# REGULATION OF HUMAN BFK

by

Serkan UĞURLU

M.D., Istanbul Medical Faculty, Istanbul University, 2005

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Graduate Program in Molecular Biology and Genetics
Boğaziçi University

2014

# ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor Assoc. Prof. Nesrin Özören for the opportunities which she provided me, her guidance and patience during this study. I would like to thank the other members of the thesis committee, Assoc. Prof. Batu Erman and Asst. Prof. Necla Birgül İyison for valuable contributions and evaluation of data. I also would like to express my appreciation to Asst. Prof. Tolga Emre and Asst. Prof. Tuğba Bağcı Önder for evaluating the thesis.

I appreciate Cemalettin Bekpen, Ph.D. for ideas and teaching me the techniques of bioinformatics for gene evolution part of my thesis and all TUBİTAK-MAM GMBE members, especially Assoc. Prof. Fatıma Yücel, for co-operation during development of anti-BFK monoclonal antibody.

I would deeply thank to Alican Sahillioğlu, M.Sc. and Yetiş Gültekin, M.Sc. for their contribution in cloning and other techniques. I would like to express my special thanks to ex lab members Burcu Sümer, M.Sc., Damla Erdoğan, M.Sc., Metehan Çifdalöz, M.Sc. and Şahru Yüksel, Ph.D. for teaching me the basic techniques in molecular biology.

I would like to thank Elif Eren, M.Sc., Aybüke Garipcan, M.Sc., Duygu Demiröz, M.Sc., Mustafa Yalçınkaya, B.Sc., Ece Terzioğlu Kara, M.Sc., Duygu Dağlıkoca, M.Sc., Tuncay Şeker, M.Sc., Gizem Gül, B.Sc., Ulduz Sobhi Afhsar, B.Sc. and all Vivarium staff for their great friendship and support.

Last but not least, I very much thank to my dear family for their patience, support and for being there for me during this study.

Finally, I am grateful to TUBITAK, EMBO-SDIG and Boğaziçi BAP for the financial support for the project during my Ph.D.

# ABSTRACT


## REGULATION OF HUMAN BFK

Bcl-2 protein family members are critical regulators for apoptosis. Reduced levels of apoptotic Bcl-2 family members are detected in gastrointestinal cancers which are responsible for a considerable part of the deaths from cancer. BFK (Bcl-2 Family Kin) is a novel pro-apoptotic Bcl-2 family member specifically expressed in the human gastrointestinal tract. BFK has the characteristic BH3 domain, which was shown to be essential for the apoptosis inducing activity of pro-apoptotic Bcl-2 family members. In the colon four alternatively spliced isoforms were identified. Human and mouse BFK genes share 70% homology at the DNA level and 68.7% homology at the aminoacid levels. Interestingly, human and mouse BFK genes show distinct expression patterns. To explain these differences, we performed gene evolution analyses on the promoter region as well as coding region of these genes. We found that the human BFK promoter experienced positive selective pressure and acquired a distinct set of repetetive elements and transcription factor binding sites. In this study, we identified several novel transcription factor candidates which may have roles in the transcriptional regulation of human BFK. As transcription factors, PARbZIP family members (especially TEF and NFIL3) regulate BFK upon binding to its promoter region. NFIL3 supresses TEF induced BFK transcription in HCT116 cells. We also studied hormonal regulation of human BFK. Tamoxifen, as a mixed agonist/antagonist of estrogen, upregulates human BFK levels in SW707 cells. We hope this study contributes to a better understanding of Bcl-2 family.

# ÖZET


## İNSAN BFK GENİNİN DÜZENLENME MEKANİZMALARI

Bcl-2 protein ailesi apoptoz için kritik düzenleyicilerdir. Kanserden ölümlerin önemli bölümünden sorumlu olan mide ve bağırsak kanserlerinde, apoptoz tetikleyici Bcl-2 protein ailesinin üretiminin düştüğü tespit edilmiştir. Yeni tanımlanmış olan apoptoz tetikleyici insan BFK (Bcl-2 family kin) proteini de özellikle mide ve bağırsaklarda üretilmektedir. BFK, apoptoz tetikleyici Bcl-2 protein ailesinde, apoptoz tetikleyici fonksiyonu olan BH3 yapısına sahiptir. Kalın bağırsakta BFK'nin dört eş-yapısı (izoform) tanımlanmıştır. İnsan ve fare BFK arasında, DNA düzeyinde %70 ve protein düzeyinde %68.7 oranında benzerlik bulunmaktadır. İlginç bir şekilde, insan ve fare BFK genleri farklı dokularda ifade edilmektedir. Bu farklılıkları açıklamak üzere, ilgili genler için hem promotör hem de gen kodlayan dizi düzeyinde evrimsel analizler yaptık. İnsan BFK geninin, pozitif seçilime maruz kaldığını ve değişik tekrarlayan elementler ile transkripsiyon faktör bağlanma bölgelerine sahip olduğunu tespit ettik. Projemizde, BFK geninin düzenlenmesinde rol alan birçok yeni adayı tespit ettik. PARbZIP transkripsiyon faktörü ailesi (özellikle TEF ve NFIL3), promotör bölgesine bağlanma sonrası BFK geninin seviyelerini düzenlemektedir. NFIL3, TEF'in arttırdığı BFK transkripsiyon seviyesini baskılamaktadır. Aynı zamanda, BFK'nin hormonal düzenlenmesi üzerine de çalıştık. Östrojenin bazı dokularda agonisti bazı dokularda antagonisti olan tamoksifenin, SW707 hücrelerinde BFK seviyelerini arttırdığını gözlemledik. Çalışmamızın, Bcl-2 ailesinin daha iyi anlaşılmasına katkıda bulunacağını umut ediyoruz.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF SYMBOLS

| | |
|---|---|
| A | Adenine |
| C | Cytosine |
| D | Aspartic acid |
| E | Glutamic acid |
| F | Phenylalanine |
| G | Guanine |
| H | Histidine |
| I | Isoleucine |
| K | Lysine |
| kDa | Kilodalton |
| L | Leucine |
| m | Meter |
| M | Methionine |
| mg | Miligram |
| min | Minute |
| ml | Mililiter |
| mM | Millimolar |
| N | Asparagine |
| ng | Nanogram |
| P | Proline |
| pmol | Picomole |

| | |
|---|---|
| Q | Glutamine |
| R | Arginine |
| S | Serine |
| T | Thymine |
| V | Valine |
| v | Volume |
| W | Tryptophan |
| w | Weight |
| Y | Tyrosine |
| µg | Microgram |
| µl | Microliter |

# LIST OF ACRONYMS / ABBREVIATIONS

| | |
|---|---|
| Aa | Amino acid |
| $AgNO_3$ | Silver nitrate |
| APS | Ammonium persulphate |
| BH3 | Bcl-2 Homology Domain |
| Bcl-2 | B Cell Lymphoma 2 |
| bp | Basepair |
| Ca | Calcium |
| Chr | Chromosome |
| del | Deletion |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide Triphosphate |
| ECD | Extracellular domain |
| EGR | Epidermal growth factor |
| ER | Endoplasmic reticulum |
| GST | Glutathione-S-transferases |
| HCl | Hydrochloric acid |
| HD | Hydrophobic domain |
| Het | Heterozygous |
| Hom | Homozygous |
| HR | Heptad repeat |
| KCl | Potassium chloride |
| KHCO 3 | Potassium bicarbonate |
| LRR | Leucine-rich repeats |
| LRT | Likelihood ratio test |

| | |
|---|---|
| MAM | Mitochondrial associated membrane |
| $MgCl_2$ | Magnesium chloride |
| mRNA | Messenger RNA |
| $Na_2EDTA$ | Disodium ethylenediamine tetraacetate |
| $NaBH_4$ | Sodium borohydride |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| NCBI | National center for biotechnology information |
| $NH_4Cl$ | Ammonium chloride |
| Nt | Nucleotide |
| OMM | Outer mitochondrial membrane |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PCR | Polymerase chain reaction |
| PR | Proline-rich |
| RBC | Red blood cell |
| ref | Reference |
| RNA | Ribonucleic acid |
| SDS | Sodium dodecyl sulfate |
| TBE | Tris-Borate-EDTA |
| TE | Tris-EDTA |
| TEMED | Tetramethylethylenediamine |
| Temp | Temperature |
| tRNA | Transfer RNA |
| UTR | Untranslated region |
| Var | Variant |
| ZnF | Zinc-finger motif |

# 1. INTRODUCTION

## 1.1. Apoptosis

Apoptosis is the process of programmed cell death (PCD), which occurs in multicellular organisms. Apoptosis includes blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. Apoptosis is controlled by different cell signals, which may originate either extra- or intracellularly. Extracellular signals may include death ligands, hormones, toxins, nitric oxide or cytokines. Cells may also initiate apoptosis via intracellular signalling in response to stress. Glucocorticoids, radiation, heat, hypoxia, nutrient deprivation, viral infection and increased intracellular calcium concentration have the potential to trigger apoptosis.

### 1.1.1. Extrinsic Pathway of Apoptosis (Death Receptor Mediated Apoptosis)

The extrinsic apoptotic pathway is activated by death receptors on the plasma membrane such as tumour necrosis factor receptor 1 (TNFR1), Fas/CD95, DR4 and DR5. The binding of TNF to TNF-R1 initiates the pathway that leads to caspase activation via the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) (Chen *et al*., 2002).

The Fas receptor binds the Fas ligand (FasL), a transmembrane protein part of the TNF family (Wajant *et al*., 2002). The interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), containing the FADD, caspase-8 and caspase-10 (Kischkel *et al*., 1995). Caspase-8 directly cleaves other members of the caspase family and triggers the execution of apoptosis in some cell types (Figure 1.1.). In other types of cells, the Fas-DISC combination starts a feedback loop, increases release of proapoptotic factors from mitochondria and the amplified activation of caspase-8. Binding

of TRAIL to its death receptors, DR4 and DR5/KILLER, activate the same apoptotic cascades as FasL (Zhang *et al*., 2005).

In recent years, another extrinsic pathway has been noticed in several toxicity studies. As a consequence of drug activity, calcium concentration increases within the cell and this increase has the ability to cause apoptosis via a calcium-binding calpain protease (Wood *et al*., 1998).

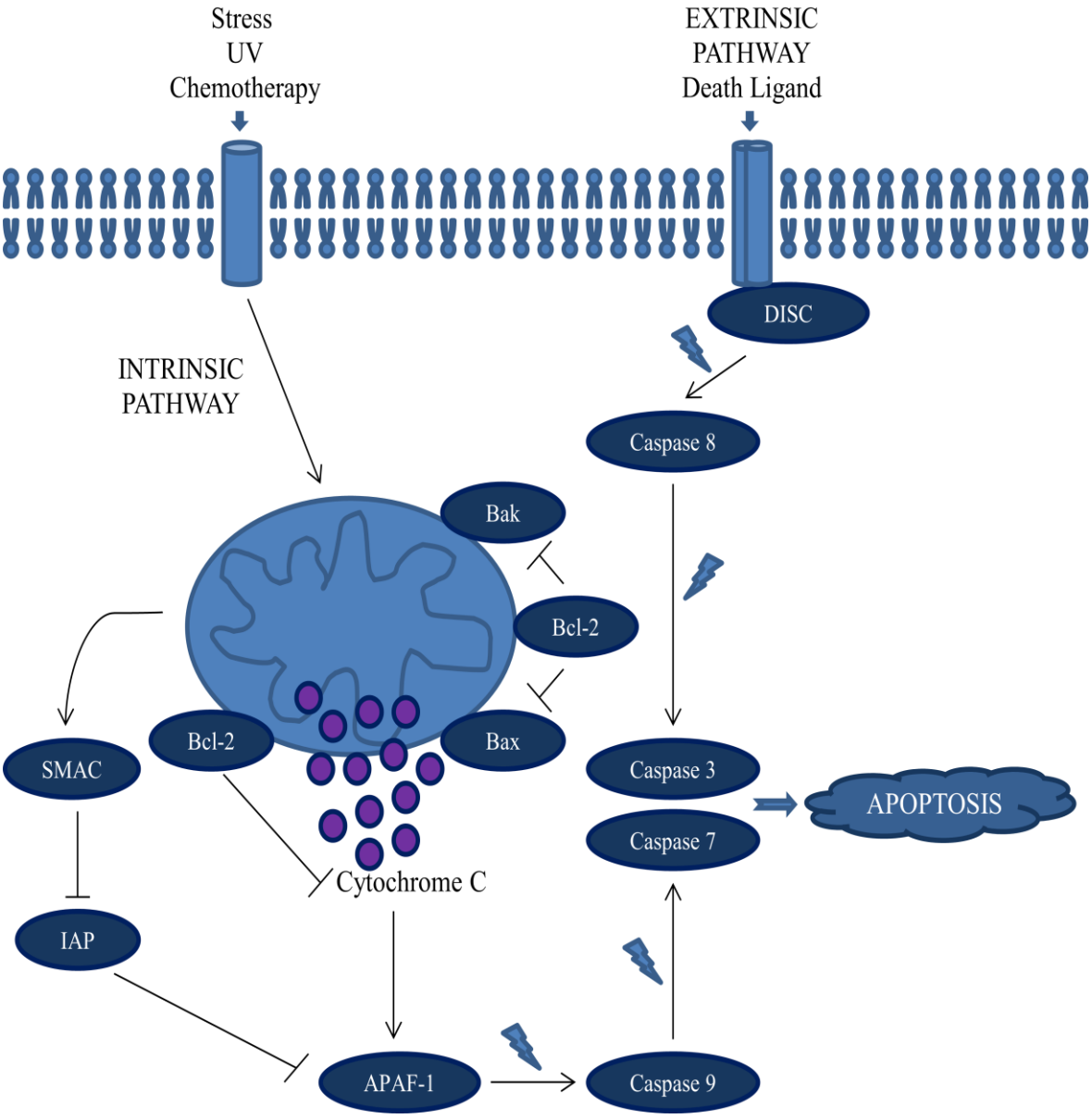


Figure 1.1. Pathways of programmed cell death (apoptosis).

### 1.1.2. Intrinsic Pathway of Apoptosis (Mitochondria Mediated Apoptosis)

The Bcl-2 protein family controls the intrinsic pathway of apoptosis with the coordination of pro-survival (Bcl-2, Bcl-W, Bcl-XL, etc.) and pro-apoptotic (Bax, Bak, BFK, etc.) members. When pro-apoptotic members inhibit the activity of pro-survival members, apoptosis is induced. The intrinsic apoptotic pathway is characterized by membrane permeabilisation of the mitochondria and this results the release of SMACs (second mitochondria derived activator of caspases) and cytochrome c into the cytoplasm **(**Laurent *et al*., 2006). SMAC binds and deactivates the inhibitor of apoptosis proteins (IAPs) in order to prevent IAPs arresting function of apoptosis, therefore allowing apoptosis to proceed (Du *et al*, 2000). Once cytochrome c is released, it forms a multi-protein complex known as the 'apoptosome' by binding with apoptotic protease activating factor 1 (Apaf-1) and ATP (Zou *et al*., 1999). The apoptosome cleaves the pro-caspase 9 to its active form of caspase 9, which in turn activates the effector caspase 3.

## 1.2. Bcl-2 family

The Bcl-2 family of proteins (Figure 1.2.) regulate critical checkpoints in the intrinsic pathway of apoptosis. This family is composed of pro-survival (Bcl-2, Bcl-W, Bcl-XL, etc.) and pro-apoptotic proteins (Bax, Bak, Bid, Noxa, etc.). The founding member, Bcl-2, was first identified at the chromosomal breakpoint of t(14;18) in human follicular B cell lymphoma (Cleary *et al*., 1985, Bakhshi *et al*., 1985 and Tsujimoto *et al*., 1985). Apoptotic processes including, plasma membrane blebbing, volume contraction, nuclear condensation and endonucleolytic cleavage are blocked by Bcl-2, localized on the mitochondrial membrane. This indicates the role of mitochondria in apoptosis (Hockenbery *et al*., 1990). Bcl-2 has function in maintaining normal cellular homeostasis. Loss of function analysis in Bcl-2 deficient mice displayed apoptosis of lymphocytes, developmental renal cell death and loss of melanocytes (Veis *et al*., 1993).

Bax, the first proapoptotic Bcl-2 family member, was identified by its interaction with Bcl-2 (Oltvai *et al*., 1993). As expected, Bax deficient mice showed selective

expansion of cell populations. The balance in the ratio of Bcl-2/Bax sets the threshold of susceptibility to apoptosis for the intrinsic pathway via controling caspase 3 activity (Danial *et al*., 2004).

Bcl-2 family members can also be redefined according to their Bcl-2 homology regions (BH1-4). Prosurvival members such as Bcl-2, Bcl-XL, Bcl-W and A1 have conserved motifs for all four BH1-4 domains. Because of having a hydrophobic pocket as a result of carrying BH1, BH2 and BH3 domains in close proximity, Bcl-XL can interact with BH3 domain of a proapoptotic protein (Muchmore *et al*., 1996; Sattler *et al*., 1997). Proapoptotic Bax and Bak, located on mitochondrial membrane, have BH1, BH2 and BH3 domains and they may require an activation event before interacting with antiapoptotic proteins. Bax and Bak double knock out cells are resistant to all tested intrinsic death pathway stimuli (Lindsten *et al*., 2000; Wei *et al*., 2001). The last group has the ability to interact with both prosurvival and proapoptotic proteins but they only contain BH3 domain and they are called as 'BH3-only' proteins (Wang *et al*., 1996). BH3 only protein, Bid, can bind both Bax and Bcl-2 and acts as a proapoptotic protein.

The precise mechanism how Bcl-2 family members trigger apoptosis is still under investigation. One possible model is that inactive Bax and/or Bak that resides at the mitochondria undergo an allosteric conformational activation in response to death signals, which includes their oligomerization, and oligomerized Bax or Bak may form pores at the mitochondrial outer membrane capable of releasing cytochrome c from intermembrane space. This thesis has origins in the structural similarity between BCL-2 family molecules and the pore-forming helices of bacterial toxins (Muchmore *et al*., 1996).

### 1.3. Caspases

Caspases, cysteine dependent aspartate directed proteases**,** are a family of cysteine proteases that play essential roles in programmed cell death, necrosis and inflammation. Caspases are produced as inactive zymogens possessing a large and a small subunit preceded by an N-terminal prodomain. Two Asp cleavage sites are processed sequentially. The large and the small subunits associate to provide the active site of the enzyme.

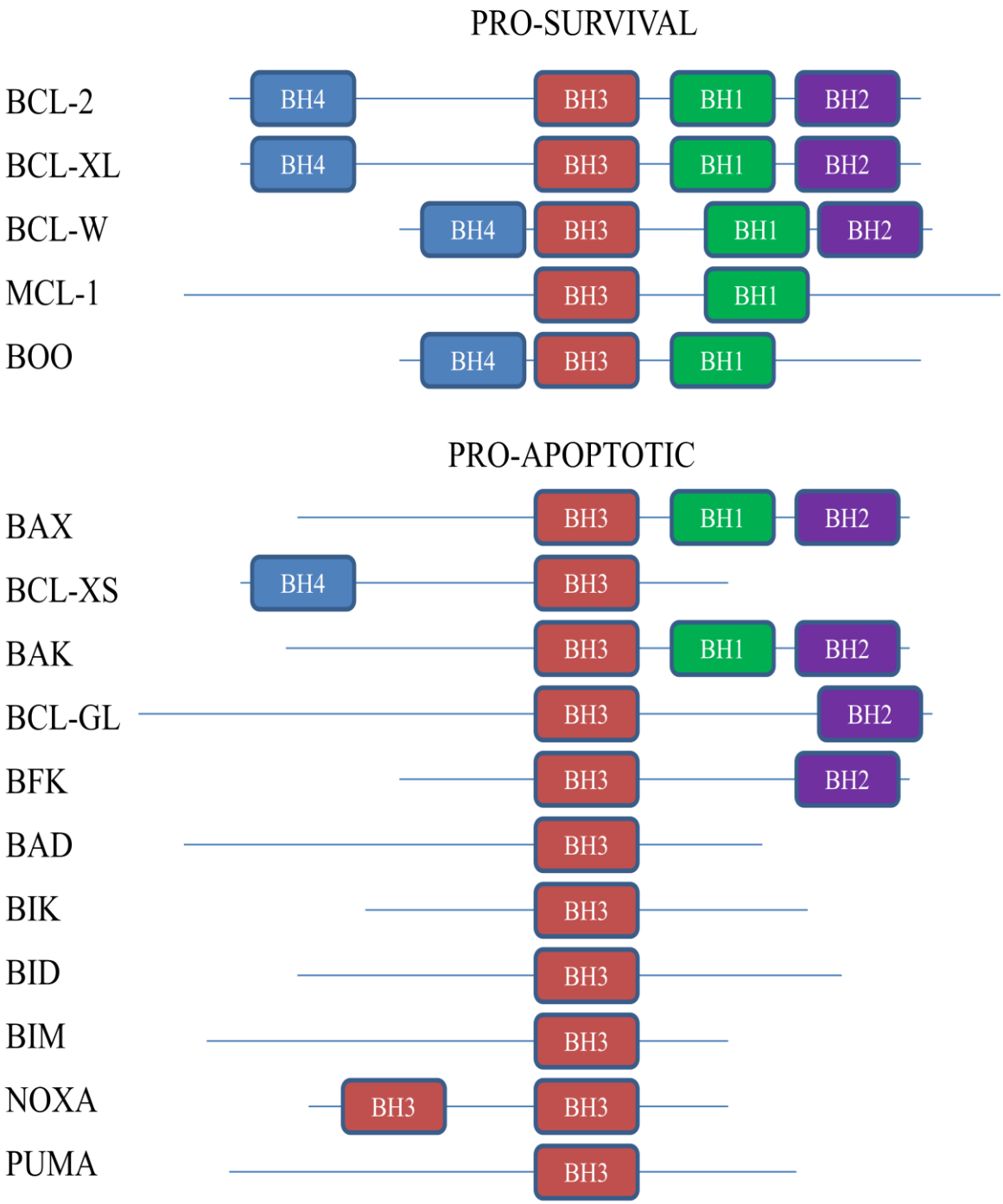


Figure 1.2. A diagrammatic representation of the mammalian B-cell lymphoma 2 (BCL-2) family. Adapted from Gross *et al.*, 1999.

Caspases are seperated into two group: initiator (apical) caspases and effector (executioner) caspases. Initiator caspases (e.g., caspase-2, caspase-8, caspase-9 and caspase-10) are capable of autocatalytic activation, generally have a long prodomain and they cleave inactive pro-forms of effector caspases, thereby activating them. On the other hand, effector caspases (e.g., caspase-3, caspase-6, and caspase-7) cleave other protein substrates within the cell, to trigger the apoptotic process (Korsmeyer *et al.*, 2004).

So far, twelve caspases have been identified in humans. The ced-3 gene product (caspase-1) is a cysteine protease with similar properties to the mammalian interleukin-1-beta converting enzyme (ICE), identified during cell death process in the development of the nematode *C. elegans* (Yuan *et al*., 2004). Depending on the lack of development defects in caspase 1 deficient mice, involvement of caspase 1 in regulating apoptosis is not a direct effect (Kuida *et al*., 1995, Li *et al*., 1995). However caspases cause cells to die by a mechanism more direct than of a hormone or a transcription factor via initiating a death cascade (Miura *et al*., 1993).

Caspase-8 and 10 can be activated by death receptors like Fas and TRAIL; caspase-9 can be activated by apoptosome; caspase-3 and 7, are proteolytically activated by the initiator caspase-8 and 9 during death receptor and DNA damage induced apoptosis, respectively. They can also be activated by granzyme B (released by cytotoxic T lymphocytes and NK cells). Caspase-7 activation requires caspase-1 inflammasomes under inflammatory conditions, while caspase-3 processing proceeds independently of caspase 1 (Kanneganti *et al*., 2010). Human version of BFK protein contains DEVD aminoacid site, which is a potential target for caspase-3 or 7.

### 1.4. Studies on Mouse Bfk

Bcl-2 Family Kin (BFK) is one of novel pro-apoptotic members of Bcl-2 protein family. It was firstly defined in mouse tissues by Coultas et al. So far, there are only two papers showing a consistent pattern of Bfk expression in mouse tissues. Bfk is expressed in the mouse bone marrow, stomach, ovary, uterus and mammary gland (Coultas *et al*., 2003). In the uterine tissue, Bfk expression was only observed in virgin but not in pregnant mice, possibly due to menstruation when proliferation and apoptosis rates for uterine cells

increase. On the contrary, Bfk expression was detected in mammary tissues of mice during pregnancy and lactation, which may again depend on increased proliferation and apoptosis rates in that term. These two findings give clues about the sex hormone dependency of mouse Bfk. One study revealed the fact that after gonadectomy (removal of gonads of mice), the levels of Bfk were decreased in the epididymis and exogenic testosterone treatment helped in regaining of Bfk levels (Pujianto *et al*., 2007).

After infection of NIH-3T3 cells with HA-Bfk containing MiT retroviral plasmid, Bfk protein is detected in the cytosol with anti-HA antibody and fractionation assay also confirmed cytosolic localization of Bfk protein (Coultas *et al*., 2003). However, one of the papers on mouse Bfk had additional findings based on a photobleaching experiment, which concluded that Bfk protein is shuttling between the nucleus and the cytoplasm (Pujianto *et al*., 2007).

### 1.5. Studies on Human BFK

Although Bfk is expressed in bone marrow, stomach, ovary, uterus and mammary gland in the mouse, it is mostly expressed in the gastrointestinal tract of human; especially in small intestine, colon and rectum (Ozoren *et al*., 2009). HT-29, DLD-1, Sw480, Sw620, HCT116, Ls174 and LoVo colon cancer lines were shown to express BFK via RT-PCR (Dempsey *et al*., 2005).

Another interesting data came from cancer profiling array of human BFK comparing normal and tumoral human tissues. A double stranded radiolabelled cDNA probe for BFK isoform a was used in this array. In colon, rectum and small intestine tissues, decreased levels of BFK were observed in tumor samples, when compared to the normal tissues (Dempsey *et al*., 2005). This reduction may be related to the inhibitory function of BFK on tumors. After considering the reduction of BFK levels in gastrointestinal tumors, colon cancer cell lines were selected as the most convenient experimental model for the human version of BFK.

Colon cancer is the fourth most common form of cancer in the United States and the third leading cause of cancer related death in the Western world according to the World Health Organisation (WHO).

### 1.6. Pro-apoptotic Activity of BFK

BFK is a member of pro-apoptotic BH3-only proteins in Bcl-2 protein family and classified as an apoptosis inducing protein (Coultas *et al*., 2003). Human BFK have a DEVD site which is the potential target of caspase-3 or 7 (Figure 1.3.) and cleavage may be required for activation of hBFK to induce apoptosis (Ozoren *et al*., 2009).

Overexpression studies in HEK293T human embryonic kidney cells (Coultas *et al*., 2003), A549 adenocarcinomic human alveolar basal epithelial cells (Dempsey *et al*., 2005) and MEF mouse embryonic fibroblast cells (Ozoren *et al*., 2009) showed weak pro-apoptotic activity of BFK. None of these cells endogenously express BFK and the study on HEK293T cells was done with mouse version Bfk, so these all may give us ideas about the apoptotic activity but may not reflect the endogenous apoptotic potential of BFK. Another important finding about human BFK was the susceptibility to cleavage via caspase activity. After DNA damaging drug doxorubicin treatment, human BFK is cleaved and addition of pan-caspase inhibitor zVAD prevents this cleavage (Ozoren *et al*., 2009).

