

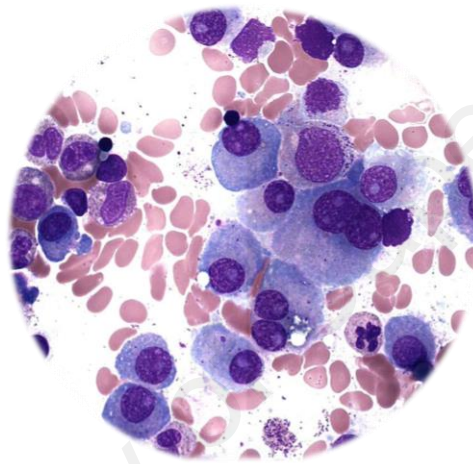


UNIVERSITY OF CAPE TOWN
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DEFINING THE FUNCTIONAL ROLE OF MAGE-C1 IN MULTIPLE MYELOMA

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STNMAR050



Submitted to the University of Cape Town in fulfilment of the requirements for the degree of Master of Science in Medicine in the Discipline Haematology.

12 February 2024

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ACKNOWLEDGEMENTS

Firstly, I would like to thank all faculty staff, colleagues and friends who have been alongside me on this journey. To Adri Rust, Kashief Khan and Leonardo Alves de Souza Rios I would like to extend my sincerest gratitude for their support, encouragement, friendship and endless cups of coffee.

Thank you to my family, who have always supported me. To my mother, Adèle Stone, thank you for your counsel, inspiration and love. Without you I would not be where I am today. To my partner, Gareth Swart, thank you for walking this path with me.

And finally, a special thank you to my supervisor, Associate Professor Karen Shires, who has guided me and been a true mentor – without your guidance I would be lost. I will forever be grateful for all you have done for me.

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LIST OF SCIENTIFIC ABBREVIATIONS AND ACRONYMS

aa	Amino acid
Ab	Antibody
ABL	Abelson tyrosine-protein kinase 1
Ant	Tetraploid cells
ASCT	Autologous stem cell transplantation
ASO-qPCR	Allele-specific oligonucleotide quantitative polymerase chain reaction
AU	Airy unit
BCA	Bicinchoninic acid
BCMA	B-cell maturation antigen
BCR/ABL	Breakpoint cluster region-Abelson
BM	Bone Marrow
bp	Base pairs
BSA	Bovine serum albumin
CA	Chromosomal abnormalities
CAR	Chimeric antigen receptor
CAR-T	Chimeric antigen receptor T-cell
CD	Cluster of differentiation
CDK	Cyclin dependant kinases
cDNA	Complimentary deoxyribonucleic acid
cm	Centimeter
Co-IP	Co-immunoprecipitation
CO ₂	Carbon dioxide
CR	Complete response
CRBN	Cereblon
CRL4	Cullin-4 RING E3 ligase
CT	Computed tomography
Ct	Crossing point(s)
CTA	Cancer/testis antigen
C-terminal	Carboxyl-terminal
Dip	Diploid cells
dL	Decilitre
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
dNTP	Deoxynucleotide(s)
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
E2-U	Ubiquitinated E2 ligase
FBS	Foetal bovine serum
Fl-Ab	Fluorescent labelled antibody
FLC	Free light chain(s)
FSC	Forward scatter
g	Gram
G-CSF	Granulocyte-colony stimulating factor
HAT	Histone acetyltransferases
HDAC	Histone deacetylases
HRP	Horse-radish peroxidase
iFISH	Interphase fluorescence <i>in situ</i> hybridisation
IFM	Immunofluorescence microscopy
IgA	A-type immunoglobulin heavy chain
IgG	G-type immunoglobulin heavy chain
IGH	Immunoglobulin heavy-chain
IKZF	Ikaros family zinc finger
IMiDs	Immune modulators
IMWG	International Myeloma Working Group
ISS	International Staging System
kDa	Kilodalton
kg	Kilogram
kV	Kilovolt
L	Litre
LDH	Lactate dehydrogenase
Lys (K)	Lysine
M	Molar
MAGE	Melanoma antigen

MAGE_N	Melanoma associated antigen family N-terminal domain
MFC	Multiparametric flow cytometry
mg	Milligram
MgCl ₂	Magnesium chloride
mg/dL	Milligram per decilitre
MGUS	Monoclonal gammopathy of undetermined significance
MHD	MAGE homology domain
min	Minute(s)
mL	Milliliter
mm	Millimeter
mM	Millimolar
MM	Multiple Myeloma
mmol/L	Millimoles per litre
MRD	Measurable residual disease
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B-cells
ng	Nanogram
NGS	Next generation sequencing
NIC	Notch1-intracellular domain
nm	Nanometer
N-terminal	Amino-terminal
N ϵ	Epsilon amino group
O/N	Overnight
OS	Overall survival
PB	Peripheral blood
PBMNC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Plasma cell
PCR	Polymerase chain reaction
PET	Positron emission tomography

PFS	Progression-free survival
pH	Potential of hydrogen
PI	Propidium iodide
Pi	Proteasome inhibitors
pmol	Picomole
PS	Penicillin-Streptomycin
PtI	Protease inhibitor
P-TP53	Phosphorylated tumour antigen P53
p38	Anti-p38 MAP kinase
qRT-PCR	Quantitative real-time polymerase chain reaction
RBX1	RING Box Protein-1
RING	Really Interesting New Gene
RIPA	Radioimmunoprecipitation assay
R-ISS	Revised international staging system
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
rRT-PCR	Real-time reverse transcription polymerase chain reaction
RT	Reverse transcription
RTP	Room temperature
SC	Stem cell(s)
SCF	Skp-Cullin-F-box
sCR	Stringent complete response
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Seconds
Ser (S)	Serine
sGC	Synthetic glucocorticoids
SKIP	Ski-interacting protein
SMM	Smouldering multiple myeloma
SPEP	Serum protein electrophoresis
SSC	Side scatter
TAE	Tris-acetate-EDTA

TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
Thr (T)	Threonine
TP53	Tumour antigen P53
TRIM28	Tripartite motif-containing 28
Tris	Trisaminomethane
Tyr (Y)	Tyrosine
U	Units
UK	United Kingdom
UPEP	Urine protein electrophoresis
USA	United States of America
V	Volt
VAD	Vincristine, doxorubicin and dexamethasone regimen
VCD	Bortezomib, cyclophosphamide and dexamethasone regimen
VDJ	Variable region of the immunoglobulin heavy-chain
via	By means of
VMP	Bortezomib, melphalan and prednisone regimen
VP	Bortezomib and prednisone regimen
VRD	Bortezomib, lenalidomide and dexamethasone regimen
vs	Versus
VT	Bortezomib and thalidomide regimen
VTD	Bortezomib, thalidomide and dexamethasone regimen
VTP	Bortezomib, thalidomide and prednisone regimen
v/v	Volume per volume
WH	Winged helix
WHD	Winged helix domain
w/v	Weight per volume
%	Per cent/percentage
°C	Degree Celsius
<	Less than
>	Greater than
±	Plus-minus
≈	Almost equal to

α	Alpha
β	Beta
β 2-M	β 2-Microglobulin
β -ME	β -mercaptoethanol
γ	Gamma
Δ	Delta
κ	Kappa
λ	Lambda
μ g	Microgram
μ L	Microliter
μ M	Micromolar
μ m	Micrometre
μ mol/L	Micromole per liter

ABSTRACT

The expression of Cancer/Testis antigens (CTAs) in various cancers, including multiple myeloma, makes them attractive targets for therapy and biomarker development. Despite intensive studies, their potential has not been fully realized due to lack of knowledge regarding their pathogenic role. To start elucidating the function of melanoma antigen (MAGE) protein-C1 (MAGE-C1) in multiple myeloma, this project aimed to firstly establish the suitability of culture cell line RPMI-8226 as an *in vitro* multiple myeloma model. Additionally, the project aimed to establish relevant reagents and techniques to study MAGE proteins by comparing MAGE-C1 to better described CTAs: MAGE-C2 and MAGE-A1. Firstly, the expression of MAGE-C1, MAGE-A1, MAGE-C2 and their respective binding partners, Ski-interacting protein (SKIP) and Ring-box 1 (RBX1), was confirmed in RPMI-8662. Next, functional similarity was investigated through co-immunoprecipitation, to determine MAGE-C1 interaction with RBX1 or SKIP, and immunofluorescence microscopy, to assess co-localisation of these proteins. Lastly, cellular pathways influenced by MAGE-C2 were evaluated using heat shock to induce a stress response and activate the pathways of interest. The expression of MAGE-C1, MAGE-A1, MAGE-C2 and their binding partners were confirmed at the mRNA level. Unfortunately, interaction with their binding partners could not be demonstrated using co-immunoprecipitation, although immunofluorescence microscopy showed co-localisation of MAGE-C1 with both SKIP and RBX1. An abnormal stress response, via the E3 ligases known to interact with MAGE-C2 was observed in RPMI-8226. An array of reagents and techniques for future MAGE investigations were established during this study. However, the findings of this project indicated that RPMI-8226 may not be an ideal model for further MAGE investigations as initially hypothesized.

CHAPTER 1: UNDERSTANDING THE ROLE OF CANCER TESTIS ANTIGEN EXPRESSION IN MULTIPLE MYELOMA

1.1. MULTIPLE MYELOMA

1.1.1. What is Multiple Myeloma?

Multiple Myeloma (MM) is a haematopoietic, B-cell malignancy caused by the malignant proliferation of a clonal population of terminally differentiated plasma cells (PCs) in the bone marrow (BM), that results in over production of a specific monoclonal immunoglobulin, referred to as a “paraprotein” or “M-protein”.^{1,2,3,4,5}

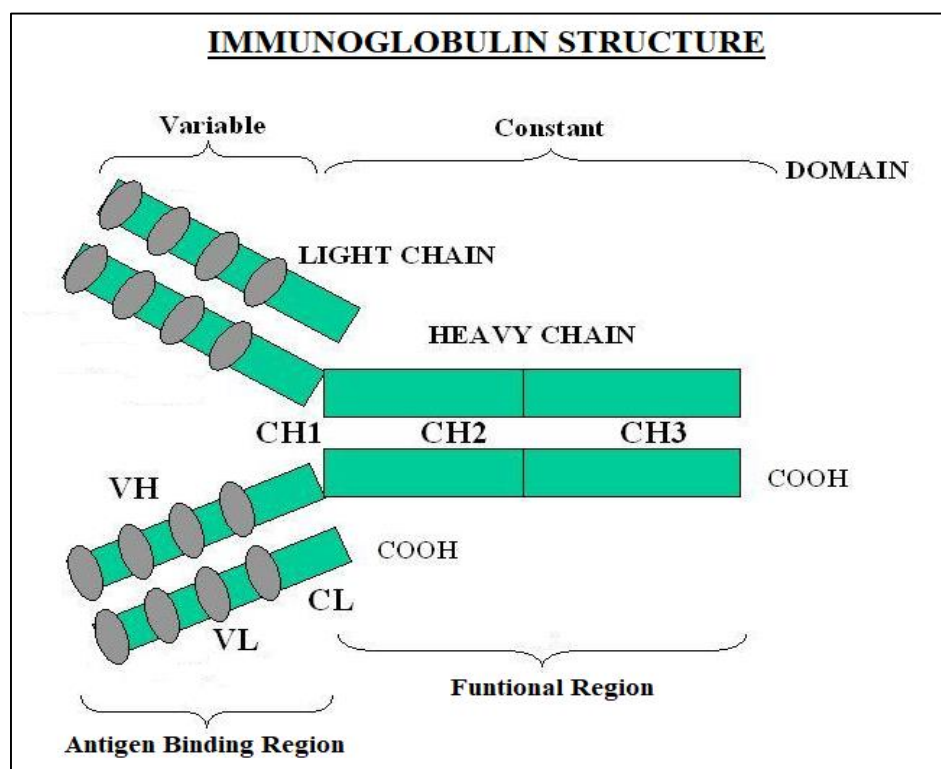


Figure 1: Structure of immunoglobulins.

This figure illustrates how light chains bind to heavy chains to form a complete immunoglobulin. Both heavy chains and light chains have constant (C) and variable regions (V). The variable regions allow the expression of various different heavy chains (IgA, IgG, IgM, IgD, or IgE) or light chains (κ or λ). (modified from Berlot et al.⁸)

PCs are a normal part of the adaptive immune response. They differentiate from B-cells, which are activated by an antigen-presenting pathogen and produce immunoglobulins (antibodies) specific to the antigen that triggered the PC differentiation. There is thus normally a variety of PCs in the blood stream, secreting a variety of immunoglobulins as initiated by the immune response.⁶ As shown in Figure 1 above, complete immunoglobulins are made up of a heavy chain (most commonly IgG or IgA) and a

bound kappa (κ) or lambda (λ) light chain. Non-malignant PC secrete mostly complete immunoglobulins (antibodies), with small amounts of free (unbound) light chains (FLC).⁷

As mentioned, in MM there is malignant expansion of a specific PC and a subsequent domination of a specific, single M-protein or myeloma protein.^{6,7} While the majority of MM cases exhibit the production of intact immunoglobulins, with a smaller fraction of FLC, some MM cases, known as light chain myeloma, have PCs that only synthesize and secrete FLC (oligo-secretory MM).⁷ In a small number of cases (1-5%) no M-protein can be detected in the peripheral blood (PB) or urine and these are known as non-secretory MM.

1.1.2. Diagnosis and Staging of MM

1.1.2.A. Classical Clinical Presentation Features of MM

Symptomatic MM has very distinct clinical features that stem from the accumulated PCs and abnormal proteins in the haematopoietic compartment (PB and BM) called CRAB features.^{9,10,11} CRAB features include end-organ damage such as hypercalcemia (C), renal failure (R), anaemia (A) and bone lesions (B).^{12,13} The painful osteolytic lesions associated with MM occur most frequently in the thoracic vertebrae, ribs, skull, pelvis and femur.¹² These are caused by the activation of osteoclasts by the PCs, which leach calcium from the bone, leading to hypercalcemia and weakened bone structure. Compression of the spinal cord and consequential neuropathy may also occur, which when combined with the increased risk of skeletal-related events, such as fractures, contribute to the morbidity and mortality of MM.⁴ Anaemia is caused by the loss of haematopoietic potential in the BM, as a result of the abnormal PCs overwhelming the stromal microenvironment and preventing normal haematopoiesis.¹⁴ This BM overcrowding also results in a compromised immune system, leading to frequent infections, which are commonly seen.¹⁴ Renal failure is caused by the accumulation and precipitation of the FLCs, resulting in renal distal tubule blockages and is indicated through elevated creatine levels.^{5,14}

Most MM patients present with symptoms that stem from the underlying CRAB features upon diagnosis. The most common symptoms include back pain or musculoskeletal pain due to bone lesions, fatigue and dizziness from anaemia and nausea, vomiting, confusion or constipation from hypercalcemia.^{11,15} Due to the accumulation of paraproteins, microscopic and macroscopic destruction of the haematopoietic compartment, and extramedullary disease associated with MM a wide range of symptoms can be observed, including neuropathy, hyperviscosity syndrome, recurrent infections, weight loss, and amyloidosis to name a few.^{11,15}

1.1.2.B. Diagnosis of MM

The International Myeloma Working Group (IMWG) are the most influential clinical organisation in advising clinicians on MM management. A summary of their latest guidelines (2014)³ to diagnose MM is shown in Table 1. According to their criteria a patient must present with at least a singular MM lesion or have more than 10% clonal PC present in the BM along with at least one myeloma defining event as described in the table.

It should be noted that PCs are defined as clonal when demonstrating light chain restriction (κ or λ) using flow cytometry, immunohistochemistry, or immunofluorescence.³ While the following techniques can be used to assess bone damage: radiography, computed tomography (CT) scan, positron emission tomography (PET) or magnetic resonance imaging (MRI), the IMWG suggest that that MRI is used for the initial assessment of patients with suspected MM due to its sensitivity.³

Table 1: Diagnostic criteria for MM according to the IMWG (modified from Rajkumar et al.³)

1.	Involvement of $\geq 10\%$ clonal PCs* in the BM [#] . or Presence of bony or extramedullary plasmacytoma.
2.	One or more of the following myeloma defining events. a. Evidence of end organ damage arising from the underlying PC disorder • Hypercalcaemia: Serum calcium > 3 mmol/L (> 12 mg/dL) • Renal Insufficiency: Creatine clearance < 40 mL per min or Serum creatine > 177 μmol/L (> 2 mg/dL) • Anaemia: Haemoglobin < 100 g/L or more than 20 g/L below the lower normal limit. • Bone lesions: One or more lesions on skeletal radiography, MRI^{\$}, CT^{&}, or PET-CT[@] b. Biomarkers of malignancy • Clonal BM PC percentage $\geq 60\%$ • A ratio of involved/uninvolved serum FLC ≥ 100 • More than one focal lesion on MRI

*Plasma cells; #Bone marrow; \$ Magnetic resonance imaging; & Computed tomography scan; @ positron emission tomography and computed tomography scans.

MM is preceded by a premalignant stage, called monoclonal gammopathy of undetermined significance (MGUS).^{3,16,17} Clinically, MGUS is distinguished from MM by the absence of end-organ damage (no CRAB features), BM PC count of $< 10\%$ of all mononuclear cells, and serum M-protein levels less than 30 g/L.^{5,16,18} The reported progression rate of MGUS to MM is 1% per year.¹⁷ According to Rajkumar et al.¹⁹ more than 50% of patients diagnosed with MM have had this pre-malignant condition for more than 10 years pre-diagnosis. MGUS usually progresses to asymptomatic or smouldering MM (SMM), which demonstrates an increase in immunoglobulin secretion (serum M-protein ≥ 3 g/dL), and/or a larger population of clonal plasma cells ($> 10\%$), but again no CRAB features.^{18,20} SMM has a risk of progression into symptomatic MM at a rate of about 10% per year for the first five years after

diagnosis,²⁰ where after the progression rate slows down.¹⁹ Treatment of these pre-MM conditions is currently not standard practise, with the “wait and see” approach usually taken and patients monitored closely for progression to active MM, due to the serious side effects of active MM therapy.^{18,20}

1.1.2.C. Staging of MM Disease

Traditionally the severity of MM was measured using the International Staging System (ISS),²¹ which has three stages based on serum albumin and serum β 2-Microglobulin (β 2-M) levels as summarised in Table 2. below, where high β 2-M levels indicate a larger tumour burden and reduced renal function and lowered serum albumin indicates increased inflammation.²¹

Table 2: Staging criteria for MM according to the ISS (modified from Cancer.Net²¹)

Stage	β 2-M [#]		serum albumin	OS* (months)
Stage I	< 3.5 mg/L	and	$\geq$ 3.5 g/dL	62
Stage II	3.5 mg/L - 5.5 mg/L	or	< 3.5 g/dL	44
Stage III	> 5.5 mg/L			29

β 2-Microglobulin; *Overall survival

Staging was revised in 2015²² by the IMWG to include additional prognostic factors: chromosomal abnormalities (CA) and serum lactate dehydrogenase (LDH), as these affect overall survival (OS). CA are investigated using interphase fluorescence *in situ* hybridisation (iFISH), with del(17p), and t(4;14)(p16;q32) and t(14;16)(q32;q23) translocations being associated with high-risk MM.²² Palumbo et al.²² reports an OS of 24.5 months for patients with CA (high risk MM) compared to standard-risk MM in the absence of CA with an OS of 50.5 months. Studies have shown that abnormally high levels of LDH are associated with aggressive MM and shorter OS.^{22,23} The revised staging system (R-ISS) is summarised in Table 3.

Table 3: R-ISS for MM (modified from Palumbo et al.²²)

Prognostic Factor	Criteria
ISS* stage	
I	Serum $\beta 2\text{-M}^{\wedge} < 3.5 \text{ mg/L}$ and Serum albumin $\geq 3.5 \text{ g/dL}$
II	Not ISS stage I or III
III	Serum $\beta 2\text{-M}^{\wedge} \geq 5.5 \text{ mg/L}$
CA# by iFISH@	
High risk	Presence of del(17p) and/or t(4;14) and/or t(14;16)
Standard risk	No high-risk CA
LDH\$	
Normal	Serum LDH < the upper limit of normal
High	Serum LDH > the upper limit of normal
R-ISS& stage	
I	ISS stage I and standard-risk CA by iFISH and normal LDH
II	Not R-ISS stage I or III
III	ISS stage III and either high-risk CA by iFISH or high LDH

*International staging system; $\wedge$ $\beta 2$ -Microglobulin; #Chromosomal abnormalities; @ Interphase fluorescence *in situ* hybridisation; \$Lactate dehydrogenase; &Revised staging system.

1.1.3. Treatment of MM

1.1.3.A. Autologous Stem Cell Transplantation

Treatment protocols for MM patients are dependent upon the stage of the disease, prognostic factors, and transplant eligibility, but autologous stem cell transplantation (ASCT) is generally accepted as the best option for patients younger than 65 years of age, with no co-morbidities.^{24,25} This therapy is aimed at achieving the best OS and progression-free survival (PFS) for the patient, although a complete cure using this approach is not yet achievable. The ASCT approach is divided into an induction phase, stem cell (SC) harvest, further chemotherapy to reduce the “tumour burden”, re-introduction of the patients’ SCs and then consolidation and maintenance therapy.²⁵ The important premise behind this type of therapy (including allogenic stem cell transplantation) is that the PCs are the only malignant cell type and that the SCs are not part of the malignancy.²⁶ This is however a contentious issue^{27,28} and will be discussed later.

1.1.3.A.i. Induction treatment

Prior to ASCT, patients undergo high-dose induction chemotherapy that include immune modulators (IMiDs) such as thalidomide and lenalidomide, proteasome inhibitors (Pi) such as bortezomib and carfilzomib, and synthetic glucocorticoids (sGC) such as prednisone and dexamethasone.^{29,30} These chemotherapy drugs work in a multitude of complicated ways to eradicate MM-cells (abnormal PCs) as briefly summarised: 1) IMiDs target Cereblon (CRBN), a receptor protein, as its primary binding partner.^{31,32} CRBN combines with other proteins to form a powerful E3 ligase complex called cullin-4

RING E3 ligase (CRL4)^{31,32} that marks other protein for cell death through ubiquitination.³³ Binding of IMiDs to CRBN makes the protein resistant to its own degradation that results in increased CRL4-mediated ubiquitination activity and subsequent degradation of various proteins including transcription factors such as Ikaros family zinc finger (IKZF) 1 and IKZF3 which contributes to the anti-tumour effect of IMiDs on MM cells;^{32,33} 2) Pi target MM cells by inhibiting proteasome 26S that results in subsequent inhibition of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B).³⁴ Protein folding is dependent on proteasome function. In this way Pi results in build-up of misfolded protein and subsequent stress signalling called the unfolded protein response which eventually causes the cell to signal for apoptosis.³⁴ Glucocorticoids counteracts MM pathogenesis by binding to GC receptors to form a GC-complex that activates annexin I and mitogen-activated protein kinase phosphatase.³⁵ The GC-complex also indirectly represses transcription factors such as NF- κ B which obstructs proliferation and causes apoptosis of MM cells.^{35,36}

During induction therapy, chemotherapy agents are usually used in combination with one another, with the addition of corticosteroids³⁷ to reduce tumour burden by selectively targeting MM PCs for cell death.³⁴ Induction therapy allows for a deeper response to ASCT and increases the chance of full engraftment with the least effect on healthy haematopoietic cells.²⁴ The regimes used to be dexamethasone based, such as the VAD regimen (vincristine, doxorubicin and dexamethasone), however novel drugs incorporated into clinical trials show that 3-drug regimens including either or both bortezomib and thalidomide have better outcomes,^{24,30,38} are now regarded as standard induction therapy for newly diagnosed MM patients.^{24,30,38}

1.1.3.A.ii. SCs and reinfusion

Following the initial reduction in the number of abnormal PCs, the patients haematopoietic SCs are stimulated to replicate and move from the BM to the PB to be harvested.^{24,39} The ideal SC count needed for harvest is 5×10^6 CD34+/kg with the minimum being 2×10^6 CD34+/kg.⁴⁰ Mobilisation can either be steady-state or chemo-based.²⁴ Steady state mobilisation uses granulocyte-colony stimulating factor (G-CSF) cytokines,⁴⁰ while chemo-mobilisation uses cyclophosphamide in addition to G-CSFs.^{24,41} The SCs are removed at this stage using apheresis so that the patient can be treated with a final myeloablative therapy, high dose melphalan (200 mg/m^2) as the last phase of induction therapy,^{24,42} without damage to these cells. The harvested cells are cryopreserved and can be re-infused within 24-hours following the melphalan therapy (optimal) or saved for a later stage (i.e: salvage therapy after relapse). This is often the recommended route for patients that respond exceptionally well to chemotherapy.¹⁹

1.1.3.A.iii. Consolidation and maintenance therapy

VRD, a combination of bortezomib, lenalidomide, and dexamethasone is used for short term therapy for patients post-ASCT. Studies found that consolidation therapy after ASCT had better complete

response (CR) rates and prolonged PFS than those without.^{24,30} Sadly, ASCT does not cure MM and relapse is an unfortunate certainty³⁰ even if a CR is obtained. Maintenance therapy is designed for long-term use to prevent relapse and prolong OS. Immune modulators such as lenalidomide are preferably used for maintenance therapy in low doses taken orally.²⁴ With the current therapies OS and PFS have both improved with ~ 4.7 years and ~ 30 months, respectively.⁴³

1.1.3.A.iv. Alternate treatment strategies

1.1.3.A.iv.a. Chemotherapy only

Since MM is an incurable disease, treatment strategies have always focused on improving quality of life and extending OS as long as possible.³⁸ In patients non-legible for ASCT (due to age and comorbidities) the goal is still to achieve the deepest response for as long as possible through use of chemotherapeutic agents alone.³⁸ Patients are treated with induction therapy (usually three-drug regimens), immediately followed by maintenance therapy. A study on the elderly (≥ 65 years) performed by Mateos et al.⁴⁴ reported an increase in MM patients who achieve define CR (responses discussed below) and longer PFS with tolerable toxicity levels when treated with combinations of bortezomib, melphalan, thalidomide and prednisone as maintenance therapy.⁴⁴

1.1.3.A.iv.b. Chimeric antigen receptor T-cell immunotherapy

B-cell maturation antigen (BCMA) chimeric antigen receptor T-cell (CAR-T) immunotherapy is currently in clinical trials as a treatment for MM.⁴⁵ This treatment is based on the ability to genetically modify the patients T-cells to express a chimeric antigen receptor (CAR) against the cancer cells.⁴⁵ CARs are artificially made receptors that contain an antigen receptor (usually from a monoclonal antibody (Ab)) and a T-cell receptor (CD3 ζ). BCMA is a surface antigen expressed solely on B-cells, including malignant MM cells and is therefore the favoured target antigen for MM.⁴⁵ During the treatment, the patient's own lymphocytes are harvested using leukapheresis, genetically modified to include BCMA CAR-T, expanded, and infused back to the patient.⁴⁵ Patients are conditioned with chemotherapy targeting lymphocytes prior to re-infusion with CAR-T.⁴⁵ Although, CAR-T therapy has shown promising results, side effects such as cytokine release syndrome and neurotoxicity has been noted.⁴⁵

1.1.3.A.iv.c. Monoclonal antibodies

CD38 is a transmembrane glycoprotein, normally expressed on lymphoid, myeloid and some non-haematopoietic cells in low levels,⁴⁶ but in PCs and MM cells it is over expressed.^{13,47-49} Various functions have been attributed to this protein, one of which, is its function as a receptor protein for ligand CD31.⁴⁶ Upon binding with CD31, CD38 releases an intracellular signal that activates T-lymphocytes and ultimately leads to the release of interleukin 6, granulocyte-macrophage-colony-stimulating factor, interferon- γ , and interleukin 10 cytokines⁴⁶ which supports the role of CD38 in cell

proliferation signalling. The function of CD38, along with the elevated expression levels on MM cells makes it an attractive therapeutic target for MM.^{46,47} There are two main monoclonal Ab in use as therapeutic agents i.e. daratumumab and isatuximab.⁴⁶ Anti-CD38 Ab targets diseased cells for cell death through complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, antibody-dependent cellular phagocytosis, and apoptosis of the cell.^{13,46,47} Both these agents can be used as a single-drug therapy, but have proven to be more effective when used in combination with other types of drugs such as IMiDs and Pi.^{13,46,47} Unfortunately side effects can be quite severe and include neutropenia, thrombocytopenia and pneumonia.^{49,50} Studies have shown that the use of these drugs in combination can produce relatively high rates of 12-month PFS,^{13,51}

1.1.4. Monitoring Therapy Responses in MM

1.1.4.A. Important Milestones in MM Therapy

There is a clear relationship between the PFS and OS of the patient, and the level of response to treatment, with a deeper response providing a better chance of a good quality of life.^{24,38,52} The IMWG have continuously developed and updated treatment response definitions, with the latest described in Table 4. Responses are measured through a variety of laboratory techniques,⁵³ which have differing sensitivities and limitations. The methodologies are described in more detail in section 1.4.B. to 1.4.C. of this review.

Table 4: Response criteria for MM according to the IMWG (modified from Durie et al.⁵³)

Response	IMWG criteria
Stable disease*	• Not meeting the criteria for CR[#], partial response, or progressive disease.
Partial response	• $\geq 50\%$ reduction of serum M-protein and $\geq 90\%$ reduction in 24-hour urine M-protein (or < 200 mg). • If the serum and urine M-protein are unmeasurable, a $\geq 50\%$ decrease in the difference between involved and uninvolved FLC levels is required in place of the M-protein criteria. • If serum and urine M-protein are unmeasurable, and serum free light assay is also unmeasurable, $\geq 50\%$ reduction in plasma cells is required in place of M-protein, provided that the baseline BM plasma cell percentage was $\geq 30\%$. • In addition to the above listed criteria, if present at baseline, a $\geq 50\%$ reduction in the size of soft tissue plasmacytomas is also required.
Very good partial response	• Serum and urine M-protein detectable by immunofixation but not on electrophoresis, or • ≥ 90 reduction in serum M-protein, and • < 100 mg per 24-hour urine M-protein.
CR	• Negative immunofixation on the serum and urine, and • disappearance of any soft tissue plasmacytomas, and • $\leq 5\%$ plasma cells in the BM.
Stringent complete response	• CR[#] in conjunction with normal FLC ratio and the absence of clonal cells in the BM by immunohistochemistry or immunofluorescence.

*Not an indicator of response; #Complete response.

While a CR was the key response to achieve up until 2015, which is defined as $< 5\%$ PCs in the BM, and no detectable serum/urine M-protein, as the newer technologies have become available and clinical trials have been able to assess the effects of deeper responses, new responses have been added. These include a “stringent CR” (sCR) that requires additional negative results for FLC assays and immunohistochemical clonality assessments, as well as measurable residual disease (MRD) status.⁵²

MRD in MM is classified as disease levels not detectable by assays used to define sCR and is defined by a minimum detection threshold of 0.01% (1 diseased cell in 10 000 cells), however most of the

current MRD monitoring techniques have a deeper detection range over 5-6 logs of disease.^{52,54} However, despite achieving deep responses such as MRD negativity, patients still relapse. This paints a picture of underlying, undetectable MRD that current methods are not sensitive enough to detect, or another disease component not monitored by current techniques.^{55,56}

The techniques currently implemented to detect MRD include multi-parametric flow cytometry (MFC), allele-specific oligonucleotide quantitative polymerase chain reaction (ASO-qPCR) and next-generation sequencing (NGS).^{52,57} MRD is primarily used as a prognostic indicator after treatment, with patients who have achieved MRD negativity having significantly longer PFS and OS than patients who achieved CR but still had detectable MRD.^{52,58,59} MRD has primarily been used immediately after therapy completion to predict possible relapse events, but recent clinical trials suggest monitoring MRD in MM patients periodically (e.g. once a year), or between treatment phases, to evaluate treatment response and thus adjust treatment accordingly, including initiating salvage therapy at an earlier time.⁶⁰ MRD assessment in MM may very well become an important pathology monitoring tool in the future.

1.1.4.B. Techniques Used to Monitor Initial Therapy Responses in MM

1.1.4.B.i. M-protein detection

Quantifying immunoglobulins in MM patients is used as a diagnostic technique^{3,61,62} to differentiate between SMM (> 3 g/dL) and MGUS,^{18,20} as well as monitoring patient response to treatment.⁵³ As previously mentioned, clonal PCs overproduce immunoglobulins which can be detected in serum or urine via protein electrophoresis.^{62,63} The protein contained in the serum or urine is separated via an electric current on a matrix (e.g. agar or cellulose acetate) and stained for visualisation (e.g. with Coomassie brilliant blue or Ponceau S) to produce 5 bands: albumin-, α 1-, α 2-, β -, and γ -globulin. The densities of the bands are then electronically analysed to produce a graph indicating the concentration of immunoglobulins in g/dL (see Figure 2).⁶²⁻⁶⁴ An increase in γ -globulins will be indicated as a spike on the graph, known as the M-spike (see Figure 2).⁶³ Serum/urine protein electrophoresis (SPEP/UPEP) is limited in its ability to identify the subtype of the overproduced γ -globulin and needs further immunofixation electrophoresis to do so.^{63,65} This technique is not very sensitive with a lower detection limit of 0.1 g/dL in serum and 200 mg/24-hour in urine.^{53,65}

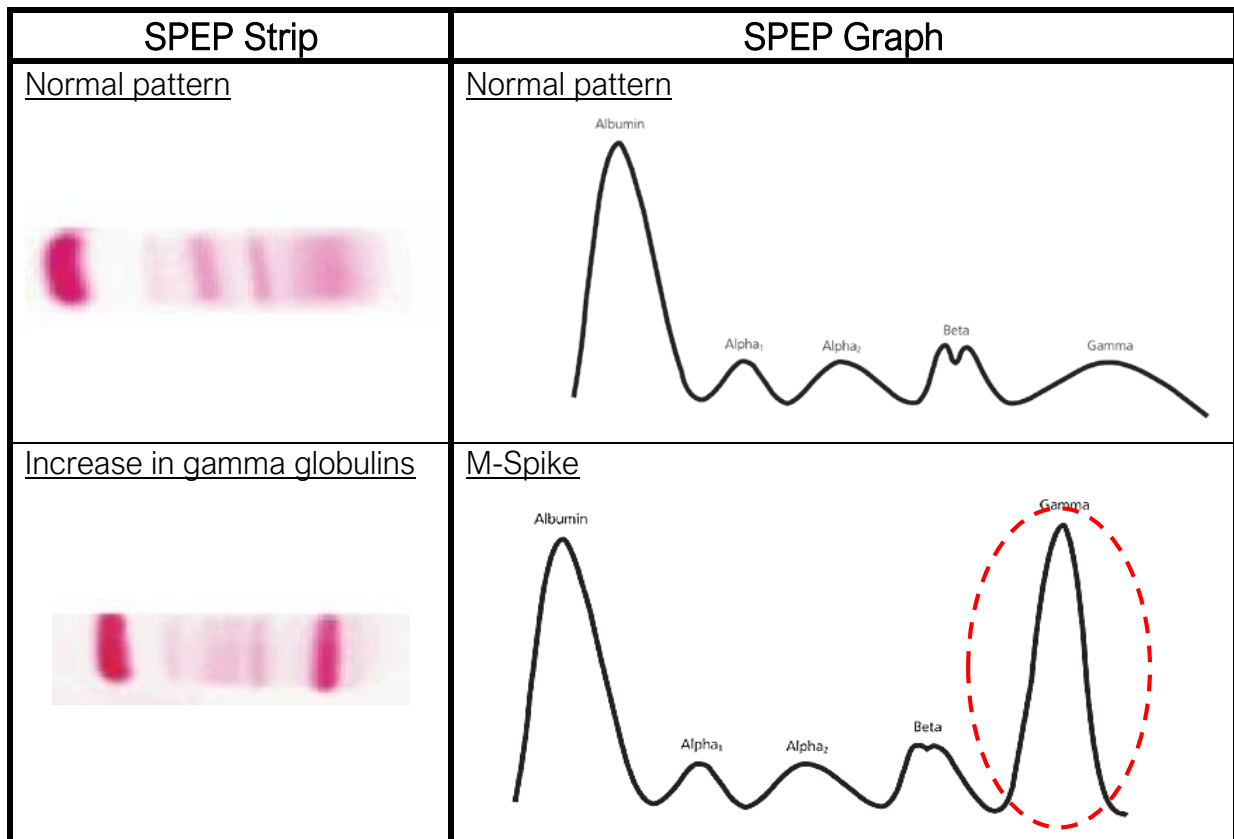


Figure 2: SPEP results illustrating a M-spike.

