (Donehower 1996). The propensity in which mice and humans expressing mutated p53 develop tumours owes in part to p53's function as a transcription factor of genes associated with cell cycle arrest, senescence, and apoptosis. Correspondingly, p53 mutations are most commonly missense mutations located within the DNA-binding domain of p53, resulting in an inability of p53 to bind to DNA and function as a transcription factor (Petitjean *et al.* 2007).

1.2.2 p53 function as a transcription factor

P53 is capable of suppressing tumour development primarily through its function as a mediator of cell response to stress signals. Normally p53 protein is maintained at low levels in the cell through ubiquitylation by E3 ubiquitin ligases which mark p53 for proteasomal degradation (Kubbutat *et al.* 1997; Rodriguez *et al.* 2000). In response to cell stresses, such as DNA damage, nutrient starvation, telomere erosion, and hypoxia, p53 undergoes significant post-translational modifications leading to its stabilization. Stabilized p53 proteins form a tetramer and bind to promoters at a p53 response element (p53RE) sequence of PuPuPuC(A/T)(A/T)GPyPyPy N{0,13} PuPuPuC(A/T)(A/T)GPyPyPy. Binding of p53 to target gene promoters stimulates transcription through recruitment of transcriptional activators (El-Deiry *et al*. 1992). Depending on the degree and type of stress, p53 is preferentially recruited to promoters of genes associated with apoptosis or cell cycle arrest. Upregulation of p53 target genes thus activates downstream pathways that allow for prevention/repair of damaged cells during cell cycle arrest or removal of damaged cells via apoptosis. P53’s stabilization and function as a transcription factor has been summarized in brief in Figure 2.

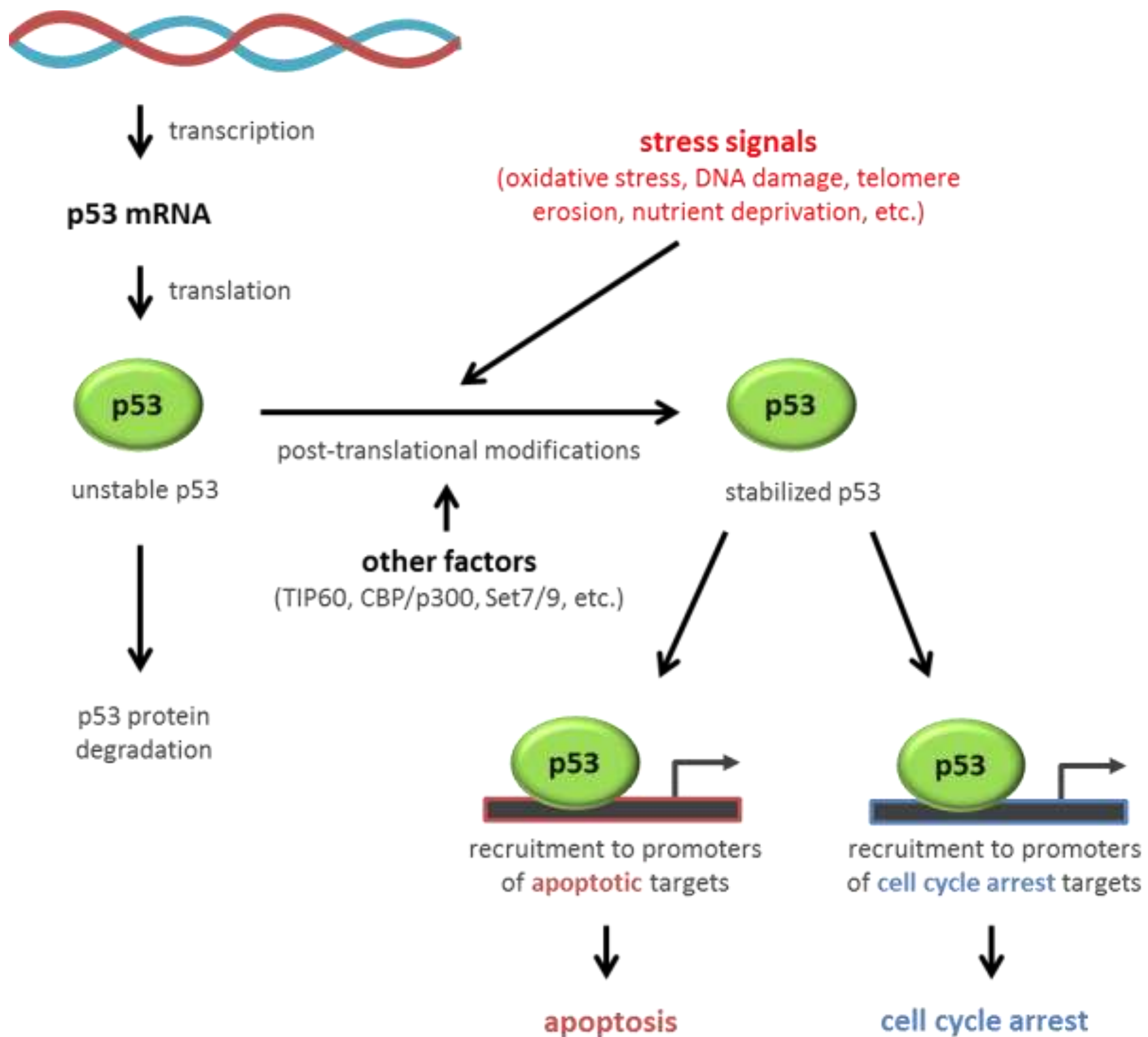


Figure 2: P53's function as a transcription factor. P53 protein is maintained at low steady-state levels via constant protein degradation. In response to stress signals, p53 undergoes post-translational modification which leads to its stabilization and tetramerization. Stabilized p53 is recruited to promoters of apoptotic and cell cycle arrest targets containing a p53 consensus sequence. Binding of p53 results in upregulation of transcription and activation of downstream pathways associated with cell cycle arrest or apoptosis.

1.2.3 p53 stabilization, activation, and promoter recruitment

P53 undergoes many post-translational modifications (PTMs) in response to stress stimuli, leading to its stabilization and activation (Kruse and Gu 2009). Some post-translational modifications that have been found associated with p53 include acetylation, sumoylation, phosphorylation, neddylation, and methylation. Different stresses result in different modifications to p53 and allow for the preferential recruitment of p53 to promoters of genes involved in apoptosis and cell cycle arrest (Meek and Anderson 2009).

A large component of the stabilization of p53 involves the uncoupling of the association between p53 and E3 ubiquitin ligases which target p53 for degradation. One of the most extensively studied PTM pathways involved in this process are the ataxia telangiectasia mutated-checkpoint kinase 2 (ATM-Chk2) and ataxia telangiectasia-and RAD3-related-checkpoint kinase 1 (ATR-Chk1) pathways which respond to doubled stranded and single stranded DNA breaks respectively. Upon DNA damage, ATM and/or ATR phosphorylate mouse double minute homolog-2 (MDM2), an E3 ubiquitin ligase that targets p53, at various serine and threonine residues located near its C-terminal domain (CTD). Phosphorylation of these sites on MDM2 results in inhibition of MDM2 and its ability to ubiquitinate p53. ATM and ATR also phosphorylate Chk2 and Chk1 respectively, which in turn phosphorylate residues on p53 that help to retain it in the nucleus and contribute to disassociation of p53 from MDM2 (Smith *et al.* 2010). Additionally, $p14^{ARF}$, an inhibitor that binds to MDM2 and blocks its ubiquitin ligase function, is frequently mutated in cancers and is one of the mechanisms by which cancers circumvent WTp53 expression (Bates *et al.* 1998).

PTMs and cofactors associated with p53 work in concert to not only activate and stabilize p53, but to facilitate its recruitment to promoters. Phosphorylation of p53 by ATM/ATR has been found to serve as a nucleation event that promotes the recruitment of histone/lysine acetyltransferases, such as p300 and CREB-binding protein (CBP), to p53. Acetylation of p53, particularly at its CTD, is believed to help stabilize p53 by blocking ubiquitylation of the CTD and contribute to uncoupling p53 from MDM2 (Meek and Anderson 2009; Sakaguchi *et al.* 1998). These modifications are also thought to contribute to p53 association with response elements on target genes. Tip60, also known as histone acetyltransferase KAT5, has been shown to acetylate p53 at K120, which is essential for p53-dependent apoptosis but is dispensable for cell cycle arrest (Tang *et al*. 2006). Methylation of p53 by methyltransferase Set7/9 was also found to mediate the acetylation of p53 by Tip60, however methylation was not required for p53-mediated cell cycle arrest and apoptosis (Campaner *et al.* 2011; Kurash *et al.* 2007). These PTM interactions represent only a few of numerous cofactors and modifications that act on p53, but display the intricacy in which p53 is regulated. Analysis of p53 modifications via mass spectrometry revealed over 150 possible PTMs present on p53, suggesting that the PTMs that govern p53 stability, activation, and promoter recruitment are very complex (DeHart *et al.* 2013).

Cofactors and PTMs that act on p53 not only aid in its recruitment to promoters but also act on the promoters themselves to help upregulate gene transcription. P53-mediated recruitment of histone acetyltransferases p300/CBP and Tip60 serve to acetylate histones within the vicinity of p53REs, which relaxes chromatin structure and allows for active transcription to occur. P53 is also capable of recruiting components of the pre-initiation complex (PIC) to the promoter of target genes, allowing for initiation of transcription (Beckerman and Prives 2010). Altogether this highlights the complexity of p53's function as a transcription factor and the network of

PTMs and cofactors involved in its stabilization, activation, and recruitment to promoters (Figure 2).

1.2.4 p53 degradation

Under normal conditions p53 protein is maintained at low levels via constant degradation and only upregulated upon cell stress. In cancers, enhanced WTp53 degradation via ubiquitylation and proteasomal degradation may allow for circumvention of WTp53 and subsequent progression in tumourigenesis. Considering p53's role in regulating cell cycle arrest and apoptosis in response to stress, post-translational modifications associated with p53 that govern its stability and degradation are highly important to suppressing tumourigenesis.

The most characterized interaction involved in the control of p53 degradation is its ubiquitylation by E3 ubiquitin ligases. Ubiquitylation involves the conjugation of ubiquitin to a protein. Depending upon the degree of ubiquitylation, proteins can be marked for proteasomal degradation (poly-ubiquitylated) or nuclear export (mono-ubiquitylated), amongst other protein fates (Lohrum *et al.* 2001; Rodriguez *et al.* 2000). MDM2 is the first and most well-known E3 ubiquitin ligase discovered to target p53 (Kubbutat *et al*. 1997). Since MDM2's discovery, several other E3 ligases have been reported, including but not limited to mouse double minute 4 homology (MDM4) and p53-induced RING-H2 domain protein (Pirh2) (Chao 2015; Leng *et al.* 2003). MDM2 is capable of binding to p53 and ubiquitylating lysines K370, K372, K373, K381, K382, and K386 located at p53's CTD (Lohrum *et al.* 2001). Poly-ubiquitylation of these sites marks p53 for degradation by the proteasome. Notably it was observed that a p53 mutant expressing Lysine to Arginine mutations at the 6 targeted lysines (p53-6KR) showed no increase in stability, suggesting that although important for degradation, the ubiquitination of these

lysines may not be essential for p53 degradation (Feng *et al.* 2005). MDM2 is also transcriptionally regulated by p53, resulting in a negative feedback loop. This auto-regulatory loop allows for upregulated p53 levels to return to homeostatic levels after a stress response (Wu *et al.* 1993).

There has been interest in developing drugs that target interactions involved with WTp53 degradation in cancers, allowing for rescue of WTp53 expression. Two such drugs are proteasomal inhibitors and E3 ubiquitin ligase inhibitors like MG-132 and nutlin-3 respectively. MG-132 is a proteasomal inhibitor which targets the 20S proteasome subunit, whereas nutlin-3 is a cis-imidazoline analog which inhibits the interaction between MDM2 and p53, thus stabilizing p53 (Guo and Peng 2013; Vassilev *et al.* 2004).

