

The mechanisms of the anti-tumour effect of inactivating ICMT in BRAF-induced cancer

Master of Science Thesis

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Abstract

B-Raf, an important component of the Mitogen-Activated Protein Kinase (MAPK) pathway, has been shown to be mutated in 66% of malignant melanomas as well as other types of cancer leading to hyper-activation of the MAPK pathway. B-RAF inhibitors have been introduced as potential therapy treatments for these cancer patients, showing promising initial results but a resistance to the drug is soon formed and the disease progresses. This has turned research towards other potential therapeutic targets such as the enzyme Isoprenylcysteine carboxyl methyltransferase (ICMT) which has been shown to reduce the growth of both *KRAS* and *BRAF* induced cancers by means which are still not fully understood. Previous work in our group has shown *ICMT* knockouts to accumulate an age related protein, Prelamin A, in their nuclear membrane. Through use of Cre/Lox technology we show *in vitro* that Prelamin A accumulation mediates the effect *ICMT* inhibition has on *BRAF* transformed cells. Our results also show that knocking out *ICMT* abolishes the PI3K/Akt pathway downstream of *KRAS* in transformed cells however the MAPK pathway is unaffected and remains constitutively active. Thus, by inhibiting *ICMT* we seem to be able to induce premature senescence in transformed cancer cells by accumulating the precursor protein; Prelamin A and by down-regulating the PI3k/Akt pathway.

Keywords: ICMT, BRAF, Prelamin A, Lamin A, Malignant Melanoma, CAAX proteins.

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Abbreviations

ABCG2	ATP-Binding Cassette G2
β-ME	β- Mercaptoethanol
BP	Basepair
DMEM	Dubelcco's Modified Eagle Medium
EGF	Endothelial Growth Factor
ERK 1/2	Extracellular-signal Regulated Kinases 1/2
ESCs	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FTase	Farnesyl Transferase
FTI	Farnesyl Transferase Inhibitor
GGTase	Geranyl-Geranyl Transferase
HGPS	Hutchinson-Gilford Progeria Syndrome
ICMT	Isoprenylcysteine Carboxymethyl Transferase
LCO	Lamin C only
MAPK	Mitogen Activated Kinase Pathway
MEFs	Mouse Embryonic Fibroblasts
MEK 1/2	Mitogen-activated ERK Kinases 1/2
MOI	Multiplicity of Infection
MSCs	Mesenchymal Stem Cells
NEAA	Non-Essential Amino Acids
PMSF	Phenyl Methanesulfonyl Fluoride
UV	Ultraviolet
VMSCs	Vascular Smooth Muscle Cells

List of Alleles

<i>BRAF</i>⁺	Wild type <i>BRAF</i> allele
<i>BRAF</i>^{CA}	<i>BRAF</i> conditional allele
<i>BRAF</i>^{V600E} / <i>BRAF</i>^{VE}	<i>BRAF</i> allele with V600E substitution mutation
<i>ICMT</i>⁺	Wild type <i>ICMT</i> allele
<i>ICMT</i>^{flx} / <i>ICMT</i>^{fl}	<i>ICMT</i> allele flanked by LoxP sites
<i>LMNA</i>⁺	Wild type <i>LMNA</i> allele
<i>LMNA</i>^{LCO}	<i>LMNA</i> allele encoding LCO (Lamin A knockout)

Introduction & Background

Cancer is one of the leading causes of death worldwide. Each year, cancer is diagnosed in around 13 million patients and is the cause of death in almost 8 million patients, which accounts for 13% of all deaths⁽⁷⁾.

Malignant Melanoma

Malignant melanoma is a cancer that occurs in skin melanocytes and is highly related to skin type and exposure to sunlight. Under homeostatic conditions, keratinocytes in the skin control the growth and behaviour of melanocytes through paracrine cellular communication. Through a number of different ways, this cellular communication can be lost and malignant melanoma can develop⁽⁸⁾.

In the world today, it is estimated that one person dies every hour from malignant melanoma and around 86 percent of these cases are caused by ultraviolet (UV) light exposure from the sun, the remaining 14% for reasons unknown^(9, 10). The incidence of melanoma has increased by 800% in females and 400% in males between the years 1970 and 2009 and the disease has been shown to be more common in coastal areas⁽¹¹⁾. The coastal city of Gothenburg, Sweden for example has 35% more melanoma cases in men and 60% more in women when compared to inland areas⁽¹²⁾.

The burden of malignant melanoma not only falls on the individual patients but also on the society as a whole. An estimated annual productivity loss from melanoma between years 2000 and 2006 was shown to be \$3.5 billion in the U.S. alone⁽¹³⁾. Melanoma, being a rapidly growing disease, is becoming more and more of a financial burden on the healthcare system as well. One study estimated that the direct cost of treatment of melanoma patients in the United States was as high as \$2.36 billion each year, highlighting the need for more successful methods of treatment⁽¹⁴⁾.

The MAPK pathway

The MAPK pathway mediates cellular responses to growth signals, controlling cellular functions such as proliferation, apoptosis and cell growth⁽¹⁵⁾. Six different groups of MAPK pathways exist in mammals although here, only the ERK 1/2 MAPK pathway will be discussed⁽¹⁶⁾.

Signalling of the MAPK pathway begins with a growth factor, such as Epidermal Growth Factor (EGF), binding a receptor tyrosine kinase⁽¹⁷⁾. This leads to the activation of the membrane bound protein RAS. Three different isotypes of RAS exist which are H-RAS, N-RAS, K-RAS⁽¹⁷⁾. Upon activation, the GDP-bound K-RAS molecule is converted to GTP-bound and a conformational change takes place. This leads to the recruitment of the kinase, RAF, to the cell membrane⁽¹⁷⁾. The RAF family is made up of *A-RAF*, *B-RAF* and *C-RAF*⁽¹⁸⁾. After being activated, RAF then phosphorylates and activates the Mitogen-activated Erk Kinases 1/2 (MEK 1/2). This, in turn, leads to the phosphorylation and activation of Extracellular-signal Regulated Kinases 1/2 (ERK 1/2). Downstream of ERK 1/2 are numerous transcription factors, such as c-Jun and c-Fos, which are then activated and go on to directly or indirectly alter gene expression of the cell⁽¹⁸⁾. This pathway is much more complex than explained here and the kinases may play additional roles in other pathways as well. K-

RAS, for example, also plays a role in another pathway called the PI3K/Akt pathway which also regulates many cellular functions such as proliferation and differentiation ⁽¹⁹⁾. **Figure 1** shows an overview of the MAPK pathway.

