CHALMERS

Inactivation of isoprenylcysteine carboxyl methyltransferase reduces proliferation of BRAF and NRAS mutated human melanoma cells

Master of Science Thesis in the Master Degree Program, Biotechnology

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ABSTRACT

Malignant melanoma is the deadliest form of skin cancer, with an increasing incidence worldwide. Surgical removal of the tumors can be an effective first line of defense. Nonetheless, when the tumors have metastasized, the prognosis is extremely poor and not influenced by systematic therapy with cytotoxic drugs. The two most common mutations in malignant melanoma are *BRAF* and *NRAS*, with the prevalence of 47% and 21%, respectively. Newly developed selective inhibitors have shown profound clinical results in patients that carry the $BRAF^{V600E}$ mutation. However, the patients develop secondary resistance and subsequent disease progression. Also, tumors caused by mutations of *NRAS* or other oncogenes are accelerated by the BRAF selective inhibitors. It is therefore of paramount importance to explore alternative or combinatorial therapies to mitigate the incurred resistance mechanism. Isoprenylcysteine carboxyl methyltransferase (ICMT), is an endoplasmic reticulum membrane protein that catalyzes post-translational carboxyl methylation of proteins encoding a C-terminal CAAX motif (C, cystein, A, aliphatic amino acids, X, any amino acid). CAAX proteins have proved important for the function of various cancer cells, and preclinical studies have shown that inactivation of ICMT might be a potential target for anticancer drugs.

In this study we evaluated the impact of inactivating *ICMT* in *BRAF* and *NRAS* mutated human melanoma cell lines, alone and in combination with *BRAF* inactivation and thereby determine if *ICMT* inactivation or co-inactivation of *ICMT* and *BRAF* would reduce the proliferative ability of these cells. By using lentiviruses expressing *ICMT*- and *BRAF*- specific short hairpin (sh) RNA, we knocked down expression of *ICMT* and *BRAF* independently and in combination in these mutated melanoma cell lines.

Inactivation of *ICMT* or *BRAF* alone significantly reduced the proliferation of *BRAF* and *NRAS* mutated human melanoma cell lines. The simultaneous inactivation of *ICMT* and *BRAF* resulted in a significant reduction of proliferation in these cells. However, the reduction did not exceed independent inactivation of *ICMT* or *BRAF*. These results indicate that targeting ICMT in human malignant melanoma could be an attractive strategy, which will be further explored.

Key words: ICMT, BRAF, NRAS, human malignant melanoma

INTRODUCTION

Malignant melanoma

Malignant melanoma arises from melanocytes, which are specialized pigment cells that produce melanins, the pigments that determine the skin and hair color [1]. In the skin, melanocytes are located in the basal layer of the epidermis and in the hair follicles. Their homeostasis is regulated by epidermal keratinocytes [2]. Melanocytes play an instrumental role in protecting our skin from the harmful effects of ultraviolet (UV) radiation and by that skin cancer. When the skin is subjected to UV radiation, keratinocytes will secrete factors that regulate melanocyte survival, proliferation, differentiation and motility, by stimulating melanocytes to produce melanin. People who lack functional melanocytes in various pigmentary disorders are hypersensitive to UV radiation [3]. However, melanocytes are the precursors of melanoma, one of the most aggressive and treatment resistant types of cancer.

Statistics have shown that melanoma incidences have more than tripled in the Caucasian population in the last 20 years and melanoma is currently the 6th most common cancer in USA, with estimated deaths of 8700 (5670 men and 3030 women) in 2010 [4]. If melanoma is diagnosed in an early stage it can be cured by surgery, and about 80% of cases are dealt with this way. However, progression occurs in around 20% of cases to an aggressive metastasizing form of the disease that is refractory to current therapies and is associated with extremely poor prognosis, with a median survival rate of 6 months and a 5-year survival rate of less than 5% [5].

Current therapies and their limitations

As it stands today, there are very few FDA-approved treatments for metastatic melanoma [6, 7]. None of the current treatments of biological agents and conventional chemotherapy agents clearly alter the progression of the disease. Interferon a2b (IFN-a2b) is the most commonly used adjuvant immunotherapy for stage III melanoma in the USA. In large scale randomly observed drug trials, IFN-a2b only demonstrated 10-20% improvement in relapse-free survival, but with no clear effect on the mortality rate of the disease [6, 8]. Interleukin-2 (IL-2) is approved to battle stage IV melanoma, based on its durable response in 10-20% of patients; it is however linked to sever toxicities [9, 10]. The only FDA-approved conventional cytotoxic chemotherapy is the alkylating agent dacarbazine (also termed DTIC), where responses are seen in about 5-10% of patients but are generally short-lived [6].

Development and progression of cutaneous melanoma

Mutations in genes responsible for growth regulation, production of growth factors and the loss of adhesion receptors all lead to a dysfunctional intracellular signaling in melanocytes, thus allowing them to avoid the clutches of keratinocytes who regulate them [11]. Therefore, these mutated melanocytes can proliferate and spread. According to the Clark model (Figure 1), which describes the progression from normal melanocytes to the malignant melanoma state [12], the first phenotypic change is the formation of a nevus or common mole. The proliferation of melanocytes can be localized to the epidermis (junctional nevus), the dermis (dermal nevus) or overlapping both parts (compound nevus). Nevi are conventionally benign but can develop into a dysplastic form, often a precursor of malignant melanoma. Dysplastic nevi can advance to the radial-growth-phase (RGP) melanoma, which entails the ability to proliferate intra-epidermally. RGP cells can advance to the vertical-growth-phase (VGP), a more aggressive and menacing stage where the cells have metastatic capability, with nests of cells invading the dermis and widening the papillary dermis. Numerous tumor-promoting events, including activation of oncogenes and inactivation of tumor suppressor genes, lead melanocytes through this transformation.

The need to pass through all of these individual phases in the model is not altogether necessary; isolated melanocytes or nevi can develop into either RGP or VGP phase and from there both can advance directly to metastatic malignant melanoma [13].

