Study on Inactivation of Tumor Suppressor Protein p53 Transcriptional Activity by Gene Mutation and Starvation Stress

（遺伝子変異と飢餓ストレスによる癌抑制タンパク質 p53 の転写活性化能の不活性化に関する研究）

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Abbreviations

Abbreviations:

AAVS1	adeno-associated virus integration site 1
BAX	BCL-2-associated X protein
BD	basic domain
Cas	CRISPR-associated
CDK	cyclin dependent kinase
CFP	cyan fluorescent protein
CNS	central nervous system
CORE	consumption and release
CRISPR	clustered regularly interspaced short palindromic repeat
DAPI	4′,6-diamidino-2-phenylindole
DBD	dna binding domain
DMEM	Dulbecco's modified Eagle medium
EAA	essential amino acid
FBS	fetal bovine serum
HRP	horseradish peroxidase
iPS	induced Pluripotent Stem
IR	ionizing radiation
MDM2	mouse double minute 2
NEAA	non-essential amino acid
NLS	nuclear localization signal
PRD	proline rich domain
p53RE	p53 response element

RRM2B	ribonucleoside-diphosphate reductase subunit M2 B
SV40	simian virus 40
TAD	transactivation domain
TD	tetramerization domain
TIGAR	*TP53*-induced glycolysis and apoptosis regulator
TSS	transcription start site
UV	ultra violet
YFP	Yellow fluorescent protein

1. General Introduction

1.1. Tumor suppressor protein p53

Tumor suppressor protein p53 was first discovered in a complex with the SV40 tumor virus-derived large T-antigen [1, 2]. After determination of p53 protein as a tumor suppressor [3, 4], studies of p53 have become more important. To date, more than 40,000 articles containing “p53” in their title have been published as listed in Web of Science. Currently, the importance of p53 as a guardian of the genome in tumor biology is well recognized by researchers [5]. Cells maintain their homeostasis by selecting an appropriate response to various cellular stresses. p53 is a crucial protein responsible for transcriptional regulation of target genes and maintaining genomic integrity in response to these diverse cellular stresses such as hypoxic, oxidative, and endoplasmic reticulum stresses [6–9]. As representative target of p53, p21 (*CDKN1A*) induces cell cycle arrest in G1 phase by inhibiting CDK, BAX promotes the release of cytochrome C for apoptosis, RRM2B induces DNA repair, TIGAR suppresses glycolysis for glycolytic metabolism, and MDM2 controls the p53 protein level by negative feedback regulation [10–14] (**Figure 1-1**). Moreover, the p53-p21 pathway acts as a regulator of cellular senescence [15, 16].

1.2. Function of p53 protein

p53 mainly acts as a transcriptional factor in various cellular pathways including cell cycle arrest and apoptosis for tumor suppression [17]. In response to genotoxic

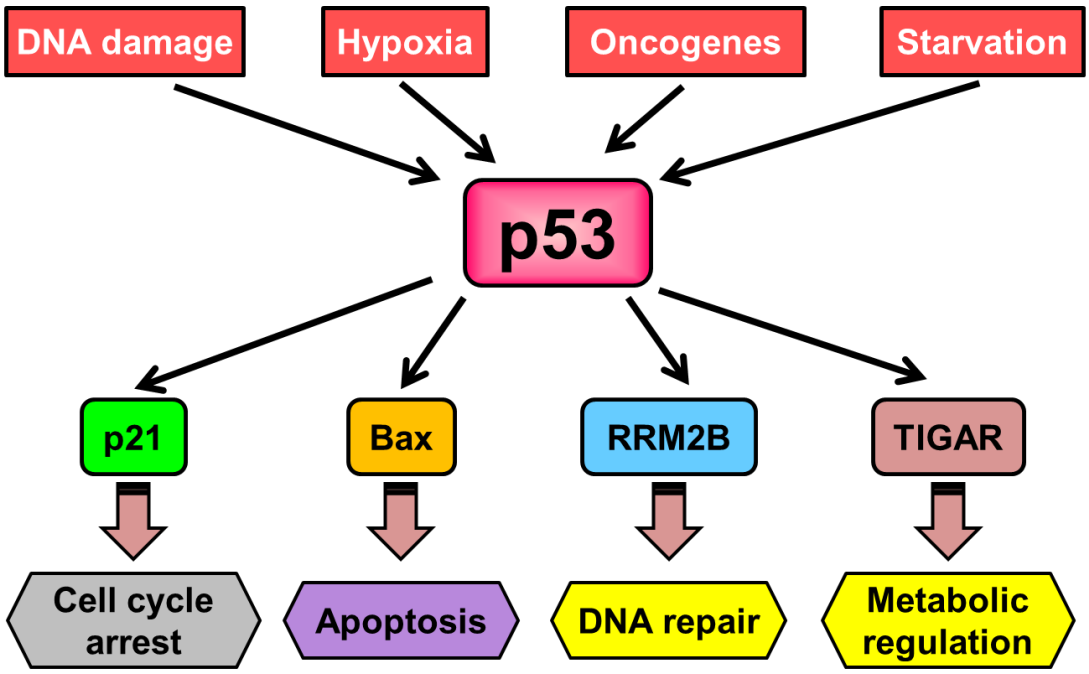

Figure 1-1. p53 is a central hub in multiple cellular pathway. p53 acts as a transcriptional factor in regulating the transcription of target genes, such as *CDKN1A* (p21) for cell cycle arrest, *BAX* for apoptosis, *RRM2B* for DNA repair, *TIGAR* for glycolytic metabolism.

stresses such as ionizing radiation (IR) and ultraviolet (UV) radiation, p53 is stabilized, tetramerized and activated through qualitative and quantitative regulation mainly by post-translational modification (**Figure 1-2**) [18]. The active form of p53 binds to response elements in target genes and regulates transcriptional levels. Cytoplasmic p53 also physically interacts with many other proteins including those involved in homologous recombination [19]. In mitochondria, p53 regulates transcription-independent apoptosis by direct protein-protein interaction [20]. It has also been reported that p53 is involved in microRNA processing by interacting with Drosha [21] and the p53-p21 pathway suppresses establishment of induced pluripotent stem (iPS) cells [22].

1.3. Structure of p53 protein

Human p53 protein is composed of the 393 amino acids. p53 protein consists of five domains: the N-terminal transactivation domain (TAD), proline-rich domain (PRD), central DNA-binding domain (DBD), tetramerization domain (TD), and the C-terminal basic domain (BD) (**Figure 1-3A**) [23]. The TAD (amino acid residues 1–42) has a binding site for many proteins, such as general transcriptional factors TFIID and TFIIH, and is responsible for transcriptional activity [24, 25]. The E3 ubiquitin ligase MDM2 binds to the TAD region for p53-negative feedback regulation [26]. The DBD (amino acid residues 101–300) binds directly to the p53 consensus DNA-binding site that usually comprises four pentanucleotide repeats [27, 28]. Tetramer formation of p53 is essential for its function [29]. The TD (amino acid residues 326–356) regulates the

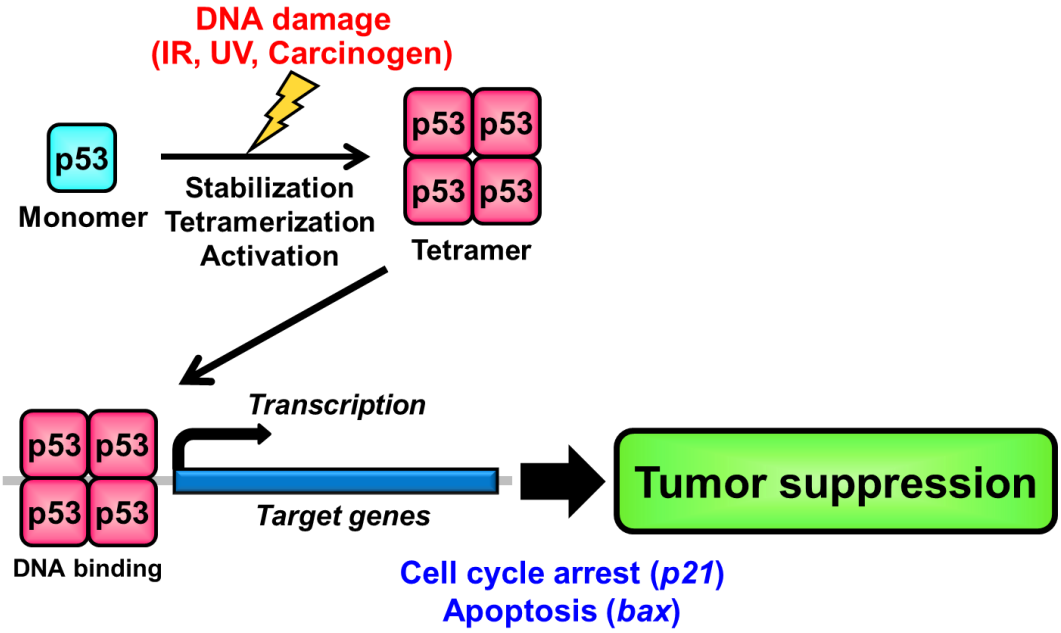

Figure 1-2. Schematic representation of p53 activation for tumor suppression

p53 is stabilized and tetramerized and activated in response to DNA damage. p53 regulates the transcription of target genes by binding to DNA in a sequence-specific manner, and induces cell cycle arrest and apoptosis for tumor suppression.

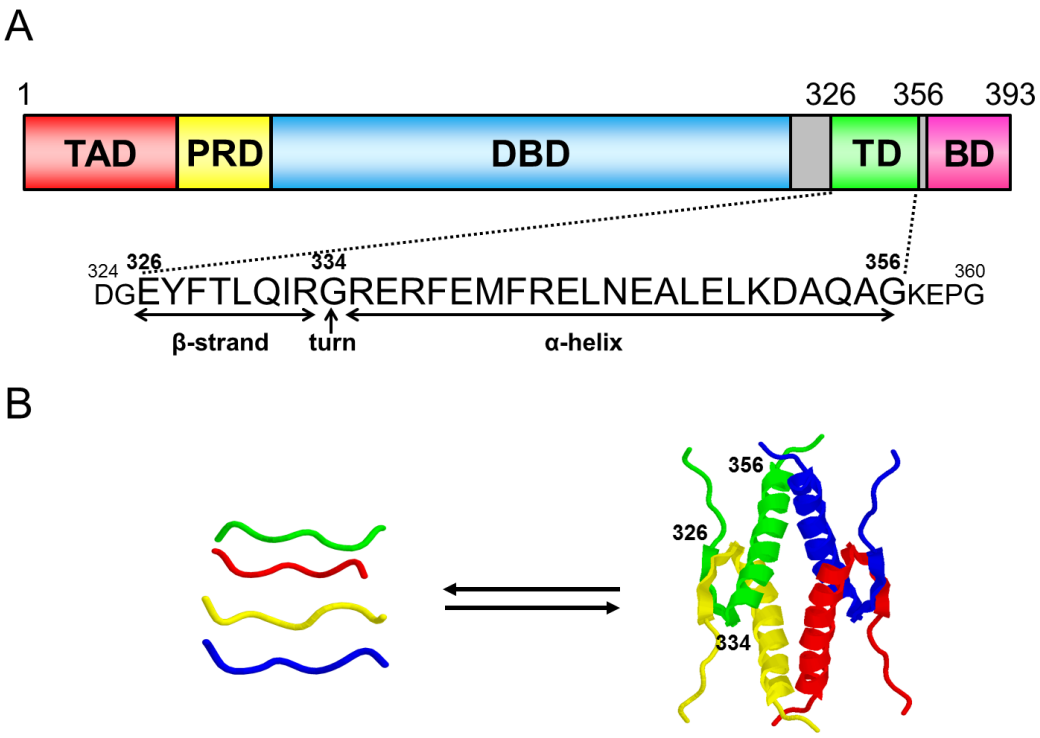

Figure 1-3. Schematic domain structure of p53 and amino acid sequence and Steric structure of p53 tetramerization domain (A) p53 consists of five domains: the N-terminal transactivation domain (TAD), the Proline-rich domain (PRD), the central DNA binding domain (DBD), the tetramerization domain (TD), and the C-terminal basic domain (BD). (B) The TD is located at C-terminal region (amino acid residue 326 to 356). Tetramer formation of p53 is in equilibrium with four monomers.

oligomeric state of p53 [30]. Two monomers form a primary dimer via an antiparallel β-sheet and helix packing [23]. Two dimers form a tetramer through a four-helix bundle. The TD consists of β-strand (residues 326–333), tight turn (residue 334), and α-helix (residues 335–356). Tetramer formation of p53 occurs in equilibrium with four monomers (**Figure 1-3B**) [31].

1.4. Response elements of p53 target genes

Tumor suppressor protein p53 binds to a specific DNA sequence in target genes via the DBD and regulates their transcription [32–34]. The p53 response element (p53RE) is usually found in the non-coding region on the target gene. The p53RE is most commonly located at the promoter region upstream from the transcription start site (TSS) of target gene. It also can be found close to the TSS, in intronic sequences and even exons. The latent transcriptional ability of p53RE generally depends on its distance from the TSS [35]. The p53-binding site in target genes originally comprises two half-sites (RRRCWWGYYY) with a spacer usually consisting of 0–21 base pairs where R is C or G, W is A or T, and Y is C or T (**Figure 1-4A**) [35, 36]. The sequence of the consecutive half site is reported most frequently. Each DBD of the p53 tetramer binds to a pentanucleotide sequence to regulate the transcription of target genes (**Figure 1-4B**) [28].

1.5. *TP53* mutation in tumors

TP53, encoding tumor suppressor protein p53, is the most frequently mutated

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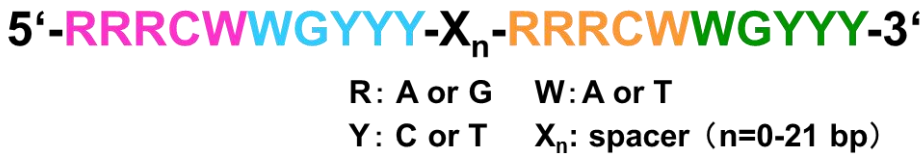

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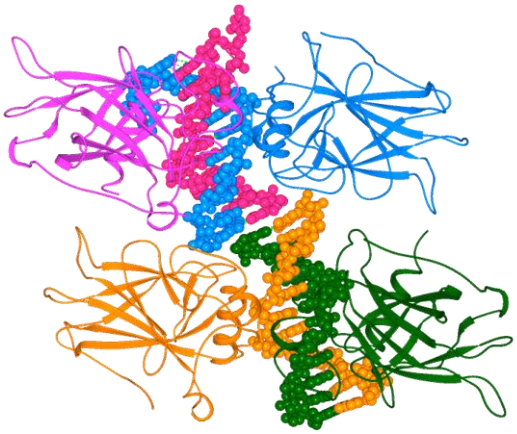

Figure 1-4. Consensus DNA sequences of p53 and complex of DNA-binding domain in p53 and DNA (A) The p53-binding site in the target genes comprises two half-sites (RRRCWWGYYY) with a spacer (X_n). R is C or G, W is A or T and Y is C or T, X_n (n=0-21 bp) is a spacer. (B) Each four colored DNA represents the sequence to which each DBD binds.

gene in various human malignant tumors [37, 38] (**Figure 1-5**). As the type of mutation, missense mutation, which are point mutations of nucleotides resulting in a change of the coding amino acid, are the most frequent (75%), followed by frameshift mutations, nonsense mutations, silent mutations, splice site mutations, in-frame deletions, and insertions (**Figure 1-6**) [39]. In somatic cells, the distribution of *TP53* point mutations is concentrated within the DBD, especially severe hotspots such as R273 (**Figure 1-7**) [37]. In germline cells, *TP53* point mutations are often found in the TD, including R337. The R337 mutation is associated with Li-Fraumeni syndrome (LFS) and Li-Fraumeni-like syndrome (LFL) that are autosomal dominantly inherited cancer predisposition disorders [40, 41].

In cases of heterozygous mutation of p53, which occurs in the early stage of tumorigenesis, hetero-tetramers should form between wild-type (WT) and mutant (Mut) p53 proteins. It is thought that p53 forms three kinds of hetero-tetramer species containing one to three WT proteins. It has been described that the dominant-negative effect of p53 mutation is very strong and abolishes the function of WT-p53 [42–44]. However, it is difficult to analyze the structure and function of each hetero-oligomer species of WT and Mut p53 proteins.

1.6. Hallmarks of cancer

Cancer cells have distinct hallmarks such as sustained proliferative signaling, cell death resistance, and abnormal energy metabolism [45] (**Figure 1-8**). Notably, the major characteristic of cancer cells is very rapid proliferation. For cell division, cell has

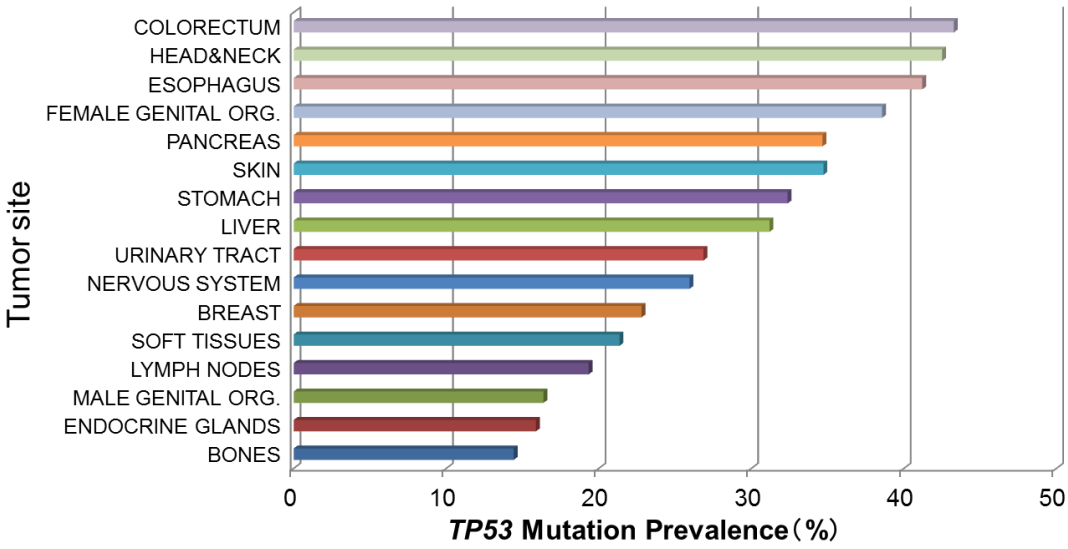

Figure 1-5. *TP53* mutation prevalence in human tumor site. *TP53* mutations have been reported in various tissues [37]. *TP53* is the most frequently mutated gene in human malignant tumors.