1.2.5 p53 and its coding and non-coding RNA network

Since its discovery, the p53 network has expanded extensively to include genes not only associated with apoptosis and cell cycle arrest, but implicated also in metabolism, angiogenesis, and fertility. A large number of p53 target genes however, are involved in cell cycle arrest (ex. p21, B-cell translocation gene 2 (BTG2)) and apoptosis (ex. p53 upregulated modulator of apoptosis (PUMA), Noxa, p53-induced protein with a death domain (PIDD)) (Vogelstein *et al*. 2000). P53's transcriptional activation of p21 in particular, a cyclin-dependent kinase (CDK) inhibitor, is a well-known target involved in the p53-dependent mediation of G1-arrest. P21 inhibits the activity of cyclin-CDK complexes cyclin-CDK1, cyclin-CDK2, and cyclin-CDK4/6 and allows for regulation of cell cycle progression (Abbas and Dutta 2009). BTG2 has also been found to contribute to arrest at the G1-S checkpoint via inhibition of cyclin D1 transcription (Guardavaccaro *et al.* 2000). In terms of apoptotic genes, PUMA and Noxa are both Bcl-2 homology domain (BH3)-only protein family members and indirectly inhibit Bcl-2 family members Bax and/or Bak to promote mitochondrial membrane permeabilization and consequently apoptosis (Ploner *et al.* 2008; Yu and Zhang 2008). PIDD's role by contrast, still remains elusive, but has been implicated with activation of caspase-2 (Bock *et al.* 2012).

In addition to the numerous coding RNA targets that p53 regulates, a number of non-coding RNAs (ncRNAs) have been recognized as being part of the p53 network. NcRNAs are capable of regulating gene expression but do not code for a specific protein (Acunzo *et al*. 2015). MicroRNAs (miRNA) are a small class of ncRNAs (~22nt) that have differential effects on gene regulation through imperfect binding to a complementary sequence located in the 3'UTR of a target mRNA. This typically results in the inhibition of translation or degradation of the mRNA target. In 2007, multiple groups independently identified members of the microRNA-34 (miR-

34) family as being regulated by p53. MiR-34a in particular has been found to have differential context-dependent effects when overexpressed, ranging from cell cycle arrest to apoptosis (Bommer *et al*. 2007; Chang *et al*. 2007; He *et al*. 2007; Raver-shapira *et al*. 2007; Tarasov *et al*. 2007). MiR-34b/c, another member of the miR-34 family, shares similar target mRNAs with miR-34a and has also been implicated with cell cycle arrest, apoptosis, and metabolism (Corney *et al.* 2007). Deregulated expression of miRNA is frequently reported in cancer, resulting in an interest in determining the role of miRNAs in tumorigenesis (Acunzo *et al*. 2015). P53 has also been found to regulate the expression of long intergenic ncRNAs (lincRNAs), which are >200 nucleotides in length and are also implicated in the regulation of gene expression. In 2010 lincRNA-p21 was discovered and found to be located 15kb upstream of the p21 gene. Reports on lincRNA-p21's function however, have been mixed, with studies showing conflicting reports on lincRNA-p21's function involving either apoptosis or cell cycle arrest (Dimitrova *et al.* 2014).

1.2.6 p53, cytokines, and apoptosis

Apoptosis is governed by the complex integration of both survival and death signals. Growth signals such as EPO promote the evasion of apoptosis in part by coordinating the upregulation of survival signals (Dranoff 2004). Considering EPO has been found to aid cancer cells in evasion of apoptosis when treated with chemotherapeutic drugs, our lab focused on studying the possible intersection between EPOR signal transduction and p53-dependent apoptosis. Previously our lab has found that EPO is capable of rescuing DP16.1/p53ts cells from p53-dependent apoptosis with a shift towards G1-arrest and a concomitant increase in p21 levels. DP16.1/p53ts cells express a temperature-sensitive p53 (p53ts) mutant that adopts a mutant conformation at 37°C but wild-type (WT) conformation at 32°C. These cells also express the spleen focus-forming virus-encoded env-related glycoprotein gp55 that is capable of binding and activating EPOR, thus mimicking the natural EPO/EPOR signalling pathway (Brown and Benchimol 2006; Johnson and Benchimol 1992; Lin *et al.* 2002). Quelle *et al.* reported similar findings in WTp53 expressing 32D and DA3-EPOR cells, with EPO showing capability of rescuing cells from apoptosis after gamma-irradiation which required JAK2 but not PI3K activation (1998).

Similar to EPO, other cytokines such as the interleukin (IL) family and macrophage inhibitor factor (MIF) have also been found to have a role in rescuing cells from p53-dependent apoptosis. Interleukins are cytokines produced by leukocytes and are involved in regulation of immune responses. IL-6 treatment has been connected to enhanced p53 protein degradation by MDM2 via increased ribosomal RNA transcription which reduced the availability of ribosome proteins for MDM2 binding (Brighenti *et al.* 2014). IL-7 was also observed to decrease p53-dependent apoptosis in A549 and human bronchial epithelial cells through upregulation of Bcl-2 and downregulation of Bax expression (Liu *et al.* 2014). Decreased p53 expression and inhibition of

apoptosis has also been observed in irradiated B lymphoma cells in the presence of IL-17 (Li *et al*. 2015). MIF treatment was also observed to suppress p53 binding to p53-responsive luciferase promoters, overcome p53-mediated apoptosis and cell cycle arrest induced by doxorubicin (Dox) and estradiol respectively, and decrease p53 protein levels (Brock *et al.* 2014; Hudson *et al*. 1999). These findings represent only a few of the many reports linking cytokines with the inhibition of p53-dependent apoptosis, and underscore the importance of understanding the contribution of survival signalling to the evasion of apoptosis in cancers.

1.3 Thesis rationale

The current work focuses on further examining EPO's ability to rescue cancer cells from stress-induced apoptosis in the DA3-EPOR cell line and its effects on the p53 network. DA3-EPOR is a WT p53 expressing murine leukemic cell line expressing EPOR obtained from Dwayne Barber at the Princess Margaret Hospital and serves as an appropriate system to observe EPO's involvement with WT p53 in a more physiological context (Miura *et al.* 1991). Although much work has been done to elucidate the mechanism of EPO enhancement of tumourigenesis, there exists little in the literature connecting EPO to p53-dependent apoptosis. Other cytokines have been examined more extensively in terms of their roles associated with p53-dependent apoptosis and may serve as support to there being a potential connection between EPO and the p53 network. The objective of this research project was determine if EPO is capable of rescuing cells from p53-dependent apoptosis induced by daunorubicin (DNR) and doxorubicin (DOX) in the more physiologically-relevant DA3-EPOR system via a p53-dependent pathway. Further observations on EPO's effect on the p53 network and p53 itself may serve to elucidate the mechanism by which WTp53 leukemic cells evade apoptosis in the presence of EPO.

2. Materials and methods

2.1 Cell culture

DA3-EPOR murine leukemic cells were maintained at 37°C with 5% CO_2 in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 1 U/mL recombinant human erythropoietin (EPO). DA3-EPOR/p53DD cells were established via nucleofection of DA3-EPOR cells with a pCMV vector containing p53DD, kindly provided by Dr. Moshe Oren. For treatment, cells were washed with RPMI 1640 with 10% FBS and incubated at 37°C for one hour without EPO. Cells are then either treated with 0.25 μM DNR, 0.25 μM Dox, and/or 1 U/mL recombinant human EPO. Cell viability was assessed routinely via exclusion of trypan blue. For inhibitor treatments, MG-132 (Sigma-Aldrich Canada Ltd., Toronto, ON, Canada) and cycloheximide (Sigma-Aldrich Canada Ltd., Toronto, ON, Canada) were added directly to culture media and harvested at appropriate timepoints.

2.2 Nucleofection

Nucleofection of DA3-EPOR cells were performed according to the instructions provided by the manufacturer (Lonza). $2x10^6$ DA3-EPOR cells were resuspended in Cell Line Nucleofector Solution V and combined with 2 μg of plasmid vector comprised of a 9:1 ratio of pCMV/p53DD plasmid (Bowman *et al.* 1996) to pSUPER-puromycin. Cells were then pulsed with the Nucleofector X-001 program using a Nucleofector 2b device kindly provided by Dr. Peter Cheung. Pulsed cells were transferred to pre-warmed RPMI1640 media containing 10% FBS and 1 U/mL EPO and allowed to recover for 48 hrs. 19 clones were then isolated for puromycin selection over 4 weeks (1 μg/mL, Sigma-Aldrich Canada Ltd., Toronto, ON, Canada). Clones were subjected to immunoblotting and flow cytometry to validate for p53DD expression after 4

weeks. Clones #16, 18, and 19 were selected for continued experimentation due to their high expression of p53DD.

2.3 Cell cycle analysis

Cells were pelleted and washed with PBS and fixed in cold 70% ethanol before freezing at -20°C until use. Fixed cells were washed with PBS at room temperature and resuspended in Staining Buffer (0.2% Triton X-100, 1 mM EDTA, in PBS) followed by incubation with 50 µg/mL RNaseA (ThermoScientific) at 37°C for 30 minutes. Cells were then stained with 50 µg/mL propidium iodide per treatment for one hour. Propidium iodide fluorescence was measured using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and data analysis was performed using the BD CellQuest Pro software.

2.4 Western blotting

Cells were directly lysed in 1X SDS lysis buffer (10% SDS, 10% glycerol, 88 mM Tris-HCl pH 6.8, in water) and boiled at 97°C for 10 minutes followed by addition of 0.1% bromophenol blue and 0.1M DTT. Cell lysates were re-boiled and allowed to cool before loading onto SDS-polyacrylamide gels and subjected to electrophoresis. Gels were transferred onto nitrocellulose membranes and incubated with the appropriate antibodies. The following primary antibodies were used in this study: p21 (Santa Cruz Biotechnology, Santa Cruz, CA), PAb421 which recognizes the c-terminal domain of p53 (Harlow *et al.* 1981), FL393 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Protein quantification was measured using a Typhoon Trio scanner (GE Healthcare). The following secondary antibodies were used in this study for protein quantification using a phosphoimager: murine and rabbit anti-Cy 3 and anti-Cy5. Protein loading was assessed using Coomassie blue staining of polyacrylamide gels after transfer and β-actin quantification.

2.5 Cycloheximide treatment

After treatment of cells with DNR and/or EPO, 40 μg/mL cycloheximide (Sigma-Aldrich) was added directly to culture media. Cells were harvested at 0 minutes, 30 minutes, 60 minutes, 120 minutes, and 240 minutes after cycloheximide addition and subjected to immunoblotting. Relative p53 and β-actin protein expression was quantified using a Typhoon Trio (GE Healthcare) and ImageJ software. Relative p53 expression was normalized to β-actin levels and plotted relative to initial p53 levels at 0 minutes after cycloheximide addition for each individual treatment.

2.6 Quantitative Real-Time PCR

Template RNA was prepared from treated cells using TRIzol Reagent (Life Technologies) and synthesized into cDNA according to the manufacturer's instructions using the High Capacity RNA-to-cDNA Kit (ThermoFischer Scientific).

Template RNA was mixed with RNAse/DNase-free water and RevoScript RT Premix tubes and subjected to cDNA synthesis using an Applied Biosystems GeneAmp PCR System 9700 (ThermoFischer Scientific). cDNA synthesis was performed at 50°C for 60 minutes and RTase inactivation at 95°C for 5 minutes. Prepared cDNA was stored at -20°C until use and 0.5 μM of cDNA was used per qRT-PCR reaction. SsoFaster EvaGreen Supermix Kits and an Applied Biosystems 7500 Real-Time PCR System (ThermoFischer Scientific) were used according to the manufacturer's instructions. Obtained Ct values were normalized to the endogenous control 18S using the Pfaffl method (Pfaffl 2001). qRT-PCR products were validated via northern blots for appropriate product size (data not shown).