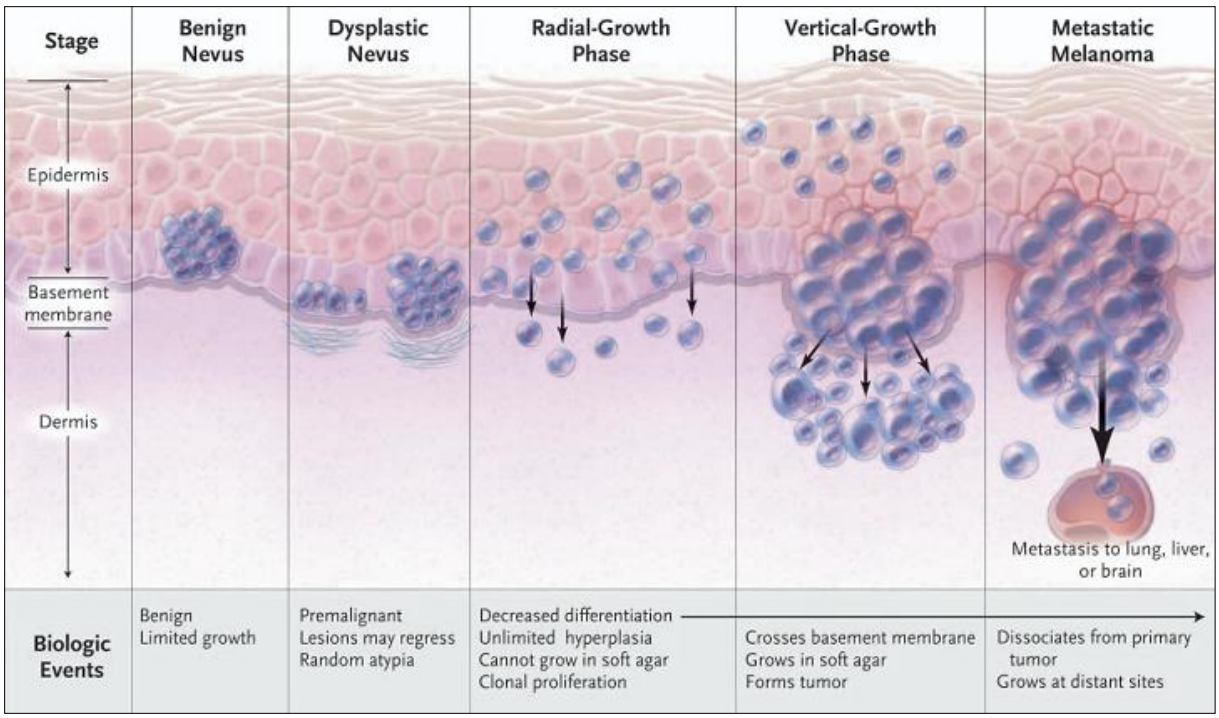

Figure 1: The Clark model [13]

The MAPK pathway activation in melanoma

Since entering the era of high-throughput genomic analyses, there has been an exponential growth in the understanding of the biology and molecular genetics of malignant melanoma, with the discovery of signaling pathways instrumental to melanoma initiation and development.

The tightly regulated mitogen-activated protein kinase (MAPK) pathway, also known as RAS/RAF/MEK/ERK pathway, plays an important role in mediating extracellular signals from the cell membrane to the nucleus via a cascade of phosphorylating events. In normal melanocytes, the MAPK pathway regulates senescence, differentiation, survival and proliferation, while in melanoma it is constitutively activated, and the favored outcomes are survival and proliferation [1].

The MAPK signaling pathway is triggered by growth factor stimulation of membrane-bound receptor tyrosine kinases (Figure 2A). This activates a small transmembrane G protein called RAS. The activation of RAS initiates the downstream signaling pathway by recruiting RAF, a serine/threonine protein kinase. Once RAF is activated, it phosphorylates and activates mitogen and extracellular signal-regulated protein kinase kinase (MEK), which consecutively activates a third protein kinase called extra cellular signal-regulated protein kinase (ERK).

There are three *RAS* (*HRAS*, *KRAS*, *NRAS*) and three *RAF* (*ARAF*, *BRAF*, *CRAF*) genes in humans that encode protein kinases carrying the same name [1]. The most recent sequencing data on somatic mutations in cancer, reveal that 47% of melanoma carry oncogenic mutations in *BRAF* [14]. The most common *BRAF* mutation (Figure 2B), in 90% of cases, is a glutamic acid substitution for valine at position 600 (V600E) [15]. *BRAF* has been shown to be mutated in a wide range of other cancers, where the mutation has been detected in about 40% of papillary thyroid cancer, 10% of colorectal cancers (CRC) and it has also been discovered in small percentages in several other tumor types [15, 16]. *NRAS* is mutated in 21% of melanomas (Figure 2B), the most prevalent substitution being leucine for glutamine at position 61 (Q61L) [14]

These recent genetic discoveries of the melanoma disease have fueled the search for therapeutics that selectively targets this pathway.

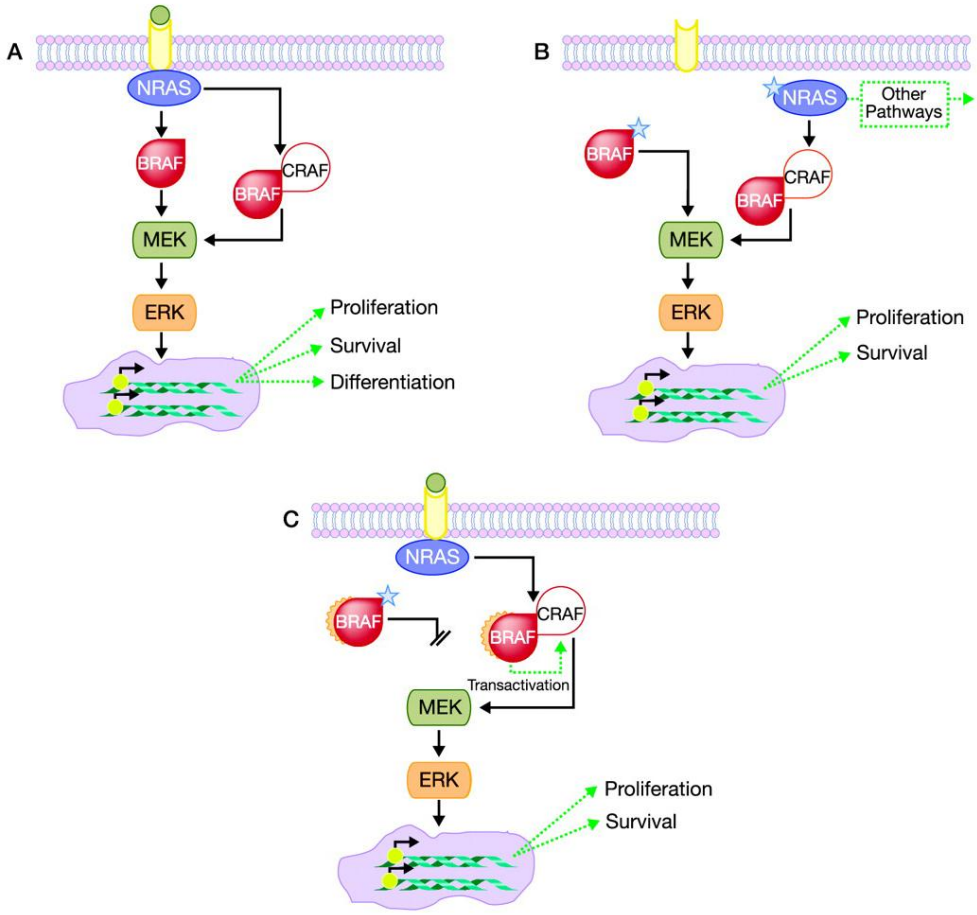

Figure 2: Targeting BRAF in the MAPK pathway [28]

(A) The MAPK pathway signaling, is initiated by extracellular signaling that acts through membrane bound RAS that activates BRAF, which signals through MEK (mitogen-activated protein kinase kinase) to ERK (extracellular-signal-regulated kinase) resulting in the activation of transcription factors involved in cell fate determination.