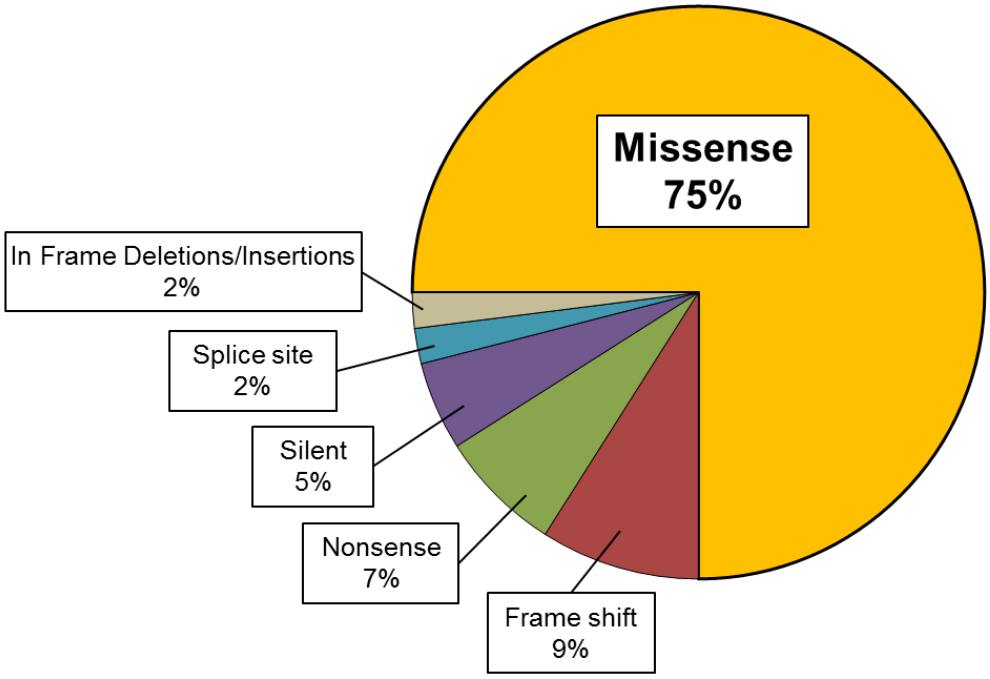

Figure 1-6. Mutation types of *TP53* in human malignant tumors

The most common of mutation type is missense mutation (75%) followed by frameshift, nonsense, silent, splice site, in frame deletions and insertions.

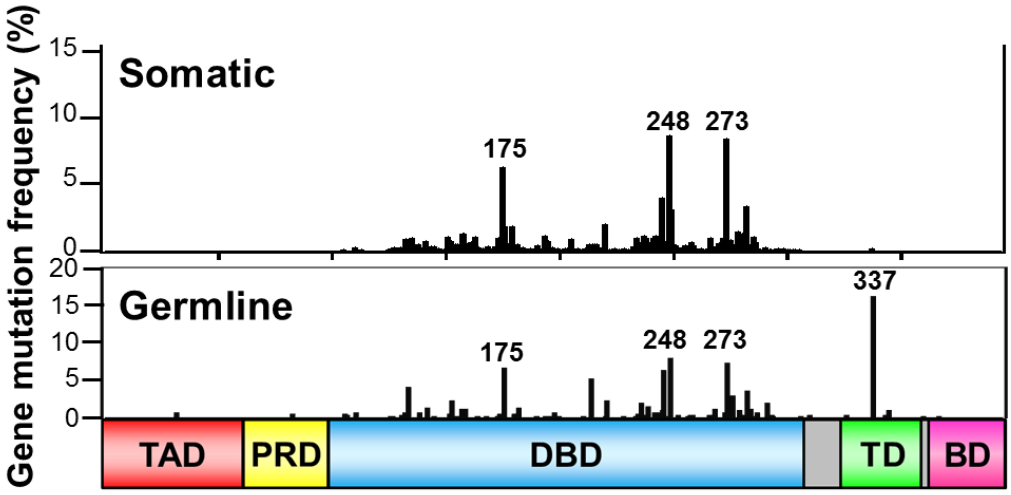

Figure 1-7. Codon distribution of *TP53* mutation in human malignant tumors. Figure represents the distribution and percentage of point mutation in somatic and germ line cells.

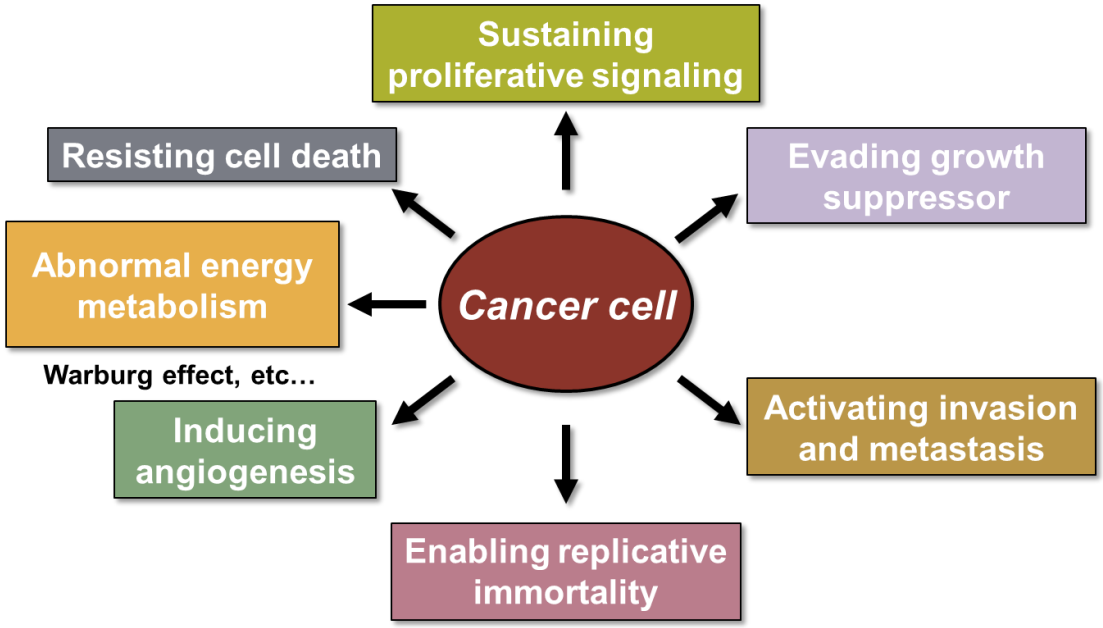

Figure 1-8. Hallmarks of cancer. Cancer cell acquired the hallmarks such as resisting cell death, sustaining proliferative signaling, evading growth suppressor, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, abnormal energy metabolism.

take up extracellular nutrients and subsequently synthesize all cellular components including nucleotides and proteins. Compared with normal cells, cancer cells have active metabolic mechanisms such as a high glycolytic flux referred to as the Warburg effect [46]. Recent studies have shown that the majority of the cancer cell mass is derived from amino acids [47]. Therefore, the metabolism in cancer cells has attracted attention as a target for cancer therapy. However, the mechanism of metabolic adaptation in response to nutrient stress remains largely unknown. It has been reported that cancer cells acquire anti-cancer drug resistance during the process of tumorigenesis [48]. One possibility is that metabolic adaptation results from changes in the tumor environment such as a poor nutrient condition. However, the mechanism of such drug resistance is not well understood.

1.7. Starvation stress and p53

Nutrients such as amino acids, glucose, vitamins, and growth factors are indispensable for cell growth and maintaining homeostasis. Starvation, which is defined as the deficiency of these nutrients, is a serious stress for cells and individuals. It is well known that cells exposed to starvation show the starvation response of metabolic remodeling, cell cycle arrest, and autophagy. In recent years, the function of p53 in the starvation response has been reported, such as inhibition of glycolysis and induction of autophagy [13, 49, 50]. In the case of amino acid starvation, p53-dependent metabolic alteration occurs. This metabolic alteration supports the cancer cell survival in response to serine starvation [51]. However, the cellular response mediated by p53 in response to

amino acid starvation is not fully understood.

1.8. Aims of this study

It is often described that p53 is the most important protein for tumor suppression through transcriptional control of target genes. Mutation of *TP53* in the early stages of carcinogenesis results in co-expression of WT and Mut-p53. Mutant p53 is expressed in the early stages of carcinogenesis. Therefore, inactivation of p53 is crucial for carcinogenesis. However, quantitative study of p53 inactivation via hetero-oligomerization is not fully understood. In this study, I investigated the effect of a p53 peptide containing the TD on p53 transcriptional activity of hetero-tetramers. As a further step, I investigated the effect of amino acid starvation on p53 transcriptional activity and cellular responses. In addition, I propose novel drug resistance induced by amino acid deprivation in lung carcinoma cells in combination with anti-cancer drug etoposide.

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2. Quantitative Analysis for Transcriptional Activity of p53 Hetero-tetramers between Wild-type Protein and Oligomerization Domain

2.1. Abstract

p53 acts as a transcriptional factor for tumor suppression via tetramerization. Although the dominant-negative effect occurs by hetero-oligomerization between wild-type and mutant p53, the precise mechanism remains unclear. Here, I report analysis of the transcriptional ability of each hetero-tetramer p53 at the physiological protein expression level in single cells. Quantitative fitting analysis showed that hetero-tetramers, which contain more than two WT-p53 proteins, have a substantial transcriptional ability. These results suggest that the two DNA-binding domains are important for transcription.

2.2 Introduction

The tumor suppressor protein p53 is well known as the most important transcriptional factor involved in preventing carcinogenesis [1]. p53 plays a central role in various cellular responses including cell cycle arrest, apoptosis, and DNA repair. p53 protein tetramerized and activated in response to genotoxic stresses such as UV radiation and oxidative stress [2]. The active form of p53 binds to the p53 consensus sequence for transcription of p53 target genes such as *CDKN1A* [3, 4]. Thus, p53 acts as a tumor suppressor responsible for maintaining genomic integrity. Tetramer formation of p53 protein is essential for its transcriptional activity and this tetrameric structure is in equilibrium with four monomers [5, 6].

TP53 encodes the tumor suppressor protein p53, and is the most frequently mutated gene in human malignant tumors [7]. In *TP53*, the most frequent somatic mutation occurs in the DNA binding domain (DBD) [8]. Mutation of p53 in the DBD, such as R273H, results in the ability to oligomerize, but mutant p53 (Mut-p53) is often unable to bind to the p53 target DNA sequence [9, 10]. In cases of heterozygous mutation of p53, which occurs in tumorigenesis, it is well established that hetero-tetramers are formed between wild-type p53 (WT-p53) and Mut-p53. It has been described that the dominant-negative effect exerted by Mut-p53 abolishes the function of WT-p53 [11–13]. However, the relationship between transcriptional activity and hetero-tetramers of WT-p53 and Mut-p53 has not yet been investigated at the physiological expression level in single cells. Complete of loss of heterozygosity occurs in the late stage of tumorigenesis [7]. Therefore, precise understanding of the

transcriptional activity of p53 hetero-oligomer species at initial step of carcinogenesis is of paramount importance. Induced pluripotent stem (iPS) cells are believed to be important for the development of regenerative therapies [14]. It has been reported that the p53-p21 pathway is involved in iPS cell generation in which p53 acts as a barrier [15]. Our laboratory previously reported that inhibition of p53 using the p53 tetramerization peptide is useful for transient p53 inactivation to generate iPS cell efficiently [16]. It has also been reported that a C-terminal p53 fragment including the oligomerization domain interacts with WT-p53 to form a hetero-oligomer [17]. p53 tetramerization peptides have also been utilized to control the metal nanostructure with biominerarization peptides [18, 19]. Here, I aim to measure the transcriptional activity of the hetero-tetramer p53 formed between WT and a p53 peptide fragment containing tetramerization domain.

2.3. Experimental procedures

2.3.1. Plasmid construction

I constructed the reporter plasmid, p(p53RE)-mCherry-nuclear localization signal (NLS)-(TetO)-TetOn Adv-Neomycin^R shown in **Figure 2-1A**. This plasmid carried the artificial p53RE, consisting of three tandem repeats of the p53-binding motif (5′-AGACATGTCCGGACATGTCT-3′) with a 16 bp spacer (5′-actagcggctgtcact-3′). Using this plasmid, red fluorescent protein (mCherry) with NLS from simian virus large T-antigen was p53-dependently expressed in the nucleus. This plasmid also contained the expression unit of the Tet-On Advanced transactivator (Tet-On Adv.) from the plasmid pTet-On Advanced (Clontech Laboratories, Inc.). Its expression was controlled by the $(TetO)_7$ promoter from the pTet-tTAk [20]. I also constructed a Tet-On expression plasmid, p(TRE)-Venus-p53-Zeocin^R, as shown in **Figure 2-1B**. This plasmid carried the P_{Tight} inducible promoter from the pTRE-Tight vector (TRE) (Clontech Laboratories, Inc.), which controls expression of a yellow fluorescent protein-p53 fusion protein (Venus-p53). The p(p53RE)-mCherry-NLS-(TetO)-TetOn Adv-Neomycin^R and p(TRE)-Venus-p53-Zeocin^R contained one or two repeats of the mRNA degradation signal, an AU-rich element [21–23], in the 3′-noncoding regions of mCherry-NLS and Venus-p53.

2.3.2. Cell culture and reporter assay

H1299 human non-small cell lung carcinoma cells (p53 null) were obtained from American type tissue culture collection (ATCC) (Rockville, MD, USA). Based on

the reporter system for p53-dependent transcriptional activity in a single cell, I established the stable cell line derived from H1299 cells by transfection of the p53 responsive reporter plasmid [p(p53RE)-mCherry-NLS-(TetO)-TetOn Adv-Neomycin^{R}] and Venus-WT-p53 expression plasmid [p(TRE)-Venus-p53-Zeocin^{R}]. Cells were selected in the presence of G418 and Zeocin and cloned by serial dilution in 96 well plates.

Stable cells were cultured on a 35-mm dish in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS). In the reporter assay, stable cells were transfected with 0.8 μg Cerulean-Mut-p53 expression plasmids [p(hCMV)-Cerulean-p53 (R273H) or p(hCMV)-Cerulean-NLS-p53[324-393] peptide or p(hCMV)-Cerulean-NLS] using lipofectamine 2000 (Invitrogen) in Opti-MEM. After 1–3 h of incubation, the medium was changed to RPMI 1640 supplemented with 10% (v/v) FBS. After 72 h of incubation, 25 ng doxycycline was added to the cells to induce Venus-WT-p53 expression. After 24 h of incubation, the cells were fixed with 3.7% formaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining. The cells were thereafter observed by fluorescence microscopy (BIOREVO BZ-9000, Keyence, Osaka, Japan). I used four fluorescent mirror units for observation. YFP-BP (excitation filter: 500/25 nm; dichroic mirror: 520 nm; absorbance filter 542/27 nm) was used for Venus, CFP-2432C (excitation filter: 438/24 nm; dichroic mirror: 458 nm; absorbance filter 483/32 nm) was used for Cerulean, TRITC (excitation filter: 540/25 nm, a dichroic mirror: 565 nm, absorbance filter 605/55 nm) was used for mCherry, and DAPI-BP (excitation filter: 360/40 nm; dichroic mirror: 400 nm;

absorbance filter 460/50 nm) was used for DAPI.

2.3.3. Fitting analysis

Fitting analysis was performed using Prism 5 (GraphPad Software, Inc.). The fitting equation is shown in the Results section. The fitted curve was calculated by non-linear least squares regression.

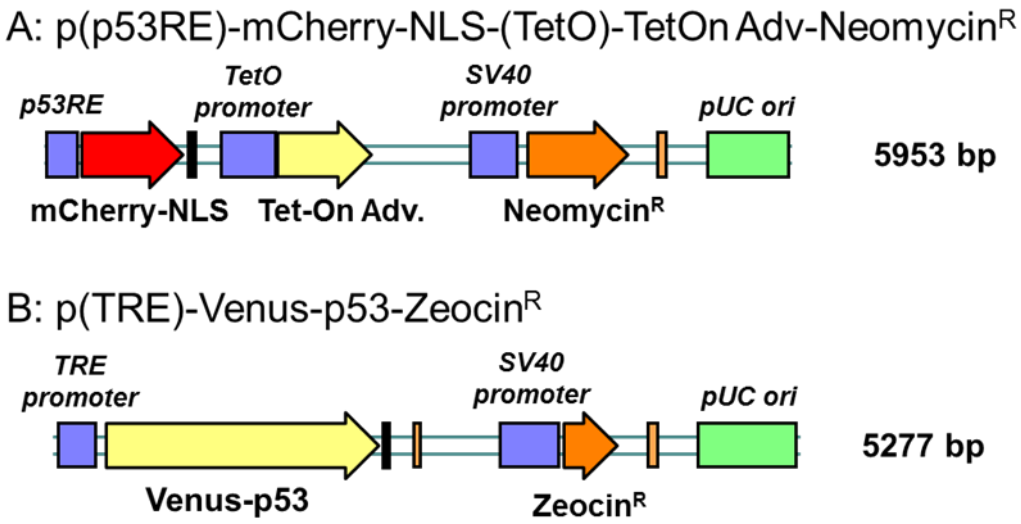

Figure 2-1. Constructed plasmids used in the p53 reporter assay. (A), p(p53RE)-mCherry-NLS-(TetO)-TetOn Adv-Neomycin^R. (B), p(TRE)-Venus-p53-Zeocin^R.

