

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.

**How does the interaction between the  
Filamin A repeat 10 domain and F-actin lead  
to severe OPD skeletal disorders?**

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science

in

Biochemistry

at Massey University, Palmerston North,

New Zealand.

**Fareeda Maged Barzak**

**2014**



## *Abstract*

The cytoskeleton network allows cells to differentiate, divide, and move in response to the external environment creating a mechanoprotection system against cell stress. The actin cytoskeleton is stabilised and tightly regulated by various actin-binding proteins, one of which are the family of Filamin (*FLN*) proteins that crosslink F-actin into three-dimensional networks. Filamins also link the actin cytoskeleton to the cellular membrane through interactions with transmembrane proteins and function as a molecular scaffold for signalling molecules. In addition to an actin binding domain, each monomer contains a rod region of 24 immunoglobulin-like repeat domains with dimerisation of the monomers occurring at repeat 24. The human filamin family contains three *FLN* isoforms; *FLNA*, *FLNB*, and *FLNC* which are differentially expressed where *FLNA* is identified as the dominant isoform located on the X-chromosome essential for mammalian development.

Mutations in Filamin A (*FLNA*) have been identified to cause distinctly different human diseases affecting the central nervous system, vascular system, or skeletal muscles; however, the molecular mechanisms of *FLNA* leading to these diseases remain unclear. Mutations cluster in distinct *FLNA* domains, suggesting their functional importance for mediating correct functions. Mutations in the *FLNA* repeat 10 domain are correlated with severe forms of the skeletal disorders Otopalatodigital syndrome spectrum disorders (OPD) thought to be due to an altered or gain-of-function phenotype. The aim of this study was to provide an insight into the biochemical properties of *FLNA* repeat 10 domain by better understanding how mutations in this domain lead to OPD.

Initially, recombinant wildtype (Wt) and mutant (V1249A and A1188T) *FLNA* repeat 10 domain proteins (FLNAR10) were purified then compared by *in vitro* biochemical studies to investigate secondary structure, stability, and affinity towards F-actin. The FLNAR10 protein was revealed to have relatively weak binding affinity towards F-actin, consistent with being an additional contributor in the filamin protein to bind F-actin. Mutations in the FLNAR10 protein exhibited a slight increase in affinity towards F-actin, accompanied by a slight reduction of thermostability in comparison to the Wt protein, but no significant changes in the secondary structure were observed. This slight increase in the affinity of the mutant *FLNA* repeat 10 proteins towards F-actin is

consistent with a gain-of function mechanism for the disease phenotype. Overall, these results contribute towards a better understanding of the FLNA function, providing further evidence towards a gain-of function mechanism for OPD.

# *Acknowledgements*

Individually, we are one drop, but together, we are an ocean. There are a number of people who I am greatly indebted to and would like to thank, but I wish to particularly thank the following:

Primarily, I would like to thank my supervisor Dr. Andrew Sutherland-Smith, for his continuous support throughout my MSc study and research, and for this amazing opportunity, support, advice, confidence and technical discussions. Thank you for guiding me and believing in my ability.

A special thanks to Dr. Gill Norris, Dr. Mark Patchett, and Prof. Geoff Jameson for all the advice and input throughout the years. Thank you to my fellow labmates, colleagues, friends, and the Structural Biology group for their stimulating discussions, advice, and technical assistances.

I would also like to thank Ann, Cynthia, Colleen, Tara, and Paul for all their help and behind the scenes organisation. Also, thank you to Kathryn Stowell for her constant support and advice throughout my years at Massey University.

Thank you to all my family and parents Maged and Sohair who continue to be a source of encouragement and inspiration throughout my life. I am grateful for all the love, support, and encouragement to always reach for the stars whilst putting up with me through the highs and lows of this journey. I could not have done any of this without everyone's belief in me.

Finally thank you to the Institute of Fundamental Sciences (IFS) and the Royal Society of New Zealand Marsden Fund for this opportunity.