The MAPK pathway in cancer

Since the MAPK pathway controls cellular functions such as proliferation and cell growth it plays a critical role in the development and progression of cancer. The MAPK pathway has been reported to be hyper-activated in breast cancer, hematologic malignancies, melanoma, colorectal cancer and many others ⁽²⁰⁻²²⁾. Lesions can occur at various sites in the pathway leading to a constitutive expression of ERK 1/2. These consist mainly of activating mutations or over-expression of receptor tyrosine kinases, sustained production of activating ligands, RAS mutations and RAF mutations ⁽¹⁶⁾.

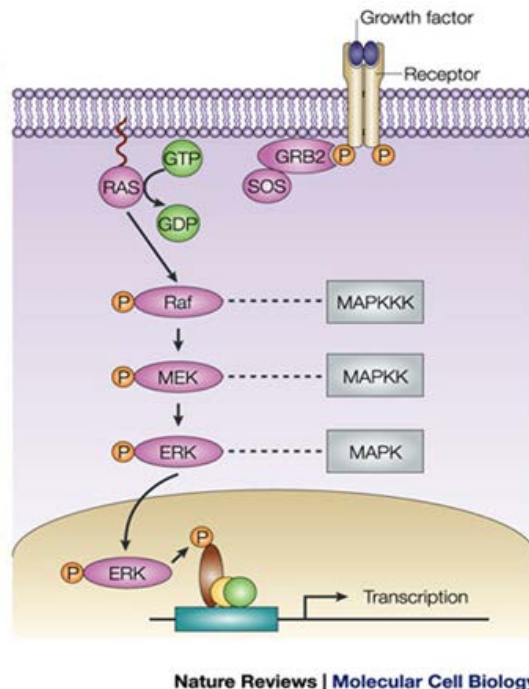


Figure 1 –An overview of the MAPK pathway ⁽²⁾

BRAF - mutated cancer

It is estimated that 8% of all cancers carry a *BRAF* mutation although its prevalence differs greatly amongst the different types of tumours ⁽²²⁾. *BRAF* mutations are most commonly associated with malignant melanoma, where they occur in 66% of all cases ⁽²³⁾.

All oncogenic mutations in *BRAF* are found in the kinase domain, with the most common being a single substitution of Valine to Glutamic acid at amino acid position 600 (*BRAF*^{V600E}), which accounts for 80% of all mutations ^(23, 24). These mutations leave B-RAF with an elevated kinase activity, hence hyper-activating the MAPK pathway. In mice, widespread expression of *BRAF*^{V600E} is embryonic lethal, with embryos dying at 7.5 days old ⁽²⁴⁾. This indicates the importance of a functional *BRAF* allele during development and the effect it can have when mutated. One study showed that a functional RAS is not required in maintaining a hyper-activated MAPK pathway in *BRAF*^{V600E} cells and that transformed cells continued to proliferate in culture after being treated with a RAS neutralizing antibody. It was reasoned that *BRAF*^{V600E} cells overcome the RAS-dependent activation of B-RAF by mimicking phosphorylation ⁽²³⁾.

B-RAF can also play indirect roles in the development of cancer. One study showed that in the presence of oncogenic RAS, when treated with B-RAF inhibitors, cells had an increased C-RAF, MEK and ERK activation, inducing melanoma. From this study they concluded that kinase-dead *BRAF* mutation, *BRAF*^{D594A} (i.e. one which leads to a non-functioning B-RAF), cooperates with oncogenic

RAS to induce melanoma in mice ⁽²⁵⁾. Another study showed that $BRAF^{V600E}$ and $BRAF^{G496A}$ mutations were found to cause EGF-Receptor (EGFR) inhibitor resistance in lung tumours ⁽²⁶⁾. The role of $BRAF$ mutations in malignancies is yet to be fully understood and several mouse models have been developed to study the effects of this mutation *in vivo*.

BRAF - mutated cancer models

To study the effects of various drugs and knockouts on cancer growth it is essential to first have an efficient model to work with. One widely used approach for researchers to manipulate the expression and suppression of specific genes is known as the Cre-lox recombination system ⁽²⁷⁾.

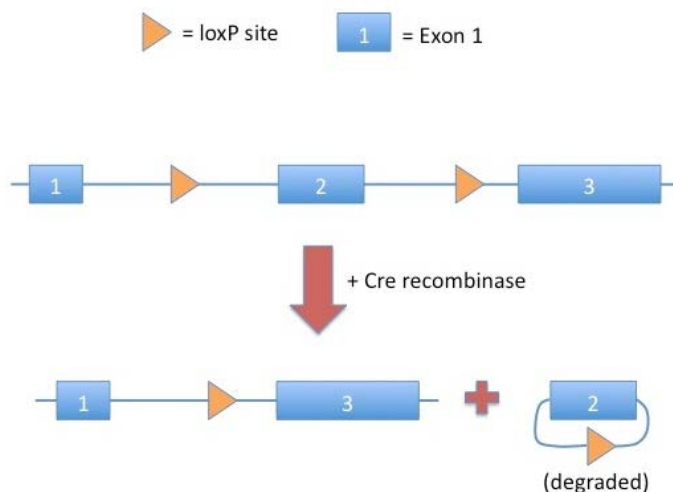


Figure 2 – The Cre-lox method used for the excision of a gene of interest, in this case Exon 2 ⁽⁴⁾

cells (ESCs) are created to express wild type $BRAF$ under normal conditions. The wild type $BRAF$ allele however, along with a downstream stop codon is here flanked by $loxP$ sites. Downstream of the $loxP$ site is a mutated $BRAF$ allele (called $BRAF^{CA}$ for $BRAF$ Conditional Allele) but due to the stop codon, this allele is inert in the absence of Cre. These ESC's are then used to give rise to transgenic mice. After lung inhalation of Cre-encoding adenovirus the $loxP$ sites are cleaved, excising the wild type $BRAF$ allele and stop codon. This leads to the activation of the mutated $BRAF$ allele (now called $BRAF^{VE}$ for $BRAF^{V600E}$) which leads to the development of lung tumours. **Figure 3** shows the concept of using Cre-recombinase to activate $BRAF^{V600E}$ by removal of a stop codon.

