AN INVESTIGATION INTO THE ROLE OF C-TERMINAL TENSIN-LIKE PROTEIN (CTEN) IN MELANOMAGENESIS

by

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Abstract

C-terminal tensin-like protein (Cten) is a focal adhesion protein with no or limited protein expression in normal tissues, which has recently been reported to be overexpressed and act as an oncoprotein in numerous cancers. Since its expression status in human cutaneous melanoma is currently unknown, I used tissue microarrays and immunohistochemical staining to examine the protein expression of Cten throughout melanoma progression. I found that Cten was significantly up-regulated in dysplastic nevi (DN) compared to normal nevi (NN), and in primary melanoma (PM) compared to both DN and NN. Strong Cten staining was associated with a poorer 5- and 10-year overall and disease-specific survival for PM patients, and was an adverse independent prognostic factor for the 5-year survival of the same patients. *In vitro* studies using two melanoma cell lines supported these findings and indicated that Cten functions as an oncogene in melanoma.

Since relatively little is known about how Cten contributes to tumorigenesis, I next investigated the expression profile of the RhoGAP Deleted in Liver Cancer-1 (DLC1), the only protein known to bind to Cten, in melanomas. Both cytoplasmic and nuclear DLC1 were detected, and both were down-regulated in metastatic melanoma (MM) compared to PM and nevi, with nuclear DLC1 expression additionally being reduced in PM compared to nevi. Both cytoplasmic and nuclear DLC1 were associated with the 5-year overall and disease-specific survival of all melanoma and MM patients, and with the disease-specific 10-year survival of all melanoma patients. Combined analysis of

cytoplasmic and nuclear DLC1 revealed that for MM patients, concurrent loss of both cytoplasmic and nuclear DLC1 was associated with the worst survival outcome, with loss of either or both forms being a significant adverse independent prognostic factor for the 5-year survival of all melanoma and MM patients. A preliminary investigation into the relationship between Cten and DLC1 indicated that the effects of Cten on patient survival were dependent on the levels of DLC1, as expected.

In summary, I here provide an initial characterization of the expression status and role of Cten in melanomagenesis, and speculate that it functions partly via interactions with the tumour suppressor DLC1.

Preface

Chapters 3.2.1 – 3.2.5 are based on the following manuscript, which has been submitted and accepted: Sjoestroem C, Khosravi S, Zhang G, Martinka M, Li G (2013) C-terminal tensin-like protein is a novel prognostic marker for primary melanoma patients. PLOS One [in press].

For the manuscript, G.L. provided the research facilities and materials, and contributed to the research design, S.K. helped perform one experiment for the revision of the paper, G.Z. and M.M. scored the TMA slides, and I designed and performed all experiments, analyzed the data, as well as prepared the manuscript and all figures and tables. For the unpublished *in vitro* data, I performed all experiments and analyzed all data, with the help of Dr. Mehdi Jafarnejad and Shahram Khosravi who taught me the cell proliferation, invasion and migration assays, as well as how to do the cell cycle analysis.

Chapters 4.2.1 – 4.2.6 are largely based on the following manuscript, which has been submitted and accepted: Sjoestroem C, Khosravi S, Cheng Y, Safaee Ardekani G, Martinka M, Li G (2013) DLC1 expression is reduced in human cutaneous melanoma, and correlates with patient survival. Modern Pathology [in press].

G.L. provided the research facilities and materials, Y.C., G.S.A. and M.M. scored the TMA slides, and S.K. provided helpful discussion on the manuscript. I designed the study, performed all experiments, analyzed the data, and prepared the manuscript.

All work was performed at the Skin Cancer Biology Lab at the University of British Columbia, and all aspects of this study, including the use of human tissues and the waiver of patient consent, were performed in accordance with the Declaration of Helsinki guidelines, as approved by the Clinical Research Ethics Board of the University of British Columbia, Vancouver, Canada (certificate number H09-01321).

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List of abbreviations

6-4PPs	Pyrimidine pyridine-(6-4)-photoproducts
A	Adenine
ABD	Actin binding domain
AJCC	American Joint Committee on Cancer
α-MSH	α-Melanocyte-stimulating hormone
Akt	Thymomo viral proto-oncogene; Protein kinase B
BAD	BCL2-associated agonist of cell death
BCAR1	Breast cancer anti-oestrogen resistance 1
BRAF	V-raf murine sarcoma viral oncogene homologue B1
C	Cytosine
c-AMP	Cyclic-adenosine monophosphate
c-MPL	Myeloproliferative leukemia virus oncogene
C1	Protein kinase C conserved region 1
Cdc42	Cell division cycle 42
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CPDs	Cyclobutane pyrimidine dimers
Cten	C-terminal tensin-like protein
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DLC1	Deleted in liver cancer-1
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERBB2	V-erb-b2 erythroblastic leukaemia viral oncogene homologue 2
ERK	Mitogen-activated protein kinase
FAB	Focal adhesion binding
FACS	Fluorescence-activated cell sorting
FAT	Focal adhesion targeting
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
G	Guanine
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
IFN	Interferon
IGF	Insulin-like growth factor
IKK	IκB kinase
IL	Interleukin
ILK	Integrin-linked kinase

IRS	Immunoreactive score
IRS-1	Insulin receptor substrate 1
ITGB1	Integrin VLA-4 beta subunit
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin 1 receptor
Mdm2	E3 ubiquitin protein ligase
MEK	Mitogen-activated protein kinase kinase
MITF	Microphthalmia-associated transcription factor
MMP	Matrix metalloproteinase
mTOR	Mechanistic target of rapamycin (serine/threonine kinase)
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NGF	Nerve growth factor
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
NSCLC	Non small cell lung carcinoma
p53	Tumour protein p53
PDGF	Platelet-derived growth factor
PDK1	Pyruvate dehydrogenase kinase, isozyme 1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-(3,4,5)-trisphosphate
PTB	Phosphotyrosine binding
PTEN	Phosphatase and tensin homolog
PTP	Protein-tyrosine phosphatase
RhoA/B/C	Ras homologue family member A/B/C
ROCK	Rho Kinase
RTK	Receptor-tyrosine kinase
SAM	Sterile alpha motif
SCF	Stem cell factor
SH2	Src homology 2
SOS	Son of sevenless homologue (Drosophila)
START	Steroidogenic acute regulatory protein (StAR)-related lipid-transfer
STAT3	Signal transducer and activator of transcription 3
T	Thymine
TGF	Transforming growth factor
TNS4	Tensin4; C-terminal tensin-like protein
UVR	Ultraviolet radiation
VEGF	Vascular endothelial growth factor

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Dedication

I dedicate this thesis to my soul mate, the love of my life, and the reason I came to Vancouver and UBC in the first place; my husband Chris.

1. General introduction

1.1 Cutaneous melanoma

1.1.1 Melanocyte transformation and melanoma progression

Melanocytes are specialized cells originating from the embryonic neural crest. From there, the melanoblasts - the precursors of melanocytes - can migrate into the basal epidermis where they differentiate into mature cutaneous melanocytes, and are responsible for skin pigmentation and for providing ultraviolet radiation (UVR) damage protection (Jimbow, 1995; Wood *et al.,* 1999; Gupta *et al.,* 2005; Gray-Schopfer *et al.,* 2007; Ibrahim & Haluska, 2009). These properties can be accredited to the production of melanin pigments; the brown/black eumelanin, and the less common, phototoxic yellow/red pheomelanin (Jimbow, 1995; Wood *et al.,* 1999; Lin & Fisher, 2007; Volkovova, 2012). Upon production, the melanin is subsequently exported from the melanocytes to surrounding keratinocytes, the predominant type of cell in the epidermis*,* located in lysosome-like granules called melanosomes (Wood *et al.,* 1999; Lin & Fisher, 2007). On average, one melanocyte transfers melanosomes to 36 nearby keratinocytes, via its dendritic tips (Seiberg, 2000).

Once there, the melanin serves to absorb free radicals and UVR, protecting the keratinocytes from UV-induced DNA damage (Wood, 1999; Ibrahim & Haluska, 2009). The phenotypic variations in skin and hair colours observed between individuals from various ethnic backgrounds are not due to differences in the number of melanocytes, but rather due to differences in

melanin production and distribution, with fair-skinned, blonde or red-haired, individuals having less protective eumelanin pigment, rendering them more susceptible to UV-induced damage, commonly manifesting as sun-burnt skin (Lin & Fisher, 2007).

Under normal circumstances, melanocyte proliferation is tightly controlled by keratinocytes through a combination of cell-cell adhesion molecules, keratinocyte-derived paracrine growth factors, and intracellular communication via second messengers (Haass *et al.,* 2005). The molecular mechanisms through which loss of melanocyte homeostasis occur are not fully understood, but are hypothesized to be due to either down-regulation of factors important for melanocyte-keratinocyte communication, such as E-Cadherin; up-regulation of factors important for melanocyte-fibroblast interaction; loss of anchorage to the basement membrane; or a combination of two or more of these (Meier *et al.,* 1998; Haass *et al.,* 2005).

Uncontrolled melanocyte proliferation can result in the formation of acquired cutaneous nevi or melanomas. Melanoma progression can been broadly categorized into six steps, all associated with the accumulation of a number of genetic alterations (Hussein, 2004): (1) common nevus; (2) dysplastic nevus; (3) early *in situ* radial growth phase (RGP) primary melanoma; (4); early invasive RGP primary melanoma; (5) advanced vertical growth phase (VGP) primary melanoma; and (6) metastatic melanoma (Guerry *et al.,* 1993; Meier *et al.,* 1998; Hussein, 2004; Croteau *et al.,* 2013).

Common - or normal - nevi are small with well-defined borders and

uniform colour (Hussein, 2005a), whereas dysplastic nevi are larger, with ill-defined borders, irregular colouration, and mild-to-severe architectural and cytological atypia; features sometimes rendering them difficult to distinguish from early stage cutaneous melanomas (Halpern *et al.,* 1991; Hussein, 2005a). There is some controversy regarding the role of dysplastic nevi in melanoma development. What we do know is that the presence of dysplastic nevi is associated with a significantly higher risk of an individual developing melanoma, but far from all dysplastic nevi will eventually develop into melanoma, and not all melanomas start off as nevi (Gruber *et al.,* 1989; Hussein, 2005a; Cummins *et al.,* 2006).

The next step of melanoma progression - the radial growth phase - is usually slow, and considered relatively benign, with next to no metastatic potential. Unlike dysplastic nevi however, their proliferation is generally not self-limiting. Although primary melanomas are considered fully curable by surgical excision at this stage, if the melanoma cells are incompletely excised, there is a high risk of recurrence and progression (Guerry *et al.,* 1993; Hussein, 2004; Laga & Murphy, 2010). Most, but not all, tumours go through this phase, and for them to progress to the next phase – the vertical growth phase – a clone capable of expandable growth must develop, an event that requires further genetic alterations (Herlyn *et al.,* 1985; Guerry *et al.,* 1993; Hussein, 2004; Laga & Murphy, 2010; Croteau *et al.,* 2013).

The vertical growth phase is characterized by the formation of tumour nodules, and the metastatic potential of these tumours is closely related to their

thickness. Once the tumour reaches the vascular or lymphatic networks, metastasis generally follows (Guerry *et al.,* 1993; Fidler, 2002; Laga & Murphy, 2010; Braeuer *et al*., 2011).

1.1.2 Incidence and epidemiology

Cutaneous malignant melanoma (CMM) is a highly aggressive type of skin cancer, and even though it accounts for less than 5% of all skin cancers, it is responsible for over 80% of all skin cancer-related deaths (Cummins *et al.,* 2006; American Cancer Society, 2011). Due to the high metastatic potential of malignant melanoma, the median survival reported for patients diagnosed with distant metastases is only 6-8 months, with an overall 5-year survival rate as low as 5-16% (Cummins *et al.,* 2006; American Cancer Society, 2011; Wasif *et al.,* 2011). However, with early detection followed by surgical excision, the 5-year survival rate is close to 100% (Cummins *et al.,* 2006; American Cancer Society, 2011).

CMM is one of very few types of cancers with a steadily increasing rate of incidence in the non-Hispanic white population throughout the world, with non-Hispanic whites having an approximately 10-fold increased risk of developing CMM compared to dark-skinned populations such as African-Americans (Diepgen & Mahlen, 2002; Giblin & Thomas, 2006; Rigel, 2010; American Cancer Society, 2011; Berwick, 2011). In the United States, the incidence rates have increased by 2.8% per annum since 1992 for this group (American Cancer Society, 2011). Worldwide, incidence rates vary from 0.2/100,000 per year for

Indian females to approximately 55-65/100,000 per year for white males in Queensland, Australia (Garbe & Leiter, 2009; Berwick, 2011; Whiteman & Green, 2011)**.** Other high-risk areas include New Zealand, the US, Canada, and Scandinavia **(**Diepgen & Mahlen, 2002; Giblin & Thomas, 2006; Garbe & Leiter, 2009; Erickson & Driscoll, 2010; Canadian Cancer Society, 2012).

In addition to skin colour and geographic location, other, less well-known, factors that influence the incidence rates include: sex, with males generally having a higher incidence rate than females; and age, with a higher age being associated with an increased incidence rate (Rigel, 2010; American Cancer Society, 2011; Whiteman & Green, 2011). Moreover, melanoma rates vary by anatomical sites. In males, the majority of tumours are found on the trunk, whereas in females, most tumours occur on the lower extremities (Garbe & Leiter, 2009). That said, an increase specifically of melanomas of the trunk has recently been reported for young females in the US, likely representing a shift in the intermittent sun-exposure pattern and behaviour for this group over the past few decades (Bradford *et al.,* 2010). In the older population, independent of sex, tumours are predominantly found on the head and neck (Garbe & Leiter, 2009). When adjusted for area however, the incidence of melanoma has been found to be highest on the ears, followed by head, neck, shoulders, and back in males, and on the face and shoulders in women (Green *et al.,* 1993).

Interestingly, in the non-Hispanic white population, the incidence of *in situ* and thin melanomas is increasing at a much higher rate than that of thick, invasive, and metastatic melanomas. Whether this observation represents a true

phenomenon or is due to increased early detection secondary to improved public awareness campaigns is however highly debated (Lipsker *et al.,* 1999; de Vries & Coebergh, 2004; Garbe & Leiter, 2009; Linos *et al.,* 2009; Erickson & Driscoll, 2010).

Conversely, in Hispanic and black populations, melanoma is more frequently diagnosed at a deeper thickness and later stage, and is hence associated with a poorer survival outcome. Likely, this is at largely due to a lack of melanoma awareness in these groups, with public health campaigns generally being aimed at the high-risk, fair-skinned population (Berwick, 2011).

Whereas melanoma incidence has been increasing in most non-Hispanic white sub-populations at a staggering rate, the corresponding mortality rates are increasing at a much slower pace, and are even plateauing or decreasing in some groups. This has again been accredited to increased public awareness and subsequent earlier detection, especially for young females, the group with the highest increase in incidence, but slowest increase in mortality rates (Miller *et al.,* 1996; Jemal *et al.,* 2000; de Vries & Coebergh, 2004; Purdue *et al.,* 2008; Linos *et al.,* 2009; Coelho & Hearing, 2010; Rigel, 2010).

1.1.3 Subtypes and staging of cutaneous melanoma

Cutaneous melanoma can be broadly sub-classified into four major categories; superficial spreading, lentigo maligna, nodular, and acral lentiginous melanomas, all exhibiting different, albeit sometimes overlapping, morphological and

histological features (Clark *et al.,* 1969; Arrington *et al.,* 1977; Porras & Cockerell, 1997; Cummins *et al.,* 2006).

Superficial spreading melanoma (SSM) is the most common subtype, accounting for approximately 70-80% of all melanomas in the non-Hispanic white population. Clinically, superficial spreading melanomas generally adhere to the "ABCD" rule (Asymmetry, Border irregularity, Colour variegation, and Diameter ≥ 6 mm), and present as large, flat or slightly raised marks with irregular borders and variegated colour (Clark *et al.,* 1969; Porras & Cockerell, 1997; Cummins *et al.,* 2006; Longo *et al.,* 2012).

Lentigo maligna melanomas (LMM) represent about 5% of all cutaneous melanoma cases, and are commonly found in fair-skinned individuals in their seventh or eighth decades of life, in areas chronically exposed to the sun such as the head and neck. They arise from precursor *in situ* lesions known simply as lentigo maligna and present clinically as large, irregular flat patches with colour variegation (Clark *et al.,* 1969; Porras & Cockerell, 1997; Cummins *et al.,* 2006; Reed & Shea, 2011; Pralong *et al*., 2012).

Nodular melanomas (NM) account for 10-15% of all melanomas, and roughly 50% of melanomas > 2 mm thick in the non-Hispanic white population, and differ considerably in presentation from the other major subtypes. Unlike SSM, nodular melanomas have no radial growth phase, and do not follow the "ABCD" rule. They are commonly described as being relatively small, elevated, symmetrical, uniformly coloured marks with round borders (Porras & Cockerell, 1997; Demierre *et al.,* 2005; Geller *et al.,* 2009; Kalkhoran *et al.,* 2010). Nodular

melanomas are faster growing and more invasive compared to the other melanoma subtypes, and are due to this generally thicker at diagnosis. Since tumour thickness is directly associated with patient survival, nodular melanomas, despite of only representing a subset of tumours, are responsible for the majority of melanoma deaths in the non-Hispanic white population (Mar *et al.,* 2012).

Of the four major melanoma subtypes, Acral lentiginous melanomas (ALM) are the least frequently occurring, accounting for only about 1-5% of all melanomas in the non-Hispanic white population. Even though ALM occurs at a similar rate in all races, it accounts for the majority of melanomas in dark-skinned people (up to 36% of all cutaneous melanomas for blacks), who have a lower overall incidence of melanomas. As the name suggests, ALMs are generally found on the palms and soles or under finger and toenails, where they present as irregular lesions with variegated colour (Porras & Cockerell, 1997; Bradford *et al.,* 2009; Piliang, 2011).

Melanoma staging has been established by the American Joint Committee on Cancer (AJCC), and was most recently revised in 2009. It divides melanomas into four main stages, encompassing several important prognostic factors such as tumour thickness, ulceration, lymph node involvement, site of metastasis, and now also mitotic rate and serum Lactate dehydrogenase (LDH) levels (Balch *et al.,* 2009).

Stage I tumours are classified as being localized and ≤ 2 mm thick with no ulceration or ≤ 1 mm with ulceration present, whereas localized tumours > 2 mm thick without ulceration, and tumours > 1 mm thick with ulceration, are

classified as AJCC Stage II tumours (Balch et al., 2009). Regional metastatic melanomas are classified as AJCC Stage III tumours, and are usually diagnosed by sentinel lymph node biopsies. Late-stage, distant metastatic melanomas are classified as AJCC Stage IV, and can be further sub-classified based on the location of the metastatic deposit and the serum LDH levels. Patients with metastases in the skin, subcutaneous tissue or distant lymph nodes, and normal LDH levels have a relatively favourable outcome, whereas patients with metastases to visceral tissues other than the skin, subcutaneous tissue, distant lymph nodes and lungs, and/or with elevated LDH levels have the least favourable outcomes (Balch *et al.,* 2009).

1.1.4 Current treatment options

Whereas early-stage, localized melanomas can generally be successfully treated by surgery alone (Lee *et al.,* 2013), there is currently no universal cure or treatment option for late stage melanoma. The standard treatment for patients with primary melanomas ≥ 2 mm thick with or without regional lymph node metastasis (Stage II and III melanomas) is surgery, including lymphadenectomy if needed, followed by adjuvant therapy or enrolment in a clinical trial (Garbe *et al.,* 2011). Adjuvant therapy usually comprises of a biological agent such as interferon-α2b (IFN-α2b); the first high-risk melanoma treatment to display a significantly improved disease-free survival in a randomized clinical trial (Mocellin *et al.,* 2009; Garbe *et al.,* 2011). Although there have been countless studies aimed at determining the mechanism of action, and the best dose and duration of

IFN-α2b treatment, many of these questions remain unanswered. Most studies have moreover failed to find an association between IFN-α2b treatment and overall patient survival, independent of dosage. However, a meta-analysis by Mocellin *et al.* (2009) did find that IFN-α2b had a positive effect on overall survival compared to observation only, but they were again unable to determine what dose had the greatest effects.

Metastatic melanoma is notoriously resistant to chemotherapy, radiation and other traditional cancer treatments. The most common chemotherapeutic drug used for melanoma is Dacarbazine. Despite a complete response rate of only about 5%, Dacarbazine is still used for Stage IV melanoma patients as palliative care, either as a single agent or together with IFN-α2b or high-dose interleukin-2 (IL-2) (Soengas & Lowe, 2003; Schopfer-Gray *et al.,* 2007; Marti *et al.,* 2011; Garbe *et al.,* 2011; Velho, 2012).

Over the last few years, several new, single-target therapies have emerged as promising treatment options for late-stage melanoma. Melanoma is a highly immunogenic tumour, and drugs targeting the immune system are becoming increasingly common. One such drug is Ipilimumab, an FDA-approved monoclonal antibody targeting CTLA-4, a T-cell receptor that negatively regulates T cell activation and proliferation, thus leading to a hyper-activated T-cell response (Hodi *et al.,* 2010; Garbe *et al.,* 2011; Velho, 2012). In a Stage III clinical trial, Ipilimumab use resulted in a significant increase in overall survival, with long-term 1- and 2-year survival rates of 45.6% and 23.5%, respectively, for metastatic melanoma patients (Hodi *et al.,* 2010).

Over half of all metastatic melanomas carry a single point V600E mutation in the BRAF gene (discussed in more detail in Chapter 1.2.3). In brief, the BRAF V600E protein exhibits a high level of constitutive phosphotransferase activity, likely due to the negative charge of the glutamic acid residue mimicking a phosphorylation site in the activation loop (between the kinase catalytic subdomain VII and VIII regions). The hyperactive BRAF V600E protein results in constitutive activation of the downstream MAPK pathway, and uncontrollable cell proliferation (Davies *et al.,* 2002; Flaherty *et al.,* 2010; Chapman *et al.,* 2011; Sosman *et al.,* 2012). This observation has lead to the development of Vemurafenib, an FDA-approved small-molecule protein-serine/threonine kinase inhibitor specifically targeting BRAF V600E. The first Stage III clinical trial on Vemurafenib conducted in 2010 revealed some truly promising results. Six months into the study, the overall survival was 84% for the Vemurafenib group compared to 64% in the control group treated with Dacarbazine, and in the interim analysis, Vemurafenib treatment was found to be associated with a 63% relative reduction in death and a 74% relative reduction in either death or disease-progression compared to the control group, resulting in a recommended crossover for the Dacarbazine group to Vemurafenib before the planned end of the study (Chapman *et al.,* 2011).

However, despite these initially promising results, the majority of patients relapse with a highly aggressive, drug-resistant disease after prolonged Vemurafenib use (Luke & Hodi, 2011; Das Thakur *et al.,* 2013), a phenomenon that has prompted extensive research efforts into ways to circumvent this issue.

One proposed strategy is to administer Vemurafenib discontinuously (to take so-called “drug holidays”), since drug-resistant cells may contradictory also display drug-dependency (Das Thakur *et al.,* 2013). This study, published in Nature earlier this year is now the basis for numerous ongoing studies examining the effects of discontinuous Vemurafenib use in combination with other targeted therapies.

In conclusion, although there is currently no cure for melanoma, and despite dismal survival outcomes for late-stage melanoma patients, the advances made during the last decade are encouraging, and there is every reason to believe that the melanoma mortality rates will continue to decline.

1.2 Melanomagenesis

1.2.1 Genetic factors

Between 4-10% of all cases of cutaneous malignant melanoma can be attributable to having an affected first- or second-degree family member, with these individuals having an approximate two-fold increase in the risk of developing CMM (Bisio *et al.,* 2010; Olsen *et al.,* 2010; Rigel, 2010; Volkovova *et al.,* 2012). The major genetic determinants for familial melanoma are germ-line mutations in the high-penetrance susceptibility genes CDKN2A and CDK4, which have both been linked to early-onset melanoma, as well as in variants of the low-penetrance susceptibility gene MC1R (Goldstein *et al.,* 2000; Bishop *et al.,* 2002; Hayward, 2002; Olsen *et al*., 2010; Rigel, 2010; Ghiorzo *et al.,* 2012). CDKN2A is a tumour suppressor gene that codes for two separate cell cycle proteins:

$p16^{INK4a}$ and $p14^{ARF}$. Whereas $p16^{INK4a}$ has been implicated in in the activation of the Rb tumour suppressor pathway via inhibition of the cyclinD1-CDK4 complex, $p14^{ARF}$ has been linked to p53 signalling; truly emphasizing the role CDKN2A mutations could play in melanomagenesis (Goldstein *et al.,* 2000; Bisio *et al.,* 2010). CDK4, by contrast, is a proto-oncogene and a positive regulator of cell cycle control, known to affect the same downstream targets as $p16^{INK4a}$; a likely explanation as to why the clinical characteristics associated with CDKN2A and CDK4 mutations are indistinguishable (Goldstein *et al.,* 2000; Hayward, 2003).

The MC1R gene (Melanocortin 1 receptor) encodes a G protein-coupled trans-membrane receptor with high affinity for α-melanocyte stimulating hormone (α-MSH). Under normal circumstances, binding of α-MSH to MC1R upon sun exposure would result in adenylate cyclase activation and in an increase in cAMP production, subsequently leading to a switch in melanin production from the photo-reactive yellow-red pheomelanin to the photo-protective brown-black eumelanin, and to a tanning response (Kennedy *et al.,* 2001; Hayward, 2003; Raimondi *et al.,* 2008**;** Katensky *et al.,* 2010). Individuals carrying MC1R variants appear to be compromised in their ability to make this switch, and thus commonly present with red hair, fair skin, freckling and an inability to tan, four of the major risk factors for melanoma (Beral *et al.,* 1983; Hayward, 2003; Rigel, 2010; Ghiorzo *et al.,* 2012). In addition to increasing the risk of melanoma through these pigment-associated phenotypes, there is evidence supporting a role for MC1R variants in melanomagenesis largely independent of skin or hair colour, as demonstrated by an association between MC1R and melanoma in individuals not

otherwise at high risk (Kennedy *et al.,* 2001; Raimondi *et al.,* 2008; Kanetsky *et al.,* 2010).

Besides familial melanoma there are a number of other genetic conditions and diseases that significantly increase the risk of melanoma. It has been well established that the major risk factor for melanoma is having a high number of normal and dysplastic nevi (Bauer & Garbe, 2003; Gandini *et al.,* 2005; Whiteman & Green, 2011), so it comes as no surprise that Familial Atypical Multiple Mole-Melanoma (FAMMM) syndrome, a syndrome which, as the name suggests is associated with having an abnormally high mole count, correlates with an increased risk of getting melanomas (Lynch *et al.,* 1978; Lynch *et al.,* 1980; Rigel, 2010). Another disease of interest is Xeroderma Pigmentosum (XP), a disorder defined by life-threatening sensitivity to the sun due to defective DNA-repair, which is associated with a 1000-fold higher risk of melanoma compared to the average population; a fact that is commonly used to highlight the role of ultraviolet radiation in melanomagenesis (Spatz *et al.,* 2001; Gray-Schopfer *et al.,* 2007; Lehmann *et al.,* 2011; Whiteman & Green, 2011).

