Regulation and function of tumor suppressor p53 isoforms beta and gamma in Acute Myeloid Leukemia

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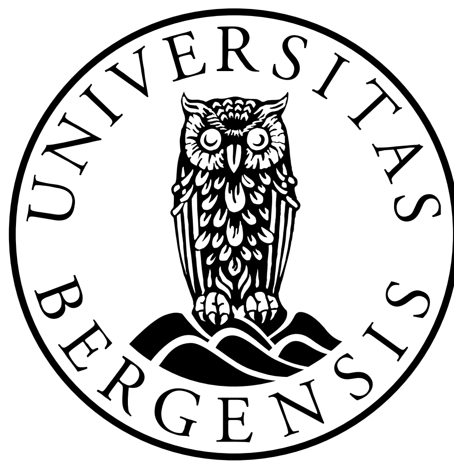

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Abbreviations

AML	Acute Myeloid Leukemia
ARF	Alternative Reading Frame
Bax	Bcl-2 associated X protein
COX	Cytochrome c Oxidase
ECGFP	Enhanced Cyan Green Fluorescent Protein
FAB	French-American-British
FACS	Fluorescence Activated Cell Sorting
FL	full-length
FLT3	FMS-like Tyrosine Kinase 3
ITD	Internal Tandem Duplication
Mdm2	Murine double minute 2
MW	Molecular weight
NPM1	Nucleophosmin isoform 1
PTMs	post-translational modifications
ROS	Reactive Oxygen Species
RT	room temperature
SEM	Standard Error of the Mean
SRSF3	Serine/Arginine-Rich Splicing Factor 3
WHO	World Health Organization
wt	wild-type

Summary

Research has proposed that the C-terminal truncated p53 isoforms β and γ enhance chemosensitivity in $p53^{null}$ cancer cell lines. Their high expression in acute myeloid leukemia (AML) patients correlates positively with increased survival and response to chemotherapy as well as with the good prognostic marker Nucleophosmin isoform 1 (NPM1) mutation. However, it is ambiguous whether they have independent functions or act in concert with full-length p53 (FLp53) in AML. Furthermore, the generation of reactive oxygen species (ROS) has been shown to play roles in p53 regulation but its mechanism is still elusive. Resazurin which acts through the generation of ROS has been demonstrated to have cytotoxic effects in leukemia cells. In this thesis, the functional roles of p53β and p53γ in response to chemotherapy and resazurin have been investigated in AML cell lines. Also, the effect of resazurin as well as wild-type and mutated NPM1 on the regulation of p53 isoforms expression has been examined.

Stable expression of p53β and p53γ in $p53^{null}$ HL-60 AML cell line was established to explore possible isoform-directed functions independent of FLp53. This thesis showed that p53β and p53γ do not affect proliferation and viability of $p53^{null}$ AML cells in response to chemotherapy, but disclosed that p53β and p53γ may act in concert with FLp53 in a ratio-dependent manner. These are based on the facts that stable expression of p53β and p53γ can be established in $p53^{null}$ HL-60 cell line, but a higher level of p53β and p53γ is unfavorable to wild-type *TP53* MOLM-13 AML cell line and a high level of p53γ is cytotoxic. Moreover, it has been revealed that p53β increases apoptosis of HL-60 cells in response to resazurin. Resazurin has been shown to cause p53-independent down-regulation of Mdm2 and increased apoptosis in HL-60 cells. In MOLM-13 cells, it does not primarily affect the cells through induction of apoptosis. However, the up-regulation of $p21^{CIP1/WAF1}$ was observed in MOLM-13 cells after resazurin exposure. Resazurin exposure has distinct impacts on the level of Mdm2 in AML cell lines depending on the expression level of p53. A lower p53 level has more attenuation of Mdm2 in response to resazurin. In addition, although the expression of wild-type and mutated NPM1 has been generated in a wild-type *TP53* cell line, p53 isoforms modulation was not determined.

In conclusion, this thesis has shown the interaction of p53β and p53γ with FLp53 and the cytotoxic effect of p53γ which suggest a prospective therapeutic target of p53 isoforms. Also, it has partly elucidated the mechanism of resazurin sensitivity which may contribute to the development of a novel therapy in AML.

1. Introduction

1.1. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a clonal, malignant disease of hematopoietic tissues which is represented by the accumulation of abnormal (leukemic) blast cells, primarily found in the bone marrow, and the impaired production of normal blood cells [1]. As a consequence, this deficiency results in anemia, mortal infection, hemorrhage, or organ infiltration of leukemic cells [2].

AML is the most common myeloid leukemia accounting for nearly 30% of all adult leukemias, and approximately 0.6% of all cancers [3]. The median age at the time of diagnosis is about 70 years, and the number of males acquiring AML is higher than of females [2]. Risk factors associated with the disease consist of exposure to ionizing radiation, benzene, and cytotoxic chemotherapy [2]. Whereas standard chemotherapy for patients being younger than 60 years might cure 20–75%, it yields such a result in less than 10% of elderly patients by virtue of their reduced ability to tolerate the treatment in addition to disadvantageous cytogenetics regarding chromosomes 5 and 7 [2]. Therefore, the demand for novel targeted therapies with lower toxicity is imperative in this group of patients.

1.1.1. Classification

Two classification systems of AML were established based on the properties of the disease. The first system is the French-American-British (FAB) classification system (Table 1) which organized AML into different categories according to the morphology and differentiation of AML blasts [4, 5]. World Health Organization (WHO) has developed a more clarified system (Table 1) relying on the criteria of FAB system in addition to genetic, immunophenotypic, biological and clinical characteristics of myeloid neoplasms [6-8]. WHO classification has a clearer impact on prognosis of AML compared to the FAB classification [9], and includes previous cancer therapy, myelodysplasia, recurrent chromosomal aberrations and gene mutations that allow relapse risk stratification [7, 10].

Table 1. FAB and WHO classification of AML.

FAB	WHO
M0 AML with no differentiation	**AML with recurrent genetic abnormalities**
M1 AML without maturation	AML with t(8;21)(q22;q22), RUNX1-RUNX1T1
M2 AML with granulocytic maturation	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;p22); CBFB-MYH11
M3 Acute promyelocytic leukemia	Acute promyelocytic leukaemia with t(15;17)(q22;q12);PML-RARA
M4 Acute myelomonocytic leukemia	AML with t(9;11)(p22;q23)MLLT3-MLL
M5 Acute monocytic leukemia	AML with t(6:9)(p23;q34); DEK-NUP214
M6 Acute erythroleukemia	AML with inv(3)(q21q26.2) or t(3.3)(q21;q26.2); RPN1-EVl1
M7 Acute megakaryoblastic leukemia	AML (megakaryoblastic) with t(1:22)(p13;q13); RBM15-MKL1
	AML with mutated NPM1
	AML with mutated CEBPA
	AML with myelodysplasia-related changes
	Therapy-related myeloid neoplasms
	Acute myeloid leukaemia, not otherwise specified
	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukaemia
	Acute monoblastic and monocytic leukaemia
	Acute erythroid leukaemia
	Acute megakaryoblastic leukaemia
	Acute basophilic leukaemia
	Acute panmyelosis with myelofibrosis
	Myeloid sarcoma
	Myeloid proliferations related to Down syndrome
	Transient abnormal myelopoiesis
	Myeloid leukaemia associated with Down syndrome
	Blastic plasmacytoid dendritic cell neoplasm

Table adapted from [5, 8].

1.1.2. Molecular pathogenesis

AML is the consequence of multiple somatic mutations in either a hematopoietic multipotential cell or a more differentiated, lineage-restricted progenitor cell. It has been recently revealed that nearly all AML cases have mutations in the cancer cells, but AML has fewer somatic mutations than other adult cancers and these mutations can be classified into nine categories of genes that are related to pathogenesis [11]. In many patients, the somatic mutation originates from a chromosomal translocation [12]. This translocation contributes to the rearrangement of a crucial area of a proto-oncogene leading to mutation. The protein product of the mutated gene, for example, core binding factor (CBF), retinoic acid receptor-(RAR-), HOX family or MLL, is usually a transcription factor or a constituent of the transcription pathway which may interrupt the differentiation and maturation of blood progenitor cells once it is expressed. Nonetheless, these primary mutations cannot evoke AML sufficiently if they work alone. The demand for additional activating mutations, for instance, in *FLT3, c-KIT, N-RAS* or *K-RAS*, is essential to result in an advantage in proliferation or survival of the affected cells [12-14]. A "two-hit" hypothesis has been proposed for the generation of AML in which two classes of gene mutations are required. While the class I leads to a proliferative and survivable advantage to the cells, the class II interacts with the class I and disturbs differentiation and maturation patterns on the mutated cells [14]. Recently, the detection of NPM1 mutations has revealed a third class of mutations affecting genes implicated in cell-cycle regulation or apoptosis [15, 16].

The receptor tyrosine kinase FLT3 has a role in normal myeloid and lymphoid progenitors. Internal tandem duplications of FLT3 on chromosome 13 have been shown to occur in approximately one-third of adult AML cases, but frequently in cases with normal cytogenetics, monocytic phenotype, and PML-RAR or DEK-CAN translocations [17]. Many studies have presented that *FLT3*-internal tandem duplication (ITD) mutation is associated with a poor prognosis if the ratio of mutant to wild-type expression is high [18, 19].

Chromosomal mutations like deletions of all or part of a chromosome (e.g., chromosome 5, 7, or 9) or additional chromosomes (such as trisomy 4, 8, or 13) are also common. In older patients and cases of AML following cytotoxic therapy, the frequency of deletions in chromosomes 5 and 7 is raised in comparison with *de novo* cases [20]. Since the mutation of genes remaining on the undeleted homologous segment of chromosome 5 is not detected, an epigenetic lesion like hypermethylation of a gene allelic to one on the deleted segment on chromosome 5 may induce the leukemogenesis.

1.1.3. Treatment

1.1.3.1. Conventional therapy

Standard treatment of AML consists of two phases, the induction and the consolidation phase. The objective of the induction phase is to attain complete remission (CR) determined as less than 5% of blasts present in both the bone marrow and normal peripheral blood counts [21]. The vital purpose of the consolidation phase after CR acquired is to prolong CR and avoid relapse [2]. In the induction phase, the combination of an anthracycline such as daunorubicin or idarubicine and cytarabine (AraC) is given to the patient targeting highly proliferative cells which also comprise the leukemic blasts. When CR is obtained, the treatment of the patient comes in the consolidation phase. This phase is fulfilled by applying high dose of AraC, allogeneic stem-cell transplantation (allo-SCT) or autologous SCT [22]. However, because allo-SCT has been related to fatality, it should only be utilized in young patients who are with a high risk of relapse or relapsed after the induction therapy [23].

For elderly patients, the available therapeutic options consist of standard treatment, investigational treatment and palliative care. The main aim of treatment is to avoid the progression of the disease, with focus on improving the quality of life of the patient [2].

1.1.3.2. Novel therapeutic strategies

In spite of the fact that the advance in regime for acute promyelocytic leukemia (APL) is outstanding, with nearly 90% of patients may be cured in the long term [24], the therapeutic results of patients diagnosed with other types of AML are still not pleasing. Therefore, the need of more effective therapies is evident for the majority of AML patients. Furthermore, the incidence of AML is increased with the aging population, the preclusion of intensive chemotherapy as well as the development of less toxic regimens are imperative in patients with co-morbid conditions [25]. Enhanced knowledge in molecular pathogenesis of the heterogeneous disease "AML" has extended the prognostic factors of the disease and provided new targets in treatment for patients.

Clinical trials with novel therapeutic agents in the treatment of AML are increasing. For example, some researchers have been interested in the utilization of c-kit inhibitors such as imatinib and dasatinib in AML treatment. However, the results of treatment with imatinib alone were disappointing [26], and could be foreseen based on the lack of efficiency of imatinib monotherapy in Ph+ CML blast crisis [27]. Recently, the combination of chemotherapy to either imatinib or dasatinib has been investigated and initial results indicate

a limited benefit of this combination [28]. Since FLT3-ITD is a poor prognostic element in AML, it has been considered as a therapeutic target which led to great attraction in the development of FLT3 inhibitors such as AC220 (quizartinib) and sorafenib for therapeutic application [29, 30]. In addition, the use of monoclonal antibodies as a molecularly targeted therapy has been demonstrated to be an effective treatment for either hematologic malignancies or solid tumors [26]. Recently, it has been presented that the NPM1-mutated AML cases have a significantly higher CD33 intensity compared with the non-mutated ones, which proposes a basal background for the utilization of anti-CD33 antibodies as a therapeutic agent in NPM1-mutated AML [31]. Generally, the employment of targeted therapies is currently limited to those ones conducted within a well-designed clinical trial. Nevertheless, by virtue of increasing acquirement in understanding molecular aspects of AML, the success of novel agents in the treatment of AML has not been probable so far [26].

1.1.3.3. Reactive oxygen species therapy through Resazurin - a potential new AML drug

Reactive oxygen species (ROS) are a heterogeneous group of molecules and free radicals created from diatomic oxygen which have a broad range of reactivity [32]. Excessive ROS production can result in oxidative stress [33], a condition that has been detected in AML [34] which may represent a new potential therapeutic target through compounds promoting its generation [35].

Resazurin which is the principle component of Alamar Blue used for cell viability assay [36, 37] has been shown to have cytotoxic effects on leukemic cells [38, 39]. This blue-colored compound is also utilized as an oxidation–reduction indicator for examination of sperm viability [40], cell proliferation [41], toxicity [42], and mitochondrial metabolism [43, 44]. In these assays, the conversion from the blue color of resazurin to the pink color of its fluorescent product, resorufin, which has an excitation maximum at 572nm and an emission maximum at 585nm [45] is detected [39]. This conversion, in living cells, is referred to the reduction of resazurin by various oxidoreductase enzyme systems employing NAD(P)H as the main electron donor [37, 46]. The biochemical reactions caused by resazurin exposure have been demonstrated to involve the production of reactive oxygen species (ROS) which is mainly generated by mitochondrial respiration [47]. Furthermore, resazurin has also been presented to have a connection with oxidation–reduction reactions in the cytosol and nucleus [48]. Taken together, it can be seen that the exposure of living cells with resazurin might influence cellular redox conditions and energy homeostasis [15]. Thus, there could be a case for saying that resazurin activates ROS generation through its robust cross-reactivity with the

constituents and metabolites of the cells, triggering a cellular stress response which is likely to provoke the dysfunction of mitochondria and the latter degradation of the cell [39].

Despite the fact that the biochemical properties of resazurin have been characterized, the information relating to the physiological outcomes of resazurin exposure *in vivo* is still scarce [39]. Because resazurin is ordinarily accepted to be low toxic within the timeframe of the assays [49, 50], in addition to the well tolerance of rats with the compound [51], the potential effects of resazurin as a treatment are regularly not assessed in cell culture applications. It has become clear that targeting the redox pathways is a practicable way to treat cancer [52, 53]. Many approved drugs for cancers' treatment such as bleomycin, bortezomib, cisplantin and anthracyclines have been revealed to influence cells by generating ROS [52, 54]. In AML, increased levels of ROS are supposed to play an important role [55]. For instance, FLT3-ITD expressing cell lines have been shown to have elevated levels of endogenous ROS [56]. By virtue of this critical relation between AML and ROS, targeting ROS production has been presumed to be fruitful as treatment against AML [55, 57, 58]. Therefore, resazurin appears to be a promising substance which can be further developed as a treatment against AML with low toxicity.

1.2. The tumor suppressor protein p53

The *TP53* gene, first described in 1979, encodes the tumor suppressor protein p53 which is a universal nuclear transcription factor in most of cell types [59, 60]. By virtue of being a transcription factor, p53 carries out its functions by binding specifically to response elements (REs) of target genes and increasing the transcription rate of these genes [61]. The protein has been considered as the "guardian of the genome" because of its essential roles in maintaining genetic stability, inducing cellular differentiation and delaying the development of cancer [60, 62]. The importance of p53 in genome protection was revealed by the fact that nearly 50% of all cancers bear mutations in the *TP53* gene [63, 64]. In the remaining cancers, disruption of the p53 pathway such as overexpression of Mdm2, the negative regulator of p53 has been shown [65, 66]. AML is a typical example of these cancers. The incidence of *TP53* mutations in AML is less than 10% of all patients [67, 68], but Mdm2 has been found to be overexpressed frequently [69, 70]. Although *TP53* mutations in AML are not common, they are strongly connected with a complex aberrant karyotype [71] and are a prognostic factor of poor survival [68, 72].

1.2.1. Downstream responses to p53 activation

The p53 protein is normally preserved in an inactive form [60]. It is activated and stabilized when cells are exposed to diverse stress signals endangering the genomic integrity of the cells. These kinds of cellular stress consist of DNA damage, oncogenic activation, ribosomal stress, loss of cell-cell contacts and hypoxia [73]. Once p53 is activated, it is able to regulate a complex transcriptional program which commences numerous biological responses comprising cell-cycle arrest, senescence, apoptosis, DNA repair, control of mitochondrial respiration and differentiation [73, 74] (**Figure 1-1**).

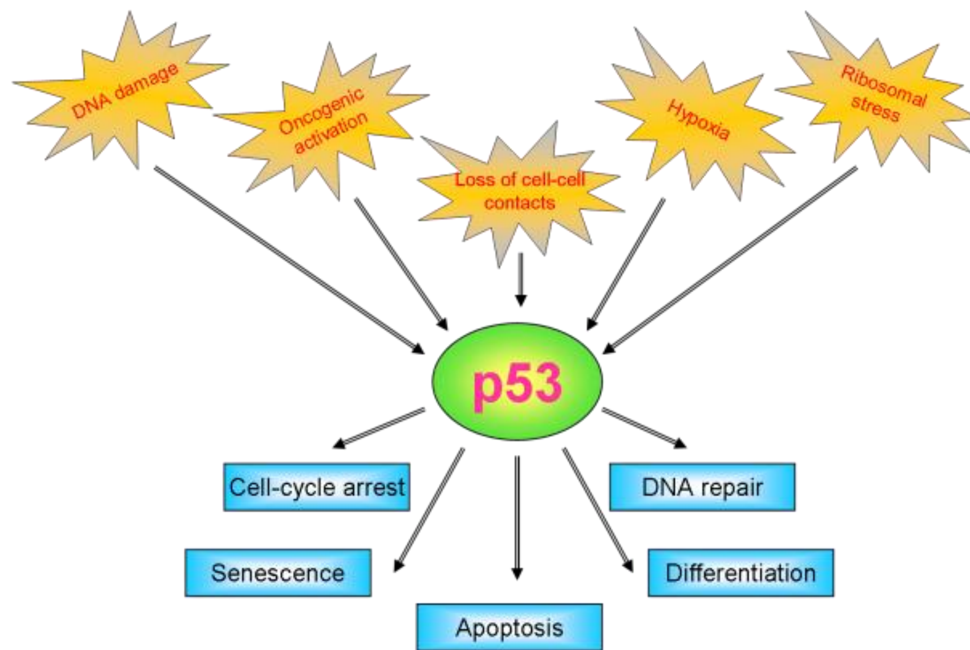


Figure 1-1 p53 activation and downstream responses. p53 is activated by numerous cellular stress signals which can initiate various responses resulting in genomic stability. Figure adapted from [73].

One of the substantial effects of p53 is that it provokes the cyclin-dependent kinase (CDK) inhibitor p21$^{WAF1/CIP1}$ expression [75]. CDKs, together with cyclin proteins, are fundamental in the regulation of cell-cycle progression. The binding of cyclin to CDK results in the activation of the enzyme which ensures the progression of cell cycle, for instance, from G1 phase to S phase or from G2 phase to M phase. The negative effect of p21$^{WAF1/CIP1}$ on CDKs' activity has been shown that it causes an arrest in transition from G1 phase to S phase of cell-division-cycle [76]. Not only does p21$^{WAF1/CIP1}$ evoke cell-cycle arrest, but it also stimulates cellular senescence [77]. As a result, p53 can suppress the proliferation of stressed cells and prevent the propagation of mutations effectively.

Another significant response to p53 activation is the induction of apoptosis [73]. The binding of p53 to the regulatory region of *BAX* gene activates directly the transcription of the gene which leads to the overexpression of pro-apoptotic Bax protein [78]. Furthermore, some genes encoding pro-apoptotic BH3-only proteins such as Noxa and Puma are also activated by p53 [79, 80]. Once these proteins are overexpressed, they induce the release of cytochrome *c* and other proteins from the intermembrane space of the mitochondria into the cytosol and further activate the caspase cascade which leads to apoptosis [81]. Interestingly, it has also been presented that p53 can bind directly to the anti-apoptotic protein Bcl-2 or the pro-apoptotic protein Bax and induce apoptotic cell death [82, 83]. Taken together, it would indicate that p53 is significant in inducing apoptosis in both a transcriptional and non-transcriptional manner [84].