The enzyme Cre, encoded by Bacteriophage P1, is a recombinase which catalyzes recombination between the 34 base pair (bp) specific sites called $loxP$ sites ⁽²⁸⁾. By flanking a gene of interest with $loxP$ sites, Cre recombinase can be added to cut these sites, hence leading to a gene knockout (or knock in). **Figure 2** shows the general concept of how Cre recombinase is used to excise genes of interest.

By using this approach, researchers were able design a mouse model for $BRAF^{V600E}$ - induced lung tumours ⁽²⁹⁾. Through use of a vector, transgenic embryonic stem

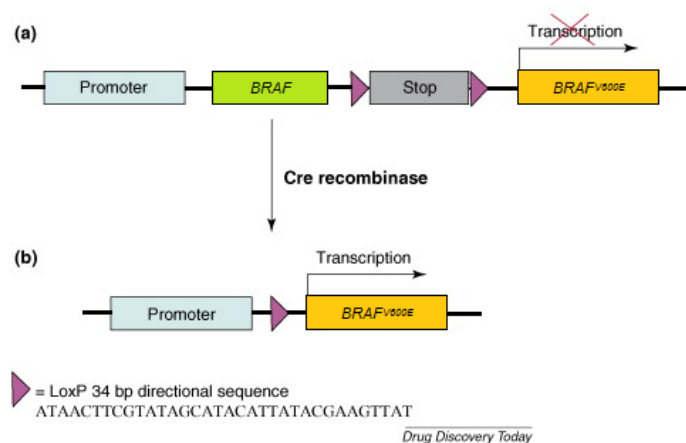


Figure 3 – The Cre-lox method used to express $BRAF^{V600E}$ (Adopted ⁽⁵⁾).

A more recent model has been developed which makes use of the $BRAF^{V600E}$ mutation in combination with silencing of the *Pten* tumour suppressor gene⁽³⁰⁾. Melanocyte specific activation of the $BRAF^{V600E}$ allele in mice leads to melanocytic hyperplasia formation but these do not progress to malignant melanoma. However, by combining the activation of $BRAF^{V600E}$ with the silencing of *Pten*, melanoma formed with 100% penetrance, short latency and metastasis (i.e. translocation of tumour cells) in the lymph nodes and the lungs. By using this, the authors created a mouse model which, when topically administered the agent 4-hydroxytamoxifen, developed malignant melanoma⁽³⁰⁾. As this model is malignant and specific to melanocytes, it is more representative of the disease than the $BRAF^{V600E}$ lung cancer model.

$BRAF^{V600E}$ drugs and drug resistance

Detection methods for the $BRAF^{V600E}$ mutation in cancer are advancing fast with new methods arising constantly⁽³¹⁾. However, the treatment of cancers with $BRAF^{V600E}$ mutation driven proliferation have not been nearly as successful and much research has been put into finding a way to inhibit or slow down the progression of these diseases.

Prior to 2011, patients suffering with malignant melanoma were treated with one of the two approved drugs; Dacarbazine or recombinant human interleukin 2. Response rates were fairly low (15% and 16%, respectively) and only the latter managed to improve the survival of the patients⁽⁶⁾. Research into the MAPK pathway led to the development of the drug Vemurafenib which was approved by the Food and Drug Administration (FDA) in 2011. Acting as a specific inhibitor of $BRAF^{V600E}$, Vemurafenib leads to a decrease in the MAPK pathway activity, which causes a decrease in cell proliferation and survival (**Figure 4**)⁽⁶⁾. Other drugs used today include the MEK inhibitor Trametinib and the immunotherapy drug Ipilimumab⁽⁶⁾.

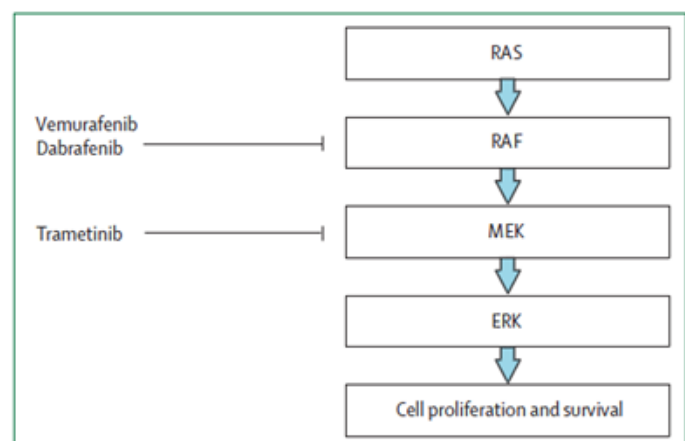


Figure 4 – Various drugs and their mechanisms of action⁽⁶⁾

Vemurafenib showed excellent tumour response with tumour shrinkage in 81% of $BRAF^{V600E}$ patients. However, after a median treatment time of 7 months, the tumours gained resistance to the drug and the disease progression continued^(6, 32). How the cancer-mutated cells develop a resistance to B-Raf inhibitors is not fully understood although several possible mechanisms have been suggested, such as the expression of the anti-apoptotic oncogene BCL2A1, ATP-Binding cassette G2 (ABCG2) and the activation of the PI3K/AKT pathway⁽³³⁻³⁵⁾. This suggests combinatorial treatments involving B-Raf inhibitors and other drugs a popular concept in the field of cancer treatment.

CAAX Proteins

The term CAAX protein refers to a group of over one hundred different proteins, each containing a 'CAAX box' on their C-terminus⁽³⁶⁾. The CAAX represents an amino acid sequence with C; Cysteine residue, A; Aliphatic amino acid residues and X; any C-terminal amino acid. All proteins with this C-terminus sequence undergo a similar post translational modification process (explained below). CAAX proteins play major roles in cell functions such as proliferation and differentiation and include proteins such as the RAS family and nuclear lamin proteins⁽³⁶⁾.

CAAX Protein Processing

After being translated, the C terminus of a CAAX protein is subjected to a number of modifications. The first step is the addition of a hydrophobic prenyl moiety to the cysteine residue; a step known as prenylation. This step is carried out by either one of the two enzymes; farnesyl transferase (FTase) or geranyl-geranyl transferase type 1 (GGTase-I) depending on the length of the prenyl moiety added⁽³⁾. Proteins modified by FTase will have a 15-carbon long moiety added whereas proteins modified by GGTase-I will be prenylated with a 20-carbon long moiety. Some CAAX proteins can be prenylated by both FTase and GGTase whereas others can only be prenylated by one or the other⁽³⁾.