1.2.2 Environmental factors

1.2.2.1 Ultraviolet radiation

It is widely accepted in the scientific community that ultraviolet radiation (UVR) is the major environmental factor responsible for skin cancer carcinogenesis. UVR can be sub-classified based on their respective wavelengths as UVA (320-400 nm), UVB (290-320 nm) and UVC (200-290 nm) (Hussein, 2005b; Platz *et al.,*

2008; von Thaler *et al.,* 2010). UVC radiation, although highly carcinogenic, does not contribute to melanomagenesis as it is completely absorbed by the ozone layer in the earth's atmosphere. In contrast, UVB is only partly absorbed in the atmosphere, and accounts for approximately 5% of the UVR that reaches the surface (with the other 95% being UVA). However, it is the biologically most active wavelength, largely due to it being directly absorbed by DNA (Atillasoy *et al.,* 1998; Rünger, 1999; Budden & Bowden, 2013).

The link between UVB and non-melanoma skin cancers (namely basal cell carcinomas and squamous cell carcinomas) is direct and clear-cut, and whereas there are overwhelming data supporting a role for UVB in melanoma as well, the relationship is not quite as straightforward. What we do know is that UVB radiation leads to the formation of cyclobutane pyrimidine dimers (CPD's) and pyrimidine pyridine 6-4 photoproducts (6-4PPs), which in turn can lead to C-T or CC-TT transitions if incorrectly repaired. If these mutations occur in tumour suppressor- or oncogenes, abnormal cell growth and tumour formation may ensue (Hussein, 2005b; von Thaler *et al.,* 2010; Volkovova *et al.,* 2012; Budden & Bowden, 2013).

The role of UVA, which accounts for the majority of UVR reaching the surface, in melanomagenesis is even more debated. It is unknown whether it causes direct damage to the DNA or indirect oxidative damage (Hussein, 2005b; von Thaler, 2010; Budden & Bowden, 2013), and until recently it was assumed that it did not play a role in melanoma development at all.

1.2.2.2 Epidemiological evidence for a role of UV in melanoma development

The epidemiological data supporting a role for sun exposure and UV radiation in melanomas are compelling. Firstly, the incidence of melanomas is about 10 times higher in non-Hispanic whites compared to non-whites, and within that group, melanomas are about twice as common in fair-skinned, red-haired and blonde individuals with a tendency to freckle and burn (Skin Types I and II), compared to people with darker features who tan well (de Vries & Coebergh, 2004; Garbe & Leiter, 2009; Rigel, 2010; Whiteman & Green, 2011). Secondly, among whites, the incidence generally increases the shorter the distance to the equator is, with the only exception being in Europe where melanomas are more common in the north (e.g. Scandinavia) compared to the southern Mediterranean countries where the population usually are classified as Skin Types III or IV (de Vries & Coebergh, 2004; Garbe & Leiter, 2009; Rigel, 2010; Whiteman & Green, 2011). The highest melanoma incidence in the world is found in whites living in northern Queensland, Australia, an area not only located close to the equator, but that is also affected by severe ozone layer depletion, resulting in higher levels of UVB reaching the surface (Berwick, 2011; Volkovova *et al.,* 2012).

Moreover, an important role for childhood sun exposure in melanoma development has been demonstrated not only by a significant correlation between childhood sunburns and risk of melanoma (Whiteman *et al.,* 2001; de Vries & Coebergh, 2004; Le Marchand *et al.,* 2006; Giblin & Thomas, 2007; Veierød *et al.,* 2010), but also by the fact that the melanoma risk is higher in immigrants (≥ 20 years old at arrival) from low-latitude countries such as

Australia to high-latitude countries such as the UK compared to ethnically similar native-born residents (Diepgen & Mahler, 2002; Le Marchand *et al.,* 2006; Garbe & Leiter, 2009; Rigel, 2010; Whiteman & Green, 2011). Multiple sunburns in adults have also been associated with an increased melanoma risk, further reinforcing the role of UVR in melanomagenesis (Elwood & Jopson, 1997; Whiteman *et al.,* 2001; Garbe & Leiter, 2009; Rigel, 2010; Veierød *et al.,* 2010; Whiteman & Green, 2011; Volkovova *et al.,* 2012;).

The proposed role of UVA in melanoma development is supported by the fact that since the 1970-80's, tanning beds have been made to emit high doses of UVA instead of UVB and UVC. Although there are a lot of controversies and divided opinions surrounding the correlation between indoor tanning and melanoma, the overall consensus is that there *is* a significant association between the two, and that the relative risk is increased with frequent visits, especially before the age of 35 (Rünger, 1999; Westerdahl *et al.,* 2000; Ting *et al.,* 2007; Coelho & Hearing, 2010; Cust *et al.,* 2010; Lazovich *et al.,* 2010**;** Rigel, 2010; Veierød *et al*., 2010).

As mentioned, the relationship between melanoma and UV exposure is, however, not all that straightforward. Numerous studies have found that while intermittent sun exposure, especially in non-Hispanic white indoor workers, is strongly associated with an increased risk of melanoma, chronic exposure in outdoor workers is not, and may even have a weak protective effect (Nelemans *et al.,* 1993; Elwood & Jopson, 1997; Bulliard, 2000; Platz *et al.,* 2008; Cicarma *et al.,* 2010). Another argument against UV as the sole environmental factor in

melanomagenesis is the frequent appearance and increased incidence of melanomas on so-called sun-protected sites (sites not chronically exposed to the sun, such as the trunk and legs). This can be explained partly by the intermittent sun exposure theory briefly mentioned above, as well as by changing behavioural and fashion-related sun exposure patterns (Bradford *et al.,* 2010; Cicarma *et al*., 2010**;** Fuglede *et al.,* 2011). That said, melanomas do occur on completely sun-protected sites such as the rectum, anus, vulva and mucosal membranes in addition to the skin, indicating that there must be other factors besides UV involved in melanoma development (Mason & Helwig, 1966; Manolidis & Donald, 1997**;** Ragnarsson-Olding *et al.,* 1999; Ragnarsson-Olding, 2011).

1.2.3 Deregulation of apoptosis, cell cycle regulation and proliferation

One of the eight hallmarks of cancer, as defined by Hanahan & Weinberg (2011), is the ability of cells to sustain proliferative signalling. It is the most fundamental characteristic of cancer cells, and can be achieved in a number of different ways, including activation of cell survival pathways or by disruption of negative feedback mechanisms of the same pathways. For melanomas, the most important signalling pathway appears to be the MAPK (Ras/Raf/MEK/ERK) pathway (Fig.1.1), which is deregulated in 80-90% of melanomas (Omholt *et al.,* 2003; Haluska *et al.,* 2006; Saldanha *et al.,* 2006; Gray-Schopfer *et al.,* 2007; Platz *et al.,* 2008). More specifically, somatic mutations of the proto-oncogene BRAF, one of the three human Raf genes, have been implicated in over half of all

cases of melanoma (Davies *et al.,* 2002; Omholt *et al.,* 2003), and have been associated with childhood- and intermittent sun-exposure (Thomas *et al.,* 2007; Platz *et al.,* 2008). In melanocytes, the MAPK pathway is activated by growth factors such as stem-cell factors (SCF) or fibroblast growth factors (FGF), which normally would only transiently activate the extracellularly-regulated/MAP kinases ERK1 and ERK2, leading to a moderate increase in cell proliferation and survival (Gray-Schopfer *et al.,* 2007). By contrast, in the majority of melanomas, ERK1 and ERK2 are hyper-activated, either as a result of somatic mutations in BRAF or in the upstream GTPase NRAS, leading to continuous stimulation of mitogenesis and cell survival (Fig. 1.1; Omholt *et al.,* 2003; Saldanha *et al.,* 2006; Gray-Schopfer *et al.,* 2007; Platz *et al.,* 2008). Approximately 80-90% of all BRAF mutations in melanomas consist of a valine to glutamic acid substitution at position 600 (V600E, previously V599E) (Davies *et al.,* 2002; Omholt *et al.,* 2003) and occur almost exclusively in tumours with wild-type NRAS expression (Omholt *et al.,* 2003**;** Goel *et al.,* 2006). Similarly, 90% of NRAS mutations in melanomas occur at codon 61 (Haluska *et al.,* 2006; Platz *et al.,* 2008), and both BRAF and NRAS mutations appear to be associated with the early stages of melanoma transformation (Omholt *et al.,* 2003).

One of the many downstream targets of ERK1 and ERK2 (and hence also of NRAS and BRAF) is the transcription factor MITF (Fig.1.1). MITF is an important factor for normal melanocyte biology, and is responsible for the regulation of melanoblast survival, lineage commitment, melanocyte development and pigmentation, as well as for the expression of numerous

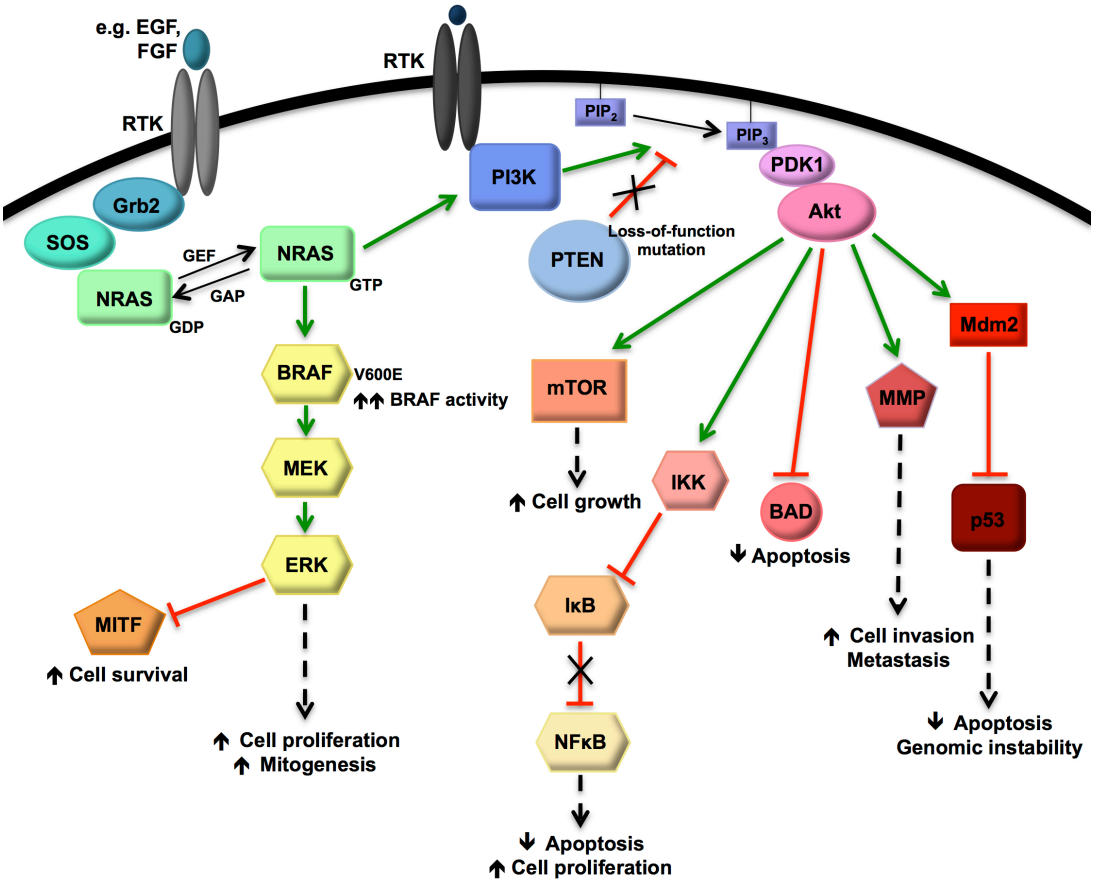
melanogenic proteins (Levy *et al.,* 2006; Gray-Schopfer *et al.,* 2007; Wellbrook *et al.,* 2008; Cronin *et al.,* 2009). At the basal level, MITF expression is essential for the survival of melanocytes and melanoma cells, and although amplifications of MITF have been detected in a subset of melanomas (Garraway *et al.,* 2005; Yokoyama *et al.,* 2011), *too low* levels of MITF interestingly also results in *increased* cell survival (Gray-Schopfer *et al.,* 2007; Wellbrook *et al.,* 2008). BRAF V600E mutations have been demonstrated to result in constant down-regulation of MITF in melanoma, and the two have moreover been found to cooperate, indicating a key role of MITF in BRAF-driven melanomagenesis (Wellbrook & Marais, 2005; Gray-Schopfer *et al.,* 2007; Wellbrook *et al.,* 2008).

In addition to its crucial role in regulating cell proliferation via the MAPK pathway and via MITF down-regulation, NRAS furthermore lies directly upstream of the PI3K/Akt pathway, which plays an important role in cell survival and apoptosis (Haluska *et al.,* 2006; Gray-Schopfer *et al.,* 2007). The oncogenic actions of PI3K are counteracted by PTEN (Fig.1.1), a phosphatase and tumour suppressor responsible for degrading PIP_3; the phospholipid product of PI3K phosphorylation (Wu *et al.,* 2003; Gray-Schopfer *et al.,* 2007; Courtney *et al.* 2010). PTEN protein expression is lost in over 60% of melanomas (Mirmohammadsadegh *et al.,* 2006), and loss-of-function mutations occur in between 5-30% of all melanomas, exclusively in tumours with non-mutated NRAS, but sometimes simultaneously with BRAF mutations. This is not surprising, as concurrent NRAS and PTEN mutations, similar to concurrent NRAS and BRAF mutations, are redundant. However, simultaneous BRAF and

PTEN mutations are highly advantageous for tumours, resulting in both increased cell proliferation and decreased apoptosis, ensuring the continued survival and spread of the tumours (Tsao *et al.,* 2004; Goel *et al.,* 2006; Haluska *et al.,* 2006; Gray-Schopfer *et al.,* 2007).

It is important to note that despite the prominent roles of these pathways, there are still several downstream targets remaining to be identified, and there are many other proteins and signalling pathways important for melanomagenesis as well. And whilst targeting BRAF or other MAPK pathway components therapeutically have had some success compared to traditional therapies, there is still no cure for late-stage melanoma, indicating just how important it is to gain a better understanding of the molecular events involved in the development, progression and metastasis of this malignancy.

Figure 1.1. Simplified diagram of the major signalling pathways implicated in melanomagenesis. The MAPK (Ras/Raf/MEK/ERK) pathway is frequently deregulated in melanoma. Somatic mutations in the NRAS or BRAF genes can result in constitutive activation of the pathway and increased cell proliferation and mitogenesis. BRAF V600E mutations have been associated with reduced levels of MITF, an important protein implicated in melanocyte development, leading to increased cell survival. Another major pathway involved in melanomagenesis is the PI3K/Akt pathway. Mutations in the tumour suppressor PTEN can result in uncontrolled activation of the oncogene Akt and subsequent activation or inhibition of downstream targets and enhanced melanoma progression and metastasis.



1.3 C-terminal tensin-like protein

1.3.1 The tensin family of proteins

The tensin family of proteins consists of four members: tensin1, tensin2, tensin3, and C-terminal tensin-like protein (Cten, also known as tensin4), all of which localize to focal adhesions (Lo, 2004). Focal adhesions are specialized areas of the plasma membrane formed around trans-membrane cores of α-β integrin dimers that bind the extracellular matrix (ECM) and create an anchor point between the ECM and the actin cytoskeleton. Because integrins cannot bind actin or relay cellular signals directly, all focal adhesion signalling is likely mediated by proteins attached to the cytoplasmic tails of the integrins, such as the tensins (Lo, 2006).

The basic structures of the tensin proteins can be seen in Figure 1.2. Briefly, all members contain C-terminal Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains, and tensin1-3 also contain N-terminal actin-binding domains (ABDs), allowing them to interact with actin at several sites, leading to cross-linking of the actin filaments. Both the ABDs and the C-termini of all tensins contain focal adhesion-binding sites, and the ABDs of tensin1-3 moreover contain PTEN-related protein-tyrosine phosphatase (PTP) sequences, although these are believed to be catalytically inactive (Lo, 2004; Lo, 2006).

Aside from focal adhesion localization, the biological functions of the tensins seem to vary. Tensin1 has been implicated in cell migration (Chen *et al.,* 2002; Chen & Lo, 2003), and *in vivo* experiments in tensin1-null mice indicate a

role for tensin1 in renal health, with older mice developing multiple cysts on their kidneys and dying from renal failure (Lo *et al.,* 1997). It moreover appears to play a role in platelet-derived growth factor (PDGF)-induced cytoskeleton reorganization and subsequent cell migration and proliferation via the PI3K/Akt pathway (Auger *et al.,* 1996).

Similarly to tensin1, tensin2 also plays a role in cell migration (Chen *et al.,* 2002), as well as in thrombopoietin-induced cell proliferation, by serving as a scaffolding protein between c-MPL; the receptor for thrombopoietin, and the PI3K/Akt pathway (Broxmeier *et al.,* 2011; Jung *et al.,* 2011; Tyner, 2011). Despite the belief that the tensins do not contain active phosphatase regions (Lo, 2006), this may not be the case for tensin2. A study by Hafizi *et al.* (2010) found that it is capable of dephosphorylating PIP_3, indicating an additional role for tensin2 in PI3K/Akt signalling. Another study by Koh *et al*. (2013) likewise detected tensin2 phosphatase activity under catabolic conditions, but this time against insulin receptor substrate-1 (IRS-1), another activator of the PI3K pathway, leading to muscle atrophy.

While tensin3 seems to be essential for the normal development and functions of the lungs, bones and small intestine, with tensin3-null mice displaying growth retardation or postnatal lethality (Chiang *et al.,* 2005), the biological function of Cten outside of carcinogenesis is less well studied. However, we know that its expression in normal tissues is highly restricted, with low or no detectable expression in most tissues except for the prostate and placenta (Lo & Lo, 2002), indicating that its functions are tissue-dependent.

Conversely, the roles and expression profiles of the full-length tensins in cancer are ill-defined. All tensin family members are down-regulated and proposed to function as tumour suppressors in renal carcinomas (Martuszewska *et al.,* 2009), and tensin3 is down-regulated in thyroid tumours compared to paired normal tissues as well (Maeda *et al.,* 2006), but displays oncogenic properties in cell lines derived from breast carcinomas, NSCLCs and melanomas (Qian *et al.,* 2009). Likewise, a variant of tensin2 is overexpressed and associated with tumorigenesis in liver cancer (Yam *et al.,* 2006). Collectively, these findings indicate that the tensins do play roles in carcinogenesis, but that they need to be examined more closely. Since its discovery in 2002, the role of Cten in cancer has however been extensively studied, and although there are still many more questions to be answered, a decade later we now have a much better understanding of this protein and its role in carcinogenesis.

1.3.2 Cten in human cancer

1.3.2.1 Cten as a tumour suppressor protein

Cten was first characterized as a putative tumour suppressor in prostate cancer, where it was found to be down-regulated in prostate cancer cell lines and patient tumour samples at the mRNA and protein levels, by Northern and Western blots, respectively. It was mapped to chromosome 17q21; a region frequently deleted in prostate cancer (Lo & Lo, 2002), and was later found to be cleaved specifically by caspase-3 during apoptosis (Lo *et al.,* 2005). It is largely unknown how Cten exerts its tumour suppressor functions, but it has been proposed that, in the

prostate, Cten functions by interacting with the known tumour suppressor Deleted in Liver Cancer-1 (discussed in Chapter 1.4) via its SH2 domain but independent of tyrosine-phosphorylation, leading to localization of DLC1 to the focal adhesions (Liao *et al.,* 2007). In prostate cancer, Cten loss has moreover been implicated in resistance to the chemotherapeutic agent paclitaxel (Li *et al.,* 2010), further supporting its proposed role as a tumour suppressor protein, and indicating that Cten expression has the potential to serve as a prognostic indicator for prostate cancer.

The only other cancer for which Cten expression has been found to be down-regulated is renal cell carcinomas, where a trend, albeit non-significant, was also seen for a correlation between loss of Cten mRNA expression and high tumour grade (Martuszewska *et al.,* 2009).

1.3.2.1 Cten as an oncoprotein

In contrast to its status in prostate and kidney cancers, Cten has been reported to be *up*-regulated and to function as an oncogene in a number of other cancers, including thymomas, breast, colorectal, gastric, lung, and pancreatic cancers (Sasaki *et al.,* 2003a; Sasaki *et al.,* 2003b; Sakashita *et al.,* 2008; Albasri *et al.,* 2009; Liao *et al.,* 2009; Albasri *et al.,* 2011a; Albasri *et al.,* 2011b; Al-Ghamdi *et al.,* 2013). It is currently unclear exactly how Cten is activated and regulated in these cancers, but there seem to be multiple pathways involved, and it appears to be largely tissue- and context-dependent.

In breast cancer-derived cell lines, as well as in the non-neoplastic mammary epithelial cell line MCF10, Cten has been identified as one of the most consistently up-regulated downstream targets of STAT3 signalling, mediated by IL-6. Cten overexpression upon STAT3 activation has been found to result in increased cell migration, invasion and metastasis, and correlates with high tumour grade, aggressive and invasive inflammatory tumours, and lymph node metastasis (Barbieri *et al.,* 2010; Pensa *et al.,* 2012). STAT3 is constitutively activated in a subset of breast tumours, or is activated by IL-6 as part of the inflammatory response in many others, indicating a key role of Cten in breast cancer progression and metastasis (Barbieri *et al.,* 2010; Pensa *et al.,* 2012). It has been previously reported that increased integrin-linked kinase (ILK)-induced IL-6 expression leads to increased activity of STAT3 in melanomas, resulting in VEGF up-regulation and enhanced angiogenesis (Wani *et al.,* 2011). It is possible that Cten represents yet another target of this pathway in melanomas, a theory that should be investigated in more detailed.

Intriguingly, in colorectal cancer, Cten lies upstream of, and signals through ILK, thereby promoting cell migration and metastasis (Albasri *et al.,* 2011a). If this is the case also in melanomas, and Cten is in fact downstream of STAT3, this would imply a potential signalling loop. It is noteworthy that these observations were made in different tumour types, and could simply represent tissue-specific characteristics of Cten signalling. Accordingly, in a recent study in pancreatic cancer, no significant effect on ILK by Cten was observed (Al-Ghamdi *et al.,* 2013). The same study instead identified E-Cadherin, an adhesion

molecule implicated in cell motility, as a downstream target of Cten. This has been previously described in colorectal cancer, where Cten induction was linked to post-transcriptional repression of E-Cadherin, and to a subsequent stimulation of cell motility. Exactly how Cten and E-Cadherin interact is, however, currently unclear (Albasri *et al.,* 2009; Al-Ghamdi *et al.,* 2013)

Furthermore, in colorectal cancers, high Cten expression positively correlates with the presence of KRAS/BRAF mutations, and this association has been confirmed to be functionally relevant in colon and pancreatic cancers, in which Cten was identified as a downstream target of KRAS signalling, playing a role in cell motility (Al-Ghamdi *et al.,* 2011). However, unlike in colorectal cancer where KRAS mutations have been described in up to 42% tumours, KRAS mutations in melanoma are rare, with NRAS mutations being far more commonly observed (Demunter *et al.,* 2001; Brose *et al.,* 2002; Yuen *et al.,* 2002; Karapetis *et al.,* 2008). As discussed in Chapter 1.2.3, BRAF mutations are conversely common features of melanoma (Davies *et al.,* 2002).

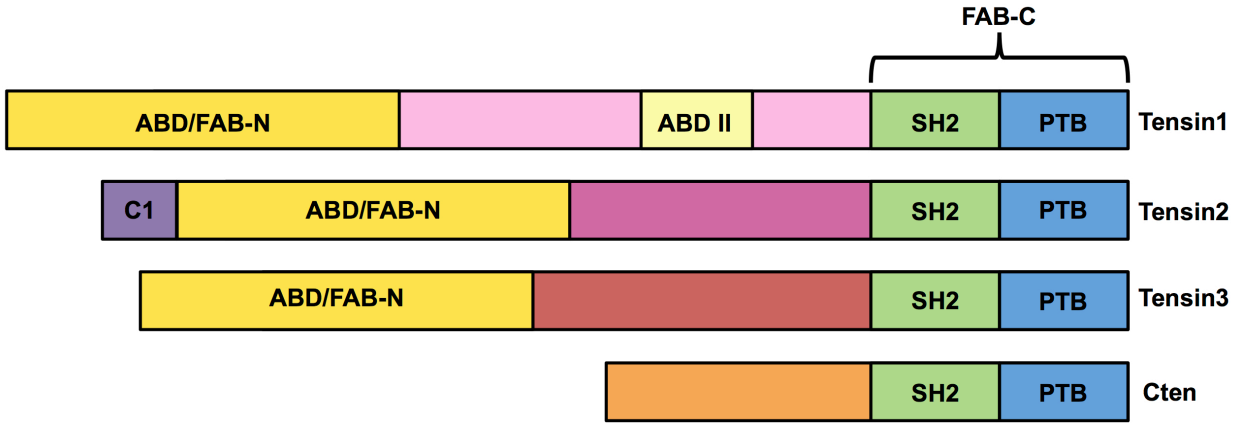
One of the most informative studies on Cten regulation to date was published earlier this year, and investigated the effects of several cancer-associated growth factors and cytokines on Cten expression (Hung *et al.,* 2013). The authors not only confirmed previous reports that IL-6 induces Cten expression (Barbieri *et al.,* 2010; Pensa et al., 2012), but also identified several new upstream activators of Cten gene transcription, including FGF2, IL-13, IGF-1 NGF, PDGF and TGF-β (Hung *et al.,* 2013). FGF2 is of particular interest since fibroblast growth factors are known to activate the MAPK pathway in melanoma

(Gray-Schopfer *et al.,* 2007), and accordingly, in their study, Cten up-regulation was found to be dependent mainly on the MAPK and PI3K/Akt pathways (Hung *et al.,* 2013).

Lastly, a study by Katz *et al.* (2007) found that Cten was highly up-regulated after treatment with EGF in MCF10A cells, and that this was followed by a corresponding down-regulation of tensin3, leading to decreased Actin-binding potential and increased cell migration (Katz *et al.,* 2007; Pylayeva & Giancotti, 2007). These results were later confirmed by Hung *et al.* (2013) and by Cao *et al.* (2012), who further showed that the EGF-driven up-regulation of Cten in MCF10A cells resulted in auto-inhibition of the RhoGAP activity of the known tumour suppressor DLC1.

Taken together, these results not only emphasize just how complex Cten signalling is, and how little we actually know about it, but also indicate that once Cten, and its role in cancer, has been better characterized, it has the potential to provide an attractive therapeutic target for treatment of a number of human cancers.

Figure 1.2. The basic structures of the tensin family member proteins. All tensin family members contain C-terminal Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains capable of binding phosphorylated tyrosine residues, and this region also contains focal adhesion-binding sites (FAB-C), responsible for localizing the tensins to the focal adhesions. Tensin1-3 contain N-terminal actin-binding domains (ABDs), responsible for binding to F-Actin, which also encompass additional focal adhesion binding sites (FAB-N). Tensin1 has a second actin-binding domain (ABD II) in the centre region capable of binding to the barbed end of F-acting, and tensin2 contains a C1 (protein kinase C conserved region 1) domain. The centre regions show very little sequence homology. Figure adapted from Lo (2004).