2.4. Results

2.4.1. Effect of the p53 peptide containing C-terminal and tetramerization domain on p53 transcriptional activity.

To evaluate the effect of the p53 peptide containing C-terminal region and TD on p53 transcriptional activity, I used the modified reporter assay system based on previous reports [16, 24] (**Figure 2-2**). In this system, WT-p53 protein with yellow fluorescent protein (Venus) is expressed by a tetracycline-inducible system (**Figure 2-3**). p53[324-393] peptide or Mut-p53R273H with cyan fluorescent protein (Cerulean) are expressed by transient transfection of a plasmid. The p53-dependent transcriptional activity is quantified by the fluorescent protein (mCherry) intensity derived from the p53RE reporter plasmid. DAPI was used for nuclear staining in this analysis.

First, I analyzed the effect of Mut-p53R273H on transcriptional activity. I selected cells that expressed the same level of total p53 at the physiological level for the analysis. The results showed that transcriptional activity clearly decreased to about 30% in the presence of Mut-p53R273H (**Figure 2-4, 5**). The p53 transcriptional activity was hardly affected by Cerulean-NLS protein in this assay. p53[324-393] peptide also decreased the transcriptional activity as well as Mut-p53R273H that showed a dominant-negative effect via hetero-oligomerization. These results indicated that the p53[324-393] peptide inhibited p53 transcriptional activity via hetero-oligomerization with WT-p53.

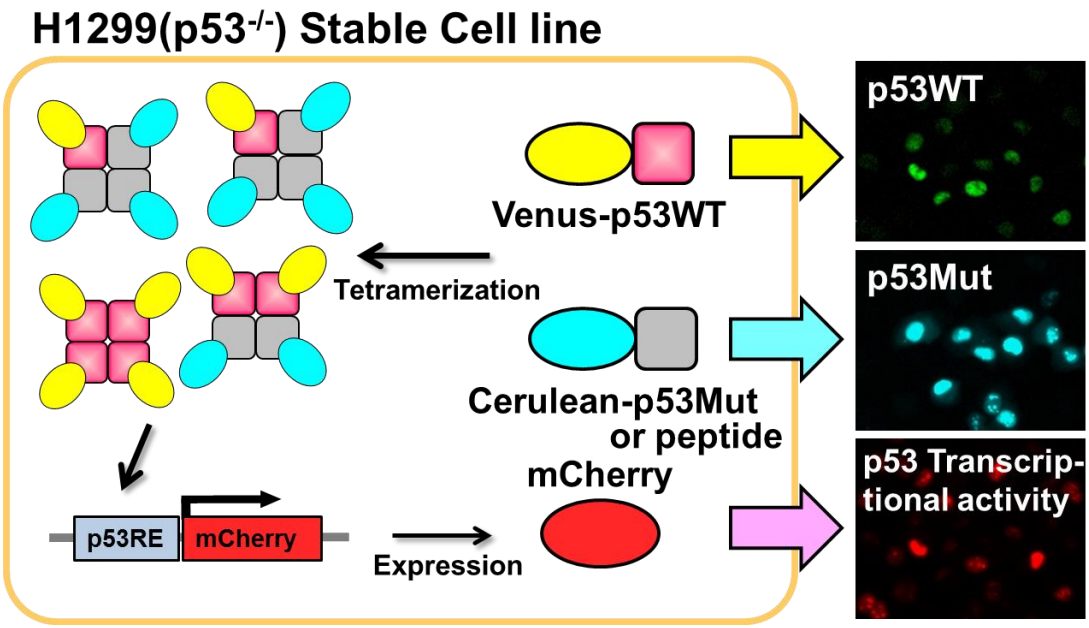

Figure 2-2. Schematic representation of the p53 reporter system.

WT-p53 protein with yellow fluorescent protein (Venus) is expressed at the physiological protein level by the tetracycline inducible system. Mut-p53 protein with cyan fluorescent protein (Cerulean) is expressed by transient transfection of a plasmid. When p53 hetero-tetramers bind to p53RE in a reporter gene, red fluorescent protein (mCherry) is expressed. This system simultaneously monitors the protein levels of WT- and Mut-p53, and p53-dependent transcriptional activity by measuring the intensities of the three fluorescent proteins in cells.

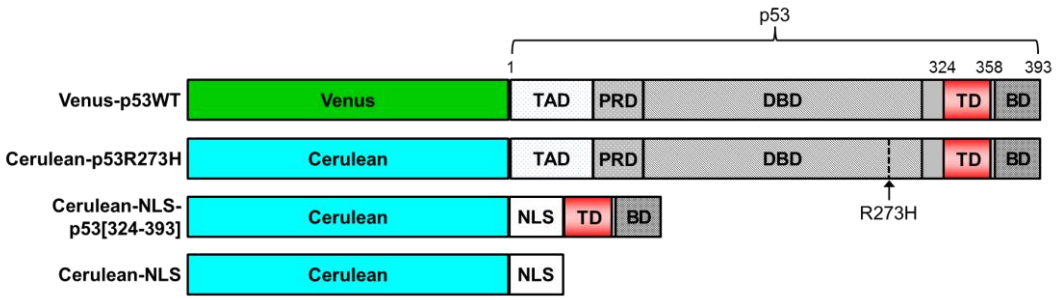

Figure 2-3. Schematic representation of proteins which are used in reporter assay.
TAD: Transactivation domain, PRD: Proline rich domain, DBD: DNA binding domain, TD: Tetramerization domain, BD: Basic domain, NLS: Nuclear localization signal. Allow indicates the location of p53 mutation (R273H).

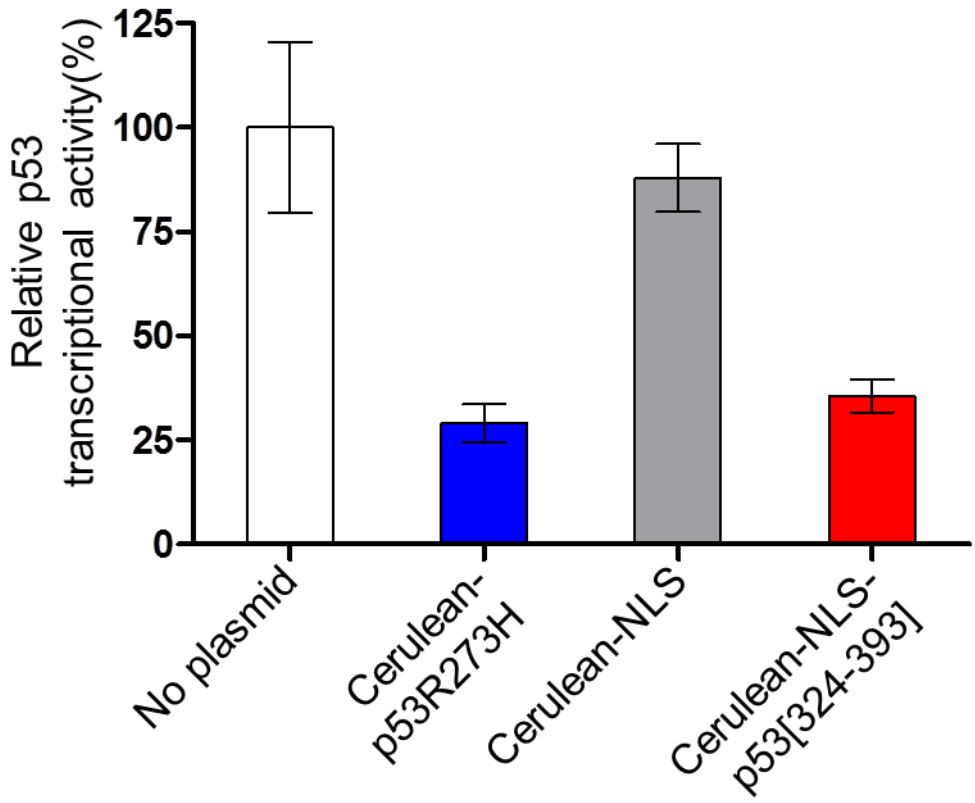

Figure 2-4. Relative p53 transcriptional activity in single cell expressed WT-p53 only (No plasmid) or the same expression level of WT-p53 and Cerulean-NLS-Mut-p53R273H or Cerulean-NLS or Cerulean NLS-p53[324-393]. Data represent the mean ± S.E. from three experiments.

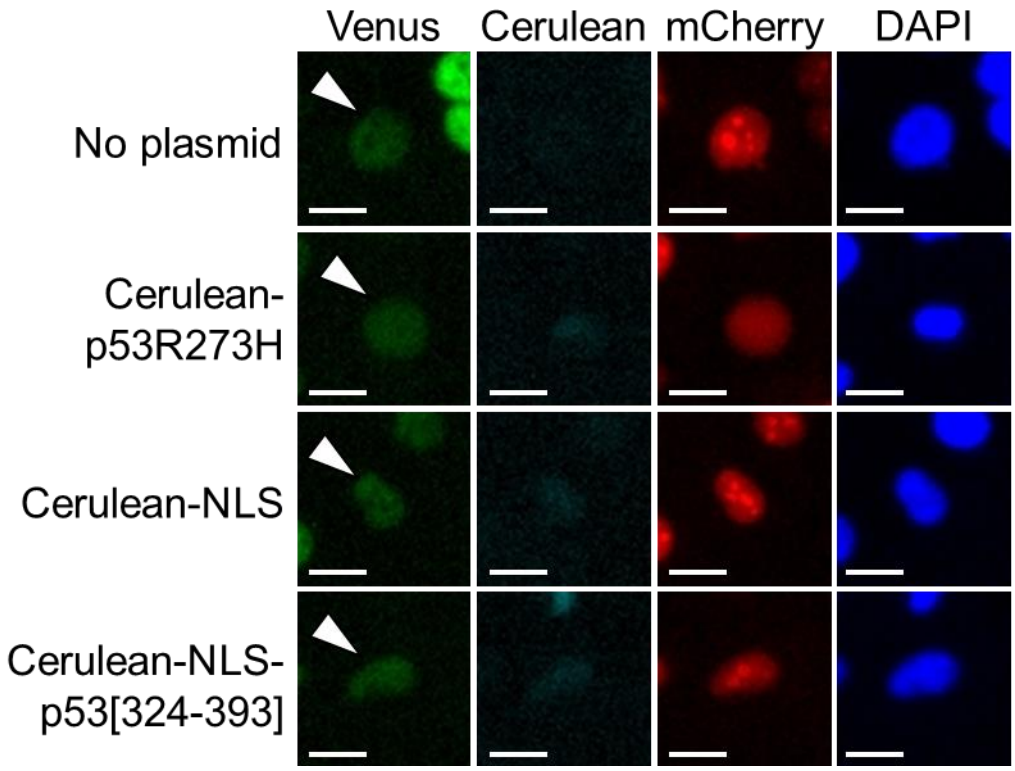

Figure 2-5. Fluorescence images. WT-p53 (Venus), p53R273H or p53[324-393] peptide (Cerulean), p53-dependent transcriptional activity (mCherry) were visualized by green, cyan and red using fluorescence microscopy. Allow heads showed the representative cells in each condition. Scale bars are 25 μm.

2.4.2. Effect of the hetero-tetramer p53 formed by WT and p53 peptide on transcriptional activity.

To determine the transcriptional ability of each hetero-tetramer p53 species, I analyzed p53 transcriptional activity at the physiological protein level of WT-p53 and various expression levels of the p53[324-393] peptide. As a result, the p53 transcriptional activity decreased as the amount of p53[324-393] peptide increased (**Figure 2-6, 7**). To estimate the transcriptional ability of the hetero-tetramer species formed by WT-p53 and p53[324-393] peptide, I constructed the fitting model shown below.

$$y = 100(1+x)((1/(1+x))^4 + 4T_1(1/(1+x))^3(x/(1+x)) + 6T_2(1/(1+x))^2(x/(1+x))^2 + 4T_3(1/(1+x))(x/(1+x))^3 + T_4(x/(1+x))^4) \quad \text{(i)}$$

This model with equation (i) estimates the quantitative ratio of WT homo-tetramers and p53[324-393] peptide homo-tetramers and hetero-tetramers between the WT-p53 and p53[324-393] peptide in terms of transcriptional activity. y means p53 transcriptional activity in a single cell. x means the quantitative ratio of the amount of p53 peptide compared with WT-p53. T_1 means the transcriptional ability of hetero-tetramers including one p53 peptide. T_2 means the transcriptional ability of hetero-tetramers including two p53 peptides. T_3 means the transcriptional ability of hetero-tetramers including three p53 peptides. T_4 means the transcriptional ability of p53 peptide homo-tetramers. The fitting model included three obvious assumptions, as

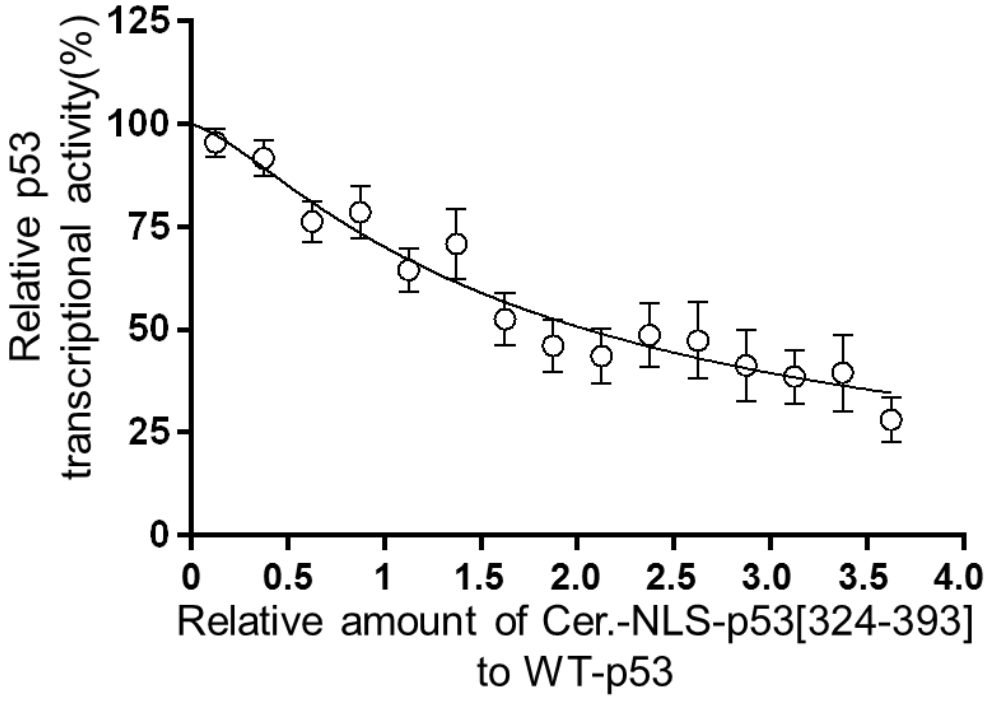

Figure 2-6. Relative p53 transcriptional activity in single cell which is expressed at the physiological protein level of the WT-p53 and various expression levels of the p53[324-393] peptide. Fitting value (R^2) is 0.947. The cell number of each point represents from at least 20. Data represent the mean ± S.E.

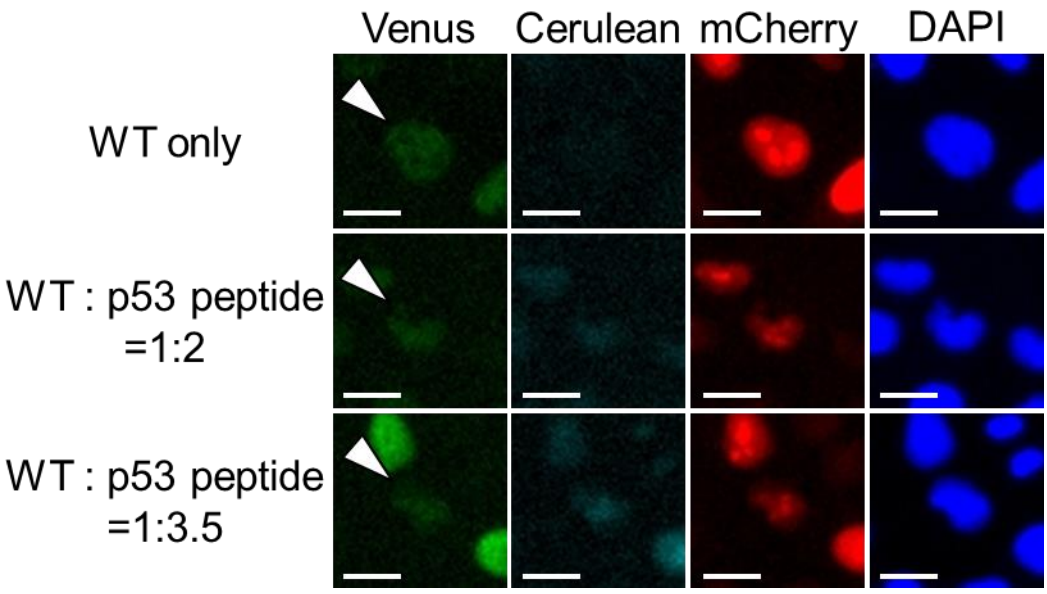

Figure 2-7. Fluorescence images of reporter assay. WT-p53 (Venus), p53[324-393] peptide (Cerulean), p53-dependent transcriptional activity (mCherry) were visualized by green, cyan and red using fluorescence microscopy. Allow heads showed the representative cells. Scale bars are 25 μm.

follows. (1) WT-p53 and p53 peptide randomly form hetero-tetramers in the same manner as p53R273H [13]. (2) The amount of hetero-tetramers formed depends on the p53 protein level. (3) T_1 is higher than T_2 (T_1>T_2) and T_2 is higher than T_3 (T_2>T_3) respectively. The transcriptional ability of homo-tetramers formed by p53 peptides (T_4) was set as 0 in this analysis.

I determined the transcriptional ability of each tetramer species by fitting analysis. The quantitative data of p53 transcriptional activity in single cells were well-fitted to the model (R^2=0.947). Interestingly, the fitting results showed that the transcriptional ability of the hetero-tetramer with three WT-p53 proteins and one p53[324-393] peptide was estimated as 72.0% compared with homo-tetrameric WT-p53 (**Table 2-1**). Although the transcriptional activity of the hetero-tetramer with two WT-p53 proteins and two p53 peptides was estimated as 28.9%, the hetero-tetramer with one WT-p53 protein and three p53[324-393] peptides was estimated as 0.0%. These quantitative results indicated that more than two DNA-binding sites in the p53 tetramer are needed to maintain a substantial transcriptional ability.

