Study of vaults in skin biology and host defence - from in vitro to in vivo

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TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**Study of vaults in skin biology and host defence – from *in vitro* to
*in vivo***

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NTU Institute for Health Technologies (HealthTech NTU)**

2018

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in vitro to *in vivo***

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A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of
Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution

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Abbreviations

ADP	Adenosine diphosphate
AKT	Protein kinase B
AP-1	Activator protein 1
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BCL-2	B-cell lymphoma 2
BiP	Binding immunoglobulin protein
C/EBP β	CCAAT-enhancer-binding proteins beta
CDDP	Cisplatin
c-FOS	FBJ murine osteosarcoma viral oncogene homologue
c-Jun	Jun proto-oncogene
COP1	Constitutively photomorphogenic 1
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cells
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELK-1	ELK1, ETS Transcription Factor
EM	Electron microscopy
EPPK	Epiplakin
ER	Endoplasmic reticulum
Erk 1/2	Extracellular signal-regulated kinases 1/2
FBS	Fetal Bovine Serum
GAGE6	G antigen 6
GFP	Green fluorescence protein
GMM	Generalized method of moments
GRP78/	78 kDa glucose-regulated protein
GTP	Guanidine triphosphate
HDAC	Histone Deacetylases
HIF1- α	Hypoxia-inducible factor1- alpha
HIV TAT	Human immunodeficiency virus transactivator of transcription
HM	Hybridisation media
HMW	High molecular weight
IF	Immunofluorescence
IFN- γ	Interferon-gamma
IL-6	Interleukin 6
IL-8	Interleukin 8

IMI	Imiquimod
InlK	Internalin K
IP	Immunoprecipitation
IRE-1	Inositol-requiring enzyme 1
ISH	<i>in situ</i> Hybridisation
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Krt8	Keratin 8
Krt18	Keratin 18
LB	Luria-Bertani
LMW	Low molecular weight
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MOI	Multiplicity of infection
MVP	Major vault protein
MYH9	Myosin Heavy Chain 9
NBT	Nitro-Blue Tetrazolium
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPC	Nuclear pore complex
NS1	Non-structural protein 1
NSCLC	Non-small cell lung cancer
Nsun2	NOP2/Sun RNA Methyltransferase Family Member 2
OAS	2'-5'-oligoadenylate synthetase
OD	Optical density
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCDHA	Protocadherin α
PDGF	Platelet-derived growth factor
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase
PFA	Paraformaldehyde
PHD2	prolyl hydroxylase domain-containing protein 2
PKR	Protein kinase RNA-activated
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern recognition receptors
PTEN	Phosphatase and tensin homologue
PTU	1-phenyl-2-thiourea
pVHL	von Hippel–Lindau protein
qPCR	Quantitative polymerase chain reaction
RGD	Arginyl-glycyl-aspartic acid/arginine-glycine-aspartate
RPM	Revolution per minute
RPMI 1640	Roswell Park Memorial Institute 1640 media
RT-PCR	Reverse transcription Polymerase Chain Reaction

SH2	Src Homology 2
SHP-2	Tyrosine-protein phosphatase non-receptor type 11
siRNA	Small interfering RNA
SMEB	sucrose magnesium electroporation buffer
SNP	Single nucleotide polymorphisms
Sp-1	Specificity protein 1
SR-A	Scavenger receptor A
Src	Src Proto-Oncogene
ssRNA	Single-stranded ribonucleic acid
STAT	Signal transducers and activators of transcription
SWATH-MS	Sequential window acquisition of all theoretical mass spectra
T4P	Type IV Pili
TEP1	Telomerase-associated protein 1
TGF β 1	Transforming growth factor beta 1
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor
USF1	Upstream stimulatory factor 1
UV	Ultraviolet
VCR	Vincristine
vPARP	Vault Poly (ADP-ribose) Polymerase
Vps34	Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3
vtRNA	Vault Ribonucleic acid
WT	Wild-type
YB-1	Y-box-binding protein 1
ZMAT2	Zinc finger matrin-type 2

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Abstract

Vault, an approximately 13 MDa ribonucleoprotein first isolated from rat liver, consists of 3 proteins, TEPI, vPARP and MVP, and a group of non-coding RNAs known as vtRNAs. Vault particles have a capsular barrel-like structure which can be formed by MVP alone. MVP, the structural protein of vaults, is its most well-studied component. MVP is highly conserved amongst eukaryotes. Yet, according to genomic sequence analysis, MVP is missing in some lineages including model organisms such as *C. elegans*, *D. melanogaster* and *S. cerevisiae*. Despite the involvement of MVP in a wide variety of cellular functions such as signalling stress responses and immune responses, its biological function remains unknown. Using a combination of zebrafish and cell lines, the expression and localisation of MVP under basal and stimulated conditions were characterised in an attempt to elucidate the function(s) of vaults. MVP expression was enriched in the epithelial tissues of zebrafish larvae. Upregulation of *krt8*, *krt18* and *eppk1*, which are involved in stress responses, was observed in developing *mvp*^{-/-} larvae. In studies using cell lines, cytoplasmic staining pattern of MVP in cells was observed. When mammalian cells were treated with bacteria, a novel extracellular interaction of MVP with these bacteria was observed. This interaction was also observed between a truncated MVP fragment in plasma, and bacteria. These interactions were found in various bacteria. In particular, Psl of *P. aeruginosa* was shown to be responsible for the binding to MVP. Binding of MVP to *P. aeruginosa* did not affect the survival of bacteria but seemed to affect the aggregation of *P. aeruginosa*. These results suggest that MVP is upregulated by the presence of bacteria and acts as a sentinel of the innate immune response, modulating host-pathogen responses.

Chapter 1. Introduction

1.1. Vaults

Vaults, a type of ribonucleoprotein complexes, were first discovered as contaminants in preparations of rat liver extract coated vesicles (Kedersha and Rome, 1986). More than 30 years later, their biological function(s) remains a mystery. With an approximate mass of 13 MDa, a single vault particle is made up of multiple copies of its constituent proteins which form an internal cavity with a volume three times that of a ribosome. The complex and unique structure of vaults can be visualised by EM imaging and have been shown to be conserved in eukaryotes, from unicellular slime mould to multi-cellular organisms like mammals (Kedersha et al., 1990). This suggests that the function of vaults is linked to its structure. Early hypotheses were that vaults are involved in intracellular transport and storage. Cryo-EM imaging has been used to study the structure of vaults since its discovery. In recent years, atomic force microscopy has also been employed to study the structure of vaults in an attempt to understand their function(s) (Xia et al., 2010, Llauro et al., 2016). Despite the high level of conservation, MVP is intriguingly missing in many model organisms; *Drosophila melanogaster*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. The loss of MVP in certain lineages or organisms suggests compensation by other genes or redundancy in vault function which is contradictory to the high levels of conservation of MVP. These conflicting observations are intriguing and further shroud the function of vaults in mystery.

1.2 Structure of vaults and MVP

The large size of vaults allows for easy isolation by ultracentrifugation. Vault particles have been isolated from a variety of organisms including chickens (*Gallus gallus*), brown rats (*Rattus norvegicus*), American bullfrogs (*Rana catesbeiana*), African clawed frogs (*Xenopus laevis*), slime mould (*Dictyostelium discoideum*) (Kedersha et al., 1990) and sea urchins (*Strongylocentrotus purpuratus*) (Hamill and Suprenant, 1997). The isolation of vaults permits their visualisation by negative staining EM (Kedersha and Rome, 1986) and determination of protein composition using SDS-PAGE. Vaults were found to be made up of three proteins, major vault protein (MVP) (Kedersha and Rome, 1986), vault Poly(ADP-ribose) polymerase (vPARP) (Kickhoefer et al., 1999a) and telomerase-associated protein 1 (TEP1) (Kickhoefer et al., 1999b) with a Mr of 100, 193 and 240 kDa respectively, and 88–141 bases long untranslated RNA molecules (vtRNA) (van Zon et al., 2001, Kedersha and Rome, 1986). However, the presence of vPARP and TEP1 independent of vaults (Kickhoefer et al., 1999b, Kickhoefer et al., 1999a), coupled with their ability to move in and out of formed vaults (Zheng et al., 2005, Poderycki et al., 2006), makes estimation of their number in vault particles impossible. In addition, mRNAs have been shown to be incorporated into vault complexes (Paspalas et al., 2009), further complicating the estimation of the composition of vault components. However, as the expression of MVP alone is sufficient to form intact vault particles, structural analysis of MVP-only vault using cryo-EM and X-ray allowed for the determination of the structure and the number of MVP in vault complexes.

Figure 1.1. **Overall structure of the vault shell.** One molecule of MVP is colored in tan, and the others are colored in purple. **(Left)** Side view of the ribbon representation. The whole vault shell comprises a 78-oligomer polymer of MVP molecules. The size of the whole particle is ~ 670 Å from the top to the bottom and ~ 400 Å in maximum diameter. The particle has two protruding caps, two shoulders, and a body with an invaginated waist. Two half-vaults are associated at the waist with N-terminal domains of MVP. **(Right)** Top view of the ribbon representation. The maximum diameter of the cap is ~ 200 Å. The outer and the inner diameters of the cap-ring are shown. (Reproduced with permission from The American Association for the Advancement of Science)

Structural analysis of MVP-only vaults determined that vaults are hollow structures with dimensions of approximately 40 nm x 70 nm and a mass of approximately 13 MDa. A single vault particle consists of two halves which form a characteristic barrel-shaped structure consisting of two cap regions at each end and a waist region with a wall thickness of 15 to 25 Å (Tanaka et al., 2009). The volume of the cavity of vaults is approximately 50 million Å³, which is 2-3 times the volume of a single ribosome. An initial model, comprising 96 copies of MVP, 8 copies of vPARP, 2 copies of TEP1 and approximately 16 copies of vtRNAs, was initially proposed as the composition of single vault complex (Kedersha et al., 1991). Subsequently, the number of MVP per vault particle was corrected to

78 copies (Tanaka et al., 2009) when the x-ray structure of rat vaults was resolved at 3.5 Å (Figure 1.1). However, the number of the other vault components remains undetermined. The discrepancy in the number of MVP per vault particle started from the preliminary structure of vaults collected by cryo-EM (Kong et al., 1999). The initial observation of unfolded vaults with eight petals in freeze-etch preparation (Kedersha et al., 1991) resulted in the authors choosing a model with C8 symmetry. This was due to the poor agreement of correlation values between particle images. The relatively low resolution of 31 Å might also prevent the detection of features that would allow for correct determination of symmetries. This C8 symmetry was later changed to D8 symmetry (Kong et al., 2000), and the new symmetry was influenced by the calculation of molecular weight by estimation of the number of MVP, TEP1, vPARP and vtRNA. In a separate study on the structure determination of CP-MVP (Mikyas et al., 2004), the authors observed apparent 48-fold symmetry in their images and thus imposed a 48-fold rotational symmetry to their model. Subsequent 2D and 3D crystal structure analyses of isolated native vaults extracted from rat livers suggest that vault barrel region have 6-fold or 3 fold symmetry (Querol-Audi et al., 2005). Together, these results seem to support a 96-mer vault particle model. In the case of Anderson et al, they used the same CP-MVP used by Kong et al. Furthermore, they were attempting to refine the previous model of the 48-fold symmetry vault complex. In the case of Tanaka et al, they used purified vaults isolated from rat liver. Initial rotational function analysis did not give consistent results and a phase refinement using NCS refinement was adopted, assuming a wide range of rotational symmetry from 2 to 48-fold. This analysis led to the observation of high correlation peaks at 3-, 13- and 39-fold symmetries, giving

rise to the model with 39 copies per vault half. Figure 1.1 shows the arrangement of all 78 copies of MVP in a vault complex. The location of the minor vault proteins and vtRNAs are not depicted in the model. Each single MVP molecule spans the entire height of a vault half. The N-terminus beta-fold domains are arranged in parallel and make up the body of the vault particle while the C-terminus alpha-helical coiled-coil domain forms the cap region. To aid in visualisation, the authors have highlighted a single MVP molecule (in tan) with respect to the other MVP molecules (in blue).

A stereoscopic structure of MVP monomer is shown in Figure 1.2 (Tanaka et al., 2009). Each MVP monomer is separated into 4 distinct segments. The first segment consists of nine vault repeats (aa 1-519). These repeats consist of a series of loops and antiparallel β -strands (Kozlov et al., 2006) and have been shown to have some level of motility to allow movement of proteins into formed vault particles (Poderycki et al., 2006). The second segment is the shoulder domain (aa 520-646) which resembles stomatin/prohibitin/flotillin/HflK/C (SPFH) domains found in many membrane proteins and have been involved in interaction with lipid rafts (Browman et al., 2007). Mouse podocin contains a SPFH domain capable of binding cholesterol, providing a molecular basis for the interaction of SPFH domain-containing protein with lipid rafts (Huber et al., 2006). This SPFH-like shoulder domain might be responsible for the interaction of MVP with lipid rafts in epithelial cells (Kowalski et al., 2007). The shoulder domain has also been suggested to facilitate intermolecular interactions between adjacent MVP monomers due to low energy scores obtained when sequences containing the shoulder domain were analysed by RosettaDock (Daly et al., 2013a).

Figure 1.2. **Stereoscopic ribbon drawing of the overall fold of an MVP monomer.** The MVP monomer is folded into nine structural repeat domains, a shoulder domain, a cap-helix domain, and a cap-ring domain. Each domain is depicted in a different color: domain 1 (Met¹-Pro⁵⁵), purple; domain 2 (Arg⁵⁶-Thr¹¹⁰), pink; domain 3 (Pro¹¹¹-Ile¹⁶³), light green; domain 4 (Gln¹⁶⁴-Val²¹⁶), coral; domain 5 (Asp²¹⁷-Val²⁷¹), light blue; domain 6 (Pro²⁷²-Asp³²²), magenta; domain 7 (Val³²³-Gln³⁷⁸), yellow; domain 8 (Ala³⁷⁹-Arg⁴⁵⁶), red; domain 9 (Val⁴⁵⁷-Gly⁵¹⁹), cyan; shoulder domain (Pro⁵²⁰-Val⁶⁴⁶), green; cap-helix domain (Asp⁶⁴⁷-Leu⁸⁰²), purple; and cap-ring domain (Gly⁸⁰³-Ala⁸⁴⁵), dark red. (Reproduced with permission from The American Association for the Advancement of Science)

The third segment is the alpha-helical ‘cap’ region (aa 647-802) which consists of an alpha-helical coiled-coil domain. Yeast two-hybrid experiment using various truncated MVP fragments demonstrated that this C-terminus coiled-coil domain is responsible for maintaining the structure of the vault complex. This is mediated by extensive lateral intermolecular interactions between adjacent stands of MVP monomer in the coiled-coil domain (van Zon et al., 2002). The last segment (aa803-893), known as the cap-ring domain, consists of the C-terminus tail which folds in to form a pore. This pore is the docking site for TEP1, which binds and anchor vtRNAs to vaults (Kong et al., 2000). The unique architecture of the vault complex requires specific intermolecular interactions between adjacent MVP monomers. A total of 74 intermolecular interactions was reported,

with 41 of them within the 160 amino acid long cap-helix domain. This gives an average of one interaction per four amino acid residues, of which two-thirds are hydrophobic interactions. Side groups of charged amino acids in the cap-helix domain tend to point into the cavity or out towards the external environment while hydrophobic side groups are oriented towards adjacent strands. These extensive hydrophobic interactions give the cap-helix region high structural stability (Tanaka and Tsukihara, 2012). In contrast, the β -folds at the N-terminus have the lowest number of intermolecular interactions per amino acid residue and truncated N-terminal repeats was unable to form stable interactions with MVP monomers (van Zon et al., 2002). Using AFM, the barrel wall of vaults was shown to undergo reversible fracture (Llauro et al., 2014). These relatively weak interactions might be responsible for the flexibility of the barrel wall of vaults which allow the incorporation protein cargo (Poderycki et al., 2006) and possibly mRNA cargo (Paspalas et al., 2009) into the central cavity. VPARP is able to incorporate into preformed MVP-only vaults (Poderycki et al., 2006) and this was found to be mediated by the C-terminus of vPARP (residues 1563–1724). This C-terminal portion of vPARP was termed as M-INT (minimal size interaction domain). The 19 kDa M-INT alone or proteins tagged with M-INT domain was shown to interact with the luminal side of the waist of the vault particle, corresponding to the MVP repeat domain 3-5 (Mikyas et al., 2004, Yu et al., 2017, Kozlov et al., 2006, Kickhoefer et al., 2005). Incorporation of M-INT fragment, cloned with a thrombin cut site, into the interior of vaults reduced its cleavage by thrombin. The cargos incorporated into the cavity is 193 kDa for vPARP and 19 kDa for M-INT. This suggests either presence of the interaction domain is sufficient to enter or passive diffusion will occur for any proteins 193

kDa and below. However, the 23 kDa thrombin was unable to access the cavity to digest the M-INT fragment, which has a thrombin cut site gained from cloning procedure. As the size of thrombin is smaller than that of the 193 kDa vPARP, it suggests that entry to the vault cavity is not due to passive diffusion alone. Furthermore, other proteins tagged with M-INT domain has been shown to enter the cavity of vaults (Goldsmith et al., 2009, Han et al., 2011). This suggests that INT is important for entry into the cavity.

Furthermore, incorporation of GL-INT, a M-INT domain-tagged Green lantern protein (GFP codon optimised for expression in mammalian cells), into vault particles reduced the rate of quenching of GL-INT fluorescence by KCl (Poderycki et al., 2006). Together, these suggest that there is a discrimination between various molecules that can enter the luminal cavity of vaults.

Based on all the above-mentioned observations, the ability to control the entry of molecules into its cavity might be important for the function of vaults. This requires the correct intermolecular interactions between amino acids and conservation of amino acid sequences is essential to ensure that MVP monomers fold and interact correctly to form functional vault particles.

1.3. Proposed functions of vaults

Since the discovery of vaults, there have been many proposed function of vaults. The hollow cavity of vaults has led to hypotheses implicating vaults in intracellular transport or storage of various molecules (van Zon et al., 2006, Slesina et al., 2006). The structural similarity between vault complex and the nuclear pore plug that docks onto the pore of nuclear pore complexes (NPC) (Dickenson et al., 2007) has also led to a hypothesis of vaults acting as nuclear-

cytoplasmic cargo shuttles. The tapered cap region of the vault particle and the pore of NPC both have a diameter of approximately 30-35 nm and a mass of 13 MDa. MVP antibody staining showed the association of MVP with the nuclear membrane and nuclear pore complexes. This led to Chugani et al putting forward the proposal that vaults are nuclear pore plugs. (Chugani et al., 1993). The proposed function of vaults was further expanded to encompass intracellular transport across the cytosolic space when the interaction of vaults with cytoskeleton was discovered (van Zon et al., 2006, Paspalas et al., 2009).

The vault complex and its components have also been implicated in cancers (Dingemans et al., 1996, Izquierdo et al., 1996a, Hart et al., 1997, Berger et al., 2000, Berger et al., 2001, Lotsch et al., 2013, Lee et al., 2017). Vault components was often upregulated in high grade tumours and drug-resistant tumours (Kickhoefer et al., 1998, Komarov et al., 1998, Rimsza et al., 1999, Scheffer et al., 2000, Berger et al., 2000, Mossink et al., 2003b, Scheffer et al., 1995, Izquierdo et al., 1996b, Szaflarski et al., 2011). In many of these cases, high levels MVP were correlated with cancer development and poorer prognosis (Izquierdo et al., 1996b, List et al., 1996, Silva et al., 2007, Xu et al., 1999, Gopinath et al., 2010, Lotsch et al., 2013, Lee et al., 2017). This has led to a hypothesis of vaults being involved in malignancy and drug resistance in tumours. However, a subsequent experiment showed that MVP upregulation was not sufficient to confer drug resistance (Siva et al., 2001) and MVP knockouts mice were not hypersensitive to cytostatic drugs (Mossink et al., 2002b). While the functions of vault complexes as a whole remain unknown, research into the function and biology of individual vault protein and vtRNAs have provided some clues on the function of vaults. These research are summarised in the following sections.

1.4. Vault RNAs

Vault RNAs (vtRNAs) are a class of non-coding RNA 88-150 nt long (van Zon et al., 2001) that associate with vault complexes via binding to TEP1 (Kong et al., 2000, Kickhoefer et al., 2001). vtRNAs make up approximately 5% by mass of a single vault complex (Nandy et al., 2009, Kong et al., 2000) and their functions are largely unknown. However, only 5% of all vtRNAs are associated with vaults, suggesting vault-independent function of vtRNAs. vtRNAs are a class of polymerase III transcripts that can be processed into small RNAs that bind Argonaute proteins and have miRNA-like functions (Vilalta et al., 1994, Minones-Moyano et al., 2013). Cleavage of vtRNAs into smaller fragments is dependent on cytosine-5-methylation mediated by Nsun2 (Hussain et al., 2013). vtRNA genes are generally found in two genomic loci, one between ZMAT2 and PCDHA genes (VTRNA1 locus) and the other between TGFB1 and SMAD5 genes (VTRNA2 locus). In humans, there is an additional pseudogene found on the X chromosome. The VTRNA2 locus is conserved in most organisms but there are some highly derived sequences in cow (*Bos taurus*) and rabbit (*Oryctolagus cuniculus*) and loss of the VTRNA2 loci in mouse and rat. The VTRNA1 locus is less conserved, with duplication events leading to multicopy clusters of vtRNA1. These are found in primates, guinea pigs (*Cavia porcellus*), teleost fish and others. *Homo sapiens* possesses 3 vtRNA genes at the VTRNA1 locus with VTRNA1-2 and VTRNA1-3 being recent duplicates (Stadler et al., 2009). vtRNAs have a low level of sequence homology to each other and across species. Homologies in vtRNAs are found mainly in the box A and box B regions, both of which are internal polymerase III promoter elements. Interestingly, all vtRNAs are reported to form a conserved pan-handle like secondary structure with a stem-

loop structure and a highly variable loop region (Mosig et al., 2007). This loop is responsible for interaction with chemotherapeutic compound mitoxantrone (Gopinath et al., 2010).

The low level of sequence homology between various vtRNAs, substantial length variations, duplications and losses in the various loci have made identification of vtRNAs difficult. This is further compounded by incompletely assembled or low coverage genomic sequences. One example is the contentious proposal of assigning miR886 as vtRNA (Stadler et al., 2009, Nandy et al., 2009) due to the sequence homology of pre-miR886 around the A and B box regions. However, in a separate paper, the authors argued miR886 is not a vtRNA. miR886 sequences were divergent from the homologous sequences of the other vtRNAs that extend beyond the box regions and miR886 was also not functionally or physically associated to the vault complex (Lee et al., 2011).

Despite these difficulties, researchers have managed to associate vtRNAs with certain functions in human cells and tissues. Thus far, vtRNAs expression has also been shown to protect against apoptosis (Amort et al., 2015), induce neuronal dysfunction (Minones-Moyano et al., 2013) and is involved in innate immunity, where it represses activation of PKR (Li et al., 2015). vtRNAs have also been shown to bind to anti-tumour drugs, such as mitoxantrone (Gopinath et al., 2010), and induce GAGE6 expression by binding to PSF, providing a mechanism by which vtRNAs contributes to the drug resistance of certain cancer cells (Chen et al., 2018).

1.5. TEP1

Human telomerase-associated protein 1 (TEP1) is a 240 kDa protein that binds to the cap of vault complexes and is responsible for the binding of vtRNAs to the vault complexes (Kong et al., 2000). TEP1 binds vtRNAs and telomerase RNAs via a conserved *tetrahymena* p80 homology domain (Poderycki et al., 2005). vtRNAs and telomerase RNAs compete for the same binding site on TEP1, which was subsequently defined as a TROVE module, commonly found in ribonucleoparticles (Bateman and Kickhoefer, 2003). Despite competing for the same binding site, vtRNAs and telomerase RNAs are targeted to vault complexes and telomerase complexes respectively. This suggests the presence of other factors regulating the interaction of these RNAs to TEP1. Knockout of TEP1 led to a lack of vtRNAs incorporation into vault complexes, vault particles isolated from *Tep1*^{-/-} mice have a reduction in density of material at the two caps. This shows that TEP1 is necessary for the interaction of vtRNA with vaults (Kickhoefer et al., 2001). Interestingly, TEP1 is not required to target telomerase RNAs to the telomerase complex (Liu et al., 2000). The mechanism for this difference in interactions is unknown. Despite being associated with the telomerase complex, TEP1 is not necessary for telomerase activities. *Tep1*^{-/-} mice did not show altered telomere length or any apparent developmental defects (Liu et al., 2000). Interestingly, TEP1 also mediates the binding of vPARP to the telomerase complex in a vault-independent manner. However, vPARP association with vaults is independent of TEP1 (Liu et al., 2004).

1.6. vPARP

vPARP (also known as PARP4/ARTD4/PH5P (Jean et al., 1999)/ADPRTL1 (Still et al., 1999)) is a 193 kDa vault-associated protein that belongs to the poly(ADP-ribose) polymerase family. (Kickhoefer et al., 1999a). PARPs are an ancient family of proteins that utilise NAD⁺ to add ADP-ribose to target proteins and PARP1 is the most studied member of the family. Canonically, PARPs are thought to generate both branched and linear chains consisting of up to 200 units of ADP-ribose linked via glycosidic bonds (Alvarez-Gonzalez and Jacobson, 1987). The catalytic activity of PARPs is due to the H-Y-E catalytic triad which was originally identified in the active site of bacteria mono-ADP-ribose transferase toxins (Marsischky et al., 1995, Papini et al., 1989). Poly-ADP-ribosylation modulates functions of target proteins including DNA damage repair (Wang et al., 2012, Ko and Ren, 2012), cellular stress response (Fujimoto et al., 2018, Nicoletti and Stella, 2003, Mathews and Berk, 2008, Ma et al., 2011, Jwa and Chang, 2012), cell division (Muramatsu et al., 2007, Ha et al., 2012), transcription regulation (Ko and Ren, 2012) and protein degradation (Arnold and Grune, 2002, Zhang et al., 2011). Substitution mutations in the catalytic domain resulted in certain members of the PARP family being only able to generate mono(ADP-ribose) modification of their targets (Kleine et al., 2008). In addition, there are some substitutions that result in a catalytically inactive domain (Vyas et al., 2014).

In humans, there are 17 known PARPs (Ame et al., 2004) and other than the catalytic ADP-ribose transferase domain, there exists little sequence homology between the members. The differences in sequence might be due to a requirement for different interaction domains specific to their targets. vPARP (also known as

PARP4) is exceptional with regards to the location of its catalytic domain. All 16 other PARPs have the catalytic domain at their C-termini while vPARP has an internal catalytic domain. The C-terminus of vPARP is instead occupied by the vault interaction domain. The vPARP gene is located on human chromosome 13 (Still et al., 1999) and its promoter contains Sp1, MyoD and GATA consensus sequence binding (Emre et al., 2004). vPARP, with 1724 amino acids, is the second largest member of the PARP family and consists of a BRCT domain (BRCA1 carboxy-terminal domain), a VIT (vault protein inter-alpha-trypsin) domain, a vWA (von Willebrand type A) domain and several PRD (PARP regulatory domain)-like domains (Kickhoefer et al., 1999a). BRCT domains are found in many cell cycle checkpoint and DNA damage repair proteins (Bork et al., 1997). This, coupled with the presence of vPARP in the telomerase complex, suggests a possible role of vPARP in DNA damage response repair. The VIT and vWA domains are protein-protein interaction domains (Colombatti et al., 1993) that possibly facilitate the binding of vPARP to its targets, whilst the PRD-like domain resembles domains that are thought to regulate the branching of ADP-ribose chains (Simonin et al., 1993).

Interestingly, vPARP displayed both poly-ADP-ribosylation and mono-ADP-ribosylation activities and the type of ADP-ribosylation it carries out is dependent on its association with vaults; vault-bound vPARP has poly-ADP-ribosylation activity (Kickhoefer et al., 1999a), while the free form has mono-ADP-ribosylation activity (Vyas et al., 2014). In the cell, vPARP can be found in the nucleus and the mitotic spindles independent of vault complexes (Kickhoefer et al., 1999a). This suggests different activities of vPARP depending on its cellular localisation, where it mediates different functions through differential ADP-

ribosylation modification of target proteins. Despite the implication of vPARP in telomerase activity and DNA repair, vPARP knockout mice did not show any defects. This shows that vPARP is not required for the proper development of mice, telomerase activity or telomere length maintenance (Liu et al., 2004). The only difference observed in vPARP knockout mice was an increased risk of tumorigenesis induced by carcinogens (Raval-Fernandes et al., 2005).

1.7. MVP

MVP is a 100 kDa protein that makes up >70% mass of a single vault particle. Each vault particle consists of 78-copies of MVP arranged into two equal halves (Tanaka et al., 2009) and expression of MVP alone is sufficient to form the characteristic architecture of vault complexes without any requirement for other vault components (Stephen et al., 2001). Vaults are generated from MVP monomers as they are being transcribed off polyribosomes complexes and all MVP are incorporated into vault complexes (Mrazek et al., 2014). Many studies have used MVP as the proxy for the study of vault function, making it the most well-studied vault component. MVP homologs are highly conserved and can be found throughout the eukaryote kingdom. Intact vault particles from various organisms have been isolated, proving that homologs are also structurally similar (Kedersha et al., 1990). *In silico* reconstruction, using amino acid sequences and structure predictions, predicts that MVP is present in the last eukaryotic common ancestor (Daly et al., 2013b). This suggests MVP serves important functions in fundamental processes. The high level of conservation in amino acid sequences of MVP might be due to the extensive intermolecular interactions required to maintain the tertiary vault structure (Tanaka and Tsukihara, 2012), implying that

the structure of vault is important for its function. However, despite the conserved nature of MVP, the *mvp* gene has been lost in certain lineages such as insects, plants and fungi. There is also a duplication of *mvp* gene in slime moulds (Vasu and Rome, 1995).