After prenylation the CAAX protein undergoes modification by the enzyme RCE1⁽³⁶⁾. In this step, the last three amino acids (-AAX) are cleaved off, a process known as endoproteolysis. This leaves the cysteine moiety free for the final enzyme in the process, ICMT, to add a carboxyl group⁽³⁶⁾. **Figure 5** shows the overall CAAX protein post translational modification process. After the processing, CAAX proteins are left with a hydrophobic C terminus which can be anchored into a hydrophobic cell membrane, where the CAAX proteins are biologically active⁽³⁶⁾.

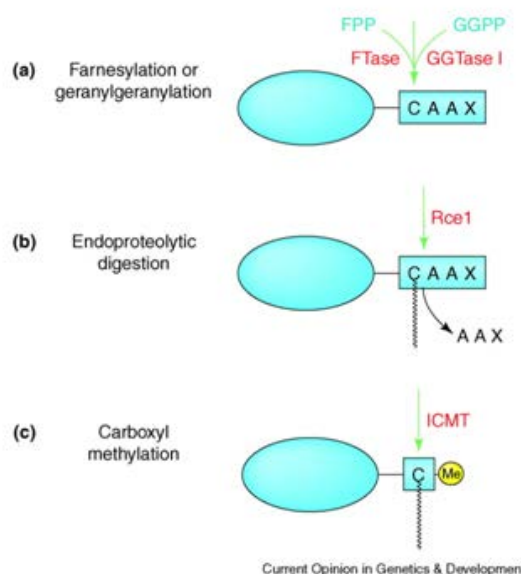


Figure 5 - The three main steps of CAAX protein processing⁽³⁾

CAAX proteins as anti-cancer targets

The CAAX protein K-RAS is an oncoprotein altered in around 30% of human cancer⁽³⁷⁾. Since attempts to specifically inhibit mutated K-RAS have failed, indirect methods of RAS inhibition have been investigated⁽³⁸⁾. One method of doing this is to target the post-translational modifications of KRAS to render it biologically inactive. This led to the development of a class of drugs known as farnesyl transferase Inhibitors (FTIs) which inhibit the action of FTase and give rise to a non-functional K-RAS⁽³⁹⁾. FTIs as anti-cancer drugs may look promising but have so far been unsuccessful.

This has led to the search for other drug targets, one of which is the enzyme ICMT. ICMT is the only enzyme acting in the methylation of K-RAS making it a potential therapeutic target⁽⁴⁰⁾. Upon inactivation of ICMT, K-RAS is mis-localized in the cell and no longer biologically active, leading to the inhibition of growth of *KRAS* - transformed cells *in vitro* and *in vivo*⁽⁴¹⁾. Surprisingly, a previous study showed that ICMT inactivation has the same inhibitory effect on *BRAF*^{V600E} transformed cell lines⁽⁴¹⁾. As B-RAF is directly downstream of K-RAS it is easy to assume that ICMT inactivation is effecting K-RAS and hence B-RAF. This is not the case because, as mentioned earlier, a functional RAS is not required for the growth of cancer cell lines with the *BRAF*^{V600E} mutation. As, in both *KRAS* and *BRAF*- transformed cells the MAPK pathway is still hyper-activated after ICMT inhibition, this suggests that the mechanism is through inactivation of another molecule, and not *KRAS*.

The Nuclear Lamina Protein; Lamin A

The CAAX protein Lamin A is a component of the nuclear lamina and is encoded by the gene *LMNA*. The *LMNA* gene is alternatively spliced to give rise to both Lamin A and Lamin C variants which together form the nuclear lamina⁽⁴²⁾. Lamin A is widely known for its involvement in a number of premature ageing syndromes and muscular dystrophies also known as laminopathies, the most well known being Hutchinson-Gilford Progeria Syndrome (HGPS)⁽⁴²⁾.

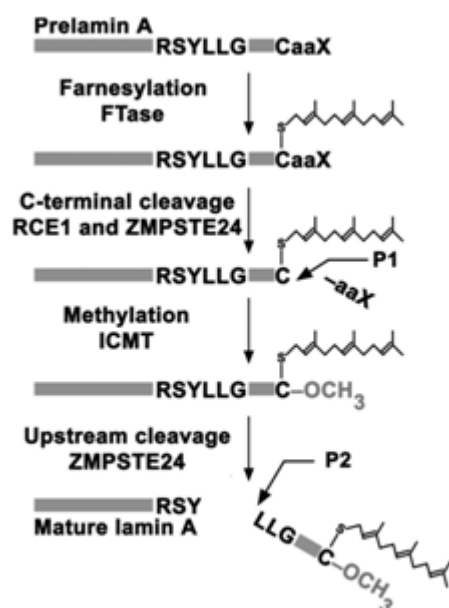


Figure 6 – The processing of Lamin A⁽¹⁾

Lamin A processing

As a CAAX protein, Lamin A is processed in a similar way to the method explained above but with an additional step. After being methylated by the enzyme ICMT another endoproteolytic cleavage step occurs in which 15 amino acids upstream of the C-terminal are cleaved off, giving rise to the final product Lamin A⁽⁴³⁾. This is performed by the enzyme known as FACE1 (or Zmpste24 in mice) which also can play the role of RCE1 in the earlier steps of processing. Prior to this final step, the transcript product is known as Prelamin A; the precursor to Lamin A⁽⁴³⁾. The accumulation of farnesylated-Prelamin A in cells (for example in the presence of Zmpste24 knock-down cells) leads to dysmorphology of the nucleus, severe growth retardation and premature death in mice⁽⁴⁴⁾. The accumulation of

a truncated version of Prelamin A, known as 'Progerin' is the underlying cause of HGPS^(44, 45). **Figure 6** shows an overview of the processing of Lamin A.