(B) Mutations in either BRAF or NRAS lead to constitutive activation of the pathway. These mutations are mutually exclusive. While mutationally activated BRAF depends on MEK/ERK for signaling, mutant RAS signals through CRAF to activate MEK/ERK and through various other pathways.

(C) BRAF specific inhibitors block kinase activity of BRAF. However, transactivation of CRAF can occur in cells with WT BRAF or activating mutations in RAS leading to reactivation of the pathway and resistance to the inhibitor.

Targeting BRAF in malignant melanoma

The breakthrough in molecular genetics and the discovery that BRAF mutations are found in a majority of melanoma incidences [15], motivated the research community to focus its efforts on targeting cancer-specific oncogenes and design compounds that inhibit their activity.

The first clinical BRAF inhibitor candidate to emerge from that effort, sorafenib (Naxayar, Bayer), gave poor results, even when coupled with conventional chemotherapeutics. The

failure of sorafenib is associated with its non-specific broad-spectrum (Pan-RAF) kinase inhibitor activity and its developed toxicity [17, 18]. Therefore, a search for a highly selective and active BRAF inhibitor took off. A structure-guided design approach was employed based on the fact that a significant proportion of BRAF-mutated melanomas have the same mutant allele (V600E) [15]. PLX4072 was developed and proved to have a considerable effect by tumor regression in xenograft animal models and growth delay in animal cell models, without significant toxicity [19]. For clinical trials in human melanoma an oral drug similar in structure, PLX4032, was developed [20]. The phase I trials of this drug, with patients harboring BRAF V600E induced metastatic melanoma, resulted in complete or partial tumor regression in 81% of patients with an estimated median progression-free survival rate of 7 months [21]. These groundbreaking results provide the first convincing evidence that oncogene-targeted approach is valid for metastatic melanoma. Side effects still occur with the drug, such as rashes, photosensitivity, fatigue, hair-loss, joint pain and cutaneous squamous cell carcinomas (more than 30% of patients) [21], but they are generally manageable.

Despite some promising clinical results, it is transient, and resistance eventually develops in all patients (Figure 2C), typically within a year following treatment [21]. Recent studies have shed light into the phenomena of the acquired resistance melanoma develops against the BRAF inhibitor, and revealed that the resistance is based on multiple mechanisms. Resistant melanomas can bypass BRAF by switching to CRAF and reactivate the MAPK pathway [22]. The resistance is acquired by overexpression of tyrosine kinase receptors that promote survival and acquired mutations in NRAS [22, 23]. It has also been shown that MEK can be activated directly by COT kinase, where these resistant clones overexpressed the kinase, circumventing the need for RAF activation [24]. Furthermore, the inhibitor paradoxically increases RAS downstream signaling in cells lacking BRAF mutations; non cancerous cells and tumor cells with wild type BRAF [25-27].

The recent clinical data in patients with metastatic melanoma [21] demonstrate the promise of BRAF drugs, but also emphasize the need to fully understand this pathway to overcome the many mechanisms involved for the development of effective alternative or combination therapies tailored to the specific genetic profile of future patients.

Post-translational processing of CAAX-proteins

A number of proteins encoding a C-terminal CAAX motif (C, cystein, A, aliphatic amino acids, X, any amino acid) that play key roles in biological regulatory events undertake a complex series of post-translational modifications[29]. This post-translational pathway (Figure 3) is initiated by the addition of an isoprenoid lipid to a cysteine residue, the C of the CAAX motif. Either a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid is covalently attached to this cysteine by protein farnesyltransferase (FTase) or protein geranylgeranyltransferase-I (GGTase-I), respectively [30]. The isoprenylated CAAX-proteins are directed to the endoplasmic reticulum (ER), where the three C-terminal amino acids (-AAX) are cleaved off by the membrane bound protein, RAS converting enzyme (RCE1). Finally, following the endoproteolysis step, the newly exposed isoprenylated cystein is methylated by Isoprenylcystein carboxylmethyltransferase (ICMT), another integral ER

membrane protein [31]. The result of this process is a protein that contains a prenylated and methylated cysteine at its C terminus. The modified CAAX-proteins are then directed to their appropriate subcellular location. Several studies have shown that the post-translational processing renders the protein hydrophobic and stimulates interactions with membranes and with other proteins [32, 33].

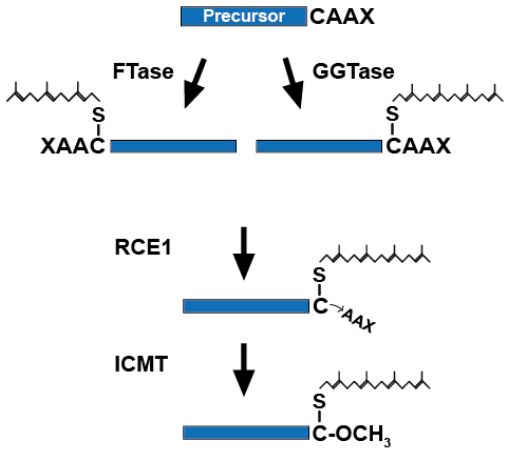

Figure 3: Post-translational modification of CAAX proteins

CAAX proteins undergo three post-translational processing steps at a carboxyl-terminal CAAX motif: 1. isoprenylation by farnesyltransferase (FTase) or geranylgeranyltransferase (GGTase-I); 2. removal of the –AAX by RAS converting enzyme (RCE1); and 3. methylation of the cysteine residue by isoprenylcysteine methyltransferase (ICMT). The processing renders the protein hydrophobic and stimulates interactions with membranes and with other proteins.