MVP is expressed in a wide variety of tissues. In humans, MVP is ubiquitously expressed in all cell type with high levels of MVP proteins in macrophage and mucosal epithelial (Izquierdo et al., 1996a). In rats, MVP is found in the liver (Kedersha and Rome, 1986), but its expression in other tissues has not been documented. MVP reacts to various stimuli by translocating to specific locations within the cell, such as ruffling edges (Herrmann et al., 1999), neuritic tips (Herrmann et al., 1999), lipid rafts (Kowalski et al., 2007), nucleus (Slesina et al., 2005) and lysosomes (Herlevsen et al., 2007). MVP also localises to the cell surface of circulating hepatocellular carcinoma cells (Lee et al., 2017). Recently, MVP was shown to be expressed in macrophages, epithelial and endothelial cells of the kidneys, gills, spleen and liver in rainbow trout. MVP expression was also detected in fish lymphoid cells (Margiotta et al., 2017).

1.7.1. MVP involvement in intracellular trafficking

The biological function of vaults and MVP have been filled with contradictions since their discovery. The unique hollow barrel-shaped structure of vaults led to early speculations that they are involved in intracellular transport or storage (Rome et al., 1991). As the expression of MVP alone is sufficient to form intact vaults and all MVPs are concurrently incorporated into vaults while they are being translated on the polyribosomes, there has been an initial focus on understanding the function of MVP in transport. GFP-tagged MVP forms intact

vaults and has been used to understand the localisation and transport of MVP within the cytoplasm (Slesina et al., 2006). MVP-GFP was initially thought to diffuse freely in the cytoplasm (van Zon et al., 2003). However, a separate investigation estimated that MVP-GFP moves at approximately 10 $\mu\text{m/s}$ in the cytoplasm (Slesina et al., 2006), suggesting an active transport of MVP. MVP is regularly found to be associated with cytoskeletal elements, actin and microtubules, further supporting the hypothesis of active transport of intracellular vaults (Eichenmuller et al., 2003, van Zon et al., 2006). Additionally, observation of MVP localisation at both proximal and distal ends of the crush site of *Torpedo marmorata* electricity-generating nerves suggests both retrograde and anterograde transport (Li et al., 1999). These all point to a regulated transport that is cytoskeleton-dependent.

MVP is involved in the transport of transcription factors and signalling molecules. MVP enhances nuclear localisation of PTEN, leading to an accumulation of PTEN in the nucleus of cells and thus modulates PTEN signalling (Yu et al., 2002). In MCF7 cells, MVP binds oestrogen receptor to facilitate its shuttling from the cytoplasm to the nucleus when exposed to estradiol (Abbondanza et al., 1998). MVP also enhanced nuclear translocation of transcription factors c-Fos and C/EBP β in response to influenza A virus or stimulation with the combination of poly(I:C) and IFN- γ (Peng et al., 2016). Recently, MVP was found to participate in exosome signalling. Proteomic analysis demonstrated the presence of MVP in purified exosomes of colon carcinoma cells where it binds and facilitates the sorting of miR-193a, a tumour suppressor, into exosomes. The packaging of miR-193a into exosomes led to a reduction in intracellular miR-193a concentration which led to increased

proliferation of cancer cells, enhanced growth and metastasis (Teng et al., 2017). In a separate study, MVP was found to interact with miRNAs and package them into exosomes (Statello et al., 2018). MVP has also been shown to bind mRNAs in neurites (Paspalas et al., 2009) Together, it suggests that MVP acts as a shuttle that can transport a wide variety of cargo to various compartments of the cell.

1.7.2. MVP is linked drug resistance and prognosis of cancers

One early attributed property of MVP was the conferment of drug resistance on cells. A 110 kDa protein (named lung resistance protein) was previously found to be overexpressed in multidrug-resistant P-glycoprotein-negative tumour cell lines (Scheper et al., 1993) and this 110 kDa protein was also found to be upregulated in other multidrug-resistant tumours (Izquierdo et al., 1996b, List et al., 1996, Xu et al., 1999, Scheffer et al., 2000). Subsequent sequencing identified this lung resistance protein as MVP (Scheffer et al., 1995).

Most of the studies on the drug resistance conferred by MVP involved the correlation of MVP levels detected through immunohistology, western blot and qPCR with the intrinsic drug resistance of the cell lines/tumours tested. For example, MVP was found upregulated in glioma cells and the levels of MVP was inversely correlated to chemosensitivity towards antineoplastic drugs (Berger et al., 2001). Drug resistance in U937 cells was also shown to follow the induction of MVP upregulation (Hu et al., 2002). However, there are several studies excluding the role of MVP in drug resistance. Vault upregulation was necessary yet insufficient for multi-drug resistance in AC16 cells (Siva et al., 2001). In ovarian cancers, conflicting data exist, suggesting that drug resistance might not be due to MVP overexpression (Izquierdo et al., 1995, Arts et al., 1999). High

MVP levels were often correlated to poor treatment outcomes in acute myeloid leukaemia (List et al., 1996, Xu et al., 1999), multiple myeloma (Filipits et al., 1999, Rimsza et al., 1999) and oropharyngeal carcinoma (Silva et al., 2007).

Some studies have attempted to determine the molecular mechanisms by which MVP confers drug resistance. MVP colocalises with doxorubicin in cytoplasmic vesicles in A549, a non-small cell lung cancer (NSCLC) cell line. Using siRNA against MVP, it was demonstrated that MVP knock-down inhibited the sequestration of doxorubicin into perinuclear lysosome (Herlevsen et al., 2007). This led to an increase in doxorubicin localisation in the nucleus and increased cytotoxicity to the treated cells. However, there were no differences in the efflux of daunorubicin between daunorubicin-sensitive SW1573 cells and MVP-GFP transfected SW1573. Knock-down of MVP in doxorubicin-selected SW1573-2R120 cell line also did not restore chemosensitivity. Together, these observations indicate vaults are not involved in the efflux of anthracyclines from the nucleus or its sequestration in vesicles (van Zon et al., 2004). Stem cells and bone marrow cells from *mvp*^{-/-} mouse also did not show hypersensitivity to antineoplastic drugs and daunomycin nuclear transport and intracellular distribution was not altered in *Mvp*^{-/-} mouse embryonic fibroblasts when compared to wild-type fibroblasts (Mossink et al., 2002b).

These contradictory observations are one of the many encountered by researchers when investigating the function of MVP. However, the results suggest that MVP is not directly involved in multi-drug resistance of cells and tumours. Instead, MVP upregulation might be a stress response in response to drug treatments.

1.7.3. MVP protects cells against various cellular stress

MVP is regularly involved in the modulation of stress response pathways against various stressors in different cell types (Table 1). Studies in various cell types also demonstrated that MVP knockout and knockdown had negative impacts on the viability of cells, especially under stressful conditions. MVP expression can be regulated by PMA (Komarov et al., 1998), HDAC inhibitors (Steiner et al., 2004) and also cytotoxic drugs. Chromatin remodelling (Emre et al., 2004) and alternative splicing (Holzmann et al., 2001) have also been suggested to regulate MVP expression. Investigation of MVP promoter regions has further suggested the involvement of MVP in stress responses. The -700 bp promoter sequence of human MVP and -449 bp promoter sequence of mouse MVP had 58% sequence identity (Mossink et al., 2002a). However, there are stark differences in terms of transcription factors binding sites. In human MVP promoter, there are binding sites for Sp-1, p53, MyoD, STAT and GATA (Lange et al., 2000, Steiner et al., 2006) whereas mouse promoter has a binding site for smad3 (Mossink et al., 2002a). This difference suggests the presence of conserved basal regulators of MVP expression with different induction mechanisms in mouse and humans. Transcription factors Sp-1 (Steiner et al., 2004, Liu et al., 2012), USF1 (Ikeda et al., 2014) and YB-1 (Stein et al., 2005, Tian et al., 2011) have been experimentally shown to modulate MVP expression in cells. Involvement of these transcription factors in stress responses (Wang et al., 2002, Ryu et al., 2003, D'Addario et al., 2006, Kim et al., 2013, Tanaka et al., 2014, Lyons et al., 2016, Galibert et al., 2001, Virolle et al., 2002), hypersensitivity of *MVP*^{-/-} cells to serum starvation-induced apoptosis and defects of MVP knock-out slime mould under nutritional stress (Vasu and Rome, 1995) led to a hypothesis of MVP being

involved in cellular stress responses.

Table 1.1. Summary of stress responses MVP is implicated in:

Type of cellular stress	Cell Type	Ref
Hyperosmotic stress	SW620 (colon carcinoma)	(Ikeda et al., 2008, Tajitsu et al., 2013, Ikeda et al., 2014)
Hypoxic stress	ACHN (renal adenocarcinoma)	(Iwashita et al., 2010)
Apoptosis	Huh7 Human bronchial Smooth muscle cells Human diploid fibroblast	(Ryu et al., 2008, An et al., 2009)
Serum starvation	Huh7 H7 (glioblastoma)	(Lotsch et al., 2013)
Low-density seeding	Huh7 H7 (glioblastoma)	(Lotsch et al., 2013)
Senescence	Human diploid fibroblast MCF7	(Ryu et al., 2008, An et al., 2009)
Oxidative stress	Human diploid fibroblast	(Ryu et al., 2008, An et al., 2009)
ER stress	Human diploid fibroblast	(Ryu et al., 2008, Zhang et al., 2015)
Anti-neoplastic drugs	Glioblastoma broncho - epithelial cell line Non-small cell lung cancer human embryonic lung fibroblasts	(Komarov et al., 1998, Shimamoto et al., 2006)
UV stress	HEK293, HeLa	(Yi et al., 2005)
DNA damage	SW620	(Shimamoto et al., 2006)

The structural similarity of the shoulder domain of MVP to toxic anion resistance protein family and subsequent experiment showing the aggregate formation of vaults in toxic anion tellurite treated cells provide evidence for the involvement of MVP in stress responses (Suprenant et al., 2007). This led to subsequent studies investigating the role of MVP in the response to and protection of cells against various stresses; these stresses include hypoxia (Iwashita et al., 2010), hyperosmolarity stress (Ikeda et al., 2008, Tajitsu et al., 2013), DNA damage

(Lloret et al., 2009), senescence (Pasillas et al., 2015, Ryu et al., 2008), oxidative stress (Ryu et al., 2008) and ER stress (Zhang et al., 2015). In ACHN, MVP binds PHD2/pVHL/HIF1- α complex and negatively regulates its levels through increased degradation via the ubiquitin/proteasome pathway (Iwashita et al., 2010). In fibroblast, the protective property of MVP was observed in senescent fibroblasts but not young fibroblasts. Upon apoptotic stress, MVP was downregulated in young fibroblasts but not in senescent fibroblasts, resulting in increased susceptibility of young fibroblasts to apoptotic stress. Continued expression of MVP in aged fibroblasts led to sustained BCL-2 level through negative modulation of the JNK pathway (Ryu et al., 2008). This difference between old and young fibroblasts was subsequently found to be dependent on p53 levels (An et al., 2009). In SW620 cells, osmotic stress leads to activation of the p38/MAPK pathway which increased MVP level. PI3K/AKT pathway was shown to be the downstream pathway by which MVP exerts its protective property (Ikeda et al., 2008). Follow-up study demonstrated that upon stress, degradation of Sp-1 transcription factor was reduced, resulting in increased binding to MVP promoter thus leading to increased MVP expression (Tajitsu et al., 2013). MVP was also demonstrated to have protective functions when cells experience DNA damage (Shimamoto et al., 2006). When cells were treated with a panel of anti-cancer drugs (Adriamycin, VP-16, SN-38, CDDP, VCR and Taxol), MVP was upregulated only in response to DNA-damaging agents, Adriamycin, VP-16, SN-38, and CDDP but not when treated with VCR and Taxol. In addition, treatment of cells with UV irradiation and ethidium bromide also led to the upregulation of MVP. In a separate study, MVP was proposed to affect non-homologous end joining through ku70/80 (Lloret et al., 2009).

Together, the data suggest MVP plays a role in DNA damage response.

In bronchial smooth muscle cells, MVP acts as a cell survival factor inhibiting heat-induced cell death. This protection was initiated by S-glutathionylation of MVP which led to the interaction of MVP and MYD9/Vps34/Beclin-1 complex, resulting in the sequestration of Beclin-1 and preventing Beclin-1-dependent cell death (Shults et al., 2016). In *D. discoideum* and *T. brucei*, MVP was shown to be involved in the resistance towards nutritional stress. Disruption of MVP gene in these two organisms led to morphological changes under nutritional stress (Vasu and Rome, 1995, SHAIK, 2013). In accordance, overexpression of MVP enhanced the growth and survival of cells (Lotsch et al., 2013, Das et al., 2016, Losert et al., 2012, Lee et al., 2017). Together, these observations suggest MVP is important in mediating cellular stress responses.

1.7.4. Modulation of signalling pathways by MVP

In many of the studies investigating the involvement of MVP in stress resistance, MVP was often linked to the regulation of intracellular signalling pathways (Table 2). MVP either functioned as a shuttle to facilitate nuclear-cytoplasmic translocation, as a signalling scaffold for the various components to interact or as a vessel to contain signalling components. In a yeast two-hybrid screen, MVP was found to bind the C2 domain of PTEN in a Ca^{2+} -dependent manner (Minaguchi et al., 2006), resulting in enhanced nuclear transport of PTEN and activation of p44/42 MAPK (Yu et al., 2002). Binding of MVP to PTEN was also observed during ER stress response. Inactive PERK, an ER stress mediator, was shown to sequester vaults, preventing it from transporting PTEN to the nucleus.

Table 1.2. Signalling pathways MVP is involved in:

Type of Signaling	Proposed role	Ref.
PTEN	Nuclear-cytoplasm shuttle	(Yu et al., 2002, Chung et al., 2005, Minaguchi et al., 2006, Zhang et al., 2015)
Estrogen receptor signalling	Nuclear-cytoplasm shuttle	(Abbondanza et al., 1998)
JNK	Nuclear-cytoplasm shuttle	(Yi et al., 2005, Ryu et al., 2008, Tajitsu et al., 2013)
EGFR/MAPK	Scaffold protein	(Lotsch et al., 2013, Kolli et al., 2004)
cFos/C/EBP β	Nuclear-cytoplasm shuttle	(Peng et al., 2016)
JAK/STAT	Scaffold protein	(Steiner et al., 2006, Rivera-Rivera et al., 2012)
TLR signalling	Scaffold protein	(Liu et al., 2015)
IFN	Nuclear-cytoplasm shuttle	(Steiner et al., 2006, Liu et al., 2012, Rivera-Rivera et al., 2012)
TNF- α /p38/JNK	Scaffold protein	(Ben et al., 2013)
Exosome	Transport vessel?	(Teng et al., 2017, Statello et al., 2018)

Activation of PERK results in the dissociation of MVP, which in turns binds and facilitates the nuclear transport of PTEN (Zhang et al., 2015). MVP also interacted with estrogen receptor when MCF-7 cells were treated with estradiol (Abbondanza et al., 1998). During senescence, Bag3 binding to MVP resulted in a reduction in proteasomal degradation of MVP, leading to increased MVP stability. This stabilisation resulted in an increased nuclear translocation of MVP which sustains the activation of Erk1/2 (Pasillas et al., 2015). This suggests a role of MVP in the mediation of nuclear signalling through nuclear receptor transport. Another evidence supporting the involvement of MVP cell signalling is the phosphorylation of MVP. Early studies of the amino acid sequences of MVP in electric ray, human, rat and slime mould revealed conserved phosphorylation sites for tyrosine kinase, casein kinase and protein kinase C. MVP was found to

be phosphorylated in isolated synaptic vesicles (Herrmann et al., 1996) and microsomal fractions (Ehrnsperger and Volkandt, 2001). Experiments demonstrated the phosphorylation of MVP *in vivo* by protein tyrosine kinase (Herrmann et al., 1998) and *in vitro* by casein kinase II (Ehrnsperger and Volkandt, 2001) and protein kinase C (Herrmann et al., 1998). It was suggested that differential regulation of the phosphorylation states of MVP leads to the recruitment of different signalling mediators. Activation of the EGF receptor leads to tyrosyl-phosphorylation of MVP and phosphorylated MVP binds with the SH2 domains of SHP-2. The interaction of pMVP with SHP-2 leads to the activation of Erk and down-stream Elk-1 transcriptional activities. The increased binding of Erk to MVP-SHP-2 complex suggests that MVP acts as a scaffold protein to increase signalling efficiency (Kolli et al., 2004). In addition, MVP has been shown to upregulate levels of EGFR on cell membrane resulting in enhanced EGFR signalling in glioblastoma. This could be the mechanism responsible for the correlation between MVP levels and malignant progression in cancers (Lotsch et al., 2013) and suggests a positive regulation of EGF-MVP signalling.

Intriguingly, MVP also binds to the SH2 domain of Src where it inhibits the kinase activity of Src, resulting in reduced Erk activation (Kim et al., 2006). These data exemplify the contradictory observations with MVP and suggest differential functions of MVP in different organisms or cell types.

In a separate study, affinity purification isolated MVP as a novel interaction partner of COP1. COP1 is an E3 ubiquitin ligase responsible for the targeting of p53 and c-Jun for proteasomal degradation. MVP binds to cytoplasmic COP-1 under basal conditions and UV radiation leads to MVP phosphorylation which

resulted in the dissociation of COP-1. This dissociation led to the loss of COP-1 activity and accumulation of c-Jun which enhances AP-1 transcriptional activities (Yi et al., 2005). The regulation of c-Jun by MVP was also observed in fibroblasts. MVP downregulation in fibroblasts results in the phosphorylation of c-Jun NH(2)-terminal kinase (JNK) which enhances accumulation of c-Jun, resulting in reduced Bcl-2 level (Ryu et al., 2008). In airway smooth muscle cells treated with PDGF, MVP was found to be S-glutathionylated. S-glutathionylated MVP interacts with the MYH9/Beclin-1/Vsp34 complex, leading to the sequestration of the complex and preventing it from inducing cell death (Das et al., 2016). In *Trypanosoma brucei*, MVP forms a complex with *T. brucei* target of rapamycin 4 (TbTOR4) which regulates the switch between the slender and stumpy forms of *T. brucei* in the blood (Barquilla et al., 2012). TOR proteins are involved in cell growth, stress resistance, and protein synthesis. Knock-down of MVP in *T. brucei* showed morphological defects during nutritional stress (SHAIK, 2013). All these findings point towards the involvement of MVP in the regulation of signalling cascades.

Studies of human, porcine and sea urchin embryos have shown controlled regulation of MVP during embryo development. An increase in nuclear localisation of MVP was observed as sea urchin embryos develop (Stewart et al., 2005) while defective human and porcine embryos showed increased accumulation and proteasomal degradation of MVP (Sutovsky et al., 2005). If MVP is such an important regulator of signalling cascades and is involved in the proper development of embryos, one would expect developmental defects in MVP knockout organisms. However, when *Mvp*^{-/-} mice were generated, no observable phenotype was reported even when histological examination of

organs was carried out (Mossink et al., 2002b).

MVP levels also increased as cells became more confluent (Steiner et al., 2006).

As cells have been shown to undergo differentiation when confluent (Artursson et al., 2001, Sambuy et al., 2005, Aldo et al., 2013), it is tempting to hypothesise that MVP expression is important in cell differentiation. In accordance with this, MVP was upregulated when monocytes were differentiated into dendritic cells (Schroeijers et al., 2002). However, in *Mvp*^{-/-} mouse, there is no impairment of dendritic functions, suggesting that MVP might be involved in other undetermined functions (Mossink et al., 2003a). This suggests that MVP is not essential for signalling but might be an enhancer of signalling pathways.

1.7.5. Involvement of MVP in innate immunity

In the past decade, there is a growing interest in the role of MVP in the innate immune response. *Mvp*^{-/-} mice showed increased susceptibility to intra-nasal infection of *Pseudomonas aeruginosa* (Kowalski et al., 2007). The author showed that upon exposure to *P. aeruginosa*, MVP was rapidly recruited to lipid rafts of epidermal cells. This recruitment is augmented by CFTR and MVP was shown to be essential for the optimal clearance of bacteria through internalisation by epithelial cells. MVP affected the bacterial uptake but not NFκB activation, IL-8 secretion or apoptosis of epithelial cells. Proteomics analysis of lipid rafts of MVP knockdown cells detected a significant reduction in the proteins involved in lipid raft formation and cell signalling. The authors proposed that MVP either stabilizes raft micro-domains or mediates extracellular signal-regulated kinase activity. *Listeria monocytogenes*, a pathogenic bacteria, was also shown to interact with intracellular MVP after escaping from the internalisation vesicles

(Dortet et al., 2011). This interaction is mediated by *L. monocytogenes* surface protein, Inl_k, and protects *L. monocytogenes* from autophagy. This leads to increased survival of *L. monocytogenes* and thus increased pathogenesis (Dortet et al., 2011).

In addition to mediating host response towards bacteria, work published by other groups showed that MVP is important in mediating inflammatory responses to viruses (Li et al., 2015, Peng et al., 2016). Using A549 cells and PBMCs, MVP was demonstrated to facilitate translocation of c-Fos and C/EBP β transcription factors into the nucleus to upregulate expression of pro-inflammatory cytokines IL-6 and IL-8 (Peng et al., 2016). Interestingly, the NS1 protein of influenza A virus induces expression of vtRNAs which in turn inhibit PKR signalling, an important innate immunity signalling pathway. This vtRNA inhibition of PKR was also observed when cells were infected with Sendai virus and herpes simplex virus (Li et al., 2015). vtRNAs were also upregulated when cells were infected with Epstein-Barr virus (EBV) and Kaposi's sarcoma virus (HHV8) (Nandy et al., 2009). As only 5% of vtRNAs are associated with vault particles, whether this function of vtRNAs is dependent on their association with vaults is unknown. MVP expression was also found to be elevated in monocytes of HIV patients and this was correlated with an increase in STAT-1 expression (Rivera-Rivera et al., 2012).

Work by Ying Zhu lab demonstrated that compared to healthy individuals, patients infected with Hepatitis B virus (HBV) (Liu et al., 2015) or Hepatitis C virus (HCV) (Liu et al., 2012) have elevated levels of MVP proteins in their sera and livers. The PBMCs of HBV- and HCV-infected patients also showed upregulation of *MVP* mRNA. In HCV-infected patient, HCV NS5A induces

MVP expression through the NF κ B and SP-1 pathways. This upregulation of MVP expression leads to the suppression of HCV protein expression and RNA replication. The antiviral response exerted by MVP was also shown to be dependent on type I IFNs expression. MVP upregulates IFN- α and IFN- β expression, leading to enhanced expression of IRF7 and other IFN-stimulated genes (STAT1/2, OAS and PKR). This MVP-dependent IFN-stimulated genes induction was also observed when cells were infected with vesicular stomatitis virus and enterovirus 71 (Liu et al., 2012). In HBV-infected patients, MVP upregulation is mediated by HBV X protein. MVP binds Myd88 and enhances Myd88 signalling resulting in increased expression of NF κ B and IFN- β . Interestingly, HBV antigens, HBsAg and HBeAg, bind MYD88 and prevent the association of MVP with MYD88, reducing downstream signalling (Liu et al., 2015). In mouse peritoneal macrophages, MVP interacts with SR-A and is essential for SR-A-caveolae-p38/JNK mediated TNF- α production. MVP positively regulates SR-A induced apoptosis (Ben et al., 2013) which is contrary to the previously mentioned pro-survival MVP function. In Hep3B, KB-3-1, HeLa and THP-1 cells, MVP expression is induced by IFN- γ -mediated binding of STAT-1 to the promoter region of MVP. This led to a 6- to 13-fold increase MVP proteins by increased translation. In small-cell lung cancer cell line, H65, MVP overexpression interferes with IFN- γ mediated STAT-1 signalling to downregulate IFN- γ -regulated genes, CD13, ICAM-1 and CD36 (Steiner et al., 2006). This resulted in a negative feedback loop in the MVP- IFN signalling pathway.

1.7.6. Vault nanocage as drug delivering platform

Due to its ability to incorporate specific proteins into its cavity and subsequent protection of incorporated cargo, vault has been explored as a nanoparticle to delivery therapeutics into cells (Lai et al., 2009, Buehler et al., 2011, Kar et al., 2012, Rome and Kickhoefer, 2013, Buehler et al., 2014, Champion et al., 2009). Investigation on the physical properties of vaults revealed the ability of vaults to exchange vault-halves (Yang et al., 2010) and the opening of vault particles under low pH (Goldsmith et al., 2007, Querol-Audi et al., 2009). This ability to transit between open and closed conformations suggests the possibility of encapsulating and releasing loaded cargo without the INT domain. Specific incorporation of cargo can also be achieved by the addition of INT domain to target proteins (Kickhoefer et al., 2005). Bioengineered vaults were shown to fold properly and can be taken up by cells in a non-specific manner. Cloning of modified vaults with epitopes derived from HIV TAT, Z-domain and RGD tag leads to enhanced uptake by cells and increases specificity to target cell types (Kickhoefer et al., 2009, Han et al., 2011, Kar et al., 2011, Yang et al., 2013, Zhu et al., 2015). Importantly, vaults are resistant to enzymatic degradation (Kedersha and Rome, 1986), allowing therapeutic vaults to reach their target before being degraded. Most importantly, as vaults are endogenous proteins, they do not trigger any allergen responses (Kar et al., 2012). Together, these properties make vault nanoparticle attractive as a drug delivery system and have led to the development of vaults for use in vaccines against chlamydia infections (Zhu et al., 2015) and other therapeutic purposes (Buehler et al., 2014)

1.7.7. Challenges in vault biology

MVP has been proposed to mediate a wide variety of functions, from stress response to cell signalling. However, no function of vaults that is ubiquitous amongst all the different organisms has been found.

In view of the various functions that MVP is involved in, it is tempting to think of MVP as a jack of all trades in mediating cellular processes. Despite the many cellular processes linked to vaults and MVP, its biological relevance remains unknown. MVP enhances signalling in different pathways, induces gene expression and is highly conserved. However, there remains no consensus on the function of MVP and vaults in biology, with relevant literature remaining disparate and disjointed. MVP modulates both up- and downregulation of Erk activities suggesting highly complex regulation of MVP function. A drawback of previous experiments was their use of cancer-derived cell lines or immortalised lines that might have unknown mutations affecting the expression of MVP.

In order to elucidate the function(s) of vaults, *in vivo* systems involving model organisms are critical. The lack of phenotypic changes in *Mvp*^{-/-} mice, aside from increased susceptibility to *P. aeruginosa* and influenza A virus in intra-nasal infections, suggests that vaults are dispensable under basal conditions. Additionally, mutant slime moulds exhibited morphological and growth defects under nutrient deprivation. This further suggests that vaults are important under stressful conditions. However, the limited representation of vaults in model organisms hampers *in vivo* studies of vaults. Use of alternative model organisms which can be efficiently screened for responses to stress might aid the study of the function of vaults.

1.8. Zebrafish as a model organism

In view of the need for an alternative model organism to investigate the function of vaults, we have turned our attention to zebrafish (*Danio rerio*). Zebrafish, a freshwater fish that barely grows beyond 40 mm in length, originated from the waters of East India and Burma. Their small size, coupled with their ability to live in high density, make rearing zebrafish more economical than mice. Zebrafish reach sexual maturity as early as two months of age, however, clutch size and successful crossing increases over time (Lawrence et al., 2012). Zebrafish breed all year round in the laboratory, giving a large clutch size of 169 ± 146.0 , with the maximum clutch size of 765 (Spence and Smith, 2006). Zebrafish eggs are transparent and less than 1 mm in diameter. The eggs are fertilized externally and develop rapidly, allowing for easy observation of developmental and cellular processes. The large clutch size, coupled with the small embryo size, allows for the use of zebrafish in high-throughput screens using 96- or 384- well plates. Together, these properties have made zebrafish the most common fish model in scientific research (Grunwald and Eisen, 2002).

Zebrafish was first used by Dr George Streisinger for developmental biology (Streisinger et al., 1981). Subsequent development of various genetic techniques such as cloning (Streisinger et al., 1981), transgenesis (Stuart et al., 1988) and mutagenesis (Grunwald and Streisinger, 1992, Walker and Streisinger, 1983) enabled the modification of the zebrafish genome. This led to two large mutagenesis screens (Driever et al., 1996, Haffter et al., 1996) that discovered and characterized mutations resulting in defects in almost all aspect of developmental biology. The phenotypes of the mutations identified in these two mutagenesis screens correlated well with human diseases (Haffter and Nusslein-

Volhard, 1996, Dooley and Zon, 2000, Lieschke and Currie, 2007), establishing zebrafish as an important model organism for developmental biology and human disease.

The anaemic mutant zebrafish, *sauternes* (*sau*), with a mutation in the erythroid-specific isoform of δ -aminolevulinate synthase phenocopies the congenital sideroblastic anaemia caused by mutation of the same gene in humans (Brownlie et al., 1998). This ability to phenocopy human disease caused by mutations in the same gene makes zebrafish a viable model organism for modelling human diseases.