1.4 Deleted in Liver Cancer-1

1.4.1 DLC1 in human cancers

The only protein known to *physically* interact with Cten is Deleted in Liver Cancer-1 (DLC1; also known as ARHGAP7 and STARD12). DLC1 is a tumour suppressor protein that has been comprehensively researched and characterized in human cancers as an ubiquitously expressed protein lost or down-regulated in approximately 50% of all primary hepatocellular carcinomas (Yuan *et al.,* 1998; Liao & Lo, 2008). It was originally mapped to chromosome 8p21.3-22, a region frequently deleted in many solid tumours, including liver cancer (Yuan *et al.,* 1998).

Since its discovery in 1998, DLC1 has been found to be down-regulated in numerous other solid and haematological cancers including breast, renal, lung, nasopharyngeal, oesophageal, cervical, prostate, colorectal, oral squamous cell, urothelial, and gastric carcinomas, as well as in multiple myelomas and lymphomas (Kim *et al.,* 2003; Plaumann *et al.,* 2003; Yuan *et al.,* 2003**;** Yuan *et al.,* 2004; Guan *et al.,* 2006; Seng *et al.*, 2007; Ying *et al.,* 2007; Ullmannova-Benson *et al.,* 2009; Zhang *et al.,* 2009; Feng et al., 2011; Tripathi *et al.,* 2012; Chen *et al.,* 2013; Peng *et al.,* 2013). These studies, together with recent *in vitro* and *in vivo* studies, have confirmed the role of DLC1 as a *bona fide* tumour suppressor in human cancer (Ng *et al.,* 2000; Zhou *et al.,* 2004; Goodison *et al.,* 2005; Xue *et al.,* 2008).

In addition to genomic deletions, promoter hypermethylation of DLC1 has emerged as the main mechanism through which DLC1 is silenced in human

cancers (Yuan *et al.,* 1998; Kim *et al.,* 2003; Wong *et al.,* 2003, Yuan *et al.,* 2004; Seng *et al.,* 2006; Song *et al.,* 2006; Durkin *et al.,* 2007; Zhang *et al*., 2007; Peng *et al.,* 2013). Other important, but less commonly reported, epigenetic mechanisms of DLC1 silencing include histone deacetylation (Guan *et al.,* 2006; Durkin *et al.,* 2007) and serine phosphorylation (Ko *et al.,* 2010). Interestingly, in liver cancer cell lines, DLC1 has been found to be a target of Akt isoforms, resulting in phosphorylation of DLC1 at S567 (Ko *et al.,* 2010).

DLC1 is a Rho-GTPase activating protein (RhoGAP), responsible for catalyzing the hydrolysis of GTP bound to either RhoA, RhoB, RhoC or Cdc42, to GDP, thus rendering the Rho-GTPase inactive, leading to uncoupling of stress fibres and focal adhesions, and cell rest (Wong *et al.,* 2003; Durkin *et al.,* 2007; Healy *et al.,* 2008; Lahoz & Hall, 2008; Liao *et al.,* 2008; Cao *et al.,* 2012). In addition to containing a RhoGAP domain, responsible for the hydrolysis of GTP to GDP, DLC1 also contains a steroidogenic acute regulatory (StAR)-related lipid transfer (START) domain, a sterile alpha motif (SAM) domain, and a focal adhesion-targeting (FAT) domain (Fig. 1.3; Durkin *et al.,* 2007; Liao & Lo, 2008). The FAT domain is of particular interest, since it has been shown to enhance the tumour suppressor activities of DLC1 (Liao *et al*., 2007**;** Liao *et al*., 2008), and it has been established that DLC1 is recruited to the focal adhesions via interactions between its FAT domain and the SH2 domains of the tensin proteins (Liao *et al.,* 2007; Liao & Lo, 2008; Cao *et al.,* 2012).

1.4.2 Cten-DLC1 interactions

As mentioned, DLC1 can interact with the tensins via its FAT domain and the SH2 domains of the tensins in a phosphotyrosine-independent manner, leading to focal adhesion-localization of DLC1. It has furthermore also been shown to interact with the PTB domain of the tensins, but this interaction is less well-characterized (Durkin *et al.,* 2007; Liao *et al.,* 2007; Qian *et al.,* 2007; Liao & Lo, 2008; Chan *et al.,* 2009; Kim *et al.,* 2009; Lukasik *et al*., 2011; Cao *et al.,* 2012). Binding to one of the full-length tensins has been shown to increase the tumour suppressor activities of DLC1 in a number of cell types, and mutations in the DLC1 FAT domain have accordingly been associated with decreased RhoGAP-activity and increased tumorigenesis (Durkin *et al*., 2007; Qian *et al.,* 2007; Liao & Lo, 2008; Chan *et al.,* 2009; Kim *et al.,* 2009). In prostate cancer, where Cten has been proposed to function as a tumour suppressor protein, it has been hypothesized that the ability of Cten to bind DLC1 and recruit it to the focal adhesions plays an essential role in the tumour suppressor activities of Cten (Liao *et al.,* 2007).

In other types of cells however, *in vitro* studies have found that DLC1 activity is differentially regulated by the tensins. Binding of DLC1 to one of the full-length tensins (such as tensin3 in MCF10A cells, but likely tensin1 in melanomas; discussed in Chapter 4) leads to enhanced DLC1 RhoGAP activities, while DLC1-Cten binding conversely attenuates the tumour suppressor activities of DLC1 (Cao *et al.,* 2012). The proposed mechanism of Cten regulation of DLC1 is shown in Figure 1.4. In short; under resting conditions,

there is a relative abundance of the full-length tensins compared to Cten, but upon stimulation by EGF or other growth factors such as FGF, Cten expression is induced via stimulation of the MAPK pathway and ERK1 and ERK2 up-regulation, resulting in a relative abundance of Cten compared to tensin. When DLC1 is bound to tensin, its SAM domain is furthermore bound to the N-terminal ABD of tensin, leaving its RhoGAP domain free and available to interact with RhoA/B/C or Cdc42. But when DLC1 is bound to Cten instead, which lacks the N-terminal ABD of tensin, this leads to the SAM domain of DLC1 interacting with, and causing auto-inhibition of, its RhoGAP domain, subsequently leading to increased ROCK-mediated formation of stress fibres and focal adhesions, and enhanced cell migration (Cao *et al.,* 2012).

Figure 1.3. Deleted in Liver Cancer-1 structure. DLC1 contains an N-terminal sterile alpha motif (SAM) domain responsible for protein-protein interactions, including binding to the actin-binding domains (ABDs) of tensin1-3. The centre region contains a focal adhesion-targeting (FAT) region required for focal adhesion localization, and a RhoGAP domain responsible for catalyzing the hydrolysis of GTP bound to RhoA, RhoB, RhoC, and Cdc42. The C-terminal contains a steroidogenic acute regulatory (StAR)-related lipid transfer (START) domain. START domains are generally believed to bind lipids, but the role of the DLC1 START domain has not been fully characterized. Figure adapted from Liao & Lo (2008).

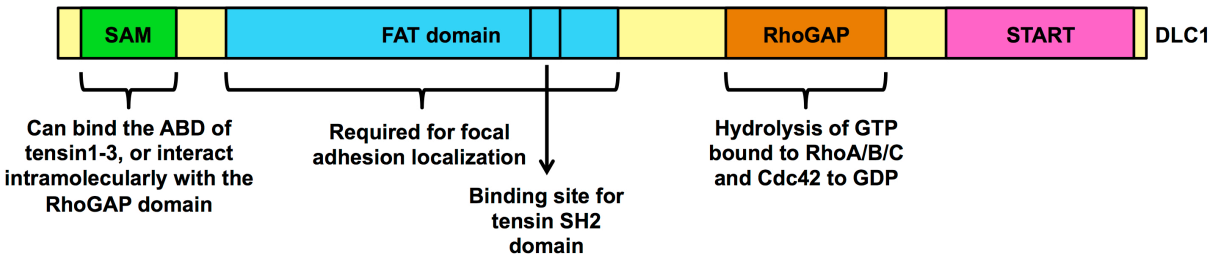
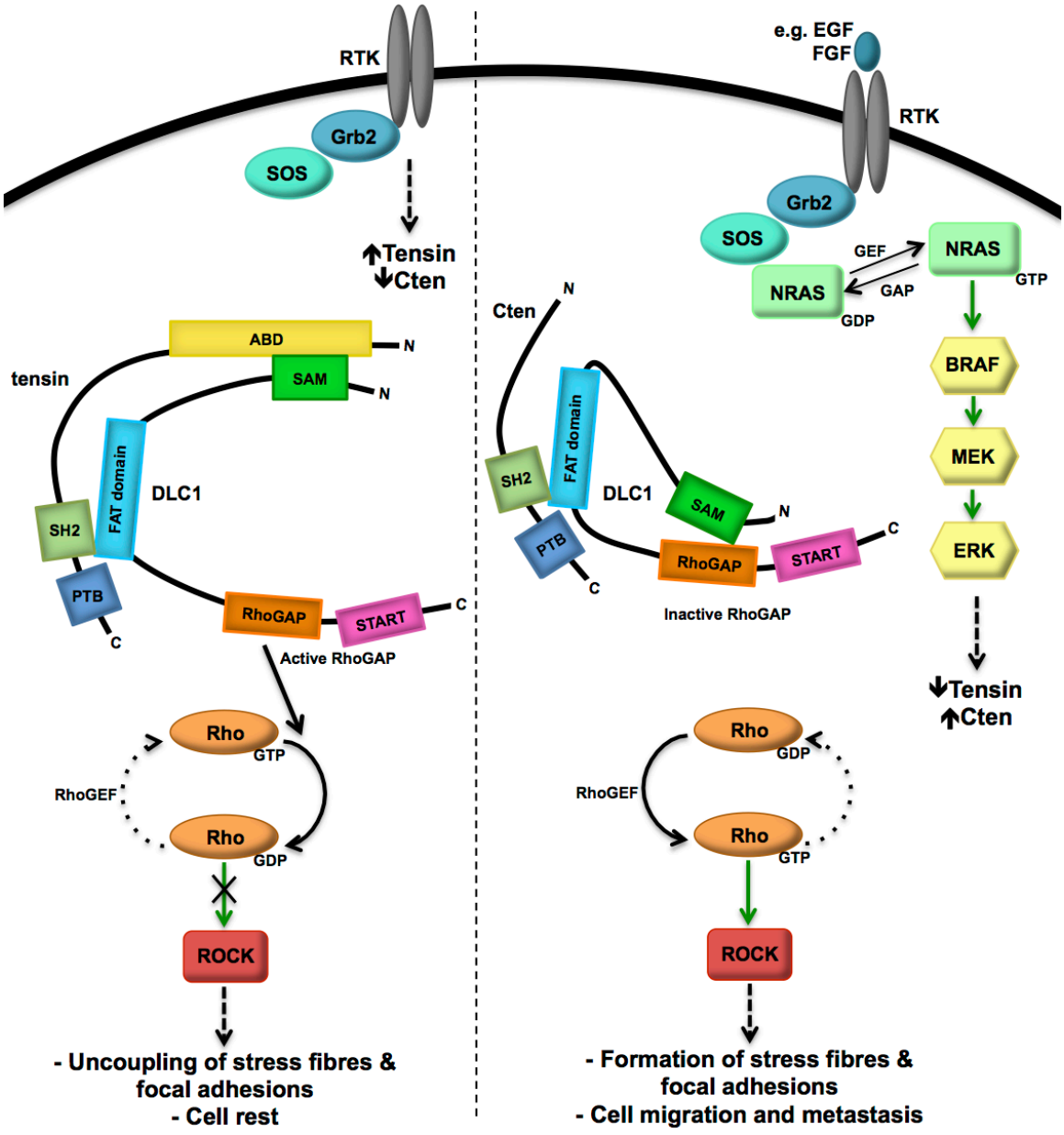


Figure 1.4. Proposed mechanism of Cten-mediated regulation of DLC1 activity. Under resting conditions, there is a relative abundance of the full-length tensins compared to Cten. Binding of the tensin ABD to the DLC1 SAM domain stops the SAM domain from binding to the RhoGAP domain of DLC1 and inhibit its activity. This results in an active RhoGAP and in hydrolysis of Rho-bound GTP to GDP. Upon stimulation by growth factors such as EGF or FGF, there is a relative abundance of Cten compared to tensin. This is hypothesized to be mediated partly via the MAPK pathway. Since Cten lacks the ABD of tensin1-3, when Cten binds DLC1 there is no release of the auto-inhibition of the DLC1 RhoGAP activity by the DLC1 SAM domain. This results in sustained activation of RhoA/B/C and Cdc42, and enhanced ROCK-mediated formation of stress fibres and focal adhesions, and ultimately to cell migration and metastasis. Figure adapted from Cao *et al.* (2012).


RTK
Grb2
SOS
↑Tensin
↓Cten
tensin
ABD
SAM
N
N
SH2
FAT domain
DLC1
PTB
C
RhoGAP
START
C
Active RhoGAP
Rho
GTP
RhoGEF
Rho
GDP
ROCK
- Uncoupling of stress fibres &
focal adhesions
- Cell rest
e.g. EGF
FGF
RTK
Grb2
SOS
GEF
NRAS
GTP
NRAS
GAP
GDP
BRAF
MEK
ERK
↓Tensin
↑Cten
Cten
N
SH2
FAT domain
DLC1
SAM
N
PTB
C
RhoGAP
START
C
Inactive RhoGAP
Rho
GDP
RhoGEF
Rho
GTP
ROCK
- Formation of stress fibres &
focal adhesions
- Cell migration and metastasis

1.5 Objectives and hypotheses

The main objectives of this study were to evaluate the expression status and prognostic significance of C-terminal tensin-like protein (Cten) in melanoma, and to perform a preliminary investigation into its role in melanomagenesis. Initial real-time quantitative PCR and Western blotting results indicated that Cten was overexpressed at both the mRNA and protein levels in melanoma cell lines compared to normal melanocytes. Based on this, **I hypothesized that Cten expression would be increased during melanoma progression when examined in a large number of melanoma patient samples, and that transient knockdown of Cten would result in reduced tumorigenicity *in vitro*.**

Since it is largely unknown how Cten functions, I also examined the expression status of the only protein known to physically interact with Cten; the *bona fide* tumour suppressor Deleted in Liver Cancer-1 (DLC1), in melanoma. **I hypothesized, in accordance with the numerous studies that have reported DLC1 down-regulation in cancer, that DLC1 expression would be decreased in the progression of melanoma, and that any effects of Cten on patient survival would be at least partly dependent on DLC1 expression**.

2. Materials and methods

2.1 TMA construction

For the tissue microarray (TMA) study, 748 formalin-fixed, paraffin-embedded tissues were obtained from Vancouver General Hospital, Department of Pathology, between 1992 and 2009 in accordance with the Declaration of Helsinki guidelines, as approved by the Clinical Research Ethics Board of the University of British Columbia, (Vancouver, Canada). Tissues with insufficient tumour cells or lost cores were excluded from the studies. The TMAs were assembled using a tissue-array instrument (Beecher Instruments, Silver Spring, MD), and duplicate 0.6 mm thick tissue cores were taken from each biopsied tissue. Multiple 4 μm sections were cut using a Leica microtome (Leica Microsystems Inc., Bannockburn, IL), and transferred to adhesive-coated slides using standard procedures. From each TMA set, one section was stained with haematoxylin and eosin as per standard protocol, and the remaining sections were reserved for immunohistochemical staining.

2.2 Immunohistochemistry of TMAs

The TMA slides were deparaffinized by heating at 55°C for 20 minutes followed by three 5-minute washes with xylene, and rehydrated by consecutive 5-minute washes in 100%, 95% and 80% ethanol, and twice in distilled water. Antigen retrieval was accomplished by heating the samples at 95°C in 10 mM sodium citrate at pH 6.0 for 30 minutes. Endogenous peroxidase activity was blocked by

incubation of the slides in 3% hydrogen peroxide for 30 minutes. The tissues were blocked with Dako antibody diluent (Dako Diagnostics, Glostrup, Denmark) for 30 minutes to prevent non-specific binding, followed by incubation with the primary antibody overnight at 4°C. Incubation in the antibody diluent without the primary antibody served as the negative control. Next, the samples were incubated with a universal biotinylated secondary antibody followed by streptavidin-HRP (Dako Diagnostics, Glostrup, Denmark) for 30 minutes each, and developed using 3,3'-diaminobenzidine substrate (Vector Laboratories, Burlington, ON, Canada). Haematoxylin counterstaining was performed to visualize the nuclei, and the slides were immediately washed twice in distilled water for 5 minutes, and then dipped in 2% sodium bicarbonate for 1 minute. Dehydration of the slides was accomplished by incubation in 80%, 95% and 100% ethanol for 5 minutes each, followed by three washes in xylene, and lastly the slides were sealed with cover slips.

2.3 Evaluation of immunostaining

Protein staining was evaluated and given an immunoreactivity score (IRS) based on the intensity of staining (0-3) and the percentage of antigen-positive cells (1 (0-25%); 2 (26-50%); 3 (51-75%); or 4 (76-100%)) by two independent observers. The IRS was calculated by multiplying the intensity score and the percentage of staining, and was identified as: negative (0); weak (1-3); moderate (4-6) and; strong (8-12). In the event of two duplicate cores having different staining, the higher score of the two was used for statistical analysis. Both Cten

and DLC1 expression were defined as having either negative to moderate (neg-mod; 0-6) or strong (8-12) expression, based on the level of staining.

2.4 Statistical analysis of TMAs

Differences in demographic and clinicopathological characteristics, and Cten and DLC1 expression between subgroups were evaluated by χ^2 tests (degrees of freedom (df) = 1, unless otherwise stated). Kaplan-Meier and log-rank tests were used to evaluate the correlation between Cten and DLC1 expression and overall and disease-specific 5- and 10-year survival outcomes for melanoma patients. Univariate and multivariate Cox regression analyses were used to determine the crude and adjusted hazard ratios, respectively, and their 95% confidence intervals. For all tests, a p-value < 0.05 was considered significant. SPSS versions 16.0 and 20.0 software (SPSS Inc., Chicago, IL, USA) was used for all analyses.

2.5 Cell lines and cell culture

All melanoma cell lines (MEWO, MMAN, MMRU, PMWK, RPEP, SK110 and SK3) were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml Amphotericin B (Invitrogen, Burlington, ON, Canada). Immortalized human melanocytes were maintained in melanocyte growth media supplemented with 5 µg/ml bovine pituitary extract, 1 ng/ml basic fibroblast growth factor, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 10 ng/ml

phorbol myristate acetate and 4% FBS (PromoCell, Heidelberg, Germany). All cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

2.6. siRNA transfection

MMAN and MMRU cells were grown to 40-70% confluency prior to siRNA transfection. Cten siRNA (SI04144350) and non-specific scrambled control siRNA (Qiagen, Mississauga, ON, Canada) were transfected using siLentFect Lipid Reagent (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions. To confirm the knockdown efficiency, cells were collected 48 hours after transfection for RNA and protein extraction.

2.7 RNA extraction

Directly upon removal of all media, cells were collected in 1 mL QIAzol lysis reagent (Qiagen, Mississauga, ON, Canada). Tubes containing the collected cells were left in room temperature for 5 minutes to promote dissociation of nucleoprotein complexes. Chloroform (200 µl) was added to each tube and the tubes were shaken vigorously for 15 seconds, incubated at room temperature for 3 minutes and centrifuged at 12,000 x g for 15 minutes in 4°C. After centrifugation, the samples separated into three distinct layers, and the colourless upper layers containing the RNA were transferred to new tubes. Isopropanol (500 µl) was added to each tube and mixed by vortexing, and after 10 minutes incubation at room temperature, the tubes were again centrifuged at 12,000 x g for 10 minutes in 4°C, and the supernatant was discarded. One (1) ml

of 70% ethanol was added to each tube and the tubes were centrifuged at 7,500 x g for 5 minutes in in 4°C. Lastly, the supernatant was aspirated, and the remaining RNA was re-dissolved in RNase-free water. The concentration of RNA was measured using a plate reader and Gen5 software version 2.01 (BioTek, Winooski, VT, USA).

2.8 Reverse transcriptase quantitative (RT-q)PCR

Reverse transcriptase (RT)-PCR was carried out according to the manufacturer's instructions (Roche Diagnostics, Laval, QC, Canada), using an anchored-oligo$(dT)_{18}$ primer, and an incubation time of 30 minutes at 55°C followed by 5 minutes at 85°C and immediate cooling at 4°C.

SYBR PCR Master Mix with the StepOne Software version 2.2.2 (Life Technologies, Burlington, ON, Canada) was used for quantitative (real-time) PCR analysis, together with the following primers: Cten, 5'-AGAGAACTGGGAGGTGCAGA-3' (forward), 5'-AGTCAGAGTGATGCCCTGCT-3' (reverse); DLC1, 5'-GGATGGATGAGGAGAAGCTGAA-3' (forward), 5'-GGTCTGCGTGGAGTTGGAAA-3' (reverse); TNS1, 5'-GGCTTAGAGCGAGAGAAGCA-3' (forward), 5'-CCCGTCCAGAGAAGAGAGTG-3' (reverse); TNS2, 5'-GAATGAACAGCAGCCCTCTC-3' (forward), 5'-TACCATGACATCGCCTTTGA-3' (reverse); TNS3, 5'-GGACGCATAGGAGTGGTCAT-3' (forward), 5'-GGGAGAGGCATTCATTTTCA-3' (reverse).

2.9 Protein extraction and Bradford assay

Upon removal of all media, the cells were washed in PBS twice followed by collection of the cells in 1 ml of PBS. Whole cell proteins were extracted by triple detergent buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulphate, 1% IGEPAL CA-630, 0.5% sodium deoxycholate) with protease inhibitors (Roche Diagnostics, Laval, QC, Canada) added at the time of extraction. Next, the samples were sonicated and kept on ice for 10 minutes, followed by centrifugation at 12,000x g. The supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad, Missisauga, ON, Canada).

For each sample, triplicates each containing 1 µl of protein extract added to 9 µl of distilled water and 190 µl of Bio-Rad protein assay dye reagent (diluted 5x; Bio-Rad Laboratories Inc., Hercules, CA, USA) were pipetted into a 96-well plate and quantified at 595 nm absorbance. Triplicates of 0, 1, 2, 3, 4 and 5 µl of 1 µg/µl bovine serum albumin (BSA) in 190 µl of Bio-Rad protein assay dye reagent and distilled water to make up 200 µl were used as standards to determine the protein concentration of the samples.

2.10 Cell fractionation

Cells were washed in PBS twice followed by collection of the cells in 1 ml of PBS, pelleted and aspirated. Next, the cells were re-suspended in 4 volumes of cell lysis buffer ((50 mM Tris, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.5% IGEPAL CA-630) with 1 mM DTT, 1 mM PMSF and 1x protease inhibitor added fresh) for 5

minutes in 4°C followed by centrifugation for 5 s at 15,000 x g. The supernatant containing the cytoplasmic extract was removed and transferred to new tubes.

The remaining pellets were rinsed once in the cell lysis buffer and briefly centrifuged to obtain nuclear pellets. Nuclear proteins were solubilized by suspension of the pellet in 4 volumes of nuclear extract buffer ((20 mM Hepes, pH 7.9, 0.35 M NaCl) with 1x protease inhibitor added fresh), sonication (4 strokes of 1 second each), incubation for 30 minutes at 4°C, and centrifugation at 14,000 x g for 15 minutes in 4°C. Bradford assay (described in Chapter 2.9) was performed to determine the protein concentrations.

2.11 Antibodies

Primary monoclonal mouse anti-Cten antibody was purchased from R&D systems (Minneapolis, MN, USA). For immunohistochemistry, a dilution of 1:50 was used, and for immunoblotting, a dilution of 1:500. Primary monoclonal mouse anti-DLC1 antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and used at a dilution of 1:50 for immunohistochemistry, and at 1:100 for Western blotting. Mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) was diluted to 1:5000 for Western blotting, and the secondary anti-mouse antibody (Sigma-Aldrich, St. Louis, MO, USA) was used at a 1:10,000 dilution.

2.12 Western blot analysis

The protein extracts (40 µg) were separated on SDS-polyacrylamide gels and

blotted onto PVDF membranes (Bio-Rad, Mississauga, ON, Canada) at 90 mA overnight in 4°C. The PVDF membranes were blocked with 5% BSA in TBST for 1 hour at room temperature before incubation with the primary antibodies (prepared in 5% BSA in TBST) overnight at 4°C. The next day, the membranes were washed in PBST three times for 5 minutes and incubated with the secondary antibodies for one hour in room temperate. The blots were washed another 3 times in PBST and visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

2.13 SRB cell proliferation assay

Cells were transfected with Cten or scrambled control siRNA as described in Chapter 2.6. The day after transfection, the cells were washed twice in autoclaved PBS, and collected by trypsinization. The cells were counted using a haemocytometer, and 50,000 cells/well were transferred to 1 mL of fresh media in 4 x 12-well plates (in triplicates). The first plate of cells was fixed with 10% trichloroacetic acid (TCA) after 6 hours (Time = 0), and the remaining three plates were fixed 24, 48 and 72 hours later and stored in 4°C. After the last plate had been kept for a minimum of 1 hour in 4°C, the plates were washed with tap water to remove any residual TCA. Next, the cells were air-dried completely and stained with 0.057% sulphorhodamine B (SRB) for 15 minutes in room temperature. Plates were washed with 1% acetic acid five times to remove unbound dye, and left to air-dry. Lastly, the cells were dissolved in 10 mM Tris HCl at pH 10.5 for 20 minutes, and 100 µl triplicates *for each triplicate* were

transferred to a 96-well plate and quantified at absorbance 550 nm.

2.14 PI staining and FACS analysis

After siRNA transfection, 100,000 cells were transferred to each well of a 6-well plate. After 48 hours, the media was collected to collect all dead cells, followed by trypsinization to collect all living cells. The tubes containing the cells were centrifuged at 1500 rpm for 5 minutes and the pellets were re-suspended in 500 µl PBS, and 500 µl of cold 70% ethanol were slowly added to each tube while vortexing, and the tubes were stored in 4°C.

Thirty (30) minutes before Fluorescence-activated cell sorting (FACS) analysis (using the BD FACSdiva software; BD Biosciences, Mississauga, ON, Canada), the cells were centrifuged at 1800 rpm for 3 minutes and re-suspended in 20 µg/ml propidium iodide (PI) and 80 µg/ml RNase A in PBS supplemented with 0.1% Triton X-100 and 0.1 mM EDTA. All samples were analyzed three times, with 10,000 events being recorded each time.

2.15 Wound healing assay

Cells transfected with Cten and control siRNA were grown to 100% confluency, and wounds were made to each plate by pressing a P2 pipette tip against the bottom of the plate, removing the melanoma cell monolayer. The debris was removed by carefully washing the plates twice with autoclaved PBS. Fresh media was added to the plates and cell migration was recorded using a microscope.

After 24 and 48 hours the procedure was repeated. The number of migrated cells was recorded in 3 different microscopic fields, and a mean value was calculated.

2.16 Boyden chamber invasion assay

The upper compartment polycarbonate membranes (with 8.0 μm pores) of a 24-well Transwell culture chamber were coated with 20 μl of 5 mg/ml Matrigel (BD Biosciences, Mississauga, ON, Canada) in serum-free DMEM and were incubated at 37°C in 5% CO_2 for 1 hour. The lower compartment was filled with 750 μl of DMEM containing 10% FBS, and 50,000 cells seeded in 250 μl of serum-free medium were transferred to the upper compartment. After 24 hours, cells were fixed by 10% TCA and kept at 4°C for minimum 1 hour. After air-drying in room temperature, the membranes were stained with 0.5% crystal violet dye for 2 hours, and non-invading cells from the upper surfaces of the membranes were removed using cotton swabs. The filters were photographed, and the dye was extracted with 30% acetic acid and read at 590 nm.