In order to determine the apoptotic activity of cleaved human BFK, mouse embryonic fibroblasts (MEFs) were transfected with cleaved BFK plasmid construct and the result of this experiment, again showed weak apoptotic activity of BFK in MEFs. To test Bax/Bak dependency of BFK, Bax and Bak Double Knock Out (DKO) MEFs were used in another experiment of this study. When overexpressed, cleaved human BFK reduced the survival rates of wild type MEFs when compared to the Bax/Bak DKO MEFs (Ozoren *et al*., 2009). These results suggest that BFK, somehow depend on Bax and/or Bak to induce apoptosis.

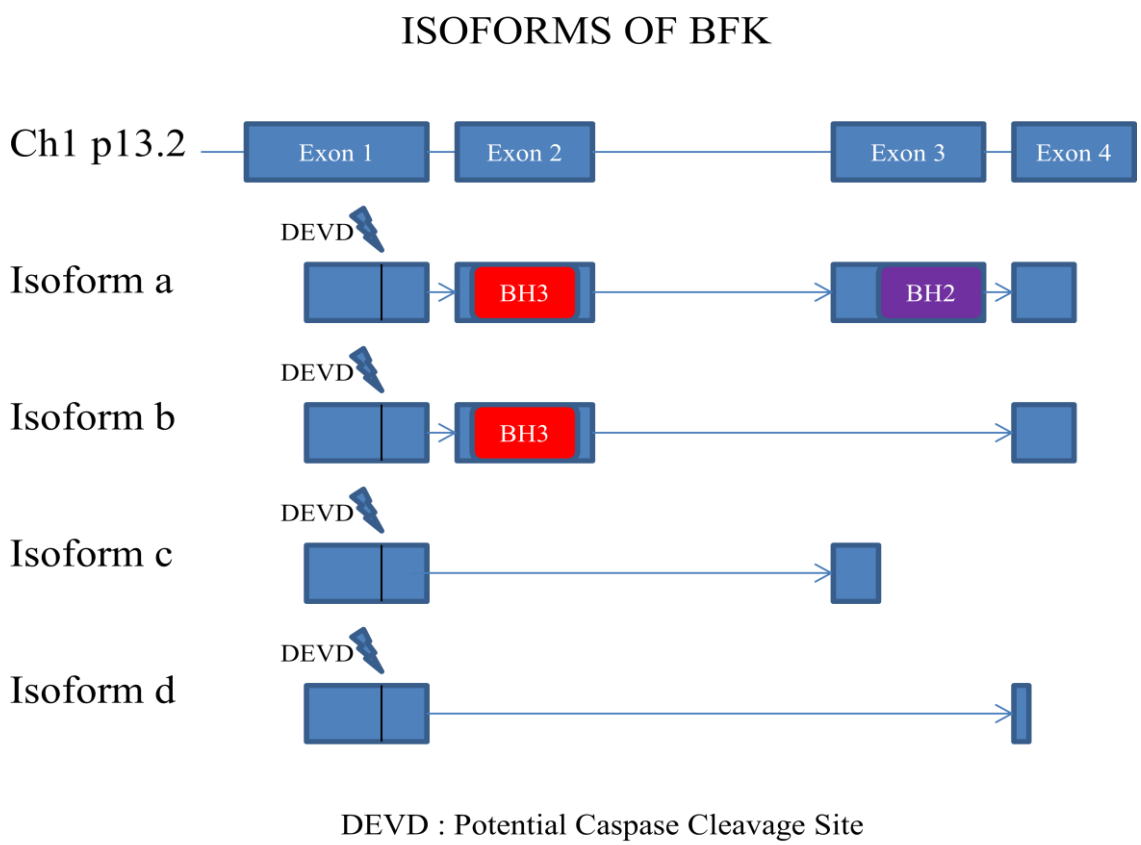


Figure 1.3. Isoform a and b of human BFK contain BH3 domains. DEVD site is a potential target of caspase 3 or 7. Adapted from Dempsey *et al*., 2005.

### 1.7. Interaction Partners of BFK

Heterotypic interactions between prosurvival and proapoptotic members of the Bcl-2 family play critical regulatory roles in the control of apoptosis. To identify potential binding partners of mouse Bfk via co-immunoprecipitation from lysates, Bfk carrying an N-terminal HA epitope tag was transiently overexpressed in metabolically labelled 293T cells together with various anti-apoptotic or pro-apoptotic FLAG-tagged Bcl-2 family members. As expected, the BH3-only protein BimL strongly interacted with Bcl-2, but they found no binding of mouse Bfk with Bcl-2, Bcl-xL, Bax, Bmf, BimEL, Bad or Puma/Bbc3 (Coultas *et al*., 2003).

BH3 domain-only members are hypothesized to relieve the inhibition of the anti-apoptotic BCL-2 family members (BCL-2, BCL-XL and BCL-W) on the pro-apoptotic members such as BAX, BAK by disrupting their interactions. Another interaction study done with human BFK showed the interaction of hBFK with the anti-apoptotic BCL-XL and BCL-W but not BCL-2 or BAD in co-immunoprecipitation experiments carried out in HEK 293T cells (Ozoren *et al*., 2009). These results confirmed the interaction differences between the human BFK and mouse Bfk proteins.

# 2. AIM OF THE STUDY

The aim of this thesis project is to enlighten regulatory mechanisms controlling human BFK expression. Since mouse Bfk and human BFK have different expression profiles in tissues and interaction partners of Bcl-2 family members, we wanted to understand the cause of these differences. In order to achieve this goal, we studied control mechanisms of BFK in two stages; transcriptional and post-transcriptional levels. The evaluation of transcriptional level control mechanisms of BFK covers investigation of promoter region of human BFK and identification of potential transcription factor binding sites determined via Transfac, Alibaba2 and Genome Browser databases. We identified PARbZIP as a potential transcription factor for human BFK promoter. We enlightened evolutionary past of BFK among species. We also focused on hormones which possibly regulate human BFK expression; three candidate steroid hormones were selected for treatment of colon cancer cell lines and we found that tamoxifen upregulates BFK expression. To study post-translational regulation of human BFK, we produced a monoclonal anti-BFK antibody to evaluate protein expression profiles of BFK in different cancer cell lines. We also checked hormonal regulation profile of human BFK protein. We tried to identify the role of valine aminoacid in DEVD site on cleavage activity. Reduced expression of pro-apoptotic isoforms of human BFK has been described in many human gastrointestinal tumours that may be related with its protective role against the development of human gastrointestinal malignancy so our research may be helpful to understand the role of Bcl-2 family members in cancer.

# 3. MATERIALS

## 3.1. Cell Lines

Table 3.1. Cell lines used in this study.

| Cell Line | Catalog No | Main Source | Provider |
|---|---|---|---|
| HEK 293 FT | R700-07 | Invitrogen, USA | Kindly provided by Maria Soengas |
| HT 29 | HTB-38 | ATCC, USA | Kindly provided by Erkan Mert |
| SW 707 | CCL-228 | ATCC, USA | Kindly provided by Erkan Mert |
| HCT 116 | CCL-247 | ATCC, USA | Kindly provided by Gabriel Nunez |
| SW 620 | CCL-227 | ATCC, USA | Kindly provided by Gabriel Nunez |
| F0 | CRL-1646 | ATCC, USA | Kindly provided by Fatıma Yücel |

## 3.2. Chemicals, Plastic and Glassware

Chemicals were purchased from either Sigma-Aldrich (USA), Merck (Germany) or AppliChem (Germany), plasticware for cell culture, tips and tubes from TPP (Switzerland), Isolab (Turkey) or Axygen (USA). All glassware, tips and tubes were autoclaved at 121$^{o}$C for 20 minutes for sterilization prior to use.

## 3.3. Buffers and Solutions

### 3.3.1. Cell Culture

Table 3.2. Solutions and media used in cell culture.

| | |
|---|---|
| 0.5% Trypsin-EDTA 10X | Gibco Invitrogen, USA |
| DMSO | AppliChem, Germany |
| Dulbecco's Modified Eagle Medium (DMEM) | Gibco Invitrogen, USA |
| Fetal Bovine Serum (FBS) | Gibco Invitrogen, USA |
| MEM Non-essential amino acid (NEAA) 100X | Gibco Invitrogen, USA |
| Penicillin/Streptomycin 100X<br>(5000 u Penicillin + 5000 μg Streptomycin per ml) | Gibco Invitrogen, USA |

Table 3.3. Buffers used in cell culture.

| | |
|---|---|
| Freezing Medium | 20% FBS<br>1X Pen/Strep<br>100 μM MEM-NEAA<br>7.5% DMSO |
| PBS 10X | 80 gr NaCl<br>2 gr KCl<br>2.4 gr KH2PO4<br>14.4 gr Na2HPO4 Add ddH2O up to 1 lt<br>(pH 7.2) |

### 3.3.2. Polymerase Chain Reaction (PCR)

Table 3.4. Materials used for PCR.

| | |
|---|---|
| 10 X $MgCl_2$ Free *Taq* Buffer | 750 mM Tris-HCl (pH 8.8 at $25^0$C)<br>200 mM $(NH_4)_2SO_4$<br>0.1% (v/v) Tween 20 (Thermo Scientific, USA) |
| Magnesium Chloride ($MgCl_2$) | 25 mM $MgCl_2$ (Thermo Scientific, USA) |
| Deoxyribonucleotide Triphosphates (dNTPs) | 100 mM of each dNTP (Fermentas, Lithuania) |

### 3.3.3. Agarose Gel Electrophoresis

Table 3.5. Materials used for agarose gel electrophoresis.

| | |
|---|---|
| 5 X Tris-Acetic Acid-EDTA (TBE) Buffer | 0.89 M Tris-Base<br>0.89 M Acetic Acid<br>20 mM $Na_2EDTA$ (pH 8.3) |
| 1 % Agarose Gel | 1 % Agarose (w/v) (Sigma Aldrich, USA)<br>in 0.5 X TBE Buffer |
| Ethidium Bromide | 10 mg/ml |
| 6 X Loading Dye | 2.5 mg/ml Bromophenol Blue<br>1% SDS (w/v) in 2 ml glycerol |

### 3.3.4. Polyacrylamide Gel Electrophoresis (PAGE)

Table 3.6. Materials used for PAGE.

| | |
|---|---|
| 5 X TBE Buffer | 0.89 M Tris-Base<br>0.89 M Boric Acid<br>20 mM Na2EDTA (pH 8.3) |
| 30% Acrylamide Stock (29:1) | 29% Acrylamide (w/w)<br>1% N, N'-methylenebisacrylamide(w/w) |
| Ammoniumpersulfate (APS) | 1% APS (w/v) |
| 6 X Denaturing Loading Dye | 95% Formamide (w/v)<br>20 mM EDTA<br>0.05% Xylene Cyanol (w/v)<br>0.05% Bromophenol Blue |
| Glycerol | 99% Glycerol |
| Tetramethylethylenediamine (TEMED) | 1% TEMED |

### 3.3.5. Cloning and Analytic Digestion

Table 3.7. Enzymes used for cloning.

| | |
|---|---|
| Restriction Enzymes (BamHI, XhoI, EcoRI, HindIII) | NEB, USA |
| T4 DNA Ligase | NEB, USA |
| High fidelity polymerase (Phusion) | NEB, USA |

### 3.3.6. Culture of Bacteria

Table 3.8. Solutions for bacterial culture.

| | |
|---|---|
| LB Agar (Solid culture, autoclaved) | 1 L LB medium<br>15 g Agar |
| LB Medium (1 L) (Liquid culture, autoclaved) | 10 g Tryptone<br>5 g Yeast Extract<br>5 g NaCl |
| Ampicillin (1000X = 100 mg/ml in 70% EtOH) | AppliChem, Germany |
| Kanamycin (1000X = 50 mg/ml in ddH20) | Sigma-Aldrich, USA |

### 3.3.7. Transfection of cells with calcium phosphate and liposome method.

Table 3.9. Buffers and solutions used in transfection.

| | |
|---|---|
| 2X HBS Buffer | 50 mM HEPES pH 7.0<br>280 mM NaCl<br>1.5 mM Na2HPO4 |
| Chloroquine | AppliChem, Germany |
| HEPES | Gibco Invitrogen, USA |
| HP Xtreme Gene Transfection Reagent | Roche, France |

## 3.4. Fine Chemicals

### 3.4.1. Antibodies

Table 3.10. Antibodies used in this thesis.

| | |
|---|---|
| anti-FLAG | F3165, Sigma Aldrich, USA |
| anti-BFK (monoclonal) | AKIL (homemade), Turkey |
| anti-BFK (polyclonal) | AKIL (homemade), Turkey |
| anti-His | Vatoz Biyoteknoloji, Turkey |
| anti-Actin | 4967S, CST, USA |
| anti-Rabbit IgG, HRP | 7074S, CST, USA |
| anti-Mouse IgG, HRP | 7076S, CST, USA |

### 3.4.2. Oligonucleotide Primers

All the primers used in this study were designed using the program Primer BLAST from NCBI Database and were synthesized by Macrogen (Seoul, Korea) .

Table 3.11. Primers used in this thesis.

| | |
|---|---|
| BFK SDM VA F | CTGTGTAGATGAAGCAGATTCAGGAGAGCC |
| BFK SDM VA R | GGCTCTCCTGAATCTGCTTCATCTACACAG |
| BFK SDM PA F | GAAGTAGATTCAGGAGAGGCTTGTTCTTTTGATGTG |
| BFK SDM PA R | CACATCAAAAGAACAAGCCTCTCCTGAATCTACTTC |
| BFK SDM IA F | GTAGTGGGACAGGTGGCTGCTCCCATGACGGGTATGATC |
| BFK SDM IA R | GATCATACCCGTCATGGGAGCAGCCACCTGTCCCACTAC |
| BFK SDM F | GGATCCATGAAGAGCTCCCAAACTTTTG |
| BFK SDM R | GAATTCTCAGCTCTCCAGATTTTCCC |
| TEF RTPCR F | CGGGCGGCGGAAAGAA |
| TEF RTPCR R | CGTCCTCCTCCAGCTTTTCC |

***** F: Forward primer, R: Reverse primer

Table 3.11. Primers used in this thesis (cont.).

| | |
|---|---|
| NFIL3 RTPCR F | AGCTGAGAAAAATGCAGACCG |
| NFIL3 RTPCR R | TCCTCCGACATGCAGAAGATT |
| NFKB F | CTCTCGAGGGAAAATCCTTTTGCTAGACAGAG |
| NFKB R | CTAAGCTTTGGGACTTCTCAGAAGGATTACC |
| NFKB DEL F | CTCTCGAGTTGCTAGACAGAGAATCTCAGATCTC |
| PARbZIP F | CTCTCGAGATTATGGCAAATAAATTCCATAAACA |
| PARbZIP DEL F | CTCTCGAGAAGTTGAACGCTGGTCCACT |
| 5UTR DEL R | CTAAGCTTTTTAGATGTTTGCTGTCAAGTTTTG |
| NFKB2 F | CTCTCGAGTCCTTCTGAGAAGTCCCACC |
| NFKB2 DEL F | CTCTCGAGCCTTTCTGAGCAGCTGTGTTT |
| NFKB3 F | CTCTCGAGTAGTGGGAAAAGTTCCAGGATT |
| NFKB3 DEL F | CTCTCGAGAGGATTACATGTCAGGAAACTACAAG |
| PAR ChIP 1F | AGTTGAACGCTGGTCCACTG |
| PAR ChIP 1R | CCTCGACTTCTTAGGTCCTGCAC |
| PAR ChIP 2F | ACTCCAGAGGCTCTTCCTGAACT |
| PAR ChIP 2R | TCTCAGACCACACCCTAACCACA |
| PAR ChIP 3F | ACAGAAGTTGAACGCTGGTCC |
| PAR ChIP 3R | CCTCGACTTCTTAGGTCCTGCAC |

### 3.4.3. DNA Ladders

Size markers used in this study were 100 bp DNA ladder with a range of 100-1000 bp and 1kb DNA ladder with a range of 250-10000 bp (Fermentas, Lithuania).

### 3.4.4. Kits

Table 3.12. Kits used in this thesis.

| | |
|---|---|
| High Pure PCR Purification Kit | Roche, Switzerland |
| High Pure Plasmid Isolation Kit | Roche, Switzerland |
| Genopure Plasmid Midi & Maxi Kits | Roche, Switzerland |

## 3.5. Equipment

Table 3.13. Equipment used in this study.

| | |
|---|---|
| Autoclave | Model MAC-601 (Eyela, Japan) |
| Centrifuge | Allegra X-22R Centrifuge (Beckman Coulter, USA)<br>Himac CT4200C, Hitachi Koki, Japan |
| Deep Freezers | 2021D (Arçelik, Germany)<br>4250T (Arçelik, Turkey) |
| Documentation System | GelDoc Documentation System (Bio-Rad, USA) |
| Electrophoresis | PROTEAN Vertical Electrophoresis System (Bio-Rad, USA)<br>Mini Trans-Blot® Electrophoretic Transfer Cell BIO-RAD, USA |
| Incubators | Hepa ClassII Forma Series, Thermo, USA |
| Laminar Flow Cabinets | Class II A, Tezsan, Turkey |
| Magnetic Stirrer | Yellowline MSH Basic, USA |
| Microscopes | Nikon, Eclipse TS100, Japan |
| Oven | Microwave Oven (Arçelik, Turkey) |
| Plate Reader | VersaMax, Molecular Devices, USA |
| Power Supplies | Power Pac Universal, BIO-RAD, USA |
| Shaker | Heildophl, Germany |
| Spectrophotometer | NanoDrop ND-1000 (NanoDrop, USA) |
| Thermal Cyclers | C 1000 Thermal Cycler (Bio-Rad, USA)<br>ExiCycler 96 (Bioneer, USA) |
| Vortex | GmcLab, Gilson, USA |
| Western blot visualization | Stella, Raytest, Germany |

# 4. METHODS

## 4.1. Gene Cloning

### 4.1.1. Plasmid DNA Isolation and Sequencing

Selected colonies from plates were grown overnight in 10ml LB medium containing 100 μg/ml ampicillin at 37$^{o}$C with shaking at 200 rpm and plasmids were isolated with MiniPrep kit (Qiagen, USA). Briefly bacterial cells were lysed and chromosomal DNA was denatured under strong alkaline conditions (pH 13.0), cell debris and chromosomal DNA were removed by centrifugation at 10000 xg for 10 minutes. Supernatant was applied to the QIAprep spin columns where DNA binds to the silica gel membrane in the presence of high salt. Impurities were removed by washing with buffer containing absolute ethanol and plasmid DNA was eluted with TE buffer (pH 8.0). Plasmids were analyzed in 1 per cent agarose gels and were sequenced at Macrogen (South Korea) using sequencing primers. Stocks of bacteria carrying plasmids with the desired fragments were prepared by adding 10% glycerol into overnight bacterial cultures and stored -80$^{o}$C until used.

### 4.1.2. PCR Reaction

DNA fragments to be cloned into plasmid vectors were amplified using the Phusion® High-Fidelity DNA Polymerase (NEB, USA). PCR reactions were carried out according to manufacturer's instructions. The protocol is summarized in Table 4.1.

Table 4.1. PCR protocol using the Phusion polymerase.

| Step #no | Description | Temperature (°C) | Duration |
|---|---|---|---|
| 1 | Initial denaturation | 98 | 30 s |
| 2 | Denaturation | 98 | 5 s |
| 3 | Annealing | Primer specific Tm | 30 s |
| 4 | Elongation | 72 | 20 s per 1 kb |
| 5 | Return to step 2 (32x) | 5 | |
| 6 | Final elongation | 72 | 5 min |

### 4.1.3. Restriction digestion of plasmids and PCR products

Plasmid DNA or purified PCR products were digested with suitable restriction enzymes for molecular cloning purposes. Restriction enzymes from NEB (USA) were preferred and digestion reactions were carried out according to manufacturer's instructions using the appropriate digestion buffers and temperatures, specific for each restriction enzyme.

### 4.1.4. Agarose Gel Electrophoresis

Amplification products or other DNA samples were mixed with 1/6 volume of 6X loading buffer (250 mg bromophenol blue, 550 mg xylene cyanol in 33 ml 150 M Tris, pH 7.6, 60 ml glycerol and 7 ml H2O) and loaded onto 1 or 2 per cent agarose gels prepared in 1X Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 1 mM EDTA, 20 mM acetic acid) and addition of 0.5 μg/ml ethidium bromide. The gels were run in the same buffer at 120 V. Molecular weight markers, Gene Ruler 1kb DNA ladder and DNA Ladder Mix were purchased from Fermentas (Lithuania). The DNA bands were visualized under UV light and the images were documented with GelDoc imaging system (BioRad, USA).

### 4.1.5. PCR Purification and Agarose Gel Extraction

PCR products and enzyme digested DNA fragments were purified with spin columns using the PCR purification kit according to manufacturer's instructions (High Pure PCR Purification Kit, Roche, Switzerland), when the PCR products or the digested DNA fragments were composed of one type of DNA fragment. Primer dimers or other small DNA fragments smaller than 100 bp do not efficiently bind to spin columns, so that DNA fragment of interest can be purified from small DNA fragments using PCR purification method. In other cases, where there are several DNA fragments larger than 100 bp, DNA sample was run on agarose gel and the DNA fragment of interest was cut using a razor under UV light. The DNA was extracted using the agarose gel extraction protocol according to manufacturer's instructions (High Pure PCR Purification Kit, Roche, Switzerland).

### 4.1.6. Ligation of DNA fragments

Restriction enzyme digested plasmid vectors and inserts were ligated using T4 polymerase (NEB, USA). In all ligation reactions, vector and insert concentrations were adjusted in a way that 1:5 (vector : insert) ratio was established. In each ligation reaction 10-30 ng of vector and corresponding amount of insert DNA were added, without exceeding the threshold of 100 ng (vector + insert) per 10 µl ligation reaction. 1 µl of 10X ligation buffer and 0.5 µl T4 ligase were added and reaction was added up to 10 µl using $ddH_2O$. The ligation reaction was incubated at 4°C overnight.

### 4.1.7. Competent Cell Preparation

Stock of frozen DH5α or Top10 bacterial cells were streaken on LB agar plates (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl and 18 g/l agar) and incubated overnight at 37$^{o}$C. A well isolated individual colony was transferred and grown in 5 ml of liquid LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) overnight at 37$^{o}$C. This overnight culture was used to inoculate 50 ml LB medium with 1:100 ratio and grown at 37$^{o}$C until OD600 reached 0.3-0.4. Then, the cultures were chilled on ice for 30 minutes and centrifuged for 5 minutes at 5000 rpm. The bacterial pellet was resuspended in 12.5 ml of ice-cold 50 mM $CaCl_2$, incubated on ice for 15 minutes. The bacteria were collected as before, resuspended in 2 ml of ice-cold 50 mM $CaCl_2$ containing 15% glycerol. The samples were aliquoted, 200 µl each, stored at -70$^{o}$C until used.

### 4.1.8. Transformation of Bacteria with Plasmids

For DH5α transformation, 100 µl of frozen DH5α competent cells were mixed with 50-100 ng of plasmid. They were kept on ice for 30 minutes, heat-shocked at 42$^{o}$C for 90 seconds and cold-shocked on ice for 2 minutes. Subsequently, 900 µl of SOC medium (2 g tryptone, 0.5 g yeast extract, 1 ml 1 M NaCl, 0.25 ml 1 M KCl, 1 ml 1 M MgCl2, 1 ml 2 M glucose in 1 l $ddH_2O$) was added and cells were allowed to grow at 37$^{o}$C for an hour with shaking at 250 rpm. Subsequently, 100 µl of bacterial suspension was plated on LB plates containing 100 µg/ml ampicillin and grown overnight at 37$^{o}$C.

### 4.1.9. Colony PCR

To verify that the colonies grown upon transformation contain the desired gene fragments, individual colonies were transferred using sterilized toothpicks into PCR tubes with 10 µl $ddH_2O$, boiled for 10 minutes to lyse the cells and the amplifications were carried out in the presence of 0.2 mM dNTP mixture, 3mM $MgCl_2$, 0.25 µM primer pair, 1X Taq polymerase buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl) and 0.5 units of Taq polymerase in a total volume of 50 µl. The amplification reaction was started with initial denaturation at 95$^oC$ for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 59$^oC$ for 40 seconds and extension at 72°C for 90 seconds.

### 4.1.10. Analytic Digestion

Positive colonies identified with the colony PCR procedure were screened via analytical digestion reactions. Briefly, positive colonies were picked with a sterile tip from the backup plate and put into LB with the suitable antibiotics (liquid bacterial culture). Bacteria were grown at 37°C for 16 h with rocking agitation. Mini-scale DNA isolation was executed and isolated plasmid DNA was digested via using a restriction enzyme pair that gives rise to a pattern that can be distinguished from negative colonies.

## 4.2. Cloning of Transcription Factor Binding Sites into pGL3 Basic Vector

Using the appropriate primers (Table 3.11), transcription factor binding sites were amplified with PCR. Amplified PCR products were gel extracted and purified from gel using QIAquick gel extraction kit (Qiagen, USA). Briefly, sliced gel was solubilized in a buffer supplied with the kit containing pH indicator, loaded on a spin column, cetrifuged at 10000g for 1 minute. Then the column was washed with buffer containing absolute ethanol, DNA was eluted with Tris-EDTA (TE) buffer containing 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA. DNA concentrations were obtained by measuring optical density at 260 nm (OD260 value of 1 corresponds to 50 µg/ml of DNA).

pGL3 basic vector (Promega, USA) and the gel purified PCR products were

incubated with 1 unit XhoI (Fermentas, USA) and 1 unit HindIII (Fermentas, USA) restriction enzymes per 1 μg of DNA in buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl at 37$^oC$ overnight to generate ends for directional cloning. Samples were fractionated on 1 or 2 per cent agarose gels and appropriate DNA fragments cut out of the gel under UV light and extracted using QIAQuick Gel Extraction Kit (Qiagen, Germany) according to manufacturers instructions.

XhoI/HindIII cut pGL3 basic vector and fragments were ligated in 1:3 end ratio. The reactions were carried out using 50 ng vector in DNA ligase buffer (400 mM Tris-Cl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP), 1 unit of T4 DNA ligase and the appropriate amount of amplification fragments at 16$^oC$ overnight.

### 4.3. Luciferase Assay

$2x10^6$ HCT116 colon cancer cell lines were seeded into 6 well plates in 2 ml DMEM as triplicates and incubated overnight. Next day, DMEM was replaced with Opti-MEM and cells were transfected with 1 ug of each luciferase constructs, 200 ng pRL-TK renilla luciferase vector and 100 ng pEGFP vector as transfection efficiency control via liposome mediated transfection reagent in 1:2 ratio. After transfection, cells were incubated for 48 hours at 37$^oC$.

Cells were lysed with lysis buffer from luciferase kit, supernatants were collected and put into 96 well luciferase plate. 75 ul of firefly luciferase substrate was added into each sample and luminescense of mixtures were measured immediately with luminometer. After that, 75 ul stop and glo substrate reagent was added into mixture and luminescense of mixtures were measured again.

