

**The Role of Lemur Tyrosine Kinase 3 (LMTK3)
in DNA Damage Repair**

Lei Cheng Lit

Thesis submitted for the degree of Doctor of Philosophy

Department of Surgery and Cancer

Division of Cancer

Imperial College London

Hammersmith Campus

United Kingdom

2017

I have no special talent.

I am only passionately curious.

~ Albert Einstein

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DECLARATION OF ORIGINALITY

All the experimentation presented in this thesis has been conducted by myself, aside from the following:

In the results section, the *in vitro* kinase assays were performed by Dr João Pedro Farinha Garção Nunes. Meanwhile, the *in vivo* animal studies were conducted by Dr Yichen Xu, Dr Adam E. Frampton, Dr Ylenia Lombardo and Dr Hua Zhang. Immunohistochemistry and histopathological analysis of tissue samples isolated from mouse mammary primary tumour was performed by Dr Pritesh Trivedi and Dr Aleksandra Filipovic.

I am appreciative of the help from the aforementioned.

Lei Cheng Lit

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ABSTRACT

The lemur tyrosine kinase 3 (LMTK3) is a member of the receptor tyrosine kinase family, previously identified as an oestrogen receptor alpha regulator that involve in endocrine resistance. Importantly, LMTK3 expression has been found in colorectal cancer, non-small cell lung cancer and particularly elevated in high-grade breast cancer. Indeed, LMTK3 overexpression promotes cancer progression and regulates gene transcription of LMTK3-bound tumour suppressor-like genes. Nonetheless, the function and the molecular mechanism of this kinase to overcome drug cytotoxicity in chemotherapy, remain poorly defined. The aims of this thesis are to discover whether LMTK3 plays a role in DNA damage response to doxorubicin and elucidate its mechanism of actions. To study LMTK3 function, we generated stably overexpressed-LMTK3 cell lines. Next, we found that in doxorubicin treatment, these cell lines were more resistant as compared with its control cells. In response to doxorubicin, at the early response, the overexpression of LMTK3 reduced phosphorylation of ATM at serine 1981, which led to decrease serine 428 KAP1 and serine 139 H2AX phosphorylation. In addition, overexpressed-LMTK3 cells reduced H2AX monoubiquitination. These series of events attenuate doxorubicin-induced DNA damage, protecting cells from death. Then we demonstrated that LMTK3 facilitates homologous recombination repair rather than non-homologous end-joining repair. We determined that LMTK3 directly binds to RAD51 and maintains the RAD51 protein level. Overexpression of LMTK3 maintained RAD51 in the nuclear compartment and may recruit RAD51 to filaments which enhance the repair of damaged DNA. Our present study provides a mechanistic insight of LMTK3

role in protecting cells from doxorubicin-induced DNA damage and promoting homologous recombination repair pathway through RAD51 binding.

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The second phase turned to be a mix feeling. I was glad to be home. After spending 18 months at London, I got to go back to University of Malaya. Guess it was not as smooth as I thought it could be. However, my days become better with help from this group of friends. Thank you to Dr Sek Peng Chin, Dr Sik Loo Tan, Sue Ping Eng, Mr Mohan, Dr Melanie, Tony Quah and Dr Elan Selvi that make my day better.

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PUBLICATIONS

All the publications below have been published during this Ph.D period.

1. Stebbing, J, Zhang, H, Xu, Y, **Lit, L C**, Green, A R, Grothey, A, Lombardo, Y, Periyasamy, M, Blighe, K, Zhang, W, Shaw, J A, Ellis, I O, Lenz, H J and Giamas, G (2015) *KSR1 regulates BRCA1 degradation and inhibits breast cancer growth*. *Oncogene*, 34 (16). pp. 2103-2114. ISSN 1476-5594
2. Xu, Yichen, Zhang, Hua, **Lit, Lei C**, Grothey, Arnhild, Athanasiadou, Maria, Kiritsi, Marianna, Lombardo, Ylenia, Frampton, Adam E, Green, Andrew R, Ellis, Ian O, Ali, Simak, Lenz, Heinz-Josef, Thanou, Maya, Stebbing, Justin and Giamas, Georgios (2014) *The kinase LMTK3 promotes invasion in breast cancer through GRB2-mediated induction of integrin β_1* . *Science signaling*, 7 (330). ra58. ISSN 1937-9145
3. Stebbing, J*, **Lit, L C***, Zhang, H, Darrington, R S, Melaiu, O, Rudraraju, B and Giamas, G (2014) *The regulatory roles of phosphatases in cancer*. *Oncogene*, 33 (8). pp. 939-953. ISSN 1476-5594
4. Stebbing, J, Filipovic, A, **Lit, L C**, Blighe, K, Grothey, A, Xu, Y, Miki, Y, Chow, L W, Coombes, R C, Sasano, H, Shaw, J A and Giamas, G (2013) *LMTK3 is implicated in endocrine resistance via multiple signaling pathways*. *Oncogene*, 32 (28). pp. 3371-3380. ISSN 0950-9232
5. **Lit, Lei Cheng**, Scott, Susan, Zhang, Hua, Stebbing, Justin, Photiou, Andrew and Giamas, Georgios (2013) *LATS2 is a modulator of estrogen receptor alpha*. *Anticancer Research*, 33 (1). pp. 53-63. ISSN 0250-7005
6. Zhang, H, Xu, Y, Filipovic, A, **Lit, L C**, Koo, C-Y, Stebbing, J and Giamas, G (2013) *SILAC-based phosphoproteomics reveals an inhibitory role of KSR1 in p53 transcriptional activity via modulation of DBP1*. *British Journal of Cancer*, 109 (10). pp. 2675-2684. ISSN 1532-1827

TABLE OF CONTENTS

DECLARATION OF ORIGINALITY	1
COPYRIGHT DECLARATION	1
ABSTRACT	2
ACKNOWLEDGEMENTS	4
PUBLICATIONS	7
TABLE OF CONTENTS	8
LIST OF FIGURES	13
LIST OF TABLES	15
LIST OF ABBREVIATIONS	16
Chapter 1 INTRODUCTION	21
1.1 Cancer	21
1.1.1 Global cancer incidence	23
1.1.2 Breast cancer incidence	25
1.2 Breast cancer and treatments	27
1.2.1 Luminal subtypes and hormonal therapies	29
1.2.2 HER2 ⁺ subtype and targeted therapies	31
1.2.3 Basal-like subtype and treatments	32
1.3 DNA damage response	33
1.3.1 DNA double-strand break signalling	36
	8

1.3.2	DNA double-strand break repair	39
1.3.2.a	Non-homologous end joining	41
1.3.2.b	Homologous recombination	42
1.4	Protein kinase	44
1.4.1	Protein kinase catalytic domain	45
1.4.2	Classification of human kinome	46
1.5	Lemur tyrosine kinase	53
1.5.1	LMTK1	57
1.5.2	LMTK2	60
1.5.3	LMTK3	64
1.6	Aim of this study	74
Chapter 2	EXPERIMENTAL	75
2.1	Materials	75
2.1.1	Mammalian cell lines	75
2.1.2	Small interference oligonucleotide sequences	75
2.1.3	Plasmid vectors	76
2.1.4	Primers	77
2.1.5	Buffers and solutions	78
2.1.6	Antibodies and recombinant proteins	79
2.1.7	Chemicals and consumables	82
2.1.8	Equipment / Instruments	84
2.2	Methods	85
2.2.1	Culture of breast cancer cell lines	85
2.2.1.a	Culture media	85

2.2.1.b	Thawing frozen cells	85
2.2.1.c	Subculturing and propagation of cells	86
2.2.1.d	Freezing cells	86
2.2.1.e	Counting of viable cells	87
2.2.2	Small interference RNA transfection	88
2.2.3	Bacterial plasmid transformation	89
2.2.4	Plasmid preparation	89
2.2.5	Transient transfection of plasmid DNA	90
2.2.6	Generation of stable cell lines	91
2.2.7	Cell proliferation assay	92
2.2.8	Extraction of total RNA	93
2.2.9	Complementary DNA (cDNA) preparation by reverse transcription	94
2.2.10	Quantitative real-time polymerase chain reaction	94
2.2.10.a	TaqMan quantitative RT-PCR	95
2.2.10.b	SYBR Green quantitative real-time PCR	96
2.2.11	Protein lysate preparation	96
2.2.11.a	Radioimmune Precipitation Buffer (RIPA) buffer extraction	96
2.2.11.b	Protein quantification	97
2.2.12	Western blotting	98
2.2.13	Co-immunoprecipitation	99
2.2.14	Glutathione S-Transferase pull-down assay	100
2.2.15	Nuclear and cytoplasmic extraction	101
2.2.16	Protein stability assay – Protein Synthesis Inhibition	101
2.2.17	Protein stability assay – Proteasome Inhibition	102
		10

2.2.18	Immunofluorescence staining	102
2.2.19	Homologous and non-homologous end joining recombination assays	103
2.2.20	Cell cycle analysis	103
2.2.21	Comet assay	104
2.2.22	<i>In vitro</i> kinase assay	105
2.2.23	Xenograft mouse model	105
2.2.24	Statistical analysis	106
Chapter 3	RESULTS	107
3.1	Generation of stable cell lines expressing LMTK3	107
3.2	Overexpression of LMTK3 protects against doxorubicin-induced cytotoxicity	110
3.3	Overexpression of LMTK3 inhibits the efficacy of doxorubicin <i>in vivo</i>	115
3.4	Effect of doxorubicin on LMTK3 protein and mRNA levels in MCF7 cells	118
3.5	Effect of LMTK3 expression on ATM and KAP1 in response to doxorubicin	120
3.6	Overexpression of LMTK3 delays resolution of γ H2AX foci	126
3.7	LMTK3 does not colocalise with γ H2AX	134
3.8	LMTK3 impedes monoubiquitylation of H2AX	135
3.9	High levels of LMTK3 accelerate DNA breaks repair	137
3.10	LMTK3 is restricted to homologous recombination pathway	139
3.11	Overexpression of LMTK3 affects cell cycle distribution after DNA damage	143
3.12	LMTK3 regulates DNA repair proteins	145
3.13	LMTK3 facilitates RAD51 foci formation and nuclear accumulation	148
3.14	RAD51 does not affect LMTK3 expression	154
3.15	LMTK3 interacts with RAD51	158
3.16	LMTK3 kinase domain does not phosphorylate the RAD51	161
		11

3.17 LMTK3 regulates the stability of RAD51 protein	162
Chapter 4 DISCUSSION	166
4.1 Expression of LMTK3 in MCF7 and T47D cells	167
4.2 LMTK3 and DNA DSB signalling	169
4.3 LMTK3 and DNA DSB repair	174
Chapter 5 CONCLUSIONS	178
Recommendation of future research	180
REFERENCES	184
RESEARCH PRESENTATION	211

LIST OF FIGURES

Figure 1.1	The hallmarks of cancer.	22
Figure 1.2	Global cancer incidence and mortality.	24
Figure 1.3	ASR incidence and mortality of the United Kingdom and Malaysia.	26
Figure 1.4	The general outline of the DNA damage response network.	35
Figure 1.5	Mammalian DSB repair pathways.	40
Figure 1.6	The eukaryotic protein kinase catalytic domain.	45
Figure 1.7	A dendrogram of human protein kinases.	52
Figure 1.8	The LMTK family comprises transmembrane and kinase domains.	54
Figure 1.9	Multiple sequence alignment of human LMTK1, LMTK2 and LMTK3.	56
Figure 1.10	An illustration of ER α regulation by LMTK3.	66
Figure 1.11	Model of the LMTK3 promotes invasion through integrin signalling network during breast cancer progression and metastasis.	70
Figure 3.1	MCF7 and T47D cells stably expressing LMTK3.	109
Figure 3.2	Effect of overexpression of LMTK3 on doxorubicin-treated MCF7 cells.	112
Figure 3.3	Effect of overexpression of LMTK3 on doxorubicin-treated T47D cells.	114
Figure 3.4	Overexpression of LMTK3 impedes the effectiveness of doxorubicin in vivo.	117
Figure 3.5	LMTK3 protein and mRNA levels in doxorubicin-treated MCF7 cells.	119
Figure 3.6	Effect of LMTK3 expression on ATM and ATR in response to doxorubicin treatment.	122
Figure 3.7	Effect of LMTK3 expression on Ser1981 ATM and Ser824 KAP1 in response to doxorubicin treatment.	123
Figure 3.8	Effect of ATM and ATR silencing on LMTK3 expression in response to doxorubicin.	125
		13

Figure 3.9	Up-regulation of LMTK3 impairs phosphorylation of H2AX upon doxorubicin.....	127
Figure 3.10	Overexpression of LMTK3 reduces gamma H2AX foci.	131
Figure 3.11	Effect of overexpressed-LMTK3 on the phosphorylation of H2AX.	132
Figure 3.12	Effect of LMTK3 knockdown on the phosphorylation of H2AX.....	133
Figure 3.13	Localisation of LMTK3 and γ H2AX.	134
Figure 3.14	Effects of LMTK3 on the monoubiquitination of H2AX.	136
Figure 3.15	Comet images of alkaline gel electrophoresis demonstrating DNA damage in the MCF7 and MCF7-LMTK3 cells.	138
Figure 3.16	LMTK3 is involved in homologous recombination repair.....	142
Figure 3.17	Overexpression of LMTK3 affects cell cycle progression after treated with doxorubicin.....	144
Figure 3.18	Effect of LMTK3 expression on DNA repair factors.	147
Figure 3.19	Effect of LMTK3 silencing on the number of RAD51 foci per cell.	149
Figure 3.20	Effect of overexpressed-LMTK3 on RAD51 foci formation.....	150
Figure 3.21	Induction of nuclear RAD51 protein in MCF7 over-expressed LMTK3 cells.....	153
Figure 3.22	RAD51 does not affect the expression of LMTK3 protein.....	157
Figure 3.23	LMTK3 exists in a complex with RAD51 in vivo.....	159
Figure 3.24	LMTK3 binds RAD51 in vitro.....	160
Figure 3.25	LMTK3 is not a kinase of RAD51.....	161
Figure 3.26	Increased RAD51 protein stability in cells with over-expressing LMTK3.	163
Figure 3.27	LMTK3 represses proteasome-induced RAD51 degradation.	165
Figure 5.1	Schematic representation of the proposed model is given.	179

LIST OF TABLES

Table 1.1	A list of exogenous agents that induce diverse spectrum of toxic lesions with their mechanism of actions and type of DNA damage-induced errors	34
Table 1.2	Classification of human eukaryotic protein kinases.....	47
Table 2.1	Cell lines used in this study.....	75
Table 2.2	List of small interference (siRNA) oligonucleotide sequences.....	75
Table 2.3	List of plasmid vectors	76
Table 2.4	Transfection media and reagents.....	77
Table 2.5	List of TaqMan quantitative real-time PCR assays.....	77
Table 2.6	List of SYBR Green quantitative real-time PCR assays.....	77
Table 2.7	List of solutions for Western blotting	78
Table 2.8	List of SDS-PAGE Acrylamide gel solutions.....	79
Table 2.9	List of antibodies for Western blotting	79
Table 2.10	List of recombinant proteins	82
Table 2.11	Microplate microassay dilutions for 2 mg/ml BSA	98
Table 3.1	The absorbance values of treated and untreated MCF7 and MCF7-LMTK3 cells with 0.2 µg/ml of doxorubicin for 2, 4 and 6 days.	111
Table 3.2	The absorbance values of treated and untreated T47D and T47D-LMTK3 cells with 0.2 µg/ml of doxorubicin for 2, 4 and 6 days.	113
Table 3.3	Percentage of DNA tail moments in MCF7 and MCF7-LMTK3 cells after doxorubicin treatment	137

LIST OF ABBREVIATIONS

AATYK	apoptosis-associated tyrosine kinase
ADP	adenosine diphosphate
aPK	atypical protein kinase
APS	ammonium persulphate
ASR	age-standardised rate
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad3-related
ATRIP	ATR interacting partner
BREK	brain-enriched protein kinase
BRCA1	Breast cancer type 1
BRCA2	Breast cancer type 2
BSA	bovine serum albumin
CDC42	cell division cycle 42
CDK	cyclin-dependent kinase
CFTR	cystic fibrosis transmembrane conductance regulator
ChIP-seq	chromatin immunoprecipitation sequencing
CHK1	checkpoint kinase-1
CHK2	checkpoint kinase-2
CHX	cycloheximide
Co-IP	co-immunoprecipitation
Cprk	cyclin-dependent kinase 5/p35

CRC	colorectal cancer
CtIP	CtBP-interacting protein
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DDX5	DEAD-box RNA helicase p68
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
dNTPs	deoxynucleoside triphosphates
Dox	doxorubicin
DSB	double-strand break
E2	oestradiol
EGFR	epidermal growth factor receptor
ePK	eukaryotic protein kinase
ER	oestrogen receptor
ER α	oestrogen receptor alpha
ER β	oestrogen receptor beta
ERK	extracellular signal-regulated kinase
ESR1	oestrogen receptor 1
FACS	fluorescence-activated cell sorting
FOXO3	forkhead box O3
GFP	green fluorescence protein
GRB2	growth factor receptor bound protein 2
GREB1	growth regulation by estrogen in breast cancer 1
GSK3 β	glycogen synthase kinase-3 beta

HER1	Human epidermal growth factor receptor 1
HER2	human epidermal growth factor receptor 2
HDR	homology-directed repair
HR	homologous recombination
HRP	horseradish peroxidase
iGFP	internal green fluorescence protein
IR	ionizing radiation
ITGA5	integrin subunit alpha 5
ITGB1	integrin subunit beta 1
KAP1	KRAB-associated protein 1
kDa	kilo Dalton
KLC	kinesin-1 light chain-2
KPI-2	kinase/phosphatase/inhibitor-2
LMR	lemur kinase
LMTK	lemur tyrosine kinase
LMTK1	lemur tyrosine kinase 1
LMTK2	lemur tyrosine kinase 2
LMTK3	lemur tyrosine kinase 3
MAPK	mitogen-activated protein kinase
miRNA / miR	microRNA
MRN complex	MRE11-RAD50-NBS1 complex
NGF	nerve growth factor
NHEJ	non-homologous end joining
NSCLC	non-small cell lung cancer

qRT-PCR	quantitative real-time polymerase chain reaction
OH	hydroxyl group
PBST	Tween 20 in PBS
PBS	phosphate buffered saline
PCR	polymerase chain reaction
phos-b	glycogen phosphorylase
PGR	progesterone receptor
PI-3K	phosphoinositide-3-kinase
PIKK	phosphoinositide-3-kinase-related protein kinase
PP1 α	phosphatase PP1-alpha catalytic subunit
PTEN	phosphatase and tensin homolog
RNF8	RING finger protein 8
RNF168	RING finger protein 168
RPA	replication protein A
RT	room temperature
RT-PCR	real-time polymerase chain reaction
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	serine
siRNA	small interference RNA
SNP	single nucleotide polymorphism
S.O.C.	Super Optimal Broth with Catabolite repression
SRB	sulforhodamine B

SRF	serum response factor
ssDNA	single-stranded DNA
TAM	tamoxifen
TCGA	The Cancer Genome Atlas
TEMED	tetramethylethylenediamine
TFF1	trefoil factor 1
Thr	threonine
TGF β	transforming growth factor beta
TNBC	triple-negative breast cancer
TNM	tumour-node-metastasis
TP53	tumour protein P53
Tyr	tyrosine
U.S.	the United States
VEGF	vascular endothelial growth factor
XLF	XRCC4-like factor

Chapter 1 INTRODUCTION

1.1 Cancer

When a normal cell behaves unusual such as does not die and keep growing in an uncontrolled manner, it turns to become abnormal cell. These abnormal cells begin to divide without stopping as if the standard control mechanism stop working and spread into surrounding tissues to form growths called tumour.

In the human body, the cells are dividing and reproducing in a strictly controlled and coordinated fashion. During DNA replication, the cell makes an identical copy of its genome and gene mutation(s) can occur during this process. If the DNA repair mechanism unable to fix the mistake, a new cell of changed genome being made which can lead to tumour formation and cancer. The majority of cancers are solid tumours. There are also cancers of the blood, such as leukaemias.

With the advanced technology and remarkable progress in cancer research, now, we know that tumours are composed of multiple distinct cell types that have undergone several gene mutations and affected by not only intracellular but also extracellular components. Herein, Hanahan and Weinberg (2000) proposed and revised (2011) the hallmarks of cancer from six to ten biological capabilities to fully understand the complex biology of cancer. Figure 1.1 illustrates the ten hallmarks capabilities.

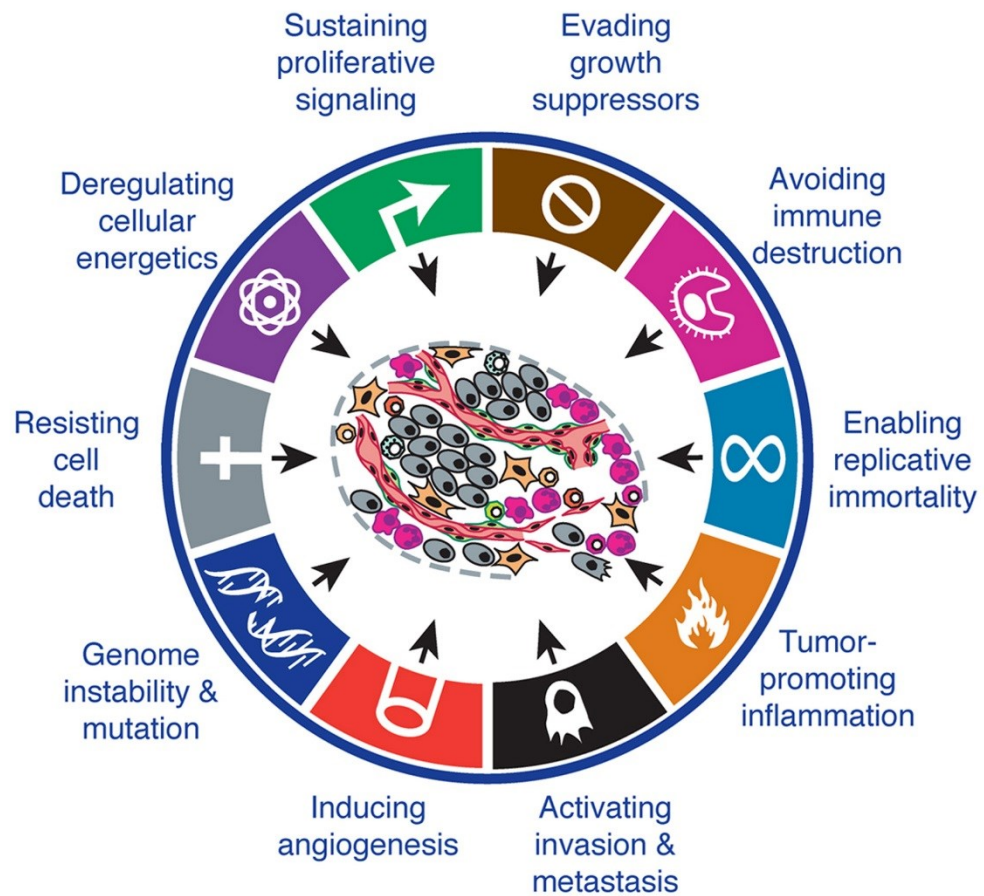


Figure 1.1 The hallmarks of cancer.

The old and new biological capabilities as hallmarks of cancer. Figure adapted from Hanahan and Weinberg (2011).

1.1.1 Global cancer incidence

According to World Health Organization estimates for 2012, cancer was one of four major noncommunicable diseases responsible for 38 million from a total of 56 million deaths occurred worldwide. As the size of the population rises, annual cancer deaths from 8.2 million in 2012 is projected to increase to 12.3 million in 2030 (World Health Organization, 2014).

There were 14.1 million new cancer cases and 32.6 million of people living with cancer (within 5 years of diagnosis) in 2012 worldwide (Figure 1.2) (Ferlay *et al.*, 2013). The most common cancer in the world is lung cancer (1.8 million cases), followed by breast cancer 1.7 million cases), colorectal cancer (1.4 million), prostate cancer (1.1 million cases), stomach cancer (951,000 cases) and liver cancer (782,000 cases) (Ferlay *et al.*, 2015).

In 2013, there were around 352,000 new cases of cancer reported in the United Kingdom, with an incident rate of 960 cases diagnosed every day. This figure is projected to rise by 2% between 2014 and 2035, to 742 cases per 100,000 people by 2035. On the flip side, the mortality rates for all cancers are projected to fall by 15% between the 20 years, to 280 deaths per 100,000 people by 2035 (Cancer Research UK, 2016). While in Malaysia, 37,400 cancer cases were diagnosed in 2012. It comprises of 18,100 males and 19,300 females. More than half of new cases of cancer leading to death. The death age-standardized rate (ASR) for all cancers in the year 2012 regardless of sex was 85.8 per 100,000 persons per year (Ferlay *et al.*, 2013).

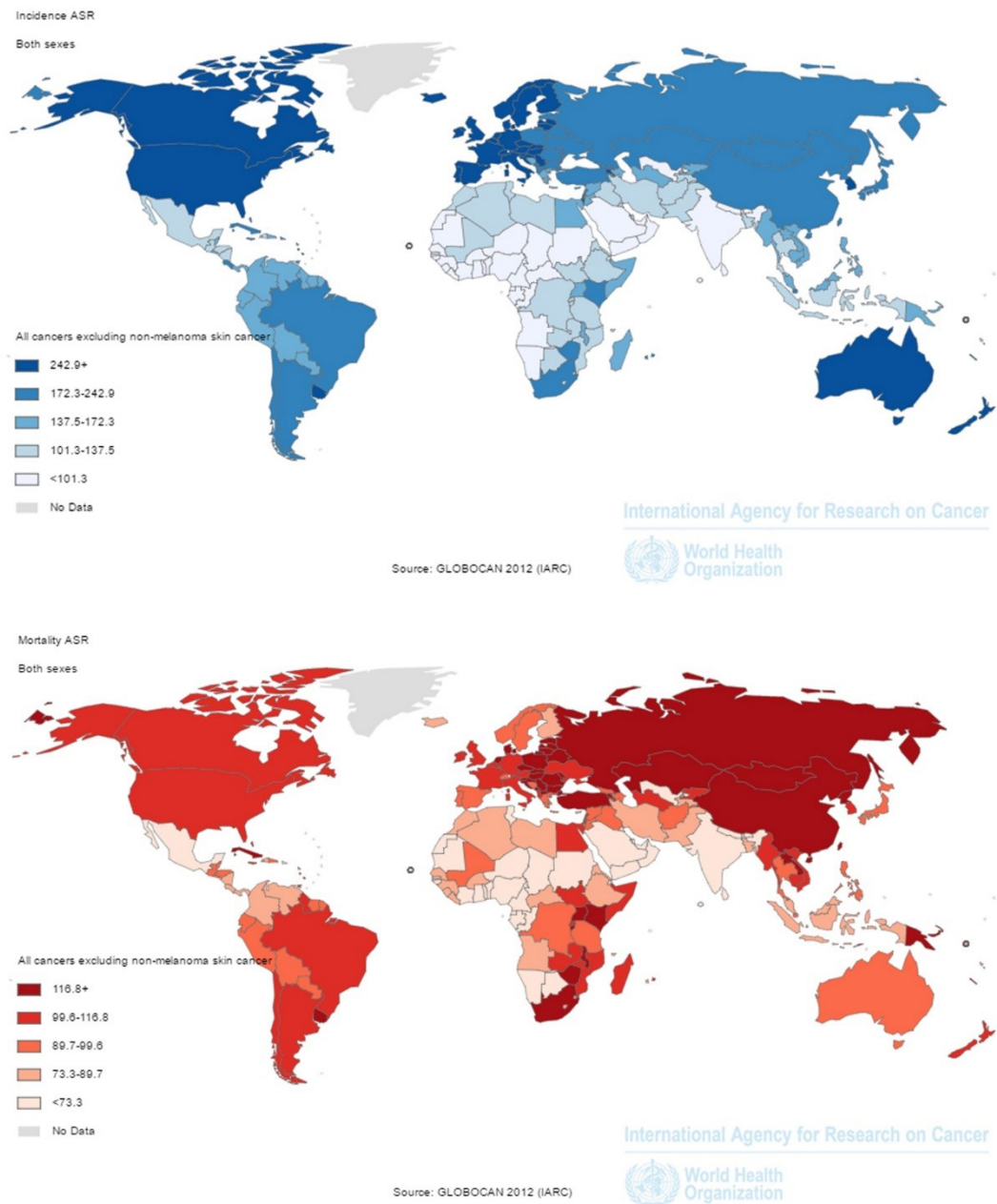


Figure 1.2 Global cancer incidence and mortality.

The incidence and mortality of all cancers (excluding non-melanoma skin cancer) in the world. Figure adapted from GLOBOCAN 2012: Estimated cancer incidence, mortality and prevalence worldwide in 2012 (Ferlay *et al.*, 2013).

1.1.2 Breast cancer incidence

Breast cancer is the most common cancer worldwide for females, with an estimated 1.67 million new cases diagnosed in 2012. It is by far the most common cancer in the United Kingdom and Malaysia for both sexes.

In the United Kingdom, there were 52,399 cases in females and reported none in males (Ferlay *et al.*, 2013). Nevertheless, Cancer Research UK (2016) reported in 2013, there were 53,252 females and 344 males diagnosed with breast cancer. The incidence ASR for the United Kingdom females with breast cancer was 95 per 100,000 (Ferlay *et al.*, 2013). According to Smittenaar *et al.* (2016), projections of incidence rates for breast cancer will rise by 2% in the United Kingdom between 2014 and 2035, to 210 cases per 100,000 females by 2035. Based on Cancer Research UK (2016), on average each year 46% of female breast cancer cases were diagnosed after 65 years old in 2011- 2013.

In Malaysia, the incidence ASR of breast cancer was estimated by the International Agency for Research in Cancer (GLOBOCAN) 2012 as 38.7 per 100,000 with 5410 new cases in 2012 (Ferlay *et al.*, 2013). Similarly, no record in males with breast cancer. A cohort conducted by Pathy *et al.* (2011) found more than 50% of the breast cancer cases from Malaysia and Singapore populations were diagnosed before the age of 50 years old. Yip *et al.* (2014) suggested that the United Kingdom has an older demographic with a median age of 39.8 years, compared with 26.1 years in Malaysia. Thus, younger mean age at presentation in Malaysia. Besides that, the current older

generation in Malaysia experiences lower-risk lifestyles such as more children, more breast-feeding and lower urbanisation.

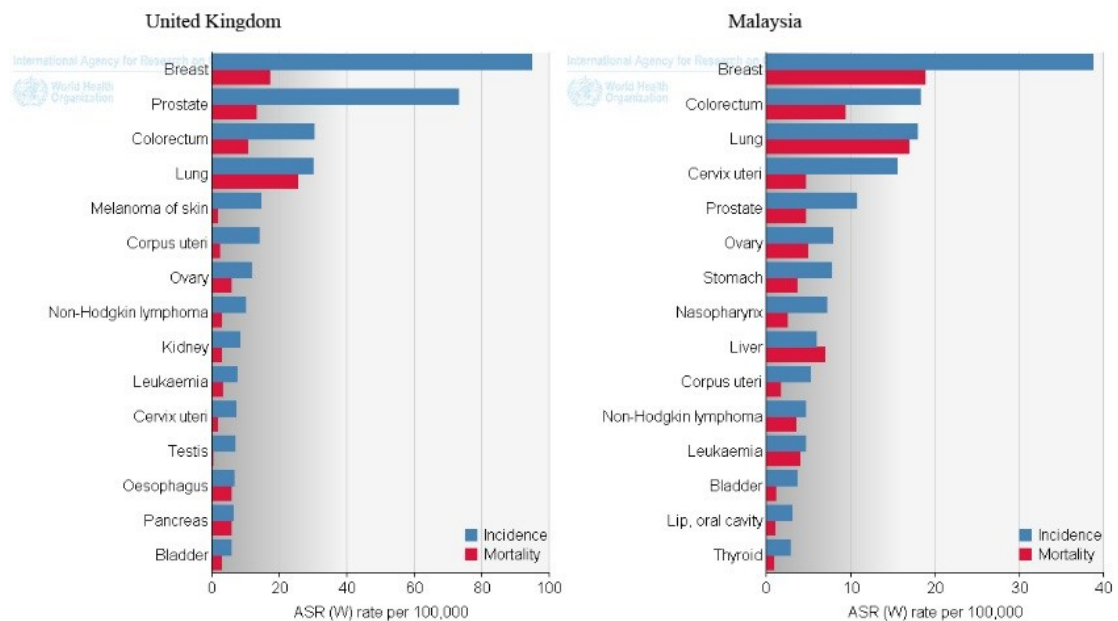


Figure 1.3 ASR incidence and mortality of the United Kingdom and Malaysia.

Estimated age-standardised incidence and mortality rate of the United Kingdom and Malaysia for both sexes in 2012. Figure adapted from GLOBOCAN 2012: Estimated cancer incidence, mortality and prevalence worldwide in 2012 (Ferlay *et al.*, 2013).

1.2 Breast cancer and treatments

Breast cancer is an uncontrolled growth of breast cells which can develop from different parts of the breast. Uncontrolled growth of breast cells can occur as a result of the mutation in genes that can be either inherit from a parent or due to genetic abnormalities.

Relatively 15% of breast cancer patients affects with hereditary breast cancer predisposition genes, the *BRCA1* and *BRCA2* genes (Kang *et al.*, 2010, Lynch *et al.*, 2008). *BRCA1* and *BRCA2*, which are tumour suppressor genes, involves in a diverse range of cellular functions to maintain genomic integrity and transcription regulation. Mutations in *BRCA1* and/or *BRCA2* impairs cells' ability to repair DNA damage and causes a cell to change into a cancerous cell (Scully and Livingston, 2000). Of breast cancer patients, who test negative for germline BRCA mutations can opt to test for *TP53* (Alsner *et al.*, 2000) and *PTEN* (Nelen *et al.*, 1996) mutations as these are another two common predisposition genes in breast cancer. The remaining majority of breast cancer cases are considered as sporadic breast cancer, which mutations occur in an individual who is not from high-risk families (Fackenthal and Olopade, 2007).

Other risk factors for developing breast cancer include age, age at menarche and menopause, overweight, breast-feeding history, menstrual history, exposure to different carcinogens and radiation, using hormone replacement therapy, alcohol intake, lack of exercise and smoking (McPherson *et al.*, 2000).

Often, surgery is carried out to remove the tumour and to determine the stage of diseases. Following treatments such as radiation therapy, chemotherapy, hormone therapy and targeted-therapy are determined based on the histopathological characteristics of the tumours.

Radiation therapy is treatment with high-energy rays or particles that can destroy cancer cells. In generally, radiation therapy is recommended after lumpectomy and may be recommended after mastectomy, to abolish remaining breast cancer cells at the tumour site. These procedures have shown to benefit the breast cancer patients (Fisher *et al.*, 1995, Fisher *et al.*, 1989, EBCTCG, 1995).

Bonadonna *et al.* (1976) introduced the first effective chemotherapy regimen, consists of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) for breast cancer. In 1990, the first anthracycline-containing doxorubicin and cyclophosphamide (AC) became a benchmark of chemotherapy regimen for breast cancer as this regimen significantly decreases the risks of disease recurrence and death (Fisher *et al.*, 1990). Later on, methotrexate in CMF regime was substituted with an anthracycline, doxorubicin or epirubicin to make either FAC (5-fluorouracil, doxorubicin and cyclophosphamide) or FEC (5-fluorouracil, epirubicin and cyclophosphamide) and generally accepted as appropriate adjuvant regimens for the treatment of early breast cancer (Martin *et al.*, 2003, Levine *et al.*, 1998). Consecutive, the development of the taxanes, such as docetaxel and paclitaxel were included in various adjuvant chemotherapy regimens and shown to be more effective than without the taxane (Bear *et al.*, 2006, Henderson *et al.*, 2003, Mamounas *et al.*, 2005, Martin *et al.*, 2005). Later, 5-fluorouracil in FAC was replaced

by docetaxel to become TAC regimen (Martin *et al.*, 2005) and Roche *et al.* (2006) added docetaxel after the FEC regimen to become FEC-T, which this regimen is significantly improved disease-free and overall survival in node-positive breast cancer patients. Chemotherapy drugs are given depending on situations either before surgery (neoadjuvant chemotherapy) or after surgery (adjuvant chemotherapy). In advanced breast cancer situation, chemotherapy drugs are used as the main treatment.

Although breast cancer is the second most common cancer overall but it ranks fifth as the cause of death because an increased understanding of the molecular subtypes that contribute to better prognosis (Ferlay *et al.*, 2015). Breast cancer consists of a heterogeneous group of neoplasms. Disease classification is essential for prognosis and treatment. Breast tumours are classified into at least four major subtypes: luminal A, luminal B, human epidermal growth factor receptor-2 positive (HER2⁺) and basal-like tumours (Desmedt *et al.*, 2008, Perou *et al.*, 2000, Sorlie *et al.*, 2001, Tamimi *et al.*, 2008).

1.2.1 Luminal subtypes and hormonal therapies

Most breast cancers are luminal tumours. The luminal type of tumour is positive for oestrogen and/or progesterone receptors. The difference between luminal A and B is based on their growth rate (low or high Ki67) (Inic *et al.*, 2014). Patients with luminal tumours usually respond well to hormone therapy. Oestrogen is a hormone produced by the ovaries which promote the growth of breast cancers. One of the ways to inhibit

oestrogen synthesis is ovarian ablation by surgical oophorectomy or ovarian radiation. This method is proven significantly improved survival compared to those who received chemotherapy alone (EBCTCG, 1996). However, it is not suitable for breast cancer patients with postmenopausal.

Another way to impede the progression of breast cancer is by inhibiting oestrogen receptor alpha (ER α) activation with anti-oestrogens and aromatase inhibitors (Brueggemeier *et al.*, 2005, Howell *et al.*, 2004). Over the past 35 years, tamoxifen has been the most widely used drug in breast cancer (Cole *et al.*, 1971, EBCTCG, 1998). It is an anti-oestrogen that compete with oestradiol for the ER α ligand binding site and inhibits ER α activity. However, many concerns were raised regarding health risks of tamoxifen such as endometrial cancer and blood clots in the lungs as well as develop resistance to tamoxifen. Thus, approaches to improve tamoxifen structure, by altering either the side chain or the non-steroidal triphenylethylene ring, to produce non-steroidal anti-oestrogens such as toremifene (Kangas *et al.*, 1986), idoxifene (Chander *et al.*, 1991), benzothiophene derivative raloxifene (Palkowitz *et al.*, 1997) and the benzopyran derivative EM-800 (Gauthier *et al.*, 1997). These anti-oestrogens are classified as selective oestrogen receptor modulators.

Another class of anti-oestrogens is selective oestrogen receptor down-regulators, for instance, fulvestrant (Faslodex). Fulvestrant binds to ER α with high affinity and initiates a series of events which includes trigger ER degradation and reduces ER dimerization rate as well as reduces binding of ER to oestrogen response element (Osborne *et al.*, 2004).

Women with hormone receptor-positive breast cancer in the postmenopausal stage are generally treated with aromatase inhibitors. Aromatase inhibitors, such as exemestane, anastrozole and letrozole, bind strongly to the aromatase enzyme, inhibiting the production of oestrogen from androgens and suppressing circulating oestrogen in the body (Fabian, 2007, Brueggemeier *et al.*, 2005). Hence, limit the oestrogen-ER interactions and consequently hinder the progression of breast cancer cells.

1.2.2 HER2⁺ subtype and targeted therapies

HER2⁺ tumours have amplification and overexpression of *ERBB2* gene and can be treated successfully with targeted therapies against HER2. Drugs, like trastuzumab (Herceptin), pertuzumab (Perjeta) and trastuzumab-DM1 are monoclonal antibodies that specifically block HER2 dimerization and downstream signalling to halt the growth of cancer cells (Li and Li, 2013). Some of these drugs are given along with chemotherapeutic drugs. A regimen of doxorubicin and cyclophosphamide with the addition of trastuzumab and paclitaxel improved outcomes among women with surgically removed-HER2⁺ breast cancer (Romond *et al.*, 2005). Docetaxel (Taxotere) is prescribed along with trastuzumab and pertuzumab to patients who have not yet received chemotherapy, showed significantly prolonged progression-free survival (Baselga *et al.*, 2012).

Besides anti-HER2 monoclonal antibodies, tyrosine kinase inhibitors, such as lapatinib is a small molecule that binds reversibly to the intracellular adenosine

triphosphate (ATP)-binding pocket of HER1 and HER2. This binding obstructs HER autophosphorylation, subsequently prevents the activation of downstream cellular signal that promotes cancer cell survival and proliferation (Medina and Goodin, 2008, Tevaarwerk and Kolesar, 2009).

1.2.3 Basal-like subtype and treatments

The majority of basal-like tumours are called triple-negative breast cancer (TNBC) because these tumours are lack of oestrogen and progesterone receptor as well as HER2⁺ (Badve *et al.*, 2011, Gazinska *et al.*, 2013). Till now, only a small percentage of these tumours respond well to standard chemotherapeutic (The Cancer Genome Atlas Network, 2012, Desmedt *et al.*, 2008). Studies have shown that patients with TNBC predominantly expressed epidermal growth factor receptor (EGFR) (Nielsen *et al.*, 2004) and vascular endothelial growth factor (VEGF) (Linderholm *et al.*, 2009) as well as BRCA1 mutation and dysfunction (Foulkes *et al.*, 2003, Turner *et al.*, 2007). A number of targeted therapies for TNBC, such as, cetuximab, bevacizumab and olaparib are at various stages of development to be used for improving patient condition (Alluri and Newman, 2014). More research is carried out in developing effective treatments for TNBC.