Lamin A's role in cells

As previously mentioned, Lamin A, together with its splice variant Lamin C, form the 'meshwork' of the nuclear lamina, giving structure to the nucleus. But, this is not the only role that Lamin A plays and recent studies are beginning to show the extent of this 70 kDa proteins importance. It is no surprise that, due to its related syndromes, Lamin A plays a role in the differentiation of cells. One study showed that the accumulation of Prelamin A was seen in ageing Vascular Smooth Muscle Cells (VMSC's) and these cells also showed nuclear morphology defects which were reversed by treatment with FTL's ⁽⁴⁶⁾. Another study showed that by knocking down the *LMNA* gene in Mesenchymal Stem Cells (MSC's) the authors were able to lower Osteoblast differentiation but enhance adipocyte differentiation ⁽⁴⁷⁾. Other than playing the roles Pre-/Lamin A plays in somatic cells and MSC's it has also been shown to play a role in cancer cells also. Yet another study showed that knocking down the *LNMA* gene in Neuroblastoma cells altered the expression of many differentiation and proliferation factors, hence leading to milder phenotypes of the disease ⁽⁴⁸⁾. These are merely samples of the many studies addressing Pre-/Lamin A's role in cellular differentiation/senescence but none-the-less, what exactly its role is, is still not fully understood.

Aims

Previous work by our group has shown that knocking out *ICMT* in a lung tumour mouse model with *BRAF*^{V600E} mutations significantly reduces the amount of tumours formed. The aim of this study is to replicate this *in vitro* and to determine the possible mechanism of action.

It has also been previously shown in our group that *ICMT*^{-/-} *BRAF*^{V600E} cells (*ICMT* knockouts) have an accumulation of the precursor protein; Prelamin A. For this reason this study aims to test Prelamin A accumulation as a possible mechanism of action.

Specific Aims of the study:

- i. To test Prelamin A accumulation as a possible mechanism of action *in vitro*.
- ii. To test if *ICMT* knockout affects the MAPK pathway signalling.
- iii. To test if *ICMT* knockout affects the PI3K/AKT pathway signalling.

Materials & Methods

All work for this study was performed at the Sahlgrenska Cancer Center in Gothenburg, Sweden where all protocols and lab materials were kindly provided. The following section describes in detail the methodological procedures used.

Protein Extraction

Lysis buffer was made by preparing 9.5 ml of Urea (Sigma-Aldrich®) in MilliQ water. The following protease inhibitors were then added: x1 Roche cOmplete EDTA-free protease inhibitor cocktail tablet, Phenylmethanesulfonyl Fluoride (PMSF), Tris-EDTA Buffer and Sodium Fluoride (Sigma-Aldrich®).

Cell cultures at 90% confluence on 100 mm petri-dishes were first washed 3 times in cold PBS to remove all traces of proteases before lysis. Freshly made urea buffer was then added to the plate (between 300-500 µl) and a cell scraper was used to detach and collect lysates. Lysates were then sonicated for 10-15 seconds before being centrifuged at maximum speed for 10 minutes. The supernatant was then taken and used for western blot analysis.

Western Blots

Protein concentrations were quantified using the BioRad DC™ Protein Assay kit. Proteins lysates were diluted according to their concentrations in a mixture of 90% NuPage® LDS Sample Buffer (Life technologies™) and 10% β-Mercaptoethanol (β-ME) (Sigma-Aldrich®). Samples were then heated at 70°C for 10 minutes before being loaded onto NuPage® 4-12% Gels (NOVEX®, Life technologies™) alongside Odyssey® molecular weight markers 928-40000 (LICOR®). Gels were then suspended in MOPS SDS Running Buffer (NuPage®, Life technologies™) and run at 170 V on a Power Pac™ HC (Bio-Rad) for 1-2 hours. The protein was then transferred to a 0.2 µm Nitrocellulose membrane (Bio-Rad) using NuPage® Transfer Buffer (NOVEX®, Life technologies™) using the wet-transfer method for 1 hour. After the protein had transferred, membranes were incubated in Rockland™ Blocking Buffer for 40 minutes to avoid unspecific binding of anti-bodies.

Primary anti-bodies were prepared in various concentrations in Rockland™ Blocking Buffer and membranes were incubated in this solution overnight at 4°C on a rocking platform. Antibodies used were: p-MEK 1/2 (S217/221) (#9121L, Cell Signalling Technology®), MEK 1/2 (#9122s, Cell Signalling Technology®), P-p44/42 MAPK (#4377s, Cell Signalling Technology®), p44/42 MAPK (ERK 1/2) (#9102s, Cell Signalling Technology®), P-Akt (s473) (#4060s, Cell Signalling Technology®), Akt (#9272s, Cell Signalling Technology®), Lamin A/C (N-18, Santa Cruz Biotechnology, inc).

The next day, membranes were washed every 15 minutes for a total of 45 minutes in PBS-Tween™ (Medicago) before being inoculated with the relevant secondary anti-body (Odyssey® Infrared Imagine System) diluted in Rockland™ Blocking Buffer for 1 hour at room temperature on a rocking platform. Membranes were then washed again in PBS-Tween™ every 15 minutes for a total of 45 minutes before being scanned on a fluorescent Odyssey® scanner (LI-COR® Biosciences).

Cell Culture Conditions

Mouse Embryonic Fibroblasts (MEF's) were grown in low glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (Gibco®), 1% Non-Essential Amino Acids (NEAA), 1% Glutamine, 1% Penicillin/Streptomycin and 0.5% of 0.1M B-ME. Cells were kept at 37°C in a humidified, 5% CO₂ incubator and split at 85% confluence. All media components were supplied by PAA Laboratories, GE Healthcare unless stated otherwise.

Proliferation Experiments

Cells were isolated from embryos at embryonic day 12.5 to 14.5. Each cell line was split and 1×10^6 cells were seeded onto two 100 mm Petri dishes each; one for treatment with a β -Gal Adenovirus control and one for treatment with Cre Adenovirus. The following day, cells were treated by changing the media to a media containing the suspended virus. The virus was added at 200 MOI (Multiplicity of Infection) as this was found to be the optimal ratio. Cells were then incubated at 37°C for 48 hours before having media changed to regular DMEM.

Cells were then kept to proliferate with regular media changes until signs of transformation became noticeable. Signs of transformation were taken to be a significant change in the morphology of cells treated with Cre Adenovirus such as a higher refraction of light when looked at through a microscope, a more regular shape and the ability to grow on the Z-plane. This took approximately 3-5 days.

Cells were then split and seeded in triplicates on 12 well plates (Fisher Scientific Inc.) at either 1×10^4 or 2×10^4 cells/plate depending on their growth rates. Some cells were also kept for DNA extraction (genotyping) and for protein extraction (western blots). Cells were counted 3, 6 and 9 days prior to seeding using a NucleoCounter cell counter (Chemometec, Allerød, Denmark). Cell images were taken using Axiovision software (Version 4.00, Axiovision).