Table 2-1. Relative p53 transcriptional abilities of the hetero-tetramers.

	Tetramer species				
Tetramer element (WT : p53 peptide)	4: 0	3 : 1	2 : 2	1 : 3	0 : 4
Relative p53 transcriptional ability (%)	100	72.0±7.2	28.9±5.6	0.0±2.3	0

The value for the relative transcriptional ability of each hetero-tetrameric species was expressed as 100 for the homo-tetramer of WT-p53 with a residual error.

2.5 Discussion

There are only few studies about the effect of hetero-tetramer species on transcriptional activity in single cells. Here, I report quantitative p53 transcriptional activity in the presence of p53[324-393] peptide that can form a tetramer with WT-p53. I showed a decline in p53 transcriptional activity in the presence of p53[324-393] peptide, suggesting that a dominant-negative effect via hetero-oligomerization [17]. Fitting analysis showed that hetero-tetramers with more than two WT-p53 proteins had a substantial transcriptional ability compared with WT-p53 homo-tetramers (**Figure 2-8**). Mutant p53A347T has been reported as a dimer mutant [25, 26]. Our laboratory has previously reported that transcriptional activity of mutant p53A347T is about 22% compared with that of WT-p53 [24]. This result is in good agreement with my results indicating that the transcriptional ability of hetero-tetramers formed between two WT-p53 proteins and two p53[324-393] peptides was about 28.9%. These results suggest that more than two DBDs in the p53 tetramer are important for maintenance of the p53 transcriptional ability.

This is the first report that measured the transcriptional ability of each hetero-tetramer p53 species in single cells. Our laboratory has previously suggested that transient inhibition of p53 transcriptional activity using a p53 peptide fragment is expected to enhance the efficiency of iPS cell generation [16]. In this study, quantitative analysis showed that the p53 peptide containing the TD is useful for efficient inhibition of p53 transcriptional activity. Depending on the p53 target genes which are reported to exceed a hundred, the binding affinity for p53 is widely different [4]. Further study is

needed to reveal the transcriptional ability of hetero-tetramers formed by WT-p53 and Mut-p53 for p53 target genes.

In summary, the results suggest that hetero-tetramer species containing the p53 peptide have a substantial transcriptional ability in single cells. I quantitatively showed that the p53 peptide is useful to inhibit p53 transcriptional activity. My findings should be considered as a potent clue for the elucidation of the mechanism behind the dominant-negative effect.

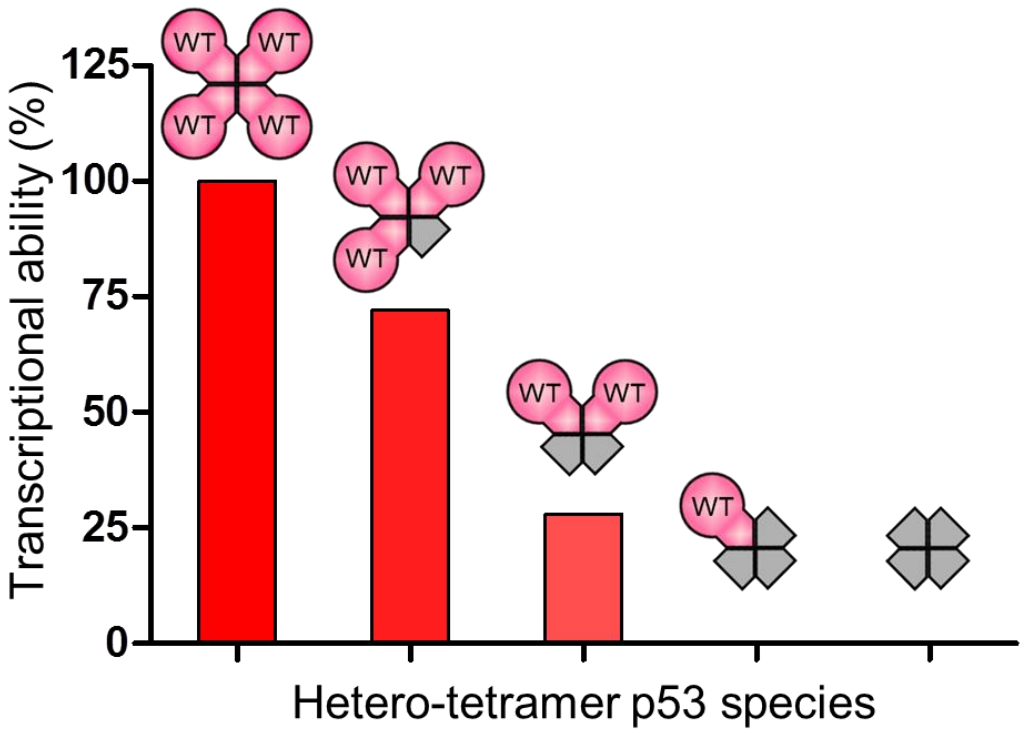

Figure 2-8. Transcriptional ability of hetero-tetramer p53 species determined by quantitative fitting analysis.

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3. Effect of amino acid starvation on the p53-mediated cellular response associated with cancer drug resistance

3.1. Abstract

Cancer cells acquire altered characteristics such a resisting cell death, sustaining proliferation signaling, and abnormal metabolism in tumorigenesis. These hallmarks are hindrances to cancer treatment. p53 is a transcriptional factor that plays a central role in various cellular responses including those to DNA damage. Recent accumulated evidence has shown that p53 is involved in the metabolic response to starvation stress such as nutrient deprivation. Notably, the importance of amino acids for proliferation of cancer cells has been reported. However, the cellular response under amino acid deficiency is still unclear. In this study, I investigated the effect of amino acid deprivation on p53 responses. By using p53 reporter assay, I showed that lysine deprivation suppressed p53 transcriptional activity among deprivations of each single amino acid. I demonstrated that lysine deprivation decreased the protein and phosphorylation levels of p53. Furthermore, lysine deprivation suppressed the induction of cell death by anti-cancer drug etoposide. These results suggest that cancer cells have a potential mechanism of p53 inactivation in response to lysine deprivation.

3.2 Introduction

Cancer cells acquire the capabilities of resisting cell death, sustaining proliferative signaling, and inducing angiogenesis in the process of tumorigenesis [1]. Recent studies showed that the metabolic alteration in cancer cells occurs and also supports to meet the nutrient demand for their growth and proliferation [2]. Glucose and glutamine are recognized for key nutrients for cancer cells. While non-essential amino acids such as glutamine can be produced by *de novo* synthesis, essential amino acids need to be taken up from external sources in mammalian cells. Thus, it has been reported that cancer cells also actively use extracellular proteins by lysosomal degradation as a nutrient source to sustain their growth [3]. However, essential amino acids lysine and histidine have a lower abundance compared with adjacent benign pancreatic tissue according to metabolomics analysis [3]. Therefore, it is considered that cancer cells are likely exposed to a poor nutrient condition in tumors such as essential amino acid starvation.

Tumor suppressor protein p53 is a transcriptional factor that plays a pivotal role in various cellular responses [4]. p53 is activated through its tetramerization in response to diverse stress signals including genotoxic stress, nutrient starvation, and ribosomal stress [5, 6]. Activated p53 leads to cell cycle arrest and apoptosis via transcriptional activation of target genes such as *CDKN1A* (*p21*) to maintain genomic integrity [7, 8]. Cellular senescence induced by p53 is also linked to tumor suppression in some contexts [9, 10]. In response to DNA damage, p53 acts as a regulator of cellular senescence. *TP53*, which encodes p53 protein is the most frequently mutated gene in

human malignant tumors [11]. Thus, it is well recognized that p53 is the most important protein for tumor suppression. There is accumulated evidences that p53 modulates the metabolic pathway in response to amino acid starvation [12–16]. However, the p53-mediated cellular response under amino acid starvation is not fully understood.

Here, I aimed to investigate the relationship between p53 transcriptional activity and amino acid starvation. First, I analyzed the consumption and excretion rates (CORE) in cancer cell lines using the metabolome analysis investigated by Jain *et al.*[17]. I found that lysine is the most consumed essential amino acid in cancer cell lines. Next, I performed exhaustive analyses of p53 transcriptional activity in response to single amino acid deprivations using the fluorescent reporter assay. Surprisingly, I observed that p53 activity was most effectively suppressed by lysine. I also investigated the effect of lysine starvation on the cancer cell survival in the presence of anti-cancer drug etoposide. Moreover, I examined the cell cycle and cell growth in response to lysine deprivation in the presence of the anti-cancer drug. Taken together, these findings indicated that cancer cells have the mechanism of survival for nutrient starvation and anti-cancer drug resistance.

3.3. Experimental procedures

3.3.1. Plasmid construction

I constructed two kinds of reporter plasmids, p((*p53RE-1*)-Cerulean-NLS-(*SV40)*-mCherry-NLS-2A-Puromycin^{R} and p(*p53RE-2*)-Venus-NLS-Neomycin^{R}, as shown in **Figure 3-1A, B**. The former plasmid carried the artificial p53RE consisting of three tandem repeats of the p53 binding motif (5′-AGACATGTCCGGACATGTCT-3′) with a 16 bp spacer (5′-actagcggctgtcact-3′). Using this plasmid, I estimated the activation of p53 based on the expression level of cyan fluorescent protein with three tandem repeats of the NLS (Cerulean-NLS). This plasmid also contained the expression unit of (*SV40)*-mCherry-NLS-2A-Puromycin^{R}. The 2A peptide sequence interrupts the normal peptide bond and leads to ribosomal skipping [18–20]. Therefore, the red fluorescent protein (mCherry-NLS) and puromycin resistance gene can be separately expressed in a p53-independent manner. The latter plasmid carried *p53RE-2* derived from the *CDKN1A* (*p21*) promoter [21]. Using this plasmid, yellow fluorescent protein (Venus-NLS) was expressed in the nucleus through p21 promoter activity. Both plasmids contained the left and right homology arms for the AAVS1 genomic integration site derived from AAVS1 SA-2A-puro-pA donor (Addgene plasmid # 22075) [22]. The mRNA degradation signal, an AU-rich element, was inserted in the 3′-noncoding regions of Venus-NLS and Cerulean-NLS to monitor the rapid p53 response [23–25]. The Cas9 and *AAVS1*-targeted

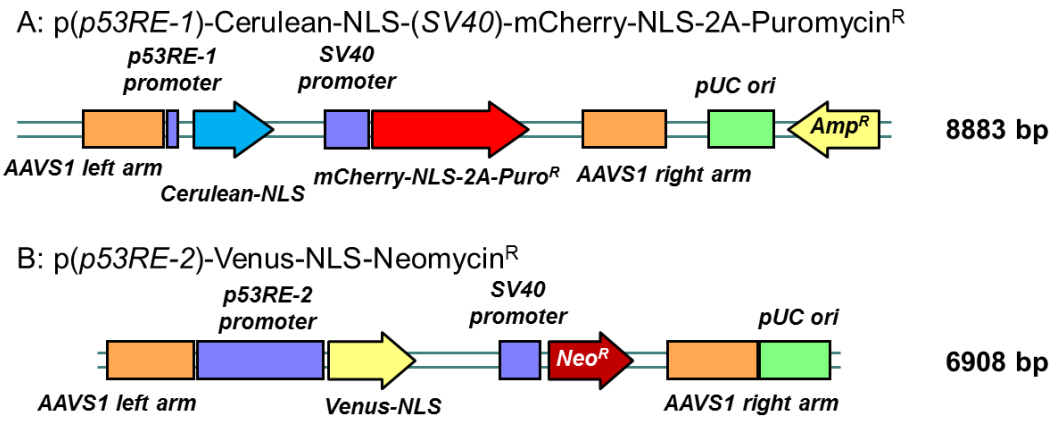

Figure 3-1. Constructed plasmids used in the p53 reporter assay. (A), p(*p53RE-1*)-Cerulean-NLS)-(SV40)-mCherry-NLS-2A-Puromycin^{R}. (B), p(*p53RE-2*)-Venus-NLS-Neomycin^{R}.

guide RNA expression plasmid (pX330-AAVS1) was constructed from pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid # 42230) [26].

3.3.2. Establishment of a p53 reporter stable cell line by CRISPR-Cas9

Based on the reporter system for p53-dependent transcriptional activity in a single cell [27, 28], I established the p53 reporter stable cell line derived from A549 cells by CRISPR-Cas9. A549 cells were transfected with the reporter plasmids, p(*p53RE-1*)-Cerulean-NLS)-(*SV40*)-mCherry-NLS-2A-Puromycin^{R} and p(*p53RE-2*)-Venus-NLS-Neomycin^{R}, and the Cas9 and *AAVS1* targeted guide RNA expression plasmid (pX330-AAVS1) using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were selected in the presence of G418 (1 mg/ml) and puromycin (0.5 μg/ml).

3.3.3. Cell culture

A549 human lung carcinoma cells (p53 wild-type) were obtained from ATCC. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (D5796, Sigma) supplemented with 10% (v/v) FBS in a 37°C incubator with 5% CO_2. Amino acid deprived-media were formulated with the same nutrient composition as DMEM without each amino acid or phenol red. For amino acid starvation experiments, cells were washed with the amino acid-deprived medium with 10% dialyzed-FBS twice. Then, cells were received each amino acid-deprived medium with 10% dialyzed-FBS and were incubated for the indicated times.

3.3.4. Reporter assay

In the reporter assay, the cells were observed by fluorescence microscopy (BIOREVO BZ-9000, Keyence). I used three fluorescence mirror units for observation. YFP-BP (excitation filter: 500/25 nm; dichroic mirror: 520 nm; absorbance filter 542/27 nm) was used for Venus, CFP-2432C (excitation filter: 438/24 nm; dichroic mirror: 458 nm; absorbance filter 483/32 nm) was used for Cerulean, and TRITC (excitation filter: 540/25 nm; dichroic mirror: 565 nm; absorbance filter 605/55 nm) was used for mCherry.

3.3.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized from 500 ng total RNA using a Primer script II 1st strand cDNA Synthesis Kit (TaKaRa) with random hexamer primers. Quantitative RT-PCR was performed with a CFX96 Touch Real-time PCR detection system (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad). Primer sequences for qRT-PCR are shown in **Table 3-1** [29]. Fold changes were calculated by the Ct method.

3.3.6. Western blotting and antibodies

Cell lysates were prepared with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 50 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1mM sodium molybdate, 1mM phenylmethylsulfonyl fluoride, and a protease inhibitor

Table 3-1. Primer sequences for qRT-PCR of *TP53, CDKN1A* (*p21*), and *GAPDH*.

Target gene	Sequence
TP53 forward	5′- CCCAAGCAATGGATGATTTGA-3′
TP53 reverse	5′-GGCATTCTGGGAGCTTCATCT-3′
CDKN1A(*p21*) forward	5′- TTGTACCCTTGTGCCTCGCT-3′
CDKN1A(*p21*) reverse	5′- TTGGAGAAGATCAGCCGGC-3′
GAPDH forward	5′- ACCCACTCCTCCACCTTTGA-3′
GAPDH reverse	5′- CTGTTGCTGTAGCCAAATTCGT-3′

mixture). Equivalent amounts of total cellular proteins were separated by 12% SDS-PAGE and transferred to PVDF membrane. Proteins were detected using mouse monoclonal anti-p53 (DO-1, Santa Cruz Biotechnology), mouse monoclonal anti-p53 (Ser15P) (16G8, Cell Signaling), and mouse monoclonal anti-actin (clone C4, Millipore) antibodies. HRP-conjugated anti-mouse secondary antibody was used. Protein signals were detected with super signal west pico chemiluminescent agent by a Lumi vision (AISIN).

3.3.7. Cell cycle analysis

Cells were incubated under the indicated conditions for 24 h. The cells were fixed with then 70% ethanol, collected, and incubated in a propidium iodide/RNase staining solution (BD Pharmingen). Cell cycle analysis was performed with a Gallios flow cytometer (Beckman Coulter). Data were analyzed using Flow Jo v7.6.5 software (Tree Star).

3.3.8. Cell growth assay

Stable A549 cells were incubated under the indicated conditions. The cells were then counted by nuclear detection of fluorescent proteins in fluorescence images. The growth rate was calculated from day 0 to 3.

3.3.9. SA-β-galactosidase staining assay

Senescence was determined using senescence-associated β-galactosidase assay basically in accordance with the previously described report [30]. Cells were fixed with neutral buffered 4% formaldehyde for 3 min. Then, the cells were incubated the SA-β-gal staining solution for 24 to 28 h. After blue color is fully developed, the cells were treated with 0.2% Triton X-100, and stained DAPI solution for nuclear staining. Cells with blue-color staining were counted under a bright-field.

3.4. Results

3.4.1. Lysine deprivation decreases p53 transcriptional activity in lung carcinoma cells.

To begin with examination of amino acid deprivation, I validated the consumption rate of essential amino acids in cancer cells using consumption and release (CORE) profiles investigated by Jain *et al.*[17]. As a result, lysine was the most consumed essential amino acid in 60 cancer cell lines including lung, breast, central nervous system, colon, leukemia, melanoma, ovarian, prostate, and renal cancers (**Figure 3-2A**). Consistent results were observed in lung cancer cell lines including A549 (**Figure 3-2B**). These results indicated that lysine is an important amino acid for cancer cells.

To evaluate the effect of amino acid deprivation on p53 transcriptional activity, I used a reporter cell line derived from A549 cells based on previous reports [27, 28]. This assay system quantifies *p21* transcriptional activation derived from the *p21* promoter, p53-dependent transcriptional activity derived from the p53-binding motif, and p53-independent transcriptional activity derived from the *SV40* promoter in single living cells (**Figure 3-3**). During cell culture in the amino acid-deprived medium as indicated in **Figure 3-4A**, I analyzed basal p53 activities and p53-independent transcriptional activity. The results showed that the single amino acid deprivation altered *p21* transcriptional activation and p53-dependent transcriptional activity.

