ANNA JANSSON

Constitutive activation of the RAF-MEK-ERK pathway in cancer development

Master’s degree project
Constitutive activation of the RAF-MEK-ERK pathway in cancer development

We performed a saturating screen for activating mutations in the protein kinase BRAF (one of three RAF isoforms, RAF=Ras Activated Factor) that can elicit oncogenic transformation of mammalian cells in tissue culture, and we investigated the role of constitutively activated BRAF-MEK-ERK signaling on the pro-apoptotic protein BIM and on apoptosis in melanoma cell lines. In the screen for activating mutants, the positive control and the random mutants failed to transform any of the cell lines used. The results from the melanoma cell lines demonstrate that expression of the BIM is affected by BRAF-MEK-ERK signaling. The results also show that the presence of BIM alone in insufficient for induction of apoptosis in melanoma cells.

Keywords
BRAF, activating mutants, genomic screen, melanoma, BIM, apoptosis

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Constitutive activation of the RAF-MEK-ERK pathway in cancer development

Sammanfattning
Anna Jansson


Ett annat projektmål var att testa hypotesen att förhöjd BRAF signalering är inblandad resistans hos melanomceller mot programmerad celldöd, apoptos. Vi ville även se om den signalväg som BRAF är del av reglerar det pro-apoptotiska proteinet BIM i melanomceller. Detta gjordes genom att blockera signalvägen med en specifik inhibitor.

Screeningen efter aktiverande BRAF gav inga resultat, då varken den positiva kontrollen eller mutanterna transformerade däggdjurscellerna. Vi kunde påvisa att blockering av signalvägen höjer nivåerna av BIM protein i cellerna, och att detta gör vissa av melanomcellinjerna mer mottagliga för induktion av programmerad celldöd.

Examensarbete 20p Molekylär Bioteknik
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1. Introduction

1.1 Cancer

Cancer is a generally characterized by uncontrolled cell division leading to aberrant tissue growth. It is believed that cancers arise from both genetic and environmental factors that lead to abnormal regulation of cell growth. The balance between proliferation and cell death is carefully regulated to ensure the integrity of organs and tissues. Mutations in DNA, due to external or hereditary factors, that lead to cancer appear to disrupt this equilibrium. The rapidly growing number of cells leads to the formation of either a benign tumor, or a malignant tumor – cancer. Benign tumors do not have the ability to spread and are rarely life-threatening. Malignant tumors however, can spread to other locations of the body and invade other organs, (metastasize) and shut down the function of the invaded organs, which, if not stopped, will lead to death.

1.2 Melanoma

Melanoma is notorious for its striking resistance to all kinds of therapeutic treatment. In the US, approximately 50 000 people are diagnosed with the disease and it claims the life of about 8000 people every year\(^1\). The resistance to therapy in patients is reflected in the strong resistance of melanoma cells to various apoptosis inducing treatments to induce apoptosis \textit{in vitro}\(^2\). Melanoma cells may acquire apoptotic resistance either by genetic/epigenetic means or by alteration in signaling pathways that regulate key components of the apoptotic machinery. However, the precise mechanism(s) by which melanoma cells can escape apoptosis is largely unknown, but it is likely to be the result of various biochemical mechanisms.

1.3 RAS-RAF-MEK-ERK signaling

The RAS-RAF-MEK-ERK signal is a transduction pathway that transduces signals from the cell surface to the cytoplasm and the nucleus (Figure 1). This pathway regulates cell growth, differentiation and proliferation. The membrane bound small G-protein Ras is activated by growth factors, hormones and cytokines. Activated Ras recruits Raf (Ras activated factor), a serine/threonine kinase, to the membrane and activates it by phosphorylation. Raf in turn activates a second kinase, MEK (Map-ERK Kinase). MEK in turn phosphorylates and activates ERK (extracellular regulated kinase) resulting in alterations of transcription factor activity, thought to be involved in cell cycle regulation. ERK has a large number of substrates both in the cytoplasm and in the nucleus. Through effects on a wide variety of substrates, activated ERKs can regulate gene expression, cytoskeletal rearrangements, and metabolism to coordinate responses to extracellular signals to regulate proliferation, differentiation, senescence and apoptosis. Moreover, the RAS-RAF-MEK-ERK pathway is found to be activated by somatic mutations in either RAS or RAF in approximately 30% of all human cancers.
1.4 BRAF – identified as an oncogene

The *BRAF* gene contains 18 exons and encodes a series of proteins ranging from 70 to 100kDa that are generated as a consequence of differential usage of exons 1 and 2, and exons 8b and 10. BRAF is a serine/threonine kinase that is an integral part of the RAS-RAF-MEK-ERK pathway. There are three *RAF* genes in mouse and humans, *ARAF*, *BRAF* and *CRAF* (also referred to as Raf-1). Human and mouse RAFs contain three conserved regions in common, CR1-CR3 (Figure 2). Two of these, CR1 and CR2, are located in the N-terminus of the protein, and the third, CR3, is the kinase domain, in the C-terminus. Of the three isoforms, BRAF is the only one that is oncogenic. All three forms are dependent on phosphorylations within their activation segments for activity. However, ARAF and CRAF may require additional phosphorylations in the kinase domain for full activity, whereas BRAF has a much higher basal kinase activity and does not require these additional phosphorylations. RAF was the first effector identified downstream of RAS.

1.5 BRAF and human cancer

A common feature of numerous cancers is the constitutive activation of the RAS-RAF-MEK-ERK pathway. RAS was identified as a *bona fide* human oncogene in the 1980s and to date activating RAS mutations are found in about 20% of human cancers. Recently, somatic mutations in RAF were found in a screen for mutated genes in cancer. Somatic BRAF mutations most frequently occur in malignant melanomas (in 60-70% of the cases), but also common in colorectal, ovarian, and papillary thyroid carcinomas, indicating the potential general importance of the BRAF-MEK-ERK pathway in the initiation and progression of human cancer.
Sequencing of the \textit{BRAF} gene from various human cancer cell lines and primary patient specimens has revealed over 30 point mutations, most of which lead to constitutive activations of the protein’s kinase activity. Most of the mutations in \textit{BRAF} are clustered to two regions – the glycine rich P-loop of the N-lobe and the activation segment and adjacent regions. The most common mutation found in \textit{BRAF} is a T→A transversion that replaces the valine in position 600 with a glutamic acid. This particular mutation is responsible for 90% of the \textit{BRAF} mutations in human cancers. The V600E mutation is positioned in the activation segment. This mutation has all the important characteristics of a conventional oncogene. The mutant protein has significantly elevated kinase activity, constitutively stimulates ERK activity in vivo independent of Ras, and it potently transforms NIH3T3 cells. In 2004, Wan et al presented structures of normal \textit{BRAF} and \textit{BRAF}_{V600E} in complex with the general RAF inhibitor \textit{BAY43-9006} \cite{wan2004crystal}. These structures suggest that many of the residues that have been found mutated in cancer contribute to stabilization of an inactive conformation of the Brat kinase domain. For the majority of these mutations, this means stimulation of enhanced \textit{BRAF} kinase activity toward MEK.