Research has also demonstrated that p53 plays a direct role in DNA repair which helps to maintain genomic stability and prevent tumor development. When activated by genotoxic stress, the protein directly initiates the transcription of repair genes such as *MSH2, DDB2* and stimulates removal of alkyl adducts by O^6-methyl-guanine-DNA-methyl-transferase (MGMT), mismatch repair (MMR), and nucleotide excision repair (NER) respectively [85]. In addition, it has been revealed that activated p53 induces the expression of target genes diminishing the level of intracellular ROS which mainly cause DNA damage and genetic instability [86]. Also, the p53 protein promotes differentiation which does not result in the abolishment of damaged cells, but removes them from proliferation instead. Thus, it aids the organism in maintaining the stability of the genome. In human cancers, the presence of mutated p53 is usually correlated with a poor grade of differentiation [74].

These responses to the activation of p53 suggest that the severity of stress determines cell fate through the action of p53. Low levels of stress cause a temporary block in cell cycle which gives time for removing intracellular ROS and repairing DNA, whereas the severe ones initiate apoptotic cell death that eradicates irreparable damaged cells [87]. Both consequences result in the maintenance of genetic stability and the suppression of tumor formation.

1.2.2. The regulation of p53

1.2.2.1. Regulation of p53 functions

Because the activation of p53 decides the cell fate, it is clear that p53 activities must be very strictly regulated to ensure normal development of the cells [59, 73]. The regulation of p53 has been described to consist of numerous different forms which comprise post-translational

modifications (PTMs) of p53, regulation of p53 stability or direct inhibition of p53's transcriptional activity [73]. While PTMs such as phosphorylation, acetylation, methylation, glycosylation and ribosylation [88, 89] have been shown to have merely modulatory functions in p53 regulation *in vivo*, stabilizing p53 or inhibiting directly p53 has revealed insightful roles in the regulation of p53's functions [90].

The cellular quantity of p53 protein is principally controlled by the rate of degradation [60]. The degradation of p53 is primarily performed via the core 26S proteasomes by an ubiquitin-dependent mechanism [91]. The E3 ubiquitin ligase Mdm2 which human homologue is Hdm2 has been presented as the key negative regulator of p53 [92]. Mdm2 is responsible for attaching ubiquitin groups to the C-terminal tail of p53 which targets the protein for subsequent proteasomal degradation [93]. In spite of the fact that Mdm2 is the main negative regulator of p53, the gene encoding it is also one of the transcriptional targets of p53 [94]. This creates a negative feedback loop in which p53 initiates the transcription of *MDM2* gene which is later translated into Mdm2. Then, the protein promotes the degradation of p53 which helps to keep the low level of p53 in physiological condition [73]. In addition to Mdm2, other ubiquitin ligases, for example, Cop1, Pirh2, HectH9/MULE/ARF-BP1, E6AP and CHIP which also promote the degradation of p53 have been recognized [73]. Although Mdm2 is able to down-regulate p53 sufficiently, it does not necessarily follow that it works alone. Some proteins have been described to work in cooperation with Mdm2 for the regulation of p53 such as p300/CBP [95] and gankyrin [96, 97].

Not only is p53 degraded by the ubiquitin-dependent mechanism, but it can also be degraded via an ubiquitin-independent mechanism. This degradation is performed through the core 20S proteasomes and is regulated by the NAD(P)H quinone oxidoreductase 1 (NQO1) [98]. NQO1 is found mainly in the 20S proteasomes and it prevents the degradation of p53 from the 20S proteasomes by directly binding to p53 [99].

Research has revealed that MdmX (Mdm4), a structural relative of Mdm2, is another important negative regulator of the tumor suppressor protein p53 [100]. Nevertheless, while Mdm2 acts as an E3 ubiquitin ligase which promotes the degradation of p53, MdmX does not have the activity of E3 ligase and functions by binding directly to p53 and inhibiting its transcriptional activity [101, 102]. The fact that MdmX has a Mdm2-independent function in the regulation of p53 does not necessarily mean that it works distinctly. In contrast, these two proteins have been shown to interact mutually [102].

1.2.2.2. Redox regulation of p53

While it is the case that p53 plays a fundamental role in the control of mitochondrial respiration, a main cellular process generating ROS which is pivotal to redox signaling, it is also regulated by redox signals which decide the destinies of the cell through selective transcription of p53 target genes [103]. For instance, whereas nuclear oxidative stress stimulates DNA repair dependent on p53 activity [104], the excess production of ROS in mitochondria caused by chemotherapeutic agents results in apoptosis [105, 106]. This proposes that there are several pathways existing in redox and p53 signaling network which lead to many different types of response. The mechanism of how ROS regulates p53's activities; however, is still elusive [103].

Besides the effects on p53 through the signaling networks, ROS may also directly affect the activities of p53 via PTMs. For example, the implication of ROS in the phosphorylation of p53 by protein kinases such as p38α MAPK (mitogen-activated protein kinase) [107], ATM (ataxia-telangiectasia mutated protein) [108], and ERK (extracellular signal-regulated kinases) [109] has been presented. Nonetheless, the activation of these enzymes is not necessarily specific to ROS. Moreover, how p53 stability is influenced by redox modification still remains mysterious [103, 110].

1.3. The isoforms of p53

1.3.1. Background

The tumor suppressor protein p53 is the member of a family which also consists of p63 and p73, two transcription factors sharing homologies in structure, biochemistry and biology with p53 [111, 112]. Many protein isoforms have been shown to be expressed by *TP63* and *TP73* genes [112]. Similarly, the human *TP53* gene has also been described to encode 12 different p53 protein isoforms via various mechanisms [113, 114] (**Figure 1-2**).

Some clinical cancer studies have revealed that the aberrant expression of some of the p53 isoforms may induce the inactivation of p53 functions [115-117]. The documentation that p53 isoform expression is deregulated in human cancers is arising. The isoforms of p53 may have functions in all biological activities that are regulated by both p53-dependent and p53-independent mechanisms. Therefore, it should not be thought that the p53 pathway is regulated by only p53 anymore, but by the interaction of p63, p73 and p53 isoforms [118].

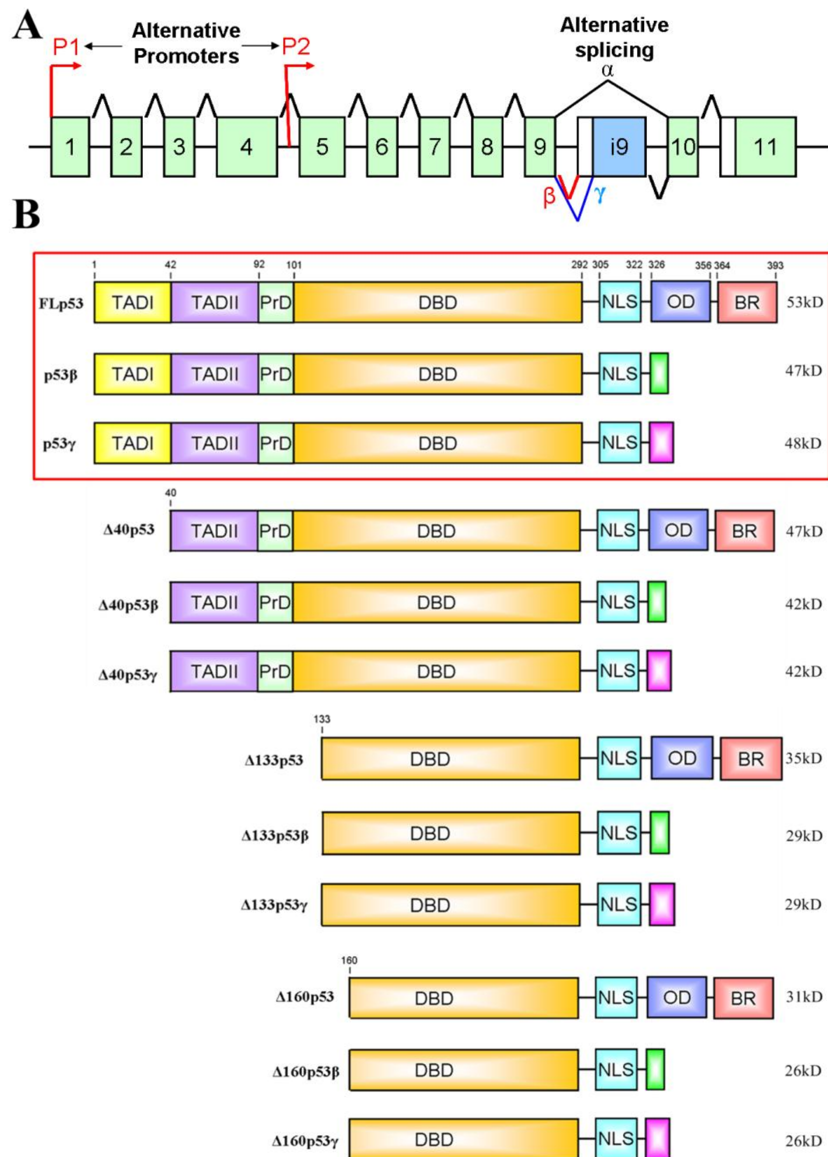


Figure 1-2 Human *TP53* gene and p53 isoforms. (A) The human *TP53* gene which contains 11 exons expresses numerous p53 isoforms due to the use of alternative promoters, splicing sites or translational initiation sites. **(B)** The full-length version of p53 (FLp53) consists of 393 amino acids and is composed of separate structural and functional domains such as transactivation domains (TADI and TADII), proline rich domain (PrD), DNA binding domain (DBD), nuclear localization signal (NLS) domain, oligomerization domain (OD) and basic region (BR) regulatory domain. Alternative splicing of intron-9 (exon 9b) produces 2 different C-terminal truncated isoforms, p53β and p53γ, which replaced the OD of FLp53 with 10 or 15 additional amino acids, respectively [115, 119, 120]. Alternative splicing of intron-2 results in the N-terminal truncated isoforms Δ40p53, Δ40p53β, Δ40p53γ. The internal promoter (P2) in intron-4 regulates the expression of Δ133p53, Δ133p53β and Δ133p53γ isoforms [115]. The isoforms Δ160p53, Δ160p53β and Δ160p53γ are created by the usage of alternative translational initiation [114]. The C-terminal domains of p53β (DQTSFQKENC) and p53γ (MLLDLRWCYFLINSS) are demonstrated with a green and pink box, respectively. The molecular weight of each p53 isoform is indicated on the right. The red box points out p53 isoforms studied in this thesis. Illustration adapted from [118, 121].

1.3.2. p53 isoforms β and γ

1.3.2.1. The functions of p53β and p53γ

It is unclear whether the C-terminal truncated p53 isoforms p53β and p53γ have their own functions or they function together with full-length p53 (FLp53). While Graupner and colleagues have reported that p53β and p53γ do not have any effect on senescence, apoptosis, and transcription [122], Bourdon and his fellows have found that they have transcriptional and direct protein interaction ability as well as specific intracellular localization [115, 118, 121]. It has been revealed that p53β is mainly localized in the nucleus, while p53γ is either in the nucleus or in the cytoplasm, proposing that p53γ shuttles between these two cellular compartments [115]. The p53β isoform was shown to be able to bind to the *BAX* or p21 promoter and enhance the transcriptional activity of FLp53 on the p21 promoter [115]. Moreover, p53β may induce apoptosis independently of FLp53, though the efficiency is lower than FLp53 [121]. It was also revealed that p53β acts in concert with FLp53 to increase replicative cellular senescence of human normal fibroblasts [123]. The p53γ isoform can bind to the internal promoter of FLp53 and improve the transcriptional activity of FLp53 on the *BAX* promoter, but not on the p21 promoter [115]. Both p53β and p53γ were found to affect an optimized p53-responsive element [124]. In previous studies, difficulties in generating cells with functional p53γ led to the conclusion that p53γ is cytotoxic [121]. This finding has been recently supported by stable low-level expression of p53γ protein in $p53^{null}$ NCI-H1299 lung cancer cell line retrovirally transduced with p53γ construct [124].

1.3.2.2. Regulation of p53β and p53γ

As mentioned before, p53β and p53γ proteins are generated by the alternative splicing of intron-9 of the human *TP53* gene. However, how this alternative splicing is controlled has not been elucidated [118]. The RNA-binding protein SRSF3 (also named SRp20) is the smallest member of the highly conserved serine/arginine-rich splicing factor family which has been shown that its down-regulation will force the splicing of *TP53* pre-mRNA towards the p53β isoform [125]. Recent studies have proposed that SRSF3 has pro-oncogenic activity based on its up-regulation in different kinds of human cancers [126, 127], and its capability in stimulating neoplastic alteration when overexpressed [126], signifying its importance in tumorigenesis. This evokes a question as whether a correlation between the expression of SRSF3 and the p53 isoforms expression exists.

The OD of p53 is the main site of PTMs which regulates p53 stability, for instance, the ubiquitination of lysine residues by Mdm2 [128]. By virtue of the presence of a stop codon in exon 9b, both p53β and p53γ isoforms lack the OD [121]. This absence led to the study as to whether Mdm2 regulates the stability of p53β and p53γ. It was shown that p53β appears to be regulated by Mdm2-mediated ubiquitination, whereas p53γ seems to be ubiqutinated by other E3 ligases and very unstable because of proteasomal degradation [124, 129]. In addition, the NAD(P)H quinone oxidoreductase (NQO1) enzyme was suggested to play roles in p53γ stability [124]. These findings have revealed that the stability of p53β and p53γ are differentially regulated [124]. Therefore, clarifying the mechanisms how p53β and p53γ are degraded may help to comprehend how these isoforms of p53 are deregulated in cancer.

It has been shown that redox plays roles in the regulation of p53, but how it regulates p53 functions as well as stability has not been well understood. Therefore, it will be interesting to investigate if redox modulates the expression of p53 isoforms as a mechanism for this regulation.

1.3.2.3. p53β and p53γ in cancer

Numerous clinical studies have reported that the expression of p53 isoforms is abnormal in various types of human cancers such as breast cancer, AML, head and neck cancers, melanoma, renal cell carcinoma, and colon, ovarian, and lung cancers, proposing that the aberrant expression of the p53 isoforms could contribute to the formation and progression of cancer [115, 116, 130-135]. The expression of p53β and p53γ was proved to be altered in cancers and had diverse roles. For example, in colon carcinomas, the expression of p53β was shown to be decreased [123]. In mutant p53 breast cancer patients, the expression of p53γ is connected with a prognosis as good as in wild-type p53 breast cancer patients [134]. Moreover, p53β expression in primary ovarian cancers was linked with worse recurrence-free survival in patients expressing wild-type p53 [135]. In AML patients, the expression of p53β and p53γ was correlated with increased chemotherapy-response and longer survival [136]. In addition, the ectopic expression of p53β and p53γ in $p53^{null}$ osteosarcoma and lung cancer cell lines has been shown to augment chemosensitivity [124]. Therefore, it is interesting to investigate the functional roles of these isoforms in AML models.

1.4. Nucleophosmin (NPM1)

1.4.1. Classification, function and regulation

Nucleophosmin (NPM1), also called B23 or Numatrin, is a member of the nucleoplasmin family of nuclear chaperones and is expressed in all tissues [137]. In humans, it is encoded by the *NPM1* gene located on chromosome 5q35 [138]. By virtue of alternative splicing, three different isoforms of NPM1 are created. The dominant isoform NPM1 (or B23.1) is a phosphoprotein of about 37 kDa comprising 294 amino acids [139]. Whereas NPM1 protein is only localized in the nucleolus, the second isoform NPM1.2 (B23.2) is principally limited in the nucleoplasm [139-142]. Despite nucleolar localization, NPM1 shuttles steadily between nucleus and cytoplasm [143] as well as between nucleoplasm and nucleolus [144].

NPM1 is a fundamental protein for embryonic development [145]. Additionally, it also plays an essential role in numerous critical cellular functions including regulation of ribosome biogenesis, stabilization of the oncosuppressor p14Arf protein in the nucleolus and control of centrosome duplication regarding its function in the establishment of many protein-protein interactions as a molecular chaperone [146]. The characteristics of a nuclear-cytoplasmic shuttling protein are crucial for most of its functions [146].

Trafficking of NPM1 inside the nucleus is primarily conducted by diffusion, a rapid, non-directional process, which often happens via energy-independent mechanisms [147]. The transferring of NPM1 protein to the nucleolus is also assisted by its ability to form homodimers [148]. In addition, post-translational modifications of NPM1 consisting of phosphorylation, ubiquitination and sumoylation, are probable to give a contribution to the regulation of its cellular trafficking [149-154].

1.4.2. NPM1 mutations in AML

Translocation or mutation of the *NPM1* gene has been detected in numerous hematological malignancies [155], and these genetic transformations frequently disturb the normal traffic of NPM1 inside the cells. The aberrant expression of mutated NPM1 in leukemic cell cytoplasm is approximately 35% of adult AML [156], and this ectopic expression is usually seen in the entire leukemic cell population [144]. Cytoplasmic NPM1 is also the immunohistochemical hallmark of AML with mutated *NPM1* [157], which is specific for recognition of AML cells with a *NPM1* mutation [144].

Despite the fact that around 50 variants of *NPM1* mutation have been identified until now, all of them cause a shift of the reading frame creating common alterations at the C-terminal end of NPM1 leading to the abnormal localization of the mutated NPM1 in the cytoplasm of leukemic cells [158]. Approximately 80% of *NPM1* mutations in AML are type A which is created by a duplication of a TCTG tetranucleotide at positions 956–959 in the *NPM1* gene [159]. The extraordinary accumulation in the cytoplasm of mutated NPM1 is likely to play a critical role in the generation of AML, yet the mechanism is still unknown [144].

Leukemic NPM1 mutants induce the dislocation of their various protein partners into the cytoplasm. One of them is the tumor suppressor protein p14ARF which is a critical positive regulator of the p53 protein in response to oncogenic activation [160]. ARF binds to Mdm2 and inhibits its activity, thereby increases the level of p53. Because the interaction of NPM1 and ARF is mostly studied in murine cell lines, the focus is on its murine ortholog p19Arf. The protein p19Arf forms a complex with wild-type NPM1 which makes them localize together in the nucleolus [161]. Since this interaction helps p19Arf have a stable structure [162], it seems to protect p19Arf from the rapid proteasomal degradation. Mutated NPM1 may contribute to the inactivation and dislocation of p19Arf and further cause AML development because the stability and subcellular distribution of p19Arf play crucial roles in maintaining its basal levels and functional activity [163]. Research has shown that when p19Arf makes a complex with the mutated NPM1, its stability is substantially prejudiced and the p53-dependent cell-cycle arrest becomes weaker [164].

Interestingly, many studies have found that AML with mutated NPM1 often bears normal karyotype and is associated with good prognosis when FLT3-ITD is not present [165-170]. Moreover, the presence of *NPM1* mutation was connected with high expression of p53β and p53γ proteins in AML [136]. It appears to be the case that the alterations of NPM1 have an effect on p53β and p53γ expression. By virtue of the involvement of NPM1 in ribosomal biogenesis, it is probable that such implication contributes to the regulation of p53 isoform expression [146]. The actual mechanism, however, is still unknown.

2. Aims

The main aims of this thesis have been to characterize the functional roles of p53 isoforms β and γ and to investigate the modulation of full-length p53 (FLp53), p53β and p53γ in acute myeloid leukemia (AML), with particular objectives as follows:

1) Investigate functions of p53β and p53γ independent of FLp53 with respect to chemotherapeutic sensitivity in a $p53^{null}$ AML cell line;
2) Establish an increased expression of p53β or p53γ in wild-type *TP53* AML cell line in order to study their functions in concert with FLp53 and endogenous p53β and p53γ;
3) Examine the effect of p53β and p53γ on AML cell line and the modulation of p53 isoforms in response to reactive oxygen species through treatment with resazurin;
4) Examine the correlation between wild-type NPM1, mutated NPM1 and p53 isoforms modulation in a wild-type *TP53* cell line.