This figure illustrates what a normal SPEP result looks like compared to that with an increase in γ -globulins. The M-spike is indicated with a red circle. (modified from O'Connell et al.⁶² and Tripathy et al.⁶³)

1.1.4.B.ii. FLC ratio determination

Serum FLC assays are performed to determine the amount and ratio of unbound κ/λ -light chains in the serum of MM patients, as opposed to monitoring the number of intact immunoglobulins as in immunofixation.³ Normally there is a balance between κ and λ FLC production, with a κ/λ ratio of 0.26-1.65 mg/mL being normal,^{66,67} but in MM the secretion of a single FLC is enhanced due to the expansion of a monoclonal PC and ratio changes.⁶⁸ If the ratio is skewed below 0.26 lambda is the involved FLC, where kappa is over expressed if the ratio is > 1.65 ⁶⁶ with a lower detection limit of nearly 0.001 mg/mL.⁶⁹ While several commercial FLC assays exist to monitor FLC levels, only the Freelite® assay (Thermo Fisher Scientific, United States of America (USA)) is mentioned in the 2014 IMWG guidelines what year as it is was the only assay validated at a diagnostic level at the time.^{3,68} This assay works on the principle of Ab association with FLCs in the solution. The more immune complexes that form because of the antibody-antigen interactions, the more turbid the solution becomes. The amount of FLC can then be quantified by either measuring the turbidity (amount of light absorbed by the solution) or nephelometry (light scattered by the solution) of the sample.⁷⁰⁻⁷² Freelite® uses polyclonal Abs that are designed to bind solely to epitopes only exposed in FLC.^{68,69}

FLC assays were first implemented for screening and treatment monitoring of patients with oligo-secretory (FLC only), and non-secretory (non-producers) MM as they produce low (SPEP < 10g/L; UPEP < 200 mg/24-hour) or unmeasurable levels of M-protein respectively, that cannot be assessed with protein electrophoresis.^{19,68,73} However, the increased sensitivity of the serum FLC assay, compared to SPEP/UPEP made it useful in all cases³ and the serum FLC ratio is now included in the IMWG diagnostic criteria for MM (see Table 1), used as a prognostic indicator, and used to define the “stringent CR”, as shown in Table 3, in treatment monitoring.^{68,74}

1.1.4.C. Techniques Used to Detect MRD in MM

1.1.4.C.i. MFC use in MM

MFC enumerates malignant PCs in the patient sample by identifying an abnormal phenotype in comparison to the norm.⁷⁵ For MFC MRD detection the IMWG 2016 guidelines, suggest the EuroFlow 8-color 2-tube protocol.⁵² This relies on an 8-colour, 2-tube panel, with the first tube containing only surface staining antibodies, while the second tube analyses both surface antigens and cytoplasmic staining of immunoglobulin light chains κ and λ to assess clonality (CD138, CD38, CD45, CD19, CD56, CD27, CD81, CD117, CyIg κ and CyIg λ).^{52,57,75,76} This technique is very sensitive with detection limits varying between 10^{-4} and 10^{-6} diseased cells.^{52,57,75} A drawback of this technique is that MFC analyses should be performed on the BM aspirate sample within hours of harvest due to the propensity of PCs to internalise CD138, a key PC marker.⁵⁷ Another limitation of MFC is that the results only represents the state of the BM where the aspirate was taken and no other potentially affected areas or extramedullary disease, thus MRD can easily be missed.⁵⁷ It is also a time consuming, requires extensive expertise and is expensive.^{52,57}

1.1.4.C.ii. ASO-qPCR use in MM

ASO-qPCR in MM is used to quantify the amount of clonal PC deoxyribonucleic acid (DNA) in the BM aspirate, as a way of determining the level of BM disease and is considered to be the most sensitive polymerase chain reaction (PCR) based technique used for MRD in MM currently, rivalled only by NGS.^{77,78} Allele specific primers are used to amplify patient specific clonal mutations within the variable region (VDJ) of the immunoglobulin heavy-chain (IGH) gene.⁷⁹ To do this, the entire VDJ region of an enriched PC patient sample is amplified and sequenced to establish the specific dominant clonal rearrangement. Patient-specific primers are designed to enable amplification of these patient-specific rearrangements, which are then incorporated with hydrolysis probes, to allow real-time quantitative PCR of this region and quantification. It is possible to detect clonal DNA alleles to a sensitivity limit of 1×10^{-5} with this assay, which makes this technique suitable for MRD detection.⁷⁹ However, this technique has many limitations; patient-specific primers are expensive and initially time

consuming to design and can be difficult to identify in BM samples with a low PC content.^{57,79} The sensitivity limit is dependent on the quantity of DNA used, which means haemodilution and insufficient PCs in the BM can lower the sensitivity significantly.⁵⁷ MM patients often have somatic hypermutations in the VDJ region which can disrupt annealing of consensus primers and consequent mutation detection.⁷⁹ These hypermutations prevent the application of this monitoring technique in up to half of all MM patients.^{52,78}

1.1.4.C.iii. NGS use in MM

MRD detection in MM using NGS is an alternate way to evaluate the clonal PC VDJ rearrangement and thus the diseased PC content of the BM. Unlike the ASO-qPCR technique, NGS of the VDJ region does not require the use of patient specific primers/probes.^{80,81} Instead, the parallel sequencing approach allows for the identification of all the different VDJ rearrangements (different PCs) at the time of diagnosis, where the dominant clone can be identified, which is then specifically looked for in the analysis of follow-up samples. Sensitivity of this type of assay varies between 1×10^{-5} and 1×10^{-6} ,^{57,80} with enrichment of the PC from the BM sample required to achieve the deeper sensitivity.^{57,81} NGS is attractive when it comes to MRD detection in MM patients as it is very sensitive and can identify monitorable patient specific IGH mutations in all patients, unlike ASO-qPCR. The main drawback of NGS is that the kits and equipment are currently very expensive (especially here in South Africa) and the bioinformatics extensive, which has limited its use in the past in most routine pathology settings.^{52,57}

1.1.4.C.iv. Limitations of current MRD techniques

The above-mentioned MRD techniques all monitor clonal PCs within the BM microenvironment which require BM aspirate samples. This is a very painful and invasive procedure, and due to the recovery time and associated health risks of this procedure, there is a limit on how often it can be performed. This makes regular monitoring, once every one-to-three months, difficult⁵⁷ and is probably one of the reasons that MRD is currently not routinely used for disease monitoring in MM. With the use of BM samples there is always a risk of reporting false negative results, due to the hemodilution and scattered tumour burden within the BM microenvironment⁷⁵ and in extramedullary niches, such as the liver and the spleen.⁵⁷ The current MRD techniques are also all specialised, expensive and time consuming. These limitations restrict the widespread application of MM MRD disease monitoring, especially in resource-limited environments.^{52,56}

Unfortunately, despite the use of these newer, more sensitive techniques and advanced treatment protocols, patients achieving MRD-negative results still eventually relapse.^{19,52,57} This suggests that either the current therapies are not necessarily eradicating all of the residual malignant cells, which may in part be due to the lack of techniques to detect all of the malignant cells, or that the current MRD techniques all assume that the malignant population is confined to the PC compartment. If other cell

types, such as a SC progenitor are involved in the pathogenesis of MM, which is strongly suspected, the current monitoring techniques are insufficient.⁸² The involvement of a malignant SC in MM has long been suspected, driven by relapse after standard ASCT, and the development MM in immunodeficient mice transfected with MM SC.^{83,84} While a MM malignant SC has yet to be universally defined in the MM community, recent studies by Shires and Wienand et al.^{28,85} have demonstrated the abnormal expression of the cancer/testis antigen (CTA), melanoma antigen (MAGE)-C1, which is usually highly restricted to malignant tissue, in a population of SCs in both the BM aspirate and PB of MM patients,^{28,85} raising the possibility of a SC disease progenitor in MM.

The concept that PCs are not the only abnormal cell in MM pathogenesis and the need for a PB-based monitoring assay has stimulated research into alternative disease biomarkers in MM, as reviewed by Shires and Van Wyk in 2018.⁵⁶ Research in our laboratory over the last few years has concentrated on the use of CTAs in this MM biomarker capacity, with MAGE-C1 appearing to be an attractive target, as it is found to be expressed in early SCs circulating in the PB of all MM patients (expressed in all stages and subtypes of MM), can be monitored using either basic flow cytometry or quantitative real-time PCR (qRT-PCR) and appears to predict impending relapse.^{28,56,82,85}

1.2. CTAs

1.2.1. Introduction to CTAs

CTAs are a group of proteins with expression in normal tissue restricted to male germ cells and placental tissue, yet showing wide expression in different cancers.⁸⁶ To date, more than 250 different CTAs have been described⁸⁷ and grouped into 70 families based on sequence homology.⁸⁸ CTAs can be subdivided into two types: CT-X antigens and non-X CTAs, depending on whether the gene is encoded by the X-chromosome or the autosomal chromosomes.⁸⁹ Sequencing has revealed that over 50% of CTAs are located on the X-chromosome, occupying 10% of entirety of the chromosome (see Figure 3).⁸⁹

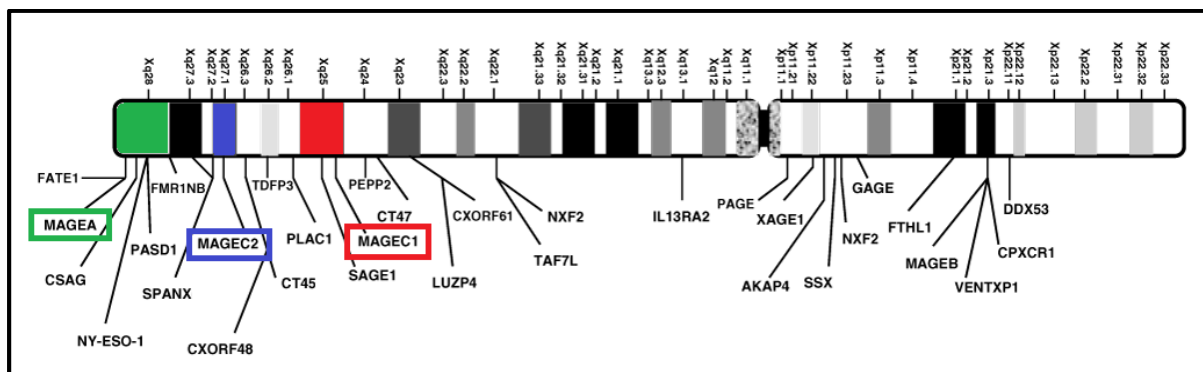


Figure 3: Arrangement of CTAs on the X-chromosome.

Mapping of identified CTAs on the X-chromosome that indicate the location CTAs of interest of this study in green, blue and red. (modified from Caballero et al⁹⁰)

The expression of CTAs in germ cells link these proteins to the gametogenesis process, where they have been shown to play a role in cellular processes like cell-cycling, apoptosis and steroid receptor pathways.^{91,92} Gametogenesis and cancer development share similar attributes such as migration, immunogenic and anti-apoptotic abilities,^{90,93} thus it is not surprising that CTA expression has also been linked to the development of many different cancer types.⁸⁶ However, the true functions of CTAs and the exact mechanism by which they lead to abnormal cell function is poorly understood due to a lack of detailed investigation.

1.2.2. The MAGE Family

1.2.2.A. MAGE Gene Family Characteristics

A subset of 40 CT-X antigens that share a 160-170 amino acid homology domain belong to the MAGE family.^{94,95} This protein group contains 10 subfamilies that are further ordered into two types based on their expression pattern and function.⁹⁵ Type I genes are expressed in highly proliferative cells, such as germ line cells and tumour cells, and belong to the subfamilies *MAGE-A*, *-B* and *-C*.⁹⁵ Their highly restricted expression identifies them as true CTAs. In contrast, type II *MAGE* genes are not characterised

as true CTAs as they are ubiquitously expressed in somatic cells and include genes from subfamilies *MAGE-D*, *-F*, *-H*, *-L2*, *NDN* and *NDNL2*.⁹⁵

The phylogeny of *MAGE* genes indicates that all three subfamilies of type I genes stem from a single origin-gene that is different from type II *MAGE* genes.⁹⁵ Sequence similarity suggests that *MAGE-C* was produced through DNA-based gene duplication of *MAGE-A*, with both of these *MAGE* subfamilies being expressed in similar tumour types.⁹⁵ Additionally, within the *MAGE-C* subfamily *MAGE-C1* and *MAGE-C2* display 73 - 77% nucleotide homology.⁹⁶ The noteworthy difference between these genes is the presence of a sizable repetitive sequence region in the coding region of *MAGE-C1* that is absent in *MAGE-C2*. Even so, the translated proteins are 57% identical.⁹⁶ Therefore, we propose that the function and mechanisms of *MAGE-C1* could be similar to that of the *MAGE-C2* and/or *MAGE-A* subfamilies due to their genetic similarity.

1.2.2.B. Function of *MAGE-A1* in *in Vitro* Models

The *MAGE* family appears to regulate signalling pathways related to cell-cycling and apoptosis in both spermatogenesis and cancer pathogenesis, yet only some family members being studied in some detail.^{56,91,92} *MAGE-A1* acts as a transcriptional repressor of Cyclin D1 expression by negating the function of the transcriptional co-regulator/adaptor protein, ski-interacting protein (SKIP), as illustrated in Figure 4. Under normal circumstances, SKIP is involved in the regulation of gene expression as it is a key binding protein that allows formation of protein complexes.⁹⁷ Laduron et al.⁹⁸ showed that *MAGE-A1* competitively binds to SKIP via the carboxyl-terminus (C-terminal) to create a heterodimer that obstructs the formation of the usual protein signalling complexes. An example of a pathway affected in this manner is the Notch1-intracellular domain (NIC) transcription activation signalling pathway.⁹⁹ Notch1 is a transmembrane receptor that regulates transcription. Its signalling is dependent on ligand-induced cleavage of, and subsequent release, of NIC.⁹⁸ In the absence of NIC (and ligand signalling) SKIP binds a repressor complex which recruits histone deacetylases (HDACs) and prevent transcription from taking place. When NIC is present (due to ligand signalling) SKIP binds NIC (replacing the repressor complex) which initiates transcription through the further recruitment of histone acetyltransferases (HAT) and co-activators.⁹⁸ In the presence of both NIC and *MAGE-A1*, *MAGE-A1* binding to SKIP is favoured which blocks activation of the SKIP/NIC transcription activation signalling pathway and subsequently represses transcription.⁹⁸

Additionally, *MAGE-A1*, in complex with SKIP, recruit's HDAC, which removes acetyl groups from histones, resulting in more condensed chromosome structures. This prevents opening of the chromosome, restricting ribonucleic acid (RNA) polymerase binding, and leading to repressed transcription from that specific promoter.⁹⁸

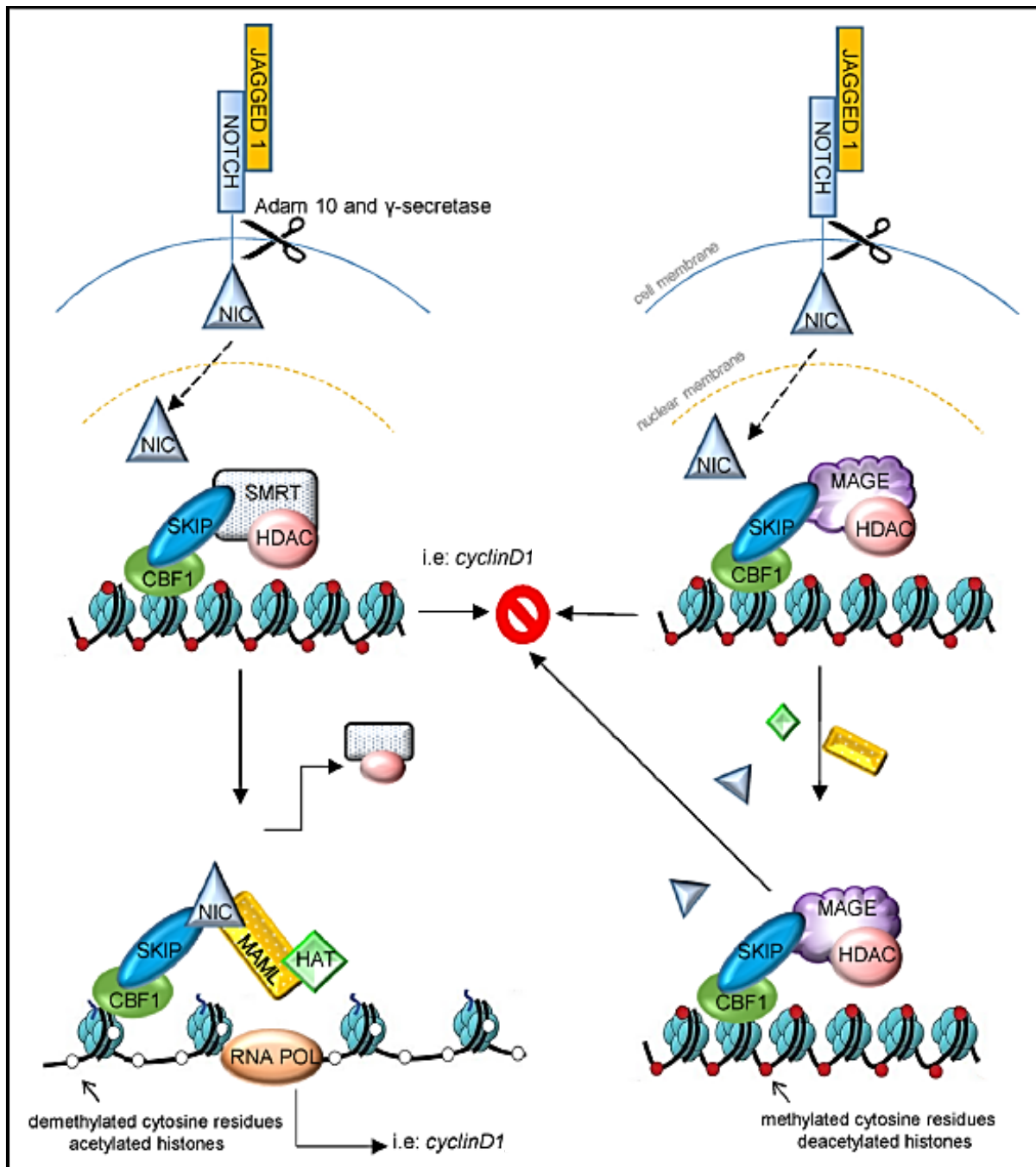


Figure 4: Binding of MAGE-A1 to SKIP acts as a transcriptional repressor.

This figure aims to illustrate how MAGE-A1 acts as a transcriptional repressor by competitively binding with SKIP in the presence of NIC and interfering with the NIC transcription activation signalling pathway. Under normal circumstances (shown on the left) the NIC of Notch1 is cleaved after cell signalling via ligand interaction, in this case Jagged 1. NIC then proceeds to bind to SKIP, knocking off the repressor complex, and recruiting HAT and co-activators leading to transcription activation. If present, MAGE-A1 binding to SKIP is favoured, which interrupts the transcription activation pathway (shown on the right). (copied from Shires et al.⁵⁶)

1.2.2.C. Function of MAGE-C2 in *In Vitro* Models

Other CTAs, such as MAGE-A3 and MAGE-C2 have been shown to have a post-translational influence on proteins by manipulating protein degradation pathways through complex formation with E3 ubiquitin ligases, that result in altered expression levels of functional proteins.^{56,100,101}

MAGE-C2 interacts with the Skp-Cullin-F-box (SCF) complex, interfering with the degradation of Cyclin E1 and affecting cell cycle progression (as shown in Figure 5). The SCF complex is a multi-protein E3 ubiquitin ligase that has a major role in regulating cell cycle protein levels by ubiquitination and targeting them for protein degradation.¹⁰² MAGE-C2 influences this pathway by binding to Really Interesting New Gene (RING) Box Protein-1 (RBX1),¹⁰² a protein in the SCF complex, preventing the addition of ubiquitin to Cyclin E1 and resulting in elevated intracellular Cyclin E1 levels. This in turn promotes cell cycle progression from G1 (resting) to S-phase (DNA replication) and cellular proliferation.¹⁰²

MAGE-C2 has also been shown to bind to E3 ligase (see Figure 5), Tripartite motif-containing 28 (TRIM28).^{100,101,103} TRIM28 is a repressor protein that suppresses protein expression levels in various ways, including acting as a binding platform for other effector proteins through its various binding domains.¹⁰⁰ The MAGE-C2/TRIM28 complex enhances ubiquitin activity, ramping up protein degradation. The exact mechanism of this is still unknown, but research suggests that it is due to enhanced stabilisation and/or recruitment of ubiquitinated E2 ligase by the MAGE-C2/TRIM28 complex.¹⁰¹ It has also been shown that TRIM28 targets tumour antigen P53 (TP53), a key tumour suppressor, for degradation through ubiquitination.¹⁰⁴ As illustrated in Figure 5, in the absence of MAGE-C2, ubiquitinated E2 ligase binds to the RING domain of TRIM28, while TP53 binds to the PB domain.¹⁰⁴ This brings the two proteins in close enough proximity for TP53 to be polyubiquitinated and degraded, regulating the levels of TP53 in the cell. In the presence of MAGE-C2, the CTA binds to the RING domain of TRIM28 via the MAGE Homology Domain (MHD) allows a second ubiquitinated E2 ligase to bind, which enhances the rate at which TP53 is polyubiquitinated and degraded.^{56,100,101,103} In the presence of lower TP53 protein levels, p21 expression is reduced which weakens the repressor effect it has on Cyclin dependant kinases (CDK) and Cyclin D, which ultimately results in cell cycle progression through the G1 checkpoint.^{90,101}

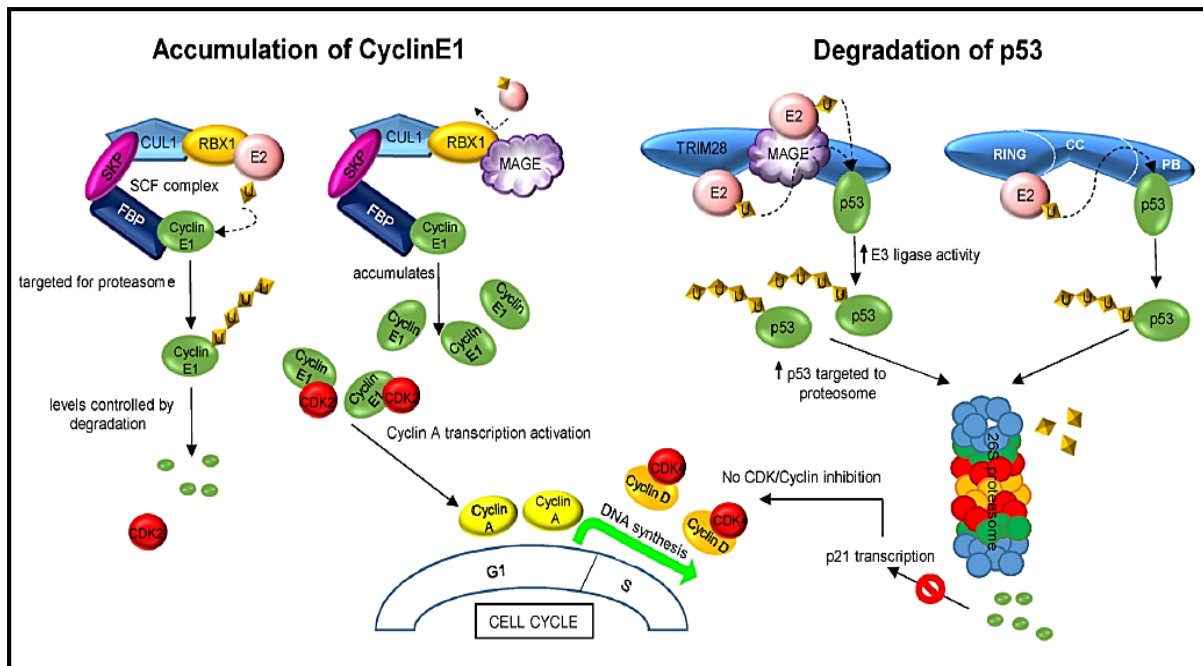


Figure 5: MAGE-C2 promotes cell-cycle progression by association with E3 ligases at post translational level.

Illustration showing the effects of MAGE-C2 on abnormal cell-cycle progression through either binding to the SCF E3 ligase complex (shown on the left), or by association with the TRIM28 E3 ligase complex (on the left). (copied from Shires et al⁵⁶).

1.2.3. Role of MAGE Proteins in MM Disease Pathogenesis

The hallmarks of MM pathogenesis include activated cell cycle progression via the promotion of Cyclin activity, and abnormal cell survival specifically through TP53 reduced activity and caspase inhibition.^{90,91,99} As described above, this quite obviously points to a possible role for CTAs, specifically MAGE proteins in the development and progression of MM. However, studies into CTA and MAGE function in MM have been superficial at this stage.

CTAs that have been found to be expressed in the BM and/or PB of MM patients are: *MAGE-C1*, *MAGE-A3/6*, *MAGE-A12*, *SP-17*, *PRAME*, *NYES01*, *SSX-1,2,5* and *BAGE*.^{82,90,105,106} Co-ordinated co-expression of MM-associated CTAs suggest that these antigens follow a cascade-like expression pattern, and that the number and type of CTAs specifically correlates with the stage of disease.^{82,105-110}

MAGE-A3 expression has previously been reported in MM patients and has been linked to increased cell survival.^{56,82,111,112} Through knockout studies it was found that MAGE-A3 binds to pro-caspase-12.¹¹² This inhibits the activation of caspase-12 from the endoplasmic reticulum (ER) stress response apoptotic pathway and so blocks down-stream activation of caspase-9 and caspase-3, making the MM cells resistant to ER-stress related cell death.¹¹² No further information about the role of MAGE-C2 or MAGE-A1 in MM is currently available, including the expression of these genes in MM patients.

With regards to MAGE-C1 expression and function in MM, our studies as well as those of other groups have shown expression in 80-100% of all symptomatic MM patients,^{56,82,87} which when combined with our research showing expression in a SC population,²⁸ suggests that this CTA potentially plays an important role in early pathogenesis of MM.⁸⁵ In MM cell lines specifically, gene expression knockdown has been used to link MAGE-C1 with increased cell-cycle activation via G1 to S-phase promotion and enhancement of anti-apoptotic processes.^{87,106,108} The mechanisms of how this occurs has however not yet been described.

To date, studies have highlighted the importance of CTAs in MM. However, few studies have investigated the exact function of these CTAs in MM to determine how they play a role in disease pathogenesis. To effectively use these interesting proteins as biomarkers of disease and develop assays for MRD detection, it is crucial to determine these mechanisms, specifically in the setting of MM disease pathogenesis. Due to gene sequence and protein similarities pointed out above, we suspect MAGE-C1 to have a similar function and mode of action as MAGE-C2, and/or MAGE-A1 and MAGE-A3.

1.3. RATIONALE FOR THE STUDY

MM is routinely treated with a combination of drug regimens and ASCT. However, this malignancy is still incurable, with hematological relapse an unfortunate certainty. Relapse after ASCT indicates that the current therapies are not eradicating the whole malignant clone and that MRD monitoring techniques are not detecting all residual diseased cells to predict relapse effectively. This suggests a SC progenitor component in the development of MM that needs to be potentially monitored.

Previous research in our laboratory focused on CTAs as biomarkers for MM disease development, and more specifically on MAGE-C1. MAGE-C1 expression was found in early SCs circulating in the PB of all MM patients at diagnosis,²⁸ with levels being able to be monitored in PB liquid biopsy at the protein or messenger RNA (mRNA) levels using a simple flow cytometric approach or qRT-PCR respectively. While we have other projects to refine the use of this biomarker as an MRD tool, it is essential that we link its expression directly to MM pathogenesis, which would strengthen its relevance as a disease biomarker. While little is known currently about its mechanism of action specifically, other MAGE proteins such as MAGE-C2, MAGE-A1 and MAGE-A3 have been studied as mentioned above. Due to the monophyletic evolution and markedly high homology of the MAGE proteins there is a strong possibility that MAGE-C1 has a similar mode of function to at least one of these other MAGE proteins.

1.4. AIMS AND OBJECTIVES

1.4.1. Aims

While the overall aim of the larger study (of which this is part) is to elucidate the function of MAGE-C1 in MM by investigating proposed functions similar to those described for MAGE-C2 and MAGE-A1, it was essential to first establish a suitable model to study these pathways. The primary aim of this study was thus to establish if we could utilise an *in vitro* approach, using the MM cell line RPMI-8226, by ensuring that all three MAGE and MAGE-binding proteins were expressed in this cell line and associated with one another. The secondary aim was to establish successful techniques and select relevant reagents that could be used in the next phase of the larger project.

1.4.2. Objectives

1.4.2.A. Bioinformatics Analysis of MAGE-A1, MAGE-C2 and MAGE-C1

- Investigate homology of MAGE-C1 to MAGE-C2 and MAGE-A1 to identify key functional domains/motifs to predict function of MAGE-C1.

1.4.2.B. Investigate Association of MAGE-C1 with MAGE-A1 Functions

- Establish if MAGE-A1, MAGE-A1 binding partner SKIP and MAGE-C1 were expressed in MM cell line RPMI-8226 at both the protein and mRNA levels (western hybridisation and rRT-PCR).
- Investigate if SKIP directly associated with MAGE-C1 using co-immunoprecipitation (Co-IP) technology, with the known MAGE-A1 association as a positive control.
- Establish cytoplasmic and/or nuclear localisation of the proteins of interest using immunofluorescence microscopy (IFM).
- *The original final objective was to activate the Notch 1 transcription activation signalling pathway, to assess if this pathway was affected in this cell line (in line with the proposed functions of MAGE-A2). However, we were unable to source Jagged 1 or any other molecule that could activate this specific pathway and thus could not complete this objective.*

1.4.2.C. Investigate Association of MAGE-C1 with MAGE-C2 Functions

- Establish if MAGE-C2, MAGE-C2 binding partners RBX1 and TRIM28 were expressed in MM cell line RPMI-8226 at both the protein and mRNA levels (western hybridisation and rRT-PCR).
- Investigate if RBX1 directly associated with MAGE-C1 using Co-IP technology, with the known MAGE-C2 association as a positive control.

- Establish cytoplasmic and/or nuclear localisation of the proteins of interest using IFM.
- The ER-stress response was induced to investigate if the cell-cycle status of the MM cell line changed in an abnormal manner to this cellular stress.
 - Cell cycle analysis post ER-stress using PI staining and flow cytometry.
 - The levels of p21, Cyclin A1 and Cyclin A2 mRNA were evaluated using rRT-PCR to establish their possible role in the stress response (in line with a MAGE-associated response).
 - The levels of Cyclin E and TP53 protein were evaluated to establish if these key proteins were affected by the external stress, as predicted by the association of MAGE-C2 with E3 ligases (western hybridisation)

CHAPTER 2: MATERIALS AND MEHODS

2.1. BIOINFORMATICS ANALYSIS

The following human MAGE reference protein sequences from the National Center for Biotechnology Information (NCBI)¹¹³ were used in the comparison bioinformatics section of the study: MAGE-A1: NP_004979.3, MAGE-C2: NP_057333.1 and MAGE-C1: NP_005453.2. Additional information from the UniProt protein database¹¹⁴ was acquired using the following sequences: MAGE-A1: P43355, MAGE-C2: Q9UBF1 and MAGE-C1: O60732.

To predict homology, structure and function of the protein, the amino acid (aa) sequences of the proteins were pairwise aligned to score identity. The sequences were analysed using the BioEdit (version 7.2.5)¹¹⁵ sequence alignment editor programme. Pairwise alignment of two sequences was used to score identity that may indicate structural/functional/evolutionary relationships.^{116,117} When the whole protein aa sequences were aligned local pairwise alignment was chosen because of the differences in length between MAGE-C1 (1142 aa), and MAGE-A1 (309 aa) and MAGE-C2 (373 aa), respectively.¹¹⁸ When the shorter MHD, comprised of winged helix (WH) 1 and WH2 domains, was aligned, global pairwise alignment was used as it was more appropriate for sequences more closely related in terms of length, than local pairwise alignment.¹¹⁸ ClustalW multiple alignment, the most commonly used multiple alignment tool using a hidden Markov model alignment algorithm,^{119,120} was used to investigate the homology between the 35 aa repeat units in MAGE-C1.

In addition, aa that have been predicted or confirmed as post translational modification sites, were identified in all three MAGE proteins, using the following databases: PhosphoSitePlus®, NCBI, UniProt and Interpro.^{113,114,121,122}

2.2. SAMPLES USED IN THE STUDY

2.2.1. Cultured Cells

MM cell line RPMI-8226 (passage number unknown), kindly donated by Professor Djordje Atanackovic (Atanackovic laboratory, University Cancer Centre, Hamburg) was used as the experimental cell line in this study, along with Jurkat (obtained from the American Type Culture Collection), a cancerous human T-lymphocytic cell line¹²³ used as a negative control in several experiments. The donated RPMI-8226 cell line was previously genotyped upon implementation in our laboratory using the PowerPlex® 16 System multiplex STR system (Promega, USA). Cells were cultured in complete RPMI-1640 culture media: RPMI-1640 media (SIGMA, USA)/10% foetal bovine serum (FBS) (SIGMA, USA)/1% Penicillin-Streptomycin (PS) (SIGMA, USA), with incubation at 37 °C with 5% carbon dioxide (CO₂). The cells were counted every two to three days using 0.5% trypan blue (distinguishes live/dead cells) and a hemocytometer.¹²⁴ Complete media was added accordingly to maintain the cell density of the RPMI-8226 cell line between 5×10^5 and 2×10^6 viable cells/mL and the Jurkat cell line at $1-2 \times 10^5$ viable cells/mL for optimal expansion. Growth curve analysis was done to confirm the logarithmic growth phase of RPMI-8226 cells two to three days post passage.

Cultured cells were tested for mycoplasma contamination via Hoechst (fluoresces blue at 460-490 nm) staining (modified from Young et al.¹²⁵). Before staining, 2 mL of each culture was plated in a 35 mm dish in PS-free media (media with added FBS (10%)) and incubated for 48 hours at 37 °C with 5% CO₂ to allow mycoplasma to multiply in the absence of the antibiotic cocktail. A sample (10 µL) of culture was spread onto a sterile microscope slide and allowed to air dry, after which they were fixed by flooding the slide with fixing solution (1:3 ratio of glacial acetic acid (MERCK, USA) and methanol (LAB-SCAN, Poland)) and incubated at room temperature (RTP) for 2 minutes (min). Following washing of the slide with distilled water, the fixed cells were covered with Hoechst staining solution (5 ng/mL) Hoechst stain (SIGMA, USA) in $1 \times$ phosphate buffered saline (PBS) (pH 7.3 ± 0.2) (OXOID, Ireland)) and incubated at RTP for 5 min before rinsing with distilled water. After the excess water was removed, the cells were covered with mounting fluid (0.1 M citric acid (Sigma-Aldrich, USA) 0.2 M disodium phosphate (Glenthams Life Sciences, United Kingdom (UK)), 50% glycerol (May&Baker, England)) and covered with a standard coverslip. The slides were viewed under $\times 100$ magnification using a fluorescent microscope (Olympus BX41) and a triple filter (U-DM-DA/FI/TR2) (excitation: 400-550 nm, emission: 455-570 nm). Photographs were taken using the Olympus DP73 camera mounted to the microscope and cellSens Dimension software (version 1.15) (OLYMPUS, JAPAN).

2.2.2. Peripheral Blood Mononuclear Cells

Human peripheral blood mononuclear cells (PBMNC) were extracted from ethylenediaminetetraacetic acid (EDTA) PB collected from the same, healthy individual (multiple collections) using Ficoll

Histopaque (SIGMA, USA), according to the manufacturer's instructions.¹²⁶ The volume of the freshly drawn PB (5-10 mL) was increased to 10 mL with 1 × PBS (pH 7.3 ± 0.2) (OXOID, Ireland) and carefully layered on top of 5 mL Ficoll Histopaque (Sigma-Aldrich, USA) in a sterile 15 mL conical tube. The density gradient was created by centrifugation of the layered sample at RTP for 30 min at 3000 revolutions per minute (rpm). The band of enriched PBMNC was transferred to a new tube and gently washed with 15 mL 1 × PBS, followed by centrifugation for 5 at 1500 rpm. The supernatant was carefully removed, and the cell pellet re-suspended in 1 mL 1 × PBS, to allow counting with a hemocytometer¹²⁴ prior to treatment with methanol fixation or RNA isolation, depending upon the requirements (described below).

These samples were obtained from a healthy participant following informed consent and the study (including the use of this particular cell line) was approved by the Human Research Ethics Committee of the University of Cape Town Faculty of Health Sciences (ethics number: HREC REF No.989/2023).

2.2.3. Commercially Sourced CTA RNA Positive Control

The main positive control for the expression of CTAs used in this study was commercially available human testis RNA (Clontech, USA) (1 µg/µL). The RNA was stored in single use aliquots at -80 °C until required.

2.3. mRNA EXPRESSION STUDIES

2.3.1. Primer Design

PCR primers used to analyse gene expression were designed specifically for this project to amplify regions of mRNA and checked for amplification specificity using the NCBI primer Basic Local Alignment Search Tool (BLAST+ 2.9).¹²⁷ The primers were designed to be approximately 20 bases long, amplify products < 300 base pairs (bp), no formation of internal hairpins or primer-dimers. To prevent amplification from co-extracted contaminating DNA and limit it to amplification from transcripts only, at least one primer per pair was designed to bind across an exon junction. Table 5 shows the primer pairs that were designed and used for this study (synthesized by either Integrated DNA Technologies™ (USA) or Inqaba Biotec™ (South-Africa)).

Table 5: Primer sets used in this study.

Gene	Primer sequence	Amplicon size	Reference sequence
<i>MAGE-A1</i>	F#: GAG GAG GCA AGG TTT TCA G R\$: GGA GAG GAG GAG GAG GTG	259 bp	NM_004988.5
<i>MAGE-C2</i>	F: TGC CGT GAG ACT GGT GCT R: GAA TGG AAC GCC TGG AAC	200 bp	NM_016249.4
<i>MAGE-C1*</i>	F: GAG CTG TAA GCC GGC CCT TT R: GAG AGG ATA CAG GGT GTC GTC	250 bp	NM_005462.2
<i>SKIP</i>	F: AAT TGC TCG ACA AGG ACA G R: TTA TCT CTT TAA TAG CTT CTT CAT C	130 bp	NM_001318844.2
<i>TRIM28</i>	F: CCA TAC TGT GCG CTC TAC TG R: CTG GTA CTG GTG GTC CTT G	163 bp	NM_005762.3
<i>RBX1</i>	F: GGT TGA TAA CTG TGC CAT CTG R: GCG AGA GAT GCA GTG GAA G	145 bp	NM_014248.4
<i>CCND1</i>	F: CAG ATC ATC CGC AAA CAC R: CGG TAG TAG GAC AGG AAG TT	157 bp	NM_053056.3
<i>p21</i>	F: AGG AGG CGC CAT GTC AGA AC R: CCT CCA GTG GTG TCT CGG TGA	191 bp	NM_000389.5
<i>Cyclin A1</i>	F: TTT GCC TGG CAA ACT ATA CTG R: CTC CAT GAG GGA CAC ACA CA	197 bp	NM_001111045.4
<i>Cyclin A2</i>	F: ATC AGT TAT TGC TGG AGC TGC R: CTC TGG TGG GTT GAG GAG AGA	229 bp	NM_001237.5
<i>ABL*</i>	F: GGAGATAACACTCTAAGCATAACTAAAGGT R: CGT GAT GTA GTT GCT TGG GAC	127 bp	NM_14752.1

Forward sequence;\$ Reverse sequence; *Previously validated primer set.