2.17 Statistical analysis for *in vitro* studies

All *in vitro* experiments were performed in triplicates, and the data are presented as mean ± SD, unless otherwise stated. Student's t test (two-tailed) was used for the statistical analyses, and a p-value < 0.05 was considered significant.

2.18 Short bioinformatics study

Using the terms *Cten* or *TNS4*, I searched the NCBI gene

(http://www.ncbi.nlm.nih.gov/gene); Uniprot (http://www.uniprot.org); Catalogue of somatic mutations in cancer (COSMIC) (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/); and STRING functional protein association network version 9.05 (http://string-db.org) databases for information on the status of Cten in human cancers.

3. C-terminal tensin-like protein is a novel oncogene and prognostic marker for primary melanoma patients

3.1 Rationale

Malignant melanoma is a highly invasive malignancy, and due to its high metastatic potential, the median survival reported for patients diagnosed with distant metastases is only 6-8 months, with an overall 5-year survival rate as low as 5-16%. However, if found and removed at their early stages, melanomas are almost 100% curable (Cummins *et al.,* 2006; American cancer society, 2011; Wasif *et al.,* 2011), demonstrating the importance of early detection, diagnosis, and prognosis. When used in combination with traditional prognostic tools, proteins that are differentially expressed in nevi and tumour tissue could help create more reliable prognoses. However, despite numerous protein biomarkers having been identified for melanomas, none are currently routinely used clinically to improve risk stratification (Gould Rothberg *et al*., 2009), which indicates a need to identify dependable molecular prognostic factors for this disease.

C-terminal tensin-like protein (Cten) is a novel protein that has been reported to be overexpressed in a number of cancers, where it has also been implicated in tumorigenesis. Importantly, Cten expression is low or absent in most normal tissues (Lo & Lo, 2002), making it an ideal tumour marker. To my knowledge, no one has yet examined the expression of Cten in melanoma, and I hence decided to do so. Using immunohistochemical staining and tissue microarrays containing a large set of melanocytic lesions, I examined the

expression profile of Cten as a first step to elucidate its role in melanoma development.

3.2 Results

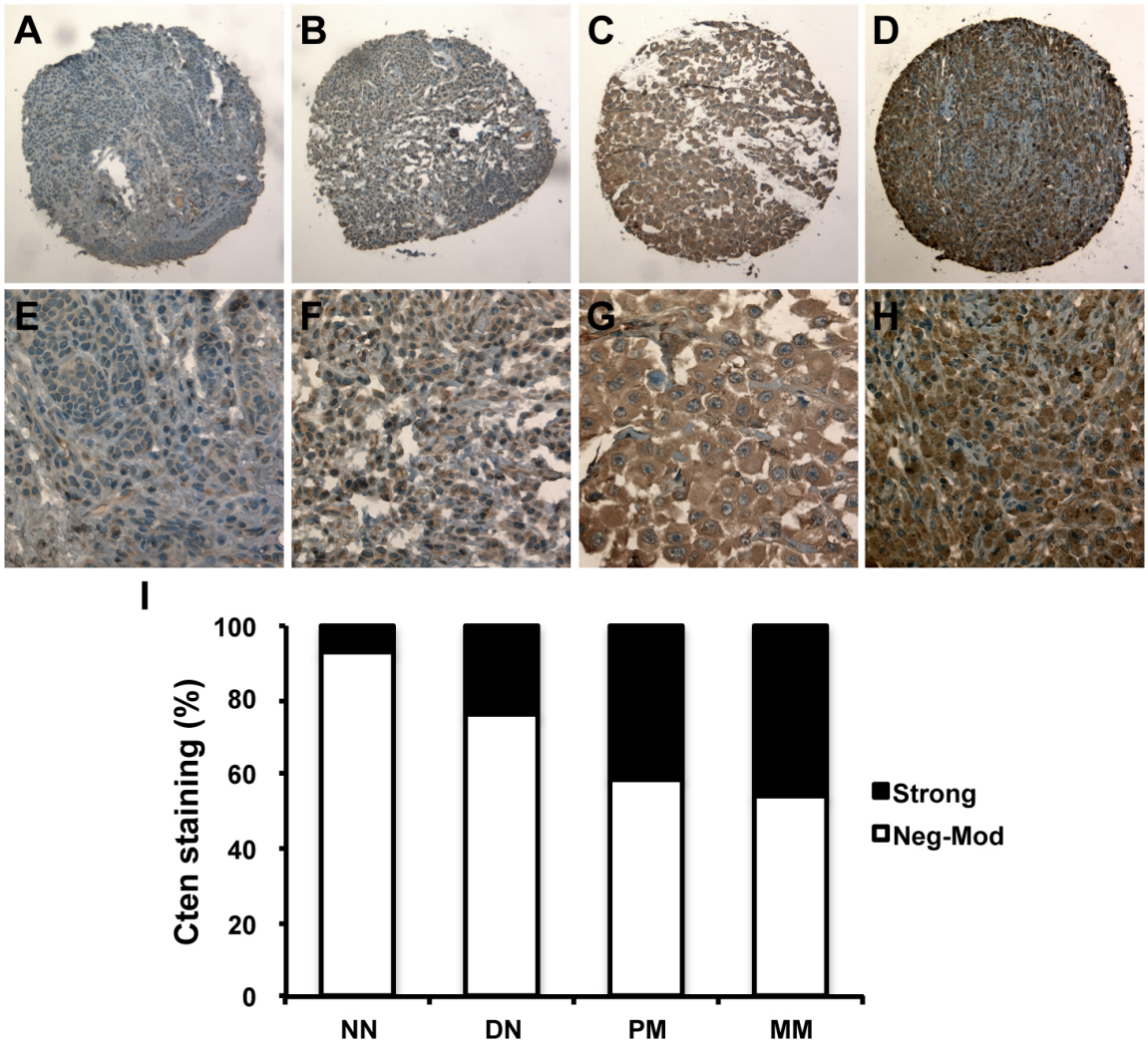
3.2.1 Clinicopathological features of TMAs

After exclusion of tissues with insufficient tumour cells or lost cores from the study, a total of 562 tissues were available for evaluation of Cten staining, including 29 normal nevi (NN), 88 dysplastic nevi (DN), 297 primary melanomas (PM) and 148 metastatic melanomas (MM). For the 445 melanoma cases, there were 263 males and 182 females, with a median age of 60 (ranging between 7 and 95 years). Of these, 170 tumours were classified as AJCC Stage I, 127 as Stage II, 62 as Stage III, and 83 as Stage IV. Three samples lacked information regarding AJCC stage. For primary melanoma, the tumours were sub-classified as acral lentiginous (n = 9), desmoplastic (n = 11), lentigo maligna (n = 63), nodular (n = 44), superficial spreading (n = 109) and other (unclassified, spitz-like, and nevoid, n = 61) melanomas. Of these, 83 tumours were biopsied from the head and neck, and 211 from sun-protected sites. Ulceration was present in 53 cases, 99 tumours were < 1.0 mm thick or *in situ*, 73 tumours were 1.0 - < 2.0 mm, 64 were 2.0 – 4.0 mm, and 61 were > 4.0 mm thick. For metastatic melanoma, 64 tumours were cutaneous, 55 were biopsied from lymph nodes, and 26 were obtained from visceral organs (Table 3.1).

3.2.2 Cten expression is increased in melanoma progression

As seen in Fig. 3.1A-H, Cten staining was found to be exclusively cytoplasmic. Strong Cten staining was observed in 7%, 24%, 41%, and 46% of normal nevi, dysplastic nevi, primary melanoma, and metastatic melanoma samples, respectively. Cten protein expression was found to be significantly higher in dysplastic nevi compared to normal nevi ($p = 0.046$, χ^2 test), in primary melanoma compared to dysplastic and normal nevi ($p = 0.003$ and < 0.001, respectively, χ^2 tests), and in metastatic melanoma compared to dysplastic and normal nevi ($p < 0.001$ for both, χ^2 tests), but there was no significant difference between metastatic and primary melanoma ($p = 0.328$, χ^2 test, Fig. 3.1I), indicating that Cten may be involved in the early stages of melanoma development, rather than in metastasis.

Figure 3.1. Representative images of Cten protein expression at 100x (A-D) and 400x (E-H) magnification. (A, E) Weak Cten staining in normal nevi (NN). (B, F) Moderate Cten staining in dysplastic nevi (DN). (C, G) Strong Cten staining in primary melanoma (PM). (D, H) Strong Cten staining in metastatic melanoma (MM). (I) Correlation between Cten expression and melanoma progression.



3.2.3 Correlation between Cten and clinicopathological features

Strong Cten expression was observed in 35% of AJCC Stage I melanomas compared to 47% of melanomas belonging to AJCC Stages II-IV ($p = 0.015$, χ^2 test). For primary melanoma, Cten staining was found to be significantly higher in tumours ≥ 1 mm thick (47% strong staining), compared to *in situ* and thin tumours < 1 mm (28% strong staining, $p = 0.002$, χ^2 test), but no correlation was seen for age, sex or ulceration status. Strong Cten expression was detected in 61% of nodular melanomas compared to only 37% of all other primary melanoma subtypes ($p = 0.003$, χ^2 test), and was also found to be significantly lower in other, mainly unclassified, melanomas ($p = 0.045$, χ^2 test). A significant difference in Cten expression was moreover observed between primary tumours found at sun-protected sites (47% strong staining) compared to tumours from the head and neck (27%, $p = 0.001$, χ^2 test). No correlations between Cten expression and tumour location or patient age and sex, for metastatic melanoma patients were detected (Table 3.1).

Table 3.1. Cten staining and clinicopathological characteristics of 445 melanoma patients

Variables	Cten staining Neg-Mod	Strong	Total	P-value[1]
Primary Melanoma (n=297)				
Age				
≤ 60	85 (58.2%)	61 (41.8%)	146	0.808
> 60	90 (59.6%)	61 (40.4%)	151	
Sex				
Male	101 (61.6%)	63 (38.4%)	164	0.300
Female	74 (55.6%)	59 (44.4%)	133	
Tumour thickness (mm)				
< 1.0	71 (71.7%)	28 (28.3%)	99	0.002[2]
1.0 - < 2.0	40 (54.8%)	33 (45.2%)	73	
2.0 - 4.0	36 (56.25%)	28 (43.75%)	64	
> 4.0	29 (47.5%)	32 (52.5%)	61	
Ulceration				
Present	30 (56.6%)	23 (43.4%)	53	0.705
Absent	145 (59.4%)	99 (40.6%)	244	
Tumour subtype				
Acral Lentiginous	6 (66.7%)	3 (33.3%)	9	
Desmoplastic	6 (54.5%)	5 (45.5%)	11	
Lentigo Maligna	44 (69.8%)	19 (30.2%)	63	
Nodular	17 (38.6%)	27 (61.4%)	44	0.003[3]
Superficial Spreading	60 (55.0%)	49 (45.0%)	109	
Other[4]	43 (70.5%)	18 (29.5%)	61	0.045[5]
Location[6]				
Sun-protected	112 (53.1%)	99 (46.9%)	211	0.001
Sun-exposed	61 (73.5%)	22 (26.5%)	83	
Metastatic Melanoma (n =148)				
Age				
≤ 60	47 (54.7%)	39 (45.3%)	86	0.865
> 60	33 (53.2%)	29 (46.8%)	62	
Sex				
Male	53 (53.5%)	46 (46.5%)	99	0.858
Female	27 (55.1%)	22 (44.9%)	49	
AJCC stage				
I	110 (64.7%)	60 (35.3%)	170	0.015[7]
II	65 (51.2%)	62 (48.8%)	127	
III	30 (48.4%)	32 (51.6%)	62	
IV	49 (59.0%)	34 (41.0%)	83	
Location				
Cutaneous	34 (53.1%)	30 (46.9%)	64	0.226[8]
Lymph nodal	27 (49.1%)	28 (50.9%)	55	
Visceral	18 (69.2%)	8 (30.8%)	26	

AJCC indicates American Joint Committee on Cancer. [1]χ^2 test, df = 1 unless otherwise stated. [2]Tumours < 1.0 mm vs. tumours ≥ 1mm thick. [3]Nodular melanoma vs. all other subtypes. [4]Other: Unspecified subtypes (n = 58), spitz-like melanoma (n = 1) and nevoid melanoma (n = 2). [5]Other melanomas vs. all other subtypes. [6]Sun-protected locations: back, trunk, arms, hands, legs, feet, and vulva; Sun-exposed sites: head and neck. Cases with unspecified location (n = 3) were excluded from analysis. [7]AJCC Stage I vs. Stages II-IV. Samples with unspecified AJCC stages (n = 3) were excluded from analysis. [8]Df = 2. Samples lacking information about the location of the metastatic deposit (n = 3) were excluded from the study.

3.2.4 Correlation between Cten and melanoma patient 5- and 10-year survival

A total of 418 melanoma patients (271 primary melanoma and 147 metastatic melanoma patients) had complete 5-year follow-up and clinical information. Survival time was calculated as time from diagnosis to last follow-up or death. Kaplan-Meier analyses revealed that there was no significant association between Cten expression and overall and disease-specific 5-year survival for all melanoma patients ($p = 0.079$ and 0.072, respectively, Fig. 3.2A). However, when separated into primary and metastatic melanoma patient groups, analyses showed that Cten expression was significantly associated with the overall ($p = 0.008$) and disease-specific ($p = 0.004$) 5-year survival for primary melanoma patients (Fig. 3.2B), but not for metastatic melanoma patients ($p = 0.434$ and 0.367 for overall and disease-specific survival, respectively, log-rank tests, Fig. 3.2C).

Furthermore, 335 patients (224 primary melanoma and 111 metastatic melanoma patients) also had complete 10-year follow-up and clinical information. Again, no association was seen between Cten expression and the 10-year overall and disease-specific survival of all melanoma patients ($p = 0.180$ and 0.217, respectively) or metastatic melanoma patients ($p = 0.063$ and 0.081, respectively, data not shown), but strong Cten expression was significantly associated with a worse 10-year overall and disease-specific survival for primary melanoma patients ($p = 0.030$ and 0.046, respectively, log-rank tests, Fig. 3.3).

Figure 3.2. Kaplan-Meier analyses for the correlations between Cten expression and 5-year survival in melanoma patients. (A) All melanoma patients (n = 418). (B) Primary melanoma patients (n = 271). (C) Metastatic melanoma patients (n = 147).

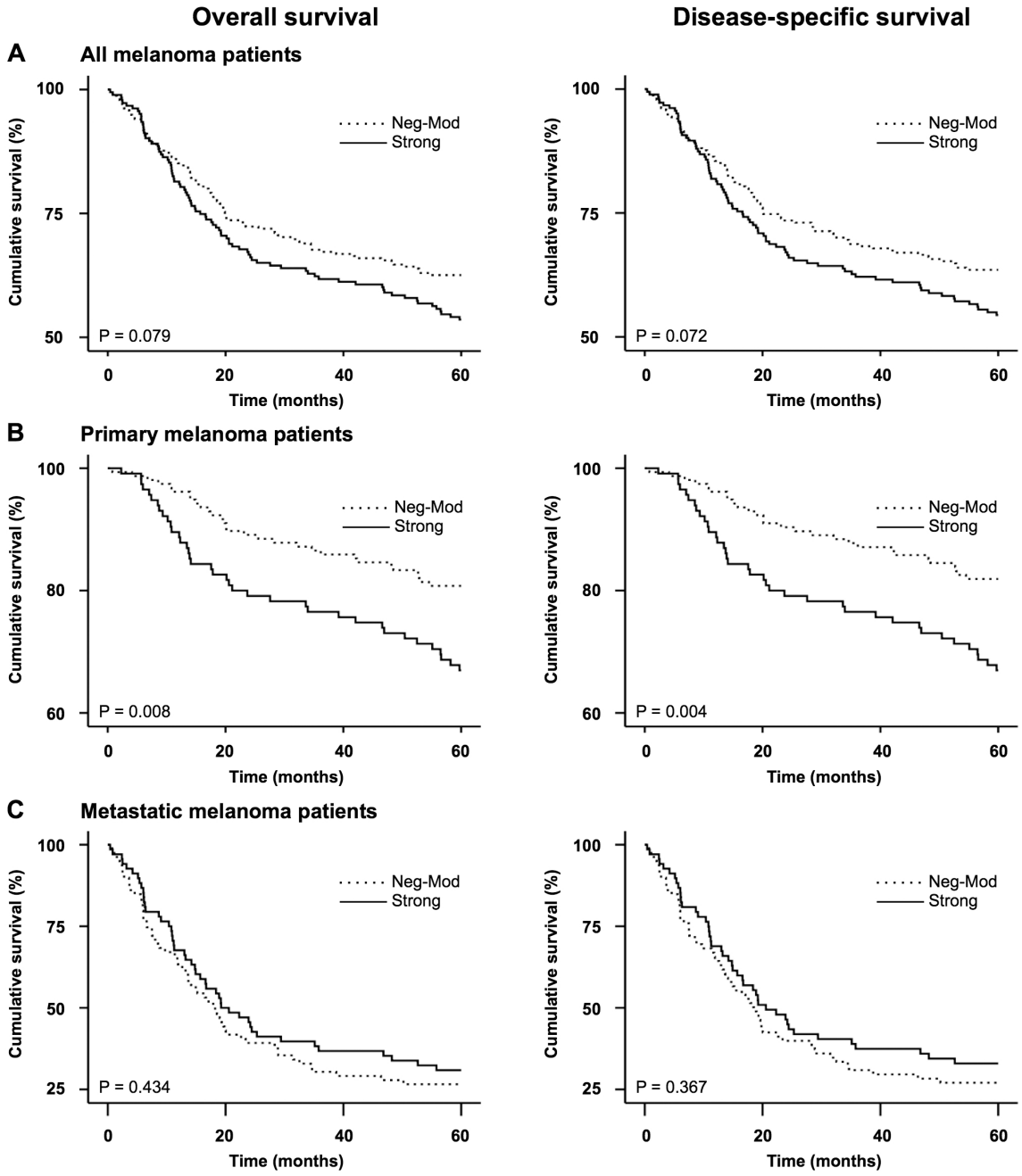
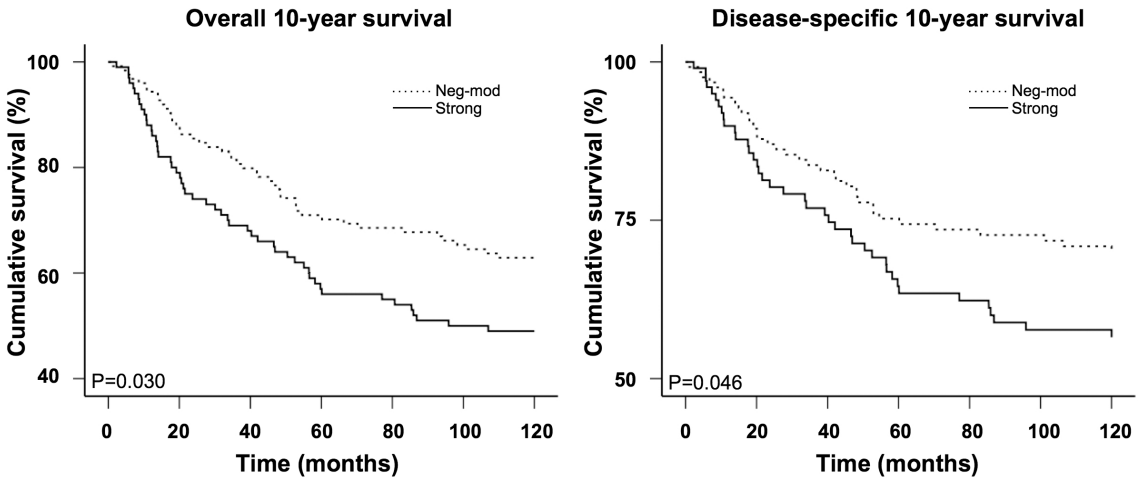


Figure 3.3. Kaplan-Meier analyses for the correlations between Cten expression and 10-year survival in 224 primary melanoma patients. Strong Cten expression was associated with a significantly poorer overall and disease-specific 10-year survival for primary melanoma patients (p = 0.030 and 0.046, respectively, log-rank tests).



3.2.5 Cten is an independent prognostic factor for primary melanoma patients

Univariate Cox proportional hazard regression analysis revealed that strong Cten expression in primary tumours was a significant adverse prognostic factor for those patients ((Hazard Ratio (HR), 1.89, 95% Confidence Interval (CI), 1.17-3.05, p = 0.009) for overall 5-year survival, and (HR, 2.03, 95% CI, 1.24-3.30, p = 0.005) for disease-specific survival; Table 3.2). Next, I examined whether Cten was also an independent prognostic marker for primary melanoma patient 5-year survival using multivariate Cox regression analysis. Sex, age, tumour thickness, ulceration status, tumour location, and Cten expression were included in the analysis, and the results showed that Cten expression was significantly associated with the overall (HR, 1.69, 95% CI, 1.03-2.77, p = 0.038) and disease-specific 5-year survival of primary melanoma patients (HR, 1.82, 95% CI, 1.10-3.01, p = 0.021; Table 3.3), and that Cten was an independent prognostic factor for the 5-year survival of these patients.

Table 3.2. Univariate Cox regression analysis on 5-year overall and disease-specific survival of 271 primary melanoma patients.

Variables	Patients	Overall survival				Disease-specific survival			
		Deaths	Death Rate	HR (95% CI)	p-value[1]	Deaths	Death Rate	HR (95% CI)	p-value[1]
Age									
≤ 60	134 (49.4%)	24	17.9%	1.00	0.007	22	16.4%	1.00	0.003
> 60	137 (50.6%)	44	32.1%	1.99 (1.21-3.27)		44	32.1%	2.17 (1.30-3.62)	
Sex									
Male	148 (54.6%)	36	24.3%	1.00	0.726	35	23.6%	1.00	0.743
Female	123 (45.4%)	32	26.0%	1.09 (0.68-1.75)		31	25.2%	1.08 (0.67-1.76)	
Thickness (mm)									
< 1.00	79 (29.2%)	7	8.9%	1.00	< 0.001	6	7.6%	1.00	< 0.001
≥ 1.00	192 (70.8%)	61	31.8%	4.23 (1.93-9.24)		60	31.3%	4.85 (2.10-11.23)	
Ulceration									
Absent	221 (81.5%)	39	17.6%	1.00	< 0.001	37	16.7%	1.00	< 0.001
Present	50 (18.5%)	29	58.0%	4.56 (2.81-7.38)		29	58.0%	4.81 (2.95-7.85)	
Location[2]									
Sun-protected	198 (73.9%)	51	25.8%	1.00	0.602	49	24.7%	1.00	0.703
Sun-exposed	70 (26.1%)	16	22.9%	0.86 (0.49-1.51)		16	22.9%	0.90 (0.51-1.58)	
Cten staining									
Neg-Mod	156 (57.6%)	30	21.4%	1.00	0.009	28	19.8%	1.00	0.005
Strong	115 (42.4%)	38	33.0%	1.89 (1.17-3.05)		38	33.0%	2.03 (1.24-3.30)	

[1] Log-rank test.
[2] Sun-protected locations: back, trunk, arms, hands, legs, feet, and vulva; Sun-exposed sites: head and neck. Cases with unspecified location (n = 3) were excluded from analysis.
Abbreviations: HR, hazard ratio; CI, confidence interval.

Table 3.3. Multivariate Cox regression analysis on 5-year overall and disease-specific survival of 271 primary melanoma patients.

Variables[1]	Overall survival					Disease-specific survival				
	β[2]	SE	HR	95.0% CI	P-value[3]	β[2]	SE	HR	95.0% CI	P-value[3]
Primary melanoma										
Sex	0.034	0.247	1.03	0.64-1.68	0.891	0.028	0.251	1.03	0.63-1.68	0.912
Age	0.380	0.265	1.46	0.87-2.46	0.152	0.447	0.272	1.56	0.92-2.67	0.100
Thickness	0.934	0.420	2.55	1.12-5.80	0.026	1.040	0.449	2.83	1.17-6.82	0.021
Ulceration	1.185	0.262	3.27	1.96-5.47	<0.001	1.212	0.265	3.36	2.00-5.64	<0.001
Location	0.042	0.299	1.04	0.580-1.88	0.889	0.096	0.301	1.10	0.61-1.99	0.749
Cten	0.524	0.253	1.69	1.03-2.77	0.038	0.596	0.258	1.82	1.10-3.01	0.021

[1]Coding of variables: Age was coded as 1 (≤ 60 years) and 2 (> 60 years); sex was coded as 1 (male) and 2 (female); tumour thickness was coded as 1 (< 1mm) and 2 (≥ 1mm); ulceration was coded as 1 (absent) and 2 (present); location was coded as 1 (sun-protected) and 2 (sun-exposed); Cten was coded as 1 (negative-moderate expression) and 2 (strong expression).
[2]β = regression coefficient.
[3]Log-rank test.
Abbreviations: SE, standard error; HR, hazard ratio; CI, confidence interval.

3.2.6 Cten knockdown results in decreased cell proliferation and increased apoptosis

To examine if Cten behaves as an oncogene *in vitro,* two cell lines – MMAN cells, which highly express Cten, and MMRU cells, which display relatively low levels of Cten mRNA and protein, were used for all experiments (Fig. 3.4). siRNA transfection resulted in a partial knockdown of Cten in both cell lines (Fig. 3.5A). SRB cell proliferation assays showed that Cten knockdown resulted in a significant reduction in cell proliferation after 24 ($p = 0.004$), 48 ($p < 0.001$) and 72 hours ($P < 0.001$) in MMAN cells, and after 48 ($p = 0.002$) and 72 hours ($p < 0.001$) in MMRU cells (Fig. 3.5B). FACS analysis after PI staining revealed that Cten knockdown in MMAN cells resulted in a significant increase in the sub-G1 population (6.6 ± 0.1% vs. 2.2 ± 0.2% for the control group, $p < 0.001$) and this was accompanied by a small, but statistically significant decrease in the S (9.4 ± 0.3% vs. 10.5 ± 0.8%, $p = 0.037$) and G2/M populations (22.7 ± 0.6% vs. 26.1 ± 1.0%, $p = 0.001$, Fig. 3.5C-D). Similarly, for MMRU cells, Cten knockdown resulted in a significant increase in the sub-G1 population (12.7 ± 6.0% vs. 2.7 ± 0.9% for the control group, $p = 0.047$), and a significant decrease in the G2/M population (10.6 ± 0.8% vs. 16.9 ± 3.6%, $p = 0.042$, Fig. 3.5C-D).

Figure 3.4. Cten expression in melanoma cell lines. (A) Western blot results. Predicted molecular mass of Cten, 72kDa; predicted molecular mass of β-Actin, 42kDa. (B) Real time quantitative PCR results, normalized to GAPDH. Both experiments were repeated three times with comparable results.

Abbreviations: MC, melanocyte.