1.3 DNA damage response

The DNA damage response (DDR) is an intricate network of signalling transduction cascades that able to distinguish lesion in DNA and stress during DNA replication; transmit this information to regulate cellular responses to repair the DNA damage (Ciccia and Elledge, 2010, Jackson and Bartek, 2009). DNA is continuously exposed to damage due to the action of endogenous and exogenous factors. The endogenous factors such as depurination, deamination, replication errors and oxygen or nitrogen free radicals can cause spontaneous DNA alterations (Hoeijmakers, 2009, Lindahl and Barnes, 2000). Besides reactive oxygen and nitrogen species, which resulting from normal cellular metabolism, free radicals species are also generated from exogenous physical agents such as ionising radiation and ultraviolet light from sunlight (Hoeijmakers, 2009). Additionally, chemical agents used in cancer chemotherapy can also produce a variety of DNA lesions. For example, type I topoisomerases create single-strand breaks in DNA and type II create double-strand breaks (Champoux, 2001, Nitiss, 2009, Wang, 2002). An example of exogenous agents, particularly agents that involve in cancer therapy, their mechanism of actions and the type of DNA damage-induced errors are listed in Table 1.1.

Concisely, when there is an aberrant in DNA structure, the sensor proteins sense the damage, initiate and convey a signal to the transducer proteins, which are the phosphoinositide-3-kinase-related protein kinases (PIKK). The PIKK family magnify the damage signal and activate a host of effector proteins that decide whether to undergo DNA repair, enter terminal differentiation senescence or apoptosis (Figure 1.4).

Table 1.1 A list of exogenous agents that induce diverse spectrum of toxic lesions with their mechanism of actions and type of DNA damage-induced errors

Type of agent	Example of drug	Mechanism of action	Type of DNA damage
Alkylators	Temozolomide Nitrogen mustard Mitomycin C Methyl methanesulfonate Nitrosoureas	Base alkylation Mono or bi-functional DNA adducts Inter, intra and DNA-protein crosslinks	Base damage Replication lesions Bulky adducts
Platinum drugs	Cisplatin Carboplatin Oxaliplatin	Bifunctional DNA adducts Inter, intra and DNA-protein crosslinks	DSBs DNA crosslinks Replication lesions Bulky adducts
Antimetabolites	5-fluorouracil Thiopurines Folate analogues	Misincorporates into DNA Depletes deoxynucleoside triphosphates	Base damage Replication lesions
Topoisomerase inhibitors	Doxorubicin Camptothecin Etoposide	Inhibits topoisomerase enzymes in complex with DNA	SSBs DSBs Replication lesions
Replication inhibitors	Hydroxyurea	Inhibits DNA synthesis	DSBs Replication lesions
Radiotherapy and radiomimetics	Ionising radiation Bleomycin	Direct and indirect production of reactive oxygen species	SSBs DSBs Base damage

Adapted from: Helleday *et al.* (2008)

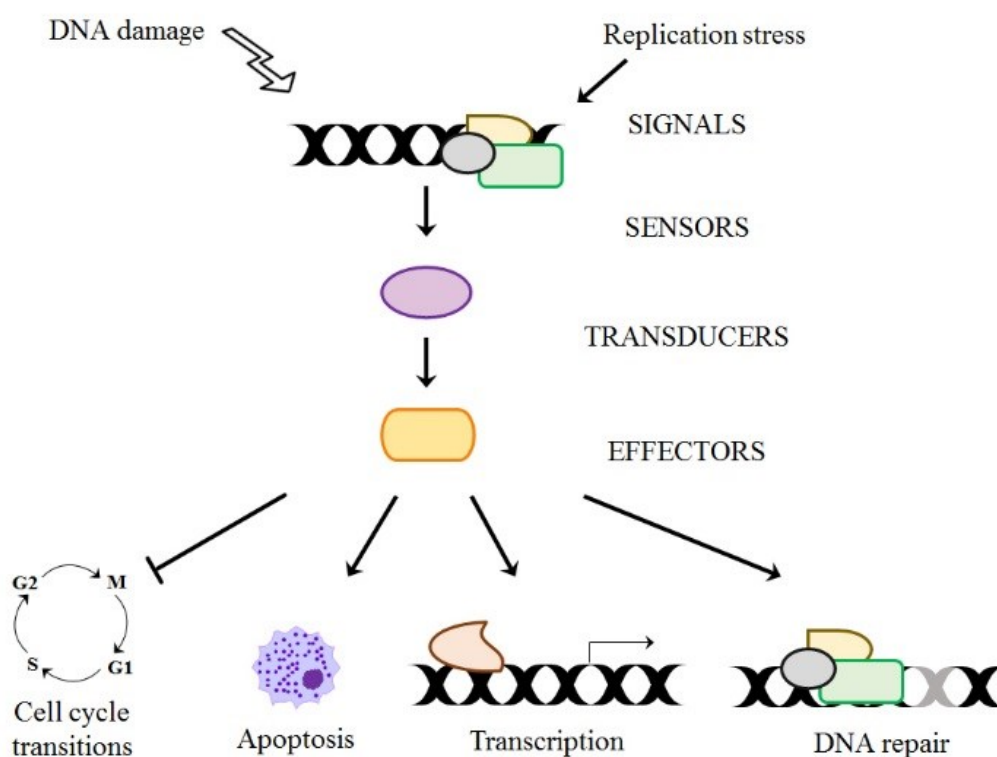


Figure 1.4 The general outline of the DNA damage response network.

Arrowhead represents activating events, and perpendicular end represents inhibitory event. The complex signalling system is depicted as a linear pathway consisting of the signal, sensors, transducers and effectors. Adapted from Zhou and Elledge (2000).

1.3.1 DNA double-strand break signalling

DNA double-strand breaks (DSBs) are highly toxic lesions, where both strands of the DNA duplex are broken. In point of fact, generation of DSBs and faulty in DSB repair induce mutations, promote genomic instability and presumably tumorigenesis (Khanna and Jackson, 2001, Lengauer *et al.*, 1998, Richardson and Jasin, 2000, Vamvakas *et al.*, 1997). The major cause of DSBs formation is when DNA replication forks encounter unrepaired DNA lesions, triggering fork collapse (Chapman *et al.*, 2012). DSB can also be induced by exogenous agents, such as ionising radiation, cisplatin (platinum drug) and doxorubicin (topoisomerase II inhibitor) (Cheung-Ong *et al.*, 2013).

Upon DSB induction, forms DNA lesion, it is followed by phosphorylation of H2AX at Ser139 by PIKK family proteins (Rogakou *et al.*, 1998, Furuta *et al.*, 2003, Stiff *et al.*, 2004). Variant H2AX is a member of histone protein H2A family in a histone octamer (H2A, H2B, H3 and H4) that forms a nucleosome, together with 147 base-pairs of DNA (Rogakou *et al.*, 1998). The phosphorylated H2AX protein is termed γ H2AX (Kuo and Yang, 2008). It is phosphorylated rapidly within minutes following DSB generated-ionising radiation (IR) and a number of foci formation are proportional to the severity of the damage (Rogakou *et al.*, 1999). The accumulation of γ H2AX, along with other damage response proteins, such as MDC1, NBS1 (MRN complex) and RAD51, localised at the damage site, forms foci (Paull *et al.*, 2000, Lowndes and Toh, 2005).

The phosphorylation of MDC1 is subsequently recognised by the E3 ubiquitin ligase, RING finger protein 8 (RNF8) that work together with E2 enzyme, Ubc13 to

ubiquitylate histones H2A and γ H2AX (Messick and Greenberg, 2009, Bergink and Jentsch, 2009). RNF8 then recruits second E3 ubiquitin ligase, RING finger protein 168 (RNF168) to promote polyubiquitination on Lys13 (H2AK13ub) and/or Lys15 (H2AK15ub) (Citterio, 2015). Another E3, RING1b/BMI1, belongs to the polycomb repressive complex 1, recruits to the DSB sites, where it monoubiquitinates H2A on Lys119 (H2AK119ub) to repress transcription locally and contributes to mediate homologous recombination repair. Loss of BMI1, impaired the recruitment of those proteins that require ubiquitylation to DNA damage sites and hinder repair of DNA DSB (Ginjala *et al.*, 2011, Ismail *et al.*, 2010). Therefore, the ubiquitination of H2A and γ H2AX is also an important event in DNA DSB signalling.

The formation of γ H2AX foci gives the advantage to analyse the DSBs formation. It can be detected by using γ H2AX specific antibody and analysed using fluorescence or cell lysate probed by Western blotting. Lately, phosphorylation of H2AX is not only recognize as the indicator of DSB but also participate in cell cycle checkpoints, repair machinery and chromatin remodelling at the sites of DNA damage (Celeste *et al.*, 2003, Fernandez-Capetillo *et al.*, 2004a, Fernandez-Capetillo *et al.*, 2004b, Paull *et al.*, 2000, Morrison *et al.*, 2004, van Attikum and Gasser, 2005). Thus far, the relationship between the kinetics of γ H2AX foci disappearance and DSBs repair is disputable (Rothkamm and Lobrich, 2003, Taneja *et al.*, 2004). Chowdhury *et al.* (2005) showed that dephosphorylation of γ H2AX by protein phosphatase 2A catalytic subunit resolved the foci and facilitated DNA DSB repair. However, Bouquet *et al.* (2006) showed that only at the low cytotoxic doses of calicheamicin γ 1, the loss of γ H2AX foci was correlated

with DSB repair activity and suggested redistribution towards the chromatin may cease the γ H2AX signal.

Histone H2AX is a substrate of several PIKKs, such as ataxia-telangiectasia mutated (ATM), ATM-and Rad3-related (ATR) kinase and DNA-dependent protein kinase (DNA-PK). Primarily, DSBs generated by doxorubicin is triggered through ATM, and the signal is further amplified by ATM-dependent activation of the downstream kinase checkpoint kinase-2 (Chk2) (Kurz *et al.*, 2004). In the absence of ATM, DNA-PK compensates ATM function in NBS1 and ATM mutant cell lines and also responds to replication associated-DSBs (Furuta *et al.*, 2003, Stiff *et al.*, 2004). Meanwhile, ATR, together with ATR-interacting partner (ATRIP) is activated following recruitment of replication protein A (RPA)-coated single-stranded (ss) DNA regions that are generated primarily in S-phase at stalled DNA replication forks (Ciccio and Elledge, 2010), eliciting ATR-dependent checkpoint signalling by checkpoint kinase-1 (Chk1) (Cimprich and Cortez, 2008, Reinhardt *et al.*, 2007).

However, recent data indicates that activation of ATR by IR-induced DSB also occurs in an ATM-dependent manner (Davalos *et al.*, 2004) and IR-induced Chk1 phosphorylation is restricted to late S and G₂ cells (Cuadrado *et al.*, 2006). Additionally, in the similar manner of DSB induction, ATR activation is possibly under the control of ATM and/or Ku70/80 (Tomimatsu *et al.*, 2007).

It is widely accepted that tri-protein MRE11/RAD50/NBS1 (MRN complex) is earliest proteins to be recruited at the sites of DNA damage (Carson *et al.*, 2003, Uziel *et*

al., 2003, Difilippantonio *et al.*, 2005). MRN complex subsequently recruits and activates ATM to the damaged DNA ends. This enables the activated ATM to phosphorylate H2AX, creating binding sites of the FHA domain of NBS1, thereby recruit additional MRN complexes and further activate ATM, leading to signal amplification (Stavridi and Halazonetis, 2005). An additional role of MRN complex lies downstream of ATM in DNA damage response pathway (Uziel *et al.*, 2003), which it involves in DSB repair, particularly in the homologous recombination pathway (Kanaar and Wyman, Stracker *et al.*, 2004).

1.3.2 DNA double-strand break repair

There are two main pathways for DNA DSB repair: non-homologous end joining (NHEJ) and homologous recombination (HR). These two pathways are biochemically distinct but both complements one another to effect DSB repair (Essers *et al.*, 2000, Haber, 2000, Takata *et al.*, 1998). During NHEJ, there is no need for extensive sequence homology between the DNA ends to ligate the two DNA DSBs. In contrast, HR requires genetic information from an undamaged DNA molecule with which it shares extensive sequence homolog when the damaged chromosomes enter into synapsis (Jackson, 2002). In mammalian cells, the NHEJ pathway functions in both proliferating and differentiating cells particularly in G₀-, G₁- and early S- phase, while the HR pathway operates mostly in proliferating cells, during S- and G₂- phases (Johnson and Jasin, 2000, Takata *et al.*, 1998).

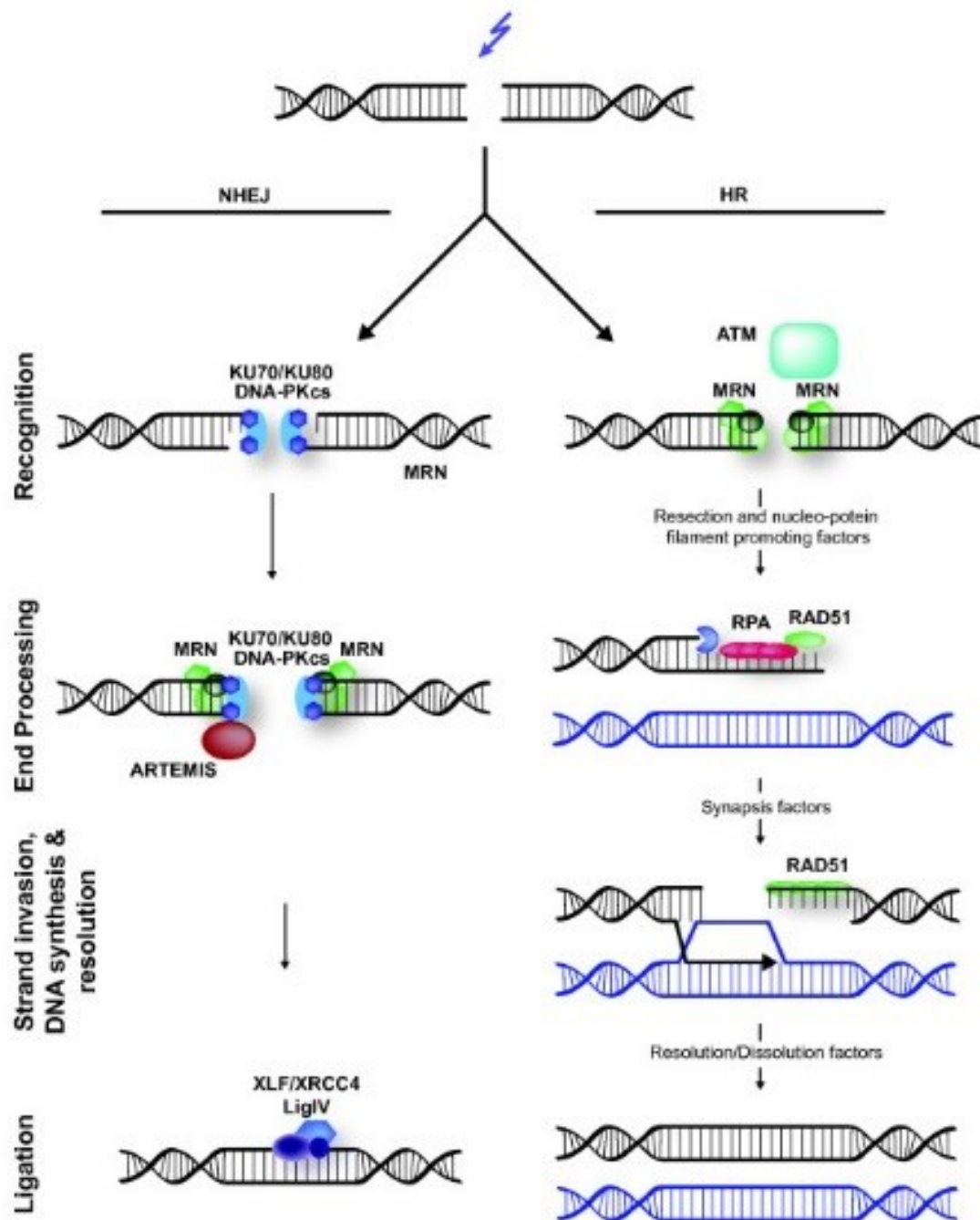


Figure 1.5 Mammalian DSB repair pathways.

A DNA DSB can be repaired by NHEJ (left branch of pathway) or HR (right branch of pathway). Adapted from Lans *et al.* (2012).

1.3.2.a Non-homologous end joining

The main factor in NHEJ repair pathway is the Ku complex, a heterodimer of two subunits named Ku70 (~70 kDa) and Ku80 (~83 kDa) (Lieber, 1999, Smith and Jackson, 1999). The Ku complex binds to the broken DNA ends and recruits the DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) to form DNA-PK. This binding results in translocation of the Ku complex inward on the double strand, which eventually activates the DNA-PKcs kinase activity (Smith and Jackson, 1999, Davis and Chen, 2013). DNA-PKcs phosphorylates and activates several repair proteins, as well as Artemis nuclease and MRN complex, which are involved in processing the DNA termini, before ligation (Lamarche *et al.*, 2010). Second NHEJ factor is DNA repair protein XRCC4 that may be responsible for the recruitment of other NHEJ factors to the DSB ends. XRCC4 interacts with DNA ligase IV, as well as XRCC4-like factor (XLF) to mediate ligation of the DSB (Davis and Chen, 2013, Lamarche *et al.*, 2010, Lieber, 2010). This pathway leads to error-prone repair of the DSB (Figure 1.5, left branch of pathway) because the restoration of DSB is without the guide of the original template which can lead to base substitutions, insertions and deletions during enzymatic modifications at the two non-compatible ends (Kuhfittig-Kulle *et al.*, 2007).

1.3.2.b Homologous recombination

When a DSB occurs in one of two sister chromatids, the ends of the DSB are recognised by the MRN complex, which promotes the activation of ATM and the preparation of DNA for HR (Williams *et al.*, 2007). MRN complex together with CtBP-interacting protein (CtIP) promote DNA end resection and lead to the generation of 3' single-stranded DNA (ssDNA) overhangs. The invasion of 3' ssDNA into a homologous duplex is a crucial step for DSB repair pathway choice (Kass and Jasin, 2010, Shrivastav *et al.*, 2008).

CtIP recruits RPA to ssDNA and promotes RAD51 and RAD52 recruitment to the DSB. Both RPA and RAD52 help to load RAD51 onto ssDNA for form ssDNA-RAD51 nucleoprotein filament (San Filippo *et al.*, 2008). The formation of ssDNA-RPA also recruits ATR/ATRIP, eliciting ATR-dependent checkpoint signalling by the CHK1 (Sartori *et al.*, 2007). The formation of RAD51 nucleoprotein filament represses single strand annealing and alt-NHEJ, eventually become a key point in promoting HR repair pathway (Jasin and Rothstein, 2013).

Following, during synapsis, the RAD51 nucleoprotein filament interacts with the sister chromatid of the homologous region and RAD51 catalyses strand-exchange events where the 3' end of the damaged molecule invades the other DNA duplex, displacing one strand as a D-loop. Next, during post-synapsis, RAD51 dissociates from double-stranded DNA to expose the 3'-OH for DNA synthesis (Krejci *et al.*, 2012). DNA is synthesised by DNA polymerases, and there are at least three different routes to form the new DNA.

First is the DSB repair model that lead to the formation of an intermediate structure called Holliday junctions formed (West, 2003). Second, the synthesis-dependent strand annealing mode of HR, which is preferred during mitosis (Allers and Lichten, 2001). Third, the break-induced replication happens when there is only one DNA end (Llorente *et al.*, 2008). Finally, to complete this error-free repair event, the ends are ligated by DNA ligase I so as to yield two intact DNA molecules. Figure 1.5 shows a schematic diagram of HR (right branch of pathway).

1.4 Protein kinase

Protein kinases are enzymes that modulate the function of proteins by adding phosphate groups to them. Concisely, protein kinases bind substrate proteins and transfer a phosphate group from ATP to amino acids with free hydroxyl groups such as serine, threonine or tyrosine, in a process called phosphorylation. The products from this kinase reaction are the phospho-protein and ADP. There is 518 putative protein kinase genes have been identified in the human genome, which comprises about 1.7% of all human genes (Manning *et al.*, 2002b). Most kinases act on serine or threonine, while some are specific to tyrosine. There are also kinases that act on all three residues (Marin *et al.*, 1999, Cohen, 2002, Ubersax and Ferrell Jr, 2007). Once the phosphate group is transferred to the substrate protein, it can alter the substrate protein activity, localisation, turnover or interaction with other proteins.

Kinases are main controllers of the overall function of many proteins by coordinating of complex functions in the cell. Most protein kinases are dephosphorylated and inactive in the basal state and are activated by phosphorylation. Protein phosphorylation is a reversible post-translation modification that is mediated by intramolecular (autophosphorylation) or intermolecular kinases and phosphatases (dephosphorylation) (Smith *et al.*, 1993, Stebbing *et al.*, 2014).

1.4.1 Protein kinase catalytic domain

Protein kinases are cluster based on their homologous kinase domains. These kinase domains are also known as catalytic domains, which consist of ~ 250 – 300 amino acid residues, equivalent to about 30 kDa (Hanks *et al.*, 1988, Hanks and Quinn, 1991). Canonical catalytic domain made up of a small N-terminus fold of β -sheets and a larger C-terminus fold of α -helices (Ubersax and Ferrell Jr, 2007). There is 12 conserved subdomains in eukaryotic protein kinase (ePK) catalytic domain (Hanks and Hunter, 1995). Two of these subdomains are mainly essential for catalytic function. First subdomain, which is located in the N-terminus of the catalytic domain, is the invariant lysine and aspartate that function in ATP binding and the second subdomain, which is located in the central part of the catalytic domain, contains the invariant aspartate that is the likely catalytic base in the phosphotransfer reaction (Knighton *et al.*, 1991, Hanks, 2003).

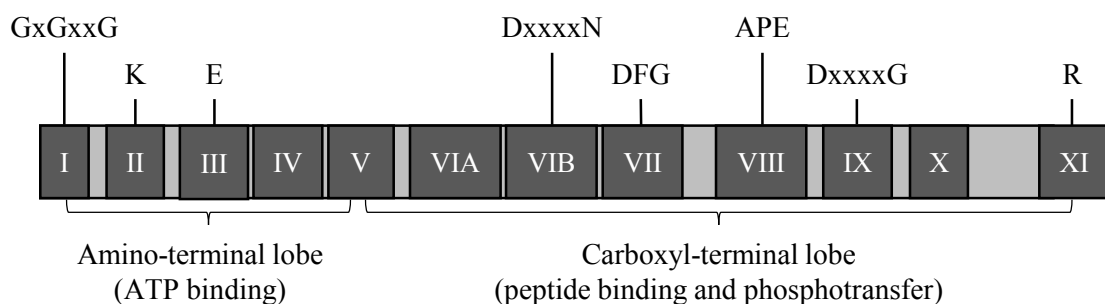


Figure 1.6 The eukaryotic protein kinase catalytic domain.

Twelve subdomain residues are recognised as being invariant throughout the eukaryotic protein kinase superfamily. Figure adapted from Hanks (2003).

1.4.2 Classification of human kinome

Protein kinases are one of the largest families of genes in eukaryotes and estimated that 30% of all cellular proteins are phosphorylated (Cohen, 2000). The Nomenclature Committee of the International Union of Biochemists has endorsed that protein kinases be categorised based on the acceptor amino acid specificity instead of protein substrate specificity (Hunter, 1991).

Protein kinases are classified into two superfamilies, ePK that share a conserved catalytic domain and the atypical protein kinases (aPKs) that have been shown to have protein kinase activity and have structural similarity but lack sequence similarity to the ePK catalytic domain (Manning *et al.*, 2002b). Based on the sequence of ePK domains, they are divided into nine major groups and are subdivided into 134 families and 196 subfamilies (Manning *et al.*, 2002b, Manning *et al.*, 2002a). Table 1.1 shows the nine main groups with examples of kinases.

Table 1.1 Classification of human eukaryotic protein kinases

Group	Family	Subfamilies	Family members
AGC – name after the protein kinase A, G and C families	AKT, DMPK, GRK, MAST, NDR, PKA, PKB, PKC, PKG, PKN, RSK, RSKL, SGK, YANK	Alpha, BARK, Delta, Eta, GEK, GRK, Iota, MSK, p70, ROCK, RSK	AKT1, AKT2, AKT3, BARK1, BARK2, CRIK, DMPK1, DMPK2, GPRK4, GPRK5, GPRK6, GPRK6ps, GPRK7, LATS1, LATS2, MAST1, MAST2, MAST3, MAST4, MASTL, MRCKa, MRCKb, MRCKps, MSK1, MSK2, NDR1, NDR2, p70S6K, p70S6Kb, p70S6Kps1, p70S6Kps2, PDK1, PKACa, PKACb, PKACg, PKCa, PKCb, PKCd, PKCe, PKCg, PKCh, PKCi, PKCips, PKCt, PKCz, PKG1, PKG2, PKN1, PKN2, PKN3, PRKX, PRKXps, PRKY, RHOK, ROCK1, ROCK2, RSK1, RSK2, RSK3, RSK4, RSKL1, RSKL2, SGK, SGK2, SGK3, SgK494, YANK1, YANK2, YANK3
	CDK, CDKL, CLK, DYRK, GSK, MAPK, RCK, SRPK		CDC2, CDK2, CDK3, CDK5ps, CDK10, CDK4, CDK6, CDK4ps, CDK5, CDK7, CDK7ps, CDK8, CDK11, CDK8ps, CDK9, CHED, CRK7, DYRK1B, DYRK1A, DYRK2, DYRK4, DYRK3, Erk1, Erk2, Erk3, Erk4, Erk5, Erk3ps1, Erk3ps3, Erk3ps4, Erk3ps2, Erk7, HIPK1, IPK3, HIPK2, HIPK4, JNK1, JNK2, JNK3, NLK, p38a, p38b, p38g, p38d, PITSLRE, PRP4, PRP4ps, PCTAIRE1, PCTAIRE2, PCTAIRE3, PFTAIRE1, PFTAIRE2, CCRK, CDKL2, CDKL1, CDKL5, CDKL4, CDKL3, CLK1, CLK2, CLK3, CLK4, CLK3ps, CLK2ps, GSK3A, GSK3B, MAK, ICK, MOK, SRPK1, SRPK2, MSSK1, SRPK2ps
CMGC – name after cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinase 3 and Cdc2-like kinase families			

Table 1.1 Classification of human eukaryotic protein kinases (continued)

Group	Family	Subfamilies	Family members
CAMK – an abbreviation for the Ca ²⁺ /calmodulin-dependent protein kinase	CAMK1, CAMK2, CAMKL, CAMK-Unique, CASK, DAPK, DCAMKL, MAPKAPK, MLCK, PHK, PIM, PKD, PSK, RAD53, RSKb, Trbl, Trio, TSSK	AMPK, BRSK, CHK1, HUNK, LKB, MAPKAPK, MARK, MELK, MNK, MSKb, NIM1, NuaK, PASK, QIK, RSKb, SNRK	AMPKa1, AMPKa2, BRSK1, BRSK2, CaMK1a, CaMK1b, CaMK1d, CaMK1g, CaMK2a, CaMK2b, CaMK2d, CaMK2g, CaMK4, caMLCK, CASK, CHK1, CHK2, CHK2ps1, CHK2ps2, DAPK1, DAPK2, DAPK3, DCAMKL1, DCAMKL2, DCAMKL3, Domain2_MSK1, Domain2_MSK2, Domain2_Obscn, Domain2_RSK1, Domain2_RSK2, Domain2_RSK3, Domain2_RSK4, Domain2_SPEG, DRAK1, DRAK2, HUNK, LKB1, MAPKAPK2, MAPKAPK3, MAPKAPK5, MAPKAPKps1, MARK1, MARK2, MARK3, MARK4, MARKps01, MARKps02, MARKps03, MARKps04, MARKps05, MARKps07, MARKps08, MARKps09, MARKps10, MARKps11, MARKps12, MARKps13, MARKps15, MARKps16, MARKps17, MARKps18, MARKps19, MARKps20, MARKps21, MARKps22, MARKps23, MARKps24, MARKps25, MARKps26, MARKps27, MARKps28, MARKps29, MARKps30, MELK, MNK1, MNK1ps, MNK2, NIM1, NuaK1, NuaK2, Obscn, PASK, PHKg1, PHKg1ps1, PHKg1ps2, PHKg1ps3, PHKg2, PIM1, PIM2, PIM3, PKD1, PKD2, PKD3, PSKH1, PSKH1ps, PSKH2, QIK, QSK, SgK085, SgK495, SIK, skMLCK, smMLCK, SNRK, SPEG, SSTK, STK33, STK33ps, Trad, Trbl, Trb2, Trb3, Trio, TSSK1, TSSK2, TSSK3, TSSK4, TSSKps1, TSSKps2, TTN, VACAMKL

Table 1.1 Classification of human eukaryotic protein kinases (continued)

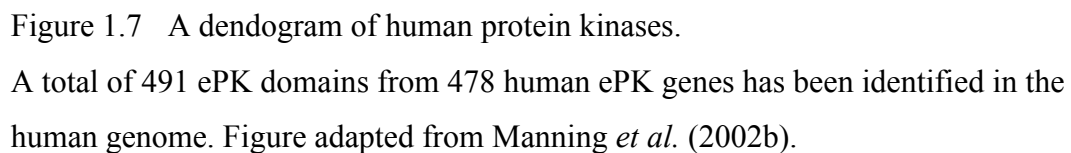
Group	Family	Subfamilies	Family members
CK1 – originally known as Casein Kinase I	CK1, VRK, TTBK,		CK1aps3, CK1a, CK1d, CK1e, CK1g2, CK1g3, VRK1, VRK2, TTBK2, TTBK1, VRK3, CK1a2, CK1aps1, CK1aps2, CK1g2ps, CK1g1, VRK3ps
RGC – receptor guanylate cyclases	RGC		ANPa, ANPb, CGDps, CYGD, HSER, KSGCps, SgK050ps
STE – consists of Ste7, Ste11 and Ste20	STE11, STE20, STE7, STE-Unique	FRAY, KHS, MSN, MST, NinaC, PAKA, PAKB, SLK,STLK, TAO, YSK	COT, Domain2_GCN2, GCK, HPK1, KHS1, KHS2, LOK, MAP2K1, MAP2K1ps, MAP2K2, MAP2K2ps, MAP2K3, MAP2K4, MAP2K5, MAP2K6, MAP2K7, MAP3K1, MAP3K2, MAP3K3, MAP3K4, MAP3K5, MAP3K6, MAP3K7, MAP3K8, MST1, MST2, MST3, MST3ps, MST4, MYO3A, MYO3B, NIK, OSR1, PAK1, PAK2, PAK2ps, PAK3, PAK4, PAK5, PAK6, SLK, STLK3, STLK5, STLK6, STLK6ps1, STLK6-ts, TAO1, TAO2, TAO3, YSK1, ZC1/HGK, ZC2/TNIK, ZC3/MINK, ZC4/NRK

Table 1.1 Classification of human eukaryotic protein kinases (continued)

Group	Family	Subfamilies	Family members
TK – phosphorylates almost exclusively on tyrosine residues	Abl, Ack, Alk, Axl, CCK4, Csk, DDR, EGFR, Eph, Fak, Fer, FGFR, InsR, JakA, JakB, Lmr, Met, Musk, PDGFR, Ret, Ror, Ryk, Sev, Src, Syk, Tec, Tie, TK-Unique, Trk, VEGFR		ABL, ACK, ALK, ARG, AXL, BLK, BMX, BRK, BTK, CCK4, CSK, CTK, DDR1, DDR2, Domain2_JAK1, Domain2_JAK2, Domain2_JAK3, Domain2_TYK2, EGFR, EphA1, EphA10, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphB1, EphB2, EphB3, EphB4, EphB6, FAK, FER, FERps, FES, FGFR1, FGFR2, FGFR3, FGFR4, FGR, FLT1, FLT1ps, FLT3, FLT4, FMS, FRK, FYN, HCK, HER2/ErbB2, HER3/ErbB3, HER4/ErbB4, IGF1R, INSR, IRR, ITK, JAK1, JAK2, JAK3, KDR, KIT, LCK, LMR1, LMR2, LMR3, LTK, LYN, MER, MET, MUSK, PDGFRa, PDGFRb, PYK2, RET, RON, ROR1, ROR2, ROS, RYK, RYKps, SRC, SRM, SuRTK106, SYK, TEC, TIE1, TIE2, TNK1, TRKA, TRKB, TRKC, TXK, TYK2, TYRO3, TYRO3ps, YES, YESps, ZAP70
		HH498, ILK, LIMK, LZK, MLK, TAK1, TESK, Type1, Type2	ACTR2, ACTR2B, ALK1, ALK2, ALK4, ALK7, ANKRD3, ARAF, ARAFps, BMPR1A, BMPR1Aps1, BMPR1Aps2, BMPR1B, BMPR2, BRAF, BRAFps, DLK, HH498, ILK, IRAK1, IRAK2, IRAK3, IRAK4, KSR1, KSR2, LIMK1, LIMK2, LIMK2ps, LRRK1, LRRK2, LZK, MISR2, MLK1, MLK2, MLK3, MLK4, MLKL, RAF1, RAF1ps, RIPK1, RIPK2, RIPK3, SgK288, TAK1, TESK1, TESK2, TGFbR1, TGFbR2, ZAK
TKL – similar to members of the TK group but generally lack the TK-specific motifs of TK group	IRAK, LISK, LRRK, MLK, RAF, RIPK, STKR, TKL-Unique		

Table 1.1 Classification of human eukaryotic protein kinases (continued)

Group	Family	Subfamilies	Family members
Other – an ePK domain that does not fit into any of the other major group	AUR, BUB, Bud32, CAMKK, CDC7, CK2, Haspin, IKK, IRE, MOS, NAK, NEK, NFK1, NKf2, NKf3, NKf4, NKf5, NRBP, Other-Unique, PEK, PLK, SCY1, Slob, TBCK, TLK, TOPK, TTK, ULK, VPS15, WEE, Wnk	GCN2, PEK, Meta	AAK1, AurA, AurAps1, AurAps2, AurB, AurBps1, AurC, BIKE, BUB1, BUBR1, CaMKK1, CaMKK2, CDC7, CK2a1, CK2a1-rs, CK2a2, CLIK1, CLIK1L, Fused, GAK, GCN2, Haspin, HRI, HRJps, IKKa, IKKb, IKKe, IRE1, IRE2, KIS, MOS, MPSK1, MPSK1ps, MYT1, NEK1, NEK10, NEK11, NEK2, NEK2ps1, NEK2ps2, NEK2ps3, NEK3, NEK4, NEK4ps, NEK5, NEK6, NEK7, NEK8, NEK9, NRBP1, NRBP2, PBK, PEK, PIK3R4, PINK1, PKR, PLK1, PLK1ps1, PLK1ps2, PLK2, PLK3, PLK4, PRPK, RNASEL, SAKps, SBK, SCYL1, SCYL2, SCYL2ps, SCYL3, SgK069, SgK071, SgK110, SgK196, SgK223, SgK269, SgK307, SgK384ps, SgK396, SgK424, SgK493, SgK496, Slob, TBCK, TBK1, TLK1, TLK1ps, TLK2, TLK2ps1, TLK2ps2, TTK, ULK1, ULK2, ULK3, ULK4, Wee1, Wee1B, Wee1Bps, Wee1ps1, Wee1ps2, Wnk1, Wnk2, Wnk3, Wnk4



1.5 Lemur tyrosine kinase

Lemur tyrosine kinase (LMTK) or lemur kinase (LMR), named after the Madagascar primates with long tails because these membrane kinases have a long carboxyl-terminus cytoplasmic tail (Manning *et al.*, 2002b). There are three members in the LMR family: LMTK1, LMTK2 and LMTK3. In contrast to classical receptor tyrosine kinases (RTKs), LMTK contains a short extracellular domain and lengthy intracellular domain (Figure 1.3). All three members are large proteins composed of 1300 – 1500 amino acids that consist of highly conserved sequence N-terminus tyrosine kinase domain and C-terminus proline-rich region (Figure 1.4).

Originally, LMTKs have been predicted to be tyrosine kinases based on its sequence homology to receptor-type tyrosine kinase (Robinson *et al.*, 2000). However, many papers have been reported its serine (Ser) / threonine (Thr) kinase activity (Kawa *et al.*, 2004, Wang and Brautigan, 2002). Therefore, all LMTK-family kinases are now considered as Ser/Thr kinases.

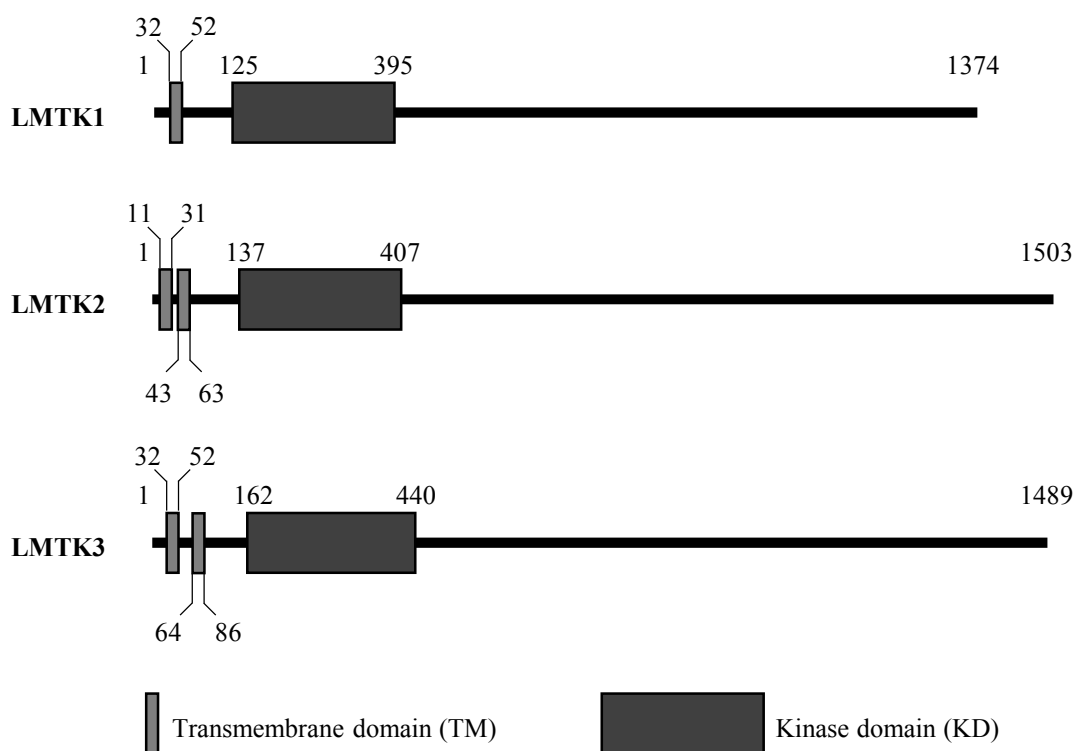


Figure 1.8 The LMTK family comprises transmembrane and kinase domains. LMTK are classified by their sequence homology, structures and the ligands they bind.