2.3.2. RNA Extraction from Cultured Cells and PBMNC

Total RNA was extracted from all human cell types described in section 2.2.1. and 2.2.2. using the silica-column based QIAamp® RNA Blood Mini Kit (Qiagen, The Netherlands), according to the manufacturer's instructions.¹²⁸ Samples were processed quickly, solutions kept on ice and ribonuclease (RNase) free tips and tubes used to try to limit possible mRNA degradation. Briefly, 1×10^7 cells (described above in section 2.2.) were pelleted via centrifugation (1500 rpm/5 min) and the supernatant

removed via aspiration. Culture cell and PBMNC pellets (section 2.2.1 and 2.2.2.) were immediately re-suspended in 600 μ L Buffer RLT/1% β -mercaptoethanol (β -ME) (MERCK, USA) (β -ME added according to manufacturer's instructions to inhibit RNases) to lyse the cells and make RNA accessible for extraction, while cultured cells were first washed twice to remove residual FBS (which would interfere with the extraction process), by re-suspending the pellet in 10 mL ice-cold RPMI-1640 media (Sigma, USA), without FBS or PS, and collecting the cells again via centrifugation (1500 rpm/5 min).

These cell/lysis buffer mixes were then homogenised by passing through a QIAshredder Spin Column (Qiagen, The Netherlands) (12000 rpm for 2 min) that shears high molecular weight DNA into smaller fragments and also breaks up high molecular weight macromolecules, lowering the viscosity of the lysate and allowing more efficient binding of the RNA to the silica. Next, 70% ethanol (KIMIX, South-Africa) (600 μ L) was added to the homogenised cell lysate to dehydrate the nucleic acids and enhance silica binding, before loading onto the silica QIAamp Spin Column (Qiagen, The Netherlands). RNA was trapped on the silica membrane via centrifugation of the sample for 15 seconds (sec) at 10 000 rpm. The silica bound RNA was washed to remove DNA and other cellular components by the addition of 500 μ L RW1 and RPE buffers sequentially, followed by centrifugation of the column for 15 sec at 10 000 rpm for each wash. As these buffers and residual ethanol can interfere with down-stream applications, the column was spun for a final 1 min at 12 000 rpm. Lastly, 30 μ L of RNase-free water (to change pH and salt concentration) was carefully pipetted directly onto the QIAamp silica membrane and the column centrifuged for 1 min at 10 000 rpm to elute the RNA. Spectrophotometry, using a Nanodrop (Nanodrop Technologies, USA) was used to determine the quantity of RNA extracted (approx. 0.5-1 μ g/ μ L per 1×10^7 cells), with 260 nm:280 nm ratios of > 1.8 indicating protein-free RNA preparations. Due to the instability of RNA, 10 μ L single use aliquots were prepared and stored at -80 °C until required.

2.3.3. Reverse Transcription

Complimentary DNA (cDNA) was prepared for all subsequent PCR analysis using the following method,¹²⁹ with care taken to work quickly on ice, with filtered deoxyribonuclease/RNase-free pipette tips and nuclease-free reagents to preserve RNA integrity and prevent contamination. A total of 1 μ g of previously prepared RNA (section 2.3.2.) or Human Testis RNA, in a total volume of 10 μ L nuclease-free water was denatured at 70 °C for 10 min, followed by immediate incubation on ice for 10 min, to prevent reformation of secondary structures. Next, 10 μ L reverse transcription (RT) reaction mix was added to each denatured RNA sample (10 μ L), containing the following components: 5 μ M dithiothreitol (DTT) (Invitrogen, USA), 1 $\times$ First Strand buffer (Invitrogen, USA), 1 μ g random hexamers (Inqaba Biotec, South-Africa), 1 mM deoxynucleotide (dNTP) mix (Promega, USA), 100 U Superscript III (Invitrogen, USA), 20 U RNase inhibitor (Invitrogen, USA). Samples were immediately

mixed and placed into a pre-heated thermocycler (GeneAmp PCR System 9700, Applied Biosystems) for the following cycles: 10 min at 22 °C (to allow random hexamer primer annealing), 45 min at 42 °C (to allow for the efficient action of Superscript III) and 3 min at 99 °C (to denature the RT enzyme as it will inhibit downstream PCR applications). Completed RT reactions were either used immediately (held on ice until addition to PCR reaction) or stored at -80 °C until required (with no freeze-thaws to prevent sample degradation).

2.3.4. Confirmation of PCR Primer Specificity

2.3.4.A. Primer Amplification

To establish the specificity of each primer set, Human Testis cDNA (prepared according to section 2.3.3.) was used as a positive control to test the primer pairs in a basic PCR reaction. Resulting amplicons were then analysed via Sanger sequencing to assess specificity of the amplified product (described below in section 2.3.4.B.) The following PCR reaction mix (25 µL total) was prepared for each primer pair: 1 × GoTaq Green reaction buffer (Promega, USA), 1.5 mM magnesium chloride (MgCl₂) (Promega, USA), 25 pmol of each primer, 1.5 U GoTaq HotStart (Promega, USA), 1.6 mM dNTP mix (Promega, USA) and 2 µL cDNA from section 2.3.3. The following PCR cycling conditions were used on a GeneAmp™ PCR System 9700 (Applied Biosystems, USA): The initial denaturation cycle was 2 min at 96 °C, then 35 cycles with a denaturation phase of 20 sec at 95 °C, an annealing phase of 30 sec at 60 °C (56 °C for SKIP primer pair) and an extension phase of 30 sec at 72 °C. The reaction was concluded with a final extension cycle of 10 min at 72 °C to ensure complete strand extension. The buffer contained glycerol and loading dyes so the PCR products were loaded directly onto a 2% w/v agarose (Bioline, UK)/0.1% ethidium bromide (BioRad, USA)/1 × Tris-acetate-EDTA (TAE) (40 mM trisaminomethane (Tris) (Glenthams Life Sciences, UK), 1 mM EDTA (Sigma-Aldrich, USA), 0.1% v/v glacial acetic acid (MERCK, USA)) gel and electrophoresed at 70 V for 70 min in 1 × TAE buffer. A low molecular weight DNA ladder (New England Biolabs, USA) was used as a size marker. An image of the separated amplicons was generated by ultraviolet exposure using the UVItect gel documentation system (Uvitec, UK).

2.3.4.B. Amplicon Sequence Analysis

The amplicons of interest were excised from the agarose gel. Following manufacturer instructions, a silica-based column method (Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel, USA) was used to extract the DNA.¹³⁰ To dissolve the excised agarose gel it was incubated at 50 °C for 10 min (vortex periodically) together with 200 µL Buffer NTI. Buffer NTI aids in gel dissolution and creates optimal binding conditions for the double-stranded DNA to adhere to the silica. The dissolved gel

mixture was loaded onto the NucleoSpin® Gel Clean-up column and the DNA forced onto the silica disk via centrifugation (30 sec/12 000 rpm). A washing step followed where 650 µL ethanol-based wash buffer NT3 was added to the membrane and centrifuged (30 sec/12 000 rpm). The ethanol enhances DNA binding to the silica membrane, by dehydrating the phosphate backbone. After discarding the flow-through, the washing step was repeated before a final centrifugation (1 min/12 000 rpm) to remove any remaining solution (especially ethanol which interferes with down-stream applications). The DNA was eluted in 25 µL Buffer NE (an alkaline tris-EDTA buffer that stabilises the DNA and promotes DNA-silica disassociation) placed directly onto the membrane, incubating for 1 min at RTP, and centrifuging (1 min/12 000 rpm). Spectrophotometry (260 nm) was used to determine the DNA concentration of the cleaned product (Nanodrop, Nanodrop Technologies, USA).

PCR-products were Sanger sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) to perform the sequencing reaction. PCR products were sequenced using both forward and reverse primers as follows: 1:10 dilution Big Dye sequencing enzyme, 1 × Big Dye buffer (contains fluorochrome-labelled dNTPs), 5 pmol PCR primer and 100 ng DNA (cleaned PCR-product). The sequencing reaction was run on a ProFlex PCR System thermocycler (Applied Biosystems, USA) as follows: 2 min at 96 °C, 30 cycles consisting of 10 sec at 96 °C, 15 sec at 55 °C, and 3 min at 60 °C per cycle, and a final hold of 7 min at 60 °C.

The sequencing reaction was purified using the Sodium acetate/ethanol precipitation method¹³¹ prior to loading on the ABI 3500 Genetic Analyser (Applied Biosystems, USA) to remove excess primers and labelled dNTPs. To do this, 10 µL PCR product was added to a well on a 96-well plate (Applied Biosystems, USA) and mixed with 2 µL 3M Sodium acetate (Sigma-Aldrich, USA) and 25 µL ice-cold 100% ethanol (KIMIX, South-Africa), to precipitate the sequencing products. These were pelleted by centrifugation (30 min/2000 rpm/RTP), and the pellets washed twice by the addition of 50 µL ice-cold 80% ethanol (KIMIX, South-Africa) and repeated centrifugation (2000 rpm for 5 min). Finally, to ensure all ethanol was removed, the plate was centrifuged inverted on tissue paper for 5 sec at 400 rpm. The DNA precipitates were re-dissolved and denatured by adding 10 µL Hi-Di™ Formamide (Applied Biosystems, USA), sealing the plate with the appropriate rubber septa and heating the plate on the GeneAmp 9700 thermocycler (Applied Biosystems, USA) at 95 °C for 2 min before cooling to 4 °C for 5 min. The products were separated on a ABI 3500 Genetic Analyser (Applied Biosystems, USA) using 36 cm capillaries filled with POP7 and the following run conditions: Oven temperature: 60 °C, Pre-run voltage: 15 kV, Run voltage: 15 kV, Injection voltage: 1.2 kV, pre-run time: 180 sec, run time: 1080 sec, injection time: 8 sec. The sequences were analysed using the BioEdit 7.2.5¹¹⁵ sequence alignment editor programme. ClustalW Multiple Alignment was used to align sequences to the reference sequences (listed in Table 5).

2.3.5. rRT-PCR Analysis Using SYBR-Green

Expression of the various genes of interest in the different cell preparations was assessed using real-time PCR with SYBR-Green incorporation. For all assays, cDNA from each sample type was prepared according to section 2.3.3. Human Testis RNA was used as a positive control, a reagent blank was included as a control for contamination, along with the expression of the house-keeping/reference gene Abelson tyrosine-protein kinase 1 (*ABL*) that acted as a control for RNA integrity and cDNA reaction efficiency. The general rRT-PCR reaction used was as follows: 20 μ L reaction mix containing 1 $\times$ QuantiNova SYBR-Green PCR master mix (Qiagen, The Netherlands), nuclease-free water (Promega, USA), 25 pmol of each primer (detailed in Table 5), and 2 μ L cDNA reaction. The PCR cycling and fluorescent detection was performed on a Rotor-Gene Q thermocycler (Qiagen, The Netherlands), with Rotor-Gene Q Pure Detection software (version 2.3.5). PCR program: 2 min at 95 °C, 40 cycles of 20 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C per cycle. Fluorescence was acquired during the extension phase (as SYBR-Green is an intercalating dye) in the green channel using an excitation wavelength of 470 ± 10 nm and a detection wavelength of 510 ± 5 nm. This was followed by a melt curve analysis that started at 72 °C and ramped up to 95 °C in 0.5-degree intervals.

Table 6: Example of how the Livak and Schmittgen (2001)¹³² $2^{-\Delta\Delta Ct}$ calculations are performed.

Ct* from rRT-PCR experiment:		
	Experimental Cell line (R)	Control cell line (H)
Experimental gene (X)	16,57	21,34
Housekeeping gene (A)	20,86	22,24
Step 1: Normalise expression of the experimental gene transcript (X) to the house-keeping gene transcript (A) in the control (H) and experimental (R) cell line, respectively. Do this by subtracting the house-keeping transcript (A) Ct from the experimental transcript(X) Ct. Delta Ct: $\Delta Ct(R) = Ct(X) - Ct(A)$ $= 16,57 - 20,86$ $= -4,29$ $\Delta Ct(H) = Ct(X) - Ct(A)$ $= 21,34 - 22,24$ $= -0,9$		
Step 2: Normalise the expression of the experimental gene in the experimental cell line ($\Delta Ct(R)$) to the experimental gene in the control cell line ($\Delta Ct(H)$) by subtracting $\Delta Ct(H)$ from $\Delta Ct(R)$. Delta Delta Ct: $\Delta\Delta Ct(R) = \Delta Ct(R) - \Delta Ct(H)$ $= -4,29 - (-0,9)$ $= -3,39$		
Step 3: To determine the fold difference of experimental gene expression (X) in experimental cell line (R) to the experimental gene expression (X) in the control cell line (H), plug the Delta Delta Ct value in the equation of Livak and Schmittgen (2001)¹³²: $2^{-\Delta\Delta Ct}$ Fold difference in expression: $= 2^{-\Delta\Delta Ct(R)}$ $= 2^{-(-3,39)}$ $= 10,48 \text{ times more of transcript X in cell line R than in cell line H}$ If result is <1 indicating a reduction then: $= 1/2^{-\Delta\Delta Ct(R)}$ $= Y \text{ times less of transcript X in cell line R than in cell line H}$		

* = Crossing point (Ct)

The real-time amplification graphs were analysed in linear scale by switching on ‘Dynamic Tube’ and ‘Slope Correct’. The samples were individually checked for amplification efficiency by checking for a sigmoidal amplification curve and amplification of *ABL* transcripts. The melt curves were performed to ensure the amplification of a single product only. The threshold was set to a fluorescence level where all the samples were in the logarithmic phase of amplification (0.02) to determine crossing points (Ct) for either Delta Ct (ΔCt) or Delta Delta Ct ($\Delta\Delta Ct$) calculations¹³² to determine normalised expression levels of relevant genes, using the following calculation: $2^{-\Delta\Delta Ct}$ as explained in Table 6.

2.4. PROTEIN EXPRESSION STUDIES THROUGH WESTERN HYBRIDISATION

2.4.1. Protein Extraction

Protein was extracted from prepared cells using Radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor (Ptl) cocktail to limit protein degradation during extraction.¹³³ All extraction steps were also performed on ice to limit proteinase activity. For each extraction approximately 1×10^7 cells (prepared in section 2.2.1. and 2.2.2.) were washed in 1 mL ice-cold $1 \times$ PBS (OXOID, Ireland) buffer to remove any residual buffers, collected via centrifugation (5 min/1500 rpm/RTP) and the pellet re-suspended in 100 μ L RIPA solution (150 mM sodium chloride (NaCl) (Glentham Life Sciences, UK), 1% Triton X-100 (SIGMA, USA), 0.1% sodium dodecyl sulfate (SDS) (SIGMA, USA), 10 mM Tris pH 7.5 (Glentham Life Sciences, UK), 1% deoxycholate powder (Sigma-Aldrich, USA) and $1 \times$ Ptl cocktail (ROCHE, Switzerland). To aid in the cell lysis process, the mixture was frozen at -80 °C overnight (O/N) and thawed on ice the following day. The remaining cell debris was pelleted by centrifugation (20 min/12 000 rpm/4 °C) and the protein-containing supernatant was collected. The protein content was quantified using the Bicinchoninic acid (BCA) assay (described below in section 2.4.2.), before being stored at -80 °C in aliquots until needed.

2.4.2. BCA Assay

Protein concentrations were determined using the BCA assay (Thermo Fisher Scientific, USA), and compared to a bovine serum albumin (in RIPA solution) standard curve (2000 μ g/mL, 1000 μ g/mL, 500 μ g/mL, 125 μ g/mL, and 0 μ g/mL) for quantification. Samples and prepared standards (10 μ L) were added to separate wells in a 96-well plate (in duplicate), and 200 μ L freshly prepared working solution (50 + 1 ratio of BCA-reagent A to BCA-reagent B) added according to the assay manufacturer's instructions.¹³⁴ The plate was incubated at 37 °C for 30 min, allowing time for the biuret reaction and subsequent chelation of BCA with the cuprous ions from the biuret reaction. Formation of the BCA/copper complex results in the purple colour change, with an absorbance at 562 nm, that is directly proportional to the amount of protein present in the sample.¹³⁴ After incubation, the plate was allowed to cool to RTP and then the absorbance of each standard and sample measured on a spectrophotometer (GloMax Multi+ Detection system (Promega, USA)) at 562 nm. A standard curve was created manually using Excel (Microsoft), using the average absorbance reading of each standard, plotted against the known bovine serum albumin (BSA) concentrations, with a trend line and line equation then used to determine the concentration of the unknown samples using the average of the duplicate readings.

2.4.3. Protein Immunoblotting

2.4.3.A. Protein Separation and Membrane Transfer

Protein expression was analysed by western hybridisation, using chemiluminescence detection of Ab binding using the Clarity™ Western ECL Substrate kit (Bio-Rad Laboratories, USA). Acrylamide gels used for the separation of proteins ranged from 10-15% (w/v) acrylamide for the resolving gel depending upon the size of the protein of interest, with a 5% stacking gel. The resolving gel contained the following: 10, 12 or 15% w/v acrylamide (SIGMA, USA)/bis-acrylamide (AMRESCO, USA), 0.375 M Tris (pH 8.8) (Glenthams Life Sciences, UK), 0.1% SDS (SIGMA, USA), 0.1% ammonium persulfate (Glenthams Life Sciences, UK), and 0.1% tetramethylethylenediamine (TEMED) (SIGMA, USA). The stacking gel contained the following: 5% w/v acrylamide/bis-acrylamide, 0.19 M Tris (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, and 0.1% TEMED. The relevant volume of quantified protein RIPA extract (section 2.4.1 and 2.4.2.) (25-50 µg) was mixed with RIPA buffer (without PtI) to a final volume of 23 µL, mixed with 1 µL 100 mM DTT (Promega, USA) (to aid in protein denaturation), and 6 µL 5 × SDS loading dye (10% SDS (SIGMA, USA), 0.04% Bromophenol blue (SIGMA, USA), 0.25 M Tris pH 6.8 (Glenthams Life Sciences, UK), 30% glycerol (May&Baker, England) and 5% β-ME (MERCK, USA)) and boiled for 5 min to ensure complete protein denaturation. The entire 30 µL denatured sample was loaded into the relevant prepared well of the gel, along with a 20-120 kDa pre-stained protein low molecular weight ladder (PepLab Biotechnologie, Germany) and electrophoresed at 100 V for 2 hours in 1 × running buffer (3.5 mM SDS (SIGMA, USA), 25 mM Tris (Glenthams Life Sciences, UK), 0.2 M Glycine (Glenthams Life Sciences, UK)) using the Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, USA).

The separated proteins were transferred from the gel onto 0.2 µm nitrocellulose membrane (Bio-Rad Laboratories, USA) using the Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, USA), in ice-cold 1 × transfer buffer (30 mM Tris (Glenthams Life Sciences, UK), 0.2 M Glycine (Glenthams Life Sciences, UK) in 20% v/v Isopropanol (KIMIX, South-Africa)) for 1 hour at 100 V before proceeding to Ab hybridisation. The success of the protein transfer was assessed by staining the membrane with Ponceau S stain (0.1% w/v Ponceau S (Sigma-Aldrich, USA) in 5% v/v acetic acid (MERCK, USA)) for 2 min. The presence of pink bands along the length of the well indicated successful protein transfer.

2.4.3.B. Ab Hybridisation and Detection

For Ab binding, the area of the membrane not coated with protein was blocked with ice-cold blocking buffer (5% milk protein (Parmalat Everfresh UHT Fat free milk (Parmalat, Italy))) in 1 × PBS (OXOID, Ireland), 0.1% Tween-20 (Sigma-Aldrich, USA) for 1 hour at RTP with constant shaking (± 50 rpm), to prevent non-specific binding of the Ab to the membrane. The pre-blocked membrane was incubated

on a shaker at ± 50 rpm at 4 °C for at least 12 hours with 2-5 mL single unlabelled primary Ab in blocking buffer at the dilutions indicated in Table 7.

Table 7: Ab used during this study. ⁽¹³⁵⁻¹⁴⁵⁾

Primary Antibodies						
Antibody	Brand and product number	Stock Concentration	Host	Mono/ Polyclonal and clone number*	Molecular weight of target	Dilution used
Anti-MAGE-C1	Abcam (UK): ab207304	0.89 mg/mL	Rabbit	Monoclonal EPR18067	124 kDa	1:1000
Anti-MAGE-A1	Abcam (UK): ab193330	0.2 mg/mL	Mouse	Monoclonal MA454	34 kDa	1:200
Anti-MAGE-C2	Sigma-Aldrich (USA): HPA062230	0.05 mg/mL	Rabbit	Polyclonal	41 kDa	1:1000
Anti-SKIP	Abcam (UK): ab67715	50 μ g/mL	Mouse	Polyclonal	51 kDa	1:500
Anti-RBX1	Abcam (UK): ab133565	0.183 mg/mL	Rabbit	Monoclonal EPR6850(B)	12 kDa	1:1000
Anti-cyclin E2	Cell Signaling Technology (USA): #4132	132 μ g/mL	Rabbit	Polyclonal	48 kDa	1:1000
Phospho-p53 (Ser15)	Cell Signaling Technology (USA): #9286	765 μ g/mL	Mouse	Monoclonal	53 kDa	1:1000
Anti-P38 MAP Kinase	Sigma-Aldrich (USA): M0800	12.6 mg/mL	Rabbit	Polyclonal	38 kDa	1:5000
Secondary Antibodies						
Antibody	Brand and clone	Stock Concentration	Dilution used			
Goat anti-rabbit IgG (HRP linked)	Cell Signaling Technology (USA): #7074	60 μ g/mL	1:5000			
Horse anti-mouse IgG (HRP linked)	Cell Signaling Technology (USA):#7076	184 μ g/mL	1:5000			
Rabbit anti-Human IgG Fc Secondary Antibody, (HRP linked)	Thermo Fisher Scientific (USA):31423	0.8 mg/mL	1:1000			

*Where available

After incubation, the unbound primary Ab was washed off by completing two 5 min and two 10 min washes on the shaker at ± 50 rpm at RTP with ice-cold washing buffer (1 $\times$ PBS (OXOID, Ireland)/0.1% v/v Tween-20 (Sigma-Aldrich, USA). For MAGE-C1, MAGE-C2 and RBX1 analysis, the secondary Ab used was goat anti-rabbit IgG conjugated to horse-radish peroxidase (HRP), while MAGE-A1 and SKIP analysis required the use of horse anti-mouse IgG HRP conjugate. All secondary Ab incubations were for 1 hour on the shaker at ± 50 rpm at RTP. The previous four-step washing cycles with ice-cold washing buffer were repeated to wash off unbound secondary Ab.

Following Ab incubations, chemiluminescence detection was performed in a dark room, by flooding the membrane for 5 min at RTP with a 1:1 mixture of Clarity Western ECL Peroxide Reagent (Bio-Rad Laboratories, USA) (substrate for HRP) and Clarity Western ECL Luminol/Enhancer Reagent (Bio-Rad Laboratories, USA). The HRP (conjugated to the secondary Ab) catalyses the oxidation of luminol (the chemiluminescent reagent) by hydrogen peroxide which results in light emission.¹⁴⁶ The membrane was placed in an exposure cassette and exposed to electromagnetic radiation film (Agfa-Gevaert, Belgium) for enough time to develop clear, solid bands (5 sec to 10 min). A glow-in-the-dark sticker was used to mark the low molecular weight protein ladder. The film was developed by completely covering the film in developer (G150 developer, Agfa-Gevaert, Belgium) for 15-20 sec until bands are visible and then in fixative (G354 Manual fixing bath, Agfa-Gevaert, Belgium) for the same amount of time, before rinsing the film with distilled water and air drying. The top right corner of the film was cut to orientate the film.

Densitometry was performed on selected blots by converting the developed film to a digital image using an image scanner (Canon PIXMA MG2140) and importing the image to the ImageJ software (1.53t).¹⁴⁷ First the image was converted to a 32-bit grey-scale image, then the bands of interest were selected and converted to a numerical value based on pixel density of the band.

2.4.3.C. Staining with Anti-P38 Map Kinase Ab

As a loading control for the different samples, the membranes and thus separated proteins in each lane were re-probed with anti-p38 MAP kinase (p38), which is regarded as a house-keeping/reference protein and can be used to normalise expression results of the proteins of interest. Following the chemiluminescence detection of the protein of interest, the membranes were treated to strip off the bound antibodies (primary and secondary), before incubating with a new Ab. The membranes were incubated for 30 min at 50 °C in pre-heated stripping buffer (100 mM β -ME (MERCK, USA), 2% SDS (SIGMA, USA), 62.5 mM Tris-HCL (pH 6.7) (Glentham Life Sciences, UK)), with frequent agitation. The membrane was then washed twice in washing buffer (10 min at a time on the shaker ($\pm$ 50 rpm at RTP)) to ensure all β -ME was removed from the membrane (as can denature the primary Ab used in subsequent experiments). At this point, the membrane was incubated with p38 Ab, as described above using the goat anti-rabbit secondary antibody (Table 7). The expression of multiple antibodies could be tested by stripping the membranes in this manner.

2.5. CO-IP

To investigate potential protein complexes formed with MAGE-A1, MAGE-C2 or MAGE-C1, Co-IP was performed using the magnetic Dynabeads® M-270 Epoxy system (Life Technologies, USA). These beads are supposed to have a large Ab-binding capacity and low non-specific protein binding, with easy retrieval due to their magnetic nature, thus simplifying the Co-IP process.¹⁴⁸ This involved binding of the magnetic beads to the anti-MAGE Ab of interest and then using these beads to “pull out” or enrich MAGE-associated complexes, which could then be assessed by western hybridisation.

2.5.1. Dynabead-Ab Coupling

Ab-bead coupling was performed according to the Dynabeads® Co-Immunoprecipitation Kit (Invitrogen, USA) user guide.¹⁴⁸ Briefly, 1.5 mg Dynabeads® M-270 Epoxy beads were washed with 1 mL C1 buffer, using a magnetic stand to isolate the beads, which were then again re-suspended in C1 buffer (to a total of 250 µL, minus the volume of Ab added), Ab added (MAGE-C1: 10.5 µg; MAGE-A1: 10.5 µg; MAGE-C2: 2.25 µg; Rabbit anti-Human IgG: 10.5 µg), followed by the addition of 250 µL C2 buffer. The buffer/Ab mix was incubated on a roller ($\pm$ 50 rpm) at 37 °C for > 12 hours. The buffers and heat created optimal conditions for covalent bonding of the Ab to the epoxy bead. It was important that the beads did not settle during this conjugation period, to ensure optimal surface exposure of the beads to the Ab and subsequent antibody coupling.

Following the O/N incubation of Ab with the beads/buffers, the beads were washed to remove any unbound Ab. The beads were first washed in 800 µL HB buffer followed by 800 µL LB buffer by placing the tube on the magnet for 1 min, removing the supernatant, removing the magnet and re-suspending the beads. As recommended by the user manual, both HB and LB wash buffers were adjusted to contain 0.05% Tween-20 (Sigma-Aldrich, USA) to improve stringency of the buffers. This was followed by two short washes in 800 µL SB buffer and a further 15 min incubation on a roller ($\pm$ 50 rpm) at RTP in SB buffer, the Ab-bead complexes were finally resuspended in 100 µL SB buffer, labelled accordingly and stored at 4 °C until use.

2.5.2. Cell Lysis and Co-IP

Co-IP requires protein complexes to be maintained during the incubation of cellular components with the Ab-bead complexes. However, cell lysis must still be achieved as optimally as possible, without causing total protein denaturation. We chose to use the recommended low stringency buffer system supplied with the kit, which contains NaCl to help lyse the cells and Ptl to help prevent proteinase activity. A total of 0.1 g RPMI-8226 cells ($\pm$ 2×10^7 cells) was used per Co-IP reaction. Following

gentle washing in 5 mL ice-cold 1 × PBS (OXOID, Ireland) to remove culture media, FBS and PS that might interfere with the subsequent lysis and Co-IP process (1500 rpm/5 min/RTP), the washed cells were re-suspended in 1 mL 1 × PBS, transferred to 1.5 mL Eppendorf tubes and the cells collected again by centrifugation (1300 rpm /5 min/4 °C). The cell pellets were re-suspended in 900 µL of the lower stringency extraction buffer (1 × IP buffer (Invitrogen, USA), 100 mM NaCl (Glenthams Life Sciences, UK), 1 × Ptl (EDTA free) (ROCHE, Switzerland) (1:9 ratio of cell mass to extraction buffer) followed by a 15 min incubation on ice. To remove cell debris from the cell lysate the tubes were centrifuged at 5300 rpm for 5 min at 4 °C and the supernatant transferred to a clean tube. To prevent further protein complex denaturation, these extracts were held on ice prior to same-day Co-IP. A more stringent extraction buffer (1 × IP (Thermo Fisher Scientific, USA), 100 mM NaCl (Glenthams Life Sciences, UK), 2 mM MgCl₂ (Merck, USA), 1 mM DTT (Promega, USA), 1×PI) was prepared and used as above to compare to the less stringent buffer during initial Co-IP experiments, but was not used for the investigations.

Co-IP reactions were performed using the Ab-bead complexes prepared in section 2.5.1. Before use in the Co-IP reaction the beads were washed with 900 µL of the low stringency extraction buffer (as above) to remove SB buffer which might disrupt the Co-IP reaction. The beads (1.5 mg coupled beads per cell lysate) were then re-suspended in the prepared cell lysate prepared and allowed to hybridise to protein complexes in the extract by incubation on a roller (± 50 rpm) for 30 min at 4 °C. The epoxy beads are designed for rapid Ab:protein interaction which is why the incubation time is so short.¹⁴⁸ Again it was important that the lysate/bead mixture did not settle during the incubation to allow optimal protein binding to the AB-labelled beads. Post incubation, the beads (with attached protein complexes) were removed via the magnet and the protein-depleted lysate (flow through) kept for analysis. The bead/protein complexes were washed three times gently in 200 µL low stringency extraction buffer, with a final wash in 200 µL 1 × LWB (Invitrogen, USA) for 5 min at RTP on a rotor (± 50 rpm) and bead/protein complex magnetic retrieval. The bound protein complexes were de-coupled from the magnetic beads by resuspension in 60 µL EB buffer, incubation for 5 min at RTP with agitation, followed by the removal of the decoupled magnetic beads using the application of the magnet. The supernatant, containing purified protein complexes was held on ice until protein separation on an acrylamide gel.

2.5.3. Western Hybridisation Analysis of Co-IP

2.5.3.A. Protein Separation and Membrane Transfer

Proteins, including the lysate, the flow-through from each Co-IP, the Co-IP protein yield itself and the IgG Co-IP were analysed by western hybridisation as described in section 2.4.3. Protein could not be quantified using the BCA assay due to buffer interference. Thus the maximum amount of protein was

used per loading reaction (23 μ L protein preparation, 6 μ L 5 $\times$ SDS (SIGMA, USA), 1 μ L 100 mM DTT (Promega, UK)) and denatured for 5 min at 95 $^{\circ}$ C before loading. The lysate and flow-through for each Co-IP was included to see if there is a reduction in the protein of interest in the flow-through, as it should have been removed from the lysate during the Co-IP reaction. The IgG Co-IP was included as a control for non-specific protein binding and pull-down during the Co-IP reaction.

2.5.3.B. Ab Hybridisation and Detection

Ab were bound to the Co-IP membranes according to section 2.4.3.B. and 2.4.3.C. The Ab used in for the pulldown reaction was first tested to see if the Co-IP reaction worked before testing for binding partners.

2.6. IFM

2.6.1. Labelling of Primary Ab

For IFM, primary Abs for MAGE-A1, MAGE-C2, MAGE-C1, SKIP, and RBX1 (details in Table 7) were labelled with fluorochromes using Mix-n-Stain™ Antibody Labelling Kits (Sigma-Aldrich, USA), where the fluorochromes are conjugated to the Ab via aminolysis. Anti-MAGE-A1, MAGE-C2 and MAGE-C1 Ab were labelled with CF™488A, while anti-SKIP and anti-RBX1 Ab were labelled with CF™647. The proteins were labelled according to the manufacturer's recommendations,¹⁴⁹ with Ab solutions containing > 10% glycerol requiring an initial filtration step to replace the contaminant with 1 × PBS.¹⁴⁹ For all Ab labelling experiments 9 µL of Ab (irrespective of Ab concentration) was mixed with 1 µL of 10 × reaction buffer and then the entire mix was added to the dye vial, gently mixed, and incubated in the dark for 30 min at RTP. Labelled Abs were aliquoted into amber tubes in 2 µL aliquots and stored at -20 °C until needed. The final concentration of the fluorescent labelled Ab (Fl-Ab) was as follows: MAGE-A1 CF488A = 0.18 mg/mL; MAGE-C2 CF488A = 0.045 mg/mL MAGE-C1 CF488A = 0.80 mg/mL; SKIP CF647 = 0.045 mg/mL and RBX1 CF647 = 0.165 mg/mL, calculated by multiplying the initial Ab concentration by the dilution factor of 0.9.

2.6.2. Methanol Fixation of Cells

Cultured RPMI-8226 cells and enriched PBMNC were prepared for IFM using methanol fixation and permeabilization, according to a modified Abcam immunocytochemistry and immunofluorescence protocol.¹⁵⁰ Methanol fixation was chosen as it simultaneously fixes and permeabilises cells while preserving cellular and protein structure. Cells totalling 2×10^6 cells per investigation, were pelleted by centrifugation (5 min/1000 rpm/RTP) and the cell pellet gently washed with 10 mL ice-cold 1 × PBS (pH 7.2) (OXOID, Ireland) to remove all culture media, before repeat centrifugation. The supernatant was carefully removed, and the cell pellet was briefly re-suspended in the remaining < 50 µL 1 × PBS by gently flicking the tube. In a dropwise manner, while vigorously vortexing the tube to prevent clumping of the cells, 2 mL ice-cold 100% methanol (MERCK, USA) was added. The re-suspended cells were then incubated at -20 °C for 10 min to allow thorough fixation and permeabilisation of cells. It was important to incubate at such a low temperature to slow down the preservation effects of methanol that would otherwise be too destructive on cellular structures.^{151,152} After this incubation period, the cells were washed three times in 1 mL ice-cold 1×PBS (5 min/1000 rpm/RTP) to remove all residual methanol and the cell pellets kept on ice before proceeding to the next step.

2.6.3. Labelling Cells with Specific Nuclear Staining

To prevent non-specific binding of Fl-Ab, the fixed cells were first re-suspended in 1 mL ice-cold blocking buffer (1% BSA (Sigma-Aldrich, USA), 0.1% Tween-20 (Sigma-Aldrich, USA) in 1 × PBS (OXOID, Ireland)) and incubated for 2 hours at RTP. Cells were pelleted (5 min/1000 rpm/RTP) and the supernatant removed. The Fl-Abs were diluted in 200 µL blocking buffer as follows: 1:50 dilutions for anti-MAGE-A1, anti-MAGE-C2 and anti-MAGE-C1 Ab; 1:100 dilutions for anti-SKIP and anti-RBX1. Ab dilutions were optimised using datasheet information and immunocytochemistry results from The Human Protein Atlas database as guidelines.^{153,154} These Ab solutions were used to re-suspend the cell pellets and the suspension incubated for > 12 hours at 4 °C on a rotor (± 50 rpm) to ensure thorough exposure of cells to Fl-Ab at a low temperature to prevent non-specific binding.¹⁵⁵ The tubes were wrapped in foil to protect Fl-Ab from light during incubation. To be able to distinguish between the nucleus and the cytoplasm of the cells, the cells were co-stained with Hoechst (Sigma-Aldrich, USA), a blue nuclear stain (distinguishable from the fluorescence of the green CFTM488A and far red CFTM647 Fl-Ab). Hoechst was added to the cells in blocking buffer after the > 12-hour incubation period to a final concentration of 5 µg/mL and incubated at RTP for 5 min with agitation (± 50 rpm). The cells were then finally washed three times in ice-cold 1 × PBS (OXOID, Ireland) (centrifugation: 5 min/1000 rpm/RTP) to wash off unbound Fl-Ab and excess nuclear staining and re-suspended in ± 30 µL of 1 × PBS.