3. Materials and Methods

3.1. Cell culture

3.1.1. Culturing cells

The FAB class M2 p53^{null} HL-60 AML cell line [171] was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and the FAB class M5 wild-type *TP53* MOLM-13 AML cell line [172] was bought from ATCC (American Type Culture Collection, Manassas, VA, USA). MOLM-13 cells transduced with vector control (shControl) and short hairpin RNA against p53 (shp53) were kindly provided by Dr. Gro Gausdal and Professor Stein Ove Døskeland (University of Bergen, Norway). HL-60 cells transduced with p53β-tdTomato, p53γ-tdTomato and tdTomato alone (vector control) were generously supplied by Dr. Elisabeth Silden (University of Bergen, Norway). All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, Inc., St. Louis, MO, USA). Human Embryonic Kidney 293 (HEK293) cell line (ATCC) and HEK293T-based Phoenix Amphotropic packaging cell line (generous gift from Professor Jim Lorens, University of Bergen, Norway) were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich). Cell culture media were supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (PAA Laboratories GmbH, Pashing, Germany), 50 UI/mL Penicillin – 50 µg/mL Streptomycin and 2 mM L-glutamine (all from Sigma-Aldrich). The cells were kept in humidified incubators at 37°C with 5% CO_2 and observed under light microscope daily to ensure optimal proliferative and bacterial free conditions.

All suspension cell lines were maintained at 0.2-1.0×10^6 cells/mL for the duration of experiment and split at a ratio of 1:5 thrice weekly as required. The adherent cells were grown in 10 cm culture dishes and split at approximately 80% confluence by trypsinization twice weekly as required. Trypsinization was performed by the removal of medium, followed by washing two times with 5 mL of 0.9% NaCl (Fresenius Kabi, Bad Homburg, Germany). 1 mL of 1X trypsin (Sigma-Aldrich) was added to the cells for 5 minutes at room temperature. The cells were then added 9 mL of medium for neutralization of the trypsin and three different volumes (1, 0.5 and 0.25 mL) of cells were separately added into medium with a total volume of 10 mL.

3.1.2. Thawing cells

Cells were rapidly thawed by heating the vial containing approximately 5.0×10^6 cryopreserved cells in hands until it was almost defrosted. 1 mL of medium was added, and the vial was left at room temperature (RT) for 5 minutes. Then, the cells were transferred to a 15 mL tube containing 10 mL of medium and centrifuged at 500g × 5 min. The supernatant was discarded to remove the cryoprotectant dimethyl sulfoxide (DMSO) and the pellet was resuspended in 10 mL of medium. Thereafter, the cell suspension was transferred to a 25 cm^2 flask. The cells were cultured under normal growth conditions and the medium was changed after 2-3 days.

3.1.3. Cryopreserving cells

Cells were counted and followed by centrifugation at 500g × 5 min. The supernatant was discarded and the pellet was dissolved in freezing medium (70% medium, 20% FBS and 10% DMSO (Scharlab S.L., Sentmenat, Spain)) at a concentration of 5.0×10^6 cells/mL. Then, each 1mL of cell suspension was transferred to a cryogenic vial (Sarstedt, Nümbrecht, Germany). The vials were stored at -80°C for at least 24 hours before transferring to a liquid nitrogen tank (-196°C) for long-term storage.

Counting cells was achieved by using a hemocytometer. All cell work was carried out under sterile conditions through the utilization of a laminar flow bench with a high efficiency particulate air (HEPA) filter.

3.2. Plasmid production

3.2.1. Plasmid construction

In order to study the correlation between NPM1 and p53 isoforms modulation, the utilization of recombinant plasmid containing *NPM1* DNA as well as an empty vector control is required. Because of the lack of vector control, we created it from the recombinant plasmid.

Approximately 3 µg of pCDNA3$^+$-*NPM1*-ECGFP plasmids (generously provided by Dr. Marikki Laiho, Johns Hopkins, USA) containing wild-type *NPM1* gene were digested by 2 µL Fermentas FastDigest *Eco*R*I* (Thermo Scientific, Wilmington, DE, USA) at 37°C for 1 hour. After digestion, 1% agarose gel electrophoresis was performed to separate the *NPM1* gene from the digested plasmid. The remaining part of the digested plasmid was collected by cutting the gel under a UV light and purified by QIAquick® Gel Extraction Kit (Quiagen

Inc., Valencia, CA, USA) according to the protocol of the manufacturer. Thereafter, the vector control was created by ligation reaction using USB Ligate-IT™ Ligation Kit (Affymetrix Inc., Santa Clara, CA, USA) following the producer's instructions.

3.2.2. Transformation of E.coli cells

The ligation mixture was chemically transformed into EO-Top10 competent *Escherichia coli* cells (kindly provided by Dr. Line Wergeland, University of Bergen, Norway) using the heat shock method [173]. The transformed *E. coli* cells were grown on Luria-Bertani (LB) (Sigma-Aldrich) plates with 50 µg/mL Ampicillin (Sigma-Aldrich) at 37°C overnight. Three random single colonies of *E. coli* were scraped off the selective LB plates the following day and grown in LB medium with 50 µg/mL Ampicillin at 37°C overnight.

3.2.3. Purification and digestion of plasmids

Bacteria were harvested by centrifuging at 10,000g × 5 min. Then, they were lysed and the plasmid was purified by using QIAprep® Spin Miniprep Kit (Quiagen) following the manufacturer's protocol. The concentration of the plasmid was measured by NanoDrop UV-Vis spectrophotometer (Thermo Scientific). The plasmids from three chosen colonies and the mother vector (control) were digested by restriction enzymes *Bam*HI (New England Biolabs Inc., Beverly, MA, USA) or *Eco*R*I* according to producers' instructions. Agarose gel electrophoresis was performed after digestion in order to examine the successful construction of vector control. 1Kb Plus DNA Ladder (Invitrogen) was used. Wild-type *NPM1-ECGFP* vector, mutated *NPM1-ECGFP* vector and the successfully generated vector control were exaggerated and purified by QIAfilter™ Plasmid Maxi Kit (Quiagen) using the protocol of the vendor.

3.2.4. DNA sequencing

The vector control, wild-type *NPM1-ECGFP* construct and mutated *NPM1-ECGFP* construct (kind gift from Dr. Randi Hovland, University of Bergen, Norway) were sequenced prior to transfection into HEK293 cells to confirm correct *NPM1* sequence. Sequencing PCR was performed by using BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with a forward primer for *T7* (5'-TAATACGACTCACTATAGGG-3') which is subsequently followed by *NPM1* sequence and a forward primer towards the middle of *NPM1* sequence (5'-CATCAACACCAAGATCAAAA-3') (Sigma-Aldrich) to guarantee that the sequencing reaction detects the whole segment. PCR was carried out as follows: an initial denaturing step at 96°C for 10 min, then 30 cycles of 96°C for 10s, 50°C

for 5s, 60°C for 4 min, and a final extension at 72°C for 7 min. PCR products were sequenced at Sequencing Facility, MBI, University of Bergen, Norway. DNA sequences were translated into amino acid sequences by using http://web.expasy.org/translate/ and then aligned with known protein sequences (downloaded from http://www.ncbi.nlm.nih.gov/pubmed) by using http://www.ebi.ac.uk/Tools/msa/clustalw2/.

3.3. Transduction and transfection of cells

3.3.1. Retroviral transduction of cells

3×10^6 HEK293T-based Phoenix Amphotropic packaging cells were seeded in each 10 cm culture dish with 6 mL of growth medium and grown at 37°C with 5% CO_2 for 24 hours. Thereafter, the cells were transfected with vector control (L335, kindly supplied by D.R. Micklem, University of Bergen, Norway), p53β-tdTomato vector or p53γ-tdTomato vector (generously provided by Dr. Elisabeth Silden) using 50 μM Chloroquin (Sigma-Aldrich) and 2 mL of a transfection mixture consisting of 128 mM $CaCl_2$ (Sigma-Aldrich), 4 μg of the retroviral vectors and 1X HBS (0.75 mM Na_2HPO_4, 8 g/L NaCl, 6.5 g/L HEPES sodium salt, pH 7.0) for 8 hours. They were then grown in 7 mL of fresh growth medium for 40 hours to produce retroviral particles. After 48 hours of transfection, the media containing retroviral particles were collected.

100×10^3 MOLM-13 cells were plated in each well of 6-well plates. 1 mL of medium containing viral particles and 50 μg/mL Protamine Sulfate (Sigma-Aldrich) was added into each well. The infection of cells was performed by centrifuging at 1200g × 90 min. The medium was changed after 24 hours of infection and the cells were grown up to prepare for assessing tdTomato expression by flow cytometry (see section 3.4.2).

3.3.2. Transient transfection of cells

3×10^6 HEK293 cells were seeded in each 10 cm culture dish and grown at 37°C with 5% CO_2 for 24 hours. Thereafter, the cells were transiently transfected with 6 μg of vector control, wild-type *NPM1-ECGFP* or mutated *NPM1-ECGFP* construct using XtremeGENE 9 DNA Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. After 48 hours of transfection, transfected cells were examined by flow cytometry (see section 3.4.2) and Leica DM IRB fluorescence microscope (Leica, Bensheim, Germany).

3.4. Flow cytometry

Flow cytometry permits the examination of both morphological and fluorescent properties of individual cells in suspension. Therefore, it can be exerted for evaluating fluorescent protein expression, sorting cells as well as analyzing cell viability.

3.4.1. Fluorescence Activated Cell Sorting (FACS)

10×10^6 transduced HL-60 cells or 30×10^6 transduced MOLM-13 cells were collected, washed once with sterile 0.9% NaCl and resuspended in sterile 0.9% NaCl at a concentration of 5×10^6 cells/mL. Cells expressing tdTomato were isolated by BD FACSAria™ Cell Sorter (BD Biosciences, San Jose, CA, USA) using a 532 nm laser. Transduced HL-60 cells were re-sorted prior to each experiment to guarantee that the population of cells mostly contains $tdTomato^+$ cells.

3.4.2. Assessment of tdTomato or ECGFP expression

The expression of tdTomato in transduced MOLM-13 cells was evaluated by BD LSRFortessa™ cell analyzer (BD Biosciences) using a 561 nm laser. Red fluorescence was discovered through a 585/42 nm bandpass filter. The percentage of $tdTomato^+$ cells was analyzed by FlowJo software (Tree Star Inc., Ashland, OR, USA).

The expression of tdTomato in transduced HL-60 cells and the expression of ECGFP in transfected HEK293 cells were assessed by Guava® EasyCyte™ flow cytometer (Millipore Corp., Hayward, CA, USA) using a 488 nm laser. The detection of tdTomato was achieved via a 583/26 nm bandpass filter whereas ECGFP expression was exposed through a 525/30 nm bandpass filter. Data were analyzed by InCyte™ software (Millipore).

3.4.3. Annexin V – To-pro-3 flow cytometric analysis

Cells stained with both Annexin V – Alexa Fluor 488 and To-pro-3 (see section 3.6.3) were analyzed by the Guava® EasyCyte™ flow cytometer using both 488 nm and 640 nm lasers. Green fluorescence (Alexa Fluor 488) was detected through a 525/30 nm bandpass filter while far-red fluorescence (To-pro-3) was revealed via a 661/19 nm bandpass filter. The assay works in the following manner: Viable cells are both Annexin V-Alexa 488 and To-pro-3 negative, while cells which are in early apoptosis are Annexin V-Alexa 488 positive and To-pro-3 negative, and cells which are in late apoptosis or already dead are both Annexin V-Alexa 488 and To-pro-3 positive (**Figure 3-1**). A minimum of 5,000 events were analyzed to acquire the percentage of viable, apoptotic and dead cells using the InCyte™ software.

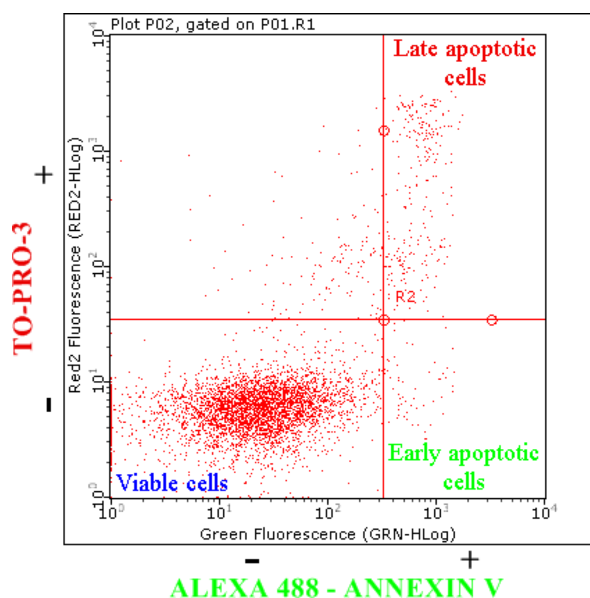


Figure 3-1 Example of Annexin V – To-pro-3 flow cytometric analysis. MOLM-13 cells were stained with Annexin V-Alexa Fluor 488 and To-pro-3. Quadrant gates divide the sample into groups of interest: Viable cells (Annexin-V -, To-pro-3 -), early apoptotic cells (Annexin-V +, To-pro-3 -) and late apoptotic cells (Annexin-V +, To-pro-3 +).

3.5. Colony formation assay

HL-60 cells and MOLM-13 cells were plated in triplicate at a density of 1×10^3 cells in 0.4 mL methylcellulose-based H4433 Methocult® medium (StemCell Technologies Inc., Vancouver, Canada) per well on a 24-well plate. Six wells of the plate were used while the remaining wells were filled with 0.4 mL sterile 0.9% NaCl to prevent the Methocult medium from drying out. The cells were grown in a humidified incubator at 37°C with 5% CO_2. After 7 days, the number of colonies which contain at least 40 cells was counted and images were taken by using Nikon TE2000 fluorescence microscope (Nikon Corp., Tokyo, Japan).

3.6. Cell proliferation and cell viability analysis

3.6.1. WST-1 based cell proliferation assay

Proliferation of cells was assessed by using the tetrazolium salt WST-1 which is cleaved to chromogenic formazan dye by mitochondrial dehydrogenases in viable cells. HL-60 cells were seeded in triplicate at a density of 20×10^3 cells in 100 µL of medium per well on 96-well plates, treated with 0.5 µM Doxorubicin (Pfizer Inc., NY, USA) or 0.5 µM Camptothecin (Sigma-Aldrich) or DMSO (vehicle) for 8 or 24 hours, and WST-1 (Roche Ltd, Basel, Switzerland) was added into each well with the dilution 1:11 at the last 4 hours of treatment period. The absorbance of the samples was measured by Spectramax Plus 384 Spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA) and the percentage of

proliferation was calculated by using the following formula: (Arbitrary units (AU) of treated sample - AU of background control)/(AU of untreated sample – AU of background control).

3.6.2. Nuclear morphology cell death assay

Cells were seeded in triplicate on 96-well plates. The cells were treated before they were fixed and stained with 4% formaldehyde (Sigma-Aldrich) and 10 µg/mL Hoechst 33342 (Enzo LifE Sciences AG, Lausen, Switzerland). Hoechst 33342 is a cell-permeable fluorescent dye which specifically binds and stains DNA at adenine-thymidine residues. After fixing and staining for at least 24 hours, the nuclear morphology of cells was examined and taken pictures by using the Leica DM IRB fluorescence microscope. Abnormal nuclei were discriminated from normal ones by their hypercondensed chromatin and fragmented nucleus [174] (**Figure 3-2**). 200-300 cells were counted in each well to calculate the percentage of normal nuclei.

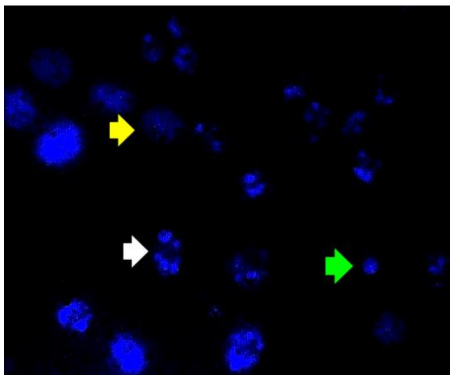


Figure 3-2 Hoechst 33342 staining. Fluorescence microscopy image of HL-60 cells treated with 0.5 µM Camptothecin for 8 hours and then stained with Hoechst 33342. Yellow arrow indicates a normal nucleus. White and green arrows indicate fragmented and hypercondensed nuclei respectively.

3.6.3. Annexin V – To-pro-3 cell death assay

When a cell begins to undergo apoptosis, phosphatidyl serine (PS) is translocated from the cytoplasmic surface of the cell membrane to the outer extracellular facing layer. This specific marker can be utilized to recognize apoptotic cells by conjugating the PS binding protein, Annexin V, to a fluorophore. Additionally, by simultaneously staining cells with To-pro-3, a fluorescent dye, selectively binding to the DNA of dead cells, a comprehensive assessment of apoptosis and cell death can be obtained by flow cytometry.

Approximately 100×10^3 cells from each sample were directly transferred to a flow tube and pelleted by centrifugation at 500g for 5 min at 4°C. The cells were then washed twice with cold 0.9% NaCl and resuspended in 50 µL 1X Annexin Binding Buffer (Invitrogen, Carlsbard, CA, USA) containing 1.25 µL Annexin V- Alexa Fluor 488 (Invitrogen). Samples

were left on ice and incubated in complete darkness for 15 min. Thereafter, 200 μL 1X Annexin Binding Buffer containing 1.5 μL of 5 μM To-pro-3 (Invitrogen) was added to each tube. The samples were then analyzed by flow cytometry (see section 3.4.3).

3.7. Western blot analysis

Cells were harvested by centrifugation. Then, they were washed twice in cold 0.9% NaCl and lysed in Lysis Buffer (10 mM Tris pH 7.5, 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% NP40, 5 mM NaF, 0.5 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). Following the addition of the lysis buffer, the cell lysate samples were homogenized in 1.5 mL tubes by pipetting and then centrifuged for 10,000g × 15 min at 4°C. Lysates were then maintained on ice for measuring protein concentration or stored at -80°C until required. Protein concentrations were determined by the Bradford method [175]. Lysate for each sample was diluted in loading buffer (final concentration 1% SDS, 10% Glycerol, 12 mM Tris-HCl pH 6.8, 50 mM DTT, and 0.1% Bromophenol Blue) to create a total protein amount of 50 μg per sample. Samples were boiled for 15 min and then briefly spun down prior to loading onto gel.

Proteins were separated by sodium dodecyl suphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% SDS-polyacrylamide gels and 1X TGS running buffer (25 mM Tris-base, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3) for approximately 2.5 hours at 100V. The standard used was Precision Plus Protein™ All Blue standards (Bio-Rad Laboratories, Hercules, CA, USA). Thereafter, electroblotting was carried out on Polyvinylidene fluoride (PVDF) membranes (HybondP, Amersham Biosciences, Oslo, Norway) using blotting buffer (10% methanol, 10% 10X TG, 80% H_2O) for 1 hour at 100V. The membranes were blocked with WBII blocking solution (2 g Tropix® I-Block (Applied Biosystems) dissolved in 1L 1X TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.5); the solution was warmed up to 70ºC and cooled down to 20ºC before the addition of 1 mL Tween® 20 (Sigma-Aldrich) and 2.18 g $MgCl_2x6H_2O$ (VWR International AS)) for 1 hour at RT to prevent unspecific binding of the primary antibody. They were then placed in sealed plastic bags containing the primary antibody diluted in WBII blocking solution with a final volume of about 3 mL. The bags were placed under moderate agitation overnight at 4°C. The p53 antibody Bp53-12, the Mdm2 antibody SMP-14, the Bax antibody 2D2, the β-actin antibody C4, the Caspase-3 antibody H-277 and the SRSF3 antibody 7B4 were purchased from Santa Cruz Biotechnology, CA, USA. The p21 antibody EA10 and the COX IV antibody (ab16056) were

bought from Abcam plc, Cambridge, UK. The NPM1 antibody (32-5200) was from Invitrogen. The Mdm2 antibody SMP-14, the Caspase-3 antibody H-277 and the p21 antibody EA10 were diluted 1:500. The COX IV antibody (ab16056) was diluted 1:2000. Other antibodies were diluted 1:1000.

After washing 4 times × 10 min in TBS-T (1L 1X TBS with 1mL Tween® 20), the secondary antibodies conjugated to horse radish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were diluted 1:1000 in 5% fat-free dry milk (Bio-Rad Laboratories) in TBS-T and subsequently added to the membranes and incubated for 1 hour under agitation at RT. The membranes were again washed 4 times × 10 min in TBS-T. Thereafter, they were visualized by using Supersignal® West Pico or Femto Stable peroxide solution and luminal/enhancer solution (Thermo scientific) with the ratio 1:1. Protein bands were detected by the Kodak Image Station 2000R (Eastman Kodak Company, Lake Avenue, Rochester, NY, USA) and quantified by using Carestream MI analysis software (Carestream Molecular Imaging, Woodbridge, CT, USA). Data were exported to Excel spreadsheet and fold induction was calculated as follows: the value of protein of interest/the value of loading control (β-actin or COX IV).