Primer efficiencies were evaluated and calculated prior to use in qRT-PCR. The following oligonucleotides were used as qRT-PCR primers: *Pri-miR-34a* (He *et al.* 2007), F 5'

CTG TGC CCT CTT GCA AAA GG 3’, R 5’ GGA CAT TCA GGT GAG GGT CTT G 3’; *Pri-miR-34b/c*, F 5’ CTC GGT TTG TAG GCA GTG TA 3’, R 5’ TTG ATG GCA GTG GAG TTA GTG 3’; *lincRNA-p21*, F 5’ CAT TCC GTC TCC AGT TCC TAA C 3’, R 5’ CGA AGA GAC AAC GGC ACA CTT 3’; *PIDD*, F 5’ TCC AGC AAG ATG TGA GCT TAT G 3’, R 5’ GGT CAT TCC AGG TGT TGT TAC T 3’; *PUMA*, F 5’ CGG AGA CAA GAA CC AGC AAG ATG TGA GCT TAT G 3’, R 5’ GGT CAT TCC AGG TGT TGT TAC T 3; *NOXA*, F 5’ TCG CAA AAG AGC AGG ATG AG 3’, R 5’ CAC TTT GTC TCC AAT CCT CCG 3’; *p21*, F 5’ CCA GAC ATT CAG AGC CAC AGG 3’, R CGA AGA GAC AAC GGC ACA CTT 3’; *p53*, F 5’ TAG GTA GCG ACT ACA GTT AGG G 3’, R 5’ CAT GGC AGT CAT CCA GTC TT 3’; *18S*, F 5’ GTG TTG AGG AAA GCA GAC AT 3’, R 5’ CAG TCT GGG ATC TTG TAC TG 3’.

2.7 ChIP-qPCR

Treated cells were cross-linked through addition of formaldehyde to a final concentration of 1% and incubated at room temperature for 8 minutes, followed by addition of 0.125 M glycine for 5 minutes at room temperature. Cells were pelleted at 1200 RPM at 4°C for 8 minutes and washed with ice cold Phosphate-Buffered Saline 1X (Corning), followed by washes with Wash Buffer I (0.25% Triton X-100, 10 mM EDTA pH 8.0, 0.5 mM EGTA pH 7.5, 10 mM Hepes pH 7.5, protease inhibitor cocktail tablet (Roche)) and Wash Buffer II (12.5 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 7.5, 10 mM Hepes, protease inhibitor cocktail tablet), and resuspension in Lysis buffer (0.15 M NaCl, 25 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 1% Triton X-100, 0.1% SDS, 0.5% Sodium deoxycholate, protease inhibitor cocktail tablet) respectively. Resuspended cells were sonicated to a size between 100-400bp using a Sonic Dismembrator Model 500 (ThermoFischer Scientific) kindly provided by Dr. Robert Tsushima. Sonicated cells were then

centrifuged at 14000 RPM at 4°C to pellet cell debris. Cell lysates were pre-cleared with pre-washed Protein-A agarose beads (Bioshop Canada Inc.) for 1 hour at 4°C.

1 mg of protein from pre-cleared cell lysates were then incubated with 2 µg of either FL393 or IgG antibody at 4°C overnight. 40 µg of protein from pre-cleared cell lysates were set aside for use as input. Cell lysates were incubated with 40 µL of prewashed Protein-A agarose beads for 1 hour at 4°C and then pelleted at 4000 RPM for 5 minutes at 4°C. Protein-Agarose bead complexes were washed with RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% SDS, 0.5% Sodium deoxycholate, 1% NP-40, protease inhibitor cocktail), High Salt buffer (0.5 M NaCl, 50 mM Tris pH 8.0, 0.1% SDS, 1% NP-40, protease inhibitor cocktail), LiCl buffer (0.25 M LiCl, 50 mM Tris pH 8.0, 0.5% Sodium deoxycholate, 1% NP-40, protease inhibitor cocktail), and TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, protease inhibitor cocktail). Washed beads were then incubated with IP elution buffer (2% SDS, 10 mM DTT, 0.1 M $NaHCO_3$) for 30 minutes at room temperature. Eluted protein complexes were collected and boiled at 65°C overnight after addition of 20 µL of 4 M NaCl to reverse crosslinks.

1 µL of 10 mg/mL RNAseA was added to each sample and incubated at 37°C for 30 minutes. 20 µL of 10X proteinase K buffer (0.1 M Tris-HCl pH 8.0, 0.05 M EDTA pH 8.0) and 20 µL of 20 mg/mL proteinase K was added to each reaction and incubated at 42°C for 1 hour. DNA was then separated from each reaction via phenol-chloroform extraction followed by precipitation via incubation at -80°C with 1/10 reaction volume of 3 M NaOAC pH 5.2, 2X reaction volume of cold 95% ethanol, and 1 µL of glycogen per reaction. Samples were pelleted at 14000 RPM for 15 minutes at 4°C and DNA pellets were washed with 80% ethanol and stored in RNAse/DNAse free water at -80°C until use.

ChIP DNA samples were subjected to quantitative real-time PCR using SsoFaster EvaGreen Supermix Kits and an Applied Biosystems 7500 Real-Time PCR System (ThermoFischer Scientific) according to the manufacturer's instructions. Obtained Ct values were normalized to non-IP input samples for each treatment and expressed as enrichment compared with input (%input). Primer efficiencies were evaluated and calculated prior to use in ChIP-qPCR.

The following oligonucleotides were used as ChIP-qPCR primers: *miR-34a*, F 5' CCA CTT TTT CTT CCC AGG TG 3', R 5' CCC CAA TCT GTG CAG TTA CC 3'; *miR-34b/c* (He *et al*. 2007), F 5' GTT GAT CCT GCC CAC AGT TAC TAG A 3', R 5' ATT AAA ACA TGA GTC TCC CTG GTC TCT 3'; *p21*, F 5' ACC AGC AGC AAA ATC GGA GC 3', R 5' CCC ACA GCT GGT AGT TGG GTA TC 3'.

2.8 Data analysis

Statistics were performed using Microsoft Excel and SPSS software.

3. Results

3.1 EPO rescues DA3-EPOR cells from DNR and Dox-induced apoptosis

Previously the Benchimol Lab has observed that EPO is capable of rescuing DP16.1/p53ts cells from p53-dependent apoptosis (Lin *et al.* 2002). To determine whether EPO is also capable of rescuing cancer cells from apoptosis in a more physiological system, DA3-EPOR cells were treated with DNR, Dox, with or without EPO for 16 hours and assessed by flow cytometry after PI staining. Apoptotic cells appear as a population of cells with < 2 N DNA content (sub-G1 population). Under normal conditions, DA3-EPOR cells are cultured in media containing EPO and display low Sub-G1 population levels of 3.56% (Figure 3). Under genotoxic stress, cells treated with Dox or DNR displayed an increase in Sub-G1 population levels of 38.76% and 67.46% respectively (Figure 3). In the presence of EPO, cells showed a significant decrease in Sub-G1 population levels despite Dox ($p=0.0050$) and DNR treatment ($p=0.0025$). Sub-G1 population levels of Dox and DNR treated cells in the presence of EPO compared with Dox and DNR treatments alone were decreased by -0.90 and -0.89 fold respectively (Figure 3A). Representative cell cycle profiles from one replicate in Figure 3A can be observed in Figure 3B. These data suggest that EPO's ability to rescue cells from apoptosis is not restricted to DP16.1/p53ts cells. For the following experiments we chose to focus on DNR treatment as the Sub-G1 population levels and EPO rescue observed for DNR treatment was more robust, and served as a more appropriate system to further investigate the mechanism of EPO rescue.

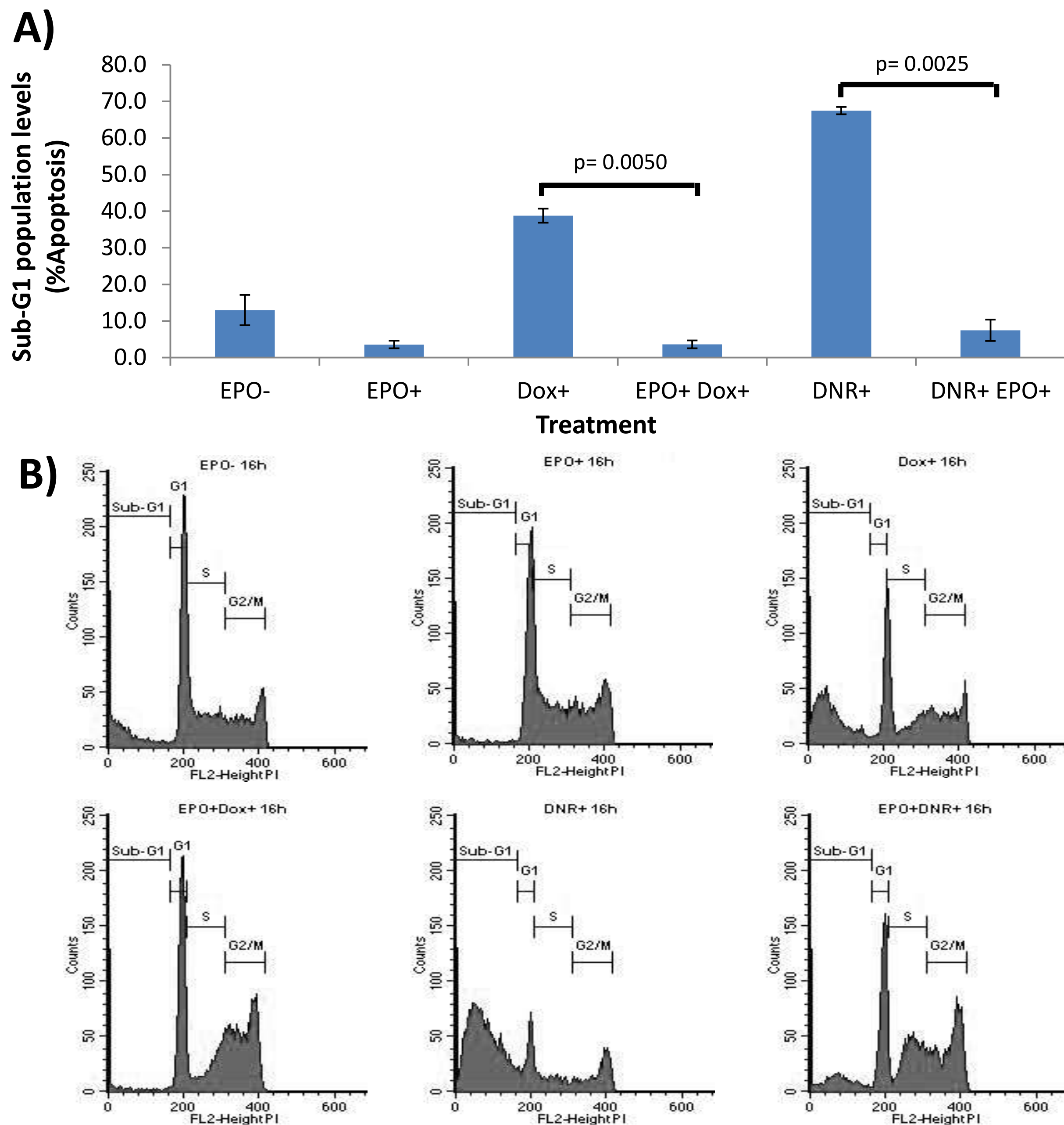


Figure 3: EPO rescues DA3-EPOR cells from Dox and DNR induced apoptosis. A) Cells were starved of EPO for 1hr followed by 16hrs of treatment with Dox (0.25μM), DNR (0.25 μM), and/or EPO (1 U/mL). Treated cells were fixed with 70% ethanol and then subjected to propidium iodide staining and flow cytometry. Error bars represent SEM (N=3 biological replicates) and p-values were obtained via Student's two-tailed t-test. B) Representative of cell cycle profiles after DNR, Dox, and EPO treatment. Contributed by Weili Ma and Thuc-Nghi Pham.