2.6.4. Slide Preparation and Microscopy

A 5 µL sample of the Fl-Ab labelled/Hoescht stained cells were dropped onto microscope slide, 5 µL mounting fluid (0.1 M citric acid (Sigma-Aldrich, USA), 0.2 M disodium phosphate (Glenthams Life Sciences, UK), 50% glycerol (May&Baker, England)) added and a cover slip lowered on top. The whole slide was protected from light with foil before microscopy at the University of Cape Town Confocal Core Facility (University of Cape Town, Medical School). All slides were imaged using the Zeiss LSM 880 confocal laser scanning microscope (Zeiss, Germany) with ×10 plus ×63 dual magnification. All images were captured in three different channels i.e. green, blue and far red, which were then overlaid into one image. Ab labelled with CFTM488A and controls were analysed using the green channel (Ch1: green, pinhole: 2.02 AU, excitation: 488 nm, emission: 562 nm, Gain: 718). Ab labelled with CFTM647 and controls were analysed using the far red channel (Ch3: far red, pinhole: 1.57 AU, excitation: 633 nm, emission: 655 nm, Gain: 885). Hoechst nuclear stain and controls were analysed using the blue channel (Ch2: blue, pinhole: 1.73 AU, excitation: 405 nm, emission: 450 nm, Gain: 475). Z-stacks were taken to assess the three-dimensional shape of the cells. The upper and lower limit of the focal plane was set just outside the boundaries of the cell. Slices were taken at 569.48 nm intervals.

2.7. ACTIVATION AND ANALYSIS OF THE E3 LIGASE PATHWAYS KNOWN TO BE AFFECTED BY MAGE-C2 INTERACTIONS

2.7.1. Heat Shock Activation of the Ubiquitination/proteasome Pathway

Ubiquitination and protein degradation via the proteasome is known to be activated via heat-shock stress, through the endoplasmic reticulum (ER) apoptotic pathway.¹⁵⁶ From a prior RPMI-8226 study,⁸⁵ our laboratory group determined that incubation at 52 °C for 2 min, followed by a 4 to 8 hour incubation at 37 °C induced an apoptotic response of 20-30%, indicating activation of the heat-shock response. We assumed this was an adequate level of induced apoptosis to induce the stress response without killing too many cells.

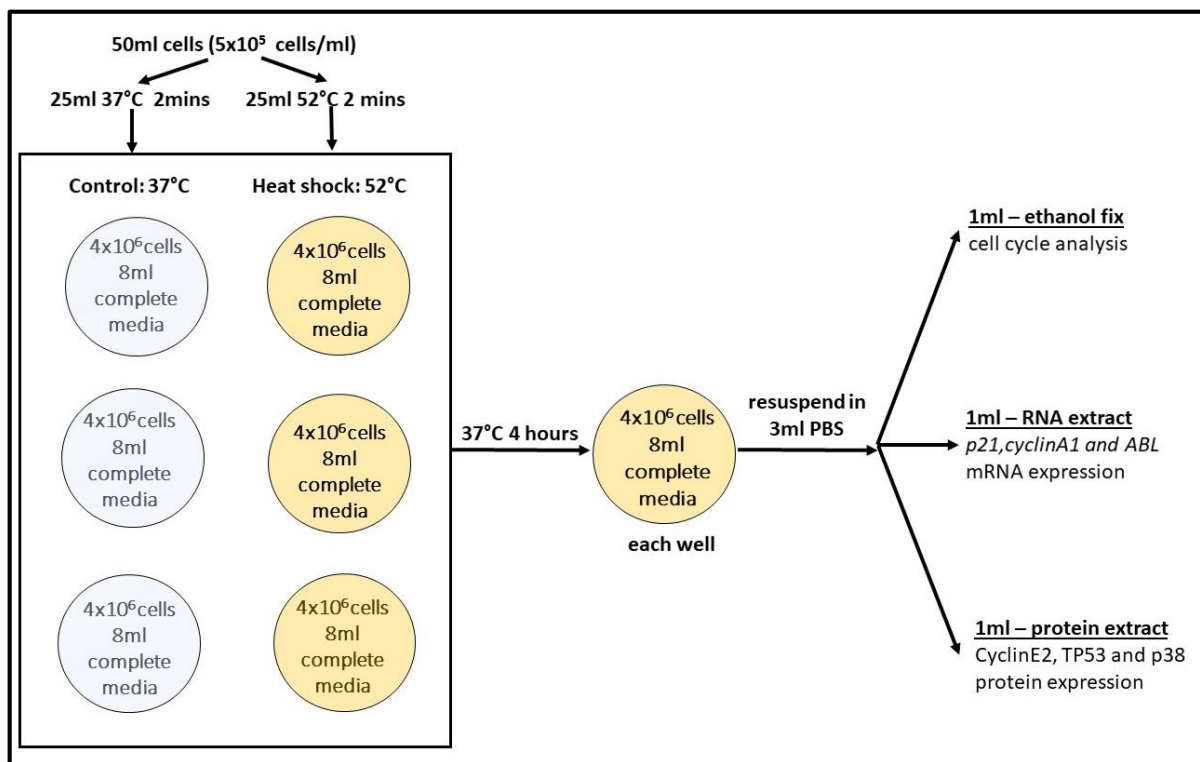


Figure 6: Experimental design for heat shock activation of the ubiquitination/proteasome pathway.

Cells (5×10^5 cells/mL) were suspended in 50 mL complete media and split into two for groups i.e. heat shock and control. After the correct heat exposure, cells from each group were split into three repeats and returned to culture conditions for 4-hours to allow signalling pathways to progress. Each repeat yielded three preparations of equal cell concentration: ethanol fixed cells, extracted RNA and extracted protein.

PBMNC and cultured RPMI-8226 (2 days post passage) were prepared according to section 2.2.1. and 2.2.2. and finally re-suspended in 50 mL complete RPMI-1640 culture media, to a concentration of 5×10^5 cells/mL. These were then split into two 25 mL aliquots, with the control tubes transferred to a 37 °C incubator for 2 min, while the experimental heat-shock tubes were placed into a 52 °C water bath for 2 min to stress the cells. Following this initial incubation, 8 mL from each tube was plated into three wells of a 6-well plate (3 × control and 3 × heat-shock wells/plate/cell type) and incubated at 37 °C, 5%

CO₂ for 4-hours to allow signalling pathways to progress. Finally, the contents of each well was transferred to a 15 mL conical tube, pelleted (1500 rpm/5 min/RTP) and washed with ice-cold 1 × PBS (OXOID, Ireland) to remove all culture media that might interfere with downstream extraction and analysis processes, before being gently re-suspended in 3 mL 1 × PBS/well. The cell suspensions were held on ice while three different sample types were prepared: ethanol fixation (1 mL), RNA extraction (1 mL) and protein extraction (1 mL) for each well sample. Figure 6 explains the experimental design.

2.7.2. Cell Cycle Analysis Using PI

2.7.2.A. Ethanol Fixation of Cells

Heat-shocked and control group cultured RPMI-8226 cells and enriched PBMNC were prepared for cell cycle analysis using 70% ice-cold ethanol to fix and permeabilise cells according to the FxCycle™ PI/RNase Staining Solution protocol.¹⁵⁷ Ethanol fixation was chosen as it simultaneously fixes and permeabilises cells, and is most accommodating for intercalating dyes.¹⁵⁸ Cells (1 mL $\approx 1.3 \times 10^5$ cells) from each well preparation were pelleted by centrifugation (1500 rpm/5 min/RTP), the supernatant carefully removed and the pellet re-suspended in 50 μ L 1 × PBS (OXOID, Ireland). In a dropwise manner, while vigorously vortexing the tube to prevent clumping of the cells, 1 mL ice-cold 70% ethanol (MERCK, USA) was added and then stored immediately at -20 °C to slow down the fix and permeabilising action for a minimum of 12 hours.

2.7.2.B. PI Staining

Cells were stained with FxCycle™ PI/RNase Staining Solution (Invitrogen, USA) according to the manufacturer's instructions.¹⁵⁷ PI fluorescently labels the nucleic acids within the cell, but as RNase is included, the amount of fluorescence generated is indicative of the quantity of DNA and thus chromosome copies. Briefly, ethanol fixed (section 2.7.2.A.) cells were pelleted via centrifugation (1500 rpm/5 min/RTP) and washed once with 1 mL 1 × PBS (OXOID, Ireland). The pellet was re-suspended in 500 μ L FxCycle™ PI/RNase staining solution (Invitrogen, USA), the tubes covered in foil to protect them from light and analysed immediately using flow cytometry.

2.7.2.C. Cell Cycle Analysis on Flow Cytometer

Cell cycle analyses was assessed by measuring the levels of PI fluorescence per cell. Fluorescence peaks represent G1 (single chromosome set/cell) and G2 (two copies of the chromosome set/cell just prior to cell division). The S-phase, which is the fluorescence in between the two peaks, represents the phase in

the cell cycle where DNA is being copied.¹⁵⁹ This analysis was performed on a BD FACSCalibur™ Flow Cytometer (BD Biosciences, USA) using the 488 nm laser, with PI having an excitation/emission spectra of 535/617 nm (FL2-A). Prior to sample analysis, Nile Red Particles (BD Biosciences, USA) (10000 events), which have a consistent level of fluorescence following 488 nm activation were run to ensure the laser is working correctly.

The PBMNC and RPMI-8226 PI-labelled cells were analysed using the settings in Table 8. The settings between the two cell lines varied slightly as they have different size and cellular content properties, but the settings were kept constant for analysing the control vs. heat shock groups within each cell line. During the process, a gate (R1) was set up on the side scatter (SSC) vs Forward scatter (FSC) dot plot, to include only the cell population of interest (lymphocytes in the case of PBMNC) and exclude cell debris. Cells within the R1-gate were then analysed on a dot plot of FL2-W vs PI (FL2-A), which is used to exclude doublets and ensure that only single cells (constant width) (R9-gate) are analysed for DNA content. The single cells included in the R9-gate were then plotted on a histogram of events vs PI (FL2-A), with the voltage adjusted to ensure that the G1-peak was placed at around 200. A minimum of 10 000 events in the R9-gate were analysed.

Table 8: Flow cytometer settings used for cell cycle analysis

Cell cycle analysis settings							
PBMNC				RPMI-8226			
Detector	Voltage	Amp/Gain	Mode	Detector	Voltage	Amp/Gain	Mode
P1: FSC	E00	1.0	Lin	P1: FSC	E00	1.0	Lin
P2: SSC	397	1.0	Lin	P2: SSC	305	1.0	Lin
P4: FL2	355	1.0	Lin	P4: FL2	320	1.0	Lin
P6: FL2-A		1.0	Lin	P6: FL2-A		1.0	Lin
P7: FL2-W		4.92	Lin	P7: PL4		4.92	Lin

After acquisition, the raw data was analysed using ModFit LT™,¹⁶⁰ which allows for auto analysis and annotation of debris, cell aggregates, G1-, G2- and S-phase on the histogram, and calculates the percentage of cells in each phase.

2.7.3. mRNA Expression Analysis of *p21*, *Cyclin A1*, *Cyclin A2* and *ABL*

Total RNA from 1 mL of cells (1.3×10^5) from each well preparation was extracted using the silica-column based QIAamp® RNA Blood Mini Kit (Qiagen, The Netherlands), according to the manufacturer's instructions¹²⁸ as described in section 2.3.2. Samples were processed quickly, solutions kept on ice and RNase-free tips and tubes used to try to limit possible mRNA degradation. Total RNA was finally eluted in 20 µL nuclease-free water (Qiagen, The Netherlands) and a single RT reaction (using 10 µL extracted RNA) was prepared according to section 2.3.3. cDNA reactions were stored at -80 °C until rRT-PCR analysis.

Expression of *p21*, *Cyclin A1* and *Cyclin A2* transcripts in the different cell cDNA preparations was assessed using rRT-PCR with SYBR-Green incorporation (QuantiNova SYBR® Green PCR kit, Qiagen, The Netherlands), as described in section 2.3.5. along with the expression of *ABL* as a control for RNA integrity, cDNA reaction efficiency and as a normalisation for the $2^{-\Delta\Delta C_t}$ calculations.¹³² All PCR reactions were performed in duplicate using 2 μ L of the single cDNA reaction for each well and 25 pmol of each relevant primer (designed and verified as described in section 2.3.1, Table 5). The PCR cycling and fluorescent detection was performed on a Rotor-Gene Q (Qiagen, The Netherlands), with Rotor-Gene Q Pure Detection software (version 2.3.5, 1990-1999©) PCR program: 2 min at 95 °C, 40 cycles of 20 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C per cycle. Fluorescence was acquired during the extension phase (as SYBR Green is an intercalating dye) in the green channel using an excitation wavelength of 470 ± 10 nm and a detection wavelength of 510 ± 5 nm. This was followed by a melt curve analysis with a temperature range of 72 to 95 °C in 0.5 degree intervals.

The real-time amplification graphs were analysed in linear scale by switching on ‘Dynamic Tube’ and ‘Slope Correct’. The threshold was set at 0.09 (where all the samples are in the logarithmic phase of amplification) and C_t values calculated. The average C_t values of the amplicons of interest were normalised using the *ABL* C_t value and then fold changes determined between the control and heat-shock samples using the $2^{-\Delta\Delta C_t}$ method.¹³² Fold change > 2 were considered significant.¹⁶¹

2.7.4. Western Hybridisation Analysis of Cyclin E2 and Phosphorylated TP53 Protein Levels

Effects of the heat-shock treatment on the levels of phosphorylated TP53 (P-TP53) and Cyclin E2 was analysed through western hybridisation using chemiluminescence detection of Ab binding using the Clarity™ Western ECL Substrate kit (Bio-Rad Laboratories, USA), with RIPA buffer protein extractions, as described in section 2.4.1. For each of the wells, 1 mL (as indicated in Figure 6) of the cell suspension was re-suspended in 30 μ L RIPA solution (150 mM NaCl (Glenthams Life Sciences, UK), 1% Triton X-100 (SIGMA, USA), 0.1% SDS (SIGMA, USA), 10 mM Tris pH 7.5 (Glenthams Life Sciences, UK), 1% deoxycholate powder (Sigma-Aldrich, USA) and 1 $\times$ Pti cocktail (ROCHE, Switzerland) and frozen at -80 °C O/N to aid the lysis process. Protein concentrations were not determined, as the same cell numbers were used in each well. Instead a constant 23 μ L of RIPA extract was mixed with 1 μ L 100 mM DTT (Promega, USA) and 6 μ L 5 $\times$ SDS loading dye and then boiled for 5 min (95 °C) before loading into the relevant well of a 12% w/v acrylamide resolving gel (5% stacking gel) (as described in section 2.4.3.A.). Cell-specific sample sets (three control and three heat shock samples) were loaded onto the same gel. The samples, along with a 20 to 120 kDa pre-stained protein low molecular weight ladder (PepLab Biotechnologie, Germany), were electrophoresed at 100 V for 2 hours in 1 $\times$ running buffer (3.5 mM SDS (SIGMA, USA), 25 mM Tris (Glenthams Life Sciences, UK),

0.2 M Glycine (Glenthams Life Sciences, UK)) using the Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, USA).

The separated proteins were transferred from the gel onto 0.2 μ m nitrocellulose membrane (Bio-Rad Laboratories, USA) using the Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, USA), as described in section 2.4.3.A. Following blocking of protein-free parts of the membrane (as described in section 2.4.3.B.) the membranes were incubated on a shaker at $\pm$ 50 rpm at 4 °C for at least 12 hours with 3-5 mL single unlabelled primary Ab in blocking buffer at the following dilutions as recommended by the manufacturer of each Ab (details in Table 7): Anti-cyclin E2 – 1:1000, anti-phospho-p53 – 1:1000 and p38 – 1:5000 (loading control). After incubation, the unbound primary Ab was washed off by completing two 5 min and two 10 min washes on the shaker at $\pm$ 50 rpm at RTP with ice-cold washing buffer (1 $\times$ PBS/ 0.1% v/v Tween-20 (Sigma-Aldrich, USA)). Anti-cyclin E2 and p38 required the use of goat anti-rabbit (HRP linked) secondary Ab with a 1:5000 dilution into ice-cold blocking buffer, while anti-phospho-p53 required horse anti-mouse IgG HRP conjugate as the secondary Ab at 1:5000 dilution. Incubation, washing and chemiluminescence steps were performed as previously described (section 2.4.3.B.), with the notable exception of analysis using the phospho-p53 Ab, which required buffers using Tris buffered saline (TBS) instead of 1 $\times$ PBS, as PBS will interfere with the binding of the phosphorylated Ab.¹⁶²

All nitrocellulose membranes were first probed with anti-phospho-p53, then anti-cyclin E2 and finally anti-p38 Abs, following membrane stripping between Abs, as previously described (see section 2.4.3.C.). Densitometry was performed on the bands on the electromagnetic radiation film by first converting the images to digital format using an image scanner (Canon PIXMA MG2140) and analysed using ImageJ software (version 1.53t)¹⁴⁷ that converts band intensity (expressed as pixel density) into a peak, with the area of the peak proportional to the intensity of the band. The peak area of Cyclin E1 and P-TP53 specific bands were then divided by the p38 specific peak area/sample to produce a normalised ratio.

CHAPTER 3: RESULTS

3.1. BIOINFORMATIC ANALYSIS OF MAGE PROTEIN SEQUENCES

Based on the available information regarding phylogeny, homology and expression of MAGE proteins, as discussed in section 1.2.2.A, we proposed that MAGE-C1 may have similar functions to those of MAGE-A1 and/or MAGE-C2. To strengthen this hypothesis, we looked at the protein sequences of these MAGE proteins in more detail, to establish if any of the aa associated with specific functions were shared.

3.1.1. Comparison of MAGE-A1 to MAGE-C1

Protein sequence alignment can be used to predict homology, structure and function in proteins.^{116,117} Pairwise alignment of sequences is a method used to calculate identity and similarity between protein sequences.¹¹⁶ Identity between two sequences indicates the number of exact aa matches in relation to the length of the shorter sequence.^{163,164} While it is generally accepted that an identity score of at least 30% between sequences is required to even consider possible similar structure and function of the proteins,^{116,117,165,166} identity levels > 40% more accurately deduce structural similarities and > 60% identity accurately deduces functional similarities.^{116,165} Sequence similarity calculations on the other hand include identical aa matches, aa that have similar physio-chemical properties and allows for the introduction of gaps for alignment.^{163,164} Similarity scores from aa alignment are generally used to infer homology between sequences and requires detailed analysis to determine the significance of the similarity to verify the implied homology.¹⁶⁷⁻¹⁷⁰

As described in the Section 2.1, the aa sequences of MAGE-A1 and MAGE-C1 were aligned using BioEdit.¹¹⁵ For the analysis a total of four alignments were performed, whole protein sequences and three individual known domains: the MHD, the conserved domain of approximately 200 aa, which defines the members of the MAGE family of proteins,¹⁰¹ and the two WHD, WH1 and WH2 which belong to the helix-turn-helix family and span the MHD.^{101,171} A WHD is recognised by its secondary structure of three α -helices and three β -strands that occur in the unique order of $\alpha 1$, $\beta 1$, $\alpha 2-3$, $\beta 2-3$.¹⁷² These domains are known to be associated with protein:DNA interactions and protein:protein complex binding.^{173,174} While MAGE-A1 has been shown to interact with and disrupt the function of SKIP, the exact aa acids involved in these interactions have not yet been defined, but the MHD has been implicated.⁹⁸

Table 9 shows the identity of the four alignments between MAGE-A1 and MAGE-C1. Due to the larger size of MAGE-C1 (1142 aa) compared to MAGE-A1 (309 aa), the very low identity (10.9%) between these two MAGE proteins was not unexpected. However, analysis of MHD and WHD regions have >

40% identity across all three domains. Based on the prediction values mentioned earlier, these values indicate that a structural similarity over these regions may be implied, while the functional similarity cannot be guaranteed but is worth investigating.

Table 9: Pairwise alignment of MAGE-A1 (NP_004979.3) and MAGE-C1 (NP_005453.2) protein sequences.

	Whole Protein	MHD*	WH1 [#]	WH2 ^{\$}
MAGE-A1 aa	1 - 309	102 - 301	72 - 183	186 - 309
MAGE-C1 aa	1 - 1142	908 - 1106	877 - 988	991 - 1114
Identity	10,9%	49,5%	41,6%	49,2%

*Mage homology domain; [#]Winged helix 1; ^{\$}Winged helix 2.

In addition to the MHD, there are specific aa residues that are known to influence protein structure and functions in general by being sites for post-translational modifications. Thus, we searched the appropriate databases for the serine (Ser, S), threonine (Thr, T), tyrosine (Tyr, Y) and lysine (Lys, K) residues in the three MAGE proteins that have been previously reported as predicted/proven ubiquitination, phosphorkylation and acetylation sites. This was performed to compare the position and number of these site between the proteins to aid in determining similar functionality. Figures 7 and 8 show the locations of the post-translational modification sites, as well as the MHD, WH1 and WH2 domains, for MAGE-A1 and MAGE-C1 respectively.

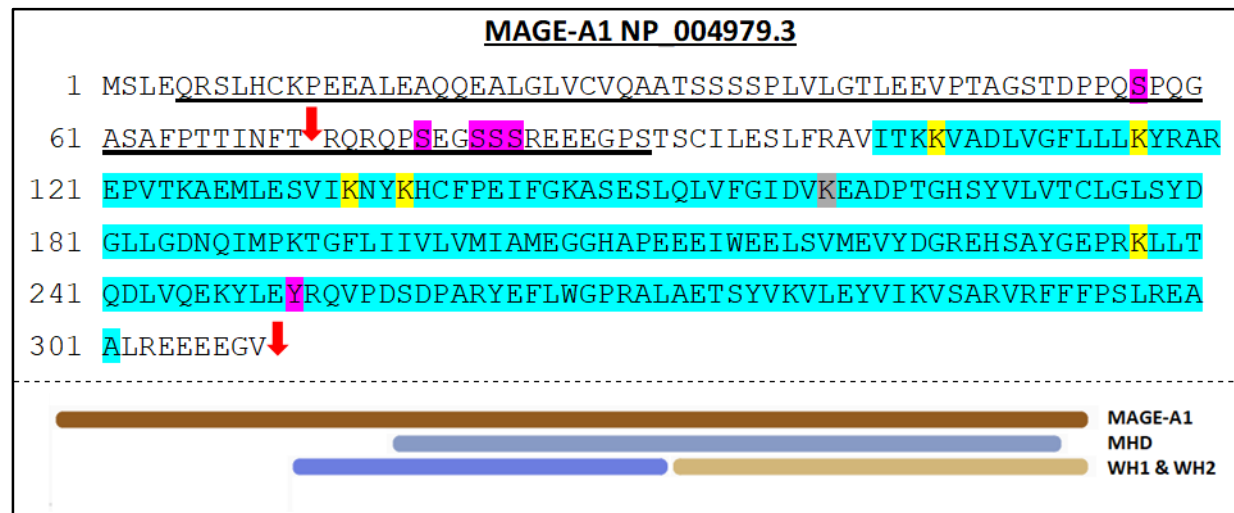


Figure 7: MAGE-A1 protein sequence and regions of interest.

The top schematic shows the following features found within the entire MAGE-A1 protein (NP_004979.3) sequence. The MHD is highlighted in turquoise, with the start and end of the WHD (containing the WH1 and WH2 subdomains) indicated by red arrows. AA residues that have been predicted/proven to be phosphorylation sites are highlighted in pink, with ubiquitination sites highlighted in yellow and acetylation sites highlighted in grey. The underlined section shows the region known as the Melanoma associated antigen family N-terminal domain. The bottom section shows a simplified image of the relative positions of the MHD and WHD within the whole protein.

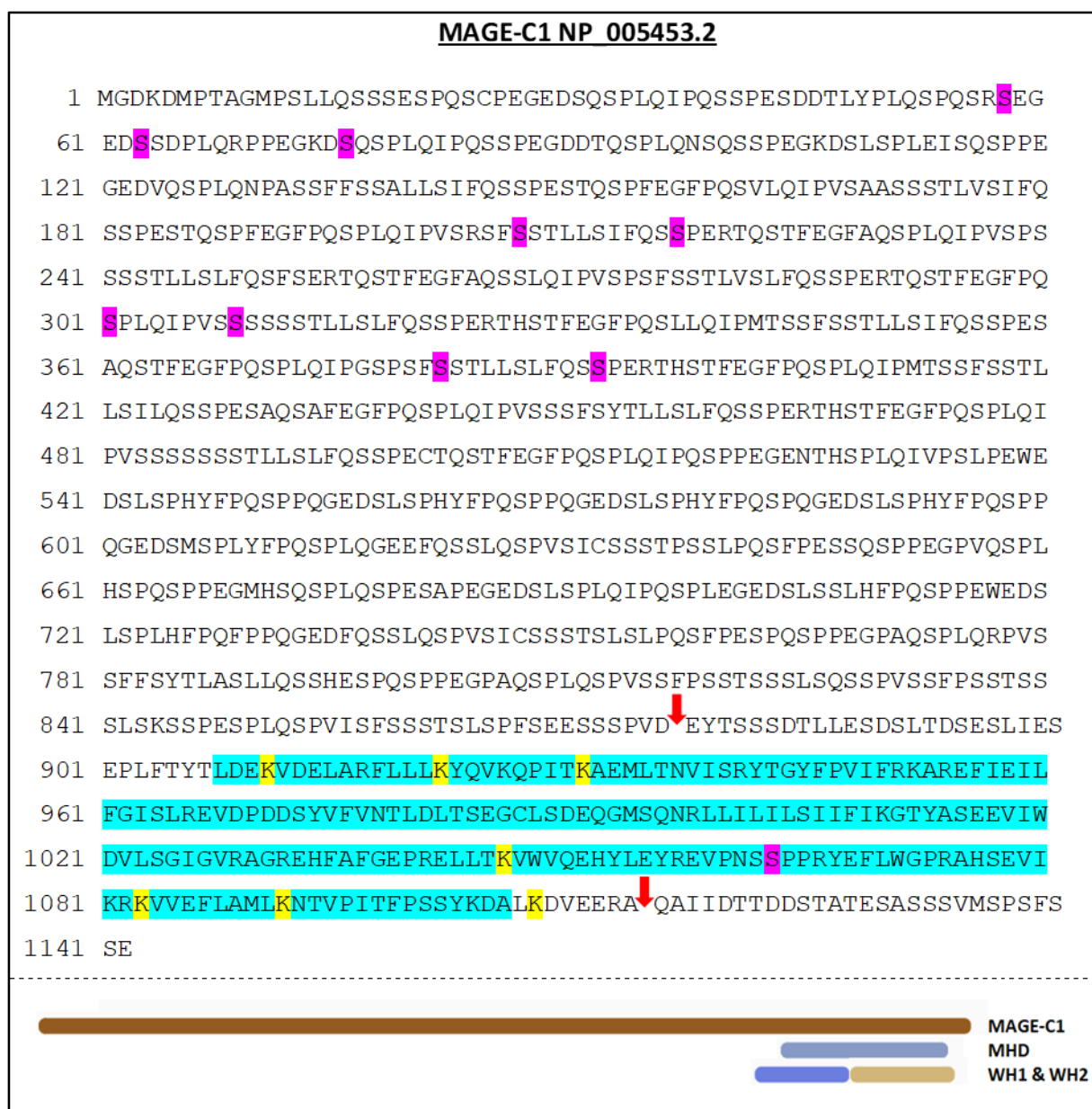


Figure 8: MAGE-C1 protein sequence and regions of interest.

The top schematic shows the following features found within the entire MAGE-C1 protein (NP_005453.2) sequence. The MHD is highlighted in turquoise, with the start and end of the WHD (containing the WH1 and WH2 subdomains) indicated by red arrows. AA residues that have been predicted/proven to be phosphorylation sites are highlighted in pink and ubiquitination sites highlighted in yellow. No acetylation sites have been indicated for this protein according to the databases used. The bottom section shows a simplified image of the relative positions of the MHD and WHD within the whole protein.

In both proteins, the predicted ubiquitination sites occurred within the WH1/WH2 domains, although MAGE-C1 had an extra two sites. Interestingly, MAGE-A1 has one potential acetylation site within the MHD (K160), while there were no predicted sites within MAGE-C1. Both proteins had various phosphorylation sites which are mainly grouped within the WHD of MAGE-A1 in contrast to MAGE-C1 where they were located outside of the WHD towards the amino terminal (N-terminal).

3.1.2. Comparison of MAGE-C2 to MAGE-C1

As described above, the human protein reference sequence of MAGE-C2 was aligned to the MAGE-C1 human reference sequence, to establish the degree of identity between the different domains. In addition to the three known domains, the C-terminal region of MAGE-C2 that is known to interact with TRIM28¹⁰¹ was also compared to the C-terminal region of MAGE-C1. Table 10 shows the results of this analysis.

Table 10: Pairwise alignment of MAGE-C2 (NP_057333.1) and MAGE-C1 (NP_005453.2) protein sequences.

	Whole Protein	MHD*	WH1[#]	WH2^{\$}	TRIM28[@]
MAGE-C2 aa	1 - 373	141 - 336	111 - 219	186 - 309	135 - 373
MAGE-C1 aa	1 - 1142	908 - 1106	877 - 988	991 - 1114	904 - 1142
Identity	19.1%	69.8%	51.8%	77.4%	69.0%

*MAGE homology domain; #Winged helix 1; \$ Winged helix 2; @ C-terminal region known to interact with the TRIM28 protein.

Again, due to the size difference the pairwise alignment of the whole proteins was not very informative (< 30% identity). However, the key domains showed significant identity, with the MHD and WH2 domains both showing > 60% identity and thus a relatively accurate prediction of similar structure and function of these domains. Additionally, the C-terminal (239 aa) of MAGE-C1 shared > 60% identity with the C-terminal (239 aa) of MAGE-C2 known to interact with TRIM28. Figure 9 show the locations of the post-translational modification sites, as well as the MHD, WH1 and WH2 domains, and the position of the TRIM28-interacting site for MAGE-C2, respectively.

In contrast to MAGE-A1 and MAGE-C1, MAGE-C2 only had one ubiquitination site which was located at the C-terminal region of the WH2 domain. The position of the phosphorylation sites also differed considerably between MAGE-C1 and MAGE-C2, with the MAGE-C2 sites all occurring within the MHD. However, MAGE-C2 also had no predicted acetylation sites.

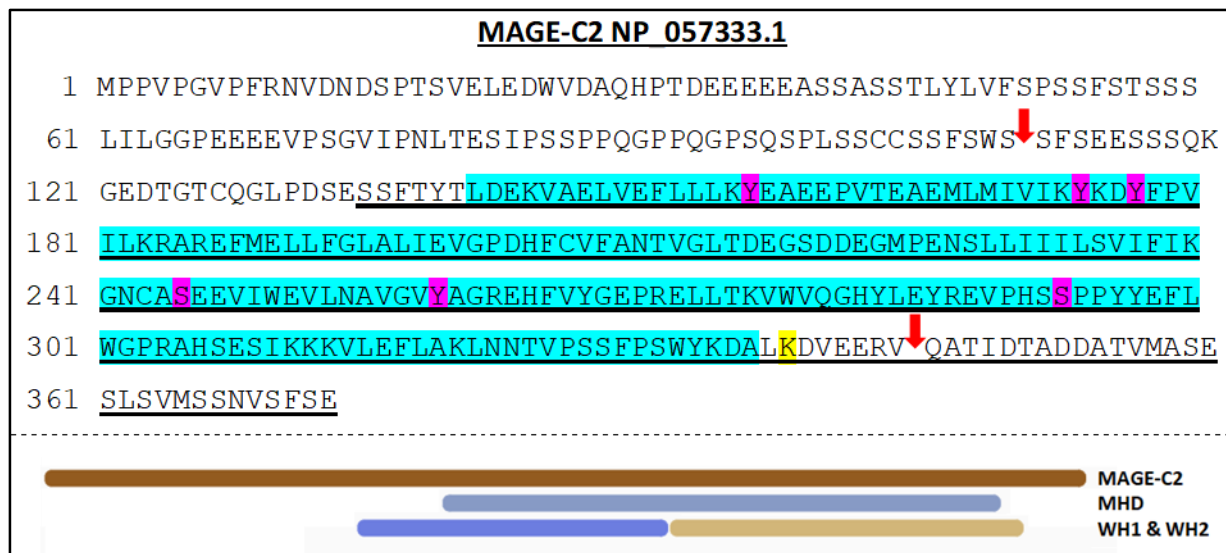


Figure 9: MAGE-C2 protein sequence and regions of interest.

The top schematic shows the following features found within the entire MAGE-C2 protein (NP_057333.1) sequence. The MHD is highlighted in turquoise, with the start and end of the WHD (containing the WH1 and WH2 subdomains) indicated by red arrows. AA residues that have been predicted/proven to be phosphorylation sites are highlighted in pink and ubiquitination sites highlighted in yellow. No acetylation sites were found. Underlined section indicates the aa residues that have been found to interact with E3 ligase TRIM28. The bottom section shows a simplified image of the relative positions of the MHD and WHD within the whole protein.

3.1.3. Additional Motifs in MAGE-C1

While similarity was observed between the C-terminal domain of MAGE-C1 and the two other MAGE proteins (where the MHD is encoded), the N-terminal region of MAGE-C1 appeared to be novel. Additional analysis of this region showed that the first 500 aa of the N-terminal showed an interesting feature, a series of 10 repeat units consisting of 35 aa, as highlighted in Figure 10. The identity comparison of these units in Figure 11 showed 70 to 94% identity between the repeats, with an average of 81.59% which strongly suggests similar structure and function within these repeat units. The Melanoma associated antigen family N-terminal (MAGE_N) domain, shown in Figure 7 (MAGE-A1), has been identified in several MAGE proteins and is usually varies from 82 to 96 aa in length.¹⁷⁵ The 85 aa MAGE_N terminal of MAGE-A1 was aligned against 85 N-terminal aa of MAGE-C1 to include the two 36 aa repeats indicated in Figure 10. This resulted in 21% identity which suggested that the unknown repeats in the blue box (of Figure 10) were not related to the MAGE_N region in structure or function. The repeat sequences were analysed using NCBI BLAST® against the human genome protein database and were found to be limited to MAGE-C1 (with probable significance).

Repeat amino acid sequences of MAGE-C1 (NP 005453.2)	
1.	MGDKDMPTAGMPSLLQSSSESPQSCPEGEDS
32.	QSPLQIPQSSPESDDTLYPQLQSPQSRSEGEDSSDPLQRPPEGKDS
77.	QSPLQIPQSSPEGDDTQSPLONSQSSPEGKDSLSPLEISQSPPEGEDV
125.	QSPLQNPASSFFSSALLSIFQSSPESTQSPFEGFP
160.	QSVLQIPVSAASSSTLVSLFQSSPESTQSPFEGFP
195.	QSPLQIPVSRFSSTLLSIFQSSPERTQSTFEGFA
230.	QSPLQIPVSPSSSSTLLSLFQSFERTQSTFEGFA
265.	QSSLQIPVSPSFSSTLVSLFQSSPERTQSTFEGFP
300.	QSPLQIPVSSSSSSTLLSLFQSSPERTHSTFEGFP
335.	QSLQIPMTSSFSSTLLSIFQSSPESAQSTFEGFP
370.	QSPLQIPGSPSFSSTLLSLFQSSPERTHSTFEGFP
405.	QSPLQIPMTSSFSSTLLSILQSSPESAQSAFEGFP
440.	QSPLQIPVSSSFSYTLLSLFQSSPERTHSTFEGFP
475.	QSPLQIPVSSSSSSTLLSLFQSSPECTQSTFEGFPQSPLQIPQSPPE
523.	GENTHSPLQIVPSLPEWEDSLSPHYFPQSPQGEDSLSPHYFPQSPQ...

Figure 10: Repeat aa sequences of MAGE-C1 protein.

Blue box: Two 36 aa interrupted repeat sequences of MAGE-C1. Red box: Ten continuous repeat sequences of 35 aa in MAGE-C1 (NP_005453.2).¹⁷⁶ Yellow: Eighty-five aa aligned against MAGE_N of MAGE-A1.

ClustalW Multiple Alignment of the 10 repeat sequences		GLOBAL pairwise alignment	
			Identity (%)
		10 & 1	74,29
		10 & 2	71,43
		10 & 3	85,71
		10 & 4	80,00
		10 & 5	85,71
		10 & 6	94,29
		10 & 7	77,14
		10 & 8	91,43
		10 & 9	74,29
		Average	81,59

Figure 11: Analysis of the N-terminal repeat sequences of MAGE-C1.

Left: ClustalW Multiple Alignment of the 10 repeat sequences of MAGE-C1 using the 10th repeat as reference.

Dots indicate aa identical to that of the target sequence. AA that have similar physio-chemical properties are indicated by the same colour. Right: Global pairwise alignment of MAGE-C1 repeat sequence 1 to 9 with the 10th sequence as target sequence. The identity for each alignment and the group average are indicated.

Using the secondary structure prediction software Jpred,¹⁷⁷ the aa string 13-21 (of the 10 repeat sequences) was shown to have a predicted helix structure, indicating that this N-terminal region probably contains at least 10 helices in close proximity, which is likely to have functional significance.

3.2. INVESTIGATION OF MAGE-A1 ASSOCIATED FUNCTION FOR MAGE-C1

3.2.1. Experimental Design

As previously described in section 1.2.2.B, it has been shown that MAGE-A1 associates with SKIP to control the expression of Cyclin D1.^{98,178,179} To start to investigate if MAGE-C1 functions in a similar manner to MAGE-A1 in MM, the first step was to establish if MAGE-C1, MAGE-A1 and SKIP were expressed in the MM cell line RPMI-8226, which served as our MM test platform. This involved determining the expression of these genes at both the mRNA (through rRT-PCR) and protein levels (via western hybridisation). Putative protein associations were then investigated with Co-IP studies to investigate possible direct interactions and using IFM to establish if the various proteins were located within the same cellular compartments (i.e. nucleus vs cytoplasm). Unfortunately, despite concerted efforts, we were unable to source Jagged 1, the ligand required to activate the Notch1 signaling pathway and allow us to investigate the effects of MAGE proteins on this pathway

3.2.2. Co-expression of MAGE-C1, MAGE-A1 and SKIP Transcripts in RPMI-8226

3.2.2.A. Primer Specificity

As described in section 2.3.1. PCR primers were designed to determine the expression of *MAGE-C1*, *MAGE-A1* and *SKIP* in the human MM cell line RPMI-8226 mRNA. To test the specificity of each primer set, a basic PCR reaction was performed rather than rRT-PCR, as those products could not be used for sequence analysis due to possible fluorescence interference from the real-time SYBR-Green component. Transcripts derived from human testis tissue RNA, a tissue known to express high levels of all CTA's,¹⁸⁰ were first converted to cDNA (as described in section 2.3.3.) and then used as the positive control in this experiment, ensuring product amplification for further sequence analysis. The PCR reactions were performed as described in section 2.3.4.A, with the resulting PCR products electrophoresed on an agarose gel, amplicons excised and cleaned, and sequenced according to section 2.3.4.B. The resulting sequences were aligned to the corresponding human reference transcript sequences (obtained from NCBI website) using ClustalW Multiple alignment (BioEdit 7.2.5)¹¹⁵ to ascertain amplification of the correct transcripts.