LMTK1	-----MSSSFNPSFAFSSHFD	18
LMTK2	-----MPGPPALRRRLLLVLLIAGSAGAAPLPQ	31
LMTK3	MRQVLWLCNVCVTARETRHHLHPAILDKMPAPGAL-----ILAAVSASGCLASPAHP	54
	: . . . :	
	DGA--PLSELWPFSSLAVVAVSFSGLFAVIVMLAC-LCKKGGIGFKEFENAEGDEYAA	75
	TGAGEAPPAAEV-SSSFVILCVCS-LIILIVLIANCVSCCKDPEIDFKEFEDNFDDE--I	87
	DGF--ALGRAPLAPPYAVVLISCSGLLAFIFLLTC-LCKKRGDVGFKFENPEGEDCSG	111
	* : * : * : * : * : * : * : * : * : *	
	DLAQGS-PATAAQNGPDVYVPLTEVSLPMAKQPGRSVQLLKSTDVGRHSLLYLKEIGRG	134
	DETPPAEDTPSVQSPAIEVFTLSVPNISLPAPSQFQPSVEGLK-SQVARHSLNYIQEIGNG	146
	EYTPPAEETSSSQSLPDVYILPLAEVSLPMPAPQFSDMTTPLGLSRQHLSYLQEIISG	171
	: : : : * : * : * : * : * : * : * : * : * : *	
	WFGKVLGEVNSGSISSAQVVVKELQASASVQEQMFLEEVQPYRALKHSLNLLQCLAQCAE	194
	WFGKVLLEIYTGTSVARVIVVKELKASANPKEQDTFLKNGEPYIILQHPNLCVQCQVE	206
	WFGKVLGEIYFSDYTPAQVVVKELRASAGPLEQRKFISEAQFYRSLQHPNVLQCLGLCVE	231
	*****: * : : * : * : * : * : * : * : * : * : * : * : * : * : *	
	VTPYLLVMEFCPLGDLKGYLRSCRVAESMAP----DPRTLQRMACEVACGVHLHHRNNF	249
	ATPYLLVFEFCDLGDLKAYLRSEQEHMRGD----SQTMLLQRMACEVAAAGLAAMHKLHF	261
	TLPFLIMEFCQLGDLKRYLRAQRPEGLSPELPPRDLRTLQRMGLEIARGLAHLHSHNY	291
	. : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
	VHSDALRNCLLTADTLVKIGDYGLAHCKYREDYFVTADQLWVPLRWIAPELVDEVHSNL	309
	LHSDALRNCFLTSDLNVKVG DYGIGFSRYKEDIETDDKVFPLRWTAPELVTSQDRL	321
	VHSDALRNCLLTSDLTVRIGDYGLAHCKYREDYFVTADQLWVPLRWIAPELVDEVHSNL	351
	: *****: * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
	LVVDQTKSGNVWSLGVTIWELFELGTQYPYQHSQDQVLAITVREQQKLKPKQLQLTSLD	369
	LTADQTKYNSIWSLGVTLWELFDNAQPYNSLNDVLNQVIRERDTKLEKPKQLEQPYSD	381
	MVVDQSRSENIWSLGVTLWELFEFGAQPYRHLSDEEVLAFVVRQHVKLARPLKLPYAD	411
	: * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
	RWYEVMQFCWLQPEQRPTAEVHLLLSYLCAKGATEA-----EEEFERRWRSRPGG	421
	RWYEVQLQFCWLSPEKRPAAEDVHRLTYRLQSQRDS-----EVDFEQWNALKPNT	433
	YWYDILQSCWRPPAQRFPSASDLQLTLYLLSERPPRPPPPPPPPRDPGPPFPWPWPPAH--	468
	* : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
	GGVGPGPGAAGPMLGGVVELAAASSFPLLEQFAGDGFHADGDDVLTVTETSRGLNFEYKW	481
	N-----SRSSNNAAFILDFHARDLGREMEEVLTVTETSSQGLSFEYVW	478
	-----SAPRPGTLSSPFLDGFPG----ADPDDVLTVTETSSRGLNLECLW	510
	: : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
	EAGRGAEA-----F-PATLSPGRTARLQELCAPDGAPPVGP	517
	EAAKHDFHDFERSRGHLDEGLSYTSIFYPVEVFESSLSDPGPGKQDSDGQDVPLRPGVVP	538
	EKARRGAGRGGA-----PAWQPASAPPAPHANPSNPFYEALSTPSVLP	554
	* . : : * : * : * : * : * : * : * : * : * : * : * : * : *	
	VLSAHSPLSGSEYFIRLEEAAAPA-AGHD--PDCAGCAPSPATADQDDSDGSTAASLAM	574
	VFDAHNLVSGSDYYIQLEEKSGSNLELDYPPALLTTMDNPERT-----GPESQLTA	591
	VISARSPVSSEYYIRLEEHSPP-PEPLFPNDWDPLDGPVAPQAPQAPS---EVPQ---	607
	* : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
	EPLLG-HGPPVDVPWGRGDHYPRRSLARDPLCPSR---SPS-----PSAG-P	616
	LRVSELEESSTDEDFQSSSTDPKDSILPGLHVTSGPESPFNNIFNDVDKSEDLPSHQKI	651
	-----LVSETWAS-----PLFPAP---RPF-----PAQS---	628
	. . : : * : * : * : * : * : * : * : * : * : * : * : *	
	LSLAEGGAEDADWGVAAFCP--AFFEDPLGTSPLGSSGAPPLPLTGEDELEEVGARRA--	672
	FDMELNGVQADFKPATLSSS---LDNPKE-----SVITGHFEKE--KP-----	690
	--SASGFSLLSGWDPEGRGAGETLAGDP--AEVLGERGTAPVW--EEEEEEEEEGSSPGED	682
	. : : : * : * : * : * : * : * : * : * : * : * : * : *	
	-----AQRGHWSRNVSANNSG-----SRCPESWDPVSAGGH-----	704
	-----RKIFDSEPLCLSDNLMHQDNFDPLNVQELSENFLQEKNNLLKGLSSKEH	741
	SSSLGGGSPRRGFLPCPLCSREGACS---CLPLERGDVAVAGW---GHP-----	725
	: : : : * : * : * : * : * : * : * : * : * : * : * : *	
	-----AEGCPSFKQTPRA-----	717
	INDLQTELKNAGFTEAMLETSCRNSLDELQFAENKPLGSLQENVSTKGDGDDTDMVLTGD	801
	-----ALGCPHPEDDSSLRAER-----GSLADLPMAPP	754

LMTK1	-SPEPGYPGEPLLGLQAASAQ-----EPGCCPGLPHLCSAQ--GLA	755
LMTK2	-TLSTSLQSSPEVQVPPTSFEETEPRRVPPDSLPTQGETQPTCLD-----VI	848
LMTK3	ASAPPEF-LDPLMGAAAPQYPGRGPPAPPPPPPPRAPADPAASPDPSSAVASPGSGLS	813
	: . * : . : * :	
	-PAPCL-----VTPSWTETASSGGDHPQAEPKLATEAEGTTGPRLLPLSPVSPSQEGA	807
	VPEDCLHQDI----SP---D-----AVTV---FVEILS----TDA	874
	SPGPKPGDSGYETETPFSPGAFPGGG-----AAEEGVPRPRAP---PEPPDPGA	861
	* : * : . . *	
	PLPSEEASAPDAPDALPD--SP-TPATG--GEVSA-IKLASALNGSSSSPEVEAPSSSED	860
	RTHSLDNRSQDSPG-----ESEETLRLTESDSVLADDILASRVSVGSSLPGLQ-----	923
	PRPPFD-----PGPLPLPGPREKPTFV--VQVST-EQLLMSLRDVDTRNLLG----EK	907
	: * : * : :	
	EDTAEATSGIFTDTSS-DGLQARRPDVVPAFRSLQKQV-----G-----TPDS	902
	---ELHNKPFSEDHSHRRLEKNLEAVETLNQLNSKDAAKEAGLVGALSDDSTSQDLSL	979
	GATAR-ETGP---RK-AGRGPGNREKVPGLNRDPTVL-----G-----NGKQ	944
	. . : * : . *	
	LDSLDIPSSASDGGYEVFSPSA-----TGPSGGQPRALDSG-----	938
	EDSLSAPFPASEPSLETP--DSLE--SVDVHEALDSLGSHTP-----QKLVPPDKPAD	1029
	APSLSLPVN---GVTVLENGDQRAPGIEEKAENGALGSFEREEKVLENGELTPRREE	1000
	** . * . . *	
	--YDTENYESPEFVLKEAQ-EGCEPQAF--A-----ELASEGEGPGPETRLST	981
	SGYETENLESPEWTLHPAPEGTADEPATTDGDDHSGGLPPNPVIVISDAGDGHRTGETVP	1089
	KALENGELRSPPEAGEKVLVNGGLTPPKS--E-----DKVSENGG---LRFFR	1042
	: . : .*** : * :	
	SLSGLNEKNPYRDSAYFS-----DLEAEAEATSGPEKKCGGDRAPG	1022
	ETFTAGSQGSYRDSAYFSDNDSEPE-----KRSEEVPGTSPSALVLVQEQPLPE	1138
	INTERPPETGPPWRAPGFWEKTPESWGPAPTIGEPAPETSLERAPAPSAVVSSRNGGETAPG	1102
	. . : * : . :	
	PELGLPSTGQPSE-----QVCL-----RPGVSGEAQSGSGPEVLPP	1058
	PVLPE-----QSPAAQDSCLEARKSQPDESC-----LSA	1167
	PLGPAPKNGTLEPGTERRAPETGGAPRAPGAGRLDLGSGGRAPVGTGTAPGGGPGSGVDA	1162
	* : * :	
	LLQ-LEGSSPE-----PSTCPSGLVPEPPEPQGPAPKVRPGP-----	1093
	---LHNSSD-----LELRATPEPAQTGPVQVHPTEDASSPWSVLNALS	1210
	KAGWVDNTRPQPPPPPLPPPEAQPRRLEFAPPRAR--PEVAPEGEPGA-PDS-----RA	1214
	: . : * * . : * *	
	-----SPSCSQFFLLTPVPLRSEGNSSSEFQGPFG---LLSGPAPQKR	1132
	SGDDFETQDDRPCTLASTGTNTNELLAYTN-----SALDKSLSSH	1250
	GGDTALSGDGDPPKPERKGPMPRLFLDLGPP---QGNSEQIKARLSRLSLALPPLTLTP	1271
	. . . : :	
	MGGPGTPRAPRLRLALPGLP---AALEGRPEEEEDSESDS---ESD---EELRC-YSVQ	1181
	SEGPKLKEPDIEGKYLGLGV---SGMLD-LSEGDMDADEEDENSDDSDLDLRAFNLS	1305
	FFGPGPRRRPFWEGADAGAAGGEAGGAGAPGAEEDEGED---EDEEDEEAAPGAAA	1327
	** . . * * . * * . * * : . : * :	
	EPSEDSEEEAPAVPVVV--AESQSARNLRSLLKMP-SLLSETFCEDLERKKKAVSFFDDV	1238
	LSSESEDETEHPVPIILSNE---DGRHLRSLLKPTAANAPDPLPEDWKKEKKAVTFDDV	1362
	GPRGPGRARAAFPVVVSSADADAARPLRGLLKSP-RGADEPEDSELERKKMVSFHDV	1386
	** : : . * * . * * : : : : * * : * . *	
	TVYLFQDESPTRELGEPPFGAKESPTFLRGSPGSPSAPNRQQADGSPNGSTAEEGGGF	1298
	TVYLFQDETPTKELGPCGGEACGPDLSGPAPASGSPY---LSRCINSESTDEEGGGF	1417
	TVYLFQDETPTNELSVQAPPEGDTDPSTPP---APPTPPHPATPGDGFP-SNDSGFGGSF	1442
	***** : * . * . : * . * . . * * . *	
	AWDDDFPLMTAKAFAFAMAL-----DPAAPAPAAPTPTPAPFSRFTVSPAP	1343
	EWDDDFSPDPFMSKTTSNLLSSKPSLQTSKYFSPPPPARSTEQSWPHSAPYSRFSISPAN	1477
	EWADDFFLLPPPGP-----PLCFSRFSVSPAL	1469
	* : * : * * : * * :	
	TSRFSITHVSDSDAESKRGEAGAGGESKEA	1374
	IASFSLTHLTDSDIEQGGSSEDEKED----	1503
	ETPGPP-----ARAPDARPAAGPVEN-	1489

Figure 1.9 Multiple sequence alignment of human LMTK1, LMTK2 and LMTK3. The alignments were made using ClustalW software (www.ebi.ac.uk). Symbols “.” semi-conserved substitutions, “:” conserved substitutions, “*” residues are identical in all sequences in the alignment.

1.5.1 LMTK1

LMTK1 is also known as brain apoptosis-associated tyrosine kinase, CDK5-binding protein and p35-binding protein. Most leading to being recognised as AATYK. The AATYK is encoded by *AATK* gene, otherwise called *LMTK1*, *LMR1* and KIAA0641. The *AATK* gene is mapped on human chromosome 17q25.3 (Seki *et al.*, 1999, Tomomura and Furuichi, 2005). In 1991, Gaozza and colleagues observed a doublet (5.1 and 5.5 KB) of *AATK* gene mostly in brain, but also low levels in kidney, heart, lung and skeletal muscle. A shorter transcripts was also detected in the liver. Subsequently, a splicing variant of AATYK containing a transmembrane domain was reported. *AATKA* encodes a cytoplasmic protein with 1317 amino acids (LMTK1A), whereas *AATKB* comprises an additional 57 amino acids, the putative transmembrane domain at the N-terminus (LMTK1B) (Baker *et al.*, 2001, Tomomura *et al.*, 2007).

As mentioned above, AATYK is highly expressed in the nervous system, mainly in the brain (Gaozza *et al.*, 1997, Baker *et al.*, 2001, Tomomura *et al.*, 2001, Tomomura *et al.*, 2003). In the presence of active kinase activity, expression of AATYK is up-regulated when induced by low K^+ environment in cerebellar granule cells that undergoing apoptosis (Tomomura *et al.*, 2001). Also, overexpression of AATYK stimulates immature neurite extension and promotes low K^+ -induced apoptosis in cerebellar granule cells (Tomomura *et al.*, 2003). Phosphorylation state of AATYK in cellular granule neurons can switch in response to high K^+ -induced hypophosphorylation state, which is relied on the depolarization-induced Ca^{2+} signalling pathway, as well as the low K^+ -induced hyperphosphorylation state, which is partly assigned to its kinase

activity (Tomomura and Furuichi, 2005) but is not link with glycogen synthase kinase-3 pathway (Hughes *et al.*, 2010).

Honma *et al.* (2003) showed that LMTK1 activity is phosphorylated at Ser34 by cyclin-dependent kinase 5 (CDK5)/p35. The LMTK1A-Ser34 phosphorylation inhibits the tyrosine phosphorylation of LMTK1A by Src family kinases (Tsutsumi *et al.*, 2010) and regulates endocytic recycling (Takano *et al.*, 2010). Previously, they identified palmitoylation of LMTK1A at cysteines 4, 6 and 7 mediated its co-localisation with transferrin receptor-positive endosomes (Tsutsumi *et al.*, 2008). Subsequently, they reported that LMTK1 negatively regulate Rab11 activity by controlling the transport of endosomal vesicles in the growing axons and dendrites of primary neurons (Takano *et al.*, 2012, Takano *et al.*, 2014). This year, they showed LMTK1A affects microtubule organisation by changing the distribution of γ -tubulin. Moreover, the kinase activity of LMTK1A regulates vesicle transition from the microtubule to the actin at the tip of growing neurites (Sharma *et al.*, 2016).

Besides CDK5/p35, LMTK1 has been shown to interact with protein phosphatase 1, Ste20p-related proline alanine-rich kinase and Src family tyrosine kinases (Src and Fyn) (Gagnon *et al.*, 2007, Tsutsumi *et al.*, 2008).

AATYK is also involved in carcinogenesis. Overexpression of AATYK promotes neuronal differentiation of human neuroblastoma SH-SY5Y cells (Raghunath *et al.*, 2000). On the contrary, AATYK inhibits cell proliferation, anchorage-dependent growth and cell migration but promotes apoptosis of melanoma cells (Ma and Rubin,

2014). Meanwhile, *AATK* expression is found to be down-regulated in colon adenocarcinoma (Lee *et al.*, 2006). Further, *AATK* is frequently hypermethylated in human cancer cell lines as well as in primary lung and breast tumours (Haag *et al.*, 2014).

The first study to demonstrate a link between miRNAs and LMTK1 identified miR-338-3p, a miRNA generated from the seventh intron of *AATK*, regulates *AATK* expression during differentiation and degeneration of hippocampal neurons (Kos *et al.*, 2012). A separate study, miR-338-3p and *AATK* are found down-regulated in the islets and β -cells under insulin-resistant conditions. Moreover, depletion of *AATK* stimulates β -cell growth (Jacovetti *et al.*, 2015).

1.5.2 LMTK2

LMTK2 is the second member of the lemur kinase family, comprises 1503 amino acids with two predicted transmembrane domains at its N-terminus, followed by a conserved catalytic domain and a very long C-terminus tail domain (Kawa *et al.*, 2004, Kesavapany *et al.*, 2003, Wang and Brautigan, 2002). *LMTK2* or KIAA1079, which also known as *AATK2*, *KPI2*, *BREK*, *Cprk* and *LMR2*, is located at 7q21.3 of chromosome 7.

Nixon *et al.* (2013) used fluorescence protease protection technique to demonstrate that LMTK2 is an integral membrane protein where both the NH₂- and COOH-terminus are exposed to the cytoplasm. In addition, the active kinase domain was facing the cytoplasm. Intriguingly, according to ExPASy Bioinformatics Resource Portal, the theoretical molecular mass of LMTK2 is 165 kDa. However, routinely run SDS-PAGE gels in this group showed a single dominant band at a molecular mass more than 200 kDa. Presumably, LMTK2 may undergo post-translational modification processes or its COOH-terminus with proline-rich domains may yield low electrophoretic mobility that migrates slower on SDS-PAGE (Nixon *et al.*, 2013).

LMTK2 has a few alternative names. Wang and Brautigan (2002) discovered LMTK2 using phosphatase inhibitor-2 as bait in yeast two-hybrid prey from a mouse embryonic day 9.5 cDNA library. In their studies, they named the kinase as kinase/phosphatase/inhibitor-2 (KPI-2). Meanwhile, using the p35 subunit of CDK5 as bait in a yeast two-hybrid screen of a human brain cDNA library, KIAA1079 was identified as a positive clone. The kinase was named *Cprk* for cyclin-dependent kinase

5/p35-regulated kinase (Kesavapany *et al.*, 2003). Kawa and colleagues identified LMTK2 through a database search for novel kinases in the brain. They named it as the brain-enriched protein kinase (BREK) (Kawa *et al.*, 2004). These three groups have reported autophosphorylation of the kinase and showed only Ser and Thr kinase activity.

Although LMTK2 is also expressed prevalently in the brain (Kawa *et al.*, 2004, Kesavapany *et al.*, 2003, Wang and Brautigan, 2002), fewer studies related to neural function was conducted. One of the studies described LMTK2 or BREK as a negative regulator of nerve growth factor (NGF)-induced neuronal differentiation. LMTK2 was shown phosphorylated promptly upon NGF stimulation, and with a kinase-defective mutant, it augmented both extracellular signal-regulated kinase (ERK) phosphorylation and neurite outgrowth in response to NGF (Kawa *et al.*, 2004). Similar to LMTK1, LMTK2 has been reported to interact with CDK5/p35 and PP1C. CDK5/p35 phosphorylates LMTK2 at Ser1418 and the mutation at this site did not affect PP1C binding with LMTK2 but effects LMTK2 phosphorylation on Thr320 PP1C (Manser *et al.*, 2012). Previously, LMTK2 or KPI-2 was reported to bind to PP1C through a valine-threonine-phenylalanine motif and phosphorylated PP1C at the Thr320 site, which induces an inhibitory on PP1C activity (Wang and Brautigan, 2002, Manser *et al.*, 2012). Thus, Rattray (2012) summarised that LMTK2 regulation via these interaction mediates neuronal survival.

In another article published, the authors further demonstrate the phosphorylation of PP1C by LMTK2, inhibits the phosphorylation of glycogen synthase kinase-3 β (GSK3 β) on Ser9, results in decrease of kinesin-1 light chain-2 (KLC2) phosphorylation

and stimulates binding of Smad2 cargo to KLC2 in response to transforming growth factor beta (TGF β) (Manser *et al.*, 2012).

Other binding partners that have been identified for LMTK2 is myosin VI (Chibalina *et al.*, 2007, Inoue *et al.*, 2008). The authors showed that LMTK2 associates to the WWY site on the C-terminus myosin VI tail and both require for transport of transferrin from early endosomes (Chibalina *et al.*, 2007). Butler and Bradbury (2015) have identified the di-acidic endoplasmic reticulum export motif is the crucial element in the export of LMTK2 from the endoplasmic reticulum and mutation at this motif modifies transferrin recycling.

In another study shows that LMTK2 is myosin VI binding partner in LNCaP, prostate cancer cells and is linked to prostate cancer progression through the secretory pathway via the recycling endosome (Puri *et al.*, 2010). LMTK2 was identified among multiple common alleles associated with prostate cancer risk by conducting genome-wide association in a large multiethnic case-control study (Waters *et al.*, 2009). Moreover, LMTK2 has recently been recognised as a major susceptibility gene for prostate cancer (Eeles *et al.*, 2008, Harries *et al.*, 2010, Shui *et al.*, 2014). In order to understand the LMTK2 role in prostate cancer, Shah and Bradbury (2015) demonstrated that LMTK2 is negatively regulate the expression of androgen receptor transcription.

In a peptide microarray analysis, cystic fibrosis transmembrane conductance regulator (CFTR) and glycogen phosphorylase (phos-b) were selected to be substrate of LMTK2/KPI-2 (Wang and Brautigan, 2006). LMTK2 binds to CFTR and phosphorylates

CFTR at Ser737, lead to endocytosis and reduces the cell surface density of CFTR chloride ion channels in human airway epithelial cells (Luz *et al.*, 2014).

In the quest to study LMTK2 function, Kawa *et al.* (2006) generated *LMTK2* knockout mice and they learned that LMTK2 knockout mice are viable but infertile due to defects in spermatogenesis. It is a unique role as a kinase that may be involved in molecular mechanism in spermatogenesis.

1.5.3 LMTK3

LMTK3, is also known as *LMR*, *TYKLM3* or *PPP1R101*, maps to chromosome 19 (19q13.33) and is composed of 17 exons. This human gene encodes 1489 amino acids. It is also recommended as serine/threonine-protein kinase LMTK3 as it found to be able to phosphorylate Ser and/or Thr residues. Similar to other LMTKs, LMTK3 is also comprising of an N-terminus transmembrane domain, a conserved catalytic domain and a long proline-rich C-terminus domain. Perhaps it due to the proline-rich domain similar to LMTK2, another band higher than 170 kDa is regularly seen in SDS-PAGE gel. Mass spectrometry analysis was conducted and resulted that both bands (170 kDa and 240 kDa) observed in the gels were full-length LMTK3, suggesting another possibility that LMTK3 may undergo post-translational modifications (Xu *et al.*, 2014a).

Likewise other LMTKs, LMTK3 is also highly expressed in cerebral cortex, cerebellum and hippocampus (Tomomura *et al.*, 2007). In contrast, there are not much of studies on the LMTK3 role in the nervous system. Till now, only one published article describes LMTK3 physiological function by using LMTK3 knockout mice. Inoue *et al.* (2014) generated LMTK3 knockout mice and found that these mice were viable plus appeared to be completely normal. However, these mice showed abnormal phenotypes, including hyperlocomotion, decreased depression-like and anxiety-like behaviours with a high level of dopamine metabolism. In the same study, they also found evidence that LMTK3 involved in neuronal endocytic trafficking of *N*-methyl-D-aspartate receptors and contemplated that LMTK3 may be an effector of CDK5.

Tyner *et al.* (2009) were one of the first studies to demonstrate the presence of LMTK3 in cancer. Using RNA interference-assisted protein target identification technology to screen primary cells from patient samples with hematologic malignancies, they identified inhibition of LMTK3 in disease specific-sensitivity. Following in the same year, LMTK3 emerged in a high-throughput RNA interference screen as a new kinase target involved in Wnt pathway, which depletion of LMTK3 reduces beta-catenin dependent transcription (Naik *et al.*, 2009). In a MassARRAY system study, high mRNA level of *LMTK3* was detectable in the HER2-positive/ER-positive tumours but not in HER2-positive/ER-negative tumours (Tanioka *et al.*, 2014).

A greater understanding of LMTK3 function was described by Giamas *et al.* (2011). They established a novel role of LMTK3 as an ER α regulator in breast cancer. The authors performed small interfering RNA screening that targets 691 human whole-genome kinases by using trefoil factor 1 (TFF1), a well-known ER α -regulated gene, as a readout. Interestingly, by all of the genes that found regulate ER α and other LMTKs, only LMTK3 is evolved under positive Darwinian selection between chimpanzees and humans. Only LMTK3 isoform knockdown, but not LMTK1 or LMTK2, down-regulated TFF1 expression and inhibited MCF7 oestradiol-dependent cell growth. Depletion of LMTK3 also down-regulated oestrogen-regulated genes such as growth regulation by oestrogen in breast cancer 1 (GREB1) and progesterone receptor (PGR). This report demonstrated LMTK3 regulates ER α by phosphorylating and protecting ER α from proteasomal degradation. Moreover, LMTK3 affects ER α mRNA transcription by recruiting FOXO3 transcription factor and ER α protein through PKC signalling (Figure 1.5).

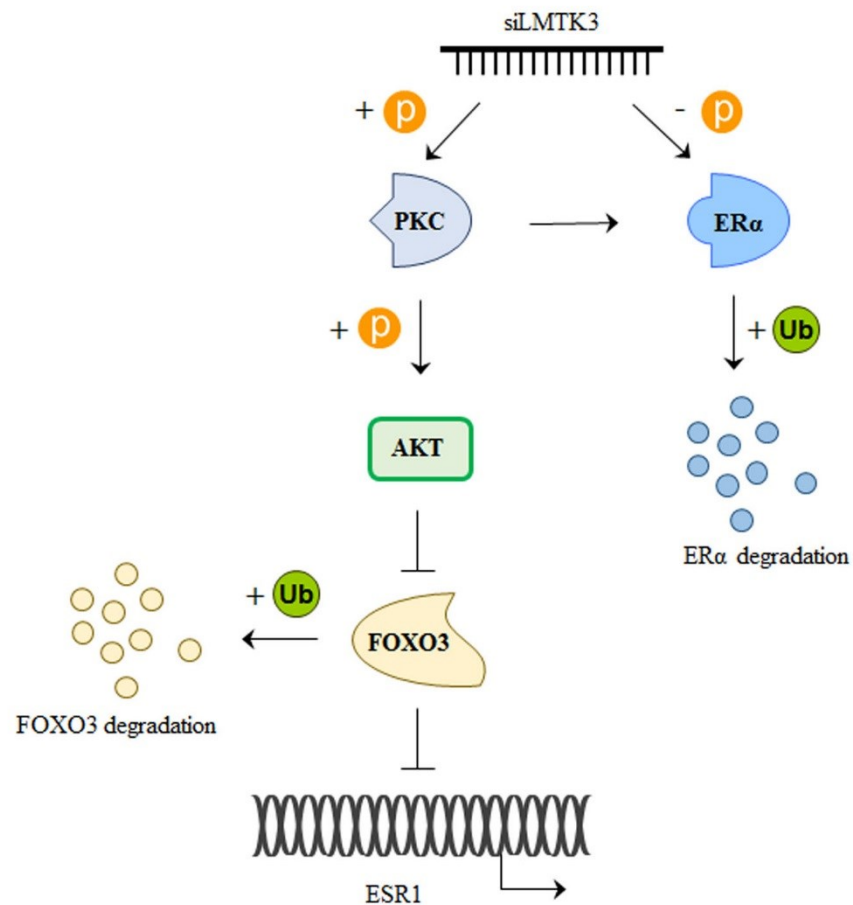


Figure 1.10 An illustration of ERα regulation by LMTK3.

Depletion of LMTK3 using siRNA leads to PKC activation which results in degradation of FOXO3 via AKT phosphorylation, therefore less FOXO3 binds to *ESR1* promoter and down-regulation of ERα protein level.

Another valuable information of this report (Giamas *et al.*, 2011) highlighted the above observation was correlated with LMTK3 abundance in 613 breast cancer patient samples. Elevated LMTK3 expression was seen more in nuclear compartment, and it increased expression or polymorphism was highly correlated with shorter disease-free survival time and overall survival time as well as a predictor of hormone-therapy resistant. They further proved that depletion of LMTK3 in tamoxifen (TAM)-resistant cell lines such as BT-474, MLET5 and LCC9 was able to re-sensitised cells to TAM treatment.

Giamas and colleagues further convince their clinical findings by comparing the European/British cohort study with the published cohort of Asian patients (Chuah *et al.*, 2011). Despite a relatively smaller sample size, the Asian cohort also showed high baseline LMTK3 expression was significantly associated with a decreased overall survivor. In addition, its expression was also mainly in nuclear. Nuclear LMTK3 expression directly correlated with Ki67, the tumour proliferation marker and the Nottingham Prognostic Index. Correspondingly, elevated LMTK3 expression was also seen in cytoplasmic with high tumour grade. This expression associated with CK5/6 expression in basal-like tumours but negatively correlated with ER α , PGR and CK18, luminal marker (Stebbing *et al.*, 2012). In a study of mRNA expression from 219 breast carcinoma samples, Asano *et al.* (2014) showed both disease-free survival time and overall survival time were significantly shorter in ER α -positive patients as well as patients receiving adjuvant endocrine therapy with high *LMTK3* mRNA expression than in those patients with low *LMTK3* mRNA expression. However, no association between *LMTK3* mRNA level with ER α -negative patients. Notably, high level of *LMTK3* mRNA was an

independent factor predictive of a poor prognosis in ER α -positive breast cancer patients (Asano *et al.*, 2014).

Although these cohorts unsuccessful to address LMTK3 association with ER α , the majority of human breast tumours express ER α . Therefore, more research and treatments focus on suppressing ER α stability, activity and/or biosynthesis. Despite the endocrine therapy has dramatically improved the treatment of breast cancer, many patients develop resistance to this treatment and relapse. In quest of LMTK3 role in endocrine resistance, Stebbing *et al.* (2013) generated TAM-resistant xenograft breast cancer mouse model with BT474 cell lines, TAM-resistant breast cancer cells which express high endogenous *LMTK3* mRNA and protein levels compared with MCF7. As predicted, depletion of LMTK3 did not change the tumour volume as compared with controls. Intriguingly, a significant reduction of tumour growth was seen when cells were treated with siRNA against LMTK3 and TAM, suggesting that LMTK3 could re-sensitise cancer cells to TAM treatment. Subsequently, microarray-based genome-wide gene expression analysis using BT474 cells was carried out to identify target genes affected by LMTK3 silencing under different treatments (oestradiol and TAM). Among those genes affected upon depletion of LMTK3, *c-Myc*, *HSPB8* and *SIAH2* were particularly known to be implicated in TAM resistance. Authors found that LMTK3 can protect breast cancer cells from TAM-induced growth inhibition by blocking autophagy through the up-regulation of *HSPB8* mRNA and protein levels.

A mass spectrometry analysis ratified that two specific bands detected on the Western blot were full-length LMTK3 proteins and further investigation showed that the

upper band (about 240 kDa) is particularly localised in the cytoplasm, meanwhile the lower band (about 170 kDa) is identified in the cytoplasm and the nucleus (Xu *et al.*, 2014a, Xu *et al.*, 2015). Xu *et al.* (2014a) found that overexpression of LMTK3 in breast cancer cell lines stimulated focal adhesion formation and actin cytoskeleton remodelling, and suggested that the abundance of the upper band of LMTK3 contributed to the cell motility. Mechanistically, LMTK3 affects integrin $\alpha 5$ and $\beta 1$ expression through direct interaction with GRB2, which then activates both RAS and CDC42. RAS activation turns on the ERK/MAPK pathway, which stimulates cell proliferation. Meanwhile, CDC42 activation triggers SRF and consequently stimulates the transcription of *ITGB1* and *ITGA5* (Figure 1.6). In addition, elevated LMTK3 expression significantly correlated with integrin $\beta 1$ subunit expression in breast cancer patient samples (Xu *et al.*, 2014a).

Interestingly, LMTK3 in nuclear has been proposed to regulate cancer progression through chromatin remodelling. Xu *et al.* (2015) used chromatin immunoprecipitation-sequencing (ChIP-seq) to map LMTK3 across the human genome. These were then validated with quantitative real time-polymerase chain reaction (qRT-PCR) and a rapid immunoprecipitation mass spectrometry of endogenous proteins to demonstrate that KAP1 was the LMTK3 binding partner in chromatin. Intriguingly, phosphatase PP1-alpha catalytic subunit (PP1 α), known as KAP1 phosphatase was found to dephosphorylate KAP1, resulting in chromatin condensation and transcriptional repression at the distal region by tethering chromatin to the nuclear periphery (Xu *et al.*, 2015). Furthermore, a positive correlation of the expression of LMTK3, PP1 α and

PPP1CA in The Cancer Genome Atlas (TCGA) breast cancer dataset strengthened the above findings (Xu *et al.*, 2015).

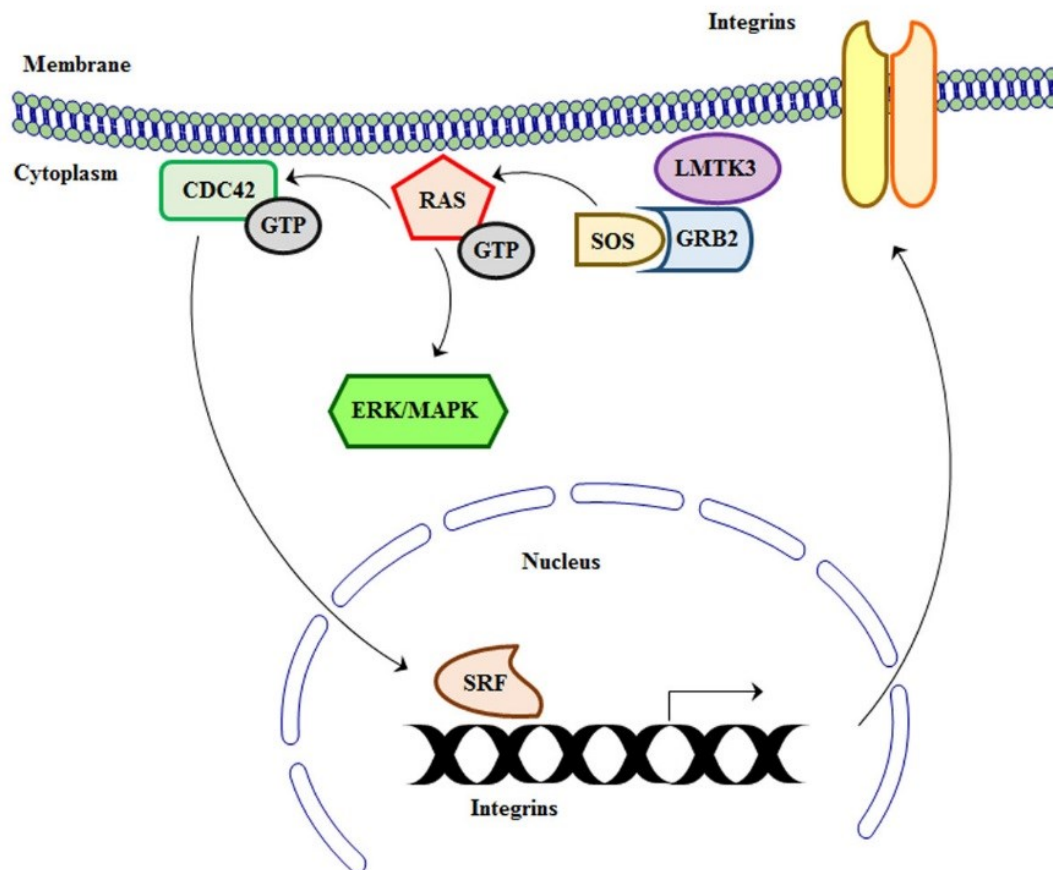


Figure 1.11 Model of the LMTK3 promotes invasion through integrin signalling network during breast cancer progression and metastasis.

LMTK3 promotes GRB2, adaptor protein binding. GRB2 association with the SOS activate RAS and ERK/MAPK kinase is one downstream target of RAS-mediated signalling events. In addition, SRF-linked signalling by CDC42 activation lead to integrin-stimulated signalling pathway. Figure adapted from Xu *et al.* (2014a).

Recent studies conducted using an *in silico* analysis showed that LMTK3 as a target of microRNA (miRNA)-34a and this was further validated by using dual luciferase reporter assay (Zhao *et al.*, 2013). Zhao *et al.* (2013) demonstrated overexpression of miR-34a inhibited MCF7 cell growth, reduced S phase ratio, suppressed tumour formation *in vivo* and abrogated the increase of LMTK3 protein and mRNA levels resulted from oestradiol (E2). This result is further proven by an article reported on March 2016. Jacob *et al.* (2016) described that LMTK3 not only induced miR-34a but also miR-196-a2 and miR-182 via interacting with DEAD-box RNA helicase p68 (DDX5), which segregate these pri-miRNAs from being process. Furthermore, overexpression of miR-34a and miR182 in highly LMTK3-expressed MCF7 and MDA-MB-231 cells hindered the proliferation, invasion and migration. Interestingly, in this study showed that the binding of miR-34a and miR-182 to 3'UTR of *LMTK3* mRNA caused a down-regulation in both protein and mRNA levels.

In breast cancer, Giamas *et al.* (2011) addressed that the *LMTK3* polymorphisms, single nucleotide polymorphism (SNP) rs9989661 (T>C) T/T genotype and rs8108419 (G>A) G/G or A/G genotype were associated with cancer outcome in European/British breast cancer patients. However, Asano *et al.* (2014) found no correlation between the genotypes of these polymorphisms and patient outcomes in 471 breast cancer tissues from the Japanese population. Similarly, Wakatsuki *et al.* (2013) presented these polymorphism were not associated with overall survival in 169 Japanese patients and 137 the United States (U.S.) patients with localised gastric adenocarcinoma. Nevertheless, multivariate analysis demonstrated that *LMTK3* rs9989661 was correlated with disease-

free survival and overall survival in the Japanese male and time to recurrence in the U.S. female. Meanwhile, *LMTK3* rs818419 was associated with overall survival in the Japanese female and the U.S. male. In addition, more than 65% of 17 sample of gastric cancer tissues were stained positive in cytoplasmic for LMTK3 protein expression (Wakatsuki *et al.*, 2013). In contrast, Li *et al.* (2014) identified LMTK3 protein was mostly expressed in the nucleus of 83 patients with postoperative gastric cancer and its expression was significantly associated with the depth of invasion and disease stages. Collectively, both cohorts agree that LMTK3 polymorphism or expression was a negative prognostic factor in patients with gastric cancer.

Concurrently, Shi *et al.* (2013) demonstrated LMTK3 protein can be found in 60 patients' blood with primary colorectal cancer (CRC) and significantly high expression compared to 53 healthy subjects. In addition, high LMTK3 level in serum was associated with histological subtypes, depth of tumour invasion and tumour-node-metastasis (TNM) classification. Correspondingly, high level of LMTK3 protein expression was identified in 62 out of 69 archival paraffin-embedded colorectal tumour tissue specimens, and it was associated with higher TNM as well as lymph node metastasis (Shi *et al.*, 2014). Both studies showed that high expression of LMTK3 either in serums or archive tissues projected a poorer overall survival rate compared with a low level of LMTK3 and addressed that LMTK3 expression was an independent prognostic factor for CRC patients.

LMTK3 protein can also be found in patients' blood with non-small cell lung cancer (NSCLC). Two clinical studies provide evidence that LMTK3 was an independent prognostic factor in the NSCLC patients. First study using 524 preoperative patients'

blood with non-small cell lung cancer (NSCLC), had higher circulating LMTK3 compared with 280 patients' blood with benign lung diseases and 100 peripheral blood of healthy donors and correlated with poor survival (Xu *et al.*, 2014b). Following, authors determined that at the cutoff value 6.85 ng/ml, LMTK3 in serum was a reliable diagnostic and differential markers for evaluating NSCLC and benign lung disease. Although the subsequent study only involved 67 patients with primary NSCLC, 28 patients with lung benign lesion and 53 healthy donors, the outcome of this study was similar as previous, which mean concentration of LMTK3 in serum was significantly higher in NSCLC group compared with lung benign group or with healthy donors group (Zhang *et al.*, 2015). Additionally, the elevated level of LMTK3 in serum was associated with age, lymph node metastasis and TNM classification. Besides measuring LMTK3 level in serum, Zhang *et al.* (2015) also assessed LMTK3 expression in tissue samples and found high expression in lung squamous cell carcinoma and adenocarcinoma.

Overall, many clinical studies have been done in establishing the LMTK3 role in different types of cancer. Although these data are promising, more mechanistic studies are required to address LMTK3 role in each type of cancer.

1.6 Aim of this study

Previous studies have identified LMTK3 is an ER α regulator implicated in endocrine resistance in breast cancer (Giamas *et al.*, 2011, Stebbing *et al.*, 2013). Recent data have established the cytoplasmic and nuclear LMTK3 roles in promoting breast tumour invasion and metastasis, as well as tumour progression through chromatin remodelling (Xu *et al.*, 2014a, Xu *et al.*, 2015). Consistently, LMTK3 is overexpressed in breast tumour, particularly in advanced stage (Stebbing *et al.*, 2012). Although overexpression of LMTK3 has been shown to stimulate breast cancer cells growth, little is known about how LMTK3 protects cancer cells from cytotoxicity induced by chemotherapeutic drugs.

The main project aim is to discover whether LMTK3 involves in DNA damage that generated from doxorubicin. Doxorubicin is widely used chemotherapeutic drug that induce DSBs as a result of topoisomerase II inhibition. Further work has been carried out to determine the potential mechanism of LMTK3 that contribute to the regulation of DNA damage repair. The working hypothesis of this project is that in response to DNA damage, overexpression of LMTK3 will mediate doxorubicin resistance by accelerating the DNA repair through one of the DSB repair pathways. Together, these aims to define the role of LMTK3 in regulating doxorubicin-induced breast cancer cell growth and determine the mechanisms of LMTK3 action.

Chapter 2 **EXPERIMENTAL**

2.1 **Materials**

2.1.1 **Mammalian cell lines**

All cell lines were purchased from the American Tissue Type Culture Collection (ATCC) and the European Collection of Cell Cultures (ECACC).