Results were calculated for each triplicate samples via using Firefly/Renilla ratio of measurements. pBVI luciferase vector, which has 7 NF-KB binding sites, was used as positive control.

## 4.4. Western Blotting Analysis

### 4.4.1. Preparation of samples

Cell culture samples were collected in lysis buffer using a scraper. 200 µl of lysis buffer was added on a well of 6-well plate and cells were collected. Subsequently, samples were lysed via vortexing in every 5 minutes for 15 minutes on ice in 15 ml falcon tubes. Subsequently, tubes were briefly spinned down at low speed to collect droplets on the walls. Samples were transferred into 1.5 ml tubes and 40 µl 6X Laemmli buffer was added. Samples were denaturated at 95°C for 5 minutes and stored at -20°C until later use.

### 4.4.2. Preparation of SDS-PAGE gels

For routine use, 1.5 mm 10-well SDS-PAGE gels were casted. Briefly, 10ml liquid 15% resolving gel solution (Table 4.2) was mixed with 100 µl 10% APS and 10 µl TEMED. The gel solution was mixed thoroughly and put between casting glasses. The gel was immediately covered with n-butanol to ensure a flat surface. Once the resolving gel was polymerized, n-butanol was drained off. Stacking gel mixture was prepared. 2 ml per gel 4% gel solution was mixed with 20 ul 10% APS and 2 µl TEMED. The mixture was added on top of the resolving gel and the comb was inserted. After polymerization was completed, the gel is ready to use.

Table 4.2. Reagents used to prepare 15% resolving solution.

| Reagents | Volume |
|---|---|
| 30% Acrylamide Stock (29:1) | 50 ml |
| 1.875M Tris pH 8.8 | 20 ml |
| 10% SDS | 1 ml |
| $dH_2O$ | 29 ml |

### 4.4.3. Protein Gel Electrophoresis

SDS-PAGE gels were run in vertical electrophoresis tanks in 1X TGS buffer. Prior to electrophoresis, samples were briefly incubated at 95°C for 5 minutes and vortexed. 40 µl of sample were loaded to each well in 1.5 mm 10-well SDS-PAGE gels. Empty wells were loaded with blank sample (PBS mixed with 6X laemmli) to avoid "smile effect". Samples were run at 60V until they enter resolving gel, then the voltage was increased to 120V. The gel electrophoresis was stopped as the dye front reached the end of gel. The glasses were removed and the gel is ready for transfer onto PVDF membrane.

### 4.4.4. Semi-dry Transfer

Proteins in the SDS-PAGE gels were transferred onto PVDF membranes using the semi-dry transfer method. Briefly, 4 Whatman filter papers were soaked in transfer buffer and PVDF membrane was soaked in methanol for 5 minutes. After 5 minutes, 2 Whatman paper, PVDF membrane, SDS-PAGE gel and other 2 Whatman filter papers were prepared in a sandwich form in transfer apparatus. Bubbles between sandwich layers were removed with a falcon tube used as a roller. Transfer was executed in the room temperature with 12V for 12 minutes.

### 4.4.5. Membrane blocking

The PVDF membrane was taken into a plastic tank for subsequent incubations with the protein side facing upwards. The membrane was washed for 5 minutes with TBST and blocked with 5% BSA for 30 min. at RT. After blocking, the membrane was washed for 5 minutes with TBST.

### 4.4.6. Antibody incubations

The membranes were incubated with the primary antibody solutions at 4°C overnight. When the incubation was finished, the membrane was washed 3 times with TBST, then incubated with a secondary antibody solution at RT for 1 hour. The secondary antibody was chosen according to the animal in which primary antibody was raised (either

mouse or rabbit in our study). After secondary antibody incubation, the membrane was washed 3 times with TBST again.

#### 4.4.7. Visualization of the membrane

Western substrate reagents (Lumi-Light Western Blotting Substrate, Roche, Switzerland) were mixed with 1:1 ratio and added on the membrane which was placed on a glass slab. The surface of membrane was covered with transparent film and bubbles were removed. Membrane was imaged using a digital visualization device (Stella, Raytest, Germany).

### 4.5. Generation of Antibodies against BFK Protein

Both polyclonal rabbit anti-BFK and monoclonal mouse anti-BFK antibodies were generated to use in our Western Blot experiments. pET30-BFK vector was used to produce pure BFK protein to inject animals. pET30 vector was selected because it has histidine tag which is used to purify proteins with nickel containing columns. Histidine bound to nickel throughout the column in purification step. Another advantage of this vector is induction control. It has LacI repressor and works with T7 polymerase produced by some modified E.coli strains. When the IPTG added to the condition, bacteria starts to express protein encoded by the vector.

pET30-BFK vector was transformed into Rosetta DE3 pLysS bacteria, inoculated into 5 ml LB and incubated at 37$^{o}$C for overnight. Next day, 5 ml LB was added into 500 ml LB and incubated at 37$^{o}$C until OD595 reaches 0.5-1. When OD595 reaches to desired value, bacteria are ready to be induced by IPTG. 1 mM of IPTG was used for induction. After four hours of induction, bacteria were lysed. For lysis, sonication was used after adding 1X binding buffer to the mixture. The supernatant of the lysate was filtered through His Bind Quick Purification Cartridges (Novagen, USA) and bound fraction was eluted with elution buffer. Purification of protein was tested with Coomassie Blue staining after running on minigel via SDS-PAGE and it was also detected with anti-His antibody after transferring to PVDF membrane. Before the injection to animals, it was further purified via membrane dialysis in PBS.

Purified hisBFK protein was sent to TUBITAK-MAM for injection into Balb-c mice and to perform the hybridoma fusion steps. Two mice, whose serum had the highest response against hisBFK protein in ELISA assay, were picked and sacrificed. Their B cells taken from their spleen were fused with immortal F0 myeloma cells (ATCC, USA). Ten days after the fusion step, hybridomas were sent to us in sixteen 96 well plates to continue the screening process. Colonies were selected by eyes and screened with ELISA. The most promising clones were selected according to their higher response against hisBfk protein compared to the response against hisGFP protein. This step was essential to guess antibody's response against the protein itself or its his tag part. The next step was isolation of single hybridoma cell, for this purpose clones were seperated to single cell, in a one cell per well form, to obtain pure monoclonal antibody producing colonies. Colonies, derived from single cell, were screened with ELISA again and five different candidate clones producing monoclonal anti-BFK antibody were stocked at -80$^{o}$C.

In a paralel work, polyclonal anti-BFK antibody was produced by injecting two rabbits with 50 ug hisBFK protein in every 15 days during a 45 day procedure. Rabbits were sacrificed to get their blood and the serum part isolated via centrifugation at 13000 rpm for 15 minutes. Anti-BFK polyclonal serum was stored at -20$^{o}$C for further studies.

## 4.6. Cell culture

### 4.6.1. Maintainance of HEK293FT cells

HEK293FT cells (Invitrogen, USA) are widely used for protein overexpression and lentiviral packaging purposes. These cells were derived from HEK293T cells which stably express the SV40 large T antigen from the pCMVSPORT6TAg.neo plasmid. HEK293FT cells were cultured with high glucose DMEM supplemented with 1x MEM Non-Essential Aminoacids, 2 mM L-Glutamine, 100 U/ml penicilin, 100 µg/ml streptomycin and 10% FBS.

### 4.6.2. Calcium Phosphate Transfection

HEK293FT cells were efficiently transfected with calcium phosphate transfection method. This procedure produces insoluble precipitates of plasmid DNA and calcium phosphate, which are subsequently engulfed by HEK293FT cells. Briefly, plasmid DNA to be transfected were mixed with $ddH_20$, 2 M $CaCl_2$ and 2X HBS solution in the given order, with the amounts indicated at Table 4.3. After 5 minutes incubation, mixture was added onto cells.

Table 4.3. Recipe for calcium phosphate transfection method.

| **Plate Type** | **DNA** | **ddH20 (add up to)** | **2M CaCl2** | **2XHBS** |
|---|---|---|---|---|
| 6-well plate | 0.5-1 µg | 219 µl | 30.5 µl | 250 µl |
| 100 mm dish | 4 µg | 439 µl | 61 µl | 500 µl |
| 150 mm dish | 12 µg | 1317 µl | 183 µl | 1500 µl |

## 4.7. Bioinformatics

DNA sequences of BFK among 15 eucaryotic species, including mouse and human, were obtained via shotgun sequencing datas from NCBI and UCSC Genome Browsers for bioinformatics studies in this thesis. Programs in bioinformatics part used with default parameters.

### 4.7.1. dN/dS Ratio

DNA sequences of BFK from rodents to primates were used to determine positively selected aminoacids during evolution. In order to do this, nucleotide substitutions were analysed. Nucleotide substitutions in genes coding for proteins can be either synonymous

which does not change amino acid (silent substitutions) or non-synonymous which changes amino acid. Usually, most non-synonymous changes would be expected to be eliminated by purifying selection, but under certain conditions Darwinian selection may lead to their retention. START (Sequence Type Analysis and Recombinational Tests) program was used to analyse our data (Jolley *et al*., 2001). This program uses a method of estimating synonymous substitutions (Nei *et al*., 1986). The first step in this protocol is enumeration of the number of synonymous and non-synonymous sites present at each codon. The next step is to determine the number of synonymous and non-synonymous changes between each pair of aligned sequences, codon-by-codon. Where there is one nucleotide difference, it is obvious whether the change is synonymous or non-synonymous. When there are two or three changes, the number of possible pathways between the codons increases to two or six respectively. Considering each of possible changes, the values of dS and dN are determined. If dN/dS ratio is over 1, that means positive selection of aminoacid during evolution.

### 4.7.2. Phylogenetic Analysis by Maximum Likelihood (PAML)

PAML is a set of programs for phylogenetic analyses of DNA and protein sequences using maximum likelihood (Yang *et al.*, 2007). PAML is commonly used for comparing and testing phylogenetic trees. DNA sequences of five primates were compared with two rodents in order to get a phylogenetic tree and maximum likelihood was calculated via PAML program. According to this program, if likelihood value of a species is higher than 1, this means gene is positively selected for this species. In other words, the gene is conserved and may have novel functions for particular species.

### 4.7.3. Phylogenetic Tree

Molecular Evolutionary Genetics Analysis (MEGA4) and Clustal W programs are used for establishing sequence alignment, phylogenetic trees and estimating divergence times (Tamura *et al*., 2007 and Larkin *et al*., 2007). MEGA program uses RelTime method and produces estimates of relative times of divergence for all branching points in any phylogenetic tree without requiring knowledge of the distribution of the lineage rate variation and without using clock calibrations and associated distributions.

# 5. RESULTS

Our project aims to clarify control mechanisms of BFK in two stages; transcriptional and post-transcriptional control. Transcriptional control mechanisms of BFK covers, investigation of promoter region of human BFK and identification of potential transcription factor binding sites determined via Transfac, Alibaba2 (http://www.gene-regulation.com/pub/databases.html) and Genome Browser databases. We identified PARbZIP as a potential transcription factor for human BFK promoter. We enlightened evolutionary past of BFK among species. We also focused on hormones which possibly regulate human BFK expression; three candidate steroid hormones were selected for treatment of colon cancer cell lines and we found that tamoxifen upregulates BFK expression. At the post-transcriptional control level, we produced monoclonal and polyclonal anti-BFK antibodies to evaluate protein expression profiles of BFK in different cancer cell lines. We checked hormonal regulation profile of human BFK protein. We tried to identify the role of valine aminoacid in DEVD site for cleavage activity and two other positively selected aminoacids for interaction with Bcl-2 family members.

## 5.1. Study of Mouse and Human BFK Gene Evolution

### 5.1.1. Comparison of Promoter Region and Coding Sequences of BFK

To enlighten the evolutionary past of BFK, we obtained promoter region sequences and BFK coding sequences of 12 species (shotgun sequences) from NCBI and UCSC genome database and we compared these sequences with MEGA4 program in order to get phylogenetic trees (Figure 5.1.). Two major diversification lineages were observed in this experiment. One of them is between rodents and other species and the other is between primates and other species. Data suggests that the promoter region and the gene have parallel evolution characteristics, meaning that when the promoter region dramatically changed, the gene also changed in accordance with that diversification.

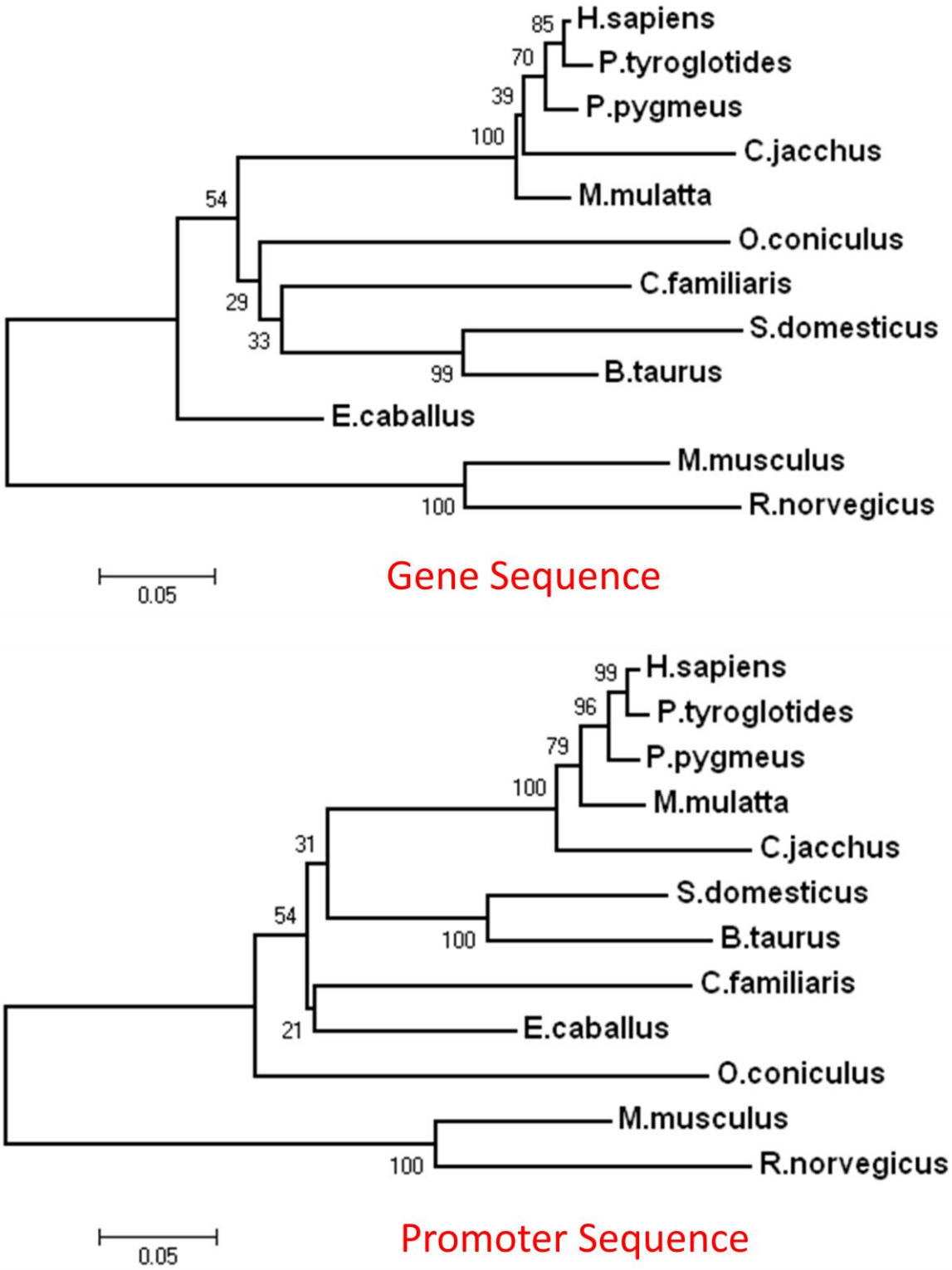


Figure 5.1. Parallel evolution of the BFK gene and the promoter region. Rodents have different evolutionary past from other species. Primates were also diversified from other species during evolution. The numbers next to each node, represent a measure of support for the node where 100 represents maximal support.

### 5.1.2. Phylogenetic Analysis

In order to determine positive selection aspects of BFK, 7 species were compared according to their BFK gene sequence with Phylogenetic Analysis by Maximum Likelihood (PAML). This analysis measures the ratio between non-synonymous (mutation of a nucleotide that causes change of an aminoacid) and synonymous changes (Yang *et al*., 2007). If ratio is equal or bigger than one, this means that gene is positively selected during evolution. We found BFK gene was positively selected for orangutan and human (Figure 5.2.). This data shows BFK may have some novel roles in humans.

### 5.1.3. Conservation of Amino Acid Sequences among Species

Amino acid sequences of 15 different species Bfk proteins were obtained from NCBI and UCSC databases and were compared with alignment and boxshade tools to find conservation motifs. We used START (Sequence Type Analysis and Recombinational Tests) program to analyse dN/dS ratios for each amino acid (Jolley *et al*., 2001). This program uses a method of estimating synonymous substitutions (Nei *et al*., 1986).

As a result, valine (40.aa, p-value=0.044) in DEVD site, proline (45.aa, p-value=0.013) and isoleucine (136.aa, p-value=0.024) amino acids were found positively selected ($p<0.05$) among primates during evolution (Figure 5.3.). DEVD site is thought to be the potential target of caspase-3 or 7. Due to this bioinformatic data, we can interpret that cleavage of BFK is valid for primates because they have a conserved DEVD motif.

We also hypothesized that proline and isoleucin amino acids may be related with the interaction of BFK with Bcl-XL and Bcl-W which is not observed for mouse Bfk. Other primates may have the same interactions because they also show conservation for these two amino acids.

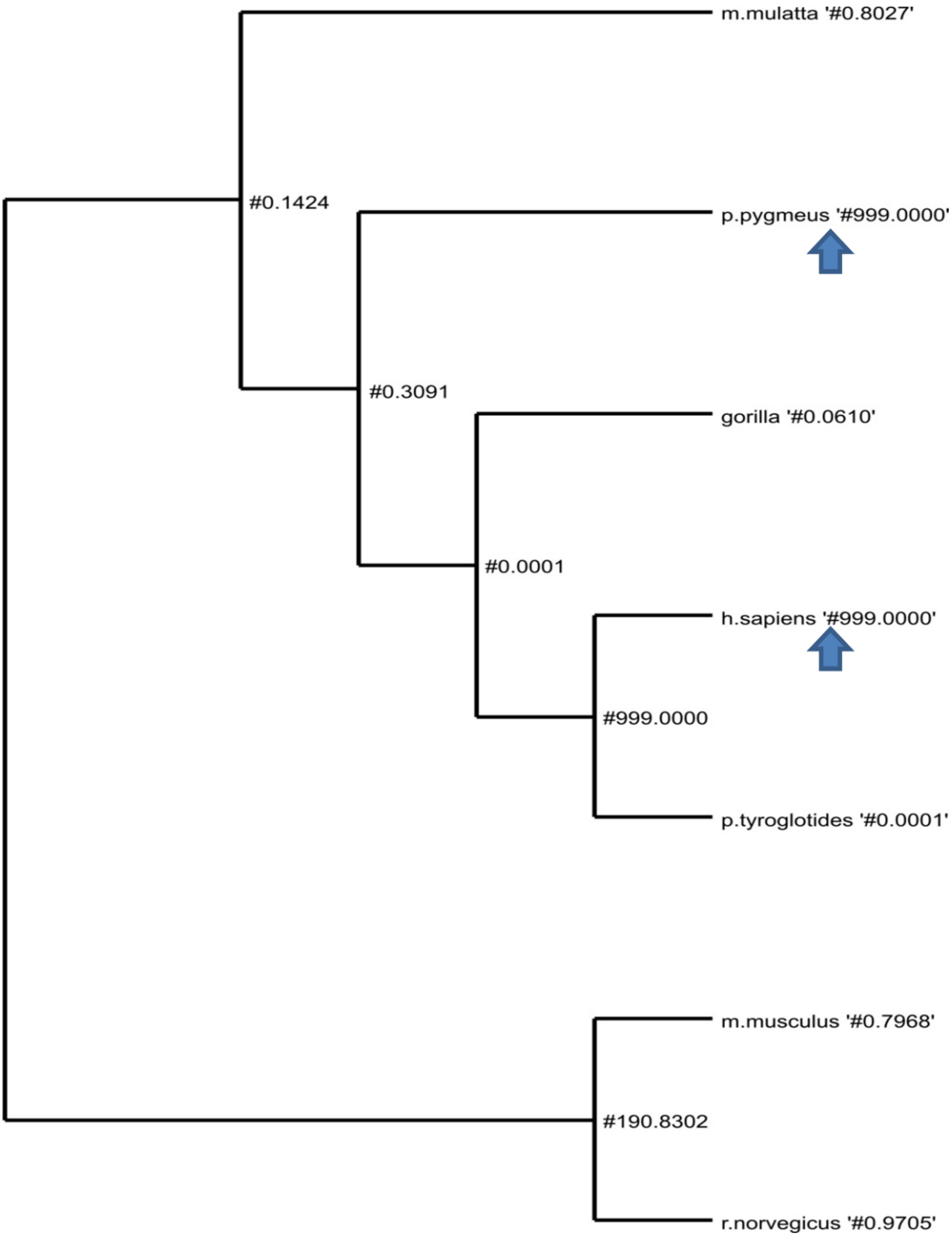


Figure 5.2. Positive selection for orangutan and human BFK.

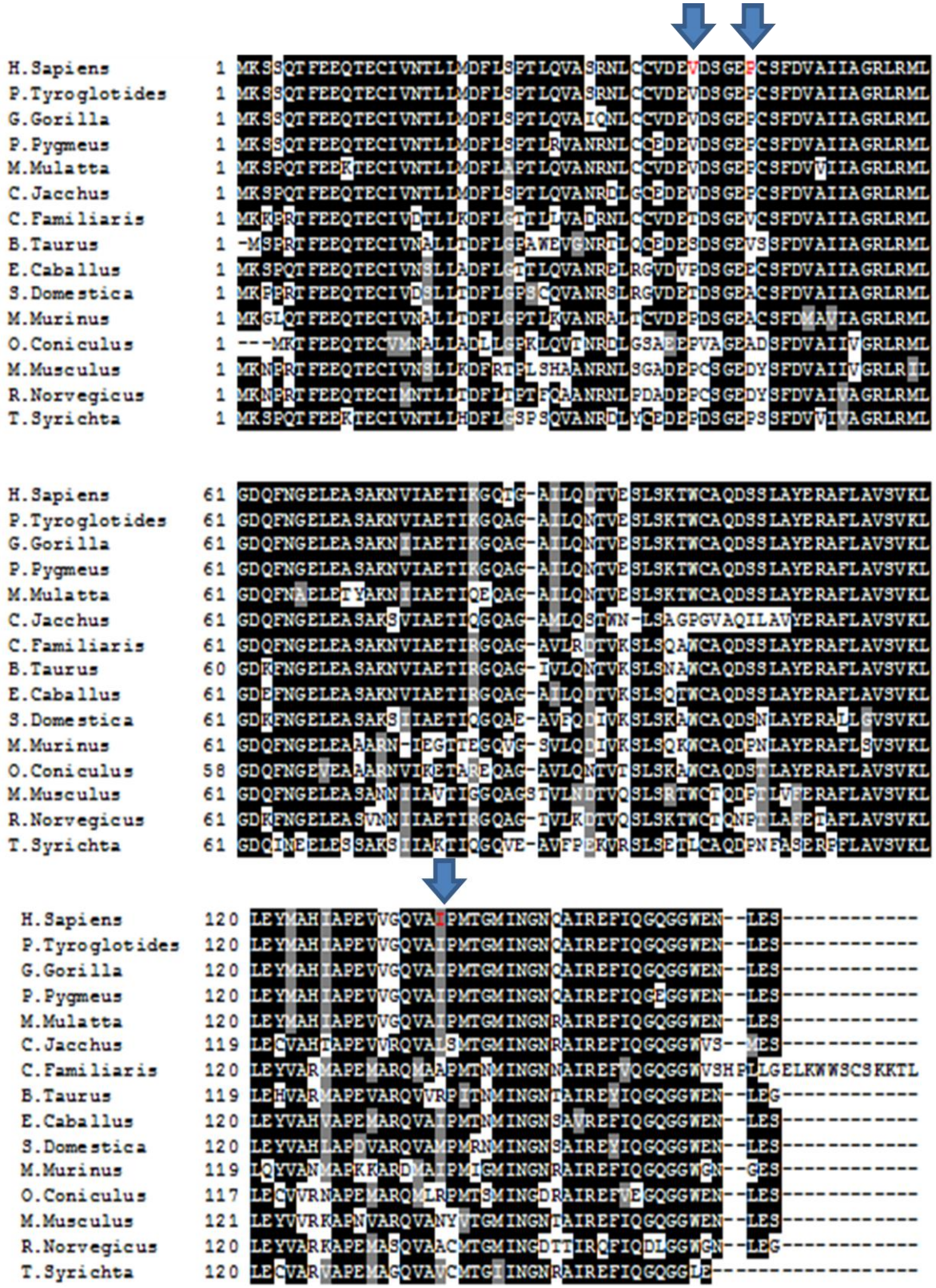


Figure 5.3. Homology alignment for amino acid sequences of 15 different species. Valine (p=0.044) in DEVD site, proline (p=0.013) and isoleucin (p=0.024) aminoacids were found positively selected among primates during evolution.

## 5.2. Transcriptional Regulation of Human BFK Gene

In this section, we tried to clarify the promoter region of human BFK, possible transcription factor binding sites and their roles using bioinformatic tools and luciferase assays. We also tested the roles of steroid hormones in the regulation of BFK transcription.

### 5.2.1. Bioinformatic Investigation of the Promoter Region of Human BFK Gene

We used bioinformatic tools such as GeneMasker, Transfac, Alibaba2, TFSearch and UCSC Genome Browser programs to identify possible repetetive elements and transcription factors of the 2 kb region of BFK.

We also compared promoter region differences between rodents and humans because they have different Bfk expression patterns in tissues and theoretically distinct apoptotic roles, such as hormone dependent homeostasis in mouse tissues and protection from tumoral transformation in human gastrointestinal tissues. We chose Clustal W (Larkin *et al*., 2007) and MEGA4 (Tamura *et al.*, 2007) programs to evaluate these differences. Besides we used the sequences of 12 different species phylogenetically located between rodents and humans to enrich our data.

The 2 kb part of the promoter region of BFK extending to the translation start site were reached via data mining from the NCBI database and whole genome shotgun sequence results for 12 different species phylogenetically located between mouse and human. The repetetive elements were determined by using RepeatMasker program (Table 5.1.). Among primates, the same LINE and Alu elements (Figure 5.4.) were found in a similar regional distribution but the same distribution pattern was not valid for other eucaryotic species. Interestingly, the 442 bp region before the transcription start site had no repetetive elements in 12 species. This region was used to find possible transcription factor binding sites in the next section.