Further development of tools/resources to consolidate zebrafish as a model organism were also carried out. For example, an online database (ZFIN) was established to serve as a community database and to facilitate the use of zebrafish in research (Howe et al., 2013). Sanger sequencing of the 1.7GB zebrafish genome started in 2001 and analysis of the resulting genome revealed 26,206 protein-coding genes (Collins et al., 2012). Comparison of zebrafish genome with human genome reveals that more than 70% of human genes have an orthologue in zebrafish (Vilella et al., 2009) and there are high levels of conserved gene synteny (Barbazuk et al., 2000). This suggests the conservation of gene functions between human and zebrafish, further proving the feasibility of using zebrafish for human biology. The sequenced genome, together with the improvement of genome-editing techniques such as zinc finger nucleases (Sander et al., 2011), transcription activator-like effector nucleases (Huang et al., 2011) and recently, CRISPRs (Hwang et al., 2013) enabled the specific modification of genes in zebrafish. These allow for the easy and specific generation of zebrafish mutants to study functions of the gene of interest.

Despite some anatomical differences, zebrafish have conserved organ systems similar to that of humans and have been used as a model organism for liver (Goessling and Sadler, 2015, Sadler et al., 2005), haematopoietic (Jing and Zon, 2011, Svoboda et al., 2014, Paik and Zon, 2010), heart (Bakkers, 2011, Bournele and Beis, 2016) and metabolic diseases (Seth et al., 2013, Santoro, 2014). Zebrafish is a unique model for the study of infections due to the temporal segregation of its innate and adaptive immunity maturation. The innate immune system is active from 25 hours post fertilisation (Herbomel et al., 1999) while the adaptive immune system is functionally active only 4-6 weeks post fertilisation (Willett et al., 1999). This allows for the study of innate immune responses with and without adaptive immunity components. The transparency of zebrafish embryo also allows for the tracking of fluorescent protein-expressing immune cells. Transgenic zebrafish lines, Tg(*mpx*:GFP) (Renshaw et al., 2006) and Tg(*mpeg1*:mCherry) (Ellett et al., 2011), drive the specific expression of GFP and mCherry in neutrophils and macrophages respectively. This allows for the study of neutrophils and macrophages dynamics in response to pathogens and wounds.

Aims

Given the high level of reported conservation of vaults in terms of the quaternary structure and amino acid sequences of MVP, one would assume that the function of vaults is important and is dependent on its structure. However, despite dynamic regulation in porcine embryos (Sutovsky et al., 2005), loss of MVP is not detrimental to development and growth of organisms (Mossink et al., 2002b, Mossink et al., 2003a, Vasu and Rome, 1995). Past research have implicated MVP in the modulation of stress responses (Yi et al., 2005, Tajitsu et al., 2013) and innate immunity via regulation of signalling processes (reviewed in (Berger et al., 2009)). However, these were carried out on cell lines, and there is a lack of *in vivo* data on the involvement of MVP in such processes.

We form the hypothesis that vaults have a role related to cell stress or immunity and this will be tested using both *in vitro* and *in vivo* methodologies.

The objectives of this thesis are thus to:

1. Investigate the effects of CRISPR-mediated knockout of MVP on zebrafish development and stress responses.
2. Characterise *in vivo* response of MVP to cellular stressors using transgenic Mvp-GFP zebrafish lines
3. Characterise changes in MVP expression and intracellular distribution in response to various cellular stressors *in vitro* using human cell lines
4. Characterise the response and function of MVP when cells are exposed to pathogen-associated molecular patterns (PAMPs) and bacteria.

Chapter 2. Methods and Materials

2.1. Homology analysis of major vault proteins

Pairwise alignment of human and zebrafish MVP was carried out using EMBOSS needle Needleman-Wunsch algorithm. Parameter used for the alignment is Blomsum62 matrix with a gap open penalty of 10, gap extension of 0.5, end gap open penalty of 10, end gap extension of 0.5. MVP sequence of various organisms was collected through searches with the BLASTp algorithm of the NCBI database using human MVP (NP_005106.2) as the query. Species with a curated MVP sequence containing the NP_ prefix were selected. In addition, non-NP prefix MVP sequence from *Dictyostelium discoideum*, *Ciona intestinalis* and *Trypanosome rangeli* SC58 were used. This resulted in a list of 17 MVP sequence. The sequences were aligned using the Clustal Omega algorithm on the Lasergene MegAlign Pro software (DNASTAR®).

2.2. Fish Husbandry and transgenic Line

Zebrafish were housed within zebrafish facility of Institute of Molecular and Cell Biology, A*STAR and Nanyang Technological University. National Advisory Committee For Laboratory Animal Research (NACLAR) Guidelines set by the Agri-Food and Veterinary Authority (AVA) of Singapore were complied with in all animal procedures. Animal procedures were overseen by the Institutional Animal Care and Use Committee (IACUC) at the A*STAR Biological Resource Centre (BRC) (IACUC No. 14924) and NTU (IACUC No. A18002). Embryos and larvae were staged according to Kimmel et al. (1995) and were raised in E3 medium (5 mM NaCl (Sigma-Aldrich), 0.17 mM KCl (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 0.33 mM MgSO₄ (Sigma-Aldrich), 0.33 mM CaCl₂

(Sigma-Aldrich) and 0.00002% methylene blue (Sigma-Aldrich), pH 7.8).

2.3. Generation of *mvp*^{-/-} zebrafish

CRISPR-mediated mutation of MVP in two different exons was carried by the lab of Dr Tom Carney. guideRNAs were designed using the ZiFiT software (Hwang et al., 2013). CRISPR/guide RNA injected fish were crossed with TLABs to generate F0 generation. F0 were crossed with wild-type AB zebrafish to generate F1 heterozygous mutants. Heterozygous F1 mutants with frameshift mutations were genotyped and crossed with wild-type to increase the number of fish containing the mutation. Heterozygous mutants were then in-crossed and screened for homozygous mutants to establish knock-out lines. GFP-tagged MVP transgenic fish were generated by the lab through injection of *krt4:Mvp-GFP* construct generated by Gateway™ cloning into AB embryos at 1-2 cell stage. Integration of construct into the zebrafish genome was facilitated by the Tol2 element in the plasmid (Kwan et al., 2007). *krt4:Mvp-GFP* construct-injected fish was then crossed with wild-types and GFP-positive offspring were used to generate stable Tg(*krt4:Mvp-GFP*) fish line.

2.4. In situ hybridisation

Embryos and larvae of 1-2 cell stage, 4 hours post fertilisation (hpf), 6 hpf, 10 hpf, 14 hpf, 24 hpf, 48 hpf and 72 hpf were fixed in 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PFA/PBS) overnight at 4 °C. Embryos before the stage of 24 hpf were fixed with their chorions intact and dechorionated using forceps. For larvae that developed past 24 hpf, 0.003% 1-

phenyl-2-thiourea (PFU) (Sigma-Aldrich) was added to the E3 medium at 24 hpf to prevent pigment formation and chorions were removed by pronase (Sigma-Aldrich) prior to PFA fixation. Fixed embryos and larvae were dehydrated in 100% methanol (Fisher Scientific) and stored at -20 °C until further processing. A 1044 bp cDNA fragment of zebrafish MVP was cloned into pGEMT-Easy (Promega). DIG-labelled anti-sense RNA *in situ* probes was generated from this plasmid using T7 RNA polymerase (Roche) after plasmid linearization by SpeI (New England Biolabs). *In situ* hybridisation was carried on embryos using the following protocol (Thisse and Thisse, 2008). Methanol dehydrated embryos were rehydrated through a series of methanol washes with an increasing amount of PBS. 24 hpf, 48 hpf and 72 hpf embryos were digested with 20 ug/ml proteinase K (ThermoFisher Scientific) for 30, 60 and 90 seconds respectively to permeabilise tissues. The digested embryos were fixed with 4% PFA for 20 min to terminate proteinase K activity, followed by four 5-min washes with PBST. The embryos were then prehybridised with hybridisation mix (HM) containing 50% deionized formamide (Life Technologies), 750 mM NaCl (Sigma-Aldrich), 75 mM sodium citrate (Sigma-Aldrich), 0.1% Tween 20, 50 mg/ml of heparin (Sigma-Aldrich), 500 mg/ml of RNase-free tRNA (Sigma-Aldrich), pH 6.0, for 2-5 h at 72 °C, followed by two high stringency 30-min washes with 0.2X SSC buffer at 70 °C. Embryos were then incubated with 200 µl HM containing 50 ng of DIG-labelled probe at 72 °C overnight. After the overnight incubation, the probe-containing HM was replaced with wash HM (HM without t-RNA and heparin). Wash HM was then changed to 2X SSC (1st BASE) (0.30 M NaCl, 0.030 M trisodium citrate) buffer through 10 min washes with an increasing amount of 2X SSC buffer at 70 °C followed by two 30-min washes with 0.2X

SSC buffer at 70 °C. The 0.2x SSC was changed to PBST through 10-min washes with an increasing amount of PBST. The embryos were then blocked for 3-4 hr with blocking buffer (1 × PBST, 2% v/v sheep serum (Merck) and 2 mg/ml BSA (Promega)). Blocked embryos were incubated overnight at 4 °C with anti-DIG alkaline phosphatase-conjugated antibody (1:5000 dilution) followed by six 15-min washes with PBST at room temperature followed by equilibration with 3 washes in alkaline Tris buffer (50 mM MgCl₂ (Sigma-Aldrich), 100 mM Tris HCl (Sigma-Aldrich), 100 mM NaCl and 0.1% Tween 20, pH 9.5). Alkaline Tris buffer was then replaced with staining solution (0.225 mg/ml NBT (Roche) and 0.175 mg/ml BCIP (Roche) in alkaline Tris buffer). Incubation with staining solution was carried out until staining was observed. To stop the staining reaction, staining solution was removed and the embryos were incubated with stop solution (1X PBS, 1 mM EDTA (Sigma-Aldrich), 0.1% Tween 20, pH 5.5).

2.5. Reverse transcription PCR of zfMVP

RNA was isolated from various fish tissues, larvae of different developmental stages and water control using TRIzol® (Life Technologies) extraction. 200 µl of TRIzol® was added to tissues/larvae before homogenising with a hand-held homogeniser, 800 µl of TRIzol® were further added to make up 1 ml. RNA purification was subsequently carried out according to the manufacturer's instruction. RNA pellets obtained were resuspended in 30 µl DEPC-treated water and the concentration determined by NanoDrop 2000 (Thermo scientific). 1 µg of extracted RNA was then converted to cDNA using oligo-dT and SuperScript® III Reverse Transcriptase (Invitrogen). PCR was carried with GoTaq® (Promega) on the synthesised cDNA using the forward primer (5'–

GACGCAGGATGAGGAATTGT) and the reverse primer (5'-ACGTTTGGGTTTGTCTCCAG) with 30s annealing at 57 °C and 20s extension at 72 °C.

2.6. ER stress induction of MVP WT and mutant fish

Tunicamycin stock (Sigma-Aldrich) was prepared by dissolving in DMSO to a concentration of 1 mg/ml and kept as aliquots at -20 °C. This stock was diluted in E3 medium to required concentrations prior to use. 24 hpf embryos were dechorionated by pronase digestion and digestion was stopped by the washing of embryos with fresh E3 medium. Zebrafish larvae were added to 12-well plates at 15-20 larvae per well. E3 medium was removed prior to treatment with tunicamycin-containing E3 medium and larvae were observed every 24 hr using a Leica stereomicroscope. Haemorrhage was observed and defined as the presence of blood accumulation outside of the vasculature. Two-way ANOVA analysis was carried out using Prism software.

2.7. SWATH-MS analysis of zebrafish larvae

30 hpf WT and *mvp*^{-/-} maternal-zygotic zebrafish larvae were euthanised by an overdose of MS-222 (Sigma-Aldrich) and de-yolked by triturating three times with Ringer's solution (calcium free): 116 mM NaCl (Sigma-Aldrich), 2.9 mM KCl (Sigma-Aldrich), 5.0 mM HEPES (Sigma-Aldrich), pH 7.2. Protein samples were reduced and alkylated using 5 mM dithiothreitol and 10 mM iodoacetamide. Proteins were digested with lysyl endopeptidase for 16 hours at 37°C, followed by trypsin digestion for 8 hours. Empore C18-SD Extraction Disk Cartridge (3M) was used to clean up the digested peptides, and the peptides were vacuum-dried before being reconstituted in 0.1% formic acid.

Swath MS was carried out using an ekspert nanoLC-425 (Eksigent) coupled with a TripleTOF 6600 system (SCIEX). Using buffer A (2% acetonitrile, 0.1% formic acid) and buffer B (95% acetonitrile, 0.1% formic acid), an online separation was carried out using a gradient of 10-18% solvent B in 55 min, followed by 18-30% solvent B in 60 min. This separation was carried out using a flow rate of 300 nL/min and mass spectrometry was carried out on 120 windows across 400-1200 m/z. Protein identification was carried out through searching generated spectra in ProteinPilot 5.0 software (SCIEX) against the zebrafish UniProt database. Peak area extraction and determination of fold changes and confidences were subsequently carried out using algorithm previously described (Lambert et al., 2013).

2.8. Maintenance of mammalian cell lines

HaCaT and HeLa (ATCC® CCL-2™) cells were cultured and maintained in DMEM (4500 mg/l glucose, with 4.0 mM L-glutamate, with sodium pyruvate, with sodium bicarbonate, with phenol red) (Life Technologies), with 10% heat-inactivated FBS (Life technologies). When the cells reached ~80% confluency, they were detached with 0.25% trypsin/EDTA and seeded with 10X dilution. THP-1 (ATCC® TIB202™) cells were cultured in RPMI-1640 (Life Technologies), supplemented with 10% heat-inactivated FBS. THP-1 cells (ATCC® TIB-202™) were maintained at a density of $0.1-1 \times 10^6$ cells/ml and THP-1 cells were spun down at 300 x g for 5 minutes and resuspended every 2-3 days in fresh media. All cells were grown at 37 °C with 5% CO₂.

2.9. Treatment of cells with various cellular stressors

Prior to treatment, cells were passaged and seeded onto 12-well plates. Cells were allowed to grow to approximately 70-80% confluency. Spent media was removed and cells were rinsed twice with sterile PBS. Fresh media containing 1 µg/ml tunicamycin, additional 100 mM NaCl, 100 ng/ml IFN-γ (Invivogen), 100 ng/ml doxorubicin (Sigma-Aldrich) and 10% *E. coli* supernatant was added to the wells and cells were treated for 24 hours. Control used was DMEM with 10% FBS.

2.10. RNA isolation and cDNA reverse transcription from mammalian cells

RNA was isolated from HaCaT cells using E.Z.N.A.® Total RNA Kit I (Omega bio-tek). 0.05×10^6 HaCaT cells were seeded in each well of 24-well plate overnight. The following day, wells were washed once with sterile PBS to remove dead cells and fresh media were added. The cells were then treated for 24 hrs. After 24 hrs, the supernatants were aspirated and cells were washed twice with PBS. Excess PBS was removed before 350 µl of TRK lysis buffer was added per well to lyse cells. 20 µl of β-mercaptoethanol was added per ml of TRK lysis buffer prior to use. Subsequent steps were carried out following the manufacturer's instruction. One microgram of each extracted RNA was reverse transcribed into cDNA using iScript™ cDNA synthesis kit (Bio-rad) following the manufacturer's protocol. This cDNA was used for downstream applications.

2.11. qPCR of samples

qPCR of cDNA was carried out on Applied Biosystems StepOnePlus™ System using PrecisionFAST qPCR mastermix (Primerdesign™). Reaction set-up was carried out as described by the manufacturer and standard qPCR cycling

conditions were applied. CT values for MVP were normalised against β -actin and represented as Δ CT. Δ CT of treatments was compared against that of the control sample and fold change was calculated from the value of $2^{(-\Delta\Delta\text{CT})}$.

2.12. Immunofluorescence analysis of MVP

Coverslips were sterilised by autoclaving at 121°C for 15 mins and stored prior to use. HaCaT and HeLa cells (0.05×10^6) were seeded on 12 mm diameter glass coverslips placed in 24-well plates and grown for 24 hours. THP-1 cells were seeded at a density of $0.15 \times 10^6/\text{ml}$ and differentiated with 5 ng/ml PMA (Sigma-Aldrich). Media from the wells were aspirated and replaced with fresh media prior to treatment. Treatments with stimuli were carried out by addition of compound/agonist into the well and any dilution required was carried out with culture media. DMSO (Sigma-Aldrich) control was used when the compounds were dissolved in DMSO. Once the required duration of incubation was reached, the media was aspirated. Cells were washed twice with sterile PBS before they were fixed with 4% PFA in PBS for 10 minutes at room temperature. After fixing, the cells were washed thrice with PBS before cells were permeabilised with PBS + 0.1% Triton X-100 for 10 minutes. After permeabilisation, the cells were washed thrice with PBS and blocked with 2% BSA in PBST (PBS+ 0.1% Tween-20) for 30 minutes before primary antibodies were added. Samples were incubated with primary antibody for 1 hour at room temperature and washed thrice with PBST. The cells were then incubated at room temperature for 1 hour with Alexa Fluor® 488- or Alexa Fluor® 568-conjugated secondary antibodies (Invitrogen). After secondary antibody incubation, coverslips were then washed thrice with PBST and mounted onto glass slides using Fluoroshield™ with DAPI

(Sigma-Aldrich).

2.13. SDS-PAGE and Western blot analysis

Cells grown in 12 well plates or 24-well plates were lysed with 50 μ L and 100 μ L 1X laemmli buffer (BioRad) with β -mercaptoethanol (BioRad) respectively. Lysates were centrifuged to pellet any debris and supernatant was collected. An equal volume of each protein lysate was then added to each well of the SDS-PAGE gel. SDS-PAGE was carried out on precast 4-20% Tris-glycine-SDS gel (BioRad) and ran using Mini-PROTEAN® Tetra Cell Systems (BioRad). For western blot analysis, proteins in the SD-PAGE gel were transferred to PVDF membrane (0.2 μ m, Biorad) and blocked with blotting grade blocker (BioRad). Blots were incubated with anti-MVP antibodies (rabbit polyclonal HPA00232, mouse monoclonal clone 2H3-1A6) (Sigma-Aldrich) (1:1500 dilution) overnight at 4°C or 1 hour at room temperature. After primary antibody incubation, blots were washed thrice with PBST and incubated with either HRP-conjugated goat anti-rabbit antibodies (Promega) or HRP-conjugated goat anti-mouse antibodies (Promega). Blots were developed using SuperSignal™ West Dura extended duration substrate (Thermo Scientific) using ChemiDoc™ MP (Biorad).

2.14. siRNA screen for Rab GTPases

ON-TARGETplus SMARTpool (Dharmacon) were dispensed into 384-plates black-walled glass bottom plates to give a final concentration of 25 nM. Hela cells were reverse transfected at 1500 cells/well using HiPerFect transfection reagent (Qiagen) for 96 hours. After 96 hours, the supernatants were removed, wells were rinsed with sterile PBS and the cells were fixed for 10 mins with 4%

PFA in PBS at room temperature. After fixing, the wells were washed thrice with PBS before cells were permeabilised with PBS + 0.1% Triton X-100 for 10 minutes. After permeabilisation, the cells were washed thrice with PBS and blocked with 2% BSA in PBST (PBS+ 0.1% Tween-20) for 30 mins before primary antibodies were added. Samples were incubated with mouse anti-MVP antibody (1:500 dilution) for 2 hours at room temperature and washed thrice with PBST before Alexa Fluor® 488-conjugated secondary antibodies (1:1000 dilution) (Invitrogen) and Hoechst 33342 (1mg/ml) were added and incubated at room temperature for 2 hours. 30 µl of PBS was then added to each well and the wells were imaged with Opera Phenix High Content Screening System (Perkin Elmer). 49 confocal images per well were taken at 400X magnification. After features and reproducibility control, such as the exclusion of mitotic cells and nucleus with non-standard morphologies, GMM unsupervised clustering was used to group and sort cells (Bishop, 2006).

2.15. Bacteria culture

PAO1 and the isogenic mutants strain, PAO1ΔpilA and PAO1ΔPslBCD were a kind gift from Prof Yang Liang (SCELSE, NTU). Bacteria cells were stored at -80 °C in 25% glycerol (Invitrogen) for long term storage. Bacteria were grown on LB (Miller) agar (1st Base) on a monthly basis to maintain a working stock. Colonies were picked using inoculating loops and grown in LB (Miller) media (1st Base) at 37°C with shaking at 200 RPM the night before experiments. On the next day, overnight cultures were diluted at least 100-fold in fresh LB (Miller) media and grown until log-phase (OD 0.3-0.6) were achieved. Bacteria cultures were subsequently diluted to reach the desired concentration for experiments. Ampicillin was used at 100 µg/ml to maintain the mCherry-expressing bacteria.

2.16. Extracellular protein isolation from mammalian cell supernatant treated with bacteria conditioned media

Bacteria were grown to stationary phase in LB (Miller) media, centrifuged and the supernatant collected and sterile filtered. Mammalian cells were treated with sterile-filtered bacteria media for one hour. Supernatants from cell culture (12-well plate) were collected and spun down at 10,000 x g for 5 mins to remove any cellular debris. The top half of the remaining supernatant was then pipetted into a new microcentrifuge tube. Ice-cold acetone was then added to collected supernatants at 5:1 ratio and precipitation was carried out overnight at -20 °C. Precipitates were spun down at 20,000 x g at 4 °C for 15 mins to pellet the precipitates. Pellets were then washed with ice-cold acetone and re-centrifuged. The acetone was then aspirated and the pellet was dried by vacufuge® (Eppendorf). The dried pellets were resuspended in 15 µl of 1 x laemmli buffer with β-mercaptoethanol and the presence of secreted MVP was determined by western blot.

2.17. Bacteria pull-down of serum proteins

Human sera were a kind gift from a colleague. Whole blood was collected from healthy individuals with written informed consent obtained. All work involving the use of human blood samples were performed in accordance with the guidelines and regulations reviewed and approved by the Nanyang Technological University Institutional Review Board, Singapore (IRB-2014-10-041). Serum was removed from coagulated blood and stored at -20 °C. Bacteria grown to log-phase were collected and washed thrice with sterile PBS. OD₆₀₀ of bacteria was

measured and bacteria were added to serum and PBS control at a final concentration of OD₆₀₀ 0.5. Bacteria and serum/PBS were incubated at 37 °C for 1 hour. After incubation, the bacteria cells were washed with PBS and pelleted. Pelleted bacteria were incubated with 1 x laemmli buffer with β-mercaptoethanol (Bio-rad) and boiled at 95°C for 5 mins followed by western blot analysis.

2.18. Competent *P. aeruginosa* preparation and transformation with mCherry-expression plasmid

An overnight culture of *P. aeruginosa*, PAO1, was pelleted by centrifugation at 4 °C for 10 min at 2,300 x g. The supernatant was discarded and the pellet was resuspended in 1 ml ice-cold SMEB buffer (1 mM HEPES, pH 7.0, 1 mM MgCl₂, 300 mM sucrose (1st Base)). Bacteria were pelleted at 4 °C for 10 min at 2,300 x g, the supernatant discarded and the pellet was resuspended in 500 µl SMEB buffer. Bacteria were pelleted at 4°C for 10 min at 2,300 x g, the supernatant discarded and the pellet was resuspended in 100 µl SMEB buffer. Finally, the pellet was resuspended in 100 µl ice-cold SMEB and aliquoted for use. Aliquots were stored at -80 °C. PAO1 was transformed with a mCherry-expression plasmid (Addgene pMF440 plasmid #62550). 30 µl of competent PAO1 was mixed with 1 µg of purified plasmid. Sterile ice-cold SMEB buffer was used to top up the volume to 50 µl. The mixture was incubated on ice for 5 mins in a 0.2 mm cuvette prior to electroporation. Electroporation was carried out using the pre-set protocol for PAO1 using Gene Pulser Xcell™ electroporator (BioRad) (C = 25 µF; PC = 200 ohm; V = 2.5kV).

2.19. Immunofluorescence of bacteria co-cultured with cells

HaCaT and HeLa cells (0.05×10^6) were seeded on 12 mm diameter coverslips placed in 24-well plates and grown overnight. Prior to the addition of bacteria, the overnight media were removed and cells were washed twice with sterile PBS before serum-free media were added to the wells. Bacteria were then added to the wells to obtain a final OD₆₀₀ of 0.001. The cells were then incubated together at 37 °C and 5% CO₂ for 1 hour. The media were aspirated and coverslips were then washed twice with PBS. Coverslips were subsequently fixed with 4% PFA for 10 mins, permeabilised using 0.1% Triton X-100 for 10 mins followed by blocking with either 2% animal serum or 2% BSA (Promega). Primary antibody staining using anti-MVP antibody (1:300 dilution) was carried out at RT for 2 hours or overnight at 4 °C. The secondary antibodies (1:1000 dilution) was incubated at RT for 1 hour. Coverslips were mounted onto glass slides using DAPI-containing Fluoroshield (Sigma). Confocal images were taken using the ZEISS LSM800 confocal system.

2.20. Biofilm assay

20 µl of overnight PAO1-mCherry culture was added with 1 µg of recombinant vaults. The mixture was then added to the centre of the chamber of chamber slides, forming a droplet. After 2 hours incubation at 37 °C, additional LB media was added until the chamber is half-full. This was further incubated for 1 hour at 37 °C. Chambers were washed thrice with PBS before PFA fixing and immunostainings were carried out.

Chapter 3. Zebrafish as a model to study MVP function

3.1. Introduction

The implication of MVP in the wide range of functions, together with the conflicting reports of MVP's involvement cellular processes, requires a model organism which allows for fast and easy screening of a wide range of conditions. Zebrafish with its small size and almost transparent embryos fulfil such criteria. In addition, zebrafish provides a few additional benefits for studying vault biology. MVP knock-outs have yielded limited phenotypic changes in tested organisms. Knockout of MVP in mouse had yielded no observable phenotype and the only phenotype observed was in *MVP*^{-/-} *D. discoideum* under nutrients starvation. Due to the absence of MVP in *C. elegans*, *D. melanogaster* and *A. thalia*, rat and zebrafish are the other common model organisms which express MVP.

Thus far, there are limited studies on vault or MVP in zebrafish. Depletion of MVP by MVP antisense oligonucleotides has been shown to reduce spinal cord regeneration and locomotor recovery after spinal injury (Pan et al., 2013), suggesting pro-regenerative properties of MVP. Crucially, *mvp* mRNA has been shown to be upregulated in the regenerating larval fin fold (Yoshinari et al., 2009). This suggests that vaults are functional in zebrafish and are regulated by some stimuli, lending strength to the use of zebrafish as a model organism to understand vault function. Another study showed that MVP knock-down led to abnormal body length and defective neural tube development (Blaker-Lee et al., 2012). However, this knock-down was achieved by morpholino and there have been reports demonstrating the poor phenotypic correlation between morpholino knock-out and reverse genetic manipulations (Kok et al., 2015). This poor

correlation makes a definitive conclusion on the function of MVP in zebrafish impossible using current data. Together, the various advantages that zebrafish confers make it attractive as a model to study *in vivo* MVP function(s), however, more work has to be carried out. This chapter describes the generation of MVP knockout zebrafish and the investigation of MVP function using wild-type, MVP knockout and transgenic MVP-GFP zebrafish lines.

3.2. Results

3.2.1. Sequence alignment and analysis of MVP

In an attempt to further validate the suitability and relevance of zebrafish (*D. rerio*) as a model organism for studying vault function, the similarity of zebrafish MVP (zfMVP) to the human MVP (hsMVP) was investigated. Using human MVP protein (Q14764) as the query sequence, Blastp analysis was carried out against the zebrafish genome (GRCz11). Three protein matches were obtained in this process. As gene duplication is common in teleosts, the chromosomal location of each protein match was checked. All three protein matches mapped to the same location on chromosome 3 of the *D. rerio* genome, suggesting that a single MVP gene in the zebrafish genome.

Pairwise alignment of zfMVP and hsMVP amino acid sequences was carried out to investigate the level of conservation between the two proteins. The alignment resulted in a length of 912 residues with 77.1% (703/912) similarity and 7.5% gaps (68/912) (Figure 3.1). Amongst the 703 similar residues, 600 of them were identical, corresponding to 65.8% identity between the two amino acid sequences. Six gaps were identified in the alignment. Compared to hsMVP

sequence, the first gap is a four amino acid insertion corresponding to aa 4-10 of zfMVP, located in the first vault repeat. The second and third gap are insertions of five and seven amino acids corresponding to aa 345-349 and aa 358-365 of zfMVP respectively. Both of this insertion lies within the seventh vault domain. The fourth gap is a deletion of two amino acid (between aa 454-455) of zfMVP which lies in the eighth vault repeat. The fifth gap is a deletion of ten amino acids (between aa 625-626) in the shoulder domain. The last gap is due to an additional 30 amino acid at the C-terminus end of hsMVP.