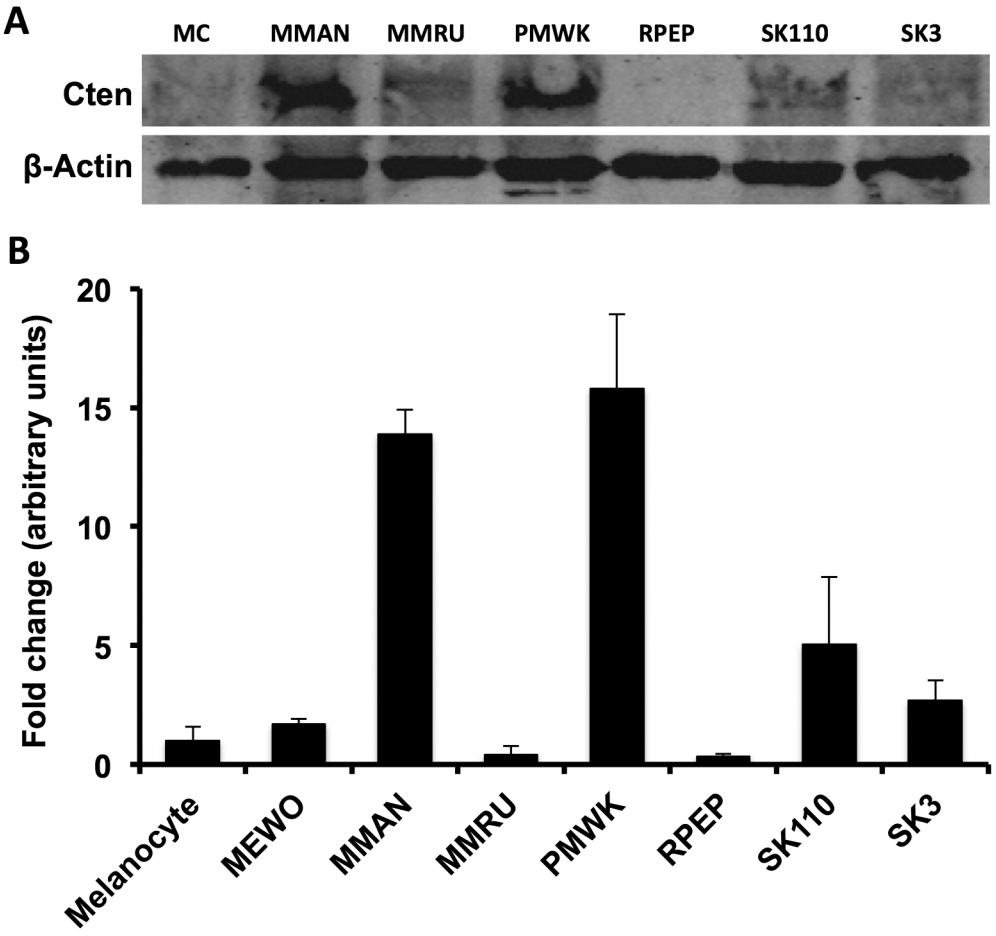
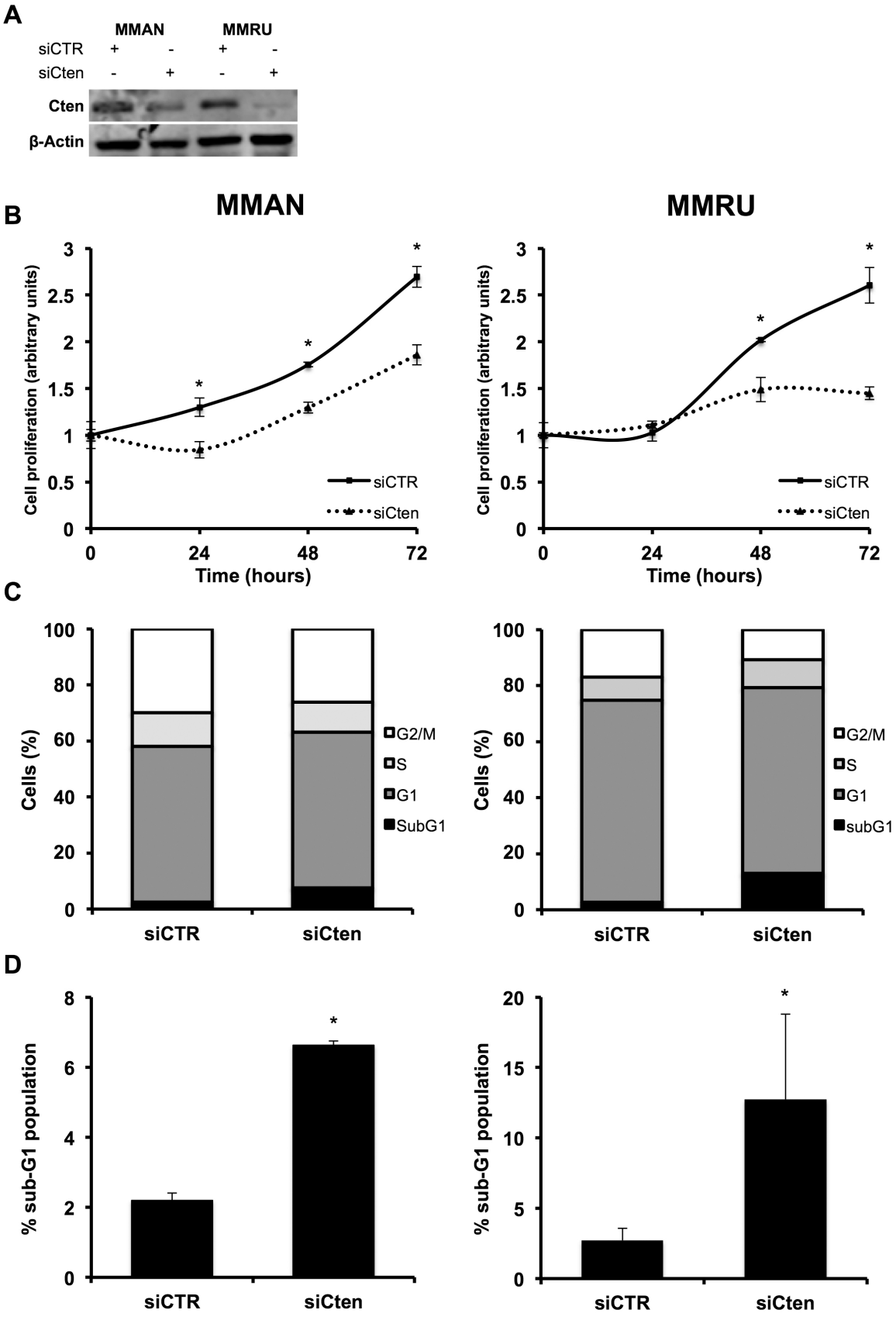


Figure 3.5 Cten knockdown results in decreased cell proliferation and increased apoptosis. (A) Western blot confirming Cten knockdown. Predicted molecular mass: Cten, 72 kDa, β-Actin, 42 kDa. (B) SRB cell proliferation assay. Cten knockdown resulted in a significant decrease in cell proliferation after 24 hours in MMAN cells and after 48 hours in MMRU cells. (C, D) FACS cell cycle analysis after propidium iodide staining. Cten knockdown resulted in a significant increase in the sub-G1 population in both cell lines. *$p < 0.05$.


A
MMAN
MMRU
siCTR + - + -
siCten - + - +
Cten
β-Actin
B
MMAN
MMRU
Cell proliferation (arbitrary units)
Time (hours)
siCTR
siCten
C
Cells (%)
G2/M
S
G1
SubG1
subG1
siCTR
siCten
D
% sub-G1 population
siCTR
siCten

3.2.7 Cten knockdown results in decreased cell invasion and a borderline decrease in cell migration

Figure 3.6 shows the results of Boyden chamber cell invasion assay in MMRU cells. After 24 hours of incubation, cells transfected with Cten siRNA displayed a significant reduction in cell invasion (54.4% relative invasion compared to the control cells, $p = 0.005$). MMAN cells have a very low invasive potential, and even after 72 hours, invasive cells were undetectable (results not shown).

The effects of Cten on cell migration were examined using wound-healing assays. After 48 hours, there was a slight, but significant decrease in cell migration after Cten knockdown, compared to cells treated with control siRNA in MMAN cells ($p = 0.040$, Fig. 3.7A), but no difference was detected in MMRU cells ($p = 0.299$, Fig. 3.7B).

Figure 3.6. The effects of Cten knockdown on melanoma cell invasion. Boyden chamber cell invasion assay in MMRU cells revealed that Cten knockdown was associated with a significant decrease in cell invasion after 24 hours. *p = 0.005.

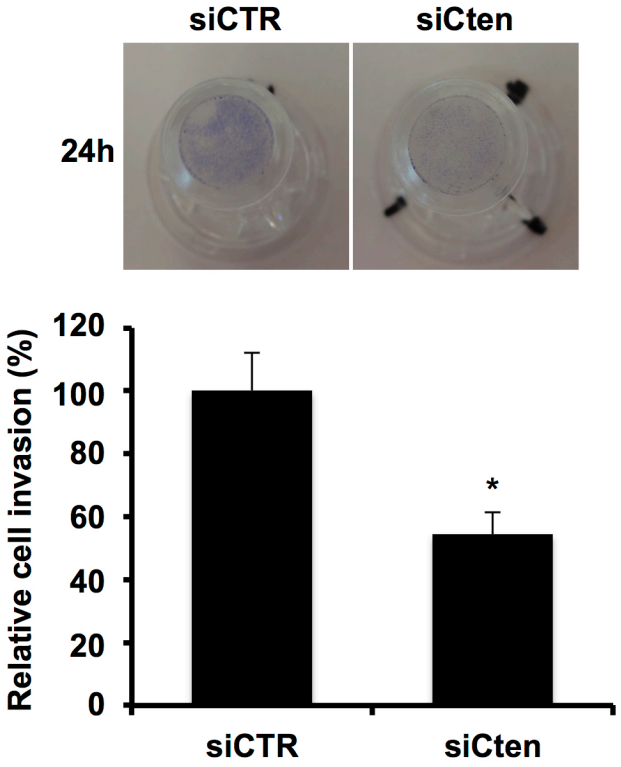
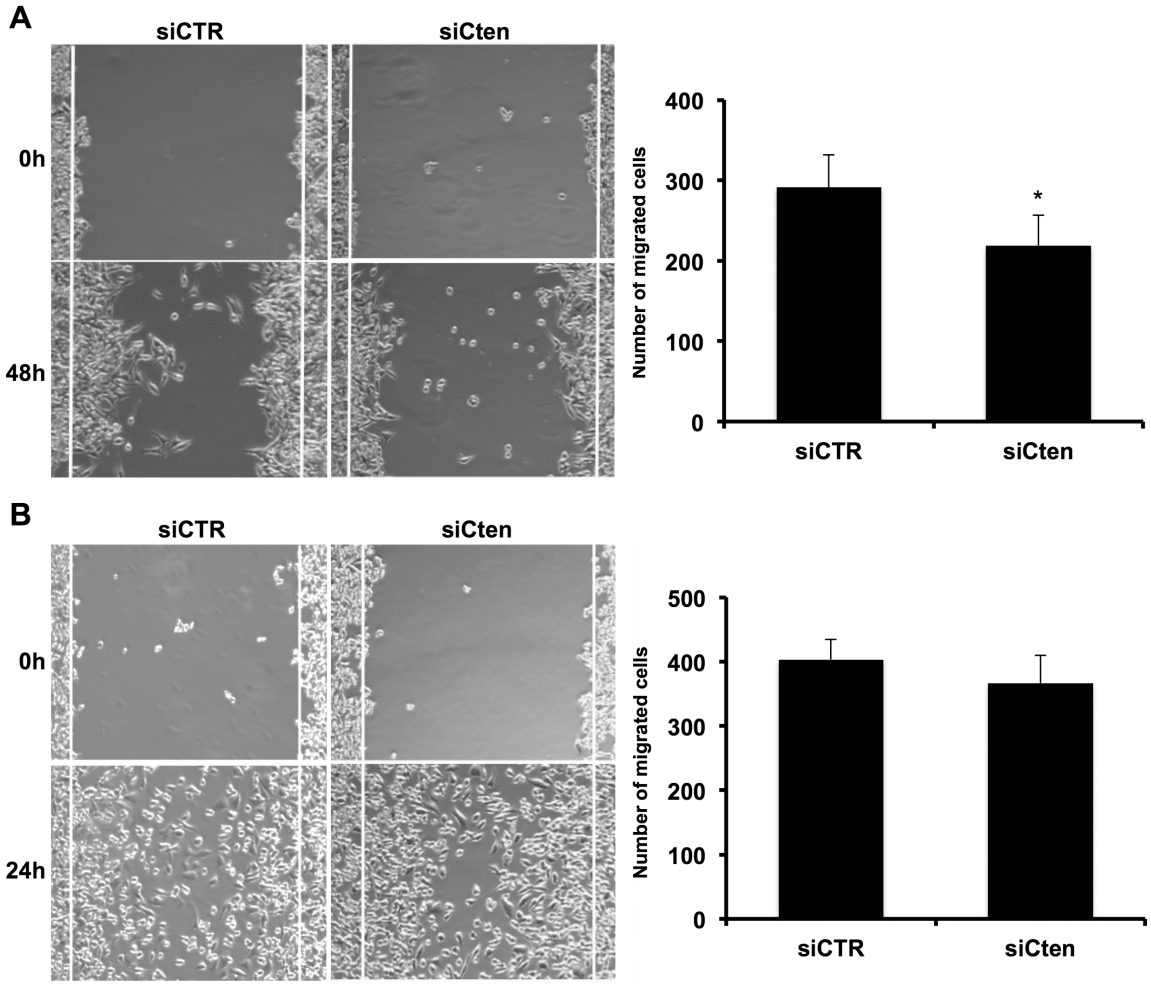


Figure 3.7. The effects of Cten knockdown on melanoma cell migration. (A) Cten knockdown resulted in a small but significant decrease in cell migration in MMAN cells after 48 hours as determined by wound healing assay. (B) No significant difference in cell migration was observed after Cten knockdown in MMRU cells. *p = 0.040.



3.2.8 A short bioinformatics study on Cten

NCBI Gene (ID number 84951) and Uniprot (ID number Q8IZW8) searches revealed little new information on Cten; the NCBI Gene database search revealed two serine residues (S82 and S248) that were found to be phosphorylated in one study, and both databases pointed to the existence of natural variants of the Cten gene, although the importance of these was unknown. The STRING network database search confirmed some of the results from the extensive literature search performed on Cten, and indicated BCAR1, DLC1, EGFR, ERBB2 and ITGB1 as potential interaction partners with Cten. It is important to note, however, that these results were mainly based on text-mining and may hence simply have been circumstantial.

The COSMIC database contained 7180 unique samples (as of 13/06/13) of tumours or cell lines with information on the status of Cten (ID number COSG63958), out of which only 52/7180 unique samples carried somatic mutations (0.007% mutation rate). Overall, no complex mutations or fusion events of the Cten gene with other genes in human cancers were found, and out of all mutations (54 mutations in 52 samples), 5 were synonymous (silent) substitutions, 5 were nonsense substitutions, 42 were missense substitutions, and 2 were frame-shift insertions. Data were available for 23 primary tissues, with mutation rates varying from 0% for samples from the biliary tract, liver, parathyroid gland, soft tissues, stomach and thyroid, to 3.95% (9/228) in samples from the skin (Table 3.4). Out of the 228 skin samples, 221 were cutaneous malignant melanoma samples, with the other 7 being squamous cell carcinoma

samples. Interestingly, none of the SCC samples had any reported mutations, meaning that the mutation rate for melanoma was in fact 4.07% (9/221 samples), which was substantially higher than the overall mutation rate of 0.007%.

The 9 mutated melanoma samples contained a total of 10 mutations, which were all missense mutations, with C>T nucleotide substitutions being most frequently observed (Table 3.5). The pattern of mutations did not differ greatly between melanoma samples and all tumour samples (Fig. 3.8).

Table 3.4. Rates of somatic mutations of the CTEN gene in human cancers.[1]

Primary tissue	Unique mutated samples	Total unique samples	% Mutated
Non-specified	2	70	2.86
Autonomic ganglia	2	362	0.55
Biliary tract	0	11	0
Breast	4	1010	0.4
Central nervous system	1	510	0.2
Cervix	1	14	7.14
Endometrium	2	227	0.88
Haematopoietic/lymphoid tissues	1	955	0.1
Kidney	1	362	0.28
Large intestine	13	725	1.79
Liver	0	71	0
Lung	6	838	0.72
Oesophagus	3	173	1.73
Ovary	2	607	0.33
Pancreas	1	365	0.27
Parathyroid	0	16	0
Prostate	2	382	0.52
Skin[2]	9	228	3.95
Soft tissue	0	15	0
Stomach	0	10	0
Thyroid	0	17	0
Upper aero-digestive tract	1	110	0.91
Urinary tract	1	102	0.98

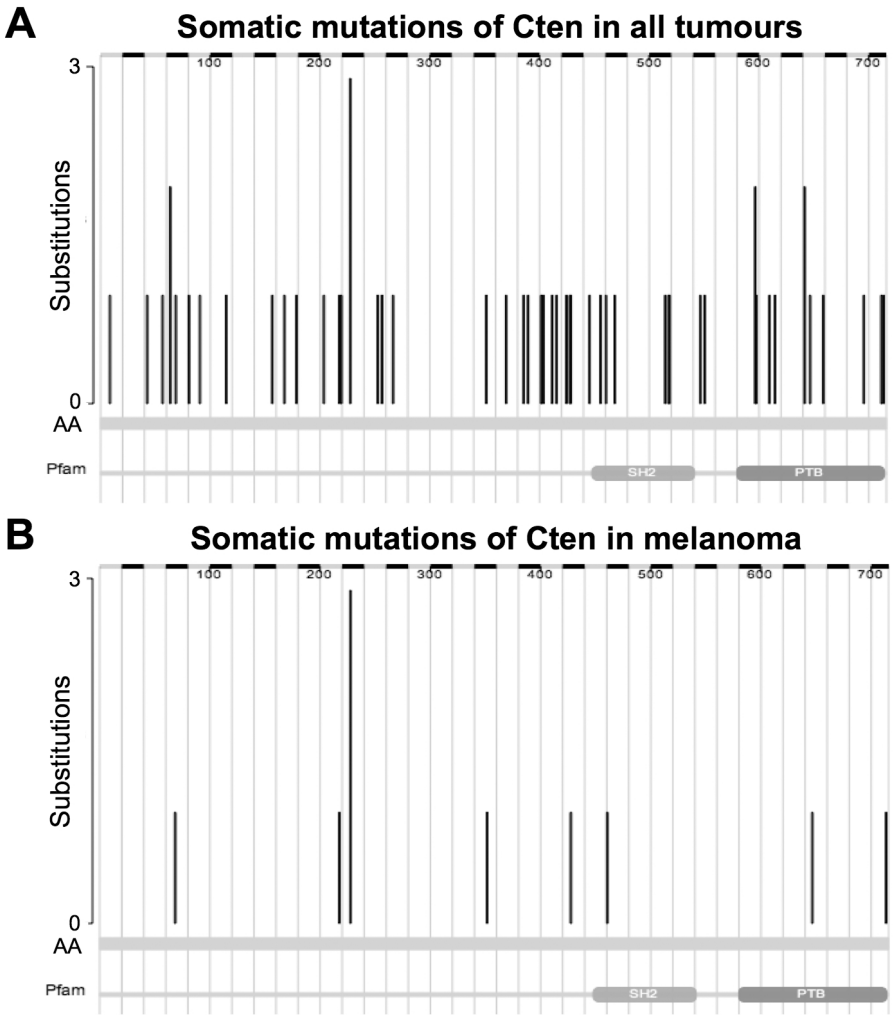
[1]Data obtained from Sanger COSMIC on 13/06/13.
[2]221 malignant melanoma samples and 7 squamous cell carcinoma samples

Table 3.5. List of confirmed somatic mutations of the CTEN gene in human cutaneous melanoma samples.[1]

Tumour location	Sample ID	Source of sample	Zygosity	AA Mutation	Nucleotide Mutation
Arm	1551811	Tumour Sample	Heterozygous	p.P428L	c.1283C>T
Face	1612192	Culture	Heterozygous	p.S218L	c.653C>T
Face	1612192	Culture	Heterozygous	p.S228F	c.683C>T
Leg	1675356	Culture	Heterozygous	p.S228F	c.683C>T
Leg	1675357	Culture	Heterozygous	p.S228F	c.683C>T
Lower leg	1612225	Culture	Heterozygous	p.A69V	c.206C>T
Neck	1612201	Culture	Heterozygous	p.T647I	c.1940C>T
Non-specified	1612180	Culture	Homozygous	p.R714K	c.2141G>A
Shoulder	1612186	Culture	Heterozygous	p.P352L	c.1055C>T
Trunk	1673894	Tumour Sample	Unknown	p.E461K	c.1381G>A

[1]Data obtained from Sanger COSMIC on 13/06/13.

Figure 3.9. Pattern of somatic mutations of the CTEN gene in all tumour types vs. melanoma. (A) Somatic mutations of Cten in all tumours. (B) Somatic mutations of Cten in melanoma samples only. The figure is adapted from two separate figures obtained from the Sanger COSMIC database on 13/06/13.



3.3 Discussion

Even though Cten was first identified as a potential tumour suppressor in prostate cancer, and later also in kidney cancer (Lo & Lo, 2002; Martuszewska *et al.,* 2009), it has since been reported to function as an oncogene in a number of other cancers, including thymomas, lung, gastric, colorectal, breast, and pancreatic cancers, but the mechanisms behind this remain controversial (Sasaki *et al.,* 2003a; Sasaki *et al.,* 2003b; Sakashita *et al.,* 2008; Albasri *et al.,* 2009; Liao *et al.,* 2009; Albasri *et al.,* 2011b; Al-Ghamdi *et al.,* 2013). Since the status of Cten expression in melanoma is currently unknown, I first investigated the protein expression of Cten in a large number of human cutaneous melanoma patient samples.

Cten protein expression was found to be significantly increased in the progression from nevi to primary melanoma, with the most dramatic increases observed between normal nevi and dysplastic nevi (7% vs. 24% strong staining), and between primary tumours < 1mm (28% strong staining) and tumours ≥ 1 mm thick (45%, 44% and 53% strong staining for tumors 1 - < 2 mm, 2 – 4 mm, and > 4 mm thick, respectively). No difference between primary tumours (especially tumours ≥ 1 mm thick, 47% strong staining) and metastatic melanomas (46% strong staining) was detected, indicating that Cten could be an oncogene, or at least a participant in oncogenic signalling, in melanoma, but that it plays a role in the early stages of melanoma development rather than in metastasis (Fig. 3.1, Table 3.1). Accordingly, Cten expression was significantly higher in tumours classified as AJCC Stages II-IV compared to AJCC Stage I tumours (Table 3.1).

Similar results have previously been observed in colorectal cancer, in which Cten overexpression was found to be an early event in cancer progression, with consistent Cten up-regulation at the mRNA and protein levels observed in all stages of colon cancer compared to normal adjacent colon tissue (Liao *et al.,* 2009). By contrast, in lung cancer, high Cten expression did not correlate with the initiation of cancer development, but was instead associated with the progression of established tumours from low to high-grade, metastatic tumours (Sasaki *et al*., 2003a). This indicates that, in addition to Cten acting as a tumour suppressor in some tissues and an oncogene in others, the oncogenic properties of Cten may also vary between tissues.

Interestingly, when I examined the correlation between Cten and other clinicopathological features of melanoma (Table 3.1), I found that Cten was significantly stronger expressed in nodular melanomas compared to all other subtypes. It can be speculated that since nodular melanomas are highly invasive (Mar *et al.,* 2012), and Cten is believed to play a role in cell motility and cell invasion, this may be the reason for why Cten expression is elevated specifically in this subtype. Additionally, Cten expression was significantly higher expressed in tumours biopsied from sun-protected sites compared to tumours from the head and neck. This could imply that induction of Cten protein expression may be mediated partly by UV-independent mechanisms, but more research will have to be performed before any conclusions regarding this, or the role of Cten in various melanoma subtypes, can be drawn.

In this study, I found that Cten expression in melanocytic lesions was

exclusively cytoplasmic (Fig. 3.1). Nuclear expression of Cten has previously been reported in a small subset of colorectal cancers (Liao *et al.,* 2009; Albasri *et al.,* 2011a), but it is likely that this discrepancy represent yet another tissue-specific characteristic of Cten, since to my knowledge, this has not been reported in any other types of cancers. Moreover, nuclear Cten does not seem to have any clinical significance, other than being higher expressed in metastatic deposits compared to primary colorectal tumours (Albasri *et al.,* 2011a).

To investigate the role of Cten in melanoma patient survival, I constructed Kaplan-Meier survival curves. Analyses showed that strong Cten expression significantly correlated with a poorer overall and disease-specific 5-year ($p = 0.008$, and 0.004, respectively, Fig. 3.2) and 10-year survival ($p = 0.030$ and 0.046, respectively, Fig. 3.3) for primary melanoma patients. These data are similar to what has been observed previously in gastric, breast and colorectal cancers, and indicate that Cten may function as an oncogene in these malignancies (Sakashita *et al.,* 2008; Albasri *et al.,* 2011a; Albasri *et al.,* 2011b). The correlation between Cten and the 5-year survival of primary melanoma patients was confirmed using univariate Cox regression analysis (Table 3.2). Subsequent multivariate Cox regression analyses showed that strong Cten expression, when adjusted to sex, age, tumour thickness, location and status of ulceration, was an adverse independent prognostic risk factor for the overall (HR, 1.69, 95% CI, 1.03-2.77, $p = 0.038$) and disease-specific (HR, 1.82, 95% CI, 1.10-3.01, $p = 0.021$) 5-year primary melanoma patient survival, compared to patients with negative to moderate Cten expression in their tumours (Table 3.3).

With the 5-year survival rate being close to 100% for melanoma patients diagnosed early, compared to only 5-16% for late-stage melanoma patients, the importance of early detection, diagnosis and prognosis is evident (American Cancer Society, 2011; Wasif *et al.,* 2011). Protein biomarkers that are differentially expressed in nevi and melanoma could help create more accurate prognoses for melanoma. The data presented here, together with the fact that Cten appears to have a highly restricted expression pattern, with negative or relatively low expression levels in all normal tissues except the prostate and placenta (Lo & Lo, 2002), indicate that Cten has the potential to be of great value as a prognostic marker for primary melanoma patients.

To determine whether or not Cten also behaves as an oncogene in melanoma *in vitro*, I used siRNAs and two melanoma cell lines; MMAN and MMRU, to investigate the effects of Cten knockdown on melanoma cell proliferation, migration and invasion. Cten knockdown resulted in a significant decrease in cell proliferation, as determined by SRB assay, and this was accompanied by a significant increase in the sub-G1 population, as determined by FACS analysis (Fig. 3.5). In normal prostate epithelial cells, where Cten has been identified as a target of caspase-3, as well as in a breast cancer cell line, it has been demonstrated that the fragments produced after cleavage of Cten by caspase-3 further promote apoptosis (Lo *et al.,* 2005). However, in a study using colorectal carcinoma cell lines, no effect on apoptosis after forced Cten expression was detected (Albasri *et al.,* 2009). Similarly, in the same study, no effects on cell proliferation after Cten overexpression was observed, which is

consistent with the results of studies on Cten in pancreatic cancer as well as in prostate cancer (Albasri *et al.,* 2009; Li *et al.,* 2010; Al-Ghamdi *et al.,* 2013).