Table 2.1 Cell lines used in this study

CELL LINE	TUMOUR TYPE	SOURCE	ER STATUS	PR STATUS	ERBB2/HER2 STATUS
MCF7	Metastatic adenocarcinoma	Pleural effusion	+	+	-
T47D	Invasive ductal carcinoma	Pleural effusion	+	+	-

2.1.2 **Small interference oligonucleotide sequences**

Table 2.2 List of small interference (siRNA) oligonucleotide sequences

Gene	Sense	Antisense	Target Sequence
ATR	CCUCCGUGAUGU UGCUUGAtt	UCAAGCAACAUC ACGGAGGtt	
ATM	GGCUAUUCAGUG UGCGAGATT	UCUCGCAACUGA AUAGCCTT	AAGGCTATTCAGT GTGCGAGA

LMTK3	Abnova	H00114783-R02
LMTK3	CGGACUACUGGU AUGACAuTdT	AUGUCAUACCAG UAGUCCGdTdT
	CCUUCAUGGUGG UGGACCAdTdT	UGGUCCACCACC AUGAAGGdTdT
	GAGAAAGUCCCG GGCCUGAdTdT	UCAGGCCCGGGA CUUUCUCdTdT
	GAGAUUGGGAGU GGCUGGUdTdT	ACCAGCCACUCC CAAUCUCdTdT
	GGACCUUCAUGG UGGUGGAdTdT	UCCACCACCAUG AAGGUCCdTdT

2.1.3 Plasmid vectors

Table 2.3 List of plasmid vectors

Vector	Insert	Source
pCM6/MYC/FLAG	Empty vector	Origene (PS100001)
pCM6/MYC/FLAG	Human LMTK3 (full length)	Origene (RC223140)
pCM6/MYC/FLAG	Human RAD51	Origene (RC218333)
pgkPURO	DR-GRP reporter	Addgene (#26475)
pCAGGS	pCBASceI	Addgene (#26477)
pim-GFP	EJ2-GFP	Jeremy Stark
pim-GFP	EJ5-GFP	Jeremy Stark

Table 2.4 Transfection media and reagents

Transfection media	Additives	Transfection reagent
Normal growth medium	No	HiPerFect Transfection Reagent (Qiagen, West Sussex, UK)
Opti-MEM [®] I Reduced Serum Medium (Gibco [®])	No	FuGENE [®] HD Transfection Reagent (Promega, Madison, USA) or Lipofectamine [®] 2000 Reagent (Invitrogen, Life Technologies Ltd, Paisley, UK)

2.1.4 Primers

Table 2.5 List of TaqMan quantitative real-time PCR assays

Gene	Assay Identification Number
GAPDH	Hs02758991_g1
LMTK3	Hs01090718_ml

Table 2.6 List of SYBR Green quantitative real-time PCR assays

Primer	Primer Assays
GAPDH	Hs_GAPDH_1_SG
RAD51	Hs_RAD51_1_SG

2.1.5 Buffers and solutions

Table 2.7 List of solutions for Western blotting

Solution	Recipe
Tris-HCl (pH 6.8)	1.5 M Tris-HCl (pH 6.8)
Tris-HCl (pH 8.8)	1.5 M Tris-HCl (pH 8.8)
SDS-PAGE Running buffer	25 mM Tris-Base, 192 mM glycine, 0.1% SDS
Semi-dry transfer buffer	48 mM Tris-base, 39 mM glycine, 20% methanol
PBST buffer	PBS, 0.1% Tween-20
Blocking buffer	5% dried skimmed milk powder (Marvel) in PBST 5% BSA in PBST
RIPA	50 mM Tris pH 8.0, 150 mM NaCl, 1% IGEPAL®CA-630, 0.5% sodium deoxycholate, 0.1% SDS
2X Laemmli buffer	120 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 0.01% (w/v) bromophenol blue
5X Loading buffer	312.5 mM Tris-HCl pH 6.8, 15% (w/v) SDS, 50% (w/v) glycerol, 16% (v/v) beta-mercaptoethanol, 0.01% (w/v) bromophenol blue

Table 2.8 List of SDS-PAGE Acrylamide gel solutions

Gel solution	Recipe
6% resolving gel	5.3 ml ddH ₂ O, 2.0 ml 30% acrylamide-bisacrylamide mix, 2.5 ml Tris-HCl (pH 8.8), 100 µl 10% SDS, 100 µl 10% APS, 8 µl TEMED
8% resolving gel	4.6 ml ddH ₂ O, 2.7 ml 30% acrylamide-bisacrylamide mix, 2.5 ml Tris-HCl (pH 8.8), 100 µl 10% SDS, 100 µl 10% APS, 6 µl TEMED
10% resolving gel	4.0 ml ddH ₂ O, 3.3 ml 30% acrylamide-bisacrylamide mix, 2.5 ml Tris-HCl (pH 8.8), 100 µl 10% SDS, 100 µl 10% APS, 4 µl TEMED
12% resolving gel	3.3 ml ddH ₂ O, 4.0 ml 30% acrylamide-bisacrylamide mix, 2.5 ml Tris-HCl (pH 8.8), 100 µl 10% SDS, 100 µl 10% APS, 4 µl TEMED
15% resolving gel	2.3 ml ddH ₂ O, 5.0 ml 30% acrylamide-bisacrylamide mix, 2.5 ml Tris-HCl (pH 8.8), 100 µl 10% SDS, 100 µl 10% APS, 4 µl TEMED
5% Stacking gel	1.4 ml ddH ₂ O, 33 µl 30% acrylamide-bisacrylamide mix, 25 µl Tris-HCl (pH 6.8), 20 µl 10% SDS, 20 µl 10% APS, 2 µl TEMED

*For two gels preparation

2.1.6 Antibodies and recombinant proteins

Table 2.9 List of antibodies for Western blotting

Primary antibodies	Catalog number	Dilution	Company
LMTK3 (I-17)	sc-100418 (M)	1:200 (WB) 1:50 (IF)	Santa Cruz Biotechnology

LMTK3 (2H6)	H00114783-M02	1 ug (IP)	Abnova
Beta actin (AC-15)	ab6276 (M)	1:100 000	ABCAM Biotech
ATM (2C1)	sc-23921 (M)	1:1000	Santa Cruz Biotechnology
ATM (2C1)	ab78 (M)	1:1000 (IP)	ABCAM Biotech
phospho-ATM (Ser1891)	CS4526 (M)	1:500	Cell Signaling Technology
ATR	CS27905 (R)	1:500	Cell Signaling Technology
phospho-ATR (Ser428)	CS2853 (R)	1:500	Cell Signaling Technology
RAD51 (D4B10)	CS8875 (R)	1:1000	Cell Signaling Technology
RAD51 (H-92)	sc-8349 (R)	1:400 (IF)	Santa Cruz Biotechnology
phospho-RAD51 (Thr309)	ab31769 (R)	1:1000	ABCAM Biotech
RAD52	CS3425 (R)	1:1000	Cell Signaling Technology
KU70 (D10A7)	CS4588 (R)	1:1000	Cell Signaling Technology
KU80 (C48E7)	CS2180 (R)	1:1000	Cell Signaling Technology
XLIF	CS2854 (R)	1:1000	Cell Signaling Technology
FEN-1	CS2746 (R)	1:1000	Cell Signaling Technology
H2A.X (D17A3)	CS7631 (R)	1:500	Cell Signaling Technology

phospho-histone H2A.X Ser139	CS2577 (R)	1:200 (WB)	Cell Signaling Technology
phospho-histone H2A.X Ser139	# 05-636 (M)	1:1000 (WB)	EMD Millipore Corporation
phospho-histone H2A.X Ser139	CS9718 (R)	1:400 (IF)	Cell Signaling Technology
p53 (DO-1)	sc-126 (M)	1:1000	Santa Cruz Biotechnology
phospho-p53 (Ser15)	CS9284 (R)	1:500	Cell Signaling Technology
phospho-p53 (Ser392)	CS9281 (R)	1:500	Cell Signaling Technology
Myc	# 05-724 (M)	1:1000	EMD Millipore Corporation
Flag-M2	F3165 (M)	1:1000	Sigma
GST	ab19256 (R)	1:1000	ABCAM Biotech
HDAC-1	sc-81598 (M)	1:1000	Santa Cruz Biotechnology
Secondary antibodies			
HRP-Goat anti-mouse IgG	P0447	1/3000	Dako, UK
HRP-Goat anti-rabbit IgG	P0448	1/3000	Dako, UK
Alexa Fluor® 555 goat anti-mouse IgG	A-21422	1/500	Invitrogen Life Technologies Ltd, Paisley, UK
Alexa Fluor® 488 goat anti-mouse IgG	A-11029	1/500	Invitrogen Life Technologies Ltd, Paisley, UK

Alexa Fluor® 555 goat anti-rabbit IgG	A-21429	1/500	Invitrogen Life Technologies Ltd, Paisley, UK
Alexa Fluor® 488 goat anti-rabbit IgG	A-11034	1/500	Invitrogen Life Technologies Ltd, Paisley, UK

Table 2.10 List of recombinant proteins

Recombinant protein	Catalog number	Company
RAD51	Ab63808	ABCAM Biotech
LMTK3 kinase domain	-	GenScript

2.1.7 Chemicals and consumables

(A) General

15 ml and 50 ml centrifuge tubes (Corning Incorporated, New York, USA)
 Microcentrifuge tubes (0.5 ml, 1.5 ml, 2 ml) (STARLAB Ltd, Milton Keynes, UK)
 Laboratory bottles (50 ml, 100 ml, 250 ml, 1 litre, 2 litres)
 Serological pipette (1 ml, 5 ml, 10 ml) (Corning Incorporated, New York, USA)
 Sterile blue and yellow pipette tips (STARLAB Ltd, Milton Keynes, UK)
 Volumetric flasks
 PBS tables, pH 7.3 (Oxoid, UK)
 beta-mercaptoethanol (Sigma-Aldrich, St. Louis, USA)

(B) Cell culture

Aspiration Pipette (Sarstedt, Nümbrecht, Germany)
 Cryovials (Corning Incorporated, New York, USA)
 Culture flask (25 cm³, 75 cm³, 150 cm³) (Corning Incorporated, New York, USA)
 Cell culture microplate (Corning Incorporated, New York, USA)
 Cell scrapers (Corning Incorporated, New York, USA)
 Culture dishes (60 mm, 100 mm, 150 mm) (Corning Incorporated, New York, USA)

Dimethyl sulfoxide (Applichem, Denmark)
Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, USA)
EDTA (Sigma-Aldrich, Poole, UK)
Fetal bovine serum (First Link, Birmingham, UK)
Fetal bovine serum, charcoal-treated (First Link, Birmingham, UK)
L-glutamine-Penicillin-Streptomycin solution (Sigma-Aldrich, St. Louis, USA)
L-glutamine (Sigma-Aldrich, Poole, UK)
RPMI-1640 (Sigma-Aldrich, Poole, UK)
Sterile syringe filter (0.22 µm) (Sartorius Stedim Biotech GmbH, Goettingen, Germany)
Sterile syringe (1 ml, 5 ml, 10 ml, 25 ml) (Corning Incorporated, New York, USA)
10X trypsin solution (Sigma-Aldrich, St. Louis, USA)

(C) Bacterial medium and plates

L-Broth and L-Broth agar capsules (MP Biomedical, LLS, France)
Ampicillin sodium salt (Sigma-Aldrich, Gillingham, UK)
S.O.C. medium (Invitrogen, Paisley, UK)
Petri dishes (Thermo Scientific, UK)
Kanamycin sulfate (Sigma-Aldrich, Gillingham, UK)

(D) Real-time PCR reagents

TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Austin, USA)
Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK)
High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK)
Microamp™ fast optical 96-well reaction plate (Applied Biosystems, Paisley, UK)
RNaseZap (Ambion, Applied Biosystems, Foster City, CA, USA)

(E) Western blotting

RIPA buffer (Sigma Aldrich, Gillingham, UK)
Phosphatase Inhibitor Cocktail Tablets (Roche Applied Science, Penzberg, Germany)
Protease Inhibitor Cocktail Tablets (Roche Applied Science, Penzberg, Germany)
ECL™ Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK)
Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK)
Immobilon™ Western (Millipore Corporation, Billerica, USA)
Thermo Scientific Spectra Multicolor Broad Range Protein Ladder #26634
ImmunoCruz™ IP/WB Optima System (Santa Cruz Biotechnology, USA)

2.1.8 Equipment / Instruments

4 °C refrigerator (Thermo Scientific, USA)
-20 °C freezer (Liebherr, Germany)
-80 °C Ultra-Low Temperature Freezer, New Brunswick (Eppendorf, Germany)
7900HT fast Real-time PCR (Applied Biosystems, Paisley, USA)
AccuBlock Digital Dry Bath (Labnet, USA)
Centrifuge (Hettich, Germany)
SorvallTM RC 6 Plus Centrifuge (Thermo Scientific, USA)
CO₂ incubator (Thermo Scientific, USA)
Ducted laminar flow hood (RS Biotech, Scotland)
Electronic balance (Ohaus, UK)
FACSCanto analyzer with FACSDiva software (Becton Dickinson, UK)
GelDoc System (Biorad, UK)
Haemocytometer (Weber, England)
Konica SRX-101A film processor (Minolta, Japan)
Laboratory water purifier, Milli-Q (Merck Millipore, UK)
Liquid nitrogen tank, CryoMed (Thermo Scientific, USA)
Magnetic stirrer (B.Braun Biotech, USA)
Microcentrifuge system (Thermo Scientific, USA)
Microplate reader with turnable filter (400 – 700 nm), (Tecan Sunrise, Switzerland)
Multi-channel pipettor (10 – 200 µl) (Fisher Scientific, UK)
NanoDrop 1000 spectrophotometer (Thermo Scientific, USA)
Olympus CK2 inverted microscope (Olympus, Japan)
pH meter (Jenway, UK)
Pipettor (1 – 10 µl, 10 – 100 µl, 200 – 1000 µl) (Fisher Scientific, UK)
Vortex mixture (Labnet, USA)
Water bath (Thermo Scientific, USA)
Leica TCS SP5 II STED laser scanning confocal microscope (Leica Microsystems, USA)
See-saw Rocker, SSL4 (Stuart, UK)

2.2 Methods

2.2.1 Culture of breast cancer cell lines

2.2.1.a Culture media

Cells were thawed, expanded in tissue culture flasks and maintained in DMEM medium supplemented with 2 mM L-glutamine, penicillin (100 Units/ml), streptomycin (100 µg/ml) and 10% heat inactivated fetal bovine serum (FBS). This medium preparation will be known as “complete culture medium”. The cells were grown at 37 °C in humidified atmosphere of 95% air/ 5% CO₂.

2.2.1.b Thawing frozen cells

The selected vial containing frozen cells was identified and quickly removed from liquid nitrogen tank or -80°C freezer to a 37°C water bath. While holding the tip of the vial, the vial was gently swirled to prevent water from penetrating the seal. When the frozen liquid started to melt, the vial was sterilised with 70% alcohol before the contents were transferred to a 15 ml centrifuge tube. Ten ml of warm complete culture medium were slowly added. The tube was then centrifuged at 1300 rpm for 3 min. The medium was decanted and the pellet was resuspended in 5 ml complete culture medium. Cells were transferred to a 25 ml culture flask and incubated at 37°C in a 5% CO₂ incubator. After more than 6 h of incubation, the culture medium was changed to remove dead cells.

2.2.1.c Subculturing and propagation of cells

Cultured breast cancer cells that grew continuously are subcultured by dilution. The cell culture flask was examined under the inverted microscope. Cell culture was grown to 70 – 80% confluency. Cell culture medium was removed from the culture flask and cells were washed with autoclaved PBS solution. Cells were trypsinised by adding 1 ml of 1X trypsin solution (Sigma) and incubated at 37°C for 3 min for cell detachment. After cell had detached, 9 ml of pre-warmed culture medium was added to neutralise the action of trypsin. A small aliquot was taken for cell counting. After cell counting, an aliquot from the original flask was taken and diluted to the appropriate cell concentration (5×10^4 to 1×10^5 cells/ml) with complete culture medium in a new flask. The flask was labelled and incubated at 37°C in a 5% CO₂ incubator. All cell lines were tested routinely to ensure they were free from mycoplasma infection.

2.2.1.d Freezing cells

The breast cancer cells in culture were harvested during late log growth phase. A small volume was taken for cell counting in a haemocytometer. The remaining was centrifuged at 1300 rpm for 3 min to obtain the pellet. The supernatant was removed. Cryoprotectant medium containing complete culture medium supplemented with 10% (v/v) DMSO (freshly prepared) was added to the pellet. Cells were resuspended to give a final cell concentration of $0.5 - 1 \times 10^7$ cells/ml. One ml of cell suspension was

transferred to labelled cryogenic vial. The vials were placed in a 'Mr Frosty' freezing container and maintained overnight in a -80°C freezer. After 24 h, the vials were transferred to liquid nitrogen tank for permanent storage.

2.2.1.e Counting of viable cells

Trypan Blue exclusion method (Rafael and Václav, 2001) was used for counting viable cells. Trypan Blue 0.4% (w/v) prepared in Hank's Balanced Salt Solution was used. First, 80 μl of complete culture medium were transferred into a microcentrifuge tube. Ten μl of cell suspension and 10 μl of 0.4% Trypan Blue solution were added (dilution factor = 10), thoroughly mixed and allowed to stand for 5 to 15 min.

A small volume of Trypan Blue-cell suspension mixture was transferred to the haemocytometer. Cells in the 1 mm center square and four 1 mm corner squares were counted. Non-viable cells were stained blue, while viable cells remained unstained.

Cell count:

Cells per ml = the average count per square $\times$ dilution factor $\times 10^4$

Total cells = cell per ml $\times$ the original volume from which cell sample was taken

Example: If the average count per square is 50 cells, dilution factor is 10,

original volume is 10 ml,

$$\text{Cells per ml} = 50 \text{ cells} \times 10 \times 10^4 = 5.0 \times 10^6 \text{ cells/ml}$$

$$\text{Therefore, total cells} = 5.0 \times 10^6 \text{ cells/ml} \times 10 \text{ ml} = 5.0 \times 10^7 \text{ cells}$$

2.2.2 Small interference RNA transfection

siRNA was resuspended in RNase-free water at a concentration of 20 μM , which is equivalent to 20 pmol/ μl . Transfection of siRNA was carried out using HiPerFect Transfection Reagent (Qiagen), with the fast forward transfection method. Cell lines were plated in culture plates at 50% of confluence and were allowed to adhere at 37°C in a 5% CO₂ incubator. For transfection experiment in 6-well plate, 4.5 μl of 20 μM siRNA oligonucleotides were diluted in 88.5 μl of culture medium without serum and 7 μl of HiPerFect was added to the diluted siRNA. The complex was gently mixed by vortexing and incubated at room temperature for 10 min to allow formation of transfection complexes. The complexes were added drop-wise onto the cells. The plate was gently swirled to ensure uniform distribution of the transfection complexes. Cells were incubated with the transfection complexes under their normal growth conditions and gene silencing was monitored after an appropriate time (e.g., 6–72 h after transfection, depending on experimental setup). The medium can be changed as required.

2.2.3 Bacterial plasmid transformation

Chemically competent Library Efficient DH5 α E. coli bacteria (Life Technologies) were used to transform DNA plasmids. A 50 μ l bacterial aliquot was thawed on ice and the 0.2 μ l of DNA plasmid was added to the cells. After 30 min incubation on ice, bacteria were heat-shocked for 20 s at 42°C and immediately placed on ice for 2 min. One ml of Super Optimal broth with Catabolite repression (S.O.C.) medium (Invitrogen) was then added and bacteria were incubated at 37°C for 1 h with 225 rpm shaking. Transformed bacteria were then plated onto LB agar supplemented with the appropriated antibiotics (100 μ g/ml of kanamycin) and cultured at 37°C overnight with the plate facing upside down.

2.2.4 Plasmid preparation

A single colony was picked from the bacterial culture plate and transferred into 5 ml of pre-warmed LB medium containing 100 μ g/ml of kanamycin. The culture was placed in a shaking incubator at 37°C with 225 rpm for 6 hr. One ml of the initial bacterial culture was added to 200 ml of LB medium containing 100 μ g/ml of kanamycin and placed in a shaking incubator at 37°C for 18 hr.

Plasmid DNA was extracted using QIAGEN Plasmid Maxi Kits, according to the manufacturer's instructions. Plasmid DNA was quantified and purity was measured

at 260 nm and 280 nm, using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

2.2.5 Transient transfection of plasmid DNA

Transient transfection of plasmid DNA was carried out using FuGENE HD (Promega) according to the manufacturer's protocol. Cell lines were plated in culture plates at 60% of confluence and were allowed to adhere at 37°C in a 5% CO₂ incubator. For transfection experiment in 6-well plate, 99.2 µl of medium prewarmed to room temperature was prepared in U- or V-bottom plate. To this 0.2 µg of 1 µg/µl plasmid DNA was added and gently mixed. For 3:1 FuGENE[®] HD Transfection Reagent:DNA ratio, 0.6 µl of FuGENE[®] HD Transfection Reagent directly into medium and immediately mixed. FuGENE[®] HD Transfection Reagent/DNA mixture was incubated for 15 min at RT. After incubation, the mixture was then pipetted dropwise into the well containing 1.7 ml of cells in growth medium without antibiotics. The plate was gently swirled to ensure uniform distribution of the transfection mixture. Cells were incubated with the transfection mixture under their normal growth conditions and transfection efficiency was assayed 24 or 72 h after transfection, depending on experimental setup.

2.2.6 Generation of stable cell lines

MCF7 or T47D cells were used to generate cell lines stably expressing human wild-type LMTK3 (pCMV6-LMTK3) and empty vector (pCMV6-vector). Sequences were confirmed before experiments. To start, a working concentration of G-418 to guarantee selection and maintenance of the neomycin resistant cells by eliminating non-transfected cells was determined as following. A titration experiment was designed by treating non-transfected MCF7 cells seeded at different densities to different doses of G-418 (0, 0.1, 0.3, 0.5, 0.8, 1 mg/ml) for 10 days, and 1 mg/ml showed to be the minimum amount of G-418 that caused 99% cell death at all densities.

Next, 1×10^6 cells were seeded in 100 mm dishes in medium in the absence of antibiotics and attached overnight. On the following day, plasmids pCMV6-LMTK3 or pCMV6-vector were transfected into cells using protocol described previously. After 48 hours, stably transfected cells were treated with normal growth media in the presence of 1 mg/ml of G-418. In 10 days from the addition of the antibiotic, non-transfected cells died and removed. Stably transfected cells were plated at a lower density to allow each single cell form a monoclonal colony with G-418. When clones were visualised by eyes, 20 clones for each transfection condition were reseeded into 24-well plates: cloning discs were dipped in trypsin-EDTA for 2-3 min, then put on top of a single cell colony and incubated for 5 min at 37°C to allow cells to detach. Each cloning disc carrying monoclonal cells was then transferred to each well of a 24-well plate in presence of G-418. Cells derived from monoclonal colonies were cultured by changing medium twice a week, and expanded to high confluence before to be transferred to a large scale plate.

Finally, selected stable cells were acquired and success of transfection was confirmed by RT-qPCR and western blotting to check the mRNA and protein expression levels.

2.2.7 Cell proliferation assay

The Sulphorhodamine B (SRB) growth assay was used to assay cell growth based on the measurement of cellular protein content (Skehan *et al.*, 1990). Cells were seeded at a density of 3,000 cells per well, in 96-well plate. Cells were fixed at 24h, 48h, 72h and 96h by gentle addition of 100 µl of ice-cold 40% trichloroacetic acid per well. Plates were incubated for 60 min at 4°C, washed five times with water followed by adding 100 µl of sulforhodamine B (Sigma) solution 0.4% (w/v) in 1% acetic acid. The plates were incubated for 30 min at RT for staining. Following SRB staining, unbound dye was removed with five times washing with 1% acetic acid. The plate was allowed to air-dry overnight. The bound stain was solubilized by the addition of 100 µl of 10 mM Tris base and placed on a shaker for 10 min at RT. Absorbance was determined at 492 nm using a Tecan Sunrise plate reader.

The percentage of proliferation was determined by using the following formula:

$$\text{Percentage of proliferation} = \left(\frac{\text{Mean absorbance}_{\text{sample}} - \text{Mean absorbance}_{\text{day0}}}{\text{Mean absorbance}_{\text{DMSO control}} - \text{Mean absorbance}_{\text{day0}}} \right) \times 100$$

2.2.8 Extraction of total RNA

For RNA preparation, cells were washed twice with ice-cold PBS to eliminate cell culture medium. For cell number less than 5 million, 350 µl of RLT buffer (QIAGEN), containing (1% v/v) β-mercaptoethanol (Sigma) was added. To avoid RNA degradation, all used equipment was cleaned with RNaseZap (Ambion). RNA extraction was carried out using the RNeasy® Mini Kit (QIAGEN) according to manufacturer's protocol. Briefly, cells were homogenised by centrifugation through a QIAshredder spin column (QIAGEN) for 2 min at 13,300 rpm and 350 µl of 70% ethanol was added, mixed by pipetting. The total mixture was transferred to RNeasy spin column (QIAGEN) placed in a 2 ml collection tube and spun at for 15 s at 13,300 rpm. The flow-through was discarded. 700 µl of buffer RW1 was added to the column and was spun for 15 s at 13,300 rpm. Again, the flow-through was discarded. Next, 500 µl of RPE buffer was added to the column and spun for 15 s at 13,300 rpm. This step was repeated with 500 µl of RPE buffer and spun for 2 min at 13,300 rpm to ensure that no ethanol contamination. Prior to elution, column was added with 30 µl of DNase using the RNase-free water and the extracted RNA was collected by centrifugation for 1 min at 13,300 rpm. RNA concentration and purity were determined by measuring absorbance value. A high purity RNAs with an OD₂₆₀/OD₂₈₀ ratio of 2, and a low salt concentration with OD₂₃₀/OD₂₆₀ ratio of at least 1.6. RNA samples were then stored at -80°C.

2.2.9 Complementary DNA (cDNA) preparation by reverse transcription

Single-stranded cDNA was prepared from total RNA by using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). In this assay, 1 µg total RNA was used for all the samples acquired. A master mix containing 2 µl of 10x RT buffer, 1 µl of dNTP Mix, 2 µl of 10x RT random primers, 1 µl of MultiScribe™ reverse transcriptase and 1 µl of RNase inhibitor was prepared per sample. Next, 1 µg total RNA with RNase-free water was added to the master mix up to 20 µl per reaction. To perform reverse transcription, the reactions were loaded into the GeneAmp 9700 PCR machine (Invitrogen) on the following conditions:

- ❖ Step 1 : 25°C for 10 min
- ❖ Step 2 : 37°C for 120 min
- ❖ Step 3 : 85°C for 5 min
- ❖ Step 4 : 4°C for ∞

When the reactions were finished, the cDNA samples were collected and diluted 1 in 10 with RNase-free water.

2.2.10 Quantitative real-time polymerase chain reaction

Gene expression analysis was carried out either using TaqMan or SYBR Green assays. Each sample for PCR was assayed in triplicates, in a Microamp™ fast optical 96-well reaction plate (Applied Biosystems). Before placing into the 7900HT fast Real-time

PCR machine, the plate was sealed with the Optical Adhesive Cover Started Kit (Applied Biosystems) and centrifuged for 30 s at RT.

2.2.10.a TaqMan quantitative RT-PCR

For TaqMan assay, each reaction mixture consisted of the following components:

- ❖ 10 µl of TaqMan® Fast Universal PCR Master Mix
- ❖ 1 µl of TaqMan assay probe
- ❖ 4 µl of DNase/RNase-free water
- ❖ 5 µl of cDNA

The thermal cycle profile:

- ❖ Stage 1 : 95°C for 20 s
- ❖ Stage 2 (for 40 cycles) : 95°C for 20 s, 60°C for 20 s

Data was analysed using the $2^{-\Delta\Delta CT}$ method and the expression of target genes was normalised to the expression of the GAPDH housekeeping gene.

2.2.10.b SYBR Green quantitative real-time PCR

For SYBR Green assay, each reaction mixture was prepared as below:

- ❖ 10 µl of Power SYBR[®] Green PCR Master Mix
- ❖ 2 µl of SYBR Green primer
- ❖ 3 µl of DNase/RNase-free water
- ❖ 5 µl of cDNA

The thermal cycle profile:

- ❖ Stage 1 : 95°C for 10 min
- ❖ Stage 2 (for 40 cycles) : 95°C for 10 s, 60°C for 30 s, 72°C for 30 s
- ❖ Stage 3 : 95°C for 15 s, 60°C for 15 s, 95°C for 15 s

Data was analysed using the $2^{-\Delta\Delta CT}$ method and the expression of target genes was normalised to the expression of the GAPDH housekeeping gene.

2.2.11 Protein lysate preparation

2.2.11.a Radioimmune Precipitation Buffer (RIPA) buffer extraction

Cells were washed twice and harvested with ice-cold PBS (1 ml per 10 cm dish). Cell lysates were collected by scraping and transferred into 1.5 ml microcentrifuge tubes. Tubes were spun at 1200 rpm for 5 min to obtain cell pellets. The supernatant was

discarded and cell pellets were frozen at -80°C until lysis was performed. Frozen pellets were thawed on ice before adding ice-cold RIPA buffer (50 mM Tris-HCl at pH8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% SDS) (Sigma), supplemented with protease and phosphatase inhibitor cocktails (Roche). Samples were incubated on ice for 30 min before centrifuging at 13,000 rpm for 10 min at 4°C. The supernatants were transferred to pre-chilled new 1.5 ml microcentrifuge tube and the pellets were discarded. The protein concentration was quantified using the Bradford Protein Assay (Bio-Rad). For western blotting, RIPA samples were mixed with 5X loading buffer and heated at 95°C for 4 min. These samples were cooled on ice prior to loading or kept in -20°C for storage.

2.2.11.b Protein quantification

The protein concentration was determined using the Bradford Protein Assay kit (Bio-Rad), according to the manufacturer's protocol. In brief, bovine serum albumin (BSA) standards were generated (1.25 – 25 µg/ml) according to the Table 2.4. 1 µl of protein lysates were added into 500 µl of Bradford dye (500X dilution). The samples were mixed and incubated for at least 5 min at RT. The reactions were then transferred into 96-well plate (triplicate well for each sample) and the absorbance was measured at 595 nm. Protein concentration of the samples was determined by multiplying the absorbance of the sample by the standard curve's regression coefficient and dilution factor.

Table 2.11 Microplate microassay dilutions for 2 mg/ml BSA

Tube	Standard volume (μl)	Source of standard	Bradford dye volume (μl)	Final [Protein] (μg/ml)
1	10	2 mg/ml stock	790	25
2	10	2 mg/ml stock	990	20
3	6	2 mg/ml stock	794	15
4	500	Tube 2	500	10
5	500	Tube 4	500	5
6	500	Tube 5	500	2.5
7	500	Tube 6	500	1.25
8 (blank)	-	-	500	0

2.2.12 Western blotting

Protein expressions were determined using SDS-PAGE resolving gels (6 to 15%), with a 5% stacking gel. A Mini-PROTEAN® Tetra Cell system (Bio-Rad) was used for electrophoresis. Samples were loaded into the wells of a mini format SDS-PAGE gel, along with molecular weight markers. Running buffer was used and electrophoresis was carried out at 80V for 30 min to allow proteins to go through the stack and then at 140V for 1 to 2 hr, until the bromophenol blue dye front reached the bottom of the gels.

The gel was transferred onto Amersham Hybond ECL nitrocellulose membrane (GE Healthcare) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) at 15V for 90

min. The membrane was stained with Ponceau S solution (Fluka, sigma-Aldrich, Dorset, UK) to ensure if the transfer was successful. The membrane was washed twice with distilled water to remove the staining and then blocked with blocking buffer [5% (w/v) skimmed milk powder in PBST] for 60 min at RT to reduce non-specific binding of primary antibody. The membrane was then incubated with the diluted primary antibody in 5% (w/v) BSA at 4°C with gentle shaking overnight.

The membrane was washed three times with PBST and incubated with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:3000 dilution) for 60 min. The membrane was washed three times with PBST. All blots were detected with ECL-plus Western Blotting Detection System (Amersham Biosciences) by GelDoc System (BioRad) or autoradiography using Hyperfilm ECL (GE Healthcare) on Konica SRX-101A film processor (Minolta). The molecular weights of the proteins were estimated by comparison with the Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific).

2.2.13 Co-immunoprecipitation

For each co-immunoprecipitation (Co-IP) reaction, 1 mg of whole cell extract prepared in RIPA buffer were used. Lysates were incubated with 50 µl of agarose beads (Santa Cruz) at 4°C with rotation for 2 hr for pre-clearing. Lysates were then centrifuged at 13,000 rpm for 30 sec at 4°C and supernatant was collected. Precleared lysates were stored on ice. To form IP antibody-IP matrix complex, 1 µg of IP antibody (anti-LMTK3

or anti-RAD51) were incubated with IP-matrix (ImmunoCruz™ IP/WB Optima System, Santa Cruz Biotechnology) at 4°C on a rotator for 2 h. After incubation, IP antibody-IP matrix complex was pelleted via microcentrifugation at 13,000 rpm for 30 sec at 4°C and washed for three times with 500 µl of PBS. Precleared lysates and IP antibody-IP matrix complex were incubated overnight rotating at 4°C. Pelleted matrix was washed three times and proteins were eluted using 2X Laemmli buffer before heated at 95°C for 4 min.

2.2.14 Glutathione S-Transferase pull-down assay

Glutathione Sepharose 4B beads (GE Healthcare) were washed with cold PBS twice to remove the 20% ethanol storage solution. Residual ethanol may interfere with subsequent procedures. A 50% slurry of beads were equilibrated in RIPA buffer supplemented with protease and phosphatase inhibitor cocktails. Glutathione S-Transferase (GST)–LMTK3 (20 µg) was incubated with 50 µl of 50% slurry of glutathione-Sepharose 4B in 250 µl of PBS at 4°C on a rotator for 2 h. The beads were washed with 1 ml of cold PBS three times and incubated with 1 mg of preclear protein lysate in 500 µl of RIPA buffer overnight at 4°C on a rotator. Beads were washed with cold PBS for four times to remove unbound proteins. Bead sample was mixed with 30 µl of 2X Laemmli buffer and heated at 95 °C for 5 min for Western Blotting.

2.2.15 Nuclear and cytoplasmic extraction

Nuclear and cytoplasmic extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotech) according to the manufacturer's protocol. Throughout extraction, lysates and reagents were on ice and spun at 4°C. Cells were harvested and pelleted by centrifugation. The supernatant was discarded and left the cell pellet as dry as possible. The cell pellet was suspended with ice-cold CER I by vortexing the tube vigorously for 15 s and then incubated on ice for 10 min. Ice cold CER II was added. Extracts were vortexed for 5 s, incubated on ice for 1 min and vortexed for 5 s again. Extracts were centrifuged for 5 min at 13,300 rpm. The supernatant was transferred to a clear pre-chilled tube and stored as cytoplasmic extract at -80°C. Pellet was washed once with 200 µl ice-cold 1X PBS, flicked and centrifuged for 5 min at 13,300 rpm. Supernatant was discarded. Pellet was resuspended in ice-cold NER, vortexed every 10 min for 22 s for 40 min and centrifuged for 10 min at 13,300 rpm. The supernatant was transferred to a clear pre-chilled tube and stored as nuclear extract at -80°C.

2.2.16 Protein stability assay – Protein Synthesis Inhibition

Cells were plated in 6-cm dishes and allowed to attach for 24 h. Cycloheximide (CHX) (Sigma) was then added to a final concentration 20 µg/ml to inhibit protein

synthesis. Cells were collected at the indicated times after the addition of CHX, and Rad51 protein levels were determined by western blotting.

2.2.17 Protein stability assay – Proteasome Inhibition

Cells were plated in 6-cm dishes and allowed to attach for 24 h. Proteasome inhibitors, MG132 or MG115 (Sigma) was then added to a final concentration 10 μ M to inhibit proteasome. Cells were harvested after 6 h of the inhibitors addition, and Rad51 protein levels were determined by western blotting.

2.2.18 Immunofluorescence staining

Cells grown on glass coverslips were fixed in 4% paraformaldehyde for 10 min, washed in PBS and incubated with 0.3% (v/v) Triton X-100 for 10 min at RT. Following, cells were washed in PBS and blocked with 10% AB serum in PBS for 30 min. Coverslips were then incubated overnight at 4°C with primary antibodies, diluted in the same buffer. Cells then were washed and incubated with Alexa Flour®-488 secondary anti-rabbit and Alexa Flour®-555 secondary anti-mouse antibodies (Invitrogen) at RT for 60 min. After washing, coverslips were mounted onto glass slide in Mowiol (Calbiochem) with 4',6-diamidino-2-phenylindole (DAPI) solution [10% (w/v) Mowiol, 1 μ g/ml DAPI]. Coverslips were examined on a Leica TCS SP5 II STED laser scanning confocal microscope (Leica Microsystems).

2.2.19 Homologous and non-homologous end joining recombination assays

DSB-induced HR frequencies were measured in MCF7 cells. Briefly, MCF7 cells were transfected with DR-GFP and EJ5-GFP with FuGENE6 (Roche) and stable colonies were selected with 1 µg/ml puromycin (Sigma). To assay GFP expression, 1 µg of pCMV6-LMTK3 or pCMV6 was used alone or co-transfected with 2 µg of pCBASceI plasmids using FuGENE6 into DR-GFP-MCF7 or EJ5-GFP-MCF7 cells. For analyses with siRNA, cells were transfected with RAD51 siRNA for 48 h before transfected with plasmids. Forty-eight hours later, two-colour fluorescence analysis revealed the percentage of GFP-positive cells scored out of 50,000 viable events using a FACSCanto analyzer with FACSDiva software (Becton Dickinson). Data was analysed by using the FlowJo software (Treestar Inc, Ashland, OR, USA). The EJ5-GFP (total-NHEJ) plasmid was a generous gift from Dr. Jeremy Stark.

2.2.20 Cell cycle analysis

Cell cycle analysis was performed using propidium iodide (PI) staining. Cells were trypsinised and collected by centrifugation at 1300 rpm for 3 min. Cells were washed with cold PBS twice before fixing in 100% cold ethanol for at least 30 min at 4°C. Samples can be stored at 4°C for up to two weeks in ethanol. Fixed cells were re-suspended in PBS containing 50 µg/ml propidium iodide (Sigma) supplemented with 100 µg/ml RNase A (Sigma) and incubated for 30 min at 37°C, protected from light. Samples were

incubated on ice prior to FACS analysis. A flow cytometry analysis was performed by using FACSCanto analyzer with FACSDiva software (Becton Dickinson).

2.2.21 Comet assay

DSB repair was analysed by neutral comet assay using the Trevigen comet assay kit (4250-050-K) according to the manufacturer's methods. Two hours after doxorubicin treatment, cells were subjected to comet analysis at the indicated time points. Cell suspension (1×10^5 cells in 50 μ l PBS) were mixed with 450 μ l of molten LMAgarose at 37°C was spread on the slide covered with agarose and allowed to solidify at 4°C in a moist box. After a clear ring appears at edge, the slides were immersed in freshly prepared cold (4°C) Lysis solution for overnight at 4°C. The slides were removed from the Lysis buffer, drained the excess buffer and transferred to an electrophoresis chamber. After equilibrated in the cold 1X Neutral Electrophoresis solution for 30 min, electrophoresis was conducted at 18 V for 45 min. The slides were then immersed in DNA Precipitation Solution for 30 min and followed by 70% ethanol for another 30 min at room temperature. The slides were drained and air dried for more than 15 min. After staining with DAPI, comet images were captured by fluorescence microscopy. The tail moment was quantified using the ImageJ software with OpenComet plug-in (Gyori *et al.*, 2014).

2.2.22 *In vitro* kinase assay

For *in vitro* kinase assay, 1-2 µg recombinant LMTK3 kinase domain was incubated with 1 ng active human RAD51 full-length recombinant protein in kinase buffer (containing 50 mM HEPES pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 0.2 mM orthovanadate, 0.25 mM ATP and 0.3 µCi/µl [γ -³²P] ATP) for 30 min at 30 °C. Samples were resolved by SDS-PAGE, stained with Coomassie, dried and subjected to autoradiography.

2.2.23 Xenograft mouse model

BALB/c athymic nude mice 7–8 weeks of age were purchased from Harlan Laboratories UK, and all procedures were carried out under Home Office license authority and local ethics. MCF7 cells stably express LMTK3 or vector cells were cultured in DMEM containing 10% FCS and 0.5 mg/ml of G418 and injected subcutaneously into mice (twenty mice/group) at a concentration of 5 X 10⁶ cells/mouse.

After 10 days, mice were randomly divided into two subgroups: control group and doxorubicin group, 10 mice for each subgroup. The mice in doxorubicin group were injected intraperitoneally with 5 mg doxorubicin per kilogram of body weight once every 7 days for a total of two times. Mice were sacrificed after day 28. Primary tumours were measured accordingly. Tumour volumes were measured every 3 or 4 days using a caliper and calculated using the formula $\frac{1}{2}$ length (mm) X width (mm)².

2.2.24 Statistical analysis

All data are expressed as means $\pm$ standard error of the mean with triplicate wells in each experiment. The data were analysed by using paired Student's t-test. Statistical differences between different doses were analysed with one-way analysis of variance (ANOVA). All statistical tests were performed with SPSS 11.0. P-value less than 0.05 and 0.01 were considered to be statistically significant.