MVP_DANRE	1	MDADHLLGASIIRIPPHYIHVLDQNTNIARVEIGPLTYIRQDNERVLF	50
MVP_HUMAN	1	MATEEF----IIRIPPYHYIHVLDQNSNVSRVEVGPKTYIRQDNERVLF	46
MVP_DANRE	51	PVRMMMVPPRHYCVVLPNPAVRDDEGQVQFDGSGQVKLRHADLEIRLAQDP	100
MVP_HUMAN	47	PMRMVTVPPRHYCTVANPVSQDAQGLVLFQVRLRHADLEIRLAQDP	96
MVP_DANRE	101	FPLYPGEEIQKDVTPQLQIVPDTALRLQALLDFEEEGEKKRVAGDEWLFE	150
MVP_HUMAN	97	FPLYPGEVLEKDITPLQVVLNPTALHLKALLDFEDKDGKVVAGDEWLFE	146
MVP_DANRE	151	GPPTYIPRKEVTVLEVIAKTVIRENQAIRLRARKEGLDRSGVQVRVTGEW	200
MVP_HUMAN	147	GPPTYIPRKEVEVEIIQATIIRQNQALRLRARKECWRDGRKERVVTGEW	196
MVP_DANRE	201	QVSKVGAYLPGAHEEVVDIVSAFILTDKKALHVRRAIRPFRDAGGRDRRTG	250
MVP_HUMAN	197	LVTTVGAYLPAVFEEVLDLDAVILTEKTALHLRARRNFRDFRGVSRRTG	246
MVP_DANRE	251	EEWLVTVADREAHIPSAEEVVGVDVTTLSNRQYCVIMDPVGADGKQQL	300
MVP_HUMAN	247	EEWLVTVQDTEAHVPDVHEEVLGVVPITTLGPHNYCVILDPVGPDKNQQL	296
MVP_DANRE	301	GQKRNVKGERSFFLMPGEHLENGIQDVVYLSEEEGLVLRAVEAFIDTQGD	350
MVP_HUMAN	297	GQKRNVKGEKSFFLQPGEQLEQGIQDVVYLSEQQGLLRALQPL-----E	341
MVP_DANRE	351	EAEERESRAKRGVQRRPGDRWMLRGPIEYVPPATVEVLLTRTAIPLD	400
MVP_HUMAN	342	EGEDEEK-----VSHQAGDHWLIRGPLEYVPSAKVEVVEERQAIPLD	383
MVP_DANRE	401	ENEGYVRDIKTGKVRVIGQTYMLTQDEELWEKELPANVESLLAQSRDP	450
MVP_HUMAN	384	ENEGYVQDVKTGKVRVIGSTYMLTQDEVLWEKELPPGVEELLNKGQDP	433
MVP_DANRE	451	LADR--SDRGRSFGQ--AERDKTRVVSYPHNPATVQVVDYREKKARVMFG	497
MVP_HUMAN	434	LADRGEKDTAKSLQPLAPRNKTRVVSYPHNAAVQVVDYREKARVVF	483

MVP_DANRE	498	PEMVMLGPDEQFTVLSLSGDKPKRPNVIKTIICLLLGPDFCTDIITIETAD	547
		: . : ... :..: . :	
MVP_HUMAN	484	PELVSLGPDEQFTVLSLSAGRPKRPHARRALCLLLGPDFFTDVIITIETAD	533
MVP_DANRE	548	HARLQLQLSYNWHFDLKQPADAAQAAALFSVPDFVGDACKAIASRIRGAV	597
		: :.... .:. . :	
MVP_HUMAN	534	HARLQLQLAYNWHFEVNDKDPQETAKLFSVPDFVGDACKAIASRVRGAV	583
MVP_DANRE	598	ASVQFDDFHKNSNRIICSAVFGFDEKLA-----VRSSLRFGQNGL	637
		. . .:. :..	
MVP_HUMAN	584	ASVTFDDFHKNSARIIRTAVFGFETSEAKGPDGMALPRPRDQAVFPQNGL	633
MVP_DANRE	638	VISSVDIQSVEPVDQRTDALQKSVQLAIEITTNSQEAAARHEAERLEQE	687
		: : : : : :	
MVP_HUMAN	634	VVSSVDVQSVEPVDQRTDALQRSVQLAIEITTNSQEAAAKHEAQRLEQE	683
MVP_DANRE	688	ARGRLERQKITDQAEAEKARKELLELEAQSAVESTGAAKAEASRAEAA	737
		. : : . . . :	
MVP_HUMAN	684	ARGRLERQKILDQSEAEKARKELLEALSMAVESTGTAKAEAESRAEAA	733
MVP_DANRE	738	RIQGEAAVEEAKLKAEAQKIEADSELARLCKAREQELNYKKQIDHLEVEK	787
		: .:. .:. : . . : .:.:.	
MVP_HUMAN	734	RIEGEGSVLQAKLKAQALAIETEAELQRVQKVRELVYARAQLELEVSK	783
MVP_DANRE	788	QQKLADIESQRFKHMDNLGTETLKEMARAGPELQVKLLQSLGLKSTLIT	837
		. : : .:. .:. : .:. : .:. : .:. : .:. : .:. : .:.	
MVP_HUMAN	784	AQQLAEVEVKKFKQMTAIGPSTIRDLAVAGPEMQVKLLQSLGLKSTLIT	833
MVP_DANRE	838	DGSSPINLFTTANGLLGSLQGKDKDE-----	863
		: :.	
MVP_HUMAN	834	DGSTPINLFNTAFGLLG--MGPEGQPLGRRVASGPSPEGISPSQAQAPQ	881
MVP_DANRE	864	-----	863
MVP_HUMAN	882	APGDNHVVPVLR	893

