Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Role of N-terminal domains of p400 ATPase in the ATM interaction and DNA damage response

A thesis presented in partial fulfillment of the requirements for a the degree of

Master of Science (Msc)

in

Genetics

At Massey University, Manawatū,
New Zealand

Lauren Elizabeth Weber

2016

Abstract

Efficient repair of damaged DNA and preservation of genomic integrity is integral in the maintenance of proper cellular function and prevention of unrestricted cell proliferation. One critical threat to the stability of the genome is the double strand break (DSB), arguably one of the most cytotoxic lesions to DNA. Interference with the DSB repair mechanism can lead to dysregulation of cellular systems and the prospective development of malignancies. Two critical proteins in DBS repair are the Ataxia Telangiectasia Mutated (ATM) kinase, a serine/threonine kinase from the Phosphatidylinositol 3-Kinase-related Kinase (PIKK) family, and p400, an ATPase chromatin remodeler. ATM is one of the first responders to DSBs and is responsible for the phosphorylation of a multitude of protein substrates including the histone variant H2AX. Beyond its phosphorylation ability, ATM has been proposed as a potential shuttle for other repair machinery, aiding in the early and efficient recruitment of proteins to the DNA damage foci. One such proposed protein is p400. The exact role of p400 in DSB repair is unknown but previous studies show that there is a decrease in repair efficiency in its absence. A prospective interaction is supported by previous studies in which p400 and p400 N-terminal derivatives co-immunoprecipitate with ATM *in vivo* in HEK293T cells.

This study aimed to confirm the interaction of ATM and p400 N-terminal derivatives *in vitro* and explore the functional implications of the association *in vivo* in U2OS cells. It was not possible to isolate full-length p400 derivatives *in vitro* and thus no conclusive results were obtained. Functional assays revealed the ability of one p400 fragment, F1, to inhibit DNA repair and cell proliferation after DNA double-strand break induction with bleomycin. Ectopic expression of the other two p400 N-terminal fragments, F2 and F3, induced an inhibition of cell proliferation under standard growth conditions. Although no conclusive results were acquired, a trend emerged suggesting that N-terminal fragment F1 is able to interfere with ATM protein-protein interactions resulting in a decrease in the efficiency of the DNA damage response and repair. These results implicate F1 as a potential target for further research in both DNA repair and cancer therapy.

Acknowledgements

No words can described how appreciative I am to everyone who has made the writing of this thesis possible. Firstly, I would like to thank my supervisor Jeong Park for all of the knowledge he shared and for helping to guide me through this degree. JP treated all of his students as an extension of his family and I will always remember the wonderful get-togethers he and his family prepared for us. I would also like to show my gratitude for Rebecca Smith. I learned more from Bex than just research skills. She always had time to help me in my times of need or confusion, which were more than I care to admit.

I would also like to thank Kathryn Stowell for her wisdom, sound advice, and quirky sense of humor. Her door was always open and I definitely popped my head to ask for assistance more than a few times. The random appearance of confectionary in the walk-in-center and chocolate Easter eggs from the "Easter bunny" added something special to the far end of Science Tower D.

I am also very appreciative for the help I received from Tracey Hale and Helen Fitzsimons for helping with my tissue culture needs as well as all of the good conversations.

A special thank you goes out to the members of ParkLand and the Twilight Zone (as well as the downstairs lab associates). Some of the study-break chats in the computer room left me in stitches. Even on the most stressful of days, I could always count on the ridiculous banter to lighten the mood.

Abbreviations

°C	degrees Celsius
-ve	negative
A	ampere
Ac	acetylation
Amp	ampicillin
APS	ammonium persulphate
AT	Ataxia Telangiectasia
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia mutated and Rad3-related protein
BCA	bicinchoninic acid
BME	β-mercaptoethanol
bp	base pair
BSA	bovine serum albumin
c-Abl	Abelson murine leukemia viral oncogene homolog 1
cDNA	complementary DNA
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
Co-IP	co-immunoprecipitation
DAPI	4', 6-diamidino-2-phenylindole
ddH_2O	double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	Deoxyribose nucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTPs	Deoxyribonucleic triphosphate
DSB	Double strand break
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
FAT	FRAP-ATM-TRRAP
FAT-C	FAT domain located at the protein's C-terminus
FBS	fetal bovine serum
g	gram
g	relative centrifugal force (force x gravity)
GSH	glutathione
GST	glutathione S-transferase
h	hours