In contrast, all of these studies, as well as additional *in vitro* studies performed in colorectal, prostate, and breast cancer models, point to a role of Cten in cell migration and metastasis (Katz *et al.,* 2007; Albasri *et al.,* 2009; Barbieri *et al.,* 2010; Albasri *et al.,* 2011a; Al-Ghamdi *et al.,* 2011; Pensa *et al.,* 2012; Al-Ghamdi *et al.,* 2013; Hung *et al.,* 2013*)*. To examine the role of Cten in melanoma cell migration, I used wound-healing assays and examined the number of migrated MMAN and MMRU cells after Cten knockdown. In MMRU cells, which are highly motile, there was a weak trend towards decreased cell migration after Cten knockdown after 24 hours, but it failed to show significance (Fig. 3.7B). After 48 hours, the migrated cells were too many to count for both the Cten and Control groups. In MMAN cells, which migrate much slower than MMRU cells, there was however a slight, but significant, decrease in cell migration after 48 hours ($p = 0.040$, Fig. 3.7A). Although all experiments will need to be repeated in a larger number of cell lines before any final conclusions can be made, these preliminary results indicate that regulation of cell migration may not be the main function of Cten in melanomas, and support the findings from the tissue microarray study that Cten is more important for the development of primary tumours than for metastasis in melanomas.

Whereas the effects of Cten knockdown on melanoma cell migration were relatively modest, the effect on melanoma cell invasion was conversely quite prominent (Fig. 3.6). As discussed, in the TMA study I found that Cten was

significantly higher expressed in nodular melanomas, which are highly invasive and aggressive, compared to all other subtypes (Mar *et al.,* 2012). The *in vitro* results now indicate that that may not be a coincidence, and together, these data support a role for Cten in melanoma cell invasion.

Database searches for Cten revealed that despite the fact that somatic mutations in the CTEN gene are highly uncommon in human cancers (0.007% overall mutation rate, Table 3.4), the mutation rate for melanoma was relatively high (4.07%, COSMIC Sanger, 2013). All mutations in melanoma were missense mutations, and even though 4.07% is still a fairly low number and the pattern of mutations was not typical of that seen for an oncogene (and instead indicated that Cten may be a so-called tumour-required protein; Fig. 3.9); and although it is possible that the mutation rate for melanomas is relatively high in general due to the effects of UVR, I believe that this is still worth investigating further, since it may represent novel means of Cten activation.

While these preliminary data strongly support a role for Cten in the promotion of melanomagenesis, it is nonetheless unclear exactly how Cten is activated and regulated in cancer. What is interesting about Cten, and one of the reasons as to why it ought to be further examined in melanoma, is the fact that it has been positively associated with BRAF V600E mutations in colorectal cancer (Al-Ghamdi *et al.,* 2011). As discussed in Chapter 1.2.3, the BRAF V600E mutation is highly prevalent in cutaneous melanoma, and is a common drug target (Davies *et al.,* 2002; Chapman *et al.,* 2011). However, with resistance to Vemurafenib (and other BRAF V600E inhibitors) developing in many cases (Das

Thakur *et al.,* 2013), the need to identify downstream effectors of BRAF that could be targeted simultaneously is critical. Evidence supporting the notion of Cten as a novel downstream target of BRAF and the MAPK pathway comes not only from the work by Al-Ghamdi *et al.* (2011) mentioned above, but also from studies showing that Cten expression is induced by EGF and FGF, as well as by other growth factors known to activate the MAPK pathway (Katz *et al.,* 2007; Cao *et al.,* 2012; Pylayeva & Giancotti, 2012; Hung *et al.,* 2013). Although these growth factors can also signal through alternative pathways, Hung *et al.* (2013) recently showed that MEK1/2 (the immediate downstream targets of BRAF) overexpression resulted in up-regulation of Cten in normal prostate and colorectal carcinoma cell lines, and that treatment with MEK inhibitors resulted in suppressed expression of Cten and in a reduction in cell migration, even after growth factor-mediated Cten induction.

The same study also identified another major signalling pathway implicated in melanomagenesis; the PI3K/Akt pathway, as a second signalling cascade responsible for Cten up-regulation in prostate and colorectal cancer cell lines, and found that PI3K inhibition significantly blocked Cten induction (Hung *et al.,* 2013). Since the PI3K/Akt pathway has been previously associated with both tensin1 and tensin2 in addition to Cten (Auger *et al.,* 1996; Hafizi *et al.,* 2010; Broxmeier *et al.,* 2011; Jung *et al.,* 2011; Tyner, 2011; Koh *et al.,* 2013), this warrants for further examination into the interrelationship between these molecules in the context of PI3K/Akt signalling. If these results are reproducible in melanomas, and Cten is in fact implicated in either or both MAPK and

PI3K/Akt signalling, I believe that Cten has the potential to be of great value in the treatment of this malignancy, either using antisense therapy, or possibly monoclonal antibody therapy, targeting the appropriate domain(s) of Cten.

In summary, in this study I showed that Cten protein expression was increased in the progression from melanocytic nevi to primary melanoma, that strong Cten expression was significantly associated with a worse 5- and 10-year survival outcome, and that Cten was a novel independent prognostic factor for primary melanoma patients. I further showed that Cten behaved like an oncogene *in vitro* in two different melanoma cell lines, and that melanoma has a relatively high rate of somatic mutations in the CTEN gene compared to other types of cancers, all supporting a role for Cten in melanomagenesis.

4. Loss of DLC1 expression correlates with poor patient survival

4.1 Rationale

Although numerous interaction partners have been suggested for Cten, the only protein *known* to physically interact with Cten is DLC1, a *bona fide* tumour suppressor protein deleted or epigenetically silenced in a number of cancers. Since relatively little is known about how Cten exerts its oncogenic effects, I hypothesized that in melanomas, this is achieved at least partly via binding to DLC1, and inhibition of its RhoGAP activity (as first described by Cao *et al.,* 2012).

Despite the vast amount of research conducted on DLC1 over the past 15 years, no one has yet reported its expression status in melanomas. Hence, I decided to investigate this, as well as to examine the correlation between DLC1 and Cten at the protein expression level. I used tissue microarrays containing an identical set of tissues as examined for Cten expression, and immunohistochemistry to investigate the protein expression of DLC1 in melanomas.

4.2 Results

4.2.1 Clinicopathological features of TMAs

TMAs containing 34 normal nevi (NN), 78 dysplastic nevi (DN), 306 primary melanomas (PM) and 121 metastatic melanomas (MM) were evaluated for DLC1 protein expression. Out of the 427 melanoma cases, 250 samples were obtained

from males, and 176 from females. One (1) sample was missing information about the sex of the patient. The median age at collection was 59 (ranging from 7 to 95 years). Out of all samples, 183 samples were classified as belonging to AJCC Stage I, 123 as Stage II, 50 as Stage III, and 68 as Stage IV. Three (3) samples lacked information about the AJCC stage. Out of the 306 primary melanoma samples, 8 were sub-classified as acral lentiginous, 12 as desmoplastic, 76 as lentigo maligna, 47 as nodular, 115 as superficial spreading, and 48 as 'other' types of melanomas (unclassified (n = 43), nevoid (n = 3), and spitz-like (n = 2) melanomas). In total, 224 tumours were taken from sun-protected sites whereas 82 were biopsied from the head and neck. Of these, 115 tumours were < 1.0 mm thick, 72 were 1.0 - < 2.0 mm, 60 were 2.0 – 4.0 mm, and 59 were > 4.0 mm thick. Ulceration was present in 52 cases and absent in 254 cases. For metastatic melanomas, 47 tumours were cutaneous, 50 were biopsied from the lymph nodes, and 21 were located in visceral organs (Tables 4.1 and 4.2).

4.2.2 DLC1 is expressed both in the cytoplasm and nuclei, and its expression is reduced in melanomas

DLC1 was expressed both in the cytoplasm and nuclei in melanocytic lesions (Fig. 4.1), and both were further analyzed. Strong cytoplasmic DLC1 expression was detected in 64.7% of NN, 67.9% of DN, 60.8% of PM, and 49.6% of MM, with a significant decrease observed in MM compared to PM and DN ($p = 0.035$ and 0.011, respectively, χ^2 test, Fig. 4.1I). Strong nuclear DLC1 expression was

observed in 82.4%, 65.4%, 56.2% and 33.9% of NN, DN, PM, and MM, respectively, with a significant difference seen between NN and PM ($p = 0.003$), NN and MM ($p < 0.001$), DN and MM ($p < 0.001$), and PM and MM ($p < 0.001$). A borderline difference was furthermore seen between NN and DN ($p = 0.070$) and DN and PM ($p = 0.143$, χ^2 test, Fig. 4.1J).

4.2.3 DLC1 expression correlates with AJCC stages

Next, the correlation between DLC1 and a number of demographic and clinicopathological characteristics was examined. Neither cytoplasmic nor nuclear DLC1 staining correlated with variables such as patient age and sex, primary tumour subtype, location, and status of ulceration, or metastatic melanoma location (Tables 4.1 and 4.2), but a significant association was found between DLC1 and AJCC stages (Fig. 4.2). Strong cytoplasmic DLC1 expression was significantly reduced in tumours classified as AJCC Stage IV (47.1%), compared to AJCC Stage I-III tumours (60.1% strong staining, $p = 0.046$, χ^2 test, Fig. 4.2A), whereas strong nuclear DLC1 staining was reduced in AJCC Stage III+IV tumours (34.7%) compared to AJCC Stage I+II tumours (56.2%, $p < 0.001$, χ^2 test, Fig. 4.2B). Nuclear DLC1 was moreover slightly, but significantly, stronger expressed in primary tumours > 4.0 mm thick compared to tumours ≤ 4.0 mm thick ($p = 0.046$, χ^2 test, Table 4.2), whereas no association was seen between cytoplasmic DLC1 and tumour thickness (Table 4.1).

Figure 4.1. Representative images of cytoplasmic and nuclear DLC1 protein expression at 100x (A-D) and 400x magnification (E-H). (A, E) Strong cytoplasmic and nuclear DLC1 staining in normal nevi (NN). (B, F) Strong cytoplasmic and moderate nuclear DLC1 staining in dysplastic nevi (DN). (C, G) Moderate cytoplasmic and weak nuclear DLC1 staining in primary melanoma (PM). (D, H) Weak cytoplasmic and negative nuclear DLC1 staining in metastatic melanoma (MM). (I) Correlation between cytoplasmic DLC1 expression and melanoma progression. (J) Correlation between nuclear DLC1 expression and melanoma progression.

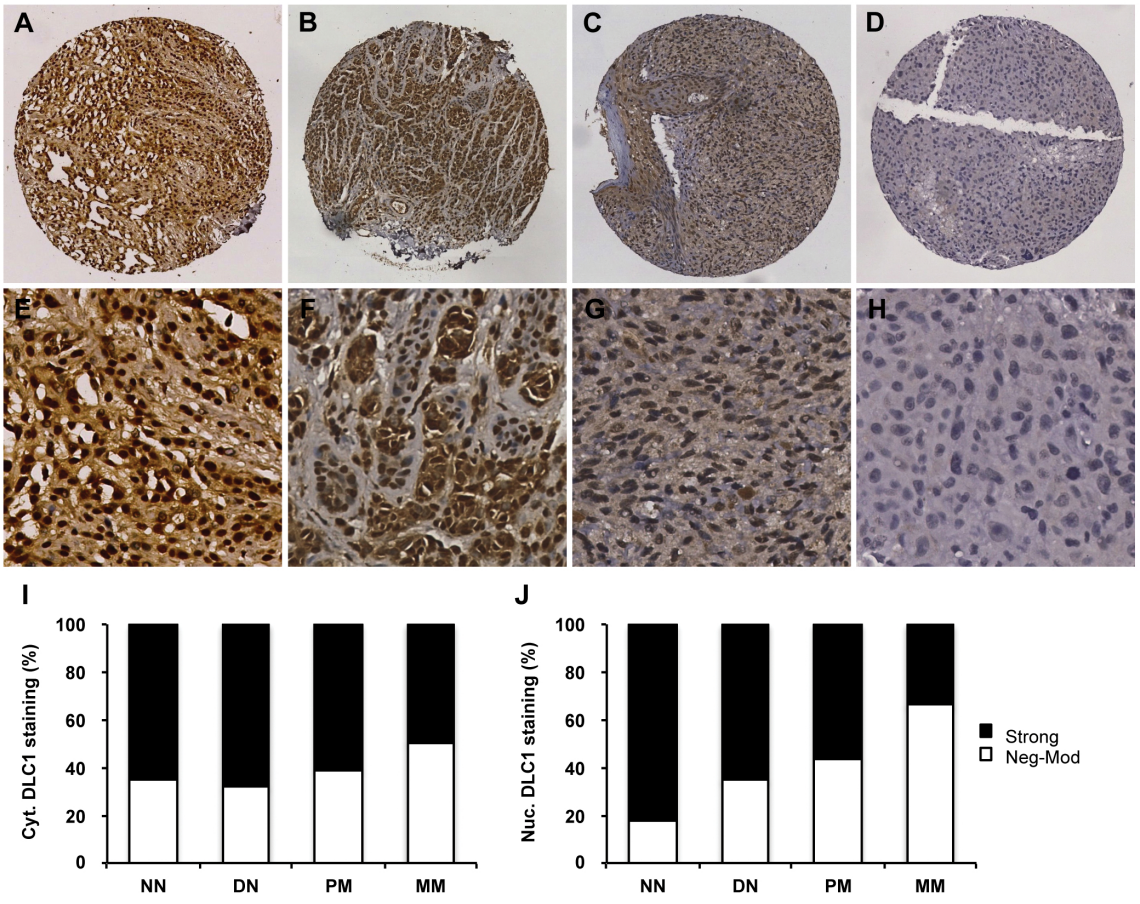


Table 4.1. Cytoplasmic DLC1 staining and clinicopathological characteristics of 427 melanoma patients.

	Cytoplasmic DLC1 staining			
Variables	**Neg-Mod**	**Strong**	**Total**	**P-value[1]**
Primary Melanoma (n=306)				
Age				
≤ 59	56 (38.4%)	90 (61.6%)	146	0.768
> 59	64 (40.0%)	96 (60.0%)	160	
Sex[2]				
Male	61 (36.3%)	107 (63.7%)	168	0.283
Female	58 (42.3%)	79 (57.7%)	137	
Tumour thickness (mm)				
< 1.0	51 (44.3%)	64 (55.7%)	115	0.144[3]
1.0 - < 2.0	22 (30.6%)	50 (69.4%)	72	
2.0 - 4.0	25 (41.7%)	35 (58.7%)	60	
> 4.0	18 (30.5%)	41 (69.5%)	59	
Ulceration				
Present	15 (28.8%)	37 (71.2%)	52	0.093
Absent	105 (41.3%)	149 (58.7%)	254	
Tumour subtype				
Acral Lentiginous	3 (37.5%)	5 (62.5%)	8	0.741[4]
Desmoplastic	6 (50.0%)	6 (50.0%)	12	
Lentigo Maligna	29 (38.2%)	47 (61.8%)	76	
Nodular	16 (34.0%)	31 (66.0%)	47	
Superficial Spreading	50 (43.5%)	65 (56.5%)	115	
Other	16 (33.3%)	32 (66.7%)	48	
Location[5]				
Sun-protected	92 (41.1%)	132 (58.9%)	224	0.272
Sun-exposed	28 (34.1%)	54 (65.9%)	82	
Metastatic Melanoma (n=121)				
Age				
≤ 59	37 (54.4%)	31 (45.6%)	68	0.319
> 59	24 (45.3%)	29 (54.7%)	53	
Sex				
Male	46 (56.1%)	36 (43.9%)	82	
Female	15 (38.5%)	24 (61.5%)	39	
AJCC stage				
I	79 (43.2%)	104 (56.8%)	183	0.046[6]
II	41 (33.3%)	82 (66.7%)	123	
III	22 (44.0%)	28 (56.0%)	50	
IV	36 (52.9%)	32 (47.1%)	68	
Location				
Cutaneous	26 (55.3%)	21 (44.7%)	47	0.536[7]
Lymph nodal	23 (46.0%)	27 (54.0%)	50	
Visceral	9 (42.9%)	12 (57.1%)	21	

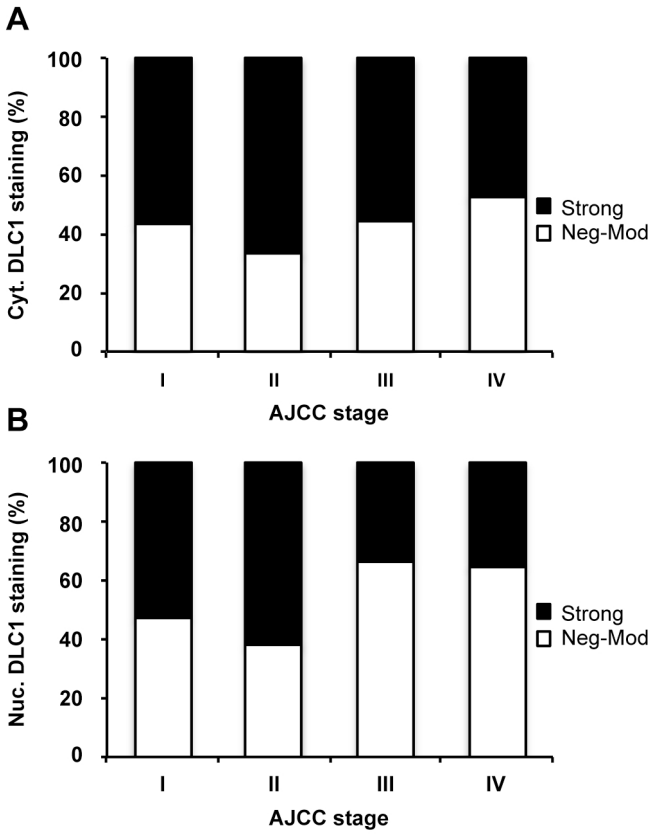
AJCC indicates American Joint Committee on Cancer. [1]χ^2 test, df = 1 unless otherwise stated. [2]One (1) patient sample was lacking information about sex and was excluded from analysis. [3]Df = 3. [4]Df = 5. [5]Sun-protected locations: back, trunk, arms, hands, legs, feet, retroauricular, and vulva; Sun-exposed sites: head and neck. [6]Tumours classified as AJCC Stages I-III vs. Stage IV tumours. Samples with unspecified AJCC stages (n=3) were excluded from analysis. [7]Df = 2. Samples lacking information about the location (n=3) were excluded from the study.

Table 4.2. Nuclear DLC1 staining and clinicopathological characteristics of 427 melanoma patients.

	Nuclear DLC1 staining			
Variables	**Neg-Mod**	**Strong**	**Total**	**P-value[1]**
Primary Melanoma (n=306)				
Age				
≤ 59	63 (43.2%)	83 (56.8%)	146	0.830
> 59	71 (44.4%)	89 (55.6%)	160	
Sex[2]				
Male	74 (44.0%)	94 (56.0%)	168	0.964
Female	60 (43.8%)	77 (56.2%)	137	
Tumour thickness (mm)				
< 1.0	56 (48.7%)	59 (51.3%)	115	
1.0 - < 2.0	30 (41.7%)	42 (58.3%)	72	
2.0 - 4.0	29 (48.3%)	31 (51.7%)	60	
> 4.0	19 (32.2%)	40 (67.8%)	59	0.046[3]
Ulceration				
Present	20 (38.5%)	32 (61.5%)	52	0.395
Absent	114 (44.9%)	140 (55.1%)	254	
Tumour subtype				
Acral Lentiginous	4 (50.0%)	4 (50.0%)	8	0.237[4]
Desmoplastic	8 (66.7%)	4 (33.3%)	12	
Lentigo Maligna	30 (39.5%)	46 (60.5%)	76	
Nodular	15 (31.9%)	32 (68.1%)	47	
Superficial Spreading	55 (47.8%)	60 (52.2%)	115	
Other	22 (45.8%)	26 (54.2%)	48	
Location[5]				
Sun-protected	103 (46.0%)	121 (54.0%)	224	0.202
Sun-exposed	31 (37.8%)	51 (62.2%)	82	
Metastatic Melanoma (n=121)				
Age				
≤ 59	47 (69.1%)	21 (30.9%)	68	0.430
> 59	33 (62.2%)	20 (37.8%)	53	
Sex				
Male	56 (68.3%)	26 (31.7%)	82	0.463
Female	24 (61.5%)	15 (38.5%)	39	
AJCC stage				
I	87 (47.5%)	96 (52.5%)	183	<0.001[6]
II	47 (38.2%)	76 (61.8%)	123	
III	33 (66.0%)	17 (34.0%)	50	
IV	44 (64.7%)	24 (35.3%)	68	
Location				
Cutaneous	34 (72.3%)	13 (27.7%)	47	0.140[7]
Lymph nodal	33 (66.0%)	17 (34.0%)	50	
Visceral	10 (47.6%)	11 (52.4%)	21	

AJCC indicates American Joint Committee on Cancer. [1]χ^2 test, df = 1 unless otherwise stated. [2]One (1) patient sample was lacking information about sex and was excluded from analysis. [3]Tumours > 4.0 mm thick vs. tumours ≤ 4.0 mm thick. [4]Df = 5. [5]Sun-protected locations: back, trunk, arms, hands, legs, feet, retroauricular, and vulva; Sun-exposed sites: head and neck. [6]Tumours classified as AJCC Stages I-II vs. Stages III-IV. Samples with unspecified AJCC stages (n=3) were excluded from analysis. [7]Df = 2. Samples lacking information about the location (n=3) were excluded from the study.

Figure 4.2. DLC1 expression correlates with AJCC stages. (A) Cytoplasmic DLC1 was significantly weaker expressed in AJCC Stage IV tumours compared to AJCC Stage I-III tumours ($p = 0.046$, χ^2 test). (B) Nuclear DLC1 is significantly weaker expressed in AJCC Stage III+IV tumours, compared to AJCC Stage I+II tumours ($p < 0.001$, χ^2 test).



4.2.4 DLC1 expression correlates with the 5- and 10-year survival of melanoma patients

In total, 396 melanoma patients (276 primary melanoma and 120 metastatic melanoma patients) had complete 5-year follow-up information. To examine the correlation between DLC1 expression and patient survival, Kaplan-Meier survival curves were constructed. Analyses revealed that both cytoplasmic and nuclear DLC1 expression were associated with the overall and disease-specific 5-year survival of all melanoma patients, with negative-moderate DLC1 expression being associated with a poorer survival outcome ($p < 0.001$ and $p = 0.001$, respectively, for cytoplasmic DLC1, and $p < 0.001$ for both overall and disease-specific survival, for nuclear DLC1, Fig. 4.3). When divided into PM and MM patient groups, a significant association was seen between DLC1 and the overall and disease-specific survival of MM patients ($p = 0.020$ and 0.008, respectively, for cytoplasmic DLC1, and $p = 0.004$ for both, for nuclear DLC1, Fig. 4.3) but not for PM patients (Fig. 4.4).

Next, I was interested to see if DLC1 was also associated with the overall and disease-specific 10-year patient survival. A total of 329 patients had complete 10-year follow-up information, and whereas no association was found for PM or MM patients alone (data not shown), both cytoplasmic and nuclear DLC1 expression were significantly associated with the disease-specific 10-year survival of all melanoma patients ($p = 0.046$ and 0.009, respectively, Fig. 4.5).

Figure 4.3. Kaplan-Meier analyses for the correlations between DLC1 expression and 5-year survival in melanoma patients. (A) Cytoplasmic DLC1. (B) Nuclear DLC1.

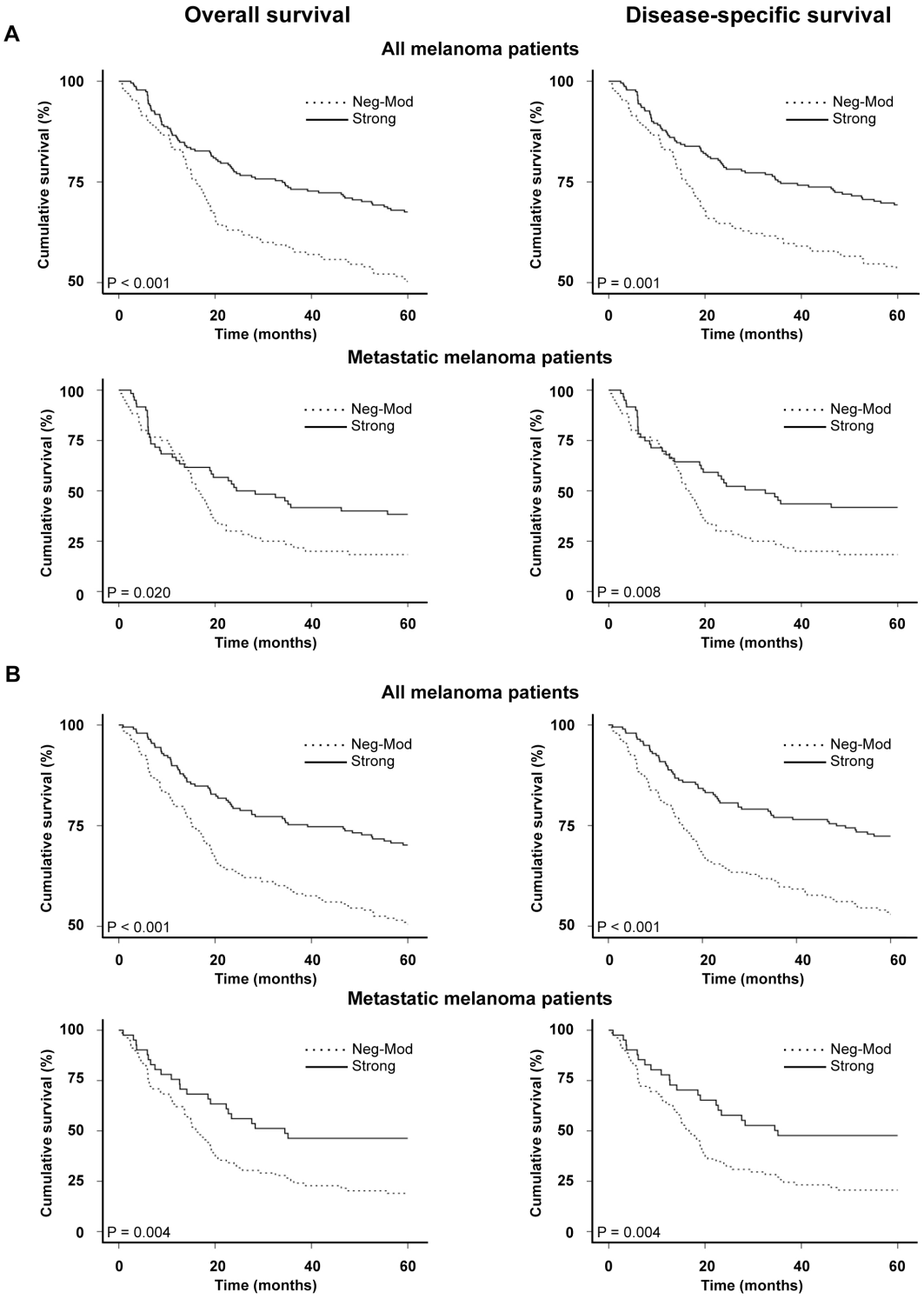


Figure 4.4. Kaplan-Meier analyses for the correlations between DLC1 expression and 5-year survival in 276 primary melanoma patients. (A) Cytoplasmic DLC1 (p = 0.089 and 0.315 for the overall and disease-specific survival, respectively). (B) Nuclear DLC1 (p = 0.360 and 0.411 for the overall and disease-specific survival, respectively).