3.8. Statistical analysis

Statistical analysis was performed by using the GraphPad PRISM® (version 5.0, GraphPad Software, Inc., La Jolla, CA, USA) software. Data were expressed as mean ± standard error of the mean (SEM). Difference in averages between two groups was determined by using a two-tailed Student's *t*-test with results considered statistically significant when $p < 0.05$. The software was also used to generate graphs. Asterisks were used to illustrate the strength of the significance as follows *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

4. Results

4.1. Functional characterization of p53β and p53γ in p53^{null} HL-60 AML cell line

4.1.1. Stable expression of p53β and p53γ in p53^{null} HL-60 cells

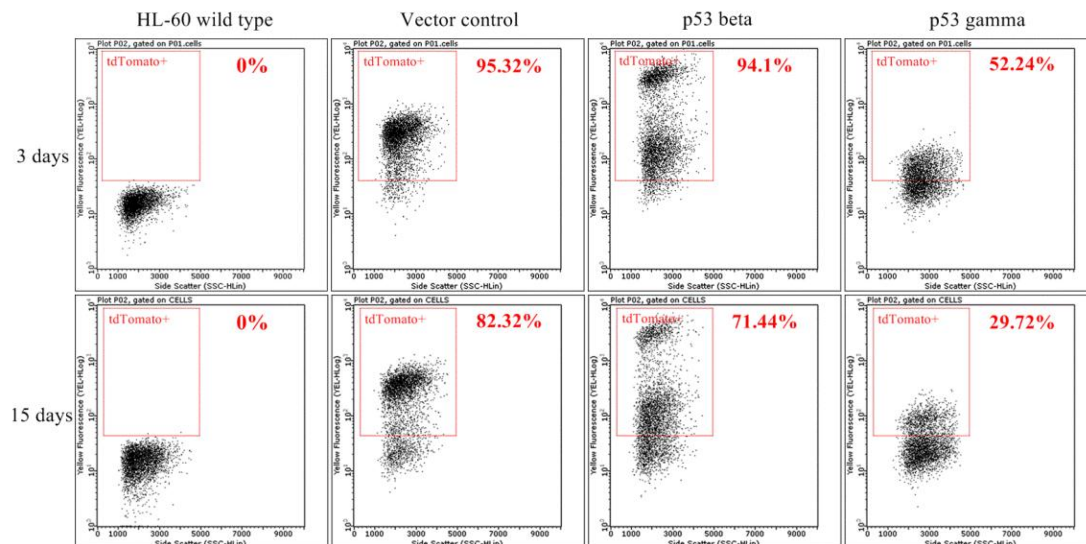
In order to investigate if the functions of p53β and p53γ are independent of FLp53, the p53^{null} AML HL-60 cells which were already transduced retrovirally with p53β-tdTomato construct, p53γ-tdTomato construct or tdTomato alone (vector control) were used. Because of the expression of tdTomato, cells successfully transduced can be isolated by FACS. **Figure 4-1A** shows the percentage of tdTomato$^+$ cells 3 and 15 days after FACS respectively. Not only did p53γ$^+$ HL-60 cells reveal a lower fluorescence intensity of tdTomato compared to p53β$^+$ cells and vector control cells, but they also displayed a faster decline of tdTomato$^+$ cells after sorting. Western blot analysis verified the expression of p53β and p53γ in HL-60 cells and presented the considerably lower expression level of p53γ in comparison with p53β (**Figure 4-1B**). Besides, the basal expression level of p21$^{WAF1/CIP1}$, Bax and Mdm2 proteins in wild-type, vector control, p53β$^+$ and p53γ$^+$ HL-60 cells was also examined by Western blot. The wild-type and p53γ$^+$ HL-60 cells had a slightly higher level of p21$^{WAF1/CIP1}$ and significantly elevated level of Mdm2 and Bax in comparison with vector control and p53β$^+$ cells. Also, the p53β$^+$ cells expressed a higher level of Mdm2 than vector control cells (**Figure 4-1C**).

4.1.2. Colony formation of p53β$^+$ and p53γ$^+$ HL-60 cells

Colony formation assay was performed to investigate the influence of stable expression of p53β and p53γ on the clonogenicity of HL-60 cells. This can point out the effect of these proteins on the differentiation status of the cells. Results revealed that there is no significant difference in the number of colonies between vector control and p53β$^+$ and p53γ$^+$ HL-60 cells (**Figure 4-2A**). The phase contrast images showed no modification in the size of colonies. They also revealed that the colonies of p53β$^+$ cells carry similar features of morphology as vector control cells. However, while the morphology of vector control and p53β$^+$ cells' colonies was delineated, the morphology of p53γ$^+$ and wild-type cells' ones was not. Wild-type and p53γ$^+$ cells' colonies were surrounded by scattered cells much more than vector control and p53β$^+$ cells' ones. The morphology of p53γ$^+$ cells' colonies included the characteristics of both wild-type and vector control cells. Moreover, the fluorescence images

showed that the colonies of p53γ+ cells have lower fluorescence intensity of tdTomato than of vector control or p53β+ cells (**Figure 4-2B**).

A



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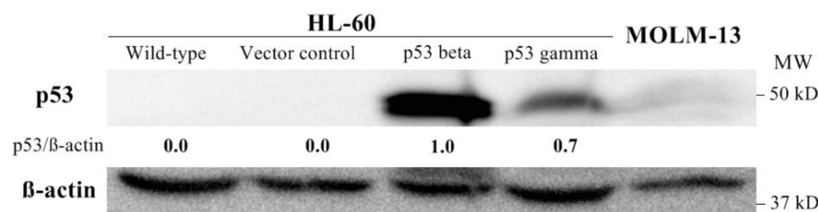

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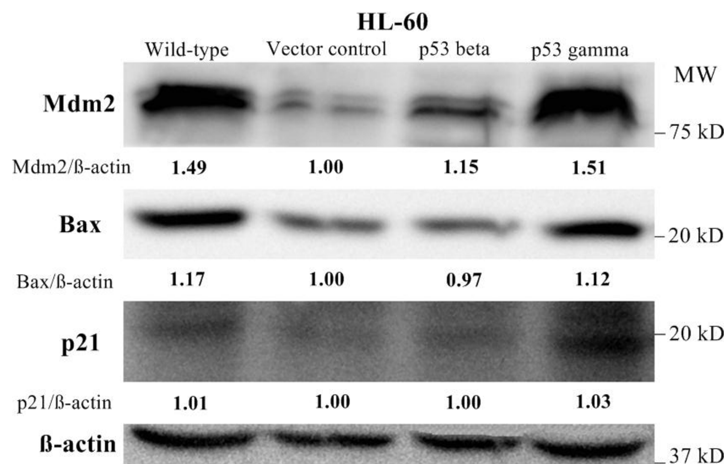
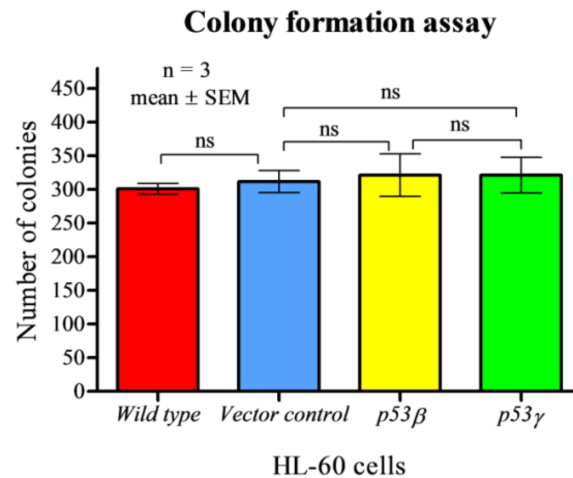


Figure 4-1 Basic characteristics of wild-type, vector control, p53β+ and p53γ+ HL-60 cells. **(A)** Flow cytometric analysis of cells 3 and 15 days after FACS. Red rectangles present tdTomato+ cells. The percentage of tdTomato+ cells is indicated in the upper right corner of each plot. **(B)** Western blot analysis to examine the presence of p53β and p53γ in HL-60 cells. The wild-type *TP53* AML cell line MOLM-13 was used as a positive control. **(C)** Immunoblot of Mdm2, Bax and p21 of wild-type, vector control, p53β+ and p53γ+ HL-60 cells. Proteins investigated are shown in bold letters to the left and molecular weight is indicated to the right of the figure. Quantified values were normalized to β-actin as loading control.

A



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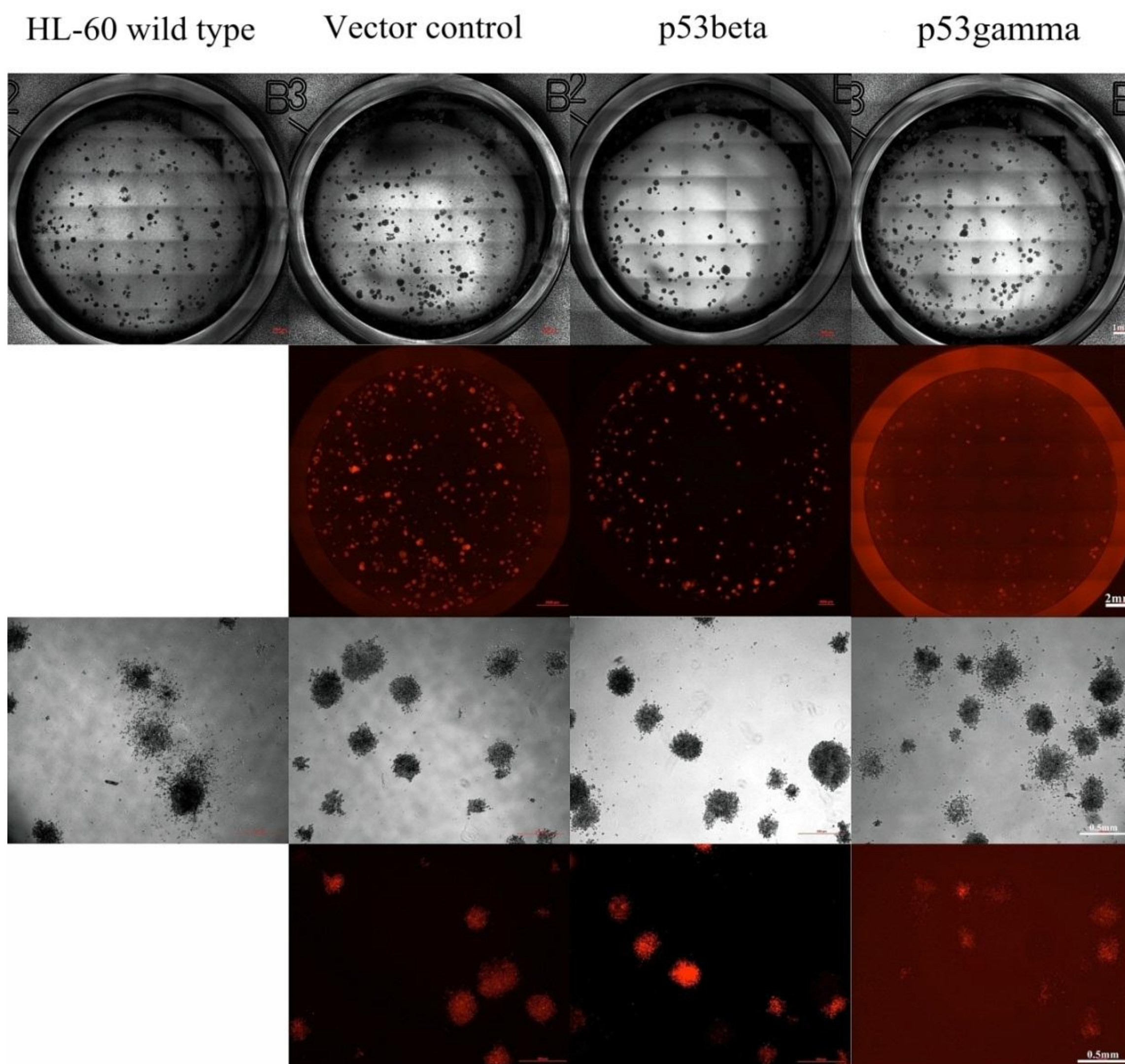


Figure 4-2 Colony formation of wild-type, vector control, p53β^{+} and p53γ^{+} HL-60 cells. (A) The numbers of colonies after culturing in MethoCult for 7 days. Results are presented as the mean ± SEM of three independent experiments (ns: not significant). **(B)** Fluorescence microscopy of colonies. First and third rows show phase contrast images, second and fourth rows display fluorescence images. Size bars are indicated at the right bottom of each image.

4.1.3. The effects of p53β and p53γ on proliferation and viability of HL-60 cells following chemotherapy

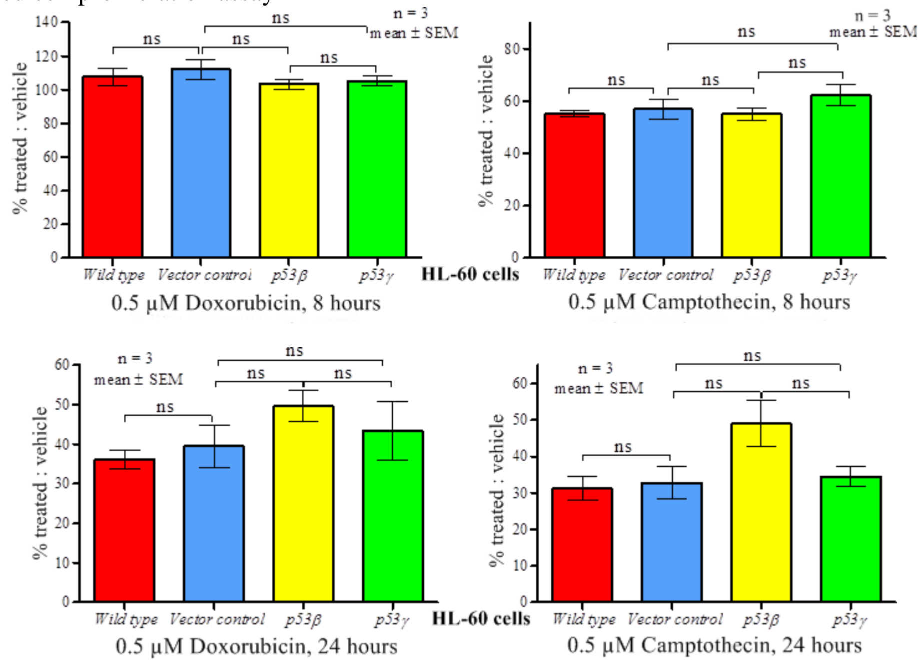
In order to examine the impacts of p53β and p53γ on the proliferation and viability of HL-60 cells in response to chemotherapy, the cells were treated by the cytotoxic anthracycline antibiotic topoisomerase II inhibitor Doxorubicin and topoisomerase I inhibitor Camptothecin. The WST-1 based cell proliferation assay was carried out to evaluate the rate of proliferation. Significant changes in the rate of proliferation following chemotherapeutic treatment were not perceived between vector control, $p53\beta^{+}$ and $p53\gamma^{+}$ cells (**Figure 4-3A**). DNA staining with Hoechst 33342 differentiates between normal and apoptotic cells' nuclear morphology allowing a calculation of the percentage of viability. The assay was utilized to assess the viability of the cells in response to Doxorubicin and Camptothecin. Nevertheless, the nuclear morphologies of $p53\beta^{+}$ and $p53\gamma^{+}$ cells did not show further abnormality in comparison with vector control cells (**Figure 4-3B,C**). These results suggest that p53β and p53γ do not affect the proliferation and apoptosis of HL-60 cells following chemotherapy.

4.2. Production of wild-type *TP53* MOLM-13 AML cell line with increased expression of p53β and p53γ

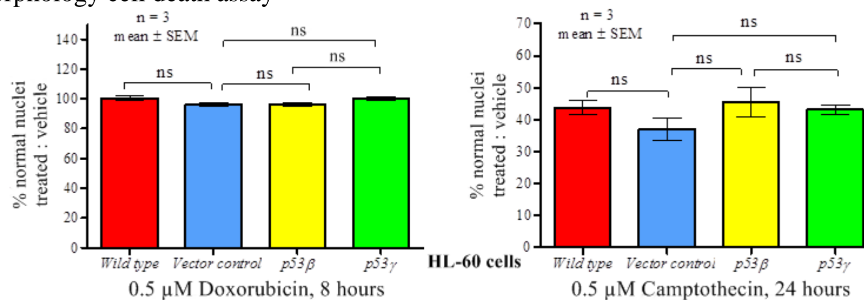
4.2.1. Retroviral transduction of MOLM-13 cells

The wild-type *TP53* MOLM-13 AML cells express FLp53, p53β and p53γ. Individually retroviral transduction of p53β-tdTomato, p53γ-tdTomato and tdTomato alone (vector control) into MOLM-13 cells was performed to generate clones having higher expression level of p53β and p53γ which can be exerted to elucidate whether or not these isoforms function together with FLp53. The strategy used for the creation of these cell clones is illustrated in **Figure 4-4A**. The cells were transduced four times and checked for the ratio of $tdTomato^{+}$ cells by flow cytometry after each transduction. After the fourth transduction, the fraction of $tdTomato^{+}$ cells for vector control was 4.54% while p53β and p53γ were 0.81% and 0.66% respectively (**Figure 4-4B**). The successfully transduced cells were sorted by FACS (**Figure 4-4C**).

A WST-based cell proliferation assay



B Nuclear morphology cell death assay



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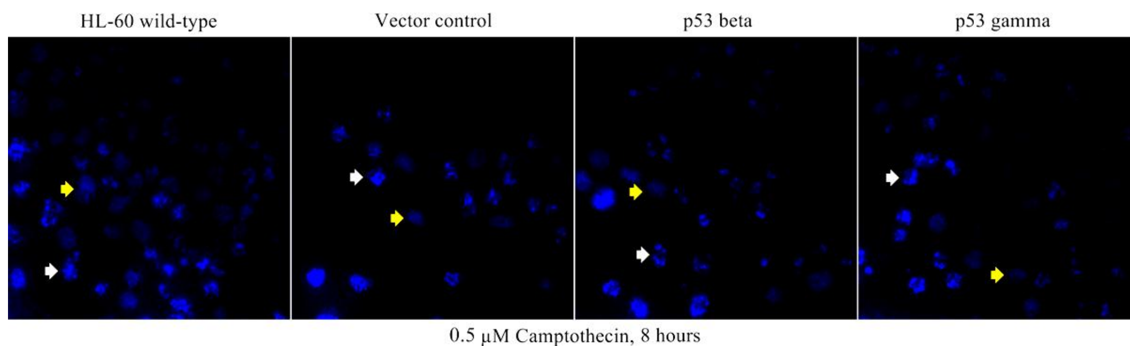
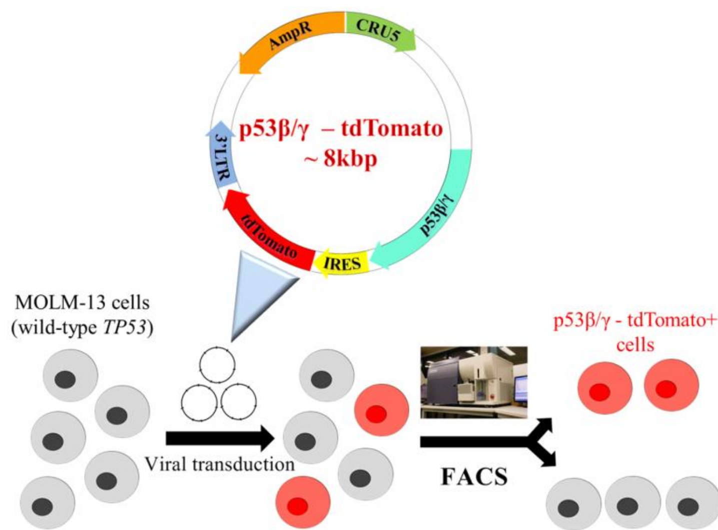


Figure 4-3 Proliferation and viability of wild-type, vector control, p53β^{+} and p53γ^{+} HL-60 cells following chemotherapy. **(A)** Proliferation of cells after treatment with 0.5 µM Doxorubicin or 0.5 µM Camptothecin for 8 and 24 hours respectively, with WST-1 added to assess oxidoreductase activity at the last 4 hours. Results were compared with untreated controls and presented as the mean ± SEM of three independent experiments (ns: not significant). **(B)** Viability of cells after treatment with 0.5 µM Doxorubicin or 0.5 µM Camptothecin for 8 hours. The percentage of normal nuclei was calculated and compared with untreated controls. Results are displayed as the mean ± SEM of three separate experiments (ns: not significant). **(C)** Nuclear staining of cells treated with 0.5 µM Camptothecin for 8 hours. Yellow and white arrows indicate normal and abnormal nuclei respectively.

