



**Investigation into the cellular function of the
Opitz Syndrome gene, *MID1* and its homologue,
*MID2***

By

Yi Zou

(Bachelor of Medicine, Master of Science)

A thesis submitted in fulfillment
for the requirements of the degree of
Doctor of philosophy

**Discipline of Genetics
School of Molecular and Biomedical Science
The University of Adelaide**

July, 2004

Table of Contents.

Acknowledgements	2
Chapter One: General Introduction	9
1.1 Opitz syndrome or BBBG syndrome.....	9
1.2 Genetics of Opitz syndrome.....	10
1.2.1 Opitz syndrome is heterogenous	10
1.2.2 OS cases with different genetic defects are clinically indistinguishable....	11
1.2.3 The OS phenotype represents a defect in tissue fusion and/or remodeling during early development.....	12
1.3 Comparison of OS with other 22q11.2 deletion syndromes.....	13
1.3.1 The clinical features of the 22q11.2 deletion syndrome	13
1.3.2 Potential causative genes for the 22q11.2 deletion syndrome.....	13
1.4 Identification of <i>MID1</i> as the causative gene in X-linked OS.....	15
1.4.1 Identification of <i>MID1</i> mutations in OS.....	15
1.4.2 The expression pattern of <i>MID1</i> also supports a OS candidate gene.....	16
1.5 Characterization of <i>MID1</i>	17
1.5.1 The RBCC protein family.....	17
1.5.2 The B30.2 domain.....	19
1.5.3 The cellular localization of <i>MID1</i>	20
1.5.4 Protein interactors of <i>MID1</i>	21
1.5.5 A potential ubiquitin E3-ligase activity for <i>MID1</i>	23
1.5.5.1 E3 ubiquitin-ligases and protein ubiquitylation.....	24
1.5.5.2 Ubiquitylation in the regulation of protein degradation.....	25
1.5.5.3 Ubiquitin and membrane trafficking.....	25
1.5.5.4 Potential target/targets for ubiquitylation.....	26
1.6 <i>MID2</i> , a homologue of <i>MID1</i> , has correlated functions.....	26
1.6.1 <i>MID1</i> and <i>MID2</i> are likely to have originated from a common ancestral gene	26
1.6.2 Functional similarities of <i>MID1</i> and <i>MID2</i>	27
1.6.3 A role for <i>MID2</i> in the pathogenesis of OS?.....	27
1.6.4 The CI subfamily: sharing a common cellular function?.....	28
1.7 <i>MID1</i> / <i>MID2</i> and microtubule-dependent cellular processes.....	29

1.7.1 Microtubule dynamics.....	29
1.7.2 Microtubules and other cytoskeletal networks.....	30
1.7.3 Connection between microtubules and membrane.....	31
1.7.3.1 Microtubule-membrane cross-linking proteins.....	31
1.7.3.2 Microtubule motors	32
1.7.3.3 Phosphoinositides and the microtubule-membrane connection.....	33
1.7.3.4 The PH-domain and PI-binding.....	34
1.7.4 Microtubule-dependent cellular processes.....	35
1.7.4.1 Microtubules and cell motility	35
1.7.4.2 Microtubules and positioning of intracellular membrane compartment	35
1.7.4.3 Microtubule-dependent vesicle trafficking.....	36
1.7.4.4 Coupling of trafficking and signalling during development.....	37
1.8 Outlines of this project.....	39
Chapter Two: Materials and Methods.....	41
2.1 Abbreviations	41
2.2 Materials	42
2.2.1 Chemical and Reagents.....	42
2.2.2 Stains and Dyes	43
2.2.3 Enzymes	43
2.2.4 Antibiotics and Indicators	44
2.2.5 Fluorescent Probes	44
2.2.6 Kits and Miscellaneous Materials	45
2.2.7 Solutions and Buffers	45
2.2.8 Nucleic acid and protein molecular weight standards.....	51
2.2.9 Cloning and expression vectors.....	51
2.2.10 Bacterial strains	51
2.2.11 Bacterial media	51
2.2.12 Yeast strains	52
2.2.13 Yeast media	53
2.2.13.1 Amino acids and Carbon source.....	53
2.2.13.2 Liquid media	53
2.2.13.3 Solid media	53

2.2.14 Libraries	54
2.2.15 Tissue Culture Cell Lines and Media.....	54
2.2.15.1 Cell Lines	54
2.2.15.2 Media	54
2.2.16 Antibodies	54
2.2.17 Oligonucleotides	55
2.2.18 MID1 and MID2 domain deletions.....	55
2.3 Methods	56
2.3.1 Ethanol precipitation of nucleic acids.....	56
2.3.2 Restriction endonuclease digestions.....	56
2.3.3 Agarose gel electrophoresis of DNA.....	56
2.3.4 Extraction of DNA fragments from agarose gels.....	57
2.3.5 Preparation of electroporation competent bacterial cells.....	57
2.3.6 Sub-cloning restriction fragments into plasmid DNA vectors.....	57
2.3.6.1 Preparation of vectors and restriction fragments.....	57
2.3.6.2 Ligation of restriction fragments in to vector DNA.....	58
2.3.6.3 Transformation of competent bacterial cell by electroporation.....	58
2.3.6.4 Plating of transformed cells.....	58
2.3.7 Determination of DNA concentration.....	59
2.3.8 Plasmid DNA preparation.....	59
2.3.8.1 Small scale preparations	59
2.3.8.2 Large scale preparations	59
2.3.9 End-filling restriction endonuclease digested DNA	59
2.3.10 Automated sequencing of PCR products.....	60
2.3.11 Polymerase Chain Reaction (PCR)	60
2.3.12 Preparation of PCR products for cloning	60
2.3.13 Yeast two-hybrid screening of cDNA library	61
2.3.13.1 Preparation of the PROQUEST™ Two-Hybrid Mouse Embryo 10.5 day cDNA library.....	61
2.3.13.2 Screening the library	61
2.3.14 Plasmid transformation into yeast	61
2.3.15 Yeast DNA preparation	61
2.3.16 Maintaining cultured cell lines	62
2.3.17 Transfection of cultured cells with Fugene	62