Figure 3.1. **Pairwise sequence alignment of zebrafish and human MVP amino acid sequences.** Pairwise sequence alignment was carried out on the amino acid sequences of human and zebrafish MVP using the Needleman-Wunsch algorithm.

```
Consensus      -----maTEESIRIPPPHYIHVLDQNSNVSRVEVGPKTYIRODNERVLF--aPvRMVTVPPRHVC
Bos_taurus      -----MSMEESIRIPPPHYIHVLDQNSNVSRVEVGPKTYIRODNERVLF--APVRMLTVPPRHVC
Canis_lupus_familiaris -----MATEESIRIPPPHYIHVLDQNSNVSRVEVGPKTYIRODNERVLF--APVRMVTVPVRHVC
Ciona_intestinalis MS-----KGRQGISVSGEEAIVRIPPPHYIHVLDQNTNVTKLEIGPKTYIRODNERVLF--GPERMTTPVRHVC
Danio_reio      -----MDADHLLGASIRIPPPHYIHVLDQNTNARVEIGPTIYIRODNERVLF--APVRMMVVPVRHVC
Dictyostelium_discoideum_a -----MADLNGVIRIKPFHEIHVLDNNTNVTREVEGPTITRODHEKLVSGPEPMIMTPQRNVVC
Dictyostelium_discoideum_b -----MATPVQASTVIRVKPHHYIHVLDNNTNVTREVEGPTITRODHEKLVNPTPLPMLIPPRQVC
Felis_catus      -----MATEESIRIPPPHYIHVLDQNSNVSRVEVGPKTYIRODNERVLF--APVRMVTVPVRHVC
Gallus_gallus    -----MEDPVIIRIPPPHYIHVLDNSNVTRVEVGPHSFIRODHERVVFSPKRMVMVPPRHVC
Homo_sapiens     -----MATEESIRIPPPHYIHVLDQNSNVSRVEVGPKTYIRODNERVLF--APVRMVTVPVRHVC
Mus_musculus     -----MLGRGLGFTMATEESIRIPPPHYIHVLDQNSNVSRVEVGPKTYIRODNERVLF--APVRMVTVPVRHVC
Pongo_abelii     -----MATEESIRIPPPHYIHVLDQNSNVSRVEVGPKTYIRODNERVLF--APVRMVTVPVRHVC
Rattus_norvegicus -----MATEESIRIPPPHYIHVLDQNSNVSRVEVGPKTYIRODNERVLF--APVRMVTVPVRHVC
Salmo_salar      MTSKNGGSLAGMKFEGLVDAFIRVPPPHYIHVLDQNTNARVEIGPTIYIRODNERVLF--SPVRMTVPVRHVC
Strongylocentrotus_purpuratus MA-----SKRGSNQDESIFRTPPVYIHVLDNDNNTTVVVGPKTYIRODHERVFGPEKMLIPPRHVC
Trypanosoma_rangeli_SC58 MDQR-----RQVKHEDAGNMGDITRIKRNHYHVLQDNTNVTGCVGPVVITRQEHCECLHGRHPGVVVPVRQVC
Xenopus_laevis   -----MSFEESIRIPPPYIYHVLQDQNSNISRVETGPKTYIRODNERVLF--HQQRMMVMVPPRHVC
Xenopus_tropicalis -----MSFEESIRIPPPYIYHVLQDQNSNISRVETGPKTYIRODNERVLF--HQQRMMVMVPPRHVC
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Consensus      vVaNPVsRDaGxVLFdVtGQVKLRHADIEIRLAQDPFFLYPGEVLekdI----TLPQVVLPTALHLKALLDfE
Bos_taurus      MVANPVARDAGQTVLFDVTGQVRLRHADLEIRLAQDPFFLPGEVLEKDI----TLPQVVLPTALHLKALLDfE
Canis_lupus_familiaris TVANPVSARDPQGSVLFdVTGQVRLRHADLEIRLTQDPFFLYPGEVLEKDI----TLPQVVLPTALHLKALLDfE
Ciona_intestinalis VIQNPVAKNADDQIQDPSGQAKLNHADQIRLAQDPFFLYPGEVLKTAV----TPIRVYTANTALRLRADLDfD
Danio_reio      VVUNPVARDDDEQVQDFDSGGVKLRHADLEIRLAQDPFFLYPGEVLEKDV----TPIQLVYPTALRLQALLDfD
Dictyostelium_discoideum_a TISNPVVRNENGSLVLDYEGQVKLRHADLEIRFSQDPFFLYPGEKISGKV----TALQVYAPKARLRALLDfD
Dictyostelium_discoideum_b I IENPTIRDKKEGKLVVDKFNQPKLRHADQEIFRSDQPFPLYPGEVIAAQP----VPLQVYQTNSALRLKQLDfV
Felis_catus      TVANPVSARDPQGLVLFdVTGQVRLRHADLEIRLAQDPFFLYPGEVLEKDI----TLPQVVLPTALHLKALLDfE
Gallus_gallus    VVUNPVARGPTGAVLFDGAGQAQLRHADLRIRLTQDPFFLYPGEVLEQGGV----TLPQVVLADTALRLALLDfE
Homo_sapiens     TVANPVSARDAGLVLFdVTGQVRLRHADLEIRLAQDPFFLYPGEVLEKDI----TLPQVVLPTALHLKALLDfE
Mus_musculus     IVANPVSARDAQGSVLFdVTGQVRLRHADLEIRLAQDPFFLYPGEVLEKDI----TLPQVVLPTALHLKALLDfE
Pongo_abelii     TVANPVSARDAGLVLFdVTGQVRLRHADLEIRLAQDPFFLYPGEVLEKDI----TLPQVVLPTALHLKALLDfE
Rattus_norvegicus IVANPVSARDTQSSVLFDTTGGQVRLRHADQIRLAQDPFFLYPGEVLEKDI----TLPQVVLPTALHLKALLDfE
Salmo_salar      VVUNPVARDDQQLVLFdVDSGQAKLRHADLEIRLTQDPFFLYPGEVLEIQGHV----TALQVYPTALRLKALLDfE
Strongylocentrotus_purpuratus I IENPVVRNDTDQVYVDNLGQVKLRHADQIRLARFPFFLYPGEVLEKQAV----TAKKVYQAANALRLALLDfE
Trypanosoma_rangeli_SC58 I IQNPVCVRDASGAPVLGANSVMRLRMGEEIRFEQDPFFLYPGEVLEKQKNEEWFLEKGVYHVRCLHNFIT
Xenopus_laevis   VIQNPVQRDSEEEKVLFDVGGAKLKHGDKIEIRLTQDPFFLYPGEVLELQGI----TPIITVVLANTALHLKALLDfE
Xenopus_tropicalis VIQNPVQRDADLRVLFdVGGTQLKSHGDKIEIRLTQDPFFLYPGEVLELQGI----TPIITVVLANTALHLKALLDfE
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Consensus      DkNG-----xKvYAGDEWLFEGPGTYIPRKEVEVVEIIQATVIRQNALRLRARKECxDRdGgkERVtGEEWLVrkv
Bos_taurus      DKNG-----KQVVAGDEWLFEGPGTYIPQKEVEVLEIIQATVIRQNALRLRARKECDRDGKERVTGEEWLVRSV
Canis_lupus_familiaris DKNG-----EKVVAGDEWLFEGPGTYIPQKEVEVLEIIQATVIRQNALRLRARKECDRDGKERVTGEEWLVRSV
Ciona_intestinalis EDD-----THRVAGDEWLFEGPGTYIPRKEVSEETIRATVIRKQNAIKLRARKECDRDGQVDRVTGEEWLVRSV
Danio_reio      EEGG-----EKRVAGDEWLFEGPGTYIPRKEVTLVEIKATVIRKQNAIKLRARKECDRDGQVDRVTGEEWLVRSV
Dictyostelium_discoideum_a EAK-----VTHVAGDEWLFEGPATYILPRDVRIEEELKATIGPNQALIKLRANKASDRSGVARKAGEWLVRSQH
Dictyostelium_discoideum_b EVKADKTTVSHIAGDEWLFEGPGTYIPRVEVQVVEPVRAVIRKENQALIKLRARLSFYDRDDVVRQVGEWLVRES
Felis_catus      DKNG-----EKVVAGDEWLFEGPGTYIPQKEVEVLEIIQATVIRQNALRLRARKECDRDGKERVTGEEWLVRSV
Gallus_gallus    DEEG-----NKVYAGDEWLFEGPGTYIPRKEVEVVEIIQATVIRKQNAIKLRARKECDRDGTRRVtGEEWLVRSV
Homo_sapiens     DKDG-----DKVVAGDEWLFEGPGTYIPRKEVEVVEIIQATVIRQNALRLRARKECDRDGKERVTGEEWLVRSV
Mus_musculus     DKNG-----DKVYAGDEWLFEGPGTYIPQKEVEVVEIIQATVIRQNALRLRARKECDRDGKERVTGEEWLVRSV
Pongo_abelii     DKDG-----DKVVAGDEWLFEGPGTYIPRKEVEVVEIIQATVIRQNALRLRARKECDRDGKERVTGEEWLVRSV
Rattus_norvegicus DKNG-----DKVYAGDEWLFEGPGTYIPQKEVEVVEIIQATVIRQNALRLRARKECDRDGKERVTGEEWLVRSV
Salmo_salar      EVGG-----EKRVAGDEWLFEGPGTYIPRKEVAVLEIKATVIRKQNAIKLRARKEGDRSGVRRVTGEEWLVRSV
Strongylocentrotus_purpuratus DGT-----QKRVAGDEWLFEGPGTYIPRKEVVVEETIRATVIRPQNAIKLRARKECDRDGTRRVtGEEWLVRSV
Trypanosoma_rangeli_SC58 DEWG-----VSRAGDEWLFEGPGTYIPRKEVEVVEVHAIITPNTALHLKALKKFTDRNGPREAGEWLVRSV
Xenopus_laevis   DESG-----KVYAGDEWLFEGPGTYIPRKEVEVVEIIQATVIRKQNAIKLRARKECDRDGKERVTGEEWLVRSV
Xenopus_tropicalis EESG-----KVVYAGDEWLFEGPGTYIPRKEVEVVEIIQASVIRKQNAIKLRARKECDRDGTRRVtGEEWLVRSV
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Consensus      GAYLPGVFEEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Bos_taurus      GAYLPAVFEEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Canis_lupus_familiaris GAYLPAVFEEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Ciona_intestinalis GAYLPGAEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Danio_reio      GAYLPGAEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Dictyostelium_discoideum_a GAYLPGVDEIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Dictyostelium_discoideum_b GAYLPGVDEIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Felis_catus      GAYLPGVDEIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Gallus_gallus    GAYLPGVDEIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Homo_sapiens     GAYLPAVFEEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Mus_musculus     GAYLPAVFEEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Pongo_abelii     GAYLPAVFEEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Rattus_norvegicus GAYLPAVFEEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Salmo_salar      GAYLPGAEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Strongylocentrotus_purpuratus GAYLPGAEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Trypanosoma_rangeli_SC58 GAYLPAVFEEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Xenopus_laevis   GAYLPGVDEIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
Xenopus_tropicalis GAYLPGVFEEVVDIVDAVILTEKIALHLRATRFRDIF--GvtrRTGEEWLVTVQDTTEAHIPDNYEEVxGVVPITTT
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Consensus      LgpRQYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLEF--GIQDVVYVLEqGGLLLRALQPLedGeee--
Bos_taurus      LGRHNYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEGK--
Canis_lupus_familiaris LGSHNYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Ciona_intestinalis LTSRQYCVILDPVGA--NGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Danio_reio      LNSRQYCVILDPVGA--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Dictyostelium_discoideum_a LNSRQYCVILDPVGA--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Dictyostelium_discoideum_b LNSRQYCVILDPVGA--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Felis_catus      LGRHNYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Gallus_gallus    LGRHNYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Homo_sapiens     LGRHNYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Mus_musculus     LGRHNYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Pongo_abelii     LGRHNYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Rattus_norvegicus LGRHNYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Salmo_salar      LNSRQYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Strongylocentrotus_purpuratus LNSRQYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Trypanosoma_rangeli_SC58 LNSRQYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Xenopus_laevis   LNSRQYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
Xenopus_tropicalis MNSRQYCVILDPVGP--DGKNQLGQKRVVKGESFFLQPGERLER--GIQDVVYVLEqGGLLLRALQPLedGEEG--
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Consensus
 Bos_taurus
 Canis_lupus_familiaris
 Ciona_intestinalis
 Danio_rerio
 Dictyostelium_discoideum_a
 Dictyostelium_discoideum_b
 Felis_catus
 Gallus_gallus
 Homo_sapiens
 Mus_musculus
 Pongo_abelii
 Rattus_norvegicus
 Salmo_salar
 Strongylocentrotus_purpuratus
 Trypanosoma_rangeli_SC58
 Xenopus_laevis
 Xenopus_tropicalis

Consensus
 Bos_taurus
 Canis_lupus_familiaris
 Ciona_intestinalis
 Danio_rerio
 Dictyostelium_discoideum_a
 Dictyostelium_discoideum_b
 Felis_catus
 Gallus_gallus
 Homo_sapiens
 Mus_musculus
 Pongo_abelii
 Rattus_norvegicus
 Salmo_salar
 Strongylocentrotus_purpuratus
 Trypanosoma_rangeli_SC58
 Xenopus_laevis
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 Felis_catus
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 Pongo_abelii
 Rattus_norvegicus
 Salmo_salar
 Strongylocentrotus_purpuratus
 Trypanosoma_rangeli_SC58
 Xenopus_laevis
 Xenopus_tropicalis

Consensus	KELLEALSAVSTGAKAEASRAEAARIEGEGVLQAKLKAEALAIETEAELGRLLKvREIElvYARAQIE
Bos_taurus	RELLEALSTAVSTGAKAEASRAEAARIEGEGAVLQAKLKAEALAIETEAELRVRKvRELELVYARAQLE
Canis_lupus_familiaris	RELLEALSTAVSTGAKAEASRAEAARIEGEGSVLQAKLKAEALAIETEAELQVRKvRELELVYARAQLE
Ciona_intestinalis	RDLEELQSSAAIESTGQAKAEASRAEAARIEGEGAVQARLKANAASIEAASELRLLKARDALKKvMKRQNE
Danio_reio	KELLEALQSAAVESTGAAKAEASRAEAARIEGEGAAVEEAKLKAEAKKIEADSELARLLKAREQELNVKKQIDH
Dictyostelium_discoideum_a	KDLELQQAQSAAVESTGQATAEAQATAEAANIAAEANVQAEKKAQATKRSSEISLLKAKRENELVQKSIDF
Dictyostelium_discoideum_b	SKLVQLQAQSAAVESTGQAVAEARARAEAAIEAASETKQARLSTRAIEIEALSEIENLAKKHTIVEIQHKSINN
Felis_catus	RELLEALSTAVSTGAKAEASRAEAARIEGEGSVLQAKLKAEALAIETEAELQVRKvRELELVYARAQLE
Gallus_gallus	RELLEALSAAVESTGAARAEAQARAEAAARIEAEEALQAKLKAEALAIETEAELARLERMQAQVRAQARAE
Homo_sapiens	KELLEALSMVAVSTGAKAEASRAEAARIEGEGSVLQAKLKAAALAIETEAELQVRKvRELELVYARAQLE
Mus_musculus	KELLEALSMVAVSTGAKAEASRAEAARIEGEGSVLQAKLKAAALAIETEAELQVRKvRELELVYARAQLE
Pongo_abelii	KELLEALSMVAVSTGAKAEASRAEAARIEGEGSVLQAKLKAAALAIETEAELQVRKvRELELVYARAQLE
Rattus_norvegicus	KELLEALSMVAVSTGAKAEASRAEAARIEGEGSVLQAKLKAAALAIETEAELRVRKvRELELVYARAQLE
Salmo_salar	KELLEALSAAVESTGAAKAEASRAEAARIEGEGAVNEAKLKAEAKKIEAEEELRLSKAREQELNVKSAAMDR
Strongylocentrotus_purpuratus	KELLEALQSAAVESTGQAKAEASRAEAARIEGEGAVDQARLKAEAAKIESESELQRLTNAREAE TKVVRQNA
Trypanosoma_rangeli_SC58	TKWLELQAQSEAVQASGQSVAEAKAKAESLLIEVESQLKQAEMLKAYRITAESELKKQRQKLDLETFVKKQNE
Xenopus_laevis	KELLEALSTIVESTGAAKAEASRAEAARIEGEGAVLQAKLKAEALAIETDAELQRLRQAASEELRYLQDKNK
Xenopus_tropicalis	KELLEALSTIVESTGAAKAEASRAEAARIEGEGAVLQAKLKAEALAIETEAELQRLRQAASEELRYTQDKNK

Consensus	LEVSKAQQLAEExEKKFKxMxEAIgpdTIRDxAvAGPEMQVKLLQSLGLKSTLITDGSSSIPNLFNTAnGLLGlg
Bos_taurus	LEVSKAQQLAEVEVKKFKQMTAELGPGSTIRDMVAVGPEMQVKLLQSLGLKSTLITDGSSTPINLFNTALGLLGLGA
Canis_lupus_familiaris	LEVSKAQQLAEVEVKKFKQMTAELGPGSTIRDLAVAGPEMQVKLLQSLGLKSTLITDGSSTPINLFNTALGLLGVGS
Ciona_intestinalis	LQLNKQDREIAEETRKFKDTIDAIGADTIRAVAVGPEMQVKLLSLGLQSTLITDGSSTPINLFNTANGLLGATA
Danio_reio	LEVKKQQLADIESQRFKHLMDNLGTEILKEMARAGPELQVKLLQSLGLKSTLITDGSSTPINLFTTANGLLGSLO
Dictyostelium_discoideum_a	LELTQSQDLAEIASKFKAIIVESIGRDTLKSIAAGNEMQAKLLQGLGLKSFMITDGSSTPINLFDTANGLLGNNN
Dictyostelium_discoideum_b	LELIKAKESATIEETTKFENYVEAIGSDTKSIAQAPPEETKAKLLAGLGLKSFMITDGSSTPINLFDTANGLLADAQ
Felis_catus	LEVSKAQQLAEVEVKKFKQMTAELGPGSTIRDLAVAGPEMQVKLLQSLGLKSTLITDGSSTPINLFNTALGLLGLGS
Gallus_gallus	AFAARAQALAAVEASKVREVAAALGPGSTIRDLAVAGPELQVKLLQGLGLQSLALITDGAAPINLFTARGLLGLAV
Homo_sapiens	LEVSKAQQLAEVEVKKFKQMTAELGPGSTIRDLAVAGPEMQVKLLQSLGLKSTLITDGSSTPINLFNTAFGLLGMGP
Mus_musculus	LEVSKAQQLADVEAKKFKEMTEALGPGSTIRDLAVAGPEMQVKLLQSLGLKSTLITDGSSTPINLFNTAFGLLGLGS
Pongo_abelii	LGVSQAQQLAEVEVKKFKQMTAELGPGSTIRDLAVAGPEMQVKLLQSLGLKSTLITDGSSTPINLFNTAFGLLGMGP
Rattus_norvegicus	LEVSKAQQLANVEAKKFKEMTEALGPGSTIRDLAVAGPEMQVKLLQSLGLKSTLITDGSSTPINLFTANGLLGLGS
Salmo_salar	LEVKKQQLAEIESNRFKKLVDSTIGADTLQEMARAGPELQVKLLQSLGLKSTLITDGSSTPINLFTANGLLGLS
Strongylocentrotus_purpuratus	LEVNKTQMSDIEETERFRNVQSIGADTLKAMAGPEMQVKLLSLGLKSTLITDGSSTPINLFTANGLLG6FS
Trypanosoma_rangeli_SC58	LELIKARQLAEIEAERVRRVAAIGRDTLVAVAGPEMQAKLLGGLGLKSYLITDGSSTPINLFTAAQGLING6V
Xenopus_laevis	LEVKKQQLAEIEIKKFKREMVESIGSGTLRDIALAGPELQVKLLRGLGISTLITDGSSTPINLFTAHGLLGITS
Xenopus_tropicalis	LEVKKQQLAEIEIKKFKREMVESIGSGTLRDIALAGPELQVKLLRGLGISTLITDGSSTPINLFTAHGLLGITG

Consensus	egQppx -----
Bos_taurus	EAGPPAKKPTGGPSVQEGLLPISTAAPLT LGNNQVVP---
Canis_lupus_familiaris	EAGHPAQKAAGMPGPREGLVLRSTSPQAVGSNHVVP---
Ciona_intestinalis	GGAAYPAVLHQTKRARKVEQESDDE-----
Danio_reio	QMVNMSNAAKQSGRRN-----
Dictyostelium_discoideum_a	RSEVDEE-----
Dictyostelium_discoideum_b	EAGPPTKKAAGMPPSPQEGLLLRSPAPQSLGDNHVVP---
Felis_catus	PEEPST-----
Gallus_gallus	EGQPLGRRVASGSPSGEGIS PQSAQAPQAPGDNHVVPVLR
Homo_sapiens	DGQPPVQK-----
Mus_musculus	EGQPLGRRVASGSPSGEGIS PQSAQAPQAPGDNHVVPVLR
Pongo_abelii	DGQPPAQK-----
Rattus_norvegicus	QSGGQ-----
Salmo_salar	AKRGIE---H-----VEEED-----
Strongylocentrotus_purpuratus	STGEHP-----
Trypanosoma_rangeli_SC58	KPAQKEKESD-----
Xenopus_laevis	KPAQKEKESD-----
Xenopus_tropicalis	KPAQKEKESD-----

Figure 3.2. Multiple sequence alignment of MVP from different species. Multiple sequence alignment of the collected 17 sequences was carried out using Clustal Omega alignment. The consensus sequence is displayed on top and all letters with white background refer to match with the consensus sequence. Sequences that are different from the consensus sequences are highlighted in black.

	<i>B. taurus</i>	<i>C. familiaris</i>	<i>C. intestinalis</i>	<i>D. rerio</i>	<i>D. discoideum a</i>	<i>D. discoideum b</i>	<i>F. catus</i>	<i>G. gallus</i>	<i>H. sapien</i>	<i>M. musculus</i>	<i>P. abelii</i>	<i>R. norvegicus</i>	<i>S. salar</i>	<i>S. purpuratus</i>	<i>T. rangeli SC58</i>	<i>X. tropicalis</i>	<i>X. laevis</i>
<i>B. taurus</i>	100	91.1	63.9	70.1	56.5	52.5	92.3	69.0	90.1	89.3	89.2	89.0	70.2	65.6	50.9	71.3	70.6
<i>C. familiaris</i>		100	63.1	69.9	56.7	51.9	95.1	68.9	91.4	90.5	90.9	90.1	70.3	65.8	50.7	72.1	71.7
<i>C. intestinalis</i>			100	66.7	60.3	56.0	62.9	60.8	63.9	64.4	63.9	64.5	67.3	72.3	49.9	67.4	66.4
<i>D. rerio</i>				100	60.9	53.9	70.1	67.6	70.1	69.3	70.0	69.6	86.1	69.7	48.8	73.6	73.5
<i>D. discoideum a</i>					100	61.2	57.1	57.3	57.2	56.9	57.0	56.9	60.6	61.2	52.2	58.5	58.4
<i>D. discoideum b</i>						100	52.1	52.7	52.7	52.7	52.2	52.8	53.5	56.1	50.3	52.7	52.8
<i>F. catus</i>							100	69.4	92.6	91.2	91.8	90.7	70.5	66.2	50.8	72.4	72.0
<i>G. gallus</i>								100	69.2	68.2	68.9	68.3	67.8	62.5	50.8	69.8	69.2
<i>H. sapien</i>									100	91.4	99.1	91.1	69.9	66.4	51.5	72.4	71.8
<i>M. musculus</i>										100	90.8	97.2	68.7	65.4	50.1	71.3	70.7
<i>P. abelii</i>											100	90.4	69.8	66.5	51.4	72.3	71.7
<i>R. norvegicus</i>												100	69.6	65.4	50.3	71.9	71.4
<i>S. salar</i>													100	70.6	48.0	74.5	74.2
<i>S. purpuratus</i>														100	51.7	70.2	69.3
<i>T. rangeli SC58</i>															100	49.5	48.7
<i>X. tropicalis</i>																100	93.9
<i>X. laevis</i>																	100

Figure 3.3. **Percentage identity matrix of Clustal Omega alignment of collected MVP sequences.** The value of the percentage identity (1 decimal place) is shown and is highlighted by a 2- colour gradient heat map where red represents 100% identity and white represents 0% identity.

In order to understand the relevance of the observed gaps and extensions between the protein alignment of human and zebrafish MVP, analysis of the conservation of MVP protein sequences was extended to include more organisms. Curated amino acid sequences of the various organisms were collected from NCBI database and subjected to Clustal Omega alignment. Clustal Omega alignment (Figure 3.2) of the collected sequences showed a high level of conservation amongst all the sequences. Conservations of amino acids were observed throughout the entire sequence, with the exception of some insertions and deletion events. In the event of substitutions, substitutions by amino acids with similar biochemical properties were generally observed. The percentage identity matrix of the Clustal Omega alignment (Figure 3.3) showed the lowest percentage identity of 48.0%, which was between *T. rangeli* and *S. salar*. *D. rerio* MVP showed a percentage identity of 48.8-86.1% when compared to the other organisms. Comparison of *D. rerio* MVP to *H. sapiens* MVP showed a percentage identity of 70.1%.

Interestingly, two lineage-specific insertions were observed (Figure 3.4.), one in mammals (Figure 3.4.A) and one in teleosts (Figure 3.4.B). When the insertions were compared to the x-ray crystal structure of rat vault (PDB 4V60), they were found to be located in regions projecting out of the vault body. An insertion in the loop at the shoulder domain, corresponding to aa 612-621 of the rat MVP sequence, was observed in mammals. All mammalian sequences examined contain this insertion with conservation of amino acid sequences. Another insertion of glutamic acid residues in the external loop of vault repeat domain 7 (corresponding to aa 316- 347 of rat MVP) was observed in the fish sequences.

Figure 3.4. Lineage-specific insertion events in MVP. Multiple sequence alignment showing the lineage-specific insertion of MVP in the fish lineage (A) and mammalian lineage (B). Using rat vault crystal structure (PDB 4V60) colour-coded to show the vault repeats (orange), shoulder domain (green), cap domain (blue) and cap ring (red), the region where the fish (cyan) and mammalian (magenta) inserts occurred was highlighted. A single monomer of MVP (C), side view (D) and top view (E) of a half vault.

3.2.2. Endogenous expression of MVP in zebrafish

The temporal and spatial expression of zfMVP in zebrafish embryos and adults were investigated to characterise zfMVP expression profiles. The endogenous expression of zebrafish MVP mRNA was investigated and characterised by RT-PCR (Figure 3.5.). RT-PCR was carried out on various tissues and organs of wild-type adult zebrafish (eye, skin, gill, gut, brain, spleen, muscle and fin) (Figure 3.5A) and on wild-type AB embryos at various stages of development (Figure 3.5B). In order to differentiate mRNA amplification from gDNA contamination, intron-spanning primers were designed. Addition of nuclease-free water in place of cDNA was used as negative control. Gel electrophoresis was carried out to visualise the size of the PCR products and a 289 bp band corresponding to zfMVP mRNA amplification was detected in tested samples. In gills and brain of adult zebrafish, a 538 bp band (Figure 3.5.A, yellow arrowhead) corresponding to amplified gDNA was detected and suggests gDNA contamination in these two samples. However, amplification of gDNA will not interfere with the interpretation of MVP mRNA expressions.

Figure 3.5. **RT-PCR of MVP in zebrafish.** RT-PCR of *MVP* from various organs of adult wild type zebrafish (A) and whole embryo of different developmental stages (B). Yellow arrowhead points to the band amplified from gDNA in the gills and brain samples.

With the knowledge that *mvp* mRNA is expressed in all tested stages of development, *in situ* hybridisation was used to visualise the spatial mRNA distribution in developing embryos (Figure 3.6.). *mvp* mRNA showed ubiquitous expression in all cells from the 2-cell stage until 6 hpf (hours post fertilisation). From 10 hpf to 14 hpf, expression of *mvp* mRNA was restricted to the superficial epidermal monolayer, known as the enveloping layer. At 24 hpf, *mvp* mRNA expression was detected in the epidermis, otic vesicle (black arrowhead, Figure 3.6.D), proctodeum (black arrowhead, Figure 3.6.E), hatching gland and yolk syncytial layer (YSL). At 72 hpf, strong *mvp* mRNA expression was observed in the developing pharynx and gut (Figure 3.6.F).

In brief, *mvp* expression zebrafish were dynamic, maternal deposition of *mvp* mRNA in the embryos resulted in a ubiquitous expression pattern followed by an epithelial- and yolk-restricted expression as development proceeds. Attempts to study MVP protein levels were unsuccessful. Anti-human MVP antibodies used in later chapters were tested on fish samples in western blots but it failed to detect any bands under the conditions tested. However, as we have subsequently shown the loss of MVP by mass spectrometry, the knock-out of MVP was verified.

Figure 3.6. **Expression of MVP in zebrafish larvae.** *in situ* hybridisation of embryos at (A, left to right) 2-cell stage, 4 hpf, 6 hpf, 10 hpf, 14 hpf, (B) 24 hpf and (F) 72 hpf. *mvp* mRNA was ubiquitously expressed until approximately 6 hpf. From 6 hpf onwards, the expression was restricted to the YSL. At 24 hpf, expression of *mvp* was observed in the YSL, epidermis, hatching gland, (D, arrowhead) otic vesicle and (E, arrowhead) proctodeum. (F) Dorsal view of 72 hpf embryo, expression of MVP was enhanced in the pharynx (empty arrowhead) and the gut (yellow arrowhead).

3.2.3. *mvp*^{-/-} zebrafish line has no observable phenotype

Figure 3.7. **Diagram depicting fish generations.** Wild-type embryos injected with CRISPR construct targeting *mvp* was annotated as F0 generation. F0 adult fish were crossed with wild-type to generate heterozygous F1 generation. F1 founders were identified by screening for frameshift mutations in the target regions. F1 founders were crossed with wild-type to increase fish population with the mutant alleles. The first zygotic mutants were generated in F3 generation through incrossing of F2 heterozygous mutant. Maternal-zygotic mutants were generated in F4 by incrossing F3 homozygous.

In an attempt to decipher the function of MVP, two *mvp* knockout zebrafish lines were generated by CRISPR-Cas9 system using guide RNAs targeting exon 3 and exon 7 respectively. The mutations lines were denoted as *mvp*^{3/3} and *mvp*^{7/7} respectively for the mutations in exon 3 and exon 7 respectively (Figure 3.9A). Injected F0 adults were out-crossed with wild-type fish to generate F1 generation. F1 adults were screened for frameshift mutations. The guide RNA target regions were amplified by PCR using primers flanking both ends of the target sites and the product was sequenced by Sanger sequencing. F1 adult fish a 5 bp deletion in exon 3 and another with a 16 bp deletion in exon 7, were identified and designated as *mvp*^{-/+} F1 founders. The F1 founders were outcrossed to wild-type to generate F2 generation to increase the number of fish carrying the mutant alleles. These F2 heterozygous mutants were in-crossed and generated offspring following Mendelian ratios. Observation of developing embryos of the F3 generation using stereomicroscope did not pick up any observable phenotypic changes and F3 homozygous mutants also grew up to become fertile adults. These demonstrate that *mvp*^{-/-} zygotic mutants are viable and fertile.

Embryos initially utilise proteins and mRNAs deposited into the oocytes by the mother, termed as maternal factors, for the earliest stages of development until the activation of the zygotic genome. Activation of zygotic gene expression occurs at the midblastula transition and in zebrafish, this stage corresponds to cleavage cycle 10, approximately at 3 hpf (Kane and Kimmel, 1993). The implication of MVP in various signalling pathways, together with the dynamically regulation of MVP localisation in developing porcine embryos (Sutovsky et al., 2005, Hamill and Suprenant, 1997), prompted us to investigate the importance of MVP in the earliest stages of embryonic development. This

was carried out by investigating the viability and development of maternal-zygotic mutants. Maternal-zygotic mutants were generated by crossing pairs of F3 *mvp*^{-/-} adult zebrafish or by crossing F3 *mvp*^{-/-} female fish with *mvp*^{-/+} male fish to generate F4 maternal-zygotic embryos devoid of MVP proteins or mRNAs. Maternal-zygotic mutants were viable, have no phenotypic differences and grow into fertile adults. Together, these data show that MVP is not essential for proper oogenesis or embryonic development.

Figure 3.8. **Diagram depicting the generation of zygotic and maternal-zygotic mutants.** Maternal-zygotic mutants were generated by crossing of *mvp*^{-/-} female fish with either *mvp*^{-/+} male fish (A) or *MVP*^{-/-} male fish (B). Zygotic mutants were generated by crossing *mvp*^{-/-} male fish with either *mvp*^{-/+} female fish (C) or by in-crossing *mvp*^{-/+} fish (D).

3.2.4. *mvp*^{-/-} zebrafish larvae are more susceptible to tunicamycin-induced brain haemorrhage

Previous studies have implicated MVP in cellular stress responses (Ryu et al., 2008, Tajitsu et al., 2013) and the relatively unchallenging laboratory environment might mask this function of vaults in zebrafish. To test if MVP is important for stress response, both wild-type and mutant embryos between 24-30 hpf were challenged with various cellular stresses. Initial screens were carried out with H₂O₂ (100, 200 µM), NaCl (100-500 mM, in multiples of 100s) and tunicamycin (1, 1.5 µg/ml). The stressor-containing media were added to 12-well plates containing approximately 20 larvae per well. For both concentrations of H₂O₂, wild-type and mutants survived till 24 hours post-treatment (hpt) but were dead at 48 hpt. For NaCl treatment, both wild-type and mutant larvae died at 24 hpt when treated with >200 mM, while those treated with 100 mM survived. The range of NaCl concentration was subsequently changed to 100, 120, 140, 160, 180 and 200 mM. No deaths were observed in wild-types or mutants over the course of 96 hours, although both wild-type and *mvp*^{3/3} larvae started showing signs of oedema around the eyes at 96 hours post-treatment (not shown).

The most obvious difference was spotted when larvae were treated with tunicamycin. Tunicamycin, a nucleoside antibiotic, inhibits GlcNAc phosphotransferase in the ER, preventing proper glycosylation of proteins. This leads to the accumulation of misfolded protein in the ER, inducing ER stress. Whilst addition of more than 1.5 µg/ml of tunicamycin resulted in deaths in both WT and mutants after 48 hours, cranial haemorrhage was consistently observed when larvae were treated with tunicamycin at lower doses of 0.125-1 µg/ml (Figure 3.9B). Cranial haemorrhage was defined by the presence of blood

accumulation outside of the vasculature. This is due to the escape of red blood cells from the blood vessels and signifies leakage or breakage of blood vessels. Embryos treated with tunicamycin were observed by stereomicroscope at 24 and 48 hpt and the number of embryos with haemorrhages (Figure 3.9 C) were recorded and presented as a percentage of total treated embryos. When mutant and wild-type embryos were treated with 0.125-0.25 $\mu\text{g/ml}$ tunicamycin, there was a statistically significant increased amount of cranial haemorrhages in the mutants. At 0.125 $\mu\text{g/ml}$, $18.0 \pm 14.2\%$ of wild-type larvae displayed signs of haemorrhage while $57.9 \pm 25.1\%$ of *mvp*^{3/3} larvae and $37.8 \pm 20.8\%$ of *mvp*^{7/7} larvae displayed signs of haemorrhage. At 0.25 $\mu\text{g/ml}$, $22.7 \pm 5.80\%$ of wild-type larvae displayed signs of haemorrhage while $69.8 \pm 18.0\%$ of *mvp*^{3/3} larvae and $56.7 \pm 31.2\%$ of *mvp*^{7/7} larvae displayed signs of haemorrhage. Large standard deviations were observed in the experiments and might be due to variation in the level of fitness of different clutches of eggs. At higher doses of tunicamycin, haemorrhage rates in wild-type embryos increased to match those of mutants and mutants start to die from the treatment (not shown). This is most probably due to an over-whelming systemic ER stress generated by the higher doses of tunicamycin.

In summary, *mvp* mutants were found to be more susceptibility to tunicamycin-induced ER stress. This susceptibility resulted in the manifestation of cranial haemorrhages at low doses of tunicamycin and deaths at higher doses of tunicamycin.

Figure 3.9. *mvp*^{-/-} mutants are more susceptible to tunicamycin-induced ER stress. (A) Screening of Cripsr-Cas9 edited founders led to two different *mvp*^{-/-} mutants lines, *mvp*^{3/3} and *mvp*^{7/7} which have a 5 bp deletion in exon 3 and a 16 bp deletion in exon 7 respectively. (B) Bright field images of (Left) lateral view and (Right) dorsal view of cranial haemorrhages after treatment of larvae with tunicamycin. (C) Quantification of tunicamycin-induced haemorrhages in WT and *mvp*^{-/-} mutants with 0.125 – 0.5 µg/ml of tunicamycin. (mean ± SD, results are from 3 separate experiments, with at least 60 larvae per condition. **** = P<0.0001, *** = P<0.0002, ** = P<0.0021, * = P<0.332, Two-way ANOVA)

3.2.5. Transgenic krt4: MVP-GFP

Figure 3.10. **Tg(krt4:Mvp-GFP) line.** (A) Dorsal view and (B) lateral view of confocal z-stack images of 3 dpf Tg(krt4:Mvp-GFP) larvae. Tg(krt4:Mvp-GFP) line larvae have a mosaic expression of Mvp-GFP in the EVL, a one cell thick layer of cells that covers the entire embryo.

in situ hybridisation data generated in the previous section of this thesis and previous histological data from human tissues (Izquierdo et al., 1996a) showed a high level of MVP expression in the epithelial cells. In addition to the challenge of wild-types and mutants with the various stressors, we also leveraged on the transparency of zebrafish embryos for real-time monitoring of MVP protein movement. This was achieved through live imaging of transgenic Mvp-GFP expressed in the epidermis of zebrafish.

The epidermis of zebrafish larvae consists of a bilayered epithelium until approximately 15 dpf (Dane and Tucker, 1985, Le Guellec et al., 2004). The outer layer of cells is a periderm monolayer, known as the enveloping cells (Kimmel et al., 1990), and can be specifically labelled with the krt4 promoter (Gong et al., 2002). The use of the krt4 promoter allows for the visualisation of MVP movement and intracellular localisation in the epidermis where it is endogenously expressed.

The generation of Tg(krt4:Mvp-GFP) zebrafish line resulted in embryos with mosaic levels of Mvp-GFP expression in the EVL (Figure 3.10). This

overexpression of Mvp-GFP did not lead to any phenotypic differences and did not affect the growth and development of embryos. Tg(krt4:Mvp-GFP) embryos were challenged with the same cellular stresses, H₂O₂, NaCl and tunicamycin, and monitored by confocal live imaging. However, no changes in the localisation of Mvp-GFP was observed in these analyses. Representative confocal images of treated and control larvae taken after 24 hours of treatment are shown (Figure 3.11). All larvae showed a predominantly cytoplasmic distribution of Mvp-GFP, with some cells showing aggregations present both before and after treatment.

Figure 3.11. **Challenge of *Tg(krt4:Mvp-GFP)* larvae with stressors.** Z-stack maximum intensity projections of confocal images of the head (A-B) and yolk (C-E) region of untreated *Tg(krt4:Mvp-GFP)* zebrafish larvae (A, C) and larvae treated with tunicamycin (B), NaCl (D) and H_2O_2 (E). Scale bar is 50 μm .

3.2.6. Proteomic analysis of *mvp*^{-/-} zebrafish larvae

Figure 3.12. **SWATH-MS analysis of 30 hpf zebrafish larvae.** (A) (Top) Venn diagram of the number of proteins upregulated (>1.5-fold, 70% confidence) in *mvp*^{3/3} (yellow) and *mvp*^{7/7} (purple). (Bottom) List of common upregulated protein in the two lines of *mvp*^{-/-}. (B) (Top) Venn diagram of the number of proteins downregulated (> 1.5-fold, 70% confidence) in *mvp*^{3/3} (yellow) and *mvp*^{7/7} (purple). (Bottom) List of common downregulated protein in the two lines of *mvp*^{-/-}.

Due to the lack of observable phenotypic difference between wild-types and mutants, the molecular impact of *mvp* knockout was investigated. Proteomic analysis was adopted to compare the protein expressions of mutants and wild-types embryos. In addition, we hope to verify the loss of MVP protein in the mutants as we do not have antibody available to verify the loss of MVP in our mutants. 30 hpf wild-type and maternal-zygotic embryos, *mvp*^{3/3} and *mvp*^{7/7}, from different parents were collected and pooled together. Downstream processing, such as protein extraction and digestion, was carried out by a colleague. Proteomic analyses were carried out using SWATH-MS by a collaborator. Comparison of both *mvp*^{-/-} lines to wild-type was used to prevent hits that arise due to Cripsr-Cas9 off-target effects and also to minimise any founder effects that could arise from the in-crossing. This redundancy allows for a more robust and accurate determination of affected proteins. Lists of differentially regulated proteins were compiled by the colleague/collaborator based on an arbitrary cut-off of 1.5-fold difference with at least 70% confidence to reduce the chance of false positives. The lists of differentially regulated proteins between *mvp*^{3/3} vs WT and *mvp*^{7/7} vs WT were compared and common up- or down-regulated proteins were selected. This resulted in a list of nine down-regulated proteins and eight up-regulated proteins (Figure 3.12).

Down-regulated proteins consist of Sord, Fam213b, Gpia, Hbbe3, Csrp1a, Mvp, pPha1b, Myl3 and Si:dkey-261m9.19. Sord, Gpia, and Fam213b are enzymes involved in sorbitol, glucose-6-phosphate and arachidonic acid metabolism respectively. Hbbe3 is an embryonic form of β -like globulin which is homologous to ϵ -globulin in humans (Song et al., 2004). Csrp1a acts on non-canonical Wnt signalling pathways in a cooperative manner and is involved in

the orchestration of cell behaviour during development (Miyasaka et al., 2007). P4ha1b is orthologous to human P4H which is involved in the modification of proline into hydroxyproline (Vuori et al., 1992). Mylz3 is fast skeletal myosin light chain 3. Most importantly, a drastic downregulation of Mvp protein levels (>13-fold) in samples from both mutant alleles when compared to wild-types was observed. This validates the CRISPR mutants as Mvp knockout.

Figure 3.13. SNP in detected H2B fragment. The presence of a SNP in H2B possibly account for fold change observed. The position of peptide fragment picked up by SWATH-MS with respect to full-length H2B in *mvp*^{3/3} (A) and *mvp*^{7/7} (B). (C) Ensembl data on SNP rs501382785 which give rise to C78A mutation (C) Genotyping of the corresponding genomic sequence from random sampling of *mvp*^{3/3} homozygous and WT adult fish shows the presence of the SNP in fish population (D).

One other striking difference in the proteomic analysis was the strong down-regulation of histone H2B (-5.73-fold and -13.75-fold for *mvp*^{3/3} and *mvp*^{7/7} respectively). Further investigation on the H2B peptides detected showed a single peptide fragment in histone H2B that was exclusively detected in wild-types embryos (Figure 3.13). The loss of this single peptide resulted in the drastic fold-change observed in our analysis. Subsequent analysis through Ensembl (Zerbino et al., 2018) database identified a nonsynonymous SNP in the detected peptide. The c.232a>g nucleotide resulted in a missense mutation leading to a C78R mutation in the H2B protein. To investigate the possibility of this SNP affecting our analysis, the corresponding region of the H2B gene in random *mvp*^{3/3} and WT adults were sequenced. The detection of the c.232a>g SNP suggests that this SNP might have led to the difference in the levels of H2B detected by the proteomic analysis. Indeed, the subsequent search through the SWATH-MS results identified the altered peptides. This concludes that drastic H2B protein fold-change was potentially due to the presence of the SNP and thus was not pursued further.