Genotyping

Genotyping was performed by PCR amplification of genomic DNA from mouse tail biopsies and cultured cells. The *ICMT*^{fix} allele was detected with forward primer 5'-GGGCGGGACGGACAG-3' and reverse primer 5'-ATGCGCATCTGCCTAAGCTG-3', yielding a 600-bp fragment from the *ICMT*^{fix} allele and a 538-bp fragment from the *ICMT*⁺ allele. The *BRAF* allele was detected with forward primer 5'-TGAGTATTTTTGTGGCAACTGC-3' and reverse primer 5'-CTCTGCTGGGAAAGCGGC-3', yielding a 185-bp fragment for *BRAF*, a 308-bp fragment for *BRAF*^{CA}, and a 335-bp fragment for *BRAF*^{V600E}.

Graphs and Statistics

Proliferation curves were plotted using Graphpad Prism (Version 6.00, GraphPad Software). Data points were plotted as Mean \pm SEM.

Results

Cells used in the experiments were isolated from embryos with varying genotypes and were the results of breeding a variety of parents. Table 1 shows the genotypes of the cells used in the experiments.

Table 1: Genotypes for the cell lines used in experiments when untreated (or β -Gal treated) and when Cre treated.

Cell line	Genotype	Genotype (after Cre treatment)
Ba-2	$BRAF^{CA/+} ICMT^{flx/+}$	$BRAF^{V600E} ICMT^{+/-}$
Bil-6-5	$BRAF^{CA/+} ICMT^{flx/+}$	$BRAF^{V600E} ICMT^{+/-}$
Be-1	$BRAF^{CA/+} ICMT^{flx/flx}$	$BRAF^{V600E} ICMT^{-/-}$
Bil-6-6	$BRAF^{CA/+} ICMT^{flx/flx}$	$BRAF^{V600E} ICMT^{-/-}$
Bil-4-3	$BRAF^{CA/+} ICMT^{flx/+} LMNA^{LCO/LCO}$	$BRAF^{V600E} ICMT^{+/-} LMNA^{LCO/LCO}$
Bil-7-1	$BRAF^{CA/+} ICMT^{flx/flx} LMNA^{LCO/LCO}$	$BRAF^{V600E} ICMT^{-/-} LMNA^{LCO/LCO}$

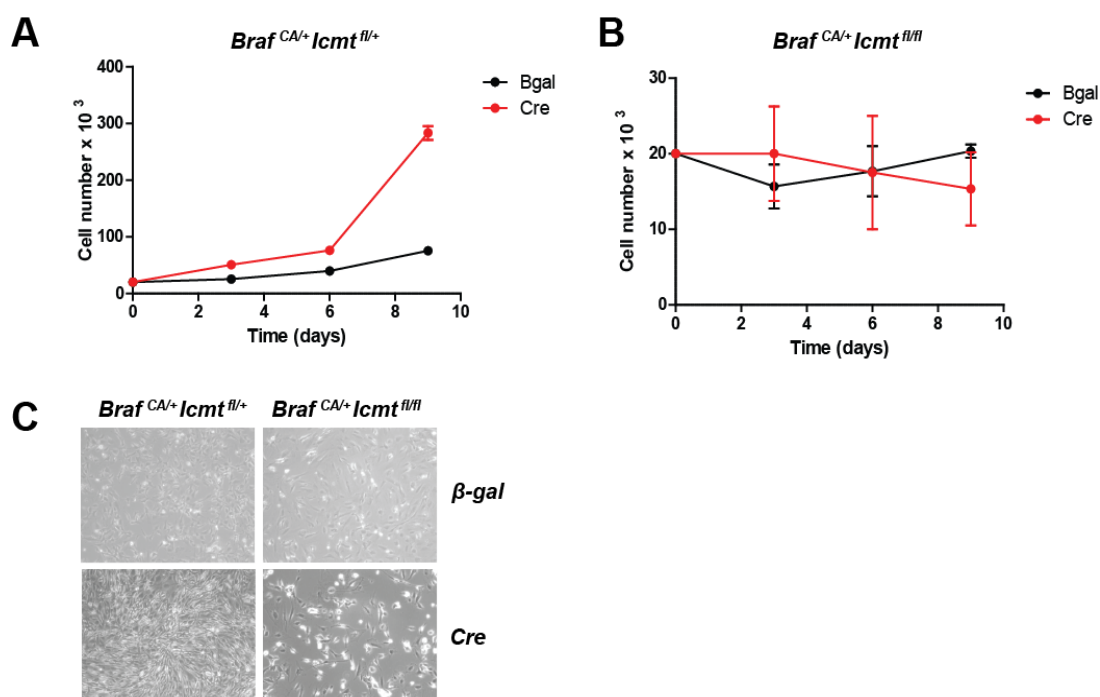


Figure 7: Inactivation of ICMT in BRAF mutated Mouse Endothelial Fibroblasts (MEF's) leads to a significant decrease in proliferation

- Growth curves of Ba-2 ($BRAF^{CA/+} ICMT^{flx/+}$) and Be-1 ($BRAF^{CA/+} ICMT^{flx/flx}$), 3, 6 and 9 days after being treated with β -Gal or Cre Adenovirus. Measurements were taken in triplicates. Data points show Mean value \pm SEM.
- Pictures of Ba-2 and Be-1 9 days after treatment with β -Gal or Cre Adenovirus.

An initial study was first performed to test whether *ICMT* knockout effects the proliferation of *BRAF*^{V600E} cells *in vitro*. After infecting the MEF cell lines Ba-2 (*BRAF*^{CA/+} *ICMT*^{flx/+}) and Be-1 (*BRAF*^{CA/+} *ICMT*^{flx/flx}) with β -Gal and Cre-Adenovirus their growth was measured in a proliferation experiment over a 9 day period (Figure 7a & 7b). Being heterozygous for *ICMT*, the cell line Ba-2 showed the typical behaviour of a wild type cell with a *BRAF*^{V600E} mutation. Cre treated cells grew exponentially with a significant increase in proliferation when compared to the β -Gal treated control cells. This was also confirmed by a change in morphology (Figure 7c) whereby transformed cells began refracting light differently, became more regular in shape and were seen to lose contact inhibition, shown by the ability to grow on top of each other. Be-1 cells, however, lacking in *ICMT*, showed no significant difference in growth when comparing Cre treated cells to β -Gal treated cells (7b). This confirmed that *ICMT* knockout does, indeed, reduce *BRAF*^{V600E} cells' growth *in vitro* as well as preventing a transformed-like change in their morphology (7c).