All the relevant primer sets amplified single amplicons of the expected size, namely, MAGE-C1: 250 bp, MAGE-A1: 260 bp and SKIP: 130 bp. The amplicon sequences aligned to the relevant sections of the human reference transcripts: MAGE-C1: NM_005462.2; MAGE-A1: NM_004988.5 and SKIP: NM_001318844.2 indicating each primer set specificity. Figure 12 shows this analysis using the MAGE-A1 primer set. Mismatches on alignments were confirmed to be artifacts on the chromatogram.

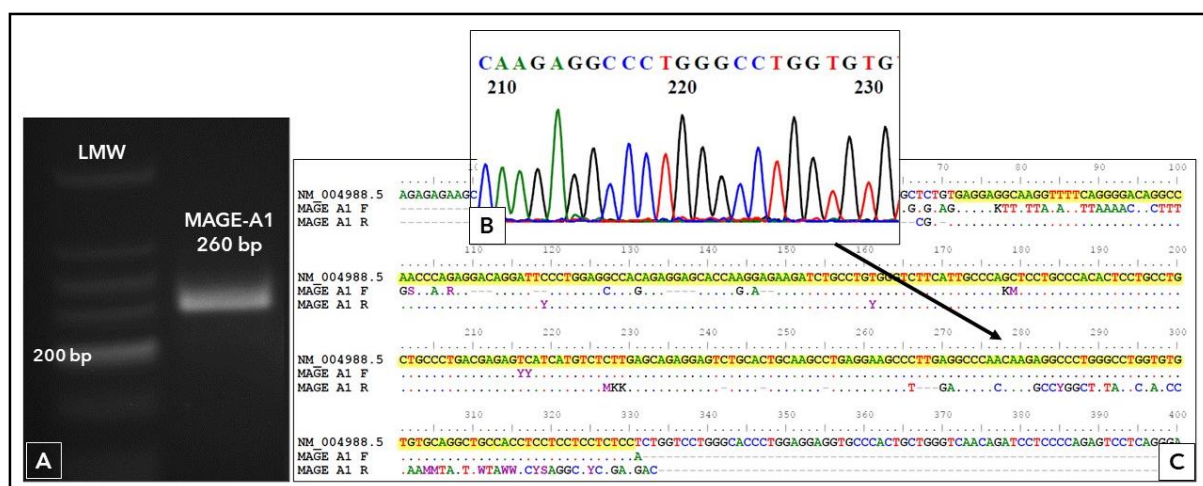


Figure 12: Example of primer specificity analysis using the MAGE-A1 primer pair.

A: Photograph of an ethidium bromide stained agarose gel showing the amplicon produced with the MAGE-A1 primer set using human testis cDNA as a template. B: An example of the raw capillary electrophoresis data (arrow indicates relevant sequence) from the Sanger sequencing of the MAGE-A1 PCR product, showing the quality of the resulting fragment peaks. C: ClustalW multiple alignment of the sequenced MAGE-A1 PCR product (using both the forward (F) and reverse (R) primers) against the NCBI MAGE-A1 mRNA reference sequence NM_004988.5. The amplified region is highlighted in yellow and the coloured dots indicate 100% base identity.

3.2.2.B. Co-expression of MAGE-C1, MAGE-A1 and SKIP Transcripts in RPMI-8226 and Controls

While basic PCR/gel electrophoresis could have been used to qualitatively assess the co-expression of the genes of interest in this study, rRT-PCR was selected instead, to additionally assess if it could be used as a tool in the future analysis of the MAGE pathways. This rRT-PCR was also used to establish which cell types could be used as suitable negative controls, for the subsequent experiments. RPMI-8226 is a human cell line derived from abnormal B-lymphocytes, with predominant PC characteristics (i.e. PC markers CD38⁺, CD138^{+/++}; B-cell markers CD19⁻, CD20⁻, CD27⁻).¹⁸¹ Therefore, the most suitable negative control, for expression studies involving this cell line, would be non-diseased PC that do not express any of the CTAs. Unfortunately, PC are very difficult to isolate as they are not usually present in significant proportions in the PB of healthy individuals and they cannot be cultured as they are terminally differentiated and thus non-dividing.¹⁸² Instead, to identify a suitable negative control for the studies, the expression of the genes of interest was assessed in: 1) Commercially sourced healthy tonsil tissue RNA, which contains an enriched PC population;¹⁸³ 2) Enriched healthy PBMNC preparations (as described in section 2.2.2.), which usually contain 70-90% lymphocytes (5-10% B-cells and 70-85% T-cells);¹⁸⁴ 3) Cultured Jurkat cells, a human lymphoblastic acute T-cell leukemic cell line,¹²³ which could potentially provide a substantial source of material for all of the required studies.

RNA was extracted from the various cell preparations as described in section 2.3.2. and, in combination with the commercial pre-extracted tonsil and testis RNA, was converted to cDNA, and then amplified with the MAGE-A1, SKIP, MAGE-C1 and ABL primers.²⁸ ABL expression served as a control to ensure efficient cDNA synthesis and PCR reaction conditions, as well as acting as a normalization factor for relative expression comparisons.¹³² Amplification of all the products was performed using the same RT preparation/sample type and the PCR was performed using the SYBR-Green protocol (as described in section 2.3.5.). All experiments were performed in duplicate and repeated to confirm results.

Figure 13 (panels A and B) show the separate amplification curves of *MAGE-C1*, *MAGE-A1*, and *SKIP* PCR products (*ABL* amplification not shown), indicating simultaneous expression of all these genes in both RPMI-8226 and human testis tissue (as expected). Melt curve analysis of the amplicons served as confirmation of the amplification of a single amplicon with each primer set, with peaks at different temperatures based upon amplicon size. The melt temperatures in RPMI-8226 and human testis, respectively were as follows: *SKIP* (130 bp) 78.75 °C in both; *MAGE-C1* (250 bp) at 83.50 °C and 83.25 °C; *MAGE-A1* (260 bp) at 85.65 °C and 85.35 °C.

While multiplex PCR, in a single PCR tube, would have been the best way of looking at relative expression, all of the transcripts (from each cell line) in this study were amplified using the same cDNA, a primer-set specific master-mix was used, and the expression of *ABL* transcripts was also amplified at the same time to allow for expression normalisation, we cautiously used the $2^{-\Delta\Delta C_t}$ method (described in section 2.3.5.) to look at relative expression differences between the cancer cell line RPMI-8226 and human testis. Figure 13 panel C shows that there was no significant difference between the two sample types with regards to *SKIP* expression (< 2-fold difference), while *MAGE-C1* potentially showed slightly reduced expression. However, a large 7.9-fold increase in the expression of *MAGE-A1* in RPMI-8226, compared to human testis was an interesting finding, and deserves further investigation (not in the scope of this study).

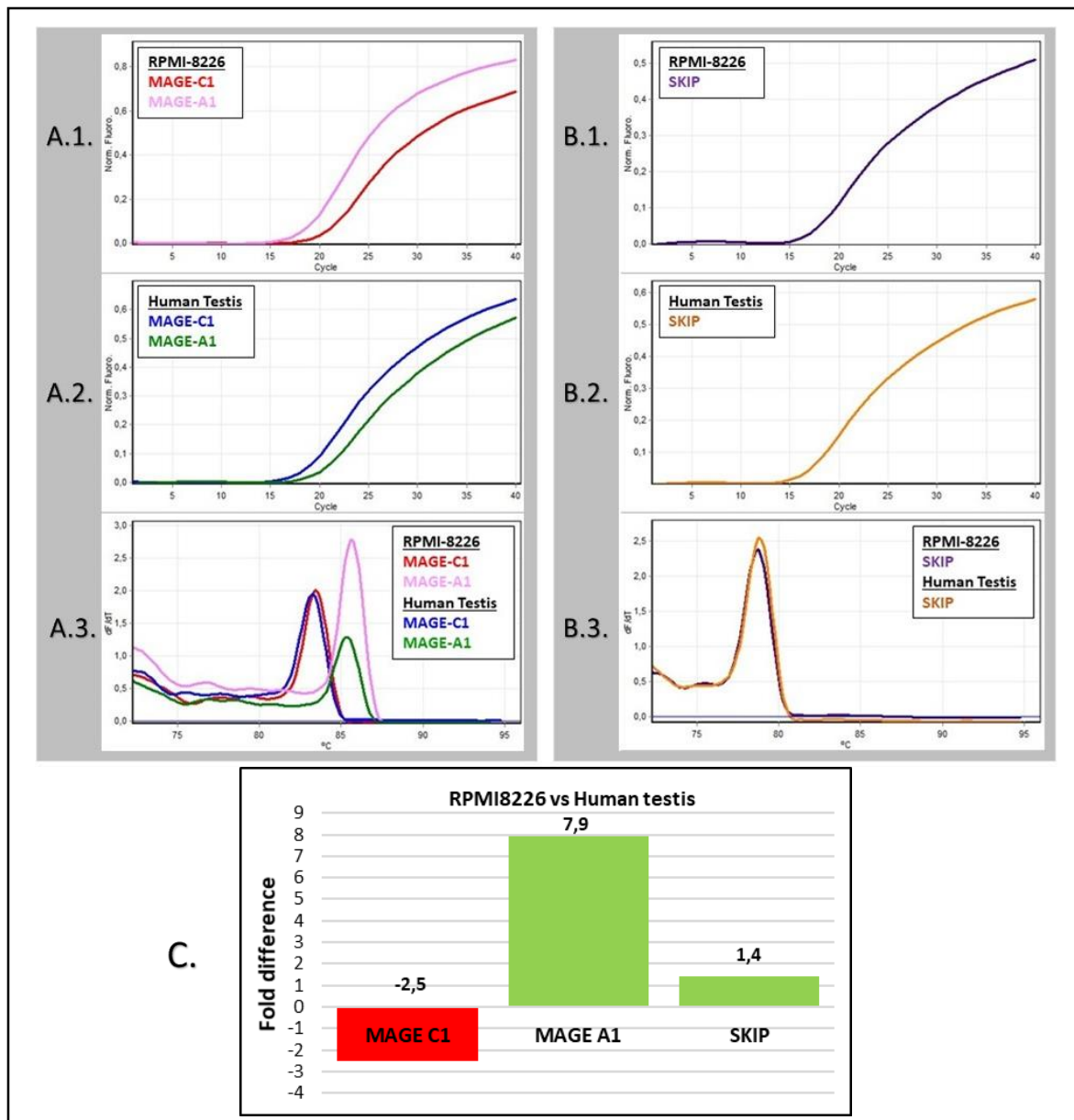


Figure 13: Co-expression of MAGE-C1, MAGE-A1 and SKIP transcripts in RPMI-8226 and human testis tissue.

Panel A: Expression of MAGE-C1 and MAGE-A1: A.1. and A.2. show amplification curves using the relevant primer sets with RPMI-8226 and human testis respectively. A.3. Melt curve analysis of the four amplicon types produced. Panel B Expression of SKIP: B.1. and B.2. show amplification curves using the relevant primer sets with RPMI-8226 and human testis respectively. B.3. Melt curve analysis of the two SKIP amplicons produced. Panel C: The difference in expression levels of MAGE-C1, MAGE-A1 and SKIP in RPMI-8226 compared to human testis using normalisation with ABL Ct values and the $2^{-\Delta\Delta C_t}$ method. Green columns indicate relative increased expression, while red indicates reduced expression.

Figure 14 shows MAGE-C1, MAGE-A1, SKIP and ABL amplification results from the potential CTA-negative control samples: Jurkat, PBMC and human tonsil RNA, with human testis RNA as the positive control in the experiment. As described above, all PCR reactions were performed in a single experiment, using the same cDNA preparation/sample type. As expected, all these samples showed

similar expression levels of *ABL* transcript (Figure 14 D), indicating that all samples contained similar levels of quality cDNA. As *SKIP* is involved in normal cellular function, similar levels of expression of these genes were also expected in all these different RNA samples, and this is what was indicated by Ct values within one Ct of each other (would be less than 2-fold difference in expression).

The expression of the two *MAGE* CTAs (Figure 14 A and B) in these samples was however very different, with very high Ct values for Jurkat, PBMNC and tonsil tissue cDNA, compared to the human testis positive control, indicating either low-level expression or non-specific amplification. Melt curve analysis of these products indicated that the primers may have amplified a mix of *MAGE-C1* specific product and non-specific product with the Jurkat cDNA, with non-specific amplification likely to be the cause of increased fluorescence in both the PBMNC and tonsil samples for *MAGE-C1*. The same seems apparent for the *MAGE-A1* amplification. This is commonly seen in experiments using SYBR-Green (due to non-specific binding into double stranded DNA products, such as primer dimers) and thus Ct cut-offs would be required if quantification were performed in additional studies to prevent false positive reporting. The results from this analysis showed that PBMNC (which contains non-diseased B and T lymphocytes) and tonsil tissue (predominately PC) samples could be used as negative controls for CTA (*MAGE-C1*/*MAGE-A1*) rRT-PCR expression. Jurkat, a neoplastic cell line, appeared to express very low levels of *MAGE-C1* and *MAGE-A1*, and while this may be useful as a low-level *MAGE* positive control, it would not be a suitable negative control for further rRT-PCR experiments unless the true sensitivity limit of detection of the assay is established.

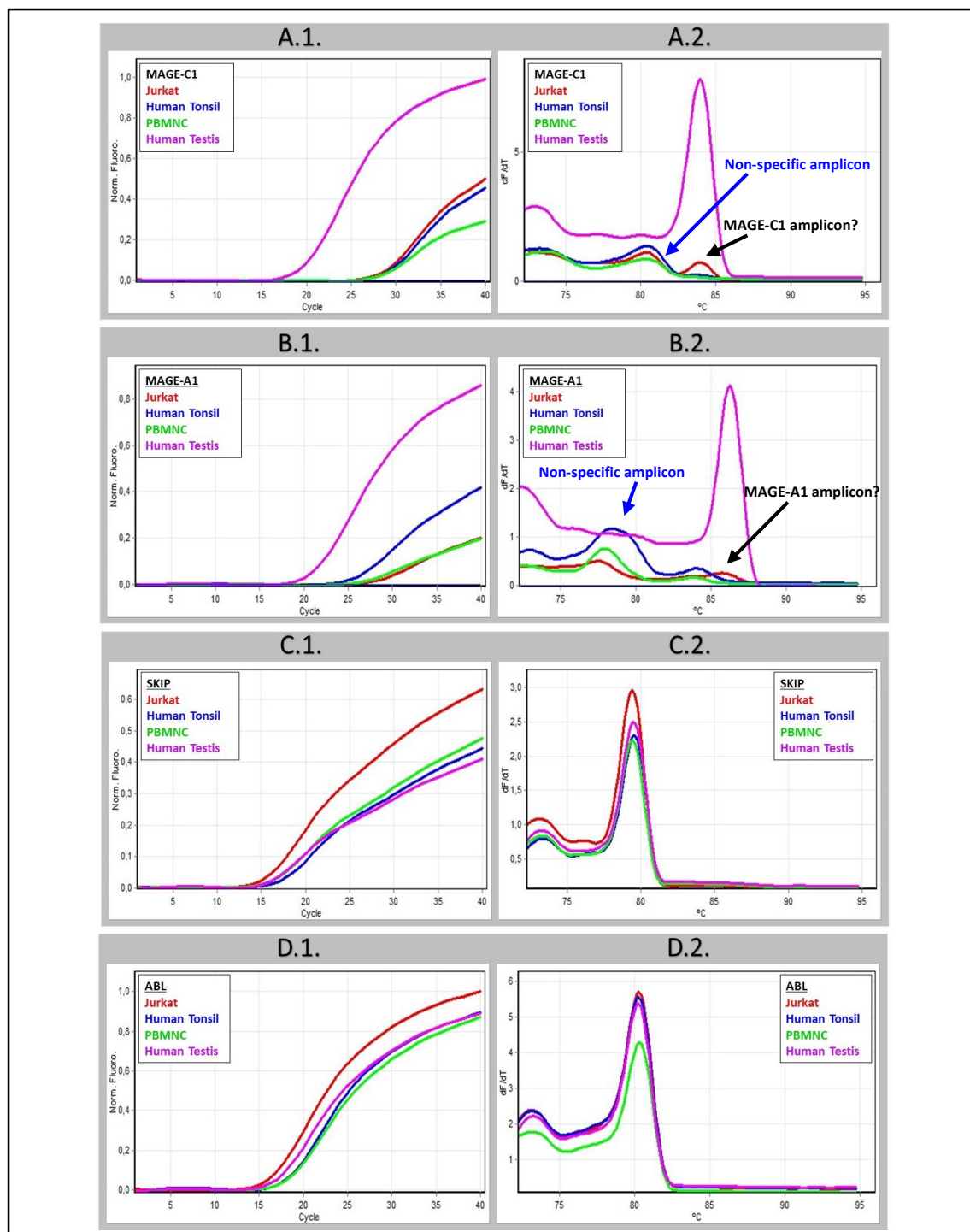


Figure 14: Amplification of MAGE-C1, MAGE-A1, SKIP and ABL transcripts in potential control samples.

Panel A: rRT-PCR amplification (A.1.) and melt curves (A.2.) of MAGE-C1 in Jurkat, human tonsil tissue, PBMNC and human testis. Panel B: rRT-PCR amplification (B.1.) and melt curves (B.2.) of MAGE-A1 in Jurkat, human tonsil tissue, PBMNC and human testis. Panel C: rRT-PCR amplification (C.1.) and melt curves (C.2.) of SKIP in Jurkat, human tonsil tissue, PBMNC and human testis. Panel D: rRT-PCR amplification (D.1.) and melt curves (D.2.) of house-keeping gene ABL in control lines Jurkat, human tonsil tissue, PBMNC and human testis. All real-time experiments were performed using the Quantinova SYBR-Green PCR kit (Qiagen, The Netherlands). The black arrows indicate possible MAGE amplicons. The blue arrows indicate non-specific amplicons.

3.2.3. Co-expression of MAGE-C1, MAGE-A1 and SKIP Protein in RPMI-8226

After successfully showing that *MAGE-C1*, *MAGE-A1* and *SKIP* mRNA transcripts were expressed in the mRNA extracted from cultured RPMI-8226 cells, the next step was to confirm the co-expression of the respective translated proteins. To do this, protein extracts were prepared using RIPA buffer (section 2.4.1.), and consequently analysed via western hybridisation (described in section 2.4.3.). RIPA buffer was specifically chosen due to its ability to lyse all cellular membranes, including nuclear membranes, thus allowing for the detection of nuclear proteins, which may include MAGE-C1.^{153,154,185} RPMI-8226 served as our experimental MM cell line, with PBMNC as a negative control cell line for the two MAGE antibodies. SKIP expression was expected in all cell samples.^{153,154} Jurkat was also included to determine its suitability as a possible low positive or negative control in the subsequent immunoprecipitation and pathway investigations. All membranes were subsequently re-probed with p38 Ab as a loading control.

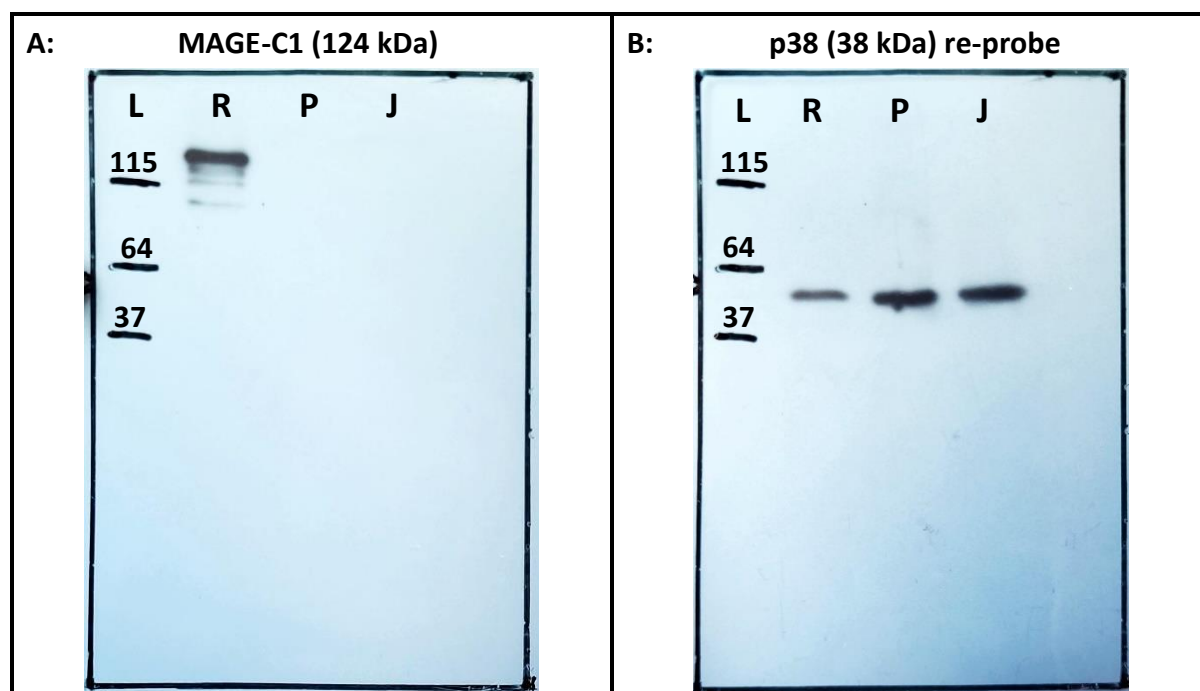


Figure 15: Expression of MAGE-C1 protein in RPMI-8226, PBMNC and Jurkat cells. Full length film images of the nitrocellulose membranes probed with various Ab via western hybridisation and fluorescence detection. **A:** Protein extracts probed with primary anti-MAGE-C1 rabbit monoclonal Ab, (1:1000) and Goat anti-rabbit IgG secondary HRP-linked Ab, (1:5000). **B:** Membrane in panel A was stripped and re-probed with primary p38 rabbit polyclonal Ab, (1:5000) and Goat anti-rabbit IgG secondary HRP-linked Ab, (1:5000). R: RPMI-8226, P: PBMNC, J: Jurkat, L: Benchmark™ Pre-Stained Protein Standard (Life Technologies, USA) (6-180 kDa).

Figure 15 A above shows the detection of the expected dominant single ± 124 kDa band in the RPMI-8226 protein extract, but not in the PBMNC or Jurkat extracts using the anti-MAGE-C1 Ab. The levels of p38 in all three cell extracts confirmed that sufficient protein was loaded and that the whole western hybridisation process was applied to all relevant samples equally. Technical issues were therefore unlikely to be the cause for the lack of detectable expression in the Jurkat and PBMNC extracts. Although Jurkat extracts potentially demonstrated low levels of *MAGE-C1* transcript, this was not detected at the protein level, possibly due to disproportionate expression of protein to mRNA,¹⁸⁶ mutational changes to the protein, or that the rRT-PCR result observed above was non-specific amplification. These results thus indicated that RPMI-8226 expresses detectable MAGE-C1 at both the mRNA and protein levels, while PBMNCs and Jurkat (with caution) can be used as negative controls.

Detection of MAGE-A1 in the RPMI-8226 extracts proved more difficult. As the anti-MAGE-A1 Ab we used had not previously been tested in a western hybridisation setting,¹³⁶ we first tried using standard conditions, which included 25 μ g protein extract/well, primary Ab at 1:1000 dilution and film exposure ranging from 30 sec to O/N. However, no signals were obtained for any of the extracts. This was despite the p38 loading control protein clearly being detected (results not shown). Figure 16 A shows the results using the primary anti-MAGE-A1 Ab at a higher concentration (1:500 dilution), which again yielded no positive signal. This was completely unexpected, as MAGE-A1 transcript analysis (section 3.2.2.B.) clearly demonstrated a relatively high level of expression of the transcript (MAGE-A1:ABL Ct = 0.93). It was however possible that the amount of translated protein was actually quite low, thus we repeated the analysis with very high levels of protein/lane (100-150 μ g, maximal amount that could be loaded) and even higher levels of anti-MAGE-A1 Ab (1:200 dilution). The results of this analysis are shown in Figure 16 B. As can be seen, this combination resulted in the obvious non-specific binding of the Ab to the protein extracts. No MAGE-A1 protein was expected in the PBMNC extract (due to no mRNA expression detected) with the expected protein size being 34 kDa (can however vary due to modifications). Because of this, the single bands of > 37 kDa detected in the RPMI-8226 and Jurkat extracts cannot be confirmed as MAGE-A1 under these circumstances. Due to these issues, the expression of MAGE-A1 in RPMI-8226 could not definitely be established.

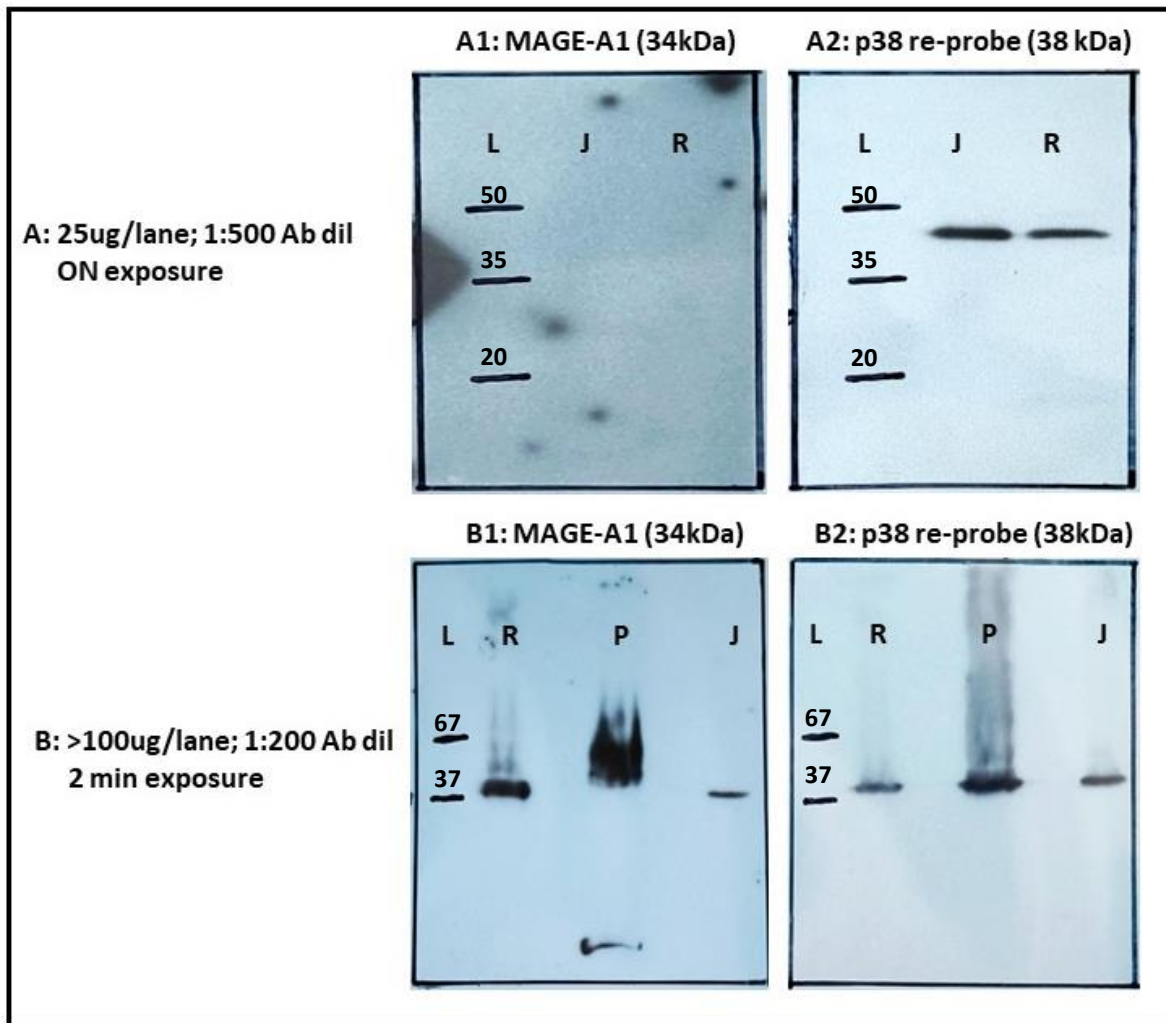


Figure 16: Expression of MAGE-A1 protein in RPMI-8226, PBMNC and Jurkat cells.

Full length film images of the nitrocellulose membranes probed with various antibodies via western hybridisation and fluorescence detection. **A1:** Protein extracts (25 μ g/lane) probed with primary anti-MAGE-A1 mouse monoclonal Ab, (1:500) and Horse anti-mouse IgG secondary HRP-linked Ab, (1:5000), with an O/N exposure. **A2:** Membrane from A1 was stripped and re-probed with primary p38 rabbit polyclonal Ab, (1:5000) and Goat anti-rabbit IgG secondary HRP-linked Ab, (1:5000). **B1:** Protein extracts (150 μ g for R and P and 100 μ g for J) probed with primary anti-MAGE-A1 mouse monoclonal Ab, (1:200) and Horse anti-mouse IgG secondary HRP-linked Ab, (1:5000), with a 2-minute exposure. **B2:** membrane from B1 was stripped and re-probed with primary p38 rabbit polyclonal Ab, (1:5000) and Goat anti-rabbit IgG secondary HRP-linked Ab, (1:5000). R: RPMI-8226, P: PBMNC, J: Jurkat, L in A1: Peqlab pre-stained protein standards (20-120 kDa), L in B1: Benchmark™ Pre-Stained Protein Standard (Life Technologies) (6-180 kDa).

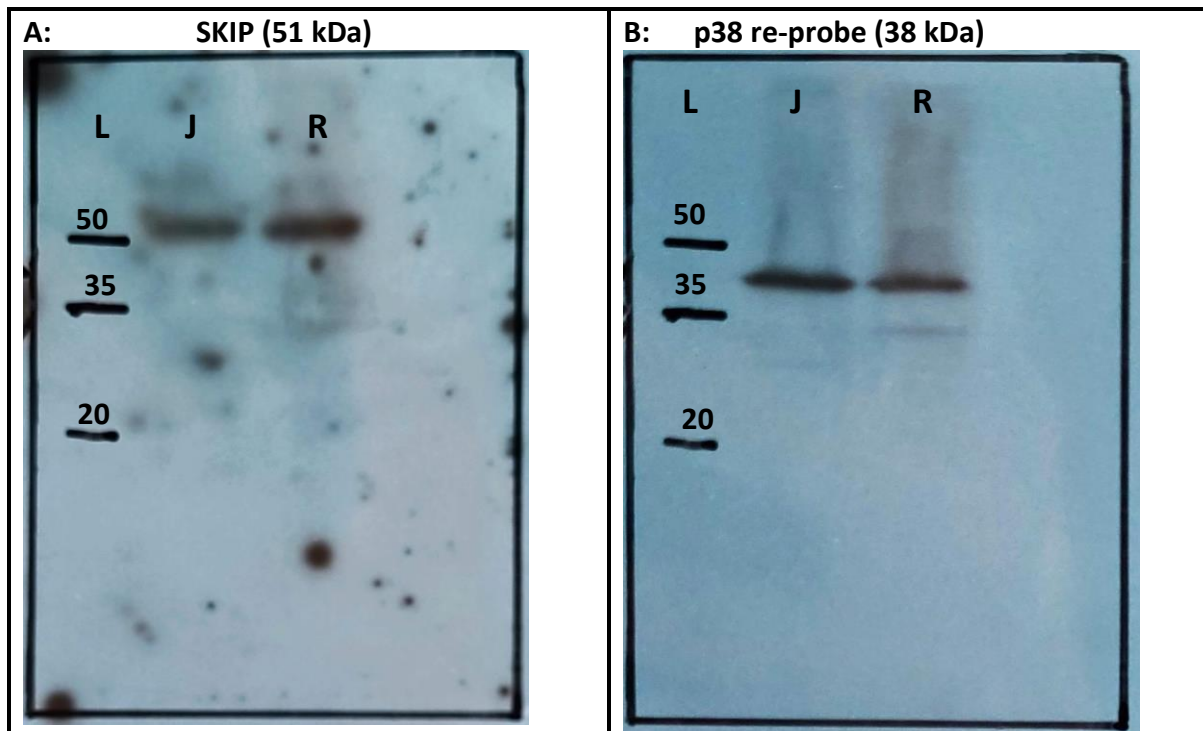


Figure 17: Expression of SKIP protein in Jurkat and RPMI-8226 cells.

Full length film images of the nitrocellulose membranes probed with various antibodies via western hybridisation and fluorescence detection. **A:** Protein extracts (30 µg/lane) probed with primary anti-SKIP mouse polyclonal Ab, (1:500) and Horse anti-mouse IgG secondary HRP-linked Ab, (1:5000). **B:** Membrane in panel A was stripped and re-probed with primary p38 rabbit polyclonal Ab, (1:5000) and Goat anti-rabbit IgG secondary HRP-linked Ab (1:5000). R: RPMI-8226, J: Jurkat, L: Peqlab pre-stained protein standards (20-120 kDa).

Figure 17 depicts the expected detection of SKIP protein expression in both RPMI-8226 and Jurkat RIPA protein extracts. A single band of approximately 51 kDa in each lane indicated specific Ab binding, indicating the potential use of this Ab in further western hybridisation experiments. PBMNC protein extracts also showed the expression of SKIP protein using this Ab (results not shown).

3.2.4. Co-IP of MAGE-C1, MAGE-A1 and SKIP Protein in RPMI-8226

After the co-expression of *MAGE-C1* and *SKIP* in RPMI-8226 was established at both the mRNA and protein levels, the next step was to determine if they directly interacted with one another, as previously shown for MAGE-A1 and SKIP (although not in our MM cell line).⁹⁸ We decided to look for direct interactions using the Co-IP with magnetic Dynabeads. The success of this technique is highly dependent upon maintaining the cellular proteins in their functional tertiary structures, including complex protein complexes.

3.2.4.A. Testing the Co-IP process

We had initially wanted to use the known MAGE-A1:SKIP association as a positive control in this analysis, but as the methodology involved western hybridisation detection of relevant proteins, we were unable to investigate this particular association due to the lack of a suitable anti-MAGE-A1 Ab. Instead, as a more generic positive control for this experimental process, Co-IP of RPMI-8226 cells with anti-Human IgG Ab was tested. MM cells express large amounts of immunoglobulins, thus we used the technique to “pull down” IgG, using the anti-IgG Ab as the bait. This also allowed us to confirm that the process does not result in non-specific enrichment of MAGE-C1. As different stringency conditions were recommended for the cell lysis, we used this control experiment to examine the effects of the different extraction buffers.

The Co-IP process was performed as described in section 2.5. This opportunity was also used to compare stringent (1 × IP (Thermo Fisher Scientific, USA), 100 mM NaCl (Glentham Life Sciences, UK), 2 mM MgCl₂ (Merck, USA), 1 mM DTT (Promega, USA), 1 × PI) and less stringent (1 × IP (Thermo Fisher Scientific, USA), 100 mM NaCl (Glentham Life Sciences, UK), lysis buffer. Briefly, beads for two Co-IP reactions (one for each lysis buffer) were prepared by coupling 1.5 mg Dynabeads to 10.5 µg Rabbit anti-Human IgG secondary Ab as recommended (section 2.5.2.). Two RPMI-8226 pellets (0.1 g = 2 × 10⁷ cells each) were prepared and lysed using each of the buffer preparations according to section 2.5.2. Following cell lysis, the lysate and Ab-coupled beads were incubated together for 30 min at 4 °C and then the protein complexes detached from the beads (as described in section 2.5.2.). As controls for the Co-IP reaction two additional cell pellets of 0.01 g were prepared and lysed for input analysis. Unfortunately, the control/input pellet for the stringent buffer was lost during the experimental process, and only input lysate from the less stringent buffer could be analysed. There was no apparent difference in the results of the Co-IP reactions between the two lysis buffers, so less stringent was selected. After Co-IP, the BCA assay was used to quantify the protein content of the isolated protein (section 2.4.2.) but was ineffective due to interference of the lysis buffer with the BCA assay.

The maximum amount of protein (23 µL) was used for the loading reaction, the proteins denatured, and the three preparations loaded onto a 12% acrylamide gel followed by protein separation via electrophoresis, as described in section 2.4.3.A. The membrane was first stained with Ponceau S to confirm successful transfer, and then probed with anti-Human IgG Ab HRP-linked, followed by anti-MAGE-C1 Ab, after the membrane had been stripped (section 2.4.3.B and 2.4.3.C.). The following fractions were collected and analysed via western hybridisation, as described in section 2.5: input cell lysate, final eluted protein fraction from the beads. Figure 18 shows the results of this analysis.