Carboxyl methylation of CAAX-proteins

The biological role of prenylation is quite well known, while the impact of C-terminal methylation in CAAX-protein activity is yet to be fully characterized. Methylation has been associated as an important contributor in protein stability, interactions with other proteins and localization [34]. Cell migration and cell adhesion have been noted to be impaired in cells with reduced levels of ICMT. This is caused by a decreased activation in RHOA and RAC1, where lower levels of ICMT yield increased expression of RHO GPD-dissociation inhibitor, who inhibits both RAC1 and RHOA [35].

The generation of a mouse model with a knockout allele for *Icmt*; gene that encodes for Icmt, has given further information regarding the function of ICMT [36]. Inactivation of *Icmt* with conditional deletion of *Icmt* alleles was shown to mislocalize all RAS oncoproteins and reduce RAS-transformation *in vitro* and *in* vivo [37, 38]. In the same study they also found that *Icmt* inactivation dramatically reduces transformation of mouse embryonic fibroblast (MEF) cells induced by $BRAF^{V600E}$ [37]. Interestingly, BRAF is not a CAAX-protein which means that ICMT deficiency affects the cells via CAAX-proteins downstream of BRAF.

ICMT is a therapeutic target

Pharmacological targeting of enzymes involved in the post-translational modification of CAAX-proteins has promise of an effective anticancer strategy. Much of the effort has been

spent on designing inhibitors against prenyltransferases, predominantly FTase [39]. The results from the clinical trials have been disappointing. The failure of FTase inhibitors (FTIs) has been attributed to 'alternate prenylation', where GGTase can prenylate the substrates of FTase when the activity of the latter is blocked [40].

Because of the relative failure of FTIs, the focus has moved towards the downstream enzymes involved in post prenylation, RCE1 and ICMT. These downstream enzymes are responsible for post-translational modifications of both farnesylated and geranylgeranylated CAAX-proteins, so blockade at these post-prenylation processing steps would not be subject to alternate modification bypass. However, inactivation of RCE1 has been shown to accelerate the growth of some malignancies, such as myeloproliferative disease [41].

Several ICMT inhibitors have been developed, and the most promising inhibitor to emerge is called Cysmethynil. It is a small molecule, indole-based inhibitor, which has shown significant antitumor activity in cancer cells and in a prostate cancer xenograft mouse model [42, 43].

Icmt inactivation inhibits the development of Braf-induced lung tumors

In a previous preliminary experiment by this group, to investigate the impact of inactivating *Icmt* in the pathogenesis of Braf induced lung cancer in mice. They bred mice with a conditional *Icmt* knockout allele ($Icmt^{fl}$) with mice harboring a *Cre*-inducible $Braf^{CA/CA}$ allele to generate $Braf^{CA/+}$ $Icmt^{fl/+}$ and $Braf^{CA/+}$ $Icmt^{fl/fl}$ mice. *Cre* recombinase was used to activate $Braf^{V600E}$ expression and inactivate one or two copies of *Icmt*, respectively. The knockout of *Icmt* significantly reduced tumor formation (Fig. 4).

However, several answers have still yet to be address. First, the lung tumors are benign adenomas, and not cancer. Second, *Braf* mutations are not common in human lung tumors. Finally, the tumors are derived from mouse cells, and it is not clear whether human cancer cells will respond in the same way.

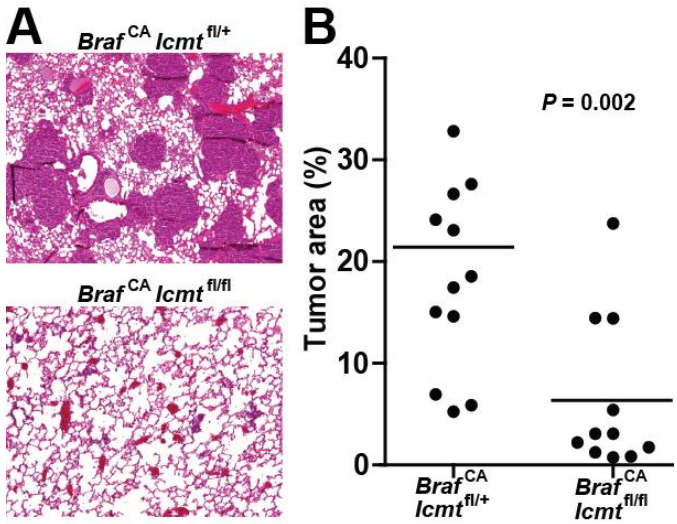

Figure 4: *Icmt* inactivation reduced Braf induced lung tumor growth in mice

(A,B) Initial experiment showing reduced tumor area in lungs of *Braf*$^{CA/+}$ *Icmt*$^{fl/fl}$ compared to *Braf*$^{CA/+}$ *Icmt*$^{fl/+}$ mice, 8 weeks after inhalation of Cre-adenovirus to activate *Braf*^{V600E} expression and inactivate the expression of two or one copy of *Icmt*, respectively.

SPECIFIC AIM

BRAF kinase inhibitor, PLX4032, has shown promising clinical results but resistance develops in all patients. Due to the fact that ICMT methylation affects both BRAF and NRAS signaling, the aim is to test the efficacy of knocking down *ICMT* expression with shRNA lentiviruses, alone and in combination with knocking down *BRAF* expression in both BRAF and NRAS human melanoma cell lines.

The specific aims for the experiments were:

- **To test the hypothesis, that inactivation of *ICMT* reduces the proliferation of BRAF and NRAS mutated human melanoma.**
- **To test the hypothesis, that a combination of *BRAF* and *ICMT* inactivation reduces the proliferation of BRAF and NRAS mutate human melanoma to a greater extent than either inactivation alone**

MATERIALS AND METHODS

Cell lines and culture conditions

All human melanoma cell lines were maintained in low glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 1% Non Essential Amino Acids (NEAA), 1% glutamine, 1% penicillin/streptomycin, 0,5% of 0,1 M β-mercaptoethanol in 37°C humidified, 5% CO_2 incubator.

Cell proliferation assay and shRNA transfections

The cell proliferation experiments were performed on a panel of mutated BRAF and NRAS human melanoma cell lines. For transient transfection of each cell line, a total of 3×10^5 cells were seeded in four unique 60 mm plates covered in DMEM/10% serum for 18 hours. From there, these four plates were transfected at the range of 3-4 Multiplicity Of Infection (MOI) with lentiviruses expressing; control-, *ICMT*-, *BRAF*- or a combination of *ICMT/BRAF* specific short hairpin (sh)RNA in the presence of 5 μg ml^{-1} polybrene, followed by media change to growth media after 24 hours. The cells were maintained in growth media for 2 days, after which the transfected cells were seeded in 12-well plates (triplicates, 2×10^4 cells/well), each 12-well plate accounting for a unique cell counting day in the cell proliferation assay. Stable transfection was carried out in the same manner but in the presence of 4 μg ml^{-1} puromycin for selection. The cells were counted and assayed for viability, size and circularity via trypan blue exclusion on every two day intervals, using a VI-CELL Cell Viability Analyser (Beckman Coulter), following manufacturer's specifications. The cells were photographed by using a Leica DMI light microscope with Leica DC200 digital camera (Leica Microsystems Ltd, Switzerland).