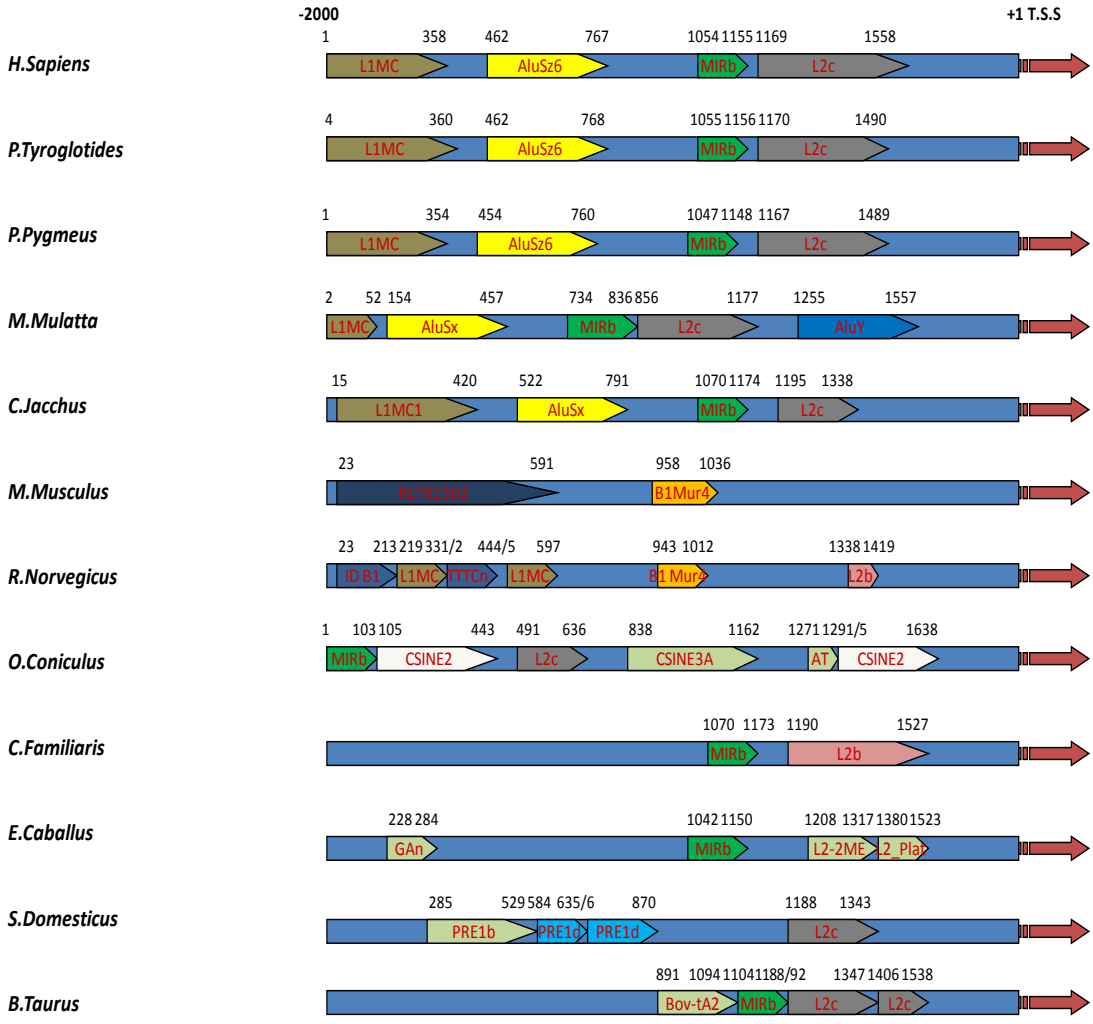


Figure 5.4. Repetetive elements in the promoter region of BFK as determined by Repeatmasker.

### 5.2.2. Identification of Potential Transcription Factor Binding Sites

The 442 bp regions found after comparison of promoter regions of 12 species, were further investigated for potential transcription factor binding sites with Alibaba2, TFSearch, USCS Genome Browser programs. Possible transcription factors were selected after comparison of human and mouse sequences (Figure 5.5. and 5.6.). NF-KB binding motif was one of the transcription factors, detected in the programs and it was interesting to work with, because a possible relation with immune mechanisms may help to find the

link between BFK and gastrointestinal tumors. Two potential binding sites were found in 442 bp region and one found in a region upstream of 442 bp part. One PARbZIP transcription factor binding site was also found upstream of this region. PARbZIP has been shown to regulate another BH3 only protein Bcl-gS (Ritchie *et al*., 2009).

Table 5.1. Repetetive elements in BFK promoter region.

| Total length: | 2000 bp | | |
|---|---|---|---|
| GC level: | 39.65 % | | |
| | Number of elements* | Length occupied | Percentage of sequence |
| SINEs: | 2 | 408 bp | 20.40 % |
| ALUs | 1 | 306 bp | 15.30 % |
| MIRs | 1 | 102 bp | 5.10 % |
| LINEs: | 2 | 748 bp | 37.40 % |
| LINE1 | 1 | 358 bp | 17.90 % |
| LINE2 | 1 | 390 bp | 19.50 % |
| L3/CR1 | 0 | 0 bp | 0.00 % |
| LTR elements: | 0 | 0 bp | 0.00 % |
| ERVL | 0 | 0 bp | 0.00 % |
| ERVL-MaLRs | 0 | 0 bp | 0.00 % |
| ERV_classI | 0 | 0 bp | 0.00 % |
| ERV_classII | 0 | 0 bp | 0.00 % |
| DNA elements: | 0 | 0 bp | 0.00 % |
| hAT-Charlie | 0 | 0 bp | 0.00 % |
| TcMar-Tigger | 0 | 0 bp | 0.00 % |
| Unclassified: | 0 | 0 bp | 0.00 % |
| Total interspersed repeats: | | 1156 bp | 57.80 % |
| Small RNA: | 0 | 0 bp | 0.00 % |
| Satellites: | 0 | 0 bp | 0.00 % |
| Simple repeats: | 0 | 0 bp | 0.00 % |
| Low complexity: | 0 | 0 bp | 0.00 % |

* Most repeats fragmented by insertions or deletions have been counted as one element.

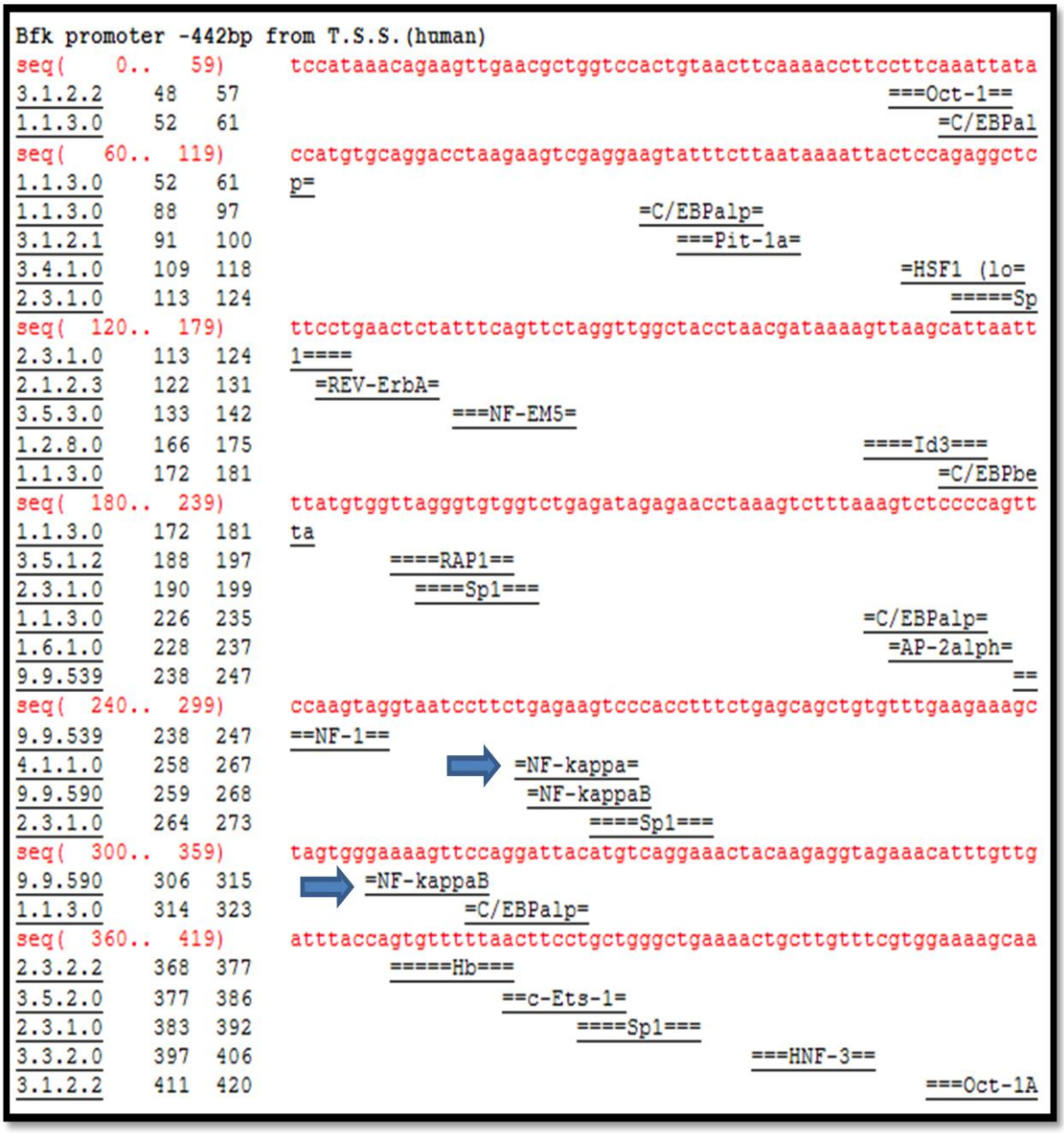


Figure 5.5. Last 442 bp region of human BFK promoter. Two potential NF-KB binding sites were found in the promoter region with Alibaba2 program (shown with arrows).

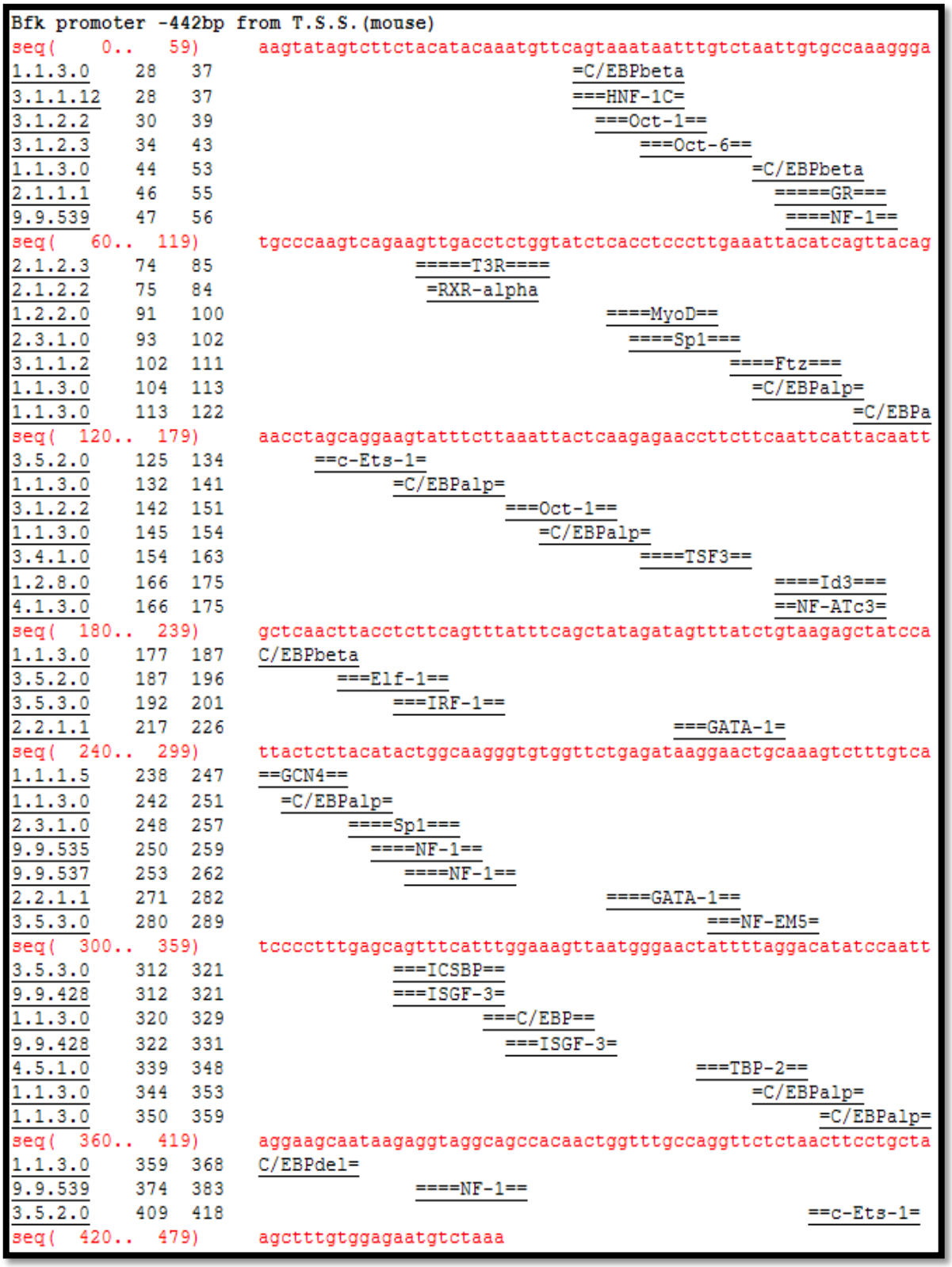


Figure 5.6. Last 442 bp region of mouse Bfk promoter. No potential NF-KB binding sites were found in the promoter region with Alibaba2 program.

### 5.2.3. Possible Role of NF-KB3 Binding Site on the Human BFK Promoter Region

Binding sites of transcription factors were determined after data mining from bioinformatic tools. In order to check transcription factor activity, we created wt and deleted constructs for transcription factor binding sites. Spesific primers (Figure 5.7.) for wild type and deleted versions of sequences of 4 selected transcription factor binding sites, were designed by using Invitrogen primer design tools (OligoPerfect Designer) and the regions amplified with PCR.

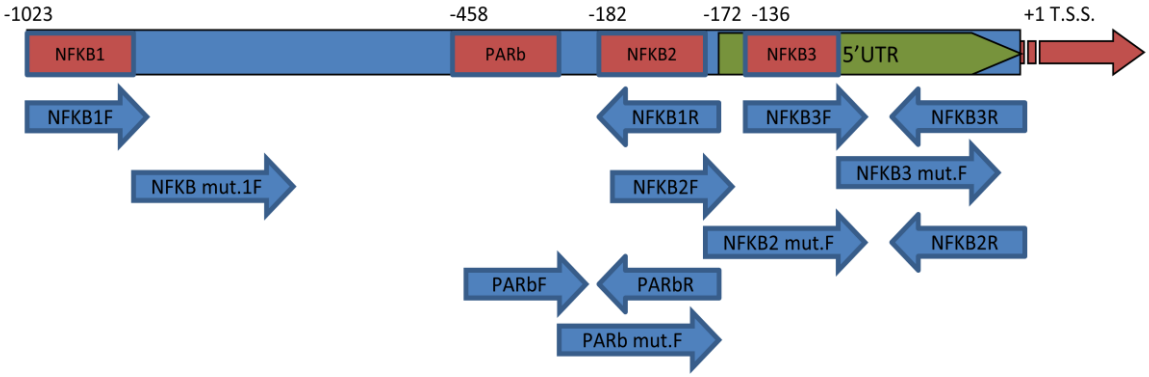


Figure 5.7. Transcription factor binding sites and the primers. F:Forward primer, R:Reverse primer.

Amplified products (Figure 5.8.) and pGL3 basic vector were cut with HindIII and XhoI restriction enzymes in sequential digestion. They were ligated with T4 DNA ligase at room temperature for 1 hour to generate wt and deleted NF-KB3 constructs (Appendix Figure 1. and 2.). Competent Top10 bacteria were transformed with these vectors and colony PCR was performed (Figure 5.9.). Positive colonies were sent to sequencing and no mutations were observed.

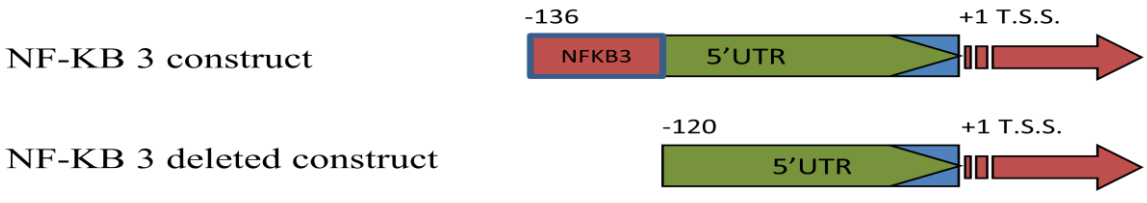


Figure 5.8. Schematic view of wtNF-KB3 and NF-KB3 deleted constructs.

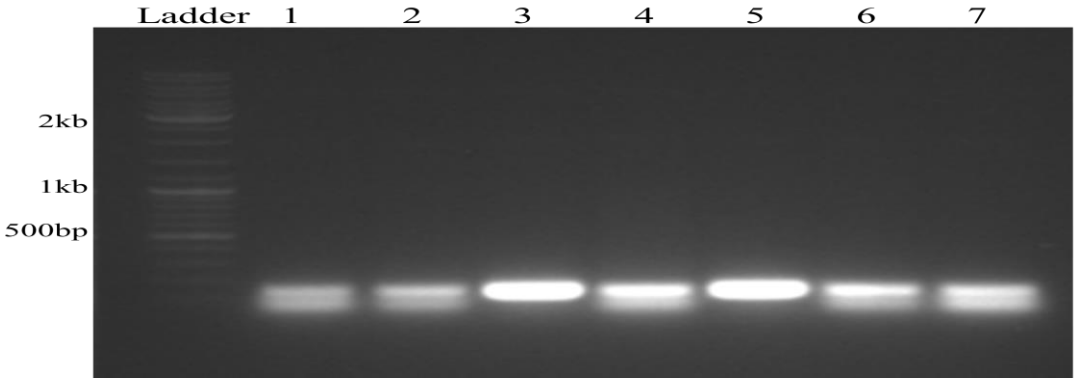


Figure 5.9. Colony PCR results for NF-KB3 construct. Lane 3 and 5 show the positive colonies.

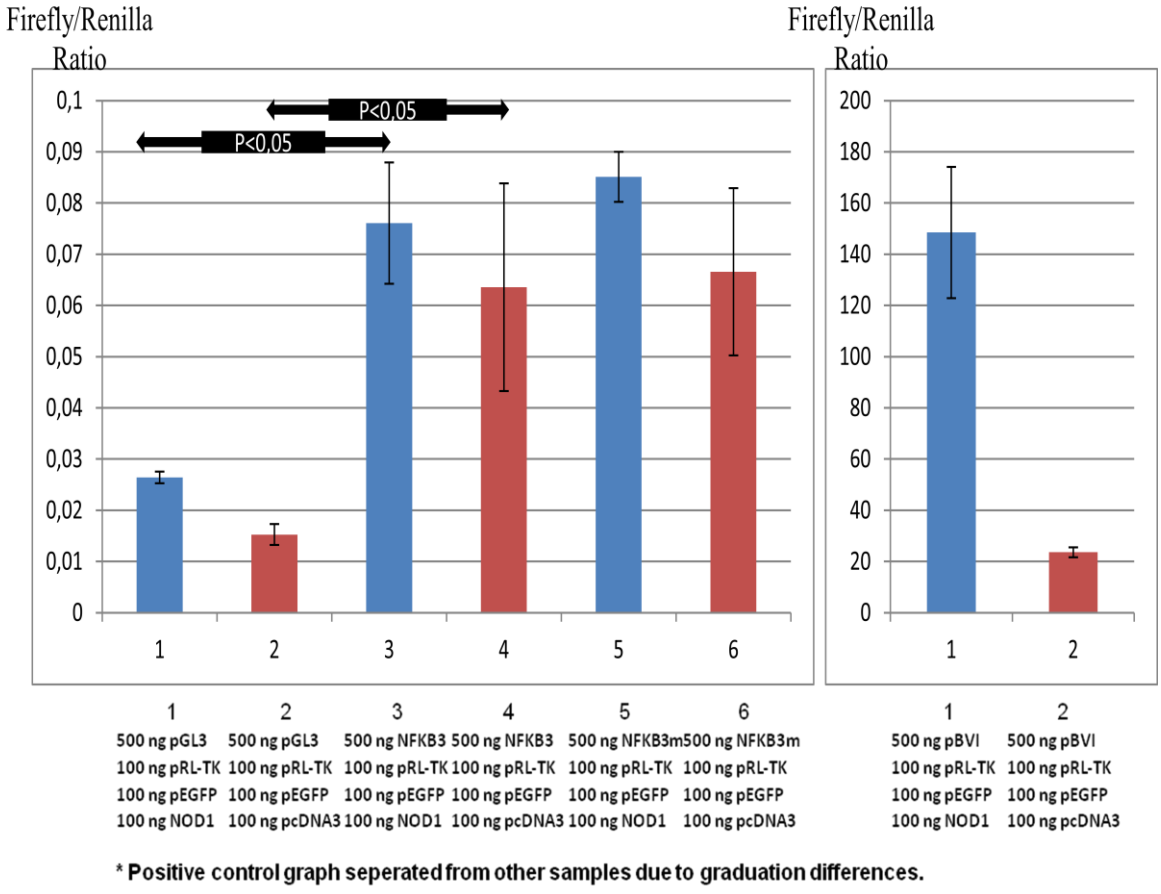


Figure 5.10. NF-KB3 binding site does not affect transcriptional activity of hBFK. Y axis shows firefly/renilla ratio, NOD1 positive conditions are shown in blue. Column 1 and 2 represent negative controls, column 3 and 4 represent wt versions, column 5 and 6 represent deleted NF-KB3 constructs. Error bars represent standard deviations. Figure shows representative result of three independent experiments.

pGL3 basic vector was selected for cloning because it is promoterless and has the luciferase coding region downstream of the multiple cloning site. Luciferase activities were measured with or without NOD1 which is an inducer of NF-KB (Figure 5.10.). pBVI-Luc vector, which has 7 NF-KB binding sites, was used as positive control. No significant effect of NF-KB3 binding site was observed in HCT116 cells.

### 5.2.4. Possible Role of NF-KB2 Binding Site on the Human BFK Promoter Region

wtNF-KB2 and NF-KB2 deletion constructs (Appendix Figure 3. and 4.) were cloned into promotorless luciferase vector, pGL3 basic, in order to evaluate the effect of NF-KB2 binding site on promoter region of BFK gene. These two constructs also have the NF-KB3 binding site (Figure 5.11.).

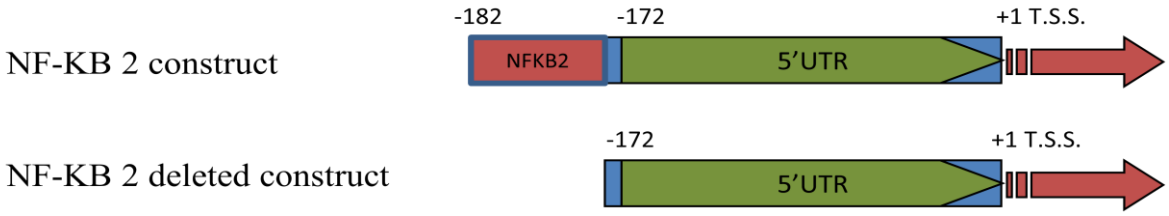


Figure 5.11. Schematic view of wtNF-KB2 and NF-KB2 deleted constructs.

Competent Top10 bacteria were transformed with these vectors and colony PCR was performed (Figure 5.12.). Positive colonies were sequenced and no mutations observed.

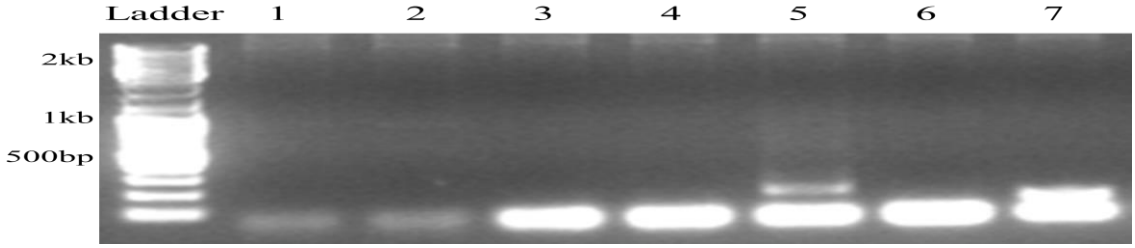


Figure 5.12. Colony PCR results for NF-KB2 construct. Lane 5 and 7 show the positive colonies.

Constructs were transfected into HCT116 colon cancer cell line with Fugene HD transfection reagent (Figure 5.13.). pEGFP vector was used for transfection efficiency control.

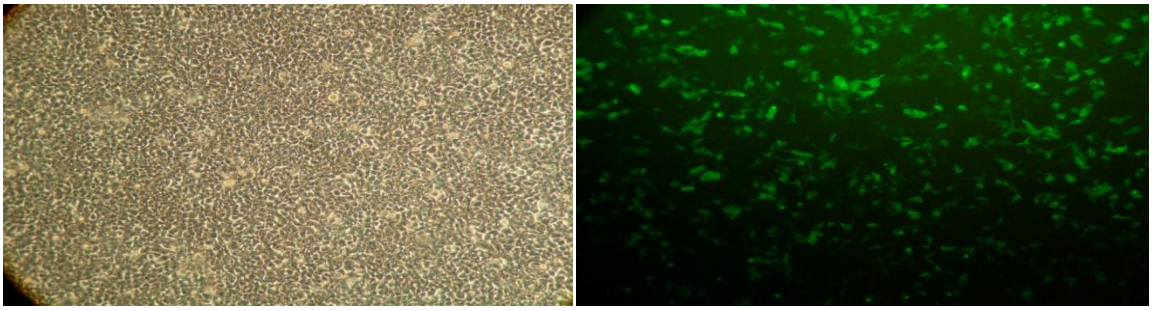

Figure 5.13. GFP expression of HCT116 cells after transfection with Fugene HD. (Left:Bright field visualisation, Right:Fluorescent Microscopy).