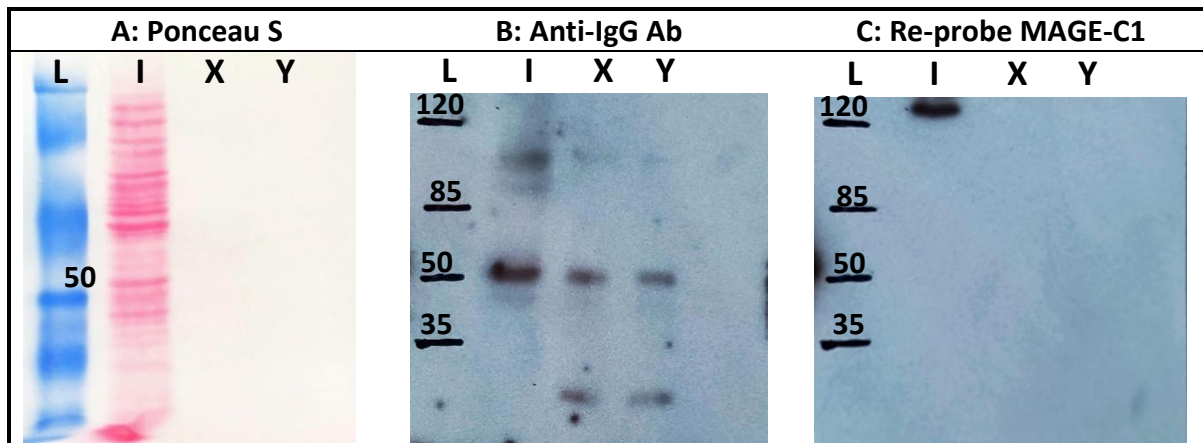


Figure 18: Detection of IgG and MAGE-C1 in the RPMI-8226 Co-IP fractions.

This figure shows the staining of the Co-IP fractions obtained from incubation of RPMI-8226 cell lysate with anti-Human IgG Ab coated Dynabeads, following separation of 23 μ L of protein on an acrylamide gel and transfer to a nitrocellulose membrane. A: The first image shows the nitrocellulose membrane stained with Ponceau S. B: The second is a blot resulting from the staining/probing of the same membrane with probed with secondary anti-Human IgG HRP-linked Ab, (1:1000) (25 and 50 kDa). C: The last image is the blot following the stripping of the same membrane and staining/probing with primary anti-MAGE-C1 rabbit monoclonal Ab (1:1000) (124 kDa) and Goat anti-rabbit IgG secondary HRP-linked Ab (1:5000). I: Input protein extracted with less stringent lysis buffer, X: Isolated protein from IgG Co-IP extracted with less stringent buffer, Y: Isolated protein from IgG Co-IP extracted with more stringent buffer, L: Peqlab pre-stained protein standards (20-120 kDa).

The Ponceau S stained nitrocellulose allowed us to confirm that there was no obvious non-specific binding/elution of protein during the process, with a very obvious reduction content between the input protein lane (I) and the two final elution lanes. The presence of IgG antigens in the cell lysate (I: strong 50 kDa band), as well as in the Co-IP protein isolates (X and Y: 50 and 25 kDa bands, size of the human IgG heavy and light chains) indicated that the Ab-bead coupling reaction worked and that the bait protein (termed this although we did not look for the co-isolation of any target proteins in this case) was successfully “pulled down” during the Co-IP reaction. There was no obvious difference between the results in lane X and Y (e.g. non-specific bands, band density) showing that there was no difference between the effectiveness of the more or less stringent lysis buffer in these. Therefore, the less stringent buffer was used to lyse all further samples for Co-IP analysis. The membrane was stripped and re-probed with anti-MAGE-C1 Ab to control for non-specific pulldown of the specific protein by the beads (as would not expect binding to the IgG to occur). A band in Figure 18 C, lane I, indicates that MAGE-C1 was present in the cell lysate before the Co-IP reaction, while the absence of bands in lanes X and Y confirms that it was not pulled down non-specifically during the Co-IP reaction.

3.2.4.B. MAGE-C1:SKIP Co-IP analysis

RPMI-8226 cell pellets with a mass of 0.1 g were lysed with the less stringent buffer, and the process conducted as described in section 2.5, using the recommended 10.5 µg anti-MAGE-C1 Ab. During the Co-IP process, the flow-through (which is the supernatant post cell lysate hybridisation to the coupled beads, representing protein that doesn't bind) and the isolated/enriched protein fraction (which is the buffer containing the proteins detached from the Ab-beads and should include the bait protein and any protein that it formed a complex with) was collected for analysis. As a control for this analysis, 0.1 g of the same cell preparation was lysed, but not incubated with the beads, to demonstrate the proteins of interest in the original cell preparation. Finally, a separate Co-IP reaction using anti-IgG Ab-coupled beads was performed at the same time, with the same cultured cells (0.1 g) and the same buffers, to demonstrate specificity of the experimental conditions (only the Co-IP enriched protein fraction was included in the western hybridisation analysis). The four relevant protein preparations (23 µL in each loading reaction and denatured) were then loaded onto a 12% acrylamide gel and the proteins separated via electrophoresis, as described in section 2.4.3.A. The membrane was first stained with Ponceau S and then probed with anti-MAGE-C1 Ab, followed by anti-SKIP Ab, after the membrane had been stripped (section 2.4.3.B and 2.4.3.C.).

Figure 19 panel A shows the results of this first Co-IP attempt using 0.1 g of cells per preparation. The Ponceau S image showed that multiple proteins were present in the input (I) and flow-through (F) fractions, as expected, but no obvious staining occurred in the lanes of the final enriched proteins (as expected, due to low levels). Probing with the anti-MAGE-C1 Ab indicated that MAGE-C1 protein was present in the original cell preparation (I) (as expected), and while it was also detected in the flow-through (F), some of the MAGE-C1 had been “caught” by the Ab-coupled beads, as shown by its detection in the C1 (enriched fraction lane), demonstrating the ability of the Ab-coupled beads to purify MAGE-C1 from the lysate. Again, the IgG experiment showed that no MAGE-C1 was enriched when different abs were used in the process (IgG). Unexpectedly, we were unable to detect SKIP in any of the fractions indicating that the use of 0.1 g RPMI-8226 cells may not have been enough to be able to detect SKIP. As found in section 3.2.3. our previous western hybridisation with the SKIP Ab required 140 µg of input protein. We therefore repeated the Co-IP experimental set using 1.2 g of RPMI-8226 cells. The results of this repeat CO-IP are shown in Figure 19B.

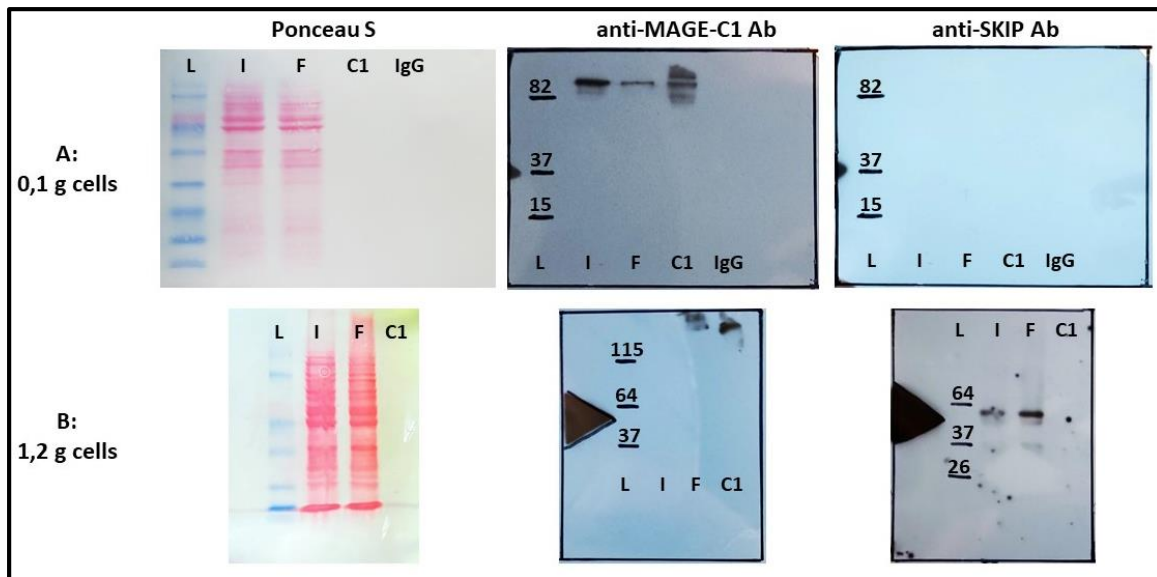


Figure 19: Detection of MAGE-C1 and SKIP in the RPMI-8226 Co-IP fractions.

The combined figures show the staining of the Co-IP fractions obtained from incubation of RPMI-8226 cell lysate with anti-MAGE-C1 Ab coated Dynabeads, following separation on an acrylamide gel and transfer to a nitrocellulose membrane. Panel A: This deals with the analysis of the Co-IP fractions using an input of 0.1g cells. The first image shows the nitrocellulose membrane stained with Ponceau S. The second is the blot resulting from the staining/probing of the same membrane with probed with primary anti-MAGE-C1 rabbit monoclonal Ab (1:1000) (124 kDa) and goat anti-rabbit IgG secondary HRP-linked Ab (1:5000). The last image in the set is the blot following the stripping of the same membrane and staining/probing with primary anti-SKIP mouse polyclonal Ab (1:500) (51 kDa) and horse anti-mouse IgG secondary HRP-linked Ab (1:5000). Panel B: This deals with the analysis of the Co-IP fractions, using an input of 1.2g cells. The three images are as described for Panel A, using a different membrane. I: Input protein lysate, pre bead incubation; F: Flow-through after the incubation of the lysate with anti-MAGE-C1 Ab coated beads C1: Isolated/enriched protein fraction following elution from the anti-MAGE-C1 Ab coated beads. IgG: Isolated/enriched proteins following elution from the anti-IgG Ab coated beads., L: Benchmark™ Pre-Stained Protein Standard (Life Technologies, USA).

Figure 19 Panel B shows the Co-IP results of this second attempt, with the Ponceau S stained membrane again showing that a wide range of proteins were present in the input (I) and flow-through (F) lanes and that at least MAGE-C1 was again detected in the flow through and in the final eluted fraction. It must be noted that the separation of the larger proteins, including MAGE-C1, was not optimal and the expected MAGE-C1 band in the initial input sample was not seen, possibly due to a loading error, or cut off while trimming the membrane. The use of the larger input of cell lysate and thus cellular protein did allow for the detection of SKIP in the same input and flow through fractions (single bands of the expected $\pm$ 51 kDa size). However, no SKIP-specific band was detected in the final fraction with the co-isolated proteins, possibly indicating that under the experimental conditions the MAGE-C1 protein that bound to the Ab-coated beads, was not co-bound to SKIP. Unfortunately we could not conclude that SKIP did not interact with MAGE-C1 from this experimental set, as the following could be responsible for this result: 1) Protein levels were still too low to detect the small amount of SKIP that

may have been bound to MAGE-C1; 2) experimental conditions, such as wash buffer stringency and harsh physical conditions may have prevented the complexes from staying intact; 3) The physiological state of the cells may not be conducive to the MAGE-C1:SKIP complex forming. The investigation of the MAGE-A1:SKIP interaction via Co-IP could have potentially provided a clearer indication of the reason for the lack of detectable SKIP.

3.2.5. IFM of MAGE-C1, MAGE-A1 and SKIP

While the Co-IP analysis of MAGE-C1 and SKIP was not conclusive, the investigation of where the proteins were localised within the cell (i.e. either nucleoplasm or cytoplasm) could potentially still provide some information regarding possible interactions and more importantly confirm that the expression of these proteins occurred in the same subcellular fractions as the models used for MAGE-A1 function. We chose to use IFM to co-locate the proteins of interest in RPMI-8226, with PBMNC preparations acting as negative controls. As this was a different Ab technique, using whole fixed cells, the anti-MAGE-A1 Ab, previously used for western hybridisation, was also used in this analysis, potentially as a control for the localisation staining. This Ab appears to have only been validated for use in paraffin-imbedded slides by AbCam.¹³⁶

As described in section 2.6.1 human anti-MAGE-A1 Ab, anti-MAGE-C1 Ab and anti-SKIP Ab were permanently fluorescently labelled with either CF™488 (MAGE-A1 and MAGE-C1; green) or CF™647 (SKIP; red). Actively cultured RPMI-8226 cells and freshly prepared PBMNC cells were fixed and permeabilised with methanol before being incubated with combinations of the FL-Abs (MAGE-A1 – 1:50 dilution, MAGE-C1 – 1:50 dilution, SKIP – 1:100 dilution),¹⁵⁴ attached to slides and finally co-stained with Hoechst nuclear stain (blue) (as described in according to section 2.6.2. and 2.6.3.). The role of the PBMNC preparations was to act as a negative control for the process, as we had already established that they were not expressing either MAGE protein or SKIP. Thus in addition to the triple-labelled preparations, the following combinations were also analysed in parallel to allow us to establish the correct settings for each required channel (excitation nm/emission nm/gain) (to exclude autofluorescence, but not actual FL-Ab binding) and to assess any non-specific Ab-binding. PBMNC labelled with only Hoechst stain; PBMNC labelled with Hoechst stain and Anti-MAGE-A1 FL-Ab; PBMNC labelled with Hoechst stain and Anti-MAGE-C1 FL-Ab; RPMI-8226 labelled with only Hoechst stain. The final channel settings were then used for the triple-stained samples, to allow direct comparison.

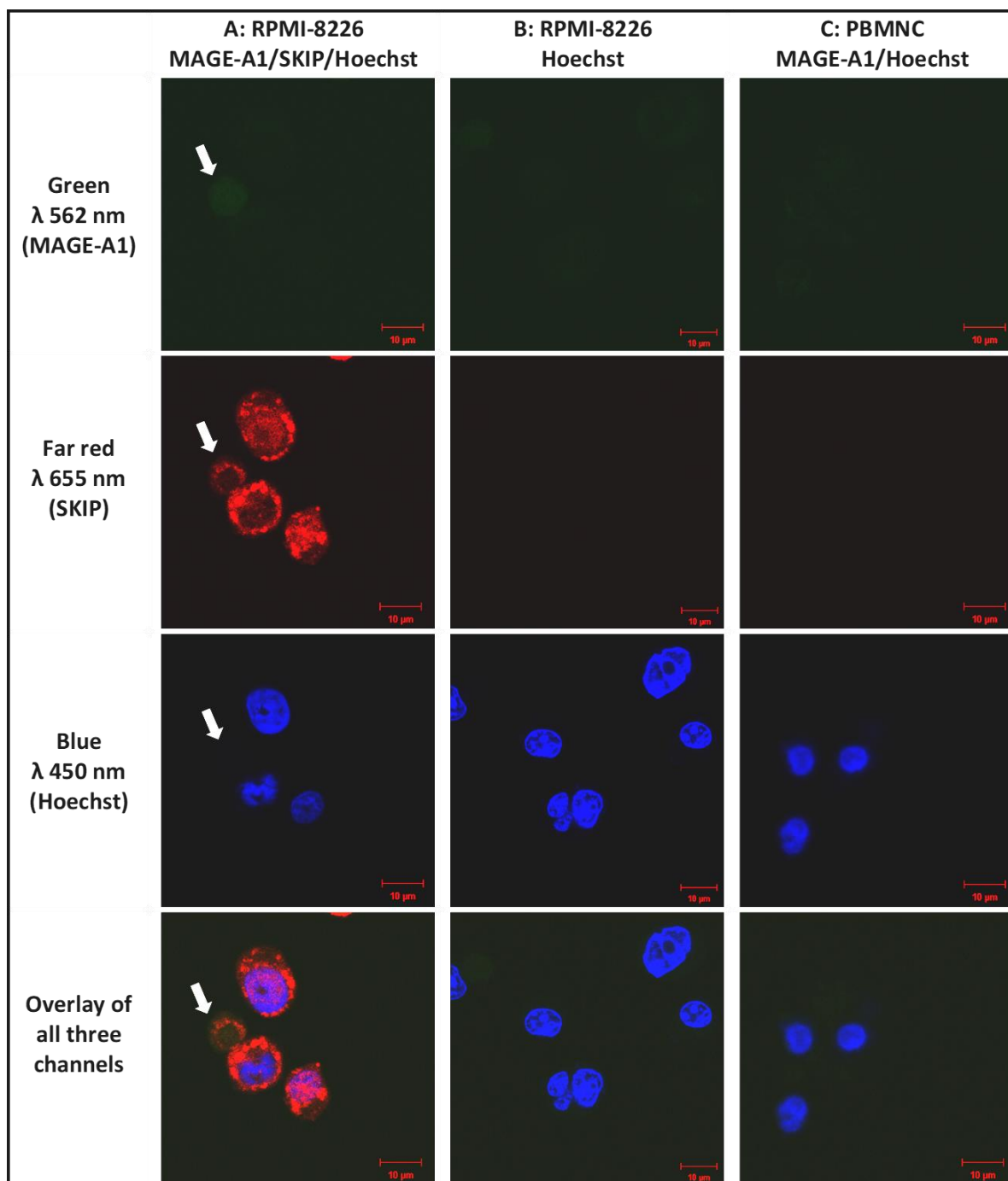


Figure 20: Microscopy analysis of RPMI-8226 and PBMNC stained with fluorescently labelled anti-MAGE-A1, anti-SKIP Ab and Hoechst stain.

Panel A: RPMI-8226 cells co-stained with anti-MAGE-A1 Ab (CF488A-tagged), anti-SKIPAb (CF647-tagged) and Hoechst nuclear stain, showing fluorescence in each relevant channel. Panel B: RPMI-8226 cells stained with Hoechst stain only, indicating the levels of autofluorescence observed with the selected gain settings in each channels. Panel C: PBMNC co-stained with anti-MAGE-A1 (CF488A -tagged) and Hoechst nuclear stain, showing fluorescence in each relevant channel. The same settings were used for all microscopy analysis. The arrows indicate artifacts/necrotic cells.

Figure 20 shows the IFM results of anti-MAGE-A1 and anti-SKIP Ab co-staining of both RPMI-8226 and PBMNC cell preparations. Panel A shows SKIP protein (far red channel) staining in the cytoplasm and nucleoplasm of RPMI-8226 (confirmed using the z-stacking function, which allowed us to scan for Fl-Ab binding in front, behind and within the nucleus separately, as expected. Panels B and C show that there was no false-signal in either the far-red (SKIP) channel or the green (MAGE-A1) channels for either RPMI-8226 or PBMNC preparations, indicating the specific binding shown in Panel A. Unfortunately, under the fixation and staining conditions, background fluorescence in the green channel (562 nm) did not increase significantly in the presence of the Fl-Ab anti-MAGE-A1 in RPMI-8226. However, non-specific binding of the Ab to dead cells/debris was noted (indicated with the white arrow in panel A, contained no nucleus). We therefore again could not confirm MAGE-A1 expression in RPMI-8226.

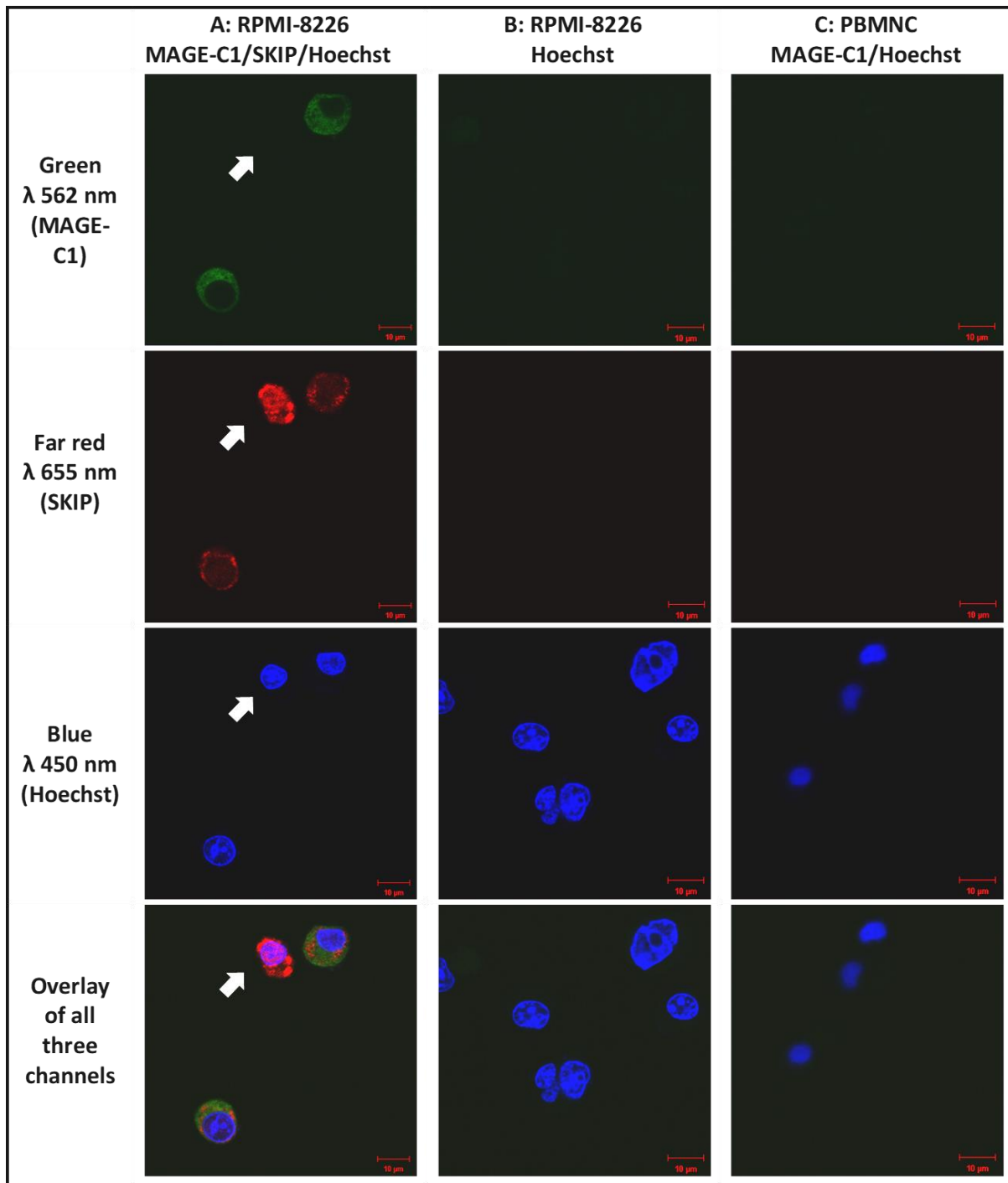


Figure 21: Microscopy analysis of RPMI-8226 and PBMNC stained with fluorescently labelled anti-MAGE-C1, anti-SKIP Ab and Hoechst stain.

Panel A: RPMI-8226 cells co-stained with anti-MAGE-C1 Ab (CF488A -tagged), anti-SKIP Ab (CF647-tagged) and Hoechst nuclear stain, showing fluorescence in each relevant channel. Panel B: RPMI-8226 cells stained with Hoechst stain only, indicating the levels of autofluorescence observed with the selected gain settings in each channel. Panel C: PBMNC co-stained with anti-MAGE-C1 (CF488A -tagged) and Hoechst nuclear stain, showing fluorescence in each relevant channel. The same settings were used for all microscopy analysis. The arrows indicate a cell not labelled with MAGE-C1, but showing high expression of SKIP.

Figure 21 shows the IFM results of anti-MAGE-C1 Ab and anti-SKIP Ab co-staining of both RPMI-8226 and PBMNC cell preparations. Panel A shows SKIP protein (far red channel) again staining in the cytoplasm and nucleoplasm of RPMI-8226, as expected (although not as bright, probably due to technical variation). Additionally, it shows the successful staining of the RPMI-8226 cells with the anti-MAGE-C1 Ab. Surprisingly, this staining indicated that MAGE-C1 protein (green channel) was located in the cytoplasm only, with the clear absence of green fluorescence in the nucleoplasm. Panels B and C show that there was no false-signal in either the far-red (SKIP) channel or the green (MAGE-A1) channels for either RPMI-8226 or PBMNC preparations, indicating the specific binding shown in Panel A. These results indicated that SKIP and MAGE-C1 co-localised in the cytoplasm of RPMI-8226.

3.3. INVESTIGATION OF MAGE-C2 ASSOCIATED FUNCTION FOR MAGE-C1

3.3.1. Experimental Design

As previously described in section 1.2.2.C, it has been shown that MAGE-C2 associates with both RBX1 and TRIM28 to affect the expression levels of Cyclin E1 and TP53.^{101,102} To start to investigate if MAGE-C1 functions in a similar manner to MAGE-C2 in MM, the first step was to establish if MAGE-C2, TRIM28 and RBX1 were expressed in the MM cell line RPMI-8226, which served as our MM test platform. This involved determining the expression of these genes at both the mRNA (through rRT-PCR) and protein levels (via western hybridisation). Putative protein associations were then investigated through Co-IP studies to investigate possible direct interactions and IFM to determine cellular localisation. Finally, we investigated the proposed effects of MAGE proteins on inappropriate cell cycle progression by activating an ER-stress response, which specifically activates ubiquitination protein degradation pathways, and then looking at the expression of several proteins linked to the triggered E3 ligase pathways.

3.3.2. Co-expression of *MAGE-C1*, *MAGE-C2*, *RBX1* and *TRIM28* Transcripts in RPMI-8226

3.3.2.A. Primer Specificity

As described in section 2.3.1. PCR primers were designed to determine the expression of *MAGE-C2*, *RBX1* and *TRIM28* in the human MM cell line RPMI-8226 mRNA. The expression of *MAGE-C1* was established previously, as described in the section 3.2.2, above. The process to determine the specificity of each relevant primer set was the same as described above in section 3.3.A.

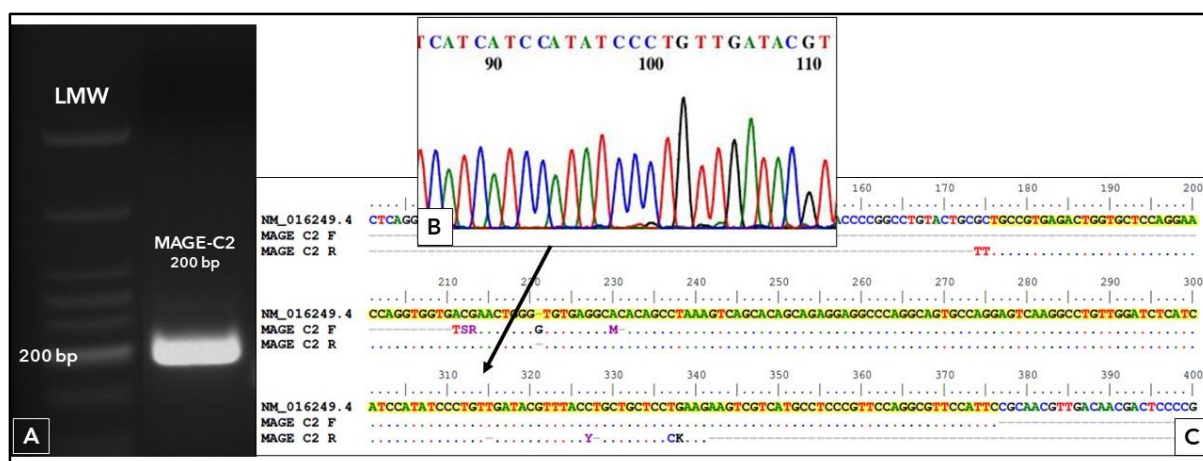


Figure 22: Example of primer specificity analysis using the MAGE-C2 primer pair.

A: Photograph of an ethidium bromide stained agarose gel showing the amplicon produced with the MAGE-C2 primer set using human testis cDNA as a template. **B:** An example of the raw capillary electrophoresis data (arrow indicated relevant sequence) from the Sanger sequencing of the MAGE-C2 PCR product, showing the quality of the resulting fragment peaks. **C:** ClustalW multiple alignment of the sequenced MAGE-C2 PCR product (using both the forward and reverse primers) against the NCBI MAGE-C2 mRNA reference sequence NM_016249.4. The amplification region is highlighted in yellow and the coloured dots indicate 100% base identity.

All the relevant primer sets amplified single amplicons of the expected size, namely, MAGE-C2: 200 bp, RBX1: 150 bp, and TRIM28: 160 bp. The amplicon sequences aligned to the relevant sections of the human reference transcripts: MAGE-C2: NM_016249.45 (76-375 bp), RBX1: NM_014248.4 (135-279 bp) and TRIM28: NM_005762.3 (1261-1423 bp), indicating each primer set specificity. Figure 22 shows this analysis using the MAGE-C2 primer pair as an example. Mismatches on alignments were confirmed to be artifacts on the chromatogram.

3.3.2.B. Co-expression of MAGE-C2, TRIM28 and RBX1 Transcripts in RPMI-8226 and Controls

To qualitatively assess the co-expression of the genes of interest in this study, rRT-PCR was selected to additionally assess if it could be used as a tool in the future analysis of the MAGE pathways. This rRT-PCR was also used to assess which cell types could be used as suitable negative controls: healthy tonsil tissue RNA (commercial product), PBMC preparations (as described in section 2.2.2.), and cultured Jurkat cells.¹²³

RNA was extracted from the various cell preparations as described in section 2.3.2. and, in combination with the commercially prepared tonsil and testis RNA, was converted to cDNA, and then amplified with the MAGE-C2, TRIM28, RBX1, MAGE-C1 and ABL primer pairs in separate tubes. As previously mentioned, ABL expression served as a control to ensure efficient cDNA synthesis and PCR reaction conditions, as well as acting as a normalization factor for relative expression comparisons.¹³⁰

Amplification of all the products was performed using the same RT preparation/sample type and all of reactions were performed as a set, using the SYBR-Green protocol (as described in section 2.3.5.). All experiments were performed in duplicate and repeated to confirm results.

Figure 23 panels A and B show the separate amplification curves of *MAGE-C1*, *MAGE-C2*, *TRIM28*, and *RBX1* PCR products (ABL amplification not shown), indicating simultaneous expression of all these genes in both RPMI-8226 and human testis tissue (as expected). Melt curve analysis of the amplicons served as confirmation of the amplification of a single amplicon with each primer set, with peaks at different temperatures based upon amplicon size. Figure 23, panel C shows that, as in the previous analysis (Figure 13.), *MAGE-C1* showed no significance in relative expression between RPMI-8226 and human testis (< 2-fold change).¹⁶¹ Logarithmic differences were however noted for *RBX1* with > 10-fold higher expression in RPMI-8226 and *MAGE-C2* showing a reduced expression of > 1.5 logs in RPMI-8226. These results indicate that RPMI-8226 and human testis show a different MAGE gene expression profile, which deserves further investigation, but is beyond the scope of the current project.

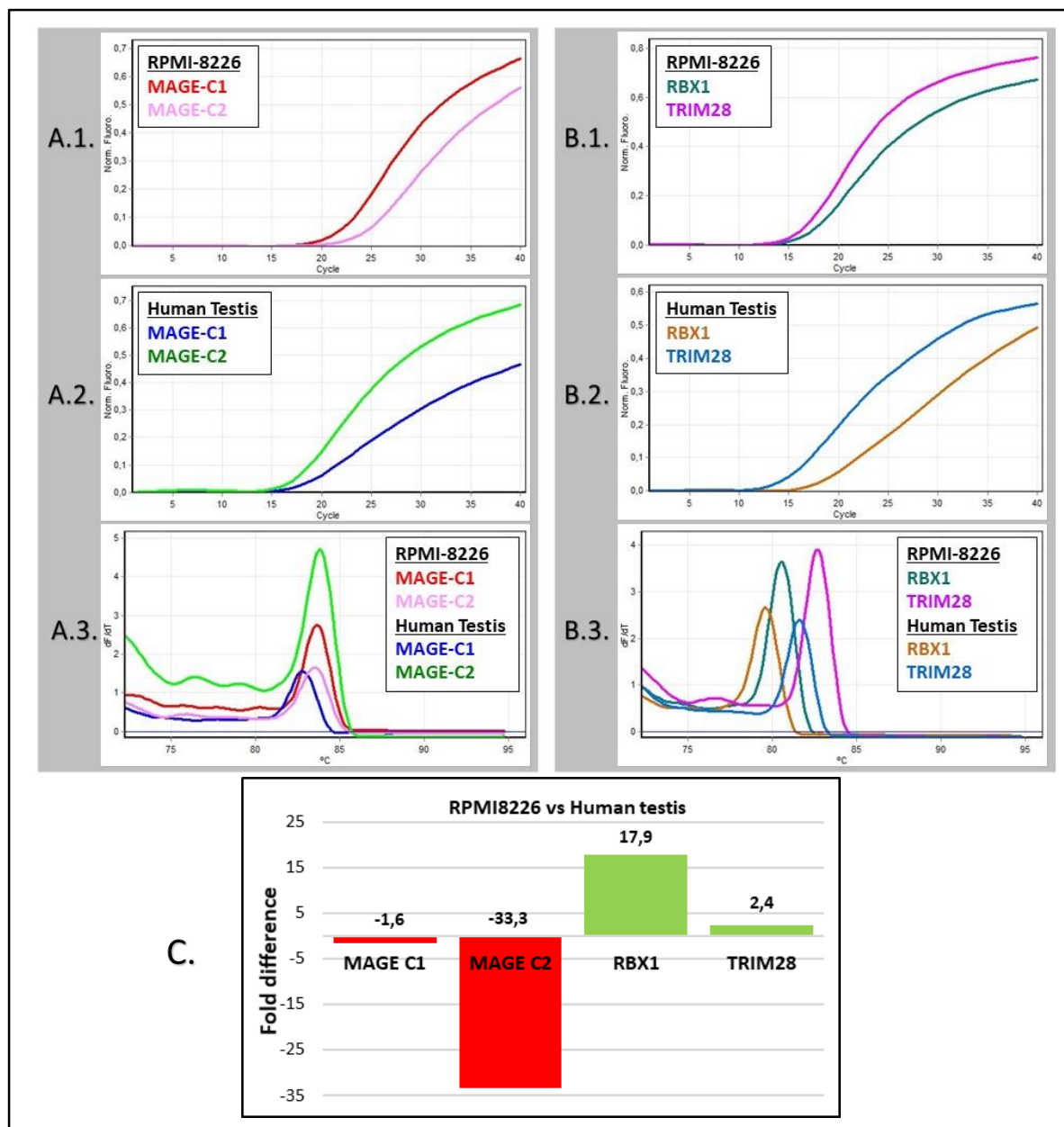


Figure 23: Co-expression of MAGE-C1, MAGE-C2, RBX1 and TRIM28 transcripts in RPMI-8226 and human testis tissue.

Panel A: Expression of MAGE-C1 and MAGE-C2. A.1. and A.2 show amplification curves using the relevant primer sets with RPMI-8226 and human testis respectively. A.3. Melt curve analysis of the four amplicon types produced. Panel B: Expression of RBX1 and TRIM28. B.1. and B.2. show amplification curves using the relevant primer sets with RPMI-8226 and human testis respectively. B.3. Melt curve analysis of the four amplicon types produced. Panel C: The difference in expression levels of MAGE-C1, MAGE-C2, RBX1 and TRIM28 in RPMI-8226 compared to human testis using normalisation with ABL Ct values and the $2^{-\Delta\Delta Ct}$ method. Green columns indicate relative increased expression, while red indicates reduced expression. The numbers indicate the fold changes compared to human testis expression.

Figure 24 shows *MAGE-C2*, *RBX1* and *ABL* amplification results using the potential CTA-negative control cDNA samples: Jurkat, PBMNC and human tonsil tissue, with human testis cDNA as the positive control in the experiment. As per the previous experiment, all PCR reactions were performed in a single experiment, using the same cDNA preparation/sample type. As expected, all these samples showed similar expression levels of *ABL* transcript (Figure 24 E), indicating that all samples contained similar levels of quality cDNA. As *RBX1* and *TRIM28* are involved in normal cellular function, relatively similar levels of expression of these genes was expected in all the different RNA samples, and this is what was observed (Figure 24 C and D).

As with the previously observed late amplification of *MAGE-C1* in this negative control set (see section 3.2.2 and shown again in Figure 24 panel A), late amplification of *MAGE-C2* appeared to occur in all of these samples (Figure 24 B1). Melt curve analysis of these products (Figure 24 B2) clearly indicated the low level amplification observed in PBMNC and tonsil RNA was definitely due to non-specific signal generation, that probably did not result in the production of amplicons. However, the product generated from Jurkat cDNA, with a similar melt profile to *MAGE-C2* in the positive control. The results from this analysis showed that PBMNC (which contains non-diseased B- and T-lymphocytes) and human tonsil tissue (predominately PC) samples could be used as negative controls for CTA (*MAGE-C1*/*MAGE-C2*) rRT-PCR expression. Jurkat, a neoplastic cell line, appeared to express very low levels of *MAGE-C1* and *MAGE-C2*, and while this may be useful as a low-level MAGE positive control, it would not be a suitable negative control for further rRT-PCR experiments.