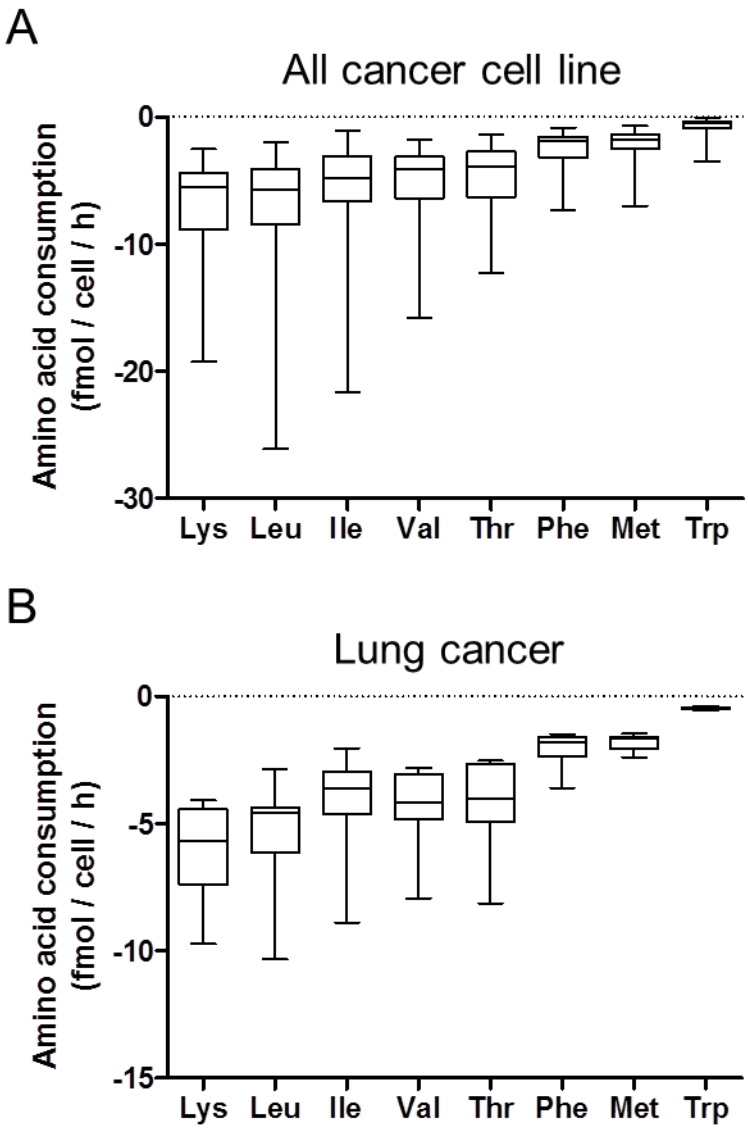

Figure 3-2. Cancer cells mostly consume lysine among essential amino acids.
A, consumption rate of essential amino acids in the NCI-60 cancer cell lines (lung, breast, central nervous system (CNS), colon, leukemia, melanoma, ovarian, prostate, and renal cancers). B, consumption rate of essential amino acids in nine cell lines derived from lung cancer (A549, EKVX, NCI-H23, HOP-62, NCI-H322M, NCI-H460, HOP-92, NCI-H522, and NCI-H226). Boxes indicate the mean and middle two quartiles, and whiskers indicate minimum and maximum values.

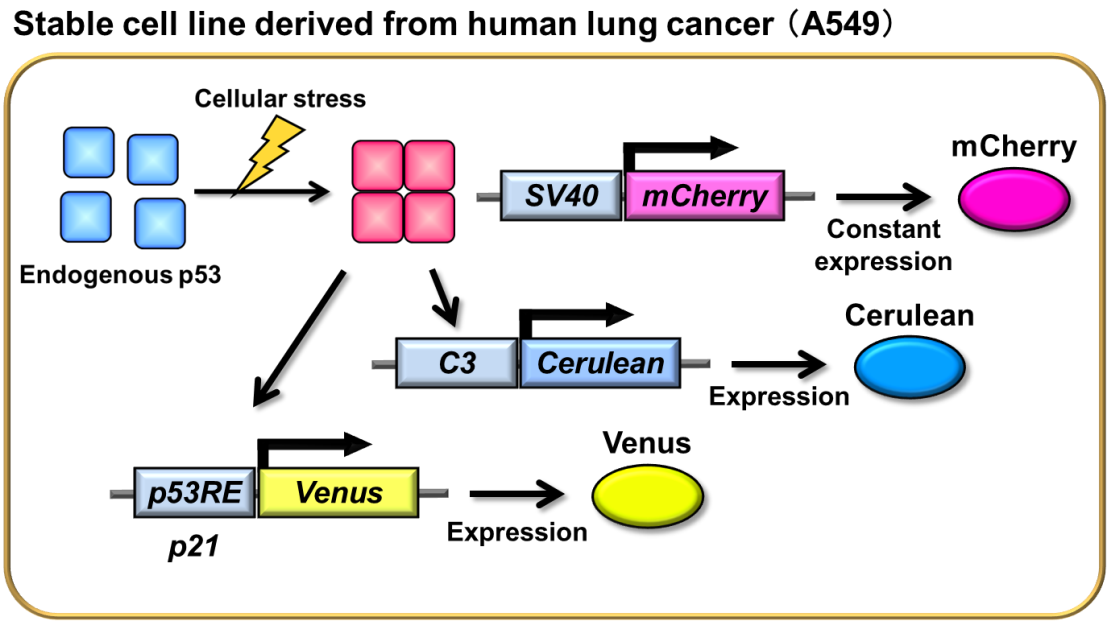

Figure 3-3. Schematic representation of the p53 reporter system. This assay system quantifies *p21* transcriptional activation (Venus), p53-dependent transcriptional activity (Cerulean), and p53-independent transcriptional activity (mCherry) by measuring the intensities of three fluorescent proteins in single living cells.

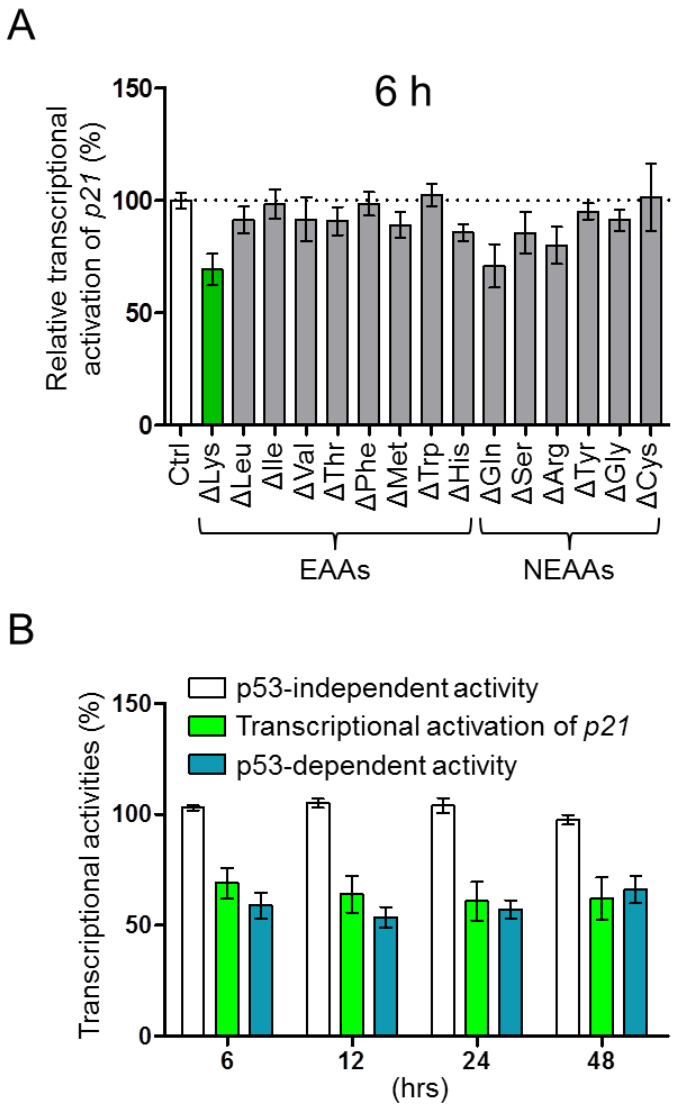

Figure 3-4. Lysine deprivation decreases p53 transcriptional activity in lung carcinoma cells. A, Relative transcriptional activation of *p21* in response to amino acid deprivation for 6 h normalized to the control. B, Transcriptional activation of *p21*, p53-dependent transcriptional activity, and p53-independent transcriptional activity in response to lysine deprivation for 48 h. Standard three-letter abbreviations are used for amino acids. EAAs, essential amino acids; NEAAs, non-essential amino acids. Data represent the mean ± S.D. from three independent experiments.

Interestingly, I observed that lysine deprivation strongly suppressed p53 activity from 6 to 48 h of starvation (**Figure 3-4A, B**). This result was also consistent with the p53-dependent transcriptional activity (**Figure 3-5**). p53-independent transcriptional activity was not changed significantly. These results implied that lysine deprivation specifically affected p53 transcriptional activity. Other apparent alterations were observed for single amino acid (histidine, isoleucine, tryptophan as essential amino acids, and arginine and glutamine as non-essential amino acids) deprivation conditions. Among them, I only observed the elevation of p53 activity in response to glutamine deprivation at 48 h (**Table 3-2, 3, 4**). This change in p53 activity was consistent with a previous report [13]. These results indicated that p53 activity could be modulated in response to single amino acid starvation.

3.4.2. Lysine deprivation suppresses p53 activation by the anti-cancer drug etoposide.

To further investigate the effect of lysine deprivation on p53 activity, I examined p53 transcriptional activity in lysine-deprived medium in combination with etoposide (10 μg/ml) treatment simultaneously (**Figure 3-6**). Reporter assay showed that lysine deprivation suppressed both the p21 transcriptional activation and p53-dependent transcriptional activity induced by etoposide at all time points from 12 to 48 h (**Figure 3-7A, B**). At 24 h, when p53 activity was the highest in response to etoposide, lysine deprivation suppressed p53 activity to nearly half compared with etoposide treatment alone. In contrast, I observed that p53-independent

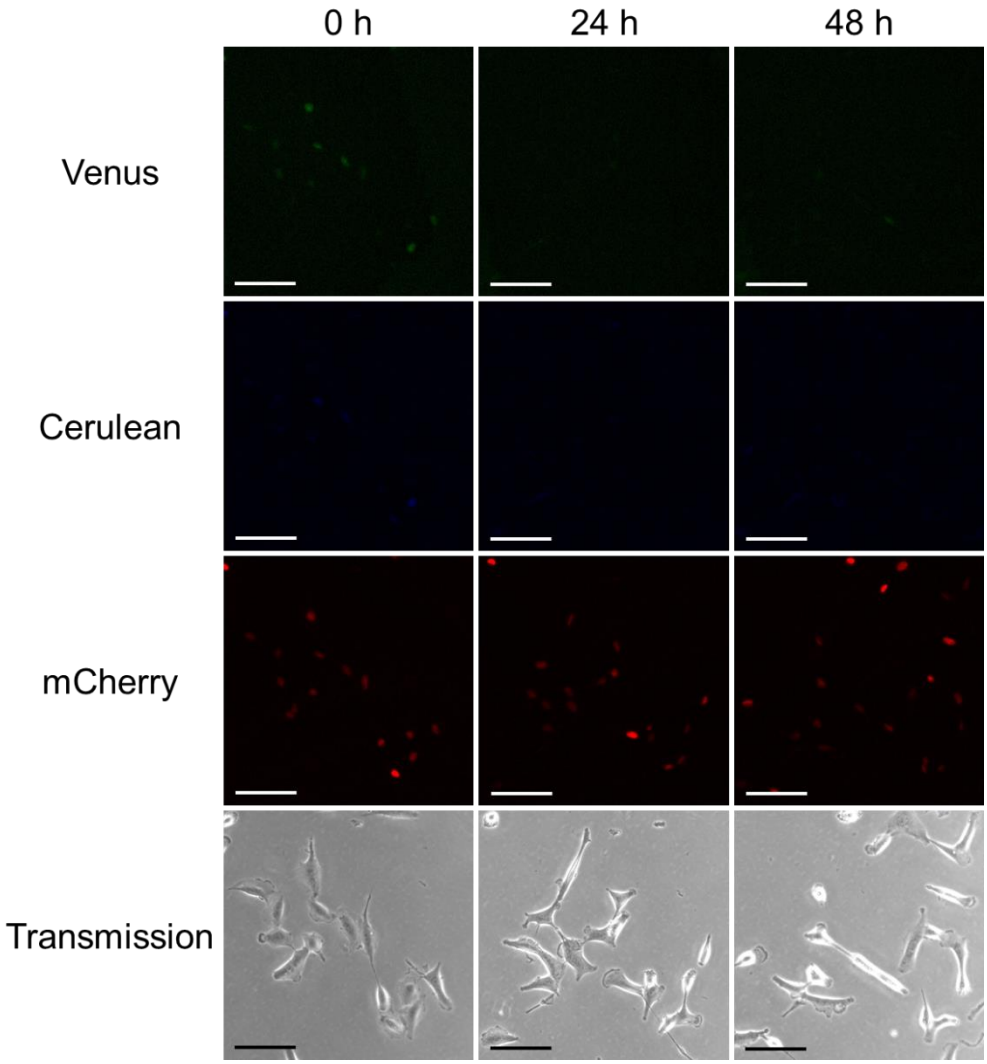

Figure 3-5. Representative fluorescence images of reporter assay. Reporter stable A549 cells were incubated in lysine-deprived medium for 48 h. The transcriptional activation of *p21* (Venus), p53-dependent transcriptional activity (Cerulean) and p53-independent transcriptional activity (mCherry) were visualized by green, blue and red respectively using fluorescence microscopy. Scale bars are 100 μm.

Table 3-2. Time course analysis of p53-dependent transcriptional activity (Venus intensity) in response to amino acid deprivation. p53 activities were normalized to control (DMEM). Data represent the mean ± S.E. from three independent experiments.

	Time (h)			
Condition	6	12	24	48
ΔLys	69.3±4.0	70.0±5.7	56.9±5.0	50.9±4.6
ΔHis	85.7±2.2	87.0±5.7	65.3±3.2	60.2±4.0
ΔIle	98.4±3.7	96.5±4.1	66.3±2.7	56.4±2.0
ΔLeu	91.3±3.4	96.0±3.8	78.8±4.4	76.3±6.5
ΔMet	89.1±3.4	100±6.1	87.0±5.2	79.2±3.4
ΔPhe	98.4±3.0	92.8±4.6	79.5±3.0	58.2±3.3
ΔThr	90.8±3.6	107±5.0	85.1±5.9	87.7±5.0
ΔTrp	102±2.9	99.1±5.1	74.9±5.6	58.0±4.9
ΔVal	91.6±5.7	99.2±5.6	70.7±2.8	74.8±3.4
ΔArg	79.9±4.8	85.6±7.2	60.7±5.0	54.5±5.5
ΔCys	101±8.6	97.2±9.1	81.7±5.2	71.2±3.2
ΔGln	70.9±5.6	88.3±8.1	97.2±7.9	125±5.5
ΔGly	91.2±2.8	89.8±4.2	73.4±3.4	57.3±2.2
ΔSer	85.6±5.5	94.3±6.1	65.0±4.1	63.7±2.7
ΔTyr	95.1±2.1	84.1±4.2	64.7±3.2	54.1±3.1
ΔSer, Gly	90.7±2.5	101±4.3	77.9±3.2	74.1±4.6

Table 3-3. Time course analysis of p53-dependent transcriptional activity (Cerulean intensity) in response to amino acid deprivation. p53 activities were normalized to control (DMEM). Data represent the mean ± S.E. from three independent experiments.

	Time (h)			
Condition	6	12	24	48
ΔLys	56.6±3.5	53.1±3.2	45.0±1.9	51.3±3.3
ΔHis	72.6±2.1	69.4±3.3	53.3±1.8	62.2±7.4
ΔIle	79.3±4.9	74.0±4.6	56.3±0.7	54.8±4.1
ΔLeu	81.3±6.8	79.2±7.0	64.9±1.3	92.2±11
ΔMet	73.0±3.7	84.8±3.7	85.0±5.7	88.4±3.2
ΔPhe	92.0±4.5	79.6±5.4	55.1±1.0	60.5±2.8
ΔThr	67.9±2.1	75.1±2.9	72.0±1.2	89.9±5.8
ΔTrp	107±3.1	104±4.6	64.2±9.5	44.5±5.8
ΔVal	67.5±3.9	70.7±3.7	73.1±3.8	87.6±9.1
ΔArg	63.9±4.8	62.1±6.0	53.1±6.0	59.1±8.8
ΔCys	102±4.5	90.9±3.1	70.4±7.6	169±18
ΔGln	60.0±4.9	65.0±5.9	73.1±4.5	124±11
ΔGly	101±5.0	99.0±7.0	79.5±5.1	61.9±5.4
ΔSer	65.5±3.6	71.0±4.9	65.6±5.1	75.3±5.4
ΔTyr	81.6±3.6	68.8±2.7	49.8±2.2	58.6±3.8
ΔSer, Gly	72.5±3.2	77.0±3.7	71.9±2.8	89.9±6.8

Table 3-4. Time course analysis of p53-independent transcriptional activity (mCherry intensity) in response to amino acid deprivation. Transcriptional activities were normalized to control (DMEM). Data represent the mean ± S.E. from three independent experiments.