\textbf{1.6 \textit{BRAF} and Melanoma}

Dysregulation of intracellular signaling pathways is common to most human malignancies \cite{braaten2002melanomas}. For over 20 years, \textit{RAS} has been known to be mutated in various human cancers, including melanoma. Approximately 20% of human melanomas express a mutationally activated form of \textit{NRAS} (one of three different \textit{RAS} genes) \cite{drake2013melanoma}. In addition, the gene encoding \textit{BRAF}, a prominent \textit{RAS} effector protein, has also been found to be mutated in human cancers. Recently, activating mutations in \textit{BRAF} were found by numerous investigators at high frequency, 60-70\%, in malignant melanomas \cite{braun2003melanoma}. These observations suggest that melanocytes, the cells from which melanoma arises, have a special susceptibility to the sustained activation of \textit{BRAF}. However, mutationally active \textit{BRAF} is also expressed in a high percentage (~90\%) of benign melanocytic nevi, which may be progenitors of malignant melanoma. This would suggest that \textit{BRAF} activation alone is insufficient for melanomagenesis, and that it needs to be combined with other genetic events, for example loss of tumor suppressor genes, such as \textit{Apoptotic activation factor 1 (Apafl)} or \textit{PTEN}.

Although the high frequency of \textit{BRAF} mutations suggests a critical role of the \textit{BRAF-MEK-ERK} pathway in melanomagenesis, the mechanism(s) by which this pathway influences the aberrant behavior of melanoma cells are largely unknown. Here we test the hypothesis that one mechanism by which \textit{BRAF} might influence the survival of melanoma cells may be through effects on programmed cell death. In particular, we suspect that these effects may be mediated in part by inhibitory effects of \textit{BRAF} on the expression/activity of a pro-apoptotic member of the BCL-2 family known as BIM. Indeed, BIM had been has been reported to be a target for ERK phosphorylation in a wide variety of cell systems \cite{fesik2003apoptosis}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{braf_structure.png}
\caption{Generic figure of \textit{BRAF} primary structure and point mutations identified in association with cancer. The illustration was adapted from Wan et al, 2004\cite{wan2004crystal}.}
\end{figure}
1.7 Apoptosis signaling

Apoptosis, or programmed cell death, is a physiologic process essential for embryonic development, the maintenance of tissue homeostasis and for an effective immune system. One hallmark of many cancer cells is the acquired ability to evade apoptosis and thereby promoting cell proliferation under conditions when normal cells would die. Apoptosis is controlled by a variety of signaling pathways and the key distinguishing features of such pathways are outlined below (Figure 3).

Apoptosis is often accompanied by activation of a family of cysteine directed aspartate specific proteases known as Caspases. There are at least two pathways by which Caspases can be activated. The first pathway is induced by the binding of ligands to transmembrane death receptors, that via FADD (Fas-associated death domain) activates Caspase-8 and Caspase-10, with subsequent activation of the effector Caspase-3 and Caspase-7. This is known as the extrinsic pathway. At least six distinct death receptors are known, but only three of them have been extensively studied.

The second apoptotic pathway can be initiated by various cellular stress signals, for example inadequate cytokine support and diverse types of intracellular damage, and it is known as the intrinsic pathway. This pathway requires caspase-2-dependent disruption of the mitochondrial membrane and release of mitochondrial proteins, such as cytochrome c. Mitochondrial membrane integrity is believed to be maintained due to a balance between the activities of pro-apoptotic BCL-2 family members (e.g. BAX, BAK, BIM and BAD) and the anti-apoptotic members such as BCL-2, BCL-XL and MCL-1.

BCL-2 is found on many intracellular membranes, including the mitochondria. BCL-2 is membrane bound even in healthy cells, whereas another anti-apoptotic family member, MCL-1 is normally cytosolic and is translocated to the mitochondrial membrane in response to cytotoxic signals.
BAX and BAK are critical pro-apoptotic proteins, and also members of the BCL-2 family of proteins. In their absence, cells do not readily undergo apoptosis. Although their precise mechanism of action is unclear they are reported to form a pore that promotes the permeabilization of the outer mitochondrial membrane, allowing apoptogenic proteins such as cytochrome-c to enter the cytoplasm. BAX is cytosolic in healthy cells, and in response to apoptotic signals it undergoes a conformational change and translocates to the mitochondrial membrane. BAK is present in the mitochondrial membrane even in healthy cells, but changes conformation in apoptotic cells and forms larger aggregates. In vitro, BAX and BAK form oligomers that can form “pores” mitochondria, allowing the exit of cytochrome c, but the nature of these channels through which mitochondrial proteins are released remains controversial.

It is reported that in healthy cells the pro-apoptotic activity of BIM is suppressed either by silencing of BIM expression or by its sequestration as a complex with a microtubule motor protein complex. However, in response to stress, levels of BIM increase in the cytoplasm through release from sequestration\textsuperscript{19}. In the cytoplasm BIM inhibits BCL-2 activity. This leads to cytochrome c release from the mitochondria. Released cytochrome c in turn induces a conformational change in the scaffold protein Apaf-1, allowing the recruitment and oligomerization of procaspase-9, and the resulting heptamer, the “apoptosome”. Activated Caspase-9 in the apoptosome then promotes activation the effector caspases-3 and -7. Under certain conditions, crosstalk between the two apoptosis networks exists. This involves another pro-apoptotic member of the BCL-2 protein family, Bid. Bid is cleaved by active caspase-8 following death receptor stimulation and the activated part, tBid, translocates to the mitochondria, where it initiates the mitochondrial apoptosis pathway\textsuperscript{18}. There are also reports that Caspase-8 can directly activate Caspase-3 and vice versa.