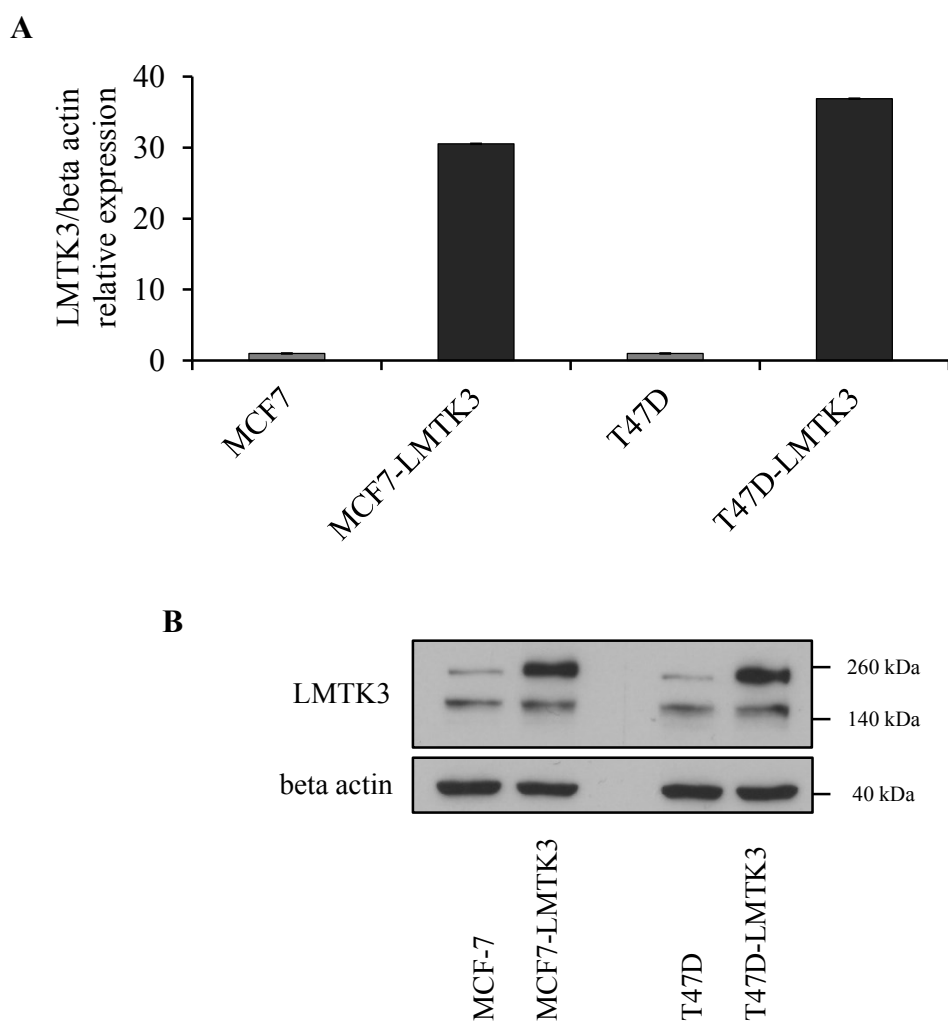
Chapter 3 RESULTS

3.1 Generation of stable cell lines expressing LMTK3

In order to access the impact of one or several of the gene's function(s), the manipulation of the expression of genes of interest in selected cell lines can be done by using transient or stable overexpression or knockdown. Experiments using transient transfection protocol usually manage to produce peak gene expression between 24 to 96 hours after transfection. If continued gene expression is required for longer periods of time, the stable cell line can be generated. Stable cell line generation is produced by using positive selection markers such as neomycin, G418 (geneticin), zeocin, hygromycin, and puromycin antibiotic resistance.

To elucidate the function of LMTK3 in DNA damage repair and to examine the epistatic relationship between LMTK3 with DNA damage repair proteins, MCF7-LMTK3 and T47D-LMTK3 stable cell lines were generated. *NEO* gene was delivered in using the pCMV vector with C-terminus Myc-DDK tag. This approach is easier and has a higher likelihood of producing drug-resistant stable transfection. A stable polyclonal population of cells expressing DDK-tagged Myc was selected by using G418 (analogue of neomycin) as the positive selection marker. A control population of MCF7 or T47D cells, transfected with the empty vector alone, was established in parallel. The expression of the LMTK3 was confirmed by qRT-PCR (Figure 3.1A) and Western blotting using an antibody raised against LMTK3. As shown in Figure 3.1B, more than one LMTK3 protein

bands (about 170 and 240 kDa) were detected in the cells. The cellular localisation of LMTK3 was also examined by immunofluorescence microscopy using anti-Myc antibody and anti-LMTK3 (Figure 3.1C). The ectopic LMTK3 protein was found to be mostly in the cytoplasm by Western blotting and immunostaining analyses with the LMTK3 antibody (Figure 3.1B and 3.1C).



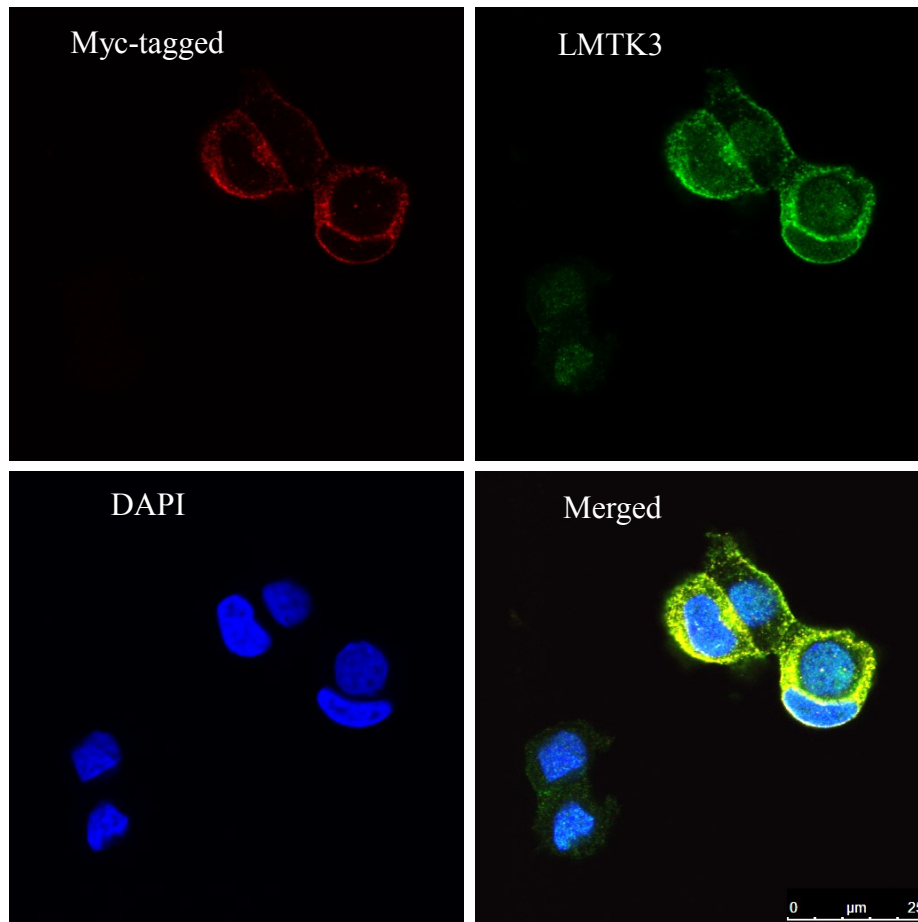
C**MCF7-LMTK3 cells**

Figure 3.1 MCF7 and T47D cells stably expressing LMTK3.

MCF7 and T47D cells were transfected with pCMV vector constitutively expressing LMTK3. **A)** qRT-PCR was performed to verify LMTK3 gene expression level. The expression levels have been normalised with the average expression of the endogenous control gene GAPDH. The bar graph represents the means and the standard error of the mean for three replicates. **B)** Western blot analysis of protein lysates prepared MCF7 and T47D cells was carried out using LMTK3 antibody. Immunoblotting for beta actin was used as a protein loading control. **C)** MCF7-LMTK3 cells were subjected to indirect immunofluorescence analysis using antibodies against Myc (red), LMTK3 (green) and DAPI (blue).

3.2 Overexpression of LMTK3 protects against doxorubicin-induced cytotoxicity

The previous study has shown that stably overexpressed-LMTK3 in MCF7 and T47D cell lines significantly stimulated the growth rate compared to parental cell lines, respectively (Xu *et al.*, 2014a). To study the physiological role of LMTK3 in regulating the DNA damage response, the effect of LMTK3 expression on response to the chemotherapeutic drug was examined. Doxorubicin was used as the anthracycline to induce DNA damage.

To assess the effects of LMTK3 on the response, the viability of cells was evaluated using SRB assay by incubating cells with various doses of doxorubicin. At the end of the incubation period: day 2, 4 or 6, the absorbance values were measured. The absorbance value is directly proportional to the cell number in each well (Vichai and Kirtikara, 2006) (Table 3.1 and 3.2). The percentage of growth cell was determined, and the finding is depicted in Figure 3.2 and 3.3.

In MCF7 cells, overexpression of LMTK3 significantly increased cell survival compared to the control cells after 2, 4 or 6 days incubation with 0.2 µg/ml of doxorubicin (Figure 3.2A, $P < 0.05$). This difference persists after treated with 0.4 µg/ml for 6 days ($P = 0.036$). Although both control and overexpressed-LMTK3 cells were killed by doxorubicin in a dose-dependent manner, cells overexpressing LMTK3 significantly showed greater resistance than the control cells (Figure 3.2B, $P = 0.025$). For instance, in order to achieve 75% inhibition of cell growth, MCF7-LMTK3 cells required more than 0.2 µg/ml doxorubicin as compared to less than 0.1 µg/ml for control cells. At a low dose

of doxorubicin ($< 0.025 \mu\text{g/ml}$), there was no statistically significant difference in cytotoxic to doxorubicin between MCF7-LMTK3 and control cells.

Similarly, a significant difference in doxorubicin-induced cytotoxicity is observed between T47D-LMTK3 overexpressing or control cells (Figure 3.3B, $P = 0.0039$). These data indicate that cells overexpressing LMTK3 are corresponding positively in resistance toward doxorubicin.

Table 3.1 The absorbance values of treated and untreated MCF7 and MCF7-LMTK3 cells with $0.2 \mu\text{g/ml}$ of doxorubicin for 2, 4 and 6 days.

Day	0	2		4		6	
Dox	-	-	+	-	+	-	+
MCF7	0.19 ± 0.013	0.57 ± 0.080	0.25 ± 0.030	1.12 ± 0.078	0.24 ± 0.024	1.43 ± 0.039	0.26 ± 0.028
MCF7-LMTK3	0.22 ± 0.025	0.63 ± 0.087	0.32 ± 0.031	1.14 ± 0.067	0.34 ± 0.046	1.30 ± 0.081	0.37 ± 0.053

Data are expressed as the means and the standard error of mean for six experiments.

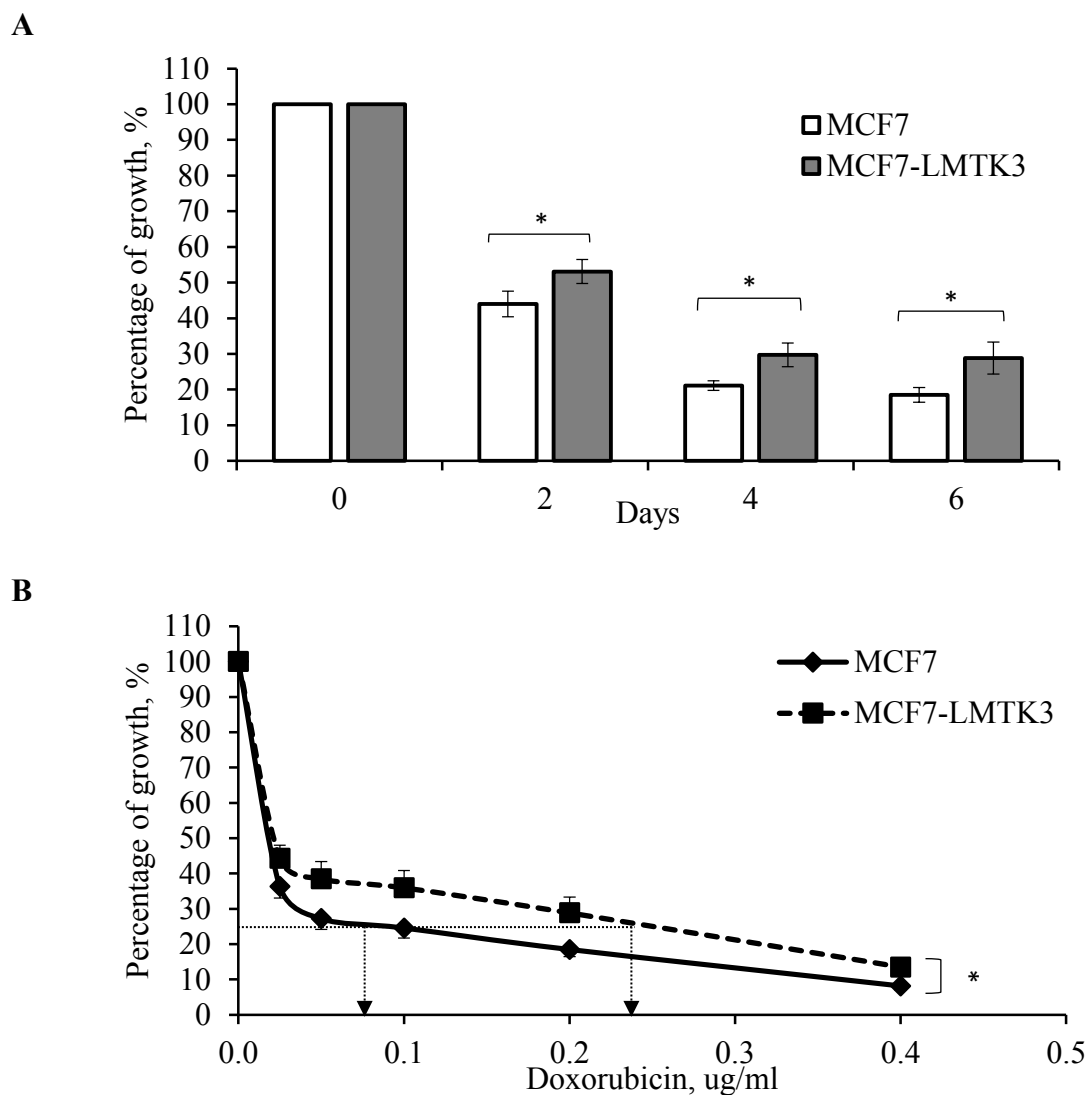


Figure 3.2 Effect of overexpression of LMTK3 on doxorubicin-treated MCF7 cells.

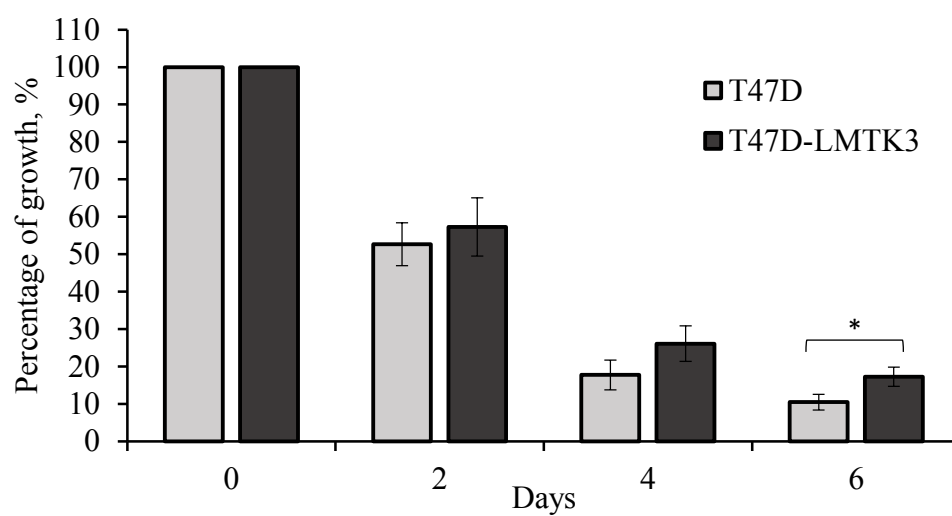
A) The percentage of growth was evaluated by SRB assay in MCF7 cells overexpressing LMTK3 and their control treated with doxorubicin at 0.2 $\mu\text{g/ml}$ for 2, 4 and 6 days. **B)** MCF7-LMTK3 and MCF7 cells were treated with various concentrations of doxorubicin (0.025, 0.05, 0.1, 0.2 and 0.4 $\mu\text{g/ml}$) and the number viable cells was measured by SRB assay at day 6. Error bars represents the means and the standard error of mean for six experiments. Significant differences ($P < 0.05$) are marked by *.

Table 3.2 The absorbance values of treated and untreated T47D and T47D-LMTK3 cells with 0.2 µg/ml of doxorubicin for 2, 4 and 6 days.

Day	0	2		4		6	
Dox	-	-	+	-	+	-	+
T47D	0.21 ± 0.008	0.49 ± 0.080	0.27 ± 0.068	0.96 ± 0.118	0.17 ± 0.05	1.30 ± 0.092	0.13 ± 0.028
T47D-LMTK3	0.21 ± 0.250	0.42 ± 0.260	0.24 ± 0.341	0.86 ± 0.315	0.21 ± 0.255	1.08 ± 0.069	0.19 ± 0.122

Data are expressed as the means and the standard error of mean for four experiments.

A



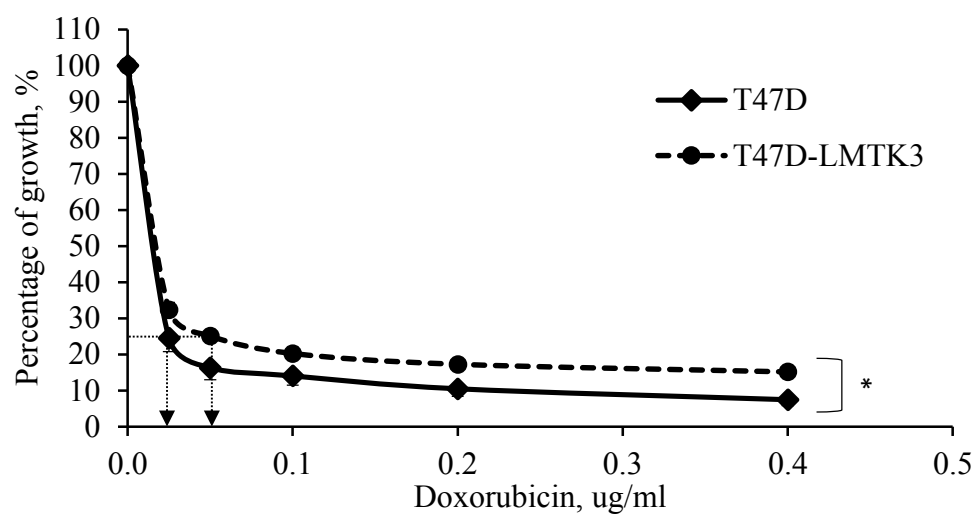
B

Figure 3.3 Effect of overexpression of LMTK3 on doxorubicin-treated T47D cells.

A) The percentage of growth was evaluated by SRB assay in T47D cells overexpressing LMTK3 and their control treated with doxorubicin (0.2 $\mu\text{g/ml}$) for 2, 4 and 6 days. **B)** The line graph represents the growth rate of T47D cells treated with different concentrations of doxorubicin by SRB assay at day 6. Error bars represents the means and the standard error of the mean for four experiments. Significant differences ($P < 0.05$) are marked by *.

3.3 Overexpression of LMTK3 inhibits the efficacy of doxorubicin *in vivo*

To explore the *in vivo* role of LMTK3 in the efficacy of doxorubicin, the xenograft models were established via subcutaneous injection of stable MCF7 cells overexpressing LMTK3. The tumour growth was monitored every three to four days before and after administration of doxorubicin. On 28th day, mice were sacrificed and the tumours were dissected and measured. Figure 3.4A shows the tumour volume and size between MCF7 and MCF7-LMTK3 tumours with or without doxorubicin treatment at Day 28. The results showed that the tumour growth was significantly inhibited by the doxorubicin treatment in both groups.

Furthermore, at day 24th, MCF7-LMTK3 tumours have a nearly twofold increase in volume over MCF7 tumours, suggesting LMTK3 has a tumorigenic function (Figure 3.4B), in agreement with previous studies (Xu *et al.*, 2014a). Importantly, treatment with doxorubicin inhibited the growth of MCF7 tumours significantly four times more effective than that of MCF7-LMTK3 tumours (Figure 3.4B). These results indicate that tumours with overexpressing LMTK3 are less sensitive to doxorubicin.

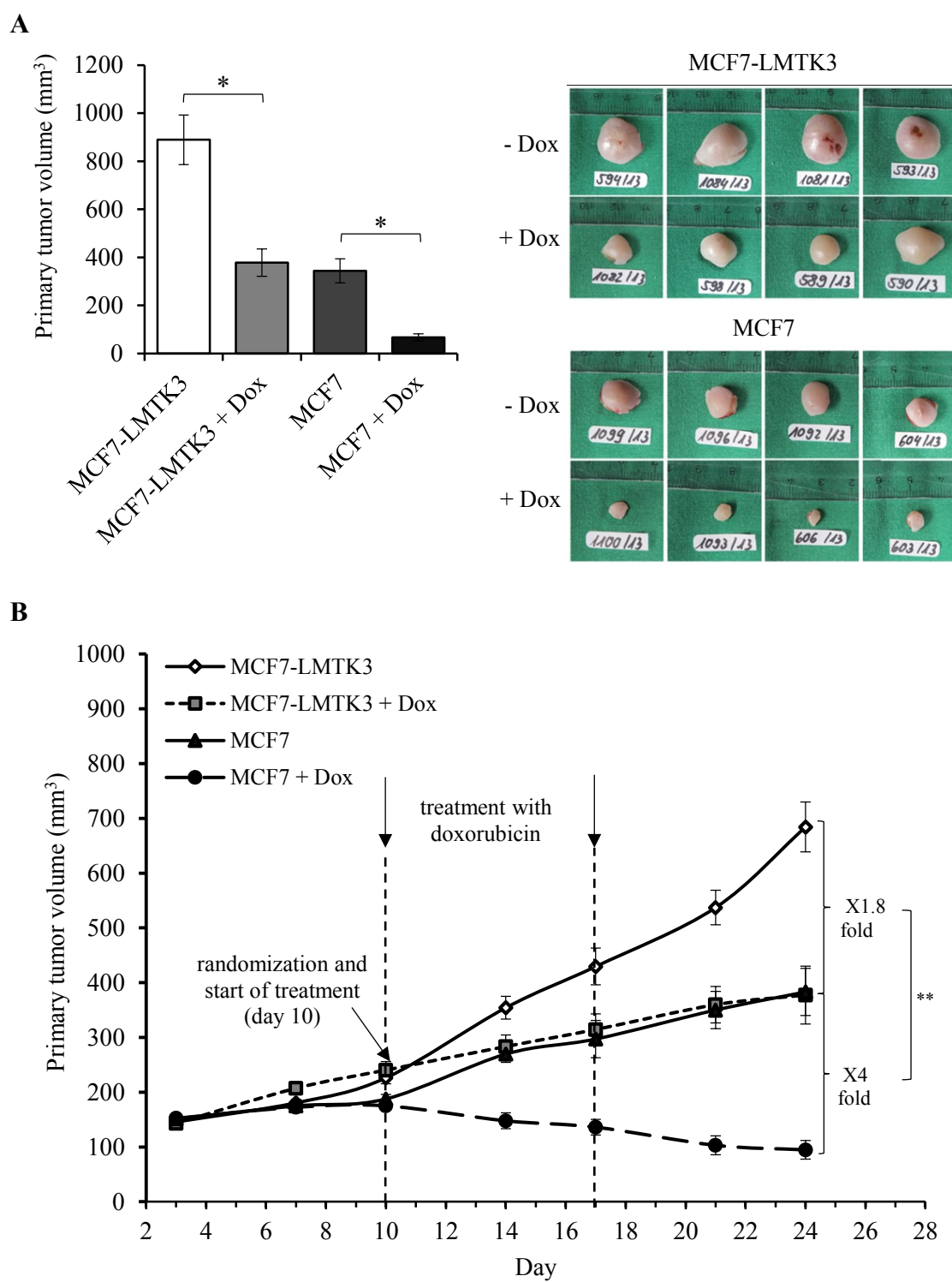


Figure 3.4 Overexpression of LMTK3 impedes the effectiveness of doxorubicin in vivo. **A)** (Left) Quantification in primary tumour size between MCF7 and MCF7-LMTK3 xenografts with or without doxorubicin treatment at Day 28. The means and the error bars show the standard error of the mean for ten MCF7-LMTK3, nine MCF7-LMTK3 + Dox, ten MCF7 and seven MCF7 + Dox –derived xenografts. (Right) Representative images of tumours at Day 28 are shown. **B)** Tumour xenografts volumes develop in mice subcutaneously injected with MCF7 or MCF7-LMTK3 cells. After 10 days, mice were randomly divided and treated with doxorubicin once every seven days for twice. The means and the error bars show the standard error of the mean for nine MCF7-LMTK3, nine MCF7-LMTK3 + Dox, ten MCF7 and seven MCF7 + Dox–derived xenografts. * $P < 0.05$ and ** $P < 0.01$.

3.4 Effect of doxorubicin on LMTK3 protein and mRNA levels in MCF7 cells

To determine the effects of doxorubicin on endogenous LMTK3, total RNA and protein lysates from MCF7 cells treated with doxorubicin for specified time periods (0, 2, 4, 8, 16 or 24 h) were prepared. Western blot analysis of LMTK3 protein levels in whole cell lysate of doxorubicin-treated MCF7 cells indicated that LMTK3 levels transiently increase, whereas at 24 h, LMTK3 protein levels decrease slightly (Figure 3.5A).

Quantitative real-time RT-PCR was performed to examine whether the doxorubicin-dependent upregulation of LMTK3 protein is attributed to alterations at a transcriptional level. After an 8-hour exposure to doxorubicin, a slight increment in the LMTK3 mRNA levels was observed in Figure 3.5B. LMTK3 transcript levels are significantly increased more than two-fold at 16 h exposure ($P = 0.0006$) and attained nearly three-fold at 24 h exposure ($P = 0.0001$). Together, these results indicate that LMTK3 was activated during the response to DNA damage.

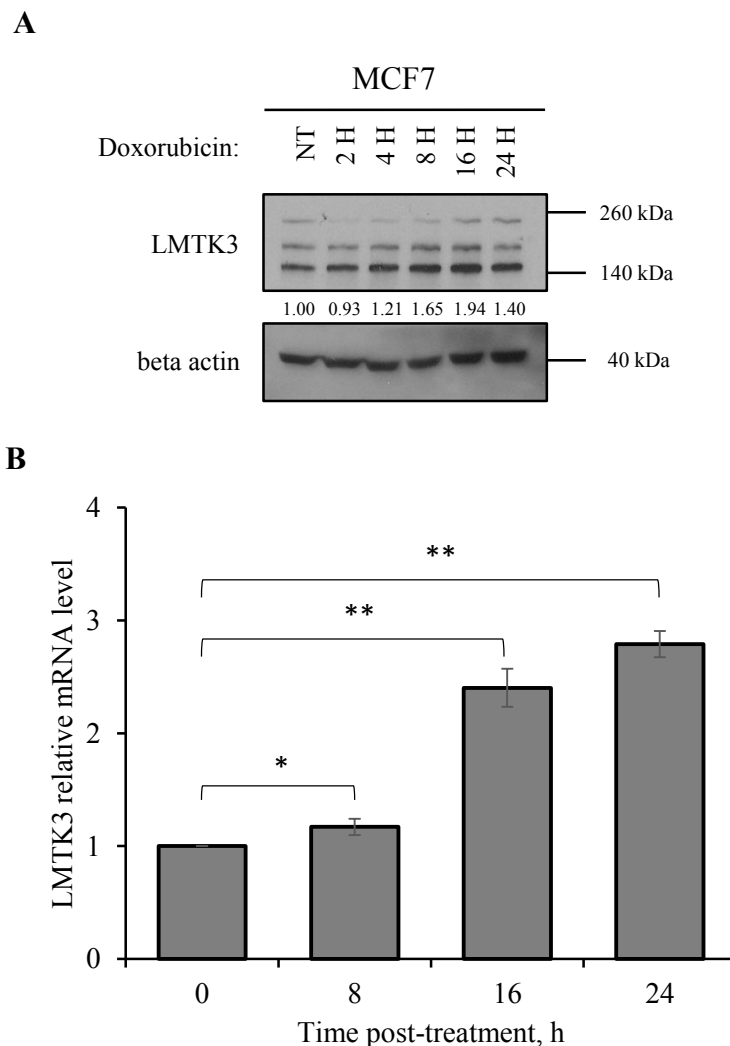


Figure 3.5 LMTK3 protein and mRNA levels in doxorubicin-treat MCF7 cells.

MCF7 cells were treated with 0.5 $\mu\text{g/ml}$ of doxorubicin for specified time periods.

A) Cells were harvested at 0, 2, 4, 8, 16 or 24 h and analysed for the expression of LMTK3 by Western blot. Beta actin was used as a loading control. One representative Western blot of three is presented. **B)** Cells were harvested at 0, 8, 16 or 24 h and analysed for the mRNA level by quantitative RT-PCR. GAPDH was used as a control. Error bars represents the means and the standard error of the mean for three experiments.

* $P < 0.05$ and ** $P < 0.01$.

3.5 Effect of LMTK3 expression on ATM and KAP1 in response to doxorubicin

Given that ATM is first activated following doxorubicin treatment, a possible role of ATM in regulating LMTK3 was evaluated. In the meantime, another PIKKs member, ATR was also assessed. MCF7 and MCF7-LMTK3 cells were exposed to doxorubicin for up to 24 h and ATM phosphorylated status was examined. Consistent with previous reports (Lee *et al.*, 2007, Li *et al.*, 2007), ATM was phosphorylated within 2 h in control cells after exposure to 0.5 µg/ml of doxorubicin (Figure 3.6). The phosphorylation of ATM on Ser1981 status was slightly reduced to a noticeable signal till 8 h and after 16 h, it was insignificant low. Whilst, in MCF7-LMTK3, the ATM phosphorylation reached its peak at 4 h and diminished at the later time points. The phosphorylated ATM signal in MCF7-LMTK3 was scarcely low as compared with the control cells. Nonetheless, LMTK3 did not affect ATR and its Ser428 status. Thus, these results suggest that LMTK3 is involved in the activation of ATM in response to doxorubicin.

Next, KAP1 Ser824 phosphorylation, a novel ATM substrate that involves in DNA damage was examined. Since the ATM is involved in the early DNA damage response, cells were treated and harvested at a shorter time points; 15 min, 30 min, 1 h, 2h and 4 h. Similar to Figure 3.6, phosphorylation of ATM at Ser1981 in MCF7-LMTK3 cells were reduced in the initial 2 h of treatment as compared with the control cells. Plus, this effect was seen on Ser824 of KAP1. Phosphorylation of KAP1 at Ser824 in MCF7-LMTK3 cells was decreased half as compared with its control cells at 2 h of doxorubicin

treatment (Figure 3.7). These data imply that overexpression of LMTK3 may delay the induction of DNA damage through the ATM-KAP1 signalling pathway.

To assess the relationship between LMTK3 and ATM, LMTK3 expression was analysed in silenced-ATM and silenced-ATR cells. MCF7 and MCF7-LMTK3 cells were transfected with ATM or ATR siRNAs and treated with doxorubicin for 2 h. Cells were continue cultured for another 3 h in fresh medium before harvesting. Silencing of ATM reduced LMTK3 expression, while doxorubicin treatment was further reduced LMTK3 expression in the absence of ATM. Nonetheless, LMTK3 expression recuperated to its baseline after cells under for 3 h repair mechanism (Figure 3.8A). On the other hand, cells depleted-ATR did not appear to regulate LMTK3 expression before or after DNA damage (Figure 3.8B). These data indicate that LMTK3 regulation requires ATM in response to doxorubicin-induced DNA damage.

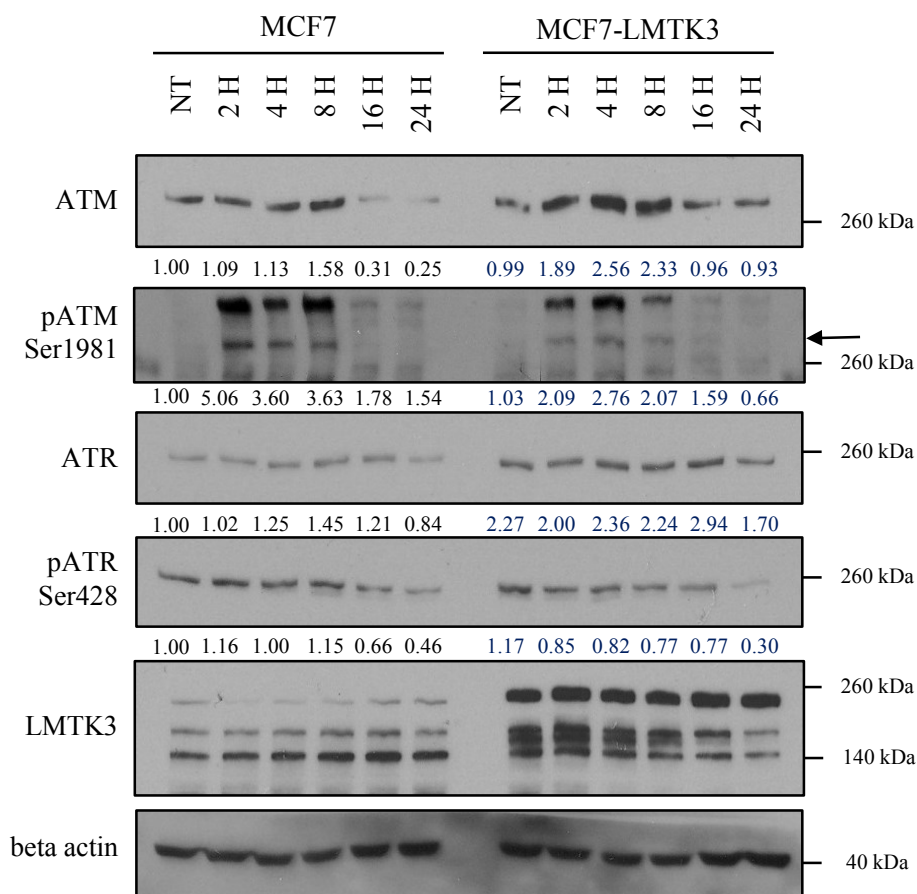


Figure 3.6 Effect of LMTK3 expression on ATM and ATR in response to doxorubicin treatment.

MCF7 and MCF7-LMTK3 cells were treated with 0.5 $\mu\text{g/ml}$ of doxorubicin and collected at the indicated time points after treatment. Whole cell lysates were analysed by Western blotting using respective antibodies. Beta actin was used as a loading control.

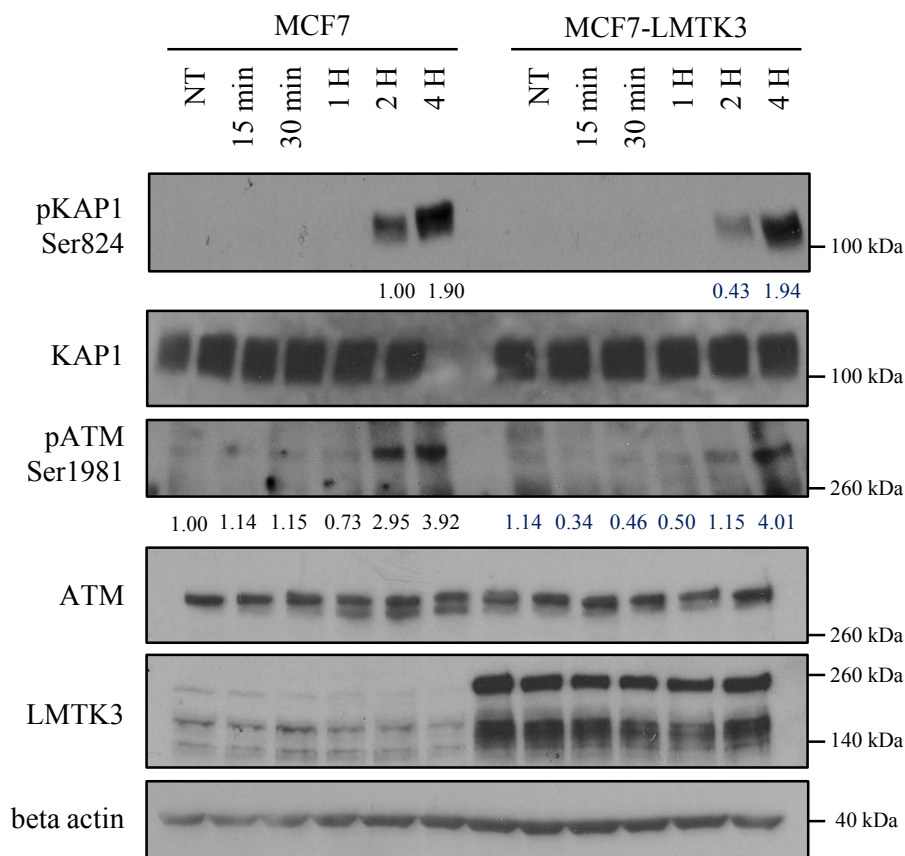


Figure 3.7 Effect of LMTK3 expression on Ser1981 ATM and Ser824 KAP1 in response to doxorubicin treatment.

MCF7 and MCF7-LMTK3 cells were treated with 0.5 $\mu\text{g/ml}$ of doxorubicin and collected at the indicated time points (0 – 4 h) after treatment. Whole cell lysates were analysed by Western blotting using respective antibodies. Beta actin was used as a loading control.

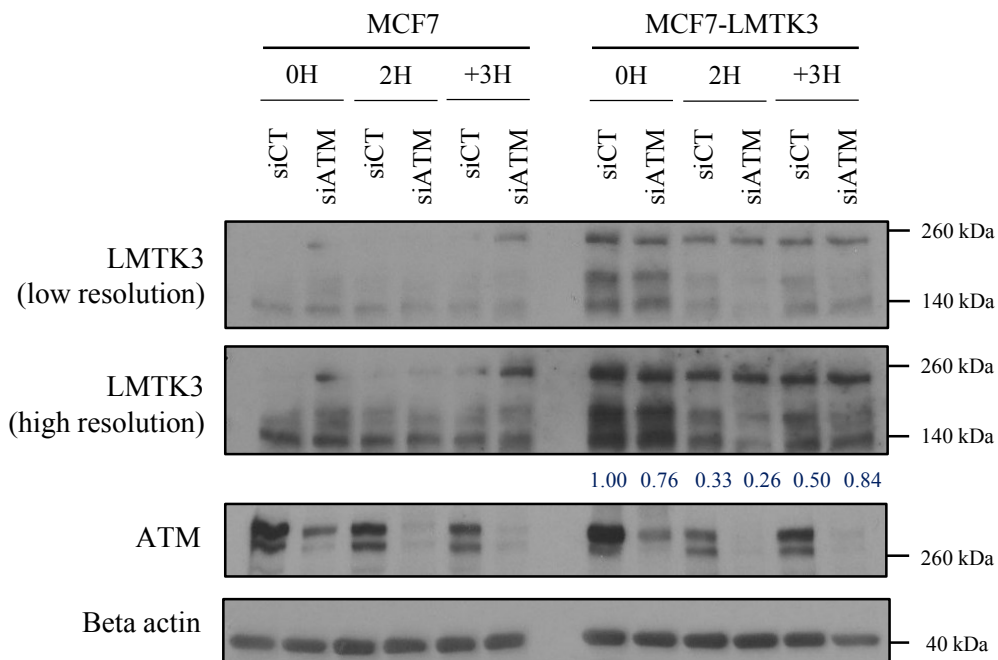
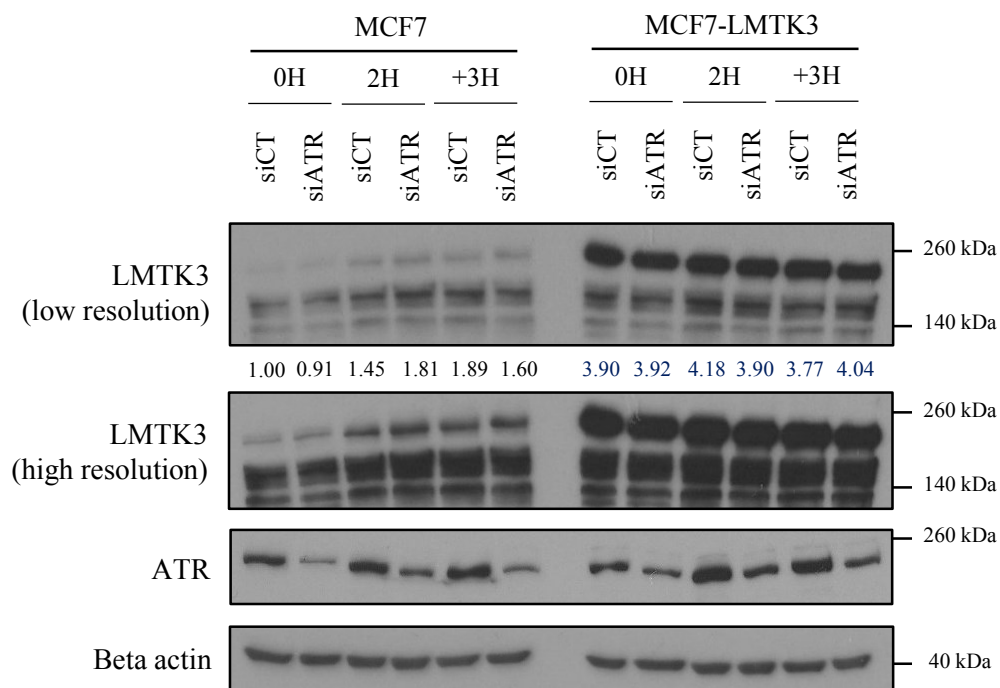
A**B**

Figure 3.8 Effect of ATM and ATR silencing on LMTK3 expression in response to doxorubicin.