	Time (h)			
Condition	6	12	24	48
ΔLys	102±3.1	101±4.0	106±2.3	91.2±2.3
ΔHis	104±3.2	105±4.0	115±1.5	105±2.6
ΔIle	101±3.8	100±4.5	108±2.4	93.5±2.8
ΔLeu	101±3.6	99.0±3.8	103±1.5	93.5±3.5
ΔMet	100±3.3	96.1±3.8	101±2.0	99.1±3.0
ΔPhe	105±4.6	103±5.4	107±3.5	101±3.5
ΔThr	103±3.1	101±3.9	111±1.9	108±3.2
ΔTrp	106±3.2	102±4.2	107±4.5	97.2±7.5
ΔVal	102±3.4	102±3.9	112±1.6	97.2±3.5
ΔArg	100±3.0	98.1±3.8	103±1.9	88.8±2.5
ΔCys	103±3.3	100±3.8	93.6±5.5	40.9±6.0
ΔGln	99.0±3.0	95.6±3.9	97.0±2.0	86.1±4.5
ΔGly	104±3.8	102±4.5	109±2.4	117±5.4
ΔSer	100±3.4	99.0±3.8	103±1.3	94.4±5.3
ΔTyr	104±3.5	101±4.0	108±1.4	106±2.4
ΔSer, Gly	101±3.2	98.1±4.2	106±2.3	92.8±4.0

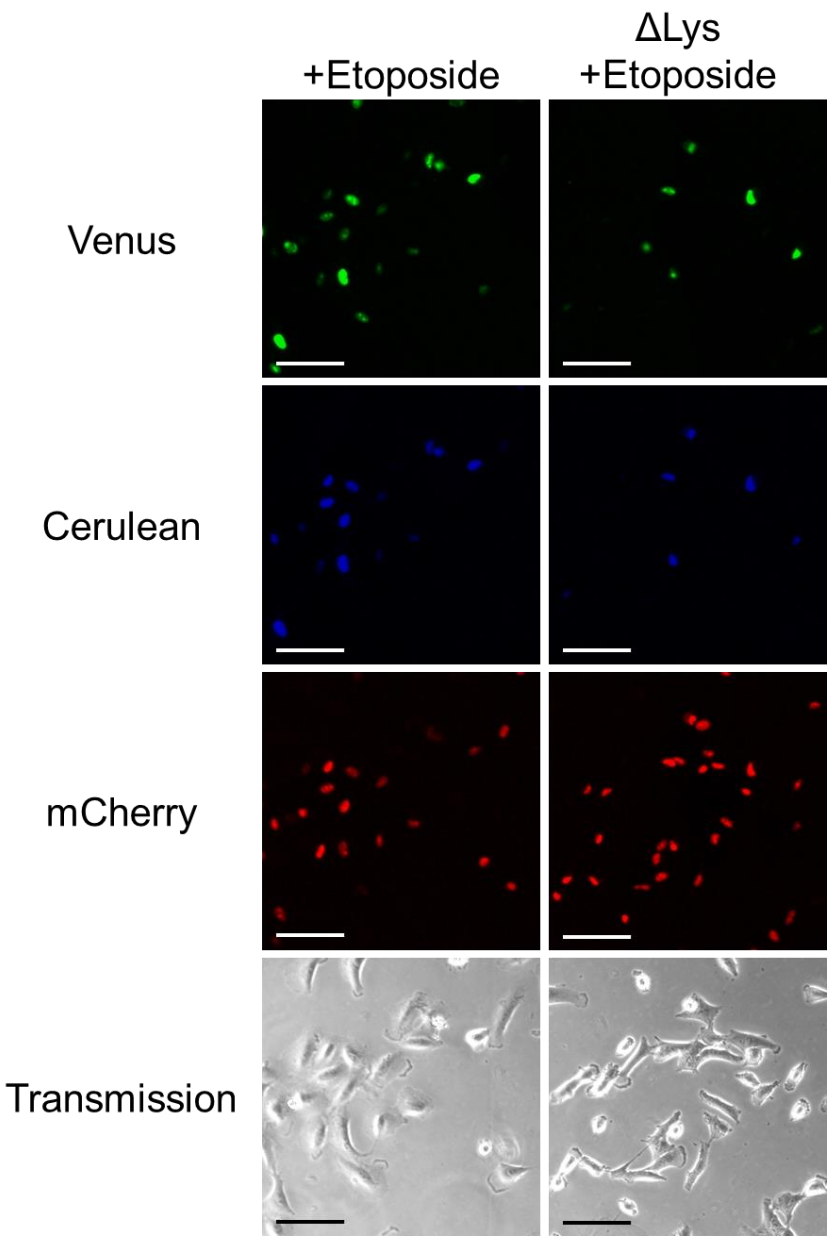

Figure 3-6. Representative fluorescence images of reporter assay. Reporter stable A549 cells were incubated in lysine-deprived medium in combination with etoposide (10 μg/ml) at 24 h. The transcriptional activation of *p21* (Venus), p53-dependent transcriptional activity (Cerulean) and p53-independent transcriptional activity (mCherry) were visualized by green, blue and red respectively using fluorescence microscopy. Scale bars are 100 μm.

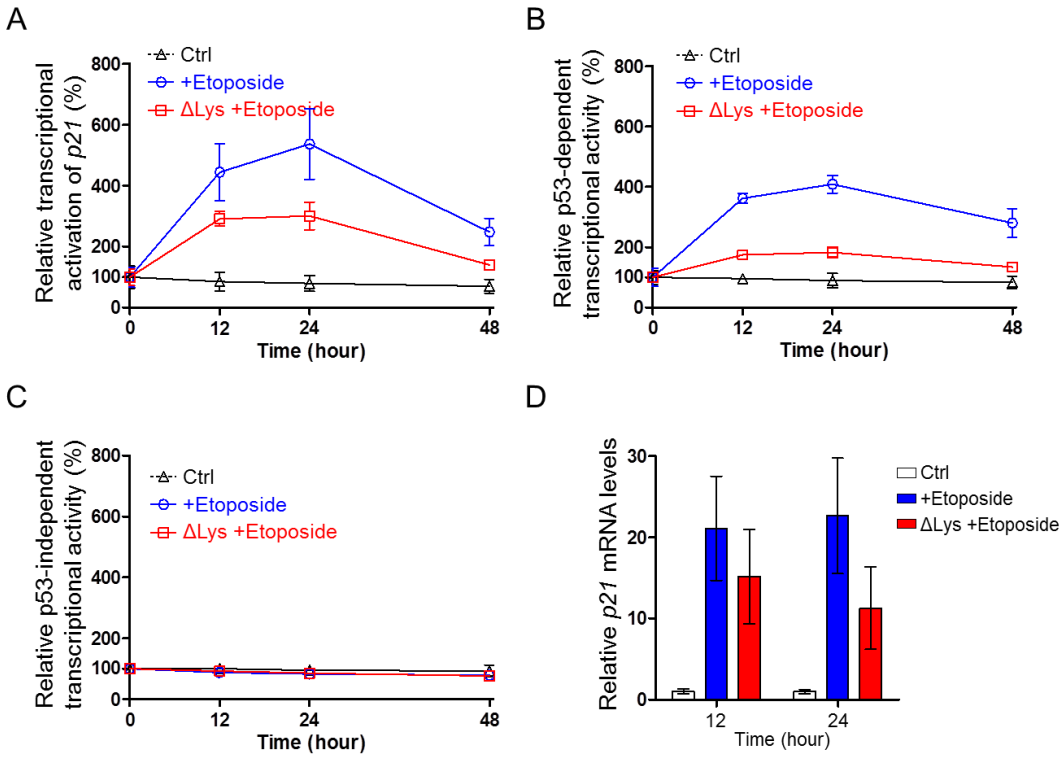

Figure 3-7. Lysine deprivation suppressed the p53 activation by an anti-cancer drug etoposide. A, Relative transcriptional activation of p21. B, Relative p53-dependent transcriptional activity. C, Relative p53-independent transcriptional activity. Data represent the mean ± S.D. from three independent experiments. D, qRT-PCR analysis of mRNA level of *p21* (*CDKN1A*). The mRNA expression level of *p21* was normalized to *GAPDH*. Data represent the mean ± S.D. from four independent experiments.

transcriptional activity was not significantly changed under lysine deprivation in combination with etoposide or etoposide treatment alone (**Figure 3-7C**). I also investigated the endogenous transcriptional level of *p21* as a p53 target gene by qRT-PCR. As expected, similar to the results from reporter assays, the mRNA expression level of endogenous *p21* was decreased under lysine deprivation with etoposide treatment compared with etoposide treatment alone (**Figure 3-7D**). These results indicated that lysine deprivation suppressed p53 transcriptional activity in the presence of the anti-cancer drug.

3.4.3. Lysine deprivation down-regulates protein and phosphorylation levels of p53, but does not change the mRNA level of *TP53*.

I examined the effect of lysine deprivation on p53. First, I performed western blotting (WB) to evaluate the p53 protein level and phosphorylation level of p53 at serine 15 (Ser15P) under lysine deprivation in combination with etoposide treatment in A549 cells. Ser15P of p53 is a well-known post-translational modification for p53 activation. The results demonstrated that the combination of lysine deprivation and etoposide treatment clearly decreased the protein level of p53 and the phosphorylation level of Ser15P (**Figure 3-8**). Moreover, I observed that even lysine deprivation alone decreased the p53 protein level. I observed double bands (upper and lower) under lysine deprivation as well as lysine deprivation in combination with etoposide treatment. To determine whether this effect occurred at the mRNA level, I investigated the mRNA

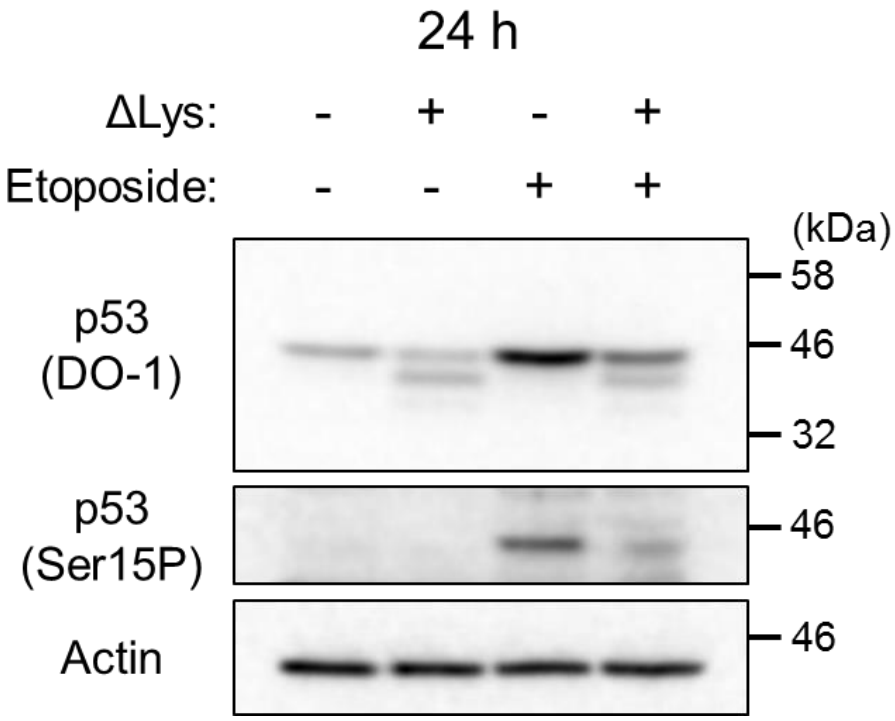

Figure 3-8. Western blot analysis for protein expression levels of p53 and actin, and the phosphorylation level of p53 Ser15P in A549 cells. Cells were incubated under control, lysine deprivation, etoposide treatment (10 μg/ml), or lysine deprivation in combination with etoposide treatment (10 μg/ml) conditions for 24 h.

level of *TP53*. The results showed that the mRNA level of *TP53* did not significantly change under both lysine deprivation alone or the combination of lysine deprivation and etoposide treatment (**Figure 3-9**). I also observed that relative p53-independent transcriptional activity as an internal control in the p53 reporter assay was not changed under both lysine deprivation alone and the combination of lysine deprivation and etoposide treatment (**Figure 3-7C**). Therefore, the results suggested that lysine deprivation specifically affected the protein level of p53 under etoposide treatment. These results suggest that lysine deprivation induces the inactivation of p53 through decreasing the p53 protein level and phosphorylation level of Ser15P.

3.4.4. Cancer cells have a proliferative potential under lysine deprivation.

To evaluate the effect of lysine deprivation on the cell cycle and cell death, I performed the cell cycle analysis by flow cytometry using PI staining. The results showed that lysine deprivation for 24 h caused an accumulation of cells in G1 phase, but not in the sub-G1 phase (**Figure 3-10, Table 3-5)**. Interestingly, the combination of lysine deprivation and etoposide treatment (10 μg/ml) for 24 h decreased the sub-G1 population compared with etoposide treatment alone (**Figure 3-11)**. These results indicated that lysine deprivation decreased the p53-mediated cell death via p53 inactivation under etoposide treatment.

To evaluate the effect of lysine deprivation on cell proliferation, I analyzed cell growth rate under lysine deprivation. As a result, lysine deprivation strongly suppressed the cell growth for 3 days, although an apparent decrease in the cell number was not

observed (**Figure 3-12A, B, 3-13**). Interestingly, cells started to proliferate a day later when they were incubated in control medium (DMEM) after lysine deprivation for 24 h. These results suggested that the cells exposed to lysine deprivation had a repopulation capacity.

3.4.5. Cancer cells exposed to lysine deprivation circumvent cellular senescence and repopulate via p53 inactivation under etoposide treatment.

To further examine the effect of lysine deprivation on cell growth in the presence of etoposide, I analyzed the cell proliferation after incubation with lysine-deprived medium and etoposide treatment. The cells were incubated with lysine-deprived medium for 24 h and then exposed to etoposide (10 μg/ml) for 6–24 h. Finally, I analyzed the cell proliferation when the cells received control DMEM (**Figure 3-14A**). Under the control condition, the cell proliferation was not observed for all etoposide-exposed conditions (**Figure 3-14B, 15**). However, surprisingly, I observed that the cells incubated with lysine-deprived medium in advance proliferated for 48 and 72 h. These results suggested that the cells incubated with lysine-deprived medium enable to proliferate by attenuating p53 activation induced by etoposide.

To investigate the involvement of lysine deprivation in cellular senescence, I performed a SA-β-galactosidase staining assay in the presence of etoposide in the same way as cell proliferation assay (**Figure 3-16A**). The cells were incubated with lysine-deprived medium for 24 h, and then exposed to etoposide (10 μg/ml) for 12 h. Finally I performed the SA-β-galactosidase staining and analyzed the SA-β-galactosidase-

positive cells after cells were incubated with control DMEM for 24 h. As a result, lysine deprivation markedly decreased the number of SA-β-galactosidase-positive cells (**Figure 3-16B, 17**). Furthermore, I observed that the pre-incubation with lysine-deprived medium strongly suppressed the transcriptional activation of *p21* in response to etoposide treatment (**Figure 3-16C**). These results showed that cells incubated with lysine-deprived medium circumvented drug-induced cellular senescence via *p21* inactivation.

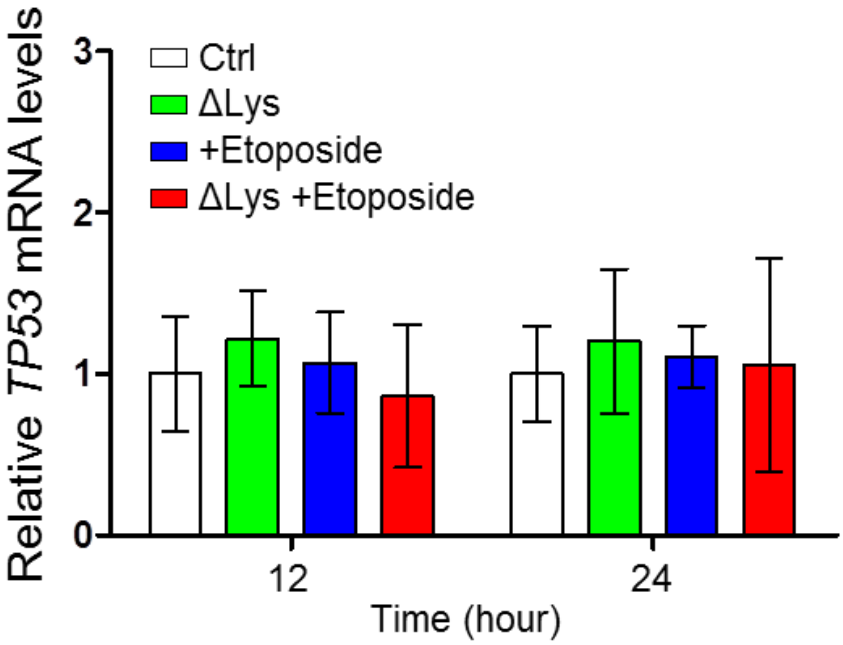

Figure 3-9. qRT-PCR analysis of *TP53* mRNA levels. The mRNA expression level of *TP53* was normalized to *GAPDH* expression. Data represent the mean ± S.D. from four experiments.

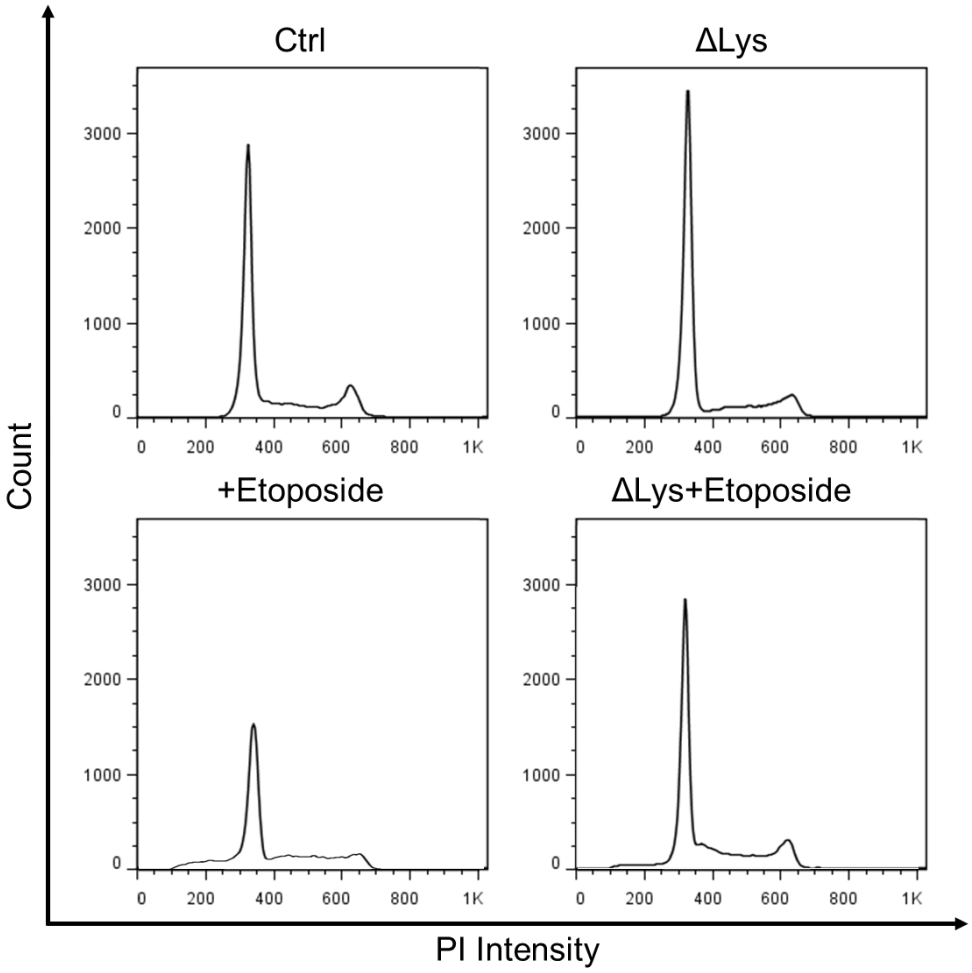

Figure 3-10. Cell cycle analysis by flow cytometry using PI staining. Cells were incubated under control, lysine deprivation, etoposide treatment (10 μg/ml), or lysine deprivation in combination with etoposide (10 μg/ml) conditions for 24 h.