3.2 EPO rescues DA3-EPOR cells from p53-dependent apoptosis

Although it has been previously shown that EPO is capable of rescuing DP16.1/p53ts cells from p53-dependent apoptosis, we wanted to determine if DNR-induced cell death was also dependent on p53 in DA3-EPOR cells. To this end, DA3-EPOR clones that express p53DD, a dominant double-negative truncated p53, were generated via nucleofection to determine whether DNR-induced apoptosis is p53-dependent. 19 DA3-EPOR/p53DD clones were generated in total, of which clones 16, 18, and 19 were chosen for continued use due to their high expression of p53DD (Figure 4). DA3-EPOR/p53DD clones showed an overall significant decrease in Sub-G1 population levels, with percent apoptotic cells ranging from 26.85-29.60% for each p53DD clone,compared with DA3-EPOR cells when treated with DNR for 16 hours ($p<0.0001$) (Figure 5), suggesting that DNR treatment induces p53-dependent apoptosis in DA3-EPOR cells.

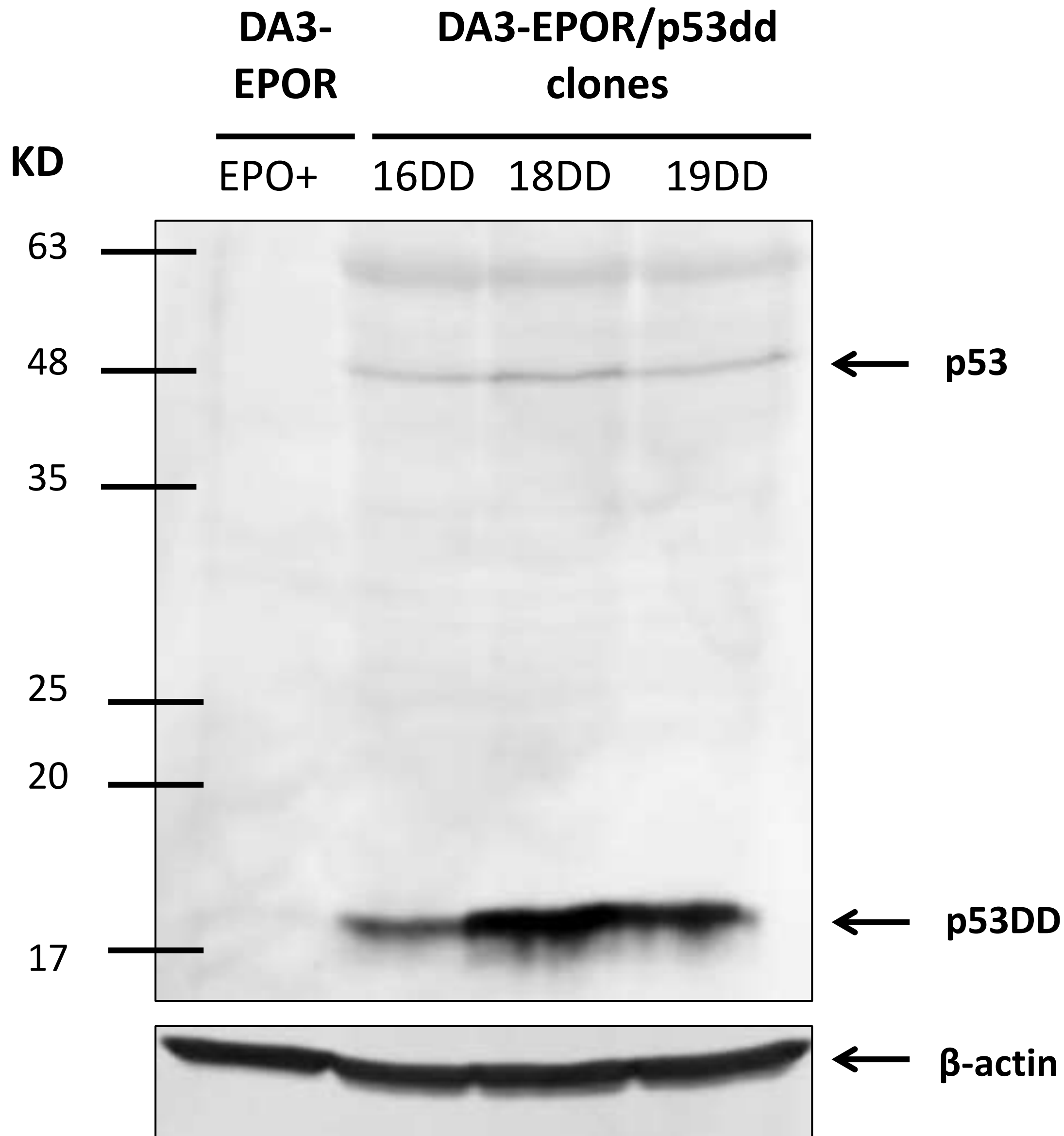


Figure 4: DA3-EPOR cells nucleofected with pCMV/p53DD vector express p53DD. DA3-EPOR cells were co-nucleofected with pCMV/p53DD and pSUPER-puromycin vectors and 19 clones were selected after puromycin selection over four weeks. Clones 16, 18, and 19 displayed the strongest expression of p53DD. Cells were harvested under normal culturing conditions containing EPO (1 U/mL) and subjected to immunoblotting.

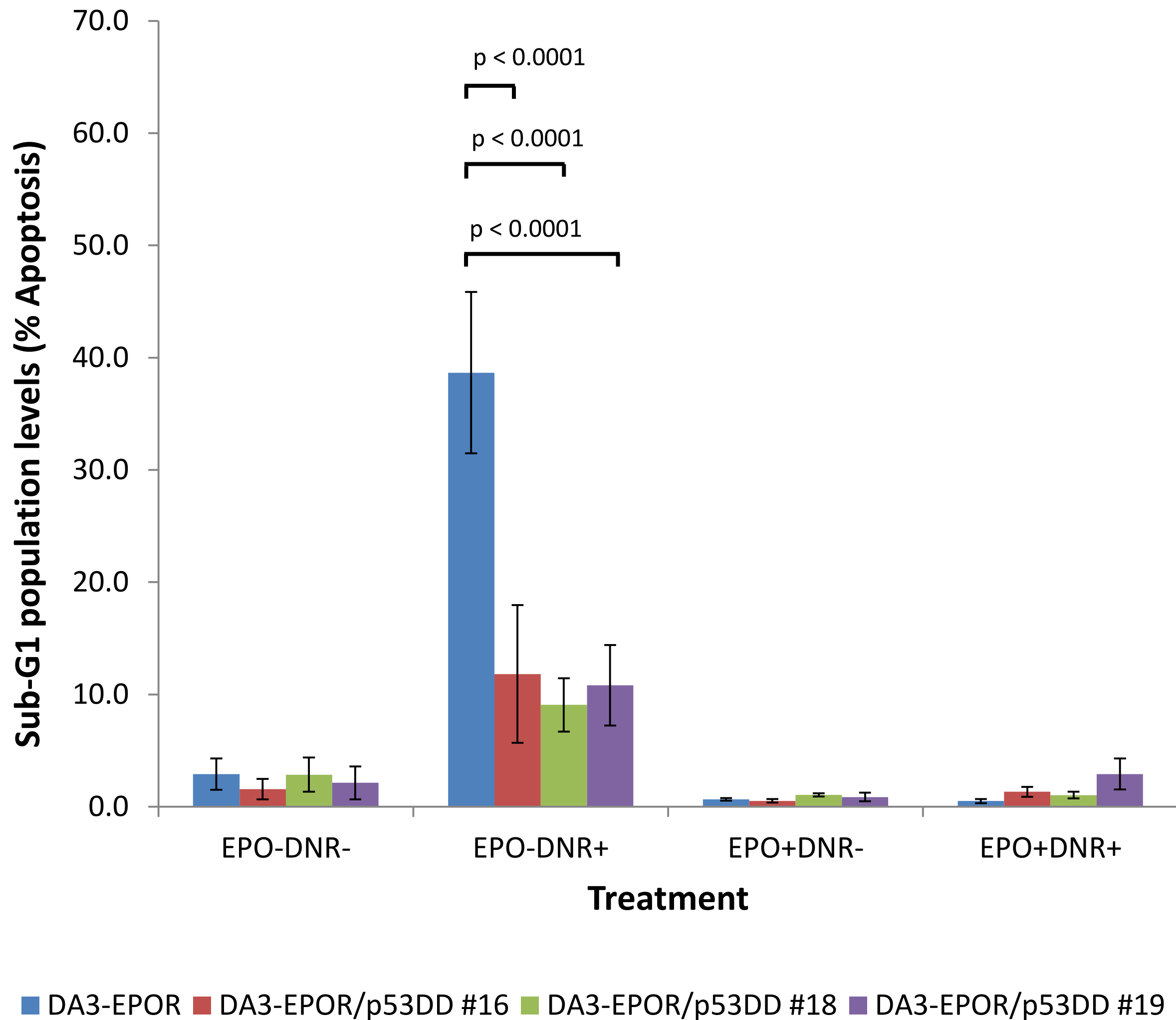


Figure 5: DA3-EPOR/p53DD clones show a reduction in sub-G1 population levels compared with DA3-EPOR cells when treated with DNR. DA3-EPOR cells were co-nucleofected with a pCMV/p53DD and pSUPER-puromycin vector and clones # 16, 18, and 19 were selected out of a total 19 clones after puromycin selection over four weeks. Cells were starved of EPO for 1hr followed by 16hrs of treatment with DNR (0.25 μM) and/or EPO (1 U/mL). Treated cells were fixed with 70% ethanol and then subjected to propidium iodide staining combined with flow cytometry. Error bars represent SEM and p-values were calculated through one-way ANOVA analysis followed by Tukey's HSD posthoc test (N=3).

3.3 EPO decreases p53 protein stability via the proteasomal pathway but does not affect p53 mRNA stability

In our investigation of EPO's effect on the p53 pathway, we observed that p53 protein levels decrease with EPO and DNR treatment compared with DNR treatment alone (Figure 6). The decrease in p53 protein levels also appeared to be much more pronounced with the usage of monoclonal antibody PAb421, which recognizes the C-terminal domain of p53, compared with polyclonal FL393 (Figure 6). There was also an observed decrease in p53 mRNA with DNR treatment for 6 hours, but no considerable change in the presence of EPO (Figure 7). Considering this, we decided to investigate if EPO's effect on p53 protein levels was associated with changes in p53 protein stability via an analysis of p53 protein levels after treatment and addition of cycloheximide, an inhibitor of protein translation, in DA3-EPOR cells. As expected, p53 protein levels were stabilized upon DNR treatment and showed only a 20.6% decrease in protein levels after 4 hours of CHX treatment (Figure 8). In the presence of EPO and DNR however, p53 protein levels show a 75.7% decrease in relative expression after 4 hours of CHX treatment compared with initial levels (Figure 8). No change in p53 protein level was observed in cells treated with DMSO-vehicle control. These novel data indicate that EPO destabilizes p53 protein in DNR-treated cells.

The proteasomal pathway is arguably one of the most well-known pathways involved in the degradation of proteins. As such, through using MG-132, an inhibitor that binds to the 20S proteasomal subunit thus blocking proteasomal degradation, we sought out to determine if p53 protein stability could be rescued from EPO treatment in the presence of MG-132. MG-132 treatment of DA3-EPOR cells treated with DNR and EPO rescues p53 protein expression compared with cells treated without MG-132 (Figure 9). Together, these findings suggest that

EPO may affect p53 protein stability through the proteasomal degradation pathway in DNR-treated cells. To our knowledge, this is the first instance of EPO being reported to decrease p53 protein stability via a proteasomal degradation pathway.