Alterations in the balance between the pro- and anti-apoptotic BCL-2 family proteins involved in these pathways have been noted in the course of melanoma progression.\textsuperscript{17}

1.8 Target-directed chemotherapy, an example

In most cases of CML (chronic myeloid leukemia) the leukemic cells share a chromosome abnormality that is the reciprocal translocation between one chromosome 9 and one chromosome 22. This translocation results in one chromosome 9 longer than normal and one chromosome 22 shorter than normal. The latter is called the Philadelphia chromosome (designated Ph\textsuperscript{1}). The DNA removed from chromosome 9 contains most of the proto-oncogene designated c-ABL, a tyrosine kinase. The break in chromosome 22 occurs in the middle of a gene designated BCR. The resulting Philadelphia chromosome has the 5' section of BCR fused with most of c-ABL. The hybrid BCR-ABL gene produces an abnormal "fusion" protein that constitutively activates a number of cell activities that normally are turned on only when the cell is stimulated by a growth factor. This unrestrained activation increases the rate of mitosis and protects the cell from apoptosis.

In recent years, the target-directed cancer chemotherapy has taken a big step forward when the molecule STI-571, a highly specific kinase inhibitor, was found to target the tyrosine kinase Bcr-Abl. Mutations that disrupt the kinase activity results in a loss of all transforming functions of Bcr-Abl. This suggests that a small molecule kinase inhibitor would be a potent agent in treating leukemia, and this is exactly what has been found. The course of events that has been detected in patients with CML, is that when treated with STI-571 in the indolent chronic phase, the drug induces complete remission in almost all patients that were treated.
directly upon diagnosis. However, the patients that were in the more aggressive blast crisis stage when treated are likely to become resistant to the drug. The majority of the patients that developed drug-resistance were found to have mutations within the Bcr-Abl kinase domain.

In the paper by Azam, Latek and Daley, they describe how they undertook an unbiased in vitro screen to identify a complete set of variants of BCR-ABL that were resistant to the kinase inhibitor STI-571, using the *E. coli* strain XL1-red to induce random mutagenesis through out the *BCR-ABL* gene.

A common feature of kinases is their plastic nature, where active and inactive states are closely linked to open and closed conformations. Amino acids in the positions that are frequently mutated in constitutively active proteins seem to have a stabilizing effect to keep the protein in an inactive state. The small molecule BRAF inhibitor BAY43-9006, have been found to block the activity of both normal BRAF, and the constitutively activated form *BRAF*<sub>V600E</sub>. However, by acquiring mutations that prevents BAY43-9006 from making a complex with the BRAF, it can evade the inhibition. As a first step, we would like to do a genomic screen to map the activating mutations in the *BRAF* gene. The second step would be to perform a screen for resistance to the BAY43-9006 among these activating mutants. However, in this report we deal only with the first step.

2. **Aim**

The RAS activated RAF-MEK-ERK signaling pathway has a central role in regulating cell growth and survival of cells as seen in numerous human cancers. Specifically, Raf has been shown to inhibit apoptosis in cultured cells. This has led to that this pathway is being considered an attractive target for anti-cancer therapies. However, the mechanism by which the Ras-Raf-MEK-ERK pathway transforms, or contributes to the transformation of, normal cells into cancer cells remain largely unknown. Small-molecule inhibitors of the kinase components of the MAPK cascade have reached clinical trials. Two of these are BAY43-9006 and CI-1040, the first being an inhibitor of Raf, and the second being an inhibitor of MEK.

BAY43-9006 has been shown to block activity of both wild type BRAF and, the constitutively activated form, *BRAF*<sub>V600E</sub>. There is a range of mutations giving rise to constitutively activated forms of BRAF that might or might not be inhibited by BAY43-9006. If more information was obtained about which mutations that can evade inhibition by BAY43-9006, it would facilitate the design of the next generation of inhibitor drugs.

One of our objectives was to perform a saturating screen for all possible activating mutations in human *BRAF* which can elicit oncogenic transformation of mammalian cells in tissue culture. These mutations will be induced by random mutagenesis in a DNA repair defective strain of *E. coli*.

Furthermore, it is of highest interest to assess the effect of these small-inhibitor drugs. The MEK inhibitor CI-1040 is known to be an inhibitor with high specificity. Since *BRAF* mutations leading to the hyperactivation of the Ras-Raf-MEK-ERK pathway are found at high frequency in melanomas, we were particularly interested in assessing the effect of MAPK pathway inhibitors on apoptosis in melanoma cell lines. Based on experiments performed on other cell lines, we have reason to believe that Bim is a potential key player in the induction of apoptosis.
Specifically, our objective was to investigate the effects of the Raf-MEK-ERK pathway on Bim expression and apoptosis in various melanoma cell lines, by blocking MEK activity with or without an additional stress induction.

3. Materials and Methods

3.1 Cell culture

Rat1a fibroblasts, NIH3T3 fibroblasts, RIE-1 epithelial cells and LNXE packaging cells were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) without phenol red (Cellgro), supplemented with 10% FCS, and 1x Penicillin-Streptomycin-Glutamine (Invitrogen). All the melanoma cell lines were cultured in DME-H16 media with 3 g/l Glucose, and 0.584 g/l L-Glutamine, 0.11 g/l Na Pyruvate and 3.7 g/l NaHCO₃, with 10% Fetal Calf Serum, 5µg/ml of insulin and Penicillin-Streptomycin (both from UCSF, Cell Culture Facility). To trophic factor deprived media, only Penicillin-Streptomycin was added. The dishes with cells that were deprived of trophic factors were washed twice with PBS to remove traces of serum.

The cells were cultured in 100mm dishes, so as to still be sub-confluent when harvested. In the experiments involving CI-1040 or Etoposide, the controls were treated with diluent alone. All the cells were cultured at 37°C and 5% CO₂.

3.2 The plasmid constructs

The plasmids used, pLXSP3 BRAFwt and pLXSP3 BRAFV599E, were previously made. Human BRAF cDNA was cloned as an EcoRI-XhoI fragment into the EcoRI-SalI site of the pLXSP3 plasmid using Gateway-mediated recombination (insert is shown in Figure 4). The plasmid encodes ampicillin resistance in E. Coli and Puromycin resistance in mammalian cells.

Figure 4. Generic map of the insert containing BRAF

3.3 Chemicals

The CI-1040 was a gift of Dr Philip Cohen, University of Dundee. Etoposide was purchased from Sigma. (Additional information in Appendix 1) CI-1040 was dissolved in DMSO (Dimethyl sulfoxide). Working concentration was 2µM. Etoposide was dissolved in ethanol. Working concentration was 40µM.