A



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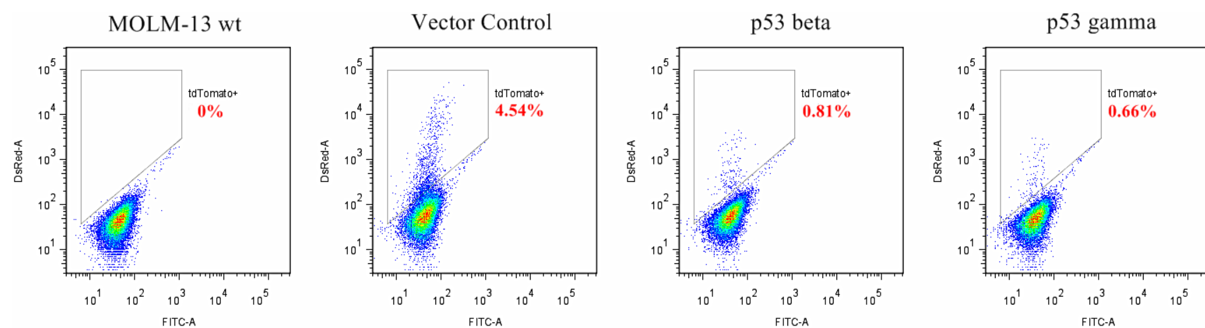

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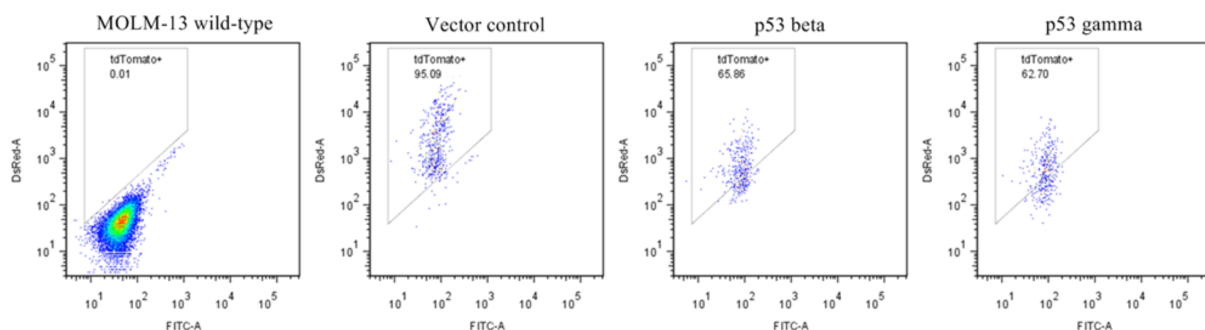


Figure 4-4 Retroviral transduction of MOLM-13 cells. (A) Schematic representation of transduction of MOLM-13 cells. The cells were transduced with p53β/p53γ-tdTomato construct or vector control. Cells successfully transduced were isolated by FACS based on the expression of tdTomato. **(B)** Flow cytometric analysis of MOLM-13 cells transduced with p53β/p53γ-tdTomato or vector control after the fourth transduction. **(C)** Flow cytometric analysis of MOLM-13 cells transduced with p53β/p53γ-tdTomato or vector control after FACS. The gates present tdTomato^{+} cells. The percentage of tdTomato^{+} cells is indicated in each plot.

4.2.2. The increase of p53γ is toxic to MOLM-13 cells

After FACS, the MOLM-13 cells transduced with the p53γ construct were selectively lost when analyzing these cells by Annexin V-Alexa 488 – To-pro-3 cell death assay at day seven

(**Figure 4-5A**). As clearly indicated, the majority of p53γ transduced MOLM-13 cells were positively stained with both Annexin V and To-pro-3, indicating that they are already dead. Furthermore, p53γ transduced cells were unable to form colonies at the seventh day of colony formation assay (**Figure 4-5B,C**). Taken together, these results demonstrate that up-regulation of p53γ in MOLM-13 cells is cytotoxic. This is in contrast to p53γ transduced HL-60 cells (**Figure 4-2**), a cell line with deleted *TP53*.

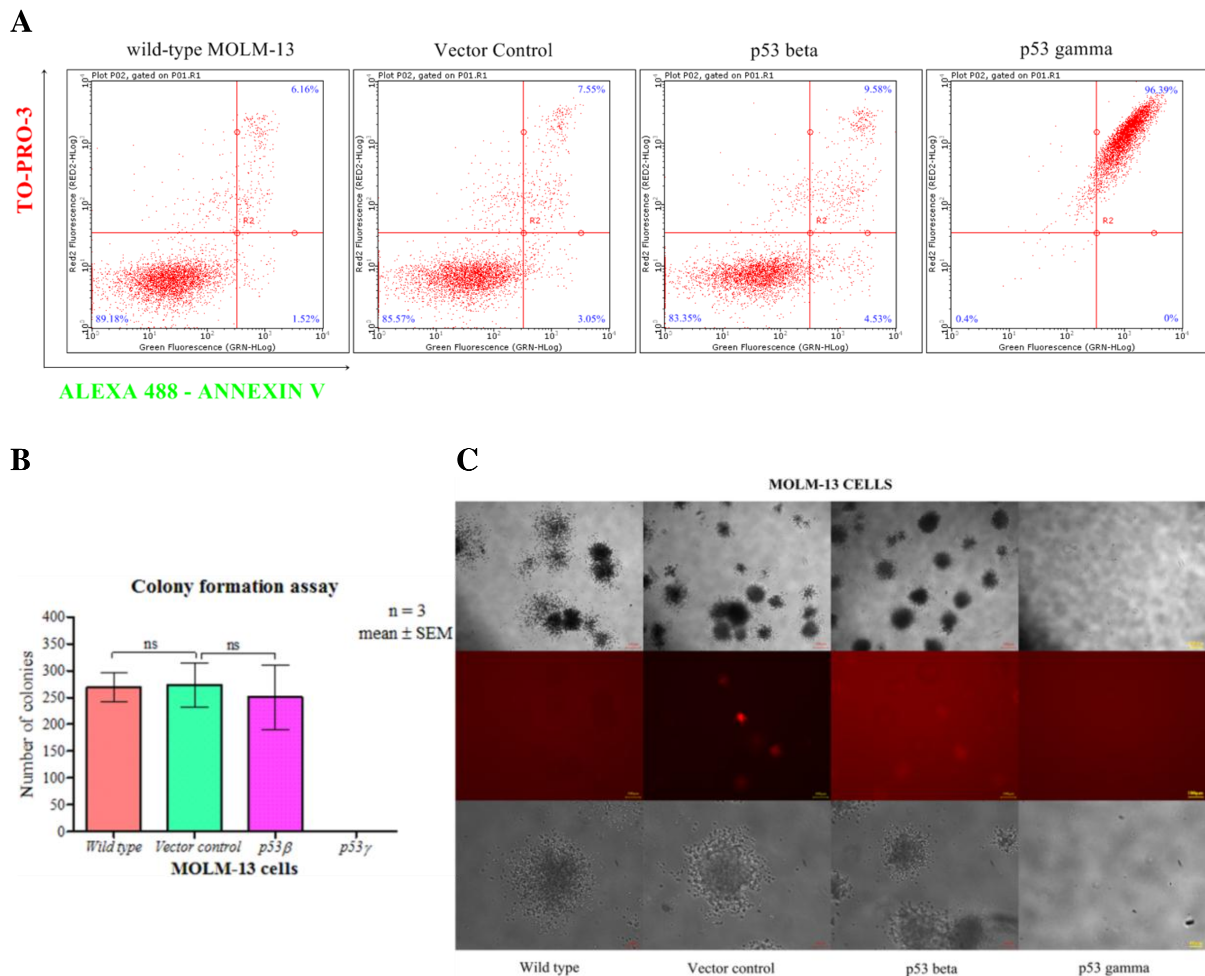


Figure 4-5 Cell viability and colony formation of wild-type MOLM-13 cells and MOLM-13 cells transduced with vector control, p53β or p53γ constructs. **(A)** Annexin V-Alexa 488 – To-pro-3 cell death assay of cells at day 7. The lower left rectangle of each plot indicates viable cells, the lower right one presents cells in early apoptosis and the upper right one displays late apoptotic or dead cells. The percentage of cells is indicated. **(B)** Colony formation of cells. The numbers of colonies are presented as the mean ± SEM of three independent experiments (ns: not significant). **(C)** Fluorescence microscopy of colonies. First and third rows show phase contrast images, second row displays fluorescence images. Size bars are indicated at the right bottom of each image.

The MOLM-13 cells were transduced with p53γ construct again. After five times of transduction, they were sorted and visualized daily by a bright field microscope. A difference in morphology between p53γ transduced cells and wild-type MOLM-13 cells as well as a gradual loss of p53γ transduced cells was observed (**Figure 4-6**). As can be seen, the p53γ transduced cells contained much more vacuoles in the cytoplasm compared to wild-type cells and the amount and size of these vacuoles increased progressively when the cells lost gradually.

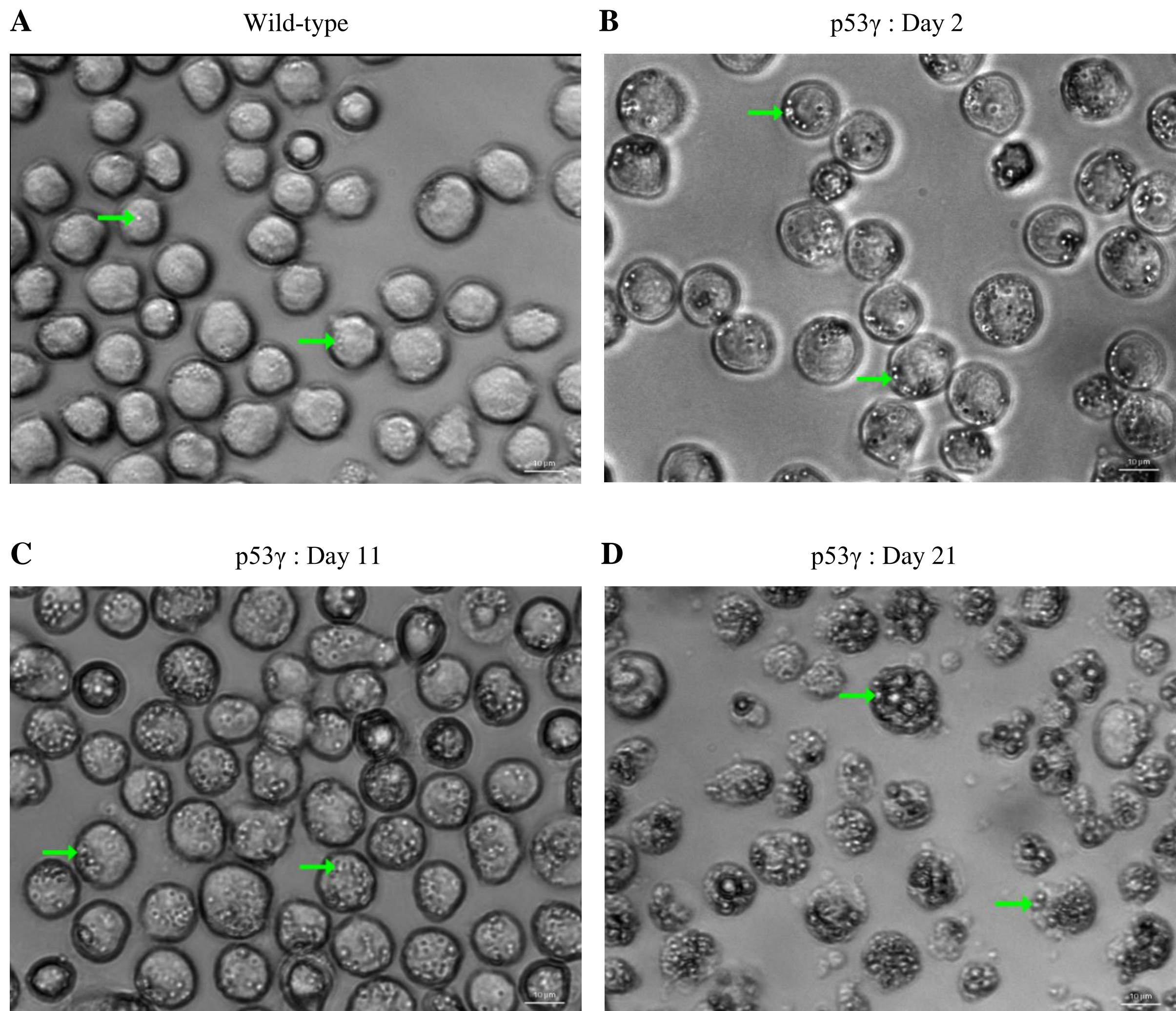


Figure 4-6 Bright field microscopy of wild-type MOLM-13 cells and MOLM-13 cells transduced with p53γ construct. (A) Image of wild-type MOLM-13 cells. **(B, C, D)** Images of p53γ transduced MOLM-13 cells after 2, 11, 21 days of FACS. Green arrows indicate vacuoles. Size bars are indicated at the right bottom of each image.

4.2.3. Increased expression of p53β in wild-type TP53 MOLM-13 cells and the formation of colonies

The increased expression of p53β in MOLM-13 cells was investigated by Western blot analysis of cell extracts utilizing specific antibody against the N-terminus of p53 (Bp53-12). The result revealed a considerable up-regulation of p53β protein in the MOLM-13 cells transduced with p53β construct in comparison with wild-type and vector control cells. Nonetheless, the basal expression level of Bax and p21 did not change significantly in these cells (**Figure 4-7**).

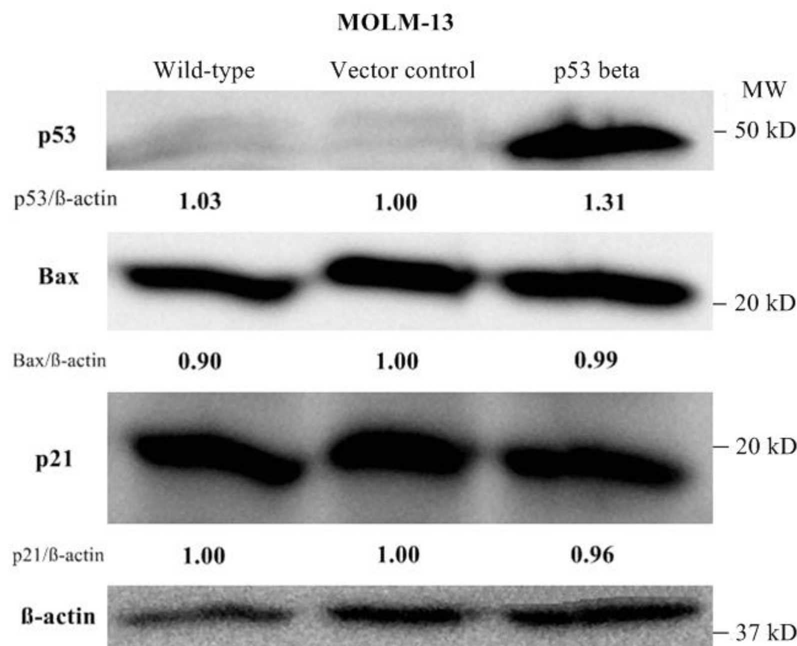


Figure 4-7 Western blot analysis of wild-type MOLM-13 cells and the MOLM-13 cells transduced with vector control or p53β construct. Proteins investigated are shown in bold letters to the left and molecular weight is indicated to the right of the figure. Quantified values were normalized to β-actin as loading control and compared to the vector control cells' values set to 1.00.

Besides, the ectopic expression of p53β was examined for effect on the clonogenicity of MOLM-13 cells. Both morphology and number of colonies were equal between wild-type, vector control and p53β-tdTomato transduced MOLM-13 cells, but the p53β transduced cell colonies showed a lower fluorescence intensity of tdTomato in comparison with the vector control (**Figure 4-5B,C**). Screening for altered monocytic and granulocytic differentiation by immunophenotyping did not reveal any significant change between wild-type and p53β-tdTomato transduced cells (data not shown). Therefore, we conclude that the increased expression of p53β do not affect the differentiation status of MOLM-13 cells.

4.3. Oxidative stress by resazurin treatment: impact of p53β and p53γ expression, modulation of p53 isoforms and Mdm2

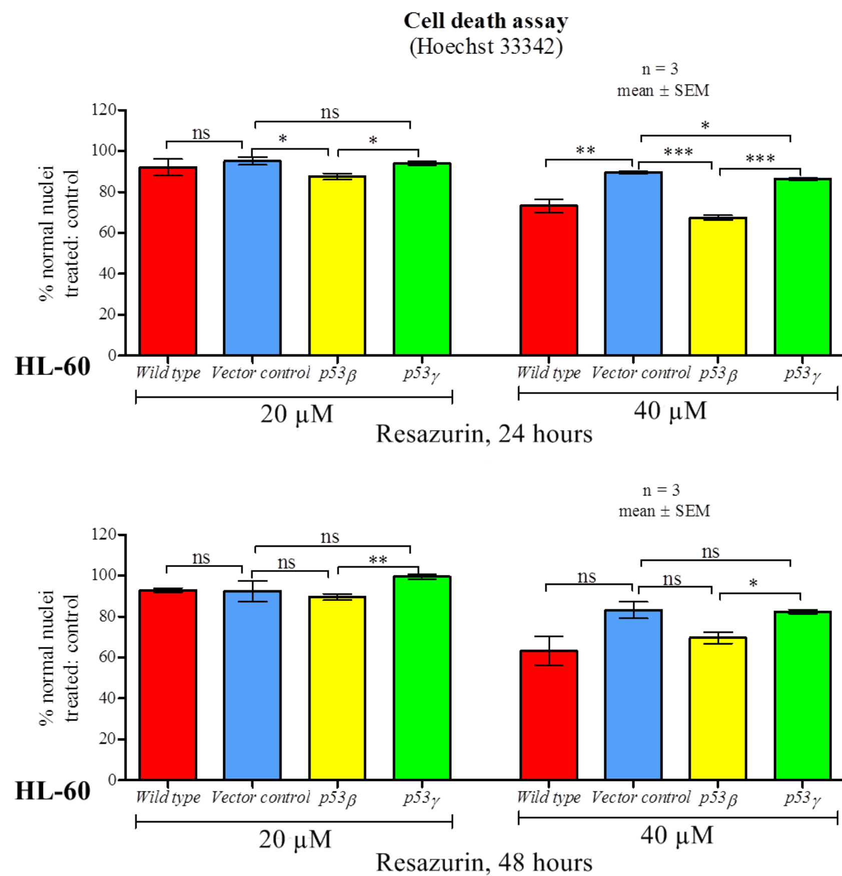
4.3.1. p53β increases the apoptosis of HL-60 cells in response to resazurin

In order to explore the relation of p53β or p53γ and resazurin's impingement, the wild-type, vector control, p53β$^+$ and p53γ$^+$ HL-60 cells were exposed to resazurin in two different concentrations for two distinct periods and examined for viability by nuclear morphology cell death assay. For the 24 hours exposure, significant differences in the ratio of normal nuclei between p53β$^+$ and p53γ$^+$ cells, p53β$^+$ and vector control cells were found, even at the low concentration of resazurin (20 μM). Interestingly, a significant difference between wild-type and vector control cells was also displayed when the cells were treated with 40 μM resazurin. Furthermore, when the cells were exposed for 48 hours, an expressive difference between p53β$^+$ and p53γ$^+$ cells was still observed (**Figure 4-8A**). Further investigation by Western blot for Caspase-3 cleavage, a marker for apoptosis, of vector control, p53β$^+$ and p53γ$^+$ cells treated with 40 μM resazurin for 48 hours also indicated an increase of cleaved Caspase-3 in p53β$^+$ cells compared to the vector control and p53γ$^+$ cells (**Figure 4-8B**). Taken together, it can be seen that the presence of p53β promotes the sensitivity of HL-60 cells for apoptosis in response to resazurin.

4.3.2. Examining the modulation of p53 isoforms and related proteins following resazurin exposure

In order to answer the question whether resazurin exposure causes a modulation of FLp53, p53β and p53γ expression, the p53β$^+$ and p53γ$^+$ HL-60 cells exposed to 40 μM resazurin for 48 hours were inspected by Western blot analysis for the expression level of p53 isoforms. Nonetheless, an alteration of p53 isoforms' expression level was not revealed (**Figure 4-9A**). In addition, the shControl and shp53 (control) MOLM-13 cells treated with 40 μM resazurin for 24 hours did not show a regulation in the expression level of these p53 isoforms (**Figure 4-9B**), indicating that resazurin affects neither the level of FLp53 nor the level of p53β and p53γ.