Quantitative Real-Time PCR

RNA extracted from the human malignant melanoma cells by RNeasyKit (Qiagen) was reverse transcribed to generate cDNA using iScript cDNA synthesis kit (BioRad). Quantitative real-time PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems) and TaqMan Gene Expression Assay probes (Applied Biosystems) on a 7900HT Fast Real Time PCR system (Applied Biosystems). TaqMan probes used for detection were *ICMT* Taq, *BRAF* Taq and *GAPDH* Taq, all specific for human samples.

Data were normalized to reference levels in the samples in duplicates. Relative expression was calculated using the delta-*C*t method using the following equation: ΔCt (Sample) = *C*t (Target) – *C*t (Reference); relative quantity = $2^{-\Delta Ct}$

shRNA constructs used (pLKO.1)

The shRNA constructs used were shICMT(1) (TRCN0000035269 , NM_012405.2-464s1c1, CCGGGCTGCTCTTTCTTCTTGGTTACTCGAGTAACCAAGAAGAAAGAGCAGCTTTTTG), shICMT(2) (TRCN0000035272, NM_012405.2-797s1c1, CCGGGATCGAACAGAAGAAGAAGAACTCGAGTTCTTCTTCTTCTGTTCGATCTTTTTG), shICMT(3) (TRCN0000303341, NM_012405.3-1139s21c1, CCGGCATGGGTGAAAGACGAGTAAGCTCGAGCTTACTCGTCTTTCACCCATGTTTTTG), shICMT(4) (TRCN0000303342, NM_012405.3-269s21c1, CCGGATAGCCATCCGAGCTTGTTTCCTCGAGGAAACAAGCTCGGATGGCTATTTTTTG), shICMT(5) (TRCN0000310659, NM_012405.3-887s21c1, CCGGACGGGCCTGCCTTTCATAAAGCTCGAGCTTTATGAAAGGCAGGCCCGTTTTTTG), shBRAF (1) (TRCN0000006290, NM_004333.2-838s1c1, CCGGCCGCTGTCAAACATGTGGTTACTCGAGTAACCACATGTTTGACAGCGGTTTTT), shBRAF (2) (TRCN0000195066, NM_004333.3-1960s1c1, CCGGCAGCTTTCAGTCAGATGTATACTCGAGTATACATCTGACTGAAAGCTGTTTTTTG), shBRAF (3) (TRCN0000196844, NM_004333.3-1931s1c1, CCGGGTCATCAGAATGCAAGATAAACTCGAGTTTATCTTGCATTCTGATGACTTTTTTG), shBRAF (4) (TRCN0000196918, NM_004333.3-236s1c1, CCGGGAACATATAGAGGCCCTATTGCTCGAGCAATAGGGCCTCTATATGTTCTTTTTTTG), shBRAF (5) (TRCN0000231130, NM_004333.4-1277s21c1, CCGGTTACCTGGCTCACTAACTAACCTCGAGGTTAGTTAGTGAGCCAGGTAATTTTTG), pLKO.1-puro Control Transduction Particles.

Graphs and statistics

Data were plotted as mean $\pm$ SEM by using GraphPad Prim 5 software (version 5.02, GraphPad Software). Differences between groups were determined by one-way ANOVA. Graphs were made with GraphPad Prim 5 software and Adobe Illustrator.

RESULTS

Selection of a specific *ICMT*-shRNA

An independent pilot experiment was conducted to select a construct that efficiently reduced mRNA levels of *ICMT*. A panel of five shRNA lentiviral transduction clones targeting *ICMT* was used to transfect a single human BRAF mutated cell line (SKMEL28). The sh5272 construct achieved the highest knock down efficiency of 85% (Figure 5) and was chosen to use for further experiments. The shRNA constructs targeting *BRAF*, where previously validated by the vendor, and the constructs who reached the highest knock down efficiency according to the vendor where chosen.

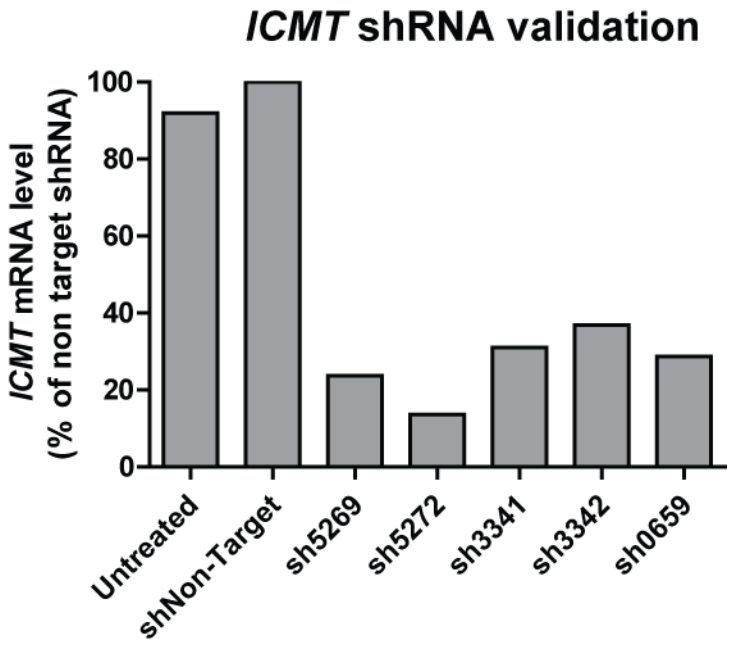

Figure 5: Selection of shRNA constructs targeting *ICMT*
The mRNA levels of *ICMT* in BRAF mutated human melanoma cell line transfected with a panel of shRNA constructs targeting *ICMT*. The levels of mRNA are normalized to control. The construct sh5272 gave the highest knock down efficiency of 85% and was selected for continuation of the knock down experiments.