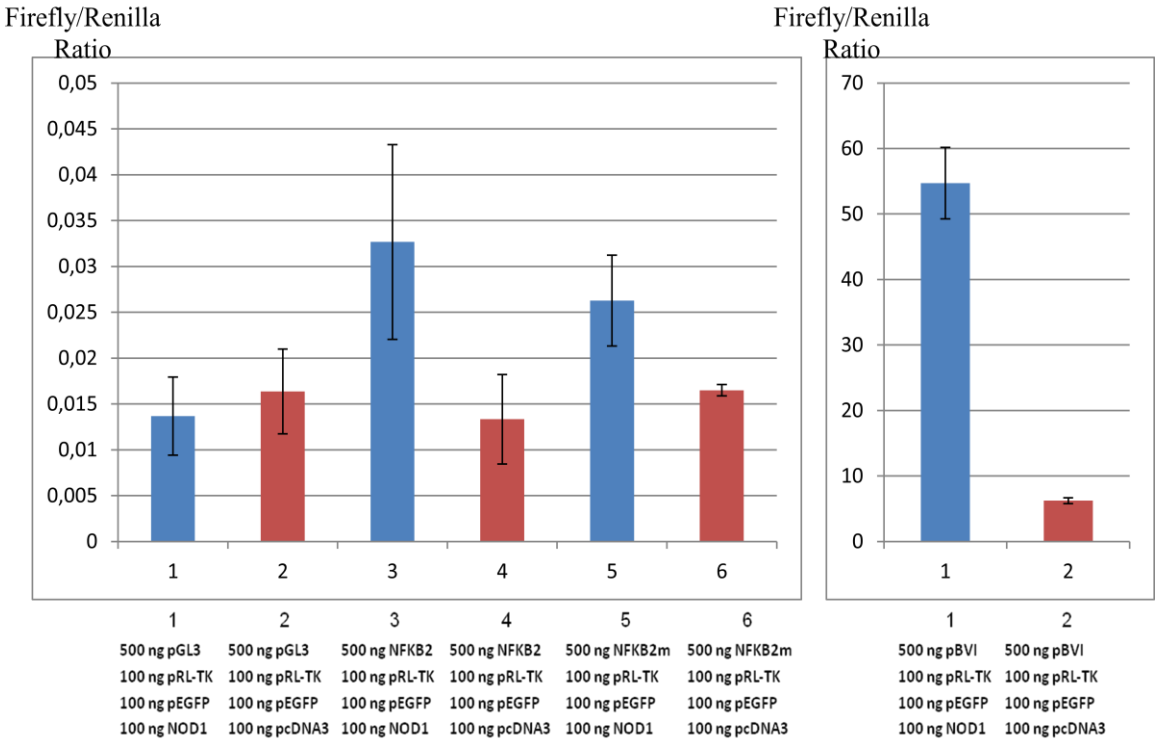


Figure 5.14. NF-KB2 binding site does not affect transcriptional activity of hBFK. Y axis shows firefly/renilla ratio, NOD1 positive conditions are shown in blue. Column 1 and 2 represent negative controls, column 3 and 4 represent wt versions, column 5 and 6 represent deleted NF-KB2 constructs. Error bars represent standard deviations. Positive controls are shown at the right graph because of graduation differences.

Luciferase activities were measured with or without NOD1 (Figure 5.14.). No significant effect of NF-KB2 binding site was observed in this experiment.

### 5.2.5. Possible Role of PARbZIP Binding Site on the Human BFK Promoter Region

Members of PARbZIP (Proline- and Acid-rich Basic Region Leucine Zipper) proteins; thyrotroph embryonic factor (TEF), D-site binding protein (DBP), and hepatic leukemia factor (HLF) have been shown to be involved in neurotransmitter homeostasis and amino acid metabolism (Benito *et al*., 2006). PARbZIP proteins are able to transactivate the promoter of Bcl-gS. This promoter is particularly responsive to TEF activation and is silenced by NFIL3, a repressor that shares the consensus binding site with PARbZIP proteins (Benito *et al*., 2006).

One of the Bcl-2 family member, Bik, has been shown to have a transcriptional pathway related with PARbZIP, which mediates oxidative stress induced apoptosis. Bik is downregulated in tail fibroblasts of PARbZIP knock-out mice (Ritchie *et al*., 2009).

In our project, the potential transcription factor PARbZIP binding site and the downstream sequences till translation start site, were cloned into promoterless pGL3 basic vector. Prepared constructs have luciferase downstream of cloned sequences in order to measure promoter activity (Appendix Figure 5. and 6.). Potential PARbZIP transcription factor binding site is located in the upstream region of 5'UTR of Bfk gene, starting 458 bp before translation start site. The difference between pGL3-PARbZIP construct and pGL3-PARbZIP deleted version is 27 bp (Figure 5.15.).

At the beginning of our experiment, we transfected our constructs into HCT116 colon cancer cell lines with pRL-TK vector coding for renilla luciferase and pEGFP vector as transfection efficiency control. We checked the luciferase activities of these constructs (Figure 5.16.). Results showed 9 fold decrease of luciferase activity in PARbZIP vector when compared with PARbZIP deleted one. This means one of PARbZIP family member or its antagonist NFIL3 may be binding and repressing the capacity on hBFK promoter region.

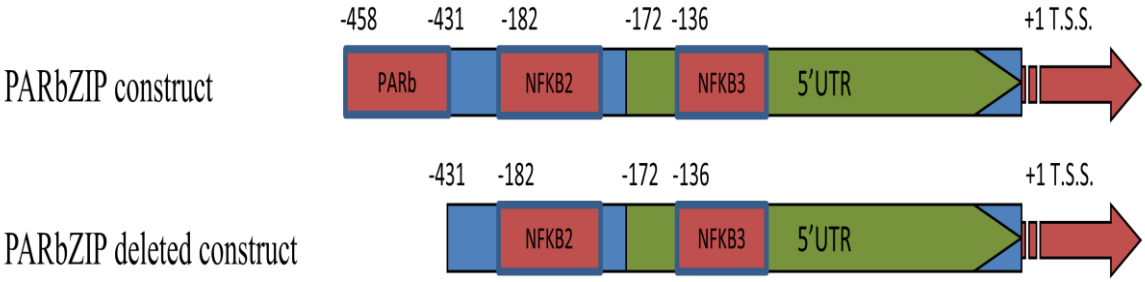


Figure 5.15. Schematic view of wtPARbZIP and PARbZIP deleted constructs.

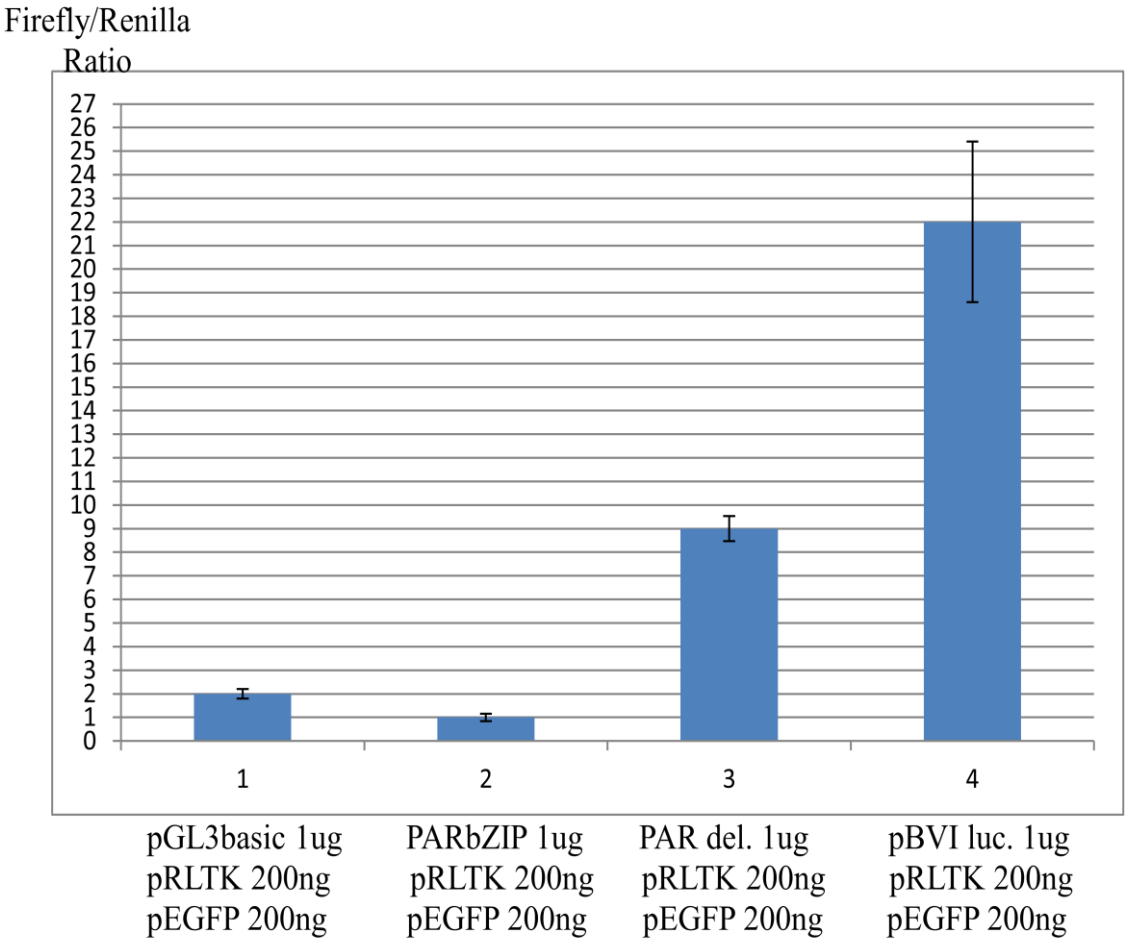


Figure 5.16. PARbZIP binding site suppresses transcriptional acitivity of hBFK. Y axis shows firefly/renilla ratio as normalized fold increase. pGL3 basic in column 1 represent negative control, pBVI Luc in column 4 represent positive control. Error bars represent standard deviations.

After determining the effect of PARbZIP DNA motif on BFK promoter region in pGL3 vector with luciferase assay, we decided to check mRNA levels of PARbZIP family (TEF, HLF, DBP) and antagonist NFIL3 in our colon cancer cell lines (SW707, HCT116 and HT29). TEF is expressed in all three cell lines but NFIL3 is only expressed in SW707 and HCT116 colon cancer cell lines (Figure 5.17.). DBP is expressed only in HT29 cells.

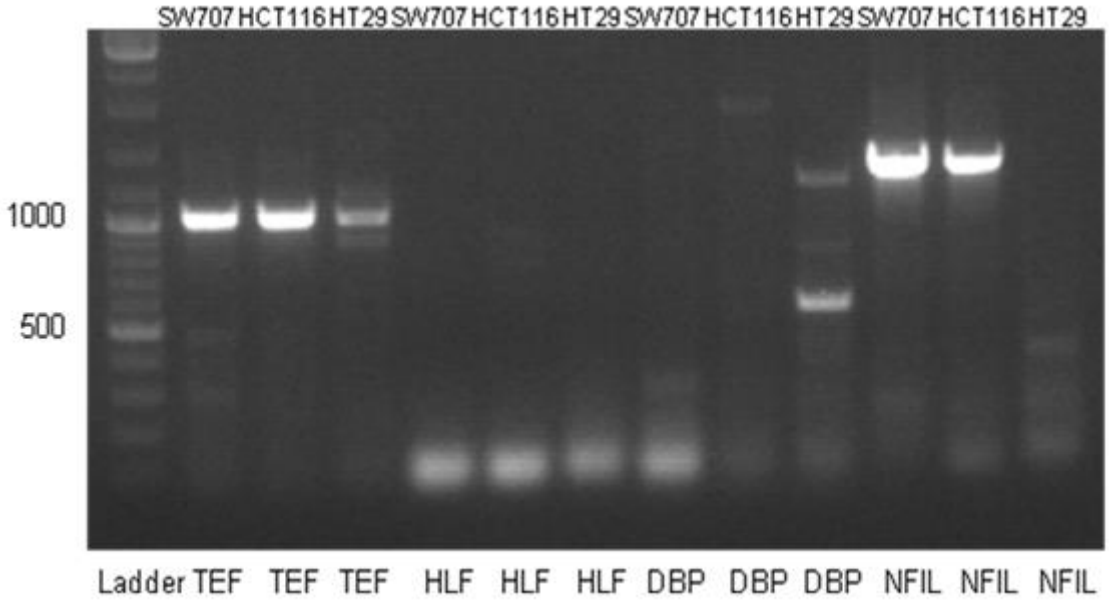


Figure 5.17. RT-PCR results of PARbZIP family mRNA levels in colon cancer cell lines. Equal amount of RNA was used for generation of cDNAs.

We decided to use PARbZIP family members (TEF, HLF, DBP) and antagonist NFIL3 to clarify their roles on the regulation of BFK promoter region so we obtained FLAG tagged pcDNA3-TEF and pcDNA3-NFIL3 as a gift from Fernandez Luna, Ph.D.

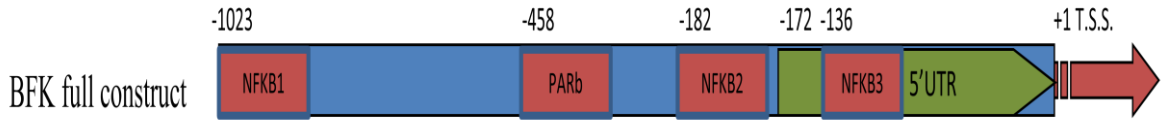


Figure 5.18. Schematic view of BFK full promoter construct.

First, we transfected HCT116 colon cancer cell lines with pGL3 BFK full construct (Figure 5.18.) with or without pcDNA3-FLAG-TEF and pcDNA3-FLAG-NFIL3 via using

Roche HP Extreme Transfection Reagent. In this experiment, we showed that TEF transcription factor induces luciferase activity while NFIL3 supresses TEF induced transcription (Figure 5.19.).

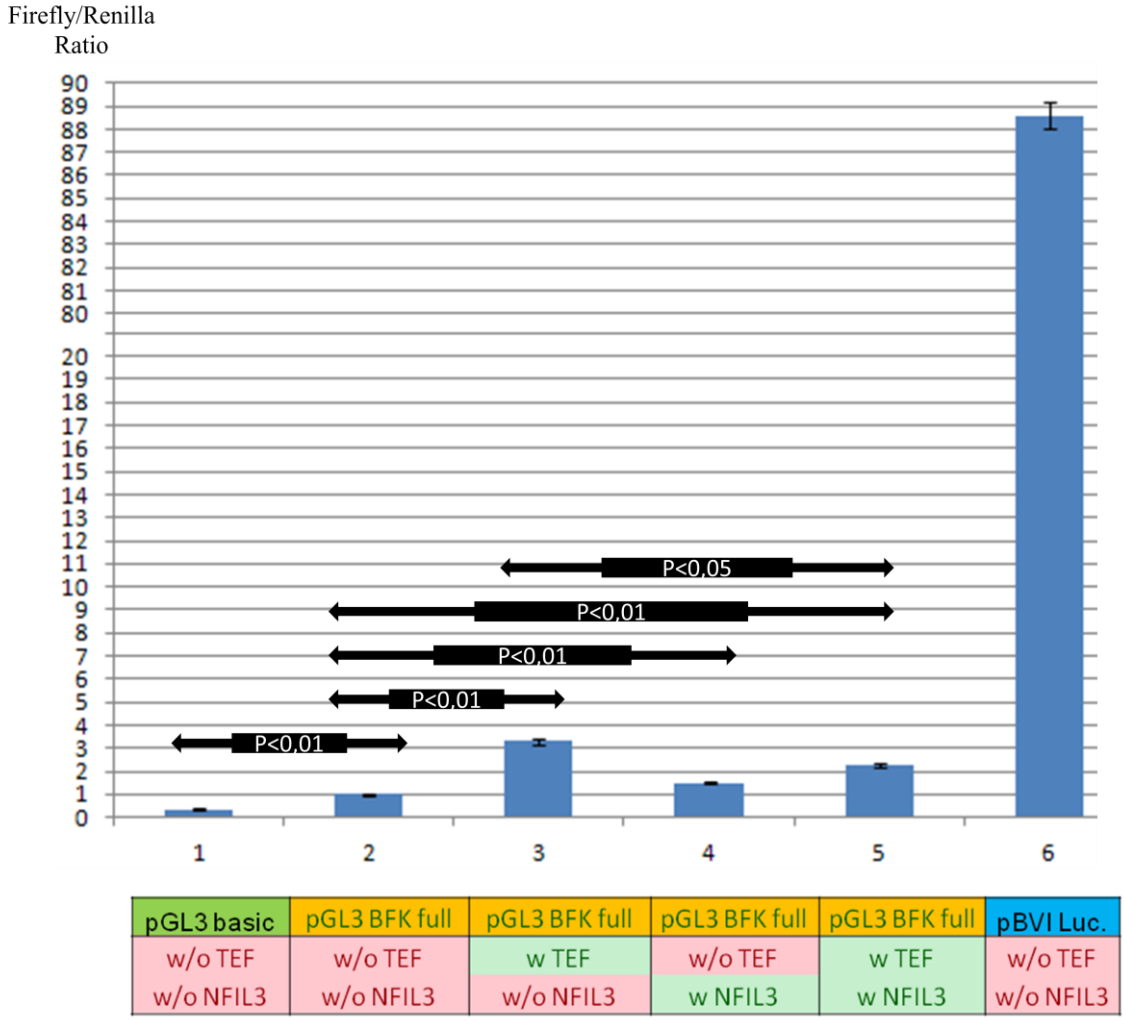


Figure 5.19. NFIL3 supresses TEF induced transcription in HCT116 colon cancer cells. Column 1 represents negative control, column 2 includes only pGL3 BFK full vector while columns 3 and 4 have pcDNA3-FLAG-TEF and pcDNA3-FLAG-NFIL3 respectively. Column 5 includes pGL3 BFK full with both pcDNA3-FLAG-TEF and pcDNA3-FLAG-NFIL3. Column 6 represents positive control. Error bars represent standard deviations.

In the next step, we co-transfected pGL3-PARbZIP and pGL3-PARbZIP deleted constructs with or without pcDNA3-FLAG-TEF and pcDNA3-FLAG-NFIL3 vectors in order to measure luciferase activities. We found that, when pcDNA3-FLAG-TEF was

added, it significantly induced luciferase activity while NFIL3 has a negative regulatory effect (Figure 5.20.). Either with pGL3-PAR or pGL3-PARdel, TEF can induce luciferase activity but with potential PARbZIP binding site, induction of TEF is higher. On the other hand, NFIL3 supresses transcription whether there is a potential PARbZIP binding site or not. This data supports another possible binding site for TEF and NFIL3 to regulate transcription of human BFK.

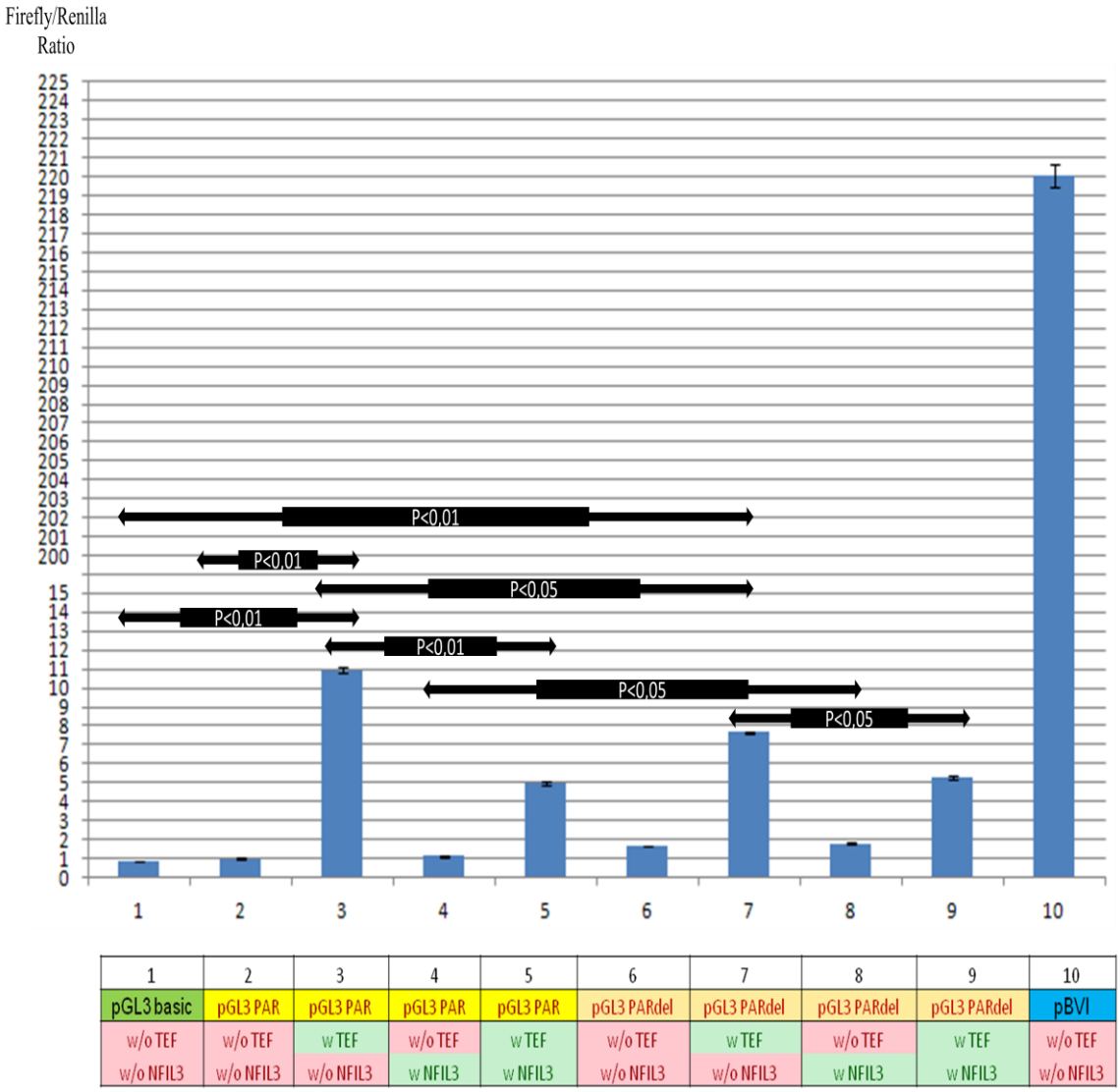


Figure 5.20. TEF induces transcription of human BFK whereas NFIL3 supresses this induction. Column 2 includes only pGL3 PARbZIP vector while columns 3, 4 and 5 have pcDNA3-FLAG-TEF, NFIL3 or both TEF and NFIL3 respectively. Column 6 includes only pGL3 PARbZIP deleted construct while columns 7, 8 and 9 have pcDNA3-FLAG-TEF, NFIL3 or both TEF and NFIL3 respectively. Error bars represent standard deviations.

### 5.2.6. Estrogen May not Have Effect on Human BFK Expression

In this part of our project, we investigated hormonal regulation of human BFK since mouse BFK is prone to be regulated by testosterone and estrogen. First of all, we wanted to determine which cell line to be used so we checked endogenous unstimulated BFK mRNA levels in our colon cancer cell lines (Figure 5.21.). For this purpose mRNAs were isolated from equal number of cells of each cell lines and cDNAs were amplified with BFK specific primers from equal amount of mRNAs. We decided to use SW707 colon cancer cell line because it expresses relatively more BFK mRNA than HCT116 and HT29 cell lines.

Estrogens are a group of compounds known for their importance in both menstrual and reproductive cycles. They are the primary female sex hormones. Natural estrogens are steroid hormones, like all steroid hormones, estrogens readily diffuse across the cell membrane. Once inside the cell, they bind to and activate estrogen receptors, which in turn modulate the expression of many genes.

We tested the effect of estrogen on hBFK expression via qRT-PCR. We treated SW707 and HT29 cells with 100 nM estrogen for 2 hr, 4 hr and 6 hours, respectively, then we checked mRNA levels of BFK with qRT-PCR. We didn't observe any effect of estrogen on BFK expression but we didn't have any positive control here so we don't have an exact conclusion from this experiment (Figure 5.22.).

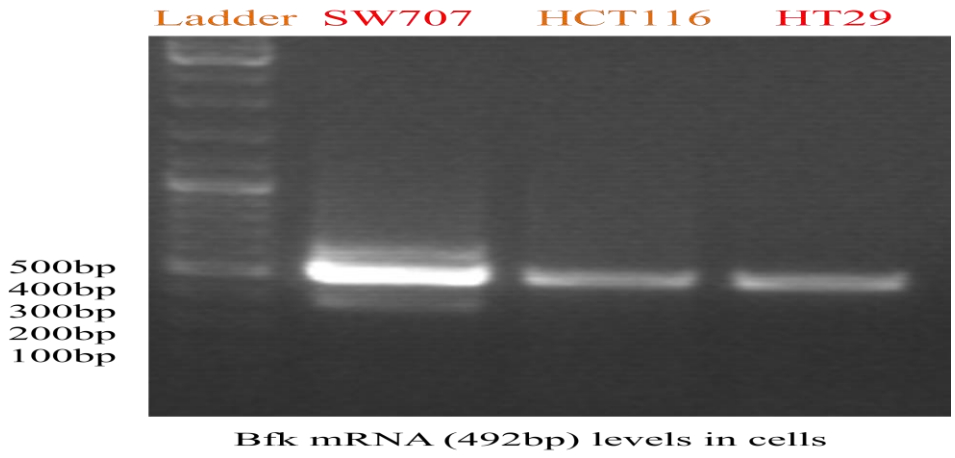


Figure 5.21. Colon cancer cells express hBFK mRNA. RT-PCR results of BFK mRNA levels in three colon cancer cell lines, SW707, HCT116 and HT29.

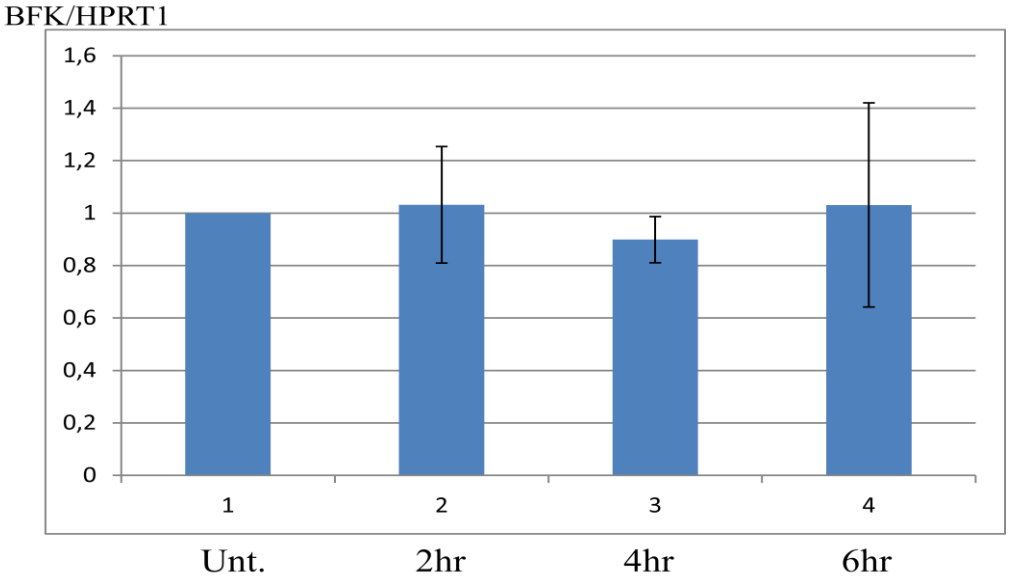


Figure 5.22. Estrogen may not effect hBFK transcription. qRT-PCR results for BFK after 100 nM estrogen treatment. Y axis shows fold increase. HPRT1 was used as internal control. Column 2 represents BFK levels (1,03+/-0,22) after 2 hr estrogen treatment, Column 3 represents BFK levels (0,89+/-0,08) after 4hr treatment, Column 4 represents BFK levels (1,03+/-0,38) after 6 hr treatment. Error bars represent standard deviations.