3.4 BRAF mutagenesis, Library generation

0.5µg of plasmid (pLXSP3 BRAFwt) was used per 100µl of cells for transformation of the E. Coli strain XL1-Red (Stratagene). The transformants were selected for on Ampicillin-agar bacterial plates. After 24 hours incubation at 37°C, followed by 1 hour at 4°C, the cells were collected by scraping. The cells were frozen prior to DNA extraction (Maxi Prep Kit, Qiagen).
10\(\mu\)g of the plasmid library was used to transfect LNXE packaging cells (~5\(\times\)10^6 cells / 100mm plate). Viruscontaining supernatants were isolated and used to infect 10^6 Rat1a and NIH3T3 fibroblasts and RIE-1 epithelial cells. 24hrs post infection, the cells were split into full media containing 4\(\mu\)g/ml of Puromycin. The cells were in selection for ~72hrs.

### 3.5 Transformation screen in Soft Agar

The cells were plated onto 100mm plates in 16ml DMEM upplemented with 0.3% bicarbonate, 10% FCS, 20mM Heps buffer (pH 7.4), Penicillin/Streptomycin/Glutamine, Fungizone, 50\(\mu\)g/ml Gentamycin, and 0.45% FMC SeaPlaque Low Melting Temperature agarose. The plates were coated with an 8ml bottom layer consisting of DMEM with 0.9% FMC SeaPlaque Low Melting Temperature agarose. An additional 8ml of the same composition was poured on top of the growth layer. The plates were put at 4°C for 3x10 min to solidify the agar. 8ml of liquid DMEM full media was poured on top of the solidified agar. The liquid media was changed every 5-7 days. After 20 days, the negative and positive controls, along with one set of cells expressing the randomly mutated BRAF, were stained with MTT (100\(\mu\)g/ml, Sigma), 5ml per 100mm dish, for 2hrs at 37°C. The stained plates were scanned with an EPSON scanner.

### 3.6 RT-PCR – RNA quantification

RNA was extracted with the RNAeasy kit from Qiagen. RT-PCR was performed with assistance from the genome core at UCSF, with the TaqMan technique, using the following primers from Applied Biosystems:

- **Bim** cat #: Hs00197982-m1
- **Bcl-2** cat #: Mm00519268-m1
- **Mcl-1** cat #: Hs00172036-m1

### 3.7 Antibodies

Antibodies were used in this study as follows:

- A mouse monoclonal (\(~p42 MAP Kinase\)) against ERK2 (Cell Signal),
- A rabbit polyclonal against Bim (Axxora),
- A rabbit polyclonal against Caspase-3 (Cell Signal),
- A mouse monoclonal against Mcl-1 (Biosource International),
- A mouse monoclonal against Bcl-2 (BD Transduction Labs),
- A rabbit polyclonal (Phospho-p44/p42 MAP Kinase Tyr202/Tyr204) against Phospho-ERK (Cell Signal).

### 3.8 Western blotting

Cells were lysed in RIPA buffer \{150mM NaCl, 1% NP40(v/v), 0.5% DOC, 0.1% SDS, 50mM Tris pH8.0, and a mixture of protease inhibitors (Roche)\}. Equal amounts of protein were loaded onto and separated by Tris-Glycine gels (12 or 16%) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P from Millipore). After incubation with the relevant antibody, the antigen-antibody complex was visualized with SuperSignal West Dura Extended Duration Substrate (Pierce), according to the manufacturer’s instructions. To ensure equal loading and transfer, membranes were probed for ERK2.
3.9 Cell viability assay

Cell death was quantified by Annexin-V-FITC staining (BD Biosciences), according to the manufacturer’s protocol, followed by flow cytometric analysis by using a FACScan (Becton Dickinson) and CellQuest software.
4. Results

4.1 Outline of an in vitro screen to identify activating mutants

The pLXSP3 BRAF plasmid was constructed by introducing the cDNA of human BRAF in to the pLXSP3 backbone. The BRAF plasmid was randomly mutagenized by introducing it into the E. coli strain XL1-Red (Stratagene) that is deficient in three major pathways for DNA repair, mutS (error-prone mismatch repair), mutD (deficient in 3’- to 5’- exonuclease of DNA polymerase III), and mutT (unable to hydrolyze 8-oxodGTP). The random mutation rate is ~5000 times higher than that in the wild type. This produced a library with full representation of mutations (Appendix 2). Mutant plasmids were transfected into LNXE packaging cells, and Rat1a fibroblasts, NIH3T3 fibroblasts and RIE-1 epithelial cells were infected with the viral supernatants. A negative and a positive control were propagated along side with the population of randomly mutagenized BRAF. BRAF wt was used as a negative control, and BRAF V600E, that has been documented to transform NIH3T3 fibroblasts at 138-fold higher frequency than wild type BRAF12, was used as a positive control. After ~72 hours in Puromycin selection, pictures of the NIH3T3 cells (wild type, cells expressing BRAF wt and cells expressing BRAF V600E) were taken (Figure 6). The cells were then put in soft agar to select for colony formation. The cells (wild type, cells expressing BRAF wt, and cells expressing BRAF V600E) were plated at densities ranging from 10^7 to 10^8 cells per 100mm plate, with a 10-fold increase in each step (Figures 8 and 9). The cells expressing the randomly mutagenized BRAF were plated at 10^7 cells per 100mm plate in quadruplicates (Figure 5).

Figure 5. Strategy for activating mutant screen
Step 1: pLXSP3 BRAF wt was transformed into XL1-Red E.Coli cells to generate a library of random mutants; Step 2: Transfect LNXE packaging cells with the plasmid, and recover virus titer; Step 3: Infect the recipient cells (rat or mouse fibroblasts, or epithelial cells) with the virus; Step 4: Screen for transforming cells in a soft agar assay; Step 5: Recover transformed colonies from the agar, expand them, and isolate genomic DNA; Step 6: PCR-amplify the region of interest and sequence. The illustration was adapted from Azam, Latek and Daley, 2003."
4.2 Changed cell morphology in the target cells

As seen in Figure 6, a change in cell morphology was observed in the NIH3T3 cells expressing BRAF\textsubscript{V600E}, compared to the wild type NIH3T3 cells, and the NIH3T3 cells expressing wild type BRAF. A corresponding morphology change could also be observed in the Rat1a fibroblasts and the RIE-1 epithelial cells. However, the RIE-1 cells expressing BRAF\textsubscript{V600E} showed low viability and this cell line was not propagated to the transformation screen in soft agar.