# *Table of Contents*

<i>Abstract</i> .....	<i>i</i>
<i>Acknowledgements</i> .....	<i>iii</i>
<i>Table of Contents</i> .....	<i>iv</i>
<i>List of Figures</i> .....	<i>ix</i>
<i>List of Tables</i> .....	<i>xi</i>
<i>List of Abbreviations</i> .....	<i>xii</i>
<b>1. Introduction</b> .....	<b>16</b>
Foreword.....	17
1.1 Actin cross-linking proteins.....	19
1.2 Filamins.....	19
1.2.1 Structural components of Filamin A.....	20
1.2.2 Dimerisation of filamin.....	21
1.2.3 Actin binding domain of filamin.....	21
1.2.4 Structure of filamin repeats.....	22
1.3 Regulation of Filamin A.....	24
1.4 Roles of Filamin A on cell function.....	25
1.4.1 Interactions with transmembrane proteins.....	25
1.4.2 Scaffold for signalling molecules.....	28
1.4.3 Mechanoprotection.....	30
1.4.4 Interaction with actin cytoskeleton.....	31
1.5 Filamin A associated with Diseases.....	32
1.5.1 Periventricular nodular heterotopia.....	33
1.5.2 Otopalatodigital spectrum malformation disorders.....	34

1.6	FLNA repeat 10 and diseases .....	36
1.7	Aim .....	40
<b>2.</b>	<b><i>Materials and Methods</i></b> .....	<b>41</b>
2.1	Chemicals and media .....	42
2.1.1	Chemicals .....	42
2.1.2	Sterilisation .....	42
2.1.3	Luria-Bertani (LB) medium .....	42
2.1.4	Ampicillin .....	42
2.2	Electrophoresis methods .....	42
2.2.1	Agarose Gel Electrophoresis .....	42
2.2.2	SDS-PAGE Gel Electrophoresis .....	43
2.3	Measurement of Nucleic Acid Concentration .....	45
2.4	Measurement of Optical Density of cultures .....	45
2.5	Measurement of Protein Concentration .....	45
2.6	Plasmids and Bacterial Strains used .....	45
2.7	Transformation of <i>E. coli</i> Cells .....	46
2.7.1	Preparation of Chemically Competent cells .....	46
2.7.2	Transformation of chemically competent cells .....	46
2.7.3	Plasmid Isolation .....	47
2.8	Cloning .....	47
2.8.1	Bioinformatics and sequence analysis .....	47
2.8.2	PCR primer design .....	47
2.8.3	PCR .....	47
2.8.4	Restriction digest .....	48
2.8.5	Ligation .....	49
2.8.6	Transformation .....	49

2.8.7	Colony PCR Screening .....	49
2.8.8	Sequencing .....	50
2.8.9	Whole plasmid PCR mutagenesis .....	50
2.9	Protein Expression and Solubility Trials .....	52
2.10	Scaled-up Protein Expression and Purification .....	53
2.10.1	Induction and Expression .....	53
2.10.2	Cell Lysis .....	53
2.10.3	Ni <sup>2+</sup> -NTA Affinity Chromatography .....	53
2.10.4	Size Exclusion Chromatography .....	54
2.11	Mass Spectrometry .....	54
2.11.1	Colloidal Coomassie Staining .....	54
2.11.2	In-Gel Tryptic Digest .....	55
2.12	High-performance liquid chromatography (HPLC) .....	56
2.13	Protein temperature instability analysis .....	56
2.14	His-tag removal .....	57
2.15	Protease resistance analysis .....	57
2.16	Circular dichroism spectroscopy .....	57
2.17	F-actin co-sedimentation assay .....	58
<b>3.</b>	<b><i>Cloning of Filamin A repeat 10 domains</i></b> .....	<b>59</b>
3.1	Introduction .....	60
3.2	FLNA Repeat 10 boundary .....	60
3.3	Cloning of human FLNA repeat 10 domain .....	61
3.4	Constructing mutant FLNA repeat 10 domain .....	65
3.5	Summary .....	67

<b>4. <i>FLNA repeat 10 protein expression and purification</i></b> .....	68
4.1 Introduction .....	69
4.2 Expression and solubility trials .....	69
4.3 Scaled up protein expression.....	70
4.4 Purification of FLNA R10 proteins.....	71
4.5 Affinity chromatography.....	71
4.6 Size Exclusion Chromatography.....	77
4.7 Secondary band issue .....	81
4.8 Summary .....	86
<b>5. <i>In vitro biochemical studies of Wildtype and mutant FLNA repeat 10 domains</i></b>	87
5.1 Introduction .....	88
5.2 Resistance to proteolytic cleavage .....	88
5.3 Secondary structure analysis .....	92
5.4 Circular dichroism (CD) spectroscopy.....	93
5.5 CD Thermal Denaturation.....	98
5.6 FLNAR10 and F-actin binding.....	103
5.7 Summary .....	112
<b>6. <i>Conclusions</i></b> .....	113
6.1 Introduction .....	114
6.2 FLNA repeat 10 domain and F-actin.....	115
6.3 Severe OPD Disorder mechanism and FLNA repeat 10.....	116
6.4 FLNA repeat 10 importance.....	117
6.5 Conclusion.....	119
<b>7. <i>Future Directions</i></b> .....	120