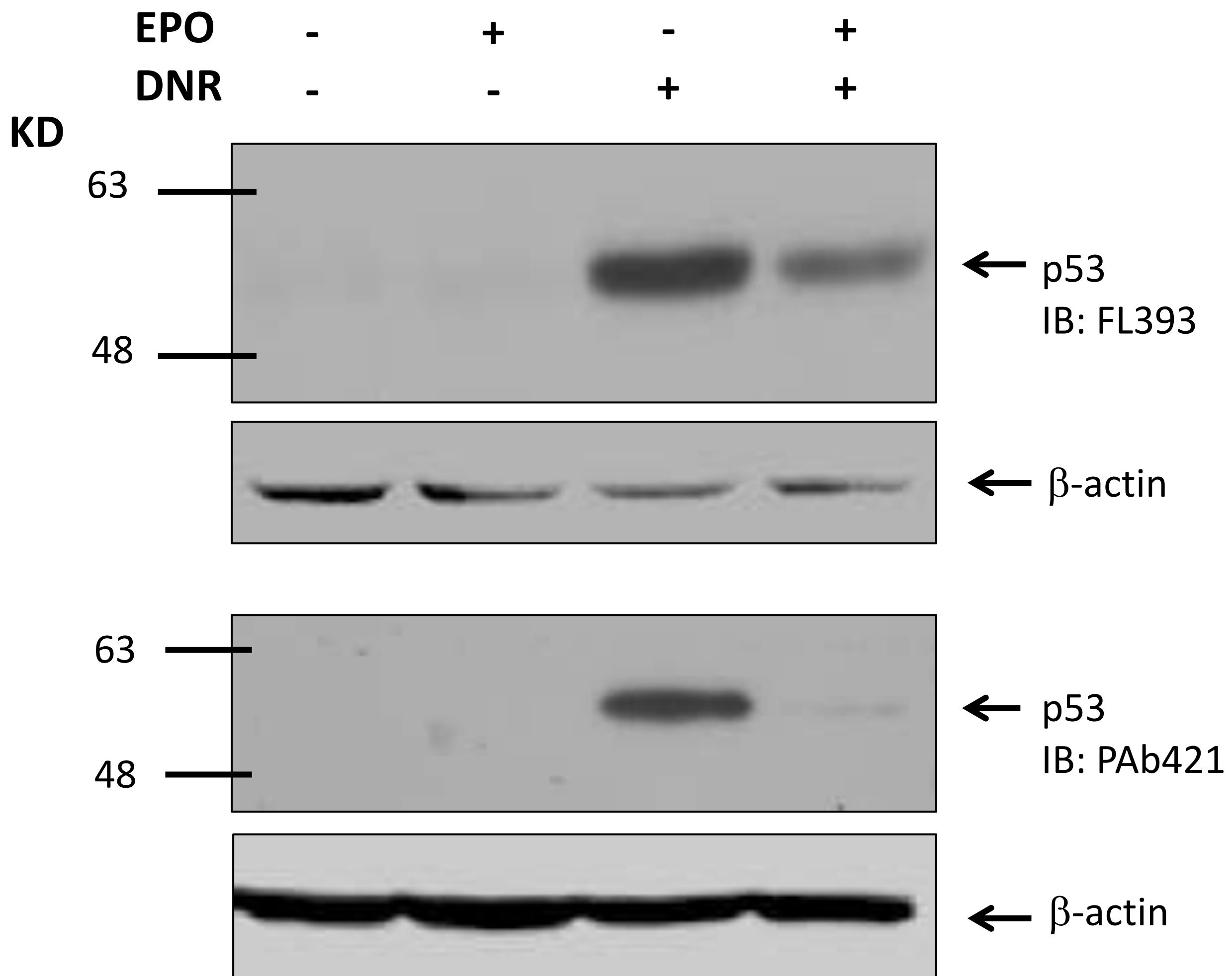


Figure 6: EPO treatment decreases p53 protein level in the presence of 6hr DNR treatment in DA3-EPOR cells. Cells were starved of EPO for 1hr followed by 6hrs of treatment with DNR (0.25 μM) and/or EPO (1 U/mL). Treated cells were harvested and subjected to immunoblotting. Contributed by Weili Ma.

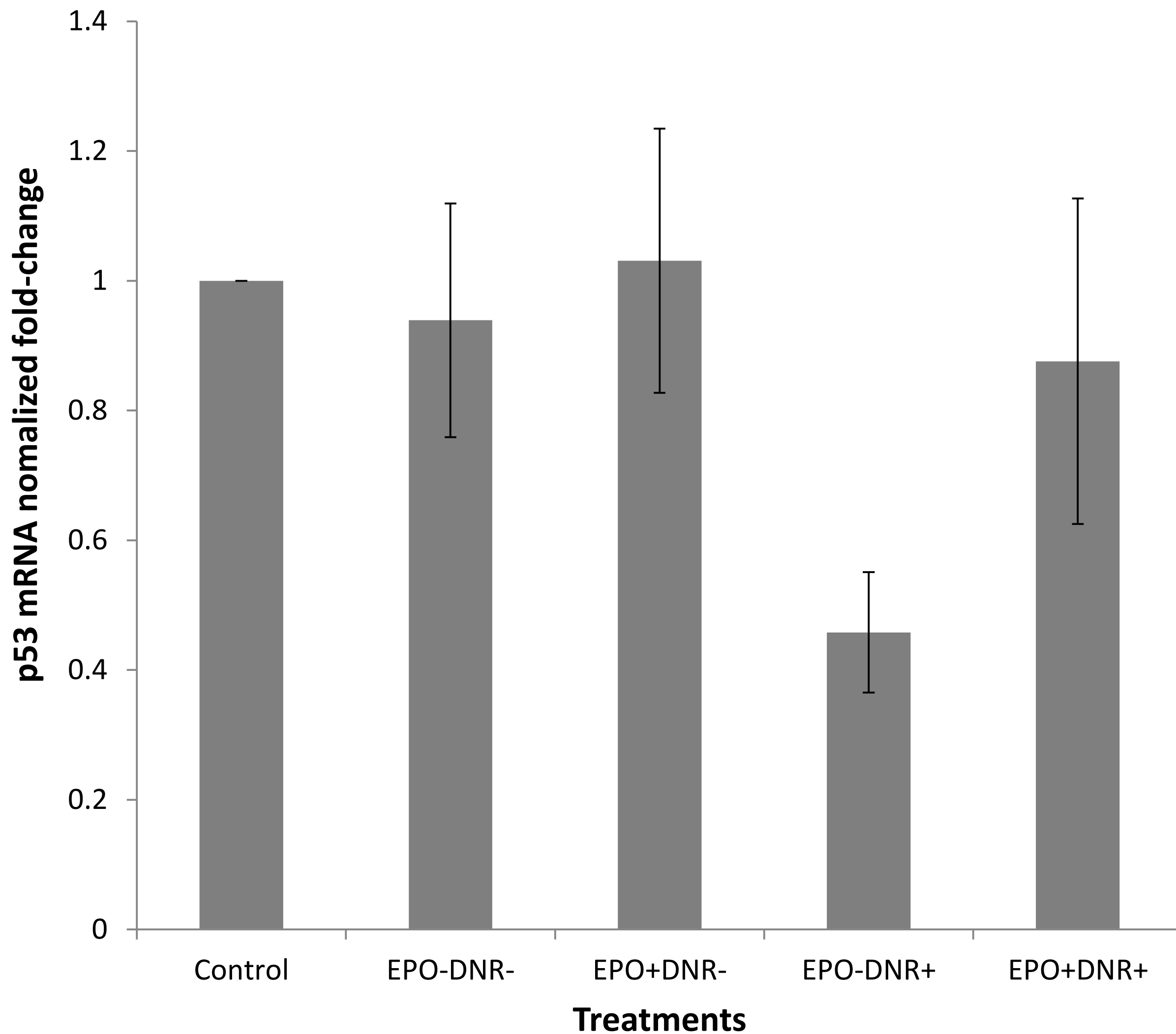


Figure 7: EPO treatment does not affect p53 mRNA levels in DA3-EPOR cells. Cells were starved of EPO for 1hr followed by 6hrs of treatment with DNR (0.25 μM) and/or EPO (1 U/mL). RNA was harvested from treated cells and 0.5μM of cDNA was used per qRT-PCR reaction. Obtained Ct values were normalized to the endogenous control 18S using the Pfaffl method. Error bars represent SEM (N=3 biological replicates). Contributed by Weili Ma.

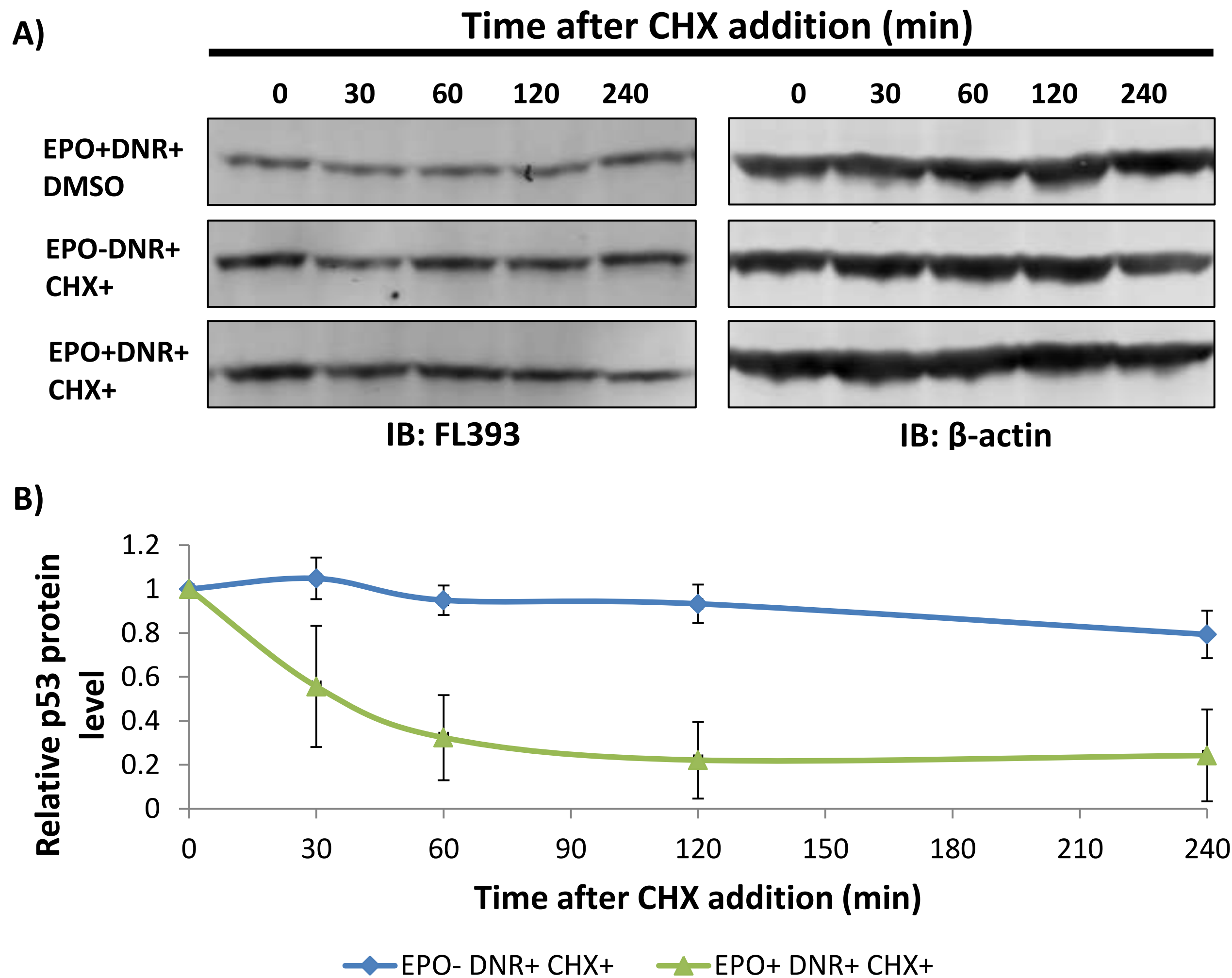


Figure 8: EPO decreases p53 protein stability in DA3-EPOR cells. Cells were starved of EPO for 1hr followed by 6hrs of treatment with DNR (0.25 μM) and/or EPO (1 U/mL). A) Cycloheximide (40 μg/mL) was added after 6hrs of treatment with DNR and/or EPO. Treated cells were harvested at the given time points after cycloheximide addition and subjected to immunoblotting. The data shown is representative of two independent experiments. B) Relative p53 proteins levels were quantified using ImageJ and normalized to β-actin levels. Normalized p53 protein levels were then plotted as a relative value compared with normalized p53 protein levels at 0min after cycloheximide addition (N=2).