![Figure 6. Morphology of NIH3T3 cells, wild type and with the BRAF\textsubscript{wt} and the BRAF\textsubscript{V600E} insert.](image)

4.3 Transformation assay in soft agar aiming to select for activating mutants

In both cell lines that were propagated to the transformation assay in soft agar, the positive control failed to produce distinct colonies and, hence were unable to in an indisputable way distinguish itself from the negative control (Figures 8 and 9). The cells were plated at a range of $10^3$ to $10^7$ cells per 100mm plate, to enable calculations of recovery frequency of the positive control. However, the cells expressing BRAF\textsubscript{V600E} did not distinguish itself in any way from the negative control (BRAF\textsubscript{wt}) or the cells without a human BRAF insert in any of the cell densities plated. Clusters of small numbers of cells can be observed, but the expected colonies of several hundred cells were absent. The population of cells expressing the randomly mutated BRAF did not produce any colonies in soft agar (Figure 7).

![Figure 7. MTT stain of soft agar assay with NIH3T3 cells expressing randomly mutated BRAF. The experiment was performed in quadruplicates.](image)
Figure 8. Soft Agar Assay stain. Ranges from $10^3$-$10^7$ cells were plated per 100mm plate. A. Rat1a wild type cells B. Rat1a $BRAF_{wt}$ C. Rat1a $BRAF_{V600E}$

Figure 9. Soft Agar Assay stain. Ranges from $10^3$-$10^7$ cells were plated per 100mm plate. A. NIH3T3 wild type cells B. NIH3T3 $BRAF_{wt}$ C. NIH3T3 $BRAF_{V600E}$
4.4 Melanoma

Based on the hypothesis that the BRAF-MEK-ERK pathway is involved in the regulation of BIM protein, and the resistance to apoptosis, in melanoma, we wanted to assess this by blocking the pathway with the MEK-inhibitor CI-1040. In the experiments, melanoma cell lines derived from tumors from different stages of the disease were used (Table 1). All of the cell lines had the BRAF\textsubscript{V600E} mutation.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sample</th>
<th>Lesion type</th>
<th>\textit{BRAF} digest result</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM9</td>
<td>Met</td>
<td>\textit{BRAF\textsubscript{V599E}}</td>
<td>heterozygous</td>
</tr>
<tr>
<td>WM35</td>
<td>RGP</td>
<td>\textit{BRAF\textsubscript{V599E}}</td>
<td>heterozygous</td>
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<td>\textit{BRAF\textsubscript{V599E}}</td>
<td>heterozygous From the same patient</td>
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<tr>
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<td>Met</td>
<td>\textit{BRAF\textsubscript{V599E}}</td>
<td>homozygous same patient</td>
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RGP – (Radial Growth Phase) – early stage of the disease  
VGP – (Vertical Growth Phase) – more advanced stage of the disease  
Met – (Metastases) – late stage of the disease

Table 1. Properties of the melanoma cell lines. Lesion type tells from which type of tumor that the cell line was derived from. All the cell lines carry the BRAF\textsubscript{V600E} mutation, heterozygous or homozygous. The WM278 and WM1617 cell lines were derived from different tumor types from the same patient.

4.5 Analysis of the effects of CI-1040 on cell signaling in melanoma cells

We hypothesize that the Ras-Raf-MEK-ERK pathway is involved in the regulation of BIM expression in melanoma cells and that hyperactivation of the Ras-Raf-MEK-ERK pathway downregulates BIM expression in the cells. Initially we aimed to investigate what effects a block of the pathway with the MEK inhibitor CI-1040 might have on cell signaling, specifically the pro-apoptotic BCL-2 family member BIM. Melanoma cell lines (WM278, WM1617, WM35 and WM9) representing different stages of the disease (Table 1) were exposed to CI-1040 (2\textmu M) for 1, 4, 8, 16, 24 and 48 hours at what time cell extracts were prepared. Western blots were prepared and probed with antisera to detect the expression of (ERK2) and activity of the ERK MAP kinases and the expression of BIM and BCL-2 as indicated. As expected, CI-1040 leads to decreased activity of ERKs (P-ERK) in all cell lines tested without changing the overall expression of the proteins (ERK). Consistent with our hypothesis, MEK inhibition led to an induced expression of BIM in all cell lines (Figure 10).

The kinetics of the BIM induction between the cell lines was however different. The WM35 cell line showed detectable levels of BIM protein as early as after 1hr, in the WM278 BIM was detected at 4hrs, but BIM was not present in WM1617 until after 24hrs after treatment. The WM9 cell line BIM appeared to be present at all the time points as well as the control.

In both the WM278 and the WM1617, Caspase-3 levels appeared to fluctuate between the samples in the same way as the total ERK, which would mean that there were no significant changes in the Caspase-3 levels. BCL-2 levels appeared to be low and not changing when the cells were exposed to CI-1040.
4.6 Analysis of the effects of CI-1040 on mRNA expression in melanoma cells

BIM has been shown to be regulated at both transcriptional levels and post-translational levels in other tissues\(^{22-23}\), and to illuminate the means by which BIM is regulated in melanoma cells, we wanted to investigate whether or not changes in mRNA levels could be detected. We were also interested in the mRNA expression of the anti-apoptotic proteins BCL-2 and MCL-1, even though we did not expect the MAPK cascade to be involved in the mRNA expression of these genes.

The cells (WM9, WM35, WM278 and WM1617) were exposed to CI-1040 (2µM) for 4, 24 and 48 hours, followed by isolation of RNA. RNA levels were assessed using quantitative PCR. The experiment was performed in duplicate. As can be seen in Figure 11A, expression of BIM mRNA was induced when the cells were exposed to CI-1040. The mRNA expression of both BCL-2 and MCL-1 showed much less of a change (Figure 11B & C), and no common trend could be seen in the cell lines tested.
Figure 11. The cells were exposed to the MEK inhibitor CI-1040 for 4, 24 or 48 hours. RNA was extracted from the cells, and mRNA levels were measured for BIM (A), BCL-2 (B), and MCL-1 (C) using quantitative PCR.

Raw data and calculations from the quantitative PCR can be found in Appendix 3 and Appendix 4.
4.7 Trophic Factor Deprivation of melanoma cells in combination with MEK inhibition

Melanoma cells have earlier been reported not to respond to trophic factor deprivation (TFD) with apoptotic cell death as does melanocytes. However, we hypothesized that an additional stress factor would sensitize the cells to the inhibition of the RAF-MEK-ERK pathway, or vice versa, and that this would lead to further induction of pro-apoptotic proteins such as BIM, and also increased sensitivity to apoptotic cell death.

The cells were exposed to either CI-1040 (2µM), or deprived of trophic factors, or both, for 24 and 48 hours. At these times, cell lysates we prepared from cell lines WM1617 and WM9 for western blotting (Figure 12 A and B), or the cells were stained with AnnexinV, cell lines WM278 and WM1617, and apoptotic cell death was assessed using flow cytometry (Figure 13).