A) ATM or **B)** ATR were silenced from MCF7 and MCF7-LMTK3 cells, and treated with 0.5 µg/ml of doxorubicin. After 2 h, culture medium was removed and cells were continue cultured in a fresh medium for another 3 h before harvesting. Whole cell lysates were analysed by Western blotting using respective antibodies. Beta actin was used as a loading control.

3.6 Overexpression of LMTK3 delays resolution of γ H2AX foci

To further understand the doxorubicin-resistance phenotype of MCF7-LMTK3 cells, γ H2AX assay was employed. Exposure to doxorubicin generates DSBs, leading to the phosphorylation of histone H2AX on Ser139. The γ H2AX formation is a rapid and sensitive marker of DSB induction. The increase in phosphorylation and disappearance of phosphorylation at Ser139 can be quantified by immunofluorescence with a highly specific antibody for phospho-histone H2AX and directly proportional to the kinetics of DSB repair (Rogakou *et al.*, 1998, Pilch *et al.*, 2003, Kurz *et al.*, 2004).

In the initial assessment, cells were exposed to 0.2 μ g/ml of doxorubicin and incubated for 12, 24 or 48 h. Untreated cells contained few γ H2AX foci (8.8 ± 1.21 foci per cell in MCF7 cells, 11.8 ± 2.01 in MCF7-LMTK3 cells; Figure 3.9) with no statistically significant difference between MCF7-LMTK3 and control cells. As expected, γ H2AX intensity increased after doxorubicin exposure relative to respective untreated cells. Interestingly, when cells were treated with doxorubicin, accumulation at various time points during exposure was significantly lesser in MCF7-LMTK3 as compared to MCF7 cells (Figure 3.9).

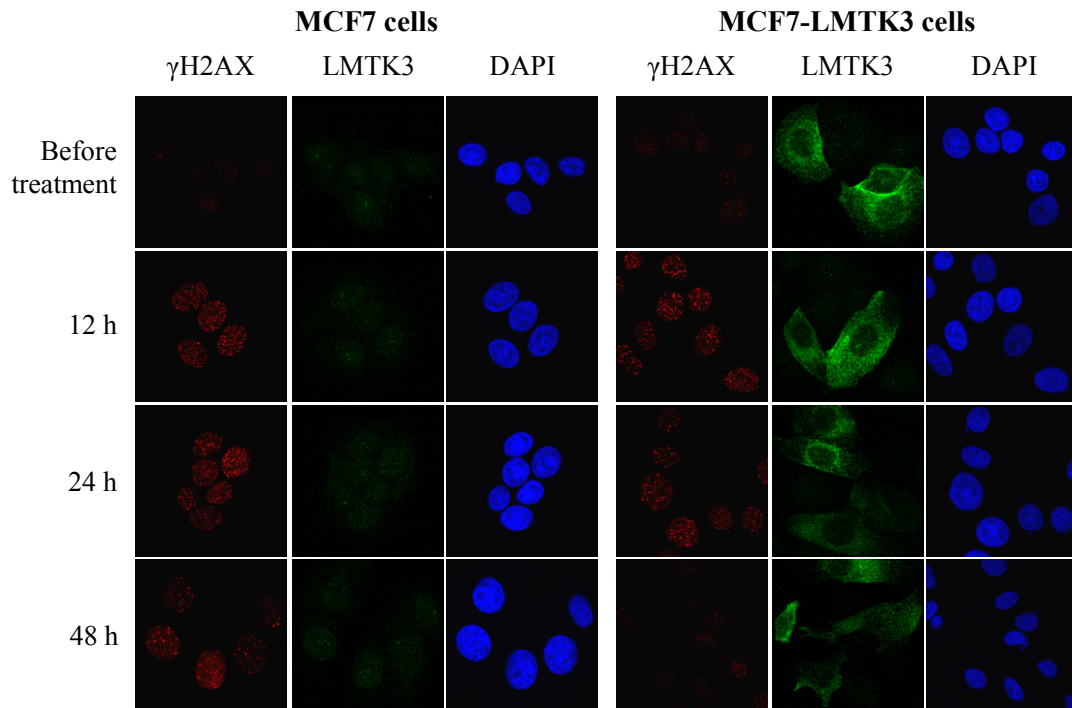
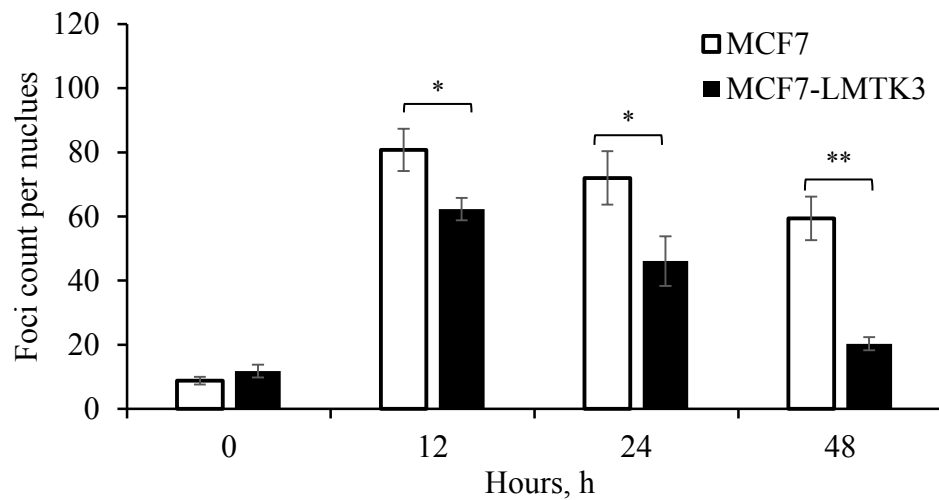
A**B**

Figure 3.9 Up-regulation of LMTK3 impairs phosphorylation of H2AX upon doxorubicin.

A) MCF7 and MCF7-LMTK3 cells were treated with 0.2 μ g/ml of doxorubicin and 12, 24 and 48 h later, γ H2AX and LMTK3 were visualised by immunofluorescence.

B) γ H2AX foci formation induced by 0.2 μ g/ml of doxorubicin was quantified for 50 cells per each time point. * P<0.05 and ** P<0.01.

To determine whether the rapid resolution in focus formation at 12, 24 or 48 h was due to delayed resolution of damage or increased induction of DNA repair, foci were quantified at earlier time points. Cells were treated with 0.5 $\mu\text{g/ml}$ of doxorubicin for 2 h to induce DNA damage.

The γH2AX kinetics followed a biphasic curve, a rapid phase of foci formation followed by an exponential γH2AX foci decay phase. A surge in H2AX phosphorylation was observed within 30 min of doxorubicin treatment and assemblage of nuclear foci was fully evident at 120 min (51.42 ± 1.64) in the MCF7 cells (Figure 3.10).

In contrast, although the initial number of foci was nearly identical at 30 min, indicating the similar levels of DNA damage induced, the number of foci present at 120 min (35.98 ± 1.57) after doxorubicin treatment was clearly lesser in MCF7-LMTK3 cells. Significantly, LMTK3 delayed this response in that the foci increased gradually and culminated at 180 min (48.58 ± 1.48) (Figure 3.10). Similarly, in Western blot analysis, the level of γH2AX was examined at the indicated time points from 0 to 120 min and 0 to 24 h after doxorubicin treatment. Overexpression of LMTK3 caused a diminution in the level of global γH2AX (Figure 3.11). In line with the above observations, depletion of LMTK3 results in an increase of H2AX phosphorylation at Ser139 upon DNA damage (Figure 3.12). These results, demonstrating that doxorubicin induces H2AX phosphorylation, albeit delayed, in MCF7-LMTK3 cells, suggest that LMTK3 may play an important role at early times following DNA damage.

In order to investigate the kinetics of DSB repair, doxorubicin was removed after 2 h and cells were continue cultured in fresh complete media for 10 h. Coverslips were collected and fixed at indicated time points for immunostaining.

Although the decay curve appears similar, analysis of the time course for the disappearance of foci in MCF7 and MCF7-LMTK3 cells after exposure to doxorubicin showed that the foci in MCF7-LMTK3 cells disappear more rapidly, compared with that observed in MCF7 cells (Figure 3.10B).

Despite the shift in the peak observed, there was no statistically significant difference between the two peaks. By 10 h post γ H2AX foci peak formation, cells over-expressing LMTK3 show significantly less number of foci (18.32 ± 1.15) as compared to the MCF7 cells ($>24.44 \pm 1.35$). These data suggest that the rate of DNA repair is accelerated by overexpression of LMTK3 upon doxorubicin exposure.

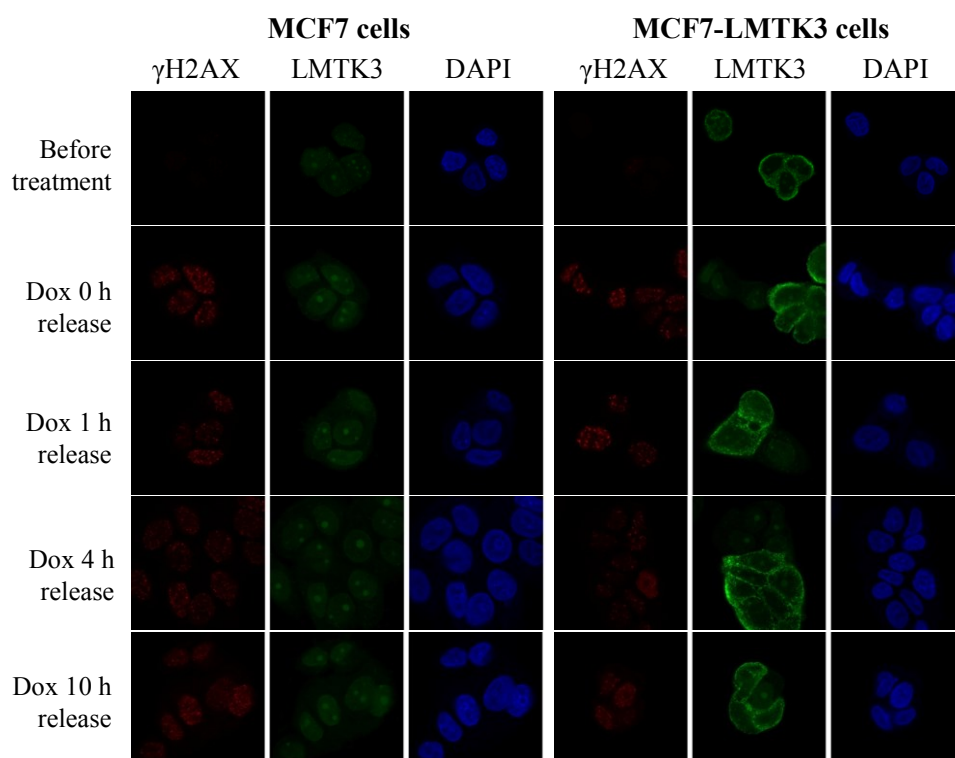
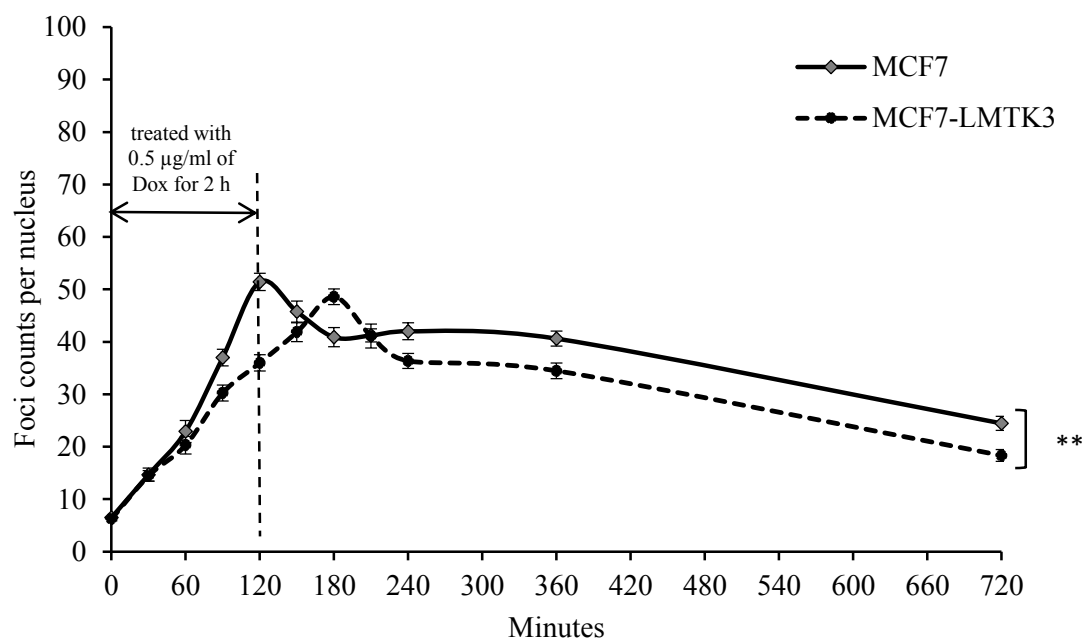
A**B**

Figure 3.10 Overexpression of LMTK3 reduces gamma H2AX foci.

A) MCF7 and MCF7-LMTK3 cells were treated with 0.5 $\mu\text{g/ml}$ of doxorubicin for 2 h and incubated for another 10 h. γH2AX and LMTK3 were visualised by immunofluorescence. **B)** γH2AX foci formation induced by 0.5 $\mu\text{g/ml}$ of doxorubicin was quantified for 50 cells per each time point. Data represent the means and the standard error of mean of two independent experiments. Significant differences ($P < 0.01$) are marked by **.

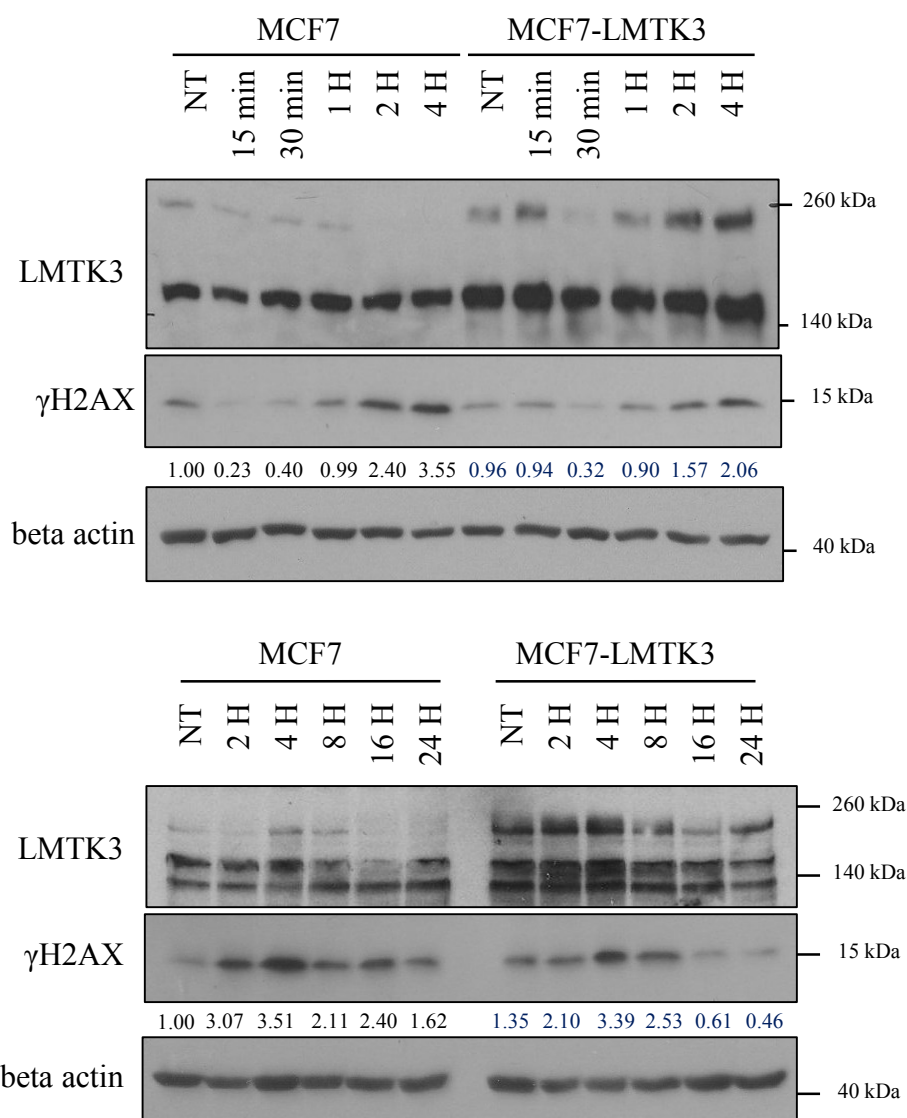


Figure 3.11 Effect of overexpressed-LMTK3 on the phosphorylation of H2AX.

MCF7 and MCF7-LMTK3 cells were treated with 0.5 µg/ml of doxorubicin and collected at the indicated time points (Top: 0 to 120 min; Bottom: 0 to 24 h) after treatment. Whole cell lysates were analysed by Western blotting using the anti-γH2AX antibody. Beta actin was used as a loading control.

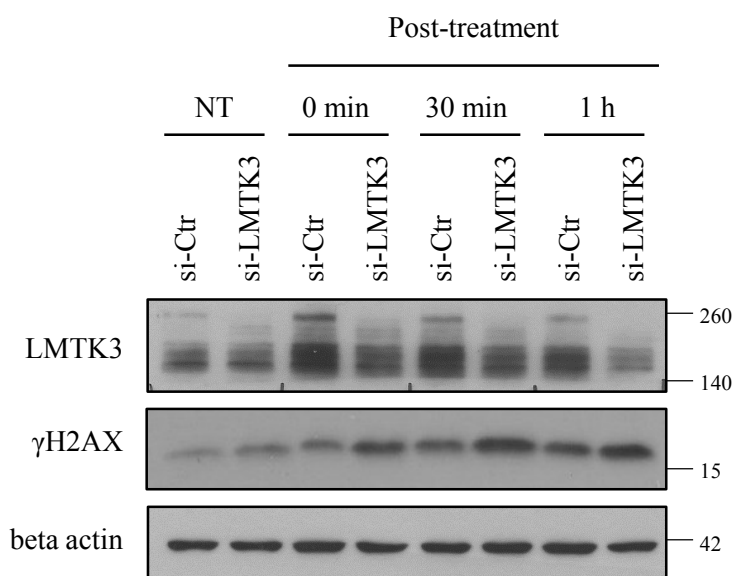


Figure 3.12 Effect of LMTK3 knockdown on the phosphorylation of H2AX.

MCF7 cells were transfected with LMTK3 or scrambled siRNA. After 48 h, cells were treated with 0.5 $\mu\text{g/ml}$ of doxorubicin. After 2 h treatment, cells were washed and cultured in complete media. Cells were harvested at the indicated times and analysed by Western blot with anti- γH2AX antibody. Beta actin was used as a loading control.

3.7 LMTK3 does not colocalise with γ H2AX

In the interest of exploring LMTK3 function at the early phase of DNA damage, the role of LMTK3 in the formation of γ H2AX foci was determined. Primarily, the localisation of LMTK3 in response to doxorubicin-induced DSBs was analysed. Figure 3.13 shows that LMTK3 does not accumulate at γ H2AX foci before or after doxorubicin treatment. Similar results using MCF7-overexpressing LMTK3 cells were obtained. These findings suggest that LMTK3 may regulate H2AX indirectly.

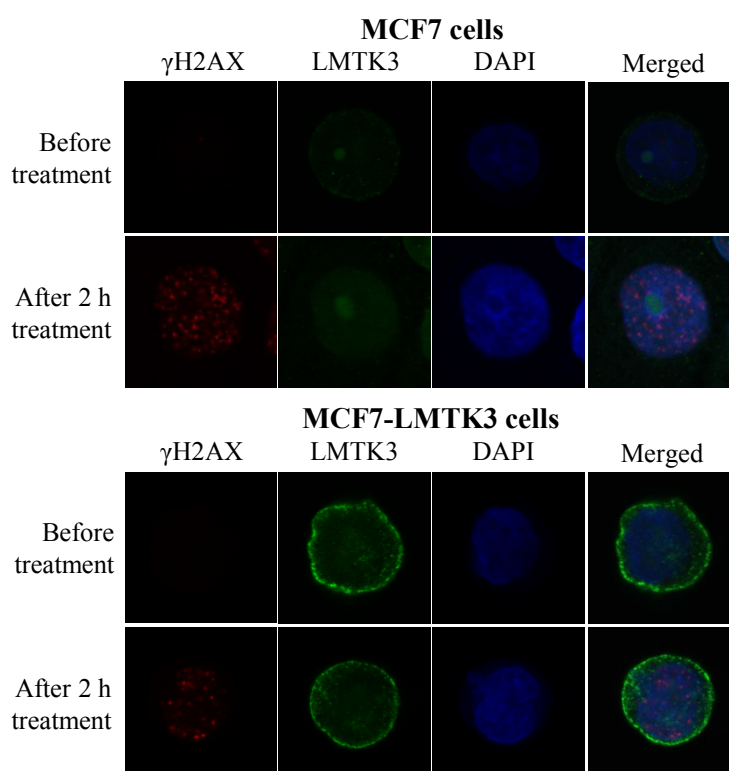


Figure 3.13 Localisation of LMTK3 and γ H2AX.

Immunofluorescence images showing the localization of LMTK3 and γ H2AX. MCF7 cells (top) and MCF7-LMTK3 cells (bottom) were treated with 0.5 μ g/ml of doxorubicin for 2 h, fixed and stained with anti-LMTK3 and anti- γ H2AX antibodies. Nucleus was counterstained with DAPI.

3.8 LMTK3 impedes monoubiquitylation of H2AX

H2AX is thought to be an essential sensor that can identify DSBs to launch the early DDR. In addition to phosphorylation of H2AX, ubiquitination of H2AX is an important marker for DNA lesions in DDR (Bergink and Jentsch, 2009). From the previous results (Figure 3.11 and 3.12), we identified that LMTK3 regulates H2AX phosphorylation in response to DNA damage. Therefore, we explore whether LMTK3 affects the H2AX ubiquitination.

Western blot analysis shows the total level of γ H2AX and the monoubiquitination of H2AX were significantly reduced 4 h after exposure to doxorubicin in cells overexpressing LMTK3 (Figure 3.14A). While cells depleted-LMTK3 showed more monoubiquitinated H2AX (Figure 3.14B). These findings indicate that LMTK3 hinders monoubiquitination of H2AX, suggesting that LMTK3 may delays the DNA damage response by inhibiting the monoubiquitination of H2AX. Further experimentation will be required to elucidate the mechanism by which ubiquitination mediates by LMTK3.

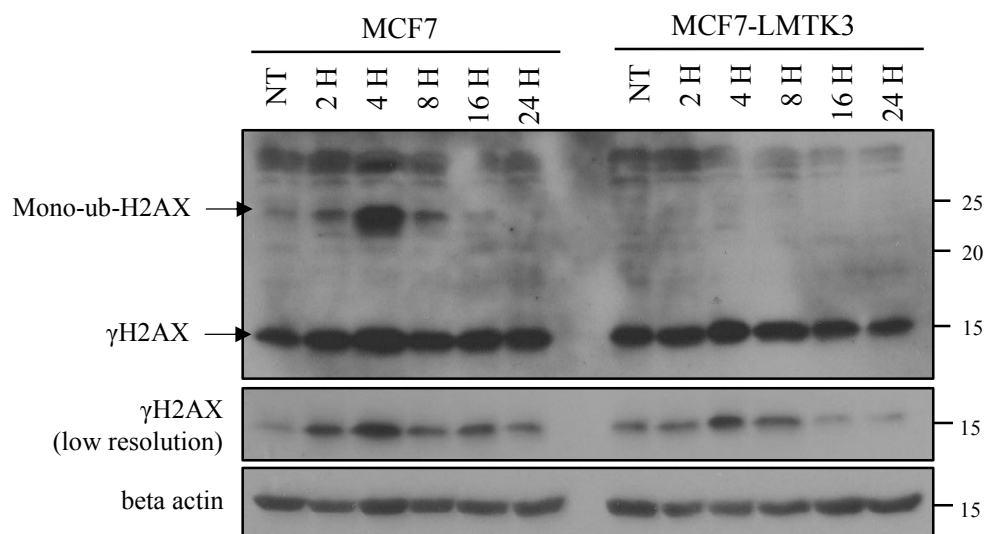
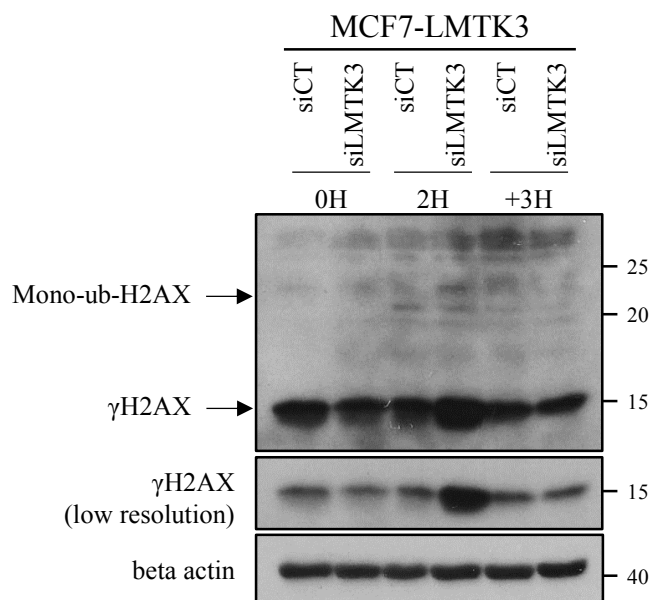
A**B**

Figure 3.14 Effects of LMTK3 on the monoubiquitination of H2AX.

A) MCF7 and MCF7-LMTK3 cells were treated with 0.5 $\mu\text{g/ml}$ of doxorubicin upto 24 h. Cells were harvested at the indicated times and analysed by Western blot with anti- γH2AX antibody. **B)** LMTK3 were depleted from MCF7-LMTK3 cells and treated with 0.5 $\mu\text{g/ml}$ of doxorubicin. After 2 h, culture medium was removed and cells were continue cultured in a fresh medium for another 3 h before harvesting. Blot was analysed with anti- γH2AX antibody.

3.9 High levels of LMTK3 accelerate DNA breaks repair

To assess whether the disappearance of γ H2AX in MCF7-LMTK3 cells was due to repaired DNA breaks, comet assay was performed. Doxorubicin acts via the induction of either single- or double-stranded DNA breaks by stabilising the topoisomerase II-DNA complex (Gewirtz, 1999). Cells were treated with 0.5 μ g/ml of doxorubicin for 2 h to induce DNA breaks. Following drug treatment, the media was removed and incubated with fresh complete media for another 6 and 24 h.

As expected, overexpression of LMTK3 led to significantly lower DNA tail moments when compared to MCF7 cells (Figure 3.15). MCF7-LMTK3 have a much shorter comet tail at 6 and 24 h after 2 h exposure to doxorubicin than MCF7 cells [12.89 ± 1.10 vs. 21.71 ± 1.35 for 6 h ($P < 0.0001$), and 8.49 ± 0.80 vs. 12.10 ± 1.00 ($P = 0.003$)]. This result is consistent with the reduced γ H2AX foci that were conducted previously. Thus, the MCF7-LMTK3 show reduced numbers of DNA breaks compared to MCF7 cells after doxorubicin exposure, implying that LMTK3 promotes the repair of damaged DNA leading to a doxorubicin-resistant phenotype.

Table 3.3 Percentage of DNA tail moments in MCF7 and MCF7-LMTK3 cells after doxorubicin treatment

	MCF7 cells	MCF7-LMTK3 cells
Before treatment	1.67 ± 0.26	1.85 ± 0.28
Dox 6 h release	21.71 ± 1.35	12.89 ± 1.10
Dox 24 h release	12.10 ± 1.00	8.49 ± 0.80

Mean tail moment percentage, with standard error of mean, are reported.

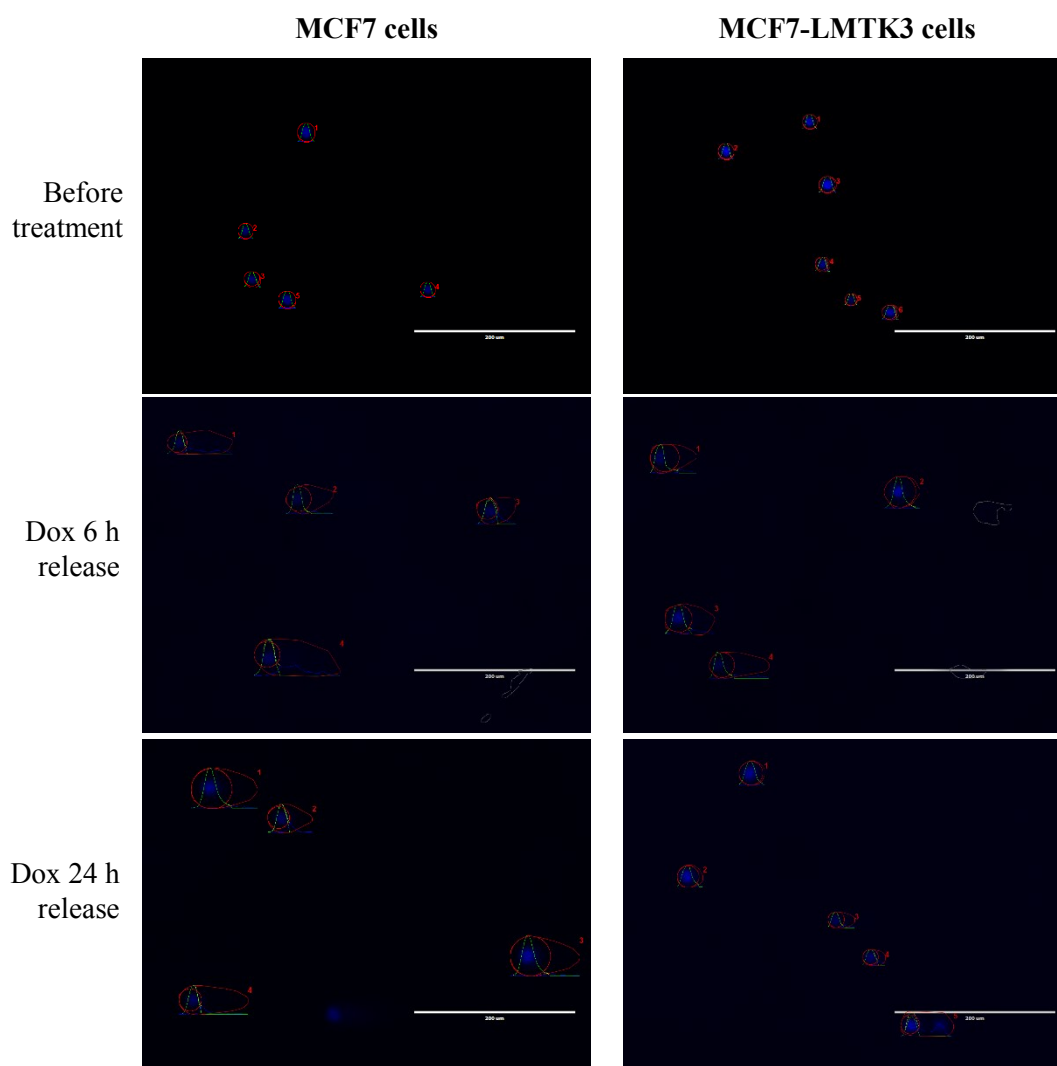


Figure 3.15 Comet images of alkaline gel electrophoresis demonstrating DNA damage in the MCF7 and MCF7-LMTK3 cells.

MCF7 and MCF7-LMTK3 cells were exposed to 0.5 $\mu\text{g/ml}$ of doxorubicin. After 2 h treatment, the drug was removed and cells were incubated with fresh culture media for another 6 and 24 h before the Comet assay. The tail moment was quantified using the ImageJ software with OpenComet plug-in.

3.10 LMTK3 is restricted to homologous recombination pathway

To more directly address the role of LMTK3 in DNA DSBs repair, the biological consequence of overexpressed-LMTK3 on the repair of DSBs was examined by utilising two well-characterized GFP-based reporter systems.

DSBs repair involves two distinct pathways: HR and NHEJ. Thus, the HR- and NHEJ-mediated DSB repair systems were established by stably integrated into MCF7 cells. DR-GFP was constructed using the homology-directed repair (HDR) product that contains an upstream GFP gene with I-SceI recognition site and a downstream internal GFP repeat. DSB is induced by I-SceI endonuclease and iGFP is used as the template for nascent DNA synthesis during HR repair, which results in the restoration of a GFP expression cassette (Pierce *et al.*, 1999). Whereas, EJ5-GFP was constructed using a non-functional GFP gene carrying a puromycin gene flanked by two I-SceI recognition sites that separate the GFP coding sequence from its promoter. Transfection of plasmid encoding I-SceI induces two DSBs flanking the puromycin gene. The promoter is ligated to the GFP coding gene when the puromycin gene is excised by NHEJ repair, leading to GFP expression (Bennardo *et al.*, 2008).

DR-GFP-MCF7 and EJ5-GFP-MCF7 cells with or without prior overexpressing LMTK3 were transfected with pCBA*SceI* or empty vector plasmids to induce DSBs. After 48 h, the cells were analysed by flow cytometry to detect GFP-positive cells.

Notably, MCF7 cells overexpressing LMTK3 exhibited a significant increase (42.7%, $P = 0.017$) in the fraction of GFP-positive DR-GFP cells but not the EJ5-GFP cells (Figure 3.16), suggesting that LMTK3 primarily participates in the HR-mediated DNA damage repair and the clearance of DNA breaks.

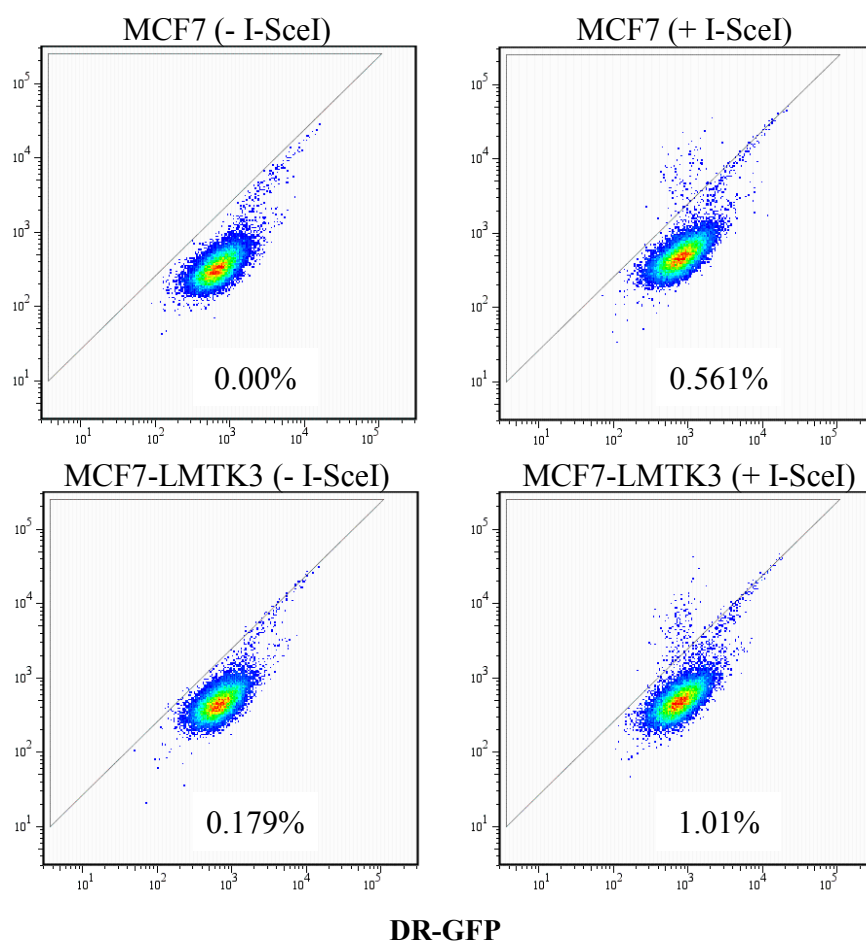
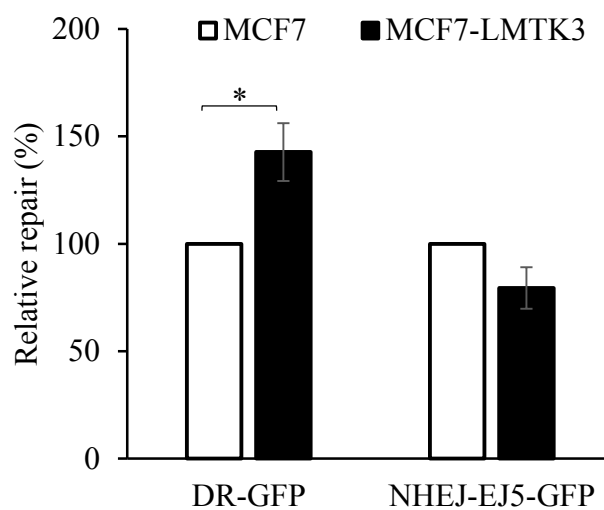
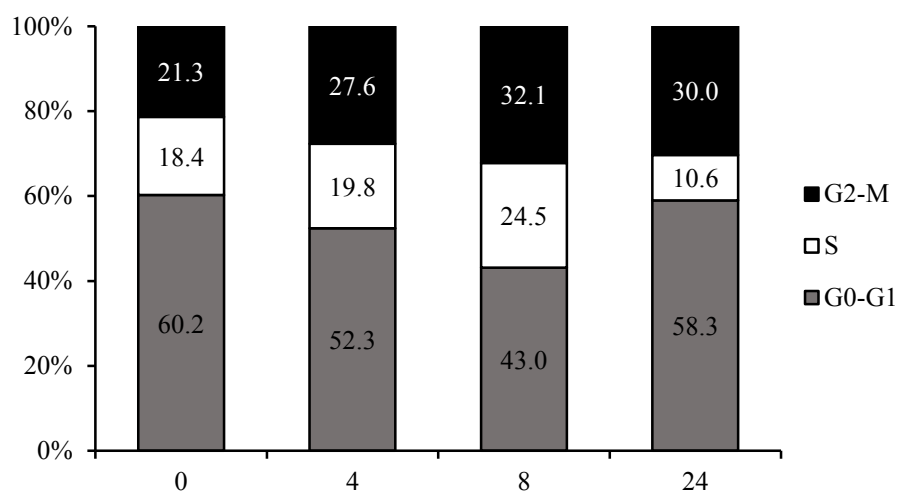
A**B**

Figure 3.16 LMTK3 is involved in homologous recombination repair.

Homologous recombination (HR) and non-homologous end joining (NHEJ) were assayed in MCF7 cells using DR-GFP reporter system bearing integrated direct green fluorescent protein. Cells were transfected with plasmids (pCMV6-LMTK3 or pCMV6 only) or co-transfected with pCBASceI plasmids into DR-GFP-MCF7 or EJ5-GFP-MCF7 cells. Forty-eight hours after transfection, cell were assayed for two-colour fluorescence analysis. **A)** Representative fluorescence plots of viable MCF7 DR-GFP cells transfected with indicated plasmid reagents, with percent GFP+ cells indicated. **B)** Percentage GFP-positive cells are indicated for each plasmid reagent, with standard error of mean based on three independent experiments. A total of 50,000 viable events were analysed for each experiment. Significant differences ($P < 0.05$) are marked by *.