The eight upregulated proteins are Eppk1, Fn1a, Ck1, Eif4ea, Zgc:101000, Faah2b, Krt8 and Krt18. Eppk1 is a protein that binds and reorganises intermediate filaments (Jang et al., 2005) and have been implicated in motility of cells (Kokado et al., 2016, Shimada et al., 2013). Eif4ea, a homologue of human eIF4e, binds m⁷GTP cap of mRNAs and regulates mRNA translation (Robalino et al., 2004). Fn1a is an extracellular matrix component involved in heart formation (Trinh and Stainier, 2004, Garavito-Aguilar et al., 2010), muscle development (Snow et al., 2008) and stress responses (Okech et al., 2016, Dhanani et al., 2017). Faah2b is an enzyme involved in the catabolism of fatty

acid amides such as N-acylethanolamines. Ck1 and zgc:101000 are type 1 cytokeratin and Dnase1l4.1 respectively, however, their functions are still unknown. Krt8 and Krt18, an ancient pair of keratin, have been implicated in cell survival and stress response in epithelial tissues (Baek et al., 2017, Zatloukal et al., 2000, Schaffeld et al., 2003, Helenius et al., 2016). Interestingly, Eppk1 have also been shown to bind Krt8/18 heterodimer to stabilise them (Szabo et al., 2015).

3.3. Discussion

Protein sequence alignment of zebrafish MVP with human MVP showed a high level of conservation with some gaps. In order to determine if these gaps could potentially affect vault particle formation, sequence alignment of MVP from other organisms, especially those with experimentally determined functional vaults, was carried out. Sequence alignment of MVP protein sequences of these various organisms demonstrated a high level of conservation across the entire sequence. The only exception to this is at the N- and C-terminus where there were more variabilities. Given the unique architecture of vault complex and the high number of intermolecular bonds (Tanaka and Tsukihara, 2012), it is not a surprise for the amino acids to be conserved to ensure the proper formation of the vault complex. The N- and C-terminus are flexible and the addition of various epitopes to the termini was able to allow proper folding of vault particles (Kickhoefer et al., 2009, Kar et al., 2011, Matsumoto et al., 2013). This suggests that the termini are not important for the maintenance of structural stability. As *D. discoideum* MVPa with a percentage identity of 57.2 % when compared to *H. sapiens* MVP, are able to form function vaults, *D. rerio* MVP with a percentage identity of 70.1%, when compared to *H. sapiens* MVP, should also be able to form intact vaults.

Interestingly, possible lineage-specific insertion events in teleost fishes and mammals were observed. These insertions suggest that there might be lineage-specific adaptations for as yet unknown functions, such as binding to protein partners, over the course of evolution or it is not strongly selected against. The insertion in salmon consists of multiple glutamic acids and could be an artefact due to sequencing slippages. Furthermore, only two fish sequences were

investigated, additional MVP sequences from other fish have to be collected and compared before any conclusion with regards to the fish-specific insertion event can be drawn. In mammals, the insertion was observed with all collected mammalian MVP sequences, suggesting a true lineage-specific insertion event. Using the x-ray crystal structure of rat vault (PDB 4V60), the insertions were mapped to loop regions with unresolved structure (Tanaka et al., 2009), suggesting a high level of flexibility of the loop. As EM analysis of various eukaryotes including non-mammals, such as slime moulds and sea urchin, have demonstrated intact vaults (Kedersha et al., 1990), these insertion events should not interfere with the proper folding of vaults. The insertions being on the external face of the vault barrel together with the flexibility of the loops most probably will not interfere with the proper folding of zebrafish vaults. Together, these findings suggest that zebrafish is a suitable model for studying vault biology.

Characterisation of endogenous MVP expression showed the presence of *mvp* mRNA at all stages of zebrafish development. The presence of *mvp* mRNA in 2-cell stage embryos means that *mvp* mRNAs are deposited in oocytes as a maternal factor. Even after the expression of zygotic genes at approximately 4 hpf, *mvp* has ubiquitous expression based on its presence in all cells at the 6 hpf stage. This ubiquitous expression switched to a more epithelia-restricted expression from 10 hpf onwards after the occurrence of epiboly. This restricted expression in epithelia is similar to that described in human tissue (Izquierdo et al., 1996a). It is important to note that despite the absence of *mvp* mRNA in the other parts of the embryo, it does not necessarily point to the absence of vaults complexes as the half-life of vaults have been estimated to be more than 3 days (Zheng et al.,

2005). However, as we were unable to obtain working antibodies for the detection of zebrafish MVP protein, a conclusion on the distribution of zebrafish MVP protein expression cannot be made. Using RT-PCR, expression of *mvp* mRNA was also observed in examined organs of adult zebrafish. However, the exact type of cell(s) expressing *mvp* cannot be determined based on this analysis. Recently, immunohistochemical staining for MVP in rainbow trout showed high levels of MVP protein in the epithelium of gills and intestine, macrophages of the head kidney and secretory cells of the renal kidney (Margiotta et al., 2017). These data showed the same conserved expression of MVP in cells exposed to xenobiotics in various organisms. Presence of MVP expression in zebrafish in similar tissue types together with the well-conserved protein sequences suggests that zebrafish is a suitable model for study vault biology.

Using CRISPR mediated gene editing, *mvp* mutant zebrafish was generated. Maternal-zygotic *mvp* mutants were viable and produced off-spring according to Mendelian ratio, suggesting that MVP mutants are fertile and viable. In addition, no observable phenotypic differences were seen in mutant larvae or adults under basal conditions. This observation is true for both mutant alleles and suggests that *mvp* is not essential for the normal embryonic development of zebrafish. As MVP is often implicated in cellular stress response (Suprenant et al., 2007, Ryu et al., 2008, Ikeda et al., 2008), wild-type and mutant zebrafish larvae were exposed to various stressors (NaCl, H₂O₂ and tunicamycin). Under the conditions tested, mutant larvae did not show any changes in sensitivity towards NaCl or H₂O₂. The killing of embryos by H₂O₂ after 48 hours, coupled with the unstable nature of the molecule, led to the abandonment of using H₂O₂ to induce stress. For NaCl, the concentrations were titrated to intervals of 20 mM but no difference

in sensitivity was observed in the *mvp*^{-/-} larvae when compared to wild-types. A possible reason is that presence of Na⁺ ions transporting ionocytes found on the epidermis of larval zebrafish (Liao et al., 2007, Wang et al., 2009) might be the key regulator of ion concentration to buffer and protect the larvae against osmotic stress. Previous observation of MVP protecting against osmotic stress was in SW620 cell line. This epithelial cell line was derived from colon cancer cells and might not possess the relevant ion transporters required to maintain homeostasis of salts. *in vivo* testing using zebrafish larvae allows for the study of MVP under complex homeostatic regulation involving multiple organs such as the ionocytes located in the epidermis. This systemic homeostatic regulation might not require MVP and the roles of Na⁺ transporters in controlling Na⁺ levels might have a stronger influence in the response to osmotic stress. In this case, any benefits of MVP would be masked.

Interestingly, when 30 hpf WT and mutant larvae were treated with tunicamycin, increased the susceptibility of *mvp*^{-/-} larvae to tunicamycin-induced haemorrhage was observed. MVP was previously shown to bind inactive PERK (Zhang et al., 2015), a receptor involved in ER stress signalling. Upon activation of PERK by ER stress, MVP dissociates from PERK. PERK, in turn, phosphorylates eIF2 α , leading to its inhibition and thus attenuating protein translation (Harding et al., 1999). It was also previously shown that tunicamycin affects the permeability of the blood-brain barrier and endothelial cells of the blood vessels (Finnie and Mukherjee, 1987, Finnie and O'Shea, 1990). This effect of tunicamycin on endothelial cells of blood vessels was due to ER stress-induced death (Leaver et al., 1988).

During development, the primordial hindbrain channel starts to form projections towards the midline and the central arteries start to form around 32 to 36 hpf (Ulrich et al., 2011). The brain haemorrhages in zebrafish observed after tunicamycin treatment could be due to induction of ER stress and/or apoptosis in the endothelial cells that form the blood vessels, resulting in poor vessel formation and/or leakages. As the blood-brain barrier starts forming only after 3 dpf (Fleming et al., 2013), we considered the probability of haemorrhages arising due to the lack of a protective blood-brain barrier resulting in accumulation of tunicamycin in the brain. Indeed, when tunicamycin was added to the larvae at 3 dpf, no haemorrhage formation was observed in both mutant and wild type embryos (data not shown). This suggests either the blood-brain barrier is able to shield the brain from tunicamycin, or the formed blood vessels are now less susceptible to perturbations by tunicamycin. This results in a small developmental window where an observable phenotype is present and the role of MVP in protection against tunicamycin-induced ER stress remains inconclusive. Future studies need to be carried out to validate the role of MVP in ER stress response.

In addition to MVP mutants, a transgenic zebrafish line expressing Mvp-GFP under the epidermis-specific promoter, Tg(Krt4:Mvp-GFP), was generated to serve as a tool to visualise the movement of MVP. The Tg(Krt4:Mvp-GFP) zebrafish line generated had a mosaic expression that was not corrected by out-crossing with wild-types. Possible explanations include insertion of the transgene into the region(s) with chromosomal regulation that prevents an even expression in all cells or differential regulation of Mvp-GFP degradation/stabilisation resulting in cells with different level of expression. Treatment of Tg(Krt4:Mvp-

GFP) larvae with the various stressors did not result in any significant changes in the localisation of Mvp within the cells. MVP either do not response to these stimuli or the overexpression of Mvp-GFP might have masked any subtle changes in Mvp-GFP intracellular distribution. In our transgenic construct, the GFP tag is added at the C-terminus of MVP and there exists a possibility that the translocation of MVP might be dependent on interactions involving the C-terminus. Immunofluorescent (IF) staining of endogenous MVP in cells might be more accurate in studying changes in localisation upon exposure to stressors.

The lack of phenotypic differences in our zebrafish lines, together with the lack of suitable antibodies to study zebrafish MVP protein level/localisation, prompted us to compare the proteome of MVP mutants and wild-types. This has two aims, firstly, to confirm the loss of MVP protein in the mutants and secondly, to determine if MVP mutants showed loss of proteins in any molecular pathways. The latter might help uncover a function for vaults as changes in the level of proteins on the same pathway would provide a clue to the possible function of vaults. Comparison of the proteomes between both *mvp*^{-/-} mutant lines and wild-types resulted in nine common downregulated proteins and eight common upregulated proteins. The nine down-regulated proteins were mainly enzymes and they do not map to any single or overlapping pathways. Furthermore, most of the function of these proteins are poorly characterised in the zebrafish, making it hard to infer any information on their functions and pathways. This makes the interpretation of the significance of their downregulation difficult. Most importantly, the loss of MVP peptides was observed, validating the knockout fish lines as MVP nulls.

Amongst the upregulated proteins, an interesting trend in Fn1a, Eppk1, Krt8 and

Krt18 was observed and that is the involvement of these proteins in stress responses. Human fibronectin has recently been shown to be responsive to cellular stress (Okech et al., 2016, Dhanani et al., 2017). Fn1a(fibronectin-1) is essential for the timing of myocardial progenitors in zebrafish embryonic development. Fn1a expression is positively regulated by transcription factor Mtx1 (Sakaguchi et al., 2006) and negatively regulated by transcription factor hand2 (Garavito-Aguilar et al., 2010). Loss of fibronectin results in the loss of tissue integrity, defective adherent junctions and lack of cardiomyocytes migration to the midline (Trinh and Stainier, 2004). On the other hand, overexpression of Fn1a in hand2 knockout zebrafish lines resulted in the decreased anterior-posterior spreading of the early endocardium. Fn1a was proposed to impede cell movement through the stiffening of the ECM. The excessively high levels of Fn1a might also modulate integrin signalling to restrict cell migrations (Sakaguchi et al., 2006). Although we do not see any defects due to dysregulation of cells migratory, this data suggests modulation of the extracellular matrix by MVP. Given that MVP increased the migratory capability of cells (Lotsch et al., 2013), it would be interesting to understand the relationship between fibronectin and Mvp in regulating cell migration. This fits the hypothesis of Mvp being involved in stress responses and the loss of Mvp might have led to an increase in compensatory stress responses involving fibronectin. However, based on *in situ* data from zfin and this thesis, fibronectin and Mvp are not expressed in the same tissues. This suggests the effect of Mvp or Fn1a on cell migration is indirect and due to the modulation of signalling pathways rather than the direct conferment of physical properties.

Similar to the involvement of Fn1a in stress responses, Krt8, Krt18 and Eppk1

have also been implicated in stress response in mouse (Alam et al., 2013, Helenius et al., 2016) and human (Omary et al., 2002, Baek et al., 2017). Krt8/18 pair is thought to be the oldest keratin pair during phylogenesis (Blumenberg, 1988), forming obligate heterodimer intermediate filaments in simple epithelia (Franke et al., 1981, Owens and Lane, 2003). Zebrafish Krt8/Krt18 pair was previously identified as the homolog of human Krt8/Krt18 (Schaffeld et al., 2003), suggesting the conservation of Krt8/Krt18 and its function. Krt8 and Krt18 protected against mechanical, toxic and apoptotic stress in hepatocytes (Tao et al., 2009, Zatloukal et al., 2000, Moll et al., 2008) and association studies showed that Krt8 and Krt18 variants have a three-fold increase in risk of end-stage liver disease (Ku et al., 2005). In addition, stress response related post-translation modification of Krt8 was increased in mouse colitis model (Helenius et al., 2016) and Krt8/Krt18 mutations or deletions caused pathological defects in the gut (Misiorek et al., 2016). Such defects are most likely due to perturbations of the tight junction as the expression of mutant Krt18 led to disrupted ZO-1 and claudin-4 distribution (Zupancic et al., 2014).

Eppk1, a member of the plakin protein family, binds to intermediate filaments and stabilises them (Jang et al., 2005). *Eppk1*^{-/-} mice displayed an increased rate of keratin network breakdown upon exposure to stress (Szabo et al., 2015). Importantly, Eppk1 binds Krt8 and Krt18 in hepatocytes and its expression mimics that of Krt8 and Krt18 in liver stress models (Szabo et al., 2015). *eppk1* (ZFIN), *krt8* (Thisse et al., 2005) and *krt18 mRNA* (Wang et al., 2006) are known to be expressed in the epidermis of developing zebrafish embryos. This coincides with the expression of *mvp* at this stage of development. It would be reasonable to propose that upregulation of the Eppk1/Krt8/Krt18 stress response pathway as

a mechanism to compensate for the loss of Mvp. Thus far, there exists limited literature on zebrafish Krt8/18. A 4.4 kb promoter of zebrafish Krt18 was shown to specifically label biliary cells in zebrafish larvae (Wilkins et al., 2014) but Krt 8/18 expression in zebrafish hepatocytes are unknown. Whether the zebrafish Krt8/18 pair is orthologous to mammalian Krt8/18 pair has to be further verified. Proteomic analysis of zebrafish liver tissue exposed to tunicamycin might provide evidence for the possible synergism between MVP and Krt8/18 in the protection against liver diseases. Given the expression of MVP in fish liver, implication of mouse and human Krt8 and Krt18 in liver diseases, (Margiotta et al., 2017) and the frequent involvement of ER stress in liver diseases (review in (Malhi and Kaufman, 2011)), it is possible that MVP plays a role in ER stress response in the liver.

3.4. Conclusion

The lack of observable phenotypes in both *mvp*^{3/3} and *mvp*^{7/7} is in agreement with the previous findings in mice that MVP is not required for the proper development of embryos and organisms. When challenged with stressors, both MVP mutant lines demonstrated increased susceptibility to tunicamycin, a GlcNAc phosphotransferase inhibitor which induces ER stress. Upregulation of stress-related genes was also observed under basal condition in MVP mutants. Together, the data in this chapter provide evidence for the involvement of MVP in stress response, in particular, ER stress. However, more work has to be carried out to verify the cross talk between MVP and Eppk1/Krt8/Krt18 stress response pathway to verify the relationship between these two pathways.

Chapter 4. *in vitro* characterisation of MVP function

under basal and stressed conditions

4.1. Introduction

In parallel to using zebrafish as an *in vivo* model for vault biology, cell lines were used to elucidate the *in vitro* function of vaults at the cellular level, to observe outcomes outside of systemic homeostasis. The availability of antibodies against human MVP aids in the study of endogenous MVP protein levels and intracellular localisation. Using immunohistochemistry, the expression of MVP in normal human macrophages and epithelial cells were previously characterised (Izquierdo et al., 1996a). As these cells are often exposed to xenobiotic stress, MVP was hypothesised to be involved in stress responses or innate immunity as bacteria are a source of xenobiotic stress. Correspondingly, there are increasing implications of MVP in innate immune responses (Kowalski et al., 2007, Liu et al., 2014, Peng et al., 2016) and stress responses (Ikeda et al., 2008, Yi et al., 2005). In order to understand the endogenous function of vaults, we decide to study the role of vaults in macrophage and epithelial cells. As such, commonly used macrophage and epithelial cell lines, HaCaT, HeLa and THP-1 were used.

HaCat is a spontaneously immortalised keratinocyte cell line (Boukamp et al., 1988) achieved by propagation at low Ca²⁺ levels and high temperature (38.5 °C). Although HaCaT cells are immortalised, they are not tumorigenic and retained the capacity to form well-structured epidermis *in vivo*. They display normal morphological differentiation and expression profiles of differentiation markers such as keratins 1 and 10 identical to explanted keratinocytes. Thus, HaCaT is a

common cell line for the study of keratinocytes biology. HeLa, derived from adenocarcinoma tumour from Henrietta Lacks, is the first cell line to be developed (Gey et al., 1952). HeLa cells were first used to propagate poliomyelitis viruses (Scherer et al., 1953) in order to study it and is now the most commonly used cell line in scientific research. THP-1 is a monocytic cell line derived from the peripheral blood of an acute myeloid leukaemia patient (Tsuchiya et al., 1980). THP-1 has been extensively used as a model to study monocyte/macrophage functions and activities (Auwerx, 1991, Chanput et al., 2014). As THP-1 is derived from a less matured stage of leukocyte development, it is also a useful tool to understand the maturation of monocytes to macrophages. Suspension THP-1 cells in the monocytic state can be easily differentiated into adherent macrophage-like cells through stimulation with PMA (Auwerx et al., 1992). Differentiation with PMA as low as 5 ng/ml for 48 hours was sufficient to induce the maturation of THP-1 cells with gene expression profiles similar to those of primary macrophages. The low PMA concentration used minimises excessive upregulation of inflammatory markers. This prevents the masking of THP-1 response to stimuli. THP-1 has been shown to express markers similar to PMBCs and PBMC-monocytes derived macrophages, and response to stimuli in a similar manner to both PMBCs and PBMC-monocytes (Park et al., 2007). Together, these properties make THP-1 one of the most commonly used cell lines for monocytes/macrophage biology. Using these cell lines as model systems, we aim to uncover factors that ubiquitously affect the localisation or level of MVP in the different cell lines. This will allow us to decipher a conserved biological function of MVP in cells/organisms. By understanding the events regulating MVP expression, we aim to gain further insights into its function.

4.2. Results

4.2.1. MVP is expressed basally in cells

In order to assess the suitability of the chosen cell lines for studying MVP function, the expression of MVP in HaCaT, THP-1 and HeLa cells was first characterised. This was achieved by western blot and IF analysis. Two antibodies against human MVP raised in different hosts (mouse and rabbit) were used. The mouse monoclonal antibody was raised against a full-length recombinant human MVP protein and the rabbit polyclonal antibody was raised against a recombinant human MVP protein fragment corresponding to aa 208-327. Antibodies from different hosts were tested to ensure more flexibility in co-staining protocols. Furthermore, colocalisation of staining using the two different antibody would give us much higher confidence that immunoreactivity observed is due to specific recognition of MVP. Antibody specificity was validated through a series of experiments including immunoprecipitation, western blot, MVP siRNA treatment and IF (Figure 4.1). Immunoprecipitation and western blot resulted in a single 100 kDa band (Figure 4.1 A-B), MVP siRNAs lead to a reduction in the intensity of 100 kDa band in western blot (Figure 4.1 C) and co-staining using both MVP antibodies showed colocalisation of fluorescent signals in a punctate cytoplasmic manner (Figure 4.1 D). This correlates well with the current understanding in literature. Together, these evaluations demonstrate that the antibodies recognise MVP in a specific manner.

With the successful validation of the MVP antibodies, expression and intracellular distribution of MVP in various cell lines were investigated by IF (Figure 4.2). In all cells tested, MVP displayed punctate cytoplasmic staining.

However, variable nuclear localisation of MVP was sometimes observed under basal conditions. Analysis of MVP localisation in neutrophils showed dense cytoplasmic staining. However, the small cytoplasmic space of neutrophils makes the determination of precise subcellular localisation difficult.

Figure 4.1. Validation of anti-MVP antibodies. (A) Silver stain of (L-R) PrecisionPlus ladder, pulldown of cell lysate with anti-MVP antibodies, remaining cell lysates (B) Western blot analysis of HeLa whole cell lysates. (C) Western blot of lysates from HeLa cell treated with control siRNA and three different MVP siRNA. siRNA 1 and 3 showed the loss of MVP protein. (D) IF of HeLa cells with DAPI, two different anti-MVP antibodies, anti-mouse AF488 and anti-rabbit AF568 secondary antibody.

Figure 4.2. **Basal expression of MVP in cells.** Confocal microscopy of IF staining of three different cell lines, HaCaT, HeLa and THP-1 and primary human neutrophils. Cells were stained with mouse monoclonal anti-MVP antibody and anti-mouse AF488 secondary antibody, nuclei were counterstained with DAPI. HaCaT, HeLa and THP-1 cells were taken at 400X magnification and neutrophils at 600X magnification. Scale bar = 50 μ m.

4.2.2. MVP level and localisation in cells are not affected by tested stressors

Although MVP was previously implicated in various cellular stress, the observations were often reported in one specific cell line (Vasu and Rome, 1995, Yi et al., 2005, Suprenant et al., 2007, Ikeda et al., 2008). No survival differences were observed when wild-type and mutant zebrafish were treated with various cellular stressors (previous chapter). As such, investigation of the impact of cellular stress on level/localisation of MVP in cell lines were carried out. Published data (Zhang et al., 2015) together with our zebrafish data suggest the involvement MVP in ER stress response. HaCaT cells were treated with varying levels of tunicamycin to induce ER stress and qPCR analysis of the treated cells showed a dose-dependent increase of MVP mRNA. However, the corresponding increase in the protein levels of MVP was not observed when western blot was carried out on cell lysate. On the contrary, a 47-67% reduction of MVP protein level was observed in the various treated condition (Figure 4.3). ER stress response was monitored using BiP/GRP78 levels. BiP/GRP78, a chaperone protein found in the ER, is the key regulator of unfolded protein response and its expression is induced under ER stress (Kozutsumi et al., 1988). Thus, BiP/GRP78 is frequently used as a marker of ER stress induction. With the exception of treatment of cells with 0.25 µg/ml tunicamycin, tunicamycin protein levels in the other condition were increased to 1.24-5.53 times, verifying the induction of ER stress under these conditions.

As no consistent differences in the levels of MVP in response to tunicamycin was observed, changes in the intracellular distribution of MVP were investigated. Although MVP shows a predominantly punctate cytoplasmic staining, it has been shown to localise to specific intracellular regions. MVP underwent nuclear

translocation upon induction of PTEN and c-FOS signalling (Peng et al., 2016, Yu et al., 2002), localised to lipid rafts during infections (Kowalski et al., 2007), formed aggregates in cells treated with toxic anions (Suprenant et al., 2007) and was found at membrane protrusions (Slesina et al., 2006).

Figure 4.3. Effects of tunicamycin on MVP expression. Tunicamycin treatment affected the level of expression of MVP mRNA and protein. (A) Changes in MVP mRNA level after treatment with 1 μ g/ml tunicamycin for 24 hr. (B) Changes in MVP protein level after treatment with a varying concentration of tunicamycin for different treatment time (O/N=overnight).

The diversity of intracellular localisations of MVP suggests the targeting of MVP to the various intracellular locations by different mediators. IF staining of MVP was carried out on HaCaT treated with 100 ng/ml NaCl, 1 μ g/ml tunicamycin, 100ng/ml doxorubicin and serum starvation (Figure 4.4), and HeLa cells treated with 100 mM NaCl, 1 μ g/ml tunicamycin and serum starvation to study the level and intracellular localisation of MVP (Figure 4.5). In addition, IF provides information on the levels of intracellular MVP. IF analysis showed that cells treated with the various stressors displayed the same punctate cytoplasmic staining observed under basal condition and did not show any observable changes in MVP intracellular levels or distribution. Some nuclear localisation of MVP in HaCaT cells under basal conditions was observed (Figure 4.4 B, C). However,

the factor responsible for this enhanced nuclear localisation in unstimulated HaCaT remains unknown. Some clonal upregulation of MVP protein were also observed in both basal and serum starved HaCaT cells (Figure 4.4 C, L, white arrowhead).

Figure 4.4. HaCaT MVP intracellular localisation is not affected by tested stressors. IF of untreated HaCaT cells (A-C) treated with 100mM of NaCl (D-E), 1 μ g/ml tunicamycin (G-H), serum starvation (J-L) and 100 ng/ml doxorubicin (M-O). (B, C, E, F, H and I) white arrows show nuclear localization of MVP protein. (C and L) clonal

upregulation of cells in Hacat cell cluster (white arrowhead). IF staining using mouse monoclonal anti-MVP antibody and anti-mouse AF488 secondary antibody counterstained with DAPI shows that there no change when cells are treated. Massive upregulation of MVP levels can be seen in some cells. (B, K) Scale bar = 20 μ m

Figure 4.5. HeLa MVP intracellular localisation is not affected by tested stressors. IF of untreated HeLa cells (A-C) treated with 100mM of NaCl (D-E), 1 μ g/ml tunicamycin (G-H), serum starvation (J-L). IF staining using mouse monoclonal anti-MVP antibody and anti-mouse AF488 secondary antibody counterstained with DAPI shows that there no drastic change when cells are treated. Scale bar = 20 μ m

4.2.3. Rab siRNAs regulate MVP intracellular protein level

As finding a stressor that is able to induce observable changes in cellular localisation of MVP proved to be challenging, a complementary analysis looking at factors affecting the localisation of MVP was carried out. MVP has previously been shown to be specifically localised to various compartments of the cells, suggesting the presence of a specific transport mechanism that drives this localisation. Rab proteins are a family of small GTPases which mediates such transports and each Rab protein has a specific subdomain that mediates their specific subcellular compartmentalisation (Schwartz et al., 2007).

Together with our collaborator, the possibility of Rab GTPases being regulators of MVP intracellular distribution was investigated by a siRNA screen. Reverse transfection of HeLa with a library of siRNAs (Appendix Table 1) against Rabs was carried out to determine their impact on MVP protein levels and localisation. PLK siRNA, which results in cell death, was used as positive control for transfection. AlexaFluor 488 fluorescent intensity was reduced only in presence of MVP siRNA but not non-targeting siRNA controls (Appendix Figure 1). To improve the efficiency of siRNA knockdown, SMARTpool, consisting of a pool of 4 siRNAs against each target were used. As a positive control and to determine MVP stability, MVP siRNA which reduced MVP immunoreactivity in HeLa were used. MVP siRNA resulted in a decrease of MVP after 96 hours of incubation, likely due to the long half-life of MVP proteins. The experiments were therefore optimised for a treatment duration of 96 hr and experiment were carried out in quadruplicate. Confocal images of treated wells were captured and unsupervised clustering of the images was carried out using a Gaussian Mixture Model (GMM). The staining patterns were grouped based on various parameters

such as spot density and intensity at various distances from the nucleus. Montages of confocal images from treated cells that gave reproducible differences are shown (Figure 4.6). Treatment with Eight siRNA constructs (Rab6B, Rab17, Rab18, Rab21, Rab25, Rab28, Rab37 and Rab38) led to increased levels of intracellular MVP staining. The use of Rab34 siRNAs (Figure 4.6.C) reduced intracellular MVP levels to levels similar to that of MVP siRNAs (Figure 4.6.B) in both runs. Rab44 siRNA only resulted in a reduction in one of the repeats. The rest of the siRNAs did not lead to any reproducible changes in intracellular localisation of MVP.

Figure 4.6. **Key hits of the Rab siRNA screen.** Montage of IF images of mock-treated HeLa cells (A) and cells treated with siRNA against MVP (B), RAB34 (C), RAB6B (D), RAB17 (E), RAB18 (F), RAB21 (G) RAB25 (H), RAB28 (I), RAB37 (J) and RAB38 (K). Each image is a montage of the 49 images taken from each individual well of treated cells and all images are displayed with intensities based on the same parameters.

4.3. Discussion

The expression of MVP in human (Izquierdo et al., 1996a) and zebrafish (this work) tissues exposed to xenobiotics together with the involvement of MVP in a variety of cellular stress responses (Suprenant et al., 2007, Ryu et al., 2008, Tajitsu et al., 2013, Lee et al., 2017) provides a basis for the hypothesis that biological function of vaults is related to stress responses. Our work with zebrafish also hints at the possibility of MVP being involved in tunicamycin-induced ER stress. Treatment of HaCaT with tunicamycin gave conflicting results when comparing changes in mRNA and protein levels. Although MVP mRNA showed a dose-dependent increase, we saw a reduction in MVP protein levels. On the other hand, the increase in GRP78/BiP protein levels showed the induction of ER stress. During ER stress, the unfolded protein response is induced to protect cells by reducing the accumulation of misfolded protein. GRP78/BiP is a chaperone that is upregulated during ER stress as part of the unfolded protein response and thus used as a marker of ER stress (Lee, 2005). GRP78/BiP binds PERK and IRE-1 under basal conditions and is dissociated under ER stress (Bertolotti et al., 2000). This dissociation is dependent on ADP-ribosylation by ARTC1 (Fabrizio et al., 2015). Activation of PERK inhibits further mRNA translation by phosphorylating eIF2a (Harding et al., 2000). Phosphorylation of eIF2a prevents its exchange of GDP to GTP, mediated by eIF2B, preventing the formation of new eIF2-GTP-tRNA^{Met} complexes (Sonenberg and Hinnebusch, 2009), preventing translation initiation. IRE1, another ER-bound ER stress sensor also gets activated and degrades mRNAs, especially those that are ER-localised (Hollien and Weissman, 2006). A possible explanation is the preferential degradation of the beta-actin mRNA during ER-

stress resulting in the perceived increase in MVP mRNA. Subsequent observation of the extracellular release of MVP (see next chapter) suggests the extracellular release of MVP upon exposure to tunicamycin as an alternative explanation for the reduced levels of MVP.

Despite the strong upregulation of MVP in SW620 cells when exposed to hyperosmotic conditions (induced by NaCl and sucrose) (Ikeda et al., 2008), no difference was observed when HeLa and HaCaT cells were treated with 100 mM NaCl. This suggests that the upregulation of MVP might be cell type-specific. SW620 was demonstrated to have a very low amount of MVP expression at the basal state with strong induction of MVP through the activation of its promoter activity (Ikeda et al., 2008). This was later shown to be dependent on the binding of Sp1 (Tajitsu et al., 2013) and USF1 (Ikeda et al., 2014) transcription factors. Sp1 is a transcriptional activator involved in stress responses (Wang et al., 2002, Ryu et al., 2003) and was thought to be responsible for the basal expression of MVP (Steiner et al., 2004). A possible explanation is the binding of Sp1 to the promoter regions of MVP in HeLa and HaCaT cells under basal conditions. Thus, when osmolarity was further increased, there was no further activation of the already bound MVP promoter sequence. Alternatively, there could be stringent transcriptional regulation of MVP transcription which could be cell type or cell line specific. The differences in the regulation and basal levels of MVP between the cell lines used in our work compared to those used in the reported studies could explain the contradictory results observed in vault biology. Treatment of additional stressors such as H₂O₂ to induce oxidative stress, and serum starvation also did not lead to any significant changes in intracellular localisation or levels of MVP.