![Figure 12. A. WM1617 B. WM9 The cells were exposed to CI-1040 and/or TFD for 24 and 48 hrs. Cell lysates were prepared and western blots were probed for P-ERK and BIM.](image)

BIM protein was induced when the cells were exposed to CI-1040, either alone or in combination with the trophic factor deprivation. WM1617 showed a delayed BIM response in that only very weak expression was detectable after 24 hours of treatment but increased at 48 hours.

In the WM9 cell line, BIM was clearly induced both by the CI-1040 alone and together with trophic factor deprivation at 24hrs. Despite BIM protein levels being increased at 48 hours following MEK inhibitor treatment alone, the protein was not detected in cells treated with CI-1040 in combination with TFD. This is however most likely not a real observation, but an artefact of technical problems. Inconsistencies in the P-ERK blots were observed, these too are most likely the results of technical issues.
When looking at the cell death assay, at 24 hours neither CI-1040 or trophic factor deprivation appeared to have any effect on the cells. Following the treatment of cells with MEK-inhibitor CI-1040 alone for 48 hours, we observed a modest increase in the number of apoptotic cells. When exposed to trophic factor deprivation for the same amount of time, levels of apoptosis did not seem to change significantly in any of the cell lines tested.

However, when cells were deprived of trophic factors in the presence of the MEK inhibitor we were able to detect slightly elevated levels of cell death at 24 hours. Moreover, at 48 hours we could see significant synergy in the number of cells undergoing apoptosis. There was no significant difference in the death of the WM1617 when they were treated with only CI-1040, compared to when they were both deprived of trophic factors and treated with CI-1040. We could also see that trophic factor deprivation alone did not seem to have any effect on the cells.
4.8 Exposing melanoma cells to Etoposide in combination with MEK-inhibition

A drug that has been used for treatment of certain cancers is the DNA-damage inducing agent Etoposide. We wanted to investigate whether or not this death stimulus might have a sensitizing effect on the melanoma cells, both alone and in combination with exposure to CI-1040. This is also important when determining if signal pathway inhibitors may be used in combination with other cytotoxic agents.

The cells were exposed to either CI-1040 (2\(\mu\)M), or 40\(\mu\)M of Etoposide, or both, for 48 hours. The cells were then either lysed and western blots were prepared (cell lines WM1617 and WM35, Figure 14), or they were stained with Annexin V and apoptotic cell death was assayed with flow cytometry (cell lines WM278 and WM1617, Figure 15).

Looking at the proteins levels (Figure 14), activity of ERK did not appear to be affected by Etoposide alone. Phosphorylation of ERK appeared to be blocked by the CI-1040 alone, and in combination with the Etoposide. MCL-1 levels appeared to be unaffected by CI-1040 or Etoposide alone, but protein levels went down dramatically when exposed to a combination of the two.

A clear induction of BIM protein could be seen both when treated with CI-1040 alone, and in combination with Etoposide. In the control, and when treated with Etoposide alone, BIM seemed to be phosphorylated as indicated by the mobility shift upwards. Due to technical difficulties, the BIM blot is missing for the WM35 cell line.

When looking at the ability of the different agents to induce apoptotic cell death, we could observe that treating the cells with Etoposide alone, induced a distinct killing effect in the WM278 cell line. When treating the same cell line with Etoposide in combination with CI-1040 compared to treating the cells with either CI-1040 or Etoposide alone, a legible increase in apoptotic cell death was observed. However, the increase looks like it’s additive rather than synergistic. Etoposide appeared to have no effect on apoptotic cell death in the WM1617 cell line, neither alone, nor in combination with CI-1040 (Figure 15).
Figure 15. Cell death assay with Annexin V staining. The cells were exposed to CI-1040 and/or Etoposide for 48 hours.
5. Discussion

5.1 Screen for activating mutants – yet to be done

Due to the fact that we were not able to find a cell line where we could observe a clear difference between our negative (cells expressing BRAF<sub>wt</sub>) and positive control (the cells expressing BRAF<sub>V600E</sub>), we were not able to go through with the screen. However, what we would have done had the screening process worked, was to pick and expand the transformed colonies from the random mutant population from the soft agar. Genomic DNA would then have been extracted, and the individual clones would have been subjected to PCR amplification of the BRAF gene, and the amplified segment would then have been sequenced. To establish whether the clones have one or several viral inserts of the randomly mutated BRAF gene, we would have done southern blots with genomic DNA from all the clones, where the DNA would have been cut with BamHI. This enzyme would cut the DNA asymmetrically, i.e. if more than one insert exists, the fragments containing the BRAF gene will be of different sizes. Then using a puromycin probe, the southern blot would give the same number of bands as the number of inserts for each clone. In the ideal case, we would only have one insert in each clone, since this would facilitate the process of determining which mutation that gives rise to the transformation of the cell. In some cases there might be several mutations, even though only one insert. To verify which mutation that ultimately causes the transformation of the cell, we would assess the effect of each mutation through site directed mutagenesis. For each mutation found in the screen we would induce a single site mutation in the pLXS P<sub>3</sub> BRAF<sub>wt</sub> plasmid, transfect and infect recipient cells and then assessing the potential in transforming the cells. These mutations would be our final candidates for mutations causing constitutive activation of BRAF.

5.2 Why did we not have any colony formation in soft agar?

So, how can it be explained that no difference could be detected between the recipient cells expressing wild type BRAF and the ones expressing BRAF<sub>V600E</sub>? One explanation is that the recipient cells were of bad quality and hence did not display the typical characteristics for that particular cell line, perhaps due to that the cells were kept through too many passages before they were frozen down. Considering that the same result was obtained with several cell lines, the probability of that explanation can be questioned.

A more plausible explanation to why the cells expressing BRAF<sub>V600E</sub> did not form colonies is that in fibroblasts, the level of ERK signaling is critical to the cells ability to proliferate. ERK signaling stimulates proliferation, but when ERK signaling is too strong, the cells with stop differentiate or senesce<sup>24</sup>. It seems that sustained ERK signaling is necessary for platelet-derived growth factor (PDGF)-induced proliferation in fibroblasts, but that when strong ERK signaling is induced, cell-cycle arrest occurs due to transcriptional up-regulation of cell-cycle inhibitors such as p21<sup>5</sup>. In our construct, BRAF is expressed under the viral LTR promoter, which is a high expression promoter. However, cells in the tumors where BRAF mutations are common, for example melanoma, are rather different to fibroblasts. Perhaps it is so that ERK signaling mediated by human BRAF under the viral LTR promoter is too strong to induce transformation in NIH3T3 cells.