Table 3-5. Cell cycle distribution under control, lysine deprivation, etoposide treatment (10 μg/ml), or lysine deprivation in combination with etoposide (10 μg/ml) conditions for 24 h. Data represent the mean ± S.D. from three independent experiments.

	G1 (%)	S (%)	G2/M (%)	Sub-G1 (%)
Ctrl	52.9±4.8	32.1±2.9	10.1±1.8	4.9±0.6
ΔLys	66.7±4.8	21.9±3.9	7.1±1.3	4.3±4.0
+Etoposide	45.8±6.2	31.7±4.5	4.8±2.4	17.6±2.3
ΔLys +Etoposide	47.1±8.5	34.0±4.6	7.4±1.8	11.5±2.3

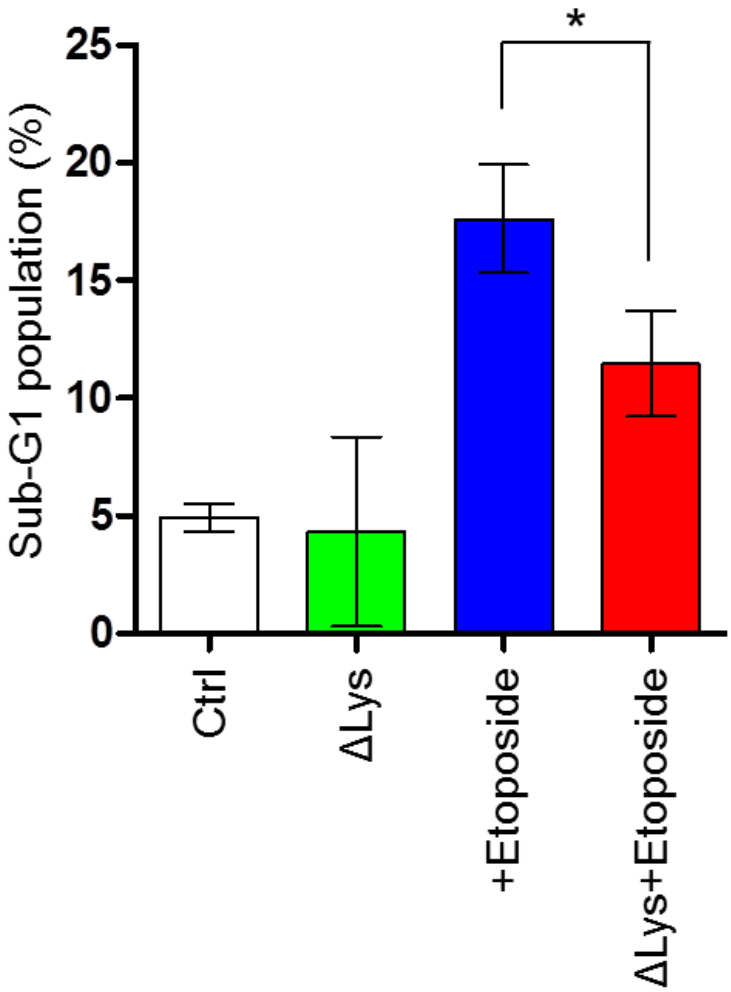

Figure 3-11. Sub-G1 population under control, lysine deprivation, etoposide treatment (10 μg/ml), or lysine deprivation in combination with etoposide (10 μg/ml) conditions for 24 h. Data represent the mean ± S.D. from three independent experiments. *, $p < 0.05$ as determined by the Student's t-test.

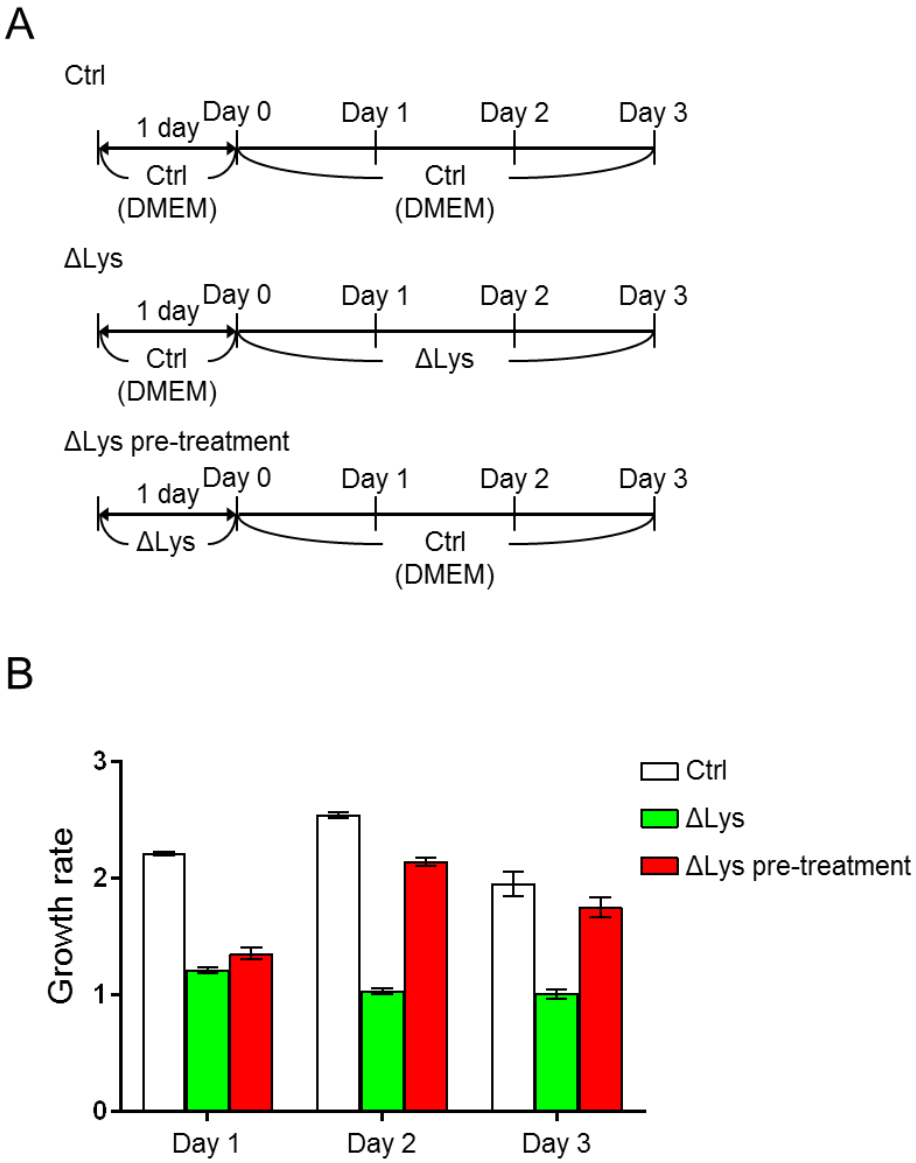

Figure 3-12. A, schematic time course of medium conditions in the cell growth assay. Cell growth rate was analyzed from day 0. B, Growth rate of stable A549 cells in control medium (DMEM), lysine-deprived medium (ΔLys), or control medium after ΔLys pre-treatment (24 h).

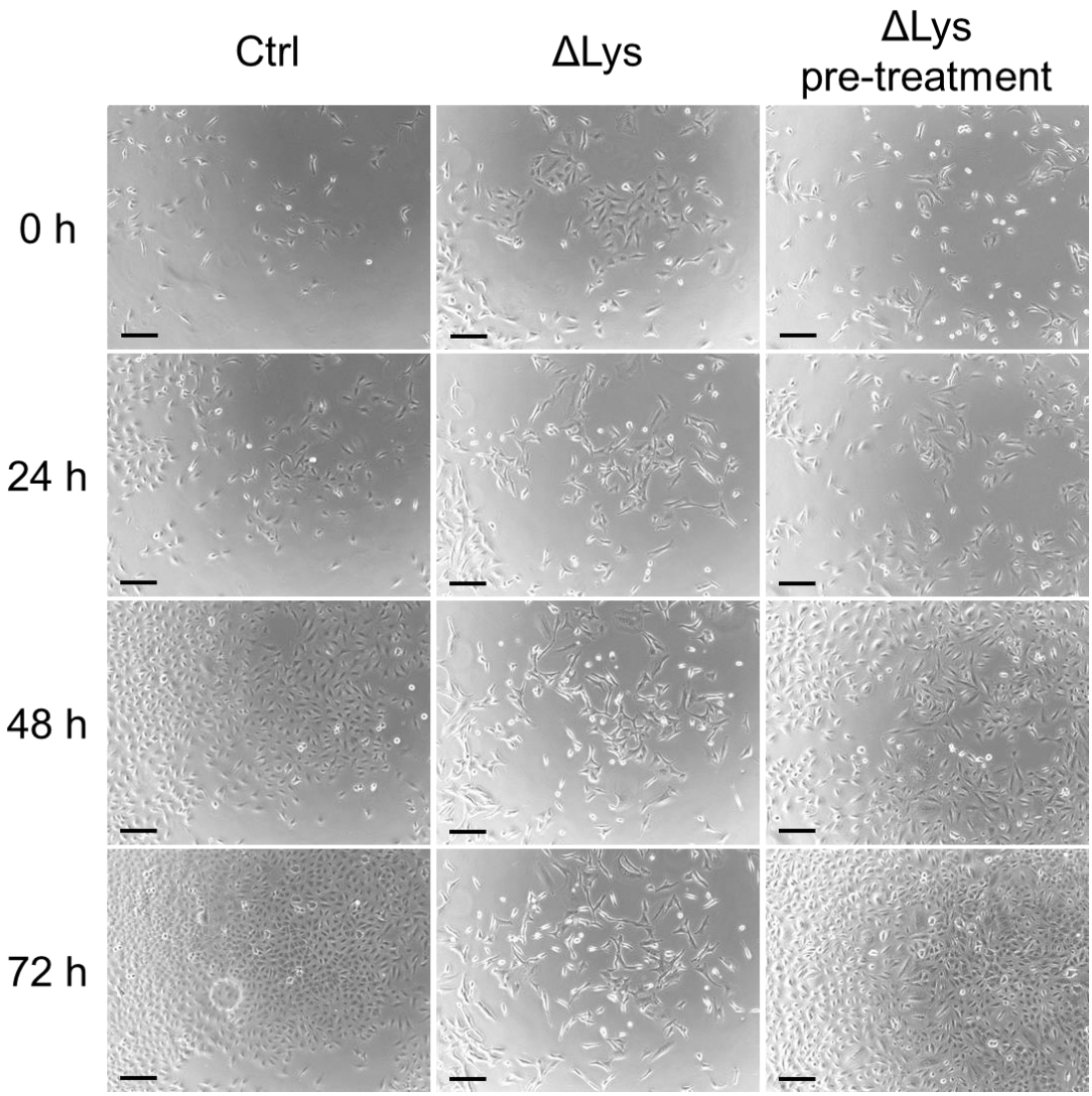

Figure 3-13. Representative phase contrast images of stable reporter A549 cells incubated in control medium (DMEM) or lysine deprivation (ΔLys) medium after ΔLys pre-treatment (24 h). Scale bars indicate 100 μm.

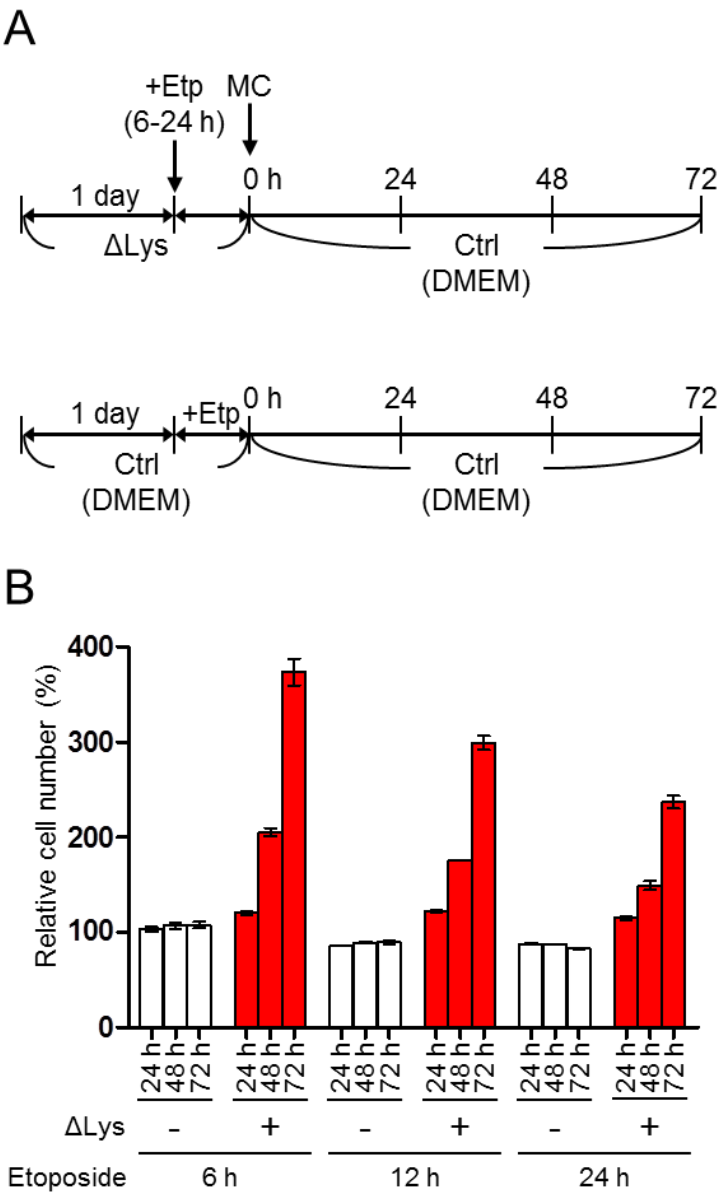

Figure 3-14. A, schematic time course of the cell proliferation assay with medium (ΔLys or DMEM) and etoposide incubation (6, 12, and 24 h). Cells were incubated in lysine-deprived medium or control DMEM. Then, the cells were incubated in the presence of etoposide for 6, 12, and 24 h. Cell proliferation was analyzed from 0 h when the medium was replaced with control DMEM. MC: medium change.
B, cell proliferation assay from 0 to 72 h under the indicated conditions. Relative cell numbers were normalized to 0 h. Data represent the mean ± S.E. from three experiments.

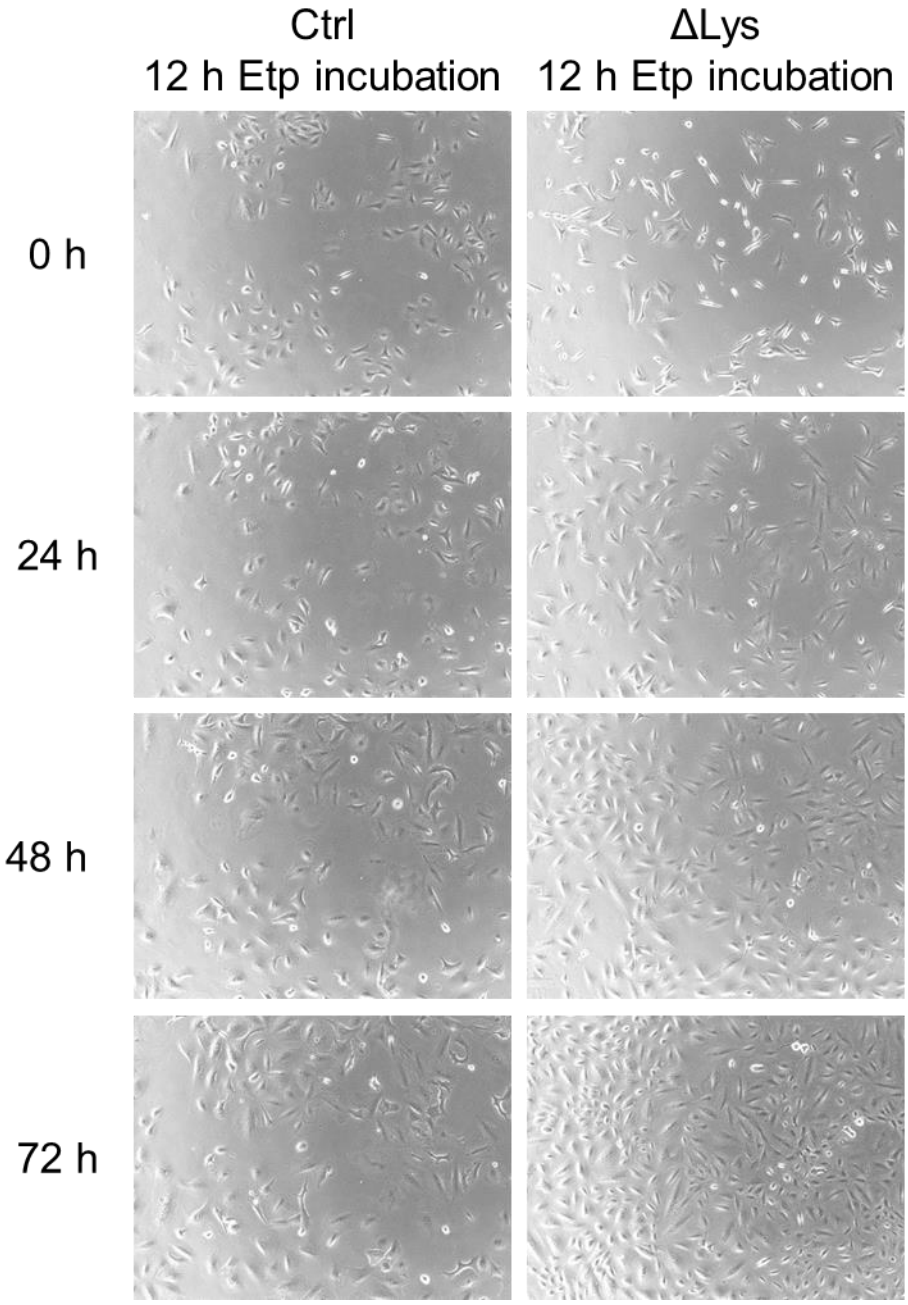

Figure 3-15. Representative phase contrast images of stable reporter A549 cells in the cell proliferation assay.