<b>8. Bibliography.....</b>	<b>122</b>
<b>9. Appendices.....</b>	<b>130</b>
9.1 Cloning.....	131
9.2 CD spectroscopy analysis.....	134
9.3 Actin Co-sedimentation assay.....	135
9.4 Phosphorylation sites.....	138

## *List of Figures*

Figure 1.1 Structure of filamin.....	20
Figure 1.2 The crystal structure of the human FLNA repeat 10 domain. ....	24
Figure 1.3 Model of FLNA interactions. ....	25
Figure 1.4 Filamins interactions and functions. ....	27
Figure 1.5 Model of $\beta$ -integrin and FilGAP binding differentially to <i>FLNA</i> . ....	29
Figure 1.6 Actin co-sedimentation assay of different recombinant FLNA constructs. .	32
Figure 1.7 Filamin A mutations associated with different diseases.....	33
Figure 1.8 Fitting the Filamin A repeat 10 domain crystal structure within the N-terminal F-actin. ....	38
Figure 3.1 FLNA repeat 10 domain boundary alignments. ....	61
Figure 3.2 Agarose gel of PCR reaction products. ....	62
Figure 3.3 Agarose gel of the digested pPROEX-HTb expression vector.....	63
Figure 3.4 Vector Map of pPROEX HTb with FLNAR10 cDNA cloned between <i>Bam</i> HI and <i>Sal</i> I restriction sites. ....	64
Figure 3.5 Plasmid digests of FLNA repeat 10 constructs.....	65
Figure 3.6 FLNA repeat 10 domain crystal structure. ....	67
Figure 4.1 Wt FLNA repeat 10 protein expression trials.....	70
Figure 4.2 Wt FLNAR10 IMAC protein purification.....	73
Figure 4.3 Wt FLNAR10 IMAC protein purification SDS-PAGE gel.....	74
Figure 4.4 V1249A FLNAR10 IMAC protein purification.....	75
Figure 4.5 V1249A FLNAR10 IMAC protein purification SDS-PAGE gel.....	75
Figure 4.6 FLNAR10 A1188T IMAC protein purification .....	76
Figure 4.7 A1188T FLNAR10 IMAC protein purification SDS-PAGE gel. ....	76
Figure 4.8 Wt FLNAR10 size exclusion protein purification using Superdex 75 10/300 GL column. ....	78
Figure 4.9 Wt FLNAR10 SEC protein purification SDS-PAGE gel.....	78
Figure 4.10 V1249A FLNAR10 size exclusion protein purification using Superdex 75 10/300 GL column. ....	79
Figure 4.11 V1249A FLNAR10 SEC protein purification SDS-PAGE gel.....	79

Figure 4.12 A1188T FLNAR10 size exclusion protein purification using Superdex 75 10/300 GL column. ....	80
Figure 4.13 A1188T FLNAR10 SEC protein purification SDS-PAGE gel. ....	80
Figure 4.14 HPLC chromatogram of Wt FLNAR10 protein sample. ....	82
Figure 4.15 HPLC of Wt FLNAR10 protein samples SDS-PAGE gel. ....	82
Figure 4.16 SDS-PAGE gel of temperature instability analysis and His-tag removal of Wt FLNAR10 protein samples. ....	83
Figure 4.17 Wt FLNA repeat 10 protein Mass spectroscopy analysis of bands. ....	84
Figure 4.18 Map of possible trypsin cleavage sites. For His-tagged for Wt FLNAR10. ....	85
Figure 5.1 SDS-PAGE gel of subtilisin resistance trials. ....	90
Figure 5.2 SDS-PAGE gel of chymotrypsin resistance trials. ....	91
Figure 5.3 Map of possible chymotrypsin cleavage sites. ....	92
Figure 5.4 Secondary structure predictions for FLNA repeat 10 domain. ....	92
Figure 5.5 Far-UV CD spectra of associated types of secondary structure. ....	93
Figure 5.6 CD spectrum analysis of the lysozyme protein sample. ....	94
Figure 5.7 Far-UV CD spectra of FLNAR10 domain protein. ....	95
Figure 5.8 CD spectrum analysis of the Wt FLNAR10 protein sample. ....	96
Figure 5.9 Filamin repeat domains CD spectra. ....	97
Figure 5.10 CD thermal denaturation spectra of Wt FLNAR10 domain protein. ....	99
Figure 5.11 CD thermal denaturation spectra of V1249 FLNAR10 domain protein. ..	100
Figure 5.12 CD thermal denaturation spectra of A1188T FLNAR10 domain protein. ....	100
Figure 5.13 Normalised CD spectra of FLNAR10 domain proteins at 206 nm fitted to Boltzmann sigmoid model. ....	102
Figure 5.14 Example actin co-sedimentation assay. ....	104
Figure 5.15 Actin co-sedimentation assay data. ....	105
Figure 5.16 Actin co-sedimentation assays fitted with 1:1 binding model (0-200 $\mu$ M FLNAR10 protein). ....	107
Figure 5.17 FLNAR10 domain structure showing possible F-actin binding groove. ....	109
Figure 5.18 FLNAR10 domain mutations associated with severe OPD disorders effects on the FLNAR10 domain structure. ....	111