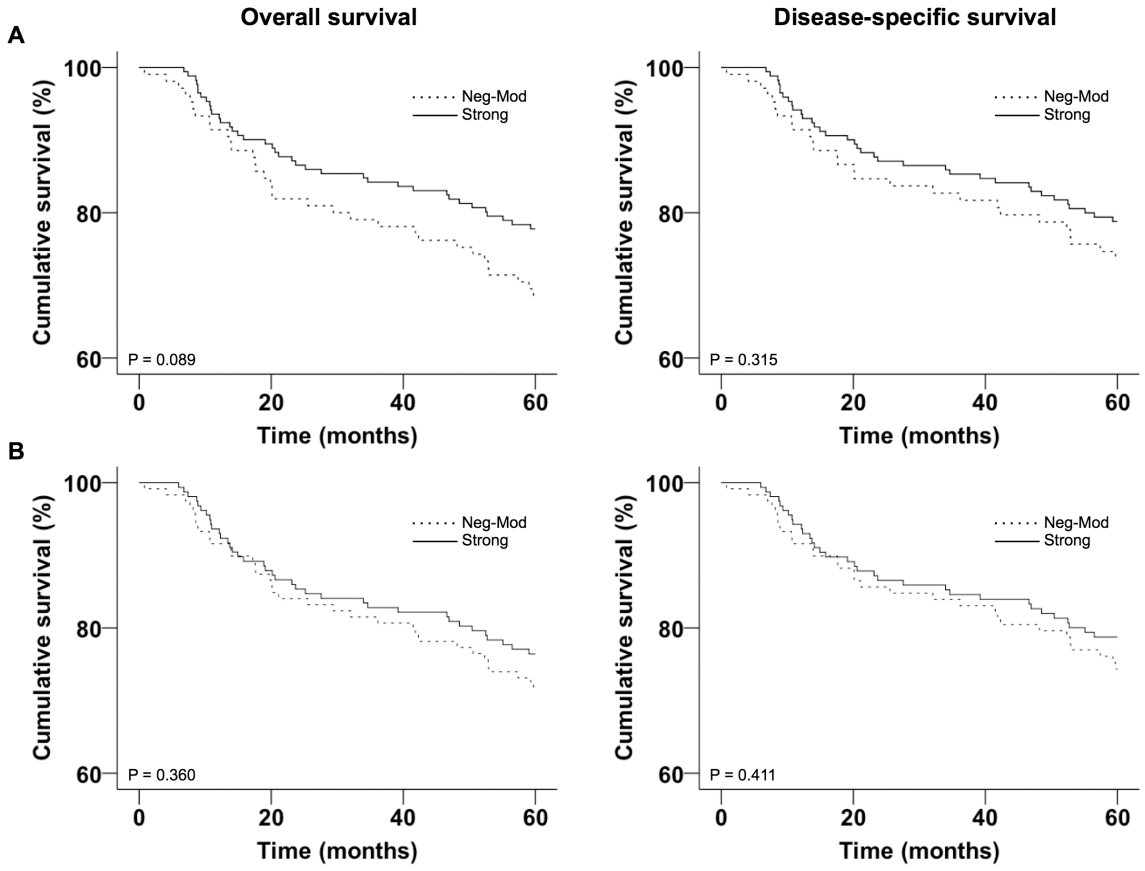
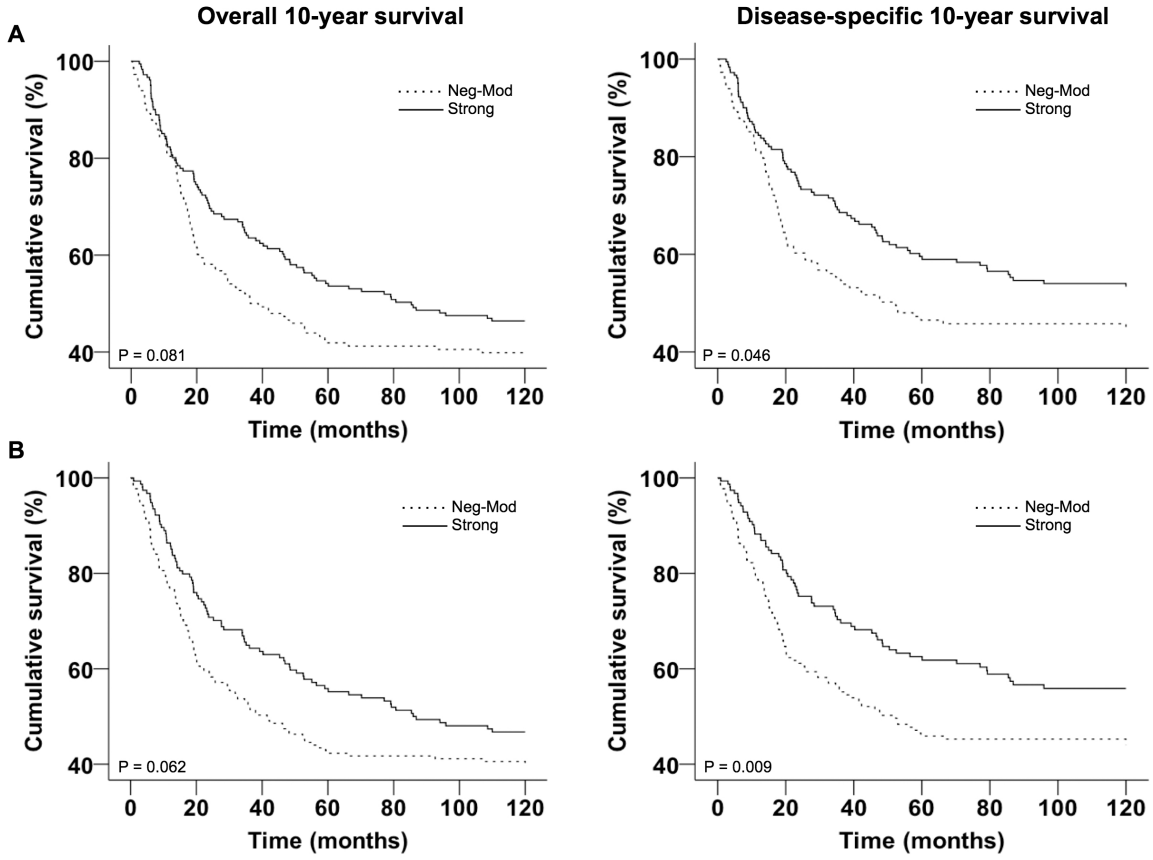


Figure 4.5. Kaplan-Meier analyses for the correlations between DLC1 expression and 10-year survival in 329 melanoma patients. (A) Loss of cytoplasmic DLC1 correlated with a significantly worse disease-specific 10-year survival (p = 0.046) but not overall survival (p = 0.081). (B) Nuclear DLC1 also correlated with the disease-specific 10-year survival (p = 0.009) but not the overall survival of all melanoma patients (p = 0.062, log-rank tests).



4.2.5 Concurrent loss of cytoplasmic and nuclear DLC1 expression is associated with a worse 5-year survival for metastatic melanoma patients

To study the correlation between cytoplasmic and nuclear DLC1, and their effects on patient survival, the samples were divided into four groups based on their staining: (1) negative-moderate cytoplasmic and nuclear DLC1; (2) negative-moderate cytoplasmic DLC1 and strong nuclear DLC1; (3) strong cytoplasmic DLC1 and negative-moderate nuclear DLC1; and (4) strong cytoplasmic and nuclear DLC1 (Fig. 4.6A). Cytoplasmic and nuclear DLC1 expressions were found to positively correlate in melanoma patients (Fig. 4.6B). Figure 4.7 shows the results of Kaplan-Meier analyses. For all melanoma patients, loss of either or both cytoplasmic and nuclear DLC1 (Categories 1-3) was associated with a poorer survival outcome compared to patients with strong cytoplasmic and nuclear DLC1 expression (Category 4) in their tumours ($p < 0.001$ for both overall and disease-specific survival, Fig. 4.7A). However, for MM patients, I found that while patients classified as Category 4 still had the most favourable survival outcome (51.7% and 55.2% for overall and disease-specific 5-year survival, respectively), this time there was a clear difference between patients classified as Categories 2 and 3 (33.3% and 25.8% overall, and 33.3% and 32.3% disease-specific survival, respectively) and patients belonging to Category 1 (14.6% overall and disease-specific survival, $p = 0.013$ and 0.008, respectively, Fig. 4.7B).

Figure 4.6. Correlation between cytoplasmic and nuclear DLC1 expression in melanocytic lesions. (A) Samples were categorized based on cytoplasmic and nuclear DLC1 expression as: (1) negative-moderate cytoplasm and nuclei; (2) negative-moderate cytoplasm but strong nuclei; (3) strong cytoplasm but negative-moderate nuclei and; (4) strong cytoplasm and nuclei. (B) Cytoplasmic and nuclear DLC1 protein expression positively correlate in melanomas ($p < 0.001$ for PM, MM, and all melanomas).

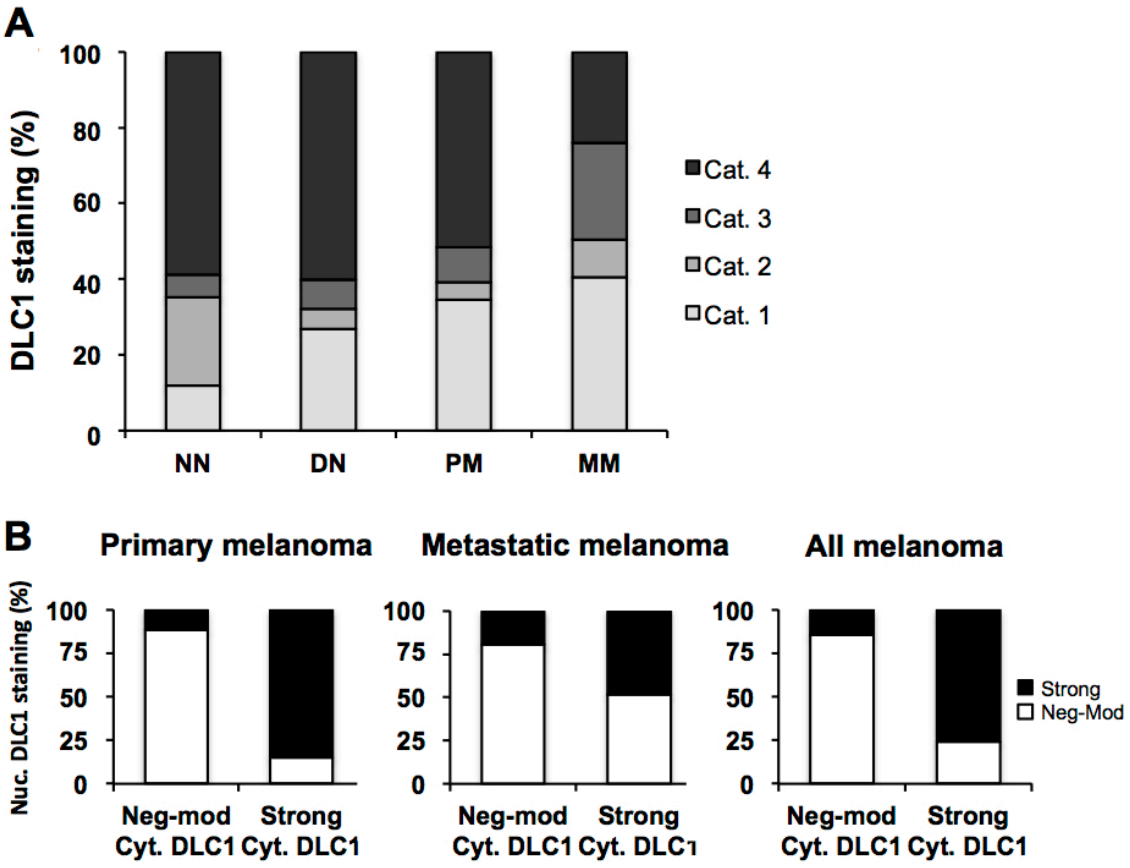
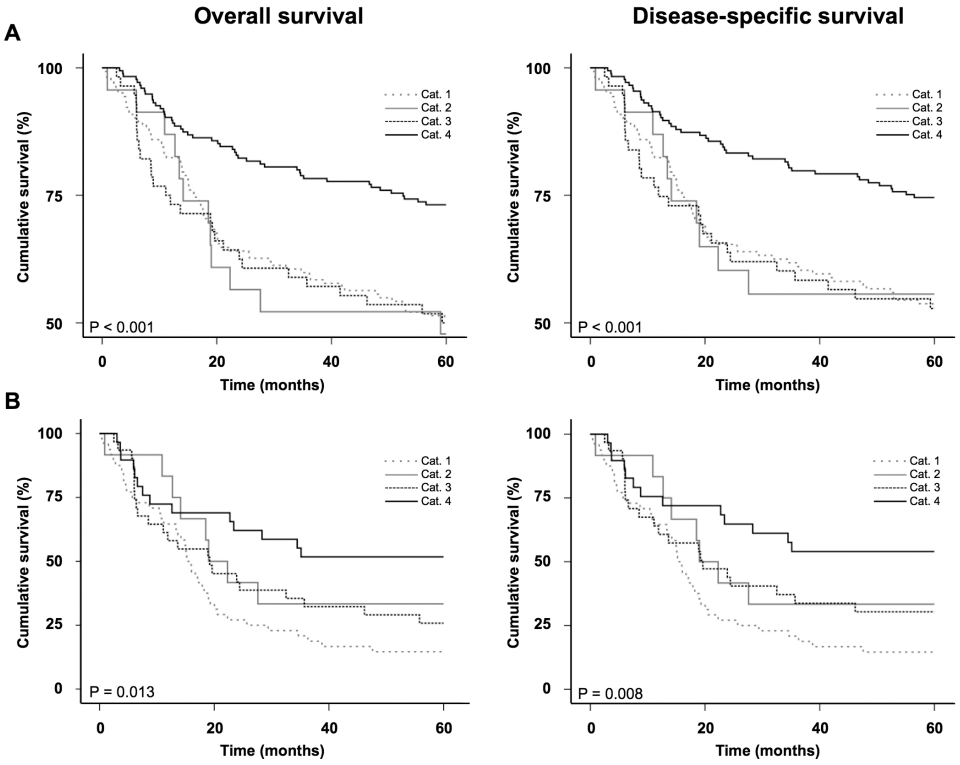


Figure 4.7. Simultaneous loss of cytoplasmic and nuclear DLC1 correlates with a poorer 5-year survival. (A) Strong cytoplasmic and strong nuclear DLC1 expression (Cat. 4) was associated with a significantly better 5-year survival outcome compared to loss of either or both cytoplasmic and nuclear DLC1 expression (Categories 1-3) for all melanoma patients ($p < 0.001$). (B) For metastatic melanoma patients only, strong cytoplasmic and nuclear DLC1 expression (Cat. 4) was associated with the most favourable 5-year survival outcome, whereas negative-moderate cytoplasmic and nuclear DLC1 expression (Cat. 1) was associated with the worst outcome, and negative-moderate cytoplasmic and strong nuclear DLC1 (Cat. 2) or strong cytoplasmic and negative-moderate nuclear DLC1 expression (Cat. 3) were associated with an intermediate survival rate ($p = 0.013$ and 0.008 for overall and disease-specific survival, respectively).



4.2.6 Strong DLC1 expression is a favourable independent prognostic factor for all melanoma patients

Both cytoplasmic and nuclear DLC1 were found to be significant risk factors for all melanoma patients and MM patients, as determined by univariate Cox regression analyses (Table 4.3). Subsequent multivariate Cox regression analyses, adjusted to patient sex, age and AJCC stage, showed that both cytoplasmic and nuclear DLC1, when analyzed separately, were independent prognostic factors for the 5-year overall and disease-specific survival of all melanoma and MM patients (Tables 4.4 and 4.5).

Importantly, when analyzed together, multivariate Cox regression analysis showed that strong expression of both cytoplasmic and nuclear DLC1 (Category 4) was a favourable independent prognostic factor for all melanoma patients ((HR, 0.59, 95% CI, 0.41-0.84, p = 0.003) for overall survival and (HR, 0.61, 95% CI, 0.42-0.88, p = 0.008) for disease-specific survival) and metastatic melanoma patients ((HR, 0.44, 95% CI, 0.25-0.79, p = 0.006) for overall and (HR, 0.42, 95% CI, 0.23-0.77, p = 0.005) for disease-specific survival, Table 4.6) when compared to loss of either or both cytoplasmic and nuclear DLC1.

Table 4.3. Univariate Cox regression analysis on 5-year survival of 396 melanoma patients

Variables	Patients	Overall survival				Disease-specific survival			
		Deaths	Death Rate	HR (95% CI)	P-value[1]	Deaths	Death Rate	HR (95% CI)	P-value[1]
All melanoma (n=396)									
Age									
≤ 59	196 (49.5%)	76	38.8%	1.00	0.713	70	35.7%	1.00	0.642
> 59	200 (50.5%)	81	40.5%	1.06 (0.77-1.45)		76	38.0%	1.08 (0.78-1.49)	
Sex									
Male	229 (57.8%)	95	41.5%	1.00	0.478	88	38.4%	1.00	0.534
Female	167 (42.2%)	62	37.1%	0.89 (0.65-1.23)		58	34.7%	0.90 (0.65-1.25)	
AJCC stage									
I+II	276 (69.7%)	71	25.7%	1.00	<0.001	63	22.8%	1.00	<0.001
III+IV	120 (30.3%)	86	71.7%	4.50 (3.27-6.20)		83	69.2%	4.85 (3.48-6.77)	
Cyt. DLC1 staining									
Neg-Mod	165 (41.7%)	82	49.7%	1.00	0.001	76	46.1%	1.00	0.001
Strong	231 (58.3%)	75	32.5%	0.58 (0.42-0.79)		70	30.3%	0.58 (0.42-0.80)	
Nuc. DLC1 staining									
Neg-Mod	198 (50.0%)	98	49.5%	1.00	<0.001	92	46.5%	1.00	<0.001
Strong	198 (50.0%)	59	29.8%	0.51 (0.37-0.71)		54	27.3%	0.50 (0.36-0.70)	
MM (n=120)									
Age									
≤ 59	67 (55.8%)	49	73.1%	1.00	0.872	47	70.1%	1.00	0.935
> 59	53 (44.2%)	37	69.8%	0.97 (0.63-1.48)		36	67.9%	0.98 (0.64-1.52)	
Sex									
Male	80 (66.7%)	57	71.3%	1.00	0.654	54	67.5%	1.00	0.501
Female	40 (33.3%)	29	72.5%	1.11 (0.71-1.73)		29	72.5%	1.17 (0.74-1.84)	
Cyt. DLC1 staining									
Neg-Mod	60 (50.0%)	49	81.7%	1.00	0.022	49	81.7	1.00	0.009
Strong	60 (50.0%)	37	61.7%	0.60 (0.39-0.93)		34	56.7%	0.56 (0.36-0.86)	
Nuc. DLC1 staining									
Neg-Mod	79 (65.8%)	64	81.0%	1.00	0.005	62	67.5%	1.00	0.005
Strong	41 (34.2%)	22	53.7%	0.50 (0.31-0.81)		21	51.2%	0.49 (0.30-0.81)	

[1]Log-rank test. Abbreviations: HR, hazard ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer; MM, metastatic melanoma

Table 4.4. Multivariate Cox regression analysis, including cytoplasmic DLC1, on 5-year overall and disease-specific survival of 396 melanoma patients.

Variables[1]	Overall survival					Disease-specific survival				
	β[2]	SE	HR	95.0% CI	P-value[3]	β[2]	SE	HR	95.0% CI	P-value[3]
All melanoma (n=396)										
Sex	0.130	0.167	1.14	0.82-1.58	0.436	0.157	0.173	1.17	0.83-1.64	0.363
Age	0.223	0.164	1.25	0.91-1.72	0.173	0.250	0.170	1.28	0.92-1.79	0.143
AJCC	1.523	0.167	4.59	3.31-6.36	<0.001	1.605	0.174	4.98	3.54-6.99	<0.001
Cyt. DLC1	-0.474	0.164	0.62	0.45-0.86	0.004	-0.461	0.170	0.63	0.45-0.88	0.007
MM (n=120)										
Sex	0.165	0.230	1.18	0.75-1.85	0.474	0.231	0.233	1.26	0.80-1.99	0.321
Age	0.109	0.226	1.12	0.72-1.74	0.631	0.155	0.230	1.17	0.74-1.84	0.500
Cyt. DLC1	-0.549	0.229	0.58	0.37-0.91	0.017	-0.652	0.235	0.52	0.33-0.83	0.006

[1]Coding of variables: Age was coded as 1 (≤ 59 years) and 2 (> 59 years); sex was coded as 1 (male) and 2 (female); AJCC was coded as 1 (Stages I+II) and 2 (Stages III+IV); DLC1 was coded as 1 (negative-moderate expression) and 2 (strong expression).
[2]β = regression coefficient.
[3]Log-rank test.
Abbreviations: SE, standard error; HR, hazard ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer; Cyt, cytoplasmic; MM, metastatic melanoma.

Table 4.5. Multivariate Cox regression analysis, including nuclear DLC1, on 5-year overall and disease-specific survival of 396 melanoma patients.

Variables[1]	Overall survival					Disease-specific survival				
	β[2]	SE	HR	95.0% CI	P-value[3]	β[2]	SE	HR	95.0% CI	P-value[3]
All melanoma (n=396)										
Sex	0.140	0.167	1.15	0.83-1.60	0.402	0.168	0.173	1.18	0.84-1.66	0.333
Age	0.183	0.162	1.20	0.87-1.65	0.261	0.211	0.169	1.24	0.89-1.72	0.211
AJCC	1.477	0.167	4.38	3.15-6.08	<0.001	1.556	0.174	4.74	3.37-6.67	<0.001
Nuclear DLC1	-0.473	0.168	0.62	0.45-0.87	0.005	-0.484	0.175	0.62	0.44-0.87	0.006
MM (n=120)										
Sex	0.152	0.230	1.16	0.74-1.83	0.510	0.207	0.232	1.23	0.78-1.94	0.373
Age	0.051	0.221	1.05	0.68-1.62	0.817	0.075	0.225	1.08	0.69-1.67	0.738
Nuclear DLC1	-0.718	0.251	0.49	0.30-0.80	0.004	-0.735	0.256	0.48	0.29-0.79	0.004

[1]Coding of variables: Age was coded as 1 (≤ 59 years) and 2 (> 59 years); sex was coded as 1 (male) and 2 (female); AJCC was coded as 1 (Stages I+II) and 2 (Stages III+IV); DLC1 was coded as 1 (negative-moderate expression) and 2 (strong expression).
[2]β = regression coefficient.
[3]Log-rank test.
Abbreviations: SE, standard error; HR, hazard ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer; MM, metastatic melanoma.

Table 4.6. Multivariate Cox regression analysis on 5-year overall and disease-specific survival of 396 melanoma patients.

Variables[1]	Overall survival					Disease-specific survival				
	β[2]	SE	HR	95% CI	P-value[3]	β[2]	SE	HR	95% CI	P-value[3]
All melanoma (n=396)										
Sex	0.142	0.167	1.15	0.83-1.60	0.393	0.167	0.173	1.18	0.84-1.66	0.333
Age	0.201	0.163	1.22	0.89-1.68	0.217	0.226	0.169	1.25	0.90-1.75	0.181
AJCC	1.436	0.169	4.21	3.02-5.86	<0.001	1.522	0.176	4.58	3.25-6.47	<0.001
DLC1	-0.529	0.180	0.59	0.41-0.84	0.003	-0.497	0.187	0.61	0.42-0.88	0.008
MM (n=120)										
Sex	0.151	0.230	1.16	0.74-1.82	0.511	0.208	0.232	1.23	0.78-1.94	0.369
Age	0.080	0.221	1.08	0.70-1.67	0.718	0.107	0.225	1.11	0.72-1.73	0.634
DLC1	-0.816	0.298	0.44	0.25-0.79	0.006	-0.869	0.308	0.42	0.23-0.77	0.005

[1]Coding of variables: Age was coded as 1 (≤ 59 years) and 2 (> 59 years); sex was coded as 1 (male) and 2 (female); AJCC was coded as 1 (Stages I+II) and 2 (Stages III+IV); DLC1 was coded as 1 (Cat. 1,2,3) and 2 (Cat. 4).
[2]β = regression coefficient.
[3]Log-rank test.
Abbreviations: SE, standard error; HR, hazard ratio; CI, confidence interval; MM, metastatic melanoma.

4.2.7 A preliminary examination into the relationship between Cten and DLC1 in melanomas

Figure 4.8 shows the correlation between Cten and DLC1 staining in 322 melanoma samples. For both cytoplasmic and nuclear DLC1, there was a positive correlation with Cten expression in primary tumours ($p = 0.005$ and 0.020, respectively). For all melanoma patients, there was a significant positive correlation between cytoplasmic DLC1 and Cten ($p = 0.017$), and although there was a trend for nuclear DLC1 and Cten, it was not significant.

Next, the samples were divided into 4 categories; (1) negative-moderate staining of Cten and DLC1; (2) negative-moderate DLC1 and strong Cten; (3) strong DLC1 and negative-moderate Cten; and (4) strong DLC1 and Cten staining. Kaplan-Meier survival analyses revealed a significant association between DLC1, Cten and the overall and disease-specific 5-year survival of all melanoma patients ($p = 0.019$ and 0.040, respectively for cytoplasmic DLC1, and $p = 0.002$ for nuclear DLC1, Fig. 4.9). Interestingly, for both cytoplasmic and nuclear DLC1, two main clusters were observed. Samples classified as Categories 1 and 2 (negative-moderate DLC1 staining but either negative-moderate or strong Cten staining) had a significantly worse overall and disease-specific 5-year survival compared to samples classified as Categories 3 and 4 (strong DLC1 expression but either negative-moderate or strong Cten expression).

As an initial investigation into whether or not Cten regulates the expression of DLC1, I used siRNAs to transiently knock down Cten in MMRU and

MMAN cells, and examined the effects on Cten and DLC1 mRNA and protein levels. As expected, Cten knockdown did not result in any changes in DLC1 expression (Fig. 4.10), supporting the theory that Cten does not regulate the expression of DLC1, but rather its activity.

Lastly, I also studied the mRNA expression of tensin1, tensin2 and tensin3 in a panel of melanoma cell lines as a first step to identify the tensin family member(s) most likely to bind DLC1 under normal conditions and counteract the actions of Cten, and found that while tensin2 expression was mostly unchanged, tensin1 expression was reduced, and tensin3 expression was increased, in melanoma cell lines compared to normal melanocytes (Fig. 4.11).

Figure 4.8. Correlation between DLC1 and Cten protein expression in 322 melanoma patient samples. (A) Cytoplasmic DLC1 and Cten protein levels positively correlated in primary melanomas (n = 228, p = 0.005) and in all melanomas (n = 322, p = 0.017), but not in metastatic melanomas (n = 94, p = 0.975, χ^2 tests). (B) Nuclear DLC1 and Cten protein levels positively correlate in primary melanomas (p = 0.020), but not in metastatic or all melanomas (p = 0.295 and 0.112, respectively, χ^2 tests).