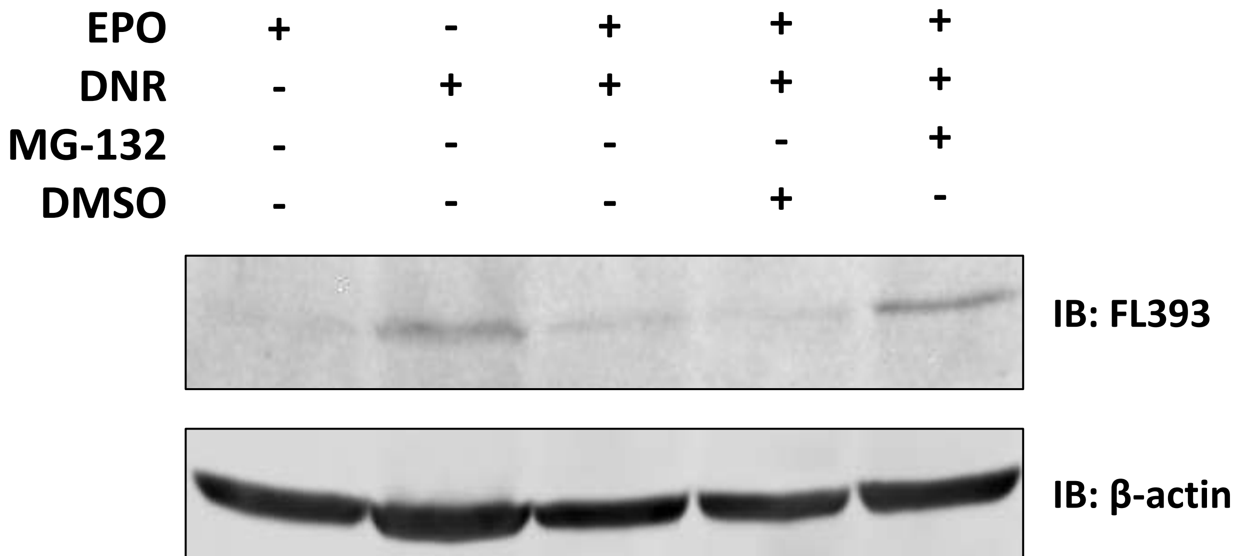
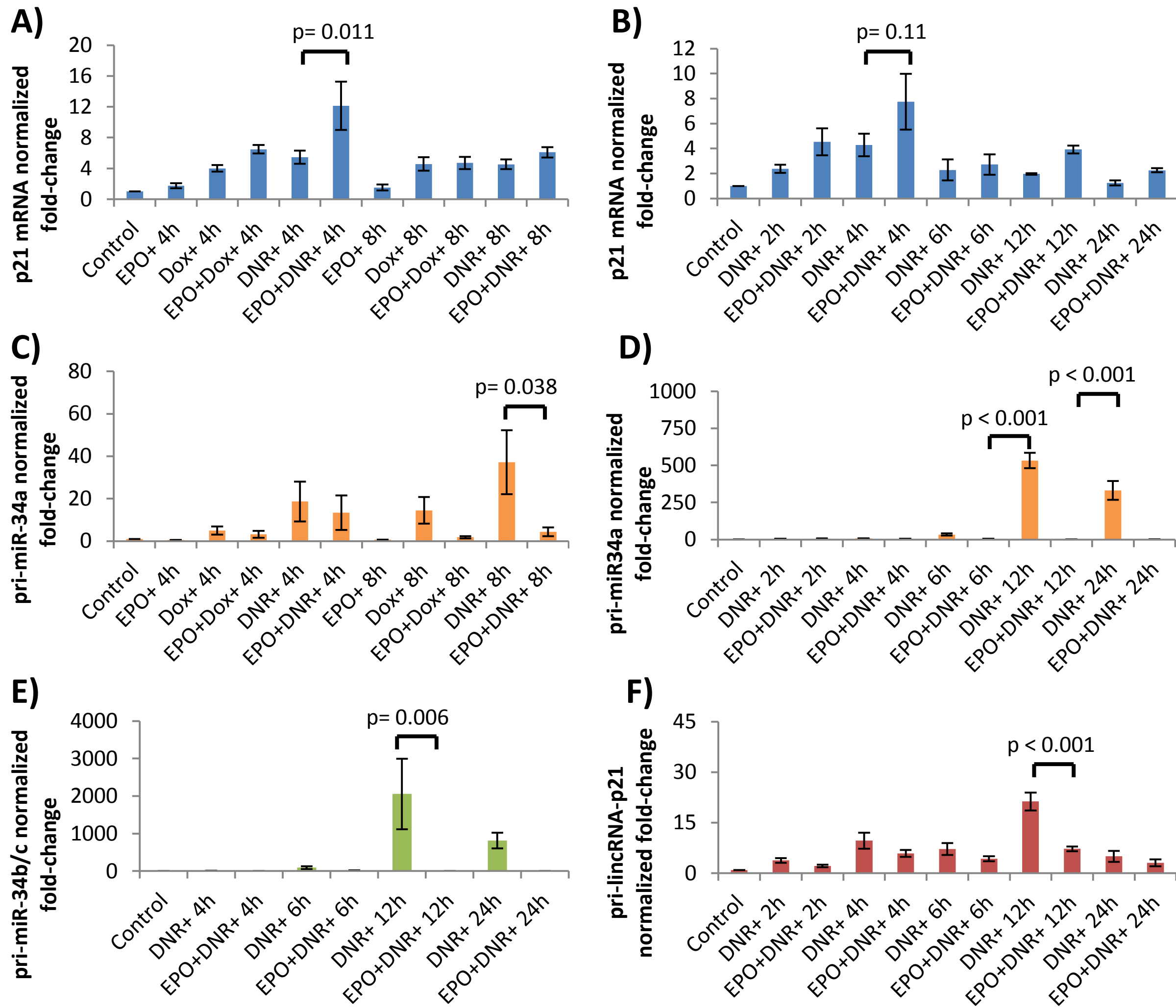


Figure 9: EPO promotes proteasomal degradation of p53 in DA3-EPOR cells. Cells were starved of EPO for 1hr followed by 6hrs of treatment with DNR (0.25 μM) with or without EPO (1 U/mL). After 6hrs of treatment, MG-132 (5 μM) was added for an additional 4hrs and harvested cells were subjected to immunoblotting (N=1).

3.4 EPO modulates the RNA expression of some p53 target genes

Upon genotoxic stress in cells, p53 undergoes substantial post-translational modifications which result in its recruitment to and subsequent upregulation of target genes involved in cell cycle arrest and apoptosis. Considering the observed effect of EPO on both p53-dependent apoptosis and p53 protein stability in DA3-EPOR cells, we next assessed EPO's potential effect on the expression of p53 target genes via qRT-PCR. EPO was observed to have an enhancing effect on p21 mRNA expression at 4 hours of treatment with EPO and DNR compared with DNR alone (Figure 10A and Figure 10B), but was only observed as significant in Figure 10A. An increase in p21 protein levels with EPO treatment was also confirmed via immunoblotting (data not shown). By contrast, EPO significantly decreased the expression of non-coding RNAs pri-miR-34a, pri-miR-34b/c, and pri-lincRNA-p21 (Figure 10C-F). Under DNR treatment alone, pri-miR-34a, pri-miR-34b/c, and pri-lincRNA-p21 RNA levels are enhanced significantly after 12 hours, but are significantly decreased in the presence of EPO (Figure 10D-E). However, we did not observe any significant changes in the mRNA expression at any timepoints of treatment for PIDD, PUMA, or Noxa (Figure 11). Together these findings suggest that EPO significantly modulates the expression of some but not all p53 target genes.



<u>Figure 10: EPO with DOX and/or DNR treatment modulates the RNA expression of p53 target genes A/B) p21, C/D) miR-34a, E) miR-34b/c, and F) lincRNA-p21 in DA3-EPOR cells.</u> Cells were starved of EPO for 1hr followed by treatment with Dox (0.25 µM), DNR (0.25 µM), and/or EPO (1 U/mL) for the given timepoints. RNA was harvested from treated cells and 0.5 µM of cDNA was used per qRT-PCR reaction. Obtained Ct values were normalized to the endogenous control 18S using the Pfaffl method. Error bars represent SEM (N=3 biological replicates) and p-values were obtained via one-way ANOVA analysis followed by Tukey's HSD post hoc test. Contributed by Weili Ma, David Miller, Yael Spiegel, and Thuc-Nghi Pham.