H2Av	Drosophila H2A.Z and H2A.X homologue
H2AX	histone variant of H2A
γH2AX	histone variant H2A.X phosphorylated at serine 139
HA	hemagglutinin
HAT	histone acetyl transferase
HCl	hydrochloric acid
HEK293T	human embryonic kidney cell line
HD	high definition
HF	high fidelity
HP1	heterochromatin binding protein 1
HR	homologous recombination
HRP	horseradish peroxidase
HSA	helicase and SANT associated
IF	immunofluorescence
IP	immunoprecipitation
ITPG	isopropyl β-D-1-thiogalactopyranoside
K	lysine
kb	kilobase
kDa	kilodalton
L	liter
LB	Luria-Bertani bacteriological media
M2 agarose	α-FLAG antibody immobilized on agarose beads
mA	milliampere
Mb	megabase
MCS	multiple cloning site
mg	milligram
min	minute
mL	milliliter
mM	millimol
mRNA	messenger ribonucleic acid
MRN	Mre11-Rad3-Nsb1
Mre11	meiotic recombination 11
MW	molecular weight
NBS	Nijmegen breakage syndrome
ng	nanogram
NHEJ	non-homologous end joining
NLS	nucelar localization sequence
nm	nanometer
NP-40	tergitol-type nonyl phnoxypolyethocyethanol 40 (Igapal CA-630)
OD	optical density
PAGE	polyacrylamide gel electrophoresis
P3	generation 3 of baculovirus
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PEG	polyethylene glycol
pen/strep	penicillin-streptomycin solution
PFA	paraformaldehyde
PHYRE2	protein homology/analogy recognition engine V 2.0
PI3K	phosphatidylinositol 3-kinase
PIKK	phosphatidylinositol 3-kinase-like protein kinase
pmol	picomol
PMSF	phenylmethanesulfonyl fluoride solution
PVDF	polyvinylidene fluoride transfer membrane
qPCR	quantitative real-time PCR
RE	restriction endonuclease
RNA	ribose nucleic acid
RNase	ribonuclease
RT	reverse transcription/reverse transcriptase
RT-qPCR	reverse transcription quantitative real-time PCR
S	serine
s	second
SANT	SWI3-ADA2-N-CoR-TFIIIB
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf9	clonal isolate of *Spodoptera frugiperda* Sf21 cells
siRNA	small interfering RNA
SWI2/SNF2	switch 2/sucrose non-fermentable 2
TAT	transactivator of transcription
TBE	tris boric acid EDTA
TBS/T	tris-buffered saline with tween-20
TE	tris EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
TIP48	transactivation-domain interacting protein of 48 kDa
TIP49	transactivation-domain interacting protein of 49 kDa
TIP60	HIV-I TAT interacting protein of 60 kDa
Tris	2-amino-2-hydromethyl-1,3-propanidol
TRRAP	transformation/transcription domain-associated protein
μg	microgram
μL	microliter
UV	ultraviolet light
V	volts
v/v	volume per volume
w/v	weight per volume
WT	wild type