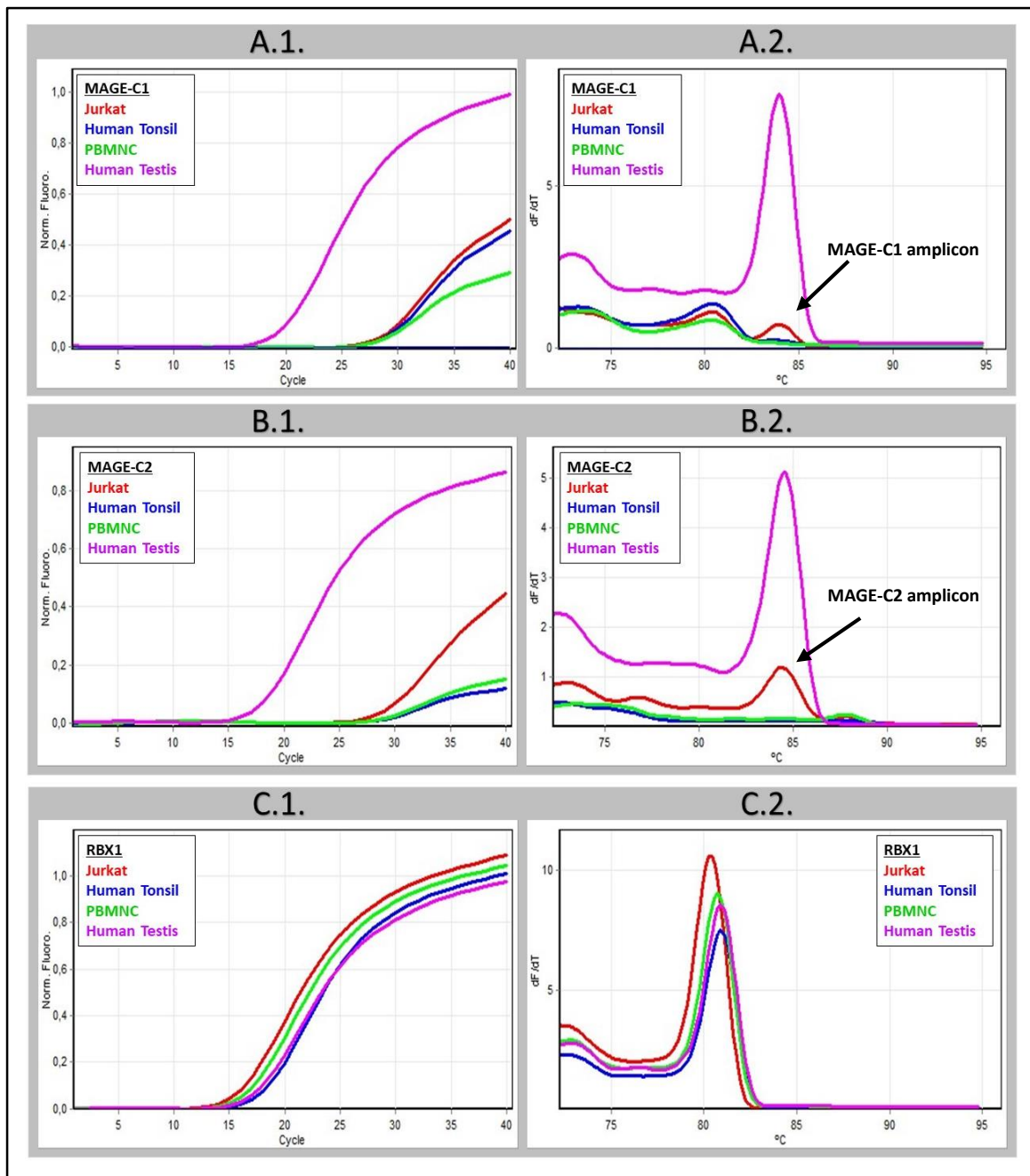


Figure 24: Amplification of MAGE-C1, MAGE-C2, RBX1, TRIM28 and ABL transcripts in potential control samples.

rRT-PCR amplification (graphs on the left) and melt curves (graphs on the right) using the Quantinova SYBR-Green PCR kit (Qiagen, The Netherlands) and a Rotorgene platform using the following cDNA preparations: Jurkat, human tonsil, PBMNC and human testis cDNA. Panel A: Analysis of MAGE-C1 amplification. Panel B: Analysis of MAGE-C2 amplification. Panel C: Analysis of RBX1 amplification. Panel D: Analysis of TRIM28 amplification. Panel E: Analysis of ABL transcript amplification. The arrows indicate possible MAGE amplicons.

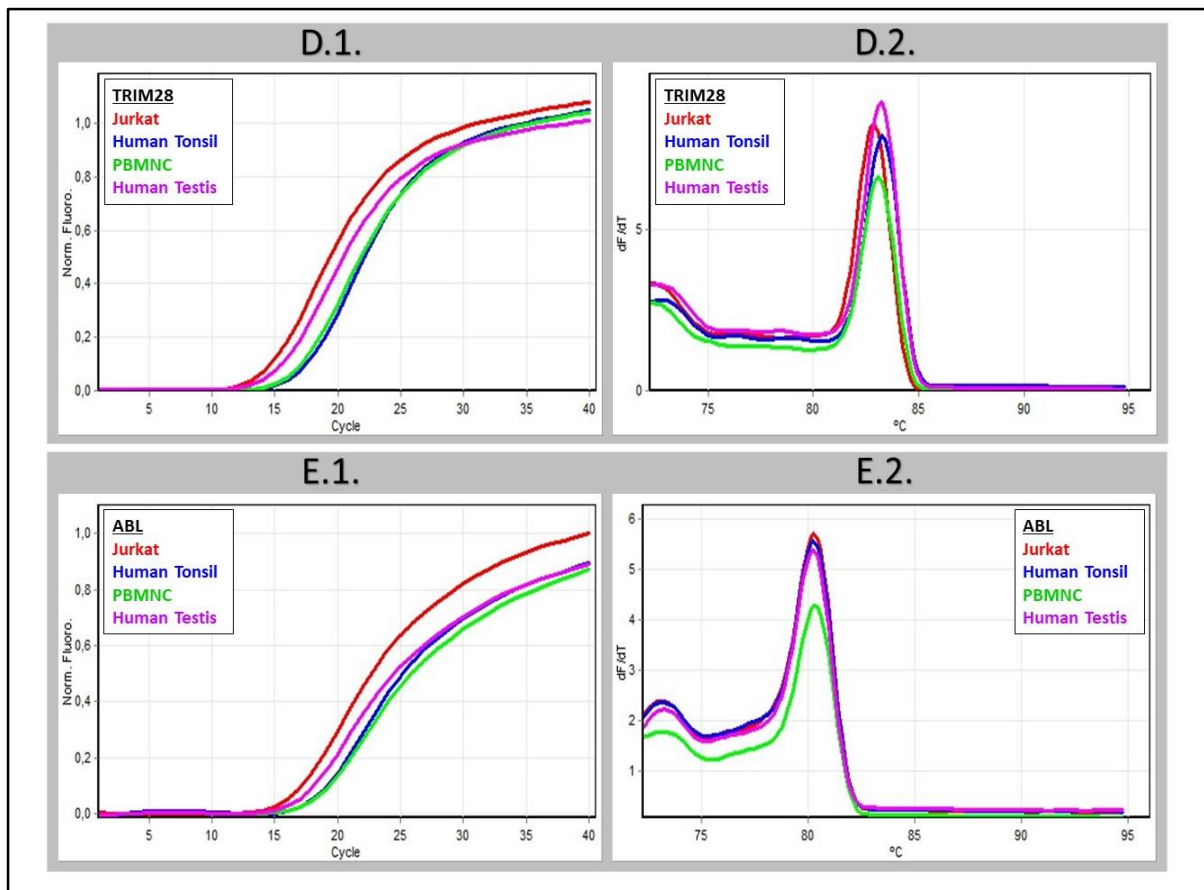


Figure 24: Amplification of *MAGE-C1*, *MAGE-C2*, *RBX1*, *TRIM28* and *ABL* transcripts in potential control samples.

*rRT-PCR amplification (graphs on the left) and melt curves (graphs on the right) using the Quantinova SYBR-Green PCR kit (Qiagen, The Netherlands) and a Rotorgene platform using the following cDNA preparations: Jurkat, human tonsil, PBMNC and human testis cDNA. Panel A: Analysis of *MAGE-C1* amplification. Panel B: Analysis of *MAGE-C2* amplification. Panel C: Analysis of *RBX1* amplification. Panel D: Analysis of *TRIM28* amplification. Panel E: Analysis of *ABL* transcript amplification. The arrows indicate possible MAGE amplicons.*

3.3.3. Co-expression of *MAGE-C1*, *MAGE-C2* and *RBX1* Protein in RPMI-8226

After successfully showing that *MAGE-C2*, *RBX1* and *TRIM28* mRNA transcripts were expressed in the mRNA extracted from cultured RPMI-8226 cells, the next step was to confirm the co-expression of the respective translated proteins. To do this, protein extracts were prepared using RIPA buffer (section 2.4.1.), and consequently analysed via western hybridisation (described in section 2.4.). As with the analysis of *MAGE-A1* expression, expression of these proteins was tested in RPMI-8226, with PBMNC extracts as a negative control for the expression of *MAGE-C2*. *RBX1* expression was expected in all cell samples.^{153,154} Jurkat was also included to determine its suitability as a possible positive or negative control in the subsequent immunoprecipitation and pathway investigations, despite low level expression

of *MAGE-C2* at the transcription level. Unfortunately, we were unable to source a suitable antibody for TRIM28 during the study period, thus protein expression in RPMI-8226 could not be verified.

Figure 25 shows the western hybridisation analysis of equal amounts of RPMI-8226, PBMNC and Jurkat extracted protein in an attempt to confirm *MAGE-C2* expression in the experimental cell line. While a single band of the expected size (41 kDa) was detected in the RPMI-8226 protein extract (as expected due to the high transcript expression), an equivalent sized band was also detected in the PBMNC and Jurkat. The difference in band intensity was found to be due to differences in the actual protein loaded in each lane (possible quantification error). These results were not expected and the fairly high levels of expression in these extracts strongly indicates that the Ab binding under these experimental conditions was not *MAGE-C2* specific. We did try altering the amount of Ab used, incubation times and washing stringency, but there was no change in the binding specificity (results not shown). The anti-*MAGE-C2* Ab we used is a polyclonal Ab that has not previously been tested in a western hybridisation setting,¹³⁷ thus non-specific binding during this technique may be possible. Unfortunately, we were unable to source a different Ab to reassess the expression of *MAGE-C2* at the protein level. Due to these issues, this anti-*MAGE-C2* Ab could not be used for further investigations that used western hybridisation, namely Co-IP.

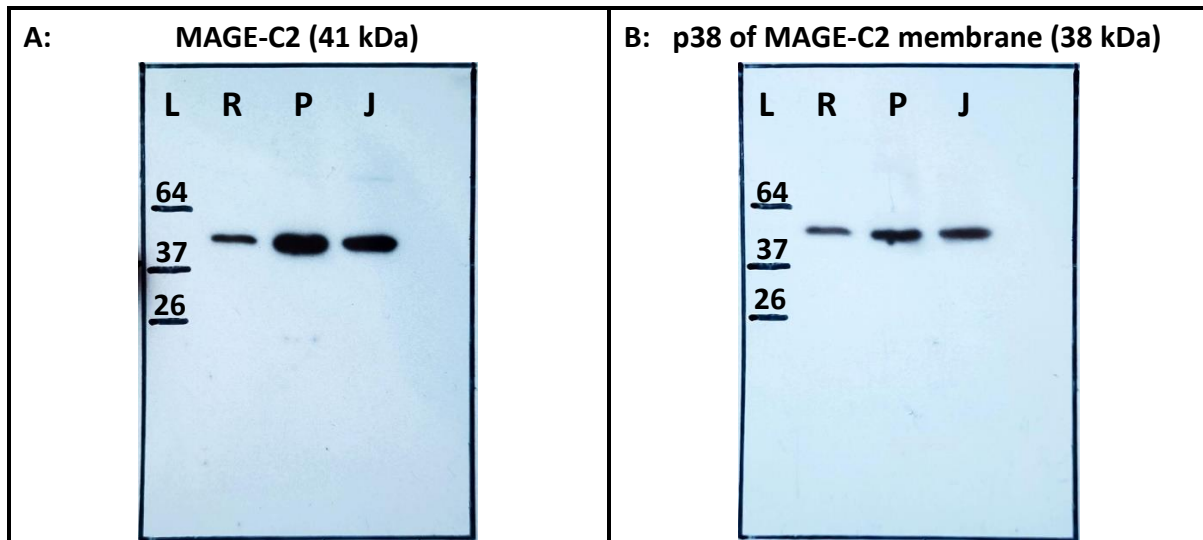


Figure 25: Detection of *MAGE-C2* protein in RPMI-8226, PBMNC and Jurkat cells.

Full length film images of the nitrocellulose membranes probed with various Ab, followed by fluorescence detection. A: Protein extracts probed with primary anti-*MAGE-C2* rabbit polyclonal Ab (1:1000) and goat anti-rabbit IgG secondary HRP-linked Ab (1:5000). B: Membrane in panel A was stripped and re-probed with primary p38 rabbit polyclonal Ab (1:5000) and goat anti-rabbit IgG secondary HRP-linked Ab (1:5000). R: RPMI-8226 (30 μ g protein), P: PBMNC (30 μ g protein), J: Jurkat (30 μ g protein), L: Benchmark™ Pre-Stained Protein Standard (Life Technologies, USA) (6-180 kDa).

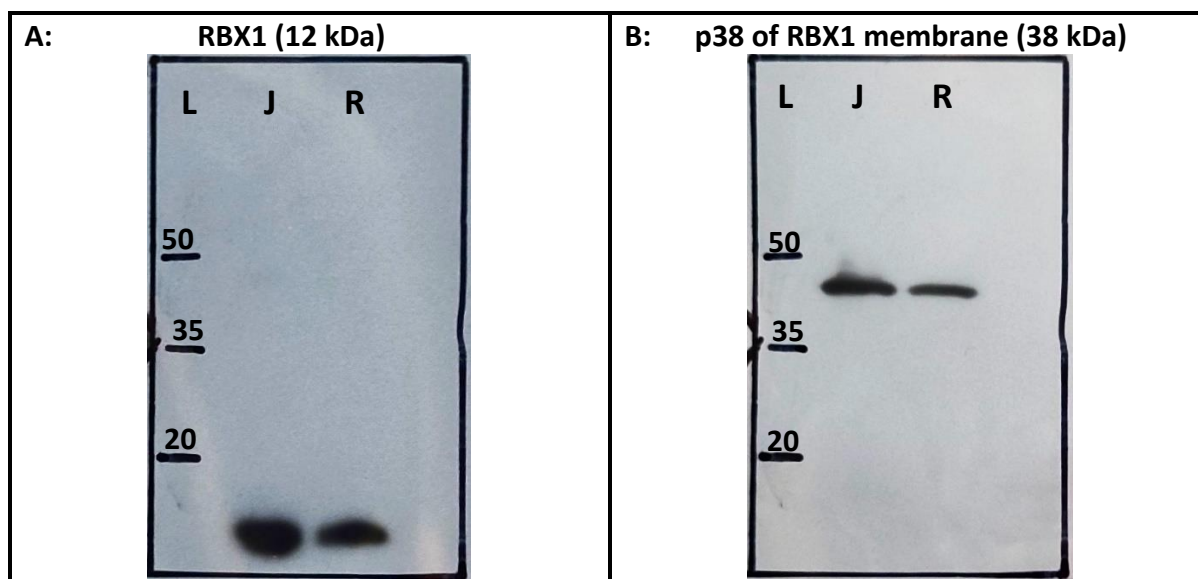


Figure 26: Detection of RBX1 protein in Jurkat and RPMI-8226 cells.

Full length film images of the nitrocellulose membranes probed with various antibodies via western hybridisation and fluorescence detection. A: Protein extracts (25 µg/lane) probed with primary anti-RBX1 rabbit monoclonal Ab (1:1000) and goat anti-rabbit IgG secondary HRP-linked Ab (1:5000). B: Membrane in panel A was stripped and re-probed with primary p38 rabbit polyclonal Ab (1:5000) and Goat anti-rabbit IgG secondary HRP-linked Ab (1:5000). R: RPMI-8226, J: Jurkat, L: Peqlab pre-stained protein standards (20-120 kDa).

Figure 26 depicts the expected detection of RBX1 protein expression in both RPMI-8226 and Jurkat RIPA protein extracts. A single band of approximately 12 kDa in each lane indicated likely specific Ab binding, justifying the use of this Ab in further Ab-based experiments.

3.3.4. Co-IP of MAGE-C1 and RBX1 Protein in RPMI-8226

After co-expression of *MAGE-C1* and *RBX1* in RPMI-8226 was established at both the mRNA and protein levels, the next step was to determine if they directly interact with one another, as previously shown for *MAGE-C2* and *RBX1* (although not in RPMI-8226)¹⁰² using Co-IP with magnetic Dynabeads. We had initially intended to use the known *MAGE-C2:RBX1* association as a positive control in this analysis, but as the methodology involves western hybridisation detection of relevant proteins, we were unable to investigate this association due to the lack of a suitable specific anti-*MAGE-C2* Ab. As a more generic positive control for this experimental process, Co-IP of RPMI-8226 cells (human cell line) with anti-Human IgG Ab was tested and confirmed previously as described above (section 3.2.4).

As the Co-IP analysis initially involved the same process as described above for the *MAGE-C1:SKIP* interaction (section 3.2.4), the same nitrocellulose membrane containing the separated Co-IP fractions using 0.1 g of cells as the input was stripped and re-probed with anti-RBX1 Ab (1:1000).

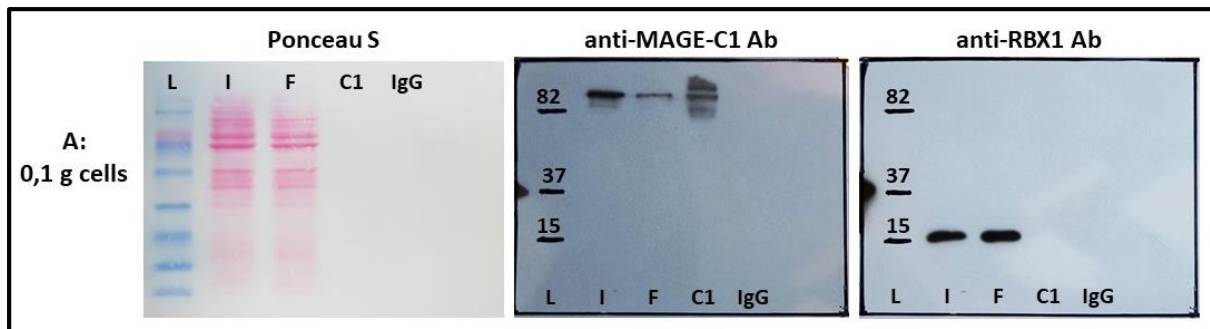


Figure 27: Detection of MAGE-C1 and RBX1 in isolated protein from RPMI-8226 MAGE-C1 Co-IP.

The combined figures show the staining of the Co-IP fractions obtained from incubation of RPMI-8226 cell lysate (0.1g cells) with anti-MAGE-C1 Ab coated Dynabeads, following separation on an acrylamide gel and transfer to a nitrocellulose membrane. The first image shows the nitrocellulose membrane stained with Ponceau S. The second is an film resulting from the staining/probing of the same membrane with primary anti-MAGE-C1 rabbit monoclonal Ab (1:1000) (124 kDa) and goat anti-rabbit IgG secondary HRP-linked Ab (1:5000). The last image in the set is the blot following the stripping of the same membrane and staining/probing with primary anti-RBX1 rabbit monoclonal Ab (1:1000) (12 kDa) and goat anti-rabbit IgG secondary HRP-linked Ab (1:5000). I: Input protein lysate, pre bead incubation; F: Flow-through after the incubation of the lysate with anti-MAGE-C1 Ab coated beads C1: Isolated/enriched protein fraction following elution from the anti-MAGE-C1 Ab coated beads. IgG: Isolated/enriched proteins following elution from the anti-human IgG Ab coated beads., L: Benchmark™ Pre-Stained Protein Standard (Life Technologies, USA) (6-180 kDa).

Figure 27 shows the results of this analysis, specifically showing the results of re-probing of the membrane with primary anti-RBX1 Ab. While a protein of the expected size (± 12 kDa) was detected in the input complete cell lysate (I) and flow-through (F) lanes (as expected), no co-enrichment of this protein was detected in the final eluted fraction (C1). In this experimental set, RBX1 did not bind tightly with the Ab-bound MAGE-C1 and co-elute. However, experimental conditions and the physiological state of the cells may influence MAGE-C1:RBX1 complex detection/forming. The investigation of the MAGE-C2:RBX1 interaction via Co-IP would have provided a clearer indication of the reason for the lack of MAGE-C1:RBX1 Co-IP.

3.3.5. IFM of MAGE-C1, MAGE-C2 and RBX1

While the Co-IP analysis of MAGE-C1 and RBX1 did not show complex formation under the experimental conditions, investigation of where the proteins were localised within the cell (i.e. either nucleus or cytoplasm) were performed using IFM. While the anti-MAGE-C2 Ab (HPA062230) did not appear to bind specifically during the western hybridisation process, it has been shown to work during immunocytochemistry analysis of the human myelogenous leukemic cell line K-562 cells (1:32 dilution),^{137,153,154,187} thus we felt that it was appropriate to try to use it in this experimental setting. As

described in section 2.6.1. anti-MAGE-C2 Ab, anti-MAGE-C1 Ab and anti-RBX1 Ab were permanently fluorescently labelled with either CFTM488 (MAGE-C2 and MAGE-C1, green channel) or CFTM647 (RBX1, far red channel). Actively cultured RPMI-8226 cells and freshly prepared PBMNC cells were fixed and permeabilised with methanol before being incubated with combinations of the Fl-Ab (MAGE-C2 – 1:50 dilution, MAGE-C1 – 1:50 dilution, RBX1 – 1:100 dilution),^{153,154} attached to slides and finally co-stained with Hoechst nuclear stain (as described in section 2.6.2. and 2.6.3.). The controls described in the IFM studies using anti-MAGE-A1 and anti-SKIP Ab (section 3.2.5) were also applied here to set the relevant detection settings

Figure 28 shows the microscopy results of anti-MAGE-C2 and anti-RBX1 Ab co-staining of both RPMI-8226 and PBMNC cell preparations. Panel A shows RBX1 protein (far red channel) staining strongly in the cytoplasm and nucleoplasm of RPMI-8226, which was unexpected as is reported to favour nuclear expression nuclear expression with very limited expression in the cytoplasm of human embryonic kidney (HEK293 and HEK293T) cells.^{102, 188} Under the fixation and staining conditions, fluorescence in the green channel (562 nm) did not increase significantly in the presence of the anti-MAGE-C2 Ab in RPMI-8226. This may reflect the inappropriateness of this Ab for this technique, as with the western hybridisation analysis, or the lack of MAGE-C2 expression in RPMI-8226, which would be unexpected. Unfortunately, due to the very limited supply of anti-MAGE-C2 Ab, alternate immunofluorescent conditions could not be investigated.

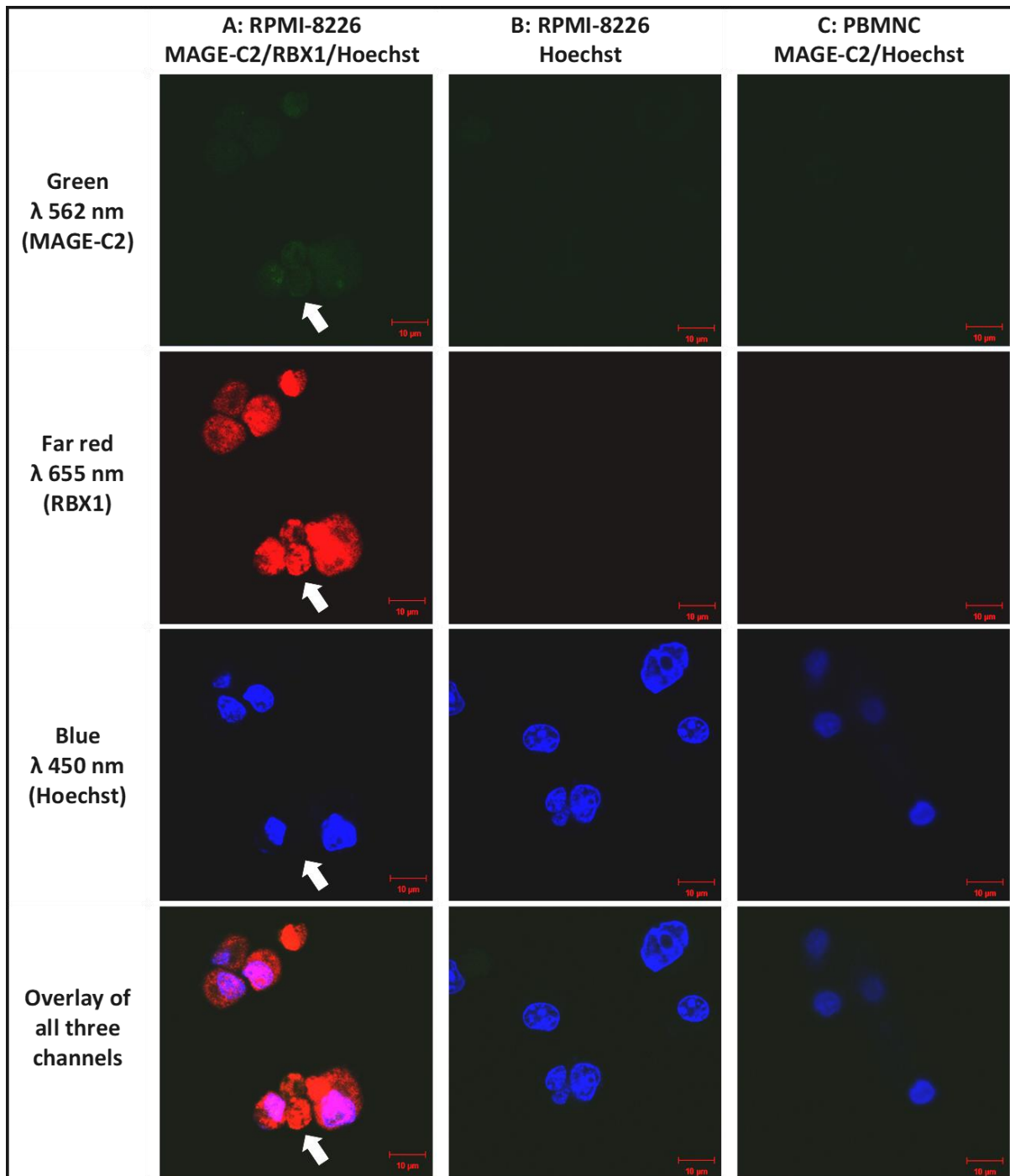


Figure 28: Microscopy analysis of RPMI-8226 and PBMNC stained with fluorescently labelled anti-MAGE-C2, anti-RBX1 Ab and Hoechst stain.

Panel A: RPMI-8226 cells co-stained with anti-MAGE-C21 Ab (CF488A -tagged), anti-RBX1 Ab (CF647-tagged) and Hoechst nuclear stain, showing fluorescence in each relevant channel. Panel B: RPMI-8226 cells stained with Hoechst stain only, indicating the levels of autofluorescence observed with the selected gain settings in each channel. Panel C: PBMNC co-stained with anti-MAGE-C2 (CF488A -tagged) and Hoechst nuclear stain, showing fluorescence in each relevant channel. The same settings were used for all microscopy analysis. The arrows indicate dead/necrotic cells.

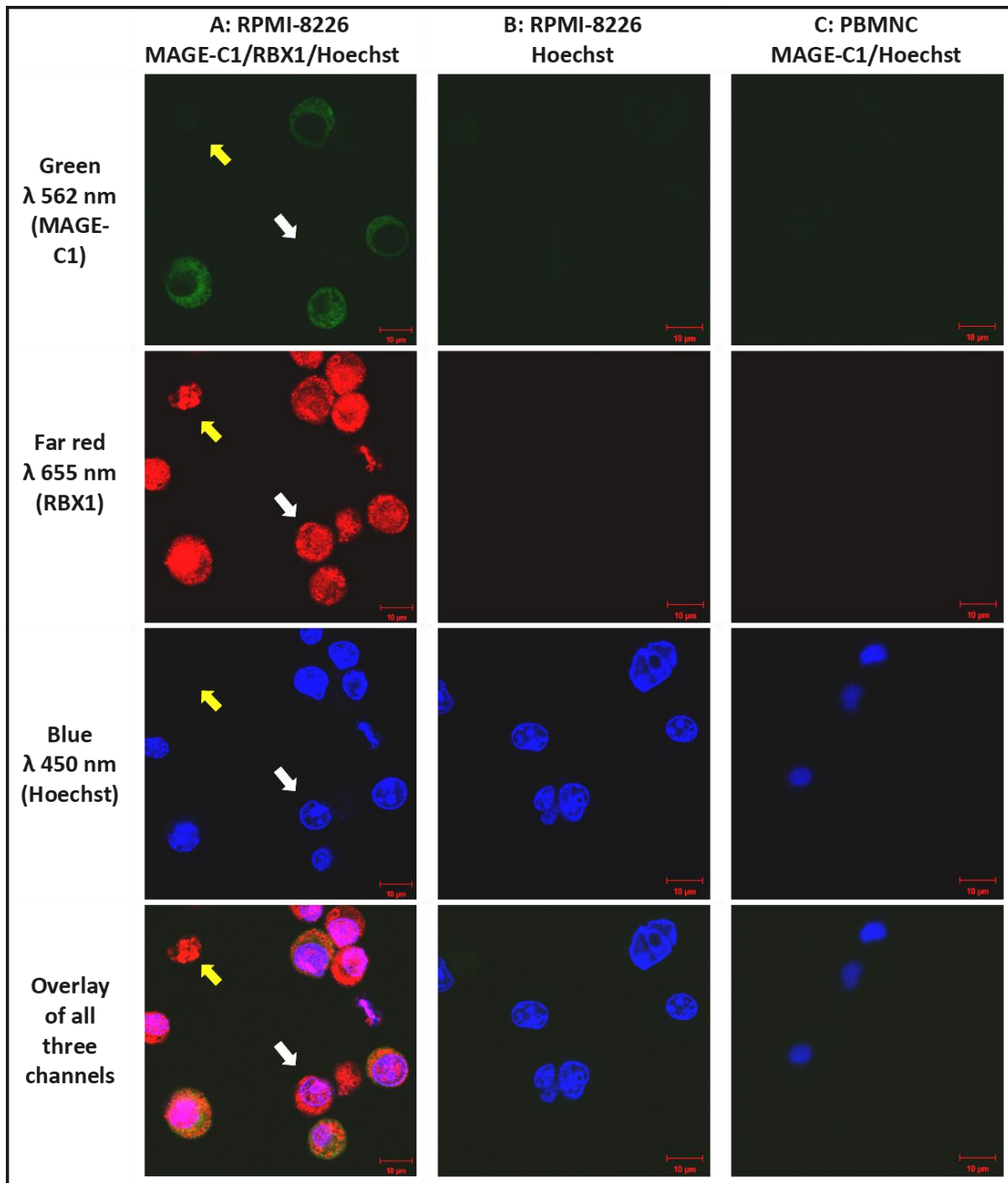


Figure 29: Microscopy analysis of RPMI-8226 and PBMNC stained with fluorescently labelled anti-MAGE-C1, anti-RBX1 Ab and Hoechst stain.

Panel A: RPMI-8226 cells co-stained with anti-MAGE-C1 Ab (CF488A -tagged), anti-RBX1 Ab (CF647-tagged) and Hoechst nuclear stain, showing fluorescence in each relevant channel. Panel B: RPMI-8226 cells stained with Hoechst stain only, indicating the levels of autofluorescence observed with the selected gain settings in each channel. Panel C: PBMNC co-stained with anti-MAGE-C1 (CF488A -tagged) and Hoechst nuclear stain, showing fluorescence in each relevant channel. The same settings were used for all microscopy analysis. The white arrows indicate a cell not labelled with MAGE-C1, but showing high expression of RBX1. The yellow arrows indicate artifacts/necrotic cells.

Figure 29 shows the microscopy results of anti-MAGE-C1 and anti-RBX1 Ab co-staining of both RPMI-8226 and PBMNC cell preparations. Panel A shows RBX1 protein (far red channel) staining very brightly throughout the cytoplasm and nucleoplasm of RPMI-8226 (confirmed by performing Z-stacking analysis), and MAGE-C1 protein (green channel) staining the cytoplasm of RPMI-8226 cells with the clear absence of fluorescence in the nucleoplasm (as previously shown section 3.2.5). Interestingly, MAGE-C1 staining is not observed in all RPMI-8226 cells (also seen during IFM analysis of MAGE-C1 and SKIP), which deserves further investigation. Based on previous studies^{102,153,154} RBX1 was expected to be localised to the nucleoplasm, while MAGE-C1 was expected to be visualised in both the cytoplasm and the nucleoplasm.^{153,154,189} This analysis does however indicate co-localisation at least in the cytoplasm.

3.3.6. Investigation of Activation and Analysis of the E3 Ligase Pathways Known to be Affected by MAGE-C2 Interactions

3.3.6.A. Experimental Approach

Based on experimental studies in human testis tissue, it has been proposed that MAGE-C2 is involved in several pathways that affect cell cycle activation indirectly through the regulation of the levels certain key signalling proteins such as Cyclin E1 and TP53, specifically through altering the ubiquitination and degradation certain proteins (discussed in section 1.2.2.C and illustrated in Figure 5). Unfortunately, these specific MAGE-C2 functions have not yet been confirmed in MM (or the MM cell line RPMI-8226 specifically). By investigating how activation of these protein degradation pathways affects some key end points, we were hoping to determine if MAGE-C2 and thus possibly MAGE-C1 act in a similar manner in RPMI-8226, as shown in human testis tissue.

Designing this experiment was difficult due to the lack of available tissue that could act as suitable positive (human testis tissue) and negative controls (PC or SC). We were thus only able to compare the expression of the key proteins in RPMI-8226 and PBMNC (enriched lymphocytes), following the activation of the ubiquitination/proteasome pathway via heat-shock treatment. The ER-apoptosis pathway, which includes the ubiquitination processes is known to be activated by this stressor¹⁹⁰ and previous studies by our research group indicated that 52 °C for 2 min was sufficient to activate apoptosis via this pathway in RPMI-8226, resulting in 20-30% cell death (Dr Kirsty Wienand, PhD thesis, 2016).¹⁹¹ We thus compared levels of certain transcripts and proteins and the overall effect on cell cycle activation in heat-shocked and non-treated cells as described in section 2.7.

3.3.6.B. TP53 and Cyclin E Protein Analysis in RPMI-8226 and PBMNC

Activation of the SCF-ligase complex during stress, such as heat-shock, should induce degradation of Cyclin E1 protein via ubiquitination, thus lowering available protein in the cell and preventing cell cycle progression. In the presence of MAGE-C2, the levels of Cyclin E1 protein should not change or even accumulate in the cytoplasm.^{56,100} Activation of the TRIM28 E3 ligase complex during stress should induce the ubiquitination of P-TP53 targeting it for proteasome degradation and thus lowering cellular levels. The presence of MAGE-C2 should theoretically greatly increase this process, leading to significantly lower levels of cellular P-TP53.^{56,100} As we successfully demonstrated that PBMNCs do not express MAGE-C1 or MAGE-C2, it was used as a negative control, which should respond in an appropriate manner to the stressor.

RPMI-8226 and PBMNC were stressed using heat-shock and total protein extracted 4-hours post stress, with cells maintained at 37 °C acting as the untreated control group (as described in section 2.7.1.). The levels of cellular Cyclin E and P-TP53 were assessed via western hybridisation (section 2.7.4.), with the levels of p38 acting as a loading control (determined to not be affected by the treatment). Cyclins E1 and E2 both promote cell cycle progression from G1- to S-phase through CDK2 activation and are often grouped together during investigations,¹⁹² thus we used the available anti-Cyclin E2 to assess Cyclin E levels. Figure 30 shows the results of this analysis.

Unfortunately, no P-TP53 was detected in the PBMNC protein extracts from either the untreated or heat shocked groups, which was not completely unexpected due to a low level of expression in this non-cancerous cell type.¹⁹³ Cyclin E2 was however detected in both the treated and untreated cells. As can be seen in Figure 30 (panel A) the heat-shock treatment did result in the expected lower level of Cyclin E2 protein. The difference between the two normalised groups was however not significant (1.25 vs 1.12), but as the standard deviation (SD) was quite high, repeat analysis and more replicates may have improved the significance of the lower Cyclin E level. The stress applied may also not have been sufficient to allow for a significant triggering of the ubiquitination pathway in this particular cell type.