A



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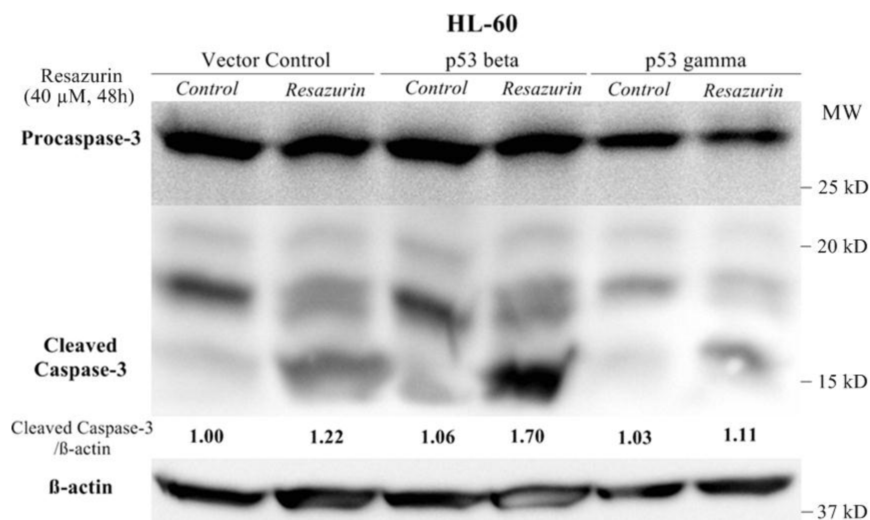
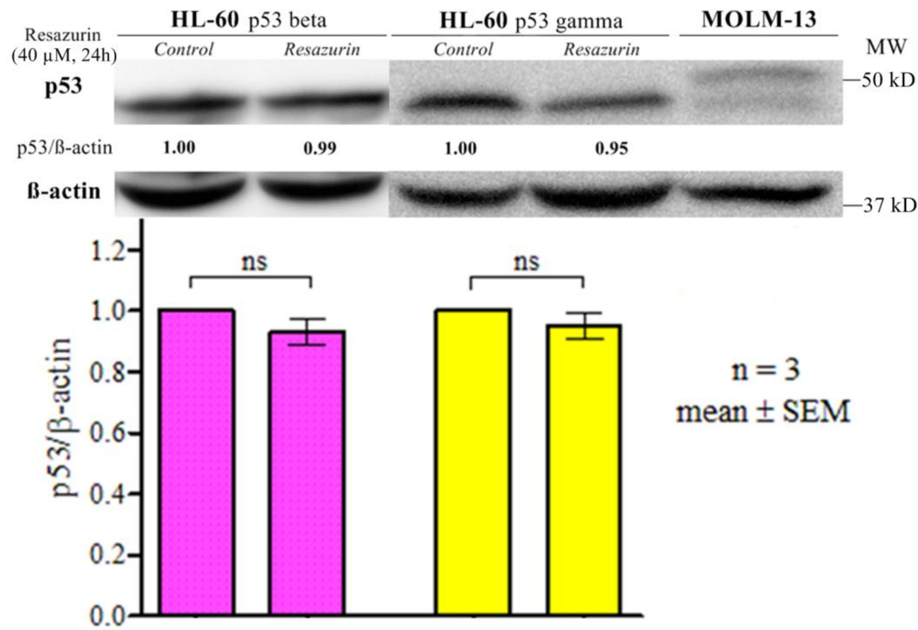


Figure 4-8 Cell viability of wild-type, vector control, p53β+ and p53γ+ HL-60 cells following resazurin exposure. (A) Viability of cells after treatment with 20 µM or 40 µM resazurin for 24 or 48 hours respectively. The percentage of normal nuclei was calculated and compared to untreated controls. Results are displayed as the mean ± SEM of three separate experiments (ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). **(B)** Western blot analysis of vector control, p53β+ and p53γ+ HL-60 cells treated with 40 µM resazurin for 48 hours. Proteins investigated are shown in bold letters to the left and molecular weight is indicated to the right of the figure. Quantified values were normalized to β-actin as loading control and compared to the untreated vector control cells' value set to 1.00. The result represents a typical experiment.

A



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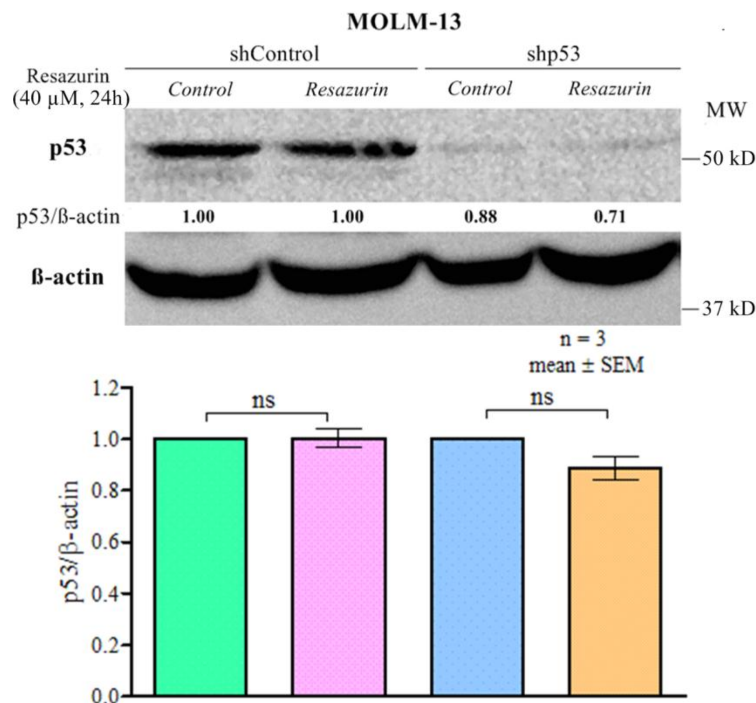
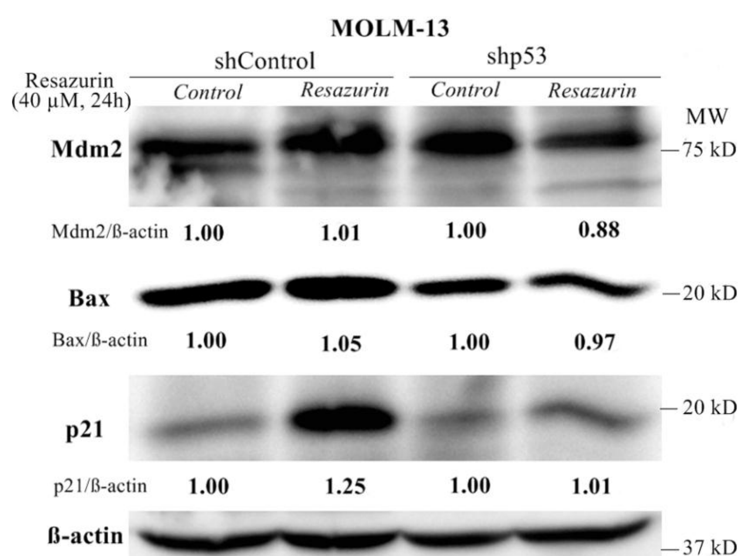


Figure 4-9 Western blot analysis of p53 in HL-60 and MOLM-13 cells following resazurin exposure. (A) Immunoblot analysis of p53β^{+} and p53γ^{+} HL-60 cells exposed to 40 µM resazurin for 48 hours. The wild-type MOLM-13 cells' lysate was used as a positive control. **(B)** Immunoblot analysis of MOLM-13 cells transduced with vector control (shControl) and short hairpin RNA against p53 (shp53) subjected to treatment with 40 µM resazurin for 24 hours. Proteins investigated are shown in bold letters to the left and molecular weight is indicated to the right of the figure. Quantified values were normalized to β-actin as loading control and compared to the untreated control cells' value was set to 1.00. Bar graphs present the mean of quantified values of three separate experiments. Error bars show SEM (ns: not significant).

Further investigation of Bax expression level by Western blot analysis in both shControl and shp53 MOLM-13 cells did not reveal a modification. However, the shControl cells showed an up-regulation of $p21^{CIP1/WAF1}$, whereas the shp53 cells which have the lower level of p53 did not. In contrast, the Mdm2 level of the shp53 cells was down-regulated slightly while of the shControl cells remained unchanged (**Figure 4-10A**). Additionally, all wild-type, vector control, $p53\beta^+$ and $p53\gamma^+$ HL-60 cells presented a substantial down-regulation of Mdm2 expression level following resazurin treatment while the modulation of Bax in these cells was not perceived (**Figure 4-10B**).

A



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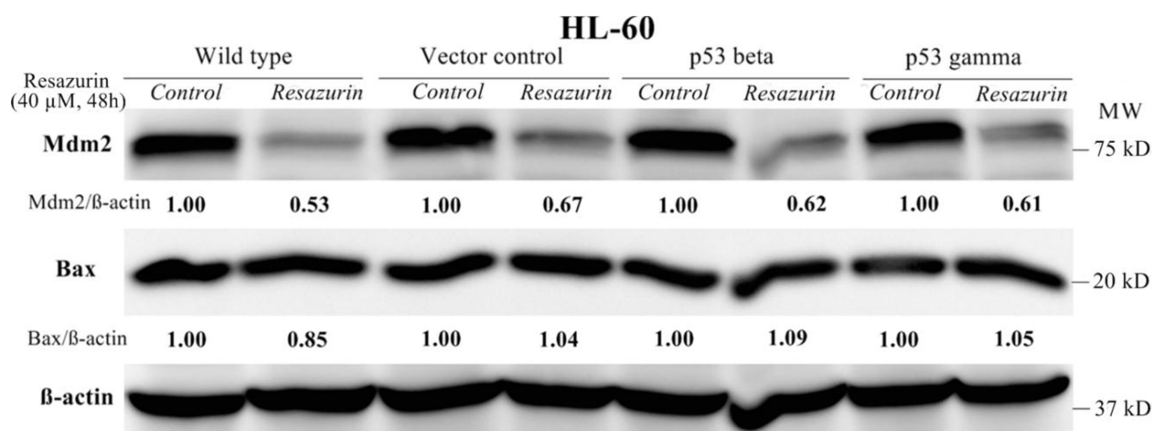


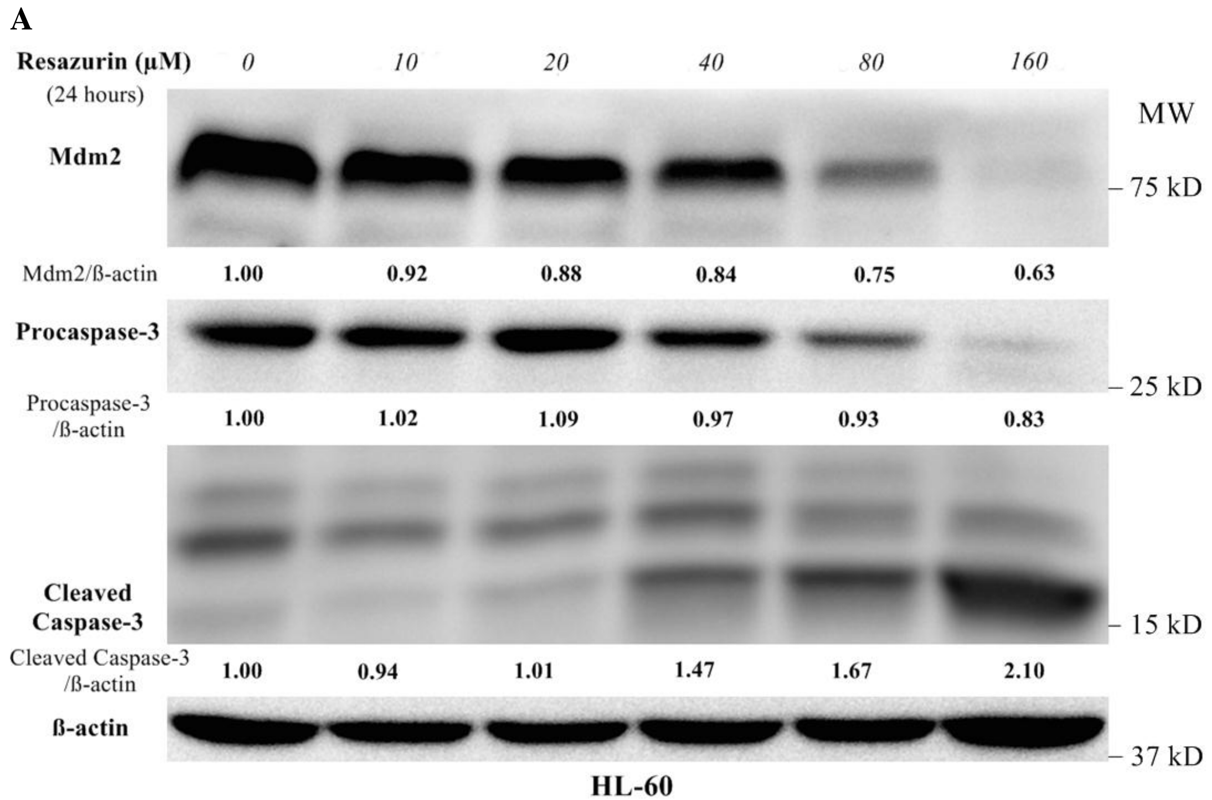
Figure 4-10 Western blot analysis of HL-60 and MOLM-13 cells following resazurin exposure. (A) Immunoblot analysis of shControl and shp53 MOLM-13 cells after treated with 40 µM resazurin for 24 hours. **(B)** Immunoblot analysis of wild-type, vector control, $p53\beta^+$ and $p53\gamma^+$ HL-60 cells exposed to 40 µM resazurin for 48 hours. Proteins investigated are shown in bold letters to the left and molecular weight is indicated to the right of the figure. Quantified values were normalized to β-actin as loading control and compared to the untreated control cells' value set to 1.00. The result represents three different experiments.

4.3.3. p53-independent down-regulation of Mdm2 and increased apoptosis in HL-60 cells by resazurin

The finding that resazurin causes an increased apoptosis and an attenuation of Mdm2 in all wild-type, vector control, p53β+ and p53γ+ HL-60 cells led to an inquiry whether a p53-independent attenuation of Mdm2 by resazurin exists. Western blot analysis of Mdm2 and Caspase-3 and nuclear morphology cell death assay were performed in wild-type HL-60 cells exposed to resazurin in a dose-response manner to clarify this. The cells were incubated with 0 (control), 10, 20, 40, 80 and 160 µM of resazurin for 24 hours. **Figure 4-11A** reveals that the expression level of Mdm2 declined progressively when the concentration of resazurin rose gradually. Similarly, a progressive decrease of normal nuclei was observed with Hoechst 33342 staining (**Figure 4-11B**). However, the stepwise decreasing of Procaspase-3 and the increasing of cleaved Caspase-3 were only detected at the concentration of 40 µM resazurin (**Figure 4-11A**). In summary, these data confirm that resazurin evokes a p53-independent down-regulation of Mdm2 in HL-60 cells.

4.3.4. Further investigation of resazurin response in MOLM-13 cells

Based on the finding that resazurin induces the up-regulation of $p21^{CIP1/WAF1}$ in shControl MOLM-13 cells and the slight down-regulation of Mdm2 in shp53 MOLM-13 cells, we examined if a difference in p53 level of the cells influences their response to resazurin. Both shControl and shp53 MOLM-13 cells were incubated with 40 µM resazurin for 24 hours and the viability of the cells was examined simultaneously by nuclear morphology cell death assay and Annexin V-Alexa 488 – To-pro-3 cell death assay as a comparison. Nevertheless, data showed that the exposure to resazurin does not effectively decrease the viability of the cells. Moreover, no significant difference in the percentage of viable cells between shControl and shp53 cells was detected in either of the assays (**Figure 4-12**). These results propose that resazurin does not primarily affect the MOLM-13 cells through the induction of apoptosis.



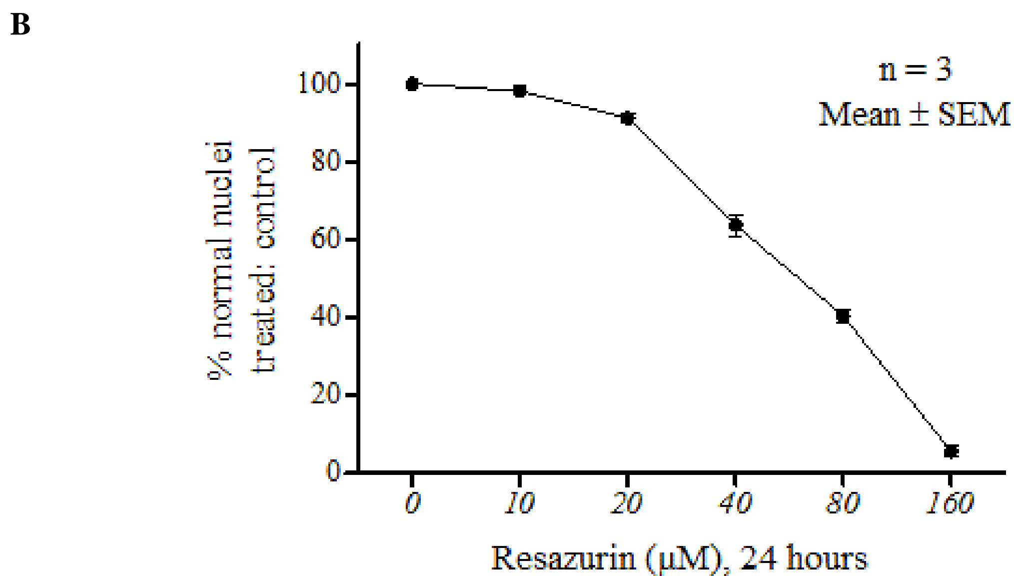
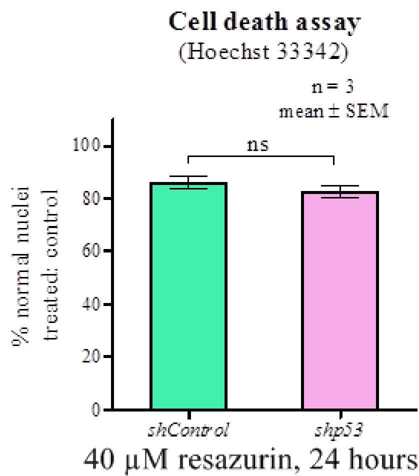
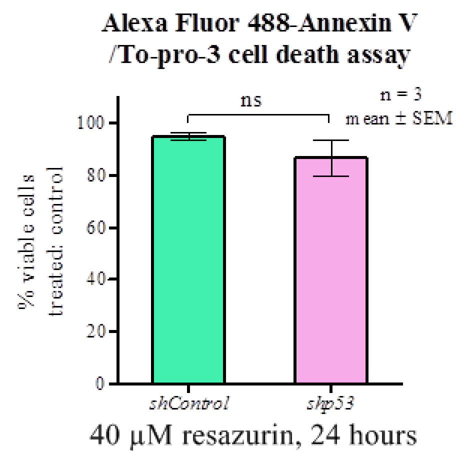


Figure 4-11 Down-regulation of Mdm2 and increased apoptosis in HL-60 cells following resazurin exposure. (A) Western blot analysis of HL-60 cells treated with resazurin (0-160 µM) for 24 hours. Proteins investigated are shown in bold letters to the left and molecular weight is indicated to the right of the figure. Quantified values were normalized to β-actin as loading control and compared to the untreated control cells' value set to 1.00. The result represents three different experiments. **(B)** Viability of cells after treatment with resazurin (0-160 µM) for 24 hours. The percentage of normal nuclei was calculated and compared with untreated control. Results are displayed as the mean ± SEM of three separate experiments.