Inactivation of *ICMT* significantly reduces cell proliferation in BRAF and NRAS mutated human melanoma cell lines

To assess the impact of *ICMT* inactivation, alone and in synergy with *BRAF* inactivation on human melanoma cell proliferation, we introduced shRNA constructs targeting *ICMT*, *BRAF* and *ICMT/BRAF* into BRAF and NRAS mutated human melanoma cell lines. The proliferation assay (Figures 6B and 7B) demonstrates that a stable knockdown of *ICMT* leads to a dramatic reduction in proliferation in both BRAF and NRAS mutated cell lines compared to control. The growth difference between the groups starts to show significance on day four of the experiment, and at day 6 the *P* value is <0.001. TaqMan analyses (Figure 6C and 7C), determined that the knock down efficiency of *ICMT* was between 85-90% and remained constant from the early time point (Day 2) to the late time point (Day 6). Interestingly, knock down of *BRAF* in the BRAF mutated melanoma cell line reduced the proliferation slightly

more (Figure 6B), with a statistical significance noted on day 6, compared to *ICMT* and *BRAF/ICMT* knockdown of the same cell line. In the NRAS mutated melanoma cell line, the reverse was seen. Where the *BRAF* knock down in that cell line led to a lower reduction of proliferation (Figure 7B) compared to *ICMT* and *BRAF/ICMT* knockdown. The co-inactivation of *BRAF* and *ICMT* did not prove to have a greater effect on the reduction of the proliferative ability in these two cell lines than the depletion of *ICMT* alone (Figure 6B and 7B). The knock down efficiency of *BRAF* in the BRAF mutated cell line, reaches 50% level on the later time point (Day 6) (Figure 6D). While the knock down efficiency of *BRAF* in the NRAS mutated cell line drops from 65% in the earlier time point to 45% in the later one (Figure 7D).

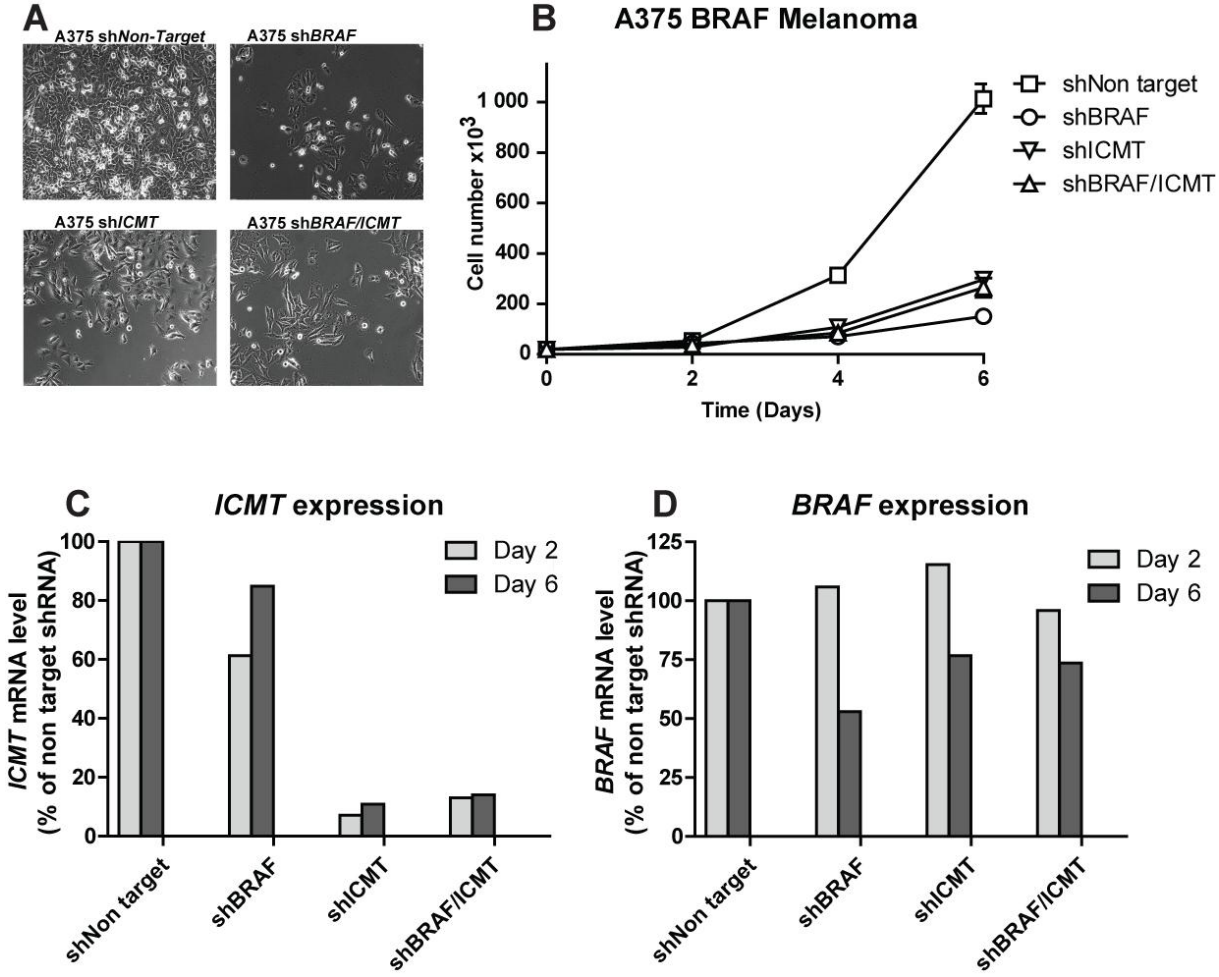

Figure 6: Inactivation of *ICMT* significantly reduces cell proliferation in BRAF mutated human malignant melanoma cell line

(A) Photographs of A375 BRAF malignant human melanoma cell line at day 7 after introduction of shRNA targeting empty vector, *BRAF*, *ICMT* and *BRAF/ICMT*.

(B) Proliferation assay of A375 BRAF malignant human melanoma cell line treated with shRNA targeting empty vector, *BRAF*, *ICMT* and *BRAF/ICMT*. Data show the mean of a single treatment assayed in triplicates, error bar represent the standard deviation of the triplicates.

(C) *ICMT* expression at day 2 and day 6 of A375 BRAF malignant human melanoma cell line treated with shRNA targeting empty vector, *BRAF*, *ICMT* and *BRAF/ICMT*.

(D) *BRAF* expression at day 2 and day 6 of A375 BRAF malignant human melanoma cell line treated with shRNA targeting empty vector, *BRAF*, *ICMT* and *BRAF/ICMT*.