It has previously been published that BRAF<sub>V600E</sub> potently transforms NIH3T3 cells at 138-fold higher frequency than wild type BRAF<sup>12</sup>. However, the construct used was different, and although a high expression promoter was used here too (CMV), the plasmid was directly
transfected in to the target cells, i.e. the BRAF gene was not integrated in the genome of the recipient cells as in our experiment. Since a viral insert can integrate anywhere in the genome it might end up positioned in a region of the chromosome with either very low or very high transcriptional activity, which can affect the actual level of expression.

5.3 Alternative screening methods

Although growth in soft agar is considered a stringent test for cell transformation, it does have the draw back of being a time consuming method. For future experiments, a screening method that gives a more direct result would be to prefer. One alternative method to screen for activating mutants is Rhodamine 123 staining. It was shown by Zarbl, Latreille and Jolicoeur in 1987, that v-fos transformed Rat1 fibroblasts displayed prolonged retention of Rhodamine 123 when stained for 30 minutes, and then cultures dye-free for another 24 hours, compared to its non-transformed counterparts\textsuperscript{25}. This is a potential method to sort for transformed cells in a very time effective manner. Another alternative screening method is the detection of BRAF induced surface markers, e.g. β\textsubscript{3}-integrin\textsuperscript{26}.

5.4 Apoptosis signaling in melanoma cells

In the set of experiments with melanoma cell lines, I have focused on comparing the WM278 and the WM1617 cell lines, since these are derived from the same patient, but from different stages of the disease: WM278 is from a vertical growth phase tumor and the WM1617 is from a metastatic tumor. This makes an interesting comparison in their resistance to cell death and what differences in apoptotic proteins can be detected. However, the WM278 cell line is more difficult to culture, and have a more “spread out” morphology, which has made protein extractions more difficult, and hence, for some of the experiments, results from the WM278 cells line are missing. Although focus has been on the WM278 and the WM1617 cell lines, I have attempted to do each experiment with multiple cell lines, to permit comparisons of the different disease stages. Problems with culturing cells, extracting proteins and contaminations have necessitated the use of different cell lines in certain instances, but interesting comparisons may still be made. Trends that can be detected in the results are presented here. Obviously, a range of further experiments would have to be made to be able to put more weight on the hypothesis presented here.

5.5 Possible BIM regulation by ERK

Blocking the Ras-Raf-MEK-ERK pathway induces BIM protein in all cell lines. This suggests that a possible mechanism for BIM regulation is mediated by ERK, for example by phosphorylation. It has been shown in other cell types that BIM is regulated in this manner – a phosphorylation would induce a conformational change that would shorten the half-life of the protein. When looking at levels of BIM mRNA as a result of MEK-inhibition, an induction can be seen also here. After 48 hours the mRNA levels were between 2 and 3 times higher compared to the control. For further comparisons to be made, the experiment would have to be repeated and also performed with a larger number of time points. However, preliminary data do not indicate that transcriptional regulation alone is responsible for the observed induction of BIM protein levels. The data do however suggest that one way that the Ras-Raf-MEK-ERK pathway may regulate BIM in melanoma cells is through transcriptional control. Additionally, the data do not rule out the possibility that there is a post-translational regulation of BIM expression through ERK mediated effects on protein stability.
As expected, depriving the cells of trophic factors alone does not induce BIM, nor does it have any effect on the killing of the cells. Melanoma cells have been shown to express different growth factors, and cytokines and their receptors, which by autocrine and paracrine effects enable them to proliferate independently of external trophic factors. However, in the WM278 cell line, cell death can be observed in response to CI-1040 treatment, and the effect in increased when the cells are deprived of trophic factors in combination with CI-1040 treatment. In the WM1617 cell line, apoptotic cell death is not observed at any time. Unfortunately, protein expression data is not available for the WM278 cell line. This result suggests that trophic factor deprivation sensitizes melanoma cells to the effects of CI-1040, or conversely, that inhibition of MEK signaling sensitizes melanoma cells to the withdrawal of trophic factors. The RAF-MEK-ERK signaling pathway therefore appears to be involved in the resistance of these cells to the apoptotic signals following trophic factor deprivation.

Similar responses in BIM induction are seen in the CI-1040/Etoposide experiments, where BIM in clearly induced in the WM1617 both when exposed to CI-1040 alone, and when they are exposed to the combination of CI-1040 and Etoposide. Again, this does not affect the viability of the WM1617 cells. However, the WM278 cell line is more susceptible to Etoposide as a death stimulus, and induction of cell death can clearly be seen, although moderate. When exposing the WM278 cell line to both CI-1040 and Etoposide, cell death is significantly increased. The fact that the BIM protein is showing up when the WM1617 cells are treated with Etoposide alone appear to be phosphorylated, could possibly indicate that Etoposide affects another part of the pathway, or the same pathway downstream of BIM. Also, BIM expression does not seem to increase when exposed to both CI-1040 and Etoposide, compared to when exposed to CI-1040 alone.

6. Conclusions

Constitutive activation of the RAS-RAF-MEK-ERK pathway appears to down-regulate BIM protein in melanoma cells, and its regulation is in one or several ways under the control of the RAS-RAF-MEK-ERK pathway. BIM is a key component in apoptotic signaling in melanoma cells, and its absence appears to be one factor in the evasion of programmed cell death in melanoma cells. These data also suggest that there is a correlation between the kinetics of the BIM induction and the sensitivity to induction of apoptotic cell death. Also, it is clear that BIM alone is not enough to induce apoptosis in the melanoma cells. Furthermore, the MEK-inhibitor CI-1040 is not an effective apoptosis inducing agent alone, but had potential when combined with other chemotherapeutics.
7. Acknowledgements

I would like to thank Dr. Martin McMahon for giving me the opportunity to do my degree project in his lab, for his enthusiastic supervision both in the experimental process and in the writing of the report. I would also like to thank Dr David Dankort, in the McMahon lab, for always being encouraging, for his patience with my questions and for taking the time for interesting discussions, and Dr Ruth Thomas, in the McMahon lab, for collaboration in the melanoma project and for constructive dialogue in my writing of the melanoma part of the project. Also, many thanks to everyone in the lab for their support during my time in the lab, and for making me enjoy my stay at the Cancer Center very much. Finally, many thanks to my scientific reviewer, Dr. Nils-Erik Heldin at Uppsala University, for valuable feedback on the report.
8. References


Appendix 1: Drug information

CI-1040
CI-1040 is an inhibitor of MEK, and should hence block the Raf-MEK-ERK pathway by preventing phosphorylation and activation of ERK.