A



B



C

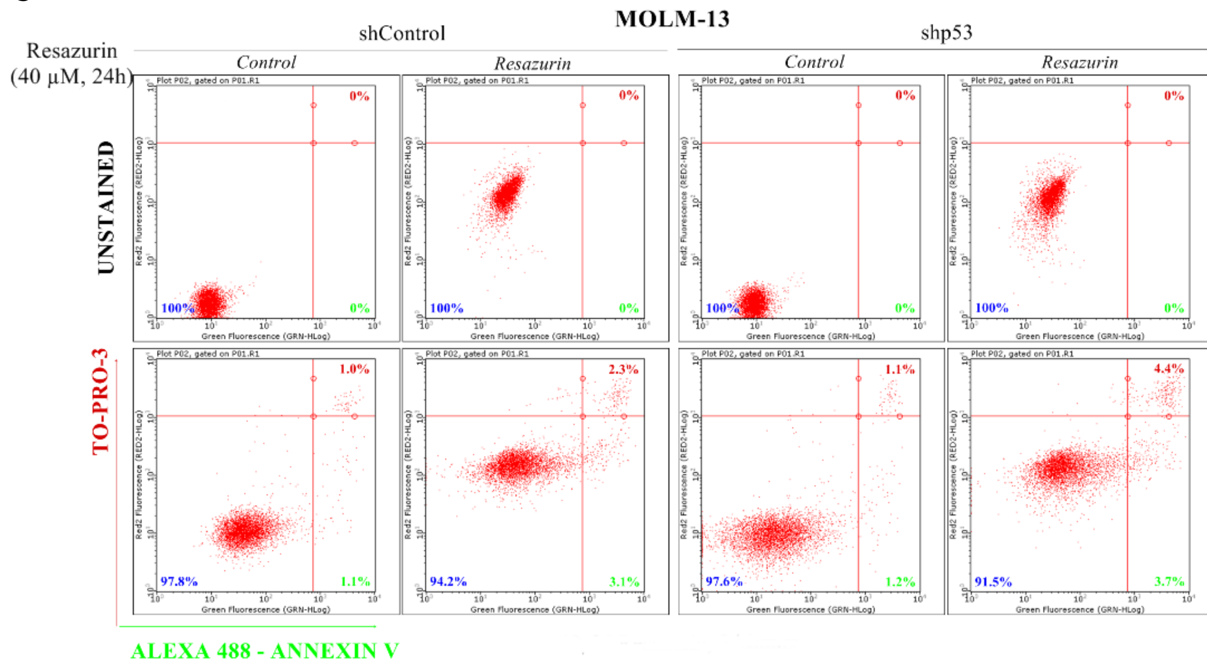


Figure 4-12 Cell viability of shControl and shp53 MOLM-13 cells following resazurin treatment. (A) Nuclear morphology cell death assay of cells after treatment with 40 µM resazurin for 24 hours. The percentage of normal nuclei was calculated and compared with untreated controls. Results are displayed as the mean ± SEM of three separate experiments (ns: not significant). **(B)** Annexin V-Alexa 488 – To-pro-3 cell death assay of cells following exposure to 40 µM resazurin for 24 hours. The percentage of viable cells was calculated and compared with untreated controls. Results are shown as the mean ± SEM of three distinct experiments (ns: not significant). **(C)** Flow cytometric analysis of a typical Annexin V-Alexa 488 – To-pro-3 cell death assay. The lower left rectangle of each plot indicates viable cells, the lower right one presents cells in early apoptosis and the upper right one displays late apoptotic or dead cells. The percentage of cells is indicated. The first row shows unstained cells and the second one reveals cells stained with both Annexin V-Alexa Fluor 488 and To-pro-3.

4.4. The investigation of p53 isoforms modulation by wild-type NPM1 and mutated NPM1

4.4.1. Production of vector control

Since mutation of *NPM1* was correlated with high expression of p53β and p53γ proteins in AML, we investigated if wild-type NPM1 or mutated NPM1 plays roles in the modulation of these p53 isoforms' expression. For this investigation, recombinant plasmids containing wild-type *NPM1* and *NPM1* mutant type A DNA were utilized. Nevertheless, because of the lack of vector control, we generated the vector control from the recombinant plasmid. **Figure 4-13A** illustrates the structure of the recombinant plasmid pCDNA3$^+$-*NPM1*-ECGFP. The plasmid contains the coding sequences of antibiotic resistance (Ampicillin and Neomycin), Enhanced Cyan Green Fluorescent Protein (*ECGFP*) gene and *NPM1* gene inserted at the N-terminus of *ECGFP* which expresses a fusion protein with ECGFP attached to the C-terminus of NPM1. The restriction sites of *Bam*HI, *Xho*I and *Eco*RI restriction enzymes are also revealed. It can be seen that *NPM1* can be cut by *Eco*RI while *NPM1-ECGFP* can be cleaved by *Bam*HI and *Xho*I. As shown in **Figure 4-13B**, the recombinant plasmids holding wild-type *NPM1* or mutated *NPM1* showed DNA bands at different sizes when they were digested by *Bam*HI and *Xho*I or *Eco*RI only. The result of a DNA band at 1.4 kb or 0.7 kb size indicated clearly *NPM1-ECGFP* or *NPM1* respectively. **Figure 4-13C** presents the strategy exerted for the generation of vector control. After digestion with *Eco*RI, *NPM1* gene was split by running agarose gel and the cleaved plasmid was cut out from the gel, purified and ligated to form the vector control which contains *ECGFP* without the attachment of *NPM1*. The ligation product was transformed into competent *E.coli* cells and then three bacterial colonies were randomly chosen from selective LB plates for plasmid purification. The successful production of vector control was verified by cutting these plasmids with *Bam*HI or *Eco*RI. **Figure 4-14A** shows that the plasmids from the bacterial colonies have a smaller DNA band size in comparison with pCDNA3$^+$-*NPM1*-ECGFP (control) when they were digested by *Bam*HI. In contrast, when they were cut by *Eco*RI, the control revealed a smaller DNA band size and the presence of *NPM1* at an expected size of 0.7 kb (**Figure 4-14B**). Additionally, the lack of *NPM1* and the presence of *ECGFP* in the vector control as well as the existence of wild-type *NPM1* or mutated *NPM1* in the recombinant DNA construct were confirmed by DNA sequencing (data not shown).

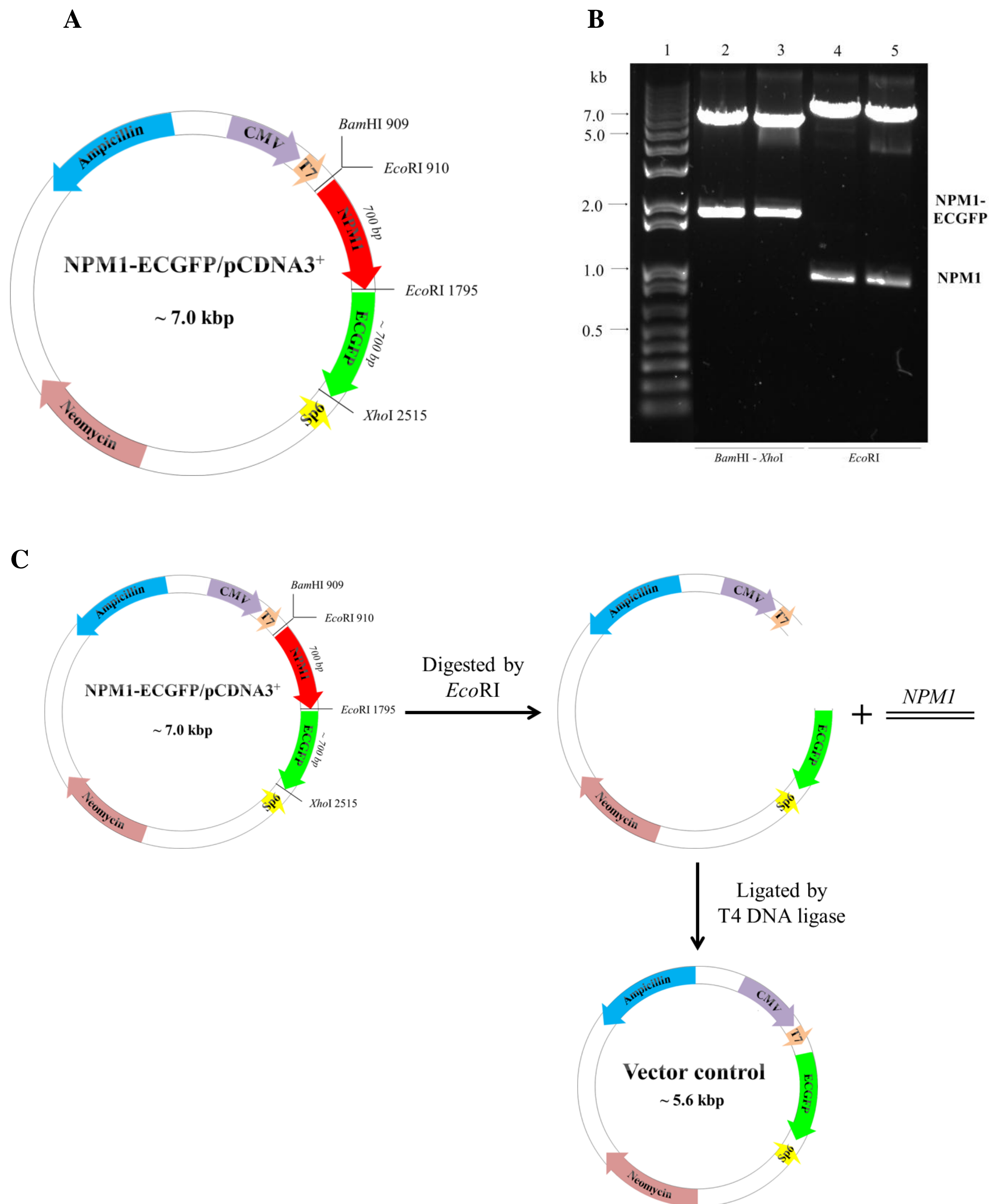


Figure 4-13 Production of pCDNA3^{+}-ECGFP (vector control). **(A)** Illustration of the structure of pCDNA3^{+}-*NPM1*-ECGFP. **(B)** Agarose gel of pCDNA3^{+}-*NPM1*-ECGFP cut with *Bam*HI and *Xho*I (lane 2 and 3) or *Eco*RI only (lane 4 and 5). Both recombinant plasmids containing wild-type *NPM1* (lane 2 and 4) and mutated *NPM1* (lane 3 and 5) were used. Molecular weight standards (lane 1) are shown in kb on the left of the figure. DNA bands at about 1.4 kb or 0.7 kb size indicate *NPM1-ECGFP* or *NPM1* respectively. **(C)** Schematic delineation of production of pCDNA3^{+}-ECGFP. The pCDNA3^{+}-*NPM1*-ECGFP recombinant plasmid was digested by *Eco*RI to separate *NPM1* gene. The cleaved plasmid was ligated by T4 DNA ligase resulting in pCDNA3^{+}-ECGFP (vector control).

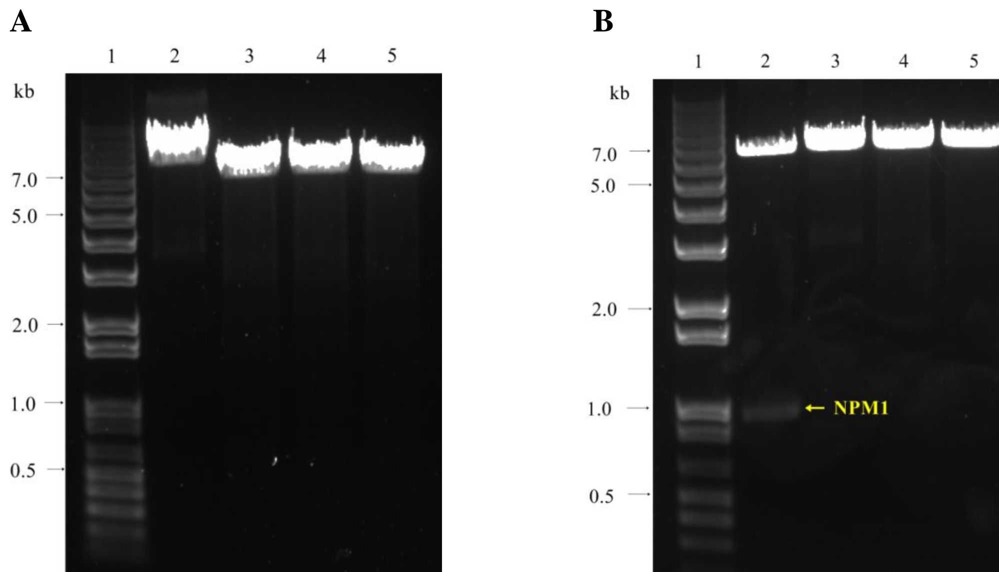


Figure 4-14 Agarose gels of ligated vectors after bacterial transformation and plasmid purification. (A) The vectors were digested by *Bam*HI. **(B)** The vectors were digested by *Eco*RI. Three colonies (lane 3 to lane 5) were randomly selected after bacterial transformation for plasmid purification. pCDNA3^{+}-*NPM1*-ECGFP (lane 2) was used as a control. Molecular weight standards (lane 1) are shown in kb on the left of the figure. The yellow arrow indicates *NPM1*.

4.4.2. Expression of wild-type NPM1-ECGFP and mutated NPM1-ECGFP in HEK293 cells

In order to examine the modulation of p53 isoforms by wild-type NPM1 and mutated NPM1, wild-type *TP53* human embryonic kidney (HEK293) cells were transiently transfected by wild-type *NPM1-ECGFP*, mutated *NPM1-ECGFP* or *ECGFP* alone (vector control). The expression of wild-type NPM1-ECGFP (NPM1wt) and mutated NPM1-ECGFP (NPM1mut) in HEK293 cells was indirectly demonstrated by the presence of ECGFP^{+} cells through flow cytometric analysis. Also, the results revealed that the percentage of ECGFP^{+} cells is low for all vector control, NPM1wt and NPM1mut (**Figure 4-15A**). Fluorescence microscopy did not only verify the expression of ECGFP in HEK293 cells, but it also signified the expression of NPM1wt and NPM1mut via the intracellular localization of the proteins: NPM1wt was localized in nucleolus, while NPM1mut was in cytoplasm (**Figure 4-15B**). Western blot analysis of cell extracts from wild-type (not transfected), vector control, NPM1wt and NPM1mut HEK293 cells utilizing specific NPM1 antibody confirmed that NPM1wt and NPM1mut cells express the fusion proteins while wild-type and vector control cells do not. Furthermore, the result exposed that the expression level of fusion proteins is profoundly low in comparison with the expression level of wild-type NPM1 of the cells (**Figure 4-15C**).

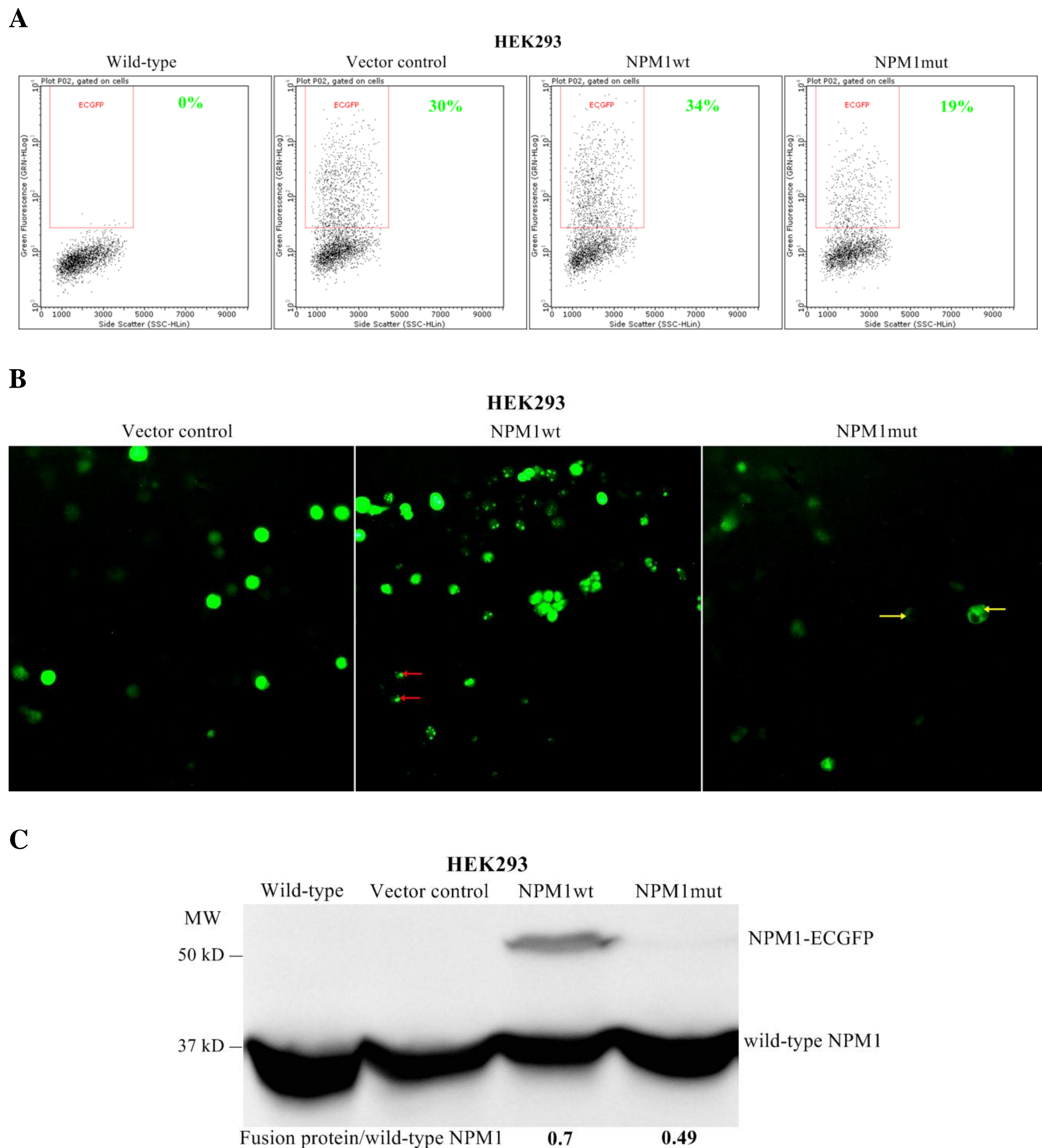


Figure 4-15 The expression of wild-type NPM1-ECGFP (NPM1wt) and mutated NPM1-ECGFP (NPM1mut) in HEK293 cells. (A) Flow cytometric analysis of cells after transfection. From the left: The plot of wild-type (not transfected), vector control, NPM1wt and NPM1mut HEK293 cells. Red rectangles present ECGFP^{+} cells. The percentage of ECGFP^{+} cells is indicated in the upper right corner of each plot. **(B)** Fluorescence microscopy of vector control, NPM1wt and NPM1mut HEK293 cells after transfection. Red and yellow arrows indicate nucleolus and cytoplasm respectively. **(C)** Western blot analysis of NPM1 in wild-type, vector control, NPM1wt and NPM1mut HEK293 cells. The expression of NPM1-ECGFP fusion proteins was verified by a protein band at an expected size of 65 kD (lane 3 and 4). The wild-type and vector control HEK293 cells did not express the fusion proteins (lane 1 and 2). Molecular weight is indicated on the left; the ratio of quantified values of fusion protein and wild-type NPM1 of the cells is shown at the bottom of the figure.

4.4.3. Assessing the modulation of p53 isoforms and related proteins following transfection of wild-type NPM1 and mutated NPM1

The modulation of p53 and related proteins such as Mdm2 and SRSF3 was investigated by Western blot analysis of wild-type, vector control, NPM1wt and NPM1mut HEK293 cells after 48 hours of transfection. Nonetheless, a significant change in the expression level of p53, Mdm2 and SRSF3 in NPM1wt and NPM1mut cells was not observed (**Figure 4-16**).