List of Figures

Figure 1.1 Chromatin Folding Mechanisms..3
Figure 1.2 Protein alignment of histone H2A variants 6
Figure 1.3 The DNA double strand break repair response...9
Figure 1.4 Schematic Representation of ATM and its Phosphorylation Sites 12
Figure 1.5 ATM activation mechanism 14
Figure 1.6 Hypothesized structure of p400 complex 17
Figure 3.1 Schematic representation of full-length p400 and its derivatives..................41
Figure 3.2 Cloning strategy for GST-p400 fragment fusion protein expression 43
Figure 3.3 Cloning of pGTK-FLAG-p400 domain constructs......................................44
Figure 3.4 Expression and purification of GST-p400 fragment fusion proteins.............46
Figure 3.5 Expression of GST-p400 derivatives..47
Figure 3.6 Expression of FLAG-ATM..49
Figure 3.7 Co-immunoprecipitation between ATM and GST-p400 derivatives 50
Figure 4.1 Cloning strategy for lentiviral vector containing FLAG-p400 derivatives ...57
Figure 4.2 Cloning of pCDH1-p400 domain constructs 59
Figure 4.3 Schematic diagram for production of lentiviral particles in HEK293t cells .61
Figure 4.4 Expression of FLAG-p400 from pCDH1 lentivirally infected U2OS cells ..63
Figure 4.5 ATM activity assay...65
Figure 4.6 Rate of cell proliferation of U2OS stable cell lines......................................68
Figure A1.1 Confirmation digest of *E. coli* BL21 plasmids...91
Figure A1.2 Confocal imaging of control U2OS cells..92
Figure A4.1 CβF Plasmid Map...103
Figure A4.12 CβF-F1 Plasmid Map...104
Figure A4.3 CβF-F2 Plasmid Map...104
Figure A4.4 CβF-F3 Plasmid Map...105
Figure A4.5 CβF-F4 Plasmid Map...105
Figure A4.6 CβF-F5 Plasmid Map...106
Figure A4.7 CβF-F6 Plasmid Map...106
Figure A4.8 pGTK-F1 Plasmid Map...107
Figure A4.9 pGTK-F2 Plasmid Map...107
Figure A4.10 pGTK-F3 Plasmid Map...108

Figure A4.11 pGTK-F4 Plasmid Map..108
Figure A4.12 pGTK-F5 Plasmid Map..109
Figure A4.13 pGTK-F6 Plasmid Map..109
Figure A4.14 pCDH1 Plasmid Map...110
Figure A4.15 pCDH1-F1 Plasmid Map..111
Figure A4.16 pCDH1-F2 Plasmid Map..111
Figure A4.17 pCDH1-F3 Plasmid Map..112
Figure A4.18 pCDH1-F6 Plasmid Map..112

List of Tables

Table 2.1 RT-qPCR lentivirus titration protocol..27
Table 2.2 Antibodies used during western blotting 36
Table 2.3 Antibodies used during confocal microscopy 39
Table 2.4 Excitation of dyes used during confocal microscopy 39
Table A2.1 Primary and Secondary Antibodies...93
Table A2.2 p400 codon analysis for expression in E. coli...93
Table A2.3 Protein stabilities of GST-p400 protein fragments using ExPASy...............94
Table A2.4 Descriptive Statistics – Day 7 Samples after Bleomycin Exposure..............94
Table A2.5 Analysis of Variance (ANOVA) One-Way – Day 7 Samples after Bleomycin Exposure...94
Table A2.6 T-Test – Day 7 pCDH1 and F1 Samples after Bleomycin Exposure............95
Table A2.7 T-Test – Day 7 pCDH1 and F6 Samples after Bleomycin Exposure............95
Table A2.8 T-Test – Day 7 F1 and F6 Samples after Bleomycin Exposure....................95
Table A2.9 ANOVA of pCDH1, F1, and F6 Samples under Standard Conditions..........95
Table A2.10 ANOVA of pCDH1, F1, and F6 Samples after Exposure to Bleomycin...96
Table A2.11 T-test – pCDH1, F1, and F6 Samples after Bleomycin Exposure...............96
Table A2.12 T-test – pCDH1, F1, and F6 Samples under Standard Conditions.............97

Table of Contents

1 Introduction 1
1.1 Cancer and Current Cancer Therapies 1
1.2 Chromatin and Gene Regulation 2
1.2.1 Role of histones in chromatin accessibility 3
1.2.2 Alteration of chromatin structure by chromatin remodeling complexes 4
1.3 H2A Histone Variants 5
1.4 DNA repair 7
1.4.1 Double Strand Break Repair 7
1.5 ATM kinase 10
1.5.1 Structure and Function of ATM 11
1.5.2 ATM Activation 13
1.5.3 Role of ATM in DNA Damage Response 15
1.6 The ATPase chromatin remodeler p400 16
1.6.1 Role of p400 in H2AZ exchange 17
1.6.2 Role of p400 in Gene Regulation 18
1.6.3 Role of p400 in DNA repair 18
1.6.4 Disruption of p400 19
1.7 Thesis outline and hypothesis 19
1.7.1 Hypothesis 20
1.7.2 Objectives 20