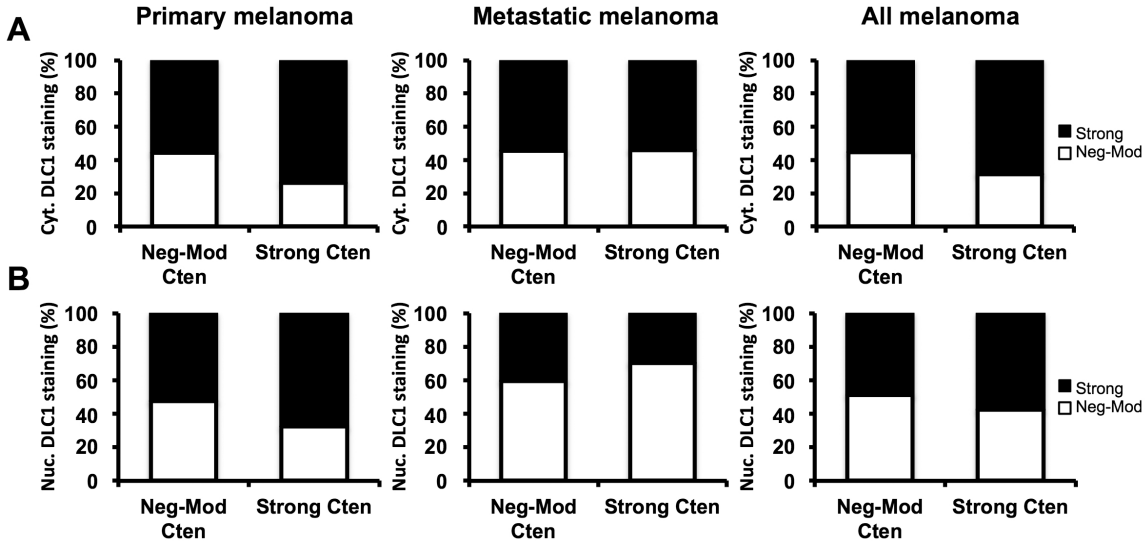


Figure 4.9. Correlation between DLC1, Cten and the 5-year survival of 305 melanoma patients. (A) Correlation between cytoplasmic DLC1 and Cten. Negative-moderate DLC1 expression (Cat. 1-2) was associated with a significantly poorer overall and disease-specific patient survival compared to strong DLC1 expression (Cat. 3-4), regardless of Cten expression (p = 0.019 and 0.040, respectively). (B) Correlation between nuclear DLC1 and Cten (p = 0.002).

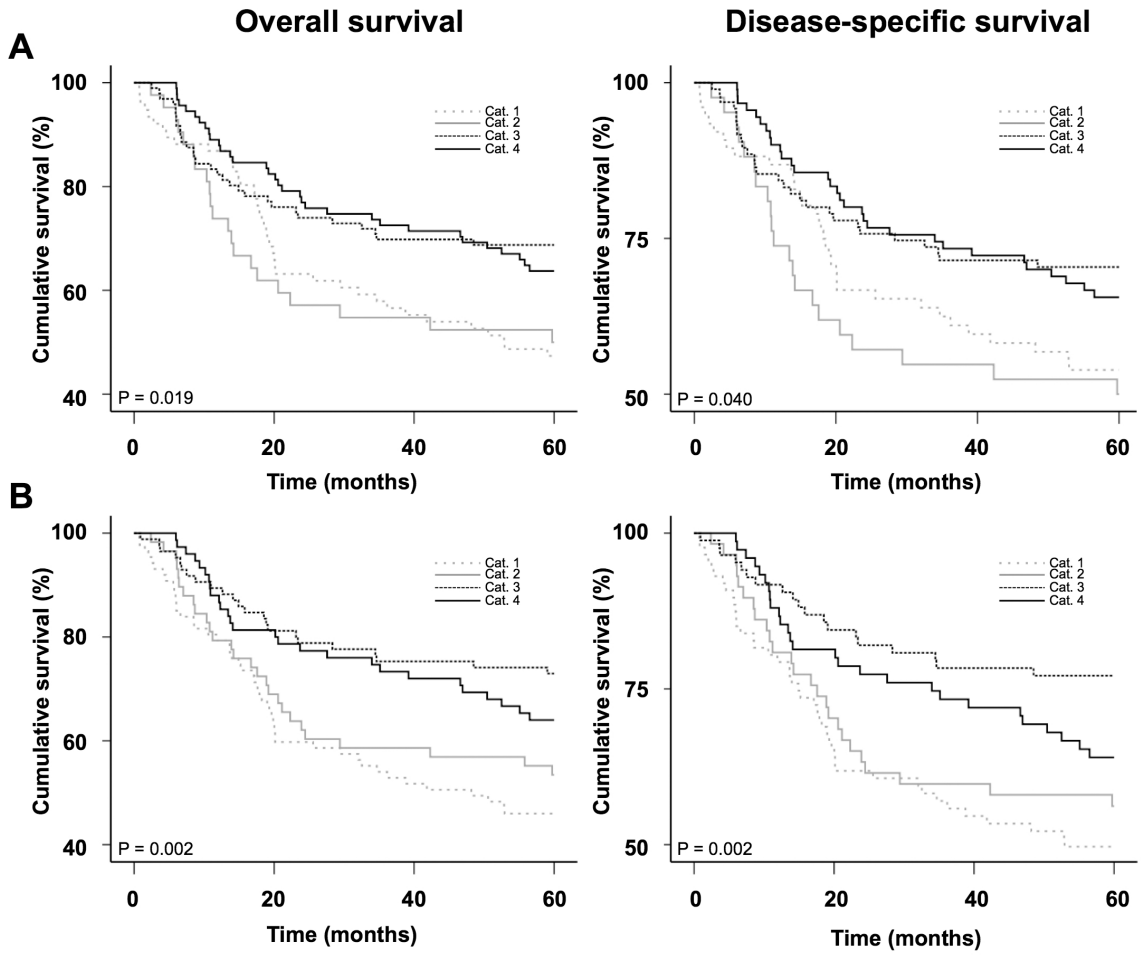


Figure 4.10. Effects of Cten knockdown on DLC1 mRNA and protein expression in MMAN and MMRU cells. (A) Western blot. Predicted molecular mass: Cten, 72 kDa, DLC1, 123 kDa, β-Actin, 42 kDa. (B) RT-qPCR results.

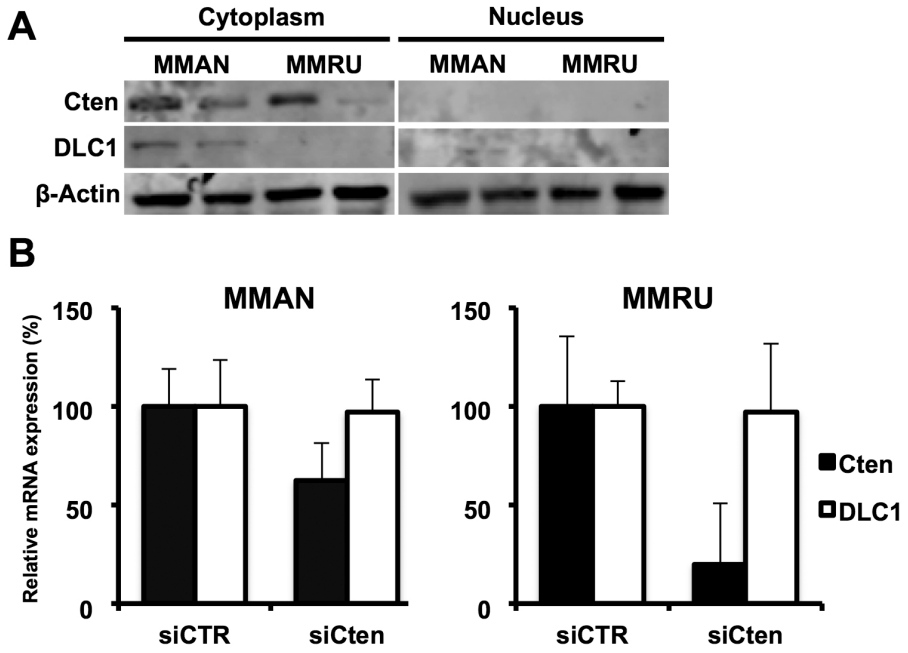
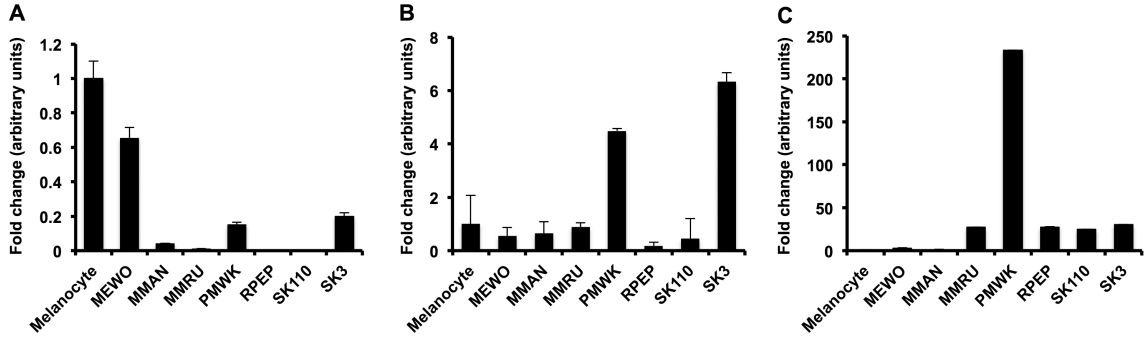


Figure 4.11. Tensin family member mRNA expression in melanoma cell lines. (A) Tensin1 mRNA expression is reduced in melanoma cell lines compared to melanocytes. (B) Tensin2 mRNA expression remains largely unchanged in melanoma cell lines. (C) Tensin3 mRNA levels are increased in melanoma cell lines compared to normal melanocytes.



4.3 Discussion

In the present study, I examined the expression status of DLC1, a known tumour suppressor, in melanoma progression. To my knowledge, this is the first report on DLC1 down-regulation in melanoma. While the vast majority of publications regarding DLC1 have focused on its expression in the cytoplasm, I here discovered that DLC1 was expressed both in the cytoplasm and nuclei in human melanocytic lesions (Fig. 4.1), and decided to investigate both further. Nuclear-localized DLC1, albeit less studied than its cytoplasmic counterpart, has recently been described in a handful of reports. Yuan *et al*. (2007) first reported that nuclear translocation of DLC1 preceded, and was required for, apoptosis in NSCLCs, and that, similar to our results for normal nevi, nuclear DLC1 was highly expressed in human non-neoplastic alveolar epithelial cells. Scholz *et al.* (2008) later showed that DLC1 was continuously shuttled between the cytoplasm and nuclei in cell lines, and yet another study from Chan *et al.* (2011) confirmed these results, and further reported that DLC1 localized to the focal adhesions did not partake in the shuttling, and that nuclear DLC1 was less efficient in exerting its tumour suppressor activities compared to cytoplasmic DLC1.

Supporting the proposed role of DLC1 as an inhibitor of cell migration and metastasis, as previously described in a number of cancers (Goodison *et al., 2005;* Ying *et al.,* 2007; Healy *et al.,* 2008; Ullmannova-Benson *et al.,* 2009; Cao *et al.,* 2012; Chen *et al.,* 2013)**,** I here found that DLC1 expression was reduced in metastatic melanoma compared to primary melanoma and nevi. Moreover, nuclear DLC1 expression was also down in PM compared to NN (Fig. 4.1),

indicating that loss of nuclear DLC1 may be an earlier event than cytoplasmic DLC1 loss in melanoma. If there is in fact a continuous shuttling of DLC1 between the cytoplasm and nuclei as suggested by Scholz *et al.* (2008) and Chan *et al*. (2011), and if Cten localized to the focal adhesions really does not participate in the shuttling, this could explain why we see a relatively stagnant expression of cytoplasmic DLC1 throughout melanoma progression (Fig. 4.1).

The notion that nuclear DLC1 loss precedes cytoplasmic DLC1 loss was further supported by the fact that cytoplasmic DLC1 expression was significantly lower in AJCC Stage IV tumours compared to Stage I-III tumours, whereas nuclear DLC1 expression was reduced in AJCC Stage III tumours already (Fig. 4.2). The importance of, and the mechanisms behind this phenomenon will, however, need to be more closely examined before any firm conclusions about the interplay between cytoplasmic and nuclear DLC1 in melanoma can be drawn. A weak association between nuclear DLC1 and primary tumour thickness was also observed, with tumours > 4.0 mm thick having *stronger* DLC1 expression than tumours ≤ 4.0 mm thick ($p = 0.046$, Table 4.2). While this might seem counterintuitive, it is possible that DLC1 expression is induced in a subset of thick primary tumours as an attempt to restore homeostasis in the cells and prevent metastasis. To be certain whether this is a true phenomenon, or whether it reflects the relatively low sample number for this group ($n = 59$), the analysis would have to be repeated in a larger cohort.

Both cytoplasmic and nuclear DLC1 were furthermore associated with the overall and disease-specific 5-year survival of all melanoma and metastatic

melanoma patients (Fig. 4.3), as well as with the disease-specific 10-year survival of all melanoma patients (Fig. 4.5). Univariate Cox regression analysis indicated that loss of either form of DLC1 was a risk factor for the 5-year survival of all melanoma and metastatic melanoma patients (Table 4.3), and multivariate Cox regression analyses showed that, when analyzed separately, both cytoplasmic and nuclear DLC1 were independent prognostic markers for the overall and disease-specific 5-year survival of all melanoma and metastatic melanoma patients (Tables 4.4 and 4.5).

Next, the correlation between cytoplasmic and nuclear DLC1, and their combined effects on patient 5-year survival was examined. For all melanoma patients, loss of either or both cytoplasmic or nuclear DLC1 (Categories 1-3) was associated with a significantly poorer survival compared to strong expression of both forms (Category 4, Fig. 4.7A). Interestingly, for metastatic melanoma patients, concurrent loss of both forms (Category 1) was associated with the worst survival outcome, whereas loss of either cytoplasmic *or* nuclear DLC1 (Categories 2 and 3) was associated with an intermediate survival outcome (Fig. 4.7B). This could imply that, similar to what has been reported in NSCLC (Yuan *et al.,* 2007), the tumour suppressor properties of cytoplasmic and nuclear DLC1 differ from one another in melanoma. Thus, simultaneous loss of cytoplasmic and nuclear DLC1 could confer additive or synergistic effects on cancer progression, metastasis and patient survival.

Even though both cytoplasmic and nuclear DLC1 were found to be independent prognostic risk factors for the 5-year survival of all melanoma and

metastatic melanoma patients when analyzed separately (Tables 4.4 and 4.5), the Kaplan-Meier analyses of their combined effects on patient survival indicate that strong expression of both cytoplasmic and nuclear DLC1 compared to loss of either or both forms is associated with an even better outcome (Fig. 4.7). Consequently, I repeated the multivariate Cox analysis using the combined data. I divided the samples into two groups; (1) loss of either or both forms of DLC1 (Categories 1-3); and (2) strong expression of both cytoplasmic and nuclear DLC1 (Category 4), and found that strong expression of both forms was a significant independent prognostic marker for the 5-year overall and disease-specific survival of all melanoma and metastatic melanoma patients (Table 4.6). In short, this means that while, for example, strong expression of cytoplasmic DLC1 is associated with a better survival outcome compared to negative-moderate cytoplasmic DLC1 expression, if nuclear DLC1 expression is concomitantly lost, this is then instead associated with a relatively poor outcome. Altogether, this indicates that for prognostic purposes, a combination of cytoplasmic and nuclear DLC1 protein expression would give more reliable results than the use of just one of the two.

As mentioned, DLC1 is the only protein known to physically interact with Cten in human cancers, and it has been proposed that Cten-DLC1 binding is the main mechanism through which Cten exerts its oncogenic properties. To examine if there was an association between Cten and DLC1 protein expression in melanoma, I divided all samples containing information regarding both DLC1 and Cten expression into 4 groups: (1) negative-moderate staining of Cten and

DLC1; (2) negative-moderate DLC1 and strong Cten; (3) strong DLC1 and negative-moderate Cten; and (4) strong DLC1 and Cten staining, and analyzed these groups using Kaplan-Meier survival curves (Fig. 4.9). Interestingly, for both cytoplasmic and nuclear DLC1, I observed two main clusters, with samples with negative-moderate DLC1 staining but *either* negative-moderate or strong Cten staining (Categories 1-2) having a significantly worse overall and disease-specific 5-year survival compared to samples with strong DLC1 expression but *either* negative-moderate or strong Cten expression (Categories 3-4), indicating that the effects of Cten on patient survival are dependent on the level of DLC1 protein expression. Thus, it can be speculated that even when Cten is strongly expressed, if cytoplasmic DLC1 levels are high, this is sufficient to saturate all Cten-DLC1 interactions, leaving some DLC1 molecules free to bind the full-length tensins and hydrolyze Rho-GTPases. Conversely, if DLC1 levels are low, it would only take low levels of Cten to interact with all available DLC1 molecules and inhibit their RhoGAP activity. Since there was a strong positive association between cytoplasmic and nuclear DLC1 levels in melanoma ($p < 0.001$, Fig. 4.6B), and since it has been shown that DLC1 is continuously shuttled between the cytoplasm and nucleus (Scholz *et al.,* 2008; Chan *et al.,* 2011), I hypothesize that the correlation between Cten and nuclear DLC1 may be an indirect effect of the direct binding of Cten to cytoplasmic DLC1, since, as discussed in Chapter 3.2.2, I did not detect nuclear Cten expression in melanocytic lesions.

As expected, I did not detect any effects on DLC1 expression after Cten knockdown, indicating that Cten does not regulate the expression of DLC1,

instead supporting the model proposed by Cao *et al.* (2012), who suggested that Cten regulates the *activity* of DLC1. While my overall results support this model, the preliminary RT-qPCR findings (Fig. 4.11) indicate that, unlike in MCF10A cells, Cten induction does not lead to a displacement of tensin3, but rather tensin1 in melanoma. This is supported by previous studies that have demonstrated that tensin3 behaves as an oncogene in melanoma cell lines (Qian *et al.,* 2009), and that tensin1 is down-regulated in clinical melanoma samples and immunoprecipitates with DLC1 in a melanoma cell line (Qian *et al.,* 2007; Hall *et al.,* 2009).

In addition to the TMA results, there are other data supporting a role for DLC1 in melanomas. Firstly, DLC1 is known to specifically interact with RhoA, RhoB, RhoC and Cdc42 (Healy *et al.,* 2008; Liao *et al.,* 2008), and RhoA, RhoC, and Cdc42 have all been positively associated with a metastatic phenotype in melanomas (Gómez del Pulgar *et al.,* 2005; Boone *et al.,* 2006). Secondly, as previously discussed, the PI3K/Akt signalling pathway is one of the major pathways implicated in melanomagenesis, and DLC1 has been found to be silenced by phosphorylation by the protein-serine/threonine kinase Akt both *in vitro* and *in vivo,* leading to disruption of its tumour- and metastasis suppression activities (Ko *et al.,* 2010). In breast cancer, DLC1 down-regulation has been found to cooperate with loss of the tumour suppressor PTEN, leading to enhanced cell migration, and the two proteins were found to physically interact *in vitro,* and to co-localize at the plasma membrane in MCF7 cells (Heering *et al.,* 2009). PTEN loss or inactivation is a relatively common event in melanoma

(Mirmohammadsadegh *et al.,* 2006), and it would be interesting to examine whether or not PTEN and DLC1 protein expressions correlate in melanoma. Intriguingly, in the same study it was also found that DLC1 down-regulation resulted in a slight increase in the phosphorylation, and hence activation, of Akt (Heering *et al.,* 2009), further indicating a role for DLC1 in PI3K/Akt signalling.

Lastly, as briefly mentioned in Chapter 1.1.1, loss of E-cadherin, a factor important for melanocyte-keratinocyte communication, has been associated with a loss of melanocyte homeostasis (Haass *et al.,* 2005). A recent study in prostate cancer cells found that DLC1 is capable of inducing E-cadherin expression via Rho signalling pathways, leading to suppressed cell invasion (Tripathi *et al.,* 2013). This is especially interesting since E-cadherin has been identified as a downstream target of Cten in two other studies (Albasri *et al.,* 2009; Al-Ghamdi *et al.,* 2013), leading me to speculate that the mechanism through which Cten regulates E-cadherin involves inactivation of DLC1 RhoGAP activity.

In conclusion, I showed here that DLC1 was expressed both in the cytoplasm and nuclei at all stages of melanocytic lesions, and that its expression was reduced in the progression from nevi and primary melanoma to metastatic melanoma. Loss of both forms of DLC1 correlated with the overall and disease-specific 5-year survival of all melanoma and metastatic melanoma patients, and concurrent loss of both cytoplasmic and nuclear DLC1 was associated with the worst survival outcome in metastatic melanoma patients. Furthermore, I demonstrated that loss of DLC1 expression correlated with a poorer 5-year melanoma patient survival irrespective of Cten expression, indicating that any

effects of Cten on patient survival are largely dependent on the level of DLC1 protein expression. While more work is still needed to elucidate the mechanisms behind the down-regulation of DLC1 as well as the modes of action of both cytoplasmic and nuclear DLC1, these results strongly support the notion that DLC1 is a tumour suppressor in melanoma.

5. General conclusions

5.1 Summary of findings and future directions

In Chapter 3, I analyzed the expression of C-terminal tensin-like protein (Cten), a focal adhesion protein expressed specifically in the prostate and placenta that has been recently found to be up-regulated and function as an oncogene in a number of cancers. Using tissue microarrays I showed that Cten was overexpressed in primary melanomas compared to normal and dysplastic nevi (Fig. 3.1), and correlated with AJCC stages, primary tumour thickness, location, and subtypes (Table 3.1). Strong protein expression of Cten was associated with a significantly poorer 5- and 10-year survival of primary melanoma patients compared to negative-moderate Cten expression (Figs. 3.2 and 3.3). Furthermore, I identified Cten as a novel independent prognostic marker for primary melanoma patients (Table 3.3), and *in vitro* studies supported the findings from the TMA study and indicated that Cten plays a role in cell proliferation, apoptosis, cell invasion and cell migration, and behaves like an oncogene in melanomas.

Since it is currently unknown exactly how Cten functions in carcinogenesis, in Chapter 4, I next investigated the protein expression of the RhoGAP and *bona fide* tumour suppressor Deleted in Liver Cancer-1 (DLC1) - the only protein known to physically interact with Cten. I detected both cytoplasmic and nuclear-localized DLC1, and found that while cytoplasmic DLC1 expression was reduced in metastatic melanomas compared to primary

melanomas and nevi, nuclear DLC1 expression was down in primary melanomas compared to nevi and then further down in metastatic melanomas (Fig. 4.1). Both cytoplasmic and nuclear DLC1 correlated with AJCC stages, with cytoplasmic DLC1 protein expression being decreased in AJCC Stage IV tumours compared to Stage I-III tumours, and nuclear DLC1 expression being down in Stages III+IV tumours compared to Stages I+II already, indicating that nuclear DLC1 loss may precede cytoplasmic DLC1 loss (Fig. 4.2). Down-regulation of either cytoplasmic or nuclear DLC1 was associated with a significantly worse 5-year survival for all melanoma and metastatic melanoma patients when analyzed separately (Fig. 4.3) and intriguingly, when analyzed together, I found that for metastatic melanoma patients, strong expression of both cytoplasmic and nuclear DLC1 was associated with the most favourable survival outcome, loss of either cytoplasmic or nuclear DLC1 was associated with an intermediate survival, and concomitant loss of both cytoplasmic and nuclear DLC1 was associated with the worst outcome (Fig. 4.7), indicating that the two forms could have different functions and may cooperate in suppressing melanoma. Accordingly, I found that loss of either or both cytoplasmic and nuclear DLC1 was an adverse independent prognostic factor for all melanoma and metastatic melanoma patients (Table 4.6).

As a preliminary investigation into the relationship between Cten and DLC1 in melanoma, I examined the correlation between their protein expressions and melanoma patient survival. In agreement with my hypothesis that the effects of Cten on patient survival are dependent on DLC1, I found that negative-moderate expression of DLC1, independent of Cten expression, was significantly

associated with a poorer 5-year survival compared to strong expression of DLC1 and either negative-moderate or strong expression of Cten (Fig. 4.9). As discussed in Chapter 4, Cten has been hypothesized to function by interacting with DLC1 and inhibit its RhoGAP activity, rather than its expression (Cao *et al.,* 2012), and this was supported by RT-qPCR and Western blot analyses, which revealed no significant changes in DLC1 mRNA or protein expression after Cten knockdown (Fig. 4.10).

I conclude that while all of these results strongly support a role for both Cten and DLC1 in the development and metastasis of human cutaneous melanomas, much more research in still needed to clarify their exact roles in this malignancy. The *in vitro* experiments performed for Cten in Chapter 3, would need to be repeated in a larger number of melanoma cell lines, and with an overexpression plasmid in addition to siRNA, and the same experiments would have to be performed for DLC1 as well. Co-immunoprecipitation of Cten and DLC1 would need to be performed to determine whether or not they interact in melanoma cell lines. The modes of action of cytoplasmic and nuclear DLC1 will need to be studied in more detail, as well as the mechanism through which DLC1 is down-regulated in melanomas. If these initial experiments were successful, the next step would be to examine the effects of Cten and/or DLC1 overexpression and knockdown *in vivo.*

Since the most commonly deregulated pathway in melanomas is the MAPK signalling pathway (Omholt *et al.* 2003; Haluska *et al.* 2006; Saldanha *et al.* 2006; Gray-Schopfer *et al.* 2007; Platz *et al.* 2008), and since Cten has been

positively associated with BRAF V600E mutations in colorectal cancer (Al-Ghamdi *et al.,* 2011) and has been implicated in MAPK signalling *in vitro* in various normal and cancer cell lines (Katz *et al.,* 2007; Cao *et al.,* 2012; Pylayeva & Giancotti, 2012; Hung *et al.,* 2013), I propose that experiments designed to elucidate the effects of knockdown/overexpression of the different components of this pathway on Cten expression and DLC1 activity should be performed. The effects of added growth factors known to activate this pathway, or targeted inhibitors against NRAS, BRAF, MEK or ERK (including Vemurafenib and other drugs currently on the market) on Cten expression should be investigated *in vitro*, and if successful, the effects of drug treatments in combinations with Cten and DLC1 knockdown or overexpression should be examined *in vivo.*

Similarly, both Cten and DLC1 have been associated with PI3K/Akt signalling (Heering *et al.,* 2009; Ko *et al.,* 2010; Hung *et al.,* 2013) another major signalling pathway frequently deregulated in melanoma, and this will also have to be examined more closely.

Lastly, I propose that the expression status and roles of the full-length tensin family members, especially tensin1 and tensin3, which I found to be down- and up-regulated, respectively, at the mRNA level in melanoma cell lines compared to normal melanocytes, should be examined more closely.

I truly believe that Cten and DLC1, either together or separately, have the potential to be of great value, not only as prognostic markers for melanoma patient survival, but one day as therapeutic targets in combination with some of the promising FDA-approved drugs already on the market, and hope that the

work presented here can support future research efforts focused on these proteins and their roles in melanomagenesis.

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