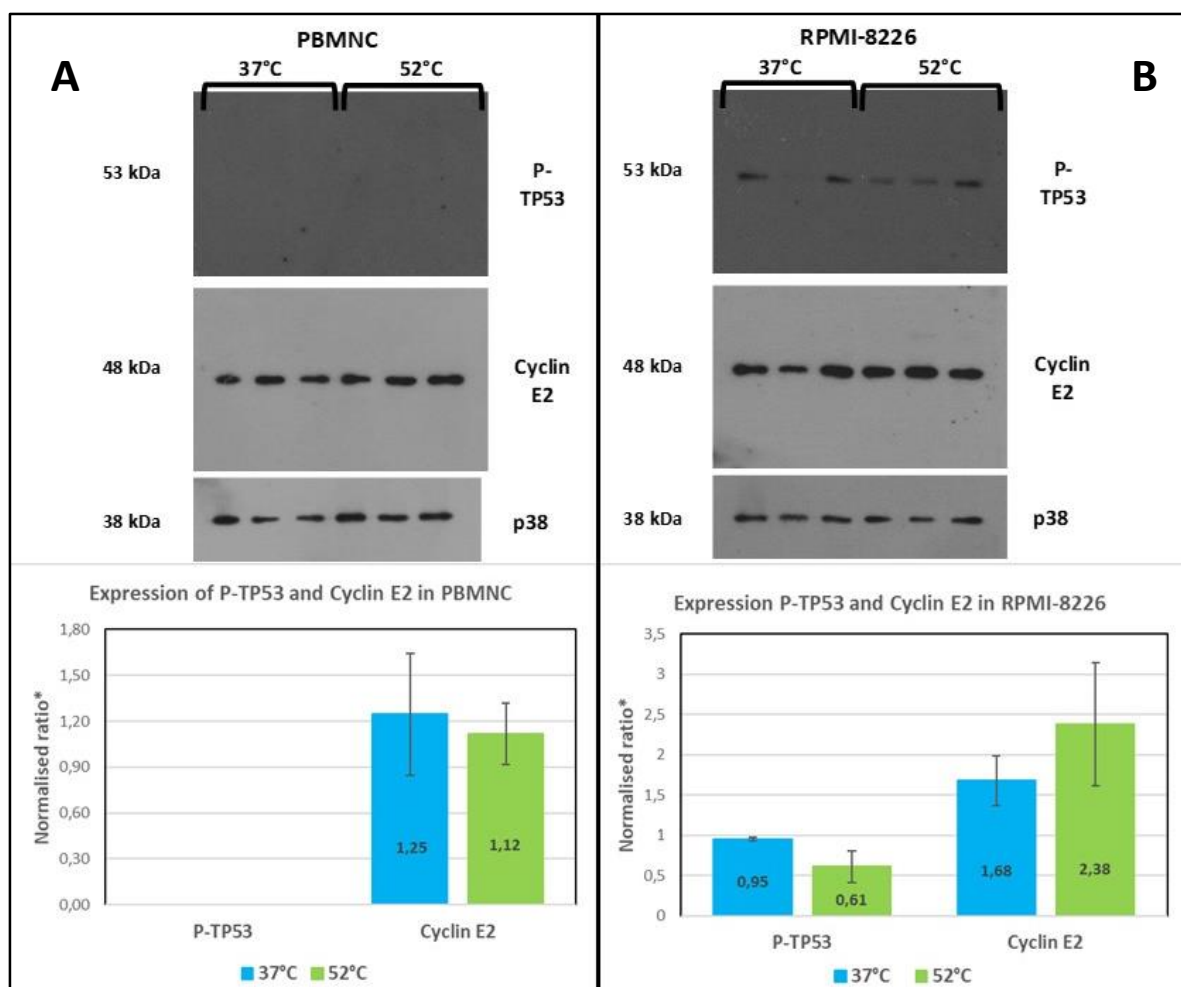


Figure 30: Effects of heat-shock stress on the levels of P-TP53 and Cyclin E2 protein in RPMI-8226 and PBMNC protein extracts.

Panel A: Film of the nitrocellulose membrane containing protein extracts from PBMNC heat-shocked to 52 °C for 2 min and the control cells held at 37 °C probed, in a sequential manner, with 1) Primary Ab anti-phospho-p53 (1:1000), secondary Ab HRP-linked horse anti-mouse (1:5000) (53 kDa expected size); 2) Primary Ab anti-Cyclin E2 (1:000), secondary Ab HRP-linked goat anti-rabbit (1:5000) (48 kDa); and 3) Primary p38 Ab (1:5000), secondary Ab HRP-linked goat anti-rabbit Ab (1:5000) (38 kDa). Resulting bands were analysed using densitometry with the IMAGEJ (version 1.54d)¹⁹⁴ software. Normalised ratios of the proteins were determined by the following calculation (to account for loading errors): density value of protein of interest/ density value of corresponding p38 band in the same lane. These ratios are graphically presented in the graph, with error bars indicating SD of the experimental replicates. Panel B shows the results of this experiment and analysis using RPMI-8226, treated and untreated, cells.

Both P-TP53 and Cyclin E proteins were successfully detected in RPMI-8226 of both groups under the experimental conditions (see Figure 30, panel B). In contrast to the PBMNC response, RPMI-8226 actually showed an increase in the levels of Cyclin E following the stress (1.68 vs 2.38, 1.4 fold increase), which is what we expected if MAGE-C2 interacted as expected with this particular pathway. Again, while the increase was not significant (< 2 fold increase), the SD may be contributing to this and

repeat analysis may improve this significance. The levels of P-TP53 also decreased as expected if MAGE-C2 interacted with the TRIM28 E3 ligase complex. Heat-shock resulted in a decrease in normalized levels from 0.93 to 0.61, a 34% reduction. A 50% reduction would have represented a significant change, but this may be achieved if more replicates were performed. An increased time at the elevated temperature may have also increased the extent of the response,

3.3.6.C. *p21*, *Cyclin A1* and *Cyclin A2* Transcript Expression in RPMI-8226 and PBMNC

While the P-TP53 and Cyclin E levels reacted in a way possibly indicative of MAGE-C2 involvement, we wanted to look at the expression of additional signalling molecules, further along the pathway to altered cell cycle progression to confirm the involvement of MAGE-C2 (and potentially MAGE-C1) in these specific pathways. This included looking at the levels of *Cyclin A1* transcripts, which are directly affected by levels of Cyclin E, as well as levels of *p21* transcripts which are directly affected by P-TP53 levels in the cell (see Figure 5 for details).^{56,90,101,102}

As part of the experimental set described in Figure 6 (Chapter 2) following the heat-shock (or control incubation), protein and RNA were simultaneously extracted. The RNA was subsequently converted to cDNA and then used to determine the levels of *p21*, *Cyclin A1* and *Cyclin A2* transcripts, via SYBR-Green rRT-PCR (as described in section 2.7.3.). Cyclin A2 is ubiquitously expressed and controls the S- and M-phases of the cell cycle, while Cyclin A1 is confined to meiotic cells, haematopoietic SC and leukemic cells and thought to only regulate the M-phase of the cell cycle,^{195,196} which is why both levels were investigated. Figure 31 and 32 shows the raw real-time PCR data for both cell lines, indicating the Ct values and amplification curves. Surprisingly, no changes in expression levels were observed for any of the transcripts in RPMI-8226 or PBMNC.

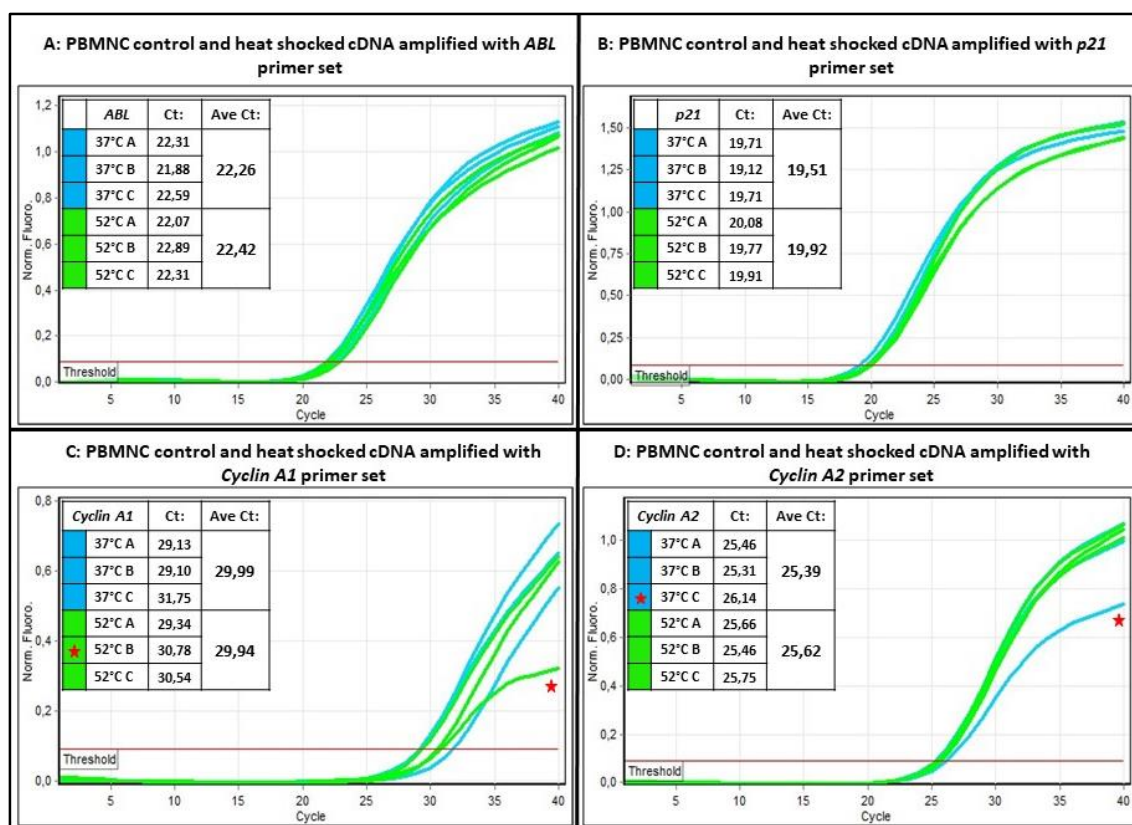


Figure 31: rRT-PCR amplification raw data of *ABL*, *p21*, *Cyclin A1* and *Cyclin A2* following heat-shock (vs control) of PBMNC.

A: Real-time amplification curves of *ABL* in cDNA from control (37 °C) and heat shock (52 °C) treated PBMNC. B: Real-time amplification curves of *p21* in cDNA from control (37 °C) and heat shock (52 °C) treated PBMNC. C: Real-time amplification curves of *Cyclin A1* in cDNA from control (37 °C) and heat shock (52 °C) treated PBMNC. D: Real-time amplification curves of *Cyclin A2* in cDNA from control (37 °C) and heat shock (52 °C) treated PBMNC. The inserts show the Ct values achieved for each sample set, using a threshold of 0.09. Curves excluded from analysis due to abnormal amplification curves are indicated with a red star. Melt curve analysis was performed on all samples post amplification to confirm the amplification of a single product (not shown).

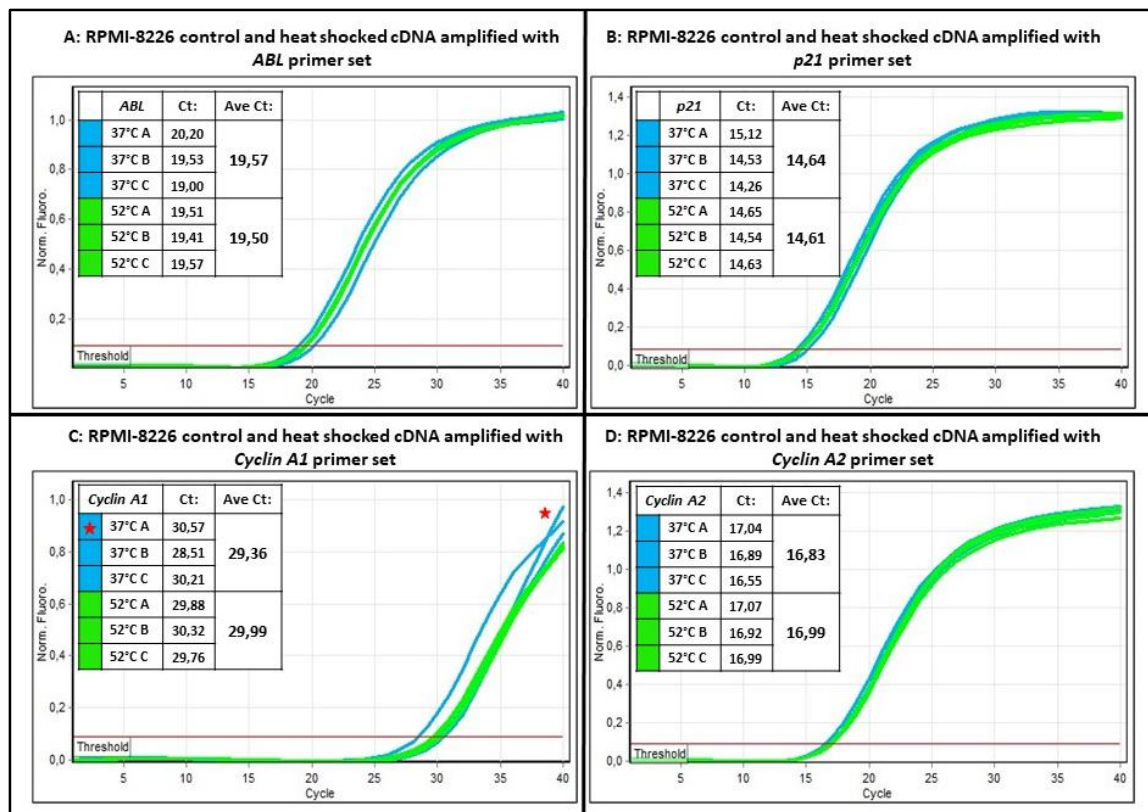


Figure 32: rRT-PCR amplification raw data of ABL, p21, Cyclin A1 and Cyclin A2 following heat-shock (vs control) of RPMI-8226.

A: Real-time amplification curves of ABL in cDNA from control (37 °C) and heat shock (52 °C) treated RPMI-8226. B: Real-time amplification curves of p21 in cDNA from control (37 °C) and heat shock (52 °C) treated RPMI-8226. C: Real-time amplification curves of Cyclin A1 in cDNA from control (37 °C) and heat shock (52 °C) treated RPMI-8226. D: Real-time amplification curves of Cyclin A2 in cDNA from control (37 °C) and heat shock (52 °C) treated RPMI-8226. The inserts show the Ct values achieved for each sample set, using a threshold of 0.09. Curves excluded from analysis due to abnormal amplification curves are indicated with a red star. Melt curve analysis was performed on all samples post amplification to confirm the amplification of a single product (not shown).

3.3.6.D. Activation of cell cycle G1-S phase progression in heat-stressed RPMI-8226 and PBMNC

Despite the lack of detectable changes to the transcription of key cell cycle modulators, namely *p21* and *Cyclin A*, we still investigated the effects of the heat-shock stress on the number of cells in each stage of the cell cycle, using PI staining and flow cytometry. As described in Figure 6, protein, RNA and whole cells were collected from each triplicate well, for both cell types, with and without exposure to the elevated temperature stress. The whole cell fractions were fixed according to section 2.7.2 and the DNA content of each cell determined with flow cytometry. Figure 33 shows some of the results from this analysis.

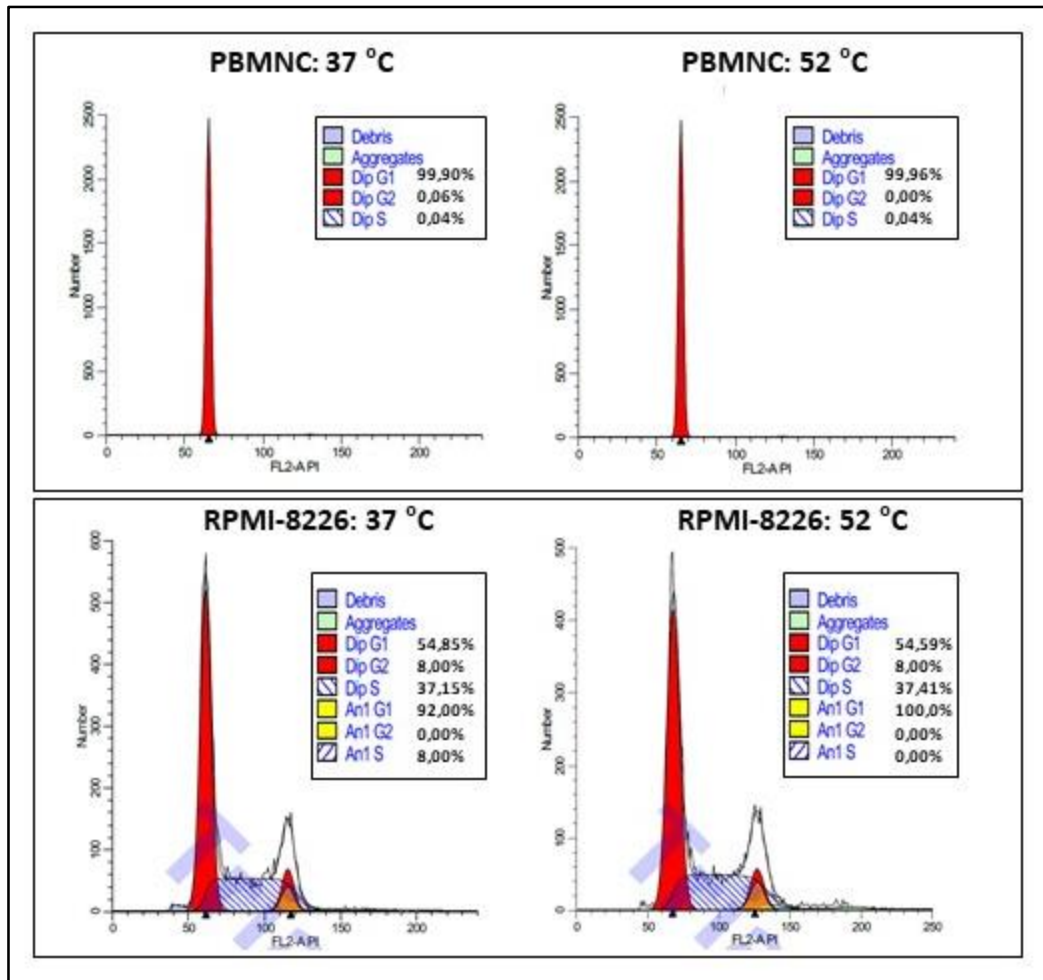


Figure 33: Cell cycle analysis of PBMNC and RPMI-8226 cells following heat-shock stress.

Modfit analysis of the PI-staining of cells harvested after the heat-shock and control. The top panel shows the diploid cell population of the PBMNC cells before after treatment, while the bottom panel shows the more complex diploid (95% of all cells analysed) and tetraploid (5% of all cells analysed) cell populations within the RPMI-8226 cultured cells. The values indicate the percentage of diploid cells (Dip) in the G1, S and G2 phases of the cell cycle, as well as percentage of tetraploid cells (Ant) in each of the three phases.

The first thing to note from this analysis was the obvious difference in the cell cycling status of the two cell types, with PBMNC showing only 0.04% of the cells in S phase, as expected unless the T-lymphocytes were activated by an immune response, while 37.15% of the RPMI-8226 diploid cells were actively synthesizing DNA in preparation for cell division. Although these results were expected, it did confirm the abnormal cycling progression of the cancerous cell line, and even a potential favouring of G1-S progression over the S-G2 progression. Unfortunately, we didn't see any changes in the proportion of the cells entering the S phase following heat-shock in either cell line, again suggesting that the stressor was not sufficient to elicit a large enough response.

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1. DISCUSSION

Our research group has previously indicated that MAGE-C1 could be used as a potential biomarker for MRD monitoring in patients with MM.²⁸ However, as little is currently known about the function of MAGE-C1 and how it contributes to the pathogenesis of MM, its validity as a biomarker has yet to be confirmed. This project forms part of a larger study to establish the function of this CTA by comparing its functions to those of better defined MAGE CTAs, namely MAGE-A1 and MAGE-C2. This project was focused on establishing if we could utilise an *in vitro* approach to compare these proteins, using the MM cell line RPMI-8226, by ensuring that all three MAGE and MAGE-binding proteins were expressed in this cell line and associated with one another as previously identified. The secondary aim was to establish successful techniques and select relevant reagents that could be used in the next phase of the larger project.

4.2. MAGE-A1 COMPARED TO MAGE-C1

4.2.1. Bioinformatics Comparison

Bioinformatics analysis aimed to predict key structural protein similarities between MAGE-A1 and MAGE-C1 to strengthen the basis for the investigation into functional similarities. Protein sequences were pairwise aligned to obtain an identity score and domains or aa residues of interest, potentially associated with function of the protein(s), were annotated to visually compare the proteins.

The alignment of the shared MHD (inclusive of the WHD) showed > 40% identity between the domains which indicates the common ancestry of the MHD and predicts structural similarity of the MHD (identity > 30%).^{116,165} This was unsurprising, as MAGE proteins are collectively grouped based on the presence of this highly homologous domain.⁹⁴⁻⁹⁶ They are, however, involved in various pathways through different modes of interaction,^{96,101} which rationalises the lack of functional similarity prediction (> 60% identity needed) when the whole proteins or just the MHD were compared.^{116,117,165,166}

Ubiquitination, phosphorylation, and acetylation are all post-translational modifications that contribute towards protein function. Identification of sites indicated that these proteins could share some functions within the cell as both MAGE-A1 and MAGE-C1 contained > 5 potential ubiquitination sites within the MHD, suggesting the involvement of the MHD in the ubiquitination process. Ubiquitination occurs when ubiquitin is coupled to the target protein at an ubiquitination site.^{197,198} There are three types of ubiquitination, mono-ubiquitination, multiple mono-ubiquitination and poly-ubiquitination.^{197,198} Mono-ubiquitination and multi mono-ubiquitination of a protein alters the stability and function of the target protein e.g. enhances protein:protein interactions,^{197,198} while poly-ubiquitination marks the target protein for degradation.^{197,199} Multiple potential phosphorylation sites (specific S and Y residues) were also identified in both proteins, clustered within the WHD of MAGE-A1, but outside of the WHD for MAGE-C1. Phosphorylation of specific S, T and Y aa residues, by an activated protein kinase, can promote protein:protein binding, the formation of protein complexes and the continuation of critical signalling pathways.^{200,201} The third modification, protein acetylation at specific K residues, occurs when an acetyl group from coenzyme A is transferred to the epsilon amino group (Nε) of the K residue. This process has been shown to play a key role in the formation of protein complexes, especially during the regulation of transcription and apoptosis.^{202,203} A single potential acetylation site (specific K residue) was identified in the MHD of MAGE-A1, while none were predicted in MAGE-C1. Collectively the post-translational modification sites identified in the MHD of MAGE-A1 most likely allow for the formation of protein complexes via this domain, which then acts on target proteins via phosphorylation. This is supported by its function as a transcriptional repressor, through its direct interaction with SKIP.⁹⁸ MAGE-A1 has also been shown to increase phosphorylation of target proteins in melanoma cell lines.²⁰⁴ While the MHD of MAGE-C1 specifically lacked these acetylation and phosphorylation sites, the ubiquitination sites infer that some functions may be similar to MAGE-A1.

Bioinformatics analysis pointed towards possible additional functions of the N-terminal of MAGE-C1, which was found to contain 10 highly homologous repeat units, all of which contain a possible helix element. While we initially identified these repeats through our own analysis, we subsequently discovered that they had been described by other groups as well.^{96,205} Functions of proteins with helical structures are varied and can include transmembrane signal transduction, signal receptors and DNA:protein interactions,^{206,207} although secondary structure alone, is not enough to accurately deduce function.²⁰⁸ Interestingly, most of the phosphorylation sites identified in MAGE-C1 were present within these repeat units, which further supports the idea that N-terminal is involved in protein:protein interaction.^{96,205}

4.2.2. Cellular Expression Comparison

Based on the findings of the bioinformatics analysis, investigations of the functional similarity of the proteins were attempted. Firstly, it was key to establish expression of MAGE-C1, MAGE-A1 and its known binding partner, SKIP, in the MM cell culture model, RPMI-8226. We also needed to establish the expression of these various proteins in potential negative controls, that could then be used in the functional assays. Expression was investigated at two levels, namely mRNA transcript expression via rRT-PCR using SYBR-Green, followed by protein expression through western hybridization. This initial expression analysis also allowed us to assess the value of the reagents, such as primer sets and Ab specificity, for use in studies relating to these MAGE proteins.

The expression of *MAGE-C1*, *MAGE-A1* and *SKIP* mRNA transcripts in RPMI-8226 was established, indicating its possible use as a model. While our methodology was not optimized for quantitative analysis of the expression levels, it appeared that *MAGE-C1* was expressed at a similar level in the diseased RPMI-8226 and the CTA-expressing healthy human testis tissue RNA extract, potentially indicating a similar role in the two cell types. However, this analysis clearly showed a considerably higher level of *MAGE-A1* mRNA in RPMI-8226 RNA extracts than in human testis total RNA. This was quite an interesting finding and may indicate that these two MAGE proteins play different roles in the MM cell line compared to human testis tissue. Previous studies, examining MAGE-A1 expression in different cells/tissues/cancers have only reported positive/negative results and have not addressed the possible importance of the variation in expression levels.²⁰⁹⁻²¹² These results definitely deserve further investigation, but would require quantitative analysis either through the use of transcript-specific probes that could either be amplified together with a housekeeping/reference gene in a multiplex PCR reaction and analysed via the $2^{-\Delta\Delta C_t}$ method by Livak and Schmittgen (2001),¹³² or amplified along with an amplicon standard set and normalized copy numbers calculated.²¹³

In addition to the human testis tissue RNA extract, which we used as a positive control for CTA and CTA-partner gene expression, three other RNA extracts were assessed for their use as MAGE-negative

controls for *MAGE-C1*, *MAGE-A1* and *SKIP* expression: human tonsil tissue, Jurkat and PBMNC. These controls were not completely appropriate, due to the nature of MM which is fundamentally a B-cell disorder, which we have previously shown has a SC and PC malignant component.²⁸ A non-diseased B-cell cell line, isolated B-cells from the PBMNC fraction or isolated SC would be more appropriate, but all of these sample types are very difficult to obtain. Instead, we chose the tonsil tissue (only RNA available), which is enriched with PC, PBMNC, which predominately contains lymphocytes and Jurkat, a cultured T-lymphocyte cell line. As expected, human tonsil RNA and PBMNC RNA expressed *SKIP*, but none of the disease-associated *MAGE* transcripts. Jurkat, a T-ALL cancerous cell line,¹²³ did however, show the expression of relatively low levels of *MAGE-C1* transcripts, while showing no detectable levels of *MAGE-A1* mRNA. This *MAGE-C1* expression appears to be a novel finding and may be of interest regarding the role of MAGE proteins in T-ALL pathogenesis.^{153,154,214} Overall, analysis of these extra sample types indicated that human tonsil tissue and PBMNC could be used as negative controls for *MAGE* mRNA expression (potentially in gene knock-out studies), but Jurkat was not suitable for these studies.

At the protein level, *MAGE-C1* and *SKIP* proteins were detected in RPMI-8226, with only *SKIP* being detected (as expected), using this less sensitive technique in Jurkat and PBMNC controls. Unfortunately, the anti-*MAGE-A1* Ab that we had acquired, did not detect specific single products in RPMI-8226, despite numerous attempts at trouble-shooting. Further trouble-shooting could include: shortening primary Ab incubation time at higher temperatures, e.g. 2-4 hours at RTP as opposed to 4 °C O/N, which has been shown to enhance specific Ab binding in some cases.²¹⁵ If the method is more sensitive, it could be further adjusted to use less protein and less Ab which should further minimize non-specific binding.²¹⁶ An alternative solution would be to source an alternate Ab. The anti-*MAGE-A1* Ab used in this study was monoclonal,¹³⁶ which makes Ab-antigen binding highly specific and is advantageous for detecting a specific protein in a closely related protein family.²¹⁷ However, it is less sensitive and more prone to not binding to the protein of interest due to epitope loss/damage during processing of protein preparations compared to poly-clonal preparations.²¹⁷ Unfortunately, the availability of *MAGE* sub-type specific Ab is currently very restricted, especially as they share strong identity in the C-terminal region. Several Abs are available that can detect several *MAGE* proteins simultaneously, but this was not appropriate for our study.

4.2.3. Association with SKIP

Co-IP, using magnetic beads, is a useful tool to extract protein complexes from a cellular extract¹⁴⁸ and can be used in a variety of ways, depending upon the Ab that is used to coat the beads. In our study, due to quantities of available antibody, we used anti-*MAGE-C1* Ab to coat the beads and thus *MAGE-C1* present in the cellular extract as the bait to extract co-bound proteins. While the experimental conditions indicated that we were able to specifically extract *MAGE-C1* from the RPMI-8226 cellular

preparation, SKIP did not appear to be a protein that co-eluted. As we were unable to perform the MAGE-A1/SKIP Co-IP, we could not definitively conclude that SKIP does not associate with MAGE-C1.

There are many aspects of this complex methodology that could be resulting in a false negative result. One major challenge of Co-IP is optimization of the extraction/lysis buffer. The stringency of the buffer should be adjusted to optimally extract proteins, while not denaturing the protein complexes of interest or inhibiting Ab-bead interaction with the bait protein.^{218,219} Certain protein complex formation is dependent on membrane interaction and/or the concentration of binding partners and co-factors in the cell, all of which are disrupted by the addition of lysis buffer.²²⁰ It could also be necessary to include additives such as ATP, DTT, MgCl₂, glycerol, phosphatase and/or phosphatase inhibitors to facilitate and stabilize protein interactions, while for some proteins the addition of these reagents will actually prevent complex formation.^{148,218,220} Optimisation of this step is thus key to a successful outcome.^{148,219} Protein abundance should also be taken into account.^{218,219} Some Co-IP protocols are only successful when proteins are overexpressed via plasmid-construct transfection.^{98,101,102} However, artificially expressed proteins may be subject to miss-folding and post-translational modification alterations (which will affect protein-protein interactions), while overexpression shifts endogenous expression levels and potentially cellular localization of the protein, leading to skewed results.²²⁰ Other protein interactions may rely on cellular signals or may be transient, both of which could be missed through Co-IP analysis.²¹⁹ It is still possible that MAGE-C1 interacts with SKIP, but an alternative Co-IP approach may be required. In addition to trying alternate cellular lysis conditions, Co-IP could also be performed with SKIP as the bait, as it also associates with other complexes²²¹ and may thus provide an alternate internal positive control as well.

While the direct interaction between MAGE-C1 to SKIP using Co-IP was not proven, IFM allowed us to determine the rough cellular localization of the proteins, indicating that both proteins could potentially interact in the cytoplasm. However, the RPMI-8226 cells did not show the expected localisation of MAGE-C1, which has been shown to be expressed in both the nuclear and cytoplasmic fractions in the MM cell line ARK,¹⁸⁹ as well as varied localisation (cytoplasmic, nuclear or combined) in the PC of MM patients.¹⁸⁹ MAGE-A1 interaction with SKIP and the SMRT–corepressor complex, previously shown by Laduron et al. occurs in the nucleus,⁹⁸ thus, in this cell line, MAGE-C1 cannot have the same function as MAGE-A1, unless activation of the Notch 1 pathway is required for MAGE-C1 to move into the nucleus. These results demonstrate that RPMI-8226 may not be suitable to compare these two MAGE functions. We did try to use the anti-MAGE-A1 Ab in this IFM technique, to see where MAGE-A1 actually localized in this cell line, but again, no specific binding was observed. Suboptimal staining in this setting could be due to epitope loss during the methanol fixation²²² and a fixative such as formaldehyde could be considered as both methanol and ethanol denature protein structures during the fixation process,²²² while formaldehyde preserves cellular structures.²²³

4.3. MAGE-C2 COMPARED TO MAGE-C1

4.3.1. Bioinformatics Comparison

As with the bio-informatics analysis of MAGE-C1 vs MAGE-A1, the similarity between MAGE-C2 and MAGE-C1 were compared. The WH1 and WH2 domains of MAGE-C2 and MAGE-C1 showed > 50% and > 69% identity, respectively, which confidently predicted structural (> 30%) and functional (> 60%) similarity of the MHD domains of these two proteins.^{116,165} These results showed that MAGE-C1 has a higher similarity to MAGE-C2 than MAGE-A1. This was not unexpected, as MAGE-C1 and C2 are part of the same sub-family due to protein and gene identity scores.

The number, type and position of post-translational modification sites within MAGE-C2 was significantly different from MAGE-C1, although the WHD contained one ubiquitination site and six phosphorylation sites. MAGE-C2 has been shown to manipulate downstream ubiquitination of proteins, such as TP53, to increase their degradation, through association with E3 ligases, which triggers the poly-ubiquitination and degradation of the protein.¹⁰¹ Hao, et al. confirmed that MAGE-C2 binds to E3 ligase RBX1 via the MHD.¹⁰² In contrast to MAGE-C2, the majority of phosphorylation sites identified in MAGE-C1 were outside of the WHD (within the first 400 aa of the protein), with all the ubiquitination sites contained within the WHD. This could indicate potential additional or different functions of MAGE-C1 compared to MAGE-C2, despite the high level of identity.

Overall the bio-informatics analysis showed greater similarity of MAGE-C1 to MAGE-A1 when looking at the number and position of post-translational modifications, but a higher identity score with MAGE-C2 that indicated functional similarity. It is thus possible that MAGE-C1 shares functions with both of these proteins.

4.3.2. Cellular Expression Comparison

We assessed the expression of known binding partners of MAGE-C2 in RPMI-8226 at both mRNA and protein levels where possible. *MAGE-C2*, *RBX1* and *TRIM28* mRNA expression was confirmed, but due to the unsuitability of the anti-MAGE-C2 Ab that we were using and the lack of a suitable, specific anti-TRIM28 Ab, we were only able to successfully demonstrate RBX1 expression at the protein level. Of interest was the relatively low levels of *MAGE-C2* mRNA transcribed in RPMI-8226, compared to human testis total RNA. Expression in these cell types does not appear to have been previously compared and may indicate different functions in these two tissues. A study by Zhao et al. that examined *MAGE-C2* expression levels in triple-negative breast cancer tissue (at transcript and protein levels), found that differential expression carried prognostic significance.²²⁴ Deeper investigation into *MAGE-C2* expression levels would require optimisation for quantitative analysis, as mentioned previously. Human tonsil tissue RNA and PBMNC RNA extracts were found to be suitable negative controls for

MAGE-C2 expression studies, with PBMNC protein extracts a suitable negative control for protein analysis studies. Jurkat was not suitable for use as a negative control, due to low levels of both *MAGE-C1* and *MAGE-C2*.

4.3.3. Association with RBX1

Unfortunately, we were unable to establish a direct binding association between *MAGE-C1* and *RBX1* using our Co-IP strategy, but we did confirm that they both co-localised in the cytoplasm of RPMI-8226 cells. *RBX1* has previously only been located in the nucleoplasm, where its interaction with *MAGE-C2* and the SCF-complex occurs,^{102,153,154} thus our finding of *RBX1* staining in both the cytoplasmic and nuclear compartments in both PMBNC (results not previously shown) and RPMI-8226 cells, brings into question the specificity of binding of this Ab and we will need to find an alternate strategy to confirm that this Ab is specific. If it is, and the staining accurately reflects the localisation of *RBX1* in RPMI-8226, then again this would indicate that this cell line is not a suitable model for studies involving the SCF/*MAGE* complex.

4.3.4. Pathways Potentially Affected by MAGE-C2

For the RPMI-8226 cell line to be suitable as a model to study *MAGE* functions, the pathways that have been shown to be affected by *MAGE-C2* in other cell lines do need to be defective/abnormal in this cell line. We investigated the effects of heat-shock on the activation of the ER-stress response, which leads to the ubiquitination and degradation of specific proteins. In non-malignant cells, this should all finally result in a halting of the cell cycle at the G1-checkpoint, to allow for DNA repair prior to cell division. In the presence of *MAGE-C2*, two separate processes involving E3 ligases result in the overall increase in the number of cells progressing into the S-phase.^{56,101,102} We therefore first investigated the effects of the heat shock on the main targets of the E3 ligases, namely Cyclin E1 and TP53. We found definite changes in the levels of these proteins in line with the involvement of *MAGE-C2*, namely elevated Cyclin E1 and reduced TP53 protein levels. However, the results indicated that the response was not particularly pronounced and a more aggressive heat-activation or longer period post stress may be needed to improve the significance of the protein level changes.

Following this finding, which was in line with *MAGE-C2* involvement in these pathways and them being abnormal in RPMI-8226, the expression of additional key genes along the pathway was investigated. For cell division to progress, it must pass through the G1-check point, which is controlled by TP53 (amongst other regulatory proteins). Upon activation (such as heat shock), TP53 promotes p21 synthesis, which then inhibits CDK and Cyclin D, causing arrest at this check point.¹⁵⁹ When subjected to stress, such as heat-shock, cells respond by either initiating cell death if the stress is too severe, or

they initiate recovery systems which favour expression of protective proteins while non-essential transcription and translation is halted, as well as pathways such as cell division.²²⁵ If MAGE-C2 is present, the lower levels of TP53, which we observed post heat shock in RPMI-8226, should lead to lower levels of the CDK inhibitor p21.^{56,101,102} Unexpectedly, we saw no change in the levels of p21 mRNA following the heat-stress in RPMI-8226. We also didn't observe an increase in Cyclin A1/2 levels, that should have resulted from the increased Cyclin E1 we detected in RPMI-8226. In retrospect we should have changed the stress conditions, or increased the time before protein collection, to allow the specific pathways to be activated. We are confident that an extended experimental system would have shown the MAGE-C2 associated changes in these genes, as well as a final impact on the cell cycle status of the cells, confirming the likely involvement of MAGE-C2 in E3 ligase binding in RPMI-8226.

4.4. CONCLUSION

Overall, despite some technical difficulties, we were able to establish a plethora of techniques that can be used to study the MAGE function in future studies. This included the successful design of primer sets that can be used in quantitative real-time PCR methodology to look at transcript expression with greater accuracy; successful validation of the anti-MAGE-C1 Ab, anti-SKIP Ab and partial validation of the anti-RBX1 Ab for use in western hybridization, IFM, and Co-IP studies. Western hybridisation, IFM and cell cycle analysis methodologies were successfully developed for use in our laboratory. However, our experiments provided enough information to be able to conclude that RPMI-8226 is not suitable for the studies we would like to do to determine the function of MAGE-C1. We therefore need to find an alternate model, which is a better representation of the MM primary cell of interest for MAGE-C1 expression studies, namely SC collected from MM patients. As SC are also routinely collected from non-diseased donors, these would provide the most appropriate negative control for all experiments moving forward. We have recently been granted permission to use residual frozen SC collections from deceased patients, and plan to repeat several of the experiments described in this study with this sample set, before proceeding to gene knock-out studies, using the reagents and techniques we have tested here.

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