2 Materials and Methods 21
2.1 Materials 21
2.2 DNA Methods 22
2.2.1 Restriction Enzyme Digestion 22
2.2.2 DNA Fill in of Cohesive Ends 22
2.2.3 PCR Purification 23
2.2.4 Calf Intestinal Phosphatase Digestion 23
2.2.5 Agarose Gel Electrophoresis 23
2.2.6 DNA Purification from Agarose Gel 24
2.2.7 DNA Ligation 24
2.2.8 Preparation of Competent Escherichia coli (E. coli) cells 24
2.2.9 Transformation of DH5α E. coli 25
2.2.10 Plasmid Purification from E. coli 25
2.2.11 DNA Sequencing 27

2.2.12 DNA Quantification *27*
2.2.13 RT-qPCR Lentivirus titration *27*
2.3 Protein Methods 27
2.3.1 IPTG induced Protein Expression *27*
2.3.2 BCA Assay *28*
2.3.3 GST-fusion Protein Purification *28*
2.3.4 ATM Cell Lysate Preparation from Sf9 Cells *29*
2.3.5 GST-Pull-Down Assay *29*
2.3.6 Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) *30*
2.3.7 Western Blotting *31*
2.3.8 Coomassie Blue Staining *31*
2.3.9 Immunoprecipitation *32*
2.4 Cell Culture 32
2.4.1 Subculturing SF9 Cell Cultures *32*
2.4.2 SF9 Infection with Baculovirus *32*
2.4.3 Subculturing Mammalian Cell Cultures *33*
2.4.4 Mammalian Cell Transient Transfection *33*
2.4.5 Lentivirus Production *34*
2.4.6 Lentivirus Concentration *34*
2.4.7 Mammalian Cell Infection with Lentivirus *35*
2.4.8 Induction of DNA Damage by Bleomycin *35*
2.4.9 ATM Activation Assay *35*
2.4.10 Cell Lysis *36*
2.4.11 Histone Extraction *36*
2.4.12 Bleomycin Sensitivity Assay *37*
2.4.13 Mammalian Cell Freezing *37*
2.4.14 Mammalian Cell Reanimation *38*
2.4.15 Confocal Microscopy *38*

3 Examination of in vitro interaction between p400 and ATM 40
3.1 Introduction 40
3.2 Cloning and expression of GST-p400 fragment fusion protein 41
3.3 Expression of ATM from Sf9 cells 48
3.4 GST Pull-Down Assay 49
3.5 Discussion 51

4 Functional analysis of interaction between p400 and ATM 55
4.1 Effect of p400 fragment expression on ATM function and DNA repair 55

4.1.1 Introduction *55*
4.1.2 Cloning and isolation of FLAG-p400 fragments *56*
4.1.3 Production of lentivirus containing FLAG-p400 fragments *60*
4.1.4 Examination of p400 N-terminal fragment expression on ATM activity *64*
4.1.5 Examination of F1 expression on cell survival after DNA damage *66*
4.2 Discussion 68
4.2.1 Effect of N-terminal p400 fragments on ATM activity and γH2AX phosphorylation .. *68*
4.2.2 Effect F1 ectopic expression on cell proliferation after induction of DSBs *70*
5 Summary and Future Directions 74
5.1 Summary 74
5.2 Limitations 75
5.3 Future Directions 76
5.3.1 Confirmation of in vitro interaction between ATM and p400 derivatives *76*
5.3.2 Confocal imaging to elucidate γH2AX phosphorylation and protein localization *77*
5.3.3 Examination of F1 functional effect on ATM in other human cell lines *78*
5.3.4 Competition assay between p400 and p400 N-terminal derivatives for ATM *78*
5.3.5 Examination of F2 and F3 under inducible promoters *79*
5.4 Conclusion 80
6 References 81
Appendix 1 Supplementary Figures 91
Appendix 2 Supplementary Tables 93
Appendix 3 Bleomycin Sensitivity Assays 98
Appendix 4 Plasmid Maps 103