MVP was shown to accumulate within intracellular location such as the nucleus, membrane ruffles and lipid rafts (Suprenant et al., 2007, Slesina et al., 2005, van Zon et al., 2003, Herrmann et al., 1999), yet little is known about the mode of transport of MVP (Li et al., 1999, Slesina et al., 2006). Thus, possible mechanisms that could regulate MVP localisation were investigated. Presence of MVP in exosomes (Teng et al., 2017, Statello et al., 2018), lipid rafts (Kowalski et al., 2007) and plasma membrane (Lee et al., 2017), suggests that some of the vaults particles could be transported in vesicles. A Rab GTPases siRNA screen was chosen to investigate the involvement of Rabs in MVP intracellular localisation. Rab GTPases, the largest family of small GTPases, are found on specific intracellular membranes (Schwartz et al., 2007, Pereira-Leal and Seabra, 2001) where they regulate the trafficking of membrane-bound vesicles (Stenmark, 2009) between cellular compartments. Rab GTPase activity is dependent on conformational changes induced by the binding of either GDP or GTP (Pfeffer, 2005). Their specific localisation (Chavrier et al., 1990) allows for the identification of the exact compartment of the vesicle trafficking pathway if they are shown to affect MVP localisation. From our siRNA screen, no difference in the intracellular distribution of MVP was observed. However, treatment of cells with Rab34 siRNA resulted in the downregulation of MVP. Rab34 is involved in macropinosomes formation (Sun et al., 2003), phagosome maturation and fusion with lysosomes (Kasmapour et al., 2012), lysosome positioning (Wang and Hong, 2002) and integrin $\beta 3$ recycling (Sun et al., 2018a). Interestingly, Rab34 has been proposed as a prognosis marker for gliomas (Wang et al., 2015) and overexpression of Rab34 was associated with tumour progression in hepatocellular carcinoma (Wu et al., 2017). This is similar to MVP

as MVP too has been linked to treatment resistance in brain tumours (Berger et al., 2001, Sasaki et al., 2002, Ramani and Dewchand, 1995), NSCLCs (Dingemans et al., 1996) and osteocarcinoma (Uozaki et al., 1997). Expression of constitutively active Rab34 increased the migratory ability of HeLa cells through localisation to membrane ruffles induced by EGF (Sun et al., 2018a). Similarly, MVP has been shown to interact with the EGF signalling pathway to increase migration of glioblastoma (Lotsch et al., 2013). These suggest that Rab34 modulation of MVP levels as a mechanism for the above observation. Rab34 could additionally be involved in the observed uptake of MVP when exogenous recombinant vaults are added to HeLa cells (Kickhoefer et al., 2005). Nine Rab siRNAs (Rab6B, Rab17, Rab18, Rab21, Rab25, Rab28, Rab37 and Rab38) led to an increase in intracellular MVP levels. Rab6B is involved in retrograde transport from Golgi to ER (Opdam et al., 2000). Rab17 is localised to the apical recycling endosomes of epithelial cells (Zacchi et al., 1998) and regulates transcytosis (Hunziker and Peters, 1998). Rab17 was also involved in melanosome trafficking and release from cells (Beaumont et al., 2011). Rab18 is involved in retrograde transport of Golgi vesicles to ER (Dejgaard et al., 2008) and inhibits the secretion of vesicles in neuroendocrine cells (Vazquez-Martinez et al., 2007). Rab21 is found on early endosomal vesicles (Simpson et al., 2004) and is involved in the internalisation of $\beta 1$ integrin (Pellinen et al., 2006) and gamma secretase to late endosomes/lysosomes (Sun et al., 2018b). Rab25 regulates recycling and apical trafficking of vesicles to the plasma membrane (Casanova et al., 1999) and displayed both oncogenic (Mitra et al., 2016) and tumour suppressing activities (Tong et al., 2012). Rab28 regulates NF κ B nuclear transport (Jiang et al., 2013) and the turnover of endocytosed protein through

targeting to lysosomes (Lumb et al., 2011). Rab37 is involved in the secretion of TIMP1 (Tzeng et al., 2017), TNF α (Mori et al., 2011), regulation of autophagosome formation (Song et al., 2018, Sheng et al., 2018) and is localised to secretory granules in mast cells (Masuda et al., 2000). Rab38 is involved in melanogenesis through regulation of TYRP1 transport to melanosomes (Loftus et al., 2002, Wasmeier et al., 2006) and is involved in lamellar bodies secretion in alveolar cells (Osanai et al., 2008).

One common function of the nine Rab GTPases that upregulate MVP levels is involved in protein secretion, lysosomal targeting and phagosome activities. The secretory activities of these Rab GTPases could explain the presence of MVP in secreted exosome (Teng et al., 2017, Statello et al., 2018) and MVP release by the protozoan *Raphidiophrys contractilis* to capture food (Bhadra et al., 2017). The secretion of MVP could potentially be modulated by the above-mentioned Rab GTPases and the lysosome targeting of these Rab GTPases suggests a possibility of MVP turnover by the lysosomal degradation pathway.

4.4. Conclusion

In conclusion, we did not see any ubiquitous response of MVP to mediators of cellular stress. This suggests that any observations previously described were most likely restricted to specific cell types or specific conditions. The siRNA screen against Rab GTPases suggests the involvement of Rab GTPases in the intracellular transport and localisation of MVP. Rab GTPases might also be responsible for the targeting of MVP into the extracellular space. However, further experiments have to be carried out to validate the role of Rab GTPase in governing MVP intracellular transport and localisation.

Chapter 5: Vaults are implicated in innate immunity

5.1 Introduction

The first major finding of MVP being involved in immune response the rapid recruitment of MVP to the lipid rafts of cells in a CFTR-dependant manner when lung epithelium cells were infected with *P. aeruginosa*. MVP knock-out resulted in reduced clearance of *P. aeruginosa* from the lungs of mice, leading to increased bacterial load and mortality in knockout mice (Kowalski et al., 2007). Subsequent *Mvp*^{-/-} mice nasal infection with influenza A virus also showed increased susceptibility. However, in the case of influenza, MVP was shown to facilitate nuclear transport of transcription factor c-Fos and C/EBP β , resulting in the activation of *IL6* and *IL8* promoter (Peng et al., 2016)

In HBV (Liu et al., 2014) and HCV (Liu et al., 2012) infected patients, the levels of MVP in the peripheral blood mononuclear cells and sera were found to be upregulated. This upregulation was found to be dependent on type-I IFN signalling and hampered viral replication via downstream signalling. When cells were infected with HBV, MVP interacts with MyD88 and acts as a signalling scaffold protein. Interestingly, HBV antigens were able to compete with Myd88/MVP interaction (Liu et al., 2014). Together, these observations led to the hypothesis of MVP being involved in host immunity. With this in mind, activators of innate immune response were tested to investigate their ability to affect the level or localisation of MVP. PAMPs activate the immune system through binding to PRRs. This binding induces downstream signalling, resulting in either the release of antimicrobial molecules, chemokines and cytokines or the induction of cellular activities that prevent the propagation of intracellular pathogens such as degradation of non-self DNA or induction of cell death. TLRs

are the first class of PRRs to be discovered and thus are the best characterised. In humans, there are 10 known members of TLRs found on both the cell surface and also on intracellular compartments. With the exception of TLR10, the other ligands of the TLRs have been identified and characterised (reviewed in (Kawasaki and Kawai, 2014)). The well-established molecular mechanism of the TLR pathways, together with the availability of specific activators and inhibitors, makes it an attractive starting point to study the role of vaults in innate immune responses.

5.2. Results

5.2.1 MVP levels increased in the presence of LPS in THP-1 cells

As no drastic changes in localisation were seen in our previous analysis with HaCaT, qPCR analysis of MVP gene was used to measure the response of MVP to challenges. THP-1 was used instead of HaCaT cells as THP-1 cells are professional immune cells and might be more responsive to PAMPs. THP-1 cells were treated with PAMPs, specific activators of the innate immune system and changes in MVP mRNA levels were characterised (Figure 5.1). Addition of TLR agonists Pam3CSK4, HKLM, poly I:C (HMW) and FSL, which activates TLR1/2, TLR2, TLR3 and TLR6/2 respectively, to THP-1 for 24 hours led to an increase in *MVP* mRNA expression. Western blot analysis on the protein lysates from treated cells also detected an increase of MVP protein by 1.29-, 1.06- and 2.87-fold in the cells with Pam3CSK4, HKLM and LPS respectively. Amongst the TLR agonists tested, LPS which binds and stimulates TLR4 activation led to the most drastic induction of MVP at the mRNA level (approximately 6-fold) and

protein level of MVP (2.87-fold).

Figure 5.1. **MVP expression is induced by LPS in THP-1.** (A) mRNA expression levels in THP-1 cells treated with various TLR agonists. (B) Western blot analysis of intracellular MVP protein levels. Fold changes based on densitometry analysis of the bands as a ratio with respect to β -tubulin was used.

5.2.2 MVP interacts extracellularly with *E.coli*

With the observation that addition of LPS led to upregulation of MVP, we hypothesised that MVP is involved in anti-bacterial responses of cells. As the loss of MVP was previously shown to affect the capacity of lung epithelial to phagocytose *P. aeruginosa* (Kowalski et al., 2007). The impact of bacteria on MVP localisation and levels within cells was investigated. When cells were co-cultured with *E. coli* for an hour, no observable changes in the intracellular localisation was seen. Surprisingly, an extracellular colocalisation of MVP antibody signal with the bacteria cell was constantly observed (Figure 5.2). In the absence of human cells, this immunoreactivity on *E. coli* was absent.

Figure 5.2. **Extracellular interaction of MVP with *E. coli*.** (Top) 400X magnification confocal images of HaCaT co-culture with *E. coli*. Insert at the bottom right of each image is a magnification of white box. Scale bar = 50µm. (Bottom) 1000X magnification IF confocal images of THP-1 co-culture with *E.coli* stained with two different antibodies. Bacteria are visualised by DAPI staining. Scale bar = 10µm.

5.2.3. Blocking with serum led to enhanced immunoreactivity

As MVP was previously reported to be present in human serum in the range of (~ 50-150 ng/ml) (Liu et al., 2012), the contribution of MVP from serum was investigated. To our surprise, a higher intensity of MVP immunoreactivity on bacteria was observed when samples were blocked with sheep serum compared to blocking of samples with BSA (Figure 5.3). Due to the high levels of sequence conservation (Daly et al., 2013b), there exists a possibility that the serum MVP might contribute to the increased signal observed or there is a component in serum that binds non-specifically to bacteria. To prevent the presence of serum factors from giving a false positive readout, all experiments were carried using serum-free media. This observation of increased intensity of MVP antibody staining on bacteria also led to a hypothesis that MVP in serum could act as a bacteria binding molecule.

Figure 5.3. Blocking of slides with animal serum increased MVP IF signal. 400X magnification confocal images of HaCaT co-culture with *E. coli*. Slides were blocked with either BSA (Top) or goat serum (Bottom). Scale bar = 20 μ m.

5.2.4. MVP in serum binds bacteria as a 50 kDa fragment

With the previous result of MVP co-localising with extracellular bacteria, experiments were carried out to test the hypothesis that MVP in serum could be a novel bacteria recognising/binding molecule. Using bacteria as bait, the pulldown of human serum proteins by bacteria was carried out. Proteins eluted from the bacteria pellet were tested for the presence of MVP by western blot (Figure 5.4). Full-length MVP was never detected by western blot. Instead, a truncated MVP fragment, approximately 50 kDa in size, was detected. This 50 kDa was not observed when PBS was used instead of plasma, eliminating the possibility of the band being a non-specific recognition of bacterial protein.

Figure 5.4. **Plasma protein pulldown with bacteria.** Western blot analysis of plasma protein pulldown by different bacteria

The presence of this 50 kDa band suggests the proteolytically processing of serum MVP when exposed to bacteria or the presence of endogenous truncated MVP in the human serum. Using an online bioinformatics platform, ExPASy peptide cutter (Wilkins et al., 1999), the prediction of thrombin digestion sites

and digestion products were carried out. Thrombin was predicted to cut after Arg452 and Arg621, this results in the generation of three fragments of 51 kDa, 12 kDa and 29 kDa in size. When the recombinant vault was digested with thrombin *in vitro*, two bands of around 50 kDa (Figure 5.5); no 12 kDa or 39 kDa fragments was observed. The efficiency of thrombin digestion of recombinant vault is low. Overnight digestion at 37°C was insufficient to complete digestion all MVP, resulting in most of the MVP remaining intact at 100 kDa.

Figure 5.5. **Thrombin digestion of MVP.** Coomassie blue staining of SDS-PAGE analysis of thrombin digestion of recombinant vaults shows the degradation of MVP into 2 fragments (yellow arrowhead). The black arrowhead is recombinant thrombin.

In order to decipher the identity of the 50 kDa band pulled down by *S. aureus*, Mass spectrometry analysis of the 50 kDa band was carried out. Eluted proteins from bacteria pulldown were subjected to SDS-PAGE and the gel from the 50 kDa region was excised and subjected to mass spectrometry analysis. Mass spectrometry analysis detected three unique peptides which correspond to human MVP aa 307-335, 512-536 and 657-674 respectively (Figure 5.6). However, the

fragments detected were low in abundance, resulting in poor confidence in the peptide identity. Intriguingly, the peptides are found on all three fragments predicted to be generated by the two thrombin cut sites, suggesting that thrombin is not the enzyme responsible for the proteolytic processing of MVP.

Gel Idx/Pos		309/M11		Instr./Gel Origin		ak072/170918		Process Status <td colspan="2">Analysis Succeeded</td>		Analysis Succeeded	
Plate [#] Name		[1] mw1		Instrument Sample Name				Spectra <td colspan="2">11</td>		11	
Rank	Protein Name	Species		Accession No.		Protein MW		Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion Confirmed C. I. %
1	Major vault protein OS=Homo sapiens GN=MVP PE=1 SV=4			sp Q14764 MVP_HUMAN		99551		10	77.611		.T.
Peptide Information											
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. %	Modification	Rank	Result Type
	1873.9866	1873.7427	-0.2439	-130	657	674 SVQLAIEITTSQEAAAK					Mascot
	1873.9866	1873.7427	-0.2439	-130	657	674 SVQLAIEITTSQEAAAK					Mascot
	2844.4712	2844.1633	-0.3079	-108	512	536 RALCLLLGPDFFTDVITIE TADHAR			Carbamidomethyl (C)[4]		Mascot
	2844.4712	2844.1633	-0.3079	-108	512	536 RALCLLLGPDFFTDVITIE TADHAR			Carbamidomethyl (C)[4]		Mascot
	3334.7317	3334.3191	-0.4126	-124	307	335 SFFLPGEQLEQGIQDVY VFSEQGLLLR					Mascot
	3334.7317	3334.3191	-0.4126	-124	307	335 SFFLPGEQLEQGIQDVY VFSQDGLIIR					Mascot

Figure 5.6. **MVP peptides detected from 50 kDa band.** Mass spectrometry analysis of excised gel from the 50 kDa band region detected only three distinct peptides from human MVP.

5.2.5 MVP is released into extracellular space in response to microbial antigens

The ability of MVP to bind bacteria extracellular, coupled with the 50 kDa serum MVP fragment pulldowned by bacteria, suggest that MVP bound to extracellular bacteria could be a truncated form of MVP. Determination of the length of MVP released when exposed to bacterial antigen was therefore investigated. Western blot analysis of proteins secreted by mammalian cells when exposed to bacteria conditioned media detected the presence of 100 kDa full-length MVP. The 100 kDa MVP band was sometimes observed in the untreated cell culture supernatant. Interestingly, a strong band of approximately 50 kDa band was detected by western blots (Figure 5.7 A). This suggests a proteolytic cleavage of MVP in presence of bacteria-conditioned media. To determine if the production of the 50 kDa band is due to bacteria protease activity, bacteria conditioned media was

heated at 95°C for 30 mins prior to addition to cells (Figure 5.7 B). Heat treatment abrogated the production of the 50 kDa MVP reactivity band. Interestingly, the drastic reduction of 50 kDa band in heat-treated bacteria conditioned media did not lead to an increase in 100 kDa full-length MVP.

Figure 5.7. **Extracellular release of MVP.** EC, SE and SA conditioned media induced release of a 50k Da MVP band. (A) Western blot MVP on supernatant and cell lysate of THP-1 cells treated with various bacteria conditioned media. (B) Preheating of bacteria conditioned media for 30 mins at 95°C reduced the amount of 50 kDa in the supernatant.

5.3. Discussion

Upregulation of MVP upon exposure to bacterial antigen LPS was detected at both mRNA (approximately 6-fold) and protein levels (2.87- fold) (Figure 5.2). This upregulation suggests the involvement of MVP in the innate immune response towards bacteria pathogen. The upregulation observed is in line with recently described involvement of MVP in innate immune responses. MVP protein level in both PBMC and plasma of HBV (Liu et al., 2014) and HCV (Liu et al., 2012) infected patients were upregulated. MVP also improved the survival of DCs when activated by LPS and TNF- α (Schroeijers et al., 2002). Thus far, the role of MVP in immune responses have been in the regulation of signalling pathways. Upregulation of MVP expression by IFN- γ signalling during influenza A virus infection resulted in enhanced nuclear localisation of cFos transcription factor (Steiner et al., 2006, Peng et al., 2016). IFN- γ induced MVP was also shown to interfere with IFN- γ activated JAK/STAT, forming a negative feedback loop. (Steiner et al., 2006). Interestingly, when mammalian cells were treated with *E. coli*, a novel extracellular interaction between MVP and *E. coli* was observed. MVP IF signal on bacteria was greatly increased when animal serum was used in the experimental procedure, supporting that the previous report of MVP in serum (Liu et al., 2015). However, in order to prevent a false positive due to MVP in serum, all experiments shown was carried out under serum-free conditions and the blocking of cells was achieved by BSA. This also led to the hypothesis that MVP is found in serum and is able to bind bacteria.

As MVP was demonstrated to be present in plasma (Liu et al., 2012, Liu et al., 2014), we tested the ability of MVP in the plasma to interact with bacteria. Western blot analysis of the proteins pulldowned by bacteria did not detect any

full length 100 kDa MVP. Surprisingly, only an approximately 50 kDa band was detected by western blot, suggesting either MVP is found in the blood as truncated fragments or there is proteolytic processing of vaults when exposed to bacterial antigens. The absence of the 50 kDa band when bacteria were incubated with PBS instead of human serum proves that the 50 kDa band is not due to cross-reactivity against a bacterial antigen. An inconsistent presence of the 50 kDa MVP band was sometimes observed when *E. coli* were used to pulldown serum protein (data not shown). This might be due to cross-contamination of bacteria samples or the presence of the binding target of the 50 kDa band in *E. coli* could be specific to growth stages.

In order to gain more insights into the identity of the 50 kDa band, mass spectrometry analysis was carried out. Mass spectrometry detected only three MVP fragments with low confidence scores due to the low abundance of these fragments. This low abundance of MVP fragment is most likely due to the presence of abundant bacteria proteins in the sample. The issue of low abundance might also be further complicated by sample preparation for mass spectrometry. Trypsin is commonly used to digest proteins into peptides before mass spectrometry analysis. Trypsin cuts after positively-charged amino acids (lysine and arginine) and 12% of amino acids in human MVP is lysine and arginine. This gives an average of 1 trypsin digestion every eight amino acids, potentially leading to small peptides which might be hard to detect.

The three MVP fragments detected span the region from aa 307-674 and calculation of the mass of this aa 307-674 region resulted in a mass of 42.5 kDa, which is smaller than the observed mass. The two predicted thrombin cut sites, at Arg452 and Arg621, are located between the three fragments detected by mass

spectrometry. Peptide fragments predicted for thrombin digestion of MVP would be aa 1-452, aa 452-621, aa 621-893, giving a mass of 51 kDa, 12 kDa and 29 kDa respectively. Possible reasons for this discrepancy are differential digestion of MVP by human and bacterial proteases, physiological host digestion of serum MVP is mediated by other proteases and the *in silico* prediction of thrombin might not reflect the physiological activity of thrombin on MVP. Furthermore, the *in vitro* digestion of vaults by thrombin in two smaller fragments of MVP in the range of 50 kDa suggests that thrombin cuts MVP at only one site. However, due to the low abundance and confidence scores of the detected MVP fragment, there exists a possibility that the detection of fragments is not accurate. In order to obtain a more accurate analysis of MVP in serum, depletion of highly abundant serum proteins such as albumin and IgG might result in a sample that would allow for the detection of more MVP peptides.

In line with the observation of proteolytically processed MVP in serum, the presence of a 50 kDa fragment of MVP was also detected in the supernatant of mammalian cells treated with bacteria-conditioned media. When bacteria conditioned media was added to mammalian cells, a slight increase in the full-length MVP and a massive amount of a 50 kDa MVP band were constantly detected by western blot. Possible explanations for the low amount of full-length MVP in the mammalian cell supernatant cell death due to bacteria conditioned media, however, this has to be validated further. Possible reasons are that the 50 kDa MVP fragment is more immunogenic or MVP is released from the cell membrane into the extracellular space. MVP localises to the cell membrane under serum starvation (Lee et al., 2017), and is found in lipid rafts (Kowalski et al., 2007). The RIPA buffer used for cell lysis might not be efficient in the

extraction of membrane-bound protein. Assuming that MVP is indeed localised to the cell membrane, RIPA might lead to a low yield of full-length membrane-bound MVP. Once MVP is cleaved, they are readily released into the supernatant and the released MVP fragments then precipitated by the acetone precipitation method resulting in a higher yield. Alternatively, there could be an upregulation of MVP at the transcript level. However, it is hard to imagine that the induction of mRNA expression is responsible for such a massive amount of the 50 kDa form of MVP. Experiments were only carried out for an hour and this time frame might be too short for the transcription, translation and cleavage of full-length MVP into the shorter fragment. Analysis of mRNA levels and inhibition of transcription by actinomycin (Sobell, 1985) would determine if the presence of the 50 kDa MVP band is due to MVP transcription. Finally, there might be alternative slicing of MVP mRNA to produce the smaller fragment, but, there is no evidence thus far for the alternative splicing of MVP mRNA.

Reduction of the 50 kDa band after heat treatment of bacteria conditioned media suggests cleavage of MVP by bacteria protease in the conditioned media. Previously, MVP was found to translocate to the cell-surface under stressful environments (Lee et al., 2017). This might expose MVP to bacteria proteases in the conditioned media such as V8 proteases (Drapeau, 1976), aureolysin (Banbula et al., 1998) and staphopains (Dubin, 2002). Another possibility is the presence of a heat labile antigen in bacteria conditioned media which led to the induction of proteolytic cleavage of MVP by endogenous proteases, followed by its release into the extracellular space. During the revision of this thesis, it was reported that caspase-1 cleaved MVP at asp441 (Grossi, 2018). This cleavage at asp441 gives an N-terminal fragment of 49.9 kDa and a C-terminal fragment of

49.4 kDa which is similar in mass to the 50 kDa we observed. Caspase-1 is part of the inflammasome and can be activated by microbial antigens. Activated caspase-1 cleaves Pro-IL1 β into the mature IL1 β to initiate immune responses (review in (Franchi et al., 2009)). Together, the data suggest that MVP could be proteolytically modified and this proteolytic product might be able to interact with bacteria cells. However, how this interaction occurs and the outcome of such interactions remains unknown. Further experiments using protease inhibitors or protease-deficient strains of bacteria would determine proteolytic cleavage is due to bacterial proteases. Further studies on the impact of MVP interaction with *S. aureus* and the impact of the degradation of MVP would allow a better understanding of MVP function and vault biology. Interestingly, MVP was taken up by cells through clathrin-dependent endocytosis (Galbiati et al., 2018). Together with our results of Rab34 siRNA, there exists strong evidence of constant MVP release and uptake.

5.4. Conclusion

In this chapter, the ability of MVP to response to bacterial antigens was demonstrated. LPS led to an increase in MVP at both mRNA and protein levels. MVP did not show any changes in localization when exposed to bacterial antigens but MVP was found to bind *E. coli* extracellularly. This extracellular MVP could possibly be a truncated 50 kDa that is released by cells. A 50 kDa MVP band was isolated from serum when *S. aureus* was used to pull down serum proteins, suggesting a role of MVP as a bacteria opsonizing molecule. More work has to be carried out to determine if the 50 kDa bands in serum and in mammalian cell supernatant are identical. However, there are signs that MVP is proteolytically processed in response to pathogens. This suggests a differential

regulation for the release of MVP or truncation of MVP by various bacteria/host proteases and might be a novel form of host-pathogen response. More work has to be done to decipher the mechanism driving the observations reported.

Chapter 6. MVP binds exopolysaccharide Psl of *P.*

aeruginosa

6.1 Introduction

During our investigation on MVP function, an extracellular interaction of MVP with *E. coli* was observed. To investigate if this interaction is specific to *E. coli*, another gram-negative bacteria, *Pseudomonas aeruginosa*, were tested. *P. aeruginosa* was chosen as MVP was previously shown to mediate host resistance to *P. aeruginosa* lung infection (Kowalski et al., 2007). MVP enhanced the epithelial cell internalisation of *P. aeruginosa* through the recruitment of CFTR to lipid rafts. *P. aeruginosa* is a versatile gram-negative bacteria which thrives in varied environments. It is clinically important as it is one of the most common causes of nosocomial infections and it can cause disease in a healthy individual (Obritsch et al., 2005). *P. aeruginosa* causes a plethora of infections including urinary tract infections (Mittal et al., 2009), wound infections (Serra et al., 2015), lung infections, and dermatitis (Silverman and Nieland, 1983). In cystic fibrosis patients, *P. aeruginosa* is the most common cause of chronic pulmonary infection, with a prevalence of up to 80%. *P. aeruginosa* can colonise various surfaces in the hospital including sinks, railing, contaminated fruits and epithelial surfaces of healthy individuals, making it impossible to remove from the hospital environment. *P. aeruginosa* is intrinsically resistant to some antibiotics and can rapidly develop resistance to different antibacterial agents (Lister et al., 2009). This antibiotic resistance is in part due to the formation of biofilms. Production of extracellular matrix (ECM) driving biofilm formation is one strategy *P. aeruginosa* employs to provide itself with a survival advantage by protecting the

bacteria from environmental stresses (DeVault et al., 1990, Pier et al., 2001) and host immune system (Jones and Wozniak, 2017). Biofilm ECMs consist of proteins, polysaccharides and extracellular DNAs and together they help in the formation and stabilisation of biofilms.

Figure 6.1. **Structures of Alginate and Psl polysaccharide.** (A) Alginate is composed of D-mannuronic acid and L-guluronic acid residues. Acetylation occurs at random D-mannuronic acid, resulting in a random structure for alginate. (B) Psl polysaccharide is composed of a repeating pentamer consisting of D-mannose, L-rhamnose, and D-glucose residues. (Adopted from Franklin et al, 2011)

The best-studied components of *P. aeruginosa* ECM are the three exopolysaccharides, alginate, Psl and Pel. The first exopolysaccharide discovered was alginate, a polysaccharide with nonrepetitive β -1,4 linked D-mannuronic and L-guluronic acids monomers (Evans and Linker, 1973). Alginate is a structural polysaccharide that scavenges free radicals (Simpson et al., 1989) and has strong water retention properties (Sutherland, 2001) to protect bacteria

from desiccation. Alginate producing strains are often found in patients with cystic fibrosis and overproduction of alginate is due to mutations in MucA, resulting in slimy looking colonies of bacteria isolated from CF patients. MucA sequesters the sigma factor driving alginate production and mutations in MucA results in the activation of alginate production by the sigma factor (Martin et al., 1993). However, most environmental *P. aeruginosa* isolates predominantly express either Psl or Pel (Friedman and Kolter, 2004b).

Pel is the polysaccharide responsible for pellicle formation in *P. aeruginosa* strain, PA14 (Friedman and Kolter, 2004a). Its structure is currently unknown but is proposed to be a cationic polysaccharide consisting of partially acetylated galactosamine and glucosamine residues linked by 1,3 glycosidic linkages (Jennings et al., 2015). Pel serves a structural role by mediating cell-to-cell interaction in PA14 biofilms and also protects encapsulated bacteria from antibiotics (Colvin et al., 2011). Pel is also important for the initial attachment and colonization of abiotic surfaces (Vasseur et al., 2005).

Psl is a neutral, branched polysaccharide that consists of repeating pentamers of D-mannose, L-rhamnose, and D-glucose residues linked by β -1,3 glycosidic linkages (Figure 6.1) (Byrd et al., 2009). Psl is found in two forms, a high molecular weight membrane-bound form and a low molecular weight released form (Byrd et al., 2009). In planktonic bacteria, Psl forms a helical pattern that surrounds the surface of *P. aeruginosa* (Ma et al., 2009) and aids in cell-cell contact and adhesion (Ma et al., 2006). Psl can also be secreted and laid down in trails through a T4P-dependent mechanism (Wang et al., 2013) to guide and recruit planktonic bacteria to form micro-colonies (Zhao et al., 2013). As micro-colonies start developing into biofilm, Psl provides structural support (Colvin et

al., 2012, Ma et al., 2012) and the aforementioned protection from stress and host immune response. Based on our previous observation of an extracellular interaction of MVP with *E. coli* and the interaction of MVP with *P. aeruginosa* in lung infection model (Kowalski et al., 2007), we hypothesise that extracellular MVP is also able to bind to *P. aeruginosa*. The study of MVP interaction with *P. aeruginosa* might further explain the protective function of MVP against *P. aeruginosa* lung infection. Exploitation of such a mechanism might possibly lead to better therapeutics against *P. aeruginosa* infection and protect CF patients from *P. aeruginosa* infections.

6.2. Results

6.2.1. MVP interacts with *P. aeruginosa*

Understanding host interaction with *P. aeruginosa* could lead to greater understanding of the immune response towards *P. aeruginosa* and how *P. aeruginosa* circumvents the immune effectors. PAO1 was used to investigate the possible interaction of MVP with *P. aeruginosa*. PAO1, a clinical isolate turned laboratory strain, is well-characterised with published genome sequence (Stover et al., 2000). To aid in its observation, wild-type PAO1 was transformed with a mCherry-expressing plasmid driven by the Trc promoter (Addgene plasmid # 62550). Transformed PAO1 were plated onto agar plates with ampicillin as a selection marker. However, since the PAO1 used was intrinsically resistant to ampicillin, it resulted in a lawn of non-transformed bacteria harbouring few mCherry-positive clones. The mCherry-positive area was picked and serially inoculated on to new plates with ampicillin until individual mCherry-positive PAO, namely 1 colony can be isolated (Figure 6.2). One such colony was picked and designated as PAO1-mCherry.

Figure 6.2. **PAO1-mCherry agar plate colony.** Agar plate showing colonies of mCherryPAO1.

To investigate the ability of MVP to bind PAO1, differentiated THP-1 cells were challenged with live PAO1-mCherry at a MOI of 5 and analysed by IF microscopy (Figure 6.3). Experiments were carried out either in the absence and presence of 1 μ g/ml of recombinant MVP. IF staining with both anti-MVP antibodies resulted in colocalisation of both antibodies in fibril-like connections between individual bacteria (Figure 6.3.E-F, H, H'). Addition of 1 μ g/ml purified recombinant human vaults led to a drastic increase in signal intensity of these fibrils (Figure 6.3 I-J, L), further confirming the specificity of antibodies against MVP. These MVP fibrils have not been reported in the literature and their unique staining pattern and close association to PAO1-mCherry suggest a *P. aeruginosa*-dependant determinant for the observed patterning. Interestingly, the observed MVP fibrils were similar to the web-like radial pattern of HHA-FITC stained Psl-fibres (Wang et al., 2013). This led to the hypothesis that MVP interacts with Psl or related ECM component of *P. aeruginosa*.

Figure 6.3. **MVP forms extracellular fibres with PAO1.** Addition of PAO1 to THP-1 cells leads to extracellular localisation of MVP with bacteria (H and H'). This interaction forms fibres joining individual bacteria. No fibres were observed in the absence of bacteria cells (A-D). Addition of recombinant vaults to the media resulted in an increase in IF staining intensity of the fibres (I-L). Scale bar is 20 μ m.