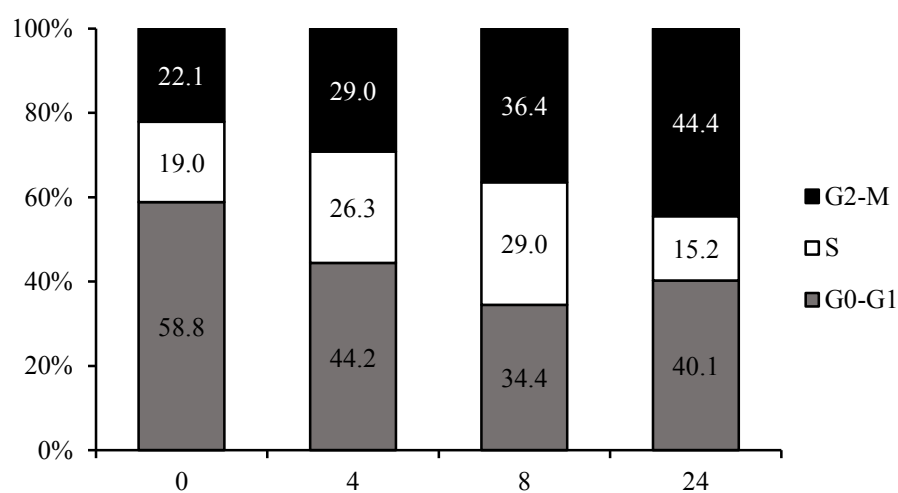
3.11 Overexpression of LMTK3 affects cell cycle distribution after DNA damage

To assess the impact of overexpressed-LMTK3 in cell cycle distribution after DNA damage, the flow cytometric analysis was carried out by using propidium iodide staining. It is known that doxorubicin blocks topoisomerase II in a state where causes attenuation at G₂ phase (Downes *et al.*, 1994). In this study, MCF7 cells treated with doxorubicin shows accumulation at G₂ phase. MCF7 cells overexpressing LMTK3 have a significant effect on cell-cycle distribution after doxorubicin exposure. At 0 h after doxorubicin release, a slight number of MCF7-LMTK3 cells accumulate in S and G₂ phases. More obvious after 4 h release of doxorubicin, MCF7 cells overexpressing LMTK3 arrest in S and G₂ phases compared to control cells (Figure 3.17). These observation are indicating that the effect of LMTK3 on DNA repair is more pronounced in S phase, in agreement with a role in homologous recombination repair.



release after Dox treatment

MCF7



release after Dox treatment

MCF7-LMTK3

Figure 3.17 Overexpression of LMTK3 affects cell cycle progression after treated with doxorubicin.

Cell cycle analysis of MCF7 (top) and MCF7-LMTK3 (below) cells was done at 0, 4, 8 and 24 h recovery periods after treated with 0.5 $\mu\text{g/ml}$ of doxorubicin for 2 h.

3.12 LMTK3 regulates DNA repair proteins

Since DNA damage response associated with promoting tumorigenesis, it is important to investigate whether LMTK3 affects DNA repair proteins in MCF7 cells. Here, LMTK3 was down-regulated with specific siRNA against LMTK3 or by overexpression of LMTK3.

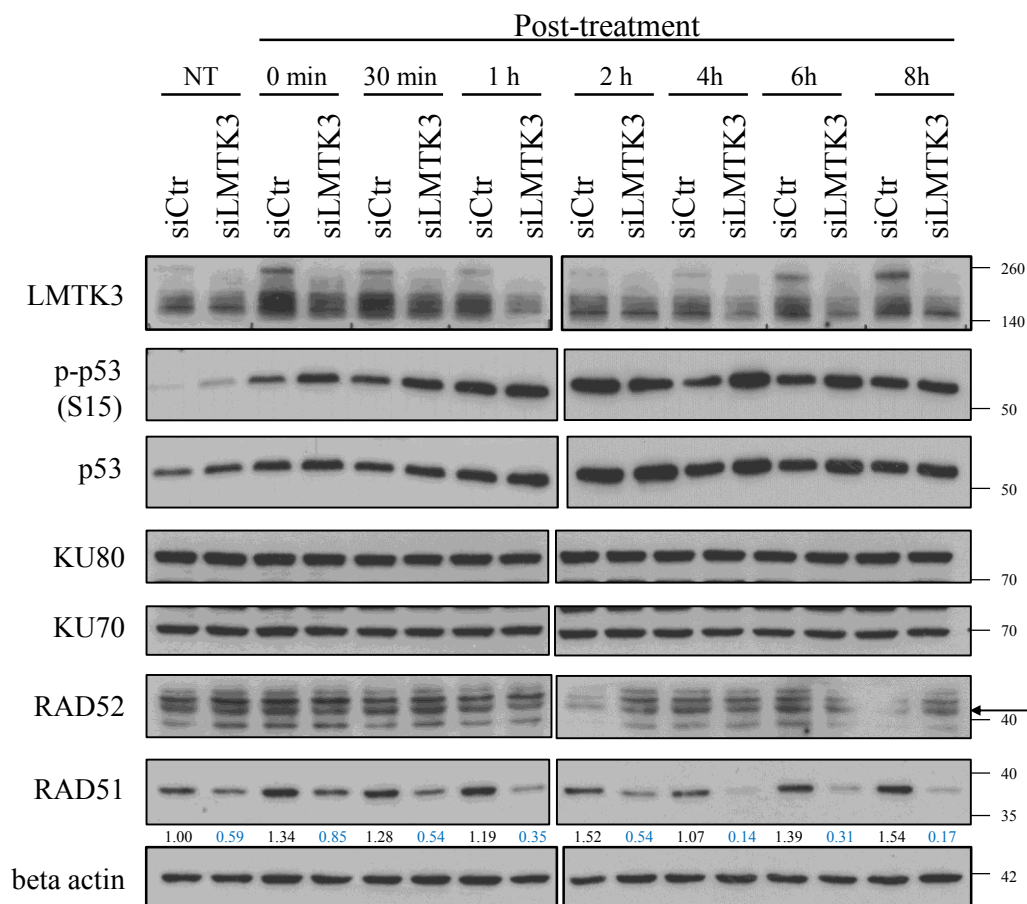
Cells were treated with 0.5 µg/ml of doxorubicin for 2 h to induce DNA breaks. Following drug treatment, the media was removed and incubated with fresh complete media for another 8 h. Cells were harvested according to indicate time points.

To ensure the changes in the events triggered by doxorubicin-induced DNA damage, phosphorylation of p53 Ser15 was measured. Phosphorylation of Ser15 is the primary target of the DNA damage response on the p53 protein mediated principally by the ATM and ATR protein kinases in response to genotoxic stress (Meek and Anderson, 2009, Meek, 2009). Similar to Kurz *et al.* (2004), treatment of cells with doxorubicin led to the phosphorylation of p53 Ser15. The increased level of phosphorylation of p53 Ser15 and total protein of p53 was irrespective of LMTK3 status (Figure 3.18).

To expand upon a possible role for LMTK3 in intracellular redistribution of repair proteins in response to DNA damage, the levels of four key repair proteins that involve for the repair of DNA DSBs: RAD51, RAD52, Ku70 and Ku80 were examined. LMTK3 knockdown reduced the expression of RAD51 and RAD51 protein levels were significantly decreased during the post-treatment period (Figure 3.18A). Conversely, an

increment of RAD51 protein expression was seen in MCF7 cells overexpressing LMTK3 compared with the wild-type (Figure 3.18B). Meanwhile, RAD52 protein that bind and wrap single-stranded DNA did not show apparent changes before or after treatment with doxorubicin. Neither the LMTK3 protein expression nor the doxorubicin treatment on the cells affects the KU heterodimer proteins expression. Demonstrably, RAD51, a central role player in homologous recombination mechanism is affected by LMTK3 expression.

A



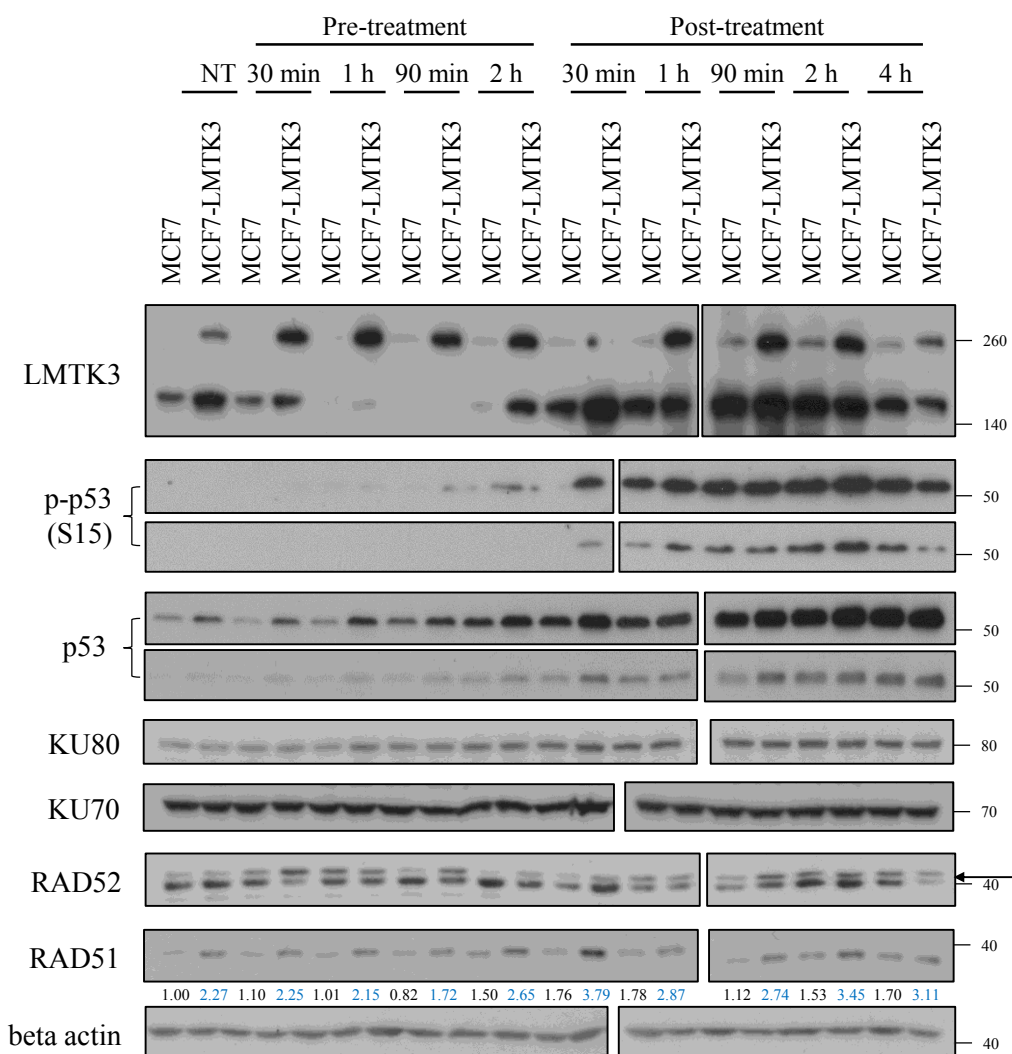
B

Figure 3.18 Effect of LMTK3 expression on DNA repair factors.

Cells were treated with 0.5 $\mu\text{g/ml}$ of doxorubicin. After 2 h, cells were washed and cultured in complete media. Cells were harvested at the indicated times and analysed by Western blot with the indicated antibodies. Beta actin was used as loading controls. **A)** LMTK3-silenced cells. **B)** LMTK3-overexpressing cells.

3.13 LMTK3 facilitates RAD51 foci formation and nuclear accumulation

In response to DNA damage, the RAD51 forms distinct nuclear foci that appear to associate with DNA breaks (Tashiro *et al.*, 2000b). Since Western blot analysis detected a net increase in RAD51 protein in over-expressed LMTK3 cells, it is important to analyse the nuclear pattern of RAD51 foci.

LMTK3-silenced as well as control mock-silenced cells were treated with 0.5 µg/ml doxorubicin. After 2 h, cells were grown in fresh medium for up to 4 h. Coverslips were collected, fixed, permeabilised and incubated with RAD51 antibody.

Notably, LMTK3 knockdown resulted in a significant decrease in the formation of RAD51 foci per cell. Furthermore, in cells treated with doxorubicin, LMTK3 ablation led to 59% reduction in RAD51 foci in comparison with the control cells after 2 h exposure (Figure 3.19). Figure 3.19 showed that RAD51 foci per cell were reduced in control cells after 4 h post-treatment.

As anticipated, MCF7 cells transfected with pCMV-LMTK3 showed higher RAD51 foci formation compared with the control cells. Upon doxorubicin treatment, the LMTK3-overexpressing cells displayed at least 30% more RAD51 foci than the control cells. The number of RAD51 foci per cell increased to about 75% in MCF7-LMTK3 as compared with the control cells after 4 h post-treatment (Figure 3.20), suggesting that LMTK3 is required for RAD51 foci formation in cells with DNA damage.

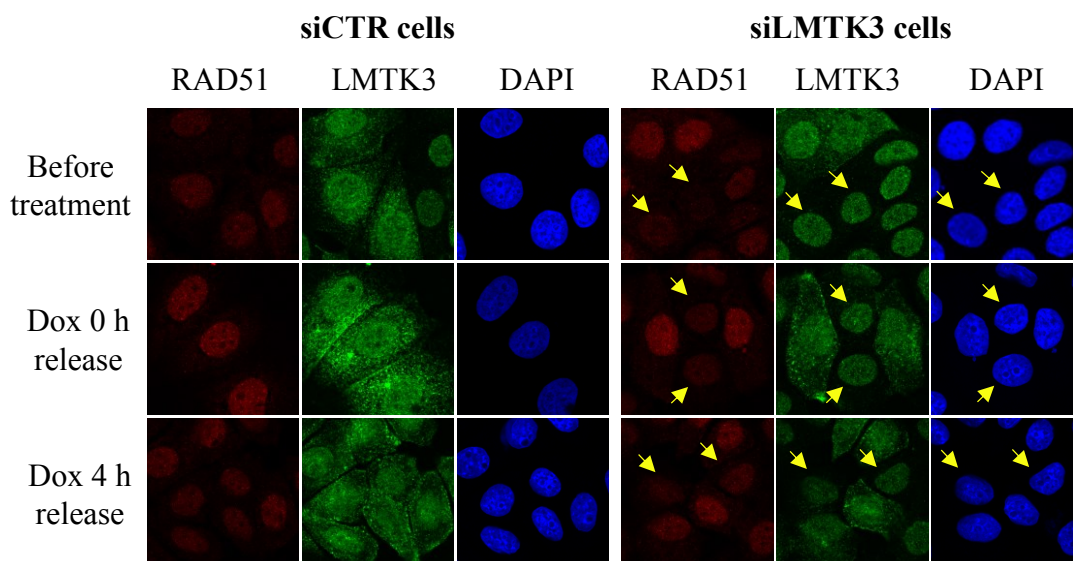
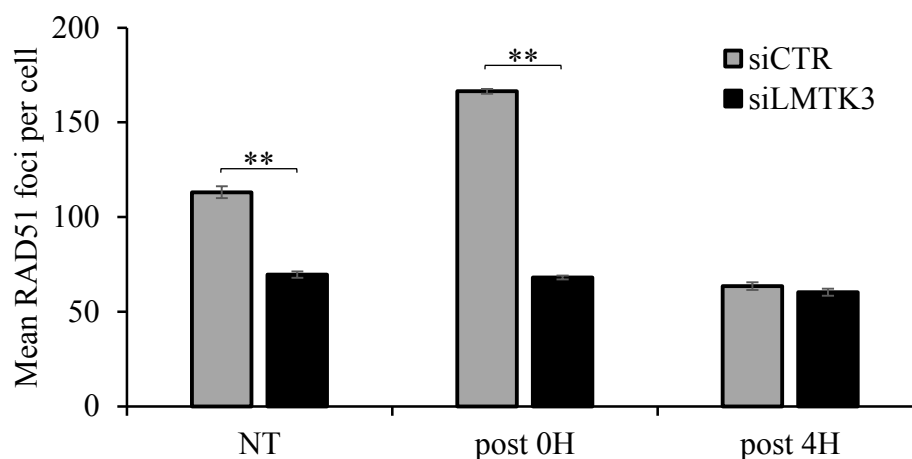
A**B**

Figure 3.19 Effect of LMTK3 silencing on the number of RAD51 foci per cell.

MCF7-LMTK3 cells were transfected with LMTK3 or CTR-siRNA and exposed to 0.5 $\mu\text{g/ml}$ of doxorubicin for 2 h. Cells were washed, grown in complete media and fixed with paraformaldehyde at the time points indicated. **A)** RAD51 (red) and LMTK3 (green) were visualised by immunofluorescence. Data quantification is presented in **B**. **B)** The bar graph represent mean of RAD51 foci per cell, for 50 cells per each time point. Significant differences ($P < 0.01$) are marked by **.

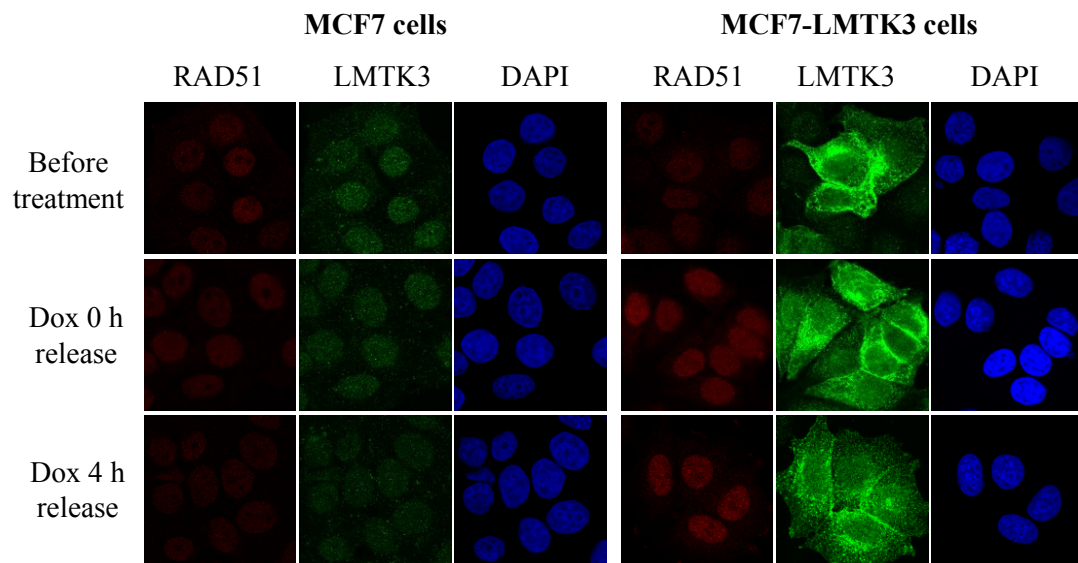
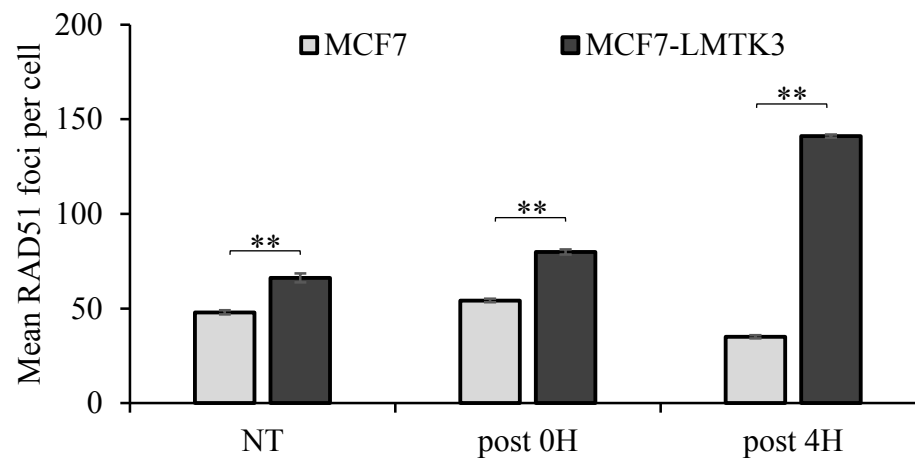
A**B**

Figure 3.20 Effect of overexpressed-LMTK3 on RAD51 foci formation.

MCF7 cells were transfected with pCMV-LMTK3 or empty vector and exposed to 0.5 $\mu\text{g/ml}$ of doxorubicin for 2 h. Cells were washed, cultured in complete media and fixed with paraformaldehyde at the time points indicated. **A)** RAD51 (red) and LMTK3 (green) were visualised by immunofluorescence. Data quantification is presented in **B**. **B)** The bar graph represent mean of RAD51 foci per cell, for 50 cells per each time point. Significant differences ($P<0.01$) are marked by **.

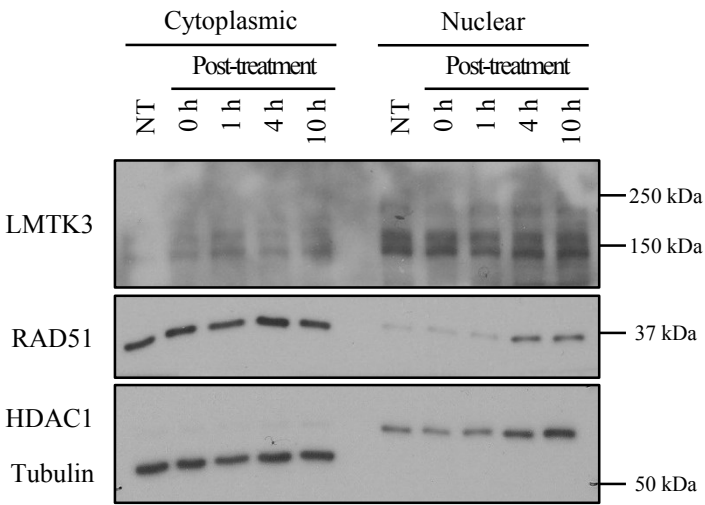
Next, to examine the effect of overexpressed-LMTK3 on the intracellular distribution of RAD51, immunoblotting analyses of proteins from the nuclear and cytoplasmic fractions were carried out.

The significant increase of the nuclear RAD51 levels was observed until 4 h after the induction of DNA damage. Subsequently, the decrease of nuclear RAD51 was observed at 10 h after doxorubicin removal (Figure 3.21). Based on the Figure 3.21A, the expression of cytoplasmic RAD51 showed only a slight increment. These results suggest that LMTK3 plays a role in the nuclear accumulation of RAD51 after the induction of DNA damage.

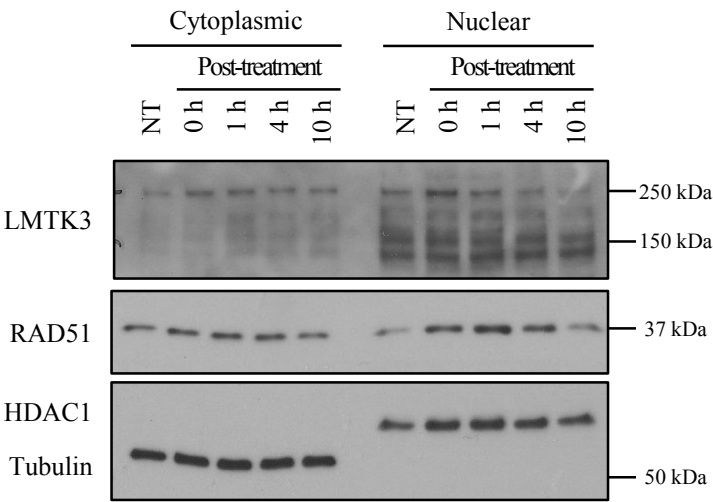
Together, these findings suggest that LMTK3 is pertinent for both the foci formation and nuclear accumulation of RAD51 in cells with DNA damage.

A

MCF7



MCF7-LMTK3



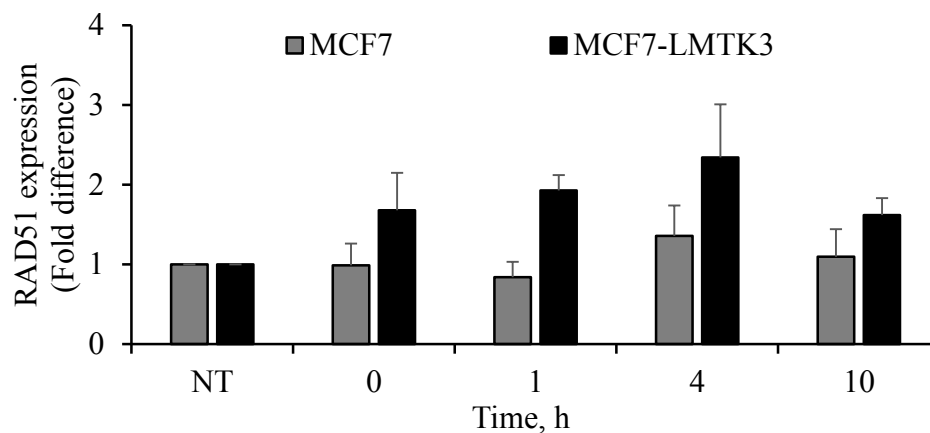
B

Figure 3.21 Induction of nuclear RAD51 protein in MCF7 over-expressed LMTK3 cells.

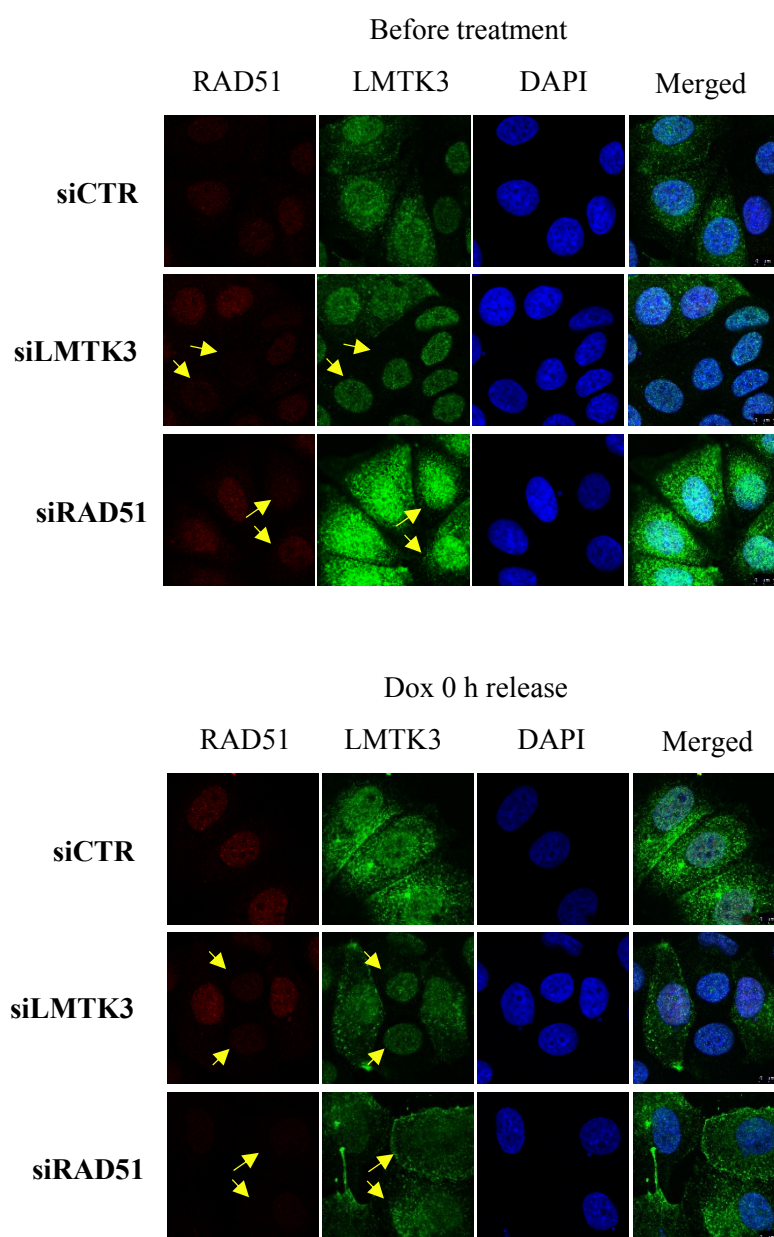
A) Increase of nuclear RAD51 in LMTK3 over-expressed cells. Western blot shows levels of RAD51 protein in cytoplasmic or nuclear fractions; NT and 0, 1, 4, 10 h after treatment with 0.5 $\mu\text{g/ml}$ of doxorubicin for 2 h, from MCF7 and MCF7-LMTK3 cells. Antibody against HDAC1 was used as a control to confirm the quality of the nuclear fraction. Tubulin was used to confirm equal protein loading. **B)** The bar graph represents RAD51 protein expression in nuclear fraction. The means and the error bars show the standard error of the mean for three independent experiments.

3.14 RAD51 does not affect LMTK3 expression

To access whether the loss of RAD51 affects the level of the LMTK3 protein, MCF7-LMTK3 cells were transfected with RAD51 siRNA and LMTK3 expression was determined by immunofluorescence and immunoblotting.

As shown in Figure 3.22, silencing of RAD51 had no effect on LMTK3 protein expression in the cells without doxorubicin treatment, which was lacked DNA damage. There was a slight increase of LMTK3 protein after the release of doxorubicin, during the DNA repair phase. Conversely, RAD51 protein expression was reduced by 65% in cells transfected with LMTK3 siRNA compared to mock-transfected cells (Figure 3.22B). Notably, the reduction in the RAD51 level was facilitated by the depletion of LMTK3 in the cells after treated with doxorubicin. It is important to note that neither silencing of RAD51 nor LMTK3 had an impact on KU70 protein expression (Figure 3.22B).

Collectively, the data attest that LMTK3 is not involved in non-homologous end joining pathway. Plus, these observations suggest that LMTK3 interacts with RAD51 and RAD51 acts downstream of LMTK3. In addition, RAD51 levels or stability may depend on the status of LMTK3 protein.

A

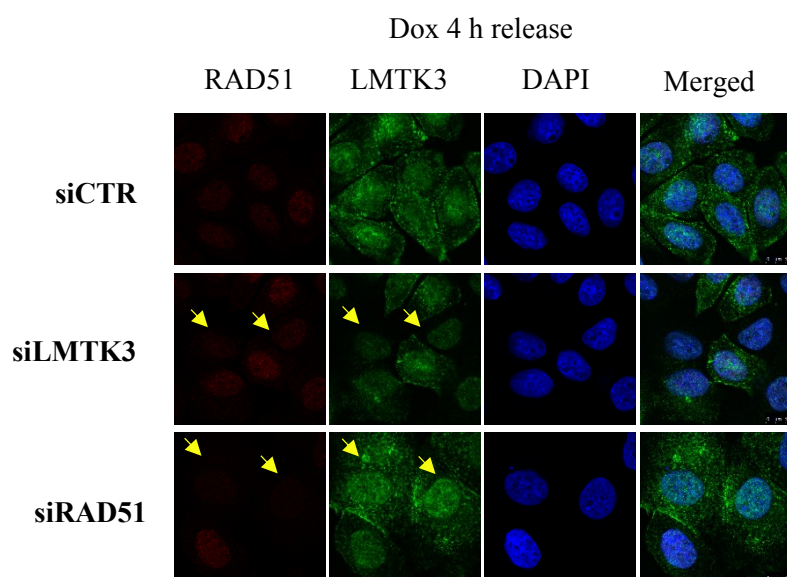
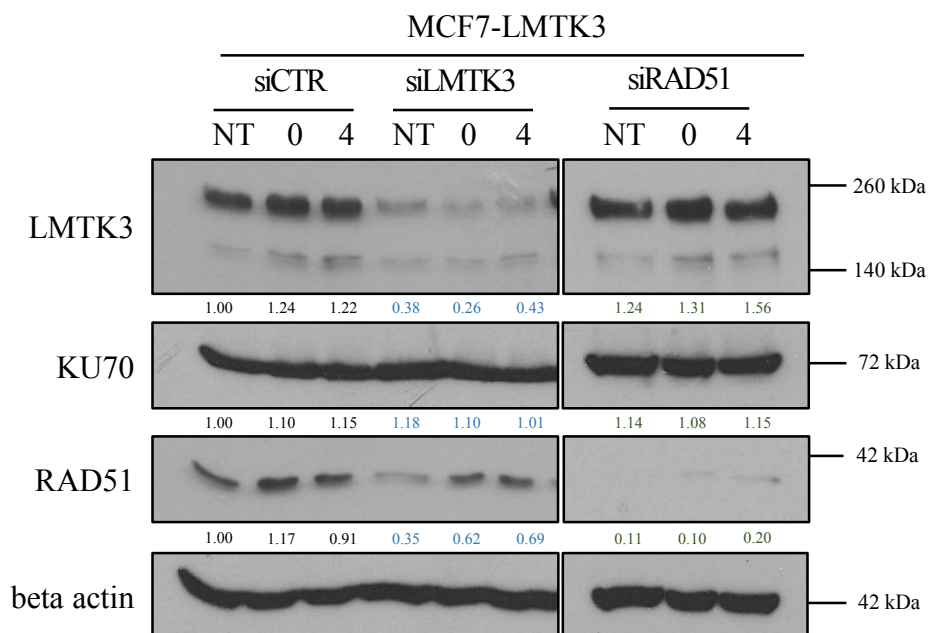
**B**

Figure 3.22 RAD51 does not affect the expression of LMTK3 protein.

Cells were transfected with RAD51 or LMTK3 or scrambled siRNA for 72 h. After exposure to 0.5 µg/ml of doxorubicin for 2 h, cells were cultured in normal medium and left to recover for 0 and 4 h before analysed. **A)** Immunofluorescence detection of RAD51 (red) and LMTK3 (green) expressions before and after RAD51 or LMTK3 depletion. **B)** Western blot shows levels of LMTK3, KU70 and RAD51 proteins upon silencing of RAD51 or LMTK3 in MCF7-LMTK3 cells and mock. Equal loading of protein lysates was confirmed with beta actin.

3.15 LMTK3 interacts with RAD51

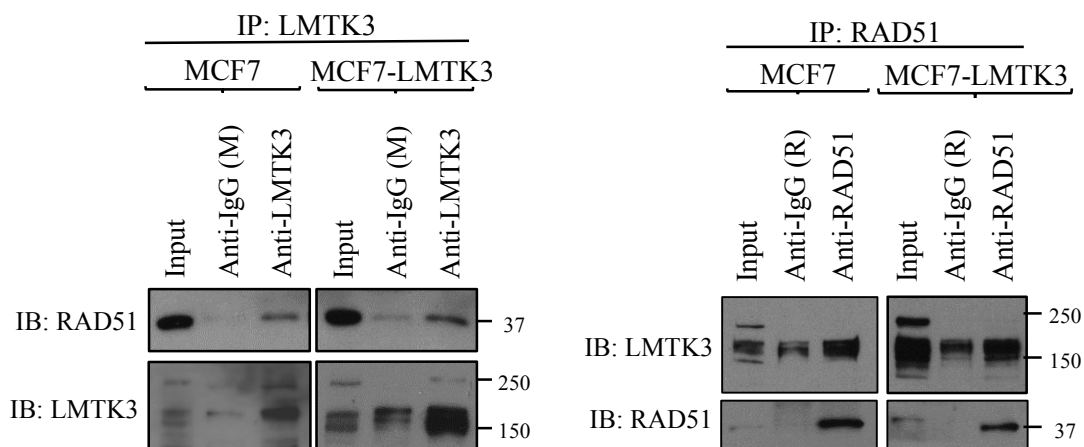
In order to verify whether LMTK3 associates with RAD51, immunoprecipitation of endogenous LMTK3 or RAD51 from MCF7 cells were performed. As a result, LMTK3 indeed links with endogenous RAD51 (Figure 3.22A). This was reconfirmed by the reciprocal immunoprecipitation / immunoblotting analysis of the exogenously expressed LMTK3 in MCF7 cells (Figure 3.23A).

To address whether the association between LMTK3 and RAD51 is affected by DNA damage response, MCF7-LMTK3 cells were treated with 0.5 $\mu\text{g/ml}$ of doxorubicin and protein lysates were prepared after 2 h of treatment and after 4 h post-treatment for immunoprecipitation analysis with anti-LMTK3, anti-RAD51 or anti-IgG control antibodies, followed by Western blot analysis with the indicated antibodies. As shown in Figure 3.20B, the association between LMTK3 and RAD51 was still intact during DNA damage response. This result implies that these two proteins formed a complex before the existence of DNA damage and was not dissociated by the induction of DNA damage and DNA repair processes.

To determine whether the interaction between LMTK3 and RAD51 is direct, a series of LMTK3 fragments fused to glutathione S-transferase (GST) were used (Figure 3.24A). GST-LMTK3 fragments were incubated with whole MCF7 cell lysate to performed *in vitro* GST pull-down assay. The blot was probed with an antibody against RAD51, revealing that part of the structurally disordered domains of LMTK3 ($\Delta 6$ and $\Delta 3$) were noticeably bind to endogenous RAD51 protein, whereas weak association was

detected with $\Delta 1$ (kinase and catalytic domain), $\Delta 2$ and $\Delta 5$ (Figure 3.24B). Thus, these findings suggest that as a kinase, LMTK3 might phosphorylate RAD51 protein.

A



B

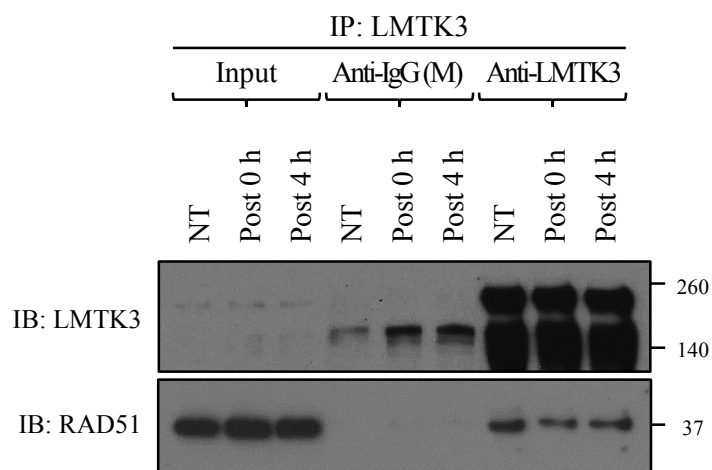


Figure 3.23 LMTK3 exists in a complex with RAD51 in vivo.

A) Lysates from MCF7 and MCF7-LMTK3 cells were immunoprecipitated with antibodies to LMTK3 (left) or RAD51 (right). The immunoprecipitates were analysed by Western blot with antibodies to RAD51 (left) or LMTK3 (right). **B)** MCF7-LMTK3 cells were treated with 0.5 μ g/ml of doxorubicin and harvested after 2 h of treatment (Post 0 h) and after 4 h post-treatment for sequential IP/Western blot analysis with the indicated antibodies.

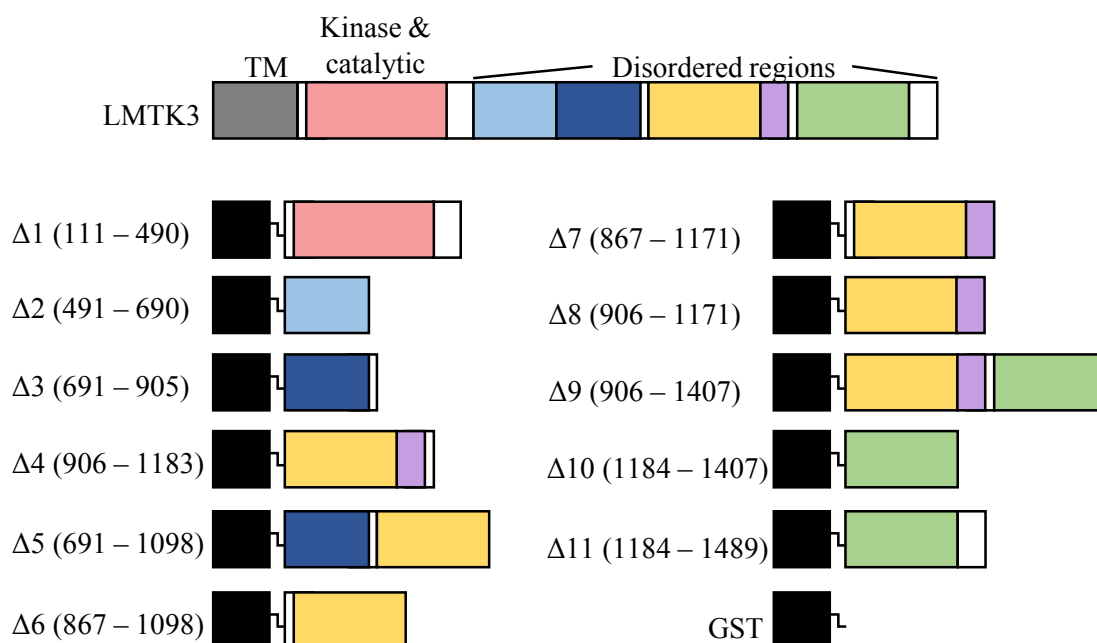
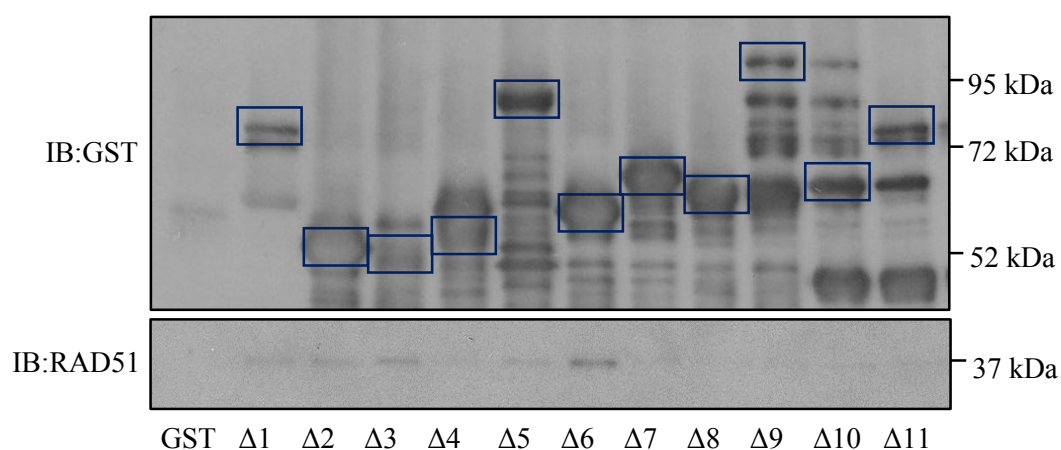
A**B**

Figure 3.24 LMTK3 binds RAD51 in vitro.