### 5.2.7. Testosteron Downregulates hBFK mRNA Expression Transiently at 4 hours

Testosterone is a steroid hormone from the androgen group and is found in mammals and other vertebrates. In mammals, testosterone is secreted primarily in the testicles of males and the ovaries of females, although small amounts are also secreted by the adrenal glands. It is the principal male sex hormone and an anabolic steroid.

We tested the effect of testosteron on BFK expression with qRT-PCR. We treated SW707 and HT29 cells with 100 nM testosteron for 2 hr, 4 hr and 6 hours, respectively, then we checked mRNA levels of BFK with qRT-PCR. We didn't observe any effect of testosteron on BFK expression for 2 and 6 hours but we observed that BFK mRNA levels are significantly downregulated after 4 hours testosteron treatment (Figure 5.23.).

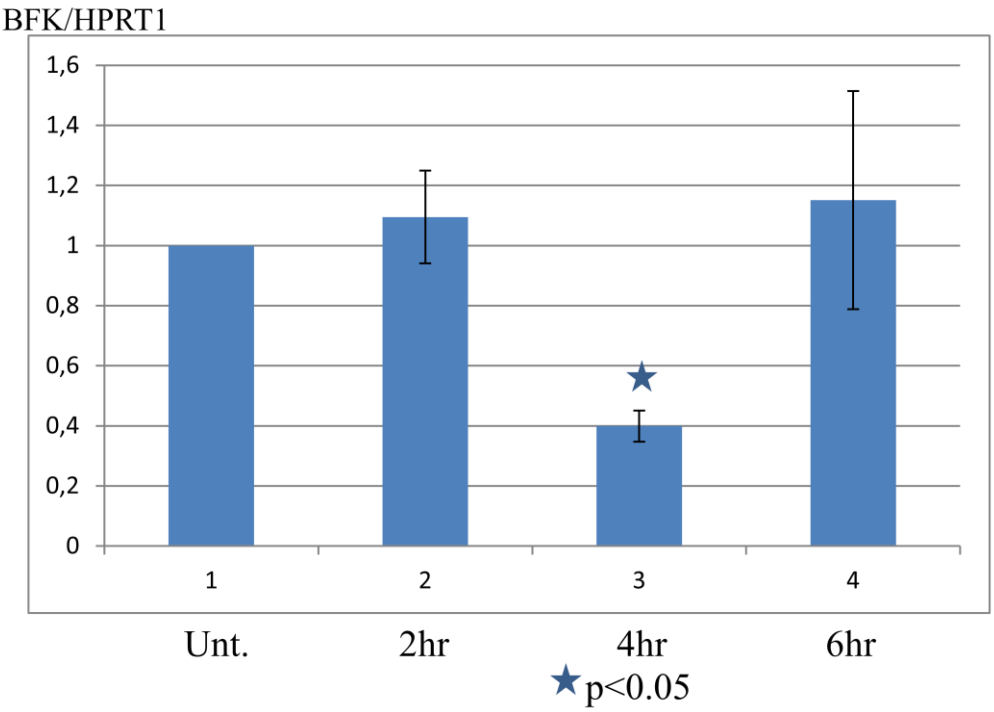


Figure 5.23. Testosteron treatment reduces hBFK transcription transiently. Y axis shows fold increase and untreated conditions are normalized to 1. Column 2 represents BFK levels (1,09+/-0,15) after 2 hr testosteron treatment, Column 3 represents BFK levels (0,39+/-0,05) after 4 hr testosteron treatment, Column 4 represents BFK levels (1,15+/-0,36) after 6 hr testosteron treatment. Error bars represent standard deviations.

### 5.2.8. Tamoxifen Upregulates Human BFK Expression

Tamoxifen is an antagonist of the estrogen receptor in breast tissue via its active metabolite, hydroxytamoxifen. In other tissues such as the endometrium, it behaves as an agonist, and thus may be characterized as a mixed agonist/antagonist. Its role in colon tissue is not clear, so we decided to check its activity on our colon cancer cell lines. We treated three colon cancer cell line with 25 nM, 50 nM and 100 nM tamoxifen for 6 hours, respectively (Figure 5.24.). We observed that 100 nM of tamoxifen treatment is required for inducing cell death visually.

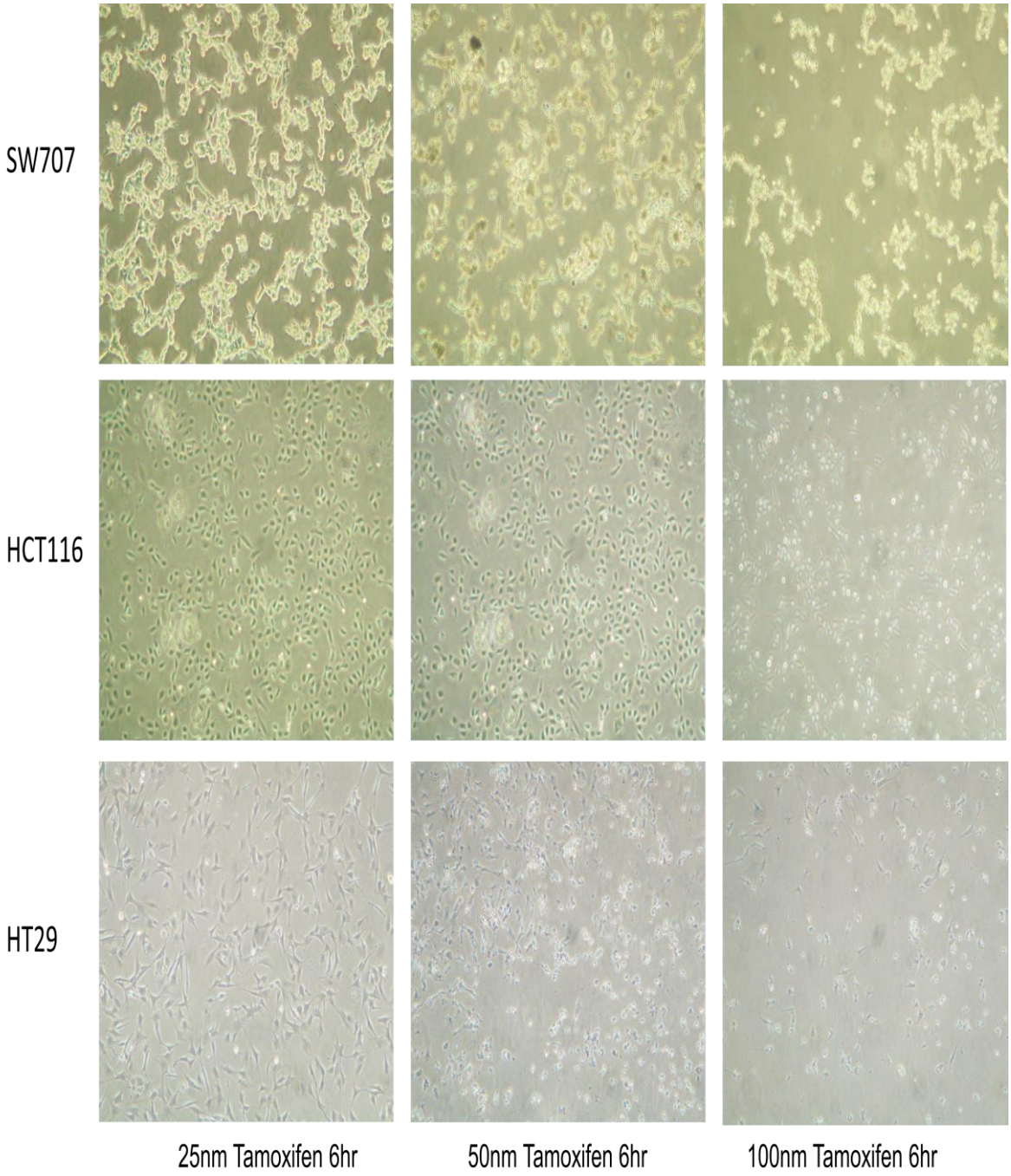


Figure 5.24. Tamoxifen treatment induces cell death in colon cancer cell lines.

We treated SW707 cell lines with 100 nM tamoxifen for 2 hr, 4 hr and 6 hr, respectively and then we isolated mRNAs to generate cDNAs in order to use in qRT-PCR experiments. We checked four BCL-2 family member (BFK, Bcl-2, Bcl-XL and BAX) expression levels with qRT-PCR after tamoxifen treatment. We used Relative Expression

Software Tool (REST) for calculation of Ct values (Michael *et al*., 2002) and we found that for all time points BFK mRNA levels are significantly ($p<0.01$) upregulated (Figure 5.25.). Besides, after 6 hour tamoxifen treatment, BAX mRNA levels are downregulated ($p<0.01$).

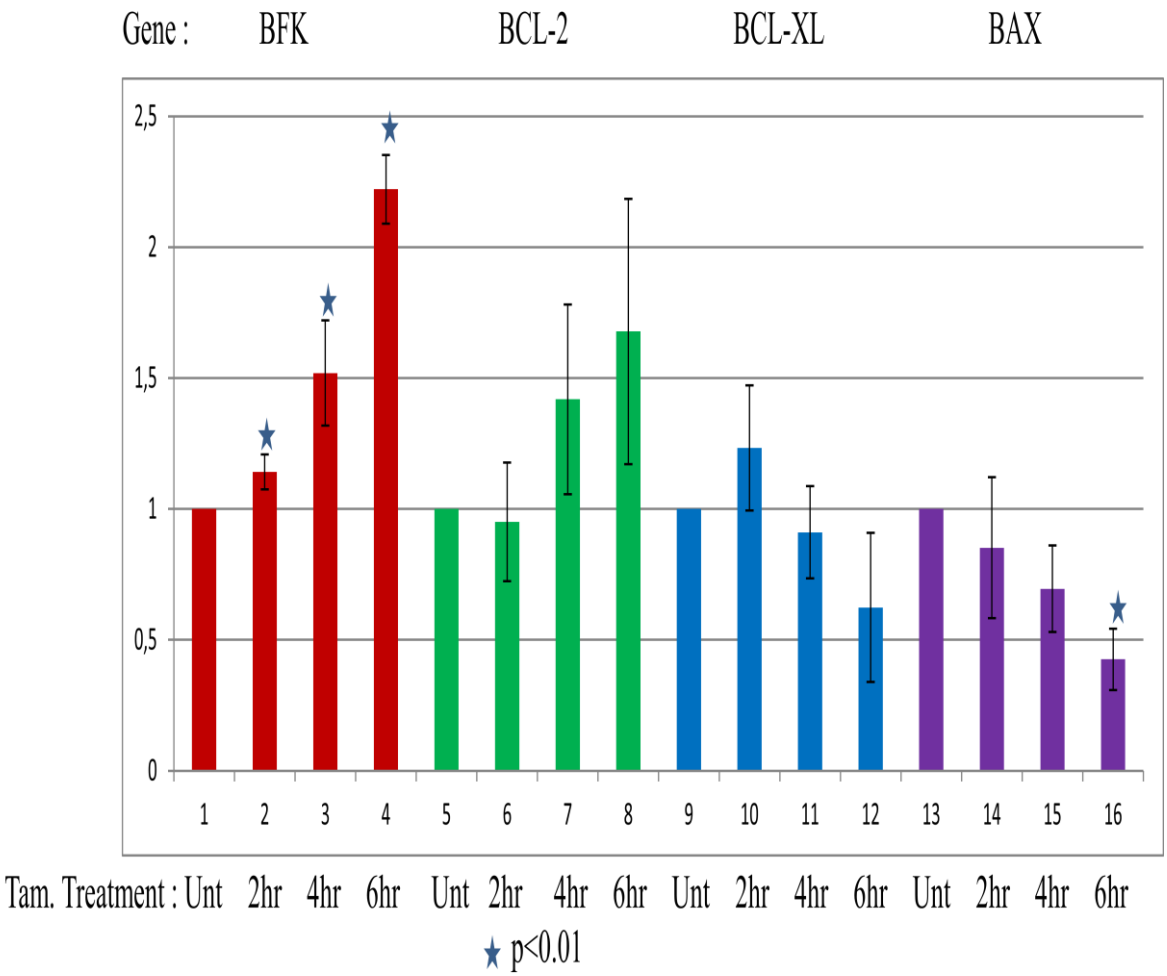


Figure 5.25. Tamoxifen upregulates hBFK transcription ($p<0.01$). HPRT1 was used as internal control in qRT-PCR. Column 2 represents BFK levels (1,14+/-0,06) after 2 hr tamoxifen treatment, Column 3 represents BFK levels (1,51+/-0,2) after 4 hr treatment, Column 4 represents BFK levels (2,22+/-0,13) after 6 hr treatment. Column 16 represents BAX levels (0,42+/-0,11) after 6 hr treatment. Error bars represent standard deviations.

## 5.3. Post-Transcriptional Regulation of Human BFK Protein

### 5.3.1. Generation of anti-hBFK Polyclonal and Monoclonal Antibodies

To work with endogenous BFK protein in our experiments, we produced anti-BFK monoclonal and polyclonal antibodies. The first step was hisBFK protein production and purification. For this purpose, we transformed Rosetta DE3 pLYsS bacteria with pET30-BFK plasmid and induced protein production with IPTG. We purified hisBFK protein with Nickel column, then we confirmed the protein with western blotting using anti-his antibody (Figure 5.26.). We performed Bradford assay to measure the amount of hisBFK protein (Appendix Figure 7.). We were able to produce 1 mg protein per cartridge.

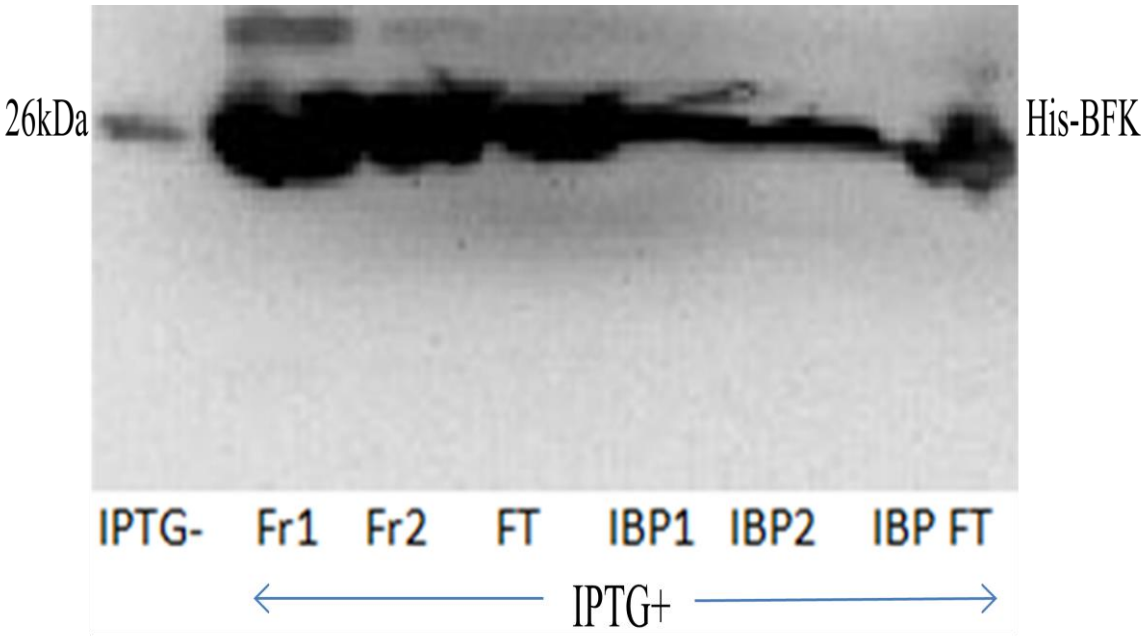


Figure 5.26. Confirmation of hisBFK protein with anti-his antibody. Fr: Fraction, IBP: Inclusion Body Protocol, FT: Flow Through. Fraction 1 represents the first elute, fraction 2 represents the second elute from same cartridge.

Purified hisBFK protein was sent to TUBITAK-MAM for injection into Balb-c mice and to perform fusion steps of hybridoma generation. Two mice, whose serum had the highest response against hisBFK protein in ELISA assay, were picked and sacrificed. Their B cells taken from their spleen were fused with immortal F0 myeloma cells. Ten days after the fusion step, hybridomas were sent to us in sixteen 96 well plates to continue the screening process. We screened them to find a clone that responds well against hisBFK but not against hisGFP. We selected clone L1H3 because of its' specificity (Figure 5.27.). We also checked the anti-BFK antibody taken from that clone via Western blotting procedure.

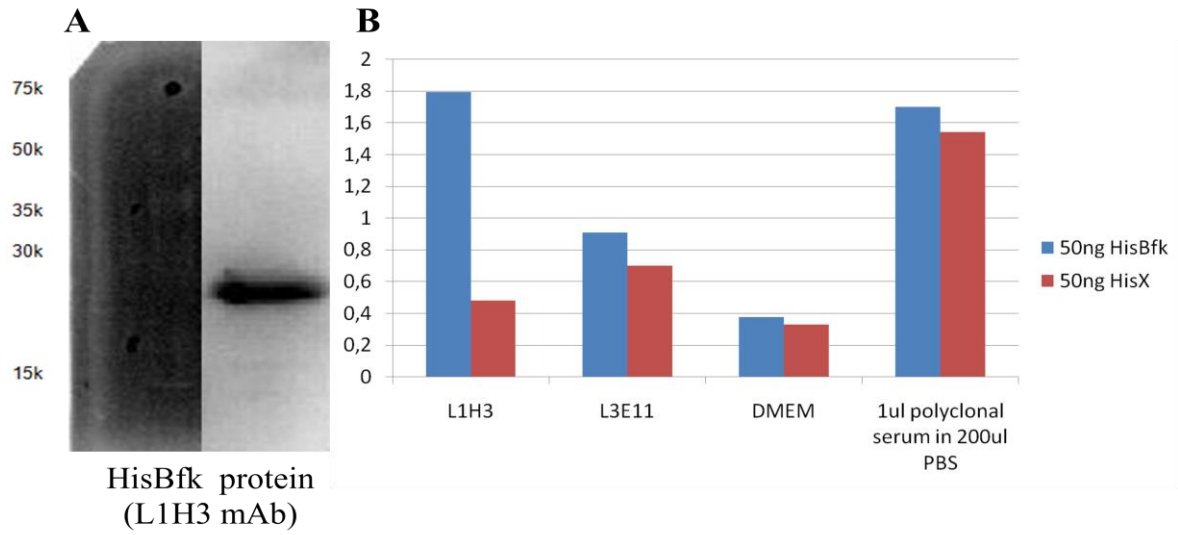


Figure 5.27. A. HisBFK protein detected with monoclonal anti-BFK antibody obtained from clone L1H3. B. High response observed against hisBFK protein compared to the other his tagged protein with ELISA. Y axis shows $OD_{450}$ value. HisX stands for HisGFP protein.

The next step to get pure anti-BFK antibody producing clone, was the isolation of single hybridoma cells. In this step, we seperated every single cell from L1H3 main clone to one well of 96 well plates and incubated at 37$^{o}$C with 5% $CO_2$ until new colonies formed. Then we checked the activities of subclones against hisBFK and hisGFP proteins with ELISA and Western blotting (Figure 5.28.). We chose L1H3-A8 clone to use in our experiments because of high response and performance in ELISA and Western Blot studies.

In a parallel work, we were able to produce polyclonal anti-hBFK antibody in rabbits. 50 ml of blood was collected from rabbit and serum part was seperated via centrifugation. Activity of polyclonal serum was tested with Western blotting (Figure 5.29.).

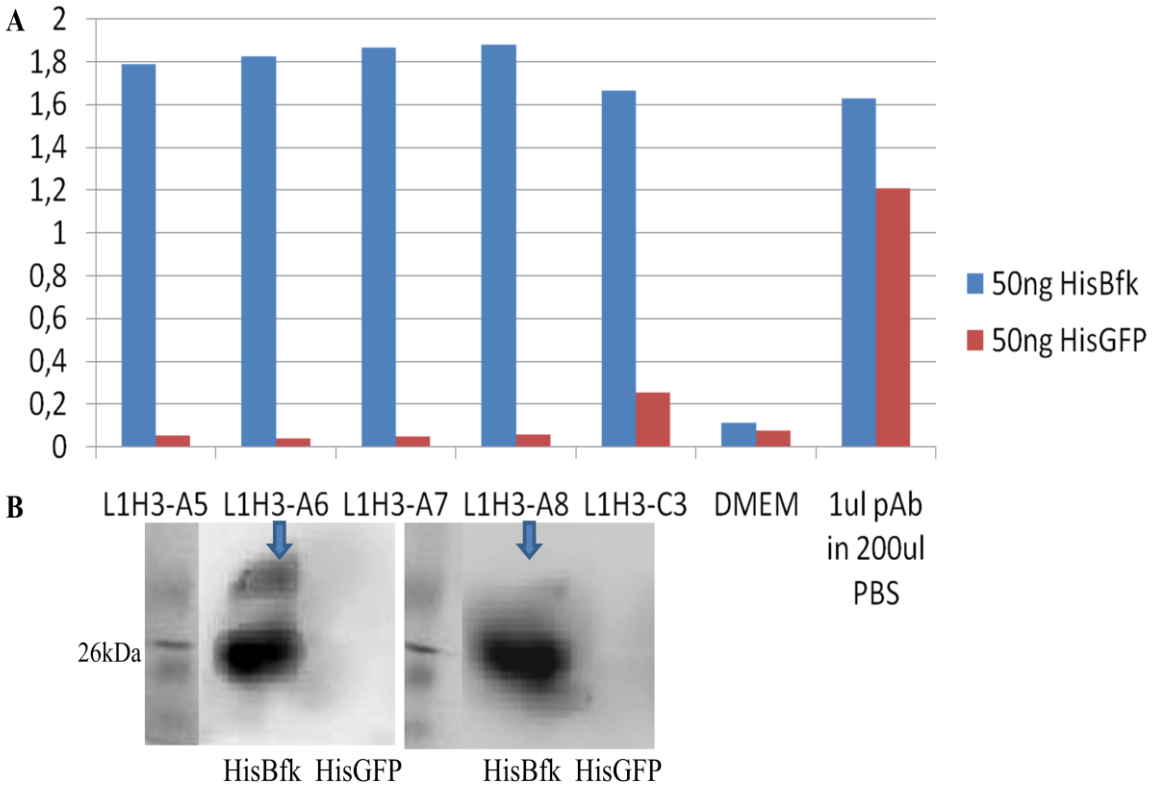


Figure 5.28. A. Pure hybridoma clone L1H3 selection via ELISA. Y axis shows $OD_{450}$ value. B. Activities of clones were confirmed with Western blotting.

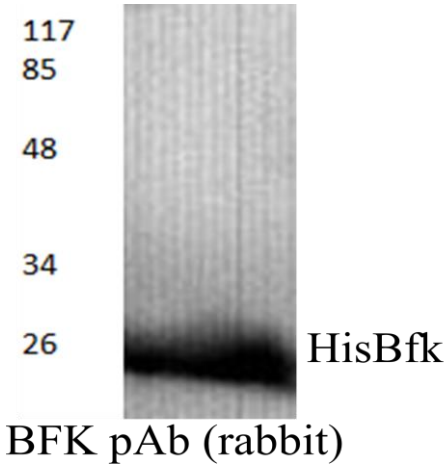


Figure 5.29. Polyclonal anti-BFK serum also detected hisBFK protein.

### 5.3.2. Site Directed Mutagenesis and Cleavage of Human BFK

To check the role of three evolutionary conserved amino acids in cleavage of hBFK, we substituted Valine (40.aa), Proline (45.aa) and Isoleucin (136.aa) to Alanine with site directed mutagenesis (SDM). Eight different primers were designed for site directed mutagenesis. Valine/Alanine (V/A) substitued BFK construct amplified as forward (with BFK F and V/A R primers) and reverse parts (with BFK R and V/A F primers) with Phusion Taq and ligated with another PCR reaction (with BFK F and BFK R primers) after purification of these samples with DNA gel extraction kit (Figure 5.30.).

**A.**


**B.**

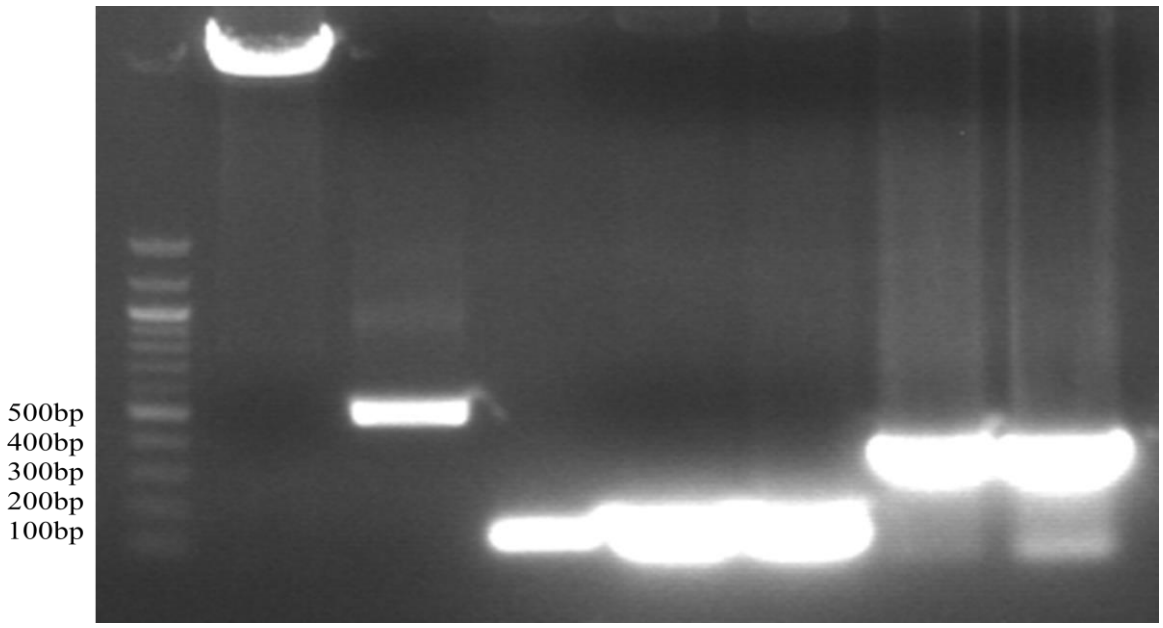


Figure 5.30. A. Primers for Valine/Alanin site directed mutagenesis. B. Lane 1: BamH1 and EcoRI double digested pcDNA3 empty vector, Lane 2: Double digested Valine/Alanin substitued BFK after second step PCR, Lane 3,4,5: Forward part of BFK amplified with BFK F and V/A R primers before second step PCR, Lane 6,7: Reverse part amplified with BFK R and V/A F primers before second step PCR. Full lenght BFK is 492 bp.

The same procedures were performed for Isoleucine/Alanine and Prolin/Alanine substitutions. Constructs were cloned into pcDNA3 vector and transformed into competent Top10 bacteria. All constructs were confirmed with both colony PCR and analytical double digestion before sending for sequencing (Figure 5.31.). Protein expression of these constructs were also tested via Western blotting after $Ca_3PO_4$ transfection into HEK293FT cells (Figure 5.32.).