## *List of Tables*

Table 2.1 Preparation of Separating Gel for SDS-PAGE. ....	43
Table 2.2 Preparation of Stacking Gel for SDS-PAGE. ....	44
Table 2.3 Preparation of 6× Sample treatment buffer. ....	44
Table 2.4 Preparation of 5X running buffer. ....	44
Table 2.5 PCR reaction components. ....	48
Table 2.6 Thermal cycling PCR protocol. ....	48
Table 2.7 Restriction digest protocol. ....	49
Table 2.8 Colony PCR protocol. ....	50
Table 2.9 Whole plasmid PCR mutagenesis components. ....	51
Table 2.10 Whole plasmid PCR mutagenesis Thermal cycling protocol. ....	51
Table 2.11 Whole plasmid PCR mutagenesis ligation reaction. ....	52
Table 2.12 1 × PBS buffer components. ....	53
Table 2.13 Colloidal coomassie stock solution buffer. ....	55
Table 2.14 Actin co-sedimentation assay buffers. ....	58
Table 5.1 Deconvolution of the CD spectrum of FLNAR10 protein using CDNN software. ....	96
Table 5.2 Analysis of the Boltzmann sigmoid model to the normalised unfolding data at 206 nm. ....	102

# *List of Abbreviations*

× g	Multiples of gravitational force
Å	Angstrom ( $10^{-10}$ m)
A1188T	FLNA repeat 10 mutation Alanine to Threonine leading to FMD
A <sub>280</sub>	Absorbance at 280 nm
aa	Amino acid
ABC	Ammonium bicarbonate
ABD	Actin binding domain
AEX	Anion Exchange Chromatography
Ala	Alanine
Amp	Ampicillin
ATP	Adenosine-5'-triphosphate
BLAST	Base local alignment search tool
bp	Base pair
CD	Circular Dichroism
cDNA	Complementary DNA
CV	Column Volumes
CH	Calponin homology domain
C-terminal	Carboxyl terminal
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
dsDNA	Double-stranded Deoxyribonucleic acid

DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
F-actin	Filamentous actin
FLN	Filamin
FLNA	Filamin A
FLNAR10	Filamin A repeat 10 protein
FMD	Frontometaphyseal dysplasia
G-actin	Globular (monomeric) actin
His <sub>6</sub>	Hexa-histidine tag
HPLC	High-performance liquid chromatography
Ig	Immunoglobulin
IPTG	Isopropyl-β-D-thio-galactoside
K <sub>d</sub>	Dissociation constant
kb	Kilobase pairs (of DNA)
LB	Luria Bertani media
LB-Amp	Luria Bertani media with 100 μg/ml ampicillin
mAU	Milli absorbance units
MeCN	Acetonitrile
Min	Minute
MNS	Melnick–Needles syndrome
MOPS	3-(N-morpholino) propanesulfonic acid

MW	Molecular Weight
N-terminal	Amino terminal
OD	Optical density at 600 nm
OPD	Otopalatodigital spectrum malformation disorders
OPD1	Otopalatodigital syndrome type 1
OPD2	Otopalatodigital syndrome type 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PH	Periventricular nodular heterotopia
pH	Negative decadal logarithm of proton concentration
KOAc	Potassium Acetate
RPM	Revolutions per minute
rTEV	Recombinant tobacco etch virus protease
S1199L	FLNA repeat 10 mutation Serine to Leucine leading to FMD
SEC	Size exclusion chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA buffer
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
Thr	Threonine
Tm	Melting temperature

TPCK	Tosyl phenylalanyl chloromethyl ketone
Tris	Tris-(hydroxymethyl)-aminomethane
U	Units
UV	Ultraviolet
V1249A	FLNA repeat 10 mutation Valine to Alanine leading to MNS
Val	Valine
v/v	Volume/volume ratio
Wt	Wild-type
w/v	Weight/volume ratio
w/w	Weight/weight ratio