A) Schematic representation of GST-tagged LMTK3 fragments derivatives. **B)** GST-tagged LMTK3 fragment proteins attached to glutathione sepharose beads were incubated with 1 mg of MCF7 cell lysates and washed. The binding proteins were detected by SDS-PAGE and Western blot analysis with antibody against RAD51 or GST.

3.16 LMTK3 kinase domain does not phosphorylate the RAD51

To investigate whether the LMTK3/RAD51 interaction is a kinase-substrate process, *in vitro* kinase assay was performed. No phosphorylation was observed after performing the assay by using recombinant LMTK3 kinase domain (encompassing amino acids 149 - 444) as a source of enzyme activity and active human RAD51 full-length recombinant protein as substrate (Figure 3.25). This finding suggests that LMTK3 kinase domain may not function to regulate RAD51 phosphorylation.

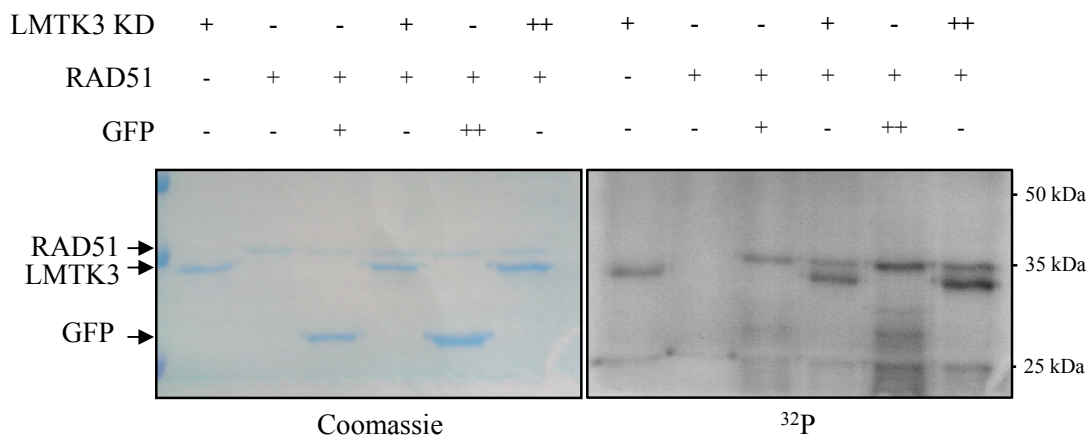


Figure 3.25 LMTK3 is not a kinase of RAD51.

Active RAD51 was incubated with purified LMTK3 kinase domain or GFP. The reaction products were analysed by Coomassie Brilliant Blue staining (left) and autoradiography (right).

3.17 LMTK3 regulates the stability of RAD51 protein

Next, to gain insight whether LMTK3 is involved in the regulation of RAD51 protein stability, cells were incubated with cycloheximide (CHX) to block new protein synthesis. Cells were then collected at various times after the addition of CHX, and RAD51 protein expression was determined by Western Blotting.

Immunoblotting analysis revealed that the overexpression of LMTK3 affects the degradation of RAD51 (Figure 3.26A). In the absence of *de novo* protein synthesis, levels of RAD51 protein decreased by approximately 45%, 70% and 90% at 5, 8 and 12 h after addition of CHX in the control MCF7-treated cells. In contrast, in MCF7-LMTK3-treated cells, RAD51 protein levels decreased by 30%, 50% and 73% at the same time points (Figure 3.26B).

Half-life of RAD51 protein was calculated using $N_t = N_0(1/2)^{t/t_{1/2}}$, where

N_0 is the initial quantity

N_t is the quantity that still remains after a time t

$t_{1/2}$ is the half-life

The half-life of RAD51 in MCF7 cells is 3.35 h, while in MCF7-LMTK3 is 6.11 h, implicating that LMTK3 remarkably enhanced RAD51 protein stability. These results suggest that LMTK3 may play a role to maintain the level of the RAD51 protein in cells.

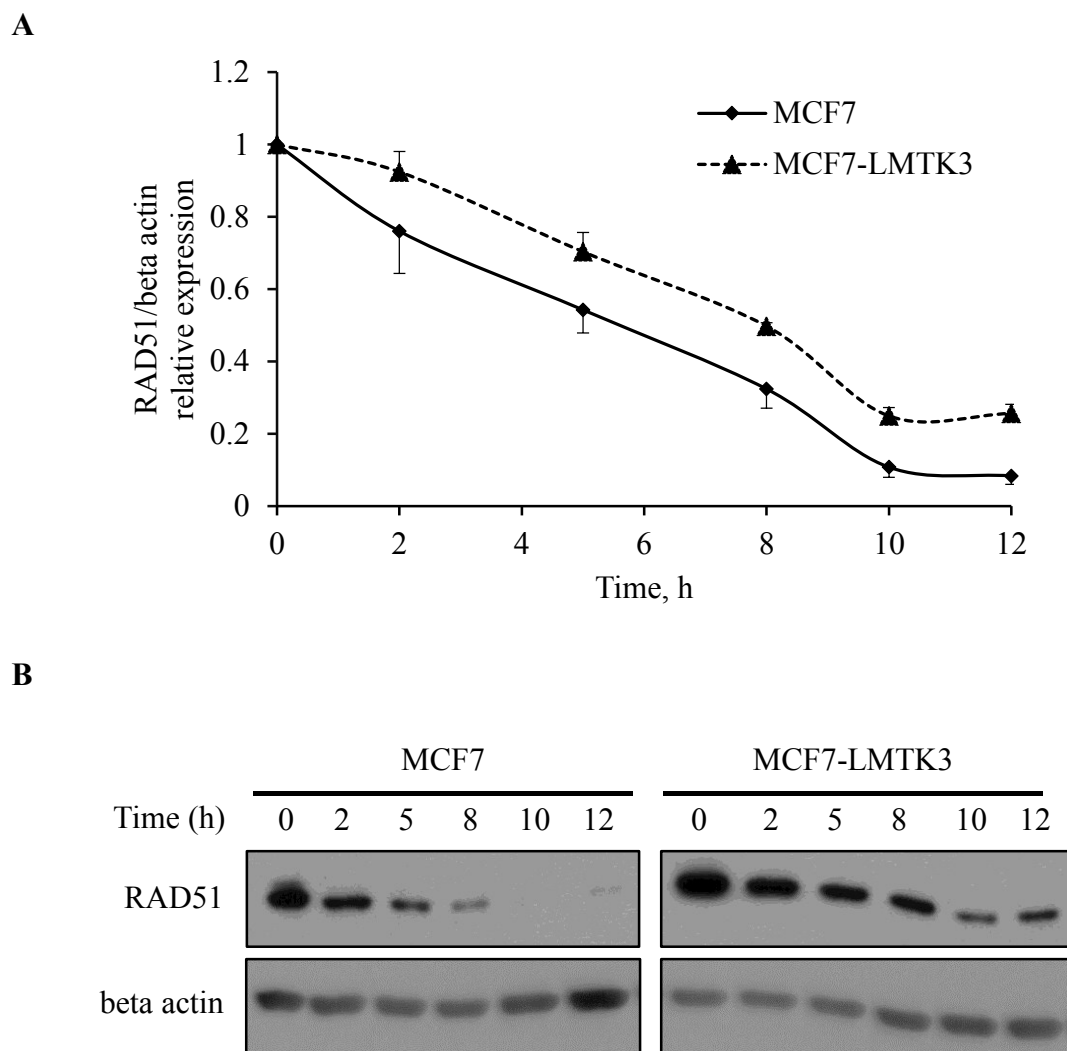


Figure 3.26 Increased RAD51 protein stability in cells with over-expressing LMTK3.

A) To access the relative protein stability of RAD51, MCF7 and MCF7-LMTK3 cells were collected at the indicated times after exposure to CHX (20 μ g/ml), and RAD51 protein expression was determined by Western blot analysis. The same blot was reprobbed with antibody against beta actin. **B)** Half-life of RAD51 protein was calculated using half-life formula. The line graph represents the means and the error bars show the standard error of the mean for three independent experiments.

To confirm this, the effect of the proteasome inhibitor on the RAD51 protein level in cells with or without overexpressing LMTK3 was examined. MG132, a 26S proteasome inhibitor and MG115, a 20S and 26S proteasome inhibitor were added for the final 6 h before harvesting MCF7 and MCF7-LMTK3 cells. As shown in Figure 3.27, both MG132 and MG115 affect basal RAD51 levels in MCF7 cells. Meanwhile, in MCF7-overexpressed-LMTK3 cells, the MG132 treatment did not change the RAD51 protein level. Similar results were obtained using MG115. These results revealed that LMTK3 controlled RAD51 protein stability through proteasome-mediated degradation. Taken together, these findings suggest that LMTK3 may functionally stabilise the RAD51 protein by repressing its degradation.

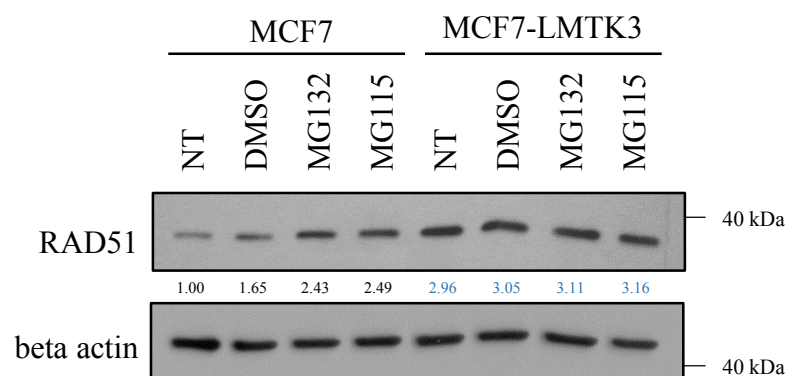
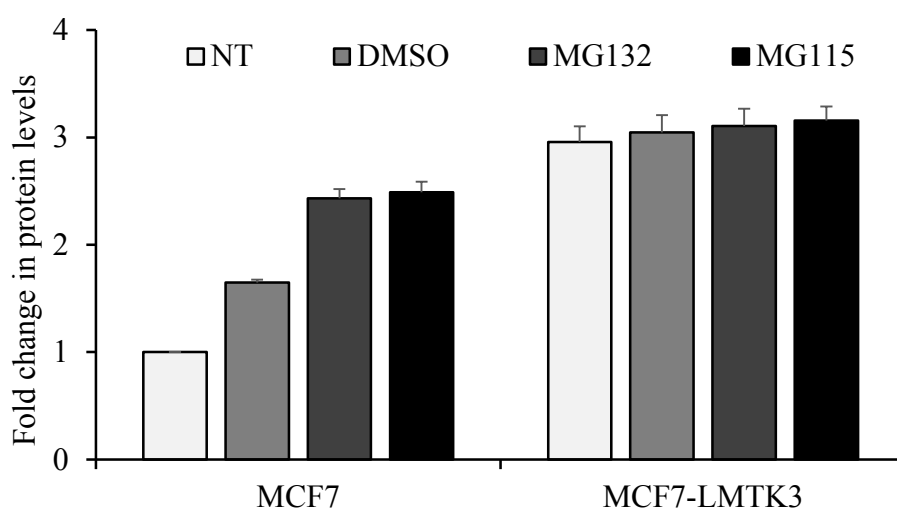
A**B**

Figure 3.27 LMTK3 represses proteasome-induced RAD51 degradation.

A) MCF7 and MCF7-LMTK3 cells were treated with proteasome inhibitors; MG132 or MG115 (10 μ M) for 6 h before harvesting. The amount of RAD51 protein was determined by immunoblotting using anti-RAD51 antibody. Beta actin was used as a loading control. **B)** The histogram shows the relative amounts of RAD51 protein from each drug treatment. The means and the error bars show the standard error of the mean for three independent experiments.

Chapter 4 DISCUSSION

LMTK3 acts as an oncoprotein which drives the progression of cancer cells. It has been described to regulate ER α transcription and its stability (Giamas *et al.*, 2011). The LMTK3 expression has been found to be significantly elevated in high-grade breast tumour and associated with poor survival in breast cancer patients (Stebbing *et al.*, 2012). While genome-wide studies have revealed that LMTK3 plays an important role in endocrine resistance via diverse signalling pathways (Stebbing *et al.*, 2013). Recently, a study has demonstrated that LMTK3 regulates integrins transcription, results in promoting breast cancer cell motility, migration and invasion (Xu *et al.*, 2014a). Furthermore, LMTK3 has been shown to mediate chromatin binding and gene regulation via its scaffold behaviour (Xu *et al.*, 2015) and also has been implicated in miRNA biogenesis (Jacob *et al.*, 2016).

The first objective of this study is to express LMTK3 in MCF7 and T47D cell lines. The *NEO* gene transiently co-transfect cells was selected with G418 to obtain an enriched pool of LMTK3 transfected cells. This enables the use of LMTK3 overexpressing cells to study the LMTK3 function.

As for the second and third objectives, overexpressed-LMTK3 cells were treated with doxorubicin to induce DNA DSB. In this study, LMTK3 is found to be a modulator of DNA damage response, when overexpressed, it decreased the phosphorylation of Ser1981 ATM, which eventually decreased Ser824 KAP1 and Ser139 H2AX and persist in doxorubicin treatment. In addition, overexpression of LMTK3 reduced H2AX

monoubiquitination, which is essential for H2AX phosphorylation. The present study provides evidence that LMTK3 is involved in DSB repair mechanism, particularly in HR and this pathway is depended on RAD51, which it protein stability regulated by LMTK3.

4.1 Expression of LMTK3 in MCF7 and T47D cells

This study was started with generating stable cell lines expressing abundant LMTK3. To this objective, the LMTK3 expression was studied in MCF7 and T47D cells, which both cell lines express a low level of endogenous LMTK3 and are less invasive. The overexpression of LMTK3 in these two cell lines have shown to promote invasive behaviour (Xu *et al.*, 2014a). In agreement with the previous study, these stably overexpressing LMTK3 in MCF7 and T47D cells exhibited more resistant towards doxorubicin treatment.

MCF7 or T47D cells were initially transfected with a construct carrying the human cDNA fused C-terminally to a Myc-DDK epitope. As compared to other transfection methods such as electroporation, nucleofection and calcium phosphate precipitation, cationic lipid based transfection by FuGENE HD reagent was used in this study. This transfection method is suitable because of simplicity, economical, less time consuming and produce a low level of cell toxicity (Asgharian *et al.*, 2014, Zaragosi *et al.*, 2007). Cells incorporated the plasmid containing kanamycin-neomycin marker into their genome were selected by using G418, rather than neomycin because normally

mammalian cells are resistant to neomycin, but are sensitive to its analogue, G418 (Borrebaeck and Hagen, 1989).

Immunoblotting analysis of MCF7-LMTK3 and T47D-LMTK3 cell lysates using anti-LMTK3 antibody demonstrated more than one LMTK3 bands (Figure 3.1B), which similar observation was seen in LMTK2 (Nixon *et al.*, 2013). The identity of these two bands was confirmed by mass spectrometry analysis as full-length LMTK3 proteins (Xu *et al.*, 2014a). The different molecular weight probably derived from the translation of alternatively spliced transcripts or alternative transcription initiation sites. This analysis also revealed that ectopic LMTK3 was expressed at levels equivalent to endogenous LMTK3 in both cell lines.

Cellular immunostaining against Myc and LMTK3 demonstrated predominantly in the cytoplasm compartment of MCF7-LMTK3 cells (Figure 3.1C). The presence of LMTK3 in both nucleus and cytoplasm raised the possibility that the protein shuttles between the two compartments. It is the responsible of the protein transport machinery in cells to ensure the right amount of protein is present at the right time and place. Mislocalisation of protein can be due to mutation, deregulation of proteins trafficking machinery, alteration of cargo proteins or oncogenic signalling (Hung and Link, 2011, Alkarain and Slingerland, 2003). Interestingly, one example of cancer-associated proteins, p27, switches its function from hindering proliferation when it localises in the nucleus, to promoting tumour progression when it expresses in the cytoplasm (Wander *et al.*, 2011). Indeed, previous studies have shown aberrant LMTK3 expression in the cytoplasm of high tumour grade breast cancer (Stebbing *et al.*, 2012) and its expression in the

cytoplasm is responsible for actin cytoskeleton remodelling that promotes tumorigenesis (Xu *et al.*, 2014a). Further study is required to define the signalling pathways regulates LMTK3 localisation.

4.2 LMTK3 and DNA DSB signalling

Previously, studies have shown that depleted-LMTK3 reduces cell proliferation in breast cancer cells and stably overexpressed-LMTK3 in MCF7 and T47D cells promotes the cells growth rate as compared to its parental cells (Giamas *et al.*, 2011, Xu *et al.*, 2014a). It is in the best interest to discover whether different levels of LMTK3 in cells are sufficient to affect their sensitivity against cytotoxicity.

In this present study, parental MCF7 and T47D cells were readily killed by doxorubicin, MCF7-LMTK3 and T47D-LMTK3 cells are resistant as determined by the SRB assay (Figure 3.2 and 3.3). Similarly, overexpression of LMTK3 promotes resistance of breast cancer cells to doxorubicin in mouse xenograft models (Figure 3.4), suggesting that LMTK3 may display in a similar way on primary tumours overexpressing LMTK3 in human.

Treatment with the DNA topoisomerase II inhibitor, doxorubicin increased transcriptional expression of LMTK3 after 16 h of treatment. Consistently, exposure to doxorubicin, induced LMTK3 protein expression in MCF7 wild-type cells (Figure 3.5). Earlier Xu *et al.* (2014a) has demonstrated the LMTK3 activates Ras and CDC42 through

the binding of GRB2. The activation of Ras leads to a notable increase in ERK/MAPK pathway, while activation of CDC42 promotes integrin activity, especially integrin subunits α_5 and β_1 . Several studies have shown that the activated Ras is not only involved in tumour progression but also in the development of resistance of tumour cells to chemotherapeutic drugs as well as radiation treatment (Gupta *et al.*, 2001, McCubrey *et al.*, 2007). Activation of Ras in breast cancer cells after exposure of doxorubicin was reported favouring phosphoinositide-3-kinase (PI-3K)/Akt pathway than MAPK pathway (Jin *et al.*, 2003, Tari *et al.*, 2001). In addition, Morozevich *et al.* (2011) showed that depleted- $\alpha_5\beta_1$ integrin in MCF7-Dox cells distinctly reduced its resistance to doxorubicin. The question of what molecular executioner leads to the activation of Ras or integrin after chemotherapy, especially in doxorubicin still remain unknown. Some studies postulated that HER2 or FAK might activate Ras to induce resistance to doxorubicin (Luo and Fu, 2014). Ras and integrin are effectors pathways of LMTK3 in regulating cell growth and cytoskeleton reorganisation. Therefore, it could be possible that LMTK3 is an upstream activator of Ras and integrin in combating against doxorubicin-induced cytotoxicity.

In order to elucidate the underlying mechanistic role of LMTK3 in doxorubicin-induced DNA damage, the most upstream protein kinases against DNA damage, ATM and ATR, were examined. Numerous studies have shown that doxorubicin generates DSBs and ATM is activated by DSBs (Lee *et al.*, 2007, Li *et al.*, 2007, Reinhardt *et al.*, 2007). Consistent with previous reports, exposure to doxorubicin-induced phosphorylation of ATM at Ser1981 in MCF7 control cells (Figure 3.6). Phosphorylation and its autophosphorylation at Ser1981 leads to dissociation of an inactive ATM dimer

to generate a catalytically active monomer form and initiates ATM kinase activity as well as stabilise ATM at the sites of DSBs (Bakkenist and Kastan, 2003, So *et al.*, 2009). Interestingly, phosphorylation of ATM at Ser1981 and phosphorylation of KAP1 at Ser428 were significantly impaired in cells overexpressing LMTK3 (Figure 3.6 and 3.7). Following, silencing of ATM reduced LMTK3 expression. Upon induction of DSBs by doxorubicin, the loss of ATM further decreased LMTK3 expression (Figure 3.7A). These observations imply that a crucial role of ATM to stabilise LMTK3 and how this interaction hinders ATM phosphorylation in response to DNA damage is still remain elusive.

A decade ago, a novel ATM signalling pathway defined transcriptional co-repressor in regulating the expression of a subset of genes linking to genome surveillance was demonstrated. KAP1, previously known as a co-repressor of gene transcription is phosphorylated by ATM exclusively at DNA lesions, which leads to chromatin relaxation and stimulate the cells sensitivity to DSB-inducing agents (White *et al.*, 2006, Ziv *et al.*, 2006). Doxorubicin-mediated ATM activation elicits phosphorylation of KAP1 on Ser824 and inhibits KAP1 sumoylation. Subsequently, ATM-dependent KAP1 Ser824 phosphorylation de-represses transcription of p21, Gadd45 α , Bax, Puma and Noxa which promoting cell cycle control and apoptosis in response to doxorubicin (Li *et al.*, 2007, Lee *et al.*, 2007). Recently, LMTK3 has been shown as a transcriptional co-repressor through interacting with PP1 α and KAP1. Overexpression of LMTK3 suppresses KAP1 phosphorylation at Ser824 (Xu *et al.*, 2015). Taken together, doxorubicin-induced KAP1 Ser824 phosphorylation may be overwhelmed by the massive expression of LMTK3 that

keeps KAP1 as an active co-repressor. These may disturb the balance between Ser824-phosphorylated KAP1 and KAP1 that eventually delays the induction of DNA damage and the de-repression of LMTK3-bound tumour suppressor-like genes, cell cycle control as well as apoptosis genes. Therefore, overexpression of LMTK3 reduces cells sensitivity to doxorubicin treatment.

Although, ATR is often activated by RPA-coated ssDNA, its activation at DSB is also ATM-dependent (Myers and Cortez, 2006, Cuadrado *et al.*, 2006, Jazayeri *et al.*, 2006). Despite ATR total protein was increased in MCF7-LMTK3 as compared with control cells, its phosphorylation at Ser428 did not change following doxorubicin treatment (Figure 3.6). Similarly, Huelsenbeck *et al.* (2012) showed that phosphorylation of ATR at Ser428 in rat cardiomyoblast cells was also not evidently increased in response to doxorubicin. In fact, several reports indicated that phosphorylation at Ser428 is not correlated with ATR activity and demonstrated that phosphorylation at Thr1989 of ATR is the marker of active ATR in response to DNA damage (Liu *et al.*, 2011, Nam *et al.*, 2011). In this study, silenced-ATR did not regulate LMTK3 expression before or after doxorubicin treatment (Figure 3.8B). These results suggest that LMTK3 is not regulated by ATR, but maybe LMTK3 regulates ATR expression due to an increase in ATR total protein in MCF7-LMTK3 cells.

In the early DNA damage response, activated ATM initiates phosphorylation of H2AX at Ser139 and forms foci at the sites of DNA lesions. Therefore, a formation of foci is an indicator of DSB (Rothkamm and Horn, 2009, Kuo and Yang, 2008). It is important to note that γ H2AX foci may also presence in small numbers even in the

absence of exogenous damage. It can be due to DNA damage that can happen during replication, viral infection, senescence, carcinogenic adducts, exposure to reactive oxygen species and other cellular processes (Bonner *et al.*, 2008). As observed, doxorubicin exposure induced accumulation of γ H2AX in overexpressed-LMTK3 and its control cells. Interestingly upon doxorubicin treatment, the focus formation was lesser (Figure 3.9) and the kinetic of foci formation was lagged (Figure 3.10) in overexpressed-LMTK3 cells as compared with its control cells. Furthermore, abrogation of LMTK3 was shown to promote expression of γ H2AX (Figure 3.12). Here, the findings presented indicate that LMTK3 is involved in DNA damage response. Although LMTK3 is shown to modulate γ H2AX expression, these two proteins did not colocalise (Figure 3.13). Several studies have shown doxorubicin treatment in cells led to the phosphorylation of H2AX at Ser139 with dependent on ATM (Bakkenist *et al.*, 2015, Brum *et al.*, 2012, Jung *et al.*, 2008, Kurz *et al.*, 2004). Following DNA DSBs, ATM phosphorylates serine residue 139, which is located in the carboxy-terminal tail of H2AX (Rogakou *et al.*, 1998). The reduction of Ser139-phosphorylated H2AX in overexpressed-LMTK3 cells can be explained by the previous observation that overexpression of LMTK3 reduces phosphorylation of ATM at Ser1981 at the initial response. Another possibility can also be due to ubiquitination of H2AX. Recent studies have highlighted the importance of ubiquitylation of H2AX in DNA damage which involves RNF8 and RNF168, RING-finger ubiquitin ligases (Bergink *et al.*, 2006, Bergink and Jentsch, 2009, Lukas and Bartek, 2009, Messick and Greenberg, 2009). In addition, Pan *et al.* (2011) showed another RING-finger ubiquitin ligase, RNF2 mediated H2AX monoubiquitination that is required for γ H2AX formation and subsequently recruitment of early DDR proteins. It is

possible that the decrease in monoubiquitination of H2AX and γ H2AX (Figure 3.14) that is not only mirrored by ATM Ser1981 phosphorylation but may be due to the regulation of LMTK3 on this ubiquitination event. Further studies are needed to address this possibility.

4.3 LMTK3 and DNA DSB repair

As observed, Figure 10 shows in the absence of doxorubicin, the kinetics of γ H2AX foci in cells with replenishing fresh media decrease gradually. Strikingly, the disappearance of these foci is more rapid in cells overexpressing LMTK3, suggesting LMTK3 expression modulates DNA repair mechanism. Since the relationship between the kinetics of γ H2AX foci disappearance and DSBs repair is debatable, the comet assay is used to measure the repair activity of cells (Collins and Azqueta, 2012). The comet assay also known as single cell gel electrophoresis detects DNA strand breaks and these single nucleoids are subjected to electrophoresis that results in comet structure with the intensity of the tail relative to the head reflects the number of DNA breaks (Azqueta *et al.*, 2014). The results from the comet assay strengthen the data previously, indicating overexpression of LMTK3 was contributed to the rapid repair of damaged DNA.

DNA DSBs in mammalian cells are primarily repaired by HR and NHEJ. The mechanisms that influence the choice between competing for DSB repair pathways can be regulated by DSB-responsive factors that enriched at DSB sites, cell cycle phases

involved, the presence of an identical sister chromatid and probably by chromosomal abnormalities that lead to aberrant of certain proteins (Chapman *et al.*, 2012). The present study showed that LMTK3 promotes DSB-induced homologous recombination (Figure 3.16). These findings show that LMTK3 has an important role in DSB repair by homologous recombination and that this accounts for the resistant of overexpressed-LMTK3 cells to doxorubicin.

Currently, there is evidence show that cell-cycle functions and the repair are associated genetically (Taniguchi *et al.*, 2002, Xu *et al.*, 2002, Zhang *et al.*, 2004). In addition, factors that modulate cell-cycle checkpoints may affect DNA repair and contribute to drug resistance. In this study, doxorubicin induced the accumulation of control cells in the G2 phase, while in the similar condition, overexpressed-LMTK3 cells were seen more in S and G2 phases (Figure 3.17). At present, there are no data links between LMTK3 expression and cell-cycle checkpoints regulation. Nonetheless, this current results demonstrated doxorubicin-induced overexpressed-LMTK3 cells were arrested more in S and G2 phase was coherent with it selection to undergo the HR repair mechanism. Studies have shown that HR is more prevalent during S and G2 phases of the cell cycle when sister chromatids are more easily available (Branzei and Foiani, 2008, Yun and Hiom, 2009, Sartori *et al.*, 2007).

Consistently, in Western blot analysis, silencing of LMTK3 reduced the expression of RAD51 in response to doxorubicin or vice versa (Figure 3.18). Mammalian RAD51 is a highly conserved RecA-like protein that has a key role in homologous recombination. Upon the occurrence of DSBs, RAD51 is recruited to sites of damage,

where it promotes DNA homologous pairing and strand exchange. RAD51 also involves in induced sister chromatid exchange in mammalian cells (Baumann and West, 1998, Lambert and Lopez, 2001). It has been reported that RAD51 nuclear foci are the sites of repair of DNA damage (Raderschall *et al.*, 2002, Tashiro *et al.*, 1996, Tashiro *et al.*, 2000a). In this study, LMTK3 was required for RAD51 foci formation in the nucleus (Figure 3.19 and 3.20) and controlled the nuclear level of the RAD51 protein (Figure 3.21), suggesting that LMTK3 regulates HR by controlling the amount of RAD51 to sites of HR. Notably, the depletion of RAD51 did not change LMTK3 expression but in another way around, depletion of LMTK3 significantly reduced RAD51 expression (Figure 3.22). Importantly, LMTK3 has identified directly binds to RAD51 protein under both conditions, normal and doxorubicin-induced (Figure 3.23 and 3.24). Previous studies demonstrated several proteins that interact physically with RAD51 can modulate its function (Popova *et al.*, 2011, Yuan *et al.*, 1998). For example, phosphorylation of RAD51 at Tyr54 by c-Abl inhibits RAD51 function in DNA strand exchange (Yuan *et al.*, 1998). However, the *in vitro* kinase assay analysis conducted showed that LMTK3 kinase domain did not phosphorylate RAD51 (Figure 3.25). Taken together, these findings suggest that LMTK3 directly binds to RAD51 and regulates RAD51 foci formation and expression in the nuclear but is not through RAD51 phosphorylation.

In this study, LMTK3 was required to maintain the level of RAD51, especially in the nuclear compartment. Overexpression of LMTK3 resulted in a decrease of RAD51 proteasome-mediated degradation, stabilise RAD51 protein (Figure 3.26 and 3.27). This explains the increment of RAD51 in nuclear was a result of LMTK3 overexpression or

vice-versa. The possible mechanism involved in maintaining the level of RAD51 in the nuclear compartment is mediated by direct binding between LMTK3 and RAD51, which directs RAD51 to the nucleus (bind to chromatin) and hence protects it from being degraded. As RAD51 accumulates at DSB sites and forms a nucleoprotein filament on the ssDNA (Atwell *et al.*, 2012, Wyman and Kanaar, 2006), it is possible that LMTK3 facilitates the binding of RAD51 and recruits RAD51 to filaments. This suggestion was supported by the observations that LMTK3 was not only required to form RAD51 foci in response to doxorubicin treatment, but also for RAD51 stability. LMTK3 promotes RAD51-dependent HR by regulating RAD51 through maintaining its protein level. Therefore, these findings show overexpressed-LMTK3 cells can become resistant due to the enhanced repair of DNA.

Chapter 5 CONCLUSIONS

This work aimed to identify the LMTK3 role in DNA damage repair. Initial work to generate stably overexpressed-LMTK3 cells lines were successful although it took longer than expected. Using the stably overexpressed-LMTK3 cell lines in the current studies demonstrated that it displayed doxorubicin-induced phenotype. The studies showed that:

- 1 LMTK3 is involved in DNA damage response. The observed phenotype shown by overexpressed-LMTK3 cells can be due to the interaction between LMTK3 and ATM that delays Ser1981 ATM phosphorylation leads to the reduction in phosphorylation of KAP1 at Ser428, ultimately continue to repress the transcription of tumour suppressor genes, cell control as well as apoptosis genes.
- 2 Despite the fact that the decline of ATM activation caused the reduction of γ H2AX foci in overexpressed-LMTK3 cells during doxorubicin treatment, it is also possible that reduction of H2AX phosphorylation is facilitated by the down-regulation of LMTK3 on H2AX ubiquitination.
- 3 In the initial investigation, overexpression of LMTK3 reduced the number of γ H2AX foci and DNA breaks, suggesting that LMTK3 may involve in DNA repair. This notion was proved by using GFP-based reporter systems. LMTK3 promoted DSB-induced homologous recombination and its choices of DSB repair pathway was consistent with the regulation of cell cycle.

- 4 Next, LMTK3 promoted homologous recombination by regulating RAD51 via the maintenance of RAD51 protein levels.

Herein, our work provides an overview of the molecular understanding of how cells with overexpressing LMTK3 able to survive in the doxorubicin treatment. Although there are still many questions to address, the current data in this study provide a platform for LMTK3 to be an attractive therapeutic target in cancer therapy.

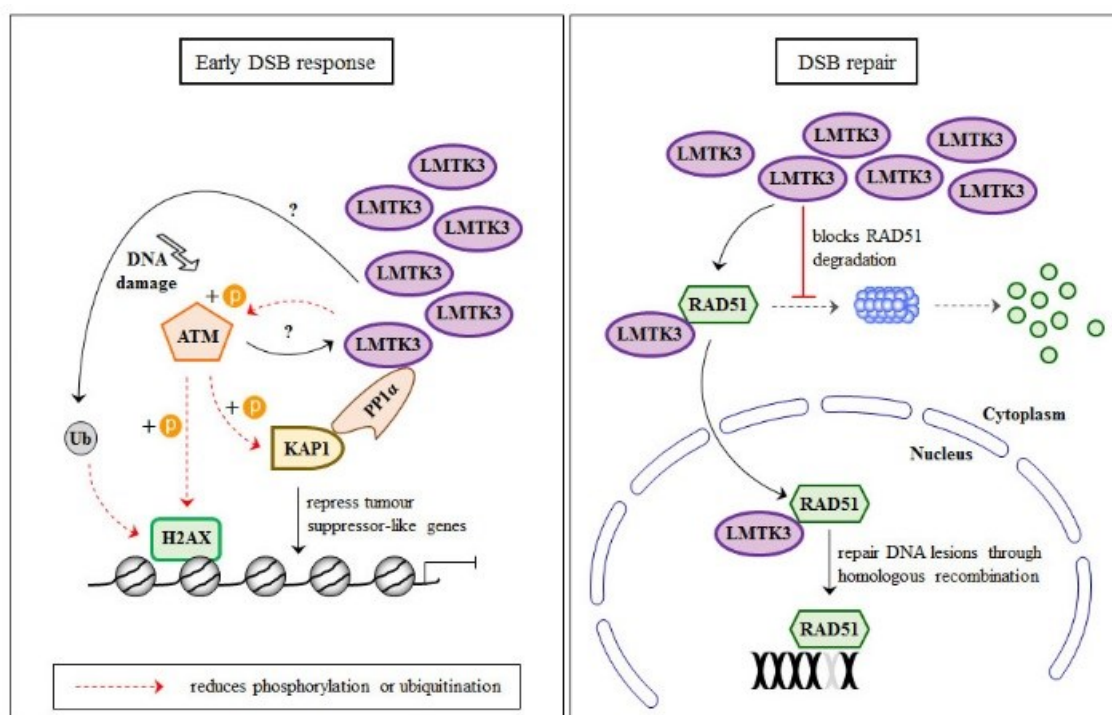


Figure 5.1 Schematic representation of the proposed model is given.

Immediate upon DSB formation, ATM is phosphorylated at Ser1981. Overexpression of LMTK3 delays Ser1981 phosphorylation and H2AX monoubiquitination, which eventually reduce phosphorylation of KAP1 at Ser428 and H2AX at Ser139. Ultimately, these events continue to repress the transcription of tumour suppressor-like genes and decelerate recruitment of DNA damage signalling proteins. During DSB repair, LMTK3 interacts with RAD51 stabilising its protein levels. RAD51 is then translocated to the nucleus to repair the DNA lesions through homologous recombination mechanism.

Recommendation of future research

Cellular response to DSBs involves a variety of pathways that require further investigation. We describe for the first time that LMTK3 is involved in DNA damage response. In this study, overexpression of LMTK3 has been shown to suspend the DNA damage processes. Tian *et al.* (2009) demonstrated that CDK5 activated by DNA damage, directly phosphorylates ATM at Ser794 and CDK5-ATM signal regulates phosphorylation and function of ATM targets such as H2AX. A recent study revealed that CDK5 also phosphorylates LMTK3 *in vitro* (Lucchiari et al., 2016). Therefore, it is possible that doxorubicin-induced DNA damage phosphorylates LMTK3 through activated CDK5. This event could negatively regulate the ATM phosphorylation at Ser1981. Although activated CDK5 phosphorylates ATM at Ser794 and causes ATM autophosphorylation at Ser1981, overexpression of activated LMTK3 could overwhelm the Ser1981 phosphorylation activity.

- In order to determine if LMTK3 prevented phosphorylation is PIKK-dependent, MDA-MB-231 cells (endogenous LMTK3 expressed abundantly) will be pretreated with wortmannin, an inhibitor of several PIKK family members, including ATM and then probe for ATM Ser1981 phosphorylation. Anticipated results will show that wortmannin strongly inhibits the phosphorylation of ATM of Ser1981 in siLMTK3 cells, while pretreatment with staurosporine, a broad-spectrum protein kinase inhibitor, should not affect ATM protein kinase activity.

- To confirm whether LMTK3 delays autophosphorylation of ATM, GFP-labeled wild-type ATM (ATM^{WT}), GFP-kinase-dead ATM (ATM^{KD}) or GFP-vector alone constructs will be transfected in to the ATM-deficient cell line AT1ABR and cells will then be treated with doxorubicin. Neither siLMTK3 cells nor doxorubicin induce ATM Ser1981 phosphorylation in cells expressing ATM^{KD}.
- If the above results show that LMTK3 negatively regulates ATM Ser1981 phosphorylation, the following thing will be to investigate whether LMTK3 interact with ATM. Endogenous ATM will be immunoprecipitated from untreated cells or cells treated with doxorubicin and immunoblotted for LMTK3. The reciprocal experiment will be conducted as well.

As shown in this thesis, overexpression of LMTK3 delays the resolution of γ H2AX foci but it does not colocalise with γ H2AX foci. Interestingly, LMTK3 impedes monoubiquitylation of H2AX. Ubiquitination of H2AX is an important marker for DNA lesions in DDR.

- In unpublished data from SILAC (stable isotope labeling with amino acids in cell culture) experiment, there are several other LMTK3-regulated proteins involved in the regulation of DNA damage induced by doxorubicin, such as E3 ubiquitin-protein ligase UBR5. UBR5 is known as a regulator of DNA damage response by suppressing RNF168, an E3 ubiquitin-protein ligase that promotes accumulation

of Lys-63-linked histone H2A and H2AX at DNA damage sites (Gudjonsson *et al.*, 2012). It could be possible that LMTK3 positively regulates UBR5 and eventually hampers H2AX monoubiquitylation through suppressing RNF168. In order to examine whether LMTK3 binds with UBR5, immunoprecipitation of endogenous LMTK3 or UBR5 from cells can be performed. To address whether the binding between LMTK3 and UBR5 is affected by DNA damage response, cells will be treated with doxorubicin before conducting the immunoprecipitation experiments. Following, if there is a binding, in order to determine whether the interaction is direct *in vitro* GST pull-down assay will be carried out using different GST-LMTK3 fusion protein that are available in our lab.

We also demonstrated that asynchronous MCF7 cells overexpressing LMTK3 accumulate more in S and G₂ phases. In G₀ and G₁, DNA DSBs are repaired by NHEJ, where as in S and G₂ phases, they are also repaired by homologous recombination.

- In order to confirm whether LMTK3 recruitment to Dox-induced DSBs occurs only in S and G₂ phases, serum starvation and treatment with other arresting agents for synchronisation of cells can be carried out. Cells will be released and treated with doxorubicin to induce DSBs. The expression of different cyclins as a marker for different phases will be used. Anticipated results will include an LMTK3 abundance in S phase, in support of the cell-cycle control of HR repair and while probably absent or less LMTK3 levels will be present in cells arrested

in G₁ phase. These anticipated findings will be consistent with studies showing that cells arrested in G₁ favour NHEJ over HR. Importantly, LMTK3 remains abundant in G₂ phase to show that the disappearance of LMTK3 in G₁ is not an indirect consequences of growth arrest. To explore the absence of LMTK3 in G₁-arrested cells is due to rapid turnover, cells can be synchronised in early S phase by hydroxyurea treatment and then released from the arrest.

- CDKs are playing important roles in regulating cell cycle. In order to examine whether LMTK3 is a CDKs substrate, a phospho-CDK substrates antibody can be utilised. To confirm the finding, samples can be treated with λ phosphatase which will block the phosphorylation of LMTK3. Similar set up with the induction of DSBs with doxorubicin can be carried out to verify LMTK3 might be phosphorylated by CDK in S phase. It has been reported that CDK2 plays a crucial role in the S-phase. Therefore, next thing will be to examine whether LMTK3 is phosphorylated by CDK2 by using *in vitro* kinase assay. Following, if CDK2 phosphorylates LMTK3, candidate sites of LMTK3 phosphorylation will need to be clarified.
- Subsequently, to examine whether the identified phosphorylation sites of LMTK3 will reduce the RAD51 loading if the sites are mutated by measuring the RAD51 foci formation.

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RESEARCH PRESENTATION

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