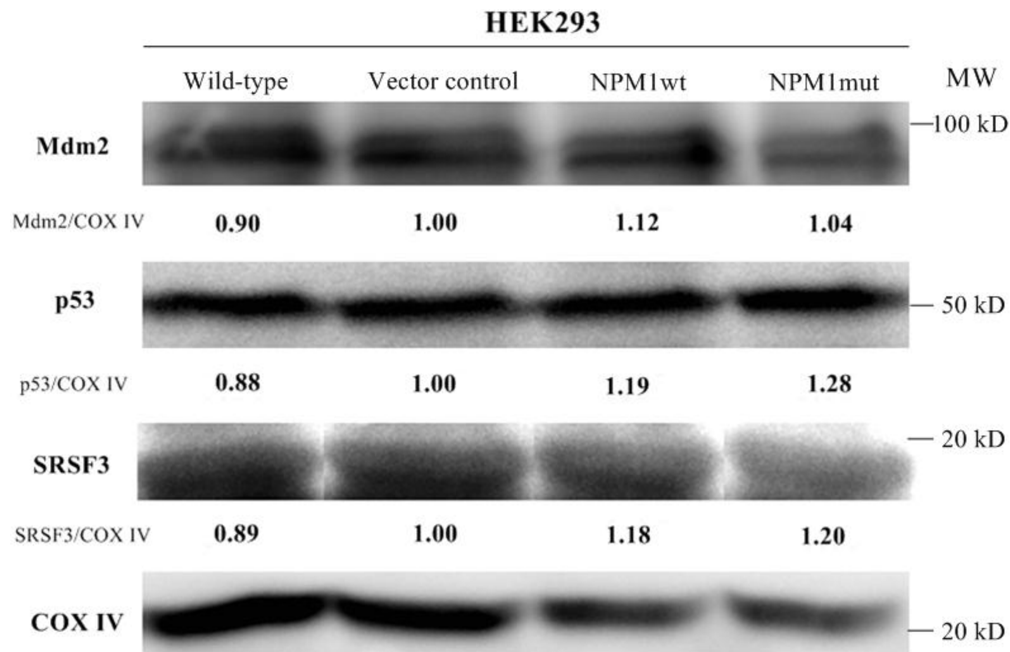


Figure 4-16 Western blot analysis of wild-type, vector control, NPM1wt and NPM1mut HEK293 cells. Proteins investigated are shown in bold letters to the left and molecular weight is indicated to the right of the figure. Quantified values were normalized to COX IV as loading control and compared to the Vector control cells' value set to 1.00. The result represents two different experiments.

5. Discussion

This thesis consists of three different parts mostly concerned in the function and modulation of p53β and p53γ isoforms in AML. The first part focused on the functional characterization of p53β and p53γ in AML cell lines to clarify if p53β and p53γ have individual roles or they interact with FLp53 to function. In the second one, the effect of p53β and p53γ on AML cells and the modulation of p53 isoforms and Mdm2 in response to oxidative stress by resazurin were evaluated. Assessing the correlation between wild-type or mutated NPM1 and p53 isoforms modulation was the target of the last part. At the end of the thesis, future perspectives will be discussed.

5.1. Functional characterization of p53β and p53γ in AML cell lines

Given that high expression of p53β and p53γ is associated with increased response to chemotherapy and longer survival in AML patients [136] and their single expression enhances chemosensitivity in $p53^{null}$ cancer cell lines [124], we hypothesized that p53β and p53γ may have individual functions independently of FLp53 in AML. Proposing that stable expression of these p53 isoforms in a $p53^{null}$ AML cell line may indicate their distinct functions, the *TP53* deleted ($p53^{null}$) HL-60 AML cell line [171] was selected after validation of *TP53* deletion (E. Silden, unpublished data). HL-60 cells were transduced with p53β or p53γ construct to establish singly stable expression of p53β or p53γ protein. The stable expression of these isoforms in HL-60 cells was successfully created. This is shown by the result of Western blot analysis of p53 in wild-type, vector control, $p53\beta^{+}$ and $p53\gamma^{+}$ HL-60 cells (**Figure 4-1B**). The expression level of p53γ protein, as can be seen, is considerably lower than of p53β. The $p53\gamma^{+}$ HL-60 cells also reveal lower fluorescence intensity of tdTomato and less stability in comparison with $p53\beta^{+}$ cells and vector control cells (**Figure 4-1A**). Moreover, the basal level of Bax and $p21^{CIP1/WAF1}$ proteins in these cells is higher than in vector control and $p53\beta^{+}$ cells (**Figure 4-1C**). These data propose that a higher expression level of p53γ protein can be toxic to the cells. Furthermore, $p53\beta^{+}$ HL-60 cells showed a higher expression level of Mdm2 than vector control cells did and the level of this protein in $p53\gamma^{+}$ cells is substantially higher than in vector control as well as in $p53\beta^{+}$ cells (**Figure 4-1C**). This suggests that the presence of p53β or p53γ in HL-60 cells clearly modulates the expression of Mdm2 and proposes a possible role of Mdm2 in the regulation of these p53 isoforms. Interestingly, a difference in the basal level of Bax and Mdm2 between

vector control and wild-type HL-60 cells was observed (**Figure 4-1C**). The vector control transduced cells have a substantially lower level of Bax and Mdm2 proteins compared to wild-type cells. A possible explanation is that retroviral transduction through viral insertion of DNA has changed the phenotype of the cells. Studies showed that the viral insertion of transgenes into chromosomal DNA in hematopoietic cells may result in the insertional activation of proto-oncogenes leading to a proliferative advantage and possible leukemogenesis [176, 177].

Colony formation assay did not display any significant difference in the number as well as the size of colonies between wild-type, vector control, p53β$^+$ and p53γ$^+$ HL-60 cells. Nonetheless, while vector control and p53β$^+$ cells exposed similarity in the morphology of colonies, a variation was seen between them and p53γ$^+$ cells (**Figure 4-2**). This finding suggests that p53γ may have roles in the differentiation of the cells. Furthermore, the colonies of wild-type and vector control cells showed a difference in morphology, indicating one more time different properties between wild-type and vector control cells.

In some previous studies, both p53β and p53γ were demonstrated to increase chemosensitivity of cancer cells [124, 136]. Therefore, we investigated the response of wild-type, vector control, p53β$^+$ and p53γ$^+$ HL-60 cells to chemotherapy by examining the proliferation and viability of the cells after treatment with the cytotoxic drugs Doxorubicin and Camptothecin. However, no significant changes in cell proliferation and apoptosis were found (**Figure 4-3**). This observation does not support the finding that p53β or p53γ enhances Doxorubicin and Camptothecin sensitivity in cancer cell lines [124]. Our finding that p53β and p53γ do not affect the proliferation and apoptosis of HL-60 cells following Doxorubicin and Camptothecin treatment does not necessarily mean that the isoforms do not affect the response of HL-60 cells to chemotherapy. In the context of this study, we only inspected the growth and survival of the cells in response to two genotoxic drugs at one concentration (0.5 μM) and at two different time points (8 and 24 hours). Thus, it is unclear whether these isoforms cause distinct responses to different doses, timeframes or types of chemotherapeutic agents. Furthermore, because we only utilized the WST-1 based cell proliferation assay which indirectly evaluates the proliferation of cells through measuring metabolic activity to examine chemotherapeutic response of the cells, it cannot be concluded firmly that p53β and p53γ do not inhibit cell proliferation. More investigations are required.

In a previous study, it has been proposed that the high expression of p53β and p53γ combined with the low expression of FLp53 in AML correlates with longer patient survival and

chemosensitizing enhancement [136]. This has elicited a question if p53β and p53γ interact with FLp53 in a ratio-dependent manner in AML. In order to elucidate this query, the generation of AML cells having low FLp53 protein level and high p53β and p53γ protein level is a prerequisite. The FAB class M5 wild-type *TP53* MOLM-13 AML cell line [172] expressing FLp53, p53β and p53γ was transduced with retroviral vector containing p53β-tdTomato or p53γ-tdTomato to establish higher expression of p53β or p53γ. Nonetheless, the fact that only 0.81% or 0.66% of cells were successfully transduced with p53β-tdTomato or p53γ-tdTomato construct respectively after four times of transduction (**Figure 4-4B**) suggests that a greater expression level of p53β or p53γ may be unfavorable to the cells. This is confirmed by the fact that cells successfully transduced with p53γ-tdTomato construct died gradually after FACS (**Figure 4-5, 4-6**), signifying that p53γ up-regulation is toxic to the MOLM-13 cells. This finding corresponds with prior reports that p53γ may be cytotoxic [121, 124] and strengthens the observations of p53γ's toxic effect on HL-60 cells. Furthermore, the p53γ transduced MOLM-13 cells showed a morphological change compared to wild-type MOLM-13 cells (**Figure 4-6**), indicating that the increase of p53γ may affect differentiation status of the cells. We were successful in producing MOLM-13 cells with a higher expression of p53β. This is demonstrated by Western blot analysis of p53 in the MOLM-13 cells transduced with p53β construct in comparison with wild-type and vector control cells (**Figure 4-7**). Although the colonies of these cells did not show a significant difference in quantity, size or morphology, they displayed a much lower fluorescence intensity of tdTomato than vector control transduced cells' colonies did (**Figure 4-5B,C**). This reinforces the suggestion that the higher expression of p53β in MOLM-13 cells may be unfavorable.

In summary, based on the observations of stable expression and function of p53β and p53γ in HL-60 cells as well as the impact of higher p53β and p53γ expression level on MOLM-13 cells, we propose that p53β and p53γ may act in concert with FLp53 in a ratio-dependent manner. The fact that the stable expression of p53γ can be established in p53^{null} HL-60 cells but its greater expression level cannot be created in wild-type *TP53* MOLM-13 cells verifies this conclusion. Also, this is strengthened by the fact that the p53β construct was readily transduced into HL-60 cells (E. Silden, unpublished data) but was not in MOLM-13 cells. In this thesis, our goals were to investigate if the functional roles of p53β and p53γ are independent of FLp53 in a p53^{null} AML cell line and to establish a higher expression of p53β or p53γ in a wild-type *TP53* AML cell line in order to study their functions in concert with

FLp53 and endogenous p53β and p53γ. We basically examined individual functions of p53β and p53γ in clonogenicity, cell proliferation and apoptosis in response to chemotherapy. Additionally, we partly clarified the interaction of p53β and p53γ with FLp53 in performing their functions when we tried to establish the up-regulation of p53β and p53γ in wild-type *TP53* MOLM-13 cells. Although our efforts to up-regulate p53γ expression level in MOLM-13 cells were unsuccessful, the up-regulation of p53β in these cells was established.

5.2. Modulation of p53 isoforms and Mdm2 in response to resazurin

Resazurin, a redox indicator, has been demonstrated to have cytotoxic effects in leukemic cells [38]. It has been proposed to act through the activation of ROS generation, leading to a cellular stress response which probably initiates mitochondrial dysfunction followed by degradation of the cell [39]. In addition, the generation of ROS which is critical to redox signaling has been shown to play roles in p53 regulation [103]. Based on these data, we investigated if resazurin exposure regulates p53 through the modulation of p53 isoforms full-length, β and γ as the mechanism of its action and if the single expression of p53β and p53γ affects resazurin's cytotoxic effects in AML cell lines. We found that the stable expression of p53β in HL-60 cells significantly increases cell apoptosis in comparison with p53γ^+ HL-60 cells following resazurin exposure. A considerable difference in the ratio of apoptotic cells between p53β^+ cells and vector control cells was also observed after 24 hours of resazurin exposure (**Figure 4-8A**). Interestingly, wild-type HL-60 cells were more sensitive than vector control cells to an exposure of 40 μM resazurin for 24 hours, strengthening the observation that vector control cells bear distinct characteristics from wild-type cells. Western blot analysis of Caspase-3 cleavage, an important marker for apotosis, in vector control, p53β^+ and p53γ^+ cells exposed to 40 μM resazurin for 48 hours also confirm that p53β increases apoptosis of HL-60 cells in response to resazurin (**Figure 4-8B**). However, it is not clear whether this effect of p53β on the cells depends on dose or time of resazurin exposure. In addition, p53β and p53γ may influence the cells by other mechanisms than apoptosis in response to resazurin. Further studies are needed to elucidate these inquiries.

The modulation of p53 isoforms by resazurin exposure was examined in p53β^+ and p53γ^+ HL-60 cells as well as MOLM-13 cells. However, Western blot analysis of p53 in these cells showed that resazurin exposure does not affect FLp53 as well as p53β and p53γ level (**Figure 4-9**), suggesting that the modulation of p53 isoforms full-length, β and γ is not the mechanism of resazurin activity. Interestingly, Western blot analysis of other proteins in the

p53 pathway such as Mdm2, Bax and $p21^{CIP1/WAF1}$ in shControl and shp53 MOLM-13 cells treated with resazurin revealed significant changes. The shControl MOLM-13 cells which contain a normal expression level of p53 presented an up-regulation of $p21^{CIP1/WAF1}$ protein and unchanged Mdm2 protein level, whereas the shp53 MOLM-13 cells which bear a much lower level of p53 showed a nearly unalterable $p21^{CIP1/WAF1}$ level and slight down-regulation of Mdm2 (**Figure 4-10A**), proposing that the exposure to resazurin clearly affects the MOLM-13 cells and these effects may depend on the expression level of p53. Nonetheless, examining the viability of the these cells simultaneously by nuclear morphology cell death assay and Annexin V-Alexa 488 – To-pro-3 cell death assay showed that resazurin does not primarily affect the MOLM-13 cells through the induction of apoptosis (**Figure 4-12**). This corresponds with the observation that the expression level of Bax protein in both shControl and shp53 MOLM-13 cells remained the same after resazurin exposure (**Figure 4-10A**). The discovery that resazurin exposure enhances $p21^{CIP1/WAF1}$ level in shControl MOLM-13 cells proposes that resazurin may affect the cells through other mechanisms such as cell cycle arrest or cell senescence but it is not clear if this up-regulation of $p21^{CIP1/WAF1}$ is dependent or independent of p53. Despite the fact that resazurin exposure did not change the expression level of p53 in these cells, it does not necessarily mean that this $p21^{CIP1/WAF1}$ enhancement is p53-independent. In this case, resazurin may activate p53 through other mechanisms, for instance, post translational modifications (PTMs). The activated p53 may cause transcription of the gene encoding $p21^{CIP1/WAF1}$ protein resulting in the up-regulation of $p21^{CIP1/WAF1}$.

Western blot analysis of Mdm2 protein in all wild-type, vector control, $p53\beta^{+}$ and $p53\gamma^{+}$ HL-60 cells following resazurin exposure revealed a substantial attenuation of this protein (**Figure 4-10B**), suggesting a p53-independent down-regulation of Mdm2 by resazurin. Moreover, Western blot analysis and cell viability evaluation of wild-type HL-60 cells exposed to resazurin in a dose-response manner showed a progressive attenuation of Mdm2 corresponding to a stepwise increase of apoptosis (**Figure 4-11**) consolidating the conclusion that resazurin can induce down-regulation of Mdm2 via a p53-independent pathway. However, it is still ambiguous whether or not resazurin induces the apoptosis of HL-60 cells through this p53-independent attenuation of Mdm2. Many researchers have demonstrated that Mdm2 may regulate proliferation and survival of cancer cells via p53-independent pathways [178-180]. On the other hand, the increase of Caspase-3 has been shown to cause the down-regulation of Mdm2 [181]. Therefore, making this query clear is essential.

To summarize, resazurin exposure has been revealed to have distinct impacts on Mdm2 level in AML cell lines depending on the expression level of p53 in these cells. In this study, we found that shControl MOLM-13 cells holding a normal p53 level have the same Mdm2 level while the level of Mdm2 in the shp53 MOLM-13 cells which carry a low level of p53 is slightly down-regulated following resazurin exposure. When the p53-defective HL-60 cells were exposed to resazurin, they presented a substantial attenuation of Mdm2, suggesting a p53-independent down-regulation of Mdm2 by resazurin. Furthermore, we discovered that the stable expression of p53β in HL-60 cells augments cell apoptosis after resazurin exposure, proposing that p53β may have functional roles in response to resazurin.

5.3. Correlation between wild-type or mutated NPM1 and p53 isoforms modulation

The finding that *NPM1* mutation, a good prognostic marker in AML, was associated with high expression of p53β and p53γ proteins [136] has led to an enquiry if wild-type and mutated NPM1 play roles in the modulation of p53 isoforms expression. We transiently established the expression of wild-type and mutated NPM1 in a wild-type *TP53* cell line and investigated the modulation of p53 isoforms full-length, β and γ in these cells in comparison with control cells. In order to perform this study, the utilization of recombinant plasmids containing wild-type or mutated *NPM1* DNA as well as vector control is required. The recombinant DNA constructs bearing wild-type or mutated *NPM1* DNA which express NPM1 tagged ECGFP was already constructed. However, since the vector control was lacking, we generated it from the recombinant plasmid with *NPM1*. We were successful in creating the vector control pCDNA3^{+}-ECGFP which expresses only ECGFP. This was shown by the result of agarose gel electrophoresis of ligated vectors after removing *NPM1* gene (**Figure 4-14**) and confirmed by DNA sequencing.

The expression of ECGFP tagged wild-type or mutated NPM1 was established in wild-type *TP53* HEK293 cells by transient transfection. It was revealed by the presence of ECGFP^{+} cells via flow cytometric analysis and fluorescence microscopy (**Figure 4-15A,B**). Fluorescence microscopy also confirmed the presence of wild-type NPM1-ECGFP in the nucleolus and mutated NPM1-ECGFP in the cytoplasm (**Figure 4-15B**). Furthermore, the expression of wild-type NPM1-ECGFP or mutated NPM1-ECGFP in these cells was verified by Western blot analysis of NPM1 using specific anti-NPM1 as the primary antibody. The

expression of these fusion proteins, however, was much lower than the wild-type NPM1 protein of the cells (**Figure 4-15C**).

In this study, our primary aim has been to investigate the role of wild-type and mutated NPM1 in p53 isoforms modulation in human wild-type *TP53* cells. The modulation of p53 and related proteins consisting of Mdm2 and SRSF3 in HEK293 cells transfected with wild-type *NPM1* or mutated *NPM1* construct was examined by Western blot analysis in comparison with the cells transfected with vector control. A significant difference in the modulation of these proteins was not observed (**Figure 4-16**). This does not support the previous finding that *NPM1* mutation was associated with high expression of p53β and p53γ. Moreover, mutated NPM1 was supposed to contribute to the inactivation and dislocation of ARF which lead to the increase of Mdm2 further resulting in the decrease of p53 level [163]. Our observation showed that there is no significant difference in the expression level of p53 between the cells transfected with mutated *NPM1* construct and the cells transfected with vector control. However, we could not conclude that wild-type and mutated NPM1 do not affect the modulation of p53 based on this observation because of the facts that the efficacy of transfection was low (**Figure 4-15A**) and the expression level of wild-type NPM1-ECGFP or mutated NPM1-ECGFP in HEK293 cells was much lower than the wild-type NPM1 protein of the cells. Otherwise, the dominant expression of FLp53 in HEK293 cells may be an obstacle. Additional studies are clearly needed to illuminate the correlation between wild-type or mutated NPM1 and p53 isoforms modulation.

5.4. Future perspectives

The achieved results of this thesis have partially uncovered the individually functional roles of p53 isoforms β and γ as well as their functional interaction with FLp53 in AML. However, they have also proposed several inquiries requiring additional investigations to elucidate such as the influence of p53β and p53γ on AML cells in response to chemotherapy as well as differentiation. More specific methods should be considered, for example, ^{3}H-Thymidin incorporation assay to evaluate cell proliferation and immunophenotyping to examine cell differentiation status. Besides, the dose-response as well as time-course to different types of chemotherapeutic agents should be clarified. The functions of p53β and p53γ in controlling cell cycle progression and cell senescence will also have to be investigated.

The finding that the up-regulation of p53γ in wild-type *TP53* MOLM-13 cells is cytotoxic represents that it may be a new therapeutic target in AML. Moreover, a further characterization of MOLM-13 cells with increased p53β is needed to better understand its functions. Future studies are needed to interpret the mechanism why p53γ is cytotoxic as well as to inspect whether or not the ratio-dependent interplay between FLp53, p53β and p53γ exists. Knocking down p53β and p53γ by shRNA to inactivate their functions in addition to viral transduction of p53 isoforms full-length, β and γ construct into cells to alter the ratio of these isoforms will make p53 isoform-specific functions clearer.

The discovery that the presence of p53β makes HL-60 cells more sensitive in response to resazurin requires further investigation. The effects of p53β and p53γ on cell proliferation, cell cycle progression and senescence following resazurin exposure will have to be explored. Moreover, the mechanisms how resazurin up-regulates $p21^{CIP1/WAF1}$ in wild-type *TP53* MOLM-13 cells as well as how it down-regulates Mdm2 in a p53-independent pathway need to be clarified. The cause and effect relationship between Mdm2 and Caspase-3 in addition to the relationship between Mdm2 and other proteins than p53 which regulate their activity will also have to be investigated.

As discussed, the impact of wild-type and mutated NPM1 on the modulation of p53 isoforms expression was inconclusive by virtue of the low efficacy of transfection, very high expression level of wild-type NPM1 protein and the prevalent expression of FLp53 in HEK293 cells. In order to avoid this problem, optimization of transfection should be considered and another mammalian cell line should be used for transfection.

It has been mentioned that the down-regulation of SRSF3 will force the splicing of *TP53* pre-mRNA towards the p53β [125]. Hence, it will be exciting to examine the correlation between SRSF3 expression and p53 isoforms expression. Furthermore, FLT3-ITD, an important prognostic factor for short survival in AML, was correlated with the expression of FLp53 [136]. This correlation should also be clarified.

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