![Chemical structure of CI-1040](image)

Figure x. Chemical structure of CI-1040

Etoposide
Etoposide can induce apoptosis in drug sensitive cells by cytochrome c release, caspase activation, DNA fragmentation and cleavage of poly(ADP-ribose)polymerase. The mechanism by which this is achieved is not clear, but it is believed to involve blocking of the topoisomerase II. This enzyme induces transient breaks in double stranded DNA to resolve topological problems in DNA replication. Etoposide is believed to block religation of the DNA strands and prevent enzyme release. DNA double strand breaks induced by topoisomerase II-poisons can induce apoptosis in tumor cells. In addition, the presence of covalent topoisomerase II-DNA complexes arrests the replication fork, which also contributes to the antineoplastic effects.

![Chemical structure of Etoposide](image)

Figure x. Chemical structure of Etoposide
Appendix 2: Calculations on library representation

A density of minimum 75 colonies/cm$^2$ was observed, which gave a total minimum of 160,000 colonies. The total amount of DNA that was recovered with the MaxiPrep was 1685 µg.

The pLXSP$_3$ BRAF is ~7500bp in size. The mass of 1bp is ~660 Da. The mass of one plasmid molecule is then $5 \times 10^6$ Da. Based on the correlation between Daltons, grams and Avogadro’s number, this means that $6.023 \times 10^{23}$ plasmids have the mass $5 \times 10^6$ grams, which equals $5 \times 10^{12}$ µg.

This means that in each transfection when using 10 µg of plasmid DNA, we are transfecting $\frac{6.023 \times 10^{23} \times 10}{5 \times 10^{12}} = 1.2 \times 10^{12}$ plasmids. Starting with $1.6 \times 10^5$ colonies, means that we should have a complete representation of the library of randomly mutated BRAF in each transfection. To verify this we use the Clark and Carbon formula$^{28}$.

$$N = \frac{\ln(1 - P)}{\ln(1 - \frac{1}{f})}$$

$N$ = number of clones to be screened  
$P$ = desired probability of finding the rare event  
$f$ = percentage abundance of your clone in the population

$f$ is $1/1.6 \times 10^5$ and we choose $P=0.99$. This gives the equation

$$N = \frac{\ln(1 - 0.99)}{\ln(1 - \frac{1}{1.6 \times 10^5})} \approx 740,000$$

This means that in order to find something that is represented just once in the library of $1.6 \times 10^5$ colonies we would need to screen 740,000 individual plasmids. Infection efficiency was measured to 26% (data not shown), which means that $3 \times 10^{11}$ of the $1.2 \times 10^{12}$ plasmids used in the transfection will actually reach the recipient cells. However, considering that we would have to screen 740,000 plasmids to have full representation, when transfecting with 10 µg of DNA, we have enough margin.
### Appendix 3: Raw data from RT-PCR

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Appendix 4: Calculations on RT-PCR data

Constants:  
\( Ct \) (cycle threshold)

Variables:  
\( \# \text{ of cycles (test)} \)
\( \# \text{ of cycles (control)} \)
\( x_{\text{test}} = \# \text{ of templates initially of test mRNA} \)
\( x_{\text{control}} = \# \text{ of templates initially of control mRNA} \)

Example:  
Average Ct (\# of cycles to reach the threshold fluorescence)
Internal control (Gus) = 26.83 = \# of cycles (control)
Test (Bim) = 34.35 = \# of cycles (test)

\( \Delta Ct = \# \text{ of cycles (test)} - \# \text{ of cycles (control)} = 34.35 - 26.83 = 7.52 \)

Schematic graph of \# of cycles against measured fluorescence by RT-PCR reaction.

Due to the fact that each PCR cycle leads to a doubling in the number of products;

\[ 2^{\# \text{ of cycles}} = \# \text{ of products / template} \]

This gives the following expression;

\[ x_{\text{control}} * 2^{\# \text{ of cycles (control)}} \propto Ct \quad (1) \]
\[ x_{\text{test}} * 2^{\# \text{ of cycles (test)}} \propto Ct \quad (2) \]

(1) and (2) \rightarrow \[ x_{\text{control}} * 2^{\# \text{ of cycles (control)}} = x_{\text{test}} * 2^{\# \text{ of cycles (test)}} \quad (3) \]

{if \# of cycles (test) > \# of cycles (control), then \( x_{\text{test}} < x_{\text{control}} \)}

Taking \( \log_2 \) of both sides of equation (3) gives;

\[ \log_2(x_{\text{control}} * 2^{\# \text{ of cycles (control)}}) = \log_2(x_{\text{test}} * 2^{\# \text{ of cycles (test)}}) \]

{logarithmic law states that \( \log(xy) = \log(x) + \log(y) \)}

\[ \log_2(x_{\text{control}}) + \log_2(2^{\# \text{ of cycles (control)}}) = \log_2(x_{\text{test}}) + \log_2(2^{\# \text{ of cycles (test)}}) \]

{Algebraic manipulations in several steps:}
\[ \log_2(x_{\text{control}}) + \# \text{ of cycles (control)} = \log_2(x_{\text{test}}) + \# \text{ of cycles (test)} \]

\[ \log_2(x_{\text{control}}) - \log_2(x_{\text{test}}) = \# \text{ of cycles (test)} - \# \text{ of cycles (control)} = \Delta C_t \]

\{\text{logarithmic law states: } \log(x) - \log(y) = \log\frac{x}{y}\}\]

\[ \log_2\left(\frac{x_{\text{control}}}{x_{\text{test}}}\right) = \Delta C_t \]

\[ -\log_2\left(\frac{x_{\text{control}}}{x_{\text{test}}}\right) = -\Delta C_t \]

\[ \log_2\left(\frac{x_{\text{test}}}{x_{\text{control}}}\right) = -\Delta C_t \]

\[ 2^{\log_2\left(\frac{x_{\text{test}}}{x_{\text{control}}}\right)} = 2^{-\Delta C_t} \iff \frac{x_{\text{test}}}{x_{\text{control}}} = 2^{-\Delta C_t} \]

\[ \frac{x_{\text{test}}}{x_{\text{control}}} \times 100 = 2^{-\Delta C_t} \times 100 \quad (4) \]

Equation (4) is in the RT-PCR raw data (appendix 3) referred to as “relative expression normalized to Gus”.

Assuming that the internal control is more or less constant;
When \( x_{\text{test}} \) is increasing \( \rightarrow \Delta C_t \) is decreasing \( \rightarrow \) “Relative expression normalized to human Gus” is increasing.