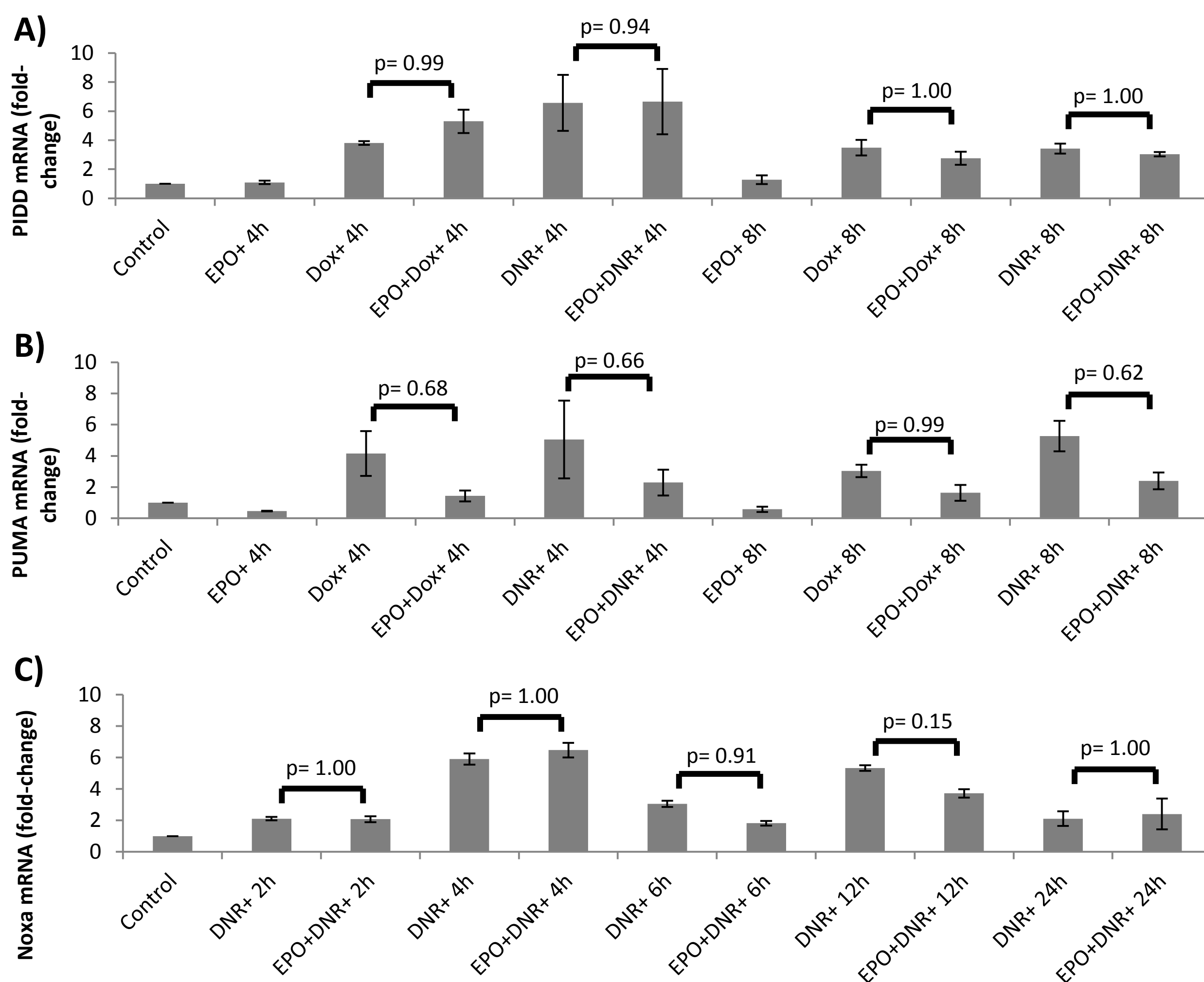
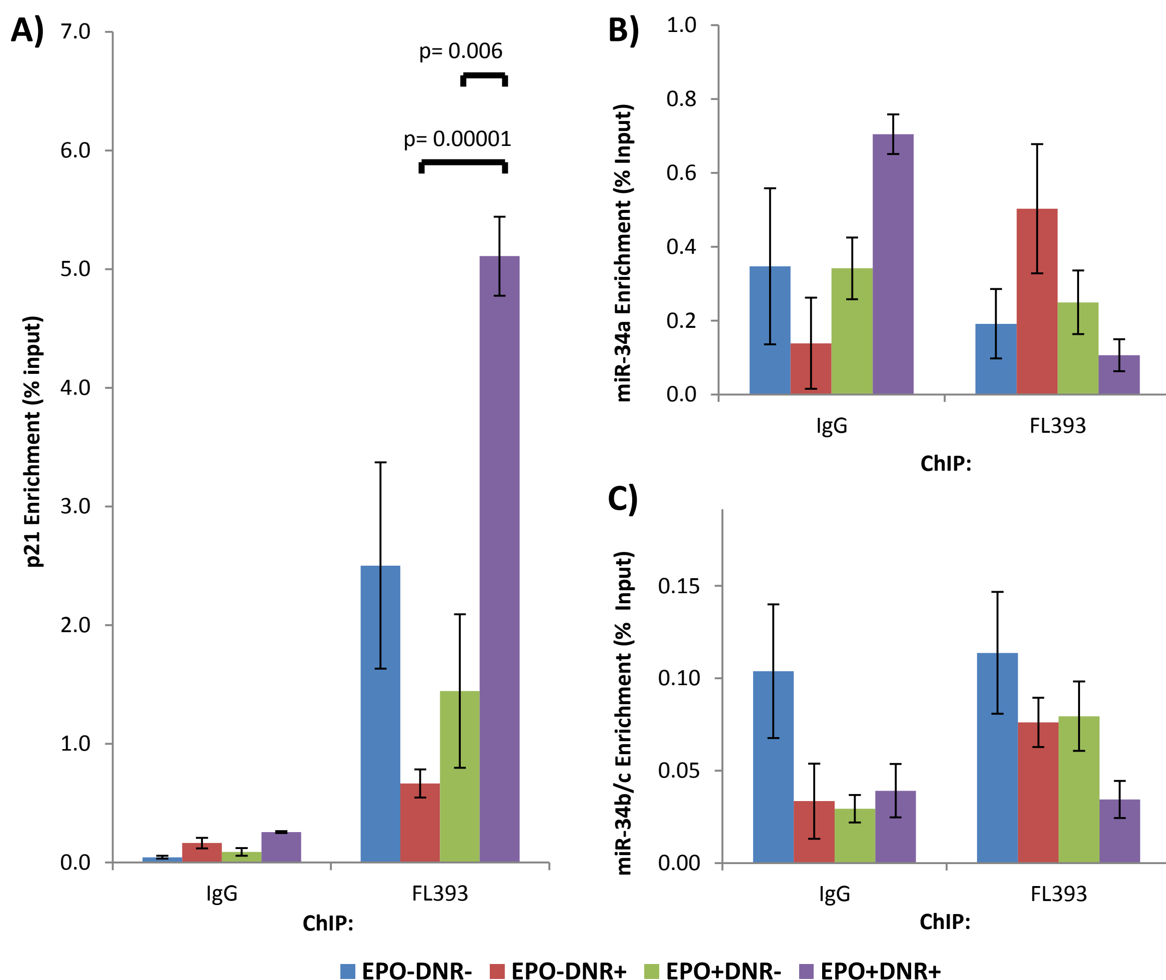


Figure 11: EPO with Dox and/or DNR treatment does not modulate the mRNA expression of p53 target genes A) PIDD, B) PUMA, and C) Noxa in DA3-EPOR cells. Cells were starved of EPO for 1hr followed by treatment with Dox (0.25 μM), DNR (0.25 μM), and/or EPO (1 U/mL) for the given timepoints. RNA was harvested from treated cells and 0.5μM of cDNA was used per qRT-PCR reaction. Obtained Ct values were normalized to the endogenous control 18S using the Pfaffl method. Error bars represent SEM (N=3 biological replicates) and p-values were obtained via one-way ANOVA analysis followed by Tukey's HSD post hoc test. Contributed by Weili Ma, David Miller, and Thuc-Nghi Pham.

3.5 EPO enhances p53 recruitment to the p21 promoter

To determine if EPO modulates p53-target gene expression through changes in the recruitment of p53 to target gene promoters, we assessed the recruitment of p53 to the p21, miR-34a, and miR-34b/c promoters under different treatments via ChIP-qPCR. Compared with DNR treatment alone, we observed a significant increase in p53 recruitment to the p21 promoter in the presence of EPO after 16 hours of treatment (Figure 12A). P53 recruitment to the p21 promoter was also observed under EPO treatment alone and with EPO withdrawal, suggesting that p53 may be present at the p21 promoter under normal conditions in the absence of genotoxic stress (Figure 12A). Unfortunately we were unable to determine any significant changes in p53 recruitment to the miR-34a and miR-34b/c promoters due to signal levels falling below or on par with IgG background levels (Figure 12B and Figure 12C). Together these findings suggest that EPO enhances p53 recruitment to the p21 promoter. Further experimentation is required to determine EPO’s effect on p53 recruitment to the miR-34a and miR-34b/c promoters.



<u>Figure 12: EPO treatment enhances p53 recruitment to the p21 promoter in DA3-EPOR cells.</u> Quantitative PCR analysis of ChIPs performed against p53 at the A) p21 and B) miR-34a and C) miR-34 b/c promoters after EPO and DNR treatment. DA3-EPOR cells were starved of EPO for 1hr followed by 16hrs of treatment with DNR (0.25 uM) and/or EPO (1 U/mL). Obtained Ct values were normalized to non-IP input and p53 levels for each sample and are expressed as Enrichment (% /input). Error bars represent standard error of the mean (N=3 biological replicates) and p-values were obtained from one-way ANOVA followed by Tukey's HSD post hoc test.

4. Discussion

Our findings help to elucidate the mechanism by which EPO rescues DA3-EPOR cells from p53-dependent apoptosis in addition to underlying effects on p53 and the p53 network. The possibility that EPO decreases p53 protein stability via a proteasomal pathway has not been reported to our knowledge. This is particularly exciting considering the majority of blood-borne cancers that express EPOR retain WTp53 expression (Canman *et al.* 1995; Petitjean *et al.*2007). Although previous work on EPO's involvement with p53-dependent apoptosis has helped to further our understanding on the subject, there has mainly been a focus on downstream effectors of p53 or EPOR as opposed to p53 itself (Lin *et al.* 2002; Quelle *et al.* 1999). Another caveat of some of these papers are their utilization of a p53ts system, which despite allowing for determination of a p53-dependent response, may also result in non-physiological p53 levels of expression (Quelle *et al*. 1999). Quelle *et al*. also observed EPO rescue from p53-dependent apoptosis in irradiated DA3-EPOR cells, but unfortunately did not definitively show p53-dependence or relative p53 protein expression (1999). As such, in this study we have focused on further investigating the effects of EPO on the p53 network and elucidating the mechanism by EPO rescues DA3-EPOR cells from DNR and Dox-induced apoptosis.

4.1 EPO rescues DA3-EPOR cells from p53-dependent apoptosis

In agreement with our previous findings in DP16.1/p53ts cells (Lin *et al.* 2002), we have found that EPO is also capable of rescuing DA3-EPOR cells from apoptosis triggered by DNR and Dox treatment. Unlike DP16.1/p53ts cells, DA3-EPOR cells represent a more physiological system that expresses EPOR and WTp53, and thus provide support to the idea that EPO may affect a larger scope of leukemic cancer systems. To determine if EPO rescues DA3-EPOR cells from p53-dependent apoptosis, we transfected DA3-EPOR cells with the dominant negative p53DD

fragment and observed for changes in cell cycle profiles after DNR treatment. DA3-EPOR/p53DD cells showed a decrease in apoptotic cells with DNR treatment compared with DA3-EPOR cells, confirming that EPO rescues the cells from p53-dependent apoptosis. Considering p53's role in apoptosis in DA3-EPOR cells, as well as their established role in 32D and DP16.1/p53ts cells (Lin *et al.* 2002; Quelle *et al*. 1999), it is likely that EPO's involvement in the evasion of apoptosis in leukemic cell lines must somehow circumvent WTp53.

4.2 EPO affects the stability of p53 protein but not mRNA

In our efforts to further understand EPO's effect on p53 we observed a decrease in p53 protein but not mRNA levels in the presence of DNR and EPO compared with DNR alone. To our knowledge, this has not been observed previously and suggests that EPO affects p53 protein levels. Interestingly, probing with the monoclonal antibody PAb421 showed a more substantial decrease in p53 levels compared with probing the same blot with polyclonal FL393. Considering PAb421 recognizes the C-terminal end of p53 this may suggest a decrease in availability of the c-terminal domain of p53 possibly results from PTMs or cofactor binding after EPO treatment (Poyurovsky *et al*. 2010). Another possibility could be due to the strength/affinity of PAb421 for p53, resulting in an overall weaker signal compared with FL393. By comparison, we observed no change in p53 mRNA levels with EPO treatment, suggesting that EPO targets p53 at the protein level and not mRNA level. We did however, observe a decrease in p53 mRNA levels with DNR treatment. It is possible that severe DNA damage may have affected p53 mRNA transcription but not p53 protein translation or stabilization (Siemer *et al*. 1999). Regardless, the reason behind the observed p53 mRNA decrease with DNR treatment remains unclear.

4.3 EPO decreases p53 protein stability via the proteasomal pathway

Considering EPO's effect on p53 protein levels, we wanted to determine whether EPO was directly targeting p53 post-transcriptionally or post-translationally. DA3-EPOR cells were treated with DNR and/or EPO followed by CHX treatment and imaged with immunoblotting to observe changes in p53 protein stability. We observed a decrease in p53 protein stability in the presence of DNR and EPO treatment compared with DNR alone, suggesting that EPO regulates p53 protein levels post-translationally. It has previously been shown that p53 is post-translationally regulated via ubiquitination by MDM2, marking it for proteasomal degradation (Kubbutat *et al.* 1997; Rodriguez *et al.* 2000). To determine whether EPO enhances p53 degradation mediated by the proteasomal pathway, we treated EPO and/or DNR treated DA3-EPOR cells with MG-132, a proteasomal inhibitor that binds to the 20S proteasome subunit (Guo and Peng 2013). We observed that MG-132 treatment was capable of rescuing p53 protein levels after EPO treatment, suggesting that EPO increases p53 association with the proteasome, leading to its degradation. This suggests that EPO may promote survival through enhanced degradation of p53. This is very exciting considering many blood-borne cancers still retain WTp53 status, and may serve as a potential mechanism for how these cancers may circumvent WTp53 expression. EPO has also been previously observed to mediate the degradation of Bcl-2 interacting mediator of death (BIM) via proteasomal degradation in erythroid cells, which supports the possibility that the EPO pathway may be connected to proteasomal degradation (Abutin *et al.* 2009). Future work utilising Nutlin-3, an inhibitor that abrogates the interaction between MDM2 and p53, may allow for further elucidation of the mechanism by which p53 is degraded in the presence of EPO (Vassilev *et al.* 2004). Together with the decrease in protein

levels of p53 mentioned earlier, this suggests that EPO acts on p53 post-translationally to decrease the expression of p53 via enhanced proteasomal degradation.