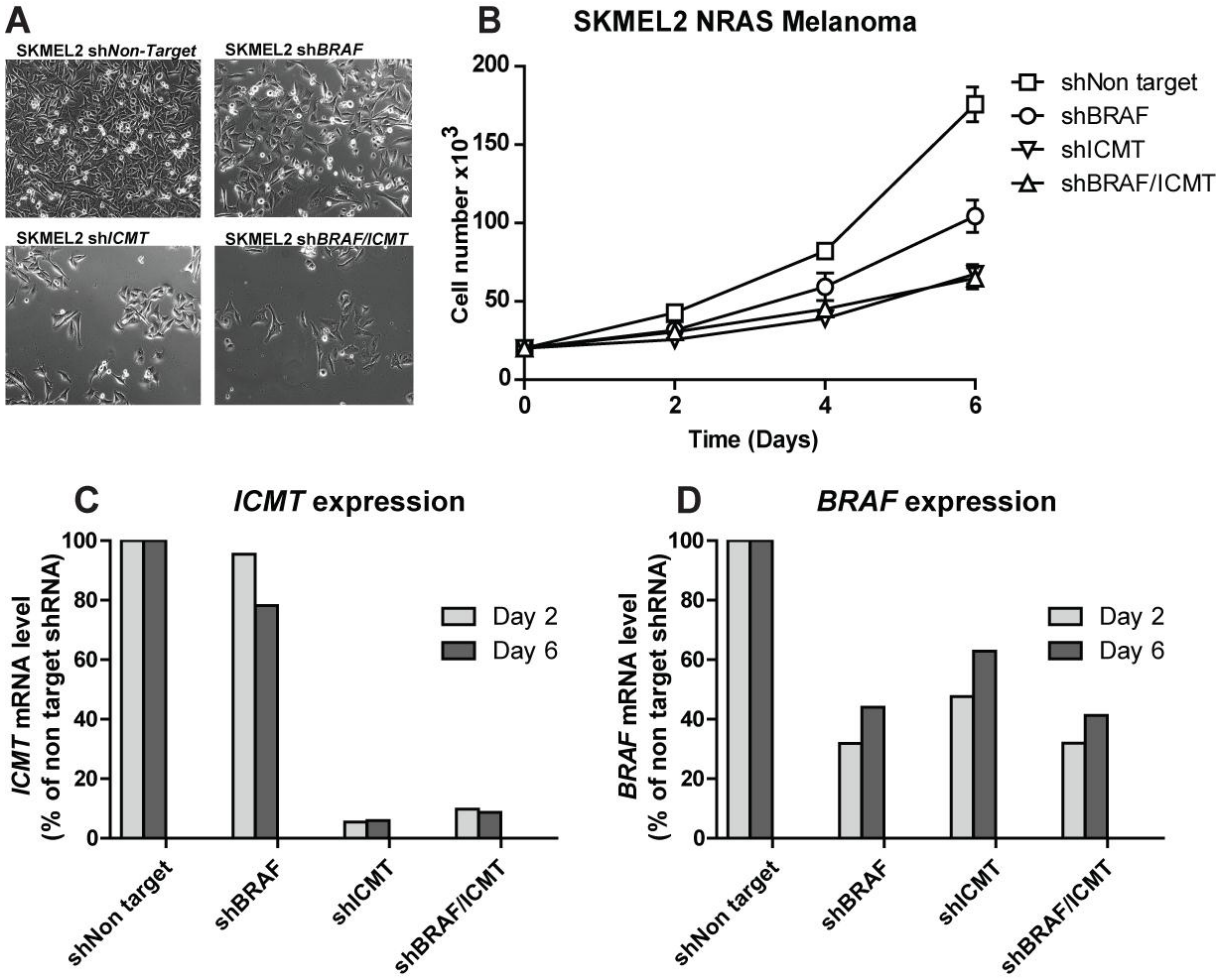

Figure 7: Inactivation of ICMT significantly reduces cell proliferation in NRAS mutated human malignant melanoma cell line

(A) Photographs of SKMEL2 NRAS malignant human melanoma cell line at day 7 after introduction of shRNA targeting empty vector, *BRAF*, *ICMT* and *BRAF/ICMT*.

(B) Proliferation assay of SKMEL2 NRAS malignant human melanoma cell line treated with shRNA targeting empty vector, *BRAF*, *ICMT* and *BRAF/ICMT*. Data show the mean of a single treatment assayed in triplicates, error bar represent the standard deviation of the triplicates.

(C) *ICMT* expression at day 2 and day 6 of SKMEL2 NRAS malignant human melanoma cell line treated with shRNA targeting empty vector, *BRAF*, *ICMT* and *BRAF/ICMT*.

(D) *BRAF* expression at day 2 and day 6 of SKMEL2 NRAS malignant human melanoma cell line treated with shRNA targeting empty vector, *BRAF*, *ICMT* and *BRAF/ICMT*.

Cell viability and phenotypic markers

Cell viability, circularity and diameter were measured in the cell proliferation assay, and determined as mean for all the counting days for both the BRAF and NRAS mutated cell lines (Figure 8A-F). The viability of the *ICMT* depleted NRAS mutated cell line (SKMEL2) did show a tendency of lowered viability compared to control (Figure 8B), while other transfected cells did not show any difference compared to control (Figure 8A and B). Measurements of cell diameter and cell circularity did also point to a phenotypic change in the *ICMT* depleted NRAS mutated cell line (Figure D and F), compared to the control.