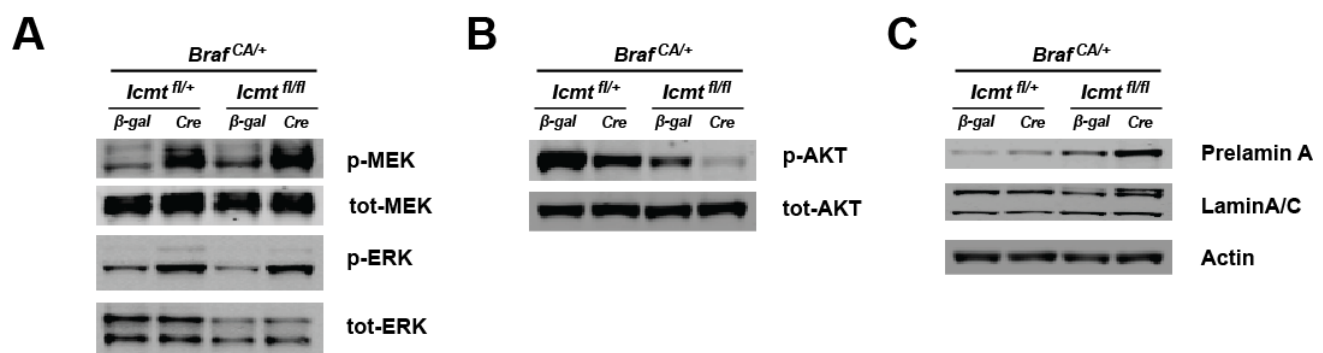


Figure 8: MAPK pathway and PI3K/Akt pathway remain unchanged in *ICMT*^{-/-} *BRAF* mutated MEF's

- Western blot showing hyper-activation of the MAPK pathway in *BRAF*^{V600E} activated cells (Cre treated) by an increase in pMEK and pERK when compared to β -Gal controls.
- Western blots showing a reduction in the activation of the PI3K/Akt pathway in *BRAF*^{V600E} activated cells by a reduction in pAkt.
- Western blot showing an accumulation of Prelamin A in *BRAF*^{V600E} activated *ICMT*^{-/-} cells but not in *BRAF*^{V600E} activated *ICMT*^{flx/+} cells.

Protein lysates were extracted from Ba-2 and Be-1 cell lines treated with β -Gal and Cre and westerns were performed to analyse the effect of *ICMT* knockout on the MAPK pathway (Figure 8a) as an up-regulated MAPK pathway is correlated with high proliferation. Results showed, however, that even in *ICMT* knockout cells (Be-1) the MAPK pathway remained up-regulated. From this we concluded that knocking out *ICMT* does not slow the proliferation of *BRAF*^{V600E} cells by down-regulating the MAPK pathway and instead, its effect originates from another factor, unrelated to this pathway.

It was also of interest to us to test the activation of the PI3K/Akt pathway as it is also regulated by the protein K-RAS (Figure 8b). We saw that in $BRAF^{V600E}$ activated cells, there was a reduction in the activity of Akt when compared to control β -Gal cells. This was shown to occur regardless of the presence of $ICMT$ in cells, indicating that the PI3K/Akt pathway also does not play a role in the inhibitory effect $ICMT$ knock-out has on $BRAF$ mutated cells.

As previous work in our group has shown that the pre-cursor to the CAAX protein Lamin A; Prelamin A, accumulates in the nuclear membrane of $ICMT$ knockout MEF's, we wanted to confirm this in a western blot. Indeed, knocking out $ICMT$ in cells led to the accumulation of Prelamin (Figure 8c). This indicated that Prelamin A accumulation may mediate the effect of $ICMT$ knockout on $BRAF$ mutated cells.