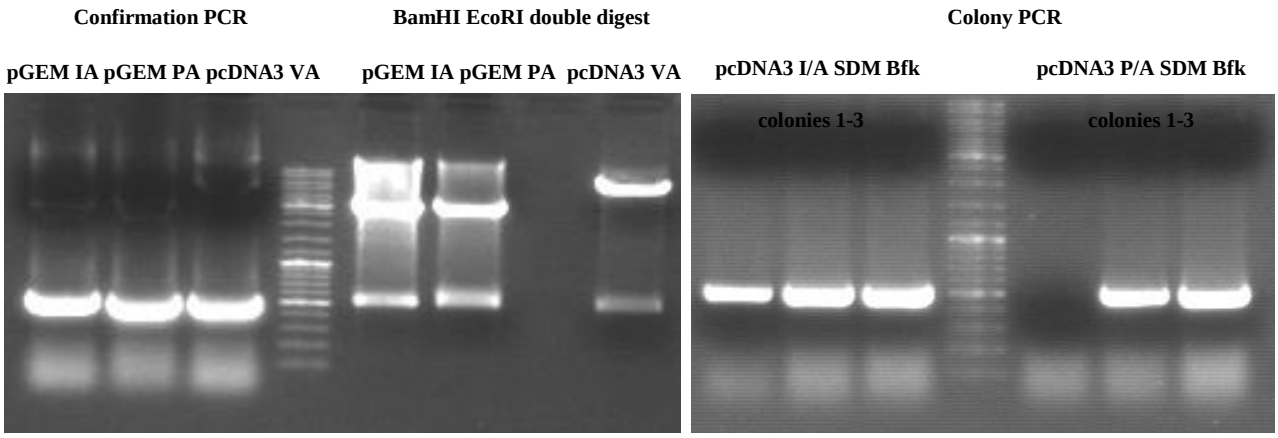


Figure 5.31. Colony PCRs and analytical double digestion for pcDNA3-V/A BFK, pcDNA3-I/A BFK, pcDNA3-P/A BFK and their subcloning versions pGEM-I/A BFK, pGEM-P/A BFK.

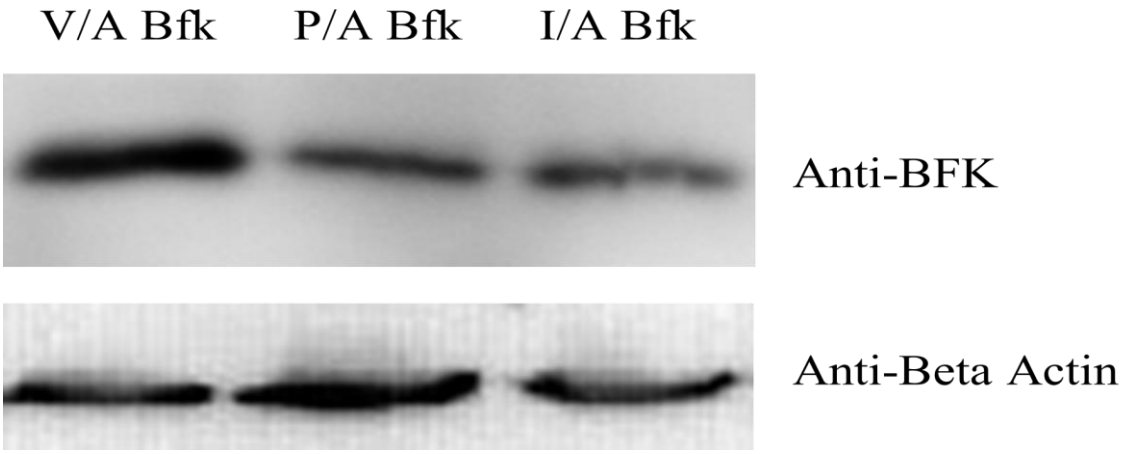


Figure 5.32. Mutated constructs express hBFK protein.

In a previous paper (Ozoren *et al*. 2009), BFK was shown to be cleaved after doxorubicin treatment, a DNA damaging drug. In our experiment, we tried to identify possible cleavage site in aminoacid sequence so we used site directed mutagenesis constructs. All constructs were transfected into HEK293FT cells via calcium phosphate transfection method. After transfection, cells were treated with 0.8 ug/ml doxorubicin and 1 ug/ml etoposide for 24 hours, we used both drugs together to increase the level of DNA damage. Many of the cells were expected to die after treatment with DNA damaging drugs but only 30% of cells died after treatment. Cell lysates were collected and used for Western blotting (Figure 5.33.).


Figure 5.33. Western blot of site directed mutagenesis constructs of BFK after DNA damaging drug treatment of HEK293FT cells.

In our western study, a band was detected, which can be cleaved BFK in FLAG-BFK transfected cell lysate. FLAG-BFK construct contains wt-BFK and has nearly 1kDa difference from other constructs. FLAG-BFK construct also has intact DEVD site, which is the putative caspase-3 or 7 target. VA-BFK construct has DEAD site instead of DEVD. IA-BFK and PA-BFK constructs may be useful for protein interactions or folding studies. If this band is cleaved BFK, it is not expected to observe the same band at VA-BFK construct. Changing in protein folding because of Isoleucin to Alanin (IA-BFK) and Prolin to Alanin (PA-BFK) substitutions may be the reason of not detecting the band in these conditions.

### 5.3.3. Hormonal Regulation of BFK Protein

In this section, we wanted to check hormonal regulation of human BFK protein levels. In order to do this, $10^7$ SW620 (male) human colon adenocarcinoma cells were seeded into 10 cm plates and treated with estradiol or testosterone hormones in a time dependent manner (Figure 5.34.). BFK protein levels were same after estrogen treatment as in qRT-PCR data. But western blotting result after testosterone treatment was not convincing enough to make a conclusion because the actin bands were fainted in the middle of the membrane, which may depend on insufficient substrate dispersal or misfocusing of Stella device.

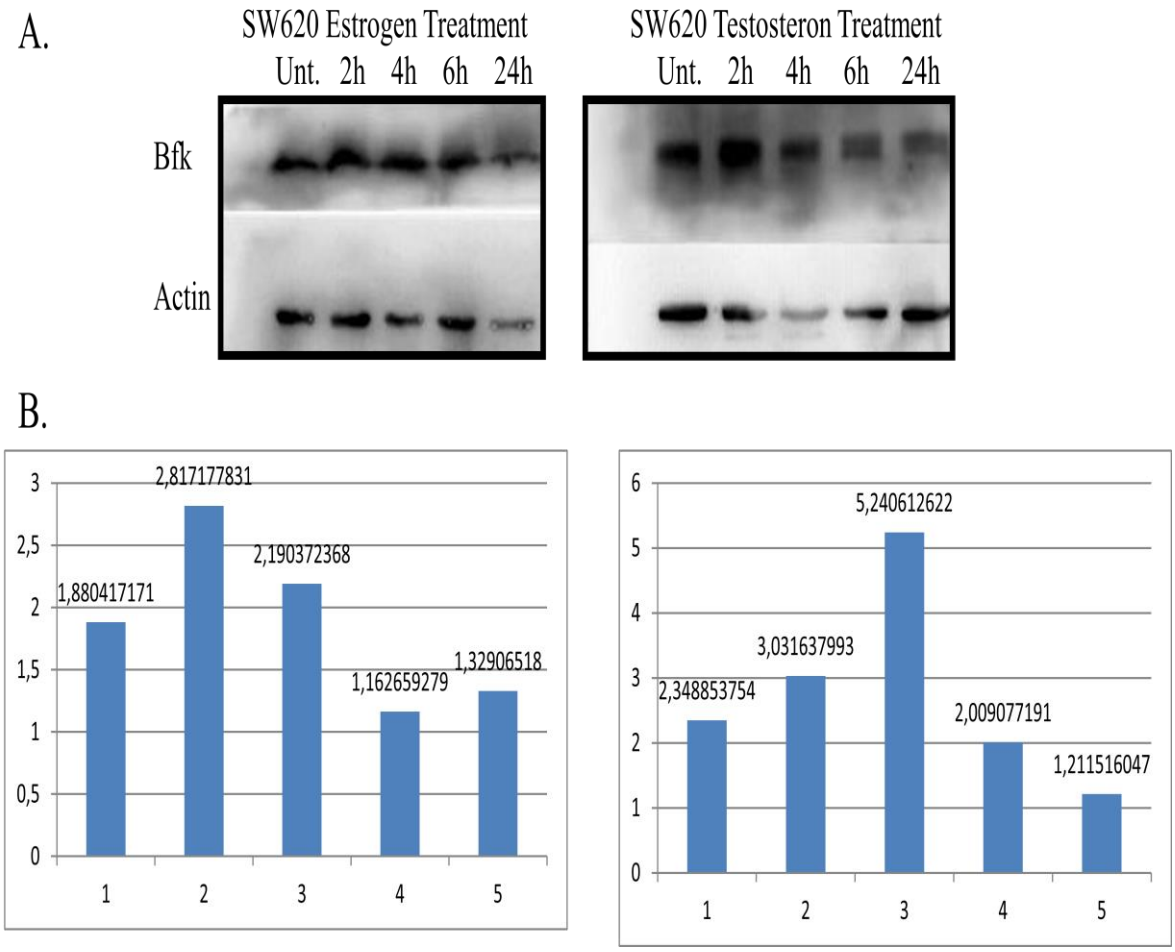


Figure 5.34. A. Hormonal regulation of BFK expression in SW620 colon cancer cell line at different time points. B. Quantification of the bands with ImageJ program.

# 6. DISCUSSION

The human BFK (Bcl-2 Family Kin) is a pro-apoptotic Bcl-2 family member especially expressed in the gastrointestinal tract. Although BFK is a pro-apoptotic protein, it has been shown that BFK only weakly induces apoptosis. On the other hand, our bioinformatics research revealed that BFK is positively selected during evolution. This situation drives us to three conclusions, firstly, BFK may be an inducible or cleaved protein to show its real apoptotic potential; secondly, BFK may have a role in development, so it may be redundant in adult tissues and thirdly, BFK may have distinct roles other than in apoptosis. These questions form the basis of our research to enlighten the regulatory mechanisms of BFK. In order to achieve this goal, we studied control mechanisms of BFK in two stages; transcriptional and post-transcriptional control.

We started our study with evaluation of putative promoter region of human BFK. Our bioinformatic data on DNA sequence showed that, among the primates, the BFK promoter regions have the same LINE and Alu elements (Figure 5.1.) in a similar regional distribution but the same distribution pattern was not valid for other eukaryotic species. Among primates, having the same repetetive elements may show that BFK has similar functions in these species because repetetive elements and possible transcription factors, which bind to this region regulate the function of a gene. The 442 bp region before the translation start site had no repetetive elements in 12 species, making this region a potential target for transcription factor binding sites, so we mainly focused on this region during the determination of putative transcription factors and their binding sites. NF-KB consensus sites were one of the potential transcription factors, detected in the bioinformatics programs. It was interesting to consider NF-KB because a possible relation with immune mechanisms may help to find the link between BFK and gastrointestinal tumors. Two potential binding sites were found in the 442bp region and one was found in a region upstream of 442 bp part. Unfortunately, we didn’t manage to show any effect of NF-KB binding sites on BFK transcription as measured by luciferase assays. NF-KB binding sites may have effect when three putative binding sites are together in a construct so we have to use BFK full promoter construct to check the activity again.

PARbZIP transcription factor binding site was also found in this 442 bp part PARbZIP (proline and acidic amino acid-rich basic leucine zipper) proteins are consist of three members: thyrotroph embryonic factor (TEF), D-site binding protein (DBP), and hepatic leukemia factor (HLF). They represent a subfamily of circadian transcription factors belonging to the bZIP family. PARbZIP proteins control expression of genes coding for enzymes involved in metabolism (Gachon *et al*., 2007). They have been shown to be involved in neurotransmitter homeostasis, amino acid metabolism and transactivation of the promoter of Bcl-gS (Benito *et al*., 2006). Our luciferase data suggests that PARbZIP regulate BFK promoter. We found TEF (Thyrotroph Embryonic Factor) and NFIL3 (Nuclear Factor Interleukin 3 or E4BP4) to have roles in this process. While TEF induces luciferase activity upon binding to BFK promoter region, NFIL3 may reverse this activity. PARbZIP proteins have role in the transcriptional regulation of pro-apoptotic Bik (Ritchie *et al*, 2009). Our result also shows the involvement of PARbZIP family in the regulation of Bcl-2 family members so it will be a great contribution to identify other targets of PARbZIP family in apoptotic pathways.

We also found that the promoter region and the coding sequence of BFK have parallel evolution characteristics after comparing phylogenetic trees. When the promoter region had dramatically changed, the gene was also changed in accordance with that diversification. There are two major diversifications in the phylogenetic trees, first one is between rodents and other species and second one is between primates and other species. These diversifications may be the reason of differences in tissue expression and interaction profile between mouse and human. Because promoter region changes may effect the transcription factors related with this gene thus characteristics of the gene may be changed.

In another bioinformatic research, we obtained the aminoacid sequences of 15 different species from databases and compared them with alignment and boxshade tools to find conservation motifs. Valine in DEVD site, proline and isoleucine aminoacids were found positively selected among primates during evolution (Figure 5.36.). Due to the DEVD motif, we can interpret that cleavege of BFK may be valid for primates. BFK becomes activated through caspase dependant cleavage during DNA damage induced apoptosis, an ideal caspase 3 target sequence, DEVD amino acids 37 through 41, is evident N terminal to the BH3 domain. The cleaved form of the protein is dependent on the presence of BAX or BAK to induce apoptosis (Ozoren *et al*, 2009). We also hypothesized

that proline and isoleucine aminoacids may be related with the interaction of human BFK with Bcl-XL and Bcl-W, which is not observed for mouse Bfk.

In previous studies, mouse Bfk was shown to be regulated by estrogen in the menstrual cyclus of mice (Coultas *et al*., 2003) and testosterone reverse the conditions after gonadectomy in male mice (Pujianto *et al*., 2007). However, estrogen treatment may have no effect on human BFK mRNA expression levels and human BFK mRNA levels appear to be downregulated 4 hours post-testosteron treatment. Reduced expression of pro-apoptotic isoforms of BFK has been described in many human gastrointestinal tumours (Dempsey *et al*., 2005) and may be related with its protective role against the development of human gastrointestinal malignancy so downregulation of BFK levels after testosteron treatment may help cancer cells to escape from apoptosis. This process may be one of the contributing mechanisms for encountering higher insidence of gastrointestinal tumours in males when compared to females.

BFK interacts with anti-apoptotic Bcl-XL and pro-apoptotic BAX (Ozoren *et al*., 2009) so we checked four BCL-2 family member (BFK, Bcl-2, Bcl-XL and BAX) expression levels with qRT-PCR after tamoxifen treatment. We found that for all time points BFK mRNA levels are significantly upregulated (Figure 5.29.). Besides that after 6 hour tamoxifen treatment, BAX mRNA levels are downregulated. This data is the first link between hormones and human BFK. Tamoxifen is a therapeutic drug, which is used in breast cancer therapies. It is an antagonist of the estrogen receptor in breast tissue. Tamoxifen induced apoptosis in breast cancer cells relates to downregulation of Bcl-2 (Zhang *et al*., 1999). In other tissues such as the endometrium, it behaves as an agonist, and thus may be characterized as a mixed agonist/antagonist drug depending on affected tissues. The role of tamoxifen on colon cancer cells is unknown. Increase levels of human BFK after tamoxifen treatment, with potential downregulation of Bcl-2, may induce apoptosis in colon cancer cells so tamoxifen treatment may be one of the option in colon cancer therapies in the future. Downregulation of pro-apoptotic BAX seems contradictory in this process but other pro-apoptotic Bcl-2 family member, BAK may compensate this downregulation. We have to check the levels of all related Bcl-2 family members after tamoxifen treatment before making an exact conclusion.

To work with endogenous BFK in our experiments, we produced anti-hBFK monoclonal and polyclonal antibodies. Monoclonal anti-hBFK antibody was produced in collaboration with TUBITAK-MAM. We used our monoclonal anti-hBFK antibody (clone L1H3-A8) in our Western studies. We didn't identify the binding region of our monoclonal antibody on human BFK. Human BFK is thought to be cleaved from Valine (40.aminoacid) in DEVD site. If our antibody binds to N terminal region of human BFK that means, no cleaved bands will be observed in Western blotting experiments. To detect cleaved form of human BFK, we need our antibody to recognize a region after cleavage site.

In order to test the cleavage activity of BFK protein, we generated 3 constructs with site directed mutagenesis and Alanine aminoacid was selected for substitutions since it is a non-polar aminoacid. We transfected HEK293FT cells with these constructs and treated cells with DNA damaging drugs. A band was detected which can be cleaved BFK in FLAG-BFK transfected cells via Western study. FLAG-BFK construct contains wt-BFK and has nearly 1 kDa difference from other constructs. FLAG-BFK construct also has intact DEVD site, which is the putative caspase-3 or 7 target. If this is a cleaved band of BFK, it is unexpected to observe same band at VA-BFK construct because VA-BFK construct has DEAD site instead of DEVD. IA-BFK and PA-BFK constructs may be useful for protein interactions or folding studies. Changing the protein folding because of Isoleucine to Alanine (IA-BFK) and Prolin to Alanine (PA-BFK) substitutions may be the reason for not detecting the band in these conditions. The results of the Western blot experiments was not clear enough to make a conclusion about the cleavage site. We have to increase the band's visibility and other hybridoma subclones will be tested for this purpose.

Depending on BFK studies done so far, we thought that hBFK may cleaved by caspase-3 or 7 from DEVD site and if tBFK overcomes the anti-apoptotic effect of Bcl-XL and Bcl-W, it interacts with pro-apoptotic BAX and BAK on the mitochondrial membrane to induce cytochrome c release and eventually apoptosis (Figure 6.1.).

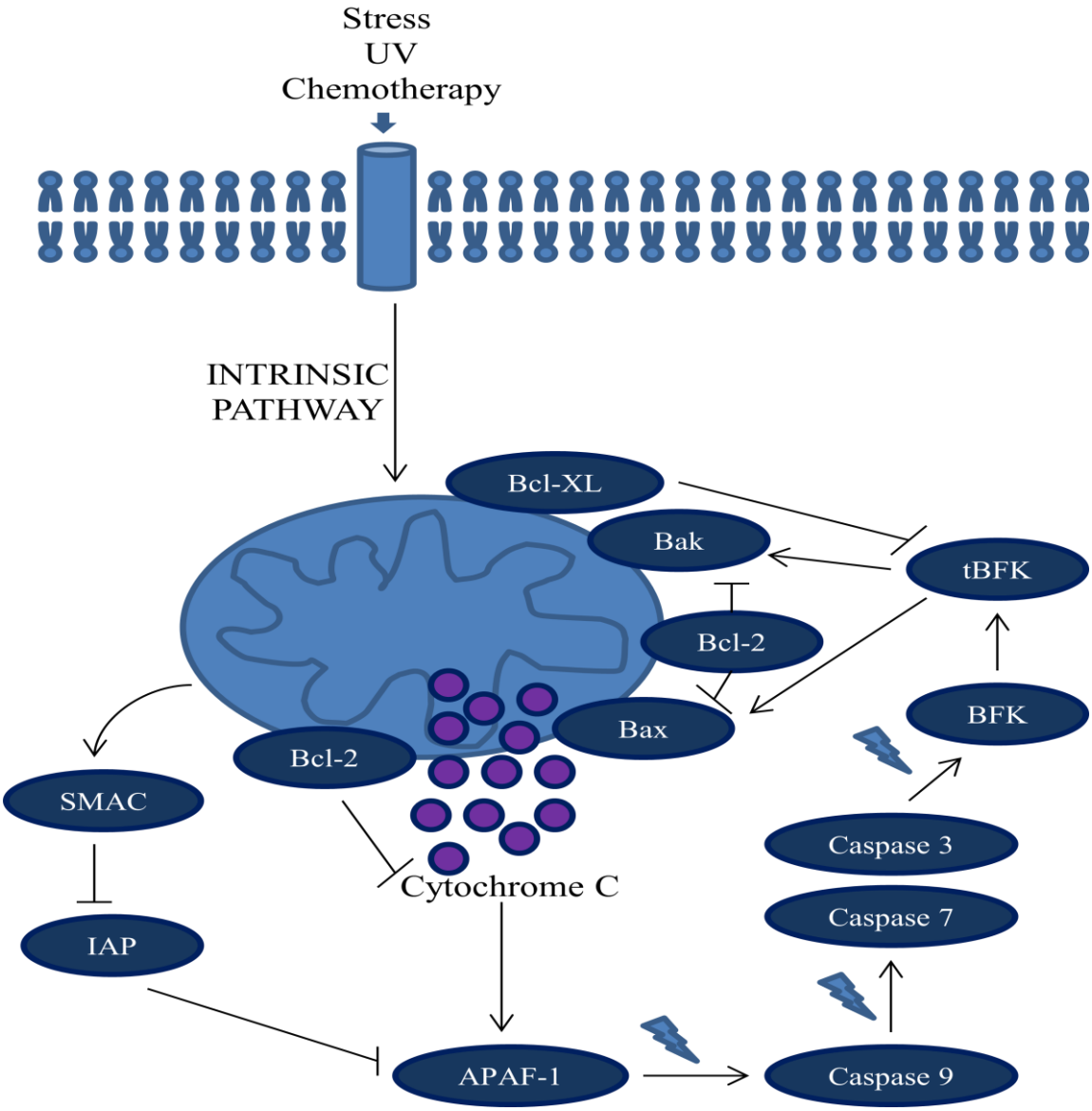


Figure 6.1. Putative diagram for human BFK.

# 7. CONCLUSION

In this study, we identified transcription factors as novel regulators of human BFK. PARbZIP family members (especially TEF and NFIL3) appear to regulate human BFK upon binding to promoter region. Tamoxifen also upregulates BFK levels. Further studies should be performed to understand how these novel regulators affect endogenous BFK expression levels. In addition, the possible link between tamoxifen and PARbZIP family members should be analyzed via qRT-PCR after treating colon cancer cells with tamoxifen. On the other hand, this data should be confirmed with other techniques such as Western blotting and ChIP. Although consequences at RNA level is revealed, the same results may not be observed at protein levels due to other regulators, such as microRNAs. We hope this study can contribute to illumination of the role of BFK and help to understand Bcl-2 family members.

# APPENDIX A: Plasmid Maps

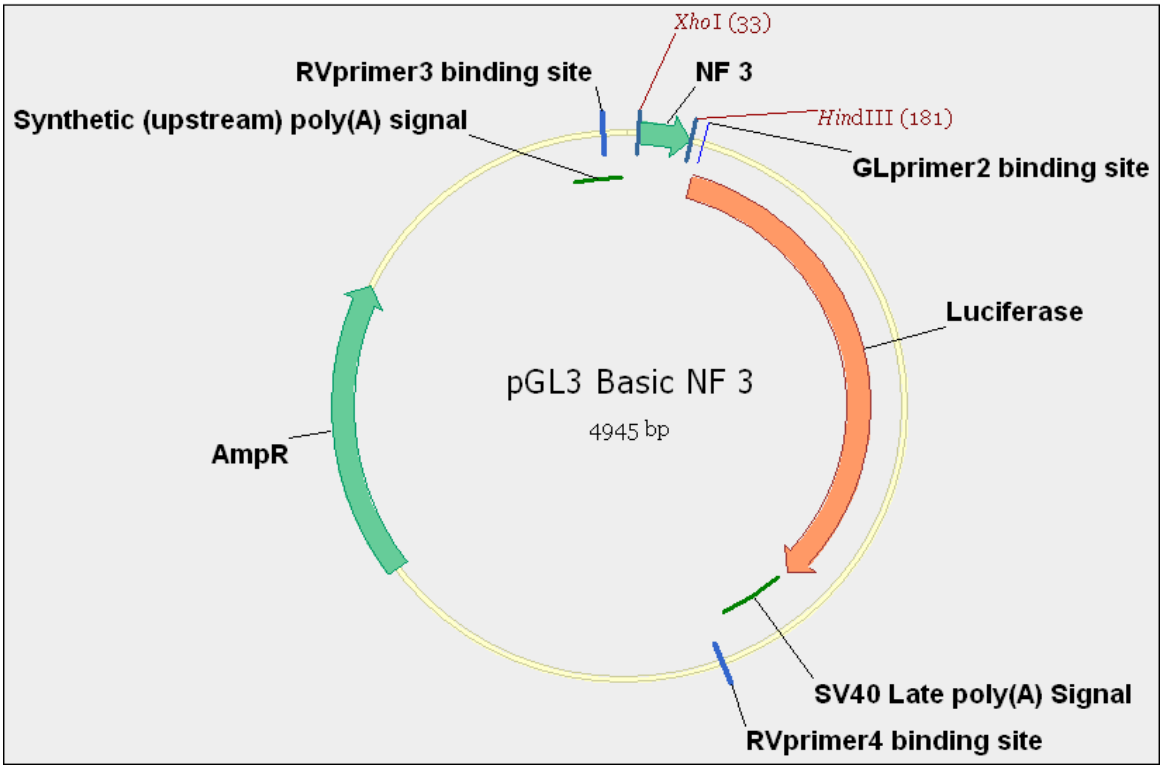


Figure 1. Cloning construct for NF-KB3 in pGL3 basic vector.

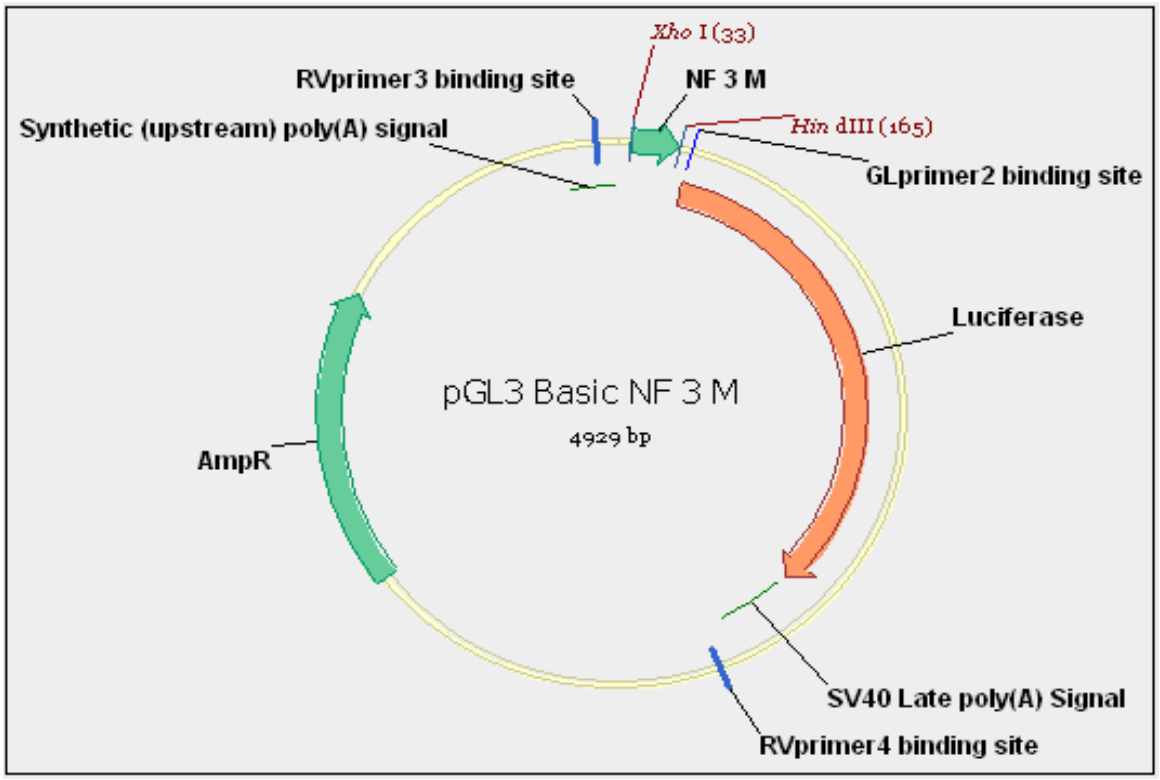


Figure 2. Cloning construct for NF-KB3 deleted version in pGL3 basic vector.