2.3.18 Immunofluorescent analysis of cultured cells	63
2.3.19 Cryo-Immunogold Electron Microscopy	63
2.3.20 Wortmannin treatment	63
2.3.21 Golgi staining with WGA-Alexa Fluor 350	64
2.3.22 Estimation of Fluid-phase endocytosis	64
2.3.23 Receptor-mediated endocytosis analysis	64
2.3.24 FRAP analysis for membrane microviscosity	65
2.3.25 Non-denaturing protein extraction from cultured cells	65
2.3.26 Protein concentration: Bradford assay	65
2.3.27 Co-Immunoprecipitation	66
2.3.28 Protein gel electrophoresis and western blotting	66
2.3.29 Protein sample preparation from cultured cells for gel filtration	66
2.3.30 Gel filtration	67
2.3.31 Protein sample concentration	67
2.3.32 Two-dimensional protein electrophoresis	67
2.3.32.1 Sample rehydration	67
2.3.32.2 Isoelectric focusing (first dimension)	68
2.3.32.3 Protein separation on SDS-PAGE (second dimension)	68
2.3.33 Coomassie blue staining and silver staining	68
2.3.34 Protein recognition	69
2.3.35 GST fusion protein expression and purification	69
Chapter Three: Functional redundancy between MID1 and MID2?	71
3.1 Introduction	71
3.2 Results	72
3.2.1 MID1 and MID2 heterodimerize	72
3.2.2 The normal microtubule-association of MID2 is disrupted in an OS derived cell line	73
3.2.3 MID2A358D – a polymorphism or a OS-causative mutation	74
3.3 Discussion	75
Chapter Four: PEPP2 was identified as a MID2 interactor.....	81
4.1 Introduction	81
4.2 Results	82
4.2.1 Yeast two-hybrid screen for MID2 interactors	82
4.2.2 Identifying the potential interactors	84

4.2.3 Validation of the interaction between MID2 and the “positives” identified in the Y2H	85
4.2.3.1 NY-CO-3 is unlikely to be a interactor of MID2	85
4.2.3.2 PEPP2 was identified as a potential interactor of MID2	86
4.2.4 The interaction between PEPP2 and MID2 was verified in mammalian cells	87
4.2.4.1 Co-localization of MID2 and PEPP2	87
4.2.4.2 Regulation of MID2 and PEPP2 interaction by MID1/MID2 heterodimerization	88
4.3 Discussion	89
Chapter Five: The cellular function of MID2 and PEPP2	95
5.1 Introduction	95
5.2 Results	96
5.2.1 The non-uniform distribution of membrane-associated PEPP2	96
5.2.1.1 The membrane-associated PEPP2 is enriched sites of at cell-cell contact	96
5.2.1.2 The membrane-association of PEPP2 is directed by phosphoinositide-binding	97
5.2.1.3 Membrane-associated PEPP2 is enriched at the actin polymerisation site	98
5.2.2 PEPP2 mediates redistribution of MID2 Δ BB to the Golgi	99
5.2.3 MID2/PEPP2 affect membrane microviscosity	99
5.2.4 The potential role of PEPP2 and MID2 in regulating endocytosis	100
5.2.4.1 The influence of MID2 and PEPP2 on fluid-phase endocytosis	101
5.2.4.2 Receptor-mediated endocytosis is affected by an overexpressed MID2 truncating mutation	102
5.3 Discussion	103
Chapter six: Unpacking MID1/MID2 protein complexes	115
6.1 Introduction	115
6.2 Results	117
6.2.1 Immuno-precipitation of the MID1 protein complexes	117
6.2.1.1 1-D electrophoresis of MID1 complexes purified by immunoprecipitation	117

6.2.1.2 2-D electrophoresis of MID1 complexes purified by immunoprecipitation	117
6.2.2 MID1 and MID2 exist in high molecular weight complexes	118
6.2.2.1 Determination of protein complexes using FPLC	118
6.2.2.2 MID1/MID2 protein complexes determined by FPLC	119
6.2.3 Decoding the MID protein complexes	120
6.2.3.1 The presence of Dynein heavy chain in MID1 complexes	120
6.2.3.2 Identification of eukaryotic translation elongation factor 1 beta in MID1 complexes	121
6.3 Discussion	123
Chapter Seven: Final Summary	127
Bibliography	137

Human congenital disorders impose a large impact not only on the affected individuals and their immediate families but also on communities, often inflicting great healthcare burdens. This thesis concentrates on one congenital disorder, Opitz Syndrome, which is a genetic disorder caused by mutations in *MIDI*. Opitz Syndrome (OS) patients present an array of clinical features including some of the more commonly found congenital structural anomalies, such as cleft lip and palate and hypospadias. The information gained from an enhanced understanding of the important cellular and molecular processes and pathways involved in Opitz Syndrome will subsequently aid in the elucidation of the basis of the individual clinical features. Only through an increased understanding of the underlying mechanisms of these congenital malformations can advances be made in prevention, diagnosis and ultimately treatment of them.