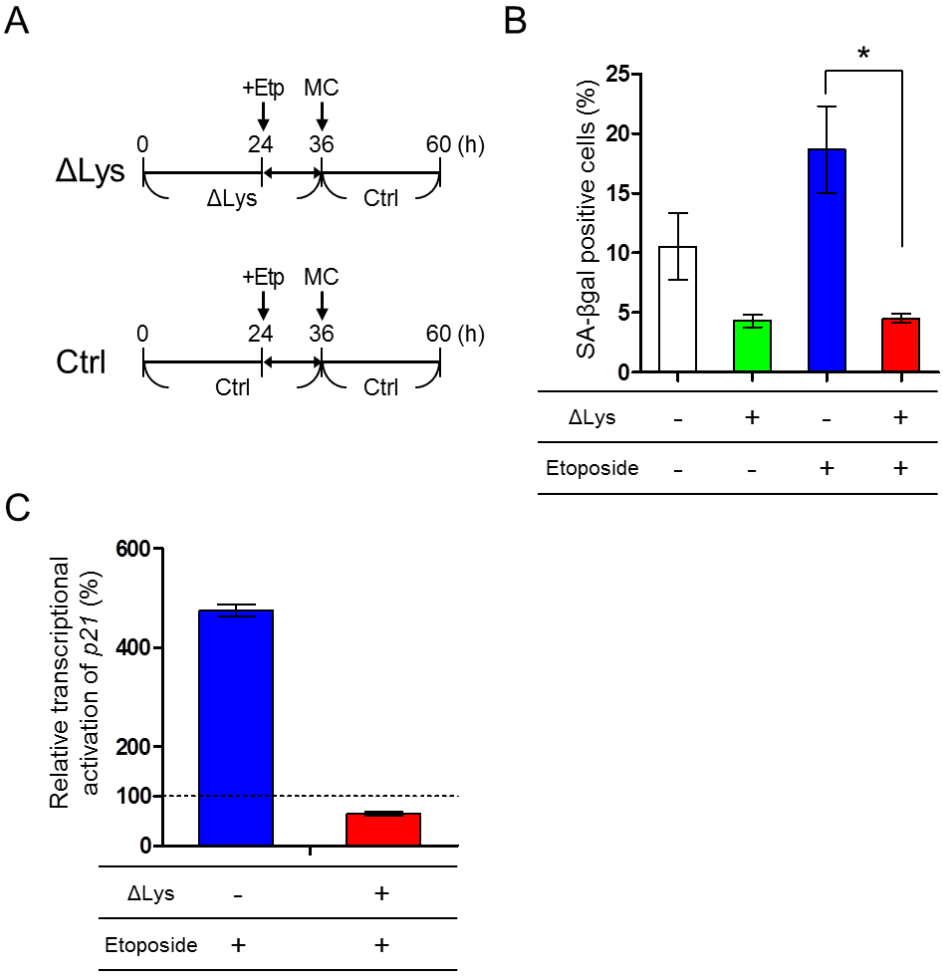

Figure 3-16. A, schematic time course of the SA-β-galactosidase staining assay. Stable A549 cells were pre-incubated in lysine-deprived medium or control medium for 24 h. Then, the cells were incubated in the presence of etoposide (10 μg/ml) for 12 h. SA-β-galactosidase staining was performed after the cells received fresh control medium for 24 h. MC: Medium change. B, quantification of the percentage of SA-β-galactosidase positive cells. C, Relative transcriptional activation of *p21* at 36 h before medium change. Data represent the mean ± S.D. from three experiments. *, $p < 0.01$ as determined by the Student's t-test.

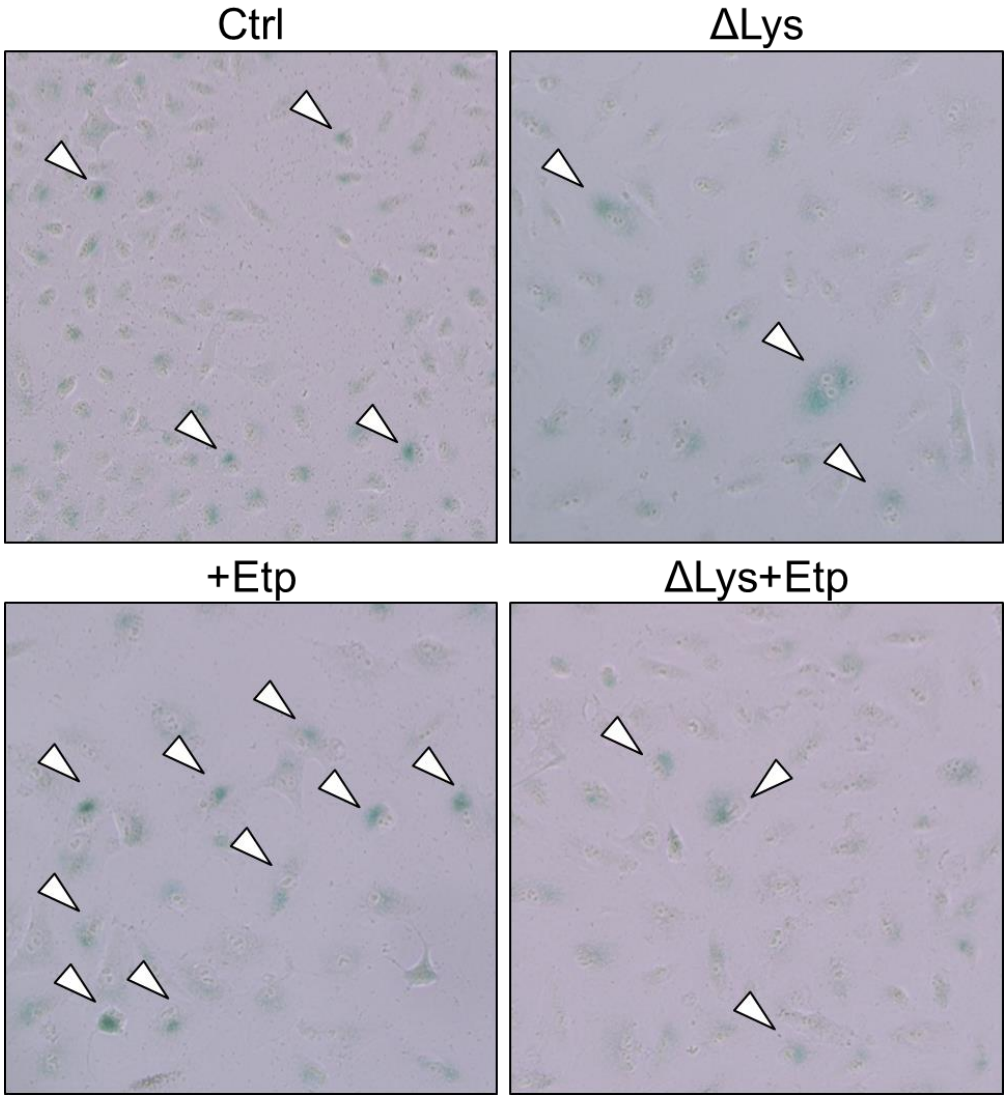

Figure 3-17. Representative bright field images of stable reporter A549 cells in the SA-β-galactosidase staining assay.

3.5. Discussion

The relationship between starvation and cancer cells is becoming visible based on recent accumulating evidence [2, 31, 32]. However, the p53-mediated cellular response to amino acid starvation remains largely unknown. In this study, I evaluated that the lysine is the most consumed essential amino acid in many types of cancer cell lines including lung carcinoma using CORE profiles [17]. *In vivo* analysis has been showed that the lysine concentration in human pancreatic tumor is the most lowest amount of ratio compared with adjacent benign tumor among essential amino acids [3]. While non-essential amino acids such as glutamine can be produced by *de novo* synthesis, essential amino acids need to be taken up from external sources by mammalian cells. Therefore, it is suggested that cancer cells are likely exposed to lysine deprivation and have a mechanism of metabolic adaptation to lysine starvation for their survival. Single cell analysis demonstrated that lysine deprivation most effectively suppressed *p21* transcriptional activation and p53-dependent transcriptional activity in response to individual amino acid starvations. Hence, these results together with previous reports suggest that cancer cells have a potential mechanism for p53 inactivation in response to lysine starvation.

Etoposide is widely used as an anti-cancer drug that induces p53-mediated cell death. Drug resistance to etoposide has been reported in human lung, ovarian, and breast cancers, which prevents cancer treatment [33–35]. Although one possibility is considered that metabolic adaptation results from changes in tumor environment, such as poor nutrient conditions, the drug resistance induced by starvation is not fully

understood. To reveal the mechanism, I analyzed the effect of lysine starvation on the *p21* transcriptional activation and p53-dependent transcriptional activity in combination with etoposide treatment. The results clearly showed that lysine starvation suppressed the p53 transcriptional activation caused by etoposide in the reporter assay. Under the same conditions, I observed that the *p21* mRNA expression level was decreased by lysine starvation and etoposide treatment compared with etoposide treatment alone in qRT-PCR analysis. To address the p53 inactivation mechanism, I analyzed the protein and phosphorylation levels of p53 and the mRNA level of *TP53* under lysine deprivation in combination with etoposide treatment. The results clearly demonstrated that the p53 protein and phosphorylation levels were decreased in the combined condition, but the *TP53* mRNA level was unchanged. These results suggest that lysine starvation may suppress the p53 activation caused by etoposide through decreasing the p53 protein level after transcription. I observed the lower band under the lysine-deprived condition in WB. Since WB was performed using a p53-specific antibody, expression of p53 isoforms or p53-specific degradation may occur under lysine deprivation.

I found that the number of cell death induced by etoposide was decreased by the combination of lysine deprivation and etoposide. The cell growth assay showed that lysine-starved cells proliferate after receiving fresh medium. I demonstrated that lysine starvation caused the accumulation of cells in G1 phase, suggesting that lysine-starved cells have a repopulation capacity. These results suggested that lysine starvation decreased the p53-mediated cell death through p53 inactivation in response to

etoposide. My findings suggest a new drug resistance mechanism through inactivation of p53 by amino acid starvation (**Figure 3-18**).

To date, almost all studies that have investigated the relationship between p53 and starvation are about p53 activation in response to starvation stress [6, 36, 37]. In this study, I obtained novel findings about the suppression of p53 activity in response to starvation. Moreover, I showed that single amino acid starvation may contribute to anti-cancer drug resistance. The repopulation capacity of the cells implied suppression of cellular senescence. It has been reported that the cellular senescence is induced by the p53-p21 pathway and p16 independently [38]. Drug-induced senescence by etoposide has been reported in lung carcinoma cells [39, 40]. SA-β-galactosidase assay results suggested that cells incubated with lysine-deprived medium circumvented drug-induced cellular senescence via *p21* inactivation. SA-β-galactosidase assay data had a critical meaning by linking the starvation stress to cancer drug resistance. Further studies are required to elucidate the precise mechanisms of p53 inactivation in response to lysine starvation.

My findings provide valuable knowledge for p53-mediated cellular adaptation of cancer cells in response to amino acid starvation. In conclusion, my study revealed a novel insight into anti-cancer drug resistance caused by a poor nutrient environment for cancer therapy.

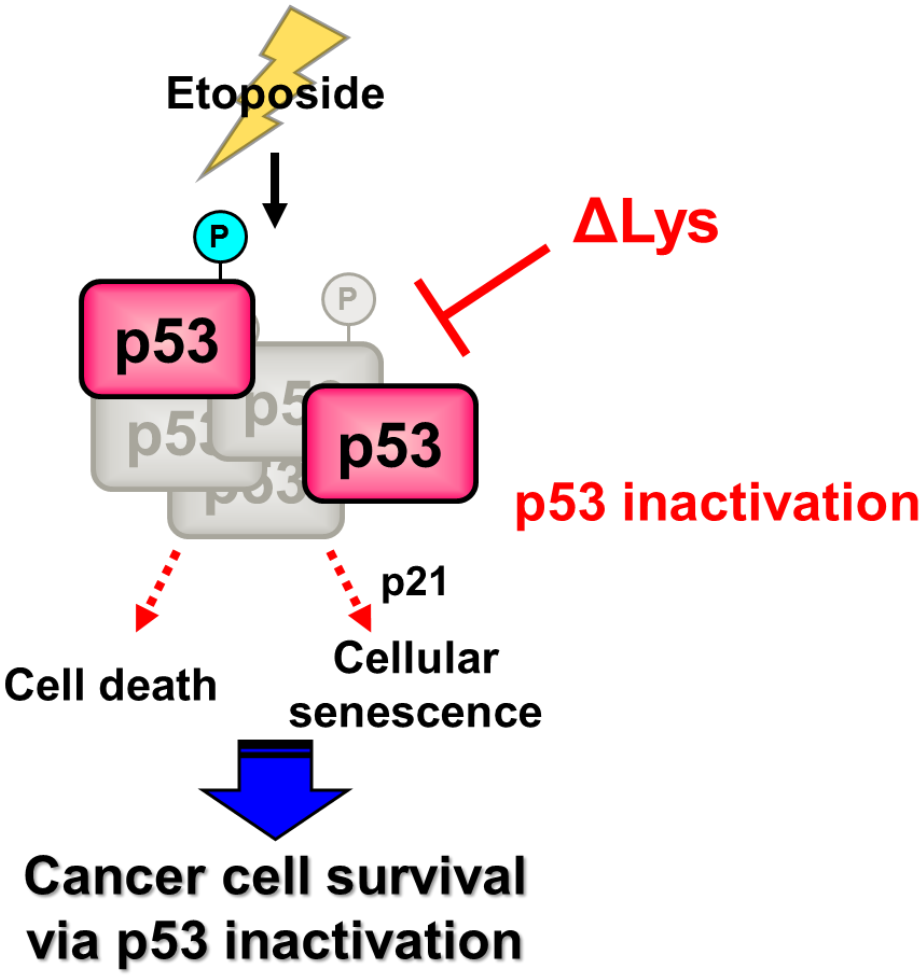

Figure 3-18. Schematic model of cancer cell survival toward anti-cancer drug via p53 inactivation under lysine deprivation

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4. Conclusions

p53 is the most important transcriptional factor for tumor suppression. In response to a wide variety of cellular stresses, p53 performs its ability for maintaining the genomic integrity. Gene mutation of *TP53* often occurs in tumors. Inactivation of p53 as a transcriptional factor caused by mutation or stress signaling is thought to be a crucial factor in carcinogenesis and tumorigenesis. Therefore, investigation of p53 inactivation is extremely important to understand the process. In this study, I revealed a mechanism of p53 inactivation by hetero-oligomerization under a physiological condition in individual cells. I also found a new mechanism for inactivation of p53 by amino acid deprivation.

In chapter 2, I investigated p53 transcriptional activity in the presence of WT and Mut-p53 or the oligomerization domain peptide. I established a new p53 reporter assay for quantitative single cell analysis of p53 transcriptional activity at the physiological protein expression level. By using this assay system, I showed that the dominant-negative effect occurred by hetero-oligomerization between WT and Mut-p53 or the oligomerization domain peptide. To estimate the transcriptional ability of each hetero-tetramer p53 species, I constructed a fitting model. Interestingly, the quantitative fitting analysis showed that the hetero-tetramers including more than two WT-p53 proteins have a substantial transcriptional ability. These results indicated that the two DBDs are crucial for the p53 transcriptional ability.

In chapter 3, I investigated the effect of single amino acid deprivation on p53 responses. I validated the consumption rate of essential amino acids to demonstrate the

importance of essential amino acids in cancer cells. As a result, lysine is the most consumed in cancer cells. By using the reporter assay, I found that lysine deprivation decreased the p53 transcriptional activity in each single amino acid deprivation condition. To determine the effectiveness of lysine deprivation on p53 responses, I analyzed transcriptional activity in the presence of anti-cancer drug etoposide. The results clearly demonstrated that lysine deprivation strongly suppressed the p53 transcriptional activity in the presence of etoposide, and that lysine deprivation downregulated the p53 protein and phosphorylation levels. Lysine deprivation in the presence of etoposide also decreased cell death. These results suggest that lysine deprivation induces inactivation of p53 through decreasing the protein and phosphorylation levels of p53 and exerts the anti-cancer drug resistance in cancer cells.

In summary, I have demonstrated the inactivation effect of hetero-tetramers by the oligomerization domain on p53 transcriptional activity in quantitative single cell analysis. Furthermore, I propose that cancer cells have a potential mechanism for p53 inactivation in response to lysine starvation and resistance to treatment with anti-cancer drug. My study of p53 inactivation has significance and contributes to elucidating the global discovery of p53 responsible for tumor suppression.

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