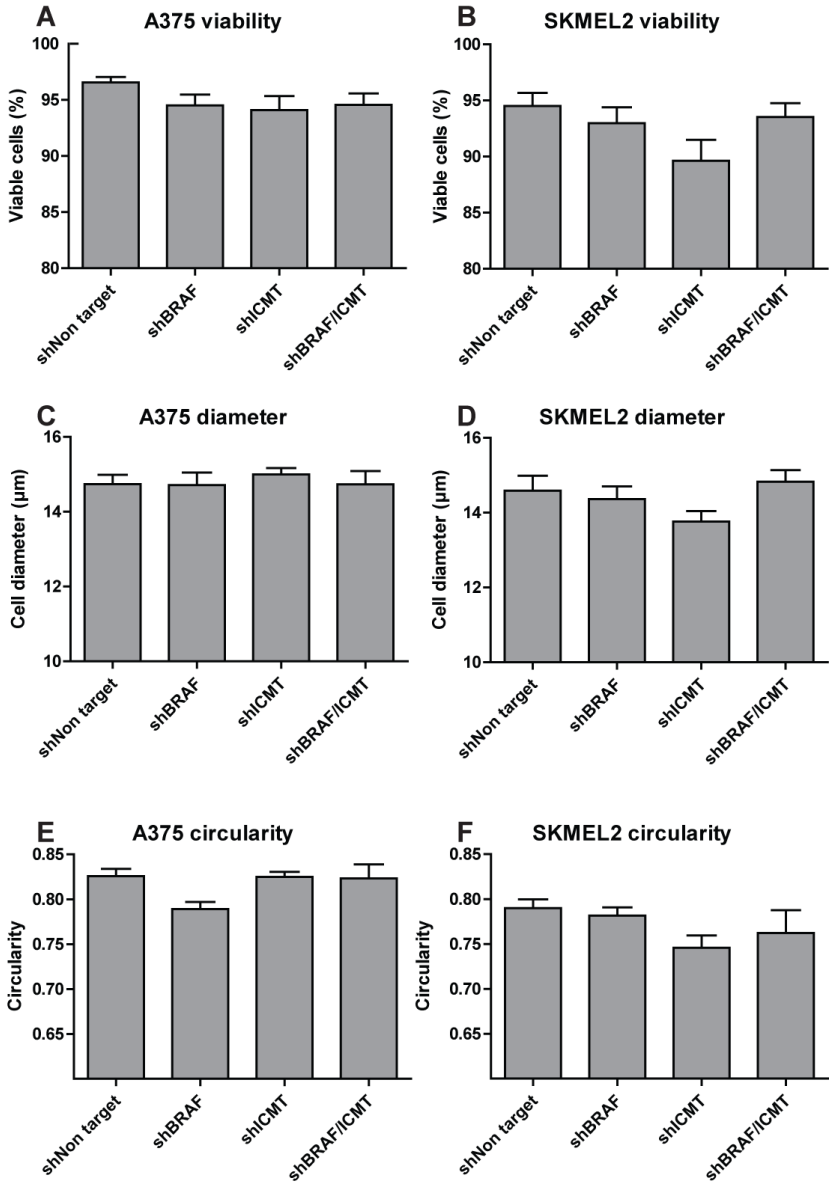

Figure 8: Cell viability and phenotypic markers of BRAF and NRAS mutated human malignant melanoma

(A-B) Viability of the BRAF and NRAS cell lines, respectively. **(C-D)** Cell diameter of the BRAF and NRAS cell lines, respectively. **(E-F)** Cell circularity of the BRAF and NRAS cell lines, respectively. Data show the mean of counting days 4 and 6 assayed in triplicates, error bar represent the standard deviation of the triplicates.

DISCUSSION

BRAF^{V600E} is the most common mutation in human malignant melanoma. The mutation renders BRAF protein kinase and MEK-ERK signaling constitutively active. A new RAF kinase inhibitor has recently emerged with remarkable activity in patients with malignant melanoma. The inhibitor is exclusively active in cells carrying the V600E mutation; this specificity makes the inhibitor more attractive than MEK or ERK inhibitors which block kinase activities in all cells. Unfortunately, resistance to the inhibitor develops fast through multiple mechanisms, mainly by trans-activation of MEK-ERK signaling through CRAF and acquired mutations in NRAS. Therefore, it is important to develop additional methods to target BRAF mutant melanoma cells.

Preclinical results on the genetical disruption and pharmacological inhibition of the CAAX processing enzyme ICMT have suggested that it might be suitable as anticancer target [37, 42, 43]. In this study, we show that the depletion of *ICMT* in BRAF and NRAS mutated human melanoma cell lines leads to a significant reduction in their proliferative ability. This inhibitory effect is due to the effect of *ICMT* depletion rather than based on cytotoxic effect related to lentiviral transfection, since the control cells were treated with an empty vector virus. Proliferative ability of these cell lines expressing oncogenic BRAF and NRAS treated with the empty vector virus was compared to untreated versions of these cell lines and showed no difference (data not shown). The co-depletion of *ICMT* and *BRAF* expression in these cells did not result in a dramatic reduction of proliferation compared to independent depletion of *ICMT* or *BRAF*. However, the co-depletion of *ICMT*/*BRAF* and the independent depletion of *ICMT* both gave a significantly higher reduction of proliferation in the NRAS mutated melanoma cell line.

The effects of *ICMT* depletion on the reduction of the proliferative ability of *BRAF* and *NRAS* mutated human melanoma are clear, and the mRNA expression levels of *ICMT* were reduced by 90-95% compared to control. The same consistent depletion was not achieved by targeting *BRAF* expression, only reaching 50% depletion. Interestingly, these cell lines also displayed a lowered *BRAF* expression when *ICMT* was independently targeted. This could mean that inactivation of *ICMT* reduces the transcription of oncogenic *BRAF*, which might contribute to the reduced proliferation of the *BRAF* mutated cell line. However, this result needs to be confirmed in several cell lines and the mechanism by which ICMT stimulates *BRAF* transcription remains unclear.

BRAF is not a CAAX protein, suggesting that ICMT affects the cells by one or several CAAX-proteins important for the transformation induced by oncogenic *BRAF*^{V600E}. We do not know the exact mechanism behind it, but we speculate that members of the RHO GTPase family might be involved, since RHO GTPase plays a role in cell-cycle progression and cell transformation. RHOA and RAC1 activity has been shown to be impaired as a consequence of ICMT inhibition [35]. If this is the case in our model as well, we hypothesize that not only proliferation, but also cancer cell migration and invasion will be reduced by ICMT inactivation. This hypothesis is based on the fact that RHOA and RAC1 are crucial for cell movement.

We cannot state with certainty whether the proliferation arrest caused by ICMT depletion is mediated by apoptosis, autophagy or cell cycle arrest. Both of the cell lines, with depleted *ICMT* expression, displayed normal viability compared to control. The NRAS mutated cell line did however show tendency of lower cell diameter and cell circularity compared to control, which might suggest a cell cycle arrest event occurring in these cells. We need to explore that effect more carefully in these cells by monitoring for apoptosis (with western blotting for cleaved caspase 3 and probing for annexin A5) and cell cycle analysis (with FACS).

In this study, we have established that the knockdown of *ICMT* expression with a lentivirus targeting *ICMT* significantly reduces the proliferation of BRAF and NRAS mutant human melanoma cell lines. Our results do not indicate that a combination of inhibitors of BRAF and ICMT would affect the proliferation to a greater extent than either treatment alone. Since the knockdown efficiency of the lentivirus targeting *BRAF* was limited, we suggest that a pharmacological inhibition of the BRAF mutation might present a more stable model to further investigate this issue. We would then introduce shRNA targeting *ICMT* in BRAF and NRAS mutated human melanoma cell lines, with the knock down efficiency established by TaqMan, and treat these *ICMT* depleted cell lines with the BRAF inhibitor PLX4032.

Nevertheless, our findings in this study support the notion that targeting ICMT could be an attractive anticancer therapy for BRAF induced malignant melanoma.

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