6.2.2. MVP interacts with Psl of *P. aeruginosa*

To test the hypothesis that MVP interacts with Psl, differentiated THP-1 cells were co-cultured with wild-type PAO1, Psl-deficient strain (Δ PslBCD) and T4P-deficient strain (Δ PilA) (Figure 6.4). T4P is responsible for deposition of the Psl trail, thus Δ PilA cells would have normal production of Psl but do not deposit Psl fibres on surfaces (Wang et al., 2013). Recombinant vaults were added to investigate if the interaction of MVP and Psl occurs in a dose-dependent manner. Using IF, an extracellular fibrillar staining pattern was observed with wild-type PAO1 (Figure 6.4., row 2). This staining was observed in both the absence and presence of recombinant vaults showing that exogenous MVP is able to bind Psl. Addition of recombinant vaults resulted in a higher fluorescent intensity of the fibrils. In the Δ PilA and Δ PslBCD treated cells, fewer bacteria were bound to the glass coverslips. This is consistent with the role of Psl and T4P in adhesion of

bacteria to surfaces. In Δ PilA mutants, extracellular MVP was associated with the bacteria cell but MVP fibres were absent. Addition of recombinant vaults did not lead to extracellular staining of Δ PslBCD mutants. When the experiments were repeated with HaCaT cells and the various *P. aeruginosa* strains, similar staining patterns were observed (Figure 6.5). Additionally, when PA14, a Psl-deficient strain which uses Pel for biofilm formation (Colvin et al., 2011), was incubated with recombinant MVP, colocalisation of MVP with bacteria was observed (Figure 6.6). Altogether, these data suggest that the MVP binds specifically to Psl and this interaction is a conserved mechanism that is not limited to specific cell types.

Figure 6.4. **Co-culture of THP-1 cells with PAO1.** (Previous page) IF analysis of THP-1 cells co-cultured with WT and mutant *P. aeruginosa* show that MVP (red) bind to both secreted and cell-bound Psl polysaccharide of *P. aeruginosa*. No colocalisation was observed in Psl deficient Δ PslBCD mutants. Type 4 pili deficient Δ PilA which is unable to secrete Psl shows MVP localisation only on the bacteria. DNA was counter-stained with DAPI (green). Scale bar is 20 μ m.

Figure 6.5. **Co-culture of THP-1 cells with PAO1.** IF analysis of THP-1 cells co-cultured with WT and mutant *P. aeruginosa* show that MVP (red) binds to both secreted and cell-bound Psl polysaccharide of *P. aeruginosa* (B and B'). Type 4 pili deficient $\Delta\PilA$ which is unable to secrete Psl shows MVP localisation only on the bacteria (C and C'). No colocalisation was observed in Psl deficient $\Delta\PslBCD$ mutants (D and D'). DNA was counter-stained with DAPI (blue), keratin (green), B', C' and D' are magnified images of inset area of B, C and D respectively. Scale bar is 20 μm .

Figure 6.6. **Anti-MVP staining of PAO1 and PA14.** Anti-MVP antibody staining of PAO1 (first and second row) and PA14 (third and fourth row) show that immunoreactivity of antibody is only seen when vaults are added to PAO1 (second row). No immunoreactivity is observed when only PAO1 is stained. (First row). PA14, both in the absence (third row) and presence of vaults (Fourth row), also did not show any immunoreactivity towards anti-MVP antibody staining. Scale bar is 10 μ m.

6.2.3. MVP does not affect the growth of *P. aeruginosa*

Having shown the interaction between MVP and Psl of *P. aeruginosa*, experiments were carried out to understand the impact of the MVP interaction with Psl. Impact of MVP on the growth rate of the various *P. aeruginosa* strains,

PAO1, PAO1 Δ pilA and Δ PslBCD, was investigated by monitoring of OD₆₀₀ absorbance over a period of 16 hours in LB media, with varying levels of recombinant vaults (Figure 6.7.). Analysis of growth kinetics did not show any significant difference between control LB media and up to 10 μ g/ml of recombinant MVP treated *P. aeruginosa*. This suggests that MVP does not have any bacteriostatic or bactericidal effects at concentrations up to 10 μ g/ml.

Figure 6.7. **Growth kinetics of *P. aeruginosa*.** Growth kinetics of PAO1 (A), Δ pilA (B) and Δ pslBCD (C) grown in 96-well plates with varying concentration of MVP from 0.125-1 μ g/ml measured by OD₆₀₀ did not show any effect of MVP on their growth kinetics.

6.2.4. MVP is incorporated into biofilm and microcolony

During the co-culture experiments, bacteria were occasionally found to form biofilms and incorporation of MVP into the biofilm was observed (Figure 6.8). IF staining showed that MVP is incorporated into the bacteria microcolony and MVP reactivity is found at the periphery of the bacteria mass with limited penetration into the centre of the mass of the biofilm. MVP reactivity was also shown to form fibrils spreading out from the base of biofilm to contact nearby

bacteria. However, it is hard to determine if the biofilm was formed after binding to MVP or before binding to MVP had occurred. In order to determine this and if this biofilm binding is dependent on the presence of mammalian cells, mCherry-expressing PAO1 was grown as a droplet on the surface of the chamber slides in the presence and absence of MVP before additional LB was added to cover the entire chamber (Figure 6.9). No MVP positive staining was observed when PAO1-mCherry were grown in the absence of recombinant vaults (Figure 6.9 B). When PAO1-mCherry was grown with 1 μ g of recombinant vaults, IF revealed a dense network of fibres crossed linking bacteria in the initial zone of inoculation (Figure 6.9 C and D). The dense network of MVP fibres was observed at both the centre (Figure 6.9 C) and edge (Figure 6.9 D) of the initial zone of inoculation. MVP strands can also be seen extending out of the initial zone of inoculation ((Figure 6.9 D, white arrows). Together, these data show that MVP can be incorporated into bacteria biofilms through the interaction with Psl.

Figure 6.8. MVP is incorporated in PAO1 microcolony. Bacteria biofilm were observed when THP-1 cells were infected with *P. aeruginosa*. Z-stack maximum projection confocal imaging of IF of MVP (green) and PAO1 (magenta) shows that MVP is incorporated into microcolony in a mesh-like manner (A). Selected planes from the base (B), centre (C) and top of microcolony (D) shows that MVP is predominately found at the periphery of the microcolony (B) with tendrils reaching out to nearby bacteria. Scale bar is 20 μm .

Figure 6.9. **MVP forms mesh-like network in PAO1 biofilm.** Figure depicting the experimental procedure. PAO1-mCherry biofilm was allowed to form in a 20 μ L drop of overnight culture with and without vaults (A). PAO1-mCherry grown without recombinant vaults shows negative MVP staining (B) while PAO1-mCherry grown in the presence of recombinant vaults shows incorporation of vaults into biofilm, forming a mesh-like network (C and D). The MVP network in the centre of the biofilm was more defined (C) while those at the edges showed some defused staining in addition to the network (D). Psl strands can also frequently be seen extending out of the edge of the biofilm (white dotted lines). Scale bar is 20 μ m

6.2.5. *P. aeruginosa* forms microcolony on cell monolayer in presence of extracellular vaults

Psl has previously been shown to be important for the attachment of *P. aeruginosa* to A549 cells (Byrd et al., 2010) and for the initialisation and maintenance of biofilms (Colvin et al., 2012). Using A549 cell monolayer, the effects of extracellular MVP on the interaction of PAO1-mCherry with biotic

surfaces were investigated. By not permeabilising cells prior to antibody staining, selective staining of extracellular MVP was achieved (Figure 6.10). At one-hour post-infection, colocalisation of extracellular MVP with PAO1-mCherry in samples with addition of 0.5 μ g recombinant vaults was observed. There were spots of MVP not associated with bacteria on the surface of A549 cells. Without the addition of recombinant vaults, there is no colocalisation of extracellular MVP with bacteria. At 3 hours post-infection, an increase in MVP staining of A549 cell surface (Figure 6.10 C and D) and bigger PAO1-mCherry microcolony was observed. The orthogonal projection of Z-stack confocal images of PAO1-mCherry infected A549 monolayer (Figure 6.11.) show robust microcolony formation by PAO1-mCherry in the presence of 0.5 μ g recombinant vaults. PAO1-mCherry formed a columnar biofilm on the surface that was more than 4 times the thickness of mammalian cells.

Figure 6.10. **mCherry PAO1 forms microcolony on A549 using MVP.** IF staining for extracellular MVP with AF488 (green) at 1 hour of infection shows an absence of extracellular MVP (A). When recombinant vaults were added, a strong the colocalization of extracellular recombinant vaults with mCherry-PAO1 (red) on cell monolayer was observed (B, white arrowhead). At 3-hour post infection (C and D), an increase in MVP positive signal on A549 cells (green) was observed in both vault treated and untreated wells. However, in the presence of recombinant vaults, a strong colocalisation of MVP with larger microcolony was observed (D, white arrowhead).

Figure 6.11. **Orthogonal projection of mCherry PAO1 microcolony on A549.** PAO1 microcolony on the surface of A549 visualised by confocal image orthogonal projection composed of 16 optical Z-planes, with a total thickness of 7.5 μm. PAO1 forms a MVP-rich columnar microcolony on the surface of A549 cell.

6.3. Discussion

In this chapter, the observation of fibrillary extracellular staining of MVP with PAO1 is described, with MVP fibre joining individual bacteria in a mesh-like network. Staining intensity increased with the addition of 1 μ g of recombinant vaults, further supporting that the staining is indeed due to the presence of MVP. A similar staining pattern was reported when PAO1 was stained with HHA-lectin (Wang et al., 2013), which binds the exopolysaccharide, Psl, of PAO1 (Ma et al., 2007). Using Psl-deficient and T4P mutants, MVP was demonstrated to bind to both secreted and membrane-bound forms of Psl. Furthermore, incubation of PAO1 with PA14, a Psl-deficient strain that predominantly uses Pel for biofilm formation (Colvin et al., 2012, Colvin et al., 2011), demonstrated that MVP is unable to bind to Pel (Figure 6.6). This demonstrates the MVP specifically binds Psl and not other polysaccharides.

MVP was previously found to be secreted by the protozoan, *R. contractilis*, to bind curdlan (Bhadra et al., 2017). Curdlan is a neutral, linear, (1 $\rightarrow$ 3)- β -glucan (McIntosh et al., 2005), that was first isolated from agrobacteria (Harada and Harada, 1996). This suggests that MVP might have some selectivity for neutral polysaccharide chains, most likely containing (1 $\rightarrow$ 3)- β -glucan as Psl is also a neutral polysaccharide with 1,3-glycosidic bonds. β -glucans are absent in humans but have been found in bacteria, yeast, fungi and even plants. β -glucans confer protection against pathogenic microbes through adaptive immune responses (Zhou et al., 2009), enhanced proinflammatory cytokines production in macrophages (Kerekgyarto et al., 1996) and enhanced dendritic cell maturation (Chan et al., 2007). β -glucans are thus classified as PAMPs and studies have identified TLR2 (Sato et al., 2003), complement receptor 3 (Ross et al., 1985),

lactosylceramide (Zimmerman et al., 1998) and Declin-1 (Brown and Gordon, 2001) as receptors for host recognition of glucan antigens. This seems to be in line with our observation of upregulation of MVP by LPS (Figure 5.2) which activates TLR4. MVP is involved in the epithelial-mediated clearance of *P. aeruginosa*; MVP knockout-mice had a lesser clearance rate of *P. aeruginosa*, resulting in greater bacteria load and mortality (Kowalski et al., 2007). We hypothesise that MVP could possibly be released as part of immune responses, such as NETosis, to trap *P. aeruginosa* in a localised biofilm. This might prevent the spread of the bacteria into other parts of the tissue and reduce the number of infection sites, allowing for an increased clearance by immune cells such as neutrophils and macrophages.

However, there is also a possibility that binding to MVP is a strategy that *P. aeruginosa* has evolved to exploit. The binding of MVP to Psl might mask the antigens on the bacteria and its biofilm. This might be the molecular mechanism for the reported roles of Psl in the suppression of the host immune response (Mishra et al., 2012). The binding of MVP to Psl of *P. aeruginosa* might affect host-pathogen interactions. Psl has also been shown to help *P. aeruginosa* evade host immune response. Binding of MVP to Psl on planktonic bacteria or microcolonies could be the molecular mechanism to mask the presence of *P. aeruginosa* antigens from the host innate immune system, allowing it to form part of the commensal community. *Pseudomonas* is capable of suppressing the growth of fungal (Kerr, 1994) and bacteria species (Sutherland et al., 1985, Krausse et al., 2005). This is achieved through the production of molecules such as 3-oxo-C12 homoserine lactone (Hogan et al., 2004), pseudomonic acid A (Sutherland et al., 1985), pyocyanin and 1-hydroxyphenazine. (Kerr et al., 1999).

The selection for keeping *P. aeruginosa* might have an advantage for the host. The incorporation of MVP onto the microcolony of *P. aeruginosa* might be a mechanism for increasing the strength and stability of Psl biofilms. Understanding how MVP affects and interacts with *P. aeruginosa* will allow us to better understand how *P. aeruginosa* persists or is selected in various epithelia. This is especially critical in cystic fibrosis patients, where they often acquire *Pseudomonas* infections (Govan and Deretic, 1996). *P. aeruginosa* frequently colonises chronic wounds (Gjodsbol et al., 2006) and form biofilm at the wound bed (James et al., 2008), preventing normal healing of the wounds. Understanding how MVP modulates the behaviour of *P. aeruginosa* in these setting could allow us to exploit MVP as a way to deliver therapeutics to disrupt *P. aeruginosa* biofilm to eradicate infections. Psl have previously have been targeted using an anti-Psl antibody, leading to disruption of *P. aeruginosa* biofilm (Ray et al., 2017). The binding of MVP to Psl component of the biofilm provides a possibility of using vaults as a therapeutic platform for the targeting and destruction of *P. aeruginosa* biofilms. MVP has been developed as a nanoparticle for drug delivery and vaccination (Champion et al., 2009, Kar et al., 2012, Rome and Kickhoefer, 2013, Zhu et al., 2015). The main problems faced with biofilms is that drugs are unable to penetrate effectively through them to reach the bacteria. If MVP can penetrate the biofilm ECM, antimicrobials, such as antibiotics or antimicrobial peptides, can be encapsulated within the cavity and can be delivered by vault deep into the ECM. This might potential circumvent the resistance of biofilm towards antimicrobials. Alternatively, vaults can be bioengineered with amphipathic antimicrobial peptides such as LL-37, protegrins, indolicins or defensin. This will results in sort of a peptide dendrimer,

leading to high local concentration and thus penetration of bacteria membrane. However, there is a variable expression of Psl and Pel in different strains of *Pseudomonas aeruginosa*, limiting the use of vault nanoparticles for only strains expression Psl. In addition, the ability of vaults to bind and penetrate matured biofilms is unknown and more work have to be carried out to determine if this strategy is feasible.

In a separate study, MVP was observed to be recruited by *Listeria monocytogenes* to escape the autophagosome (Dortet et al., 2011). In that study, the authors used GFP-tagged MVP which was not observed to be released. The presence of a GFP tag at the C-terminus of MVP might prevent its release from the cells, however, the endogenous form might still be released to bind extracellular *L. monocytogenes* and facilitate its entry into the cells. Together, the data hint at MVP being able to interact with various bacteria and modulate host-pathogen interactions.

6.4. Conclusion

This chapter describes the extracellular interaction of MVP to Psl polysaccharide of *P. aeruginosa*. MVP has the ability to bind both secreted and membrane-bound Psl, forming a network between individual PAO1 on glass coverslips. MVP was found to be neither bacteriostatic nor bactericidal but might instead act as a crosslinker to cross-link Psl strands, especially in the case of biofilms. When MVP was added to bacteria, they were incorporated into biofilm of PAO1, forming a mesh-like network. PAO1 also formed columnar biofilms on A549 monolayer by using incorporation of MVP into its biofilm.

Chapter 7: Summary and future work

Since their discovery in the 1980s, vaults have been studied by many researchers. However, despite all the studies carried out so far, the biological function of vaults remained undetermined. Investigation on vaults has been focused on the major vault protein (MVP) as it is the structural protein that gives rise to the unique vault structure (Stephen et al., 2001). MVP is implicated in a variety of cellular stress responses (Yi et al., 2005, Ikeda et al., 2008, Tajitsu et al., 2013), the modulation of signalling pathways in cells (Kolli et al., 2004, Liu et al., 2015) and drug-resistance in cancers (Scheffer et al., 1995, Izquierdo et al., 1996b, Siva et al., 2001). However, literature in this aspect remains disparate and contradictory. Human MVP promoter contains binding sites for stress-related transcription factor Sp-1 (Lange et al., 2000) and MVP expression has also been shown to be induced by transcription factors involved in stress responses (Sp-1 (Steiner et al., 2004), USF (Ikeda et al., 2014) and YB-1 (Stein et al., 2005)). MVP was shown to protect senescent fibroblast against H₂O₂, thapsigargin and staurosporine (Ryu et al., 2008) and to protect SW620 cells against osmotic stress (Tajitsu et al., 2013). However, most of these studies were carried out on specific cell lines/cell type and the relevance of this setting in an *in vivo* setting is still unknown. Establishing a definitive role of MVP in protection against cellular stress responses *in vivo* still remains untested.

MVP-knockout mice were generated previously but they did not show any developmental defects or phenotypes, showing that MVP is not a central component of any major signalling pathway nor essential for proper development and growth of organisms (Mossink et al., 2002b). If MVP is essential for critical signalling processes, defects in development should be observed.

A 110-kDa protein, termed as lung resistance protein, was often upregulated in many multidrug-resistant cell lines (Scheper et al., 1993) and this lung resistance protein was subsequently identified as MVP (Scheffer et al., 1995). This led to the many investigations into the role of MVP in drug resistance and malignancy in cancers (Kickhoefer et al., 1998, Hu et al., 2002, Silva et al., 2007, Lotsch et al., 2013). The role of MVP in resistance towards doxorubicin was carried out on the *MVP*^{-/-} mice generated, and the study concluded that MVP does not confer resistance towards chemotherapeutics (Mossink et al., 2002b). This result was in line with previous cell line studies, suggesting that MVP alone is not sufficient to confer resistance towards chemotherapeutics. However, despite the implication of MVP in cellular stress response, no *in vivo* study on the role of MVP in cellular stress responses have been reported.

MVP has also often been implicated in the control or modulation of innate immune responses (reviewed by (Berger et al., 2009). MVP was shown to be upregulated in the serum and monocytes of hepatitis C virus-infected patients (Liu et al., 2012). The upregulation of MVP is demonstrated to be induced by NF-κB and Sp-1 and leads to enhanced expression of IRF7, resulting in antiviral responses. In a separate study, MVP was shown to bind MyD88, to trigger type-I IFN expression (Liu et al., 2014). An *in vivo* influenza A lung infection model involving *MVP*^{-/-} mice demonstrated that MVP protected mice against influenza A virus. This is mediated by the enhanced translocation of transcription factors c-Fos and C/EBPβ which resulted in enhanced IL-6 and IL-8 production (Peng et al., 2016). Interestingly, MVP was also previously shown to protect mice against *P. aeruginosa* infections of the lungs (Kowalski et al., 2007). In this case, the mechanism proposed was the translocation of MVP to the lipid raft where

they bind to *P. aeruginosa* to enhance its uptake. In the event of viral and bacterial infections, MVP was shown to have a protective function. MVP knockout resulted in an increase in pathogen load and increased mortality of infected mice. In these two infection models, the proposed mechanism by which MVP confers protection is different, suggesting that the observed protective properties of MVP might be an adaption rather than a conserved biological function. Contrarily, MVP was shown to be exploited by *Listeria monocytogenes* to avoid autophagy (Dortet et al., 2011). Inlk, a protein found on *L. monocytogenes* was found to bind MVP and this interaction prevents the binding of autophagy pathway members, p62 and LC3, to *L. monocytogenes*, thus allowing *L. monocytogenes* to evade the autophagy pathway. Thus there is some evidence in the literature that vaults may bind bacteria.

In this thesis, a cell type-independent extracellular interaction of MVP with the polysaccharide, Psl, of *P. aeruginosa* was observed. MVP binds to both membrane-bound and secreted Psl that was deposited on plastic and glass surfaces. This interaction is specific as MVP was unable to bind Pel, another polysaccharide found on *P. aeruginosa*. The interaction of MVP with Psl of *P. aeruginosa* was neither bacteriostatic nor bactericidal but seems to affect the microcolony formation of *P. aeruginosa* on cell monolayers. This suggests that MVP might affect the behaviour, adhesiveness or motility of *P. aeruginosa*.

We demonstrated that although vaults bind to Psl, a polysaccharide containing β -1,3 linked glucose (Byrd et al., 2009), they did not bind to Pel, a separate *P. aeruginosa* polysaccharide that contains β -1,4 linked N-Acetylglucosamine and N-Acetylgalactosamine (Jennings et al., 2015). The observation of the interaction of MVP with Psl only suggests a specificity of MVP binding to sugar moieties.

Interestingly, it was previously shown that MVP of the protist, *R. contractilis* is secreted into the extracellular milieu in the presence of prey. Here, MVP was demonstrated to bind curdlan, a linear polymer of β -1,3 linked glucose (Bhadra et al., 2017).

Together, these data suggest a conserved MVP function in terms of binding to β -1,3 linked glucose. β -1,3 glucans are produced by plants, bacteria and fungus but not vertebrates and are microbes associated molecular patterns which can invoke immune responses (Brown and Gordon, 2001, Chan et al., 2007). The ability of MVP to bind β -1,3 glucans suggests that it might play a role in recognition of and/or immune response against foreign antigens/microbes. MVP may be an opsonisation molecule similar to other opsonins such as IgM, pentraxins and collectins that facilitate the phagocytosis of microbes (reviewed in (Litvack and Palaniyar, 2010). Alternatively, binding of vaults to polysaccharides in the cell wall of *P. aeruginosa* may alter its propensity to form microcolonies, biofilms or its dispersal therefrom.

Interestingly, we observed the presence of an extracellular truncated 50 kDa fragment of MVP when cells were treated with supernatant of *E. coli* and *S. aureus*. The release of the cleaved MVP is dependent on a heat-labile component in the bacterial supernatant. This suggests that there is an active attempt by the bacteria to degrade or destroy intact MVP/vaults, further lending strength to the suggestion of MVP being involved in the binding, recognition and impediment of microbe propagation in the host. Assuming that MVP is an opsonin, degradation of MVP/vaults might result in the reduced clearance of microbes such as that reported in the study investigating the impact of MVP on mouse lung infection by *P. aeruginosa*.

In this thesis I showed that in bacterial/cell co-cultures, MVP binds bacteria extracellularly. This challenges the current dogma that vaults must act intracellularly due to their size and lack of clear evidence of secretion. We propose that vaults act as sentinels in epithelial and innate immune cells that encounter bacterial challenges from the environment. We suspect that they are not secreted actively, rather are released following necrosis or lysis of host cells at the infection site, analogous to the release of anti-bacterial Nets during Netosis. This proposal suggests that the function of vaults is uncoupled from their source, and implies a multicellular assay system is required to understand their biology. For this reason we also used another model organism, zebrafish, to investigate the *in vivo* role of MVP in cellular stress responses and innate immune responses. As the external development of zebrafish embryo allows for easy mutagenesis and transgenesis, we generated *mvp*^{-/-} and transgenic Mvp-GFP zebrafish lines using CRISPR-cas9 mediated mutagenesis and Tol2-mediated transgenesis respectively. *mvp*^{-/-} zebrafish generated were viable and fertile and did not show any phenotypic differences or developmental defects. This further cements the fact that MVP is redundant for proper growth and development of complex organisms. Using *in situ* hybridisation, MVP was demonstrated to be expressed in the epithelial cells of the developing zebrafish embryo. This expression of MVP in tissues exposed to xenobiotics is consistent with the previous report of MVP expression in human tissues (Scheper et al., 1993), and also supports our hypothesis outlined above. However, when *mvp*^{-/-} and WT zebrafish larvae were treated with various cellular stressors, there was no observable difference in survival except for treatment with tunicamycin. *mvp*^{-/-} larvae were found to be more susceptible to tunicamycin-induced haemorrhage. Interestingly, proteomics

analysis of *mvp*^{-/-} larvae also showed the upregulation of proteins involved in stress (krt8, krt18 and eppk1) (Szabo et al., 2015, Baek et al., 2017). However, the expression of MVP and the upregulated stress-related protein were not in the brain where haemorrhage was found and the observation of haemorrhage was specific to a window in development. Tg(krt4:Mvp-GFP) zebrafish larvae also did not show any changes in the localisation of Mvp-GFP when treated with the various cellular stressors. We conclude that there is limited to no evidence of a role for MVP in the protection against cellular stress.

The *in vitro* interaction of MVP with *P. aeruginosa* led us to the hypothesis that MVP plays a role in the opsonisation or recognition of microbes. The *mvp*^{-/-} and Tg(Mvp-GFP) zebrafish lines provide us with a unique platform for the real-time investigation of cellular responses towards microbes. Zebrafish embryos are transparent and allow for live-imaging of cells labelled with fluorescent proteins using conventional light and confocal microscopes. There are also many specific promoter-driven fluorescent protein-expressing lines, such as Tg(mpeg:mCherry) (Ellett et al., 2011) and Tg(mpx:GFP) (Renshaw et al., 2006) which labels macrophage and neutrophils respectively. The transparent larvae and specific labelling of immune cell types will allow for the investigation of innate immune responses towards microbes and labelled vault particles. The attraction of phagocytes and the phagocytosis of microbes by the phagocytes can be compared between *mvp*^{-/-} and WT larvae to study the impact of MVP on such processes. Injection of fluorescently labelled bacteria will also allow us to determine if the enhanced microcolony formation of *P. aeruginosa* can be capitulated *in vivo* and the effect of this phenomena on host survival using the vaultless zebrafish lines. Once a robust assay of vault function can be identified,

the function of the different components of vaults, such as v-PARP and TEP-1, can be tested.

In summary, this thesis provided *in vivo* data that suggests that MVP does not play any major role in most cellular-stress responses. We have developed *mvp* knockout and transgenic zebrafish lines which serve as a valuable platform to investigate vault biology. The thesis has also demonstrated that biological function might be related to the interaction of MVP with sugar moieties on bacterial cell walls. Here, MVP might act as an opsonin that binds or recognises microbes and modulates the immune responses towards such detected microbes. This opens a new direction into the investigation of vault biology that might finally provide answers to the biological function of vaults.

7.2. Future work

The results reported in this thesis open up new possibilities in vault biology. Result from the zebrafish experiments suggests that MVP is involved in stress response in the developing embryo. More experiments could be carried out to investigate what stressors MVP can provide protection against and the molecular mechanisms involved. This can be achieved by the use of MVP mutants in determining immune function against bacteria.

MVP was shown to be released upon bacteria antigen exposure, which suggests an innate immune response role for MVP. Using siRNA screen against Rab GTPases, we have shortlisted few candidates that might be involved in the release/uptake of MVP. Future work confirming the activity of individual Rab GTPase will allow a better understanding of vault biology. As vaults are being

developed as nanoparticle in vaccine (Champion et al., 2009, Kar et al., 2012, Rome and Kickhoefer, 2013) and for cancer therapy, the understanding of the uptake and release mechanism can allow for the optimisation of the targeting of vaults to cell and thus increase efficacy of designed therapy.

In addition, the release of MVP was rapid and resulted in the interaction with PAO1 through binding with Psl. It was also previously reported that MVP binds to β -glucans (Bhadra et al.). This suggests that MVP might have an affinity towards certain polysaccharide moieties and can either bind or interact with specific polysaccharide-containing targets. However, the polysaccharide specificity has to be further validated to determine suitable bacteria targets. β -glucans are not found in humans and are xenobiotic antigens that can be specifically recognised by the innate immune system (Brown and Gordon, 2001, Chan et al., 2007). As the data show that vaults are not directly bacteriostatic or bactericidal, it would be reasonable to postulate that MVP is a novel opsonisation molecule that helps in the detection or capture of such antigens, further implicating MVP in innate immune responses. It would be interesting to see if MVP interacts with other microbes such as bacteria and fungi. MVP might be also involved in the tolerance or recognition of microbes and could be a factor in the recognition by the immune system as commensal or pathogen. One way to study this would be by comparing microbiome of *mvp*^{-/-} and WT organisms to see if there is any shift in bacteria populations or metagenomics analysis that would expand this analysis to other microbes such as fungi and archaeobacteria. As Psl is a signal to mediate exploration and migration of individual bacteria towards microcolonies (Zhao et al., 2013), experiments on the impact of MVP binding to Psl on the motility of *P. aeruginosa* can be investigated. However, the

relatively large amount of bacteria in agar plate assays might overcome the stoichiometric binding of MVP particles and might mask any effects exerted by the limited number of MVP particles. Microscopic analysis of the movement of individual bacteria might provide clues regarding the impact binding of MVP to Psl, on motility.

Finally, further research can be carried out to determine if tagging vault particles with antibacterial compounds can give rise to potential therapeutics against *Pseudomonas* biofilm by targeting and delivering the compounds into the biomass of the biofilms.

Appendix

Appendix Table 1. Plate map of siRNA screen

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
A	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock
B	PLK	RAB1A	RAB2A	RAB2B	RAB3A	RAB3B	RAB3C	RAB3D	RAB4A	RAB4B	RAB5A	RAB1A	RAB2A	RAB2B	RAB3A	RAB3B	RAB3C	RAB3D	RAB4A	RAB4B	RAB5A	PLK
C	NT	RAB5B	RAB5C	RAB6A	RAB6B	RAB7A	RAB7B	RAB8A	RAB8B	RAB9A	RAB9B	RAB5B	RAB5C	RAB6A	RAB6B	RAB7A	RAB7B	RAB8A	RAB8B	RAB9A	RAB9B	NT
D	NT	RAB10	RAB11A	RAB11B	RAB12	RAB13	RAB14	RAB15	RAB17	RAB18	RAB19	RAB10	RAB11A	RAB11B	RAB12	RAB13	RAB14	RAB15	RAB17	RAB18	RAB19	NT
E	MVP	RAB20	RAB21	RAB23	RAB24	RAB25	RAB26	RAB27A	RAB27B	RAB28	RAB7L1	RAB20	RAB21	RAB23	RAB24	RAB25	RAB26	RAB27A	RAB27B	RAB28	RAB7L1	MVP
F	MVP	RAB30	RAB31	RAB32	RAB33A	RAB33B	RAB34	RAB35	RAB36	RAB37	RAB38	RAB30	RAB31	RAB32	RAB33A	RAB33B	RAB34	RAB35	RAB36	RAB37	RAB38	MVP
G	Mock	RAB39A	RAB39B	RAB40A	RAB40B	RAB40C	RAB40AL1	RAB41	RAB42	RAB43	RAB44	RAB39A	RAB39B	RAB40A	RAB40B	RAB40C	RAB40AL1	RAB41	RAB42	RAB43	RAB44	Mock
H	Mock	RASEF	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	RASEF	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock
I	Mock	RAB1A	RAB2A	RAB2B	RAB3A	RAB3B	RAB3C	RAB3D	RAB4A	RAB4B	RAB5A	RAB1A	RAB2A	RAB2B	RAB3A	RAB3B	RAB3C	RAB3D	RAB4A	RAB4B	RAB5A	Mock
J	Mock	RAB5B	RAB5C	RAB6A	RAB6B	RAB7A	RAB7B	RAB8A	RAB8B	RAB9A	RAB9B	RAB5B	RAB5C	RAB6A	RAB6B	RAB7A	RAB7B	RAB8A	RAB8B	RAB9A	RAB9B	Mock
K	Mock	RAB10	RAB11A	RAB11B	RAB12	RAB13	RAB14	RAB15	RAB17	RAB18	RAB19	RAB10	RAB11A	RAB11B	RAB12	RAB13	RAB14	RAB15	RAB17	RAB18	RAB19	Mock
L	Mock	RAB20	RAB21	RAB23	RAB24	RAB25	RAB26	RAB27A	RAB27B	RAB28	RAB7L1	RAB20	RAB21	RAB23	RAB24	RAB25	RAB26	RAB27A	RAB27B	RAB28	RAB7L1	Mock
M	Mock	RAB30	RAB31	RAB32	RAB33A	RAB33B	RAB34	RAB35	RAB36	RAB37	RAB38	RAB30	RAB31	RAB32	RAB33A	RAB33B	RAB34	RAB35	RAB36	RAB37	RAB38	Mock
N	Mock	RAB39A	RAB39B	RAB40A	RAB40B	RAB40C	RAB40AL1	RAB41	RAB42	RAB43	RAB44	RAB39A	RAB39B	RAB40A	RAB40B	RAB40C	RAB40AL1	RAB41	RAB42	RAB43	RAB44	Mock
O	PLK	RASEF	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	RASEF	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	PLK
P	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock

Appendix Figure 1. Quality control of siRNA screen. (Left) The number of nuclei in each well was calculated in the images captured and siRNA against PLK was used as a positive control to end efficiency of transfection. (Right) Reduction in intensity of AF488 fluorescence signal using siRNA against MVP validated the knockdown of MVP. Scrambled siRNA did not show any changes in fluorescence intensity.

Appendix Figure 2. **siRNA screen staining morphology and distribution of morphology types.** Images taken of the siRNA treated HeLa cells were subjected to Gaussian Mixture Model clustering in duplicates (GMM1 and GMM2). (A-D) Cells in the first analysis were segregated into 17 groups and the distribution of mock cells (B), MVP siRNA (C) and Rab34 siRNA (D) treated cells were displayed as spider plots. (E-F) Cells in the second analysis were segregated into 14 groups and the distribution of mock cells (F), MVP siRNA (G) and Rab34 siRNA (H) treated cells were displayed as spider plots.

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