4.4 EPO modulates the expression of some but not all p53-target genes

Since we observed EPO's effect on p53-dependent apoptosis and p53 protein levels, we next investigated potential downstream effects on the p53 network. Despite the decrease in p53 protein levels observed, we only observed some modulation in expression of p53-target genes via qRT-PCR. This modulation included the ncRNAs miR-34a, miR-34b/c, lincRNA-p21 and coding RNA p21, but not coding RNAs PUMA, Noxa, and PIDD. It is unclear whether any of these changes are causally related to the pro-survival function of EPO. Although PUMA, Noxa, and PIDD mRNA levels did increase with DNR treatment as expected (Bock *et al.* 2012; Ploner *et al.* 2008; Yu and Zhang 2008), there was no significant change observed in the presence of EPO. It is possible that activation of pro-survival pathways by EPO may have circumvented any initiation of apoptosis by PUMA, Noxa, or PIDD. Further investigation into the protein expression of these genes and potential preservation of mitochondrial integrity may shed more light into their role in these cells.

We also observed a decrease in expression for ncRNAs miR-34a, miR-34b/c, and lincRNA-p21 with EPO treatment, in addition to inconclusive ChIP-qPCR results for p53 presence at the miR-34a and miR-34b/c promoters. MiR-34a, miR-34b/c, and lincRNA-p21 have previously been associated with p53-dependent apoptosis (Bommer *et al.* 2007; Chang *et al.* 2007; Corney *et al.* 2007; Dimitrova *et al.* 2014), but we were unable determine whether their decreased expression is essential for evasion of apoptosis or whether it is a result of decreased p53 expression and recruitment to promoters. Due to the ability of ncRNAs to regulate transcription of multiple targets, the potential role for ncRNAs in EPO's evasion of apoptosis is particularly

exciting (Acunzo *et al*. 2015). Future work determining potential changes in expression of downstream targets of these ncRNAs would provide more insight into their role in EPO rescue from p53-dependent apoptosis.

An increase in p21 levels and increased p53 recruitment to the p21 promoter was observed in the presence of EPO and DNR compared with DNR alone, suggesting that EPO enhances p21 expression via increased p53 recruitment to the p21 promoter. Considering EPO's enhancement of p21 expression occurs concomitantly with decreased p53 expression, it is possible that p21 expression may in part also be stimulated independent of p53. Another possibility is that EPO-dependent changes in p53 PTMs, such as a loss of Tip60-mediated acetylation, results in preferential recruitment of p53 to the p21 promoter. This may explain the decrease in p53 levels yet increased p53 recruitment to the p21 promoter. Interestingly, there was also a decrease in p53 recruitment to the promoter with DNR treatment as well as a small basal amount of p53 recruited the promoter under all conditions over IgG background. This is consistent with the idea that some p53 is found present on the p21 promoter in unstressed cells (Kaeser and Iggo 2002) and that DNR treatment may preferentially shift p53 recruitment to apoptotic target genes, resulting in a decrease in p53 recruitment to p21. There is also an increase in p53 recruitment to the p21 promoter with EPO withdrawal, which may be the result of an EPO withdrawal stress that induces G1-arrest. These findings support our previous work suggesting that EPO is mediating survival of these cells from stress-induced apoptosis dependent on p53 by shifting the cells from apoptosis to G1-arrest (Lin *et al.* 2002).

4.5 Conclusions

Altogether this work suggests and supports the concept that EPO rescues cells from p53-dependent apoptosis caused by DNR/Dox treatment in addition to increased proteasomal degradation of p53 and an orchestrated shift from p53-dependent apoptosis to cell cycle arrest. EPO was also observed to have an effect on expression of p53 target genes, which included decrease in expression of ncRNAs in the p53 network but increase in p21 expression and recruitment of p53 to the p21 promoter (Figure 13). This work helps to broaden our understanding of EPO's effect in cancer cells and potentially target the pathway by which cancers utilize EPO for apoptosis evasion.

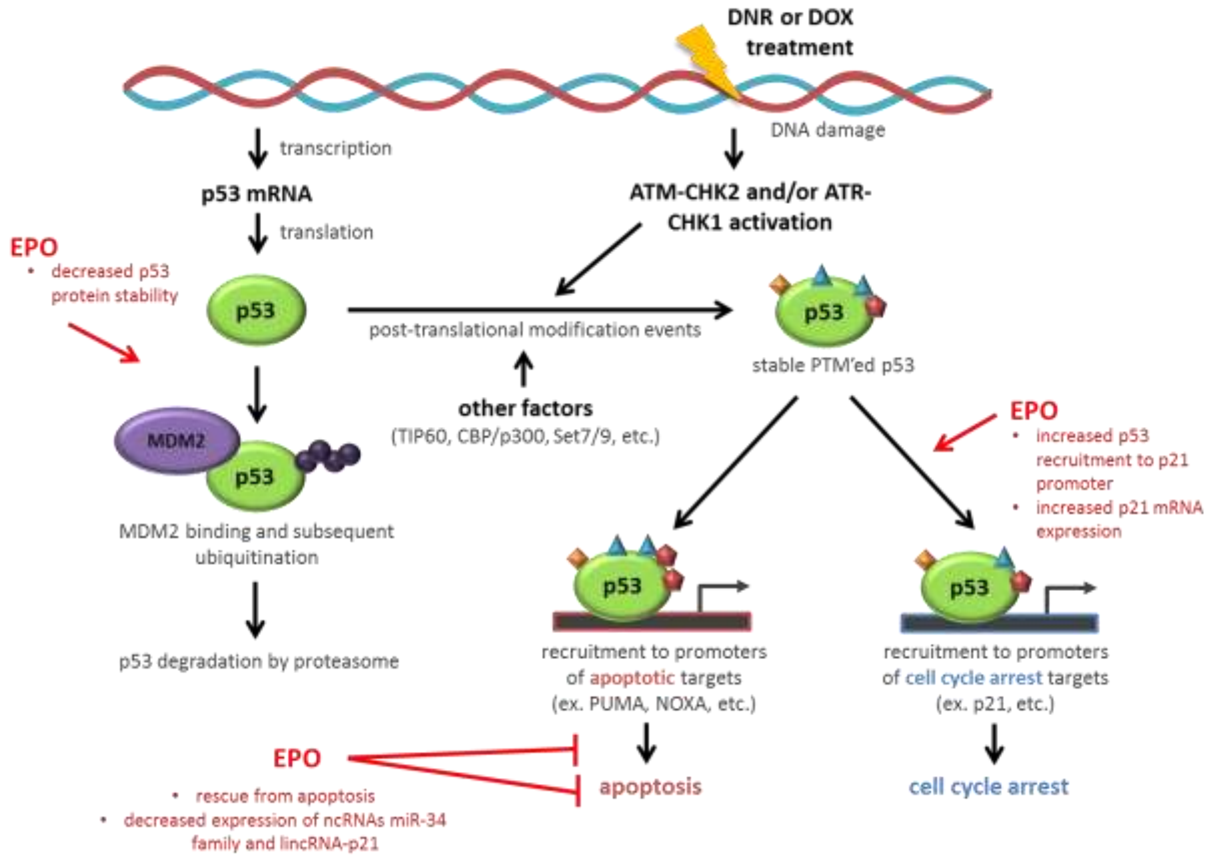


Figure 13: Schematic of the effects of EPO on p53-dependent apoptosis and the p53 network in DA3-EPOR cells. EPO rescues cells from p53-dependent apoptosis and enhances proteasomal degradation of p53 with a concomitant decrease in miR-34a, miR-34b/c, and lincRNA-p21 expression. EPO was also observed to increase p53 recruitment to the p21 promoter followed by increased p21 expression.

5. Future directions

Although we have observed a number of changes in the p53 network with EPO treatment, it is important to determine what factors are fundamental in EPO's ability to rescue cancer cells from apoptosis. Future work would require delineating the mechanism of EPO's rescue from apoptosis and dissecting where this may involve p53 and its network.

5.1 Determination of factors involved in p53 degradation with EPO treatment

We have observed increased p53 proteasomal degradation with EPO treatment, however it has yet to be determined what other factors may be involved in shuttling p53 protein towards the proteasome. Potentially, p53 is being ubiquitylated, leading to its recruitment to the proteasome. Utilization of Tandem Ubiquitin Binding Entity (TUBE) technology, which allows for the characterization and isolation of ubiquitylated proteins, may allow for identification of ubiquitin levels on p53 with EPO treatment (Hjerpe *et al.* 2009). If p53 is found to have elevated ubiquitylation with EPO treatment, it is likely that an E3 ubiquitin ligase is involved in its ubiquitylation. MDM2 has previously been established as the primary E3 ubiquitin ligase to target p53 in the literature (Kubbutat *et al*. 1997). To determine whether ubiquitylation of p53 may be associated with MDM2, treatment of cells with Nutlin-3, an inhibitor of the interaction between MDM2 and p53, would elucidate whether MDM2 contributes to p53 degradation. Co-immunoprecipitation of MDM2 bound to p53 would also help to determine if there is increased interaction between the two factors. However, if ubiquitylation of p53 proves to be MDM2-independent, other E3 ubiquitin ligases, such as Pirh2, could be examined (Leng *et al.* 2003).

5.2 Determination of the importance of p53 degradation for EPO-induced G1 arrest and evasion of apoptosis

It still remains unclear as to whether the enhanced degradation of p53 with EPO treatment is sufficient for evasion of apoptosis and subsequent induction of G1-arrest. Although p53DD clones showed reduced apoptotic cells with DNR treatment, this demonstrates the effect of p53 inactivation as opposed to p53 knockdown in these cells. To determine if the partial knockdown of p53 protein levels by EPO treatment is sufficient for evasion of apoptosis, cells expressing short hairpin RNA (shRNA) constructs to partially knockdown p53 expression in the presence of only DNR treatment could be generated and evaluated for influence on cell cycle profile. The usage of multiple shRNA constructs with varied ability to reduce p53 levels has previously been used to evaluate the effects of differing levels of p53 expression on cell phenotype (Hemann *et al.* 2003). A similarly designed experiment in DA3-EPOR cells should determine the importance of partial p53 knockdown to EPO evasion of apoptosis.

5.3 Mechanistic study of p53 recruitment to the p21 promoter

Although we observed enhanced p53 degradation as a result of EPO treatment, it is unclear how there is also enhanced recruitment of p53 to the p21 promoter and an increase in p21 levels. It is possible that p53 is post-translationally modified and/or associated with cofactors in such a way that stable forms of p53 that are not actively degraded are preferentially recruited to the p21 promoter. To determine whether post-translationally modified p53 and/or recruiters are present at the p21 promoter under EPO treatment, Global ExoNuclease-based Enrichment of Chromatin-Associated Proteins for Proteomics (GENECAPP) may potentially be used. GENECAPP is a new technique developed by Wu *et al.* which allows for the capture of proteins cross-linked to a specific DNA sequence via sequence-specific hybridization present on a solid support (2011).

This allows for a DNA-centric approach to examining proteins bound to a specific DNA sequence. Protein-DNA complexes are digested via exonucleases, rendering the bound DNA sequence single stranded and capable of binding the sequence-specific hybridization complementary DNA oligonucleotide array. Captured complexes associated with the p21 promoter can thus be analysed via mass-spectrometry for present p53 PTMs and cofactors.

Additionally, it is also possible that p21 levels increase independently of p53. To determine if EPO induces p21 expression independent of p53, DA3-EPOR/p53DD cells could be immunoblotted for p21 expression in the presence of EPO.

5.4 Translational studies in other systems

It is important to examine whether the observed effects of EPO treatment on apoptosis and p53 in DA3-EPOR cells can be replicated in other systems to give support to the translatability of our findings. Future work could examine the effects of EPO in human cell lines that express EPOR in addition to other murine cell lines. A bioinformatics study could also be performed to determine the relative correlation between EPOR expression, WTp53 protein status, and patient prognosis and overall survival in clinics. Finally, the usage of mouse models would allow for examining the effects of EPO on tumourigenesis and the p53 network in a more physiological system.

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