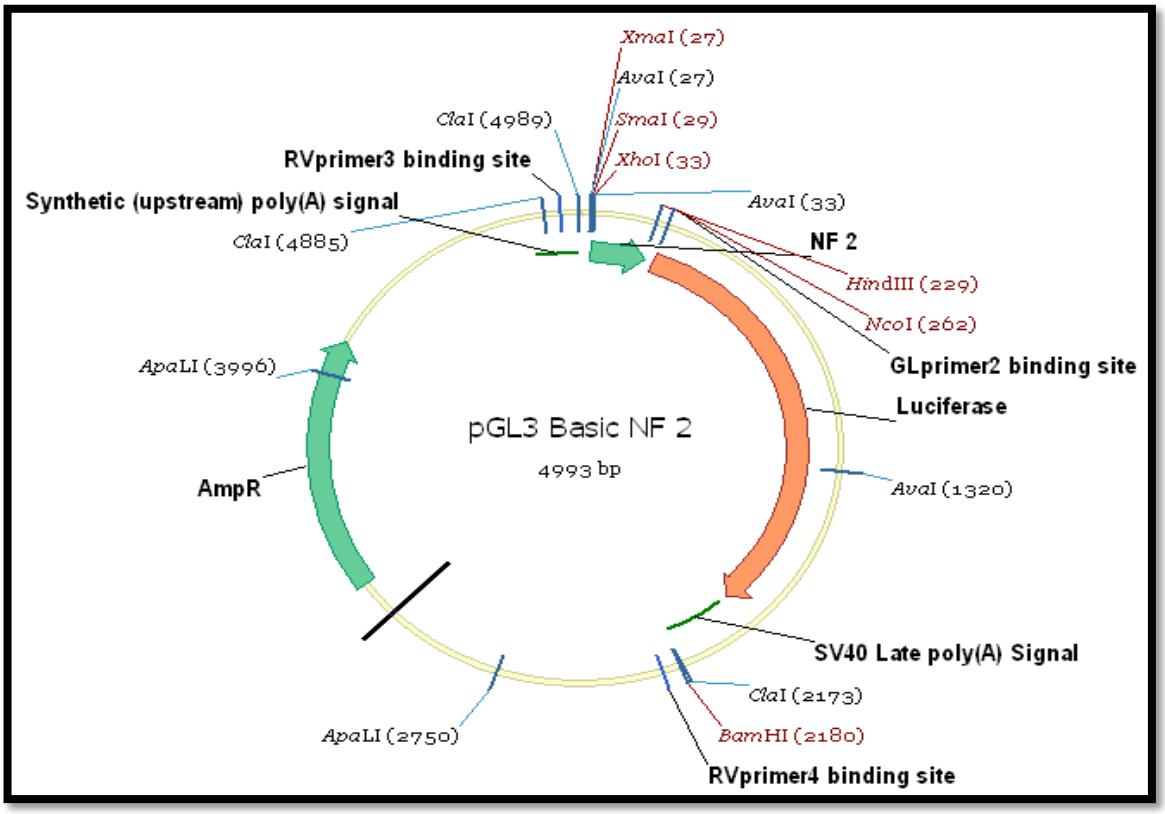


Figure 3. Cloning construct for NF-KB2 in pGL3 basic vector.

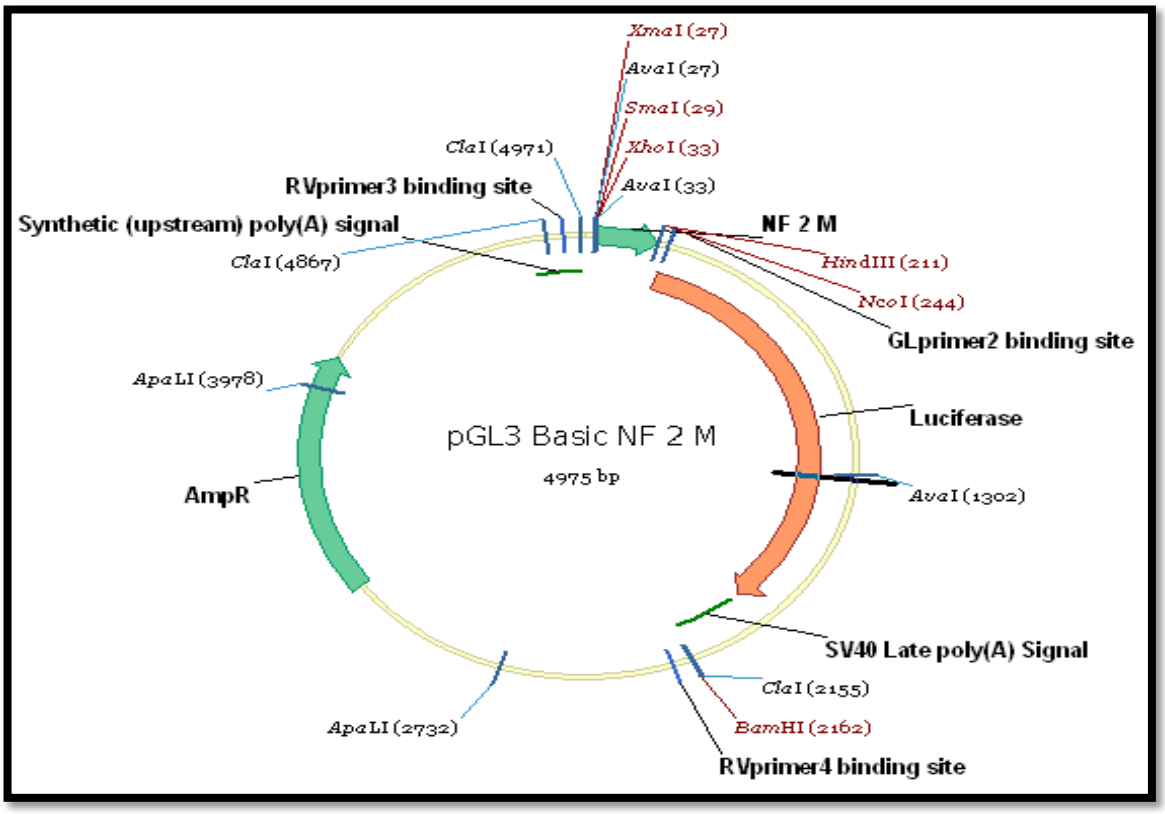


Figure 4. Cloning construct for NF-KB2 deleted version in pGL3 basic vector.

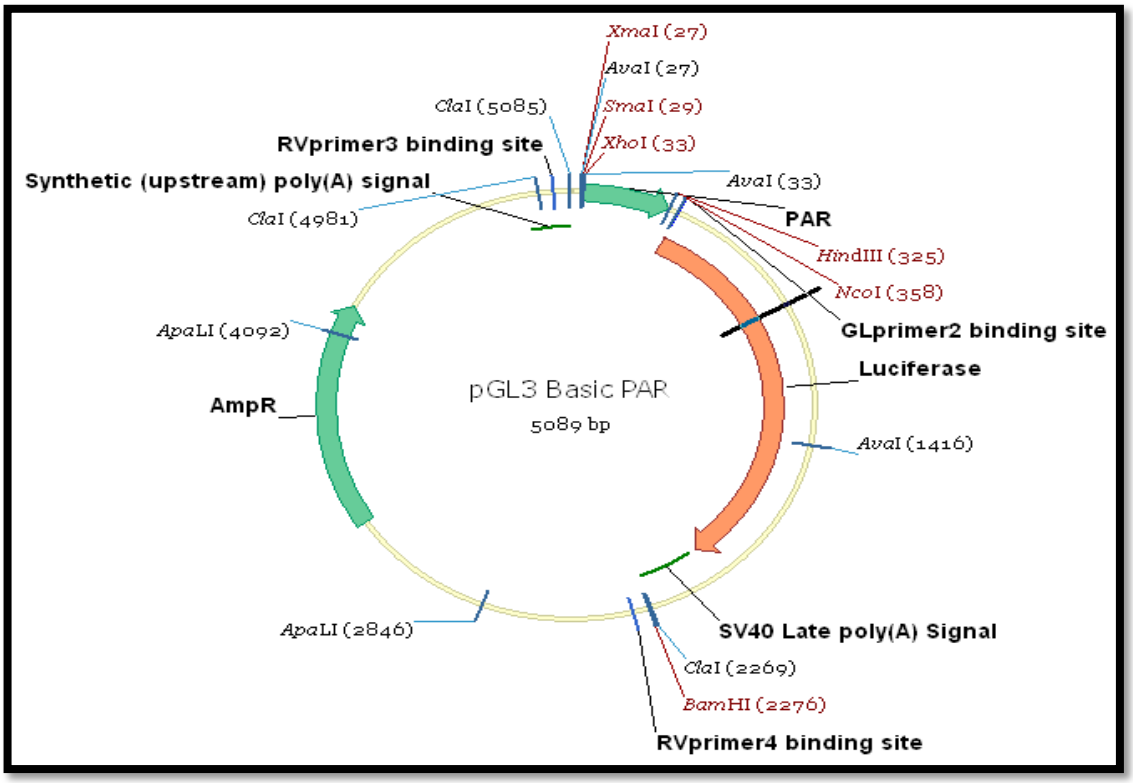


Figure 5. Cloning construct for PARbZIP in pGL3 basic vector.

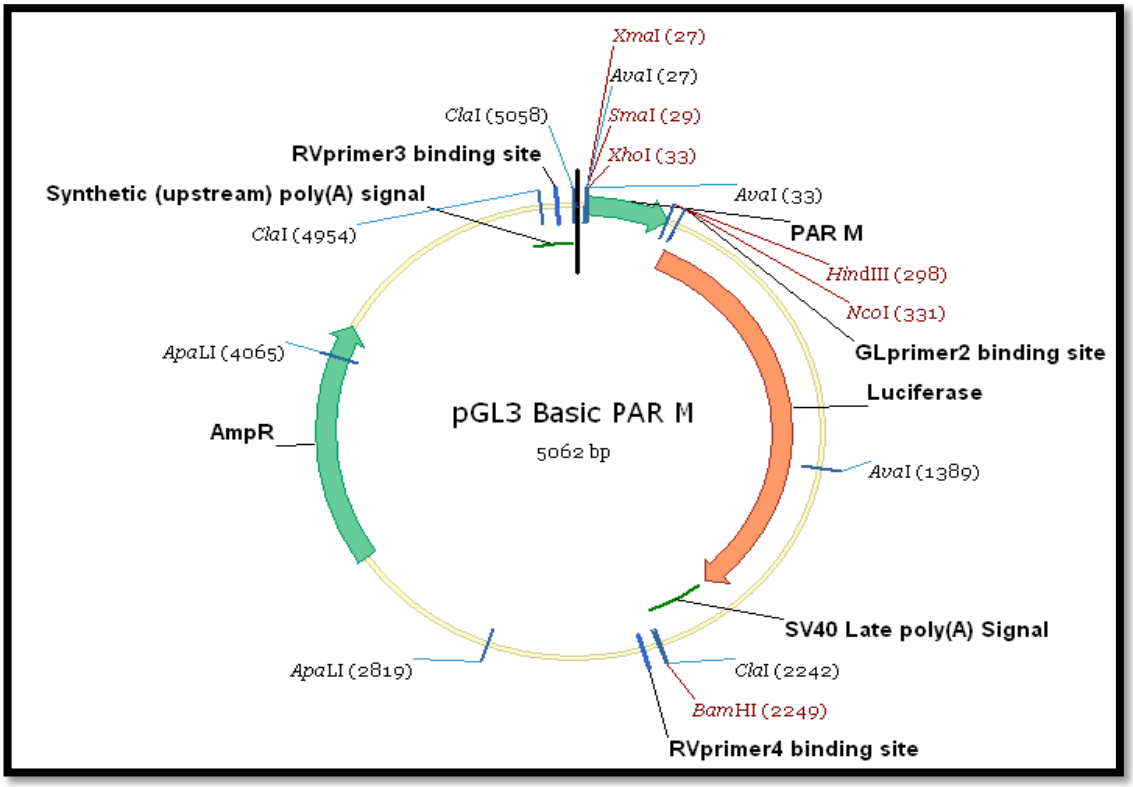


Figure 6. Cloning construct for PARbZIP deleted version in pGL3 basic vector.

Unknowns

**Unknowns**

| Sample | Wells | OD_Values | Concentration | MeanConc | SD | CV | Dilution | AdjConc |
|---|---|---|---|---|---|---|---|---|
| CE | F10 | 0.608 | 412.367 | 396.474 | 22.476 | 5.7 | 0.0 | 0.000 |
| | G10 | 0.583 | 380.581 | | | | | |
| EB1 | D11 | 0.702 | 550.688 | 514.180 | 51.630 | 10.0 | 0.0 | 0.000 |
| | E11 | 0.657 | 477.672 | | | | | |
| EB2 | D12 | 0.627 | 436.715 | 403.122 | 47.508 | 11.8 | 0.0 | 0.000 |
| | E12 | 0.575 | 369.528 | | | | | |
| IBP CE | H9 | 0.639 | 452.283 | 423.726 | 40.386 | 9.5 | 0.0 | 0.000 |
| | H10 | 0.595 | 395.168 | | | | | |
| IBP EB1 | F11 | 0.364 | 58.990 | 53.798 | 7.344 | 13.7 | 0.0 | 0.000 |
| | G11 | 0.353 | 48.605 | | | | | |
| IBP EB2 | F12 | 0.356 | 51.464 | 30.882 | 29.108 | 94.3 | 0.0 | 0.000 |
| | G12 | 0.309 | 10.300 | | | | | |
| IBP UNF. | H11 | 1.055 | 1562.995 | 1656.838 | 132.714 | 8.0 | 0.0 | 0.000 |
| | H12 | 1.085 | 1750.681 | | | | | |
| IPTG | D10 | 0.531 | 312.558 | 349.106 | 51.687 | 14.8 | 2000.0 | 698211.940 |
| | E10 | 0.587 | 385.654 | | | | | |

Unknowns_NoDiln

StandardCurve Fit: Cubic Spline

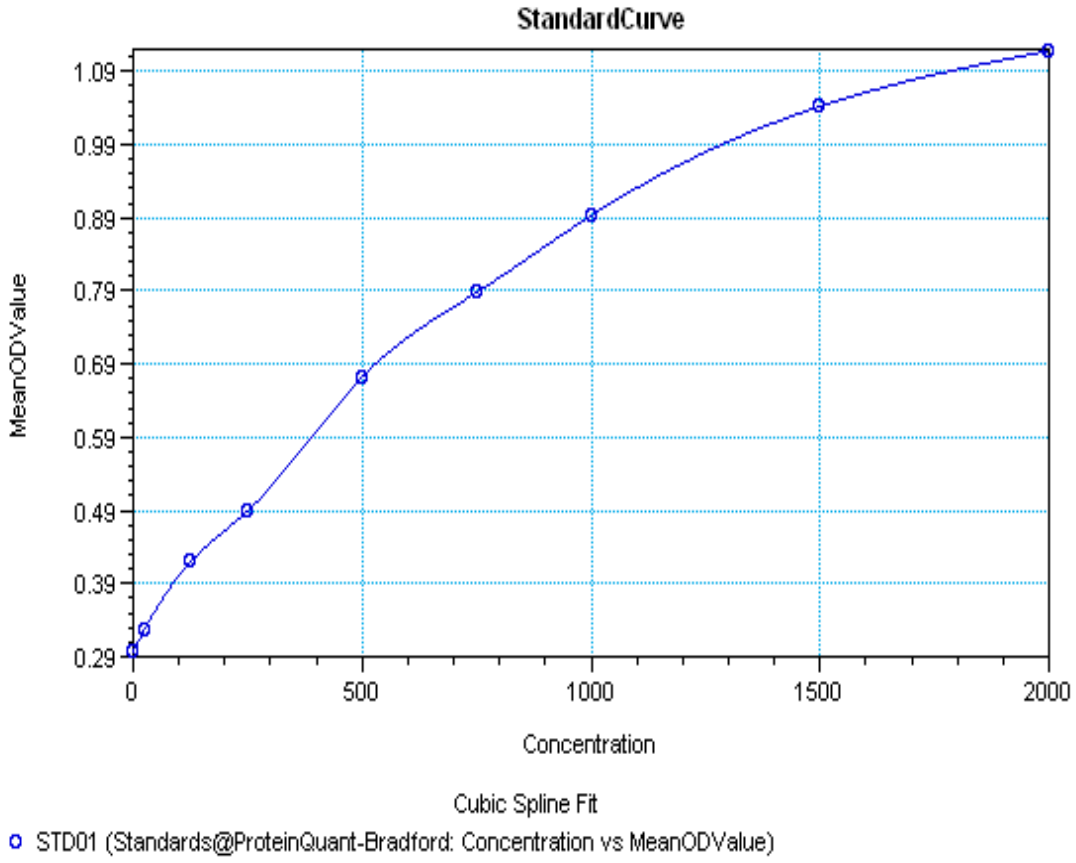


Figure 7. Bradford assay to measure the amount of hisBFK protein.

# REFERENCES

Bakhshi A., Jensen J., Korsmeyer S., 1985, "Cloning the Chromosomal Breakpoint of t(14;18) Human Lymphomas: Clustering around JH on Chromosome 14 and near a Transcriptional Unit on 18", *Cell*, Vol.41, pp.899-906.

Benito A., Gutierrez O., Fernandez L., 2006, "A Novel Role for Proline- and Acid-rich Basic Region Leucine Zipper (PAR bZIP) Proteins in the Transcriptional Regulation of a BH3-only Proapoptotic Gene", *Journal of Biological Chemistry*, Vol.281, pp.38351–38357.

Chen G., Goeddel V., 2002, "TNF-R1 Signaling: A Beautiful Pathway". *Science*, Vol.296, pp.1634–1635.

Cleary M. L., Sklar J., 1985, "Nucleotide Sequence of a t(14;18) Chromosomal Breakpoint in Follicular Lymphoma and Demonstration of a Breakpoint-cluster Region near a Transcriptionally Active Locus on Chromosome 18", *Proceedings of National Academy of Science USA*, Vol.82, pp.7439-7443.

Coultas L., Pellegrini M., Visvader J., Lindeman G., Chen L., Adams J., Huang D., Strasser A., 2003, "Bfk: A Novel Weakly Proapoptotic Member of the Bcl-2 Protein Family with a BH3 and a BH2 Region", *Cell Death and Differentiation*, Vol.10, pp.185-192.

Danial N., Korsmeyer S., 2004, "Cell Death: Critical Control Points", *Cell*, Vol.116, pp.205-219.

Dempsey C., Dive C., Renshaw S., 2005, "Expression of Pro-apoptotic Bfk Isoforms Reduces during Malignant Transformation in the Human Gastrointestinal Tract", *FEBS Letters*, Vol.579, pp.3646-3650.

Du C., Fang M., Li Y., Wang X., 2000, "Smac, a Mitochondrial Protein that Promotes Cytochrome c–Dependent Caspase Activation by Eliminating IAP Inhibition", *Cell*, Vol.102, pp.33-42.

Gachon F., 2007, "Physiological Function of PARbZIP Circadian Clock Controlled Transcription Factors", *Annals of Medicine*, Vol.39, pp.562-571.

Gross A., Korsmeyer S., 1999, "Bcl-2 Family Members and the Mitochondria in Apoptosis", *Genes and Development*, Vol.13, pp.1899-1911.

Hockenbery D., Nunez G., Korsmeyer S., 1990, "Bcl-2 is an Inner Mitochondrial Membrane Protein that Blocks Programmed Cell Death", *Nature*, Vol.348, pp.334-336.

Jolley K., Feil E., Chan M., Maiden M., 2001, "Sequence Type Analysis and Recombinational Tests", *Bioinformatics*, Vol.17, pp.1230-1231.

Kanneganti T., Lamkanfi M., 2010, "Caspase-7: A Protease Involved in Apoptosis and Inflammation", *Biocel*, Vol.42, pp.21-24.

Kischkel F.C., Hellbardt S., Peter M., 1995, "Cytotoxicity dependent APO-1 associated proteins form a death inducing signaling complex with the receptor", *EMBO Journal*, Vol.14, pp.5579-5588.

Korsmeyer S. J., Danial N., 2004, "Cell Death: Critical Control Points", *Cell*, Vol.116, pp.205-219.

Kuida K., Lippke J., Ku G., Flavell R., 1995, "Altered Cytokine Export and Apoptosis in Mice Deficient in Interleukin 1 beta Converting Enzyme", *Science*, Vol.267, pp.2000-2003.

Laurent D., Caballero M., Kinnaly K., 2006, "Is MAC the Knife that Cuts Cytochrome c from Mitochondria during Apoptosis?", *Cell Death and Differentiation*, Vol.13, pp.1387–1395.

Larkin M., Blackshields G., Higgins D., 2007, "Clustal W and Clustal X Version 2.0", *Bioinformatics*, Vol.23, pp.2947-2948.

Lindsten T., Ross A., King A., Zong W., Rathmell J., Shiels H., Ulrich E., Thompson C., 2000, "The Combined Functions of Pro-apoptotic Bcl-2 Family Members Bak and Bax are Essential for Normal Development of Multiple Tissues", *Molecular Cell*, Vol.6, pp.1389–1399.

Li P., Allen H., Banerjee S., Franklin S., Herzog L., Johnston C., Paskind M., Salfeld J., 1995, "Mice Deficient in IL-1 beta Converting Enzyme are Defective in Production of Mature IL-1 beta and Resistant to Endotoxic Shock", *Cell*, Vol.80, pp.401-411.

Michael W., Graham W., Leo D., 2002, "Relative Expression Software Tool for group wise comparison and statistical analysis of relative expression results in real-time PCR", *Nucleic Acids Research*, Vol.30, pp.36-37.

Miura M., Zhu H., Yuan J., 1993, "Induction of Apoptosis in Fibroblasts by IL-1 beta Converting Enzyme, a Mammalian Homolog in the *C.elegans* Cell Death Gene ced 3", *Cell*, Vol.75, pp.653-660.

Muchmore S., Sattler M., Liang H., Meadows R., Fesik S., 1996, "X-ray and NMR Structure of Human Bcl-xL, an Inhibitor of Programmed Cell Death", *Nature*, Vol.381, pp.335–341.

Nei M., Gojobori T., 1986, "Simple Methods for the numbers of synonymous and non-synonymous nucleotide substitutions", *Molecular Biology and Evolution*, Vol.3, pp.418-426.

Ozoren N., Inohara N., Nunez G., 2009, "A Putative Role for Human BFK in DNA Damage Induced Apoptosis", *Biotechnology Journal*, Vol.4, pp.949-1092.

Pujianto D. A., Damdimopoulos A., Poutanen M., 2007, "Bfk, a Novel Member of the Bcl-2 Gene Family, is Highly Expressed in Principal Cells of the Mouse Epididymis and Demonstrates a Predominant Nuclear Localization", *Endocrinology*, Vol.148, pp.3195-3204.

Ritchie A., Gutierrez O., Fernandez L., 2009, "PAR bZIP-bik is a Novel Transcriptional Pathway that Mediates Oxidative Stress Induced Apoptosis in Fibroblasts", *Cell Death and Differentiation*, Vol.16, pp.838-846.

Sattler M., Liang H., Fesik S., 1997, "Structure of Bcl-xL-Bak Peptide Complex: Recognition between Regulators of Apoptosis", *Science*, Vol.275, pp.983–986.

Strasser A., 2005, "The Role of BH3-only Proteins in the Immune System", *Nature Reviews Immunology*, Vol.5, pp.189-200.

Tamura K., Kumar S., 2007, "MEGA4: Molecular Evolutionary Genetic Analysis Software Version 4.0", *Molecular Biology and Evolution*, Vol.24, pp.1596-1599.

Tsujimoto Y., Cossman J., Jaffe E., Croce C., 1985, "Involvement of the Bcl-2 Gene in Human Follicular Lymphoma", *Science*, Vol.228, pp.1440-1443.

Veis D. J., Sorenson C., Korsmeyer S., 1993, "Bcl-2 Deficient Mice Demonstrate Fulminant Lymphoid Apoptosis, Polycystic Kidneys and Hypopigmented Hair", *Cell*, Vol.75, pp.229- 240.

Wajant H., 2002, "The Fas Signaling Pathway: More than a Paradigm", *Science*, Vol.296, pp.1635–1636.

Wang K., Yin X., Korsmeyer S., 1996, "BID: a Novel BH3 Domain-only Death Agonist", *Genes Development*, Vol.10, pp.2859-2869.

Wei M., Zong W., Cheng E., Korsmeyer S., 2001, "Pro-apoptotic BAX and BAK: a Requisite Gateway to Mitochondrial Dysfunction and Death", *Science*, Vol.292, pp.727–730.

Wilson K. P., Black J., Thomson J., Raybuck S., 1994, "Structure and Mechanism of Interleukin-1 beta Converting Enzyme", *Nature*, Vol.370, pp.270–275.

Wood D.E., Thomas A., Newcomb E., 1998, "Bax cleavage is mediated by calpain during drug-induced apoptosis", *Oncogene*, Vol.17, pp.1069-1078.

Yang Z., 2007, "PAML4: Phylogenetic Analysis by Maximum Likelihood", *Molecular Biology and Evolution*, Vol.24, pp.1586-1591.

Yuan J., Horvitz H.R., 2004, "A First Insight into the Molecular Mechanisms of Apoptosis", *Cell*, Vol.116, pp.53–56.

Zhang G., Kimijima I., Onda M., Takenoshita S., 1999, "Tamoxifen-induced Apoptosis in Breast Cancer Cells Relates to Down-Regulation of Bcl-2, but not Bax and Bcl-$X_L$, without Alteration of p53 Protein Levels", *Clinical Cancer Research*, Vol.5, pp.2971-2972.

Zhang L., Fang B., 2005, "Mechanisms of Resistance to TRAIL Induced Apoptosis in Cancer", *Cancer Gene Therapy*, Vol.12, pp.228-237.

Zou H., Yuchen L., Wang X., 1999, "An APAF-1 Cytochrome *c* Multimeric Complex Is a Functional Apoptosome that Activates Procaspase-9", *Journal of Biological Chemistry*, Vol.274, pp.11549-11556.