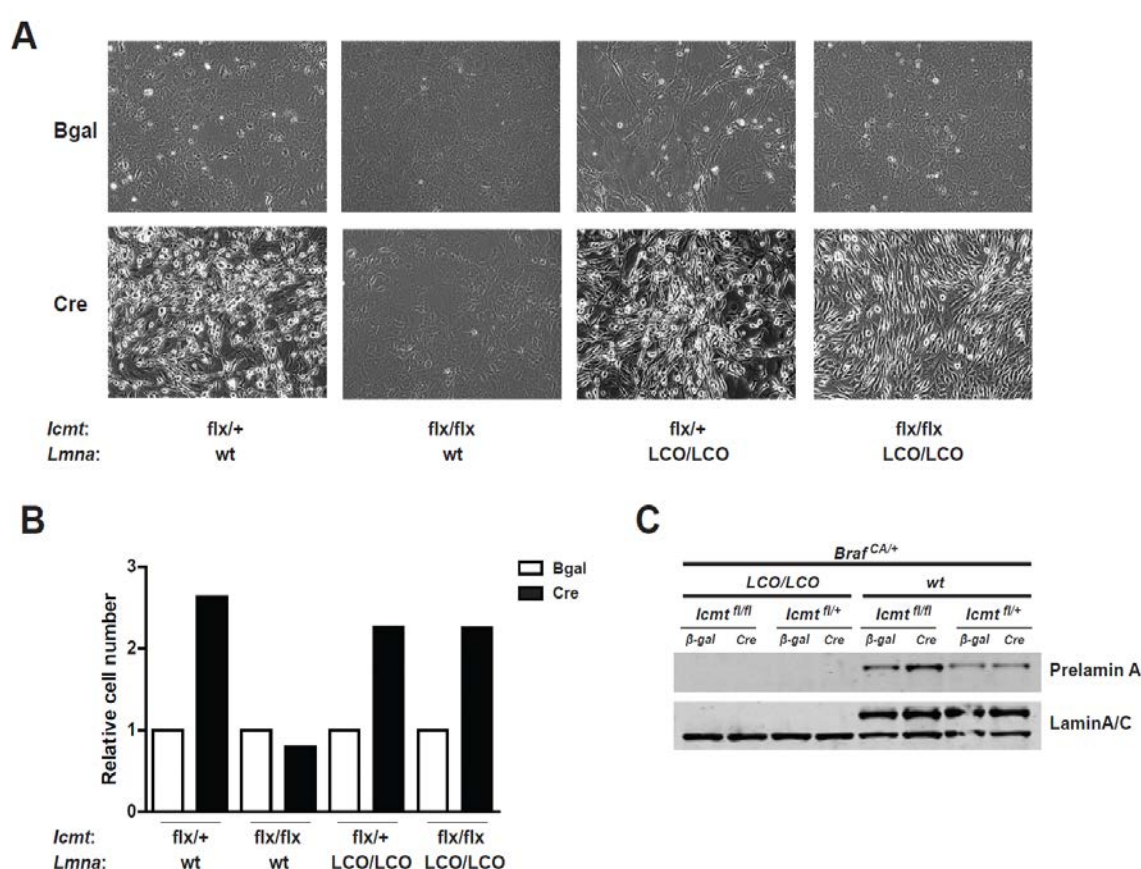


Figure 9: Knocking out Lamin A mediates the effect of reduced proliferation in $ICMT^{-/-}$ $BRAF^{V600E}$ cells

- Pictures of Bil-6-5 ($BRAF^{CA/+}$ $ICMT^{flx/+}$), Bil-6-6 ($BRAF^{CA/+}$ $ICMT^{flx/flx}$), Bil-4-4 ($BRAF^{CA/+}$ $ICMT^{fl/fl}$ $LMNA^{LCO/LCO}$) and Bil-4-3 ($BRAF^{CA/+}$ $ICMT^{flx/+}$ $LMNA^{LCO/LCO}$) 9 Days after treatment with β -Gal or Cre Adenovirus.
- Bar graphs showing $BRAF^{V600E}$ $ICMT^{flx/+}$ (Bil-6-5), $BRAF^{V600E}$ $ICMT^{-/-}$ (Bil-6-6), $BRAF^{V600E}$ $ICMT^{-/-}$ $LMNA^{LCO/LCO}$ (Bil-4-4) and $BRAF^{V600E}$ $ICMT^{flx/+}$ $LMNA^{LCO/LCO}$ (Bil-4-3), 9 days after being treated with β -Gal or Cre Adenovirus.
- Western blot showing Lamin A knockout in $LMNA^{LCO/LCO}$ cells and Prelamin A build up in $BRAF^{V600E}$ $ICMT^{-/-}$ cells only.

A number of MEF cell lines were isolated with varying genetic backgrounds (Table 1). Western blots confirmed the Lamin A knockout in $BRAF^{CA/+} ICMT^{flx/+} LMNA^{LCO/LCO}$ and $BRAF^{CA/+} ICMT^{flx/flx} LMNA^{LCO/LCO}$ cells, leaving only Lamin C (i.e. LCO) (Figure 9c). Western blots also confirmed the accumulation of Prelamin A in new $BRAF^{V600E} ICMT^{-/-}$ cells after treatment with Cre.

After being treated with β -Gal and Cre-Adenovirus cells began to show a change in morphology after 9 days (Figure 9a). The morphology indicated that $ICMT^{-/-}$ cells lacking in Lamin A experienced a transformation when $BRAF^{V600E}$ was activated. Also, it was evident that cells lacking Lamin A, but with functional ICMT were also experiencing a transformation. This showed that the effect of knocking out Lamin A alone (without $ICMT$ knockout) had no inhibitory effect on $BRAF^{V600E}$ activated cells. These results were then confirmed by a cell count showing over a 2-fold increase in growth when comparing Cre treated cells to β -Gal treated control cells (Figure 9b).

Discussion

Prelamin A accumulation appeared to be the factor mediating the anti-cancer effect when knocking out *ICMT* both through proliferation and cell morphology. This could possibly pave the way for new drugs in the treatment of malignant melanoma and other types of cancer. This effect must first be replicated in other cell lines of the same genotype as well as *in vivo* before confirming it. Future studies would need to have the same genetic setup in lung tumour model mice or more ideally in malignant melanoma model mice where we would expect to see reduced malignant melanoma in *ICMT* knockout mice but more aggressive cancer in *ICMT* knockout lacking in Lamin A.

As this study involves knocking out *ICMT* and hence Lamin A one could question if there would be negative effects on the host. This has been answered in a study where the authors produced LCO mice. In this study a number of tests were performed such as looking for bone abnormalities (as Lamin A deficiencies involve excessive bone degradation), weight gain and the mice's ability to grip. It was concluded that Lamin A and its precursor, Prelamin A, are dispensable and removal of this protein has no significant effect on the host⁽⁴⁹⁾. *ICMT* knockout also has no effect as *ICMT*^{-/-} mice are healthy, indicating that if also the case *in vivo* *ICMT* inhibitors may prove an effective anti-cancer drug.

We saw that in *ICMT* knockout cells, the PI3k/Akt pathway was abolished; shown by little to no p-Akt. Although the PI3k/Akt pathway is not downstream of *BRAF*, it is considered to be frequently altered in human cancer and play a main role in the development of cancer⁽⁵⁰⁾. If by knocking out *ICMT* we can significantly reduce the PI3k/Akt pathway, this may also be acting to reduce the cell growth and induce senescence. The idea of targeting the PI3k/Akt pathway for cancer treatment is not a new one⁽⁵¹⁾. A lot of research is now looking into using inhibitors of this pathway either alone or in combination with other drugs to treat cancer patients hence supporting the use *ICMT* inhibitors. These results, too, must first be replicated in many more cell lines and seen *in vivo* before drawing final conclusions.

The MAPK pathway remained hyper-activated in cells with impaired growth from knocking out *ICMT*. This suggests that the anti-tumour effect of knocking out *ICMT* is not specific to the MAPK pathway and so, not specific to *BRAF* induced cancer. Thus, *ICMT* inhibition may prove to reduce the aggressiveness of other types of cancer and possibly be used as a more general treatment for cancer.

In conclusion, both Prelamin A accumulation and the PI3k/Akt pathway were shown to play a role in *ICMT* inhibition's anti-tumour effect, and not just one or the other. This is an example of the complexity of cancer development and it may easily prove to be more complex once tested *in vivo*. Determining Lamin A, and its precursor Prelamin A, to be the reason behind such a dramatic anti-cancer effect will hopefully draw more attention to these molecules and open doors to new research into their role in other types of cancer.

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